Edited by Wolfgang Krämer
and Ulrich Schirmer

WILEY-VCH

Modern Crop Protection Compounds

Volumes 1 - 3

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Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

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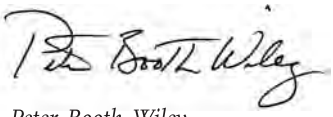

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WILEY-VCH Verlag GmbH & Co. KGaA

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Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the Internet at ⟨http://dnb.d-nb.de⟩.

Printed in the Federal Republic of Germany
Printed on acid-free paper

Composition Asco Typesetters, Hong Kong
Printing betz-druck GmbH, Darmstadt
Bookbinding Litges & Dopf GmbH, Heppenheim
Cover Design Adam Design, Weinheim
Wiley Bicentennial Logo Richard J. Pacifico

ISBN 978-3-527-31496-6

Contents

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Volume 3

Preface

Modern market economies are not able to abandon modern crop protection as a basis for efficient and economical agriculture to nourish their growing population and the growing population in developing countries. Instead, they have to intensify the use of modern science to improve R&D processes for new cultivars or new crop protection compounds that fulfill worldwide registration demands and have no cross resistance to older ones, and to develop ecological and safety standards for real risk assessment.

To meet this challenge, scientists in universities, multinational organizations, like UNO and OECD, governmental authorities and in agricultural chemical companies and agricultural seed companies are working together to invent, develop and bring forward new solutions for effective, sustainable crop protection and the production of high-quality food.

This book aims to stimulate these processes by bringing together knowledge gained by modern biology, including genetics, biochemistry and chemistry, in crop protection during the last two decades, and by discussing the invention and development of modern crop protection compounds, whether unique in their chemistry or mode of action or as substance classes with a similar mode of action and similar or different chemistries.

Therefore, the contributions on new crop protection compounds are arranged not only under the headings new herbicides, fungicides and insecticides but also in respect of their biochemical mode of action.

Each of the main Sections, ''Herbicides'', ''Fungicides'', and ''Insecticides'', is introduced by a contribution of authors of the respective Resistance Committee, reflecting the common responsibilities of the crop protection industry for maintaining the efficacy of marketed crop protection compounds and supporting sustainable agriculture and improved public health.

These introductions allow us to mention, in a short overview, those compounds and compound classes that are not described in detail because they are dealt with *in extenso* in standard books such as *Chemistry of Plant Protection* (Springer, Berlin, Heidelberg, New York, Tokyo) and *Chemistry of Pesticides* (John Wiley and Sons, New York).

Our general target for ''new'' crop protection compounds was to include such compounds that have come to the market in the 15 years between 1990

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ISBN: 978-3-527-31496-6

and 2005 along with the development compounds of the ''new millennium'' up to 2006.

This book would not have been realized without the support of all major agricultural chemical companies and their research and development divisions, nor without the highly committed authors from them and from universities. We appreciate the tremendous work involved in collecting the literature and in writing and then submitting excellent manuscripts on time. We also especially appreciate that all of the chapter are written in a very individual manner, reflecting that all authors are, over many years, inventors or researchers or developers of plant protection compounds. As readers can see from the literature, many of the authors are the inventors of the compound or compound class they describe.

Consequently, we express our deepest gratitude to all our authors and their companies for their excellent contributions. We are sure the readers will enjoy this book and will use it as a compendium on plant protection research, in much the same way as we ourselves have experienced research on crop protection for about 30 years: stimulating, enjoyable, but also challenging. During the time period covered, tremendous market changes through new genetically modified crops have taken place, which have influenced research targets. The high demands of the public and registration authorities for safer, more ecologically compatible compounds, with tremendous increasing costs in research and development, have led to a strong concentration in the agricultural industry. From 20 companies with their own research and development in the 1980s only about five continue to carry out research in all main application fields (herbicides, fungicides, insecticides) and, additionally, in the seed business. In parallel, a deeper understanding of the efficacy and activity of crop protection compounds, their side effects on the basis of physicochemical properties and mode of action, has led to lower application rates, more selectivity of activity, and special uses, together with improved formulations.

We hope this book will also contribute to a better understanding between biologists, chemists, biochemists, agronomists, geneticists and conservationists dealing with plant protection science.

Note

The authors have named products/compounds preferably by their common names. Sometimes registered trademarks are cited. Their use is not free for everyone.

In view of the number of trademarks it was not possible to indicate each particular case in each table and contribution.

We accept no liability for this.

January 2007

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ISBN: 978-3-527-31496-6

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Part I
Herbicides

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Overview

Wolfgang Krämer and Ulrich Schirmer

The Section on *Herbicides* reflects not only the changes in herbicide markets worldwide but also the changes in importance of the different herbicide classes and modes of action for the market as well as for research and development.

With the invention of the aceto-hydoxy-acid synthesis inhibitors (AHAS) the dominance of herbicides that act as photosynthesis inhibitors was dramatically broken – as it was also by the development of genetically modified herbicide tolerant crops. These especially important areas of research and development, from the 1990s up to now, are exemplified in Chapters 3 and 7. The development of 12 new sulfonyl urea herbicides launched since 1995 and the invention of four development compounds of the same chemical class, after the introduction to the market of twenty compounds already between 1980 and 1995, reflects the importance of this biochemical mode of action for the herbicide market as well as the different chemistries found to be active at this target, such as imidazolinones, triazolopyrimidines, pyrimidinyl-carboxylates, and sulfonylaminocarbonyl-triazolinones.

One of the most important reasons for the success of the AHAS inhibitors is their extremely low application rates, in the range of 10 g-a.i. ha^{-1}, corresponding to 1 mg-a.i. m^{-2}, allowing farmers a flexible use of such herbicides with reduced market prices.

The success story of genetically modified herbicide crops, with market shares, e.g., $\geq 80\%$ in the soybean herbicide markets of USA and Argentina, reflects two facts: The low manufacturing and application costs of the herbicides used in those crops and, due to the activity of these compounds as total herbicides, the extremely broad spectrum against nearly all weeds, either broad leaf weeds or grassy weeds and also perennial ones.

Even if public opinion, especially in Europe, hindered the introduction and use of genetically modified crops in EU countries, the ability of these methods to protect crops from weed competition will not be prohibited worldwide for long. The more active (i.e., broader efficacy and spectrum, higher selectivity) and cheapest solution in solving weed problems will be used by farmers trying to survive under the pressure of low selling prices for their goods such as cereals, corn, soybeans and other crops.

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However, the introduction of new herbicides, either from AHAS biochemistry or others such as HPPD inhibitors, ACC-ase inhibitors and others, shows that selective herbicides, sometimes together with safeners, will find their markets when they are competitive with older solutions and when they offer advantages to farmers, such as one application a season.

Thus, the contributions in the *Herbicides* section also aim to discuss the importance of the different biochemical pathways in the search for new herbicides.

Chapter 5, entitled "Safeners for Herbicides", demonstrates the progress in this research field, bringing out new compounds that create highly competitive products for the farmers out of only partly selective herbicides having a very broad weed spectrum and very low application rates ("the chemical answer to genetically modified herbicide resistant crops").

Chapter 10, entitled "Photosynthesis Inhibitors", discusses also regulatory aspects and the reregistration process in Europe, along with compounds as an example of the impact of political and public requests for safer and ecobiologically more selective compounds, and the impact on markets for producers and sellers of generic products. This example demonstrates that markets for generic compounds not only grow through compounds losing patent protection but that they can also shrink by losing registration in countries who are opinion leaders in registration requests. Conversely, registration demands and the fulfilling of the regulatory requests by the producer/seller will protect the compounds/products longer than the patent protection lasts.

Chapter 1 ("HRAC Classification of Herbicides and Resistance Development") describes the importance of resistance development of weeds for the herbicide developers and users. This impact on herbicidal activity under field situations also shows the necessity of continuous further research for new herbicides with new modes of action, at least by the global-acting agrochemical companies.

Plant growth regulators – having their own market as growth retardants, fruit thinning agents for better quality and fruit size, sprout suppressants, defoliants, stress protectants and harvesting help, for example by suppressing dwarfing, – are only of research and development interest in a small group of agrochemical companies. However, because they influence the biochemistry of plants we have included them in this section.

1
HRAC Classification of Herbicides and Resistance Development

Hubert Menne and Helmut Köcher

1.1
Introduction

The first cases of herbicide resistance were reported around 1970. Since then resistance of mono- and dicotyledonous weeds to herbicides has become an increasing problem world-wide.

In March 2006 the International Survey of Herbicide-Resistant Weeds recorded 305 herbicide-resistant biotypes with 182 weed species – 109 dicotyledonous and 73 monocotyledonous weeds [1]. The relatively steady increase in the number of new cases of resistance since 1980 accounts for the increasing importance of herbicide resistance in weeds in the major agricultural regions (Fig. 1.1).

In the period 1970–1990 most documented cases were concerning triazine resistance. The introduction of new herbicides with different modes of action (MoA) resulted in a shift, so that more recently ALS- and ACCase resistant weeds have been reported (Fig. 1.2).

The rapid adoption of glyphosate resistant crops in North and South America and the use of glyphosate as a pre-sowing treatment in different cropping systems has resulted in increasing cases of glyphosate resistance [1]. The probability of resistance development to glyphosate had been expressed as being likely, though less frequently in comparison with most mode of action classes [2].

1.2
HRAC Classification System of Herbicides

The global HRAC group proposed a classification system for herbicides according to their target sites, modes of action, similarity of induced symptoms or chemical classes (Table 1.1).

It is the most comprehensive existing classification system of herbicides globally. With the WSSA Code System and Australian Code System two similar classification systems were developed earlier for regional needs. The usage of differ-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

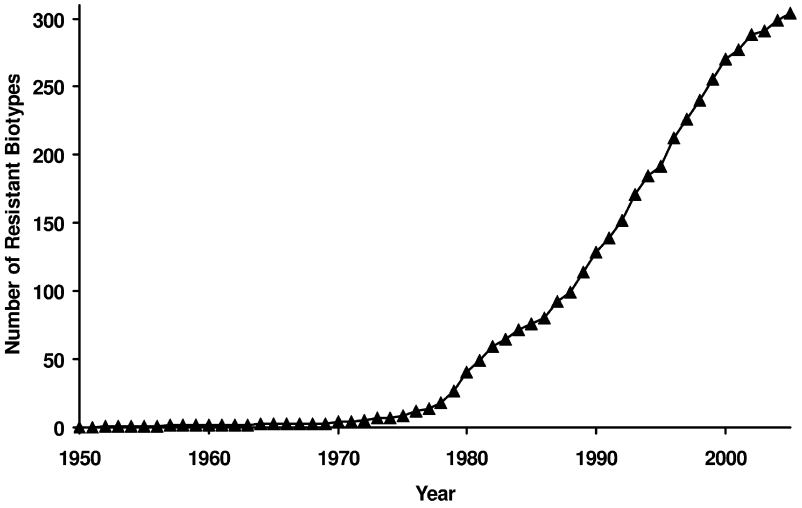

Fig. 1.1. Chronological increase in the number of herbicide-resistant weeds worldwide [3].

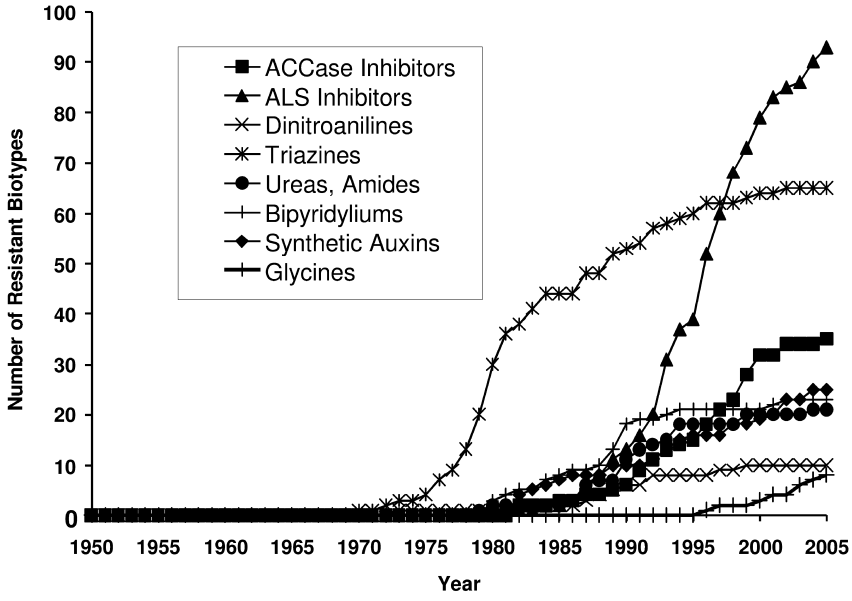

Fig. 1.2. Chronological increase in the number of herbicide-resistant weeds for the different herbicide classes [3].

Table 1.1 HRAC classification system in comparison to WSSA and Australian code system. (Adapted from Refs. [3–5].)

Mode of action	Chemical family	HRAC group	WSSA group[a]	Australian group[a]
Inhibition of acetyl CoA carboxylase (ACCase)	Aryloxyphenoxy-propionate, cyclohexanedione, phenylpyrazoline	A	1	A
Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	Sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl(thio)benzoate, sulfonylaminocarbonyl-triazolinone	B	2	B
Inhibition of photosynthesis at photosystem II	Triazine, triazinone, triazolinone, uracil, pyridazinone, phenyl-carbamate	C1	5	C/K
	Urea, amide	C2	7	C
	Nitrile, benzothiadiazinone, phenyl-pyridazine	C3	6	C
Photosystem-I-electron diversion	Bipyridylium	D	22	L
Inhibition of protoporphyrinogen oxidase (PPO)	Diphenyl ether, phenylpyrazole, N-phenylphthalimide, thiadiazole, oxadiazole, triazolinone, oxazolidinedione, pyrimidindione, other	E	14	G
Inhibition of the phytoene desaturase (PDS)	Pyridazinone, pyridinecarboxamide, other	F1	12	F
Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	Triketone, isoxazole, pyrazole, other	F2	27	F
Inhibition of carotenoid biosynthesis (unknown target)	Triazole, diphenylether, urea (also C2)	F3	11	F
Inhibition of 1-deoxy-D-xylulose 5-phosphate synthase (DOXP synthase)	Isoxazolidinone	F4	13	F
Inhibition of EPSP synthase	Glycine	G	9	M

Table 1.1 *(continued)*

Mode of action	Chemical family	HRAC group	WSSA group[a]	Australian group[a]
Inhibition of glutamine synthetase	Phosphinic acid	H	10	N
Inhibition of DHP (dihydropteroate) synthase	Carbamate	I	18	K
Inhibition of microtubule assembly	Dinitroaniline, phosphoroamidate, pyridine, benzamide, benzoic acid	K1	3	D/K
Inhibition of mitosis/ microtubule organisation	Carbamate	K2	23	E
Inhibition of VLCFAs (inhibition of cell division)	Chloroacetamide, acetamide, oxyacetamide, tetrazolinone, other	K3	15	K
Inhibition of cell wall (cellulose) synthesis	Nitrile	L	20	K
	Benzamide	L	21	
	Triazolocarboxamide	L		
	Quinoline carboxylic acid	L	26	
Uncoupling (membrane disruption)	Dinitrophenol	M	24	
Inhibition of lipid synthesis – not ACCase inhibition	Thiocarbamate, phosphorodithioate	N	8	E
	Benzofuran	N	16	K
	Chloro-carbonic-acid	N	26	J
Action like indole acetic acid (synthetic auxins)	Phenoxy-carboxylic-acid, benzoic acid, pyridine carboxylic acid, quinoline carboxylic acid, other	O	4	I
Inhibition of auxin transport	Phthalamate, semicarbazone	P	19	
Unknown	Arylaminopropionic acid	Z	25	K
Note: While the mode of action of herbicides in Group Z is unknown it is likely that they differ in mode of action between themselves and from other groups	Pyrazolium	Z	26	
	Organoarsenical	Z	17	
	Other	Z	27	

[a] Not all chemical classes are classified.

ent numbers and letters in the different classification systems lead very often to confusion and misunderstanding on the global level. One common global system would be highly desirable for all users and for better understanding of differences between molecular classes. All single systems should give support and advice to all users of herbicides. This advice should state how to apply the individual active compounds to achieve the best results in terms of weed control and resistance management.

The classification system is describing not only the chemical family belonging to a specific mode of action but all compounds via their common names counted to each family, as shown in Table 1.2 for the modes of action such as ''Inhibition of DHP (dihydropteroate) synthase'', ''Microtubule assembly inhibition'', ''Inhibition of mitosis/microtubule organization'', ''Inhibition of VLCFAs (Inhibition of cell division)'' and ''Inhibition of cell wall (cellulose) synthesis'' as examples (not mentioned in other chapters of this book) (for a detailed table see www.plantprotection.org/HRAC/). The scheme ''The World of Herbicides'' available under this internet address also shows all chemical structures of the different herbicides belonging to the different chemical families.

1.3 Herbicide Resistance

In the weed population, herbicide resistance in weeds is a natural phenomenon that occurs at a low frequency and which has evolved over millions of years. Herbicide applications only select for these weeds in a population but they do not cause resistance. Increasing problems with herbicide resistant weed populations have predominantly occurred in countries with intensive agriculture cropping systems. The reliance on few of the available weed management tools with disregard of the principles of Integrated Weed Management (IWM) are closely related to changes in the weed population community. Changes in the farming environment and specifically the economic pressure on farmers are key factors that force farmers to change their practices to those that encourage resistance development.

The limitation in cropping systems, lack of rotation of herbicide chemistry or mode of action, limitation in weed control techniques, reduction of dose rates, etc. are major drivers for the selection of herbicide resistances. Regular country based surveys often make clear that farmers are aware of the problems and their causes. A survey in Germany in 2004 showed that 94% of the farmers are aware that the repeated use of the same herbicide, and 89% that the reduction of dose rates, causes the development of herbicide resistance. However, 86% of the farmers are forced to reduce their costs and they do not have a lot of scope with their weed management techniques [6].

As mentioned previously, the planting of herbicide resistant crops worldwide, which increased from 1.7 mio ha^{-1} in 1996 to around 90 mio ha^{-1} in 2005, has changed the farmers weed control tactic completely [7]. These systems have provided the farmers favorable economic advantages as well as more cropping flexi-

Table 1.2 Selected groups of the HRAC classification system with examples of the active ingredients, which are not mentioned in following chapters. (Adapted from Refs. [3–5].)

Mode of action	Chemical family	Active ingredient	HRAC group	WSSA group	Australian group[a]
Inhibition of DHP (dihydropteroate) synthase	Carbamate	Asulam	I	18	K
Microtubule assembly inhibition	Dinitroaniline	Benefin = benfluralin *Butralin* *Dinitramine* Ethalfluralin Oryzalin Pendimethalin Trifluralin	K1	3	D
	Phosphoro-amidate	*Amiprophos-methyl* *Butamiphos*			
	Pyridine	Dithiopyr Thiazopyr			
	Benzamide	Propyzamide = pronamide *Tebutam*			K
	Benzoic acid	DCPA = chlorthal-dimethyl		3	D
Inhibition of mitosis/microtubule organization	Carbamate	*Chlorpropham* *Propham* Carbetamide	K2	23	E
Inhibition of VLCFAs (inhibition of cell division)	Chloroacet-amide	Acetochlor Alachlor Butachlor *Dimethachlor* Dimethanamid Metazachlor Metolachlor *Pethoxamid* Pretilachlor Propachlor *Propisochlor* Thenylchlor	K3	15	K
	Acetamide	*Diphenamid* Napropamide *Naproanilide*			
	Oxyacetamide	Flufenacet Mefenacet			

Table 1.2 *(continued)*

Mode of action	Chemical family	Active ingredient	HRAC group	WSSA group	Australian group[a]
	Tetrazolinone	Fentrazamide			
	Other	Anilofos			
		Cafenstrole			
		Piperophos			
Inhibition of cell wall (cellulose) synthesis	Nitrile	Dichlobenil	L	20	K
		Chlorthiamid			
	Benzamide	Isoxaben		21	
	Triazolocarboxamide	*Flupoxam*			
	Quinoline carboxylic acid	Quinclorac (for monocots) (also group O)		26	

[a] Not all chemical classes are classified.

bility. In Canada the adoption of herbicide resistant cropping systems has already reached 5.2 million ha (95%) out of 5.5 million ha for canola production [8].

The reliance on one herbicide has reduced the number of applications and the number of MoA used. In 2004, glyphosate was applied on 87% (62% in 2000, 25% in 1996) of the whole acreage of soybeans in the US [9]. No other herbicide was applied on more than 7% of the acreage (four herbicides with more than 10% in 2000) [9].

The continued use of herbicide resistant cropping systems with over-reliance on single weed management techniques selects for weeds that have already evolved resistance to the herbicide. Additionally, in the population, specific weed species can become dominant that were less frequent before but naturally resistant and, therefore, more difficult to control. It was suspected that a weed population shift will have a bigger impact on the cropping system than the selection of resistant weeds [10, 11]. Recent research studies and findings suggest that resistance in weeds and weed populations shifts are occurring more quickly than expected [12]. Statistical observations have shown that the use, dose rates and application frequency have already changed. In the US in 1996 glyphosate was applied in soybeans 1.1 times with 773 g-a.i. ha^{-1}. The usage increased to 1.3 applications and 1065 g-a.i. ha^{-1} in 2000, to 1.5 applications and 1211 g-a.i. ha^{-1} in 2004 [9]. Similar trends can be observed for corn and cotton and also for soybeans in other countries like Argentina and Brazil.

Intensive soil cultivation techniques and stubble burning were always common weed control techniques in agricultural areas in the past. The increasing limitation or ban of stubble burning caused increasing weed coverage, an increasing

soil seed bank and the development of herbicide resistance in many agricultural regions. Different investigations have shown that the burning of straw drastically decreased weed densities, e.g., the number of waterplants (*Echinochloa* spp.) in comparison to the incorporation of rice straw into the soil [13]. Australian farmers in particular look for alternative weed control techniques during the harvest operation because of the limited choice of chemical solutions during the growing season. Balling of straw methods, such as trailing baler attached to the harvester or destroying of weed seeds physically during the harvesting operation (''Rotomill''), gives additional possibilities [14].

The economic pressure to farmers to produce at lowest costs and the changing environmental influences, like soil erosion or water availability, have led to the adoption of no-till practices in recent years. The use of no-till is expected to further increase globally. In most cases the shift to no-till systems causes an over reliance on herbicides. The price erosion of herbicides during the last years played a significant role in adoption of no-till practices. Survey studies showed that farmers are aware that no-till practices increase herbicides costs, herbicide resistance and in particular glyphosate resistance. Nevertheless, the acreage for no-till is expected to increase, especially in areas where no-till is still of low proportion [15]. However, growers with increasing herbicide resistance problems were planning to reduce the use of no-till.

Simulation studies showed that the risk of adopting no-till and the development of herbicide resistances can be reduced by alternating between minimum and no-tillage systems or by alternating between non-selective herbicides for pre-sowing weed control [16]. The most efficient weed control strategy for conserving susceptibility in no-tillage systems was the ''double knockdown'' pre-sowing application scheme of glyphosate and paraquat in sequence.

One of the most effective tools in the management of herbicide resistance and weed density is a diverse crop rotation practice. Weed species are typically associated with crops, and crop rotations compose their specific weed populations over time [17]. A high diversity provides the farmer more opportunities with more flexibility with respect to growing conditions, tillage practices and planting time, selecting of crop cultivars, rotating herbicides with different modes of action, varying the application timings of herbicides across years to a specific weed emergence period and/or including nonchemical management techniques etc. [18]. These practices give farmers opportunities to prevent or to slow down the selection and development of herbicide resistance. Selected resistance can remain in field populations for many years. They are stable until resistant weed seeds disappear from soil seed banks, which is very seldom. Investigations with triazine resistant weed strains showed that resistant weed seeds remained in soil despite changes in crop rotation and absence of triazine herbicides [19]. Similar results were obtained in studies that evaluated the effect of management practices on ACCase resistant *Alopecurus myosuroides* in the field [20]. The percentage of resistant plants did not change during a three-year period even without herbicide applications of ACCase inhibitors. The density of blackgrass plants was decreased, however, especially when spring crops were part of the crop rotation.

Neither cropping systems nor single weed management tactic can solve specific weed problems on a long-term basis. The use of all possible practices to prevent and to manage herbicide resistances in an integrated fashion should be the long-term goal for agricultural production.

As already mentioned, continuous application of a herbicide selects rare genotypes of weeds that are resistant to the herbicide and eventually at the same time already cross-resistant to other herbicides. These genotypes may already exist in a weed population in very low frequency before the introduction of the selecting herbicide.

1.3.1 Biochemistry of Herbicide Resistance

Resistance can be based on one of the following biochemical mechanisms [21]:

Target-site resistance is due to reduced or lost ability of the herbicide to bind to its target protein. This is usually an enzyme with a crucial function in metabolic pathways or the component of an electron transport system. As a further possibility, target-site resistance could also be caused by an overproduction of the herbicide-binding protein.

Nontarget-site resistance is caused by mechanisms that reduce the amount of herbicidally active compound reaching the target site. An important mechanism is enhanced metabolic detoxification of the herbicide in the weed, with the effect that only insufficient amounts of herbicidally active substance will reach the target site. Furthermore, reduced uptake and translocation or sequestration of the herbicide may lead to insufficient herbicide transport to the target site.

Cross-resistance means that a single resistance mechanism causes resistance to several herbicides. The term *target-site cross-resistance* is used when these herbicides bind to the same target site, whereas *nontarget-site cross-resistance* is due to a single nontarget-site mechanism (e.g., enhanced metabolic detoxification) that entails resistance across herbicides with different modes of action.

Multiple resistance is a situation where two or more resistance mechanisms are present within individual plants or within a population.

1.3.1.1 Target-site Resistance

Cases analyzed to date show that herbicide resistance is very frequently based on a target-site mutation. Within the past 35 years weed species have developed target-site resistance to most known herbicide chemistries. Those of major importance are discussed below.

Inhibitors of Photosystem II (PS II) Early reports on resistance of weeds to PSII inhibitors of the triazine group appeared around 1970. Since then triazine resistance was reported for numerous, mainly dicotyledonous, weed species.

Research on the mechanism of resistance to triazines revealed that in most cases it is due to a mutation which results in a modification of the target site which is known to be the Qb site of the D1 protein in the PSII reaction center.

The triazine herbicides bind to this site and thus inhibit the photosynthetic electron flow. In the resistant mutants triazine binding is markedly reduced. As an example, the concentration of atrazine needed to obtain a 50% inhibition of photosynthetic electron flow in isolated chloroplasts of *Chenopodium album* was found to be at least 430× higher for chloroplasts from an atrazine-resistant mutant than for chloroplasts from wild-type plants [22].

In many cases, mutants of weed species with target-site resistance to triazines showed lower growth rate and ecological fitness than the susceptible wild type, when analyzed in absence of a triazine herbicide as selection agent. The quantum yield of CO_2 reduction in resistant biotypes was decreased. Furthermore, the electron transfer between the primary and secondary quinones in the PS II reaction center was slowed, which may have been the cause of increased susceptibility to photoinhibition in the resistant biotypes [23, 24].

The D1 protein is encoded by the chloroplast *psb*A gene, which is a highly conserved gene in higher plants, algae and cyanobacteria [25]. In almost all cases of investigated resistance of weed species in the field to triazines, resistance was attributed to a mutation in the *psb*A gene with a resultant serine 264 to glycine change in the herbicide binding niche of the D1 protein. Hence this resistance is usually maternally inherited. Though herbicides of the phenylurea group are also inhibitors of the PS II system, cross-resistance of atrazine-resistant mutants with a serine 264 to glycine change has not been observed to phenylureas. It was proposed that the binding sites of triazines and phenylureas are not identical but overlapping [26, 27]. Serine 264 provides a hydrogen bond to atrazine or other herbicides of the triazine group. Substitution of serine 264 by glycine removes this bond, which is important for binding the triazines. According to the concept of overlapping binding sites, hydrogen bonding to serine 264 is not important for phenylureas, due to a different binding geometry, hence phenylurea binding will not be affected by the serine 264 to glycine mutation.

In 1999 Masabni and Zandstra reported on a mutant of *Portulaca oleracea* with a resistance pattern to PS II inhibitors that was different to most triazine resistant weeds [28]. This mutant was resistant to the phenylureas linuron and diuron, but also cross-resistant to atrazine and other triazines. Sequencing of the D1 protein revealed that in the resistant biotype the serine 264 was replaced by threonine and not by glycine. This was the first report on a serine 264 to threonine mutation on a whole plant level. It was proposed that the serine-to-threonine mutation modified the conformation of the herbicide binding niche at the D1 protein in a way, which resulted in reduced binding of phenylureas and triazines as well.

Another novel mutant was identified, when field accessions of *Poa annua* with resistance to PS II inhibitors, collected in Western Oregon, were analyzed after amplification of the herbicide-binding region (933 base pair fragment) of the chloroplast *psb*A gene using PCR.

Sequence analysis of the fragment from a mutant with resistance to diuron and metribuzin (resistance factors between 10 and 20) revealed a substitution from valine 219 to isoleucine in the D1 protein encoded by the *psb*A gene. This amino

acid substitution was previously identified after mutagenesis of laboratory cultures of algae and cell cultures of *Chenopodium rubrum*. The finding of a valine-219 to isoleucine substitution in *Poa annua*, however, was the first reported case of a weed species with resistance to PS II inhibitors in the field, due to a *psb*A mutation other than at position 264. As previously mentioned, electron transfer processes in the PS II reaction center of weeds with a mutation at position 264 were slowed and the ecological fitness of the mutants was reduced. In contrast, no effect on electron transfer in the PS II reaction center was found for the *Poa annua* mutant with the valine 219 to isoleucine change, and it was supposed that this mutant may be ecologically as fit as the wild type [29].

Inhibitors of Acetyl-CoA Carboxylase (ACCase) Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA, which results in the formation of malonyl-CoA. In the plastids this reaction is the initial step of *de novo* fatty acid biosynthesis and hence of crucial importance in plant metabolism. Species of the Poaceae family (grasses) have in their plastids a homomeric, multifunctional form of ACCase with the following domains: biotin carboxy carrier protein (BCCP), biotin carboxylase (BC) and carboxyltransferase (CT). Other monocotyledonous species, so far examined, and most dicotyledonous species have in their plastids a heteromeric, multisubunit type of ACCase with the BCCP, BC and CT domains encoded on separate subunits. In addition all di- and monocotyledons, including the Poaceae, have a cytosolic ACCase, which belongs to the homomeric type. The ACCase-inhibiting herbicides inhibit only the plastidic homomeric ACCase in grasses (Poaceae), but not the plastidic heteromeric form of other mono- and dicotyledonous species nor the homomeric ACCase in the cytosol. Therefore, these herbicides selectively have a lethal effect only on grass species, while they are tolerated by other monocotyledonous and by dicotyledonous species. There are two different chemical groups of ACCase inhibitors, the aryloxyphenoxypropionates (APPs) and the cyclohexanediones (CHDs), which have developed in the past 15 to 20 years to a very important herbicide family with selective action on a broad spectrum of grass weed species.

Target-site resistance of biotypes to ACCase inhibitors has up to now been confirmed for quite a few grass weed species of economic importance. The earliest cases of target-site based resistance were reported for biotypes of *Lolium multiflorum* from Oregon, USA [30] and of *Lolium rigidum* from Australia [31].

ACCase prepared from the resistant *L. multiflorum* biotype, which had been selected by field use of diclofop, was inhibited by the APPs diclofop, haloxyfop and quizalofop with IC_{50}s (herbicide conc. needed for 50% enzyme inhibition) that were 28-, 9- and 10-times higher than for ACCase from a susceptible biotype. There was no cross-resistance to the CHD herbicides sethoxydim or clethodim [32]. ACCase resistance was subsequently also confirmed for *L. multiflorum* biotypes from other countries. In a resistant biotype selected by diclofop in Normandy, the resistance factor (ratio of the IC_{50} for ACCase from the resistant to the IC_{50} for ACCase from the susceptible biotype) was 19 for diclofop and 5 for haloxyfop, but only 2 for the CHDs clethodim and sethoxydim [33]. Interestingly,

a different ACCase resistance pattern was found for the resistant *L. multiflorum* biotype Yorks A2, though field selection was apparently also mainly by diclofop. Resistance factors were 3 and 9, respectively, for the APPs diclofop and fluazifop, but 20 for the CHD herbicide cycloxydim [34].

First biotypes of *Lolium rigidum* with target-site resistance to ACCase inhibitors were identified in the early 1990s in Australia. Selection either with an APP or a CHD herbicide resulted in target-site cross-resistance to both herbicide groups. But, regardless of whether selection was by an APP or a CHD compound, the level of resistance in these biotypes was higher to APP than to CHD herbicides. ACCase resistance factors were 30–85 for diclofop, >10–216 for haloxyfop and 1–8 for sethoxydim [31, 35, 36].

Biotypes with target-site-based resistance to ACCase inhibitors were also selected in wild oat species (*Avena fatua*, *A. sterilis*). The resistance patterns were found to be variable. For example, the resistance factors for ACCase from the Canadian *A. fatua* biotype UM1 were 105 for sethoxydim, 10 for tralkoxydim, and 10 for diclofop and fenoxaprop, whereas for the *Avena fatua* biotype UM33 from Canada the ratios were 10.5 for fenoxaprop, 1.2 for diclofop, 5 for sethoxydim and 1.7 for tralkoxydim. It was proposed that this was due to different point mutations, each being associated with a characteristic resistance pattern [37]. Another reason could be the frequency of homozygote and heterozygote resistant and susceptible plants within a tested population.

During resistance studies with *Alopecurus myosuroides* populations from the UK two biotypes, Oxford A1 and Notts. A1, were identified, which were highly resistant to fenoxaprop, diclofop, fluazifop and sethoxydim due to an insensitive ACCase. Genetic studies revealed that the target-site resistance in the two *A. myosuroides* biotypes was monogenic and nuclear inherited, with the resistant allele showing complete dominance [38].

Target-site based resistance to ACCase has also been reported for several other grass weeds, e.g., two biotypes of *Setaria viridis* from Manitoba, Canada, one of them (UM8) conferring high levels of ACCase insensitivity to fenoxaprop and sethoxydim, while the ACCase of biotype UM 131 was highly insensitive to sethoxydim, but only moderately to fenoxaprop (reviewed in Ref. [36]). Biotypes of *Setaria faberi* and *Digitaria sanguinalis*, obtained in a vegetable cropping system in Wisconsin, USA, had an ACCase highly insensitive to sethoxydim and moderately insensitive to clethodim and fluazifop [39].

Based on the patterns of target-site-based cross-resistance of weeds to APP and CHD herbicides it was postulated that the two classes of ACCase inhibitors do not bind in identical manner to the target site (“overlapping binding sites”), and that different point mutations at the target enzyme account for variable resistance patterns. Molecular research with chloroplastic ACCase from wheat indicated first that a 400-amino acid region in the carboxyl transferase (CT) domain is involved in insensitivity to both APP and CHD herbicides [40]. Follow-up research with chloroplastic ACCase of *Lolium rigidum* showed that resistance to ACCase inhibitors was due to a point mutation which resulted in an isoleucine to leucine change in the CT domain of the enzyme [41]. Tal and Rubin have investigated the molec-

ular basis of ACCase resistance in a *Lolium rigidum* biotype from Israel with resistance to CHD and APP herbicides [42]. After amplification by PCR of a 276-bp DNA encoding the CT domain of ACCase they found also in this resistant biotype a substitution of a single isoleucine by leucine. Inheritance studies of the same authors suggested that the alteration of ACCase in *L. rigidum* was governed by a single nuclear co-dominant gene.

It was shown that a point mutation resulting in an isoleucine to leucine substitution within the chloroplastic ACCase CT domain is also responsible for target-site resistance of *Avena fatua* [43] and of *Alopecurus myosuroides* [44]. Furthermore, in *Setaria viridis* biotype UM 131 a point mutation resulting in an isoleucine to leucine change of ACCase was detected [45]. The mutant leucine ACCase allele in this species was characterized to be dominant. No negative effect was detected on ACCase function of the mutant. It was suggested that the change in ACCase conformation caused by the isoleucine to leucine mutation is only minor, but sufficient to prevent or strongly reduce herbicide binding to the enzyme. Finally, also in *Alopecurus myosuroides*, an isoleucine to leucine substitution in the ACCase is associated with resistance to ACCase inhibitors [44]. These authors also pointed to the very interesting fact that the leucine found in the plastidic homomeric ACCase of mutated resistant grass weeds is also found in the heteromeric plastidic enzyme of non-grass species and in the cytosolic homomeric enzymes that are ''naturally'' resistant to these herbicides. Hence the selective action of ACCase-inhibiting herbicides appears to reside at this enzyme site.

Further studies by Délye and coworkers with *Alopecurus myosuroides* accessions from different sites in France shed more light on the molecular basis of the different resistance patterns to ACCase inhibitors. The isoleucine to leucine mutation (position 1781) resulted in resistance to fenoxaprop, diclofop and cycloxydim, but not to clodinafop and haloxyfop, while a newly discovered mutation of isoleucine to asparagine in position 2041 conferred resistance to fenoxaprop, diclofop, clodinafop and haloxyfop, but not to cycloxydim. Both resistance alleles can occur in the same plant and both are dominant, thus giving rise to plants that are resistant to the total spectrum of the above-mentioned herbicides [46]. Meanwhile, additional point mutations were identified in *Alopecurus myosuroides* that gave rise to insensitive ACCase due to exchange of one amino acid: Trp to Cys (pos. 2027), Asp to Gly (pos. 2078) and Gly to Ala (pos. 2096). The resistance patterns originating from these mutations gave further support to overlapping binding sites for APP and CHD herbicides at the ACCase enzyme [47].

Recently PCR amplification and sequencing of plastidic ACCase domains involved in herbicide resistance has been employed to screen a spectrum of 29 grass species for target-site-based resistance to ACCase inhibitors by direct comparison of the sequences of plastidic ACCase around the critical codons [48]. The authors found that, in *Poa annua* and *Festuca rubra*, a leucine residue occurred at position 1781, while the wild types of all other grass species had an isoleucine in this position. *Poa annua* and *F. rubra* are already known from enzyme inhibition tests to possess a plastidic ACCase that is markedly less susceptible to ACCase inhibitors than the ACCase of other grass species. Thus, the leucine in position

1781 can clearly be regarded as the basis or a substantial part of the natural inherent tolerance of both species to ACCase-inhibiting herbicides.

A different mechanism of target-site resistance to ACCase inhibitors to be mentioned here was identified in a *Sorghum halepense* biotype from Virginia, USA, which was selected in the field by quizalofop applications. The resistance level of this biotype *in vivo* was relatively low, with resistance factors (based on ED_{50} values) ranging between 2.5 and 10 for quizalofop, sethoxydim and fluazifop. No difference was found between herbicide susceptibility of ACCase from the resistant biotype and a susceptible standard. However, the specific activity of ACCase in the resistant biotype was found to be 2–3× greater than in susceptible plants. The results suggested that an overproduction of ACCase was the mechanism that conferred a moderate level of resistance to these herbicides. Owing to the enzyme overproduction the resistant biotype was, presumably, able to sustain a level of malonyl-CoA production necessary for survival of herbicide treatment. This was so far the only reported case for this mechanism in a naturally occurring biotype [49].

Inhibitors of Acetolactate Synthase (ALS/AHAS) The enzyme acetolactate synthase (ALS) plays in plants an essential role in branched-chain amino acid biosynthesis. In the pathway leading to valine and leucine, ALS catalyzes the formation of 2-acetolactate from two pyruvate molecules, and in the pathway to isoleucine the formation of 2-acetohydroxybutyrate from 2-ketobutyrate and pyruvate. Due to this double function the enzyme is also called with a more general term acetohydroxyacid synthase. ALS is inhibited by several groups of herbicides, mainly the sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylthiobenzoates(PTBs) and sulfonylaminocarbonyltriazolinone (SCTs) (see Chapter 2.1, M. E. Thompson).

Resistant biotypes being reported in the early 1990s were selected by chlorsulfuron or metsulfuron-methyl in wheat-growing areas or by sulfometuron-methyl in non-crop areas. While resistance of *Lolium rigidum* to ALS-inhibitors was attributed to enhanced herbicide metabolism [50] it was shown, for *Lolium perenne* and dicotyledonous species like *Stellaria media*, *Kochia scoparia*, *Salsola iberica* and *Lactuca serriola*, that resistant biotypes had a mutated ALS with reduced susceptibility to ALS-inhibiting herbicides [51–53]. The IC_{50}s for sulfonylureas, which were determined *in vitro* with ALS isolated from *Stellaria media*, *Salsola iberica* and *Lolium perenne*, increased 4- to 50-fold in the resistant biotypes. Smaller increases, about 2- to 7-fold, were determined in the same biotypes for the imidazolinone herbicide imazapyr [53].

Later ALS-inhibitors were developed for selective use in rice and led to the selection of resistant rice weed biotypes. A biotype of *Monochoria vaginalis*, discovered in Korea, showed high levels of cross-resistance to bensulfuron-methyl, pyrazosulfuron-ethyl and flumetsulam. Resistance factors determined for ALS *in vitro* were 158 to bensulfuron-methyl and 58 to flumetsulam, but only 1.6 to imazaquin [54]. In rice fields in Japan a biotype of *Scirpus juncoides* was selected, which exhibited a high degree of resistance to imazasulfuron (resistance factor

of 271, calculated from ED_{50}s for growth inhibition). Inhibition tests with isolated ALS revealed an IC_{50} of 15 nM for the enzyme from susceptible plants, but of more than 3000 nM for ALS isolated from the resistant biotype, suggesting that resistance was due to an altered ALS enzyme [55].

It appears that reduced sensitivity of the target enzyme is the predominant cause of resistance to ALS inhibitors, and that resistance is conferred by a single, dominant or at least partial dominant, nuclear-encoded gene. Molecular studies revealed that resistance is caused by single substitution of one of five highly conserved amino acids in the ALS enzyme. These are the following (amino acid number standardized to the *Arabidopsis thaliana* sequence): Pro197, Ala122 and Ala205, located at the amino-terminal end, Trp574 and Ser653, located near the carboxy-terminal end [56]. For more details see also Chapter 2.1 (M. E. Thompson).

In the ALS of a *Lactuca serriola* biotype, highly resistant to SUs and moderately resistant to IMIs, Pro197 was substituted by His. The pyruvate binding domain on the ALS enzyme was not found to be altered by the mutation [57]. From *Kochia scoparia* it was reported that several substitutions of Pro197 by another amino acid (Thr, Arg, Leu, Gln, Ser, Ala) will confer resistance to sulfonylureas [58]. In the same species, it was found later that a substitution of Trp574 by Leu will also cause resistance to sulfonylureas and in addition cross-resistance to imidazolinones [59]. The latter substitution was also detected in resistant biotypes of several other dicotyledonous weed species.

In a biotype of *Amaranthus retroflexus* from Israel, resistance was caused by a change of Pro197 to Leu. This biotype exhibited cross-resistance to sulfonylureas, imidazolinones, triazolopyrimidines and to pyrithiobac-sodium *in vivo* and on the ALS enzyme level [60]. In mutations of *Amaranthus rudis*, Ser653 was found to be exchanged by Thr or Asn. These were only resistant to imidazolinones [61].

From the multiplicity of amino acid substitutions it was concluded that the herbicide-binding site of the ALS can tolerate substitutions of each of the five conserved amino acids without major consequences to normal catalytic functions. It was, therefore, speculated that the herbicide-binding site and the active site of ALS are different, though they are probably in close proximity. In absence of herbicide selection, the weed biotypes with mutated ALS showed, in most cases, no reduction or only negligible reduction of fitness (reviewed in Ref. [56]).

Glyphosate Resistance to glyphosate has now appeared in several weed species. In resistant accessions of *Eleusine indica* from Malaysia it was found to be due to point mutations of the target enzyme EPSP synthase. By PCR amplification and sequence analysis of an EPSP synthase fragment an exchange of Pro106 by Ser was found in two resistant accessions, and an exchange of Pro106 by Thr in a third resistant accession [62].

1.3.1.2 Nontarget-site Resistance by Enhanced Metabolic Detoxification

Crop and weed species dispose of enzyme systems that catalyze the metabolic conversion of herbicides. The metabolites, which are usually more polar than the

parent compound, are either non-phytotoxic at all or have a reduced phytotoxic potential. Among the various enzyme systems involved in metabolic herbicide detoxification, two are of particular importance in weeds and crops. One is the cytochrome-P450 monooxygenase system, which catalyzes oxidative transformations of the herbicide molecule (e.g., hydroxylations and oxidative dealkylations). Actually, it is a large enzyme family consisting of multiple cytochrome-P450 monooxygenases with diverse substrate specificities. The other enzyme is the glutathione-S-transferase (GST) family, catalyzing conjugation reactions that result in a nucleophilic displacement of aryloxy moieties, chlorine or other substituents by the tripeptide glutathione. Also the GSTs occur in various isoforms which differ in their catalytic properties.

The herbicide tolerance of crop species has been found to be based frequently on differential rates of metabolic herbicide detoxification in crop and weed species: while rates of herbicide detoxification in the weed species are too low to prevent binding of a lethal herbicide dosage at the target site, the tolerant crop is able to metabolically detoxify the herbicide with such a high rate that binding of the herbicide at the target site in sufficient amounts to cause irreversible herbicidal effects will be prevented. If weed biotypes with an improved ability for herbicide detoxification, comparable to the tolerant crop species, occur in a population they will survive herbicide application and will thus be selected.

To date quite a few weed biotypes have been described for which herbicide resistance was related to enhanced metabolic herbicide detoxification. Several cases have been published for *Lolium rigidum*. An early paper of Christopher et al. reported that excised shoots of biotype SLR 31 from Australia, which was resistant to diclofop, exhibited cross-resistance to the sulfonylureas chlorsulfuron, metsulfuron-methyl and triasulfuron [63]. The metabolite pattern of chlorsulfuron was identical in the resistant biotype and a susceptible standard, but the resistant biotype metabolized the herbicide more rapidly. The pathway of chlorsulfuron detoxification in *Lolium rigidum* was similar to the one described for wheat, ring hydroxylation being followed by glucose conjugation. The time course of chlorsulfuron metabolism in the *Lolium rigidum* biotype SR 4/84 (resistant to diclofop and cross-resistant to chlorsulfuron) was analyzed separately in shoots and roots. The half-life of chlorsulfuron in susceptible plants was longer in the roots (13 h) than in the shoots (4 h) and was reduced in the resistant biotype to 3 and 1 h, respectively. Detoxification of the herbicide by ring hydroxylation, likely catalyzed by a cytochrome-P450 monooxygenase with subsequent glucose conjugation was enhanced in the resistant biotype [50].

Two other *Lolium rigidum* biotypes from Australia (WLR2 and VLR69) developed metabolism-based resistance to PSII inhibitors. WLR2 came from a field with selection pressure by atrazine and amitrole, but never by phenylureas, and VLR69 from a field with selection pressure by diuron and atrazine. Both biotypes were resistant to triazines, and, despite the field selection by atrazine, resistance was more pronounced to the structurally related simazine. Furthermore, both biotypes were resistant to chlorotoluron, though only VLR69 was previously exposed to phenylureas. Analytical work revealed that in both resistant biotypes

metabolism of chlorotoluron and simazine was enhanced, and that the main route of metabolism was via N-dealkylation reactions. This type of reaction and the fact that herbicide metabolism was inhibited by 1-aminobenzotriazole (ABT), an inhibitor of cytochrome-P450 monooxygenases, suggested increased activity of cytochrome-P450 monooxygenases in the resistant biotypes [64, 65]. The mechanism of phenylurea resistance of *Lolium rigidum* biotypes from Spain has been studied [66]: A biotype (R3) selected in the field by applications of diclofop plus isoproturon or plus chlorotoluron had *in vivo* resistance factors (ED_{50} R/ED_{50} S) of about 9.3 and 5.5 to chlorotoluron and isoproturon, respectively, and was also resistant to a broad spectrum of other phenylureas. Metabolism studies with chlorotoluron, in absence and presence of the cyochrome-P450 monooxygenase inhibitor 1-aminobenzotriazole, suggested that resistance was due to enhanced ability to degrade the molecule to non-toxic ring-alkylhydroxylated intermediates suitable for follow-up conjugation reactions. Several biotypes of *Lolium multiflorum* from the UK with resistance to diclofop have been analyzed [34]. While one biotype had an insensitive ACCase, resistance of three other biotypes could be attributed to enhanced metabolism of this herbicide.

The resistance of the grass weed *Phalaris minor* to isoproturon and of the dicotyledonous weed species *Abutilon theophrasti* to atrazine has also been attributed to enhanced metabolism. GST was the enzyme responsible for atrazine detoxification in *A. theophrasti* [67], whereas in *P. minor* the cytochrome P450 monooxygenase was probably involved in the enhanced detoxification of isoproturon [68].

The increasing occurrence of *Alopecurus myosuroides* resistance to herbicides in several European countries prompted research on resistance mechanisms also in this species. Aside from target-site-based resistance, cases of resistance due to enhanced herbicide metabolism had also been reported. Two biotypes, Peldon A1 and Lincs. E1, with *in vivo* resistance factors to isoproturon of 28 and 2.6, respectively, metabolized this herbicide faster than a susceptible standard. The rate of metabolism was higher in Peldon than in Lincs. Addition of the cytochrome-P 450 monooxygenase inhibitor 1-aminobenzotriazole decreased the rate of chlorotoluron metabolism and correspondingly increased phytotoxicity, suggesting the involvement of the cytochrome-P450 monooxygenase system in the detoxification of the herbicide. The major detoxification reaction in these biotypes appeared to be the formation of a hydroxymethylphenyl metabolite [69].

The same biotypes, Peldon A1 and Lincs. E1, are also resistant to the graminicide fenoxaprop, which is used for selective control of *A. myosuroides* and other grass weeds in cereals, mainly wheat. On a whole plant level, Lincs. E1 was more resistant than Peldon A1. The selectivity of this herbicide has been attributed to rapid detoxification by GST-catalyzed conjugation in the cereal species. In both resistant *A. myosuroides* biotypes GST activities towards fenoxaprop were found to be increased to a similar degree, when compared with a susceptible biotype. This was due to increased expression of a constitutive GST and to expression of two novel GST isoenzymes. Furthermore, glutathione levels were increased in the resistant biotypes, in Peldon more than in Lincs. The data pointed to an involve-

ment of GST activity and glutathione levels in the resistance to fenoxaprop, though the lack of correlation to whole plant resistance of these biotypes did not permit definite conclusions [70]. A range of European *A. myosuroides* biotypes with resistance to fenoxaprop has been investigated [71]. Several of these biotypes, in particular one from Belgium, detoxified this herbicide with increased rates. The biotype from Belgium had also the highest GST activity towards the unspecific substrate CDNB, but GST activity towards the herbicide was not tested.

Studies on the mode of inheritance of metabolic herbicide resistance in *Alopecurus myosuroides* did not result in a uniform picture. It was reported that a single gene was responsible for metabolism-based resistance in a biotype resistant to fenoxaprop and flupyrsulfuron [72], while in another biotype resistance to chlorotoluron was attributed to more than one gene [73].

Different to the cases described above, the herbicide propanil is detoxified in rice and weed species by the action of an aryl acylamidase (aryl-acylamine amidohydrolase). High activity of this enzyme in rice confers crop tolerance. In Colombia, a biotype of *Echinochloa colona* was found that is resistant to propanil. Enzyme tests with extracts from this biotype revealed an about three-fold higher activity of aryl acylamidase in the resistant than in a susceptible biotype. It was concluded that resistance of the *E. colona* biotype is based on enhanced propanil detoxification [74].

1.3.1.3 **Nontarget-site Resistance by Altered Herbicide Distribution**

Cases of nontarget-site resistance by altered herbicide distribution have been reported for two important herbicides, paraquat and glyphosate.

Intensive use of the herbicide paraquat has resulted in the evolution of resistance in various weed species. Intensive research on the resistance mechanisms was mainly carried out with resistant biotypes from *Hordeum* spp. and *Conyza* spp., and altered distribution of the herbicide in the resistant weeds was suggested as the cause – or at least the partial cause – of resistance. In resistant *Conyza canadensis* it was supposed that a paraquat inducible protein may function by carrying paraquat to a metabolically inactive compartment, either the cell wall or the vacuole. This sequestration process would prevent the herbicide from getting in sufficient amounts into the chloroplasts as the cellular site of paraquat action. Inhibitors of membrane transport systems, e.g., *N*,*N*-dicyclohexylcarbodiimide (DCCD), caused a delay in the recovery of photosynthetic functions of the paraquat-resistant biotype, when given after the herbicide. These transport inhibitor experiments supported the involvement of a membrane transporter in paraquat resistance [75].

Translocation studies with two paraquat-resistant biotypes of *Hordeum leporinum* revealed that the basipetal transport of paraquat in resistant *H. leporinum* was much reduced compared with susceptible plants. It was concluded that the resistance to paraquat was the result of the reduced herbicide translocation out of the treated leaves [76]. One can suppose that also in this species herbicide sequestration may have been the primary cause for the altered long-distance transport.

Independent populations of *Lolium rigidum* with resistance to glyphosate have been reported from different locations in Australia. One of them, with a ca. 10-fold *in vivo* resistance to glyphosate, was used for intensive investigation of the mechanism of resistance. Neither a modification of the target enzyme EPSP synthase nor of herbicide metabolism contributed to the resistance in this case. Translocation studies after foliar application revealed, however, that in the resistant biotype glyphosate accumulated preferentially in the leaf tips, while in susceptible plants accumulation was stronger in the leaf bases and the roots. This result suggested a shift of glyphosate transport in the resistant plants from the phloem to the xylem system. It was speculated that the resistant biotype might have lost in efficiency to load glyphosate into the symplast. Thus more of the herbicide would remain in the apoplast and be translocated acropetally with the transpiration stream, while the concentration of glyphosate in the plastids of the sensitive meristematic tissues at the shoot base and in the roots would be reduced [77].

1.3.1.4 **Multiple Resistance**

As defined above, multiple resistance means that more than one resistance mechanism occurs in a weed population or an individual plant. This can either mean that a target site-based and a nontarget-site based mechanism occur in the same biotype, or that a biotype is resistant to herbicides with different mechanisms of action. Multiple resistance can result in resistance of a weed biotype to a very broad range of herbicide chemistries. Multiple resistance has been reported for several weed species, particularly for *Lolium rigidum*, *Alopecurus myosuroides*, *Kochia scoparia*, *Conyza canadensis* and *Amaranthus rudis*. It developed to a serious extent particularly in Australian biotypes of *Lolium rigidum*, probably as a result of agricultural conditions paired with biological characteristics of this weed (cross pollinating species with high genetic variability and seed production and high plant numbers per area).

Multiple resistance can develop by selection with a single herbicide or by selection with several herbicides, which are either used sequentially or simultaneously. Furthermore, cross-pollinating species can become multiple resistant, when two individuals, each with a different resistance mechanism, cross. An example for the selection of multiple resistance by a single herbicide (the ALS inhibitor chlorsulfuron) is the *Lolium rigidum* biotype WLR1. This biotype had as main mechanism of resistance an ALS with reduced sensitivity to chlorsulfuron, sulfometuron and imazamethabenz, and as additional mechanism enhanced metabolism of chlorsulfuron [78]. Extreme cases of multiple resistance, due to an application history of many herbicides, were reported from Australia for several *Lolium rigidum* biotypes. As an example, biotype VLR69 possessed the following mechanisms: enhanced metabolism of ACCase-inhibiting herbicides, resistant form of the ACCase enzyme, enhanced metabolism of the ALS-inhibitor chlorsulfuron, and in addition a resistant form of the ALS enzyme in 5% of the population [36].

Selection of multiple resistance after sequential use of different herbicides has been described for a biotype of *Kochia scoparia* from North America. Many years

of triazine usage resulted in the selection of a biotype with target-site resistance of the D1 protein in photosystem II. By subsequent usage of ALS inhibitors, a point mutation in the gene encoding for ALS was selected in addition, which made this biotype also target-site-resistant to sulfonylureas and imidazolinones [59].

Obviously, multiple resistance leads to complex patterns of broad herbicide resistance, particularly in cross-pollinating weed species. This seriously restricts the remaining options of chemical weed control in agricultural practice.

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2
Acetohydroxyacid Synthase Inhibitors (AHAS/ALS)

2.1
Biochemistry of the Target and Resistance

Mark E. Thompson

2.1.1
Acetohydroxyacid Synthase (AHAS)

The first committed step in the biosynthetic pathway of the branched chain amino acids is catalyzed by the enzyme acetohydroxyacid synthase (AHAS, EC 2.2.1.6), which is also referred to as acetolactate synthase (ALS). As depicted in Fig. 2.1.1, the pathway leading to valine and leucine begins with the condensation of two molecules of pyruvate accompanied by loss of carbon dioxide to give (*S*)-2-acetolactate. A parallel reaction leading to isoleucine involves the condensation of pyruvate with 2-ketobutyrate to afford (*S*)-2-aceto-2-hydroxybutyrate after loss of carbon dioxide. Both reactions are catalyzed by AHAS, which requires the cofactors thiamin diphosphate (ThDP) and flavin adenine dinucleotide (FAD). A divalent metal ion, most commonly Mg^{2+}, is also required. Several excellent reviews of AHAS have appeared that describe the biochemistry, genetics, inhibition, and active site modeling of the enzyme [1–4].

Many authors have used the acronym ALS to refer to the enzyme that catalyzes the reaction of Fig. 2.1.1. However, since ALS refers specifically to the pathway leading to valine and leucine through the intermediate (*S*)-2-acetolactate, the designation AHAS better describes all products of the reaction. AHAS is present in bacteria, fungi, and plants. Many of the early kinetic, mechanistic, and structural studies were carried out with AHAS isolated and purified from enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*. Eukaryotic AHAS has proven more difficult to isolate and purify because of its reduced stability. Three AHAS isozymes have been characterized in bacteria – AHAS I, II, and III – whereas only one isozyme is known in fungi and plants.

Figure 2.1.2 shows the mechanism of the AHAS reaction [5, 6]. The first step involves removal of the proton attached to the 2-carbon atom of the thiazolium ring of ThDP to form an ylide. This ionization is followed by addition of the thiazolium 2-carbanion to the carbonyl group of pyruvate to give lactyl-ThDP, which

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

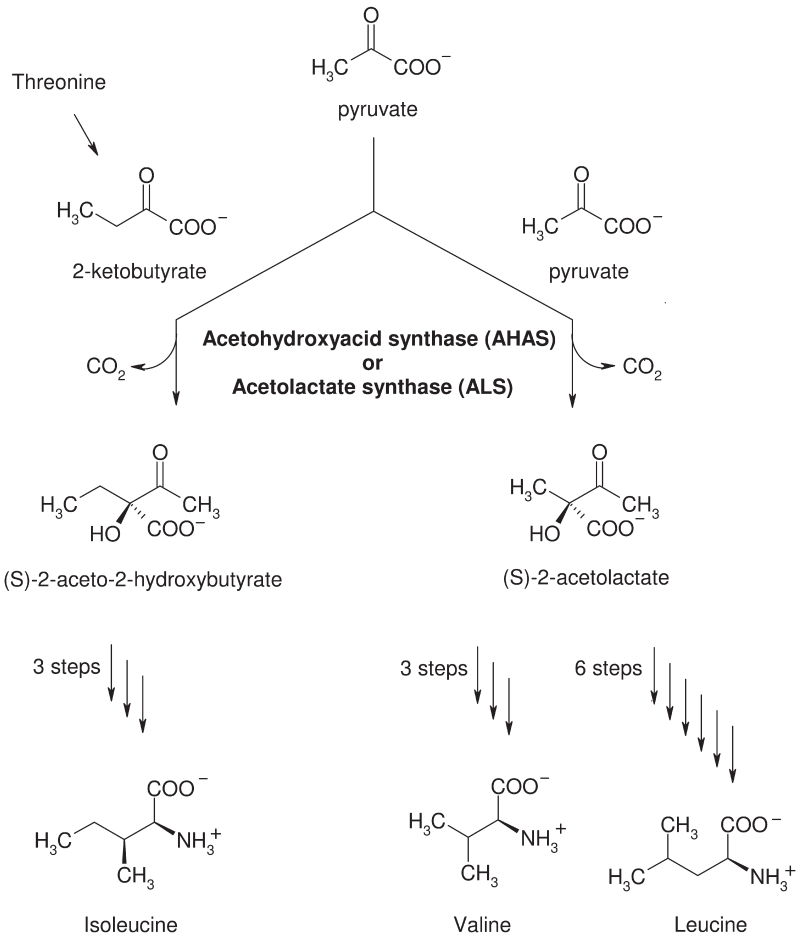

Fig. 2.1.1. Branched chain amino acid biosynthetic pathway.

loses carbon dioxide to generate the intermediate, hydroxyethyl-ThDP (HEThDP). The deprotonation is the only common step for all ThDP-dependent enzymes, and crystallographic studies combined with site-directed mutagenesis have identified a highly conserved glutamate residue as a key feature in ThDP catalysis [7]. NMR spectroscopic analysis of ThDP-ylide formation in yeast pyruvate decarboxylase (PDC) has provided convincing evidence that interaction of the glutamate with the 1′-nitrogen atom of the ThDP pyrimidine ring activates the 4′-amino group to facilitate removal of the thiazolium ring C-2 proton in an intramolecular process [8]. The exact details of the AHAS mechanism have not been completely resolved, especially with respect to the formation of the reactive ylide in the first step and whether it is a discrete or concerted process.

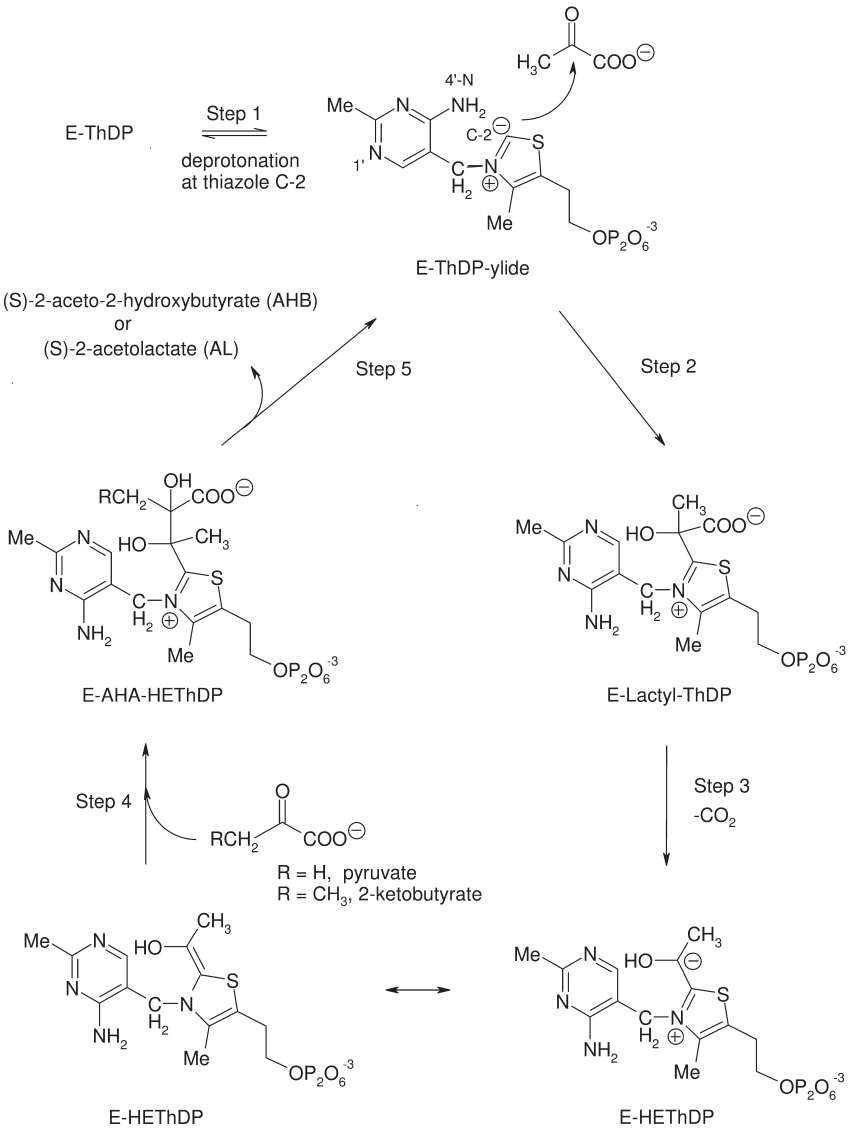

Fig. 2.1.2. Mechanism of the AHAS-catalyzed reaction.

The HEThDP intermediate reacts with either a second molecule of pyruvate or with 2-ketobutyrate to give acetolactate (AL) or acetohydroxybutyrate (AHB), respectively. In-depth studies of the kinetics of this reaction have been conducted on *E. coli* AHAS III [9]. Comparison of these results with earlier data for AHAS I and II indicate that all three bacterial isozymes catalyze similar mechanisms. However, AHAS II and III show a much more pronounced preference for reac-

tion with 2-ketobutyrate in step 4. The substrate specificity in step 4 is an intrinsic property of the enzyme and is unaffected by pH or feedback inhibitors such as valine [10]. Recent work using a new NMR method for detecting covalent reaction intermediates has enabled the calculation of microscopic rate constants for both wild-type and mutant AHAS II from *E. coli*. These studies showed that addition of the ThDP C-2 anion to pyruvate is the rate-limiting step and that all other steps in the reaction are comparatively fast [6, 11].

The dependence of AL formation on pyruvate concentration in all three bacterial AHAS isozymes obeys Michaelis–Menten kinetics in the absence of 2-ketobutyrate. Such behavior implies that there is an irreversible step between the addition of the first and second pyruvate molecules [9, 12, 13]. Steady-state experiments confirmed that the rate-determining and product-determining steps in the mechanism are different. The observation that a wide range of substrate concentrations, changes in pH, or the presence of feedback inhibitors do not affect the specificity of the enzyme supports the idea that the first steps in the mechanism – preceding the binding of the second substrate – are rate determining [9]. Modulation of these first steps would be expected to affect the turnover rate of the enzyme without affecting the choice of products.

The role of FAD in the AHAS reaction is not fully understood since no oxidation or reduction occurs. Several hypotheses have been put forth, but so far no experimental evidence has conclusively supported any single explanation. One possibility is that FAD plays a structural role only and is likely an evolutionary remnant from a pyruvate oxidase (POX)-like ancestor [14]. The divalent metal ion does not play a direct role in the reaction, but serves to anchor the ThDP molecule to the protein by coordinating the diphosphate group and certain amino acid side chains [15].

The genes *ilv*BN, *ilv*GM, and *ilv*IH, which code for *E. coli* AHAS I, II, and III, respectively, have been cloned and sequenced [16–19]. Use of bacterial clones has enabled the production of significant quantities of each isozyme and purification essentially to homogeneity. The bacterial holoenzymes are heterotetramers composed of two types of subunits: Two large, identical catalytic subunits of approximately 60 kDa, which contain all of the catalytic machinery, and two small, identical regulatory subunits of molecular weight 9–17 kDa [12, 20, 21]. The catalytic subunit alone possesses low or no activity, but reconstitution *in vitro* with the regulatory subunit restores full enzymatic activity [22]. While AHAS I and III of *E. coli* are regulated by feedback inhibition from branched chain amino acids, especially valine, isozyme II is insensitive. The binding sites for the branched chain amino acid feedback modulators are located in the AHAS regulatory subunits [23].

Cloning and sequencing of the yeast AHAS gene, *ilv2*, and comparison of the amino acid sequence with that of *E. coli* AHAS showed a great deal of homology [24]. However, the subunit composition of eukaryotic AHAS is not as well characterized. In *Saccharomyces cerevisiae*, the *ilv2* gene encodes a peptide that is homologous to the bacterial large (catalytic) subunit. Expression of *ilv2* in *E. coli* gave a

putative catalytic subunit, which showed considerably diminished enzymatic activity after isolation and purification [25]. Expression of the *ilv6* gene from *S. cerevisiae* in *E. coli* and reconstitution with the yeast large subunit substantially enhanced the catalytic activity and conferred sensitivity to valine inhibition, thereby providing the first evidence for a eukaryotic small (regulatory) subunit [26].

Early studies in plants demonstrated that AHAS is a nuclear-encoded, chloroplast-localized enzyme [27, 28]. The first genes from higher plants encoding AHAS were isolated from *Arabidopsis thaliana* and *Nicotiana tabacum* using a yeast AHAS gene as a heterologous hybridization probe [29]. Comparison of the DNA and amino acid sequences from the two plants showed approximately 70% nucleotide homology and 85% homology in the mature proteins. Alignment of the plant DNA sequences with those from *E. coli* and yeast showed many regions of high homology interspersed with regions of divergence. One region of low homology between the plant proteins occurred in the first 85 amino acids, which were presumed to comprise N-terminal chloroplast transit peptides.

The first isolation of a regulatory subunit from plant AHAS was reported by Hershey et al. [30]. A cDNA clone was used to express the peptide from *Nicotiana plumbaginifolia* in *E. coli*. Based on homology with various bacterial AHAS small subunits and the observation of enhanced enzymatic activity when reconstituted with the large subunit of AHAS from either *N. plumbaginifolia* or *A. thaliana*, the authors concluded that they had isolated the regulatory subunit. Identification of the regulatory subunit of *A. thaliana* was subsequently reported by Lee and Duggleby [22], who showed that *in vitro* reconstitution not only enhanced the activity of the catalytic subunit, but also conferred sensitivity to regulation by all three branched-chain amino acids.

Several early structural models of AHAS were proffered based on homology to other known ThDP-dependent enzymes, such as POX from *Lactobacillus plantarum*, and carefully planned site-directed mutagenesis studies. Many of the features of the early models were borne out by the first X-ray crystal structure at 2.6-Å resolution of the dimeric catalytic subunit of AHAS from *S. cerevisiae* [15, 31]. Pang et al. thus confirmed that AHAS shares many structural features in common with other ThDP-dependent enzymes. Figure 2.1.3 depicts one of the monomers of the AHAS protein and the organization of the α-, β-, and γ-domains. The positions of the cofactors, ThDP, FAD, and Mg^{2+} are clearly defined. In this representation, the second ThDP molecule has been included to show the location of the enzyme active site, which is formed at the interface of the α-domain of one monomer and the γ-domain of the second monomer. The crystal structure shows that the active site without bound substrate or inhibitor is quite accessible to solvent. The two rings of ThDP are held in a bent conformation by interactions with Met525, Met555, Tyr113, Gly523, and Ala551 residues and by two hydrogen bonds [32]. ThDP is anchored to the γ-domain through its phosphate groups, which interact with the Mg^{2+} and several amino acids. FAD is most closely associated with, and binds with high affinity to, the β-domain. The cofactor is secured through numerous hydrogen bonds and van der Waals

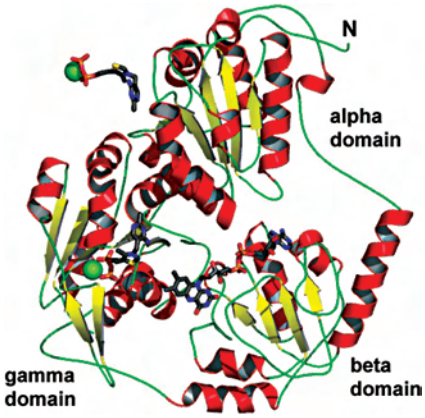

Fig. 2.1.3. Structure of a single monomer of the yeast AHAS catalytic subunit. ThDP and FAD molecules are represented as stick models. Mg^{2+} anchored to the ThDP is shown as a green sphere. ThDP of the γ-domain of the partner subunit has been added to more clearly illustrate the position of the enzyme active site at the interface of the dimer. (Reproduced with modification from Ref. [15], Figure 3, with permission from the publisher, Elsevier.)

interactions in an extended planar conformation [25, 32]. The crystal structure sheds no light on the functional role of FAD in the AHAS reaction, but does indicate that the cofactor appears to be too far removed from ThDP to be a direct participant.

2.1.2
Herbicides that Target AHAS

The discovery that certain synthetic small organic compounds inhibit AHAS and cause plant death has contributed significantly to the attention garnered by this enzyme. The first class of herbicides known to inhibit AHAS was the sulfonylureas (SUs) [33, 34]. The first commercial example of a sulfonylurea was chlorsulfuron, which was introduced by DuPont in 1982 under the trade name Glean®. This product provided highly effective control of dicotyledonous weeds in postemergence applications with excellent selectivity toward wheat [35]. Almost simultaneously, researchers at American Cyanamid discovered a structurally distinct family of herbicides, the imidazolinones (IMIs), which were also shown to inhibit the AHAS enzyme [36, 37]. Since then, three additional classes of AHAS-inhibiting herbicides have been discovered and commercial products introduced: triazolopyrimidines (TPs) from Dow AgroSciences [38], pyrimidinyl(thio)benzoates from Kumiai [39, 40], and sulfonylaminocarbonyltriazolinones from Bayer CropScience [41]. The AHAS inhibitors have proven to be among the most successful and widely used herbicides, with more than 50 active ingredients

commercialized to date. This tremendous success can be attributed to the compounds' generally high bioefficacy, low field application rates, selectivity to many agronomically important crops, favorable environmental profiles, and ultra-low mammalian toxicity [42].

Early mode-of-action studies on the SUs revealed that treatment of plants with chlorsulfuron resulted in rapid cessation of cell division [43]. A further key observation in the laboratory was that growth inhibition of *S. typhimurium* caused by the SU herbicide, sulfometuron methyl, could be reversed by the addition of isoleucine, which pointed to the branched chain amino acid pathway as the biological process that was being disrupted. The mode of action of SUs in bacteria was confirmed by LaRossa and Schloss who showed that AHAS activity in extracts from wild-type *S. typhimurium* was completely inhibited by sulfometuron methyl [44]. These findings were subsequently confirmed in plants by Ray, who reported I_{50}s for chlorsulfuron inhibition of the AHAS enzyme from several plant species ranging from 18.5 (wheat) to 35.9 nM (Johnsongrass) [45]. Chaleff and Mauvais isolated tobacco mutants resistant to chlorsulfuron and sulfometuron methyl from tissue cell cultures and conclusively demonstrated that inhibition of the AHAS enzyme was the mode of herbicidal action in plants [46]. At about the same time, Shaner et al. reported that the phytotoxic effects of three IMI herbicides on corn tissue culture could be reversed by addition of valine, leucine, and isoleucine. The authors also showed that IMIs were potent inhibitors of the AHAS enzyme from *Zea mays*, with K_i values ranging from 1.7 to 12 μM [36].

2.1.3
Binding Site for AHAS-inhibiting Herbicides

The inhibition of AHAS by herbicidal compounds is a time-dependent process that is complex and not well understood [4, 47]. Since AHAS II is most similar to the enzyme in higher plants with respect to its sensitivity to various herbicides, most enzymological studies on the effects of synthetic small molecule inhibitors have been carried out on that isozyme [14, 48]. Early experiments showed that sulfometuron methyl exhibited slow, tight-binding inhibition of AHAS II from *S. typhimurium* with an initial, apparent K_i of 1.7 μM (50 mM pyruvate), followed by a time-dependent increase in potency to a final, steady-state K_i of 82 nM. The final, steady-state rate in the presence of excess herbicide indicated that the inhibition process is reversible. Although sulfometuron methyl binds to AHAS II in the absence of pyruvate, it only forms the reversible, tight-binding complex observed under turnover conditions and is competitive with pyruvate at both initial and final inhibition levels. Thus, pyruvate is required for the slowly reversible form of inhibition, but competes with sulfometuron methyl for binding to the enzyme. This observation has been explained in the context of the AHAS reaction mechanism by assuming that sulfometuron methyl binds most tightly to the enzyme following addition of the ThDP-ylide to the first molecule of pyruvate and decarboxylation (Fig. 2.1.2, steps 1–3) [49]. Evidence in support of this hypothesis was obtained from chemical quench experiments with AHAS II using ^{14}C-labeled

pyruvate and ThDP, which showed that the level of HEThDP obtained by quenching steady-state reaction mixtures increased in the presence of sulfometuron methyl. Thus, while the SU virtually eliminated the enzymatic reaction, it increased the level of the HEThDP intermediate by inhibiting the binding and condensation of the second molecule of pyruvate [50].

The IMI herbicides also exhibit complex interactions with AHAS. When enzyme activity was measured over an extended period in the presence of various concentrations of imazapyr, inhibition increased with time, thereby suggesting that the equilibrium between the herbicide and AHAS was reached slowly, a characteristic of tight-binding inhibitors [51]. In contrast to SUs, substrate–inhibitor studies suggested that inhibition by imazapyr is uncompetitive with respect to pyruvate, which implies that the synthetic molecule binds to AHAS only after formation of the ternary enzyme–pyruvate–ThDP complex [52]. However, noncompetitive binding has also been reported for the IMIs, which underscores the complexity of the kinetics of AHAS inhibition [49].

The lack of obvious structural similarities among the AHAS inhibitors and the substrates or intermediates in the reaction catalyzed by AHAS suggested early on that the herbicides might not bind at the active site of the enzyme. Furthermore, the lack of obvious structural similarities among the different classes of AHAS-inhibiting herbicides, coupled with the differences in binding kinetics, has led to the speculation that the various classes of herbicides bind to different, albeit overlapping sites in the enzyme [4]. Because of the similarities between AHAS and POX, and the fact that the former enzyme requires FAD even though the reaction it catalyzes does not involve oxidation or reduction, Schloss et al. proposed that the SUs bind at a site that is distinct from the active site and is an evolutionary vestige of the ubiquinone binding site [14].

Early attempts to elucidate the herbicide binding site of AHAS were based on the similarity of AHAS to other ThDP-dependent enzymes for which X-ray crystallographic data existed. For example, a herbicide binding site structural model was postulated on the basis of homology between AHAS and POX, and an IMI molecule was positioned in the binding pocket using structure–activity information [53]. A significant milestone that greatly advanced the state of knowledge of the AHAS herbicide binding site was the publication by Pang et al. of the crystal structure at 2.8-Å resolution of yeast AHAS bound with chlorimuron ethyl, a commercial SU herbicide that is a potent inhibitor of the enzyme [54]. This crystal structure showed that the overall features of the AHAS•SU complex are quite similar to those of the free enzyme. The location and bent conformation of ThDP, which defines the enzyme active site at the interface of the α-domain of one monomer and the γ-domain of the second monomer, remain essentially unchanged vis-à-vis the free enzyme. Chlorimuron ethyl is positioned near FAD, which is in the same general location as in the free enzyme. However, the flavin ring of FAD has been displaced by several angstroms to avoid unfavorable steric interactions with the herbicide molecule.

One noteworthy difference between the crystal structures of the AHAS•SU complex and the free enzyme is that the volume of the protein in the region in

which the active and herbicide binding sites are located has been reduced. A second, more significant difference is that a new substrate access channel has been formed in the AHAS•SU complex as a result of the ordering of two relatively short sequences of amino acids near the active and herbicide binding sites. As a result, ThDP exposure is substantially reduced with only the C-2 position of the thiazolium ring readily accessible to solvent. Numerous hydrophobic interactions between chlorimuron ethyl and amino acid residues along with four hydrogen bonds to the molecule's "bridge" ($-SO_2NHCONH-$ moiety) anchor the SU in the substrate access channel in such a way that the herbicide completely blocks access to the enzyme active site. The authors showed through molecular modeling that a cavity in the herbicide binding site that is normally occupied by a single water molecule will accommodate the reaction intermediate, HEThDP, with no unfavorable interactions and a stabilizing hydrogen bond to the 4′-amino group of the ThDP. This structural feature is consistent with the hypothesis that SUs bind most tightly to the AHAS enzyme following addition of the ThDP-ylide to the first pyruvate molecule and decarboxylation.

Crystal structures of four additional SUs bound to yeast AHAS were subsequently published by McCourt et al. [55]. Figure 2.1.4 shows the chemical structure of chlorsulfuron and key contact points with various amino acids in the binding site. While the conformations of all four bound SUs were similar, the authors were able to relate certain differences to structural features of the molecules and their respective binding affinities. For example, structure–activity studies had previously demonstrated the importance of the substituent in the *ortho*-position of the phenyl ring adjacent to the SU bridge for optimal herbicidal activity [34]. In the case of chlorsulfuron, this substituent is chloro. The crystal structures show that the chlorine atom of chlorsulfuron does not fit as tightly into the binding site as the carboxylic ester *ortho* groups of the other three SUs. This observation could account for the 39-fold lower binding affinity of chlorsulfuron for yeast AHAS and would be consistent with the earlier finding that the size of the *ortho* group is the most important attribute in determining the potency of SU inhibition of the enzyme [56]. The four SUs in this study differ somewhat in the nature of the extensive hydrophobic interactions that each makes with the highly conserved amino acid side-chains lining the substrate access channel. Mutation of several of the residues disrupts those interactions and has previously been shown to confer resistance to the four SUs although there was considerable variability [57]. The authors point out that, of the 13 amino acid residues making contact with the SUs, 11 are highly conserved across bacterial, fungal, and plant AHAS sequences, thereby suggesting that they play important roles in the AHAS reaction.

Twenty-two years after the introduction of the first commercial AHAS inhibiting herbicide, a preliminary crystal structure at 3.0-Å resolution of the AHAS catalytic subunit from a plant, *A. thaliana*, was published by Pang et al. [58]. This was followed two years later by crystal structures of the same enzyme complexed with five SUs and one IMI at 2.5- and 2.8-Å resolution, respectively [59]. The latter achievement by McCourt et al. represented the first reported X-ray crystal

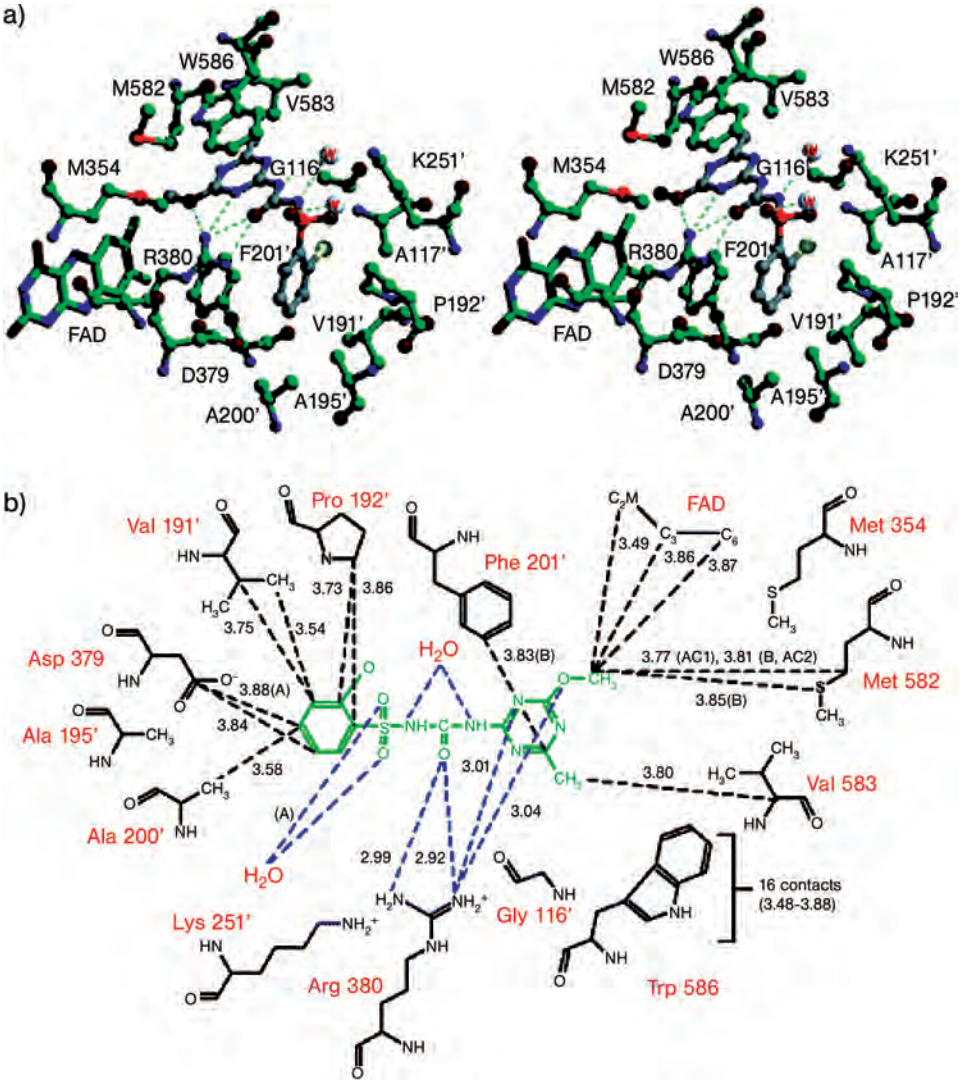

Fig. 2.1.4. Chlorsulfuron in the herbicide binding site of yeast AHAS. (a) The herbicide and nearby amino acids are shown as ball-and-stick models. The isoalloxazine ring of FAD is shown as a stick model. (b) Key contact distances (a) from chlorsulfuron to nearby amino acids. Hydrophobic contacts are broken black lines and hydrogen bonds are broken blue lines. Prime numbers denote residues from the other monomer. (Reproduced from Ref. [55], Figure 5, with permission from the publisher, American Chemical Society.)

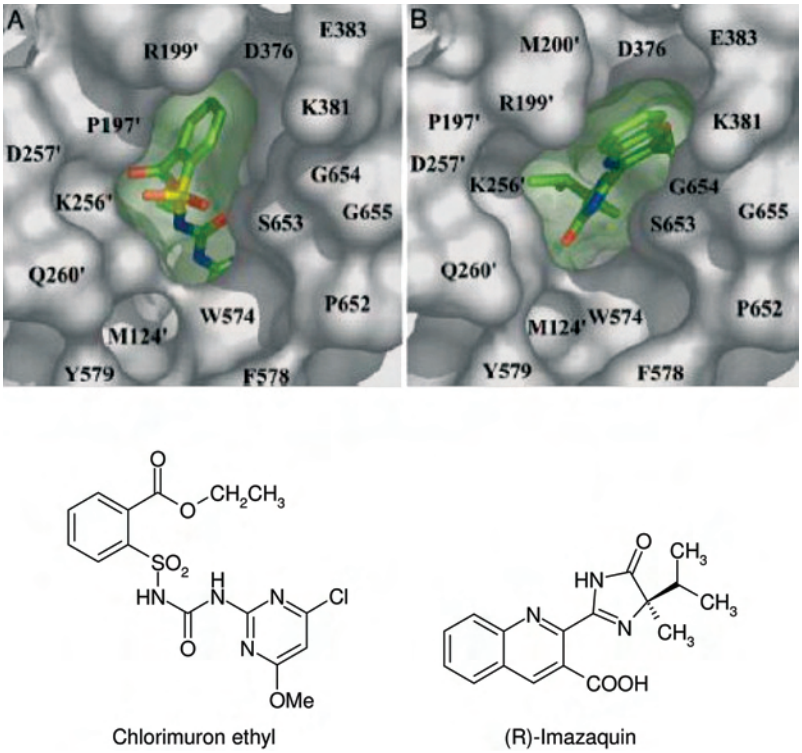

Fig. 2.1.5. Herbicides bound to the AHAS protein of *Arabidopsis thaliana*, showing blockage of the channel leading to the enzyme active site. The herbicides are shown as stick models and the residues lining the channel are gray surfaces. Prime numbers denote residues from the other monomer. (Reproduced from Ref. [59], Figure 3, with permission from the publisher, National Academy of Sciences, USA.)

structures of plant protein–herbicide complexes. The overall structural features of the AHAS•SU and AHAS•IMI enzymes are similar, including a region consisting of short amino acid sequences in the vicinity of the active site that the authors hypothesize becomes more ordered in the presence of the inhibitors to form the substrate access channel in analogy to yeast AHAS. In addition, the conformations of the SU molecules bound to the plant AHAS are similar to those in yeast.

Figure 2.1.5(A) shows chlorimuron ethyl bound in the AHAS substrate access channel. The phenyl ring is located at the entrance to the channel with both the ortho-carboxylic ester group and the SU bridge pointing toward the enzyme active site. The pyrimidine ring is barely visible and is inserted deeper into the active site. Key interactions with several amino acid residues are apparent, including Trp574, Pro197, and one that is not present in yeast, Ser653. Figure 2.1.5(B) shows the IMI imazaquin positioned in the AHAS substrate access channel, with the imidazolinone ring directed toward the enzyme active site, as is the car-

boxylate substituent in the 3-position of the quinoline ring. Although racemic imazaquin was used in the crystallization, only the (R)-enantiomer was observed bound to the enzyme, which is consistent with the known higher herbicidal efficacy of this isomer versus the (S)-enantiomer [52].

The crystal structures provide excellent insight into the known higher AHAS binding affinity of SUs versus IMIs. For example, the apparent K_i values for inhibition of AHAS from *A. thaliana* for chlorimuron ethyl and chlorsulfuron are 10.8 and 54.6 nM, respectively, while that for imazaquin is 3 μM [60]. These differences can be attributed to the significantly greater number of van der Waal contacts and hydrogen bonds between the SUs and the enzyme versus imazaquin, and by the fact that the SUs are positioned closer to ThDP in the active site. While many of the residues interacting with the SUs and imazaquin are the same, there are six that only make contact with SUs and two that interact only with imazaquin. Thus, the crystal structures confirmed earlier suppositions that the two classes of herbicides occupy partially overlapping, but different, binding sites.

2.1.4 Molecular Basis for Resistance to AHAS Inhibitors

Several plants and cultured plant cells resistant to AHAS-inhibiting herbicides have been generated using both conventional mutation breeding and tissue culture cell selection. Mutants resistant to chlorsulfuron and sulfometuron methyl were first isolated from cultured cells of *Nicotiana tabacum* that were grown in the presence of one of the herbicides [61]. Crosses of fertile plants from several isolates established that resistance resulted from single dominant or semidominant nuclear mutation and the isolates were cross-resistant to both compounds. Co-segregation of resistance to the herbicides demonstrated that both resistances resulted from the same, or closely linked, mutations. Tobacco plants regenerated from the sulfometuron methyl-derived mutant cell lines showed resistance to high concentrations of chlorsulfuron.

Cloned yeast and bacterial genes were used to investigate the molecular basis for resistance to the SU herbicides [62]. Spontaneous mutations that conferred resistance to sulfometuron methyl were obtained in cloned genes for AHAS from *S. cerevisiae* and *E. coli*. The DNA sequences of the mutant AHAS genes showed single nucleotide differences from their wild-type counterparts, resulting in single amino acid substitutions in the corresponding proteins. The yeast mutant, Pro192Ser, resulted in reduced levels of enzyme activity, reduced sensitivity to sulfometuron methyl, and unaltered resistance to feedback inhibition from valine. The bacterial mutant, Ala26Val, resulted in unaltered levels of enzyme activity, greatly reduced sensitivity to sulfometuron methyl, and slightly reduced sensitivity to valine.

In yeast AHAS, spontaneous mutations at ten separate sites have each been shown to confer resistance to SUs [63, 64]. The X-ray crystal structure of yeast AHAS bound with chlorimuron ethyl revealed that nine of those residues make direct contact with the herbicidal molecule [54]. The authors studied the effects

of several mutations on SU sensitivity in the context of molecular interactions in the herbicide active site. For example, the crystal structure showed that the indole ring of Trp586 is involved in aromatic π-orbital stacking interactions with the pyrimidine ring of chlorimuron ethyl. The mutation Trp586Leu in yeast AHAS is known to result in a >6000-fold reduction in sensitivity to chlorimuron ethyl, which can be understood in terms of the total disruption of the aromatic ring interactions.

A systematic study was carried out in which ten active mutants of yeast AHAS were constructed by mutagenesis and the resultant enzymes evaluated for their resistance to six SU and three IMI herbicides. The results were interpreted in terms of the herbicide binding site that was revealed by the X-ray crystal structure of AHAS from *S. cerevisiae* [57]. All ten mutants were resistant to some degree to the six SUs, although the levels of resistance spanned a range of nearly 10^4 and there was considerable variability in several mutants. The most consistent and highest levels of resistance were observed with Trp586Leu. The Pro192Ser mutant also displayed relatively high levels of resistance to all six Sus, and the crystal structure of yeast AHAS supports this observation in that Pro192 interacts with the phenyl rings of all of the bound herbicides. Eight of the mutants were resistant to the IMI, imazethapyr, although several of these were only barely affected and Asp379Asn was more sensitive than the wild-type enzyme. As represented schematically in Fig. 2.1.6, the positions in AHAS from various sources where

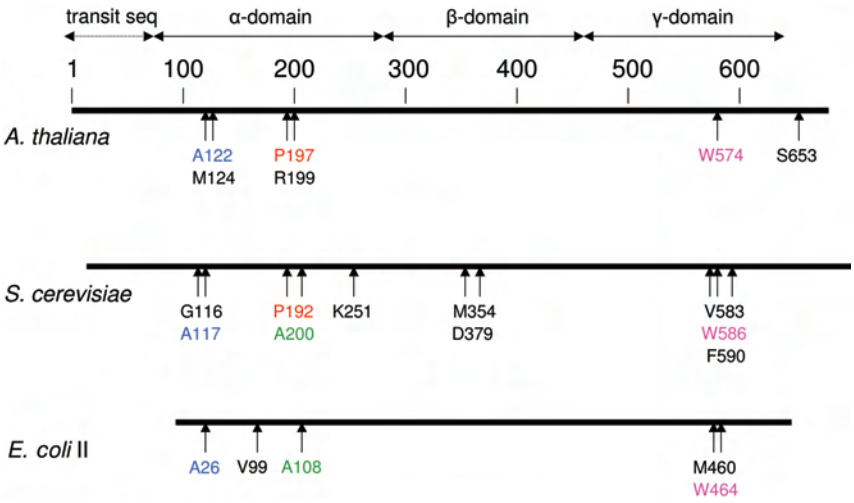

Fig. 2.1.6. AHAS mutations conferring herbicide resistance. Arrows point to positions in the sequences of AHAS from plant (*Arabidopsis thaliana*), yeast (*Saccharomyces cerevisiae*), and bacterial (*Escherichia coli*, isozyme II) sources where spontaneous or induced mutations result in an herbicide-insensitive enzyme. Colors designate substitutions occurring in more than one species.

mutations are known to confer resistance to one or more herbicides are distributed across the three domains of the protein [4, 32, 65]. At some sites, virtually any amino acid substitution confers resistance, while at others only a few substitutions are permitted.

Genes that specify herbicide-resistant forms of the AHAS enzyme were isolated from mutant *N. tabacum*, sequenced, and characterized [66]. The authors showed that a single amino acid change, Pro196Gln, in one of two distinct tobacco AHAS genes conferred resistance to SU herbicides. In the other tobacco AHAS gene, a double mutation of Pro196Ala and Trp573Leu resulted in significantly enhanced resistance to SUs. Transgenic plants carrying these mutant genes were highly resistant to chlorsulfuron treatments. Hattori et al. later showed that a single mutation in plant AHAS can confer resistance to multiple classes of AHAS-inhibiting herbicides [67, 68]. Thus, tobacco lines transformed with a plant AHAS gene specifying a single mutation, Trp557Leu, were strongly resistant to SUs, TPs, and IMIs. The tryptophan residue is conserved in virtually all wild-type AHAS proteins.

2.1.5 Resistance to AHAS-inhibiting Herbicides in Weeds

Certain characteristics of the AHAS target have played roles in the development of weeds resistant to herbicides that inhibit the enzyme: Target-based resistance is inherited as a single, semidominant trait that is carried on a nuclear gene; AHAS is the single site of action; there are multiple sites in AHAS that can be mutated to confer resistance; and mutant AHAS enzymes can possess full catalytic activity, which leads to resistant weeds that are fit [69]. Certain characteristics of the molecules themselves also contributed to resistance development, such as their potency and the relatively long soil residual of some of the early products. The first examples of resistance to chlorsulfuron occurring in the field were discovered in 1987 in the U.S. The fields containing those resistant weeds had been treated continuously with chlorsulfuron for five years. By 1992, there were numerous examples of weeds that had developed resistance to AHAS-inhibiting herbicides. Many additional observations of weeds resistant to AHAS-inhibiting herbicides have been reported since then, and there are now 93 known weed species with confirmed resistance to one or more of the five chemical classes of these compounds [70]. In 1998, the AHAS-inhibiting herbicides surpassed all other classes of herbicides in terms of the number of weed species for which at least one resistant population had been reported. At least two excellent reviews of resistance in weeds to AHAS-inhibiting herbicides have appeared [71, 72].

Studies have shown that AHAS resistance-conferring mutations can have subtle effects on plant growth and development, but they do not consistently reduce plant fitness. For example, the catalytic efficiencies of AHAS enzymes isolated from both resistant and susceptible biotypes of *L. serriola*, *K. scoparia*, *S. iberica*, *S. media*, and *L. perenne* have been shown to be virtually identical [71, 73].

In weed biotypes where the mechanism of evolved resistance has been

confirmed, the majority has been due to reduced sensitivity of AHAS to the herbicide. One exception is *Lolium rigidum*, which first developed metabolism-based resistance to the ACCase inhibitor, diclofop-methyl, and then showed metabolism-based cross resistance to SUs and other classes of herbicides [74]. The identities of specific mutations in weed species that had evolved resistance to AHAS inhibitors were first determined by Guttieri et al. [75]. A Pro173His mutation was identified in resistant *L. serriola* and a Pro173Thr mutation was identified in *K. scoparia*. Mutations of five amino acid residues are known to be involved in causing resistant weed species: Ala122, Pro197, Ala205, Trp574, and Ser653 (*A. thaliana* numbering) [76]. These five amino acid residues are highly conserved across all known plant AHAS sequences [72]. Multiple substitutions have been identified for Pro197 with resistance primarily to SUs and, to a lesser extent, TPs and IMIs. The mutation, Pro197Thr, results in resistance to at least one herbicide from all five classes of AHAS inhibitors in *Chrysanthemum coronarium*, although the levels are only moderate for IMIs and TPs. The substitution Pro197Leu confers high levels of resistance to four classes of AHAS inhibitors in *Amaranthus retroflexus*. Six different amino acid substitutions in Pro197 have been linked to resistance in *K. scoparia* alone. Substitutions of Ala122 or Ser653 result in resistance to IMI, but not SU, herbicides. The mutation Trp574Leu confers resistance to several plant species and the levels of resistance are all high against IMIs, SUs, and TPs. No other evolved Trp574 mutations have been reported.

The patterns of mutation that confer evolved resistance to AHAS-inhibiting herbicides are quite understandable in light of the laboratory site-directed mutagenesis studies and the X-ray crystal structures of AHAS from yeast and *A. thaliana* that were described earlier. For example, Fig. 2.1.5 shows that Trp574 is strategically located at the opening of the herbicide binding site and interacts extensively with both chlorimuron ethyl and imazaquin. A leucine substitution alters many of those interactions and modifies the shape of the substrate access channel [59]. Figure 2.1.5 also shows that the Pro197 residue contacts the phenyl ring of chlorimuron ethyl, but is further removed from imazaquin. This structural feature explains why nearly any Pro197 replacement will hinder SU access to the channel and confer resistance while only the most bulky amino acid substitutions will displace IMIs. Conversely, Ser653 lies in close proximity to the quinoline ring of imazaquin so that replacement with a bulky amino acid would be expected to interfere with the compound's binding in the channel and confer resistance whereas this residue does not interact as strongly with chlorimuron ethyl. Not only is there wide variability in cross resistance to the different classes of AHAS-inhibiting herbicides, but resistance to one compound within a particular class does not necessarily guarantee cross resistance to all members of that family. This is particularly true of the SUs for which differential resistance has been reported in several species [77–80].

Despite the evolution of resistance to AHAS-inhibiting herbicides, these products are still among the most efficacious and widely used weed control agents in the world [72]. Commercial AHAS-inhibiting herbicides accounted for approxi-

mately 17.5% of the total worldwide herbicide market in 1997 [81]. The total sales of AHAS-inhibiting herbicides grew from 1.86 to 2.56 $billion during the decade 1994–2004, and these products still account for about 17.5% of the total worldwide herbicide market [82]. Within the past five years, eight new active ingredients have been introduced with this mode of action. Innovations in delivery methods have also recently appeared with the advent of homogeneous blends, which allow for customized mixtures of two or more different granular herbicides [83].

In cases where resistance to AHAS inhibitors has been selected, it has typically been after five to eight years of repeated, if not continuous, use of herbicides with that mode of action. Resistance has generally not been selected where AHAS-inhibiting herbicides have been used as part of an integrated program [84]. Resistance can be effectively managed by following several well-documented best practices such as rotating herbicides or using mixtures of herbicides with different modes of action with the same spectrum of weeds controlled [71, 81, 85, 86]. For example, the judicious use of glyphosate in combination with AHAS inhibitors may provide a powerful tool for managing resistance to both classes of herbicides. With this approach, the rate of increase of AHAS-resistant weed species in the USA may slow [81]. By following a well-planned weed management program, growers should be able to use the environmentally friendly AHAS-inhibiting herbicides for many years to come to achieve effective, broad-spectrum weed control.

Acknowledgments

The author is grateful to Drs. Hugh Brown, Josephine Cotterman, Ronald Duggleby, Jerry Green, Steven Gutteridge, Jennifer McCourt, Robert Pasteris, and Leonard Saari for their critical review of the manuscript, to Drs. Ya-Jun Zheng and John Andreassi for assistance with Figs. 2.1.3–2.1.5, to Mr. Thomas Dougherty for access to the global sales figures for AHAS-inhibiting herbicides, and to Ms. Debbie Carman and Ms. Susan Titter for assistance with the literature references.

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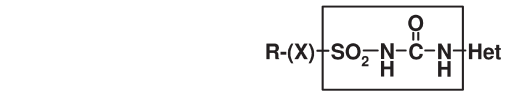
2.2
Newer Sulfonylureas

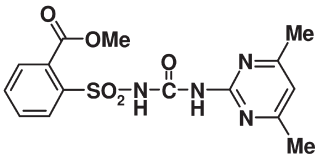
Oswald Ort

2.2.1
Introduction

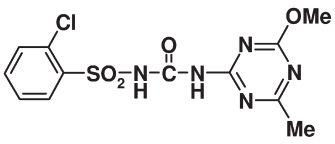
Most commercial sulfonylurea herbicides are characterized by the typical sulfonylated urea bridge connecting a nitrogen-containing heterocycle with an ortho-substituted aryl or heteroaryl moiety (Fig. 2.2.1). To date, sulfonylurea herbicides have been developed and commercialized worldwide in over 80 countries, in all major agronomic crops and for many specialty uses (e.g., rangeland/pasture, forestry, vegetation management applications).



typical sulfonylurea bridge



Sulfometuron-methyl (DuPont)



Chlorsulfuron (DuPont)

Fig. 2.2.1. First generation sulfonylurea herbicides.

Herbicidal sulfonylureas have a unique mode of action: they interfere with a key enzyme required for plant cell growth – acetohydroxyacid synthase (AHAS, EC 2.2.1.6) [1, 2, 3] (see also Mark E. Thompson in this volume, Chapter 2.1 ''Biochemistry of the Target and Resistance''). AHAS is the enzyme responsible for the synthesis of the branched-chain amino acids valine, leucine and isoleucine. Inhibition of this enzyme disrupts the plant's ability to manufacture proteins, and this disruption subsequently leads to the cessation of all cell division and eventual death of the plant.

The visible signs of herbicide action after postemergent application of sulfonylurea herbicides are an almost immediate arrest of growth, followed by leaf yellowing (chlorosis), stimulation of anthocyanin production (leading to the typical reddish coloration of weed leaves), and finally, progressive shoot death. Depending on the weed species and environmental conditions, plant death will usually occur between seven and twenty days after herbicide application.

Since the initial discovery of the first sulfonylurea herbicides by George Levitt at Du Pont in 1975, this compound class has attracted, and indeed continues to attract, very high interest and much activity within the agrochemical research domain.

This continued research interest can largely be attributed to the fact that the herbicidal activity levels demonstrated by this class of compounds remain unsurpassed today – with the most active compounds able to control undesired vegetation at rates lower than 10 g-a.i. ha^{-1} (= 1 mg-a.i. m^{-2}).

This, in turn, then contributes to a reduction in environmental burden by replacement of older higher-rate herbicides and provides an attractive return on investment to both the farmer and the producing company. In addition, the favorable environmental properties and low acute mammalian toxicology shown by the sulfonylureas usually provide a large margin of safety with regard to ecological and toxicological effects (cf. Tables 2.2.1 and 2.2.2).

Table 2.2.1 Acute toxicity for birds and water organisms (LD_{50}, LC_{50} or EC_{50}).

Compound	Birds[a] (mg kg^{-1})	Fish (96 h, mg L^{-1})	*Daphnia magna* (48 h, mg L^{-1})
Azimsulfuron	>2250	>154[b]	941
Cyclosulfamuron	Not available	>100	Not available
Ethoxysulfuron	>2000	>78.4	307
Flucetosulfuron	Not available	>10[c]	>10
Flupyrsulfuron	>2250	470	721
Foramsulfuron	>2000	>100	>100
Iodosulfuron	>2000	>100	>100
Mesosulfuron	>2000	>100	>100
Oxasulfuron	>2250	>100	>89.4
Sulfosulfuron	>2250	>91	>96
Trifloxysulfuron	>2000	>120[c]	>108
Tritosulfuron	Not available	>100	>100

[a] Mallard duck/Bobwhite quail.
[b] *Oncorhynchus mykiss*.
[c] *Cyprinus carprio*.

Table 2.2.2 Acute toxicity for mammals (LD_{50} or LC_{50}).

Compound	Oral rat (mg kg^{-1})	Dermal rat (mg kg^{-1})	Inhalation rat (mg L^{-1})
Azimsulfuron	>5000	>2000	Not available
Cyclosulfamuron	>5000	>4000[a]	>5.2
Ethoxysulfuron	>3270	>4000	3.6
Flucetosulfuron	>5000	>2000	>5.03
Flupyrsulfuron	>5000	>2000	>5.8
Foramsulfuron	>5000	>2000	>5.0
Iodosulfuron	2678	>2000	>2.8
Mesosulfuron	>5000	>5000	1.3
Orthosulfamuron	>5000	>5000	>2.2
Oxasulfuron	>5000	>2000	>5.1
Sulfosulfuron	>5000	>5000	>3.0
Trifloxysulfuron	>5000	>2000	>5.0
Tritosulfuron	3310	>2000	5.9

[a] Rabbit.

The objective of this Chapter is to give an overview of the sulfonylurea herbicides that either have been introduced to the market since 1995 or are currently in their later stages of development. These include flupyrsulfuron-methyl-sodium, sulfosulfuron, iodosulfuron-methyl-sodium, mesosulfuron-methyl, tritosulfuron and monosulfuron for use in cereals; ethoxysulfuron, azimsulfuron, cyclosulfamuron, flucetosulfuron, TH 547 and orthosulfamuron in rice; foramsulfuron in maize; oxasulfuron in soybeans; and trifloxysulfuron-sodium in sugarcane and cotton.

2.2.1.1 History and Development

Since George Levitt's landmark discovery of herbicidal sulfonylurea herbicides at Du Pont in 1975 many hundreds of patents have been granted to Du Pont and, in addition, to over twenty other agrochemical companies.

Numerous review articles about sulfonylurea herbicides are available. Amongst those particularly recommended for further reading is George Levitt's original description of his work [4]. This monograph contains seven more papers on sulfonylurea herbicides covering the literature up to 1991. Another, earlier standard text for sulfonylurea enthusiasts is that of Beyer et al. [5]. More recent sulfonylurea reviews can be found in Ref. [6].

The first generation of crop selective sulfonylurea herbicides, e.g., chlorsulfuron and metsulfuron-methyl, was found to be mainly active against broadleaf weeds. At that time it was thought that this herbicide class would be specifically applicable for controlling broadleaf weed species in a wide range of crops. This view changed with the appearance of the second generation of sulfonylureas bearing pyridylsulfonamide moieties, such as nicosulfuron and rimsulfuron, for use in maize. These compounds were active not only against broadleaf weeds but also against a broad spectrum of grasses. A further important break-through was achieved with the advent of the third generation of sulfonylurea herbicides: grass-killer experts such as mesosulfuron, blackgrass specialists such as flupyrsulfuron, and cross spectrum compounds such as iodosulfuron (cf. Table 2.2.6) and foramsulfuron (cf. Table 2.2.20).

Table 2.2.3 gives an overview of those sulfonylurea herbicides that were introduced prior to 1995.

Before going on to describe the compounds and their uses in more detail, the following section will provide a short overview of the most commonly applied synthesis methods that can be used in the production of these compounds.

Table 2.2.3 Sulfonylurea herbicides introduced before 1995 (in alphabetical order).

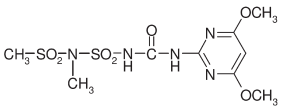
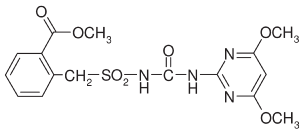
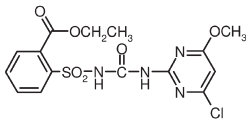
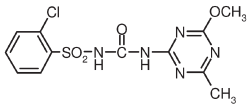
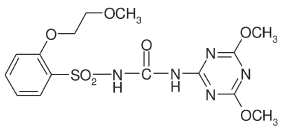
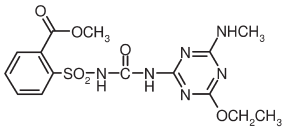
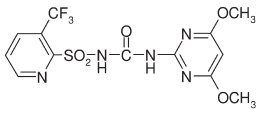
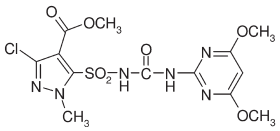
Chemical structure	Main crop	Common name (company)	Application rates (g-a.i. ha^{-1})	Ref.
	Cereals	Amidosulfuron (Bayer CropScience)	30–60	6, 60
	Rice	Bensulfuron-methyl (Du Pont)	20–75	61
	Soybeans	Chlorimuron-ethyl (Du Pont)	8–13	62
	Cereals	Chlorsulfuron (Du Pont)	9–25	63
	Rice	Cinosulfuron (Syngenta)	20–40	6
	Oilseed rape	Ethametsulfuron-methyl (Du Pont)	15–20	6, 64
	Turf, vegetation management	Flazasulfuron (Ishihara)	25–100	6, 65
	Maize, turf	Halosulfuron (Nissan)	18–35	6, 66

Table 2.2.3 *(continued)*

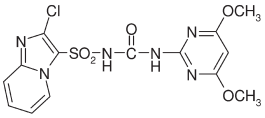
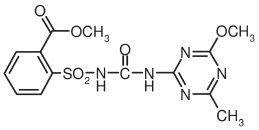
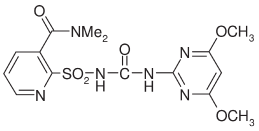
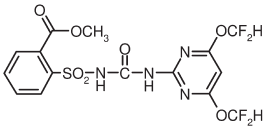
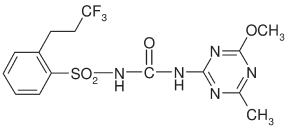
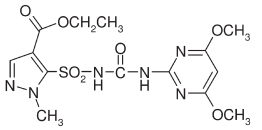
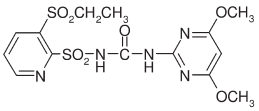
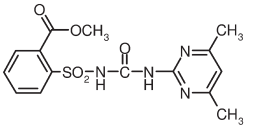
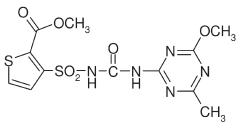
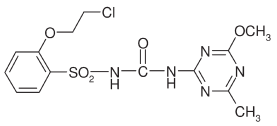
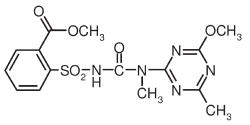
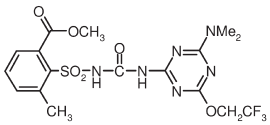
Chemical structure	Main crop	Common name (company)	Application rates (g-a.i. ha^{-1})	Ref.
	Rice, turf	Imazosulfuron (Takeda)	50–100	6
	Cereals, rice vegetation management	Metsulfuron-methyl (Du Pont)	3–8 14–168	6, 67
	Maize	Nicosulfuron (Du Pont/ Ishihara)	35–70	68, 69
	Maize	Primisulfuron-methyl (Syngenta)	20–40	70
	Cereals, maize	Prosulfuron (Syngenta)	20–40	6, 71
	Rice	Pyrazosulfuron-ethyl (Nissan)	15–30	6
	Maize	Rimsulfuron (Du Pont)	5–35	72
	Vegetation management	Sulfometuron-methyl (Du Pont)	26–420	73

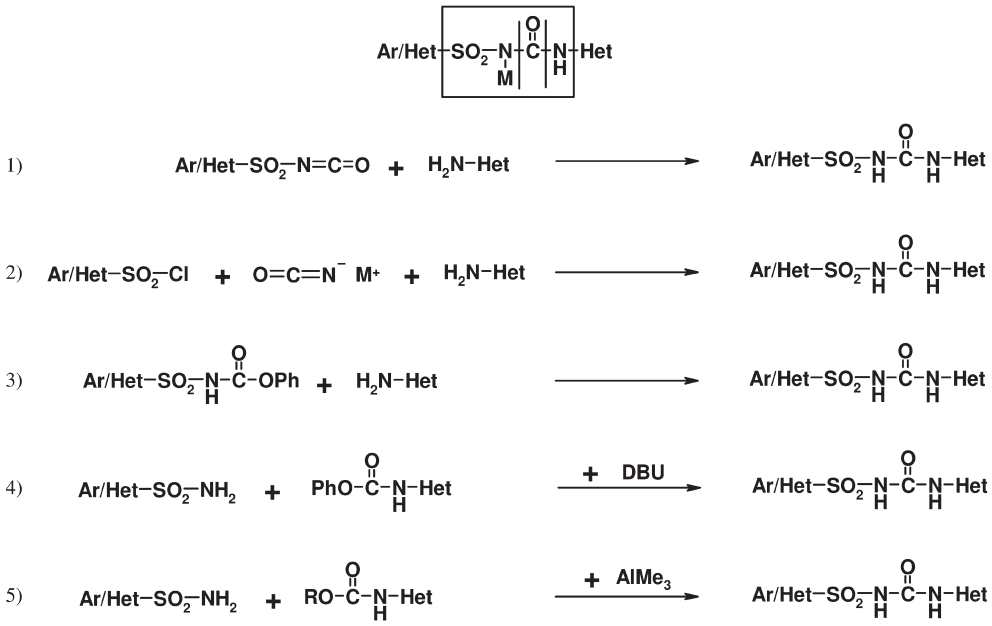
Table 2.2.3 *(continued)*

Chemical structure	Main crop	Common name (company)	Application rates (g-a.i. ha^{-1})	Ref.
	Cereals, maize, soybeans	Thifensulfuron-methyl (Du Pont)	2–30	74
	Cereals	Triasulfuron (Syngenta)	10–30	75
	Cereals	Tribenuron-methyl (Du Pont)	9–18	6, 76
	Sugar beet	Triflusulfuron-methyl (Du Pont)	15–30	6, 77

2.2.1.2 **Synthesis**

The five synthetic approaches shown in Scheme 2.2.1 have been employed most widely for the construction of the typical sulfonylurea bridge [7, 8].

Of these methods, number 1 is the most commonly used for the commercial production of sulfonylureas. The reaction is high yielding and highly atom efficient, giving advantages for downstream waste processing. In addition, the required sulfonylisocyanates are readily accessed from the corresponding sulfonamides by reaction with phosgene under several conditions. The second method has the advantages of saving two reaction steps to prepare the sulfonylisocyanate and allowing the reaction to proceed in one pot. This method is particularly useful in cases where the sulfonylisocyanate is difficult to isolate or where formation of a saccharin as by-product is problematic. Whilst methods 3 and 4 result in good conversion into the targeted products, they suffer from the undesirable production of phenol as a by-product. This can be overcome by employing alkoxy N-heterocyclycarbamates in the presence of $AlMe_3$, leading to the generation of more innocuous alcoholic by-products.



Scheme 2.2.1. Basic construction routes to the sulfonylurea bridge.

2.2.2 Agricultural Utility

In 2004 the global crop protection market (excluding seeds & biotechnology) amounted to 25.9 bio €, with herbicides accounting for roughly half of this value at 12.3 bio € sales. The herbicide subtotal is relatively evenly spread over the three main crops: cereals, maize and soybeans (19%, 18% and 16%, respectively); rice 8% and others 39% (including non-selective/non-crop use), with the main markets for herbicides in North America and Europe [9]. Table 2.2.4 gives an overview of the global acreage and production of the world's major arable crops.

The following sections give an overview, split by crop segment, of the new sulfonylurea herbicides that have either been introduced since 1995 or are currently in their later stages of development.

2.2.2.1 Cereals

Cereals (wheat, barley, sorghum, oats, rye and triticale) are the most important of the arable crops (Table 2.2.4). In 2005, global cereal production was approximately 870 mio tonnes on 340 mio hectares of land, with wheat (*Triticum aestivum*) being the most important cereal grain, accounting for more than two-thirds of the total production (Table 2.2.5).

Geographically, the largest cereal production areas are in regions with temperate conditions, such as Europe, North America and cooler parts of Australia and China.

Table 2.2.4 Major crops of the world, average 2004–05/source FAO [78].

Crop	Mio (ha)	Mio MT[a] production
Cereals[b]	333	882
Rice, paddy	152	613
Maize (grain)	147	709
Soybeans	91	208
Sugarcane	20	1309
Rapeseed	26	46
Sunflower	22	29
Potatoes	19	325
Sugar beets	6	246

[a] MT = metric tonnes.
[b] Wheat, rye, oats, triticale, barley, sorghum.

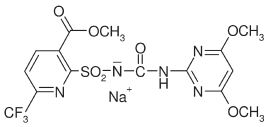
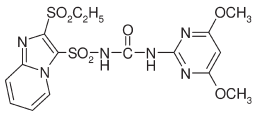
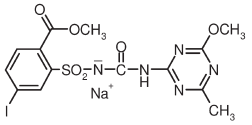
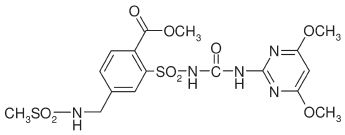
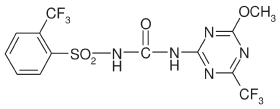
A significant area of cereal fields world-wide are infested with grass weeds. More than 40 major grass weed species can be found in cereal fields and these weeds are often highly competitive with the crop, causing substantial losses in both yield and quality. As mentioned above, sulfonylurea herbicides have been used in cereals, mainly as herbicides against broadleaf weeds, since they were first introduced in the early 1980s. Tank-mixtures with these first generation sulfonylureas or spraying programs involving different grass weed herbicides remained the most widely employed chemical control strategy up to the late 1990s. The introduction of the new generation of sulfonylurea herbicides, such as flupyrsulfuron-methyl-sodium, iodosulfuron-methyl-sodium or mesosulfuron-methyl with their broad-spectrum grass and broadleaf performance, provided the farmer with a single, innovative and easy one-pass solution saving both time and cost (Table 2.2.6).

In the following sub-sections, each of the compounds listed in Table 2.2.6 are described in more detail.

Table 2.2.5 Major cereal crops of the world, average 2004–05/Source FAO [78].

Crop	Mio (ha)	Mio MT production
Wheat	212	624
Barley	55	144
Oats	12	25
Rye	7	17
Triticale	4	14
Sorghum	43	58

Table 2.2.6 Cereal sulfonylurea herbicides in order of market introduction.

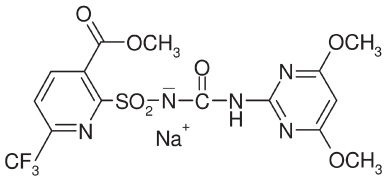
Chemical structure	Common name (company, launch year)	Agricultural utility	Application rate (g-a.i. ha^{-1})
	Flupyrsulfuron-methyl-sodium (Du Pont, 1997)	Grass weeds and select broadleaf weeds	8–10
	Sulfosulfuron (Takeda/ Monsanto, 1997)	Grass weeds and broadleaf weeds	10–35
	Iodosulfuron-methyl-sodium (Bayer CropScience, 1999)	Broadleaf and grass weeds	5–10 + safener Mefenpyr
	Mesosulfuron-methyl (Bayer CropScience, 2001)	Grass weeds and select broadleaf weeds	6–15 + safener Mefenpyr
	Tritosulfuron (BASF, 2004)	Broadleaf weeds	30–50

2.2.2.1.1 **Flupyrsulfuron-methyl-sodium**

Flupyrsulfuron-methyl-sodium (DPX-KE459) (Table 2.2.7) [10] is a postemergent cereal herbicide designed for the control of problem grass weeds, such as *Alopecurus myosuroides* and *Apera spica-venti*, and a wide range of broadleaf weeds with application rates of 8–10 g-a.i. ha^{-1}. It is commercialized by Du Pont [11] under the trade name "Lexus® 50DF" as a stand-alone product or as "Lexus® Class" in a 1:2 ratio in combination with carfentrazone-ethyl and was launched in 1997. At the 10 g-a.i. ha^{-1} rate, the following broadleaf weeds are well controlled: *Chenopodium album*, *Lamium purpureum*, *Matricaria* sp., *Polygonum aviculare*, *P. convolvulus*, *Senecio vulgaris* and *Sinapis arvensis*.

Ciral® is a combination of flupyrsulfuron-methyl-sodium (33.3%) and metsulfuron-methyl (16.7%) for the control of *Alopecurus myosuroides*, *Apera spica-venti*, *Poa annua* and annual broadleaf weeds (except *Galium aparine*) such as *Thlaspi arvense*, *Capsella bursa-pastoris*, *Galeopsis* spp., *Matricaria* spp., *Papaver rhoeas*, *Centaurea cyanus*, *Brassica napus*, *Viola arvensis*, *Lamium* spp., *Myosotis ar-*

Table 2.2.7 Physicochemical properties of flupyrsulfuron-methyl-sodium.



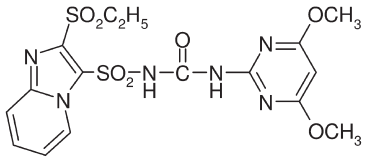
Common name (ISO)	Flupyrsulfuron-methyl-sodium	
CAS-No.	144740-54-5	
Code numbers	DPX-KE459	
Melting point	Not determined (decomposition at 165–170 °C)	
Vapor pressure	$<1 \times 10^{-9}$ Pa (20 °C)	
Dissociation constant (at 20 °C)	pK_a 4.94 (93.4%)	
Solubility in water (g L^{-1} at 20 °C)	0.06 (93.4%) (pH 5) 0.61 (pH 6) Instability of the solution (pH 7)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone	3.1 (93.4%)
	Acetonitrile	4.3
	Benzene	0.028
	Dichloromethane	0.60
	Hexane	<0.001
	Methanol	5.0
	n-Octanol	0.19
Partition coefficient (log P_{OW}) in octanol–water (at 25 °C)	0.96 (pH 5.0) 0.11 (93.4%) (pH 6.0)	

vensis and *Stellaria media*. Ciral® is a flexible product that can be applied in autumn and spring in wheat and in flax at a dose rate of 25 g ha^{-1} [12] of formulated product containing 8 g-a.i. flupyrsulfuron-methyl-sodium.

2.2.2.1.2 **Sulfosulfuron**

Sulfosulfuron (MON 37500) (Table 2.2.8) [13] is a postemergent herbicide for the control of grass (especially *Bromus* species) and broadleaf weeds in cereal crops at rates of 10–35 g-a.i. ha^{-1}. Sulfosulfuron was jointly developed [14, 15] by Monsanto Company and Takeda Chemical Industries and launched in 1997. Barley and oats are sensitive to applications of sulfosulfuron and so use in these crops is not recommended. At rates of 20–30 g-a.i. ha^{-1} the following weeds are controlled with at least 85% efficiency: *Elymus repens*, *Apera spica-venti*, *Agrostis*

Table 2.2.8 Physicochemical properties of sulfosulfuron.



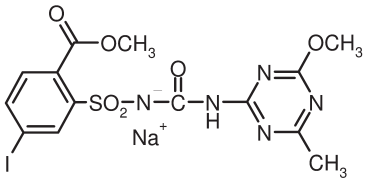
Common name (ISO)	Sulfosulfuron	
CAS-No.	141776-32-1	
Code numbers	MON 37500 TKM 19	
Melting point	201.1–201.7 °C	
Vapor pressure	3.05×10^{-8} Pa (20 °C)	
Dissociation constant (at 20 °C)	pK_a 3.51	
Solubility in water (g L^{-1} at 20 °C)	0.018 (pH 5) 1.627 (pH 7) 0.482 (pH 9)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone Ethyl acetate Dichloromethane *n*-Heptane Methanol Xylene	0.71 1.01 4.35 <0.001 0.33 0.16
Partition coefficient (log P_{ow}) in octanol–water	0.73 (pH 5.0) −0.77 (pH 7.0) −1.44 (pH 9.0)	

stolonifera, *Avena fatua* (North America), *Bromus commutatus*, *B. japonicus*, *B. mollis*, *B. rigidus*, *B. secalinus*, *B. sterilis*, *B. tectorum*, *Poa bulbosa* and *Poa trivialis*, *Ambrosia artemisiifolia*, *Amsinckia lycopsoides*, *Atriplex patula*, *Brassica nigra*, *Capsella bursa-pastoris*, *Claytonia per*, *Descurainia pinnata*, *D. sophia*, *Fumaria officinalis*, *Galium aparine*, *Helianthus* sp., *Matricaria chamomilla*, *M. inodora*, *Polygonum aviculare*, *P. persicaria*, *Sinapis arvensis*, *Sisymbrium altissimum*, *Stellaria media*, *Thlaspi arvense* and *Viola arvensis*. In Europe sulfosulfuron is commercially available under the trade name "Monitor".

2.2.2.1.3 **Iodosulfuron-methyl-sodium**

At a rate of 2.5–10 g-a.i. ha^{-1}, iodosulfuron-methyl-sodium (AE F115008) (Table 2.2.9) [16] controls more than 50 different broadleaf weed species, including some very competitive weeds that cause a substantial reduction of cereal productivity, e.g., *Galium aparine*, *Matricaria chamomilla*, *Stellaria media*, *Raphanus* ssp.,

Table 2.2.9 Physicochemical properties of iodosulfuron-methyl-sodium.



Common name (ISO)	Iodosulfuron-methyl-sodium	
CAS-No.	144550-36-7	
Code numbers	AE F115008	
Melting point	148–152 °C	
Vapor pressure	2.6×10^{-9} Pa (20 °C) 6.7×10^{-9} Pa (25 °C)	
Dissociation constant (at 20 °C)	pK_a 3.22 (under strong acidic conditions – pH 2 – formation of iodosulfuron-methyl)	
Solubility in water (g L^{-1} at 20 °C)	0.16 (pH 5) 25.0 (pH 7) 65.0 (pH 9)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetonitrile Ethyl acetate *n*-Heptane Methanol 2-Propanol Toluene	52 23 0.001 12 4.4 2.1
Partition coefficient (log P_{OW}) in octanol–water (at 20 °C)	1.96 (pH 4.0) 1.22 (pH 9.0)	

Cirsium arvense, *Lamium* ssp. Whilst application of iodosulfuron-methyl-sodium at the lower end of the suggested use-rate is usually sufficient for control of broadleaf weeds, a higher rate is needed for consistent grass weed control. Major grass weeds controlled with a 7.5–10 g-a.i. ha^{-1} dose rate applied at the three-leaf stage up to end of tillering are *Agrostis gigantea*, *Apera spica-venti*, *Lolium multiflorum*, *L. perenne*, *L. persicum*, *L. rigidum*, *Phalaris brachystachys*, *P. canariensis*, *P. paradoxa*, *Poa annua*, and *P. trivialis*.

Iodosulfuron-methyl-sodium was the first safened sulfonylurea herbicide on the market [17, 18] when introduced in 1999 and has been commercialized by Bayer CropScience for use both in cereals and maize. A safener such as mefenpyr-diethyl (cf. Fig. 2.2.2) is a chemical that, when applied to crop plants, reduces the injury caused by herbicides to an acceptable level. A safener ideally does not

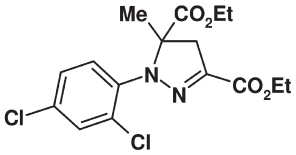


Fig. 2.2.2. Cereal safener mefenpyr-diethyl (AE F107892).

reduce activity against the target weeds. A series of experiments were conducted to compare the behavior of iodosulfuron-methyl-sodium with and without the safener mefenpyr-diethyl. The findings suggest that the safener acts by specific catalytic enhancement of herbicide degradation in cereals but not in target weeds such as wild oat. The topic concerning safeners is dealt with later in more detail (see Chris Rosinger and Helmut Koecher in this volume, Chapter 5 ''Safener for Herbicides'').

In cereals, iodosulfuron-methyl-sodium is commercialized under the trade name ''Hussar'' as a straight product in a 1:3 ratio with the safener mefen-

Table 2.2.10 Iodosulfuron-based products, formulations and composition.

Iodosulfuron-based products	Formulation type	Iodosulfuron	Mesosulfuron	Fenoxaprop-ethyl	Amidosulfuron	Mefenpyr-diethyl
Hussar®, Husar®, Huzar®, Huszar®, Al Fares®, Wipe®	WG[a]	50[b]				150[b]
Sekator®, Grodyl® Ultra	WG[a]	12.5[b]			50[b]	125[b]
Sekator® OD	OD[c]	25[d]			100[d]	250[d]
Chekker®, Hoestar® Super	WG[a]	12.5[b]			125[b]	125[b]
Hussar® OF Evolution	SC[e]	8[d]		64[d]		24[d]
Hussar® OD	OD[c]	100[d]				300[d]
Atlantis® WG	WG[a]	6[b]	30[b]			90[b]
Pacifica®	WG[a]	10[b]	30[b]			90[b]
Archipel®, Cossack®, Chevalier®, Hussar maxx®	WG[a]	30[b]	30[b]			90[b]

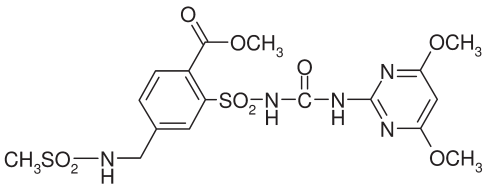
[a] WG: water dispersible granules.
[b] Units: g-a.i. kg^{-1}.
[c] OD: oil dispersion.
[d] Units: g-a.i. L^{-1}.
[e] SC: suspension concentrate.

pyr-diethyl. The compound is also commercialized in various combinations with other mixing partners such as "Hussar® OF" (+ fenoxaprop-P-ethyl + mefenpyr-diethyl), "Sekator®"/"Chekker®" (+ amidosulfuron + mefenpyr-diethyl), "Cossack®" (+ mesosulfuron + mefenpyr-diethyl) and "Atlantis®" (+ mesosulfuron + mefenpyr-diethyl) (cf. Table 2.2.10).

2.2.2.1.4 **Mesosulfuron-methyl**

Mesosulfuron-methyl (AE F130060) (Table 2.2.11) [19] was the second safened sulfonylurea herbicide for cereal crops to be commercialized. This compound was introduced in 2001 and has been commercialized by Bayer CropScience [20, 21]. Its strength is broad-spectrum post-emergence grass weed control. Mesosulfuron-methyl, at a dose rate of 4.5–15 g-a.i. ha^{-1}, reliably controls 24 different grass weed species from 12 different families. Among the commercially

Table 2.2.11 Physicochemical properties of mesosulfuron-methyl.



Common name (ISO)	Mesosulfuron-methyl	
CAS-No.	208465-21-8	
Code numbers	AE F130060	
Melting point	195.4 °C (98.7% purity)	
Vapor pressure	1.1×10^{-11} Pa (25 °C)	
Dissociation constant (at 20 °C)	pK_a 4.35	
Solubility in water (g L^{-1} at 20 °C)	0.007 (pH 5) 0.483 (pH 7) 15.39 (pH 9)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone Ethyl acetate Dichloromethane *n*-Hexane Toluene	13.66 2.0 3.8 <0.0002 0.013
Partition coefficient (log P_{OW}) in octanol–water (at 25 °C)	1.39 (pH 5.0) −0.48 (pH 7.0) −2.06 (pH 9.0)	

important grass weed species, it provides good control of *Agrostis* spp., *Alopecurus myosuroides*, *Apera spica-venti*, *Avena* spp. *Lolium* spp., *Phalaris brachystachis*, *P. minor*, *P. paradoxa*, *Poa annua*, *Poa trivialis*, *Pucciniella* spp. and *Sclerochloa kengiana*. Additionally mesosulfuron-methyl controls, or has a strong suppressive effect on, some very persistent grass weed species, such as *Bromus catharticus*, *B. diandrus*, *B. erectus*, *B. japonicus*, *B. mollis*, *B. tectorum*, *B. secalinus*, *B. sterilis* and *Vulpia* spp.

The compound is applied on soft and durum wheat, triticale and rye, together with the safener mefenpyr-diethyl (Fig. 2.2.2) as the straight products "Atlantis® OF", "Silverado®" and "Osprey®" or in combination with iodosulfuron-methyl-sodium ("Atlantis® WG", "Cossack®", "Pacifica®"), diflufenican and propoxycarbazone-sodium (Table 2.2.12).

"Atlantis® WG" is positioned in market segments where grass weeds are the main target, whereas "Cossack®" is a cross spectrum product, active against grasses and against a large number of important broadleaf weeds. Mesosulfuron-methyl belongs to the group of modern OnePass® products. It predominantly acts via the leaves of treated weeds; however, highly susceptible grasses, such as

Table 2.2.12 Mesosulfuron based products, formulations and composition.

Mesosulfuron-based products	Formulation type	Mesosulfuron-methyl	Iodosulfuron-methyl sodium	Diflufenican	Propoxycarbazone	Mefenpyr-diethyl
Atlantis® OD	OD[a]	30[b]				90[b]
Atlantis®	WG[c]	30[d]	6[d]			90[d]
Pacifica®	WG[c]	30[d]	10[d]			90[d]
Archipel®, Cossack®, Chevalier®, Hussar maxx®	WG[c]	30[d]	30[d]			90[d]
Silverado®	WG[c]	20[d]				120[d]
Osprey®	WG[c]	45[d]				90
Alister®	OD[a]	9[b]	3[b]	150[b]		27[b]
Othello®	OD[a]	7.5[b]	2.5[b]	50[b]		22.5[b]
Olympus flex®	WG[c]	45[d]			67.5[d]	90[d]

[a] OD: oil dispersion.
[b] Units: g-a.i. L^{-1}.
[c] WG: water dispersible granules.
[d] Units: g-a.i. kg^{-1}.

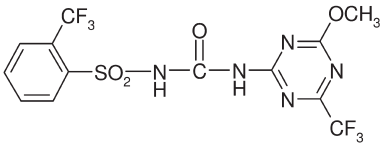
Apera and *Alopecurus*, are also successfully controlled by uptake of mesosulfuron-methyl via the soil and the roots.

The safener mefenpyr-diethyl, as with iodosulfuron-methyl-sodium, selectively accelerates the degradation of the active ingredient to non-phytotoxic compounds in cereals but not in weeds.

2.2.2.1.5 **Tritosulfuron**

Tritosulfuron (BAS-635) (Table 2.2.13) [22] is a broad-spectrum postemergent dicot herbicide mainly for use in cereals, rice, maize and turf with application rates of 40–75 g-a.i. ha^{-1}. In cereals it was commercialized by BASF in 2004 under the trade name "Biathlon®" [23] as a WG formulation containing 714 g kg^{-1} tritosulfuron and is applied at a rate of 50 g-a.i. ha^{-1}. The following weeds are well controlled: *Thlaspi arvense*, *Mercurialis annua*, *Urtica urens*, *Cirsium arvense*, *Veronica hederifolia*, *Chenopodium* spp., *Sinapis arvensis*, *Capsella bursa-pastoris*, *Galeopsis*

Table 2.2.13 Physicochemical properties of tritosulfuron.



Common name (ISO)	Tritosulfuron	
CAS-No.	142469-14-5	
Code numbers	BAS-635	
Melting point	167–169 °C	
Vapor pressure	1.0×10^{-5} Pa (20 °C)	
Dissociation constant (at 20 °C)	pK_a 4.69	
Solubility in water (g L^{-1} at 20 °C)	<0.001 (pH 1.7) 0.04 (pH 7.0) 78.32 (pH 10.2)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone Acetonitrile Ethyl acetate Dichloromethane *n*-Heptane Methanol Toluene	– – 83.0 25.0 <0.001 23.0 4.2
Partition coefficient (log P_{OW}) in octanol–water (at 20 °C)	2.85 (pH 4.0) 0.62 (pH 7.0) −2.38 (pH 10.0)	

tetrahit, *Matricaria* spp., *Galium aparine*, *Polygonum* spp., *Centaurea cyanus*, *Lamium* spp., *Myosotis arvensis*, *Stellaria media*, *Vicia* spp., *Convolvulus arvensis*, *Sonchus arvensis*, *Brassica napus*. Tritosulfuron acts mainly through the treated leaves and not via the soil. The compound has the advantage of having a short soil half-life, which allows re-cropping after 60 days without plowing [24, 25].

Tritosulfuron is selective in the following cereal crops: wheat, rye, barley, triticale, oat, durum wheat and spelt. The application window of tritosulfuron in all winter and summer cereals ranges from vegetation start up to ES 39. Sold in maize as "Tooler®", it can be applied from ES 12 to ES 18.

2.2.2.1.6 **Cereals Development Candidates**

Two compounds, monosulfuron and NPC-C9908, from research in China are currently in the early market introduction phase or late development stage in China. Thus there is only limited public knowledge available about these compounds.

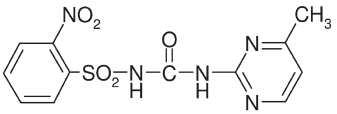


Fig. 2.2.3. Monosulfuron.

Monosulfuron (CAS-No.: 155860-63-2) (Fig. 2.2.3) is a new herbicide for the control of weeds in wheat (*Triticum aestivum*) and millet (*Panicum miliaceum*), with application rates ranging from 15–60 g-a.i. ha^{-1}. The molecule was discovered by Nankai University in 1993 [26] and recently registered in China. Monosulfuron provides effective control of various broadleaf and grass weeds, such as *Leptochloa chinensis*, *Amaranthus retroflexus*, *Chenopodium album*, *Abutilon theophrasti*, *Xanthium sibiricum* Patrin., *Portulaca oleracea*, *Acalypha australis*, *Solanum nigrum*, *Digitaria sanguinalis*, *Descurainia sophia*, *Echinochloa phyllopogon*, *Eriochloa villosa* and *Puccinellia distans*. Further properties and environmental data of monosulfuron are detailed in several papers by Fan [27].

HNPC-C9908 [2-(4-methoxy-6-methylthiopyrimidin-2-yl) carbamoyl sulfonyl benzoate] (CAS-No.: 441050-97-1) (Fig. 2.2.4) is a novel sulfonylurea herbicide [28, 29] discovered by the Hunan Branch of the National Pesticide R&D South Center, Changsha, China, and is reported to be effective in controlling various broadleaf weeds and some grasses in wheat.

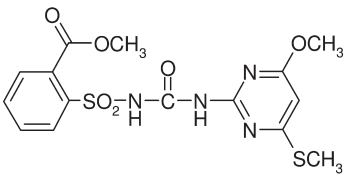
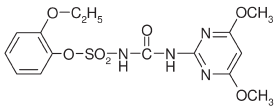
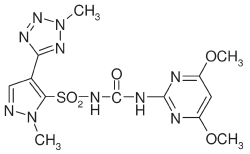
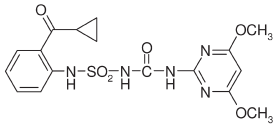
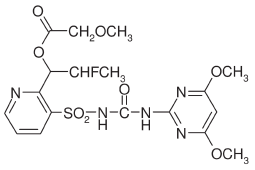


Fig. 2.2.4. HNPC-C9908 herbicide.

Table 2.2.14 Rice sulfonylurea herbicides in order of market introduction.

Chemical structure	Common name (company, launch year)	Agricultural utility	Application rate (g-a.i. ha^{-1})
	Ethoxysulfuron (Bayer CropScience, 1996)	Annual and perennial broadleaf and sedge weeds	6–60
	Azimsulfuron (Du Pont, 1996)	Annual and perennial broadleaf and sedge weeds	6–25
	Cyclosulfamuron (BASF, 1997)	Annual and perennial broadleaf and sedge weeds	10–60
	Flucetosulfuron (KRICT/LG Chem., 2004)	Annual and perennial broadleaf and sedge weeds	15–60

2.2.2.2 **Rice**

Around 60% of the global population, particularly in Asia, rely on rice (*Oryza sativa*) as a major food source. Rice is grown mainly in the humid and sub-humid tropics of the Far East. Rice production on ca. 154 mio hectares totaled 618 mio tonnes in 2005, with the biggest two producers, China and India, being responsible for more than half of the global total [78]. However, value-wise Japan is the largest rice market with >40% of the total market value.

It is estimated that, on average, weed infestation in tropical rice areas accounts for 10–20% of yield loss, but there are studies that show that some problem weed species, such as red rice (*Oryza sativa* ssp.) and barnyard grass (*Echinochloa crus-galli*), can cause even higher losses. Red rice (the term "red rice" is used synonymously for weedy rice because its grains frequently have a red pigmented pericarp) is in the same genus and species as cultivated conventional rice, which makes it very difficult to eliminate in rice fields. Fisher and Ramirez [30, 31] found that a 5% density of red rice decreased conventional rice yields by up to 40%.

Typically herbicides in rice are used in combinations of active ingredients and, as labor is becoming more expensive, the trend is towards single application products. These products usually contain sulfonylureas as the main active ingredient (Table 2.2.14).

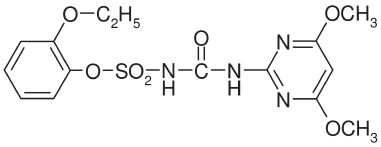
The following sub-sections describe in more detail each of the compounds listed in Table 2.2.14.

2.2.2.2.1 **Ethoxysulfuron**

Ethoxysulfuron (HOE 095404) [32] is a very flexible herbicide for the control of broadleaf and sedge weed species (Table 2.2.15).

Although rice is the main use crop, the compound can also be applied in cereals and sugar cane [33]. Selectivity is achieved due to a differential metabolism in the target crops to that in the weeds [34]. With an application rate of 15–60 g-a.i. ha^{-1} a wide range of important annual and perennial rice weeds are controlled,

Table 2.2.15 Physicochemical properties of ethoxysulfuron.



Common name (ISO)	Ethoxysulfuron	
CAS-No.	126801-58-9	
Code numbers	HOE 095404	
Melting point	144–147 °C	
Vapor pressure	6.6×10^{-5} Pa (20 °C)	
Dissociation constant (at 20 °C)	pK_a 5.28	
Solubility in water (g L^{-1} at 20 °C)	0.026 (pH 5) 1.353 (pH 7) 9.628 (pH 9)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone Ethyl acetate Dichloromethane *n*-Hexane Methanol Poly(ethylene glycol) Toluene	36.0 14.1 107.0 0.006 7.7 22.5 2.5
Partition coefficient (log P_{OW}) in octanol–water (at 21 °C)	2.89 (pH 3.0) 0.004 (pH 7.0) −1.22 (pH 9.0)	

Table 2.2.16 Ethoxysulfuron-based products, formulations and composition.

Ethoxysulfuron-based products	Formulation type	Ethoxy-sulfuron	Anilofos	Fenoxaprop-ethyl	Safener
Gladium, Grazie, Hero, Skol, Sunrice	WG[a]	600[b]			
Sunrice, Sunstar	WG[a]	150[b]			
Ricestar®, Ricestar® Xtra, Tiller® Gold, Turob®	OD[c]	20[d]		69[d]	75[d]
Sunrice Plus	SC[e]	15[d]	300[d]		

[a] WG: water dispersible granules.
[b] Units: g-a.i. kg^{-1}.
[c] OD: oil dispersion.
[d] Units: g-a.i. L^{-1}.
[e] SC: suspension concentrate.

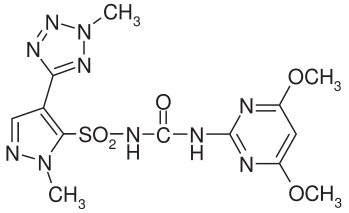
such as *Cyperus* spp., *Aeschynomene* spp., *Eleocharis* spp., *Sagittaria* spp., *Scirpus* spp., *Amannia* spp., *Lindernia* spp., *Ludwigia* spp. and *Monochoria vaginalis*. Ethoxysulfuron is fully selective in all types of seeded rice (dry drilled, pre-germinated wet seeded, pre-germinated water seeded) and all types of transplanted rice. The selectivity is not influenced by the rice growth stage at application time, the water management or other environmental factors. Ethoxysulfuron was introduced in rice in 1996 (Vietnam) and has been commercialized by Bayer CropScience as a straight product under the trade name ''Sunrice® WG'' and as SC formulated products in combination with anilofos as ''Riceguard®'', ''Benefiter®'', ''Sunrice® Super'' and ''Sunrice® Plus'' (Table 2.2.16).

2.2.2.2.2 **Azimsulfuron**

Azimsulfuron (DPX-A8947) [35] is a new rice herbicide introduced in 1996 by Du Pont [36] for the control of broadleaf weeds (including hard-to-control perennials) (Table 2.2.17). At rates of 8–20 g-a.i. ha^{-1} it gives superior weed control, including *Echinochloa crus-galli*, when compared with the first-generation sulfonylurea bensulfuron at 50–75 g-a.i. ha^{-1}. Azimsulfuron is targeted to replace or supplement bensulfuron in some applications. In Japan, in planted rice, azimsulfuron is used as a pre-mixture with bensulfuron (6 + 30 g-a.i. ha^{-1}) to boost the activity against perennial weeds. Good control has also been reported of other members of the Echinochloa family, such as *E. hispidula*, *E. oryzicola* and *E. oryzoides*. Other weeds controlled include *Alisma lanceolatum*, *A. plantago-aquatica*, *Butomus umbellatus*, *Cyperus difformis*, *Scirpus maritimus*, *S. mucronatus*, *S. supinus*, *Heteranthera limosa*, *Potamogeton nodosus*, *Ammannia coccinea*, *A. robusta*, *Bergia capensis* and *Lindernia dubia*.

Azimsulfuron is sold under the trade names ''Gulliver'' and ''Azin''.

Table 2.2.17 Physicochemical properties of azimsulfuron.

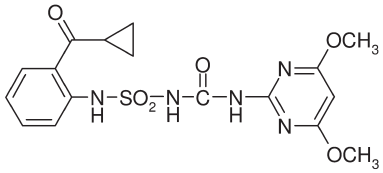


Common name (ISO)	Azimsulfuron	
CAS-No.	120162-55-2	
Code numbers	DPX-A8947	
Melting point	170 °C	
Vapor pressure	4.0×10^{-9} Pa (25 °C)	
Dissociation constant (at 20 °C)	pK_a 3.6	
Solubility in water (20 °C)	0.072 (pH 5) 1.050 (pH 7) 6.536 (pH 9)	
Solubility in organic solvents (g L^{-1} at 25 °C)	Acetone Acetonitrile Ethyl acetate Dichloromethane *n*-Hexane Methanol Toluene	26.4 13.9 13.0 65.9 <0.2 2.1 1.8
Partition coefficient (log P_{OW}) in octanol–water (at 25 °C)	0.646 (pH 5.0) −1.367 (pH 7.0) −2.076 (pH 9.0)	

2.2.2.2.3 **Cyclosulfamuron**

Cyclosulfamuron (AC 322,140) herbicide was launched in 1997 and is commercialized by BASF for control of a wide range of broadleaf weeds and sedge species in rice, wheat and barley [37] (Table 2.2.18). Rice weeds controlled with greater 90% efficiency at an application rate of 45–60 g-a.i. ha^{-1} include *Cyperus serotinus*, *C. difformis*, *Elatine triandra*, *Eleocharis congesta*, *E. kuroguwai*, *Lindernia annua*, *L. procumbens*, *Monochoria vaginalis*, *Rotala indica*, *Sagittaria pygmaea*, *S. trifolia* and *Scirpus juncoides*. Selectivity in the rice paddy is achieved due to various factors, including rapid metabolic degradation of the herbicide in rice shoots, placement of rice seedlings during transplanting and the compound's soil binding properties, which retain cyclosulfamuron in the upper soil layer of the paddy [38].

Table 2.2.18 Physicochemical properties of cyclosulfamuron.

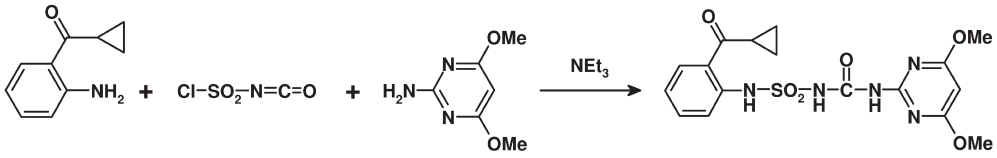


Common name (ISO)	Cyclosulfamuron	
CAS-No.	136849-15-5	
Code numbers	AC 322,140	
Melting point	170–171 °C	
Vapor pressure	$<2.2 \times 10^{-5}$ Pa (20 °C)	
Dissociation constant (at 20 °C)	pK_a 5.04	
Solubility in water (25 °C)	0.001 (pH 5) 0.003 (pH 6) 0.006 (pH 7) 0.032 (pH 8)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone Ethyl acetate Dichloromethane *n*-Hexane Methanol Toluene	21.0 5.0 50.0 <0.001 1.5 1.0
Partition coefficient (log P_{OW}) in octanol–water (at 25 °C)	1.58 (pH 3.0) 2.05 (pH 5.0) 1.69 (pH 6.0) 1.41 (pH 7.0) 0.70 (pH 8.0)	

In rice, cyclosulfamuron is commercialized under the trade name ''Ichiyonmaru'' and ''Saviour''. In combinations with daimuron and cafenstrole it is commercialized as ''Nebiros'' and in combination with pentoxazone as ''Utopia''. ''Shakariki'' is the trade name for the mixture with esprocarb.

At rates of 25–50 g-a.i. ha^{-1} cyclosulfamuron can also be used in cereal crops for pre- and postemergent control of several important broadleaf weeds, such as *Veronica persica*, *V. hederifolia*, *Galium aparine*, *Matricaria* spp. and *Polygonum convolvulus* [38].

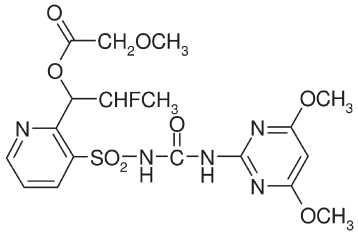
Cyclosulfamuron cannot be synthesized by any of the general methods depicted in Scheme 2.2.1. From the methods published in the patent literature, Brady et al. [40] describe a straightforward reaction of 2-amino-4,6-



Scheme 2.2.2. CSI route to cyclosulfamuron.

dimethoxypyrimidine with chlorosulfonylisocyanate (CSI) at 0 °C with a mixture of 2-aminophenyl cyclopropyl ketone and triethylamine to yield 70% of the desired herbicide (Scheme 2.2.2). This synthesis method of cyclosulfamuron and its intermediate products can also be found in the paper by Tan from 2005 [41].

Table 2.2.19 Physicochemical properties of flucetosulfuron.



Common name (ISO)	Flucetosulfuron	
CAS-No.	412928-75-7	
Code numbers	LGC-42153	
Melting point	178–182 °C	
Vapor pressure	$<1.86 \times 10^{-5}$ Pa (25 °C)	
Dissociation constant (at 20 °C)	pK_a 3.5	
Solubility in water (25 °C)	0.114 g L^{-1} (pH 7)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone	22.9
	Ethyl acetate	11.7
	Dichloromethane	113.0
	Dimethylformamide	265.0
	Dimethyl sulfoxide	211.7
	n-Hexane	0.006
	Methanol	3.8
Partition coefficient (log P_{OW}) in octanol–water (temperature not published)	n.a. (pH 3.0) 1.05 (pH 7.0) n.a. (pH 9.0)	

2.2.2.2.4 Flucetosulfuron

Flucetosulfuron (LGC-42153) [42] was presented at the BCPC Conference in 2003 by researchers from LG Life Sciences Ltd. and KRICT [43, 44] and was commercialized in 2004 (Table 2.2.19). It can be used for the control of broadleaf weeds, some grass weeds and also sedges in rice and cereal crops. In rice, flucetosulfuron provides excellent control of *Echinochloa crus-galli*, which is usually not controlled by other commercial rice sulfonylurea products. In addition, the following weeds are controlled at a rate of 10–20 g-a.i. ha^{-1}: *Alisma* spp., *Ammannia coccinea*, *Cyperus difformis*, *Fimbristylis* spp., *Lindernia* spp., *Monochoria vaginalis*, *Rorippa silvestri*, *Rotala indica*, *Scirpus juncoides*, *S. mucronatus* and *S. maritimus*. At a higher rate of 20–30 g-a.i. ha^{-1}, greater than 90% control of *Aeschymene indica*, *Butomus umbellatus*, *Eleocharis kuroguwai*, *Sagittaria pygmaea*, *S. trifolia* and *Sparganium erectum* is achieved by flucetosulfuron with a high crop safety margin when applied to soil or foliage in direct-seeded or transplanted rice.

In cereal crops, flucetosulfuron at a 20–30 g-a.i. ha^{-1} rate shows excellent activity against *Galium aparine* and other broadleaf weeds, such as *Capsella bursa-pastoris*, *Galeopsis tetrahit*, *Lamium purpureum*, *Matricaria* spp., *Myosotis arvensis*, *Papaver rhoeas*, *Raphanus raphanistrum*, *Senecio vulgaris*, *Sinapis arvensis*, *Stellaria media* and *Thlaspi arvense*, while being safe to use in wheat and barley at up to three times the recommended application rate.

2.2.2.2.5 Rice Development Candidates

In rice there are currently two compounds, TH 547 from research at Sumika-Takeda, and orthosulfamuron from Isagro, shortly before market introduction.

TH 547 (Fig. 2.2.5) is a new sulfonylurea under development by Sumika-Takeda and is currently in official trials in Japan. Although the structure has not been officially confirmed, it is believed to be related to the imazosulfuron class [45, 46]. The compound is expected to be introduced in 2008–2009 and is considered to be a new generation sulfonylurea for the control of annual and perennial broadleaf weeds and sedges, especially against ALS-resistant weed biotypes. At rates of 70 g-a.i. ha^{-1}, TH 547 controls *Cyperus serotinus*, *C. difformis*, *Elatine triandra*, *Eleocharis congesta*, *E. kuroguwai*, *Lindernia annua*, *L. procumbens*, *Monochoria vaginalis*, *Rotala indica*, *Sagittaria pygmaea*, *S. trifolia* and *Scirpus juncoides*. At a higher rate of 90 g-a.i. ha^{-1}, it gives total weed control, including *Echinochloa* spp.

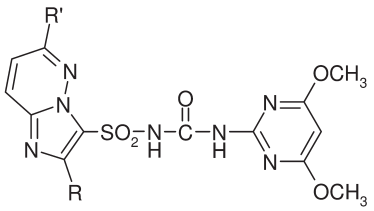


Fig. 2.2.5. Tentative structure of TH 547 (R = Cl; R′ = *n*-Pr).

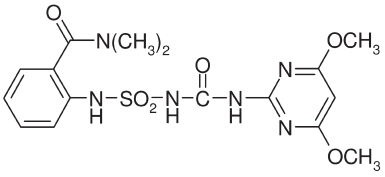


Fig. 2.2.6. Orthosulfamuron herbicide.

Orthosulfamuron (IR-5878, CAS-No.: 213464-77-8) (Fig. 2.2.6) [47], is a broad-spectrum pre- and postemergent rice herbicide developed by Isagro for the control of annual broadleaf and sedge weeds. There is only limited public knowledge available about this compound but it is assumed to be applied at a use rate of 25–150 g-a.i. ha^{-1} and could be on the market by 2007.

2.2.2.3 **Maize**

Approximately 700 mio tonnes of maize were produced worldwide in 2005 on more than 140 mio hectares land [78]. Maize (*Zea mays*), occupies third place in world production as a source of food, forage and processed products for industry. The main producing countries are the USA, China and Brazil, which together account for ca. two-thirds of global production. Maize is most commonly grown for animal feed use, although it is a dietary staple in some areas such as Mexico and other Latin American countries.

Maize, with its shallow root system, is particularly prone to competition by other plants in its early growth stages. While older generations of maize herbicides are predominantly used as preemergent herbicides, e.g., atrazine from the triazines class, there are now modern, postemergent sulfonylurea products available to the farmer for cost-effective and time-flexible weed control. The latest compound to be introduced after 1995 is discussed below (Table 2.2.20).

Table 2.2.20 Maize sulfonylurea herbicides.

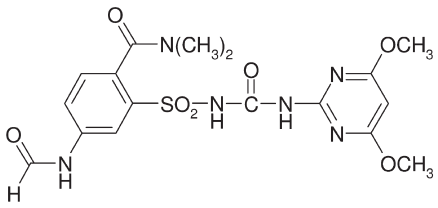
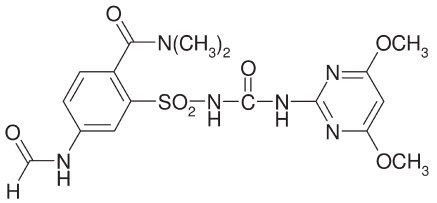
Chemical structure	Common name (company, launch year)	Agricultural utility	Application rate (g-a.i. ha^{-1})
	Foramsulfuron (Bayer CropScience, 2001)	Grass and broadleaf weeds	30–45 + Safener Isoxadifen

Table 2.2.21 Physicochemical properties of foramsulfuron.



Common name (ISO)	Foramsulfuron	
CAS-No.	173159-57-4	
Code numbers	AE F130360	
Melting point	194.5 °C (98.4% w/w)	
Vapor pressure (Pa)	4.2×10^{-11} (20 °C) 1.3×10^{-10} (25 °C)	
Dissociation constant (at 21.5 °C)	pK_a 4.6	
Solubility in water (g L^{-1} at 20 °C)	0.037 (pH 5) 3.293 (pH 7) 94.577 (pH 8)	
Solubility in organic solvents (g L^{-1} at 20 °C)	Acetone	1.925
	Acetonitrile	1.111
	1,2-Dichloroethane	0.185
	Ethyl acetate	0.362
	Heptane	<0.01
	Methanol	1.660
	p-Xylene	<0.01
Partition coefficient (log P_{OW}) in octanol–water (20 °C)	1.44 (pH 2.0) 0.60 (pH 5.5–5.7) −0.78 (pH 7.0) −1.97 (pH 9.0)	

2.2.2.3.1 **Foramsulfuron**

Foramsulfuron (AE F130360) [48] is a postemergence sulfonylurea herbicide for the control of major grass species and certain broadleaf weeds in maize (Table 2.2.21). It is applied with the safener isoxadifen-ethyl (AE F122006) (Fig. 2.2.7) and in some products in combination with small quantities iodosulfuron-methyl-sodium [49].

Introduced in 2001 and subsequently commercialized by Bayer CropScience, the three-way mixture of foramsulfuron with iodosulfuron-methyl-sodium and isoxadifen-ethyl is used for postemergent weed control in maize. Foramsulfuron,

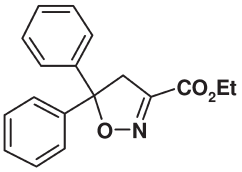


Fig. 2.2.7. Maize safener isoxadifen-ethyl (AE F122006).

at a dose rate of 30–45 g-a.i. ha^{-1}, offers a minimum of 90% weed control on most grassy weeds, such as *Echinochloa crus-galli*, *Setaria* spp., *Agropyron repens*, *Apera spica-venti*, *Alopecurus myosuroides*, *Lolium multiflorum*, *Panicum dichotomiflorum*, *Poa annua* and *Sorghum halepense*, and a wide selection of broadleaf weed species, such as *Abutilon theophrasti*, *Amaranthus* spp., *Galinsoga parviflora*, *Lamium purpureum*, *Solanum nigrum* and *Stellaria media* [50]. The addition of 1–2 g-a.i. ha^{-1} of iodosulfuron-methyl-sodium improves the level of weed control on broadleaf weed species, including *Chenopodium album*, *Galium aparine*, *Fallopia convolvulus*, *Ipomoea* spp., *Polygonum aviculare*, *P. lapathifolium*, *Sonchus arvensis* and *Xanthium strumarium*.

The basis of selectivity of foramsulfuron in the presence of the safener isoxadifen-ethyl is a more rapid rate of metabolic detoxification in maize compared with target weeds, in which little or no degradation of the parent sulfonylurea occurs [51]. Three main routes of metabolism have been established in maize – a hydrolytic cleavage of the sulfonylurea bridge, a deformylation of the amino group and oxidative metabolism of the dimethoxypyrimidine ring.

Foramsulfuron is commercialized with the safener isoxadifen-ethyl under the trade names ''Option®'' and ''Equip®'', whereas in combination with iodosulfuron-methyl-sodium the ternary mixture is sold as ''MaisTer®'', ''Mester®'', ''Fortuna®'' or ''Equip® Plus'' and ''Option® 360'' (Table 2.2.22). The

Table 2.2.22 Foramsulfuron-based products, formulations and composition.

Foramsulfuron-based products	Formulation type	Foramsulfuron	Iodosulfuron	Isoxadifen-ethyl
Option®$_{WG}$	WG 70[a]	350[b]		350[b]
MaisTer®$_{WG}$, Mester®, Fortuna®	WG 61[a]	300[b]	10[b]	300[b]
Equip®	OD 05[c]	22.5[d]		22.5[d]
Equip®Plus, Option® 360	WG 62[a]	300[b]	20[b]	300[b]

[a] WG: water dispersible granules.
[b] Units: g-a.i. kg^{-1}.
[c] OD: oil dispersion.
[d] Units: g-a.i. L^{-1}.

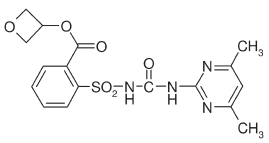
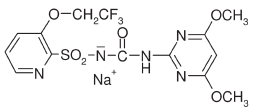
combination of the two herbicides probably make "MaisTer®" and "Mester®" the widest-spectrum maize herbicides used in Europe today.

2.2.2.4 Other Crops

Soybeans are the number one oilseed crop world-wide. In 2005, a total of 210 mio metric tonnes of soybean were produced. Relatively few countries produce soybeans: the USA accounts for more than 40% of the world production, with Brazil, Argentina and China together accounting for an additional 55%. In Europe, Italy, Russia and the Ukraine are the main producer countries. In the USA, Brazil and Argentina, the most widely planted soybeans are genetically modified varieties (GMO), which are tolerant against the herbicide glyphosate.

Sugarcane and cotton also represent important crops that benefit from newer sulfonylurea herbicides. Table 2.2.23 shows the most recent compounds introduced after 1995.

Table 2.2.23 Other sulfonylurea herbicides for use in soybeans, cotton and sugarcane.

Chemical structure	Common name (company, launch year)	Crop	Agricultural utility	Application rate (g-a.i. ha^{-1})
	Oxasulfuron (Syngenta, 1996)	Soybeans	Broadleaf weeds	45–90
	Trifloxysulfuron-sodium (Syngenta, 2001)	Sugar cane, cotton, turf	Sedges and broadleaf weeds	5–23

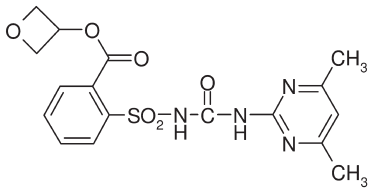
2.2.2.4.1 Oxasulfuron

Oxasulfuron (CGA 277476) (Table 2.2.24) [52] was launched in 1996 by Syngenta as a preemergent and postemergent herbicide. At application rates of 66–92 g-a.i. ha^{-1}, it provides greater than 80% control of *Abutilon theophrasti*, *Xanthium strumarium*, *Amaranthus* spp., *Ambrosia artemisiifolia*, *A. trifida*, *Bidens pilosa*, *Cyperus esculentus*, *Polygonum pensylvanicum*, *Sorghum bicolor*, *Echinochloa crus-galli*, *Helianthus annuus*, *Sesbania exaltata* and *Ipomoea* spp. [53] in soybeans. The observed selectivity is due to rapid metabolization in the target crop.

2.2.2.4.2 Trifloxysulfuron-sodium

Trifloxysulfuron-sodium (CGA 362622) (Table 2.2.25) [54] is a post-emergence herbicide commercialized by Syngenta in 2001 for use in all major cotton and

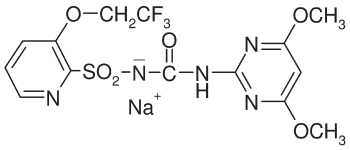
Table 2.2.24 Physicochemical properties of oxasulfuron.



Common name (ISO)	Oxasulfuron
CAS-No.	144651-06-9
Code numbers	CGA 277476
Melting point	158 °C (decomposition)
Vapor pressure	$<2 \times 10^{-6}$ Pa (25 °C)
Dissociation constant (temperature not published)	pK_a 5.10
Solubility in water (g L^{-1} at 25 °C)	0.052 (pH 5.1) 0.063 (pH 5.0, buffer solution) 1.70 (pH 6.8, buffer solution) 19.0 (pH 7.8, buffer solution)
Solubility in organic solvents (g L^{-1} at 25 °C)	Acetone 9.3 Ethyl acetate 2.3 Dichloromethane 69.0 *n*-Hexane 0.0022 Toluene 0.32
Partition coefficient (log P_{OW}) in octanol–water (25 °C)	0.75 (pH 5.0) −0.81 (pH 7.0) −2.2 (pH 9.0)

sugarcane production areas [55]. In cotton, it is formulated as a WG 75 and can be applied postemergent at 5–7.5 g-a.i. ha^{-1} in conventional or GMO cotton and at higher rates of 10–15 g-a.i. ha^{-1} postemergent directed. At the lower rates, the following weeds are controlled: *Acanthospermum hispidum*, *Ambrosia artemisiifolia*, *Bidens pilosa*, *Senna obtusifolia*, *Cassia occidentalis*, *Chenopodium album*, *Euphorbia heterophylla*, *Ipomoea* spp., *Melochia corchorifolia*, *Mollugo vertillata*, *Sesbania exalta*, *Trianthema portulacastrum*, *Xanthium strumarium*. With post-directed sprays and higher dosages, additional control is achieved of *Ageratum conyzoides*, *Amaranthus hybridus*, *A. palmeri*, *Cyperus esculentus* and *Tridax procumbens*. Application of trifloxysulfuron-sodium may be made after cotton (picker-type varieties only) has reached a minimum of five true leaves, with applications continuing until 60 days before harvest. Due to reduced crop tolerance, the product is not recommended as a postemergent over-the-top spray on stripper-type cotton varieties.

Table 2.2.25 Physicochemical properties of trifloxysulfuron-sodium.



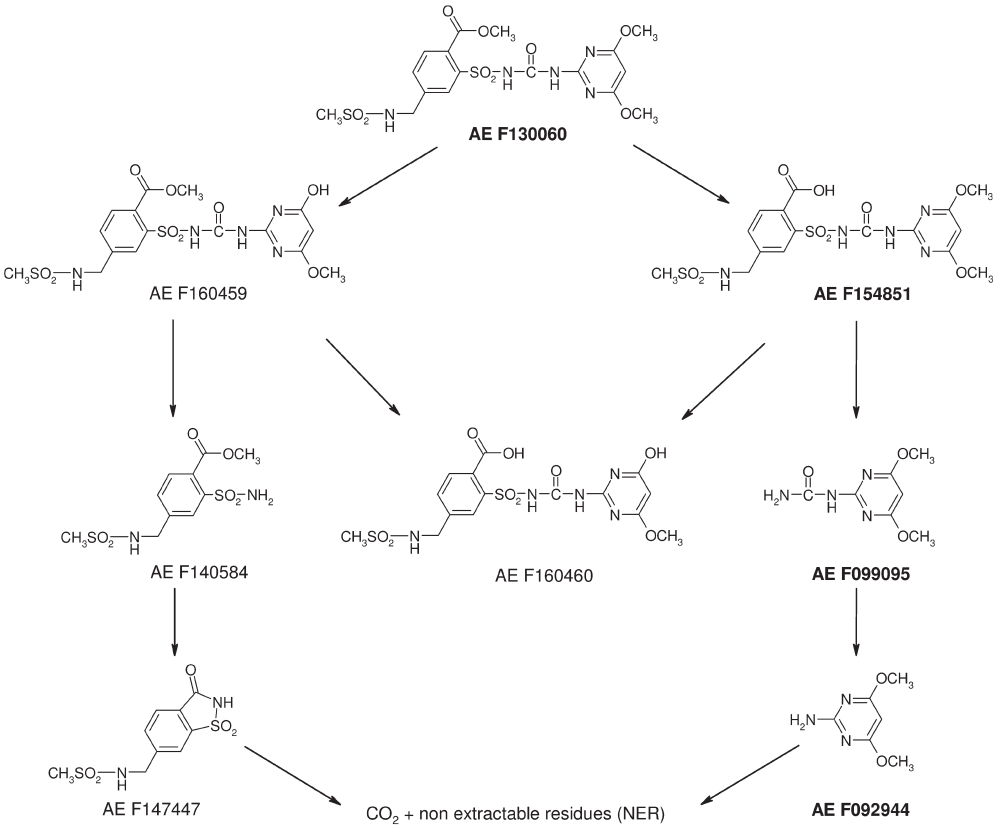
Common name (ISO)	Trifloxysulfuron-sodium	
CAS-No.	199119-58-9	
Code numbers	CGA 362622	
Melting point	170.2–177.7 °C	
Vapor pressure	$<1.3 \times 10^{-6}$ Pa (25 °C)	
Dissociation constant (at 20 °C)	pK_a 4.76	
Solubility in water (g L^{-1}) (25 °C)	0.063 (pH 5.0) 5.016 (pH 7.0) 25.7 (pH 7.4)	
Solubility in organic solvents (g L^{-1} at 25 °C)	Acetone Ethyl acetate *n*-Hexane Methanol Octanol Toluene	17.0 3.8 <0.001 50.0 4.4 <0.001
Partition coefficient (log P_{OW}) in octanol–water (25 °C)	1.4 (pH 5.0) −0.43 (pH 7.0)	

In cotton and sugarcane trifloxysulfuron-sodium is commercialized under the trade name "Envoke®" as a straight product. In cotton it is used in combination with prometryn as "Suprend®" and in sugarcane in combination with ametryn as "Krismat®". In sugarcane "Envoke®" can be used for a maximum of three applications pre-spiking, post-emergence over-the-top, and/or post-emergence directed at a total rate of 78 g-a.i. ha^{-1} per season. The product may be applied to sugarcane at a plant height of 45–60 cm up to 100 days before harvest. At a dose rate of 16 g-a.i. ha^{-1} the following weeds are controlled with greater than 85% efficacy: *Alternanthera philoxeroides*, *Acanthospermum hispidum*, *Panicum adspersum*, *Mollugo vertillata*, *Xanthium strumarium*, *Cassia occidentalis*, *Gnaphalium pensylvanicum*, *Eupatorium cappilliforium*, *Desmodium tortuosum*, *Trianthema portulacastrum*, *Sesbania exaltata*, *Rottboellia cochinchinensis*, *Chenopodium album*, *Ipomoea* spp., *Cyperus esculentus*, *C. rotundus*, *Amaranthus* spp., *Ambrosia artemisiifolia*, *Melochia corchorifolia*, *Senna obtusifolia*, *Bidens bipinnata*, *Linaia canadensis*, *Abutilon theophrasti* and *Euphorbia heterophylla*.

2.2.3
Metabolic Fate and Behavior in the Soil

There is abundant knowledge about the metabolic fate of sulfonylurea herbicides. However, especially with regard to animal data to support product registrations, most of this information is as yet unpublished. For readers who are interested in more information on plant metabolism and crop selectivity, reference is given to articles by Brown et al. [56]. Another excellent review article on the metabolic fate of sulfonylurea herbicides is found in Part 1 of the *Metabolic Pathways of Agrochemicals* series also authored by Brown et al. at Du Pont [57].

In the soil, there are two major pathways of sulfonylurea degradation [58]: (a) chemical hydrolysis and (b) microbial degradation. The breakdown of sulfonylureas in sterile soils is solely attributable to chemical hydrolysis, whereas breakdown in non-sterile soils is a combination of both microbial degradation and



Scheme 2.2.3. Metabolic pathway of mesosulfuron-methyl in soil under aerobic and anaerobic conditions (compounds labeled in bold were detected at >10% of the applied radioactivity).

chemical hydrolysis. The relative importance of the microbial degradation can then be calculated from the differential rate.

The main soil degradation pathways of sulfonylurea herbicides are cleavage of the sulfonylurea bridge, *O*- and *N*-dealkylation reactions, aryl and aliphatic hydroxylation reactions, dehalogenation and ester hydrolysis. It is not within the scope of this chapter to discuss each of these in detail for all of the above-mentioned new sulfonylureas. Instead mesosulfuron-methyl is taken below as a general illustration of commonly found soil degradation pathways established within the sulfonylurea family.

Mesosulfuron-methyl is degraded in soil and water via hydrolysis and *O*-demethylation reactions. Its metabolites are also readily degraded to non-extractable-residues (NER) and CO_2 [59]. During soil metabolism studies with radiolabeled mesosulfuron-methyl, major metabolites found, representing more than 10% of the applied radioactivity, were mesosulfuron acid AE F154851, pyrimidinyl urea AE F099095 and aminopyrimidine AE F092944 (Scheme 2.2.3). *O*-Demethylation of the methyl ether in the pyrimidine moiety to yield hydroxypyrimidine derivative AE F160459 proved to be of minor relevance in soil. Other minor soil metabolites were the sulfonylurea AE F160460, the sulfonamide AE F140584 and the saccharin derivative AE F147447. Carbon dioxide and unidentified non-extractable residues (NER) bound to the soil matrix were the final products of the degradation in the soil.

2.2.4
Concluding Remarks

Twelve new sulfonylurea herbicides for all major crops have been commercialized since 1995 and four new compounds from this class are currently in their late development stage. These together with the 20 sulfonylurea products that already have been on the market prior to 1995 give a remarkable figure, outnumbering any other herbicidal class in modern crop protection. The reason for this is a combination of the environmental friendliness of the products, their versatility as regards applicable crops and timing flexibility and also their cost/benefit performance. It remains to be seen whether the market can accept yet further innovations from this class, and whether resistant weed development will one day become an issue despite hitherto successful resistance strategies employed by the agro-industry.

In conclusion, it is fascinating to see the development that began with George Levitt's pioneering work at Du Pont over 30 years ago. In "Gulliver's Travels" (*Voyage to Brobdingnag*, Ch. 6), the Irish author Jonathan Swift (1667–1745) wrote that

> Whoever could make two ears of corn or two blades of grass grow upon a spot of ground where only one grew before, would deserve better of mankind, and do more essential service to his country than the whole race of politicians put together.

It is against this background that the achievements of George Levitt and all other colleagues involved in the world's agrochemical industry should be viewed.

Acknowledgments

The author is pleased to acknowledge Drs. Darren Mansfield, Klaus-Helmut Mueller, Graham Holmwood, Arno Schulz and Shinichi Shirakura for their critical review of the manuscript and many helpful suggestions, and Mrs. Tong Lin and Mr. Tetsuya Murata for translations of Chinese and Japanese publications.

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2.3
Imidazolinone Herbicides

Dale L. Shaner, Mark Stidham, and Bijay Singh

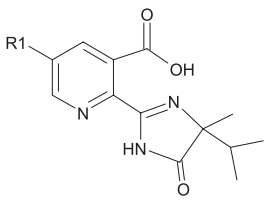
2.3.1
Overview

The imidazolinone herbicides (Table 2.3.1) are a family of six compounds that were discovered and developed by American Cyanamid Corporation. Readers may obtain comprehensive and detailed information in *The Imidazolinone Herbicides* [1], a book authored by the researchers who discovered and developed the herbicides. The herbicides as a class are broad spectrum and are active both pre- and postemergence. Imidazolinones are absorbed and moved through both xylem and phloem, eventually accumulating in the meristematic tissue. Activity is characterized by rapid cessation of growth followed by plant death days or weeks after treatment. Selectivity is based most often on metabolic inactivation except for selection-developed target site based resistance.

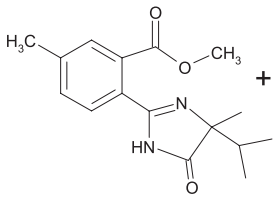
Synthesis methodology for numerous imidazolinones is described in the patent literature [2–6]. Figure 2.3.1(A) shows a simple one-step method [7].

Imidazolinones are generally formulated as the amine salts. Perhaps because of their high potency, broad spectrum, and high water solubility, the imidazolinones have been co-formulated with many other herbicides.

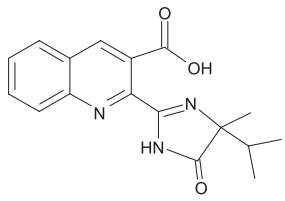
Table 2.3.1 Structure of commercialized imidazolinones.



R1	Common Name
H	imazapyr
CH_3	imazapic
CH_2CH_3	imazethapyr
CH_2OCH_3	imazamox



Imazamethabenz-methyl



Imazaquin

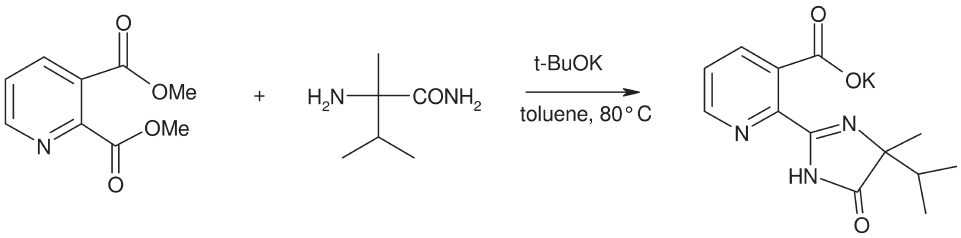


Fig. 2.3.1. Synthesis method for imidazolinones.

2.3.2 History of Discovery

The imidazolinone herbicides were discovered through a long process of observation, exploration, and optimization. The account here has been presented in greater detail elsewhere [2, 3]. The initial lead molecule **1** was synthesized in the 1950s by an American Cyanamid Medical Division chemist working on anticonvulsives (Fig. 2.3.2). The compound came years later to the Agricultural Division for random screening, where it showed herbicidal activity at 4 kg ha^{-1}, sufficient for additional synthesis effort. The mode of action was not known or even investigated at the time, but years after the discovery of the imidazolinones, this original phthalimide was shown to be an inhibitor of acetohydroxyacid synthase (AHAS).

Initial modifications did not improve the herbicidal activity, but derivative **2** showed interesting plant growth regulant activity similar to gibberellic acid (Fig. 2.3.2) [4, 5]. This new compound was further optimized for plant growth regulation, resulting in **3**.

Associated work to enable production of field trial samples produced a tricyclic compound, and in the spirit of comprehensive exploration (and thorough patent coverage), the same reaction was attempted on the original herbicide lead compound, resulting in **4** (Fig. 2.3.3). This compound showed broad-spectrum herbi-

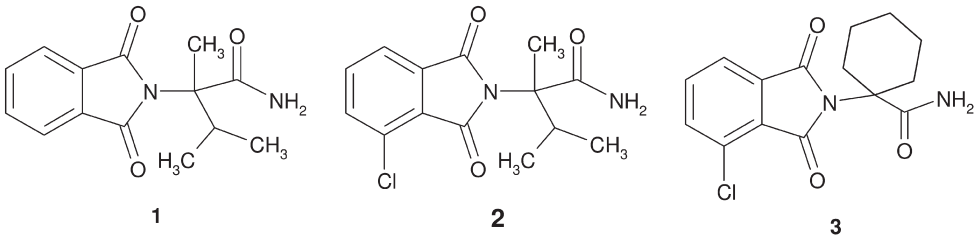


Fig. 2.3.2. Early lead compounds that led to the imidazolinones.

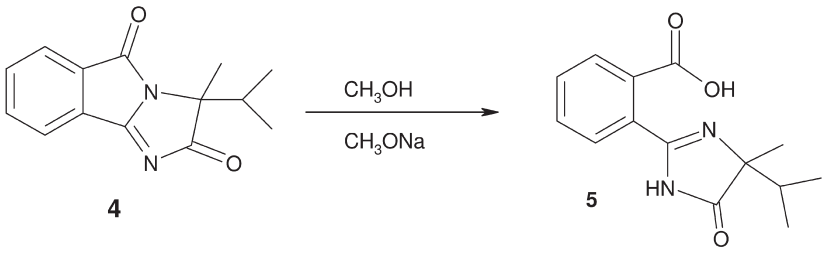


Fig. 2.3.3. Synthesis of first imidazolinone lead.

cidal activity, and continued exploration in the series resulted in the first imidazolinone **5** (Fig. 2.3.3). This compound had markedly improved herbicidal spectrum and potency with some selectivity in rice.

Work continued in this program, eventually resulting in **6**, the isomer mixture imazamethabenz-methyl, a wheat-selective herbicide (Fig. 2.3.4).

A quantum leap in herbicidal potency and spectrum occurred when the benzene ring was replaced with a pyridine ring. The resulting compound had pre- and postemergence activity at doses in the range 10–100 g ha^{-1} in greenhouse tests. Exploration of this new series demonstrated that the picolinic acid and isonicotinic acid had far less herbicide activity than the nicotinic acid. Also, high activity is maintained only in derivatives with substituents at the 5- and 6-position of the pyridine ring. Thus, unlike the other major classes of AHAS-inhibiting herbicides, the imidazolinones have a relatively narrow structure–activity pattern for weed control [8].

2.3.3
Physical Chemical Properties

The imidazolinone salts have high water solubility, ranging from >57% (imazapyr/isopropylamine salt) to 17% (imazaquin ammonium salt). Imazapyr has two sites for protonation, namely the imidazolinone secondary nitrogen and

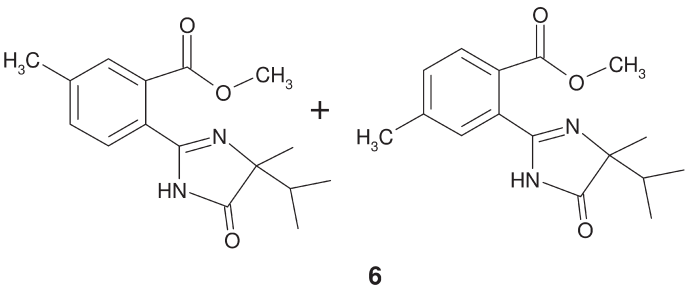


Fig. 2.3.4. Structure of imazamethabenz-methyl.

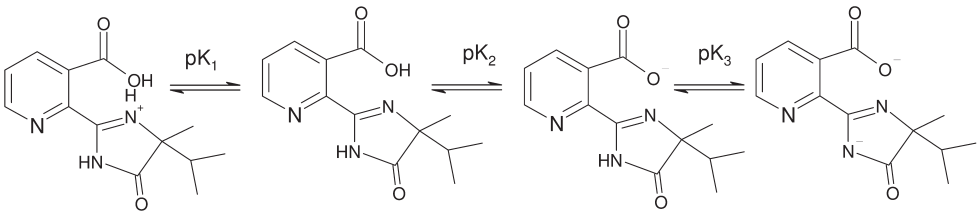


Fig. 2.3.5. Ionization states of imidazolinones.

the carboxylic acid substituent on the pyridine ring. The ionization constants are relatively similar for the pyridine imidazolinone herbicides; for imazapyr, pK_1 is 1.9 and pK_2 is 3.6 (Fig. 2.3.5). A third ionization on the primary imidazolinone nitrogen occurs at pH ~ 11 (Fig. 2.3.5) [9]. The pK_2 is important for concentrating the herbicide inside the cell through a weak acid trapping mechanism. Outside the cell in the apoplast, a relatively low pH allows a substantial proportion of the imidazolinone to exist in an uncharged state, with enough lipophilicity to passively cross cell membranes. Once inside the cell, the pH is much higher and the charged form predominates, effectively trapping the herbicides inside the cell [10].

2.3.4
Structural Features of Herbicidal Imidazolinones

The structural features of imidazolinones important for target site and herbicide activity have been summarized [11–14]. The orientation of the imidazolinone ring ortho to the acid equivalent is critical. Derivatives of the acid equivalent are herbicidally active if they can be metabolized to the acid either in the soil or in the plant. Likewise, tricyclic derivatives such as **4** are pro-herbicides that must be metabolized to the acid-imidazolinone form.

The commercial herbicides are a mixture of R and S isomers at the chiral center where the methyl and isopropyl substituents are placed, but the (R)-isomer is approximately ten-fold more potent both as an enzyme inhibitor and as an herbicide. Substituents other than methyl and isopropyl are substantially weaker enzyme inhibitors an herbicides [14].

The aromatic ring component illustrates the relative contributions of enzyme inhibition and physicochemical properties to herbicidal activity. The benzene imidazolinones are approximately ten-fold more potent than the corresponding pyridine derivatives as enzyme inhibitors but are less potent as herbicides.

The primary factor that determines the biological activity of the imidazolinones besides the inhibition of AHAS is their ability to translocate to meristematic tissue. AHAS, the target site for these herbicides, functions primarily in rapidly dividing tissue and decreases rapidly as tissue matures [15]. Thus, the difference in herbicidal activity among the six commercial imidazolinones depends on differences in their ability to be absorbed and translocate within the plant. Imazaquin

is primarily used as a soil applied herbicides. This imidazolinone is the most lipophilic of all the commercial herbicides and is the most readily absorbed by roots and translocated to the shoot [16, 17]. Imazapyr and imazamox, in contrast, are the least lipophilic and are also the most active when applied to the foliage [16, 17]. Imazethapyr and imazapic fall in between these two extremes.

The differences in herbicidal activity among these analogs appear to be related to their ability to be trapped in the phloem. The imidazolinones are absorbed into phloem via an ion-trapping mechanism as described previously. Thus, all imidazolinones can penetrate the phloem and will be carried to meristematic tissue. However, the concentration of the herbicide that actually reaches the meristems is a function of how rapidly the chemicals diffuse out of the phloem as it moves through the plant. Imazaquin will diffuse out of the phloem more readily than imazamox or imazapyr because it is more lipophilic. Thus, it will not be carried as far and has limited postemergent activity compared with imazamox or imazapyr. As mentioned previously, the benzene imidazolinones are not as herbicidally active as the pyridine imidazolinones, although the former analogs are more potent inhibitors of AHAS. The benzene imidazolinones are more lipophilic than the pyridine imidazolinones and hence are not trapped in the phloem as well.

There may be other factors governing the herbicidal activity of the imidazolinones. The position of the nitrogen in the pyridine ring in relation to the carboxylic and imidazolinone ring substitution is critical, although inhibition of AHAS is unaffected by the relative position of the nitrogen in the imidazolinone ring in relation to the substitutions [16]. Cellular uptake of the imidazolinones is affected by the relative position of the nitrogen to the carboxylic acid moiety. Hawkes et al. have shown that if the nitrogen in the pyridine ring is not ortho to the carboxylic acid group, the compound is not absorbed by the cell [18]. The mechanism of this differential uptake is not known. If an imidazolinone is not absorbed or translocated well within the plant, it is not herbicidal.

2.3.5 Mode of Action of Imidazolinones

Although plant growth stops soon after application of the imidazolinone herbicides, death of the whole plant may take 2–3 weeks. Meristematic tissues exhibit chlorosis and necrosis first followed by slow necrosis of the mature tissues. Physiological changes resulting from herbicide treatment include changes in metabolite concentrations [19, 20], reduction of assimilate transport [21, 22], inhibition of DNA synthesis [23, 24] and cell division [23, 25]. These physiological effects in plants result from inhibition of AHAS, the first enzyme in the biosynthesis of branched chain amino acids, valine, leucine and isoleucine. Supplementation of plants with branched chain amino acids reverses the effects of herbicide [21] which suggests that starvation for branched chain amino acids is the primary cause of plant death [26].

The I_{50} for various commercial imidazolinone herbicides *in vitro* AHAS assays varies between 0.1 and 10 μM depending upon the assay conditions [27]. Under

in vivo conditions, binding of imidazolinones seems to cause irreversible loss of AHAS activity [28]. The level of AHAS activity extracted from plants treated with lethal dose of an imidazolinone herbicide is reduced more than 80%. This effect of inhibitors can be discerned within an hour after treatment and the loss of AHAS activity is proportional to the concentration of the inhibitor in the plant tissue. There are several possible reasons for the loss of extractable AHAS activity in the imidazolinone treated plants. The imidazolinones may interact with the enzyme in such a way *in vivo* that the herbicide does not easily separate from the enzyme during the extraction procedure; the herbicide causes a change in the protein structure such that it is enzymatically inactive; or the inhibitor bound enzyme is easily degraded by the proteases. The last possibility was ruled out by immunoassay studies (Bijay Singh, unpublished). Binding of imazethapyr with AHAS appears to stabilize the AHAS protein in relation to other proteins that are degraded after the herbicide treatment [29].

2.3.6
Imidazolinone-tolerant Crops

Owing to many desirable properties of imidazolinone class of chemistry, development of imidazolinone-tolerant crops began in early 1980s, the same time when different imidazolinone herbicides were being discovered and developed for commercialization. This example is probably the first in which selection for a herbicide tolerant crop began so early in the development of a class of herbicides. During this research, Anderson and Georgeson [30] were successful in obtaining imidazolinone-tolerant maize plants through tissue culture selection and regeneration. Subsequent research showed that resistance at the whole plant was a semidominant trait that resulted from an alteration in the gene encoding AHAS. This early work not only proved that imidazolinone-tolerant crops could be selected, but it also led to the discovery of the site of action of this class of herbicides and to the development of other imidazolinone-tolerant crops.

Plants tolerant to imidazolinones have been produced by both transgenic and non-transgenic mechanisms. However, all of the imidazolinone-tolerant crops currently being sold have been developed by non-transgenic methods. The first imidazolinone-tolerant crop (maize) was introduced in 1992. Subsequently, four additional imidazolinone-tolerant crops (canola, rice, wheat and sunflower) have been commercialized [31]. All of the imidazolinone-tolerant crops are being sold under the CLEARFIELD™ trade name.

These imidazolinone tolerance traits in different crops were developed by various methods. These methods included tissue culture selection (maize), pollen mutagenesis (maize), microspore selection (canola), seed mutagenesis (wheat and rice) and incorporation of resistance trait from a weedy relative (sunflower). Details of these methods have been previously reviewed [31, 32]. In all of these cases, the basis of tolerance is due to the presence of an altered form of AHAS that is resistant to inhibition by imidazolinones. The resistant enzyme is produced due to a single base pair change in the gene encoding the large subunit

of AHAS that results in a single amino acid change in the mature protein. Several mutations in the gene encoding the large subunit of AHAS have been identified that confer tolerance to imidazolinones [31, 33]. Specifically, the amino acid changes identified in different imidazolinone-tolerant crops are Ala205Val (sunflower; amino acid number in reference to AHAS sequence from *Arabidopsis thaliana*), Trp574Leu (maize and canola), Ser653Asn (maize, canola, wheat and rice), and Gly654Glu (rice). The amino acid changes that confer tolerance to imidazolinones are distributed over the entire primary structure of the AHAS protein. However, these amino acids reside in a pocket of the folded protein in the quaternary structure of the enzyme [34, 35].

From the imidazolinone family, four different molecules, imazapyr, imazapic, imazethapyr and imazamox, have been registered for weed control in various imidazolinone-tolerant crops in different regions of the world. These herbicides are applied alone or in combination with other imidazolinones or with other classes of herbicides for a broad spectrum, season-long weed control. A combination of different imidazolinone tolerance traits and multiple herbicide options provides an effective weed management tool for farmers around the world.

2.3.7 Commercial Uses of the Imidazolinone Herbicides

Six imidazolinones are commercially available. These herbicides have extremely low toxicity or are non-toxic to mammals, birds, invertebrates and fish [36]. The crops on which these herbicides are registered and whether or not they are applied to foliage or to the soil is determined by the structure of the chemical (Table 2.3.2). When applied to the foliage of plants, a non-ionic surfactant or oil adjuvant is required for maximum activity. The addition of either urea or another form of nitrogen can also increase herbicidal activity.

Imazamethabenz methyl is strictly applied postemergent to most major varieties of wheat (spring and winter), barley (spring and winter) and rye as well as some varieties of winter triticale and sunflower and safflower. Imazamox is used postemergent in leguminous crops, including soybeans, alfalfa and edible beans, as well as in imidazolinone resistant wheat, sunflower, rice, and canola. Imazaquin, though, is primarily a soil applied herbicide that is used in soybeans, established bermudagrass, centipedegrass, St. Augustinegrass, zoysiagrass, and selected landscape ornamentals. Imazethapyr is used both postemergent and preemergent in soybeans, edible beans, alfalfa, peanut, and imidazolinone resistant maize, rice, and canola. Imazapic is also applied both to the foliage and the soil in peanuts, rangeland, sugarcane, and imidazolinone resistant canola, maize, wheat, and rice. Imazapyr controls the broadest spectrum of weeds of the imidazolinones, but has selectivity on many coniferous species as well as date and oil palms. It is used for weed control and site preparation in pines and date and oil palms. It is also used in non-crop sites for control of weedy vegetation and/or maintenance of bare ground as well as in imidazolinone resistant maize and sunflower.

Table 2.3.2 Registered uses of imidazolinone herbicides in the U.S.A.

Imidazolinone	Application	Crop	Imidazolinone-resistant crop
Imazamethabenz methyl	Foliar	Barley, wheat, sunflower	
Imazethapyr	Foliar and soil	Edible beans, peas, soybean, lentils, alfalfa, peanuts, clover, birdsfoot trefoil, crown vetch, lupine, switchgrass, wheatgrass, little bluestem, orchardgrass, western wheatgrass, big bluestem, canarygrass	Maize, rice, canola
Imazamox	Foliar	Soybeans, chicory, peas, edible beans, alfalfa, clover	Canola, wheat, sunflowers
Imazapyr	Foliar and soil	Forest lands, wetlands, noncrop areas, roadsides, bahiagrass, bermudagrass	Maize
Imazaquin	Foliar and soil	Soybeans, yucca, hosta, bermudagrass, centipedegrass, mondo grass, pachysandra, St. augustinegrass, zoysiagrass, liriope, crape myrtle, gardenia, Indian hawthorn, wax-myrtle, dwarf yaupon, holly, Fraser photinia, Pfitzer juniper	
Imazapic	Foliar and soil	Peanuts, sugar cane, pastures, rangeland, ornamental turf, ditch banks, conservation reserve program land, noncrop areas	

2.3.8 Mechanisms of Selectivity

Crop selectivity of the imidazolinones is primarily dependent on differential metabolism of the herbicide between the crops and targeted weeds. For the 5′-substituted imidazolinones (i.e., imazethapyr, imazamox, imazapic, and imazamethabenz-methyl) detoxification of the herbicides is through a mixed function oxidase that hydroxylates the substitution followed by conjugation of the metabolite to glucose through the hydroxyl group [36] (Fig. 2.3.6). Imazapyr and imazaquin are metabolized via a different route in which there is a condensation between the carboxylic acid on the aromatic ring to nitrogen in the imidazolinone ring followed by cleavage of the imidazolinone ring (Fig. 2.3.6). The half-life of

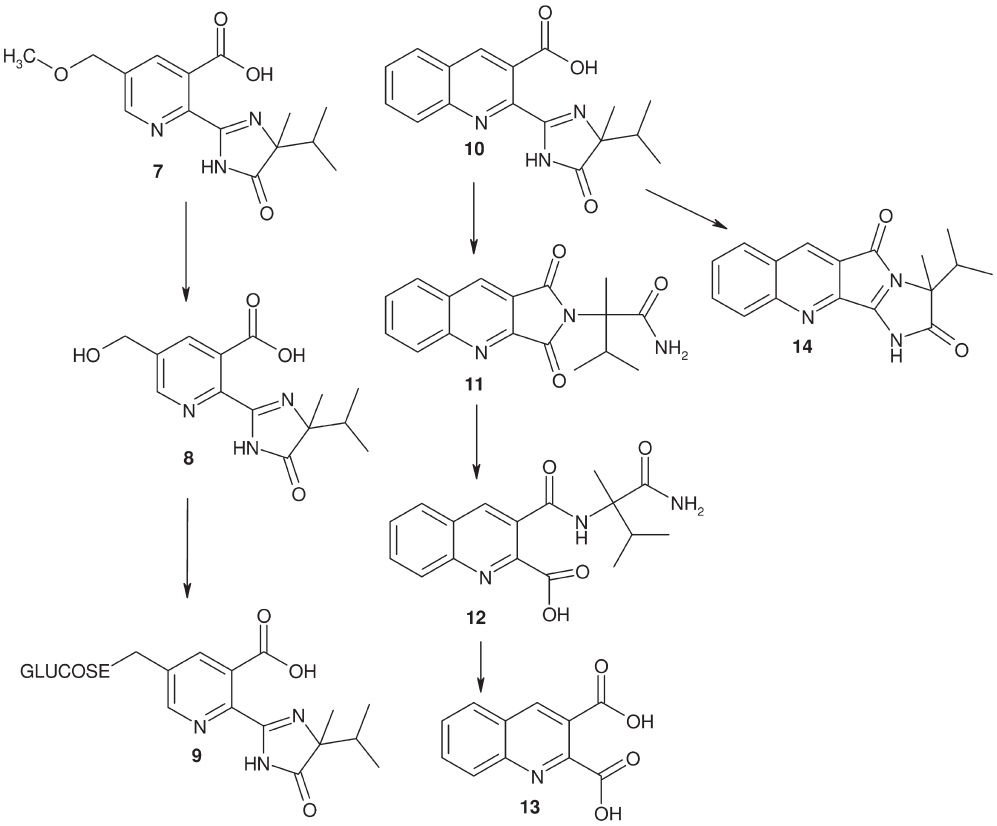


Fig. 2.3.6. General routes of metabolism of imidazolinones [36].

imidazolinones in naturally tolerant crops is less than 24 h [37]. Imidazolinone resistant crops contain a natural mutation in the AHAS gene which encodes an enzyme that no longer binds these herbicides, although metabolism may play a role in determining the level of tolerance of the resistant crop.

The weed spectrum of the imidazolinones is dependent on differential metabolism. Imazethapyr controls many broadleaf weeds and some grasses, but has limited activity on legumes and many composites. Imazamox, in contrast, has much better activity than imazethapyr on grasses. Imazapyr controls the broadest spectrum of weeds of all the imidazolinones, although it is not as active on legumes and composites. The reason for these differences is due to the ability of weeds to metabolize the herbicides. The half-life of imazethapyr in many grasses is less than 24 h because they can rapidly hydroxylate the 5′-ethyl substituent of imazethapyr [37]. However, most grasses are unable to rapidly hydroxylate the 5′-methoxyethyl substituent on imazamox [37]. Legumes and many composites can hydroxylate the 5′ substituent of both imazethapyr and imazamox. Since imaza-

pyr does not have any substituents on the pyridine ring, most species are unable to metabolize the herbicide. However, legumes and some composites can metabolize imazapyr via the mechanism described above [37].

2.3.9 Conclusion

The imidazolinone herbicides have been and continue to be highly successful products. The ability to mix and match different imidazolinones to take advantage of their differing weed control spectrum and pre- and postemergent activity has proved invaluable in designing products for imidazolinone-resistant crops throughout the world. Although the number of imidazolinone analogs that were commercialized is extremely small compared with other ALS inhibiting herbicides, these compounds fill vital niches in many weed management programs.

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2.4 Triazolopyrimidines

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2.4.1 Introduction

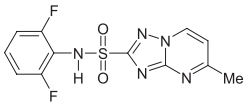
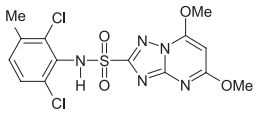
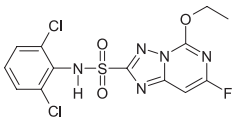
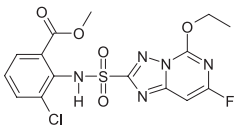
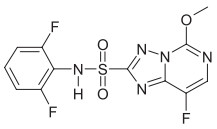
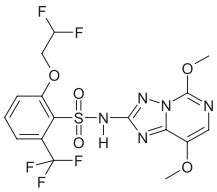
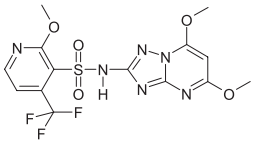
Triazolopyrimidine sulfonamides and related compounds have been studied extensively since their discovery in the early 1980s. The initial lead was discovered while examining bioisosteric relationships to the sulfonyl ureas [1]. Further investigations of structure–activity relationships around this lead eventually led to the triazolo[1,5-*a*]pyrimidine sulfonanilides and the discovery of flumetsulam (**1**) and metosulam (**2**) (Table 2.4.1). Flumetsulam was developed for use in maize and soybeans and metosulam was developed for use in maize and cereals. Studies have shown the triazolopyrimidine sulfonamides to be competitive with the amino acid leucine for binding to acetohydroxyacid synthase (AHAS) isolated from cotton (*Gossypium hirsutum*) [2]. The same study showed similar results for the sulfonylurea and imidazolinone herbicides. In addition, analysis of *Arabidopsis thaliana* mutants with resistance to AHAS-inhibiting herbicides identified a mutation that conferred resistance to triazolopyrimidine sulfonanilide and sulfonylurea herbicides but not to the imidazolinone herbicides [3]. Since those discoveries, additional work has led to the development of diclosulam (**3**) and cloransulam-methyl (**4**) for broadleaf weed control in soybeans and florasulam (**5**) for broadleaf weed control in cereals. Research efforts of new N-aryltriazoloazinyl sulfonamides, which include the triazolo[1,5-*a*]pyridine, the triazolo[1,5-*a*]pyrazine, N-triazolo[1,5-*c*]pyrimidine and N-triazolo[1,5-*a*]pyrimidine sulfonamides, led to the discovery of penoxsulam (**6**) and DE-742 (**7**). Penoxsulam was developed for broadleaf, grass and sedge weed control in rice and DE-742 is being developed for broadleaf and grass weed control in wheat.

2.4.2 N-Triazolo[1,5-*c*]pyrimidine Sulfonanilides

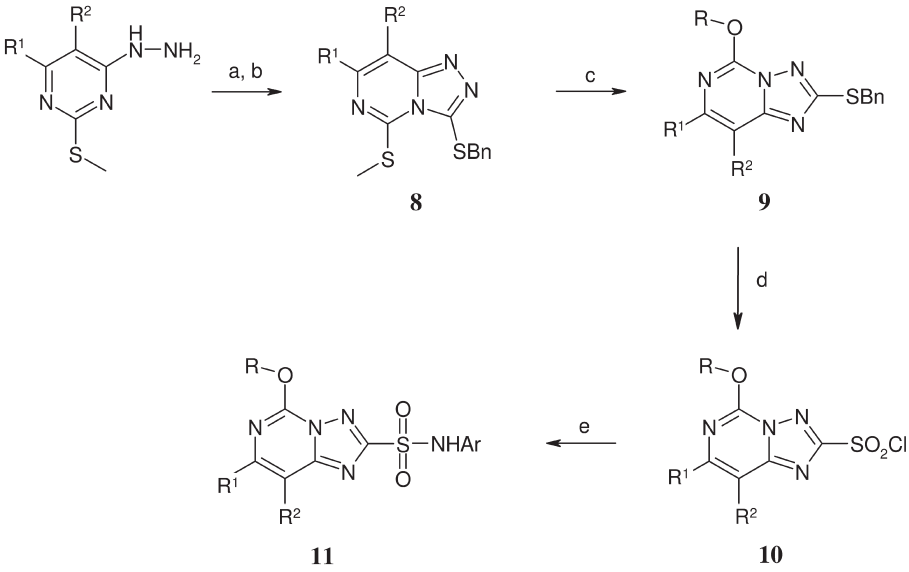
2.4.2.1 Synthesis

Synthetic routes leading to triazolo[1,5-*c*]pyrimidine sulfonanilides have been reviewed [4]. Scheme 2.4.1 shows a general synthetic route to the triazolo[1,5-*c*]pyrimidine sulfonanilides [5]. An appropriately substituted 4-hydrazino-2-methylthiopyrimidine is reacted with carbon disulfide followed by benzyl chloride to afford 3-benzylthio-5-methylthio-1,2,4-triazolo[4,3-*c*]pyrimidine (**8**). Compound **8** is then treated with methoxide to afford 2-benzylthio-5-methoxy-1,2,4-triazolo[1,5-*c*]pyrimidine (**9**). The benzyl sulfide (**9**) is oxidized to the sulfonyl chloride (**10**) by treatment with chlorine and water. The sulfonyl chlorides

Table 2.4.1 Commercial and developmental triazolopyrimidine sulfonamides.

	Chemical structure	Common name	Launch date	Log *P*	Melting point (°C)
1		Flumetsulam	1994	−0.68	251–253
2		Metosulam	1994	0.98	210–211
3		Diclosulam	1997	0.98	216–218
4		Cloransulam-methyl	1997	0.85	218–221
5		Florasulam	1999	−1.22	193–230
6		Penoxsulam	2004	−0.35	212
7		DE-742		1.83	194–195

are then reacted with N-trimethylsilylanilines in the presence of a catalytic amount of dimethyl sulfoxide or with anilines in the presence of a catalytic amount of dimethyl sulfoxide and pyridine to afford the desired sulfonanilides (**11**).



Scheme 2.4.1. (a) CS_2, dioxane, Et_3N; (b) BnCl; (c) NaOMe, MeOH, ethyl acrylate; (d) Cl_2, H_2O; (e) $ArNHSi(Me)_3$, DMSO (cat), CH_3CN or $ArNH_2$, pyridine, DMSO (catalytic), CH_3CN.

2.4.2.2 **Biology**

Unless otherwise noted, the *in vivo* greenhouse screening data presented in the following sections is a tabulation of postemergence foliar applied results and expressed as a "percent in growth reduction" (GR) for treated plants compared with untreated plants, where the rate identified provides the level of weed control or crop injury. The broadleaf weed activity (BW) is given as an average percent reduction in growth at a given concentration, as indicated, over five to eight broadleaf weeds chosen from the following: *Xanthium strumarium*, *Datura stramonium*, *Chenopodium album*, *Helianthus* spp., *Ipomoea* spp., *Amaranthus retroflexus*, *Abutilon theophrasti*, *Veronica heteraefolia*, *Ipomoea hederacea*, *Stellaria media* and *Polygonum convolvulus*. The grass weed activity (GW) is averaged over five weeds chosen from *Alopecurus* spp., *Echinochloa crus-galli*, *Setaria fabarii*, *Sorghum halapense*, *Digitaria sanguinalis* and *Avena fatua* and expressed in a manner similar to broadleaf weeds.

The general structure–activity relationships (SAR) for triazolo[1,5-*c*]pyrimidine sulfonanilides (**11**) have been described [4]. The SAR identified compounds with alkoxy in the 5-position (**11**, OR) and halogen or alkoxy in the 7- and 8-position (**11**, R^1 and R^2) as having the highest levels of activity. Further investigation identified compounds with halogen in the 7-position as having good levels of activity on broadleaf weeds and selectivity to soybeans. In addition, compounds with halogen in the 8-position were identified as having good activity on broadleaf weeds with selectivity to wheat.

2.4.2.2.1 Cloransulam-methyl and Diclosulam Crop Utility

Cloransulam-methyl and diclosulam are members of the triazolo[1,5-*c*]pyrimidine sulfonanilide family of AHAS-inhibiting herbicides. Both compounds show excellent crop selectivity, broad-spectrum broadleaf weed control and low toxicity. The herbicidal utility of cloransulam-methyl in soybeans was first presented in 1994 [6, 7] and further described in 1995 [8, 9] and 1996 [10–12]. Diclosulam was first described for use in soybeans and peanuts in 1997 [13] with additional description in 1998 [14] and 1999 [15–17].

Cloransulam-methyl was commercialized in the United States under the trade name FirstRate (Trademark of Dow Agrosciences, LLC) herbicide for the control of annual broadleaf weeds and certain perennial sedges in soybeans. Applications can be made preplant surface, preplant incorporated, preemergence and postemergence for the control of broadleaf weed species. Postemergence applications of cloransulam-methyl at 17.5 g-a.i. ha^{-1} or soil-applied treatments at rates of 35–44 g-a.i. ha^{-1} provide control of a large number of soybean relevant weeds. Cloransulam-methyl does not provide control of annual and perennial grass weeds or certain broadleaf weeds such as *Solanum* spp. [18].

Diclosulam is registered in the United States and in Latin America for use in peanuts and soybeans. Applications can be made preplant surface, preplant incorporated and preemergence at rates of 17.5–26 g-a.i. ha^{-1} for the control of numerous broadleaf weed species. Diclosulam does not provide control of annual and perennial grass weeds or certain broadleaf weeds such as *Solanum* spp.

2.4.2.2.2 Florasulam Crop Utility

Florasulam (**5**) provides excellent postemergence selectivity in turf and small grain cereal crops such as wheat, barley, oats, rye and triticale [19, 20]. The European and North American cereal markets are of primary commercial interest for florasulam due to its specialized spectrum of broadleaf weed control. Florasulam is highly active on economically important species in the Compositae, Caryophyllaceae, Cruciferae, Rubiacea and Leguminosae plant families at a typical use rate of 5 g-a.i. ha^{-1} [21]. Owing to the relatively short half-life in soil, only postemergence applications are used in commercial practice [22].

2.4.2.3 Mechanism of Crop Selectivity

2.4.2.3.1 Cloransulam-methyl and Diclosulam Mechanism of Crop Selectivity

The metabolism of triazolopyrimidine sulfonanilides (**1**–**4**) in plants has been reviewed [23, 24]. It has been shown that diclosulam (**3**) and cloransulam-methyl (**4**) are rapidly metabolized in soybeans by facile conjugation with homoglutathione which displaces the 7-fluoro substituent (Fig. 2.4.1) [25]. This mechanism was found to only occur in soybeans for **3** and **4**. Oxidation at the 4-position of the aniline ring occurs rapidly in maize for **3** and **4**. In wheat, **4** undergoes O-dealkylation of 5-ethoxy followed by glucose conjugation and oxidation at the 4-position of the aniline ring occurs for **3** [25].

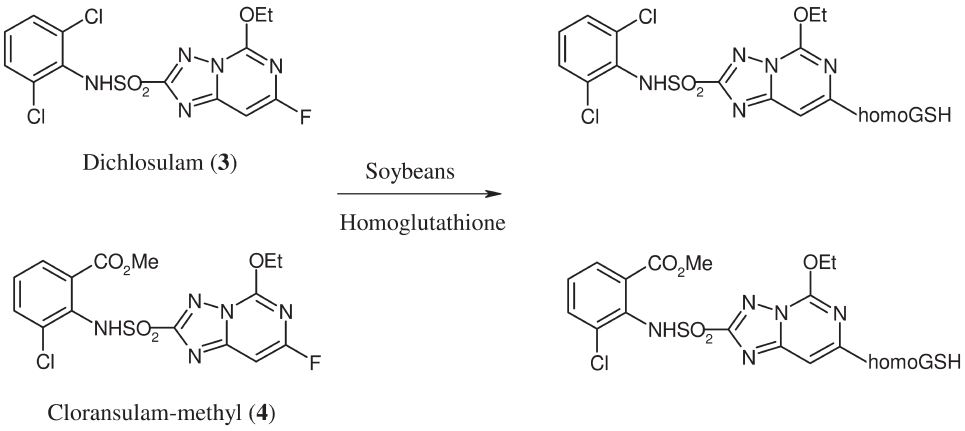


Fig. 2.4.1. Metabolism of diclosulam (**3**) and cloransulam-methyl (**4**) in soybeans (*Glycine max*).

2.4.2.3.2 Florasulam Mechanism of Crop Selectivity

The selectivity of florasulam (**5**) to wheat and the high level of herbicidal activity on important weeds are related primarily to the difference in rates of metabolism [26]. Florasulam has a half-life in wheat of 2.4 h as compared with a half-life in *Galeopsis tetrahit* L., *Polygonum papathifolium*, and *Galium aparine* L. of 19.8 h, 43.6 h and >48 h, respectively (Table 2.4.2). In wheat, florasulam has been shown to undergo rapid metabolism at the 4-position of the phenyl ring to give the 4-hydroxy metabolite which, in turn, is conjugated to glucose (Fig. 2.4.2). In contrast, slow metabolism is observed in *Galeopsis tetrahit* L. and *Polygonum papathifolium* with little degradation of florasulam observed in *Galium aparine* L., even at 48 h after treatment. Similar differences in the rate of metabolism in wheat compared with broadleaf weeds accounted for the sensitivity of broadleaf weeds to closely related analogs [24].

Table 2.4.2 Herbicidal activity and metabolism of **5**.

Species	GR_{50} (g ha^{-1})	$T_{1/2}$[a] (h)
Triticum aestivum	≫32	2.4
Galium aparine L.	≪2.5	>48 (202.4)
Galeopsis tetrahit L.	<2.5	19.8
Polygonum papathifolium	<2.5	43.6

[a] Time required for plants to metabolize 50% of the applied compound.

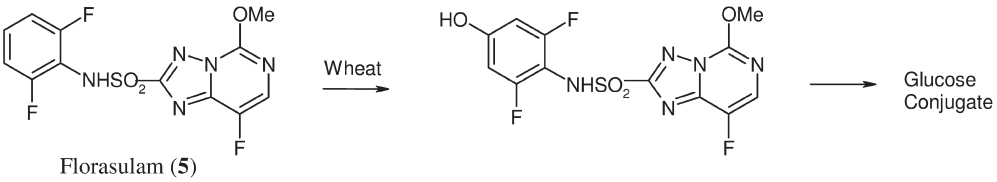
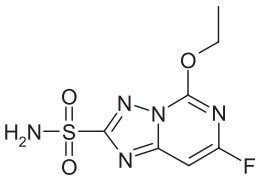


Fig. 2.4.2. Metabolism of florasulam (**5**) in wheat (*Triticum aestivum*).

2.4.2.4 Environmental Degradation, Ecotox and Tox

2.4.2.4.1 Cloransulam-methyl and Diclosulam Environmental Degradation

Metabolism in aerobic soils is a significant dissipation mechanism for both cloransulam-methyl (**4**) and diclosulam (**3**). Analysis of soil samples from bare-ground applications of cloransulam-methyl and diclosulam gave half-life ranges of 3–11 days and 13–43 days, respectively [27, 28]. Organic matter content and soil temperature were found to be the two factors that most influenced the soil degradation rates. Dealkylation of the 5-ethoxy on the triazolopyrimidine ring to form the associated 5-hydroxytriazolopyrimidine is a metabolic manipulation shared by both compounds. The shared aminosulfonyl triazolopyrimidine (**12**) was a metabolite identified in soil degradation studies for both compounds [29, 30]. Both compounds underwent additional, unique metabolic manipulations in the soil. Unextractable residues accounted for a significant amount of the final metabolite distribution for both cloransulam-methyl and diclosulam. Photolysis in water was also shown to be a significant avenue of degradation for cloransulam-methyl, with a half-life of less than 1 h [31]. Diclosulam does not show significant degradation by photolysis (half-life $>$ 100 days).



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Both cloransulam-methyl and diclosulam have low acute toxicology profiles and no indication of any chronic toxicology issues. Both compounds do show slight toxicity to *Daphnia* but are considered practically non-toxic to birds, insects, aquatic organisms and earthworms.

2.4.2.4.2 Florasulam

Florasulam (**5**) dissipates primarily through microbial degradation [22]. Other patterns of degradation or dissipation contribute minimally to the loss of florasu-

lam in the agricultural field environment. As florasulam degrades in the soil, several metabolites of the herbicide are formed. The primary soil metabolite, the 5-hydroxytriazolo[1,5-*c*]pyrimidine sulfonanilide analog of **5** (**5**-OH), has been shown to have limited plant activity (a factor of 100× or greater) relative to the parent while other metabolites are inactive. The field half-life of florasulam in soil ranges from 2 to 18 days. While soil pH, texture and level of organic matter influence rate of degradation, temperature has the greatest impact on soil half-life. In natural sediment and surface water in the dark at 20 °C, florasulam is degraded to the **5**-OH metabolite with a half-life of 9 to 29 days. In anaerobic conditions, the half-life was approximately 13 days. In water, the aqueous photolytic half-life was 4.9 days.

The overall toxicological profile of florasulam is very favorable. It is not acutely toxic, does not pose an inhalation hazard, nor is it a skin sensitizer. No evidence of mutagenic or carcinogenic potential was obtained from any study. It showed no teratogenic effects in either rats or rabbits. Tests also indicate that florasulam is not a reproductive hazard or concern.

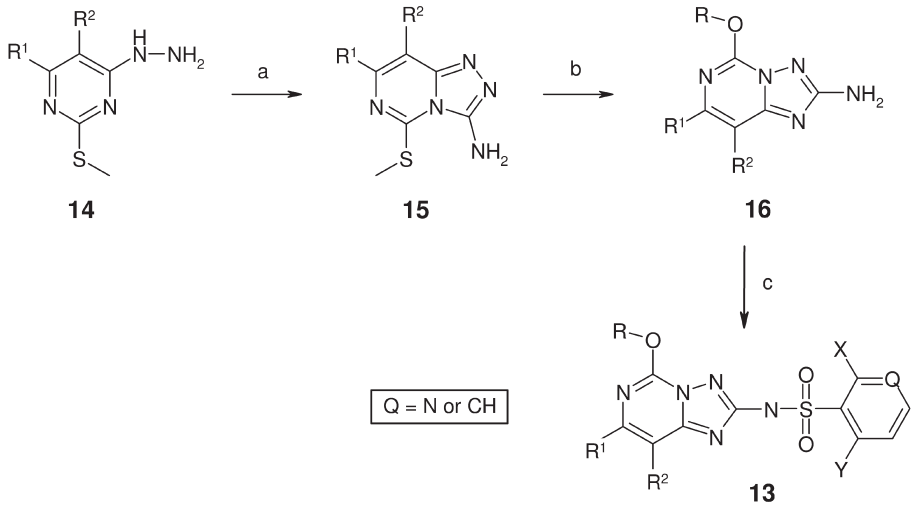
2.4.3
N-Triazolo[1,5-*c*]pyrimidine Sulfonamides

The N-triazolo[1,5-*c*]pyrimidine sulfonamides and related compounds differ from their sulfonanilide counterparts by the orientation of the linkage between the triazoloazine and the aryl or heteroaryl ring. However, in most cases synthesis of sulfonamides is similar to the sulfonanilides, as the target molecules are formed by reaction of a sulfonyl chloride and an amine in the final step. Notably, the synthesis of arylsulfonyl chlorides account for much of the diversity in these molecules [32–36].

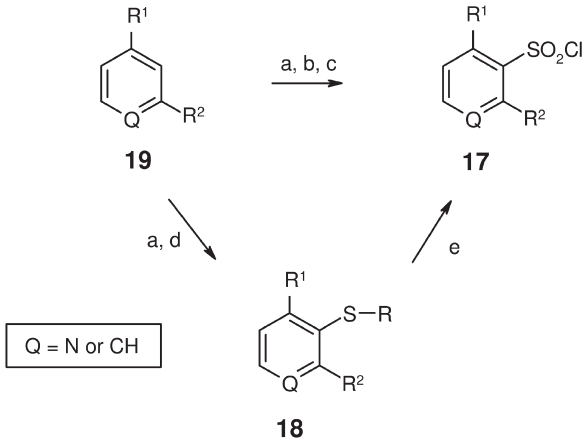
2.4.3.1 **Synthesis**

Scheme 2.4.2 outlines a straightforward and general route for the synthesis of N-triazolo[1,5-*c*]pyrimidine sulfonamides (**13**) [32, 35]. 4-Hydrazino-2-methylthiopyrimidines (**14**) are reacted with cyanogen bromide to give the 3-amino-5-methylthiotriazolo[4,3-*c*]pyrimidines (**15**), usually as the hydrogen bromide salt. Treatment of **15** with sodium methoxide affords the 2-amino-5-methoxytriazolo[1,5-*c*]pyrimidine ring system (**16**). The sulfonamides (**13**) are prepared by reacting **16** with arylsulfonyl chlorides (**17** Scheme 2.4.3) in the presence of pyridine and a catalytic amount of dimethyl sulfoxide.

Several substituted benzene and pyridine sulfonyl chlorides from which to prepare sulfonamides have been investigated. However, with respect to crop selectivity, most interest has focused on 2,6-disubstituted benzenesulfonyl chlorides and 2,4-disubstituted pyridine-3-sulfonyl chlorides. A general method for the preparation of various benzene and pyridine sulfonyl chlorides is via ortho directed metalation [32, 37]. The sulfonyl chlorides (**17**) can be prepared directly from the aryl lithium species by reacting with sulfur dioxide followed by sulfuryl chloride (Scheme 2.4.3). Alternatively, reaction of the aryl lithium species with a disulfide,



Scheme 2.4.2. (a) BrCN, *i*-PrOH; (b) NaOR, ethyl acrylate; (c) $ArSO_2Cl$, pyridine, DMSO (catalytic), CH_3CN.



Scheme 2.4.3. (a) Excess **19**, BuLi, TMEDA, i-Pr_2NH, THF or Et_2O; (b) SO_2, Et_2O; (c) SO_2Cl_2; (d) $(n\text{-}PrS)_2$; (e) Cl_2, H_2O, HOAc.

most commonly propyl disulfide, gives an alkyl aryl sulfide (**18**: R = *n*-Pr) which can be converted into the sulfonyl chloride using chlorine and water. The later method is commonly used when further manipulation on the aryl ring is required.

2.4.3.2 **Biology**

The structure–activity trends for triazolo[1,5-*c*]pyrimidine sulfonamides (**13**) have been studied extensively [32–36]. Table 2.4.3 summarizes the herbicidal activity

Table 2.4.3 Herbicidal activity for analogs of **13** (R = Me) (structure shown in Scheme 2.4.2).

X	Y	Q	R^2	R^1	Average GR_{80} BW (ppm)	Average GR_{80} GW (ppm)
Cl	Cl	CH	OMe	H	3	11
Cl	Cl	CH	OEt	H	216	>500
Cl	Cl	CH	Me	H	<15	>500
Cl	Cl	CH	Cl	H	1	15
Cl	Cl	CH	H	OMe	>1000	>1000
OMe	CF_3	CH	OMe	H	<0.2	1
OMe	OMe	CH	OMe	H	0.5	<0.1
OMe	F	CH	OMe	H	1	3
OMe	Me	CH	OMe	H	1	3
OMe	CO_2Me	CH	OMe	H	10	2
OMe	Cl	N	OMe	H	12	15
OMe	OMe	N	OMe	H	<1	2
OMe	CF_3	N	OMe	H	4	16
OEt	CF_3	N	OMe	H	2	64
CF_3	OMe	N	OMe	H	>250	>250

for **13** with various substitutions on the triazolo[1,5-*c*]pyrimidine ring and 2,6-disubstitutions on the aryl ring. Analogs with substitution in the 8-position of the triazolopyrimidine ring (R^2) are more active than those with substitution in the 7-position (R^1). The 8-methoxy analog has the best activity on both grass and broadleaf weeds. Halogen substitutions in the 8-position have good levels of activity on broadleaf species with somewhat reduced levels of activity on grass species. High levels of activity are achieved with 2,6-disubstitutions on the phenyl ring, especially when one of the substituents is methoxy. The highest levels of activity are achieved when both the 2- and 6-positions are methoxy, although good levels of activity are achieved with various substituents in the 6-position when there is a methoxy in the 2-position. For substitutions on the pyridine ring, good levels of activity are achieved when at least one of the substituents is methoxy. The best levels of activity on both grass and broadleaf species is gained with the dimethoxy analog (**13**, Q = N, X = Y = OMe, R = Me, R^2 = OMe). The 4-methoxy analog (**13**, Q = N, X = CF_3, Y = OMe) has very little activity on either grass or broadleaf weeds.

Several 2-trifluoromethylphenyl analogs of **13** have been prepared with various alkoxy and substituted alkoxy groups in the 6-position of the phenyl ring and some of these molecules demonstrated trends for selectivity toward rice (*Oryza sativa*) with activity on barnyard grass (*Echinochloa crus-galli*) [32, 35]. Tables 2.4.4 and 2.4.5 summarize the activity observed on rice and key rice weeds when applied as a water-injected treatment (Table 2.4.4) and as a postemergence foliar treatment (Table 2.4.5) in the greenhouse to 1–3 lf rice and weeds for 2-alkoxy-6-trifluoromethylphenyl substituted analogs that were identified as having activity of interest. Particularly noteworthy are the 2,2-difluoroethoxyphenyl (**13**, Q = CH,

Table 2.4.4 Herbicidal activity on transplanted paddy rice and weeds for analogs of **13** (Q = CH, R = Me, R^1 = H, R^2 = OMe).

X	Y	*Oryza sativa* GR_{20} (g-a.i. ha^{-1})	*Echinochloa crus-galli* GR_{80} (g-a.i. ha^{-1})	*Monochoria vaginalis* GR_{80} (g-a.i. ha^{-1})	*Scirpus juncoides* GR_{80} (g-a.i. ha^{-1})	*Cyperus difformis* GR_{80} (g-a.i. ha^{-1})
CF_3	OCH_2CH_2F	14	10	5.2	9	8
CF_3	OCH_2OMe	124	16	4	15	18
CF_3	OCH_2CF_3	51	19	5	21	36
CF_3	OCH_2CF_2H	75	12	<2	12	14
CF_3	$OCH(CH_2F)_2$	140	14	1	9	31

Table 2.4.5 Herbicidal activity on direct-seeded rice and weeds as a postemergence foliar application for **13** (Q = CH, R = Me, R^1 = H, R^2 = OMe).

X	Y	*Oryza sativa* GR_{20} (g-a.i. ha^{-1})	*Echinochloa crus-galli* GR_{80} (g-a.i. ha^{-1})	*Scirpus juncoides* GR_{80} (g-a.i. ha^{-1})
CF_3	$O(CH_2)_2F$	4	1	3
CF_3	OCH_2OMe	>70	12	5
CF_3	OCH_2CF_3	>70	9	–
CF_3	OCH_2CF_2H	>140	20	30
CF_3	$OCH(CH_2F)_2$	>70	10	–

X = CF_3, R = Me, R^2 = OMe) and 2-fluoroethoxyphenyl (**13**, Q = CH, X = CF_3, R = Me, R^2 = OMe) analogs which showed high levels of activity on all weeds species, particularly barnyard grass, with good selectivity to rice.

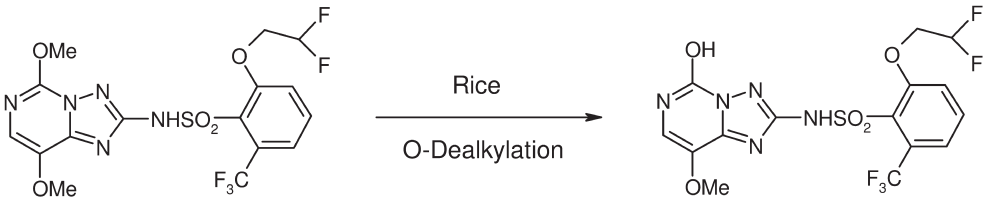
2.4.3.3 Penoxsulam Crop Utility

Based on the above greenhouse results, several 2-trifluoromethyl-6-alkoxyphenyl analogs of **13** were tested in key rice growing countries from 1997 to 1999 to characterize their activity. From these analogs, the 2,2-difluoroethoxyphenyl analog of **13** (**6**) was identified as having good rice tolerance, broad spectrum weed control (*Echinochloa* spp. and many key broadleaf and sedge weeds) and providing good residual weed control depending on the rates applied. Other analogs tested were not selected for several reasons, such as being too injurious to rice, providing poor weed control, or having short residual activity, when compared with **6**. Additionally, it was discovered that **6** could be co-applied with the grass herbicide cyhalofop-butyl which can not be tank-mixed with commercially available ALS or auxin mode of action herbicides without antagonizing the control of *Echinochloa* spp. Based on the ability to meet many of the commercial rice herbicide needs

(crop tolerance, broad-spectrum weed control, residual weed control activity, and tank-mix ability) in transplanted rice, direct-seeded rice and water-seeded rice in over 25 rice countries, **6** was identified for development as a new rice herbicide with the code number DE-638 and the common name "penoxsulam" [38–46].

2.4.3.4 Penoxsulam Mechanism of Crop Selectivity

Metabolism studies conducted on **6** showed that *O*-dealkylation of one heterocycle methoxy group was occurring in rice (Fig. 2.4.3) [47]. A comparison of metabolic degradation rates and activity on indica rice, japonica rice and barnyardgrass for **6** indicates that degradation rates explain the major differences observed in activity (Table 2.4.6). Other factors, such as site of uptake and transport, which are modulated by plant structure and metabolism, may contribute to additional rice selectivity observed for penoxsulam.



Penoxsulam (**6**)

Fig. 2.4.3. Metabolism of penoxsulam (**6**) in rice (*Oryza sativa*).

Table 2.4.6 Herbicidal activity and metabolism of **6**.

Species	GR_{80} (ppm)	$T_{1/2}$[a] (h)
Indica rice	>250	14.4
Japonica rice	>250	38.4
Echinochloa crus-galli	0.24	106

[a] Time required to metabolize 50% of the applied compound.

2.4.3.5 Penoxsulam Environmental Degradation, Ecotox and Tox

Dissipation of penoxsulam occurs primarily through microbial degradation. Other patterns of degradation or dissipation (e.g., photolysis, volatility, leaching and chemical hydrolysis) contribute to the loss of penoxsulam in the agricultural field environment. As penoxsulam degrades in the soil, several metabolites of the herbicide are formed. The primary soil metabolite, the 5-hydroxytriazolo[1,5-*c*]pyrimidine sulfonamide analog of **6**, has been shown to have very limited plant activity (a factor of >100×) relative to the parent, while other metabolites are inactive. The half-life of penoxsulam under field conditions averaged 6.5 days (4 to 10 days) under flooded water-seeded rice conditions, and averaged 14.6 days (13

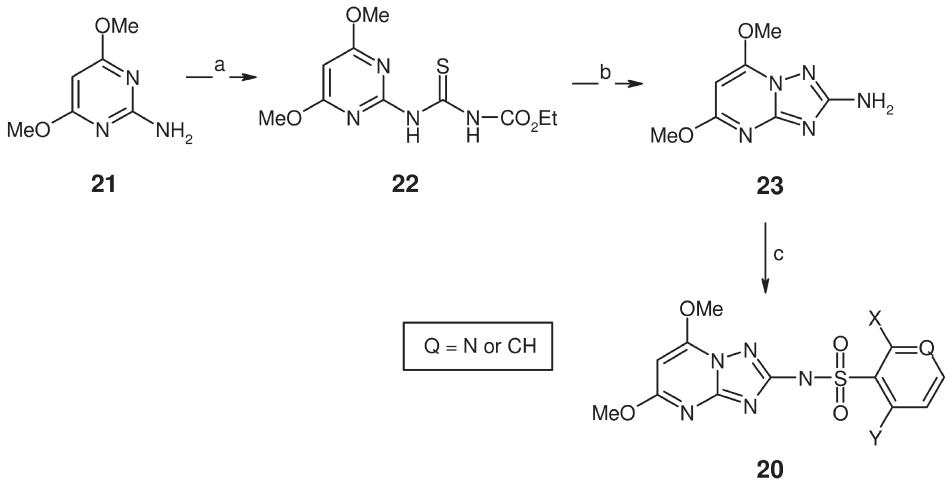
to 16 days) under non-flooded, dry-seeded rice conditions. Soil pH, texture and level of organic matter will influence the rate of degradation. Half-lives for aerobic aquatic conditions averaged 25 days (11–34 days) while half-lives for anaerobic conditions averaged 7 days (5–11 days). The major route of degradation in water is photolysis (half-life in water from photolysis was 2 days; summer sunlight, 40° N latitude).

Penoxsulam has a toxicological profile similar to other triazolopyrimidine sulfonamides. There was no indication of acute or chronic toxicity issues to mammalian and non-target organisms such as fish, fresh water invertebrates, honey bees, earthworm and beneficial arthropods.

2.4.4 N-Triazolo[1,5-*a*]pyrimidine Sulfonamides

2.4.4.1 Synthesis

Scheme 2.4.4 outlines a general route for the synthesis of N-triazolo[1,5-*a*]pyrimidine sulfonamides (**20**) [48, 49]. 2-Amino-4,6-dimethoxypyrimidine (**21**) is reacted with thiocarbonyldiimidazole followed by hydroxylamine in the presence of a base to give **23**, which is then reacted with various substituted benzene and pyridine sulfonyl chlorides (**17** Scheme 2.4.3) in the presence of a catalytic amount of dimethyl sulfoxide and a base to give **20**.



Scheme 2.4.4. (a) $SCNCO_2Et$; (b) $HONH_2$, $Et(i\text{-}Pr)_2N$; (c) $ArSO_2Cl$, pyridine, DMSO (catalytic).

2.4.4.2 Biology

Structure–activity trends for analogs of **20** have been reported previously [1]. For substitutions on the triazolo[1,5-*a*]pyrimidine ring, these studies showed that better herbicidal activity was achieved when the 5- or 7-position is substituted with

Table 2.4.7 Herbicidal activity for analogs of **20** (structure shown in Scheme 2.4.4).

X	Y	Q	Average GR_{80} BW (ppm)	Average GR_{80} GW (ppm)	*Alopecurus myosuroides* GR_{80} (ppm)	*Triticum aestivum* GR_{20} (ppm)
Cl	Cl	CH	4	4	4	<1
OMe	OMe	CH	8	8	4	<1
OMe	CF_3	CH	15	1	<1	<1
$O(CH_2)F$	CF_3	CH	2	16	27	13
OCH_2CF_2H	CF_3	CH	2	30	28	>250
OCH_2CF_3	CF_3	CH	4	62	240	46
OMe	CF_3	N	2	2	2	2
OMe	CF_2CF_3	N	>250	125	164	3
OMe	I	N	31	8	1	<1
OEt	CF_3	N	15	62	10	62

methoxy than when the 5- or 7-position is substituted with alkyl, halogen or halo alkyl. Recent efforts have shown that when both the 5- and 7-position are substituted with methoxy superior levels of herbicidal activity are achieved. With respect to the phenyl ring, previous efforts showed that 2,6-disubstitutions have superior herbicidal Activity over other substitution patterns. Much of the recent work has focused on 2,6-disubstituted phenyl and 2,4-disubstituted 3-pyridyl analogs of **20** with a 5,7-dimethoxy substitution on the triazolo[1,5-*a*]pyrimidine ring [48]. Table 2.4.7 summarizes general trends in activity on grass weeds, broadleaf weeds, blackgrass (*Alopecurus myosuroides*) and wheat (*Triticum aestivum*) for phenyl and pyridyl analogs of **20**. Good levels of herbicidal activity are achieved on both grass and broadleaf weeds with 2,6-substitutions on the phenyl ring (**20**, Q = CH) and in particular when one of the substituents is methoxy. Superior levels of activity on grass species is achieved with the 2-methoxy-6-trifluormethylphenyl analog of **20** (Q = CH, X = OMe, Y = CF_3). However, these analogs cause significant injury to wheat. With higher alkoxy substitutions (e.g., **20**, Q = CH, X = OMe, Y = OCH_2CH_2F) the activity on blackgrass decreases. With 2-methoxy-4-trifluoromethyl substitution on the pyridyl ring of **20** (Q = N, X = OMe, Y = CF_3) excellent activity is achieved on both grass and broadleaf weeds. Replacing methoxy with ethoxy (**20**, Q = N, X = OEt, Y = CF_3) results in a loss of herbicidal activity which is more significant on grass than broadleaf species. Good levels of activity on blackgrass are observed for 4-trifluoromethylpyridyl analogs of **20** (Q = N, X = OMe, Y = CF_3) and this analog shows a trend for wheat selectivity. Based on grass and broadleaf weed control in field studies, this analog was identified for development as a new herbicide for wheat with the code name DE-742.

2.4.4.3 **DE-742 Crop Utility**

DE-742 (**7**) requires addition of a safener to achieve commercial levels of postemergence selectivity in small grain cereal crops, the main target market. Com-

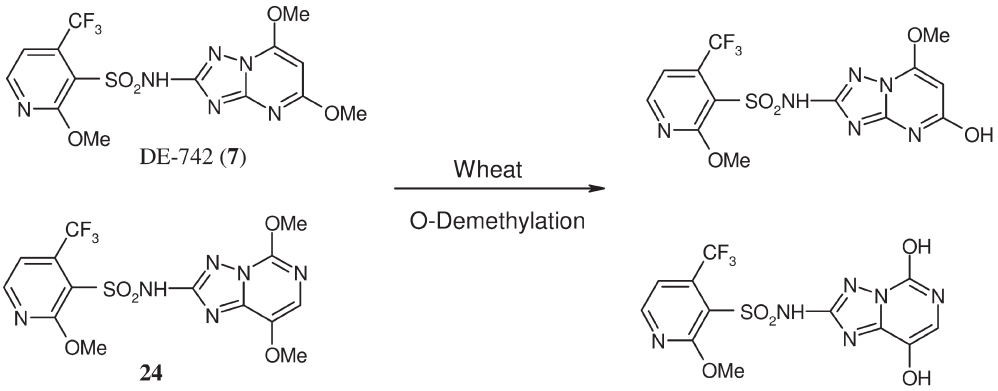


Fig. 2.4.4. Metabolites identified for DE-742 (**7**) and **24** in wheat (*Triticum aestivum*).

mercial selectivity is limited to wheat, rye and triticale varieties. DE-742 is broadly active on annual grass and broadleaf weeds, with some activity on certain perennial weed species. It derives most of its activity from foliar application but has some ability to provide soil residual control of emerging weeds. DE-742 controls economically important grass and broadleaf weed species in the global cereals markets.

2.4.4.4 DE-742 Mechanism of Crop Selectivity

Metabolism studies have been conducted on **7** and the closely related triazolo[1,5-*c*]pyrimidine analog **24** (Fig. 2.4.4). The metabolites identified in wheat (*Triticum aestivum*) for **7** and **24** are shown in Fig. 2.4.4. These studies showed that *O*-dealkylation of one heterocycle methoxy groups was occurring with **7** in wheat (Fig. 2.4.4). In comparison, both methoxy groups on the heterocycle of **24** underwent *O*-dealkylation. Table 2.4.8 compares the metabolism rates and activity on wheat and blackgrass (*Alopecurus myosuroides*) for **5**, **7** and **24**. The order of rank-

Table 2.4.8 Herbicidal activity and metabolism of **5**, **7** and **24**.

Herbicide	*Triticum aestivum*		*Alopecurus myosuroides*	
	GR_{20} (ppm)	$T_{1/2}$ (h)[a]	GR_{80} (ppm)	$T_{1/2}$ (h)[a]
Florasulam (**5**)	7.8	2.4	15.6	NA
24	12.2	5.7	4.4	51.6
DE-742 (**7**)	2.9	14	0.31	46

[a] Time required for plants to metabolize 50% of the applied compound.

ing is **5** > **24** > **7**, with respect to rate of metabolism in wheat, and **7** > **5** > **24**, with respect to activity on wheat. The slower rate of metabolism in wheat along with the higher levels of activity most likely account for the injury observed when **7** is not used in conjunction with a herbicide safener, such as cloquintocet. However, **7** is significantly more active on blackgrass than either **5** or **24**, with a rate of metabolism in blackgrass comparable to **24**.

2.4.4.5 DE-742 Environmental Degradation, Ecotox and Tox

Under aerobic conditions, laboratory studies have shown that DE-742 degrades rapidly in soil. The average laboratory half-life was 4 days at 20 °C across 20 different soils from Europe, the United States, and Canada. The principal metabolites are 7-hydroxytriazolo[1,5-*a*]pyrimidine sulfonamide (**25**), 5-hydroxytriazolo[1,5-*a*]pyrimidine sulfonamide (**26**), 7-hydroxy-6-chlorotriazolo-[1,5-*a*]pyrimidine sulfonamide (**27**), 5,7-dihydroxytriazolo[1,5-*a*]pyrimidine sulfonamide (**28**), and the corresponding sulfonic acid of DE-742 (**29**) (Fig. 2.4.5). All soil metabolites have little or no phytotoxicity compared with DE-742. DE-742 degrades at a moderate rate under anaerobic conditions, with a half-life of 47 days determined on a single soil. DE-742 does not photodegrade at a measurable rate on soil surfaces. Studies indicate overall toxicological profile of DE-742 is very favorable and similar to other triazolopyrimidine sulfonamides.

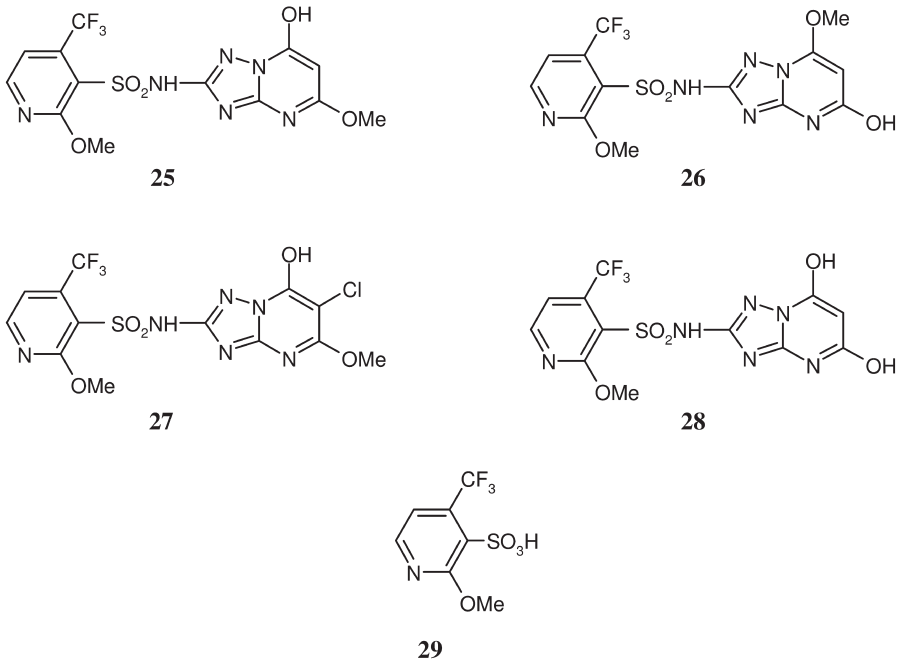


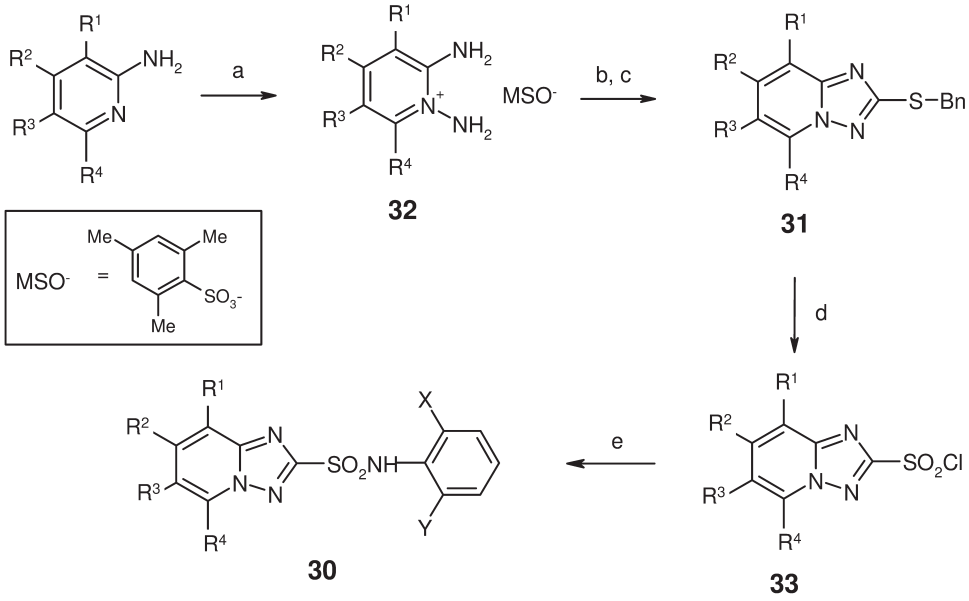
Fig. 2.4.5. Soil metabolites of DE-742 (**7**).

2.4.5 Other Systems

2.4.5.1 Synthesis

2.4.5.1.1 N-Aryl-triazolo[1,5-*a*]pyridine Sulfonanilides

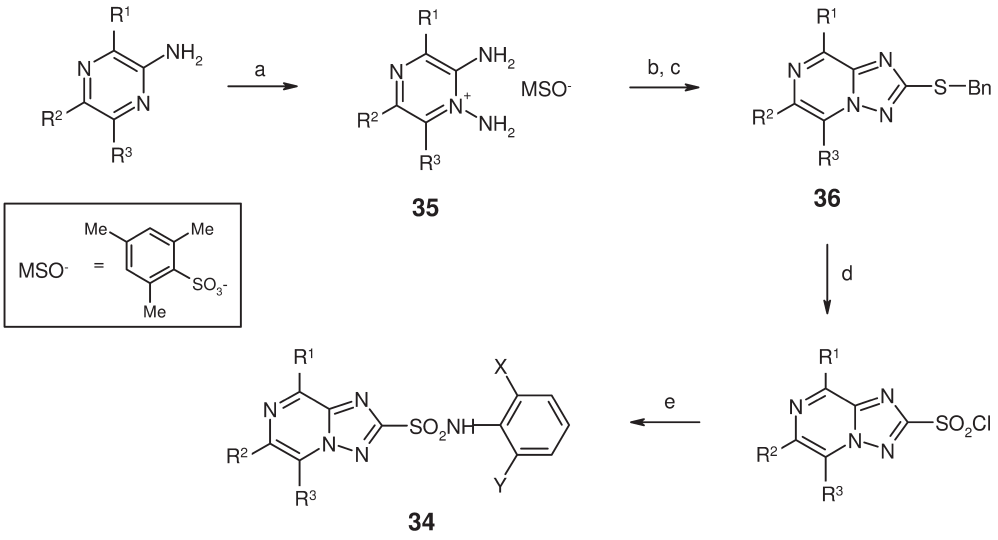
The triazolo[1,5-*a*]pyridine-2-sulfonanilides (**30**) can be prepared by the general route as outlined in Scheme 2.4.5 [49, 50]. The intermediate 2-benzylthio-triazolo[1,5-*a*]pyridine (**31**) is prepared starting from an appropriately substituted 2-aminopyridine. The 2-aminopyridine is reacted with O-mesitylenesulfonylhydroxylamine to give the N-aminopyridinium mesitylate (**32**). Compound **32** is then reacted with thiocarbonyldiimidazole followed by benzyl chloride to give **31**. Conversion of the benzyl sulfide, **31**, into the corresponding sulfonyl chlorides (**33**) is accomplished with chlorine and water. The sulfonyl chlorides are converted into the desired sulfonanilide (**30**) by reaction with aniline in the presence of pyridine and a catalytic amount of dimethyl sulfoxide.



Scheme 2.4.5. (a) *O*-Mesitylenesulfonylhydroxylamine; (b) 1,1′-thiocarbonyldiimidazole; (c) BnCl; (d) Cl_2, H_2O; (e) $ArSO_2Cl$, pyridine, DMSO (catalytic).

2.4.5.1.2 N-Aryl-triazolo[1,5-*a*]pyrazine Sulfonanilides

The triazolo[1,5-*a*]pyrazines sulfonanilides (**34**) can be prepared in a manner analogous to the triazolo[1,5-*a*]pyridines, starting from 2-aminopyrazines (Scheme 2.4.6) [51]. 2-Aminopyrazines are reacted with O-mesitylenesulfonylhy-



Scheme 2.4.6. (a) *O*-Mesitylenesulfonylhydroxylamine; (b) 1,1′-thiocarbonyldiimidazole; (c) BnCl, BuOH; (d) Cl_2, H_2O; (e) $ArNH_2$, pyridine, DMSO (catalytic).

droxylamine to yield the N-aminopyrazinium mesitylate (**35**). Compound **35** is then converted into the intermediate 2-benzylthiotriazolo[1,5-*a*]pyrazine (**36**) by first reacting with thiocarbonyldiimidazole followed by reaction with benzyl chloride in hot butanol. Further manipulation of the triazolo[1,5-*a*]pyrazine ring can take place at this stage to introduce additional functionality. Compound **36** is converted, by reaction with chlorine in water, into the sulfonyl chloride, which is then reacted with substituted anilines to give **34**.

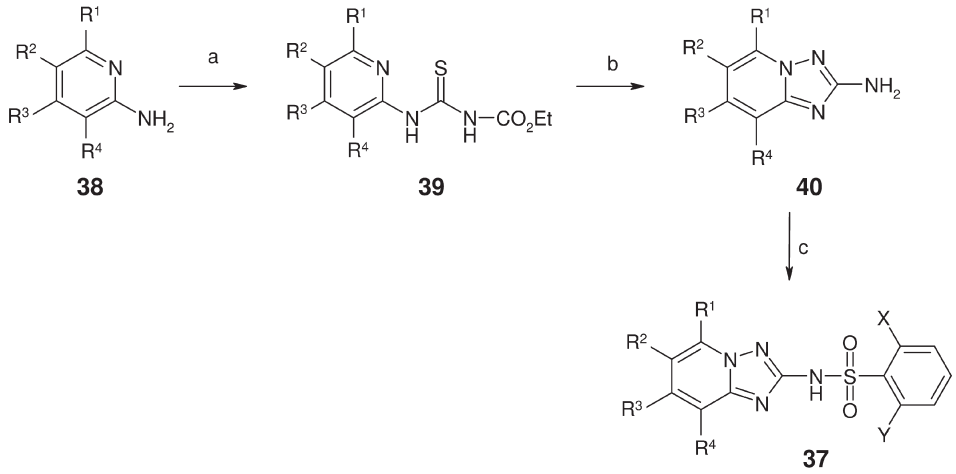
2.4.5.1.3 N-Triazolo[1,5-*a*]pyridine Sulfonamides

The N-triazolo[1,5-*a*]pyridine sulfonamides (**37**) are prepared by the general methods outlined in Scheme 2.4.7 [32, 36]. A substituted 2-aminopyridine (**38**) is reacted with ethoxycarbonylisothiocyanate to give the thiourea (**39**). Reaction of **39** with hydroxylamine in the presence of a base yields the 2-aminotriazolo[1,5-*a*]-pyridines (**40**). Compound **40** is then reacted with substituted sulfonyl chloride in the presence of a catalytic amount of dimethyl sulfoxide and pyridine to give **37**.

2.4.5.2 Biology

2.4.5.2.1 Triazolo[1,5-*a*]pyridine Sulfonanilides

The structure–activity relationships, with respect to substitutions on the phenyl ring, for the triazolo[1,5-*a*]pyridine sulfonanilides (**30**) are similar *in vivo* against broadleaf and grass weeds to the triazolo[1,5-*a*]pyrimidine sulfonanilides [4, 49, 50]. Compounds with substitutions in the 2- and 6-position of the phenyl ring



Scheme 2.4.7. (a) $SCNCO_2R$; (b) $HONH_2$, $Et(i\text{-}Pr)_2N$; (c) $ArSO_2Cl$, pyridine, DMSO (catalytic).

are more active than those with substitution in the 3- and 4-position. Substitutions on the triazolo[1,5-*a*]pyridine ring have been studied more extensively than those on the phenyl [4, 5]. Table 2.4.9 presents a compilation of herbicidal activity for substitutions on the fused heterocyclic ring. With a methoxy in the 5-position (**30**, $R^1 = OMe$) there are good levels of activity on both broadleaf and grass weeds. When the substituent in the 8-position is methoxy (**30**, $R^4 = OMe$) there

Table 2.4.9 Herbicidal activity for **30** (X = Y = Cl or X = Y = F) (structure shown in Scheme 2.4.5).

X	Y	R^1	R^2	R^3	R^4	Average GR_{80} BW (ppm)	Average GR_{80} GW (ppm)
Cl	Cl	Cl	H	Me	H	31	>500
Cl	Cl	OMe	H	Me	H	1	8
Cl	Cl	OMe	H	OMe	H	16	62
Cl	Cl	H	Me	H	OMe	62	125
Cl	Cl	OMe	H	Br	H	1	62
F	F	OMe	H	H	H	2	8
F	F	OMe	H	H	OMe	1	125
F	F	OMe	H	Me	H	1	8
F	F	OEt	H	Me	H	8	31
F	F	Cl	H	Cl	H	>2000	>2000
F	F	OMe	H	Cl	H	1	16
F	F	OMe	H	H	Br	15	31

Table 2.4.10 Herbicidal activity for **34** (X = Y = Cl) (structure shown in Scheme 2.4.6).

R^1	R^2	R^3	Average GR_{80} BW (ppm)	Average GR_{80} GW (ppm)
OMe	H	H	>250	>125
H	H	OMe	36	>125
Br	H	OMe	11	600
OMe	H	OMe	<4	27
H	Cl	OMe	4	375

are good levels of activity on broadleaf weeds, but the activity on grass species is somewhat less. The best levels of activity, on both grass and broadleaf species, are observed when the 5-position is methoxy (**30**, R^1 = OMe) and the 7-position (R^3) is methyl or chlorine.

2.4.5.2.2 **Triazolo[1,5-*a*]pyrazine Sulfonanilides**

The structure–activity trends for the triazolo[15-*a*]pyrazines have not been studied as extensively as other members of triazolopyrimidine sulfonanilides [51]. Table 2.4.10 shows the activity on broadleaf and grass species for a series of substitutions on the fused heterocyclic portion of **34**. The highest levels of activity on grass and broadleaf species are observed when both 5- and 8-positions are substituted with methoxy (**34**, $R^1 = R^3$ = OMe). However, the herbicidal activity observed for **34** is weaker than that for the triazolo[1,5-*a*]pyrimidine sulfonamides.

2.4.5.2.3 **N-Triazolo[1,5-*a*]pyridine Sulfonamides**

The structure–activity relationships for substitutions on the phenyl ring of the triazolo[1,5-*a*]pyridine sulfonamides (**37**) are similar to **13**. Disubstitutions are

Table 2.4.11 Herbicidal activity for phenyl analogs of **37** (X = Y = OMe) (structure shown in Scheme 2.4.7).

R^1	R^2	R^3	R^4	Average GR_{80} BW (ppm)	Average GR_{80} GW (ppm)
H	H	H	OMe	57	118
Cl	H	H	OMe	<4	17
OMe	H	H	Cl	15	<62
OMe	H	H	OMe	2	10
OMe	H	Me	H	>1000	>1000
OMe	H	Cl	H	>62	>62
H	OMe	H	OMe	216	>250

more active than mono-substitutions and 2,6-disubstituted analogs, especially when both are methoxy, give rise to the best levels of activity on both grass and broadleaf weeds. Structure–activity relationships for substitutions on the triazolo[1,5-*a*]pyridine ring of **37** have been studied more than those on the phenyl ring [32, 36]. Table 2.4.11 presents a compilation of activity for **37** with various substitutions on the triazolo[1,5-*a*]pyridine ring. The 5,8-dimethoxy analog (**37**, $R^1 = R^4 = OMe$) has the best level of activity on both grass and broadleaf weeds. However, these compounds are weaker herbicides than the triazolopyrimidine sulfonamides.

2.4.6 Conclusion

The triazolopyrimidine class of ALS inhibitors has grown to include several fused triazole ring systems containing a bridgehead nitrogen. Members have demonstrated control of grass, broadleaf, and sedge weeds in several agronomically important crops. The discovery of molecules with crop safety and favorable environmental profiles has led to the development of seven new herbicides for use in many crops, including corn, peanuts, soybeans, wheat, barley, oats, rye, triticale, rice, sugarcane, sorghum and turf.

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2.5 Pyrimidinylcarboxylates

Fumitaka Yoshida, Yukio Nezu, Ryo Hanai, and Tsutomu Shimizu

2.5.1 Introduction

Pyrimidinylsalicylates is the class of ALS-inhibiting herbicides disclosed in the late 1990s by Kumiai Chemical Industry and Ihara Chemical Industry. Their biological activities are as potent as those of the sulfonylureas (SUs). Further research led to the pyrimidinylglycolates, which are experimental herbicides. Since both types have the carboxyl moiety in their chemical structure, they are called pyrimidinylcarboxylates (PCs) or pyrimidinyl carboxy (PC) herbicides.

2.5.2 Discovery of the Pyrimidinylcarboxylates

The discovery of the PC herbicides started with attempts to synthesize new herbicides that incorporate a dimethoxypyrimidine [1]. During the synthetic and bioassay project, phenoxyphenoxypyrimidine **1** was found to show potent herbicidal activity with symptoms similar to Hill reaction inhibitors. In elaborating the structure to develop more systemic herbicides, a carboxylate group was introduced to give the compound **2** [2]. While the ethyl ester **2** was inactive, its ''regio-isomer'' **3** exhibited moderate activity against broadleaf weeds with pre- as well as post-emergent treatments [3]. Symptoms observed on plants after treatment with **3** were similar to those of the SUs; ALS-inhibiting herbicides and unlike those of the Hill reaction inhibitor **1**. By removing the second phenoxy group from **3**, the resulting *O*-pyrimidinylsalicylate **4** was found to exhibit highly potent herbicidal activity, characteristic of ALS inhibition (Fig. 2.5.1).

Using the skeletal structure of **4** as a new lead compound, various derivatives were synthesized to optimize the herbicidal activity. Among conventional substituents on the benzene ring, the carboxylate group ortho to the pyrimidinyloxy

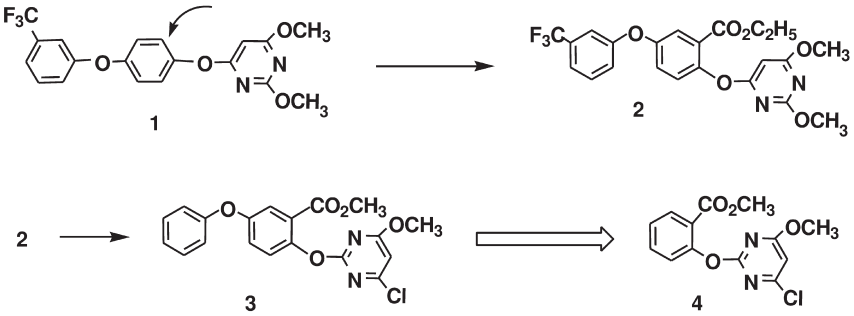


Fig. 2.5.1. Structural modification pathway towards the lead compound **4**.

one was essential for potentiating activity. A pyrimidine ring was better than any other nitrogen heterocycle. The most favorable substitution pattern was the 4,6-dimethoxy-2-pyrimidinyl **5**. At the rate of 1 kg-a.i. ha^{-1}, it controlled various grass- and broad-leave weed species pre- as well as post-emergently with phytotoxic symptoms similar to those of the SUs. Unfortunately, the safety margin of **5** for crops was narrow and unacceptable as a selective-herbicide despite a marked increase in herbicidal activity (Fig. 2.5.2) [4]. Thus, the ALS inhibitory activities of **4** and **5** were assessed. As shown in Table 2.5.1, the free acid of **5** was potent in terms of the I_{50} (nM) of ALS inhibitory activity. This compound exhibited a much higher ALS inhibitory activity than imazapyr (a representative IMI) [5].

This study of ALS inhibition demonstrated that the PCs are a novel class of ALS-inhibiting herbicides, differing from both the SUs and the IMIs. The PCs and the SUs are structurally unrelated, but possess common structural parts of a weakly acidic proton and an N-containing heterocyclic ring. On a two-dimensional hexagonal grid template, the common parts in both molecules overlap (Fig. 2.5.3) [6]. This suggested that a weakly acidic proton and an N-containing heterocyclic ring, appropriately located in a molecule are requisites for inhibiting ALS. Further modifications based on this hypothesis led to the highly active pyrimidinylglycolates in which a carboxylic and a pyrimidinyloxy group were not directly connected with a benzene ring (Fig. 2.5.4) [7].

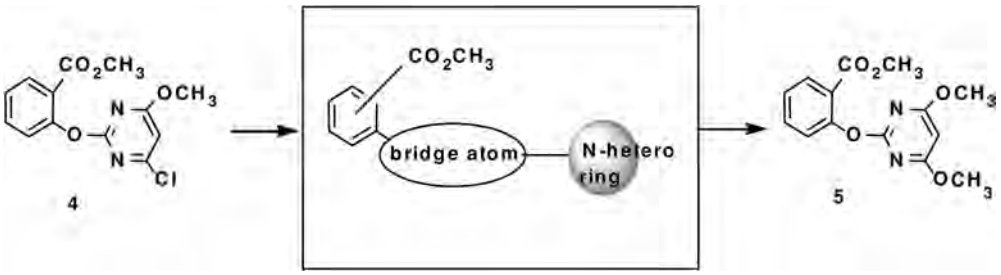


Fig. 2.5.2. Optimization from the lead compound **4**.

Table 2.5.1 ALS inhibitory activity of the acids of compounds **4** and **5**.

Compound	ALS inhibitory activity I_{50} (nM)[a]
4 (COOH)	4600
5 (COOH)	250
Imazapyr	9100
Chlorsulfuron	27

[a] I_{50}, molar concentration required for 50% inhibition of the ALS activity. ALS sample prepared from etiolated pea seedlings.

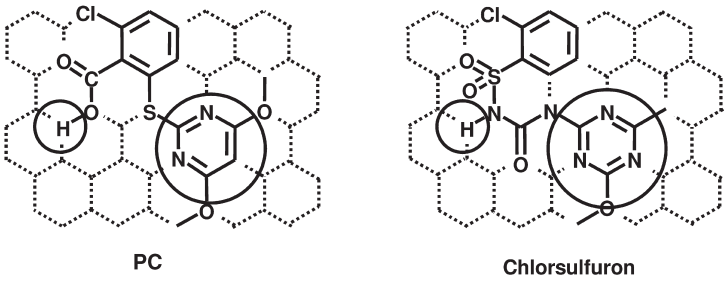


Fig. 2.5.3. Comparison of PC and chlorsulfuron on a hexagonal grid template.

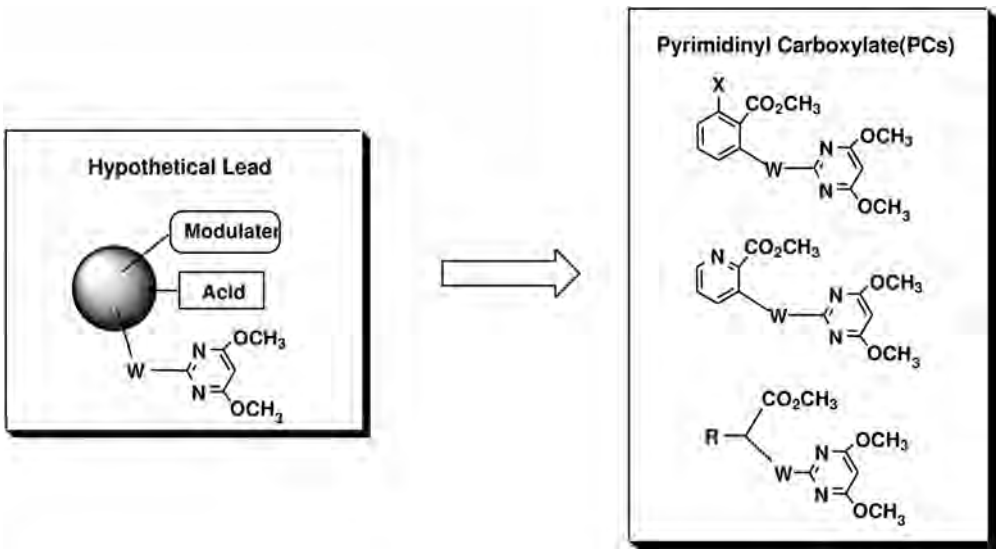


Fig. 2.5.4. Pyrimidinylcarboxylates – a new class of ALS inhibitors.

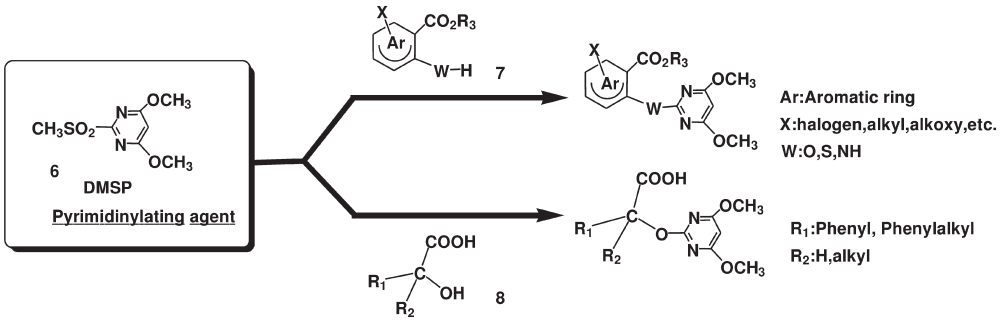


Fig. 2.5.5. Synthesis via 2-methanesulfonylpyrimidinyl intermediate **6**.

2.5.3 Structure–Activity Relationships

The first PC compound was prepared using the 2-methanesulfonylpyrimidine (OMSP) **6** and the salicylic acid ester in DMF. The sulfonyl compound **6** was a very efficient intermediate to synthesize 2-substituted pyrimidines [8]. The methanesulfonyl group in **6** is easily replaced by nucleophilic reagents like 7 and 8 (Fig. 2.5.5) [7, 9, 10]. This method was generally employed to synthesize numerous analogues aiming at new herbicides, not only with a high potency but also with an enhanced crop safety. Structural modifications were first made with the skeletal structure **9** (Fig. 2.5.6) [3].

2.5.3.1 Effects of Benzene Ring Substituents in the *O*-Pyrimidinylsalicylic Acids

The herbicidal activity of the pyrimidinylsalicylates (PSs) varied with the structure and position of ring substituents (X) (Table 2.5.2). First, the position-specific effect of ring substituents was examined. For Cl derivatives, the 6-Cl was obviously more potent than 3-Cl and 5-Cl (Table 2.5.2) [4, 11]. Also in other cases such as the methyl and fluoro derivatives, a similar positional pattern was shown. Therefore, the sequence of effects of the ring substituents in position was 6 > H (unsubstituted) > 3 > 5 ≫ 4. Thus, only substitution at the 6-position was favorable for enhancing the herbicidal activity of unsubstituted compound **5**. Among the 6-substituted derivatives, the halogeno, methyl, acetyl, phenyl, CF_3 and lower alkoxy derivatives exhibited extremely high activity at both the pre- and post-emergent

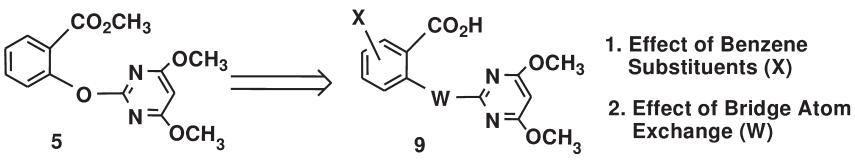
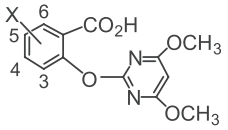


Fig. 2.5.6. Optimization for both herbicidal activities and safety to crops.

Table 2.5.2 Post-emergence herbicidal and ALS inhibitory activities of the dimethoxypyrimidinyl salicylic acids.[a]



X	pI_{50}[b]	*Ech*[c]	*Dig*[d]	*Pol*[e]	*Ama*[f]	*Che*[g]	*Cyp*[h]
3-Cl	6.3	2	1	5	5	1	5
4-Cl	4	0	0	0	0	0	0
5-Cl	5.4	0	0	2	5	3	4
6-Cl	7.6	5	5	5	5	5	5
H	6.6	5	4	5	5	5	4

[a] Applied at the dose of 250 g-a.i. ha^{-1}, and assessed with 6 grades from 0 (no effect) to 5 (complete kill).
[b] pI_{50}, ALS inhibitory activity ($-\log I_{50}$).
[c] *Ech*, *Echinochloa crus-galli*.
[d] *Dig*, *Digitaria adscendens*.
[e] *Pol*, *Polygonum nodosum*.
[f] *Ama*, *Amaranthus retroflexus*.
[g] *Che*, *Chenopodium album*.
[h] *Cyp*, *Cyperus iria*.

applications. These compounds controlled weeds completely at a dose of 250 g-a.i. ha^{-1}, but their phytotoxicity to crops could not be improved up to practical use [4].

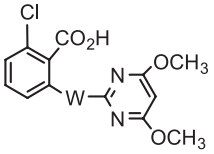
2.5.3.2 Effect of a Bridge Atom in the Pyrimidinylsalicylates

Fixing the 6-substituent as Cl, the effect of the bridge between the two rings was examined (Table 2.5.3). The *S*-bridge derivative showed excellent herbicidal activity, and the CH_2-bridge derivative was moderately active, whereas the *NH*-bridge and *SO*-bridge derivatives exhibited poor activity even at the application rate of 250 g-a.i. ha^{-1}. In an *in vitro* study of the ALS inhibition, the *S*-bridge and *SO*-bridge derivatives have inhibitory activities comparable to that of the *O*-bridge derivative, whereas those of the *NH*-bridge and CH_2-bridge derivatives decreased [4].

2.5.3.3 Pyrimidinylglycolates

As discussed in the Section 2.5.2, the pyrimidinylglycolates shown in Fig. 2.5.4, in which carboxylic and pyrimidinyloxy groups were not directly connected with a benzene ring also have high herbicidal activity [7, 9]. We attempted to examine favorable distances among important substructures, namely a carboxylic group, a pyrimidine and benzene rings (Fig. 2.5.7). First, compounds **10**, **11** were synthesized, because the distances between the carboxylic group and pyrimidine ring in these compounds were supposed to be close to that in the pyrimidinylsalicylates

Table 2.5.3 Herbicidal activities of 6-Cl pyrimidinylsalicylic acid analogues in which the *O*-bridge is modified.[a]



W	Post-emergence[b]				Pre-emergence			
	Ech	*Dig*	*Pol*	*Ama*	*Ech*	*Dig*	*Pol*	*Ama*
O	5	4	5	5	4	5	2	4
S	5	3	5	5	5	5	5	5
NH_2	0	0	0	3	0	0	0	2
SO	3	0	3	5	1	3	1	5
CH_2	4	4	4	5	4	4	5	5

[a] Applied at the dose of 250 g-a.i. ha^{-1}, and assessed with 6 grades from 0 (no effect) to 5 (complete kill).
[b] *Ech*, *Echinochloa crus-galli; Dig*, *Digitaria adscendens; Pol*, *Polygonum nodosum; Ama*, *Amaranthus retroflexus*.

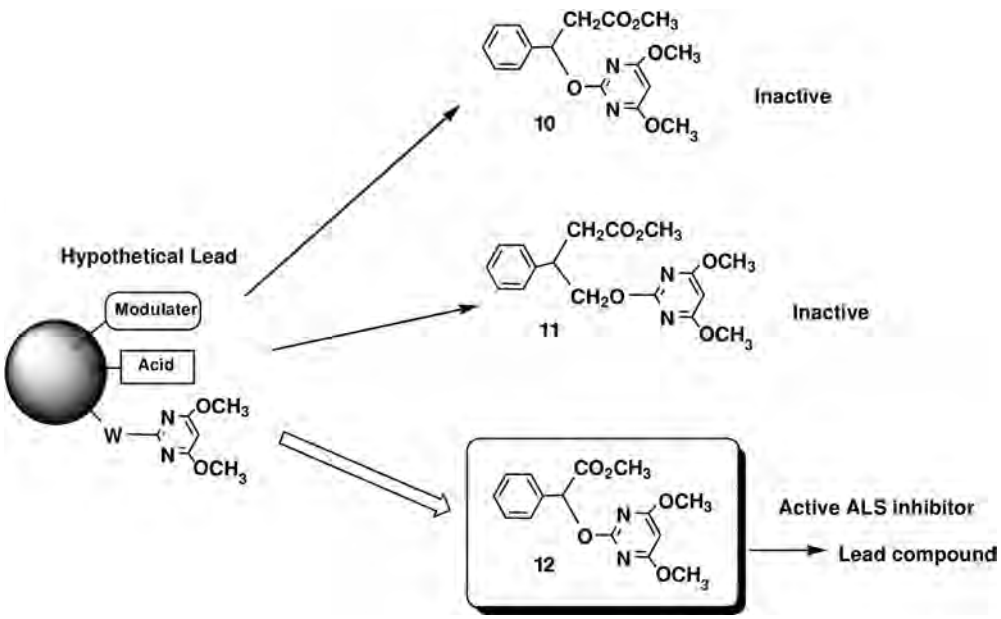


Fig. 2.5.7. Discovery of a new lead compound.

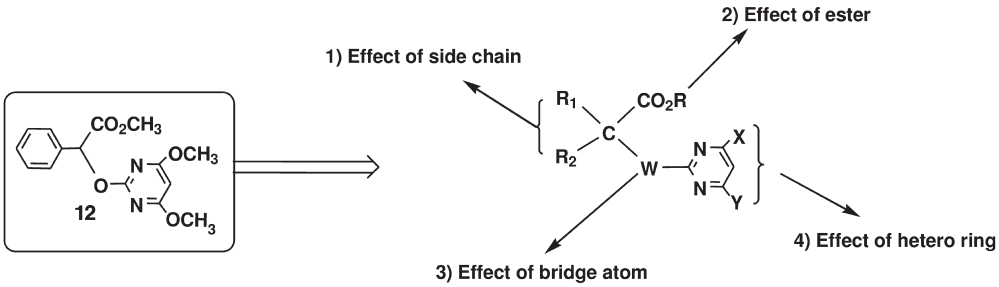
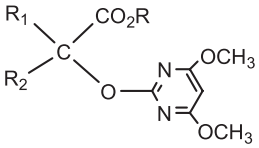


Fig. 2.5.8. Structural modifications from lead compound **12**.

having strong activity [12]. However, they were totally inactive. Next, compound **12**, with the carboxylic group placed at α position of the pyrimidinyloxy one, showed encouraging ALS inhibitory and herbicidal activities. Starting from **12**, structural modifications were made as shown in Fig. 2.5.8 to discern the effects of side chains (R_1,R_2), ester residues (R), bridge atoms (W) and pyrimidine substituents (X,Y) on herbicidal activity. As a result, the bridge atom (W) were fixed as oxygen and the pyrimidine substituents (X,Y) as methoxy in subsequent examinations. These options led to the most active compounds.

The herbicidal activities varied with substituents R_1, R_2 and R (Table 2.5.4). The α-hydrogen atom is, probably, essential for the herbicidal activity because the di-

Table 2.5.4 Herbicidal efficacy of the pyrimidinylglycolates.



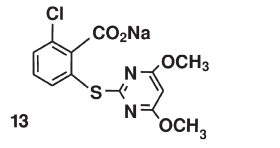
R_1	R_2	R	Pre-emergence (ED_{90}[a])		Post-emergence (ED_{90}[a])	
			Ech[b]	*Pol*[c]	*Ech*[b]	*Pol*[c]
Ph	H	H	B	A	A	B
$PhCH_2$	H	H	A	A	A	A
PhC_2H_4	H	H	B	A	A	B
Ph	CH_3	H	C	C	C	C
Ph	H	C_2H_5	C	C	A	C
$PhCH(CH_3)$	H	H	A	A	A	A
tert-C_4H_9	H	H	A	A	A	A

[a] ED_{90} (kg-a.i. h^{-1}): A, 1 or less; B, 1–4; C, 4 or more.
[b] *Ech*, *Echinochloa crus-galli*.
[c] *Pol*, *Polygonum nodosum*.

substituted compound (R_1 = Ph, R_2 = CH_3) completely lost activity. In contrast, two compounds (R_1 = $PhCH_2$, R_2 = H) and (R_1 = PhC_2H_4, R_2 = H) extended by methylene length(s) in R_1 almost retained the herbicidal activities of the starting compound **12** (R_1 = Ph, R_2 = H). Furthermore, the phenyl group in **12** was replaced by straight alkyl ones. The optimal length of the alkyl chain R_1 is around C_3 but with lower herbicidal activity. If the phenyl group in **12** is replaced by α-branching alkyl groups, *tert*-alkyl groups are more active than *sec*-alkyl groups, but again with lower herbicidal activity than **12**. The free acid is more active than the esters (e.g. R = ethyl).

2.5.3.4 **Commercialized PC Herbicides**

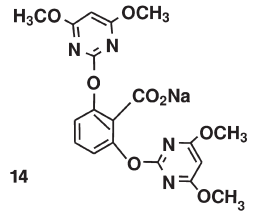
Further optimization of the pyrimidinylsalicylates led to three useful herbicides: (1) The sodium salt of 6-chloro-2-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoic acid **13** (pyrithiobac-sodium) was selected as one of the best cotton herbicides [13]. (2) The sodium salt of 2,6-bis[(4,6-dimethoxypyrimidin-2-yl)oxy]benzoic acid **14** (bispyribac-sodium) is used as a post-emergent herbicide for the control of a wide range of weeds with excellent selectivity on direct-seeded rice and for the vegetative growth reduction [14]. (3) Methyl 6-[1-(methoxyimino)ethyl]-2-[(4,6-dimethoxypyrimidin-2-yl)oxy]benzoate **15** (pyriminoibac-methyl) is a selective herbicide with outstanding efficacy on *Echinochloa* spp. in paddy rice (Fig. 2.5.9) [15].



13

"Pyrithiobac-sodium" Staple(R)
1996 market introduction (USA)

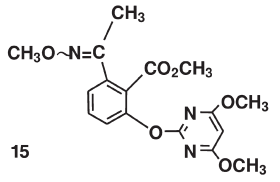
M.p., 233.8-234.2 °C (decomp.); V.p., 4.80×10^{-9} Pa; logP=0.6 (pH 5); -0.84 (pH 7)
Solubility: In water, 264 g/l (pH 5); 705 g/l (pH 7); 690 g/l (pH 9);In methanol, 270 g/ml; In n-hexane, 10 mg/ml
Acute oral: LD_{50} for male rats, 3300 mg/kg; LD_{50} for female rats, 3200 mg/kg
Eye irritation for rabbits, Irritating
TLm (96 hr) for rainbow trout, >1000 ppm; TLm (48 hr) for daphnia, >1100 ppm



14

"Bispyribac-sodium" Nominee(R)
1997 market introduction

M.p., 223-224 °C ; V.p., 5.05×10^{-9} Pa; logP=-1.03
Solubility: In water, 73.3 g/l; In methanol, 26.3 g/100l; In n-hexane, 3.56 mg/l Acute oral: LD_{50} for male rat, 4111 mg/kg; LD_{50} for female rats, 2635 mg/kg
Eye irritation for rabbits: Slightly irritating
TLm (96 hr) for rainbow trout, >100ppm;
TLm (48 hr) for daphnia, >100ppm



15

"Pyriminobac methyl" Prosper(R)
1997 market introduction

M.p.: Tech. 105 °C ; E-isomer, 107-109 °C ; Z-isomer, 70 °C
V.p.: E-isomer, 3.5×10^{-5} Pa ; Z-isomer, 2.681×10^{-5} Pa
logP of E-isomer= 2.98; logP of Z-isomer= 2.70
Solubility: E-isomer in water, 9.25 mg/ml; E-isomer in methanol, 14.6 g/ml; Z-isomer in water, 175 mg/l; Z-isomer in methanol, 14.0 g/l
Acute oral, LD_{50} for rats, > 5000 mg/kg
Eye irritation for rabbits: slightly irritating
TLm (96 hr) for rainbow trout, 21.2ppm;
TLm (24 hr) for daphnia, >20ppm

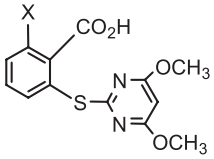
Fig. 2.5.9. Commercialized pyrimidinylcarboxy herbicides.

2.5.4
"Pyrithiobac-sodium" – Cotton Herbicide

2.5.4.1 Discovery

The effect of the 6-substituents in the thiosalicylate moiety was fine-tuned (Table 2.5.5). Halogeno and thioalkyl derivatives had good post-emergent herbicidal activity against broadleaf weeds, but were weak on *Echinochloa crus-galli*, whereas the acetyl derivative showed good herbicidal activity both against *Abutilon theophrasti* and *Echinochloa crus-galli*. This suggested that hydrophobic substituents such as halogens are favorable for killing broadleaf weeds, whereas the hydrophilic properties of the acetyl group affect positively the activity against grass weeds [4, 11]. Based on the good safety margin of the sodium salt of **13** (pyrithiobac-sodium) it was selected to be developed as a cotton herbicide against broadleaf weeds such as *Abutilon theophrasti* and *Ipomoea lacunosa* [16].

Table 2.5.5 Post-emergence herbicidal activities of 6-substituted pyrimidinylthiosalicylic acids.[a]



X	Crops[b]			Weeds[c]			
	Zea	*Gly*	*Gos*	*Ech*	*Abu*	*Ipo*	*Xan*
F	7	10	3	3	6	7	5
Cl	9	8	0	4	9	6	7
Br	9	7	0	1	7	4	6
I	10	7	0	2	9	6	8
CH_3	8	6	1	0	4	0	4
CF_3	8	5	0	0	6	2	2
$COCH_3$	10	4	1	10	9	8	4
OCH_3	9	9	0	2	5	1	6
OC_3H_7-*i*	2	4	1	0	2	0	0
SCH_3	9	8	4	0	10	10	9
NO_2	3	6	2	1	6	3	1

[a] Applied at the dose of 16 g-a.i. ha^{-1}, and assessed with 11 grades from 0 (no effect) to 10 (complete kill).
[b] Crops: *Zea*, Zea mays; *Gly*, Glycine max; *Gos*, *Gossypium hirsutum*.
[c] Weeds: *Ech*, *Echinochloa crus-galli; Abu*, *Abutilon theophrasti; Ipo*, *Ipomoea lacunosa*, *Xan*, *Xanthium strumarium*.

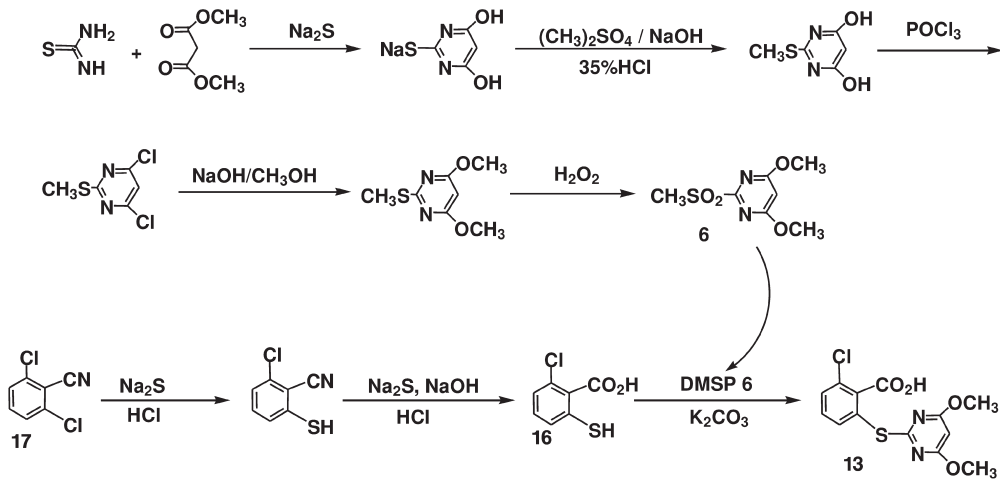


Fig. 2.5.10. Synthetic route for pyrithiobac.

2.5.4.2 **Synthesis**

Pyrithiobac-sodium is prepared by the condensation of 2-chloro-6-mercaptobenzoic acid **16** and DMSP **6**. Compound **16** was synthesized through two steps starting from 2,6-dichlorobenzonitrile **17**. The industrial process of DMSP **6** synthetic scheme (Fig. 2.5.10) has been optimized [17, 18].

2.5.4.3 **Biology**

Pyrithiobac-sodium is a herbicide for controlling a wide range of weeds in cotton [11, 16, 19]. This compound provides excellent control of troublesome weeds such as *Ipomoea* spp., *Xanthium strumarium*, *Abutilon theophrasti*, *Sida spinosa*, *Sesbania exaltata*, and *Sorghum halepense*. It can be applied pre- or post-emergently. Soil or foliar treatment with pyrithiobac-sodium at 35–105 g-a.i. ha^{-1} provides excellent control of weeds. Adjuvants such as non-ionic surfactants or some petroleum-based adjuvant oils play an important role in achieving consistent performance on several weed species when applied post-emergent. A good safety margin for cotton at rates that are effective on weeds has been observed with pre-emergence treatment in both the greenhouse and the field.

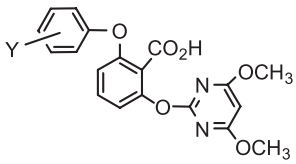
2.5.5 **"Bispyribac-sodium" – Herbicide in Direct-seeded Rice**

2.5.5.1 **Discovery**

Our previous studies on the PCs showed that substitution at the 6-position of the salicylate moiety was preferable for herbicidal and ALS inhibitory activities. Some PCs showed a strong activity against various weeds even at rate of around

10 g-a.i. ha^{-1}, but rice injury was severe. In structural modification of the 6-substitutent on the benzene ring, compounds with halogeno, alkyl or alkoxy group did not improve rice safety. With a bulky substituent such as phenoxy group, the herbicidal activity was somewhat decreased, but rice injury was significantly alleviated [20, 21]. Starting from the 6-phenoxy compound as a basic structure, various substituents Y were introduced on the 6-phenoxy. Unfortunately, no compounds gave acceptable rice safety and strong herbicidal activity at the same time (Table 2.5.6). The severe rice injury was attributed to the hydrophobic property of the phenoxy group. Thus, more hydrophilic substituents of heterocycle-oxy groups were introduced in its place. Among five- or six-membered hetero-rings, pyrimidinyloxy groups exhibited the most suitable performances as a rice herbicide in both aspects of activity and rice safety. PCs with 2 or 4-(substituted)-

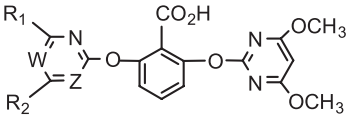
Table 2.5.6 Effect of substituent(s) Y on the benzene ring of 6-phenoxypyrimidinylsalicylic acids on herbicidal activity and rice phytotoxicity at post-emergence application.[a]



Y	Phytotoxicity *Ory.*[b]	Herbicidal activity[c]			
		Ech	*Pol*	*Ama*	*Xan*
H	4	9	10	10	7
2-Cl	3	5	7	8	2
3-Cl	0	0	7	8	2
4-Cl	6	4	4	8	0
2-F	5	4	10	9	2
3-F	0	3	9	9	4
4-F	2	0	8	8	2
2-CH_3	2	4	8	9	6
3-CH_3	0	4	9	9	6
4-CH_3	0	0	7	10	4
2-OCH_3	6	8	7	9	6
2-NO_2	4	4	9	9	0
3,5-$(OCH_3)_2$	4	7	6	7	8

[a] Applied at the dose of 16 g-a.i. ha^{-1}, and assessed with 11 grades from 0 (no effect) to 10 (complete kill).
[b] *Ory, Oryza sativa*.
[c] Weeds: *Ech*, *Echinochloa crus-galli; Pol*, *Polygonum nodosum; Ama*, *Amaranthus retroflexus*, *Xan*, *Xanthium strumarium*.

Table 2.5.7 Effect of substituents Rn on the pyrimidine ring of bis(pyrimidinyloxy)benzoic acids on herbicidal activity and rice phytotoxicity at post-emergence application.[a]



R_1	R_2	Z	W	Phytotoxicity[b] *Ory.*	Herbicidal activity[c]			
					Ech	*Pol*	*Ama*	*Xan*
H	H	N	CH	8	4	8	6	0
Cl	CH_3	N	CH	3	4	7	9	2
Cl	OCH_3	N	CH	5	9	8	9	9
CH_3	CH_3	N	CH	5	8	8	8	6
CH_3	OCH_3	N	CH	4	9	9	9	8
OCH_3	OCH_3	N	CH	1	10	9	10	9
H	H	N	CCl	7	4	8	7	2
OCH_3	OCH_3	CH	N	5	8	9	8	8

[a] Applied at the dose of 16 g-a.i. ha^{-1}, and assessed with 11 grades from 0 (no effect) to 10 (complete kill).
[b] *Ory, Oryza sativa*.
[c] Weeds: *Ech*, *Echinochloa crus-galli; Pol*, *Polygonum nodosum*, *Ama*, *Amaranthus retroflexus*, *Xan*, *Xanthium strumarium*.

pyrimidinyloxy group as a 6-substituent on the benzene ring were, furthermore, synthesized and evaluated (Table 2.5.7).

In comparison with the unsubstituted compound ($R_1 = R_2 = H$), it was, consequently, revealed that the introduction of substituents into 4 and 6 positions of the pyrimidine was favorable for improving rice safety without decreasing herbicidal activity. In particular, the 4,6-dimethoxy compound, being a bis-pyrimidinyl compound, showed both a remarkable improvement in rice safety and excellent activity against *Echinochloa* spp. and broad-leave weeds. Finally, the sodium salt of 2,6-bis[(4,6-dimethoxypyrimidin-2-yl)oxy]benzoic acid (**14**, bispyribac-sodium) was selected to be commercialized as a herbicide on direct-seeded rice [22].

2.5.5.2 Synthesis

After double pyrimidinylation by the reaction of benzyl 2,6-dihydroxybenzoate with two equivalents of DMSP **6**, the benzyl group is removed by hydrogenation to yield bispyribac (Fig. 2.5.11) [20, 21].

2.5.5.3 Biology

Bispyribac-sodium is a post-emergent herbicide for the control of a wide range of weeds with excellent selectivity on direct-seeded Indica-type rice [22, 23]. The low

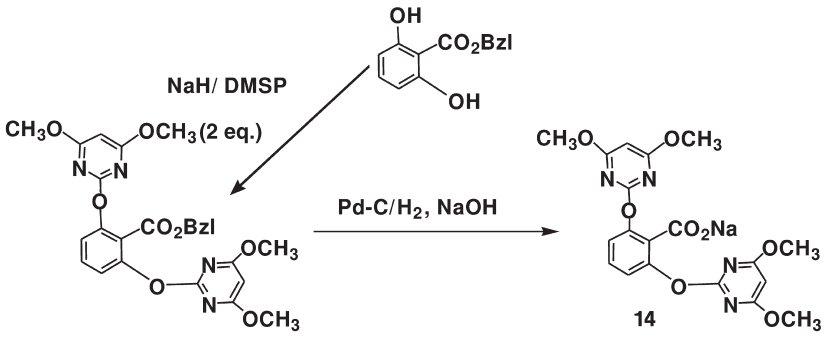


Fig. 2.5.11. Synthetic route for bispyribac-sodium.

application rate of 15–45 g-a.i. ha^{-1} with surfactant has provided outstanding efficacy on *Echinochloa* spp. and can be applied from the 1- to 7-leaf stage of the weed. It can control other troublesome weeds, including *Brachiaria* spp., *Cyperus* spp., *Scirpus* spp., *Polygonum* spp., *Sagittaria* spp., *Commelina* spp. and *Sesbania exaltata*. Adjuvants, such as non-ionic surfactants, silicon-type adjuvants or crop oil concentrate play an important role in enhancing the activity and achieving a consistent performance of this compound. Bispyribac-sodium has high selectivity between Indica-type rice and *Echinochloa oryzicola* by foliar application under dry-seeded conditions, suggesting that this compound can be used against a wide range of growth stages of *Echinochloa* spp. without rice crop injury. On the other hand, bispyribac-sodium at the rate of 150 g-a.i. ha^{-1} pre-mixed with a non-ionic surfactant reduced the vegetative growth of weeds such as *Imperata cylindrica*, *Digitaria adscendens*, *Miscanthus sinensis* and *Artemisia princes* [24]. The growth reduction persisted for 50 days after application of this compound when applied 5–10 days after mowing (at 10–20 cm plant height). Also, bispyribac-sodium controlled a wide range of weed species such as *Solidago altissima*, *Polygonum lapathifolium*, *Aeschynomene indica*, *Paspalum distichum* and *Echinochloa crus-galli* that grew in rice levees or on highway and railroad right-of-ways. The results indicated that bispyribac-sodium can reduce the frequency of mowing in paddy rice levees, and on highway and railroad right-of-ways.

2.5.6 "Pyriminobac-methyl" – Rice Herbicide

2.5.6.1 Discovery

Since ALS inhibitory and low-dose herbicides, including the SUs, were not commercially available for the effective control of *Echinochloa* spp. in transplanted rice when the pyriminobac methyl project was initiated, we focused our studies on

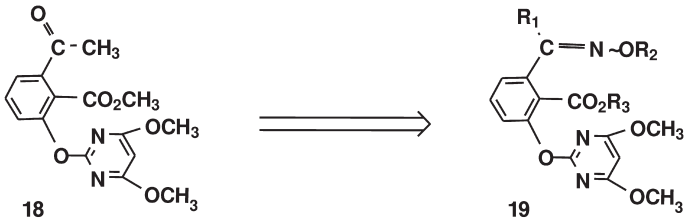
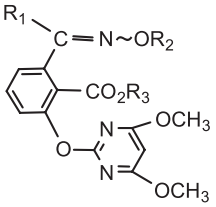


Fig. 2.5.12. Modification to an oxyimino group from an acyl one.

Table 2.5.8 Herbicidal activities of the 6-alkoxyiminosalicylate analogues against barnyard grass.



R1	R2	R3	Pre-emergence		Selectivity ED_{10}/ED_{90}
			Phytotoxicity ED_{10}(*Ory.*[a])	Herbicidal activity ED_{90}(*Ech.*)[b]	
H	CH_3	CH_3	63	16	4
CH_3	CH_3	CH_3	250	16	16
C_2H_5	CH_3	CH_3	63	63	1
C_3H_7	CH_3	CH_3	63	>1000	<1/16
CH_3	H	CH_3	4	16	1/4
CH_3	C_2H_5	CH_3	250	16	16
CH_3	C_3H_7	CH_3	250	16	16
CH_3	C_3H_7-*i*	CH_3	63	16	4
CH_3	C_4H_9	CH_3	63	16	4
CH_3	CH_3	C_2H_5	63	63	1
CH_3	CH_3	H	<4	16	<1/4

[a] Active ingredient amounts (g ha^{-1}) required for less than 10% phytotoxicity of *Oryza sativa* (*Ory.*)
[b] Active ingredient amounts (g ha^{-1}) required for more than 90% control of *Echinochloa oryzicola* (*Ech.*).

low-dose herbicides particularly effective in controlling *Echinochloa* spp. in the paddy rice. Our previous studies of pyrimidinylsalicylates had provided the following findings: substitution at the 6-position of the salicylate moiety was preferable for herbicidal and ALS inhibitory activities; electron-withdrawing groups contributed to ALS activity; and hydrophilic groups led to better activities against grass weeds than broad-leaf weeds. Therefore, 6-acyl compounds were specially interesting, since the acyl groups are both hydrophilic and electron-withdrawing. Compound **18** showed excellent control of *Echinochloa* spp., but caused unacceptable phytotoxicity to rice. However, the herbicidal profile of **18** satisfied our minimum requirements as a prototype for *Echinochloa* spp. herbicide [25].

To reduce rice injury, while keeping herbicidal activity of **18**, the introduction of an oxyimino group was attempted to give a hypothetical bio-isosteric analogue of **18**. The methoxyimino group has a similar [σ_p] (acyl group: $\sigma_p = 0.4$, methoxyimino group: $\sigma_p = 0.3$) and a steric similarity to a carbonyl group, and the hydrophilicity of the oxyimino moiety can be varied by alkylation and acylation. Extensive synthetic modifications were then made to the 6-alkyl moiety (R_1), the alkoxyimino moiety (R_2) and the ester moiety (R_3) of **19** (Fig. 2.5.12).

Structure–activity relationships of the synthesized compounds were studied by examining their herbicidal activities against *Echinochloa oryzicola* in paddy rice at various growth stages, including pre-emergence (Table 2.5.8). Compounds with $R_1 = CH_3$, $R_3 = CH_3$ and $R_2 =$ alkyl showed the best selectivity/activity relationship, but compounds with $R_3 > CH_3$ had reduced herbicidal activity at a higher growth stage [25, 26].

According to a study of the mode of action (Table 2.5.9), the ALS inhibitory activities of the methyl compound **15** against both *Echinochloa oryzicola* and rice were almost identical and about 1000× lower than that of the carboxylic acid (**20**). Besides, the metabolic transformation of **15** into **20**, which is considered to be the metabolically activated form as an ALS inhibitor, was enhanced, particularly

Table 2.5.9 ALS inhibitory activity, herbicidal activity and phytotoxicity of 6-methoxyiminosalycylate and its acid.

Compound	ALS I_{50}[a] (μM)		Phytotoxicity ED_{10}(*Ory.*)[b]	Herbicidal activity ED_{90}(*Ech.*)[c]
	Rice	Barnyardgrass		
15	59	47	6.3	25
20	0.018	0.016	<0.4	0.4

[a] Concentration required for 50% inhibition.
[b] Maximum effective dosage (g-a.i. ha^{-1}) for less than 10% phytotoxicity against transplanted *Oryza sativa* (*Ory.*) at 3 cm in depth.
[c] Minimum effective dosage (g-a.i. ha^{-1}) required for more than 90% control against *Echinochloa oryzicola* (*Ech.*) in pre-emergence.

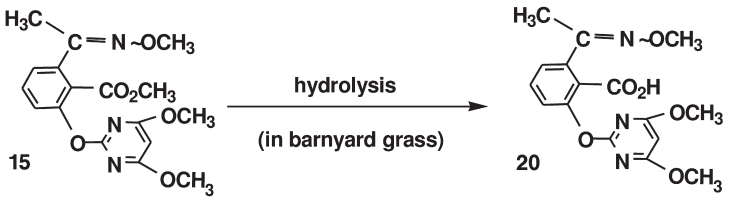


Fig. 2.5.13. Proposed metabolic pathway of **15** to the activated form **20** in barnyard grass.

in *Echinochloa oryzicola*, while not enhanced in rice (Tables 2.5.8 and 2.5.9 and Fig. 2.5.13) [26]. Methyl 6-[1-(methoxyimino)ethyl]-2-[(4,6-dimethoxypyrimidin-2-yl)oxy]-benzoate, (pyriminobac-methyl) (**15**) was, finally, selected as the candidate for commercialization as a novel barnyard-grass killer with an excellent selectivity for rice [27].

2.5.6.2 **Synthesis**

Figure 2.5.14 shows a synthetic route for pyriminobac-methyl [28]. The key step is ortho-lithiation reaction (step 3) of compound **21** protected by dimethylacetal and benzylation, followed by regioselective carbomethoxylation at the 2-position with methyl chloroformate via lithiated benzene prepared by *n*-butyllithium. Through several processes of deacetalization, methoxyimination of the acetyl group and debenzylation, compound **22** is condensed with DMSP **6** to give pyriminobac-methyl **15** [29].

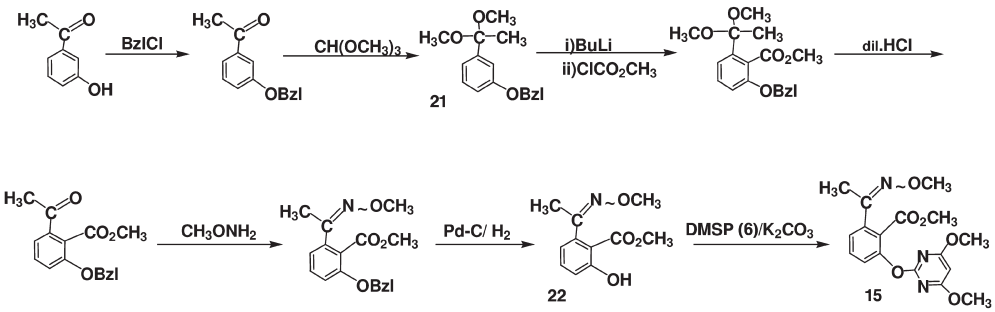


Fig. 2.5.14. Industrial synthetic route for pyriminobac-methyl.

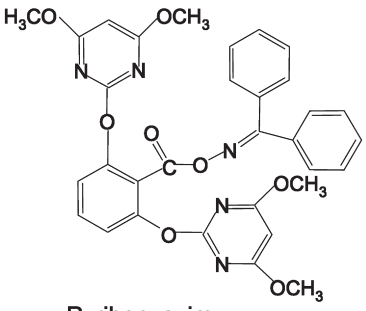
2.5.6.3 **Biology**

Pyriminobac-methyl is a selective herbicide with outstanding efficacy on *Echinochloa* spp. in paddy rice [26, 27, 30]. This compound has a specific effectiveness against *Echinochloa* spp. during a wide range of growth stages, from pre- to late post-emergence, with an excellent crop safety in rice. The use rate of pyriminobac-methyl is extremely low in comparison with the recommended rate of molinate and thiobencarb. Pyriminobac-methyl has shown excellent safety on

all eleven varieties tested of water-seeded rice and can be applied at any growth stage of rice. There was no observed significant difference in susceptibility to pyriminobac-methyl among rice varieties tested. Pyriminobac-methyl can be used alone or mixed with other rice herbicides such as bensulfuron-methyl. The residual activity of pyriminobac-methyl at 30 g-a.i. ha^{-1} was superior to thiobencarb at 3000 g-a.i. ha^{-1} under flooded conditions in the greenhouse.

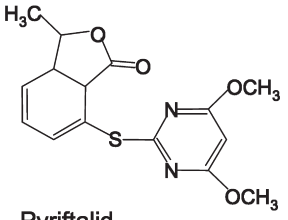
2.5.7
"Pyribenzoxim and Pyriftalid" – Rice Herbicides

Pyribenzoxim (Fig. 2.5.15) is an oxime ester of bispyribac, which has been developed by LG Chemical Ltd. for use in rice [31]. This chemical compound has postemergent activity on various grass and broadleaf weeds, including *E. crus-galli*, *Alopecurus myosuroides* and *Polygonum hydropiper*. *E. crus-galli* is controlled by pyribenzoxim at 30 to 40 g ha^{-1}, when applied alone in the field. Pyriftalid (Fig. 2.5.15) is an ALS-inhibiting herbicide categorized as a PC herbicide, which has been developed by Syngenta Crop Protection AG for use in rice [32]. This chemical compound controls grasses, especially *Echnochlora* spp., with an application rate of 100–300 g ha^{-1}. Herbicidal activities of pyriftarid are assumed to derive from conversion of the structure into the ring-open salicylic acid form.



Pyribenzoxim
1997 market introduction

M.p. 128-130 °C
V.p. < 9.9×10^{-4} Pa
logP 3.04
Solubility in water: 3.5 mg/l



Pyriftalid
2001 market introduction

M.p. 163.4 °C
V.p. 2.2×10^{-8} Pa
logP 2.6
Solubility in water: 1.8 mg/l

Fig. 2.5.15. Other pyrimidinylcarboxy herbicides.

2.5.8 Mode of Action of the PC Herbicides

The growth inhibition of rice seedlings and chlorella by the PCs were alleviated by simultaneous application of three branched-chain amino acids [5]. The PSs, including pyrithiobac, bispyribac and pyriminobac, strongly inhibited ALS in various plant species at concentrations in the nanomolar range [33]. The SU [34] and the triazolopyrimidine (TP) [35] inhibit plant ALSs activity in the mixed-type with respect to pyruvate in the steady state analysis, while the IMI inhibits in the uncompetitive manner [36]. We have shown the following kinetic results in our studies [37, 38]. Pyrithiobac and bispyribac inhibited the ALS of etiolated pea seedlings in the mixed-type with respect to pyruvate by means of a 40-min steady-state analysis. This inhibition pattern was the same as that of a SU, chlorsulfuron, but different from that of a IMI, imazapyr. Imazapyr inhibited this enzyme in an uncompetitive manner. The inhibition pattern of pyrithiobac for ALS of *Pseudomonas aeruginosa* was non-competitive with respect to pyruvate as same as that of chlorsulfuron, whereas that of imazapyr was uncompetitive [39]. The inhibition patterns of these inhibitors are different from those by feedback inhibitors, whose inhibition patterns are partially competitive. The small ALS from etiolated pea seedlings, which lost its sensitivity to the feedback inhibition, was potently inhibited by the PCs. These results indicated that the binding sites of these inhibitors on the enzyme are different from those of feedback inhibitors. Imazapyr has been demonstrated to compete with a SU, sulfometuron-methyl for the binding to ALS of *Salmonella typhimurium* [40]. In our study, chlorsulfuron competed with bispyribac for the binding to ALS of etiolated pea seedlings. This competition was more potent than that of pyrithiobac [38]. The binding site of the PCs on ALS is located on the allosteric site in a wide sense near the catalytic center. Both the SUs and the TPs might share the binding site with the PCs. Whereas, the IMIs bind to the site that is somewhat distinct from but overlaps that of the SUs, the TPs and the PSs. These sites are not on the regulatory subunit, but are considered to be in the vestige of the ubiquinone binding site on the catalytic subunit [40] that lost its role in the enzymatic reaction during the evolutionary process.

Despite their reversible nature to the inhibition of ALS, the SU and the IMI are slow-binding inhibitors of plant ALS [41, 42], which inactivate ALS irreversibly after reaching the final steady inhibitions. Irreversible inactivation of the enzyme has been found in both the presence [43] and absence [44] of pyruvate. Pyrithiobac and bispyribac inhibited the ALS of etiolated pea seedlings with slow-binding properties. Pyrithiobac showed the mixed-type pattern with respect to pyruvate in the initial inhibition. The inhibition constants in the initial inhibition by pyrithiobac and bispyribac were about 20-fold larger than those in the final steady state. The maximal first-order rate constant (k_1, 0.069 min^{-1}) for transition from the initial to the final steady state inhibition of pyrithiobac [37] was nearly identical to those of the SU and IMI. However, the dissociation constant of bispyribac to the ALS of etiolated pea seedlings after reaching the final steady inhibition was nearly identical with the inhibition constant in the initial inhibition [33].

2.5.9
Mode of Selectivity of the PC Herbicides in Crops

Despite the high selectivity of pyrithiobac for cotton and bispyribac for rice, there were no differences in the sensitivities of ALSs to pyrithiobac between cotton and other plants, and to bispyribac between rice and other plants. The selectivities of pyrithiobac and bispyribac must be determined by other factors. As for pyrithiobac, there is no published paper on its selectivity for cotton. However, oxidative demethylation of the 3,5-dimethoxy moiety has been shown to account for the tolerance of tall morning-glory to pyrithiobac [45]. Thus, the same mechanism is assumed to be involved in its selectivity between cotton and other sensitive plants. Regarding bispyribac, translocation of the compound mainly accounts for its selectivity between rice and barnyard grass [unpublished data]. Since des-methyl bispyribac was detected in a rice plant treated with bispyribac [46], and application of P-450 inhibitors such as 1-aminobenzotriazol and piperonyl butoxide reduced selectivity of bispyribac for Indica-type rice [unpublished data], the oxidative detoxification metabolism, like that of pyrithiobac, is presumed to be another factor in the selectivity of bispyribac for the rice plant.

One of the methyl ester compounds of the PC (**5** in Fig. 2.5.2), which has the same herbicidal potency as its free acid, hardly inhibited the activity of ALS separated from esterase. However, this compound inhibited the ALS activity as potently as its free acid, when the esterase was added in the reaction mixture [47]. Thus, the active forms of ester compounds are their free acids. However, pyriminobac-methyl inhibited ALS less potently than its free acid even in the presence of esterase. Pyriminobac-methyl was hardly hydrolyzed by the esterase existing in the soluble fractions of both rice and barnyard grass, whereas it was hydrolyzed by the microsomal fraction of barnyard grass [unpublished data]. Also, the free acid of pyriminobac-methyl was detected in barnyard grass treated with this compound, but not in rice [48]. These results indicate that the selectivity of pyriminobac-methyl between rice and barnyard grass depends on the difference in substrate specificity of the enzyme having esterase activity in the membrane fraction of plants.

2.5.10
PC-resistant Plants and their Mutated ALS Genes

It was expected that novel mutated ALS genes that had different mutations from those reported [2] were obtained through the selection of plant cells under the pressure of the PC herbicides. First, the callus from rice seeds was induced. The calli were then cultured with 1 μM bispyribac-sodium for about 2 months so that the bispyribac-sodium resistant cells were generated. The cells were next cultured with higher concentrations of bispyribac-sodium. Finally, several kinds of spontaneous BS-resistant cells that could grow under the pressure of 100 μM bispyribac-sodium were obtained. A wild-type ALS gene and a mutated ALS gene have been cloned from the bispyribac-sodium resistant cells using the partial cDNA that is

Fig. 2.5.16. Comparison of amino acid sequences between ALSs from the mutant and the wild type.

an expressed sequence tag obtained from the Ministry of Agriculture, Forestry and Fishery (MAFF) DNA bank of Japan as a homologous hybridization probe. Figure 2.5.16 shows a comparison of the deduced amino acid sequences between the wild-type ALS and one of the mutated ALSs. The first amino acid shows the sequences between position 361 and the C-terminal position 644 in the mutated ALS, and second amino acid sequence shows that in the wild-type ALS. The mutations involved the residues of tryptophan 548 to leucine and serine 627 to isoleucine. This double mutation on rice is a new combination of spontaneous mutations with the novel substitution at the serine position (DDBJ accession number, AB049823) [49].

One-point mutated ALS genes were then prepared to compare the sensitivities of their recombinant ALSs to the ALS-inhibiting herbicides with that of the two-point mutant. Each one-point mutant was prepared from the two-point mutant by PCR and the self-polymerase reaction. Recombinant ALSs from these ALS genes were expressed in *Escherichia coli* as GST-fused proteins and the proteins were examined for their sensitivities to herbicides. The ALS expressed from the wild-type gene showed a similar sensitivity to bispyribac-sodium and chlorsulfuron compared with that prepared from the natural source. Conversely, the ALS expressed from the two-point mutated ALS gene showed quite different sensitivities to the herbicides. This ALS showed a stronger resistance to bispyribac-sodium than to chlorsulfuron. Bispyribac-sodium had no effect on the enzyme even at 100 μM, which is an approximately 10 000-fold higher concentration than the I_{50} for the wild-type enzyme (Fig. 2.5.17). Notably, the two-point mutated gene imparted

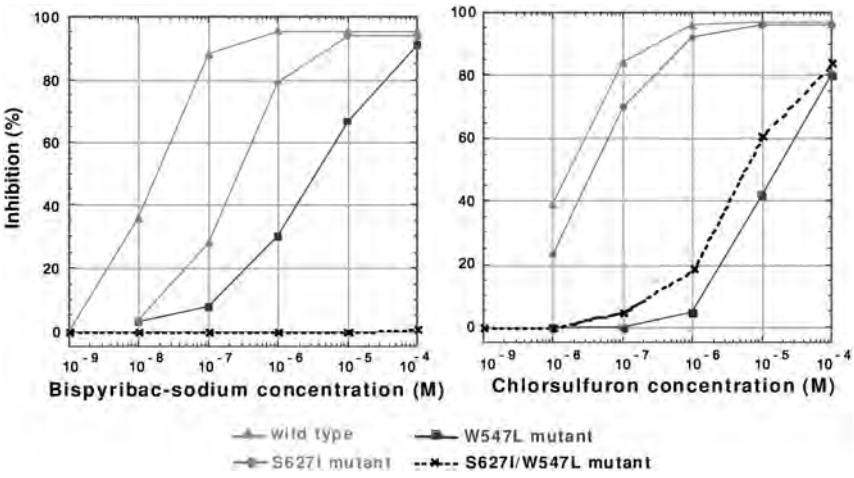


Fig. 2.5.17. Sensitivities of GST-fused ALSs to bispyribac-sodium and chlorsulfuron.

synergistic resistance to ALS against bispyribac-sodium that is stronger than the additive effect predicted from the degree of each resistance of the one-point mutated ALS [49].

2.5.11 Use of the Mutated ALS Genes for Genetic Transformation of Plants

As shown above, the novel mutated ALS gene from rice exhibited a high resistance to bispyribac-sodium. Thus we studied the use of this gene as a selectable marker for the genetic transformation of plants. Promoters and terminators derived from rice were used and a new binary vector was constructed. The two-point mutated ALS gene was driven with a rice callus specific promoter, and the GFP gene was driven with a constitutive promoter. Rice seeds were transformed with this vector by the Agrobacterium method and the transformed cells were selected by the pressure of bispyribac-sodium. As a result, fluorescence from GFP was detected only in selected cells, indicating that the two-point mutated ALS gene was an effective selection marker for rice transformation [50].

Transgenic rice plants were then generated to examine whether this gene works normally in the plant or not. The two-point mutated ALS gene was driven with a constitutive 35S promoter cassette with enhanced expression activity. Rice seeds were transformed with this vector and a transgenic rice plant was generated. This transgenic rice plant exhibited resistance to bispyribac-sodium and grew normally so that it was fertile. T_1 seeds were collected and the bispyribac-sodium resistant phenotype of T_1 plants were examined. The result showed that the phenotype was segregated by approximately 3:1 according to Mendel's law. The plants that exhibited resistance to bispyribac-sodium were cultivated on a large scale and several kinds of T_2 seeds were collected. Consequently, homozygotes for the

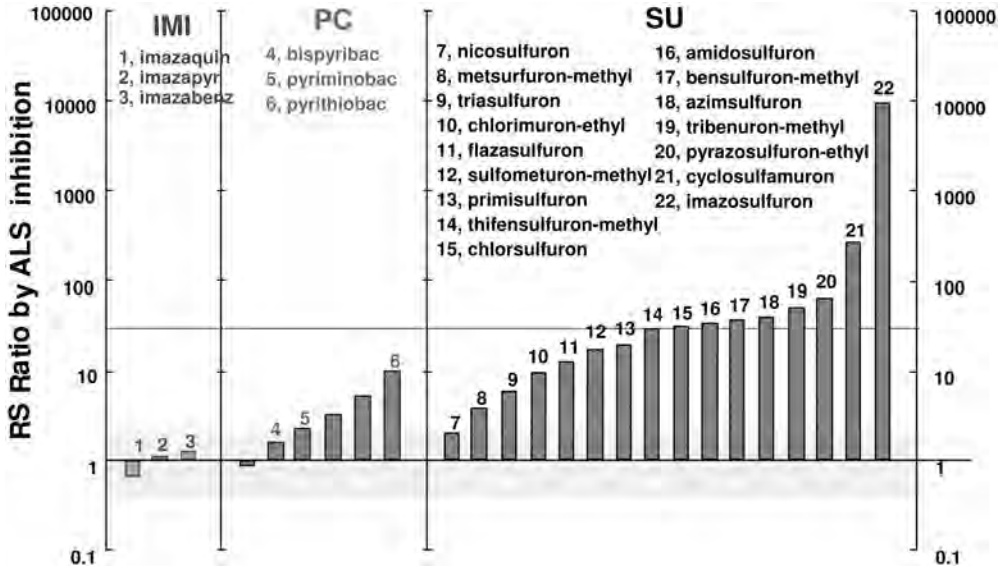


Fig. 2.5.18. Sensitivities of ALS from SU-resistant kochia to ALS-inhibiting herbicides. The data is for a kochia that has a mutation of proline to serine at position 189.

resistant trait were found in these T_2 seeds through examination of their sensitivities to bispyribac-sodium. This homozygote grew normally without bispyribac-sodium and exhibited resistance to bispyribac-sodium. These results suggested that the two-point mutated rice ALS gene functionally worked in rice and had no bad effects on rice [49].

The marker system is needed not only for the general recombinant technology but also for the gene targeting such as the homologous recombination and the mismatch repair. The mutated rice ALS gene can be used for such gene technologies. Novel binary vectors which have the S627I one point mutated ALS gene driven by the rice ALS promoter or the rice callus specific promoter have been developed and are now on the market (http://www.kumiai-chem.co.jp/).

2.5.12
Use of the Mutated ALS Genes for Resistance Management of ALS-inhibiting Herbicides

Using the accumulated knowledge concerning rice ALS genes, rice mutated ALS genes were artificially prepared [51]. Each recombinant ALS was prepared and the sensitivity of each protein to the ALS-inhibiting herbicides was examined. The mutated ALSs that have one-point mutation in proline at position 171 exhibited a high resistance to the SU herbicide, chlorsulfuron. Conversely, the resistance level of these mutated ALSs to the IMI herbicide, imazaquin, was lower

than that of chlorsulfuron. In contrast, the resistance level of the proline mutated ALSs to the PSs was moderate, between those of chlorsulfuron and imazaquin [52]. These results were correlated to the cross-resistance pattern of the proline-mutated ALS of *K. scoparia* (Fig. 2.5.18). From these results, it is considered that rice mutated recombinant ALSs are useful as resistant enzyme models for the herbicide resistance management at newly developed or developing ALS-inhibiting herbicides.

Abbreviations

ALS, acetolactate synthase
SU, sulfonylurea
IMI, imidazolinone
PS, pyrimidinylsalicylate
PC, pyrimidinylcarboxy(late)
TP, triazolopyrimidine sulfonamide
DMSP, 2-methanesulfonyl-4,6-dimethoxypyrimidine

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2.6
Sulfonylaminocarbonyl-triazolinones

Klaus-Helmut Müller

2.6.1
Introduction

The first examples of the new herbicidal class of sulfonylaminocarbonyl-triazolinones (SACTs) were reported in 1989 [1]. Following intensive chemical optimization two representatives were developed and commercialized for selective weed control in cereals. In 2000, flucarbazone-sodium (1) was introduced in the Canadian market under the trade name Everest® for the control of wild oats (*Avena fatua*) and green foxtail (*Setaria viridis*) in spring wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) (Fig. 2.6.1).

Propoxycarbazone-sodium (**2**) was first launched in Kenya in 2000. It is now registered in the major cereal producing European countries as Attribut® and in the United States as Olympus®. It is especially effective against brome (*Bromus* spp.), loose silky-bent (*Apera spica-venti*), common couchgrass (*Elymus repens*), blackgrass (*Alopecurus myosuroides*), jointed goatgrass (*Aegilops cylindrica*) and several broadleaf weeds from the mustard family (Fig. 2.6.2).

Both compounds are inhibitors of the acetolactate synthase enzyme, also known as aceto hydroxy acid synthase (AHAS) and are classified in group B by the Herbicide Resistance Action Committee HRAC. Table 2.6.1 gives the physicochemical properties of **1** and **2**.

2.6.2
Discovery of the Active Ingredients

The discovery of the herbicidal class of SACTs is outlined in detail in Ref. [2]. Starting from the concept of seeking new applications for the Nylon 6 intermedi-

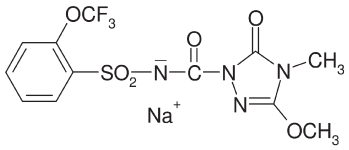


Fig. 2.6.1. Compound **1**, flucarbazone-sodium, Everest®, Vulcano®.

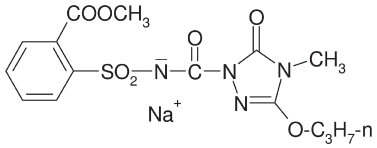
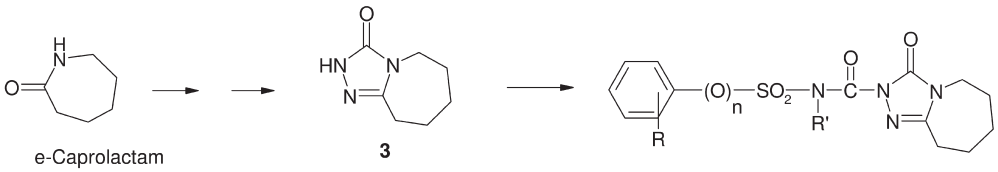


Fig. 2.6.2. Compound **2**, propoxycarbazone-sodium, Attribut®, Olympus®.

ate ε-caprolactam, the bicyclic triazolinone (**3**) was synthesized in the late 1970s as a possible intermediate for potential fungicides (Scheme 2.6.1) [3].



Scheme 2.6.1

Amongst many other derivatives (by NH-acylation, sulfonylation, alkylation, arylation) a sulfonylaminocarbonyl-triazolinone with the internal code no. BAY DAM 4493 was synthesized in 1985 (Fig. 2.6.3). It showed not only activity against rice blast (*Pyricularia oryzae*) but also phytotoxic symptoms at application rates of 500 g a.i. ha^{-1}.

About two years later this compound was identified in an *in vitro* assay as an unusual ALS inhibitor [4–6] and was the starting signal for a major synthesis program.

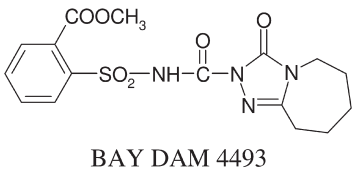


Fig. 2.6.3

Table 2.6.1 Physicochemical properties of flucarbazone-sodium and propoxycarbazone-sodium.

Property	Flucarbazone-sodium (1)	Propoxycarbazone-sodium (2)
CAS-No.	181274-17-9	181274-15-7
Code numbers	BAY MKH 6562	BAY MKH 6561
Melting point (°C)	200 (with decomposition)	230–240 (under decomposition)
Vapor pressure	Cannot be determined directly due to its extremely low value. From experimental results obtained for 70 °C a value of $<4 \times 10^{-8}$ Pa can be estimated as an upper limit. This limit would correspond to $<1 \times 10^{-9}$ Pa at 20 °C	Cannot be determined directly due to its extremely low value. From experimental results obtained for 70 °C a value of $<9 \times 10^{-8}$ Pa can be estimated as an upper limit. This would correspond to $<1 \times 10^{-8}$ Pa at 20 °C
Dissociation constant (at 20 °C)	The free acid produced by protonation under acidic conditions has a pK_a of 1.9.	The free acid produced by protonation under acidic conditions has a pK_a of 2.1.
Solubility in water (at 20 °C)	Water solubility is 44 g L^{-1} in unbuffered aqueous solutions in the range pH 4–9. Solubility is not influenced by pH in the range pH 4–9.	Unbuffered water and buffered between pH 7 and 9: 42 g L^{-1}. Solubility is not influenced by pH in the range pH 7–9. Solubility at pH 4.5: 2.9 g L^{-1}
Solubilities in organic solvents (g L^{-1} at 20 °C)	Dimethyl sulfoxide: 250 Poly(ethylene glycol): 48 Acetonitrile: 6.4 2-Propanol: 0.27 Xylene: <0.1	Dimethyl sulfoxide: 190 Poly(ethylene glycol): 5.2 Acetonitrile: 0.9 2-Propanol: <0.1 Xylene: <0.1
Partition coefficient log P_{OW} (octanol–water) (20 °C)	−2.85 (unbuffered) −0.89 (pH 4.0) −1.84 (pH 7.0) −1.88 (pH 9.0)	−2.60 (unbuffered) −0.30 (pH 4.0) −1.55 (pH 7.0) −1.59 (pH 9.0)

2.6.3 Optimization of the Lead Structure

All efforts to improve the herbicidal activity of BAY DAM 4493 by variation of the seven-membered ring were unsuccessful. Ring contraction to the five- and

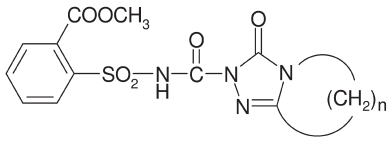


Fig. 2.6.4

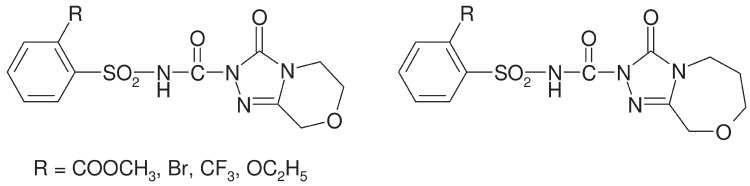


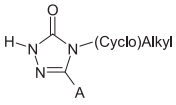
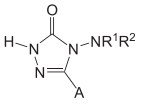
Fig. 2.6.5

six-membered analogues ($n = 3, 4$) and ring enlargement ($n = 5, 6, 7$) up to the thirteen-membered ring ($n = 11$) significantly reduced the herbicidal potency (Fig. 2.6.4) [1, 7, 8].

Similar results were obtained upon the introduction of oxygen (Fig. 2.6.5) [9].

In contrast to this observation the introduction of monocyclic triazolinones dramatically enhanced the herbicidal activity. In a systematic manner hundreds of previously known and new intermediates were prepared and transformed into SACTs. Many new synthetic procedures have been developed. To date, derivatives of the type shown in Table 2.6.2 have been synthesized and published.

Table 2.6.2 Synthesized derivatives of monocyclic triazolinones and the relevant references.

A		
A = (cyclo)alkyl	[1, 10]	[1, 10]
S-R^3	[11, 12]	[11]
Hal	[13]	[13]
NR^4R^5	[10]	[10]

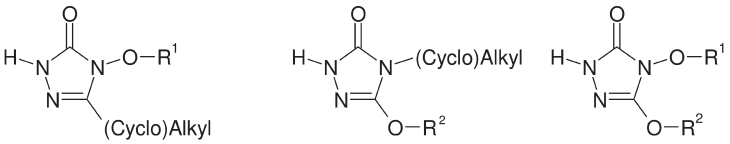


Fig. 2.6.6

The major breakthrough was the introduction of oxygen-bound residues, either on nitrogen and/or on carbon, (Fig. 2.6.6).

Special interest in the SACTs was generated by their biological profile. In the early 1990s most known ALS inhibitors like sulfonylureas had their main focus on dicotyledons. In contrast, the new class of SACTs exhibits in general high activity against grassy weeds, with sometimes rather good dicot activity and selectivity in cereals.

The first outdoor trials were undertaken in 1991 with the N-ethoxy derivative BAY MKH 4340 (internal code) (Fig. 2.6.7).

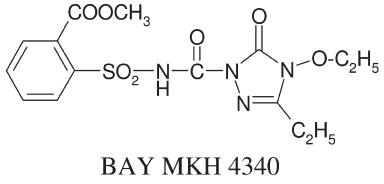


Fig. 2.6.7

In the following two years a further seven compounds were tested in parallel in the form of their sodium salts in Europe, the United States and Canada (Fig. 2.6.8).

The special biological spectrum – especially against difficult to control monocotyledonous weeds – and the selectivity in cereals led to development of two compounds, later known as flucarbazone-sodium (**1**) and propoxycarbazone-sodium (**2**).

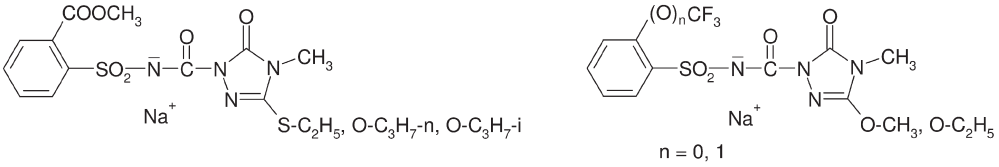
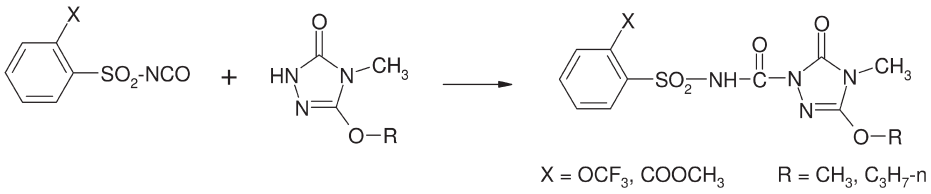


Fig. 2.6.8

2.6.4
Synthesis

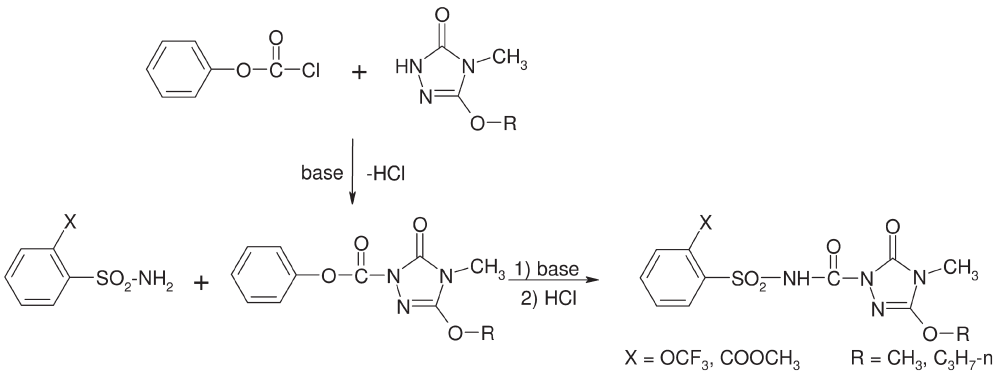
Flucarbazone and propoxycarbazone can be prepared by various methods:

(a) Sulfonyl isocyanate addition (Scheme 2.6.2) [1, 17].



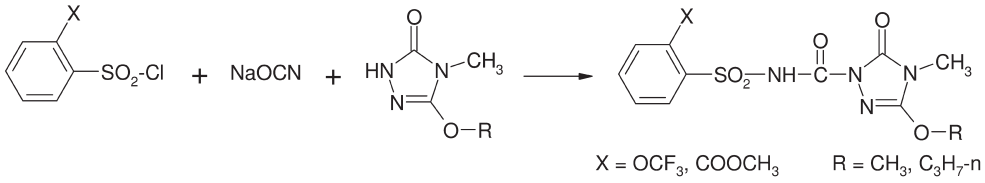
Scheme 2.6.2

(b) The phenylurethane route (Scheme 2.6.3) [10].



Scheme 2.6.3

(c) The cyanate route (Scheme 2.6.4) [18].

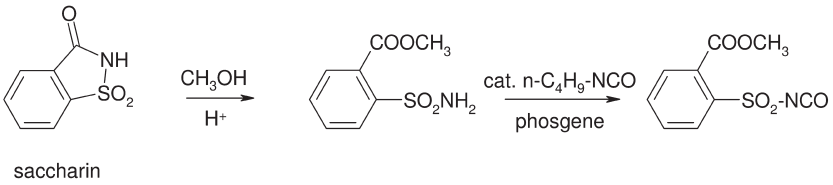


Scheme 2.6.4

For stability reasons both compounds are formulated as sodium salts. Several synthetic procedures describe the salt formation [19, 20].

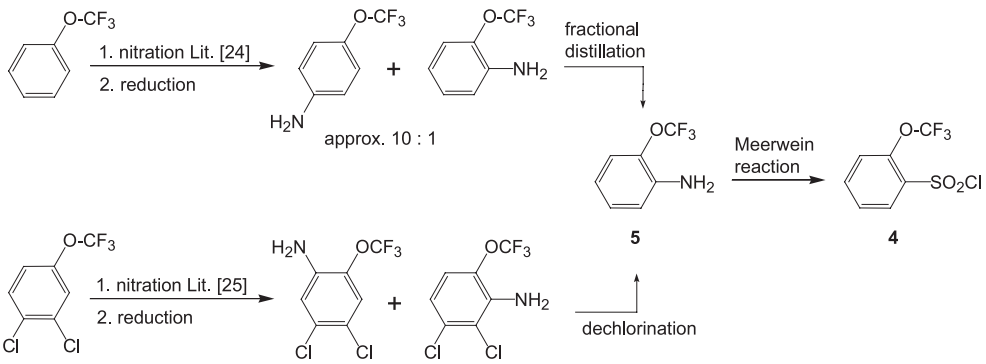
2.6.4.1 Sulfonyl Components

The sulfonyl component of propoxycarbazone-sodium (**2**) is an integral part of several commercial sulfonylureas [21]. Technical procedures exist for sulfonyl isocyanate preparation starting from saccharin (Scheme 2.6.5) [22, 23].



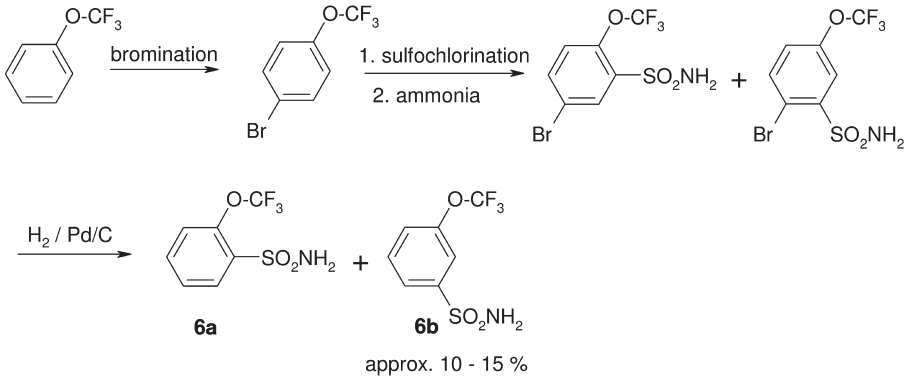
Scheme 2.6.5

The sulfonyl part **4** of flucarbazone-sodium (**1**) can be prepared by a classical Meerwein reaction from the aniline (**5**) prepared according to Refs. [24, 25] (Scheme 2.6.6).



Scheme 2.6.6

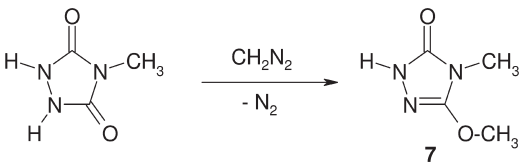
Another method is based on sulfochlorination and the different solubilities of the isomeric sulfonamides **6a** and **6b** (Scheme 2.6.7) [26].



Scheme 2.6.7

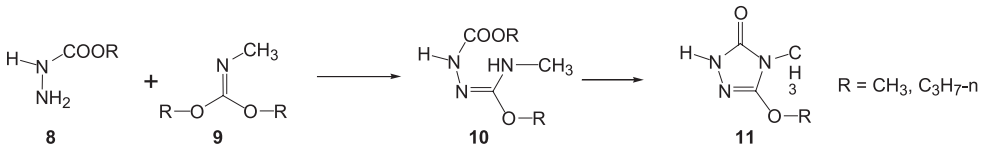
2.6.4.2 Triazolinone Synthesis

The flucarbazone-sodium intermediate (**7**) has long been known [27], but for safety reasons there was a great need for alternative procedures (Scheme 2.6.8):



Scheme 2.6.8

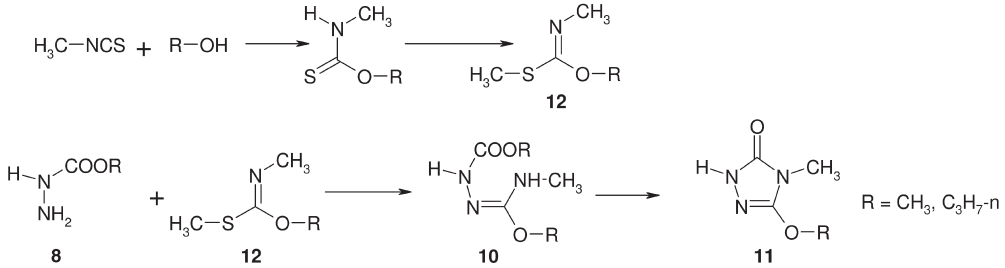
(a) Iminoester route ("tin pathway") (Scheme 2.6.9) [28–31].



Scheme 2.6.9

Here, the iminoester (**9**) reacts with carbazate (**8**) to give **10**, which cyclizes under basic conditions to the triazolinones (**11**).

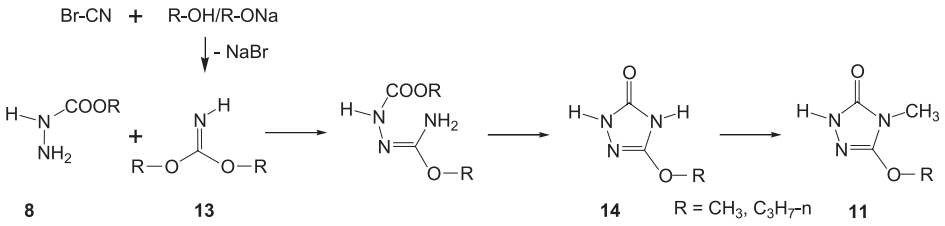
(b) Iminothioester route (Scheme 2.6.10) [32, 33].



Scheme 2.6.10

The iminothioester (**12**) is much more easily prepared than the iminoester (**9**).

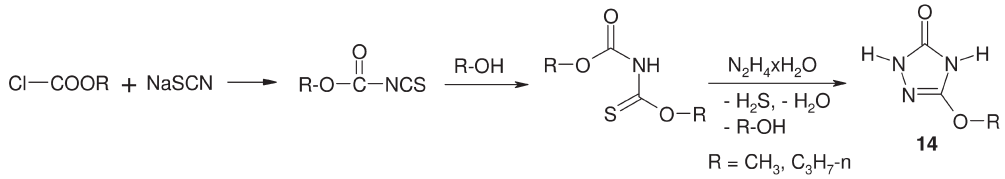
(c) Methylation of NH-triazolinones (**14**) (Scheme 2.6.11) [34, 35].



Scheme 2.6.11

The methylation of **14**, prepared via imidoester **13** [36], takes place exclusively at the amidic nitrogen.

Various patents describe alternative methods for the formation of the NH-triazolinones (**14**) (Scheme 2.6.12) [35, 37–39].



Scheme 2.6.12

2.6.5 Biology

Flucarbazone-sodium (**1**) was discovered and developed by the former Plant Protection Division of Bayer AG (now Bayer CropScience) [40–42]. Since 2002 it is commercialized by Arysta LifeScience in Canada and the USA under the trade name Everest® [43] and in Chile as Vulcano®.

It is a selective herbicide for the control of wild oats (*Avena fatua*) [44], green foxtails (*Setaria viridis*), Italian ryegrass (*Lolium multiflorum*), windgrass (*Apera spica-venti* and *A. interrupta*) and *Bromus* species like cheatgrass (*Bromus secalinus*) and Japanese brome (*Bromus japonicus*) in spring, durum and winter wheat [40–43].

In addition to these grasses, numerous broadleaf weeds are controlled, such as redroot pigweed (*Amaranthus retroflexus*), wild mustard (*Brassica kaber*), stinkweed (*Thlaspi arvense*), shepherd's purse (*Capsella bursa-pastoris*), green smartweed (*Polygonum scabrum* Moench.) and volunteer canola (*Brassica napus*). For broader spectrum control of broadleaf weeds Everest® may be mixed with broadleaf herbicides like 2,4-D (MCPA) amine or ester, bromoxynil, dicamba, fluroxpyr or sulfonylureas like metsulfuron-methyl, triasulfuron, tribenuron-methyl, chlorsulfuron, thifensulfuron-methyl or prosulfuron [43]. Currently application rates of 15–20 g a.i. ha^{-1} are registered in Canada and 21–42 g a.i. ha^{-1} in the USA.

Propoxycarbazone-sodium (**2**) is a new selective herbicide for grass control in wheat, rye and triticale [45]. In Europe it is registered as Attribut® at rates of 28 to 70 g a.i. ha^{-1}. It acts predominantly against important grasses such as *Bromus* species [46, 47], blackgrass (*Alopecurus myosuroides*) [48, 49] and loose silky-bent (*Apera spica-venti*) [49]. It is applied post-emergence in spring at a core use rate of 42 g a.i. ha^{-1}. Compared with single applications a split application or sequences following an autumn-standard treatment is more favorable, giving good to very good grass control. The following *Bromus* species can be controlled effectively [46, 47]: field brome (*B. arvensis*), meadow brome (*B. commutatus*), Japanese brome (*B. japonicus*), soft brome (*B. mollis*), rye brome (*B. secalinus*), barren brome (*B. sterilis*) and drooping brome (*B. tectorum*). Propoxycarbazone-sodium is taken up via leaves and, particularly, via roots. Especially on light soils, and under moist conditions, it controls couch grass (*Elymus repens*) at a commercially acceptable level. On heavy soils higher rates are recommended. Owing to its systemic mobility it also kills the rhizomes of *E. repens* [50].

In the USA propoxycarbazone-sodium is registered in spring, winter and durum wheat with application rates of 30 to 45 g a.i. ha^{-1} and sold under the trade name Olympus®. Bromus control is the primary target and, as in Europe, all important species, including cheatgrass (*B. secalinus*) and downy brome (*B. tectorum*), are well controlled [51]. Besides brome the following grasses can be economically reduced or suppressed [51]: loose silky-bent (*Apera spica-venti*), wild oats (*Avena fatua*), littleseed canarygrass (*Phalaris minor*) [52] and paradoxagrass (*P. paradoxa*). Suppression of jointed goatgrass (*Aegilops cylindrica*) can be

achieved by two sequential treatments of 30 g a.i. ha^{-1} in autumn and spring [53].

Besides grasses, broadleaf species belonging to the mustard complex like *Sisymbrium*, *Brassica*, *Descurainia*, *Chorispora*, *Camelina*, *Capsella* and *Thlaspi* [51] represent further target weeds for this herbicide.

Propoxycarbazone-sodium (**2**) can be applied straight or in tank mixtures with other herbicides such as triasulfuron, metsulfuron-methyl, chlorsulfuron, thifensulfuron-methyl, prosulfuron, carfentrazone, dicamba, bromoxynil, clopyralid, MCPA amine or ester, 2,4-D amine or ester, metribuzin or fluroxypyr. Olympus™ flex is a ready to use formulation with mesosulfuron.

2.6.6 Conclusion

Sulfonylaminocarbonyl-triazolinones are a new ALS inhibitor class discovered in 1987 by the former Plant Protection Division of Bayer AG. In the 1990s two compounds were developed for selective grass control in cereals. Flucarbazone-sodium (**1**) acts predominantly against green foxtail and wild oats and is registered in US and Canada as Everest® and in Chile as Vulcano®. Propoxycarbazone-sodium (**2**) is a brome specialist both in Europe (Attribut®) and the US (Olympus®). Besides other grasses, such as loose silky-bent and blackgrass, the rhizomes and the green part of couchgrass can be controlled. Additionally, there is a good activity against several broadleaf weeds from the mustard family.

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3
Protoporphyrinogen-IX-oxidase Inhibitors

George Theodoridis

3.1
Introduction

Rarely do we encounter an area of agrochemical research with both the chemical diversity and the very specific and often conflicting structure–activity relationship (SAR) rules as is the case with the herbicidal protoporphyrinogen oxidase (Protox) inhibitors. It was this incredible array of possibilities that lured every single agrochemical organization during the 1980s and 1990s in the United States, Europe, and Asia into initiating a research effort, in the hope of finding the next blockbuster herbicide. Soon, many Protox areas that were initially seen as having unlimited potential turned into dead ends, with only a handful of commercial products achieving significant market share. Part of the difficulty in exploiting the Protox area of herbicide chemistry was the fact that even though it was relatively easy to find chemistries with good biological activity, it was much harder to find clear crop selectivity, either on pre-emergently or post-emergently applied materials.

The lack of clear selectivity of several commercially significant row crops was overcome following the discovery of several highly active and selective Protox herbicides such as the post-emergence soybean selective herbicide fomesafen **7** (Flex®, Flexstar®, Reflex®) [1, 2], introduced in 1983 by ICI Plant Protection Division, and the soybean selective pre-emergence herbicides F5231, compound **14** [3–5], and sulfentrazone (**15**) (Authority®, Boral®, Capaz®) [6–8] introduced by FMC in 1991.

Research in Protox herbicides peaked in the early 1990s [9] and diminished soon after as the use of glyphosate-resistant crops gained increased market share. Glyphosate, N-(phosphonomethyl)glycine, is a broad-spectrum, post-emergence, systemic herbicide that has been used extensively over the past 30 years. This intense and prolonged use of glyphosate has resulted in documented resistance to glyphosate in several weed populations [10], which, in turn, has stimulated new interest in Protox-inhibiting herbicides.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

The mode of action of Protox herbicides has been extensively reviewed [11–17]. Protox herbicides act by inhibition of the enzyme protoporphyrinogen oxidase, the last common enzyme to both heme and chlorophyll biosynthesis [18–23]. The protoporphyrinogen oxidase enzyme catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX by molecular oxygen. Inhibition of the Protox enzyme results in the accumulation of the enzyme product protoporphyrin IX, but not the substrate, via a complex process that has not been entirely elucidated. In the presence of light, protoporphyrin IX generates large amounts of singlet oxygen, which results in the peroxidation of the unsaturated bonds of fatty acids found in cell membranes (Fig. 3.1). The end result of this peroxidation process is the loss of membrane integrity and leakage, pigment breakdown, and necrosis of the leaf that results in the death of the plant. This is a relatively fast process, with leaf symptoms such as a flaccid wet appearance observed within hours of plant exposure to the Protox herbicide under sunlight.

In this chapter, we discuss recent developments and challenges in the field of Protox-inhibiting agrochemicals and place those agrochemicals in the context of research done in this area of chemistry in the past four to five decades.

3.2 Historical Development

The diphenyl ether nitrofen (**1**) [24], introduced in 1963 by Rohm and Haas, now Dow AgroSciences; the oxadiazolinone oxadiazon (**2**) [25, 26] (Explorer®, Herbstar®, Romax®, Ronstar®), introduced in 1968 by Rhone-Poulenc; and the tetrahydrophthalimide chlorophthalim **3** [27], introduced in 1972 by Mitsubishi, represent the earliest examples of Protox herbicides (Fig. 3.2). Though all three classes are chemically quite different, they share a common mode of action, inhibition of the protoporphyrinogen oxidase enzyme, though this was not known until the late 1980s.

Each of these chemistries generated intensive work in the 1960s–1980s, which resulted in numerous diverse chemistries, from which several useful commercial products were obtained.

3.2.1 Diphenyl Ether

Following the discovery of the herbicidal activity of nitrofen (**1**) in 1963, intense research by several agrochemical companies resulted in a vast number of highly active and diverse chemistries [28, 29]. As mentioned earlier, the diphenyl ether chemistry represents the first class of Protox herbicides. Replacement of the aromatic 4-chloro group with a trifluoromethyl, as is the case with oxyfluorfen (**5**) (Goal®) [30], resulted in a significant improvement in biological activity, and 2-

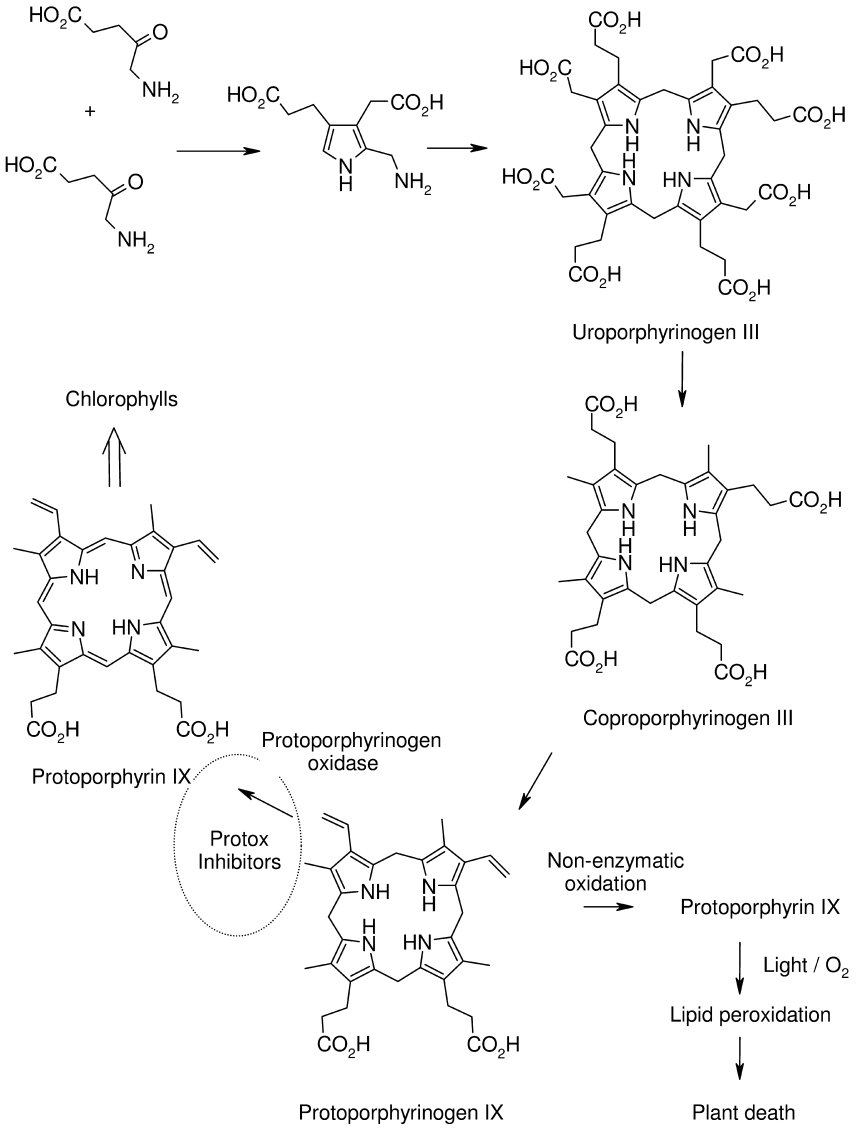


Fig. 3.1. Chlorophyll biosynthetic pathway.

chloro-4-(trifluoromethyl)benzene became the dominant substitution pattern for the second generation of diphenyl ethers (Fig. 3.3), eventually replacing products such as nitrofen (**1**) and bifenox (**4**) (Foxpro®, Modown®) [31]. As can be seen from Fig. 3.3, the 2-chloro-1-(3-substituted-4-nitrophenoxy)-4-(trifluoromethyl)-benzene became the most successful diphenyl ether chemistry scaffold, with four

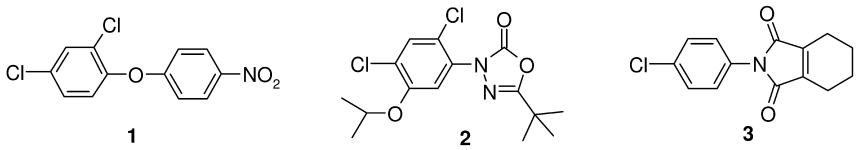
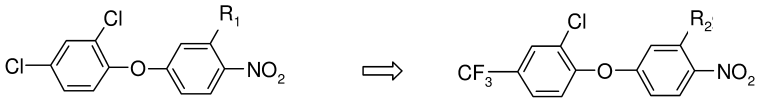


Fig. 3.2. Chemical structures of three early examples of Protox inhibitors.

significant products launched in fewer than ten years. In general, diphenyl ether herbicides such as oxyfluorfen (**5**) (Goal®), acifluorfen-sodium (**6**) (Blazer®) [32, 33], fomesafen (**7**) (Flex®, Flexstar®, Reflex®) [1, 2], and lactofen (**8**) (Cobra®) [34] are more effective when applied post-emergently, and are more effective for the control of broadleaf than of grass weeds.

Though the 1980s and early 1990s were a period of intense research in diphenyl ether chemistry, the main products described above were all introduced by 1987. By 1996, sales of diphenyl ethers had peaked at $381 million, steadily declining to $200 million by 2001 [35]. This decline was due to the introduction of more effective herbicides, as well as the increasing adoption of herbicide-tolerant crops. Despite this decline, research in diphenyl ether chemistry continued, resulting in the third generation of diphenyl ethers. This newer group of diphenyl ether herbicides consisted of compounds in which either the nitrophenyl ring was replaced by various fused benzoheterocycles, such as benzotriazole [36], benzoisoxazole [37], and indolin-2(3H)-ones [38], or the 2-chloro-4-(trifluoromethyl)benzene group was replaced by a heterocyclic ring such as pyrazole [39].

The extensive research invested by many companies in this third generation of diphenyl ether chemistry resulted in many active molecules, but no successful commercial product.



Entry	R_1	Herbicide	Entry	R_2	Herbicide
1	H	nitrofen	**5**	OEt	oxyfluorfen
4	CO_2Me	bifenox	**6**	CO_2Na	acifluorfen-sodium
			7	$CONHSO_2CH_3$	fomesafen
			8	$CO_2CH(CH_3)CO_2Et$	lactofen

Fig. 3.3. Evolution of diphenyl ether herbicides.

3.2.2
Phenyl Ring Attached to Heterocycle

Several discoveries made in the 1960s had a significant impact on our understanding of the structure–activity of Protox herbicides. The first breakthrough was the discovery of the importance of the 2,4-dihalo-5-substituted-phenyl substitution pattern. Rhone-Poulenc first introduced 3-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**9**) in 1965 [40]. Further structure–activity optimization at the phenyl ring soon led to the discovery in 1968 of the 2,4-dichloro-5-isopropoxyphenyl substitution pattern of the herbicide oxadiazon (**2**) [41, 42]. The 2,4-dihalo-5-substituted pattern at the aromatic ring would become the basis for much of the 2,4,5-trisubstituted phenyl tetrahydrophthalimide **10** [43] research that followed in the Protox area of chemistry.

Another breakthrough discovery was the boost in biological activity caused by the replacement of chlorine by fluorine at the 2-phenyl position. In 1976, DuPont introduced the first example of a 2-fluoro-4-chlorophenyl tetrahydrophthalimide Protox inhibitor (**11**) [44] (Fig. 3.4). The dramatic increase in biological activity caused by the fluorine in the 2 position of the phenyl ring would, in the next decade, the 1980s, influence work in the Protox area, such as the discovery of the 4-chloro-2-fluorophenyltetrahydrophthalimide herbicide S-23142 (**12**) [45].

The herbicide oxadiazon (**2**) is used for the pre-emergence control of annual broadleaf weeds and grasses and bindweed, and for the post-emergence control of annual broadleaf weeds in ornamentals such as carnations and roses, as well as in fruit trees, vines, cotton, rice, and turf. It requires high application rates of 1 kg-a.i. ha^{-1} for weed control in rice, and up to 4 kg-a.i. ha^{-1} for pre-emergence weed control in vines and orchards [25, 26]. The high rates of pre-emergence application, the limited selectivity in several row crops, and the introduction of

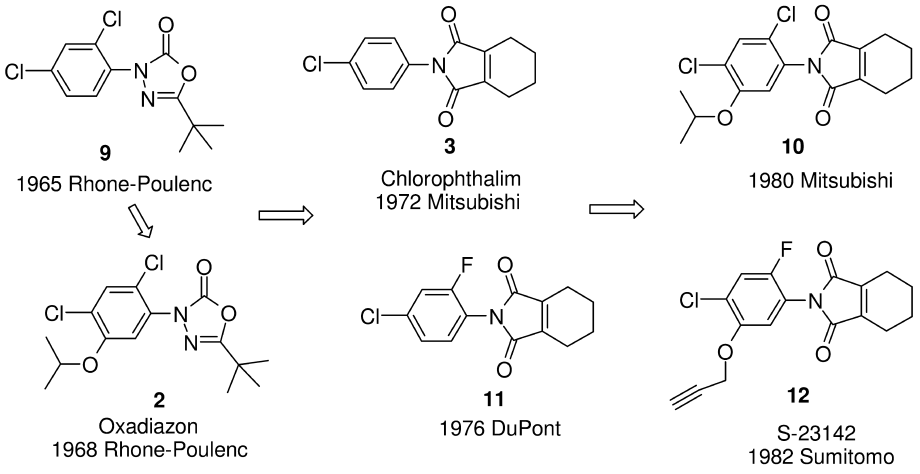
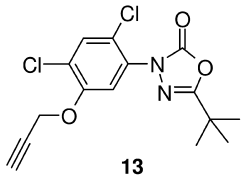


Fig. 3.4. Incorporation of the 2,4-dihalo-5-alkoxy aromatic pattern of oxadiazon into new phenyl tetrahydrophthalimide ring systems.



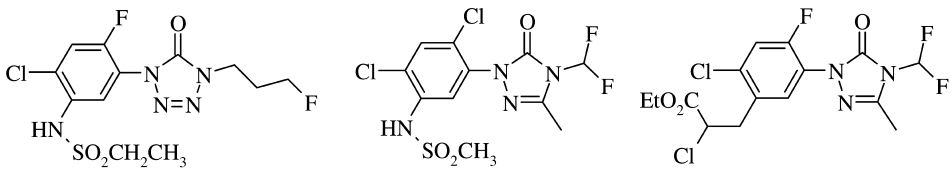
13

Oxadiargyl

Fig. 3.5. Chemical structure of oxadiargyl (**13**).

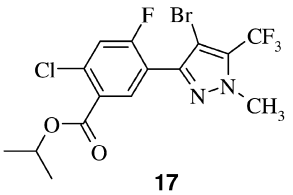
newer, more effective herbicides all have served to limit the commercial use of oxadiazon (**2**). Later, Rhone-Poulenc introduced oxadiargyl (**13**) (Raft®, Topstar®) [46, 47] (Fig. 3.5), a compound related to oxadiazon, for the control of broadleaf weeds, grass, and annual sedge in transplanted rice.

In the 1980s, several chemical changes on the 1,3,4-oxadiazol-2(3*H*)-one heterocyclic system resulted in several significant improvements in the pre- and post-emergence biological efficacy and crop selectivity of Protox herbicides. Detailed discussion of the various classes of phenyl heterocycles introduced several decades ago is beyond the scope of this chapter; they have been previously reviewed [28]. The introduction in 1985 of the 5-aminosulfonyl group in the phenyl ring of 2,4,5-trisubstituted-phenyl tetrazolinones was one such significant change. F5231 (**14**) [5], a molecule under development consideration by FMC in the late 1980s, was the first Protox inhibitor to provide excellent pre-emergence broadleaf control and clear selectivity at low application rates on several crops such as soybean, rice, corn, and wheat. FMC later replaced F5231 (**14**) with the phenyl triazolinone sulfentrazone (**15**) for soybean, sugarcane, and other crops [6–8]. Sulfentrazone (**15**) provides pre-emergence control of several broadleaf weeds – as well as several selected grass weeds – for the soybean market. A few years later, FMC introduced a second commercial phenyl triazolinone, the post-emergence cereal and corn herbicide carfentrazone-ethyl (**16**) (Aim®, Affinity®, Aurora®) [48, 49]. At low rates of 20–35 g-a.i. ha^{-1} carfentrazone-ethyl (**16**) provides excellent control of weeds in commercially important cereal crops – weeds such as bedstraw, speedwell, morning-glory, kochia, spurge, and deadnettle [50] (Fig. 3.6).



14 F5231 **15** Sulfentrazone **16** Carfentrazone-ethyl

Fig. 3.6. Chemical structure of F5231 (**14**), sulfentrazone (**15**), and carfentrazone-ethyl (**16**).



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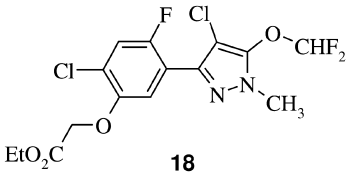
Fluazolate



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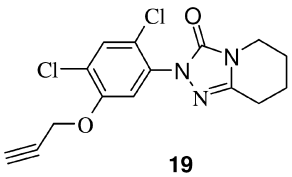
Pyraflufen-ethyl



19

Azafenidin

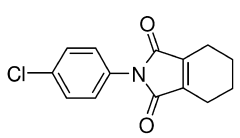
Fig. 3.7. Chemical structure of fluazolate (**17**), pyraflufen-ethyl (**18**), and azafenidin (**19**).

In addition to the oxadiazolinone, tetrazolinone, and triazolinone heterocyclic ring systems, other five-membered ring systems investigated in the 1980s included pyrazoles, such as fluazolate (**17**) [51] from Monsanto; pyraflufen-ethyl (**18**) (Ecopart®) [52, 53], introduced in 1993 by Nihon Noyaku as a post-emergence broadleaf herbicide in cereals; and fused triazolinone rings such as azafenidin (**19**) [54, 55] from DuPont (Fig. 3.7).

3.2.3 Phenyl Tetrahydrophthalimide

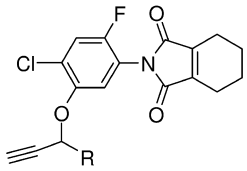
Phenyl tetrahydrophthalimides represent the third class of early Protox herbicides. They were introduced in the early 1970s, after the diphenyl ether and 1,3,4-oxadiazol-2(3H)-one chemistries. Following the introduction by Mitsubishi of chlorophthalim (**3**) [27] in 1972, incorporation of the 2,4,5-trisubstituted-phenyl pattern in the 1980s resulted in the synthesis of highly active molecules such as S-23142 (**12**) [45], and S-23121 (**20**) [45, 56] (Fig. 3.8).

The tetrahydrophthalimide area of chemistry generated a great deal of interest between 1980 and 2000, with hundreds of patents issued by a wide range of agrochemical companies [29]. Once the fluorine group at the 2-phenyl position and the chlorine group at the 4-phenyl position were established as the optimum substituents for the aromatic ring, the 5 position of the phenyl ring and the tetrahydrophthalimide heterocycle became the target of intense research. A wide variety of oxygen (**21**), sulfur (**22**), amino (**23**), and carbonyl (**24**) derivatives at the 5 posi-



3

Chlorophthalim
1972 Mitsubishi

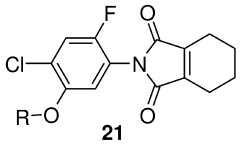


12 R = H S-23142 1982 Sumitomo
20 R = CH_3 S-23121 1982 Sumitomo

Fig. 3.8. Chemical structures of chlorophthalim (**3**), S-23142 (**12**), and S-23121 (**20**).

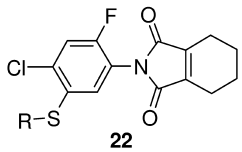
tion of the aromatic ring were introduced [29] (Fig. 3.9). Some of these phenyl tetrahydrophthalimides reached the market, such as flumiclorac-pentyl (**25**) (Resource®) [57] and cinidon-ethyl (**26**) (Lotus®) [58] (Fig. 3.10).

The phenyl tetrahydrophthalimides are primarily post-emergence herbicides for the control of broadleaf weeds, though they will show pre-emergence activity at higher rates of application. Flumiclorac-pentyl is a post-emergence herbicide for the control of broadleaf weeds such as cocklebur, common lambsquarters, jimsonweed, amaranthus, prickly sida, and velvetleaf in soybean and corn at 25–



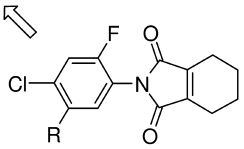
21

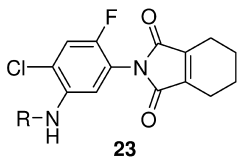
R = Propargyl (S-23142, **12**), alkyls, allyl, benzyl, acetates



22

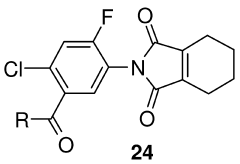
R = Alkyls, allyl, benzyl, acetates





23

R = CF_3SO_2-, acetate, alkyl, acetyl



24

R = AlkylO, benzyl, AlkylNH

Fig. 3.9. Derivatization of aromatic position 5 of phenyl tetrahydrophthalimide.

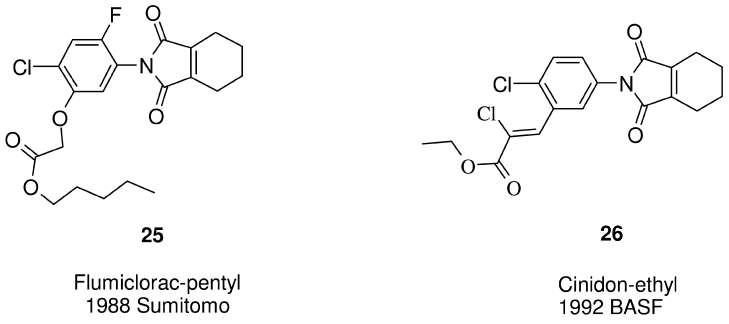


Fig. 3.10. Chemical structures of flumiclorac-pentyl (**25**) and cinidon-ethyl (**26**).

50 g-a.i. ha^{-1}. Cinidon-ethyl is used for the post-emergence control of annual broadleaf weeds, particularly bedstraw, deadnettle, and speedwell, in winter and spring small grain cereals at 50 g-a.i. ha^{-1}.

In addition to the phenyl tetrahydrophthalimides discussed, several significant Protox herbicides with a phenyl ring attached to a fused thiadiazole[3,4-*a*]pyridazine or an oxazolidinedione ring were reported. Two examples of phenyl thiadiazole[3,4-*a*]pyridazine systems are fluthiacet-methyl (**27**) (Appeal®) [59] and NCI-876-648 (**29**) [60]. These compounds are said to act as pro-herbicides, converted in the plant into the corresponding phenyl triazolo[3,4-*a*]pyridazines [61] (Fig. 3.11).

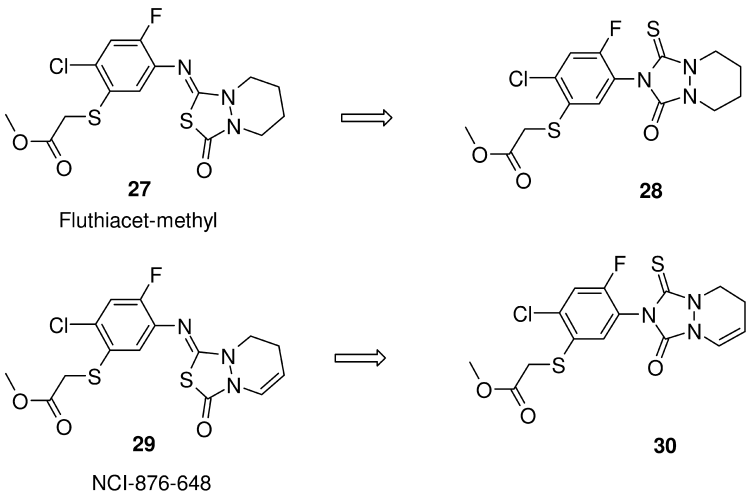
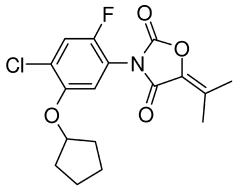


Fig. 3.11. Rearrangement of phenyl thiadiazolo[3,4-*a*]pyridazine to phenyl triazolo[3,4-*a*]pyridazines.



31

Pentoxazone

Fig. 3.12. Chemical structure of pentoxazone (**31**).

Phenyl oxazolidinedione chemistry is best exemplified by pentoxazone (**31**) (Wechser®) [62, 63] (Fig. 3.12) – discovered by Sagami for the pre-emergence control of weeds such as barnyard grass in rice at application rates of 200–450 g-a.i. ha^{-1}.

3.3 Non-classical Protox Chemistries

Several chemistries introduced in the 1990s did not conform to the established structure–activity relationships (SARs) of previous chemistries like the diphenyl ethers and the 2,4-dihalo-5-substituted-phenyl heterocycles. Figure 3.13 shows the SARs of 2-fluoro-4-chloro-5-substituted-phenyl heterocycles [5, 64]. These newer developments impacted both the aromatic and the heterocyclic portion of N-phenyl heterocycles.

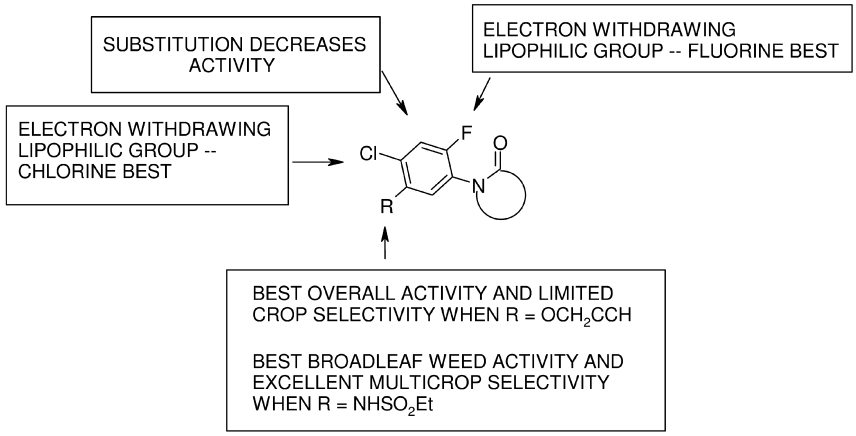
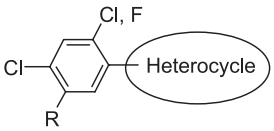


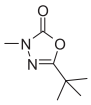
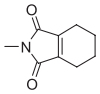
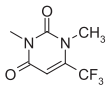
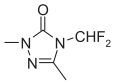
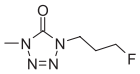
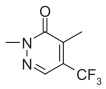
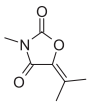
Fig. 3.13. Structure–activity relationships of the two aromatic rings of 2,4,5-trisubstituted-phenyl heterocyclic systems.

3.3.1
N-Phenyl Heterocycle – New Heterocyclic Systems

Many heterocyclic systems, usually attached to aromatic rings via a nitrogen or carbon atom, have been introduced in the past fifteen years. We have already discussed some of these heterocyclic rings, such as oxadiazolinone [25, 26], tetrahydrophthalimide [27], tetrazolinone [5], triazolinone [6–8, 48], pyrazole [51–

Table 3.1 Introduction of the uracil and pyridazinyl heterocyclic ring systems in the 1990s.



Ring system	Heterocycle		Heterocycle	Ring system
Oxadiazolinones				
Tetrahydrophthalimides				
				Uracil
Triazolinones		1990 ⇨		
Tetrazolinones				Pyridazinone
Pyrazoles				
Oxazolidinediones				

53], and oxazolidinedione [62]. Extensive reviews of these heterocyclic systems, their properties, and their synthesis have been published [28, 29].

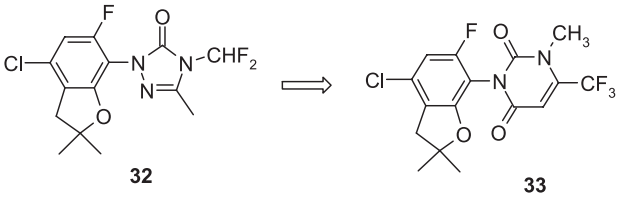
Of the several dozen new heterocyclic systems introduced during the period 1990–2005, the one that stands out as having the greatest impact in terms of significant increase in biological activity is 6-trifluoromethyl-2,4(1*H*,3*H*)-pyrimidinedione ring – commonly referred to as uracil – initially introduced by Hoffman-La Roche and Uniroyal [65, 66] (Table 3.1).

Replacement of the tetrahydrophthalimide and other heterocyclic rings such as the triazolinone **32** with the uracil **33** ring resulted in a significant improvement in biological activity, particularly when applied pre-emergently [67] (Table 3.2).

Because of this significant improvement in herbicidal activity, the uracil ring has become a standard ring in any N-aryl heterocycle Protox-related patent application. Initial SAR studies at the uracil nitrogen position showed that methyl and amino resulted in optimum activity [67]. Increasing the size and length of the R group in compound **34** resulted in a significant reduction in biological activity. Interestingly, both the lipophilic methyl group (**35**) and the hydrophilic amino group (**36**) are equally active (Table 3.3).

Three examples of molecules that contained the uracil ring and reached an advanced stage of development are benzfendizone (**41**) [68, 69], butafenacil (**42**) (Inspire®, Rebin®) [70], and flufenpyr-ethyl (**43**) [71] (Fig. 3.14). Benzfendizone is a post-emergence herbicide that provides good control of grass and broadleaf weeds in tree fruits and vines, acts as a cotton defoliant, and has applications in total vegetation control. The 6-trifluoromethyl group in the uracil ring is essential for biological activity; replacing it with methyl results in complete loss of activity.

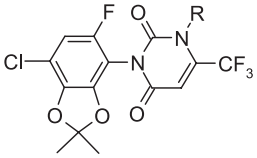
Table 3.2 Comparison of biological activity of triazolinone and uracil heterocycles.



32 **33**

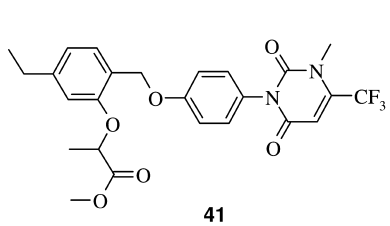
Weed species	Pre-emergent biological activity ED_{85} (g-a.i. ha^{-1})	
	32	**33**
Morning-glory	395	22
Johnson grass	300	10

Table 3.3 Effects of uracil N-substituents on herbicidal activity of analogs of compound **34**.



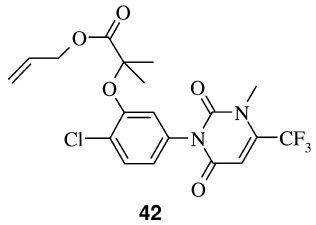
34

Compound	R	Pre-emergent biological activity ED_{85} (g-a.i. ha^{-1})		
		Velvetleaf	Morning-glory	Green foxtail
35	CH_3	3	3	3
36	NH_2	3	3	3
37	CH_2CH_3	8	17	3
38	CH_2OCH_3	18	52	44
39	$CH_2C_6H_5$	958	>1000	307
40	$CH_2CH_2CH_3$	>1000	>10000	>1000



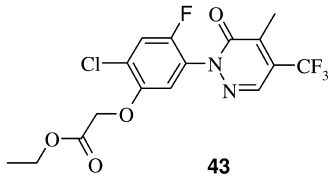
41

Benzfendizone



42

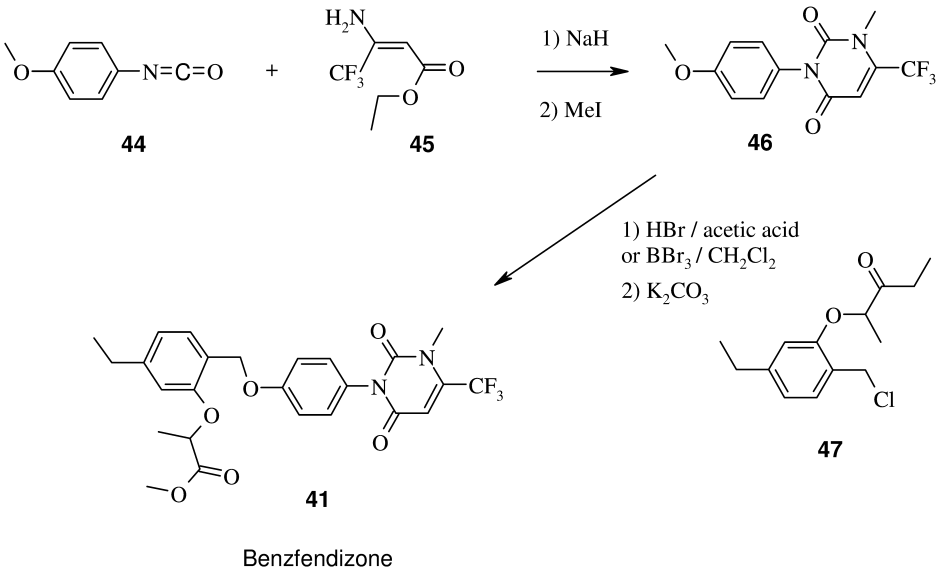
Butafenacil



43

Flufenpyr-ethyl

Fig. 3.14. Chemical structures of benzfendizone (**41**), butafenacil (**42**), and flufenpyr-ethyl (**43**).



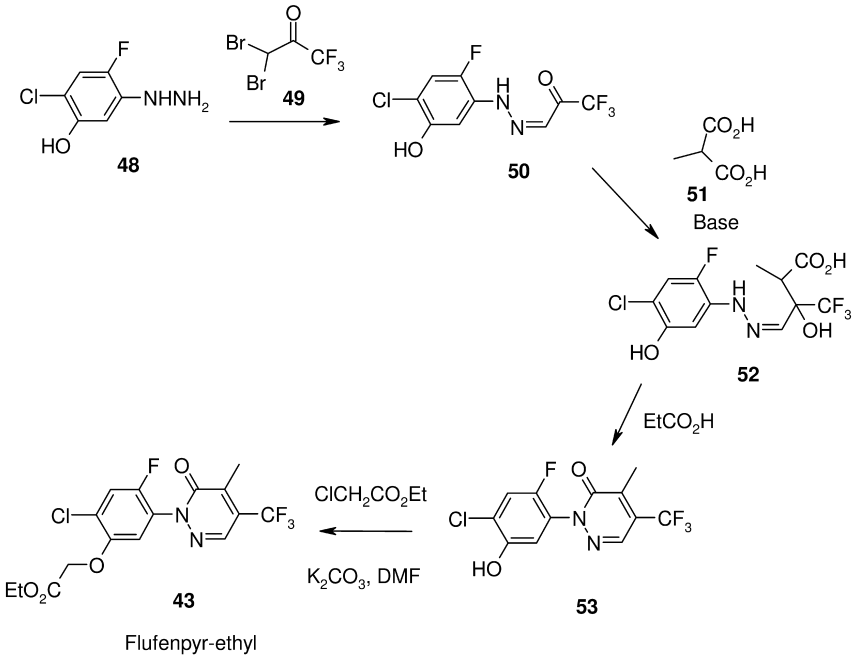
Scheme 3.1. Synthesis of the uracil heterocyclic ring of benzfendizone (**41**).

The uracil heterocycles are readily prepared in high yields from the corresponding aryl isocyanates **44** and from ethyl trifluoromethylaminocrotonate (**45**) in the presence of a base [72]. The uracil heterocycle is then directly N-methylated with methyl iodide in a one-pot reaction (Scheme 3.1). The uracil ring is stable to treatment with strong acids such as HBr and weak bases such as potassium carbonate, both reagents used in the further derivatization of the intermediate **46** with the benzyl chloride **47** to produce benzfendizone (**41**).

A less familiar ring system, but one that was part of a molecule selected for advanced testing, was the 5-methyl-6-oxo-4-(trifluoromethyl)-1-(6*H*)-pyridazinyl ring system of flufenpyr-ethyl (**43**). The pyridazinyl heterocycle can be prepared from the reaction of 4-chloro-2-fluoro-5-hydroxyphenyl hydrazine (**48**) and 1,1-dibromo-3,3,3-trifluoroacetone (**49**) to give the corresponding hydrazone **50**, which when reacted with methyl malonic acid (**51**), in the presence of a base, provides the intermediate **52**. Acid-catalyzed ring closure of **52**, followed by *O*-alkylation of **53** with ethyl chloroacetate, results in the synthesis of flufenpyr-ethyl (**43**) [73] (Scheme 3.2).

3.3.2
Phenoxyphenyl and Benzyloxyphenyl Attached to Heterocycle

Extensive modeling and quantitative structure–activity relationship (QSAR) studies of Protox herbicides have been reported [12, 74, 75]. Earlier, it was postulated that Protox herbicides act by mimicking the protoporphyrinogen oxidase



Scheme 3.2. Synthesis of the pyridazinyl heterocyclic ring of flufenpyr-ethyl (**43**).

substrate, protoporphyrinogen IX [76] (Fig. 3.15). This observation resulted in the discovery of the three-ring 2,4-dihalo-5-phenoxyphenyl propionate heteroaryl herbicide **54**, and later the heterocyclic phenoxymethylphenoxy propionate chemistry **56**, a significant improvement over the 4-chlorobenzyloxyphenyl heterocycles **55**. Both **54** and **56** are highly potent classes of Protox herbicides.

Subsequently, molecular modeling studies found good overlap between the diphenyl ether aromatic rings and protogen [77], as well as between a set of imide-type Protox inhibitors and protogen [78]. These studies were important in advancing the hypothesis that the diphenyl ethers mimicked protogen, though they were of limited practical value, having failed to reveal any new chemical structures.

A class of Protox inhibitors that redefined the accepted SARs and QSARs of the aromatic 4 position was the substituted benzyloxyphenyl heteroaryl area. As discussed earlier, SAR and QSAR studies of the phenyl ring of Protox herbicides demonstrated the need for halogens in the 2- and 4 positions of the phenyl ring, with the exception of the 4-chlorobenzyloxy group such as that of 4-chlorobenzyloxyphenyl tetrahydrophthalimide outlier **55** (Fig. 3.15) and reported by Ohta and coworkers in 1980 [79]. Chlorine at the para position of the benzyloxy was reported to provide optimum biological activity.

Further QSAR studies by Fujita [80] rationalized the high activity of this outlier 4-chlorobenzyloxy group by stating that the unexpected activity of the 4-benzyloxy ring was due to additional interactions of this group with the target enzyme.

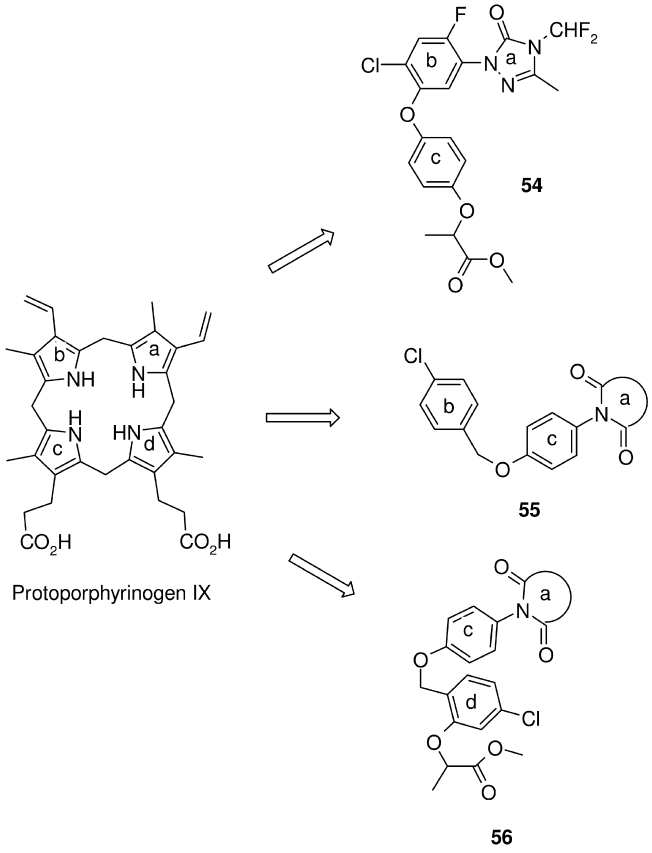


Fig. 3.15. Phenoxyphenoxy and benzyloxyphenyl as three-ring mimics of tetrapyrroles.

Given the strict steric and electronic requirements of groups at the 4 position of the phenyl ring, with chlorine as the optimum group, Fujita's explanation for the unexpected activity of a bulky electron-donating group such as the 4-benzyloxyphenyl is highly unlikely. In general, the presence of outliers in SAR or QSAR analysis indicates an unusual property of that group – such as a switch in the nature of the binding of the outlier in the enzyme site – and an opportunity for a major breakthrough. It was speculated that the activity of the 4-benzyloxy outlier was potentially due to a shift in binding mode for phenyl rings attached to heterocycles containing two flanking carbonyl rings, such as tetrahydrophthalimides and the newer uracil rings [64, 69]. Based on this new binding mode, the benzyloxy group could mimic the lipophilic portion of protoporphyrinogen IX, ring b, or the hydrophilic portion of protoporphyrinogen IX, ring d. Based on this working hypothesis, a series of compounds were prepared that contained an oxypropionate side chain, in addition to chlorine, in the benzyloxy

group [69]. This work resulted in benzfendizone, a highly active broad-spectrum post-emergence herbicide.

3.3.3
Benzoheterocyclic Attached to Heterocycle

As discussed earlier, extensive studies of the 2, 4, and 5 positions of the phenyl ring of Protox inhibitors revealed very specific electronic, lipophilic, and steric requirements for chemical groups at these positions. Thus, it was rather surprising when it was discovered that it was possible to obtain highly active molecules by linking the 4 and 5 or the 5 and 6 positions of the phenyl ring to yield a wide variety of benzoheterocycles, such as those in Figs. 3.16 and 3.17.

Linking the 4 and 5 or 5 and 6 positions of the phenyl ring of Protox inhibitors to give a new heterocyclic ring resulted in two new classes of Protox herbicides, both with increased biological efficacy and new SARs. In the first instance, previous SAR studies of 2,4,5-trisubstituted-phenyl heterocycles have shown that position 2 of the phenyl ring required a halogen group for optimum biological activity, with chlorine and fluorine generating the highest overall activity. Position 4 of

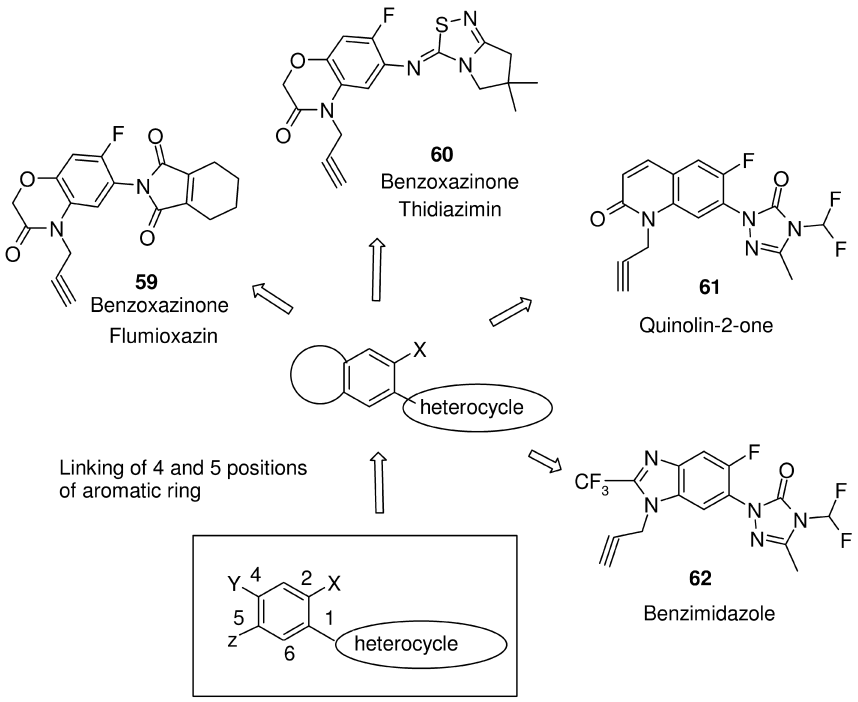


Fig. 3.16. Benzoheterocycles resulting from linking aromatic positions 4 and 5 of phenyl heterocyclic Protox inhibitors.

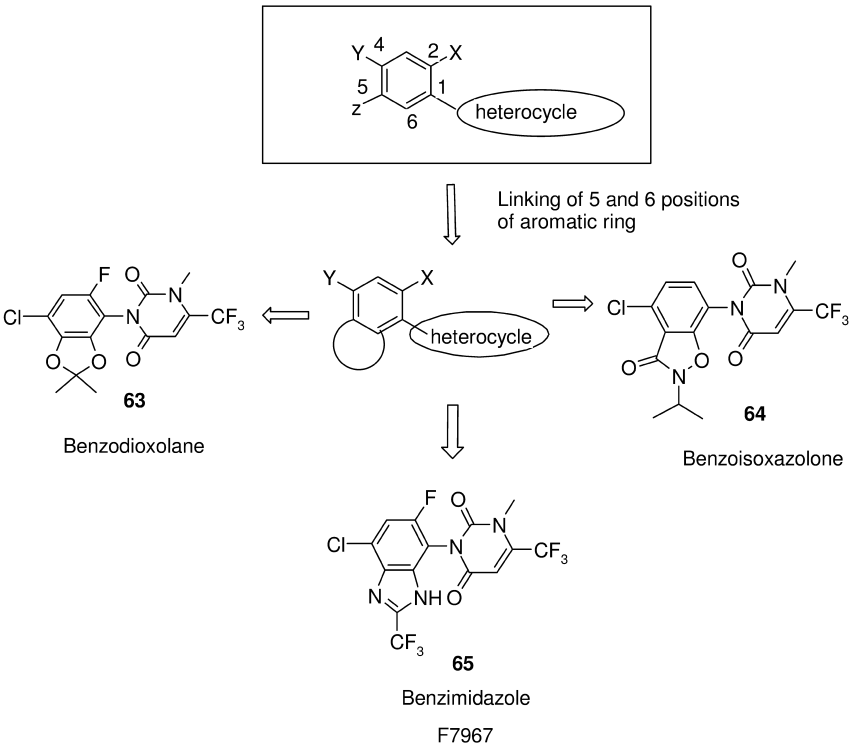


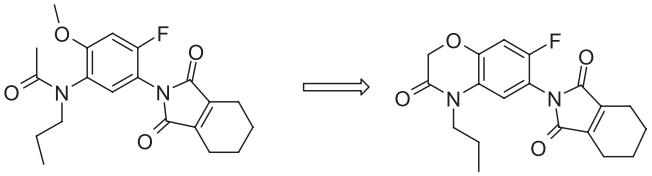
Fig. 3.17. Benzoheterocycles resulting from linking aromatic positions 5 and 6 of phenyl heterocyclic Protox inhibitors.

the phenyl ring required a hydrophobic, electronegative group such as halogen for optimum activity, with chlorine and bromine resulting in the best activity. Electron-donating groups such as methoxy resulted in significant loss of biological activity. The benzoxazinone SAR does not fit these rules, with compound **58** being far more active than its open chain analog **57** [81, 82] (Table 3.4).

Incorporating the benzoxazinone ring in Protox herbicides resulted in several commercial molecules, such as flumioxazin (**59**, Sumisoya®) [83] and thidiazimin (**60**) [84] (Fig. 3.16). Other heteroaryl rings include the quinolin-2-one **61** [82] and benzimidazole **62** [85].

The second class of benzoheteroaryl Protox herbicides are obtained when aromatic positions 5 and 6 are linked together to form various 3-(4,6-substituted benzoheterocyclyl) rings, which can be attached to a wide range of heterocycles (Fig. 3.17). The 3-(4,6-substituted benzoheterocyclyl) ring system represents a highly active area of Protox inhibitors, particularly when applied pre-emergently. The benzodioxolane uracil **63** provides complete control of pigweed, wild mustard, velvetleaf, green foxtail, and johnson grass at rates as low as 10 g-a.i. ha^{-1} when applied pre-emergently [72]. Other rings include benzoisoxazolone **64** [72]

Table 3.4 Comparison of biological activity of open vs. fused ring analogs.



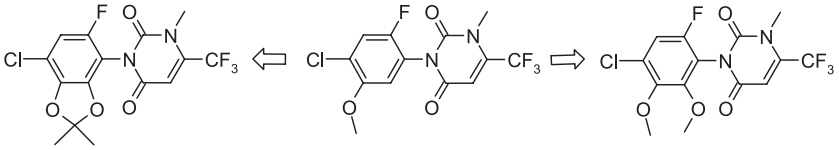
57 **58**

Compound	Pre-emergent biological activity ED_{85} (g-a.i. ha^{-1})	
	Velvetleaf	Morning-glory
57	2000	>4000
58	62.5	125

and the corn and rice benzimidazole F7967, compound **65**, a new pre-emergence herbicide from FMC [86]. In pre-emergence applications, under greenhouse conditions, **65** controlled at rates as low as 10–30 g-a.i. ha^{-1} several broadleaf weeds – velvetleaf, morning-glory, pigweed, bindweed, nightshade, kochia, and chickweed – and grass weeds such as crabgrass, foxtails, johnson grass, and shattercane.

The SARs of these 3-(4,6-substituted benzoheterocyclyl) heterocycle herbicides differ from the more traditional 2,4-dihalo-5-substituted-phenyl heterocycles discussed earlier. As shown in Table 3.5, introducing a methoxy group at position 6 of compound **66** results in a dramatic loss of biological activity, the resulting com-

Table 3.5 Comparison of biological activity of open vs. fused ring analogs.



63 **66** **67**

Compound	Pre-emergent biological activity ED_{85} (g-a.i. ha^{-1})	
	Velvetleaf	Green foxtail
63	3	4
66	6	9
67	32	143

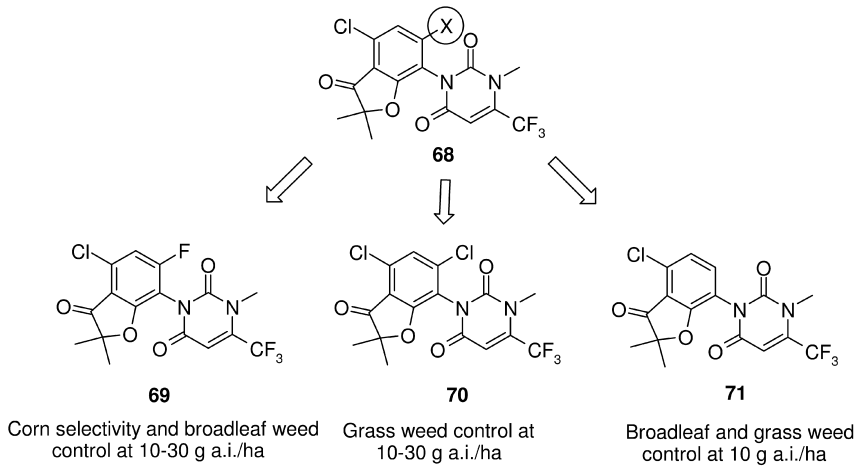


Fig. 3.18. Effect on biological activity of substituents at position 6 of the 2,3-dihydrobenzofuran ring.

pound **67** being more than five-fold less active than **66**. Linking together the aromatic positions 5 and 6 into a benzodioxolane ring resulted in compound **63**, which was more active than either compound **66** or **67** [72].

Substituents at the 6 position of the benzoheterocyclic ring had a dramatic effect on the weed spectrum and crop selectivity of these compounds when applied pre-emergently. First, as Fig. 3.18 shows, in the case of compound **68**, a fluorine at position 6 of the 2,3-dihydrobenzofuran ring gives compound **69**, which has excellent corn selectivity and control of broadleaf weeds (velvetleaf, wild mustard, and pigweed) at 10–30 g-a.i. ha^{-1}. Next, replacing the 6-fluoro group with a chlorine resulted in a compound **70**, which has good grass weed control (barnyardgrass, green foxtail, and johnson grass) at 10–30 g-a.i. ha^{-1}. Finally, compound **71**, with a hydrogen substituent at the 6 position, resulted in broad-spectrum control of both grass and broadleaf weeds at 10 g-a.i. ha^{-1}. Compounds **70** and **71** did not provide the same degree of corn selectivity as compound **69** [72].

3.3.4
Benzyl Attached to Heterocycle

This very interesting chemistry class of Protox inhibiting herbicides has received less attention than other Protox herbicides. It is the only class that introduces the use of a benzyl ring instead of a phenyl ring, and in so doing it has redefined the structure–activity of the aromatic ring. SAR studies of the benzyl uracil series resulted in compound **72**, with a 2,3,5-trisubstitution pattern of the phenyl ring, a significant difference from that of 3-phenyl-6-trifluoromethyluracils, where the optimum substitution pattern is that with substituents at the 2, 4, and 5 positions of the phenyl ring, as in **66** [87] (Fig. 3.19).

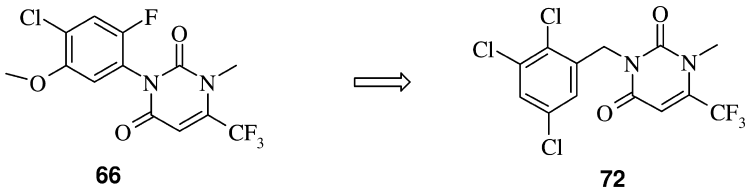
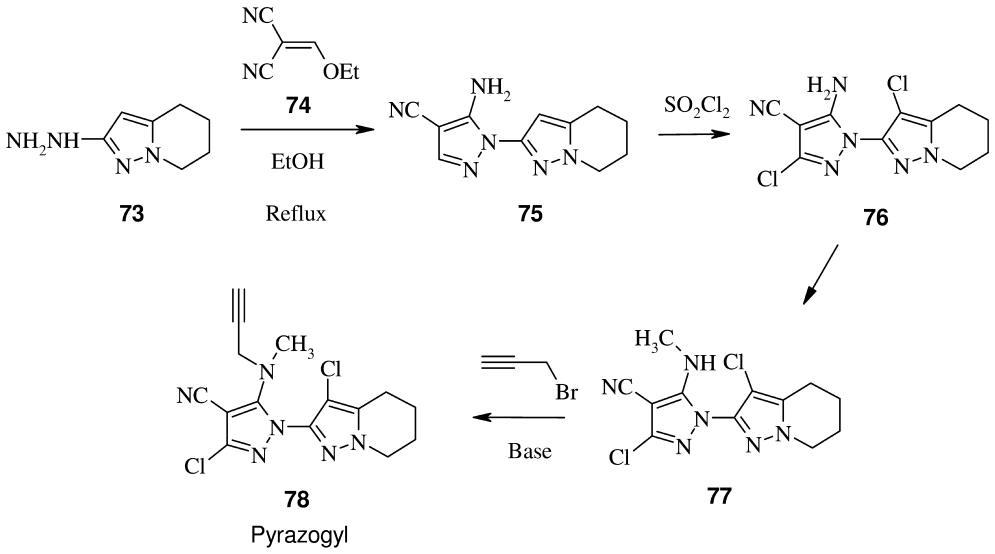


Fig. 3.19. Substitution patterns of phenyl and benzyl uracils.

3.3.5
Replacement of Phenyl Ring with Pyrazole

In this section we discuss the unusual replacement of the phenyl portion of Protox herbicides with a pyrazole ring to give pyrazogyl (**78**) [88], a rice herbicide initially discovered by Aventis, now Bayer CropScience.

There are several examples of Protox inhibitors in which the phenyl ring has been replaced with pyridine [89, 90] – a fairly common bioisosteric move – while preserving the 2,4-dihalo-5-substituted pattern in the heteroaromatic. Pyrazogyl (**78**) is unusual in that it involves several changes, such as the nature and placement of substituents, the size of the ring, and the replacement of phenyl with a heterocycle. It can be prepared in several steps from 2-hydrazino-4,5,6,7-tetrahydropyrazo[4,5-*a*]pyridine (**73**) and ethoxymethylenemalononitrile (**74**), followed by bis-chlorination of the pyrazole rings in **75**, *N*-methylation of **76**, and, finally, *N*-propargylation of **77** [88] (Scheme 3.3).



Scheme 3.3. Synthesis of pyrazogyl (**78**).

3.4 Recent Developments

Several reviews of Protox herbicides cover the period from the 1960s to 2002 [11–13, 28, 73, 91]. In this section we discuss Protox-related work conducted between 2003 and 2006. Following the momentous volume of research in all aspects of Protox herbicides – their chemistry, biology, biochemistry – in the decades between 1970 and 1990, work in this area of herbicide chemistry has significantly slowed in more recent years. Although corporations have continued to invest in Protox research, with several new structures introduced recently, none of these new chemical structures differ significantly from those already discussed.

The crystal structure of the mitochondrial protoporphyrinogen IX oxidase enzyme obtained from tobacco, and complexed with phenyl pyrazole Protox inhibitors, was published in 2004 [92]. As discussed in the introduction, the membrane-embedded protoporphyrinogen oxidase enzyme is the target of the Protox herbicides. It was also mentioned in Section 3.3.2 that molecular modeling studies of Protox inhibitors found good overlap between the diphenyl ether aromatic rings and protoporphyrinogen IX (protogen) [77], and between a set of imide-type Protox inhibitors and protogen [78]. The paper on the protoporphyrinogen IX oxidase crystal structure, a collaboration between the Max-Plank Institute, Bayer CropScience, and Proteros, discusses how the active site architecture suggests a specific substrate-binding mode that is compatible with the rare six-electron oxidation. It also proposes that the pyrazole ring of 4-bromo-3-(5-carboxy-4-chloro-2-fluorophenyl)-1-methyl-5-trifluoromethylpyrazole (**79**) matches ring A, and the phenyl ring matches ring B of protoporphyrinogen IX (Fig. 3.20).

In terms of recent patent activity related to Protox inhibitors, a series of N-substituted phenyl isothiazolone Protox herbicides were prepared to investigate the potential of the isothiazolone heterocycle ring to act as a bioisostere for comparable tetrahydrophthalimides such as compound **80** [93] (Fig. 3.21). The 2-(4-chloro-3-isopropoxycarbonyl)phenyl isothiazole-1,1-dioxide **83** was the most active

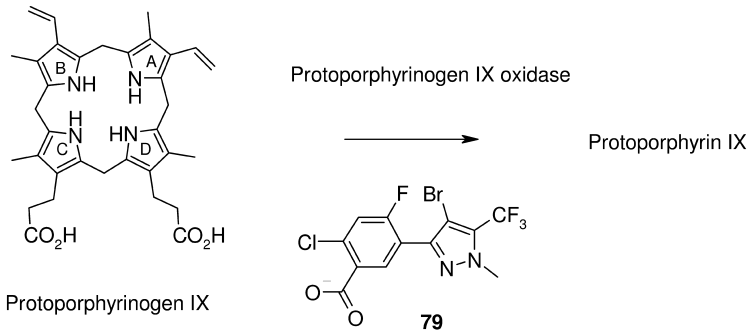


Fig. 3.20. Protox inhibitor 4-bromo-3-(5-carboxy-4-chloro-2-fluorophenyl)-1-methyl-5-trifluoromethylpyrazole (**79**) used in protoporphyrinogen IX oxidase binding studies.

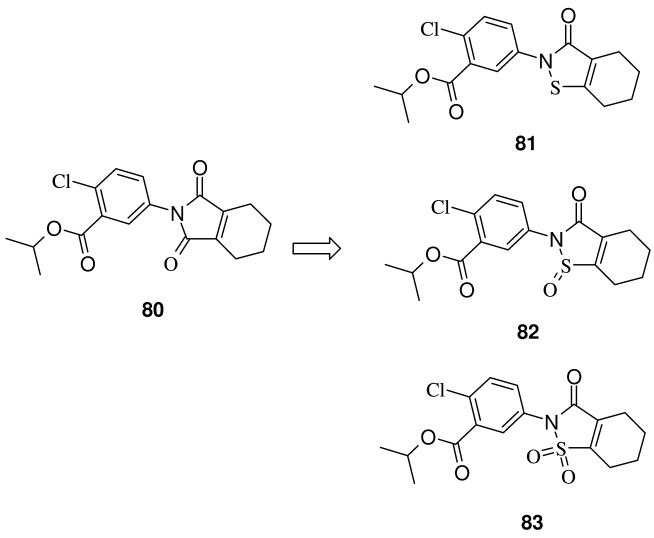
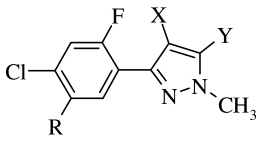
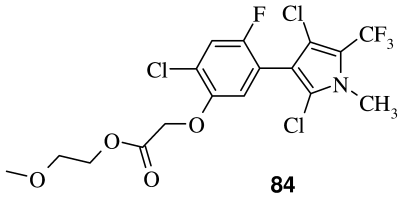


Fig. 3.21. Isothiazolone bioisosteres for tetrahydrophthalimide Protox inhibitors.

in the isothiazolone series as measured by inhibition of protoporphyrinogen IX oxidase isolated from corn, as well as by growth inhibition, chlorophyll decrease, and peroxidative destruction of cell membranes of green microalga *Scenedesmus acutus* [94]. Compound **83** was more active than either compound **81** or **82**, but about 100× weaker than the reference compound **80**.

Also published in 2004 were the synthesis and structure–activity of several 2-fluoro-4-chloro-5-substituted-phenyl pyrrole Protox herbicides, such as compound **84** [95]. This interesting pyrrole class of chemistry further extends the structure–activity of the 2-fluoro-4-chloro-5-substituted-phenylpyrazoles fluazolate (**17**) and pyraflufen-ethyl (**18**) discussed in Section 3.2.2 (Fig. 3.22).



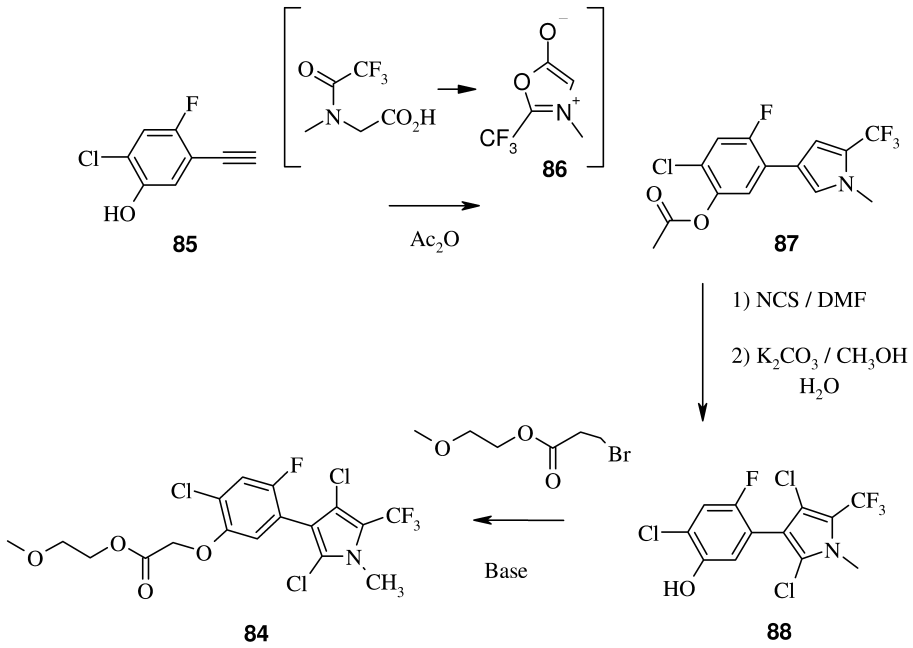


84

R	X	Y	Compound	
$CO_2CH(CH_3)_2$	Br	CF_3	Fluazolate	**17**
OCH_2CO_2Et	Cl	$OCHF_2$	Pyraflufen-ethyl	**18**

Fig. 3.22. Phenyl pyrazole and phenyl pyrrole Protox inhibitors.

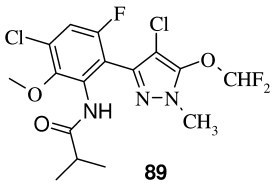
Compound **84** was extensively field tested in cereals and soybeans between 1999 and 2002 in France, Italy, and the United States. Post-emergence field application of **84** at 50 g-a.i. ha^{-1} demonstrated broadleaf weed control, with soybean tolerance, of morning-glory, redroot pigweed, and prickly sida. Soybean plants eventually outgrew initial injury at seven days after application. Field testing on winter wheat provided >80% control of several broadleaf weeds, including cleavers, at application rates of 50–60 g-a.i. ha^{-1}. The pyrazole **84** can be prepared in several steps starting from the 1,3-dipolar cycloaddition of 2-trifluoromethyl-3-methyl-1,3-oxazolium-5-olate **86** to 2-chloro-4-fluoro-5-ethynylphenol (**85**), followed by chlorination of the resulting pyrrole **87**, and reaction of **88** with the corresponding bromo acetate [95] (Scheme 3.4).



Scheme 3.4. Synthesis of phenyl pyrrole Protox inhibitors.

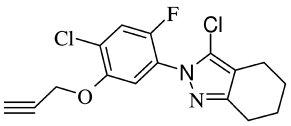
Another area related to fluazolate (**17**) and pyraflufen-ethyl (**18**) chemistry is a series of 2,4,5,6-tetrasubstituted-phenyl pyrazoles **89** (Fig. 3.23) from Ishihara Sangyo Kaisha [96]. These compounds differ from previous phenyl pyrazoles in that they have substituents at the 6 position of the phenyl ring. Pre-emergence application of **89** provided 100% control at 63 g-a.i. ha^{-1} of barnyardgrass, crabgrass, green foxtail, redroot pigweed, prickly sida, and velvetleaf. Soybean was reported to have 20% injury for compound **89** at this rate of application.

Several 2-phenyl-4,5,6,7-tetrahydro-2*H*-indazoles with several isoxazolinylmethoxy groups at the 5 position of the aromatic ring, such as compound **91**, were



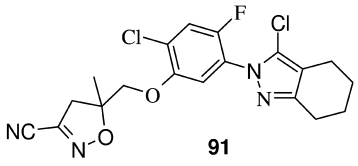
89

Fig. 3.23. Chemical structure of a tetrasubstituted-phenyl pyrazole.



90

S-275



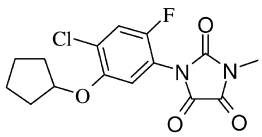
91

Fig. 3.24. Chemical structure of 2-fluoro-4-chloro-5-isoxazolinylmethoxy tetrahydroindazole **91**. Such compounds have the general structure of S-275 (**90**).

introduced by the Korea Research Institute of Chemical Technology [97, 98] as paddy rice herbicides (Fig. 3.24). These compounds have the general structure of S-275 (**90**), from Sumitomo [99]. Introduction of the isoxazolinylmethoxy groups at the 5 position of the aromatic ring is said to provide good broadleaf control with good tolerance by transplanted rice seedlings.

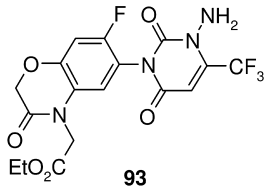
Herbicidal activity on several weeds, such as hairy beggarsticks, black nightshade, and knotweed, was reported in 2004 for a series of 2,4,5-imidazolidine triketones, such as compound **92** [100] (Fig. 3.25).

A series of four- and five-membered benzoheterocycle uracils derived from tying back the 4 and 5, as well as the 2 and 3, aromatic positions were disclosed. The benzoheterocycles obtained from linking aromatic positions 4 and 5 were developed by Bayer in 2003 [101]. The differentiating feature between these benzoheterocyclic uracils and earlier ones discussed in Section 3.3.1 is the replacement of the N-methyl group with an amino group in the uracil heterocycle, as exemplified by **93** and **94** (Fig. 3.26).



92

Fig. 3.25. Chemical structure of a 2-fluoro-4-chloro-5-alkoxy phenyl imidazolidine triketone Protox inhibitor.



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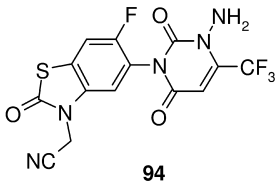
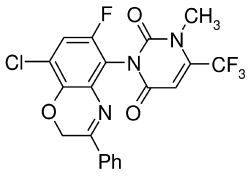


94

Fig. 3.26. Benzoheteroaryl N-amino uracil derivatives.



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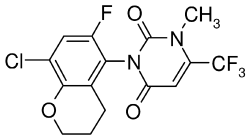


96

Fig. 3.27. Benzoheterocycle uracils.

Ishihara Sangyo Kaisha disclosed a series of benzoheterocycles derived from linking the 2 and 3 aromatic positions, such as compounds **95** [102] and **96** [103] (Fig. 3.27).

Further derivatization at the 5 position of the phenyl ring of 2,4,5-trisubsitutedphenyl heterocycles has resulted in several new Protox herbicide patents. Ishihara Sangyo Kaisha introduced several benzohydrazide derivatives such as **97** [104] for use as herbicides, desiccants, and defoliants. Pre- and post-emergence control of several weeds, such as redroot pigweed, velvetleaf, sicklepod, ivyleaf morning-glory, and cocklebur, was demonstrated at application rates as low as 63 g-a.i. ha^{-1}. BASF reported the following new chemistries: the benzoic acid derivatives **98**, with good post-emergence activity in redroot pigweed and common lambsquarter, as well as potential use as cotton desiccants or defoliants [105], and **99** [106]; aminosulfonylamino phenyl uracil derivatives (**100**) [107]; and benzosulfonamides (**101**) [108]. Figure 3.28 shows these and other Protox inhibitors with diverse groups at the aromatic meta position, which are discussed below.

Bayer introduced 2-aryl-1,2,4-triazine-3,5-diones with the 2,4-dihalo-5-aminoalkylsulfonylphenyl, such as **102** [109] and **103** [110]; the aromatic substitution pattern is reminiscent of sulfentrazone (**15**).

In addition, in 2003 Bayer introduced phenyluracil derivatives with heteroarylmethyleneoxy groups at the 5 position of the phenyl ring, as in **104** [111], and N-(thiocarbonylaminophenyl)uracils such as **105** and **106** [112].

Isagro Ricerca claimed good pre-emergence and post-emergence weed control at rates as low as 15 g-a.i. ha^{-1} for several Protox inhibitors with a wide variety of groups in the 5 aromatic position of 2,4-dihalo-5-substituted uracils, such as

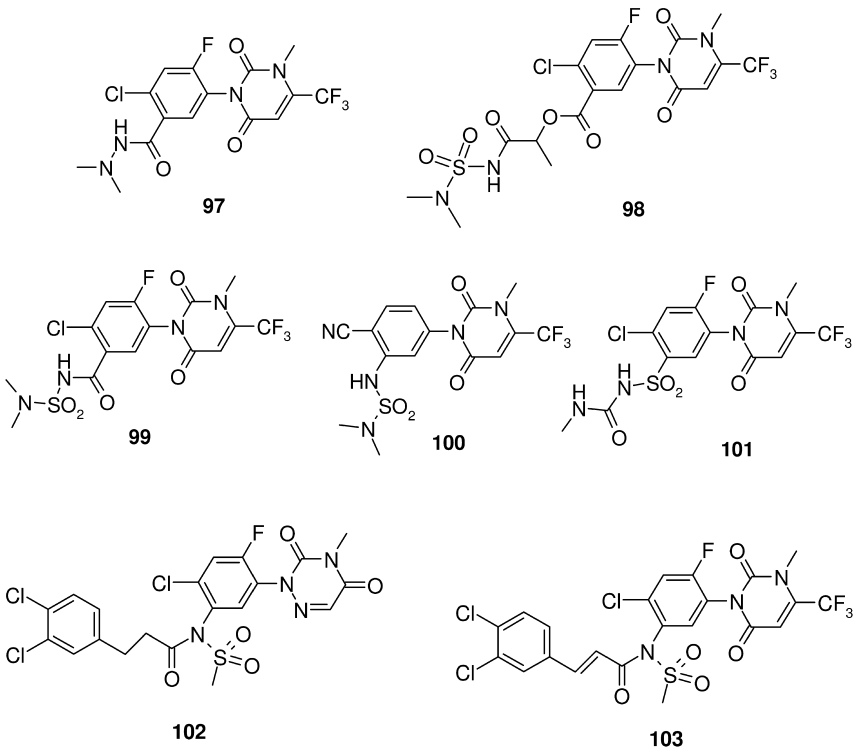


Fig. 3.28. Protox inhibitors with diverse groups at the aromatic meta position (see text for details).

107 and **108** (Fig. 3.28) [113]. Among the weeds controlled were bedstraw, barnyardgrass, redroot pigweed, prickly sida, and velvetleaf, with crop selectivity in rice, wheat, barley, corn, and soybean.

Researchers at Central South University and Hunan Research Institute of Chemical Industry in Changsha, Hunan, China have reported the herbicidal activity of several isoindoline-1,3-diones molecules such as **109**, and compared their biological activity to flumioxazin (**59**) (Fig. 3.29). Compound **109** provided >80% control at 75 g-a.i. ha^{-1} in both pre- and post-emergence treatments against broadleaf weeds such as velvetleaf, common lambsquarter, and redroot pigweed, and against grass weeds such as large crabgrass, barnyardgrass, and green foxtail. Compound **109** was reported to be safe on cotton and corn at an application rate of 150 g-a.i. ha^{-1} when applied pre-emergently, and it also provided good wheat safety when applied post-emergently at 7.5–30 g-a.i. ha^{-1}. The IC_{50} (inhibitive concentration, in g-a.i. ha^{-1}, to obtain 50% growth inhibition) values for the post-emergence control of velvetleaf and crabgrass were given for **109**, $IC_{50} = 3.6$ and 4.8, respectively, and compared to those of flumioxazin, $IC_{50} = 1.0$ and 2.5 [114].

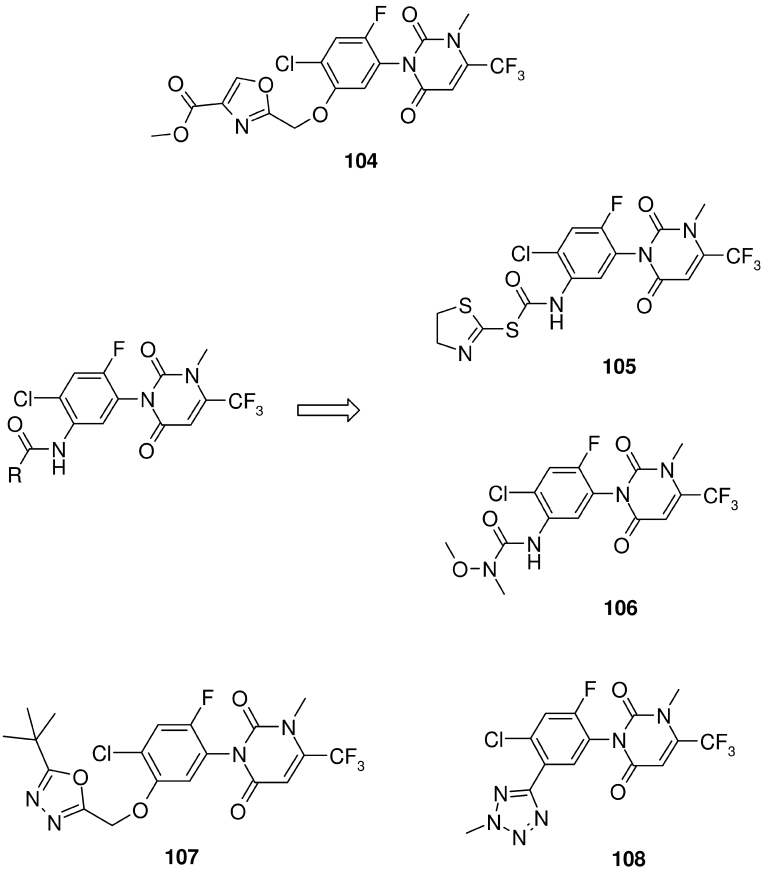


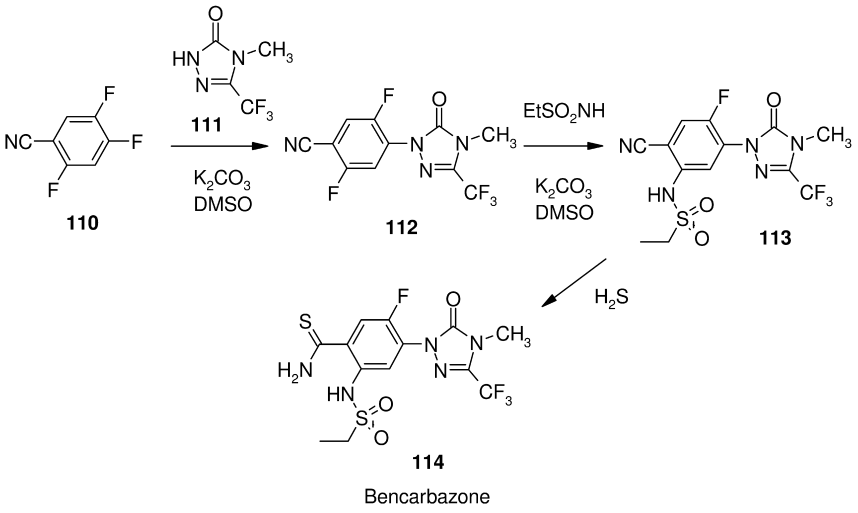
Fig. 3.28. (*continued*)

Bencarbazone (**114**) [115] is a recent Protox inhibitor triazolinone herbicide from Arvesta for the post-emergence control of broadleaf weeds in cereals and corn. It provides good control of bedstraw, velvetleaf, redroot pigweed, common lambsquarter, and speedwell at rates of application of 20–30 g-a.i. ha^{-1}. Bencarba-

Fig. 3.29. Chemical structure of benzoxazine isoindoline-1,3-diones.

zone (**114**) has many of the features associated with Protox herbicides, particularly those of the Protox herbicide sulfentrazone (**15**). The most striking chemical feature of bencarbazone (**114**) is the replacement of the phenyl 4-chloro group with a thioamide group.

Bencarbazone **114** can be prepared in several steps from the nucleophilic displacement reaction of 2,4,5-trifluorobenzonitrile (**110**) with 4-methyl-3-trifluoromethyl-1,2,4-triazolin-5-one (**111**) to give 1-(4-cyano-2,5-difluorophenyl)-4-methyl-3-trifluoromethyl-1,2,4-triazolin-5-one (**112**). Reaction of **112** with ethanesulfonamide in the presence of a base such as potassium carbonate gives **113**, which on reaction with hydrogen sulfide gives bencarbazone (**114**) [116] (Scheme 3.5).



Scheme 3.5. Chemical structure and synthesis of bencarbazone (**114**).

3.5 Toxicology

The toxicology of Protox inhibitors has been discussed previously [13, 117]. It was shown that the addition of high doses of the Protox-inhibiting herbicides fomesafen, oxyfluorfen, and oxadiazon to the diet of mice increased the porphyrin content of liver, bile, and feces. The porphyrin accumulation induced by high-dose, short-term herbicide treatment is reversible. Within days after withdrawal of herbicide treatment, porphyrin levels returned to normal. Based on these findings – the high dose required to elicit an effect and the reversible nature of that effect – the authors, Krijt et al., concluded that the toxicological risk resulting from exposure to Protox-inhibiting herbicides is small [117].

3.6 Summary

Protox-inhibiting herbicides continue to be an area of interest to agrochemical companies, with most effort focused on fine tuning the 5 position of the aromatic ring of N-phenyl uracil to gain both a particular crop/weed/application method as well as a proprietary position.

In addition to Protox herbicide activity reported in the patent literature, there is continued interest in understanding the structure–activity relationships of Protox inhibitors [118–120]. Research efforts continue to be devoted to the development of Protox inhibitor-resistant crops [121]. In 1999, Syngenta announced its discovery of a novel gene technology, under the trademark Acuron™, that provides crops with tolerance to Protox inhibitors.

Finally, weed shifts observed in genetically modified crops, caused by the development of weed resistance to the widely used glyphosate herbicide, will offer market opportunities for herbicides with other modes of action, such as Protox-inhibiting herbicides.

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4
Herbicides with Bleaching Properties

4.1
Phytoene Desaturase Inhibitors

Gerhard Hamprecht and Matthias Witschel

4.1.1
Introduction

Herbicidal activity through inhibition of phytoene desaturase (PDS) can be easily detected by a striking whitening effect of tissues in newly grown plant leaves in the light. These symptoms led to their classification as ''bleaching herbicides'', i.e., herbicides interfering with the biosynthesis of photosynthetic pigments, chlorophylls or carotenoids [1, 2]. While norflurazon, as the oldest representative, was introduced by Sandoz as a spin-off of phenylpyridazinone chemistry (see Section 4.1.4.6) as early as 1968, it took almost two decades for the Mode of Action (MoA) – inhibition of PDS and consequently carotenoid biosynthesis – to become fully known. Since then, due to their low application rates, lack of resistance in the field – which could only be introduced genetically [3, 4] – and favorable mammalian toxicity, industrial research concentrated on this new MoA, leading to several potent herbicides for modern agriculture.

4.1.2
Carotenoid Biosynthesis and Phytotoxic Effects of Bleaching Herbicides

4.1.2.1 Targets for Bleaching Herbicides

Bleaching may be a result of photooxidative events generated within the plant cell or chloroplast, leading to the destruction of the plant pigments or direct inhibition of pigment biosynthesis, whereby carotenoid and chlorophyll formation is prevented [5].

With carotenoid biosynthesis, plastoquinone is involved as an electron acceptor, which we encounter further in photosynthetic electron transport [2]. An important precursor in the synthesis of plastoquinone, which also serves as a cofactor for the PDS enzyme, is homogentisic acid, which is formed from 4-hydroxyphenyl-pyruvate by 4-hydroxyphenylpyruvate dioxygenase (HPPD)

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

[6, 7]. Inhibition of plastoquinone biosynthesis through HPPD blockade, therefore, causes herbicidal and bleaching phytotoxicity symptoms similar to those of PDS inhibition [6, 8]. However, HPPD inhibition induces reduced growth and chlorosis, which can be antagonized by homogentisic acid. Additionally α-tocopherol synthesis – a scavenger of activated singlet oxygen – is blocked, leading ultimately to oxidation of the D1 protein chain with the nonheme iron of the photosystem II reaction center, oxidative tissue damage, and bleaching [6]. The story of the discovery of HPPD herbicides and the structural requirements for herbicidal diketones have been described [9, 10]; see also Chapter 4.2 [Hydroxyphenylpyruvate Dioxygenase (HPPD) the Herbicide Target] of this book. In addition to PDS and HPPD inhibitors, other herbicides became known for their bleaching properties: amitrole – an oldtimer herbicide, applied in the 1950s – and clomazone, both inhibiting an early step in carotenoid biosynthesis [6, 11, 12].

4.1.2.2 **Carotenoids – Properties and Function**

Carotenoids are constituents of the photosynthetic reaction centers and the light-harvesting complexes of the antennae [13], playing their role as redox intermediates in electron transfer processes of photosystem II [14] and as accessory pigments in light harvesting [5, 15].

The reaction centers are rich in β-carotene and in some plant species may also contain α-carotene. In contrast, the peripheral light-harvesting complex contains several xanthophylls, including lutein, violaxanthin and neoxanthin [5].

Carotenes play a vital role in the protection of the chloroplast against photooxidative damage. At high light intensities, the chlorophyll molecules are exposed to more light than they can direct into electron transport, leading to chlorophyll fluorescence as one way of energy offtake [15] and intersystem crossing of the excited singlet chlorophyll to the longer-lived triplet state as a second way of energy offtake [5]. This triplet-state chlorophyll can use its energy to convert molecular oxygen into the highly active and destructive singlet oxygen ($^1O_2$). The latter will lead to destruction of lipids, membranes, nucleic acids and whole tissues. As a result, the degradation of chlorophyll, depending on the intensity of illumination, leads to the typical bleaching symptoms in plants and decline of photosynthetic activity. Typically, only the newly formed green leaves are affected and fade away by bleaching. Carotenoids protect against this photosensitized damage by direct quenching of the excitation energy of triplet-state chlorophyll. Secondly, the carotenoid molecule can also quench any $^1O_2$ build up, producing carotenoid triplets, which then decay harmlessly, developing heat rather than toxic products [5, 15].

Besides their function as light collectors and photoprotectors, carotenoids also have important effects as membrane stabilizers in chloroplasts. The xanthophyll violaxanthin and its enzymatic de-epoxidation products antheraxanthin and zeaxanthin partition between the light-harvesting-complexes (LHCs) of PS I and PS II and the lipid phase of the thylakoid membranes, bringing about a decrease in membrane fluidity, an increase in membrane thermostability and a lowered susceptibility to lipid peroxidation [16].

4.1.2.3 Carotenoid Biosynthesis in Higher Plants

4.1.2.3.1 The Biosynthetic Pathway

Carotenoids of higher plants, algae, and fungi are C_{40} tetraterpenes biosynthesized by the well-known isoprenoid pathway [1, 5, 6, 8, 17, 18]. The early steps, involving the formation of the C_5 isoprenoid units and the subsequent synthesis of prenyldiphosphate intermediates, are common to all classes of terpenoids.

4.1.2.3.2 Early Steps and Formation of Phytoene

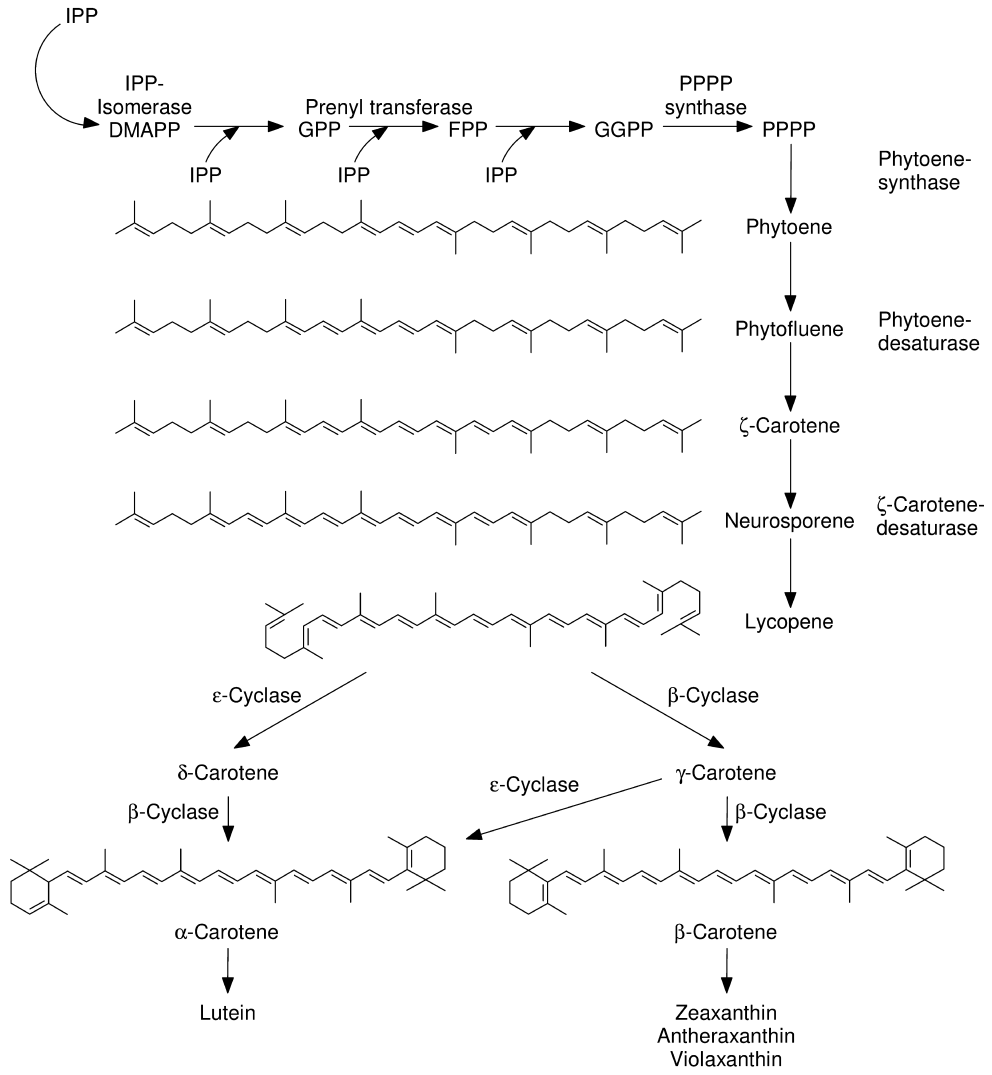
The first specific precursor for terpenoids in the cytoplasma is the C_6 molecule mevalonic acid (MVA), which is built via the classical acetate/mevalonate pathway and converted by a series of phosphorylating and decarboxylation reactions into C_5 isopentenyldiphosphate (IPP), the universal building block for chain elongation up to C_{20}. In the chloroplasts, the biosynthesis of IPP starts from glyceraldehyde-3-phosphate and pyruvate to give 1-deoxy-D-xylulose-5-phosphate (DOXP) via the non-mevalonate pathway as a recently detected alternative IPP route [19]. The reaction is catalyzed by the enzyme DOXP synthase and can be inhibited by a breakdown product of the herbicide clomazone [12].

After 1,3-allylic isomerization of IPP to dimethylallyl pyrophosphate (DMAPP) by the enzyme IPP isomerase, another IPP unit is added to yield C_{10} geranylpyrophosphate (GPP).

Subsequent addition of a second or third molecule of IPP leads to the formation of C_{15} farnesyl pyrophosphate (FPP) and the C_{20} geranylgeranyl pyrophosphate (GGPP). The chain elongation is a head-to-tail condensation process, which forms carbon–carbon bonds between C-4 of IPP and C-1 of the allylic substrate.

4.1.2.3.3 The Specific Carotene Pathway

The stages unique to carotenoid biosynthesis start with the formation of the C_{40} phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) from two molecules of GGPP via the C_{40} intermediate prephytoene pyrophosphate (PPPP), from which phytoene with its central double bond is directly derived (Fig. 4.1.1). It is colorless, being formed by head-to-head condensation of two molecules of GGPP (all-trans) and obtained in all photosynthetic organisms as the 15-*cis*-phytoene [20]. The condensation is catalyzed by the enzymes PPPP synthase and phytoene synthase. Desaturation starts from the symmetrical phytoene on both of its identical halves to give, in a first step, phytofluene as an intermediate and then ζ-carotene, catalyzed by the enzyme phytoene desaturase (PDS). Further desaturation of the latter occurs by a stepwise sequence of reactions to form neurosporene and the maximally desaturated lycopene. At each stage two *anti*-hydrogen atoms from adjacent functions are lost by oxidation to extend the chromophore by two double bonds. Starting with three conjugated double bonds in phytoene, one ends up with 11 in lycopene. The other enzyme involved is ζ-carotene desaturase (ZDS), which catalyzes a closely similar desaturation to PDS (Fig. 4.1.1) [21].



DMAPP dimethylallyl pyrophosphate; FPP farnesyl pyrophosphate; GPP geranyl pyrophosphate; GGPP geranylgeranyl pyrophosphate; IPP isopentenyl pyrophosphate; PPPP prephytoene pyrophosphate.

Fig. 4.1.1. Pathway of carotene biosynthesis from IPP to α- and β-carotene.

4.1.2.3.4 **Cyclization**

Lycopene is the starting building block for the cyclization reactions to the final α- and β-carotenes via their intermediates δ-carotene (with one ε-ionone ring) and γ-carotene (with one β-ionone ring) respectively. Two different enzymes are responsible for the β- and ε-cyclization, called lycopene β- and ε-cyclase respectively [22, 23].

In contrast to the 15-*cis* phytoene, the colored, fully desaturated carotenoids present in photosynthetic tissues are usually in the (all-ε) all-*trans* form, e.g., β-carotene or lutein. By hydroxylation of carotenes with molecular oxygen and in the presence of NADPH-dependent mixed-function oxygenase, hydroxy groups are introduced and epoxidation is another path for further derivatization, though little is known about the epoxidase involved [5]. Typical representatives are xanthophylls containing a hydroxy group at C-3 in the β- or ε-ring, violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β, β-carotene-3,3′-diol) or zeaxanthin (β,β-carotene-3,3′-diol). The importance of the violaxanthin–zeaxanthin cycle for both high rates of photosynthesis and energy dissipation has been described [5, 24].

4.1.2.3.5 Isolated Enzymes

Carotenoid biosynthesis takes place in a membrane-bound multienzyme complex, making it difficult to isolate and purify the enzymes involved. Owing to their sensitivity to detergents and low abundance, only a few have been purified from plant tissue. Many others had to be heterologously expressed in a way that high-pressure cell breaking resulted in a soluble and enzymatically active form [18, 25]. As an example, phytoene desaturase was cloned and expressed in recombinant *Escherichia coli*. To prepare the enzyme, the *E. coli* cells were disrupted by pressing them through a French Press. After centrifugation, the soluble supernatant fraction was used for enzymatic assays with HPLC recording or recording by optical absorption spectra [26].

4.1.3 Primary Targets

4.1.3.1 Inhibition of Phytoene Desaturase and ζ-Carotene Desaturase

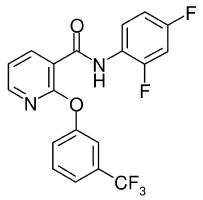
Owing to the similarity of desaturation reactions catalyzed by PDS or ZDS, differentiation in the plant is not easy to detect. Most of the herbicidal inhibitors probably inhibit both, although to a different extent [6]. If strong inhibition of PDS has taken place with accumulation of phytoene, then the compound's ability to inhibit ZDS cannot be seen. Figure 4.1.2 shows that the commercial products primarily inhibit PDS [6, 8, 27–29]. Cell-free studies exemplified by norflurazon and fluridone have shown them to act as reversible noncompetitive inhibitors of PDS [27]. Other PDS active structures are shown below in Table 4.1.2 and in Section 4.1.4.10.

Direct interaction with the enzyme ζ-carotene desaturase was shown for the dihydropyrone LS-80707 and the pyrimidine SAN 380H [8]. Later, the compound RH 1965 and substituted 4-phenyl-3-benzylthio-4*H*-1,2,4-triazoles were reported to also inhibit ζ-carotene desaturation [30, 31].

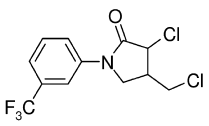
4.1.3.2 Inhibition of Lycopene Cyclase (LCC)

Amitrole (3-amino-1*H*-1,2,4-triazole) has been known to lead to some lycopene accumulation *in vivo* at a temperature-dependent rate but it is not considered to

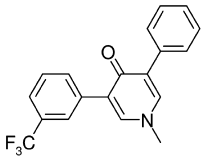
Inhibitors of phytoene desaturase (PDS)



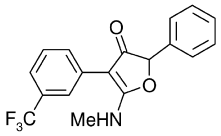
diflufenican



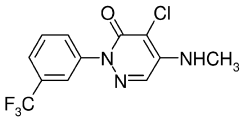
flurochloridone



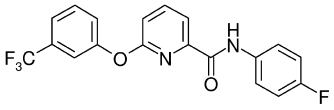
fluridone



flurtamone

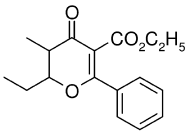


norflurazon

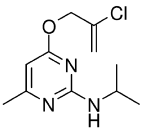


picolinafen

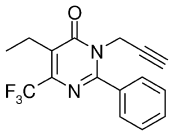
Inhibitors of ζ-carotene desaturase (ZDS)



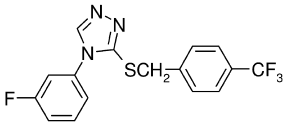
LS-80707
(cis)



SAN 380H

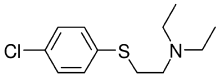


RH 1965

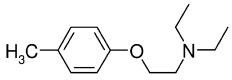


Ref. 31

Inhibitors of lycopene cyclase (LCC)



CPTA



MPTA

Fig. 4.1.2. Structure of commercial herbicides and some herbicidally active compounds that inhibit different enzymes in the biosynthetic pathway leading to the carotenoids.

be primarily an LCC inhibitor and may indirectly inhibit an early step in carotenoid biosynthesis [6, 11, 33]. The only specific more recent inhibitors are substituted diethylamines like CPTA and MPTA, which appear to inhibit both β- and ε-cyclase (Fig. 4.1.1) [6]. Their MoA is noncompetitive inhibition of lycopene cyclase versus lycopene [32].

In 2001, potent diethylamines were found as a new LCC inhibitor structural type [33]. Although very effective in seedling tests, the LCC inhibitors known so far have not shown sufficient activity for herbicide development.

4.1.3.3 Genetic Engineering of Herbicide Resistance by Modification of the Carotenogenic Pathway

The availability of numerous carotenogenic genes makes it possible to modify and engineer the carotenoid biosynthetic pathways in microorganisms and plants. Convenient tools for generation of mutants with a herbicide resistant PDS are unicellular cyanobacteria [3, 4, 7]. Various lines of resistant mutants of *Synechococcus* have been selected against norflurazon, showing not only a resistance factor of up to 70 but in most cases also cross-resistance to other PDS herbicides [7].

4.1.4 Chemical Structure and Activities of PDS Inhibitors

4.1.4.1 Enzyme Activity, Physical Data and Acute Oral Toxicity of Commercial PDS Herbicides

In recent years, structural evolution, detailed quantitative and qualitative structure–activity studies have been performed with a range of chemically different PDS inhibitors. Reference [27] reviews the early literature until about 1990. Subsequent years, to the late 1990s, is the topic of another review [34].

Table 4.1.1 presents IC_{50}s of commercial herbicides for inhibition of carotenoid biosynthesis obtained *in vivo* according to Ref. [26]. The assay is easier to run than the early radioactive approach with unicellular cyanobacteria [35], giving in three cases differing results. This may be caused by differences in target site sensitivity, uptake and translocation effects or metabolism of the herbicides in the treated bacteria cells of the early test assays. Table 4.1.1 also presents physical and acute oral toxicity data [36, 37].

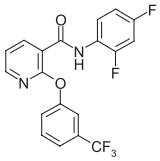
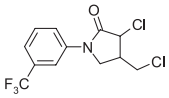
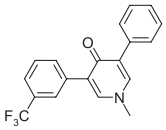
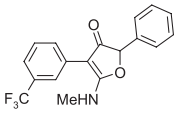
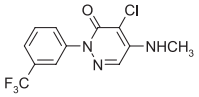
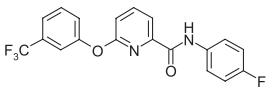
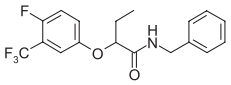
4.1.4.2 Phenoxybenzamides

Removal of a p-nitro group from peroxidative diphenyl ethers drastically reduced their peroxidative activity while increasing the inhibition of carotenoid biosynthesis, provided a substituted formamide substituent is present in the meta-position (**1**, Fig. 4.1.3). Both the o- and p-derivatives are inactive (reviewed in Ref. [27]). Lipophilicity of the phenoxy ring and chain length of the alkyl group up to five carbon atoms increases activity, while branching results in a loss of activity. QSAR equations of the effect of the carbonamide substituent have been calculated [38]. No commercial product has been developed.

4.1.4.3 Phenoxypyridincarbonamides

Phenoxypyridinecarbonamides are surprisingly flexible, when the pyridine ring is substituted (review in Refs. [27, 34]). The first active pattern consisted of nicotinamides with a 2-phenoxy substitution (**3**, Fig. 4.1.4). For the latter, the m-position (R^1) was important with 3-CF_3 and 3-Cl being most active, while double substitution led to a decrease of activity. While small substituents R^2 such as H or CH_3 gave good herbicidal activity, Br or Cl were weaker. In the amide part, N-phenyl and N-benzyl derivatives showed comparable activity; ethylene as a spacer

Table 4.1.1 IC_{50} values and physicochemical and oral toxicity data for commercial herbicides for carotenoid biosynthesis inhibition.

Structure	IC_{50} (mol L^{-1})	log *P* (pH 7.5)	Vapor pressure (mbar)	*M* (g mol^{-1})	mp (°C)	LD_{50} rats (mg kg^{-1})
 diflufenican	3.40×10^{-8}	4.90	4.25×10^{-8}	394.3	159–161	>2000
 flurochloridone	2.02×10^{-6}	3.36	4.40×10^{-6}	312.1	41 (eutectic)	4000
 fluridone	2.93×10^{-7}	1.87	1.30×10^{-7}	329.3	154–155	>10000
 flurtamone	8.21×10^{-7}	3.22	4.20×10^{-7}	333.3	152–155	500
 norflurazon	5.18×10^{-7}	2.45	3.86×10^{-8}	303.7	174–180	>5000
 picolinafen	8.98×10^{-8}	5.37	1.66×10^{-12}	376.3	107	>5000
 beflubutamid	1.75×10^{-6}	4.28	1.10×10^{-7}	355.3	75	>5000

Enzyme values obtained from BASF Agricultural Research, other values taken from *The Pesticide Manual* [36] and SRC PhysProp Database [37].

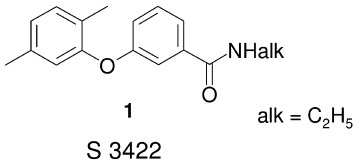


Fig. 4.1.3. Phenoxybenzamide S 3422 (**1**).

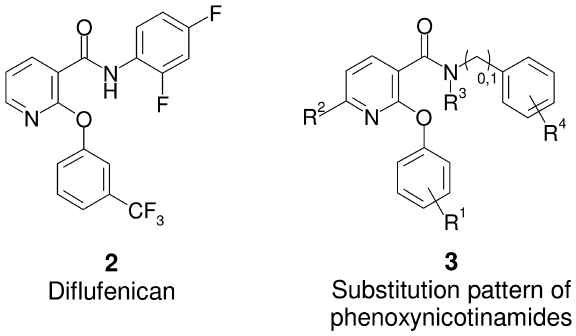


Fig. 4.1.4. Diflufenican (**2**).

strongly decreased *in vitro* activity. Thioamides were slightly less active. Substitution of the amide hydrogen (R^3) by alkyl led to a decrease of activity parallel to their length. Single substitution of the N-phenyl ring (R^4) resulted in loss of activity, with the exception of the 4-F-moiety; the 2,4-difluoro derivative showed comparable activity to the unsubstituted compound. Most SAR contributions came from the laboratory of May & Baker, where diflufenican (**2**, Fig. 4.1.4) was found and later developed by Rhône-Poulenc [39, 40].

Researchers in the Shell laboratories later discovered a gap in the diflufenican patent, the 2,6-isomer **5**, which soon became very promising and led, after the acquisition by American Cyanamid and later BASF, to the marketing of picolinafen (**4**) in 2001 (Fig. 4.1.5) [41]. The discovery of the 2,6-pyridine cluster marked the beginning of a considerable number of follow-up patents to secure the new lead [34]. It could be shown that lower alkyl amino groups ($R^2 = CH_3$) may substitute

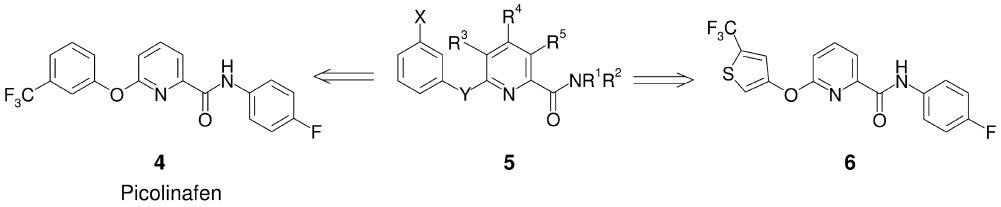


Fig. 4.1.5. Picolinafen (**4**).

the 4-F- or 2,4-difluoroanilide moiety, while R^1 has to be H or CH_3. In addition R^3–R^5 is best with H or F. The 6-phenoxy unit could be replaced by 4-oxypyridine, 5-oxypyrazole [34] and 3-oxythiophene **6** (Fig. 4.1.5) [42]. The latter all need, again, a substituent (X) meta to the ether bridge (Y) and while the pyridine ether gives similar activity with Cl, a CF_3 group will be necessary for the phenoxy, 5-oxy-pyrazole and 3-oxy-thiophene unit.

Since the amide moiety could be totally replaced by aryl or hetaryl ethers and also directly substituted by pyrazol, several new combinations became possible, which led to the discovery of the phenoxypyridine ethers (Section 4.1.4.4).

4.1.4.4 **Phenoxypyridine Ethers**

In the 6-phenoxy moiety of this lead (**8**) a CF_3 substituent X proved best for herbicidal activity, which is also the case for 5-pyrazole-oxy and 3-thiophene-oxy substitution; see Ref. [34] and Table 4.1.2. In the 4-pyridyl-oxy group m-Cl and m-difluoromethoxy were another good choice of substituent. When 2,6-bisaryloxy-pyridines were synthesized, one phenyl group could be replaced by benzyl. Only a highly lipophilic aliphatic substituent R^4 such as trifluoromethylthiopropyl **14** ($n = 1$) could compete with the (hetero)-aryloxy compounds. In general, the best substituents for R^1 and R^3 were H and F. The substituent R^2 may be H, CH_3 and CH_3O and with **16** ($R^1 = R^3 = F$) brought a rise in activity. Interestingly, activity in structures **13**, **15** and **16** is retained, even when replacing oxygen by a bond.

4.1.4.5 **Phenylfuranones**

The oldest phenylfuranone is difunon (**17**, Fig. 4.1.6), which turned out to be a rigid structure. Replacement of the 4-phenyl group by n-butyl, cyclohexyl and of the 3-CN group by carbonamide or an ester resulted in loss of activity (reviewed in Ref. [27]). Only the 3-position of the phenyl ring was tolerant of substitution such as -SCH_3, -OCH_3, -C_6H_5 and -CF_3, leading in some weeds to a rise of activity. The compound never became commercialized.

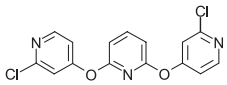
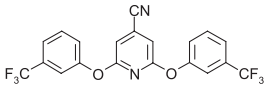
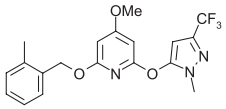
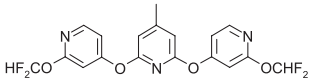
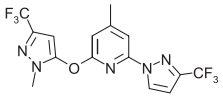
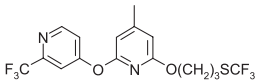
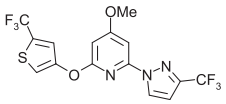
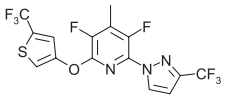
The other representative, flurtamone (**18**, Fig. 4.1.6) only has the 4-phenyl group and a basic side chain in common with difunon while the remaining substituents vary considerably. The best substituent of position 2 is phenyl; surprisingly it could be replaced by C_1–C_3-alkyl, but branching is unfavorable.

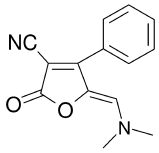
The 4-phenyl ring needs m-substitution by CF_3 whereas decreasing lipophilicity shows lower inhibition of PDS (reviewed in Ref. [27]).

4.1.4.6 **Phenylpyridazinones**

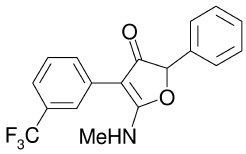
Pyridazinones turned out to be very flexible and, depending on position and substitution, show different MoA. While the cluster of the early chloridazon (**19**) is responsible for photosynthesis inhibition [51], BAS 10501W (**20**) inhibits fatty acid desaturation, changing the ratio of 18:2/18:3 fatty acids in plant membranes [27, 52], and norflurazon (**21**), finally, inhibits PDS (Fig. 4.1.7). Its inhibition went along with a CF_3-substituent R^3 in the phenyl moiety and small alkylamino groups R^2 in structure **22**. Longer chains or branching lowered activity. While position 4 of the heterocycle (R^1) needs electron-withdrawing substituents, R^2 at C-5

Table 4.1.2 Structural evolution of phenoxypyridine ethers since 1994.

No.		Ref.	Year
9		43	1994
10		44	1996
11		45	1998
12		46	1999
13		47	2001
14		48	2001
15		49	2003
16		50	2003

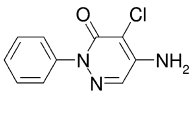


17
Difunon

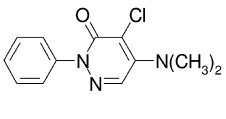


18
Flurtamone

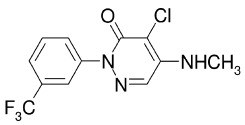
Fig. 4.1.6. Difunon (**17**) and flurtamone (**18**).



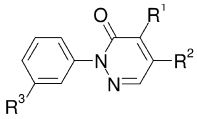
19
Chloridazon



20
BAS 10501W



21
Norflurazon



22

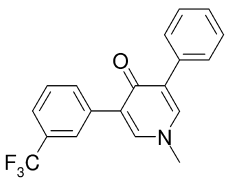
Fig. 4.1.7. Phenylpyridazinones.

has to be connected with electron-donating substituents, shifting electrons towards the heterocycle to increase activity (reviewed in Ref. [27]).

QSAR studies were performed with 2-phenylpyridazinones substituted at position 3 of the phenyl ring (R^3), where lipophilicity exerted a very strong effect on activity, counteracted by electronic properties. Steric factors did not show an influence [27, 53]. The results were subsequently confirmed by new m-substituted derivatives [54]. Replacing the CF_3-group by fluorophenoxy or a fluorophenylalkyl side chain led to superior activity in spite of their much larger size. When Cl in R^1 was substituted later by a m-CF_3-phenyl group while R^2 was retained as CH_3NH, an early member of the diaryl heterocycle PDS inhibitor type with strong herbicidal activity was found (Section 4.1.4.10).

4.1.4.7 **Phenylpyridinones**

The pyridinone structure of fluridone (**23**, Fig. 4.1.8) is biologically rather inflexible, so that the thiopyridinone and higher N-alkyl derivatives showed only little



23
Fluridone

Fig. 4.1.8. Fluridone (**23**).

activity (reviewed in Ref. [27]). A m-substitution of one phenyl ring by the highly lipophilic CF_3 group is necessary, while exchange by Cl or CO_2H decreased activity. QSAR equations with whole cell data confirmed lipophilic and inductive effects; however, *in vitro* results only correlated to π [55]. To leave the pyridinone cluster, other contributors omitted the C=O group and continued with a series of 2,4-diphenylpyrimidines.

Although the hetero ring system of fluridone and flurtamone are completely different, their three-dimensional structures and projection to a common overlay gave rise to the concept of the "diaryl heterocyclic PDS inhibitors" of Section 4.1.4.10 [34].

4.1.4.8 **Phenylpyrrolidinones**

The most prominent representative of the phenylpyrrolidinones **25** is flurochloridone (**24**, Fig. 4.1.9). Again it needs an electron-withdrawing lipophilic substituent R^1 in the 3-phenyl position, such as 3-CF_3 or SCF_3, while CN or $SO_{1-2}CF_3$ were somewhat weaker (reviewed in Refs. [27, 34]). Activity ends with NO_2, NH_2 or C=O as substituents, which are no longer lipophilic and instead more prone to hydrogen bridging, with the exception of the NO_2 group. Surprisingly, high activity could be conserved by replacing Cl in R^2 with methyl and ethyl carbonamide. For reasons of activity, the chain length of R^3 is restricted to 2 and the 5-position (R^4) must be unsubstituted. Fluridone has two asymmetric carbons in the pyrrolidinone ring and 3,4-trans stereochemistry gives better herbicidal activity than the cis form. In the early 1990s, the 3-Cl was replaced by phenyl carrying an m-CF_3 group or halides in the 3–5-positions while varying R^1 and R^3. Among them 1-(3-isopropylphenyl)-3-phenyl-4-ethyl-2-pyrrolidinone was one of the herbicidally most active. When R^2 = Cl was omitted, R^3 had to be CF_3. The lipophilic CF_3 in R^1 was also replaced by phenoxy units with different substituents or ring-anellated with the adjacent o-position into a 2′,2′-difluordioxol-2,3-benzo ring.

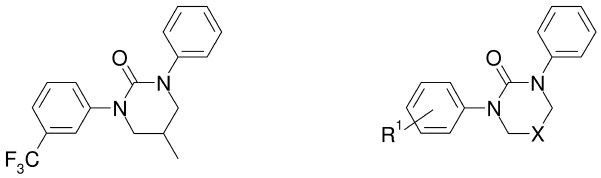
24
Flurochloridone

25

Fig. 4.1.9. Flurochloridone (**24**).

4.1.4.9 **Phenyltetrahydropyrimidinones**

Prerequisite for high herbicidal activity in phenyltetrahydropyrimidinones (**27**) is also the m-CF_3-substitution in one phenyl group (Fig. 4.1.10). Substitution of R^1 by an electron-withdrawing group shows the same biological ranking as in the other compound classes discussed before (reviewed in Ref. [27]). From the ring size of the heterocycle, a six-membered ring with X = $CHCH_3$ as optimum



26
NTN-28621

27

Fig. 4.1.10. Phenyltetrahydropyrimidinones.

shows the best results, while five- or seven-membered cyclic ureas are less effective. From their three-dimensional structure, there is a similarity between the phenyl-pyridinones (Section 4.1.4.7) and the saturated NTN-28621 (**26**, Fig. 4.1.10), which with its CH-CH_3 group indeed imitates the N-CH_3 group of fluridone and thus became a precursor for the compounds of Section 4.1.4.10 [34].

4.1.4.10 **Structural Overlay for Diaryl Heterocycle PDS Inhibitors and Newer Developments**

Structural overlay of flurtamone, fluridone and NTN-28621 led to a new pyrazolone **28** and pyridine **29**, many pyrimidines **30** and some 1,2,4-triazines **31** with the joint possession of 1,3-connected phenyl groups (“1,3-diaryl-heterocycle”) (Fig. 4.1.11) [34]. A pyrimidine **30** with $R^1 = CH_3O$, $R^2 = H$, $X = 3\text{-}CF_3$ and

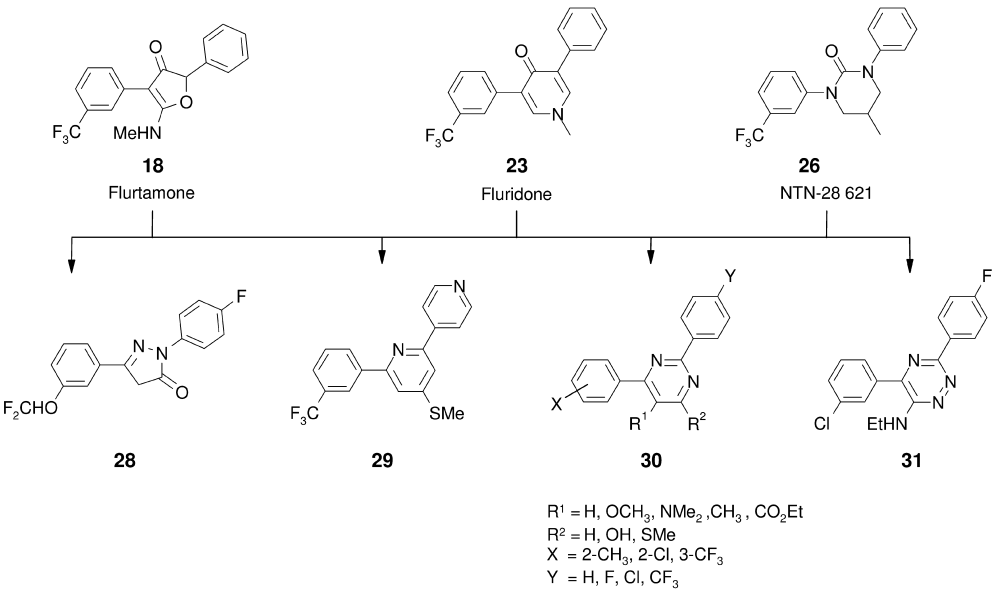


Fig. 4.1.11. Structural overlay of diaryl heterocycle PDS inhibitors and newer developments.

Y = F has been reported with application rates as low as 63 g ha^{-1}. Other substituents for R^1 are NMe_2, CH_3 and CO_2Et while R^2 was H. In another series, R^1 was kept constant with hydrogen while R^2 could vary from OH to MeS and X was 2-CH_3, 2-Cl and 3-CF_3.

Along this line, Table 4.1.3 represents recent developments, from which **32–37** pursue substituted hetarylethers with **35** integrating its ether bridge into a heterocycle. Compounds **38–40** constitute classical pyrimidines, while pyrimidines **41** and **42** are purely aliphatically substituted. Notably, both also inhibit ZDS. Compounds **44** and **45** may be viewed as substituted phenylpyrrolidinones. The ketomorpholine **43**, the carbonamide **46** and the carbamate **47** are new PDS leads. The same holds for the pyrazolethers **36** and **37**.

4.1.4.11 Models of the Active Site – Structural Requirements

Few reports on models of the PDS herbicide binding site have appeared in the literature. The early QSAR equations correlated molecular properties like σ, π and steric parameters of one lead with its enzyme activity. Good results were obtained concerning the nature and position of substituents or when optimizing the chain length of a side chain, to get an approximate impression of electronic and steric prerequisites. However, activity prediction of structurally diverse molecules would not be possible. Also it should be kept in mind that QSAR does not take into account biological uptake, or stability against light and water at different pH, nor does it consider metabolization of a molecule in the plant, when activity in the field is desired.

A first hypothetical binding site model was proposed in studies of substituted 3(2H)-furanones [69]. Later, a steric model for the binding site of the PDS enzyme was developed by superposition of five commercial, structurally diverse inhibitors assumed to bind in the same way [64]. Conformational analysis was performed with the aid of three molecular mechanics programs to investigate three common regions in an orthogonal view: region **X** (phenyl ring preferentially substituted by the lipophilic CF_3 group), **Y** (central heterocyclic ring with an amide, vinylogous amide CO group), in which steric and electronic requirements appear to be relatively well defined and region **Z**, which appears to be sterically more tolerant. This model is similar to Fig. 4.1.12 when **A**, **B** and **C** are represented by **X**, **Y** and **Z**. It was used to predict the likely levels of activity of some analogues of the 6-ketomorpholine **43** and was able to show that the inhibitory activity resides almost exclusively with the (2R),(5S) form.

In 1999, another contribution described PDS active structure **34** and **35**, showing an overall similarity to the leads mentioned earlier [57]. In computational studies with SYBYL and a pharmacophore mapping approach putative receptor-bound conformations of benzoxazole and benzothiazoles differing from these were obtained, supporting coplanar geometry over the tilted conformation. In addition to previous findings, a functional group at the enzyme opposite to the central ring acting as either a hydrogen bond donor or an acceptor was suggested for interaction with the sp^2 hybridized nitrogen of the benzoxazole or the benzothia-

Table 4.1.3 Diaryl heterocycle PDS inhibitors and recent developments.

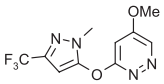
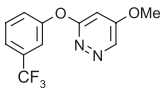
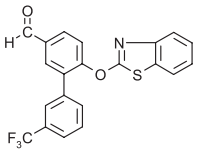
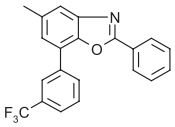
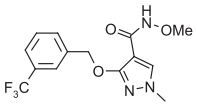
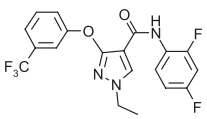
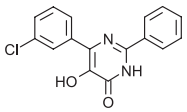
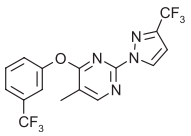
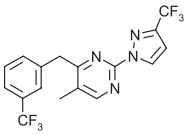
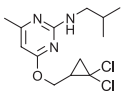
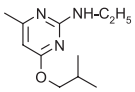
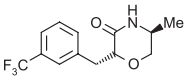
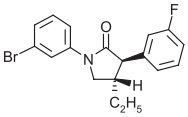
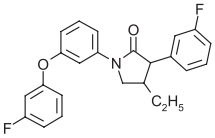
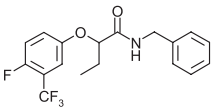
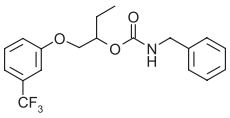
No.	Type	Ref. (year)	IC_{50} (μM) Dose
Hetarylethers			
32		56 (1998)	17–140 g ha^{-1}
33		56 (1998)	
34		57 (1999)	0.75×10^{-9}
35		57 (1999)	0.35×10^{-8}
36		58 (2004)	9.6×10^{-7}
37	 KPP-856	59 (2004)	
Pyrimidines			
38		60 (2001)	1.4×10^{-6}
39		61 (2002)	5–10 g ha^{-1}

Table 4.1.3 *(continued)*

No.	Type	Ref. (year)	IC_{50} (μM) Dose
40	 DPX-MY926	62 (2002)	10–15 g ha^{-1}
41		63 (2002)	8.6×10^{-7} ZDS 4.2×10^{-6}
42		63 (2002)	7.9×10^{-6} ZDS 6.5×10^{-5}
Saturated heterocycles			
43	 (2R), (5S)	64 (1995) 65 (2001)	1.7×10^{-7}
44		66 (2001)	1×10^{-9}
45		66 (2001)	4.8×10^{-7}
Aliphatic scaffolds			
46	 beflubutamid	67 (1999)	
47		68 (2003)	1.3×10^{-7}

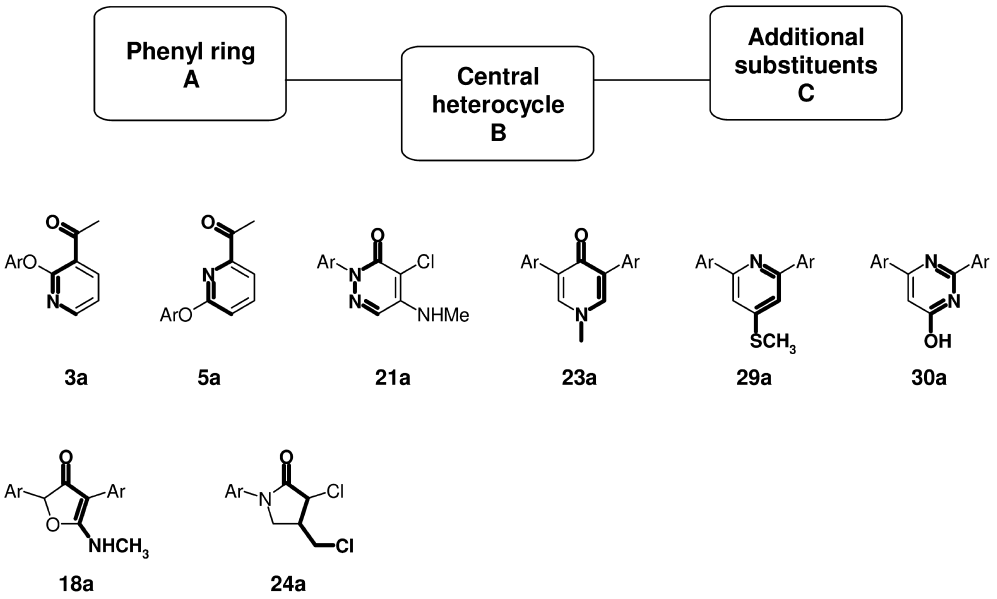


Fig. 4.1.12. Common structural elements of PDS inhibitors. (Modified from Ref. [7].)

zole heteroatoms. The herbicide binding site thus generated would be large and defined enough to fit most of the other inhibitors.

When comparing compounds with a modified central ring, it was concluded that the optimum inhibition of PDS is further reflected by a similar diagonal length from the negatively charged regions across the central heterocycle B carrying one or two substituted phenyl rings A and C (Fig. 4.1.12) [7]. A region of a total of six C- and hetero-atoms spans from one end of the substituted central heterocycle with C=O and N=C groups to the opposite site. In the nonplanar ketomorpholine **43**, the optimum length is only five atoms with the 5-methyl group in the (5S)-form. The stereospecific inhibitory property of certain substituents of this heterocycle is another indication for the spatial requirement of this model. Most PDS inhibitors fit this description quite well.

However, some exceptions have to be noted. Norflurazon (**21**) is highly active and does not contain a ring C. In beflubutamid (**46**, Table 4.1.3) and in structure **47**, an oxycarbonamide and oxyalkanecarbamate moiety, respectively, replace the central ring B by constituting a polar scaffold for rings A and C to interact with the binding niche, and in structures **41** and **42** only one central ring, without aromatic substituents, is left.

Finally, the question was raised whether inhibitors of PDS and ZDS can be modeled as analogues of plastoquinone because of their competitive behavior with the latter [70].

4.1.5
Biology and Use Pattern [36]

Diflufenican was introduced by May&Baker Ltd. (now Bayer CropScience). It is applied at 125–250 g ha^{-1} pre- or early post-emergence in autumn sown wheat and barley for the control of broad-leaved weeds. Degradation proceeds via the metabolites 2-(3-trifluormethylphenoxy)nicotinamide and 2-(3-trifluormethylphenoxy)nicotinic acid to bound residues and CO_2. DT_{50} varies from 15 to 30 weeks, depending on soil type and water content.

Fluorochloridone was introduced by Stauffer Chemical Co. (now Syngenta AG). Rights were acquired by Agan Chemical Manufacturers Ltd. in 2002. It is applied pre-emergence at 250–750 g ha^{-1} for the control of weeds in winter wheat and winter rye, sunflowers and potatoes. It degrades in the soil under laboratory conditions, mostly forming CO_2 and a bound residue. DT_{50} in the field is 9–70 days.

Fluridone was introduced by Eli Lilly&Co. (now Dow Agrosciences), and later sold to SePRO. It is used as an aquatic herbicide for control of most submerged and emerged aquatic plants in ponds, lakes, reservoirs, irrigation ditches, etc. Application rates are 45–90 ppb in ponds and 10–90 ppb in lakes. As an upland crop, only cotton has been found selective. In aquatic environments, degradation occurs mainly by photolytic processes, but microorganisms and aquatic vegetation are also factors. DT_{50} in water (anaerobic) 9 months, (aerobic) about 20 days.

Flurtamone was introduced by Chevron Chemical Co. and later acquired by Rhône-Poulenc Agrochemical (now Bayer CropScience). It is incorporated preplant, pre-emergence or applied post-emergence for the control of broad-leaved and some grass weeds in small grains, peanuts, cotton, peas and sunflowers at 250–375 g ha^{-1}. The main metabolite is trifluoromethylbenzoic acid and, 10 months after application, no residues could be detected. Field DT_{50} 46–65 days.

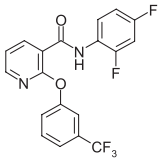
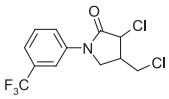
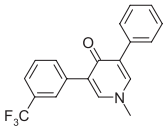
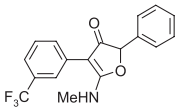
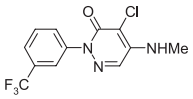
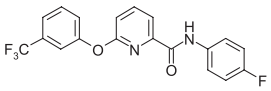
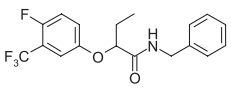
Norflurazon was introduced by Sandoz Ag (now Syngenta AG). It is used at 0.5–2 kg ha^{-1} for the pre-emergence control of grasses and sedges as well as broad-leaved weeds in cotton, Soya beans and peanuts, and at 1.5–4 kg ha^{-1} in nuts, citrus, vines, pomefruit, stone fruit, ornamentals, hops and industrial vegetation management. It dissipates in soil by photodegradation and volatilization, DT_{50} about 6–9 months.

Picolinafen is the youngest commercial PDS herbicide and was discovered by Shell International Research, acquired by American Cyanamid Company (now BASF AG). It is used at 50 up to 100 g ha^{-1} as a cereal herbicide for the post-emergence control of broad-leaved weeds and marketed in mixtures with other cereal herbicides such as pendimethalin, isoproturon, and MCPA. It shows strong synergistic properties, for instance with pendimethalin, to also control grasses [71]. It is further registered in lupines. Metabolism proceeds via rapid hydrolytic cleavage of the amide bond. It is photochemically degraded in the environment; DT_{50} 1 month.

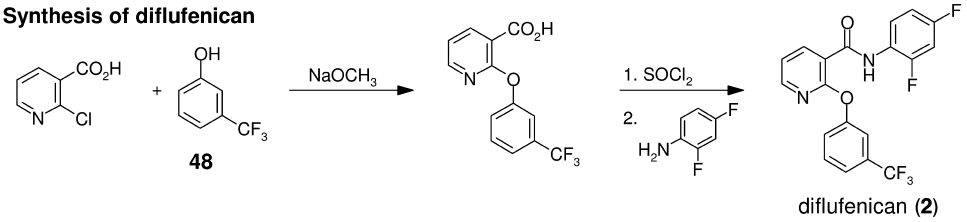
Beflubutamid is under development by Ube Industries Ltd. Its intended use is alone or in mixture with isoproturon for pre- and early post-emergence control of broad-leaved weeds in wheat and barley at 170–255 g ha^{-1}. Soil DT_{50} 5.4 days; the main metabolite is the corresponding butanoic acid, which itself is rapidly degraded in soil.

Table 4.1.4 summarizes an overview.

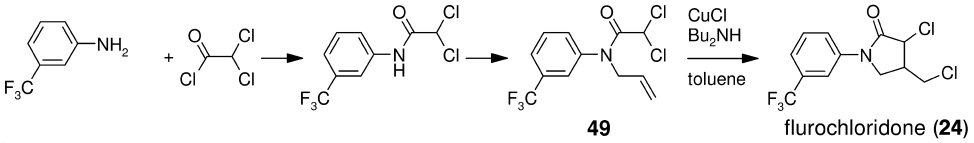
Table 4.1.4 Summary of application data of commerical PDS herbicides.

Chemical structure	Tradename (year)	ISO name Code No. Company	Dose (g ha^{-1}) Applic. method Target crops	Ref.
	Fenikan Tigrex (1987)	Diflufenican MB-38544 May & Baker Bayer CropScience	125–250 Pre, post Cereals	39
	Racer (1985)	Flurochloridone R-40244 Stauffer Chemical Syngenta	250–750 Pre Cereals, cotton, sunflowers, potatoes	72
	Sonar (1977)	Fluridone EL171 Elanco SePRO	500–2000 Pre, post Cotton aquatic herbicide 45–90 ppb	51 73
	Bacara (1997)	Flurtamone RE-40885 Chevron Bayer CropScience	250–375 Pre, post Cereals, peanuts, peas, cotton, sunflowers	74
	Solicam Zorial (1968)	Norflurazon H 9789 Sandoz Syngenta AG	500–2000 Pre Cotton, peanuts, soybeans, orchard	75 76
	Pico (2001)	Picolinafen AC 90001 ACC BASF AG	50–100 Post Cereals, lupines	41
	Herbaflex (2003)	Beflubutamid UBH-820 Ube under development	170–255 Pre, early post Wheat, barley	77

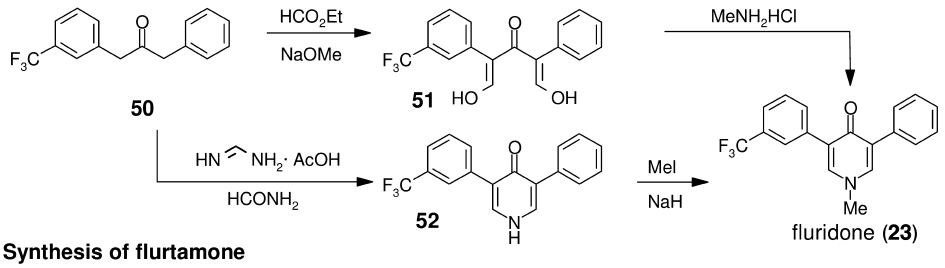
Synthesis of diflufenican



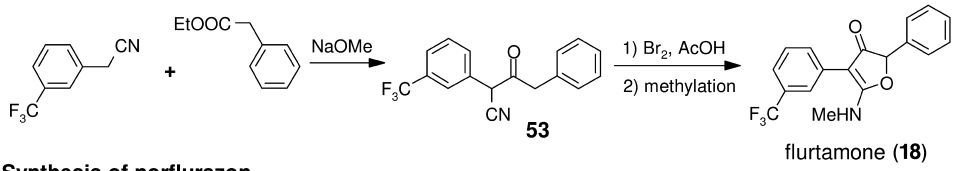
Synthesis of flurochloridone



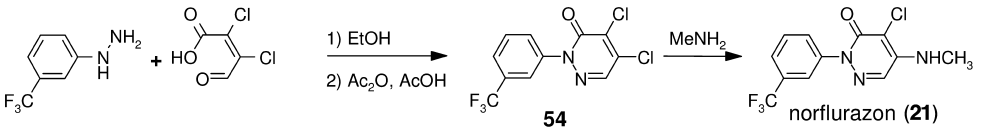
Synthesis of fluridone



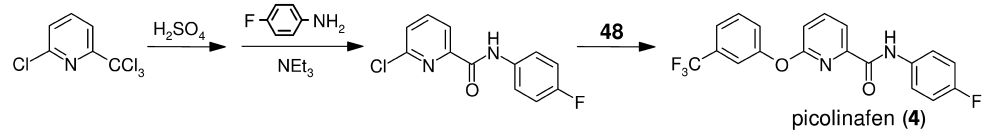
Synthesis of flurtamone



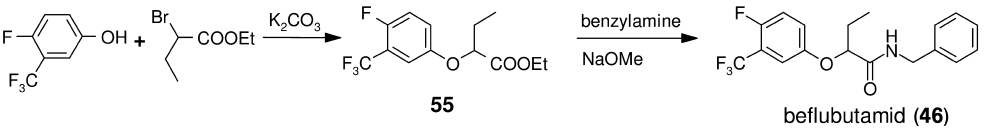
Synthesis of norflurazon



Synthesis of picolinafen



Synthesis of beflubutamid



Scheme 4.1.1. Major synthetic routes for diflufenican (**2**), flurochloridone (**24**), fluridone (**23**), flurtamone (**18**), norflurazon (**21**), picolinafen (**4**) and beflubutamid (**46**).

Engineering of resistance opens the possibility for obtaining tolerant plants to increase the crop spectrum beyond the scope described above [78]. Not only was it possible to confer resistance to tobacco but also to increase the carotenoid content in tomato fruits, rapeseed and rice [18].

4.1.6 Major Synthetic Routes for Phytoene Desaturase Inhibitors

Scheme 4.1.1 depicts the major synthetic routes, which are described below.

Diflufenican is synthesized by nucleophilic substitution of 2-chloronicotinic acid with 3-hydroxybenzotrifluoride and further reaction with thionyl chloride and 2,4-difluoroaniline to the final product [39].

Flurochloridone is made by copper chloride-catalyzed cyclocondensation of N-allyl-(3-trifluormethylphenyl)dichloroacetamide **49** [34].

Fluridone is accessible in two ways. 1-(3-Trifluormethylphenyl)-3-phenyl-2-propanone (**50**) is reacted with ethyl formate in the presence of a base to yield the diformyl derivative **51**, which is cyclized with methylamine to the final product. Alternatively, **50** is condensed with formamidine acetate in the presence of formamide to the 4(1*H*)-pyridone intermediate **52** and then methylated to give fluridone [34].

Flurtamone is prepared by cyclization of 4-phenyl-2-(3-trifluormethylphenyl)-3-oxobutyronitrile (**53**) with bromine in the presence of acetic acid and methylation to the final heterocycle [34].

Norflurazone production is based on the condensation of 3-trifluormethylphenylhydrazine and mucochloric acid followed by cyclization with acetic anhydride to give 4,5-dichloro-2-(3-trifluormethylphenyl)pyridazin-3-one (**54**). Nucleophilic substitution of **54** with methylamine yields norflurazon [34].

Picolinafen is built on the partial hydrolysis of 2-chloro-6-trichlormethylpyridine, reaction with 4-fluoroaniline and subsequent nucleophilic substitution with 3-hydroxybenzotrifluoride [79].

Beflubutamid is synthesized from ethyl 2-(4-fluoro-3-trifluormethylphenoxy)butanoate **55** and benzylamine in the presence of a base [34].

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4.2
Hydroxyphenylpyruvate Dioxygenase (HPPD) – the Herbicide Target

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The corn herbicide sulcotrione and destosyl pyrazolate (DTP), the active hydrolysis product of the rice herbicides pyrazolate and pyrazoxyfen (Fig. 4.2.1), were known [1, 2] as bleaching herbicides before their HPPD mode of action was recognized. Loss of chlorophyll and accumulation of phytoene suggested possible sites of action in protochlorophyllide biosynthesis or at the phytoene desaturase (PDS) step in carotenoid biosynthesis [3]. However, these polar acids neither resemble typical PDS inhibitor herbicide types [4] nor inhibit PDS *in vitro* [5]. The clue to the true mode of action came from toxicological studies indicating that rats fed with experimental benzoyl cyclohexane-1,3-dione (CHD) herbicides such as nitisinone (Fig. 4.2.1) exhibit increased levels of tyrosine in blood and

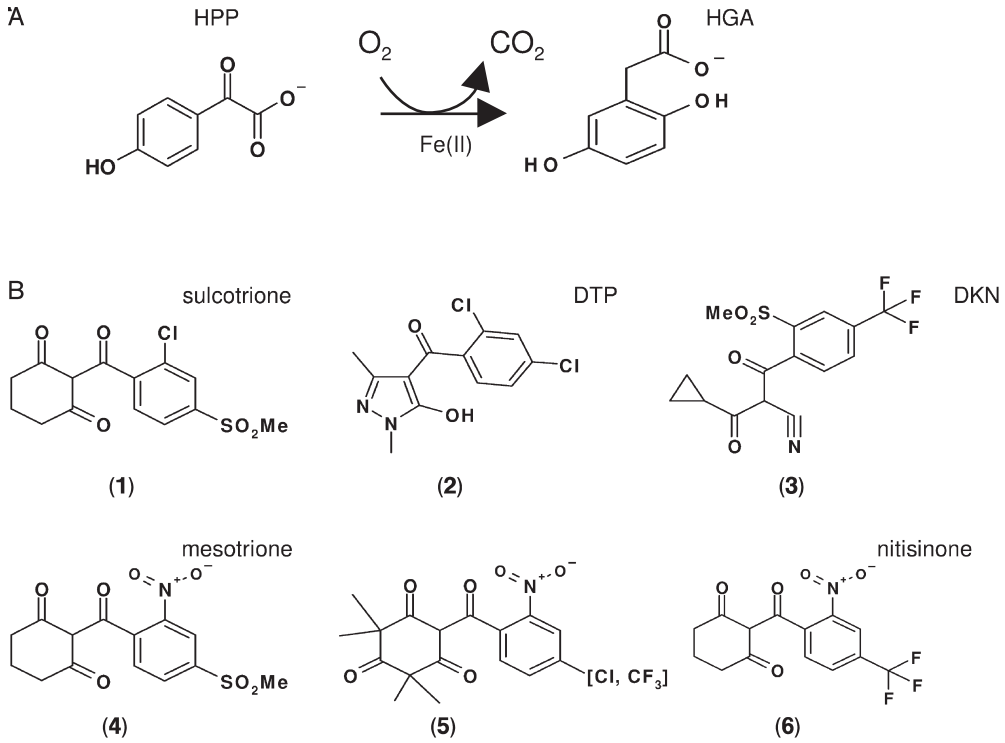

Fig. 4.2.1. (A) HPPD reaction and (B) the structures of some inhibitors.

p-hydroxyphenylpyruvate (HPP) in urine. This suggested a block in the catabolic degradation of tyrosine, and further investigative work [6–8] indicated that nitisinone is a potent inhibitor of mammalian HPPD, an enzyme that catalyzes the oxidative decarboxylation and rearrangement of HPP to homogentisate (HGA). The discovery that CHDs also inhibit HPPD in plants and the evidence firmly linking this to their herbicidal effect followed soon after [8, 9]. HGA was found to be a specific antidote for HPPD inhibitor-induced bleaching [8, 9] and a phytoene accumulating *Arabidopsis* mutant, PDS1, exhibiting a homozygous lethal bleaching phenotype rescuable by HGA mapped to a lesion in the plant HPPD gene [10]. Expression of heterologous HPPDs in transgenic plants resulted in specific tolerance to "HPPD-inhibitor" herbicides [11].

HPPD catalyzes an early step in a tyrosine degradation pathway [12] that is widely distributed in nature [13] and thus, as in animals, treatment of plants with inhibitors causes significant accumulation of tyrosine [8, 14]. HPP derived from transamination of tyrosine, is converted into HGA via HPPD, HGA is oxidized via HGA oxidase to 4-maleylacetoacetate, which is further degraded via 4-maleylacetoacetate isomerase and 4-fumarylacetoacetate lyase to fumarate and acetoacetate. In microbes the pathway provides assimilable carbon from tyrosine

and phenylalanine; in higher mammals defects cause hereditary diseases [12] such as tyrosinemia type I where a lesion in 4-fumarylacetoacetate lyase causes a build up of toxic and liver-carcinogenic keto acids. Nitisisone, which blocks HPPD and thereby prevents toxin accumulation [7], is now an FDA approved treatment for tyrosinemia type I and may also find use in ameliorating other diseases arising from defects in tyrosine degradation [12].

HPPD inhibitors are only acutely toxic to photosynthetic organisms. In these HGA is not only an intermediate in tyrosine degradation but also in the biosynthesis of plastoquinone (PQ) and tocopherols [15, 16]. HPPD provides HGA from HPP, which is derived from transamination of tyrosine in the cytosol. Southern and sequence analysis of the *Arabidopsis* genome indicates the presence of only a single HPPD gene [10] for which no transit peptide leader is predicted and which is expressed only in the cytosol [17]. HGA made in the cytosol diffuses to the plastid where alternative prenyl transferase enzymes HGA solanyltranferase (HST) or HGA phytyltransferase (HPT) located in the inner plastid envelope convert it into the precursors 2-methyl-6-solanyl-1,4-benzoquinone (MSBQ) or 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ), respectively. These are then further methylated (by a single enzyme, MSBQ/MPBQ methyltransferase, common to both pathways) to yield, respectively, PQ or, in the case of the tocopherol pathway, 2,3-dimethyl-6-phytyl-1,4-benzoquinone, which then gives rise to γ- and α-tocopherol (the dominant tocopherol in leaves) via further steps of cyclization and methylation.

Of the two deficiencies, lack of PQ and lack of tocopherols, which result from inhibition of HPPD, the significance of the former was more obvious [8, 9]. *In vitro* studies [18] as well as genetic mapping of phytoene accumulating mutants of arabidopsis [10] identified PQ as an essential electron acceptor in the PDS step of carotenoid biosynthesis. Herbicides such as norflurazon compete with PQ for binding to PDS [4] whereas HPPD inhibitors would appear to act indirectly by preventing PQ from being made. Consistent with this notion, PQ levels decrease in plants treated with HPPD herbicides [8] well before [14] the onset of bleaching and phytoene accumulation. Both types of herbicide ultimately cause depletion of carotenoids. Carotenoids act as accessory light-harvesting pigments and precursors of abscisic acid but their main role is in photo-protection [4]. Their extended conjugated double-bond system makes them effective quenchers of high energy triplet states of chlorophyll that would otherwise generate singlet oxygen. They mainly comprise part of the light harvesting antenna structure but β-carotene bound to the D2 protein is also a structural part of the photosystem II core. Herbicide-induced depletion of carotenoids is associated with light-dependent generation of singlet oxygen which damages lipids and proteins and causes disassembly of the photosynthetic complex and release of free chlorophyll. Free chlorophyll is photodynamically photodestructive and itself generates further singlet oxygen, eventually leading to the destruction of all leaf pigments and the characteristic white bleaching.

HPPD inhibitors might be expected to deliver the same herbicidal effects as direct inhibitors of PDS. Both are most effective in newly developing tissues that

emerge bleached, presumably as a consequence of a failure to properly assemble photosynthetic units in the absence of carotenoids [19] and because those that do form bleach upon first exposure to light. Tissue damage is slower in mature tissue since it depends upon light intensity and carotenoid turnover. HPPD inhibitors are more effective applied post-emergence than are PDS inhibitors and exert greater effects on growth while PDS inhibitors cause more damage to mature leaves. These differences likely arise from differences in translocation. However, HPPD inhibitors cause some distinct phytotoxic effects. In mature cotyledons the effect of sulcotrione on both PSII quantum yield and pigment content appears intermediate between that of the PDS inhibitor fluridone and the PSII herbicide, diuron and it was thus suggested that direct inhibition of electron transport from PSII due to depletion of PQ may contribute to sulcotrione phytotoxicity [20]. Synergy between PSII and HPPD inhibitor herbicides further supports this notion. Mesotrione control of several weeds is synergistically improved with the addition of low rates of atrazine; in addition a significantly faster rate of tissue necrosis is observed in the mixture [21]. This could be rationalized purely in terms of improved competitive binding of PSII herbicides when PQ is depleted. However, there is also evidence that PSII effects might be mediated via depletion of tocopherol. Under high light and when electron transport from PSII is blocked (as by PSII herbicides) the PSII P680 reaction centre becomes over-reduced and the chlorophyll partitions toward the triplet state. Unquenched, this would generate singlet oxygen, damage the adjacent D1 protein and lead to PSII disassembly, chlorophyll release and photodynamic bleaching. Evidence from studies in *Chlamydomonas* [22] suggests that tocopherol has a key role in quenching. With careful poising of the concentration of HPPD inhibitor it was possible to partly inhibit photosynthesis in a culture of *Chlamydomonas* such that, at low light levels, PQ did not limit the photosynthetic rate and tocopherol levels were only 50% diminished. On transfer to high light the tocopherol pool diminished sharply and, within less than 3 h, PSII was inactivated and the D1 protein virtually disappeared. These effects were prevented or slowed by direct addition of short-chain cell-permeable analogues of tocopherol or diphenylamine, another direct chemical quencher of singlet oxygen. However, various tocopherol-deficient mutants of *Arabidopsis*, vte2 (HPT), vte1 (tocopherol cyclase) and vte3-1 (an MSBQ/MPBQ point mutant deficient in α- and γ-tocopherols) exhibit quite normal phenotypes [16, 23]. Only under rather drastic conditions of short exposure to high light and low temperatures could bleaching and lipid damage be induced. Under continuous high light other protective mechanisms, the xanthophyll cycle (which HPPD inhibitors would effectively block) and non-photochemical energy dissipation appear to provide compensatory protection. Overall, it seems likely that depletion of tocopherol [14] could only contribute significantly to the phytotoxic effects of HPPD herbicides under photo-inhibitory conditions. Nevertheless, it may underpin synergy between PSII and HPPD inhibitors where the two effects, of generating singlet oxygen and removing the means of protection, combine to cause a new phytotoxic symptom via rapid damage to the D1 protein.

Clearly, to be useful as herbicides in crops, HPPD inhibitors need be selective. Thus far, commercial HPPD inhibitor herbicides have been for use in corn (sulcotrione [1], mesotrione [24] and isoxaflutole [25] or rice (pyrazolate, pyrazoxyfen [2]). Development compounds, Bayer AE 0172747 (proposed name tembotriazone) and BAS670 (BASF, proposed name topramezone), are also mainly for use in corn although potential for cool-climate weed control in wheat is also indicated. Pyrazolate, pyrazoxyfen and isoxaflutole are proherbicides, with the former being detosylated [2] and the latter, (5-cyclopropylisoxazol-4-yl 2 mesyl-4-trifluoromethylphenyl ketone) quickly non-enzymically hydrolyzed to the corresponding diketonitrile (DKN) [14]. The crop safety of DKN depends on degradation to an inactive benzoic acid derivative [14]. Post-emergence safety of mesotrione in corn arises from favorable differential uptake and rapid P450-mediated hydroxylation of the cyclohexane ring [26]. In addition, mesotrione, which has a mainly broad-leaved weed spectrum, is a significantly more potent inhibitor of arabidopis HPPD (K_d 15 pM) than of HPPD from, for example, wheat (K_d 5 nM) a species to which it is much less herbicidal. Allowing for the 3–4-fold difference in the K_m for HPP this translates to about a 100-fold difference in effective potency. Accordingly, transgenic tobacco expressing wheat HPPD is highly resistant to mesotrione [26]. Where natural mechanisms of crop selectivity are inadequate such genetic engineering provides an alternative route. As yet, no transgenic HPPD-herbicide resistant crops have been commercialized although a good deal of work has been described in the patent and academic literature [11, 27]. Analogous to the engineering of resistance to glyphosate [28] mechanisms include increased expression of the target site and expression of altered target site HPPDs having enhanced resistance (e.g., by mutation or through natural tolerance). More novel are recent examples where (i) HPPD is bypassed through the expression of a three-enzyme algal pathway that provides an alternative route to provide HGA (and explains why *Synechocystis* should be insensitive to HPPD inhibitors) and (ii) resistance is considerably enhanced through co-expression of prephenate dehydrogenase with HPPD [11]. Herbicides also need to be selected for pharmacokinetic and kinetic properties that minimize impact in mammals. The pharmacokinetics of, for example, nitisinone selected to maintain a long-term block on tyrosine degradation, contrast markedly with those of the herbicide mesotrione where tyrosine accumulation effects are weak and transitory [29]. The recently solved crystal structures of rat and *Arabidopsis* HPPDs with inhibitors bound [30] promises to facilitate inherent selectivity by design.

In recent years understanding of the structure and mechanism of HPPD has progressed rapidly. A major highlight has been the X-ray crystallographic elucidation of the structure of plant, microbial and mammalian enzymes both with and without inhibitors bound [30–33]. Many aspects of HPPD structure and catalysis have been reviewed [12]. As a non-heme Fe(II)-containing dioxygenase, HPPD is a member of a wider group which couple oxidation of a substrate by dioxygen to oxidative decarboxylation of an α-keto acid (commonly α-keto-glutarate). Of the four electrons required to reduce dioxygen, two derive from oxidative decarboxyla-

tion and the other two from the substrate itself. For the HPPD reaction (Fig. 4.2.1) the decarboxylated 2-keto acid (pyruvyl) is not a separate cosubstrate but a side chain of the phenyl ring substrate that is oxidized. The pyruvyl side chain is decarboxylated to a carboxymethylene group which then migrates to the adjacent carbon of the phenyl ring while the ring is hydroxylated on the carbon at which the side chain was originally attached.

HPPD has a subunit polypeptide mass of 40–50 kDa and is typically a tetramer in bacteria or a dimer in eukaryotes (including mammals and plants) [11]. Conserved residues are found only in the C terminus and from recently solved X-ray structures (*P. fluorescens*, *Arabidopsis thaliana*, *Zea mays*, *S. avermitilis* and rat) the Fe and its associated inhibitor/substrate-binding site are clearly structurally well conserved and located within the C terminal part of the protein that folds as a discrete domain. At the primary sequence level, plant proteins appear somewhat distinct because they include a 15 amino acid insertion but, at a structural level, the core active site region remains similar to that in HPPDs from other phylla. As in all non-heme Fe(II) oxygenases this core consists of an active site Fe(II) coordinated by a triad of 2 histidine residues and one carboxylate [34]. In HPPD, the overall peptide fold as well as the disposition of these three residues through the primary sequence is similar to extradiol dioxygenases [12, 32] and it is suggested that HPPD exemplifies a 2-keto acid type dioxygenase that arose by convergent evolution from an extradiol type. In all HPPDs the Fe(II) is located at the centre of a cavity, between 8 and 14 Å wide, that is formed by an eight-stranded twisted half open β barrel. The three residues coordinating the Fe are located on three of these strands and the surrounding cavity environment is almost entirely conserved and dominated by hydrophobic amino acid residues within rigid secondary structural elements.

Current understanding of the catalytic mechanism derives from a combination of structural information, spectroscopy, kinetics and, since many of the proposed intermediates are too short-lived to be observed directly, also theoretical considerations. Figure 4.2.2 is based upon one current view [35].

The proposed nature of early intermediates in catalysis is consistent with the ordered mechanism observed in steady state kinetic studies [12, 36]. HPP binds first and CO_2 is the first product released. In α-ketoglutarate dioxygenases, α-ketoglutarate initially associates as a bidentate ligand of the Fe(II) and HPP appears to coordinate Fe(II) in HPPD in a similar way [12]. The initial, enzyme–Fe(II)–HPP complex (isolable under anaerobic conditions) exists as a mixture of five- and six-coordinate Fe [37] similar to the complex with inhibitor [12, 33]. In resting enzyme, the Fe(II) is relatively unreactive and, again, analogous to the other dioxygenases, HPP coordination primes it to react rapidly with dioxygen [38]. Binding of dioxygen is endergonic and the reactive Fe(III)–O_2 species thus formed predicted to be short-lived. Withdrawal of electrons into the Fe-bound dioxygen facilitates nucleophilic attack on the α carbonyl carbon resulting in decarboxylation and the generation of a theoretical short-lived Fe(II)-peracid species that heterolytically disproportionates to yield the oxo-Fe(IV) electrophile. This key species has not been detected directly but its existence is inferred from the reac-

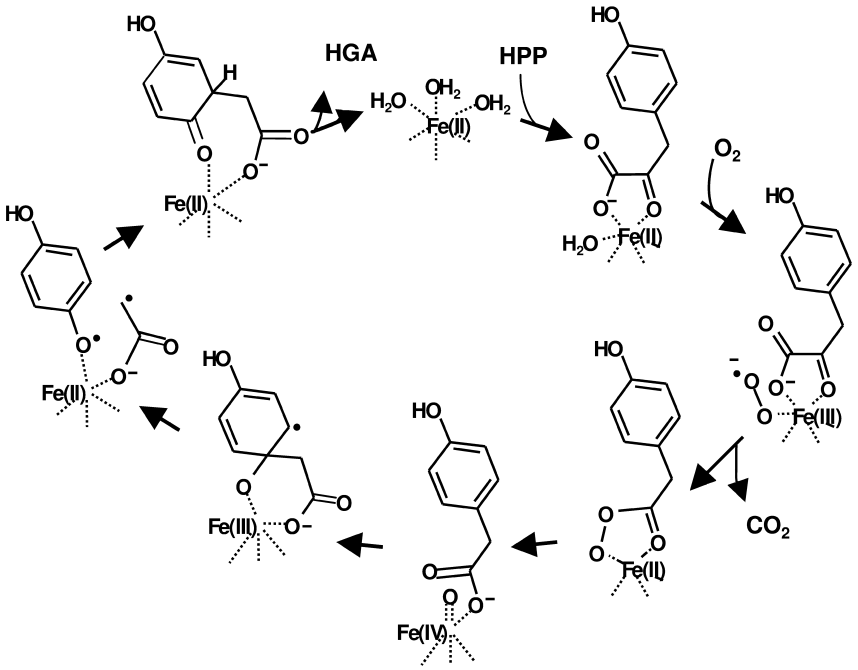

Fig. 4.2.2. Proposed intermediates in the HPPD reaction. (Based on Ref. [35].)

tion chemistry, by analogy with similar enzymes and, also, experimentally, from the observation [39] that the alternative substrate (4-hydroxyphenyl)thio-pyruvate is hydroxylated on sulphur rather than the ring. Proposals for the subsequent steps are varied. Some involve the oxo-Fe(IV) species abstracting two electrons from the aromatic ring to generate an arenium cation or benzene oxide [12]. Alternatively [35], it is argued that with two negatively charge ligands in the iron coordination shell the transfer of a second electron from the ring to the iron would be hindered and that a single electron process is more likely. This yields a radical sigma complex (that could potentially generate arene oxide via non-productive side reactions) which, in one suggested mechanism (Fig. 4.2.2) for side chain migration, undergoes homolytic C–C bond cleavage to yield a highly unstable biradical species that decays to form the new C–C bond and the ketone species. In the finals steps, re-aromatization and tautomerization to HGA could equally take place in solution as enzyme-bound.

X-Ray crystallography has provided considerable insight into how inhibitors bind. In the *S. avermitilis* HPPD/nitisinone complex, Fe is five/six-coordinate with bidentate chelation from the 5′ and 7′ oxygens of the inhibitor and a water weakly occupying the 6'th position [33]. Inhibitor binding shifts a C terminal helix to provide one of two phenylalanines that sandwich the phenyl ring of the inhibitor in a π-stacking interaction. No other energetically significant interactions between inhibitor and enzyme surfaces are evident other than exclusion of

waters through space-filling Van der Waal contacts. The site around the inhibitor is highly conserved and phenylpyrazole binding into rat and plant HPPDs appears similar [30]. It is not immediately obvious how inhibitor binding should be selective between one HPPD and another. However this clearly is possible [26, 30] and 100-fold differences in K_d (12 kJ mol^{-1}) or greater may originate from the sum of structural and orientation differences too subtle to discern.

In principle inhibitors could coordinate Fe(II) or Fe(III) in HPPD. In free solution inhibitor complexes with Fe(III) have the lower dissociation constants [40]. However DKN and nitisinone bind significantly only to Fe(II) forms of the carrot and *S. avermitilis* enzyme [41, 42]. Iron(II) enzyme inhibitor complexes are highly stable, unreactive to oxygen and, similar to the anaerobic Fe(II) enzyme HPP complex, weakly colored due to charge transfer transitions [12, 42]. Spectroscopic studies of the Fe(II) centre have provided quantitative insight into the relative contribution of metal coordination to overall binding. Magnetic and non-magnetic circular dichroism spectroscopy combined with calculations based on density function theory indicate that nitisinone interacts with the Fe(II) somewhat more weakly than the substrate [43]. Thus, the π-stacking interaction with the enzyme phenylalanines makes a major contribution to inhibitor binding. Inhibitors may act as mimics of a reaction intermediate and analogous interactions may help drive catalysis. The π-stacking may electronically stabilize the putative arenium cation [12] or, equally, a phenyl radical intermediate (Fig. 4.2.2). In either case, tight binding of inhibitors as compared with substrate may be understood in terms of favorable interactions with electron deficient aromatic rings. Spectroscopic and kinetic studies [12, 43] have also provided insight into the steps involved in inhibitor binding. While di- and triketone inhibitors exist in solution as an equilibrium between several tautomers, for nitisinone at least the exocyclic enol(ate) predominates in solution at pH 7 whereas the keto form of HPP is the substrate [44]. Pre-steady state spectroscopic studies of nitisinone binding to anaerobic *S. avermitilis* HPPD indicate at least three substeps before tight complex formation, a rapid weak non-chromophoric complex, a shift to a chromophoric complex (8 s^{-1}) and finally a slower chromophoric shift (0.76 s^{-1}). A solvent deuterium isotope effect of three on the latter is consistent with a proton shift being involved in final complex formation.

Whatever the sub-steps involved the orphan drugs and herbicides clearly form remarkably tight complexes with Fe(II) HPPD. Initial studies with nitisinone and sulcotrione indicated half-times for dissociation from the rat enzyme of approximately 10 and 63 h [6]. Studies with HPPD from carrot indicated single step competitive binding of DKN to form a similar tight slow-dissociating complex with the plant enzyme [41]. The value of K_i can be evaluated from the ratio of the rate constants governing dissociation (k_{off}) and formation (k_{on}) of the enzyme inhibitor complex although relatively few measurements have been reported (Table 4.2.1).

Formation rate constants, k_{on}, have been estimated on the basis of enzyme assay [6, 26, 27, 41, 45] or quenched physical binding studies using labeled inhibitors [26, 27] and dissociation rates, k_{off}, based on rates of inhibitor exchange [26,

Table 4.2.1 Estimated inhibition constants of HPPD inhibitors.

Inhibitor (Fig. 4.2.1)	HPPD	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (pM)	Ref.
(3)	Carrot	1.5×10^4	9×10^{-5}	6000	41
(3)	*P. fluorescens*	1.6×10^4	1.8×10^{-4}	11000	27
(3)	Wheat	6.9×10^4	6.2×10^{-5}	900	27
(3)	*Arabidopsis*	1.8×10^5	8.3×10^{-6}	46	27
(4)	*Arabidopsis*	2.3×10^5	3.3×10^{-6}	14	26
(4)	*Pseudomonas*	1.8×10^4	2×10^{-6}	114	27
(4)	Wheat	1.8×10^5	1.0×10^{-3}	5500	26
(5) [Cl]	*Arabidopsis*	3.0×10^5	1.2×10^{-6}	4	27
(5) [Cl]	*Pseudomonas*		$>2 \times 10^{-4}$		27
(5) [Cl]	Wheat	3.0×10^5	4.2×10^{-6}	12	27
(5) [CF3]	Rat	1.5×10^4	1.9×10^{-6}	125	45
(6)	Rat	9.9×10^4	3.2×10^{-6}	32	6
(1)	Rat	3.3×10^{-4}	1.9×10^{-5}	575	6

27, 41] or activity recovery [6, 45]. Concentrations of HPPD active sites have been determined via titration with labeled inhibitors in both crude and purified preparations of enzyme. While pure preparations are preferable for kinetic studies, particularly with plant HPPDs, purification and reconstitution with Fe(II) leads to activity loss and, in principle, the possible generation of damaged species with altered binding kinetics (k_{cat}/K_m values can appear 3–10-fold greater in part- than in fully-purified preparations of *E. coli*-expressed recombinant plant HPPDs [26, 27, 41]). Certainly differences in assay and preparational procedures make all comparisons difficult and the absolute accuracy of, especially, the faster on and slower off rates in Table 4.2.1 is not guaranteed. Nevertheless, it is striking that many inhibitor are highly potent with K_ds in the pM range and also that there are significant, several hundred-fold, species-dependent differences in inhibitor K_d and k_{off} values with the *Arabidopsis* enzyme being the most sensitive of those tested. It is difficult to know which kinetic parameter to take as most predictive of biological activity. "Stickiness" (k_{off}) may be key in maintaining persistence of the pathway blockade since, once inhibited, HPPD will stay inhibited for days (until new enzyme is synthesized) and this may be important in achieving good herbicidal activity.

Interestingly, it appears that inhibition requires the binding of only a single inhibitor molecule per HPPD dimer. While only a single DKN molecule bound per dimer of carrot HPPD this "half-site" binding was nevertheless associated with complete enzyme inhibition [41]. Similar was observed in preliminary studies with part-pure *Arabidopsis* HPPD [27] with equilibrium binding of DKN being half that observed with CHDs (e.g., mesotrione). Mesotrione binding to *Arabidopsis* HPPD was biphasic with (presumptive) half-site binding and complete en-

zyme inhibition occurring in an initial rapid phase (1.8×10^5 M^{-1} s^{-1}) and the remaining 50% then binding much more slowly. Thus, even with CHDs that eventually bind one per monomer it appears that only the initial rapid half-site binding may be required for inhibition.

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4.3
Hydroxyphenylpyruvate Dioxygenase (HPPD) Inhibitors: Triketones

Andrew J. F. Edmunds

4.3.1
Introduction

This chapter aims to give an insight into the discovery of the triketone class of herbicides and their continuing development. A very qualitative picture of structure–activity relationships will be discussed and currently commercialized triketones, in terms of their use, weed spectrum, crop selectivity, environmental and toxicological profiles, and manufacture will be described. This chapter also contains an overview of the major companies' activities in the field in the last two decades, focusing on compounds that are likely to be brought to the market, or were putatively close to development.

4.3.2
Discovery

In 1977, at the Western Research Centre in California, scientists in Stauffer (a former legacy company of Syngenta) noticed that relatively few weeds grew under the bottle brush plant *Callistemon citrinus*. Analysis of soil samples where *C. citrinus* was growing revealed that the herbicide the plants were excreting was leptospermone (**1**) [1]. This natural product had previously been isolated from the volatile oils of Australian myrtacious plants [2]. Pure samples of leptospermone (**1**) showed unique bleaching symptoms on several weed species albeit at relatively high (5 kg ha^{-1}) rates. This herbicidal activity was patented in 1980 [3].

Independent of this discovery, in 1982 scientists from the same company were working on a project aimed at preparing novel Acetyl-CoA carboxylase inhibitors, based upon the typical cyclohexanedione structure known for this class. The first targeted compound (**2**), prepared as shown in Fig. 4.3.1, showed some herbicidal activity and they thus attempted preparation of a phenyl analogue in a similar manner. This led not to the expected product (**3**), but to the triketone (**4**). This compound was devoid of herbicidal activity, but (luckily!) in safener screens the compound showed antidotal effects in Soya for thiocarbamate herbicides. A further round of synthesis optimization was undertaken and it was found that the compound (**5**) with an ortho-chloro substituent showed reasonable herbicidal activity. Furthermore, they noticed that it exhibited the same unique bleaching symptomology observed for leptospermone (**1**, Fig. 4.3.1) [4]. Further optimiza-

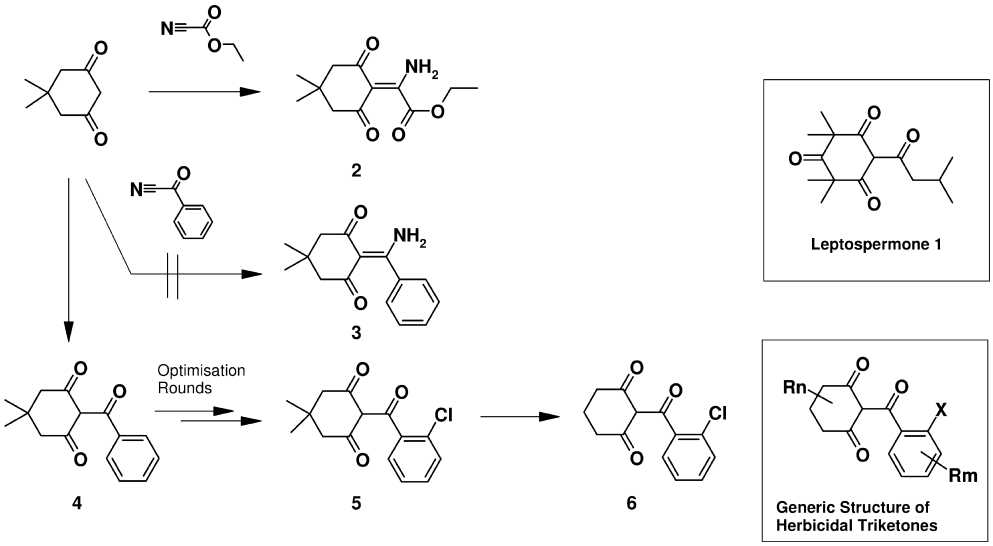


Fig. 4.3.1. Discovery of the triketone herbicides.

tion showed that removal of the methyl groups at the 5-position of the cyclohexanedione moiety (**6**) resulted in significantly enhanced herbicidal activity against a wide range of broad-leaved weeds, with good corn tolerance, when applied pre- and post-emergence at rates of about 2 kg ha^{-1}. The first patent was filed [5] and the discovery of the benzoylcyclohexanedione herbicides had been made. These events, and the generic structure of herbicidal triketones are summarized in Fig. 4.3.1.

4.3.3 Mode of Action

As discussed in detail in Chapter 4.2, triketones exert their herbicidal mode of action by inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD) [6]. Triketones are not the only herbicide class that have this mode of action, and it has retrospectively been shown that apparently structurally non-related heterocyclic commercial herbicides such as isoxaflutole (**7**, BALANCE® and MERLIN®), and the rice herbicides pyrazolate (**8**, SANBIRD®) and benzobicyclon (**9**, SHOWACE®) also cause these bleaching symptoms by the same mode of action. However, a common feature of these herbicides, after metabolic activation to the active metabolites (**7′**) [7], (**8′**) [8] and (**9′**) [9] is the presence of an acidic 1,3-dicarbonyl moiety, which is also present in triketones (Fig. 4.3.2). Triketones and related her-

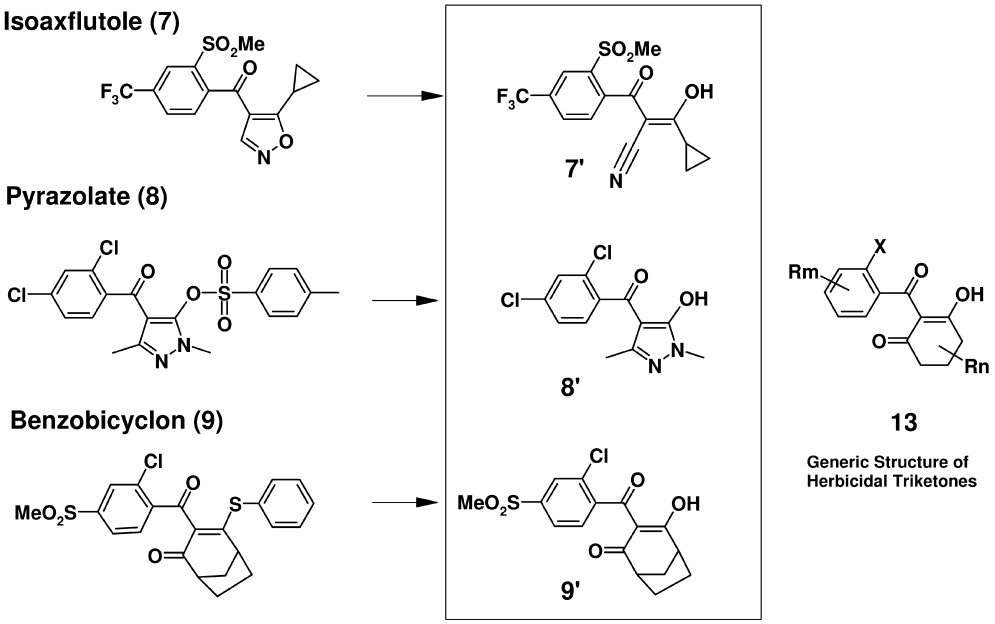
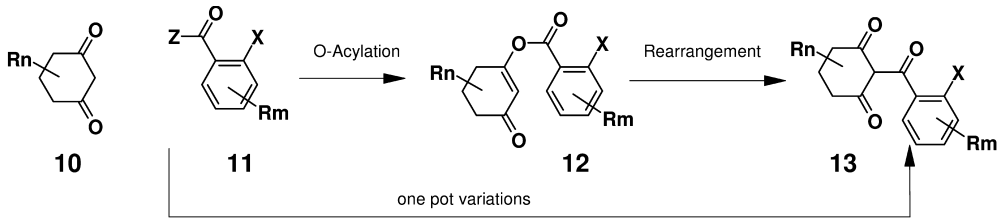


Fig. 4.3.2. Commercial herbicides with a HPPD mode of action.

bicides mimic the -keto acid group of the HPPD substrate hydroxyphenyl pyruvate, and competitively bind to the iron at the active site of the enzyme, causing its inhibition. Homology models of all structure types bound to this enzyme [10] and crystal structures of pyrazoles [11] and triketones [12] bound to the same enzyme have been published.

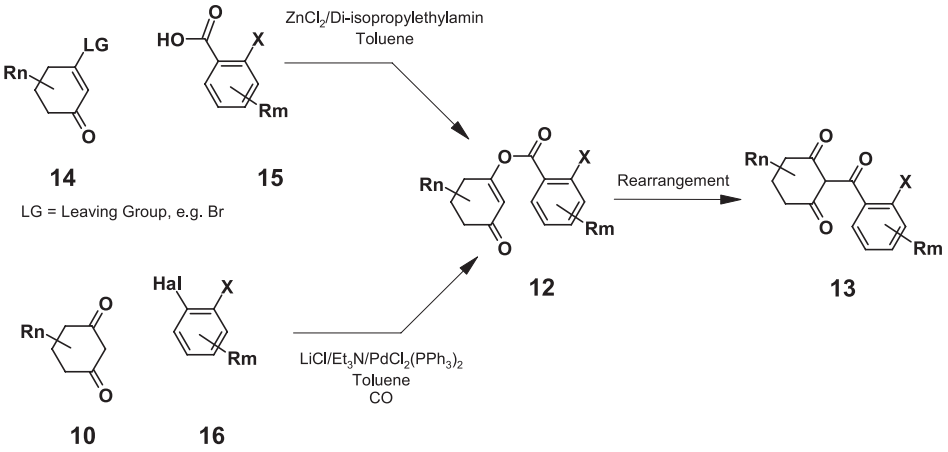
4.3.4 Synthesis of Triketones

Most triketone herbicides (**13**) reported in the literature are synthesized by O-acylating a cyclic 1,3-dione (**10**) with an activated aroyl acid (**11**), and then carrying out an O- to C-acyl rearrangement of the O-acyl intermediate (**12**) in the presence of a catalyst (Scheme 4.3.1). The O-acylation is generally achieved using an acid chloride in the presence of a base, but other reagents such as dicyclohexylcarbodiimide (DCC) [13], *N*-methyl-2-chloropyridinium iodide (Mukaiyama coupling agent) [14], 2-chloro-1,3-dimethylimidazolinium chloride [15], have been used in triketone synthesis. Typical catalysts used for the rearrangement are cyanide [16], aluminum trichloride [17], 1,2,4-triazole [18], potassium fluoride [19] and azide salts [20] whereas the cyanide source (including acetone cyanohydrin) induced O–C rearrangement has been generally the method of choice. The reaction may be carried out in a stepwise fashion (i.e., isolation of **12**) but one-pot variations have been developed in many cases by choice of the correct solvent [21]. There is also an isolated report of direct C-acylation (**10**) with an aroyl acid chloride (**11**, Z = Cl) using potassium carbonate in acetonitrile [22]. Alternatively, triketones can be obtained directly by acylation of (**10**) with the appropriate benzoyl cyanide (**11**, Z = CN) [23].



Scheme 4.3.1

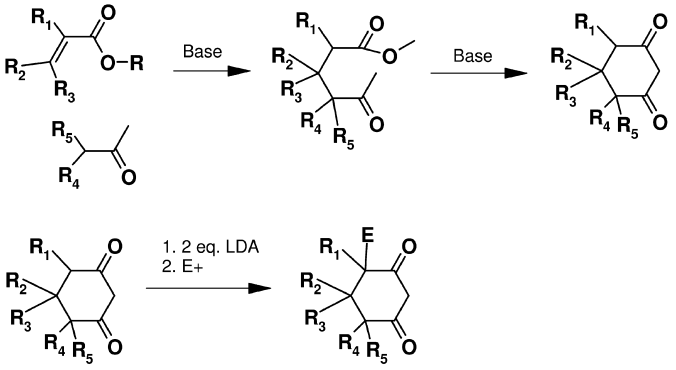
Other syntheses that have been developed for preparation of triketones include activating the dione portion (to give **14**) and coupling this with an aroyl acid (**15**) in the presence of a Lewis acid catalyst [24] followed by O-acyl rearrangement, or by palladium-catalyzed carbonylation of an aroyl halide (**16**) in the presence of a dione (**10**) [25] and subsequent rearrangement of the O-acyl product (**12**) formed into the triketone (**13**) (Scheme 4.3.2).



Scheme 4.3.2

Cyclohexane diones with various substitution patterns can be readily synthesized by the two general routes shown in Scheme 4.3.3 [26, 27].

The synthesis of the benzoyl portions of triketones can not be so generalized, and specific syntheses have been developed for developmental and commercialized compounds.

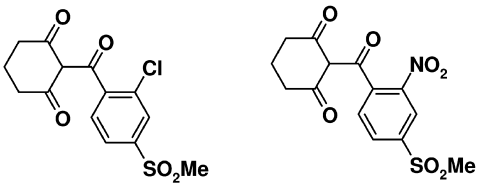


Scheme 4.3.3

4.3.5 Structure–Activity Relationships

The triketones can be separated into two parts for analysis of the structure–activity relationships, namely the benzoyl and the dione moieties. Each part can be examined independently, as they appear to play distinct and different roles in the overall expression of herbicidal activity [1]. Apart from the necessity of an ortho-substituent on the phenyl ring, it was established that 2,4,- or 2,3,4-benzoic acid substitution patterns were required for optimal activity, with the 2,5-

pattern(s) being the least effective. After more than 20 years of HPPD research, this original optimal substitution pattern still seems to be valid based on analysis of published patents. A correlation was found between the pK_a of triketones (which can be viewed as vinylogous benzoic acids) and herbicidal activity [28], with a pK_a of <6 being required for activity, as this will affect not only binding to the iron at the active site of the enzyme but also transport and translocation within the plant. As the pK_a will be affected by substituents on the aromatic ring, those that generally reduce the electron density of the aromatic ring lead to compounds with a reduced pK_a and improved herbicidal activity. A survey of the patent literature and reported SAR studies [4, 29] suggest that small ortho electron-withdrawing substituents such as Cl, NO_2 and CF_3 are particularly favored. An ortho methyl substituent is also tolerated as long as the total electron density of the aromatic ring is kept low. The para substituent is generally also an electron-withdrawing moiety, particularly halo, haloalkyl and alkylsulfonyl, with some restraints on size according to published data [4]. Zeneca (now Syngenta) arrived at several compounds with these types of substitution patterns, such as sulcotrione (**17**) and mesotrione (**18**, Scheme 4.3.4), at an early stage in triketone research. Both compounds have since reached the marketplace (Section 4.3.6).

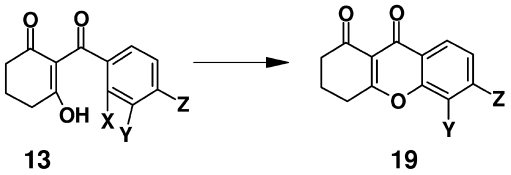


Sulcotrione (**17**) Mesotrione (**18**)

Scheme 4.3.4

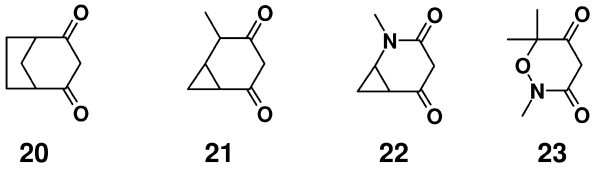
At the meta position, a multitude of functionalities have been reported to lead to herbicidally active compounds. One problem that, however, often leads to reduced potency is the presence of an electron-withdrawing substituent at the meta position combined with a potential leaving group at the 2-position (e.g., nitro or chloro) as this may give rise to dihydroxanthenones (**19**), which are known to be much less herbicidally active (Scheme 4.3.5) [4].

Two strategies have been generally used to remedy this situation: One has been to use meta substituents such as alkoxy or thioalkyl, which are reasonably electron donating at their ortho positions, thus hindering formation of **19**, but inductively electron withdrawing at the carbonyl, thus increasing overall acidity [4]. The more frequently used strategy, however, is to have a small non-leaving group at the ortho-position, such as methyl, whilst having substituents at the 3,4-positions that make the aromatic ring overall electron deficient. Many fused ring types have been reported in the patent literature and it would appear that para,meta-fused ring systems are generally more favored than ortho,meta-fused systems.



Scheme 4.3.5

The effect of adding substituents to the cyclohexanedione ring is to block site(s) of metabolism by plants [29, 30]. This results in greater herbicidal activity, as the plants have greater difficulty in detoxifying the molecule. Studies using model compounds indicate that the principal routes of metabolism of the benzoylcyclohexanediones in plants are hydroxylation at the 4-position of the cyclohexanedione (if this position is blocked, then hydroxylation takes place at the chemically equivalent 6-position), and hydrolytic cleavage of the benzoyl group. It has been demonstrated that placing two methyl groups at the cyclohexanedione 4-position slows the rate of both of these metabolic processes in plants. As the sites for hydroxylation are sequentially blocked, an increase in overall activity against grasses is observed. Some of the most active triketones known contain the 2,2,4,4-tetramethyl-cyclohexane-1,3,5-trione moiety, also found in leptospermone (**1**, Fig. 4.3.1) [29]. However, reducing the potential for metabolism has other consequences, such as reduced corn selectivity and a dramatic increase in soil persistence [1]. To compensate for this effect, several important diones have regularly appeared in the patent literature, which have strained bicyclic rings and/or heterocyclic atoms (Scheme 4.3.6 compounds **20**–**23**), thus putatively being more easily metabolized.



Scheme 4.3.6

4.3.6
Review of the Patent Literature

Some of the important and typical structural types patented by the various companies are discussed in this section. Generic structures are simplified and thus not necessarily those that appeared in the referenced patents.

Stauffer (later Zeneca, now Syngenta) patented extensively after their initial discovery and were able to gain good intellectual property advantage over competitor companies, particularly in terms of important 2,4- and 2,3,4-substituted benzoic

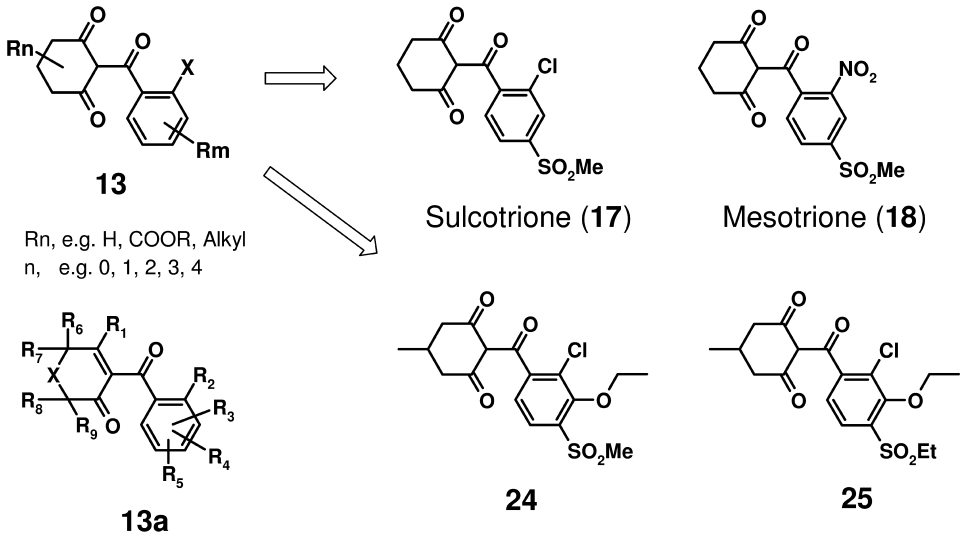


Fig. 4.3.3. Typical Zeneca patents.

acid types (e.g., **17** and **18**, respectively **24** [23b] and **25** [31]), as well as important triketones with more substituted cyclohexane diones (e.g., **13a** X = C(O) [5, 31], and heteroatom containing diones (e.g., **13a** X = O, Fig. 4.3.3) [32].

They were also granted a patent that covered cyclohexane diones coupled to heteroaroyl acids (e.g., **26**–**28**, Fig. 4.3.4) with very broad scope [34], which made patenting rather difficult for companies following Zeneca.

Pyridines and pyrimidines were patented separately, to complete an impressive array of protection for the heterocyclic triketones [35]. Nevertheless, after the first patent appeared regarding this novel substance class, most of the major companies started programs in the field. There were basically two strategies: Some companies searched for novel diones that were at the time outside the scope of the Zeneca published patents, while other companies searched for novel aromatic acids. For example, Sandoz (now Syngenta) concentrated on the search for novel diones, and several compounds containing bicyclo[3.2.1]octane-2.4-dione, such as **29** [36, 37] and **30** [38], as well as the oxazinedione types (**31**) [39, 40], were important compounds for use in corn (Fig. 4.3.5). A collaboration between Sandoz and SDS Biotech has also led to the identification of proform triketones containing bicyclo[3.2.1]octane-2.4-diones for use in rice, such as benzobicyclon (**9**) [41].

Nippon Soda also initially investigated the dione portion of triketones, and patented extensively compounds containing bicyclo[4.1.0]heptane-2,4-diones such as **32**–**35** (Fig. 4.3.6) [42].

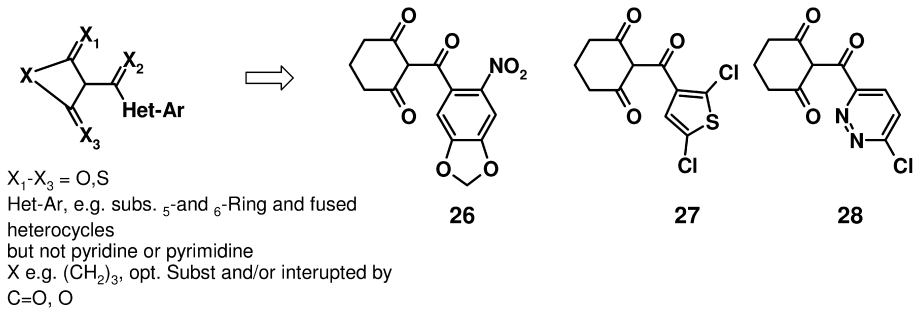


Fig. 4.3.4. Zeneca heteroaroyl triketones.

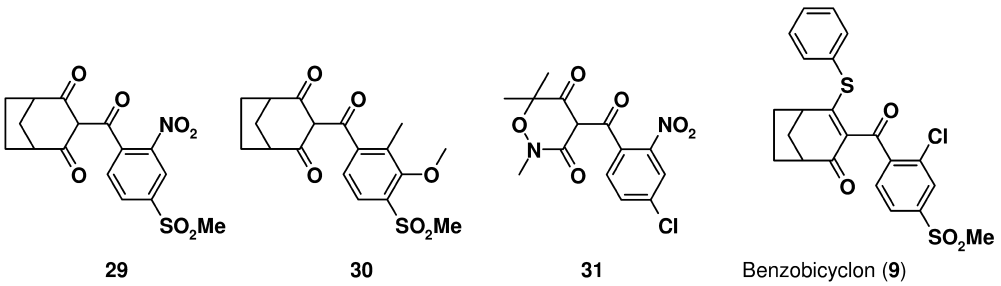


Fig. 4.3.5. Typical Sandoz compounds.

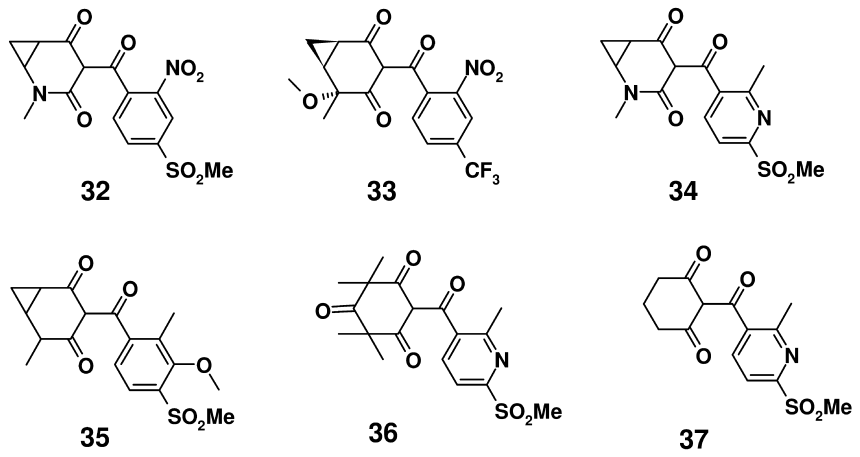


Fig. 4.3.6. Nippon soda types.

They also, apparently, were very interested in triketones with a nicotinoyl acid moiety, based on the number of applications filed in this area (**34**, **36**, and **37**, Fig. 4.3.6) [42, 43]. Nissan noticed the similarity of the triketones to the pyrazole type herbicides such as pyrazolate (**8**), and secured intellectual property freedom in

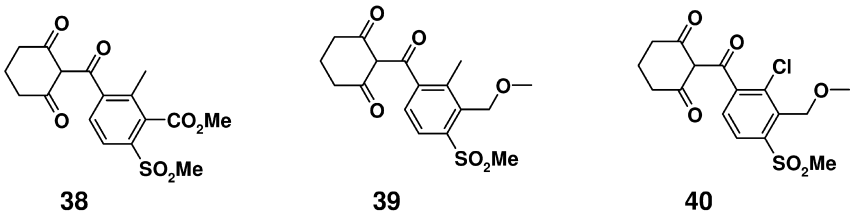


Fig. 4.3.7. Nissan triketones.

this area by patenting pyrazoles with the optimally substituted aroyl acids discussed previously [44]. Some important triketones containing novel trisubstituted acids were also first patented by Nissan (**38**–**40**, Fig. 4.3.7) [45].

BASF initially attempted to conquer some intellectual property by using proprietary diones from their DIMS chemistry (**41** and **42**, Fig. 4.3.8) [46]. However,

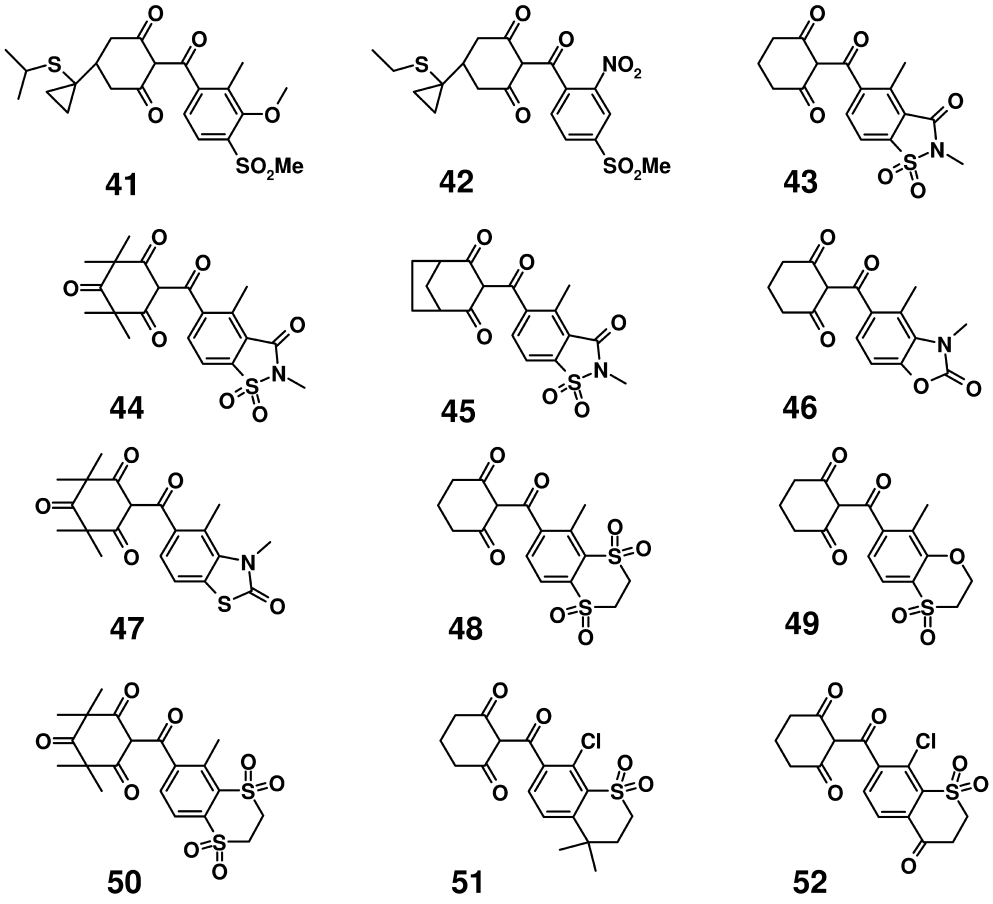


Fig. 4.3.8. BASF triketones.

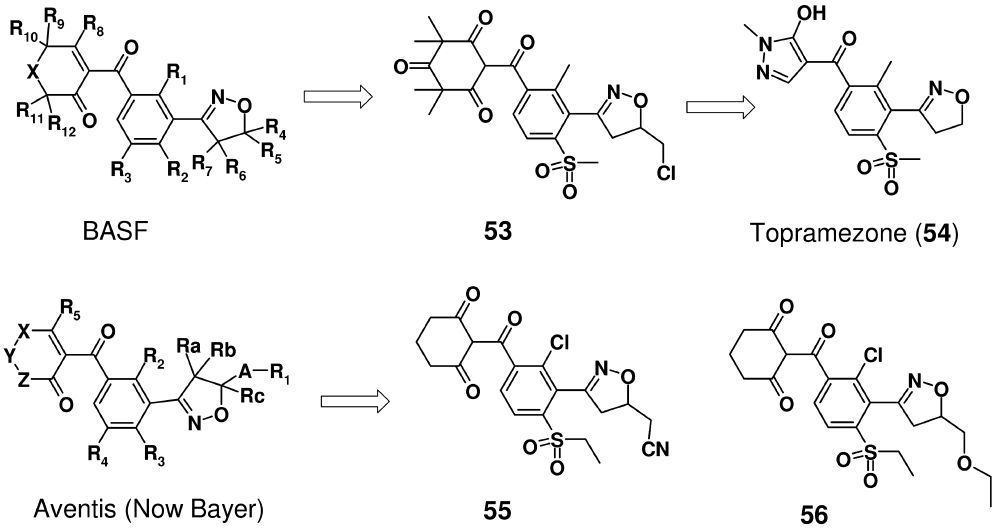


Fig. 4.3.9. BASF and Bayer meta-heterocyclic substituted triketones.

after probably realizing that large substituents at the 5-postion of cyclohexanediones are not optimal for herbicidal activity, they switched their attention to the search for novel acids. Particularly prominent acids from BASF that have appeared in the patent literature are the saccharin's (e.g., **43**–**45**, Fig. 4.3.9) [47] and other fused 3,4-aroyl acids, such as **46** and **47** (Fig. 4.3.8) [48], and especially those in which the alkylsulfonyl group is incorporated into a fused ring at the 3,4-positions (**48**–**52**, Fig. 4.3.8) [49, 50].

BASF also explored patent free examples of triketones with novel meta-substituents, particularly acids containing heterocyclic rings at this position (e.g., **53**, Fig. 4.3.9). The 4,5-dihydro-isoxazole containing pyrazole corn herbicide topramezone (**54**, IMPACT®, CLIO®) [50, 51] has resulted from this work (Fig. 4.3.9).

With regard to triketones of this structure type, Aventis (now Bayer) patented substituted 4,5-dihydro-isoxazole compounds [52] prior to BASF [53], and two compounds from Bayer (**55** and **56**, Fig. 4.3.9) have frequently appeared in mixture patents with safeners and other herbicides for use in corn [54].

Idemitsu also concentrated their efforts on new acids, with emphasis on those in which the alkylsulfonyl substituent at the 4 position was joined into a ring at the 3-postion (typical Idemitsu types **57**–**61** are shown in Fig. 4.3.10) [55]. Although they received a patent for compounds of this type with 2-chloro substituent (e.g., **59**) they were also forced to switch to more complicated substituted heteroaromatic systems after the publication of interfering patents from Zeneca [33], or to pyrazoles [56]. They now appear to have a pyrazole compound (generic structure **61**, Fig. 4.3.10) in development for use in corn, based on recently published mixture patents [57].

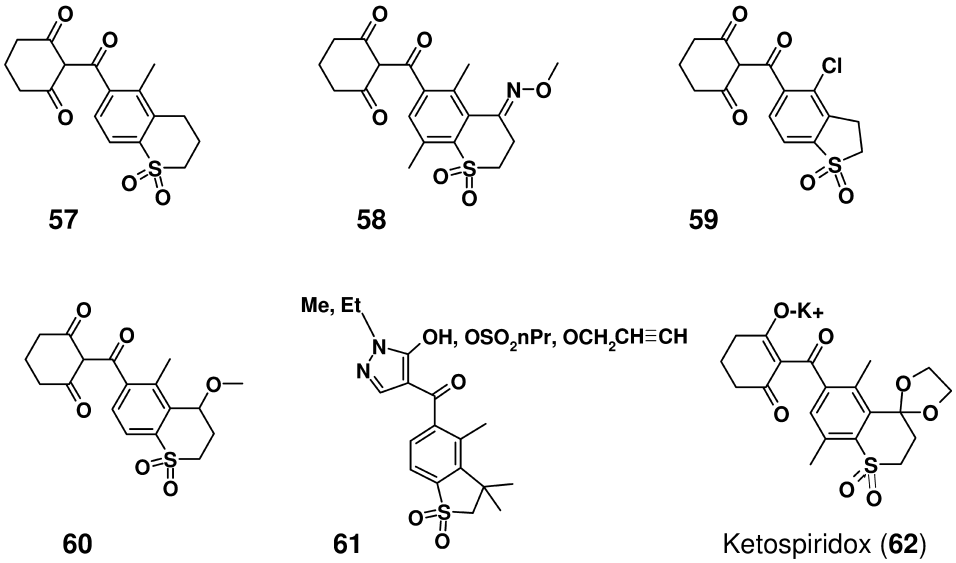
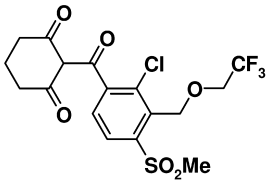


Fig. 4.3.10. Idemitsu and Du Pont triketones.

Du Pont started relatively late in the triketone field, and directed their efforts toward novel fused bicyclic acids. As a result of their work in this area, they discovered the broad leaf weed cereal herbicide ketospiridox [58] (**62**, proposed common name, Fig. 4.3.10). Ishihara, inspired by earlier work of Hokko [59], identified some new benzoyl analogues with cyclic acetal meta-substituents (**63** and **64**, Fig. 4.3.11) that they claim have good pre-emergent activity in flooded rice paddy fields without damaging the rice seedlings [60].

Fig. 4.3.11. Ishihara rice triketones.

Bayer have published recently – mainly after the successful merger with Aventis – a multitude of patents [61], in which they have basically explored in more detail the effect of several novel meta-substituents on the biological activity of triketones, especially those with substituted 3-alkoxyalky-2-chloro-4-alkylsulfonyl substituents in the aromatic ring. From this work, the post-emergence corn herbicide



Tembotrione (**65**)

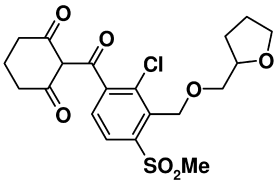


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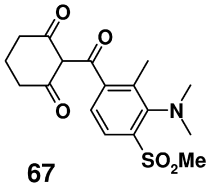
Fig. 4.3.12. Bayer development compounds.

tembotrione (**65**, proposed common name, Fig. 4.3.12) has resulted [62]. Tembotrione is being promoted as a direct competitor to mesotrione (**18**) in the corn herbicide market. Where tembotrione differentiates itself from mesotrione is improved grass control according to tests reported by Bayer [62]. Several mixture patents have also been disclosed, which suggest Bayer has a compound to be developed in paddy rice [63]. Figure 4.3.12 shows the structure of the putative development product (**66**), and, as can be seen, it is very similar to the Ishihara's rice compounds shown in Fig. 4.3.11.

DOW invested virtually all their effort in the field of pyrazoles and has recently published several reviews in this area (Chapter 4.4) and only a few triketone patents appeared. Those that did had meta-amino substituents such as **67** and **68** (Fig. 4.3.13) [64].



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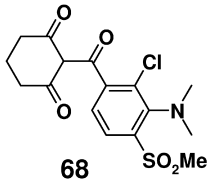

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Fig. 4.3.13. Dow triketones.

Despite the broad granted scope of the initial Zeneca heteroaroyl triketone patent [34], the pyridyl triketones were not further pursued by Zeneca, neither (behind the earlier Stauffer claims [36]) by Sandoz [27b] nor by Ciba-Geigy [65] (all now Syngenta). Nippon Soda too also left gaps in their patents in claiming pyridyls [42, 43]. All of this was exploited then once more by Novartis (now Syngenta). A series of patents around novel pyridyl acid containing triketones were published [66], and on the basis of mixture [67] and process [24, 37, 68] patents it appears that Syngenta found some very interesting new compounds for use in corn (**69** and **70**) and cereals (**71** and **72**, Fig. 4.3.14).

Particularly interesting here is that **70** and **71** containing larger ortho substituents than the usual patented types (e.g., Cl or Me) are tolerated at the enzyme site, and that the picolinic acid (**72**) has no ortho-substituent, which suggests that the lone pair on the pyridyl nitrogen can act as such.

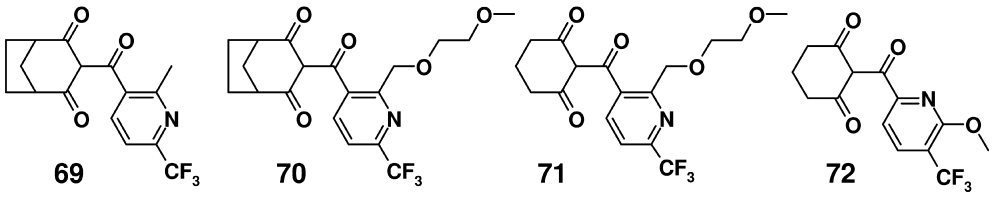


Fig. 4.3.14. Syngenta pyridyl triketones.

4.3.7
Commercialized Triketone Herbicides

The first triketone herbicide to be commercialized was sulcotrione (**17**). It was discovered by Stauffer (one of the legacy companies of Syngenta) and was registered for use in 1993 [69]. The product is sold under the trade name of MIKADO® in Europe and had sales in 2004 of $60 million (the compound is not registered for use in the USA) [70]. It was sold by Syngenta in 2001 to Bayer to satisfy conditions imposed by the European Commission in connection with the merger of Novartis Agribusiness and Zeneca Agrochemicals to form Syngenta. Bayer are apparently now seeking registration in the USA [70]. It is used for post-emergence control of (particularly) broadleaf weeds and some grass weeds (*Digitaria sanguinalis*, *Echinochloa crus-galli*, and *Panicum miliaceum*) in corn with application rates being 300–450 g ha^{-1}. Sulcotrione is readily absorbed through the leaves and also via the roots. The metabolism in soil is as shown in Fig. 4.3.15 [71]. Soil half-lives of 15–74 days have been measured, which causes no threat to cereals, the usual rotational crops to corn in Europe [72].

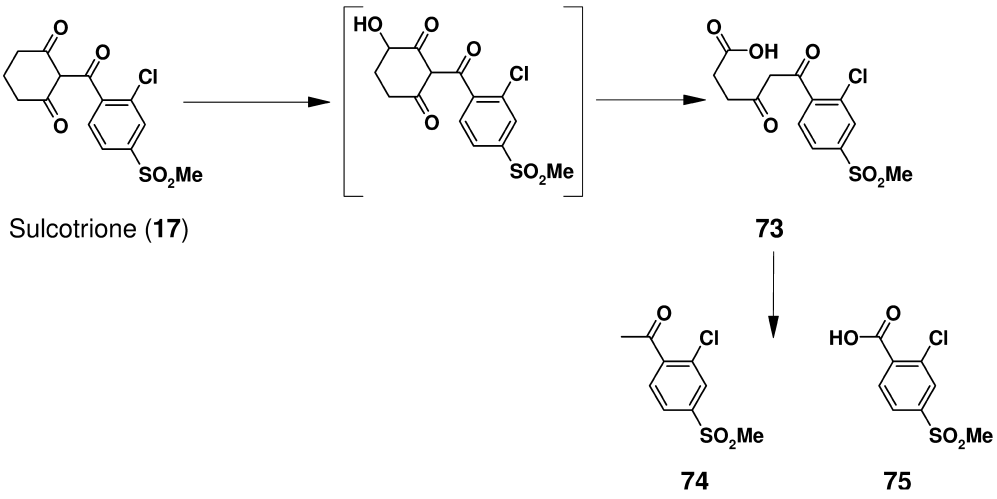
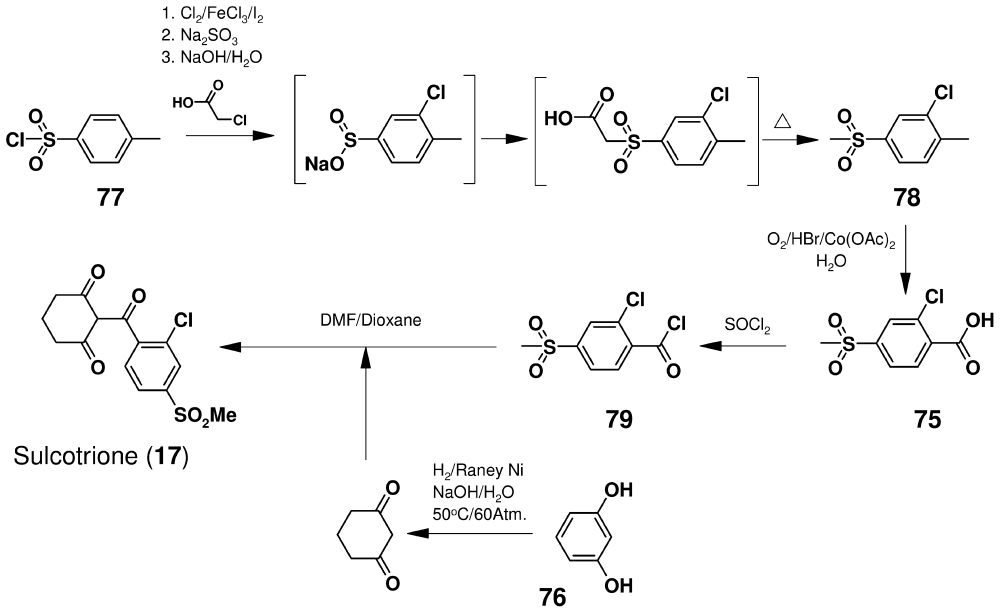


Fig. 4.3.15. Sulcotrione soil metabolism.



Scheme 4.3.7 Synthesis of sulcotrione.

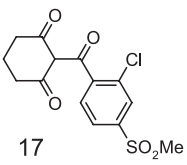
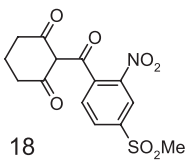
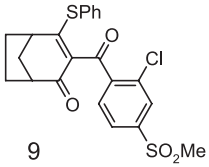
Scheme 4.3.7 shows a recently published possible technical synthesis that gave a yield of 59–62% starting from resorcinol (**76**) and *p*-toluenesulfonyl chloride (**77**) [73].

Selected physical chemical, toxicological, and environmental properties of sulcotrione are listed in Table 4.3.1, column 1.

Mesotrione (**18**) is a second-generation triketone product developed by Zeneca (now Syngenta) as CALLISTO® for pre-emergence and post-emergence control of all the important broad-leaved weeds in corn together with some of the annual grass weeds (*Digitaria* and *Echinochloa* sp.), which are important in European corn production [1]. Typical use rates range from 100 to 225 g ha^{-1} when applied pre-emergence, and 70 to 150 g ha^{-1} for post-emergence applications. Broad-leaved weeds controlled include *Xanthium strumarium*, *Abutilon theophrasti*, *Ambrosia trifida*, together with *Chenopodium*, *Amaranthus* and *Polygonum* sp. Selectivity in corn is given by its ability to rapidly metabolize (detoxify) mesotrione into the inactive metabolites (**80**) and (**81**) (Fig. 4.3.16).

This metabolism is mediated by cytochrome P450 enzymes in both corn and weeds. In corn the detoxification process is so rapid that mesotrione is not translocated away from the directly treated area. However, the P450 enzymes in susceptible weeds can only metabolize mesotrione slowly. This allows extensive translocation throughout the weed (uptake occurs through the leaves, roots and shoots) and allows inhibition of HPPD [1]. It has also been suggested that a secondary effect contributing to corn selectivity may be the fact that foliar uptake of

Table 4.3.1 Selected physical chemical, toxicological, and environmental properties of commercial triketones.

Compound	Sulcotrione	Mesotrione	Benzobicyclon
Structure	 17	 18	 9
Major product names	MIKADO®	CALLISTO® Other Products (mixtures): CAMIX®, CALARIS®, LEXAR®, LUMAX®	SHOW-ACE® Other products (mixtures): FOCUS SHOT®, KUSAKONTO®, SMART®
Melting point (°C)	139	165	187.3
Vapor pressure (mPa)	5×10^{-3} (25 °C)	5.69×10^{-3} (20 °C)	$<5.6 \times 10^{-2}$ (25 °C)
K_{ow} log P	<0	0.11	3.1
Solubility in water (mg L^{-1})	165 (25 °C)	15 (25 °C); 2.2 (20 °C) (pH 4.8)	0.00052 (20 °C)
Stability in water	Stable	Stable	Rapidly hydrolyzed
pK_a	3.13	3.12	–
Rat (acute oral) LD_{50} (mg kg^{-1})	>5000	>5000	>5000
Birds dietary LC_{50} for bobwhite quail (mg kg^{-1})	>5620	>2000	>2250
Fish LC_{50} (96 h) for rainbow trout (mg L^{-1})	227	>120	LC_{50} (48 h) for carp > 10 ppm
Algae	EC_{50} (96 h) for *Selenastrum capricornutum* 1.2 mg L^{-1}	EC_{50} (72 h) for *S. capricornutum* 4.5 mg L^{-1}	EC_{50} (72 h) for *S. capricornutum* > 1 ppm
Bees LD_{50} (oral and contact) (μg per bee)	>200	>9	>200
K_{oc}	44 (high pH) to 940 (low pH)	19 (pH 7.7) to 387 (soil pH 4.6)	
Soil deg DT_{50} (days)	1–11	4–31.5	

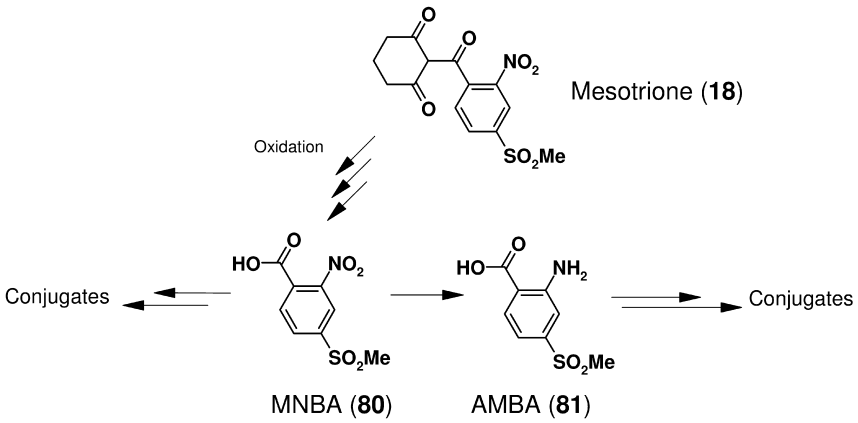
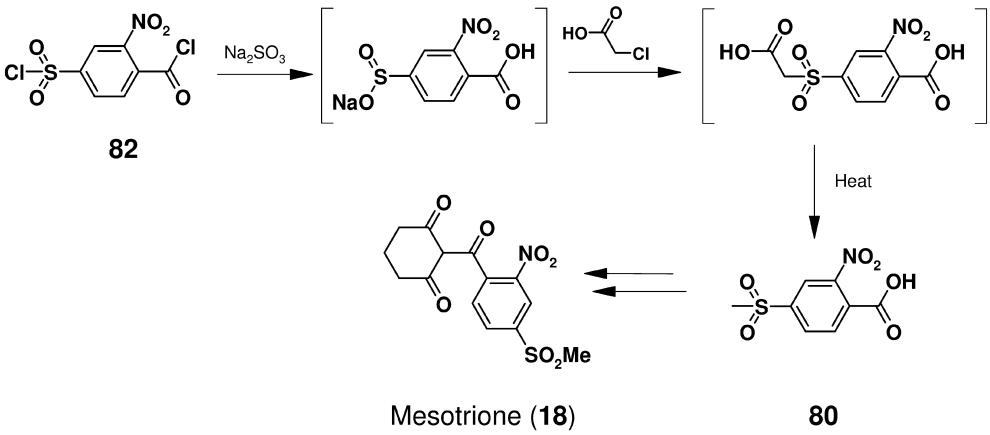


Fig. 4.3.16. Mesotrione *in planta* metabolism.

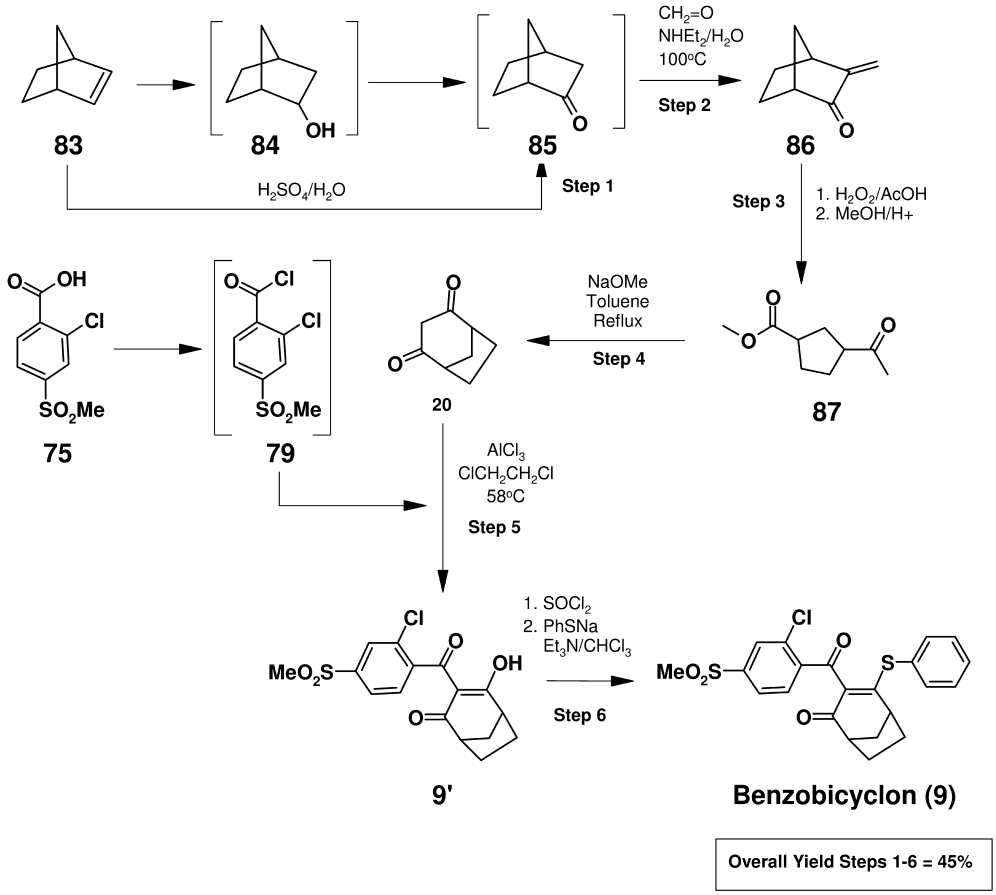
mesotrione is slower for corn than for weed species. Recent studies suggest the selectivity of sulcotrione may also be rationalized by similar arguments [74]. In extensive field tests in the USA and Europe, no corn injury has been observed with pre-emergence applications, and injury averages $\leq$ 3% for post-emergence applications. In contrast, soybean is extremely sensitive, developing bleaching symptoms when treated with mesotrione post- emergence at application rates as low as 4 g ha^{-1}. Nevertheless, there is no risk of carry-over in rotational soybean crops due to the rapid degradation of mesotrione in soils. Mesotrione is also sold in mixtures with other herbicides to complete its spectrum (notable gaps are pre-emergent grass control and *Setaria* sp. in general). Some important brand products are LEXAR® and LUMAX® (various mixtures of mesotrione, S-metolachlor and atrazine, or alternatively terbuthylazine in countries/regions where atrazine use is prohibited). These mixtures are used as a pre-emergence broad spectrum weed control product in corn (one-shot treatment). The products have a high S-metolachlor content for areas where *Setaria* species are a major problem. Some formulations of LUMAX® also contain the S-metolachlor safener benoxacor. CAMIX® (mesotrione plus S-metolachlor) is a product that has been developed to give broad spectrum pre-emergence control of broadleaf and grass weeds in corn, where triazine herbicides are not permitted or desired, and CALARIS® (mesotrione plus terbuthylazine) is used as an early post-emergence weed control herbicide in corn (dicots and some grass weeds) for countries where atrazine use is forbidden. Mixtures with inhibitors of photosystem II such as atrazine and terbuthylazine are truly synergistic [75], which is a consequence of the complementary mode of action of triketones and PS-II inhibitors. Mesotrione has been a major success since its introduction in 2001 into the USA. Sales of mesotrione-based products have steadily increased ($270 million in 2004) [70], and it is now also a major product in Europe. Mesotrione can be synthesized similarly to the synthesis shown for sulcotrione. Scheme 4.3.8 shows a possible technical synthesis of the required benzoic acid (**80**) starting from (**82**) [76, 77].



Scheme 4.3.8 Synthesis of mesotrione.

Selected physical chemical, toxicological, and environmental properties of mesotrione are listed in Table 4.3.1, column 2.

Benzobicyclon (**9**, SHOW-ACE®, Table 4.3.1, column 3) is a new triketone niche product herbicide that has been developed for control of broadleaf weeds (especially sulfonyl urea resistant weeds, e.g., *Lind* sp., *Lindernia attenuata*, *Monochoria vaginalis*) and some important grasses [e.g., *Scirpus juncoides* (sulfonyl urea resistant), *Echinochloa oryzicola* and other *Echinochloa* sp.] in paddy rice [78]. Inspection of the structure shows that it contains the sulcotrione acid moiety combined with a bicyclo[3.2.1]octane-2,4-dione, which in turn has been further elaborated to a pro-herbicide (attachment of a hydrolytically labile phenyl sulfide group to the vinylogous acid hydroxyl moiety). The latter imparts some positional selectivity to rice by decreasing the water solubility of the molecule. The phenylsulfide moiety slowly hydrolyses in water, or is metabolized in the plant to generate the active principle (**9′**, see Fig. 4.3.2). Benzobicyclon is reported to be very selective in rice and environmentally friendly due to its low water solubility (3000× less than sulcotrione) and low fish toxicity [LD_{50} (48 h), Carp > 10 ppm] [78]. Low water solubility is important for paddy rice herbicides as herbicide-containing water flowing out of the paddy field is minimized. Benzobicyclon arose out of a joint venture with SDS Biotech and Sandoz Crop Protection (now Syngenta). The presence of Sandoz's bicyclo[3.2.1]octane-2,4-dione moiety (BIOD, **20**, Scheme 4.3.9) posed a major problem for the development of this compound as the initial synthesis of benzobicyclon was cost prohibitive, particularly the synthesis of BIOD. However, after extensive process work, chemists at SDS were able to reduce the synthesis of BIOD to four steps [78, 79]. Another critical breakthrough in the technical synthesis was the finding that aluminum trichloride mediated C-acylation could be achieved in high yield directly from BIOD and the acid chloride (**79**), as the commercial use of cyanide catalyzed O-



Scheme 4.3.9 Synthesis of benzobicyclon.

to C-acylation was prohibited by competitor patents [16]. Scheme 4.3.9 shows the industrial synthesis of benzobicyclon [78, 79].

Selected physical chemical, toxicological, and environmental properties of benzobicyclon are listed in Table 4.3.1, column 3.

4.3.8 Summary

Since their discovery in the early 1980s, the triketone herbicides have been extensively studied over the last two and half decades. In view of this, it may surprise the reader that only three commercial products have appeared to date. However, as has been described, other triketone products are due to appear on the market (e.g., tembotrione), and related compounds with this mode of action (see Chapter

4.4) are likely to play an important role in weed control over the coming years, as there is to date no known case of resistance to HPPD inhibitors.

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4.4
Hydroxyphenylpyruvate Dioxygenase (HPPD) Inhibitors: Heterocycles

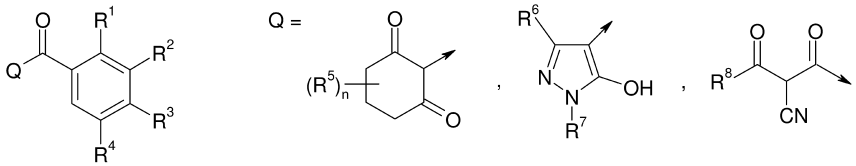
Andreas van Almsick

4.4.1
Introduction

As already mentioned in Chapter 4.2, all known HPPD inhibitors are chelating agents. To exhibit not only *in vitro* but also *in vivo* activity additional requirements such as uptake, transport and metabolic stability in plants (especially weeds) are necessary. The market compounds and the long list of published HPPD molecules with the general structure **1** (Fig. 4.4.1) fulfill, normally, all these needs [1].

There are many Q moieties but 1,3-cylohexanediones, pyrazolones and diketonitriles are the most important examples. It is essential to know that all compounds of the general structure **1** could exist in different tautomeric forms, as shown in Fig. 4.4.2 for Q = 1,3-cyclohexanedione.

Regarding the substitution pattern of **1**, the 2,4-disubstitution and the 2,3,4-trisubstitutions are particularly important (see Edmunds, Chapter 4.3, Section 4.3.5). *In vitro* activity is strongly connected with a substitution in 2-position ($R^1 \neq H$).



1

R^{1-4} = H, alkyl, haloalkyl, halogen, etc.
R^{5-6} = H, alkyl, etc.
R^{7} = alkyl
R^{8} = alkyl, cycloalkyl

Fig. 4.4.1. Markush structure of many HPPD inhibitors.

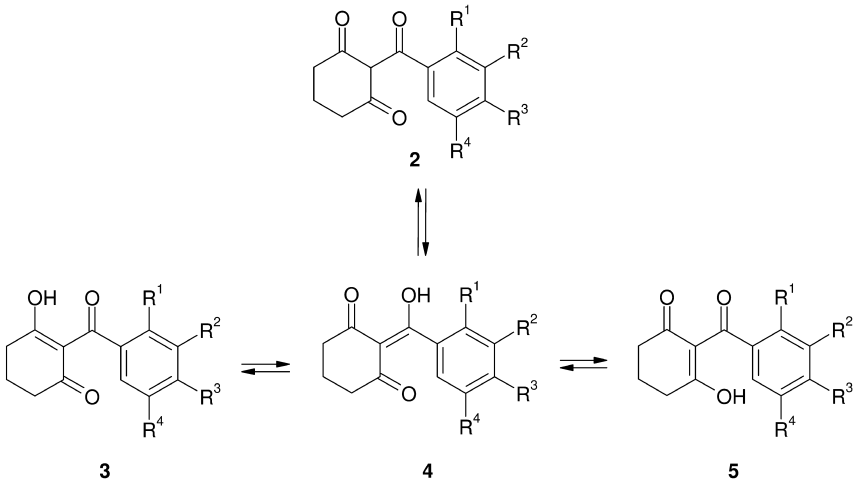
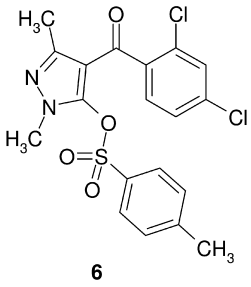


Fig. 4.4.2. Tautomeric forms of HPPD inhibitors of type 1,3-cyclohexanedione.



pyrazolynate

Fig. 4.4.3. Structure of pyrazolynate (**6**).

Whilst compounds of the general structure **1** are active *in vitro* and, therefore, drugs, for every Q different prodrugs are also known (see Edmunds, Chapter 4.3, Fig. 4.3.2).

For simplification, the HPPD inhibitors with Q different from 1,3-cyclohexanedione and its prodrugs are summarized here as heterocycles; and it was the heterocyclic HPPD-inhibitor pyrazolynate (**6**, Fig. 4.4.3) that was the first HPPD product launched into the market.

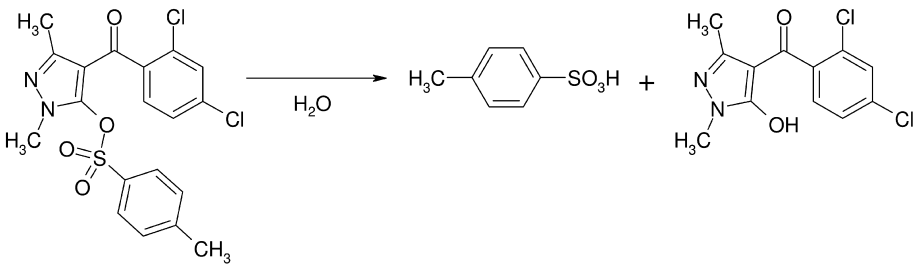
4.4.2 Market Products

4.4.2.1 Pyrazolynate (Pyrazolate)

When pyrazolynate (pyrazolate) (**6**, Fig. 4.4.3) was launched in 1980 by Sankyo Co., Ltd in Japan the world's first HPPD compound entered the herbicide market even though at that time the target site was unknown. Two years earlier, Sankyo had presented its activity on this area at the Fourth International Congress of Pesticide Chemistry in Zurich, Switzerland [2] but had already patented the main compounds in 1974 [3]. Interestingly, this all happened without the knowledge of the precise mode of action. Pyrazolynate and two analogues were previously classified as Protox inhibitors [4]. Pyrazolynate is not new and modern but it is included in this review as it is relatively unknown outside of Japan.

The herbicide with the trade name Sanbird® is able to control both annual and perennial weeds in paddy fields [5] with application rates of 3–4 kg ha^{-1}. As a very selective herbicide in rice it was a good innovation for the Japanese rice market. This product reached peak sales in Japan in 1986 with 650 000 ha (28.6% market share) [6]. This declined with the introduction of sulfonylurea herbicides such as bensulfuron-methyl in the year 1987. In 2005 Sanbird® was only used on 101 200 ha (5.9% market share) in Japan [7].

Pyrazolynate is a prodrug and itself not herbicidally active. It has low solubility in water [0.056 mg L^{-1} (25 °C)] and in solution it is hydrolyzed to give *p*-toluenesulfonic acid (**7**) and 4-(2,4-dichlorobenzoyl)-1,3-dimethyl-5-hydroxypyrazole (**8**), the herbicidal entity of pyrazolate [8–10] (Scheme 4.4.1).

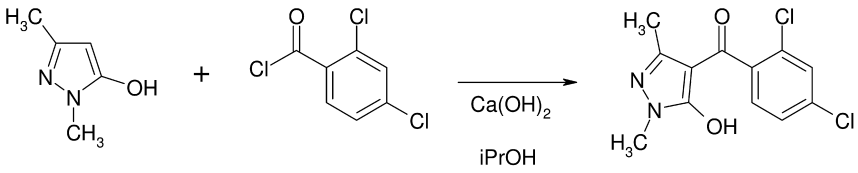


6 **7** **8**

Scheme 4.4.1

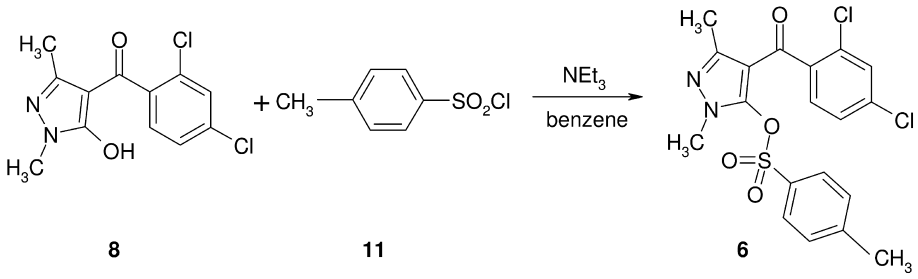
The half-lives of pyrazolynate in water at 25 °C are: 52.7 h at pH 3; 17.5 h at pH 1; 25.0 h at pH 7; and 4.3 h at pH 9 [11]. In soil a DT_{50} of 8–10 days is observed [12].

Schemes 4.4.2 and 4.4.3 show the synthesis of pyrazolynate. 1,3-Dimethyl-5-pyrazolon (**9**) and 2,4-dichlorobenzoyl chloride (**10**) react in the presence of calcium hydroxide in isopropanol to give 4-(2,4-dichlorobenzoyl)-1,3-dimethyl-5-hydroxypyrazole (**8**) [3].



9 **10** **8**

Scheme 4.4.2



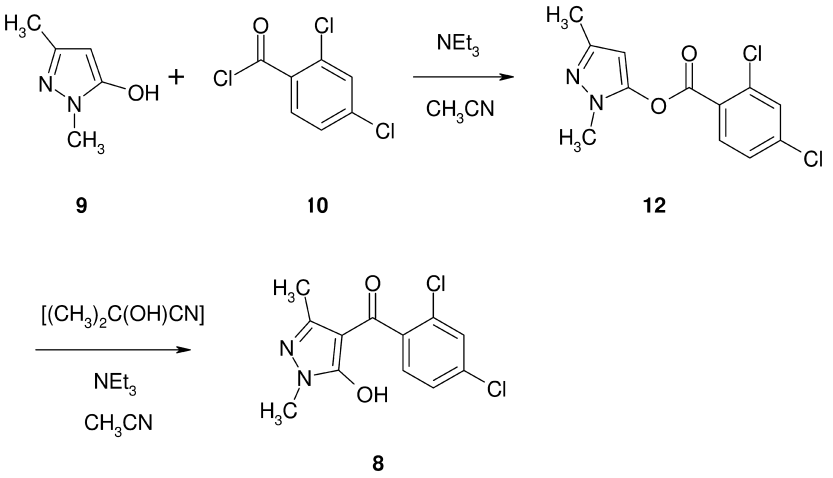
8 **11** **6**

Scheme 4.4.3

4-Methylbenzenesulfonyl chloride (**11**) is then added to a solution of **8** and triethylamine in benzene [3].

Two points should be highlighted. Firstly, today instead of benzene other solvents such as toluene are used and, secondly, for the formation of substituted 4-benzoyl-1-alkyl-5-hydroxyxpyrazole like **8** other routes are also known. Scheme 4.4.4 shows the most popular one, with **8** as an example [13].

Both, 1,3-dimethyl-5-pyrazolon (**9**) and 2,4-dichlorobenzoic acid are commercial available, which allows a few steps synthesis of pyrazolynate. However, owing to the high application rate of 3–4 kg ha^{-1}, the treatment costs are very high. In theory the application rate could be lower by using the drug 4-(2,4-dichlorobenzoyl)-1,3-dimethyl-5-hydroxypyrazole (**8**) instead of the prodrug. Another important factor for the Japanese rice market is season-long weed control of a herbicide, which is not possible with the more polar and more water-soluble



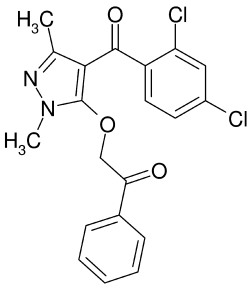
Scheme 4.4.4

drug **8** but is with the prodrug pyrazolynate. The effect is similar to that of a slow release formulation, the active ingredient is released over a long period of time and is, therefore, present at lethal dose rates for the weeds for a longer period of time.

4.4.2.2 **Pyrazoxyfen**

Pyrazoxyfen (**13**, Fig. 4.4.4) is a very close analogue of pyrazolynate and was lunched by Ishihara Sangyo Kaisha Ltd in 1985 for the Japanese rice market.

The herbicide was patented in 1977 [15] and reported in 1984 by F. Kimura [14]. The trade name is Paicer® and the herbicide has a broad weed control spec-



13

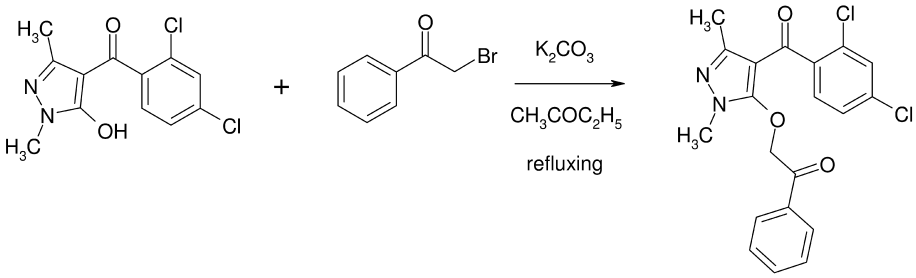
pyrazoxyfen

Fig. 4.4.4. Structure of pyrazoxyfen (**13**).

trum under flooded field condition, including for many annual and perennial weeds, with application rates of 3 kg ha^{-1}. It is very selective to transplanted rice and also to direct-seeded rice at temperatures < 35 °C. At higher temperatures temporary crop damage may occur [14].

Paicer® reached peak sales in Japan in 1988 with 45 000 ha (2.2% market share) [6]. In 2005 the product was only used on 6911 ha (0.4% market share) in Japan [7]. As the second product to reach the Japanese market for the same segment as pyrazoynate and with the same mode of action, Paicer® was and remains much less successful.

To synthesize pyrazoxyfen (**13**), 2-bromoacetophenone **14** is added to a solution of **8** and anhydrous potassium carbonate in methyl ethyl ketone (Scheme 4.4.5) [15].



8 **14** **13**

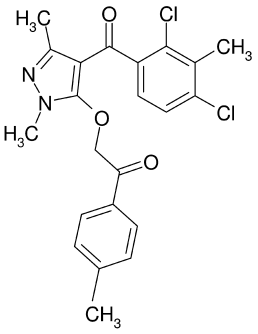
Scheme 4.4.5

The difference between pyrazoxyfen and pyrazolynate is only the chosen prodrug system. In plants, both herbicides were metabolized to 4-(2,4-dichlorobenzoyl)-1,3-dimethyl-5-hydroxypyrazole (**8**). Pyrazolynate is only slightly soluble in water, but, once dissolved, is rapidly hydrolyzed to the herbicidally active metabolite [11]. In contrast, pyrazoxyfen shows considerable stability in aqueous solutions [16].

4.4.2.3 **Benzofenap**

As a third compound of this series benzofenap, (**15**, Fig. 4.4.5) was lunched by Mitsubishi Petrochemical Co. Ltd. (now Mitsubishi Chemical Corp.) and commercialized by Rhône-Poulenc Yuka Agro KK, a joint venture of Mitsubishi Chemical Corp. and Rhône-Poulenc Agro (now part of Bayer CropScience) in 1987 for the rice market. Interestingly, benzofenap is not only applied in Japan as Yukawide® but also in Australia as Taipan®. The new herbicide was patented 1982 [17] and reported 1991 [18].

Yakawide® reached peak sales 1998 in Japan with 180 000 ha (10% market share) [6]. In 2005 the product was only used on 62 000 ha (3.6% market share)



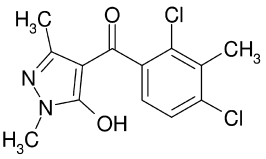
15

benzofenap

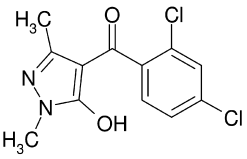
Fig. 4.4.5. Structure of benzofenap (**15**).

in Japan [7]. This third product for the Japanese rice market with HPPD mode of action Yakawide® was much more successful than its closest analogue Paicer®.

The differences between benzofenap and pyrazoxyfen are the additional methyl groups on the biologically active metabolite 4-(2,4-dichloro-3-methylbenzoyl)-1,3-dimethyl-5-hydroxypyrazole (**16**) (Fig. 4.4.6) and the prodrug moiety 4′-methylacetophenone.



16



8

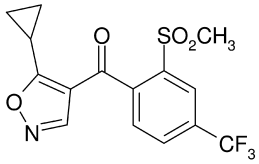
Fig. 4.4.6. Biologically active metabolites, **16** and **8**, of benzofenap and pyrazoxyfen, respectively.

These result in a different environmental behavior and different herbicidal activity [14, 18]. The half-lives in paddy field soil rose from 4 to 15 days for pyrazoxyfen and to 38 days for benzofenap. The application rate of 3 kg ha^{-1} is as high as for both the other rice herbicides but benzofenap allows a longer weed control of up to 50 days compared with 21–35 days with pyrazoxyfen. Importantly, benzofenap is a more crop selective herbicide. Another advantage of benzofenap over pyrazoxyfen is that it is not temperature-dependent. Even at higher temperatures no phytotoxicity is observed.

None of the three HPPD rice herbicides are able to control all annual and perennial weeds in rice, thus they need mixture partners, especially to fill gaps such as barnyard grasses or *Cyperus* spp. Common mixture partners are butachlor, pretilachlor, thiobencarb [19], piperophos [14], pyribaticarb and bromobutide [18].

4.4.2.4 **Isoxaflutole**

With the introduction of isoxaflutole (IFT) (**17**, Fig. 4.4.7) new crops, corn and sugarcane, came in the focus of HPPD inhibitors of type heterocycles. IFT is not the first HPPD compound for corn, this was sulcotrione in 1990, but it was the first for pre-emergence application. Reported 1995 by Luscombe et al. [20] the compound had been initially patented 1991 [21] by Rhône-Poulenc Agriculture Limited (now Bayer CropScience).



17

isoxaflutole

Fig. 4.4.7. Structure of isoxaflutole (**17**).

The herbicide with the trade names Merlin®, Balance®, Provence® and others was first launched 1996 in South America for broadleaf weed and grass control in corn and sugar cane. In corn, IFT is a selective pre-emergence herbicide. Applications are usually made in spring in post sowing/pre-emergence of the crop, but it is also possible to apply isoxaflutole in early pre-plant up to 3 weeks before the planting of the crop. The application rate of 75 g ha^{-1} is very low compared with other conventional pre-emergence herbicides for corn (e.g., S-metolachlor 0.8–1.6 kg ha^{-1}).

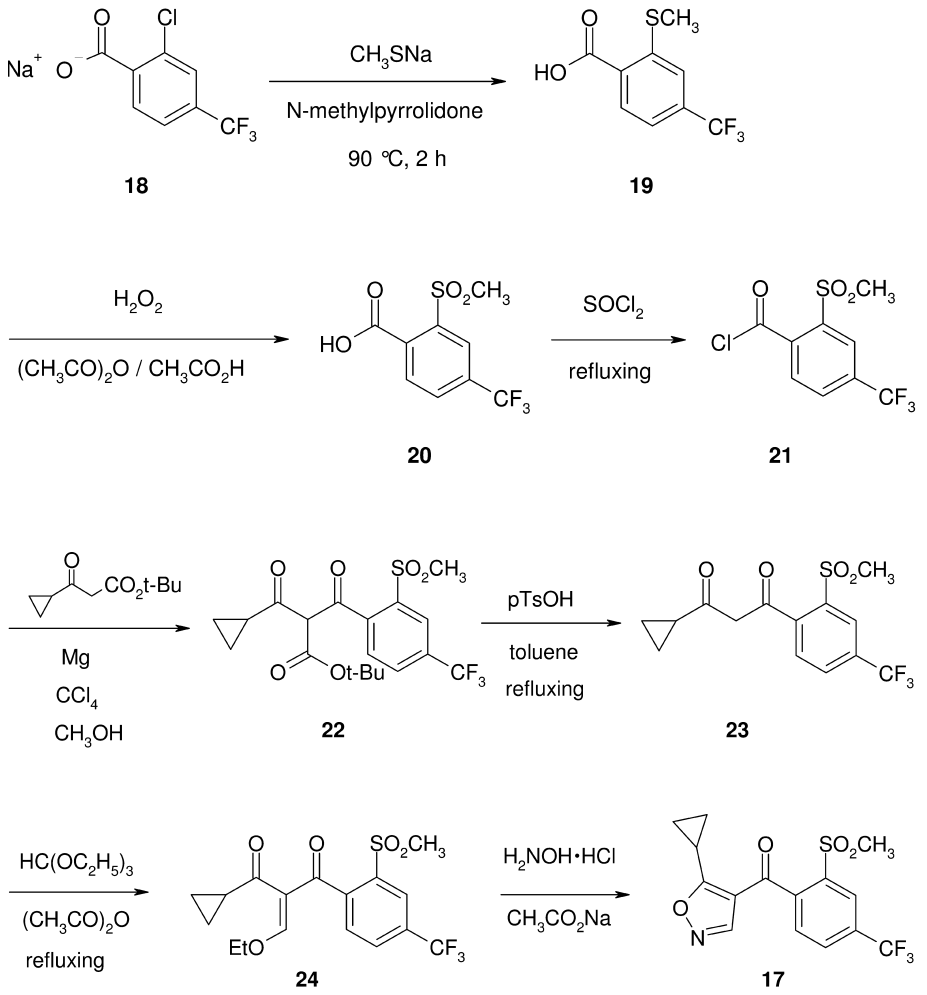
Common mixture partners in corn are flufenacet, aclonifen, terbuthylazine and, especially, atrazine to complete the weed spectrum.

In sugar cane, isoxaflutole controls annual grasses and some key annual broadleaf weeds. It may be applied pre- or post-emergence but normally pre-emergence is the preferred option. The application rate of 140 g ha^{-1} is still very low compared with other pre-emergence products [22, 23].

In sugarcane IFT may be tank-mixed with paraquat formulations, diuron, atrazine and Actril® DS.

In some countries isoxaflutole is also registered for weed control in other crops such as chick peas, poppy seed and some nurseries.

IFT is a much more complex compound than the three previously described rice compounds and needs, therefore, a longer synthesis route [21, 24]

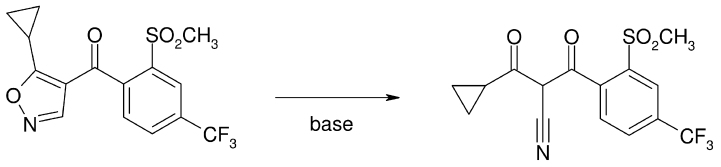


Scheme 4.4.6 Synthesis of isoxaflutole.

(Scheme 4.4.6). One possible educt is 2-chloro-4-trifluoromethylbenzoic acid sodium salt (**18**) to get 2-methylthio-4-trifluoromethylbenzoic acid (**19**). Further treatment with hydrogen peroxide and acetic anhydride in acetic acid yields 2-methylsulfonyl-4-trifluoromethylbenzoic acid (**20**). With thionyl chloride the corresponding benzoyl chloride **21** is available, which will be transformed into *t*-butyl 2-(2-methylsulfonyl-4-trifluoromethylbenzoyl)-3-cyclopropyl-3-oxopropionate (**22**) via the magnesium enolate of *t*-butyl 3-cyclopropyl-3-oxopropionate in methanol. To remove the *t*-butyl carboxylate group, **22** is refluxed in toluene in the presence of toluenesulfonic acid. The so-formed 1-(2-methylsulfonyl-4-trifluoromethylphenyl)-3-cyclopropylpropan-1,3-dione (**23**) is used to obtain 1-(2-methylsulfonyl-4-trifluoromethylphenyl)-3-cyclopropyl-2-ethoxymethylenepropan-

1,3-dione (**24**) in a mixture of triethylorthoformate and acetic anhydride. Finally, the addition of sodium acetate and hydroxylamine hydrochloride yields IFT (**17**).

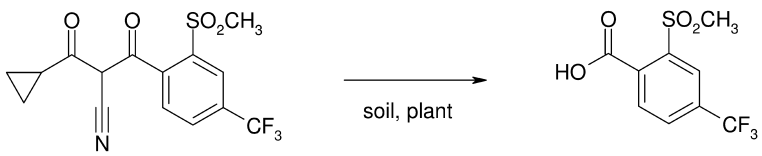
As already mentioned, isoxazoles such as IFT are prodrugs and are not sufficiently persistent in plants to inhibit the HPPD enzyme. It is the first metabolite of isoxaflutole, the so-called DKN (diketonitrile) 3-cyclopropyl-2-[2-(methylsulfonyl)-4-(trifluoromethyl)benzoyl]-3-oxopropanenitrile (**25**) that is the herbicidally active entity. In soil, and also in plants, IFT undergoes rapid conversion into DKN [25]. In aqueous solutions there is an influence of temperature and pH on the chemical hydrolysis of IFT to DKN. The hydrolysis increases with increasing pH and temperature: for 295 K and pH 9.3 the rate of degradation was 100-fold faster than at pH 3.8. (Scheme 4.4.7) [26]. The DT_{50} in water is 11 days at pH 5, 20 h at pH 7 and 3 h at pH 9 [22].



17 **25**

Scheme 4.4.7

The DT_{50} of IFT in soil is also very low and in the range of 12 h to 3 days under laboratory conditions. This is, however, once more dependant on several factors such as temperature, pH, moisture and soil type [27]. Moreover, the half-life of IFT in aqueous sterile solutions is higher than in soil at the same temperature and pH and confirms the catalytic effect of the soil reported by Taylor-Lovell et al. [28]. Used under normal agricultural conditions the rate of DKN formation will be affected by the quantity and frequency of rainfall. The log *P* of IFT is 2.19 and the water solubility is 6.2 mg L^{-1} compared with values for DKN of 0.4 and 326 mg L^{-1}, respectively. These properties restrict the mobility of IFT, which is retained at the soil surface, where it can be taken up by surface-germinating weed seeds. DKN, which has a laboratory DT_{50} of 20–30 days, is more mobile



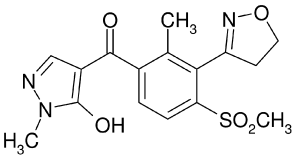
25 **26**

Scheme 4.4.8

and is taken up by the roots. In addition to influencing the soil behavior of IFT and DKN, the greater lipophilicity of IFT leads to greater uptake by seed, shoot and root tissues. In both plants and soil, DKN is converted into the herbicidally inactive benzoic acid **26** (Scheme 4.4.8). This degradation is more rapid in corn than in susceptible weed species and this contributes to the mechanism of selectivity, together with the deeper sowing depth of the crop [27].

4.4.2.5 **Topramezone**

The launch of topramezone (**27**, Fig. 4.4.8) for the post application corn market was for 2006 under the trade names Impact® in USA and Canada and Clio® in Germany and Austria. The compound is based on a BASF patent from 1995 [29].



27

topramezone

Fig. 4.4.8. Structure of topramezone (**27**).

In 2005 BASF granted rights to develop, register and commercialize topramezone in North America to Amvac Chemical, whilst rights in Japan have been granted to Nippon Soda. The new corn compound will only be marketed in Latin America and Europe [30] by BASF.

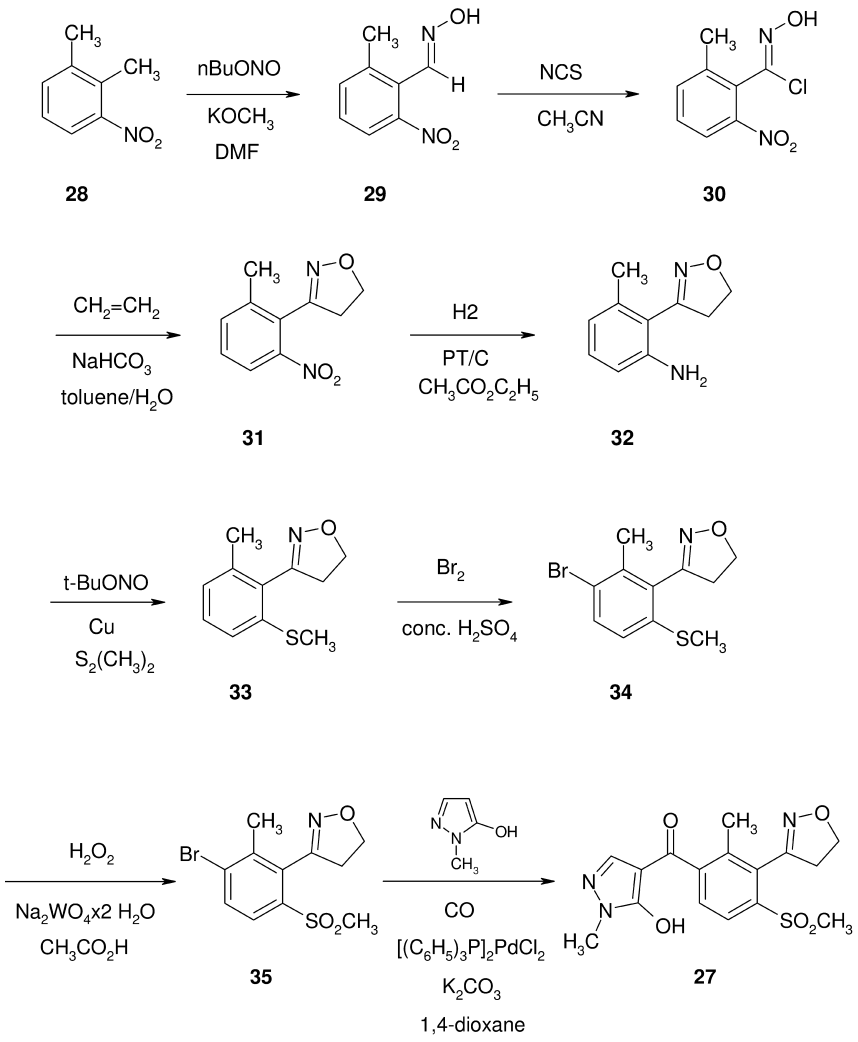
Topramezone is aimed at the post-emergence control of major grass and broadleaf weeds in corn crops worldwide. This means that this new corn compound differentiates itself from sulcotrione and mesotrione in that it shows real cross spectrum activity like isoxaflutole and it is not limited to mainly broadleaf weed control.

Clio® is a 336 g L^{-1} SC-formulation with recommended application rates of 50–75 g ha^{-1} topramezone [32]. Like IFT for pre-application topramezone defines a new level of biological activity for HPPD compounds in post-application.

Topramezone is, like pyrazolynate, pyrazoxyfen and benzofenap, a pyrazolone but without a protective group and is therefore not a prodrug. Also noticeable is the 4,5-dihydroisoxazol group in 3-position of the benzoyl moiety.

Different synthesis routes have been published [29, 31]. Scheme 4.4.9 shows only one of them.

Starting with 3-nitro-*o*-xylene (**28**), 3-(2-methyl-6-nitrophenyl)-4,5-dihydroisoxazole (**31**) is synthesized via the benzaldehyde oxime **29**. Subsequent reduction of the nitro group, replacement of the corresponding amino group by methyl

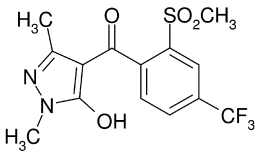


Scheme 4.4.9 Synthesis of topramezone.

sulfide, bromination to 3-[3-bromo-2-methyl-6-(methylthio)phenyl]-4,5-dihydroisoxazole (**34**), and then oxidation affords the sulfone **35**. Finally, topramezone (**27**) is available by conversion of **35** with 1-methyl-5-hydroxypyrazol in the presence of carbon monoxide and a suitable palladium catalyst, an alternative process to those described in Schemes 4.4.2 and 4.4.4.

4.4.2.6 **Pyrasulfotole**

During the Analyst & Investor Days in Lyon on September 5–6, 2005 Bayer CropScience announced the development of a new pyrazolone called pyrasulfo-



36

pyrasulfotole

Fig. 4.4.9. Structure of pyrasulfotole (**36**).

tole (**36**, Fig. 4.4.9) for the cereals market. The compound had been initially patented 2000 [33] by Aventis CropScience (now Bayer CropScience).

Pyrasulfotole would be the first HPPD compound for cereals and therefore a new mode of action for this crop. It is described as an innovative tool for resistance management with excellent broad-spectrum activity and excellent crop compatibility due to combination with proprietary safener technology [34]. Application rates and environmental behavior are so far not reported.

Interestingly, pyrasulfotole uses the same benzoic acid **20** like IFT and the also the well-known 1,3-dimethyl-5-pyrazolon (**9**) and, like topramezone, it is not a prodrug.

4.4.3 Conclusion

HPPD inhibitors of the heterocycle type are represented in rice, corn, sugar cane and, in future, also in the cereals market. Even if the three rice compounds have passed their commercial peak, HPPD inhibitors are very successful, especially in corn.

Interestingly, most of the compounds described here are prodrugs, but this does not mean that it is a prerequisite for this type of HPPD inhibitor, as can be seen with topramezone and pyrasulfotole. Moreover, the prodrug concepts used are chemically quite different. Whereas the three rice compounds leave their prodrug moiety as waste in the environment, IFT undergoes a conversion by opening its isoxazole ring without changing its molecular mass. All these HPPD inhibitors share the feature that they have a relatively higher log *P* and, therefore, lower water solubility and are metabolized to an active metabolite with a much lower log *P* and higher water solubility.

Other more important differences are the application rates. The three rice compounds are used on the kg scale whereas IFT and topramezone are used about 100 g ha^{-1} and lower.

Chemically, the compounds described are quite similar, with the exception of topramezone. The substitution patterns of the benzoyl moieties bare resemlance, even though there is of course a big difference between Cl, CH_3, CF_3 or SO_2CH_3.

Benzofenap and, in particular, topramezone also show that in 3-position a substitution is allowed and obviously important for good biological activity. Also important are the different Q moieties, but, as already mentioned, both pyrazolone and diketonitrile are chelating agents for Fe(II).

It is also fascinating to see the quite different biological activity already achieved with the shown variations. The future will show what will be at the end of the HPPD story regarding crop, application rate and profile.

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5
Safener for Herbicides

Chris Rosinger and Helmut Köcher

5.1
Introduction

Herbicide safeners (also referred to as herbicide antidotes or protectants) fulfill an important role in crop protection. Safeners are chemicals that protect crop plants from unacceptable injury caused by herbicides. Either by placement on the crop seed or by way of a physiological selectivity mechanism, safeners in commercial use do not negatively impact the weed control of the herbicide. Although many herbicides have been developed for use without a safener, some of the strongest and most broad-spectrum herbicides tend towards border-line crop selectivity, which may completely preclude use in a particular crop or at least limit maximum use rates or the crop varieties that can be safely treated. It is for such situations that safeners have been developed. Several books and reviews of safeners have been written over the past 20 years [1–3]. It is not the intention of this chapter to cover in detail older safeners, but rather to focus on more recently developed commercial safeners as well as some of the older compounds still in wide commercial usage.

The story of herbicide safeners began in 1947 with an accidental observation by Otto Hoffmann, a researcher in the Gulf Oil Company. On entering his greenhouse on a hot summer afternoon he saw that tomato plants had suffered injury that he presumed was from 2,4-D vapor drift. However, plants treated with 2,4,6-trichlorophenoxyacetic acid showed no symptoms of this injury [4]. Hoffmann recognized the potential use of such an effect and started research into compounds that could protect crops from herbicide injury.

A fundamental problem for safener discovery and development is to find safeners that do not also antagonize weed control. The fruits of Gulf Oil Company research (reported by Hoffmann in 1969) was 1,8-naphthalic anhydride (NA), which works best as a seed treatment, whereby antagonism of weed control is not an issue. To the authors' knowledge just over a dozen further safeners have been commercialized in the years since NA was introduced, although several of the early safeners have since been superseded and/or withdrawn. This subse-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

quent period of safener commercialization may be informally split into three phases; the first, mainly seed treatment safeners; the second, pre-emergence tank mix safeners, and the third post-emergence tank mix safeners. Table 5.1 shows the chemical structures and usage of these safeners. In all these cases the crops are monocotyledons (maize, sorghum, rice, and cereals such as wheat and barley). Figure 5.1 shows the effect of one of them, mefenpyr-diethyl. To date no comparable safeners have been commercialized for broad-leaved crops. However, the ''extender'' dietholate is used by FMC to help protect cotton against the herbicide clomazone (US patent application 20050009702). In addition, several compounds (daimuron, cumyluron and dimepiperate) generally considered herbicidal are included in some products principally because they reduce crop injury from another herbicidal component. These are of relevance, particularly in rice and the structures are also shown in Table 5.1. Because they are relatively old compounds they will not be covered here.

To ensure maximum crop safety, safeners that are applied in mixture with the herbicides need to act quicker than the herbicide injury develops. The mechanism of action of safeners has received much scientific attention and will be dealt with in some detail in this chapter (Section 5.3).

Safeners, like pesticides, must be registered before use. However, the regulatory situation for safeners is complex, in particular when considered on a global basis. For example, whereas several European countries require for safeners full data packages like those for pesticides, safeners do not fall under Annex I of the European Union pesticide directive 91/414. In the USA safeners are treated under inert legislation as opposed to pesticide legislation. However, full data sets (like those for active ingredients) are actually required for evaluation by the environmental protection agency (EPA) to establish a residue limit for the federal food, drug and cosmetic act. In Canada and Australia, safeners are now treated legally as pesticides. In other parts of the world safeners are legally treated as formulation additives.

Fig. 5.1. Post-emergence safening of wheat by mefenpyr-diethyl against mesosulfuron-methyl. (A) Untreated; (B) mesosulfuron-methyl at 60 g-a.i. ha^{-1}; and (C) mesosulfuron at 60 g-a.i. ha^{-1} plus mefenpyr-diethyl at 30 g-a.i. ha^{-1}.

Table 5.1 Structures of commercial safeners.

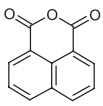
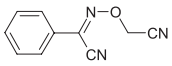
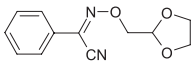
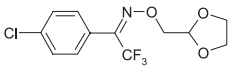
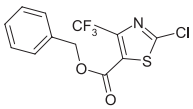
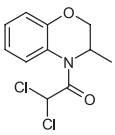
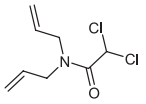
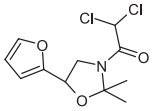
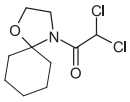
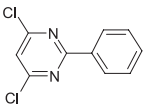
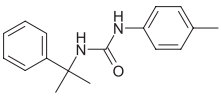
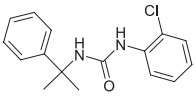
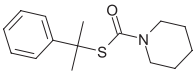
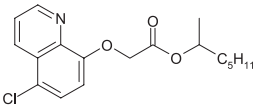
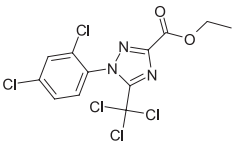
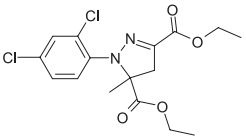
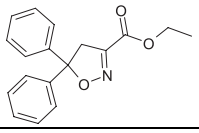
Common name (development codes)	Chemical structure	Application/Crop/Herbicides
1,8-Naphthalic anhydride (NA) Protect®		Seed treatment Maize Thiocarbamates
Cyometrinil (CGA43089) Concep I®		Seed treatment Sorghum Thiocarbamates/chloroacetamides
Oxabetrinil (CGA92194) Concep II® Superseded Concep I®		Seed treatment Sorghum Thiocarbamates/chloroacetamides
Fluxofenim (CGA133205) Concep III® Superseded Concep II®		Seed treatment Sorghum Thiocarbamates/chloroacetamides
Flurazole (MON4606) Screen®		Seed treatment Sorghum Chloroacetamides
Benoxacor (CGA154281)		Spray Pre, PPI Maize Chloroacetamides
Dichlormid (R25788)		Spray Pre, PPI Maize Thiocarbamates/Chloroacetamides
Furilazole (MON13900)		Spray pre-emergence Maize Chloroacetamides (Acetochlor)
AD-67 MON4660		Spray pre-emergence Maize Chloroacetamides (Acetochlor)

Table 5.1 *(continued)*

Common name (development codes)	Chemical structure	Application/Crop/Herbicides
Fenclorim (CGA123407)		Spray pre-emergence Rice Pretilachlor
Daimuron (K223, SK-23)		Water application post-emergence Rice Sulfonylureas
Cumyluron (JC-940)		Water application post-emergence Rice Sulfonylureas
Dimepiperate (MY-93)		Water application post-emergence Rice Sulfonylureas
Cloquintocet-mexyl (CGA185072)		Spray post-emergence Cereals Clodinafop-propargyl
Fenchlorazole-ethyl (AE F070542)		Spray post-emergence Cereals Fenoxaprop-ethyl
Mefenpyr-diethyl (AE F107892)		Spray post-emergence Cereals ACCase and sulfonylureas
Isoxadifen-ethyl (AE F122006)		Spray post-emergence Maize/rice ACCase and sulfonylureas

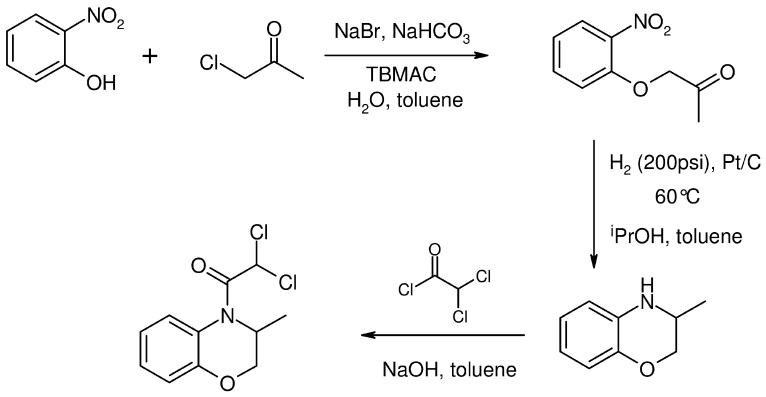
5.2
Overview of Selected Safeners

5.2.1
Dichloroacetamide Safeners

This class contains several important commercial safeners as well as a range that were reported (and patented) but not launched. They are all of relatively low molecular weight (MW $\leq$ 300) with the -N-C(O)-$CHCl_2$ substituent in common. Although, the first member of this class was commercialized in the early 1970s, three compounds are still of considerable commercial importance; benoxacor, dichlormid and furilazole.

5.2.1.1 **Benoxacor**

Commercially, benoxacor is one of the most important members of this class of safeners. It is included in products containing metolachlor as racemate or as the single isomer S-metolachlor (subsequently "(S-)metolachlor" indicates both are being referred to). These products are principally used in maize pre-plant, pre-plant incorporated and pre-emergence. Benoxacor was developed under the code CGA 154281 by Ciba-Geigy AG (now Syngenta) and was first reported in 1988 [5]. It was specifically claimed in the US patent US4601745 (filed 18th March 1985) but a priority date of 12th December 1983 relates back to general claims for the structure class (EP 149974). The synthesis of benoxacor, as disclosed in WO2001090088, involves a three-step process (Scheme 5.1).



Scheme 5.1. Synthesis of benoxacor.

There are now numerous products that contain benoxacor and (S-)metolachlor, with and without further herbicide components (Table 5.2). As the patents for

Table 5.2 Product examples containing benoxacor.

Herbicide(s)	Trade name examples
S-metolachlor	Dual II magnum®, Cinch®
S-metolachlor + atrazine	Bicep II magnum®, Cinch® ATZ, Cinch® ATZ lite
Metolachlor + atrazine	Stalwart® Xtra
Metolachlor	Stalwart®, Parallel® Me-Too-Lachlor II®
S-metolachlor + mesotrione	Camix®
S-metolachlor + mesotrione + atrazine	Lexar®, Lumax®

Table 5.3 Physicochemical properties of benoxacor and metolachlor.

Property	Benoxacor	Metolachlor
Log P	2.6	2.9
K_{oc}	42–176	121–309

benoxacor and metolachlor have both expired, this product list also contains several from generic producers.

The use of benoxacor with (S)-metolachlor is particularly necessary under stress conditions for maize. Injury to corn from (S)-metolachlor is greater under cool or wet soil conditions [6–8] where both the availability of the herbicide may be increased and the ability of maize to metabolize metolachlor reduced [9]. Benoxacor and metolachlor have similar chemical properties, influencing there behavior in soil, and this tends to ensure that the safener and herbicide are taken up together, hence providing safening under various weather conditions.

Products such as Dual-II-magnum® and Cinch® are also labeled for use on sorghum seed treated with fluxofenim or flurazole. This is a notable example of safeners used in sequence so as to obtain optimal crop safety. Table 5.4 indicates that benoxacor has a favorable toxicological profile.

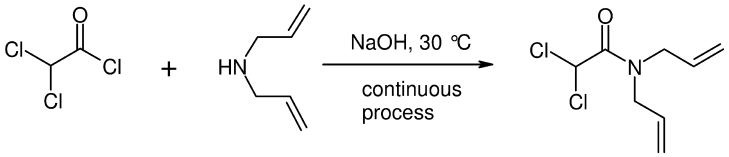
Table 5.4 Toxicological and soil degradation data for benoxacor.

Rat, oral	$LD_{50} > 5000$ mg kg^{-1}
Rat, inhalation	$LC_{50} > 2000$ mg m^{-3}
Rabbit, skin and eye irritation	Not irritant
Guinea pig, skin sensitizing	Slightly sensitizing
DT_{50} in soil	Rapid, ca. 5 days

5.2.1.2 **Dichlormid**

Of the safeners covered separately in this chapter, dichlormid is the oldest still in use. It was developed under the code number R25788 by Stauffer (now Syngenta) and first reported in 1972 [10]. It is used to safen maize against injury from acetochlor. Products include Surpass®, TopNotch®, Volley®, and Confidence®. Stalwart C® is a metolachlor product that contains dichlormid instead of benoxacor. Dichlormid is also present in several acetochlor products that also contain atrazine (e.g., Confidence Xtra®, Keystone®, Volley® ATZ).

The simple one-step synthesis of dichlormid (claimed in US 4278799) is shown in Scheme 5.2.



Scheme 5.2. One-step synthesis of dichlormid.

As described for benoxacor, dichlormid also has similar physicochemical properties to those of the herbicide components, allowing for similar plant uptake profiles for good safening potential. Further extensive coverage of dichlormid can be found in *Crop Safeners for Herbicides* [1].

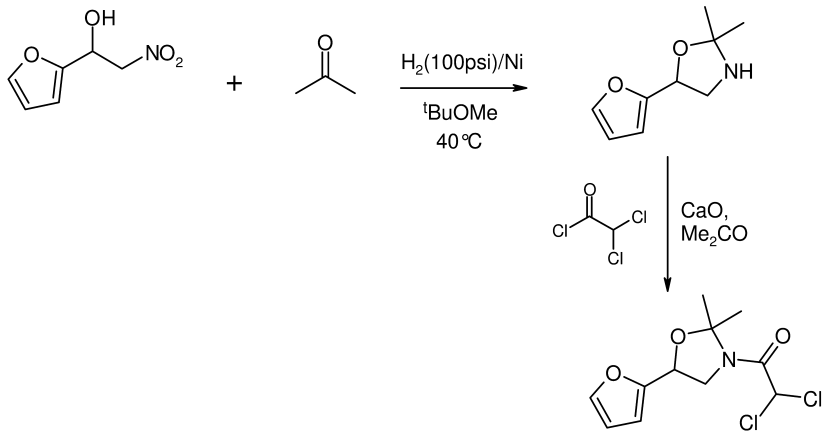
5.2.1.3 **Furilazole**

Furilazole was developed by Monsanto Co. under the code number MON13900 and first reported in 1991 [11]. In that publication it was claimed to safen many herbicides from diverse classes, but detailed efficacy was only presented for the combination with the sulfonylurea herbicide halosulfuron-methyl (NC-319). Since its launch in 1995 furilazole has been marketed with halosulfuron-methyl in the products such as Battalion® and Permit® used pre- and post-emergence in corn and sorghum. It is also used in pre-emergence maize products containing acetochlor (e.g., Degree®, Degree Extra®, Harness®, Guardian®).

Note – Acetochlor can be safened by several dichloroacetamide safeners other than dichlormid and furilazole. For example, the product Acenit® contains the safener AD67 (MON4660) which has no assigned common name (see Table 5.1 for chemical structure).

The two-step synthesis of furilazole (claimed in patent EP 648768) is shown in Scheme 5.3.

The toxicological profile of furilazole is quite favorable (Table 5.5).



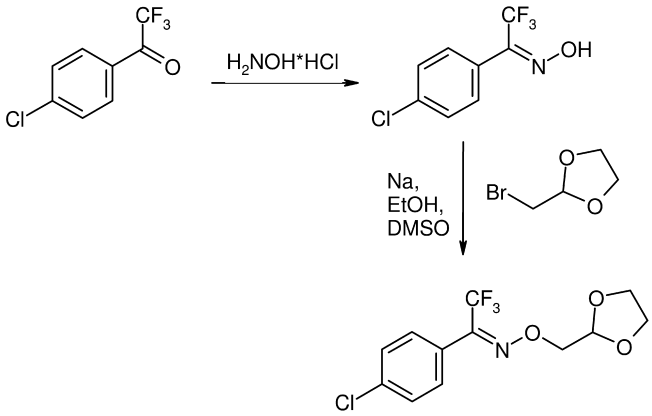
Scheme 5.3. Two-step synthesis of furilazole.

Table 5.5 Toxicological and soil degradation data for furilazole.

Rat oral	$LD_{50} > 869$ mg kg^{-1}
Rat inhalation	$LC_{50} > 2300$ mg m^{-3}
Rabbit skin and eye irritation	Not irritant to skin/slight eye irritant
Guinea pigskin sensitizing	Non-sensitizing
DT_{50} in soil	Rather rapid, ca. 10–20 days

5.2.2 Oxime Ethers

Three oxime ethers have been commercialized by Ciba Geigy (now Syngenta) as seed treatment safeners for sorghum; protection being provided against thiocarbamate and chloroacetamide herbicides (in particular metolachlor). The first (cyometrinil) was launched in 1978 as Concep I®. It was replaced in 1982 by oxabetrinil (Concep II®), which had less potential for negative crop effects from the seed treatment. Concep II® was in turn superseded by fluxofenim (Concep III®), which is still in commercial use. In this case, the reason for replacement is not fully clear, but was reportedly due to an undesirable interaction of Concep II® with downy mildew disease in sorghum [1]. Fluxofenim was developed under the code CGA133205 and first reported in 1986 [12]. The physical chemistry of fluxofenim (log $P = 2.9$) allows rapid uptake into seeds at use rates of 0.3–0.4 g-a.i. kg^{-1}.

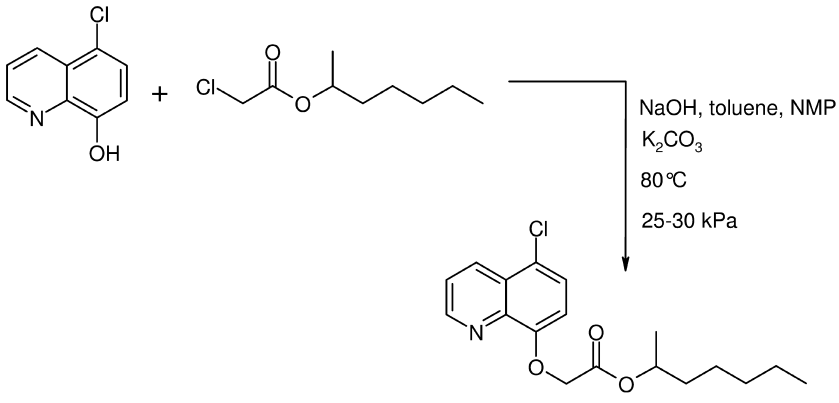


Scheme 5.4. Synthesis of fluxofenim.

Nonetheless the commercial market is limited by use only in the relatively minor crop sorghum. Scheme 5.4 shows the two-step synthesis route for fluxofenim.

5.2.3
Cloquintocet-mexyl

Cloquintocet-mexyl was developed under the code CGA 185072 by Ciba-Geigy (now Syngenta) and is used post-emergence in cereals. The basic patent (EP 94349) has a priority date of 7th May 1982. Various other country patents followed (e.g., US4902340 and US 5102445). It was first reported in 1989 [13] alongside the ACCase inhibitor clodinafop-propargyl, and till now the main use of



Scheme 5.5. Synthesis of cloquintocet-mexyl.

Table 5.6 Toxicological data for cloquintocet-mexyl.

Rat, oral	$LD_{50} > 2000$ mg kg^{-1}
Rat, dermal	$LD_{50} > 2000$ mg kg^{-1}
Rat, inhalation	$LD_{50} > 935$ mg m^{-3}
Rabbit, skin and eye irritation	Not irritant

cloquintocet-mexyl is still in mixtures with this ACCase-inhibiting herbicide. Products include Topik®, Horizon®, Discover® (US), Celio®, Hawk®, Magestan®. The first launch was in 1991 on Switzerland, South Africa and Chile, with the US registration of the safener/herbicide combination in 2000. The greatest safening is observed in wheat, with less safening in barley. Rye and triticale can also be safened. WO2002000625 claims a single-step synthesis route for cloquintocet-mexyl (Scheme 5.5).

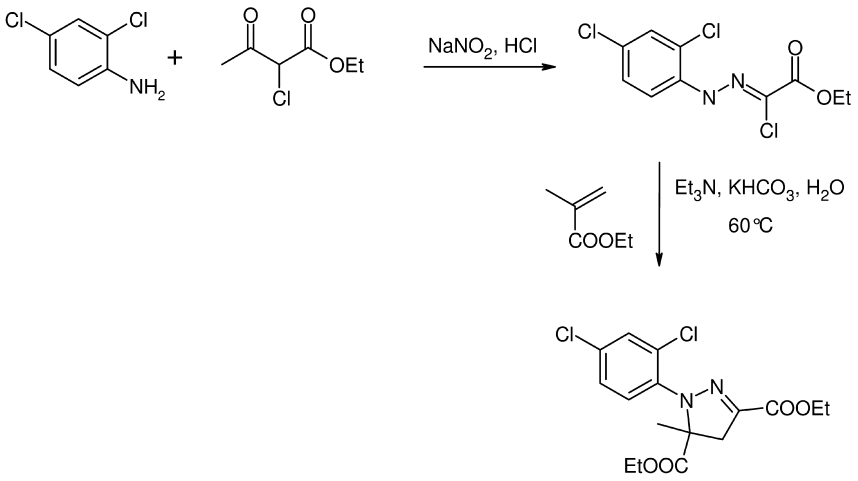
Cloquintocet-mexyl has a favorable toxicological profile (Table 5.6).

In the soil cloquintocet-mexyl degrades rapidly to the free acid ($DT_{50} < 3$ days) with further degradation and mineralization within weeks or a few months. The parent safener and major metabolites are reported to bind strongly to soil and hence have low leaching potential.

5.2.4
Mefenypr-diethyl

Mefenpyr-diethyl is, like cloquintocet-mexyl, used post-emergence to safen cereals. It is used in combination with various aryloxyphenoxypropionates and sulfonylurea herbicides in wheat, rye, triticale and some varieties of barley. It was developed under the code AE F107892 by AgrEvo (now Bayer CropScience) and was first reported alongside iodosulfuron-methyl in 1999 [14] and has replaced its predecessor fenchlorazole-ethyl. Mefenpyr-diethyl had the advantage over fenchlorazole-ethyl of providing post-emergence selective grass weed control not only in wheat and rye but also in spring barley. A further, very important advantage of mefenpyr-diethyl was the property to act as a safener for a wider range of herbicides used post-mergence in cereal crops. The priority date for patent coverage of the pyrazoline safeners was November 1989 (WO9107874) and the first registration of mefenpyr-diethyl was in 1994. It is prepared using a two-step synthesis (Scheme 5.6).

As already pointed out, mefenpyr-diethyl is a versatile safener and it has been commercialized in combinations with several single or mixed herbicides, including fenoxaprop-P-ethyl (e.g., Puma S®), iodosulfuron-methyl-sodium (e.g., Hussar®) and mesosulfuron-methyl (Atlantis®). In general, the quantity of mefenpyr-diethyl required to provide adequate safening lies between 20 and 100 g-a.i. ha^{-1}, and there is no set ratio between the rates of the herbicides and



Scheme 5.6. Two-step synthesis of mefenypr-diethyl.

mefenpyr-diethyl. At this point it is worth mentioning some general considerations with regards to the dose rates required for safeners. Of course, from a commercial and safety standpoint the safener rate should be the lowest needed to obtain crop safety. Seed treatment rates can be selected independent of the subsequent herbicide dose. However, the maximum rate on the seed may sometimes be limited by negative phytotoxic effects. This is exemplified well by the germination inhibition in sorghum caused by cyometrinil, which eventually lead to its replacement by oxabetrinil. For products containing a mixture of safener and herbicide, significant development effort is needed to define the required herbicide/safener ratio. This ratio should be adequate to ensure crop safety and weed control at all recommended rates. A farmer that reduces the product rate to below the minimum that is recommended on the product label runs the risk of not only inadequate weed control (due to insufficient herbicide) but also possible crop injury due to insufficient safener. For mefenpyr-diethyl, a wide range of products exist globally, in which this critical herbicide/safener ratio is tuned to the specific herbicide(s) and agronomic conditions.

Mefenpyr-diethyl has a highly favorable toxicological and ecotoxicological profile (Table 5.7).

In the environment, mefenpyr-diethyl dissipates rapidly with a soil DT_{50} of <10 days. Complete mineralization occurs due to photolysis, hydrolysis and microbial degradation. There is no leaching risk, with the parent compound and soil metabolites not exceeding 0.1 ppb at 1 m soil depth in lysimeter trials.

Mefenpyr-diethyl is most probably a pro-safener, a term introduced by Rubin in 1985 [15]. With mefenpyr-diethyl a decarboxylation occurs rapidly in plants and soil and it is likely that the safening activity comes from mefenpyr-ethyl. How-

Table 5.7 Toxicological data for mefenpyr-diethyl.

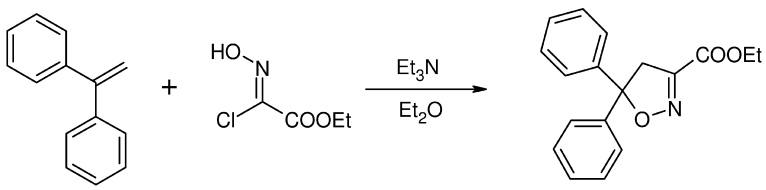
Rat, oral	$LD_{50} > 5000$ mg kg^{-1}
Rat, dermal	$LD_{50} > 4000$ mg kg^{-1}
Rabbit, skin and eye irritation	Not irritant
Guinea pig, skin sensitizing	Not sensitizing
Mutagenicity *in vitro* and *in vivo*	Non-mutagenic

ever, the good post-emergence performance of mefenpyr-diethyl depends upon its physicochemical characteristics (log $P = 3.83$ @ pH 6.3, 21 °C), which lead to better leaf uptake than from mefenpyr-ethyl. The biochemical mode of action of mefenpyr-diethyl is covered in Section 5.3.

5.2.5
Isoxadifen-ethyl

The most recently commercialized safener is isoxadifen-ethyl. It is used post-emergence to safen maize and rice. It was developed under the code AE F122006 by AgrEvo (now Bayer CropScience) and was first reported in 2001 [16–18]. It was launched in US in 2002 in maize in combination with foramsulfuron (Option®). It is also used in combinations with foramsulfuron plus iodosulfuron-methyl-sodium (Equip®, Maister®). In rice it is used with fenoxaprop-P-ethyl (Ricestar®, Starice®) and ethoxysulfuron (Tiller Gold®). From this it can be seen that isoxadifen-ethyl takes safeners to a new level; being able to safen multiple herbicides (of various modes of action) in multiple crops. The priority date for patent coverage of the isoxazoline safeners was 16th September 1993 (DE4331448, US9507897, US5516750).

The synthesis of isoxadifen-ethyl claimed in WO 1995007897 is via a one-step route (Scheme 5.7).



Scheme 5.7. One-step route to isoxadifen-ethyl.

Isoxadifen-ethyl has a favorable toxicological profile according to the U.S. Environmental Protection Agency notice of filing (Table 5.8) [19].

Table 5.8 Toxicological data for isoxadifen-ethyl.

Rat, oral	LD_{50} 1740 mg kg^{-1}
Rat, dermal	$LD_{50} > 2000$ mg kg^{-1}
Rat, inhalation	$LD_{50} > 5000$ mg m^{-3}
Rabbit, skin and eye irritation	Not irritant/slightly irritating

5.3 Mechanisms of Herbicide Safener Action

When applied alone, safeners generally have little visible effects on crop or weed species. This was found, for example, for the safeners fenchlorazole-ethyl and mefenpyr-diethyl [20, 21]. In contrast, fenchlorazole-ethyl exerted an immediate protective effect on wheat and prevented even a transient inhibition of leaf growth by fenoxaprop-ethyl [22]. The same was observed subsequently for combinations of mefenpyr-diethyl with fenoxaprop-P-ethyl.

Potentially, a safener could increase the tolerance of the crop by reduction of herbicide uptake and translocation, or by enhancement of metabolic herbicide inactivation in the crop tissue. Furthermore, a safener could counteract the effect of a herbicide at its biochemical target site, with a resultant reduction of crop susceptibility. Evidence for and against these potential modes of action is presented in the following sub-sections. In addition, aspects of safener specificity (crop versus weed) are covered for situations where the safener is applied in tank mix with the herbicide.

5.3.1 Safener Interactions with the Herbicide Target Site

Potentially a safener could exert its effect in crop species by interference with herbicide binding at the herbicidal target site. This possibility was tested, for example, in wheat chloroplast suspensions with combinations of the ACCase inhibitor fenoxaprop (herbicidally active free acid of the herbicide fenoxaprop-ethyl) and the safener fenchlorazole-ethyl. Even very high concentrations of fenchlorazole-ethyl (100 μM) did not alter the IC_{50} for fenoxaprop at the target enzyme (0.6 μM). The same result was obtained when instead of fenchlorazole-ethyl the corresponding free acid fenchlorazole was tested in this assay. This showed that no herbicide/safener interaction occurred at the herbicidal target enzyme [22]. Analogous *in vitro* assays with the ALS inhibitor chlorsulfuron and the safener 1,8-naphthalic anhydride (NA) were carried out with target enzyme extracts from maize tissue. Also in this case, no herbicide/safener interaction was found at the target enzyme [23].

These findings were in contrast to a report of competitive binding of the tritiated dichloroacetamide safener R-29148 and the herbicides EPTC or alachlor at a proteinaceous component of maize seedling extracts. In addition, a good correlation was observed between competitive inhibition of [^{3}H]R-29148 binding by other dichloroacetamide compounds and their effectiveness as safeners. This was taken as support for the hypothesis that dichloroacetamide safeners act as receptor antagonists for the herbicides EPTC and alachlor [24].

One may also postulate that a safener could stimulate the activity of the herbicidal target enzyme and thus overcome phytotoxic effects of the herbicide in the crop species. In fact, Rubin and Casida [25] found a 25% increase in ALS activity in maize root or shoot tissue after application of the safener dichlormid. An increase of ALS levels in maize tissue was also reported after application of the safeners NA and oxabetrinil [26, 27]. This was in contradiction, however, to work of Barrett [28], who could not find any enhancement of ALS activity in maize and sorghum seedlings after treatment with the safeners NA, oxabetrinil, flurazole or dichlormid. Also, *in vivo* measurements of ALS activity in wheat after application of the sulfonylurea herbicide iodosulfuron-methyl sodium and the safener mefenpyr-diethyl did not indicate that the safener action could be attributed to a stimulation of target enzyme activity or to an interaction directly at the target enzyme [14].

Overall, with some exceptions, literature data suggest that herbicide/safener interactions at the herbicide target site or safener-induced effects on the activity of the herbicide target site are not the mechanism responsible for safener action. In addition, there are examples which, for circumstantial reasons, speak against a major involvement of the herbicide target site in the mechanism of safener action. One such example is the broad action of the safener NA against a spectrum of herbicides with different mechanisms of action. A more recent example is the safener mefenpyr-diethyl, which is not only a safener for ALS inhibitors of the sulfonylurea class but also an excellent safener for the ACCase inhibitor fenoxaprop-P-ethyl [21, 29]. Another point, worth mentioning in context with ALS and ACCase inhibitors, is the high similarity of herbicide binding characteristics to the herbicide target enzyme from cereal crops and from grass weed species, while the safeners for these herbicides act specifically only in cereal crop species, but not in the grass weeds.

5.3.2
Influence on Herbicide Uptake and Translocation

It is usually part of the investigations on the mechanism(s) of safener action to look for possible safener interactions with the herbicide partner at the process of herbicide uptake into the crop. Looking through the relevant literature gives a complex picture. This can also be seen in a review of Davies and Caseley [2], who present an exhaustive compilation of safener effects on herbicide uptake for relevant herbicide/safener combinations developed up to that time. Only in 20% of the cases was the uptake of the herbicide reduced in combination with the

safener; 40% showed no influence of the safener on herbicide uptake, and in the remaining 40% of cases herbicide uptake was even stimulated by the safener. But, also in the cases of reduced herbicide uptake by the safener, the question remained whether this effect was the basis for the safener action. As an example, root uptake of the imidazolinone herbicide AC 263222 by chlorophyllous maize seedlings was reduced by 19% after seed dressing with NA, suggesting a contribution of this effect to the protective action of NA. Follow-up work, however, showed that NA exhibited also a protective effect when an interaction with herbicide uptake was excluded by application of the safener one day after the herbicide. This observation, but also contradictory results of other studies, which showed either no effect or a stimulatory effect of NA on herbicide uptake, made it questionable that an interference with herbicide uptake plays a significant role for the mechanism of action of this safener [30, 31]. It should be added that contradictory results (inhibition, stimulation or no effect on herbicide uptake) can also be found in the literature for other herbicide/safener combinations.

Uptake studies were also carried out with the recently developed combinations of the safener mefenpyr-diethyl with the sulfonylurea herbicides mesosulfuron-methyl and iodosulfuron-methyl-sodium, which are used for selective post-emergence weed control in wheat crop. In both combinations the safener had no influence on herbicide uptake [29].

In summary, it can be said that from present experience only in a few cases was herbicide uptake by the crop reduced in combination with a safener, and even then doubts remained as to whether the reduction of herbicide uptake was the mechanism of safener action. It is, therefore, concluded that interference with herbicide uptake by the crop has no importance as a mechanism of safener action, though it cannot be excluded that there may be cases where it plays an auxiliary role.

5.3.2.1 **Translocation**

Many of the modern herbicides, which are used in combination with a safener for selective post-emergence weed control in cereal crops, are ALS or ACCase inhibitors. The most sensitive morphological sites of action of these herbicides are the meristematic tissues, which in the early stage of development are located at the shoot base of the grass weed as well of the gramineous crops. After foliar spraying of these herbicides, long-distance transport to the basal meristems is a requirement for herbicidal action in grass weeds, as well as the phytotoxic effects in cereal crops. Theoretically, such phytotoxic effects could therefore be prevented, if a safener would act by specific inhibition of herbicide translocation in the phloem to the site of action. So far no case is known were a safener directly interferes with the long-distance translocation of these herbicides. However, there can be indirect effects on translocation due to a safener-induced enhancement of herbicide metabolism in the leaf mesophyll, which in turn may influence the amount of herbicide and metabolites transferred into the long-distance transport system. As an example, after foliar application of ^{14}C-labeled fenoxaprop-ethyl to wheat, translocation of ^{14}C-labeled material was not influenced by combination

with the safener fenchlorazole-ethyl soon after application. However, after a period of three days the percentage of translocated ^{14}C-labeled material was lowered in the presence of the safener. This was interpreted as an effect of differential kinetics of herbicide metabolism and hence differential mobility characteristics of ^{14}C in the presence and absence of the safener [22].

Indirect effects on mobility were also reported for combinations of herbicides with safeners applied pre-emergence or by seed dressing. In corn seedlings treated with [^{14}C]metazachlor the amount of ^{14}C in the developing leaves was lowered when the seedlings had been incubated with the dichloroacetamide safener BAS 145138 (Dicyclonon). Analytical data suggested that safener-enhanced metabolism of metazachlor to a polar non-mobile metabolite in the adjacent seedling tissues reduced the amount of ^{14}C reaching the developing leaves [32]. In maize seedlings treated with the ^{14}C-labeled imidazolinone herbicide imazapic (AC 263222), the acropetal movement from root to shoot was markedly less in seedlings that had received a seed dressing with the safener 1,8-naphthalic anhydride (NA). This was attributed to the safener-enhanced formation of an immobile metabolite being retained in the seedling root [31].

5.3.3 Effects of Safeners on Herbicide Metabolism

With few exceptions, herbicides are subject to metabolic transformations both in weed and crop species, after they have penetrated the plant tissue and are under way to their target site. As a rule, the herbicide metabolites are more polar than the herbicidal parent compound, and they exhibit reduced phytotoxicity or are completely non-phytotoxic. While often the first step of herbicide metabolism entails a partial or total detoxification of the parent compound, there are other cases where the herbicidally active form is generated in the first metabolic reaction (e.g., the hydrolysis of the inactive fenoxaprop-P-ethyl to the herbicidally active free acid fenoxaprop-P) followed by detoxification of the molecule in the subsequent metabolic step.

The most important mechanisms for the detoxification of herbicides in weeds and crops are oxidative reactions (e.g., hydroxylations, oxidative dealkylations) catalyzed by the cytochrome-P450 mono-oxygenase system, and glutathione-S-transferase catalyzed conjugation reactions, which result in a nucleophilic displacement of aryloxy moieties, chlorine or other substituents by the tripeptide glutathione. It is known that the selective action of a herbicide in a certain crop is mostly based on a faster rate of herbicide detoxification in this crop than in the target weeds. Therefore, it is easy to speculate that the safener action could be due to an enhancement of herbicide detoxification in the crop. In the following this will be reviewed for the different safeners used in agricultural practice.

5.3.3.1 1,8-Naphthalic Anhydride (NA), Flurazole, Fluxofenim

These chemically diverse safeners all need to be applied to the crop (maize, sorghum) by seed dressing to obtain the selective safener effect. The oldest and best

examined of these compounds is NA, which acts as a safener in combination with several classes of herbicides. Early studies of Sweetser [33] already suggested that the action of NA as safener for chlorsulfuron and other sulfonylureas in maize was due to an enhancement effect on the oxidative detoxification of these herbicides. Later it was demonstrated with the sulfonylurea compound triasulfuron that the effect of NA as a safener for this herbicide in maize seedlings was due to induction of a specific cytochrome P450-monooxygenase that catalyzes the hydroxylation of the parent compound to the detoxification product 5-hydroxytriasulfuron [34].

NA had also a stimulatory effect on the oxidative metabolism of the herbicide bentazone. Microsomal preparations of etiolated shoots from maize, which had received a seed treatment with NA, showed activity of a bentazone hydroxylase, which was not detectable in extracts from controls without safener pre-treatment [35]. Also, the improved tolerance of maize to the imidazolinone AC263222 after NA seed treatment could be related to enhanced AC 263222 hydroxylation by stimulation of a cytochrome P450 monooxygenase [31].

Gronwald et al. [36] reported that NA and flurazole substantially increased the glutathione-S-transferase activity in corn and sorghum (17- and 30-fold, respectively), when the herbicide metolachlor was used as substrate. This was well correlated to the protective effect of these safeners against metolachlor injury. In contrast, stimulation of GST activity was less than two-fold when, instead of metolachlor, the unspecific substrate CDNB (1-chloro-2,4-dinitrobenzene) was used as substrate. Flurazole had very similar effects in combination with the herbicide metazachlor. In particular, in sorghum it strongly stimulated the conjugation of this herbicide with glutathione [37].

Seed treatment of wheat with fluxofenim increased GST activity nine-fold, when assayed with the herbicide dimethenamid as a substrate. This increase correlated well with accelerated herbicide metabolism in wheat shoots, which was observed as a response to fluxofenim treatment [38].

5.3.3.2 **Dichloroacetamides**

The safeners of the dichloroacetamide family are usually applied in combination with the herbicide, either pre-plant incorporated or pre-emergence. Ekler and Stephenson [37] investigated the mode of interaction of the dichloroacetamide dichlormid, BAS 145138 and MG-191 with the herbicide metazachlor in maize and sorghum. They found an increase of the GST-catalyzed conjugation rate of metazachlor with glutathione (5- to 11-fold), and in addition an increase in GSH levels. The influence of BAS 145138 on the behavior of metazachlor in maize was also studied by Fuerst and Lamoureux [32], who concluded that the safener protected from metazachlor injury by acceleration of the enzymatic glutathione conjugation of the herbicide. Similarly the safener benoxacor was found to induce GST isoenzymes in maize. The increase in GST activity, assayed with metolachlor as substrate, was closely correlated with the protection of maize from metolachlor injury. Resolution of total GST activity by fast protein liquid chromatography (FPLC) resulted in four major activities, which to different degrees were all stimu-

lated by the safener. One of them was only detectable in safener-treated plants [39]. Induction of GST activity, determined with alachlor as herbicide substrate, was also reported for the dichloroacetamide safener R-29148 [40].

Though this group of safeners appears to influence predominantly the GST system, Lamoureux and Rusness [41] reported that the safener BAS 145138 stimulated in maize not only the GSH conjugation but also the hydroxylation of the herbicide chlorimuron-ethyl.

5.3.3.3 **Fenclorim**

The safener fenclorim is used to prevent injury of the herbicide pretilachlor in paddy rice. Deng and Hatzios [42] analyzed GST extracts from several rice cultivars, with and without fenclorim pre-treatment. In all tested cultivars fenclorim increased GST activity with pretilachlor as substrate. FPLC elution patterns revealed multiple glutathione-S-transferases and mass spectrometry confirmed the formation of a pretilachlor conjugate with GSH. Apart from increasing the activity of the constitutive GST peaks, fenclorim also induced the formation of up to five new peaks, depending on the cultivar, which had activity towards pretilachlor.

5.3.3.4 **Fenchlorazole-ethyl, Cloquintocet-mexyl**

Fenchlorazole-ethyl/fenoxaprop-ethyl and cloquintocet-mexyl/clodinafop-propargyl were the first safener/herbicide combinations for selective post-emergence weed control in cereals. Studies of fenoxaprop-ethyl metabolism showed a more rapid decline in the level of fenoxaprop-ethyl and the free acid fenoxaprop in wheat, when applied in combination with the safener fenchlorazole-ethyl. Further studies in wheat suggested that the safener stimulated a GST-catalyzed detoxification reaction of the free acid fenoxaprop, which resulted in the formation of a glutathione conjugate with the 6-chloro-benzoxazolone moiety of the herbicide molecule. This effect was already apparent just a few hours after plant treatment and occurred only in the cereal crop, but not in the target grass weed species. The results suggested that the specificity of safener action is based on differential induction of the detoxification reaction in the crop versus the grass weed species [22, 43, 44]. Subsequently, multiple isoenzymes of GST were purified by Cummins et al. [45] from wheat shoots treated with fenchlorazole-ethyl, and it was found that only the safener-inducible isoenzymes catalyzed the detoxification of fenoxaprop-ethyl.

Also the protective effect of the safener cloquintocet-mexyl against phytotoxicity of clodinafop-propargyl in wheat was found to be based on an enhancement of herbicide detoxification in this crop. After ester hydrolysis the free acid clodinafop was metabolized in wheat by ring hydroxylation and ether cleavage with subsequent conjugate formation, while in the grass weed species metabolism was by malate ester formation. The safener specifically enhanced only herbicide metabolism in wheat, not in grass weed species [46, 47].

5.3.3.5 **Mefenpyr-diethyl**

In the above-mentioned combination, fenoxaprop-ethyl/fenchlorazole-ethyl the racemic form of the herbicide was subsequently replaced by the biologically active

optical isomer fenoxaprop-P-ethyl and fenchlorazole-ethyl was replaced by the new safener mefenpyr-diethyl. Mefenpyr-diethyl alone did not have any phytotoxic effects, even when applied in very high dosages. It was readily taken up by the foliage of the cereal crop and acted systemically. When fenoxaprop-P-ethyl, alone or in combination with mefenpyr-diethyl, was applied to the foliage of wheat, durum wheat or barley, it was – after foliar penetration – in both cases rapidly hydrolyzed to the free acid fenoxaprop-P. However, the rate of the subsequent conversion of the herbicidally active free acid into polar non-phytotoxic products was significantly faster in the presence than in absence of the safener [21]. The key step leading to the detoxification of fenoxaprop-P was again (as described above for the racemate fenoxaprop) the GST-catalyzed attack of glutathione at the fenoxaprop-P molecule, resulting in the formation of 4-hydroxyphenoxypropanoic acid and of a glutathione conjugate of 6-chlorobenzoxazolone (Fig. 5.2A). Both products were subject to further transformation reactions. Notably, mefenpyr-diethyl, as with the older safener fenchlorazole-ethyl, acted exclusively by enhancement of the detoxification reaction, but did not alter the pathway of herbicide metabolism or the metabolite pattern in the crop species. Furthermore, mefenpyr-diethyl did not significantly influence the rate of fenoxaprop-P metabolism in wild oats (*Avena fatua*), as an example of a representative grass weed species, hence it acted specifically only in the cereal crops.

Determinations of GST activity against 1-chloro-2,4-dinitrobenzene (CDNB) and fenoxaprop in barley plants revealed that the exposure to mefenpyr-diethyl increased the conjugation rate with the unspecific substrate CDNB about two-fold, while a 12-fold increase was determined for the conjugation rate with fenoxaprop [48]. It was suggested that this was due to the specific induction by mefenpyr-diethyl of GST isoenzymes with fenoxaprop-conjugating ability. Analogous findings were previously described after application of the safener fenchlorazole-ethyl.

As already mentioned, it is a major advantage of mefenpyr-diethyl to act as a safener also in combination with sulfonylurea herbicides in cereal species. Combinations have been developed with the sulfonylureas iodosulfuron-methyl-sodium and mesosulfuron-methyl.

Studies on the mode of safener action in wheat indicated that the safener enhanced the metabolic degradation of both herbicides in the crop species, while it did not significantly alter their rate of degradation in the target weed species wild oats and blackgrass (*Alopecurus myosuroides*) [14, 21, 49].

In analogy to the findings for fenoxaprop-P-ethyl, mefenpyr-diethyl influenced in wheat only the rate of metabolism of the sulfonylurea compounds, but did not lead to any changes in the metabolite pattern. However, in contrast to fenoxaprop-P-ethyl, GSTs were not found to be involved in the metabolic detoxification of iodosulfuron-methyl sodium or mesosulfuron-methyl. The results of plant metabolism studies suggested instead that specific cytochrome P450 monooxygenases are responsible for catalyzing early detoxification reactions. A metabolite of mesosulfuron-methyl, which appeared first after application of the herbicide to wheat plants, was identified as methyl 2-[3-(4-hydroxy-6-methoxypyrimidin-2-yl)ureidosulfonyl]-4-methanesulfonamidomethyl-benzoate and was likely formed

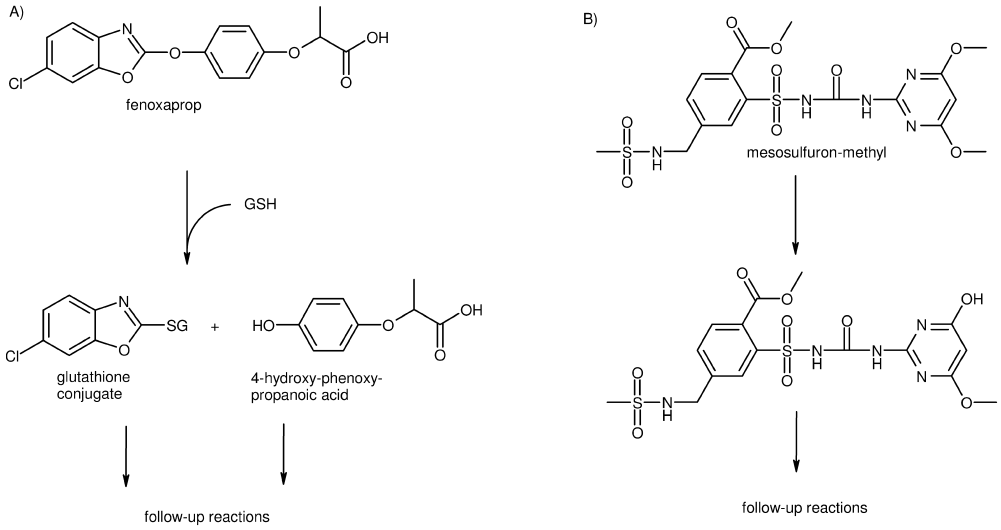


Fig. 5.2. Examples of herbicide detoxification reactions stimulated by the safener mefenpyr-diethyl. (A) Conjugation reaction of fenoxaprop with glutathione (GSH). (B) Oxidative demethylation of mesosulfuron-methyl.

by a P450 monooxygenase-catalyzed oxidative demethylation of the parent compound. The formation of this metabolite was markedly stimulated in wheat by the safener mefenpyr-diethyl [29] (Fig. 5.2B).

5.3.3.6 **Isoxadifen-ethyl**

This compound was recently developed as a safener for the sulfonylurea herbicide foramsulfuron in maize. Metabolism studies revealed that isoxadifen-ethyl also acts by enhancement of foramsulfuron metabolism in maize, while it does not influence the rate of metabolism of this herbicide in susceptible weed species [17, 18].

5.3.4 **Conclusions**

From the presented research data herbicide safeners obviously act in crops predominantly by enhancement of herbicide metabolism to non-phytotoxic degradation products. Notably, all safeners investigated so far only influenced the rate of herbicide metabolism, but did not alter the metabolic pathway. Hence safeners never altered the pattern of herbicide metabolites, but only led to quantitative shifts in the ratios between the phytotoxic parent compound and the metabolites of the herbicide, when compared with control plants without safener application. These quantitative differences between plants with and without safener treatment

are mainly apparent the first days after plant treatment with the herbicide/safener combination. However, since the metabolic degradation of the herbicide takes place not only in safener-treated plants but also in plants without safener treatment, though there with lower rate, the quantitative differences in metabolite levels between the two treatments will become less and less with progressing disappearance of the phytotoxic parent compound.

Theoretically, a safener could act by direct activation of metabolic enzymes, but there are no data to support such a mechanism. Experimental findings rather suggest that safeners are a group of chemical compounds that act at a transcriptional level by regulating gene expression, as pointed out in reviews by Gatz [50] and by Davies and Caseley [2]. An early example of research along this line was the work of Wiegand et al. [51], who reported that in maize treatment with the safener flurazole caused a three- to four-fold increase of mRNA levels coding for a subunit of the GST I enzyme. In a further example, Hershey and Stoner [52] reported on two cDNAs (In2-1 and In2-2) from a 2-chlorobenzenesulfonamide (2-CBSU) induced maize cDNA library. In2-1 and In2-2 mRNA was found in the roots and shoot of maize seedlings after 2-CBSU application via hydroponic solution, maximum levels being reached after 6 h in the roots and after 12 h in the leaves. Both mRNAs were undetectable in seedlings without 2-CBSU induction.

While it appears now well established that safeners act at the gene expression level, it still needs to be elucidated what happens in detail at the molecular level after safener treatment of a plant. This may also help to better understand the molecular basis of the crucially required safener specificity, which results in full protection of the crop while retaining the efficacy of the herbicide on target weeds.

5.4 Concluding Remarks

Since their discovery and first introduction in the 1960–1970s herbicide safeners have provided a valuable tool for agriculture, enabling highly effective herbicides to be used in situations that would otherwise be impossible. Although this technology now competes with herbicide tolerant genetically modified (or naturally selected) crops, safeners still underpin an important part of the herbicide market in maize, cereals and rice. As described in Section 5.3, studies to identify the mechanism of safener action have also provided valuable information to help increase our understanding of herbicide metabolism in crops and weeds. Many of the commercial safeners are now off-patent, offering a chance for generic suppliers to enter the market together with off-patent herbicides. In contrast, recent mixture patents with new herbicides still allow exclusive usage by the patent holder. The number of patents for new safener classes has declined dramatically over the past 10 years, suggesting either a diminishing research success rate or, more likely, discontinued safener research in most research-based ag-chem companies. Nonetheless, because crop safeners allow the use of highly active and thus commercially competitive herbicides in situations not otherwise possible, it is ex-

pected that safeners will continue to play a valuable role in world agriculture for the foreseeable future.

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6
Genetically Modified Herbicide Resistant Crops

6.1
Overview

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6.1.1
Introduction

Herbicides are classified as either selective or broad spectrum. Selective herbicides can be used in-crop to control weeds without significant crop damage. Broad spectrum herbicides such as glyphosate and glufosinate are limited to preplant or post-directed applications. The technology to engineer herbicide resistance has enabled in-crop use of broad spectrum herbicides for improved weed control and yield.

Herbicide resistance can be generated through introduction of a gene or through a selection process. Crops generated via introduction of a gene are referred to here as genetically modified (GM) crops. Crops generated through a selection process were developed by identification of the desired herbicide resistant trait from a natural or mutagenized population of cells or plants. Where data is available, we will consider all herbicide resistant crops in this section regardless of the process by which they were generated.

In 2005, GM crops were cultivated in 21 countries with 71% of those acres being accounted for by herbicide resistant traits in soybean, corn, canola, and cotton. This percentage increases to 82% if one includes herbicide resistance trait acres that are stacked with other biotechnology traits. Globally, GM herbicide resistant soybean, cotton, canola, and corn were grown on 134.4 (60%), 12.1 (14%), 11.4 (18%), and 24.5 (7%) million acres, respectively. (Fig. 6.1.1) [1].

The growing global use of GM crops has had several positive agronomic, economic, and environmental impacts. In the United States in 2004 alone, the use of GM crops reduced pesticide use by 62 million pounds, with 55.5 million pounds of that accounted for by the use of herbicide resistant crops. GM crops also produced significant environmental benefits. In addition to reduced pesticide use, increased no-till practices have reduced water runoff, greenhouse gas emissions,

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

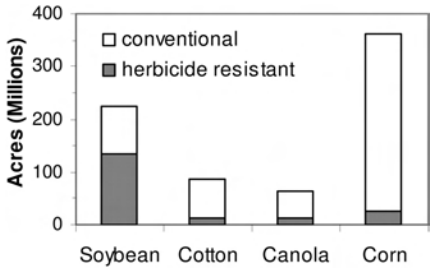

Fig. 6.1.1. Global cultivation of conventional or GM herbicide resistant crops in 2005.

and improved habitats for birds and animals [2]. These benefits are even greater if one includes all herbicide resistant crops and are expected to continue to expand the adoption of biotechnology crops.

6.1.2 Mechanisms for Engineering Herbicide Resistance

In general, herbicide resistance can be achieved through four primary strategies: detoxification of the herbicide to a non-phytotoxic metabolite; expression of an herbicide insensitive target; overexpression of the herbicide target; and cellular sequestration of the herbicide away from the target. Of these, only the first two strategies have been successfully used to develop commercial products to date. Readers are referred to other reviews on herbicide resistance [3, 4].

6.1.2.1 Detoxification of Herbicide

This strategy has led to commercial development of herbicide resistance for glufosinate, glyphosate and bromoxynil. Glufosinate and glyphosate resistance will be discussed in detail in later sections of this chapter (see also Chapter 6.2). Bromoxynil's herbicide activity is due to inhibition of electron transport in photosystem II. Crops engineered with bromoxynil nitrilase metabolize the herbicide to a non-phytotoxic compound [5].

Detoxification has also been utilized to engineer resistance against several other herbicides, including various auxins such as 2,4-D and dicamba, diphenyl ethers (DPEs) such as oxyfluorfen and acifluoren, pyridines such as thiazopyr, and chloroacetanilides such as alachlor. To date, none of these have been developed into commercial products [3].

6.1.2.2 Expression of an Insensitive Herbicide Target

This strategy was used to engineer resistance against glyphosate, and imidazolinones and sulfonylureas that inhibit acetolactate synthase (ALS), a key enzyme in the biosynthesis of branched chain amino acids. ALS resistant crops have primarily been generated through selection for an herbicide insensitive ALS allele from natural or mutagenized cell or plant populations [3].

Expression of an herbicide insensitive target has also been reported to provide resistance to diclofop and sethoxydim (ACCase inhibitors), various dinitroanilines, and inhibitors of phytoene desaturase, lycopene cyclase and hydroxyphenylpyruvate dioxygenase. None of these traits are currently incorporated into commercial products.

There are no commercial herbicide resistant crops that function by increased expression of the protein target, although some level of plant resistance has been reported for glyphosate, glufosinate, some DPEs and inhibitors of hydroxyphenylpyruvate dioxygenase. Similarly, cellular sequestration of the herbicide from the target has been reported with some DPEs, auxins and photosystem I inhibitors, but none have been developed commercially [3].

6.1.3
Commercialized Herbicide Resistant Crops

This section includes data for herbicide resistant crops generated by both selection and biotechnology processes. The first commercially available herbicide resistant crop in the United States was imidazolinone resistant corn introduced in 1992. This was followed by glyphosate resistant soybean and canola in 1996. Since then, the cultivation of herbicide resistant crops has grown globally with multiple herbicide resistant traits available in many large-acre crops (Table 6.1.1). The acreages of herbicide resistant traits in wheat and rice are insignificant and therefore not included in the table. The United States cultivates the greatest acreage of herbicide resistant crops and will be the primary focus of our discussions.

Table 6.1.1 Herbicide resistance traits by crop, the associated trade names, and manufacturers.

Crop	Herbicide resistance	Trade name	Company
Soybean	glyphosate	Roundup Ready	Monsanto
	sulfonylurea	STS	DuPont
Cotton	glyphosate	Roundup Ready	Monsanto
	glufosinate	Liberty Link	Bayer
	bromoxynil	BXN	Stoneville Cotton
Corn	glyphosate	Roundup Ready	Monsanto
	glufosinate	Liberty Link	Bayer
	imidazolinone	Clearfield	BASF
	sethoxydim	SR	BASF
Canola	glyphosate	Roundup Ready	Monsanto
	glufosinate	Liberty Link	Bayer
	imidazolinone	Clearfield	BASF

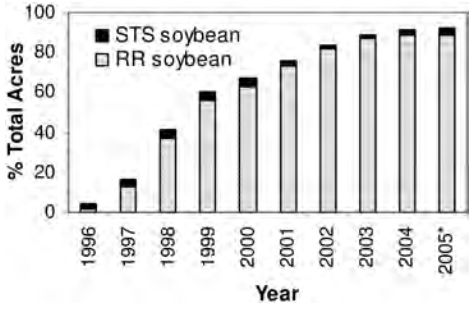

Fig. 6.1.2. Percentage of total acres of herbicide resistant soybeans by trait. *The value given for 2005 is a forecast.

6.1.3.1 Herbicide Resistant Soybeans

There are two commercially available herbicide resistant traits in soybean, glyphosate resistance (Roundup Ready®, RR) and sulfonylurea resistance (STS®). Herbicide resistance now accounts for over 90% of approximately 73 million total soybean acres grown in the United States (Fig. 6.1.2) [6].

RR soybean was developed using a glyphosate insensitive CP4-EPSPS (5-enolpyruvyl shikimate 3-phosphate synthase). Since its introduction in 1996, RR soybean has increased steadily from 2 to 89% of the total soybean acres grown in 2005 (Fig. 6.1.2). The widespread adoption of RR soybeans has resulted in significant grower and environmental benefits. In 2004 alone, RR soybeans reduced grower production costs by $1.37 billion and pesticide use by 22.4 million pounds. Furthermore, there has been about a 64% increase in the number of no-till soybean acres that decreased soil erosion, dust and pesticide run-off, and improved soil moisture, air and water quality [2].

STS soybean was introduced in 1993. Crop resistance was achieved by traditional breeding for an insensitive ALS allele. Since its introduction in 1993, STS soybean has accounted for 2 to 4% of the total soybean acres in production (Fig. 6.1.2).

6.1.3.2 Herbicide Resistant Cotton

There are three commercially available herbicide resistant traits in cotton: glyphosate resistance (Roundup Ready®, RR), glufosinate resistance (Liberty Link®, LL), and bromoxynil resistance (BXN®). In 2005, herbicide resistance traits were cultivated on over 80% of approximately 14 million total cotton acres in the United States (Fig. 6.1.3) [6]. This acreage represents a combination of herbicide resistance alone or stacked with other traits such as insect control traits.

RR cotton, engineered with CP4-EPSPS, was commercialized in 1997 and has since grown from 5 to 80% of the total cotton acres in 2005 (Fig. 6.1.3). Roundup Ready Flex cotton, a second generation product, was commercialized in 2006. The new technology enables glyphosate applications over-the-top from emergence through seven days prior to harvest and represents a significant improvement from the first generation product that limited glyphosate application through the four-leaf stage.

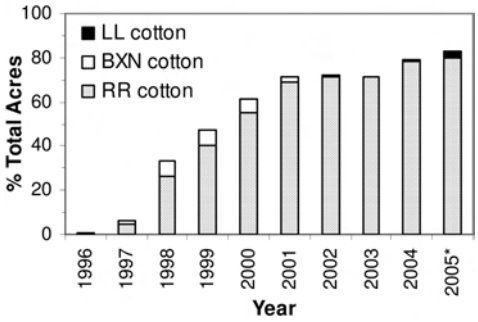

Fig. 6.1.3. Percentage of total acres of herbicide resistant cotton by trait. *The value given for 2005 is a forecast.

Glufosinate or phosphinothricin (Liberty™ and Basta®) inhibits glutamine synthetase that results in the toxic build-up of ammonia in plant cells. LL cotton, commercialized in 2004, was achieved via detoxification by phosphinothricin acetyl transferase (PAT). LL cotton grew from 1 to 3% of the total cotton acres in 2005 (Fig. 6.1.3).

Bromoxynil (Buctril®) inhibits electron transport in photosystem II by binding to the D1 protein. BXN cotton, achieved via detoxification and introduced in 1996, peaked at about 7% of the total cotton acres in 1998 but has steadily declined in use and was last sold in 2004 (Fig. 6.1.3).

As with soybean, the adoption of herbicide resistant cotton has resulted in significant grower and environmental benefits. Use of these traits in 2004 alone has resulted in a reduction in crop production costs of $264 million and pesticide use of 14 million pounds. A major effect of herbicide resistant cotton has been the increase in the adoption of no-till production. The percent increase in no-till acres has been higher in cotton than any other crop and resulted in about $20 per acre savings in fuel and labor costs [2].

6.1.3.3 Herbicide Resistant Corn

There are four commercially available herbicide resistant traits in corn: glyphosate resistance (Roundup Ready®, RR), glufosinate resistance (Liberty Link®, LL), imidazolinone resistance (Clearfield®, CF), and sethoxydim resistance (SR). In 2005 herbicide resistant traits were grown on almost 45% of approximately 81 million total corn acres in the United States (Fig. 6.1.4) [6]. This total represents a combination of herbicide resistance alone or stacked with other traits.

RR corn was commercialized in 1998. Resistance was achieved via expression of a glyphosate insensitive TIPS-EPSPS, which is a maize enzyme with two amino acid mutations that conferred glyphosate insensitivity (see Chapter 6.2, Section 6.2.2). A second-generation RR corn trait with CP4-EPSPS showed improved glyphosate resistance and was introduced in 2001. Since its introduction in 1998, RR corn has grown to 31% of the total corn acres in production for 2005 (Fig. 6.1.4).

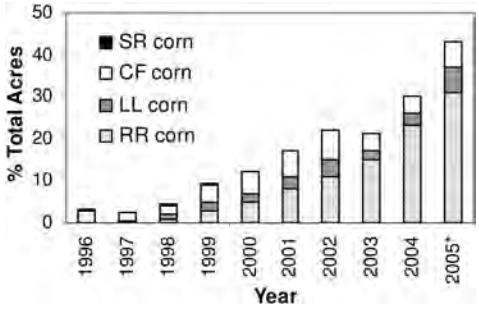

Fig. 6.1.4. Percentage of total acres of herbicide resistant corn by trait.
*The value given for 2005 is a forecast.

Imidazolinones (e.g., imazethapyr and imazapyr), like sulfonylureas, inhibit ALS. CF corn, commercialized in 1992, was developed by mutagenesis and selection of an imidazolinone insensitive ALS allele. Since its introduction in 1992, CF corn has been used in approximately 4 to 7% of the total corn acres (Fig. 6.1.4).

LL corn was developed via detoxification with the PAT gene and was introduced commercially in 1997. LL corn has grown to about 6% of the total corn acres in 2005 (Fig. 6.1.4).

Sethoxydim (e.g., Poast®) inhibits acetyl CoA carboxylase (ACCase) which is the first committed step in *de novo* fatty acid synthesis. SR corn was achieved by traditional breeding and selection for the herbicide insensitive ACCase allele and was introduced in 1996. SR corn accounted for less than 0.3% of corn acres in any one year and is no longer commercially available in field corn (Fig. 6.1.4).

Until 2004, the adoption of herbicide resistant traits in corn has been slower than in other crops mainly due to trade restrictions in export markets for GM products. Nevertheless, the adoption of herbicide resistant corn has resulted in significant grower and environmental benefits. Use of glyphosate and glufosinate resistance traits in 2004 alone resulted in a reduction in crop production costs of $139 million and pesticide use of 18.5 million pounds with numerous positive environmental attributes from increased no-till acres [2].

6.1.3.4 **Herbicide Resistant Canola**

Canada cultivates 90% of the canola acres in North America and is the focus of this survey. The three primary commercial herbicide resistant traits in canola are glyphosate resistance (Roundup Ready®, RR), glufosinate resistance (Liberty Link®, LL), and imidazolinone resistance (Clearfield®, CF). There are two other herbicide resistant traits that are used on a relatively small number of acres and these are bromoxynil resistance (BXN®) and triazine tolerance (TT). In 2004, herbicide resistant traits were grown on over 90% of the 12–13 million total canola acres in Canada (Fig. 6.1.5) [7, 8].

RR canola was introduced in Canada in 1996. Unlike other RR crops, RR canola was achieved by a combination of an insensitive enzyme (CP4-EPSPS)

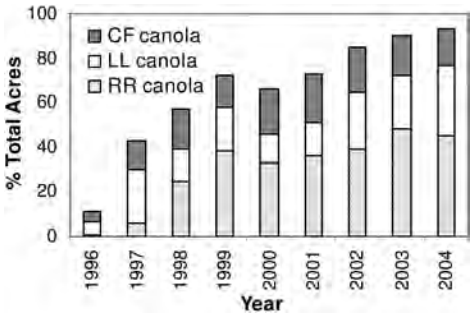

Fig. 6.1.5. Percentage of total acres of herbicide resistant canola by trait.

and a detoxification enzyme (GOX, glyphosate oxidase) that catalyzes the degradation of glyphosate to aminomethylphosphonic acid (AMPA) [9]. Since commercialization, RR canola has grown from about 1 to 45% of the total Canadian canola acres in 2004 (Fig. 6.1.5).

LL canola, introduced commercially in 1995, was achieved via detoxification by PAT. Use of LL canola has grown from about 6 to 30% of the total Canadian canola acres in 2004 (Fig. 6.1.5).

CF canola, introduced commercially in 1995, was achieved by mutagenesis and selection of an imidazolinone insensitive ALS allele. CF canola has grown from about 4 to 20% of the total Canadian canola acres (Fig. 6.1.5).

BXN canola was used in less than 100 000 acres at its peak and has since been withdrawn from the market. Triazine resistant canola, achieved through a selection process, has been used on a limited number of acres and never gained popularity.

The adoption of herbicide resistant canola has resulted in significant grower and environmental benefits. Herbicide resistant varieties allow farmers to plant earlier and control weeds better, resulting in greater yields. GM canola varieties have allowed farmers to save an estimated 8.2 million gallons of fuel and earned, on average, $10 more per acre. Their use has also increased the adoption of low and no-till framing, savings millions of acres from soil erosion [10].

There are several other commercially available herbicide resistant crops. The imidazolinone resistant trait is also available in rice, wheat, sunflower, and lentil. Glufosinate resistance is available in rice and glyphosate resistance was recently commercialized in alfalfa in 2005.

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6.2
Inhibitors of 5-enolpyruvyl Shikimate 3-phosphase Synthase (EPSPS)

Claire A. CaJacob, Paul C.C. Feng, Steven E. Reiser, and Stephen R. Padgette

6.2.1
Introduction

EPSPS is the sixth enzyme in the shikimate pathway that leads to the biosynthesis of aromatic amino acids, tryptophan, tyrosine, and phenylalanine. These aromatic amino acids along with intermediates of the pathway give rise to important secondary metabolites commonly referred to as phenylpropanoids that include phenolics, lignins, tannins, phytoalexins, etc. [1]. The shikimate pathway is localized in plastids and EPSPS is a key enzyme in regulating the flux through the pathway.

EPSPS catalyzes the transfer of phosphoenol pyruvate (PEP) to shikimate 3-phosphate (S3P) to produce 5-enolpyruvyl shikimate 3-phosphate (EPSP). This reaction starts with the binding of S3P to EPSPS to form a binary complex followed by binding of PEP to produce EPSP. Glyphosate (N-phosphonomethyl glycine) is an uncompetitive inhibitor of EPSPS and competes favorably with PEP for binding to the EPSPS/S3P binary complex to form a dead end complex. The binding specificity of glyphosate to EPSPS is extremely high [2]. Thousands of structural homologs, analogs, and derivatives were synthesized and screened for inhibition of EPSPS with most of them showing little to no activity. To date, glyphosate is the only commercialized molecule whose herbicidal activity is attributed to inhibition of EPSPS.

EPSPS is found in plants, fungi and bacteria [3]. As a result glyphosate shows broad spectrum activity against most plant species. Owing to the absence

of EPSPS, glyphosate exhibits little to no toxicity in mammals, birds or fish [1]. Glyphosate was commercialized in 1975 and is the active ingredient of numerous commercial formulations, including Roundup®. The mechanism of action of glyphosate is attributed to inhibition EPSPS, resulting in the buildup of shikimate, and depletion of aromatic amino acids and phenylpropanoid metabolites.

6.2.2 Factors that Impact Glyphosate Efficacy

The success of glyphosate as an herbicide goes well beyond its ability to inhibit EPSPS. Glyphosate as a salt is highly water soluble; it is one of the most systemic herbicides and is readily translocated in the phloem thereby accessing difficult-to-control underground tissues such as the roots [4]. Glyphosate is applied post-emergence to foliar tissues and its efficacy is dependent on the efficiency of absorption. Glyphosate exhibits little pre-plant activity as the molecule is tightly bound to soil and not available to plants or leaching through the soil. Glyphosate in soil is quickly metabolized by microfilaria, primarily to AMPA, with an average half-life of less than a week to months depending on soil type [2].

6.2.2.1 Foliar Absorption

Studies have consistently shown that foliar absorption through the cuticle is the biggest barrier to glyphosate efficacy [5–7]. Over the years, glyphosate formulations have been developed to facilitate its absorption into the plant. Proprietary formulations have been developed using surfactants to maximize the amount and the speed of absorption; however, overly aggressive surfactants that cause excessive local tissue injury may antagonize glyphosate translocation [5, 8].

The standard method for studying plant uptake is to apply droplets of radiolabeled glyphosate to a single leaf in a plant. This method of application is drastically different from field application where a formulation after dilution to the desired volume is atomized and sprayed over-the-top of plants through a nozzle (Fig. 6.2.1). The leaf droplet method ignores potential variables in foliar spray interception, retention, and coverage. Furthermore, studies have shown that foliar absorption of glyphosate is affected by concentrations of glyphosate and surfactant, as well as spray droplet size [6, 8]. The leaf droplet method typically uses droplet size of 1 μL, which is equivalent to a diameter of 1200 μm. In comparison, a typical flat fan nozzle generates a broad range of droplet sizes from less than 100 to greater than 1000 μm with a considerably smaller volume median diameter of only 173 μm [9]. Studies have shown that glyphosate uptake is improved with large size droplets; however, this is offset by reduced foliar retention and coverage during spray application. Increasing the spray volume increases the foliar coverage but at the expense of reduced surfactant and glyphosate concentrations. It is apparent that plant absorption of glyphosate is affected by numerous interdependent variables that cannot be adequately modeled by the leaf droplet method [8].

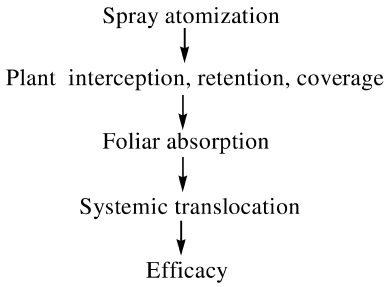

Fig. 6.2.1. Schematic representation of events that characterize an over-the-top spray application of glyphosate formulation *in planta*.

Our initial study in velvetleaf plants employed the standard leaf droplet method to compare glyphosate absorption among commercial formulations [5]. Realizing the limitation of the leaf droplet method, we cautiously began using a track sprayer for over-the-top spray application of formulations augmented with radiolabeled glyphosate [8, 10–13]. The track sprayer method required much greater care and preparation during experimentation but allowed us to generate data on absorption of glyphosate under realistic field use rates and parameters.

In our subsequent study, we used the track sprayer method to compare the absorption of ^{14}C-glyphosate among different commercial formulations in velvetleaf [10]. Plants were sprayed with a field use rate (0.2 kg-a.e. ha^{-1}) at a volume of 93 L ha^{-1} using a commercially available flat fan nozzle. We observed similar spray retention in plants among the three formulations, indicating that differential spray retention contributed little to no difference in efficacy. Following spray application, plants were harvested at various times to measure the levels of glyphosate remaining on the foliar surfaces, absorbed into the leaves, and translocated to the roots. With spray application, the applied dose varied, depending on plant size; therefore, the absorption efficiency was expressed as percentage of total recovered dose from each plant. Significant differences were observed among the formulations in the rate of glyphosate absorption (Fig. 6.2.2). The most efficient formulation (formulation A) rapidly absorbed 28% of the applied dose by 24 h after treatment (HAT) and plateaued thereafter. Formulation B showed much reduced absorption at only 16% by 24 HAT. Formulation C showed slow initial (13% at 24 HAT) but more prolonged absorption [10]. These results illustrated the subtle differences in glyphosate absorption among commercial formulations in velvetleaf under realistic field application parameters.

Not surprisingly, glyphosate absorption is also affected by species differences in cuticle structure and leaf morphology. Using the track sprayer method, foliar absorption of ^{14}C-glyphosate at field use rates with a flat fan nozzle ranged from 20 to 36% in velvetleaf, prickly sida, kochia and RR corn [10, 14]. These results indicated that studies on foliar absorption of glyphosate not only need to employ a relevant method (i.e., track sprayer) but also be conducted in the species of interest.

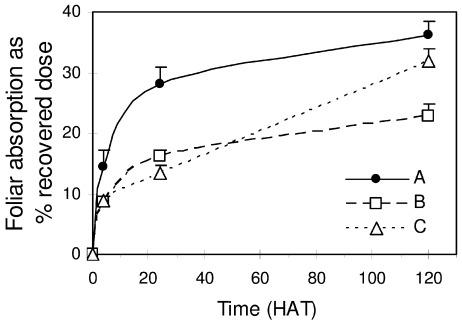

Fig. 6.2.2. Comparison of foliar absorption of ^{14}C-glyphosate with time (hours after treatment, HAT) in commercial formulations A, B, and C using over-the-top track spray application in young velvetleaf plants.

6.2.2.2 **Systemic Translocation**

Most plant species do not metabolize glyphosate [2], with the exception of soybean, which slowly metabolizes glyphosate to AMPA [15]. As a result, once glyphosate enters the phloem, it is translocated along the sucrose gradient from source to sink tissues [16]. The accumulation of glyphosate in sink tissues produces local injuries that diminish the sink strength and sucrose demand, resulting in reduced glyphosate translocation; thus glyphosate imposes a self-limitation on its own efficacy [17, 18]. Sink tissues can sense the effect of glyphosate within a few hours after application, although visible injury may take 7 to 10 days to appear [17]. For these reasons, efficacious glyphosate formulations generally exhibit rapid uptake and translocation to avoid self-limitation [10]. At the same time, fast absorption also results in favorable rainfastness for greater application flexibility.

With the track sprayer method, systemic translocation of absorbed glyphosate among commercial formulations was measured in roots that were shielded from the spray in velvetleaf plants. Formulation A, which showed the highest absorption (28%, Fig. 6.2.2), showed 6% translocation to roots at 24 HAT (Fig. 6.2.3) [10]. Root translocation was proportional to foliar absorption and followed the ranking of formulation A > C > B, which is also the ranking of overall plant efficacy. These results showed that, even with efficient absorption, only about one-third of the applied dose was absorbed, and only a fraction of that was translocated to the roots at 24 HAT. Since the amount translocated was proportional to that absorbed, increasing absorption would increase overall efficacy as long as translocation is not hindered in the process.

Our initial microscopy studies in velvetleaf plants showed that large 1-µL droplets used in the leaf droplet method caused localized spot necrosis on the leaf [5, 19, 20]. In contrast, smaller droplets as encountered in spray application caused little to no visible local injury [21]. A recent study in RR cotton also showed that glyphosate distribution to bolls differed between over-the-top spray versus manual

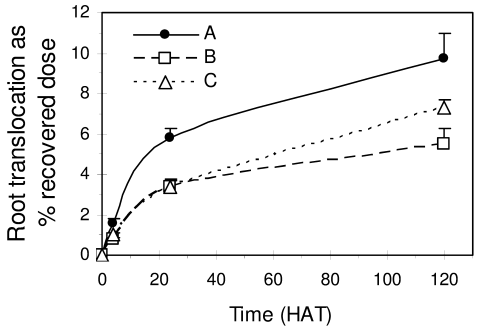

Fig. 6.2.3. Comparison of root translocation of ^{14}C-glyphosate with time (hours after treatment, HAT) in commercial formulations A, B, and C following over-the-top spray application in young velvetleaf plants.

leaf droplet application [12]. This difference was caused by the fact that different leaves, depending on age and position, source different bolls in plants; therefore, glyphosate translocation was dependent on which leaves intercepted the spray. These results further demonstrate that glyphosate application to a single leaf may produce misleading data on absorption and translocation of glyphosate with little relevance to spray application in the field. Nevertheless, the leaf droplet method continues to be used by most researchers when studying herbicide absorption and translocation in plants.

6.2.3
Development of Glyphosate Resistant Crops

A major milestone occurred in 1996 with the commercial introduction of glyphosate resistant soybean [22]. Since then, the glyphosate resistant trait (Roundup Ready®, RR) has been introduced into canola, cotton, corn, alfalfa, sugar beet, and others. All RR crops thus far contain a glyphosate insensitive EPSPS derived either from the plant or bacteria.

The X-ray crystal structures of *E. coli* EPSPS showed that the enzyme consists of two domains that undergo a conformational change upon ligand binding [23, 24]. The resulting closure of the two domains forms the catalytic pocket at the interface [25]. Glyphosate binding appeared to be modulated by several key amino acids near the vicinity of the catalytic pocket [23]. These key amino acids are highly conserved across species and in fact have been used to predict the sensitivity of the EPSPS to glyphosate [26].

Kinetic analysis showed that the endogenous maize EPSPS has a K_m-PEP of 27 μM with a K_i of 0.5 μM (Table 6.2.1). Mutations were introduced at the T102I and P106S positions to produce the variant TIPS [26]. The TIPS-EPSPS showed a K_m of 10.6 μM with a K_i of 58 μM. The double mutant enzyme preserved the EPSPS function while reducing sensitivity to glyphosate. Analysis of single mutations

Table 6.2.1 Comparison of steady state kinetics of EPSPS enzymes from plant and bacteria that are used to engineer glyphosate resistance in crops.

EPSPS	K_m-PEP (μM)	K_i (μM)	K_i/K_m
Maize wild type	27.0 ± 4.0	0.50 ± 0.06	0.02
Maize TIPS	10.6 ± 1.6	58.0 ± 14	5.5
Agrobacterium CP4	12	2720	227

(T102I or P106S) showed increased K_m or sensitivity to glyphosate, thus the desired kinetic properties were obtained only in the double mutant enzyme [26].

Bacterial sources of EPSPS were screened for insensitivity to glyphosate [27]. The EPSPS that is most insensitive to glyphosate was isolated from *Agrobacterium* species CP4. This enzyme showed desirable K_m-PEP (12 μM) but much greater K_i (2720 μM) with a K_i/K_m ratio that is 41× higher than that of TIPS-EPSPS [26]. CP4-EPSPS, which is kinetically superior to TIPS-EPSPS, is currently utilized in all RR crops. The TIPS-EPSPS was utilized in the first generation RR corn (GA21) [28], which has since been replaced by CP4-EPSPS in the second generation RR corn (NK603) [29].

6.2.3.1 Alternative Mechanisms for Engineering Glyphosate Resistance

Attempts were made to engineer glyphosate resistance by increased expression of a glyphosate sensitive EPSPS in *E. coli*, petunia and *Arabidopsis* [30–32]. Transgenic petunia with >20-fold increased expression of EPSPS showed limited resistance to glyphosate with growth inhibition at field use rates [31]. These results suggested that increased expression of a sensitive EPSPS is not likely to generate commercial level resistance to glyphosate in crops.

An alternative mechanism is to use enzymes that catalyze glyphosate detoxification. Glyphosate oxidoreductase (GOX) was cloned from *Achromobacter* sp. strain LBAA and was shown to catalyze the degradation of glyphosate to AMPA (Fig. 6.2.4) [33]. Glyphosate resistance was observed in plants expressing the GOX gene; however, at a level insufficient for commercialization. GOX is currently utilized in conjunction with CP4-EPSPS in RR canola. Recent reports have shown that AMPA may exhibit some plant toxicity of its own [15], which makes GOX less desirable for engineering glyphosate resistance.

Reports by Castle et al. [34] and Siehl et al. [35] described a glyphosate acetyl transferase (GAT, Fig. 6.2.4) useful for engineering plant resistance to glyphosate. GAT was originally cloned from *Bacillus licheniformis* but conferred no resistance to glyphosate when expressed in any host. The catalytic efficiency of GAT was improved through 11 iterations of DNA shuffling and the resulting gene, when expressed in tobacco and maize, conferred resistance to field use rates of glyphosate. A recent patent publication [36] describes yet another bacterial enzyme

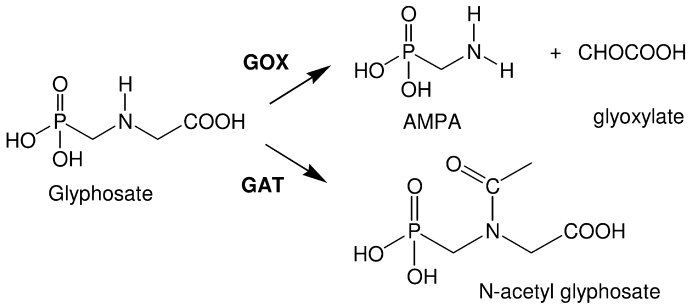

Fig. 6.2.4. Detoxification enzymes that may be useful for engineering glyphosate resistance in crops.

(GDC) suitable for engineering glyphosate resistance. This enzyme is described as having homology to decarboxylases and presumably catalyzes the decarboxylation of glyphosate. Both GAT and GDC are reportedly under development; however, neither gene has been utilized in a commercial crop to date.

6.2.3.2 Disease Control Benefits of Glyphosate Resistant Crops

Although EPSPS is also found in fungi and bacteria, glyphosate was previously shown to have very weak fungicidal activity (ED_{50} 100 to >1000 mg g^{-1}). Recent reports by Feng et al. [13], and Anderson and Kolmer [37] showed that glyphosate reduced the incidence of leaf and stripe rusts in RR wheat and of Asian rust in RR soybean. These fungi (*Puccinia triticina*, *P. striiformis*, and *Phakopsora pachyrhizi*) are obligate pathogens and have been responsible for major yield losses in wheat and soybean.

Studies in RR wheat showed that glyphosate, at a spray dose typically recommended for weed control (i.e., 0.84 kg-a.e. ha^{-1}), provided both preventive and curative activities for a period of 2–4 weeks against leaf and stripe rusts. Disease control was minimal in formulation blanks without glyphosate and was directly correlated to the level of systemic glyphosate in leaves. Field tests under natural heavy rust pressure further confirmed the activity of glyphosate [13]. Current results suggest that glyphosate may provide beneficial effects of rust control in RR wheat and RR soybean. Studies are underway to determine whether glyphosate has activity against other diseases in other RR crops.

From the stand point of engineering glyphosate resistance, either an insensitive EPSPS or a detoxification gene should be equally feasible; however; the disease control benefits of glyphosate is expected to be mostly associated with the use of an insensitive EPSPS due to preservation of glyphosate in crops. This has been observed in glufosinate resistant crops. Glufosinate has also shown fungicidal and disease control activities in glufosinate resistant plants [38–40]. Glufosinate showed only a 2–3 day disease control window [38], which is much shorter than that observed with glyphosate [13]. This can simply be explained by the fact that glufosinate resistant plants are engineered with PAT, which effectively detoxified the herbicidal and fungicidal activities of glufosinate. Presumably plants engi-

neered with a glufosinate insensitive glutamine synthetase would demonstrate a longer disease control window.

6.2.4 Effects of CP4 Expression on Plant Resistance

The lack of plant metabolism and the use of a glyphosate-insensitive EPSPS translate to persistence of glyphosate, which continues to mobilize from source to sink tissues in RR crops [26]. The sink tissues in a plant vary, depending on the growth stage, as a result, the timing of glyphosate spray, which is determined by best weed control, will impact which sink tissues are at risk for glyphosate injury. Our experiences in developing RR crops have taught us that male reproductive tissues are strong sinks and vulnerable to glyphosate injury [26].

Monsanto's approach to second generation RR traits in crops such as corn, cotton, and soybean has been to improve upon first generation products by coordinating expression of the highly glyphosate insensitive CP4-EPSPS in tissues that are at-risk to glyphosate injury. Therefore, the development of second generation RR cotton, corn, and soybean has shifted from strong constitutive viral promoters to regulatory elements with enhanced expression, both spatial and temporal, in the at-risk tissues such as the developing pollen and tapetum. These regulatory expression elements have been engineered as part of a second CP4 EPSPS expression cassette in the case of second generation RR corn and RR Flex cotton. These second generation products have shown enhanced field performance compared to their forerunners. The different promoters used in first and second generation RR crops are highlighted in Table 6.2.2.

Table 6.2.2 Genetic elements used to engineer glyphosate resistance in first and second generation Roundup Ready crops.

Roundup ready crops	Event(s)	Expression cassette 1		Expression cassette 2	
		Promoter/ intron	Coding region	Promoter/ intron	Coding region
RR Corn-1	GA21	Os.Act1/ Os.Act1	TIPS-EPSPS	None	None
RR Corn-2	NK603	Os.Act1/ Os.Act1	CP4-EPSPS	e35S/hsp70	CP4-EPSPS
RR Cotton-1	1445	FMV	CP4-EPSPS	None	None
RR Flex Cotton-2	MON88913	FMV/TSF1	CP4-EPSPS	35S/ACT8	CP4-EPSPS
RR Soybean	40-3-2	e35S	CP4-EPSPS	None	None
RR Canola	RT73	FMV	CP4-EPSPS	FMV	GOX
RR Alfalfa	J101 & J163	eFMV	CP4-EPSPS	None	None

6.2.4.1 **Roundup Ready Cotton**

The second generation RR Flex cotton was commercialized in 2006 to provide growers with greater flexibility in the amount and timing of glyphosate application [41]. Both first and second generation products employ CP4-EPSPS; however, RR Flex cotton will employ two CP4-EPSPS expression cassettes [42–45]. In particular, the first cassette uses a chimeric promoter composed of the *Arabidopsis thaliana* TSF1 gene promoter that encodes elongation factor EF-1alpha [46–48] and enhancer sequences from the Figwort Mosaic virus 35S promoter [49], together with a *cis*-acting TSF1 intron. The second cassette utilizes another chimeric promoter composed of the ACT8 gene promoter of *Arabidopsis thaliana* [50] combined with the enhancer region of the cauliflower mosaic virus (CaMV) 35S promoter [51] together with intron sequences from the ACT8 gene. These chimeric promoters provide strong vegetative expression from the viral enhancer elements and at the same time boost expression in key male reproductive organs via the promoter elements from TSF1 and ACT8 (Fig. 6.2.5B and C). The result is that RR Flex cotton plants are able to withstand glyphosate applications with excellent boll retention throughout the growing season (Fig. 6.2.5A).

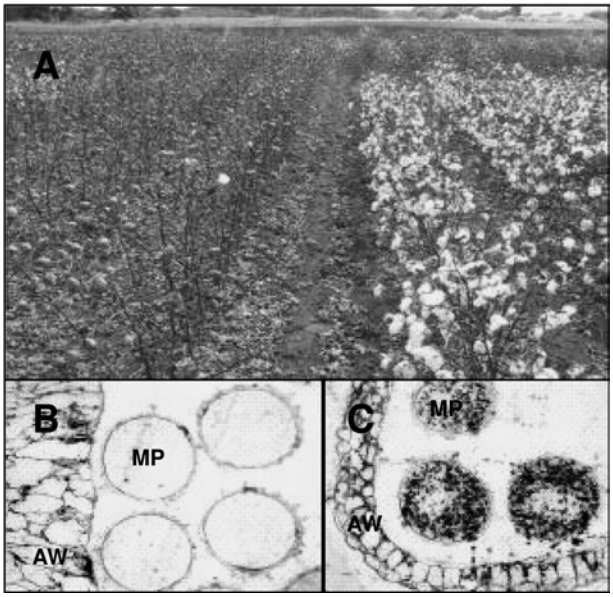

Fig. 6.2.5. Field performance and tissue expression profiles of CP4-EPSPS in first- and second-generation Roundup Ready (RR) cotton. (A) Comparison of boll retention between RR Flex cotton-2 (right) and RR cotton-1 (left) treated with Roundup (2.5 kg-a.e. ha^{-1}) at 4, 6, 10, and 14-node stages. (B) Immunolocalization of CP4-EPSPS protein in the anther wall (AW), but not in the mature pollen (MP), in event 1445 of RR cotton-1. (C) Strong CP4-EPSPS expression in both AW and MP in event RR60, a predecessor of RR Flex cotton-2.

6.2.4.2 **Roundup Ready Corn**

Expression of CP4-EPSPS by the e35S promoter produced corn plants that exhibited vegetative tolerance but poor reproductive tolerance (i.e., male sterility) when challenged with commercially applicable rates of glyphosate [26, 29]. The use of rice actin 1 promoter (Os Act1) and intron elements [52] boosted expression in key male reproductive tissues and produced male fertility. The first generation RR corn (GA21) employs the Os Act1 promoter and introns with the TIPS-EPSPS [28]. The second generation RR corn (NK603) [29] employs CP4-EPSPS in two expression cassettes driven by Os Act1 and e35S promoters for high expression in both male reproductive and vegetative tissues, thus giving rise to robust and consistent field performance.

6.2.4.3 **Roundup Ready Soybean**

The current RR soybean event utilizes CP4-EPSPS under the transcriptional regulation of the 35S promoter [22]. RR soybeans demonstrate excellent reproductive fertility from application of labeled rates of glyphosate; however, reproductive fertility could not be demonstrated from 35S expression of CP4-EPSPS in *Arabidopsis*, tomato or tobacco. The second generation RR soybean is being developed using a modified version of the gene and a new chimeric promoter in a single expression cassette to enhance expression in male reproductive and vegetative tissues.

6.2.5 **Stacking Traits in Roundup Ready Crops**

New RR traits will likely be commercialized as part of a stack with other biotechnology traits to deliver multiple attributes and benefits simultaneously. Traits can be stacked by breeding via cross pollination of lines containing different traits. Alternatively, traits can be stacked in the transformation vector generating multiple traits in one transformation event. Breed stacking has the advantage of utilizing existing events without the need to generate new events; however, as the number of genetic loci increases so does the complexity of event management and selection. Vector stacking contains multiple traits in one genetic loci, which simplifies breeding but requires *de novo* transformation. Trait stacking is a complicated decision that needs to take into account the market demand and grower needs.

Bollgard® (BG) cotton, which protects cotton plants from lepidopteran pests, was introduced in 1996. RR cotton was commercialized in 1997 as a single trait but also with limited availability of the RR/BG stack. The adoption of RR cotton peaked at about 2001 while RR/BG stack has continued to grow and accounted for more acres in 2005 than any of the single trait events (Fig. 6.2.6) [53, 54]. A second-generation lepidopteran product in cotton, Bollgard® II, was introduced exclusively with a RR stack in 2004. These stacked products were generated though breeding. Undoubtedly new trait stacks can be expected from the introduction of RR Flex cotton in 2006.

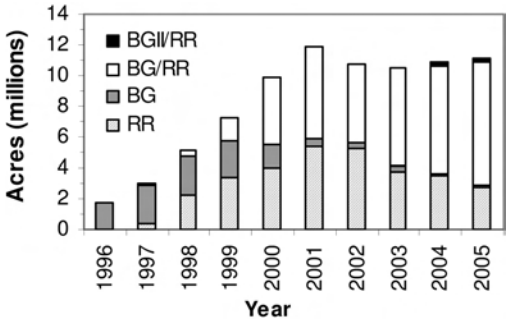

Fig. 6.2.6. Distribution of RR cotton acres as single or stacked traits with Bollgard (BG) since its commercial introduction in 1997.

In RR corn, three different trait stacks are commercially available. RR corn was stacked with YieldGard® Corn Borer (YGCB) for control of lepidopteran pests. In 2005, RR/YGCB was grown on more than 11 million acres, which was similar to that of RR alone [55]. RR corn has also been stacked with YieldGard® Root Worm (YGRW) for rootworm control. The first triple stack, which combines RR, YGCB and YGRW, was introduced in 2005 for glyphosate resistance with lepidopteran and rootworm control. These stacked products were produced through breeding. There are no RR stacks available to date in other crops such as soybean and canola, but that is expected to change in the future.

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6.3
Glutamine Synthetase Inhibitors

Günter Donn

6.3.1
Introduction

Despite the fact that the atmosphere consists of 78% of nitrogen, plants evolved in contrast to animals under conditions where accessible nitrogen sources were

growth limiting due to the chemical inertness of the molecule. Whereas in animals effective pathways evolved to detoxify and to excrete surplus ammonia as urea or ureides, plants are dependent on perfect mechanisms of ammonia recycling. The key enzyme in plants to assimilate, reassimilate and to detoxify ammonia is glutamine synthetase which converts ammonia and glutamate into glutamine under consumption of ATP. Especially in photosynthetically active cells, considerable amounts of ammonia are released in the photorespiratory C_2 cycle which have to be recycled with high efficiency to prevent the build up of high ammonia levels that eventually are toxic or may cause the loss of the volatile NH_3.

Phytopathogenic *Pseudomonas* strains were the first organisms that exploited this Achilles heel of plants: *P. syringae* pv tabaci produce the glutamine synthetase inhibitor tabtoximine-β-lactam, which enables the pathogen to colonize the host tissue killed by the toxin.

In the late 1960s/early 1970s *Streptomyces* strains were discovered that produce a tripeptide consisting of two molecules alanine and an unusual amino acid containing a phosphino group. The latter compound was named L-phosphinothricin and the tripeptide is known as bialaphos (syn. bilanaphos). L-Phosphinothricin was recognized as a glutamate analogue and potent inhibitor of bacterial glutamine synthetases. In the mid-1970s it was recognized that the natural tripeptide as well as the amino acid L-phosphinothricin and the synthetic racemate named glufosinate reveal high herbicidal potential as post-emergent nonselective herbicides. For two decades glufosinate as well as the natural tripeptide have been commercialized and recognized as valuable tools in post-emergent weed control strategies.

Twenty years ago it became evident that the phosphinothricin producing *Streptomyces* strains have in their genomes highly specific acetyltransferase genes that after transfer into transgenic crop plants protect these in a perfect manner from herbicidal activity of phosphinothricin and glufosinate. This opened up fascinating opportunities to use these glutamine synthetase inhibitors as selective herbicides in transgenic crops.

6.3.2
Role of Glutamine Synthetase in Plant Nitrogen Metabolism

Amongst the plant enzymes that use ammonia as substrate, glutamine synthetase (GS; E.C. 6.3.1.2) has the highest affinity (K_m 3–5 μM) for this nitrogen source. Ammonia is released in plant tissues by nitrite reduction and amino acid catabolism but the highest amount, up to 90%, originates in photosynthetic tissues from the photorespiratory C2 cycle [1].

In photosynthetic tissues under atmospheric conditions the oxygenase activity of Rubisco leads to the formation of 2-phosphoglycolate in the chloroplasts, which is cleaved into inorganic phosphate and glycolate. In peroxisomes this intermediate is oxidized by glycolate oxidase to glyoxylate and H_2O_2. Glyoxylate is rapidly metabolized by the enzymes glutamate-glyoxylate-aminotransferase and serine-glyoxylate-aminotransferase. In both cases glycine is the end product. In

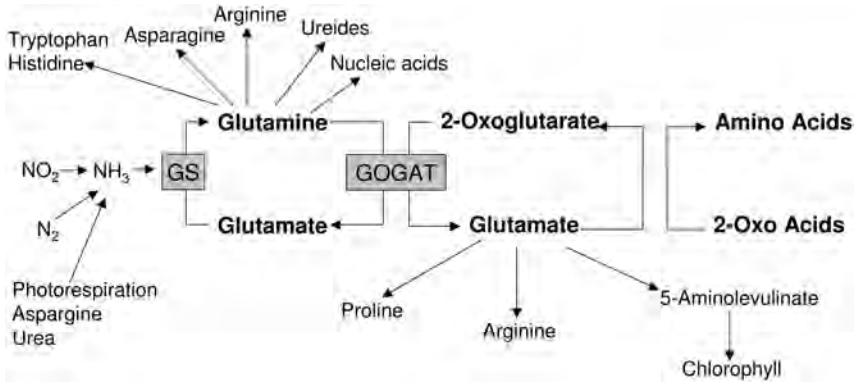


Fig. 6.3.1. Central role of the GS/GOGAT cycle in plant N-metabolism.

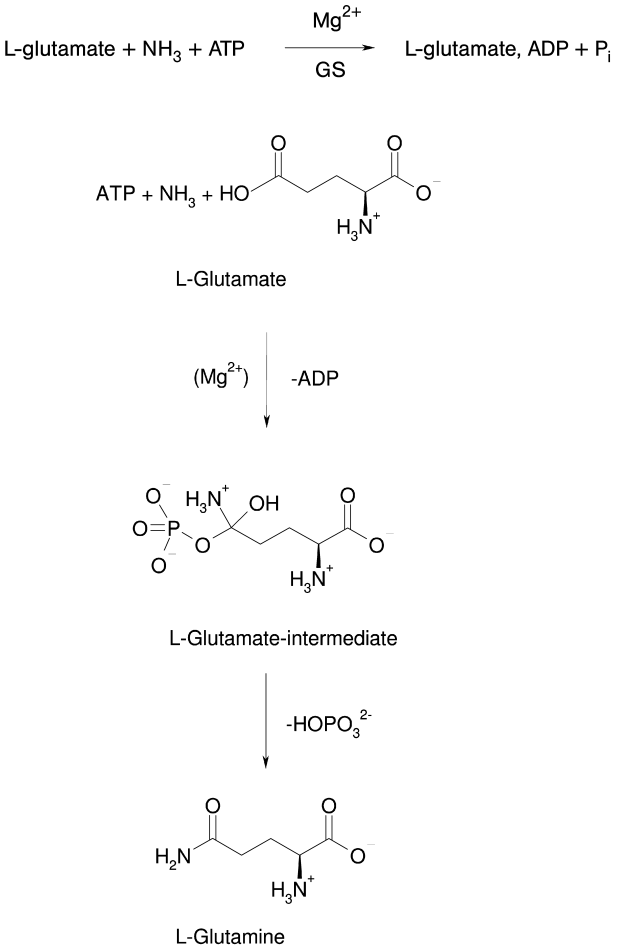
mitochondria two molecules of glycine are converted into one molecule of serine and $CO_2 + NH_3$ are released, which are reassimilated in the chloroplasts [2].

Glutamine synthetase (GS) uses glutamate and ammonia as substrates (Fig. 6.3.1). The resulting glutamine is the substrate for glutamate synthase (glutamine-2 oxoglutarate-aminotransferase, GOGAT), which transfers the amido group from glutamine to 2-oxoglutarate, synthesizing two molecules of glutamate [3]. The GS-GOGAT cycle enables plants to assimilate and to recycle ammonia with high efficiency. The end products of both enzymes are substrates for the respective partner enzyme as well as amino donors for the synthesis of amino acids, purines and pyrimidines [4].

Due to its central role in nitrogen metabolism, plants typically confer several GS genes. They code for GS isoforms that are differentially expressed. Plant GS enzymes consist, like all known eukaryotic GS enzymes, of eight subunits [5]. The molecular weight of the subunits varies in the range 38–45 kDa, depending on the species and the subcellular localization of the respective isoform. At least one cytosolic isoform (GS1) and a chloroplast specific (GS2) can be distinguished in most higher plants, whereas their relative abundance varies considerably between species [6]. The expression of the gene is enhanced by high light intensity [7] and high sucrose levels. In some species a root specific isoform (GSR) can be distinguished and in legumes at least one nodule specific isoform has been discovered [8].

Each subunit of the enzyme has an active center with high binding affinity for the substrates. Glutamate is activated by the enzyme via formation of glutamyl phosphate and consequently this intermediate is amidated with ammonia. For the activation, ATP and Mg^{2+} are required (Scheme 6.3.1).

GS_2 deficient barley mutants isolated under conditions that suppress photorespiration grow without phenotypic aberrations under nonphotorespiratory conditions (2% O_2, 0.7% CO_2), but mutants with less than 40% of the wild-type GS_2 activity show severe phytotoxic symptoms, mainly chlorophyll destruction, when



Scheme 6.3.1. Reaction catalyzed by glutamine synthetase (GS).

grown under normal atmospheric conditions (20% O_2, 0.03% CO_2) in full light [9]. The mutants show a significant increase in the level of free ammonia in their leaves, depending on the light intensity. Interestingly, under photorespiratory conditions this increase of the ammonia level is correlated with the development of phytotoxic symptoms, whereas these symptoms were not observed under conditions where photorespiration was suppressed, even though in both cases the ammonia level was elevated [10].

These mutants demonstrate that glutamine synthetase is a potential target for herbicidal compounds and they indicate that photosynthetic tissue is most vulnerable for herbicidal damage caused by GS inhibitors.

Some 50 years ago it was discovered that certain phytopathogenic *Pseudomonas* strains, namely *P. syringae* pv. tabaci, release a toxic metabolite at the site of leaf

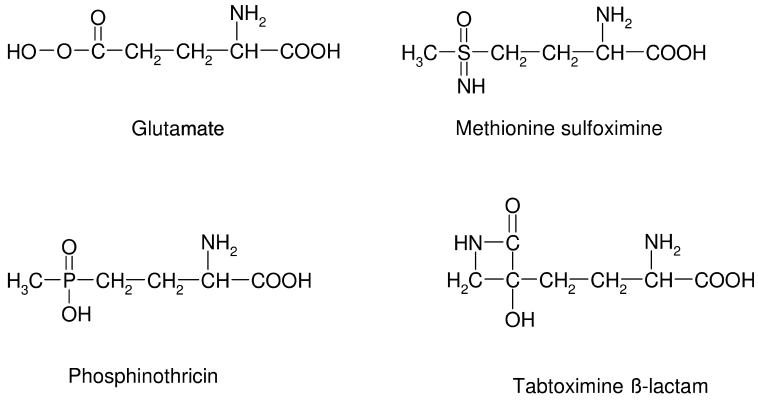


Fig. 6.3.2. Glutamate and some analogues described as GS inhibitors.

infection. The metabolite causes the formation of a chlorotic halo at the infection site and the damaged tissue of the host is then colonized by the bacteria. The molecular structure of the toxic metabolite was identified [11]. The toxic compound is known as tabtoxinin-β-lactam (Fig. 6.3.2), a strong inhibitor of plant GS [12]. If the toxin is applied on tobacco leaves it causes the same symptoms as virulent toxin producing bacteria. Similar symptoms develop after local administration of methionine sulfoximine (Fig. 6.3.2), which was known at that time as a strong GS inhibitor [13].

6.3.3
Phosphinothricin, a Potent GS Inhibitor

In 1972 the team of Professor Zaehner at Tübingen described a *Streptomyces* strain producing a novel compound with antibiotic properties. The antibiotic tripeptide produced by *Streptomyces viridochromogenes* consists of two alanine residues and a novel amino acid that was named phosphinothricin [14] (Fig. 6.3.2). Owing to its structural analogy to glutamate Bayer et al. [14] tested and proved the hypothesis that phosphinothricin acts as an inhibitor of bacterial GS enzyme, whereas the tripeptide phosphinothricyl-alanyl.alanine did not inhibit the isolated GS enzyme. Nevertheless the tripeptide was 1.000–10.000-fold more active in its growth inhibitory effect on different bacteria. The discrepancy is explained by the observation that free phosphinothricin cannot be taken up efficiently by bacteria, whereas the tripeptide is taken up into the bacteria by peptide carriers and, subsequently, the tripeptide is cleaved.

Independent of the research activities in Germany, a Japanese research team discovered the same tripeptide produced by a *Streptomyces* isolate from a Japanese soil sample. This isolate was named *S. hygroscopicus* and the tripeptide was named bialaphos (syn. bilanaphos) [15].

In 1984 a third phosphinothricin-producing microorganism was discovered and described as *Kitasatospora phosalacinea* (syn. *Streptomyces phosalacineus*) which produces phosphinothricyl alanyl-leucine (phosalacine) [16, 17].

6.3.4
Discovery of the Herbicidal Activity of Phosphinothricin

In the mid-1970s, DL-phosphinothricin was synthesized in Hoechst central research laboratories and tested for its biological activity in the biological research unit of the Agricultural Division. Whereas the compound did not show a significant herbicidal activity in the screening for preemergent herbicides, it showed a strong and broad activity against almost all weeds after foliar application in the PO screening, but the compound did not show selectivity in field crops. Field experiments confirmed the excellent broad spectrum weed control potential of DL-phosphinothricin and the development of the compound as a non-selective post-emergent herbicide was initiated [18].

In 1984 the ammonium salt of the compound was introduced to the market under the common name glufosinate-ammonium as a post-emergent herbicide for directed spray application in vineyards. In the following years the label was extended for using glufosinate-ammonium in orchards and plantation crops and subsequently further uses were developed [19].

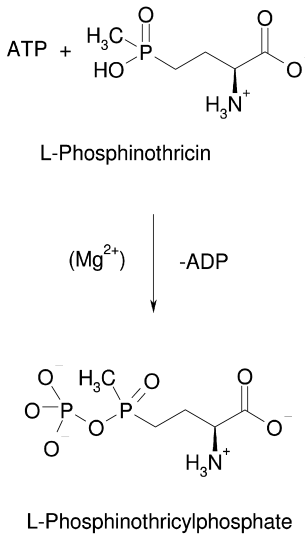
In Japan, Meiji Seika discovered the herbicidal activity of bialaphos. Owing to its good performance as a natural product for weed control after foliar application, the tripeptide was developed as a foliar non-selective herbicide for the Japanese market, where it was introduced in 1984 under the trade name Herbiace [20].

To date, all attempts to synthesize more potent structural analogues of glutamate with herbicidal activity failed despite the research efforts dedicated to the herbicide target glutamine synthetase [19].

6.3.5
Mode of Glutamine Synthetase Inhibition

In 1968 Ronzio and Meister had already developed a model for GS inhibition by methionine sulfoximine (MSO) [21]. They postulated that MSO inhibits GS in two steps. The first step is reversible when the inhibitor competes with glutamate at the binding site. In the second step the substrate analogue is phosphorylated (Scheme 6.3.2) and then irreversibly bound to the enzyme. Manderscheid and Wild [22] confirmed the two-step hypothesis using L-phosphinothricin in their inhibition studies with GS from wheat. They found that the phosphorylated phosphinothricin was irreversibly bound to the enzyme. Furthermore, they concluded that each subunit of the enzyme is able to bind one molecule of phosphinothricin.

Only the L-enantiomer of the racemic glufosinate (DL-homoalanin-4-yl(methyl)phosphonic acid) acts as an GS inhibitor. The tripeptide bialaphos



Scheme 6.3.2. Formation of the phosphorylated intermediates of L-phosphinothricin.

does not inhibit the GS enzyme directly. After foliar uptake the tripeptide is cleaved and then the released L-phosphinothricin inhibits the GS enzyme. Therefore, at the GS target site both herbicides act identically.

6.3.6 Physiology of the Herbicidal Activity of Phosphinothricin

6.3.6.1 Herbicidal Symptoms of Phosphinothricin

One–three days after the herbicide is applied the first symptoms become visible, depending on weed species and climatic conditions. Chlorotic spots and necrotic zones increase rapidly. These symptoms develop either simultaneously as in most dicot weeds or subsequently as in grasses. In grasses intensive chlorosis usually precedes wilting and desiccation. Usually, the treated plants are killed within 7–10 days. Low temperatures delay the herbicidal activity significantly. Sublethal doses or unfavorable climatic conditions may lead to regrowth, especially on older plants.

6.3.6.2 Physiological Effects of GS Inhibition in Plants

When plants are kept in the light, already 1 h after foliar application of glufosinate an increase in free ammonia is measurable. Within 24 h the ammonia level is 100-fold increased, whereas in plants kept in constant darkness, the level of free ammonia is only slightly increased after 24 h [23]. This observation is in agreement with the fact that the vast majority of the released ammonia is generated in the photosynthetic C_2 carbon cycle and to a smaller degree by nitrite re-

duction in the light or amino acid metabolism in darkness. It is known from growth chamber experiments as well as from field observations that glufosinate-ammonium causes fast and strong symptoms at high light intensities, whereas under low light and in darkness the symptom development is delayed [24]. The amino acid pools of glufosinate treated plants undergo dramatic changes in parallel to the ammonia accumulation. Glutamine, glutamate, asparagine, aspartate, glycine, serine and alanine are depleted shortly after glufosinate treatment, while arginine and aromatic amino acids increase in parallel [25]. It was concluded that the relative increase of the latter amino acids is a consequence of the depleted *de novo* synthesis of the amino acids, showing a rapid turnover as well as a result of protein breakdown, especially of proteins showing a high turnover rate like Rubisco. A decrease in protein content in treated plants was indeed observed [26]. Photosynthetic carbon fixation is inhibited by glufosinate within hours as well, whereas the photosynthetic electron transport in chloroplasts prepared from glufosinate treated plants did not decrease within 48 h after herbicide application. Ammonia at high concentrations is regarded as toxic for plants [27], leading to a perturbation of membrane transport processes, most probably due to a collapse of the pH gradient normally maintained by membranes [28]. Originally it was thought that ammonia accumulation as the consequence of glutamine and glutamate depletion caused by GS inhibition is the main reason for phytotoxicity of GS inhibitors in plants, but not all experimental data can be explained by this hypothesis. The results of chlorophyll fluorescence measurements on glufosinate treated plants do not support the hypothesis of ammonia induced uncoupling of photophosphorylation [29]. In addition, *Sinapis* plants kept under nonphotorespiratory conditions (0.1% CO_2, 2% O_2) did not show an inhibition of photosynthesis even though ammonia accumulated to levels that were strongly inhibitory under normal atmospheric conditions causing photorespiration [30]. Furthermore, in detached *Sinapis* leaves kept under photorespiratory conditions, feeding of glutamine or glutamate drastically reduced the inhibition of photosynthesis even though the ammonia accumulation was more pronounced than in leaves that did not get additional glutamine or glutamate [31]. These observations indicate that interrupting the GS-GOGAT cycle causes glyoxylate accumulation due to the depletion of glutamate [32] which acts as substrate for glutamate-glyoxylate-aminotransferase which converts glyoxylate into glycine. Either glyoxylate itself or the arrested glycine–serine conversion, arresting the re-import of C_3 skeletons back into the Calvin cycle, may be the cause for the rapid breakdown of photosynthetic CO_2 fixation [33]. Wild and Wendler [34] showed evidence that glyoxylate inhibits, at very high concentrations, Rubisco directly and inhibits at lower concentration Rubisco-activase, which would explain the rapid breakdown of photosynthetic carbon fixation as well. The latter hypothesis together with the observation the rapid depletion of the pools of crucial amino acids necessary for purine and pyrimidine synthesis as well as for protein synthesis explain the severe and eventually irreversible metabolic disturbance, leading to inhibition of photosynthetic carbon fixation, *de novo* protein synthesis, and finally to the death of the plant tissue.

6.3.6.3 Modulation of Herbicidal Activity of Glufosinate by Environmental Conditions

As a highly polar and water-soluble compound that is insoluble in epicuticular wax and lipid bilayers it is explicable that environmental factors as air humidity and temperature strongly influence the uptake and herbicidal efficacy of phosphinothricin. Uptake is significantly higher at high air humidity [35] even though this effect is less pronounced in the formulated commercial product. The compound is more active above 25 °C than below 10 °C. Hot, humid weather conditions at high light intensities give excellent weed control results even for weed species that are hard to control under less favorable conditions. When glufosinate is applied on plants at temperatures below 10 °C, the translocation as well as the development of herbicidal symptoms is delayed [36], which eventually may lead to reduced herbicidal activity under adverse environmental conditions.

6.3.6.4 Uptake and Translocation of Glufosinate-ammonium

More than 50% of the active ingredient that can penetrate into the leaf is taken up within the first 4–6 h after foliar application and more than 90% is taken up within 24 h [37, 38]. Ullrich et al. [39] showed evidence for active uptake of the compound which is mediated by amino acid carriers. As already mentioned, air humidity modulates the uptake. Under conditions that favor rapid symptom development (high temperatures and high light intensity), the translocation of the compound is limited, whereas in plants kept in the dark after application the active ingredient shows a considerable phloem mobility. Under field conditions glufosinate-ammonium is regarded as a contact herbicide with partial phloem mobility [40].

6.3.7 Use of Phosphinothricin-containing Herbicides in Agriculture and Horticulture

Its mode of action and its slow metabolization in plants explains why phosphinothricin has a very broad herbicidal activity and lacks any selectivity. Herbicides containing this active ingredient originally were developed and brought to market as non-selective post-emergent herbicides for vegetation management in orchards, vineyards, plantation crops, reforested areas and tree nurseries. The selectivity can be generated by directed spraying of the herbicide on the weed canopy and careful avoidance of drift on leaves of the respective crop. Also, in field crops or in horticultural indications in vegetables and ornamentals the crop can be protected from herbicidal damage either by shielded spraying or by application before planting of the crop. In both cases, exposure of the crop to the active ingredient is prevented.

Further registered applications of glufosinate-ammonium cover its use as harvest aid [19], especially for pre-harvest leaf and vine desiccation in potatoes [41].

6.3.8 Attempts to Generate Crop Selectivity for Glufosinate

Due to its activity against a broad weed spectrum, its unique mode of action, its complete biodegradability and low toxicity against non-target organisms [42], attempts were initiated to explore approaches that may allow the use of glufosinate as a selective herbicide in major field crops. In parallel to the genetic approaches outlined in the following paragraph, special spraying devices were developed that allow directed spraying between the crop rows whilst protecting the crop from the sprayed herbicide. Even though the selective use of glufosinate in conventional maize varieties with the help of a directed spraying device was registered in 1993, the necessity to invest in the specific application equipment limited the use of this system considerably.

6.3.8.1 Genetic Approaches to generate Glufosinate-Selectivity in Crops: Target-based Approaches

In the mid-1980s attempts were initiated to select glufosinate tolerant mutants by exposure of regenerable tissue cultures from crops to inhibitory concentrations of the herbicide. When plants were regenerated from *in vitro* selected tobacco and maize cell cultures conferring a 4–8-fold increased glufosinate tolerance, the regenerants showed only marginally improved tolerance to glufosinate after foliar application, which was not worthwhile to be used in breeding programs (Donn, unpublished). No significant changes in GS activity were observed in regenerants of both crops. In contrast to these negative results a phosphinothricin tolerant alfalfa cell line was obtained by stepwise increase of the inhibitor concentration in the culture medium [43]. The resulting mutant cell population tolerated a 20-fold increased herbicide dose, but failed to regenerate to plants. The resistant alfalfa cell line overexpressed GS up to 10-fold compared with the wild type cells due to an amplification of a GS_1 gene.

The constitutive overexpression of the alfalfa GS1 gene in transgenic tobacco led to a significant accumulation of alfalfa GS protein in the tobacco plants, leading to a up to 10-fold increased GS activity in these plants but, nevertheless, these plants were only partially tolerant against foliar application of glufosinate [44]. These plants did show symptoms of leaf chlorosis after treatment with agronomical relevant glufosinate doses of 1–2 kg-a.i. ha^{-1}.

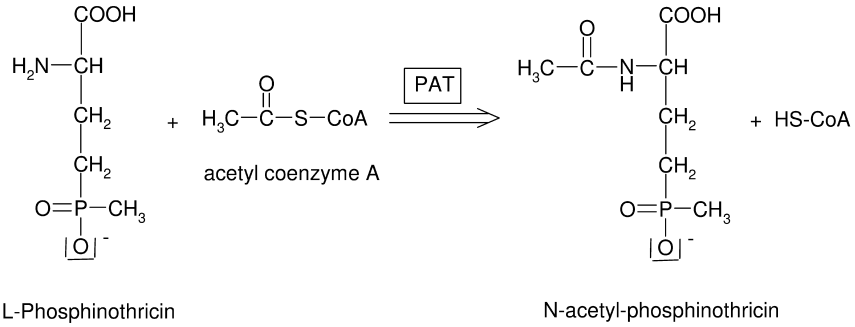
In parallel, attempts were made to mutate the alfalfa GS c-DNA. Even though it was possible to complement a GS deficient *E. coli* mutant by the alfalfa GS_1 cDNA [45], all attempts to generate in the *E. coli* system glufosinate tolerant GS_1 mutants failed. The few mutants that showed a reduced binding affinity for glufosinate lost their binding affinity for glutamate as well.

In summary, to date, all attempts to generate glufosinate tolerance either by target overexpression or by target mutation were unsuccessful. This fact, together with the observation that in weed populations which were exposed to glufosinate for two decades no target based mutants were found, is a strong hint that also

in future it will be unlikely that glufosinate resistant weeds based on target site mutations will evolve.

6.3.8.2 Crop Selectivity by Expression of Phosphinothricin Acetyl Transferase

Twenty years ago Japanese and German research groups characterized, independently of each other, two genes belonging to the biosynthesis gene clusters of phosphinothricin producing *Streptomyces* strains. Both genes conferred bialaphos resistance to *E. coli* and consequently both genes were successfully used as selectable marker genes in gene transfer experiments in crops. The **b**ial**a**phos **r**esistance (bar) gene from *S. hygroscopicus* has been described by Thompson et al. [46] and has been widely used in plant transformation experiments. A similar widespread use as selectable marker experienced the homologous gene from *S. viridochromogenes*, which was described by Wohlleben et al. [47] as **p**hosphinothricin-**a**cetyl-**t**ransferase (pat) gene in 1988. The two genes and their gene products share a high degree of homology. On the DNA level they show 85% homology and they code for proteins that share 87% homology. The biochemical properties of the two proteins in respect of pH and temperature optimum, and their substrate specificity, are very similar [48]. Both enzymes N-acetylate with a high specificity phosphinothricin (Scheme 6.3.3) and desmethylphosphinothricin, a precursor molecule in the biosynthetic pathway of this natural substance, whereas they do not acetylate proteinagenous amino acids.



Scheme 6.3.3. Inactivation of L-phosphinothricin by N-acetylation.

Owing to the high substrate affinity of both enzymes, trace amounts of the proteins are sufficient to protect the transgenic plants from herbicidal damage. Even if less than 0.1% of the total protein consists of Bar or Pat protein, the respective plants efficiently acetylate phosphinothricin quantitatively when it enters the plant cells. These plants do not show any signs of GS inhibition even after application of high doses of glufosinate, which exceed the field application rate 5–10-

fold. Natural evolution provided the phosphinothricin producing *Streptomyces* strains with a perfect mechanism to keep the level of free phosphinothricin within their cells extremely low. The responsible enzymes protect crop plants as well against this herbicidal substance in a perfect manner when the responsible *Streptomyces* gene is transferred and expressed in crops under the control of appropriate promoters.

Because *Streptomyces* show a different codon usage profile than higher plants, a synthetic pat gene was synthesized, coding for the same protein but using the preferred plant codons [49]. The synthetic gene is characterized by a GC content of 50% whereas the natural pat gene has a GC content of 70%. The expected advantage of the synthetic gene was to minimize the risk of gene silencing due to the lower GC content. After 20 years of coexistence of both gene versions in transgenic crops it is evident that the natural gene did not reveal a higher probability of pat gene silencing than with the synthetic version. Both genes allowed the breeding of glufosinate tolerant crop varieties that are expressing the transgene since more than 20 generations.

6.3.8.3 **Bar and Pat Gene in Plant Breeding**

Both genes facilitated the development of efficient gene transfer protocols for various crops. They were successfully used to establish gene transfer protocols for maize [50–52] and rice [53, 54], regardless of the transformation method used by the respective researchers. These genes are still attractive in crop transformation experiments because they are good selectable marker genes *in vitro* to select the transgenic offsprings of the few transgenic cells scattered in the cultured plant tissue in media that contain inhibitory concentrations of phosphinothricin either in the form of the tripeptide or glufosinate. The regenerated transgenic plants are easily distinguishable from nontransgenic siblings by leaf application of the GS inhibitor, which leaves the transgenic regenerants unaffected whilst the nontransgenic siblings develop severe herbicide symptoms. Both genes enabled researchers to develop clean gene constructs conferring solely agronomical useful genes to crops and avoiding the use of antibiotic resistance genes in plant transformation experiments.

Since 1995, transgenic glufosinate tolerant (Liberty Link) canola varieties have been grown commercially in Canada and in 1997 Liberty Link maize was introduced to North American agriculture. Whereas in 2005 Liberty Link canola was grown on more than 25% of the Canadian canola acreage, approximately 6% of the maize acreage in the US was planted with Liberty Link corn at that time. In maize the ratio reflects the predominance of Bt corn amongst the transgenic varieties. In canola the Liberty Link varieties are high yielding hybrid varieties sold under the brand name In Vigor canola.

Furthermore the success story of transgenic canola in Canada which is currently grown on more than 80% of the Canadian canola acreage is explained by the fact that weedy cruciferous relatives of canola are controlled by the 2 complementary herbicides more precisely than with traditional selective canola herbi-

cides. The improved control of the weedy relatives helps to produce a high quality oil which is almost free of glucosinolates and erucic acid [55].

6.3.9
Use of N-Acetyl-Phosphinothricin as Proherbicide

Whereas N-acetyl-phosphinothricin is not deacetylated in plants, bacterial enzymes have been described that can remove the acetate residue from the molecule [56, 57]. Transgenic plants conferring a bacterial deacetylase gene under the control of a constitutive promoter show herbicidal symptoms when sprayed with N-acetyl-phosphinothricin. Therefore, transgenic plants expressing an appropriate deacetylase gene can be selectively eliminated in plant canopies [58]. If the deacetylase gene is linked to tissue specific promoters, specific cells can be ablated in transgenic plants, conferring the gene construct. For example, a deacetylase gene from *Stenotrophomonas* sp. linked to a tapetum specific promoter was successfully used to generate facultatively male sterile tobacco flowers after treatment of the plants with N-acetylphosphinothricin in the flower bud stage (Fig. 6.3.3).

6.3.10
Conclusions

The natural glutamine synthetase inhibitor phosphinothricin as well as its synthetic racemate glufosinate are broad spectrum post-emergent herbicides that will play a role in future agriculture due to the unique mode of action. Because these GS inhibitors fully control weeds that have evolved resistances against other types of herbicides, the use of phosphinothricin-containing herbicides in tolerant crops will remain an important option for future sustainable agriculture.

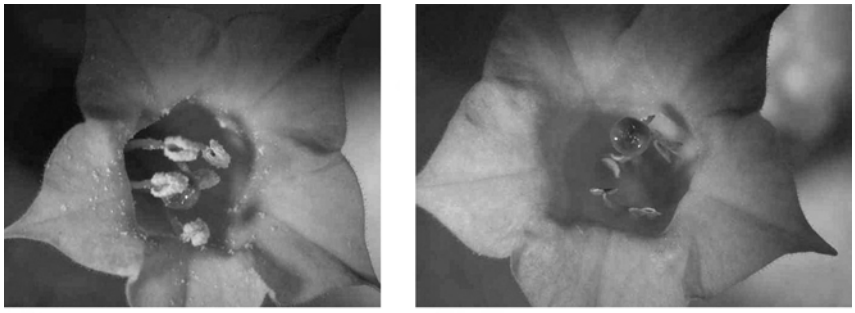


Fig. 6.3.3. Tobacco flowers treated with N-acetylphosphinothricin.
(a) Nontransgenic control; (b) transgenic tobacco conferring a bacterial deacetylase gene under the control of a tapetum specific promoter.

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7
Microtubulin Assembly Inhibitors (Pyridines)

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7.1
Introduction

Herbicides with the microtubulin assembly inhibitor [1, 2] (MAI) mode of action are generally applied pre-emergence for control of annual grasses and small-seeded broadleaf weeds, causing swelling in meristematic regions such as root tips. Susceptible plants may show thickened or swollen hypocotyls or internodes [3]. The MAIs are grouped into five chemical families: the dinitroanilines, the phosphoroamidates, the pyridines, the benzamides, and the benzoic acids (Herbicide Resistance Action Committee class K1). The most popular family of the MAIs is the dinitroanilines, which includes herbicides such as trifluralin, benefin, oryzalin, pendimethalin, and prodiamine. In the 1980s the demand for pre-emergence herbicides that were more efficacious, colorless and dependable at lower application rates led to the investigation of potential compounds in the pyridine family. In the pyridine family, there are only two herbicides being marketed today – dithiopyr and thiazopyr – so they are the focus of this chapter. Both herbicides were initially patented by Monsanto [4, 5] before subsequently being sold to Rohm & Haas Company (1994). Ultimately the products became the property of Dow AgroSciences through the acquisition of the Rohm & Haas Agricultural Chemical business by The Dow Chemical Company (2001). These products can be used by professional turf, ornamental, perennial tree & vine and *Oryza* growers to control a broad range of troublesome broadleaf and grass weeds.

7.2
Biology of Microtubulin Assembly Inhibitors (Pyridines)

Dithiopyr is a pre-emergence and early postemergence herbicide primarily used in turf, ornamentals and *Oryza* in the United States, Canada, Japan, China, Aus-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

tralia, Egypt, South Korea, Taiwan and Puerto Rico. It is applied pre-emergence or postemergence to turf at 150–560 g-a.i. ha^{-1} per application. Early postemergence applications can be utilized to control *Digitaria* spp. seedlings in their early stage and prior to emergence of a second tiller [6]. Adjuvants have low influence on postemergent control because translocation from treated leaves is minimal [7].

Another turf use pattern is selective pre-emergence control of *Poa annua* L. in overseeded warm-season turf. A common practice in warm climates is to overseed warm-season turfgrass species with cool-season turfgrass species such as *Lolium perenne* L. to maintain a green color through the winter months when warm-season grasses typically go dormant. Dithiopyr has been proven effective for selective control of *Poa annua* for 4–6 months after treatment while not injuring *Lolium perenne* that was seeded 8 weeks prior to treatment.

Applications of Dithiopyr to paddy grown *Oryza* are targeted to control *Echinochloa* spp. Dithiopyr can be formulated into several different formulations, including an emulsion in water (EW) containing 240 g a.i. L^{-1}, an emulsifiable concentrate (EC) up to 120 g a.i. L^{-1}, and a wettable powder (WP) with 40% a.i. In addition, granular formulations are available.

Dithiopyr controls key annual monocot species and many dicot species, including *Digitaria* spp., *Poa annua* L., *Eleusine indica* (L.) Gaertn., *Oxalis* spp., *Euphorbia* spp., *Medicago lupulina* L., and *Stellaria media* (L.) Vill. In warmer climates or while seeking control of more challenging weed species such as *Eleusine indica* (L.) Gaertn., sequential applications may be necessary [8, 9].

Most species of cool-season and warm-season turfgrasses are tolerant when the root system is well established. However, some species (such as *Agrostis tenuis*) and some varieties (such as *Cynodon. dactylon* × *C. transvaalensis* "Tifgreen") are not tolerant. Dithiopyr should not be applied to new perennial turf until the root system is well established [10]. It should also not be applied to sod within three months of harvest. Dithiopyr's effect on rooting of established turfgrass species was shown to be minimal and not significantly different than most other pre-emergent herbicides with an MAI mode of action [11, 12].

Thiazopyr is a pre-emergence herbicide that is currently used in non-crop areas, tree, vine and *Oryza* crops and has demonstrated selectivity in *Medicago* spp., *Gossypium* spp., *Arachis* spp., *Glycine* spp., and *Saccharum* spp. [13–15]. It is effective on most annual grasses and certain broadleaf weeds. Thiazopyr is presently registered in 13 countries in North America, Latin America, Europe, Australia and Asia.

A key strength of thiazopyr is its long residual control of annual grass weeds when used at its typical rate range of 0.56–1.12 kg-a.i. ha^{-1}. Also of note is the high level of *Cyperus* spp. suppression when applied pre-emergence. The use of thiazopyr in the United States is as a residual herbicide in permanent crops and in non-crop areas. Citrus, tree-nuts, vines, pomefruit and stonefruit are of primary importance, primarily for control of *Panicum maximum*.

7.3 Environmental Fate of Microtubulin Assembly Inhibitors (Pyridines) [16]

Dithiopyr is strongly adsorbed to soil (K_{oc} average: 1638 mL g^{-1}), but can be desorbed in soils low in organic matter. Soil half-life in field studies ranged from 3 to 49 days (17 day average) with degradation resulting mostly from microbial activity [17]. The major metabolites detected were the diacid and two forms of a monoacid. These metabolites dissipated within one year. Dissipation from field soils can also occur through volatilization. Dithiopyr is stable to photolysis on treated soil. Leaching or runoff, even from highly permeable golf course putting greens, has been shown to be minimal [18–20].

The photolytic half-life in water was 17.6 days, indicating a moderate rate of degradation and a potential for degradation in surface water. The two monoacids and the diacid were the primary metabolites observed. The potential movement in water would be limited due to the low water solubility of dithiopyr and its strong adsorption to soil particles and plants.

Thiazopyr is considered to be relatively immobile in soils due to a low water solubility and high affinity for soil organic matter. Soil microorganisms and hydrolysis are the primary routes of degradation in soil. The average DT_{50} was 64 d (8–150 d) following soil dissipation studies on various soils. The monoacid metabolite applied at normal use rates also had limited mobility. In aqueous solutions the DT_{50} was 15 d, indicating surface water contamination should not be an issue.

In plants, oxygenases metabolize the dihydrothiazole ring to the sulfoxide, sulfone, hydroxyl derivative and thiazole. Thiazopyr is also deesterified to its carboxylic acid.

Table 7.1 Toxicology of dithiopyr and thiazopyr [16].[a]

Organism	Administered	Measure	Dithiopyr value	Thiazopyr value
Rats/mice	Oral	LD_{50} (mg kg^{-1})	>5000	>5000
Rats	2 yr	NOEL (mg kg^{-1})	≤10	0.36
Dogs	1 yr	NOEL (mg kg^{-1})	≤0.5	0.5
Bobwhite quail	Acute oral	LD_{50} (mg kg^{-1})	2250	1913
Rainbow trout	96 h	LC_{50} (mg L^{-1})	0.46	3.2
Honeybee	Topical	LD_{50} (μg per bee)	81	>100
Earthworm	14 d	LC_{50} (mg kg^{-1})	>1000	>1000

[a] Dithiopyr and thiazopyr are non-mutagenic and non-genotoxic. EPA toxicity class is III.

7.4 Toxicology of Microtubulin Assembly Inhibitors (Pyridines) [16]

In rats, dithiopyr is rapidly absorbed, extensively metabolized and rapidly excreted (Table 7.1). Eye irritation in rabbits was slight while skin irritation was non-irritating. Following three weeks of repeated skin exposure to dithiopyr technical, mild transient skin irritation and increased liver weights were the only effects observed in rats.

Animals quickly metabolize and eliminate thiazopyr (Table 7.1). Rat-liver microorganisms use sulfur and carbon oxidation along with deesterification for degradation. Studies in bluegill sunfish demonstrated 98% elimination within 14 days.

7.5 Mode of Action of Microtubulin Assembly Inhibitors (Pyridines)

Dithiopyr is not systemic and is absorbed by roots and to some degree by the foliage of susceptible plants. The most important site of uptake appears to be the meristematic regions since dithiopyr translocation is limited and the primary site of action is meristematic tissues. Efficacy symptoms are most evident by a swelling of the meristematic regions such as root tips in susceptible plants where mitosis is inhibited. This mode of action is disrupting spindle microtubule formation in late prometaphase. Dithiopyr does not bind to tubulin but to another protein that may be a microtubule associated protein (MAP) [1, 2]. These MAPs function in microtubule stability and the action of this molecule results in shortened microtubules that cannot form spindle fibers normally responsible for separating chromosomes to the poles of the cell during mitosis. Cortical microtubules, which normally prevent isodiametric cell expansion, are also essentially absent, resulting in club-shaped roots tips of susceptible plants. Thiazopyr also inhibits microtubule assembly in roots of emerging seedlings but is not effective as an early postemergence treatment, like dithiopyr.

There have been no cases of weed resistance to dithiopyr or thiazopyr reported. In one study, *Digitaria ischaemum* that was resistant to fenoxaprop-p was controlled by dithiopyr [21]. However, cross-resistance to other biotypes resistant to the MAI mode of action could probably occur.

7.6 Synthesis: Dithiopyr and Thiazopyr [22, 23]

Dithiopyr (**1**) and thiazopyr (**2**, Fig. 7.1) are pyridine-based herbicides. These compounds, which are accessed via the pyridine **3**, are synthesized by a similar route (Scheme 7.1).

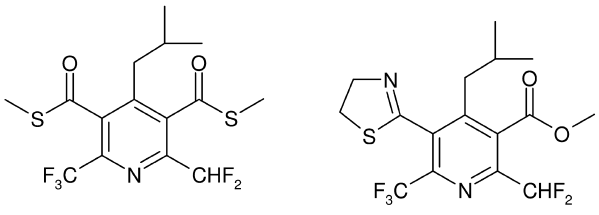
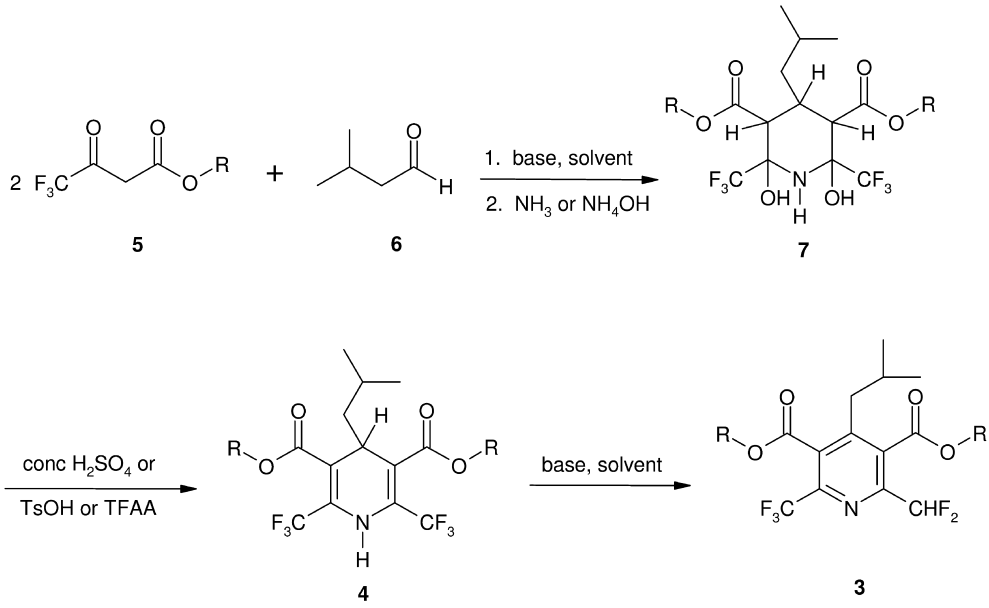


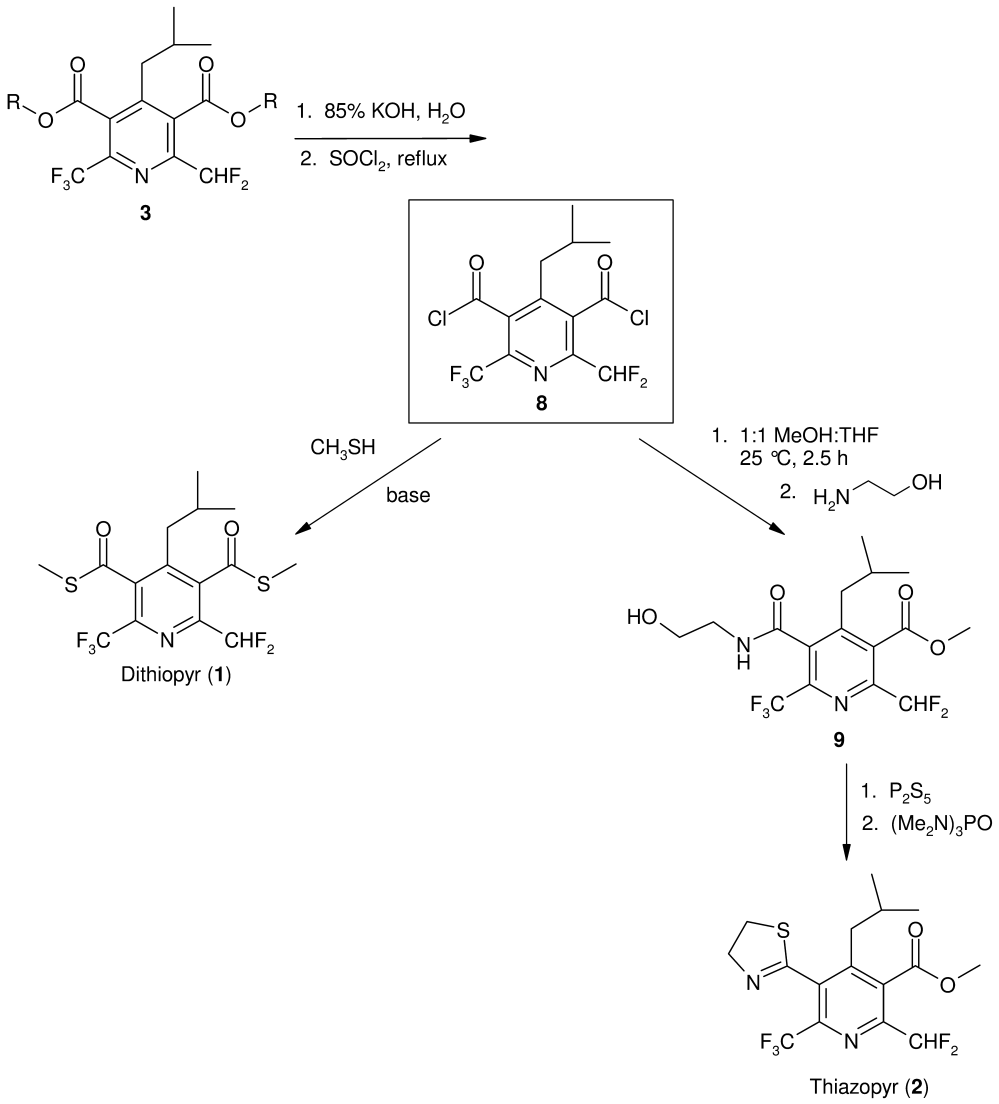
Fig. 7.1. Structures of dithiopyr (**1**) and thiazopyr (**2**).



Scheme 7.1. Synthetic route to the common pyridine intermediate **3** [4, 5].

The syntheses of dithiopyr (**1**), thiazopyr (**2**) and related compounds [4, 5] begin with a Hantzsch-type base-catalyzed intermolecular cyclization [22], which provides the dihydropyridines **4** (R = Me or Et). Two equivalents of methyl or ethyl trifluoroacetoacetate (**5**, R = Me or Et) are allowed to react with one equivalent of isovaleraldehyde (**6**) in the presence of a base, like piperidine, in a suitable solvent at temperatures varying from room temperature to reflux. The intermediate dihydroxytetrahydropyran (structure not shown) is converted into the dihydroxypiperidine **7** by reaction with a nitrogen source, such as ammonium hydroxide or ammonia gas. Reaction of **7** with a dehydrating agent, such as concentrated sulfuric acid, toluenesulfonic acid, or trifluoroacetic anhydride, gives a mixture

of the 1,4-dihydropyridine **4** and its 3,4-isomer. In the case of both **1** and **2**, the major isomer is the 1,4-isomer and it is isolated cleanly. Regiochemical preference for the 3,4-isomer is determined by the choice of dehydrating agent as well as the group in the 4-position of the dihydroxypiperidine **7**. The dihydropyridine **4** is then allowed to react with a base, such as DBU, tributylamine, triethylamine,



Scheme 7.2. Syntheses of dithiopyr (**1**) and thiazopyr (**2**) from the pivotal intermediate bis-acid chloride **8** [4, 5].

or 2,6-lutidine, either neat or in a suitable solvent to provide the dehydrofluorinated [23] pyridine **3**, a common intermediate in the syntheses.

Saponification of the esters is accomplished with 85% potassium hydroxide in aqueous media, providing the 3,5-diacid. The diacid is converted into the pivotal intermediate, the bis-acid chloride **8**, by reaction with neat thionyl chloride at reflux. The acid chloride is treated with methanethiol in the presence of a base to give dithiopyr (**1**) (Scheme 7.2).

Thiazopyr (**2**) is synthesized in a similar fashion. Treatment of the bis-acid chloride **8** in methanol:THF (1:1) at room temperature for 2.5 h affords the 5-chlorocarbonyl-3-methyl ester selectively [24], which is allowed to react with 2-hydroxyethyl amine to form the corresponding 2-hydroxyethyl amide **9**. The hydroxyethyl amide **9** is subsequently treated with phosphorus pentasulfide and hexamethyl phosphoramide, resulting in sulfurization and cyclization to form the 4,5-dihydrothiazole in thiazopyr (**2**).

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8
Inhibition of Cell Division (Oxyacetamides, Tetrazolinones)

Toshio Goto, Akihiko Yanagi, and Yukiyoshi Watanabe

8.1
Introduction

Oxyacetamides and tetrazolinones are new classes of herbicides characterized by excellent efficacy against many major annual grass weeds and certain dicotyledonous weeds, with pre- and post-emergent activity and long lasting weed control.

Oxyacetamides and tetrazolinones inhibit early plant development by disturbing cellular and biochemical level functions. The induced morphological and physiological symptoms are very similar to those of the well-known chloroacetamide herbicides. According to the symptomatic similarity, the Herbicide Resistance Action Committee (HRAC) classifies the herbicides into an action group K_3. The K_3 herbicides are described as inhibitors of cell division or inhibitors of very long-chain fatty acid (VLCFA, >18 carbon chain in length) synthesis. The mode of action of the K_3 group remains unclear.

The selected herbicides flufenacet and mefenacet from the class of oxyacetamides and fentrazamide from tetrazolinones are introduced here.

8.2
Mode of Action

Oxyacetamides and tetrazolinones taken up via the soil provide a strong effect on meristem bearing cell division in the root and shoot tips. Complete arrest of cell division results in cessation of growth and distortion of elongated tissue, leading to plant death.

The mode of action of K_3 herbicides has been reported from biochemical and physiological studies with chloroacetamide herbicides [1]. The findings propose the involvement of the inhibition of VLCFA biosynthesis through a reaction involving covalent binding between herbicide and target enzyme. However, the target site of group K_3 is not sufficiently clarified by binding studies.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

In plants, VLCFAs are synthesized by the membrane-bound, multienzyme acyl-CoA elongase system on the endoplasmic reticulum [2]. The synthesis involves sequential addition of a C2-unit from malonyl-CoA to a fatty acid acceptor by a four-step reaction analogous to *de novo* fatty acid synthesis in the plastid. The first step is the condensation of an acyl-CoA primer (fatty acids > 16 carbon long) with malonyl-CoA to form β-ketoacyl-CoA followed by reduction to β-hydroxyacyl-CoA, dehydration to 2-enoyl-CoA, and a second reduction forming longer chain acyl-CoA . The substrates of acyl elongation are esterified to CoA rather than to acyl carrier protein (ACP) by fatty acid synthase [3]. VLCFAs are essential biological components or precursors of cuticular waxes [4], seed storage triacylglycerols [5], and glycosphingolipids in the plasma membrane [6].

Much investigation with chloroacetamides has focused on fatty acid metabolism, especially fatty acid elongation to elucidate the mode of action.

Phytotoxic chloroacetamides provided a linear relationship between severe inhibition of growth and inhibition of the incorporation of [^{14}C]oleic acid into VLCFAs in *Scenedesmus acutus* [7]. In higher plants, the incorporation of [^{14}C]stearic acid or malonyl-CoA into VLCFAs was inhibited by chloroacetamides while the formation of fatty acids up to C_{18} was not influenced [8]. Acyl elongation with 20:0-CoA and 18:0-CoA primer substrates was inhibited by the active (S)-enantiomer of metolachlor but not by the (R)-isomer [1, 9]. Inhibition of VLCFA formation was also observed in metazachlor-resistance mutant (Mz-1) cells of *S. acutus* [9]. Thus, the phytotoxic action of chloroacetamide herbicides is most likely by the inhibition of VLCFA synthesis.

Inhibition of 20:0-CoA elongation increased with time- and temperature-dependency on preincubation. The findings indicate that formation of the enzyme–inhibitor complex is as an irreversible chemical reaction [10]. The enzyme–inhibitor bond is formed by nucleophilic attack of an enzyme. Chloroacetamides bind covalently to cysteines *in vitro* [11]. Condensing enzymes contain one essential, highly reactive cysteine, which covalently binds the acyl primer substrate before the condensing reaction; mutagenesis studies show the enzymatic similarity of the fatty acid elongase [12]. Based on the peptide mapping analysis of the covalent binding between chloroacetamide and chalcone synthase or stilbene synthase, the active site cysteine residue in condensing enzymes was recently concluded to be the primary common target of the herbicides [13].

The above investigations imply (1) a high affinity of the condensing elongation enzyme to its inhibitors in each step, (2) an increase of inhibition of elongation step with the decrease of acyl-CoA substrate concentration, and (3) a tight binding of inhibitors with the target enzyme [10].

The inhibition reaction is due to the nucleophilic attack of the elongase-condensing enzyme. Inhibitors should have an electrophilic C-atom. Chloro- or oxyacetamides have an active methylene formed by the leaving Cl or heterocycle-oxy. Tetrazolinones bind with a target enzyme through nucleophilic addition eliminating the tetrazolinone moiety. Nucleophilic interaction of the elongase-condensing enzyme with inhibitors is assumed to be an inhibitor–enzyme binding mechanism [1].

Genomics studies with gene encoding VLCFA-elongases from *Arabidopsis* and heterologous expression in *Saccharomyces* support the biochemical and physiological arguments for the molecular target of K_3 herbicides [14, 15].

8.3
Chemistry and Biology of Oxyacetamides and Tetrazolinones

8.3.1
Chemistry of the Compounds

8.3.1.1 Oxyacetamides/Flufenacet, Mefenacet

The first compound of the heteroaryloxyacetamide class (simplified as oxyacetamides) launched in 1986 was mefenacet (FOE 1976; Fig. 8.1), as a paddy rice herbicide. Whereas FOE 1976 was synthesized at Bayer (now Bayer CropScience), its good performance was investigated by biologists of Nihon Tokushu Nouyaku Seizou K.K. (now Bayer CropScience K.K.) through primary, secondary and field trial tests.

The physicochemical (water solubility of 4 mg L^{-1} at 20 °C) and biological properties of FOE 1976 were confirmed as highly suitable for paddy rice [16].

Continuous study of oxyacetamide chemistry shifted research from the paddy herbicide to an upland herbicide with increasing water solubility that is suitable for such upland use. To this end, benzanellated analogues such as the benzothiazole moiety of mefenacet were changed to simple five-membered heterocycles that contain at least one nitrogen atom to increase water solubility, and sulfur or oxygen atom to decrease lipophilicity, for instance thiazoles, thiadiazoles, oxazoles and oxadiazoles (Fig. 8.2). Consequently, many patent applications of the new class of heteroxyacetamide herbicides were disclosed [17, 18].

Through structure–activity correlation studies with the new oxyacetamide substances, only 1,3,4-thiadiazole derivatives with specified substituents provided high herbicidal activity. Requisite properties of the compound for selection were (1) very good efficacy against grassy weeds, (2) very good compatibility for maize and soybeans, and (3) suitable water solubility (56 mg L^{-1} at 25 °C). Based on these results, flufenacet (FOE 5043; Fig. 8.1) was selected and developed as a second-generation heteroxyacetamide class for use as an upland herbicide.

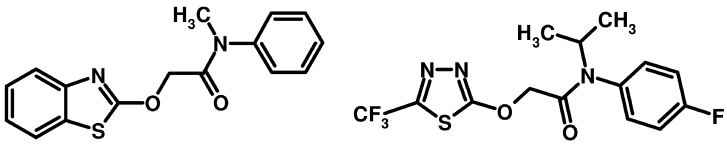
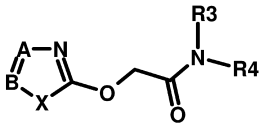


Fig. 8.1. Products from oxyacetamides.



General formula : **A=N, C-R1**
B=N, C-R2
X=O,S

Fig. 8.2. General formula based on the concept of using oxyacetamides as upland herbicides.

Figures 8.3 and 8.4 show the synthetic pathways for mefenacet [19] and flufenacet [20], respectively. The new key intermediates to produce flufenacet are acetoxyacetamide derivative **3**, derived from acetoxyacetylchloride (**2**) and *N*-isopropyl-4-fluoroaniline (**1**), and 2-methylsulfonyl-5-trifluoromethyl-1,3,4-thiadiazole (**5**), derived from trifluoroacetic acid.

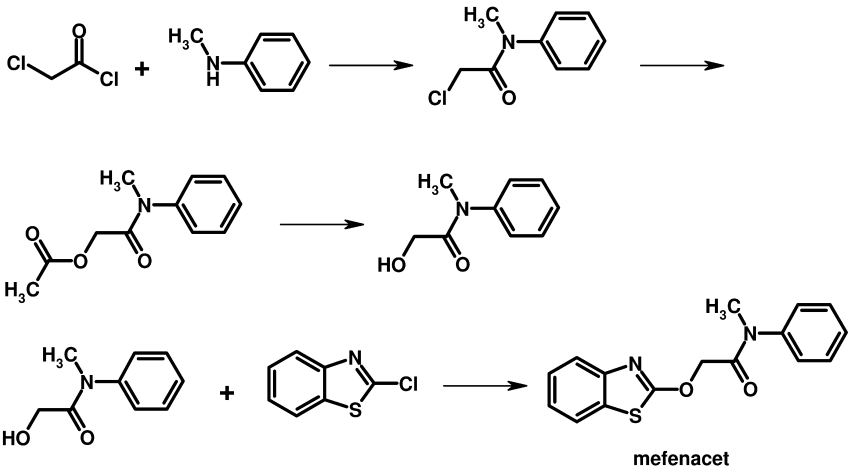


Fig. 8.3. Synthetic pathways to mefenacet.

In summary, to date, two heteroxyacetamide class compounds have been launched on the market, i.e., mefenacet and flufenacet.

8.3.1.2 Tetrazolinones/Fentrazamide

Tetrazolinones were relatively unknown in herbicide chemistry until 1985, when Uniroyal Chemical applied for a patent describing the herbicidal action of carbamoyl tetrazolinones [21]. Several companies have explored this chemistry, and in 1999 Bayer CropScience launched the first practical tetrazolinone herbicide, ''fentrazamide'', for grass control in rice (Fig. 8.5).

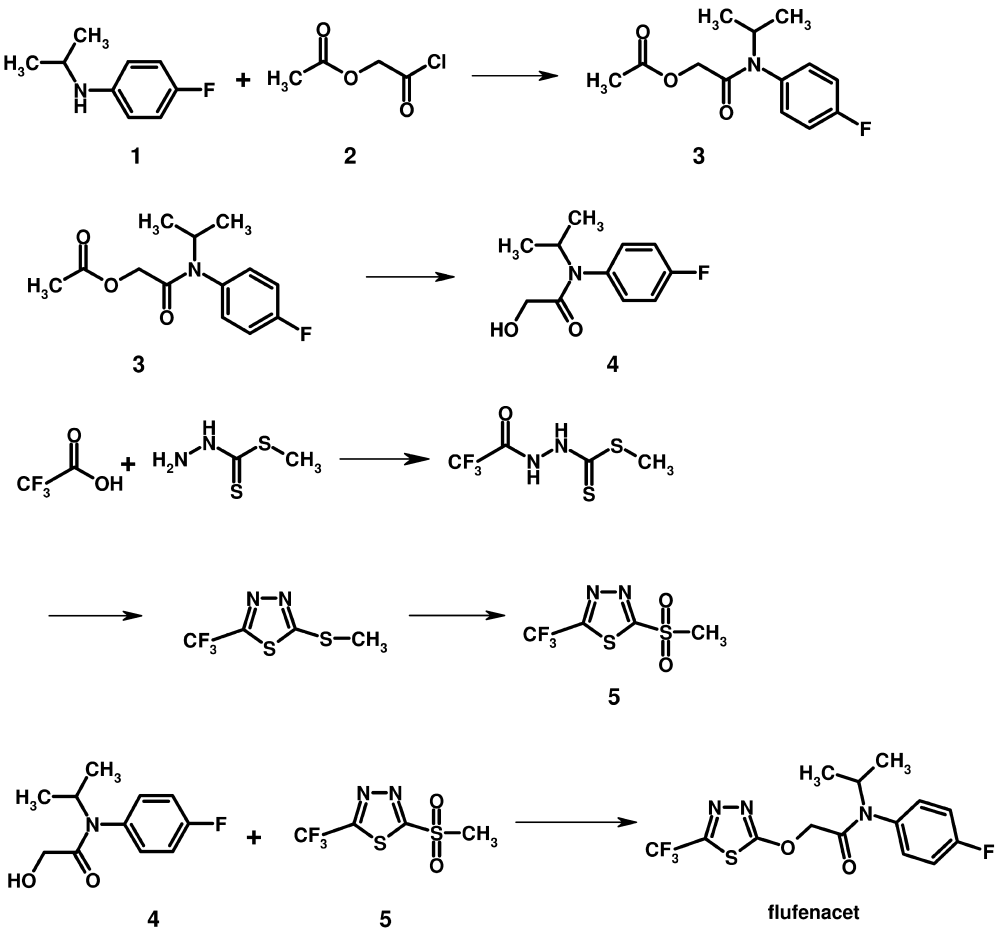


Fig. 8.4. Synthetic pathways to flufenacet.

In 1991, Nihon Bayer Agrochem K. K. (now Bayer CropScience K. K.) started a program for the synthesis and optimization of carbamoyl tetrazolinones. Early in the program, research focused on possible usage in rice because of the high activity of the chemical group to barnyard grass. In contrast, 4-phenyl analogs among

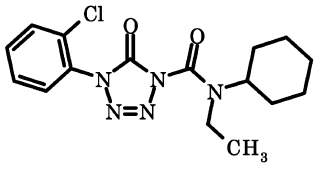


Fig. 8.5. Fentrazamide.

various 4-substituted-1-carbamoyl-tetrazolinones showed some selectivity to transplanted rice. These findings led to 4-phenyl-1-carbamoyl tetrazolinones as a lead structure for the development of new rice herbicides.

A thorough investigation of phenyl substitution patterns revealed that (1) ortho-substitutions with one or two small group(s) such as methyl, ethyl, F, Cl and Br made a significant contribution to activity, whereas substitution at the 3 or 4 position had a weak effect; but (2) some substituents, such as electron-releasing groups (methyl, methoxy, ethyl) and certain electron-withdrawing groups (F, CF_3), resulted in phytotoxicity to rice. In other words, methyl and/or Cl group introduced at the ortho positions of the phenyl ring were most suitable for providing good herbicidal performance. Furthermore, evaluation of the various carbamoyl groups attached to ortho-substituted phenyl tetrazolinones indicated that (1) lower mobility in soil resulted in better crop compatibility, which is due to decreasing mobility with increasing total number of carbon atoms in the N-alkyl group; and (2) a significant decrease in activity was observed when either a linear alkyl group of C_4 or longer was introduced, or the total number of carbon atoms in an N-alkyl group exceeded eight, while the existence of a C_5 or C_6 cycloalkyl group had a positive effect on herbicidal action.

Based on the results, fentrazamide, with high activity to barnyard grass, an excellent safety to rice seedlings and a lower mobility in soil, was selected [22–24].

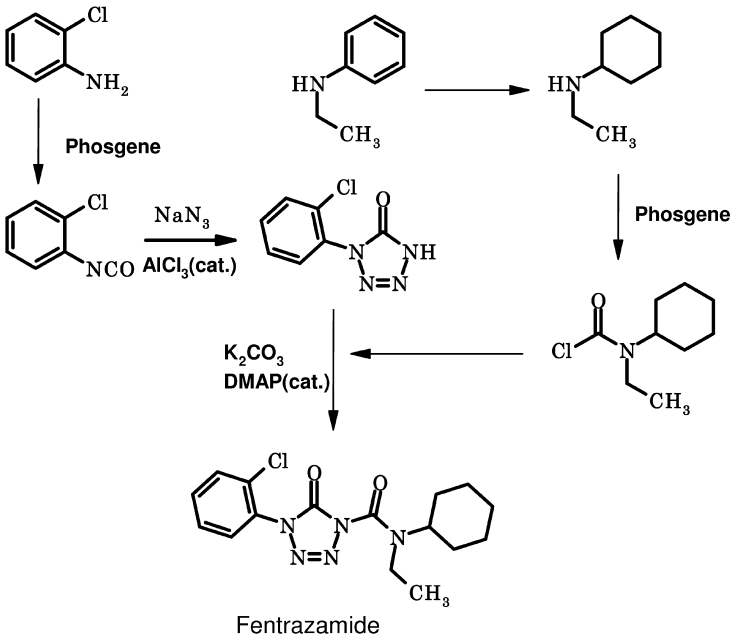


Fig. 8.6. Synthetic pathways to fentrazamide.

As shown in Fig. 8.6, the manufacturing processes of fentrazamide involve the conversion of two inexpensive run-of-the mill anilines, 2-chloroaniline and *N*-ethyl aniline, into 2-chlorophenyl isocyanate and *N*-cyclohexyl-*N*-ethylcarbamoyl chloride, respectively. 1-(2-Chlorophenyl)-5(4*H*)-tetrazolinone can be provided quantitatively by reacting equimolar amounts of 2-chlorophenyl isocyanate and sodium azide in the presence of catalytic amounts of aluminum trichloride in dimethylformamide [25]. The tetrazolinone reacted with *N*-cyclohexyl-*N*-ethylcarbamoyl chloride, in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP), to afford fentrazamide with no formation of its O-carbamoylated isomer [26].

Subsequent investigations by our research group have revealed that, in general, non-aromatic substituted tetrazolinones with an N-phenyl isopropyl carbamoyl group and phenyl- or heteroaryl-substituted tetrazolinones with a dialkyl carbamoyl group are active against barnyard grass [27, 28]. However, none of these tetrazolinones has reached the market as a K_3 herbicide.

8.3.2 Biology of the Compounds

8.3.2.1 Flufenacet

Flufenacet is a selective pre- and early post-emergence herbicide. It is taken up mainly through the root system and xylem-transported to the meristematic tissue of the roots and young shoot to cause growth inhibition. In the greenhouse, flufenacet at 250 g-a.i. ha^{-1} controls >95% of grasses, including *Echinochloa crus-galli*, *Digitaria sanguinalis*, *Setaria viridis*, *Panicum miliaceum* and *Alopecurus myosuroides*, and also >80% of dicots, such as *Amaranthus retroflexus*, *Chenopodium album* (CHEAL), *Galium aparine* and *Galinsoga parviflora* [29].

The crop tolerance of flufenacet is attributed to rapid detoxification by glutathione S-transferases [30].

8.3.2.2 Mefenacet and Fentrazamide

Mefenacet and fentrazamide are used at pre- and post-emergence of weeds, mainly in transplanted rice. They provide stable efficacy against *Echinochloa* sp. (ECHSS) and other dominant weeds in paddy with long-lasting control. The proper application timings are from before emergence of weeds up to the 3 leaf stage (LS) of ECHSS. Mefenacet at 1000–1200 g-a.i. ha^{-1} and fentrazamide at 200–300 g-a.i. ha^{-1} effectively control ECHSS and annual sedges with good compatibility to transplanted rice [31, 32]. The plant compatibility is derived from a low mobility of the herbicides in soil. Almost all active ingredients of mefenacet and fentrazamide applied are detected within 0.5 cm of the soil surface.

Possible application of fentrazamide at 0-DAT (0 Days After Transplanting, that is to say simultaneous application with transplanting of young rice seedlings before emergence of weeds) is basically due to the strong adsorption of the active ingredient to surface layer of the soil. The 0-DAT application technique achieves efficient labor saving in rice cultivation.

8.4 Biology of the Marketed Products and use Pattern

8.4.1 Marketed Products

8.4.1.1 Flufenacet Products

More than 80% of the value of flufenacet-containing products is currently generated in maize (US and Europe) and autumn uses in winter cereals (Europe mainly).

Flufenacet single product (Define™) can control most annual grasses and selected annual broadleaf weeds in maize and soybeans by the treatment of the herbicide alone or as its recommended tank mixes. Possible applications are preplant surface, preplant incorporated or pre-emergence. In cereals, flufenacet is used in ready mixtures with either diflufenican or pendimethalin. A premix with metribuzin (Axiom®) is more effective than other grass herbicides for early-season suppression of *Ambrosia elatior* and *Polygonum* sp. in maize. A premix with isoxaflutole (Epic®) controls major grasses and broadleaf weeds, including *Digitaria* sp., *Setaria* sp., *Panicum dichotomiflorum*, CHEAL, *Amaranthus* sp. and *Eriochloa villosa* control in maize. Epic acts season-long by recharge-action to provide one-pass weed control.

8.4.1.2 Mefenacet Products

Despite the launch of many new one-shot rice herbicides, mefenacet products maintained ca. 16% of Japanese total one-shot application area in 2005 PY (Japanese pesticide sales year from Oct. 2004 to Sep. 2005). The inherent performance of mefenacet – high activity to ECHSS, broad application period and long lasting efficacy – offered a platform for creating a so-called one-shot herbicide. To ensure wide weed control spectrum in paddy rice, mefenacet has been mixed with proper antidicotyledon partners, especially with sulfonylurea (SU) rice herbicides. Typical mefenacet combination products are as a plus bensulfuron-methyl (Zark®), a plus pyrazosulfuron-ethyl (Act®), and a plus imazosulfuron (Batl®). They stably control ECHSS, *Cyperus difformis* (CYPDI), *Scirpus juncoides* (SCPJU), *Monochoria vaginalis* (MOOVP), annual broad-leaved weeds (BBBBB), *Eleocharis acicularis* (ELOAL), *Sagittaria pygmaea* (SAGPY) and *Cyperus serotinus* (CYPSE) with a good safety to transplanted rice at application from 3 DAT up to the 3 LS of ECHSS. In contrast to known sequential application with certain other herbicides, such one-shot products reduced weeding time in rice fields.

The combinations formulated to GR type are conventionally used.

8.4.1.3 Fentrazamide Products

The application area of fentrazamide products has constantly increased since its launch and was up to ca. 14% of total one-shot application area in 2005 PY.

Fentrazamide products such as a plus bensulfuron-methyl (Innova®), a plus pyrazosulfuron-ethyl (Doublestar®) and a plus imazosulfuron (Leading®) provide

the same performance as mefenacet–SU combinations. In addition, these products are applicable at 0-DAT due to their outstanding safety to young rice seedlings [33]. New combinations with HPPD inhibitors like benzobicyclon are under development as a countermeasure for SU-resistance weeds. GR formulations of the products are conventionally used. Special easy-to-use formulation types such as SC and floating granules (GF) packed in water-soluble poly(vinyl alcohol) (PVA) poach and throw-in type application technique as well as 0-DAT application satisfy the farmer's demand for labor saving [34].

In seeded paddy rice, fentrazamide mixture with propanil (Lecspro®) is used as an early post-emergence herbicide for controlling ECHSS, CYPDI, *Cyperus iria*, *Fimbristylis miliacea*, *Leptochloa chinensis* and *Sphenoclea zeylanica* [35].

8.5 The Future of Flufenacet, Mefenacet and Fentrazamide

Increasing generic pressure has influenced the use of flufenacet products, especially in soybeans. Although turning the tide is obviously difficult, flufenacet may be applied either alone or in tank mixtures in cereals, potatoes, sunflowers and vegetables. The occurrence of resistance weeds on using flufenacet is extremely rare, so that the combination of VLCFA-synthesis and HPPD inhibitors may allow the control of glyphosate-, triazine-, and ALS-resistant species of weeds.

The global rice herbicide market has steadily declined, which is due to changes in Japanese farming conditions, such as reducing rice acreage, reducing demand for rice, diversifying consumption patterns, aging and reducing farming populations. Such farming conditions seem to be common to other developing countries. Genetically modified herbicide-tolerant rice varieties are not likely to have significant effects on herbicide sales until GM-rice gains global social acceptance. An increasing demand for rice, reducing farming labor and changing land usage in populous and developing countries are noticeable trends. Therefore, low application volume, less-toxic, one-shot use and value-added rice herbicides seem to be essential in the longer term.

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9
Acetyl-CoA Carboxylase Inhibitors

Jean Wenger and Thierry Niderman

9.1
Introduction

Acetyl-CoA carboxylase (ACC) catalyzes the first step in fatty acid biosynthesis. Owing to its role it has been exploited as an important herbicide target. Two chemical classes, the aryl-oxy-phenoxy-propionate (AOPP or fop) and the cyclohexanedione (CHD or dim) herbicides are widely used to control a broad selection of grass weeds in dicot crops and some of them even in cereals or in rice [1, 2]. Their frequent use has resulted in the development of resistance in several grass species [3].

Dicot tolerance is based on the inherent insensitivity of broadleaves to these herbicides, whereas in monocot crops the selectivity is usually due to higher rates of herbicide detoxification [3, 4].

AOPP and CHD herbicides are well described in the literature and are known to inhibit the carboxylate transferase (CT) function of homomeric ACC found in the plastids of grasses [1, 5].

Over a decade ago, 2-aryl-1,3-diones emerged in the literature as a new, weakly active class of ACC inhibitors [6]. Recently, pinoxaden was reported as a novel cereal graminicide that belongs to this class [7].

This chapter presents an insight into recent developments in biochemistry and resistance mechanisms of ACC and gives an overview of the aryl-diones (ADs) as a novel class of ACC inhibitors. In addition, the industrial synthesis, biology and metabolism of pinoxaden is described.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

9.2 Biochemistry

9.2.1 Overview

Acetyl-CoA carboxylase (ACC; EC 6.4.1.2) is a biotin-dependent carboxylase that produces malonyl-CoA from bicarbonate as a source of carboxyl group and ATP as a source of energy. The reaction catalyzes the conversion of acetyl-CoA into malonyl-CoA through the incorporation of a carboxyl group into the acetyl radical of the acetyl-CoA. This transcarboxylation reaction is performed following the three-step process followed by all biotin-dependent transcarboxylases (Scheme 9.1)

$$HCO_3^- + \text{enzyme-biotin} + \text{ATP-Mg} \rightarrow \text{enzyme-biotin-}CO_2^- + \text{ADP-Mg} + \text{Pi} \quad [1]$$

$$\text{Enzyme-biotin-}CO_2^- + \text{acceptor} \rightarrow \text{acceptor-}CO_2^- + \text{enzyme-biotin} \quad [2]$$

$$HCO_3^- + \text{acceptor} + \text{ATP-Mg} \rightarrow \text{acceptor-}CO_2^- + \text{ADP-Mg} + \text{Pi} \quad [3]$$

Scheme 9.1

The overall ACC transformation is the result of the cooperation of different catalytic activities: [1] carbamoyl-phosphate synthase, [2] biotin-carboxylase and [3] acetyl-CoA transcarboxylase.

In prokaryotes and in plastids of some plants, the ACC is a multisubunit enzyme, whereas in eukaryotes the cytosolic isozyme and, in some instances also the plastid isozyme, are multidomain proteins. The latter contain three major functional domains, which account for the biotin carboxylase (BT), biotin carboxyl-carrier (BCC) and carboxyltransferase (CT) activities and, which are organized in one large polypeptide.

The chloroplastic ACC is responsible for the synthesis of malonyl-CoA then metabolized to a fatty acid chain up to C_{18}. This is in part exported to the cytoplasm, thus contributing to the control of flux through the plant's *de novo* fatty acid biosynthetic pathway [8].

The cytoplasmic malonyl-CoA pool is dispatched into the following:

- Long and very long chain fatty acids, which are elongation products of the C_{18} lipids,
- A large group, consisting of flavonoids, pigments and stilbene derivatives through to the synthesis of naringenin.
- N-malonyl-D-amino acids.

Figure 9.1 summarizes the role of acetyl-CoA carboxylase in plants.

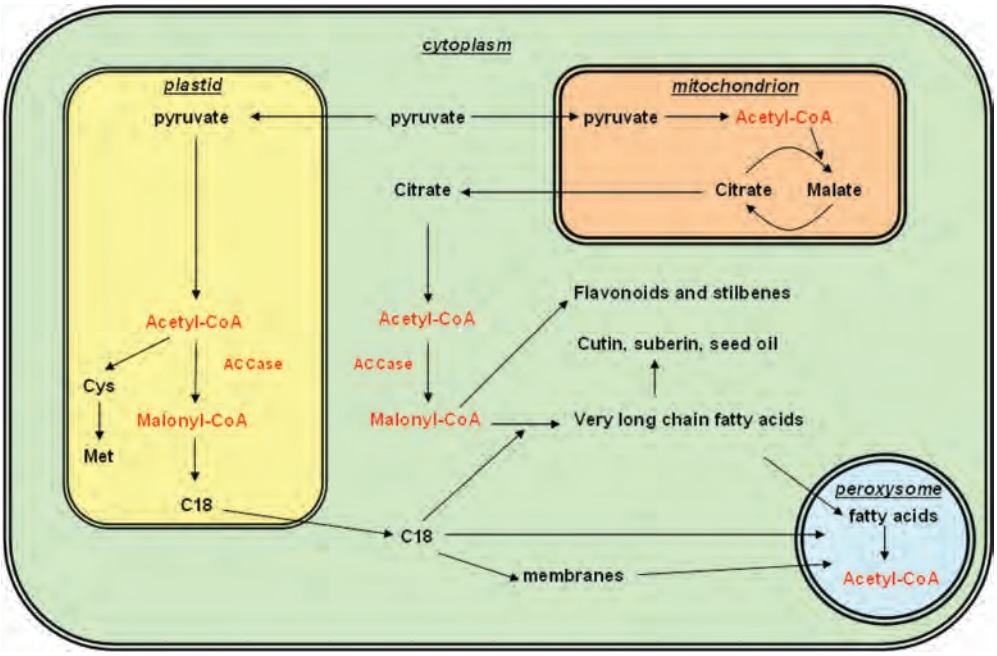


Fig. 9.1. Schematic drawing of the central metabolic role of acetyl-CoA in plants. Acetyl-CoA is the starting material for the biosynthesis of fatty acids, some amino acids, flavonoids, sterols, and isoprenoids. Acetyl-CoA does not cross membranes and is produced in the compartment where it is needed.

Grasses contain two multidomain ACC, one chloroplastic and one cytosolic, whereas dicots contain two well-differentiated forms, a cytosolic multidomain ACC and chloroplastic multisubunits ACC (Table 9.1) [9, 10].

The multisubunits enzyme is encoded by the nuclear DNA, with the exception of the β-subunit of carboxyltransferase that is encoded by a chloroplastic gene [11]. In grasses, the chloroplastic multidomain ACC is encoded by a nuclear gene, which is distinct from that coding for the cytosolic multidomain ACC.

Cytoplasmic and plastidic ACCs from wheat are 2260 amino acids and 2311 long, respectively, and their sequences are 67% identical [12]. A chloroplast targeting signal is present at the N-terminus of the multidomain plastid ACC from wheat [13], maize [14], and *Brassica napus* [15].

Numerous works have been published that attempt to analyze the quaternary structure of the multifunctional ACCs. It seems that the active enzyme has to be at least dimeric, either homodimeric or heterodimeric. Polymeric filaments (10–15 units) are also detected, which contain a heterodimeric subunit periodically interspersed throughout the otherwise homodimeric filamentous enzyme.

Table 9.1 Summary of different ACCs in plants.

Characteristics	Chloroplast	Cytoplasm
Grasses		
Type	Eukaryote type I, isoform 1	Eukaryote type I, isoform 2
Molecular mass	≈ 240 kDa	≈ 220 kDa
Native structure	Homodimer	Homodimer
Reaction	Three catalytic domains per protein	Three catalytic domains per protein
Major role	Fatty acid biosynthesis	Secondary metabolite biosynthesis
Dicots		
Type	Prokaryote type II	Eukaryote type I
Molecular mass	≈ 32–80 kDa	≈ 230 kDa
Native structure	Multicomponent enzyme	Homodimer
Reaction	One catalytic domain per enzyme	Three catalytic domains per protein
Major role	Fatty acid biosynthesis	Secondary metabolite biosynthesis

Furthermore, ACC isozymes are bound to the outer face of the mitochondria and in conjunction with other proteins, in particular carnitine phosphate transferase, a complex that regulates the flux of malonyl-CoA out of the mitochondria [16]. Recently, a specific ACC from yeast was shown to be targeted to mitochondria [17]. Moreover, Focke et al. [18] have presented biochemical evidence for a mitochondrial localized acetyl-coenzyme A carboxylase in barley.

It is not clear how many subunits and different proteins are contained in such complexes; the quaternary structure is likely to depend on the particular function of the ACC isozyme. In each organism, and even in each differentiated tissue, ACC quaternary structures will be dependent on genetic (isozymes and allelic variants), functional and activation factors. In others words, the biological role of the different isoforms of ACC determines the quaternary structure of the enzyme and associated proteins.

ACC regulation in cells is poorly understood, but redox control and phosphorylation are probably key factors. In pea, activation was found to be mediated through reduction of a disulfide bond between the α-CT and the β-CT subunits [19]. Plastid ACC is likely to be subject to redox regulation similar to that of several key enzymes of photosynthesis.

Phosphorylation of serine residue(s) of the β-subunit of the carboxyltransferase unit occurs in pea chloroplasts incubated in the light [20]. Alkaline phosphatase treatment reduces ACC activity in parallel to removal of phosphate groups from ACC. This activation by phosphorylation is opposite to the inhibition of animal ACC by phosphorylation but is consistent with the increase in ATP concentration and rates of fatty acid synthesis in chloroplasts in the light and the activation of other plastid enzymes by phosphorylation. These results suggest that the CT subunit reaction is rate determining for overall ACC activity, at least for the multisubunits enzyme of dicots.

In wheat cytosolic ACC none of the four conserved motifs containing serine residues corresponding to phosphorylation sites in rat, chicken, and human ACC [21] is present at a similar position.

9.2.2 Mode of Action of ACC Inhibitors

The first consistent study of an effect of ACC inhibitors on plant lipid biosynthesis was reported by Hoppe [22], and showed strong inhibition of incorporation of ^{14}C-labeled acetate into plant lipids. It was only in late 1987 that the two independent laboratories of Burton and Focke demonstrated that the site of action of these inhibitors was located in the acetyl-coenzyme A carboxylase. Burton et al. [23] found that ACC isolated from chloroplasts of corn seedling was inhibited by the herbicides sethoxydim and haloxyfop, with IC_{50} concentrations of 2.9 and 0.5 μM, respectively, whereas the ACC from pea chloroplasts was not inhibited by these inhibitors. Focke and Lichtenthaler [24] reported that the cyclohexane-1,3-dione derivatives cycloxydim, sethoxydim and clethodim inhibited fatty acid biosynthesis in a chloroplast enzyme preparation from barley when acetate and acetyl-CoA were the substrates, but not when malonate and malonyl-CoA were added. These results suggested that ACC was the site of action for these herbicides. Moreover, they showed that ACC from dicot species reported almost no inhibition, suggesting that the mechanism of selectivity between dicot and grass species was at the ACC site of action.

Two key papers [25, 26] established that the two types of ACC enzymes in plant correlated with the differential inhibition of the new herbicides represented by two classes: the AOPPs and the CHDs, which are strong inhibitors of the multidomain plastid ACC found in grasses. Prokaryotic-type multisubunit plastid ACC is resistant to these herbicides, as are eukaryote ACCs from animals and yeast.

Widely used commercial herbicides, represented by AOPPs and CHDs, are potent inhibitors of ACCs of sensitive plants and kill them by shutting down fatty acid biosynthesis, thus leading to metabolite leakage from the membranes and cell death [27]. AOPPs and CHDs inhibit the carboxyltransferase activity (Scheme 9.1, reaction [2]), thus blocking the transfer of the carboxyl group to acetyl-CoA [28]. They show nearly competitive inhibition with respect to the substrate acetyl coenzyme A [29].

This observation confirms that an inhibitor of the CT domain is sufficient to block the function of the ACC (Fig. 9.2), and it establishes this domain as a valid target for the development of inhibitors against these enzymes.

Interaction of AOPP and CHD inhibitors is an important tool to understand plant ACC biochemistry, and the use of chimeric genes was a significant step forward in the elucidation of differential activities for different chemical classes [12, 31]. Gornicki et al. showed that some determinants of sensitivity were located on a 400-amino acid fragment of wheat plastidic ACC in the CT domain [12]. The chimeric genes consisted of the yeast GAL10 promoter, the yeast ACC1 leader and the wheat acetyl-CoA carboxylase cDNA.

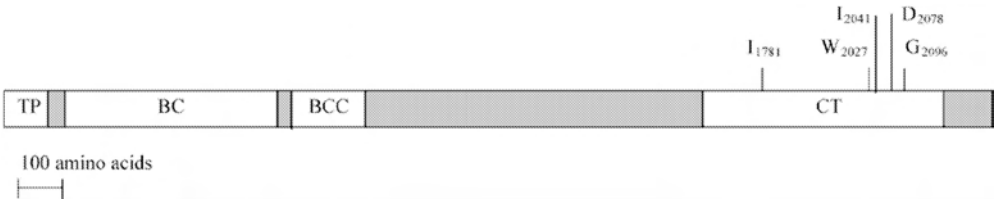


Fig. 9.2. Schematic representation of a plastidic homomeric acetyl coenzyme A carboxylase (ACC) showing the three functional domains (BC, biotin carboxylase; BCC, biotin carboxyl-carrier; and CT, carboxyl transferase) and the transit peptide (TP) that is absent in cytosolic ACC. The five amino acid residues critical for sensitivity to ACC-inhibiting herbicides have been referenced after the sequence from black-grass plastidic ACC (EMBL accession AJ310767). (From Ref. [30].)

The yeast ACC1 3′-tail was used to complement ACC1 null mutation. These genes encode a full-length plastid enzyme, with or without the putative chloroplast transit peptide, as well as five chimeric cytosolic/plastid proteins (Fig. 9.3).

Combining this yeast gene replacement strains system with kinetics values from purified plastidic proteins together provide a convenient tool to study herbicide interaction with the enzyme and a powerful screening system for new inhibitors.

Pinoxaden [32], the leading compound of the Ads, acts primarily on the plastidic homomeric ACC, but also exhibits new features.

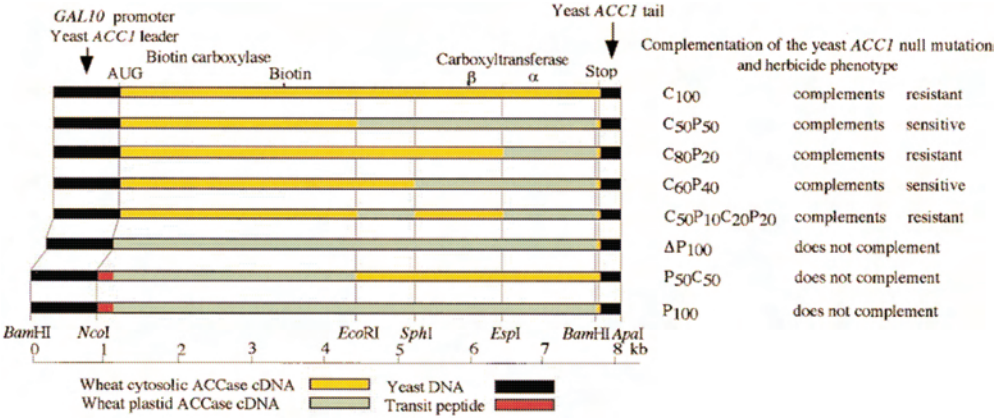


Fig. 9.3. Chimeric genes constructed for expression of wheat cytosolic, plastidic, and cytosolic/plastidic ACC in yeast. Construct names reflect the composition of the encoded proteins (C, cytosolic ACC; P, plastid ACC). Locations of key restriction sites used in the constructions are shown. (From Ref. [12].)

9.2.3 Resistance

The frequent use of AOPP and CHD graminicides has resulted in the development of resistance to these herbicides in some grass species throughout the world [33]. Up to now, 35 resistant species [34] have been reported. The species in which resistance has developed include the important grass weeds *Alopecurus myosuroides*, *Avena fatua*, *Setaria viridis*, *S. faberi*, *Lolium rigidum* and *Eleusine indica*.

Mechanisms of resistance to ACC-inhibiting herbicides can be divided into two categories: ACC-related and metabolism-based. Metabolism-based resistance is well described and reviewed in the literature [35, 36]. In most cases, resistance is due to alteration of the target enzyme, making it less sensitive to inhibition, as reviewed by Devine [37] and by Délye [38]; the latter gives an overview on homomeric plastidic ACC isoforms with altered sensitivities to AOPPs and CHDs or both [38]. Furthermore, the identification of mutations involved in altered sensitivity was achieved recently (Table 9.2) [38].

An updated version of this table is maintained at the International Survey of Herbicide Resistant Weeds Web site (http://www.weedscience.org).

A single point mutation leading to substitution of an isoleucine (Ile) by a leucine (Leu) residue at position 1781 within the CT binding domain of plastidic ACC in *Alopecurus myosuroides* (blackgrass) has been found to confer resistance to most CHDs and AOPPs [46]. A homologous mutation is responsible for target site resistance in three other grass weeds, *Lolium sp.* (Rye-grass) [43, 47], *Avena fatua* L. (wild-oat) [41], and *Setaria viridis* (green foxtail) [42].

The mutations leading to an isoleucine (Ile)-asparagine (Asn) exchange at position 2041, of tryptophan (Trp) in position 2027 to cysteine (Cys), as well as glycine (Gly) to alanine (Ala) in 2096, affects mainly the AOPPs in blackgrass and in ryegrass [44, 45]. In blackgrass again, aspartic acid (Asp) to glycine (Gly) mutation at position 2078 leads to resistance on APPs and CHDs [44].

Three-dimensional models of homodimeric ACC were reconstructed for a detailed evaluation of the effects of amino acid substitutions at positions 1781, 2027, 2041, 2078, and 2096 in black-grass ACC upon herbicide binding [48], using models built into maps obtained by electron crystallography of the yeast free ACC CT domain as templates [49].

All five amino acids given in Table 9.2 are located within the active site cavity of the ACC CT domain [48]. Only the substitution at position 2041 interferes directly with herbicide binding. It has been proposed that the other four mutations cause resistance by hampering inhibitor access to its binding site or by altering the spatial shape of the herbicide binding site [46]. Zhang et al. have determined the crystal structures of the CT domain of yeast ACC in complex with haloxyfop and diclofop [50]. The inhibitors are bound in the active site, at the interface of the dimer of the CT domain. Unexpectedly, inhibitor binding requires large conformational changes for several residues in the interface, which create a highly conserved hydrophobic pocket that extends deeply into the core of the dimer.

Table 9.2 Amino acid substitutions within plastidic, homomeric ACC and associated cross-resistance patterns observed at the whole plant level.[a]

Amino acid residue[b]			Resistance[a]									Ref.
			APPs[c]					CHDs[d]				
Wild-type	Resistant	Weed species[e]	Cd	Dc	Fx	Fz	Hx	Ct	Cx	Sx	Tk	
Ile_{1781}	Leu	*Alomy*	S	R	R	R	S	S	R	R	R	39, 40
	Leu	*Avefa*	ND	R	ND	ND	ND	ND	ND	R	ND	41
	Leu	*Setavir*	ND	R	R	ND	ND	ND	ND	R	R	42
	Leu	*Lol* sp.	S	R	R	ND	ND	ND	R	ND	ND	43
Trp_{2027}	Cys	*Alomy*	R	ND	R	ND	R	S	S	ND	ND	44
Ile_{2041}	Asn	*Alomy*	R	ND	R	ND	R	S	S	ND	ND	45
	Asn	*Lol* sp.	R	R	ND	ND	R	ND	S	ND	ND	45
	Val	*Lol* sp.	S	ND	ND	ND	R	ND	S	ND	ND	45
Asp_{2078}	Gly	*Alomy*	R	ND	R	ND	R	R	R	ND	ND	44
Gly_{2096}	Ala	*Alomy*	R	ND	R	ND	R	S	S	ND	ND	44

[a] S and R respectively indicate that plants containing at least one copy of the ACC mutant allele are sensitive or resistant to the corresponding herbicide either in the field or in bioassays (see text for comment). ND, not determined at the whole plant level.
[b] Amino acid number is standardized to *A. myosuroides* plastidic, homomeric ACC (EMBL accession AJ310767).
[c] Cd, clodinafop; Dc, diclofop; Fx, fenoxaprop; Fz, fluazifop; Hx, haloxyfop.

The mutation of two residues that are located in this binding site and affect herbicide sensitivity disrupts the structure of the domain.

9.2.4 Detection of Resistance

To date, detection and management of resistance has predominantly been carried out with bioassays. These are essentially based on comparative growth of seedlings or plants of suspected resistant and sensitive weed biotypes subjected to different herbicide treatments [51–53]. Such bioassays are simple, but do not differentiate between target site and metabolic resistance mechanisms.

ACC-based resistance is expressed in pollen, whereas metabolism based is not [53, 54].

The main I1781L mutation leading to resistance can only occur by substitution of an adenine (A) by thymine (T) or cytosine (C) at the first position in the cognate codon. As a result, it was possible to develop a polymerase chain reaction

(PCR)-based allele-specific amplification assay to detect the I1781L mutation in the plastidic ACCase of *L. rigidum* and *A. myosuroides* plants, providing a quick and efficient method for monitoring a key resistance mechanism to ACC inhibitors in these species [43, 55].

Kaundun and Windass [56] described an alternative derived Cleaved Amplified Sequence (dCAPS) method [57] that can be used on several grass weeds and that offers the additional advantage of easy discrimination between homozygous and heterozygous L1781 mutation bearing plants.

9.3
Aryl-diones as Novel ACC Inhibitors

9.3.1
Discovery

The first 2-aryl-1,3-diones (ADs) were reported in 1977 by Wheeler (Union carbide) [58]. He claimed biocidal aryl-cyclohexenyl esters **1**, **2** (Fig. 9.4) with pre- and post-emergence herbicidal effects and miticidal activity against *Tetranychus urticae*.

Ten years later R. Fischer et al. (Bayer) discovered 2-aryl-indolizine-2,4-diones with herbicidal and miticidal activity [59] and reported compound **3** (Fig. 9.5) to inhibit plastidic ACC of grasses [6].

Almost simultaneously Cederbaum (Ciba-Geigy) [60] as well as Fischer and coworkers [61] claimed the herbicidal activity of 2-mesityl-tetrahydro-pyrazolo-1,3-diones **4**.

The Bayer research group described further heterocyclic diones (Fig. 9.5), which all belong to this chemical class: 3-aryl-pyrrolidine-2,4-diones **5** [62, 63], 3-aryl-furan-2,4-diones **6** [64], 2 aryl-cyclopentan-1,3-diones **7** [65] 4-phenyl-[1,2]oxazin-3,5-diones **8** [66].

Although interesting, all these AD derivatives were substantially weaker herbicides than commercial AOPPs and CHDs. Many of these compounds with good miticidal activities are reported to be phytotoxic [67]. A major breakthrough with regard to the herbicidal activity was achieved as aryl moieties bearing ethyl, ethynyl or methoxy groups in the 2,6-positions were synthesized [68]. Such a sub-

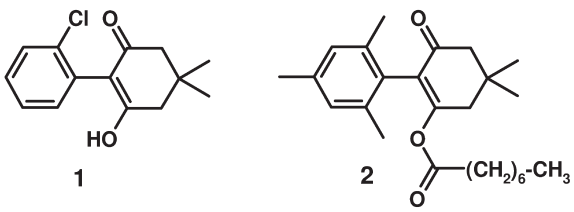


Fig. 9.4. Biocidal aryl-cyclohexenyl esters **1** and **2**.

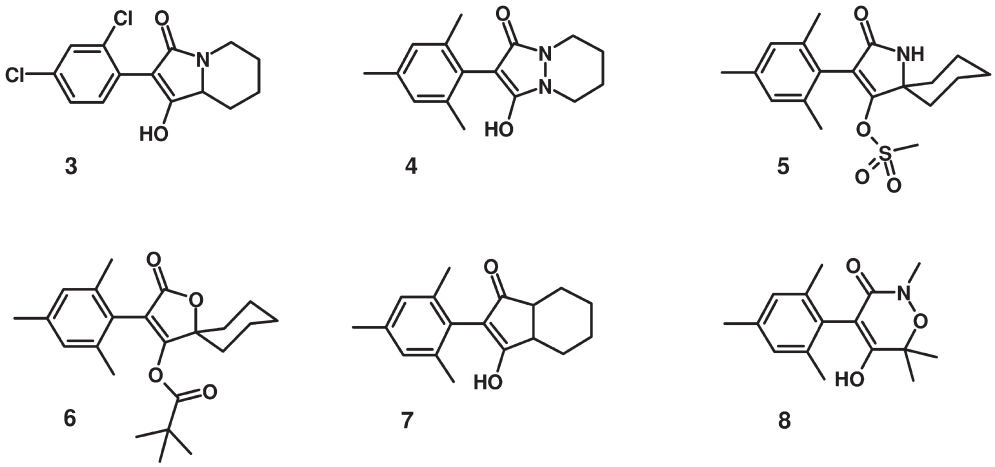


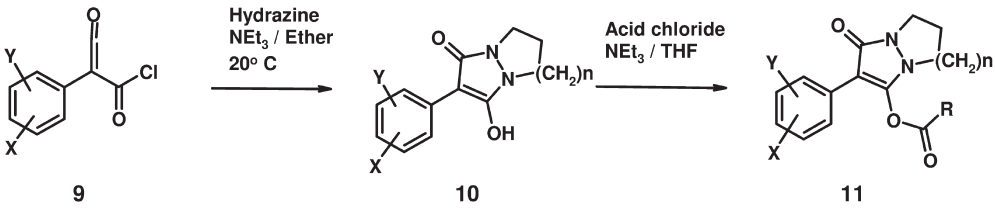
Fig. 9.5. Structures of compounds **3**–**8**.

stitution pattern boosts the herbicidal activity in combination with each type of 1,3-dione, whereas the miticidal activity is strongly reduced [69].

Further variations of the hydrazine moiety [70] ultimately led to pinoxaden (Scheme 9.5 below shows the synthesis of pinoxaden).

9.3.2 Syntheses

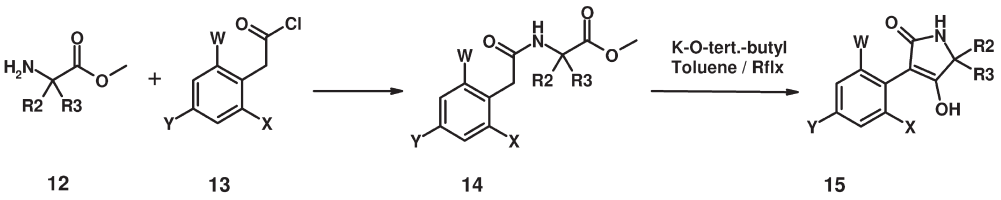
4-Aryl-pyrazolidin-3,5-diones **10** and their esters **11** were prepared as outlined in Scheme 9.2.



Scheme 9.2

Phenyl-substituted chlorocarbonylketenes **9**, first described by Nakanishi [71], represent a highly reactive equivalent of the phenyl-malonates. They react under mild conditions with hydrazines [65]. The ketoenol **10** is esterified with a standard method.

Scheme 9.3 depicts the synthetic route to tetramates **15** [60, 67].



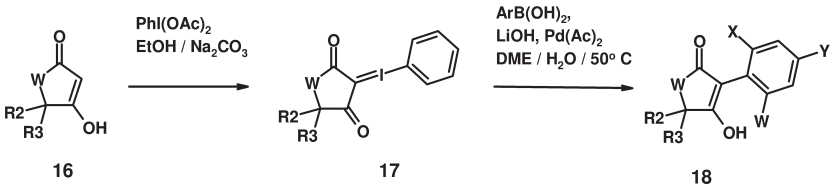
12 13 14 15

Scheme 9.3

Acylation of the amino acids **12**, (synthesized from a ketone via a Strecker amino acid synthesis) with aryl-acetyl chloride **13** leads to the intermediate **14**, which is cyclized to the tetramic acid **15** with potassium *tert*-butylate in refluxing toluene [62].

Indolizine-diones, tetronic acids, [1,2]-oxazin-3,5-diones and cyclopentane-diones were obtained with similar cyclization steps.

Alternatively, most AD derivatives can be prepared with a Suzuki coupling reaction between iodonium-ylides **17** and phenylboronates **18** (Scheme 9.4).



16 17 18

W = NH / O / S / N(Me)-O / methylene / ethylene

Scheme 9.4

The iodoniumylides **17** were obtained from diverse diones **16** and (diacetoxy)iodobenzene.

The yields of the cross-coupling reaction with sterically hindered arylboronates were modest [72]. However, many ADs were best prepared by this method since it allows convergent syntheses.

9.3.3 Structure–Activity Relationships

Very little data related to herbicidal activities of the ADs have been released [73, 74]. The overview given in this section reflects mainly the results based on studies with 4-aryl-pyrazolidin-3,5-diones [75], which were optimized towards activity and selectivity in small grain cereals.

The 2-aryl-1,3-diones can be separated into three parts for the analysis of the structure-activity relationships, namely the aryl-, the dione with its procidal forms

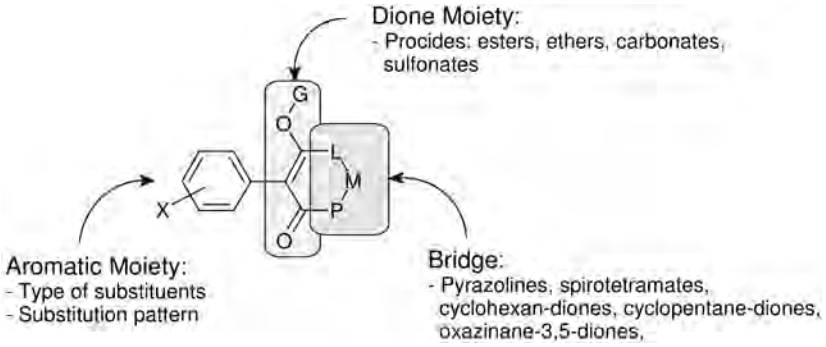


Fig. 9.6. Aryl-, dione (with its procidal forms), and bridging moieties of 2-aryl-1,3-diones.

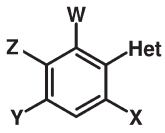
and the bridging moiety (Fig. 9.6). Each part can be examined separately as they appear to play distinct and different roles in the overall expression of the herbicidal activity.

Only the free diones are active *in vitro* on ACC [76] and are responsible for the target site activity. With a pK_a of about 3.9, free diones are highly soluble in water.

Various prodrugs have been synthesized, with the aim of increasing the penetration into the leaves. Carbamates and most ethers are weakly active. However, carbonates are less active than the free dione. Since most esters hydrolyze easily, the free dione is usually equally active. A slight increase of activity and less variability was observed with aliphatic or aromatic sulfonates and the pivaloyl-esters and their homologs.

The substitution pattern of the aryl moiety strongly influences the overall herbicidal activity (Fig. 9.7). 2,4- and 2,6-Dihalo-aryl derivatives are all very weak compounds. 2,6-dimethyl-phenyl and 2,6-diethylphenyl substitution lead to some activity, whereas the 2,4,6-trimethyl pattern give a fair control of grasses at 100 g-a.i. ha^{-1} if combined with an optimized dione.

A 2-ethyl group leads to an increased activity and a 2,6-diethyl-4-methyl substitution pattern boosts the graminicidal activity. The level of activity can even be improved with a 2-ethynyl or a 2-methoxy-substituent in a 2,4,6-pattern. However, these two functionalities induce a higher level of phytotoxicity in cereals. Interestingly, 2,6-dimethoxy or 2,6-dibromoaryl ADs were found to be almost inactive.



W : methoxy = ethinyl > ethyl = ethylen > bromo = methyl > propyl >> -CF_3

X : ethyl > methyl

Z : -H > -F >> -Cl > -Br

Y : phenyl > methyl > ethyl >> halogen

Fig. 9.7. Summary of the influence of the substitution pattern of the aryl moiety on overall herbicidal activity.

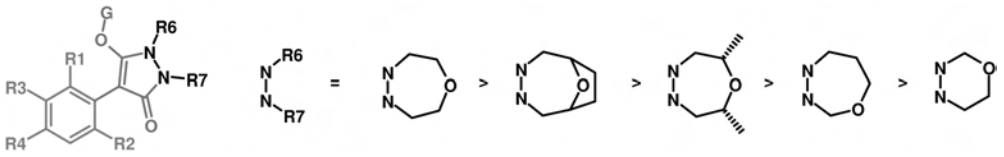


Fig. 9.8. Relative activity of cyclic hydrazines.

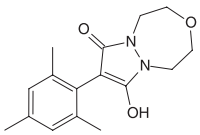
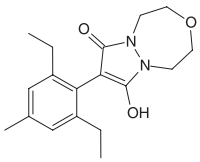
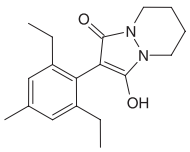
Position Y must be functionalized. Compared with methyl, a phenyl group or a thiophene leads to a broader spectrum, but also to phytotoxicity on cereals. In parallel, some activity on broadleaf weeds and a strong inhibition on cytosolic ACC are observed.

Introduction of a halogen in the position Z leads to a decrease of the herbicidal activity (Fig. 9.7).

High levels of herbicidal activity on grasses were found with many types of diones such as tetramates, cyclopentane-diones, cyclohexanediones or oxazin-3,5-diones linked to 2,6-diethyl-4-methyl-benzene.

With pyrazolines bridges, cyclic hydrazines are clearly the most active derivatives. There is not a great difference between five-, six- and seven-membered rings. However, [1,3,4]oxadiazinane and diverse oxadiazepanes derivatives were

Table 9.3 Compounds were applied with the adjuvant A12127 used at 0.5% at a rate of 60 g ha^{-1} on barley and wheat, 30 g ha^{-1} on *Alomy* (*Alopecurus myosuroide*), *Avefa* (*Avena fatua*), *Lolpe* (*Lolium perenne*), *Setfa* (*Setaria faberi*) at 2 leaf stage.

Structure	Barley	Wheat	Alomy	Avefa	Lolpe	Setfa
	0	0	10	60	80	50
	0	10	100	100	100	90
	80	80	100	100	80	90

found to increase the activity and to have better selectivity in all cereal crops, the [1,4,5]oxadiazepane being the most active (Fig. 9.8).

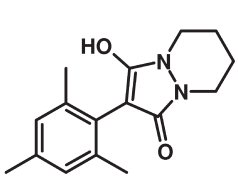
Table 9.3 summarizes the optimization in the aryl-pyrazolines series. It demonstrates the effect of the aryl substitution with regard to the level of activity and the influence of the ring oxygen atom in the bridging moiety on the selectivity.

9.3.4
AD versus AOPP and CHD on ACC

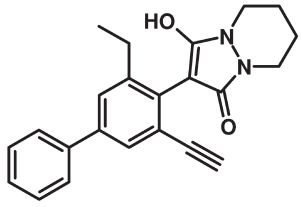
AOPP and CHD herbicides are far more potent inhibitors of chloroplastic ACC than they are of the cytosolic ACC. Quizalofop is reported to inhibit maize chloroplastic ACC (IC50 0.03 μM) some 500-fold more strongly than the cytosolic form of maize ACC (IC50 ~ 60 μM); a similar differential was reported in the same paper for fluazifop [77]. Similarly, Joachimiak et al. indicate that CHDs (sethoxydim) and AOPPs (haloxyfop) inhibit the chloroplastic form of wheat ACC at least 50-fold more potently than the cytosolic [31].

Our own unpublished data paint a similar picture in respect of clodinafop and propaquizafop, with both of these AOPPs inhibiting the cytosolic ACCase only relatively very weakly (with IC_{50}s > 300 μM) [78]. Interestingly, in contrast, some of the aryl diones appear to be quite potent inhibitors of the cytosolic enzyme. For example, the ADs SYN 271312 and SYN 436752 (Fig. 9.9) exhibit IC_{50}s of only 2 and 0.3 μM versus the cytosolic maize ACC [78]. The potency of these two compounds was further confirmed in gene replacement studies, according to Joachimiak et al. [31]. Unlike sethoxydim, clodinafop or propaquizafop, both ADs potently inhibited the growth of ACC1 null mutant yeast strains expressing the wheat cytosolic ACC (Table 9.4) [78].

At the primary sequence level the cytosolic ACC of dicot plants is similar to that in grasses and, accordingly, appears to exhibit an overall similar degree of sensitivity to inhibitors (for example quizalofop inhibits pea cytosolic ACC with a K_i of ~7 μM versus ~50 μM for maize cytosolic ACC [77]). Accordingly, the two ADs, SYN 271312 and SYN 436752 were also relatively potent inhibitors of pea cytosolic ACC, exhibiting IC_{50}s of 15 and 1 μm, respectively. Furthermore, these two compounds also exhibited significant glass house activity versus broadleaves,



CGA 271312



NOA 436752

Fig. 9.9. Inhibitors of the cytosolic enzyme.

Table 9.4 Growth inhibition (in %) by herbicides (10 μM) of yeast gene-replacement haploid strains expressing chimeric wheat ACCs. Protein names reflect composition of C (cytosolic ACC) and P (plastid ACC). Work in collaboration with P. Gornicki.

Chimeral proteins	AOPP		CHD	AD	
	Clodinafop	Propaquizafop	Sethoxydim	SYN 271312	SYN 436752
C100	0	0	0	63	99
C50/P50	98	93	99	94	100
C80/C20	0	0	0	98	100
C60/P40	98	97	100	96	99
C50/P10/C20/P20	0	0	2	99	100

indicating that AD chemistry may also offer potential for control of dicot as well as of grass weeds.

9.3.5
AD on Herbicide Resistant ACC

As discussed in Section 9.2.3, resistance is a relevant feature for a novel chemical class.

In a recent paper Shukla attempted to identify molecules that target herbicide-sensitive and -resistant forms of ACC [80]. Among several experimental and commercial compounds, all the tested substances inhibited ACC from sensitive biotypes of *Setaria viridis* (green foxtail) and *Eleusine indica* (goosegrass). The I_{50}s of

Table 9.5 I_{50}s and R/S I_{50} ratios for ACC from herbicide-sensitive and -resistant biotypes of *Setaria viridis* (S1, sensitive; R1 and R2, resistant) and *Eleusine indica* (S3, sensitive; R3, resistant) and maize.

Herbicide	*Setaria viridis*					*Eleusine indica*			*Maize*		
	I_{50} (μM)			I_{50} (μM)		I_{50} (μM)			I_{50} (μM)		
	S1	R1	R1/S1	R2	R2/S1	S3	S4	R3/S3	S4	R4	R4/S4
Cpd. 8	51	67	1.3	54	1	76	227	3	18	40	2.2
Sethoxydim	54	>100	>1.9	89	1.7	39	407	11	0.9	>100	>111
Fluazifop						56	>500	>90	1.5	55	37
Diclofop	0.7	28	47								

most compounds assayed against resistant ACCs were higher than those against the corresponding sensitive ACC, indicating reduced binding to the resistant enzyme. However, compound **8** (Fig. 9.5), which belongs to the Ads, was almost equally effective against resistant and susceptible enzymes (Table 9.5) of green foxtail (*Setaria veridis*) and goosegrass (*Eleusine indica*). Additionally, this AD had similar I_{50}s on ACC from wild-type (S4) and sethoxydim-resistant biotypes of maize.

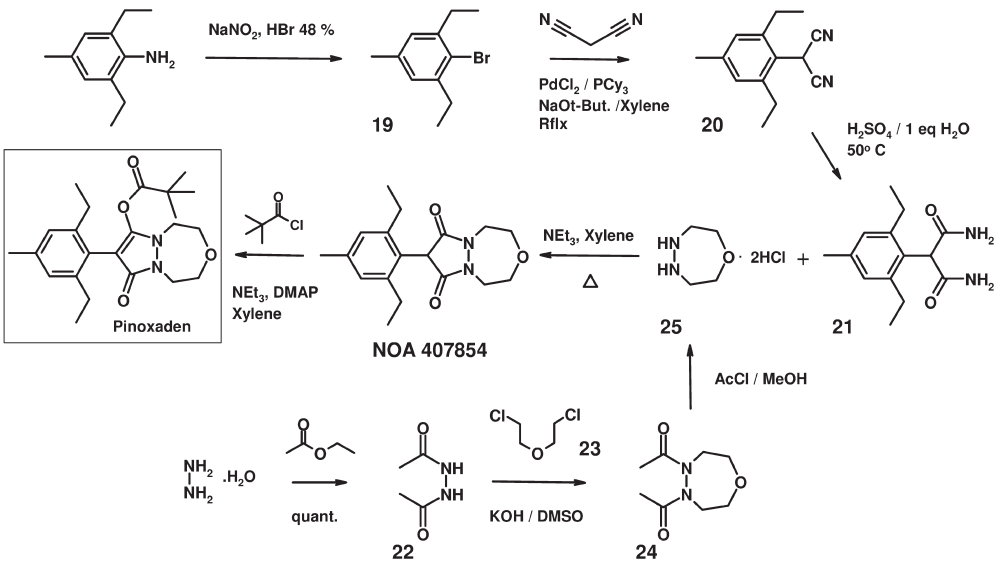
9.4 Pinoxaden

9.4.1 Characteristics

Pinoxaden is a new graminicide for cereal crops developed by Syngenta [7]. It belongs to the AD chemical class. Table 9.6 summarizes its physicochemical and toxicological data.

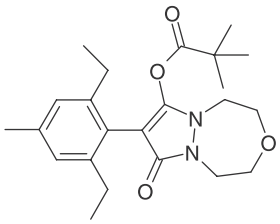
9.4.2 Technical Synthesis

A convergent synthetic route (Scheme 9.5) leads to the intermediate NOA 407854, which is esterified in the last step with pivaloyl chloride.



Scheme 9.5

Table 9.6 Properties of pinoxaden.



Physicochemical properties	
Common name	Pinoxaden (provisionally approved by ISO)
Company code	NOA 407855
Melting point	121 °C
Partition coefficient octanol–water	Log $P_{OW} = 3.2$
Solubility	200 mg L^{-1} in water
Vapour pressure	4.6×10^{-7} Pa
Toxicological profile	
Rat: Acute oral LD_{50} (mg kg^{-1} bw)	>5000
Rat: Acute dermal LD_{50} (mg kg^{-1} bw)	>2000
Rat: Inhalation LC_{50} (mg L^{-1})	5.2
Skin and eye irritation	Irritant
Ecotoxicological & environmental profile	
Birds: acute LD_{50} (mg kg^{-1} bw)	>2250: negligible risk to birds
Earthworms: LC_{50} (mg kg^{-1} dry soil)	>1000: non-toxic
Bees: LD_{50} (μg per bee; contact)	>100: safe to bees
Aquatic organisms	No risk to algae, fish and daphnia
Non-target flora and fauna	No risk to dicot plants and no adverse effects against beneficial arthropods

The precursor aryl-malonamide **21** is prepared in a three-step procedure from 2,6-diethyl-toluidine. A technically feasible cross-coupling reaction has been developed for the synthesis of aryl malononitrile **20** starting from benzene derivative **19** and malononitrile. The optimized procedure with $PdCl_2$/tricyclohexylphosphine and sodium *tert*-butoxide as base in refluxing xylene [81] was improved even further using palladium dichloride/triphenylphosphine as catalyst and sodium hydroxide as base in 1-methyl-2-pyrrolidone at 125–130 °C [82]. The aryl-malononitrile **20** is hydrolyzed to the aryl-malonamide **21** in conc. sulphuric acid.

The [1,4,5]oxadiazepane dihydrochloride **25** is obtained in three steps. Refluxing hydrazine hydrate in ethyl acetate [83] generates N,N′-diacylhydrazine **22**. A cyclocondensation with ether **23** in DMSO [84] followed by the acid-catalyzed hydrolysis of **24** provides the oxadiazepane **25**.

The pyrazoline-dione NOA 407854 is prepared by refluxing aryl-malonamide **21** with oxadiazepane **25** and triethylamine in xylene [85].

9.4.3 Biology

Pinoxaden is applied post-emergence at use rates of 30–60 g-a.i. ha^{-1} [7]. Interplay of the active compound with a safener proves essential to maximize the tolerance [7]. Methyl oleate as adjuvant enhances the level of activity without impairing the crop safety [86].

Pinoxaden is applied flexibly from the two-leaf up to the flag leaf stage of grasses [7]. Its weed spectrum covers a wide range of key annual grass species like *Alopecurus myosuroides* (blackgrass), *Apera spica venti* (silky bent grass), *Avena* spp. (wild oats), *Lolium* spp. (ryegrass), *Phalaris* spp. (canary grass), *Setaria* spp. (foxtails) and other monocot weed species commonly found in cereals [7].

In an uptake experiment, over 90% of the radiolabeled pinoxaden was incorporated into the crops within 5 h when treatment solutions were applied in droplets to the adaxial leaf surface of two-leaved plants of barley, winter wheat or durum wheat. After 24 h, about 20% is translocated out of the treated leaf by basipetal movement below the treated area [87]. Cloquintocet does not affect the absorption or the movement of the herbicide within the crop.

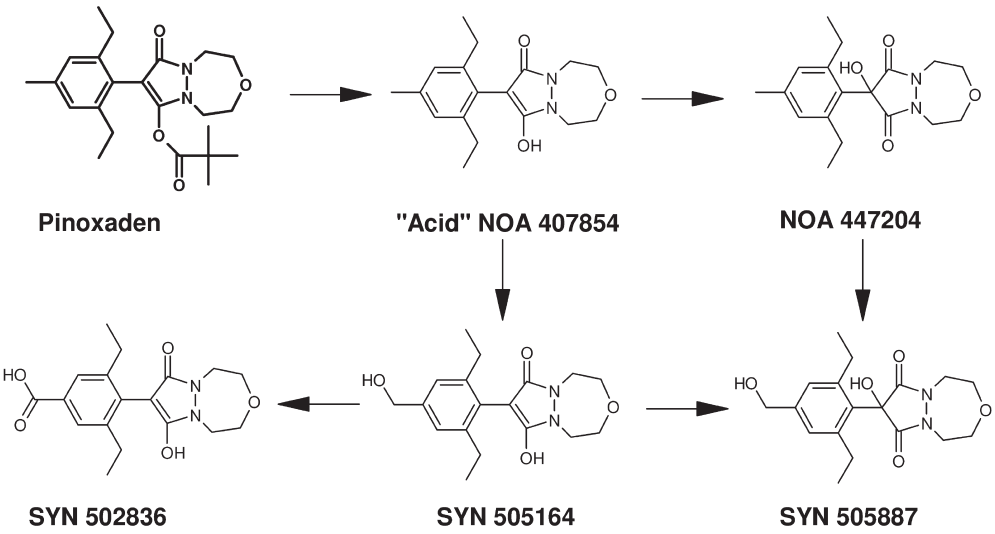
While active against certain ACC-resistant biotypes, both target site and metabolic resistant, pinoxaden is not active on all of them [7].

9.4.4 Metabolism and Selectivity

The total radioactive residues in winter wheat treated in autumn applications under out-door conditions declined rapidly in forage from 6.7 mg kg^{-1} on day 1 to 0.3 mg kg^{-1} 14 days after treatment (DAT). Ultimately, the total residues in grain, husks and straw at maturity were low. Scheme 9.6 gives the major detected metabolites and a proposed metabolic pathway [88].

Pinoxaden is hydrolyzed within a very short time to the parent acid, which is rapidly hydroxylated to the major metabolite found in plants, SYN 505164. The benzylic alcohol is oxidized to a large extent to the acid SYN 502836 or glycosylated and further conjugated. NOA 447204, which is the primary and main metabolite in soils, is also found in plants at lower levels. It is hydroxylated to SYN 505887. All the metabolites, except the parent acid NOA 407854 were inactive *in vitro* tests on plastidic wheat ACC and did not have any phytotoxic effect on emerged grasses and cereals in greenhouse trials, even at higher rates.

The effect of the safener cloquintocet-mexyl on the biokinetics and metabolism of pinoxaden in barley, winter and durum wheats, *Avena fatua* and *Lolium rigidum* was studied with the radiolabeled herbicide [89]. Safening is achieved by enhancing the metabolism of pinoxaden within the crop (Table 9.7).



Scheme 9.6

The safener mainly triggers the hydroxylation of the methyl group of NOA 407854 to SYN 505164 in all cereal crops, but does not seem to affect the hydroxylation of the dione to NOA 447204. Cloquintocet has no relevant effect on the metabolism of pinoxaden in the grass weed *Lolium rigidum* or in *Avena fatua*.

Table 9.7 Pinoxaden was applied at a rate equivalent to 90 g ha^{-1} with the adjuvant A12127 used at 0.5%. Leaves treated with 20 × 0.2 mL droplets containing ^{14}C-labeled pinoxaden at 4000 dps. Treatments were made up with or without cloquintocet-mexyl (S) added at 25% the rate of the herbicide (H). Barley (*cv Manitou*), Winter wheat (*cv Soisson*), Durum wheat (*cv Colossea*).

	Barley		**Winter wheat**		**Durum wheat**		***Lolium rigidum***		***Avena fatua***	
	H & S	**H**	**H & S**	**H**	**H & S**	**H**	**H & S**	**H**	**H & S**	**H**
Pinoxaden	1.5	2.1	0	0	0.8	0.3	0	0	0	0
SYN407854	32.3	48.3	36	81.6	48.8	79.1	84.2	86.6	84.7	90.4
Total parent	33.8	50.4	36	81.6	49.7	79.4	84.2	86.6	84.7	90.4
SYN505164	38.3	25.5	59.8	13.8	35.6	14.7	3.8	3.5	11.1	7.1
SYN447204	8.3	8.7	4.2	4.1	5.8	3.1	4.2	3.5	3.2	2.5
Others	19.6	15.4	0	0.5	9	2.8	7.8	6.4	1	0
All metabolites	66.2	49.6	59.8	18.4	50.4	20.6	15.8	13.4	15.3	9.6

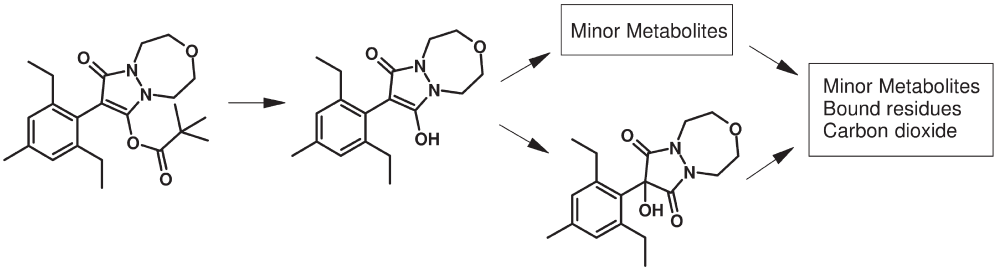


Fig. 9.10. Soil metabolism of pinoxaden.

Figure 9.10 summarizes the soil metabolism of pinoxaden [88].

Pinoxaden hydrolyses very rapidly in soil to NOA 407854 with half-lives below one day under aerobic, aerobic–anaerobic, and sterile–aerobic conditions.

NOA 407854 is highly soluble in water at neutral pH, but is rapidly hydroxylated to NOA 447204, which is almost insoluble. The half-life of NOA 407854 varies from 1.8 to 6.1 days, depending on the type of soil, whereas NOA 447204 degrades with a half-life of 6.2 to 37 days.

Diverse minor metabolites accounted for less than 5% of the applied radioactivity. The bound residues reach a maximum of 49% after 14 to 30 days.

The only identified volatile metabolite was carbon dioxide, demonstrating mineralization. Up to 47.6% of the applied radioactivity was mineralized after 100 days in laboratory soil metabolism studies. Finally, minimal dissipation has been observed in soils.

9.5 Summary and Outlook

Three classes of commercial herbicides, the AOPP, the CHD and the newly discovered AD derivatives, inhibit the CT function of the eukaryotic ACC found in the plastids of grasses [3, 4]. Interestingly, ACC is a target site of the novel insecticides spirodiclofen and spiromesifen [90, 91], which also belong to the chemical class of AD.

Progress in function elucidations of ACC inhibitors has largely contributed to the understanding and the differentiation of resistance mechanisms [30].

Considerable effort has been undertaken in recent years to elucidate the mode of action of herbicides on ACC at the molecular level. Point mutations in the

chloroplastic ACC CT domain of different monocots, principally *Lolium rigidum*, have been correlated with resistant phenotypes [44, 92, 93]. Fine mapping of these mutations in the sequence has led to the identification of those amino acids important for herbicide action in the CT domain. At the same time, the CT domain of the protein, spanning the domain where the point mutations have been found, has been crystallized [49].

Taken together, both approaches have allowed the prediction of the herbicide binding to the CT domain. In particular, it was possible to determine amino acid changes responsible for herbicide resistance to AOPP and/or CHD analogues and localize the amino acid directly involved in the binding of herbicides, but only for this domain [50].

AOPPs were shown to bind inside the active site cavity of ACC CT dimers. Binding of one AOPP molecule inside one of the two active sites of a CT dimer caused conformational changes in the structure of the whole dimer. The general binding mode of AOPP and CHD inhibitors inside the CT active site cavity is very likely to be similar for both plastidic and cytosolic isoforms given the conservation among homomeric ACC proteins. However, the emergence of a new chemical class such as the AD shows differences, which have led to the inhibition of the dicot cytosolic enzyme and the opening of new paths for research.

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10
Photosynthesis Inhibitors: Regulatory Aspects, Reregistration in Europe, Market Trends and New Products

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10.1
Introduction

Herbicides acting as inhibitors of photosynthesis by blocking of electron transport in photosystem II belong to the eldest classes of plant protection agents. These compounds are still of market relevance, especially in developing countries, but they are out of the focus of modern herbicide research due to their high application rates in response to the high enzyme concentration for photosynthesis in plants and their cross-resistance behavior.

Photosynthesis inhibitors are divided into the compound classes of triazines, triazinones, the newest one triazolinones (see amicarbazone, Section 10.6.1), uracils and phenylcarbamates belonging to the C 1 group of HRAC classification scheme, the arylureas and amides belonging to the C 2 group, and the nitriles, benzothiadiazinones and phenylpyridazines in the C 3 group of the HRAC classification [1]. Photosynthesis, which takes place in the chloroplasts, was already recognized as the principle of ''assimilation of carbon dioxide'' by plants in the mid-19th century but the individual reaction steps were evaluated and well understood with the research of Hill in 1937 and starting in the 1950s [2, 3], mainly via the investigations with these inhibitors, especially the ureas [4–7], the triazines [8–10] and the triazinones [11–13] between 1956 and 1975. Already in 1961, M. Calvin, University of California, had won the Nobel Prize in chemistry ''for his research on the carbon dioxide assimilation in plants'' and investigations of the light-dark reactions in photosynthesis [14] and the synthesis of carbohydrates from CO_2. But also the modern protein structure chemistry and the investigations via X-ray with the description of binding niches and inhibitors binding in it were started with the ubichinon binding pocket in the photosynthesis and led to the Nobel prize for chemistry in 1988 being awarded to H. Deisenhofer, R. Huber and H. Michel [15].

Since the Hill reaction (Scheme 10.1) permits the quantitative determination of the inhibitory properties of photosynthesis blockers on chloroplast systems by measurement of O_2 evolution (oxygen electrode, Warburg manometer) and thus

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

$$H_2O + A \longrightarrow AH_2 + 1/2\ O_2$$

Scheme 10.1. Hill reaction scheme.

the 50% inhibitory concentration of a photosynthesis inhibitor in the Hill reaction, these values (in their negative logarithm as pI_{50} values) can be used in quantitative structure–activity studies (QSAR) regarding the *in vitro* activity (''QSAR; Hansch approach'' [16, 17, 20–22]) and can be compared with their pI_{50} values in greenhouse trials to evaluate biochemical activities versus biological activities [describing and including transport, membrane and metabolism effects (ADME)]. Consequently, QSAR, as a method to improve the biological activities in synthesis programs for inventing new crop protection compounds, was investigated broadly and approved first in photosynthesis research programs [18, 19].

The 1,3,5-triazines were invented first in the Geigy laboratories [23, 24], with simazine (1955) as the first representative of this group followed by atrazine (Geigy, 1958), propazine (Geigy, 1960), trietazine (Geigy, 1960), terbutylazine (Geigy, 1966) and cyanazine (Shell, 1971) from the 2,6-diamino-4-chloro-1,3,5-triazines, prometryne (Geigy, 1962), ametryn (Geigy, 1964), desmetryne (Geigy, 1964) and terbutryne (Geigy, 1966) from the 2,6-diamino-4-methylmercapto-1,3,5-triazines, and terbumeton (Geigy, 1966) from the 2,6-diamino-4-methoxy-1,3,5-triazines.

The 1,2,4-triazinones [25], metamitron (Bayer, 1975) and metribuzin (Bayer, Du Pont, 1971) and the 1,3,5-triazine-2,4(1H,3H)-dione [26] hexazinone (Du Pont, 1975), resulted first from the resynthesis of university publications or analogue synthesis using uracils as starting ideas.

The uracils [27] bromacil (Du Pont, 1952), lenacil (Du Pont, 1974) and terbacil (Du Pont, 1966) were invented in the Du Pont laboratories, whereas the pyridazinones [28, 29], pyrazon (BASF, 1962) came out from research investigations in BASF and Sandoz laboratories. The phenylcarbamates [32] desmedipham and phenmedipham invented by Schering AG are also included in the C1 group (HRAC classification) (Fig. 10.1).

The herbicidal effect of aryl- and hetarylurea, systematically studied starting from first observations in 1946, was improved between 1951 [30] and 1973 [31]. From this chemistry today the compounds chloroxuron (Ciba, 1960), dimefuron (Hoechst, 1969), diuron (Du Pont, 1954), ethidimuron (Bayer, 1973), fenuron (Du Pont, 1957), fluometuron (Ciba, 1960), isoproturon (Hoechst, 1974), linuron (Hoechst, 1960), methabenzthiazuron (Bayer, 1968), metobromuron (Ciba, 1963), metoxuron (Sandoz, 1968), monolinuron (Hoechst, 1958), neburon (Du Pont, 1957), siduron (Du Pont, 1964) and tebuthiuron (Elanco, 1973) are still used.

The amides propanile [33] and pentanochlor [34], also belonging to the C2 group (HRAC classification) fulfill the general formula for photosynthesis inhibitors bearing an CONH group (Fig. 10.2).

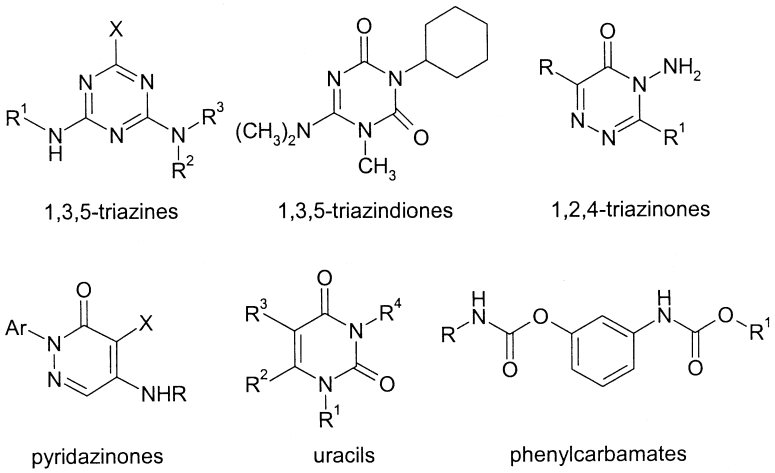


Fig. 10.1. PS II inhibitors, C1 group.

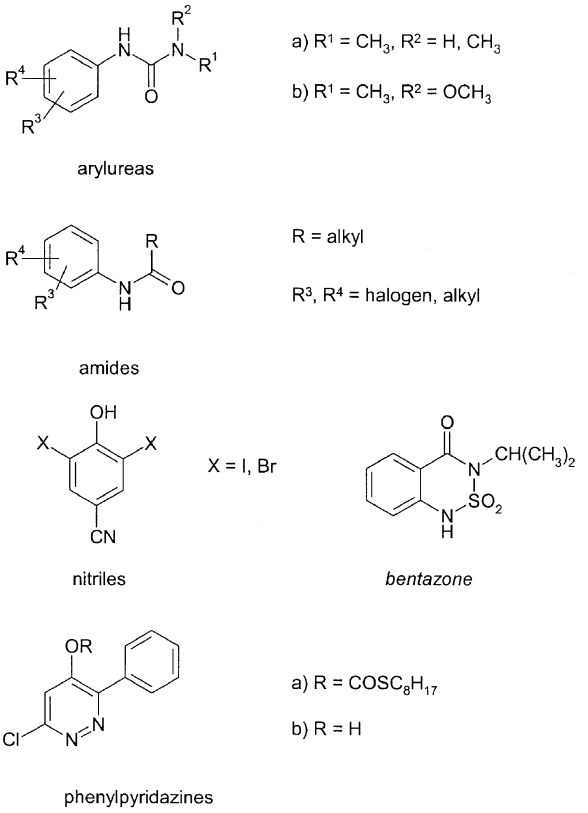


Fig. 10.2. PS II inhibitors, C2 and C3 group.

The photosynthesis inhibitors of the C3 group (HRAC classification), the nitriles [35] bromofenoxim, bromoxynil, ioxynil, as well as the benzothiadiazinone bentazone [36] and the phenylpyridazines [37] pyridate and pyridafol have completely different structures, without any CONH group (Fig. 10.2).

Whereas photosynthesis inhibitors represented nearly 50% of market share of all herbicides in 1980 [38] the situation has significantly changed, not only by the introduction of the newer ALS-inhibitors like the sulfonylureas, HPPD-inhibitors and the genetically modified crops resistant against EPSP-synthase and glutamine synthetase inhibitors but also through significant changes in reregistration requirements, especially in Europe.

10.2
The Reregistration Process in the European Union

The registration of agrochemicals falls under national laws of all the countries throughout the world were plant protection compounds are used. These national laws regulate the data requirements for active compounds as well as for formulations, mixtures etc., the risk assessment process and requirements for labeling the marketed plant protection product. Early on in the history of agrochemicals the companies inventing, developing and marketing plant protection compounds and products as well as the public were looking for harmonisation of data requirements and risk assessment for registration. Examples of supranational harmonisation activities are given in Tables 10.1–10.4.

Additionally, global harmonisation endeavors are undertaken by the FAO and WHO. The FAO supports harmonisation efforts, e.g., through the information system ''Prior Informed Consent'' (PIC). In this information system an exchange on certain hazardous pesticides and industrial chemicals in international trade takes place between member authorities. The members have agreed on an international code of conduct on the distribution and use of pesticides and on guidelines related to the development and evaluation of data considered in the registration process. Further, WHO (World Health Organisation) organizes joint meetings of their members together with the WHO on pesticide residues (JMPR) to define and organize the MRL Database on Pesticides, in which the maximum pesticide residue levels are documented. The WHO has developed the pesticides evaluation scheme ''WHOPES'' in which it establishes and publishes specifications for technical material and related formulations of public health pesticides. WHO reviews safety reports, issues, guidelines for laboratory and field evaluation of insecticides and repellents and gives recommendations on equipment and application manuals. It publishes health criteria (EHC) monographs on chemicals/pesticides, e.g., the WHO Classification of Pesticides by Hazard and the WHO/FAO Pesticide Datasheets (IPCS Inchem) [39].

The OECD published a vision document [on the occasion of the 14th meeting of the Working Group on Pesticides (WGP) held in Paris on 5th and 6th November 2002] with statements on achievements to-date in the international harmoni-

Table 10.1 Supranational Harmonisation Activities in EC, US and NAFTA.

Political union/ country	Responsible authority	Legislation	Object of registration	Time to registration
E.C./ countries of the E.C.	EU Commission, through the European Food Safety Authority (EFSA) National authorities of the different countries	Directives like Directive 91/414/ EEC, national laws like COPR, COP(A)R, PPPR, Deutsches Pflanzen-schutzgesetz etc.	Active ingredients (a.i.) regulated by EEC Directives (adopted to national laws). Products regulated by national laws	Up to 4 years until Annex 1 inclusion for a new active substance
USA/States	EPA	Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); Food Quality Protection Act (FQPA; 1996)	A.i. and products. States may register a new end use product or an additional use of a federally registered pesticide product under specific conditions	Up to 3–4 years
NAFTA	Technical Working Group of Pesticides (TWG). US-EPA, Canadian Pest Management Regulatory Agency (PMRA), a consortium of Mexican agencies (CICOPLAFEST)	Common data submissions for manufacturers – electronic harmonisation. Joint reviews. Eliminating trade problems related to differences in MRL (maximum residue limits)	A.i. and products	Subject to national timelines

sation of the regulatory approaches for agricultural pesticides (chemical and biological) and in the use of work sharing arrangements in examining and reporting on data submissions (dossiers) provided by industry as well as the use of country evaluations (monographs) to support applications for their registration or the re-registration or to support the establishment of MRLs or import tolerances for particular active substances. It also published a statement of their vision for the next ten years, including details of the specific objectives, milestones to be reached along the way, and the indicators and measures of success to be used to record and document progress achieved [40].

Table 10.2 Supranational harmonisation activities in Central- and South-America.

Political union/country	Responsible authority	Legislation	Object of registration	Time to registration
Central America				
Belize, Costa Rica, El Salvador, Honduras, Mexico, Nicaragua, Panama	Technical Regional Pesticide Working Group OIRSA	Harmonized registration data and labeling requirements FAO specifications and Codex Alimentarius Data exchange on efficacy within region	Products	Up to 2 years
South America				
(a) Andean Community Bolivia, Columbia, Ecuador, Peru, Venezuela (b) Mercosur Argentina, Brazil, Paraguay and Uruguay, Chile and Bolivia	National authorities	Common Pesticide Registration Manual (July 2002) "Norma Andina para el Registro y Control de Plaguicidas Quimicos de Uso" Agricola-Decision 436. Comision Andina. Gaceta Oficial del Acuerdo de Cartagena Ano XIV-No. 347 Lima, 17 June 1998 (based on FAO principles) "International Code of conduct for the distribution and use of Pesticides"	Products	Up to 2 years

By working together, OECD governments and industry are "sharing the burden" of testing and assessing high production volume chemicals, pesticides and, most recently new chemicals". OECD programs on harmonisation are leading to exchange of documents used in reregistration and registration in OECD countries, beginning already in 1992, by comparing pesticide data reviews, by working out OECD databases on pesticide and biocide review schedules, by issuing guidance on the preparation of dossiers and monographs, by undertaking joint reviews on new compounds like, for example, Project "Cornelia" on Bayer's corn

Table 10.3 Supranational Harmonisation Activities in Asia.

Political union/ country	Responsible authority	Legislation	Object of registration	Time to registration
Asia				
(a) Japan	MAFF	National Specific data requirements and test protocols	A.i. and products	Up to 4 years
(b) P. R. China/ Vietnam	National	Harmonisation of MRLs	A.i. and products	Up to 2 years
(c) South Korea, other	National	National Efforts in harmonisation through the Regional Network on Pesticides in Asia and the Pacific (RENPAP)	A.i. and products	Up to 2 years
India	National CIBRC	National Data generated for Indian Registration v/s Data needs of most developing countries match very well	A.i. and products	Up to two years, incl. late fixation of MRL by Ministry of Health
Australia	National	National Comparable to EU requirements	A.i. and products	Up to 2 years
New Zealand	National	National Comparable to EU	A.i. and products	Up to 2 years

herbicide foramsulfuron (Joint review between US-EPA, Canadian PMRA and German BvL, 2000–2002), by surveying best practices in the regulation of pesticides in twelve OECD countries and by recommending the electronic protocols used for data submission. Progress in harmonisation of data requirements and test guidelines are also achieved through surveying test guideline program (TGP) priorities for pesticides, minimum data requirements for establishing MRLs and import tolerances, guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies, etc. They stated the vision that by the end of 2014 the regulatory system for agricultural pesticides will have been harmonised to the extent that country data reviews (monographs) for pesticides

Table 10.4 Supranational harmonisation activities in Africa.

Political union/ country	Responsible authority	Legislation	Object of registration	Time to registration
Africa CSP (comite Sahelien des Pesticides) (Chad, Mali, Burkina Faso, Niger, Mauretania, Senegal, Cape Verde, Gambia and Guinea-Bissau)	CILSS		A.i. and products	Up to 2 years
SADC (Southern African Development Community) Angola, Botswana, Congo (DR), Lesotho, Malawi, Mauritius, Mozambique, Namibia, Seychelles, South Africa, Swaziland, Tanzania, Zambia and Zimbabwe		South Africa; Registration Act 36/ 1947 and Agricultural Remedies Registration Procedure Policy Document	A.i. and products	Up to 2 years

prepared in the OECD format on a national or regional basis (e.g., EU or NAFTA) can be used to support independent risk assessments and regulatory decisions made in other regions or countries.

In such a harmonisation process the EC enacted in 1991 the *"Council Directive of 15 July 1991 concerning the placing of plant protection products on the market" (91/414/EEC)*.

In this Directive the EC regulates the registration and reregistration of active ingredients and products for all countries in the EU. This Directive came into force on 26 July 1993 and must be implemented by national laws in all countries in the EU, e.g., in the UK by the Plant Protection Products Regulations 2003.

The main elements of the Directive are as follows:

- To harmonise the overall arrangements for authorization of plant protection products within the European Union.

This is achieved by harmonising the process for considering the safety of active substances at a European Community level by establishing agreed criteria for considering the safety of those products. Product authorization remains the responsibility of individual Member States.

The Directive provides for the establishment of a *positive list of active substances* (Annex I) that have been shown to be without unacceptable risk to humans or the environment.

New and existing active substances can be initially included to Annex I of the Directive for a period of 10 years pending their successfully passing the European Commission's (EC) review program.

Member States can only authorize the marketing and use of plant protection products after an active substance is listed in Annex I, except where transitional arrangements apply.

Before an active substance can be considered for inclusion in Annex I of Directive 91/414/EEC, companies must submit a complete data package (dossier) on both the active substance and at least one plant protection product containing that active substance. The data required is:

- Identification of an active substance and plant protection product.
- Description of their physical and chemical properties.
- Their effects on target pests.
- A comprehensive file of study reports to allow for a risk assessment to be made of any possible effects on workers, consumers, the environment and non-target plants and animals.

Detailed lists of the data required to be evaluated to satisfy inclusion in Annex I of the Directive, or the authorization of a plant protection product are set out in the Directive (Annexes II and III). Annex II data relate to the active substance and Annex III to the plant protection product. These data are submitted to one or more Member States for evaluation. A report of the evaluation is submitted to the European Food Safety Authority (EFSA). Following peer review of the report the EFSA makes a recommendation to the European Commission on whether Annex I inclusion is acceptable. This recommendation is then discussed by all Member States in the framework of the Standing Committee on the Food Chain and Animal Health (SCFA), previously the Standing Committee on Plant Health (SCPH). Where necessary, the Scientific Panel is consulted before the SCFA can deliver an opinion on whether an active substance should be included in Annex I of 91/414/EEC.

All member states are obliged to the ''*Uniform Principles*''.

The ''Uniform Principles'' (Annex VI of Directive 91/414/EEC) establishing common criteria for evaluating products at a national level were published on 27 September 1997 (OJ L265, p. 87). Application of the Uniform Principles ensures that authorizations issued in all Member States are assessed to the same standards.

The Directive states that all active ingredients should be reviewed periodically within 10 years.

This applies to all old agrochemical compounds (substances) used in a country of the EU prior to 1991 or before a country became a member of the EU (Reregistration). Thus all old photosynthesis inhibitors, for example, needed to be reviewed and the manufacturers had to apply for registration (listing on Annex I) by submitting dossiers prepared under the Directive 91/414/EEC [41]. A similar reregistration process was set from the US-EPA for all compounds on the market before 1984 in the US.

10.3 Main Changes in Guidelines regarding EU Registration

The following main changes have also to be applied on the preparing of registration data for such compounds, which have been registered in different countries based on dossiers regulated under the national laws before the reregistration procedure was enforced.

10.3.1 Good Laboratory Practice

Good Laboratory Practice (GLP) is concerned with the organizational process conditions under which studies are planned, performed, monitored, recorded; GLP ensures that the way the work is done is adequately standardized and of a sufficiently high quality to produce reliable results that can, with confidence, be compared with others carrying out the same work and applying the same general principles. Internationally accepted GLP guidelines, drawn up by the Organisation for Co-operation and Development, provided a reference point for later EU legislation [42]). The respective Directive applied to both active ingredients and formulated products, came into effect on 30 June 1988. The subsequent ''Authorizations'' Directive, 91/414/EEC, and others extended the scope of GLP, by requiring GLP compliance for all safety and efficacy studies whether conducted in the field or laboratory, and whether using formulated product or active ingredient.

10.3.2 Physical and Chemical Properties of Active Substance

Declaration of toxicological, ecotoxicological, or environmental significant *impurities* is needed. Especially hazardous chemicals like, for example, nitrosamines have to be declared and are regulated by a maximum admissible concentration. In this example, the total nitrosamine content of a pesticide formulation must not exceed 1 mg kg^{-1} of the active substance present.

Use and declaration of *analytical methods* have to be in correspondence with the ''Technical Material and Preparations: Guidance'' for generating and reporting

methods of analysis in support of pre- and post-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5). FAO guidelines (guidance on FAO specifications) and copies of specifications are available from www.fao.org/ag/agp/agpp/pesticid/ and GCPF (formally GIFAP) guidelines. Information on CIPAC can be obtained from www.cipac.org.

These guidelines apply to all studies started after *1st October 1999*.

Where an FAO specification is available for an active substance in a preparation, the tolerance limits must meet those in the FAO specification. However, where there is no appropriate FAO specification, tolerances must meet limits as accepted by the FAO Group of Experts [43, 44].

Where an active substance is present as an ester or a salt, the active substance content must be expressed as the amount of the ester or salt present (as the technical material) with a statement declaring the amount of the active principle.

The methods used for the determination of physical properties should be in accordance with the requirements of EC Directive 94/37/1.

10.3.3 Storage Stability

The data submitted must support the proposed shelf-life of the preparation. It is normally expected that a preparation should have a shelf-life of at least two years. Only where a preparation has a shelf-life of less than 2 years should the label include a ''Use by . . .'' date or other precautionary phrase.

Where a loss of $\geq$ 5% of active substance occurs then the fate of the active substance must be addressed and the breakdown products identified [45].

10.3.4 Physical and Chemical Characteristics of Preparation

The physical and chemical characteristics of preparations via parameters (e.g., explosive properties, oxidizing properties, flashpoint and other indications of flammability, acidity/alkalinity and pH, surface tension, density, wettability, suspensibility, dilution stability, dry and wet sieve test, particle size distribution and other properties of the formulation) and the corresponding methods have to be determined and reported in detail [46].

Specific new tests on viscosity and surface tension are guided by the Commission Directive 98/98/EC of 15 December 1998.

10.3.5 Operator Exposure Data Requirements

New regulations to protect the applicant of the plant protection products were brought into force, regulating data requirements, experimental details for the measurement and model calculations [47, 48].

10.3.6
Residue Data Requirements

The guidance documents embrace also the following aspects:

- Metabolism and Distribution in Plants (Appendix A).
- General recommendations for the design, preparation and realization of residue trials (Appendix B).
- Testing of plant protection products in rotational crops (Appendix C).
- Comparability, extrapolation, group tolerances and Data requirements (Appendix D).
- Calculation of maximum residue levels and safety intervals (Appendix E).

These documents are available on the European Commission website, at http://europa.eu.int/comm/food/plant/protection/resources/publications_en.htm

10.3.7
Estimation of Dietary Intakes of Pesticides Residues

Estimates of pesticide intake need to be made to compare potential consumer dietary exposure with acceptable dietary intakes derived from toxicological studies. At its most basic level, if estimates of long- and short-term intake are less than the acceptable daily intake (ADI) and the acute reference dose (acute RfD), respectively, then the risks to the consumer may be regarded acceptable.

The Guidelines and Criteria for the Preparation and Presentation of Complete Dossiers and Summary Dossiers for the inclusion of Active Substances in Annex I of Directive 91/414/EEC (Article 5.3 and 8.2) (Document 1663/VI/94) require that an estimate is made regarding the theoretical intakes of pesticide residues by consumers. Consumer risk assessment is a vital part of the approval process and it is in the applicant's interest to estimate potential intakes since intake estimates can assist in assessing whether further information is required.

Two intake calculation models are now available, one for short-term (acute) intake calculations the other for long-term (chronic) intake calculations. These two models present updates of the previous versions, with new adult, vegetarian, elderly and more detailed child consumption data incorporated. As Excel spreadsheets, they are designed to be more user friendly than the previous versions and are available with accompanying guidance notes.

10.3.8
Fate and Behavior of Agricultural Pesticides in the Environment

The data provided by applicants must permit an assessment to be made of the fate and behavior of the pesticide in the environment. This information is sub-

sequently used to assess the risk to non-target species (soil or aquatic organisms, plants, etc.) and following crops that will be exposed to the pesticide formulation, its active substance(s), and the metabolites, transformation and degradation products of the active substance(s). The information provided should therefore be sufficient to:

- Predict the distribution, fate and behavior of the pesticide in the environment, as well as the time courses involved, i.e., estimate the concentrations in soil, water and air and assess how these concentrations compare with any recognized limits or standards.
- In conjunction with other data, identify measures necessary to minimize contamination of the environment and impact on non-target species.
- In conjunction with other considerations, permit a decision to be made as to whether the pesticide can be approved, and the uses for which it can be approved.
- In conjunction with other data, classify the product as to risk.
- Specify relevant risk and safety phrases for the protection of the environment, which are to be included on labels.

As indicated above, the nature and amount of data required for pesticide approval depend on the properties and use of each active substance. A stepwise, tiered or triggered approach allows an efficient selection of tests essential to each individual contamination risk analysis. The environmental exposure to a pesticide depends primarily on the following factors.

Concentration of Chemical in the Relevant Environmental Compartment

The highest concentrations usually occur during and just after application.

Following application, the concentration of residues declines due to:

- degradation
- movement into other compartments
- dilution.

Degradation can include such processes as hydrolysis, photolysis, microbial metabolism, etc. Movement reduces the concentration in the treated compartment but transports residues to untreated compartments, e.g., from plant surface to soil or soil to water.

Bioavailability of the Chemical

For substances entering surface waters, the availability of a chemical to organisms is primarily related to its concentration in the aqueous phase. When strongly adsorbed to sediment or soil, availability will often be significantly re-

duced. Under some circumstances it would also be necessary to consider exposure of organisms via the food chain or via the atmosphere.

Nature of the System or Organism

Exposure assessment for an organism requires information on such aspects as:

- Does it live in the treated area, or in an area to which the pesticide could be transported?
- Does it actively consume treated crops or become exposed via the dermal or inhalation routes etc?

To assess the risk of contamination of the environment or exposure of non-target organisms, the potential of the pesticide for movement through the environment must be addressed. For pesticides used in, on or over soils, a study of the pesticide's breakdown in soil is required. Similarly, for pesticides intended for use in or near water, or whose entry into water cannot be ruled out, information must be supplied on mobility in soil and on the fate in the aquatic environment, including natural water/sediment systems. Such requirements and any trigger values for performing different types of study are detailed in the data requirements guidance.

10.3.9 Specific Guidance regarding Water Limits according Annexes of the Authorizations Directive

Account must be taken of fate and behavior in relation to groundwater as well as surface water.

The behavior of any environmentally significant metabolites, transformation and degradation products of the pesticide, with significant potential to contaminate water or soil and cause harm to non-target organisms, must also be investigated. The EC Authorizations Directive stipulates that this requirement applies to those products formed from the pesticide active substance and occurring at levels above 10% of the added pesticide. It may be necessary to investigate such products formed at levels $< 10\%$ where they are known to have significant effects on target and or non-target organisms. The Annexes of the Directive and EU guidance documents should be referred to for more detailed guidance on this point. Unless otherwise stated, the term pesticide in this document will be deemed to include both the active substance and any significant metabolites, transformation and degradation products. However, in the context of the 0.1 $\mu g\ L^{-1}$ drinking water limit being applied to groundwater, it is possible to make the case (with appropriate supporting data) that metabolites, transformation and degradation products are ''not relevant'' and this limit does not then apply [49, 50].

10.3.10
Ecotoxicology Requirements

The areas that need to be addressed are:

- Risk to birds and other terrestrial vertebrates;
- risk to aquatic life;
- risk to honeybees;
- risk to non-target arthropods;
- risk to earthworms;
- risk to soil microbial processes;
- risk to other soil macro-invertebrates (see above);
- risk to other non-target organisms (flora and fauna);
- risk to biological methods of sewage treatment (see above).

The guidance deals with each of these issues in turn. It addresses the basic data requirements and highlights appropriate risk assessment schemes and other sources of information that can be used in producing a good risk assessment. There may also be other references and information that can be used in support of the risk assessment. Notably, the use of such material should be scientifically justified.

EPPO Risk Assessment Schemes

The European and Mediterranean Plant Protection Organisation (EPPO) have produced several schemes that can be used to assess the risk to non-target organisms. These schemes aim to provide a basis for undertaking an appropriate risk assessment.

- Depending upon the proposed use pattern data are required on the acute, dietary and reproductive toxicity of an active substance and/or product to birds. Further details of when such studies are required are outlined in Annex II Section 8.1 and Annex III 10.1 and 10.3 of Directive 96/12/EC.
- Data are always required on the acute toxicity of an active substance to two fish species, Daphnia magna and algae.

If the active substance is a herbicide data are also required on an additional species of an alga as well as an aquatic plant. Full details of the appropriate studies are provided in Section 8.2 Directive 96/12/EC and the Aquatic Guidance Document.

Data are also required on the toxicity of the plant protection product . Further details on when these data are required can be found in the Aquatic Guidance Document, as well as Section 10.2.1 of Directive 96/12/EC.

- Depending on the persistence of the active substance in the water phase of a sediment water study, toxicity data are required to address the possible *chronic risk* of an active substance. Guidance on when these data are needed and on appropriate studies is provided in Section 8.2 and 10.2.4 of Directive 96/12/EC as well as in the Aquatic Guidance Document.
- Depending upon the partitioning and persistence of an active substance in the sediment phase of natural water sediment study, data may be required on its toxicity to sediment dwelling invertebrates. Details of when this study is required and choice of test method are given in the Aquatic Guidance Document. Information is also given in Section 8.2.7 of Directive 96/12/EC.
- Data are required on the bioconcentration potential of an active substance when the log P_{OW} is >3. Further details are given in Section 8.2.3 Directive 96/12/EC, together with the Aquatic Guidance Document.

Buffer zones and LERAPs

In certain instances it may be necessary for the product to have a buffer zone restriction added to the label to protect aquatic life.

Honeybee Risk Assessment

- Acute oral and contact toxicity tests are required in conjunction with a hazard quotient. Where the hazard quotient is greater than 50, further testing may be required. Details of the types of tests and calculation of the hazard quotient are given in Section 8.3 and 10.4 of Directive 96/12/EC. Guidance is also given in the Terrestrial Guidance Document.
- An appropriate risk assessment is required where the hazard quotient is >50, further testing may be required (see above).
- In certain cases, a bee brood feeding test may also be required. Reference should be made to Section 8.3.2 of Directive 96/12/EC and the Terrestrial Guidance Document.

Risk to Non-target Arthropods

The risk to non-target arthropods must be addressed, except where use is in situations where there is no exposure. Details of when the tests are not required are given in Section 8.3.2 of Directive 96/12/EC.

Initially, laboratory tests are undertaken, with further higher tier testing, e.g., extended laboratory tests, required if effects of >30% are seen. Tests are usually

undertaken with a representative formulation of the active substance. Details of the tests required are given in Section 8.3.2 and 10.5 of Directive 96/12/EC.

Risk for Soil Non-target Microorganisms
Key guidance on the risk assessment for soil non-target microorganisms is given in the Guidance Document on Terrestrial Ecotoxicology. European Commission Working Document 2021/VI/98 describes the same risk assessment procedure as it is applied to *soil non-target macro-organisms (earthworms, beetles etc.)*

New MRL (Maximum Residue Levels) regulation for the European Union are being established [51]. Key features of the document are:

- Several foods will be subject to MRLs for the first time.
- It provides for MRL controls to be extended to animal feeds in the future.
- A default MRL of 0.01 mg kg^{-1} (set as a limit of determination) will apply to those commodities where no specific MRL is set, unless a different default level is agreed, or until such time as an MRL is set on the basis of the evaluation of data.

Annex I is necessary for the full implementation of controls under EC Regulation 396/2005, but the new commodity list will not be employed until the EC Regulation comes into force.

10.4 Situation of PS II Inhibitors in the EC Markets

The latest submission of data for an active substance being on the market two years after the Directive 91/414/EEC was published or an active substance that was on the market before 1 May 2004 in the Czech Republic, Estonia, Cyprus, Latvia, Lithuania, Hungary, Malta, Poland, Slovenia and Slovakia and which is not included in stages one to three of the program of work and which is not covered by Regulation (EC) No 1112/2002 was implemented inter alia by COMMISSION REGULATION (EC) No 2229/2004 of 3 December 2004, laying down further detailed rules for the implementation of the fourth stage of the work referred to in Article 8(2) of Council Directive 91/414/EEC at the *latest* by November 2005 [52].

The Directive 91/414/EEC stipulates according to article 5 for inclusion of an active substance in Annex I, the following shall be taken into particular account:

1. Where relevant, an acceptable daily intake (ADI) for man;
2. An acceptable operator exposure level if necessary;
3. Where relevant, an estimate of its fate and distribution in the environment as well as its impact on non-target species.

Table 10.5 EU listed PS II Inhibitors and Specific Provisions (Status November 2005).

Number	Common name, identification numbers	IUPAC name	Purity (1)	Entry into force	Expiration of inclusion	Specific provisions
11	Bentazone CAS No 25057-89-0 CIPAC No 366	3-Isopropyl-(1*H*)-2,1,3-benzothiadiazin-4-(3*H*)-one-2,2-dioxide	960 g kg^{-1}	1.8.2001	31.7.2011	Only uses as herbicide may be authorized In their decision-making according to the uniform principles, Member States must pay particular attention to the protection of groundwater Date of Standing Committee on Plant Health at which the review report was finalized: 13.7.2000
16	Pyridate CAS No 55512-33-9 CIPAC No 447	6-Chloro-3-phenylpyridazin-4-yl S-octyl thiocarbonate	900 g kg^{-1}	1.1.2002	31.12.2011	Only uses as herbicide may be authorized For the implementation of the uniform principles of Annex VI, the conclusions of the review report on pyridate, and in particular Appendices I and II thereof, as finalized in the Standing Committee on Plant Health on 12 December 2000 shall be taken into account. In this overall assessment Member States: – must pay particular attention to the protection of groundwater – must pay particular attention to the potential impact on aquatic organisms and must ensure that the conditions of authorization include, where appropriate, risk mitigation measures

28	Isoproturon CAS No 34123-59-6 CIPAC No 336	3-(4-Isopropylphenyl)-1,1-dimethylurea	970 g kg^{-1}	1 January 2003	31 December 2012	Only uses as herbicide may be authorized For the implementation of the uniform principles of Annex VI, the conclusions of the review report on isoproturon, and in particular Appendices I and II thereto, as finalized in the Standing Committee on Plant Health on 7 December 2001 shall be taken into account. In this overall assessment Member States: – must pay particular attention to the protection of the groundwater, when the active substance is applied in regions with vulnerable soil and/or climatic conditions or at use rates higher than those described in the review report and must apply risk mitigation measures, where appropriate – must pay particular attention to the protection of aquatic organisms and must ensure that the conditions of authorization include, where appropriate, risk mitigation measures
51	Linuron CAS No 330-55-2 CIPAC No 76	3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea	900 g kg^{-1}	1 January 2004	31 December 2013	Only use as herbicide may be authorized For the implementation of the uniform principles of Annex VI, the conclusions of the review report on linuron, and in particular Appendices I and II thereof, as finalized in the Standing Committee on the Food Chain and Animal Health on 3 December 2002 shall be taken into account. In this overall assessment Member States: – must pay particular attention to the protection of wild mammals, non-target arthropods and aquatic organisms. Conditions of authorization should include risk mitigation measures, where appropriate – must pay particular attention to the protection of operators.

Under the *"Uniform Principle"* application, especially the maximum admissible concentration on drinking water of 0.1 μg L^{-1}, set by EC Directive on Drinking Water (98/83/EC) [53], was a hurdle for a listing into Annex I for many compounds belonging to the class of PS II inhibitors. Nevertheless, although this standard value does not reflect any risk under toxicological assessment it is binding for all EU member states as, for example, *The Pesticide Safety Directorate* stated [54]:

> Especially for the distribution in the environment the EC Directive on Drinking Water (98/83/EC) has set a maximum admissible concentration of 0.1 μg L^{-1} for any individual pesticide in drinking water. The figure is independent of any toxicological or environmental assessment and does not necessarily represent risk. Nevertheless it is UK Government policy to control the use of pesticides in such a way as to reduce the occurrence and levels of pesticide contamination found in drinking water (Annex VI of 91/414/EEC which is 97/57/EEC requires that this 0.1 μg L^{-1} standard for any individual pesticide applied to groundwater).

By searching under European Union, Reregistration of Plant Protection Agents, residues in Groundwater via Google.de, ≈ 21500 citations are found, indicating the political importance of this question caused by, for example, the "Grundwasserrichtlinie" in Germany. It is of eminent importance in public awareness [55].

Another politically important subject is carcinogenicity, which led to non-listing on Annex I under 91/414/EEC Directive of atrazine [56]:

> Unlike the EU the US-EPA has reregistered Syngenta's atrazine for use in maize, sugarcane, sorghum, cereals and other crops. Atrazine failed to get reregistration in Europe in October 2002 because of suggestions that it could be linked to increased cancer risks. The US-EPA concluded that there have been no studies confirming increased risk.

Other major reasons for non-listing of PS II inhibitors in Annex 1 under 91/414/EEC Directive could be: changes in buffer zones listings, withdrawals for commercial reasons and failures to meet data submission deadlines.

Examples for PSII inhibitors that have already been included into Annex I are pyridate, isoproturon and bentazone. Table 10.5 exemplifies the provisions that have been imposed for Member States' Registration Authorities to address, e.g., by implementation of national use restrictions to ensure these substances are being used safely in the EU Member States.

Additionally from the C 1 group of PS II inhibitors the phenylcarbamates desmedipham and phenmedipham are listed in Annex I and from the C 3 group bromoxynil and ioxynil. The triazinones metamitron and metribuzin are applied for listing, the uracil lenacil, the pyridazon pyrazon/chloridazon, the ureas diuron, fluometuron, methabenzthiazuron, and the amide propanil. Off label for minor use (essential use) are applied for cyanazine, dimefurone and fenuron, metobromuron and metoxuron as well as for pentanochlor.

Table 10.6 describes the status of the reregistration process of PS II inhibitors in the EU (Status November 2005).

Evidently, from these data, the most important groups of chemistry in PS II inhibitors in the 1980s, i.e., triazines and, to a large extent, ureas will not be used anymore in the European Union, with some very small exceptions (Table 10.7).

10.5 Marketshare of PS II Compound Groups Today

Whereas photosynthesis inhibitors belonged in 1980 to the most important herbicide classes [38] the market situation changed, especially in Europe, at beginning of the 1980s through, especially, the introduction of new cereal, corn and oil seed rape herbicides from other herbicide classes but also through the reregistration process in Europe up to now.

The value of PSII inhibitors sold in the EU declined from ca. 745 Mio Euro in 1995 to only ca. 441 Mio Euro in 2004, i.e., by minus ca. 40%. At the same time, the value of the total herbicide market in the EU increased from ca. 1.600 Mio Euro to ca. 2.000 Mio Euro, i.e., by plus 25%. Thus, the value share of PSII inhibitors in the total EU herbicide market decreased from ca 45% in 1995 to only 22% in 2004.

Out of a total number of 50 PSII inhibitors, sales could only be recorded in the EU for some 40 compounds. Although the total number of PSII herbicides where sales could be recorded in the EU has only slightly declined from some 40 compounds in 1995 to about 36 compounds in 2004, only ten compounds have yet been included into Annex I as per end of 2005, while the future of a total number of slightly more than 30 PSII compounds still on sale in Europe is unclear or their life has come to an end, for one of the following reasons:

1. The substance has not even being notified, i.e., no dossier has been submitted (4 compounds where sales could be recorded).
2. The substance has not yet passed the EU Review Program successfully (ten compounds where sales could be recorded).
3. The substance has passed the EU Review Program with a negative outcome (so far 19 compounds where sales could be recorded), either because the notifier has not further supported the compound or the EU Commission has taken a negative decision on Annex I inclusion (see Tables 10.6 and 10.7).

Despite the fact that some 19 PSII compounds have already ended up in a non-inclusion, many of those are still on the market and sales can be recorded in the year 2004, mainly because either time-limited essential uses have been granted, or existing stocks are being sold out.

Table 10.6 Status of Reregistration process of PS II inhibitors (Nov 2005, Source: EU Commission).

Chemical Family	A.i.	No. in list of authorization	No. of authorizations in EU	Countries of authorization	List-no. (stage)	RMS	Registration status	Status list of peer review (State of main works)	Reasons for non-inclusion/ withdrawl	Essential uses (according to regulation 2076/2002/EC authorization of PPPs until 30 June 2007	Dossier submitted by	List of uses supported by available data
Triazine	Cyanazine	not in EU-list	/	/	/	/	Not on list but essential uses (1336/2003/EC)			UK: Pea, bean, brassica, narcissi, oilseed rape, allium, forestry; SE: Oil seed rape, pickling cucumber; IR: Onion	Syngenta	
Triazinone	Metamitron	370	23	FI, SE, DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, IT, PT, EL, PL, CZ, HU, SK, SI, EE, LV, LT	3B	United Kingdom	Pending, data list	No information			BCS	

	Metribuzin	125	24	FI, SE, DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, IT, PT, EL, PL, MT, CY, CZ, HU, SK, SI, EE, LV	2	Denmark	Pending, dossier DAR	Pending		BCS	
Uracil	Lenacil	366	15	IE, UK, BE, AU, FR, ES, PT, IT, EL, PL, CY, CZ, HU, SK, EE	3B	Belgium	Pending, dossier	No information			
Phenyl-carbamate	Desmedipham	15	23	FI, SE, DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, IT, PT, EL, PL, CZ, HU, SK, SI, EE, LV, LT	1	Finland	Inclusion in Annex I (expiration: 28. Feb. 2015)	Inclusion 2004/58/EC 6.10.2004, p. 26		Agrevo, main data submitter	Sugar & fodder beet

Table 10.6 *(continued)*

Chemical Family	A.i.	No. in list of authorization	No. of authorizations in EU	Countries of authorization	List-no. (stage)	RMS	Registration status	Status list of peer review (State of main works)	Reasons for non-inclusion/ withdrawl	Essential uses (according to regulation 2076/2002/EC authorization of PPPs until 30 June 2007	Dossier submitted by	List of uses supported by available data
	Phenmedipham	34	24	FI, SE, DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, IT, PT, EL, PL, CZ, HU, SK, SI, EE, LV, LT	1	Finland	Inclusion in Annex I (expiration: 28. Feb. 2015)	Inclusion 2004/58/EC 6.10.2004, p. 26			Task Force on Phenmedipham (BCS, United Phosphorus Ltd.)	Sugar & fodder beet, red beet (beetroot)

Amide	Propanil	382	5	FR, ES, PT, IT, EL	3B	Italy	Pending, data list	No information				
	Pentanochlor	582	1	UK	3		Out 07/03; 2076/2002/EC; essential uses 1336/2003/EC	No information	Not supported anymore by the notifier: With the available data no safe use could be assessed by the Commission.	UK: Umbellifers, herbs, ornamentals		
Nitrile	Bromoxynil	11	18	DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, PT, IT, EL, PL, CZ, HU, SK, SI	1	France	Inclusion in Annex I; 04/58/EC	Inclusion, 2004/58/EC, 6.10.2004, p. 26			BCS, Makhteshim Agan	Winter/spring cereals (barley, wheat, oats, rye, triticale), maize (member state: EU)

Table 10.6 *(continued)*

Chemical Family	A.i.	No. in list of authorization	No. of authorizations in EU	Countries of authorization	List-no. (stage)	RMS	Registration status	Status list of peer review (State of main works)	Reasons for non-inclusion/ withdrawl	Essential uses (according to regulation 2076/2002/EC authorization of PPPs until 30 June 2007	Dossier submitted by	List of uses supported by available data
	Ioxynil	22	19	FI, SE, DK, IE, UK, NL, BE, LU, DE, AU, FR, ES, PT, IT, EL, PL, CY, HU, SI	1	France	Inclusion in Annex I; 04/58/EC	Inclusion, 2004/58/EC, 6.10.2004, p. 26			BCS, Makhteshim Agan, ACI International, CFPI Nufarm	Winter/ spring cereals
Urea	Metobromuron	558	8	BE, DE, AU, FR, SP, PT, IT, EL	3		Out 07/03; 2076/2002/EC; essential use 1336/2003/EC	No information	Not supported anymore by the notifier: With the available data no safe use could be assessed by the Commission.	BE: Lambs lettuce, bean, potato; ES: Potato; DE: Lambs lettuce, bean, tobacco; FR: Lambs lettuce, artichoke		

Metoxuron	559	8	IE, UK, NL, BE, LU, FR, ES, IT	3	Out 07/03; 2076/2002/EC; essential use 1336/2003/EC	No information	Not supported anymore by the notifier: With the available data no safe use could be assessed by the Commission.	BE: Carrot, potato; FR: Carrot; IR: Carrot; LU: Carrot, potato; NL: Carrot, potato, iris, gladiolus; UK: Carrot, parsnip

Table 10.7 Withdrawn PS II Inhibitors from Broad Reregistration in EU (Status Nov 2005, Source: EU Commission).

Chemical family	A.i.	Countries of authorization	RMS	Registration Status	Status list of peer review (State of main work)	Essential uses (according to regulation 2076/2002/EC authorization of PPPs until 30 June 2007
Triazines **1**	Ametryn	FR, ES, IT		Out 07/03; 2076/2002/EC		
	Atrazine	IE, UK, BE, LU, FR, ES, PT, EL, PL, HU, SK	United Kingdom	Out 10/04; 04/247/EC	Withdrawn, 2004/248/EC, 16.03.2004, page 53	
	Desmetryne	UK, AU, ES, IT		Out 07/03; 2076/2002/EC		
	Prometryne	IE, UK, AU, FR, ES, PT, IT, EL, PL, MT, CY, HU, SK, EE, LV, LT		Out 7/03 essential use, 835/04; 2076/2002/EC bzw. 1336/2003/EC		
Triazines **2**	Propazine			Out 07/03; 2076/2002/EC		
	Simazine	DK, IE, UK, BE, LU, AU, FR, ES, PT, IT, EL, PL, CY, SK	United Kingdom	Out 10/04; 04/247/EC	Withdrawn, 2004/247/EC, 16.03.2004, page 50	
	Simetryne				No information	
	Terbumeton	ES, IT		Out 07/03; 2076/2002/EC	No information	

	Terbuthylazine		United Kingdom	Pending, data list	No information	
	Terbutryne	FI, SE, IE, UK, AU, FR, ES, PT, IT, EL, PL, HU, SK		Out 7/03; 2076/2002/EC	No information	
	Trietazine	IE, UK		Out 7/03; 2076/2002/EC	No information	
	Hexazinone	IE, AU, FR, ES, IT, CZ, HU, SK		Out 7/03; 2076/2002/EC	No information	
Uracil	Bromacil	IE, UK, AU, FR, ES, PT, IT, EL		Out 7/03 essential use; 2076/2002/EC	No information	
	Lenacil	IE, UK, BE, AU, FR, ES, PT, IT, EL, PL, CY, CZ, HU, SK, EE	Belgium	Pending, dossier	No information	
	Terbacil	UK, FR, ES, EL, PL, HU		Out 7/03; 2076/2002/EC	No information	
Urea 1	Chloroxuron	FR		Out 07/03; 2076/2002/EC	No information	
	Dimefuron	IE, DE, AU, FR		Out 07/03; 2076/2002/EC; essential use 1336/2003/EC	No information	**DE**: Oil seed rape
	Diuron	DK, IE, UK, BE, LU, DE, AU, FR, ES, PT, IT, El, PL, CY, HU	Denmark	Pending, Dossier DAR	Pending, EFSA conclusion 14.01.2005	
	Ethidimuron (= sulfodiazol)	FR, ES, IT		Out 07/03; 2076/2002/EC	No information	
	Fenuron	UK, HU		Out 7/03 essential use; 2076/2002/EC bzw. 1336/2003/EC	No information	**UK**: Pea, bean, spinach
	Fluometuron	ES, EL	Greece	Pending, dossier	No information	

Table 10.7 *(continued)*

Chemical family	A.i.	Countries of authorization	RMS	Registration Status	Status list of peer review (State of main work)	Essential uses (according to regulation 2076/2002/EC authorization of PPPs until 30 June 2007
Urea 2	Metobromuron	BE, DE, AU, FR, SP, PT, IT, EL		Out 07/03; 2076/2002/EC; essential use 1336/2003/EC	No information	
	Metoxuron	IE, UK, NL, BE, LU, FR, ES, IT		Out 07/03; 2076/2002/EC; essential use 1336/2003/EC	No information	
	Monolinuron		United Kingdom	Out 9/01; 00/234/EC	**Withdrawn**, 2000/234/EC, 22.03.2000, p. 18	
	Neburon	IT		Out 07/03; 2076/2002/EC	No information	
	Siduron	FR		Out 07/03; 2076/2002/EC	No information	
	Tebuthiron			Out 07/03; 2076/2002/EC	No information	
Nitrile	Bromofenoxim	ES, IT		Out 07/03; 2076/2002/EC	No information	

The class of urea herbicides is widely being eliminated in Europe, but, more distinctively, the important class of triazine herbicides is disappearing from the EU herbicide market. None of their some ten representatives in Europe where sales can be recorded have made it into Annex I, except terbutylazine, which is still pending for the time being. The traditionally most important triazine representatives, atrazine and simazine, have not passed the EU Review Program and will have to be replaced by new chemistry in the EU, while both substances, particularly atrazine, still represent a significant importance in the US market, e.g., atrazine sales in the US accounted for some 165 Mio Euro in 2004, since this compound is widely and efficiently used in the US corn market, also in combination with Roundup-Ready.

Overall, the EU Review Program and the associated costs of maintaining substances in the market is leading to a significant streamlining in the number of PSII compounds by 50–75%, depending how many of the ten still pending compounds will be included into Annex I. The value share of the remaining PSII inhibitors in the total EU herbicide market will further decline in the years to come, and the downward trend recorded from ca. 45% market share in 1995 to only ca. 22% in 2004 will be further characteristic for this class of chemistry, since the phase-out is still ongoing.

10.6
A New Herbicide for Corn and Sugarcane: Amicarbazone – BAY MKH 3586

10.6.1
Introduction

Amicarbazone is a new herbicide for broad spectrum weed control in corn and sugarcane. It belongs to the chemical class of carbamoyl triazolinones and acts as an inhibitor of photosystem II. It was discovered 1988 by the former Plant Protection Division of Bayer AG (now Bayer CropScience) and developed under the internal code no. BAY MKH 3586 (Fig. 10.3).

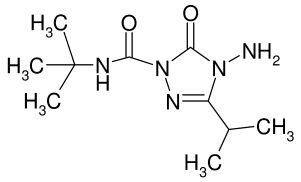


Fig. 10.3. Amicarbazone, BAY MKH 3586, Dinamic®.

Table 10.8 Physicochemical properties of amicarbazone.

Melting point	137.5 °C
Vapor pressure (Pa)	1.3×10^{-6} (20 °C) 3.0×10^{-6} (25 °C)
Dissociation constant (20 °C):	Amicarbazone has no acidic or basic properties in aqueous solutions. It is not possible to specify dissociation constants for water.
Solubility in water (g L^{-1}) (20 °C):	4.6 in unbuffered and buffered solutions; solubility not influenced by pH in the range pH 4–9
Volatility (Henry's law constant at 20 °C)	6.8×10^{-8} Pa m^3 mol^{-1}
Solubilities in organic solvents (g L^{-1}) (20 °C)	*n*-Heptane: 0.07 Xylene: 9.2 Poly(ethylene glycol) (Lutrol): 79 Dimethyl sulfoxide: >250 Dichloromethane: >250
Partition coefficient, Log P_{OW} in octanol–water (20 °C):	1.23 (pH 7) 1.14 (unbuffered)

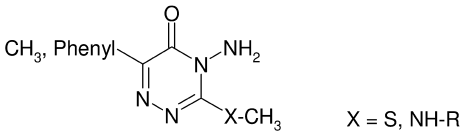
10.6.2
Physicochemical Properties of Amicarbazone

Table 10.8 gives some physicochemical data.

10.6.3
Discovery of the Active Ingredient

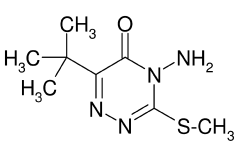
Research is in most cases a continuous process and takes place in small steps. To better understand the discovery of amicarbazone we should go back to the year 1964 when Dornow published the first examples of the hitherto unknown class of 4-amino-1,2,4-triazin-5-ones [25] (Fig. 10.4).

Research chemists at the former Farbenfabriken Bayer AG identified these compounds in 1965 as herbicides [57] and specified the mode of action as inhibition of photosystem II [11, 58].

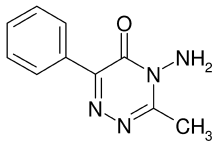


4-Amino-1,2,4-triazin-5-one

Fig. 10.4. 1,2,4-Triazinones, general formula.



1966: Metribuzin, Sencor



1971: Metamitron, Goltix

Fig. 10.5. 1,2,4-Triazinones, marketed compounds.

The optimization process led in 1966 to the discovery of metribuzin [57] and five years later to metamitron [59], two commercially very successful herbicides for soybeans and sugarbeet, respectively (Fig. 10.5).

Figure 10.6 gives a schematic representation of the essential atoms of a herbicide binding to the 32 kDa peptide of photosystem II, indicating the sp^2 hybrid with X (usually O, S or C) attached to a lipophilic group and the essential positive charge.

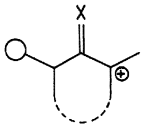
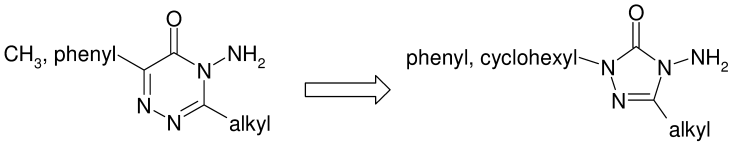


Fig. 10.6. Requirements for PS II inhibitors.

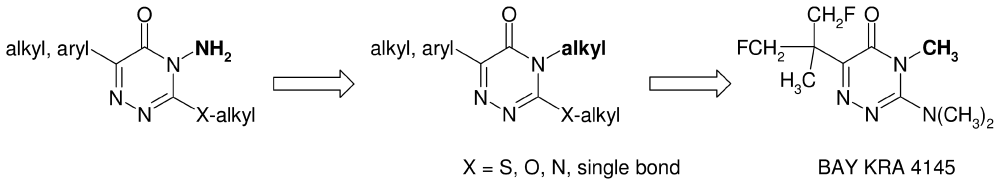
Following the above concepts of the structural requirements of PS II inhibitors [60] five-membered analogues of metamitron were synthesized and checked for their biological activity [61] (Scheme 10.2).



Scheme 10.2. Five-membered analogues of metamitron.

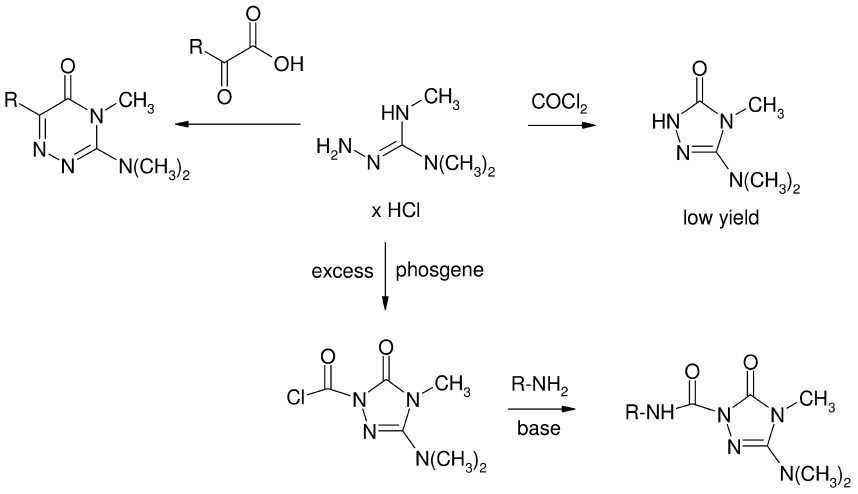
Although active *in vitro*, these compounds were rather inactive *in vivo* as herbicides.

With the goal of a corn herbicide, research efforts continued in the field of triazinones, and N-alkyl derivatives, and various sulfur [62], oxygen [62], nitrogen [62–65] and carbon substituents [66] were filed for patent. In 1981 a compound with the internal code number BAY KRA 4145 was synthesized and taken into development after intensive field tests [67] (Scheme 10.3).



Scheme 10.3. Structure elucidation to BAY KRA 4145.

To reduce the high costs of a linear synthesis a convergent approach was evaluated using trimethylaminoguanidine as intermediate. Following the concept to use a new intermediate in different ways a lot of chemistry was performed, including reaction with phosgene (Scheme 10.4).



Scheme 10.4. Trimethylaminoguanidine as intermediate.

Excess phosgene generated in good yield the chlorocarbonyl triazolinone. Reaction with various amines produced carbamoyl triazolinones, the first derivative being isolated in May 1986 [68]. They are active *in vitro* as PS II inhibitors, but show, in contrast to the directly linked triazolinones, higher herbicidal *in vivo* activity. This was the starting point of a major synthesis program, generating more than 2500 compounds of the general type shown in Fig. 10.7.

All synthetic variations showing good herbicidal activity have been published in various patents (Table 10.9).

Regarding activity, selectivity and cost of goods, BAY MKH 3586 [77, 78] represents the optimum and was developed for selective weed control in corn and sugarcane.

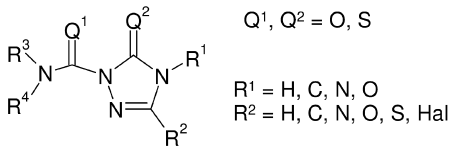


Fig. 10.7. General formula of carbamoyl triazolinones.

Table 10.9 Synthetic variations of structure shown in Fig. 10.7 and associated patents.

R^1	R^2	Ref.
Alkyl	$N(alkyl)_2$	68–72
Alkyl	S-alkyl	68–72
Alkyl	NH-alkyl	72, 73
Alkyl	Alkyl	72, 74
Alkyl	Halogen	72, 75
Alkyl	O-alkyl	72, 76
NH_2	Alkyl	72, 77–80
NH-alkyl	Alkyl	72, 81, 82
NH_2	S-alkyl	72, 83, 84
NH_2, NH-alkyl, $N(alkyl)_2$	O-alkyl	72, 85
NH_2, NH-alkyl, $N(alkyl)_2$	NH_2, NH-alkyl, $N(alkyl)_2$	72, 86, 87

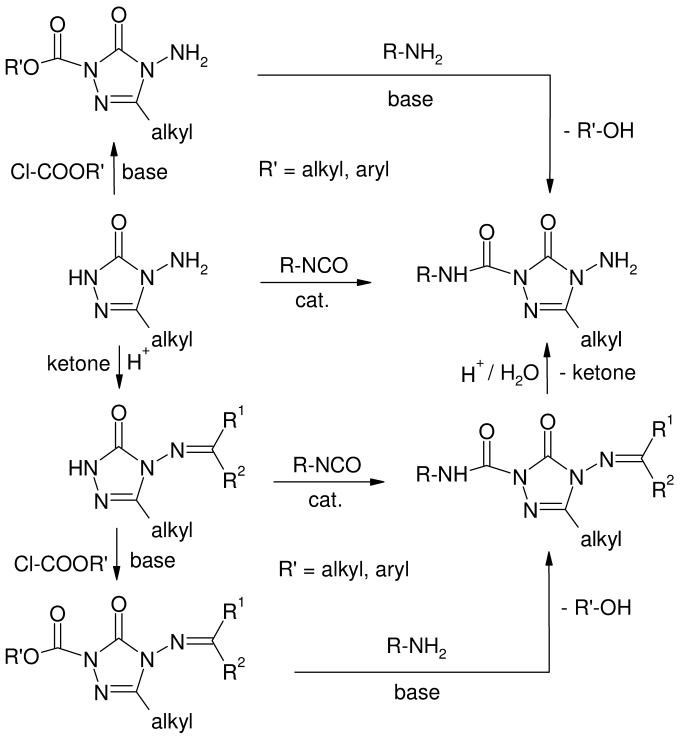
10.6.4 Synthesis

Final Product There are various methods of synthesizing carbamoyl triazolinones [68–87]. In the case of N-amino triazolinones a protecting group like a Schiff base can be helpful [77–80]. Otherwise, under suitable conditions it is possible to add isocyanates directly in a kinetically controlled reaction to the amidic nitrogen of N-NH_2-triazolinones [77, 78, 88] (Scheme 10.5).

The first synthesis of the intermediate 4-amino-3-isopropyl-1,2,4-triazol-5-one was described by F. Malbec et al. [89]. The synthesis of this known intermediate can also be achieved by several other methods:

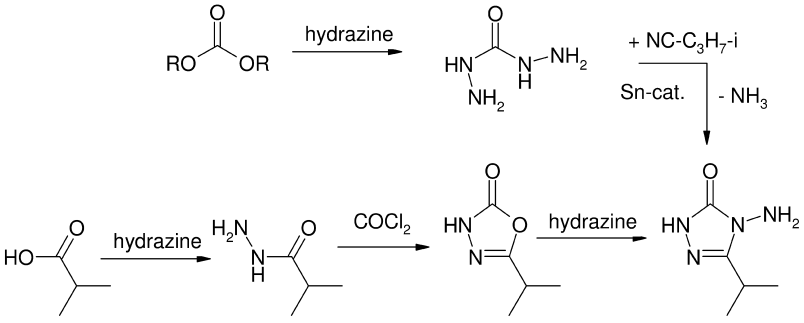
- Hydrazinolysis of acylated carbazates [90].
- Cyclization of carbohydrazide with carboxylic acids or ortho esters [90].
- Hydrazinolysis of ester carbalkoxy-hydrazones [89, 91, 92].

Owing to several disadvantages, such as low yield, long reaction time, formation of side products or number of synthesis steps, new synthetic methods were elaborated:



Scheme 10.5. Synthesis of N-amino-carbamoyltriazolinones.

- *In situ* preparation of carbohydrazide and cyclization with isobutyronitrile in the presence of a suitable tin compound as reaction auxiliary [93].
- Hydrazinolysis of 5-isopropyl-1,3,4-oxadiazol-2(3*H*)-one [94–98] (Scheme 10.6).



Scheme 10.6. Synthesis of the intermediate 4-amino-3-isopropyl-1,2,4-triazol-5-one.

10.6.5
Biological Behavior

Amicarbazone is tolerated by corn and sugarcane crops and shows excellent activity against many major annual dicotyledonous weeds that infest these crops.

In corn it may be applied up to a maximum rate of 500 g a.i. ha^{-1} to the soil at preplant or pre-emergence timings. In combination with other corn herbicides [99, 100] such as isoxaflutole the application rate can be reduced. Additionally, amicarbazone also shows contact activity on emerged weeds. The compound provides burndown as well as residual weed control, which is particularly useful in reduced and zero tillage corn production systems. Important weeds controlled by amicarbazone are, amongst others, velvetleaf (*Abutilon theophrasti*), common lambsquarters (*Chenopodium album*), pigweed (*Amaranthus* spp.), common cocklebur (*Xanthium strumarium*) and morning-glory species (*Ipomoea* spp.). In October 2005 amicarbazone was granted conditional registration by EPA in the United States [101]. From its biological spectrum and its mode of action it will compete mainly against atrazine (in all markets), but also replace the broadleaf part of the weed control spectrum of alachlor, acetochlor and metolachlor where grasses are not the dominant weeds [101].

In the Brazilian market amicarbazone was introduced in 2004 under the trade name Dinamic® by Arysta LifeScience [102] for weed control in sugarcane.

It can be applied either pre-emergence or post-emergence at application rates up to 1500 g a.i. ha^{-1} solo or with 700 g a.i. ha^{-1} in combination [103, 104] with tebuthiuron (750 g a.i. ha^{-1}) or ametryn (1500 g a.i. ha^{-1}). In tank mixtures with metribuzin (960 g a.i. ha^{-1}) the rate can be reduced to 560 g a.i. ha^{-1} (post-emergence) or 800 g a.i. ha^{-1} (pre-emergence). Besides dicot weeds like painted spurge (*Euphorbia heterophylla*) and morning-glories, many annual grasses like marmeladegrass (*Brachiaria plantaginea*), southern sandbur (*Cenchrus echinatus*), bengal commelina (*Commelina benghalensis*) and guineagrass (*Panicum maximum*) are controlled [104]. More detailed information about the biological profile was revealed at the British Crop Protection Conference – Weeds 1999 [104].

10.6.6
Metabolites

In a corn metabolism study [105] amicarbazone and two degradation products were identified as the major components in corn matrices (Fig. 10.8).

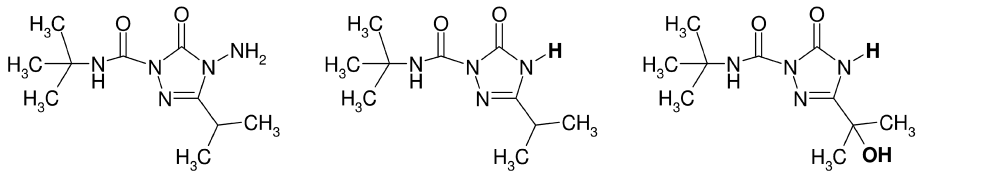


Fig. 10.8. Metabolites of aminocarbazone obtained in corn.

10.6.7
Final Remarks

Amicarbazone is the latest representative in the still economically important group of photosystem inhibitors. It belongs to the chemical class of carbamoyl triazolinones, was found and developed by Bayer AG and will be commercialized in the US in the corn market and in sugarcane growing countries by Arysta Life-Sciences.

10.7
Conclusions

PS II inhibitor herbicides were one of the most important herbicide classes. It could be shown in this chapter what influence the introduction of new herbicide classes could have on the market share of ''ripened'' herbicides but also the registration requirements in main markets. Nevertheless, new introductions could find a market and will be a good solution for weed control in corn and sugarcane markets.

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11
New Aspects of Plant Growth Regulators

Hans Ulrich Haas

Plant growth regulators (PGRs), their use, mode of action and plant-internal and -external interactions, have been the subject of intense research since they were introduced for agricultural use in the early 1930s. With new experimental results, knowledge and experience, the use and the spectrum of PGRs have increased continuously over the years, but the mode of action of growth regulators in plants is of such high complexity that even for auxins, the oldest known group of growth regulators, understanding of their mode of action is still incomplete.

This chapter gives an overview of PGRs, their current use and new developments. It is a summary of available knowledge and a tool for deeper and more intense analyses of specific items. The literature given at the end includes reviews and specialist summaries. Internet links provide detailed and up-to-date overviews, such as chemical structures, including the chemical names [1], chemistry, use and environmental aspects [2–4], and summarized overviews [5, 6].

PGRs are compounds of natural or synthetic origin used for controlling or modifying plant growth processes without apparent phytotoxic effects at the dose applied. They belong to a wide range of chemistry (Figs. 11.1 and 11.2).

Classically, there are five main categories of naturally occurring PGR, the auxins (IAA, NAA, IBA, 2,4-dichlorophenoxyacetic acid), gibberellins (GA), cytokinins (kinetin, benzyladenin, zeatin), ethylene, and growth inhibitors like abscisic acid (ABA).

Auxins were the first phytohormones detected and auxinic activity had already been observed in 1879 by J. Sachs during plant propagation. The first compounds (indole-3-acetic acid) were isolated and described by Kögl 1934 [7]. Auxins are involved in fruit ripening, phototropism, rooting, apical dominance, and cell enlargement. They are widely used as herbicides, showing activity due to an overdose and subsequent de-/regulation processes in plants. A herbicidally active auxin dose leads, for example, to an overdose of ethylene, inducing epinastic growth, tissue swelling, a stimulation of abscisic acid biosynthesis and leaf abscission [8].

Ethylene effects were firstly described by Nejebulov 1901 [9], but ethylene as a hormone could be identified only after gas chromatography was established in

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

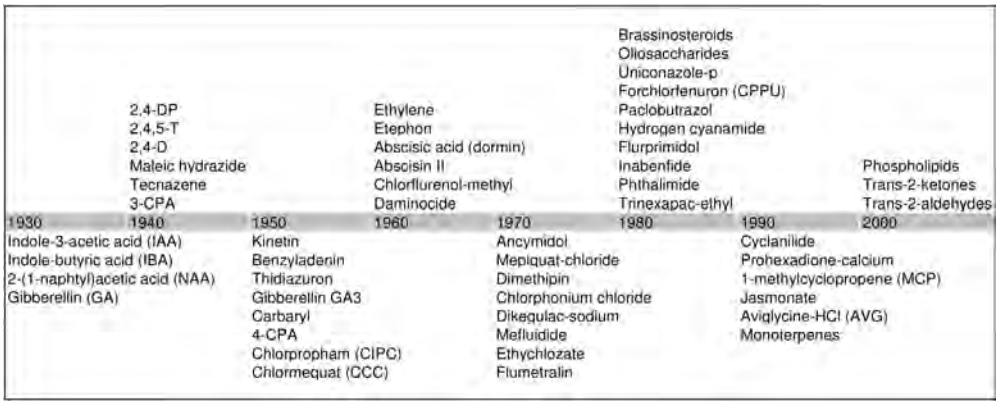

Fig. 11.1. Commercialized and new plant growth regulators and the decade of their market introduction or publication.

the 1960s [10]. Ethylene influences the balance of auxins vs. gibberellins [8], it inhibits cell division and strengthens cell walls. Furthermore, it is involved in the initiation of flowering, breaking of dormancy, abscission of parts of the plants and ripening processes. The most frequently used ethylene-based PGR is ethephon, an ethylene releaser. It breaks down in plant tissue to phosphate, chloride ions and ethylene, which acts as the PGR [11].

Gibberellins are involved in growth processes, including the elongation of internodes, flowering, dormancy, and fruit morphology. Their effects were first described by Kurosawa 1926 [12], who observed shoot elongation of rice after treatments with culture filtrates of *Fusarium moniliforme Sheld*. Yabuta was then

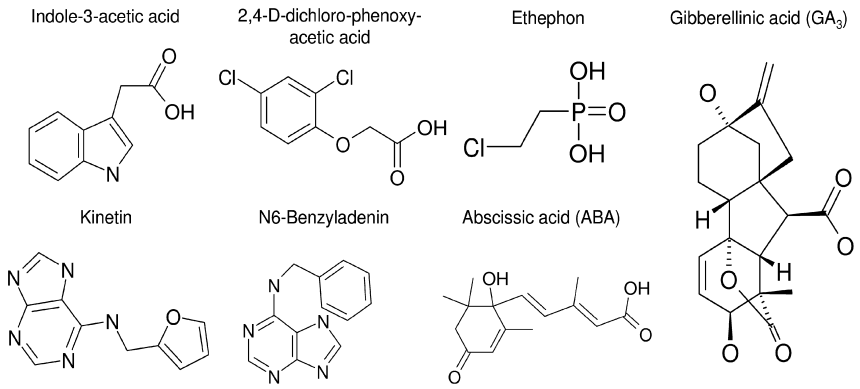

Fig. 11.2. Chemical structures of plant growth regulator compounds out of the five main PGR categories: auxins (IAA, 2,4-D), ethylene (ethephon), cytokinin (kinetin, benzyladenin), growth inhibitors (ABA), and gibberellins (GA_3).

Table 11.1 Key use areas of PGR in modern agriculture and compounds currently either in use or under development.

Factor	Example	Compounds
Plant growth	Shoot growth inhibition	Chlormequat-chloride
	Dwarfing	Ethylene, ethephon
		Mepiquat-chloride
		Mepiquat-pentaborate
		Paclobutrazol
		Prohexadione-Ca
		Promalin
		Trinexapac-ethyl
	Dormancy breaking	Ammonium nitrate
		Ca-cyanamide
		Ca-Nitrate
	Propagation	Auxins, giberellins, cytokinins
Yield and fruit quality	Flower induction	Ammonium thiosulfate
	Fruit-thinning	Ethylene, ethephon
		Gibberellinic acid
		Glutamic acid
		Paclobutrazol
		Prohexadione-Ca
	Ripening	Aviglycine HCL
	Fruit size adjustment	Benzyladenine
	Sugar accumulation	Cu-ethylenediamine
		Ethylene, ethephon
		MBTA-HCl
		Phospholipids
		Trinexapac-ethyl
Storage	Ripening	Aminoethoxyvinylglycine (AVG)
	Sprout supression	Carvone
		Chlorpropham (CIPC)
		1,2,6-DIPN
		Ethylene, ethephon
		Maleic hydrazide
		Menthol
		1-Methylcyclopropene (1-MCP)
		trans-2-Aldehyde/ketone

able to isolate a crystalline compound (5-*n*-butylpicolinic acid, fusaric acid) from the fungal culture. In 1938 Yabuta and Sumiki [13] published a first paper on the gibberellins. GA_3 was described in 1955 by Brian and Hemming [14]. Of all known gibberellins, today only gibberellic acids GA_3, GA_4 and GA_7 are of commercial importance [10].

Cytokinins act mainly through cell cycle regulation [15]. They stimulate cell division, prevent abscission, prevent rooting, enhance germination, and prevent senescence. Kinetin was firstly described in 1955 [16]. Benzyladenin was discovered by Strong in 1958 [17]. Both compounds are still the most commonly used cytokinins in plant micropropagation [7]. In addition, Strong [17] described thidiazuron, which is in use mainly to induce senescence in cotton.

Growth inhibitors like abscisic acid (dormin) and abscisin II retard growth, promote abscission, and induce dormancy. They were discovered in the 1960s [18]. They directly affect cell division and expansion and induce stomatal closure [8].

Plant growth regulators play an important role in modern agriculture. They are used to ensure and enhance quantity and quality of all parts of a crop cycle, from seed to harvest and postharvest. PGR can be grouped into main use categories, including shoot length control, yield regulation and harvest facilitation, storage control, propagation, and combined effects (Table 11.1).

Besides the auxins, which were commercialized predominantly as herbicides soon after their discovery, one of the first plant growth regulator products for shoot length control was maleic hydrazide, first described as a PGR in 1949 [19]. Since then the classical and core use of growth regulators is *shoot length control*. Growth inhibitors currently in use in cereals act mainly as gibberellin inhibitors. Chlormequat-chloride (CCC), trinexapac-ethyl, mepiquat-chloride and paclobutrazol are typical examples of such compounds (Fig. 11.3).

Rademacher [20] has summarized the chemistry of growth retardants in agronomic and orchard crops with special emphasis on gibberellin biosynthesis. Paclobutrazole and uniconazole are gibberellin inhibitors belonging to the N-containing heterocyclic triazoles. Triazoles are commonly known as fungicides, acting as demethylation inhibitors (DMI). Triazole fungicides are often reported to have growth retardant side effects [21]. Some of these PGR activities are of practical importance, such as metconazole and tebuconazole in oilseed rape.

An additional effect, which has not been given much attention, is the implication of the effects of such fungicides on the growth inhibition of weeds [22, 23]. The growth reduction of the crops and of the weeds may be about the same unless a specific weed species is selectively inhibited more than the crop.

Fruiting and growth of orchard trees is variable and dependent on climate, weather, but also plant specific factors such as alternation, the biennial fluctua-

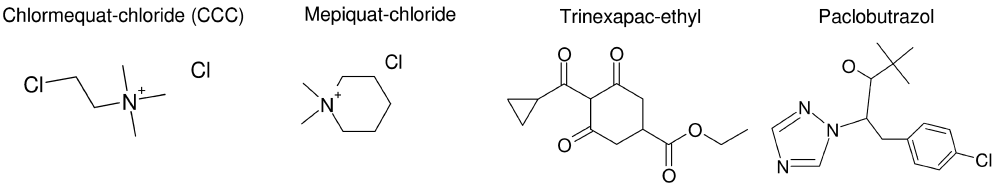

Fig. 11.3. Chemical structures of plant growth regulators mainly in use for shoot length control.

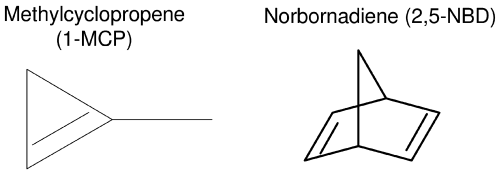

Fig. 11.4. Chemical structures of ethylene binding site inhibitors.

tion of the fruit yield in orchards. PGRs are used in this area to reduce and harmonize plant growth, to equalize and accelerate blossom and fruiting seasons (e.g. defoliation and re-growth), to precondition fruits for harvesting, and to thin fruits for better quality and fruit size and more equal yields during several years. To control tree growth in orchards but also in arable crops and flowers the gibberellin-biosynthesis inhibitors paclobutrazol [24] and prohexadione-Ca [25] are in use. Blossom thinners are ammonium thiosulfate (ATS), endothalic acid, pelargonic acid, sulfcarbamide-1-aminomethanamide, hydrogen tetraoxosulfate and hydrogen cyanamide [10]. For postbloom thinning, naphthalene acetic acid (NAA) is still important, but also a side effect of the insecticide carbaryl is used for thinning [26]. Benzyladenine (6-BA), as cytokinin, is registered for the reduction of fruits, and additionally stimulates cell division in remaining fruits [27].

A further use area of PGR is the control of *storage and ripening* of fruits and plant products, e.g., cut flowers. Ethylene is broadly utilized to induce ripening. The opposite, delayed ripening for better shelf-life, is more difficult to manage. Daminocide was the first commercial compound to delay ripening of apple fruits [28]. It was replaced by the ethylene biosynthesis inhibitor aviglycine-HCl (AVG), which also had to be applied to the fruits on the tree before harvest.

A new episode of controlled ripening started with the investigation of ethylene binding site inhibitors, like 1-methylcyclopropene (1-MCP) and norbornadiene (2,5-NBD) (Fig. 11.4), and their development for market use [29]. Meanwhile 1-MCP was commercialized for postharvest treatment to delay ripening of apples [30–32].

Sprout inhibition of potatoes has mainly been driven by the use of propham, chlorpropham and maleic hydrazide during the last few years. Whilst propham (IPC) and chlorpropham (CIPC, Fig. 11.5) are applied after harvest at the begin-

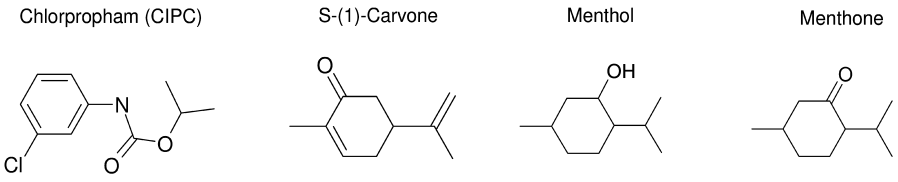

Fig. 11.5. Chemical structures of commercialized sprout suppressants.

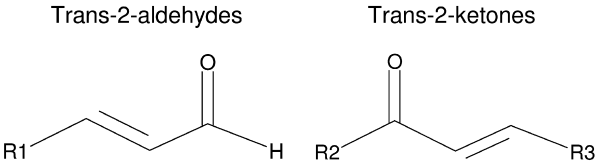

Fig. 11.6. Chemical structures of a new class of potential sprout suppressants.

ning of the storage, maleic hydrazide (MH) is applied to the potato foliage when tubers have reached a size of 40–70 mm. Tecnazene (TCNB) was also used in potato storage, but disappeared from the market due to its long degradation time. The monoterpenes S-(+)-carvone, produced from caraway seeds, has been developed commercially as a competitive product to CIPC (Fig. 11.5). Recently, menthol was commercialized for use as a sprout suppressant. Besides their sprout suppressing ability the natural terpenes also inhibit microbial growth and prevent rotting of treated potato tubers [33]. Coleman et al. [34] detected different activities of S-(+)-carvone, menthone and neomenthol, a diastereomer of menthol. The latter two showed 5–10× higher activity in suppressing tuber sprouting than S-(+)-carvone.

A patent on a new class of sprout suppressants was published recently [35]. It covers trans-2-ketones and trans-2-aldehydes (Fig. 11.6) being active as potato sprout suppressants. Known from "grass smell", trans-2-hexenal is included in this patent.

An important role of PGR is their involvement in *abiotic* and *biotic* stress defense mechanisms. Triazoles like paclobutrazole, propiconazole and tetraconazole are reported to be stress protectants [36]. Natural PGR are involved in indirect defense mechanisms of plants against herbivores [37]. Jasmonic acid, salicylic acid (Fig. 11.7) and ethylene are part of the signaling pathways of stress defense mechanisms.

Phospholipids impact the hypersensitive response and systemic acquired resistance in plants, and therefore might also be a potential new class of commercial

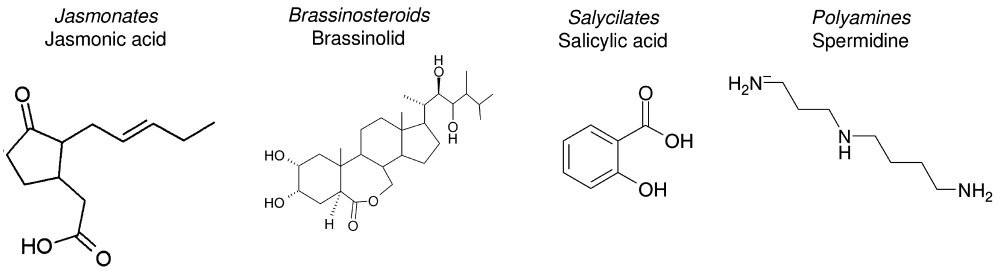

Fig. 11.7. Examples of chemicals out of potential new groups of PGR.

PGR [38]. The influence of polyamines [putrescine, spermidine (Fig. 11.7), spermine] on plant growth, including cell division, germination, till fruit development and stress response, has been reviewed by several authors [39–41]. Evans and Malmberg [42] furthermore summarized current knowledge of interactions of polyamines to commercial PGR and environmental stimuli. An interaction of polyamines with phospholipids in vesicles was described by Tadolini [43]. Polyamines may have an important role in stabilizing membranes through protection of lipid peroxidation. The metabolic link between polyamine and ethylene synthesis led to the suggestion of an impact of these PGR in abiotic and biotic interactions of the roots and the rhizosphere [44]. Romera and Alcantara [45] have summarized recent findings of ethylene involvement in the regulation of Fe-deficiency stress response. Especially in plants with iron-acquisition strategy I, the acidification of rhizosphere and subapical swelling of roots, ethylene plays a role in the regulation of stress response.

A reduction of abiotic and biotic stress in plants is also described as a side effect of quinone-outside-inhibiting (QoI)-fungicides, like the strobilurins. Wu and von Tiedemann [46, 47] reported that the strobilurin azoxystrobin and the triazole epoxiconazole exhibited strong antioxidative properties in delaying senescence and protecting barley and wheat from ozone injury. An increased resistance of tobacco against the tobacco mosaic virus *Pseudomonas syringae* pv. *tabbaci* was reported by Herms et al. [48] after a treatment of the plants with pyraclostrobin.

An abiotic stress with increasing importance in the near future is *water stress*. First reports indicate that PGRs also act as regulators under drought conditions. A positive response of kentucky bluegrass (*Poa pratensis* L.) to natural PGR was described by Schmidt in 1993 [49]. In these studies foliar application of seaweed extracts could accelerate recovery of kentucky bluegrass under serious drought conditions. Ervin and Koski [50, 51] described reduced evapotranspiration in kentucky bluegrass treated with trinexapac-ethyl. Marcum and Jiang [52] found similar effects on tall fescue (*Festuca arundinacea* S.). Zhang and Schmidt [53] and Zhang and Ervin [54] described enhanced drought tolerance of tall fescue and creeping bentgrass (*Agrostis palustris* Huds. A.) after application of humic acid or seaweed extract. In 2004, Zhang et al. [55] reported a better yield of soybeans after spray of uniconazole, brassinolide and ABA under drought conditions, compared with the untreated control. They did not detect such effects after benzyladenine (6-BA) treatment. Schubert [56] has reported effects of PGR on the yield of cereals under drought stress dependent on the PGR applied. Trinexapac-ethyl treated plants had a much higher harvest index and increased thousand-kernel weight compared with those of CCC treatments and the control.

Further new developments of commercial PGR include label extensions and mixtures of currently commercialized PGR as well as the evaluation of development of scientifically known PGR, including jasmonate [57], brassinosteroids [58], polyamines [42], phospholipids [38] and oligosaccharides [59, 60], for agricultural use.

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Part II
Fungicides

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Overview

The section on *Fungicides* embraces the chemistry, biochemistry and biology of new, different fungicidal compound classes and compounds, the resistance development against different fungicidal compound classes, and the changes in fungicide markets worldwide. The different contributions also deal with the changes in importance of the different fungicide classes and modes of action for research and development. They describe new compounds and compound classes introduced to the market during the last 20 years, including newly announced development compounds.

The contribution "FRAC Mode of Action Classification and Resistance Risk of Fungicides" (Chapter 12), starting with a short introduction on the history of fungicides, explains the importance of individual modes of action relative to their market penetration, their mechanisms and occurrence of resistance, the importance and occurrence of practical resistance on the management of fungicide resistance by FRAC (Fungicide Resistance Action Committee) and its recommendations to farmers related to the modes of action of the different fungicides.

Practical resistance occurred very early in the history of fungicides, but since the 1980s advisors from universities, regulatory authorities, farmers and farmers advisors and the agrochemical industry have learnt to deal with and to recommend practical application advice, alternating spraying recommendations and application methods and mixtures of fungicides to prevent yield losses caused by resistance development.

With the introduction of Bc 1 complex inhibitors (Chapter 13.2) in 1996, which act on the electron transport of fungi cells, the most important new compound class called *strobilurins*, derived from the natural compound Strobilurin A, was introduced in the reference period to the market. Chapter 13 describes the biochemistry of oxidative phosphorylation and of the related compounds and compound classes acting at different sites, reflecting the importance of these targets for the fungicide market nowadays. At the same time it became evident that resistance development, e.g., in the compound class of *strobilurins* must not lead necessarily to the abandonment of the marketing, research and development of such chemistry.

Although the class of *sterol biosynthesis inhibitors* (Chapter 17) was invented by the 1970s, the last 20 years has seen intensive research and development in this

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

area from all agrochemical companies, leading to further important innovations, including highly specific or broad spectrum fungicides.

Oomycetes fungi are biologically and biochemically very different to Ascomycetes and also cause very important damage in crops, especially vine grapes, potatoes, vegetables like tomatoes. However, with new compounds with different modes of action, such as antitubulin action (Chapter 16), action on perturbation of the cytoskeleton of the fungi (Chapter 19) or new compound groups like CAA (Carboxylic Acid Amides, Chapter 18), new weapons have been introduced against diseases caused by *oomycetes* fungi.

New modes of actions have been detected in the last 20 years, leading to fungicides marketed against *eye spot disease* in wheat and barley and *botrytis* like methionine biosynthesis inhibitors (Chapter 14.2), or by acting on signal transduction of fungi (Chapter 15), or melanin biosynthesis in cell wall (Chapter 20). Chapter 20 also explains research based on the target and the X-ray studies of the binding pocket and molecular modeling based synthesis of new inhibitors, leading to new rice blast disease fungicides.

The mode of action has not been detected or exactly known – of course – for all newly introduced fungicides (Chapters 21 and 22). These examples demonstrate the need for future additional biochemical research effort in universities as well as in agrochemical companies.

However, chemical innovations in stereospecific synthesis can be the key for a new fungicide based on an old (known for over 30 years) structure and long-used mode of action (Chapter 23).

This section on Fungicides reflects the research and development of recent decades, giving great insight into this area through the excellent contributions of authors from both agrochemical companies and universities.

12
FRAC Mode of Action Classification and Resistance Risk of Fungicides

Karl-Heinz Kuck and Ulrich Gisi

12.1
History of Fungicide Use

The first fungicide, discovered at the beginning of the 19th century, was lime-sulphur, introduced by William Forsyth, and recommended for the control of powdery mildew diseases of fruit trees [1]. The next milestone was the introduction of Bordeaux mixture, a copper based preparation, in 1885 by Millardet to combat the newly introduced downy mildew pathogen *Plasmopara viticola* on grapes [2]. In the early 20th century the first organic fungicides, organo-mercury compounds, were introduced for cereal seed treatment. Since the 1930s organic compounds such as the dithiocarbamates and the phthalimides became very important tools for disease control. The mode of action of these fungicides is described as "multi-site", inhibiting simultaneously a range of enzymes and cellular structures and giving preventative protection of plants against various diseases in a non-systemic way on the surface of the plant.

The first fungicides with a specific mode of action – benzimidazoles, carboxamides and early sterol biosynthesis inhibitors (SBIs) such as triforine – were discovered in the 1960s and early 1970s. During the late 1970s and the early 1980s dicarboximides, phenylamides and new SBI classes, among them the first triazoles entered the market. Specific fungicides control fungal plant pathogens more effectively and at much lower rate compared with multi-site contact fungicides. Most, but not all, of them have systemic properties and are therefore able to penetrate the plant tissue and to be further distributed via the xylem vessels into plant parts not been reached directly by the spray application. Overall, specific fungicides with systemic properties were regarded as a real progress in crop protection because they are less likely to be removed by rain but often re-distributed within the plant. As a result they allowed a considerable reduction of the dose rate and the number of applications per season. However, the specific mode of action was the origin of a new phenomenon, the selection of resistant individuals in fungal populations and the development of practical field resistance.

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ISBN: 978-3-527-31496-6

12.2
Fungicides: Importance of Individual Modes of Action

Forty-six different specific fungicide and bactericide modes of action are actually classified in the FRAC lists [3], including the unknown modes of action (Table 12.1). In addition, numerous multi-site inhibitors and plant defense inducers are available for the control of plant diseases worldwide. Therefore, a sufficient diversity of modes of action seems to be available for the control of plant diseases and for an effective resistance management. However, many fungicides are only available in a restricted number of regions and crops because they may not be registered everywhere or because the market size may not be big enough.

In addition, a use limitation of the modes of action results from a narrow spectrum of activity (25 out of 46 modes of action, Table 12.1), affecting only one systematic group of pathogens such as Oomycetes or even only one single pathogen species. For example, pencycuron and validamycin control only *Rhizoctonia solani*, the causal agent of sheath blight in rice and of stem canker and black scurf in potatoes. Furthermore, the thiophene-carboxamide derivative silthiofam specifically controls *Gaeumannomyces graminis*, the take-all pathogen of wheat. As shown in Table 12.1, there are nine specific modes of action available for Oomycetes control, four against rice blast (incited by *Magnaporthe grisea*), and four with a specific activity against powdery mildew pathogens.

Tables 12.2–12.4 give an overview of the importance of individual modes of action and the crops or regions in which they are mostly used. The total worldwide fungicide market in 2004 has been estimated to about 7.33 billion US$ [4]. A few modes of action dominate the overall fungicide market (Table 12.2):

Table 12.1 Number of modes of action mentioned in the FRAC list[a] with broad or narrow spectrum of activity.

Pathogen group(s) controlled	Broad spectrum	Narrow spectrum
Bacteria		4
Oomycetes		9
Powdery mildews		4
Rice blast		4
Take all		1
Rhizoctonia		2
Botrytis and related		1
Ascomycetes/Basidiomycetes	15	
Ascomycetes/Basidiomycetes/Oomycetes	6	
Total	21	25

[a] Specific modes of action [unknown (U) included; except host plant defense inducers (P) and multi-site inhibitors (M)].

Table 12.2 Sales of fungicide groups as a % of total fungicide market in 2004. Mode of action classifications refer to FRAC codes as given in Tables 12.6–12.18. (Data Source: Phillips McDougall 2005 (estimated total fungicide sales in 2004: US$ 7.33 billion).)

Fungicide group[a]	Mode of action group	%
DMI fungicides	G1	27.7
QoI fungicides	C3	19.1
Dithiocarbamates and related	M3	7.2
Copper and sulfur formulations	M1 and M2	4.8
Benzimidazoles and thiophanates	B1	3.6
Chloronitriles	M5	3.3
Dicarboximides	F1	2.9
Phenylamides	A1	2.8
Amines	G2	2.7
Anilinopyrimidines	D1	2.6
MBI fungicides	I1 and I2	2.6
Carboxamides	C2	1.8
Uncouplers	C5	1.8
Resistance inducers	P1, P2, P3	1.8
Phosphonates	U (33)	1.7
Other multi-site fungicides	M_x	1.7
Phthalimides	M4	1.6
CAA fungicides	F5	1.5
Cyanoacetamide oximes	U (27)	1.5
Others		7.3

[a] For an explanation of group designations see Tables 12.6–12.18.

Sterol biosynthesis inhibitors (including DMI fungicides and amines) clearly take a leading position, accounting for more than 30% of total fungicide sales. Another fungicidal mode of action, the inhibition of complex III in mitochondrial respiration, has rapidly gained importance since 1996. This fungicide group is nowadays usually known under the name QoI fungicides (Quinone outside Inhibitors) or (chemically less correct) ''strobilurins''. Most other specific modes of action shown in Table 12.2 originate from the 1960s and 1970s. In addition, two more recent groups, the anilino pyrimidines and the CAA (carboxylic acid amides) fungicides, are gaining importance but have not yet reached a prominent position.

Table 12.3 documents the dominant position of cereals within the total fungicide market, followed by the market segment ''fruits and vegetables'', which is, however, a complex segment composed of a multitude of smaller crops. Only small changes in the relative importance of specific crops are expected for the near future; however, one single pathogen, soybean rust (*Phakopsora pachyrhizi*), has created an important and totally new segment for fungicide use within recent years.

Table 12.3 Fungicide use in different crops in 2004. (Data Source: Phillips McDougall 2005.)

Crop	%
Cereals	22.0
Fruits and vegetables	19.1
Vines	10.7
Soybean	9.6
Rice	8.6
Potato	7.6
Pome fruit	5.4
Others	17.0

Table 12.4 Fungicide market in different regions. (Data: Agrowin 2004.)

Region	%
Western Europe	36.7
Asia/Pacific	24.2
South and Latin America	22.1
North America	9.0
Eastern Europe	5.4
Others	2.6

At the regional level, fungicide sales in Western Europe are outstanding because of the dominant position of the cereal crop, followed by Asian regions with important fungicide consumption in vegetable and fruit production and by the new market segment soybean rust, which is causing an increasing fungicide market in Brazil and Latin-America (Table 12.4).

12.3 Fungicide Resistance

12.3.1 Mechanisms and Occurrence of Resistance

Although some multi-site fungicides have been in use for over 200 years, resistance reports for this class of chemicals are rare and usually of low practical importance. As shown in Table 12.5, resistance of cereal pathogens to organomercury compounds was reported in 1964 and resistance of apple scab to dodine in 1969 [5].

Table 12.5 Occurrence of practical resistance to fungicides. (After Brent & Hollomon [23], supplemented.)

Date first observed	Fungicide/class	Years[a]	Crop/pathogen[b]	Practical importance[c]
1960	Aromatic hydrocarbons	20	Citrus storage rots *Penicillium* sp.	Low
1964	Organomercurials	40	Cereal leaf spot and stripe *Pyrenophora* spp.	Low
1969	Dodine	10	Apple scab *Venturia inaequalis*	Medium
1970	Benzimidazoles	2	Many target diseases incited by Ascomycetes	High
1971	2-Aminopyrimidines	2	Cucumber and cereal powdery mildews *Sphaerotheca fuliginea* and *Blumeria graminis*	Medium
1971	Kasugamycin	6	Rice blast *Magnaporthe grisea*	Low
1976	Phosphoro-thiolates	9	Rice blast *Magnaporthe grisea*	Medium
1977	Triphenyltins	13	Sugar beet *Cercospora beticola*	Low
1980	Phenylamides	2	*Phytophthora infestans* *Plasmopara viticola* and other Oomycetes in numerous crops	High
1982	Dicarboximides	5	Grey mould in grapes and other crops *Botrytis cinerea*	Medium to high
1982	DMI fungicides	7	Many pathogens of Ascomycetes in many crops	Medium
1985	Carboxamides	15	Barley loose smut *Ustilago nuda*	Low
1994	CAA fungicides	2	Grape downy mildew *Plasmopara viticola*	Low
1998	Phenylpyrroles	4	Grape *Botrytis cinerea*; apple *Venturia inaequalis*	Low
1998	QoI fungicides	2	Many pathogens of Oomycetes and Ascomycetes in many crops	High

Table 12.5 *(continued)*

Date first observed	Fungicide/class	Years[a]	Crop/pathogen[b]	Practical importance[c]
2000	Anilino-pyrimidines	5	Grape *Botrytis cinerea*; apple *Venturia inaequalis*; wheat *Blumeria graminis* and *Oculimacula* spp.	Low
2001	Quinoxyfen	4	Wheat and barley *Blumeria graminis*	Low to medium
2002	MBI-D Fungicides	6	Rice blast *Magnaporthe grisea*	Medium

[a] Estimated numbers of years after market introduction.
[b] Examples given for high risk cases only.
[c] Judged based on risk of loss of control under practical conditions.

The occurrence of resistance to single-site fungicides was reported in the 1970s and 1980s. However, important differences exist in terms of practical implications. In only a few cases were severe problems for product performance noted, such as in benzimidazoles. In other cases, the practical consequences are of limited importance due to several possible reasons:

- low resistance factors (e.g., DMIs);
- reduced fitness of resistant isolates (e.g., dicarboximides);
- limited commercial importance of the affected fungicide class;
- successful resistance management (e.g., phenylamides).

The factors described above are the result of both intrinsic properties of resistant isolates and the way in which the fungicides were used. Intrinsic properties of resistant isolates are strongly related to the biochemical mechanism that causes a reduced sensitivity. Several types of resistance mechanisms can be distinguished:

1. unspecific resistance based on ABC transporters;
2. polygenic resistance characterized by a continuous selection process;
3. monogenic resistance (mutations at target site) leading to a disruptive selection process;
4. resistance based on metabolic detoxification of fungicide.

The greatest impact on resistance is associated with monogenic mechanisms, especially mutations at the target site that result in high resistance factors and low or no fitness penalties. These factors apply for the E198A/G/K and F200Y

mutation in the β-tubulin gene conferring resistance to benzimidazoles and the G143A mutation in the cytochrome b gene delivering QoI resistance. The most prominent examples for polygenic resistance resulting in continuous, stepwise selection are those connected with SBI fungicides (DMIs and amines) [6–7]. Unspecific resistance mechanisms based on the energy-dependent export of xenobiotics by ABC-transporters are often associated with polygenic resistance but on their own play only a limited role for practical field resistance because of low resistance factors and distinct fitness penalties [8]. In contrast to the importance for herbicides and insecticides, metabolic detoxification of the active ingredient is of low importance for fungicides. The only documented cases of fungicide detoxification are those for iprobenfos, [9] fenhexamid [10] and kresoxim-methyl [11].

12.3.2 The Fungicide Resistance Action Committee (FRAC)

As a consequence of wide spread resistance problems with benzimidazoles and dicarboximides and upcoming problems with phenylamide and triazole fungicides, resistance seminars were organized at the University of Wageningen in the Netherlands in 1981 and 1982. Representatives of the chemical industry decided to establish an inter-company group that had the task of coordinating resistance management strategies. The Fungicide Resistance Action Committee (FRAC) was founded as an organization designated to discuss resistance problems and to make cooperative efforts in the prevention and management of fungicide resistance. FRAC became incorporated within GIFAP, the International Group of National Associations of Manufacturers of Agrochemical Products. This organization evolved later on – within an organization called Global Crop Protection Federation (GCPF) and then within CropLife International, the global federation representing the plant science industry.

The purpose of FRAC is to provide guidelines for fungicide resistance management to prolong the effectiveness of "at risk" fungicides and to limit crop losses should resistance occur.

In more detail the main aims of FRAC [12] are to

- Identify existing and potential resistance problems.
- Collate information and distribute it to those involved in research, development, distribution, registration and use of fungicides.
- Provide guidelines and advice on the use of fungicides to reduce the risk of resistance and to manage it should it occur.
- Recommend procedures for fungicide resistance studies.
- Stimulate open liaison and collaboration with universities, government agencies, advisors, extension workers, distributors and farmers.

If molecules from different manufacturers have the same mode of action and if this mode of action bears at the same time a significant resistance risk, a FRAC

Working Group can be established to analyze the resistance risk and to develop and publish common resistance management recommendations. There are currently FRAC Working Groups for sterol biosynthesis inhibitors (SBIs), QoI fungicides, anilinopyrimidines (APs) and carboxylic acid amides (CAAs) which meet regularly and publish yearly updated reports on the resistance status and suitable resistance management recommendations. In addition, the FRAC Banana Working Group, composed of fungicide manufacturers and fruit companies, coordinates resistance management recommendations for all specific fungicides used in banana production. For older modes of action for which regular monitoring programs are no longer performed (benzimidazoles, phenylamides and dicarboximides), so-called Expert Fora are available at the FRAC website (www.frac.info) to give advice and collect important published literature on resistance monitoring methods and resistance management.

12.3.3 Resistance Risk Assessment

The overall resistance risk is the result of the interaction of numerous independent factors. The intrinsic risk is related to all aspects of the mode of action, the biology of the pathogen and the interactions between them, whereas the extrinsic (or management) risk includes all aspects of how a product is used such as number and interval of applications, rates and type of treatments and whether the product is used as a solo formulation or in mixture or alternation with other modes of action. The intrinsic risk is composed of several elements, which have been described in more detail in different documents [13–15]. The most important elements include the analysis of base line sensitivity of field isolates [16], population structure (uni- or bimodal), cross and multiple resistance, stability of resistance, forced selection of resistant individuals over several generations, artificial mutagenesis and selection, biochemical site of action (single- or multi-site), molecular mechanism of resistance (mutations in target site gene) and genetic inheritance of resistance (mono- or polygenic resistance). Some of these elements are not easy to evaluate, especially when no resistant isolates are available from field populations. However, when the results generated for a new active ingredient are compared with those of known chemical classes, the intrinsic risk can mostly be assessed quite well and classified as low, medium or high. According to the estimated intrinsic risk, appropriate strategies can be defined as to how to use the product to minimize the management risk.

12.3.4 Resistance Management and Risk Modifiers

Based on the environmental and toxicological properties of a compound, a rather fixed frame is given that defines the maximum number of applications per season and area. The assessment of resistance risk must be done within this frame and the number of applications may be further reduced to adequately manage resistance risk.

Appropriate resistance management tools have to be chosen and validated for each individual pathogen/fungicide combination and out of all available resistance risk modifiers. The EPPO Standard on Resistance Risk Analysis [17] lists the following tools for lowering the overall resistance risk:

- Use of good plant protection practices:
  - Lower the disease pressure by all means of good plant protection practices as, for example, the use of less susceptible cultivars, crop rotation, adequate use of fertilizers, and of sanitation measures lowering the primary inoculum such as elimination of plant debris by plowing instead of minimum tillage.
- Use of mixtures/alternations of fungicides:
  - The use of mixtures of fungicide partners without cross resistance is a validated standard tool in resistance management as well as the alternation of non-cross resistant fungicides that is preferably used in longer spray schedules.
- Application frequency, timing and dose rate:
  - Beside the use of mixtures or alternations, the limitation of the number of applications per season is another important standard tool in resistance management. In addition, the timing of application (preventive or curative) and the dose rate applied are of outstanding importance.
- Negative cross resistance:
  - The use of fungicides exhibiting negative cross resistance is theoretically a suitable way to decrease the frequency of resistant isolates. Unfortunately, negative cross resistance is very rare in fungicide resistance.
- Sensitivity monitoring, reporting to the authorities and reaction to changes in performance:
  - Systematic monitoring programs that allow observation of the resistance dynamics are the basis for the development of rational resistance management concepts. The availability of detailed sensitivity profiles needs, in addition, discussion with authorities to improve resistance management programs.

12.4 Fungicide Classes and Modes of Action

The classification of fungicides according to their mode of action and cross resistance pattern became necessary to facilitate resistance management at the field level under practical agronomic conditions. If fungicides are recommended to be used in alternation or mixtures to achieve robust disease control and delaying resistance evolution, clear information on the cross-resistance behavior for each

compound is required because it is not possible to recognize whether two fungicides are cross resistant by just comparing chemical structures, common names or group names. The FRAC classification covers all important fungicides (and some bactericides and plant defense inducers) that are registered worldwide. As far as available the classification is based on the biochemical and/or molecular mode of resistance. This implies that the methods used for studying the mode of action and the mechanism of resistance are inevitably different for each new class because they depend on the availability of technical possibilities during the time of the studies. For fungicides that are used in only few countries and crops, such studies are often very limited or not published in one of the common languages. Therefore, the FRAC code list is a compromise between the need to come to a reasonable classification and pure scientific approaches. A similar compromise is often needed for the chemical group names, which are sometimes not derived from a systematic chemistry approach but have a more general character or are based on the first member in the group.

The FRAC Code list is available in two versions, one sorted by serial numbers or letters given at the time when they were registered for commercial use and the second by mode of action. Once the mode of action or cross resistance pattern of a new fungicide becomes known, the compound is given a unique serial number. All known modes of action are listed in the following tables. A similar list giving, in addition, information on resistance risk and needed resistance management measures for each mode of action is published at the FRAC homepage and is updated yearly [18].

Fungicides that interfere with nucleic acid synthesis are an important group of phenylamides that have kept a strong position in the Oomycete market despite major resistance problems (Table 12.6). Fungicides inhibiting adenosine-

Table 12.6 Group A: Fungicides interfering with nucleic acid synthesis.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
A1	4	RNA polymerase I	Phenylamides	Metalaxyl-M	Specific for Oomycetes
A2	8	Adenosine-deaminase	Hydroxy-pyrimidines	Ethirimol	Specific for powdery mildew
A3	32	DNA/RNA synthesis (prop.)	Hetero-aromatics	Hymexazol	Broad spectrum
A4	31	DNA topoisomerase type II	Carboxylic acids	Oxolinic acid	Bactericide

Table 12.7 Group B: Fungicides interfering with mitosis and cell division.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
B1	1	β-Tubuline assembly in mitosis	Methyl-benzimidazole carbamates	Benomyl, carbendazim, thiophanat-methyl	Broad spectrum fungicides; high resistance risk
B2	10	β-Tubuline assembly in mitosis	N-Phenyl-carbamates	Diethofencarb	Negative cross resistance to B1
B3	22	β-Tubuline assembly in mitosis	Benzamides	Zoxamide	Specific for Oomycetes
B4	20	Cell division (prop)	Phenylureas	Pencycuron	Specific for *Rhizoctonia solani*

deaminase are of limited market importance nowadays due to pronounced resistance problems in the powdery mildew pathogens. Hymexazol is used as a soil- and seed-treatment fungicide and oxolinic acid for the control of bacterial diseases such as fire blight in apples and pears caused by *Erwinia amylovora*.

Fungicides that interfere with β-tubuline assembly (benzimidazoles and benzimidazole generators) have lost most of their initial importance for the control of a multitude of diseases due to the wide spread distribution of resistance (Table 12.7). Also, the B2 compound diethofencarb has lost importance; it was initially developed to specifically combat benzimidazole-resistant strains as it inhibits only the mutated isolates, thus showing a negative cross resistance to benzimidazoles. Zoxamide is the first representative being specifically optimized for the inhibition of β-tubuline assembly in Oomycetes that are not affected by B1 fungicides. Pencycuron exhibits a highly specific fungicidal action against only one pathogen, *Rhizoctonia solani*.

Due to the basic importance of the target, fungicides that inhibit fungal respiration have mostly a broad spectrum of activity and control both Oomycetes and the true fungi (e.g., Ascomycetes and Basidiomycetes) (Table 12.8).

Uncouplers and most complex II inhibitors have been available for many years. For a long time carboxamides such as carboxin were restricted to the control of Basidiomycetes but, recently, new representatives of the C2 group such as boscalid have been discovered that can also be used for the control of Ascomycetes.

Owing to their broad spectrum and long-lasting activity, QoI fungicides have rapidly gained an important market share since the introduction of the first representatives (azoxystrobin and kresoxim-methyl) in 1996. However, their use has

Table 12.8 Group C: Fungicides interfering with fungal respiration.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
C1	39	Complex I (prop.)	Pyrimidine-amines	Diflumetorim	
C2	7	Complex II: succinate-dehydrogenase	Carboxamides	Carboxin, Boscalid	Mostly broad spectrum
C3	11	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site → QoI fungicides	Strobilurins and related	Azoxystrobin, Pyraclostrobin	Mostly broad spectrum; high resistance risk
C4	21	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qi site → QiI fungicides	Cyano-imidazoles	Cyazofamid	Specific for Oomycetes
C5	29	Uncoupler of oxidative phosphorylation	Diverse	Binapacryl, fluazinam, ferimzone	Broad spectrum

been recently limited in certain crops (e.g., cereals, grapes) due to the high frequency of resistant isolates in field populations.

In Group D, all compounds except the anilinopyrimidines are antibiotics of microbial origin (Table 12.9). D2 and D3 show a specific action against rice blast (*Magnaporthe grisea*), whereas D4 and D5 are specific bactericides that are used in parallel in the medical field. The anilinopyrimidine fungicides can be used against a broad range of diseases caused by Ascomycetes in fruits and vegetables, cereals and bananas.

The quinoline derivative quinoxyfen controls exclusively powdery mildews in cereals and broadleaved crops whereas phenylpyrroles such as fludioxonil are used as foliar, post harvest and seed treatment fungicides against a broad range of pathogens such as *Botrytis cinerea* and apple scab (Table 12.10).

Group F compounds interfere with lipid and membrane synthesis as well as with cell wall deposition – they cover a range of target sites, most of which have a putative status because the modes of action have not been fully elucidated (Table 12.11).

Dicarboximides are still used against *Botrytis cinerea* and related pathogens in a range of crops although resistance was reported repeatedly. However, resistance

Table 12.9 Group D: Fungicides interfering with amino acid and protein synthesis.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
D1	9	Methionine biosynthesis (prop.)	Anilino-pyrimidine fungicides (AP fungicides)	Pyrimethanil, cyprodinil, mepanipyrim	Broad spectrum (*Botrytis*, *Venturia*, *Oculimacula*, Sigatoka, etc.)
D2	23	Protein synthesis	Enopyranuronic acid antibiotic	Blasticidin-S	Rice blast
D3	24	Protein synthesis	Hexopyranosyl antibiotics	Kasugamycin	Rice blast
D4	25	Protein synthesis	Glucopyranosyl antibiotics	Streptomycin	Bactericide
D5	41	Protein synthesis	Tetracycline antibiotics	Oxy-tetracycline	Bactericide

to this class of fungicides seems to be variable in frequency and location. The mode of action of the Carboxylic Acid Amides is still speculative and resistance has been detected in *Plasmopara viticola* but not in *Phytophthora infestans*.

Since the first introduction of amines and DMIs in the 1960s and 1970s the inhibition of fungal sterol biosynthesis has rapidly become the most successful biochemical target within specific fungicides (Table 12.12). In addition to the broad spectrum of activity, covering most pathogens belonging to the Ascomycetes and

Table 12.10 Group E: Fungicides interfering with signal transduction.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
E1	13	G-proteins in early cell signaling (prop.)	Quinolines	Quinoxyfen	Specific for powdery mildew
E2	12	MAP protein kinase in osmotic signal transduction	Phenylpyrroles	Fludioxonil	Broad spectrum

Table 12.11 Group F: Fungicides interfering with lipid and membrane synthesis, cell wall deposition.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
F1	2	NADH cytochrome c reductase in lipid peroxidation (prop.)	Dicarboximides	Iprodione, procymidone, vinclozolin	Predominately used for *Botrytis* and related pathogens
F2	6	Methyl transferase in phospholipids biosynthesis	Phosphoro-thiolates/ dithiolanes	Edifenphos pyrazophos/ isoprothiolane	Mostly for rice blast control
F3	14	Lipid peroxidation (prop.)	Aromatic hydrocarbons/ thiadiazoles	Biphenyl chloroneb/ etridiazole	Diverse activity spectra
F4	28	Cell membrane permeability, fatty acids (prop.)	Carbamates	Propamocarb	Specific for Oomycetes
F5	40	Phospholipid biosynthesis and cell wall deposition (prop.)	Carboxylic acid amides (CAA fungicides)	Dimethomorph, iprovalicarb, mandipropamid	Specific for Oomycetes

Table 12.12 Group G: Fungicides interfering with sterol biosynthesis in membranes.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
G1	3	C14-**D**e**M**ethylation **I**nhibitors in sterol biosynthesis (DMI fungicides)	Piperazines, pyridines, pyrimidines, imidazoles, triazoles	Prochloraz, propiconazole, tebuconazole, epoxiconazole, prothioconazole	Broad spectrum; over 30 commercial compounds
G2	5	Δ^{14}-Reductase and $\Delta^{8} \rightarrow \Delta^{7}$ isomerase in sterol biosynthesis	Amines, (morpholines, piperidines, spiroketalamines)	Fenpropimorph, fenpropidin, spiroxamine	Used mainly against powdery mildews, rusts and Sigatoka
G3	17	3-Keto reductase in C4 demethylation	Hydroxyanilides	Fenhexamid	Specific for *Botrytis* and related pathogens
G4	18	Squalene-epoxidase	Thiocarbamates/ allylamines	Pyributicarb/ naftifine	Not used as agricultural fungicides

Table 12.13 Group H: Fungicides interfering with glucan synthesis.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
H1	26	Trehalase and inositol synthesis	Glucopyranosyl antibiotics	Validamycin	Specific for rice sheath blight (*Rhizoctonia solani*)
H2	19	Chitin synthase	Polyoxins	Polyoxin B	Broad spectrum

Basidiomycetes, a pronounced curative activity and a moderate resistance risk are characteristics for DMIs and amines. Contrary to other inhibitors of fungal sterol biosynthesis, the G3 representative fenhexamid shows a rather narrow spectrum of activity, confined to *Botrytis cinerea* and the related pathogens *Monilinia* spp. and *Sclerotinia* spp.

Inhibitors of fungal glucan synthesis are of microbial origin, produced by *Streptomyces hygroscopicus* var. *limoneus* (H1) and of *S. cacaoi* var. *asoensis* (H2) (Table 12.13) [19]. Validamycin is a specific compound for the control of rice sheath blight, whereas polyoxins inhibit a target site that, potentially, is present in all Ascomycetes and Basidiomycetes. Nevertheless, the market importance of polyoxins remains limited.

The inhibition of melanin synthesis in fungal cell walls is a rather specific target for the control of the rice blast pathogen (*Magnaporthe grisea*) (Table 12.14). The melanization of the appressorium cell wall is a pathogenicity factor of this pathogen, relying on mechanical pressure during the penetration of the host cuticle. Few other pathogens, such as *Colletotrichum* spp., use the same mechanism of penetration. As a consequence, the efficacy of melanin biosynthesis inhibitors

Table 12.14 Group I: Fungicides interfering with melanin synthesis in cell wall.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
I1	16.1	Reductase in melanin biosynthesis (MBI-R)	Melanin biosynthesis inhibitors	Tricyclazole	Specific for rice blast
I2	16.2	Dehydratase in melanin biosynthesis (MBI-D)	Melanin biosynthesis inhibitors	Carpropamid	

Table 12.15 Group P: Host plant defense inducers.

FRAC code		Target site	Chemical group	Compounds (examples)	Comments
Target no.	Serial no.				
P1	P	Salicylic acid pathway	Benzothiadiazole BTH	Acibenzolar-S-methyl	Indirect action on fungi, bacteria and viruses
P2	P	Unknown	Benzisothiazole	Probenazole	
P3	P	Unknown	Thiadiazole-carboxamide	Tiadinil	

is, theoretically, confined to both pathogens but, practically, to rice blast control only.

The induction of systemic acquired resistance (SAR) has been studied in detail during recent decades in a multitude of university groups and industrial research laboratories and is documented in many scientific papers [20]. However, only limited control is achieved by the induction of defense mechanisms against diseases under field conditions. Acibenzolar-S-methyl, better known under the trade name Bion®, is used in numerous crops and shows not only activity against fungal but also against bacterial and viral diseases (Table 12.15). Probenazole, well known under its trade name Oryzemate®, and more recently tiadinil are used to control rice blast (*Magnaporthe grisea*) but have also some antibacterial and antiviral effects.

Despite many studies, the biochemical mode of action of several fungicides and bactericides remains unclear (Table 12.16). Notably, two compounds, cymoxanil (trade name: Curzate®) and fosetyl-Al (trade name: Alliette®) have gained broad, long-term market acceptance for Oomycete control. The compounds of this table

Table 12.16 Group U: 1. Fungicides with unknown mode of action.

1-FRAC Serial no.	Chemical group	Compounds (examples)	Comments
27	Cyanoacetamide oxime	Cymoxanil	Oomycetes
33	Phosphonates/phosphorous acid	Fosetyl-Al	Oomycetes
34	Phthalamic acids	Tecloftalam	Bactericide
35	Benzotriazines	Triazoxid	Narrow spectrum
36	Benzene-sulfamides	Flusulfamide	Broad spectrum
37	Pyridazinones	Diclomezine	Broad spectrum
42	Thiocarbamate	Methasulfocarb	Broad spectrum

Table 12.17 Group U: 2. Recently introduced fungicides with unknown mode of action and resistance risk.

FRAC code	Chemical group	Compounds (examples)	Comments
U5	Thiazole carboxamides	Ethaboxam	Oomycetes
U6	Phenyl-acetamide	Cyflufenamid	Powdery mildews
U7	Quinazolinone	Proquinazid	Powdery mildews
U8	Benzophenone	Metrafenone	Powdery mildews and eyespot
U9	Acylpicolides	Fluopicolide	Oomycetes

have been classified with a serial FRAC code number because long-term sensitivity monitoring revealed that there is no cross resistance to other existing fungicide groups.

Compounds shown in Table 12.17 entered the market only recently or are going to enter soon. Because the mode of action and mechanism of resistance are still unknown, these compounds have been given a transient status. As soon as more detailed information becomes available, these compounds will be given a specific FRAC code number.

Most of the fungicides with a multi-site mode of action have been widely used for decades (Table 12.18). As already described above, this applies especially for the inorganic fungicides based on copper salts or sulfur, which are the oldest fungicides of all. Multi-site fungicides are generally considered as a group with low resistance risk without any signs of resistance development under field condi-

Table 12.18 Group M: Multi-site mode of action.

FRAC code	Chemical group	Compounds (examples)
M1	Inorganics	Copper (different salts)
M2	Inorganics	Sulfur
M3	Dithiocarbamates and related	Mancozeb
M4	Phthalimides	Captan
M5	Chloronitriles	Chlorothalonil
M6	Sulfamides	Tolylfluanid
M7	Guanidines	Dodine
M8	Triazines	Anilazine
M9	Anthraquinones	Dithianon

tions over decades. Although several reports on reduced sensitivity of pathogens to some of the multi-site compounds under laboratory conditions are available, the resistance risk under field conditions seems to be quite low. One exception is dodine for which resistance was described by Szkolnik and Gilpatrick already in 1969 [21]. They reported a distinct reduction of sensitivity of apple scab, *Venturia inaequalis*. These findings were confirmed in subsequent years by other research groups. Polygenic control of resistance to dodine and a continuous sensitivity distribution of *Venturia inaequalis* have been reported by Georgopoulos [22]. Koeller and Wilcox [23] have found evidence that dodine-resistant *Venturia* isolates were sometimes less sensitive also to other fungicide classes such as DMIs. These effects might be based on unspecific resistance mechanisms including ABC transporters.

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13
Fungicides Acting on Oxidative Phosphorylation

13.1
The Biochemistry of Oxidative Phosphorylation – A Multiplicity of Targets for Crop Protection Chemistry

Fergus Earley

13.1.1
Introduction

In all eukaryotic cells, the efficient use of carbohydrate, fat or protein as an energy source depends on the complete oxidation of constituent carbon atoms to carbon dioxide. The energy available from these oxidations is conserved through the coupled synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate (Pi) and used to drive the kinetic, biosynthetic and homeostatic processes of the cell through numerous concerted reactions involving the hydrolysis of ATP back to ADP.

The oxidation reactions involved are catalyzed by a series of nicotinamide adenine dinucleotide (NAD^+) or flavin adenine dinucleotide (FAD) dependent dehydrogenases in the highly conserved metabolic pathways of glycolysis, fatty acid oxidation and the tricarboxylic acid cycle, the latter two of which are localized to the mitochondrion, as is the bulk of coupled ATP synthesis. Reoxidation of the reduced cofactors (NADH and $FADH_2$) requires molecular oxygen and is carried out by protein complexes integral to the inner mitochondrial membrane, collectively known as the respiratory, electron transport, or cytochrome, chain. Ubiquinone (UQ), and the small soluble protein cytochrome c, act as carriers of electrons between the complexes (Fig. 13.1.1).

Before there was any understanding of the nature of the proteins involved, the overall sequence of electron transfer between NADH and oxygen could be divided into three sections by the use of exogenous substrates, specific inhibitors and by observing the oxidation state of the cytochromes. Thus the site of action of inhibitors and the sequence of electron transfer, NADH to UQ (inhibited by rotenone), UQ through cytochrome b to cytochrome c (inhibited by antimycin A), and cytochrome c through cytochrome a/a3 to oxygen (inhibited by cyanide), were defined

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

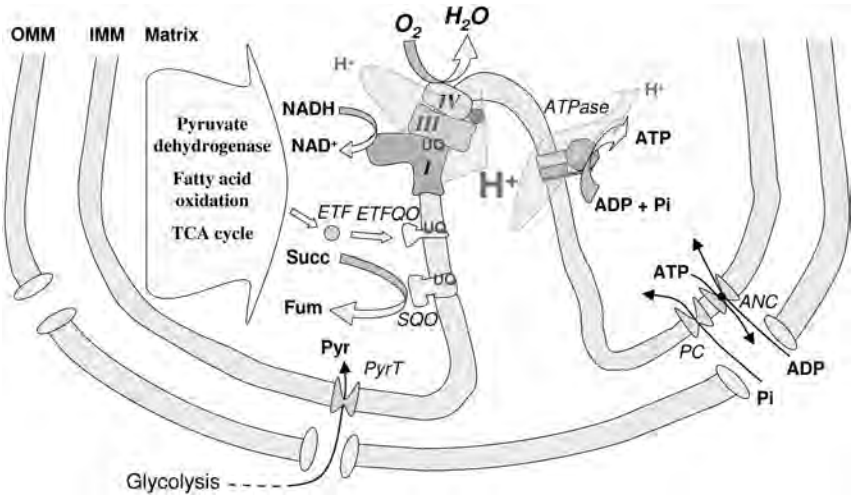


Fig. 13.1.1. Schematic overview of mitochondrial oxidative phosphorylation. A part of the mitochondrion is represented, showing the outer mitochondrial membrane (**OMM**), inner mitochondrial membrane (**IMM**) and crista (an invagination of the inner membrane). Substrates for oxidation enter the mitochondrion through specific carrier proteins, e.g., the pyruvate transporter, (*PyrT*). Reducing equivalents from fatty acyl CoA dehydrogenases, pyruvate dehydrogenase and the TCA cycle are delivered to the electron transport chain through NADH, succinate ubiquinol oxidoreductase (*SQO*), electron transfer flavoprotein (*ETF*) and its ubiquinol-dependent oxidoreductase (*ETFQO*). NADH is reoxidized by Complex I (***I***), and reduced ubiquinone (**UQ**, in blue) by electron transfer through Complex III (***III***), cytochrome c (red circle) and Complex IV (***IV***). These three complexes pump protons (H^+) across the membrane to create the electrochemical gradient that drives ATP synthesis by the F_1F_0ATP synthase (*ATPase*). Phosphate entry and ATP/ADP exchange with the cytosol is mediated by the phosphate carrier (*PC*) and ADP/ATP carrier (*ANC*), respectively. Other abbreviations not defined in the text are **Succ**, succinic acid; **Fum**, fumaric acid; and **Pyr**, pyruvic acid.

and it has since become clear that the proteins that support these sections of the electron transport chain are also physically associated in the membrane.

ATP synthesis is coupled to electron transfer at each of the sections defined above, referred to as coupling sites 1, 2 and 3, respectively. The mechanism of coupling was first proposed by Mitchell, and his ''Chemiosmotic hypothesis'' is now, in essence, universally accepted – i.e., that electron transfer through each of the coupling sites results in proton translocation from inside to outside of the inner mitochondrial membrane, and that the electrochemical gradient so generated both drives ATP synthesis and controls the rate of electron transfer (for a comprehensive overview of mitochondrial oxidative phosphorylation and the genesis of our current understanding, the reader is referred to excellent historical reviews [1, 2]).

Because these processes are essential to the survival of most aerobic organisms they have been exploited repeatedly by nature as targets for the chemical armory

of secondary metabolites used for defense or competition. Synthetic chemistry has also successfully exploited these targets for the benefit of agriculture, as fungicides, insecticides and acaricides. Indeed, our knowledge of the organization and mechanism of oxidative phosphorylation owes much to the discovery of these inhibitors and to the study of their action. They can be divided into three classes: inhibitors of electron transport, inhibitors of phosphorylation, and "uncouplers".

Both inhibitors and uncouplers have usually been detected as antimicrobial, pesticidal or cytotoxic agents that rapidly affect the rate of oxygen utilization by the cell, and then localized in their action by their effects on isolated mitochondrial preparations. The site of action of electron transport inhibitors is still initially determined from their ability to inhibit the oxidation of exogenous substrates (NADH, succinate, cytochrome c), and by their effect on the reduction or re-oxidation of the cytochromes, in crude mitochondrial or sub-mitochondrial preparations (e.g., Refs. [3, 4]). Defining an uncoupler or phosphorylation inhibitor is more demanding and requires the use of intact "coupled" mitochondrial preparations, in which oxidative phosphorylation can be monitored by the rate of oxygen consumption under controlled conditions (e.g., Ref. [5]). Mitochondria from animal tissues are generally preferred for these studies over fungal mitochondria, not only because of ease of preparation, but because the mitochondria of fungi, like those of plants, can express alternative electron transfer pathways that complicate the analysis (see Section 13.1.2.5).

Numerous structurally diverse organic chemicals have been identified as highly potent ($IC_{50} < 100$ nM) and specific inhibitors of electron transport, principally from plant and microbial secondary metabolites, but also from the screening of synthetic chemical libraries for fungicides and insecticides. Amongst the natural producers, the myxobacteria have proved a particularly abundant source of novel structural classes [6, 7], but potent inhibitors have also been isolated from plants, fungi, streptomycetes and marine organisms. Natural products have provided the inspiration for the commercially successful strobilurin class of fungicides [8] (see Chapter 13.2 of this volume), and even found commercial use themselves (rotenone has been registered for use as an insecticide since 1947 [9]).

Most potent electron transport inhibitors discovered so far are selective either for the NADH–ubiquinone oxidoreductase or the ubiquinol–cytochrome c oxidoreductase sections of the chain, very few classes are known to have similar potency against succinate–ubiquinol oxidoreductase. For cytochrome c oxidase, known potent inhibitors are limited to small molecules or ions that form coordination complexes with haem (e.g., CN^-, N_3^-, CO, PH_3). The inhibitor selectivity between UQ dependent oxidoreductases is surprising given that all inhibitors are thought to act at UQ binding sites, but is probably a consequence of the lack of sequence and structural conservation between different classes of quinone binding proteins [10–12]. In contrast to their selectivity between different sites in the respiratory chain, potent inhibitors generally do not show much selectivity between their mitochondrial targets in different species [13, 14] (although there are exceptions [15, 16]), which reflects evolutionary conservation of sequence and structure of the key functional subunits of the respiratory chain complexes.

This of course means that toxicity to non-target organisms is always a major consideration in the development of respiration inhibitors for use in agriculture.

13.1.2 Components of Mitochondrial Electron Transport Chains

When mitochondria from bovine heart were solubilized by treatment with mild detergents it was possible to separate and purify the sections of the respiratory chain referred to earlier as coupling sites 1, 2 and 3. These were named Complex I (NADH–ubiquinone oxidoreductase), Complex III (ubiquinol–cytochrome c oxidoreductase, cytochrome bc_1 complex) and Complex IV (cytochrome c oxidase) [17], and have since been characterized as independent entities, although it is now recognized that these three complexes co-assemble with specific stoichiometry to form respiratory chain ''supercomplexes'' or ''respirasomes'' in fungal, plant and mammalian mitochondria [18–20]. There is also evidence that succinate–ubiquinone oxidoreductase (which was purified alongside the other complexes and named Complex II [21]) forms a tight association with Complex III in yeast mitochondria [22].

Purification and reconstitution has revealed the full complement of proteins necessary for oxidative phosphorylation and enabled the identification of the genes encoding these proteins. The gene sequences are necessary tools for understanding the relationship between components of the mitochondrial respiratory chain and their prokaryotic ancestors and for tracking their divergence among the eukaryotes, both of which have helped to assign function to some of the subunits. They also provide the protein sequences essential for structural analysis, which has been achieved to atomic resolution for all complexes except Complex I.

The degree of conservation, in terms of subunit composition and protein sequence, between mammalian respiratory chain complexes and those characterized from fungi and other organisms depends on the subunit and complex being considered (detailed in specific sections below), but in general, those subunits which are known to have a central role in electron transport are well conserved in terms of protein sequence and, where known, tertiary structure. For these subunits, a clear relationship to bacterial respiratory chain components can also be seen, which leads to the conclusion that the mitochondrial respiratory chain complexes have evolved and adapted from those of the symbiotic bacterial ancestor of the mitochondrion [23]. Mitochondrial complexes have in most cases acquired many additional subunits whose function remains obscure.

13.1.2.1 Complex I and its Inhibitors

Complex I catalyzes the two-electron oxidation of NADH coupled to the transport of four protons across the membrane [24]. It is the largest and least well understood of the respiratory chain complexes. The form purified from bovine heart mitochondria has an aggregate molecular weight of at least 980 kDa and is made up of 46 different proteins [25]. It has also been purified from other sources, in-

cluding the filamentous mould, *Neurospora crassa*, obligate aerobic yeast, *Yarrowia lipolytica*, and the Gram negative bacterium *Escherichia coli*. Bacterial Complex I is much simpler than the mitochondrial forms, having only 14 subunits, but is functionally equivalent in terms of electron transport and proton pumping capabilities [26], and even retains some inhibitor sensitivities [14]. All 14 subunits of *E. coli* Complex I have homologues in the mitochondrial forms, and so these, or a subset, are considered as the ''core'' functional subunits [27, 28]. Complex I from *Y. lipolytica* and *N. crassa* contains 37 and at least 39 non-identical subunits, respectively, of which all but four, in each case, have homologues in the bovine heart enzyme [29, 30].

Analysis by electron microscopy shows that Complex I from bovine heart, *N. crassa*, *Y. lipolytica*, and *E. coli*, can adopt a similar L shape structure, which spans the inner mitochondrial membrane with an arm extending into the matrix compartment [31, 32] (Fig. 13.1.2). A sub-domain representing part of this arm can be isolated that contains the FMN and retains NADH dehydrogenase activity coupled to reduction of ferricyanide (which is also an enzymic activity of intact Complex I). This activity is sensitive to the now superseded fungicide fenaminosulf [33, 34]. Fenaminosulf is not a highly selective Complex I inhibitor, but its ability to inhibit ferricyanide reduction is unusual and defines its site of action

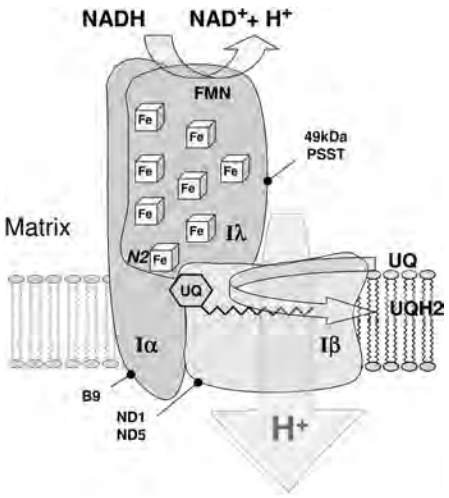


Fig. 13.1.2. Schematic representation of the structure and function of Complex I. The complex can be resolved into three sub-complexes: **I*α***, **I*γ*** (a subset of **I*α***) and **I*β***, arranged as indicated [28]. The NADH binding site, flavin cofactor (**FMN**) and all of the know iron sulfur centers (**Fe**) are carried on subunits within **I*γ***. Center ***N2*** is believed to be the oxidant for ubiquinone (**UQ**). The sub-domain assignments for subunits referred to in the text in the context of inhibitor binding is indicated. Subunit PSST is also believed to ligate iron sulfur center N2.

as being at or close to the site of initial direct electron transfer between NADH and FMN.

The application of electron paramagnetic resonance (EPR) spectroscopy has shown that electron transfer from FMN to UQ involves reduction of eight or more iron sulfur clusters, of which that with the highest redox potential, center N2, is responsible for UQ reduction [35]. EPR spectroscopy has also revealed the presence of two ubisemiquinone species, suggesting the presence of multiple quinone binding sites. There may also be other redox centers involved in electron transport through the complex [26, 36–38]. With the exception of fenaminosulf, none of the inhibitors listed in Table 13.1.1, or elsewhere in this volume, have been distinguished in their site of action based on effects on the reduction or re-oxidation of detectable redox centers – all that have been studied in detail seem to act to prevent electron transfer somewhere between center N2 and UQ [14].

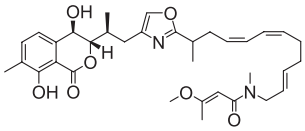
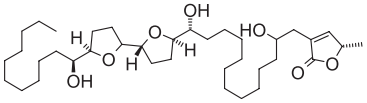
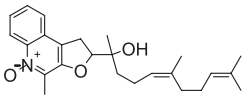
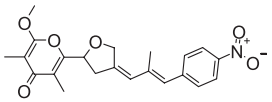
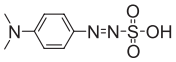
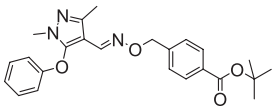
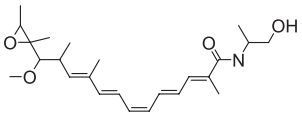
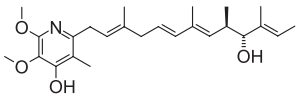
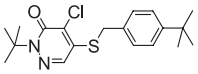
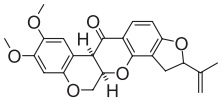
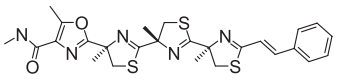
Several studies have tried to identify inhibitor binding sites through affinity or photoaffinity labeling, but they have not given entirely consistent results. Photoaffinity labels based on the structures of the unrelated acaricides fenpyroximate and pyridaben (Table 13.1.1) predominantly labeled different proteins, the ND5 and PSST subunits, respectively, although each prevented labeling by the other [39, 40]. PSST is labeled to a minor degree by the fenpyroximate analogue. Earlier studies using rotenone analogues predominantly labeled another subunit, ND1 [41, 42], which was also labeled to a minor degree by the pyridaben analogue. The pattern of labeling is sensitive to the conformational state of the complex, since it is altered in the presence of NADH and other ligands [43, 44] and some differences may be attributable to this. Another possibility is that all of these subunits are involved in the construction of one or more UQ/inhibitor binding sites and that the yield and site of crosslinking is determined by the structure, half-life and chemical reactivity of the reactive species generated by photolysis. Yet another polypeptide (known as subunit B9 in the bovine complex) is photolabeled by a UQ analogue [44, 45]. Interestingly, both ND1 and B9 react with dicyclohexylcarbodiimide, whose effect on the enzymatic activities of Complex I is similar to those of rotenone and piericidin [46]. The 49-kDa subunit has also been implicated in inhibitor binding because mutant forms of this protein are resistant to rotenone, piericidin and pyridaben [47, 48]. Thus, polypeptides belonging to all three sub-domains of Complex I have been implicated in inhibitor binding (Fig. 13.1.2). Unambiguous assignment of inhibitor and UQ binding sites will probably need to await a high-resolution structure determination.

Fungicidal, acaricidal and insecticidal Complex I inhibitors are discussed in detail in Chapters 13.5 and 28.3 of this volume.

13.1.2.2 Complex III (Cytochrome bc1 Complex) and its Inhibitors

Complex III has been purified from many sources and the structure solved by X-ray crystallography for several mitochondrial forms (reviewed in Ref. [49]). The bovine mitochondrial form contains eleven subunits [50], one of which represents a processing fragment of another, whilst fungal forms from *Saccharomyces cerevisiae* [51, 52] and *N. crassa* [53] contain nine or ten. Bacterial forms are func-

Table 13.1.1 Selected potent inhibitors of Complex I. Further fungicidal and acaricidal inhibitors are described in Chapters 13.5 and 28.3 respectively.

Name	Structure	Use	Ref.
Ajudazol B			4
Annonin VI			157
Aurachin A			14
Aureothin			14
Fenaminosulf		Fungicide	33
Fenpyroximate		Acaricide	39
Myxalamide PI (related to phenalamides)			14
Piericidin A			158
Pyridaben		Insecticide, acaricide	159
Rotenone		Insecticide	158
Thiangazole			14

tionally equivalent in terms of electron transfer and proton translocation, but are composed of only three or four subunits [54]. All forms of Complex III contain the same three highly conserved subunits, cytochrome b, the Rieske iron sulfur protein (ISP), and cytochrome c_1, which together carry all of the redox prosthetic groups. The additional subunits in mitochondrial forms are largely conserved between fungal and mammalian enzymes, but the function of most remains obscure.

The complex catalyzes electron transfer from reduced UQ to cytochrome c, coupled to the translocation of protons by a mechanism known as the Q cycle [55–57]. This involves the diversion of half of the electrons available from ubiquinol oxidation and deprotonation at a site on the outside of the inner mitochondrial membrane (Qo site) to reduce and protonate UQ at a site on the inside of the membrane (Qi site). The pathway for electron transfer across the membrane is provided by the two haem centers (b_L and b_H) of the mitochondrial gene product cytochrome b. The remainder of the electrons from ubiquinol oxidation pass along the chain to reduce first the Rieske iron sulfur protein (ISP), then cytochrome c_1 and then cytochrome c (Fig. 13.1.3).

Known potent and selective inhibitors of Complex III act at one of these two UQ binding sites (detailed in Table 13.1.2). Those acting at the Qi site are distinguished by their ability to induce oxidant dependent "super reduction" of cytochrome b in purified Complex III or mitochondrial membranes [58, 59]. In-

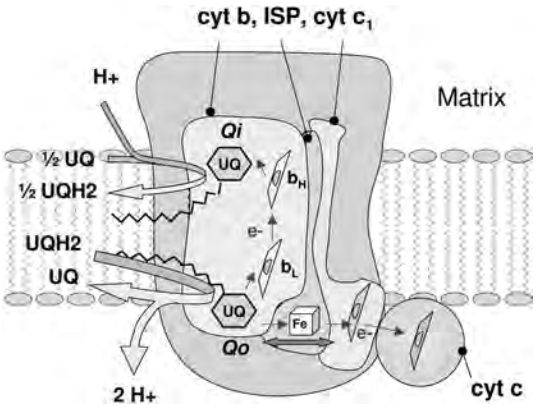
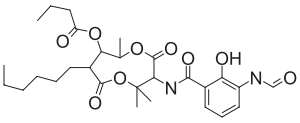
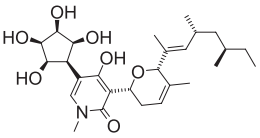
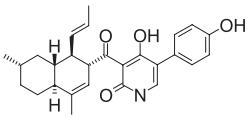
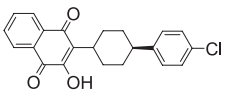
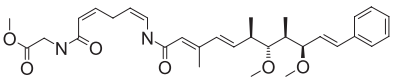
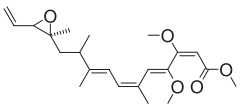
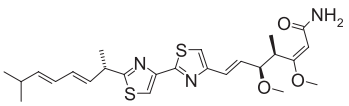
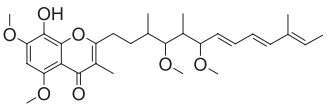


Fig. 13.1.3. Schematic representation of the structure and function of Complex III. The three catalytic subunits, cytochrome b (**cyt b**), the Rieske iron sulfur protein (**ISP**), and cytochrome c_1 (**cyt c_1**) are indicated. Two binding sites for inhibitors and ubiquinone (**UQ**), ***Qi*** and ***Qo*** are shown within cytochrome b, close to the two haems **b_H** and **b_L**. The bifurcated electron transfer pathway from the Qo site is shown by blue arrows. One electron is transferred to the iron sulfur center (**Fe**), then to the haem of cytochrome c_1 and then on to cytochrome c (**cyt c**). The other electron passes through b_L and b_H to reduce ubiquinone at the Qi site.

Table 13.1.2 Selected potent inhibitors of Complex III. Fungicidal and acaricidal inhibitors are described in Chapters 13.2 and 28.3, respectively.

Name	Structure	Binding site	Use	Ref.
Antimycin A1		Qi	Piscicide	160
Funiculosin		Qi		161
Ilicicolin H		Qi		58
Atovaquone		Qo	Anti-protozoal	162
Crocacin		Qo		163
Haliangicin		Qo		164
Myxothiazol		Qo		7
Stigmatellin		Qo		7

hibitor sites of action, and in some cases detailed modes of binding, have been confirmed by numerous crystal structures with inhibitors bound [60–63]. Although all inhibitors acting at the Qo site bind in a mutually exclusive manner, they can be classified according to the position they occupy within the site. Some (e.g., stigmatellin) bind closely to the ISP and cause a shift in its redox midpoint potential, whilst others (e.g., myxothiazol, strobilurin, famoxadone) bind close to haem b_L and influence its absorption spectrum [64, 65].

Inhibitors can also be classified by the pattern of sensitivity of variant forms of cytochrome b. Many amino acid substitutions arising through genetic mutations in the cytochrome b gene have been discovered that give rise to inhibitor insensitive forms that remain functional. Most of these mutations map to the Qi and Qo binding pockets, and distinguish between the Qi and Qo inhibitor classes. Inhibitors acting at either site can be further subdivided by cross resistance pattern; thus there are mutants that impart resistance to 2-*n*-heptyl-4-hydroxyquinoline N-oxide (HQNO) but not to antimycin and *vice versa* [63]. Similarly, at the Qo site, there are several mutations that give resistance to stigmatellin but not to strobilurins or myxothiazol [54, 66]. The effects of many of these mutations can be rationalized in terms of binding interactions seen in crystal structures (reviewed in Ref. [64]). Some of the variant forms of cytochrome b have been shown to be responsible for pathogen resistance to drugs or agrochemical fungicides, and the protein sequence differences seem to have little impact on the fitness of the organism [67–69]. Cross resistance has been observed between all seven classes of commercial fungicides acting at the Qo site [70] (see also Chapter 12).

Particular classes of Complex III inhibitors are covered in more detail in Chapters 13.2 and 28.3 of this volume.

13.1.2.3 **Complex IV**

Complex IV, or cytochrome c oxidase, was the first of the mitochondrial electron transport complexes to have its molecular structure and the internal path of electron transfer revealed by X-ray crystallography. The catalytic core of the complex consists of two subunits. Subunit II contains a binuclear copper center (Cu_A) that is directly responsible for the oxidation of cytochrome c. From there electrons are passed to haem *a* and then to the adjacent binuclear center that consists of haem a_3 and another copper ion (Cu_B), which are all held within subunit I (Fig. 13.1.4). Oxygen is bound and reduced between Cu_B and the iron of haem a_3, and access paths for protons from the inside of the membrane and for oxygen from within the membrane have been defined from several crystal structures available for bovine and bacterial enzymes. In addition to the protons taken up for the reduction of oxygen, translocation of further protons across the membrane is coupled to electron transfer by a mechanism that is not yet understood (reviewed in Refs. [71, 72]).

Bacterial cytochrome c oxidases have three or four subunits, whereas the bovine mitochondrial enzyme has 13, including closely related homologues of bacterial subunits I–III (which includes those bearing the redox centers) that are encoded by the mitochondrial genome. There is some uncertainty about the

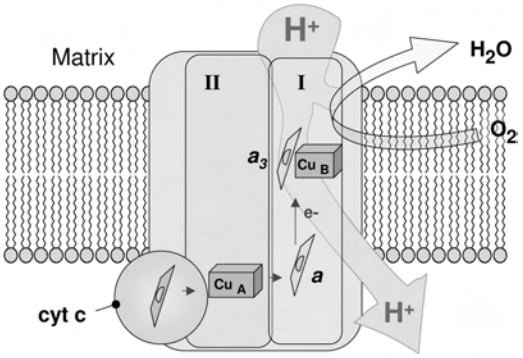


Fig. 13.1.4. Schematic representation of the structure and function of Complex IV. Electrons enter from cytochrome c (**cyt c**) and are passed through the binuclear copper center ($\mathbf{Cu_A}$) and haem *a* (***a***) to reduce oxygen at the center formed by haem a3 (***a3***) and copper ($\mathbf{Cu_B}$). Protons are taken up from the matrix side for the reduction of water and for transport across the membrane.

number of subunits in fungal mitochondria; the yeast *S. cerevisiae* has nine or eleven subunits, depending on the method of isolation [73]. Most of the nuclear encoded yeast genes have homologues in mammalian mitochondrial subunits with varying degrees of conservation. The function of the additional subunits is uncertain, except in so far as they have been shown to have a role in Complex IV assembly, or to contain binding sites for regulatory ligands. There also exist isoforms of a number of these subunits, which are selectively expressed in a tissue specific manner in mammals [71] or under different growth conditions in fungi [73].

It seems that cytochrome c oxidase is highly resistant to inhibition by secondary metabolites or synthetic organic chemistry, since no potent inhibitor from these sources has been reported. Perhaps this is due to a lack of requirement for conformational flexibility [71], or the inaccessibility of the redox centers or substrate transport channels to anything but very small molecules [74]. However, opportunities for inhibition may arise through exploiting natural control mechanisms such as the allosteric regulation by ATP [75] or phosphorylation of a highly conserved tyrosine in subunit I [76].

13.1.2.4 Succinate Dehydrogenase (Complex II) and its Inhibitors

Complex II is the succinate dehydrogenase of the tricarboxylic acid, or Krebs, cycle and catalyzes the oxidation of succinate to fumarate, coupled to the reduction of UQ to ubiquinol. It is part of a large family of related succinate quinone oxidoreductases and quinol fumarate oxidoreductases found in bacteria and mitochondria that have been classified based on subunit structure, number of cytochrome b haem centers and class of quinone substrate [77, 78]. The mitochondrial form belongs to the class that has a single *b* type haem bound to one of

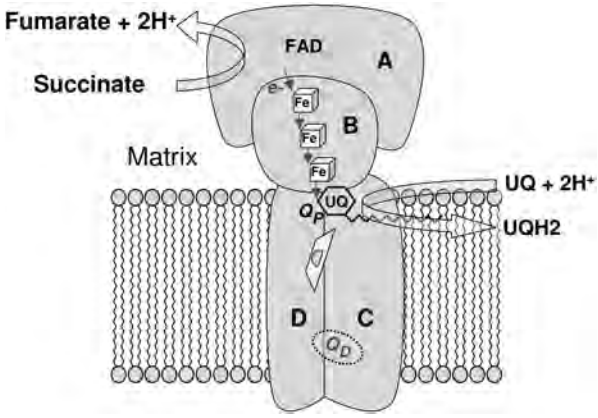


Fig. 13.1.5. Schematic representation of the structure and function of Succinate Dehydrogenase (Complex II). The diagram shows the topographical arrangement of the four subunits **A**, **B**, **C** and **D**. Subunit A binds the co-factor flavin adenine dinucleotide (**FAD**). Subunit B carries three iron sulfur clusters (**Fe**) that provide an electron transfer pathway (blue arrows) to the ubiquinone (**UQ**) and the inhibitor binding site (Q_P) indicated at the interface of subunits B, C and D. The location of a possible second UQ binding site (Q_D) is also indicated.

two membrane-spanning subunits that together support the catalytic iron protein and flavoprotein subunits on the matrix side of the membrane. The flavoprotein (subunit A) contains covalently bound flavin adenine dinucleotide (FAD), which is the primary oxidant of succinate. The iron protein (subunit B) contains three iron sulfur clusters that provide a path for conduction of electrons to the junction with the membrane domain (subunits C and D) (Fig. 13.1.5). The mitochondrial enzyme belongs to the same class as the succinate dehydrogenase from *E. coli* whose crystal structure has been solved to a resolution of 2.6 Å and which has been considered a model for the mitochondrial enzyme [79]. Recently, the 2.4-Å and 2.1-Å structures of the porcine and chicken mitochondrial complexes have also been published [80, 81]. Together the structures reveal a highly conserved UQ binding site (Q_P) at the interface between the membrane-spanning subunits and the iron protein subunit, from which the quinone can receive electrons from the iron sulfur center furthest from the FAD. The *b* haem does not seem to be required for electron transfer to the quinone at the Q_P site, since it is further from the bound UQ than the iron sulfur center and at the opposite side, towards the external face of the membrane. Its role in electron transfer within the complex is uncertain [79, 80] and it seems that much of the haem can be lost from the yeast enzyme with less than anticipated impact on function [82]. UQ binding at the Q_P site has been observed directly in both the *E. coli* and porcine structures and is supported by the results of photoaffinity labeling studies on the bovine enzyme [83]. It seems that UQ can bind in at least two overlapping positions within this site [12].

The porcine structure revealed another binding pocket in the membrane domain, towards the external surface, which could form a second UQ binding site (Q_D). It is located in a position analogous to a quinone binding site in *E. coli* quinol fumarate oxidoreductase [84]. The existence of a second binding site for UQ on mitochondrial complex II has also been suggested by inhibitor binding and site-directed mutagenesis studies [85, 86], but so far there is no direct evidence for a functional UQ interaction at this site.

Complex II is strongly inhibited by 3-nitropropionic acid, a toxic metabolite produced by plants and fungi [87], which is reported to act as a suicide substrate at the succinate binding site [88]. As expected, this inhibitor was seen to form a covalent bond with the protein close to the FAD in the chicken crystal structure [81]. Other inhibitors are known, or presumed, to compete with UQ for binding at the Q_P site (Table 13.1.3). Atpenin A5, and the less potent 2-thenoyltrifluoroacetone, have been directly observed at this site in various crystal structures [12, 80], and mutations at this site impart resistance to carboxin in *Paracoccus denitrificans* [89], and the fungi *Pleurotus ostreatus* [90], *Mycosphaerella graminicola*, *Ustilago maydis* [91] and *Coprinus cinereus* [92]. The latter was also shown to be resistant to the basidiomycete selective fungicide flutolanil. Mutations imparting carboxin resistance are found in all three subunits that form the Q_P site.

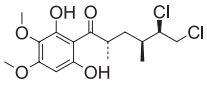
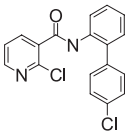
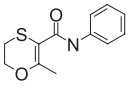
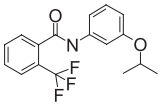
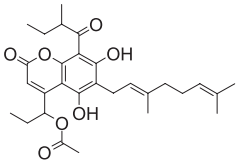
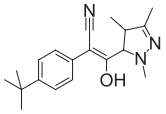
Fungicidal inhibitors of Complex II are discussed in Chapter 13.3 of this volume. Recently, the novel acaricide Cyenopyrafen, which is structurally related to an earlier insecticide [93], has been reported to act by potent inhibition of Complex II after hydrolysis of the *t*-butyl ester (Table 13.1.3) [94].

13.1.2.5 **Alternative Electron Transport Chains**

Fungal and plant mitochondria commonly contain alternative NADH–ubiquinone oxidoreductases that bypass Complex I, and a ubiquinol oxidase (usually called the alternative oxidase) that bypasses Complexes III and IV. They are considered here because their expression may be relevant to the sensitivity of fungal pathogens to Complex I and Complex III inhibitors. Neither class is known to be targets for chemical control in themselves. Several other UQ reductases are not covered in this overview, although they provide important links from metabolism to the electron transport chain. They include electron transfer flavoprotein UQ oxidoreductase (Fig. 13.1.1), essential for the function of acyl-CoA dehydrogenases [95], α-glycerophosphate dehydrogenase, which has an important role in insect flight [96], and proline dehydrogenase, of particular importance in some insect tissues [97]. These too are currently not known to be targets of antibiotics or crop protection chemicals.

The alternative oxidase is a nuclear gene product targeted to the inner mitochondrial membrane, which catalyzes the oxidation of UQ by molecular oxygen, mediated by a di-iron center [98, 99]. Since it does not transport protons across the membrane, use of the alternative electron transport chain it provides has a large cost in terms of energy conservation. Its expression is therefore tightly regulated and in many fungi its activity is low unless it is up-regulated in response to

Table 13.1.3 Selected inhibitors of Complex II. Further fungicidal inhibitors are described in Chapter 13.3.

Name	Structure	Use	Ref.
Atpenin A5			165
Boscalid		Fungicide	See Chapter 13.3
Carboxin		Fungicide	166
Flutolanil		Fungicide	167
Surangin B			168
Active hydrolysis product of Cyenopyrafen		Acaricide	169

defects in the respiratory chain [100–103]. Even when both pathways are present there is evidence that electrons flow preferentially through the energy conserving Complex III and Complex IV [104], perhaps because of electron channeling through "supercomplexes" [18].

Alternative NADH–ubiquinone oxidoreductases are also nuclear encoded single polypeptide enzymes targeted to the inner mitochondrial membrane. They catalyze the same reaction as Complex I, but again are not energy conserving. There are many related forms, varying in orientation of the NADH binding site (towards the matrix or external face), substrate specificity and sensitivity to regulatory ligands, and some fungi express more than one form (reviewed in Ref. [105]). They are dependent on FAD as the only prosthetic group and enzyme ki-

netic studies suggest that the binding sites for NADH and UQ overlap [106]. Although these alternative electron transport pathways are now well characterized at the molecular level, their physiological role in fungi is not clear. They may protect against oxidative stress or mitigate the effects of inhibition of the respiratory chain [107, 108]. Their presence may also affect the sensitivity of plant pathogens to strobilurin fungicides with respect to biological spectrum and efficacy at different growth stages [109].

13.1.3 Energy Conservation

The conservation of energy from electron transport requires not only the synthesis of ATP within the mitochondrion, but also its export to the cytoplasm as well as the import of substrates for oxidation and phosphorylation. Only the proteins responsible for synthesis of ATP and exchange of ATP for ADP across the inner mitochondrial membrane will be considered in any detail here, as they are known targets for antibiotics and pesticides. However, numerous other mitochondrial transporters identified in plants, fungi and animals may provide future opportunities for useful chemical intervention [110, 111].

Energy conservation is also dependent on chemiosmotic coupling, a process that is disrupted by a class of chemicals called ''uncouplers'', some of which have found commercial application as herbicides, fungicides and insecticides. Uncouplers collapse the electrochemical gradient across the inner mitochondrial membrane, allowing oxidation to proceed without the normal control and preventing the action of the ATP synthase. The most effective are protonophores (compounds that increase proton permeability of the membrane), whose continuous ''catalytic'' action allows them to be highly efficient disrupters of oxidative phosphorylation. Uncouplers are invariably cytotoxic to eukaryotic and prokaryotic cells, unless the cell has some means to minimize its exposure, e.g., through metabolic inactivation [112] or efflux pumps [113, 114]. Chapters 13.4 and 28.2 of this volume cover the action and application of uncouplers in more detail. There are also comprehensive reviews in the literature [115, 116].

13.1.3.1 F_1F_0ATP Synthase and its Inhibitors

The ATP synthase complex was purified from bovine heart mitochondria around the same time as the respiratory chain complexes. It is most commonly referred to as the F_1F_0ATPase or ATP synthase, but is also known as Complex V. It is made up of 15 different subunits (excluding the inhibitor protein IF_1) organized in two major subcomplexes termed F_1 and F_0. F_1 is a globular domain that extends on two ''stalks'' into the mitochondrial matrix. It consists of five different subunits in the stoichiometry $\alpha_3\ \beta_3\ \gamma_1\ \delta_1\ \varepsilon_1$ and its structure has been solved by X-ray crystallography [117, 118]. The γ, δ, and ε subunits make up the central stalk and the six α and β subunits are arranged symmetrically around extending α-helices of the γ subunit to form the globular head (Fig. 13.1.6). The isolated F_1 domain is soluble and acts as an ATPase. It contains three catalytic sites, one on

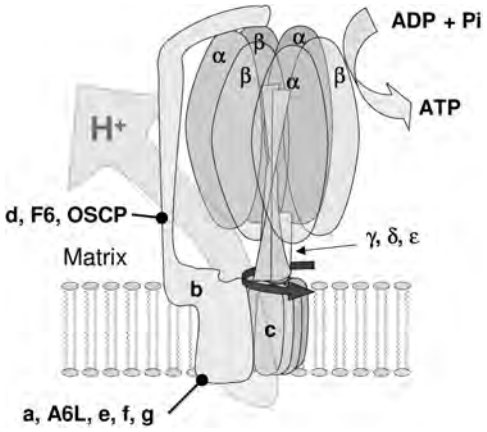
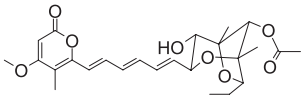
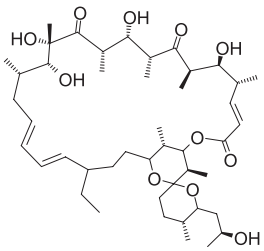
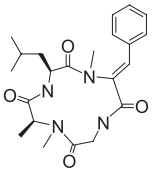
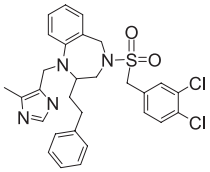
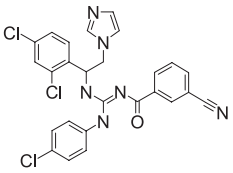


Fig. 13.1.6. Schematic representation of the structure and function of the mitochondrial F_1F_0ATP synthase (Complex V). Rotation of the **c** subunits is believed to be driven by proton conduction through the membrane domain, which in turn drives rotation of the central stalk (subunits γ, δ, ε) in the direction shown. This drives the condensation of ADP and Pi sequentially at each of the catalytic β subunits. The locations of other subunits are indicated; their function is discussed in the text.

each of the β subunits and behaves as a molecular motor; ATPase activity causes the central stalk to rotate within the oligomer of α and β subunits [117, 119]. A glutamic acid residue on the β subunit is a site of reaction of the inhibitor dicyclohexylcarbodiimide (DCCD) [118], and other inhibitor binding sites, including those for aurovertin B (Table 13.1.4), the peptide antibiotic efrapeptin, and the endogenous inhibitor protein IF1, have been localized to this globular domain by X-ray crystallography [120]. The fungal natural product tentoxin (Table 13.1.4) is a specific inhibitor of chloroplastic F_1 and binds at the interface of the α and β subunits [121].

The membrane or F_0 sub-complex is composed of seven subunit types, organized as two domains. One domain consists of ten copies of the membrane spanning, hairpin shaped, c subunit. These are arranged as a barrel with the longitudinal axis perpendicular to the membrane. The c subunits form extensive contacts with the γ and δ subunits of the central stalk, an arrangement that has been confirmed by the crystal structure of the yeast F_1-c_{10} complex [122]. The other membrane domain probably consists of the a, b, A6L, e, f and g proteins [123]. The F_0 domain is also believed to be a molecular motor. In the proposed mechanistic model for ATP synthesis, proton transport through the F_0 domain (driven by the chemiosmotic gradient) causes rotation of the c subunit oligomer that in turn rotates the central stalk within F_1, in a direction that promotes ATP synthesis (reviewed in Ref. [124]). Rotation of the α and β subunits is prevented by the peripheral stalk or ''stator'' that connects the membrane domain to the top of F_1. The b subunit also forms part of the peripheral stalk together with d, F6 and OSCP (oligomycin sensitivity conferring protein) [125]. Additional subunits

Table 13.1.4 Inhibitors of the F_1F_0 ATP synthase.

Name	Structure	Binding site	Ref.
Aurovertin B		F_1	170
Oligomycin A		F_0	170
Tentoxin		Chloroplast F_1	121
Compound **1**			135
Compound **2**			137

can be found in dimeric forms of the enzyme and may function to promote the formation of oligomers. Recent studies suggest that oligomerization of the ATP synthase drives the membrane curvature that leads to the formation of the mitochondrial cristae [126, 127].

The F_0 domain is also a site of action for inhibitors, the best known of which is oligomycin (Table 13.1.4), which acts to inhibit proton conduction [128]. The oligomycin binding site has been localized to the a and c subunits of the F_0 domain

through the characterization of resistant mutants in yeast [129, 130]. Related spiroketal macrolides, ossamycin and venturicidin A are located to the same binding site through their cross resistance profiles and it is assumed that the growing family of structurally related macrolides, including apoptolidin, dunaimycins, citovaricin and rutamycin act at the same site [131–133]. In addition to its inhibitory effect on ATP hydrolysis in F_1, DCCD also inhibits the proton conduction channel in F_0 through reaction with the c subunit [46]. The carbodiimide metabolite of the acaricide and insecticide diafenthiuron (covered in detail in Chapter 28.1 of this volume) has been shown to act in the same way [134].

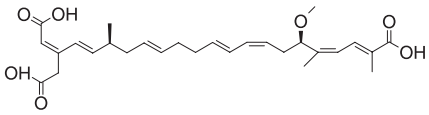
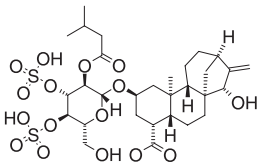
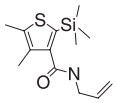
Medicinal chemistry has also successfully generated potent inhibitors of the F_1F_0ATP synthase (e.g., **1** and **2**, Table 13.1.4), although their specificity and exact site of action are unclear [135–137]. These compounds were designed as selective inhibitors of ATP hydrolysis and intended for treatment of ischemic tissue injury.

13.1.3.2 **Inhibitors of the Mitochondrial ADP/ATP Carrier**

The exchange of intra-mitochondrial ATP for extra-mitochondrial ADP plays a central role in mitochondrial function. It is controlled by a specific carrier protein that is a member of a large family of mitochondrial transporters related by sequence and structure, all of which are encoded by nuclear genes. The ADP/ATP carrier is a single polypeptide chain whose three-dimensional structure has recently been revealed by X-ray crystallography [138, 139] (reviewed in Ref. [140]). Its discovery and characterization owes much to the availability of the toxic plant secondary metabolite, atractyloside, and the bacterially produced bongkrekic acid, which proved to be potent and highly selective inhibitors (reviewed in Ref. [141]) (Table 13.1.5). Atractyloside and its analogue carboxyatractyloside are highly negatively charged at physiological pH and cannot penetrate the inner mitochondrial membrane. They bind to the external face of the transporter in a way that excludes nucleotide binding. In the crystal structure, carboxyatractyloside is bound at the bottom of an externally facing cone shaped depression [138]. Bongkrekic acid has not yet been co-crystallized with the transporter, but it is thought to bind to the internal face because binding requires it to penetrate the membrane. Its binding seems to lock the conformation of the transporter into the ADP bound state [141].

The novel fungicide silthiofam (MON 65500, Latitude^{TM}) [142], which is now marketed as a seed treatment for control of "take-all" fungus (*Gaeumannomyces graminis* var. *tritici*), has been shown to affect ATP export from mitochondrial preparations in a way that was mimicked by high concentrations of atractyloside [143] and so it has been proposed that it acts to inhibit the ATP/ADP carrier. More direct studies on the effect of this compound on carrier function have not been reported; neither have studies on its biochemical effects on mitochondria from other organisms. As a fungicide, silthiofam is highly specific for *G. graminis* and it is not acutely toxic to mammals despite its high bio-availability [144]; this is in contrast to the high mammalian toxicity of atractylosides and bongkrekic acid [145, 146]. It seems that the toxicological species selectivity of this compound may reflect the sensitivity of the biochemical target.

Table 13.1.5. Inhibitors of ATP export from the mitochondrion.

Name	Structure	Ref.
Bongkrekic acid		141
Atractyloside		141
Silthiofam		143

13.1.4 Concluding Remarks

Several recent developments have reinvigorated research into the mechanism of oxidative phosphorylation and the interaction of the mitochondrion with its host cell. Perhaps of most immediate interest from the perspective of crop protection chemistry is the increasingly successful application of X-ray crystallography in revealing the structures of many components of oxidative phosphorylation. This has dramatically improved our understanding of the catalytic mechanism and of the action of inhibitors. Many common themes are emerging, prominent amongst them the insights into the structure of UQ binding sites. Nature seems to have designed these to be relatively "open" binding pockets that have provided disproportionate success in the discovery and design of novel inhibitor classes.

Other developments are the growing understanding of the role of the mitochondrion in the pathogenesis of disease, ageing and the determination of cell fate. It is now clear that both inhibitors and uncouplers of oxidative phosphorylation have effects other than depriving the cell of ATP and that these can play an important role in their action. Many respiration inhibitors increase the rate of production of reactive oxygen species in the cell and can activate endogenous cell death or apoptotic pathways [147–149]. The defenses that the cell has developed to protect against reactive oxygen generation in the mitochondrion may themselves become targets. Indeed, this may be one of the roles of the alternative oxidase and NADH dehydrogenases found in many fungal pathogens [108], and is the most likely explanation for aspects of the control of cytochrome c oxidase

activity, the proton pumping activity of which is not always fully coupled to electron transport [150]. These systems may act to limit the mitochondrial membrane potential and so limit the degree of reduction of those redox centers that are sites of superoxide production. Another mechanism for the control of membrane potential is through ''uncoupling proteins''. First recognized for their role in mammalian thermogenesis, this class of protein is now known to be expressed in fungal, plant and animal mitochondria providing proton channels whose activity can be regulated by guanine nucleotides and free fatty acids [151, 152].

Chemical uncouplers can also influence cell fate other than through depletion of ATP [153, 154] and their action to depolarize membranes outside of the mitochondrion also needs to be considered with respect to their overall effects on the target cell or organism; for instance, they have been shown to dissipate plasma membrane potential and to destabilize lyzosomes [155, 156].

A new role for the mitochondrion has emerged, suggesting new targets for medicinal and crop protection chemistry. At the same time structural biology has greatly improved our understanding of existing targets and their inhibitors, which will allow the synthetic chemist to design around issues of species selectivity and resistance using the abundant tool set provided by natural products. It seems that the exploitation of mitochondrial targets will continue to expand to provide safer and more effective crop protection agents for the future.

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13.2
Strobilurins and Other Complex III Inhibitors

Hubert Sauter

13.2.1
Introduction

After more than 20 years of industrial research and development, strobilurins have become one of the most important classes of crop protection agents. ''With a distributor sales value of 1.3 billion US$ in 2004 [1], they currently represent approximately one-fifth of the world fungicide market (7.3 billion US$ in 2004 [1]). Within fungicides they rank – in commercial terms – second only to sterol biosynthesis demethylase inhibitors (DMI's), e.g. triazoles (see Chapter 17 of this book).'' This chapter also includes the three other Complex III inhibitors, famoxadone, fenamidone and cyazofamide, that, besides strobilurins, have achieved introduction in agricultural practice.

There are several prominent and some unique features connected with strobilurins, which should be mentioned right at the beginning:

1. They originate from academic natural products research, starting 40 years ago when biological activity was first detected, followed by isolation and structure elucidation of oudemansin A and strobilurin A. The unusual simplicity of the strobilurin A structure, together with its antifungal activity, made it a compelling inspiration for synthetic analogues.
2. Simultaneous, but distinctly different, processes operated to bring strobilurins from universities to research groups of the former ICI (now merged into Syngenta) and of BASF.
3. Very early on the physiological mode of action as mitochondrial respiration inhibitors was established. This allowed *in vitro* testing to evaluate intrinsic potency of new analogues, which gave invaluable guidance for rational and relatively rapid optimization.
4. Subsequent identification and X-ray structure determination of the exact target site, namely the mitochondrial bc_1 complex, formed the basis for understanding inhibitor–target

interaction, as well as resistance phenomena, at the molecular/submolecular level.

5. Despite potential for non-target toxicity of the mode of action, careful optimization of the biological profile led to safe products.
6. The extremely broad activity spectrum of strobilurins, with potential to control all four major classes of phytopathogenic fungi (Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes), is unique among commercial fungicides.
7. Unusually broad structural variations are possible without losing intrinsic activity. This variability includes all areas of the molecule, including even the pharmacophore.
8. During the evolutionary process of lead-structure optimization, probably well over 50 000 strobilurin analogues were synthesized by competing industrial fungicide discovery research groups worldwide. Several compound types, and even specific compounds, were made independently at almost the same time.
9. This competition also led to an unprecedented international patent race, which has produced more than 1000 patent applications, and several patent interferences with narrow differences in priority dates.
10. At present, nine different strobilurins have been introduced into the fungicide market (see Table 13.2.1 and Fig. 13.2.2 below), and one more has been announced as being developed in China (enestroburin). There is also a strobilurin commercialized in Japan as an acaricide (fluacrypyrim).
11. Unexpected events have already changed the market for strobilurins. On the one hand, surprisingly fast development of resistance in pathogens such as powdery mildew and *Septoria tritici* has limited opportunities in some key segments. On the other hand, these losses have been compensated by outstanding efficacy against a new fungal disease of extreme economic importance, the soybean rust epidemic in South America.
12. Further market opportunities have resulted from unexpected beneficial influences of strobilurins directly on the physiology of treated plants, even under conditions of little or no fungal infection, giving unprecedented yield increases, stress tolerance, and generally improved plant health. These effects pose a significant challenge for further research to optimize plant physiology.
13. All this happened during a time of far-reaching changes in industrial crop protection companies. Three points should be mentioned in this respect: first, several mergers and acquisitions, which led to the migration of strobilurin patents

and developmental or commercial products from one company to another; second, the integration of molecular biology into agrochemical research; and third, the increasing use of bioanalytical methods and biokinetic considerations in this research. Each may be said to have been encouraged by the commercial and scientific interest in strobilurins.

Table 13.2.1 Commercialized strobilurins and other Complex III inhibitors.

Fungicide	Code number	Originator	Current owner	First year of sales	Sales volume (2004, Million $)[a]
Kresoxim-methyl	BAS 490 F	BASF	BASF	1996	183
Azoxystrobin	ICI A 5504	ICI	Syngenta	1996	505
Metominostrobin	SSF-126	Shionogi	Bayer	1999	<30
Trifloxystrobin	CGA 279202	Ciba	Bayer	1999	255
Picoxystrobin	ZA 1963	Zeneca	Syngenta	2002	50
Pyraclostrobin	BAS 500 F	BASF	BASF	2002	295
Fluoxastrobin	HEC 5725	Bayer	Bayer	2004	<30
Dimoxystrobin	BAS 505 F	BASF	BASF	2004	<10
Orysastrobin	BAS 520 F	BASF	BASF	2006	–
Famoxadone	DPX JE874	DuPont	DuPont	1997	70
Fenamidone	EXP10745	Rhône-Poulenc	Bayer	2001	30
Cyazofamid	IKF 916	Ishihara	Ishihara	2001	<30

[a] From Ref. [1].

In addition to their enormous significance in crop production, strobilurins have an additional feature worth considering in detail: they represent one of the most instructive examples of how modern fungicide research can deliver tailored solutions, by combining rational and market-driven research with alertness for serendipity. In particular, the history of strobilurin research demonstrates the value of detailed structure–activity considerations, for a strategy that is focused on variation of molecular structures with the aim of optimizing biological profiles. Much can be learned from this experience that is relevant to future agrochemical optimization, not only in fungicides, and this chapter will take the opportunity to illustrate some of these aspects.

13.2.2 Evolution of Strobilurins as Agricultural Fungicides

Several aspects of strobilurin fungicides, including the natural lead-structures, have already been summarized [2–9]. The elegant path that led to the discovery

of azoxystrobin has been reported in detail by a sequence of papers from the Jealott's Hill group of the former ICI (later Zeneca, now Syngenta) [2–4]. A later review appeared 1998, including kresoxim-methyl, SSF-126 (metominostrobin), and a thorough survey of the families of related natural products known up to that time [5]. The last review from this group was published in 2002, giving a comprehensive overview of the chemical, biological and ecotoxicological properties of the six strobilurins then on the market, and the azolones famoxadone and fenamidone [6].

From the BASF group, a first review concentrated on the discovery of BAS 490 F (kresoxim-methyl), on pharmacophore variations and on structure–activity relationships at the mitochondrial target level [7]. Another paper was focused more on biokinetic features, and their affect on final biological properties [8]. In 1999 a general review summarized the historical evolution from natural products to commercial strobilurins up to trifloxystrobin, and the international R&D competition, including some dramatic patent races [9]. Additionally, strobilurins have been reviewed in Russian, Chinese and Indian journals [10–13].

The history of strobilurins started with academic research. In 1969 Musilek and coworkers in Czechoslovakia published the isolation of an antifungal antibiotic from extracts of the fungus *Oudemansiella mucida* and named it mucidin, without a proposed structure [14]. The German research groups of T. Anke and W. Steglich obtained strobilurin A from fermentations of the fungus *Strobilurus tenacellus*, and reported in 1977 its broad antifungal activity and its chemical and physical data [15]. In 1984 they reviewed some confusing earlier reports in the literature about the correct stereochemical structures of mucidin and strobilurin A, and gave the correct (E,Z,E)-structure on the basis of chemical and spectroscopic evidence [16]. The assumed identity of strobilurin A and mucidin was finally proved in a 1986 paper by direct spectroscopic comparison [17]. In 1979, the structurally closely related oudemansin A was reported by the groups of Anke and Steglich [18]. Subsequently, many natural derivatives of the strobilurin and oudemansin type have been found in several producing organisms [19, 5]. All have a common structural feature: the (*E*)-*β*-methoxyacrylate unit, linked to the rest of the molecule in the *α*-position, according to which they have been named *β*-methoxyacrylates or MOAs. This is true also for the cyrmenins, recently isolated from myxobacteria [20].

Another structurally related group of natural antifungal substances are the myxothiazols, which also contain an (*E*)-*β*-methoxyacrylate moiety, this time *β*-linked. Their discovery, characterization and structure elucidation was first performed in the German groups of H. Reichenbach and G. Höfle [21–23]. Later, the same groups discovered the closely related melithiazols [24]. For a review on the naturally occurring *β*-methoxyacrylates see Ref. [25].

Obviously, natural evolution led to fungi that synthesize and excrete substances that fight against competing fungi, while at the same time being completely insensitive to the fungicides they produce (see Section 13.2.6).

An important Rosetta Stone came from the discovery that strobilurin A, oudemansin A and myxothiazol A not only share a common structural feature, namely

the (*E*)-*β*-methoxyacrylate group, and have inhibition of mitochondrial respiration as their common physiological mode of action, but also act at a common molecular binding site, the mitochondrial bc_1 complex [26]. These findings inspired attempts in Steglich's group – and in industry – to design simplified but still active synthetic strobilurin analogues [19]. For example, the ICI fungicide research team obtained a sample of oudemansin A from T. Anke in June 1982. They found potent, broad fungicidal activity in greenhouse tests, where the substance, which lacks the destabilizing triene functionality of strobilurin A, is sufficiently stable, and soon started a synthesis program. They also focused on strobilurin A, confirming its correct E,Z,E-configuration and obtaining it in quantity by total synthesis [2].

One year later, BASF started their own activities in the field. In July 1983, during a publicly funded joint project with several university research groups on natural products as leads for new agrochemicals, BASF obtained strobilurin A. Although it showed high activity against fungi grown on artificial media in the laboratory, the results of greenhouse tests were disappointing. This led to the hypothesis that the inherent lability of its triene system might give rise to rapid photolytic, oxidative or metabolic degradation under greenhouse conditions, and thus attempts were made, in cooperation with Steglich's group, to make stabilized, more active analogues [7, 9].

Neither the ICI nor the BASF group knew about their similar research objectives, and each worked simultaneously but completely independently. Therefore, it might be considered an interesting case of ''evolutionary convergence'' that ICI and BASF, while starting from slightly different points, very soon came to the same molecule as a new, simple and stabilized synthetic strobilurin: the so-called enol ether stilbene (also called methoxyacrylate stilbene, MOA-stilbene, or MOAS). This compound differs from strobilurin A only in that the central double bond of the original triene system is incorporated into a stabilizing benzene ring. This compound showed not only the hoped-for increase in fungicidal efficiency in glasshouse tests, but also, somewhat surprisingly, even higher mitochondrial target activity. Quite soon it became obvious that, for purposes of further variations, this ''second generation lead-structure'' could be reasonably divided into three parts: the side-chain, the central linking ring, and the pharmacophore (Fig. 13.2.1). We prefer the term ''pharmacophore'', for several reasons, to other commonly used terms [27].

The variations in all the commercial strobilurins (Fig. 13.2.2) can be categorized according to this pattern. All possess the central linking ring – as ortho-disubstituted benzene – unchanged, but one finds five(!) different pharmacophores and nine different side-chains.

Having seen the excellent greenhouse results with oudemansin, the ICI research group, immediately aware of the potential of their new lead, started an extensive research program around it, and soon (October and December 1984) filed a very broad, apparently insurmountable basic patent on stabilized synthetic *β*-methoxyacrylates of the strobilurin-type [28]. BASF filed similar patent applications in May and December 1985 [29] – too late, and therefore without value.

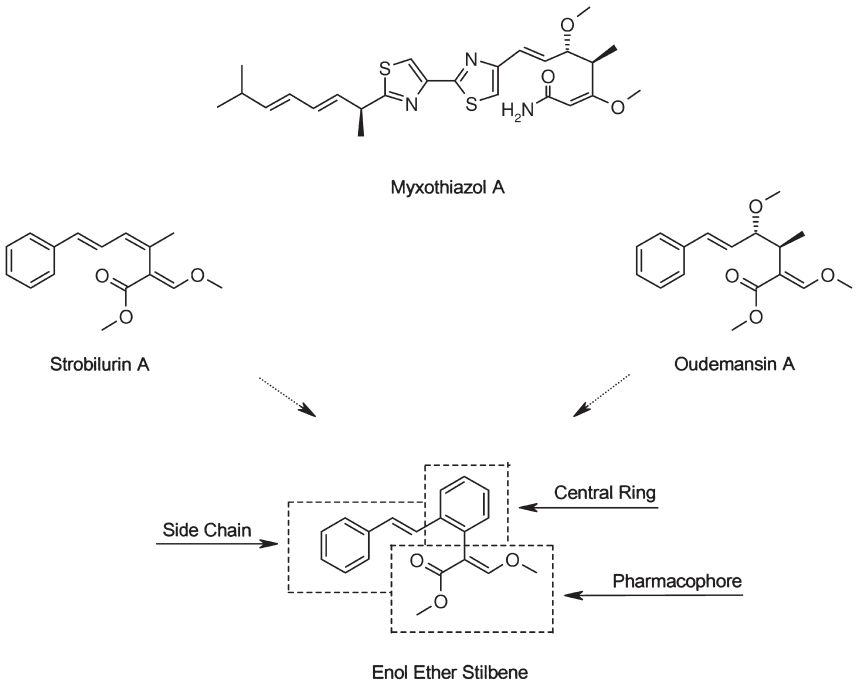


Fig. 13.2.1. From natural antifungals to the enol ether stilbene as a new synthetic lead structure.

Thus ICI was still half a year in the lead, and in a very comfortable position for further optimization work.

In contrast, we at BASF were now compelled to concentrate further research outside of the ICI claims. We decided to focus on pharmacophore variations – admittedly a risky strategy – and were rewarded by finding that oxime ethers (or oximino esters) were also active. This discovery was covered by a patent application filed in 1986, just two days before ICI filed on the same subject. So the next round of the patent race went to BASF [30, 31].

Meanwhile, the search for strobilurins with commercial potential went ahead in both companies. ICI focused from the beginning on the concept that an optimal fungicide must be "systemic"; i.e., the compound should be transportable at least in the acropetal direction, moving upward in the xylem transpiration stream of the treated plants, and thereby providing protection in remote, untreated areas of the plant. It was well known that a low octanol–water partition coefficient (log P_{OW} below 5.0, with a calculated maximum at 2.0) is one prerequisite for good acropetal mobility. The other prerequisite is sufficient metabolic stability inside the plant. ICI had already oriented their fungicide screening in this direction, and thus were able to reach their strobilurin objective quite soon. Their brilliant optimization strategy, based on sequential, rational variations of the side-

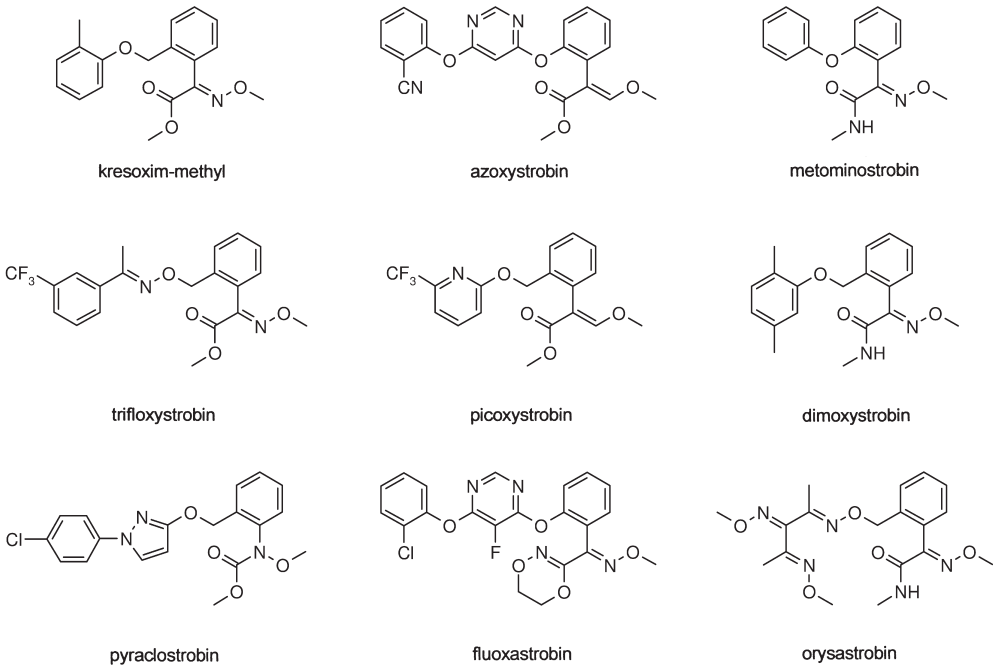


Fig. 13.2.2. Commercial strobilurin fungicides.

chain, has been comprehensively described [2–5; see also Refs. 6, 9]. The end result was azoxystrobin, first announced as a systemic fungicide with broad spectrum activity in 1992 [32].

During its early optimization efforts to find a strobilurin with market potential, BASF's options regarding chemical structures were restricted from the patent side. Thus we concentrated on pharmacophore variants other than the enol ether type, particularly on our patent-protected oxime ether (oximino ester) variants [9]. As it turned out later, strobilurins bearing the oxime ether pharmacophore do *not* have good potential for systemicity, because the pharmacophore itself is degraded relatively rapidly in plants by hydrolysis of its ester group (see Section 1.3.2.3.4). On the other hand, compounds of this type showed high intrinsic activity, which was further optimized with the aid of the mitochondrial target test. They also exhibited outstanding activity against powdery mildews in several crops, a market segment that was traditionally a main focus of BASF's fungicide biology and marketing. Thus the fungicide testing system in place at that time "welcomed" this particular type of strobilurin, and anticipated market potential for them. No wonder, then, that kresoxim-methyl, the first strobilurin from BASF research [33] and the first strobilurin to reach the market, was an oximino ester, which fit perfectly into the powdery mildew market despite not being specifically designed for that purpose. In this light, kresoxim-methyl may be seen as a case of serendipity.

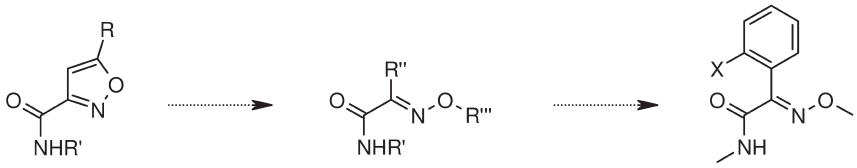


Fig. 13.2.3. Steps in Shionogi's sequence of thoughts for the synthesis of oximino amides.

Later, during the development phase, H. Köhle and his colleagues discovered the physicochemical and biokinetic basis for its particular biological activity profile, namely its episystemic distribution properties (Section 13.2.3.3), which bring it into close contact with fungi like powdery mildews that grow on the leaf surface [8, 9, 34, 35].

Interestingly, starting with the natural lead-structures was not the only way to obtain new strobilurin fungicides. Shionogi's path, which finally led to metominostrobin, originated from a completely different, chemistry-driven program. Starting from fungicidal carbamoyl isoxazoles **1**, they synthesized first ring-opened analogues **2**, and then aryl derivatives of type **3** with increased fungicidal activity (Fig. 13.2.3) [36; see also Refs. 5, 9].

In this way they found a new pharmacophore variant, the oximino amides (or oxime ether amides). They realized later the similarity with oximino ester strobilurins, established that their compounds had the same mode of action [37] and filed a basic patent application on this new structural type [38]. By varying the oximino esters, BASF came independently to the same new pharmacophore type **3** and filed a corresponding application [39], after Shionogi's application was filed, but before it was laid open. The result was that, while Shionogi obtained the basic patent, BASF was granted a selection invention with claims for individual oximino amides not specifically described in the Shionogi application. These included the compound that later became the product dimoxystrobin [40], and agreements with Shionogi allowed BASF to undertake its commercial development. Later, Shionogi's agrochemical interests merged first into Aventis and then into Bayer Crop Science.

Trifloxystrobin, like kresoxim-methyl, has the oximino ester pharmacophore. Besides that, its most interesting feature is the oxime ether side-chain, which turned out to contribute considerably to strobilurins with particularly high intrinsic activity. This side chain was not specifically claimed in the basic patent applications for enol ethers, oximino esters or oximino amides, respectively. These gaps led later to an unusual patent race, in which five competing companies were involved [9]. Despite some claim interferences, the enol ethers were granted primarily to ICI [41], the oximino esters to Ciba-Geigy [42], and the oximino amides to BASF [43]. The oximino ester trifloxystrobin was developed and announced by Novartis [44] and sold to Bayer in 2000 as part of the requirements for the merger between Novartis Agribusines and Zeneca Agrochemicals to form Syngenta.

Picoxystrobin, first announced in 2000 [45], is an outcome of ICI's optimization work on enol ethers. It combines two physicochemical properties, namely a relatively low octanol–water partition coefficient and an relatively high vapor pressure, which give it both systemic xylem mobility and episystemic distribution properties. Regarding episystemicity, it closes a gap in the biokinetic and biological properties of azoxystrobin.

Pyraclostrobin was announced at the same Brighton Conference by BASF [46a]. It contains not only a new pharmacophore type, but also a new type of side-chain, with a five-membered, oxygen-linked heterocycle. For the first time, the original carbon–carbon double bond of the enol ethers was replaced by a nitrogen–oxygen single bond, and the pharmacophore at its center has a nitrogen atom instead of carbon. In this case, too, the pharmacophore variant led to a competition of rival patent applications [47]. According to its physicochemical characteristics, pyraclostrobin has no pronounced systemic or episystemic mobility in or on plants. Very distinct are its rapid leaf uptake and translaminar movement, its extremely broad activity spectrum, and its outstanding crop safety and yield enhancements [46b, 48]. This bundle of positive properties may – or may not – have its origin in the fact that pyraclostrobin possesses the lowest melting point of all the presently commercialized strobilurins, which should favor rapid bioavailability in molecularly dispersed form (see Section 13.2.3.3).

Fluoxastrobin was announced by Bayer in 2004 [49]. It contains once more a new pharmacophore type, in which the carbonyl group of the previous ester or amide pharmacophores is now inventively replaced by an oximino moiety that, in addition, is incorporated in a six membered dihydrodioxazine ring. However, the skeleton of the remaining molecule including its side-chain closely resembles that of azoxystrobin. Like the latter, fluoxastrobin is obviously successfully designed for xylem mobility [50] and exhibits broad-spectrum activity [51]. It is also the first strobilurin marketed for seed treatment.

Orysastrobin [52], the latest of the four BASF strobilurins, was from the beginning specifically designed for use as a nursery box treatment of rice seedlings against rice leaf diseases. To fulfill the special requirements necessary for this purpose, care was taken to focus on strobilurins with low lipophilicity and high water solubility, without losing too much intrinsic activity. The structure of orysastrobin combines the relatively metabolically stable oximino amide pharmacophore with a relatively large but not too lipophilic side-chain. The metabolic stability and low lipophilicity make it possible for the molecule to be easily taken up by roots and transported acropetally into leaves. Its high water solubility leads to the low aquatic toxicity necessary in rice applications. The large spatial extension of the tris-oxime side-chain clearly contributes to the fact that sufficient intrinsic activity is obtained.

At this point, mention should also be made of two other active strobilurins with interesting structures:

The individual structure SYP-Z071 (Fig. 13.2.4) was described by a Rohm and Haas patent application [53] together with scientists of the Chinese Shenyang Research Institute of Chemical Industry and is announced to be under evaluation

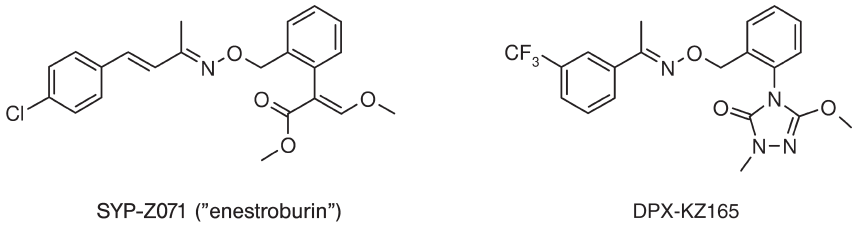


Fig. 13.2.4. Two other candidate strobilurins.

as a broadly active fungicide by the latter [54]. As the presently used name "enestroburin" (not the more conventional name "enestrobin") may suggest, its unsaturated (aralk-*en*-yl) side-chain has an en-(E)-configuration. Although enol ethers with aralkenyl side chains have been already generally claimed in the respective early ICI application, no specific claims or examples of that kind of elongated oxime ether side-chains were given [41].

DPX-KZ165 (Fig. 13.2.4) [55] represents an inventive cyclic triazolinone pharmacophore [56] and has the same side-chain as trifloxystrobin. Compounds of that pharmacophore type have been intensively investigated by DuPont scientists [57]. Interestingly, the triazolinone pharmacophore is in itself rigid and contributes to a distinctly low lipophilicity; compared with the analogous enol ethers its lipophilicity is lowered by 1.4 log P_{OW} units. Nevertheless, their intrinsic activities as well as fungicidal performance are considerably high, although no strobilurin of this cyclic pharmacophore type has achieved commercial status.

13.2.3 Structure-Activity Relationships of Strobilurins

Once a biologically active substance is defined and validated as a lead, variation of its chemical structure is the starting point for hypotheses and experiments to obtain optimum properties in respect to biological performance, ecological and toxicological safety, acceptance by regulatory agencies, and finally economic potential. All these final properties have their origin in the molecular structure of the active compound. Consequently, in a lead structure optimization process, structure–activity reflections on the different levels of consideration are of great relevance and should be included as early as possible. Later, – and I would like to emphasize this – they may also be of value for candidate selection processes. This means that structure–activity considerations are not only most helpful but are essential for rational and promising processes in crop protection R&D.

13.2.3.1 Interplay of Target Activity and Biokinetic Behavior

The activity of a fungicide and its selectivity under real-life conditions in a complex system of the fungal pathogen, the plant, the environment, and time is determined by a multitude of quite different influencing factors [58]. In the optimization process of a lead structure it is useful to separate such influencing factors

and the associated molecular properties into two parts, namely its intrinsic activity at the molecular target and its biokinetic behavior.

Target Activity

A decisive factor is the activity of the respective active substance for its molecular target in the fungus and potentially also in non-target organisms. With strobilurins, intrinsic activity is primarily determined by the binding affinity of the active substance to the Q_O site of the bc_1 complex of the respiratory chain (see Chapter 13.1 of this volume).

The relevance of a given molecular target regarding a final fungicidal effect may change during development of the treated organism. As a consequence, different development stages may exhibit different sensitivities to an individual fungicide or a fungicide class directed to a particular molecular target. The ATP energy-demanding, and therefore strongly respiration-dependent, spore germination is – compared with mycelial growth – particularly sensitive to respiration inhibitors like strobilurins. Since, among eukaryotes, spore germination occurs almost exclusively in fungi this fact contributes on the physiological level also to selective action against fungal pathogens [9].

Optimization of target activity is best done on the basis of a cell-free biochemical test that is as near to the isolated target protein as possible. If this is not possible, tests that are still "near to the target" should be used, for instance at the cellular level; such tests can also often provide valuable complementary information to that obtained from a cell-free system.

Biokinetic Behavior

Equally important for *in vivo* activity under practical conditions is how much of the active substance actually succeeds in reaching its target.

Of critical importance here are the particular characteristics of the molecule that govern its absorption, transportation, breakdown, and, where appropriate, excretion; together, they help answer a critical question: how much of the active substance is where, and when? This seems a simple question, but to answer it empirically using the techniques of analytical chemistry generally requires high expenditures, and results available during optimization are thus limited and approximate.

In this situation, invaluable help comes from a more theoretical direction: basic physicochemical characteristics of an individual compound, such as melting point, lipophilic/hydrophilic partition coefficients, water solubility, and vapor pressure are easy to measure, and usually give a reasonably good prediction for trends regarding several aspects of its real biokinetic (*dynamic*) behavior in one or another environment. This is particularly true if one considers the correlation or ranking of a series of analogous compounds according to their physicochemical parameters on one side and their complex, time-dependent "biological" effects in one and the same test on the other side.

Thus, these well defined and *static*, substance-specific parameters, if used carefully, can help to estimate by "educated guess" the time-dependent, *dynamic*

distribution processes in complex environmental systems, as a part of the substances biokinetic behavior [58], and they usually give reasonably good predictions.

An important second aspect for these predictions are the velocities of deactivation (which usually mean degradation rates) in different environments, for instance on or in soil, plants, fungi, or other organisms, even if the data are available only in semi-quantitative form, or through estimation.

Regarding fungicides, strobilurins provide an exceptionally instructive example of these principles for several reasons: the extraordinary broad variability of their chemical structures with retention of their principal activity, their extremely broad fungicidal activity spectra, and the fact that, in contrast to former fungicide classes, a simple and reliable target test was available. Using this test, the influence of structure changes on intrinsic activity became clear, and could be assessed and understood separately from the parallel influences of the structure changes on biokinetic properties and behavior. Table 13.2.2 illustrates some of these aspects for the commercialized strobilurins.

13.2.3.2 **Target Activity**

Measuring the binding constants of strobilurins directly at their membrane-bound target, the bc_1 complex, is not easily done. Easier to measure is the active substance concentration necessary for 50% inhibition of an appropriate submitochondrial enzyme preparation (called the I_{50} value), which can be used as an effective surrogate [7, 8, 59] for the more fundamental docking measurements. Tests carried out using mitochondrial preparations from various species (yeast, *Botrytis*, maize, housefly and rat) on 14 strobilurins and myxothiazole showed that the activity ranking for the compounds was fairly parallel in all species (ranking correlation coefficients ≥ 0.8). Thus, for strobilurins no appreciable contribution to species selectivity has been observed, or can be expected, at the target level [8, 59].

For routine evaluation of structure–activity relationships at the target level we used at BASF an automatized test with yeast submitochondrial preparations. To ensure that individual results were comparable, all test series included a reference standard, the enol ether stilbene, to which the I_{50} value obtained for a test substance was referred [Eq. (1)]:

$$F = I_{50}\,(\text{test substance})/I_{50}\,(\text{enol ether stilbene}) \qquad (1)$$

By definition, $F = 1$ for the enol ether stilbene, and hence the smaller F, the higher the activity. This test has proven to be an extremely useful tool, *inter alia* in the identification of new pharmacophore variants. To give an impression of resulting structure–activity relationships, Fig. 13.2.5 shows a small selection of such pharmacophore variants together with their corresponding F values. The presented compounds all contain the same side-chain, namely that of kresoxim-methyl. For a more detailed discussion, see Refs. [7, 9].

Notably, without any X-ray structure-based knowledge of submolecular details of the binding characteristics at the target enzyme, a detailed analysis of pharma-

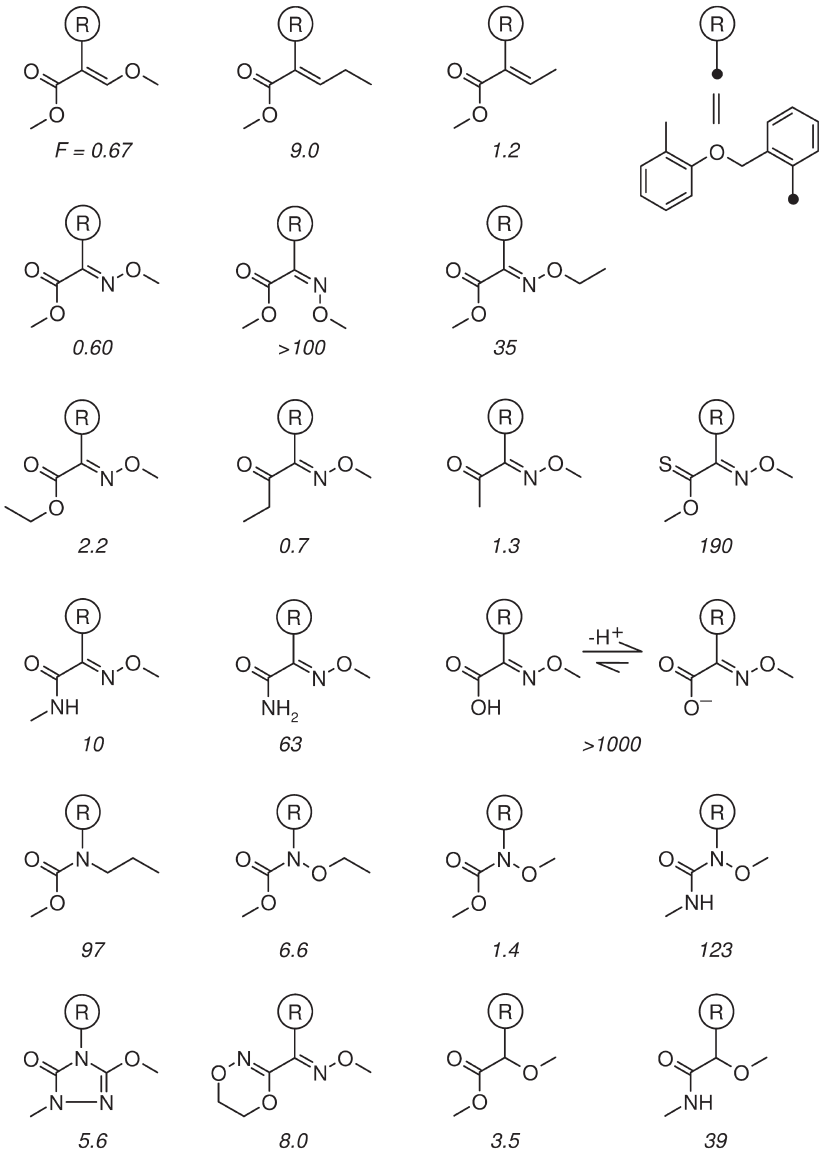


Fig. 13.2.5. Strobilurins with the kresoxim-methyl side chain and variations on the pharmacophore. Their relative activities at the target level are given below the formulas; *F* is inversely proportional to the activity (see text).

cophore structure versus target activity soon led to the central conclusion that a hydrogen bond, coming from the target enzyme as donor, and interacting with the carbonyl group as the acceptor in the strobilurin pharmacophore, contributes most to binding, and seems to be essential for activity [7]. Later, the cyclic pharmacophore of DPX-KZ 165 (left-hand formula at the bottom of Fig. 13.2.5) gave additional information about the docking conformation of the pharmacophore: the carbonyl group must have an s-(E)-orientation (''north west'', not ''south'', as suggested in Ref. [7]) regarding the remaining molecule, as indicated in the formulas of Fig. 13.2.5. Looking to the pharmacophore of fluoxastrobin (Fig. 13.2.5, bottom line, second left), clearly, the ''essential'' hydrogen-bond forming carbonyl group in the pharmacophore can be replaced by other groups that can fulfill the same electronic and spatial hydrogen acceptor function. Notably, replacement of the carbonyl group by groups that are not good hydrogen-bond acceptors – such as the thiocarbonyl group (Fig. 13.2.5, third line) – leads to drastic losses of target activity. In contrast, considerable loss of activity does not occur if one replaces the ester methoxy group with non-hydrogen-acceptor groups like alkyl, as in the ketones of Fig. 13.2.5 [7, 9].

The model of Fig. 13.2.6 accords with crystallographic data from eight cocrystallized strobilurin/bc_1 complexes, showing that an N–H proton of Glu272 (yeast enzyme numbering) is the hydrogen donor for the carbonyl group of strobilurins [60, 61]. Using beef enzyme numbering, this residue corresponds to Glu 271, and is sometimes also referred to as the ''amide N–H of Pro 270'' [62]. An alternative binding mode that favors the ester methoxy oxygen of the strobilurin pharmacophore as the hydrogen acceptor of the Glu272 (yeast) proton [63], seems to be less likely, based on the known structure–activity relationships [7, 9]. This alternative binding mode is also disfavored from a more theoretical viewpoint, considering the much higher proton acceptor potency of carbonyl (sp^2) oxygen versus methoxy ester (sp^3) oxygen in combination with the exchangeable spatial positions

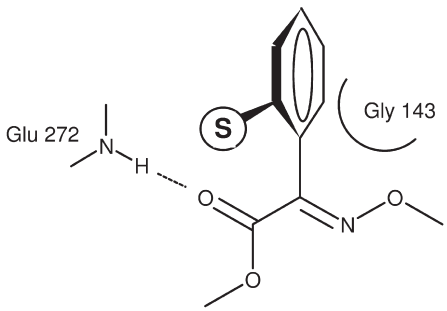


Fig. 13.2.6. Model of the hydrogen bridge between Glu 272 (yeast protein numbering) of the bc_1 complex and the carbonyl group of a strobilurin pharmacophore. (Adapted from Refs. [8, 60, 61].) Gly143 indicates the area of steric repulsion between strobilurins and the resistant Gly143Ala mutants. The torsion of the pharmacophore relative to the side chain S is adapted from the active, torsionally restricted (+)-enantiomer in Ref. [65].

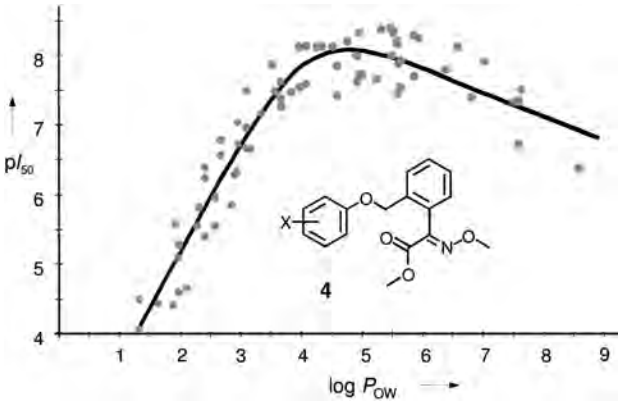


Fig. 13.2.7. Target-level activity of a series of strobilurin oximino esters as a function of the lipophilicity of the substituents X. pI_{50} is the negative logarithm of the I_{50} value from the yeast mitochondria test. Each point represents a variant of the general structure shown.

of both oxygen types in strobilurin pharmacophores, simply by a single bond rotation. In addition, a recent paper [62] clarifies this in more detail by sophisticated modeling studies for azoxystrobin, docking at its bc_1 binding site. This paper also shows clearly that only the biologically active (+)-enantiomer of the two atropisomers of a torsionally restricted analogue of DPX-KZ165 [64, cf. 65] fits into the bc_1 target.

Also for side chain variations, clear – and in this case quantitative – structure–activity relationships have been established at the target level. In a series of oximino esters of type **4**, we obtained a curve (Fig. 13.2.7) in accordance with a bilinear equation [7, 9]. Similar correlations have been deduced for enol ethers, oximino amides, crotonic esters and methoxycarbamates [66].

Thus, clearly the overall lipophilicity of the molecule – modified by the substituents X in these series of analogues – is one critical influencing factor. This may reflect the importance of the distribution between lipophilic and hydrophilic micro- (or nano-) environments before and while the strobilurin can match its docking place at the target enzyme. Significant ''underperformers'' with respect to the curve in Fig. 13.2.7 arise if, on account of certain substituents or substitution patterns X, the steric bulk of the side chain no longer permits optimal docking at the target [7]. In contrast, if a compound or a compound series is located significantly above the curve of Fig. 13.2.7, those are obviously ''outperformers'' – a much more interesting case. We found hints in that direction with the oxime ether side chain – as in trifloxystrobin – and also with other variations [66]. Such deductions and findings, once more, favor careful structure–activity analysis at the target level, whenever possible, as a powerful tool in lead optimization procedures.

Table 13.2.2 lists, for comparison, data for the commercialized strobilurin fungicides. The *F* values for mitochondrial target activity according to Eq. (1) were

Table 13.2.2 Factors affecting strobilurin properties: Intrinsic activity, physicochemical data, metabolic degradation features, biokinetic properties and biological use patterns.

	Mitochondr. factor *F*[a] *Mycosph. fijiensis*	Melting point (°C)	Aqueous solubility (mg L^{-1}) (20 °C)	Lipophilicity (Log P_{OW})	Vapor pressure (Pa) (20 °C)	Pharmacophore metabolic stability	Soil DT_{50} (days)	*Daphnia magna* EC_{50}, 48 h (µg L^{-1})	Biokinetic or biological characteristics	Typical biological targets
Metominostrobin	20[b]	87–89	128	2.3	1.8×10^{-5}[c]	High	98	14 000[d]	Root uptake, high xylem mobility	Rice diseases
Orysastrobin[a]	9.5[b]	99	81	2.4	7×10^{-7}	High	51–58	1200	Root uptake, high xylem mobility	Rice diseases
Kresoxim-methyl	2.2	102	2	3.4	2.3×10^{-6}	Low	<1–3	186	Episystemic distribution, fast degradation	Powdery mildews
Trifloxystrobin	0.26	73	0.6	4.5	3.4×10^{-6}[c]	Low	4–10[e]	16	Episystemic distribution	Powdery mildews
Picoxystrobin	0.61	75	3	3.6	5.5×10^{-6}	Medium	3–35[e]	18	Episystemic distribution and xylem mobility	Powdery mildews
Dimoxystrobin	4.1	138–140	4	3.6	6.0×10^{-7}	High	2–125	39	Long lasting, xylem systemic	*Fusarium* spp.
Azoxystrobin	4.9	116	6	2.5	1.1×10^{-10}	Medium	7–56[e]	259	Xylem systemic, translaminar, not episystemic	Very broad activity spectrum

Fluoxastrobin	2.9[b]	103–108	2.5	2.9	6×10^{-10}	High	16–119	480	Xylem systemic, translaminar, not episystemic	Very broad activity spectrum
Pyraclostrobin	0.27	64–65	1.9	4.0	2.6×10^{-8}	Medium	2–37[e]	16	Rapid leaf uptake, translaminar, long lasting	Very broad activity spectrum

Data from Ref. [68] if not otherwise noted.
[a] BASF data.
[b] Compound for mitochondrial testing re-synthesized at BASF.
[c] At 25 °C.
[d] *Daphnia pulex*.
[e] Ref. [6].

determined at BASF, using a published standard procedure with mitochondria from *Mycosphaerella fijiensis*. Measurements were carried out with purchased reference substances, if not otherwise noted. For optimal comparability of the resulting values, all compounds were tested simultaneously, and these tests were replicated three times; the average standard deviation for the I_{50}s was 32% [67]. The resulting target activities extend over two orders of magnitude. Independent from the different structures of the respective pharmacophores, the most lipophilic compounds, trifloxystrobin and pyraclostrobin, have the greatest intrinsic activity, whereas the most hydrophilic compounds metominostrobin, orysastrobin and azoxystrobin rank at the lower end of the activity scale.

13.2.3.3 **Transportation and Distribution**

Besides target activity, dosage transfer of a strobilurin from the point of application to the receiving bc_1 complex of the fungal target organism and its availability at this receptor determines its activity *in vivo*.

13.2.3.3.1 **The First Step**

To achieve efficient transportation, the active ingredient must start its journey in a molecularly dispersed state, i.e., in solution or in gas phase. If the compound is deposited as solid particulate material, for instance on a leaf surface, then it is necessary to break up its amorphous or crystalline supramolecular associations as a first step. The energy and time necessary for that depends on the melting point: the lower the melting point, the easier and faster molecular dispersion. Whatever the melting point, formulation can influence the bioavailability in the direction of faster or slower release, as desired for a particular treatment. For example, in the case of fluoxastrobin, Bayer scientists were able to show by SEM that, despite its relatively high melting point (103–108 °C, see Table 13.2.2), the compound when formulated as a particular ''EC 100'' was deposited on barley and other plant leaves as an ''amorphous layer without pronounced solid particulate material'', thus providing a deposit that is probably already molecularly dispersed, for both fast and ''prolonged foliar penetration and the associated systemic distribution'' [50].

Another example: Table 13.2.2 shows that pyraclostrobin has the lowest melting point of the commercialized strobilurins (64–65 °C). On that basis, one would expect, first, that it would be easy to formulate it in liquid form and, second, that it should provide a very fast first-step bioavailability; i.e., extremely rapid leaf uptake and translaminar penetration after leaf treatment. Both characteristic properties of the compound are observed, the latter supported also by its relatively high lipophilicity ($\log P_{OW} = 4.0$).

The importance of formulation for good performance cannot be overemphasized. Anyone who has seen comparative field experiments of one and the same active substance in different formulations (e.g., as EC versus WP) knows how drastically efficacy – and, more important, decisions to proceed with one or another substance – can be influenced by formulation.

13.2.3.3.2 The Next Steps

Regarding distribution processes and their influence on *in vivo* fungicidal efficacy, the question arises: what is the pattern of concentration in space and time of the active ingredient with regard to the host plant, and with regard to the location of the fungal pathogen and its organs in or on the plant?

Leaf Surface Distribution via Vapor Phase, Episystemicity If the volatility of the active substance is high enough, migration can start from its deposits on leaf surfaces via vaporization and gas phase transportation. For strobilurins, this phenomenon became first most prominent with kresoxim-methyl and has been named as quasi-systemic, episystemic, leaf surface systemic, or – if connected with translaminar movement – mesostemic. Experience shows that a minimum vapor pressure of approx. 10^{-7} Pa is necessary [58] to get a sufficient amount of molecules into their molecularly dispersed gaseous state in a reasonable time period. However, it is also clear that volatility should not be too high, to avoid too fast substance dissipation. If the molecular properties regarding volatility and lipophilicity are appropriate, lateral distribution/redistribution processes between the substance deposit on the leaf, the boundary air layers above it, and the waxy leaf surface can occur. Having their origin in the point the substances deposit, they will then slowly lead to time-dependent, concentric distribution patterns of the substance on the leaf surface. For episystemic strobilurins, such distribution patterns, and even transportation from one plant to another, have been indirectly observed by remote fungicidal effects [8, 35, 45, 69], and directly observed with radiolabeled substances [6, 44, 45].

A cluster of three strobilurins shows very pronounced distribution properties of this type: kresoxim-methyl, trifloxystrobin and picoxystrobin. Their vapor pressures all lie in the astonishingly narrow range between of $2.3–5.5 \times 10^{-6}$ Pa (Table 13.2.2). Regarding the observable biological consequences resulting from that, two points should be emphasized. First, vapor phase transportation enables the fungicide to attack and eradicate efficiently fungi or fungal organs that grow *on* the leaf surface, e.g., the mycelial mats of powdery mildews. Second, in protective treatments, if the substance is applied early enough, episystemic transportation can lead to a more or less uniform distribution in the waxy leaf surfaces, thereby forming a protective shield against air-borne fungal spores, by continuously releasing active ingredient. Strobilurins generally exhibit outstanding activity against fungal spore germination, which usually exceeds their activity against other fungal development stages [6, 8]. This is reflected in the tendency for strobilurins to show better protective then curative performance in field treatments [70]. Thus, combination of their extremely efficient spore germination inhibition and episystemic distribution into the waxy leaf layers makes the cluster of the three episystemic strobilurins mentioned earlier particularly well suited for protective leaf applications. The fact that there is no metabolism in the waxes also allows for metabolically more labile compounds like the oximino esters kresoxim-methyl and trifloxystrobin to be present and active for a long period, and to provide long residual activity.

Systemicity in Plants, Xylem Transportation After leaf treatment, systemic acropetal transportation in the apoplastic xylem stream is possible, if foliar penetration of the active substance occurs, if the distribution between the lipophilic and hydrophilic phases is appropriate, and if the substance is metabolically stable enough under such conditions. A – necessary but not sufficient – measure for the distribution properties is given by the octanol–water partition coefficient P_{OW}. Maximum systemicity can be obtained with log $P_{OW} \approx 2$, and below ca. 0 or above ca. 4 no appreciable xylem systemicity can be observed [71, 72]. In contrast to acropetal xylem mobility, phloem (symplastic) systemicity, which allows additionally basipetal migration, requires compounds with either some acidity or extremely high hydrophilicity. With strobilurins, both acidity and extremely high hydrophilicity are absent. Four commercial strobilurins, successfully designed for xylem systemicity, have log P_{OW} values between 2.5 and 3.6 *and* are metabolically sufficiently stable in plants (Table 13.2.2): azoxystrobin (2.5), fluoxastrobin (2.9), picoxystrobin (3.6) and dimoxystrobin (3.6). They all show a pronounced xylem systemicity [6, 40, 50]. Picoxystrobin has, in addition, episystemic mobility (see above), which favors a broader activity spectrum, including, especially, mildews. For fluoxastrobin, a simulation of its time-dependent systemic distribution in crop leaves was in a good accordance with experimental data [50]. This paper shows also the position of azoxystrobin, fluoxastrobin, picoxystrobin and trifloxystrobin in the time-continuous optimum curve for the transpiration stream concentration factor (TSCF, as a measure for xylem systemic accumulation) versus log P_{OW}, and it touches implicitly on the question of whether maximum systemicity always translates to optimum fungicidal efficacy. Because target docking is a process of dynamic equilibrium, high mobility to reach a target is also connected with high potential to dissociate away from the target.

Root Uptake Looking at Table 13.2.2, Shionogi's metominostrobin stands out as having the lowest log P_{OW} (2.3) of all the listed strobilurins, the highest water solubility and the lowest aquatoxicity. Together with the high metabolic stability of the oximino amide pharmacophore, this bundle of properties provides all the prerequisites for root uptake, acropetal movement, residual activity in leaves, and compatibility with aquatic environments. Regarding hydrophilicity, it was already well known that high water solubility and low lipophilicity of bioactive compounds both correlate positively with low aquatoxicity [73]. More concretely, we found this again, with an excellent linear correlation, by plotting 17 fungicidal strobilurins with log P_{OW} ranging from 1.8 to 4.8 against their log EC_{50} with daphnia ($r = 0.81$; $F = 24$; $S = 0.41$) [74]. However, the consequence of low lipophilicity is also a relatively low intrinsic activity, at least with strobilurins (Fig. 13.2.7 and Table 13.2.2). So, unless metominostrobin has broad spectrum activity at higher application rates, its primary biological target is a special one: water surface application in paddy rice against rice blast. Regarding this regionally important target crop, it is perhaps not surprising that this has been found by Japanese scientists.

The same molecular properties – high water solubility and a low log P_{OW}, combined with high metabolic stability in plants – were in the focus of our R & D

at BASF, with the aim of gaining entrance into Asian rice fungicide markets. Our team was also convinced that the increasingly popular use of nursery boxes – whereby rice seedlings are first grown up to a certain stage in nursery boxes before they are transplanted into the field – would soon be a major market segment for rice fungicides. Our strategy was simple and clear: we took advantage of our accumulated knowledge of strobilurin structure–activity relationships. This led to some deviations from routine screening procedures. The main principles were: (a) no compound with a log P_{OW} above 3.0 would be suitable for the targeted rice market; (b) the candidate has to be independent of patent claims outside BASF's rights; (c) metabolic stability in plants should be expected to be high (no oximino esters!); and (d) aquatoxicity *must* be low, and must be tested early. Although the latter restriction narrowed the chances of finding extremely active strobilurins, a sufficiently high mitochondrial target activity had to be maintained. We also agreed that very early we should have a realistic impression about the expected final biological performance of the candidates under conditions of practical application. In this preselection phase, considerations of the expected synthetic availability and possible production costs played *no* major role, because – according to the authors' experiences – motivated chemists can usually solve such problems with their own creative input and competence. With orysastrobin, this proved to be the case once again [75]. The preselection criteria described led to an ensemble of only 300 initial candidates (out of more than 10 000), which were then tested in specially designed, more sophisticated tests, targeted in the direction of the final application. Only four compounds out of this group reached a status of more serious concern for further development, and, of these, only one compound had – according to our criteria – the potential to reach the targeted market. This compound, orysastrobin, was announced in 2004 [52] as an effective rice fungicide against both rice blast and sheath blight for use in nursery boxes and for paddy rice application after transplanting.

Regarding efforts that have been focused on systemicity during strobilurin research worldwide, interestingly, BASF's top strobilurin, pyraclostrobin, shows no appreciable systemicity such as xylem mobility or leaf surface distribution. This ostensible disadvantage was expected from its high lipophilicity and low vapor pressure. Nevertheless, its low melting point gives rise to a fast bioavailability, and as a consequence also to a particularly fast translaminar distribution. It ranks also at the top regarding intrinsic activity and long-lasting efficacy. It possesses outstanding plant compatibility and crop safety and is – in part as a consequence of that – registered for use in more then 100 crops worldwide. In this connection it should be said that increasing the hydrophilicity and systemicity of strobilurins may also increase the possibility of incurring slight phytotoxic effects. To summarize, despite all the rationally derived, straightforward design procedures, which are undoubtedly necessary and proven to be successful, the beneficial consequences of serendipity should never be neglected.

For enestroburin, not many data are published. The technical product and the compound in the patent [53] have been characterized as an oil; the pure compound has been described as a white crystalline solid (melting point not dis-

closed). Some results from fungicide trials have been given [54]. For comparison, the compound has been resynthesized at BASF. Besides its obviously very low melting point, it has a high log P_{OW} (>4), low vapor pressure ($<10^{-7}$ Pa), and considerable target activity ($F \cong 0.4$). For experts – or for careful readers of this article – this may already give hints as to its other – e.g., biological – properties.

13.2.3.4 Metabolic Degradation Rates

Regarding the overall fungicidal activity and biokinetic behavior, active substance losses through metabolic degradation must be taken into account. Within strobilurins, degradation generally leads to deactivation, particularly if the pharmacophore is involved. There are not many exact degradation rates under the many different conditions in plants or other metabolically active environments available. As a rough and more semiquantitative measure, the individual DT_{50} value ranges of the compounds for soil degradation can be used. They may give also an idea of relative degradation rates in other environments, such as plants, and an insight into structure–activity trends for metabolism (Table 13.2.2). Obviously, the oximino esters (kresoxim-methyl and trifloxystrobin) are by far most rapidly metabolized. Many investigations have shown that the first metabolic step in both molecules is rapid hydrolysis of the methyl ester group of the pharmacophore, resulting in inactive carboxylic acids. Therefore, with strobilurin oximino esters one cannot expect long-lasting activity when they are subjected to metabolically active environments, as is the case inside plants. This is one reason for their lack of xylem systemic activity.

According to Table 13.2.2, the two enol ethers (azoxystrobin and picoxystrobin) and the methoxycarbamate pyraclostrobin rank next, thus forming a cluster of strobilurins with intermediate degradation speeds.

The highest metabolic stabilities are seen with the oximino amides (orysastrobin, metominostrobin and dimoxystrobin) and with the dihydrodioxazine (fluoxastrobin). This high level of stability is one of several prerequisites for applications where long-lasting fungicidal efficacy should be maintained while the active compound is intensively exposed to plant metabolic processes, e.g., after root uptake in rice.

The aim of this chapter is not to describe the fungicidal profiles of different strobilurins against different pathogens in detail. If the dosage is high enough, their activity spectra show considerable similarities. The aim is to demonstrate from a more global perspective some guidelines of how structural features in this class can influence the fine tuning of biological activity and use patterns. More detailed information about the biology and many other aspects of the commercial or developmental products can be found in their announcement papers or in the Pesticide Manual [68] and, most comprehensively, in the periodically updated collection "AGRO Projects" [76].

At this point, I want to present a somewhat farcical – but not entirely ludicrous – quantitative structure–activity relationship (QSAR): Each of the nine commer-

cial strobilurins of Fig. 13.2.2 contains at least once *a three-atom fragment consisting of oxygen and an imino group*, in one of two different arrangements. Across all nine molecules, this feature occurs exactly 2.00 times per molecule – on average, so to speak! Less jokingly: the nine substances contain altogether eleven examples of the oximino group –C=N–O– and seven examples of the group –N=C–O–, the latter always in conjunction with heterocyclic structures. The incorporation of so many relatively hydrophilic fragments reflects – at least to some extent – the more or less directed approach to get moderately lipophilic, xylem systemic compounds.

Regarding the oximino group of oxime ethers, several reasons favor its use as a building block for agrochemicals. Synthetically, starting with a carbonyl group, its introduction into a molecule is extremely easily performed and not connected with any C–C bond formation, and, in most cases, the thermodynamically preferred (E)-configuration can be obtained almost exclusively under acidic equilibrium conditions. From the viewpoint of physicochemical properties, the group is of intermediate polarity and can contribute considerably to the size of a molecule without enhancing its lipophilicity. Log P_{OW} is even lowered when a single bond, e.g., between two carbon atoms, is replaced by the oximino fragment (CH=N–O–), and remains approximately constant when –C(CH_3)=N–O– is introduced. Finally, biologically, the latter group possesses in general a remarkable metabolic stability.

13.2.3.5 Summary of Strobilurin Structure-Activity Relationships

A rough summary of the structure–activity experiences with strobilurins is visualized in Fig. 13.2.8. It illustrates the complex intercorrelation network between

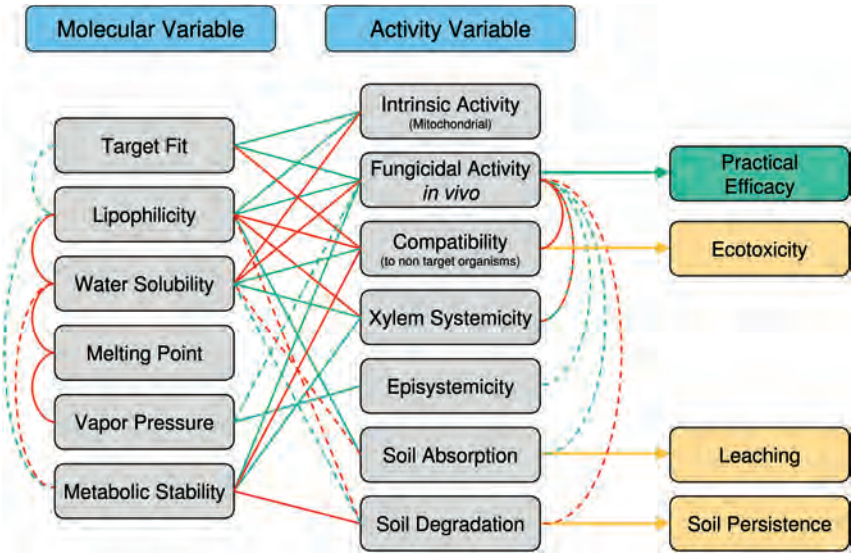


Fig. 13.2.8. Structure–activity relationships: the complex network between variables.

some molecular variables (left), activity variables (middle) and the main final output properties (features) in agricultural practice (right). Positive correlations between the variables are marked by green lines, negative correlations by red lines. Broken lines indicate that a correlation is relatively weak. Correlation in this context does not mean a strict and exclusive linear relation between the variables. It means simply that one variable has an influence on another. This influence can sometimes be only weak, and be valid only between certain limits. Naturally, a correlation between two variables does not exclude influences from other variables, as can be seen in Fig. 13.2.8. For example, looking at the molecular variable lipophilicity, it is clear from the bilinear curve of Fig. 13.2.7 that the positive correlation with target activity is valid only at values below log $P_{OW} \leq 5$. Staying with lipophilicity, there is also clearly only a weak positive connection with target fitting (lipophilic areas of the target need somewhat lipophilic side chains of the strobilurin) and with metabolic stability (more hydrophilic substances tend to be degraded faster), and that chemical or spatial changes in the molecular structure may exert a much more drastic influence on target fitting or metabolic stability than lipophilicity. Keeping that in mind, the chart can serve as a navigation aid through the complexity of strobilurin structure–activity relationships, and also – *cum grano salis* – outside strobilurins.

13.2.4
Beneficial Influences on Plant Physiology and Crop Yield

It has been reported consistently from field trials that the yield enhancement obtained after strobilurin treatments in wheat [e.g., Ref. 77] and barley exceed significantly the values that could be expected from comparative triazole treatments with similar levels of visible fungal disease control, cf. Refs. [6, 70]. Also observed were a pronounced ''greening'' effect and delayed senescence. This enables the plants to maintain green leaf area until late in the season, thereby maximizing the grain-filling period and yield. Also in other crops, and under conditions of no or very low fungal infection, unexpected beneficial effects on yield and quality and better stress tolerance after strobilurin treatments have been observed. Pyraclostrobin also seems to be the most potent strobilurin in this respect. This led, for instance, to the introduction of pyraclostrobin (Headline™) for the optimization of plant health and crop yield in corn and soybean.

These benefits, thought to be the result of direct influences on physiological processes of the treated plants, are referred as to ''physiological effects'' [48a], and have been most extensively studied with kresoxim-methyl and pyraclostrobin. These include effects like delayed senescence, altered CO_2 compensation point, reduced stomatal aperture and water consumption, and better tolerance of oxidative stress. Significantly altered levels of enzyme activities (ACC synthase, nitrate reductase, peroxidases, alternative oxidase AOX) could be observed or inferred indirectly *in vivo*, but in no attempted cases directly with isolated enzymes *in vitro*. The simplest and therefore most convincing hypothesis to explain all these many

different effects [48] is that strobilurins have a direct influence on mitochondrial respiration not only in fungi but in plants too, and that this then leads to a cascade of the different biochemical, physiological and agricultural consequences [48a]. This theory includes the generation of NO as a fully-systemic, acro- *and* basipetally-movable signal molecule [48b] for triggering – even remote – plant defense reactions [48c], even against pathogens that are not sensitive to strobilurins, e.g., the tobacco mosaic virus or the bacterial wildfire pathogen *Pseudomonas syringae* pv *tabaci* [48d].

Mitochondria from maize leaves do in fact respond to a series of strobilurins, but are in this case *less sensitive* than mitochondria from non-plant species (yeast, *Botrytis*, rat, house fly) [59]. Note that, generally, inhibition of mitochondrial respiration in plants (''dark respiration'') does not lead to severe undesired influences on plant physiology.

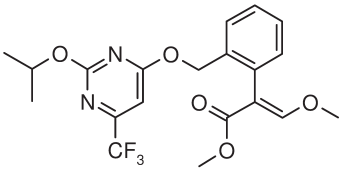
There is a second theory to explain the beneficial yield effects, which does not include direct influences on plant biochemical processes but relates exclusively to fungicidal effects. This theory says that strobilurin treatments prevent spore germination of pathogenic, non-pathogenic and saprophytic fungi and thereby stop the elicitation of energy-demanding host-defense responses with the result of higher crop yields [78].

In practice, the beneficial effects of strobilurins on plant health and crop yield have been proven over many years and under many different conditions. From a more scientific point of view, however, the reasons for this are not yet fully understood. It is certainly a challenge for future research, and may initiate the search for new compounds with optimized physiological effects. In that regard, metabolic profiling of treated plants – compared with untreated – may be a key technique for finding such effects and interpreting them on the metabolome level. Metabolic profiling as a new diagnostic method was introduced to crop science by us in the 1980s [79], and has subsequently found increasing interest, progress and industrial applications [80] in plant metabolome research [81].

13.2.5
Insecticidal and Acaricidal Activity

In the patent literature, many claims and some data can be found about insecticidal and acaricidal activities of strobilurins. Attempts to optimize insecticidal performance did not lead to a commercial product. It seems that, with strobilurins, sufficient insecticidal activity can only be obtained if the compound has very high lipophilicity and very high metabolic stability. This combination of properties gave numerous compounds with excellent insecticidal activity but also with unacceptably high acute mammalian toxicity, so that candidates were abandoned rather early in the research phase. It seems impossible to separate the tight connection between insecticidal and mammalian toxicity in this particular case. Similar problems have been reported for respiration inhibitors of Complex I [82a].

In contrast to insects, mites seem to be much more sensitive to strobilurins, so that the chances of finding selective strobilurin acaricides are better than in the



fluacrypyrim

Fig. 13.2.9. The strobilurin acaricide fluacrypyrim.

case of insecticides. Mites are much smaller than insects, have in general a greater surface per gram biomass, lack a thick cuticule, and, possibly, also have lower metabolic degradation capacities. Consequently, optimal acaricides [82b] are usually different substances than optimal insecticides.

In fact, there is one strobilurin acaricide on the market: fluacrypyrim (Fig. 13.2.9) [68, 76, see also Chapter 28.3]. The compound and its acaricidal activity were originally discovered by BASF [83], and the compound was later developed by Nippon Soda for use in fruit crops and vegetables. It has low mammalian toxicity and log $P_{OW} = 4.5$. Most interestingly, its vapor pressure (2.7×10^{-6} Pa at 20 °C) is in the same narrow range as those of the episystemic strobilurin fungicides of Table 13.2.2. Clearly, an episystemic distribution pattern can be expected from fluacrypyrim. Since adult mites and the possibly more sensitive earlier developmental stages live in close proximity to plant surfaces, vapor phase distribution should be particularly advantageous for acaricides. A recent acaricide review [82b] lists nine commercial mitochondrial respiration inhibiting acaricides. A literature survey reveals that seven of them (diafenthiuron, fluacrypyrim, fenazaquin, fenpyroximate, pyridaben, tebufenpyrad, chlorfenapyr) have vapor pressures in the astonishingly narrow range of $2–12 \times 10^{-6}$ Pa, thus being very prone for episystemic distribution patterns, and having therefore also good prerequisites for long residual activity.

13.2.6
Fungal Resistance

This topic has been comprehensively reviewed [6, 84], both from a more molecular biological and biochemical [84a] and from a more practical view point [84b]. It is also a permanently updated subject of the FRAC Q_OI Working Group as part of the Fungicide Resistance Action Committee, an inter–industry organization that monitors fungicidal resistance and coordinates resistance management (www.frac.info). For strobilurins, several quite different resistance mechanisms have been described that have only laboratory significance and are not of major importance for agricultural applications, e.g., circumvention of the bc_1 complex

by the alternative oxidase pathway or the extrusion of the fungicide by ABC transporters.

Also of limited practical importance is some resistance of *Venturia inaequalis* (causing apple scab) against kresoxim-methyl, which has its origin in the esterase-mediated metabolization of the active substance by the resistant isolates [85]. Target mutations Phe129Leu (yeast numbering) lead only to lower resistance factors in a few fungal species, and seem also to be of minor practical relevance.

In contrast, target mutation Gly143Ala (yeast numbering) is of great importance for Complex III Q_O site inhibitors. At the molecular/submolecular level it prevents docking of the active substance to its target completely [60, 84a, 86a]. This and four other mutations at different places in the amino acid sequence of the bc_1 complex in different species are the main reason why strobilurin-producing basidiomycetes like *Strobilurus tenacellus* are insensitive to their own fungicide [84a, 87]. Subject to Gly143Ala resistance are not only strobilurins, but also the other known commercial Q_O site inhibitors, famoxadone and fenamidone, in contrast to the Q_I inhibitor cyazofamid, which is not affected. The consequences of G143A are high resistance factors and an almost complete loss of fungicidal activity. In practice, resistance was observed soon, in some indications only two or three years after market introduction of strobilurins. Of severe concern was first wheat powdery mildew in cereals, somewhat later followed by *Mycospaerella graminicola* (S*eptoria tritici*). At present, both diseases are no longer main recommended targets for strobilurin applications in practice, although pyraclostrobin is an exception and still shows considerable activity against *Septoria tritici* under field conditions [70]. Notably, protective treatments with azoxystrobin were still very effective against resistant isolates in greenhouse experiments, whereas curative treatments were much less effective under similar conditions [70]. A bc_1 modeling study gives hints to explain species differences in the effect of Gly143Ala mutations at the enzyme level [86a].

Strobilurins were also severely confronted with resistance problems in other crops, such as in the case of *Plasmopara viticola* in grapes. Remarkably, however, other economically important fungal diseases, such as those caused by rust pathogens in different crops, are not at all affected by Gly143Ala strobilurin resistance, and it relates generally to all plant pathogenic Basidiomycetes up to now. This parallels the phylogenic separation of Basidiomycetes from the more Gly143Ala resistance affected Ascomycetes and Oomycetes, as demonstrated with recent phylogenic relatedness studies of several fungal species at the mitochondrial *cyt b* gene level and at the nuclear level using Internal Transcribed Spacers (ITS) in the ribosomal DNA [86b].

In general, and particularly in the threatened indications, the FRAC recommendations for strobilurin use do provide a great contribution in preventing resistance. In principle, they consist of four major points: limitation of the number of treatments per season, application of sufficiently high fungicide dosages, alternation and/or mixtures with non-cross-resistant fungicides having other modes of action.

Table 13.2.3 Selected data for azolones and cyazofamid.

	Melting point (°C)	Aqueous solubility at 20 °C (mg L^{-1})	Lipophilicity (log P_{OW})	Vapor pressure (Pa) (20 °C)	Soil DT_{50} (days)	*Daphnia magna* EC_{50} 48 h (μg L^{-1})
Famoxadone	141–142	0.05	4.7	6.4×10^{-7}	2–28[c]	12
Fenamidone	137	7.8	2.8	3.4×10^{-7}[a]	7–8[c]	190[c]
Cyazofamid	153	0.12	3.2	1.3×10^{-5}[b]	3–5	>140

Data from Ref. [68] if not otherwise noted.
[a] At 25 °C.
[b] At 35 °C.
[c] Ref. [76].

13.2.7 Other Complex III Inhibitors

Note that, despite a certain similarity in their names, neither the azolones nor the N-(N′,N′-dimethylaminosulfonyl)azoles are in any way related to the azole fungicides of the DMI type (see Chapter 17 of this book).

Data for the three commercialized products of these types can be found in Tables 13.2.1 and 13.2.3.

13.2.7.1 Azolones

As with strobilurins, the central starting point for the azolone fungicides was again academic research, this time in the group of D. Geffken in Germany. This research was basically "pure chemistry" without a specific biological target. During cooperation agreements, the lead structure, a thioxo-oxazolidinone (Fig. 13.2.10), was transferred to Du Pont research.

There, the fungicidal activity was detected and an optimization program carried out [88] that finally led "after three years of work and the preparation of over 700 analogs" [88b] to an optimized structure, famoxadone; for patents see [89]. *In vivo* structure–activity relationships have been reported with the oomycetes *Phytophthora infestans* and *Plasmopara viticola* [88]. Famoxadone was announced in 1996 as a broad spectrum fungicide for the control of diseases caused by Ascomycetes and Basidiomycetes in various crops and particularly against downy mildew diseases caused by oomycetes in potato, vines and vegetables [90].

In 1998, a second commercial fungicide out of this azolone group was announced [91]: fenamidone, originated by Rhône-Poulenc (as part of Aventis's agrochemical interests, later merged into Bayer Crop Science); for patents see Ref. [92]; for QSAR of fenamidone analogs with *Agaricus campestris* mitochondria

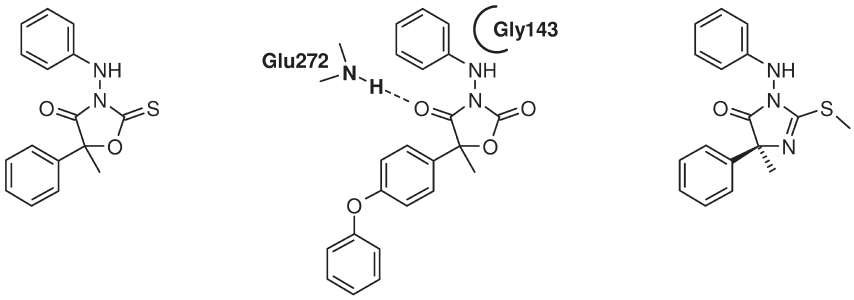


Fig. 13.2.10. Azolones: lead structure and commercial products. Target interaction sites for the hydrogen-bridge binding (Glu272), and for the point mutation at Gly 143 that causes resistance, are sketched together with the famoxadone formula.

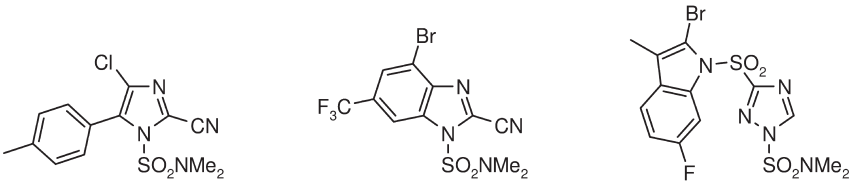
see Ref. [93]. Interestingly, only the active S-enantiomer of the chiral active ingredient [94] has been developed and is distributed as a fungicide to reduce environmental loading. As in the case of famoxadone, the main agricultural target is the control of downy mildews in various crops [91, 95].

Like strobilurins, the azolones bind to the Qo site of the bc_1 complex [96, 97]. They also form a hydrogen bridge to Glu272 (yeast numbering) and their binding is also prevented in the Gly143Ala (yeast numbering) mutants, as roughly illustrated in Fig. 13.2.10. The latter fact explains cross resistance of azolones and strobilurins in almost all cases of practical relevance up to now [98]; see also Chapter 12 of this book. In summary, azolone positioning in the enzyme niche does not completely overlap with the respective areas where strobilurins are located during bc_1 binding, cf. [99]. Kinetic studies show also differences between strobilurin (MOAS) and famoxadone binding modes. While famoxadone binds in a noncompetitive manner, MOAS is described as having mixed competitive/noncompetitive binding in relation to ubiquinole [96b].

Based on the common mode of action of strobilurins and azolones, treated fungi are particularly sensitive during their energy demanding spore germination stage. For Oomycetes, additionally, zoospore liberation and motility – also highly energy demanding processes – as well as zoospore cellular integrity [96a, 100] are extremely sensitive to Complex III inhibitors [6, 91, 95].

13.2.7.2 **N-(N′,N′-Dimethylaminosulfonyl)azoles**

This group (Fig. 13.2.11) exhibits also very high fungicidal activity against Oomycetes (now named Peronosporomycetes). The fungicidal activity is directed specifically against this fungal class and against the Plasmodoriophoromycete *Plasmo-*



cyazofamid | CGA 52232 RP (proposed common name dimefluazole) | amisulbrom

Fig. 13.2.11. Q_i site inhibitor cyazofamid and analogous compounds.

diophora brassicae. In addition, this fungicide group has, obviously, two other things in common: chemically the dimethylaminosulfonyl moiety linked with an electron-poor azole ring, and biochemically the common mode of action as Complex III inhibitors (up to now not disclosed but expected for amisulbrom).

Cyazofamid was announced [101] and then commercially introduced in 2001 as a fungicide for the control of late blight and downy mildews; for the mode of action, see Ref. [102]. Amisulbrom has been developed by Nissan for similar uses and entered official trials in Japan in 2003 [76].

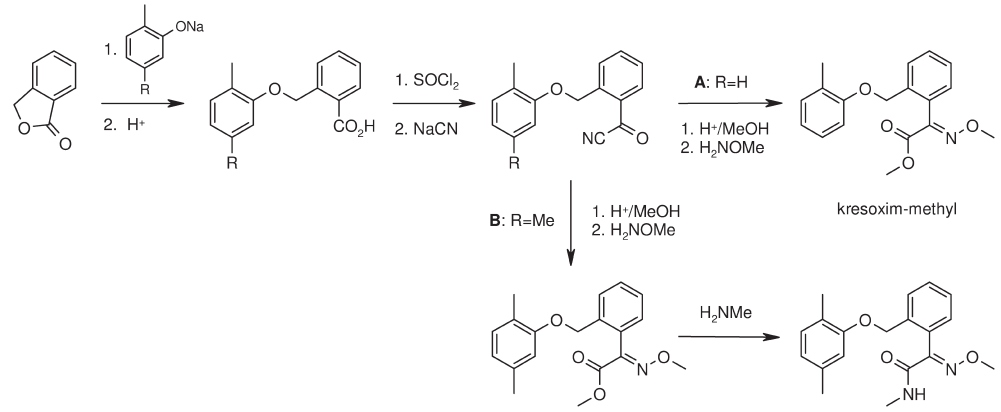
Dimefluazole did not achieve commercial status, but this compound and an analog were the subject of a detailed investigation of the mode of action [103]. The conclusion was that the azole moiety acts as a leaving group, so that the sulfonyl group of the a.i. binds covalently to a nucleophile of the Q_I-center of oomycetes [103]. The different submolecular target sites of cyazofamid and Q_O-site inhibitors lead to lack of cross resistance.

13.2.8
Synthesis Routes

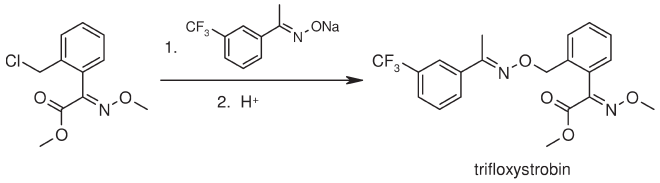
The following schemes show for each of the above-mentioned, commercialized fungicides one possible synthesis route, taken from published sources, mostly patents. For chemists, this should give an impression how individual compounds *can* be synthesized, and *can* also suggest synthesis strategies and chemical reactions on which the technical processes are based. But this is not necessarily so in each case, since the production processes usually are not published.

For synthetic organic chemists, the schemes are self-explanatory and require no detailed comment. In strobilurins, the ortho substitution pattern at the central bridging ring favors the use of easy accessible starting materials or intermediates in which a lactone-type ring is cleaved to obtain pharmacophores and side chains built up in the proper ortho connection. As already mentioned at the end of Section 13.2.3.4, the thermodynamically favored E-configuration of the differ-

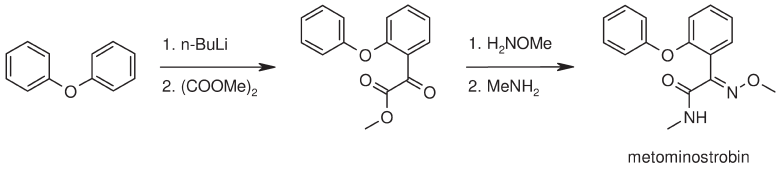
Synthesis of kresoxim-methyl and dimoxystrobin



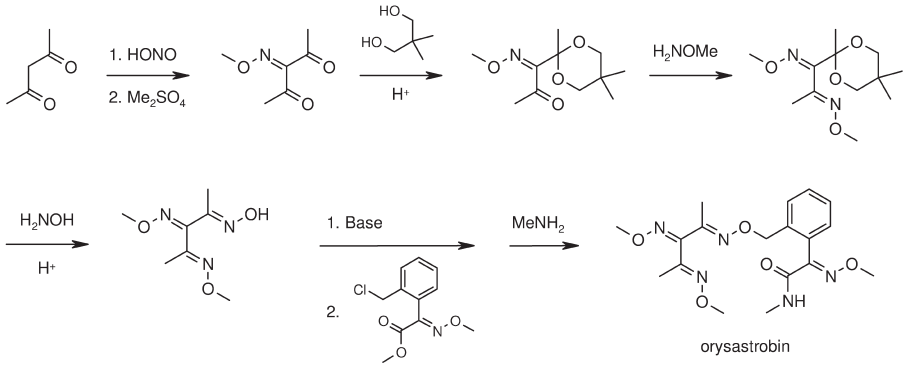
Synthesis of trifloxystrobin



Synthesis of metominostrobin

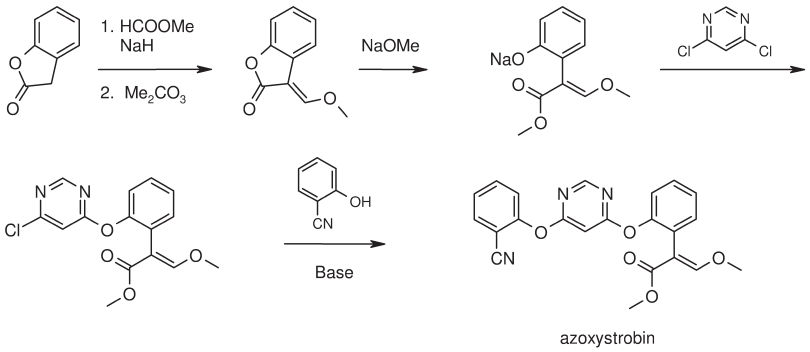


Synthesis of orysastrobin

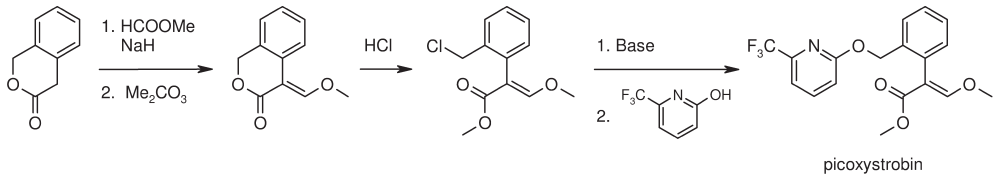


Scheme 13.2.1. Synthesis routes to strobilurins with the oximino pharmacophores.

Synthesis of azoxystrobin



Synthesis of picoxystrobin



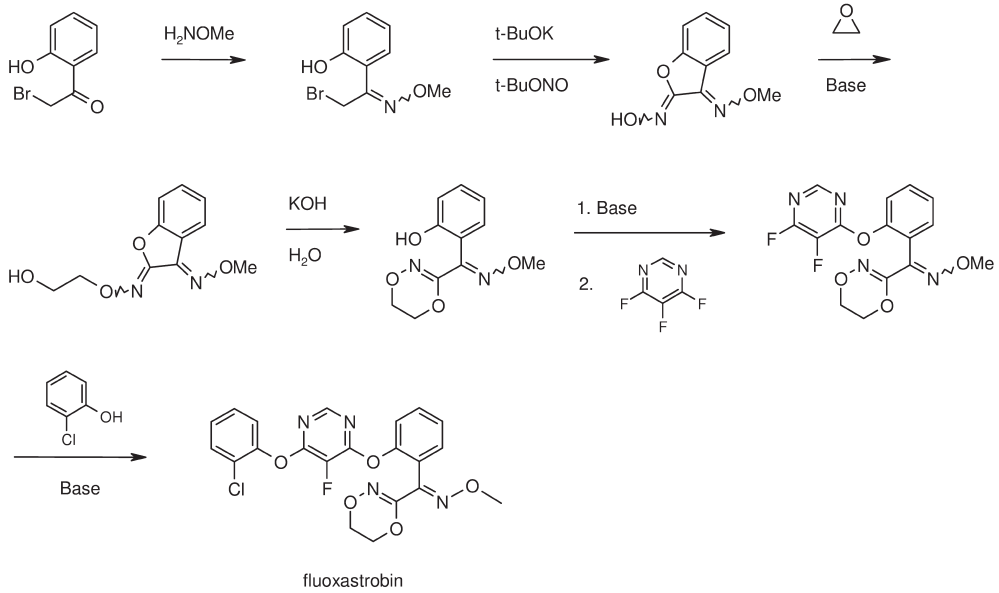
Scheme 13.2.2. Synthesis routes to strobilurins with the methoxyacrylate pharmacophore.

ent oximino groups can usually be obtained by equilibration under acidic conditions.

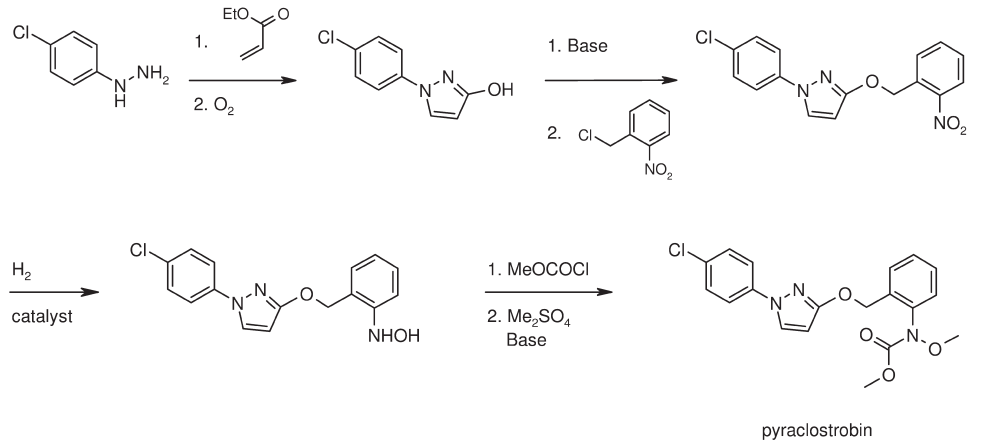
The chiral starting material S-methyl phenyl glycine in the synthesis of fenamidone (Scheme 13.2.4) can be obtained by enzymatic resolution processes. Alternatively, chirality can be introduced at a later, cyclic stage using a chiral reagent that resolves an intermediate hydantoin [94].

In the cyazofamid synthesis (Scheme 13.2.5), a nice all-in-one reaction can be used to convert **5** into **6**: it involves a dehydration, two deoxygenations and a chlorination at the imidazole ring system in an one-pot reaction [104].

Synthesis of fluoxastrobin

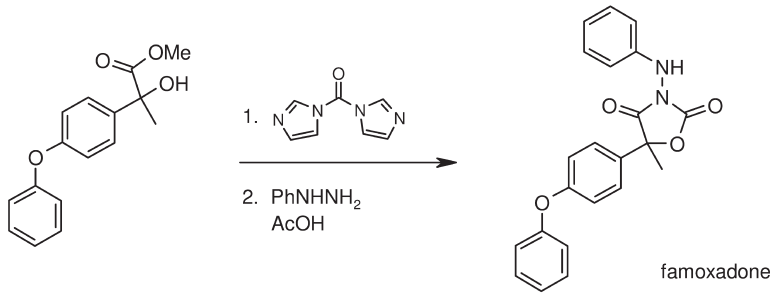


Synthesis of pyraclostrobin

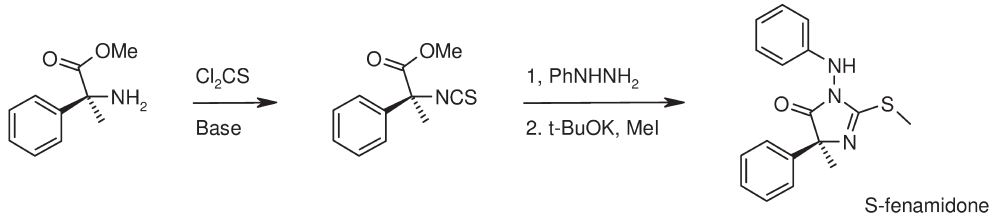


Scheme 13.2.3. Synthesis routes to fluoxastrobin and pyraclostrobin.

Synthesis of famoxadone

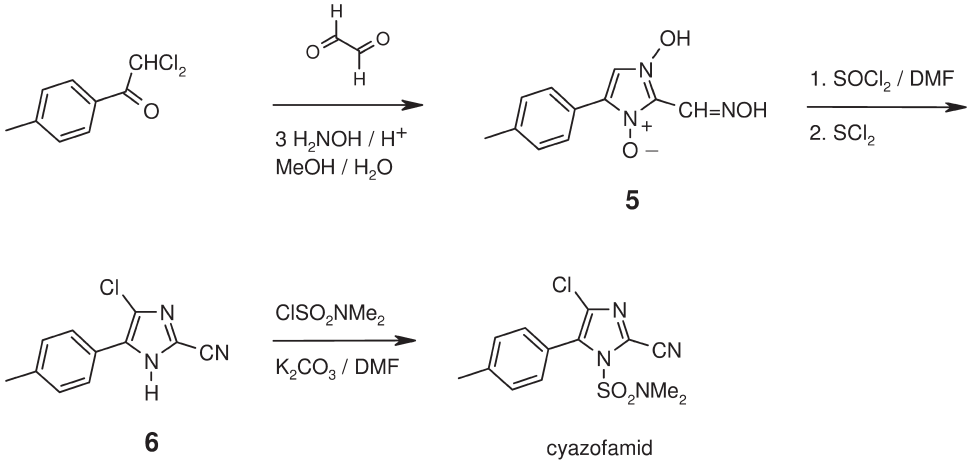


Synthesis of fenamidone



Scheme 13.2.4. Synthesis routes to the azolones.

Synthesis of cyazofamid



Scheme 13.2.5. Synthesis route to cyazofamid.

Acknowledgments

It is with great pleasure and sincere admiration that I thank the many colleagues from BASF who were engaged in strobilurin research, development, production and marketing, for their outstanding contributions to this fascinating and still inspiring field. I am also particularly grateful to William K. Moberg for many enlightening discussions and for his invaluable editorial assistance in preparing the manuscript for publication.

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13.3
Succinate Dehydrogenase Inhibitors

Joachim Rheinheimer

13.3.1
Introduction

Already in 1977 a general structure (Fig. 13.3.1) had been published for carboxylic amides as succinate dehydrogenase inhibitors, a structure that still forms the basis of most modern molecules [1].

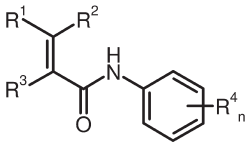


Fig. 13.3.1. General structure for carboxylic amides as succinate dehydrogenase inhibitors.

At that time about ten compounds of this structural class were developmental candidates or had been introduced into the market. Of these carboxin (**1**) and oxycarboxin (**2**) (Table 13.3.1 below) are well known examples that are still used today [2].

The main use of carboxin is for seed dressing, predominantly against *Rhizoctonia* spp. in cereals and other crops [3, 4]. *Ustilago* spp. and *Tilletia* spp. can also be treated. Oxycarboxin is active against rust diseases in cereals, turf and ornamentals [4]. Two other early compounds have similar biological properties: Benodanil (**3**) and fenfuram (**4**), the latter still in use for seed dressing. These early achievements have spurred diverse research activities by many companies, resulting in several new products or developmental candidates.

13.3.2
Active Ingredients

Table 13.3.1 compiles the four best known older molecules along with six more recent structures.

The next generation of anilides consists of benzoic acid derivatives mepronil (**5**) and flutolanil (**6**). Both have very similar structures, differing by the fluorination of a methyl group only. The biological spectrum of these active ingredients introduced during the 1980s by Nihon Nohyaku (flutolanil) [5, 6] and Kumiai (mepronil) [7, 8] is similar to that of the earlier compounds, and application is possible via seed treatment, soil incorporation or foliar spray [4].

Similar to some of the early examples like fenfuram, the amide group is attached to a five-membered heterocycle in furametpyr (**7**) (Sumitomo Chemical

Table 13.3.1 Active ingredients.

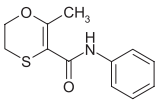
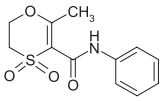
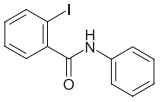
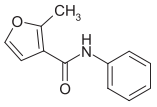
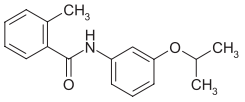
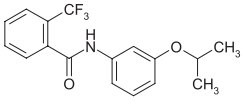
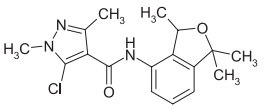
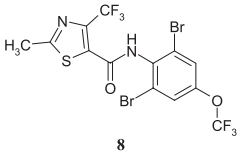
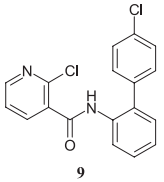
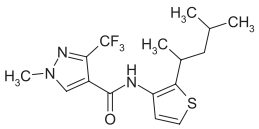
Common name Company Year of introduction Melting point (°C) K_{ow} (log P)	**Structure**
Carboxin (**1**) Uniroyal Chemical Co. [4] 1968 [43] 91–92 [4] 2.3 [4]	 **1**
Oxycarboxin (**2**) Uniroyal Chemical Co. [4] 1971 [44] 119.5–121.5 [4] 0.77 [4]	 **2**
Benodanil (**3**) BASF [4] 1974 [45] 137 [4] Not available	 **3**
Fenfuram (**4**) Shell (now Bayer CropScience) [4] 1974 [42] 109–110 [4] Not available	 **4**
Mepronil (**5**) Kumiai Chemical Industry Co. [4] 1981 [4] 92–93 [4] 3.66 [4]	 **5**
Flutolanil (**6**) Nihon Nohyaku Co. [4] 1986 [4] 104–105 [4] 3.7 [4]	 **6**
Furametpyr (**7**) Sumitomo [4] 1997 [10] 150.2 [4] 2.36 [4]	 **7**

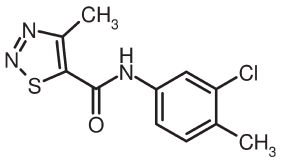
Table 13.3.1 *(continued)*

Thifluzamide (**8**) Monsanto (now Dow AgroSciences) [4] 1997 [4] 177.9–178.6 [4] 4.16 [4]	 **8**
Boscalid (**9**) BASF [4] 2003 [4] 142.8–143.8 [4] 2.96 [4]	 **9**
Penthiopyrad (**10**) Mitsui [4] Not yet introduced Not available Not available	 **10**

Co.) [9, 10] and thifluzamide (**8**) (Dow AgroSciences) [11, 12], which were introduced in the 1990s. The biology again focuses on the pathogens characteristic for this class of compounds, especially *Rhizoctonia* spp.

A structurally very similar molecule, tiadinil (**11**, Fig. 13.3.2), has been shown to have another mode of action as it is an activator of systemic acquired resistance and induces defense gene expression [13]. This illustrates that succinate dehydrogenase inhibitors cannot be recognized based on their structure alone.

The latest generation of succinate dehydrogenase inhibiting anilides consists of boscalid (**9**) (BASF) [14–16] and penthiopyrad (also known as MTF 753) (**10**) (Mitsui Chemicals) [17, 18]. Although these compounds can be considered to be closely related to the older molecules as far as their structures are concerned, their biological activity is very different. Boscalid is the first succinate dehydrogenase inhibitor, introduced into the market in 2003, to control ascomycetes on



11

Fig. 13.3.2. Tiadinil (**11**).

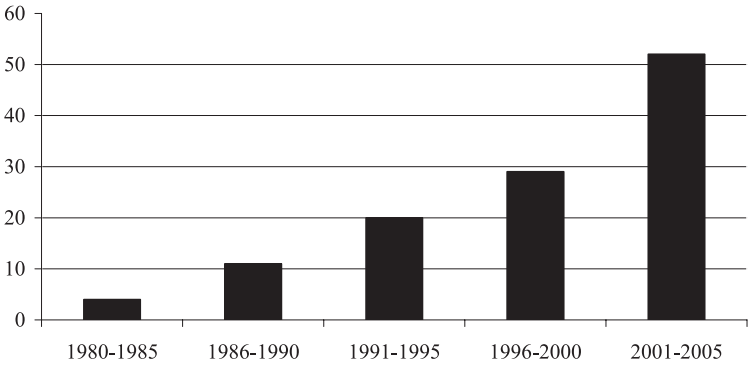


Fig. 13.3.3. Number of patent applications of active ingredients (mixtures, formulations and process related documents have not been included).

various fruits and vegetables. Penthiopyrad also is active against some pathogens of this group, in addition to the well-known *Rhizoctonia* spp.

These unexpected fungicidal activities have widened the scope of this class of compounds substantially. It can no longer be considered to be restricted to special pathogens.

13.3.3
Research Activities and Patent Situation

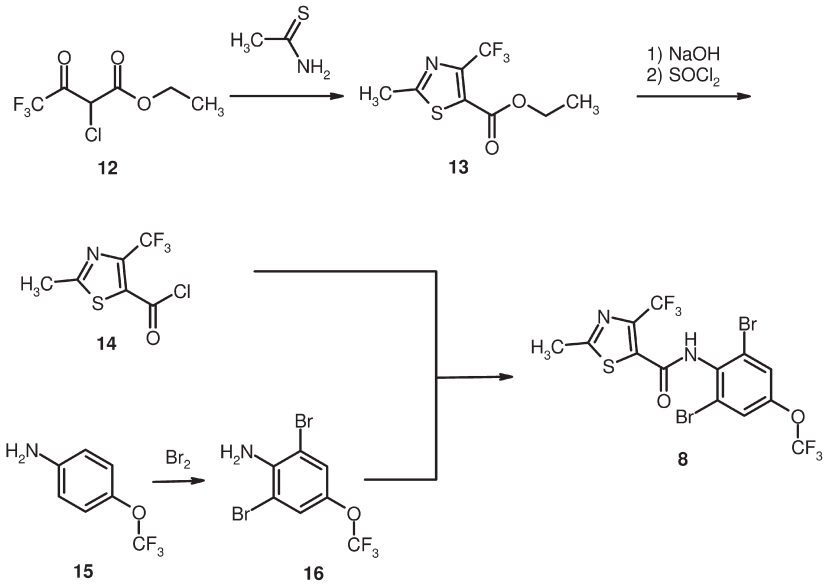
During recent years interest in these molecules has risen considerably. An increasing number of patents has been filed by many companies. An analysis has been made taking into account only those patent applications devoted to active ingredients (mixtures, formulations and process related documents have not been counted). Arranging these according to their years of publication shows the growing activities quite impressively (Figure 13.3.3). Starting from the early 1980s to the first five years of the new millennium have seen a ten-fold increase in the number of patents filed in this field.

This can be attributed to the discovery of the latest generation compounds with a substantially broader biological spectrum. More than 15 companies, including most major players and also many smaller competitors, have been involved in these efforts.

13.3.4
Synthesis

Although this class of molecules bears several striking structural similarities, the actual strategy for a large-scale synthesis depends on the specific heterocycles or aromatics involved, on their substitution pattern, and on the commercial availability of suitable precursors.

According to Alt et al. [12] thifluzamide (**8**) was prepared starting from the



Scheme 13.3.1

halogenated acetoacetate **12** and thioacetamide to yield the thiazole **13**. This intermediate was hydrolyzed and transformed into the chloride **14**, which finally gave the active ingredient **8**. The necessary aniline **16** was obtained by direct bromination of 4-trifluoromethoxyaniline (**15**, Scheme 13.3.1).

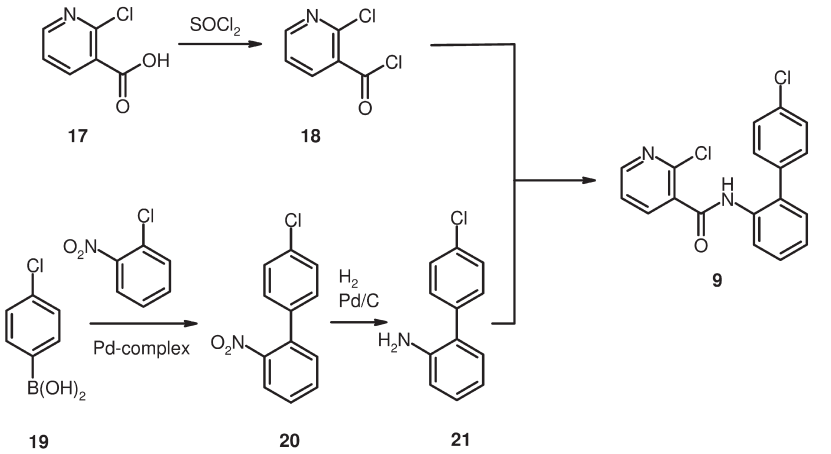
Boscalid (**9**) can be assembled starting from 2-chloronicotinic acid (**17**), the synthesis of which from 3-methylpyridine has been described [19]. The corresponding chloride **18** can then be reacted with the aniline **21** to obtain the desired product **9** [20]. The route from the boronic acid **19** via the 2-nitrobiphenyl **20** to **21** is the first example of the transfer of a palladium-catalyzed coupling reaction to large-scale agrochemicals synthesis (Scheme 13.3.2).

In both examples the heterocyclic carboxylic acid has been activated as the chloride to be combined with the aniline in a straightforward converging synthesis.

For penthiopyrad (**10**) a laboratory-scale synthesis has been published in which the branched alkyl residue on the thiophene ring was generated from an acetyl group by addition of methylmagnesiumbromide and subsequent reduction by triethylsilane [18].

13.3.5
Biological Activity and Application

Early succinate dehydrogenase inhibitors are active against basidiomycetes, above all against *Rhizoctonia* spp. This holds true for the next generations still. Thifluzamide is active against *Rhizoctonia* spp. on rice, potatoes, peanuts, strawberry, turf, wheat and cotton, *Ustilago* spp. and *Tilletia caries* on cereals, *Sclerotium* spp.,



Scheme 13.3.2

Hemileia vastatrix, and others. *Puccinia* spp. in cereals and peanuts can also be treated. This compound has preventative and curative as well as systemic activity. Formulations for seed treatment, foliar application, seedling boxes and paddy fields are known [21]. Compared to the early compounds the modes of application are apparently more diverse now (some of the older molecules were used as a seed dressing only).

The discovery of boscalid has broadened the biological spectrum of this class of compounds substantially as it is most active against ascomycetes, namely *Botrytis* spp. (vine, fruit, vegetables), *Sclerotinia* spp. (fruit, vegetables, coffee, rape seed, turf), *Alternaria* spp. (fruit, vegetables), *Phoma* spp. (rape seed, coffee), *Mycosphaerella* spp. (fruit, vegetables), *Monilinia* spp. (fruit), *Pseudocercosporella herpotrichoides* (cereals) and others, including *Rhizoctonia* in some crops [16]. Penthiopyrad also shows activity against some ascomycetes (*Podosphaera leucotricha*, *Venturia inaequalis* and *Botrytis cinerea*) and the basidiomycete *Rhizoctonia* [4, 18].

13.3.6 Structure–Activity Relationships

Several studies on structure–activity relationships of succinate dehydrogenase inhibitors have been published [22–28]. Each of the analyses has focused on specific carboxylic acid moieties of the molecule. The influence of substituents of the carboxylic acid and of the aniline has then been studied based on enzyme inhibition and biological data. Some empirical relationships have been established within each structural subclass. The importance of electron-withdrawing groups on the carboxylic acid and of lipophilic effects on the aniline has been observed. The orientation of the amide bond has also been discussed, suggesting that the cis configuration of the amide bond may be important in molecules with bulky ortho substituents [28].

Taking this into account, it can clearly be noted that the more recent com-

pounds **6**–**10** all have electron-withdrawing groups like trifluoromethyl or chlorine in the carboxylic acid part of the molecule. Compared with their predecessors, they also have more lipophilic substituents in the aniline. Of course, the complex agronomical implications cannot be derived from these analyses, as is illustrated by the shift from basidiomycete to ascomycete activity caused by subtle structural changes.

13.3.7 Resistance

Carboxin-resistant strains of *Mycosphaerella graminicola* have been shown to have an exchange of histidine at codon 267 for either tyrosine or leucine [29]. The strains involved in this study were artificially generated by UV-light mutagenesis. In resistant *Ustilago maydis* a similar replacement of histidine with leucine has been observed [30]. However, in *Coprinus cinereus* another variation (N80K) has been found [31].

Recently, carboxin-resistant *Ustilago nuda* has been reported from field applications in France and Italy [32]. This substance class has been regarded to be of medium risk of resistance and resistance management would be required if used for risky pathogens [33]. Interestingly, certain pathogenic strobilurin-resistant strains of *Alternaria solani* (having the F129L mutation in complex III) show an increased sensitivity towards boscalid [34].

Carboxin inhibits, to a different degree, succinate dehydrogenase of such diverse organisms as fungi, bacteria, plants and mammals, and the study of resistant mutants has contributed to understanding the mechanism of action [35]. Additional information stems from *Saccharomyces* and bacteria [36–38].

13.3.8 Metabolism

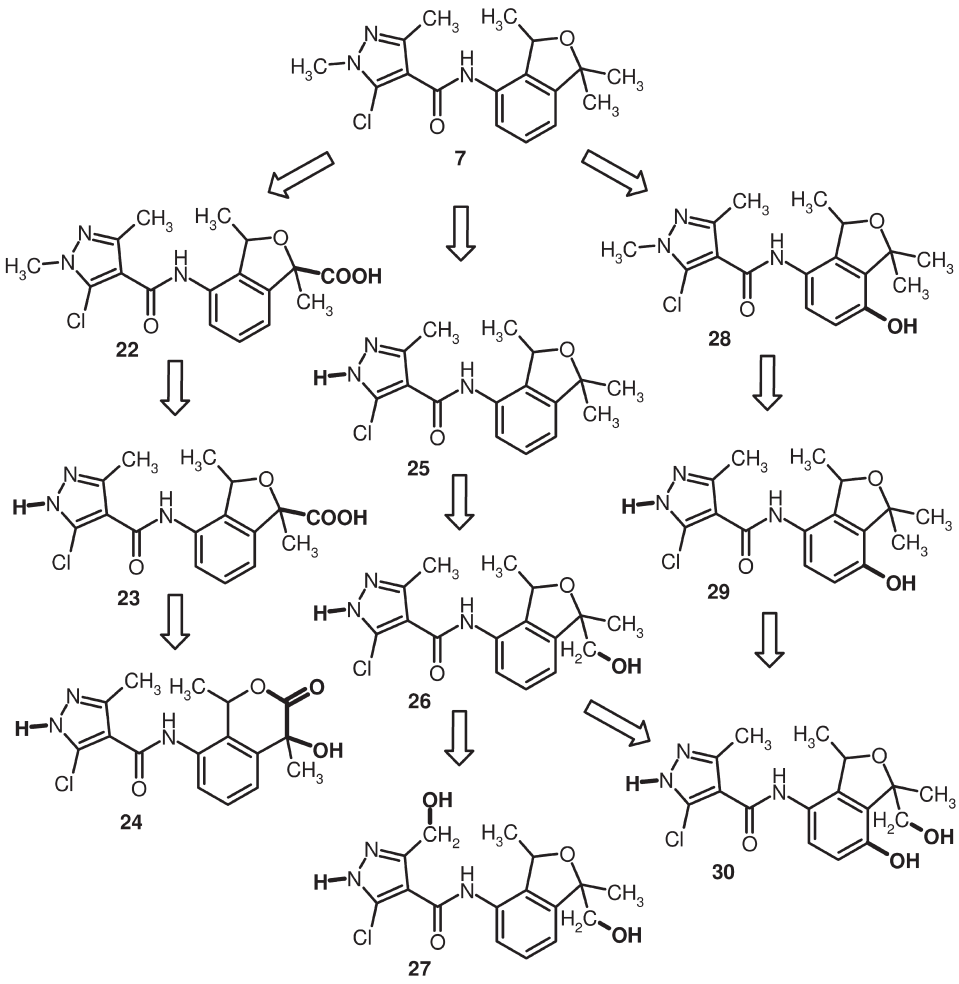
Metabolism of this compound class usually starts with hydroxylation of aromatic rings or alkyl or alkoxy groups. Cleavage of the amide bond takes place at a later stage in most cases [39]. Fourteen metabolites have been detected during a study of furametpyr (**7**) in mammals, some of which are shown in Scheme 13.3.3 [40, 41].

The most important biotransformations were N-demethylation (as seen in metabolites **23**, **25**, **29**), oxidation of methyl groups (**22**, **26**, **27**, **30**) and aromatic hydroxylation (**28**).

13.3.9 Discussion

Succinate dehydrogenase inhibitors of the anilide type are a fungicide class of agronomical and commercial importance. Although known for a long time, the biological scope has been widened significantly during recent years.

One of the reasons for the long-term research efforts is the relatively benign



Scheme 13.3.3 Metabolism of furametpyr in mammals.

toxicology profile with LD_{50} values of more than 1500 mg kg^{-1} for most land vertebrates [4]. This could not be anticipated as the target is present in mammals, too. (A comprehensive study comparing the inhibition of succinate dehydrogenase of various organisms by different anilides, and relating the results to the observed biological effects, has not yet been published.)

Another reason for the long-standing research efforts is the limited but promising fungicidal spectrum of the early examples combined with a flexible structure. This has motivated many companies to exploit various carbocyclic and heterocyclic systems decorated with numerous suitable substituents.

The understanding of the structure of the target and of the mechanism of action has increased considerably recently, as has the number of patent applica-

tions. This illustrates that scientific and commercial interests still focus on this subject.

Acknowledgments

Valuable comments and suggestions by Dr. J. Dietz, Dr. W. Grabarse, Dr. T. Grote and Dr. S. Strathmann, BASF Agrochemicals, are especially acknowledged.

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13.4
Uncouplers of Oxidative Phosphorylation

William G. Whittingham

13.4.1
Introduction

Previous chapters in this volume describe how inhibitors of the different complexes of the mitochondrial electron transport chain can produce potent antifungal effects.

As well as inhibition of the individual respiratory complexes, there are other ways in which the overall process of oxidative phosphorylation can be disrupted. One of the most effective of these is to disconnect, or uncouple, the electron

transport chain from ATP synthesis, thus allowing respiration to continue but preventing the conversion of metabolic energy into the ATP needed to operate cellular processes.

In its broadest sense, the term uncoupler has been used to describe any compound that prevents the synthesis of ATP (other than by direct inhibition of ATP synthase) but allows electrons to be accepted from NADH and succinate. Thus, menadione, which acts as an alternative acceptor for electrons, thereby diverting them from the full mitochondrial pathway, has been described as an uncoupler of oxidative phosphorylation [1]. However, for the purposes of this chapter, uncouplers are more precisely defined as compounds that break the link between the intact and functional electron transport chain and ATP synthase.

Several general reviews of uncouplers and the uncoupling process have been published [2–10].

13.4.2 Mechanism of Action of Uncouplers

The chemiosmotic theory, first proposed by Peter Mitchell in 1961 and now generally accepted, explains the coupling of respiration and ATP synthesis in terms of a proton gradient across the inner mitochondrial membrane [11–13]. The complexes of the respiratory chain accept electrons (originally from NADH and succinate) and use the energy released as they move down the potential gradient to transport protons across the membrane (see Chapter 13.1 for a more detailed discussion of this process). The resulting proton gradient and transmembrane potential drives the flow of protons through ATP synthase, and hence the production of ATP. Anything that dissipates the proton gradient across the membrane will lead to an uncoupling of oxidative phosphorylation by removing the protonmotive force required for the operation of ATP synthase. If this uncoupling occurs in an uncontrolled manner it has a drastic effect, eventually causing cell death. There are several ways in which uncoupling can be brought about.

Uncoupling proteins (UCPs) have been identified in many organisms [12, 14]. These allow a controlled flow of protons across the membrane and are thought to have several functions, including heat generation and control of the transmembrane potential.

Various chemicals cause uncoupling by increasing the permeability of the membrane to protons and other small ions. Detergents, which destabilize the membrane structure, can have this effect [15]. Several small molecules, including some ''classical'' uncouplers, have been described as affecting the membrane permeability transition (MPT), which causes a dramatic increase in the ability of ions to move across the membrane [9, 16, 17]. More specific effects that increase the permeability of the membrane to protons, as caused by the peptide gramicidin A, also lead to uncoupling [14, 18, 19].

Ionophores, such as valinomycin, can transport metal ions across the membrane. This effectively dissipates the transmembrane potential without altering the proton gradient. However, as the potential is the major driver for proton flow

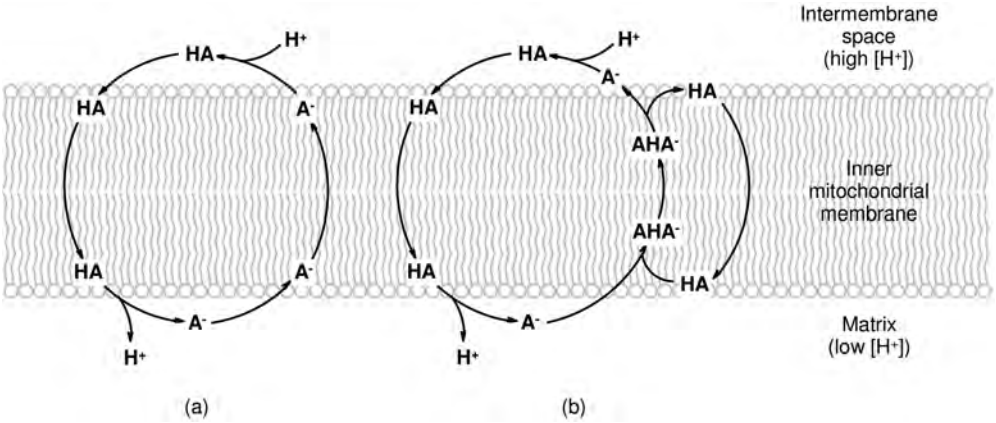


Fig. 13.4.1. Mechanism of proton transport for weakly acidic uncouplers: (a) monomolecular, (b) bimolecular.

through ATP synthase, this leads to a much reduced efficiency of coupling and results in similar effects to dissipation of the proton gradient [3, 19, 20].

Long-chain fatty acids also cause uncoupling. The mechanism appears complex, but seems to be distinct from either simple membrane disruption or a classical protonophoric effect, and appears to require the involvement of active transporters [9, 14, 19, 21–24].

A final class of uncouplers are compounds that transport protons across the membrane, leading to dissipation of the transmembrane proton gradient, and hence removing the proton-motive force that drives ATP synthase [25–27]. These are the only uncouplers of real significance so far as fungicide discovery is concerned.

The most common type of protonophoric uncouplers, sometimes referred to as classical uncouplers, are lipophilic weak acids. Figure 13.4.1(a) illustrates the simplest explanation of proton transport by these compounds. On the intermembrane side of the membrane, where the proton concentration is high, the compounds exist in their neutral, protonated form (HA). This is able to diffuse through the membrane to the mitochondrial matrix, where the pH is considerably higher. Under these conditions the proton is removed, and the resulting anion (A^-) is driven back across the membrane by the membrane potential, thus continuing the cycle. In this way, the uncoupler both dissipates the proton gradient, by proton transport from intermembrane space to matrix, and collapses the membrane potential, by negative charge transfer in the opposite direction. It produces this effect in a catalytic manner, with each molecule of uncoupler able to transfer many protons as it repeatedly proceeds through the cycle.

A limiting factor in the efficiency of the uncoupling cycle is the ability of the charged anionic form of the uncoupler to cross the lipophilic interior of the membrane bilayer. In some cases a bimolecular mechanism occurs, in which a neutral

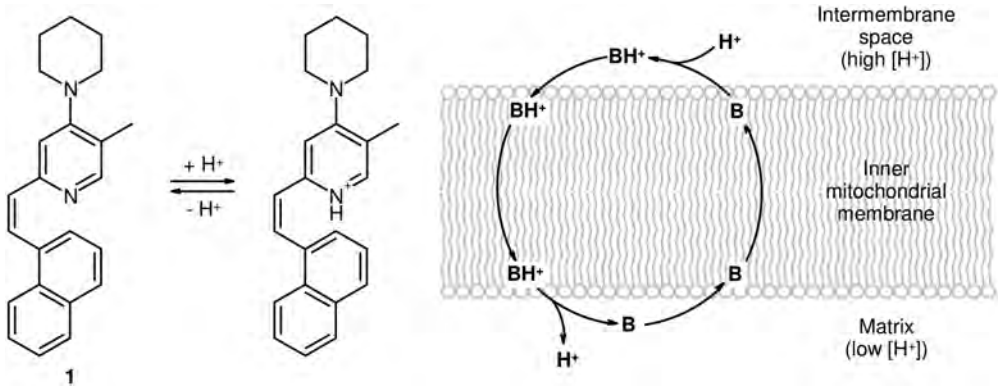


Fig. 13.4.2. Structure of AU-1421 (**1**) and general mechanism of proton transport for a weakly basic uncoupler.

molecule of the uncoupler associates with, and facilitates the movement of, the anion, presumably by increasing the delocalization of the negative charge [28]. This situation is illustrated diagrammatically in Fig. 13.4.1(b). If mixtures of uncouplers are present it has been suggested that they can interact to form heterodimers to facilitate movement across the membrane and hence act synergistically [29].

Although much less commonly observed, there have been several reports of lipophilic weak bases acting as protonophoric uncouplers, e.g., the pyridine AU-1421 (**1**) [30]. These compounds can transport protons as shown in Fig. 13.4.2. The weakly basic compound is protonated on the low pH intermembrane space side of the membrane, and the resulting cation (BH^+) crosses the membrane, driven by the transmembrane potential. In the less acidic matrix, the proton is removed, and the neutral uncoupler (B) can diffuse back across the membrane to continue the cycle.

13.4.3 Selectivity and Toxicity

By virtue of their mechanism of action, protonophoric uncouplers interact in a non-specific manner with membranes, rather than acting at a specific binding site. Because of this they also have the correct properties to interact with other membranes, both in the target organism and other species. For this reason selectivity is an issue for many compounds with this mode of action. Indeed, one of the first uncouplers to be commercialized over 100 years ago, dinitrocresol (DNOC **2**), was first used as an insecticide and later as a herbicide [31]. It has also been used as a fungicide on fruit trees and vines, and to break winter dormancy of fruit trees grown in warm climates [32], although it has now largely been replaced, due to toxicity.

In some cases the lack of selectivity can be beneficial. For example, the primary use of dinocap (**3**) is as a fungicide for control of powdery mildews, but it is also acaricidal and is used to suppress the populations of various mites [33]. In a similar way fluazinam (**4**), one of the most selective fungicidal uncouplers, has been shown to control some mites [34].

However, in general the lack of selectivity is undesirable and requires careful management. As an example, dinocap has detrimental effects on some beneficial insects, especially predatory mites [35–37]. Phytotoxicity can also be an issue on some crops [38–41] and ornamentals [42].

One particular manifestation of a lack of selectivity is toxicity to humans. Carefully controlled uncoupling mediated by uncoupling proteins is important for the regulation of cells, and uncoupling has also been suggested as a potential target for the treatment of obesity [43, 44]. However, the toxic effects of a natural weight-loss dietary supplement have been linked to the presence of the uncoupler usnic acid (**5**) [45]. Uncoupling has also been implicated in the toxic side effects of some non-steroidal anti-inflammatory drugs [46, 47].

Several toxic effects of fungicidal uncouplers have been observed, but it is not clear whether these are directly related to the uncoupling mode of action or to other, compound-specific mechanisms. Some uncouplers have a very high acute toxicity, which is almost certainly a result of the mode of action. This has been exploited in the case of bromethalin (**6**), which is used as a rodenticide. Another toxic effect that has been observed for several distinct structural types of uncoupler, and hence is likely to be a direct result of this mode of action, is oedema of the central nervous system [48]. Direct interaction with the myelin sheath membrane is probably responsible for this toxicity.

Several other toxic effects have been observed for specific fungicidal uncouplers, but these are more likely to be related to specific structural features of the compounds rather than the uncoupling mode of action. Fluazinam is a skin sensitizer that can cause allergic contact dermatitis [49–51] – an effect that is most likely to be a result of thiol reactivity rather than uncoupling properties. Several toxic effects have been seen with dinocap, most notably potent teratogenicity in mice [52, 53]; although the technical material is not teratogenic in rats or hamsters [54, 55]. Two pure regioisomers of the active ingredient do not cause teratogenic effects in mice [56], and the toxicity appears to be linked to a single isomer [57] and hence is due to a more specific effect than uncoupling.

Various strategies have been employed to minimize the potential toxicity and lack of selectivity of uncouplers. Several commercial compounds are sold as propesticides of the parent molecule, so that the active uncoupler is liberated only after metabolism. These include the insecticide chlorfenapyr (**7**; see Chapter 28.2 for a detailed description of this compound) and the fungicides dinocap (**3**) and binapacryl (**8**). This approach can be highly successful in reducing acute toxicity: dinocap has an acute oral LD_{50} of 265 mg kg^{-1} in mice, whereas the LD_{50} for its active phenolic metabolite is 29 mg kg^{-1} [58]. In this case, the active uncoupler is released by the action of esterases in the target organism but hydrolysis is slow in the acidic environment of the mammalian stomach (half-life of 229 days at pH 5

[59]). A second successful approach is to design compounds that can be readily metabolized in non-target organisms. An example is fluazinam (**4**), which reacts rapidly with glutathione and other thiols and is thus rapidly detoxified in mammals (Section 13.4.8).

13.4.4 Resistance

The development of resistance is a major issue for many classes of fungicide, including those that inhibit respiration. However, uncouplers appear to be less susceptible to the onset of resistance than many other fungicides. For example, a study of resistance development in *Sphaerotheca fuliginea* in cucumber greenhouses observed no resistance to dinocap, despite it having been used for over 30 years [60]. This was in contrast to other classes of fungicide, such as benzimidazoles, where resistance had developed over a much shorter period. Even under artificial selection pressure, no resistance to dinocap was seen [61].

Although it has not been used for such an extended period, the situation for fluazinam appears to be similar. Thus, despite being used for several seasons with up to 10 applications per season and no mixture partners, there is no indication of a change in sensitivity of *Phytophthora infestans*, despite the known ability of this pathogen to develop resistance to other classes of fungicide [62]. Similar studies of *Botrytis cinerea* in French vineyards have revealed no resistance development after several years of use [63–65]. The same situation was reported for *Botrytis* in Japan [66], but more recently there has been a report of a resistant isolate being found after 10 years repeated use in bean crops [67]. This isolated report notwithstanding, uncouplers are classed by FRAC (FRAC code 29) as having low resistance risk [68], and they are often used to control fungi that have developed resistance to other fungicides [69–73].

The reasons for the low resistance risk are not completely understood. As protonophoric uncouplers do not act at a specific binding site on a protein, the possibility of a single point mutation at the binding site giving rise to resistance does not exist. It is less clear why other possible resistance mechanisms, such as metabolism or active transport, do not seem to operate. However, uncouplers are often used to block the operation of multiple drug resistance pumps in biochemical experiments [74–76]. It may therefore be that the action of uncouplers in depleting cellular ATP also prevents the operation of energy-dependent resistance mechanisms. Notably, the ABC transporter from *Aspergillus nidulans* has been shown to have some effect in reducing the toxicity of fluazinam to this organism, although treatment with the arylhydrazone uncoupler CCCP (**9**) reduced the effectiveness of this efflux pump [77].

Although little resistance to uncouplers has been observed in fungi, several bacteria are known that have reduced sensitivity to protonophoric uncouplers [78–80]. The likelihood of similar resistance mechanisms developing in fungi is unknown, but is probably small, as no such mutations have yet been observed.

13.4.5
Physicochemical Properties of Protonophoric Uncouplers

Detailed mathematical studies of the relationship between uncoupling potency and physicochemical properties have been published for several classes of uncoupler, including phenols [81–89], arylhydrazones [90, 91], salicylanilides [81, 92, 93], pyrroles [94], benzimidazoles [81], coumarins [95, 96] and diarylamines [97–99]. Although the details of these analyses differ, there are several physicochemical parameters that are common to all and therefore appear to be the key properties required for uncoupling. These are acid strength, lipophilicity, and often a measure of the steric bulk of substituents adjacent to the acidic centre [94].

To act as an efficient protonophoric uncoupler a weakly acidic compound must have properties that allow it, in both the uncharged protonated and the anionic deprotonated forms, to enter and cross the membrane lipid bilayer. The compound must have a suitable pK_a such that on the more acidic, intermembrane side of the membrane a significant proportion is protonated, whilst on the less acidic matrix side a proportion is deprotonated. A compound that is not acidic enough may transfer a single proton across the membrane, but will not release it in the matrix, and hence cannot repeat the cycle. In contrast, too strong an acid will remain deprotonated even in the intermembrane space. For this reason 2,4-dinitrophenol (pK_a 4.04) is a stronger uncoupler than 2-nitrophenol (pK_a 7.14); and picric acid (pK_a 0.53) does not act as a protonophoric uncoupler in mitochondria, although all three compounds have a similar lipophilicity [88].

To efficiently cross the membrane, uncouplers must be reasonably lipophilic (generally log $P > 2$). However, this alone is not sufficient. A key part of the action of a protonophoric uncoupler is the ability to cross the membrane in the anionic form, and this requires the negative charge to be extensively delocalized, or shielded from the lipid interior of the membrane in some way. For this reason, many of the most potent uncouplers combine bulky lipophilic groups adjacent to the ionizable proton with extended conjugated systems through which the charge can be spread. A typical example of this is malonoben (**10**), one of the most potent phenolic uncouplers known (Fig. 13.4.3) [100].

However, many lipophilic carboxylic acids satisfy the log P and pK_a criteria for uncoupling, but the charge on the anion cannot be delocalized beyond the carboxyl group and so they do not act as effective protonophoric uncouplers. Those that do have structural features that allow the charge to be delocalized include the anacardic acids, where the adjacent phenol stabilizes the negative charge (Fig. 13.4.4a) [101]. A similar intramolecular interaction occurs in the salicylanilide class of uncouplers, of which one of the most active is S-13 (**11**, Fig. 13.4.4b).

In some cases a bimolecular mechanism allows the anion to cross the membrane, as described in Section 13.4.2. In this case the negative charge is delocalized across two molecules of uncoupler. Certain benzimidazoles, e.g., TTFB (**12**), function in this way (Fig. 13.4.5) [2, 4, 28].

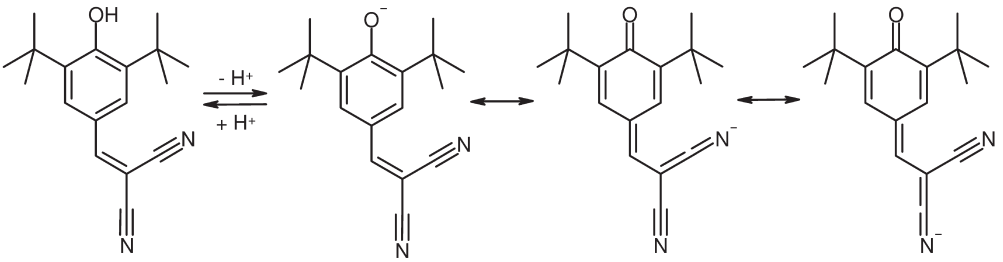


Fig. 13.4.3. Delocalization and lipophilic shielding of charge on the anion of malonoben (**10**).

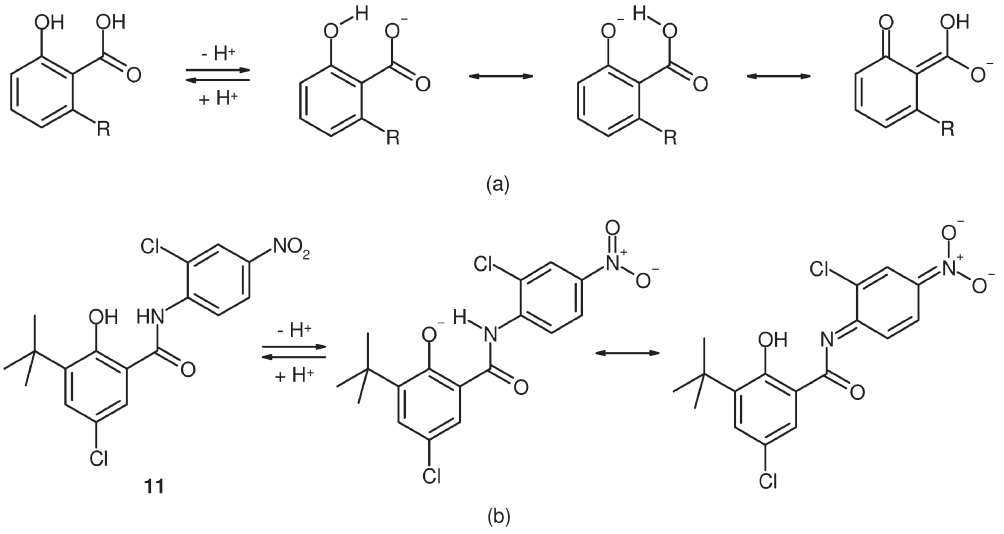


Fig. 13.4.4. Delocalization of anionic charge by an adjacent group in (a) the anacardic acids and (b) salicylanilide S-13 (**11**).

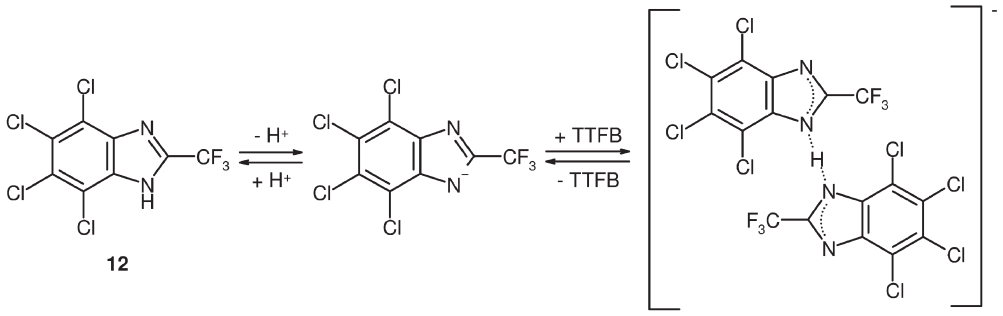


Fig. 13.4.5. Bimolecular delocalization of negative charge, illustrated for TTFB (**12**).

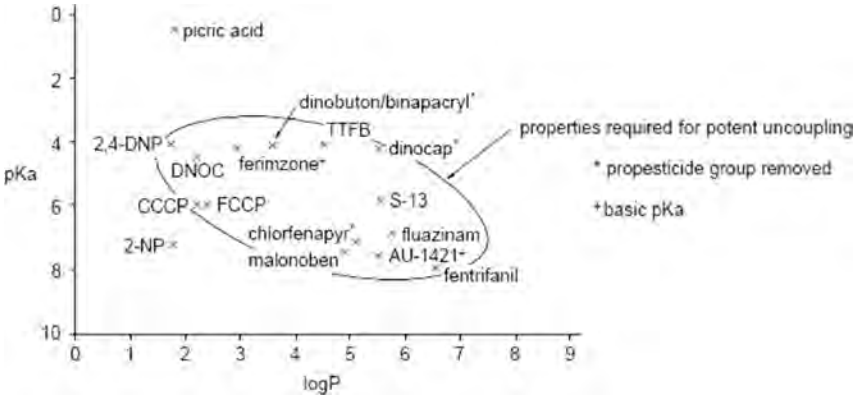


Fig. 13.4.6. Combination of log *P* and pK_a required for uncoupling activity.

It is not possible to formulate a simple set of rules that can accurately predict from physicochemical properties whether or not a compound will be an efficient uncoupler. However, some general guidelines for combinations of log *P* and pK_a that can lead to uncoupling can be devised and these are illustrated in Fig. 13.4.6, with some selected examples marked [102]. Thus, the compound should be weakly acidic, with a pK_a between 4 and 8, and have a moderately high log *P*. Notably, the properties of the basic uncoupler AU-1421 (**1**) also fall within this range (log *P* 5.5, basic pK_a 7.4) [30].

As already mentioned, this is a rather simplistic view, and the importance of charge delocalization and steric effects should not be overlooked.

13.4.6
Chemical Uncouplers

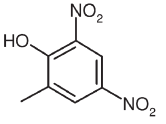
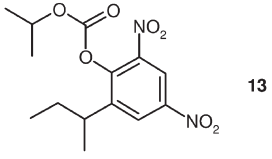
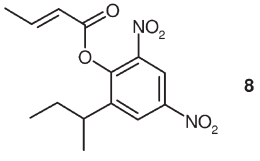
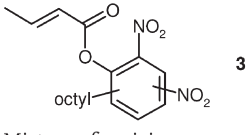
Clearly, from the discussion above, many different classes of chemistry could theoretically satisfy the physicochemical requirements for uncoupling. This is also the case in practice, with a wide range of different chemical types showing fungicidal (and wider agrochemical) effects. Table 13.4.1 lists a selection of the most important of these, along with some key references.

Although all of the chemical classes in Table 13.4.1 have activity against plant pathogenic fungi, commercially three are of particular interest: dinitrophenols, arylhydrazones and diarylamines.

The dinitrophenols were the first group of uncouplers to be commercialized, and representatives of this group have found use as herbicides, insecticides/acaricides and fungicides. However, several have been superseded due to their toxicity and lack of selectivity.

The most significant of the dinitrophenols still used as a fungicide is dinocap, marketed by Dow under the trade name Karathane. This compound is sold as a

Table 13.4.1 Structures of selected fungicidal uncouplers.

Chemical class	Structure	Trivial name (major trade names)	Commercial uses	Notes	Key references
Dinitrophenols	 **2**	DNOC (Trifocide)	Fungicide for top fruit and vines. Herbicide, insecticide	No longer used	31, 103
	 **13**	Dinobuton (Acrex)	Powdery mildews, acaricide	Pro-pesticide of active phenol (dinoseb)	104, 105
	 **8**	Binapacryl (Acricid)	Powdery mildews, acaricide	Pro-pesticide of active phenol (dinoseb); largely superseded	106
	 **3** Mixture of regioisomers (see below)	Dinocap (Karathane)	Powdery mildews, acaricide	Pro-pesticide of active phenol	106

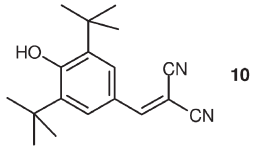
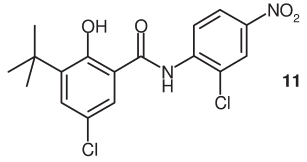
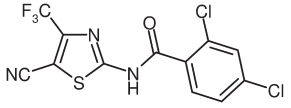
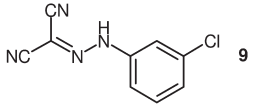
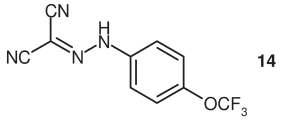
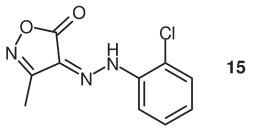
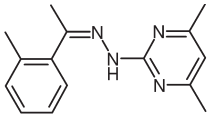
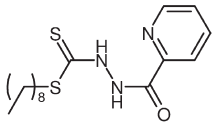
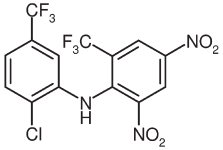
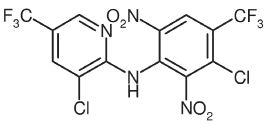
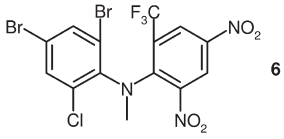
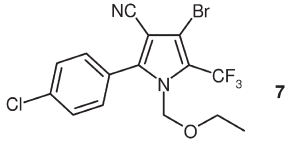
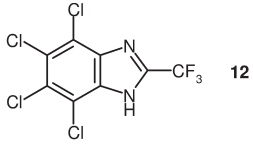
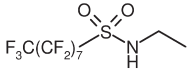
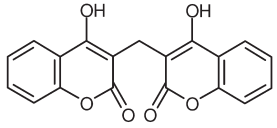
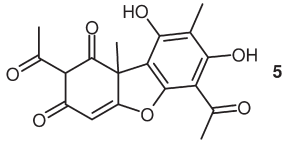
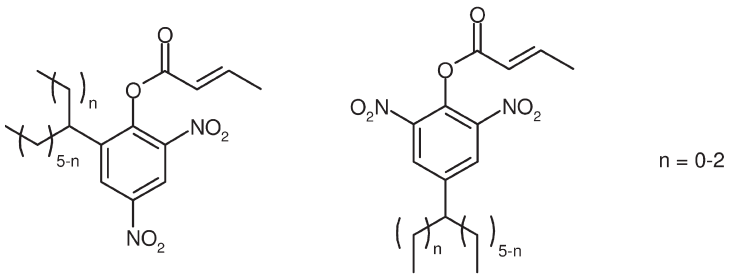
Other phenols	 **10**	Malonoben, SF6847	Acaricide	No longer used commercially. Commonly used biochemical standard uncoupler	107–110
Salicylanilides	 **11**	S-13	Not commercialized	Commonly used biochemical standard uncoupler	111, 112
Other amides			Not commercialized		113
Arylhydrazones	 **9**	CCCP	Not commercialized	Commonly used biochemical standard uncoupler	114–116
	 **14**	FCCP	Not commercialized	Commonly used Biochemical standard uncoupler	114
	 **15**	Drazoxolon (Milcol)	Broad spectrum fungicide	No longer used	117

Table 13.4.1 *(continued)*

Chemical class	Structure	Trivial name (major trade names)	Commercial uses	Notes	Key references
	 **16**	Ferimzone (Blasin)	Rice diseases	See Section 13.4.7	118–121
Dithiocarbazates		PDTC-9	Not commercialized		122–124
Diarylamines	 **17**	Fentrifanil	Acaricide; not commercialized	See Chapter 28.2 for more details	125
	 **4**	Fluazinam (Shirlan, Frowncide)	Broad spectrum fungicide	See Section 13.4.8	72, 126

	 **6**	Bromethalin (Rampage)	Rodenticide	Pro-pesticide See Section 13.4.8	127
Nitrogen heterocycles	 **7**	Chlorfenapyr (Pirate)	Insecticide	Pro-pesticide See Chapter 28.2 for more details	128–130
	 **12**	TTFB	Not commercialized	Commonly used biochemical standard uncoupler	131
Fluorinated sulfonamides		Sulfluramid (Fluramim)	Domestic insecticide	Pro-pesticide; see Chapter 28.2 for more details	132, 133
Coumarins		Dicoumarol	Not commercialized	Commonly used biochemical standard uncoupler	95, 134
Natural products	 **5**	Usnic acid	Not commercialized	Present in natural weight-loss dietary supplement	135



18 **19**

Fig. 13.4.7. Composition of commercial dinocap.

roughly 2:1 mix of regioisomers **18** and **19**, each of which contains a mixture of three isomers of the octyl group (Fig. 13.4.7).

Dinocap is used as a powdery mildewicide on a range of crops, including vines, fruit, vegetables and ornamentals [136]. It also has secondary effects as a suppressor of some mites, and a recent patent claims it to be active against whitefly [137]. To provide a good level of powdery mildew control, repeat spraying at intervals of 7 to 14 days is required, up to a maximum of five to ten treatments a year, depending on the crop [40]. Despite this type of repeated use over many years, no indication of resistance has been found, clearly demonstrating the low resistance risk with the uncoupling mode of action [60]. The reason that dinocap is only effective against powdery mildews is not clear. However, this is also true of the other two fungicidal dinitrophenol pro-pesticides, dinobuton (**13**) and binapacryl (**8**), which suggests that it may be due to either the uptake or cleavage of the pro-pesticide. Interestingly, dinobuton and binapacryl are both pro-pesticides of the commercial herbicide dinoseb, so the properties of the pro-pesticide group are clearly important for selectivity.

Although this class of chemistry has been well known for many years, work on related compounds still continues. A recent example is the arylnitrophenols disclosed by Valent [138].

The other two major classes of fungicide that have produced commercial products are discussed in the following sections.

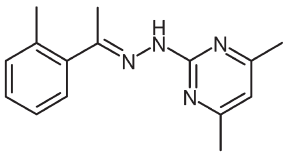
13.4.7
Arylhydrazones, including Ferimzone

Hydrazones prepared by the reaction of aryldiazonium salts with malononitrile (trivially named carbonyl cyanide phenylhydrazones, or CCPs) have been known as uncouplers for many years [114]. Studies on the relationship between physicochemical properties and uncoupling potency have been reported [90, 91], and these show that this relationship is very similar to those for other classes of weakly acidic protonophoric uncouplers. Thus, two of the most potent uncouplers of

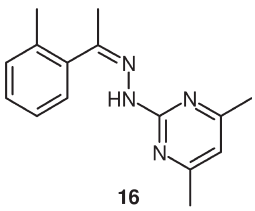
this type, CCCP (**9**) and FCCP (**14**), have an acidic pK_a of 6.0 and log *P*s of 2.19 and 2.42 respectively [90, 91]. Although these compounds are potent uncouplers in mitochondria from various organisms, including mammals, plants and insects [115], they have not found any agrochemical application. They are, however, commonly used as biochemical standards for uncoupling [74, 139].

Replacement of the malononitrile group with an isoxazolone group leads to a well known class of fungicides, of which one member, drazoxolon (**15**), has been commercialized by ICI under the trade name Milcol. This compound was used to control powdery mildews and other diseases on a range of crops and ornamentals, and as a seed treatment for control of *Pythium* and *Fusarium* species, but has now been replaced. The fungicidal effects of drazoxolon and analogues have been shown to be a result of its uncoupling activity [117].

A more distantly related series of pyrimidinylhydrazones was discovered, through random screening, to have high levels of fungicidal activity [140]. A program of analogue synthesis around the original lead culminated in the identification of meferimzone (**20**) as a promising fungicide [140].



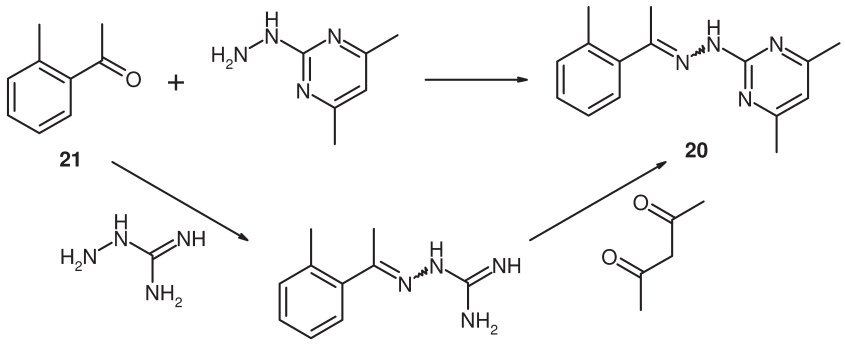
20 is a mixture of (*E*)- and (*Z*)-isomers



16

Although the compound containing the (E)-isomer of the double bond is more active when tested against fungi growing in media, there is little difference between the isomers when tested on whole plants, presumably because isomerization of the double bond occurs under the test conditions [118, 141]. Further work resulted in the development of the pure (Z)-isomer **16**, named ferimzone, as this is more stable than the (E)-isomer [142]. This compound was first commercialized by Sumitomo in 1991 under the trade name Blasin, and is used for the control of a range of diseases of rice, including *Pyricularia oryzae*, *Helminthosporium oryzae* and *Cercospora oryzae* [121]. Ferimzone has been shown to be fungistatic rather than fungicidal, and is unusual amongst uncouplers in providing curative as well as protectant activity. It is also noteworthy that, although it does not act as a pro-pesticide, ferimzone has a low acute toxicity compared with many typical uncouplers, with an acute oral LD_{50} for rats > 600 mg kg^{-1}. It is also environmentally friendly, with low toxicity to birds (acute oral LD_{50} for mallard ducks $>$ 292 mg kg^{-1}), fish (96 h LC_{50} for carp 20 mg L^{-1}) and bees (oral $LD_{50} > 140$ μg per bee), and short persistence in soil (DT_{50} 3–14 days) [143].

Two main synthetic routes have been used for the synthesis of ferimzone and its analogues (Scheme 13.4.1) [118, 140, 141]. Both start with 2′-methylacetophenone (**21**), and a new method for the industrial synthesis of this key intermediate for ferimzone has been developed [144].



Scheme 13.4.1. Synthetic routes to meferimzone (**20**).

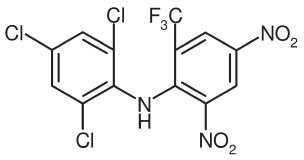
Although ferimzone is described as an uncoupler in the *Pesticide Manual* [143], and is classified as such by FRAC [68], its properties are somewhat different to the other protonophoric uncouplers described in this chapter. Most notably, ferimzone is non-acidic: the acidic pK_a of the hydrazone is about 14 [145]. However, it is weakly basic with a pK_a of 4.16 [146]. So it is possible that ferimzone is a rare example of a weakly basic protonophoric uncoupler. Several mode of action studies have been reported, and these clearly indicate that ferimzone has an effect on fungal membranes, increasing their permeability, and that this is likely to be responsible for the compound's antifungal activity [119, 120]. However, the precise details of the primary mode of action remain unclear. In field trials ferimzone shows unexpected antibacterial and antiviral activity, which is not seen in *in vitro* tests. Further testing has demonstrated that these effects are the result of a secondary activity of ferimzone in inducing systemic acquired resistance in plants [147, 148]. It therefore seems likely that the combination of effects on fungal membranes, with resultant uncoupling, and the induction of plant defense responses is responsible for the fungicidal activity of ferimzone.

13.4.8
Diarylamines, including Fluazinam (4)

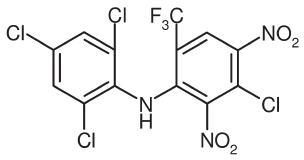
Diarylamines have been utilized as non-steroidal anti-inflammatory drugs (NSAIDs) for many years. One side-effect of this class of drugs is liver toxicity, due to uncoupling effects [46]. Structure–activity studies have shown that very potent uncoupling activity can be achieved in this chemical class [98]. These compounds have therefore also attracted attention as potential agrochemicals, and early examples proved to be good acaricides [149, 150], including the development compound fentrifanil (**17**) [125]. However, these compounds suffered from toxic effects, including severe acute toxicity and brain oedema [48], which prevented their commercialization. One positive spin-off from this work is bromethalin (**6**) which, despite being a pro-pesticide, shows such high acute toxicity that it has been commercialized as a rodenticide [127].

Further exploration of this area of chemistry by Ishihara resulted in the discovery that arylaminopyridines are also potent uncouplers [98]. These compounds possess moderate acaricidal activity, but are better fungicides than the diarylamines. Optimization of this class of chemistry resulted in the discovery of fluazinam (**4**) [151]. Not only is fluazinam one of the most potent uncouplers known [99, 126], it also shows a high level of reactivity with thiols, the chlorine atom on the highly electron-deficient phenyl ring being readily displaced [126, 152]. It is likely that the potent, broad-spectrum antifungal activity of fluazinam is a result of the combination of these two modes of action [153, 154].

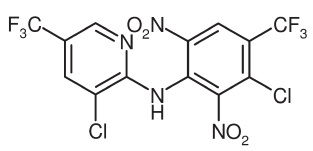
Fluazinam is unique amongst uncouplers in combining potent, broad-spectrum fungicidal activity with very low mammalian toxicity. The low toxicity is a result of the high thiol reactivity of the compound, which leads to rapid metabolism and consequent detoxification in mammals [155]. A similar safening effect has been observed in the diarylamine chemical series. Compounds **22** and **23** are potent uncouplers with similar physicochemical properties. However, **22** is highly toxic to mice (oral LD_{50} 0.9 mg kg^{-1}), whereas **23**, which contains a displaceable chlorine atom and reacts rapidly with thiols, is much safer (oral LD_{50} in mice $>$ 100 mg kg^{-1}) [155].



22



23



4

Fluazinam's unique combination of properties has resulted in its worldwide commercialization by Ishihara and Syngenta under various trade names, including Shirlan and Frowncide, and it is by far the most commercially important of the fungicidal uncouplers discovered to date.

Fluazinam is an excellent protectant fungicide, but has no systemic and little curative activity [156, 157], although it does have some anti-sporulant effect [156, 158]. It shows good residual activity and has excellent rainfastness [72, 156, 159, 160]. Fluazinam inhibits several stages of the fungal lifecycle, including spore germination and the formation of infection structures [158, 161]. It also has a very potent inhibitory effect on the motility of zoospores of *Phytophthora* species [159, 162].

Although fluazinam has a broad spectrum of fungicidal activity it is less potent on rusts and powdery mildews [72, 156] (in contrast to the dinitrophenols, which are generally most effective against powdery mildews) and has not been commercialized for use on cereal crops. It has, however, found wide application in other crops since its first launch in New Zealand in 1988.

Fluazinam is extensively used in potatoes for the control of late blight (*Phytophthora infestans*). It is especially effective against tuber blight as the potent activity against zoospores prevents them moving through the soil to infect the tubers

[156, 159, 163–165]. In this market, it is sprayed up to ten times per season, at 7 to 14 day intervals, depending on the severity of the outbreak [166–169]. Despite this intensive use as a stand-alone product, there is no indication of resistance to fluazinam developing [62] and it is especially useful for the control of *Phytophthora* that has developed resistance to other fungicides [72]. It also has some effect against other potato diseases [170, 171].

Fluazinam is also useful for the control of *Sclerotinia* sp., notably on peanuts [172–178] and turf [179]. It can be used against *Botrytis* on a wide range of crops, especially beans [66, 180], and grapes [63–65, 181], where use has been shown to significantly reduce the levels of ochratoxin present [182, 183].

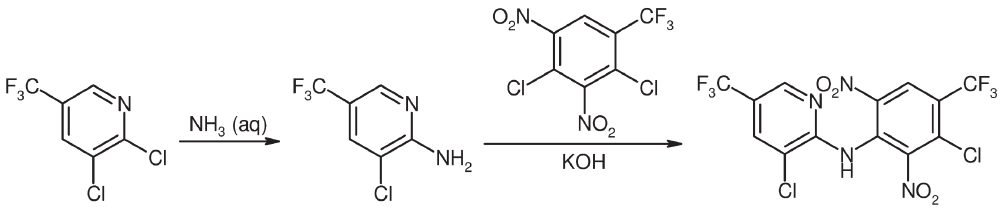
As well as use as a foliar spray, fluazinam is effective against soil-borne diseases. It has low mobility in soil [184, 185], and in some cases seed treatment has proved to be more effective than soil application [186]. Fluazinam is particularly effective for the control of brassica clubroot (*Plasmodiophora brassicae*) [187–192] and can be used against root rots in various crops [169, 193–198]. It can also be used to protect stored crops, e.g., chicory [199].

In addition to its use in crops, fluazinam is employed for the treatment of ornamental plants [200–202], and is particularly effective for the treatment of bulbs [203, 204].

As already mentioned, the thiol reactivity of fluazinam results in very low mammalian toxicity, with an acute oral LD_{50} for rats of >5000 mg kg^{-1} [205]. However, its thiol reactivity does create some issues and is likely to be the cause of the skin sensitization effect of fluazinam. In cases of repeated exposure this can result in the development of allergic contact dermatitis in sensitive individuals [49–51].

Fluazinam has low toxicity to birds (acute oral LD_{50} for mallard ducks 4190 mg kg^{-1}), bees (contact $LD_{50} > 200$ μg per bee), and worms (28 day $LD_{50} > 1000$ mg kg^{-1}) [206]. It does have high toxicity to aquatic organisms (e.g., the LC_{50} over 96 h for rainbow trout is 0.11 mg L^{-1}) [205]. However, it has a very short persistence (half-life approximately 1 day) in aquatic systems [207], and in a microcosm study based on realistic use rates in tulip cultivation it was found to have little or no adverse effect [207, 208].

Fluazinam is easily prepared from trifluoromethylpyridine intermediates that are used for the synthesis of other agrochemicals (Scheme 13.4.2) [151, 209].



4

Scheme 13.4.2. Synthetic route to fluazinam (**4**).

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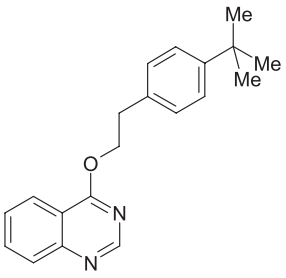
13.5
NADH-Inhibitors (Complex I)

Harald Walter

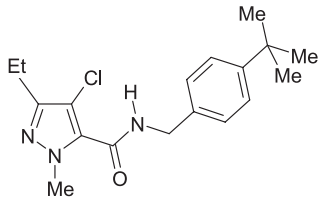
13.5.1
Introduction

Complex I inhibition is an important mode of action of pharmaceuticals and agrochemicals [1] (for a general introduction see Chapter 13.1). In the crop

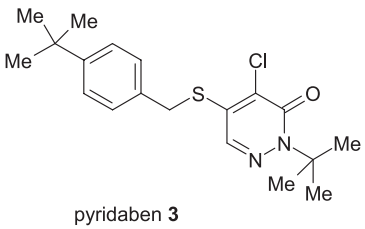
protection area mainly insecticides and acaricides with useful properties have been investigated in the last 15 years. Compounds such as fenazaquin (**1**), tebufenpyrad (**2**) and pyridaben (**3**) are examples of commercialized insecticides/acaricides (see Chapter 28.3):



fenazaquin **1**

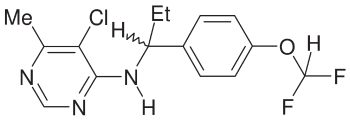


tebufenpyrad **2**



pyridaben **3**

Agrochemical fungicides with useful potency, spectrum and toxicological properties that are interesting enough for commercialization are rare. Only one compound, diflumetorim (**4**) (trade name: pyricut) [2], has been introduced into the market in the last 15 years (Fig. 13.5.1).



diflumetorim **4**

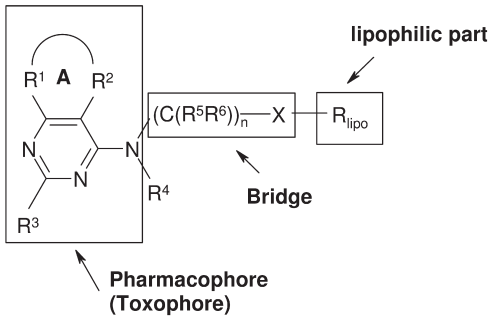
racemic

Fig. 13.5.1. Structure of diflumetorim (**4**).

Diflumetorim (**4**) is mainly used in ornamentals (Japan) with estimated sales of only 4–5 million US$ in 2004/05 (Cropnosis database) and has been sold and marketed by SDS Biotech since the beginning of 2003 [3]. This product will be discussed in more detail later in this chapter.

Diflumetorim (**4**) belongs to the class of the aminoalkylpyrimidines, the most interesting class of fungicides of the complex I mode of action (Fig. 13.5.2).

Although all the major companies pursued this lead intensively, only Ube Industries was successful in developing a commercial compound. No broad spectrum fungicide with the complex I mode of action has been found to date, despite the discovery of many compounds with a broad spectrum of activity (e.g., powdery mildew, brown rust and leaf spot diseases) and reasonable application rates in the greenhouse.



general aminoalkypyrimideine formula **I**

R^1, R^2 = C_1-C_4-alkyl, C_1-C_4-haloalkyl, C_1-C_4-alkoxyalkyl, halogen;
R^1 and R^2 together may also form an aromatic or nonaromatic 5-7 membered ring system A (e.g. phenyl, thienyl, cyclohexyl etc.)

R^3 = H, Me

R^4 = H, C_1-C_4-alkyl, COC_1-C_4-alkyl etc.

R^5, R^6 = H, C_1-C_4-alkyl

R_{lipo} = phenyl, phenoxyphenyl, benzylphenyl,naphthyl, tetrahydronaphthyl, indanyl, benzothienyl, benzofuryl, benzoxazinyl, quinolinyl, tetrahydrochinolinyl, cyclopentyl, heteroatom containing nonaromtic 5-membered ring systems, cyclohexyl, heteroatom containing nonaromatic ring systems

X = direct bond or O

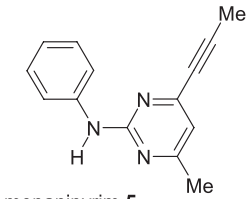
n = 0-3

(only most important substituents given)

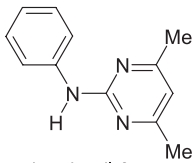
Fig. 13.5.2. General structure of the class of aminoalkylpyrimidines.

13.5.2
The Aminoalkylpyrimidine Class [4]

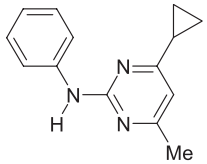
Fungicides containing pyrimidine moieties with relevant use in agriculture have been known for over 30 years. The most prominent compounds belong to the anilinopyrimidine class. Mepanipyrim (**5**), pyrimethanil (**6**) and cyprodinil (**7**) are the most important examples of this class (see Chapter 14.2):



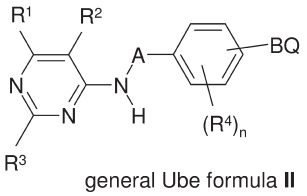
mepanipyrim **5**



pyrimethanil **6**



cyprodinil **7**



general Ube formula **II**

R^1, R^2 = C_1-C_4-alkyl, halogen etc.
R^3 = H, C_1-C_4-alkyl
R^4 = H, C_1-C_4-alkyl, halogen
A = lower alkylene
B = direct bond, O, S, lower alkylene or alkyleneoxy
Q = phenyl, heterocycle, substituted or unsubstituted alkyl, allyl, geranyl, farnesyl, glycidyl, acetonyl etc.

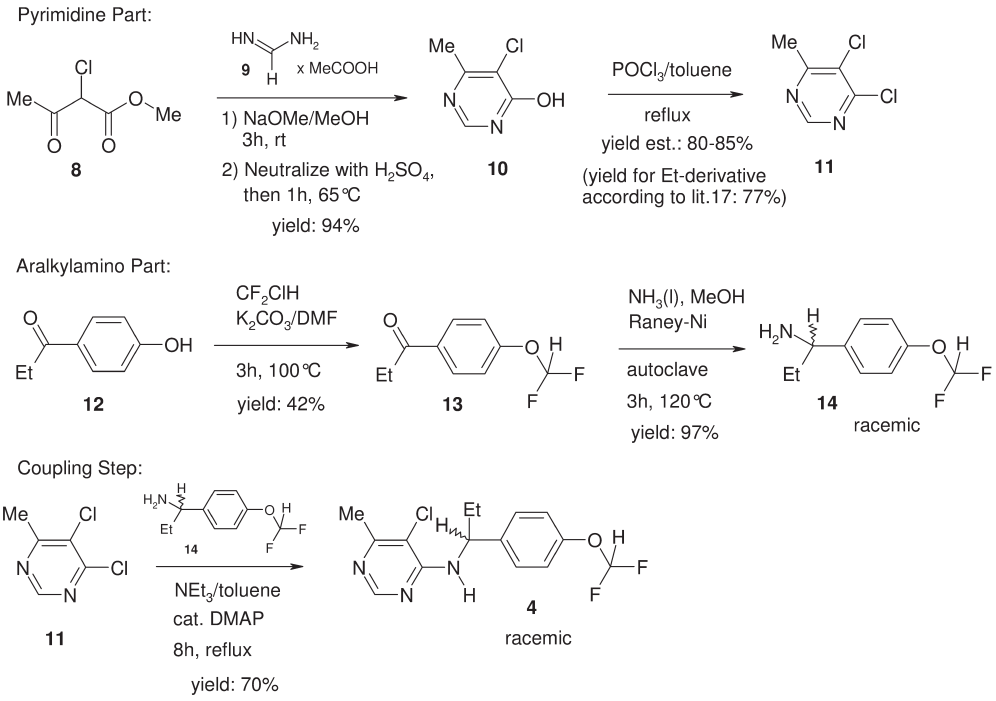
Fig. 13.5.3. First important Ube subclass.

However, these compounds do not exhibit complex I inhibition, but are reported to inhibit the biosynthesis of methionine. The pioneers of the complex I-aminoalkylpyrimidine class are chemists from Ube Industries, who were inspired by a publication of Whitehead and Traverso reporting the diuretic properties of some 4-aminopyrimidine derivatives [5]. Aminoarylalkyl-substituted pyrimidine compounds of the general formula **II** [6] (Fig. 13.5.3) were first patented in 1988, claiming both insecticidal and fungicidal activity like, for example, rice blast, powdery mildew and downey mildew. Interestingly, diflumetorim (**4**), Ube's development compound was already generically claimed in this first application but not exemplified either in the text or in the respective tables.

Further patents from Ube Industries in this area were published in subsequent years [7–12], with the last one appearing in 2003. The most interesting UBE applications belonging to the fungicide area were published in 1990 (EP 0370 704) and 1991 (EP 0432 894 – mixture patent). The development compound diflumetorim (**4**) ((*RS*)-5-chloro-*N*-1-[4-(difluoromethoxy)phenyl]-propyl-6-methylpyrimidin-4-ylamine) was exemplified for the first time in these patents. Diflumetorim (**4**) (trade name: pyricut) was registered as a new fungicide for ornamental use in Japan in April 1997 and developed by Ube Industries and Nissan Chemical Industries. The major targets are rose powdery mildew and chrysanthemum white rust. Diflumetorim (**4**) has good protective properties, some curative activity and instantly arrests fungal growth at any stage from germination of conidia to formation of conidiophore [2]. Some acute toxicity/ecotoxicity properties and environmental fate behavior of diflumetorim (technical material) have been published by Ube scientists [2]. LD_{50} values of 387 mg kg^{-1} body weight (male mouse) and 534 mg kg^{-1} body weight (male rat) are within a more favorable range for this type of compound. As a consequence of their mode of action, complex I inhibitors generally possess a higher degree of acute oral toxicity in mammals than most modern pesticides [13, 14]. The acute dermal toxicity in the rat (LD_{50} rat $>$ 2000 mg kg^{-1}), the acute inhalation toxicity in the rat (LD_{50} = 0.61 mg L^{-1}) as well as a series of negative *in vitro* tests for genotoxicity (Ames test, chromosome aberration test, mouse micronucleus test) look favorable for **4**. The ecotoxicological behavior of diflumetorim (**4**) will only be discussed very briefly. Toxicity to fish is high with a 48 h LC_{50} of 0.098 and 0.025 mg L^{-1}

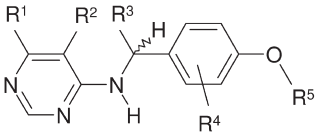
for carp and rainbow trout, respectively, and toxicity is moderate to *Daphnia* (3 h LC_{50} of 0.96 mg L^{-1}). For birds, toxicity is relatively low (LD_{50} = 1979 mg kg^{-1} for mallard duck and 881 mg kg^{-1} for quail), which indicates that risks are likely to be low. Only one point of the environmental fate behavior will be touched upon here, the soil dissipation behavior. An aerobic metabolism study in soil has shown a slow degradation of **4** (DT_{50} = 4.5 months). The major metabolism pathway elucidated in this study was the hydroxylation of **4** in the 2-position of the pyrimidine ring. Soil dissipation studies performed in the field and in the laboratory using various soil types revealed DT_{50} values of 60–140 days; absorption studies (K_{oc} = 473) indicate a low potential for mobility in soil for this compound.

The chemistry of diflumetorim (**4**) looks straightforward and a possible technical synthesis for this compound is shown in Scheme 13.5.1.



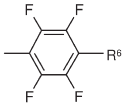
Scheme 13.5.1 Synthesis of diflumetorim.

The pyrimidine part (4,5-dichloro-6-methylpyrimidine, **11**) is synthesized in a two-step sequence starting with the condensation of 2-chloro-3-oxobutanoic acid methyl ester (**8**) and formamidine acetate **9** [15] to give 5-chloro-4-hydroxy-6-methylpyrimidine (**10**) in high yield, followed by the chlorination of the hydroxy group using standard synthesis methodology [15–18]. Synthesis of the sub-



general formula **III**

R^1 = C_1-C_4-alkyl
R^2 = halogen
R^3 = C_1-C_4-alkyl
R^4 = H, F, Cl, OMe, $OCHF_2$, 3,5-Me_2
R^5 = CF_2H: subclass **IIIa**



(R^6 = F: subclass **IIIb**)

R^6 = F, CF_2H, CF_3, Ac, CHO, CN, NO_2

Fig. 13.5.4. Structures of subclasses **IIIa** and **IIIb**.

stituted benzylamine **14** [19] starts from 4-hydroxypropiophenone (**12**), which is transformed into 4-difluoromethoxypropiophenone (**13**) by reaction of the phenol **12** with difluorochloromethane in the presence of potassium carbonate in an autoclave. Reductive amination of the resulting ketone by treatment with liquid ammonia in methanol in the presence of Raney nickel, again in an autoclave, gives α-(*RS*)-ethyl-4-difluoromethoxybenzylamine (**14**) in 97% yield. The final compound 5-chloro-6-methyl-N-(α-(*RS*)-ethyl-4-difluoromethoxybenzyl)-pyrimidine-4-amine (**4**) [19] is obtained by reacting **11** with **14** in the presence of triethylamine and a catalytic amount of dimethylaminopyridine (DMAP).

Structure–activity relationship (SAR) studies, correlating preventive fungicidal potency against wheat brown rust and barley powdery mildew, have been described only for special subclasses of N-benzyl-4-pyrimidine-amines [19, 20].

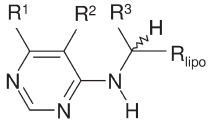
Ube scientists have published a short SAR analysis of compounds of the general formula **III** (Fig. 13.5.4) [19].

The preferred combination of substituents R^1 and R^2 on the pyrimidine ring seems to be a small alkyl group (R^1 = Me, Et) and a halogen group (R^2 = halogen, preferably Cl). The introduction of a small alkyl group (R^3 = Me, Et) at the benzyl position leads to the most active compounds. Generally, the introduction of further substituents R^4 in the benzyl-benzene moiety of the OCF_2H-subclass **IIIa** does not seem to lead to significant improvements of activity (one exception: 2′-F improves the brown rust activity). For the OC_6F_5-subclass **IIIb**, the influence of further substituents R^4 in the benzyl-benzene part was not further investigated. However, a study of the influence of substituents R^6 in the 4′-position demonstrated that replacement of the 4′-F by substituents such as CHO, Ac, CN and NO_2 leads to compounds with significantly reduced activity (with the exception of CF_2H!). In summary, substitution of the 4-position of the benzyl-benzene by electron-withdrawing groups such as OCF_2H and OC_6F_5 leads to the excellent fungicidal aminoalkylpyrimidine subclasses **IIIa** and **IIIb**.

Since diflumetorim was developed for use in ornamentals seemingly confined to the Japanese market only and not as a cereal fungicide (an unfavorable price/activity ratio may be a major reason), sales potential will remain limited.

13.5.2.1 The Competitors Contributions

Many competitor companies started synthesis programs based on this novel lead of complex I inhibitors. Among the major competitors the legacy companies of Syngenta Crop Protection AG (Ciba-Geigy AG and Sandoz Ltd.), Bayer CropScience (Hoechst AG and Hoechst Schering Agrevo) and Du Pont should be recognized. Eli Lilly, Sumitomo Ind., Shell AG and BASF published on a smaller scale on this lead.



general formula **IV**

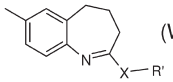
(ex. Ciba-Geigy AG)

R^1 = C_1-C_4-alkyl

R^2 = halogen

R^3 = C_1-C_4-alkyl

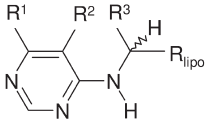
R_{lipo} = 2-naphthyl (EP 470 600): subclass **IVa**



(WO 94/20490): subclass **IVb**

R' = C_1-C_6-alkyl, C_2-C_6-alkenyl, C_3-C_7-cycloalkyl

X = O, S

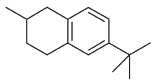


general formula **IV**

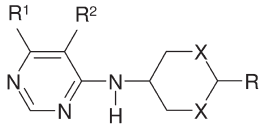
Du Pont (WO 92/08704)

R_{lipo} = 2-naphthyl (overlap with Ciba-Geigy): subclass **IVa**

2-benzothienyl: subclass **IVc**

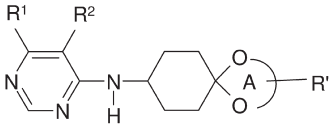


subclass **IVd**



general formula **V**

Hoechst Schering Agrevo
(WO 93/01950, WO 95/31441
and WO 97/02264)



general formula **VI**

Hoechst Schering Agrevo
(WO 96/11924)

R^1 = C_1-C_4-alkyl, CH_2OR^1; R^2 = halogen, C_1-C_4-alkoxy

R = C_1-C_8-alkyl, phenyl, subst. phenyl: subclass **Va**

COO-alkyl: subclass **Vb**

R' = alkyl, subst alkyl

A = 5 or 6-membered ring system

X = O, OH_2

Fig. 13.5.5. Some general structures of competitor subclasses.

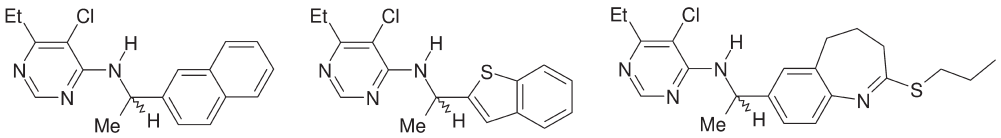


Fig. 13.5.6. Structures of the promising competitor compounds **15**–**17**.

It soon became evident that the pyrimidine moiety (toxophore) was already well optimized for fungicidal activity (best knowledge: 5-halogen, 6-Me, Et). Occasionally, substitution patterns of the pyrimidine ring such as $R^1 = CH_2OMe$ and $R^1 = OMe$ were put forward. Synthetic efforts thus concentrated on modifying the bridge link [$C(R^5R^6)_nX$] and the lipophilic part R_{lipo}. Researchers at Ciba-Geigy AG [21–25], Sandoz Ltd. [26] and Du Pont [27–29] introduced further ring systems, leading to aryl(hetaryl)methylalkylamine compounds whereas efforts at Hoechst and Hoechst Schering Agrevo [30–40] led to the introduction of nonaromatic cyclic amines such as substituted cyclohexylamines and others (Fig. 13.5.5).

(5-Chloro-6-ethylpyrimidin-4-yl)(1-naphthaleno-2-ylethyl)amine (**15**, Fig. 13.5.6), discovered at about the same time by scientists of Ciba-Geigy AG [21] and Du Pont [27], showed an interesting broad spectrum of activity.

The promising activity against powdery mildew diseases, brown rust and some leaf spot diseases of the pyrimidine **15** was also confirmed in field trials. However, due mainly to the unfavorable cost/activity ratio, the compound was dropped at an early stage in development. Replacing naphthalene by benzothiophene [22, 27] also led to active compounds, (1-benzo[*b*]thiophen-2-ylethyl)(5-chloro-6-ethylpyrimidin-4-yl)amine (**16**, Fig. 13.5.6) being a representative example. The introduction of the 4,5-dihydro-3*H*-benzo[*b*]azepine derivatives led to improved oomycete control [*P. infestans* (late blight) and *P. viticola* (downey mildew)] [24]. A representative example is (5-chloro-6-ethylpyrimidin-4-yl)[1-(2-propylsulfanyl-4,5-dihydro-3*H*-benzo[*b*]azepin-7-yl)ethyl]amine (**17**, Fig. 13.5.6). This compound showed very high activity against late blight on tomato and potato in the greenhouse but performance under field conditions was inefficient. The introduction of cyclic nonaromatic amines did not, to our knowledge, lead to fungicides with robust activity under field conditions. A patent analysis seems to suggest that the insecticidal/acaricidal activity is more pronounced in this subclass.

Further applications were published, covering aralkylamino-quinazolines and other heterocycles (Eli Lilly [41]), new benzyl-substituted pyrimidines (Sumitomo [42]), aminoalkylspiro-1,3-dioxolanes (Shell [43]) and hydrazino-substituted pyrimidine compounds (BASF [44]).

13.5.2.2 Summary – Aminoalkylpyrimidines

Aminoalkylpyrimidines represent an interesting class of novel inhibitors of complex I in the mitochondrial respiration chain. Broad spectrum activity against ma-

jor diseases in major crops have been found. However, no commercial products, with the exception of diflumetorim (**4**), have reached the market. This may be due to an unfavorable cost/activity relationship and, in certain cases, also toxicity issues. Diflumetorim (**4**) remains, hitherto the only complex I fungicide introduced into the market, but only for use in horticulture.

13.5.3 Other Leads in the Area of Complex I Inhibitors

There are only a few leads in the fungicide area worth mentioning in this chapter. Phenoxan (**18**) [45], a secondary metabolite isolated from *Polyganium* sp. strain PI VO19 with an oxazole-γ-pyrone structure is discussed as a first example (Fig. 13.5.7).

Phenoxan (**18**) inhibits the growth of agrorelevant fungi such as *Botrytis cinerea* and *Ustilago maydis*, both major diseases in agriculture, in an agar diffusion test system [45]. Other natural product leads originate from fermentations of the *Pterula* species 82168 (basidiomycete). Pterulone (**19**) and pterulinic acid (**20**, Fig. 13.5.7) [46] have been isolated. Both metabolites show antifungal activity against *Fusarium* species, *Ustilago nuda* and *Botrytis cinerea* in agar diffusion tests. To our knowledge, no significant progress was made in finding close analogues of the natural products **18**–**20** showing useful activity in glasshouse tests. The aryloxylepidines [47] (Fig. 13.5.8), closely related to the quinazoline compound fenazaquin (**1**), a commercialized acaricide and quinoxyfen (**21**, Fig. 13.5.8), a potent powdery mildewicide.

Whereas fenazaquin (**1**) is a well-known complex I inhibitor, the primary mode of action of quinoxyfen (**21**) is, as yet, unknown. Although the lepidine compound **22** (Fig. 13.5.8) shows sub-micromolar activity level (*Ustilago maydis* – IC_{50} 0.73 µmol) in the biochemical complex I assay, the activity level in glasshouse tests is not satisfying at all.

The chances of finding lepidine derivatives of sufficient biological activity in agrorelevant disease complexes are rather low [47].

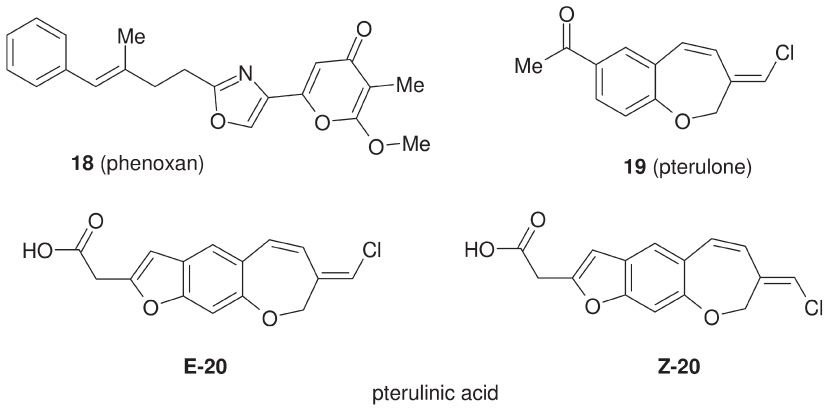
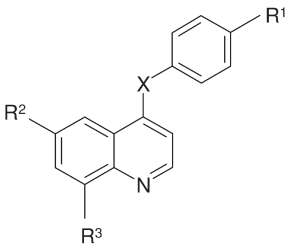


Fig. 13.5.7. Structures of natural product leads.



general formula **VII**

(lepidines: X = CH_2O)

21: R^1 = F, R^2 = R^3 = Cl, X = O

22: R^1 = F, R^2 = H, R^3 = F, X = CH_2O

Fig. 13.5.8. Structures of the general lepidine class **VII**, quinoxyfen (**21**) and lepidine (**22**).

13.5.4 Conclusions

Finding complex I inhibitors with market relevant fungicidal properties seems to be much more difficult than finding insecticides/acaricides showing market relevant properties. In the fungicide, as well as in the insecticide field, only niche products having restricted market volume seem to be possible. The reasons for this finding are not fully understood. For the fungal aminoalkylpyrimidine class it was shown that, sometimes, the cost/activity relationship and/or problems with the acute oral toxicity could be responsible for stopping promising projects at an early stage. For natural products and other leads the potential for optimization of the biological activity and spectrum turned out to be rather limited.

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14
Fungicides Acting on Amino Acids and Protein Synthesis

14.1
Natural Compounds used in Agriculture Interfering in Protein Synthesis of Fungi and Bacteria

Heinrich Buchenauer and Frank Walker

14.1.1
Introduction

Only a limited number of antifungal and antibacterial compounds that interfere in protein synthesis are used in agri- and horticulture. Interestingly, almost all inhibitors of protein synthesis are natural rather than synthetic compounds. The various processes by which proteins are synthesized will be summarized.

14.1.2
General Mechanisms of Protein Biosynthesis

The information of a gene encoded by the sequence of a DNA region is translated into a particular protein. This process involves several steps. During transcription, the DNA region encoding the gene is transcribed into a complementary messenger RNA (mRNA) [1]. In eukaryotic cells the mRNA is further modified and the mature mRNA is exported to the cytoplasm. The mRNA binds to a ribosome that uses the sequence as a template for the synthesis of a specific polypeptide. In prokaryotic cells the mRNA is not further modified and ribosomes can bind to the nascent mRNA. Protein synthesis is catalyzed by ribosomes that contain several proteins and ribosomal RNA (rRNA). Ribosomes (cytoplasmic) from eukaryotes and prokaryotes exhibit sedimentation coefficients of 80S and 70S, respectively. The particles from eukaryotes are composed of the 40S and 60S subunits, those from prokaryotes of 30S and 50S subunits. The rRNA and various protein units function as structural components of the ribosomes and play a role in the process of protein synthesis. For translation of the genetic information on the mRNA, adaptor molecules are required, the transfer RNAs (tRNAs). They consist of about 80 nucleotides and each tRNA can recognize a specific codon on the mRNA by a complementary triplet, called the anticodon. At the 3′-end of each

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

specific tRNA a particular amino acid is attached by a specific aminoacyl-tRNA synthetase. In eukaryotic cells the small ribosomal subunit first associates with an initiation tRNA (Met-tRNA) and binds the mRNA at its 5′ cap. After attachment, the complex scans along the mRNA until reaching the translation start, the AUG-codon that binds the Met-tRNA. Then, during initiation, the large ribosomal subunit is added to the complex and protein synthesis can start in the 5′ → 3′ direction. Each ribosome has three binding sites. The first tRNA binding site, the P (peptidyl)-site, contains the initiation tRNA. The second A (aminoacyl)-site is free to be occupied by an aminoacyl-tRNA that carries an anticodon complementary to the second codon. After the A site is occupied, the amino acid of the P site, which is the methionine, establishes a peptide bond with the amino group of the amino acid at the A site. The tRNA carrying the dipeptide moves to the P site, the unloaded tRNA will move to the E (exit) site and will leave the ribosome. The A site is open for another aminoacyl-tRNA that is complementary to the third codon in the sequence and, thus, the ribosome moves one codon further downstream. This process is repeated until a stop codon (UAA, UAG, UGA) is reached. Then the newly synthesized polypeptide detaches from tRNA and the ribosome releases the mRNA.

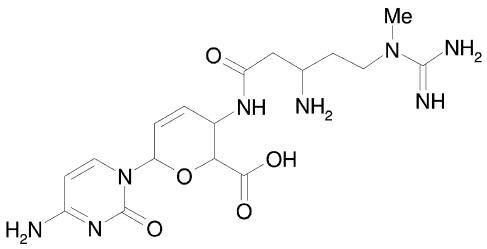
Consequently, the synthesis of proteins can be subdivided into four distinct steps: formation of aminoacyl-tRNA, initiation, elongation and termination of the polypeptide chains.

The general mechanisms by which proteins are synthesized appear to be similar in both prokaryotes and eukaryotes. There are some minor differences, particularly in chain initiation and termination and in designation and chemical characteristics of the various soluble factors.

The compounds discussed in this chapter interfere in one or more of the major steps of protein synthesis in fungi or bacteria.

14.1.3
Blasticidin S

Blasticidin S (**1**) represents the first agricultural antibiotic developed in Japan. The compound has been isolated from culture broth of *Streptomyces chriseochromogenes* [2] and the chemical structure has been elucidated [3, 4]. Blasticidin S



Blasticidin S (**1**)

shows a broad spectrum of biological activities. The antibiotic exhibits antibacterial, antiviral, antitumor and antifungal activity [5, 6]. The compound has been developed especially to control rice blast, caused by *Pyricularia oryzae* [7].

Blasticidin S, the benzylaminobenzene sulfonate derivative, proved to be least phytotoxic to rice plants and displayed a pronounced antifungal activity against *P. oryzae* [8]; this salt has been produced industrially since 1961 to control rice blast. Other derivatives of this antibiotic, blasticidin A and C proved to be inferior to the S derivative in disease control. The concentration of blasticidin S following spray treatments is 10 to 40 g ha^{-1}. The chemical caused some phytotoxic effects on rice plants when higher concentrations were applied.

Following spray treatment, using a [^{14}C]-labeled radioactive compound, most of the antibiotic remained as residue on the surface of the rice plants and only a small portion was taken up and translocated in the host tissue. In contrast, the compound was easily taken up through wounds of infected plant parts and translocated to the apexes [9].

Residues of the antibiotic on the plant surface were decomposed by sunlight and the main degradation product detected was cyteine. Following treatment of paddy field soil with radioactive labeled blasticidin S a significant degradation of the antibiotic was determined and several microorganisms colonizing the paddy field were found to diminish the biological activity of blasticidin S [9]. These findings indicate that blasticidin S is easily degraded in the environment and no environmental pollution and food contamination may be expected. Biological assays of residual levels in unpolished rice revealed less than 0.05 ppm of the antibiotic [9].

The antifungal effect of blasticidin S against rice blast may be attributed to inhibition of mycelium growth of *P. oryzae*. Studies on the mode of action indicated that blasticidin S effectively inhibited incorporation of [^{14}C]-labeled amino acids into protein using cell-free systems of *P. oryzae* and *Escherichia coli* [10]. The compound binds to 60 S and 50 S ribosomal subunits, respectively [11]. While blasticidin S is highly inhibitory to *P. oryzae* it exhibits no antifungal activity to *Pellicularia sasaki*. This differential sensitivity between both fungi to the antibiotic was reduced to differences in binding affinities of the compound to the ribosomes from both fungi [10, 11].

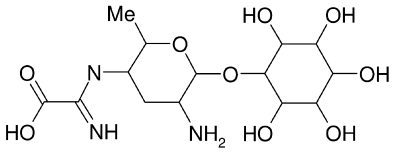
The antibiotic, when it comes accidentally in contact with eyes, especially following dust application, causes conjunctivities. Less injury is caused after application of wettable powder or solution. Blasticidin S may also cause inflammation of mucous membrane or of injured skin if they come in contact with the antibiotic. The addition of calcium acetate to blasticidin S alleviated eye irritation without affecting antiblast activity and the addition of calcium acetate to the antibiotic was used in dust applications.

Strains of *P. oryzae* resistant to blasticidin S have been easily selected on agar media. Preparations of cell-free systems from sensitive and resistant strains proved to be equally inhibited by the antibiotic and it has been shown that resistance to the antibiotic was reduced due to decreased permeability of the plasmalemma for the antibiotic [10]. Resistant mutants obtained in laboratory experiments displayed a decreased pathogenicity [12]. No emergence of resistance in

P. oryzae to blasticidin S has been observed in practice. It is not known whether the lack of resistance under field conditions might be explained by decreased fitness of resistant strains [13].

14.1.4 Kasugamycin

Kasugamycin (**2**), a water-soluble and basic antibiotic, is produced by *Streptomyces kasugaensis* [14]. Chemically it is composed of three moieties: D-inositol, kasugamine (2,3,4,5-tetradeoxy-2,4-diaminohexopyranose) and an imino acetic acid side chain [14–16].



Kasugamycin (**2**)

Kasugamycin has been developed as a specific and effective antibiotic to control rice blast since 1965. The antibiotic controls the disease when applied at 20 ppm in aqueous solution and shows both protective and curative activity [20]. Kasugamycin has been used to control rice blast on a large scale. In practice, the antibiotic is predominantly used as a dust at 0.3% of the active ingredient. In addition, seed treatment with 2% wettable powder of kasugamycin protects rice plants against blast for one month. The antibiotic exhibits a high crop safety; no phytotoxicity has been observed.

In addition, kasugamycin exhibits activity against plant diseases caused by bacteria; soil treatment of nursery boxes with the antibiotic controlled *Pseudomonas glumae*, which causes severe diseases of rice seedlings [17]. Following foliar applications, kasugamycin displayed preventive and curative activity to control cucumber angular leaf spot [18]. The antibiotic was not sufficiently effective to control citrus canker evoked by *Xanthomonas campestris* pv. *citri*; however, when the antibiotic was applied in combination with copper oxychloride an effective diseases control was obtained. It might be assumed that the antibiotic displayed a synergistic effect with copper in controlling citrus canker. In laboratory and greenhouse studies kasugamycin was effective in controlling vegetable soft rot (*Erwinia carotovora*), bean halo-blight (*P. syringae* pv. *phaseolicola*), cucumber marginal blight (*P. marginalis*) and rice sheath brown rot (*P. fuscovaginae*) [19].

Kasugamycin exhibited a low toxicity to animals (e.g., mice, rats, rabbits, dogs and monkeys). The oral LD_{50} for mice was 2 g kg^{-1}, and at a concentration of 1000 μg mL^{-1} no fish toxicity was determined. The antibiotic did not interfere with the protein synthesis of ribosomes of rat liver [21].

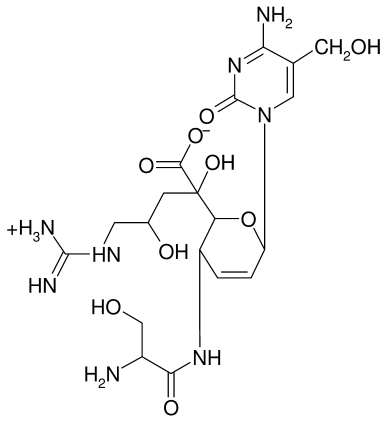
The compound inhibits mycelium growth of *P. oryzae* in media with acidic pH (pH 5.0) and is hardly inhibitory at neutral pH 7.0 [22]. Kasugamycin interferes in protein synthesis of *P. oryzae* and bacteria. In cell-free systems from *P. fluorescens* and *Escherichia coli* cells, the antibiotic reacts with the 30S ribosomal subunits. The complex between the small ribosomal subunit and the antibiotic inhibits initiation of protein synthesis by destabilizing the special initiator aminoacyl-tRNA [23]. At ribosomes derived from resistant strains, the antibiotic did not bind to the 30S subunits [24]. In a cell free system from a sensitive strain of *P. oryzae* kasugamycin inhibited binding of aminoacyl-tRNA to ribosomes. When ribosomes from a resistant strain of *P. oryzae* were used, the antibiotic did not interfere in binding of aminoacyl-tRNA to ribosomes. Thus, resistance appeared to be due to decreased affinity of the ribosomes to the antibiotic [25]. Genetic analysis of resistant strains to kasugamycin revealed that resistance may be reduced to one major gene mutation. Three loci for antibiotic resistance have been detected. One of these genes also mediates resistance to blasticidin S [26].

Resistant strains of *P. oryzae* to kasugamycin could easily be isolated *in vitro*. These strains were only weakly controlled by the antibiotic. The findings indicate that kasugamycin selects spontaneous emergence of resistant strains of *P. oryzae*. Under field conditions, when the antibiotic was used repeatedly (4–5 times per year) and exclusively over three years, a decrease of rice blast was observed [27–29]. When the treatments were stopped the portion of resistant strains decreased. The disappearance of resistant strains under field conditions may indicate that the fitness of resistant strains would be in general inferior to that of sensitive strains [30]. When a mixture of spores of a sensitive and a resistant strain (portion 1:1) was used for inoculation in pot trials in the absence of the antibiotic, the sensitive strain produced more and larger lesions than the resistant strain. This study also confirms an inferior competitive ability of the resistant strains compared with the wild-type strains [30]. On agar medium, however, the resistant and sensitive isolates did not differ in mycelium growth and sporulation [31]. Because of the lower fitness of resistant strains compared with sensitive strains, the resistant strains disappeared under field conditions in the absence of selection pressure by the antibiotic. Kasugamycin has again been successfully used to control rice blast, either in combination or by alternating application of fungicides displaying different modes of action [13, 19].

14.1.5 Mildiomycin

Mildiomycin (**3**) has been isolated from the culture filtrate of *Streptoverticillium rimofaciens* B98891. The compound is water soluble and belongs to a new nucleoside antibiotic. The pyrimidine base of mildiomycin is 5-hydroxymethylcytosine [32, 33]. Whereas the compound shows only a weak activity on agar media against Gram-positive and Gram-negative bacteria, yeast and some phytopathogenic fungi (e.g., *Cochliobolus miyabeanus*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Alternaria kikuchiana*), it proved to be highly active against powdery mildews

and, consequently, it was named mildiomycin [33–35]. All 13 powdery mildew species tested on 15 plant species were controlled by mildiomycin [35]. Mildiomycin was also active against powdery mildew on green pepper caused by the endoparasitic fungus *Leveillula taurica*. It also effectively controlled benomyl-resistant strains of cucumber [36]. In addition, the compound showed systemic activity; root treatment controlled powdery mildew on cotyledons and leaves of cucumber plants. Translaminar activity of mildiomycin against powdery mildew on tobacco and cucumber plants has been demonstrated [36].



Mildiomycin (**3**)

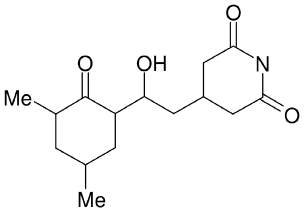
Germination of *Sphaerotheca fuliginea* conidia was inhibited at rather low concentrations of the compound and when germ tubes were formed they showed spherical or oval shaped alterations [36]. Regarding the mode of action, mildiomycin interferes in protein synthesis; at low concentration (0.02 mM) the compound inhibited incorporation of amino acids into polypeptides in a cell-free system of *E. coli*, whereas synthesis of polypeptides in mammalian cell-free systems from rabbit reticulocytes proved to be less sensitive to mildiomycin [37].

Mildiomycin exhibits low toxicity to mammals and fishes. The LD_{50} for acute toxicity in rats and mice is 500–1000 mg kg^{-1} following intravenous and subcutaneous injections as well as 2.5–5.0 mg kg^{-1} by oral administration. The compound showed no irritation to crenea and skin of rabbits [38].

14.1.6
Cycloheximide

Cycloheximide (**4**), β-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide, is a member of the glutarimide antibiotics. Cycloheximide was discovered in 1946 in a culture filtrate of *Streptomyces griseus*. The antibiotic exhibits high fungal effectiveness against a wide range of fungi and yeasts. It shows no activity

against bacteria. Cycloheximide is not a specific inhibitor of fungi, it is also toxic to plants and animals [39, 40]. Because of its phytotoxicity the compound has limited use in controlling plant diseases caused by fungi.



Cycloheximide (**4**)

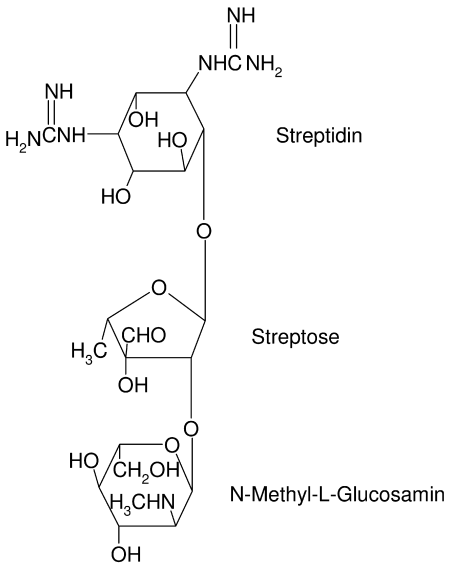
Cycloheximide is an effective inhibitor of protein synthesis in eukaryotic organisms. Kerridge [41] first showed that the antibiotic inhibited protein synthesis in the yeast *Saccharomyces carlsbergensis*. At a concentration of 0.7 μM of the compound, which inhibited growth of intact cells of *S. pastorianus*, amino acid incorporation into proteins in cell-free extracts was diminished by 50% [42]. Cycloheximide interferes in protein synthesis by inhibiting the transfer of amino acids from aminoacyl-tRNA into the protein [40, 43]. The antibiotic did not affect amino acid activation or transfer to tRNA. The binding site of the ribosomes to cycloheximide is the 60S subunit [44, 45]. Cycloheximide-resistant mutants of *S. cerevisiae* were easily isolated in *in vitro* experiments [46]. While incorporation of amino acids into the protein in a cell-free system from wild-type cells was inhibited, the compound did not interfere in amino acid incorporation into proteins in cell-free extracts from resistant cells [47]. Mutation of a single protein component of the 60S subunit led to a specific alteration of the binding site [43]. Because of the limited use of the antibiotic for control of plant diseases, no development of cycloheximide resistance has been reported.

14.1.7 Streptomycin

After successful treatments with antibiotics against bacterial diseases of humans, testing of antibiotics against bacterial pathogens of cultivated plants started in the 1950s. While many antibiotics tested proved to be highly active in inhibiting growth of *Erwinia amylovora in vitro*, few compounds were suitable to be used under practical conditions because of plant or mammalian toxicity, lack of systemic activity or short persistence on plant surfaces [48, 49].

Among the antibiotics tested against fire blight caused by *E. amylovora*, streptomycin and to some extent oxytetracycline and kasugamycin fulfilled the requirements for controlling the disease under field conditions. Streptomycin (**5**), an aminoglycoside antibiotic, is produced by strains of *Streptomyces griseus*. It was

discovered in 1944 [50] and successfully applied against tuberculosis caused by *Mycobacterium tuberculosis*. Streptomycin has also been tested against different plant-pathogenic bacteria *in vitro* and *in vivo*. It inhibited 14 species of plant pathogenic bacteria, both Gram-positive and Gram-negative species [51]. In addition, antifungal activity has been reported for species of Oomycetes (such as *Pythium*, *Phytophthora* and downy mildew species) and yeasts [52, 53].



Streptomycin (**5**)

Streptomycin when applied in a concentration range between 30 and 240 μg mL^{-1} controlled fire blight and caused no phytotoxic effects on leaves and no fruit russetting [54, 55], and the antibiotic has been used in USA since 1955. In numerous orchard trials in the 1950s and 1960s the efficiency of streptomycin treatments for control of fire blight was determined. Because of its limited systemic activity, spray treatments should completely cover all possible infection sites, such as open flowers, shoots and leaves. For fire blight control streptomycin is also used in New Zealand, some European countries and Middle Eastern countries [49].

Since the late 1950s fire blight has been spreading in Europe from country to country; depending on the country, streptomycin is used either regularly, applied on an emergency basis or not permitted. The main reason why the use of the antibiotic is not allowed in many countries is the development of resistance to streptomycin, not only in *E. amylovora* but also in other organisms on the plant surface or in soil or water, including potential human and veterinary pathogens [56].

Streptomycin is also permitted in USA on tobacco for control of wild fire (*P. syringae* pv. *tabaci*) and blue mold (*Peronospora tabacina*), which is the only fungal pathogen controlled by streptomycin [57].

The mode of action of streptomycin has been studied intensively in bacterial cell-free systems. One molecule of [^{3}H]dihydrostreptomycin binds per 30S subunit [58] and a single ribosomal 30S subunit protein (S12) has been identified as the binding site of streptomycin [59]. No binding to the 50S subunit has been determined. Streptomycin also causes misreading of the genetic message in both whole cells and cell-free systems, resulting in miscoded amino acids being incorporated into proteins [60].

Highly resistant strains of *M. tuberculosis* developed under *in vitro* conditions shortly after application of streptomycin to control tuberculosis [61]. Streptomycin-resistant strains of *E. amylovora* were first detected in 1971 in pear orchards of California [62, 63]. Subsequently, streptomycin-resistant *E. amylovora*-strains were reported from areas where the antibiotic has been applied intensively for fire blight control, such as in several western states of the USA [64–66] and outside the USA, e.g., in Egypt [67] and in New Zealand [68].

The emergence of streptomycin-resistant strains in pear orchards in California in 1971 and in Michigan in 1990 has stimulated studies on emergence, development and mechanisms of streptomycin resistance in *E. amylovora*. Mechanisms of resistance to streptomycin include alterations of the ribosomal target site, production of streptomycin-modifying enzymes and reduced uptake and thus access to the target site [69, 70].

Two phenotypes, highly resistant [minimal inhibitory concentration (MIC) of 2000 μg mL^{-1}] and moderately resistant strains (MIC 500 to 750 μg mL^{-1}) to streptomycin have been detected in the *E. amylovora* populations [64, 71, 72]. Spontaneous mutants with high level of resistance to streptomycin were isolated at a frequency of 4 of 10^9 bacteria and mutants with moderate resistance level were obtained at a frequency of 0.1 of 10^9 bacteria.

All streptomycin-resistant *E. amylovora*-strains isolated in California pear orchards showed high resistance levels [73]. Resistance in highly resistant strains has been attributed to point mutation in the *rps L* gene of the ribosomal S12 protein by which the streptomycin target site is altered and binding of streptomycin to the ribosome is prevented [74]. The *rps L* gene of *E. amylovora* is only 375 bp and mutations in this gene associated with streptomycin resistance in highly resistant strains of *E. amylovora* have been identified. The highly resistant strains contained a mutation in the codon 43. The codon encoding for lysine (AAA) in the sensitive strains was converted into a codon for arginine (AGA) in most of the highly resistant strains or in some other strains for asparagine (AAT or ACC) or threonine (ACA) [74].

Although mutation of *rps L* is the primary mechanism of streptomycin resistance, resistance in strains isolated from Michigan apple orchards exhibiting a moderate level of resistance to streptomycin was located on a 34 kb self-transmissible plasmid, pEa34 [66]. Sequence analysis of the plasmid in *E.*

amylovora strain CA11 revealed a novel 6.7 kb Tn3-type transposon, Tn*5393*, containing two linked *strA* and *strB* genes, which encode for aminoglycoside-3″-phosphotransferase and aminoglycoside-6-phosphotransferase, respectively [56, 66, 71, 72, 75]. These enzymes mediate resistance by phosphorylation of the 3″- or 6-hydroxyl position of the streptomycin molecule, by which the antibiotic is inactivated. This plasmid-mediated resistance for streptomycin is transferable by conjugation, which may result in a rapid increase of streptomycin-resistant strains. Strains with a moderate degree of resistance have been identified easily under laboratory conditions; however, they were seldom detected in nature because of reduced fitness [73]. Although pEa34 is the most common vehicle for Tn*5393* in *E. amylovora*, some moderately streptomycin-resistant strains carry Tn*5393* on a non-transmissible plasmid, pEA29, which is unrelated to pEa34 [71]. In five streptomycin-resistant strains of *E. amylovora* Tn*5393* was inserted at five different positions in pEA 29 [57]. Beside pEa34 an 8.7 kb streptomycin-resistant plasmid, pEA8.7, has been detected in isolates of *E. amylovora* from apple orchards in California. This plasmid, related to the broad-host-range plasmid RSF1010, confers resistance to both streptomycin and sulfonamide antibiotics, encoded by *strA-strB* and *sulII* genes, respectively [76].

Furthermore, it may be likely that highly resistant strains with chromosomal mutations may also have genes coding for enzymes that modify streptomycin.

Streptomycin was introduced in USA for fire blight control in the 1950s. Development of resistance of *E. amylovora* to streptomycin in western states of the USA occurred many years earlier (1971) than in eastern states (1990). This difference in emergence of resistance between regions is most likely associated with significant differences in selection pressure exerted by streptomycin. The number of applications of streptomycin in western states was between 10 and 14 applications per season [77, 78], while in eastern USA the antibiotic was used initially up to five times per season and, since the early 1960s, streptomycin was applied when environmental conditions were favorable for infection. By development of risk assessment systems ensuring optimal timing of treatments in relation to risk of infection, the selection pressure for resistance was markedly reduced in the USA [79–83] and European countries, e.g., in England [84, 85], France [86], Belgium [87] and Germany [88].

Strains of *E. amylovora* isolated in the western states of USA (California, Washington and Oregon) expressed high levels of resistance to streptomycin. After emergence of resistance the use of streptomycin was discontinued [73, 89]. These strains were not impaired in fitness and showed long-term survival [90].

Fire blight control will be very difficult in areas where highly resistant strains to streptomycin prevail. In regions of USA where streptomycin-resistant strains of *E. amylovora* are established, growers use oxytetracycline either alone or in combination with streptomycin [71]. In areas where resistance problems have not appeared, management strategies preventing disease build-up will be important. These methods include selection of cultivars and rootstocks of lower susceptibility to fire blight, sanitation methods and using good forecasting programs for precise timing of each spray during flowering [91].

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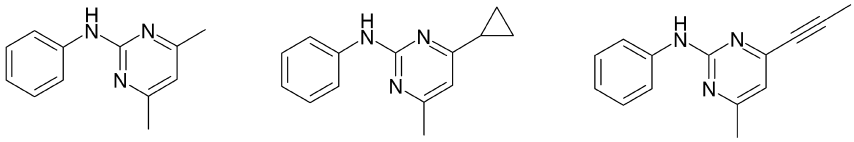
14.2
Anilinopyrimidines: Methionine Biosynthesis Inhibitors

Ulrich Gisi and Urs Müller

14.2.1
Introduction

Pyrimidines have long been known as pharmaceuticals and as crop protection agents. In plant disease control the first pyrimidines were introduced more than 30 years ago. Anilinopyrimidines were, to the best of our knowledge, first prepared in 1901 and later described by Imperial Chemical Industries, Ltd. (ICI) as having some potential anti-malarial activity [1]. As fungicides, anilinopyrimidines were first patented in 1981 by VEB Fahlberg-List, Magdeburg (former German Democratic Republic, DDR) [2]. In the late 1980s the anilinopyrimidines were rediscovered as fungicides independently by Ciba-Geigy, Schering and Kumiai/Ihara Chemical Industries. As a result of these research efforts, three anilinopyrimidines were introduced into the market as novel fungicides between 1992 and 1995: pyrimethanil (**1**) (reported 1992) [3], cyprodinil (**2**) (1994) [4] and mepanipyrim (**3**) (1995) [5] (Fig. 14.2.1, Table 14.2.1).



1 2 3

Fig. 14.2.1. Structures of the anilinopyrimidines: pyrimethanil (**1**), cyprodinil (**2**), mepanipyrim (**3**).

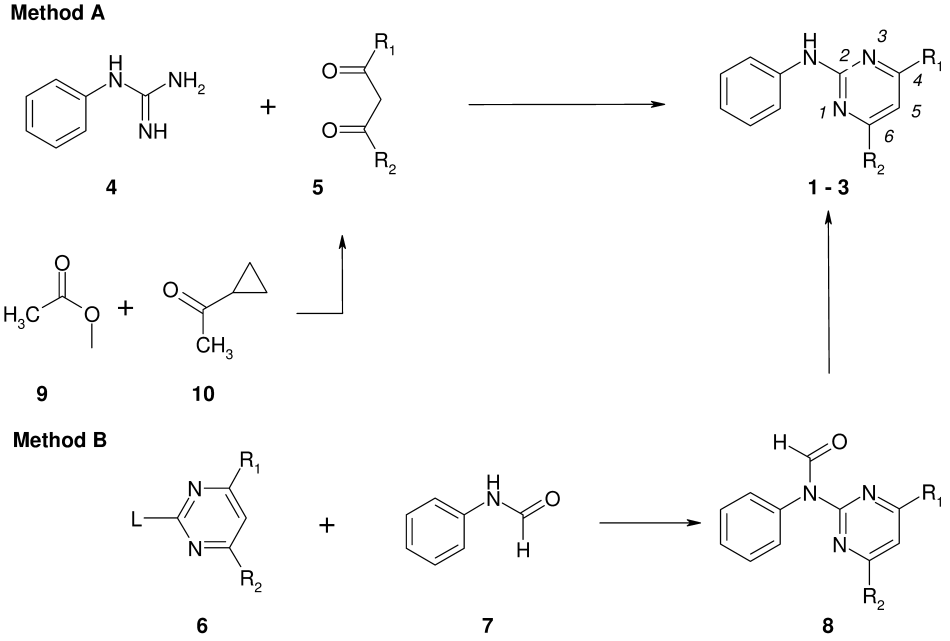
Table 14.2.1 Chemical and physical properties of anilinopyrimidines [3–5, 27].

Common name	Pyrimethanil (**1**)	Cyprodinil (**2**)	Mepanipyrim (**3**)
Patent no.		EP310550	EP224339/JP 63208581
Melting point (°C)	96.3	75.9	132.8
Vapor pressure (mPa)	2.2 (25 °C)	Crystal mod. A: 0.51 Crystal mod. B: 0.47	2.32×10^{-2} (20 °C)
K_{OW} (log P) (25 °C)	2.84 (pH 6.1)	4.0 (pH 7.0)	3.28 (20 °C)
Solubility (g L^{-1}) at 25 °C in	Water 0.121 (pH 6.1), methanol 176 (pH 6.1), acetone 389 (pH 6.1), *n*-hexane 23.7 (pH 6.1)	Water 0.013 (pH 7.0), ethanol 160, acetone 160, *n*-hexane 26	Water 0.003.10 (20 °C), acetone 139 (20 °C), methanol 15.4 (20 °C), *n*-hexane 2.06 (20 °C)
Stability	Stable: in water within relevant pH range; to heat 14 d at 54 °C	Stable: in water ($DT_{50} \gg 1$ y, pH 4–9, 25 °C); photolysis in water DT_{50} 5–30 d	Stable: in water ($DT_{50} > 1$ y, pH 4–9); to heat; to light in water (DT_{50} 12.9 d)
pK_a	3.52, weak base (20 °C)	4.44, weak base	n.d.

14.2.2 Chemistry of the Anilinopyrimidines

Two general syntheses, here named methods A and B, have been used to prepare anilinopyrimidines (Scheme 14.2.1).

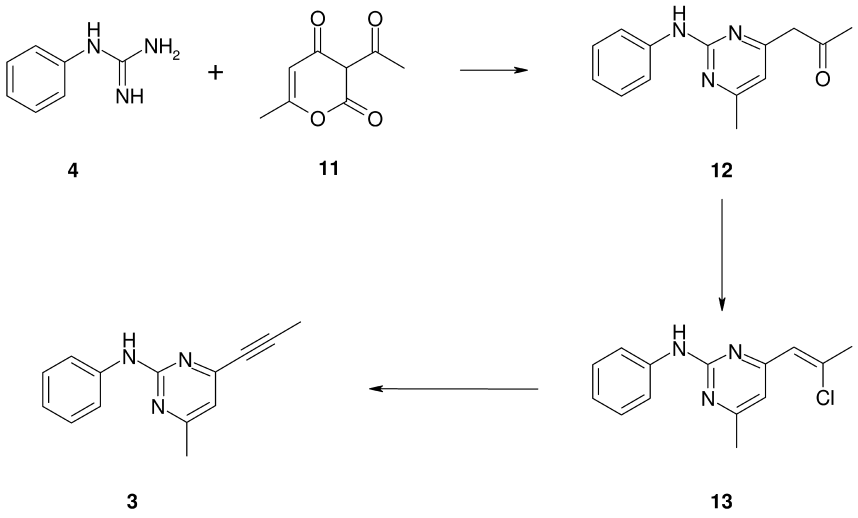
Following method A, condensation of phenylguanidine **4** with the corresponding β-di-ketones **5** gives the anilinopyrimidines in a single step [2, 6]. Both starting materials, phenylguanidines and β-diketones are easily accessible and



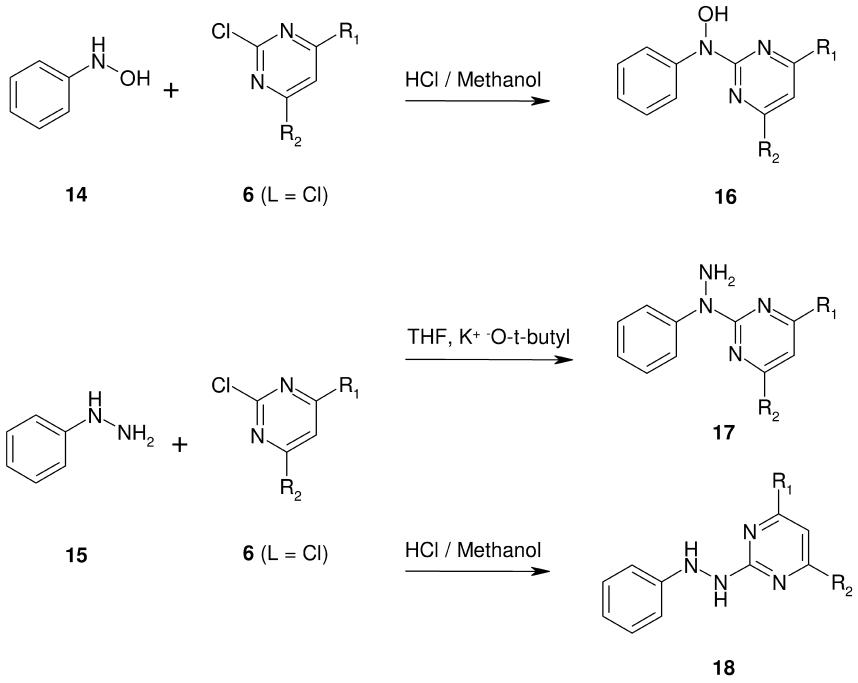
Scheme 14.2.1. Syntheses of anilinopyrimidines.

allow the preparation of a wide variety of anilinopyrimidines. As an example, the β-di-ketone, 1-cyclopropyl-butane-1,3-dione (**5** $R_1 = CH_3$, R_2 = cyclopropyl), intermediate for the preparation of cyprodinil **2,** is easily prepared by a Claisen condensation of methyl acetate (**9**) and methyl cyclopropyl-ketone (**10**) [7]. Following synthesis method B, 2-anilino-4,6-dimethyl-pyrimidine (**1**), i.e., pyrimethanil, was first prepared as early as 1901 [9]. Anilines or, as described later [10], N-formyl-anilines **7** were reacted with 4,6-disubstituted pyrimidines **6** carrying a leaving group like halogen, sulfide or preferably a sulfonyl group at position 2. As an example, mepanipyrim (**3**) was first prepared starting from 2-methansulfonyl-4-methyl-6-(1-propynyl)pyrimidine (**6**; L = $-SO_2CH_3$) and formanilide **7** in presence of a strong base to give the N-formyl-derivative (**8** $R_1 = CH_3$, R_2 = 1-propynyl), which on hydrolysis affords mepanipyrim (**3**) [10]. In analogy to method A, phenylguanidine **4** was condensed with dehydroacetic acid **11** to give the intermediate 4-(propan-2-one)-6-methyl-2-anilinopyrimidine (**12**, Scheme 14.2.2), which was then converted in two steps into mepanipyrim (**3**): first chlorination to the chloro-allyl compound **13** and second elimination of hydrochloric acid [8].

Zondler and Hubele [11] described the synthesis of N-amino- and N-hydroxy-2-anilino-pyrimidines in analogy to method B (Scheme 14.2.3). Noteworthy here are the reaction conditions for the preparation of hydroxylamino compounds **16** and the hydrazine compounds **17**. Substitution with arylhydroxylamines leads, under acidic reaction conditions, to the desired N-hydroxy-anilinopyrimidines **16**; with arylhydrazines under these conditions, however, the undesired 1-aryl-2-



Scheme 14.2.2. Synthesis of 4-alkynyl-2-anilinopyrimidines.



Scheme 14.2.3. Synthesis of *N*-hydroxy-anilinopyrimidines and 1-phenyl-1-pyrimidinyl-hydrazines.

pyrimidinyl derivatives **18** are produced. The desired 1-aryl-1-pyrimidinyl-devivatives **17** were obtained under basic reaction conditions only. Under the same conditions the N-hydroxy-anilinopyrimidines could not be prepared. These compounds were described as generally stable crystalline compounds at room temperature, which, however, decompose to the parent 2-anilino-pyrimidines when exposed to light [12]. Following the described methods, numerous anilinopyrimidines displaying a wide diversity of properties have been prepared.

14.2.3
Biological Activity

The spectrum of fungicidal activity of anilinopyrimidines is restricted to Ascomycetes, including a broad range of pathogens such as *Botryotinia fuckeliana* (*Botrytis cinerea*) on grapes, fruits, vegetables and ornamentals, *Venturia inaequalis* on apple and *Alternaria* and *Monilinia* spp causing leaf spot diseases and rot on a range of vegetables and deciduous fruits [12]. In addition to these pathogens, cyprodinil also controls a range of cereal diseases caused by *Tapesia* spp. (*Pseudocercosporella herpotrichoides*, eyespot), *Pyrenophora teres* and *Rhynchosporium secalis* in barley (net blotch and scald, respectively) and to a moderate degree also *Leptosphaeria nodorum* (*Septoria* on wheat) and *Blumeria* (*Erysiphe*) *graminis* (powdery mildew) on cereals. Pyrimethanil has additional activity against *Ascochyta* spp. in legumes, *Mycosphaerella* spp. in banana, pea and other vegetables and some of the post harvest diseases (e.g., *Aspergillus* and *Penicillium* spp.) and seed borne pathogens (e.g., *Pyrenophora graminea*, which is controlled also by cyprodinil). To broaden the spectrum of activity and delay the evolution of resistance, anilinopyrimidines are often mixed with Sterol Biosynthesis Inhibitors (SBIs) for their use in cereals and fruits (e.g., propiconazole, cyproconazole, fluquinconazole, imazalil), with multi-site inhibitors in apple and legumes (e.g., with captan, dithianon, chlorothalonil) or with fludioxonil in grapes.

Anilinopyrimidines exhibit strong preventive activity that is based on the inhibition of germ tube elongation during spore germination, the formation of appressoria and mycelial growth. Also, the penetration and infection process of the pathogen into the host tissue is affected, presumably through inhibition of the secretion of hydrolytic enzymes during pathogenesis. Anilinopyrimidines are systemic fungicides translocated in the apoplast of leaves, resulting in the inhibition of later stages in the pathogenesis such as the formation of haustoria, intercellular growth of mycelium and sporulation. Consequently, anilinopyrimidines exhibit curative activity against *V. inaequalis* in apples up to three days after infection; however, later stages and spore germination are not affected.

14.2.4
Structure–Activity Relationship

Studies on the structure–activity relationship clearly demonstrate that the biological activity generally falls sharply with the introduction of any substituents in po-

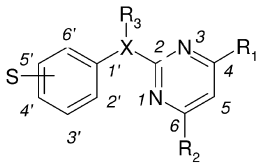


Fig. 14.2.2. General structure of anilinopyrimidines.

sitions 2′ to 6′ of the anilinobenzene ring – the exception being the 3′ or 4′ fluoro-substituted compounds, which showed some biological activity [12, 13]. Likewise, the introduction of substituents in position 5 of the pyrimidine ring greatly reduced the biological activity (Fig. 14.2.2). The photolytic and hydrolytic stabilities of the N-amino-, N-hydroxy- or N-O-alkyl or O-acetyl-2-anilinopyrimidine derivatives have to be considered when assessing the biological activity as these compounds might decompose to the parent compounds [11]. *N*-Methyl-anilinopyrimidine showed some activity, but less than the unsubstituted analogues. Higher alkyl substituents at the bridging nitrogen led to inactivity. No fungicidal activity was observed when the bridging nitrogen was replaced by sulfur or oxygen. Various substituents like alkyl, chloro, methoxy, methylamino-, cyclopropyl- and alkenyl and alkynyl are tolerated in the 4 and 6 positions of the pyrimidine ring [11, 12]. The highest potency and broadest spectrum was observed with sterically small and chemically stable combinations such as those present in pyrimethanil, cyprodinil and mepanipyrim.

14.2.5
Mode of Action and Mechanism of Resistance

Anilinopyrimidines are considered to be inhibitors of methionine biosynthesis [14, 15] (Fig. 14.2.3). They are single-site inhibitors in the amino acid biosynthesis pathway, and cross resistance was observed between cyprodinil, pyrimethanil and mepanipyrim [16], suggesting a common mode of action for the entire class.

In addition, they were reported to also affect (potentially as a consequence) the secretion of hydrolytic enzymes during penetration of the target pathogens into plant tissue [17, 18], although the biosynthesis of these enzymes was not affected. In biochemical studies, methionine and homocysteine but not cystathionine reversed the action of anilinopyrimidines [14] and the effect of the incorporation of radiolabeled sulfur starting from sulfate suggested an inhibition of methionine biosynthesis [15]. Therefore, it was proposed that either cystathionine-β-lyase (CBL) or cystathionine-γ-synthase (CGS) may be the target enzymes. However, isolated CBL was not sensitive to cyprodinil (cell-free assay) and CGS-deficient mutants of *Neurospora crassa* were insensitive to anilinopyrimidines [19].

When the sequence of the *cbl* gene in sensitive and resistant field isolates of *Botrytis cinerea* was compared, no mutations were detected conferring resistance

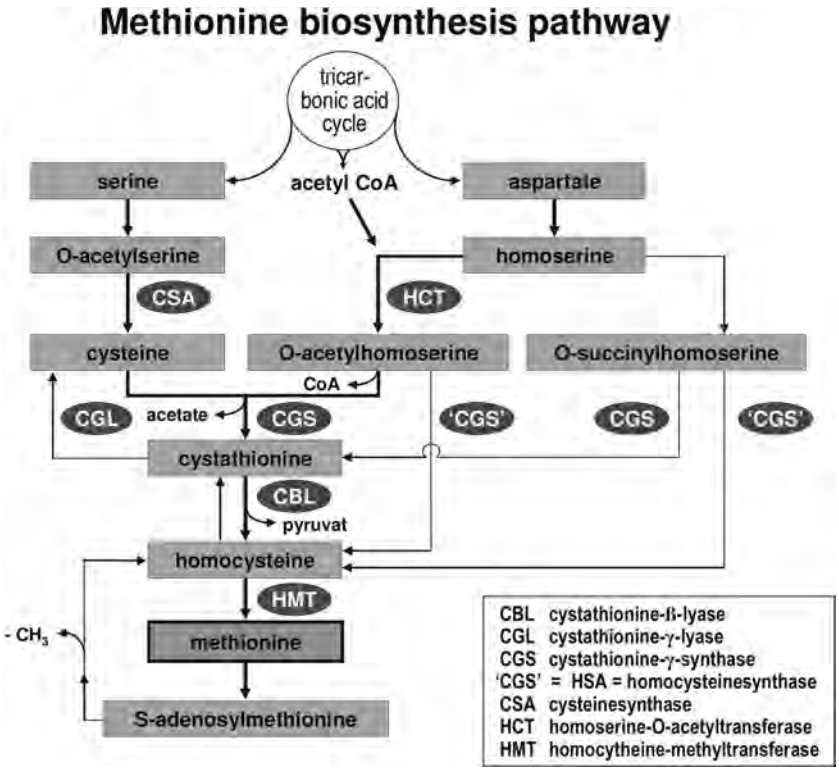


Fig. 14.2.3. Schematic representation of the methionine biosynthesis pathway [19].

to anilinopyrimidines [19]. Thus, CBL is not likely to be the target enzyme for resistance to anilinopyrimidines and is probably not the biochemical site of action. However, two different mutations (S24P and I64V) were found in the *cgs* gene of several *B. cinerea* field isolates that were resistant to anilinopyrimidines [19]. When sensitive and cyprodinil-resistant parents of *B. cinerea* were crossed and the 8 F1 ascospore progeny analyzed for resistance (per ascus in two independent crosses), the segregation of resistance was 1:1 [17] (Fig. 14.2.4). In both crosses, the mutations (S24P in cross 1 and I64V in cross 2) co-segregated with the resistant phenotype and were inherited in a 1:1 manner [19] (Fig. 14.2.4). Therefore, it can be assumed that resistance to AP fungicides in *B. cinerea* (and probably also in other target pathogens) is monogenic but multiallelic. The CGS enzyme complex is end-product inhibited and accepts different substrates circumventing cystathionine for methionine biosynthesis (Fig. 14.2.3). Comparison of the *cgs* gene sequences in *B. cinerea* revealed that the amino acid change in the resistant phenotype was located in the regulatory part of the gene [19]. It was suggested that the mutated CGS is insensitive to end-product repression and that the change in CGS regulation (overexpression) may confer resistance to anilinopyrimidines.

Cross 1				Cross 2			
Isolate	sensitivity	S24P	I64V	Isolate	sensitivity	S24P	I64V
CH 9.83 (s)	0.003	wt	wt	CH 9.83 (s)	0.003	wt	wt
Z 103.16 (r)	10	wt	mut	Z 203.21 (r)	2	mut	wt
Ascospore 1	10	wt	mut	Ascospore 1	s	wt	wt
Ascospore 2	10	wt	mut	Ascospore 2	r	mut	wt
Ascospore 3	10	wt	mut	Ascospore 3	s	wt	wt
Ascospore 4	0.007	wt	wt	Ascospore 4	s	wt	wt
Ascospore 5	0.016	wt	wt	Ascospore 5	r	mut	wt
Ascospore 6	0.012	wt	wt	Ascospore 6	r	mut	wt
Ascospore 7	7	wt	mut	Ascospore 7	r	mut	wt
Ascospore 8	0.006	wt	wt	Ascospore 8	s	wt	wt

wt: wild type, no mutated allele, sensitive to cyprodinil

S24P: exchange of serine by phenylalanine at position 24 in CGS, resistant to APs

I64V: exchange of isoleucine by valine at position 64 in CGS, resistant to APs

Fig. 14.2.4. CGS genotype (wt, S24P, I64V) and sensitivity (s, r, EC_{50}, mg L^{-1}) of parent isolates (CH 9.83, Z 103.16, Z 203.21) and F1 single ascospore progeny isolates (8 per ascus in crosses 1 and 2) of *Botrytis cinerea* to cyprodinil [19, 20].

Therefore, the level and extent of resistance evolution is estimated as moderate (see FRAC classification). However, the mode of action of AP fungicides remains speculative.

In fact, resistance to anilinopyrimidines was already detected in several pathogens, including *B. cinerea*, *V. inaequalis* and *Tapesia* spp., several years ago [21–24] but has so far not evolved to an extent that product performance is affected in practice. Resistance to anilinopyrimidines was observed at trial sites in France in 1991 for *B. cinerea* [21] and in Italy and Switzerland in 1997 for *V. inaequalis* after excessive product use (up to 14 applications in orchards) [22]. Also, in *Tapesia* spp., resistant isolates have been detected repeatedly, but their frequency remained low [23] and product performance was good. Since the current sensitivity assays are normally done with bulk samples there is no solid information on the proportion of resistance in field populations. Although there is an inherent risk of resistance evolution to anilinopyrimidines in field populations, the extent and distribution did not follow the same dynamics as was observed for other single gene mechanisms (e.g., in benzimidazoles, QoIs). Restriction of the number of applications per season (one to two in cereals and grapes, three to four in apple, fruits and vegetables) and the use of mixtures or alternations are recommended for the delay of resistance evolution (see AP-FRAC recommendations) [25].

14.2.6
Degradation and Metabolism

Anilinopyrimidines are decomposed rapidly in water when exposed to UV light (DT_{50} about 2 weeks). In soil, cleavage of the aniline–pyrimidine linkage represents the major degradation pathway. Other reactions include hydroxylations, oxidations and nitrations [26]. Based on their high K_{OW} values (Table 14.2.1), anilinopyrimidines show minimal movement to deeper soil layers. In plants, the major residual components after application are the active ingredients. Metabolization of anilinopyrimidines occurs mainly via hydroxylation at the phenyl ring, the pyrimidine or the methyl moiety [26].

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15
Fungicides Acting on Signal Transduction

15.1
Mode of Action

Andrew Corran

15.1.1
Mode of Action of Phenylpyrroles and Dicarboximides

The hyperosmolarity regulatory pathway (HOG) in the budding yeast *Saccharomyces cerevisiae* is well characterized; for recent reviews see Refs. [1–3]. In this pathway, the sole histidine kinase Sln1p, like histidine kinases in most other eukaryotes, is a hybrid protein in which the kinase domain is fused to the response regulator domain. Sln1p is a transmembrane protein that modulates its cytoplasmic kinase domain activity in response to external stimuli and conditions of low osmolarity lead to autophosphorylation of a histidine residue in the kinase domain. This phosphate group is then transferred to an aspartate residue in the Sln1p receiver domain, then to Ypd1p and finally Ssk1p (Fig. 15.1.1). In this phosphorylated form Ssk1p is inactive and unable to phosphorylate Ssk2p or Ssk22p, the two mitogen-activated protein kinase kinase kinases (MAPKKKs) in the downstream HOG MAP kinase cascade. Conversely, under conditions of high osmolarity Sln1p, Ypd1p and Ssk1p are all dephosphorylated. Ssk1p in this dephosphorylated, active form subsequently phosphorylates and activates the first kinases of the HOG MAP kinase cascade. This eventually leads to the transcription of enzymes involved in glycerol production that allow the cell to compensate for the high external osmotic pressure.

Osmotic sensitive mutants of *Neurospora crassa* have been identified, including *os-2*, a deletion mutant lacking the MAP kinase orthologous to Hog1p. These mutant strains grow normally in a low osmotic environment but cannot adapt to conditions of high osmolarity and were found to be highly resistant to phenylpyrroles such as fludioxonil and fenpiclonil and dicarboximides such as iprodione and vinclozolin [4]. In addition, both phenylpyrroles and dicarboximides stimulate glycerol production in wild-type strains of *Neurospora* and cause conidia and hyphal cells to swell and burst, probably by the generation of too high an internal turgor pressure [4, 5]. However, this bursting did not occur in the *os-2* mutant, which

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

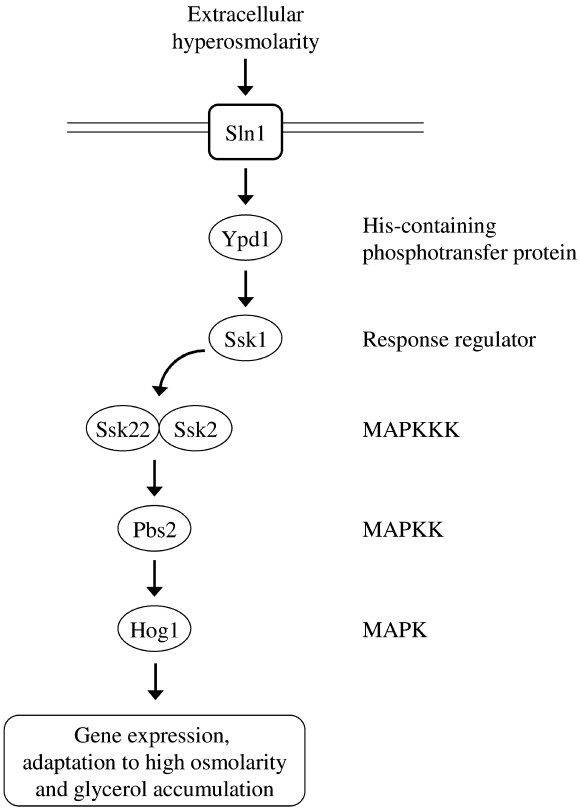

Fig. 15.1.1. Signaling events downstream of the histidine kinase *Sln1p* following hyperosmotic stress in *Saccharomyces cerevisiae*.

was found to be unable to synthesize glycerol in response to phenylpyrroles or conditions of high osmolarity [6]. This MAP kinase is therefore essential for adaptation to a high osmolarity environment and for expression of fungicide activity. Supporting this, in the fungal pathogen *Colletotrichum lagenarium*, the orthologous MAP kinase (Osc1p) was found to be rapidly activated and transported into the nucleus following treatment with either high osmotic stress or fludioxonil [7]. Kojima and coworkers were also able to demonstrate similar activation of the orthologous MAP kinases in both *Botrytis cinerea* and *Cochliobolus heterostrophus* with fludioxonil treatment, indicating an overall similarity between fungal species in pathways involved in osmotic signaling. The *C. lagenarium osc1* null mutant was fully pathogenic, showed reduced sensitivity to fludioxonil and expressed growth defects under conditions of high osmolarity [7].

In addition to the hyperosmolarity sensing MAP kinase, other upstream protein kinases in *N. crassa*, including the Os-1 histidine kinase, have been simi-

larly implicated in fungicide resistance and osmotic sensitivity [8, 9]. These data have now been widely accepted to indicate that phenylpyrroles and dicarboximides act upstream of the MAP kinase cascade involved in the hyperosmolarity response.

From the available genomic and biochemical information so far, filamentous fungi have similar signaling pathways to *S. cerevisiae* to detect and respond to changes in the osmotic environment in that both histidine kinases and a downstream MAP kinase cascade have been identified in fungi as well as fungal homologues of both Ypd1p and Ssk1p [10]. However, despite this, *S. cerevisiae* is insensitive to both phenylpyrroles and dicarboximides, indicating that there are some key differences between budding yeasts and filamentous fungi. In addition, whereas budding yeast has one essential histidine kinase, *N. crassa* has been reported to have eleven, and plant pathogenic fungi were found to contain even more, e.g., *B. cinerea* is reported to have 20 histidine kinase genes [10, 11]. These additional kinases probably reflect the greater need for plant pathogenic fungi to sense and respond to a complex changing environment. Another difference in the histidine kinases between budding yeast and filamentous fungi is that *sln1* null mutants (and *ypd1* null mutants) are non-viable in *S. cerevisiae*, presumably due to inappropriate activation of the MAP kinase cascade and glycerol synthesis causing the yeast cells to take in water, swell and burst. However, deletion of histidine kinases in filamentous fungi is not lethal in low osmolarity medium, possibly due to functional redundancy between histidine kinases and, in addition, these null mutants retain full pathogenicity.

Catlett *et al.* [10] further clustered histidine kinases into eleven families, some of which, such as the putative osmotic sensing family (group III), were found to be highly conserved between species whereas others appeared to have few homologues, suggesting they may have evolved to fulfill a specific requirement in the lifestyle of a fungus. Histidine kinases that are involved in the hyperosmotic response have now been cloned and sequenced from a range of fungi, including *B. cinerea* [12, 13], *N. crassa* [14], *C. heterostrophus* [15], *C. lagenarium* [7], *Alternaria brassicicola* [16] and *Pyricularia oryzae* [17]. Osmotic-sensing histidine kinases from filamentous fungi differ from Sln1p in that they contain six N-terminal tandem amino acid repeats that are predicted to form a coiled coil structure that is essential for functioning of the histidine kinase. These authors showed that histidine kinase null mutants as well as a range of different mutants with alterations to the tandem amino acid repeat sequences can confer high levels of resistance to dicarboximides and phenylpyrroles [14–17].

In *Botrytis*, dicarboximide and phenylpyrrole resistant laboratory strains [12, 18] and less sensitive field isolates [13, 19] are usually found to be mutated in *Daf1*, the histidine kinase homologous to Os-1. However, field isolates less sensitive to dicarboximide fungicides were reported to retain wild-type levels of sensitivity to phenylpyrroles [18–20]. Vignutelli *et al.* [21] crossed a field resistant strain of *Botrytis* with a sensitive one and found that dicarboximide resistance segregated separately from phenylpyrrole resistance, suggesting different genes regulate

field resistance for these two fungicides. In addition, they crossed a field resistant fludioxonil strain with one generated in the laboratory and again found the two resistance genes segregated independently, indicating that fludioxonil resistance is influenced by at least two different genes. Furthermore, mutations in three separate genes were reported to result in fludioxonil resistance in strains of *Ustilago maydis* generated in the laboratory [22]. These data suggest that a fully functional osmotic-sensing histidine kinase is required for expression of phenylpyrrole and dicarboximide fungicidal activity but several different genes can regulate fungal sensitivity to dicarboximides and phenylpyrroles and these genes can differ between the two different classes of chemistry.

Recently, high levels of field resistance to both phenylpyrroles and dicarboximides have been found in *A. brassicicola* [23]. These *Alternaria* strains are particularly significant because field resistant fungal strains had not previously shown high levels of cross-resistance between phenylpyrroles and dicarboximides. One particular mutant was found that was proposed to lack the first two N-terminal amino acid repeats but was thought to retain functional histidine kinase activity [16]. This mutant was found to be highly resistant to phenylpyrroles and dicarboximides, suggesting that the N-terminal tandem repeat structure itself is required for expression of fungicide activity.

The orthologous histidine kinase gene (*HIK1*) from the rice blast disease, *Pyricularia oryzae* (telomorph: *Magnaporthe grisea*), has been isolated by Motoyama and coworkers [17]. Like *N. crassa os-1* and *A. brassicicola nik1*, gene disruption of *HIK1* in *P. oryzae* was found not to be a lethal event. Appressorium formation and pathogenicity were unaffected and it was reported to be highly resistant to phenylpyrroles and dicarboximides. Interestingly, this null mutant showed increased sensitivity to high concentrations of sugars such as 1 M sorbitol but, unlike *N. crassa os-1* [14], tolerance to high levels of salt was unaffected, suggesting differences in perception of osmotic stress between fungal species. The same group went on to express *P. oryzae HIK1* in *S. cerevisiae* [24] and found that expression of this gene conferred sensitivity to phenylpyrroles and dicarboximides, leading them to suggest that Hik1p itself could be the molecular target for these chemistries. The sensitivity of the transformed yeast towards phenylpyrroles and dicarboximides was also found to be dependent upon the presence of other genes in the hyperosmotic response pathway and Hik1p was shown to interact directly with Ypd1p, suggesting that Hik1p transmits the fungicide signal to Hog1p *via* Ypd1p.

Further work needs to be done to determine the molecular target(s) of phenylpyrroles and dicarboximides and to understand the role that hyperosmolarity sensing histidine kinases play in the mode of action of these fungicides. Still unresolved is the role of the protein kinase PK-III that was isolated from *N. crassa* and found to be inhibited by phenylpyrroles but not by dicarboximides [5]. Another recently reported and surprising finding was that an inhibitor of the *N. crassa* Os-2 MAP kinase was able to antagonize the fungicidal effect of fenpiclonil but not that of vinclozolin [25], again suggesting that the mode of action of these two fungicides is different.

15.1.2
Mode of Action of Quinoxyfen

Quinoxyfen is a protectant fungicide controlling powdery mildew diseases of wheat, barley and grapes and is a potent inhibitor of appressorium formation in these fungi (Chapter 15.2) [26–28]. In barley powdery mildew (*Blumeria graminis* f.sp. *hordei*, syn. *Erysiphe graminis*), appressorial germ tubes are normally approximately 20 μm long and have a swollen tip and a hooked apical lobe but quinoxyfen treatment results in the formation of longer germ tubes (approximately 60 μm, personal communication) that remain unswollen, similar to normal hypha and no penetration of the host leaf occurs [28]. Detailed biochemical investigations of the mode of action of quinoxyfen have been hampered because *B. graminis* is an obligate plant pathogen and cannot be cultured away from its plant host. However, quinoxyfen resistant strains of barley powdery mildew have been generated in the laboratory by chemical mutagenesis and have also been isolated from quinoxyfen-treated plots [29]. These strains were found to show no cross-resistance to sterol biosynthesis inhibitors or to ethirimol, and in addition they require quinoxyfen to be present in the growth medium and display a range of unusual phenotypes, including defects in sporulation [29]. As a result of concerns over the potential for development of resistance to fungicides, the sensitivity of wheat powdery mildew in Europe to quinoxyfen has been monitored from 1995 to 2000 [30]. No significant changes were found, suggesting that the risk of a rapid shift in sensitivity to quinoxyfen is unlikely. Conidia from a resistant strain of barley powdery mildew from the quinoxyfen-treated plots were artificially maintained in the laboratory and found to germinate normally on barley leaves compared with the wild-type strain and formed appressoria even in the presence of high concentrations of quinoxyfen [27]. Interestingly, germination rates on an artificial membrane were higher in the resistant mutant than in the wild-type control, suggesting that perception of the host surface had been affected in the mutant strain. Wheeler and coworkers went on to use differential display reverse transcription PCR to identify a gene that is expressed in wild-type conidia on treatment with quinoxyfen but which was absent in the resistant mutant under the same conditions. This gene was putatively identified as a Ras-type GTPase activating protein (GAP) [27, 28]. Ras G-proteins have been reported to play a role in spore germination [31, 32] and are negatively regulated by GAPs, which stimulate their inherent GTP hydrolyzing activity [33] (Fig. 15.1.2).

After spore germination on the host surface, the concentration of the GAP declines, the Ras-type G-protein becomes active through the binding of GTP and appressorium formation proceeds. Disruption of GAP expression in quinoxyfen-resistant mutants may therefore cause the G-protein to be inappropriately activated, disrupting host recognition signals and resulting in unregulated appressorium formation. However, expression of GAP mRNA was found in later stages of the infection process, suggesting that quinoxyfen disrupts expression of GAP at a specific stage in the life cycle of this fungus. The presence of this inappropriately active G-protein, although not lethal for the fungus, may be

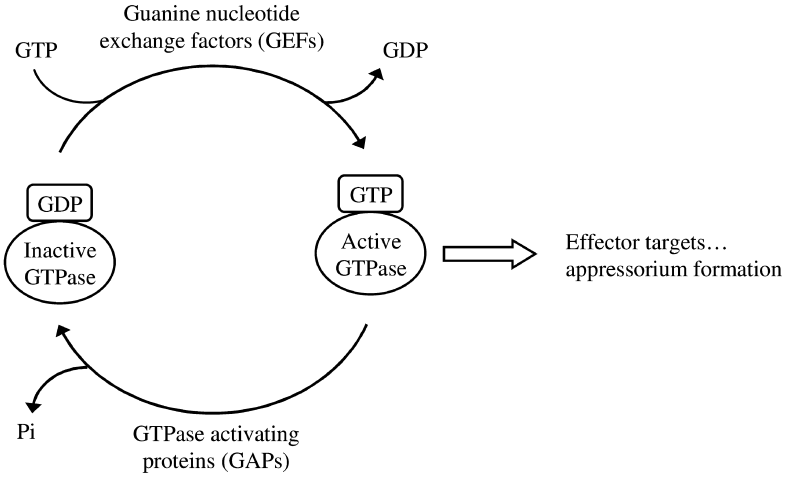

Fig. 15.1.2. The GTPase cycle showing activation of GTPase by guanine exchange factors (GEFs) in the presence of GTP and inactivation by hydrolysis of GTP to GDP in the presence of GTPase activating proteins (GAPs).

fairly disruptive and has been suggested to lead to many of the phenotypic defects seen in these mutant strains [27]. The precise mechanism of action of quinoxyfen remains to be elucidated.

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15.2
Chemistry and Biology of Fludioxonil, Fenpiclonil, and Quinoxyfen

Peter Ackermann, Gertrude Knauf-Beiter, and Ronald Zeun

15.2.1
Phenylpyrroles: Fenpiclonil and Fludioxonil

15.2.1.1 Chemistry

15.2.1.1.1 Introduction

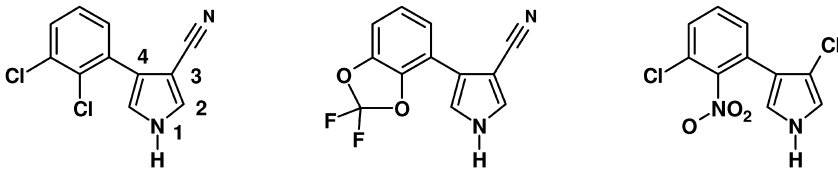
Fenpiclonil (**1**) [1] and fludioxonil (**2**) [2] belong to the class of fungicides known as phenylpyrroles (Fig. 15.2.1). These two non-systemic fungicides were developed by Ciba-Geigy. The discovery of this novel class of agrochemical fungicides is based on the synthetic optimization of the natural product pyrrolnitrin (**3**).

15.2.1.1.2 Chemistry

Pyrrolnitrin (**3**) was first isolated in 1964 by Arima from the bacterial cells of *Pseudomonas pyrocinia* [3]. This simple, secondary metabolite strongly inhibited the growth of fungi [4]. Pyrrolnitrin was developed as an antimycotic for topical application in human medicine. Owing to rapid metabolism pyrrolnitrin showed only minimal activity after oral administration [5].

The first use of a pyrrolnitrin analogue in plant protection was described in 1975 in a Japanese patent application [6]. In greenhouse tests pyrrolnitrin showed interesting activity against a range of phytopathogenic fungi. Owing to insufficient photostability, this natural compound could not be used under field conditions. Exposure of several 3-chloro-4-phenylpyrroles to sunlight revealed that photooxidation of the pyrrole ring led to inactive metabolites [7]. Optimization of the lead structure pyrrolnitrin for crop protection use focused, therefore, on the synthesis of analogues with increased photostability.

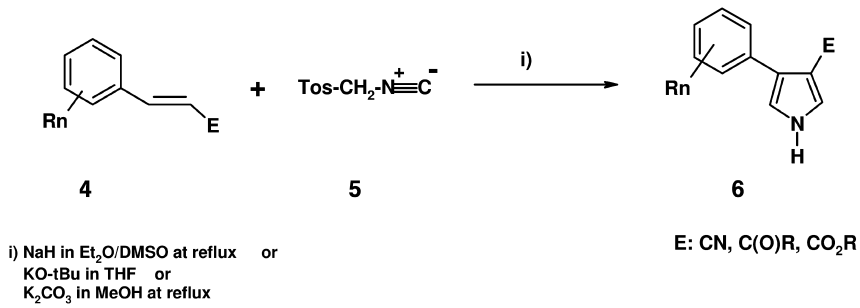
Several research groups tried to stabilize 3-chloro-4-phenyl-1*H*-pyrroles by varying the substituents on the phenyl ring [8], by acylation of the pyrrole-nitrogen [9] or by inclusion of 3-chloropyrroles into cyclodextrin [10]. No commercial crop protection compound resulted from such efforts. The use of biocontrol bacteria, which produce pyrrolnitrin as a metabolite, can protect plants from infection by soil-born fungal pathogens [11].



Fenpiclonil 1 **Fludioxonil 2** **Pyrrolnitrin 3**

Fig. 15.2.1. Structures of phenylpyrroles.

In 1972 van Leusen described a simple synthesis of 4-phenylpyrroles bearing a cyano-, a keto- or an ester-group in the 3-position of the pyrrole ring, using TosMIC (**5**) as a key reagent (Scheme 15.2.1) [12].



Scheme 15.2.1.

This approach was quickly taken up by several research groups for the preparation of new analogues of pyrrolnitrin. The structure–activity relationship (SAR) resulting from such an optimization work has been published [13]. The best biological activity was observed for the substituents E, X and R in structure **7** shown in Fig. 15.2.2.

With the discovery of 3-cyanopyrroles two major problems were solved. Such molecules are about 50 to 100× more stable towards exposure to sunlight than their 3-chloro analogues [13] and they are readily accessible. Furthermore, their activity as fungicides was comparable to pyrrolnitrin in the greenhouse but much more effective in the field.

Fenpiclonil (**1**) was the first phenylpyrrole fungicide developed by Ciba-Geigy for seed treatment [14]. Two years later fludioxonil (**2**) was developed as a foliar fungicide [15] and for seed treatment [16].

Production processes described by Ciba-Geigy for fenpiclonil [17] and fludioxonil [18] both use TosMIC (**5**) as a key reagent. Crystalline TosMIC is thermo labile and has a potential for deflagration and was therefore not available in bulk quantities. EP 378 046 [17] described a production process for TosMIC and its safe handling as a solution in organic solvents.

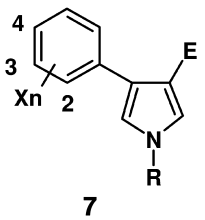
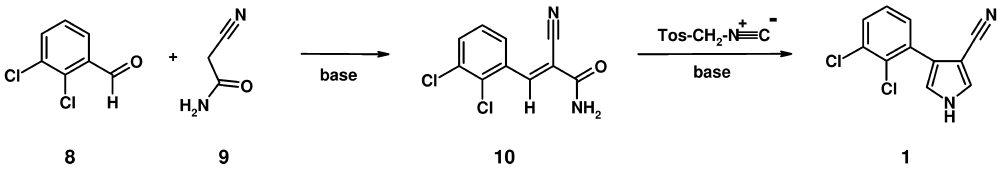


Fig. 15.2.2. SAR of phenylpyrroles.
E = CN; X = Cl, Br, CF_3, or -O-CF_2-O-;
position of X = 2-, 3- or 2,3-position; n = 1 or 2;
R = H or a group that can hydrolyze back to the parent 1*H*-pyrrole.

Synthesis of Fenpiclonil



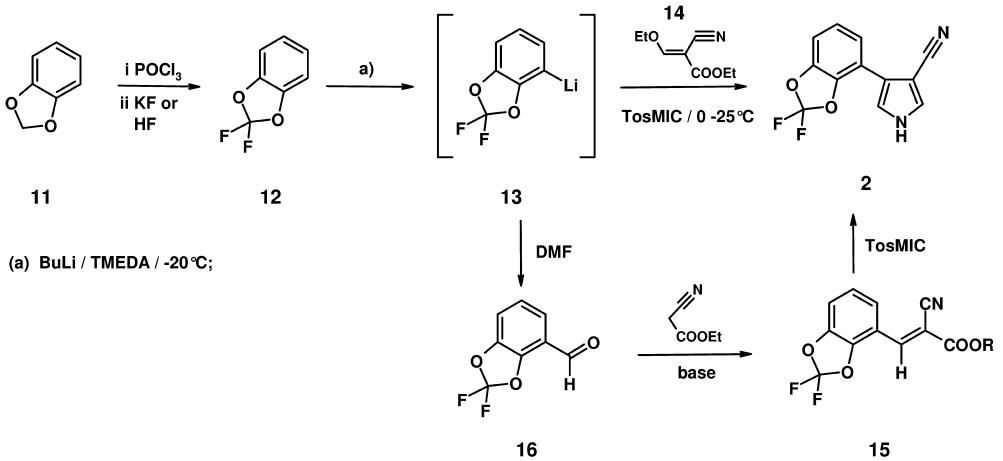
Scheme 15.2.2

Fenpiclonil (**1**) can be prepared (Scheme 15.2.2) by Knoevenagel condensation of 2,3-dichlorobenzaldehyde (**8**) with a cyanoacetic acid derivative **9**, providing an α-cyanocinnamate intermediate **10** that is then reacted with TosMIC in the presence of a base to produce the desired 1*H*-pyrrole **1** in high yield.

Researchers of Nippon Soda showed in an earlier work [19] that α-cyanocinnamates of structure type **10** render much higher yields of 3-cyano pyrroles than the corresponding phenyl acrylates such as **4**. These findings can be rationalized by big differences in pK_as of the corresponding intermediates involved in the pyrrole ring formation [20].

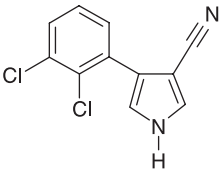
An atom economic route for the preparation of fludioxonil (**2**) has been patented [18]. Known 2,2-difluorobenzodioxole **12** is regioselectively lithiated to form **13** (Scheme 15.2.3). In a one-pot reaction intermediate **13** is directly quenched with **14** followed by conversion of the formed intermediate **15** with TosMIC into the desired fludioxonil (**2**). Alternatively intermediate **13** can be quenched with DMF to form aldehyde **16** which is, similar to the above process, stepwise reacted with a cyanoacetic acid derivative to obtain **15** followed by ring formation using TosMIC to deliver fludioxonil (**2**). Table 15.2.1 lists the chemical and physical properties of fenpiclonil (**1**) and fludioxonil (**2**).

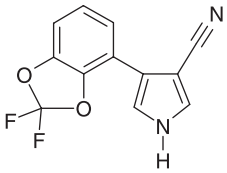
Synthesis of Fludioxonil



Scheme 15.2.3

Table 15.2.1 Chemical and physical properties of fenpiclonil and fludioxonil. (From *The Pesticide Manual*, 11th edn.) British Crop Protection Council, Farnham.





Common name:	Fenpiclonil	Fludioxonil
Melting point (°C)	144.9–151.1	199.8
Vapor pressure at 25 °C (Pa)	1.1×10^{-5}	3.9×10^{-7} Pa
Solubility in water at 25 °C (mg L^{-1})	4.8	1.8
Partition coefficient:	Log $P = 3.86$ (*n*-octanol–water)	Log $P = 4.12$ (*n*-octanol–water)
Light stability $T_{1/2}$ (hours)	48	24.9
Hydrolysis	Not hydrolyzed up to 6 h at 100 °C between pH 3 and 9	Not hydrolyzed at 70 °C between pH 5 and 9

15.2.1.1.3 **Safety Profile**

The toxicological profiles provided by fenpiclonil and fludioxonil demonstrate that both compounds can be considered to be safe without restrictions for humans, animals and the environment [21, 22].

15.2.1.2 **Biology**

15.2.1.2.1 **Introduction**

Fludioxonil was introduced in 1990 as a foliar fungicide and for seed treatment [15, 16]. It provides broad-spectrum activity across all fungal classes except oomycetes, especially against species of the genera *Aspergillus*, *Fusarium*, *Monilinia*, *Penicillium* and *Botrytis cinerea*. Table 15.2.2 gives an overview of the *in vitro* activity spectrum of fludioxonil.

15.2.1.2.2 **Foliar and Postharvest Use of Phenylpyrroles**

Fludioxonil is highly effective against *B. cinerea* on grapes [23], fruits [24], vegetables and ornamentals [25, 26]. In addition to the very efficient *Botrytis* control, fludioxonil also controls moulds such as *Penicillium*, *Aspergillus* and *Trichothecium* on grapes [27]. Furthermore, it provides protection against *Monilinia* spp. in stonefruit and *Sclerotinia* spp. [28], *Rhizoctonia* and *Alternaria* in vegetables, turf and ornamentals. Its high efficacy against different moulds makes it well suitable for

Table 15.2.2 *In vitro* activity spectrum of fludioxonil (**2**).

Fungal species	Growth inhibition [EC_{50} (mg-a.i. L^{-1})]
Alternaria solani	0.15
Aphanomyces laevis	10.3
Aspergillus carbonarius	0.18
A. niger	0.02
Botrytis cinerea	0.02
Cercospora arachidicola	0.2
Cladosporium cucumerinum	>100
Cochliobolus sativus	0.08
Fusarium culmorum	0.18
F. graminearum	0.02
F. oxysporum	0.08
F. proliferatum	3.3
F. semitectum	0.01
F. sulphureum	0.09
Magnaporthe grisae	>100
Monilinia fructicola	0.07
M. fructigena	<0.01
M. laxa	<0.01
Monographella nivale	0.15
Penicillium digitatum	0.01
Phytopthora infestans	>100
Pyrenophora teres	0.05
Pythium ultimum	>100
Rhizoctonia cerealis	0.01
R. solani AG1 (rice)	0.02
R. solani AG2 (sugarbeet)	0.03
R. solani AG3 (potato)	0.22
R. solani AG4 (cotton)	0.4
Sclerotinia sclerotiorum	0.002
Sclerotium rolfsii	0.22
Venturia inaequalis	6.05

Source: Internal data Syngenta Crop Protection AG.

use as pre- and postharvest treatment in citrus [29], grapes [30], stonefruits [31, 32] and top fruits [33–37].

Biokinetic studies on grape berries show fludioxonil to be non-systemic (Table 15.2.3). After application, the major part of the applied fludioxonil was recovered from the surface. A small percentage of the applied fludioxonil (approx. 10%) was detected in the wax layer up to 14 days after application. No uptake of active ingredient into the skin or pulp was detected [38].

The inherent risk for development of resistance towards phenylpyrroles is considered as medium [39]. No cross-resistance has been reported between phenylpyrroles and products of other chemical classes, including benzimidazoles, dicarboximides, N-phenylcarbamates and anilinopyrimidines for *B. cinerea* [40].

Table 15.2.3 Biokinetic data for treatment of grape berries with fludioxonil.

Grape berry fraction	Time after application			
	2 h	1 day	7 days	14 days
	% Recovered active ingredient			
Surface	96	96	38	48
Wax layer	4	10	3	4
Skin	0	0	0	0
Pulp	0	0	0	0

Recently, multiple resistance of *Alternaria brassicola* isolates [41] towards phenylpyrroles and dicarboximides was observed; however, the underlying mechanisms need further clarification. Although reduced sensitivity of *B. cinerea* lab mutants [42] and field isolates [43] to phenylpyrroles have been observed in a very few cases, no losses in the performance of the solo or the mixture product were observed under practical commercial conditions.

For broadening the spectrum of activity and resistance management fludioxonil is mainly applied in mixtures with the anilinopyrimidine cyprodinil [44–46].

15.2.1.2.3 Seed Treatment Use of Phenylpyrroles

Fenpiclonil was the first phenylpyrrole fungicide introduced by Ciba-Geigy in 1988 as a seed treatment in cereals [14]. The introduction of fenpiclonil was followed by the second phenylpyrrole fungicide fludioxonil, which provides improved biological properties compared with fenpiclonil and, therefore, subsequently replaced fenpiclonil as seed treatment [15, 16, 47].

Seed treatments with phenylpyrroles show limited uptake into the seed and seedling. Studies indicate that the major part of seed-applied fludioxonil remains on the surface of the seed or in the immediate vicinity of the seed. As a consequence a protective layer is built up around the seed, shielding it and seedlings against soil-borne infections, while the component of the active ingredient adhering directly to the seed acts against diseases located on the seed surface or below the seed coat. About 4% of the applied amount penetrates the seed during germination, providing control against deep-seated fungal infections, and a proportion also reaches the coleoptile, where it controls *Fusarium* spp., e.g., *Monographella nivalis* or *F. culmorum* [48].

One of the strengths of fludioxonil is its broad coverage of many different species from the *Fusarium* group, including *F. graminearum*, *F. oxysporum* and *F. solani* [49–51]. Besides causing direct effects, including reduced emergence and seedling growth, many *Fusarium* species are producers of potent mycotoxins that can lead to severe health damage when consumed by livestock or humans [52, 53].

Table 15.2.4 Uses of fludioxonil as a seed treatment.

Crop	Diseases	Rate in g-a.i. per 100 kg seed
Cereals (wheat, barley, rye)	*Claviceps purpurea*	5
	Cochliobolus sativus	5
	Fusarium culmorum	5
	Monographella nivale	5
	Phaeospheria nodorum	5
	Pyrenophora graminea	5
	Tilletia caries	5
Maize	*Fusarium graminearum*	2.5
Cotton	*Fusarium* spp.	2.5
	Rhizoctonia solani	2.5
Peanut	*Rhizoctonia solani*	2.5
Peas	*Mycosphaerella pinoides*	10
Potato	*Fusarium* spp.	2.5
	Helminthosporium solani	2.5
	Phoma exigua	2.5
	Rhizoctonia solani	2.5
Rice	*Gibberella fujikuroi*	5
Soybean	*Fusarium* spp.	2.5
	Sclerotinia sclerotiorum	2.5
	Rhizoctonia solani	2.5

Source: Internal data Syngenta Crop Protection AG.

Fludioxonil is highly active against the causal agent of snow mould in wheat and rye, *Monographella nivale*, providing excellent long-term control both in growth chamber and field trials [54]. Owing to its different mode of action no cross-resistance to benzimidazole fungicides occurs [40] and therefore benzimidazole-insensitive strains of *M. nivale* are also controlled.

On corn it provides protection against fungi of the genera *Fusarium*, *Rhizoctonia*, and *Helminthosporium* and weakly pathogenic fungi of the genera *Penicillium* and *Aspergillus* [50]. In potato, fludioxonil controls silver scurf caused by *Helminthosporium solani* [55], black leg caused by *Rhizoctonia solani* [56], gangrene caused by *Phoma exigua* and *Fusarium* dry rots [57, 58]. In oil seed rape the seedling disease complex caused by *Fusarium* spp., *Rhizoctonia* spp. and *Alternaria* spp. is controlled by fludioxonil [59]. In peas fludioxonil controls foot rot disease caused by *Mycosphaerella pinodes* [60].

For seed treatment purposes fludioxonil is generally formulated as an FS (Flowable for Seed Treatment). Fludioxonil is very safe to seeds and seedlings and has

shown no negative effects on emergence or on growth and development of cereals, maize, cotton and other plant species. Owing to its broad activity and good crop tolerance fludioxonil is used worldwide as a seed treatment in many different crops against a range of important diseases (Table 15.2.4).

Several combinations of fludioxonil with other fungicides are available to complete the spectrum of activity.

15.2.1.2.4 Biological Mode of Action

The primary effect of fludioxonil is a very efficient inhibition of conidia germination *in vitro* [61]. Experiments with *B. cinerea* on grape leaves (Table 15.2.5) confirmed the inhibitory effect of fludioxonil on conidia germination [62].

The pronounced effect on germination of *Botrytis* conidia on the leaf surface explains the excellent efficacy of fludioxonil after preventive application – application timings before inoculation.

Table 15.2.5 Inhibitory effect of fludioxonil on conidia germination for *B. cinerea*.

Rate (mg-a.i. L^{-1})	Time after inoculation (h)		
	24 h	72 h	120 h
	% Inhibition of conidia germination		% Control of leaf attack
0.1	9	2	0
1	95	59	95
10	100	100	100

Germination on untreated plants 99%; % leaf attack on untreated plants 100%.

15.2.2 Quinoxyfen

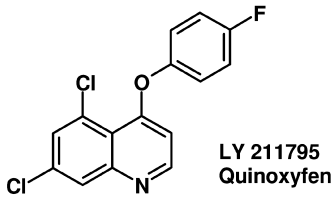
15.2.2.1 Chemistry

15.2.2.1.1 Introduction

Quinoxyfen (**17**, Fig. 15.2.3) [63], developed by Dow–Elanco, belongs to a new chemical class of fungicides, the phenoxyquinolines, possessing specific activity against powdery mildews.

15.2.2.1.2 Chemistry

The excellent control of wheat powdery mildew of LY-186054 (**18**, Fig. 15.2.3) was discovered by Eli Lilly [64] during routine screening. After proving that this



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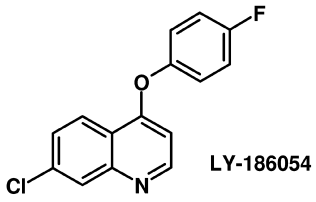

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Fig. 15.2.3. Quinoxyfen (LY 211795, **17**) and LY-1 186054 (**18**).

4-phenoxyquinoline showed no cross-resistance with known commercial mildewicides, an optimization program was started for this new lead. Systematic modifications of ring A through C as well as of the linker in **19** led to the SAR shown in Fig. 15.2.4.

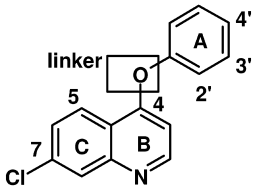

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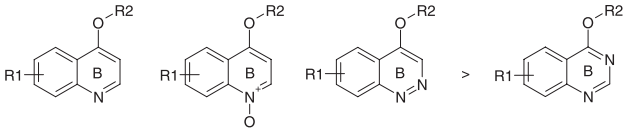
Fig. 15.2.4. SAR of 7-chloro-4-phenoxyquinoline. Best activity was seen for: A = 4′-F; 2′,4′-F_2; 2′-CF_3; 2′-NO_2; 2′-Me-4′-F; linker = O; OCH_2; $O(CH_2)_2$; CH_2; $OCH(CH_3)$. Variation of B:



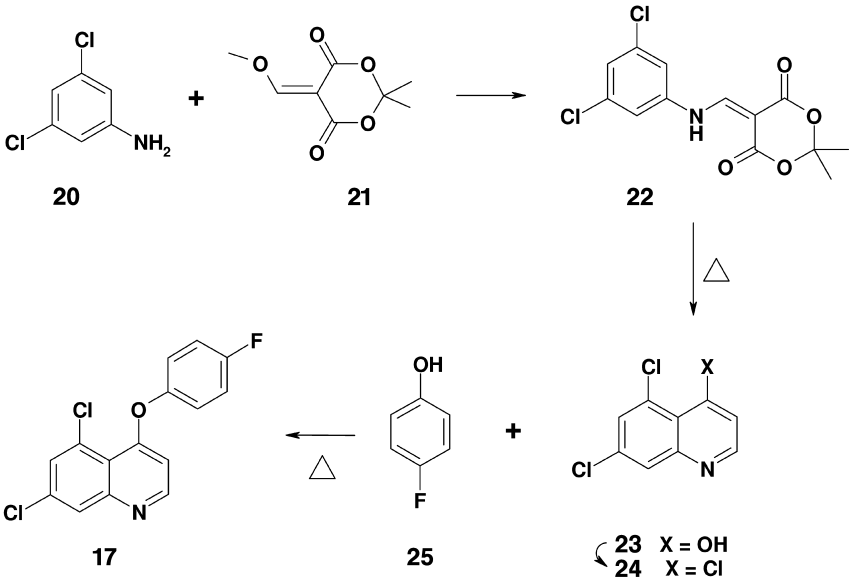
C = 5,7-Cl_2; 7-Cl; 5,7-$(Me)_2$; 6-F-7-Cl; 5,7-Cl_2-6-F; 7-Br; 5-NO_2.

Interestingly, the 8-chloro analogues of LY-186054 (**18**) showed diminished powdery mildew control yet were active against botrytis and other commercially interesting species of fungi. This shift in the biological profile is based on their different MOA; 8-haloquinolines are known inhibitors of dihydroorotate dehydrogenase [65].

Field assessment of several analogues as powdery mildewicides on various host crops led to the selection of LY-211795, quinoxyfen (**17**, Fig. 15.2.3) as a development candidate.

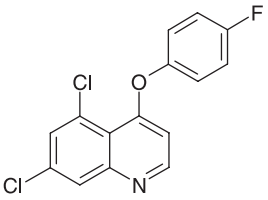
Preparation of quinoxyfen is described in EP 326 330 [63]. Synthesis of 4,5,7-trichloroquinoline (**24**) follows known synthetic procedures. Addition of aniline **20** to the Michael acceptor **21** is followed by cyclization of **22** to a 4-

Synthesis of Quinoxyfen



Scheme 15.2.4

Table 15.2.6 Chemical and physical properties of quinoxyfen (**17**). (From *The Pesticide Manual*, 13th edn.)



Common name	Quinoxyfen
Melting point (°C)	106–107.5
Vapor pressure at 25 °C (Pa)	1.2×10^{-5}
Solubility in water at 20 °C, pH 6.45 (mg L^{-1})	0.116
Partition coefficient	K_{ow} log $P = 4.66$ (pH 6.6, 20 °C)
Hydrolysis	In dark at 25 °C, stable to hydrolysis at pH 7 and 9
Light stability	Degraded more rapidly in light

hydroxyquinoline **23** (Scheme 15.2.4). Treatment of **23** with phosphoryl chloride gives **24**, which after heating to about 140 °C together with 4-fluorophenol (**25**) provides the desired ether quinoxyfen (**17**). An improvement in the last step using 4-dialkylaminopyridine as a catalyst is described in EP 569 021 [66]. Table 15.2.6 lists the properties of quinoxyfen.

15.2.2.1.3 **Safety Profile**

Quinoxyfen can be considered as a toxicological relative safe compound [67, 68]. With the exception of some aquatic species, quinoxyfen has a very desirable tox profile towards nontarget organisms in the environment.

15.2.2.2 **Biology**

Quinoxyfen was introduced in 1996 [68] by Dow-Elanco and has been in commercial use since 1997 for the control of powdery mildews. Quinoxyfen is specifically active against powdery mildews on cereals and horticultural crops [69, 70–72].

It is active after protective application but does not show any curative activity. It is recognized by a long-lasting activity that can remain on cereals up to 42 days. Quinoxyfen interferes with conidia germination and appressorium formation in the life cycle of target fungi [73] but haustorium formation and further development of the disease are not affected, thus explaining the strong preventive activity and the lack of activity in curative situations. Quinoxyfen is redistributed in and on the treated plant through the xylem and by superficial vapor movement [74].

A high frequency of wheat powdery mildew isolates resistant towards quinoxyfen have been reported from Northern Germany [75]. Field isolates of *Uncinula necator* with reduced sensitivity to quinoxyfen were found in 2003 but the efficacy level under practical field conditions remained unchanged [76].

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16
Fungicides Acting on Mitosis and Cell Division

16.1
Zoxamide, an Antitubulin Fungicide for Control of Oomycete Pathogens

David H. Young

16.1.1
Introduction

Zoxamide (**1**) [3,5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide, RH-7281, Fig. 16.1.1] was discovered and commercialized by Rohm and Haas Company in 2001 for control of Oomycete pathogens [1], and is now marketed by Dow AgroSciences LLC. The primary markets for zoxamide are late blight on potatoes and downy mildews on vines and vegetables. Inhibitors of microtubule assembly that have been used as fungicides include the benzimidazole (BZ) and thiophanate fungicides such as carbendazim and thiophanate-methyl, the N-phenylcarbamate (NPC) diethofencarb, and the antibiotic griseofulvin. Zoxamide represents the first inhibitor of microtubule assembly to be used commercially for control of Oomycetes.

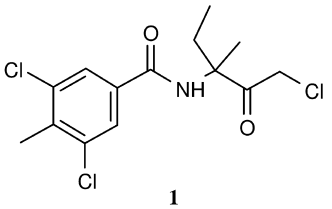

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Fig. 16.1.1. Zoxamide.

16.1.2
Mechanism of Action

Microtubules are hollow filaments of the cytoskeleton composed primarily of tubulin, a dimeric protein consisting of α- and β-subunits, each of which is ap-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

proximately 55 kDa. As components of the mitotic spindle, microtubules play a critical function in separating the daughter chromosomes during nuclear division. Since this role requires reversible assembly from free tubulin, nuclear division can be blocked by agents such as colchicine that inhibit assembly or by compounds like paclitaxel that stabilize microtubules and prevent their disassembly [2].

Zoxamide belongs to a class of benzamides that shows activity towards a broad range of organisms, including both Oomycete and non-Oomycete fungi [1], protozoan [3], plant [4] and mammalian cells [5, 6]. The relative potency of analogs towards different organisms varies widely depending on their structure [6]. At the cellular level, these benzamides arrest nuclear division and destroy the microtubule cytoskeleton [4, 6, 7]. This loss of microtubules results from inhibition of microtubule assembly caused by a highly specific covalent binding to cys-239 on the β-subunit of tubulin [6, 7].

16.1.3
Analysis of the Benzamide Binding Site using Radioligand Binding Assays

The covalent binding property of benzamides has been exploited in the development of cell-based competitive binding assays that measure the ability of other antitubulin agents to inhibit binding of radiolabeled benzamides to β-tubulin in whole cells. The tritiated S-enantiomer of zoxamide has been used to study the zoxamide binding site in the Oomycete *Phytophthora capsici* [7]. Tritiated analogs **2** and **3** (RH-4032 and RH-5854, Fig. 16.1.2) have been used in similar assays in plant [4] and mammalian cells [6], respectively.

The experimental benzamide fungicide zarilamide (**4**, Fig. 16.1.3 below) was discovered by ICI in the 1980s [8] and was found to act on microtubules [9]. Zarilamide was later shown to inhibit ^{3}H-(*S*)-zoxamide binding to β-tubulin in *Phytophthora capsici* in a competitive manner [7], indicating a common binding site with zoxamide.

In tobacco cells, the antimitotic herbicides pronamide (**5**) and the NPC chlorpropham (**6**) inhibited binding of ^{3}H-RH-4032 to tobacco tubulin, suggesting a common binding site with zoxamide-related benzamides [4]. Similarly, colchicine

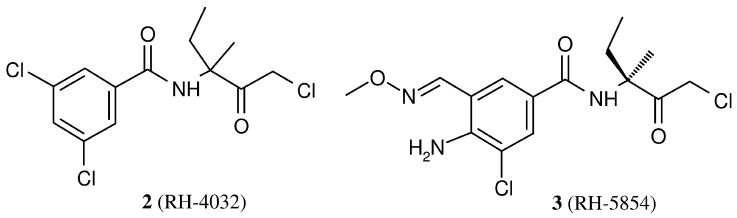

Fig. 16.1.2. RH-4032 and RH-5854.

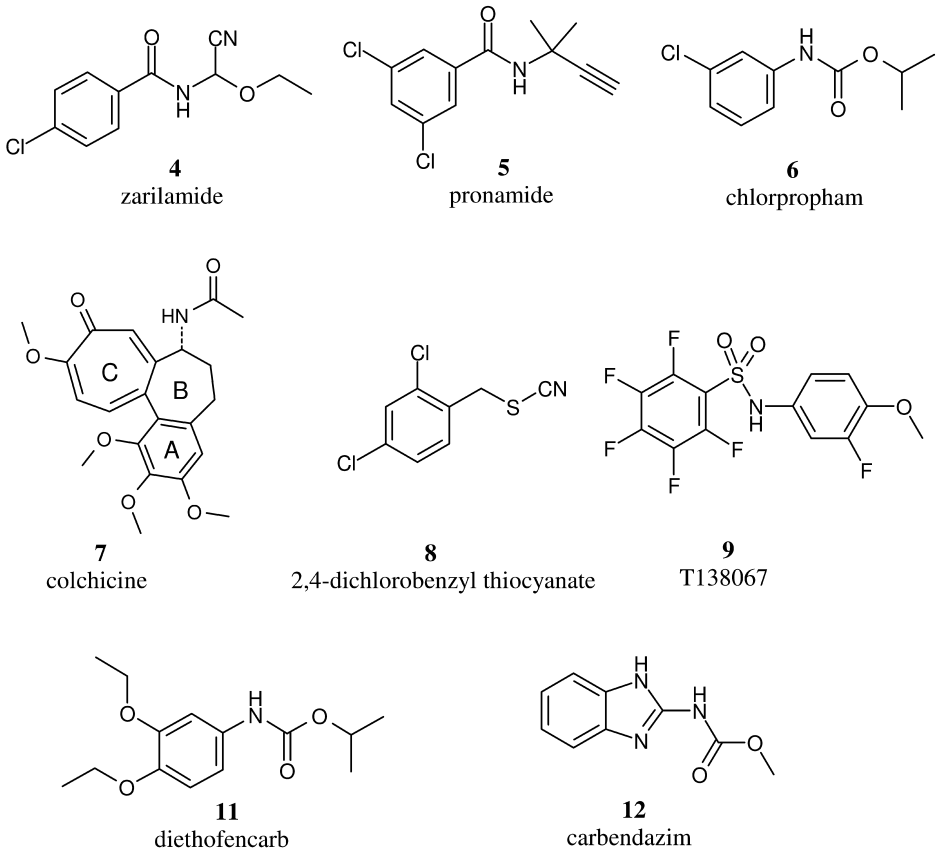

Fig. 16.1.3. Representative antitubulin compounds believed to bind at the benzamide site.

(**7**) and various other ligands of the colchicine site, including the BZ nocodazole, podophyllotoxin, tubulozole C and TN-16, were found to inhibit binding of ^{3}H-RH-5854 to mammalian tubulin [6]. In contrast, the anticancer drug vinblastine, which is known to bind to a different site from colchicine [2], enhanced ^{3}H-RH-5854 binding, presumably through an allosteric effect [6].

Covalent binding to cys-239 in β-tubulin is involved in the mechanism of action of some other antitubulin compounds. These include 2,4-dichlorobenzyl thiocyanate (**8**) [10], the experimental anticancer drug T138067 (**9**) [11], and the bifunctional reagent *N*,*N*′-ethylenebis(iodoacetamide) [12]. The latter compound forms a crosslink between cys-239 and cys-354 in a reaction that occurs in free tubulin, but not in intact microtubules [12]. As in the case of the zoxamide analog RH-5854 [6], reaction of cys-239 with these various agents was inhibited by ligands of the colchicine site [10–12], and with T138067 and *N*,*N*′-ethylenebis(iodoacetamide) was enhanced by vinblastine [11, 12]. Cys-239 has also been shown to bind

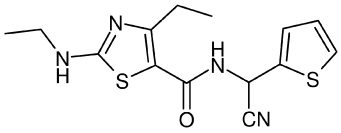

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Fig. 16.1.4. Ethaboxam.

to chemically reactive analogs of colchicine in which methoxy groups on the A-ring of colchicine are replaced by chloroacetyl groups [13]. Moreover, recent modeling studies of the colchicine binding site place cys-239 close to the A-ring [13, 14]. The location of cys-239 in the colchicine binding site correlates well with the ability of colchicine site ligands to inhibit binding of radiolabeled benzamides to tubulin and implies a common binding region for zoxamide and colchicine.

Representative compounds believed to bind at the benzamide site on the basis of evidence from competitive binding or cross-resistance studies (discussed below) are shown in Fig. 16.1.3. Although these compounds differ from zoxamide in their relative toxicity toward different organisms, they appear to bind to the same domain on β-tubulin. Selective toxicity may be governed by structural differences between organisms in this domain.

Ethaboxam **10**, a fungicide currently being developed by LG Life Sciences for the Oomycete market (Fig. 16.1.4), was recently reported to act by disruption of microtubules in *Phytophthora infestans* [15]. Ethaboxam bears some structural similarity to zarilamide; however, it has not yet been established whether ethaboxam binds to tubulin and, if so, whether it binds to the same site as other benzamides.

16.1.4
Cross-resistance Relationships between Zoxamide, Carbendazim and Diethofencarb

Although zoxamide is only used commercially to control Oomycete pathogens, it is also active against other fungi [1]. These include some pathogens in which field resistance to BZs has occurred, such as *Botrytis cinerea*, *Venturia inaequalis*, *Monilinia fructicola*, *Mycosphaerella fijiensis* and *Cercospora beticola*. Resistance to BZs in the field occurred shortly after their introduction due in part to their widespread, exclusive use [16]. Subsequently, the finding of negatively-correlated cross-resistance between NPC herbicides and BZs led to the discovery and commercialization of the NPC diethofencarb (**11**) to combat BZ-resistant fungi [17]. However, the combined use of diethofencarb and the BZ carbendazim (**12**) resulted in strains resistant to both compounds [18]. Resistance to carbendazim and diethofencarb in field isolates is determined by allelic mutations at positions 198 and 200 in β-tubulin [19].

Analysis of cross-resistance relationships between zoxamide, carbendazim and diethofencarb in such resistant isolates has provided further information about

Table 16.1.1 Cross-resistance relationships between zoxamide, carbendazim and diethofencarb in *Botrytis cinerea* field isolates.

Phenotype	No. strains	$EC_{50} \pm$ S.D. ($\mu g\ mL^{-1}$)		
		Zoxamide (1)	Carbendazim (12)	Diethofencarb (11)
Sb[a]	9	0.97 ± 0.36	0.045 ± 0.009	>50
Rb1[b]	6	0.073 ± 0.039	>50	0.077 ± 0.028
Rb2[c]	4	>50	9.3 ± 1.08	>50

[a] Sb = sensitive to benzimidazoles.
[b] Rb1 = resistant to benzimidazoles and sensitive to diethofencarb.
[c] Rb2 = resistant to both benzimidazoles and diethofencarb.

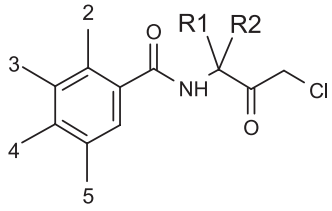
the zoxamide binding site [20]. As shown in Table 16.1.1, carbendazim-resistant isolates of *Botrytis cinerea*, which are sensitive to diethofencarb due to negatively-correlated cross-resistance (Rb1 phenotype), show greatly enhanced sensitivity to zoxamide. Furthermore, strains that are resistant to both carbendazim and diethofencarb (Rb2 phenotype) are resistant to zoxamide. The fact that mutations in β-tubulin that alter sensitivity to carbendazim and diethofencarb also affect sensitivity to zoxamide suggests a common binding domain for benzamides, BZs and NPCs. This conclusion is supported by results from binding assays that showed inhibition of benzamide binding in mammalian cells by the BZ nocodazole [6] and in plant cells by the NPC chlorpropham [4]. It is also consistent with the aforementioned binding of colchicine to the same region as benzamides, since colchicine and BZs bind to the same domain [2].

16.1.5 Structure–Activity Relationships

The α-haloketone-containing benzamide series that led to zoxamide originated in a herbicide synthesis effort to make analogs of the herbicide pronamide (**5**) [21]. Although pronamide is not fungicidal, early α-haloketone analogs such as **13** (Table 16.1.2) were found to be highly active towards Oomycete fungi as well as plants. Despite the fact that activity towards Oomycetes and plants was based on the same mode of action [4, 7] the relative potency towards Oomycetes and plants could be modulated by structural changes.

In attempts to identify analogs with high fungitoxicity and low phytotoxicity, over 300 analogs were prepared. These were tested in parallel in an *in vitro* fungitoxicity assay against *Pythium ultimum* and in a tobacco root elongation assay, with the ratio of the EC_{50} values providing a measure of selective toxicity towards Oomycetes. Appropriate substitution of the phenyl ring was essential for strong biological activity (Table 16.1.2). Substitution at the 3 and 5 positions (**13**) dramati-

Table 16.1.2 Optimization of potency and crop safety.



Compound	Substituent						Potency		
	2	3	4	5	R1	R2	*Pythium* EC_{50}[a]	Tobacco EC_{50}[b]	*Pythium* EC_{50}/ tobacco EC_{50}
13	H	Cl	H	Cl	CH_3	CH_3	0.024	0.006	4.0
14	H	H	H	H	CH_3	CH_3	12.0	ND	–
15	Cl	Cl	CH_3	Cl	CH_3	C_2H_5	3.49	0.030	3.0
2	H	Cl	H	Cl	CH_3	C_2H_5	0.007	0.004	1.7
1 (zoxamide)	H	Cl	CH_3	Cl	CH_3	C_2H_5	0.006	0.017	0.35

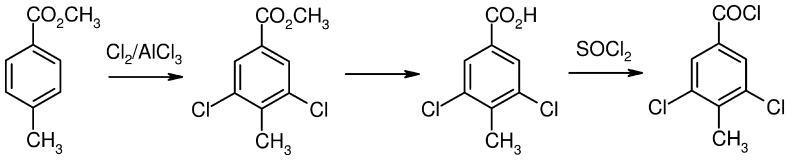
[a] EC_{50} ($\mu g\ mL^{-1}$) for inhibition of *Pythium ultimum* growth.
[b] EC_{50} ($\mu g\ mL^{-1}$) for inhibition of tobacco root elongation.

cally increased activity over the unsubstituted ring (**14**), whereas substitution at the 2-position greatly reduced activity (**15**). Methyl and Et in the R1 and R2 positions, as in **2**, were found to be optimal for fungitoxicity. A key finding was the discovery that when R1 = Me and R2 = Et, certain 4-substituents on the phenyl ring reduced phytotoxicity with little or no reduction in fungitoxicity [22]. Thus, the combination of 3,5-dichloro, 4-methyl as favorable substituents on the phenyl ring with Me and Et in the R1 and R2 positions yielded **1** (zoxamide) as an experimental compound with an improved fungitoxicity/phytotoxicity ratio that showed outstanding disease control in greenhouse and field-tests and no phytotoxicity on whole plants.

Zoxamide consists of two enantiomers; however, its biological activity is due almost entirely to the S-enantiomer [23]. Although more active than the racemic mixture, manufacture and sale of the purified S-enantiomer was not economically attractive. Consequently, the commercial product is currently sold as the racemic mixture.

16.1.6
Synthesis of Zoxamide

Key precursors in the preparation of zoxamide are 3,5-dichloro-4-methylbenzoyl chloride (**16**) and 3-amino-3-methyl-1-pentyne (**17**). Compound **16** is prepared

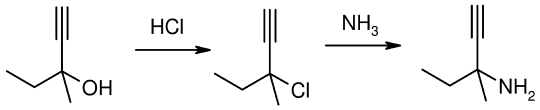


16

Scheme 16.1.1 Synthesis of intermediates of zoxamide.

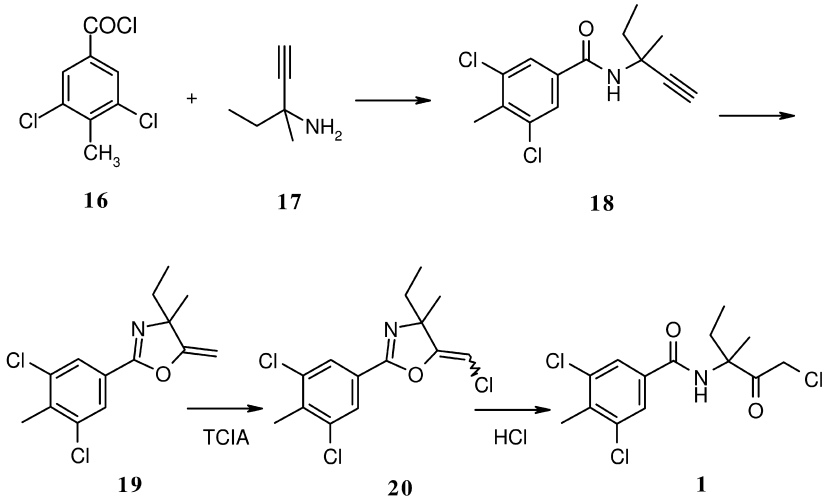
from methyl toluate (Scheme 16.1.1), and **17** from its corresponding acetylenic alcohol (Scheme 16.1.2).

Precursors **16** and **17** react to yield the alkynyl amide **18**, which is converted into the 5-methylene oxazoline **19** (Scheme 16.1.3). Chlorination of **19** employs trichloroisocyanuric acid (TCIA) as the chlorinating agent to produce the monochlorinated oxazoline **20**, which is converted into zoxamide (**1**) by acid-catalyzed hydrolysis.



17

Scheme 16.1.2 Synthesis of intermediates of zoxamide.



16 **17** **18** **19** **20** **1**

Scheme 16.1.3 Synthesis of zoxamide.

16.1.7
Resistance Risk

Since its first commercial use in 2001, there have been no reports of reduced pathogen sensitivity to zoxamide. Laboratory studies to investigate the potential for resistance development to the benzamide class have been carried out with zoxamide [24], and zarilamide [25], a benzamide that binds to the same site as zoxamide on β-tubulin [7]. In these studies, attempts to isolate resistant mutants in different Oomycetes using chemical mutagenesis, UV irradiation or adaptation were unsuccessful. These results suggest that the risk for resistance development to zoxamide in its commercial target pathogens is relatively low.

Despite the similarity between zoxamide and the BZ fungicides in their mechanism of action, the resistance risk for zoxamide in the field contrasts sharply with the serious resistance problems of the BZ fungicides [16]. A critical difference between zoxamide and the BZs is the nature of the pathogens against which these products are used. The Oomycete fungi targeted by zoxamide are diploid [26], whereas fungi in which BZ-resistance has occurred are haploid. A likely explanation for the low resistance risk of zoxamide is that a target site mutation that affects its binding would likely be recessive and would have little effect on sensitivity of diploid cells that were heterozygous with respect to the mutation [24, 27].

16.1.8
Metabolism and Toxicology

Zoxamide has low toxicity to mammals except for the potential to cause skin sensitization [28]. Based on laboratory studies, it poses very low risk to most non-target species [1, 28]. Environmental fate studies have shown that zoxamide dissipates rapidly in the environment due to hydrolysis, photodegradation in water and microbial metabolism. It has a half-life in soil of 2–10 days, low water solubility, and low soil mobility [1], resulting in little potential for leaching into groundwater.

16.1.9
Biology and Use in Agriculture

Zoxamide was developed under the trade name Zoxium®. It is sold primarily in mixtures with mancozeb under the trade names Gavel®, Electis®, Electis® Pro, Aderio®, Stimo®, Unikat® and Roxam®. Application rates are typically within the range 125–150 g-a.i. ha^{-1} in formulation with mancozeb at 1.2–1.4 kg-a.i. ha^{-1}. Spray intervals depend on the crop and disease but are usually 7–14 days. In addition to the mixtures with mancozeb, zoxamide is also co-formulated with cymoxanil and sold under the trade name Harpon®.

Zoxamide is highly active towards a broad range of Oomycete fungi and is used commercially on potatoes, vines and vegetables for control of late blight and

downy mildew diseases. Activity has also been demonstrated against certain non-Oomycete fungi, such as *Venturia*, *Sclerotinia*, *Mycosphaerella*, *Botrytis* and *Monilinia* spp. [1].

Consistent with its mode of action, the stages in fungal growth that are susceptible to inhibition by zoxamide are those dependent on nuclear division. Thus, zoxamide inhibits germ tube elongation and mycelial growth [7], and prevents the proper formation of zoospores by interfering with nuclear division in developing sporangia [29]. Zoxamide does not directly affect zoospore motility, encystment or germination, but arrests germ tube elongation coincident with the first cycle of nuclear division [7], and prevents penetration into the host plant. Zoxamide is not a systemic fungicide but does exhibit penetrant activity [1]. Good residual efficacy and excellent rainfastness result from a high affinity of zoxamide for the plant cuticle [1, 30]. Zoxamide is highly effective in controlling tuber blight [31, 32]. The mechanism of tuber blight control does not involve a direct effect on zoospore motility [29], and cannot result from a protective action in the tuber or soil since zoxamide is not systemic and has a short soil half-life. The mechanism of tuber blight control may involve reduced production of motile zoospores caused by inhibition of nuclear division in sporangia as they form on the plant surface [29].

Since zoxamide has a different mode of action from other products used in the Oomycete market, there is no likelihood of cross-resistance to existing products such as metalaxyl, dimethomorph, cymoxanil or strobilurins. Consequently, zoxamide provides a unique tool for resistance management in the Oomycete fungicide market.

Acknowledgments

The author is grateful to Brian Sheppard, Bob Ehr and Gary Roth for their helpful discussion and critical reading of the manuscript.

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16.2
Pencycuron, a Phenylurea Fungicide for *Rhizoctonia solani*

Isao Ueyama and Yoshio Kurahashi

16.2.1
Introduction

16.2.1.1 Overview of the Compound

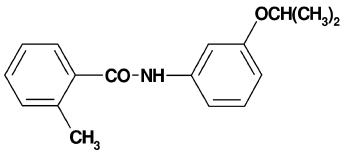
Pencycuron was invented in Nitokuno (Japanese subsidiary of Bayer CropScience) and the according application for patent was filed in 1976 [1]. This fungicide is specifically active against *Rhizoctonia solani* (perfect stage: *Thanatephorus cucumeris*), causes several important plant diseases such as rice sheath blight, potato black scurf, leaf blight of sugar beet and seedlings damping-off of various crops.

Pencycuron induces abnormal branching of the hyphae of the sensitive strains of *R. solani* and its activity is fungistatic [2]. This morphological change implied that the mode of action of pencycuron would be antimicrotubular, like carbendazime, and thus it is classified as "B4: Cell Division" in the FRAC code list. However, while carbendazime inhibits β-tubuline assembly in mitosis of *R. solani*, pencycuron does not act there but acts to destroy the cytoskeleton of microtubules. A clear-cut explanation of the mode of action of pencycuron is not yet available. Especially, the mechanistic elucidation as to why pencycuron acts only on quite limited strains of Anastomosis Groups of *R. solani* remains as an interesting theme.

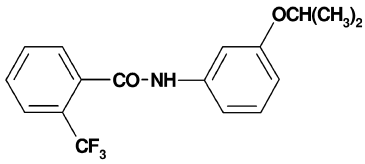
Although the compound was introduced to the market already in 1985, current turnover still achieved over 40 Mio US$; the major markets are Japan, Taiwan, Korea, Netherlands, Germany, France and UK.

16.2.1.2 Background of Pencycuron Invention

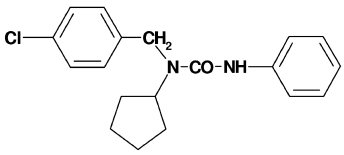
Following the rice blast diseases, sheath blight of rice is the second most troublesome disease for rice culture and significant crop damage by this disease has been reported every year, especially in Northeast Asian countries. In 1970s, organic arsine fungicides were used in Japan to control rice sheath blight. Although these fungicides showed stable efficacy, the use of arsine compounds in the environment caused concerns of safety and pollution and sales of these products were stopped. Instead of arsenic compounds, two antibiotics; Polyoxin (Kaken) and Validamycin (Takeda) were developed. But these antibiotics were insufficient in long-lasting efficacy. Therefore, the launch of new sheath blight control fungicides having satisfactory effect with good plant compatibility was keenly expected in the Japanese market. Under these circumstances, several compounds were developed in Japan. First mepronil (Kumiai) was launched in early 1980. Flutolanil (Nihon Nohyaku), pencycuron and diclomezine (Sankyo) were marketed one after another. Figure 16.2.1 depicts the chemical structures of these compounds.



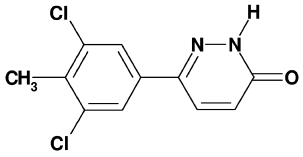
Mepronil
(Kumiai)



Flutolanil
(Nihon Nohyaku)



Pencycuron
(Nitokuno, BCS)



Diclomezine
(Sankyo)

Fig. 16.2.1. Representative rice sheath blight control fungicides developed in Japan since 1980.

Of these four fungicides, pencycuron was unique in terms of its narrow fungicidal spectra. Namely, the antifungal activity of pencycuron was extremely selective. There are both isolates sensitive and less sensitive (inherent resistant) to pencycuron even in the same Anastomosis Groups (AGs) of *R. solani*. In addition, only a quite narrow range of derivatives of the urea skeleton showed control activity against *R. solani*. Therefore, retrospectively speaking, it would seem to require a miracle to find one active molecule worth developing from this class of chemical structure.

16.2.2 Chemistry of Pencycuron

16.2.2.1 History of Pencycuron Invention

Figure 16.2.2 summarizes the perceived primary activity and each milestone compound on the way to reaching pencycuron. The compound was found in an indication shift: Normally chemists synthesize new compounds intended for a particular activity. With pesticides inquiry, however, a new finding was often seen in a different area from the anticipated one. For instance, the development of pencycuron originated from what was primarily a herbicide project.

In early 1950, many urea compounds with herbicidal activity had been studied and some of candidates were developed as practical herbicides. DCMU (*N*-3,4-

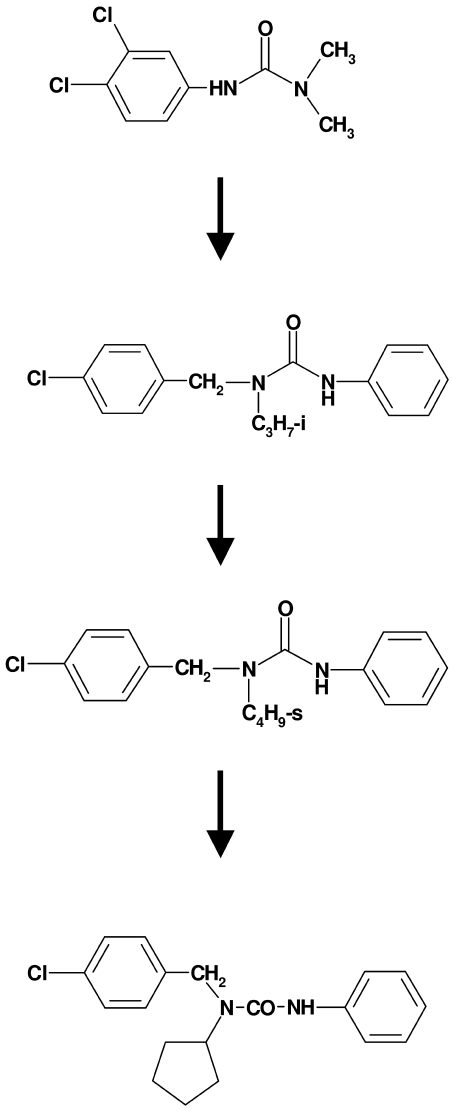
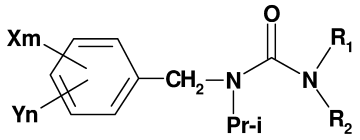


Fig. 16.2.2. Milestone compounds leading to pencycuron in chemical modification.

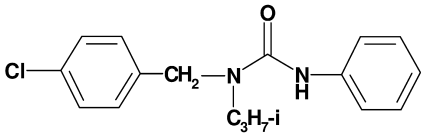
dichlorophenyl-*N*′-dimehylurea) was successfully developed by Du Pont in 1954. To find more active compounds, chemists tried to modify the urea molecule. However, most compounds were far from improving herbicidal activity. Under routine screening tests, neither fungicidal nor insecticidal activities could be seen.

Then, anticipating a new deployment, substituted benzyl was introduced instead of substituted phenyl at the N-position. A new general formula was pro-



X, Y: H, lower alkyl, halogen, CN, NO_2

R_1, R_2: H, lower alkyl, phenyl



NTN 15192

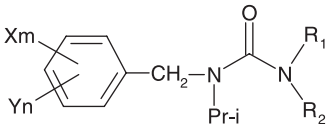
Fig. 16.2.3. General formulas for derivation to lead NTN 15192.

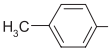
posed (Fig. 16.2.3). Based on this proposed formula, many compounds were synthesized, but, again, these did not show any biological activity, except just one molecule (NTN 15192), which showed weak active against *R. solani* (Table 16.2.1). In agar plate test results, however, NTN 15192 did not show any activity against various plant pathogenic fungi, except *R. solani*. Under higher tire screening tests (low concentration, long interval), the results were again unsatisfactory. The project was then stopped.

After two blank years, the fungicidal activity of NTN 15192 was reviewed, and the general formula for chemical modification was structured and the N-alkyl part was changed. Based on this new scheme, derivatives modified with N-alkyl and substituent at benzyl or phenyl ring were synthesized and one compound (NTN 16543) proved to be worth promoting to the next stage as it showed excellent efficacy against sheath blight of rice under greenhouse conditions (Table 16.2.2). Further studies under field trails revealed that this compound was prominent against sheath blight of rice and damping-off diseases caused by *R. solani*. However, it also showed low plant compatibility – the soil metabolism study revealed why. Namely, NTN 16543 decomposed in soil to form a des-benzyl moiety that showed slight herbicidal activity. Further efforts at compound derivation were assiduously exerted in accordance with the structure–activity relationship defined in this study. Finally, the team reached an N-cyclopentyl compound, (code: NTN 19701), overcoming many weak points shown by previous compounds. NTN 19701 was named ''pencycuron'' as common name, and the commercial name was ''Monceren®'', which was marketed in 1985 in Japan.

From these experiments we learnt that unintended small changes and scant signs observed in the study happened to be meaningful to future progress; care-

Table 16.2.1 Pot test results of the derivatives modified from urea herbicides.



X, Y	R1	R2	Effectiveness against *R. solani* on rice (0: Excellent, 5: no activity, – no trial) Concentration (ppm) in screening 1st 500	2nd tire 250	125
4-Cl	CH_3-	H	5	–	–
4-Cl		H	5	–	–
4-Cl		H	0	2	1
(= NTN 15192)					
4-CH_3		H	5	–	–
H		H	5	–	–
4-Cl		H	4	–	–
4-Cl		CH_3-	5	–	–
2, 4-Cl_2		H	5	–	–

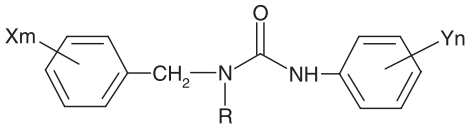
fully observing any symptoms in the tests and investigating minutely the results were very important in spotting signs of primal activity.

16.2.2.2 **Structure–Activity Relationships**

In reference to the chemical structure of NTN 15192 and NTN 16543, various N-alkyl compounds were tested (Table 16.2.2). However, none of the derivatives introducing those alkyls, such as methyl, ethyl, *n*-propyl, *n*-butyl, *i*-butyl, *t*-butyl, showed any biological activities. In contrast, *i*-propyl and *s*-butyl in the N-alkyl of the substructure showed obvious activity to *R. solani*. 4-Cl substitution at the benzyl ring was effective, but methyl or no substitution showed no effectiveness. Compounds substituted at phenyl ring were not effective as well.

Then derivatives modified at various parts of the chemical structure were elaborately synthesized and their activity evaluated. Table 16.2.3 shows representative

Table 16.2.2 Pot test results of the urea derivatives aiming for fungicidal activity (I).

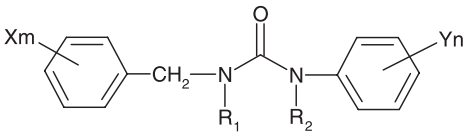


R	Xm	Yn	Effectiveness against *R. solani* on rice (0: Excellent, 5: no activity, – no trial) Concentration (ppm) in screening 1st 500	2nd tire 250	125
CH_3-	4-Cl	H	5	–	–
C_2H_5-	4-Cl	H	5	–	–
n-C_3H_7-	4-Cl	H	5	–	–
i-C_3H_7- (= NTN 15192)	4-Cl	H	0	1	2
n-C_4H_9-	4-Cl	H	5	–	–
s-C_4H_9- (= NTN 16543)	4-Cl	H	0	0	0

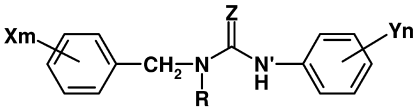
data used to elucidate the relation between the chemical structure and the *R. solani* control activity. Based on this data, a clear-cut, well-defined relation between chemical structures and biological activities was gradually unfolded. In the basic skeleton of the chemical structure (Fig. 16.2.4), substituents at benzyl (Xm) and phenyl rings (Yn) proved to be strictly limited for the chemicals to be active. Namely, ortho- or meta-substitution and multiple substitutions at the benzyl ring lead to the loss of activity. Various alkyls, including cyclo-alkyl combined at the N-atom, were tested. Although *i*-propyl and *s*-butyl were active, *n*-propyl and *n*-, *i*- or *t*-butyl showed no activity at all. Despite branching, such a structure at the first carbon atom of the alkyl may be needed, although *s*-pentyl was not active. With cycloalkyl groups, which also branch at the first carbon atom, the N-cyclopentyl compound was excellent in efficacy. However, N-cyclohexyl or N-cyclopropyl were clearly inferior to the former compound. Suitable bulkiness of the alkyl at the N-atom must be necessary.

Based on these screening results, the following rules about the relation of chemical structure and *R. solani* activity were proposed (Fig. 16.2.4): (a) Electron-withdrawing lipophilic substituents like Cl or Br at the para position of benzyl ring are necessary. But F or I atoms reduce the activity and electron-donating groups such as methyl or ethyl lead to loss of activity. (b) C_3–C_5 alkyl with branching at the first carbon atom is required (such as *i*-propyl, *s*-butyl and cyclopentyl group). (c) One proton is essential at the N′-position. (d) Substitutions at the phenyl ring lose the activity, though it remains with derivatives that are sub-

Table 16.2.3 Pot test results of the urea derivatives aiming for fungicidal activity (II).



Xm	R1	R2	Yn	Effectiveness against *R. solani* on rice (0: Excellent, 5: no activity, – no trial) Concentration (ppm) in screening 1st 500	2nd tire 250	125
4-F	*s*-C_4H_9-	H	H	1	3	–
4-Br	*s*-C_4H_9-	H	H	0	0.5	1
4-Cl	*s*-C_4H_9-	H	H	0	0	0
(= NTN 16543)						
2-Cl	*s*-C_4H_9-	H	H	5	–	–
3-Cl	*s*-C_4H_9-	H	H	5	–	–
2,3-Cl_2	*s*-C_4H_9-	H	H	5	–	–
4-Cl	*s*-C_5H_{11}-	H	H	5	–	–
4-Cl		H	H	5	–	–
4-CH_3		H	H	5	–	–
4-Cl		H	H	0	0	0
(= NTN 19701, pencycuron)						
4-Br		H	H	0.5	0.5	1
4-NO_2		H	H	0.5	1	2
4-Cl		H	H	4	–	–
3,4-Cl_2		H	H	5	–	–
4-Cl		H	4-Cl	5	–	–
4-Cl		CH_3-	H	5	–	–
4-Cl		H	3-OH-	0.5	1	2
4-Cl		H	4-OH-	1.5	2	3



Xm:	**para-position with electron withdrawing substituents**
	(such as Cl or Br, but not F or I)
R:	**3-5 alkyl with branching at the first carbon**
	(such as *i*-propyl, *s*-butyl, cyclopentyl)
N':	**One proton is essential.**
Ym:	**No substituents**
Z:	**O is slightly better than S.**

Fig. 16.2.4. Relation between chemical structure and *R. solani* control activity of various urea skeleton derivatives.

stituted at meta or para-OH. Thiourea derivatives with the same substructure were similar to but not better than the urea derivatives in efficacy.

16.2.3 Chemical Synthesis and Physicochemical Properties

16.2.3.1 Preparation of Pencycuron

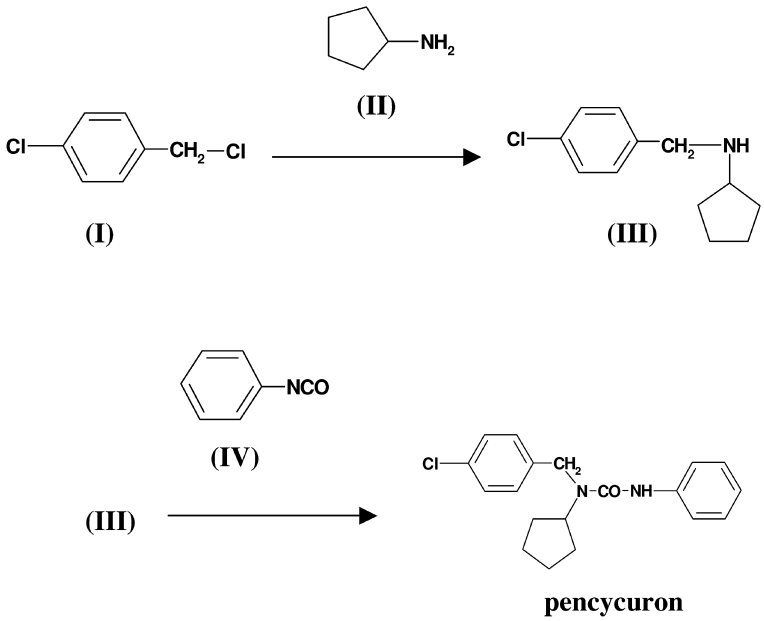
Pencycuron is obtained in two steps (Scheme 16.2.1).

4-Chlorobenzyl chloride (**I**) was added to a mixture of cyclopentylamine (**II**) and NaOH aqueous solution to give 4-chlorobenzyl-*N*-cyclopetylamine (**III**), which was isolated as a colorless oil (bp 109–110 °C/20 Pa).

Phenylisocyanate (**IV**) was then added to a solution of (**III**) in toluene, furnishing *N*-4-chlorobenzyl-*N*-cyclopentyl-*N'*-phenylurea (pencycuron) as a white solid (mp 129–134 °C).

16.2.3.2 Physicochemical Property of Pencycuron

The physicochemical properties of technical pencycuron were mentioned first in the literature in 1986 [2]; the data, including updates, are as follows. The melting points are 128 °C (form A) and 132 °C (form B). The vapor pressure is $<1.0 \times 10^{-5}$ Pa (20 °C), and the solubility in water is 0.3 mg L^{-1} at 20 °C. Log P_{ow} is 4.68 at 20 °C. In distilled water under dark conditions, pencycuron is rather stable: half-life is 76 days at pH 5, and no degradation is seen at either pH 6.6 or 8.8. But the half-life in distilled water under sun light conditions [in Japan, August, 8 h exposure, 338 W m^{-2} (300–3000 nm) at 23–27 °C] was just 2 days.



Scheme 16.2.1. Preparation of pencycuron.

16.2.4 Mode of Action and Biology

16.2.4.1 Mode of Action

^{14}C-Labeled pencycuron was applied to four strains of *R. solani* that differed in sensitivity to this fungicide [3]. Since there no metabolites existed in the medium and mycelia tested, which showed stronger fungicidal activity than the parent compound, it was suggested that pencycuron itself is ultimately the active substance, and metabolic activation or detoxification is less important in explaining the differences among the strains in sensitivity to pencycuron.

Several comparison studies were conducted by using other anti-*Rhizoctonia* chemicals such as validamycin, flutolanil and polyoxin [4, 5]. Biochemical experiments revealed that pencycuron did not affect trehalose biosynthesis, trehalase activity, and the biosyntheses of fatty acids, lipids, chitin, protein and DNA. Therefore, it was concluded that the mode of action of pencycuron was different from other existing rice sheath blight control fungicides. Sensitive strains of *R. solani* treated with pencycuron showed morphological changes of abnormal branching was observed. Since similar morphological changes are shown by benzimidazole fungicides, such as carbendazime, Leroux implied that pencycuron could produce antimicrotubular effects in fungi [6]. Using β-tubuline immunofluorescence microscopy technology, Ueyama and Araki demonstrated that while carbendazime inhibited β-tubuline assembly in the mitosis of *R. solani*, pency-

curon did not act there but acted to destroy the cytoskeleton of microtubules [7]. Later, however, Kim mentioned that pencycuron had no affect on the assembly of tubuline extracted from a sensitive strain of *R. solani* [8]. Rather, due to the high lipophilicity of pencycuron (log *P*; 4.82), this compound can be accommodated in lipid bilayers of fungal cells, resulting in a change in membrane fluidity [9]. A conclusive, clear-cut mode of action of pencycuron, especially an explanation as to why this fungicide acts on several limited strains of the AGs of *R. solani*, has not yet been discerned.

16.2.4.2 **Biology**

The primary infection of sheath blight originates from sclerotia that are floating in irrigation water in the paddy field, and come into contact with tillers of growth plants, especially at the tillering stage. Secondary infection takes place by extension of hyphae derived from the young lesions, proceeding laterally to the neighboring tillers during the periods from tillering to the heading of the rice stage and also upwards to the upper leaf sheath at the later stage. In addition, disease development is promoted under humid and high temperature conditions, and continues to the heading stage. Therefore, long-lasting efficacy is the key factor for controlling rice sheath blight disease.

Pencycuron is a non-systemic contact fungicide and is chemically stable. It exhibits sufficiently long-lasting efficacy during disease outbreak when it is used as a foliar application for rice sheath blight control.

In addition to rice sheath blight, pencycuron is effective against black scurf of potato, leaf and root rot of beet and damping-off diseases of various crop seedlings by *R. solani*. Black scurf of potato is well controlled by seed potato dipping. Leaf and root rot of sugar beet are also well controlled by foliar spray and/or soil drench application. Although damping-off diseases caused by *R. solani* can be well controlled by seed or soil treatment of pencycuron, pencycuron does not work for damping-off caused by soil and seed borne *Pythium* or *Fusarium*. In this case, mix application with other effective fungicides is recommended for the simultaneous control.

16.2.4.3 **Sensitivity to Several Anastomosis Groups (AGs) of *Rhizoctonia solani***

Pencycuron is extremely specific in fungicidal spectrum and does not show any substantial activity against all plant pathogenic fungi except *R. solani*. Besides, it was effective against only limited AGs of *R. solani*. Most of the economically important *Rhizoctonia* diseases, however, can be well controlled by appropriate applications of pencycuron. Namely, rice sheath blight (AG-1), black scurf of potato (AG-3), leaf blight and root rot of sugar beet (AG-2-2), stem rot of mat rash (AG-2-1) and main damping-off diseases of young seedlings (AG-4) are included in important diseases, which are covered by protection of pencycuron. Moreover, sensitive and insensitive strains to pencycuron are contained in the AG-2 and 4 (Table 16.2.4). Presently, 14 AGs of *R. solani* have been described in the literature [10] and a few subsets in most of the them have been reported, but the sensitivity of pencycuron was not sufficiently clear for all AGs or those subsets.

Table 16.2.4 Sensitivity of pencycuron to each Anastomosis Groups (AG) of *R. solani* and other plant pathogenic fungi.

Fungi and AG of *R. solani*	Strains code	Isolated plant	MIC ($\mu g\ mL^{-1}$)	Sensitive/ tolerant
R. solani				
AG-1	C-423	Rice	1.6	S
	R-1-2-1	Acacia	0.1	S
	HS-1	Rice	0.4	S
AG-2-1	C-121	Mat grass	0.4	S
AG-2-2	BV-30	Sugar beet	0.4	S
	I	Sugar beet	0.4	S
AG-3	C-563	Potato	0.4	S
AG-4	RC	Rice seeding	1.6	S
	Rh-131	Beet	>500	T
AG-5	SH-1	Soil	>500	T
	SH-19	Soil	>500	T
Rhizoctonia orizae	RO-23	Rice	>500	T
Pyricularia orizae	TH 67-22	Rice	>500	T
Alternaria mali		Apple	>500	T
Corticium rolfsii		Tobaccos	>500	T
Pythium sp.		Cucumber	>500	T
Sclerotinia sclerotiorum		Unknown	>500	T

16.2.5 Toxicology, Eco-toxicology and Metabolism

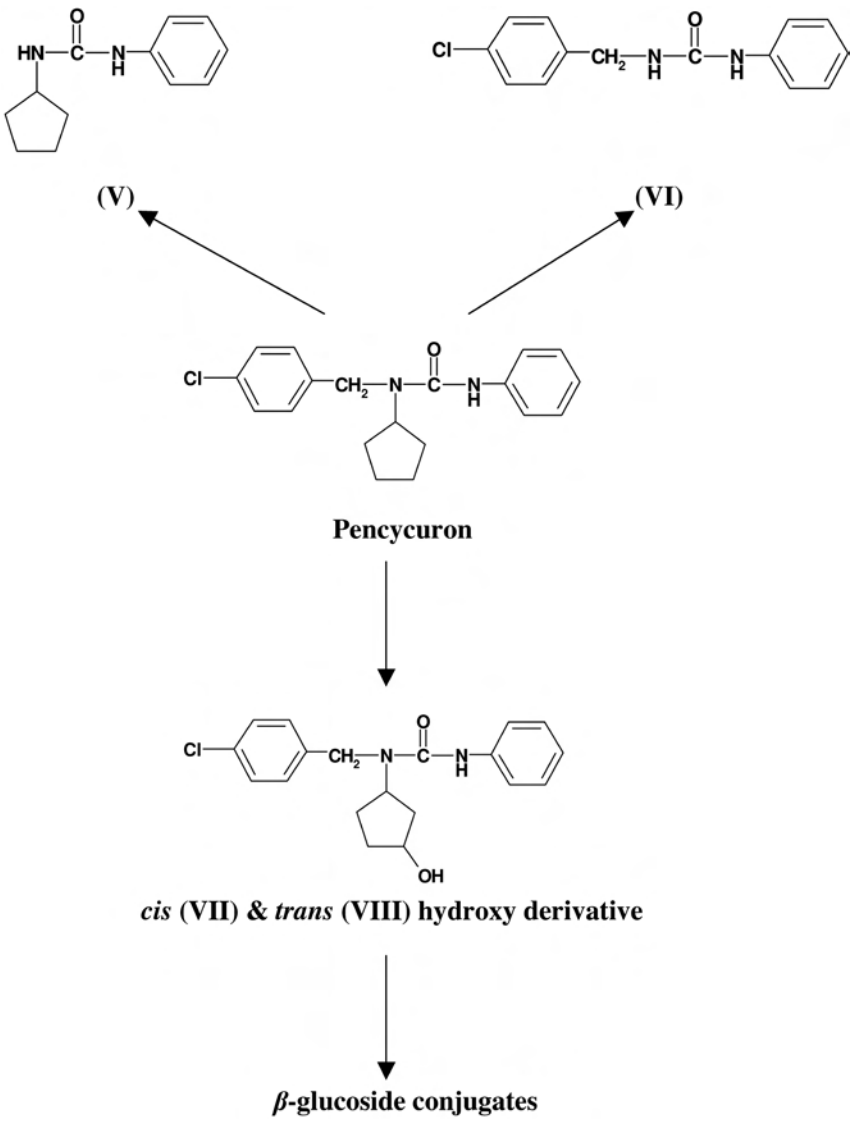
16.2.5.1 Toxicology and Eco-toxicology of Pencycuron

Several mammalian toxicological results are summarized in the literature [2]. The mammalian toxicity of pencycuron is quite low (acute oral LD_{50} for rats, mice, dogs $> 5\ g\ kg^{-1}$). Dermal, inhalational, skin function, chronic, teratogenicity and other toxicities are also quite favorable. This ''low toxicology'' tendency is also applies to other environmental biota such as fish, algae, daphnia and birds. The fact that no accidents have been reported that were caused by this fungicide during the more than 20 years since it was launched demonstrates clearly the favorable toxicological and eco-toxicological character of this compound.

16.2.5.2 Metabolism of Pencycuron

The metabolic fate of pencycuron in rice plants has been investigated with [phenyl-U-^{14}C]pencycuron [11]. When leaves were treated with [^{14}C]pencycuron the radiocarbon gradually penetrated into the leave tissues and part of them showed an acropetal movement. Most radiocarbon was, however, retained on the leave's surface. Pencycuron remained as a major component (52% of the applied

dose after 40 days) and its metabolites were relatively minor in amount (7% of the applied dose). Metabolites identified were 1-cyclopentyl-3-phenylurea (**V**), 1-(4-chlorobenzyl)-3-phenylurea (**VI**), 1-(4-chlorobenzyl)-1-(*cis*-3-hydroxycyclopentyl)-3-phenylurea (**VII**) and its trans isomer (**VIII**), and glycosides conjugates (Scheme 16.2.2). When [^{14}C]pencycuron was sprayed twice on rice plants before heading and at the heading stage, radioactive residues in rice grains were found as



Scheme 16.2.2. Metabolic pathways of [^{14}C]pencycuron in rice plants.

0.56 ppm as a pencycuron equivalent, but the radiocarbon was mainly located in the bran (85%). Intact pencycuron was detected at 0.018 ppm in hulled rice and 0.003 ppm in polished rice. Radiocarbon in grains remained as an unextractable bound residue.

In one mammalian metabolism study, ion-cluster analysis was applied to a rabbit examination [12]. Namely, after dosing with proton (= non label) and deuterium-labeled pencycuron (each 50% by volume), the urine and feces of rabbit were collected and the extracts were investigated by GC/MS. By comparison with over 20 authentic samples, it was revealed that the main degradation pathway was para-hydroxylation at the phenyl moiety and its β-glucuronic acid conjugation. Further hydroxylation occurred at the 3-position of the cyclopentyl moiety. In total, eleven metabolites were found, including five β-glucuronic acid conjugations.

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17
Sterol Biosynthesis Inhibitors

Karl Heinz Kuck and Jean-Pierre Vors

17.1
SBI Fungicides in Agriculture

Fungicides that inhibit targets within the fungal sterol biosynthesis have been the most important group of specific fungicides world-wide for over two decades. The biochemical basis of this success is the fact that fungi have specific sterols that differ from those in plants and animals, giving the chance to develop selective inhibitors.

Fungal cell membranes are characterized in most pathogens belonging to the Ascomycetes and Basidiomycetes by a common dominant sterol component, ergosterol. The designation "ergosterol" was generated by Tanret in 1889 as a result of studies with the ergot pathogen *Claviceps purpurea* [1].

Despite the general predominance of ergosterol, some exceptions have to be noted. In the important pathogen group of rust fungi, for example, fungisterol (ergost-7-enol), stigmast-7-enol and other sterols where found but no ergosterol [2]. Because ergosterol is the major sterol in most true fungi but not in all, the group name Sterol Biosynthesis Inhibitors (SBI) fungicides should be preferred to the designation EBI fungicides (Ergosterol Biosynthesis Inhibitors) which has been partly used in parallel.

Nevertheless, because ergosterol is the typical sterol in the vast majority of all fungi, the ergosterol content of food and plant material can be used as a quantitative indicator of fungal contamination or infection in all kinds of biological material. Therefore, studies are available that investigate the ergosterol content of many fungal species from different taxonomic groups [3, 4].

An important group of plant pathogens, the Oomycetes, lacks taxonomic affinity with the so-called true fungi (i.e., Ascomycetes and Basidiomycetes). Oomycetes, formerly regarded as fungi, have been excluded from the traditional "true fungi" of the kingdom Mycotae and have been included along with brown algae in the kingdom Chromista according to the newer classification [5]. More recently, Oomycetes have been classified with diatoms, the golden algae and the brown algae in a clade called Stramenophiles [6]. Important differences exist be-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

tween the Oomycetes and true fungi. For example, Oomycetes have cell walls composed mainly of β-glucans and cellulose and have only minor contents of chitin. Phytopathogenic Oomycetes are unable to perform the full *de novo* synthesis of sterols but can – to different degrees – metabolize exogenous precursors derived from plants. So, for example, some species of the order Peronosporales, which are unable to epoxidize squalene and thus to synthesize sterols, can metabolize exogenous cycloartenol to lanosterol and in some organisms to fucosterol, ergosterol, and cholesterol [7]. Consequently, SBI fungicides show no activity under field conditions against Oomycetes.

The fact that more than 40 SBI fungicides have reached the market stage is a proof of the interesting properties of inhibitors of this biosynthesis pathway. One important property is that targets within fungal sterol biosynthesis obviously give enough chemical room to synthesize a considerable diversity of highly active fungicides that are at the same time safe for treated plants and on the toxicological and environmental level. Secondly, SBI fungicides offer not only a broad spectrum of activity within the Ascomycetes and Basidiomycetes but, moreover, most SBIs are among one of the few fungicide groups that provide a pronounced curative and eradicative activity. Thirdly, the resistance risk of SBI fungicides is generally low to medium [8]. If resistance to SBIs occurs it usually has a multigenic basis resulting in a step-wise, continuous selection and not – as with benzimidazoles and strobilurins – in a disruptive selection. Stepwise selection, also designated as ''shifting'', gives good chances for a rational and effective resistance management. Moreover, gradual losses in activity can often be overcome by the introduction of new compounds exhibiting a higher intrinsic activity [9]. The possibility to compensate efficacy losses is supported by the fact that – although a general cross resistance is mostly found within the SBI classes I and II – at the same time a complete cross resistance between individual members of each class is quite rare.

17.1.1 Market Importance of SBI Fungicides

SBI fungicides have become the most important fungicide class overall. In 2004 the total fungicide market was estimated to have a total value of about 7.33 billion US$ [10]. As described in Chapter 12 (Table 12.2) over 30% of the fungicide market is covered by fungicides interfering with sterol biosynthesis. Table 17.1 clearly shows that by far the most important regional market for SBIs is in Western Europe, followed by Latin America. Taken together, both regions consume 70% of the overall SBI market. In addition, Table 17.2 shows that the success of SBI fungicides is essentially that of triazole fungicides as all other SBI mode of action classes and the chemical classes within DMI fungicides play only a limited role in terms of sales.

This regional distribution can be explained by considering the most important crops for SBI fungicides. Table 17.3 shows that the intensive wheat and barley production in Europe consumes a great part of the worldwide SBI production.

Table 17.1 Regional markets of SBI fungicides in 2004. (Data: Agrowin 2004.)

Region	%[a]
Western Europe	42
Latin America	28
North America	6
Eastern Europe	9
Asia/Pacific	12
Others	2

[a] % of total SBI market 2004.

Table 17.2 Market share (%) of individual SBI fungicides classes at the total fungicide market in 2004. (Data source: Phillips MacDougall, 2005.)

SBI class	Chemical subgroup	%[a]
DMI fungicides	Triazoles	25.7
	Imidazoles	1.3
	Others	0.8
Amines		2.7
Hydroxyanilides		0.6
		Total = 31.1

[a] % of total fungicide market 2004.

Table 17.3 Importance of individual crops for the use of SBI fungicides in 2004. (Data source: Agrowin.)

Crop	% of total SBI sales
Cereals	44.7
Soybeans	15.3
Fruits & nuts	11.7
Grapevines	6.2
Vegetables & flowers	5.9
Oilseed-rape	4.1
Rice	3.8
Beets	2.9
Other crops	5.5

Latin America has gained increasing importance for triazole fungicides because of the arrival of a new devastating disease, soybean rust (*Phakopsora pachyrhizi*), in Brazilian soybean production.

17.1.2 Biochemical Targets of SBI Fungicides

Several detailed reviews on the fungal ergosterol biosynthesis pathway and of fungicides interfering with it are available [11, 12]. Therefore, only a short, simplified overview is given here. Figure 17.1 shows the main biosynthesis steps, involving eleven enzymes, from squalene to ergosterol. Further information on the involved enzymes and the targets of agricultural fungicides is given in Table 17.4. The main primary biochemical consequences of an impaired ergosterol biosynthesis has been often a matter of debate. For an explanation, Vanden Bossche [13] cites Sisler and Ragsdale [14] who noted that a "Lack of ergosterol impedes the synthesis of new membranes and leads to deterioration of existing membranes." Associated with these changes it was observed that the fatty acid synthesis continues at a relatively high rate, resulting in a disproportion between fatty acid synthesis and utilization for phospholipids. Further, detailed studies have shown a multitude of consequences of ergosterol depletion, on one hand, and the accumulation of toxic precursors on the other hand [12].

Distinct plant growth regulatory effects have been noted with DMI fungicides since their introduction. Whereas un-specific signs of phytotoxic activity such as necrosis or leaf drop are only occasionally reported with DMI fungicides, more often pronounced plant growth regulatory side effect on plants have been noted. Typically, shorter shoots and internodes designated as "stunting", smaller dark green leaves and an improved stress tolerance of DMI treated plants are reported. Accordingly, specific plant growth regulators (PGRs) such as paclobutrazol have been developed out of the triazole group [15]. The beneficial side effects of fungicidal triazoles have been intensively studied and documented [16] and are an important part of biological profile of DMI fungicides in several crops such as oilseed rape (prevention of lodging) and cereal seed treatment (increased frost tolerance).

Investigations on the biochemical mechanisms causing these symptoms in plants have revealed that the inhibition of the biosynthesis of the plant hormone gibberellin and of plant sterols seem to be the most important targets in plants [17, 18].

17.1.3 SBI Classes

Table 17.5 gives an overview on the classes of agricultural SBI fungicides as defined in the FRAC classification. As described in Chapter 12 of this book [19], the major purpose of this classification is to facilitate resistance management on the farmer level. The table demonstrates well the need to come to a simple and

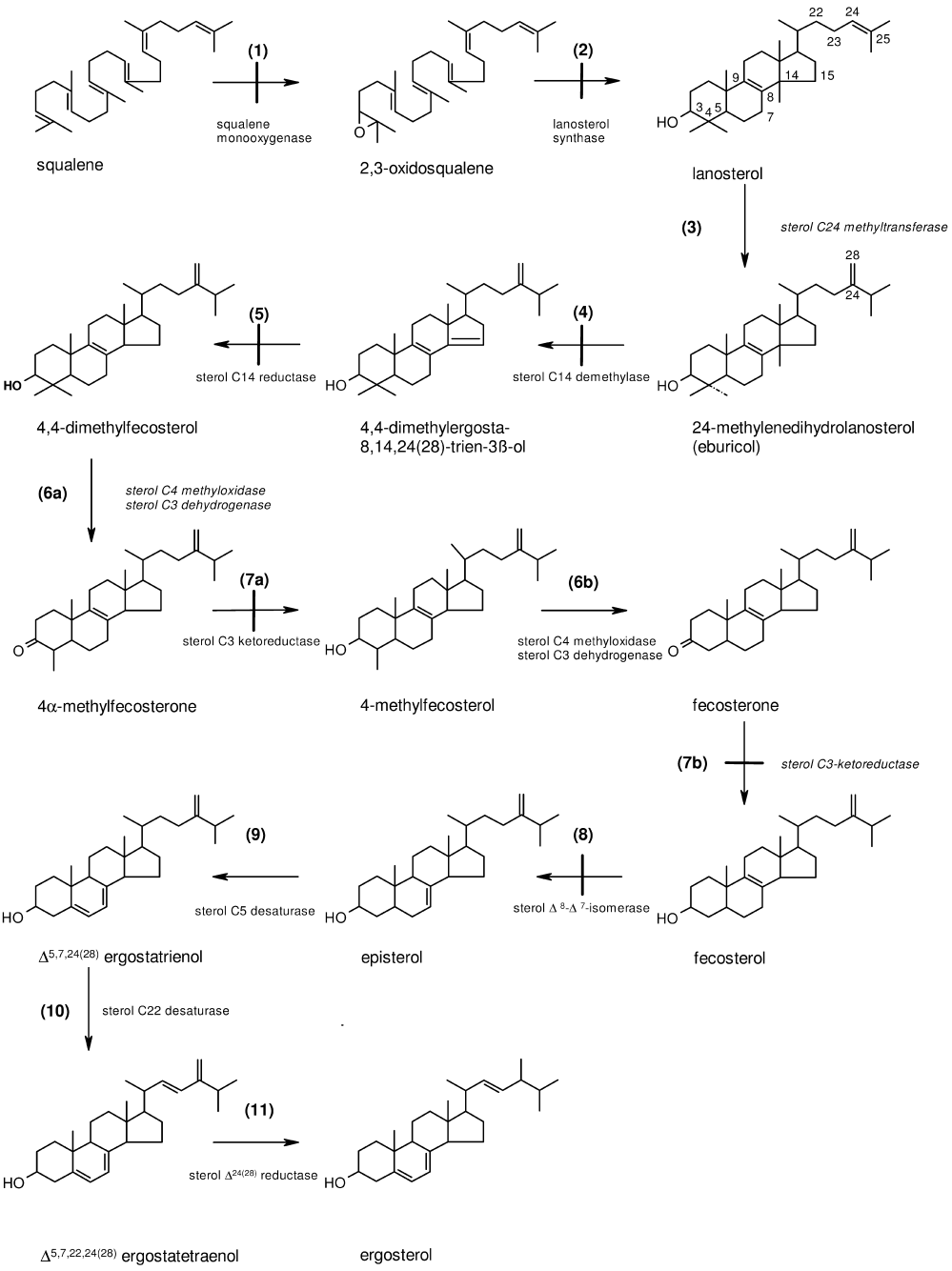


Fig. 17.1. Simplified pathway of ergosterol in most Ascomycetes and Basidomycetes, indicating sites blocked by SBI fungicides. Further information is given in Table 17.4 and the text.

Table 17.4 Enzymes involved in fungal sterol biosynthesis and targets of agricultural fungicides. Step numbers are those shown in Fig. 17.1.

Step no.	Enzyme	Gene; other enzyme; designations	Agricultural inhibitors
1	Squalene mono-oxygenase	Erg1; squalene-epoxidase; oxidosqualene synthase	Target of G4 inhibitors such as allylamines; side target of some amines (G2)
2	Lanosterol synthase	Erg 7; oxidosqualene cyclase	Side target of some amines (G2)
3	Sterol C24 methyl transferase	Erg6; sterol methyl transferase	
4	Sterol C14 demethylase	Erg11, CYP51; lanosterol 14α-demethylase	Target of the DMI fungicides (G1)
5	Sterol C14 reductase	Erg 24; sterol Δ^{14} reductase	Main target of fenpropidin and spiroxamine (G2)
6	Sterol C4 methyloxidase Sterol C3 dehydrogenase	Erg25 Erg26; sterol C4 decarboxylase	
7	Sterol C3 ketoreductase	Erg27	Target of hydroxyanilides (G3)
8	Sterol Δ^{8}–Δ^{7} isomerase	Erg2; sterol C-8 isomerase	Main target of tridemorph (G2)/secondary target of other amines (G2)
9	Sterol C5 desaturase	Erg3; C5 dehydrogenase	
10	Sterol C22 desaturase	Erg5; ergosterol Δ^{22} desaturase	
11	Sterol $\Delta^{24(28)}$ reductase	Erg 4; 24-methylene sterol, (24(28))-reductase	

Table 17.5 Grouping of SBI fungicides in the FRAC classification.

FRAC codes	G: Sterol biosynthesis inhibitors			
	G1	**G2**	**G3**	**G4**
Group name	**D**e-**M**ethylation **I**nhibitors: (**DMI**s)	Amines (formerly "morpholines")	Hydroxyanilides	Squalene-epoxidase inhibitors
Target in sterol biosynthesis	Sterol C14 demethylase	Δ^{14} reductase and $\Delta^7 \rightarrow \Delta^8$ isomerase	3-Keto reductase	Squalene-epoxidase
Chemistry	Piperazines, pyridines, pyrimidines, imidazoles, triazoles	Morpholines, piperidines, spiroketalamines	Hydroxyanilides	Thiocarbamates, allylamines

clear system to distinguish mode of action classes that are at the same time cross resistance classes. For example, the DMI group covers fungicides belonging to five different chemical classes although they all have the same biochemical target in common. A general (although mostly not complete) cross resistance within the DMI fungicide group has to be considered and is indicated by the common FRAC code number. No cross resistance has been found between different SBI classes, e.g., between DMIs and Amines. Accordingly, in some countries, e.g., in the United States, the FRAC codes are part of the label information and are the basis of resistance management programs.

Only the first three SBI classes have practical importance in plant protection. Squalene epoxidase inhibitors, although used as antimycotics in pharmaceutical applications, have until now not been launched as plant protection fungicides.

17.2 SBI Class I: DMI Fungicides

During studies on the mode of action of triarimol Ragsdale and Sisler [20] described, in 1973, for the first time fungal sterol biosynthesis in *Ustilago maydis* to be the target of this pyrimidine derivative, which has never reached the market stage but which is chemically closely related to fenarimol (Table 17.6 below). Later, Ragsdale [21] published that C14 demethylase was the most important target site affected within sterol biosynthesis by triarimol. Nowadays, C14 demethy-

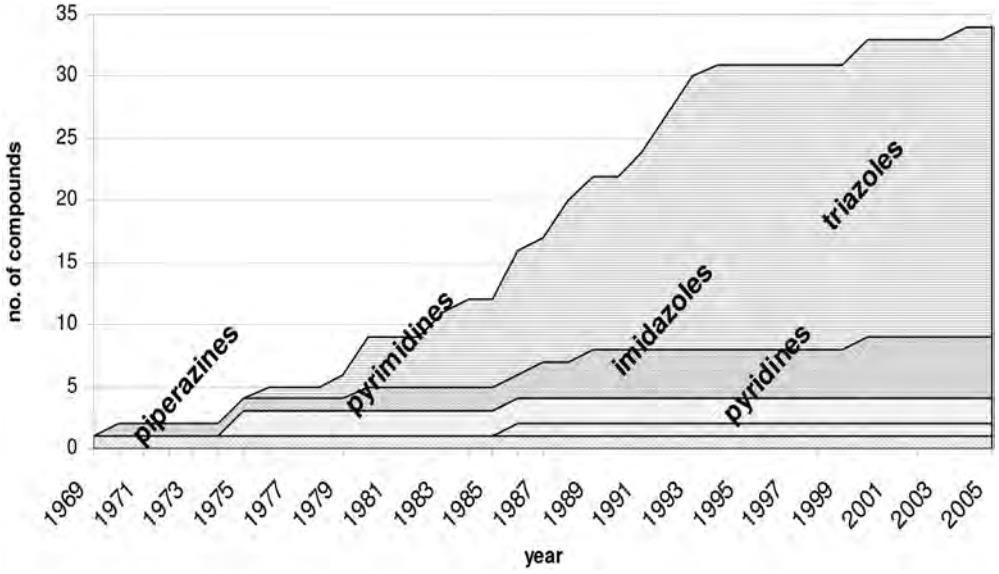


Fig. 17.2. Market launch of members of different chemical DMI groups.

lase is the common target of over 30 agricultural fungicides belonging to diverse chemical classes grouped together under the designation De-Methylation Inhibitors (abbreviated DMIs).

As already shown in Table 17.2, by their economical importance within the SBI fungicides the DMI fungicides are by far the most important mode of action class. Figure 17.2 demonstrates that – moreover – within the DMI fungicides one chemical class, the triazoles, dominates not only by their market share but also in the number of compounds having reached the market level. Another aspect becomes clear from Fig. 17.2: Although the first DMI fungicides, piperazines and imidazoles, had been brought to market level already at the end of the 1960s, the introduction of new DMI compounds still continues. During the last decade further imidazoles and, primarily, triazoles have been presented. This extraordinarily long life cycle of a fungicidal mode of action class is unique within the specific fungicides and an indirect proof of the commercial viability of this fungicide class. Although resistance to DMIs has been reported since the early 1980s with some pathogens such as powdery mildews, an adapted resistance management and the steady introduction of new compounds with a higher intrinsic activity has allowed maintenance of the efficacy of DMI fungicides on an economically highly competitive level for over 30 years [9].

As several reviews on DMI fungicides are already available [22–24], a short overview on some important DMI fungicides is given here and only more recent market introductions are treated in more detail.

17.2.1
Piperazines, Pyridines, Pyrimidines and Imidazoles

Fungicides belonging to the piperazines, pyrimidines and imidazoles were the first DMI fungicides to enter the agricultural market. The first and only pyridine compound, pyrifenox, entered the market in 1986. With the exception of the imidazole derivatives pefurazoate and oxpoconazole no further market introductions of representatives belonging to these four chemical classes could be noticed since the mid-1980s. Table 17.6 gives an overview of the most important compounds launched before 1990. One compound, prochloraz, rapidly gained a dominant position in the 1980s for the control of the cereal eyespot pathogens (*Oculimacula yallundae* and *O. acuformis* = *Tapesia yallundae* and *T. acuformis* = *Pseudocercosporella herpotrichoides*) whereas the other fungicides shown in Table 17.6 are predominately used in broadleaved crops and ornamentals against leaf spot and powdery mildew diseases.

17.2.1.1 **Pefurazoate**

In 1985 the Japanese companies Ube and Hokko published jointly a patent on new imidazole derivatives that were said to be particularly useful for disinfecting plant seeds [25].

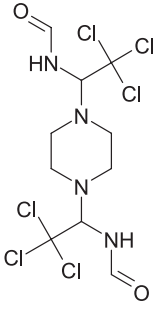
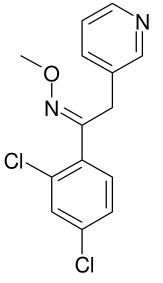
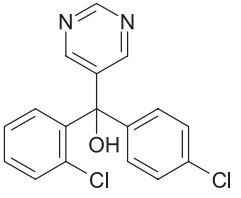
From this patent application a new fungicide with the common name pefurazoate was developed (Table 17.7). The new imidazole seed treatment fungicide exhibits effective control of major rice seed-borne pathogens, including bakanae disease (*Fusarium moliniforme*), brown spot (*Cochliobolus miyabeanus*) and rice blast (*Magnaporthe grisea*) [26, 27]. Studies on the enantioselective antifungal activity of the two enantiomers of pefurazoate revealed that the (S)-(−) isomer exhibited much higher activity against *Gibberella fujikuroi* than the (R)-(−)-isomer [28, 29].

Pefurazoate belongs to the class of imidazole DMIs. More specifically, unlike most of the DMIs where the nitrogen atom of the heterocycle is attached to an aliphatic carbon, the nitrogen of the imidazole ring is linked to a carbonyl group, making a less flexible and less basic N-acylimidazolecarbonyl group than the imidazolylmethyl counterpart in imazalil.

Pefurazoate can be synthesized [30] by transesterification of methyl 2-bromobutyrate with 4-pentenyl alcohol, substitution of the bromine by furfurylamine, and imidazolylcarbonylation of the amine by phosgene or diphosgene and imidazole (Scheme 17.1).

The registered pefurazoate is a mixture of two enantiomers, C2 of the butanoic acid backbone is chiral. The synthesis of each enantiomer of this compound has been described [23] from chiral 2-aminobutanoic acid. The respective position of the furan and imidazole rings are very different for each enantiomer and could account for the difference in biological efficacy observed, the (S)-enantiomer (Scheme 17.2) being much more active then the (R)-isomer.

Table 17.6 Important piperazine, pyridine, pyrimidine and imidazole compounds launched before 1990.

Structure			
Common name	Triforine	Pyrifenox	Fenarimol
Chemical class	Piperazines	Pyridines	Pyrimidines
Example trade name(s)	Saprol®	Podigrol®, Dorado®	Rubigan®, Rimidin®
Launch year and company	1969 Boehringer/Celamerck	1986 Hoffmann-LaRoche	1975 Eli Lilly/Dow
Distributor (example)	Celaflor	Syngenta	Gowan
Patent no.	DOS 1901421, 1968	EP 49854, 1980	Fr 1.569940, 1967

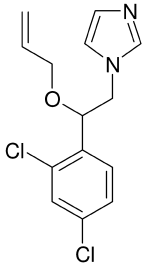
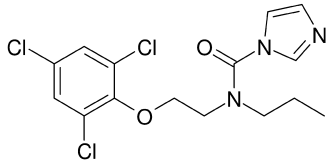
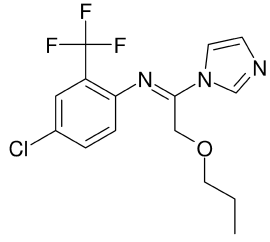
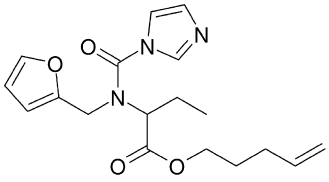
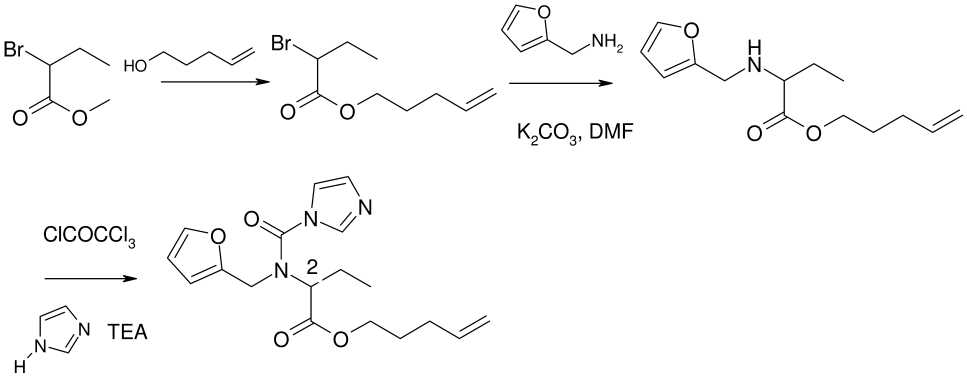
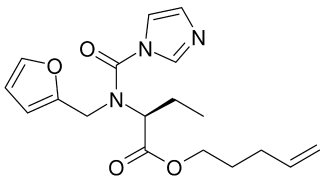
Structure			
Common name	Imazalil	Prochloraz	Triflumizole
Chemical class	Imidazoles	Imidazoles	Imidazoles
Example trade name	Fungazil®	Sportak®	Trifmine®
Launch year and company	1970 Janssen	1980 Boots/FBC/Schering	1987 Nippon Soda, Uniroyal
Distributor (example)	Janssen	Bayer	Nippon Soda
Patent no.	BP 1244530, 1969	GB 1469772, 1973	DOS 2814041, 1978

Table 17.7 Data for pefurazoate.

Structure/common name	Commercial data	Physicochemical data
 pefurazoate	Launched by Ube, Hokko in 1990 Patent no. EP00248086 (1985) Example trade names: Healseed®, Healthied® Marketed by (for example) SDS Biotech	Decomposes at 235 °C Water solubility: 443 mg L^{-1} (25 °C) Log $P_{OW} = 3.0$ Vapor pressure: 0.65 mPa (23 °C)



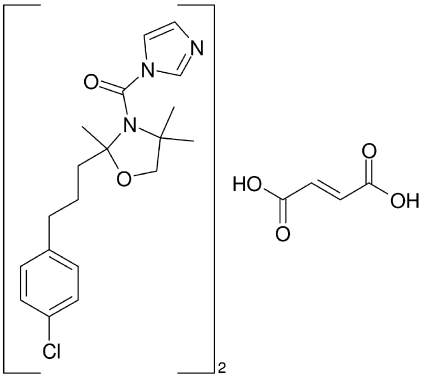
Scheme 17.1 Synthesis of pefurazoate.



(*S*) enantiomer of pefurazoate

Scheme 17.2 Biologically most active enantiomer.

Table 17.8 Data for oxpoconazole.

Structure/common name	Commercial data	Physicochemical data
 oxpoconazole fumarate	Launched by Ube, Otsuka in 2000 Patent no. JP07304774 (1994) Example trade names: All-Shine®, Oh-Shine® Marketed by (for example) SDS Biotech	Mp 123.6–124.5 °C Water solubility: 89.5 mg L^{-1} (25 °C, pH 4) Log P_{OW} = 3.69 (pH 7.5; 25 °C)

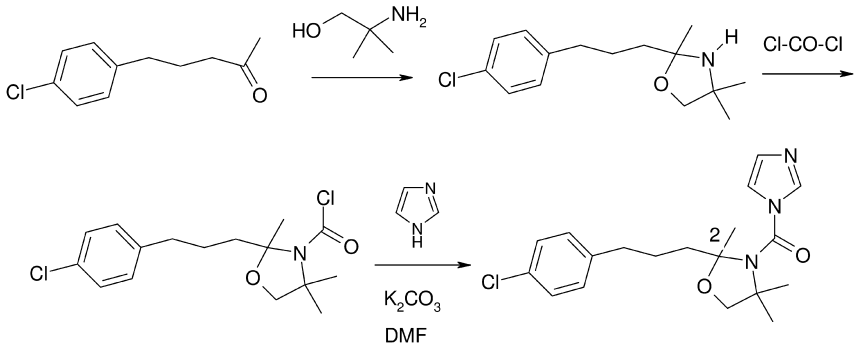
17.2.1.2 **Oxpoconazole**

Oxpoconazole is a new imidazole derivative launched by Ube and Otsuka in 2000 (Table 17.8). The compound is mainly used in fruits under the trade name All-Shine® but seems also to be suitable for the control of diseases in rice seedlings such as *Magnaporthe orzyzae* and *Rhizoctonia solani*. The compound has been developed as its fumarate salt. In addition, unlike most DMIs, oxpoconazole seems to have a field efficacy against the grey mould pathogen, *Botrytis cinerea* [31]. General reports on the synthesis and the biological activities of oxpoconazole are available from Morita et al. [32] and from Li et al. [33]. Typical use rates in apples and peaches against *Venturia* spp., *Monilinia* spp., and *Phomopsis* are around 0.01%.

Oxpoconazole is the most recent example of a DMI that includes an N-acylimidazole group like its predecessors prochloraz (1980) and pefurazoate (1990, see above). Owing to the presence of the non-acylated nitrogen of the imidazole ring, this compound is quite basic and it is sold as a fumarate salt.

The synthesis of the free base has been described from a 5-aryl-2-pentanone [34], its keto group being transformed into a 1,3-oxazolidine with α,α-dimethyl ethanolamine, the free NH then reacts firstly with phosgene and secondly with imidazole (Scheme 17.3).

The registered compound is a mixture of enantiomers, C2 of the oxazolidine ring is chiral. The synthesis and properties of the enantiomers have not been described.



Scheme 17.3 Synthesis of oxpoconazole.

17.2.2 Triazoles

17.2.2.1 Triazoles Launched before 1990

Several well-known triazole based DMI fungicides that were launched before 1990 still, mostly, have a significant market importance, although newer triazoles have largely taken over the leading position.

The first triazole introduced into the market, in 1976, was triadimefon, which became rapidly known under its trade name Bayleton®. This first representative of the triazole group was considered as real progress at that time because of excellent activity against powdery mildew and rust combined with significant activity against several leaf spot diseases.

Table 17.9 shows a selection of important further triazoles. Beside triadimefon, Bayer introduced bitertanol and triadimenol in the early 1980s. Bitertanol offers as a foliar fungicide good control of apple scab (*Venturia inaequalis*) and of the Black Sigatoka pathogen in bananas (*Mycosphaerella fijiensis*) and is also used as a seed treatment fungicide against snow mould (*Microdochium nivale*) and common and dwarf bunt (*Tilletia caries*, *T. controversa*). Triadimenol was introduced initially exclusively as a systemic seed treatment fungicide in cereals but was later also developed as a foliar fungicide in mono- and dicots. Propiconazole, also well known under its trade name Tilt®, is another successful triazole offering a broader spectrum of activity and especially good leaf spot activity in a multitude of crops such as cereals and banana. Owing to a specific strength in the control of barley diseases flusilazole (Punch®) from Du Pont was for many years a dominant product in this crop. Penconazole known as Topas® was specifically developed for broadleaved crops. Beside the simultaneous control of scab and powdery mildew in apples the product is used for the control of powdery mildew and other diseases in grapes, fruits and vegetables. Tebuconazole is a very successful triazole that has found worldwide use due to its very broad fungicidal spectrum of activity. The product was initially introduced in cereals under its trade name

Table 17.9 Important triazole fungicides launched before 1990.

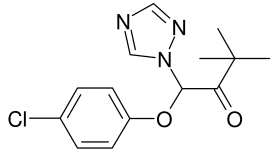
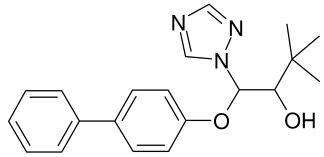
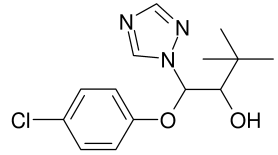
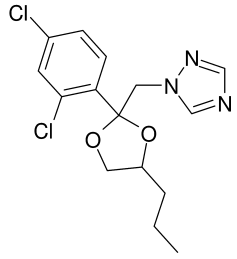
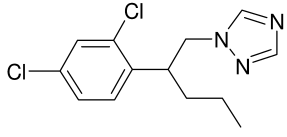
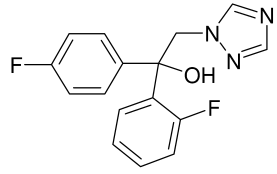
Structure			
Common name	Triadimefon	Bitertanol	Triadimenol
Trade name(s) (example)	Bayleton®	Baycor®, Sibutol® (ST)	Bayfidan®, Baytan® (ST)
Launch (year/company)	1976 Bayer	1979 Bayer	1980 Bayer
Marketed by (example)	Bayer	Bayer	Bayer
Patent no., priority	DE 02201063, 1972	DE 2324010, 1973	DE 2324010, 1973
Structure			
Common name	Propiconazole	Penconazole	Flutriafol
Trade name(s) (example)	Tilt®	Topas®	Impact®
Launch (year/company)	1980 Ciba-Geigy	1983 Ciba-Geigy	1984 ICI/Zeneca
Marketed by (example)	Janssen, Ciba-Geigy	Syngenta	Cheminova
Patent no., priority	DOS 2551560, 1974	DOS 2735872, 1976	EP 0015756, 1979

Table 17.9 (*continued*)

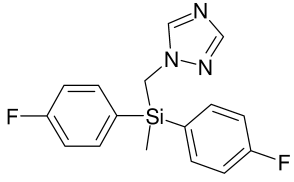
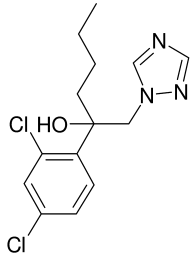
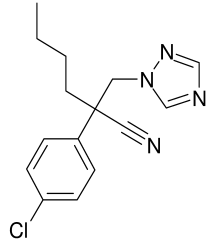
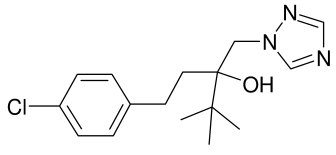
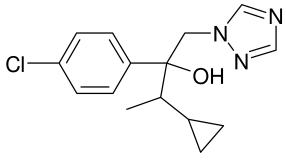
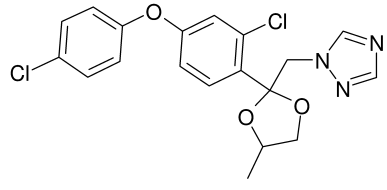
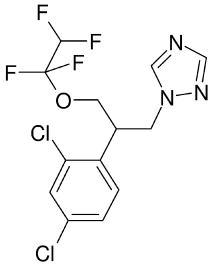
Structure			
Common name	Flusilazole	Hexaconazole	Myclobutanil
Trade name(s) (example)	Punch®, Nustar®	Anvil®	Systhane®
Launch (year/company)	1986 DuPont	1986 ICI/Zeneca	1986 Rohm & Haas
Marketed by (example)	DuPont	Syngenta	Dow
Patent no., priority	EP 0068813, 1981	EP 0015756, 1979	US 4366165, 1977
Structure			
Common name	Tebuconazole	Cyproconazole	Difenoconazole
Trade name(s) (example)	Folicur®, Raxil®	Alto®, Sentinel®	Score®
Launch (year/company)	1988 Bayer	1988 Sandoz	1989 Ciba-Geigy
Marketed by (example)	Bayer	Syngenta, Bayer	Syngenta
Patent no., priority	EP 40345, 1980	GB-A-21 36423, 1982	EP 65485, 1981

Table 17.10 Data for tetraconazole.

Structure/common name	Commercial data	Physicochemical data
 tetraconazole	Launched by Montedison in 1991 Patent no. EP0234242 (1986) Example trade name: Eminent® Marketed by (for example) Isagro	Mp 6 °C Water solubility: 156 mg L^{-1} (20 °C, pH 7) Log P_{OW} = 3.56 (20 °C) Vapor pressure: 0.18 mPa

Folicur® but is used nowadays in a multitude of crops such as peanuts, bananas and soybeans as a foliar fungicide as well as in cereal seed treatment (Raxil®).

17.2.2.2 **Triazole Fungicides Launched since 1990**

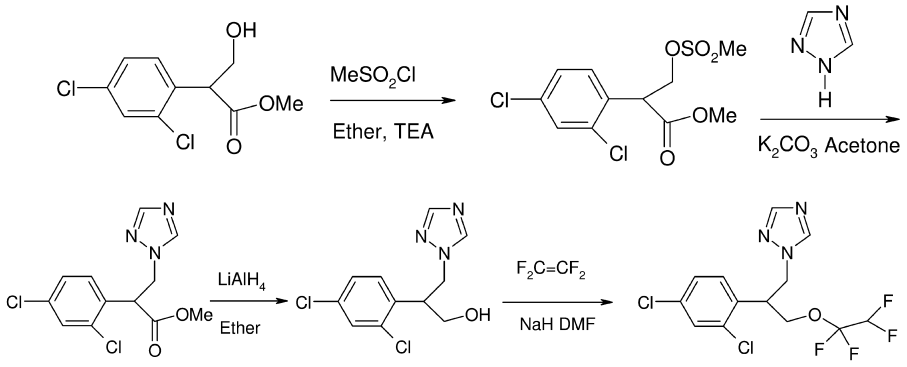
Since 1990, a further eleven new triazoles have reached the market stage. These compounds are presented below in more detail.

17.2.2.2.1 **Tetraconazole**

Tetraconazole was the first azole fungicide introduced by the Italian company Isagro Ricerca (formerly Montedison) and is nowadays distributed by Isagro (Table 17.10). Information on the comparative antifungal effects of tetraconazole is given by Carzaniga et al. [35] and studies on the stereoselective interaction of tetraconazole with C14 demethylase have been published by Gozzo et al. [36]. Its vapor pressure is comparatively high (0.18 mPa), resulting – together with its pronounced systemicity – in a good redistribution in plant tissue but may, on the other hand, also cause evaporation losses at higher temperatures. In cereals 125 g-a.i. ha^{-1} of tetraconazole (trade name: Eminent®) are used to control powdery mildew, rusts as well as *Stagonospora nodorum* and *Septoria tritici* [37]. Cercospora leaf spot (*Cercospora beticola*) in sugar beets is another indication where tetraconazole shows promising results at rates of about 100 g-a.i. ha^{-1} [38, 39]. Bianchi et al. studied the fungitoxicity of the (R)-(+) and the (S)-(−) enantiomers of tetraconazole with a series of pathogens. In all cases the R-(+)-enantiomers was more active [40].

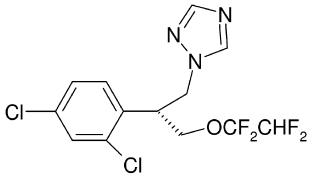
What makes the structure of this triazole different is the presence of the highly fluorinated side chain. The structure of this triazole is quite unique because of the unusual position of the oxygen gamma to the triazole instead of beta. Its synthesis [41] involves a nucleophilic substitution of the mesyl group of methyl

2-(2,4-dichlorophenyl)-3-mesylpropanoate by triazole, followed by reduction of the ester to the alcohol by lithium aluminum hydride and alkylation of the hydroxyl group so-obtained by tetrafluoroethylene (Scheme 17.4).



Scheme 17.4 Synthesis of tetraconazole.

The final compound is a mixture of two enantiomers (C2 is chiral) (Scheme 17.5). The use of lipases in 1991 to separate racemic mixture of a key synthon represents the cornerstone of the synthetic scheme leading to each enantiomer [40]. The two enantiomers of tetraconazole were separated using β-cyclodextrin-mediated capillary electrophoresis in 2001 [42].



(R)-enantiomer of tetraconazole

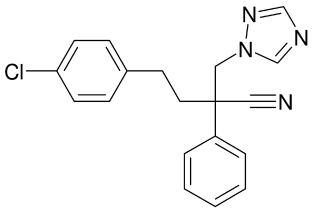
Scheme 17.5 Biologically most active enantiomer.

17.2.2.2.2 **Fenbuconazole**

Fenbuconazole (Table 17.11) is a triazole fungicide intended for use as an agricultural and horticultural fungicide spray for the control of leaf spot, yellow and brown rust, powdery mildew and net blotch on wheat and barley and apple scab, pear scab and apple powdery mildew on apples and pears. Fenbuconazole was already presented to the public in 1988 by Driant et al. [43].

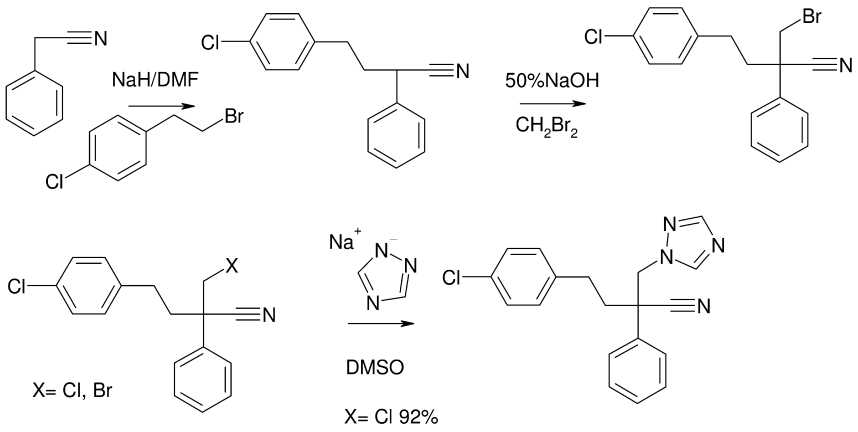
Under its trade name Indar®, fenbuconazole is one of the few fungicides registered in the USA for the control of Mummy berry, a blueberry disease, caused by

Table 17.11 Data for fenbuconazole.

Structure/common name	Commercial data	Physicochemical data
 fenbuconazole	Launched by Rohm & Haas in 1991 Patent no. DE03721786 (1986) Example trade names: Enable®, Indar® Marketed by (for example) Dow AgroSciences	Mp 124–126 °C Water solubility: 3.8 mg L^{-1} (25 °C) Log $P_{OW} = 3.23$ Vapor pressure: 3.4×10^{-1} mPa (25 °C)

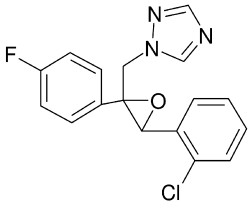
Monilinia vaccinii-corymbosi. Further, control of soybean rust (*Phakopsora pachyrhizi*) with the product Enable® is under investigation in the USA and in Latin America.

Like former triazoles from Rohm and Haas, fenapanil and myclobutanil, fenbuconazole is chemically characterized by its nitrile substituent on the quaternary C2. Its synthesis [44] starts from the phenylethylation of phenylacetonitrile by 1-(2-bromoethyl)-4-chlorobenzene (Scheme 17.6). A second alkylation at the same carbon with dibromomethane then leads to a quaternary carbon still bearing a reactive bromomethyl group. Fenbuconazole is obtained as a mixture of enantiomers by nucleophilic substitution of the halogen by triazolyl sodium.



Scheme 17.6 Synthesis of fenbuconazole.

Table 17.12 Data for epoxiconazole.

Structure/common name	Commercial data	Physicochemical data
 epoxiconazole	Launched by BASF in 1992 Patent no. EP196038 (1985) Example trade names: Opus® Marketed by (for example) BASF	Mp 136 °C Water solubility: 6.6 mg L^{-1} (20 °C) Log P_{OW} = 3.33 or 3.44 Vapor pressure: $<10^{-3}$ mPa

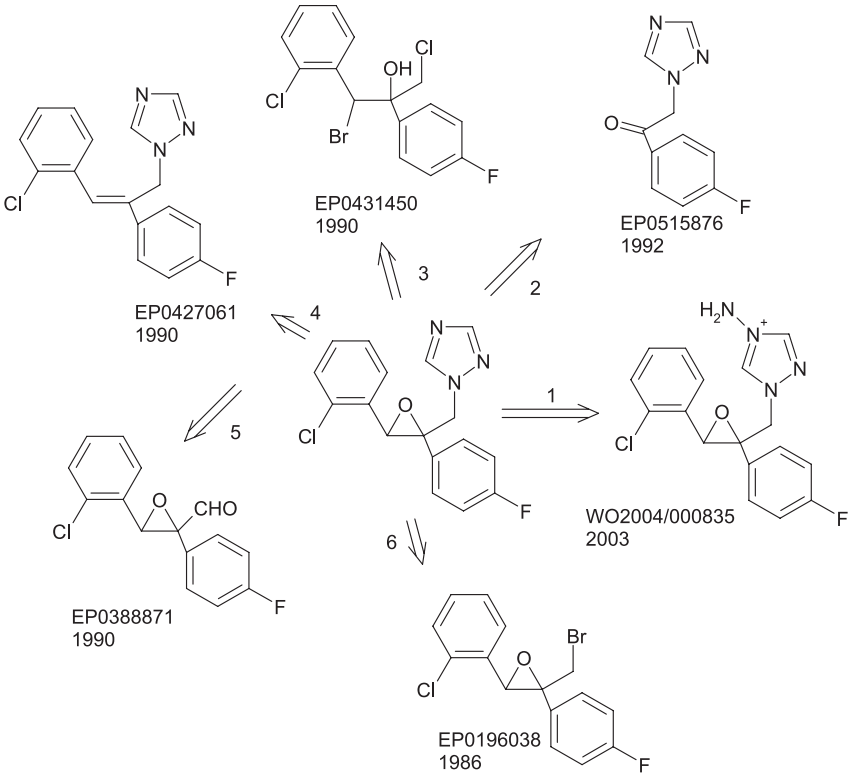
The compound on the market is a mixture of enantiomers (quaternary C2 is chiral). There are no references so far about the synthesis and biological properties of each enantiomer.

17.2.2.2.3 **Epoxiconazole**

Epoxiconazole was presented to the public in 1990 (Table 17.12) [45, 46]. It is a broad spectrum triazole fungicide with a pronounced strength against cereal leaf spots and rust fungi and a prolonged duration of activity. Owing to the increasing importance of *Septoria tritici* since the beginning of the 1990s in European wheat production epoxiconazole rapidly gained a prominent role in the cereal fungicide market were it is used alone (trade name: Opus®) at application rates of 90 to 125 g-a.i. ha^{-1} or in combination with strobilurins and several other mixing partners. Detailed investigations on the uptake and systemic translocation of epoxiconazole are available from Akers et al. [47]. The non-fungicidal effects of epoxiconazole on wheat plants have been studied in detail by Siefert and Grossmann [48]. Beside cereal applications, epoxiconazole can be used in other crops such as tea, sugar beets and coffee against a broad range of diseases.

Many synthesis methods are described in BASF patents [49]: six main N-1 intermediate can be found in the literature, which are gathered in Scheme 17.7, together with the patent application number and its year of publication:

1. By deprotection of N-aminotriazolium;
2. by a Corey–Chaykovsky reaction on the corresponding ketone;
3. by cyclization of an *erythro*-1-bromo-3-chloro-1,2-diaryl-2-propanol;
4. by epoxidation of the corresponding double bond;
5. by reductive amination of the corresponding formyl epoxide;

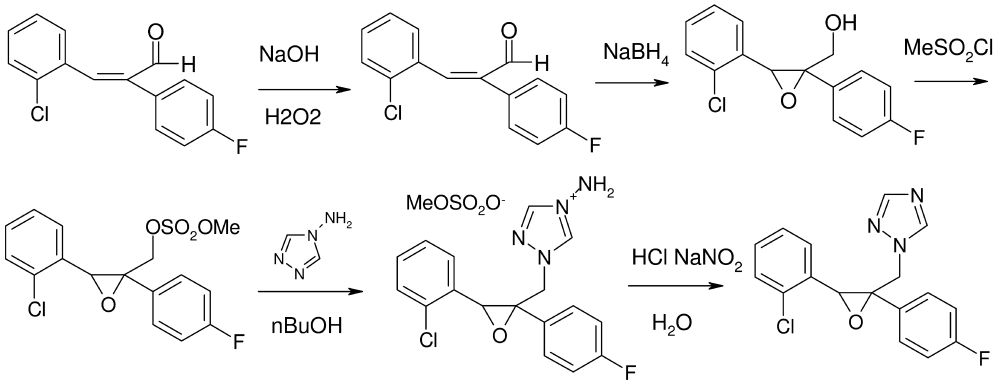


Scheme 17.7 Synthesis methods for epoxiconazole.

6. By the classical nucleophilic substitution of the bromine atom of a bromomethyl group with the triazolyl salt.

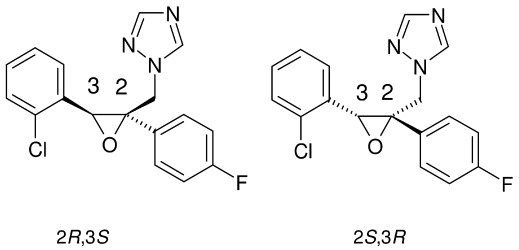
Scheme 17.8 highlights the most recent patented route [via intermediate (1) in the above list]: epoxidation of the double bond of 3-(2-chlorophenyl)-2-(4-fluorophenyl)acrylaldehyde is conducted with hydrogen peroxide under basic conditions followed by the reduction of the aldehyde to the corresponding alcohol. The free hydroxyl group is activated by mesylation to the mesyl derivative, which in turn undergoes nucleophilic substitution with symmetrical N-aminotriazole. The attacking nitrogens are the ones free from the N-amino functionality. The N-aminotriazolium obtained is then reduced with sodium nitrite under acidic conditions to afford epoxiconazole.

The active ingredient is a mixture of the *2R,3S* and *2S,3R* enantiomers (C2 and C3 are chiral), corresponding to a cis stereochemistry, meaning that both chlorophenyl and triazolylmethyl groups are on the same side of the oxirane ring



Scheme 17.8 Recent patented synthesis route of epoximazole.

(Scheme 17.9). The synthesis and plant growth regulatory activity of each enantiomer has recently been shown by Cheminova [50a]. A patent describing the synthesis of the trans epoxiconazole has recently been published [50b].



2*R*,3*S* 2*S*,3*R*

Scheme 17.9 cis stereoisomers of epoximazole.

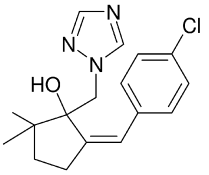
17.2.2.2.4 **Triticonazole**

Triticonazole was presented in 1991 as a new specific triazole for the control of cereal seed-borne and foliar diseases by seed treatment application (Table 17.13) [51].

Applied at rates of 150 g-a.i. (cereals) to 600 g-a.i. (maize) per 100 kg of seeds, triticonazole was reported by Mugnier et al. [52] to give good systemic control of diseases such as *Rhynchosporium secalis* in barley or of corn head smut (*Sphacelotheca reiliana*). Furthermore, spray applications with triticonazole showed activity against several turf grass diseases. Other cereal diseases controlled via seed treatment such as rusts, *Septoria tritici*, powdery mildew and the W-strains of eyespot (*Oculimacula yallundae*) have been described by Gauillard and Peron [53].

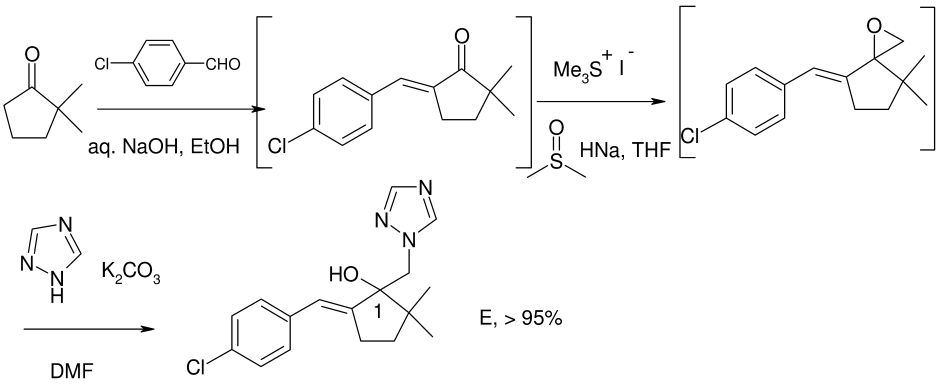
At lower dose rates around 5 g-a.i. per 100 kg of seed triticonazole provides, in products such as Kinto TS® (mostly in combination with prochloraz), good con-

Table 17.13 Data for triticonazole.

Structure/common name	Commercial data	Physicochemical data
 triticonazole	Launched by Rhone-Poulenc in 1992 Patent no. EP 00378953 (1989) Example trade names: Charter®, Real® Marketed by (for example) Bayer, BASF	Mp 139–140.5 °C Water solubility: 7 mg L^{-1} (20 °C) Log $P_{OW} = 3.29$ (20 °C) Vapor pressure: $<1 \times 10^{-5}$ mPa (50 °C)

trol of smuts, bunts and other seed and soil-borne diseases that are usually controlled by DMI fungicides [54]. Good systemic mobility is needed for systemic control of foliar diseases. Detailed studies on the uptake and distribution of triticonazole in wheat following seed treatment have been published by Querou et al. [55, 56].

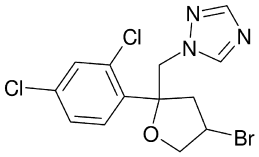
The synthesis of triticonazole is only described in patents as a one-pot sequence [57]: Knoevenagel condensation of 4-chlorobenzaldehyde on 2,2-dimethylcyclopentanone gives the α,β-unsaturated ketone, which enters a Corey–Chaykovsky epoxidation reaction to afford the epoxide which in turn is opened with the potassium salt of 1,2,4-triazole (Scheme 17.10).



Scheme 17.10

The commercially available triticonazole is a racemic mixture (C1 bearing the hydroxy group is chiral) with an E double bond. There are no literature reports on the preparation of the enantiomers and their biological properties.

Table 17.14 Data for bromuconazole.

Structure/common name	Commercial data	Physicochemical data
 bromuconazole	Launched by Rhône-Poulenc in 1992 Patent no. EP00258161 (1986) Example trade name: Granit® Marketed by (for example) Bayer CropScience	Mp 84 °C Water solubility: 50 mg L^{-1} Log $P_{OW} = 3.24$ Vapor pressure: 4×10^{-3} mPa (25 °C)

17.2.2.2.5 **Bromuconazole**

In 1990 Rhône-Poulenc presented a new triazole fungicide, bromuconazole, to the public (Table 17.14) [58].

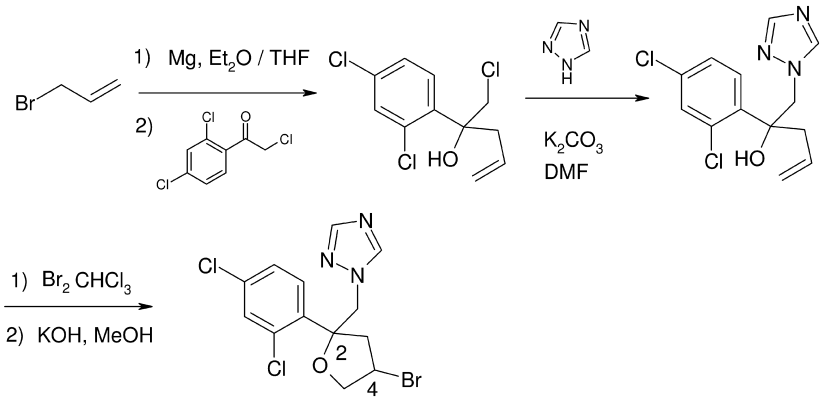
In cereals good control of eyespot (*Oculimacula yallundae*), *Microdochium nivale* and Fusarium head blight are reported beside the diseases that are usually combated by triazoles such as rusts, powdery mildew and *Septoria tritici*. Bromuconazole showed good potential in oilseed rape, vegetables and potatoes where especially *Alternaria* diseases are well controlled. Various other crops where the product shows good control at rates between 50 and 200 g ha^{-1} are, for example, fruits (*Monilinia*, *Venturia*), coffee, turf and rice. Bromuconazole as a cereal fungicide is mostly used at 133–200 g-a.i. ha^{-1} [59].

The only synthesis described [60], from Rhône-Poulenc Agrochimie, involves three steps. The initial allylmagnesium bromide adds to the carbonyl double bond of 2-chloro-1-(2,4-dichlorophenyl)ethanone to afford the corresponding alcohol (Scheme 17.11). The aliphatic chlorine is then substituted by the potassium salt of triazole and the tetrahydrofuran ring is formed by attack of the hydroxy group on the brominated double bond.

The compound contains two chiral centers (C2, C4) and exists as a mixture of roughly equal proportions of two diastereomers (*2RS,4RS:2RS,4SR*), each with two enantiomers [61]. As a consequence, the three substituents of the tetrahydrofuran are equally distributed up or down versus the heterocyclic ring. Notably, the two diastereoisomers can be separated by column chromatography.

17.2.2.2.6 **Metconazole**

The triazole fungicide metconazole was invented by Kureha and is co-distributed by BASF (Table 17.15). It was first presented by Sampson et al. in 1992 [62]. To date, its main markets are in cereals and oilseed rape [63, 64].

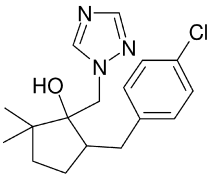


Scheme 17.11 Synthesis of bromuconazole.

In cereals, metconazole is one of the few triazoles that show a pronounced effect against Fusarium head blight caused by a complex of pathogens such as *Fusarium culmorum* and *F. graminearum*. As these *Fusarium* species can synthesize several mycotoxins such as, for example, DON (desoxynivalenol) their control has become of increasing importance. The role of metconazole and of other fungicides such as tebuconazole in lowering the mycotoxin contents of cereal grains has, therefore, been studied intensively [65, 66].

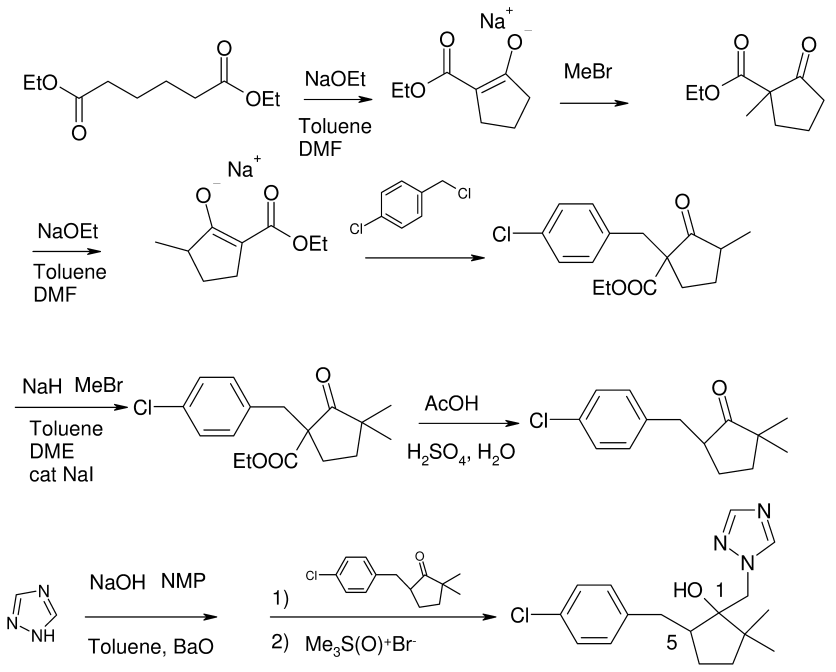
Beside Fusarium head blight, metconazole controls the disease complex in wheat and barley caused by rusts, *Septoria*, powdery mildew, *Rhynchosporium* and *Drechslera* species at low rates of between 60 and 90 g-a.i. ha^{-1}. In oilseed rape the same rates are needed to combat *Sclerotinia sclerotiorum*, *Phoma*, *Alternaria* and other pathogens.

Table 17.15 Data for metconazole.

Structure/common name	Commercial data	Physicochemical data
 metconazole	Launched by Kureha in 1993 Patent no. EP00329397 (1988) Example trade names: Caramba® Marketed by (for example) BASF, Kureha	Mp 110–113 or 100–108.4 °C Water solubility: 15 mg L^{-1} Log P_{OW} = 3.85 (20 °C)

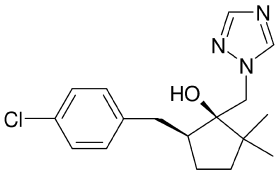
Three-dimensional modelings of C14 demethylase and its interaction with metconazole have been published [67, 68] as well as a QSAR study covering metconazole and ipconazole [69].

The synthesis scheme is described in two process patents [70]: Dieckmann condensation applied to ethyl adipate leads to the salt of 2-ethoxycarbonylcyclopentanone (Scheme 17.12). From this compound, methylation at position 2 followed by a rearrangement under basic medium brings the methyl group to position 5. The salt obtained is directly benzylated with 4-chlorobenzyl chloride. A second methylation then occurs directly at position 5 and a subsequent decarboxylation allows access to the key carbonyl cyclopentanone with all the required substituents present. The last step consists of a one-pot Corey–Chaykovsky epoxidation reaction in which are successively added to the triazolyl sodium formed *in situ*, the cyclopentanone and the trimethylsulfoxonium bromide.



Scheme 17.12 Synthesis of metconazole.

Commercially available metconazole is a mixture of cis (*1RS,5SR* major) and trans (*1RS,5RS* minor) isomers (C1 and C5 are chiral), meaning that the hydroxy and the benzyl groups are on the same side of the cyclopentane ring (Scheme 17.13).



(1*R*,5*S*) metconazole

Scheme 17.13 Metconazole, stereochemistry.

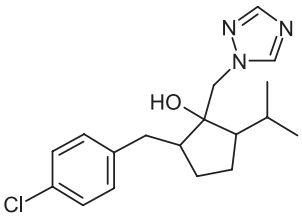
17.2.2.2.7 **Uses of Ipconazole**

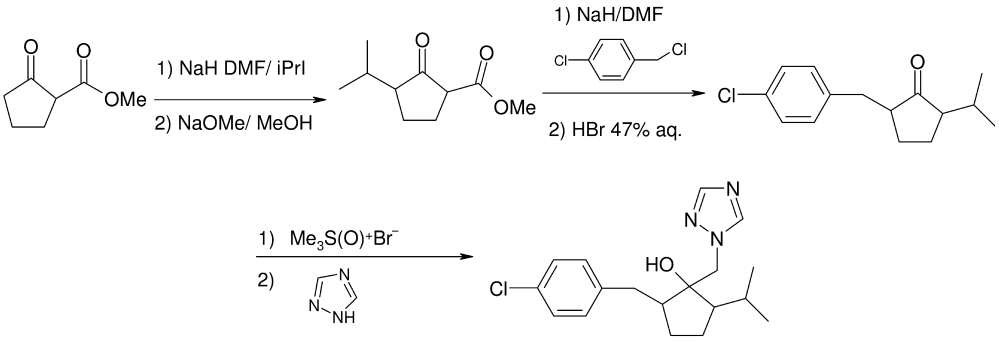
Ipconazole was launched by Kureha in 1993 (Table 17.16). It is a systemic fungicide, suitable for the control of a wide range of seed diseases in rice and other crops with seed treatment application. Trade names are Tec-Lead®, Techlead®, Vortex® and Crusoe®. Ipconazole is particularly effective against Bakanae disease caused by *Fusarium moniliforme* [71], Helminthosporium leaf spot and blast (*Magnaporthe grisea*) on rice. In combination with copper, ipconazole is marketed under the trade name TEC-LEAD C FLOWABLE®.

Information on the structure–activity relationships of enantiomers of ipconazole as well as their fungicidal and plant growth inhibitory activities has been published by Saishoji et al. [72] and Ito et al. [73]. QSARs and three-dimensional shape studies of fungicidal azoylmethyl-cyclopentanols such as ipconazole and metconazole are available from Ref. [70].

The synthesis scheme [74] (Scheme 17.14) is similar to that described in Scheme 17.12 for metconazole. Isopropylation takes place at position 2 of the easily available 2-methoxycarbonylcyclopentanone. Subsequent rearrangement leads to the less hindered isomeric 5-isopropyl. Then, the same type of condensation (without rearrangement) with the 4-chlorobenzyl chloride followed by decarboxylation gives rise to the key ketone precursor of ipconazole. Here also, the one-

Table 17.16 Data for ipconazole.

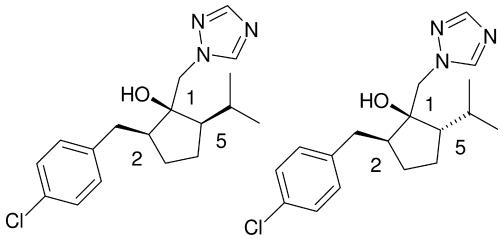
Structure/common name	Commercial data	Physicochemical data
 ipconazole	Launched by Kureha in 1993 Patent no. EP 00329397 (1988) Example trade names: Tec-Lead®, Vortex® Marketed by (for example) Kureha	Mp 88–90 °C Water solubility: 6.9 mg L^{-1} (20 °C) Log P_{OW} = 4.21 (25 °C)



Scheme 17.14 Synthesis of ipconazole.

pot final step uses a Corey–Chaykovsky epoxidation with trimethylsulfoxonium bromide.

Commercially available ipconazole is a mixture of two diastereoisomers: 1RS, 2SR, 5RS and 1RS, 2SR, 5SR, meaning that only four enantiomers are present out of the eight that are theoretically possible. Clues about the ratio of isomers of the three stereo centers (C1, C2 and C5) and their separation through chiral column can be found in another Kureha patent [75]. To illustrate the position, up or down, of the different substituents versus the cyclopentane ring, only one enantiomer of each former pair is shown in Scheme 17.15.



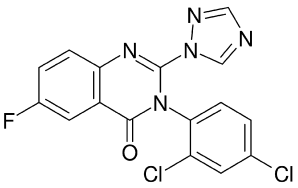
Scheme 17.15 Stereoisomers of ipconazole.

17.2.2.2.8 **Fluquinconazole**

Fluquinconazole, a quinazoline-based triazole fungicide, was introduced to the public in 1992 [76] (Table 17.17). When used as a foliar fungicide fluquinconazole is particularly active against pome fruit diseases such as *Venturia inaequalis* and *Podosphaera leucotricha*. Other diseases controlled include powdery mildews, *Monilinia* spp., *Cercospora* spp., rusts.

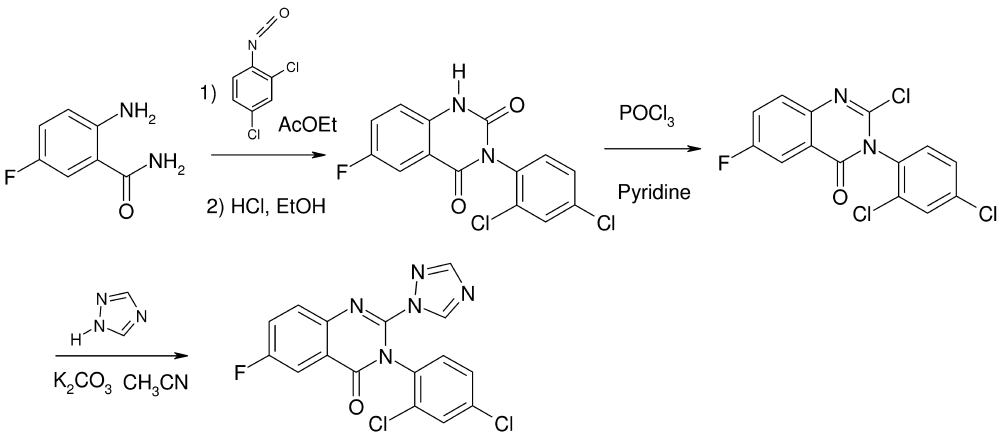
As a seed treatment fluquinconazole protects cereal roots against take-all (*Gaeumannomyces graminis*) at the rate of 75 g-a.i. per 100 kg of seeds [77]. Systemic

Table 17.17 Data for fluquinconazole.

Structure/common name	Commercial data	Physicochemical data
 fluquinconazole	Launched by FBC/ Schering in 1993 Patent no. EP00183458 (1984) Example trade names: Castellan®, Galmano® Marketed by (for example) Bayer, BASF	Mp 191.9–193.0 °C Water solubility: 1.1 mg L^{-1} (pH 6.6) Vapor pressure: Log $P_{OW} = 3.24$ (20 °C) 6.4×10^{-6} mPa (20 °C)

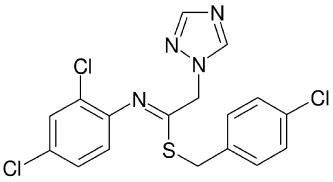
efficacy against infection by Puccinia and Septoria leaf diseases as well as control of seed-borne diseases, such as *Tilletia* spp. and *Ustilago* spp., has also been reported [78, 79].

Contrarily to the other triazoles, where there is a certain degree of flexibility in the vicinity of the triazole heterocycle, this compound is based on a unique rigid quinazolone. Furthermore, steric hindrance around the triazole is reinforced by the presence of the aryl moiety in the ortho position. The synthesis depicted below is based on the general approach patented in EP 0183458 on close analogs bearing halogens other than fluorine [80]; fluquinconazole preparation itself is not described *per se* in any patent. Starting from 5-fluoroanthranilic-amide, the quinazolone ring is formed by interaction with 2,4-dichloroisocyanatobenzene (Scheme 17.16). Subsequent treatment with phosphorus oxychloride and reaction



Scheme 17.16 Synthesis of fluquinconazole.

Table 17.18 Data for imibenconazole.

Structure/common name	Commercial data	Physicochemical data
 imibenconazole	Launched by Hokko in 1994 Patent no. DE03238306 (1981) Example trade name: Manage® Marketed by (for example) Hokko	Mp 89.5–90 °C Water solubility: 1.7 mg L^{-1} (25 °C) Log $P_{OW} = 4.94$ Vapor pressure: 8.5×10^{-5} mPa (25 °C)

of the newly formed chlorine substituent with the potassium salt of 1,2,4-triazole gives fluquinconazole.

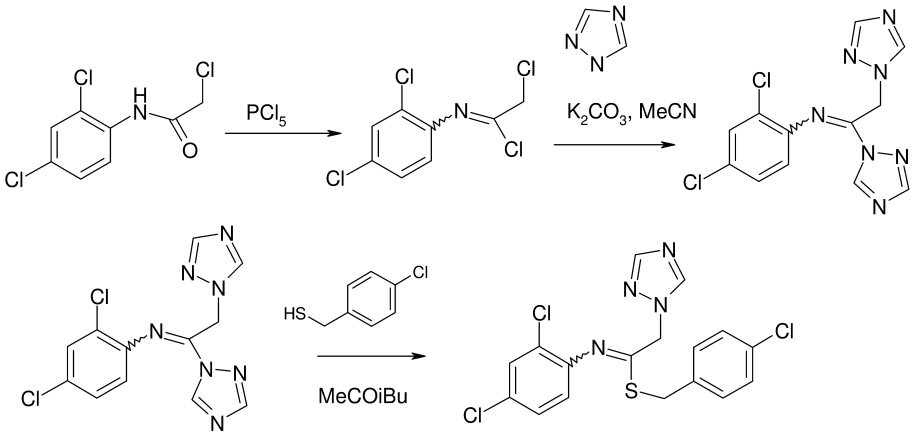
17.2.2.2.9 **Imibenconazole**

In 1988 Hokko presented HF-6305, a new triazole fungicide to the public (Table 17.18) [81]. The compound, subsequently known under its common name imibenconazole, shows a wide range of activity against diseases in fruit, turf, vegetables and ornamentals. e.g., in apple and pear diseases such as scab (*Venturia* spp.), powdery mildew (*Podosphaera leucotricha*) and rust. Furthermore, good performance against grape powdery mildew, grape anthracnose (caused by *Elsinoe ampelina*) as well as control of citrus and peach scab has been reported. With the exception of grape powdery mildew, these diseases have been difficult to control with most other triazole fungicides. Studies on the mode of action of imibenconazole have been published by Ogawa [82].

An early synthesis, published in 1983 and 1984 [83], requires three steps from 2-chloro-*N*-(2,4-dichlorophenyl)acetamide (Scheme 17.17). First, the amide group is transformed into imidoyl chloride with phosphorus pentachloride, followed by displacement of the two non-aromatic chlorine atoms by two equivalents of the potassium salt of 1,2,4-triazole. The triazolyl group borne by the imino bond being the best leaving group is replaced when reacted with a good nucleophile such as (4-chlorophenyl)methanethiol. The stereochemistry (E or Z) of the double bond is not stated in the literature.

17.2.2.2.10 **Simeconazole**

Simeconazole (Table 17.19) was developed by Sankyo and presented to the public in 2000 as a new broad-spectrum compound for seed treatment in cereals and rice [84]. In studies comparing the systemic activity of simeconazole with other DMI fungicides, Tsuda et al. [85] reported a prominent vapor-phase activity, a

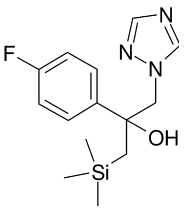


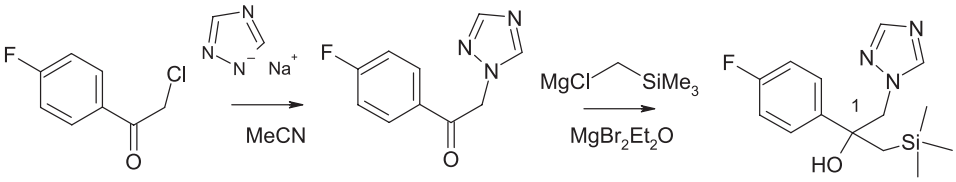
Scheme 17.17 Synthesis of imibenconazole.

good translaminar movement as well as systemic activity against barley powdery mildew after soil drench application. Accordingly, seed treatment with simeconazole achieves excellent efficacies against *Ustilago* at 4 to 10 g-a.i. per 100 kg of seed. At high doses of 50 to 100 g-a.i. per 100 kg seed it is also reported to be effective against *Rhizoctonia cerealis*, *Oculimacula yallundae* and *Blumeria graminis* on barley. In rice, simeconazole is taken up by plants after submerged application and is therefore able to give systemic control of rice sheath blight (*Rhizoctonia solani*) [86].

The quite straightforward two-step synthesis [87–89] of simeconazole involves substitution of the chlorine atom of α-chloro-4-fluoro-acetophenone by the sodium salt of 1,2,4-triazole (Scheme 17.18). Subsequent addition of trimethylsilyl-

Table 17.19 Data for simeconazole.

Structure/common name	Commercial data	Physicochemical data
 simeconazole	Launched by Sankyo in 2000 Patent no. EP00537957 (1991) Example trade names: Sanlit®, Mongarit® Marketed by (for example) Sankyo	Mp 118.5–120.5 °C Water solubility: 57.5 mg L^{-1} (20 °C) Log $P_{OW} = 3.2$ Vapor pressure: 5.4×10^{-2} mPa (25 °C)



Scheme 17.18 Synthesis of simeconazole.

methylmagnesium chloride on the carbonyl double bond gives a mixture of the two enantiomers of simeconazole. The two steps can be inverted but it seems that the order depicted in the scheme gives the best results.

The preparation of each enantiomer (C1 is chiral) has been described by a modified route involving the addition of the same Grignard reagent on chiral esters of phenylglyoxylic acid [90].

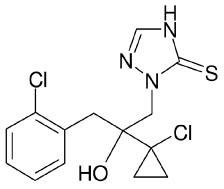
17.2.2.2.11 **Prothioconazole**

The latest introduction into the DMI market, prothioconazole, is unique among the triazole fungicides because its toxophore moiety is a 1,2,4-triazole-3-thione (Table 17.20) [91, 92].

Introduced into the market in 2004 by Bayer CropScience, the new compound rapidly gained market importance due to its broad spectrum of activity, covering all important cereal diseases.

In cereal crops prothioconazole is used at 200 g-a.i. ha^{-1} as a solo product (trade name: Proline®). In mixtures with fungicide partners such as fluoxastrobin (Fandango®), spiroxamine (Input®) or tebuconazole (Prosaro®) prothioconazole is used at rates between 125 and 200 g-a.i. ha^{-1}. The disease spectrum controlled by prothioconazole in wheat covers leaf spot diseases such as Septoria leaf spot (*Septoria tritici*) and tan spot (*Drechslera tritici-repentis*) as well as rust (*Puccinia triticina* and powdery mildew (*Blumeria graminis* f.sp. *tritici*) which are also within

Table 17.20 Data for prothioconazole.

Structure/common name	Commercial data	Physicochemical data
 prothioconazole	Launched by Bayer CropScience in 2004 Patent no. DE19528046 (1994) Example trade name: Proline® Marketed by (for example) Bayer CropScience	139.1–144.5 °C Water solubility: 5 mg L^{-1} (pH 4, 20 °C); 300 mg L^{-1} (pH 8, 20 °C) Log $P_{OW} = 3.82$ (pH 7) Vapor pressure: $\ll 4 \times 10^{-4}$ mPa (20 °C)

the spectrum of activity of the product. Further, prothioconazole is one of the rare azoles providing excellent protection against Fusarium ear blight caused by several *Fusarium* species [93].

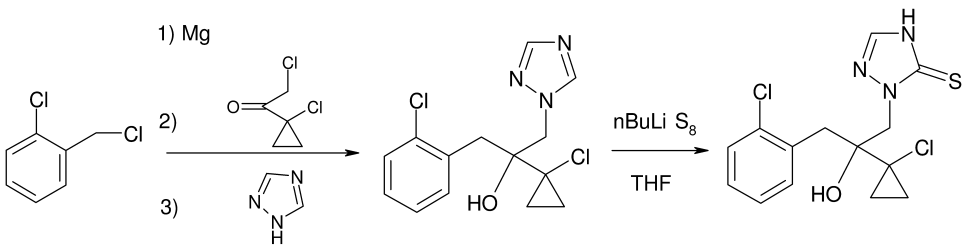
A unique feature of prothioconazole is that it shows equally good activity against both cereal eyespot species, *Oculimacula yallundae* (= *Tapesia yallundae* = *Pseudocercosporella herpotrichoides* W-type) and *O. acuformis* (= *Tapesia acuformis* = *Pseudocercosporella herpotrichoides* R-type) whereas all other triazoles used against eyespot control only *O. yallundae* effectively. Surprisingly, in cross resistance studies prothioconazole revealed a unique profile as far as no positive cross resistance to either triazole-resistant or to prochloraz-resistant isolates could be detected [94].

In barley, prothiconazole provides a high level of efficacy against diseases such as *Rhynchosporium secalis*, *Drechslera teres* as well as good activity against powdery mildew (*Blumeria graminis* f.sp. *hordei*) and barley leaf rust (*Puccinia hordei*).

In oilseed rape all economically important pathogens such as *Sclerotionia sclerotiorum*, *Phoma lingam*, and *Pyrenopeziza brassicae* fungi are controlled at 175 g ha^{-1}. Further crops where prothioconazole is under development are peanuts and pulse crops, including, for example, peas, beans and lentils. Beside foliar applications, cereal seed treatment products containing prothioconazole at rates < 10 g-a.i. per 100 kg of seed alone or in combination with fluoxastrobin and tebuconazole are under development.

One representative synthesis of prothioconazole starts [95] with the addition of the Grignard derivative of 2-chlorobenzyl chloride on the carbonyl double bond of chloromethyl 1-chloro-cyclopropyl ketone (Scheme 17.19). The untouched chlorine atom of the chloromethyl group is then classically substituted with 1,2,4-triazole. From this intermediate, one way to obtain the 2,4-dihydro-3*H*-1,2,4-triazole-3-thione of prothioconazole is by direct lithiation of the 1,2,4-triazole at position 5 with n-butyl lithium and reaction with sulfur. The commercially available compound is a mixture of two enantiomers (chirality of the quaternary carbon bearing the hydroxy group).

These enantiomers have been separated [96] by column chromatography with a chiral auxiliary [*N*-methacryloyl-L-leucin-3-(2,4-dimethylpentyl)-amide] bound to silica gel.



Scheme 17.19 Synthesis of prothioconazole.

17.3
SBI Class II: Amines

17.3.1
Morpholines and Piperidines

The first SBI fungicides to be introduced as agricultural fungicides were, chemically, morpholines such as dodemorph and tridemorph, which had already entered the market at the end of the 1960s (Table 17.21). Whereas dodemorph was mainly developed in ornamentals, tridemorph gained importance in cereal crops and bananas [97].

Owing to resistance problems of cereal powdery mildews towards triazole fungicides in the mid-1980s the morpholine fenpropimorph and the piperidin fenpropidin gained rapidly in importance as partners for the resistance management of triazole fungicides. The latest introduction within the amine group is spiroxamine from Bayer, which is the first representative of a new chemical class within fungicidal amines, the spiroketalamines.

17.3.2
Biochemical Targets of Amines

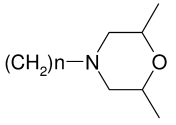
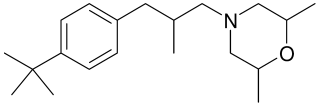
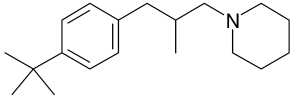
Amines are SBI fungicides that inhibit several targets within fungal sterol biosynthesis. Comprehensive reviews on the mechanism of action of cyclic amines launched until 1995 have been given by Kerkenaar [98] and Mercer [99].

The available studies demonstrate that each individual molecule shows a unique profile in regard to the strength of inhibition at the different targets sites.

In addition to the compound dependant inhibition profile, in different target pathogens different inhibition profiles are found that are characteristic for each individual species. Accordingly, the dominant site of inhibition after tridemorph application is the $\Delta^8 \rightarrow \Delta^7$ isomerase. With fenpropimorph, predominately the Δ^{14} reductase is targeted and $\Delta^8 \rightarrow \Delta^7$ isomerase is inhibited only at higher concentrations. With fenpropidin, predominately Δ^{14} reductase besides $\Delta^8 \rightarrow \Delta^7$ isomerase is inhibited [100]. At high concentrations the accumulation of squalene and 2,3-oxidosqualene indicates also the inhibition at earlier steps in sterol biosynthesis.

The complicated and manifold picture of target sites affected by amines was enlarged by Tiemann et al. [101] with spiroxamine. Generally, the biochemical profile of spiroxamine is similar to that of fenpropidin. Tiemann and coworkers found that all four isomers of spiroxamine were active on Δ^{14}-reductase – other target sites became apparent when the four stereo isomers of spiroxamine were tested separately. Inhibition of $\Delta^8 \rightarrow \Delta^7$ isomerase could be demonstrated for the B/S and B/R isomers. Secondary effects on squalene mono-oxygenase by the A/S isomer and on lanosterol synthase by the A/R isomer were detected although these activities were reported to vary in intensity according to the test fungus.

Table 17.21 Most important morpholine and piperidines compounds launched before 1990.

	 n= 10 to 13 n=12: ~ 70%		
Common name	Tridemorph	Fenpropimorph	Fenpropidin
Trade name(s) (example)	Calixin®	Corbel®	Patrol®
Chemical class	Morpholines	Morpholines	Piperidines
Launch (company/year)	BASF 1969	BASF 1980	Hoffmann-LaRoche 1985
Marketed by (for example)	BASF	BASF	Syngenta
Patent no./priority	GB 00988630, 1961	DE 02656747, 1976	DE 02752135, 1976

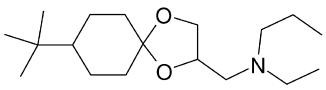
17.3.3
Spiroxamine, First Representative of the Spiroketalamines

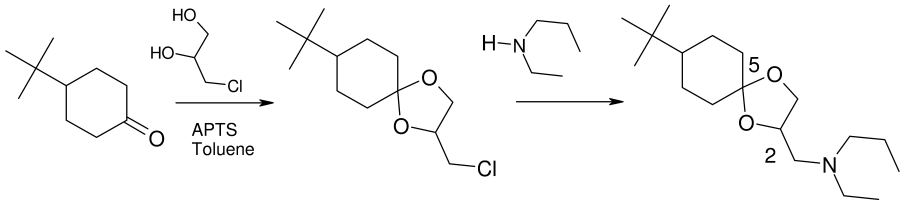
The first representative of the new chemical class spiroketalamine within the amine fungicide group is spiroxamine, which was introduced to the public in 1996 (Table 17.22) [102]. Its chemistry and stereochemistry have been described by Krämer et al. [103, 104] and the biological spectrum has been characterized by Dutzmann [105].

Similar to other amine fungicides, spiroxamine is applied either alone, under the trade name Impulse®, or in mixture with other fungicide partners for the control of powdery mildew in cereals at rates between 500 and 750 g-a.i. ha^{-1}. Beside a pronounced preventive, curative and eradicative activity against powdery mildew efficacy against cereal rusts (*Puccinia* spp.), net blotch (*Drechslera teres*) and side effects against *Septoria tritici* and *Stagonospora nodorum* are reported. In addition to the use in cereals, spiroxamine has other important fields of application, in grapes against powdery mildew (*Erysiphe necator*) at rates between 300 and 400 g-a.i. ha^{-1} ha and in bananas against the Black Sigatoka pathogen, *Mycosphaerella fijiensis* at 320 g-a.i. ha^{-1}. In grapes spiroxamine is the only amine representative with a registration in all major vines producing countries due to its favorable plant selectivity.

The synthesis involves the formation of a ketal of 4-*tert*-butyl cyclohexanone with racemic 3-chloro-1,2-propanediol, and substitution of the chlorine with ethylpropylamine, as described by W. Krämer and coworkers (Scheme 17.20).

Table 17.22 Data for spiroxamine.

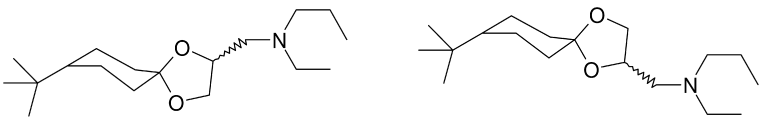
Structure/common name	Commercial data	Physicochemical data
 spiroxamine diastereomeric mixture cis:trans = 1:1	Launched by Bayer in 1997 Patent no. DE03735555 (1987) Example trade name: Impulse® Marketed by (for example) Bayer CropScience	Liquid Water solubility: 470 mg L^{-1} diastereomer A; 340 mg L^{-1} diastereomer B (20 °C, pH 7) Log P_{OW} = 2.79 diastereomer A; 2.98 diastereomer B (20 °C, pH 7) Vapor pressure: 9.7×10^{-3} Pa (mixture at 20 °C)



Scheme 17.20 Synthesis of spiroxamine.

The compound on the market is a mixture of two diastereoisomers (chiral C2 and C5), in the ratio A 49–56%/B 51–44%.

Scheme 17.21 shows the structures of diastereoisomers A and B. Notably, publication [103] also includes the preparation and the description of the biological properties of all four enantiomers.



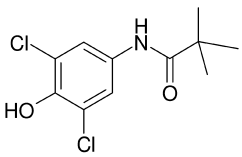
A form, 2 enantiomers B form, 2 enantiomers

Scheme 17.21 Diastereomers of spiroxamine.

17.4
SBI Class III: Hydroxyanilides

17.4.1
Fenhexamid, First Representative of the Hydroxyanilides

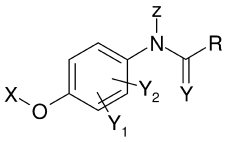
During a Bayer synthesis program directed towards photosynthesis complex II inhibitors as herbicides the biological activity of synthesis intermediates as well as of target molecules was tested also against fungi and insects. Surprisingly, 4-hydroxy-3,5-dichloro-anilides showed a weak but stable *in vitro* and *in vivo* activity against *Botrytis cinerea* in the test systems (Scheme 17.22). 1,4-Hydroxyanilides



Lead structure

Scheme 17.22

proved to be of particular interest as the starting point for chemical research, as, depending on the properties of their aromatic substituents, these molecules are easily degraded, and thus potentially have a very favorable toxicological profile and favorable environmental behavior (Scheme 17.23).



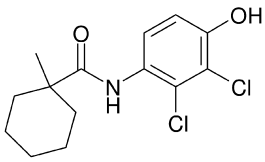
Particularly active:
X = CO-R1, H
Y1, Y2 = Halogen
Z = H
Y = O
R = tert-Cycloalkyl, tert-Haloalkyl

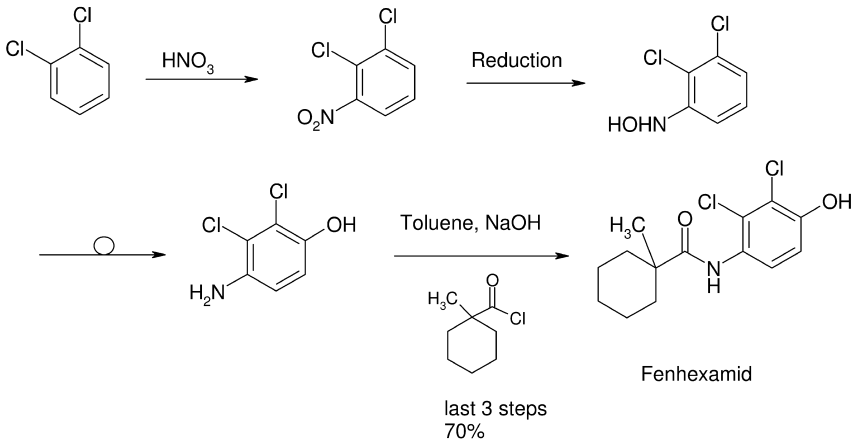
Scheme 17.23 General structure of 1,4-hydroxyanilide fungicides.

By adapting the aromatic substitution pattern, in particular by the introduction of chlorine atoms in positions 2 and 3, and the incorporation of tertiary carboxylic acids of a certain size as carboxy part of the molecule, e.g., 1-alkylcycloalkanoyl or halogen-substituted pivaloyl, highly active botryticides with additional activity against different other fungi were obtained [106].

Optimum activity was reached in a novel hydroxyanilide with the chemical name *N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide, which was presented by Bayer in 1997 as a new specific botryticide with the common name fenhexamid (Table 17.23) [107, 108].

Table 17.23 Data for fenhexamid.

Structure/common name	Commercial data	Physicochemical data
 fenhexamid	Launched by Bayer in 1998 Patent no. EP0339418 (1988) Example trade names: Teldor®, Elevate® Marketed by (for example) Bayer CropScience, Arysta LifeScience	Mp 153 °C Water solubility: 20 mg L^{-1} (pH 5–7, 20 °C) Log P_{OW} = 3.51 at 20 °C (pH 7) Vapor pressure: 4×10^{-7} Pa at 20 °C (extrapolated)



Scheme 17.24 Synthesis of fenhexamid.

The amide can be easily prepared in high yield and high purity by the reaction of 2,3-dichloro-4-hydroxy-aniline and 1-methylcyclohexane-carboxylic acid chloride, e.g., in toluene with sodium hydroxide as a base (Scheme 17.24). The starting aniline can be made by a Bamberger rearrangement of the intermediate hydroxylamine obtained by partial reduction of the corresponding nitro aromatic [109].

17.4.2
Biochemical Target of Fenhexamid

Although it became clear quite early during cross resistance studies that fenhexamid was the first representative of a new mode of action class, the exact biochemical target was unknown when the new botryticide was launched in 1998. Only in 2001 did the research group of Pierre Leroux at INRA Versailles identify fenhexamid as the first member of a new class of SBI fungicides [110].

Further studies by the same group confirmed the original findings [111]. Starting from the observation that the three 3-keto compounds 4α-methylfecosterone, fecosterone and episterone accumulated after fenhexamid application, Debieu et al. concluded that fenhexamid is a specific inhibitor of 3-keto reductase in fungal sterol biosynthesis, an enzyme involved in the C-4 demethylation. Figure 17.3 gives details on the sites of inhibition within the sterol biosynthesis pathway.

17.4.3
Biology

Fenhexamid is one of the rare SBI fungicides with a quite narrow spectrum of biological activity. *In vitro*, it shows excellent activity against *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*) and most other *Botrytis* species. Further on, the related taxon groups *Sclerotinia* and *Monilinia* are affected at low concentrations. A

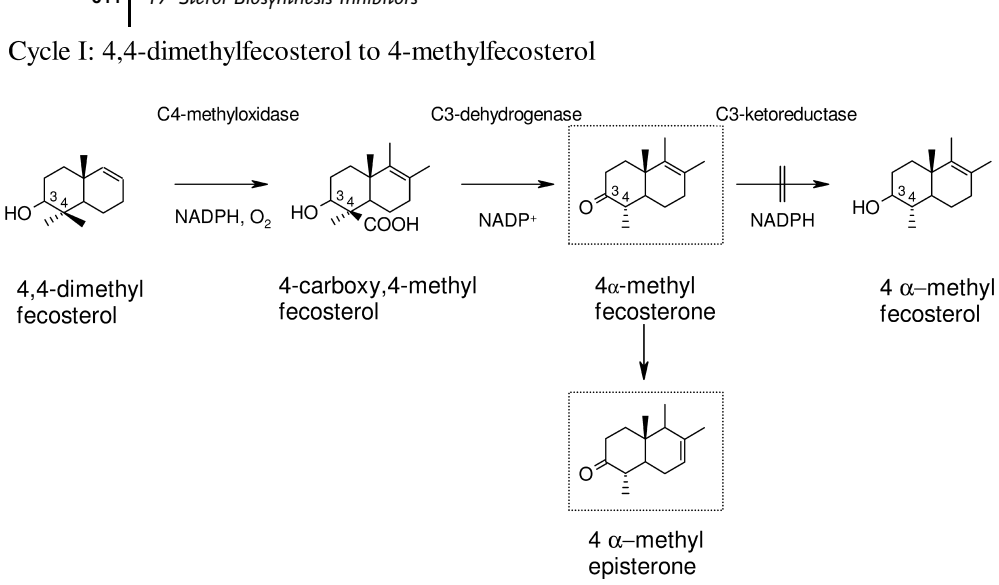
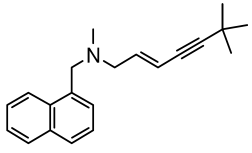
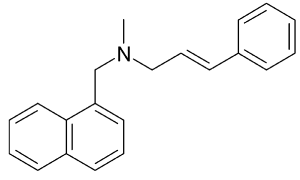
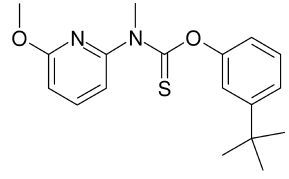


Fig. 17.3. Interference of fenhexamid in fungal sterol biosynthesis according to Debieu et al. [110]. The frames indicate accumulating sterones.

broad but moderate activity against other fungi belonging to the Ascomycetes and Basidiomycetes becomes visible only at distinctly higher concentrations under *in vitro* conditions. As with other SBI fungicides no activity against Oomycete pathogens can be detected. In good correlation with the *in vitro* results is the profile of use under field conditions. Fenhexamid is used with dosages from 375 to

Table 17.24 Squalene epoxidase inhibitors.

			
Common name	Terbinafine	Naftifine	Pyributicarb
Trade name(s)	Lamisil®	Naftin®	Toyocarb®
Chemical class	Allylamines	Allylamines	Thiocarbamates
Launch (year) company	1991 Novartis, Sandoz	Merz Pharmaceuticals	1990 Toyo Soda
Patent no.	EP 00024587, 1979		JP 85067463, 1984

1000 g-a.i. ha^{-1} in grapes, berries, stone-fruits, citrus, vegetables and ornamentals against *Botrytis cinerea* and the related pathogens *Monilinia* spp. and *Sclerotinia sclerotiorum* [112].

The main target pathogen of fenhexamid, *Botrytis cinerea*, belongs to the high risk pathogens in view of its ability to develop resistance against fungicides. Accordingly, intensive studies have been performed during the pre-market period to clarify eventual risks. The studies revealed that, already, before market introduction a small part of the population was able to metabolize fenhexamid under *in vitro* conditions [113]. However, as demonstrated by Suty et al., the metabolization took place only within long periods of undisturbed growth under optimal conditions. As these requirements are obviously not fulfilled under outdoor conditions, the practical importance of this resistance mechanism is low. Generally, these tendencies have been confirmed by the group of Pierre Leroux at INRA, Versailles [114]. Detailed studies of this group were able to differentiate three different groups with specific resistance to fenhexamid, Hyd R1 to Hyd R3.

17.5 SBI Class IV: Squalene Epoxidase Inhibitors

SBI class IV includes squalene epoxidase inhibitors that are actually not used as agricultural fungicides. Fungal squalene epoxidases are only very distantly related to their mammalian and higher plant counterparts in the phylogenetic tree [115] and are therefore principally suited as target of selective antimycotics as well as of herbicides.

Table 17.24 shows that inhibitors of squalene epoxidase belong to two different chemical classes. The allylamines consist of two compounds used as antimycotics against a wide range of fungi. Terbinafine is used in topical and oral uses whereas naftifine is restricted to topical uses.

Pyributicarb is a systemic herbicide, absorbed by roots, leaves and stem, and translocated to active growth sites where it inhibits elongation of roots and aerial plant parts. It is mainly used in rice and turf against annual and perennial grass weeds such as *Echinochloa*.

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18
Carboxylic Acid Amide (CAA) Fungicides

Ulrich Gisi, Clemens Lamberth, Andreas Mehl, and Thomas Seitz

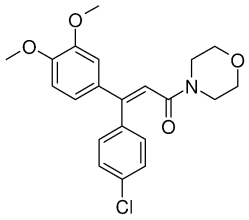
18.1
Introduction

The chemical group of Carboxylic Acid Amide (CAA) fungicides was officially announced by FRAC (www.frac.info) in 2005 as group number 40 in the FRAC code list, including the three sub-classes cinnamic acid amides (dimethomorph, flumorph), valinamide carbamates (benthiavalicarb, iprovalicarb, valiphenal) and mandelic acid amides (mandipropamid) (Fig. 18.1). The reason for this classification was a common cross resistance pattern amongst all members for the vast majority of the tested isolates of *Plasmopara viticola*. Other common features are the specific and rather narrow spectrum of activity, including, within the Oomycetes, pathogens of the families Peronosporaceae (e.g., *Bremia* on lettuce, *Peronospora* on tobacco, pea, onion, *Pseudoperonospora* on cucurbits, *Plasmopara* in grape and sunflower) and Pythiaceae (*Phytophthora* spp. on many crops such as potato, tomato, pineapple) except for the entire genus *Pythium*, which is insensitive, as are all other pathogens outside the Oomycetes. Dimethomorph (**1**) was the first in the class to be introduced in 1988 [1] followed by iprovalicarb (**3**) in 1998 [2], flumorph (**2**) in 2000 [3], benthiavalicarb (**4**) in 2003 [4] and mandipropamid (**6**) in 2005 [5]. Valiphenal (**5**) is expected to be introduced in the next few years. In addition, several experimental compounds and compound families have been described in literature within this chemical class, such as the glyoxylic acid derivatives (**53**) (related to mandelic acid amides) in 1995 [6], the mandelic acid amide SX 623509 (**34**) in 2003 [7] and the aminosulfone XR-539 (**30**) in 2005 [8]. The mode of action of CAA fungicides is not known but inhibition of cell wall deposition and phospholipid biosynthesis were described as potential targets (see below). Table 18.1 summarizes the physicochemical data of CAA fungicides.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

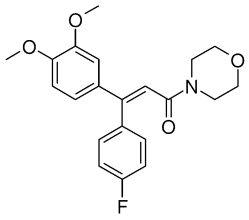
ISBN: 978-3-527-31496-6

Cinnamic acid amides:



1

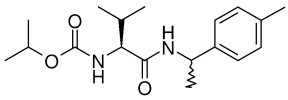
dimethomorph



2

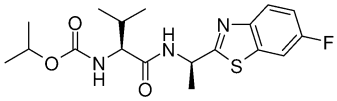
flumorph

Valinamides:



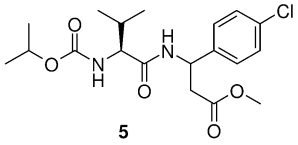
3

iprovalicarb



4

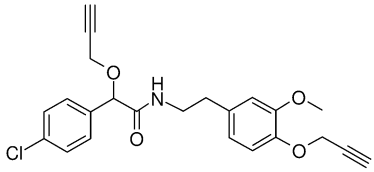
benthiavalicarb



5

valiphenal

Mandelic acid amides:



6

mandipropamid

Fig. 18.1. Carboxylic acid amides launched into the market (status 2006). Valiphenal (**5**) is expected to be introduced within the next few years.

Table 18.1 Physicochemical data of launched carboxylic acid amides (according to the *Pesticide Manual* 2006, BCPC, Alton, UK).

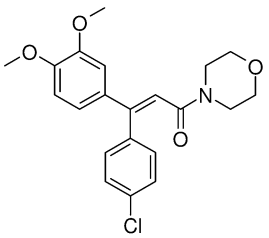
CAA	Dimethomorph (1)	Flumorph (2)	Iprovalicarb (3)	Benthiavalicarb (4)	Mandipropamid (6)
Melting point (°C)	125–149	105–110	163–165	152.0	96.4–97.3
Solubility in water (mg L^{-1})	42–81		11.0	13.1	4.2
Solubility in organic solvents (mg L^{-1})	Acetone: 106		Acetone: 22; DMSO: 42		
Vapor pressure (Pa)	9.7×10^{-8}		7.7×10^{-8}	$<3.0 \times 10^{-4}$	$<9.4 \times 10^{-7}$
Log P_{OW}	2.63	2.20	3.2 (20 °C)	2.52	3.2 (25 °C)

18.2 Chemistry of Carboxylic Acid Amides

18.2.1 Cinnamic Acid Amides

18.2.1.1 Dimethomorph

Dimethomorph (**1**) (Fig. 18.2) was discovered as a specific Oomycete fungicide in the early 1980s by the pharmaceutical research group at Celamerck. This company was subsequently acquired by Shell, whose agrochemical business was in turn acquired by American Cyanamid, which was then acquired by BASF. Dime-

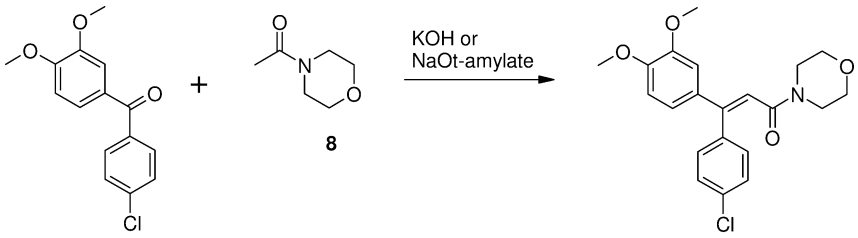


1

Fig. 18.2. Chemical structure of dimethomorph (**1**).

thomorph was described in detail by Albert et al. in 1988 [1] and is a mixture of E and Z isomers. The fungicidal activity resides exclusively in the Z isomer. However, in sunlight dimethomorph rapidly equilibrates to an E/Z mixture of about 20:80.

A concise synthesis of dimethomorph can be achieved by condensation of 4-chloro-3′,4′-dimethoxybenzophenone (**7**) and *N*-acetylmorpholine (**8**) with the aid of potassium hydroxide [9] or sodium *tert*-amylate (Scheme 18.1) [10].

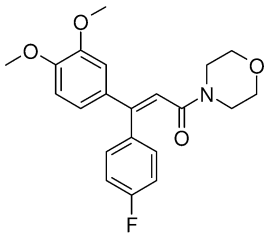


7 8 1

Scheme 18.1. Synthesis of dimethomorph (**1**).

18.2.1.2 **Flumorph**

Flumorph (**2**), a close analog of dimethomorph, was developed by Shenyang (Fig. 18.3) [3]. Herein, the replacement of dimethomorph's chloro substituent by a fluorine atom seems to further improve the antisporulant and curative activities. Flumorph is composed of a mixture of E and Z isomers in an E/Z ratio of 45:55.



2

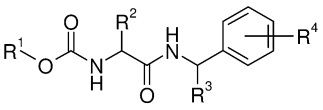
Fig. 18.3. Chemical structure of flumorph (**2**).

18.2.2 **Amino Acid Amides**

18.2.2.1 **Iprovalicarb**

Iprovalicarb (**3**) was the first fungicide introduced into the market out of the amino acid amide carbamate class of compounds with the general formula **9**,

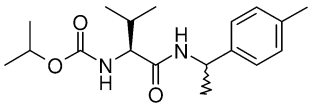
which was discovered by Bayer during a synthesis program for new fungicidal lead structures in 1988 [2]. Even first representatives of this compound class showed interesting effects on pathogens of the Oomycetes such as *Plasmopara viticola* or *Phytophthora infestans* [11]. Its structural variability permitted optimization work to be carried out on the basic chemical structure **9** (Fig. 18.4).



9

Fig. 18.4. General structure of amino acid amide carbamates.

Good efficacy is obtained in particular with α-branched alkyl residues or directly bound aromatic systems in the carbamate section of the underlying structure (R^1). The use of valine or isoleucine as the amino acid portion of the molecule (R^2 = isopropyl or *sec*-butyl) leads to highly active compounds. Finally, the use of an α-branched arylethylamine as the amine portion of the amino acid amide carbamate (R^3) ensures good efficacy. The outcome of a vast selection program was the development of iprovalicarb (**3**) (Fig. 18.5) [12].

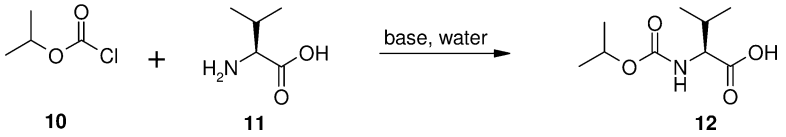


3

Fig. 18.5. Chemical structure of iprovalicarb (**3**).

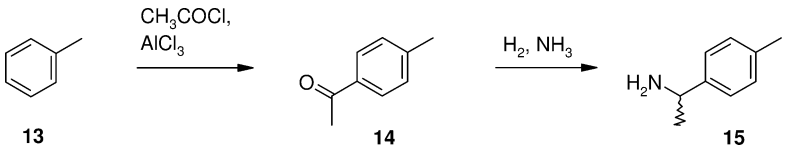
The iprovalicarb molecule contains two chiral centers; the configuration of the stereocenter in the amino acid function is defined by the use of L-valine as a natural amino acid component. The amine portion of the molecule is racemic, so the active substance contains two diastereomers (the S,S- and S,R-diastereomers). Iprovalicarb (**3**) is made up of three building blocks: the carbamate component isopropyloxycarbonyl, the natural amino acid L-valine (**11**), and the amine unit *p*-methylphenylethylamine (**15**). In the first step, isopropyl chloroformate (**10**) is treated with L-valine (**11**) in aqueous sodium hydroxide solution to give isopropyloxycarbonyl-L-valine (**12**) (Scheme 18.2).

A short parallel sequence produces the amine component *p*-methylphenylethylamine (**15**) from toluene (**13**). First, a Friedel-Craft acylation is used to selectively convert toluene (**13**) into *p*-methylacetophenone (**14**). In a second step, the reduc-



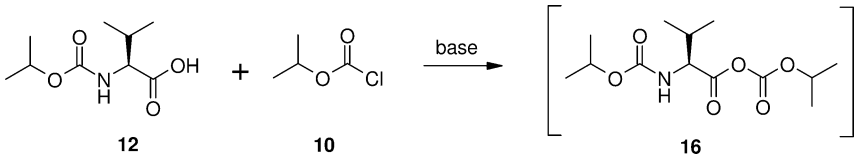
Scheme 18.2 Synthesis of iprovalicarb (final step).

tive amination of **14** with hydrogen and ammonia in the presence of a Raney nickel catalyst produces the required *p*-methylphenylethylamine (**15**) (Scheme 18.3).



Scheme 18.3

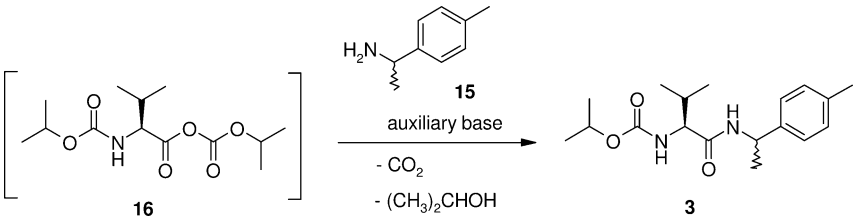
In the last step of the reaction the carboxylic acid function of isopropyloxycarbonyl-L-valine (**12**) is activated by treating it with a second equivalent of isopropyl chloroformate (**10**) under basic conditions using toluene as a solvent. This produces the reactive mixed anhydride (**16**) as an intermediate (Scheme 18.4).



Scheme 18.4

Finally, the mixed anhydride **16**, which cannot be isolated, is treated with an auxiliary base and the *p*-methylphenylethylamine (**15**), already prepared in toluene solution, as part of the same reaction step to form the active substance iprovalicarb (**3**) with the elimination of carbon dioxide and isopropanol (Scheme 18.5).

The entire reaction sequence can be carried out on a laboratory scale with an isolated yield of **3** of more than 85%, and it can also be employed on the technical scale [13–15].



Scheme 18.5

Iprovalicarb (**3**) was first registered in Indonesia in 1998. The product received approval in Germany in 2000 in combination with tolylfluanid (as Melody Multi®) and has been registered in France and Italy in combination with mancozeb (as Yorel®), folpet (as Melody Care®, Melody Combi®, Odena®, Sirbel®) and with copper (as Melody Compact®, Ocarina®). Additionally, iprovalicarb has been registered in combination with propineb (as Invento®, Melody WP®, Melody Duo®, Positron®) in many countries. A ternary mixture with mancozeb and fosetyl-Al (Melody Triplo®) was approved in Italy in 2002. These combination products provide a broad spectrum of activity under various growing conditions in different target crops and contribute to an effective anti-resistance strategy.

18.2.2.2 **Benthiavalicarb**

Benthiavalicarb (**4**, benthiavalicarb-isopropyl, KIF-230, Fig. 18.6) was discovered by Kumiai-Ihara and has been developed for the control of Oomycete diseases such as downy mildews (*Plasmopara viticola* and *Pseudoperonospora cubensis*) on grape vine and vegetables and late blight (*Phytophthora infestans*) on potatoes [4, 16]. The valinamide derivative benthiavalicarb (**4**) shows a close structural similarity to iprovalicarb (**3**).

Application for EU approval was submitted in early 2002 and the dossier was declared complete in 2003. Kumai Chemical received its first global registrations for benthiavalicarb in Switzerland and Cuba. The mixture of benthiavalicarb with mancozeb (Valbon®) was launched in Switzerland in 2004 for use against potato late blight. Concurrently, Vincare® (benthiavalicarb plus folpet) was launched for use against grape downy mildew. In 2005, Valbon® was launched in Belgium, the Netherlands and UK; in 2006, Valbon® and Vincare® were introduced in Austria.

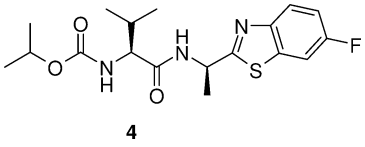
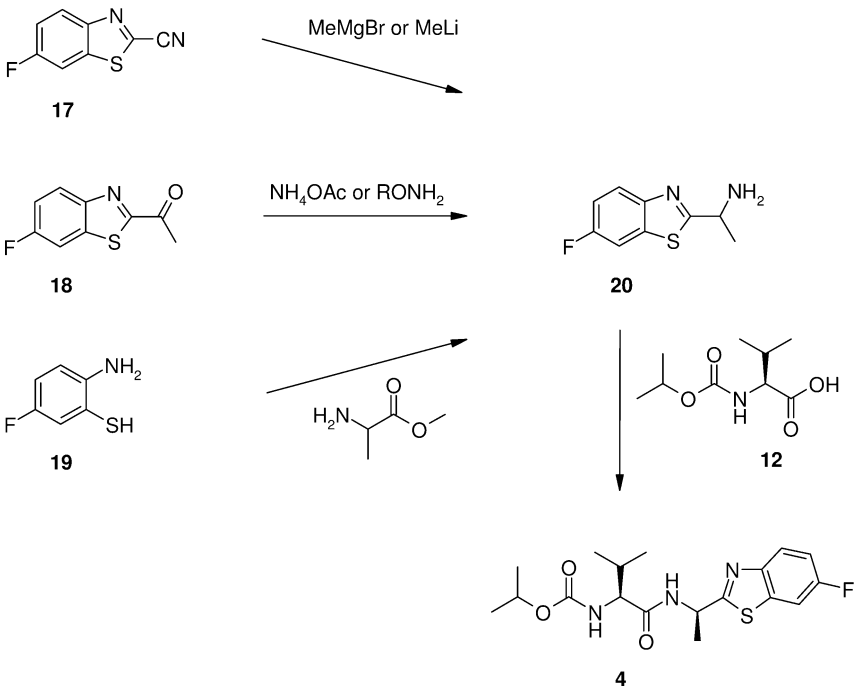


Fig. 18.6. Chemical structure of benthiavalicarb (**4**).

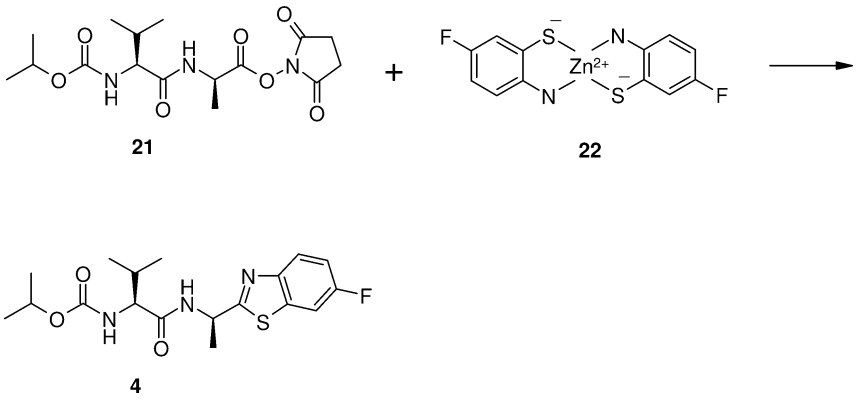
The benthiavalicarb molecule contains, similarly to iprovalicarb, two chiral centers. The configuration of the stereocenter in the first amino acid function is defined by the use of L-valine as a natural amino acid component. The second portion of the molecule contains the unnatural amino acid (*R*)-alanine.

In the first synthesis step isopropyl chloroformate (**10**) is treated with L-valine (**11**) in aqueous sodium hydroxide solution to give isopropyloxycarbonyl-L-valine (**12**), analogous to the iprovalicarb synthesis. In a parallel sequence the amine component 2-(1-aminoethyl)-6-fluorobenzothiazole (**20**) can be synthesized from 2-amino-5-fluorothiophenol (**19**) and its reaction with (*R*)-alanine ester. Other ways are the stereoselective reductive amination of 2-acetyl-6-fluorobenzothiazole (**18**) or the Grignard reaction of 2-cyano-6-fluorobenzothiazole (**17**) with methylmagnesium bromide or methyllithium (Scheme 18.6). In the last step of the reaction the carboxylic acid function of isopropyloxy-carbonyl-L-valine (**12**) is activated and, finally, the mixed anhydride **16** is treated with an auxiliary base and the 2-(1-aminoethyl)-6-fluorobenzothiazole (**20**), already prepared in toluene solution as part of the same reaction step, to form the active substance benthiavalicarb (**4**) [17]. An alternative synthesis route is the amidation reaction of isopropyloxycarbonyl-L-valine (**12**) with (*R*)-alanine, which is protected at the carbonic acid function with *N*-hydroxysuccinimide. The resulting dipeptide **21** can be converted into the target molecule benthiavalicarb (**4**) by reaction with the zinc salt of 2-



Scheme 18.6

amino-5-fluorothiophenol (**22**) in dimethylformamide and water under acidic conditions (Scheme 18.7) [18].

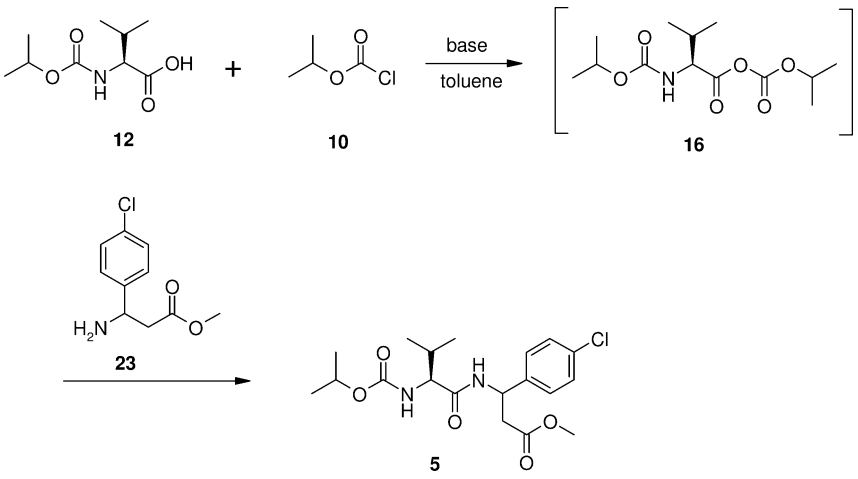


Scheme 18.7

18.2.2.3 **Valiphenal (Experimental Compound)**

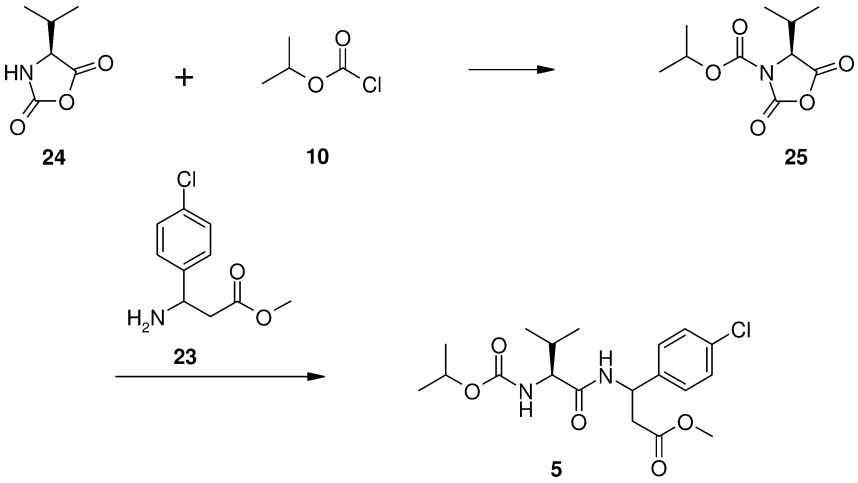
Valiphenal (**5**), currently under development by Isagro (IR 5885), is a fungicidal dipeptide of the valinamide class of compounds and is active against Oomycetes, such as *Phytophthora* sp., *Peronospora* sp. and *Plasmopara* sp., suitable for use in crops such as grapevines, potatoes and various vegetables. Detailed information on the biological characteristics of valiphenal has not yet been published.

The compound can be synthesized in a similar way to that shown for iprovalicarb, via the mixed anhydride **16** (Scheme 18.8). A second process is exemplified



Scheme 18.8

by a single preparation of the specified compound, methyl (±)-(*RS*)-3-(*N*-isopropoxycarbonyl-*S*-valinyl)amino-3-(4-chlorophenyl)propanoate (**5**). The process consists of the addition of *N*-methylmorpholine to a mixture of isopropyl chloroformate (**10**) and 4-isopropyloxazolidine-2,5-dione (**24**) in ethyl acetate. The resulting N-alkoxycarbonyloxazolidinedione intermediate **25** is then reacted with the aminoester **23** to give the desired dipeptide **5** (Scheme 18.9) [19].

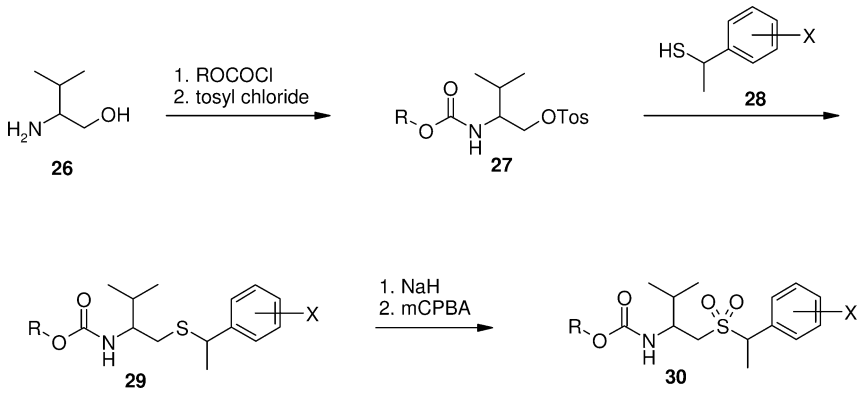


Scheme 18.9

18.2.2.4 **Aminosulfones (Experimental Compounds)**

The experimental fungicide XR-539 (**30**) discovered by Dow belongs to the aminosulfone class of chemistry showing high activity against Oomycete diseases, such as grape downy mildew and potato late blight [8]. These compounds can be synthesized as follows. In a first step the *β*-amino alcohol **26** is reacted with a formic acid chloro ester to afford the corresponding carbamate-protected amino alcohol. The free hydroxy group is then activated with tosyl chloride to give the ''tosylate electrophile'' **27**. In the central step to the desired molecule this ''tosylate electrophile'' is coupled with the ''thiol nucleophile'' phenethylthiol **28**, followed by oxidation of the resulting sulfide **29** to the sulfone **30** (Scheme 18.10) [20].

XR-539 (**30**) bears some structural similarity to the amino acid amide carbamates, i.e., iprovalicarb and benthiavalicarb-isopropyl. In radial growth inhibition assays against *Phytophthora capsici*, XR-539 (**30**) acted similarly to dimethomorph and iprovalicarb both in potency and in the shape of their dose–response curves [8]. Similar to other CAA fungicides, XR-539 is inactive against *Pythium ultimum*. Strong evidence was provided that XR-539 is cross resistant to other CAAs in *Plasmopara viticola* and thus acts by the same general mechanism as, for example, valinamides and cinnamic acid amides. It remains to be established whether the



Scheme 18.10 Synthesis of XR-539.

various compounds bind to the same target protein or, alternatively, act on different proteins in the biochemical pathway or process [8].

18.2.2.5 N-Sulfonyl Amino Acid Amides (Experimental Compounds)

The carbamate moiety of N-carbamoyl amino acid amides can be replaced by a sulfonamide function with full preservation of their biological activity. However, in this case, the α-methylbenzylamine moiety, which is typical for amino acid amide carbamates, has to be exchanged by a special dialkoxy-substituted phenethylamine. As with amino acid amide carbamates, a diverse range of different amino acids can be transformed into this kind of fungicides [21]. In general, the amino acid needs a lipophilic backbone for good fungicidal activity. Examples of suitable amino acids are valine and isoleucine, but also non-proteogenic amino acids like phenylglycines are tolerated [21]. The configuration of the chiral α-carbon atom is also important. The naturally occurring L-forms show, in most cases, higher activities than their D-enantiomers. The two highly active N-sulfonyl amino acid amides **31** and **32** demonstrate the similarities and differences between valinamides and phenylglycinamides (Fig. 18.7). Both subclasses have a

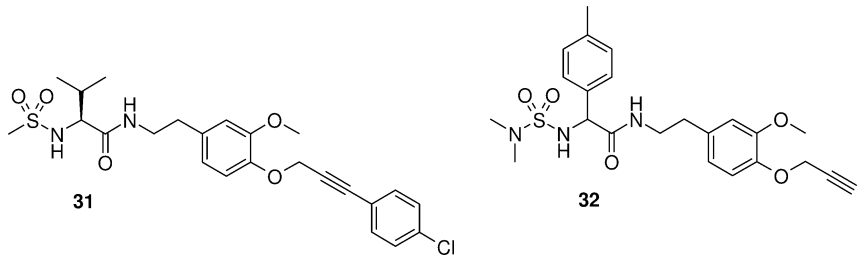


Fig. 18.7. Structures of **31** and **32**.

common characteristic, in that small sulfonyl groups, like methylsulfonyl, ethylsulfonyl and dimethylsulfamoyl, achieve the best biological activity [21]. Different structure–activity requirements affect the para-position of the phenethylamine moiety. The valinamide **31** with a chlorophenylpropargyloxy substituent demonstrates its high efficacy against *Phytophthora infestans* on tomato with the very low EC_{80} of 0.02 ppm. The much shorter propargyloxy group is the best substituent for the 4-position of the phenethylamine in phenylglycinamides like **32** [21].

18.2.3 Mandelic Acid Amides

18.2.3.1 Mandipropamid

The antifungal activity of mandelic acid amides (mandelamides) with dialkoxylated phenethylamine moieties was first discovered for human pathogens by Yu and Van Scott in the mid-1980s [22]. They found that **33**, which is the acetylated adduct of mandelic acid and homoveratrylamine, has significant activity against skin disorders like mycosis fungoides and psoriasis (Fig. 18.8). In the early 1990s, this general structure was taken up by chemists at Agrevo (now Bayer), who found that the mandelamide SX 623509 (**34**) has activity against plant pathogens, especially Oomycetes [7, 23, 24]. At Novartis (now Syngenta), replacement of the ethoxy group of **34** by a propargyloxy function resulted in the mandelamide **35** with enhanced fungicidal efficacy [25]. The exchange of methoxy and ethoxy groups by propargyloxy often leads to increased biological activity, as reported in the pharmaceutical literature for compounds with antibacterial [26] and leishmanicidal activity [27].

The introduction of a second propargyl group into the mandelic acid moiety of **35** clearly increased the fungicidal activity further, leading finally to Syngenta's

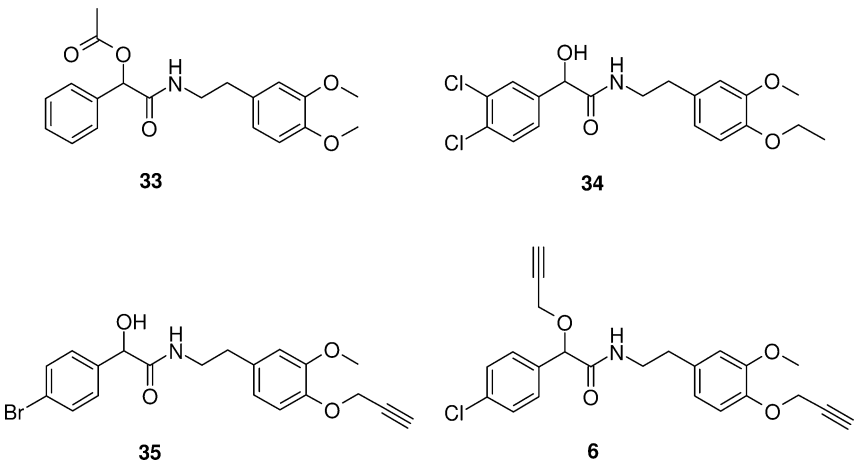
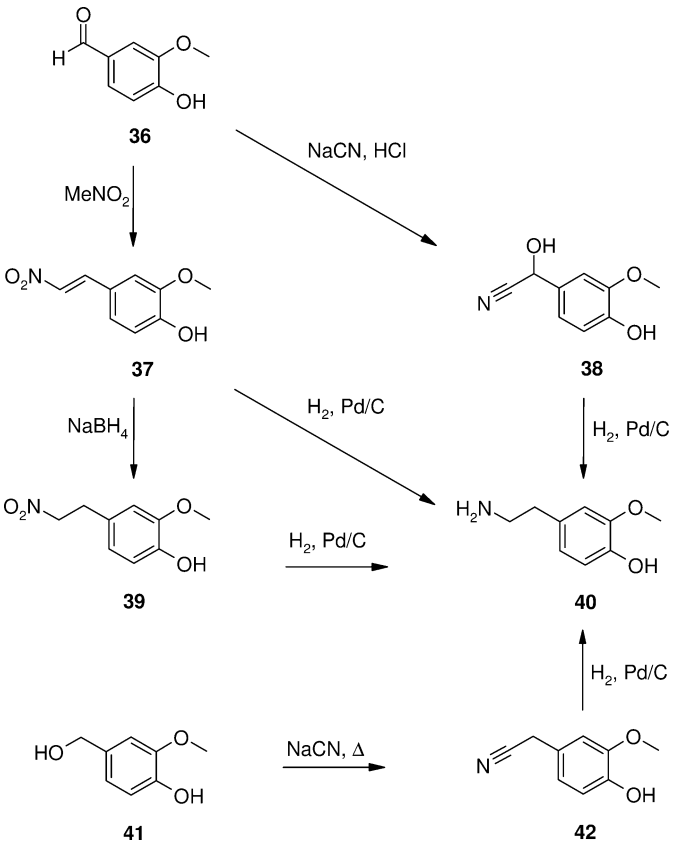


Fig. 18.8. Mandelic acid amides.

fungicide mandipropamid (**6**), which is the first derivative of the chemical class of mandelamide fungicides to be commercialized (Fig. 18.8) [5, 28, 29].

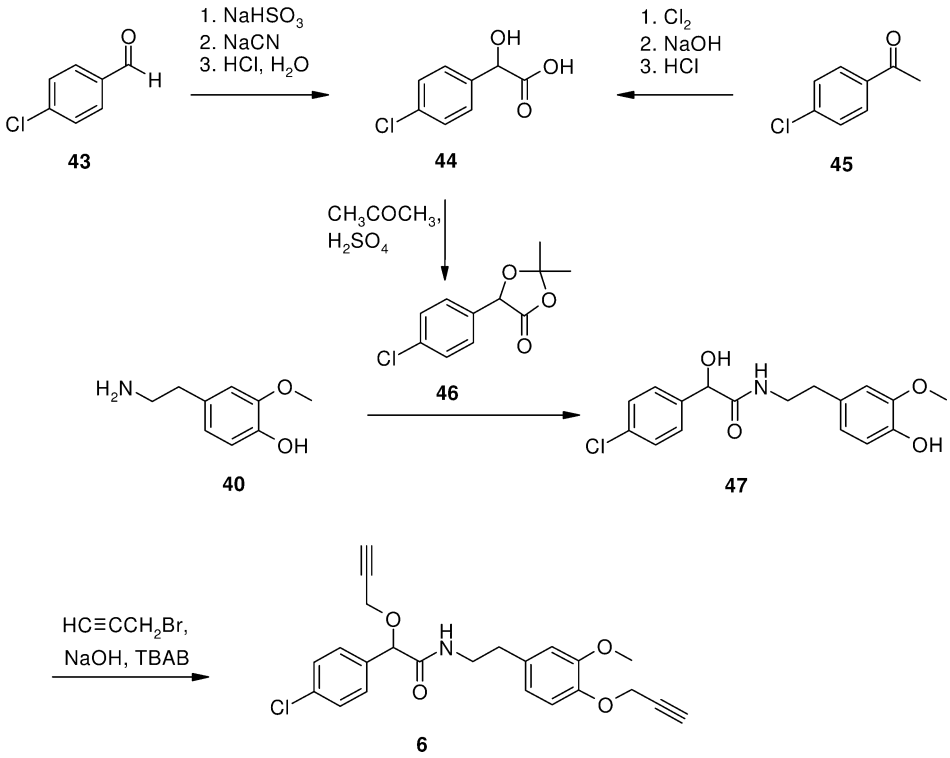
An important building block for the amine moiety of mandelic acid amides is 2-(4-hydroxy-3-methoxy)phenethylamine (**40**), also known as 3-O-methyldopamine or 3-methoxytyramine. In principle, **40** can be prepared by several different methods, three of which are highlighted in Scheme 18.11. Most widely applied is the reduction of the nitrostyrene **37**, which may be obtained by the Henry reaction of vanillin (**36**) and nitromethane. This reduction of a nitro group and an olefin function can be performed in one step, using either catalytic hydrogenation [30] or lithium aluminum hydride [31], or more reliably and avoiding undesirable highly exothermic reaction profiles, in two steps via the phenylnitroethane **39** [21]. A second strategy is the catalytic hydrogenation of vanillin cyanohydrin (**38**) [32]. The third possibility is the catalytic hydrogenation of the benzyl cyanide **42**, which can be directly obtained from vanillinol (**41**) through a quinoid transition state [33].



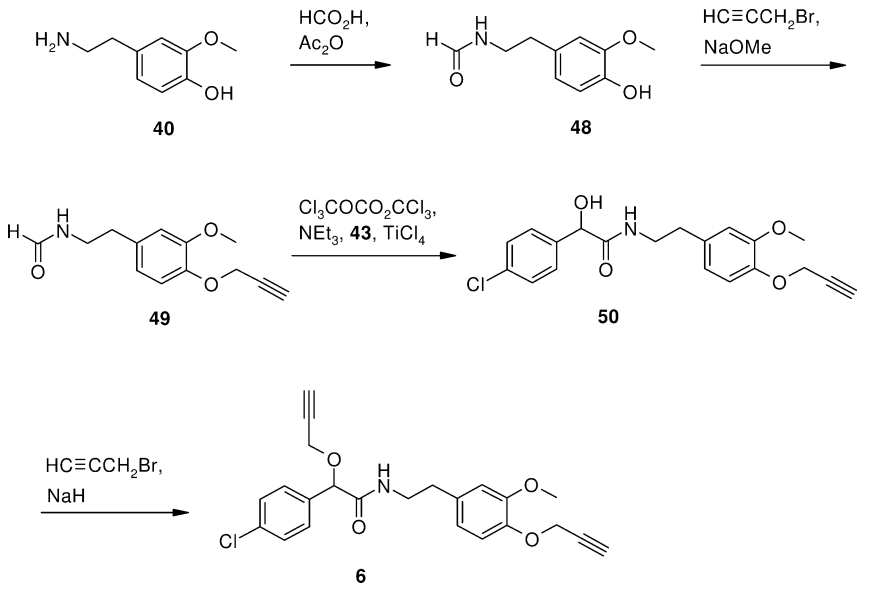
Scheme 18.11 Synthesis of 3-O-methyldopamine.

The synthesis of mandipropamid (**6**) is possible via several different routes. One important approach employs 4-chloromandelic acid (**44**), which can be prepared from 4-chlorobenzaldehyde (**43**) via Strecker-type mandelonitriles [34] as well as with chloroform and sodium hydroxide (Scheme 18.12) [35]. Alternatively, 4-chloroacetophenone (**45**) can be converted into **44** either in two steps by dichlorination and a subsequent Cannizzaro-type transformation of the intermediate dichloromethyl ketone [36] or by a ytterbium triflate promoted tandem one-pot oxidation – Cannizzaro reaction [37]. 4-Chloromandelic acid can be transformed with acetone under acidic conditions into the acetonide **46** [38], which is ring-opened [39] with 2-(4-hydroxy-3-methoxyphenethyl)amine (**40**) [21] to the mandelamide **47** [40], which bears an alcoholic as well as a phenolic hydroxy function. Both OH groups may be simultaneously propargylated with propargyl bromide and sodium hydroxide under phase transfer conditions to obtain mandipropamid (**6**).

In a different approach, the propargylated formamide **49**, available in two steps from **40**, can be directly transformed by Seebach's modification [41] of the Passerini reaction [42] into the mono-propargylated mandelamide **50** (Scheme 18.13).



Scheme 18.12 Synthesis of mandipropamid.



Scheme 18.13. Alternative synthesis of mandipropamid.

The introduction of a second propargyl group into the hydroxy function of the mandelic acid moiety of **50** leads to mandipropamid (**6**) [28].

Mandipropamid (**6**) has been developed as racemic mixture of both enantiomers. Also, the stereoselective synthesis of enantiopure mandelamide fungicides is possible via a diastereoselective Passerini reaction with a galacturonic acid derivative as acid component [43] as well as by enantioselective hydrogenation of phenylglyoxylic amides with a homogeneous Rh catalyst system [44]. However, none of the enantiomers, when used alone, offered a biological advantage over the mixture; therefore, mandipropamid was registered as racemate. Other amides that are closely related to mandelamides can also achieve high fungicidal activity (Fig. 18.9). The glyceric acid amide **51**, which bears an OCH_2

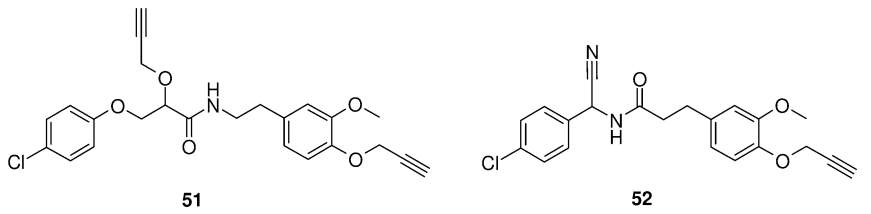
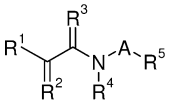


Fig. 18.9. Structures of **51** and **52**.

spacer between the 4-chlorophenyl ring and the 2-propargyloxyacetamide function of mandipropamid, has been reported by Syngenta to be highly active against *Phytophthora infestans* on tomato (EC_{80} 0.02 ppm) [45]. The hydroferulic acid amide **52**, which is a mandipropamid derivative with an inverted amide function, possesses powerful anti-oomycete efficacy and has been patented by Sumitomo [46].

18.2.3.2 Glyoxylic Acid Derivatives (Experimental Compounds)

The glyoxylic acid derivatives **53** (Fig. 18.10) were discovered by Bayer in 1994 as a new class of Oomycete fungicides by derivatization of advanced research project compounds with broad fungicidal activity [6].



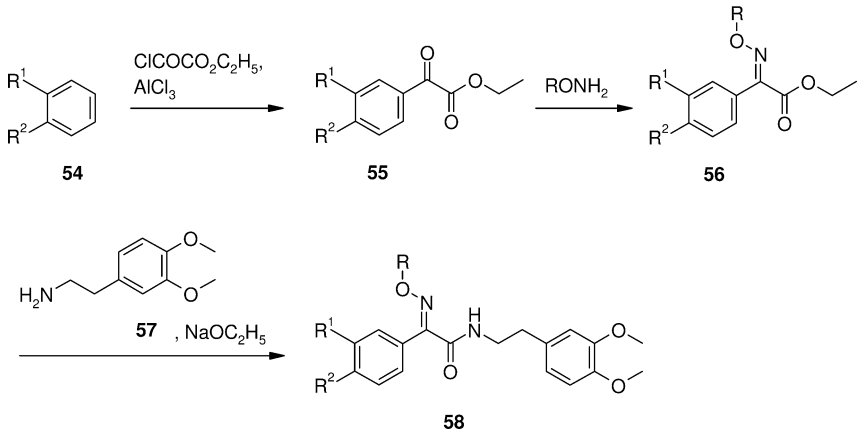
53

Fig. 18.10. General structure of glyoxylic acid derivatives.

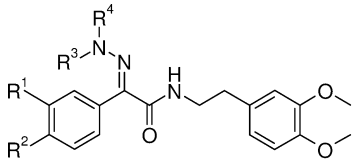
The glyoxylic acid derivatives **53** exhibit specific activity against Oomycetes, including downy mildew on grapes (*Plasmopara viticola*) and late blight on potatoes and tomatoes (*Phytophthora infestans*). In addition they are active against Oomycetes in soil such as *Phytophthora* in tobacco and citrus. The derivatives exhibit protective, curative, eradicative and antisporulant activity. This class of compounds can be synthesized with a simple synthetic approach. In the first step oxalic ester chloride is added to the substituted phenylic part via a Friedel–Crafts acylation. In the second step the keto function of the glyoxylic acid ester **55** is converted into the oxime ether function by reaction with an alkoxy amine derivative. The resulting intermediate **56** can be easily transformed into the desired product **58** via an amidation reaction with a substituted phenethylamine (**57**) under basic conditions [6].

For high fungicidal activity it is important to have small substituents like chlorine, methyl or ethyl in the meta or para position of the aromatic moiety of the glyoxylic acid molecule part (R1, R2) or a saturated or aromatic bicyclic system (with R1, R2 building a ring together). Ortho substituents lead to complete inactivity of the final compound. At the oximether moiety (R) small alkyl substituents like methyl are preferred (Scheme 18.14).

In the past decade several variations of that structural motif have been invented by different companies. All of these compound families exhibit specific activities against Oomycetes. Figure 18.11 displays a few examples [47–49].



Scheme 18.14 Synthesis route to glyoxylic acid amides-oximether.



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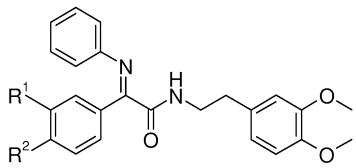
Bayer



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Bayer

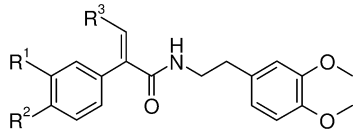

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Fig. 18.11. Examples of glyoxylic acid derivatives displaying specific activity against Oomycetes.

18.3
Biological Activity of Carboxylic Acid Amides

All CAA fungicides are specifically effective against foliar pathogens of the Oomycetes, including species of the Pythiaceae such as *Phytophthora infestans* (potato and tomato late blight) and Peronosporaceae such as *Plasmopara viticola* (grape downy mildew) and *Pseudoperonospora cubensis* (cucumber downy mildew). The entire genus *Pythium*, as well as all pathogens outside the Oomycetes, are not sensitive to CAA fungicides. CAAs inhibit the germination of cystospores and sporangia (but not zoospore release and motility), they affect growth of germ tubes and mycelium, thus preventing infection of the host tissue. After foliar application, CAAs are reported to exhibit, besides their strong preventive activity, curative activity and some eradicative effects, depending on the quantity of the fungicide taken up into the leaf and its distribution based on translaminar movement. Dimethomorph has good preventive activity; in addition, curative and antisporulant activities are very pronounced. After a soil drench or foliar application, iprovalicarb is reported to be more systemic than dimethomorph (**1**) [2, 50, 51] and mandipropamid (**6**). Iprovalicarb (**3**) is a systemic compound [52], it is distributed in the apoplast (acropetal translocation) and also protects untreated leaves against infection, especially in grapes. Autoradiographs of grape leaves treated with ^{14}C-iprovalicarb showed a high level of systemic distribution in the tissue [53]. Generally, specific ingredients in the formulation have a strong impact on uptake and fungicidal activity [2]. Elevated temperature, humidity and leaf wetness also increase the uptake and curative activity of iprovalicarb [53]. Benthiavalicarb delivers long-lasting preventive [4, 54] and some loco-systemic but low translaminar activity in grape leaves [55]. Further studies showed that benthiavalicarb, applied at 1 to 6 days post-inoculation, protected grape leaves against downy mildew and inhibited sporulation of *P. viticola* [55]. Mandipropamid binds rapidly and tightly to the wax layer of the leaf surface, providing a rainfast and long-lasting barrier against infections [56]. It delivers strong preventive and translaminar activity and provides robust control of both *Phytophthora infestans* and *Plasmopara viticola* also under severe disease pressures [5].

18.4
Mode of Action and Mechanism of Resistance for CAA Fungicides

Cytological studies have implicated that dimethomorph, iprovalicarb and benthiavalicarb inhibit processes involved in cell wall biosynthesis and assembly [57–63]. This was supported by observations that they affect regeneration of protoplasts of *P. infestans*, alter the staining of cell walls with fluorochromes, and inhibit the encystment of zoospores of various *Phytophthora* species and *Plasmopara viticola* or cause their lysis. No inhibition was observed for zoospore discharge from sporangia and zoospore motility. However, further studies showed that CAAs had no ef-

fect on the transition of zoospores into cystospores, which requires cell wall synthesis and rearrangement. These findings indicate that cell wall deposition at this stage is obviously insensitive [61]. The most sensitive developmental stage in the life cycle of Oomycetes is the germination of cystospores and sporangia. One hour of incubation of cystospores in CAAs followed by an incubation in water for another 2 h was not as effective as a continuous exposure, suggesting that the binding of CAAs to its target is not completed within 1 h, either because CAAs did not reach it, the target is not yet ready for binding, or because the binding is weak [61]. The process of cell wall synthesis in Oomycetes is rather complex and still not well investigated. The altered architecture of the cell wall after CAA treatment may be a consequence of effects on cytoskeletal elements or membrane bound components (e.g., receptors, enzymes) responsible for transport of cell wall precursors. Enzymes associated with cell wall biosynthesis, such as glucanases and synthases of β-1,3 glucans and cellulose, are not necessarily inhibited [58, 60, 61]. In studies with iprovalicarb a direct inhibition of glucan synthase could be excluded [60]. Thus, CAAs may inhibit the three-dimensional arrangement and cross-linkage of the complex glucan structure necessary for germ tube and hyphal growth. Cytological studies with *P. infestans* showed a different microtubule organization after treatment with iprovalicarb than with dimethomorph [59]. Alterations in phospholipid biosynthesis were also proposed with an inhibition of phosphatidylcholine (lecithin) biosynthesis as the main target [7]. Since rather high fungicide concentrations were used in this study, it is not clear whether the observed effects are a reaction to general cell death rather than a specific inhibition of lecithin biosynthesis. In conclusion, considering all observations published so far, the biochemical mode of action of CAA fungicides is still not elucidated.

Resistance to CAAs in *Phytophthora* (mainly *P. infestans* but also other species) has never been detected in field population even though dimethomorph has been in use for more than 15 years. The lack of resistant isolates in nature encouraged several researchers to produce artificial mutants *in vitro* [64–70]. Mutants resistant to dimethomorph (**1**), flumorph (**2**) or mandipropamid (**6**) were produced but were found to show reduced growth rates, reduced frequency of infections on leaves and tubers and lower fitness or survival over several generations compared with wild-type isolates. Accordingly, based on the lack of practical resistance and stable mutants, the resistance risk of *P. infestans* to CAA fungicides has been estimated to be low (FRAC CAA Working Group, CAA FRAC guidelines, www.frac.info). However, resistance to CAAs in *P. viticola* had already been reported in 1994, shortly after the introduction of dimethomorph (**1**) in France [69]. As a consequence, intensive sensitivity monitoring was done across European vineyards by several companies and resistant isolates were repeatedly detected mainly in some of the grape growing regions in France and Germany. Clear cross resistance was found among all CAAs for the vast majority of isolates. However, isolates were found at low frequency which showed resistance only to iprovalicarb but not to dimethomorph and *vice versa*. As to be expected, no cross

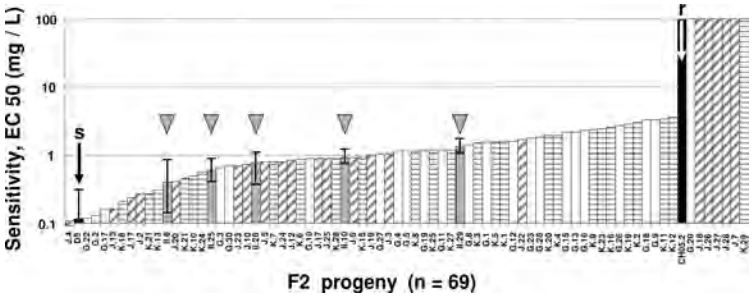

Fig. 18.12. Sensitivity to mandipropamid (**6**) of F2 progeny isolates ($n = 69$) produced from crosses (G, J, K, white and hatched columns) between sensitive F1 isolates (gray columns highlighted by triangles) of *Plasmopara viticola* (black columns highlighted by arrows are F0 sensitive and resistant parents) [71].

resistance was found between CAAs and other modes of action such as phenylamides, QoI fungicides and zoxamide. Because CAAs express different intrinsic activities, resistance factors (difference in sensitivity between wild type and resistant isolates) can vary significantly.

To obtain more information on CAA resistance, the segregation pattern was investigated in sexual crosses made between sensitive and CAA resistant isolates of

Table 18.2 Mammalian toxicology of launched carboxylic acid amides.

Compound	Dimethomorph (1)	Flumorph (2)	Iprovalicarb (3)	Benthiavalicarb (4)	Mandipropamid (6)
Acute oral (LD_{50} rat) (mg kg^{-1})	3900	>2710 (♂) >3160 (♀)	>5000	>5000	>5000
Acute dermal (LD_{50} rat) (mg kg^{-1})	>5000	>2150	>5000	>2000	>2000
Acute inhalation (LC_{50} rat) (mg L^{-1})	>4.2		>4.98	>4.6	>5.0
Other characteristics	a, c, d, f, g	d, e, g	a–i	a, c, d–g, j	a, e, f–j

[a] Non-carcinogenic.
[b] Non-genotoxic.
[c] Non-irritating to eyes.
[d] Non-irritating to skin.
[e] Non-mutagenic.
[f] Non-skin-sensitizing.
[g] Non-teratogenic.
[h] No reproductive and developmental toxicity.
[i] No evidence of neurotoxicity.
[j] No adverse effect identified on reproduction.

Table 18.3 Environmental profile and fate in soil (half-life) of launched carboxylic acid amides.

Compound	Dimethomorph (1)	Flumorph (2)	Iprovalicarb (3)	Benthiavalicarb (4)	Mandipropamid (6)
Bobwhite quail (LD_{50}) (mg kg^{-1})	>2000		>2000	>2000	>2250
Rainbow trout ($LC_{50,\,96\ h}$) (mg L^{-1})	6.2		>22.7	>10	>2.9
Earthworm ($LC_{50,\,14\ d}$) (mg per kg of soil)	>1000		>1000	>1000	>1000
Bee ($LD_{50,\,contact}$) (μg per bee)	>100	>170	200		>200
Bee ($LD_{50,\,oral}$) (μg per bee)			>199	>100	>200
Fate in soil (half-life in days)	14–50		1–17	11–19	2–29

P. viticola [71]. All F1 progeny isolates were sensitive to CAA fungicides reflecting a segregation pattern of s:r = 1:0. When F1 progeny isolates (siblings) were crossed, the segregation in the F2 progeny was about 9:1 (s:r) (Fig. 18.12) [71]. This segregation pattern suggests that resistance to CAAs may be controlled by more than one (probably two) recessive nuclear genes. In the F2 progeny, resistance co-segregated for all tested CAAs (mandipropamid, dimethomorph, iprovalicarb). In contrast, the same crosses produced offspring isolates with a segregation in F2 of about 1:3:2 (s:i:r) for metalaxyl-M, reflecting the well-known monogenic, semi-dominant nature of resistance to phenylamide fungicides. Therefore, the risk and extent of resistance in *P. viticola* is classified as high for phenylamides and as moderate for CAA fungicides. As a consequence, resistance to CAAs in *P. viticola* can be managed through appropriate use strategies such as the use of mixtures and restriction of the number of applications per season (see CAA FRAC recommendations on www.frac.info).

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19
Fluopicolide, a new Anti-oomycetes Fungicide with a New Mode of Action inducing Perturbation of a Spectrin-like Protein

Valérie Toquin, François Barja, Catherine Sirven, and Roland Beffa

19.1
Introduction

Even if oomycetes seem to share some morphological, physiological and biochemical features with fungi, they are phylogenetically distant [1]. Oomycetes such as *Phythophthora*, *Pythium*, or *Plasmopara* cause dramatic diseases in a wide variety of plant species, including potato, vegetables or grape. Because of the differences with other fungi, many effective fungicides, e.g., azoles, are not effective on oomycetes.

Fluopicolide belongs to a new chemical class of fungicides (Fig. 19.1) and exhibits high activity against a broad spectrum of oomycetes such as *Phytophthora infestans*, *Plasmopora viticola* and various *Phytium* species. It shows no cross-resistance to other commercially anti-oomycete fungicides and can inhibit the development of strains resistant to phenylamides, strobilurins, or dimethomorph, and iprovalicarb. This strongly suggests that fluopicolide acts with a new mode of action. In addition, fluopicolide affects several stages of the life cycle of the different oomycetes studied, such as the release and the motility of zoospores, the germination of cysts, the growth of the mycelium as well as the sporulation.

Detailed biochemical analysis has shown that fluopicolide does not inhibit respiration, has no direct effect on membrane composition, and does not significantly alter tubulin polymerization or tubulin and actin content in the cell.

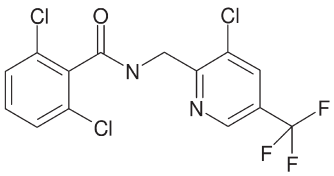

Fig. 19.1. Fluopicolide.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Table 19.1 Physicochemical properties of fluopicolide.

ISO name	Fluopicolide
Chemical class	Acylpicolides [2]
Chemical name (IUPAC)	2,6-Dichloro-*N*-{[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl}benzamide
Code number	AE C638206
Melting point	151.5 °C
Vapor pressure	3.03×10^{-7} Pa (20 °C)
Partition coefficient	2200 (log $P = 2.9$) (octanol/water)
Water solubility	2.8 mg L^{-1} (20 °C, pH 7)
DMSO solubility	183 g L^{-1} (20 °C)
Photolytic stability	Stable
Hydrolytic stability	Stable over pH range 4–9

19.2 Chemical and Physical Properties

The physicochemical properties of fluopicolide (Table 19.1) allow it to be easily redistributed via the xylem (acropetal systemic activity) and translocated within the leaf tissues, providing a translaminar activity.

19.3 Toxicology

19.3.1 Mammalian Toxicity

Table 19.2 gives the mammalian toxicity data for fluopicolide.

19.3.2 Ecotoxicological and Environmental Properties

These properties are listed in Table 19.3.

Table 19.2 Mammalian toxicity data for fluopicolide.

Acute oral toxicity (rat)	$LD_{50} > 5000$ mg kg^{-1}
Acute dermal toxicity (rat)	$LD_{50} > 5000$ mg kg^{-1}
Eye irritation, rabbit	Not irritating
Skin irritation, rabbit	Not irritating
Acute inhalation in air, 4 h, LC_{50} (rat)	>5160 mg-a.i. m^{-3}
Sensitization (guinea pig)	Not sensitizing
Carcinogenicity (mice, rat)	No carcinogenic potential
Mutagenicity	No genotoxic effects
Chronic toxicity	No embryotoxic potential
Teratogenicity, rat, rabbit	No teratogenic potential

Table 19.3 Ecotoxicological and environmental properties of fluopicolide.

Bird, acute oral, LD_{50}, quail	>2250 mg kg^{-1}
Fish, acute (96 h), LC_{50}, rainbow trout	0.36 mg L^{-1}
Fish, acute (96 h), LC_{50}, bluegill sunfish	0.75 mg L^{-1}
Water fleas, acute (48 h), EC_{50}, *Daphnia magna*	>1.8 mg L^{-1}
Algae, growth inhibition (72 h), ErC_{50}, *Selenastrum capricornutum*	>4.3 mg L^{-1}
Plant, growth inhibition (7 d), EC_{50}, *Lemna gibba*	>3.2 mg L^{-1}
Earthworm, acute (14 d), LC_{50}	>1000 mg-a.i. (kg-soil)$^{-1}$
Honeybee, contact, LD_{50}	>100 μg per bee
Typhlodromus sp., acute, LR_{50}	0.313 kg ha^{-1}
Aphidius sp., acute, LR_{50}	0.419 kg ha^{-1}

19.4 Spectrum of Activity

Fluopicolide has been tested successfully on the crops and pathogens shown in Table 19.4.

Fluopicolide is being developed worldwide, in combination with other fungicides, for use in a wide variety of crops. The first commercial launches of fluopicolide are in co-formulation with fosetyl-Al for use in vines, to be marketed as Profiler®, and with propamocarb-HCl for use in potatoes and vegetables, under the trade name Infinito®.

19.4.1 Effect on Zoospores and Mycelium Growth of *P. infestans*

Fluopicolide induced dramatic symptoms on *P. infestans* zoospores. They stopped swimming within a minute after being treated at concentration as low as

Table 19.4 Crops and pathogens on which fluopicolide has been tested successfully.

Potato	*Phytophthora infestans*
Tomato	*Phytophthora infestans*
Peppers	*Phytophthora capsici*
Leek	*Phytophthora porri*
Vines	*Plasmopara viticola*
Brassicas	*Peronospora parasitica*
Tobacco	*Peronospora tabacina*
Cucurbits	*Pseudoperonospora cubensis*
Lettuce	*Bremia lactucae*
Roses	*Peronospora sparsa*

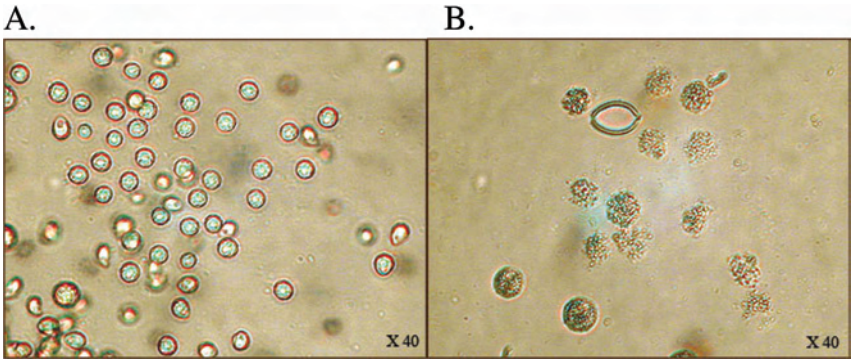

Fig. 19.2. Fluopicolide effect on *P. infestans* zoospores. (A) Control represents solvent (DMSO) treated zoospores. (B) Fluopicolide treated zoospores at 3 ppm, 10 min after treatment.

1 µg mL^{-1}. Then the zoospores swelled and burst in a few minutes (Fig. 19.2). Fluopicolide strongly inhibited *in vitro* the mycelium growth of *P. infestans*. A 80% growth inhibition was observed at a concentration as low as 0.1 µg mL^{-1} over 4 to 7 days. Distinctive symptoms were also observed on treated mycelium (Fig. 19.3). There was evidence of leakage of cellular content after the staining of treated hyphae with Blue Trypan. This showed that fluopicolide also induced mycelium lysis, preferentially observed at the apex of the hyphae.

19.5
Fluopicolide Effect on Spectrin-like Protein Distribution

These rapidly induced symptoms caused by fluopicolide led us to study in more detail the protein known to be associated to the cytoskeleton since no significant

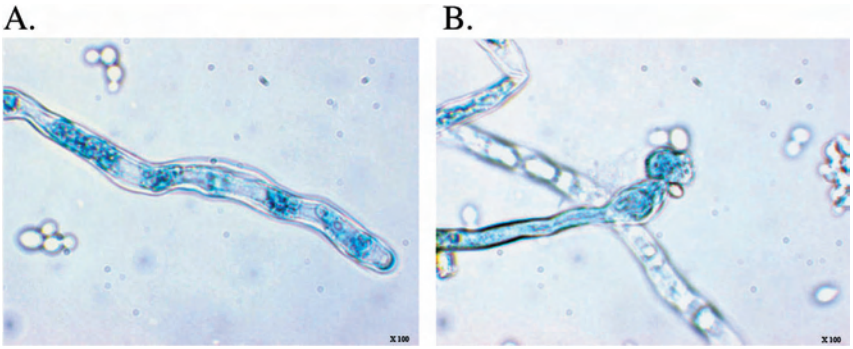

Fig. 19.3. Fluopicolide effect on *P. infestans* hyphae. *P. infestans* is colored with Blue Trypan. (A) Control treated with solvent (DMSO). (B) Fluopicolide treated mycelium at 10 ppm, 48 h post-treatment.

effect was observed on tubulin or actin. One candidate was spectrin, known to play a crucial role in membrane stability by anchorage to other cytoskeletal proteins in animal cells [3]. For this purpose, immunofluorescence studies using antibodies raised against chicken erythrocytes α/β spectrin (Sigma S1390) were first conducted on mycelium of *P. infestans*. They showed that antigen(s) cross-reacting with those antibodies were prominently localized in the peripheral regions (close to the plasma membrane) along the non-treated hyphae (Fig. 19.4). Upon fluopicolide treatment, a complete loss of plasma membrane localization of these spectrin-like protein(s) was observed and they were distributed as spherical spots in the cytoplasm of the hyphae cells. The kinetics of this effect revealed that spectrin-like protein delocalization occurred very rapidly, as early as 3 min after fluopicolide treatment (Fig. 19.4). In addition delocalization was maintained with longer treatment times. In conclusion, fluopicolide treatment induced a clear effect on cellular delocalization of spectrin-like protein(s) from the plasma membrane to the cytoplasm. A similar effect was observed on zoospores when they were just ceasing to move (1 min treatment with fluopicolide), during swelling (5 and 10 min treatment) and before cell lysis (from 15 to 20 min treatment)

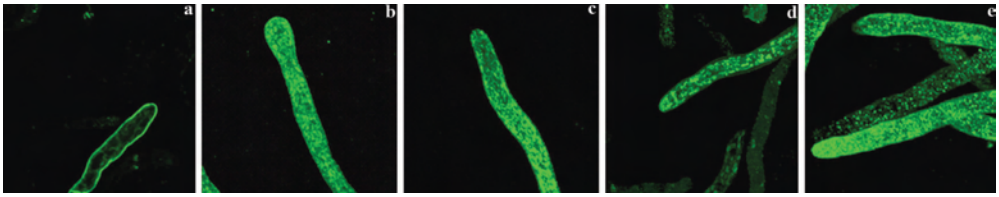

Fig. 19.4. Kinetics of the fluopicolide effect on the distribution of the spectrin-like proteins in hyphae of *P. infestans*. Control cell (a). Hyphae treated with 10 ppm fluopicolide for 3 min (b), 10 min (c), 2 h (d) and 24 h (e).

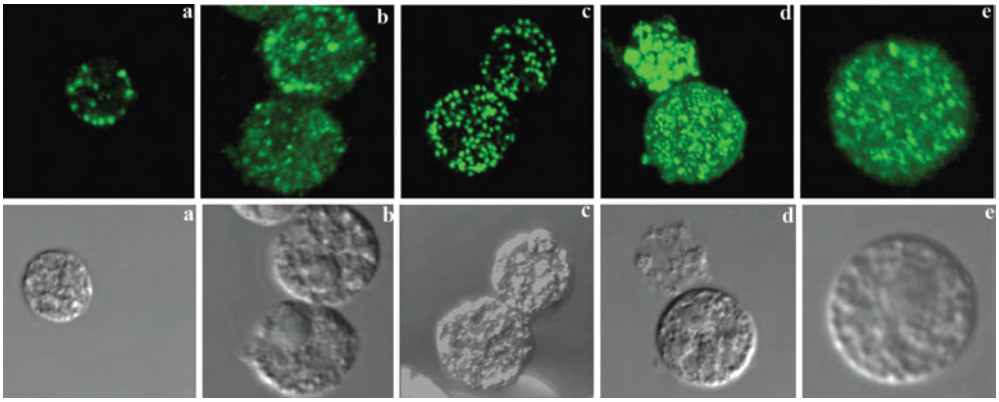

Fig. 19.5. Kinetics of the fluopicolide effect on the distribution of the spectrin-like proteins in zoospores of *P. infestans*. Control cell (a). Zoospores treated with 3 ppm fluopicolide for 1 (b), 5 (c), 10 (d) and 20 min (e).

(Fig. 19.5). Interestingly, this modification of the spectrin-like protein(s) cellular localization correlated very well with the phenotypic symptoms observed on zoospores.

The action of anti-oomycete fungicides on spectrin-like protein(s) localization was compared with that of fluopicolide. Different times of treatment were tested, from 3 min to 24 h. Figure 19.6 illustrates the results obtained after 2 h of treatment. None of the fungicides tested (iprovalicarb, fenamidone, dimethomorph, metalaxyl and zoxamide) induced spectrin-like protein redistribution. Unlike for

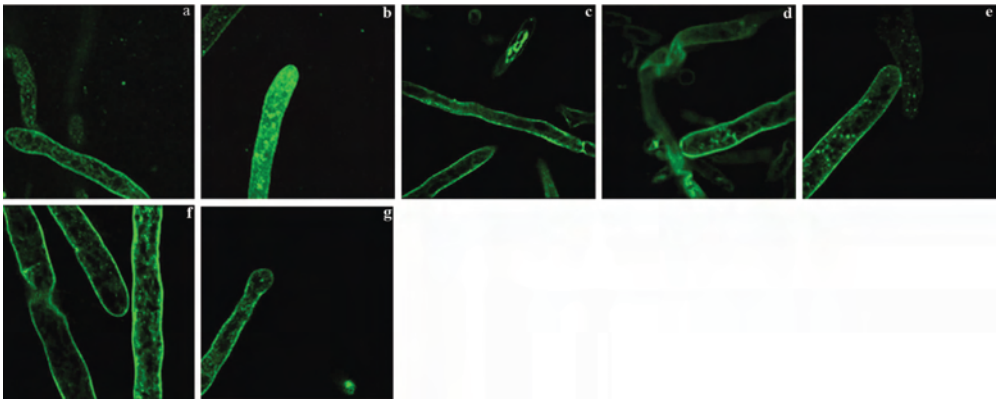

Fig. 19.6. Immunofluorescent localization of spectrin-like protein(s) in hyphae of *P. infestans* treated with fluopicolide and known anti-oomycetes. Control cell (a) or treated with 10 ppm of fluopicolide (b), 10 ppm of iprovalicarb (c), 10 ppm of fenamidone (d), 10 ppm of dimethomorph (e), 10 ppm of metalaxyl (f) and 10 ppm of zoxamide (g), 2 h post-treatment.

fluopicolide, for all fungicides tested a peripheral staining similar to the untreated control cells was observed, whatever the time of treatment.

19.5.1 Characterization of Spectrin-like Proteins in *P. infestans* by Bioanalysis

Spectrin was first discovered and described in animal cells in different tissues and cells types [3]. Interestingly, spectrin-like proteins have been also found in plant and fungi [4–7]. In both fungi and plants, this type of protein was characterized by its spatial localization close to the plasma membrane, and its size was determined on western blot using the anti-chicken α/β spectrin antibody (Sigma S1390). In none of these organisms has a protein corresponding to spectrin been purified and the amino acid sequence identified. A database search to find spectrin-like protein(s) in fungi (*M. grisea* and *N. crassa* genome sequences) or in oomycetes (*P. sojae* and *P. ramorum* genome sequences, partial EST sequences of *P. infestans*) by homology using BLAST was unsuccessful. A search by spectrin domain was then initiated. The structure of erythrocytes spectrins is composed of anti-parallele heterodimer of two sub-units α (240 kDa) and β (220 kDa) and are characterized by the presence of specialized domains: (a) a domain formed by triple-helical repeat of 106–120 amino acids, the so-called spectrin repeat (present from 4 to over 20 times); (b) an EF-hand domain, a calcium-binding domain; and (c) a highly conserved N-terminal domain responsible for binding of actin filaments. The spectrin repeat (SpR) domain was used to start a PFAM analysis [8], a system based on Hidden Markov Models. The SpR domain built only with mammalian representative domains (PF00435) gave no hit in fungal or oomycete species. To improve this search, a PFAM motif was constructed with a SMART (a simple modular architecture research tool) alignment [9]. In this system the motifs are automatically enriched by new sequences coming from worldwide databases, allowing more diversity. The ''seed alignment'' of the SMART database (SM00150) was then used to build a new PFAM motif for SpR domain. This approach provided one hit in *P. sojae*, to a protein of around 100 kDa (accession number 137006) that corresponds to a putative protein belonging to the spectrin family (α-actinin). This protein contained two SpR domains of 107 and 113 residues, showing a homology to the consensus spectrin repeat. A search with the others domains gave no better results. In fungi similar results were obtained, i.e., a 113 kDa protein and a 88 kDa protein can be found in *N. crassa* (NCU06429.1) and *M. grisea* (MG06475.4), respectively; both are closely related to a putative α-actinin.

19.6 Conclusion

Fluopicolide-induced delocalization of spectrin-like proteins represents a new mode of action that is different to that of known anti-oomycete fungicides on the market. Spectrin-like proteins are poorly characterized in fungi and oomy-

cetes. According to our knowledge this is the first indication of the presence of spectrin-like protein(s) in *Phytophthora*. The possibility of spectrin-like protein(s) as the biochemical target for fluopicolide and its role in oomycete development is under further investigation.

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20
Melanin Synthesis in Cell Wall

Michael Schindler, Haruko Sawada, and Klaus Tietjen

20.1
Biological Occurrence and Function of Melanin in Fungi

Inhibitors of melanin biosynthesis (MBIs) in cell walls constitute a minor segment of the world fungicide market but with a global market share of about 30% in rice fungicides in 2004 they play an important role in rice production in Asia, although the rice acreage treated with MBIs was only 7% (source: Bayer Business Intelligence). In Japan, among various specific fungicides for rice blast (*Pyricularia oryzae*), MBIs come a close second after host defense inducers.

Melanin is a general term used in different biological contexts for different insoluble dark or black complex polymers, the chemical structure of which is often only incompletely characterized [Butler & Day, 1998; Nosanchuk & Casadevall, 2003]. Keeping in mind the imprecise definition, melanin is found ubiquitously in the living world. Chemically, melanin commonly is a polyphenol resin. Best known in biological and medical literature is mammalian melanin, which is biosynthesized from the amino acids tyrosine and 3,4-dihydroxyphenylalanine (L-DOPA) as monomeric building blocks. In mammalians, melanins have different biological functions, sometimes, as in the neuronal system, quite enigmatic. But mammalian melanin is rather distinct from the fungal melanin that is the subject here.

Fungi like *Pyricularia* contain a melanin built from 1,8-dihydroxynaphthalene (DHN), called DHN melanin [Butler & Day, 1998]. The biosynthetic pathway is short and simple (Fig. 20.1). The origin of the building blocks is not amino acids, but a polyketide originating from acetyl coenzyme A and malonyl coenzyme A. Besides DHN this fungal melanin may contain other poorly characterized constituents. All the enzymes of the DHN melanin biosynthesis pathway are known, have been cloned, except the last one, and are more or less well described [Butler & Day, 1998].

Knowledge of the enzyme's sequences allows us to check their presence in different organisms (Fig. 20.2). BLAST sequence searches find the first specific enzyme of the pathway, polyketide synthase (PKS1), in Sordariomycetes, Eurotiomy-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

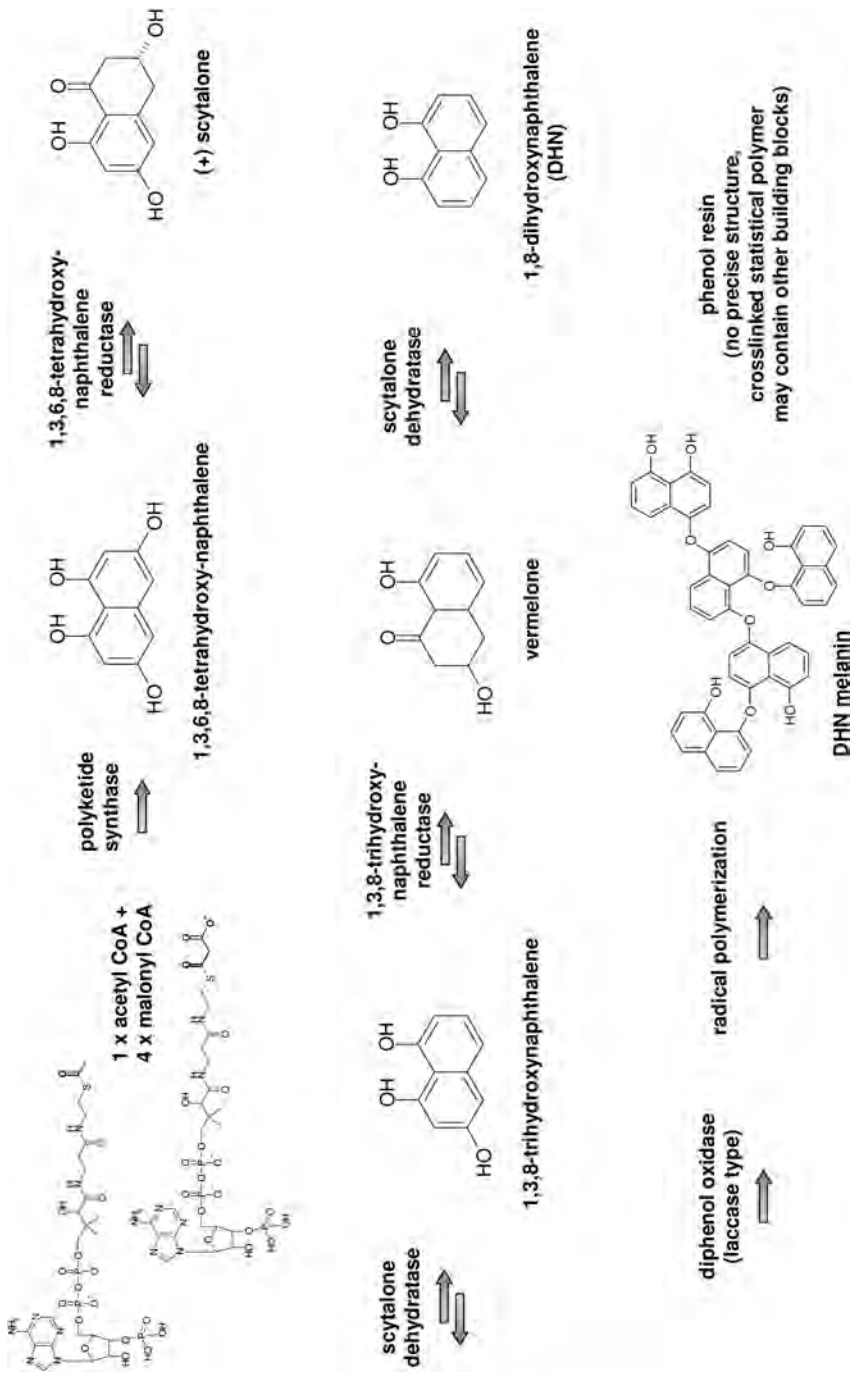
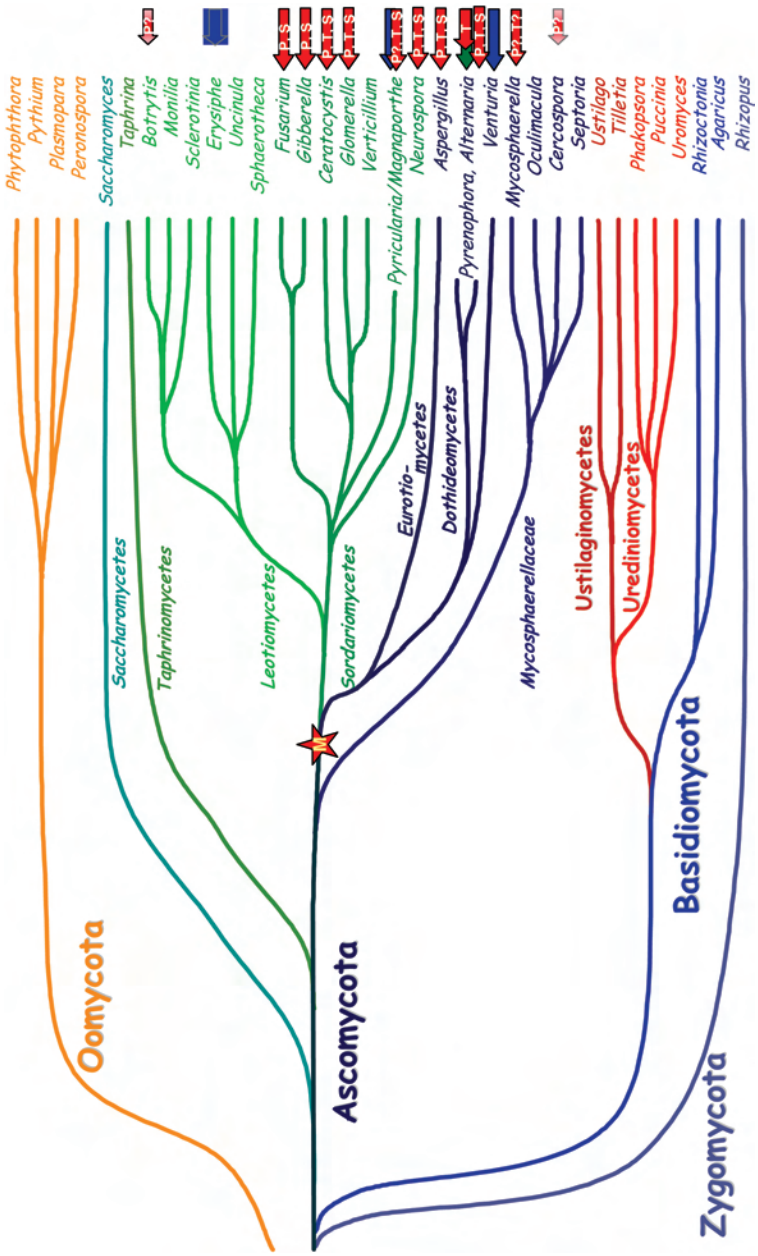


Fig. 20.1. Biosynthetic pathway of fungal DHN melanin.


Phytophthora
Pythium
Plasmopara
Peronospora
Saccharomyces
Taphrina
Botrytis
Monilia
Sclerotinia
Erysiphe
Uncinula
Sphaerotheca
Fusarium
Gibberella
Ceratocystis
Glomerella
Verticillium
Pyricularia/Magnaporthe
Neurospora
Aspergillus
Alternaria
Venturia
Mycosphaerella
Oculimacula
Cercospora
Septoria
Ustilago
Tilletia
Phakopsora
Puccinia
Uromyces
Rhizoctonia
Agaricus
Rhizopus
P.S.
P.T.S.
T
P.T.
Oomycota
Saccharomycetes
Taphrinomycetes
Leotiomycetes
Sordariomycetes
Eurotio-
mycetes
Dothideomycetes
Pyrenophora,
Mycosphaerellaceae
Ascomycota
Ustilaginomycetes
Urediniomycetes
Basidiomycota
Zygomycota

cetes and Dotideomycetes. Since various polyketide synthases can have many other functions besides their function in DHN melanin biosynthesis, more distant relatives are found in many different other organisms. The second pathway enzyme, 1,3,6,8-tetrahydroxynaphthalene reductase (THNR), is found again in Sordariomycetes, Eurotiomycetes and Dotideomycetes. Related in sequence is an enzyme in Mycosphaerella pini (Q8TFD5_MYCPJ) which belongs to aflatoxin biosynthesis and is classified as a member of further related bacterial short-chain dehydrogenases. The third enzyme, scytalone dehydratase (ScD), is found only in the same organisms as both preceding enzymes. The final enzyme, a diphenol oxidase, is known to exist, because a mutation, Pgr1$^-$ in *Cochliobolus heterostrophus*, lacks melanin and accumulates the precursor 1,8-DHN [Tanaka et al., 1992], but to date no sequence of such an oxidase has been identified. Diphenol oxidases are involved in several biological processes; fungal genomes are, therefore, rich in candidates for an oxidase participating in melanin biosynthesis. It has been shown that a laccase-type diphenol oxidase of *Colletotrichum* can synthesize DHN melanin, although a knock-out of this enzyme does not abolish melanin biosynthesis in the organism [Tsuji et al., 2001]. The clearly identified melanin biosynthesis genes occur in Sordariomycetes, Eurotiomycetes and Dotideomycetes only, but not in any other organism. This evolutionary appearance explains well the limited biological spectrum of fungicides inhibiting DHN melanin biosynthesis.

Nevertheless some phytopathogenic fungi absolutely require melanin for their pathogenicity [Sisler and Ragsdale 1995; Kurahashi & Pontzen, 1998, and references therein]. Among these fungi are *Pyricularia* causing rice blast as well as *Colletotrichum* causing anthracnoses. These fungi build appressoria for penetration into the plant epidermis. The appressoria cell walls are heavily fortified by melanin, except the tip, from where the penetration peg grows into the plant [Butler & Day, 1998]. Only the melanin bracing allows the build up of an ex-

Fig. 20.2. Occurrence of DHN melanin biosynthesis enzymes in a phylogenetic tree of fungi [unpublished assembly] and biological coverage of scytalone dehydratase inhibiting fungicides. Explanations: Red Star with yellow M: Evolutionary point of DHN melanin biosynthesis emergence. Red Arrows: Fungi where genes of melanin polyketide synthase (P), 1,3,6,8-tetrahydroxynaphthalene reductase (T) or scytalone dehydratase (S) are found in BLAST searches. Green arrow: Fungi where at least the occurrence of DHN melanin is proven. Blue arrows: Biological scope of carpropamid, a scytalone dehydratase inhibitor. Fungal DHN melanin is located in granular or fibrillar layers in the cell walls [Butler & Day, 1998]. DHN melanin as a robust polymer mechanically protects the cell walls. The dark color shields cells from UV light. Melanin absorbs poisonous heavy metals and gives further chemical protection against oxygen radicals set free during plant defense. Furthermore, melanin is resistant to lytic enzymes (glucanases, chitinases) of predators as well as of prey plants. The defensive character of melanin explains why fungicides that inhibit melanin biosynthesis do not directly kill the fungi and do not have fungicidal activity against fungi grown in liquid culture or on agar.

tremely high turgor pressure, forcing the penetration peg into the plant. Other melanin-containing fungi without pressure appressoria are not hindered by melanin biosynthesis inhibiting fungicides in their infection process. Therefore, the biological spectrum of melanin biosynthesis inhibiting fungicides is relatively narrow.

20.2
Overview: Fungicides inhibiting DHN Melanin Biosynthesis

There are not many known inhibitors of the polyketide synthase of melanin biosynthesis (Table 20.1). One rare example is aflastatin A (**1**), a pretty complex natural compound, which inhibits melanin production in *Colletotrichum lagenarium* [Okamoto et al., 2001]. Abikoviromycin (**2**) and a dihydro derivative thereof (**3**) also inhibit the polyketide synthase of melanin biosynthesis in *Colletotrichum lagenarium* [H. Maruyama et al., 2003]. Fungicide tests (i.e., pathogenicity tests) with aflastatin A or abikoviromycin have not been reported. Furthermore, KC10017 (**4**) [Kim et al., 1998] is known as fungicide inhibiting the polyketide synthase and cerulenin (**5**), better known as a fatty acid synthase inhibitor, also blocks the polyketide synthase of melanin biosynthesis [Kubo et al., 1986].

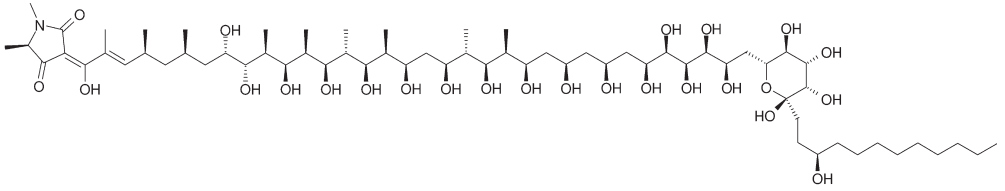
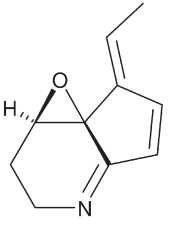
Several fungicides inhibit 1,3,6,8-tetrahydroxynaphthalene reductase (MBI-Rs) (Table 20.2). These inhibitors have been used since the 1970s, such as pentachlorobenzyl alcohol (PCBA) (**6**), tricyclazole (**7**) (Eli Lilly, 1975), pyroquilon (**8**) (Ciba, Pfizer, 1985) and fthalide (**9**) (Kureha, 1971), without any resistance problems and retain considerable economical importance, mainly in Northeast Asia. They are not discussed in more detail here.

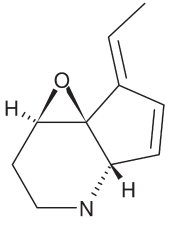
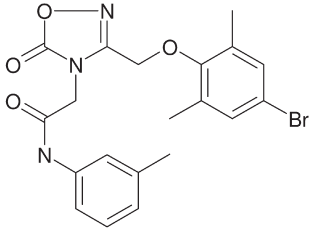
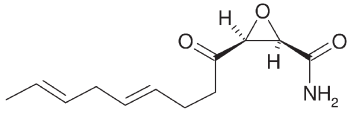
Since 1998 several rice fungicides have been introduced into the market that act by inhibiting scytalone dehydratase (MBI-Ds) (Table 20.3). These fungicides have been almost exclusively used in Japan as systemic fungicides for specialized protective application in rice nursery boxes. In the following we overview the syntheses, structural, biochemical and biological aspects of these melanin biosynthesis inhibitors. Prominent examples of these are carpropamid (Bayer, 1998) (**10**), diclocymet (Sumitomo, 2000) (**11**), and fenoxanil (American Cyanamide, Nihon Nohyaku, 2001) (**12**). They are shown in Table 20.3 together with other inhibitors, most of which have been co-crystallized with their target.

20.3
Biology of Scytalone Dehydratase Inhibitors

Common properties of MBI-Rs and MBI-Ds are protective control activity against rice blast and inhibition of appressorium pigmentation of *Pyricularia oryzae* on agar plates or cellophane membrane [Hattori et al., 1994; Soma et al., 1999; Sieverding et al., 1998]. Inhibition of spore liberation from leaf lesions is also commonly observed in all known MBI-Rs and MBI-Ds [Kitamura et al., 1976; Okuno

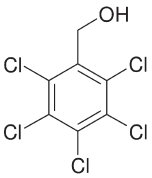
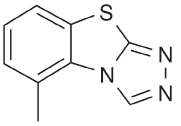
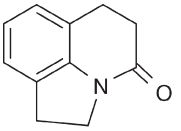
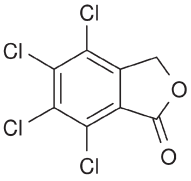
Table 20.1 Structures of inhibitors of polyketide synthase in DHN melanin biosynthesis.

Compound no.	Name and structure	Status	Biology
1	Aflastatin A 	Exptl.	C
2	Abikoviromycin 	Exptl.	C

	Dihydroabikoviromycin			
3			Exptl.	C
	KC10017			
4			Exptl.	RF
	Cerulenin			
5			Exptl.	P,C

Abbreviations used in Tables 20.1–20.3: P: inhibition activity *in vitro*, Pyricularia; C: inhibition activity *in vitro*, Colletotrichum; RF: Blast control efficacy in rice, foliar spray; RS: Blast control efficacy in rice, systemic application; MP: commercial product in 2006; Res: Field resistance authorized by FRAC.

Table 20.2 Structures of inhibitors of 1,3,6,8-tetrahydroxynaphthalene reductase in DHN melanin biosynthesis.

Compound no.	Name and structure	Status	Biology
6	Pentachlorobenzyl alcohol 	Withdrawn	RF
7	Tricyclazole 	Market	RF RS MP
8	Pyroquilon 	Market	RS MP
9	Fthalide 	Market	RF MP

et al., 1983; Shiba et al., 1983; Sakuma et al., 1999; Soma et al., 1999; Yamamoto et al., 2000] which could be considered as an indirect effect of melanin inhibition in the conidia.

Agronomically, the advantage of known MBI-Ds to MBI-Rs is their compatible systemic action combined with long-lasting control efficacy. Tricyclazole and pyroquilon are highly water soluble and show quick mobility but their action is hardly long-lasting. Fthalide has an excellent long-lasting efficacy but no systemic effect. In clear contrast to those MBI-Rs, carpropamid, diclocymet and fenoxanil show systemic effects with moderate levels of water solubility, which enables season-

Table 20.3 Structures of inhibitors of scytalone dehydratase (SD) in DHN melanin biosynthesis.

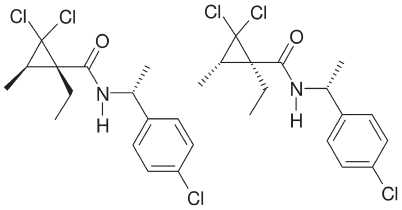
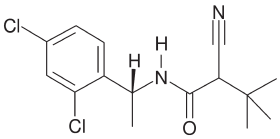
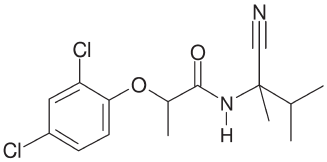
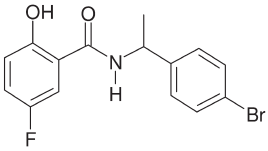
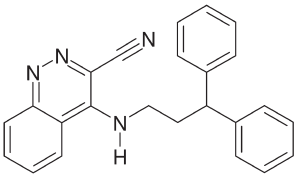
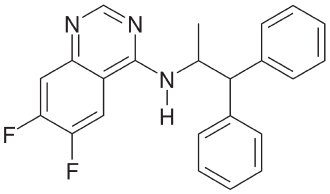
Compound no.	Name and structure	Status	Biology	Comment
10	Carpropamid 	Market	RS RF MP $K_i = 19$ pM[a]	Res
11	Dicyclomet 	Market	RS RF MP $K_i = 36$ pM[b]	Res
12	Fenoxanil 	Market	RS RF MP $K_i = 130$ pM[b]	Res
13		Exptl.	$K_i = 47$ pM[a]	
14		Exptl.	$K_i = 8$ pM[d]	
15		Exptl.	$K_i = 32$ pM[a]	

Table 20.3 *(continued)*

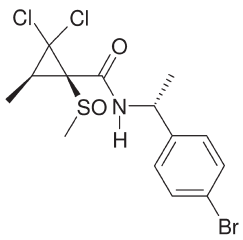
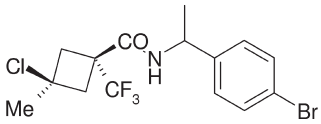
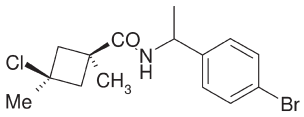
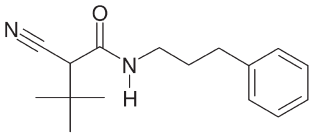
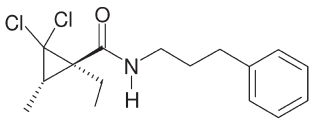
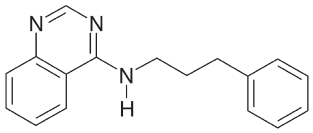
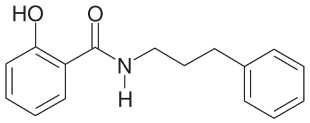
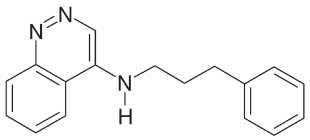
Compound no.	Name and structure	Status	Biology	Comment
16		Exptl.	$K_i = 2300$ pM[a]	
17		Exptl.	$K_i = 26$ pM[b]	Not systemic
18		Exptl.	$K_i = 100$ pM[b]	Systemic
19		Exptl.	$K_i = 12$ pM[c]	Hydrophilic variation
20		Exptl.	$K_i = 2.2$ pM[c]	Hydrophilic variation
21		Exptl.	$K_i = 13$ pM[c]	Hydrophilic variation
22		Exptl.	$K_i = 3.8$ pM[c]	Hydrophilic variation
23		Exptl.	$K_i = 3.6$ pM[c]	Hydrophilic variation

Table 20.3 *(continued)*

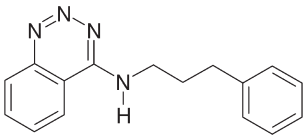
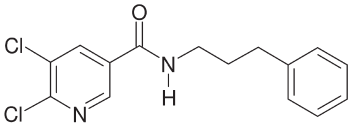
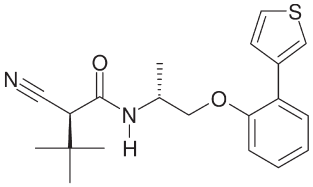
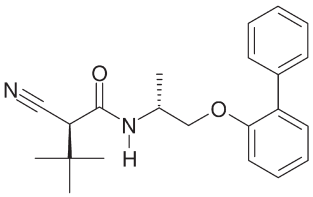
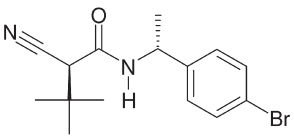
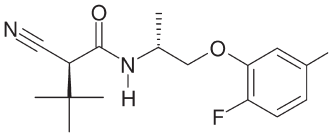
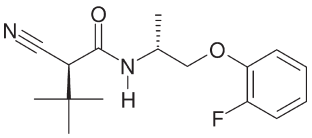
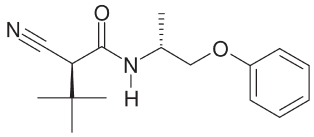
Compound no.	Name and structure	Status	Biology	Comment
24		Exptl.	$K_i = 15$ pM[c]	Hydrophilic variation
25		Exptl.	$K_i = 4.6$ pM[c]	Hydrophilic variation
26		Exptl.	$K_i = 11$ pM[c]	Lipophilic variation
27		Exptl.	$K_i = 25$ pM[c]	Lipophilic variation
28		Exptl.	$K_i = 20$ pM[c]	Lipophilic variation
29		Exptl.	$K_i = 18$ pM[c]	Lipophilic variation
30		Exptl.	$K_i = 80$ pM[c]	Lipophilic variation

Table 20.3 *(continued)*

Compound no.	Name and structure	Status	Biology	Comment
31		Exptl.	$K_i = 580$ pM[c]	Lipophilic variation

[a] [Wawrzak et al., 1999].
[b] [Jennings et al., 2000].
[c] [Jordan et al., 2000a].
[d] [Basarab et al., 1999a].

long control of leaf blast by one-shot application at transplanting and also provides comparable lasting efficacy to fthalide when sprayed. The lower water solubility is favorable by itself from the environmental viewpoint of rice cultivation. In Korea, carpropamid is used also for seed treatment with insecticides for long-lasting control of blast and pests in rice. In Japan, MBI-Ds, together with CNI insecticides, have significantly contributed to reduce farmers' labor costs and the total amount of rice pesticides by the popularization of protective one-shot application in the rice nursery.

However, the overwhelming prevalence of the one-shot application with long-lasting MBI-Ds must have enhanced the relatively early outbreak of field resistance, which had not been expected at all for these secondary metabolism inhibitors, based on the long-term experience of using MBI-Rs without resistance problems.

Another common property of the three commercial MBI-Ds that is not shared by MBI-Rs is specific systemic damage to Solanaceae plants. The relevance of this specific sensitivity and MBI-D activity has not been investigated. However, since MBI-s are exclusively used in an isolated environment for rice, this is not regarded as a serious problem in practice.

20.4
Biochemical Reaction Mechanism of Scytalone Dehydratase and Structure-based Inhibitor Design

Scytalone dehydratase catalyzes two analogous steps in DHN melanin biosynthesis (Fig. 20.1). Various biochemical aspects of the catalysis have been investigated by Douglas Jordan and coworkers from DuPont [Basarab et al., 2002; Zheng et al., 2002; Jordan et al., 2000 a; Jordan et al., 2000 b; Basarab et al., 1999; Jordan et al., 1999]. Based on crystal structure analysis, which is discussed below in much more detail with respect to inhibitor design, a biochemical reaction mechanism can be proposed (Fig. 20.3).

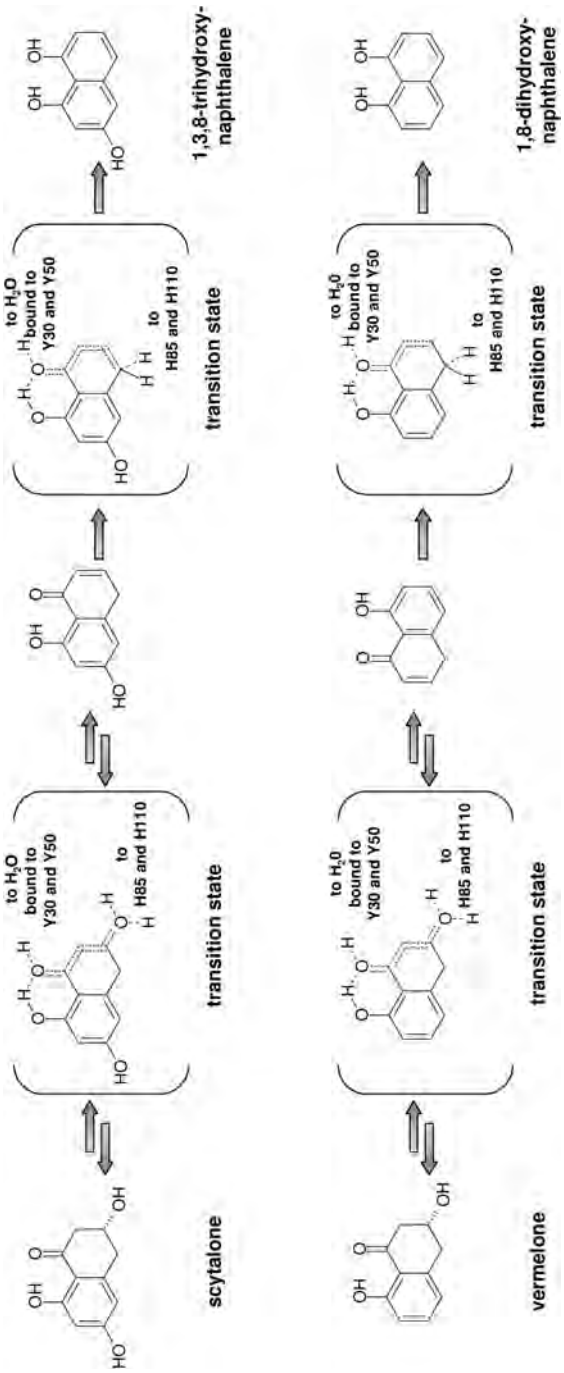


Fig. 20.3. Biochemical reaction mechanism of scytalone dehydratase in DHN melanin biosynthesis. Note that the molecules flip by 180° between the two steps.

20.4.1
X-ray Structures and the Active Site of Scytalone Dehydratase

Although one has to keep in mind that binding to a target is only a necessary requirement for biological activity but not a sufficient one, as ADME (absorption, distribution, metabolism, excretion) factors are at least as important, understanding a target's mechanism of action and the rational design of new compounds is greatly enhanced by knowledge of the three-dimensional structure of the enzyme–ligand complexes.

From published protein X-ray structures of scytalone dehydratase (SD) in its apo form and co-crystallized with several inhibitors (Table 20.4), its binding niche in the presence of inhibitors is rather well known. Protein X-ray structures with the natural substrates scytalone or vermelone, which are considerably smaller in size than the inhibitors, are not available.

SDs are symmetric trimers built from identical single domain monomers belonging to a group of folds called $\alpha + \beta$ rolls. The active site of each monomer forms a hydrophobic pocket in the interior of the central ß-barrel formed by a curved six-stranded ß-sheet. Figure shows 20.4 two representations of an SD monomer co-crystallized with carpropamid (**10**), the first member of the novel class of MBI-Ds.

Only one polar amino acid, Asn131, is available for direct interaction with the inhibitors. Two conserved water molecules can interact with the inhibitors. They are fixed by hydrogen bonds to the hydroxyl groups of Tyr30 and Tyr50 and by the imidazole nitrogens of His85 and His110. Access to the active site might be possible by a hinge bending movement of the amphiphilic carboxy-terminal helices H4 and H5 which contribute significantly to the hydrophobic part of the binding niche. In the apo-structure, the flexibility of the C-terminal renders the amino acids 156–172 invisible.

Table 20.4 Publicly available protein X-ray structures of scytalone dehydratase (SD) inhibitor complexes.

PDB code	Inhibitor class	Year	Resolution (Å)	Ref.	Inhibitor
1STD	Salicylamide	1994	2.9	[Lundquist et al., 1994]	**13**
2STD	Cyclopropanecarboxamide	1998	2.1	[Nakasako et al., 1998]	**10**
3STD	Cyanocinnoline	1998	1.65	[Chen et al., 1998]	**14**
4STD	Salicylamide	1999	2.15	[Wawrzak et al., 1999]	**13**
5STD	Norephedrine	1999	1.95	[Wawrzak et al., 1999]	**15**
6STD	Cyclopropanecarboxamide	1999	1.80	[Wawrzak et al., 1999]	**16**
7STD	Cyclopropanecarboxamide	1999	1.80	[Wawrzak et al., 1999]	**10**
1IDP	Apo Enzyme	2002	1.45	[Nakasako et al., 2002]	–

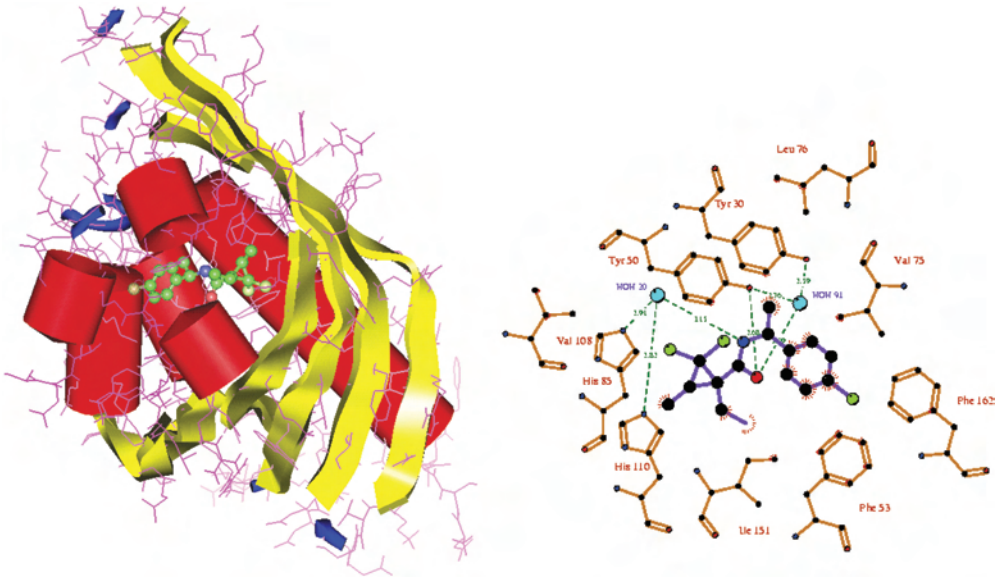


Fig. 20.4. Cartoons [SYBYL; LIGPLOT] of the 3D-structure of an SD-monomer with carpropamid in the active site.

Comparing the amino acid sequences of the SDs in different organisms – augmented by the knowledge of 3D protein structures – enables us to rationalize the reasons for specificity or failure of fungicides to be active. Especially, resistance caused by mutations in the fungal target can be explained and, perhaps, circumvented. The sequences of all SDs known by 2006 are shown in Fig. 20.5.

20.4.2
Computational Investigations of the Enzyme Mechanism

Molecular dynamics calculations exploring the mobility of the two water molecules in the SD binding pocket in the presence of the weak inhibitor *N*-isopropyl salicylamide [Jordan et al., 2000c] revealed that the water molecule associated with the inhibitor carbonyl is more labile than that associated with the inhibitor NH. Although the protein was kept fixed during the simulation and the protonation states of the two histidines are worth being discussed, these findings can help to prioritize drug design efforts.

The same holds true if the detailed enzymatic steps were known. However, as no complex of its natural substrates, scytalone or vermelone, is available the proposed mechanism of SD, the catalysis of a *syn* ß-elimination of water from scytalone and subsequent aromatization [Lundquist et al., 1994], remains to be proven experimentally.

Though much smaller and much less lipophilic, both scytalone and vermelone share some structural features with the co-crystallized competitive inhibitors. To

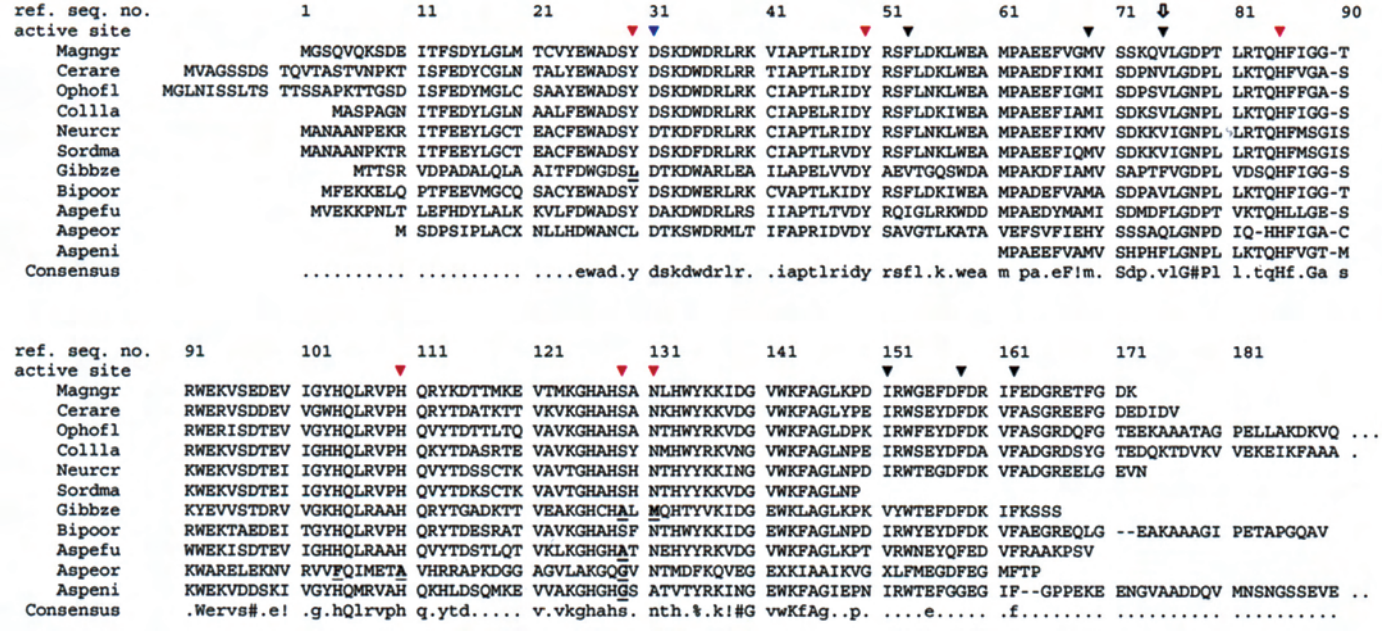


Fig. 20.5. Sequence alignment of known scytalone dehydratases. Legend: ▼ (red) = active site residues, ▼ (blue) = further hydrogen bonding residues, ▼ (black) = further hydrophobic residues; ⇩ = V75M mutation; other relevant mutant residues are marked bold and underlined in the sequences. Organisms: *Magnaporthe grisea 70-15*, *Ceratocystis resinifera SD1*, *Ophiostoma floccosum 387N*, *Colletotrichum lagenarium*, *Neurospora crassa OR74A*, *Sordaria macrospora S48977*, *Gibberella zeae PH-1*, *Bipolaris oryzae*, *Aspergillus fumigatus Af293*, *Aspergillus oryzae*, *Aspergillus nidulans FGSC A4*.

investigate the proposed enzyme mechanism, a quantum chemical model system was built based on the protein structure 4STD [Wawrzak et al., 1999], consisting of the four amino acids Tyr30, Tyr50, His85 and His110, the catalytic water molecule and vermelone, whose initial position was obtained by superposition on the salicylic ring of the inhibitor in 4STD. The second conserved water molecule found in the X-ray structures was omitted as it is supposed to be the product of the enzymatic reaction.

Keeping only the backbone atoms of the model system fixed, a geometry optimization using density functional theory (RI-DFT, BP86 functional/SVP basis set, unpublished results) as provided by the TURBOMOLE suite of programs [Turbomole] revealed that only one conformation of vermelone, with the ß-hydrogen to be abstracted in an axial position, avoids being trapped in a local minimum, preventing a subsequent reaction. During the optimization the catalytic water and the side chains change their positions only marginally, an exception being Tyr50, whose phenoxy ring rotates by more than 100° to improve its H-bond with the catalytic water. In contrast, to prepare for the reaction, vermelone moves by almost two bond lengths from its starting position, shortening the $H_{ß}$–$N_{\varepsilon 85}$ and OH–$N_{\varepsilon 110}$ distances from 3.58 and 5.59 to 2.63 and 1.84 Å, respectively. For the next step, the formation of the carbanion intermediate in the $E1_{cb}$ reaction path, we attached $H_{ß}$ to $N_{\varepsilon 85}$, keeping His85 either protonated at N-δ or not. The calculations indicate that deprotonation of N-δ is essential for the water-assisted formation of the enolate and the subsequent abstraction of the hydroxyl-group.

Although the last step in the reaction sequence, abstraction of a proton from C4, followed by aromatization of the α,β-unsaturated ketone, will occur spontaneously without assistance by a protein, it seems reasonable to assume that product formation is accelerated considerably by an enzymatic mechanism. Again, DFT calculations suggest that deprotonated His110 could accept a proton from the previously generated water molecule, which in turn accepts the C4 proton. Protonation of the ketone could occur by the catalytic water bound by the two tyrosines.

20.4.3
Comparison of Inhibitor Structures in the SD Binding Niche

With the exception of Asn131 and Asp31 the binding niche of SD is mainly hydrophobic. Its polar part consists of His85 and His110, and of Tyr30 and Tyr50 coordinating two conserved water molecules that mediate hydrogen bonds to the inhibitors in the protein–ligand complexes.

All of the five different co-crystallized inhibitor complexes of Table 20.4 and those published during rational design programs [Chen et al., 1998; Jordan et al., 1999 b; Basarab et al., 1999 a; Jennings et al., 1999] show a common pattern of H-bonds: the carbonyl oxygens accept an H-bond from the tyrosine-coordinated water, and the amide NH donates an H-bond to the histidine-coordinated water. Replacing salicylamide by quinoxaline does not change this pattern. Superposition of the protein structures shows the varying flexibility of the binding niche: While the amino acids responsible for recognition have their side chains nearly

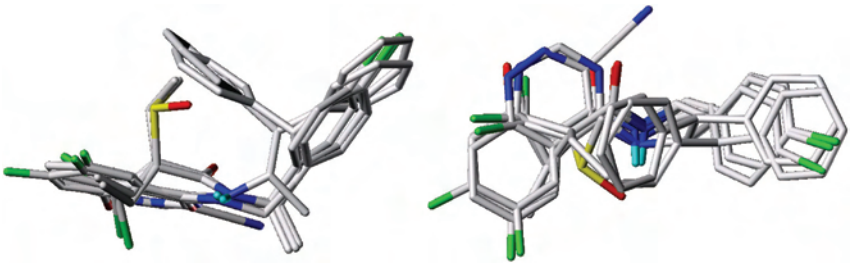


Fig. 20.6. Two views [SYBYL] of the SD-inhibitors, from backbone-superimposition of the X-ray structures 1STD-7STD.

unchanged, the hydrophobic part of the binding pocket exhibits considerable side chain flexibility. Remarkably, though, the backbone atoms show almost no structural variation. This is true even for the apo structure where only His110 adopts an outward directed side chain conformation. Obviously, the inhibitors stabilize the C-terminal helix of SD (Phe156:Lys172).

Some notable backbone differences can be found between the early X-ray structures 1STD and 2STD obtained at low (pH 4.5, pH 5.1) and those obtained later at neutral pH (7.5–8) (3STD-7STD), which can be attributed to the pH differences. Site-directed mutations (see below) support the assumption that these differences affect the lipophilic inhibitor recognition.

Superposition of the Cα-atoms of 1STD-7STD and looking at the resulting inhibitor positions gives an impression of the extensions of the active site (Fig. 20.6).

Once target protein structures are available, several computational techniques can be applied to support rational drug design. Rational design, based on the protein X-ray structures, led to several proposals that turned out to bind more effectively than the original compounds. The catalytic water molecule was successfully replaced by a part of a ligand designed for this purpose [Chen et al., 1998]. Modeling techniques can be used to place other putative or existing inhibitors into the binding site, as is outlined for the examples of diclocymet (**11**) and fenoxanil (**12**) below.

It is not too difficult to place the two isomers of diclocymet into the known binding niche as it is supplied as a mixture ((*RS*)-2-cyano-*N*-[(*R*)-1-(2,4-dichlorophenyl)ethyl]-3,3-dimethylbutyramide) where the specified (R)-configuration mimics that of carpropamid, but fenoxanil (*N*-(1-cyano-1,2-dimethylpropyl)-2-(2,4-dichlorophenoxy)proprionamide) requires some attention as no information on its stereochemistry is provided. One of the default approaches would be to start with poses proposed by a docking program, say, FLEXX [flexx], which is typically used in a high-throughput application, the so-called virtual screening approach, where libraries of millions of compounds are docked into the binding niche. In the fenoxanil case this approach is inferior to docking by hand, as fenoxanil is not only larger than the other inhibitors but also has a reversed functionality at the amide moiety. DFT optimization of the

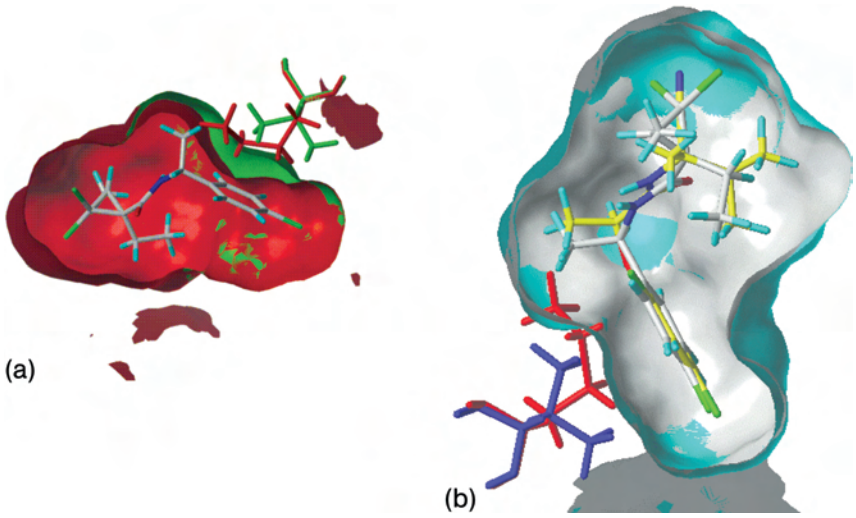


Fig. 20.7. (a) Connolly surfaces of the binding niches of native (green) and V75M (red) SD in 7STD. (b) Superposition of carpropamid and fenoxanil (yellow) in the 7STD binding niche.

SS (RR) enantiomer reveals that the conformation fitting best into the binding niche corresponds to a local minimum in energy, ~3 kcal mol^{-1} above the absolute minimum with its internal H-bond. In the binding conformation this internal H-bond is replaced by external H-bonds to water. Its proposed orientation within the binding niche of 7STD, superimposed with carpropamid, is shown below in Fig. 20.7, together with the Connolly surfaces of the wild-type and the V75M binding niches.

Highly potent (8 pM $< K_i <$ 48 pM) cyanoacetamide derivatives of norephedrine were designed [Basarab et al., 1999a] using multiple crystal structures, permitting the detection of variable regions of the active site and optimizing hydrophobic contacts with the inhibitors. In addition, by combining selected cyclic aliphatic carboxylic acids with appropriate amides combinatorial chemistry was employed to successfully identify new chemical classes [Jennings et al., 1999], the best representative of which, a cyclobutanecarboxamide with chlorine trans to the trifluoromethyl group **17**, shows an *in vitro* activity (K_i = 26 pM) comparable to carpropamid. From the X-ray structure [Jennings et al., 1999] it becomes clear why the cis isomer is less active by almost two orders of magnitude – the favorable complementary electrostatic interaction of **17** with the side-chain of Asn131 is disturbed by the "wrong" spatial arrangement of the substituents.

The importance of optimizing general physicochemical properties in addition to improving binding characteristics of the inhibitors was demonstrated by a design process where replacement of CF_3 in the exceptionally potent trifluoro-substituted compound [Basarab et al., 2002a] by a methyl group led to a slightly less potent but significantly more systemic one (**18**) [Jennings et al., 2000].

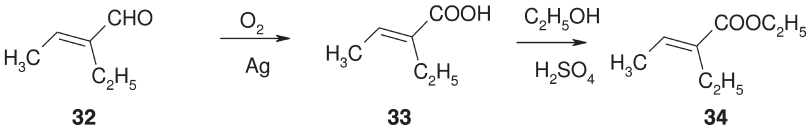
20.4.4
Complementary Information by Site-directed Mutations

Other inhibitor complexes have been solved [Basarab et al., 1999 a; Jennings et al., 1999] in the course of drug design programs changing systematically the hydrophobic and hydrophilic parts of the ligands, and numerous site-directed mutations [Jordan et al., 2000a] based on the structures served to explore the variability of the binding site and to probe the importance of selected amino acids for binding (Table 20.3, compounds **19**–**25** and **26**–**31**).

In the hydrophilic part of the active site 15 single point mutants resulted in binding affinities ranging from 10-fold enhancements to 1100-fold reductions for the ligands **19**–**25**, and five mutations in the hydrophobic part led to enhancements for the ligands **26**–**31**, ranging from 3- to 70-fold compared with the wild-type SD. From these studies one can conclude that the side chain of Phe158, whose orientation differs in the X-ray structures grown at acidic pH, where it is exposed to the solvent, and at neutral pH, where it points towards the inhibitors, is important for the hydrophobic interactions in the binding site. Val75 is critical for resistance effects as it recognizes the chiral methyl groups of some inhibitors. The hydroxyl groups of Tyr30 and Tyr50 and their H-bonds to the catalytic water molecule are much less important for inhibitor binding than the corresponding H-bond network of His85 and His110. Inhibitors with good acceptor properties interact favorably with the carboxamide of Asn131, whereas its H-bond to Ser129 does not contribute to the shape of the active site.

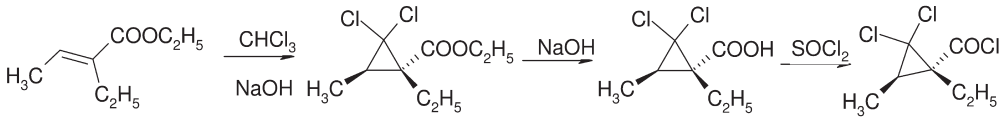
20.5
Chemistry and Stereochemistry of Carpropamid

A novel class of fungicides for rice blast control was presented in 1994 at the Brighton Crop Protection Conference [Hattori et al., 1994], and the synthesis of its most prominent representative, carpropamid (**10**), is outlined below [Kraatz et al., 1998]. The synthesis of the two other marketed MBI-Ds, diclocymet (**11**) and fenoxanil (**12**), is described elsewhere [Manabe et al., 2002; Buck and Raddatz, 1998]. Three chiral atoms of carpropamid give rise to eight stereoisomers, the most active mixture of which is (1*RS*, 3*SR*, 1′*RR*)-2,2-dichloro-*N*-[1-(4-chlorophenyl)ethyl]-1-ethyl-3-methyl-cyclopropanecarboxamide [Kagabu et al., 1998]. The free energies of the two pairs of enantiomers having alternating chiralities at the cyclopropyl ring differ by less than 0.2 kcal mol^{-1}, which is well within the computational error (DFT/TZVP/COSMO) [COSMO]. In a multi-step process the racemic *trans*-2,2-dichloro-1-ethyl-3-methyl-cyclopropane-acid chloride (**37**) is synthesized from commercially available (*E*)-2-ethyl-crotonaldehyde (**32**) via its acid **33** and ethyl ester **34** (Scheme 20.1) followed by a stereospecific addition of dichlorocarbene, saponification of the ester **35** to the acid **36** which is finally treated with thionyl chloride (Scheme 20.2).



32 **33** **34**

Scheme 20.1

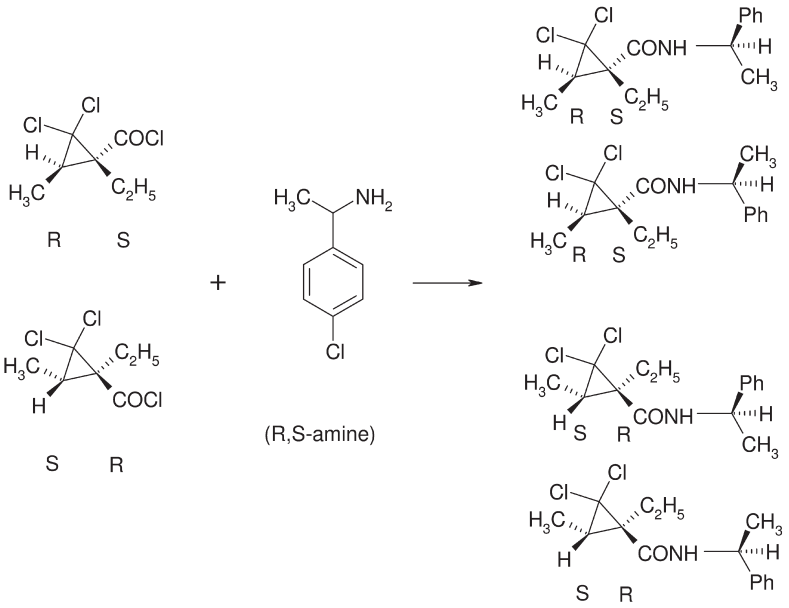


35 **36** **37**

Scheme 20.2

The necessary (*R*)-(+)-*p*-chlorophenethylamine is obtained from racemic *p*-chlorophenethylamine by racemic cleavage with optically active (*S*)-phenylcarbamate-lactic acid.

In the last reaction step the racemic *trans*-cyclopropyl-carbonic acid chloride is reacted with (*R*)-(+)-*p*-chlorophenethylamine in the presence of a base to the two main products, contaminated with small amounts originating from the (S)-amine (Scheme 20.3).



Scheme 20.3

Finally, the individual stereoisomers can be analyzed by HPLC using a chiral separation phase.

20.6 Resistance Problems and Successful Management in Japan

MBI-D fungicides (MBI-Ds) have been used in Japan since 1998. In 2001, reduced performance of MBI-Ds was first reported in a limited area [Yamaguchi et al., 2002]. *Magnaporthe grisea* isolates from this area showed decreased sensitivity to MBI-Ds *in vitro* and *in vivo* [So et al., 2002]. A single-point mutation at *Sdh1*, GenBank Accession Number AB004741, causing substitution of one amino acid in scytalone dehydratase (Valine 75 to Methionine: V75M), was found in the isolates showing decreased sensitivity to MBI-Ds while no change was observed in their metabolism activity for MBI-Ds [Takagaki et al., 2002], and a practical method for detecting V75M mutants was established by using PIRA (Primer-introduced Restriction Enzyme Analysis)-PCR [Kaku et al., 2003]. The correlation between the incidence of the V75M mutation and the efficacy of MBI-Ds was confirmed [Sawada et al., 2004]. The V75M variant enzyme retained a significant level of enzymatic activity [Yamada et al., 2004].

Based on the 3D-structures of the molecular target, the influence of the mutations on the properties of the binding niche or the direct influence on binding could be studied. It turned out that the volume of the binding niche is reduced by the more bulky side chain (Fig. 20.7a) and, though leaving the natural substrates unaffected, especially the chiral methyl groups of the inhibitors were prevented from optimally fitting into it (Fig. 20.7b).

Since 2002, monitoring studies of the resistant strains in a broader area were performed to establish the resistance management for MBI-Ds, using PIRA-PCR [Arai, 2004a]. Another SNP (single nucleotide polymorphism) diagnosis method, PCR-Luminex, [Ishii et al., in press] is also available for this mutation; however, it is not widely accepted for economic reasons. Nationwide monitoring from 2002 to 2005 revealed that the V75M mutants are distributed in wider areas in Japan [Arai, 2004b]. Genetic studies for population analysis suggest that the mutation has occurred independently in each area [Sawada et al., 2003; Sawada et al., 2004; Anonymous, 2005], with some exceptional cases where artificial transportation by infected seeds is suspected [Anonymous, 2004; Sasaki et al., 2005].

Based on the monitoring results since 2002, local governments stressed, thoroughly, seed sanitary as the first priority. This countermeasure was mostly successful, since rice blast is a seed-borne disease with rare exceptional cases under Japanese conditions. When some resistant mutants are found without problems of reduced control efficacy, each prefecture gives administrative guidance to farmers' cooperatives for complete renewal of seeds for next season with blast free seeds. In case some failure of control with MBI-D is suspected and resistant mutants are dominant at several fields in a village, replacement of MBI-D is additionally recommended to the relevant unit that is sharing a common supplier of

Table 20.5 Total treated area of MBI-D fungicides from 1998 to 2004 in Japan (ha).

Year	1998	1999	2000	2001	2002	2003	2004
Carpropamid	120 600	205 400	229 900	236 100	221 600	177 400	161 700
Diclocymet	0	0	6200	70 527	88 400	116 650	147 760
Fenoxanil	0	0	0	8987	21 670	30 523	33 233
Total	120 600	205 400	236 100	315 614	331 670	324 573	342 693

the seeds. Though MBI-Rs or probenazole show no cross resistance to MBI-Ds [So, 2003], once the blast infection rate of the seeds reached an extremely high level, they could not provide sufficient control [Nakajima et al., 2004]. In such cases, it takes several years to recover the mutants rate to a controllable level, [Yasunaga et al., 2004; Yamaguchi et al., 2005]. Therefore, a supply of healthy seeds is always prioritized in commercial rice production area [Nemoto, 2005]. Consequently, serious problems in the field have seldom been reported since 2004 in major areas. However, epidemiological investigation with V75M mutants in the western part of Japan proved that even some authorized seeds could be seriously contaminated by blast fungus [Arai, 2004b]. In contrast, regarding the major rice production area in the eastern part of Japan, MBI-Ds still successfully contribute to blast control and the total treated area of MBI-D has increased up to 2004 (Table 20.5).

This is the first successful case of fungicide resistance management in Japan based on integrated collaboration of molecular biological and epidemiological approaches.

A fitness penalty of V75M mutants is not confirmed. Temperature tolerance, ultraviolet sensitivity and virulence of V75M mutants are not significantly different from wild strains [Sawada et al., 2004; Kimura, 2005]. The mutants rate in Saga 2001 was maintained in 2002 despite no selection pressure from MBI-D [Sawada et al., 2004]. Kimura [Kimura, 2005] reported weaker competitiveness of three samples of V75M mutant isolate in comparison with three samples from wild-type strains.

20.7 Final Remarks

The development of the new MBI_D class of scytalone dehydratase inhibitors is a story of truly interdisciplinary research, ranging from the classical biology and chemistry to molecular biology, biochemistry, protein X-ray crystallography and computational chemistry. Based on the knowledge of the variability of the ligand binding site from several X-ray structures and mutation experiments, numerous variations of the inhibitors – mainly of their lipophilic parts – were explored and

resulted in several highly potent classes of novel fungicides that efficiently control rice blast.

Unfortunately, the fungi responded very quickly by mutations of the molecular target, rendering the binding niche smaller. The reasons causing this resistance could be explained convincingly, and effective resistance management strategies to overcome it were applied. This emphasizes the necessity for continuous research on new modes of action, offering new chemical classes of compounds access to new and different fungal targets.

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21
Newer Fungicides with Unknown Mode of Action

Stefan Hillebrand and Jean-Luc Zundel

21.1
Introduction

Synthetic compounds from a number of distinct chemical classes show fungicidal activity against a broad range of fungal pathogens. Selected examples would include the triazole group of fungicides or the respiration QoI inhibitors which are also described in this volume. However, sometimes single pathogens or groups of pathogens are not affected by broad-spectrum compounds such as triazoles or QoI fungicides. In other cases the application rates of the broad spectrum fungicides accepted for commercial use are too low for a sufficient control of specific pathogens. Moreover, some fungal pathogens tend to develop resistance very rapidly. To be able to offer complete solutions to farmers, plant protection companies are interested in filling these gaps within their fungicide product portfolio. Hence, there is a strong permanent demand for compounds with activity against groups of pathogens or even single pathogens that are not covered by other fungicides.

Despite intensive efforts to elucidate the mode of action of new fungicides, in some cases the biochemical target of a new compound remains unclear. This chapter describes five compounds with unknown mode of action, cymoxanil, fosetyl-aluminium, flusulfamide, diclomezine and triazoxide.

21.2
Cymoxanil

Due to important phylogenetic differences existing between Oomycetes and the so-called true fungi (i.e., Ascomycetes and Basidiomycetes) several broad spectrum fungicide classes do not show sufficient activity against Oomycetes, which are destructive pathogens in many crops of high commercial significance. Therefore, fungicides with efficacy against Oomycetes possess high market importance and several specific fungicides have been developed for this market segment. One of these substances is cymoxanil which was developed by DuPont and launched in 1977 [1].

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

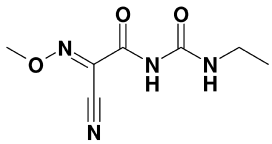


Fig. 21.1. Chemical structure of cymoxanil.

Cymoxanil belongs to the chemical class of cyanohydroxyiminoacetamides and was discovered in 1972 by DuPont [2]. The IUPAC chemical name is 2-cyano-*N*-[(ethylamino)carbonyl]-2-(methoxyimino)acetamide (CAS-RN.: 57966-95-7) (Fig. 21.1). No further compounds from this class have ever been put into development.

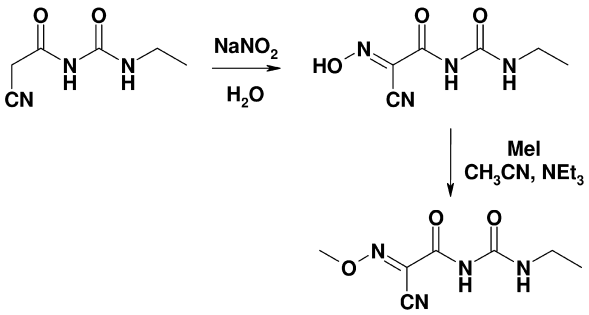
Due to its high polarity, the water solubility of cymoxanil is rather high. It is stable towards hydrolysis and unstable under UV light. Table 21.1 lists additional physicochemical properties [3].

The technical synthesis of cymoxanil [4] is rather straightforward. It starts, as described in Scheme 21.1, with the nitrosation of 1-(2-cyano-acetyl)-3-ethylurea with sodium nitrite in water, followed by methylation of the oxime with a methylating reagent such as iodomethane.

Cymoxanil is a protective and curative fungicide for foliar application; it is especially active against *Peronospora* spp., *Phytophthora* spp. and *Plasmopara* spp. in

Table 21.1 Physicochemical properties of cymoxanil.

Melting point (°C)	159–160
Hydrolysis	Stable between pH 2 and pH 7.3
UV stability	Sensitive to light
pK_a	9.7 ± 0.2
Vapor pressure (mPa) (pH 5, 20 °C)	0.15
Specific gravity (g cm^{-3}, 22.5 °C)	1.32
log $P_{O/W}$	0.59 (pH 5), 0.67 (pH 7)
Solubility (g L^{-1}, 20 °C)	Water (pH 5) = 0.9 Water (pH 7) = 0.8 Acetone = 62.4 Acetonitrile = 57.0 Dichloromethane = 133 Ethyl acetate = 28.0 Hexane = 0.037 Methanol = 22.9 Toluene = 5.29 *n*-Octanol = 1.43



Scheme 21.1. Synthesis scheme of cymoxanil.

vines, hops, tomatoes and some other vegetables as well as in potatoes where it is used for leaf and tuber treatment. Although fungicidal activity against other pathogens (e.g., *Botrytis cinerea*) has been observed in laboratory studies [20], this proved to be insufficient to allow development of this compound to control these pathogens.

Cymoxanil penetrates rapidly into plant leaves and also shows excellent contact activity via its strong sporulation inhibition properties. Due to its high water solubility it is mobile in the plant, showing favorable curative and local systemic effects. Cymoxanil degrades very quickly within the plant [5–6], leading to a lack of long-lasting activity. Therefore, it is used mainly in combination with other fungicides to improve the residual activity [7]. In these combinations the favorable curative properties of cymoxanil are emphasized. Plant diseases where cymoxanil has a high market significance are listed in Table 21.2 [8].

Several companies (i.e., DuPont, BASF, Bayer, Shanghai Zhongxi, Sipcam, Staehler, Sundat and Syngenta) sell cymoxanil in many countries in Europe,

Table 21.2 Cymoxanil – use against plant diseases of high market significance.

Plant disease	Application rates (recommended) (g-a.i. ha^{-1})	Maximum number of applications per season
Potato late blight (*Phytophthora infestans*)	110 (mixtures with mancozeb) 175 (mixtures with famoxadone)	8
Vine downy mildew (*Plasmopara viticola*)	50–200 (mixtures with famoxadone)	3
Tomato late blight (*Phytophthora infestans*)	90–180 (mixtures with famoxadone)	5
Cucumber downy mildew (*Pseudoperonospora cubensis*)	75–150 (mixtures with famoxadone)	5

Asia, and the Americas either as solo products or in mixtures, under numerous trade names, e.g., Curzate®, Blizzard®, Pulstar®, Tanos®, Equitation Pro®, Aktuan®, Horizon®, Wakil®, Evolve®, Scribe®, etc. Due to its rapid degradation in plants [5–6], animals [9] and in the environment the toxicological and ecotoxicological profile of cymoxanil is favorable (acute oral toxicity rats: $LD_{50} = 960$ mg kg^{-1} day^{-1}), leading to a classification in WHO toxicity class III (slightly hazardous).

Although cymoxanil has been intensively used for more than 20 years, no significant decrease in sensitivity of *Phytophthora infestans* has been observed [10–11]. However, some studies indicate a significant decrease in sensitivity of *Plasmopara viticola* to cymoxanil compared to baseline studies performed before market introduction [12–13].

The mode of action of cymoxanil remains unclear according to previous reviews [14–16], although some interesting results have been reported.

Cymoxanil when applied at concentrations up to 100 μg mL^{-1} had no effect on mycelium respiration and zoospore motility (*Phytophthora infestans* [17]), thus showing that energy production within the organism is not affected.

In *P. infestans* [17], cymoxanil when applied at concentrations up to 100 μg mL^{-1} did not inhibit the uptake of radiolabeled precursors of DNA (thymidine), RNA (uridine) or proteins (phenylalanine). However, after less than 2-hours treatment, although uptake was not affected thymidine incorporation was considerably reduced while uridine incorporation was slightly affected and phenylalanine incorporation was insensitive. In contrast, cymoxanil at 10 μg mL^{-1} did inhibit thymidine or uridine incorporation weakly while inhibition of mycelial growth was complete, suggesting that DNA and RNA synthesis inhibition is a secondary effect. RNA polymerase activity in isolated nuclei was not inhibited by cymoxanil.

In *P. cinnamomi* mycelium [18], short treatment (less than 2 h) by cymoxanil at up to 100 μg mL^{-1} led to moderate inhibition of both, uridine and phenylalanine (or serine) uptake and incorporation. After longer treatment (4 and 6.5 h), uridine and phenylalanine (or serine) uptake and incorporation were still inhibited in addition to acetate incorporation into lipids.

Taken together these results indicate that DNA, RNA and protein biosyntheses are not the primary target of cymoxanil.

In parallel, some interesting results were obtained in a non-target organism, *Botrytis cinerea* strain B [19], a benzimidazole-resistant strain found to be sensitive to cymoxanil with an $ED_{50} = 0.7$ μg mL^{-1} on mycelial growth. Firstly, similar to *P. cinnamomi*., short-term treatment (less than 2 h) by cymoxanil at up to 100 μg mL^{-1} had no effect on respiration and led to minimal inhibition of uridine and phenylalanine uptake and incorporation. Strong inhibition was observed after long treatment (4 and 6.5 h), the major effect being on uridine uptake and incorporation. In contrast to *P. cinnamomi*, acetate incorporation into lipids increased without change in lipid composition [19]. Secondly, the fungitoxicity of cymoxanil can be partially reversed by addition of serine and cysteine to the growth medium but not by methionine or glutathione [19]. Thirdly, cymoxanil is

quickly metabolized by the sensitive strain but not by the resistant ones, suggesting it may be a pro-fungicide. However, the fungitoxic metabolites have not yet been identified [20].

The effect of preventative or curative application of cymoxanil on host–pathogen interactions during *Phytophthora infestans* infection of tomato and potato has been studied by both light and electron microscopy [21]. Cytological analysis showed that in the presence of the pathogen and cymoxanil a hypersensitive-type response of the host cells was observed. This response was characterized by granulation, plasmolysis and yellowing of cytoplasm of invaded epidermal cells, cell wall thickening and necroses at the infection site.

In conclusion, available data suggest that cymoxanil has an unknown fungicidal mode of action and might also induce some host plant defense responses (similar hypersensitive effects are known from many fungicides, triazoles, carboxin, strobilurins, etc.).

21.3 Fosetyl-aluminium

Another fungicide of high market significance with efficacy mainly against Oomycetes is fosetyl-aluminium. It was developed by Rhône-Poulenc, now Bayer CropScience. The first market introduction was in 1977.

Fosetyl-aluminium belongs to the chemical class of phosphonates and was discovered in 1973 by Philagro [22]. The IUPAC chemical name is aluminiumethylhydrogenphosphonate (CAS-RN.: 39148-24-8). Figure 21.2 shows its chemical structure.

One related compound, the corresponding sodium salt (fosetyl-sodium), also reached an advanced stage of development as an Oomycetes fungicide, however did not make it to market introduction. Moreover, other salts and the parent compound itself, ethyl phosphite or fosetyl, are also active against Oomycetes.

Due to the fact that the compound is a salt, the water solubility of fosetyl-aluminium is extremely high. It is stable towards hydrolysis under neutral conditions and decomposes only under strong acidic or basic conditions or by exposure to strong oxidizing agents. Table 21.3 lists further physicochemical properties [23].

According to a patent application published in 1996 [24] (Scheme 21.2) fosetyl-aluminium can be prepared by combining phosphorus trichloride with a stoichiometric amount of ethanol (85–100%) at temperatures below 20 °C followed by

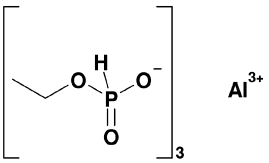
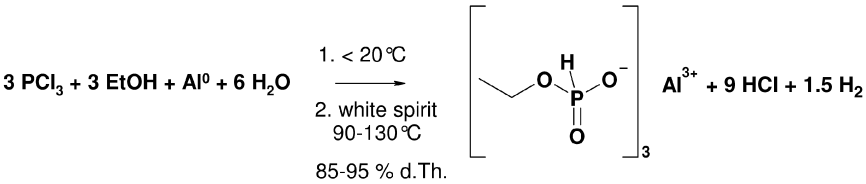


Fig. 21.2. Chemical structure of fosetyl-aluminum.

Table 21.3 Physicochemical properties of fosetyl-aluminium.

Melting point (°C)	>200 (dec.)
Hydrolysis[a] DT_{50} (70 °C)	6 h at pH 1.2 12 h at pH 12.8
Vapor pressure (mPa, 25 °C)	<0.013
log $P_{O/W}$	~2.7 (pH 4)
Solubility (mg L^{-1}, 20 °C)	Water = 120 000 Acetone = 13 Acetonitrile = 5 Ethyl acetate = 5 Hexane = 5 Methanol = 920 Propylene glycol = 80

[a] Stable under normal storage conditions.



Scheme 21.2. Synthesis scheme of fosetyl-aluminum.

dissolving metallic aluminum to give aluminum chloride. This mixture reacts *in situ* with water to yield diethyl phosphite containing traces of ethyl phosphite. The diethyl phosphite reacts with aluminum chloride in an inert solvent at 90–130 °C to give fosetyl-aluminium in good yields with HCl generated as a side product.

Fosetyl-aluminium is a protective and curative fungicide for foliar application. The compound allows a specific control of Oomycetes (*Phytophthora* spp., *Plasmopara* spp., *Bremia* spp., etc.) on lettuce, hops, strawberries, pome fruits, citrus fruits, pineapples, avocados, vines, cucurbits, onions, cocoa, rubber, tobacco, ornamental plants and shrubs. It also possesses a wider spectrum of lower level activity against other Oomycetes such as pearl millet downy mildew [25] (*Sclerospora graminicola*) and some bacteria. After spray application of fosetyl-aluminium it is rapidly absorbed, predominantly through the leaves but also through the roots. Within the plants it is translocated both acropetally and basipetally, leading to a protection of the complete plant, including roots and fruits [26]. Fosetyl-aluminium acts by inhibiting germination of spores and by blocking development of mycelium.

Table 21.4 Fosetyl-aluminium – use against plant diseases of highest market significance.

Plant disease	Application rates (recommended) (g-a.i. ha^{-1})	Maximum number of applications per year
Downy mildew on grapes (*Plasmopara viticola*)	500–2000 (fosetyl) (mixtures with fenamidone)	3
Downy mildew on hop (*Pseudoperonospora humuli*)	2000–400 (fosetyl)	8
Downy mildew on lettuce (*Bremia lactucae*)	2200 (fosetyl)	3
Downy mildew on cucumber (*Pseudoperonospora cubensis*)	2200–4400 (fosetyl)	4

Fosetyl-aluminium is mainly used in grapes, vegetables, citrus and tropical fruits such as pineapple. The use of fosetyl-aluminium to control the plant diseases of the highest market significance are listed in Table 21.4 [27].

Several companies (i.e., Bayer, Philagro, Shanghai Zhongxi, Sundat) currently sell fosetyl-aluminium as solo products or in mixtures under several trade names, including Aliette®, Mikal®, Valiant®, Proban®, Mikalix®, Cap 25®, Odyssee®, Aliziman®, Rhodax®, Almanach®, Alliance®, Artimon®, Sillage®, etc.

Due to its fast degradation in plants [5–6], animals [28] and in the environment, where it is metabolized rapidly into phosphate, the toxicological and ecotoxicological profile of fosetyl-aluminium is very favorable (acute oral toxicity rats: $LD_{50} = 5800$ mg kg^{-1} day^{-1}), leading to a classification in WHO toxicity class V (unlikely to be hazardous).

Although this compound has been intensively used for nearly 30 years, resistance development of fungal pathogens to fosetyl-aluminium is very limited. Some studies, though, indicate a significant decrease in sensitivity of *Plasmopara viticola* to fosetyl-aluminium with resistance factors between 5 and 24 [29]. Moreover, decreased sensitivity against lettuce downy mildew, *Bremia lactucae*, has been observed [30]. However, resistance development to fosetyl-aluminium seems to have only a limited impact on the overall product performance.

In some studies, the authors isolated fungal strains insensitive to fosetyl-aluminium as well as to metalaxyl [30–31]. Although there has been some speculation about possible cross resistance between the two compounds it has not been possible to show this under field conditions. It is likely that the fungal isolates show independent multiple resistance to these fungicides although the effect might be explained by other mechanisms [32]. In laboratory experiments, no metalaxyl-tolerant isolates of *Phytophthora capsici* with insensitivity against fosetyl-aluminium could be generated by mutagenesis [33]. Nevertheless, the possibility to obtain strains of *Pythium aphanidermatum* resistant to metalaxyl and fosetyl-aluminium by the exposure of a metalaxyl-resistant field isolate to a

chemical mutagen has been shown [34]. In summary, the risk of significant resistance problems with fosetyl-aluminium within the near future appears to be very low.

The abundant literature on the biochemical mode of action of fosetyl-aluminium produced during the 1980s has been thoroughly reviewed [16, 35–37], leading to the conclusion that fosetyl-aluminium and phosphonate, the *in planta* metabolite of fosetyl-aluminium, both have a direct and an indirect mode of action.

The direct mode of action affects multiple targets in phosphate transport, phosphate use and regulatory functions within the fungus. *In vitro* the efficacy is generally dependent on (low) phosphate concentration in the assay system.

Thus several enzymes of glucose metabolism [38–39] as well as inorganic pyrophosphatase [40] from *Phytophthora* spp were found to be inhibited *in vitro*. Further to this biochemical activity, physiological responses were measured in *P. palmivora* and *P. citrophthora*. Initially, in treated *P. palmivora*, a decrease in NAD and ATP was observed [41]. Longer exposure resulted in changes in phosphorus distribution and lipid composition [42] while key enzyme activities of the pentose phosphate pathway and of β-glucan biosynthesis were increased several-fold [39]. The former results point to altered fungal metabolism, whereas the latter indicate direct or indirect control of protein levels [39, 43]. Reported activities are in the millimolar range, consistent with the high concentrations needed to inhibit fungal growth.

However, inhibition of the above enzymes is unlikely to account for the specific anti-Oomycetes effect of phosphonates, since the same enzymes obtained from other sources (yeast, animals) are also affected.

In contrast, an indirect mode of action for example promotion of plant defense responses could explain the better antifungal activity observed *in planta* than *in vitro*.

Fosetyl-aluminium stimulates hypersensitive response (HR) in plants and the production of phytoalexins in a range of host–pathogen systems, e.g., in tomato infested by *P. capsici* or in grapevine infested by *Plasmopara viticola* [44]. Moreover, fosetyl-aluminium alone induces both phytoalexin and pathogenesis-related (PR) proteins production in grapevines [45]. Also, PR1 gene expression is induced by fosetyl-aluminium in wild-type *Arabidopsis thaliana* concurrently with control of the pathogen *Peronospora parasitica*. Conversely, in SAR-compromised plant mutants there is neither PR1 induction nor control of pathogen growth (SAR, salicylic acid-dependent systemic acquired resistance) [46]. This demonstrates that fosetyl-aluminium is indeed an inducer of plant defense responses. These results were recently extended and it was shown that a broader spectrum of defense-related genes were activated at mRNA level by fosetyl-aluminium, including PR1 and PR2 markers of salicylic acid-dependent systemic acquired resistance (SAR) as well as other plant response genes [47].

In conclusion, fosetyl-aluminium (phosphonate) is a potent inducer of several plant defense responses in addition to its limited direct effect on fungal metabolism.

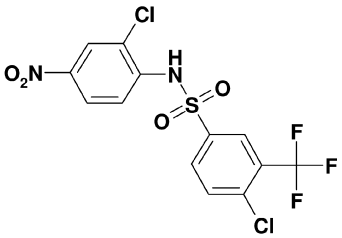


Fig. 21.3. Chemical structure of flusulfamide.

21.4
Flusulfamide

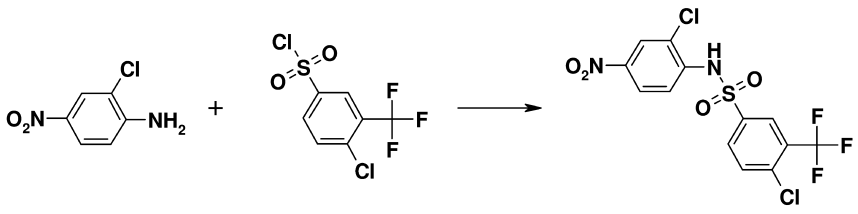
A small number of fungicides with activity against a very narrow range of target pathogens are commercially available. One of these niche products, flusulfamide, was discovered and developed by Mitsui Toatsu and in South Africa the compound was co-developed by Chemserve (formerly Kynoch). Flusulfamide was first registered in Japan in 1992 [48].

Flusulfamide belongs to the chemical class of benzenesulfonanilides and was discovered in 1972 by Mitsui Toatsu [49]. The IUPAC chemical name is 4-chloro-*N*-(2-chloro-4-nitrophenyl)-3-trifluoromethyl-benzenesulfonamide (CAS-RN.: 106917-52-6). No further compounds from this chemical class have ever been put into development. Figure 21.3 shows the chemical structure of flusulfamide.

Flusulfamide is a rather polar compound. However, due to the high melting point its water solubility is relatively low. It is stable in water. Table 21.5 lists further physicochemical properties [50–51].

Flusulfamide can be prepared by the reaction of 2-chloro-4-nitro-phenylaniline with 4-chloro-3-trifluoromethyl-benzenesulfonyl chloride [51] (Scheme 21.3).

Flusulfamide has been developed to control diseases caused by soil-borne pathogens out of the order Plasmodiophorales, the only order of the Plasmodiophorid group. In the past, this isolated group of obligate plant pathogens has been classified in either the protoctists kingdom or in the fungi kingdom. Flusulfamide is mainly used as a soil treatment for the control of the causal agent of



Scheme 21.3. Synthesis scheme of flusulfamide.

Table 21.5 Physicochemical properties of flusulfamide.

Melting point (°C)	170–172.5
Hydrolysis	Stable in acidic media; moderately stable in alkaline media
Vapor pressure (nPa, 20 °C)	358
Spec. gravity (g cm^{-3}, 23 °C)	1.739
log $P_{O/W}$	2.4
Solubility (g kg^{-1}, 25 °C)	Water = 0.0029 Acetone = 314 Chloroform = 17 Ethyl acetate = 125 Hexane = 0.05 Methanol = 24 Tetrahydrofuran = 592 Xylene = 14

clubroot disease of *Brassicae* (crucifer) crops, *Plasmodiophora brassicae*. Furthermore, it shows efficacy against another member of the Plasmodiophorales in reducing the incidence of powdery scab on potatoes (*Spongospora subterranea* var. *subterranea*) in field trials after soil treatment prior planting or after spray treatment of the seed tubers (tuber dip). Since potato powdery scab acts as vector of potato mop top virus indirect efficacy could also be shown against this disease [50]. Although flusulfamide also shows antifungal activity on cell test level against fungal plant pathogens such as *Botrytis cinerea*, *Pythium aphanidermatum* and others [51], it has never been registered for use against these diseases. Table 21.6 lists the use of flusulfamide to control the plant diseases of the highest market significance.

Mitsui & Co. Ltd. and Certis Europe, the agroscience company of Mitsui & Co. in Europe, sell flusulfamide in products for soil treatment under the trade names Nebijin®, Scablok® and Hoganna®. It is additionally distributed by Elliott.

Table 21.6 Flusulfamide – use against plant diseases of the highest market significance.

Plant disease	**Application rates (recommended) (g-a.i. ha^{-1})**
Cabbage/cauliflower clubroot (*Plasmodiophora brassicae*)	600 (soil treatment)
Potato powdery scab (*Spongospora subterranea*)	1800 (soil-borne, soil treatment) 1 g (seed-borne, seed tuber dip)

Of note is the acute toxicity of flusulfamide on mammals (LD_{50}: 180 mg kg^{-1} for male rats [52], 132 mg kg^{-1} for female rats [52], 245–254 mg kg^{-1} for mice), leading to a classification in WHO toxicity class II (moderately hazardous). However, it did not show mutagenic, teratogenic, reproductive or oncogenic effects. Studies on the translocation of flusulfamide from soil to cabbage and turnip plants showed almost no translocation. Since the compound accumulates neither in the soil nor in mammals any potential toxicological effect to the consumers and the environment is very low [51].

Flusulfamide inhibits germination of resting spores and thus prevents root-hair infection, however, spore integrity and viability is not affected [53].

The biochemical mode of action of flusulfamide is unknown since nothing is published up to now.

21.5 Diclomezine

A further specialist with fungicidal activity against several sclerotial diseases of rice plants is diclomezine. It was discovered in 1972 by Sankyo [54] and got its first registration in Japan in 1987.

Diclomezine belongs to the chemical class of pyridazinones. The IUPAC chemical name is 6-(3,5-dichloro-4-methylphenyl)-3(2*H*)-pyridazinone (CAS-RN.: 62865-36-5). Its structure is given in Fig. 21.4. Other compounds from this class have never been put into development.

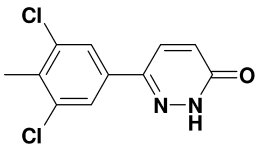
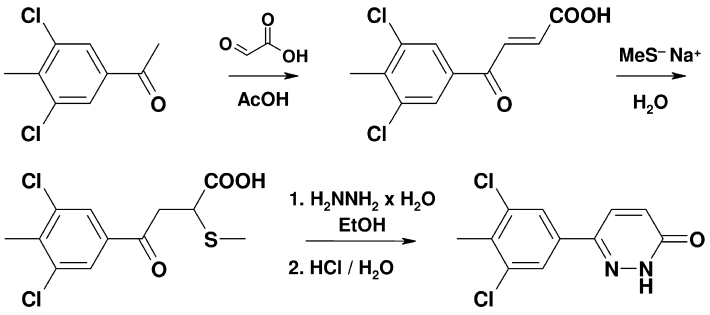


Fig. 21.4. Chemical structure of diclomezine.

Table 21.7 Physicochemical properties of diclomezine.

Melting point (°C)	254–258
Hydrolysis	Stable under acidic, neutral, and alkaline conditions
UV stability	Slow decomposition
Vapor pressure (mPa, 60 °C)	≤ 0.015
Solubility	Water (25 °C) = 0.74 mg L^{-1} Acetone (23 °C) = 3.4 g L^{-1} Methanol (23 °C) = 2.0 g L^{-1}



Scheme 21.4. Synthesis scheme of diclomezine.

The melting point of diclomezine is high, its water solubility is very low. It is stable in water and decomposes slowly under UV light. Table 21.7 lists further physicochemical data [55–56].

The synthesis of diclomezine starting from 1-(3,5-dichloro-4-methylphenyl)-ethanone, according to the literature [56], is shown in Scheme 21.4. It starts with an aldol condensation of the acetophenone with glyoxylic acid, yielding the corresponding benzoylacrylic acid. Addition of sodium methanethiolate in water affords 4-(3,5-dichloro-4-methylphenyl)-2-methylsulfanyl-4-oxo-butyric acid. The latter can be cyclized with hydrazine hydrate in ethanol to afford 6-(3,5-dichloro-4-methylphenyl)-4-methylsulfanyl-4,5-dihydro-(2*H*)-pyridazin-3-one, which can be aromatized under acidic conditions by elimination of methyl mercaptan to generate diclomezine.

Diclomezine is a highly effective fungicide against rice sheath blight caused by *Rhizoctonia solani*. Applied by foliar application at panicle initiation to heading stage at rates of 160–480 g ha^{-1} it exhibits high protective and curative activity against this and other sclerotial diseases including *Rhizoctonia oryzae*, *Sclerotium fumigatum* and *Sclerotium oryzae-sativae*. Diclomezine adheres to the sheath surfaces of rice plants and persists for a long period, thus showing long-lasting efficacy. Furthermore, it shows efficacy against white mould (*Sclerotium rolfsii*) and twig rot (*Rhizoctonia solani*) of peanuts as well as against *Rhizoctonia* diseases of turf grass. Due to the narrow range of pathogens controlled by diclomezine its market importance has remained limited. It is sold under the trade name Monguard® by Sankyo. No information about resistance development is available in the literature.

The toxicological and ecotoxicological profile [55] of diclomezine is favorable. In rats it shows no acute oral toxicity ($LD_{50} \geq 12\,000$ mg kg^{-1} day^{-1}) and it is neither mutagenic nor teratogenic. In soil it is readily adsorbed onto soil particles.

Inhibition of septum formation and leakage of cytoplasm were observed in mycelium of *Rhizoctonia solani* after 2–3 h treatment by 1 ppm diclomezine [55].

However, the primary biochemical mode of action of diclomezine remains unknown.

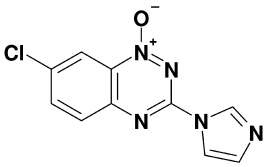


Fig. 21.5. Chemical structure of triazoxide.

21.6 Triazoxide

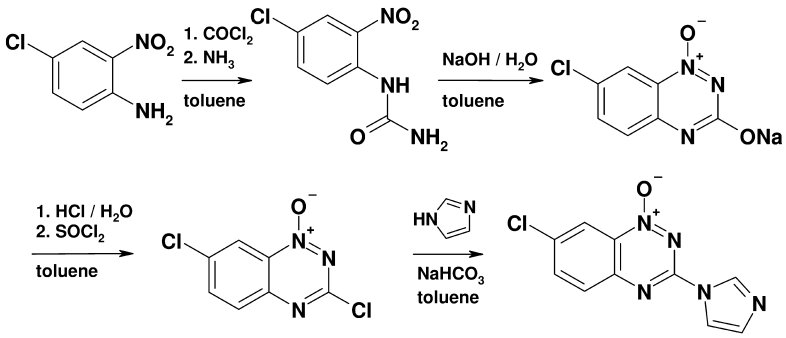
To fill a classical gap in the control of seed-borne diseases, triazoxide was developed as a fungicide with activity against one single disease complex, the seed-borne *Pyrenophora* diseases.

Triazoxide belongs to the chemical class of 1,2,4-benzotriazines and was discovered in 1978 by Bayer [57]. The IUPAC chemical name is 7-chloro-3-(1*H*-imidazol-1-yl)-1,2,4-benzotriazine-1-oxide (CAS-RN.: 72459-58-6). Although there are other 1,2,4-benzotriazine-1-oxides with fungicidal activity known from the literature, triazoxide is the only one to have been commercialized. Figure 21.5 shows the chemical structure of triazoxide.

The aqueous stability of triazoxide depends largely on the pH. Its water solubility is rather low and it is not very stable under UV light. Table 21.8 lists further physicochemical properties [58–59].

Table 21.8 Physicochemical properties of triazoxide.

Melting point (°C)	182
Hydrolysis, DT_{50} (22 °C, extrapol)	≫1 y at pH 4 3.6 y at pH 7 22.6 d at pH 9
UV stability	May be decomposed by light
Vapor pressure (Pa)	1.5×10^{-12} (20 °C) 5.2×10^{-12} (25 °C) 2.4×10^{-4} (120 °C)
Spec. gravity (g cm^{-3}, 22.5 °C)	1.577
log $P_{O/W}$ (23 °C)	2.0
Solubility (g L^{-1}, 20 °C)	Water 0.03 Dichloromethane 50–100 Hexane < 1 Isopropanol 2–5 Toluene 20–50



Scheme 21.5. Synthesis scheme of triazoxide.

The most appropriate technical synthesis of triazoxide [60], as described in Scheme 21.5, starts with phosgenation of 2-nitro-4-chloroaniline, followed by ammonolysis of the isocyanate. Cyclization of the formed arylurea occurs by treatment with aqueous sodium hydroxide to obtain the sodium salt of 3-hydroxy-7-chloro-1,2,4-benzotriazine-1-oxide. After acidification, the hydroxy group can be converted into the corresponding imidazolyl derivative after being transformed into the chloro compound with thionyl chloride.

Triazoxide has been developed for the control of soil-borne *Pyrenophora* diseases. It is used only as a seed dressing primarily for the control of *Pyrenophora graminea*, the cause of leaf stripe on barley [58], one of the most important seed-borne diseases of barley, which is difficult to control by most seed treatment compounds such as triazoles. Since the spectrum of fungicidal activity is very narrow, triazoxide is sold only in mixtures with other seed dressing compounds such as triazoles. As it is not translocated within barley plants, the fungicidal activity of triazoxide is limited to seed-borne *Pyrenophora* diseases. Therefore, *Pyrenophora teres*, the net blotch pathogen of barley, whose spores can also be dispersed by wind cannot be fully eliminated. However, control of seed-borne *Pyrenophora teres* is desirable since it may delay the onset of this disease [58]. Table 21.9 lists the recommended application rates of triazoxide.

Bayer CropScience sells triazoxide as seed dressing in mixtures with DMI inhibitors such as triadimenol (trade name Brio®) or tebuconazole (trade name Raxil S®), the latter also in mixture with the insecticide imidacloprid (trade name Gaucho Orge®).

Table 21.9 Triazoxide – use against plant diseases of market significance.

Plant disease	Application rates (recommended)
Leaf stripe on barley (*Pyrenophora graminea*)	2–4 g-a.i. per 100 kg seed (seed treatment)
Loose smut on barley (*Ustilago nuda*)	2–4 g-a.i. per 100 kg seed (seed treatment)

Of note is the acute oral toxicity of triazoxide in rats ($LD_{50} = 100–200$ mg kg^{-1} day^{-1} [58]), leading to a classification in WHO toxicity class II (moderately hazardous), although it is excreted quickly in the urine and the faeces. However, no mutagenic or teratogenic effects are observed. Since the dose required for a complete control of the named pathogens is very low and as the amount of triazoxide taken up by plants after seed treatment is negligible, no studies investigating the metabolism of triazoxide in the plant have been published. Within soil it degrades steadily, however no leaching risk of the parent could be observed as the compound is almost totally immobile in soil.

Information on the biochemical mode of action of triazoxide is not available from the literature. The FRAC Code List reports no resistance development to date.

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22
Recently Introduced Powdery Mildew Fungicides

Jochen Dietz

22.1
Introduction

Powdery mildew pathogens continue to infect numerous field and specialty crops. The appearance of strains with increased tolerance to existing mildew fungicides such as benzimidazoles, DMIs, or strobilurins has resulted in a significant drop in efficacy of these active ingredients [1, 2]. Consequently, there was a need for new mildewicides with novel modes of action to ensure highly effective control of powdery mildew pathogens and to allow for smart resistance risk management. Cyflufenamid (Nippon Soda), metrafenone (BASF Aktiengesellschaft), and proquinazid (Du Pont de Nemours) have recently been introduced as innovative solutions specifically aimed to combat powdery mildew pathogens in cereals and specialty crops.

22.2
Cyflufenamid

Cyflufenamid (**1**), a benzamidoxime fungicide, has been developed under the code NF-149 by Nippon Soda Co., Ltd. (Nisso) and has recently been introduced in the market as a powdery mildewicide for fruits, vegetables and cereals (Table 22.1) [3–6].

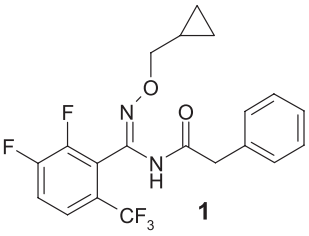
22.2.1
Discovery

Owing to the changing market environment in the 1990s for powdery mildew fungicides, Nippon Soda started reevaluating the potential of benzamidoximes for this target [4]. According to the company, the core structure of the benzamidoximes was a combination of structural features derived from several older pesticides such as the miticide benzoximate (**2**), the herbicides alloxydim-sodium (**3**)

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Table 22.1 Structure, physical, toxicological and ecotoxicological properties of cyflufenamid [3].

Structure	 **1**
Melting point (°C)	61.5–62.5
Vapor pressure (Pa) (20 °C)	3.54×10^{-5}
Water solubility (mg L^{-1}) (20 °C, pH 6.5)	0.52
log P_{OW} (25 °C, pH 6.75)	4.70
Acute toxicity (oral, rat)	$LD_{50} > 5000$ mg kg^{-1}
Eye irritation (rabbit)	Slightly irritating
Acute toxicity to fish	$LC_{50} > 1.14$ mg L^{-1} (carp, 96 h)
Acute toxicity to algae	$EC_{50} > 0.83$ mg L^{-1} (72 h)

and sethoxydim (**4**), and the fungicide metalaxyl (**5**) (Fig. 22.1). As a result of the reevaluation, compounds **6** and **7** were found to show a high efficacy against powdery mildew pathogens, combining excellent curative and residual activity. However, systemicity, crucial for preventing powdery mildew infestations on newly grown leaves, was not observed with these compounds. Further optimization then led to the discovery of the highly potent derivative **8**. Although this compound had a better low-concentration activity than its precursors, it still did not exhibit satisfactory in-plant mobility. To circumvent this obstacle, a different strategy for the distribution of the active ingredient was chosen and proved to be successful. The introduction of one or more additional fluorine atoms into the molecule eventually led to novel analogs exhibiting a sufficiently high vapor pressure to allow for a distribution to newly grown leaves via the vapor phase [3, 4]. Ultimately, the 2,3-difluoro-6-trifluoromethyl derivative, cyflufenamid, was selected as the development candidate considering synthesis simplicity, production cost, safety and influence on the environment [4, 6].

22.2.2 Cross Resistance and Mode of Action

Cross resistance of cyflufenamid to DMIs, morpholines, QoIs, benzimidazoles, cyprodinil and quinoxyfen has not been observed. Morphologically, cyflufenamid inhibits the infection process by preventing haustorium formation, haustoria development, growth of secondary hypha, and conidiospore formation, whereas

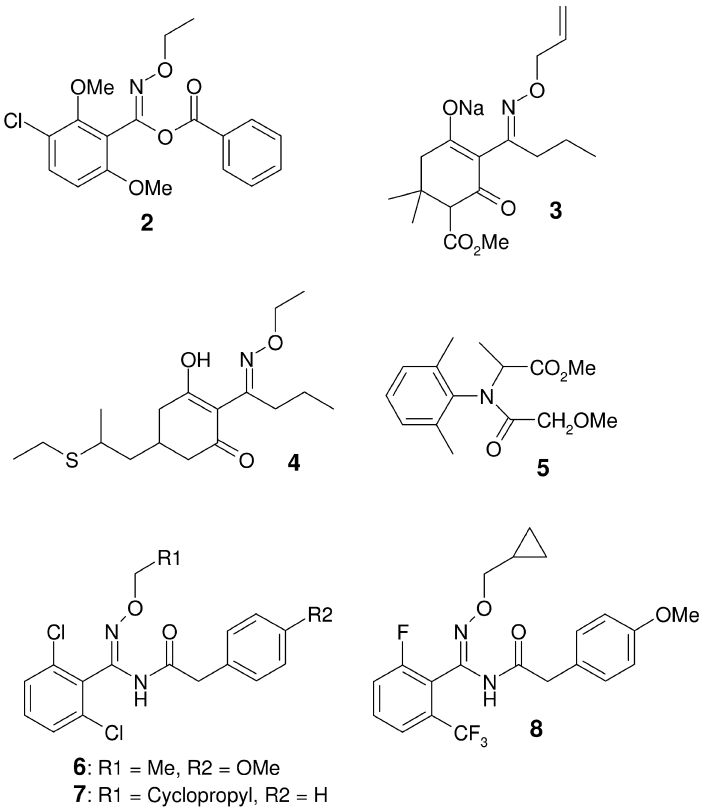
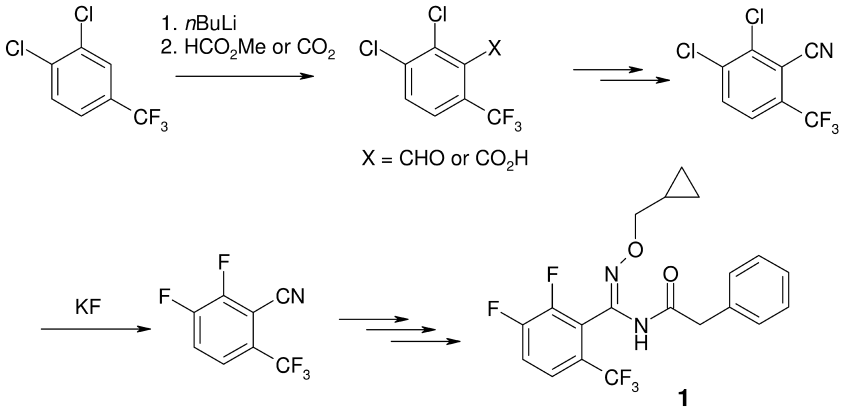


Fig. 22.1. Structural precursors (**2**–**5**) of cyflufenamid, lead compounds (**6**, **7**) and initial candidate **8**.

spore germination, germ tube elongation and appressorium formation are not inhibited [3, 4]. Although the exact biochemical target cyflufenamid interacts with has not yet been identified, several possibilities could be excluded. In a test system using *Monilinia fructicola* no influence on cell division, sterol biosynthesis, lipid biosynthesis, cell membrane functions, or respiration was observed [4].

22.2.3 Manufacturing Process

The arrangement of the four consecutive substituents on the benzene moiety was the key challenge for cyflufenamid's synthesis [4]. Several routes were examined and it was ultimately decided that an ortho-lithiation strategy would be the best method to introduce this substitution pattern (Scheme 22.1). Toward this end, 3,4-dichlorobenzotrifluoride, a raw material commercially available in bulk quan-



Scheme 22.1. Access to 2,3-difluoro-6-trifluoromethylbenzonitrile, a synthetic key intermediate for cyflufenamid (**1**).

tities, was chosen as starting point and converted into the carbaldehyde or the carboxylic acid by a metalation reaction. The latter functional groups were then converted into a carbonitrile function and subsequent chlorine–fluorine exchange with potassium fluoride afforded 2,3-difluoro-6-trifluoromethylbenzonitrile, a suitable precursor for cyflufenamid equipped with all necessary functional groups in the desired positions.

22.2.4
Fungicidal Profile

Cyflufenamid shows both preventative and curative activity on powdery mildew pathogens in cereals and specialty crops [3–5, 7]. In addition, it exhibits a good residual activity, a remarkable vapor phase activity as well as good translaminar mobility. In contrast, only poor translocation within the host plant was observed [3].

22.2.5
Registration, Products, Formulation and Crops

Cyflufenamid was first registered in 2002 for fruits and vegetables in Japan and more recently launched for cereals in the UK (2005) [3, 4, 7]. To avoid resistance development, it is not sold as a solo product for fruits and vegetables but as a mixture with triflumizole under the trade name Pancho® TF.

For cereals a 50 g L^{-1} emulsion in water formulation (Cyflamid®) was introduced [7]. Since this product is a solo formulation, a tank mix with a broad spectrum fungicide is recommended to avoid resistance development. Registrations in other European countries have been granted in 2006 (trade name e.g. Vegas® [8]).

22.2.6
Summary

Cyflufenamid, sold as the mixture product Pancho® TF for specialty crops or as the solo product Cyflamid® (Vegas®) for cereals, is a potent new powdery mildewicide. It shows both preventative and curative ability and allows for excellent pathogen control with its new mode of action.

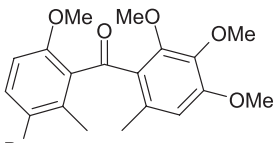
22.3
Metrafenone

Metrafenone (**9**), the first fungicide launched from the benzophenone family, was originally discovered by American Cyanamid as a result of structural considerations of previously investigated fungicides (Table 22.2) [9, 10]. It was transferred during development phase to BASF Aktiengesellschaft following the merger of the two companies in 2000. In 2004 metrafenone was introduced in the European market as a potent powdery mildewicide for the cereal and specialty crop sector [11].

22.3.1
Cross Resistance and Mode of Action

No cross resistance of metrafenone with other products has been observed to date. The mode of action is not yet fully understood but proven to be different from that of other major fungicides. Extensive investigations attempting to clarify

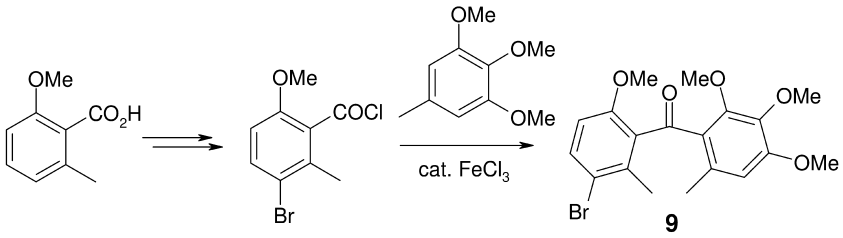
Table 22.2 Structure, physical, toxicological and ecotoxicological properties of metrafenone [12, 13].

Structure	 **9**
Melting point (°C)	99.2–100.8
Vapor pressure (Pa) (25 °C)	2.56×10^{-4}
Water solubility (mg L^{-1}) (20 °C, pH 7)	0.49
log P_{OW} (pH 4)	4.3
Acute toxicity (oral, rat)	$LD_{50} > 5000$ mg kg^{-1}
Eye irritation (rabbit)	Not irritating
Acute toxicity to fish	$LC_{50} > 94$ mg L^{-1} (96 h, *Oncorhynchus mykiss*)
Acute toxicity to algae	$EC_{50} = 2.9$ mg L^{-1} (72 h)

the biochemical target are currently ongoing [14–17]. Initial studies with barley powdery mildew indicate that metrafenone disturbs the organization or polarization of the actin cytoskeleton [14]. Morphological investigations show that metrafenone interacts at early stages of the development of wheat powdery mildew [18]. It inhibits mycelium growth and mycelial penetration into the leaf surface. Furthermore, development of appressoria, formation of haustoria, and sporulation are reduced.

22.3.2
Manufacturing Process

Processes for the synthesis of metrafenone have been described in two BASF patents [19, 20]. The benzophenone can be obtained by an iron(III) chloride catalyzed Friedel–Crafts acylation of 3,4,5-trimethoxytoluene. The appropriate benzoyl chloride is easily accessible by bromination of 2-methoxy-6-methylbenzoic acid and subsequent conversion into the acid chloride. (Scheme 22.2)



Scheme 22.2. Synthesis of metrafenone (**9**).

22.3.3
Fungicidal Profile

Metrafenone provides excellent preventative, curative and residual activity against powdery mildew pathogens in cereals, grapes and vegetables. In addition, it is effective against eye spot on wheat and barley. Metrafenone shows significant translaminar action, good acropetal translocation and, similar to cyflufenamid, distribution by vapor phase diffusion.

22.3.4
Registration, Products, Formulation and Crops

Metrafenone was first launched in the UK in 2004 to control powdery mildew in wheat and barley [18]. Subsequently, registrations for use in field crops have been granted in the Netherlands and in Germany as well as for powdery mildew control in grapes in Germany [21–23]. BASF expects to receive further registrations

for both sectors in other European countries in 2006. According to the American Environmental Protection Agency (EPA), registration as a grapevine fungicide in the United States should be expected by summer 2007 [24].

Metrafenone is sold as Flexity® (300 g L^{-1} SC) for use in cereals and as Vivando® (500 g L^{-1} SC) for powdery mildew control in grapevines. The recommended use rate is 0.25–0.5 L ha^{-1} for powdery mildew control and 0.5 L ha^{-1} to reduce eye spot. Since cereal powdery mildew is a high risk pathogen regarding resistance development; a spray program with azole fungicides for broad spectrum purposes is recommended [25]. In grapes alternate applications with non-metrafenone fungicides, e.g., strobilurin-containing products, are advised [22].

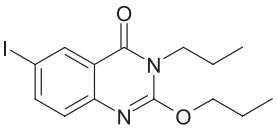
22.3.5 Summary

Metrafenone, an active ingredient with a new mode of action, was recently introduced in the market by BASF Aktiengesellschaft for preventative and curative use against powdery mildew pathogens in cereals and vines. In addition, it exhibits good activity against eye spot in wheat and barley. The compound is sold as an SC formulation in the solo products Flexity® and Vivando® for cereal and grapevine application, respectively.

22.4 Proquinazid

Proquinazid (**10**), the first fungicide belonging to the quinazolinone class, was developed under the code DPX-KQ926 by Du Pont de Nemours (Table 22.3) [26, 27] and has been very recently introduced in Europe as a potent preventative powdery mildewicide for the cereal and the grapevine markets [8, 26, 28, 29].

Table 22.3 Structure, physical, toxicological and ecotoxicological properties of proquinazid [27, 30].

Structure	 **10**
Melting point (°C)	48–49
Acute toxicity (oral, rat)	$LD_{50} > 2000$ mg kg^{-1}
Eye irritation	Irritating
Acute toxicity to fish	$LC_{50} = 2.3$ mg L^{-1} (96 h, *Oncorhynchus mykiss*)
Acute toxicity to algae	$EC_{50} = 3.3$ mg L^{-1} (72 h)

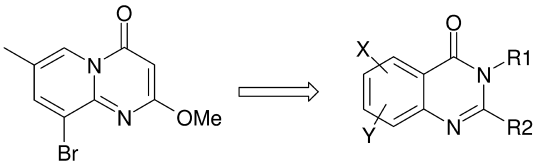


Fig. 22.2. Initial lead compound **11** for the optimization program of proquinazid and structural development.

22.4.1 Discovery

According to the inventors, proquinazid was derived from the initial lead compound **11**, coming from Du Pont's random screening program for novel fungicides (Fig. 22.2) [26]. Pyridopyrimidone **11** showed an interesting but weak activity at a high dose rate against wheat powdery mildew. The lead optimization process then led to various novel analogs of **11** with the same core structure as well as to the synthesis of novel core-modified derivates **12** [27, 31].

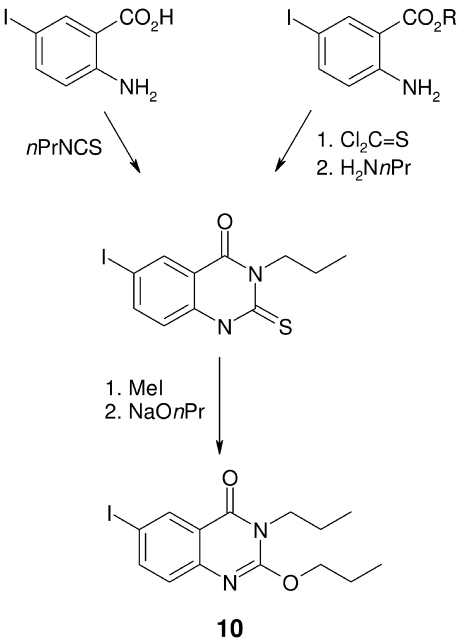
Many of these analogs were highly active in glass house screening but only the quinazolinone derivatives **12** showed also good activity in the field. Eventually, proquinazid was chosen as development candidate since it showed outstanding pathogen control in field trials in cereals, grapes and other crops at low dose rates, where it also demonstrated an excellent residual activity [26].

22.4.2 Manufacturing Process

Proquinazid can be prepared starting from 5-iodoanthranilic acid (Scheme 22.3) [27, 32, 33]. Cyclization with *n*-propyl isothiocyanate affords 2,3-dihydro-6-iodo-3-propyl-2-thioxo-4(1*H*)-quinazolinone. Alternatively, this key intermediate can be obtained by reaction of an appropriate anthranilate with thiophosgene and *n*-propylamine. Subsequent introduction of the propoxy substituent via a methylation displacement sequence concludes the synthesis.

22.4.3 Cross Resistance and Mode of Action

Proquinazid has a new and previously unknown mode of action and cross resistance to other fungicides has not been reported to date [29]. So far, the molecular target where proquinazid interacts with a biochemical pathway could not be clarified. Morphologically, the active ingredient inhibits spore germination and appressorium formation. According to Du Pont, proquinazid may also stimulate the expression of host defense genes and this may represent a second indirect



10

Scheme 22.3. Synthesis of proquinazid (**10**).

mode of action. Currently, advanced trials are underway to confirm this hypothesis [29].

22.4.4
Fungicidal Profile

Proquinazid acts in a preventative manner and does not show a significant curative activity against powdery mildew pathogens. It is locally systemic and allows, similar to cyflufenamid and metrafenone, for the protection of untreated leaves or neighboring plants by distribution of the active ingredient via the vapor phase [29].

22.4.5
Registration, Products, Formulation and Crops

Proquinazid obtained its first registration and sales as the cereal fungicide Talius® in 2005 [29]. Du Pont expects further registrations for cereal application in Western Europe in 2006/7. In addition, proquinazid could successfully be registered as the grapevine fungicide Talendo® in Hungary and Austria in 2005. Also in this sector, further registrations are expected within the next few years.

Talius® (200 g L^{-1} EC) controls powdery mildew infections on cereals at approx. 40 g-a.i. ha^{-1} and provides an excellent residual activity up to six weeks

from a single application. The product can be applied twice a season but for resistance management reasons it should be mixed with a broad spectrum fungicide or a powdery mildewicide with a different mode of action.

Talendo® (200 g L^{-1} EC) is recommended for the control of *Uncinula necator* in grapes at approx. 50 g-a.i. ha^{-1}.

22.4.6 Summary

Du Pont has recently launched proquinazid as the market products Talius® and Talendo® to control powdery mildew infections in the cereal and the grapevine sectors. With its new mode of action and its preventative ability, the active ingredient allows for excellent residual pathogen control.

22.5 Conclusion

Three new powdery mildew fungicides have been launched within the last four years for both the field and the specialty crop sector. The active ingredients cyflufenamid, metrafenone and proquinazid were designed to combat powdery mildew attacks in cereals, fruits, vegetables and grapevines. In combination with other fungicides, these innovations are particularly useful solutions for the broad-spectrum control of fungi in various crops. All three compounds have a new mode of action and do not show cross resistance to any existing market product. Thus, smart resistance risk management should avoid possible emergence of resistant strains and allow for effective powdery mildew control in cereals and specialty crops for a long time.

Acknowledgments

I would like to thank my colleagues Ms. M. Scherer, Dr. H. Van T. Cotter, Dr. T. Grote, Dr. J. Renner and Dr. S. Strathmann for stimulating discussions and their valuable input.

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23
Newest Aspects of Nucleic Acid Synthesis Inhibitors – Metalaxyl-M

Urs Müller and Ulrich Gisi

23.1
Introduction

The control of diseases caused by plant pathogens of the Oomycetes has been a major target since the beginning of modern chemical crop protection. A wide range of fungicides is available and new products are being introduced to the market at regular intervals [1–3]. The phenylamide fungicides include compounds such as metalaxyl **3**, metalaxyl-M **1** (Fig. 23.1), furalaxyl, benalaxyl, ofurace and oxadixyl [4]. Metalaxyl was the first of this class, introduced into the market in 1977. It marked a breakthrough in chemical disease control and became the most important compound of the class in this market segment. The unique properties of phenylamide fungicides such as the control of all members of the Perenosporales and Pythiales, the long-lasting preventive and curative activity, the high systemicity and the excellent safety profile have been reviewed by Gisi and Ziegler [4]. In 1996 Ciba-Geigy announced the introduction of the active enantiomer of metalaxyl [5, 6]. By introducing a pure enantiomer, replacing the racemic metalaxyl, sometimes named the "chiral switch", a new chapter in

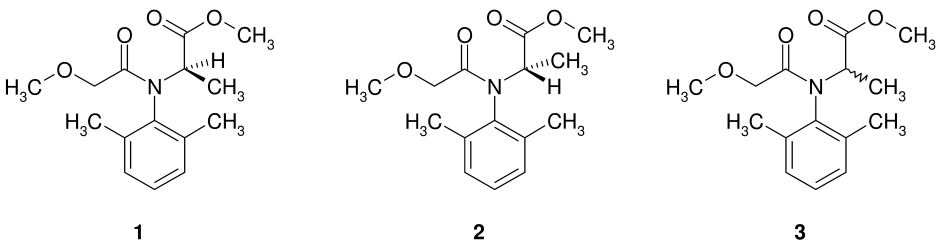


Fig. 23.1. Metalaxyl-M (USA: mefenoxam): methyl-*N*-(methoxyacetyl)-*N*-(2,6-xylyl)-D-(−)-alaninate **1**; methyl-*N*-(methoxyacetyl)-*N*-(2,6-xylyl)-L-(+)-alaninate **2**; metalaxyl: methyl-*N*-(methoxyacetyl)-*N*-(2,6-xylyl)-*rac*-alaninate **3**.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

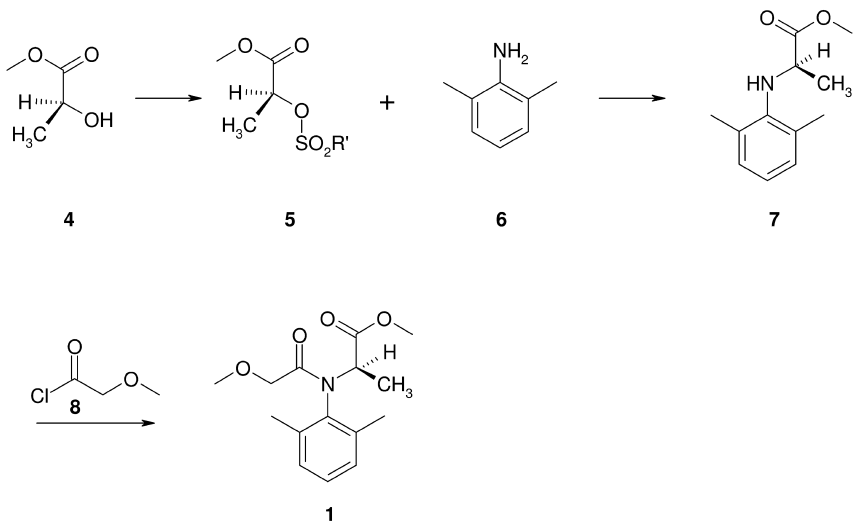
ISBN: 978-3-527-31496-6

both the control of oomycetes by phenylamide fungicides and the use of chiral crop protection agents in general was opened.

To date, metalaxyl-M, also called mefenoxam (USA) [7], is an indispensable product in the control of plant pathogenic Perenosporales and Pythiales.

23.2 Chemistry of Metalaxyl-M/Mefenoxam

Both enantiomers of metalaxyl were first prepared by the classical procedure of fractional crystallization of the salts of DL-*N*-(2,6-xylyl)-alanine with (+) and (−)-α-phenethylamine, followed by Fischer esterification and acylation with methoxyacetyl chloride in ethyl acetate in the presence of triethylamine as base, or in toluene using sodium carbonate as base, at room temperature. Optical purity was determined by NMR using chiral shift reagents and the absolute configuration was assigned by connecting the enantiomers to L-lactic acid of the chiral pool. The reaction of 2,6-dimethylaniline with the *p*-nitrobenzene sulfonate of L-methyl lactate gave methyl D-*N*-(2,6-dimethylphenyl)-alaninate through inversion at the chiral centre [8]. This process was further developed. Key is the preparation of D-*N*-(2,6-dimethylphenyl)-alanine esters, which, elaborated in a general synthesis of N-substituted α-amino acids, starts with (*S*)-2-(trifluoromethylsulfonyloxy)-carboxylic acid esters and reacts them with various amines and anilines (Scheme 23.1) [9, 10]. Sulfonylation of methyl (*S*)-lactate **4** gave pure sulfonylated ester **5** in yields up to 96%. Surprisingly, the sterically hindered 2,6-dimethylaniline **6**



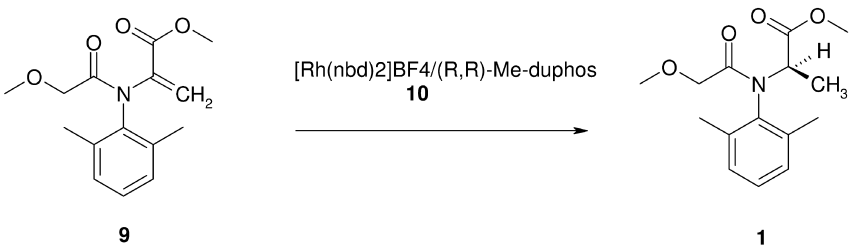
Scheme 23.1. Synthesis of metalaxyl-M **1** starting from (*S*)-lactic acid methyl ester (chiral pool).

Table 23.1 Chemical and physical properties of metalaxyl-M **1** [7].

Common name	Metalaxyl-M (BSI, E-ISO, F-ISO) mefenoxam (only in USA)
Patent no.	WO96/01559
Specific rotation	(−); $[\alpha]^{24}_{D} = -57 \pm 1$ (*c* 1.807%, H_3CCOCH_3) [9]
(S)-Isomer **2**	(+); $[\alpha]^{24}_{D} = +57 \pm 1$ (*c* 2.883%, H_3CCOCH_3) [9]
Form	Pale yellow to light brown, viscous liquid
Vapor pressure (mPa) (25 °C)	3.3
K_{OW} log *P* (25 °C)	1.71
Solubility (g L^{-1})	Water = 26 (25 °C); *n*-hexane = 59. Miscible with acetone, ethyl acetate, methanol, toluene, *n*-octanol
Stability	Hydrolytical stability pH ≤ 7 (DT_{50} > 200 d) pH 9 (25 °C) (DT50 116 d)

also reacted easily to afford the corresponding methyl (*R*)-*N*-(2,6-dimethylphenyl)-propionate **7** in good yields (90%) and high optical purity. Later this process was generalized by reacting the aniline **6** with alkyl and, especially, methyl (*S*)-2-(methylsulfonyloxy)-propionate, claiming yields of 90% (methylsulfonate) and a R/S ratio of 97.5:2.5 of **7**. Acylation of the intermediate **7** with methoxy-acetyl chloride **8** yielded metalaxyl-M **1** (properties listed in Table 23.1) in 94% chemical yield without any racemization (R/S ratio 97.5:2.5) (Scheme 23.1) [11, 12].

Obviously, the classical separation of the enantiomers by fractional crystallization as described above would hardly be economic for the large-scale production of a pesticide. A selective enzymatic hydrolysis of esters of DL-*N*-(2,6-xylyl)-alanine to the corresponding (*R*)-*N*-(2,6-xylyl)-alanine and subsequent synthesis of metalaxyl-M has recently been published [13]. After the pioneering breakthrough in the large-scale production of the herbicide *aR*/*aS*,1′*S*-metolachlor using an enantioselective catalytic hydrogenation process for the preparation of a key intermediate [14], Spindler and Blaser elaborated the basic principles governing the use of such catalytic processes [15]. Prerequisite for the successful development of such processes for the large-scale production of pharmaceuticals and agrochemicals is a broad experience in catalysis and the capacity to optimize both the chiral catalyst and the reaction conditions. This has been nicely demonstrated in the development of catalytic processes for the production of metalaxyl-M (Scheme 23.2). The easily prepared enamide **9** was subjected to catalyst screening. Of the 34 chiral Rh diphosphine catalysts tested, twelve produced enantiomeric excesses (e.e.) of >90% of metalaxyl-M but most showed insufficient activity to be considered for large-scale production. Further optimization led to the discovery of the catalyst $[Rh(nbd)_2]BF_4$/(*R*,*R*)-Me-duphos **10** and to a hydrogena-



Scheme 23.2. Enantioselective catalysis in the preparation of metalaxyl-M **1**.

tion process with hydrogen at 10 bar, 60 °C and a substrate-to-catalyst ratio of 5×10^4, resulting in 95.6% e.e. and a turnover frequency of 5.2×10^3 h^{-1} [16].

23.3
Biological Activity

Like metalaxyl, metalaxyl-M controls all pathogens of the Oomycetes in the orders Perenosporales, Sclerosporales and Pythiales such as the downy mildews of the genera *Albugo*, *Bremia*, *Peronospora*, *Peronoscelospora*, *Plasmopara*, *Pseudoperonospora*, *Sclerophtora* and *Sclerospora*, as well as Pythiales such as *Pythium* ssp and *Phytophtora* spp. In all applications, such as foliar, soil or seed treatment the outstanding level of control by metalaxyl-M is achieved at up to half the rate of its predecessor metalaxyl **3** [5, 6]. As shown in greenhouse trials the (R)-(−)-enantiomer **1** is the active, the (S)-(+)-enantiomer **2** the almost inactive isomer. This also indicates that there is no racemization, i.e., formation of the (S)-(+)-enantiomer **2** on or within the plant or pathogen after treatment, an important prerequisite for the introduction of pure enantiomers as products. Metalaxyl-M is mostly used in mixture with multi-site fungicides to protect a wide range of crops and is a major pillar product in the control of diseases caused by pathogens of oomycetes [17]. Major brand names are RIDOMIL GOLD® and APRON XL®. Metalaxyl and metalaxyl-M are extremely safe to crop plants. In a study of Singh, Mersie and Brlansky on the control of foot rot and root rot (*Phytophtora* spp.) in citrus, it was shown that the slight herbicidal effect of metalaxyl is associated with the (S)-(+)-enantiomer **2** and that such effects could not be detected for the (R)-(−)-enantiomer (metalaxyl-M) [18].

23.4
Mode of Action and Mechanism of Resistance

The phenylamide fungicides, including metalaxyl and metalaxyl-M, inhibit ribosomal RNA synthesis, specifically RNA polymerization (polymerases). In myce-

lium of *Phytophthora megasperma*, metalaxyl primarily affected the polymerase I complex of rRNA synthesis, which is considered as the primary site of action [19]. Endogenous RNA polymerase activity of isolated nuclei of *P. megasperma* and *P. infestans* was highly sensitive to metalaxyl unless the isolates were from resistant strains, suggesting that a mutation in the target site is responsible for resistance [20]. This hypothesis was further supported by the observation that (^{3}H)-metalaxyl binds to cell-free mycelial extracts of metalaxyl sensitive but not of resistant isolates [20]. Although metalaxyl, metalaxyl-M, oxadixyl, benalaxyl and ofurace exhibit different levels of intrinsic activity and rRNA polymerase inhibition [20], cross-resistance was observed between all phenylamide fungicides [21].

The phenylamide fungicides affect especially hyphal growth and the formation of haustoria and spores in oomycetes [22]. Since spores contain many ribosomes to support early growth stages, RNA synthesis is fully operational only after spore germination; therefore, later development stages are most sensitive [19]. As a consequence of RNA inhibition, the precursors of RNA synthesis (i.e., nucleoside triphosphates) are accumulated. They activate β-1,3-glucan synthetases, which are involved in cell wall formation [19] with the result that metalaxyl-treated hyphae often produce thicker cell walls than untreated ones.

Shortly after the commercial use of metalaxyl, resistant isolates were detected in *Pseudoperonospora cubensis*, *Phytophthora infestans*, *Peronospora tabacina* and *Plasmopara viticola* [23]. In most cases this was coupled to a decline in disease control. As a consequence, strict recommendations for the use of phenylamides were designed and enforced by PA-FRAC (''PhenylAmide Fungicide Resistance Action Committee'') to prevent and further delay resistance evolution [24]. These involve the preventive use of pre-packed mixtures with well-defined amounts of non-phenylamide fungicides, a limited number of applications per crop and per season and no soil use for the control of air-borne pathogens. These recommendations have been successfully implemented and products containing phenylamides remain important fungicides, offering specific advantages for the control of diseases caused by oomycetes, although resistant isolates can be found in all regions of the world and on many crops.

Phenylamide resistance has been described as monogenic. The majority of the F1 progeny produced from metalaxyl-resistant (r) and metalaxyl-sensitive (s) parental isolates of *P. infestans* had an intermediate sensitivity to metalaxyl. Crosses between two F1 isolates with intermediate sensitivity yielded a 1s:2i:1r ratio of progeny in the F2 generation [25]. This Mendelian segregation pattern reflects a single-gene (monogenic) resistance [26] based on an incompletely dominant gene [27]. Resistance to metalaxyl was also reported to be controlled by a single incompletely dominant gene in *Phytophthora capsici* [28], *P. sojae* [29] and *B. lactucae* [30]. However, a continuous sensitivity segregation pattern was observed in the F1 generation received from r $\times$ s crosses of European and Mexican *P. infestans* parents, suggesting that one semi-dominant locus, together with several minor loci may be involved in resistance [31]. Resistance in these isolates was associated

with two loci, *MEX1* and *MEX2*, the second locus mapping to the same linkage group as *MEX1* but to a distinct site [32]. Although many investigations on the mode of action and mechanism of resistance to phenylamide fungicides have been undertaken over the last 25 years, the responsible resistance gene(s) and the site of mutation(s) in the genome have not yet been elucidated.

Although phenylamides are considered to bear a high intrinsic resistance risk [33], they have failed to fully eliminate the sensitive sub-populations from nature even after 25 years of intensive use [17]. The proportion of resistant isolates in *P. infestans* and *P. viticola* fluctuates from year to year and also within the season. It increases within a season, more rapidly in fields treated with phenylamides than in untreated fields, starts to decline at the end of the season and is significantly lower at the beginning of the next season compared with the proportion at the end of the previous year [23].

23.5 Degradation and Metabolism of the two Enantiomers

One of the goals of making the "chiral switch", i.e., of developing the pure enantiomer of metalaxyl, was the reduction of chemicals dispersed in the environment. Obviously, application of half the amounts compared with metalaxyl will result in a reduction of the active ingredient in the environment. However, just as they have different biological activity, the (R) and (S) enantiomers may be expected to behave differently in the environment, since degradation processes are mostly of enzymatic nature. The metabolic pathways of metalaxyl-M in soil, plants and animals are very similar to metalaxyl [4]. The main metabolite of metalaxyl-M in soil is, like for metalaxyl, the corresponding acid of metalaxyl-M. Because of the extremely wide variation of soil and climatic conditions a uniform degradation process can not be expected for the two enantiomers under natural conditions. Nevertheless, some general trends emerge from the extensive studies carried out in this field. Racemization was neither observed in plants nor in the different soil types investigated [34–36]. Indicators of soil processes like changes of ammonium and nitrate concentrations, as well as the activity of soil enzymes like phosphatases, β-glucosidases or dehydrogenases, can be affected, but no uniform correlation to the use of metalaxyl-M could be found. The changes of these soil processes depend very much on the number of treatments, the amount of the active ingredient applied and on whether the soil investigated was previously treated with the fungicide (enhanced degradation) [37]. In conclusion, the use of metalaxyl-M is safe to the environment. No specific effects have been found that can be attributed to the (R)-(−)-optical isomer and impair the safety profile of metalaxyl-M. Reduction of the application rate to almost half compared with metalaxyl while maintaining the spectrum of activity and the level of potency clearly marks the innovative step in the development of metalaxyl M.

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Part III
Insecticides

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Overview

The use of insecticides belongs to the oldest plant protection measurements, starting with the application of natural insecticides such as nicotine, rotenone or pyrethrum. With the discovery of organophosphorus insecticides on the one hand and the insecticidal chlorohydrocarbons on the other hand the era of industrially produced insecticides started in the 1940s and 1950s. The most important innovation in the field of insecticides was the discovery and introduction of the synthetic pyrethroids in the 1970s and 1980s. Since that time research and development of new insecticides has dramatically changed. Aspects of higher selectivity, minor risk regarding acute and long-term toxicity, reduced chemical stability (persistence in the environment) and the protection of beneficial insects came more and more into the focus of modern research and development efforts of agrochemical companies, the regulatory authorities, farmers, food companies and the public. The use of beneficial insects together with insecticides or miticides or other new application methods, e.g., seed treatments against insects like aphids as vectors for virus disease was intensified with the invention of higher selective compounds. This led to the development of insecticides with smaller insecticidal spectra, e.g., only against sucking or biting insects or mites.

All these aspects are reflected in the different contributions to the Section on *Insecticides*.

The reader is given a broad overview of the different insecticide classes in Chapter 24 (IRAC; Insecticide Resistance and Mode of Action). The IRAC Mode of Action Classification v. 5.1, September 2005, presented in this chapter, enumerates the main groups of insecticides and specifies the active ingredients within the different chemical groups. This gives a survey on older insecticides, already described in standard literature like *Chemistry of Pesticides*, John Wiley and Sons, 1983 or others. At the same time the objectives of IRAC, the mission, the structure and organization and activities clearly indicate the cooperation of regulatory authorities, agrochemical companies and university researchers in preventing resistance development against insecticides by practicing alternations, sequences, rotations or mixtures of compounds from different MoA (mode of action) groups as a sustainable and effective approach to Integrated Resistance Management (IRM).

In contrast to the sections on *Herbicides* and *Fungicides*, the logical sequence of the different contributions here follows the physiology of insects, starting with in-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

secticides acting on insect molting and metamorphosis (Chapter 25) and chitin synthesis (Chapter 26) with highly specific insecticidal targets not found in mammals or other non-arthropod organisms.

Bacillus thuringiensis toxins acting on the midgut of insects are the basis of transgenic crops protected by Bt genes against damage from biting insects. With the introduction of transgenic Bt cotton, maize and potato against lepidopteras and specific coleopteras the insecticide markets have changed and will change further dramatically. Therefore, Chapter 27 (Midgut – Transgenic Crops expressing *Bacillus thuringiensis* Proteins) is indispensable in a book describing modern insecticides and modern methods for combating insects. Formulations of Bt spore-crystal mixtures as natural insecticides have been used for over 40 years and have demonstrated that Bt is a very specific, effective and safe bioinsecticide based on its crystal proteins acting highly specifically on the midgut of insects.

Inhibitors of metabolic processes in insects have been a wide area for the discovery of new insecticides since the 1970s. The main challenge in this research area was to discover compounds and compound classes with high selectivity to the insect target. Chapter 28, describing inhibitors of oxidative phosphorylation, inhibitors of mitochondrial electron transport and, especially, inhibitors of lipid synthesis, illustrates the efforts and success of agrochemical companies in these areas in the last 20 years. The discovery and development of inhibitors acting in these metabolic processes was mainly possible by using a prodrug concept. This reduced, for example, the possible high acute toxicity in mammals that can be expected from such compounds. Such prodrugs can not be metabolized in mammals but are in insects. This process, producing the toxic drug by insect metabolism, allows high differentiation between insects and other organism. Differentiation can also be attained when the metabolic process has strong biochemical diversity, leading to different bond strengths between receptors of mammals or insects and the corresponding inhibitors.

Insecticides that act on the nervous system are the most effective compounds in preventing crop damage in a short time, by killing insects or preventing them from sucking (sometimes in seconds). This is why farmers will prefer, and have preferred, such insecticides. Whereas the organophosphorus insecticides and, subsequently, the pyrethroids dominated the insecticide market in the 1980s, the introduction of neonicotinoids with their systemic properties in the 1990s, against mainly sucking insects, and the introduction of spinosyns, mainly against biting insects, changed this situation dramatically and will change the market further. This is described in Chapters 29.1–29.3. Progress also took place in the sodium channel insecticide research field with the development of a new compound also obtaining its selectivity by the prodrug concept (Chapter 29.4) or by high selectivity to insect ion channels (Chapter 29.6). With the fiproniloids a compound class was invented in 1985 as a spin-off of herbicidal research that produced insecticides with new structures that act on the known GABA channel, but which were very competitive due to their systemic properties, making them usable also as seed treatment compounds (Chapter 29.5).

Completely new synthetic inhibitors of unknown mode of action (Chapter 30.3) or new highly specific inhibitors with such a mode of action described only prior by highly toxic natural inhibitor receptors (Chapter 31) are presented by the inventors from Japanese companies, showing the highly innovative contribution of Japanese researchers to the discovery of new insecticides and miticides.

With new research approaches, but also by serendipity in highly specified biological screenings, significant progress in the battle against insects has been realized within the last twenty years. This will strongly impact the future market situation as well as research and development in international agrochemical companies. The future production of generic compounds, especially from the older insecticide classes like the organophosphates and the pyrethroids, will be changed markedly through these new solutions. The new compounds will, additionally, allow regulatory authorities of different countries to restrict the use of compounds with high acute toxicity when less toxic compounds are available to solve farmer's problems.

24
IRAC, Insecticide Resistance and Mode of Action Classification of Insecticides

Alfred Elbert, Ralf Nauen, and Alan McCaffery

24.1
Introduction

In most of the world's agriculture and horticulture, the effective management of pest insect populations depends on many inputs, including a ready supply of safe, highly efficacious chemical insecticides. Similarly, the effective control of insect vectors of diseases and a range of pests of non-agricultural or urban importance is also highly dependent on the availability of insecticidal products. Because insect populations usually have short life-cycles and are numerically abundant, they can readily develop resistance to insecticides with the result that once effective insecticides are no longer able to control the pests for which they were intended. Resistance may therefore be usefully defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". This definition differs somewhat from others in the literature, but IRAC believes it represents the most accurate, practical definition of relevance to farmers and growers. The agrochemical industry views resistance to insecticides as an extremely serious threat and combating it needs a proactive approach. Effective insecticide resistance management (IRM) is essential and the Insecticide Resistance Action Committee (IRAC) is dedicated to making this a reality [1].

24.2
Objectives of IRAC

IRAC was formed in 1984 to provide a coordinated crop protection industry response to prevent or delay the development of resistance in insect and mite pests. The mission of IRAC is to promote the development of resistance management strategies in crop protection, vector control and in other areas of insecticide use so as to maintain efficacy and support sustainable agriculture and improved pub-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

lic health. IRAC operates as an inter-company organization acting as a Specialist Technical Group of CropLife. IRAC International not only supports resistance management project teams but also provides a central coordination role to regional, country and technical groups around the world. Accordingly, the main focus of IRAC International is to facilitate communication and education on insecticide resistance and to provide technical advice and expertise to promote effective insecticide resistance management and its regulation.

24.3 Structure and Organization of IRAC

IRAC implements comprehensive strategies to confront resistance through a range of activities. Along with the other Resistance Action Committees, e.g., FRAC (Fungicide Resistance Action Committee), IRAC operates under the umbrella of CropLife International and as such is recognized by The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations (UN) as an advisory body. The group's activities are coordinated via the IRAC Executive Committee, IRAC International and Country or Regional Committees with the information disseminated through meetings, workshops, educational materials and the IRAC website (www.irac-online.org). Groups are composed primarily of key technical personnel from the agrochemical companies affiliated with CropLife through membership in the relevant National Associations (ECPA, CropLife America, etc.). Current member companies are: BASF, Bayer CropScience, Dow AgroSciences, DuPont, FMC and Syngenta.

24.3.1 Project and Functional Teams

Project Teams are set up with specific resistance management objectives and timelines to deal with a local, national or international insect resistance issue. Once the Project Team has completed its objectives, the work and outcome of the project is reported and the team then disbanded, e.g., Vector, Codling Moth and Cotton Teams. There are three functional teams that currently exist within the IRAC International Group: the Communication and Education Team, the Regulatory Team and the recently created Biotechnology Team. Additional Functional Teams are operational since 2006, e.g., Non-crop Pest Team (including disease vectors), and others may be created as deemed necessary.

24.3.2 Country Groups

IRAC International encourages the formation of Country Groups to interact with local experts and research institutions and to form Project Teams to address local resistance issues. Currently, country groups exist in the USA, Brazil, South Af-

rica, Spain, India, and Australia, but clearly there are still many resistance problems that need to be managed through the formation of new Country or Regional groups. An example of this is South East Asia where IRAC International is actively encouraging the formation of a regional group to tackle local problems. In some European countries IRAGs (Insecticide Resistance Action Groups) have been formed, such as IRAG UK, and these groups work closely with IRAC, regulators and other stakeholders. In other countries such as Germany (including participants from Austria and Switzerland), Poland and Italy, similar groups have been established very recently.

24.4 Activities

IRAC groups are actively involved in, and on certain occasions provide funding for, various resistance management projects around the world. These are generally driven or coordinated by the local country group and in some cases a specific project group is set up to lead and ultimately report results and findings into the public domain. Examples of these have been the long-term monitoring of mosquito resistance in Mexico and the monitoring of pyrethroid resistance of *Helicoverpa armigera* in West African cotton. A new project group was set up recently within IRAC International to investigate codling moth resistance in several countries around the world. A Neonicotinoid Project Group has been established to define and implement guidelines for the use of this valuable chemical class of insecticides. Other activities focus on issues relating to education, communication and regulatory approvals as well as providing expert technical support. These more general activities are wide ranging but can be grouped under the following headings.

24.4.1 Resistance Monitoring Methods

Reliable information on resistance rather than anecdotal reports or assumptions builds the cornerstone of successful resistance management. To that end, sound baseline data on the susceptibility of the target pest to the insecticide/acaricide has to be generated. Baseline data can be defined as that obtained from a strain (or several strains) with no history of selection with the toxicant or related toxicants showing cross resistance. Currently a wide range of bioassay and biochemical tests are employed to characterize the susceptibility of target pests to insecticides and acaricides. Unfortunately, the results from specific test methods may not always be comparable since they measure different parameters and this can lead to difficulties over the interpretation of monitoring data. IRAC, in fulfilling its aim of providing expert advice to CropLife International on all technical matters relating to insecticide and acaricide resistance, has addressed this issue with the aim of recommending a range of reliable and reproducible bioassay tech-

niques to monitor insecticide and acaricide susceptibility for selected pest species of economic importance. IRAC has evaluated and validated a wide range of testing methods that have been published and are also freely available on the IRAC website. IRAC has an ongoing program to test and validate additional new methods for resistance monitoring.

24.4.2 IRAC and the Regulatory Requirements of Resistance Management

IRAC (along with HRAC, Herbicide, and FRAC, Fungicide, Resistance Action Committees) has taken a leading role as an expert group providing industry responses to proposals from regulatory bodies. For example, there is now a regulatory requirement in the EU under Directive 91/414/EEC for companies to provide an assessment of the potential risk of resistance being developed by target organisms and for management strategies to be introduced to address such risks [2, 3]. The Resistance Action Committees (RACs) have been instrumental in developing workable guidelines for companies, resulting in the publication of an official Guidance Document. Similarly, the US Environmental Protection Agency and the Pest Management Regulatory Agency of Canada have been developing a voluntary pesticide resistance management labeling scheme based on mode of target site on the pest. The RACs have been heavily involved in classifying pesticides into specific groups and families to enable the scheme to work. Development has been carried out under the auspices of the North American Free Trade Association and has resulted in the issue of a Pesticide Registration (PR) Notice in the US. In addition some of IRAC's other resources are being used by Regulatory Authorities such as the IRAC Mode of Action Classification Scheme, the IRAC Monitoring Methods and the MSU Resistance Database (see below).

24.4.3 Education and Communication

For IRAC, education and communication play a key role in the global management of resistance. Many steps have been taken over the years to provide resources to academia, researchers, industry and growers. IRAC education material has been put together to provide a basic understanding of insecticide resistance and to explain how resistance can be best managed. Most IRAC Country Groups, as well as utilizing centrally developed resources, have their own educational programs in place, tailored to meet their local needs. IRAC US, for example, publishes articles on a regular basis in grower magazines, while IRAC Brazil holds training workshops in different locations. Other IRAC Groups such as Australia, South Africa, Spain and India have similar ongoing initiatives.

Workshops, seminars, conferences and exhibitions are important platforms for communication and education and are often organized, attended or sponsored by IRAC. As an output from these meetings, many papers have been published by members on behalf of IRAC. The full bibliographic listing of more than 100 key

papers provides an interesting overview on recent findings in the different areas of insecticide/acaricide resistance management. The biannual *Resistant Pest Management Newsletter* of the Centre for Integrated Plant Systems (CIPS) in cooperation with IRAC and the Western Regional Coordinating Committee (WRCC-60) updates the most recent findings on insect/mite resistance.

The existing IRAC website (http://www.irac-online.org) has now been on-line since 2001 and has become the main home for IRAC information. It has recently been completely redesigned and updated. Data available include the IRAC Monitoring Methods, the IRAC Mode of Action Classification Scheme, Project and Country Group updates, meeting minutes, member contact details and useful links, details of published articles and copies of new posters recently produced. A special section for growers and other relevant associations has been established to facilitate practical advice for resistance management. *eConnection* is IRAC's quarterly published newsletter and it has been developed in conjunction with the new IRAC website to update users about new information appearing on the website, news of ongoing IRAC activities supporting insecticide resistance management and to highlight topical insecticide resistance issues.

24.4.4
Resistance Database Managed by Michigan State University and Supported by IRAC

For many years IRAC maintained the Resistance Survey that was built up from information received from sources around the world. In recent years this has become out of date and the decision was taken in collaboration with CropLife to sponsor Michigan State University (MSU) to extend their Pesticide Resistant Arthropod Database to include and extend the information that was contained in the original survey. The database includes reports of resistance cases from 1914 to the present. The introductory text explains that pesticide resistance is a dynamic, evolutionary phenomena and a record in the database may or may not be indicative of a specific area. Similarly, the absence of a record in this database does not indicate absence of resistance. This project is a major effort and will become a valuable new resource for the management of insecticide/acaricide resistance.

24.4.5
The Mode of Action Classification Scheme

The IRAC Mode of Action (MoA) Classification provides farmers, growers, advisors, extension staff, consultants and crop protection professionals with a guide to the selection of insecticides or acaricides for use in an effective and sustainable insecticide or acaricide resistance management strategy. In addition to presenting the MoA classification, this document outlines the background to, and purposes of, the classification list and provides guidance on how it is used for IRM purposes.

24.5
Principles of Resistance

As indicated above, resistance to insecticides may be defined as

> A heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species.

Resistance arises through the overuse or misuse of an insecticide or acaricide against a pest species and results in the selection of resistant forms of the pest and the consequent evolution of populations that are resistant to that insecticide or acaricide.

24.5.1
Mode of Action, Target-site Resistance and Cross-resistance

In most cases, not only does resistance render the selecting compound ineffective but it often also confers cross-resistance to other chemically related compounds. This is because compounds within a specific chemical group usually share a common target site within the pest, and thus share a common mode of action (MoA). It is common for resistance to develop that is based on a genetic modification of this target site. When this happens, the interaction of the selecting compound with its target site is impaired and the compound loses its efficacy. Because all compounds within the chemical sub-group share a common MoA, there is a high risk that the resistance that has developed will automatically confer cross-resistance to all the compounds in the same sub-group. It is this concept of cross-resistance within chemically related insecticides or acaricides that is the basis of the IRAC Mode of Action Classification.

24.5.2
Non-target Site Resistance Mechanisms

It is fully recognized that resistance of insects and mites to insecticides and acaricides can, and frequently does, result from enhanced metabolism by enzymes overexpressed due to insecticide selection pressure. Such metabolic resistance mechanisms are not linked to any specific site of action classification and therefore they may confer cross-resistance to insecticides in more than one IRAC MoA group. Where such metabolic resistance has been characterized and the cross-resistance spectrum is known, it is possible that certain alternations, sequences or rotations of MoA groups cannot be used. Similarly, mechanisms of reduced penetration of the pesticide into the pest, or behavioral changes of the pest, may also confer resistance to multiple MoA groups. Where such mechanisms are known to give cross-resistance between MoA groups, the use of insecticides should be modified appropriately.

Where the resistance mechanism is unknown, the intelligent use of alternations, sequences or rotations of compounds from different MoA classes remains an entirely viable resistance management technique since such a practice will always minimize selection pressures.

24.6
The Mode of Action (MoA) Classification Scheme v5.1, September 2005

The following classification scheme, developed and endorsed by IRAC, is based on the best available evidence of the mode of action of available insecticides (Table 24.1). Details of the listing have been agreed by IRAC companies and approved by internationally recognized industrial and academic insect toxicologists and biochemists.

24.6.1
Rules for Inclusion of a Compound in the MoA List

- Chemical nomenclature is based on that appearing in *The Pesticide Manual*, 13th edition, 2003, Ed. C.D.S. Tomlin, published by The British Crop Protection Council. 1250 pp., ISBN 1 901396 13 4.
- To be included in the active list, compounds must have, or be very close to having, a minimum of one registered use in at least one country. Superseded, obsolete or withdrawn compounds with no current registration are listed separately (in preparation).
- In any one MoA classification sub-group, where more than one active ingredient in that chemical sub-group is registered for use, the chemical sub-group name is used.
- In any one MoA classification sub-group, where only one active ingredient is registered for use, the name of that exemplifying active ingredient is used.
- Where more than one chemical sub-group or exemplifying active ingredient appears in a single mode of action group, each is named according to the above rules; chemical sub-groups having precedence over single active ingredients.

IRAC aims to ensure that insecticide and acaricide users are aware of mode of action groups and that they have a sound basis on which to implement season-long, sustainable resistance management through the effective use of alternations, sequences, rotations or even mixtures of insecticides with different modes of action. To help to delay resistance development it is strongly recommended that growers also integrate other control methods into insect or mite control programs (further advice is given in Table 24.1).

Table 24.1 IRAC mode of action classification v 5.1, September 2005[a].

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
1 Acetylcholinesterase inhibitors	**1A** Carbamates	Aldicarb, Alanycarb, Bendiocarb, Benfuracarb, Butocarboxim, Butoxycarboxim, Carbaryl, Carbofuran, Carbosulfan, Ethiofencarb, Fenobucarb, Formetanate, Furathiocarb, Isoprocarb, Methiocarb, Methomyl, Metolcarb, Oxamyl, Pirimicarb, Propoxur, Thiodicarb, Thiofanox, Trimethacarb, XMC, Xylylcarb
	Triazamate	Triazamate
	1B Organophosphates	Acephate, Azamethiphos, Azinphos-ethyl, Azinphos-methyl, Cadusafos, Chlorethoxyfos, Chlorfenvinphos, Chlormephos, Chlorpyrifos, Chlorpyrifos-methyl, Coumaphos, Cyanophos, Demeton-S-methyl, Diazinon, Dichlorvos/DDVP, Dicrotophos, Dimethoate, Dimethylvinphos, Disulfoton, EPN, Ethion, Ethoprophos, Famphur, Fenamiphos, Fenitrothion, Fenthion, Fosthiazate, Heptenophos, Isofenphos, Isopropyl *O*-methoxyaminothio-phosphoryl) salicylate, Isoxathion, Malathion, Mecarbam, Methamidophos, Methidathion, Mevinphos, Monocrotophos, Naled, Omethoate, Oxydemeton-methyl, Parathion, Parathion-methyl, Phenthoate, Phorate, Phosalone, Phosmet, Phosphamidon, Phoxim, Pirimiphos-, ethyl, Profenofos, Propetamphos, Prothiofos, Pyraclofos, Pyridaphenthion, Quinalphos, Sulfotep, Tebupirimfos, Temephos, Terbufos, Tetrachlorvinphos, Thiometon, Triazophos, Trichlorfon, Vamidothion
2 GABA-gated chloride channel antagonists	**2A** Cyclodiene organochlorines	Chlordane, Endosulfan, gamma-HCH (Lindane)
	2B Phenylpyrazoles (Fiproles)	Ethiprole, Fipronil

Table 24.1 *(continued)*

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
3		
Sodium channel modulators	DDT	DDT
	Methoxychlor	Methoxychlor
	Pyrethroids	Acrinathrin, Allethrin, d-cis-trans Allethrin, d-trans Allethrin, Bifenthrin, Bioallethrin, Bioallethrin S-cylclopentenyl, Bioresmethrin, Cycloprothrin, Cyfluthrin, beta-Cyfluthrin, Cyhalothrin, lambda-Cyhalothrin, gamma-Cyhalothrin, Cypermethrin, alpha-Cypermethrin, beta-Cypermethrin, theta-cypermethrin, zeta-Cypermethrin, Cyphenothrin [(1R)-trans-isomers], Deltamethrin, Empenthrin [(EZ)-(1R)-isomers], Esfenvalerate, Etofenprox, Fenpropathrin, Fenvalerate, Flucythrinate, Flumethrin, tau-Fluvalinate, Halfenprox, Imiprothrin, Permethrin, Phenothrin [(1R)-trans-isomer], Prallethrin, Resmethrin, RU 15525, Silafluofen, Tefluthrin, Tetramethrin, Tetramethrin [(1R)-isomers], Tralomethrin, Transfluthrin, ZXI 8901
	Pyrethrins	Pyrethrins (pyrethrum)
4	**4A**	
Nicotinic acetylcholine receptor agonists/ antagonists	Neonicotinoids	Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid, Thiamethoxam
	4B	
	Nicotine	Nicotine
	4C	
	Bensultap	Bensultap
	Cartap hydrochloride	Cartap hydrochloride
	Nereistoxin analogues	Thiocyclam, Thiosultap-sodium
5		
Nicotinic acetylcholine receptor agonists (allosteric) (not group 4)	Spinosyns	Spinosad
6		
Chloride channel activators	Avermectins, Milbemycins	Abamectin, Emamectin benzoate, Milbemectin4

Table 24.1 *(continued)*

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
7 Juvenile hormone mimics	**7A** Juvenile hormone analogues	Hydroprene, Kinoprene, Methoprene
	7B Fenoxycarb	Fenoxycarb
	7C Pyriproxyfen	Pyriproxyfen
8 Compounds of unknown or non-specific mode of action (fumigants)	**8A** Alkyl halides	Methyl bromide and other alkyl halides
	8B Chloropicrin	Chloropicrin
	8C Sulfuryl fluoride	Sulfuryl fluoride
9 Compounds of unknown or non-specific mode of action (selective feeding blockers)	**9A** Cryolite	Cryolite
	9B Pymetrozine	Pymetrozine
	9C Flonicamid	Flonicamid
10 Compounds of unknown or non-specific mode of action (mite growth inhibitors)	**10A** Clofentezine Hexythiazox	Clofentezine Hexythiazox
	10B Etoxazole	Etoxazole
11 Microbial disruptors of insect midgut membranes (includes transgenic crops expressing *Bacillus thuringiensis* toxins)	**11A1** *B. thuringiensis* subsp. *israelensis*	*Bacillus thuringiensis* subsp. *israelensis*
	11A2 *B. sphaericus*	*Bacillus sphaericus*
	11B1 *B. thuringiensis* subsp. *aizawai*	*Bacillus thuringiensis* subsp. *aizawai*
	11B2 *B. thuringiensis* subsp. *kurstaki*	*Bacillus thuringiensis* subsp. *kurstaki*

Table 24.1 *(continued)*

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
	11C *B. thuringiensis* subsp. *tenebrionis*	*Bacillus thuringiensis* subsp. *tenebrionis*
12 Inhibitors of oxidative phosphorylation, disruptors of ATP formation (inhibitors of ATP synthase)	**12A** Diafenthiuron	Diafenthiuron
	12B Organotin miticides	Azocyclotin, Cyhexatin, Fenbutatin oxide
	12C Propargite Tetradifon	Propargite Tetradifon
13 Uncouplers of oxidative phosphorylation via disruption of proton gradient	Chlorfenapyr DNOC	Chlorfenapyr DNOC
14 Vacant		
15 Inhibitors of chitin biosynthesis, type 0, Lepidopteran	Benzoylureas	Bistrifluron, Chlofluazuron, Diflubenzuron, Flucycloxuron, Flufenoxuron, Hexaflumuron, Lufenuron, Novaluron, Noviflumuron, Teflubenzuron, Triflumuron
16 Inhibitors of chitin biosynthesis, type 1, Homopteran	Buprofezin	Buprofezin
17 Moulting disruptor, Dipteran	Cyromazine	Cyromazine
18 Ecdysone agonists/ moulting disruptors	**18A** Diacylhydrazines	Chromafenozide, Halofenozide, Methoxyfenozide, Tebufenozide
	18B Azadirachtin	Azadirachtin
19 Octopaminergic agonists	Amitraz	Amitraz

Table 24.1 *(continued)*

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
20 Mitochondrial complex III electron transport inhibitors (Coupling site II)	**20A** Hydramethylnon	Hydramethylnon
	20B Acequinocyl	Acequinocyl
	20C Fluacrypyrim	Fluacrypyrim
21 Mitochondrial complex I electron transport inhibitors	METI acaricides	Fenazaquin, Fenpyroximate, Pyrimidifen, Pyridaben, Tebufenpyrad, Tolfenpyrad
	Rotenone	Rotenone
22 Voltage-dependent sodium channel blockers	Indoxacarb	Indoxacarb
23 Inhibitors of lipid synthesis	Tetronic acid derivatives	Spirodiclofen, Spiromesifen
24 Mitochondrial complex IV electron transport inhibitors	**24A** Aluminium phosphide	Aluminum phosphide
	24B Cyanide	Cyanide
	24C Phosphine	Phosphine
25 Neuronal inhibitors (unknown mode of action)	**25** Bifenazate	Bifenazate
26 Aconitase inhibitors	Fluoroacetate	Fluoroacetate
27 Synergists	**27A** P450-dependent monooxygenase inhibitors	Piperonyl butoxide
	27B Esterase inhibitors	Tribufos (DEF)

Table 24.1 *(continued)*

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
28 Ryanodine receptor modulators	Flubendiamide	Flubendiamide
un Compounds with unknown mode of action[b]	**una** Benzoximate	Benzoximate
	unb Chinomethionat	Chinomethionat
	unc Dicofol	Dicofol
	und Pyridalyl	Pyridalyl
ns Miscellaneous non-specific (multi-site) inhibitors[c]	**nsa** Borax	Borax
	nsb Tartar emetic	Tartar emetic

Notes to be read in association with the above classification scheme:
[a] Inclusion of a compound in the list above does not necessarily signify regulatory approval.
[b] A compound with an unknown mode of action or an unknown mode of toxicity will be held in category ''un'' until evidence becomes available to enable that compound to be assigned to a more appropriate mode of action class.
[c] Category ''ns'' is used for compounds or preparations with a non-specific, multisite action.
Groups and Sub-groups – Although sharing the same primary target site, it is possible that not all members of a single major MoA class have been shown to be cross-resistant. Different resistance mechanisms that are not linked to the target site of action, such as enhanced metabolism, may be common for such a group of chemicals. In such cases, the MoA grouping is further divided into sub-groups. For the purposes of this classification it should be assumed that cross-resistance exists between compounds in any one MoA sub-class. Alternation of compounds from different sub-groups within a class *may* be an acceptable part of an IRM strategy. Consult a local resistance expert for further advice.

Products containing multiple or stacked toxins will be differentiated from those containing single toxins only. This will be done by adding a suffix of ''m'' for multiple toxin products and ''s'' for single toxin products. Products containing spores will be differentiated from those without spores by adding ''+'' for spore-containing products and ''−'' for those that do not contain spores. For example, *Bacillus thuringiensis* ssp. *kurstaki* products containing multiple toxins and spores may be designated as 11Dm+, while the same product without spores and expressing only one toxin would be designated as Group 11Ds−.

The organophosphates and carbamates as inhibitors of acetylcholinesterase (IRAC MoA Group 1A and 1B) still form the largest group of insecticides, followed by the pyrethroids, known to act on voltage-gated sodium channels. These classes are briefly covered below in terms of their mode of action and biological value.

24.6.2 Organophosphates and Carbamates

Organophosphates (OPs) were first introduced to the agrochemical market in 1944 and are economically still the most successful and diverse chemical class of insecticides ever invented [4]. Over 100 different active ingredients belonging to this class are known, with the best selling OP of all being chlorpyrifos.

All OPs act as neuroactive compounds, irreversibly binding to AChE, thus preventing the hydrolysis of the neurotransmitter acetylcholine in the central nervous system. They lead to prolonged periods of nerve excitation, resulting in paralysis and subsequently death of the treated insects [5, 6]. OPs are used to control almost all pest species from a wide variety of insect orders, including Lepidoptera, Coleoptera, Diptera, Hemiptera (including aphids) and many more [7]. Additionally, they control phytopathogenic nematodes and phytophagous mites. This advantage of fast action and broad spectrum of OPs made them a well fitting weapon for the farmers against insects. In contrast, these properties are leading to the two major disadvantages of most OPs: Their activity also against predatory and beneficial insects and their acute toxicity to vertebrates, including humans. Some of them have LD_{50} values (acute p.o. rat) of less than 5 mg kg^{-1} (e.g., disulfoton), but most show acute toxicities between 5 and 50 mg kg^{-1} (e.g., methamidophos). A few examples, such as acephate ($LD_{50} > 600$ mg kg^{-1} acute rat p.o.), exhibit lower acute toxicity values. The chemical structure makes the OP compounds easily degradable via metabolism and hydrolysis so these properties improve considerably their ecological and toxicological profiles.

Another class of AChE inhibitors of economic importance but usually less toxic to non-target organisms are the carbamates, which were introduced to the insecticide market in the late 1950s [6]. Their physicochemical properties often lead to systemic compounds such as that of the most important aphicides from this group: Pirimicarb launched in the early 1970s. Pirimicarb exhibits high activity against aphids, especially in cereals. Another widely used carbamate, aldicarb, is applied as a soil insecticide and nematicide. Its highly diluted granular formulation leads to low acute toxicity of the product for the farmer. These examples show that improvements in the ready to use products through suitable formulations can overcome the disadvantages of acute toxicity against mammals.

24.6.3 Pyrethroids

One of the most important chemical classes of insecticides are the pyrethroids, which act as ligands of voltage-gated sodium channels in nerve axons [8, 9]. In

the mid-19th century an insecticidal powder derived from dried flower heads of the genus *Pyrethrum* (*Chrysanthemum*) was introduced from Africa to central Europe. The insecticidal components were identified as pyrethrins. Owing to several asymmetric centers these compounds have many enantiomeric forms, and only a few of them are insecticidally active. Natural pyrethrins are unstable, sensitive to photodegradation and relatively expensive. These natural pyrethrins were used as templates to generate synthetic analogues, the so-called synthetic pyrethroids [10–13]. Modern synthetic pyrethroids are well-optimized compounds with respect to potency, residual activity and photostability. Considering the symptomology of poisoning induced by these contact insecticides, pyrethroids can be separated into two classes [14]. Type I pyrethroids (e.g., permethrin) cause hyperactivity and incoordination, whereas Type II pyrethroids containing an alpha-cyano substituent induce nerve depolarization and subsequently paralysis of the insect. Because of their different intrinsic activity and/or different content of active isomers the recommended application rates vary widely between 5 and 200 g a.i. ha^{-1}. The ready to use pyrethroids have low toxicity due to high dilution in formulations, having LD_{50}s against insects that render them 1000-fold more active than against rats and other vertebrates. These properties allow also the use as pharmaceuticals for pets (e.g., against parasites on dogs, rabbits and guinea pigs). Pyrethroids are highly active against lepidopteran pest species, but their speed of action, which leads to rapid knock-down, renders them useful in many cropping systems against numerous pests of different insect orders, e.g., lepidopteran, coleopteran and many sucking pests, including aphids.

24.7 Effective IRM Strategies and Approved Principles

The objective of successful Insecticide Resistance Management is to prevent or delay the evolution of resistance to insecticides, or to help regain susceptibility in insect pest populations in which resistance has already appeared. Effective IRM is thus an important element in maintaining the efficacy of valuable insecticides. It is important to recognize that it is usually easier to proactively prevent resistance occurring than to reactively regain susceptibility. Nevertheless, the IRAC MoA Classification will always provide valuable guidance to the design of effective IRM strategies.

Experience has shown that all effective management strategies seek to minimize the selection for resistance from any one type of insecticide or acaricide. In practice, alternations, sequences, rotations or even mixtures of compounds from different MoA groups provide a sustainable and effective approach to IRM. This ensures that selection from compounds in any one MoA group is minimized. The IRAC classification in this chapter is provided as an aid to insecticide selection for these types of IRM strategies.

Applications are often arranged into MoA spray windows or blocks that are defined by the stage of crop development and the biology of the pest(s) of concern.

Local expert advice should always be followed with regard to spray windows and timings. Several sprays of a compound may be possible within each spray window but it is generally essential to ensure that successive generations of the pest are not treated with compounds from the same MoA group.

Table 24.2 lists the principles endorsed by IRAC as basic tools for successful resistance management.

To assist users in the selection of insecticides for use in IRM strategies employing sequences, rotations or alternations of MoA groups, IRAC is encouraging producers to clearly indicate the IRAC MoA group number and description on the product label, and to accompany this with appropriate advice of the type indicated below. Thus, in addition to the detailed product information, handling, and safety information required by local regulations, a typical title label should clearly indicate the IRAC MoA Group number, description and brief advice in IRM.

24.8 Future Market Trends

Figure 24.1 shows the major insecticidal classes and their market share in 2003. The global insecticide market is forecast to decline in value by 1.3% per annum until 2007. This represents a fall in the overall agrochemical market share from 27.5% in 2002 to 25.7% in 2007 [15].

This forecast is based on several factors, with the expansion in the deployment of insect-resistant crops being one of the most important. The incorporation of Bt genes into plants to express intrinsic insect resistance has already had an impact on insecticide sales, particularly in the cotton and maize sectors. Whilst this impact has been modest compared with the effect of herbicide-tolerant crops on herbicide sales, we expect to see insect-resistant crops having a greater negative influence on insecticide sales as the technology improves. Currently, Bt crops control only a limited pest range, primarily the cotton bollworm and corn borer. However, this is slowly being expanded to cover other pests through stacking technology and the discovery of additional toxins, e.g., Cry1F in Herculex maize and the forthcoming Vip3A protein. With the commercial launch of corn rootworm-resistant maize, another important insecticide market is now also under pressure.

Other key factors include increasing regulatory pressure and generic competition. Regulatory restrictions in Western Europe and North America are affecting many old but still commercially important insecticides. To a certain extent, substitution with alternative products will minimize this impact, although some negative effect on sales is inevitable. Generic manufacture is affecting sales of several chemistry groups. This is particularly true in Far East markets such as China [15].

In real terms based on €s, sales of pyrethroids are projected to increase slightly up to 2014, whereas neonicotinoid sales will roughly double between 2004 and 2014 due to the introduction of new molecules. As mentioned above, due to

Table 24.2 Recommendations for successful resistance management.

1	Consult a local agricultural advisor or extension services in the area for up-to-date recommendations and advice on IPM and IRM programs
2	Consider options for minimizing insecticide use by selecting early-maturing or pest-tolerant varieties of crop plants
3	Include effective cultural and biological control practices that work in harmony with effective IRM programs. Adopt all non-chemical techniques known to control or suppress pest populations, including biological sprays such as Bt's, resistant varieties, within-field refuges (untreated areas) and crop rotation
4	Where possible select insecticides and other pest management tools that preserve beneficial insects
5	Use products at their full, recommended doses. Reduced (sub-lethal) doses quickly select populations with average levels of tolerance, whilst doses that are too high may impose excessive selection pressures
6	Appropriate, well-maintained equipment should be used to apply insecticides. Recommended water volumes, spray pressures and optimal temperatures should be used to obtain optimal coverage
7	Where larval stages are being controlled, target younger larval instars where possible because these are usually much more susceptible and therefore much more effectively controlled by insecticides than older ones
8	Use appropriate local economic thresholds and spray intervals
9	Follow label recommendations or local expert advice for use of alternations or sequences of different classes of insecticides with differing modes of action as part of an IRM strategy
10	Where there are multiple applications per year or growing season, alternate products of different MoA classes
11	In the event of a control failure, do not reapply the same insecticide but change the class of insecticides to one having a different mode of action and to which there is no [locally] known cross-resistance
12	Mixtures may offer a short-term solution to resistance problems, but it is essential to ensure that each component of a mixture belongs to a different insecticide mode of action class, and that each component is used at its full rate
13	Consideration should be given to monitor the incidence of resistance in the most commercially important situations and gauge levels of control obtained
14	Withholding use of a product to which resistance has developed until susceptibility returns may be a valid tactic if sufficient alternative chemical classes remain to provide effective control

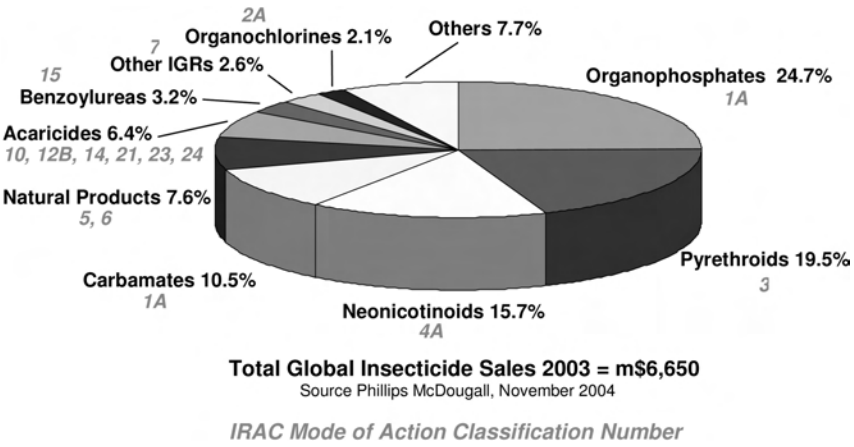

Fig. 24.1. Major chemical classes of insecticides and their market share.

increasing regulatory pressures, sales of organophosphates, carbamates and organochlorines will decline rapidly during this period.

The increasing demand for high quality vegetables will positively affect insecticide use, whereas in cotton and maize, and eventually also in rice, negative effects due to Bt-technology are to be expected. Long-term use of Bt crops will create opportunities for emerging sucking pests like aphids, whiteflies, bugs, spider mites and Lepidoptera that are currently not controlled by this technology.

24.9 Conclusions

It is becoming increasingly difficult and costly to discover new insecticidal active ingredients with novel modes of action that not only circumvent existing problems of insecticide resistance but that also pass the increasingly stringent hurdles being put in place by regulatory bodies. IRAC therefore believes that it is absolutely vital to ensure the sustained efficacy of the broad range of modern, safe and effective insecticides that the agrochemical industry currently produces. Central to this approach is the concept that susceptibility is a highly valued commodity and as such it should not be squandered indiscriminately through the misuse or over-use of insecticides. IRAC believes that effective insecticide resistance management is therefore not an option; it is clearly essential and it is one of the most challenging issues in modern applied entomology. In view of its responsibilities to the agrochemical industry and its customers, and in the interests of protecting the industry's products, IRAC is undertaking a broad range of activities to help make effective IRM possible.

Acknowledgments

The information for future market trends given by Cropnosis is acknowledged. Phillips McDougall (November 2004) is gratefully acknowledged as the source for data in Fig. 24.1.

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25
Insect Molting and Metamorphosis

25.1
Bisacylhydrazines: Novel Chemistry for Insect Control

Tarlochan Singh Dhadialla and Ronald Ross, Jr.

25.1.1
Introduction

The discovery of insecticidal bisacylhydrazine (BAH) compounds reinforced the original concept of Carrol Williams [1] for discovery and development of insect hormone mimics as a third generation of novel and reduced risk insecticides. Although this concept was proposed several decades ago, it was not until the mid-1970s that the first insecticidal compounds that mimicked insect juvenile hormone (JH) were discovered (reviewed in Refs. [2, 3]), followed by the discovery of BAH non-steroidal agonists of insect molting hormone 20-hydroxyecdysone (20E; Fig. 25.1.1; **1**) in the late 1980s (reviewed in Refs [4–6]). Initial attempts to synthesize insecticides with 20E activity had failed because compounds based on the cholesterol backbone and structural similarities to ecdysteroids are chemically and metabolically unstable [7, 8]. It was not until the late 1980s that the first non-steroidal ecdysone agonist was identified at Rohm and Haas Company by Hsu and colleagues [9] and the first prototype, RH-5849 (Fig. 25.1.1; **3**), characterized by its insecticidal spectrum of activity and ability to compete with ecdysteroids for binding to ecdysone receptor in insect cell preparations [10, 11]. Since then four members of the BAH chemistry, three discovered at Rohm and Hass Company and one at Nippon Kayaku/Sankyo Companies, have been commercially developed as insecticides (Table 25.1.1 below).

This chapter reviews the structures and biology (physiological, biochemical and molecular basis of mode of action, insect activity spectrum, and eco-toxicological safety) of the commercialized BAH non-steroidal ecdysone agonist insecticides (refer to former reviews on this topic [4–6, 12]).

25.1.1.1 Physiological and Molecular Basis of Insect Molting Hormone Action

Arthropods achieve growth and development by molting several times as immature nymphal or larval instars. The molting requirement is necessitated due to

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

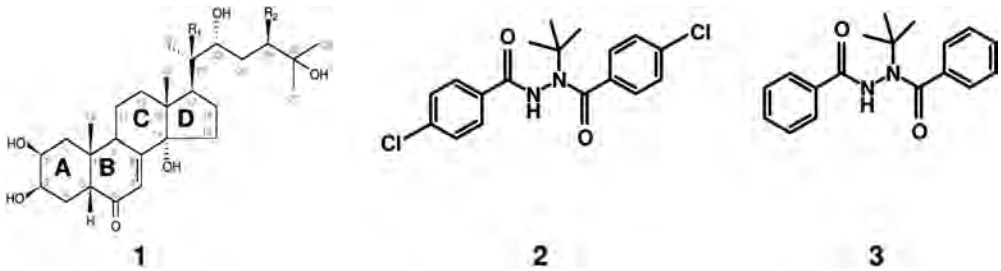


Fig. 25.1.1. Chemical structures of 20-hydroxyecdysone (20E; **1** $R_1 = OH$), the first bisacylhydrazine found to have an ecdysone agonist effect in insect assays (**2**), and the first bisacylhydrazine (RH-5849; **3**) well characterized for its ecdysone effects at cellular and whole insect level, as well as binding to *Drosophila melanogaster* cell extracts containing ecdysteroid receptor complexes. Numbers on the 20E structure represent the carbon-numbering system.

the absence of an endoskeleton. The molting process is accomplished through a sequence of steps, which include cessation of feeding, separation of the old cuticle from the underlying epidermal cells (apolysis), synthesis of a new cuticle, absorption of the old cuticle by secreted chitinolytic enzymes, and a hormone-dependent eclosion behavior that allows the molting insect stage to emerge from the old cuticle. The presence of the new cuticle before it is sclerotized allows the molted life-stage to expand and resume growth by continuing to feed.

The growth and development in insects is regulated by two primary hormones, the steroidal insect molting hormone (20-hydroxyecdysone, 20E; Fig. 25.1.1; **1**) and the sesquiterpene, Juvenile Hormone (JH), of which there are four main types (reviewed in Refs. [13, 16]). Initiation of the molting process is characterized by rising 20E titers in the insect larval hemolymph, and the cessation of feeding by the larva. As the 20E titers continue to rise, the old cuticle separates from the underlying epidermal cells (apolysis), allowing inactive chitinolytic enzymes to fill the ecdysial space and signaling the epidermal cells to secrete proteins that would form the new cuticle. As the 20E titers decline, the chitinolytic enzymes are activated to digest away the old endocuticle and the secreted proteins for the new cuticle layered systematically. By the time the 20E titers have declined to a basal level, only remnants of the old exocuticle remain, the new cuticle is fully formed, and the molting larva is ready to ecdyse from its old cuticle into a new one. At this time a neuropeptide, eclosion hormone, is released to cause the secretion of the ecdysis trigger hormone, which enables the ecdysis behavior and allows the larva to depart from its old cuticle shell. It is important that the 20E titers have declined to a basal level, otherwise, the eclosion hormone is not released, and the ecdysis or eclosion behavior will not occur [14, 17, 18]. Following the completion of larval ecdysis, the newly molted larva resumes feeding and deposition of the endocuticle continues during the intermolt period.

The manifestation of 20E effects during molting or other developmental stages (e.g., reproduction) are brought about by the interaction of 20E with the ecdysone

receptor complex. The ecdysone receptor complex is a heterodimer of the ecdysone receptor protein (EcR) and the ultraspiracle protein (USP), homolog of the vertebrate retinoid X receptor protein (RXR) (reviewed in Ref. [15]). Both EcR and USP are members of the steroid receptor super family of ligand-dependent transcription factors. Members of this family are characteristic in having DNA- and ligand-binding domains (DBD and LBD, respectively) in between transactivation domains at the N- and C-termini. Binding of ecdysteroids to the ecdysone receptor takes place only when both EcR and USP exist as heterodimers. However, recent reports suggest low affinity binding of tritiated ponasterone A, a phytoecdysteroid, to EcR proteins from rice stemborer, *Chilo suppressalis* [19] and Colorado potato beetle, *Leptinotarsa decemlineata* [20]. cDNA's encoding EcRs and USPs (or RXR like USPs) from several arthropods have been cloned and the proteins expressed for ligand and/or DNA binding (reviewed in Refs. [5, 15]), or crystal structure studies [21, 22].

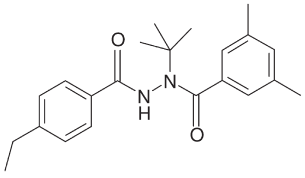
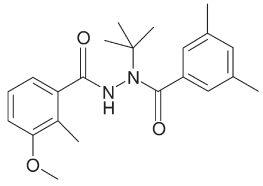
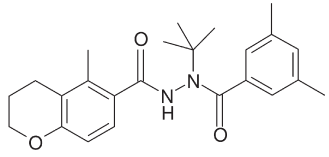
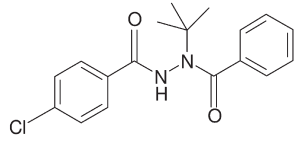
25.1.2
Discovery and Structures of Commercialized Bisacylhydrazine Insecticides

Several years after the first insecticidal bisacylhydrazine (Fig. 25.1.1; **2**) was serendipitously discovered at the Rohm and Haas Company [9] the first prototype of a bona fide non-steroidal ecdysone agonist bisacylhydrazine, RH-5849 (Fig. 25.1.1; **3**), was discovered [23]. The characterization of its spectrum of activity and binding to ecdysone receptor containing preparation from *Drosophila* Kc cells [10, 11] led to an intense chemical synthesis program at Rohm and Haas Company and the use of this compound in both *in vitro* and *in vivo* studies to further the understanding of developmental and reproductive physiology of susceptible insects. Further work on the structure–activity of RH-5849, which had broad spectrum activity against several lepidopteran, coleopteran, and dipteran insects, led to the discovery and commercialization of three bisacylhydrazine compounds as insect selective insecticides (Table 25.1.1); tebufenozide (RH-5992), methoxyfenozide (RH-2485) and halofenozide (RH-0345). Halofenozide, which is predominantly active on coleopteran and lepidopteran larvae, has been commercialized for the turf market in the USA. Both tebufenozide and methoxyfenozide are predominantly active on lepidopteran larval pests of vegetable crops, fruits, nuts and vines, corn and cotton. Methoxyfenozide is more potent and has a broader spectrum of activity for lepidopteran larval pests than tebufenozide. Finally, a fourth bisacylhydrazine, chromafenozide (Table 25.1.1) was discovered and jointly commercialized by Nippon Kayaku Company and Sankyo Company for the control of lepidopteran larval pests of vegetables, fruits, vines, tea, rice and ornamentals in Japan [24, 25].

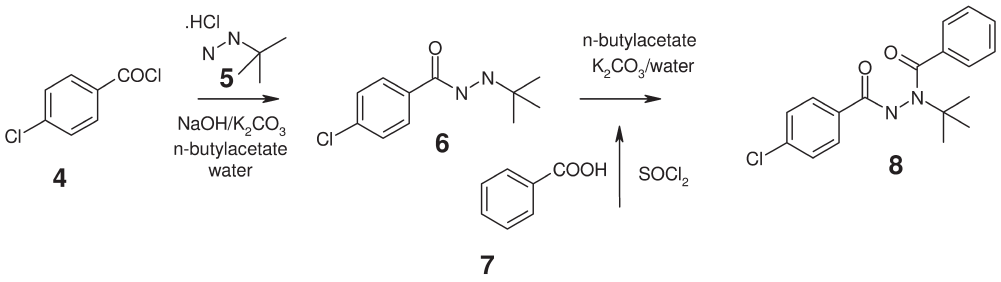
25.1.3
Synthesis of Commercial Bisacylhydrazines

Halofenozide **8** is prepared via a regioselective acylation of *t*-butyl hydrazine hydrochloride (**5**) (Scheme 25.1.1). 4-Chloro-benzoyl chloride (**4**) in butyl acetate is added to an aqueous solution of *t*-butyl hydrazine and sodium hy-

Table 25.1.1 Commercialized bisacylhydrazine ecdysone agonist insecticides: structures, common and coded names and pest spectrum.

				
Common name	Tebufenozide	Methoxyfenozide	Chromafenozide	Halofenozide
Coded AS	RH-5992	RH-2485	ANS-118; CM-001	RH-0345
Registered names	MIMIC™ CONFIRM™ ROMDAN™	INTREPID™ RUNNER™ PRODIGY™ FALCON™	MATRIC® KILLAT®	MACH2™
Melting point (°C)	191	204–205	186.4	153–155
Log *P*	4.25	3.7	2.7	3.42
Pest spectrum	Lepidoptera	Lepidoptera	Lepidoptera	Coleoptera Lepidoptera
Industry	[a]	[a]	[b]	[a]

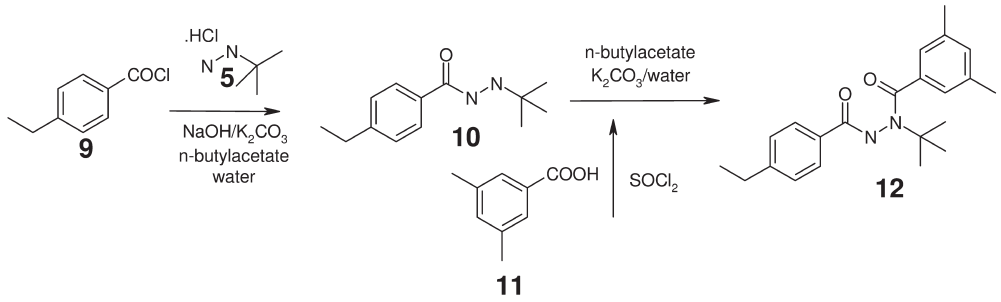
[a] Trademarks of Dow AgroSciences LLC.
[b] Nipon Kayaku Company, Saitame, Japan and Sankyo Company, Ibaraki, Japan.



Scheme 25.1.1. Synthesis of halofenozide.

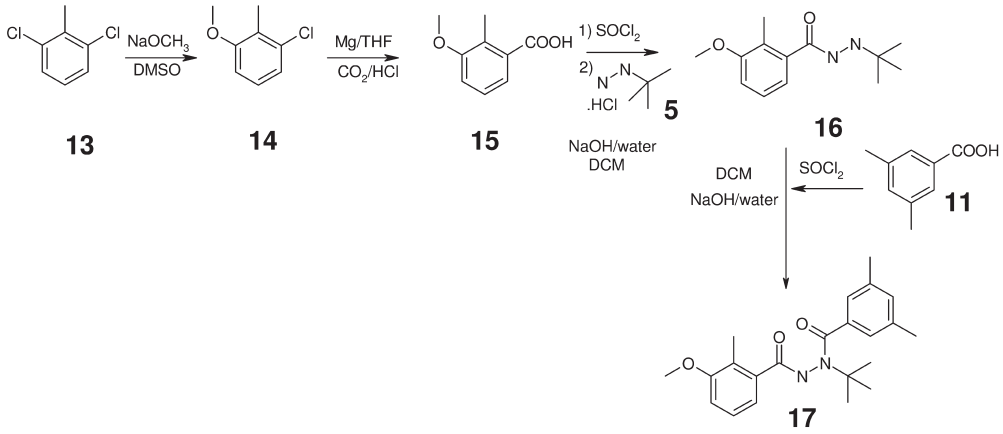
droxide and potassium carbonate. The Schotten–Baumann conditions afford the 4-chlorobenzoic acid *N′-t*-butyl-hydrazide (**6**) in high purity and regioselectivity. This material is additionally acylated with benzoyl chloride (prepared from benzoic acid **7**) to yield halofenozide in excellent yield and purity [26].

Tebufenozide (**12**) is prepared by the same process, (Scheme 25.1.2), acylating first with 4-ethylbenzoyl chloride (**9**) to produce 4-ethylbenzoic acid *N′-t*-butyl-hydrazide (**10**). A second acylation with 3,5-dimethylbenzoyl chloride produced from the corresponding benzoic acid **11**, affords tebufenozide [26].



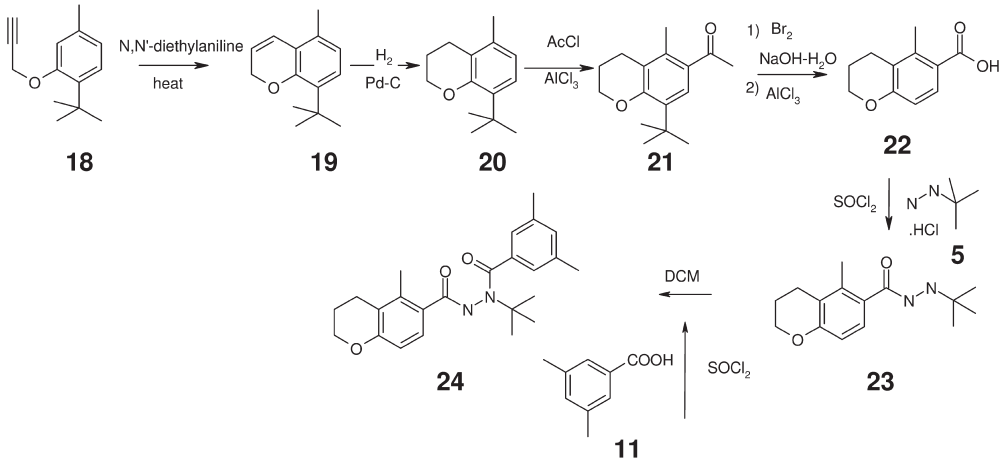
Scheme 25.1.2. Synthesis of tebufenozide.

Methoxyfenozide (**17**) is produced as shown in Scheme 25.1.3. The intermediate 3-methoxy-2-methylbenzoic acid (**15**) is prepared in two steps from 2,6-dichlorotoluene (**13**). Nucleophilic substitution of **13** with sodium methoxide in DMSO at 140–160 °C yields 1-chloro-3-methoxy-2-methylbenzene (**14**) in high yields and purity. This compound is converted into **15** by Grignard formation and subsequent quenching with carbon dioxide [27]. Preparation of the corresponding benzoyl chloride, followed by regiospecific Schotten–Baumann acylation of *t*-butylhydrazine hydrochloride (**5**) gives 3-methoxy-2-methylbenzoic acid *N′-t*-butyl-hydrazide (**16**). Finally, **16** is again acylated with 3,5-dimethylbenzoyl chloride in methylene chloride with aqueous sodium hydroxide to afford methoxyfenozide [28].



Scheme 25.1.3. Synthesis of methoxyfenozide.

Chromafenozide (**24**) is the most synthetically complex compound in the BAH class of chemistry. Scheme 25.1.4 outlines the published procedure for its production [29]. 2-*t*-Butyl-5-methylphenol is O-alkylated with propargyl bromide to give 1-*t*-butyl-4-methyl-2-prop-2-ynyloxybenzene (**18**). This material can be efficiently cyclized to the 8-*t*-butyl-5-methyl-2*H*-chromene (**19**) in refluxing *N*,*N*-diethylaniline. Catalytic hydrogenation with 5% palladium on carbon affords 8-*t*-butyl-5-methylchroman (**20**). Low temperature (0 °C) Friedel–Crafts acylation of **20** with acetyl chloride and anhydrous aluminum chloride gives the 1-(8-*t*-butyl-5-methyl-chroman-6-yl)-ethanone (**21**). Bromination of **21** in 1,4-dioxane followed by hydrolysis and subsequent de-*t*-butylation with aluminum chloride gives 5-methylchroman-6-carboxylic acid (**22**). This compound is converted into the acid chloride by typical methods, and then used to mono-acylate *t*-butyl hydrazine



Scheme 25.1.4. Synthesis of chromafenozide.

hydrochloride (**5**). The resulting 5-methylchroman-6-carboxylic acid *N′-t*-butylhydrazide (**23**) is again acylated with the acid chloride of 3,5-dimethylbenzoic acid to afford chromafenozide (**24**).

25.1.4 Structure–Activity Relation (SAR) of Ecdysteroids and Bisacylhydrazines

The SAR of the bisacylhydrazines, both during and after the discovery of the commercial compounds, has been extensively studied. This was partly driven by the novelty of the chemistry, mode of action (ecdysone agonists via interaction with the ecdysone receptor), and the availability of suitable assays (tissue, cell and target site based). Numerous papers have been published on this subject and the reader is referred to an excellent review by Dinan and Hormann [6] as a starting point. This section highlights and summarizes the collective findings by various researchers.

In considering the SAR of bisacylhydrazines for the discovery of new and novel ecdysone agonists, it is essential to understand the SAR of ecdysteroids. This helps to define the three-dimensional (3D) space of ecdysteroids in the EcR LBD and allows for overlaps and comparison with BAH or other ecdysone agonist chemotypes (described below).

25.1.4.1 Structure–Activity Relation (SAR) of Ecdysteroids

Earlier studies on the SAR of ecdysteroids based on several simple different insect bioassay results [30] were much later substantiated in most part using very comprehensive SAR based on sets of ecdysteroids and their quantitative effects in cell- and tissue-based ecdysone responsive assays, and use of comparative molecular field analysis (CoMFA) and four-dimensional qualitative SAR (4D-QSAR) approaches to analyze the data ([31–33]; also reviewed in Ref. [6]). In general, the results of the two approaches are similar in that all the hydroxyl groups, except for 14-OH and 25-OH, and the steroid side chain (Fig. 25.1.1; **1**) play an important role in hydrogen bonding and activity of ecdysteroids, respectively. The general picture that emerges from the CoMFA and 4D-QSAR analysis is that the ecdysteroid side chain lies in a sterically restricted hydrophobic cavity in the ligand binding pocket of EcR, in which position 20-OH and 22-OH provide hydrogen bonding functions. Much of these conclusions have been supported by homology modeling of insect EcR LBD based on low levels (24–27%) of sequence homology to published sequences and crystal structures of vertebrate steroid receptor LBD coupled with ecdysteroid docking studies [34, 35]. The presence of 25-OH on 20E significantly diminishes activity of 20E as compared with that of ponasterone A, which lacks 25-OH.

Publication of the crystal structure of the *Heliothis virescence* EcR (HvEcR) LBD heterodimerized with LBD of *H. virescence* ultraspiracle protein (HvUSP) with and without ponasterone A (phytoecdysteroid) or a BAH ecdysone agonist provided a more realistic conformation of the HvEcR LBD and the ligands in the LBD [21]. The most surprising result was that ponasterone A co-crystallized in the HvEcR LBD in an orientation opposite to that predicted by the CoMFA, 4D-QSAR and

homology modeling studies. In this case the steroid nucleus was furthest away from helix 12, while the steroid side chain was closest to it. The crystal structure studies revealed that *Hv*EcR LBD amino acid residues R383, E309, T343/T346, Y408 and A398 interacted with 2-OH, 3-OH, 14-OH, 20-OH and the C-6 carbonyl, respectively, for hydrogen bonding (Fig. 25.1.2; Fig. 25.1.4 below). Interestingly, all these amino residues are conserved amongst EcR LBDs of insects from different orders (see sequence review in Ref. [15]), which is consistent with the observation that the insect EcRs are similarly responsive to binding of active or potent ecdysteroids to produce biological effects.

25.1.4.2 **Structure–Activity Relation (SAR) of Bisacylhydrazines**

Following the initial discovery of the first few analogs of BAHs and their characterization as ecdysone agonists in both *in vivo* insect assays as well as *in vitro* cell based and target site based assays [10, 11], this class of chemistry continued to enjoy extensive SAR analysis (reviewed in Refs. [6, 36–38]). From over 4000 analogs synthesized, three compounds (tebufenozide, methoxyfenozide and halofenozide) were commercialized by Rohm and Haas Company, and are currently owned by Dow AgroSciences LLC. Before the crystal structure of HvEcR ligand binding domain in the absence and presence of ponasterone A or a BAH (BY-106830) in the ligand binding pocket was elucidated, most SAR using CoMFA and 4D-QSAR approaches was done based on various BAHs and internally generated data sets (extensively reviewed in Refs. [6, 37, 38]). The investigators used target site ligand binding, cell based-, tissue- and whole insect assays to predict descriptors (substitutions) for BAH responsible for lepidopteran and coleopteran activity.

Figure 25.1.2 is a summary of the SAR of BAH proposed by Rohm and Haas Company investigators based on the activity of these compounds in southern armyworm larvae.

These SAR studies led to the discovery and commercialization of two Lepidoptera-specific (tebufenozide and methoxyfenozide) and one Coleoptera and Lepidoptera specific (halofenozide) BAHs. Subsequently, Nipon Kayaku and Sankyo Co. announced the discovery of another commercial insecticidal BAH, chromafenozide [25, 39, 40] and qualitative SAR limited to structures related to this compound [41–43] were published. Sawada et al. [41–43] demonstrated that heterocycles fused to the 3,4-positions of the A-ring were insecticidal to the common cutworm, *Spodoptera litura*. The main departure in structure of chromafenozide from tebufenozide is in the A-ring, where the most active compounds have oxygen and carbon-containing, five- or six-membered fused rings devoid of bulky or hydrogen bond-donating substitutions, and two methyl groups on the B-ring (as for tebufenozide and methoxyfenozide) (Table 25.1.1).

Dinan and Hormann [6] made the following observations for a BAH pharmacophore with toxicity related features common to species from sensitive insect orders (mainly Lepidoptera and Coleoptera):

1. Two hydrogen acceptor or polar negative atoms that are ~3.5–4.0 Å apart.

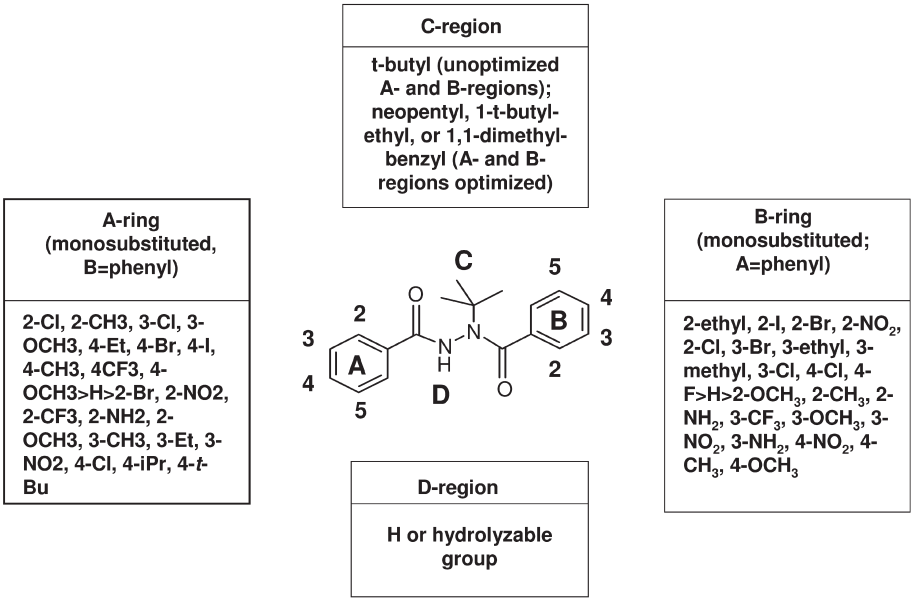


Fig. 25.1.2. Chemical structure of the first non-steroidal ecdysone agonist compound (RH-5849) with insecticidal activity. Different substitutions on this molecule led to the discovery of the four bisacylhydrazine commercial insecticides. The letters and the numbers refer to substitutions shown in the boxes around the structure.

2. A bulky, conformational-determining lipophilic group located asymmetrically between the two negative centers.
3. Moderately sized (about six carbons) groups on either side of the negative centers. Aryl groups are favored for both Lepidopteran and Coleopteran activity.
   a. Lepidopteran activity is enhanced with the following substitutions on the A-ring; 4-position with 1–2 carbon lipophilic groups or, alternatively, with a 2,3-or a 2,[3,4]-ring patterns. Substitutions on the B-ring are less specific, though substitutions at the 2-, 2,5-, 3,5-, or 3,4,5-positions can be favorable.
   b. Coleopteran activity, in contrast, is favored by one or two small group substitutions on the aryl rings, as exemplified by halofenozide, which has a 4-Cl substitution on the A-ring.
4. A hydrogen bond-donating group located near the alternate negative center.

25.1.5
Mode of Action of Bisacylhydrazine Insecticides

The discovery that RH-5849 acts as an ecdysone agonist in *Drosophila* Kc cells [10] and in whole insects [11] stimulated much research in insects and other arthropods at the cellular, tissue and whole insect levels (reviews [4, 5, 12]). Wing [10] demonstrated that in *Drosophila* Kc cells RH-5849 elicited cell aggregation and growth inhibition in a similar manner as active ecdysteroids. In the same study, competitive displacement of tritiated ponasterone A bound to Kc cell nuclear extracts containing ecdysone receptor complexes by excess amounts of RH-5849 indicated that RH-5849 manifests its ecdysone agonist effects via interaction with the same macromolecule (ecdysone receptor) as do 20E and ponasterone A. Subsequently, morphological cellular effects (cellular aggregation, clumping and inhibition of cell growth) of tebufenozide, methoxyfenozide and halofenozide similar to those induced by 20E were demonstrated for several cell lines derived from tissues or embryos of different insects [13, 44–49]. Once again, several investigators were able to demonstrate that RH-5849 and the commercial bisacylhydrazine insecticides bound to ecdysone receptors in imaginal wing discs, cellular extracts or *in vitro* expressed EcR and USP proteins from different orders of insects (reviewed in Refs. [5, 19, 20, 50]). Table 25.1.2 shows the relative binding affinities of ecdysteroids and bisacylhydrazines to ecdysone receptor complexes from different orders of insects. Clearly, while ponasterone A binds to ecdysone receptor

Table 25.1.2 Comparison of relative binding affinities of ecdysteroids, tebufenozide, methoxyfenozide and halofenozide to either cellular extracts or *in vitro* expressed EcR and USP proteins from representatives of different insect orders.

Insect (order)	K_d (nM)				
	20E	Pon A[a]	Tebu-fenozide	Methoxy-fenozide	Halo-fenozide
Drosophila melanogaster (Diptera)	145	0.9	336		
Aedes aegypti (Diptera)	28	2.8	30		
Chironomus tentans (Diptera)		0.35			
Spodoptera littoralis (Lepidoptera)	158		86	24.3	
Spodoptera frugiperda-Sf9 cells (Lepidoptera)	166	8.9	1.5	3.5	
Anthonomus grandis (Coleoptera)	247	6.1	>12 000		
Leptinotarsa decemlineata (Coleoptera)	425		1316		
Tenebrio molitor (Coleoptera)		6	>10 000	>10 000	>10 000
Locusta migratoria (Orthoptera)	1000	1.8	>10 000	>10 000	>10 000
Bemesia argentfoli		8	>10 000	>10 000	>10 000

[a] Pon A = ponasterone A.

complexes from different orders of insects with similar affinities (K_d = 0.9~9 nM range), tebufenozide binds only to lepidopteran EcR with affinity in the same range as for ponasterone A. The binding affinity of tebufenozide to receptors from dipteran and coleopteran receptors is 2–4 orders of magnitude lower than its binding to lepidopteran receptors. Moreover, binding of tebufenozide to ecdysone receptors from homopteran and orthopteran ecdysone receptors cannot be detected even at concentrations as high as 10 μM. The binding data correlate very well with the insect selective toxicity of tebufenozide and methoxyfenozide, which are predominantly active on lepidopteran insects. For at least three of the four BAH insecticides that are predominantly lepidopteran specific, the affinity of the BAH ecdysone agonists for the lepidopteran ecdysone receptor directly correlates with the toxicity manifested in that order of insects ([19, 50] and reviewed in Ref. [5]). However, a similar correlation is not revealed for halofenozide, which has a very weak binding affinity to ecdysone receptors from both Coleoptera and Lepidoptera but is toxic to select members of both orders of insects [4, 5, 20].

Although predominantly selective for lepidopteran larval pests, both tebufenozide and methoxyfenozide do show some toxicity to a few dipterans like the midge, *Chironomus tentans* [46, 51], and a few mosquito species [52, 53]. Interestingly, tebufenozide shows very disparate binding affinities to ecdysone receptors from three different dipteran species, *Drosophila melanogaster* (Dm), *Aedes aegypti* (Aa) and *C. tentans* (Ct). It binds to bacterially produced GST-fusions of DmEcR/DmUSP, AaEcR/AaUSP and CtEcR/CtUSP with determined K_ds of ~300, 30 and 3 nM, respectively, which are directly proportional to susceptibilities in that order (*C. tentans* > *A. aegypti* > *D. melanogaster*; reviews in Refs. [4, 5]). The determined K_d for tebufenozide binding to CtEcR/CtUSP equals that of tebufenozide binding to EcR/USP heterodimer from the spruce budworm, *Choristoneura fumiferana*. In both cases, tebufenozide exhibits biological potency. In discovering of new compounds based on target site assays, it is important to keep in mind that mere binding of a compound to its target site does not automatically translate into an *in vivo* biological or toxic function. For example, even though tebufenozide binds to DmEcR/DmUSP with sub-micro molar affinity (K_d), which is about two times the K_d for 20E for the same receptor (Table 25.1.2), tebufenozide is not toxic to *Drosophila* larvae. Dhadialla and colleagues ([5] and unpublished results) further investigated the significance of binding of tebufenozide to ecdysone receptors from *D. melanogaster*, *A. aegypti* and the spruce budworm, *Choristoneura fumiferana* (Cf), using limited proteolysis of different radiolabeled EcRs in EcR/USP heterodimers following equilibrium binding with either muristerone A (a potent ecdysteroid) or tebufenozide. The results demonstrated that binding of muristerone A and tebufenozide to CfEcR/CfUSP and AeEcR/AeUSP induced similar conformational changes in EcR (indicated by protease resistant EcR peptide fragments of same molecular size). In contrast, binding of tebufenozide to DmEcR/DmUSP did not afford protease resistance (indicative of a lack of ligand induced conformation), but muristerone A did. These results re-enforce the concept that mere ligand–receptor interaction is not enough for biological activity, but that such an interaction has to result in a conformational change in the recep-

tor that leads to subsequent steps important for the biological manifestation of the ligand.

Elucidation of the crystal structure of HvEcR/HvUSP heterodimeric LBDs in the absence or presence of steroidal or non-steroidal ligands [21] conclusively demonstrated the binding of BAH in the EcR LBD. These results also made it possible to verify the pharmacophore structural requirements for both an ecdysteroid (ponasterone A) and a non-steroidal ecdysone agonist BAH (BY106830) interaction with residues specifically in a lepidopteran EcR LBD (Fig. 25.1.3A and 3B,

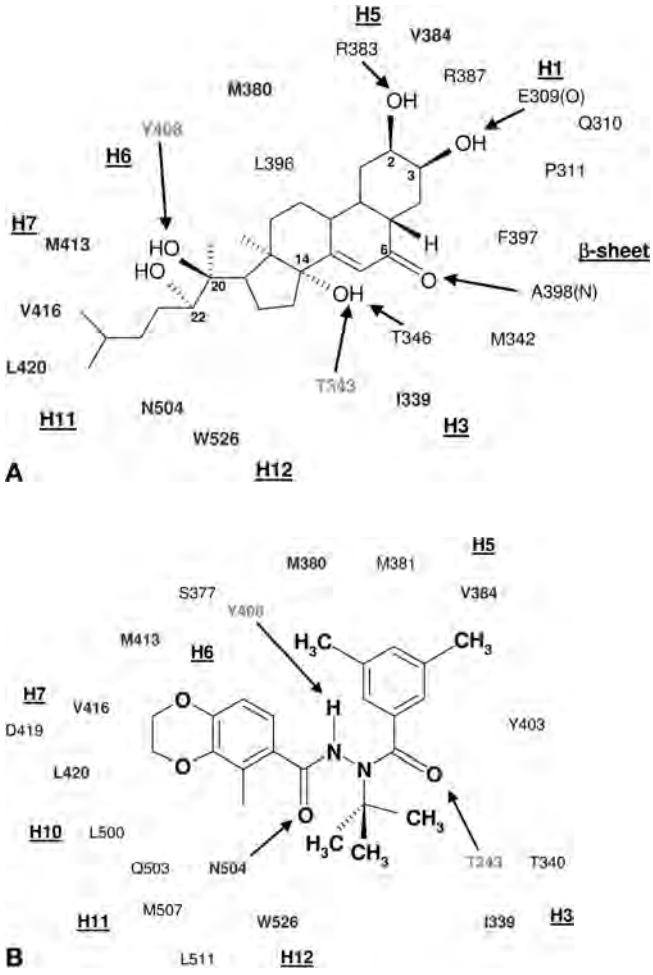


Fig. 25.1.3. Hydrogen bond interactions of ponasterone A (A) and BAH, BY106830 (B) with amino acid residues in the ligand binding cavity of *H. virescence* EcR are shown schematically. H1 . . . H12 represent the relative locations of HvEcR LBD helices. The hydrogen bonds formed by the two ligands and the amino acid residues are shown by arrows. (Adapted from Ref. [21].)

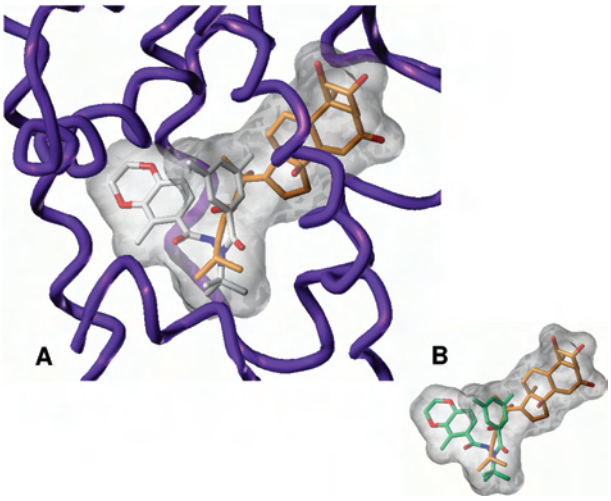


Fig. 25.1.4. Relative positions and conformations of ponasterone A (yellow) and BY106830 (green) in the ligand binding cavity of HvEcR (A). The purple ribbons represent the helices surrounding the binding cavity. The steroidal and non-steroidal molecules occupy largely different spaces in the binding cavity (more clearly shown in B) with overlapping space for the side chain of ponasterone A and the *t*-butyl group of BY106830. (Adapted from 1R1K.pdb and 1R20.pdb files deposited by Ref. [21].)

respectively). This also allowed verification of the CoMFA, 4D-QSAR and *in silico* docking of BAH or ecdysteroid molecules in homology models of EcR LBD that were developed from the crystal structures of vertebrate steroid receptor LBDs that have <30% sequence homology to insect EcR LBDs. It became apparent from the crystal structure studies of Billas et al. [21] that the two ligands occupy distinctly different but overlapping cavities in the EcR ligand binding pocket (Fig. 25.1.4). While ponasterone A localizes in a long and slender conformation and a deeply buried cavity located distal to helix 12 in EcR LBD, the BAH assumes a much more globular conformation in a bulky V-shaped cavity with an open cleft between H7 and H10 proximal to H12 in EcR LBD. The two ligands overlap over the *t*-butyl group of BAH and the side-chain of ponasterone A. The crystal structure results of EcR/USP LBD heterodimer liganded with ponasterone A showed that the steroid binds with the fused A/B rings furthest away from helix 12 and the side chain closest to helix 12. However, earlier homology modeling and ligand docking studies assumed the reverse orientation of the steroid molecule in EcR ligand binding cavity [35, 54]. Additionally, while the pre-crystal structure studies did not predict an important role for the 14-OH on the steroid molecule, the crystal structure showed the interaction of 14-OH with not only one amino acid residue but two threonine residues, T343 and T346 (Fig. 25.1.3A). The strong interaction of hydroxyl groups, 2-OH and 3-OH with residues R383 and E309, respectively, of the H1–H2 loop, H5 and the β-sheet bring stability to the conformation of H2, and interaction of 20-OH with Y408 on H6 is important (as previously predicted) for boosting the *in vivo* activity of 20E by ~100-fold over that of ecdy-

sone (E), which lacks the 20-OH moiety. The three hydroxyl groups have long been known to be important for ecdysteroid activity. The C-6 carbonyl on ponasterone A interacts with A398.

The complexing of the BAH, BY106830, with the EcR LB cavity results in slight differences in the conformation of EcR LBD, which are distinct from those induced by the steroidal ponasterone A. BY106830, which lodges in a different but slightly overlapping (with the side chain of ponasterone A) space in the EcR ligand binding cavity, destabilizes the helical conformation of H2 and disrupts the interactions between the second and third *β*-sheet strands [21]. As indicated in Fig. 25.1.3(B) (derived from1R1K.pdb and 1R20.pdb files deposited [21]) hydrogen bond interactions of this BAH are made on the A-ring carbonyl with N504, B-ring carbonyl with T343, and NH group with Y408. The *t*-butyl group lies in a hydrophobic cavity formed by residues from H3, H11, the H6/H7 and the H11/H12 loops. Billas et al. [21] propose that the basis of lepidopteran specificity of BAH, specifically BY106830, lies in the V384 residue in H5, which is conserved in lepidopteran insect EcR LBDs, but is replaced by methionine in all other insects. However, this does not explain the activity of some of the lepidopteran specific BAHs like tebufenozide that bind strongly with EcRs from a few dipterans like the midge, *C. tentans*, and the mosquito, *A. aegypti* ([42, 49], Dhadialla, unpublished data). Moreover, halofenozide, which has only 4-Cl substitution on the A-ring, binds both lepidopteran and coleopteran EcRs, albeit not as tightly as tebufenozide and methoxyfenozide bind to lepidopteran EcRs [4, 5, 19, 50].

Although tebufenozide and methoxyfenozide show a very high affinity for ecdysone receptors from Lepidopteran and a few dipteran insects, 20E binds to ecdysteroid receptors from all insects to manifest its effects. Kumar et al. [35] have demonstrated that the binding of 20E to CfEcR LBD could be eliminated by mutation of a single residue. These investigators mutated A110 (where alanine is the 110^{th} residue if numbering of residues is started from first residue in helix one of LBD, which otherwise is A393 in the full-length CfEcR) to proline. Test of the binding and responsiveness of these single point A110P mutated CfEcR LBD in ligand binding and cell based transactivation assays, respectively, indicated that both ponasterone A and 20E were ineffective. However, while there was a 30% decrease in transactivation assays for methoxyfenozide, its ability to bind the mutated EcR LBD was not effected. Interestingly, Kumar et al. [35] predicted the amino acid residues for mutational analysis based on homology models derived from the crystal structures of vertebrate steroid receptors LBDs (<30% homology to insect EcR LBDs) and ligand docking, before the crystal structure of HvEcR LBD [21] became available. This was done, even though ecdysteroid was docked in an orientation opposite to that revealed by crystal structure data.

25.1.5.1 **Whole Organism Effects**

Commercialized bisacylhydrazine insecticides manifest their toxicity to susceptible insects almost exclusively via ingestion, and have very low contact toxicity only when used at ten-fold higher doses than required for oral toxicity. The effects of bisacylhydrazines have been studied in several susceptible insects belonging to

the Lepidoptera, Coleoptera and Diptera ([20, 25, 40, 54–62], and also reviewed in Refs. [4, 5]). Owing to their much greater metabolic stability in insects than the ecdysteroids and their mode of action, intoxication of susceptible insects with bisacylhydrazines creates a condition termed ''hyperecdysonism'', first coined by Williams [1]. As a result of this, the susceptible larvae stop feeding. This cessation of feeding can occur within 3–14 h after ingestion of the BAH [55, 63, 64]. Although the intoxicated larva does not actually die till 2–4 days later, cessation of its feeding prevents further feeding damage to the host plant. Several hours after inhibition of feeding, the intoxicated larva slips its head capsule as part of the molting process prematurely induced by BAH insecticides. Owing to the premature nature of the induced molt, the intoxicated larva is unable to complete the act of molting, which normally culminates in the larva eclosing from its old, digested cuticle into a new one. The intoxicated larva remains moribund and ultimately dies as a result of starvation, hemolymph loss due to hemorrhage or predation. BAH insecticides are most active in the larval stages of susceptible insects.

The effects of lethal doses of the commercial BAH insecticides have been studied at the ultrastructural, biochemical and molecular level in tissues of intoxicated larvae. Ultrastructural studies have been conducted in larvae of the beet armyworm, *Spodoptera exigua* [57], spruce budworm, *Choristoneura fumiferana* [64], and Colorado potato beetle, *Leptinotarsa decemlineata* [5]. These studies revealed that following ingestion of tebufenozide or halofenozide by lepidopteran or coleopteran larvae, respectively, synthesis of a new cuticle could be observed as early as 3-h post-ingestion of the insecticidal BAH. This is followed by apolysis of the new cuticle from the old one. While the old cuticle is digested the new cuticle is malformed, as indicated by the disordered lamellate layering of the epicuticule proteins. The epidermal cells secreting the new cuticle proteins become vacuolated. Comparison of the integument, consisting of epidermal cells, endo- and exo-cuticle, shows that the cuticle of the intoxicated larvae is dramatically thinner than that of control larvae [5].

Differences at the biochemical and molecular level between control and BAH insecticide-treated larvae further help to understand the basis of mode of action of BAH insecticides. During a normal molt the rise and decline of 20E modulates expression and repression of certain genes and the decline to basal levels of 20E results in the release of the eclosion hormone for the eclosion behavior to occur. Unlike during a normal molt, the rapid increase in the larval hemolymph of ingested tebufenozide results in the expression of 20E dependent genes. However, owing to its much greater metabolic stability and potency than 20E, its continued presence in the hemolymph and the target tissues does not allow for the regulation of genes normally dependent on the declining titers of 20E. Additionally, owing to the continued presence of tebufenozide (and the same would most likely apply to the other BAH insecticides) the eclosion hormone is not released, which leaves the intoxicated larvae mid way through the molt process, i.e., with malformed new cuticle, slipped head capsule and the inability to eclose from its old cuticle. Retnakarn et al. [65] reported that in intoxicated spruce budworm larvae, dopadecarboxylase, an enzyme important for sclerotization and tanning of new

cuticle, is not expressed. Its expression is normally suppressed in the presence of 20E, and in this case by tebufenozide which mimics 20E.

25.1.5.2 Basis for Selective Insect Toxicity of Bisacylhydrazine Insecticides

Of the four commercial BAH insecticides, tebufenozide, methoxyfenozide and chromafenozide are selectively toxic to lepidopteran larvae. Tebufenozide and methoxyfenozide have been shown to also have insecticidal activity to mosquito species [55, 54, 66] and the midge, *C. tentans* [53]. Although the mode of action of BAH insecticides is manifested via interaction with the ecdysone receptor, the reasons for their selective insecticidal activity were at first puzzling, since all insects have ecdysone receptors and almost all of them use the same ecdysteroid, 20E, as the molting hormone. As mentioned above, 20E manifests its action via interaction with the ecdysone receptor ligand binding domain. Three major causes (metabolism, pharmacokinetics and differences in the target sites) as the basis for the selective toxicity of BAH insecticides have been investigated. Using ^{14}C-labeled RH-5992 (tebufenozide) both Smagghe and Degheele [67] and Dhadialla and Thompson (unpublished results) demonstrated that there were no differences in the pharmacokinetics and metabolism of ingested RH-5992 in insect species that are susceptible (e.g., the armyworms, *S. exigua*, *S. exempta*) and non-susceptible (Colorado potato beetle, *L. decemlineata* and Mexican bean beetle, *Epilachna verivesta*) to this insecticidal BAH. Since no differences were found for metabolism of RH-5992 in susceptible and non-susceptible insects, attention was focused on the relative binding affinities of tebufenozide, methoxyfenozide and halofenozide to either ecdysone receptor in extracts from cell lines from different insect Orders [4, 5] or proteins expressed from cloned cDNA's encoding EcRs and USPs from insect species representing different Orders of insects. The results obtained with both tebufenozide and methoxyfenozide were revealing. While these compounds had an extremely high affinity for ecdysone receptor proteins from lepidopteran insects, the binding affinities for these two compounds dropped by 1–3 orders of magnitude for EcR/USP heterodimers from insects weakly or not at all susceptible to tebufenozide or methoxyfenozide (Table 25.1.2). The very high affinity for lepidopteran ecdysone receptors correlated directly with selective toxicity towards members of this insect order. In cases where tebufenozide is active against a non-lepidopteran insect, e.g., larvae of the midge, *Chironomus tentans*, the same correlation holds. In insects like mosquitoes, where tebufenozide does not have a very high activity, its affinity for the mosquito (*Aedes aegypti*) ecdysone receptor is also intermediate between that for a susceptible and a non-susceptible insect (Table 25.1.2).

Sundaram et al. [68] have investigated other possible reasons for the selective insect toxicity of tebufenozide. They observed that lepidopteran (*C. fumiferana*) and dipteran (*D. melanogaster*) cell lines responded equally to 20E or ponasterone A for induction of ecdysone inducible genes, Hormone Receptor 3 (HR3) from *C. fumiferana* or *D. melanogaster*, respectively. In contrast, the two cell lines responded differently to RH-5992. Other than the >100-fold higher binding affinity of RH-5992 to CfEcR compared with DmEcR, lepidopteran cells retained much higher levels of RH-5992 than did *D. melanogaster* cells. The results of this study

demonstrated that this differential retention of RH-5992 in the two cell lines was due to an active efflux mechanism in dipteran cells, which was temperature dependent and could be blocked with 10^{-5} M oubain (an inhibitor of Na^+, K^+-dependent ATPase). It would be interesting to determine if similar Na^+, K^+-dependent ATPases are also present in cells of other dipterans such as *C. tentans* and *A. aegypti*, both of which are significantly more susceptible to tebufenozide than *D. melanogaster*.

However, the data to date indicate that lepidopteran ecdysone receptor affinities for methoxyfenozide and chromafenozide are most likely the primary drivers for their selective toxicity for lepidopteran larvae.

While the very high affinities of tebufenozide, methoxyfenozide and chromafenozide for lepidopteran ecdysone receptors help explain the basis for their selective lepidopteran toxicity, the same does not apply to the fourth BAH insecticide, halofenozide, which is toxic to both lepidopteran and coleopteran larvae. Halofenozide has significantly reduced affinity for ecdysone receptors from the two insect orders. It seems that the relatively weak affinity of halofenozide to the ecdysone receptor of the target susceptible insect may be compensated by its increased metabolic stability in the same insect.

25.1.6
Spectrum of Activity of Commercial Bisacylhydrazine Insecticides

The reader is referred to more in-depth reviews and specific bibliography on the BAH insecticides by Dhadialla et al. [4, 5]. A brief and important description of the spectrum of activity of the four insecticides is given below.

25.1.6.1 Tebufenozide (MIMIC™; CONFIRM™; ROMDAN™; RH-5992), Methoxyfenozide (RUNNER™; INTREPID™; PRODIGY™; FALCON™; RH-2485), and Chromafenozide (MATRIC®; KILLAT®; ANS-118; CM-001)

All three insecticides are predominantly toxic to lepidopteran larvae. While tebufenozide is toxic to most of the lepidopteran species, it lacks substantive commercial activity on *Heliothis* and *Ostrinia* species due to its lower potency and systematic activity. Both methoxyfenozide and chromafenozide, in contrast, are active on major pests from the two genera. Chromafenozide is registered for lepidopteran pests on vegetables, fruits, vines, tea, rice, arboriculture, ornamentals, and other crops in Japan. Both tebufenozide and methoxyfenozide have been registered worldwide for lepidopteran pests on the same crops, and in addition to lepidopteran pests in forests, and fruit and nut trees as well. Typical use rates for CONFIRM™ (tebufenozide) and RUNNER™ (methoxyfenozide) insecticides are in the range 60–500 g-a.i. ha^{-1}. Methoxyfenozide is about twice as potent as tebufenozide. These insecticides manifest their toxic effects primarily by ingestion, and are only weakly active when applied topically. The feeding inhibition and lethal effects of these insecticides are manifested during the larval stages.

However, examples of sub-lethal reproductive or ovicidal effects of tebufenozide and methoxyfenozide have been reported for some lepidopteran adults or when their eggs are deposited on surfaces treated with tebufenozide [58, 70–73].

25.1.6.2 Halofenozide (MACH 2^{TM}; RH-0345)

Unlike the above three BAH insecticides, halofenozide is more soil systemic and is active not only against lepidopteran larvae, but also coleopteran larvae. It has been developed primarily for the control of beetle grub and lepidopteran larval pests of turf in lawns and on golf courses. It is highly efficacious against the soil dwelling larval stages of scarbaeid beetles such as the Japanese beetle, *Popillia japonica*, the oriental beetle, *Exomala orientalis*, and *Phyllophaga*, *Cyclocephala*, and *Hyperodes* spp, as well as various soil- or sod-dwelling caterpillars such as cutworms and webworms [74–76]. Halofenozide was not active on the Asiatic garden beetle, *Maladera castanaea* (Arrow) even at high doses. The recommended rates for control of lepidopteran larvae and beetle grubs for halofenozide (MACH 2^{TM}) are in the range of 2 lb-a.i. acre^{-1}.

25.1.7 Ecotoxicological and Mammalian Reduced Risk Profiles

Table 25.1.3 shows mammalian and ecotoxicological data for the four commercial bisacylhydrazine ecdysone agonist insecticides. Methoxyfenozide was only one of the four pesticide products to be awarded the ''Presidential Green Chemistry Award'' in 1998 by the US Government to recognize outstanding chemical processes and products that reduce negative impact on human health and the environment. Both tebufenozide and methoxyfenozide were registered by EPA under its Reduced Risk Pesticide Program. Both these pesticides, as seen in Table 25.1.3, have low acute and chronic mammalian toxicity, and safety to most beneficial arthropods. In fact, considering their mode of action (ecdysone agonists) their highly selective toxicity to lepidopteran larvae is amazing. When tested on 150 insect species from different insect Orders (Lepidoptera, Hymenoptera, Coleoptera, Hemiptera, Diptera, Homoptera, and Neuroptera), both tebufenozide and methoxyfenozide were devoid of any toxicity to members of these insect orders, except for toxicity on a few of Dipteran species like the midge, *C. tentans*, and mosquito species (Glenn Carlson, unpublished data). In separate studies, these insecticidal compounds were found to have very little or no toxicity in several non-lepidopteran (Coleoptera, Homoptera, mites, and nematodes) orders [49, 77, 78]. Both tebufenozide and methoxyfenozide are also non-toxic to bees.

25.1.8 Resistance Mechanisms and Resistance Potential

The history of insecticides has shown that, depending upon how an insecticide is used, target insect species will inevitably develop some resistance to a given insecticide at one time/place or another.

Table 25.1.3 Mammalian and ecotoxicological reduced risk profiles of registered bisacylhydrazine insecticides.

	Tebufenozide	Methoxyfenozide	Chromofenozide	Halofenozide
Mammalian				
Acute oral LD_{50} (rat, mouse) (mg kg^{-1})	>5000	>5000	>5000	2850
Acute dermal LD_{50} (mg kg^{-1})	>5000	>2000	>2000	>2000
Eye irritation (rabbit)	Non-irritating	Non-irritating	Slightly irritating	Moderately irritating, positive for contact
Dermal sensitization (guinea pig)	Non-sensitizer	Negative	Mildly sensitizing	Allergy
Ames assay	Negative	Negative	Negative	Negative
Acute inhalation (mg L^{-1})	>4.3	>4.3		>2.7
Reproduction (rat)	No effect	No effect	No effect	No effect
Ecotoxicological				
Avian: mallard duck, LC_{50} (8-day dietary) (mg kg^{-1})	>5000	>5620		>5000
Bobwhite quail, LC_{50} (8-day dietary) (mg kg^{-1})	>5000	>5620	>5000 (Japanese quail, 14 day)	4522
Aquatic: bluegill sunfish, acute acute LC_{50} (96 h) (mg L^{-1})	3.0	>4.3		>8.4
Daphnia magna, acute EC_{50} (48 h) (mg L^{-1})	3.8	3.7	>189 (3 h)	3.6
Honeybee (oral and contact) acute LC_{50} μg per bee	234	100	>100 (contact) >133 (oral)	>100
Earthworm, LC_{50} (14 days) (mg kg^{-1})	1000	1213	>1000	980

Since the initial use of tebufenozide in mid-1990s, the first documented case of codling moth (*C. pomonella*) resistance to tebufenozide was reported in southern France by Sauphanor and Bouvier [79] and Sauphanor et al. [80] and subsequently in the greenheaded leafroller, *Planotortrix octo*, in New Zealand by Wearing [81]. The resistance reported by Sauphanor and Bouvier [79] seemed almost

too rapid from the initial time of launch of a product with a new insecticidal mode of action. A major contributing factor for this resistance may have been due to existing multi-resistant codling moth populations as a result of extensive use of different insecticides. Attempts to select lab colonies of beet armyworm (*Spodoptera exigua*) continuously exposed to sub-lethal amounts of tebufenozide in the diet led to the selected strain crash after 12 generations of selective pressure [82]. In this study and studies conducted in the author's laboratory with susceptible beet armyworm larvae, oxidative metabolism of tebufenozide was found to be the main route for detoxification ([83, 84]; Dhadialla, unpublished observation). Interestingly, the first few oxidative metabolites of tebufenozide (monoalcohols at the ethyl and methyl substitutions on the two phenyl rings) continue to retain affinity to the ecdysone receptor, albeit much lower than the parent (Dhadialla, unpublished observations). The oxidative metabolism of tebufenozide in beet armyworm selected over six generations with tebufenozide could be dramatically reduced with the use of piperonyl butoxide, an inhibitor of P450 monooxygenases, but not with DEF, an esterase inhibitor [84]. These results support oxidative metabolism of tebufenozide as the main mechanism for detoxification and resistance development.

After reports of a decrease in the field efficacy of MIMIC^{TM} insecticide for control of beet armyworm on vegetables outside Bangkok, Thailand, Moulton et al. [85] amplified the resistance level in generations of field collected BAW larvae to levels reaching 150-fold over the susceptible strain of the same insect. The selected strain was about 120-fold lower in its susceptibility to methoxyfenozide, suggesting a common mechanism of resistance. These regions in Thailand, like in the south of France, have seen rapid development of resistance to insecticides with new and old modes of action due to the insufficient implementation of resistance management strategies.

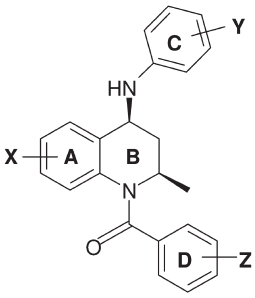
While the available data suggest oxidative metabolism as the main route for detoxification of at least two of the four bisacylhydrazine insecticides (tebufenozide and methoxyfenozide), there has not been any evidence of target site resistance.

25.1.9
Other Chemistries and Potential for New Ecdysone Agonist Insecticides

At least two other chemotypes, tetrahydroquinolines (Fig. 25.1.5, **25**; [87–88]) and amidoketones (Fig. 25.1.5, **26**; [89, 90]; also reviewed in Ref. [6]) have been shown to directly (ligand binding assays) or indirectly (cell based reporter gene transactivation assays) interact with the ecdysone receptor. These new ecdysone receptor binding chemistries could lead to new products for control of insect pests not controlled by current BAH insecticides.

25.1.10
Conclusions and Future Prospects of Ecdysone Agonist Chemistries

The bisacylhydrazines are well-understood insecticides in terms of their mode of action at the physiological, biochemical and molecular (including crystal struc-



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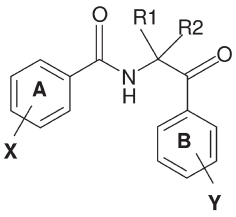

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Fig. 25.1.5. Generalized structures of two additional chemotypes, tetrahydroquinolines (**25**) and amidoketones (**26**), that bind ecdysone receptors from several insects. X, Y, and Z represent different substitutions on the phenyl rings in the two chemotypes. R1 and R2 can be 4- or 5-attached carbons, either as two acyclic substituents or, preferably, as a five- or six-membered ring (reviewed in Ref. [6]).

tures of ligand–receptor) level. The availability of the crystal structures of ecdysone receptor ligand binding domains in the absence and presence of ligands (steroidal and non-steroidal), and the availability of at least four different chemotypes (20E, BAH, tetrahydroquinolines and amidoketones), offers great potential for rational design and discovery of new non-steroidal ecdysone agonist insecticides with activities against different spectra of pests. Owing to the novel mode of action of bisacylhydrazine insecticides, their insect selectivity and reduced risk eco- and mammalian-toxicity profiles, these insecticides are ideally suited for use in integrated insect resistance and management programs.

Another utility of the registered bisacylhydrazine insecticides, not discussed in this chapter, has been the pursuit to use these chemicals in the area of gene switch application for regulation of genes or traits in mammalian or plant systems. The reader is referred to reviews by Palli et al. [91] and Dhadialla et al. [5] for greater in-depth understanding of the utility of tebufenozide and methoxyfenozide for ligand dependent gene expression in plants and mammalian systems in which the ecdysone receptor based gene switch was reconstituted.

Acknowledgments

We would like to thank David Demeter for creating Fig. 25.1.4 from the 1R1.pdf and 1R20.pdf files deposited by Billas et al. (2003), Mark Hertlein and Steve Evans for their critical reading of the manuscript, and W. Kleschick for his support and approval for us to write this chapter.

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25.2
A New Juvenoid – Pyriproxyfen

Makoto Hatakoshi

25.2.1
Introduction

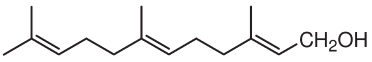
Pyriproxyfen is an insect growth regulator (IGR) that disrupts insect development at specific stages and is classified as a juvenoid among IGRs. Although insects undergo molting and metamorphosis during the growth process, which are controlled by their endocrine system, the action mechanism has been studied in detail in many species of insects, especially lepidopteran insects [1]. It has been clarified that many phenomena such as reproduction, egg development, phase polymorphism, diapause, pheromone synthesis, and so forth are controlled by the endocrine system of insects.

Since juvenoids affect the insect specific endocrine system, they are expected to become insect-specific insecticides [2]. Some naturally occurring compounds have juvenoid activity, e.g., Schmialek [3] isolated farnesol (**1**) and farnesal (**2**) with juvenoid activity from the feces of mealworm, *Tenebrio molitor*, (Fig. 25.2.1).

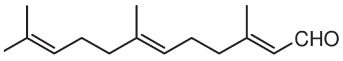
Wigglesworth [4] confirmed that farnesol and its related compounds showed the juvenoid activity against the bloodsucking bug, *Rhodnius prolixus*. Slama and Williams [5] found that paper products made from balsam fir, *Abies balsamea*, showed juvenoid activity against hemipteran bug, *Pyrrhocoris apterus*, and called the compound the paper factor. The chemical structure of the paper factor was identified as a methyl ester of todomatuic acid, and named juvabione (**3**) [6].

Although many compounds with juvenoid activity have been found in plants, they are not practical insecticides due to chemical instability and complexity of synthesis. Many compounds have been synthesized and their activities were checked against insects to find more active and stable compounds. The following describes representative juvenoids reported so far.

Farnesol **1**



Farnesal **2**



Juvabione **3**
(paper factor)

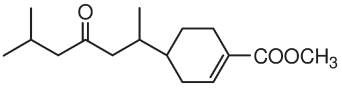


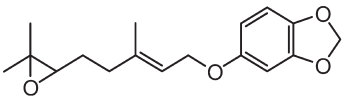
Fig. 25.2.1. Chemical structures of naturally occurring juvenoids.

25.2.2 History of Juvenoid Research

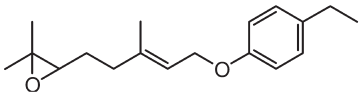
Bowers [7] found that some compounds used as insecticide synergists (e.g., piperonyl butoxide) possess juvenoid activity, and so he synthesized analogues of the synergists, e.g., some aromatic terpenoid ether compounds, and examined their morphogenetic activity against *T. molitor* and milkweed bug, *Oncopeltus fasciatus*. He found the first synthetic compound (**4**, Fig. 25.2.2) with high activity [8].

Later, many compounds that introduced various substituents into the phenyl ring and/or changed the side chain were produced, among which 4-ethylphenyl ether **5** was found to have high juvenoid activity [9].

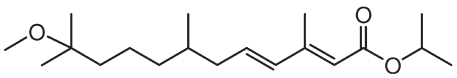
Aromatic terpenoid ether **4**



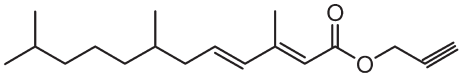
4-Ethylphenyl ether **5**



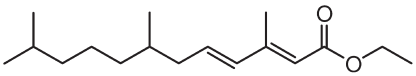
Methoprene **6**



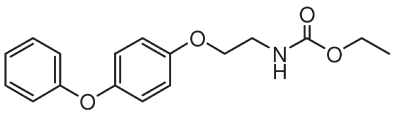
Kinoprene **7**



Hydroprene **8**



Fenoxycarb **9**



Pyriproxyfen **10**

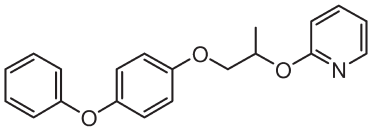


Fig. 25.2.2. Chemical structures of some synthetic juvenoids.

Table 25.2.1 Commercialized juvenoids and their target insects.

Common name	Trade name	Target insects
(*S*)-Methoprene	Altosid	Mosquitoes Horn fly (feed through)
	Apex	Sciarid flies (mushrooms)
	Diacon II	Storage pests (e.g., *T. molitor*)
	Strike	Filter flies, midges
(*S*)-Kinoprene	Enstar II	Aphids, thrips, whiteflies (greenhouse)
(*S*)-Hydroprene	Gentrol	Cockroaches
Fenoxycarb	Insegar	Lepidoptera (fruits, grapes)

In contrast, researchers at Zoecon Corporation found high juvenoid activity in alkyl (2*E*,4*E*)-3,7,11-trimethyl-2,4-dodecadienoates, some of which were commercialized as methoprene (**6**, ZR-515) [10], kinoprene (**7**, ZR-777) [11], and hydroprene (**8**, ZR-512) [10]. Since these compounds, however, possess double bonds and an ester bond in the molecule, they could not be used in the open field where stability in sunlight is required.

This defect was overcome by exchanging the unstable terpenoid structure with a 4-phenoxyphenyl group. Fenoxycarb (**9**), a carbamate with a 4-phenoxyphenyl group, was the first compound developed for agricultural use [12]. The ether compound pyriproxyfen (**10**) was found later by Sumitomo Chemical [13].

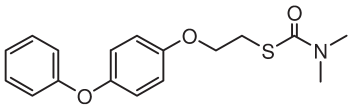
Table 25.2.1 shows the main target insects of juvenoids.

The target insect pests of methoprene are mosquitoes, sciarid flies, horn flies, storage pests, filter flies and midges, and so forth. Optical isomers are present in juvenoids since methoprene has an asymmetric carbon atom at C-7, and it seems that the (S)-form ((*S*)-methoprene) has a higher activity [14] and is now used in all products. The active ingredient in products using kinoprene is also the (S)-form; it is sold to control aphids, scales, and whiteflies. With hydroprene, (*S*)-hydroprene is used in products to control cockroaches, drain & fruit flies, bedbugs, and storage pests. Fenoxycarb is sold to control lepidopteran insects on fruits and grapes. Pyriproxyfen is sold to control mainly whiteflies on vegetables and cotton as various types of formulated products.

25.2.3 Process of Pyriproxyfen Research

In 1981 when juvenoid research was started at Sumitomo Chemical, insecticide research was focused mainly on pyrethroids that act quickly on insects and have a wide insecticidal spectrum. However, the appearance of resistant insects owing

Thiolcarbamate **11**



Oxime ether **12**

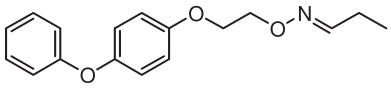


Fig. 25.2.3. Chemical structures of 4-phenoxyphenyl juvenoids discovered in the early stage of research at Sumitomo Chemical.

to the frequent use of these insecticides arose as a problem. Moreover, the development of highly selective insecticides was required from the viewpoint of integrated pest management (IPM).

We synthesized the thiocarbamate **11** (Fig. 25.2.3) in 1981.

Although this compound did not show insecticidal activity against insects other than spider mites in the primary screening, it changed the body color of the tobacco cutworm, *Spodoptera litura*, to red. The compound was presumed to be a juvenoid with a completely different action mechanism from the conventional insecticides, and this was confirmed by a biological test, the *Galleria* wax test [15].

As a result of screening many analogues after creating an evaluation system using larvae of the common mosquito (*Culex pipiens pallens*) and housefly (*Musca domestica*), which were the main target insect pests of juvenoids in those days, compound **11** was selected as the most active compound. However, **11** did not show sufficient efficacy against mosquito larvae in the field. The oxime ether compound **12** (Fig. 25.2.3), which has the same juvenoid action, was quickly identified, and it became clear that this chemical group had remarkably high inhibitory activity against adult emergence as a result of laboratory tests [16, 17]. However, residual efficacy against mosquito larvae obtained in the field test was unsatisfactory and further research was needed to find more stable compounds [17]. From the previous results, it was deduced that thiocarbamate and oxime ether groups control the stability of the compound. As one design to improve chemical stability, the synthesis of heterocyclic compounds that cyclized these groups was begun in 1982, and pyriproxyfen was selected the following year as a candidate compound possessing high IGR activity and remarkably improved stability [18]. Various laboratory tests were conducted using household insect pests [19–21] and agricultural insect pests [22–27]. Further promising target insects were then sought, and many field trials were carried out.

25.2.4 Activity of Optical Isomers

Pyriproxyfen contains an asymmetric carbon atom. When the activity of optical isomers was investigated using housefly larvae, *M. domestica*, the activity ratio of

Table 25.2.2 Inhibition of adult emergence of optical isomers of pyriproxyfen against larvae of housefly, *Musca domestica*.

Compound	e.e. (%)	R/S	IC_{50} (ppm)[a]
Pyriproxyfen	–	50/50	0.017
(*R*)-Pyriproxyfen	99.4	99.7/0.3	0.068
(*S*)-Pyriproxyfen	96.3	1.85/98.15	0.0090

[a] Half-inhibitory concentration of adult emergence.

the (R)- and (S)-forms was about 1:9 (R:S), and the (S)-form showed higher activity (Table 25.2.2).

25.2.5 Mechanism of Action

Pyriproxyfen acts only at specific growth stages to control insects by juvenoid activity [28], i.e., the early egg stage, the last instar larva, pupa, and adult. The action of pyriproxyfen involves (a) inhibition of egg hatching (ovicidal activity); (b) inhibition of metamorphosis; (c) inhibition of adult emergence; (d) inhibition of reproduction (decreased number of eggs oviposited and/or decreased hatchability), and so forth [29]. The action mechanism depends on the insect, with no general tendency, and should be identified for each insect.

Since the molecular mode of action of juvenoids is unknown, the endocrine mechanism of supernumerary larval molt [30] is shown below as an example of the mode of action of pyriproxyfen.

When various amounts of pyriproxyfen were applied to day 0 last instar larvae of tobacco cutworm, *S. litura*, they molted into pupae, 7th instar larvae (superlarva), or larval–pupal intermediates, depending on the doses (Table 25.2.3). The last instar larval period was also affected (Table 25.2.3). Although the untreated last instar larval period was 5.6 ± 0.7 days (mean $\pm$ SE), when increasing the dose of pyriproxyfen, the last instar larval periods of individuals that molted into pupae and larval–pupal intermediates were prolonged, and became 9.1 ± 1.1 days at a dose of 30 μg. On the other hand, supernumerary larval molt was observed at more than 3 μg, and all treated larvae molted into 7th instar supernumerary larvae at a dose of 100 μg. The larval period of individuals that molted into super larvae was about 4.0 days, irrespective of the doses. The obtained super larvae continued feeding and their body weight reached about 1.9 g on average (the average maximum body weight of untreated last instar larvae is about 0.8 g).

Changes in the ecdysteroid titer in hemolymph of untreated and 100 μg pyriproxyfen-treated last instar larvae were investigated [13]. In the pyriproxyfen-treated larvae, about a 100 ng mL^{-1} peak was observed on day 3, but about a 300 ng mL^{-1} peak was observed on day 4 in untreated larvae. As the prothoracic

Table 25.2.3 Effects of pyriproxyfen on the development of last instar larvae of *Spodoptera litura*.[a]

Dose (μg per larva)	*N*	% Molted into			6th instar larval period[b]	
		7th	L/P[c]	Pupa	7th	L/P + Pupa
100	14	100	0	0	4.4 ± 0.6	
30	15	60	20	20	4.0 ± 0.5	9.0 ± 1.1
10	14	7	50	43	4.0	7.9 ± 0.9
3	15	7	53	40	4.0	7.1 ± 0.7
1	15	0	80	20		6.4 ± 0.6
0.3	15	0	67	33		5.7 ± 0.5
Untreated control	20	0	0	100		5.4 ± 0.5

[a] Day 0 last instar larvae were treated with pyriproxyfen.
[b] Mean ± SE (day).
[c] Larval–pupal intermediate.

gland secretion of ecdysone in pyriproxyfen-treated larvae was only one-third that of untreated larvae one day earlier, this means that pyriproxyfen affected the activity of the prothoracic glands directly or indirectly.

Since the timing of ecdysone release was affected by pyriproxyfen, ligation between the head and thorax was conducted to investigated the timing of prothoracicotropic hormone (PTTH) release, which triggers the release of ecdysone in the untreated and 100 μg pyriproxyfen-treated larvae. Consequently, PTTH was released at 13:40 on day 3 in untreated larvae, and at 10:00 on day 2 in treated larvae (nearly 28 h earlier), i.e., pyriproxyfen stimulates the brain and accelerated PTTH release.

The location of brain cells containing PTTH was studied by staining the neurosecretory substance with paraldehyde-fuchsin. Two pairs of large neurosecretory cells (about 20 μm wide) and two pairs of small neurosecretory cells (about 10 μm wide) were recognized in the pars intercerebrum of the brain. It was shown that the staining of large cells changed with insect development. Although the staining of large cells hardly changed during days 0–2 in untreated larvae, it fell rapidly on day 3. In contrast, although the staining of large cells in the pyriproxyfen-treated larval brain was the same level on days 0 and 1 as in untreated larvae, it fell rapidly on day 2. From these results, PTTH was considered to be released from two pairs of large neurosecretory cells in the pars intercerebrum of the brain. It was reported that PHHT was present in one pair of lateral neurosecretory cells of tobacco hornworm (*Manduca sexta*) [31], five pairs of the dorsolateral part of the protocerebrum of waxmoth (*Galleria mellonella*) [32], and four pairs of dorsomedial neurosecretory cells of silkworm (*Bombyx mori*) [33].

When the pyriproxyfen titer in the hemolymph was measured by GC-MS, 144.7 ng mL^{-1} pyriproxyfen existed just after treatment, reaching a peak of 992.3

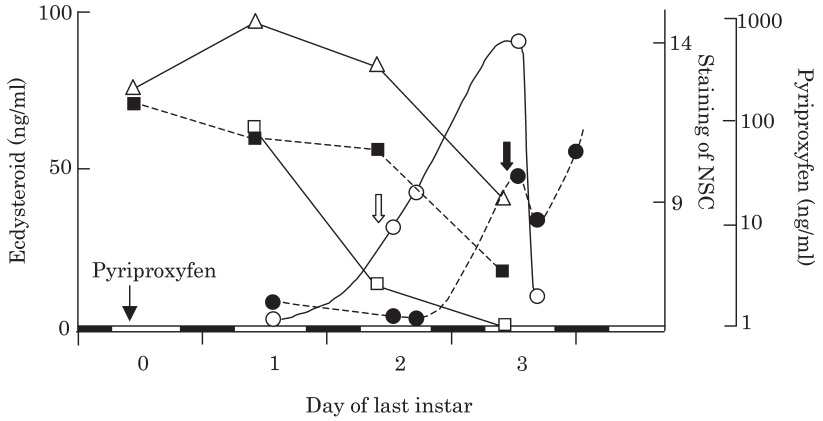


Fig. 25.2.4. Effects of topically applied pyriproxyfen (triangle) on the staining of NSC (square), PTTH release (arrow) and ecdysteroid titer (circle) in the last instar larvae of *Spodoptera litura*. Filled symbols: untreated larvae; open symbols: 100 μg pyriproxyfen was treated on day 0.

ng mL^{-1} on day 1, followed by 319.3 ng mL^{-1} on day 2 and 21.5 ng mL^{-1} on day 3. It is thought that the last instar larvae treated with pyriproxyfen had a high concentration of pyriproxyfen in the hemolymph on day 2 when PTTH was released. From these results, if a quantity of pyriproxyfen is administered to day 0 last instar larvae of tobacco cutworm and exists in the hemolymph for a time, the brain will release PTTH about one day earlier. The prothoracic glands secrete ecdysone in the same pattern as seen in 5th instar larvae in the presence of pyriproxyfen to induce supernumerary larval molt. Figure 25.2.4 summarizes these results.

25.2.6 Biological Activity

Since pyriproxyfen is mainly used to control agricultural pests, laboratory and field evaluations of these insects are described below. The biological activity against household insect pests is shown elsewhere [30].

25.2.6.1 Laboratory Evaluations

The activity of pyriproxyfen against various agricultural insects has been evaluated in Japan and other countries. Tests on adult cotton whitefly, *Bemisia tabaci*, which had become an important pest, were carried out in the Middle and Near East in 1984 [34]. The results were summarized as the following five points, (a) almost no effect on adults (no lethality toward adults); (b) although eggs were laid, most of them did not hatch; when adults were released just after spraying, no eggs hatched; (c) this tendency has little concentration dependency; (d) few eggs were oviposited just after spraying; (e) over time, the number of eggs ovipos-

Table 25.2.4 Ovicidal activity of pyriproxyfen against eggs of whitefly, *Trialeurodes vaporariorum*.

Egg stage	LC_{50} (ppm)
0∼1	0.46
1∼2	0.34
2∼3	0.21
3∼4	>30
4∼5	>30

ited increased, and development to nymphs and pupae was observed. These results indicated that pyriproxyfen should be applied preventively due to its sterilizing effect.

Following these results, the characteristics of pyriproxyfen were examined in detail in Japan, i.e., the activity against each stage was investigated in the laboratory using diamondback moth, *Plutella xylostella*, and greenhouse whitefly, *Trialeurodes vaporariorum*. Consequently, we recognized ovicidal activity at very low concentrations (Table 25.2.4) and an inhibitory activity against adult emergence when applied to larvae (Table 25.2.5) [34]. Further evaluation of greenhouse whitefly, aphids and lepidopterans was carried out in the greenhouse or in the field, and spray concentrations, spray timing, number of sprays, spray interval, and so forth were examined. In addition, since sterilizing activity and ovicidal activity by pyriproxyfen was obtained using various insects, a doubt remained that the response was specific to the test insects. The response of all developmental stages to this compound was then examined using one insect, diamondback moth (*P. xylostella*). Consequently, ovicidal activity, the inhibitory activity of pupation when applied to larvae, inhibitory activity of adult emergence, and, further, the inhibitory activity of adult emergence when applied to pupae and sterilizing activity when applied to adults was confirmed [29].

Table 25.2.6 summarizes the clarified mode of action of pyriproxyfen in some insects.

Table 25.2.5 Effects of pyriproxyfen on the development of whitefly, *Trialeurodes vaporariorum*.

Treated stage	*N*	% Adult emergence
Hatching	46	0
First instar nymph	165	0
Second instar nymph	54	0
Pupa	44	93.2
Untreated control	68	76.5

Table 25.2.6 Effects of pyriproxyfen on each stage of insects.

Insects	Treated Stage			
	Egg	**Larva/nymph**	**Pupa**	**Adult**
Bemisia tabaci [24, 27]	Most active within 24 h after oviposition	Treated to 1st to 2nd	→	No adults; eggs do not hatch
Trialeurodes vaporariorum [25]	Active within 3 days old	Treated to 1st to 3rd	→	No adults
Aonidiella aurantii [23]		High activity against 1st stage		No offspring when males or females treated
Myzus persicae [25, 26]				No offspring
Cydia pomonella [35]	Active within 24 h after oviposition			Eggs do not hatch
Spodoptera litura [36]		Matured larva		
		Just after pupation	→	Low adult emergence; decreased no. of eggs and ability to hatch
Thrips palmi [37]		Reared on treated leaf = dermal uptake from treated leaf	→	Inhibition of adult emergence

25.2.6.2 Field Evaluations

To control insects by spraying pyriproxyfen, we should reconsider (a) the target insect; (b) the target stage; (c) can pyriproxyfen reach the target insect, based on the fundamental knowledge already mentioned. The process of investigation for the definite application method is shown below.

From the information on the sterile effect on cotton whitefly (*B. tabaci*) previously shown, pyriproxyfen was sprayed three times at intervals of about three to four weeks on about a 100 m^2 experimental cotton field in Sudan. Consequently, (a) the number of adults in the treated plot was almost same as that in the untreated; (b) the number of eggs in the treated was also almost same as that in the untreated; (c) however, first instar nymphs did not increase (Fig. 25.2.5). The increase of adults, despite pyriproxyfen treatment, was thought to be due to the narrow width of the experimental field and the immigration of adults from the untreated plot. This hypothesis has checked by a report from Turkey. As a result of spraying pyriproxyfen onto a one hectare cotton field, the number of adults showed no increase just after application, but, clearly from Fig. 25.2.6, increased

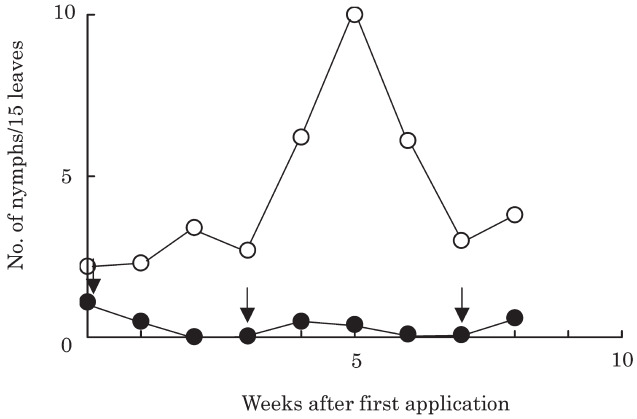


Fig. 25.2.5. Efficacy of pyriproxyfen on the nymphs of whitefly, *Bemisia tabaci* on cotton. Arrows: application timing of pyriproxyfen (100 g-a.i. ha^{-1}); (●) treated, (○) untreated.

when residual efficacy of the compound seemed to be lost. Thus, it was thought that the adults originated in the treated field by immigration from untreated areas. From the results obtained in these two countries, it was clear that (a) the application dose of pyriproxyfen is below 75 g ha^{-1}; (b) the application interval is two weeks.

Next, the spray timing was examined. The number of adults per leaf on cotton was incorporated into the spray timing index; Fig. 25.2.7 shows the changes in the number of nymphs. The results suggested that spraying should, preferably, be started when 50 or fewer adults per 100 leaves, i.e., one adult per two leaves, are counted.

However, an index of four or five adults per leaf was set up considering that the above number was still low. The spray frequency was limited to one application, having in mind the assumption that resistance to this compound may develop

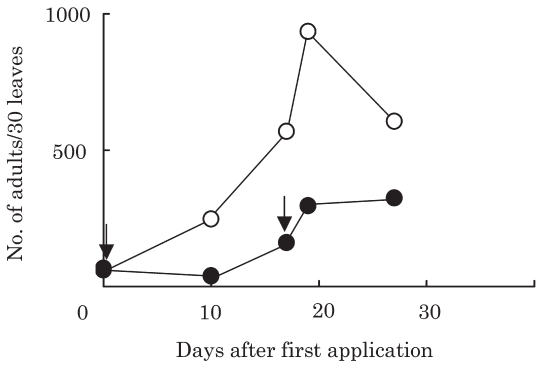


Fig. 25.2.6. Efficacy of pyriproxyfen on adult whitefly, *Bemisia tabaci* on cotton. Arrows: application timing of pyriproxyfen (100 g-a.i. ha^{-1}); (●) treated, (○) untreated.

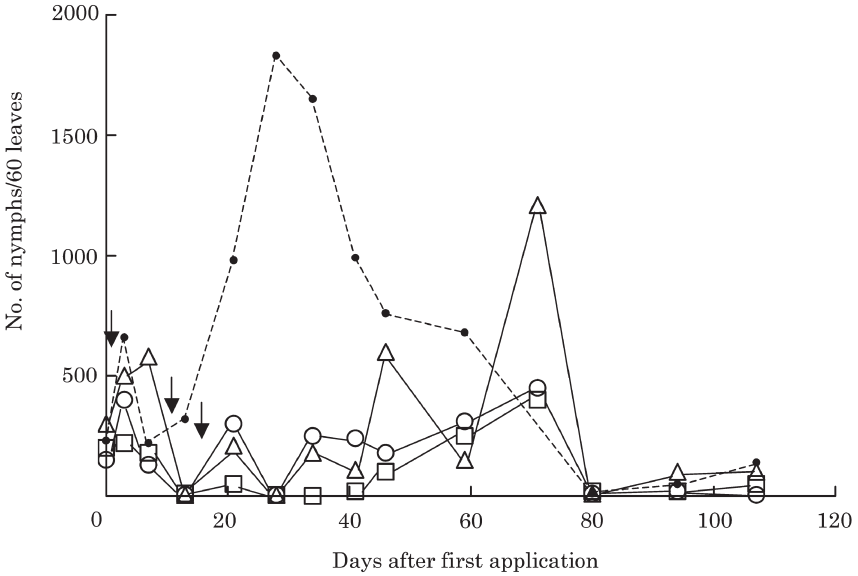


Fig. 25.2.7. Effects of the application timing of pyriproxyfen on the nymphs of whitefly, *Bemisia tabaci* on cotton. Pyriproxyfen was applied successively three times. First application was made at the time of no. of adults indicated. Arrows: application timing of pyriproxyfen (200 g-a.i. ha^{-1}); (□) 50 adults/100 leaves; (○) 200 adults/100 leaves; (△) 250 adults/100 leaves; (●) untreated.

upon repeated use, as seen in many examples of resistance development. Therefore, rotation was adopted.

Moreover, the effect of this compound was investigated not only for whitefly but also other insects. As an example, the experimental result for California red scale, *Aonidiella aurantii*, in an orchard is shown in Table 25.2.7 [34].

In this test, the characteristics of inhibiting reproduction and metamorphosis are well demonstrated. As a result of the treatment, high quality fruits without infestation of scales can be harvested (Table 25.2.8) [34].

Table 25.2.7 Efficacy of pyriproxyfen on California red scale (*Aonidiella aurantii*) on apple trees.

Conc. (ppm)	*N*	Mortality (%)[a]		
		Young stage	Females	Reproducing females
200	200	91.8	69.5	66.0
Untreated control	200	27.3	37.0	4.0

[a] Mortality was observed after 71 days.

Table 25.2.8 Effect of pyriproxyfen on fruit damage by California red scale, *Aonidiella aurantii*, on mature apple trees.

Conc. (ppm)	Clean fruits (%)
200	98
Untreated control	45

In Japan, to control greenhouse whitefly and cotton whitefly that infest vegetables and ornamentals in the greenhouse, a yellow plastic tape formulation containing pyriproxyfen was developed. The tape has been widely used as it can lower the population of whitefly for several months after installation, and the influence on natural enemies and pollinators is low, and so forth. The action mechanism in this system is that adults attracted by the yellow color touch the tape and take pyriproxyfen into the body. The hatching of oviposited eggs is strongly inhibited by ovicidal activity via adults [38, 39].

25.2.6.3 **Resistance**

Resistance to pyriproxyfen was observed only in whitefly, and has been reported in Israel [40] and the United States [41]. The resistance to pyriproxyfen in Israel is reviewed briefly as an example. Pyriproxyfen was introduced in Israel in 1991. Although it was sprayed once a season to control whitefly (*B. tabaci*), the whitefly developed a middle to high resistance up to 1996. The use of pyriproxyfen was stopped in 1996 and 1997. The resistance mechanism is unknown; it is reported that piperonyl butoxide, an oxidase inhibitor, does not have a synergistic effect [42]. The resistance is incompletely or partially dominant [43], and the susceptibility of whitefly was recovered by stopping the use of pyriproxyfen [44, 45].

25.2.7 **Synthesis**

Figure 25.2.8 shows the synthetic route to pyriproxyfen. The optical isomer of pyriproxyfen is synthesized using optically active lactic acid as a starter material [46, 47] or by using enantioselective hydrolysis with enzymes [48, 49].

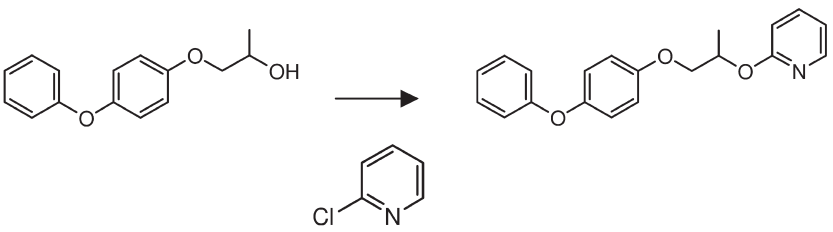


Fig. 25.2.8. Synthetic route to pyriproxyfen.

Table 25.2.9 Physicochemical properties of pyriproxyfen.

Melting point	48.1 °C
Vapor pressure	$<1.0 \times 10^{-7}$ mmHg (22.8 °C)
Log *P*	5.37 (25 °C)
Solubility	Water: 0.367 mg L^{-1} (25 °C) *n*-Hexane: 42 g L^{-1} (20 °C) Methanol: 44 g L^{-1} (20 °C) Acetone: >500 g L^{-1} (20 °C)

25.2.8
Physicochemical Properties and Formulation

25.2.8.1 Physicochemical Properties

Table 25.2.9 shows some physicochemical properties of pyriproxyfen.

The active ingredient of pyriproxyfen is an odorless white crystal with a melting point of 48.1 °C. Its vapor pressure is below 1.0×10^{-7} mmHg (22.8 °C) and its solubility in water at 25 °C is 0.367 ppm.

25.2.8.2 Stability

Pyriproxyfen is stable and hardly decomposes even if kept at 50 °C for six months. It is easily decomposed by higher pH and higher temperature. Moreover, although pyriproxyfen is promptly decomposed by ultraviolet rays, decomposition by sunlight (>290 nm) was slight.

25.2.8.3 Formulation

An emulsifiable concentrate and granules are sold as formulations containing pyriproxyfen. The tape formulation is also sold in Japan under the trade name of Lano®. The physicochemical properties of the formulations are very good and storage stability is very good. Moreover, mixtures with various insecticides have also been developed and sold.

25.2.9
Toxicology

The very favorable mammalian toxicity, the animal and plant metabolism, environmental toxicity and residue, and the effect on non-target organisms are described in a technical report [34].

25.2.10 Conclusions

The sale of pyriproxyfen was started under temporary registration in the Middle and Near East in 1988, and this registration was approved in 1991. In the United States, temporary registration was approved in 1996 and an emulsifiable concentrate named Knack® (10% content of pyriproxyfen) is sold and shows high efficacy against whitefly. The registration countries were expanded by setting whiteflies and scales on various crops as the main targets, and it is now sold to control whitefly on cotton and vegetables, thrips (*Thrips palmi*) on vegetables, scales on citrus fruits, psyla on pears, and leafroller and scales on fruit trees. In Japan, the tape formulation (containing pyriproxyfen at 1 g m^{-2}) for whitefly control in the greenhouse was registered in 1995 as Lano® tape.

It is also sold as Sumilarv® granules (0.5% content of pyriproxyfen) to control mosquito and housefly larvae for household use, and the sale was later expanded to control midges in Japan. In other countries it was also developed to control mosquitoes and houseflies, and sold as Sumilarv® 0.5% granules. In addition, it has been developed for home and PCO uses and was registered with the EPA in 1995.

Thus, although attention initially focused on its development for household use, the breakthrough to agricultural use was made by investigating the sterilizing activity of pyriproxyfen. Furthermore, this sterilizing effect was linked to the development of Lano® tape, which controls whiteflies without the need for spraying.

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26
Chitin Synthesis

26.1
Chitin Synthesis and Inhibitors

Joel J. Sheets

Chitin is the second most abundant biopolymer found in nature next to cellulose [1]. Similar to cellulose, chitin is also a carbohydrate, consisting of long unbranched chains of polymerized N-acetyl-glucosamine (poly-N-acetyl-glucosamine) monomers linked where the adjacent sugars have opposing orientations (Fig. 26.1.1). The only chemical difference between chitin and cellulose is the presence of aminoacetyl side groups found in chitin for the C-2 hydroxyl groups found in cellulose. Chitin is present in the cuticle of insects and is also found in other organisms, including the shells of all crustaceans, in protozoa, fungi, algae, and nematodes [2–4]. It is completely absent in vertebrates and higher plants, which makes its biosynthetic pathway an attractive target site for the action of insect specific insecticides [1, 5]. The cuticular exoskeleton of insects is composed of both chitin and proteins to provide a rigid support structure for muscle attachment, locomotion, and to protect the insect from environmental contaminants and desiccation [2]. Chitin is also synthesized and secreted by endodermal cells of the midgut of insects, combining with proteins and glycoproteins to form the peritrophic matrix. Chitin is a major component of the peritrophic matrix that lines the interior of the insect gut and separates its contents from the intestinal epithelium. The peritrophic matrix helps provide protection to the gut from mechanical damage, functions as a semi-permeable membrane to regulate passage of molecules between different midgut compartments, and acts as a barrier to protect insects from microbial and parasitic attack [6–13].

Insects make use of three different types of chitin (α, β, and γ) that differ in the relative orientations of adjacent chitin polymer chains. Typically, α-chitin is the most abundant form found in the insect cuticle. It is composed of polymeric chains of N-acetyl-glucosamine arranged in an anti-parallel orientation. This arrangement allows for the formation of microfibrils consisting of closely packed crystalline arrays of individual chitin chains that utilize extensive hydrogen bonding between the amine and carbonyl groups to help provide mechanical strength

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

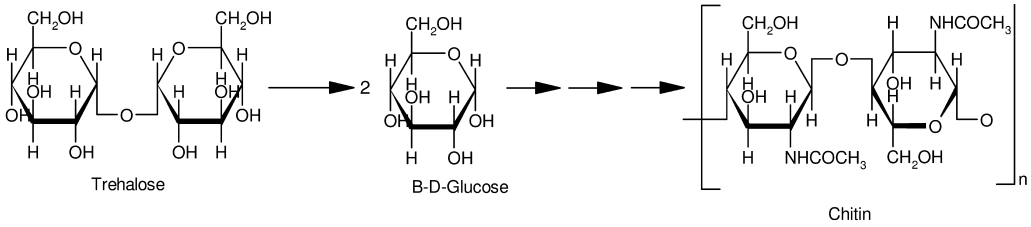


Fig. 26.1.1. Abbreviated biosynthetic pathway of the monomeric N-acetyl-glucosamine structure of chitin starting from trehalose.

[4, 14]. β-Chitin is found in the insect gut, along with α-chitin, as a component of the peritrophic matrix. The adjacent chitin polymers in β-chitin are parallel, as opposed to the anti-parallel orientation of α-chitin [15]. This results in the adjacent chitin chains forming fewer hydrogen bond linkages, allowing the structure to be less rigid and more hydrated [16]. The third and less predominant form of chitin (γ-chitin) exists primarily in cocoons and has a structure consisting of two parallel strands of chitin polymers positioned next to a single chain of chitin running in the opposite direction.

The biosynthetic pathway for chitin starts with the disaccharide trehalose, which is synthesized in the fat body and is the most predominant sugar found in insects [17–19]. Trehalose is cleaved into two glucose molecules that are phosphorylated, isomerized, and acetylated, affording the precursor UDP-N-acetylglucosamine (Fig. 26.1.1). Chitin synthetase (EC 2.4.2.26) is the final enzyme of the pathway that utilizes the activated sugar UDP-N-acetylglucosamine, polymerizing it to form chitin. Several excellent reviews describe the activity and structure of this enzyme [18–22]. Chitin synthetase is a 180-kDa protein belonging to the family of β-glycosyltransferases, and is considered to be the key enzyme involved in chitin synthesis. The enzyme has been shown to require divalent cations such as Mg^{2+}, Mn^{2+}, or Ca^{2+} for activity [23]. Investigations to localize chitin synthetase in the midgut of *Manduca sexta* by immunohistochemistry using fluorescently labeled anti-chitin synthetase antibodies show the midgut brush border membranes to be heavily labeled, with the immunofluorescence labeling localized at the apical areas of the microvilli. In addition, chitin synthetase is also localized in the apical membranes of salivary glands and tracheal cells [24]. Immunochemistry performed on the epiprot of the American cockroach *Periplaneta americana* show chitin synthetase also located in the apical region of the epidermis [4].

Based on insects so far investigated, two different forms of chitin synthetase have been found encoded by two different genes (*CHS-A* and *CHS-B*). These genes are differentially regulated and expressed in different tissues, including the integument, midgut and trachea [25–30]. *CHS-A* is expressed in the epidermis for the formation of chitin in embryonic and pupal cuticles, whereas *CHS-B* is associated with the expression of chitin associated with the peritrophic matrix of the midgut [25]. Tellam and co-workers were the first to determine the cDNA

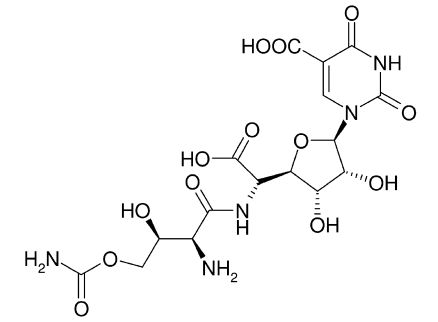
sequence of an insect chitin synthetase from the Australian sheep blowfly *Lucilia cuprina*. The amino acid sequence of the protein shows low similarities to yeast chitin synthetases and contains between 15 and 18 putative transmembrane regions, indicating that the enzyme is an integral membrane protein [29].

There is evidence that fungal chitin synthetase is initially synthesized as a zymogen, requiring proteolytic activation for full expression of its biological activity [31, 32]. Chitin synthetase in arthropods has also been proposed to be synthesized as a zymogen based upon results from tissue assays [18], but evidence for this has only recently been obtained from *in vitro* chitin synthetase preparations from the midgut of *Manduca sexta* [30]. In these studies, activation of enzymatic activity is observed when crude cell extracts from midgut tissues are treated with trypsin. However, when the 12 000 *g* membrane fraction from this preparation is treated with trypsin, no activation of chitin synthetase activity is observed even though this fraction should be enriched with chitin synthetase. Adding the soluble fraction back to the membrane fraction restores the ability of trypsin to activate chitin synthetase activity, suggesting that trypsin acts on a soluble protein or some other factor(s) that in some way activates chitin synthetase located in the membrane fraction [30].

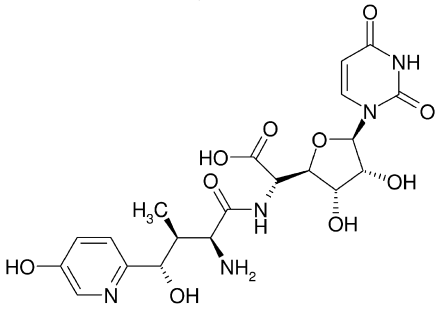
Assays designed for measuring chitin synthesis rely on several unique physical properties of chitin, including its insolubility in most solvents, combined with its ability to be deacetylated in boiling alkali to chitosan, which is also insoluble. Treatment of chitin with concentrated hot acids results in deacetylation and hydrolysis, yielding soluble glucosamine. Chitin is also sensitive to degradation by chitinases [33]. Cultured insect imaginal wing disks provide a convenient *in vitro* preparation for measuring chitin synthesis. This tissue preparation responds to the addition of ecdysteroids by stimulating the rate of incorporation of [^{14}C]GlcAc into base insoluble material that is readily digested with chitinase [34–36]. Hajjar and Casida have described an *in vitro* assay to the measure rate of incorporation of [^{14}C]glucose into [^{14}C]chitin using the abdomen of newly emerged adult milkweed bugs *Oncopeltus fasciatus* [37]. The authors show a good correlation between the activity of twenty four different benzoylphenyl urea analogs to inhibit chitin synthesis and their toxicity towards *O. fasciatus* nymphs, thus demonstrating that this is a convenient *in vitro* assay system to measure the activity of new chitin synthesis inhibitors. A non-radioactive high throughput assay for measuring chitin synthase activity in yeast has been described that could be adapted for measuring this activity in insect cell lines [38].

26.1.1 Inhibitors of Chitin Synthesis

Polyoxin D and nikkomycin Z are *Streptomyces* derived peptidyl nucleoside antibiotics that have been shown to be competitive inhibitors of chitin synthetase in both fungal and insect *in vitro* systems [39–44]. Both polyoxin D and nikkomycin Z have structural similarities to the substrate UDP-N-acetylglucosamine, which most likely accounts for the competitive nature of their ability to inhibit chitin



Polyoxin D



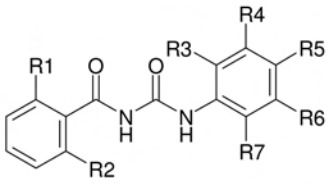
Nikkomycin Z

Fig. 26.1.2. Structure of peptidyl nucleoside antibiotic inhibitors of chitin synthesis.

synthetase (Fig. 26.1.2). Polyoxin D is a moderate inhibitor of chitin synthesis in isolated integument tissues from various different insects, whereas nikkomycin has greater inhibitory potency [40]. Using [^{14}C]glucose as a substrate, polyoxin D inhibits the formation of [^{14}C]chitin by 50% at 12 μM in an *in vitro* system using *Oncopeltus fasciatus* [37]. Diflubenzuron, however, in the same system is about 22 times more potent. Nikkomycin reversibly inhibits chitin synthesis in a dose dependant manner when injected into fifth instar larvae of the tobacco hornworm, *Manduca sexta* [45]. Much higher concentrations of nikkomycin are required to inhibit chitin synthesis when the compound is applied topically or as an oral dose, as compared with when injected directly into insects. The poor activity of these nucleoside inhibitors when applied to insects is probably due to poor pharmacokinetic properties of these compounds, which has greatly limited their agricultural use as insecticides. They have found limited use as fungicides but have proven useful as tools to study chitin synthetase [5, 23].

26.1.1.1 **Benzoylphenyl Ureas**

An early representative to the benzoylphenyl ureas includes diflubenzuron, discovered by Philips-Duphar B.V. as an inhibitor of chitin synthesis, and commercialized in 1977 for control of lepidopteran and coleopteran pests in fruit, cotton,



Chemical	R1	R2	R3	R4	R5	R6	R7
Diflubenzuron	F	F	H	H	Cl	H	H
Chlorfluazuron	F	F	H	Cl	3-Cl-5-CF_3-pyrindin-2-yloxy	Cl	H
Teflubenzuron	F	F	F	Cl	F	Cl	H
Triflumuron	H	Cl	H	H	-OCF_3	H	H
Hexaflumuron	F	F	H	Cl	-OCF_2CF_3	Cl	H
Noviflumuron	F	F	F	Cl	-OCF_2CHFCF_3	Cl	H
Novaluron	F	F	H	Cl	-$OCF_2CHFOCF_3$	H	H
Lufenuron	F	F	H	Cl	-OCF_2CHFCF_3	H	Cl
Bistrifluron	F	F	Cl	CF_3	H	CF_3	H

Fig. 26.1.3. Structure of representative benzoylphenyl ureas.

soybeans and vegetable crops [46–51] (Fig. 26.1.3). Triflumuron represents an additional early benzoylphenyl urea registered in the early 1980s by Bayer AG for control of insect pests on fruits and vegetables [52]. These older representatives have found additional roles in the urban pest management arena due to their activity against non-crop insects such as mosquitoes, termites and cockroaches [53–55].

Chlorfluazuron was introduced in the late 1980s in Australia, Hungary, Japan, Philippines, and Vietnam by Ishihara Sangyo Kaisha, for control of lepidopteran pests on cotton, tea, vegetables, and fruits, especially in situations requiring management of insecticide resistance. It was never registered in the USA. Chlorfluazuron possesses ovicidal activity against adult German cockroach (*Blattella germanica*), and the common cutworm (*Spodoptera litura*) [54, 56], and has also been evaluated for uses in non-crop applications such as fly control [57] and for use as part of a termite bait matrix [58, 59] which may extend the use of this mature compound.

Teflubenzuron was introduced in 1986 by ACC (now BASF) into parts of Europe and Africa for control of lepidopteran pests in fruits, citrus, vegetables and cotton. It also has mosquito larvacide activity and is currently being investigated for use as a feed treatment for sea lice on fish grown in captivity [60].

Hexaflumuron, originally developed by Dow as an insecticide for control of Lepidoptera, Coleoptera, Homoptera, and dipera pests on cotton, top fruit and potatoes, was first introduced into Latin America in 1987. It was later registered in the USA in 1995 as the first reduced risk pesticide for use as part of a termite bait matrix system called Sentricon® [59, 61–65]. Hexaflumuron has since been replaced with another benzoylphenyl urea, noviflumuron, which is more potent

and faster acting [66], and also has activity against other household pests such as German cockroach (*Blattella germanica*) applied either as a spray or as part of a bait matrix [67, 68].

Novaluron is the most recently introduced benzoylphenyl urea, originally developed by Isagro and subsequently sold to Makhteshim. This molecule is registered in Europe and in the USA for use on ornamentals, cotton, fruits, and vegetables. It is very potent on the cotton leafworm (*S. littoralis*) and the Colorado potato beetle (*Leptinotarsa decemlineata*), showing some contact and good residual activity [69, 70]. It is also undergoing investigations for use in vector control programs against *Aedes aegypti* [71].

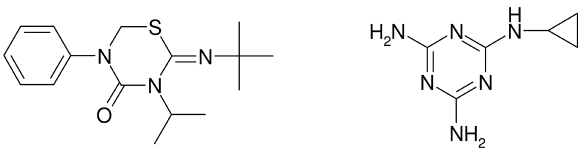
Lufeneron from Ciba-Geigy is the current market leader of the benzoylphenyl ureas [72]. It has been used in Europe and Japan on cotton and vegetables and markets are increasing in Latin America and South Korea. Its major application has been in the animal health market for the control of fleas on domestic dogs, cats and other animals [73–76]. It is also currently being developed as a bait for termite control [72].

Bistrifluron (DBI-3204) is a benzoylphenyl urea, recently introduced by Dongbu Hannong of Korea, that is active against whiteflies (*Trialeurodes vaporariorum* and *Bemisia tabaci*) and Lepidoptera pests (e.g., *Spodoptera exigua*, and *Plutella xylostella*) [77]. This compound has also shown promise for use as a bait for the control of ants and cockroaches in domestic environments [78].

26.1.1.2 **Other Chitin Synthesis Inhibitors**

Buprofezin [2-*tert*-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one. Applaud®, NNI-750 (Nihon Nohyaku)] is an insect growth regulator based on the thiadiazine class of chemistry. It was introduced in 1984 as a non-systemic contact insecticide for the Japanese rice market, having particularly good activity as a foliar spray against the brown planthopper *Nilaparvata lugens*. It inhibits molting, leading to suppression of ecdysis, presumably through inhibition of chitin synthesis, but it has little effect on egg viability [79, 80] (Fig. 26.1.4).

Cryomazine [*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine. Trigard, CGA 72662 (Ciba-Geigy now Syngenta AG)] is another member of the thiadiazine class of chemistry, introduced in 1985 as a foliar spray with systemic and translaminar activity against leaf miners in vegetables, potatoes and ornamentals. It interferes



Buprofezin Cyromazine

Fig. 26.1.4. Structures of other chitin synthesis inhibitors.

with molting, presumably due to inhibition of chitin synthesis, but the ultrastructural changes in the cuticle resulting from the action of cryomazine appear to be distinct from those that result from the action of diflubenzuron [81].

26.1.2 The Future of Chitin Synthesis Inhibitors for Crop Protection

Chitin synthesis inhibitors have the useful property of having a non-neurotoxic mode of action on a target site that is not present in vertebrates, which makes them generally safe to mammals when ingested. Having a target site distinct from that of the more commonly employed neurotoxic insecticides allows these molecules to be used as important tools in insecticide resistance management programs [70, 82]. Resistance to the benzoylphenyl ureas has been measured in both the field and the laboratory, but it is usually correlated with insects obtaining increased capacity to metabolize and eliminate the compound [83, 84] as opposed to target site resistance. These compounds can be toxic to chitin-synthesizing invertebrates such as daphnia, and crustaceans, that exist lower down the food chain, and this can result in significant environmental problems if these chemicals are inadvertently allowed to enter streams and lakes. The speed of action of the benzoylphenyl ureas is generally slow, requiring several days before observing a reduction in larval numbers, even though cessation of feeding often comes earlier. This slow activity has resulted in the need to properly educate farmers accustomed to the rapid knockdown activity of neurotoxic pesticides on the proper application timing of these compounds and the time required to observe their full activity. The lack or limited contact activity of the benzoylphenyl ureas has also limited their utility against insects that are hidden feeders.

Overall, in the 5 years up to 2004, global sales of benzoylphenyl ureas have been fairly flat, at about $230 million USD, with a growth rate of about 3% [72]. Using these compounds outside of crop protection as part of a bait matrix for the long-term control of urban pests or use as veterinarian medicinal products for flea and tick control in companion animals and livestock represents significant opportunities to extract further value from these chemistries beyond their use in crops. These opportunities typically represent higher gross margins than what is obtained in commodity agricultural markets. The continual development of transgenic crops such as cotton and corn expressing genes that produce intrinsic insect resistance will most likely continue to erode the use of these chemicals for crop protection, unless widespread resistance breaks out, either to the transgenic crops or to other insecticides having neurotoxic activity.

Chitin synthesis inhibition still remains an important and under utilized target site for the control of agricultural insect pests. Given the high turnover and important role of chitin synthesis in maintenance of the peritrophic matrix of insects, this mode of action is an accessible target in the gut of insects to inhibit through transgenic means. Discovery of proteins that inhibit chitin synthesis in the gut of insects and expressing these proteins in transgenic plants could be an

attractive strategy for controlling pests in the future. It also presents a target site that could be used to screen for new contact active chemicals or gut active proteins having the ability to inhibit this biological activity.

Acknowledgments

The author wishes to thank Dr. Thomas C. Sparks for his careful review of this manuscript, and to Gene F. Tisdell for providing his insights into the 2,6-diphenyl-heterocyclic chemistries. This work was supported by Dow AgroSciences.

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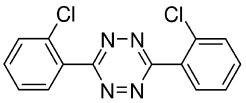
26.2
Mite Growth Inhibitors (Clofentezine, Hexythiazox, Etoxazole)

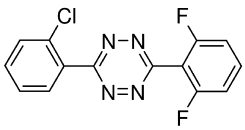
Thomas Bretschneider and Ralf Nauen

26.2.1
Introduction

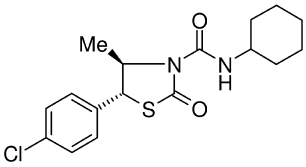
Phytophagous mites are important pests in many cropping systems worldwide, e.g., fruits, vegetables, grapes, and ornamentals. A major problem in their control is their high reproductive potential and the extremely short life cycle, both of which facilitate rapid resistance development to many acaricides, often after only a few applications. Therefore, the history of spider mite control is a head-to-head



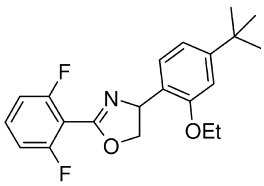
Clofentezine **1**



Diflovidazin **2**



Hexythiazox **3**



Etoxazole **4**

Fig. 26.2.1. Structures of discussed mite growth inhibitors.

race between resistance development in frequently exposed populations, suitable measures for resistance control by, for example, spray programs for commercial products and the development of acaricides with a new mode of action not affected by established resistance mechanisms.

Many different biochemical targets in mites have been addressed in recent decades for the development of new acaricides [1, 2]. One group of acaricides are known as mite growth inhibitors and are classified in group 10 of the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme (www.irac-online.org). Mite growth regulators such as clofentezine (**1**), diflovidazin (**2**) hexythiazox (**3**) and etoxazole (**4**) are presented in this chapter, because they share some common characteristics (Fig. 26.2.1).

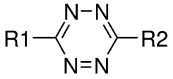
The compounds interfere with mite development and show activity against all juvenile stages of mites (eggs, larvae, nymphs) except adults. Cross-resistance occurs between some of these compounds and, therefore, they have been grouped together in the group 10 of the IRAC Mode of action classification scheme mentioned above [3].

The discovery, synthesis, structure–activity relationships, biology and biochemistry of these compounds are described in this chapter.

26.2.2
Tetrazines (Clofentezine, Diflovidazin = Flutenzine)

The mite ovicidal activity of ortho-halogen phenyl substituted tetrazines was discovered at Chesterford Park Research Station in 1976. In contrast to the inactive unsubstituted bis-phenyl derivative (entry 1, Table 26.2.1) the introduction of the 2-chloro-phenyl residue on one side of the tetrazine leads to an interesting ovici-

Table 26.2.1 Selected structure–activity data in the area of diaryl-tetrazines.



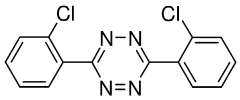
Entry	Compound no	R1	R2	Relative ovicidal mite activity (0 = weak, 4 = excellent)
1		Ph	Ph	0
2	Lead compd	2-Cl-Ph	Ph	2
3		2-Cl-Ph	4-Cl-Ph	0
4		2-Cl-Ph	3-Cl-Ph	0
5	Clofentezine	2-Cl-Ph	2-Cl-Ph	4
6		2-Cl-Ph	2-Br-Ph	3
7		2-Cl-Ph	2-I-Ph	3
8		2-Cl-Ph	CH_2-Ph	2
9		2-Cl-Ph	cyclohexyl	4
10		2-Cl-Ph	CH_2-cyclohexyl	3

dal mite activity (entry 2, Table 26.2.1) and served as a lead for further research [4]. Table 26.2.1 presents some representative structure–activity data.

Keeping the 2-chloro-phenyl substitution in position 3 of the tetrazine, many different substituents in position 6 were screened. Highly active compounds were discovered with 2-chloro-phenyl (entry 5) and a cyclohexyl derivative (entry 9). In field trials the bis-2-chloro derivative showed advantages, especially regarding its long-lasting activity, and was finally selected for development by Schering AG (later incorporated into AgrEvo and Bayer CropScience AG) under the common name clofentezine (Table 26.2.2) [5]. Clofentezine was presented by Schering at the 1981 British Crop Protection Council Conference [6] and was launched in its first countries 1983 under the main trade name Apollo®.

At the beginning of the 1990s the Hungarian company Chinoin (now Agro-Chemie) started chemical research in the area of the miticidal tetrazines with the goal of finding compounds with improved properties [7, 8]. They synthesized, especially, unsymmetrical substituted tetrazines and reported that the introduction of a fluorine atom in the ortho position of the phenyl ring resulted in acaricides with improved translaminar and transovarian properties and with higher vapor activities [9]. Of those derivatives, the 2,6-di fluoro compound **2** was selected for further development and presented under the code name SZI 121 at the 1994 British Crop Protection Council Conference [10]. The initially proposed common name flufenzine was later changed to diflovidazin (provisionally approved by ISO in 2004). The product was launched in Hungary in 1997 under the trade name Flumite 200® (Table 26.2.3).

Table 26.2.2 Data for clofentezine.



Common name	Clofentezine
IUPAC name	3,6-bis(2-Chlorophenyl)-1,2,4,5-tetrazine
Development code	NC 21314
Patent	EP5912 (priority 1978-05-02)
Launch	1983
Melting point	186 °C
log *P*	4.1
Water solubility	<1 mg L^{-1}
Toxicity (rat, oral)	>5200 mg kg^{-1}

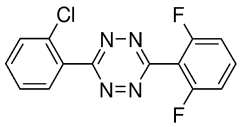
Scheme 26.2.1 shows the synthesis routes leading to clofentezine and diflovidazin.

The synthesis of clofentezine starts with a twofold acylation of hydrazine hydrate with 2-chlorobenzoyl chloride, followed by a phosphorous pentachloride mediated activation and a second reaction with hydrazine hydrate to give the 1,2-dihydrotetrazine derivative, which is finally oxidized to clofentezine using sodium nitrite [4, 11].

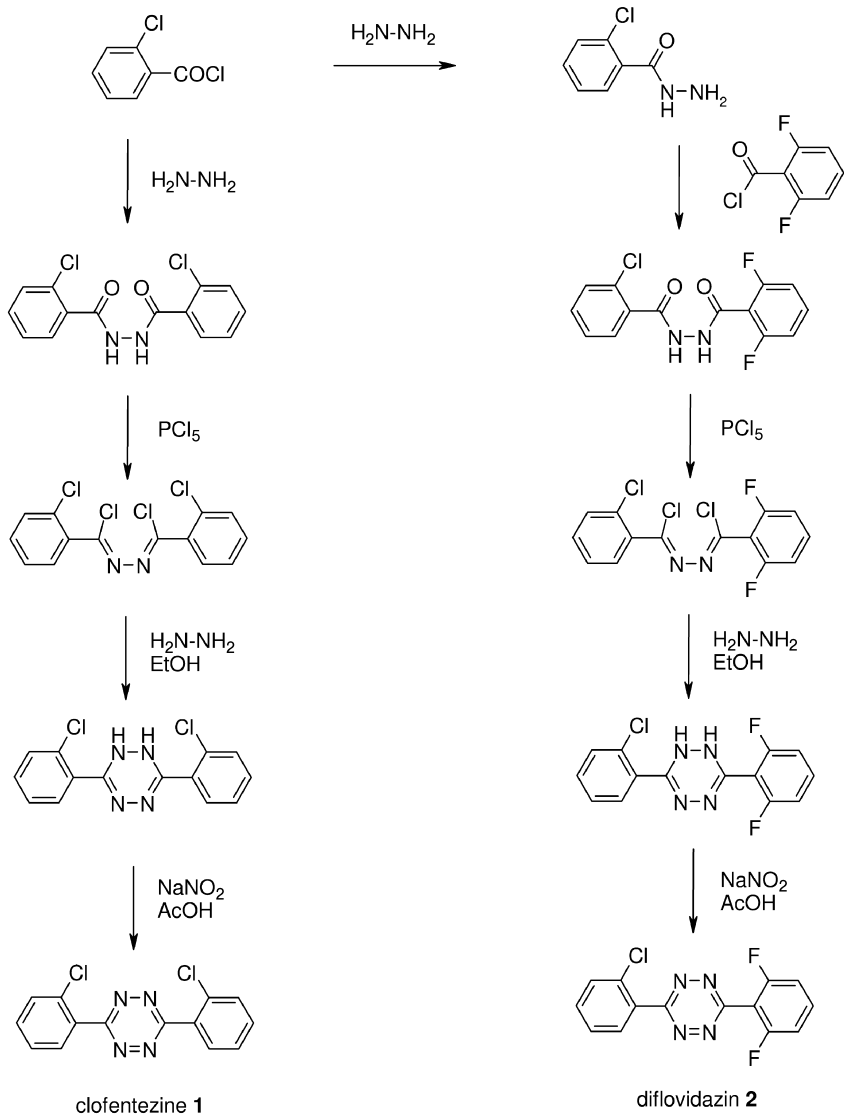
26.2.2.1 **Biology and Biochemistry**

Clofentezine and diflovidazin have been developed for the control of a wide range of spider mite species such as *Tetranychus* spp., *Panonychus* spp. and *Eriophyid*

Table 26.2.3 Data for diflovidazin.



Common name	Diflovidazin
IUPAC name	3-(2-Chlorophenyl)-6-(2,6-difluorphenyl)-1,2,4,5-tetrazine
Development code	SZI 121
Patent	EP635499 (priority 1993-07-21)
Launch	1997
Melting point	187–189 °C
log *P*	4.1
Water solubility	<1 mg L^{-1}
Toxicity (rat, oral)	>594 mg kg^{-1}



Scheme 26.2.1. Synthesis of the tetrazine acaricides clofentezine and diflovidazin.

mites, especially on topfruits and vines. Clofentezine is a specific contact acaricide acting primarily as an ovicide with some effect on young motile stages and with long residual activity, e.g., sprayed on grape leaves prior to hatching of winter eggs of *P. ulmi* gave control for >60 days [12]. It has no activity against adult mites. Clofentezine interferes with cell growth and differentiation during the final phases of embryonic and early larval development. Clofentezine is particularly effective against mite eggs, including winter eggs of the European red mite. The compound is marketed in different formulations and combinations with other

acaricides and insecticides to broaden the spectrum also against adults (e.g., Viktor CL® is effective against all stages of *P. ulmi*, *T. urticae*, and *Eotetranychus carpini*), additional insects (Torant®) or to prevent fast resistance development (Table 26.2.4).

Nevertheless resistance to clofentezine was identified in different populations of mites and areas such as the European Red Mite (*P. ulmi*) from orchards in Ontario after ca. 5 years of use [13] or populations of *T. urticae* from Australia [14, 15] with resistance factors between 770 and >2000-fold at LC_{50}- or LC_{90}-values. Observations on field populations indicated that resistance persisted for at least two seasons [13]. Cross-resistance to hexythiazox was also observed [14]. Enhanced detoxification by increased activity of mono-oxygenases (MO) and esterases is at least partially responsible for the observed resistance and cross-resistance [16].

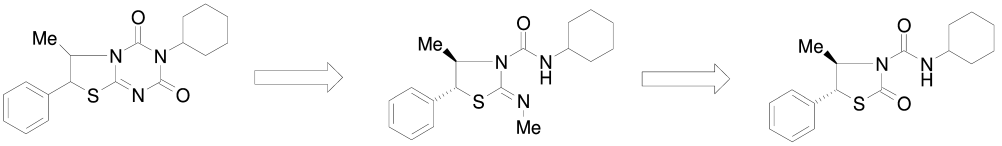
Both clofentezine and diflovidazin have very favorable ecotoxicological properties. They are especially extremely safe to beneficial arthropods: predatory mites of the genus *Amblyseius*, *Phytoseiulus*, *Typhlodromus*, *Zetzelia*; predatory insects of the genus *Anthocoris*, *Chrysoperla*, *Orius*, *Stethorus* pollinating bees and parasitic wasps. This property leads to preferred uses under integrated pest management conditions. The use rates in the different crops vary between 7.5 and 40 g-a.i. hl^{-1} depending on the water volume used for spraying in topfruits, softfruits, vegetables (basic dose rate: 100–200 g-a.i. ha^{-1}) and 150–250 g-a.i. ha^{-1} in cotton (50–100 g-a.i. hl^{-1} at 150–300 l ha^{-1}).

Diflovidazin was introduced in 1997 to East-European and Asian markets (Table 26.2.5). Furthermore, it has been submitted for registration in the EU.

In field trials, diflovidazin (SZI-121) provides long-lasting control at application rates as low as 80 g-a.i. ha^{-1} against *Panonychus*, *Tetranychus*, *Aculus* and *Calipitrimerus* spp. in apple and vine [10]. It has translaminar and transovarial activity and is also effective via vapor phase. This acaricide acts, like clofentezine, primarily as an ovicide, but was investigated also for activity against the chrysalis stage of *T. urticae* in laboratory trials. Interestingly, it showed an LC_{50} of 0.39 ppm and was much more active than clofentezine (LC_{50} > 20 ppm) [8]. In soil, diflovidazin degraded more rapidly than clofentezine.

26.2.3
Thiazolidinones (Hexythiazox)

The discovery of the thiazolidinone acaricides in the laboratories of Nippon Soda started during work on fungicidal thiazolo[2,3-*b*]triazine derivatives when a weak miticidal activity was observed with derivative **5**.



5 **6** **7**

Table 26.2.4 Different formulations and mixtures of clofentezine.

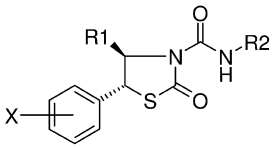
Name®	Use	Crops	Content
Apollo 50 SC (Apollo 50 SC, Acaristop 50 SC, Sa-lan, Cara) and 20 SC	Control of a wide range of mite species	Topfruit, citrus, vines, cotton, vegetables, ornamentals, softfruit, tea	500 g L^{-1} clofentezine
Apollo Plus (6SE)	Control of a wide range of mite species, especially under circumstances of developing tolerance to clofentezine	Topfruit, citrus, vines, cotton, vegetables, ornamentals, softfruit, tea	60 g L^{-1} clofentezine plus 540 g L^{-1} mineral oil (SE)
Victor	*Tetranychus* spp., *Panonychus ulmi*, *Eriophyid mites*, esp. *Calepitrimerus vitis* Leafhoppers on vines	Vegetables, vines, others	SE containing 200 g L^{-1} clofentezine + 100 g L^{-1} fenpropathrin
Torant CL, Percut	*Tetranychus* spp., *P. ulmi*, *Eriophyid* mites, esp. *Calepitrimerus vitis*; grape berry moth	Vines, vegetables; strawberries, ornamentals and roses (Percut)	SC containing 200 g L^{-1} clofentezine + 40 g L^{-1} bifenthrin
Orion CL	*Tetranychus* spp. *P. ulmi*, *Eriophyid mites*	Topfruit and vines suitable for IPM situations	Twinpack with 500 mL clofentezine 20 SC + 1000 mL propargite 57 EW
Torero	Control of mites	Vegetables, vines, others	Twinpack with 500 mL clofentezine 20 SC + 300 mL tau-fluvalinate 24 SC
Apollo/Kelthane	Control of mites, esp. *P. citri* and *T. cinnabarinus*	Citrus	Twinpack with 300 mL clofentezine 20 SC + 1000 mL dicofol 48 LE
Apollo/Sanmite	Control of mites with side effect on whitefly	Citrus	Twinpack with 500 mL clofentezine 20 SC + 500 mL pyridaben 20 SC

Table 26.2.5 Diflovidazin registrations (status 2004).

Country	Trade name	Crops	Status	Date
Hungary	Flumite	Vines, apples, peaches, plums	Launched	1997
Georgia	Flumite	Apples, grapes, citrus	Approved	1998
Kazakhstan	Flumite	Apples, cotton	Approved	1998
UAE	Flumite	Vegetables	Approved	1998
Uzbekistan	Flumite	Cotton	Approved	1998
Yugoslavia	Flumite	Apples	Launched	1998

Several triazine ring opened derivatives were screened, among which the trans configured derivatives **6** and, especially, the thiazolidinone **7** showed very interesting activities against *T. urticae* and served as a lead for a broad chemical optimization program. Table 26.2.6 shows selected structure–activity data [17].

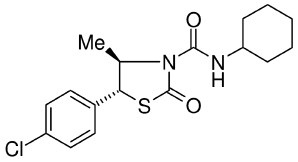
Table 26.2.6 Selected structure–activity data in the area of thiazolidinones.



Entry	R1	R2	X	Relative *Tetranychus* activity (0 = weak, 3 = excellent)
1	H	Cyclohexyl	H	0
2 Lead compound	Me	Cyclohexyl	H	2
3	Me	Cyclopentyl	H	1
4	Me	*n*-Hexyl	H	0
5	Me	*i*-Pr	H	0
6	Me	Ph	H	0
7	Me	Cyclohexyl	2-Cl	1
8	Me	Cyclohexyl	3-Cl	2
9	Me	Cyclohexyl	3,4-Cl_2	1
10 Hexythiazox	**Me**	**Cyclohexyl**	**4-Cl**	**3**
11	Et	Cyclohexyl	4-Cl	2
12	*n*-Pr	Cyclohexyl	4-Cl	0
13	*i*-Pr	Cyclohexyl	4-Cl	0
14	Me	Cyclohexyl	4-CF_3	3
15	Me	Cyclohexyl	4-Me	2
16	Me	Cyclohexyl	4-OMe	2

Score: EC_{50}: 0: >125 ppm; 1: 125–10 ppm; 2: 10–1 ppm; 3: <1 ppm.

Table 26.2.7 Data for hexythiazox.



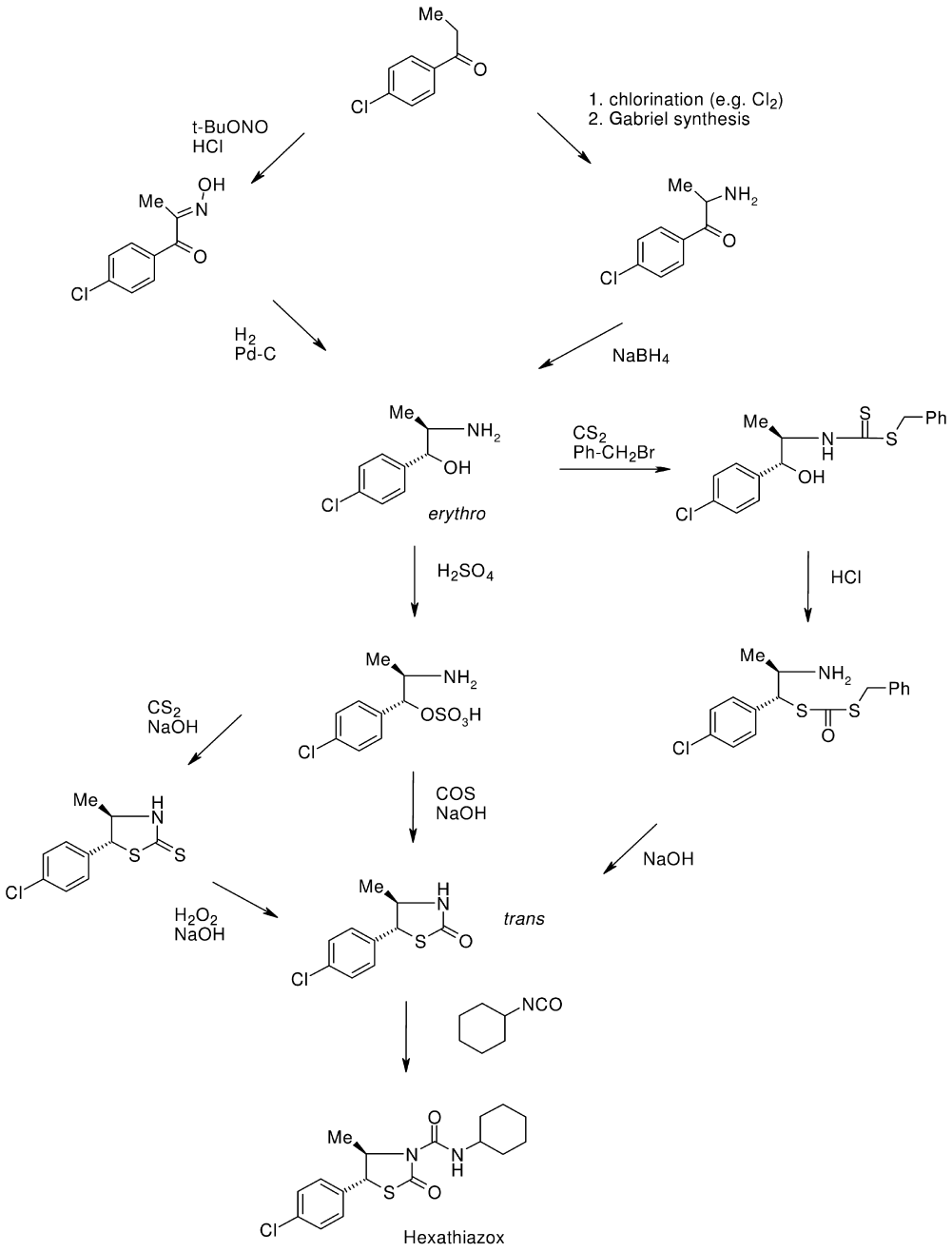
(4R,5R)-(+) Enantiomer of Hexythiazox

Common name	Hexythiazox
IUPAC name	*trans*-5-(4-Chlorophenyl)-*N*-cyclohexyl-4-methyl-2-oxo-1,3-thiazolidine-3-carboxamide
Development code	NA-73
Patent	DE 03037105 (priority 1979-10-03), US4442116
Launch	1985
Melting point	108 °C
log *P*	2.5
Water solubility	0.5 mg L^{-1}
Toxicity (rat, oral)	>5000 mg kg^{-1}

The examples illustrate the importance of a small alkyl group as R1 (methyl is the optimum), the cyclohexyl group on the amide R2 (even cyclopentyl is less active) and an electron-withdrawing group in the para position of the phenyl ring (X). Regarding these constraints after field trials with different candidates Nippon Soda choose the derivative with the internal code NA-73 (entry 10 in Table 26.2.6) for development under the common name hexythiazox (Table 26.2.7) [18]. Only the trans diastereomer shows acaricidal activity – separation of the enantiomers showed that the activity is related to the (4R,5R) enantiomer; the (4S,5S) enantiomer is inactive [17].

Commercial hexythiazox is a racemic mixture of the two trans enantiomers; Scheme 26.2.2 shows the main synthetic pathways [11, 17, 19]. Starting from 4-chloro propiophenone the key intermediate erythro amino alcohol may be obtained by stereoselective catalytic reduction of the corresponding hydroxy iminoketone or by sodium borohydride reduction of the aminoketones obtained via Gabriel synthesis. Different routes lead from this aminoalcohol to the trans-thiazolidinone system; the basis of all routes is activation of the hydroxy group, e.g., in form of the sulfonate and a ring forming reaction with carbon disulfide or carbonyl sulfide. The final acylation of the NH group with cyclohexyl isocyanate leads to hexythiazox.

Nippon Soda presented hexythiazox at the beginning of the 1980s [20] and launched the product in 1985 as, for example, Nissorun® (Nippon Soda), Cesar® (AgrEvo) and Ordoval® (BASF).



Scheme 26.2.2. Synthetic pathways to hexythiazox.

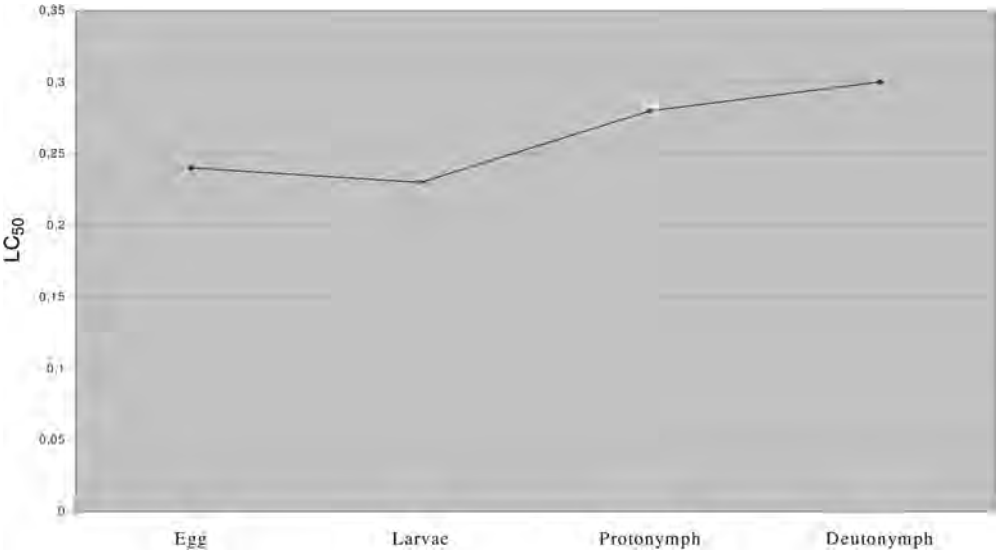


Fig. 26.2.2. LC_{50} values of hexathiazox against different development stages of *Tetranychus urticae*.

26.2.3.1 **Biology and Biochemistry**

Hexythiazox shows a broad acaricidal spectrum against different mites such as *Tetranychus* spp. and *Panonychus* spp. with LC_{50}s between 0.2 and 1.1 ppm (Fig. 26.2.2) [20]. It has ovicidal and larvicidal activity but it is poor against adult mites ($LC_{50} > 500$ ppm).

Hexathiazox is used in apples, citrus, vine, vegetables and cotton. Table 26.2.8 shows the application rates [20].

Hexythiazox has a very long-lasting activity, e.g., against *Brevipalpus phoenicis* it was efficient up to 127 days from the application date [21].

Hexythiazox has a highly favorable ecotoxicological profile being safe against predatory mites and beneficial insects. This property makes the compound especially useful in IPM situations [22] or where predatory mites were used together with an acaricide to suppress *T. urticae* populations [23]. Resistance has been developed in different strains of mites like *T. urticae* in Australia [14, 15], *P. ulmi* [14] and *P. citri* [24] against this compound, which might be based on a single major gene mutation [24].

26.2.4
Oxazolines (Etoxazole)

During its oxazoline chemistry program Yashima discovered the high acaricidal activities of 2,4-diphenyl-1,3-oxazolines of structure type shown in Table 26.2.9

Table 26.2.8 Field application hexythiazox.

Crop	Mite	Dosage (a.i. g^{-1} hl^{-1})
Apple	European red mite (*P. ulmi*) Two spotted spider mite (*Tetranychus urticae*)	3.0–5.0
Citrus	Citrus red mite (*P. citri*)	2.5–5.0
Vine	European red mite (*P. ulmi*) Two spotted spider mite (*T. urticae*) Yellow grape mite (*Eotetranychus carpini* f. *vitis*)	2.5–5.0
Other fruits	Two spotted spider mite (*T. urticae*) European red mite (*P. ulmi*)	3.0–5.0
Vegetables	Two spotted spider mite (*T. urticae*) Carmin spider mite (*T. cinnarabinus*) Desert spider mite (*T. desertorum*)	3.0–5.0 + adulticide

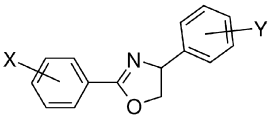
[25]. Some structure–activity data of selected compounds synthesized during the optimization program are also given in Table 26.2.9 [26].

Starting from the unsubstituted lead compound (entry 1, Table 26.2.9) it was discovered that, especially, ortho substitution X enhances the activity; the 2,6-difluoro pattern was kept constant in further optimizations due to its good aphicidal activity (entry 10, Table 26.2.9). Concerning the substituents Y in the second phenyl ring it was recognized that alkyl substituents in the para position show especially favorable acaricidal and aphicidal activities (e.g., entries 14 and 17 in Table 26.2.9). An additional ortho substituent in this ring can increase the activity, possibly via suppression of the oxidative detoxification of the oxazoline to a oxazole heterocycle. An optimal combination was found with the 2-ethoxy, 4-*t*-butyl pattern, which showed high activity against *Tetranychus*, *Plutella* and *Myzus* (entry 20, Table 26.2.9) and was therefore chosen for development under the internal code YI-5301 (common name etoxazole).

The synthesis of etoxazole is shown in Scheme 26.2.3 [19, 26]. Starting from 2-ethoxy-4-*t*-butyl acetophenone standard procedures lead to an oxime intermediate, which is reduced to the corresponding amino alcohol. Acylation of this amino alcohol with 2,6-difluorobenzoyl chloride and subsequent base-catalyzed cyclization after activation of the hydroxy group leads to etoxazole (**4**). An alternative route starts with the amino acid ester, which is first acylated using 2,6-difluorobenzoyl chloride and then reduced with sodium borohydride to the same final intermediate.

Etoxazole was first launched in 1998 by Yashima in Japan under the trade name Baroque® and is further marketed together with Sumitomo (Table 26.2.10).

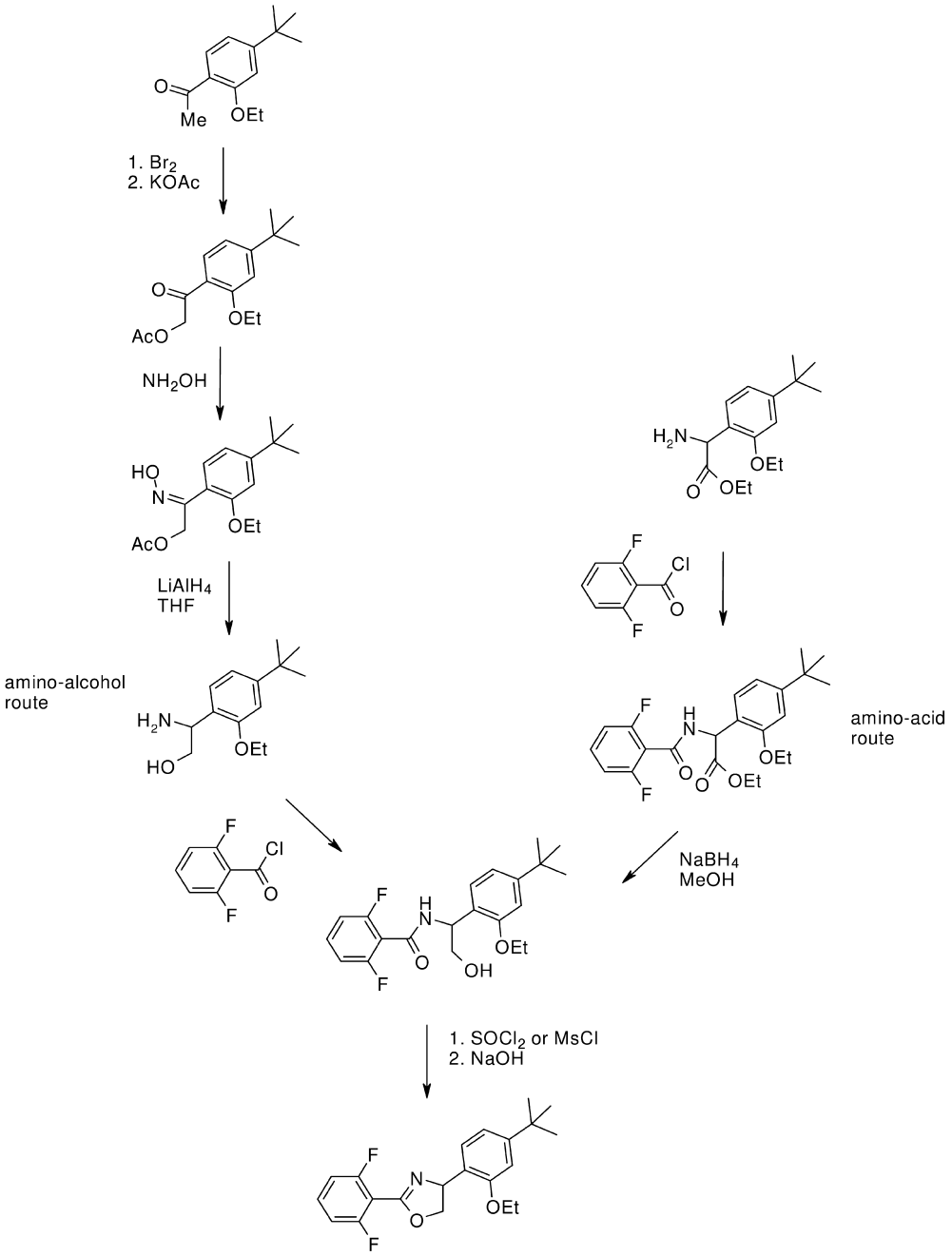
Table 26.2.9 Selected structure–activity data of diphenyl-oxazolines.



Compound no	X	Y	Relative acaricidal TETRUR activity, eggs (0 = weak, 6 = excellent)	Relative insecticidal PLUTMA activity (0 = weak, 5 = excellent)	Relative aphicidal MYZUPE activity (0 = weak, 5 = excellent)
1 Lead compound	H	H	1	3	0
2	2-Cl	H	2	2	0
3	3-Cl	H	0	3	0
4	4-Cl	H	0	3	0
5	2-Me	H	0	3	3
6	2-OMe	H	1	1	0
7	2-F	H	1	2	0
8	2,6-Cl_2	H	2	1	1
9	2-Cl,6-F	H	4	0	1
10	2,6-F_2	H	2	0	5
11	2,6-F_2	2-Cl	3	2	5
12	2,6-F_2	3-Cl	2	0	0
13	2,6-F_2	4-Cl	6	0	0
14	2,6-F_2	4-Me	3	0	5
15	2,6-F_2	2-OMe	1	1	0
16	2,6-F_2	2-OEt	1	1	0
17	2,6-F_2	4-*t*-Bu	6	0	5
18	2,6-F_2	2,4-Cl_2	4	3	5
19	2,6-F_2	2,4-Me_2	1	2	5
20 Etoxazole (**4**)	2,6-F_2	2-OEt, 4-*t*-Bu	6	5	5

26.2.4.1 **Biology and Biochemistry**

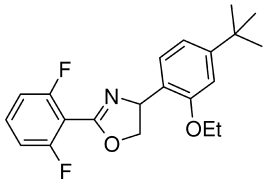
Like hexythiazox, etoxazole has excellent activity on eggs, larvae, protonymphs and deutonymphs of susceptible mites, but has no activity on adult mites. But, in contrast to all insecticides with activity on the molting process, etoxazole shows good effects on juvenile stages of aphids – their molting process was incomplete after treatment with the compound in the laboratory with LC_{50} between 0.5 and 2.2 ppm for *A. gossypii* and *M. persicae* nymphs [27] (also no activity against adult aphids). The activity of etoxazole (YI-5301) on eggs of four major spider mite spp. (*T. urticae*, *T. kanzawai*, *P. citri*, *P. ulmi*) was described to be 100× higher than for hexythiazox, which was also demonstrated by treatment of larvae, proto-



Etoxazole **4**

Scheme 26.2.3. Synthetic pathways leading to etoxazole.

Table 26.2.10 Data for etoxazole.



Common name	Etoxazole
IUPAC name	5-*tert*-Butyl-[2-(2,6-difluorophenyl)-4,5-dihydro-1,3-oxazol-4-yl]-phenetole
Development code	YI-5301 (Yashima), S-1283 (Sumitomo)
Patent	WO9322297 (priority 1992-04-28) [25]
Launch	1998
Melting point	101–102 °C
log *P*	5.59
Water solubility	0.075 mg L^{-1}
Toxicity (rat, oral)	>5000 mg kg^{-1}

nymphs and deutonymphs of these mites. (LC_{50} between 0.0008 and 0.08 ppm against the different developmental stages of the different mites). Against eggs of hexythiazox-resistant *P. citri* and *T. kanzawai* (hexythiazox $LC_{50} > 1000$ ppm) etoxazole was equally active as on susceptible ones [27]. The biochemical pathway that etoxazole effects in mites and aphids was speculated to be very similar to that of benzoylureas [26]. This is supported by the findings of Nauen and Smagghe, who describe chitin biosynthesis inhibition by etoxazole in *Spodoptera frugiperda* with similar symptomatology of poisoning to triflumuron [28]. The authors demonstrated that chitin biosynthesis is inhibited in whole larvae as well as in isolated integuments.

Under field conditions etoxazole can be used in many crops [apples, cherries, citrus, cotton, cucumbers, egg plants (aubergines), fruit, ground covers, Japanese medlar, melons, ornamental plants, ornamental trees, peas, shrubs, strawberries, tea, tomatoes, watermelons, vegetables, vines] against all important mites (*Amblyseius fallacis*, *E. carpini*, *E. lewisi*, *Oligonychus illicis*, *O. ununguis*, *P. citri*, *P. ulmi*, *Stethorus punctum*, *T. cinnabarinus*, *T. pacificus*, *T. urticae*). The field application rates vary between 5 to 10 g hl^{-1} and 100–150 g ha^{-1}, depending upon the crop and the water volume used. The smallest necessary amount is in cotton with 32–50 g-a.i. ha^{-1}.

The compound has already been launched in several countries and will soon be launched in others (Table 26.2.11).

Table 26.2.11 Marketing status of etoxazol (status 2004).

Country	Trade name	Crops	Status	Date
Japan	Baroque®	Citrus, melons, cherries, watermelons, apples, peas, peaches, tea, strawberries, cucumbers, egg plants, Japanese medlar	Launched	1998
S Korea	Zoom®	Citrus	Launched	1998
Turkey	Zoom®	Apples, citrus, tomatoes, cotton	Launched	1998
Taiwan	Zoom®		Launched	2000
US	Zeal® Tetrasan®	Cotton Outdoor uses: shrubs, ornamental trees, flowering crops, foliage crops, ground covers	Approved Launched Launched	2003 2004 2003
Australia	Paramite®	Apples, cotton	Approved	2004
EU		Fruit, vegetables, cotton	Dossier declared complete	1999>
France	Bornéo®	Vines, apples, peaches, pears, nectarines, apricots	Launched	2002
Belgium	Bornéo®	Vines, fruit trees	To launch	2004
Italy	Bornéo®	Vines, fruit trees	To launch	
Spain	Bornéo®	Vines, fruit trees	To launch	
South Africa	Smite (Philagro) Rose spider mite (Ball Straathof)	Roses, apples, tomatoes, pears	Approved	

Resistance against etoxazole has been described in *T. urticae* and demonstrated to be under monogenic control [29].

The ecobiological properties of etoxazole are not as favorable, allowing its use in combination with predatory mites. In experiments on the comparative toxicity of some acaricides to the predatory mite *Phytoseiulus persimilis* and the twospotted spider mite, *T. urticae*, etoxazole did not seriously affect the survival and reproduc-

tion of adult female predators, but caused high mortality rates in eggs and larvae of *P. persimilis* [30].

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27
Midgut – Transgenic Crops Expressing *Bacillus thuringiensis* Cry Proteins

Jeroen Van Rie and Stefan Jansens

27.1
Introduction

Worldwide preharvest crop losses have been estimated to be 13.8% from insects and other arthropods, 11.6% from disease (fungi, bacteria and viruses) and 9.5% from weeds [1]. Total crop losses in Africa and Asia, the continents with the largest annual human population increase, reach almost 50% [2]. To control insects efficiently in a sustainable way, synthetic insecticides must be integrated with alternative pest control methods. One method involves the use of resistant plant varieties obtained through "classical breeding". Another alternative is the use of biological insecticides, such as sprayable formulations based on *Bacillus thuringiensis* (Bt). However, due to their limited field stability, lack of capacity to reach cryptic insects and narrow spectrum of activity, Bt sprays still represent only a minor fraction of the insecticide market. Transgenic plants expressing Bt insecticidal crystal protein (Cry) proteins overcome the first two drawbacks. The potential benefits of Bt crops include increased crop yield, reduction in broad-spectrum insecticide use and associated application costs and energy input, reduced need for scouting, improvement of health conditions of farm workers and, time savings. These benefits should be balanced against putative safety and environmental risks, as compared with benefits and risks of insect control in conventional agriculture.

27.2
Plant Engineering

Significant progress has been made since the first successful transformations of plants. The capacity to introduce and express foreign genes in plants now extends to over 120 species, including some previously classified as recalcitrant [3]. *Agrobacterium*-mediated transformation has proven an efficient and reliable method to engineer different traits in a wide range of crops, both dicotyledonous

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

and monocotyledonous plants [4]. The main advantages of this DNA transfer method are the low level of rearrangements in the transforming DNA and the high number of plants with a single insertion of the transgene. In contrast, direct gene delivery systems such as particle bombardment or protoplast electroporation frequently result in a higher frequency of complex patterns of transgene integration. Equally important was the development of tissue culture techniques allowing the production of highly regenerable tissues from immature undifferentiated tissue and the development of tools to control the expression of a transgene in a plant. Plant transformation is still a random process with respect to the integration site of the transgene into the plant genome, sometimes resulting in suboptimal transgene expression or a negative impact on the expression of endogenous plant genes. Somaclonal variation is another aspect that can potentially lead to transgenic plants with suboptimal characteristics. Gene silencing, probably due to the presence of multiple copies of foreign gene sequences, has also been observed in transformed plants. Together, these phenomena necessitate the generation of a large number of transgenic plant lines (events) from which those plants with the best performance (elite events) have to be selected through several rounds of laboratory and field evaluations, a process sometimes referred to as elite event selection.

27.3
Insecticidal Crystal Proteins from *B. thuringiensis*

Formulations of Bt spore-crystal mixtures have been used for more than 40 years and have demonstrated that Bt is a very specific, effective and safe bioinsecticide. The insecticidal activity of Bt is mainly due to the presence of the insecticidal crystal proteins (Cry and Cyt proteins) and vegetative insecticidal proteins (Vip proteins). Vip proteins are mostly produced during the vegetative stage of growth of the bacterium. Vip1 and Vip2 binary toxins are specific for coleopteran insects, whereas Vip3 proteins are specific for Lepidoptera [5]. Knowledge on the mode of action of these toxins is rather limited [6–8]. In contrast, Cry proteins are produced during sporulation, and much data are available on their mode of action, mainly based on studies of Cry1A proteins. The following model for the pathway of toxic action has been proposed [9]: when ingested by susceptible insects, the crystals dissolve in the insect gut and the protoxins are liberated and proteolytically activated to a toxic fragment. This fragment passes through the peritrophic membrane, binds to a specific cadherin on the brush border membrane of gut epithelial cells and oligomerizes into a tetramer. The oligomer binds to an aminopeptidase N, is driven into lipid raft microdomains and (partially) inserts into the membrane, generating pores. The change in membrane permeability leads to colloid osmotic lysis of gut epithelial cells and ultimately to death of the insect. Thus, binding, at least of Cry1A proteins, appears to involve several different proteins, including aminopeptidases and cadherin-like proteins. The functional role

of aminopeptidase N in toxicity has been indicated by RNAi experiments in *Spodoptera litura* [10] and by ectopic expression in *Drosophila melanogaster* larvae [11]. The significance of cadherin-like proteins as receptors has been demonstrated by ectopic expression in different cell lines [12, 13] and is further corroborated by the presence of mutated cadherin genes in resistant *Heliothis virescens* [14, 15] and *Pectinophora gossypiella* [16, 17] insect strains. In addition, glycolipids and glycosylated alkaline phosphatase have been implicated in Cry binding [18, 19].

More than 300 Cry sequences are currently known and classified solely on the basis of sequence homology of the full-length proteins into 49 Cry classes [20, 21]. There is no simple correlation between sequence and insecticidal spectrum, but some generalizations can be made. For example, Cry1 and Cry9 proteins are active on lepidopteran larvae, whereas Cry3, Cry7 and Cry8 proteins are active on coleopteran larvae. However, within a certain class, Cry proteins may have widely differing activity spectra. This specificity is still one of the most intriguing aspects of Cry proteins. Any step of the mode of action can influence the activity spectrum.

Many Cry proteins, such as Cry1 and Cry9, are protoxins of about 120 to 140 kDa that are proteolytically processed to an active toxic fragment of about 60 to 70 kDa. Characterization of the proteolytic fragment and fragments generated by the expression of truncated *cry* genes has indicated that, while only few amino acids can be removed from the N-terminus without interfering with biological activity, about half of the protoxin can be removed at the C-terminus. Other Cry proteins, such as Cry2, are proteins of about 70 kDa and appear to require no C-terminal activation for toxicity. Upon alignment of Cry amino acid sequences, sequence variation is clearly not distributed in a random fashion. Five conserved sequence blocks can be distinguished in the activated fragment of most Cry proteins.

Today, the crystal structure of six activated Cry proteins has been solved [22–27]. These proteins, Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Bb, and Cry4Ba, have a very similar architecture and are composed of three structural domains. The N-terminal domain [residues 58–290 (in Cry3A)] contains seven α-helices with the central more hydrophobic helix (α5) encircled by six outer amphipathic helices. The second domain (residues 291–500) consists of three β sheets, packed as three sides of a prism. The third, C-terminal, domain (residues 501–644) is a β sandwich with the outer sheet facing the solvent and the inner sheet facing the other two domains. The level of amino acid sequence homology between some of the six Cry proteins is very low, yet their global structure is quite similar. This suggests that most other Cry proteins possess a similar global architecture. Based on these structures and the characterization of Cry mutants and hybrids, the following hypotheses have been put forward regarding the function of the three structural domains of Cry's: the long amphipathic helices of domain I would be responsible for pore formation; domain II would play a major role in receptor binding; domain III also plays a role in receptor binding and perhaps modulates pore formation [28, 29].

Fig. 27.1. Tobacco plants expressing a truncated *cry1Ab* gene resulting in significant levels of Cry1Ab protein and high insecticidal activity to *Manduca sexta* larvae feeding from the leaves.

27.4 Bt Plants

The first experiments to create plants expressing *B. thuringiensis cry* genes (Bt plants) used T-DNA vectors in *Agrobacterium* carrying the coding sequence for Cry1A protoxins. Only very low levels of Cry proteins and no significant insecticidal activity related to Bt was observed [30–32]. The first successes were obtained by expressing gene fragments encoding the toxic part of the Cry protein only. Expression of truncated *cry1Aa* [31] and *cry1Ab* [32] (Fig. 27.1) genes in tobacco resulted in significant levels of protein and high insecticidal activity to *Manduca sexta* larvae feeding from the leaves. Also, tomato plants engineered with a truncated *cry1Ac* gene [33] proved to be protected from feeding damage by *M. sexta* and resulted in mortality or growth inhibition of *H. virescens* and *Helicoverpa zea* larvae. Tubers from different potato varieties engineered with a truncated *cry1Ab* gene and infested with potato tuber moth (*Phthorimea operculella*) larvae did not show tunneling or feeding damage following a 2 month storage period [34].

When tested under agronomic conditions in the field, transgenic tomato [35] and tobacco plants [36] expressing truncated *cry* genes showed substantial levels of insecticidal activity against their primary pest insects. Yet, it became apparent that, for certain crops or insect pests, higher Cry protein expression levels were needed to achieve complete insect control in the field. Typically, expression levels of native truncated *cry* genes in plants, usually about 0.001% of total soluble pro-

tein, were lower than levels obtained with other transgenes. Plant genes generally have a high G+C content whereas bacterial *cry* genes typically have a high A+T content. A+T-rich regions in native *cry* genes contain cryptic intron splice sites [37] and potential polyadenylation sites [38], resulting in aberrant splicing or premature polyadenylation, both leading to non-functional mRNA. Furthermore, the codon usage in *cry* and plant genes is significantly different. The presence of rare plant codons in native *cry* genes could result in ribosomal pausing [38] and perhaps in accelerated degradation of the *cry* gene messenger. However, experimental evidence suggests that the presence of rare codons per se does not dramatically interfere with mRNA accumulation [39, 40]. On the other hand, comparison of mRNA and protein levels in plants transformed with truncated *cry1Ab* genes with different degrees of modification led Perlak et al. [41] to suggest that the presence of predominantly plant preferred codons improved the overall *cry* gene translational efficiency.

Several authors demonstrated that modifications in a specific region could result in significant improvements in expression. For example, Perlak et al. [41] found that changes in the 5′ half of the *cry1Ab* gene were more efficient in achieving increased expression levels than changes in the 3′ half. Cornelissen et al. [42] identified a region between nucleotides 785 to 1285 in *cry1Ab* where transcript elongation was retarded. Tobacco plants transformed with a *cry1Ab* gene with 63 translationally neutral substitutions in this region showed up to 20-fold higher level of *cry1Ab* transcript [43]. Furthermore, these authors demonstrated that modifications that removed cryptic splice sites caused further increases in transcript levels. Although modifications in a specific region can result in significant improvements in expression, translationally neutral nucleotide changes throughout the *cry* coding region are mostly used to obtain the highest levels of expression of *cry* genes integrated in the nuclear genome. A truncated *cry1A* gene was rendered more ''plant like'' by modifications, including the removal of potential polyadenylation signals and ATTTA sequences by changing 62 of the 1743 nucleotides [41]. Transformation of tobacco and tomato with constructs containing this modified gene resulted in a higher number of insecticidal plants and higher expression levels (0.02% of total soluble protein) than constructs containing the wild-type genes. Another modified *cry1A* gene, containing additional changes to increase overall G+C content and to introduce plant-preferred codons, increased expression levels up to about 0.2% of the total soluble proteins. Similar results were obtained for a modified truncated *cry1Ac* gene. Cotton engineered with these modified genes showed protection from feeding damage by their main lepidopteran pests [44, 45]. More recently, *cry* genes are optimized for plant expression by taking into account the above parameters and are constructed by total gene synthesis [46].

In the early 1990s the two main monocot crops, maize and rice, were successfully transformed to express Cry proteins. Maize transformed with a truncated modified *cry1Ab* gene [47], driven by either a constitutive CaMV 35S promoter or the combination of a green tissue and pollen specific promoter, was reported to result in plants with high levels of expression (up to 4 μg mg^{-1} total plant pro-

Fig. 27.2. Cry1Ab corn event (right) gives excellent European corn borer control, compared with the non-transgenic control B73 (left). Split corn stalks and ears are depicted, following artificial infestation of corn plants with more than 1000 neonate *Ostrinia nubilalis* larvae per plant.

tein). Field trials confirmed that tunneling of corn stalks by European corn borer (*Ostrinia nubilalis*) was dramatically reduced in such plants. Armstrong et al. [48] transformed maize with a modified *cry1Ab* gene driven by the constitutive CaMV 35S promoter and found excellent European corn borer control. Similarly, maize lines transformed with a modified *cry9C* or Cry1Ab gene, driven by the CaMV 35S promoter and including 5′ untranslated leader sequences, showed complete inhibition of stalk tunneling by *O. nubilalis* in greenhouse and field trials, and reduced feeding damage by *Agrotis ipsilon* [49] (Fig. 27.2). Certain modifications outside the coding region may also contribute to Cry protein expression in plants. With rice (a monocotyledonous plant), introns are frequently introduced to increase Cry protein expression levels giving rice stem borer control [50–53]. In maize, Armstrong et al. [48] also introduced an intron to increase Cry protein expression levels.

Modified or "synthetic" *cry1A* genes have now been used for the transformation of several additional plant species, including peanut, Chinese flowering cabbage, canola, broccoli and soybean [46], coffee [54] and "exotic" crops, such as persimmon [55] and walnut [56]. Similarly, a *cry1C* gene redesigned for high level expression in plants provided protection to *Spodoptera littoralis* and *Spodoptera exigua* in transgenic tobacco and alfalfa [57] and protection from *Plutella xylostella* in transgenic broccoli [58].

Using plastid transformation, high levels of expression can be obtained without the use of modified or synthetic genes. It has been observed that bacterial genes are well expressed in plant plastids without any optimization of the codon usage.

In this way, high levels of Cry1Ac [59], Cry2Aa2 [60] and Cry1Ia5 [61] were obtained in tobacco and high levels of Cry1Ab in soybean [62].

A next important step was the first registration in 1995 by the U.S. Environmental Protection Agency (EPA) of Bt maize, Bt cotton and Bt potato. Now, over ten years later, about 90 million hectares of transgenic crops are grown in the world [63]. One quarter are Bt crops, mainly Bt maize and Bt cotton. The USA and Argentina grow most transgenic crops, followed by Brazil, Canada, China, Paraguay and India [63].

The most dominant Bt crop is Bt maize, commercially grown in 2005 in the USA, Argentina, Canada, South Africa and on lesser acreage in the Philippines, Spain, Uruguay, Honduras, Portugal, Germany, France and the Czech Republic: 11.2 million hectares of Bt maize and 6.5 million hectares of Bt/herbicide tolerant maize were grown [63]. The different Bt maize events target two maize pest complexes: lepidopteran corn borers and coleopteran corn rootworms. The maize event Mon810, containing a Cry1Ab protein that is constitutively expressed, represents >85% of the Bt maize planted worldwide, followed by the maize event Bt11, which also constitutively expresses the Cry1Ab protein [64]. Both are sold under the trade name ''Yieldgard'' and have an excellent control of corn borers. More recently, in 2003 the maize event TC1507, containing a different Cry protein, Cry1F, was introduced in North America as ''Herculex I''. In addition to excellent corn borer control, it was reported to provide control of armyworms and cutworms. In 2003, the first event that controls corn rootworm (*Diabrotica* spp.), the most destructive pest of maize in North America, was commercially introduced in the USA [65]. The Mon863 event, sold as ''Yieldgard rootworm'', contains a modified *cry3Bb1* gene, optimized for expression in monocots and driven by a root-enhanced promoter. Also in the USA, the event DAS-59122-7, ''Herculex rootworm'', will was introduced in 2006. A binary delta-endotoxin Cry34/35Ab1 from the *B. thuringiensis* strain PS149B1 was introduced into the maize and was reported to give excellent corn rootworm control [66].

Bt cotton and Bt/herbicide tolerant cotton was grown in 2005 on 4.9 and 3.6 million hectares, respectively, mainly in the USA and China, and also in South Africa, Australia, India and Argentina [63]. The event Mon531, containing the *cry1Ac* gene driven by a constitutive promoter and sold as ''Bollgard'' in the US or as ''Ingard'' in Australia, has been the most important Bt cotton event in the last 10 years. It has near 100% control of square and boll damage against the tobacco budworm (*H. virescens*) [196] and pink bollworm (*P. gossypiella*). However, against the cotton bollworm (*Helicoverpa armigera* in the Old Word and *H. zea* in the New World) control was not always complete [67] and extra foliar insecticide applications were needed [68]. Since the introduction of ''Bollgard'', sprays to control lepidopteran pests have been reduced by about half. In China, besides the Mon531 event, cotton varieties GK using a modified Bt fusion gene *cry1Ab/cry1Ac* were developed by public research institutes led by the Chinese Academy of Agricultural science (CAAS) and have been grown since 1997 [69]. More recently CAAS had a new genetically engineered variety, SGK321, approved [70]. Two pesticidal genes, the Bt fusion gene *cry1Ab/cry1Ac* and the cowpea trypsin

inhibitor, were inserted in cotton. CAAS expects that the bollworm will take more generations to develop insect resistance when using these two genes. In 2003 a second generation of Bt cotton, which expresses two insecticidal Cry proteins, was introduced in the US. The ''Bollgard II'' event Mon15985 was created by inserting the *cry2Ab* gene into the ''Bollgard'' event Mon531. ''Bollgard II'' expresses more toxin and the levels of insect control are higher than in ''Bollgard'' [71]. Field studies showed that ''Bollgard II'' provided excellent cotton bollworm control and an increased efficacy against armyworms and loopers [72]. Another two insecticidal protein product, ''Widestrike'', was produced by cross-breeding two insect-resistant cotton events: DAS-24236-5, producing constitutively Cry1F protein and DAS-21023-5, producing constitutively Cry1Ac protein. Introduced in the US market in 2005, it was reported to give excellent control of bud- and bollworm [73] and the presence of the Cry1F protein in the transgenic variety increases control of fall armyworm (*Spodoptera frugiperda*) and beet armyworm (*S. exigua*) [74]. The ''VipCot'' Bt cotton event Cot102, which is likely to be grown by the US farmers in the coming years, expresses a different type of insectidal protein from Bt, Vip3A [5, 75]. It has no sequence or structural homology with the crystal proteins of *B. thuringiensis* and has a different mode of action [6, 7, 28]. It may retard resistance evolution to Cry toxins in cotton lepidopteran pests. Extensive field evaluation was reported to indicate efficacious control of bud- and bollworms, beet armyworm and soybean looper [76]. Bacheler and Mott [77] compared all the above-mentioned Bt cotton events in adjacent fields under the same cotton bollworm pressure and agronomic conditions. In this study, with high bollworm insect pressure ''Bollgard II'' gave excellent control, with 6% peak boll damage in September. ''Widestrike'' and the ''VipCot'' Cot102 event gave 15% and 14% peak boll damage, respectively – much less than the adjacent ''Bollgard''. Other ''VipCot'' events, Cot202 and Cot203, where the *vip3A* gene is driven by a stronger constitutive promoter, are under development and are being field tested under high bollworm pressure [78]. They gave around 1% boll damage – much better than with Cot102 and the non-transgenic control, which gave 10% and 73.2%, respectively.

It was expected that China would be the first country to introduce Bt rice on the market to control economically imported lepidopteran rice pests such as striped stem borer (*Chilo suppressalis*), yellow stem borer (*Scirpophaga incertulas*) and rice leaf folder (*Cnaphalocrocis medinalis*). The KMD1 and KMD2 lines, generated by transforming Xuishi 11, a commercial Chinese *japonica* rice, with a synthetic *cry1Ab* gene under control of a maize ubiquitin promoter [79] has been extensively field tested in China. Shu et al. [80] reported in 2000 that the KMD1 line was resistant to eight different lepidopteran pests. Many years of field testing [81, 82] confirmed the high level of stable resistance against stem borers and leaf folders. A construct containing a *cry1Ab*/*cry1Ac* fusion gene driven by the rice actin I promoter [83] has been introduced and field tested in an *indica* hybrid rice Shanyou 63 [84] and in an elite *indica* rice IR72 [85]. In both cases, a high level of protection was found against rice stem borers and rice leaf folders. However, the Bt rice developed by the Agricultural Biotechnology Research Institute at

Karaj was officially released in Iran in 2004 on 2000 ha and full commercialization is expected in 2006 in Iran, when 10000–20000 ha will be planted [63]. The aromatic variety Tarom Molaii was transformed with a synthetic *cry1Ab* gene under the control of the phospho-enolpyruvate carboxylase (PEPC) promoter [86]. Alinia et al. [87] showed in greenhouse tests that stem borer and leaf folder control was good in early plant stages but declined at the flowering stage. Basmati Bt rice is being field tested and developed by Pakistan: rice expressing the synthetic *cry1Ab* gene under the control of different promoters and expressing the synthetic *cry2A* gene under the control of the CaMV 35S promoter [88, 89].

Contrary to most groups that use constitutive or tissue specific promoters to drive the expression of *cry* genes such as *cry1Ab*, *cry1Ac*, *cry1Ab*/*cry1Ac* and *cry2A*, Breitler et al. [90] used the −689/+197 region of the maize protease inhibitor gene to direct wound induced expression of the *cry1B* gene in the elite *japonica* cultivar Ariete. They found satisfactory levels of striped stem borer (*C. suppressalis*) control, with a low level of stem penetration, but with higher external symptoms compared with the rice lines where the expression is constitutively driven by the maize ubiquitin promoter. The authors think that this difference is due to the time lag before the plant is protected by the Cry1B protein.

27.5 Insect Resistance to Bt

Evolution of resistance in insect populations is a serious threat to the continued success of Bt crops. In 1985, the first report on resistance to Bt was published: a 250-fold level of resistance to Bt was observed in a *Plodia interpunctella* population from grain bins that were regularly treated with Bt. Since then, a substantial number of strains of different insect species with various levels of resistance to Cry proteins have been obtained by laboratory selection experiments or established from field collections [91]. Laboratory selection experiments do not predict if resistance will develop in the field or which resistance mechanisms will be selected, but can indicate the repertoire of resistance mechanisms available in a certain population. The only species for which field-evolved resistance has been observed is *P. xylostella* (diamondback moth). Insects could, in principle, become resistant to Cry proteins due to mutations in genes encoding proteins involved in any of the different steps in the mode of action. Several mechanisms have been observed in laboratory selected insect strains [91], such as altered binding to midgut receptors, altered protoxin activation, toxin degradation, more efficient repair (or replacement) of damaged midgut cells, esterase sequestration [92] and elevated immune status [93]. In contrast, only one major mechanism, i.e., altered binding, has so far been detected in field selected resistant insects. In most of such cases, the pattern of cross-resistance parallels the pattern of binding specificity of the Cry proteins in the particular insect species. For example, a *P. xylostella* strain collected from fields in Hawaii, which had been treated with a spray-

able Bt product (DiPel) and further selected in the laboratory, had high levels of (cross-) resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja but no significant (cross-) resistance to Cry1Ba, Cry1Bb, Cry1Ca, Cry1Da, Cry1Ia or Cry2Aa [94]. Binding of Cry1Ab and Cry1Ac, but not Cry1Ca, was strongly reduced in this resistant strain. A very similar pattern of resistance and binding characteristics was observed in a *P. xylostella* strain from Pennsylvania [95], the Philippines [96] and Florida [97], as well as in two *P. gossypiella* resistant strains [98]. Indeed, while there was a complete lack of Cry1Ab binding in the *P. xylostella* strain from Florida, binding of Cry1B and Cry1C was unaltered [97]. These cross-resistance and binding data in *P. xylostella* can be understood in view of the model for the Cry binding sites in this species: according to this model, one site (site 1) is recognized only by Cry1Aa; another (site 2) is shared among Cry1Aa, Cry1Ab, Cry1Ac, Cry1F and Cry1J; and two additional sites bind Cry1Ba (site 3) and Cry1Ca (site 4) [91]. Site 1 appears to be a non-functional binding site. Resistant *P. xylostella* strains, selected with Cry1A containing Bt products, appear to have an altered site 2, explaining their cross-resistance to Cry1F and Cry1J, while their site 3 and site 4 have remained unaltered, explaining their full susceptibility towards these two Cry proteins. The lack of such cross-resistance can be exploited in resistance management strategies. Resistance, at least in field-evolved resistant insect strains, appears to be autosomally inherited and in many cases involves a single major locus, as assessed from backcross data, and reverts upon withdrawal of selection, suggesting fitness costs associated with resistance genes or closely linked loci. On a molecular genetic level, mutant alleles of a cadherin gene, encoding a putative functional Cry1A receptor, have been linked to resistance in *H. virescens* [14, 15] and *P. gossypiella* [16, 17]. The level of dominance has been tested in five resistant insect strains using transgenic plants expressing high levels of Cry proteins; in all these cases, the resistance to the expressed Cry protein was demonstrated to be functionally recessive [16, 99–102]. Interestingly, also, resistance to Cry proteins in diet or leaf dip bioassays does not necessarily enable resistant larvae to survive on Bt plants [103].

27.6
Resistance Management with Bt Plants

In insects, resistance is a pre-adaptive phenomenon that develops by selection of rare individuals of a population that can survive a certain insecticide treatment. Resistance management strategies try to prevent or diminish the selection of the rare individuals carrying resistance genes and hence to keep the frequency of resistance genes below levels that would result in inefficient insect control.

There are different strategies that at least in theory should slow down the development of insect resistance in Bt plants [104, 105], including tissue-specific Bt protein expression (protecting only the critical tissues), wound-induced Bt protein expression (if expression would only be induced at a threshold level of insect

damage), chemical-induced Bt protein expression, moderate Bt expression, ultra-high Bt protein expression (making the plant a non-host for the pest), rotation of crops expressing different types of Bt proteins, and pyramiding (expression of different Bt protein types in the same crop plant).

When the Bt crops were first commercially grown in 1996, there was a consensus among population geneticists and insect resistance experts that the best insect resistance management (IRM) tactic for Bt crops was the high-dose strategy combined with a refuge. The principle of the high-dose strategy is that the plant tissues express a Bt protein dose high enough to kill all of the most common carriers of resistance, i.e., the heterozygote resistant insects. Using modified Bt genes, a high-dose (defined as 25× the dose needed to kill all homozygous susceptible larvae) should be achieved in plants. A refuge is an area free of toxin-expressing plants that allows homozygote susceptible insects to survive. Provided that the initial allele frequency for resistance is low, any rare homozygous resistant insect – emerging from the Bt crop area – will more likely mate with susceptible insects from the refuge than with other resistant insects. Such crosses will result in heterozygous resistant progeny, which will be killed by the transgenic crop plants, and hence cause a dilution of resistance. Refugia that are temporally and spatially contiguous with the transgenic crop should ensure random mating between homozygote resistant and susceptible adults and should produce a least 500 susceptible moths for each homozygous resistant moth emerging from the Bt crop. Another prerequisite for the high-dose/refuge strategy is that the resistance is recessive or at least partially recessive. As mentioned above, resistance to Cry proteins, as tested on high dose transgenic Bt plants, is indeed functionally recessive. Although the high-dose/refuge strategy may be difficult to realize for sprayable insecticides, it seems likely to be efficacious for Bt plants. Whereas the validity of the high-dose/refuge strategy was originally only based on projections from computer models simulating insect population growth under various conditions, more recent studies have provided experimental support for this strategy. Indeed, selection of *P. xylostella* under laboratory conditions resulted in resistance in colonies without refuge more rapidly than in those provided with a refuge [106]. Furthermore, controlled greenhouse trials [107] and field trials [108] involving Cry1Ac expressing broccoli plants and artificial *P. xylostella* populations, with known Cry1A resistance allele frequencies, have demonstrated that resistance could be delayed by increasing the percentage of refuge plants. Also a 20% refuge, separated from the Bt plants, was more effective in maintaining the population of susceptible insects than a 20% mixed refuge, created by planting a mixture of seeds of Bt and non-Bt plants. This probably holds true for any Bt crop where pest larvae can move between plants to any extent. The authors also reiterated the notion that the size and potential treatment of the refuge should be such that enough susceptible adults emerge from it to "overwhelm" any resistant adults emerging from the Bt crop plants.

Unprecedented in the field of insect control, the US EPA (Environmental Protection Agency) required a compulsory insect resistance management plan, based on the high-dose/refuge strategy, in 1996 with the introduction of Bt crops. Based

on the experience with Bt crops grown under different agronomic conditions the plan is further optimized on a regular basis. Currently, the insect resistance management plan for Lepidoptera controlling Bt maize requires a structured refuge of at least 20% non-Bt maize but 50% in cotton growing areas due to the extra potential selection pressure on *H. zea* from Cry1A expressing cotton. The refuge corn can be treated with insecticides only when the level of pest pressure meets or exceeds the economic threshold and sprayable Bt insecticides must not be applied to the refuge. The refuge must be placed within 0.5 mile (0.25 mile preferred) near the Bt maize field and it can be a separate field, a block within the maize field, the field perimeters or an alternation of four or more consecutive rows of refuge maize with Bt maize [109]. For coleopteran controlling Bt maize, on each farm at least 20% non-Bt should be planted. The refuge maize can be treated for corn rootworm larvae and other soil pests and it must be planted within or adjacent to the Bt maize fields. Alternatively the refuge may be planted as in-field or perimeters strips. These strips must be at least six consecutive rows wide. Three options are possible for Bt cotton: 5% external structured unsprayed refuge, 20% external sprayed refuge and 5% embedded refuge [110, 111]. For the 5% external structured unsprayed refuge, the refuge may not be treated with any insecticide labeled for the control of tobacco budworm, cotton bollworm, or pink bollworm except only at the pre-squaring stage when the refuge may be treated with any insecticide to control foliage feeding caterpillars. The refuge must be at least 150 feet wide and be planted within 0.5 mile (0.25 mile preferred) from the edge of the Bt cotton field. The 5% embedded refuge should have the non-Bt cotton, as a contiguous block within the Bt cotton field, at least 150 feet wide and it may be treated with the same insecticides at the same moment as the Bt cotton field. However, it may not be treated independently of the surrounding Bt cotton field in which it is embedded, except only at the pre-squaring cotton stage when the refuge may be treated with any lepidopteran insecticide to control foliage feeding caterpillars. For areas affected by pink bollworm only, the refuge cotton may be planted as single rows within the Bt cotton field: one non-Bt row for every six to ten rows of Bt cotton. In the 20% external sprayed refuge option one should ensure that at least 20 acres of non-Bt cotton are planted as a refuge for every 100 acres of Bt cotton. All cotton may be treated with insecticides (excluding foliar Bt products) labeled for control of the tobacco budworm, cotton bollworm, or pink bollworm. The refuge must be maintained within 1 mile (preferably within 0.5 mile) from the edge of the Bt cotton. Other requirements of the insect resistance management plan are annual resistance monitoring, grower education, compliance assurance, research and reporting. Research can be on mode of action, pest biology and resistant insect colonies. There is also a requirement for a remedial action plan should insect resistance develop in the field [112].

After 10 years of growing Bt plants, no major insect control failure has occurred. Tabashnik et al. [113] monitored pink bollworm resistance frequency and did not find an increase from 1997 to 2004. Although the high-dose/refuge strategy has contributed to this, some of the prerequisites are not completely fulfilled and there is concern that the demanded refuge size may not be large enough

[104]: random mating may not occur when the resistant and susceptible insects do not have the same development time; incomplete or non-recessive inheritance of insect resistance may occur and certain Bt events may not be high dose for less sensitive insect species. Other issues are contaminations of the Bt seed lots with non-transgenic seeds, placement of the refuge and low grower compliance with the insect resistance management plans.

In the second generation Bt plants, such as ''Bollgard II'', the high-dose/refuge strategy is combined with the strategy of pyramiding or stacking two or more Bt toxins, with a different mode of action, into one variety [114]. In this strategy, all insects, except the extremely rare double homozygous resistant individuals (with complete resistance), will be killed and development of resistance to stacked toxins is expected to be much slower than to single toxin plants [105]. Computer models [105] have shown that refuge size can be reduced from 30–40% when using single Bt plants sequentially to 5–10% for stacked or ''dual'' Bt plants. The EPA, however, did not reduce its refuge requirements when the ''dual'' Bt cotton event ''Bollgard II'' was introduced in the USA [112]. Zhao et al. [115] showed experimentally in the greenhouse that transgenic plants expressing two Bt toxins, binding to a different site in the target insect, delays development of resistance: a population of *P. xylostella* that contains genes for resistance against Cry1Ac and Cry1C developed slower resistance to the stacked Cry1Ac/Cry1Ab broccoli with 20% refuge than to the Cry1Ac broccoli with 20% refuge. Also, compared with single Bt plants deployed in mosaics (with 20% refuge) the resistance development was delayed.

In some countries, with millions of small farmers, it is not so straightforward to implement refuges.

For Bt rice, Cohen et al. [116] propose to create refuges by limiting the number of Bt rice cultivars that can be released in a certain growing area and to focus the implementation of a refuge system on large rice growing estates, collectives and well-organized farmer organizations. He urges to release as fast as possible stacked Bt rice where fewer refuge fields will be needed. A similar option could be proposed for Bt cotton in China, which has no formal refuge requirements [104].

Clearly, Bt plants cannot be considered as a stand-alone product, but rather an additional insect control tool that should be integrated with other pest management tools, such as crop rotation, manipulation of insect predators and parasites, spray-on insecticides, destruction of larval overwintering sites, etc.

27.7 Safety of Bt Plants

The pre-market regulatory review of genetically modified crops, including Bt crops, assesses the food and environmental safety of such crops. Based on the concept of substantial equivalence, this safety assessment focuses on the proteins encoded by the genes that have been introduced in the novel variety. Principal

components of food safety assessments include evaluation of potential toxicity and allergenicity, in the context of anticipated human dietary exposure [117]. Animal toxicity studies with the Cry proteins present in currently commercial Bt crops have indicated the absence of any acute or chronic effects [118, 119]. The evaluation of potential allergenicity is mainly based on *in vitro* digestibility assays using simulated gastric fluid and amino acid sequence comparisons with known allergens. The Cry proteins in current Bt crops are all degraded rapidly by gastric fluid and do not show any sequence similarity with known allergens [118, 119]. In conclusion, none of these Cry proteins show any characteristics of toxins or food allergens [64, 117–119].

Protection from the European corn borer by Bt corn has resulted in significant reductions in fumonisins, produced by certain *Fusarium* species, in many locations [120–122]. Such reductions in fumonisins have not been observed under conditions where *H. zea*, rather than *O. nubilalis*, is the predominant pest insect on Bt corn [123, 124]. This is likely explained by the significantly lower susceptibility of *H. zea* to Cry1Ab, as compared with *O. nubilalis*. Fumonisins are acutely toxic to various animals, are carcinogenic in rats and have been associated with esophageal cancer in humans [125]. Corn borers such as *O. nubilalis* larvae can act as a vector of *Fusarium* spores from the plant surface to damaged kernels or to the interior of stalks or provide entry wounds for fungi. Thus, Bt corn has the potential to reduce the levels of fumonisin mycotoxins in field-harvested grain and hence their dietary intake, especially in regions of the world with high incidence of *Fusarium* and high levels of corn consumption [120].

The adoption of Bt crops, especially Bt cotton, has resulted in significant reductions in chemical pesticide applications [64, 126, 127]. Pray et al. [126] estimated a reduction in pesticide use of 78 000 tons of formulated pesticide in 2001 in China. Reduced pesticide exposure may benefit farmer health, especially in countries where pesticides are applied under conditions that are not always optimal with respect to worker protection. Such positive effects on farmer health have been reported for both Bt rice and Bt cotton in China [128, 129].

Bt biopesticides are generally regarded as safe for use as biological control agents and are promoted in both organic and integrated pest management systems [130]. In Bt crops, the Cry proteins are present as non-crystalline proteins, are often truncated, and are present throughout the growing season. Therefore, their impact on non-target insects may be different from the Bt sprayable insecticides and need to be assessed individually as part of a risk assessment, where risk is defined on the basis of both potential hazard and exposure. Such assessment has involved studies of different levels of complexity, going from laboratory tests under very high exposure, to studies that analyze responses of organisms under more realistic conditions, and ultimately field studies.

In view of the specificity of Cry proteins the effect of Bt crops on most non-target insects can be expected to be minimal, especially when compared with the effects of broad-spectrum insecticides. In the case of Bt cotton and Bt corn plants, both expressing lepidopteran-specific Cry proteins, some non-target Lepidoptera may be negatively affected when challenged with tissues of such Bt plants. Losey

et al. [131] found that, under laboratory conditions, Bt corn pollen dusted over milkweed plants decreased survival of larvae of the monarch butterfly, *Danaus plexippus*. The authors stated that their results have potentially profound implications for the conservation of monarch butterflies. Although criticized on methodological grounds, this report on the monarch butterfly was seen by many as an example of agricultural biotechnology, specifically pollen from Bt corn, disrupting nature [132]. However, the critical question is not whether some Lepidoptera are susceptible to the Cry protein expressed in tissue of Bt plants, but whether or to what extent the larvae are exposed to the protein under field conditions. In a subsequent experiment Jesse and Obrycki [133] placed potted milkweed plants in corn fields at different distance from the field edge. Laboratory bioassays demonstrated that disks from milkweed plants dusted with Bt corn pollen resulted in significantly higher mortality than disks from milkweed plants dusted with non-Bt corn pollen. Bioassays using pollen extracted from tassels from Bt corn and non-Bt corn and applied on milkweed leaf disks also indicated increased mortality from the Bt corn pollen. Based on these results, the authors predicted that transgenic Bt corn would have a negative impact on *D. plexippus* larvae in and adjacent to Bt corn fields. Notably, however, the pollen samples collected from tassels contained substantial amounts of plant debris and this contamination may have caused significant mortality [134]. A series of more recent studies examined the impact of Bt corn pollen more rigorously to quantify the potential risk to monarch butterflies associated with the large-scale growing of Bt corn [134–139]. The hazard posed by Cry1Ab was confirmed in laboratory bioassays, but the exposure was shown to depend significantly on the expression level of Cry1Ab in pollen in different Bt corn events [134, 137]. Also, the exposure of larvae to Bt expressing pollen varied significantly, depending on the distance from the corn field, the position of the leaf of milkweed plants (i.e., upper leaves versus middle leaves), the site of pollen deposition on the leaf (i.e., along the midrib or areas flanking the midrib) and the occurrence of rainfall events [136]. Spacial and temporal overlap between the presence of susceptible life stage of *D. plexippus* and corn pollen shed is another important determinant of exposure [135, 137]. In essence, the studies indicated that the currently registered corn events have little or no impact on monarch populations. Notably, the only Bt corn event producing pollen with substantial toxicity to the monarch butterfly larvae, Bt corn event 176, has been phased out. Gatehouse et al. [140] have stated that

> Contrary to media hype, the primary threat to the monarch population is loss of crucial winter habitats in southern California and central Mexico, rather than commercial growing of Bt-maize.

Likewise, Pimentel and Raven [141] mentioned that

> Although Bt corn pollen under certain conditions has the potential of adversely affecting the population levels of monarch butterflies and other non-target Lepidoptera, we consider these impacts to be minimal when compared with habitat loss and the widespread use of pesticides throughout the ecosystem.

Another study that attracted much media attention was the report by Hilbeck et al. [142] that demonstrated increased mortality of larvae of the predatory green lacewing (*Chrysoperla carnea*), when offered *O. nubilalis* or *S. littoralis* larvae that had been fed on Cry1Ab expressing Bt corn. In an effort to differentiate between a direct effect of the toxin and an indirect effect, due to reduced nutritional quality of the Bt fed prey, Hilbeck et al. [143] compared survival of *C. carnea* larvae developing on *Ephestia kuehniella* eggs or on artificial diet with or without Cry1Ab. The use of the artificial diet increased the mortality to 30%, compared with 8% when using eggs as the diet. Inclusion of Cry1Ab at 100 μg mL^{-1} in the diet increased the mortality to 57%. However, Dutton et al. [144] found that *C. carnea* larvae were not affected by feeding on Bt corn reared *Tetranychus urticae* spider mites while these prey insects contained higher levels of Cry1Ab than Bt corn reared *S. littoralis* larvae. These observations are in line with the fact that lepidopteran larvae are a low quality prey for *C. carnea* larvae, as compared with other prey such as aphids, spider mites or lepidopteran eggs. Recently, Romeis et al. [145] developed an improved bioassay for *C. carnea* using a sucrose-based artificial diet and demonstrated that Cry1Ab at a concentration of 1 mg mL^{-1} has no direct toxic effect on *C. carnea*. The amount of Cry1Ab consumed by *C. carnea* was calculated to be 10000× higher than that ingested through Bt corn reared *S. littoralis* larvae. These results were corroborated by data showing the absence of either direct or indirect, prey-mediated toxic effects of Cry proteins, as well as the absence of Cry1Ac binding, in *C. carnea* [146]. In conclusion, the above data demonstrated that (a) it is of crucial importance to use a high quality artificial diet when assessing direct toxic effects; (b) predators should not be forced to feed exclusively on prey species that constitute only a relatively minor portion of their natural diet in the field; and (c) the earlier reported negative effects of Bt corn were due to the low nutritional quality of the prey rather than to direct toxic effects. What do these data mean with respect for the risk of Bt corn to *C. carnea*? Since larvae of this predator species are known to prefer aphids to lepidopteran larvae in the field and aphids are not harmed by Bt corn, the risk of Bt corn to *C. carnea* is considered to be negligible [145]. This has been confirmed by results from field studies comparing densities of beneficial insects, including *C. carnea*, on Bt and non-Bt corn [147–151]. The above data on the monarch butterfly and green lacewing illustrate that considerable care should be taken in extrapolating laboratory findings to natural field conditions. Factors such as the significance of the crop as a food source and the degree of specialization of the predator or parasite species are likely to be important in estimating the impact under field conditions. Clearly, in evaluating the risk to non-target organisms, both toxicity and exposure must be taken into account. Also, any impact of Bt crops should be judged alongside conventional insect control methods. Whereas early field studies were usually performed on a rather limited scale, a series of recent field studies on the effects of Bt crops on non-target insects have been published, most of which were performed on a medium- to large-scale, during several years and on multiple locations [152–162]. Collectively, these studies showed only minor changes in abundance of a few non-target taxa, but almost all these effects

were explained by expected changes in size of target pest populations. Importantly, a five-year field trial of Bt cotton indicated essentially no effects of Bt cotton on natural enemy function and showed that minor reductions in density of several non-target taxa in Bt cotton may have little ecological meaning concerning the natural biological control of key cotton pests [158]. Probably, reductions in the abundance and associated function of any one species, especially predators, of the natural enemy complex of cotton are offset by other members of the community. The studies also demonstrated that the use of broad spectrum insecticides, as an alternative insect control measure, had a significantly larger impact on non-target arthropods [159]. According to O'Gallaghan et al. [163], the extensive testing on non-target plant-feeding species and on beneficial species that has accompanied the long-term and wide-scale use of Bt plants has not detected significant adverse effects. Romeis et al. [164] have recently evaluated all peer-reviewed studies published to date on the effect of Bt crops on predators and parasites. They concluded that (a) laboratory and greenhouse studies have revealed effects on natural enemies only when Bt susceptible, sublethally affected herbivores were used as prey or host, with no indication of direct effects; (b) field studies have only revealed minor, transient or inconsistent effects of Bt crops on parasitoids and predators as compared with non-Bt crops, with the exception of specialist natural enemies; (c) applications of conventional insecticides have usually resulted in severe negative impacts on biological control organisms; and (d) since Bt transgenic varieties can lead to substantial reductions in insecticide use in some crops, such Bt crops can contribute to a better integrated pest management with a strong biological control component [164]. With respect to the reductions in abundance of specialist natural enemies that depend on the target pest, notably, any control method, including biological control and conventional host–plant resistance, resulting in a drastic reduction in the target pest density will have similar effects. Various studies have examined the potential effect of Bt crops, through root exudates and postharvest plant residues, on soil ecosystems. Cry proteins have been detected in root exudates from Bt corn, potato and rice, but not in Bt canola, cotton and tobacco [165–167]. The degradation of Cry proteins in the soil likely depends on various parameters such as plant and soil type, composition of the soil microbial communities, agricultural practices and environmental conditions. This may partially explain the large variation observed when assessing the presence over time of Cry proteins in soils from root exudates and/or postharvest plant material. The highest potential for persistence of Cry proteins is in soils high in clay and organic matter since they can bind to these soil constituents and thereby be protected against microbial degradation and retain insecticidal activity [168, 169]. Some studies have indicated that Cry proteins degrade rather slowly in soil environments [170], whereas other studies found rapid decay of Cry proteins [171–174]. Moreover, a multi-year study could not detect Cry1Ac by either ELISA or insect bioassays in soil samples taken from fields where Cry1Ac expressing cotton cultivars had been grown for 3 to 6 consecutive years [175]. The impact of Bt corn on culturable bacterial species of soil communities [176–178], as well as on actinomyces, fungi, protozoa, nematodes, earth-

worms [178], springtails (Collembola) [153, 179] and various members of the community of soil-dwelling invertebrates [148, 152] was determined to be minimal or not significant. Cultivation-independent, more sensitive molecular techniques indicated either no effects on bacterial community structure [180] or small shifts in bacterial communities between Bt and non-Bt plant varieties [176, 181, 182]. The environmental relevance of the latter observations is unclear. Interestingly, in this context, a field study at two sites during three consecutive years of Cry1Ab expressing corn indicated that environmental factors such as field site and age of plants caused greater differences in rhizosphere communities than the expression of Cry1Ab [183]. Perhaps more important than changes in the soil microbacterial community per se are potential changes in soil microbial activity by Bt crops. A two-year field study using Cry3Bb expressing corn found no effects on microbial activity measures such as N mineralization potential, short-term nitrification rate and soil respiration rate [180]. Similarly, no or only small differences in the decomposition rate between Cry1Ab expressing and non-transgenic maize have been reported by Hopkins and Gregorich [173] and Castaldini [184], respectively. In contrast, Flores et al. [177] and Stotzky [185] did report different decomposition rates between Bt and non-Bt varieties in corn and other crop species from laboratory incubation studies using soil amended with ground biomass. An additional prerequisite for a correct interpretation of observations is the use of appropriate controls, such as plant lines from the same cultivar that have been transformed and regenerated but do not express the transgene as well as unrelated plant cultivars, since differences between the transgenic and non-transgenic plant may be smaller than the differences between different cultivars. In conclusion, while there appears to be variation in the soil decay rate of Cry proteins from Bt crops, no major impact of Cry protein residues on soil (micro)biota has been observed.

Movement of genes, both from conventional and transgenic plants, to wild relatives of the crop might result in the evolution of increasingly weedy and/or invasive plants. Pollen-mediated gene flow depends on the geographic distributions of donor crops and the recipient wild plant, the distance of pollen movement, the rate of outcrossing, the synchrony of flowering between donor and recipient plant and the sexual compatibility between both. The fertility of the hybrids and their offspring is an important factor in determining the likelihood of transgene introgression. Seed-mediated gene flow depends on seed persistence and dispersal. The consequences of gene flow will depend on the nature of the (trans)gene and its expression level in the hybrid and the biology and ecology of the recipient plants [186]. While hybridizations between crops and their wild relatives may be relatively widespread, the likelihood for such hybridizations in Bt corn and Bt cotton in, for example, the USA and Europe seem essentially non-existent since either no wild relatives of these crops occur in these regions or they are incompatible with cultivated varieties [118]. Gene flow can occur not only from crops to wild plants but also between crops, e.g., between transgenic cultivars and landraces. A recent paper suggested that transgenic DNA including a *cry* gene had

introgressed into maize landraces in Mexico, despite a ban of transgenic corn in this country [187]. The paper was later retracted since introgression per se was not shown [188]. A subsequent report failed to find evidence for the presence of such transgenes in maize landraces from the same area [189]. These authors stated that it is unlikely that the presence of a few transgenes would reduce the genetic diversity of the landraces to a greater extent than gene flow from conventional modern cultivars. Resistance traits such as insect resistance due to Cry expression could potentially confer an increased fitness on recipient plants by reducing lepidopteran damage and increasing seed production. Thus the ecological effect of a Bt gene introgressed into landraces or wild plants is likely to depend to a large extent on the importance of lepidopteran herbivores in these populations, as well as to the degree that the transgene is linked to domestication genes or any gene that would be selected against [189–191]. Studies on the effects on the fitness of the presence of a *cry* gene in a wild relative of either sunflower or oilseed rape have yielded different results, depending on the plant species and the location of the field test site [192, 193]. In this context, notably, although gene flow has been introducing pest-resistance genes from conventional crops to wild relatives for generations, there are no known examples of increased invasiveness owing to introgression of those alleles [194]. Furthermore, fitness-related measures do not necessarily predict invasiveness [191, 195]. In summary, the potential for, and the risk of, gene flow to wild relatives from commercially available Bt crop varieties in those areas where they are currently grown seems very limited. The risk of Bt transgenes being introgressed into landraces or modern crop cultivars depends on many factors, should be addressed on a case-by-case basis and can likely, at least to some extent, be minimized by (physical) containment measures, such as isolation distances and border rows.

27.8 Conclusion

Bt crops have provided farmers a valuable additional tool to control insect pests on corn and cotton. Bt rice is likely to be commercialized this year, at least in one country. At least in the USA, when growing such crops, farmers must agree to implement certain IRM tactics, aimed at preventing or delaying the development of resistance in insect populations. These tactics are being refined as our knowledge on insect pest biology and insect/crop plant interactions expands. Field evaluations conducted so far have not found negative impacts of Bt crops on non-target and beneficial insects, except for the expected reduction in specialized natural enemies. Especially in cotton, significant reductions in synthetic insecticide sprays have been realized upon adoption of Bt crops. Judicious use of this novel insect control tool should result in sustainable benefits to farmers and the environment, and, as a consequence, also to the consumer.

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28
Metabolic Processes

28.1
Inhibitors of Oxidative Phosphorylation

Josef Ehrenfreund

28.1.1
Introduction

Mitochondria produce most of the energy in cells by oxidative phosphorylation. This process combines two distinct but tightly coupled parts: Electron transport and phosphorylation of ADP to ATP – as discussed in detail in Chapter 13.1. Most modern insecticides and acaricides that disrupt mitochondrial ATP synthesis [1] interfere with the electron transport (mainly at complex I, less frequently at complex III) (see Chapter 28.3).

This chapter focuses on compounds that disrupt oxidative phosphorylation by direct inhibition of the mitochondrial ATP synthase (complex V) with the main emphasis on diafenthiuron, the only modern representative of that class.

28.1.2
Mitochondrial ATP Synthase as a Target for Insecticides and Acaricides

Within the process of oxidative phosphorylation the mitochondrial ATP synthase (also referred synonymously as complex V, F_1F_0-ATPase or F_1F_0-ATP synthase) has to fulfill two main tasks:

(a) To discharge the electrochemical potential gradient that has been generated by the expulsion of protons across the inner mitochondrial membrane. It does so by actively channeling protons across the inner mitochondrial membrane from the cytoplasmic side back to the matrix side.
(b) To catalyze the phosphorylation of ADP and to release the so-formed ATP into the cell.

It is an enzyme of enormous complexity and efficiency. For a detailed discussion of its structure and reaction mechanism see Chapter 13.1. Importantly, the mech-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

anism of the transmembrane proton conduction is highly conserved and may be common to an entire class of membrane channels [2]. It involves an essential free carboxyl group of subunit c (Asp61 in *E. coli*, Glu in all other organisms) inside the phospholipids bilayer which transfers a proton to a nearby basic Arg of the stator. The lipophilic carbodiimide *N*,*N*-dicyclohexylcarbodiimide (DCCD) binds irreversibly to this essential carboxylic group, and therefore acts as general inhibitor of ATP synthase.

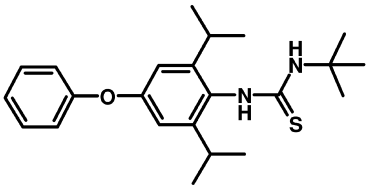
Despite the large and complex machinery of ATP synthase, its essential role in cellular bioenergetics, and the existence of inhibitors of structural diversity (see for instance Refs. [3–9]) few agrochemicals, mostly specific acaricides are reported as inhibitors of the enzyme. They include chlorfenson, tetradifon, chloropropylate, bromopropylate, flubenzimine, oxythioquinox and propargite [10]. However, for most of them there is no clear evidence that the biological activity *in vivo* is due to ATP synthase inhibition. Of these compounds only tetradifon and propargite are still commercially available [11].

For the acaricide fenazaquin [4-(4-*tert*-butylphenethoxy)quinazoline], a potent inhibitor of electron transport at complex I (see Section 28.1.3), an additional low-affinity binding site in the stalk region of ATP synthase has been recently identified. The relevance of this newly discovered binding site is unknown since the enzymatic activity of ATP synthase is not impaired [12].

Some obsolete members of the class of organotin acaricides significantly inhibit ATP synthesis at the ATP synthase level. Enzyme preparations from non-mammalian sources are reportedly more sensitive to organotins (I_{50}s in the range 0.1–100 nM) than typical mammalian enzymes (I_{50}s in the range 1000–10000 nM), which may explain their apparent selective toxicity towards invertebrates [1]. It has been suggested that some of these chemicals bind to the F_0 component of mitochondrial ATP synthase at a site different to the known inhibitors oligomycin or DCCD in such a way that the rapid rotary motion required to maintain efficiency of ATP synthesis is inhibited [13]. However, organotins exhibit a range of additional biological effects that may well be as relevant as ATP synthase inhibition for their effects *in vivo*, e.g., mitochondrial uncoupling caused by hydroxide ion shuttle across the inner membrane or inhibition of Ca^{2+} ATP-ases [1].

ATP synthase from DDT [1,1-bis-(*p*-chlorophenyl)-2,2,2-trichlorethane] susceptible insects, but not from DDT resistant strains, is inhibited by DDT, although at relatively high concentration. Recently, it has been suggested that this inhibition is associated with the presence of a specific protein in the F_0 component at a site different from the binding sites of oligomycin and DCCD. Since this specific protein is only present in insect strains that are susceptible to DDT the authors conclude that this protein is the target for DDT and that inhibition of ATP synthase is its primary mode of action [14].

For only one of the recently introduced pesticides convincing evidence is available that inhibition of mitochondrial ATP synthase is responsible for its biological activity *in vivo* and therefore represents its mode of action: The insecticide and acaricide diafenthiuron (**1**) (Fig. 28.1.1).



1; diafenthiuron (Polo™; Pegasus™)
LD_{50}: 2068 mg/kg rat,oral,acute

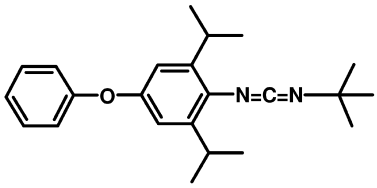
Fig. 28.1.1. Diafenthiuron (**1**): Structure and acute toxicity data.

It was introduced by Ciba-Geigy AG, now Syngenta Crop Protection AG, in 1988 [15] and launched in 1991. It is currently marketed under the main trade names Pegasus® and Polo®.

The following sections review its biochemical mode of action, outline some aspects of its chemistry and describe the important biological features that make diafenthiuron a unique and highly valuable crop protection tool for the farmer.

28.1.3
Diafenthiuron: Mode of Action

All available studies support the conclusion that diafenthiuron is a proinsecticide [16] that is activated by oxidative desulfurization to the insecticidal carbodiimide **2** (Fig. 28.1.2).



2; diafenthiuron carbodiimide (CGA140408)

Fig. 28.1.2. Structure of diafenthiuron carbodiimide (CGA140408) (**2**).

The evidence can be summarized [17] as follows:

1. Diafenthiuron (**1**) itself had no effect in biochemical and neurophysiological assay systems, including mitochondria, cuticle formation, axonal sodium channel and main neuronal receptors. However, **1** was a substrate for cytochrome P-450 [18].

2. The conversion of **1** into **2** occurs readily: photochemically on glass plates [19] or in water [20], on cotton [19] or Chinese cabbage [21] in the field, in microsomes as well as in whole organisms of insects and mammals [18, 22]. Activation of diafenthiuron to **2** and its precursor diafenthiuron S-monoxide competes with metabolic deactivation, mainly to hydroxylated derivatives and diafenthiuron-ureas. Quantitatively, the proportion of activation to deactivation varies significantly in different animals and organs. Rat liver microsomes do not accumulate significant amounts of **2** but hydroxylate diafenthiuron at the 4′-position. This efficient deactivation may explain the favorable acute oral toxicity of diafenthiuron in rats [35].
3. The carbodiimide **2**, in contrast to **1**, displays biological effects *in vitro* that may be responsible for the activity *in vivo*. In particular, **2** is a potent inhibitor of mitochondrial ATP synthesis at the ATP synthase level *in vitro* [10, 18] and *in vivo* [22–24]. Radiolabeling experiments with [^{14}C]-**2** confirm that it covalently binds to the 8-kDa proteolipid of F_0 of the mitochondrial ATP synthase in isolated mitochondria from insect flight muscle and rat liver. Because binding is competitively blocked by DCCD and partly inhibited by venturicidin it has been concluded that **2** and the classical and well-studied inhibitor DCCD [25] share the same binding site on the F_0 proteolipid [26].

   Additionally, the carbodiimide **2** as well as DCCD also bind to porin, a 30 kDa voltage-dependent anion channel located in the outer mitochondrial membrane. Again a common binding site involving covalent interaction with an essential carboxylate is postulated. However, in contrast to DCCD, **2** binds to porins from insects specifically [23, 26]. Binding of **2** to porin does not dramatically impair the channel but induces changes of its voltage dependency [27]. Porin has important biological functions that are still being explored [28]. DCCD binding reportedly affects some of these functions [29]; however, the effect of **2** on these functions has not been explored.

   Carbodiimide **2** reportedly also stimulates the octopamine-sensitive adenylate cyclase of the bulb mite *Rhizoglyphus echinopus* [30], adults of the diamondback moth *Plutella xylostella* [31] and the lantern of the firefly *Photinus pyralis*, an organ known to be a rich source of octopamine-sensitive adenylate cyclase [32]. Since poisoning symptoms of diamondback moth adults treated with diafenthiuron or **2** resembled closely those of the known octopaminergic agonist

N′-(4-chloro-*o*-tolyl)-*N*-methylformamidine [33], and differed strongly from those of DCCD, the authors suggest that **2** acts *in vivo* by affecting octopaminergic transmission [34]. However, these results could not be extended to other insects and mites [35, 36].

4. The following evidence supports the causality between oxidative activation of diafenthiuron to its carbodiimide **2**, ATP synthase inhibition by **2** and biological activity *in vivo*. Diafenthiuron as well as **2** inhibit respiration of locusts *in vivo* at low rates; however, only **2** is active *in vitro* [22, 26]. *In vivo* the onset of poisoning symptoms is more rapid for **2** than for **1** [18, 31, 34]. Photosensitizers such as Bengal Red, which promote [37] the photochemical conversion of diafenthiuron into **2**, accelerate the acaricidal effects of diafenthiuron *in vivo* [38]. Complementarily, the toxicity of diafenthiuron **1** in insects is antagonized by piperonyl butoxide (PBO) [18]. PBO is a commonly used synergist and a potent inhibitor of cytochrome P-450 dependent monooxygenases. However, this antagonistic effect of PBO was not apparent in mice [22].

In insects ATP synthesis in certain organs is strongly and progressively affected after application of **2**. When locusts were topically treated with **2**, mitochondrial ATP synthase activity in the abdominal ganglia was decreased by 46% at the onset of the first symptoms and by 63% when the animals were paralyzed. In addition, a severe block of ATP synthase in the gut (78%) and in the jumping leg muscle (83%) was noted. In all these organs, inhibition of ATP synthesis also caused a significant decrease of the actual ATP levels. On the one hand, mitochondrial ATP synthase activity of flight muscles and heads of the flies *Calliphora erythrocephala* and *Phormia regina* – both tissues that contain a high number of mitochondria – was not significantly reduced by either diafenthiuron **1** or by its carbodiimide **2** at the onset of paralysis. In addition, total energy metabolism in fly thoraces was not affected by lethal doses of diafenthiuron or **2** [23].

Evidently, therefore, mitochondria of different organs are not equally sensitive to **2**. A supportive indication that mitochondria from different tissues can be affected differently by pesticide action was observed with fenpyroximate, a complex I inhibitor of the respiration chain (see Chapter 28.3). It causes morphological changes of mitochondria in peripheral nerve cells but not in muscular cells [39].

A complementary line of evidence demonstrates that the acute toxicity of diafenthiuron in mice may also be attributed to its conversion into **2** and inhibition of mitochondrial ATP synthase in different target organs [22, 40, 41].

In summary, most of the evidence leads to the conclusion that diafenthiuron, thanks to its conversion into its carbodiimide **2**, acts strikingly similarly to the well-known carbodiimide DCCD in its reaction towards potential binding proteins [22, 35]. DCCD reportedly binds to many channels that conduct protons

[42, 43] as well as to the calcium channel of the sarcoplastic reticulum [44]. More generally, it interacts or may be expected to interact with the manifold of proteins that catalyze ATP-triggered reactions and that contain Walker sequence motifs at their active site [13]. As a theoretical consequence it may be expected that the carbodiimide **2** – similar to DCCD – may additionally affect other essential proteins. Further studies, therefore, may be necessary to clarify as to what extent other binding sites contribute to the mode of action of diafenthiuron [35].

28.1.4
Diafenthiuron: Discovery, SAR and Production Process Chemistry

Chemistry driven optimization of chemical leads that have been originally identified by competitor companies is a classical tool in crop protection discovery [45]. Inspired by patent applications filed by Bayer 1976/1977 claiming N-aryl-N′-alkyl(or cycloalkyl)-thioureas and isothioureas as insecticides and acaricides [46], it was soon established that the introduction of a (substituted) phenoxy substituent into the 4-position of the original "Bayer lead" (Fig. 28.1.3) [47] resulted in a profound shift of the biological spectrum in the greenhouse: While the systemic activity of the lead against the brown planthopper, an important pest of rice, decreased, strong activity against phytophagous mites and some lepidoptera became apparent [48].

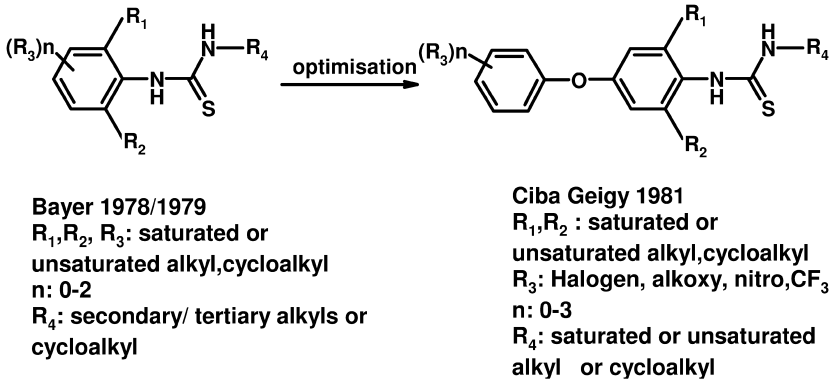
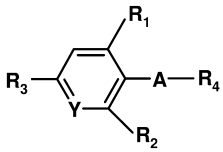


Fig. 28.1.3. Thiourea insecticides: First optimization at Ciba-Geigy.

Unexpected field results with diafenthiuron (**1**), which was selected as the most promising compound for further development, triggered chemodynamic studies: sprayed on cotton leaves in the field, **1** was quickly converted into **2** by sunlight with a half-life of 3 h [19, 48, 49]. The newly formed **2** was – in comparison with **1** – much more light stable, biologically more active against mites, but less stable against hydrolysis in acid media and, unfortunately, quite phytotoxic [48]. In accordance with this result the extensive optimization of this chemical class at Ciba-Geigy was extended to include, besides thioureas and isothioureas, also the corresponding carbodiimides (Fig. 28.1.4).



A = NHCSNH; N=C(SAlkyl)-N; N=C=N
R_1;R_2 = Alkyl, Cycloalkyl
R_3 = X - Aryl; X - Heteroaryl;
X = O,S, CH_2;$CH(CH_3)$;$C(CH_3)_2$;NH; N(Alkyl); NCHO
Y = CH, N
R_4 = secondary or tertiary alkyl

Fig. 28.1.4. Diafenthiuron optimization: Scope of Ciba-Geigy patent applications.

Although only limited detailed information has been published, some general SAR principles have been formulated [48].

For excellent potency:

- Both R_1 and R_2 should be alkyl. Isopropyl often leads to maximum activity.
- R_4 must be a sterically demanding alkyl or cycloalkyl, *t*-butyl is often best.
- Isothioureas excel against lepidopterous larvae; however, at least for one example high mammalian toxicity has been observed.
- Carbodiimides generally are the most potent acaricides; however, they are often toxic to fish and some show only limited crop tolerance.

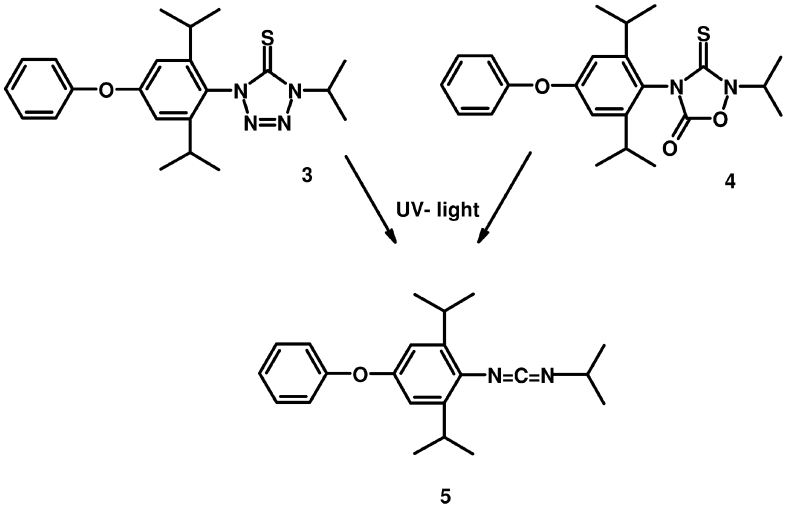


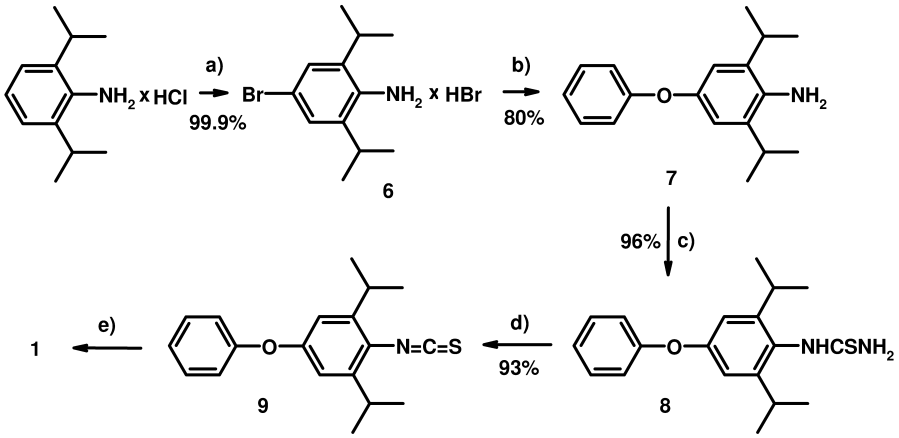
Fig. 28.1.5. 1,4-Dihydro-5*H*-tetrazol-5-thione **3** and 5-thiono-1,2,4-oxazolidinone **4** as precursors of insecticidal carbodiimide **5**.

These rules were confirmed by combined quantitative multivariate SAR and chemodynamic studies in the subclass of N-(pyrid-3-yl)thioureas and -carbodiimides (Fig. 28.1.4: Y equals N). These studies support the SAR concept that, for thiourea analogs of diafenthiuron, the rate and efficiency of carbodiimide formation and its photolytic and chemical stability governs the overall biological potency [50, 51].

This concept has been extended in two further examples. Based on a literature report that 1,4-dihydro-1-phenyl-5*H*-tetrazol-5-thiones photochemically form carbodiimides by extrusion of nitrogen and sulfur [52], and the expectation that 5-thiono-1,2,4-oxazolidinones may similarly lose carbon dioxide and sulfur to form carbodiimides, **3** and **4** were prepared as potential proinsecticides. Both compounds were efficiently converted into the carbodiimide **5** by ultraviolet light and displayed the expected strong insecticidal and acaricidal properties *in vivo* (Fig. 28.1.5) [53–55].

The large-scale production process for diafenthiuron (**1**), starting from the industrial chemical 2,6-diisopropylaniline, is outlined in Scheme 28.1.1 [56, 57].

Although the technical route involves only classical well-known reactions, important improvements were necessary to realize a viable industrial process, e.g., conventional bromination of 2,6-diisopropylaniline in acetic acid gives the desired 4′−4′ brominated product in only about 90% yield and purity. In contrast, bromination of 2,6-diisopropylaniline hydrochloride in non-polar solvents yields **6** in nearly quantitative yield and excellent purity. Since 4-bromoanilines are notoriously thermolabile and, therefore, cannot be safely distilled on a large scale, this improvement was of critical importance for successful implementation of the



a) Br_2/Cyclohexane/70°C
b) $C_6H_5OK/CuCO_3$/DMF/140°C
c) NaSCN/HCl + H_2O/o-Xylene/90°C
d) o-Xylene/150°C
e) t-$C_4H_9NH_2$

Scheme 28.1.1

overall production process. Additionally, the use of thiophosgene for the conversion of **7** into **9** [47] was not acceptable for an industrial large-scale process. An optimized version of a known [58] two-step reaction sequence had to be developed instead.

28.1.5
Diafenthiuron: Mammalian Toxicology and Ecotoxicology

The key toxicological and ecotoxicological data of diafenthiuron have been summarized [59]. Since it is very readily degraded in the environment the ecotoxicological hazard has been rated as low.

28.1.6
Diafenthiuron: Biological Activity and Significance for Crop Protection

Originally, the development of diafenthiuron at Ciba-Geigy (now Syngenta Crop Protection AG) was based on its potent activity against spider mites in cotton, citrus and deciduous fruit. Its high potential for the control of the sweetpotato whitefly *Bemisi tabaci* was first unexpectedly observed during acaricide trials in cotton [60] and was decisive for the positioning of the compound in the market. Since its commercial introduction in 1991, diafenthiuron has established itself as an important tool for crop protection, especially for multiple spray programs in cotton.

Diafenthiuron has a useful spectrum of activity that cannot be found in insecticides of other chemical classes: at the recommended rate of 300–400 g-a.i. ha^{-1} [60] it controls not only the important sucking insect complex of cotton, especially the cotton whitefly, cotton aphid, cotton leafhoppers, but also tetranychid and tarsonemid mites and young larvae of noctuids [61].

In addition, although it is not systemic it displays translaminar activity: pests located on the underside of the leaf are controlled even if they are not directly hit by the spray. This property is especially valuable in crops that produce dense canopies such as cotton, and may be the result of vapor phase activity [15, 19] and/or efficient uptake into the leaf cuticle [21].

The efficacy of diafenthiuron against different stages of whiteflies on cotton seedlings has been characterized in the laboratory [61]: progeny of female adults was highly suppressed even at the low rate of 5 mg L^{-1}. Larvae were the most susceptible stage (LC_{50} 6.5 mg L^{-1}; LC_{90} 49.2 mg L^{-1} for second instar), followed by adults (LC_{50} 23 mg L^{-1}; LC_{90} 102.4 mg L^{-1}) and pupae (LC_{50} 45 mg L^{-1}) while reduction of egg hatch was much less pronounced.

A similar stage sensitivity pattern has been observed in leaf dip tests with the mite *Tetranychus urticae*: at the rate of 200 mg-a.i. L^{-1} larvae and adult females were more susceptible than nymphs while egg hatch was insufficiently controlled [15]. More recently, the sensitivity of different developmental stages of the carmine mite *Tetranychus cinnabarinus* towards diafenthiuron has been determined and compared with dimethoate and propargite [62].

The strong translaminar activity of diafenthiuron, which is an important benefit for its overall field performance, was also confirmed in the laboratory with *Tetranychus cinnarabinus*: careful treatment of the upper surface only of cotton leaves with diafenthiuron (300 mg-a.i. L^{-1}) gave – in contrast to the commercial standard propargite applied at the same rate – good overall control of the mobile stages [21]. However, neither the translaminar activity nor the strong gas-phase activity became apparent against cotton whiteflies larvae in the laboratory. Since this effect is clearly observable under field conditions, the authors propose that the vapor phase activity might be stronger in the field because of the larger amount of vapor produced under field spray conditions [61].

Besides its main application against the sucking pest complex in Asian, Australian and Latin American cotton [15, 63–65], diafenthiuron has important specific additional uses against lepidopterous pests in brassicas in southeast Asia and the Far East. Specifically, good activity against susceptible and resistant strains of diamondback moth, the lesser armyworm, the small white butterfly and *Spodoptera litura* at rates ranging from 30–60 g-a.i. 100-L^{-1} have been recorded [15, 66–69].

Under field conditions, diafenthiuron is harmless towards the main beneficial arthropods, especially those of cotton. It is, therefore, highly compatible with IPM (Integrated Pest Management) spray programs. Neither mite stimulation nor aphid and whitefly resurgence phenomena, which sometimes are observed with less selective insecticides, have ever been reported with diafenthiuron.

Although some toxicity against nymphs and adults of the predatory bugs has been observed in the laboratory [70, 71] the effects are not significant under field conditions [72, 73]. Because diafenthiuron has a unique mode of action, no cross resistance with any other insecticide or acaricide has been reported. Most importantly, the white flies *Bemisia tabaci* and *Trialeurodes vaporiorum*, and the aphid *Aphis gossypii*, which have rapidly developed strains with tolerance/resistance against all major insecticides, including organophosphates, pyrethroids, growth regulators and neonicotinoids, remained fully susceptible to diafenthiuron [64, 65, 74, 75]. A similar lack of cross resistance has been reported for strains of the diamondback moth, which had acquired multiple resistance against organophosphates, acylureas, pyrethroids and abamectin [66, 76]. In addition, resistance development to diafenthiuron may be slow: during field cage selection pressure studies, carried out in Malaysia and Thailand, the tested populations of diamondback moth developed no observable resistance after 25 generations in Malaysia or even after 55 generations in Thailand [77]. Similarly, resistance monitoring trials in Taiwan confirmed that while *Plutella xylostella* strains in heavily treated areas have become considerably less susceptible to modern insecticides such as abamectin, emamectin benzoate, fipronil, chlorfenapyr and spinosad, the susceptibility to diafenthiuron remained unchanged [78].

In summary, diafenthiuron remains a singular active ingredient in crop protection chemistry because of its unique chemical class and biochemical mode of action. Owing to its unparalleled biological spectrum, translaminar and gas-phase activity, selectivity towards beneficial arthropods and the lack of cross resistance

with all other established insecticide classes, it continues to be an important component of rotational spray regimes, mainly in cotton and vegetable crops.

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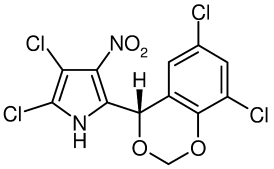
28.2
Inhibitors of Oxidative Phosphorylation via Disruption of the Proton Gradient

David Kuhn and Nigel Armes

28.2.1
Introduction

Molecules targeting mitochondrial functions represent viable alternatives to classical neurotoxicants. Several naturally occurring compounds targeting respiration processes within the cell have been identified, including the annonins [1], the anacardic acids [2] and sesquiterpenes [3]. As part of a program to identify novel microbial metabolites having insecticidal activity, Carter and coworkers, using bioassay guided fractionation, isolated dioxapyrrolomycin, a member of the pyrrolomycin family of pyrrole antibiotics (**1**, Fig. 28.2.1) from the fermentation of



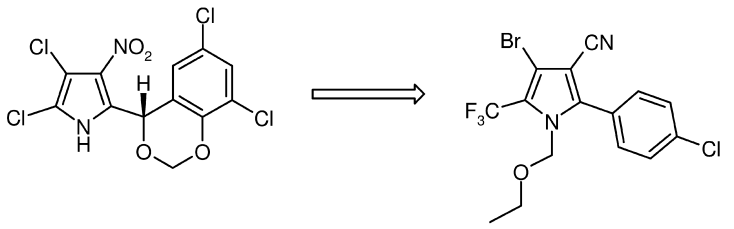
1

Species	LC_{50} (ppm)
Southern armyworm (Spodoptera eridania)	40
Tobacco budworm (Heliothis virescens)	32
Two-spotted spider mite (Tetranychus urticae)	10
Western potato leafhopper (Empoasca abrupta)	<100

Fig. 28.2.1. Structure and insecticidal activity of dioxapyrrolomycin (**1**).

Streptomyces fumanus (Sveshnikova) [4]. This compound displayed broad, moderate insecticidal and acaricidal activity [5]. However, the relatively high oral toxicity on mice precluded it from development.

The simplicity of the structure did warrant using **1** as a starting point for a synthesis program to optimize the insecticidal activity while attempting to reduce mammalian toxicity. Chlorfenapyr (**2**) is the result of this program (Scheme 28.2.1).



dioxapyrrolomycin, **1**

chlorfenapyr, **2**

m.p. = 100-101° C
vP <1.2 X 10^{-2} mPa (20° C)

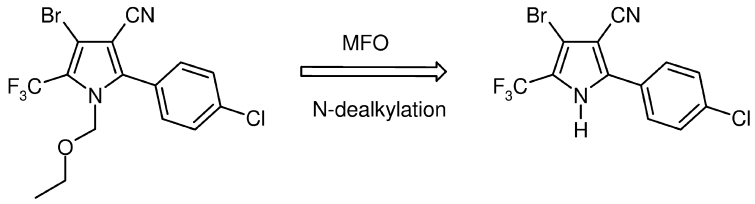
Scheme 28.2.1

28.2.2 Biochemical Mode of Action

Based on the structure of dioxapyrrolomycin and related compounds, it was postulated that the insecticidal activity of these compounds was due to the uncoupling of oxidative phosphorylation. This was subsequently confirmed through mouse-liver mitochondrial assays [6].

Uncoupling activity is dependent on two physicochemical parameters: (1) lipophilicity (log *P*) that allows the molecule to move across the mitochondrial membrane [7, 8] and (2) acidity (pK_a) that allows the molecule to disrupt the proton gradient necessary for the conversion of ADP into ATP [7–9]. Studies have shown that a log *P* of 6.0 $\pm$ 1 and a pK_a range of 7.0–7.9 are necessary for optimal insecticidal activity [10].

Examination of the structure of chlorfenapyr (**2**) reveals that, while it is a lipophilic molecule, it lacks the acidic proton necessary for potent uncoupling activity. In *in vitro* studies using intact Sf9 insect cells, chlorfenapyr did not show significant inhibition of respiration [11]. However, the N-dealkylated compound **3** was a potent uncoupler in insect mitochondria. Taken together, these studies suggest that chlorfenapyr (**2**) acts as a pro-insecticide and that **3**, liberated by metabolic dealkylation of the parent, partially by mixed function oxidases (MFOs), was the active compound in insects (Scheme 28.2.2). The fact that both the parent mole-



chlorfenapyr, **2**	**3**
Lipophilic	Lipophilic
No Acidic proton	Acidic proton present
LC_{50} (TBW): 5.9 ppm	LC_{50} (TBW): 3.3 ppm
U_{50}:>1000 nM	U_{50}: 2.4 nM

Scheme 28.2.2. U_{50} = concentration giving 50% uncoupling.

cule and the N-dealkylated compound were equipotent *in vivo* adds credence to the pro-insecticide concept.

Further support for the pro-insecticide concept was found using Colorado potato beetles that had been pretreated with piperonyl butoxide (PBO), an inhibitor of MFOs. In this study, chlorfenapyr (**2**) gave complete control at a dose rate of 10 ppm on the untreated insects. Treatment of the insects with PBO reduced the level of control to <10% [12].

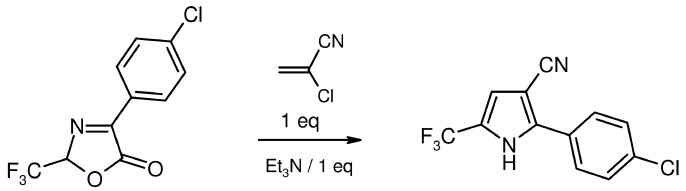
The N-ethoxymethyl group provided the best balance between metabolic activation while avoiding phytotoxic effects seen for the parent, **3**.

28.2.3 Chemistry

At the beginning of the analog program, several interesting synthetic challenges were identified. Methods for the preparation of densely functionalized pyrroles were limited. Also, the preparation of trifluoromethyl substituted pyrroles in a regiospecific manner had not been developed. Both of these issues were addressed by the development of a new cycloaddition reaction [13, 14]. Thermal cycloaddition of the oxazolinone **4** with 2-chloroacrylonitritrile in the presence of a base gave the trisubstituted pyrrole **5** in good yields (Scheme 28.2.3) [15].

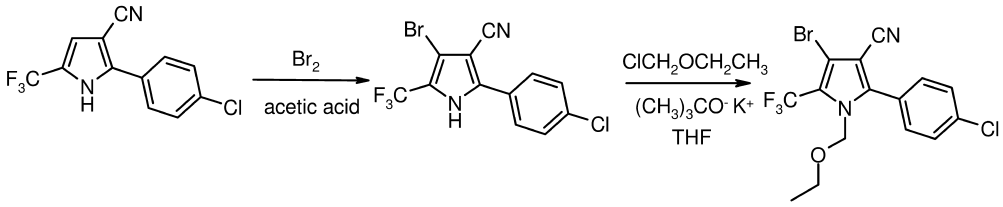
The synthesis of chlorfenapyr was completed by introduction of bromine onto **5** using standard conditions to give the **3** followed by alkylation on the pyrrole nitrogen. Scheme 28.2.4 summarizes the results.

With the discovery of the activity of chlorfenapyr, alternate routes for the preparation of the trisubstituted intermediate pyrrole **5** were investigated. The cycloaddition routes shown in Scheme 28.2.5 allowed for the regiospecific preparation of **5** while avoiding the preparation of the oxazolinone **4** [16, 17].



4 5

Scheme 28.2.3



5 3 chlorfenapyr, **2**

Scheme 28.2.4

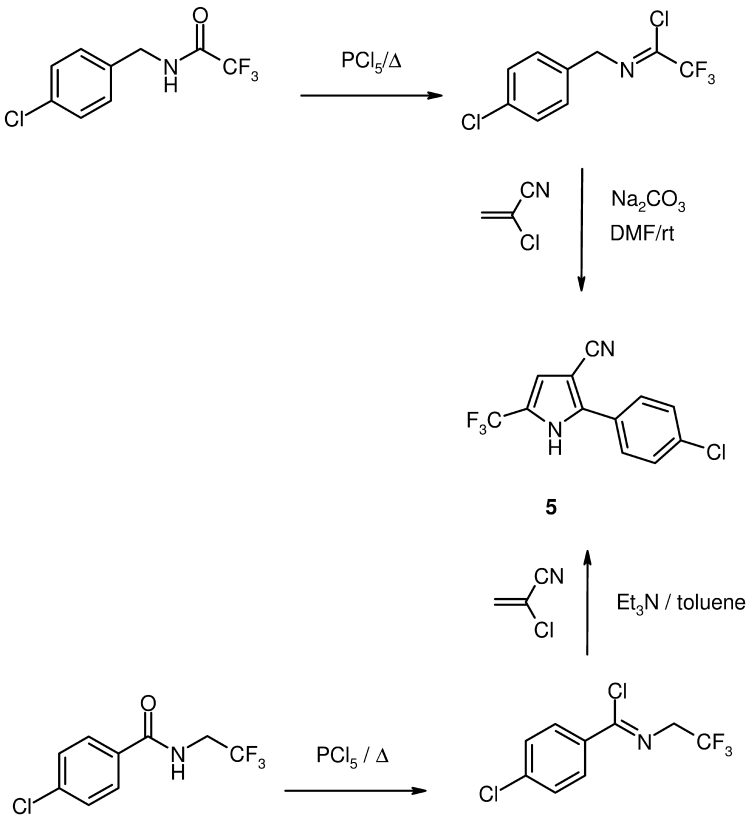
The use of the benzoyl chloride (or benzoic acid) opens manufacturing opportunities that would be limited by previous routes. Also, new intermediates such as imidoyl chlorides or amides containing fluoroalkyl groups are made available for biological screening.

The necessity to prepare densely functionalized pyrroles has led to the development of cycloaddition reactions that proceed in high yield and in a regiospecific fashion. These routes helped to facilitate structure–activity relationship studies to optimize the biological activity for this class of chemistry.

28.2.4 Pest Species and Markets

Chlorfenapyr is active against larvae and adults of many pest species, including insects and crop mites in the orders Lepidoptera, Coleoptera, Thysanoptera, Isoptera, Orthoptera, Hymenoptera and Acarina. Its broad spectrum of activity has provided commercial opportunities for use in control of pests in a wide range of crops, including vegetables, tree fruits, vines, cotton and ornamentals.

First crop registrations were achieved in Africa in early 1995, followed by Japan with registrations for major crop uses against Lepidoptera, Coleoptera and Acari in 1996. The USA non-crop registration for termite control was granted in 2001.



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Scheme 28.2.5

Uptake of chlorfenapyr is mainly by ingestion and secondarily by contact. Owing to its unique mode of action, chlorfenapyr controls pests resistant to other insecticide chemical classes and no instances of target site cross-resistance have been observed. It exhibits good translaminar movement in plants but very limited systemicity.

Chlorfenapyr is non-repellent and has therefore found particular utility in non-crop pest control. Applied as a barrier treatment around buildings, termites do not detect it in the soil and move through the treated zone picking up a lethal dose. Termite control professionals have found chlorfenapyr to be an important tool in eliminating termites from houses and providing effective residual protection from further termite attack [18]. Similarly, due to its non-repellency, chlorfenapyr is highly effective as a spray and in baits for control of cockroaches, ants and other household pests that tend to avoid irritant type insecticides.

Because of its pro-insecticide properties, requiring conversion into the active insecticidal form by the action of MFOs in the body of the insect, chlorfenapyr has proven to be relatively benign to natural enemies. Field studies in Australia and

the USA have shown that it has significantly less impact on predatory bugs and various parasitic wasps and spiders than other broad spectrum insecticides used in cotton (AmCy commissioned studies: Simpson, Lloyd and Murray, QDPI Australia, and M. Sullivan, Clemson University, USA).

28.2.5 Conclusions

This chapter summarizes the discovery and development of chlorfenapyr, a potent uncoupler of oxidative phosphorylation as an insect control agent. This compound shows activity against a broad spectrum of crop and urban pests while having relatively little impact on beneficial insects.

The manipulation and improvement of the biological activity from the natural product lead, dioxapyrrolomycin, demonstrates that the use of naturally occurring compounds as scaffolds for synthetic programs remains a viable avenue for the discovery of new insecticides.

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28.3
Inhibitors of Mitochondrial Electron Transport – Acaricides and Insecticides

Thomas C. Sparks and Carl V. DeAmicis

28.3.1
Introduction

Insect control agents, including acaricides, act through three broad mechanisms: disrupting the nervous system, insect development (insect growth regulators) or respiration [1]. The insect nervous system has long been the target for most insect control agents, past and present, as exemplified by the organophosphorus, carbamate, pyrethroid, cyclodiene, and DDT-related families of insecticides [1–3]. Until the early 1990s examples of insect control agents acting through the disruption of insect respiration had been limited to the dinitrophenols, organotins and a few natural products such as rotenone and piericidin A [3]. Interestingly, in the past 15 years there have been several new insecticidal and acaricidal molecules that exert their effects through disruption of respiratory processes, which includes mitochondrial electron transport (MET) and oxidative phosphorylation [1, 4]. The MET chain consists of a series of sequentially acting electron carriers (metalloproteins) bound to the inner membrane of the mitochondria [5, 6]. These carriers move electrons from NADH though a sequence of four metalloprotein complexes (I–IV) to, ultimately, molecular oxygen [6, 7]. Although there are potential sites for inhibition throughout the MET chain, thus far only two target sites (Complex I and Complex III) have been exploited as sites of action for insecticides and acaricides [4, 8].

This chapter focuses on insecticides and acaricides that act by inhibiting MET at these two sites. The reader is directed to Chapters 13.1 (Earley [5]) and 13.5 (Walter [9]) in the Section on Fungicides for overviews of the MET system and Complex I as a target for fungicides, respectively. Likewise, Ehrenfreund [10] and Kuhn [11], in Chapters 28.1 and 28.2, respectively, provide information on insect control agents that interfere with aspects of oxidative phosphorylation.

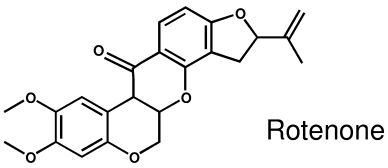


Fig. 28.3.1. Structure of rotenone.

28.3.2 Complex I Inhibitors

Rotenone (Fig. 28.3.1) is a well-known insecticidal constituent from plant species belonging to the genera *Derris* and *Lonchocarpus* [12]. The mode of action of rotenone is inhibition of MET at complex I [12, 13], and for many years rotenone and its related analogs (rotenoids) were the primary insecticidal compounds employing this mode of action. Thus, interestingly, in the early to mid-1980s research at different companies in the US and Japan, all involving unrelated chemistry, led to the discovery of acaricidal molecules, each unique and distinct in its chemistry, and yet all possessing the identical, and until that time, under-exploited mode of action, inhibition of site 1 in the MET system. Since this first group of MET-I (mitochondrial electron transport, complex I) inhibitors, other compounds and analogs that act at this target site have been explored, leading in some cases to the development of other new acaricidal and insecticidal products. Table 28.3.1 at the end of this section provides an overview of properties, toxicology and pest spectrums for these different MET compounds.

As a group, the MET-I inhibitor-based acaricides are broadly active against a diverse array of mite species, especially spider mites. In many cases rust mites are also controlled, as are other mite species. While the initial MET-I inhibitors were primarily active on mites, more recent compounds such as tolfenpyrad, and perhaps others, also provide control of an expanded spectrum of insect species as well. Compared with many older acaricides, the MET-I acaricides tend to be active on all mite stages (Table 28.3.1), thus enhancing their utility to the grower. Additionally, in contrast to some new acaricides that function via growth regulation or inhibition of fatty acid synthesis [4], the MET inhibitor chemistry tends to be fast acting with a good knock-down as well as good residual [14]. However, the MET-I acaricides and insecticides tend to be much more active against aquatic species (Table 28.3.1; fish and daphnia LC_{50} in the range of 0.001 to 0.1 mg L^{-1}), a potential consideration in some cropping systems. All of the MET-I inhibitors possess rat/mouse oral toxicities that are generally in the range 100–997 mg kg^{-1} for technical materials (formulated materials typically display improved mammalian selectivity), with dermal and avian values typically >2000 mg kg^{-1} (Table 28.3.1). Notably, mammalian selectivity in the form of acute rat oral toxicity for the MET-III inhibitors is, in general, better than observed for the MET-I inhibitors (Table 28.3.1).

Table 28.3.1 Commercial and experimental insecticidal and acaricidal MET inhibitors.

Common name; trade names; code	Manufacture (year introduced)	Physical properties	Properties (use rates in g ha^{-1})	Toxicology[a]	Spectrum (Ref.)
MET I inhibitors					
Rotenone	1848	–	Primarily foliar short residual	60–1500 ro, >2000 rbd, 2600 md, 0.031 tr	Broadly active: caterpillars, aphids, mites, beetles, thrips, hoppers, etc. also as a piscicide [3]
Fenpyroximate; Danitron®, FujiMite®, Kendo®, Acaban®, Ortus®, Akari®; NNI-850	Nihon Nohyaku (1991)	mp 101–102 °C; log K_{OW} 5.01	Primarily foliar quick knockdown (25–50)	245–480 ro, >2000 rd, >2000 md, 0.0061 cp	Mite eggs & motile forms, tetranychids, tarsonemids, tenupalpids, eriophyids, grape leafhoppers, pear physlla, grape mealy bug [21]
Pyridaben; Sanmite®, Pyramite®, Nexter®; NC-129, NCI-129	Nissan (1991)	mp 111–112 °C, log K_{OW} 6.37	Rapid knockdown, good residual (100–250)	358–435 ro, >2000 rd, >2250 md, 0.0083 cp	Mite motile forms, broad spectrum acaricide, whitefly, thrips, leafhoppers, mealybugs, plant bugs [21]
Fenazaquin; Magister®, Matador®; EL-436, XDE-436	Dow AgroSciences (now Gowan) (1993)	mp 78–80 °C, log K_{OW} 5.51	Primarily foliar, contact active (56–560)	>134 ro, 1480 mo, >5000 rbd, >2000 md, 0.004–0.034 cp	Mite eggs & motile forms, spider mites [21]

Table 28.3.1 *(continued)*

Common name; trade names; code	Manufacture (year introduced)	Physical properties	Properties (use rates in g ha^{-1})	Toxicology[a]	Spectrum (Ref.)
Tebufenpyrad, Masai®, Pyranica®; MK-239	Mitsubishi (now Nihon Nohyaku) (1993)	mp 61–62 °C, log K_{OW} 5.04	Rapid knockdown, good residual, translaminar (100–250)	595–997 ro, >2000 rd, >2000 md, 0.073 cp	Mite eggs & motile forms, broad spectrum acaricide [21, 120]
Tolfenpyrad; Hachihachi®; OMI-88	Mitsubishi (now Nihon Nohyaku) (2002)	mp 87–89 °C	Ovicidal effects, anti-feedant effects (75–200)	75–86 ro, 107–114 mo, >2000 rd, 0.0029 cp	Broad spectrum insecticide, aphids, thrips, whiteflies, lepidopteran leaf miners, some mites, Diamondback moth [121]
Pyrimidifen; Miteclean®; SU 8801, SU 9118	Sankyo/Ube (1995)	mp 69–91 °C, log K_{OW} 4.59	25–75	110–148 ro, >2000 rd, 445 md, 0.093 cp	Spider & rust mites, all stages, other pests, diamondback moth [21]
Flufenerim; S-1560	Ube in development	–	–	–	Broader spectrum development of pyrimidifen, incl. some lepidopterans [29, 63]
XR-100; LY 247356	Dow AgroSciences, discontinued	–	–	>10–<50 mo	Mites, eggs & motile forms, lepidopteran eggs [71]
LY 809460	Dow AgroSciences, discontinued	–	–	–	Broad spectrum insecticide, esp. lepidopterans [71]

LY 823089	Dow AgroSciences, discontinued	–	–	<0.0001 tr	Broad spectrum insecticide, esp. lepidopterans [72]
SAN 548A	Sandoz, discontinued	–	–	–	Ticks and possibly other insect/mite pests [39]
Hoe 11077	Hoechst, discontinued	–	–	–	Cockroaches and possibly other insect/mite pests [57, 74]
MET III Inhibitors					
Acequinocyl; Kanemite®, Shuttle®; DPX-3792, AKD-2023	DuPont (now Agro-Kanesho) (1999)	mp 59–60 °C, log K_{OW} > 6.2	Contact-oral activity, good residual (130)	>5000 ro, >2000 rd, >2000 md, 97 cp	Broad spectrum acaricide, esp. motile stages [21]
Fluacrypyrim; Titaron®; NA-83	Nippon Soda (2002)	mp 107–109 °C, log K_{OW} 4.51	–	>5000 ro, >5000 rd	Spider mites [21]

[a] ro = acute rat oral LD_{50} (mg kg^{-1}), mo = acute mouse oral LD_{50} (mg kg^{-1}), rd = acute rat dermal LD_{50} (mg kg^{-1}), rbd = acute rabbit dermal LD_{50} (mg kg^{-1}), md = acute avian toxicity, mallard duck LD_{50} (mg kg^{-1}), cp = acute aquatic toxicity, carp LC_{50} (mg L^{-1}), tr = acute aquatic toxicity, trout LC_{50} (mg L^{-1}). Data adapted from Refs. [3, 8, 14, 21, 29, 47, 112–119].

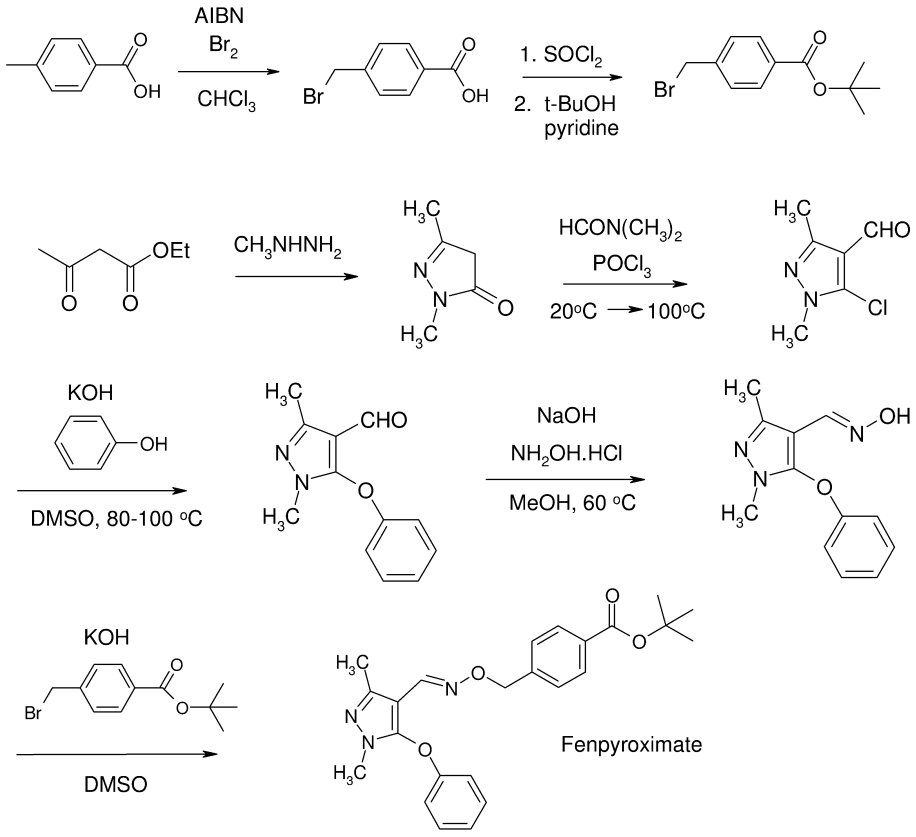
28.3.2.1 **Fenpyroximate**

Interestingly, each of the four of the initial companies that developed MET-I acaricides employed rather different methodologies in their discovery efforts. At Nihon Nohyaku, the discovery of fenpyroximate (NNI-850; Table 28.3.1) was the result of an effort focused on discovering a new acaricide, using a chemistry based approach. Here, the effort was initiated using chloroformylpyrazol as a template because it was easily synthesized and possessed several sites for substitution [15]. About 2000 analogs were synthesized and tested for acaricidal activity, including some pyrazoloxime ethers that exhibited high levels of mite activity. Further activity-directed optimization led to the identification of methyl groups in the 1,3 positions of the pyrazole along with a phenoxy group in the 5-position as being highly active. When coupled with a 4-*t*-butyl ester moiety on the benzyl ring the resulting compound appeared optimal and was selected for development as fenpyroximate [15]. Subsequent studies demonstrated that fenpyroximate inhibits MET at Complex I [16–19]. Interestingly, fenpyroximate can assume the same nonlinear molecular shape as the other MET-I inhibitors, including pyridaben and tebufenpyrad [20]. Fenpyroximate is active against a wide variety of mite species (Table 28.3.1) and is registered for a wide variety of crops, including citrus, pome fruit, vegetables, beans, vines, strawberries, melons, hops and ornamentals [21].

Scheme 28.3.1 shows the synthesis of fenpyroximate. The pyrazolone ring, formed by the condensation of ethyl acetoacetate with methylhydrazine, is then subjected to the Vilsmeier–Haack chloroformylation using DMF and $POCl_3$ to give the 5-chloro-4-formylpyrazole [22, 23]. The chloride in the 5-position of the pyrazole is substituted with phenol through a nucleophilic displacement reaction [24]. Fenpyroximate is then generated by condensation of the 4-formyl-5-phenoxypyrazole with hydroxylamine followed by a Williamson ether synthesis with the side chain *t*-butyl (4-bromomethyl)benzoate [15, 22, 25, 26]. The side chain is prepared by free radical bromination of *p*-toluoic acid followed by formation of the acid chloride and condensation with *t*-butanol [27, 28].

28.3.2.2 **Pyridaben**

In contrast to fenpyroximate, the discovery of pyridaben (NCI-129; Table 28.3.1) by Nissan Chemical Industries exemplifies where activity in one product area can lead to product level activity in other product areas. This also demonstrates the value of broad screening of chemistries as a means to unearth new activity. In this case, research into herbicidal activity of pyridazinones led to the discovery that one of the analogs possessed acaricidal activity [29]. A subsequent exploration of the structure–activity relationships (SAR) around this chemistry, now focusing on acaricidal activity, led to the identification of pyridaben [29]. Like others in this group, pyridaben inhibits insect respiration and has been shown to specifically impede Complex I oxidations [18]. More recent studies using a pyridaben derivative as a photoaffinity label demonstrate that pyridaben, as well as other MET-I inhibitors, interact with the PSST subunit of Complex I [30–32]. Pyridaben is a broad-spectrum, contact acaricide (Table 28.3.1) registered for use on various



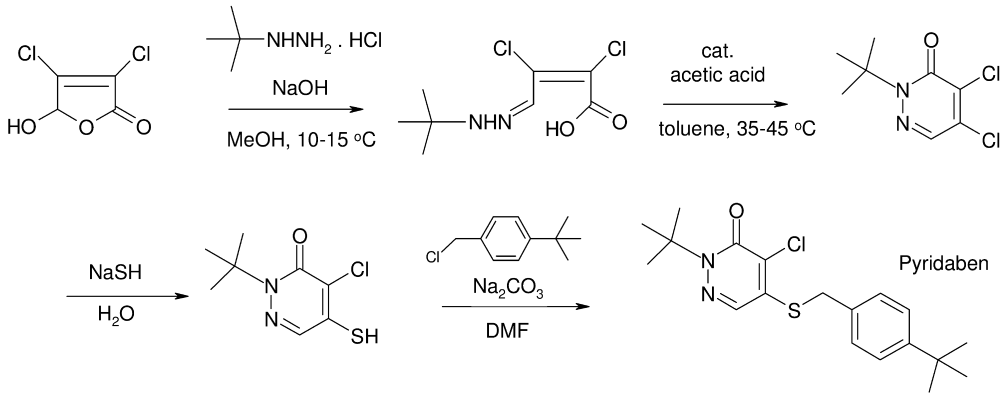
Scheme 28.3.1 Synthesis of fenpyroximate.

crops, including tree fruit (apples, almonds, cherries, plums, pears), citrus, vegetables, grapes, strawberries and ornamentals [21].

Scheme 28.3.2 shows the synthesis of pyridaben. The pyridazinone ring is formed though a two-stage process involving the condensation of mucochloric acid with *t*-butylhydrazine at low temperature to give a *t*-butylhydrazone intermediate that is then cyclized at elevated temperature with catalytic acid [33, 34]. The chlorine in the 5-position of the pyridazinone is then substituted with sodium thiolate to give a mercaptan that is reacted with *p-tert*-butylbenzyl chloride to give pyridaben [35]. The *p-tert*-butylbenzyl chloride is prepared in high yield by free radical chlorination of *p-tert*-butyltoluene [36].

28.3.2.3 **Fenazaquin**

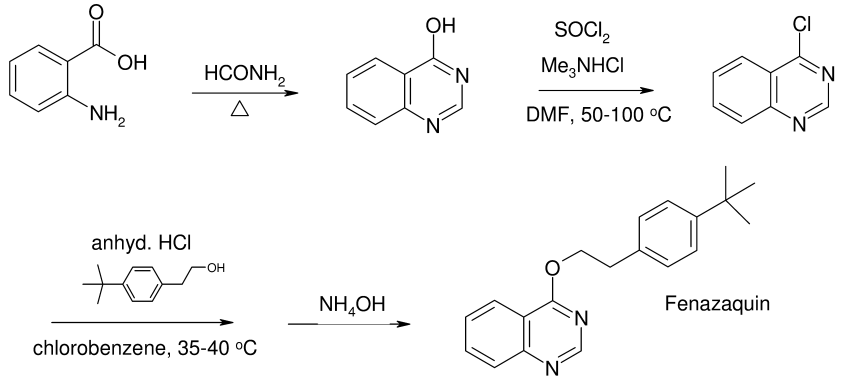
The discovery of fenazaquin (EL-436, XDE-436; Table 28.3.1) resulted from yet another approach. In the early 1980s as part of Elanco's random screening program a quinazoline, LY-176771, was found to exhibit some fungicidal activity against



Scheme 28.3.2 Synthesis of pyridaben.

grape downy mildew [37]. Based on the observed activity and an examination of prior art, a series of quinazoline ethers was investigated [38] with the goal of improving fungicidal activity. However, broad screening of these new compounds identified analogs that exhibited activity against lepidopterous insects. Further refinement of the SAR focused on acaricidal activity, ultimately leading to fenazaquin [38]. Internal and external studies demonstrated that fenazaquin inhibits MET at Complex I [18, 39–41]. Fenazaquin is particularly effective against tetranychid mite species, including two-spotted spider mite and red mites (Table 28.3.1) and is registered for use on various tree fruit (apples, citrus, pears, plums), vines, vegetables and ornamentals [21].

Scheme 28.3.3 shows the synthesis of fenazaquin. The quinazoline ring is formed by the condensation of anthranilic acid with formamide and subsequent



Scheme 28.3.3 Synthesis of fenazaquin.

halogenation at the 4-position is accomplished using the Vilsmeier reagent [42]. The 4-chloroquinazoline is coupled to the side chain 4-*tert*-butylphenylethanol with the aid of anhydrous HCl [43]. This gives fenazaquin as the HCl salt, which is liberated as the free base with aqueous ammonia.

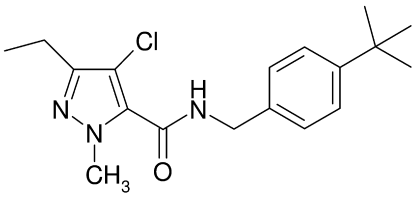
28.3.2.4 **Tebufenpyrad**

The approach that Mitsubishi Chemical Corporation took in its efforts to develop a new acaricide lies somewhere between the strict chemical based approach and simple broad screening approach, specifically targeting chemistry that possesses biological activity in one area of interest and exploring it for activity in other product arenas. It was noted that *N*-phenylpyrazole carboxamides exhibited fungicidal and herbicidal activity, and because of their interesting chemical structure and known activity in other areas, a targeted synthesis effort around pyrazolecarboxamides, focusing on acaricidal activity, was initiated [44]. The outcome was the identification of pyrazole-5-carboxamides with acaricidal activity, ultimately leading to the discovery of tebufenpyrad (MK-239; Table 28.3.1) [44]. As with the above compounds, subsequent studies were to show that tebufenpyrad functions as an inhibitor of MET Complex I [32, 39]. Tebufenpyrad is a broad spectrum acaricide, possessing translaminar activity (Table 28.3.1) registered for use on various crops, including pome and stone fruit, ornamentals, strawberries, hops, melons, citrus and tomato [21].

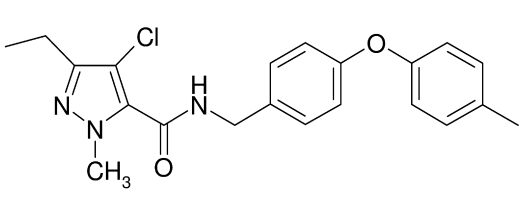
The preparation of tebufenpyrad and tolfenpyrad (see below) are outlined in Scheme 28.3.4. The pyrazole ring is prepared from a Claisen condensation of 2-butanone with diethyl oxalate followed by treating the resulting acylpyruvate with hydrazine [45, 46]. The pyrazole ring is then alkylated with dimethyl sulfate at 50–60 °C without base to give, selectively, the 1-methylpyrazole-5-carboxylate. The pyrazole is then chlorinated in the 4 position and saponified to give the pyrazolecarboxylic acid. The acid chloride of the pyrazolecarboxylic acid is formed and coupled with 4-*tert*-butylbenzylamine or 4-(*p*-tolyloxy)benzylamine to give tebufenpyrad and tolfenpyrad, respectively. The side chain compound 4-*tert*-butylbenzylamine is prepared by the reductive amination of 4-*tert*-butylbenzaldehyde with aqueous ammonia. The compound 4-(*p*-tolyloxy)benzylamine is prepared by coupling 4-fluorobenzonitrile with sodium *p*-cresol followed by reduction with Raney nickel in aqueous ammonia [47, 48].

28.3.2.5 **Tolfenpyrad**

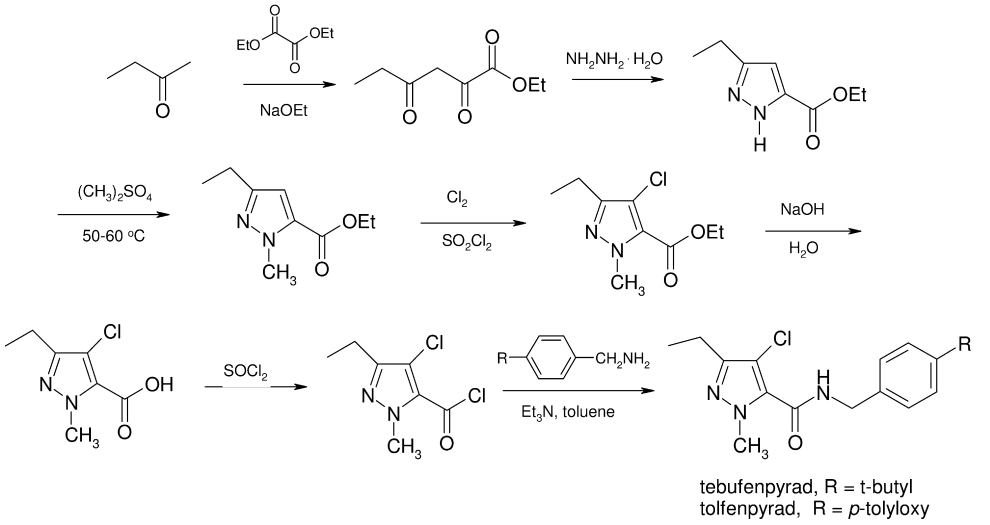
Mitsubishi Chemical Corporation's interest in the pyrazolecarboxamides did not end with the discovery of tebufenpyrad. Further synthesis was undertaken to improve and expand on the acaricidal activity, leading to identification of weak activity against some insect species for some analogs of tebufenpyrad [45, 49–51]. Further studies indicated that replacement of the *t*-butyl tail with electron-withdrawing groups greatly improved insecticidal activity against hemipterans and some lepidopterans. However, there was also an increase in mammalian toxicity [45, 51]. Further investigations [47] lead to the identification of a *N*-

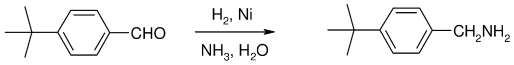


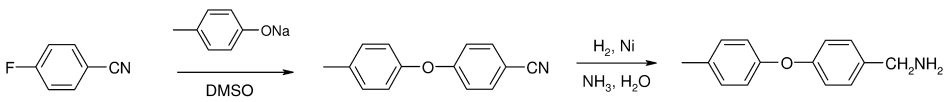
Tebufenpyrad



Tolfenpyrad







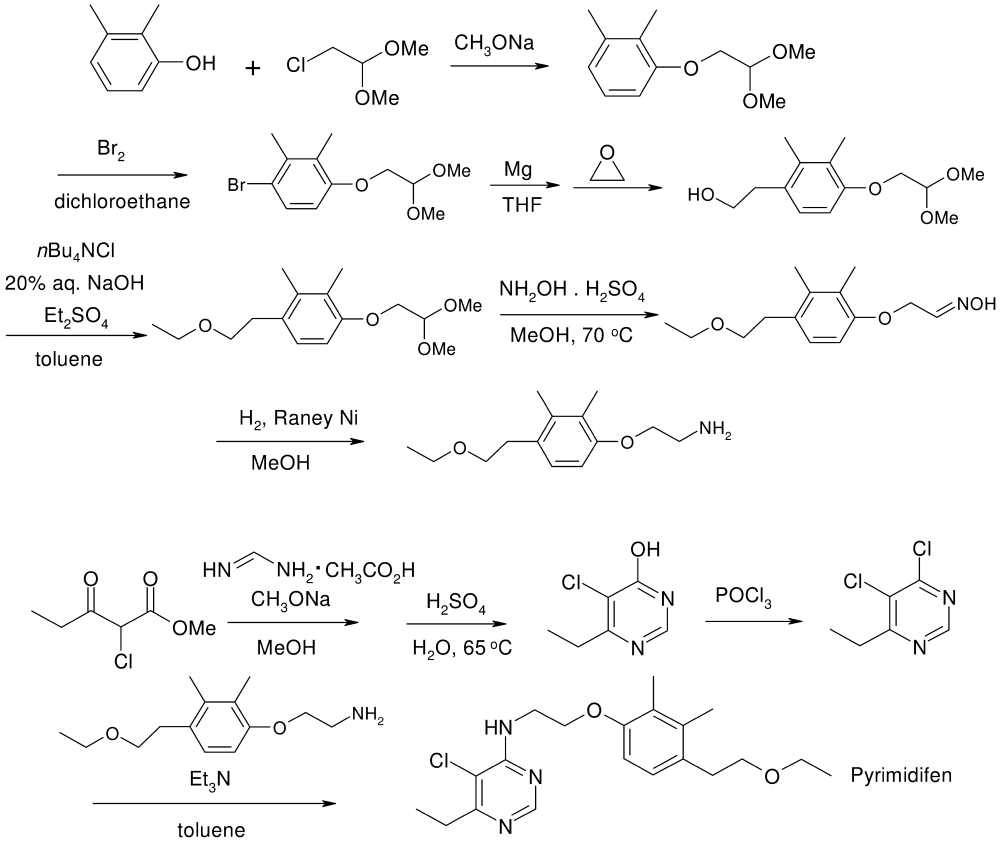
Scheme 28.3.4 Synthesis schemes of tebufenpyrad and tolfenpyrad.

tolyloxybenzyl derivative, tolfenpyrad (OMI-88; Table 28.3.1), as possessing good insecticidal activity coupled with acceptable mammalian selectivity. Tolfenpyrad is broad spectrum miticide/insecticide (Table 28.3.1), currently registered for use on vegetables and orchards in Japan [21].

28.3.2.6 **Pyrimidifen**

Pyrimidifen (SU 8801; Table 28.3.1) is an acaricide jointly patented and developed by Sankyo Company and Ube Industries. This acaricide chemistry appears to be the result of a long line of research starting with *N*-(substituted phenoxyalkyl)-4-quinazolinamines that exhibited fungicidal activity morphing into *N*-benzyl-4-pyrimidinamines displaying moderate lepidopteran and mite activity [52–56], further evolving into analogs from which pyrimidifen emerged [55]. Based on similarity to other close pyrimidine compounds and specific studies, pyrimidifen appears to act at Complex I [4, 57, 58]. Pyrimidifen is effective on various mite species, including spider and rust mites, and certain other insect pests, and has been registered for use in tree fruit (e.g., apples, pears) citrus, vegetables and ornamentals [21].

Scheme 28.3.5 shows the synthesis of pyrimidifen. The pyrimidine ring in pyrimidifen is prepared via a condensation of methyl 2-chloro-oxovalerate with



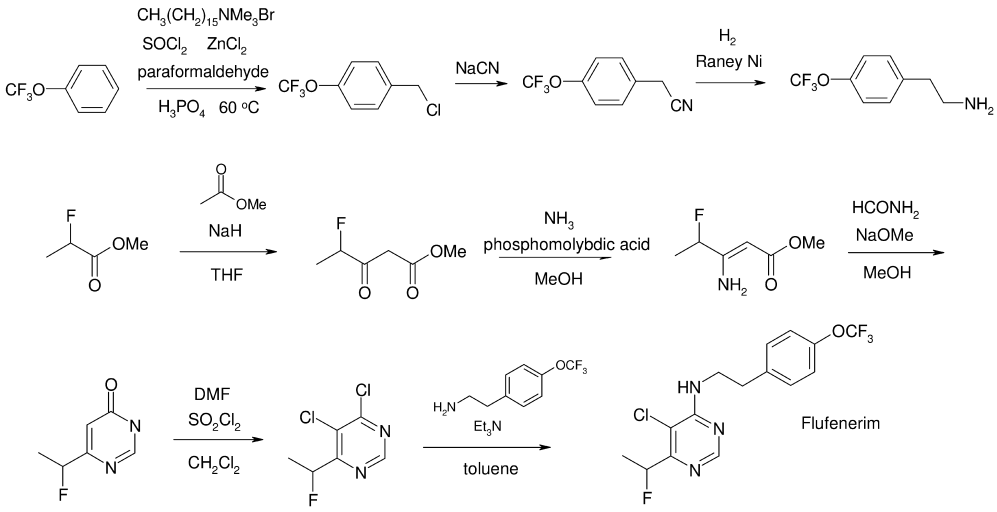
Scheme 28.3.5 Synthesis of pyrimidifen.

formamidine acetate in the presence of base [59, 60]. The resulting 4-hydroxypyrimidine is then chlorinated with phosphorous oxychloride to give 4,5-dichloro-6-ethylpyrimidine. The chlorine in the 4-position is substituted with the side chain 2-[4-(2-ethoxyethyl)-2,3-dimethylphenoxy]ethylamine through nucleophilic displacement [54, 61]. The side chain is prepared from the reaction of 2,3-xylenol with chloroacetaldehyde dimethylacetal [59]. The resulting acetal is then brominated in the 4-position, converted into the Grignard, and reacted with ethylene oxide to give the phenethyl alcohol. The alcohol is converted into the ethyl ether with diethyl sulfate under phase-transfer conditions. The resulting substituted phenoxyacetaldehyde dimethylacetal is converted into the oxime with hydroxylamine under acidic conditions and the oxime reduced with hydrogen in the presence of Raney nickel to give the substituted phenoxylethylamine side chain [62].

28.3.2.7 **Flufenerim**

Flufenerim (S-1560; Table 28.3.1) is an acaricide/insecticide under development [21] that appears to be a more recent derivative of pyrimidifen, but possessing less labile substitutions on the head and tail regions (Fig. 28.3.2). Available information suggests that flufenerim has acaricidal activity and perhaps some lepidopteran and fungicidal activity as well [21, 63, 64].

Scheme 28.3.6 shows the synthesis of flufenerim. The pyrimidine ring is formed from a Claisen condensation of methyl 2-fluoropropionate with methyl acetate [65] followed by reaction of the fluoroacetopropionate with ammonia in phosphomolybdic acid [66] and cyclization with formamide in the presence of base [67].



Scheme 28.3.6 Synthesis of flufenerim.

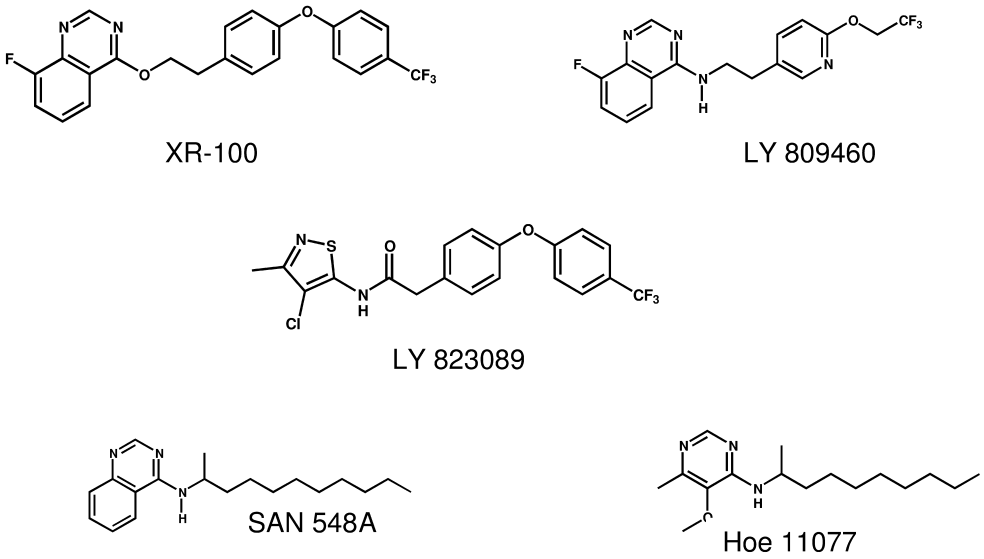


Fig. 28.3.2. Structures of experimental MET I acaricides and insecticides.

This gives the 4-pyrimidone ring, which is converted into the 4,5-dichloro-6-(1-fluoroethyl)pyrimidine by the action of sulfuryl chloride in the presence of dimethylformamide (DMF) [68]. The chlorine on the 4-position of the pyrimidine ring is substituted with the side chain 2-[4-(trifluoromethyl)phenyl]ethylamine through a nucleophilic substitution reaction [63]. The side chain 2-[4-(trifluoromethoxy)phenyl]ethylamine is prepared by chloromethylation of trifluoromethoxybenzene followed by displacement of the chlorine atom with cyanide and reduction of the nitrile with Raney nickel [69].

28.3.2.8 **Experimental Compounds**

In addition to the compounds above that are either already products or in development, other compounds of perhaps of a more experimental nature or that did not progress as far in development have also been examined. Following the discovery of fenazaquin, the structure–activity relationships (SAR) around this chemistry were explored, including fused pyrimidine derivatives of fenazaquin [38]. Additionally, pyridine and pyrimidine amides analogs were investigated [70], as were quinoline/quinazoline derivatives of fenazaquin with modifications to the tail (*t*-butyl) region [71]. These investigations lead to the identification of analogs such as XR-100 (Fig. 28.3.2) and *O*-haloalkyl pyridyl derivatives such as LY 809460 (Fig. 28.3.2; compound 11 in Hackler et al. 1998 [71]) that were more active than fenazaquin at inhibiting MET activity and exhibited an expanded spectrum, proving to be active against lepidopterans [71]. Additionally, a series of isothiazolyl phenylacetamides was also examined [72], displaying good broad spectrum insect activity. However, for both series optimization of the insecticidal

activity also lead to increases in mammalian and/or fish toxicity [71, 73] that could not be adequately remedied, thereby limiting their utility.

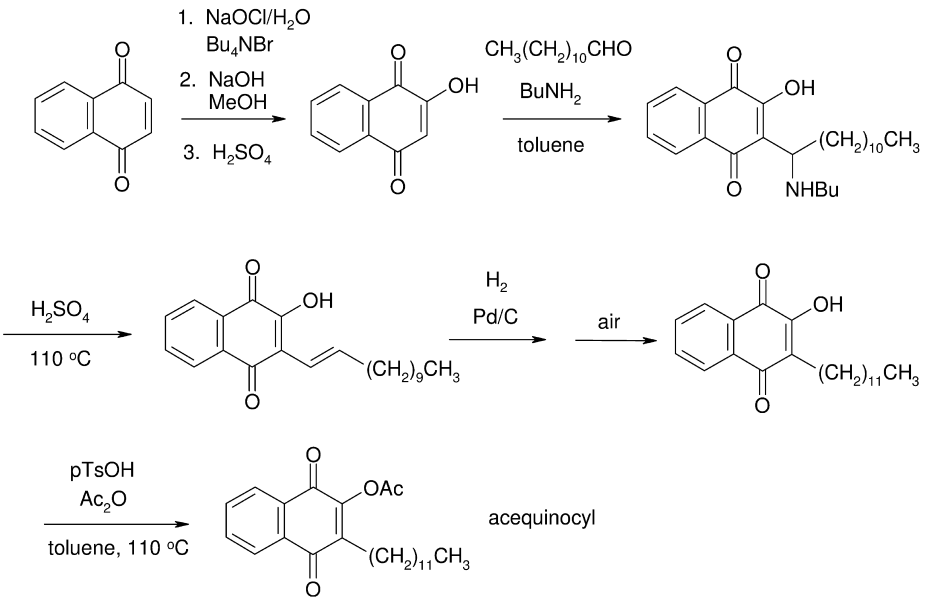
In addition to the above-mentioned efforts of Mitsubishi and Dow AgroSciences, during the early 1990s other companies also investigated chemistry acting as MET-1 inhibitors, including SAN 548A (Fig. 28.3.2, Table 28.3.1) [39], and Hoe 11077 (Fig. 28.3.2, Table 28.3.1) and related compounds [49, 57, 58, 74].

28.3.3 Complex III Inhibitors

Complex III has been very successfully exploited as a target site for fungicides such as the strobilurins, fanoxadone and fenamidone [75]. However, it has only been in the last few years that insecticidal or acaricidal products have used inhibition at Complex III as a mode of action.

28.3.3.1 Acequinocyl

Originally discovered by DuPont in the 1970s and known as DPX-3792, acequinocyl (Table 28.3.1) was subsequently licensed to and brought to market by Agro-Kanesho (ADK-2023) in the early 1990s [21]. Unlike the MET-I inhibitors, acequinocyl is a pro-insecticide. Acequinocyl is bioactivated via deacylation to its deacetyl metabolite, DHN (2-hydroxy-3-*n*-dodecyl-1,4-naphthoquinone). Studies by Koura et al. [76] demonstrate that acequinocyl, via DHN, acts at the ubiquinol



Scheme 28.3.7 Synthesis of acequinocyl.

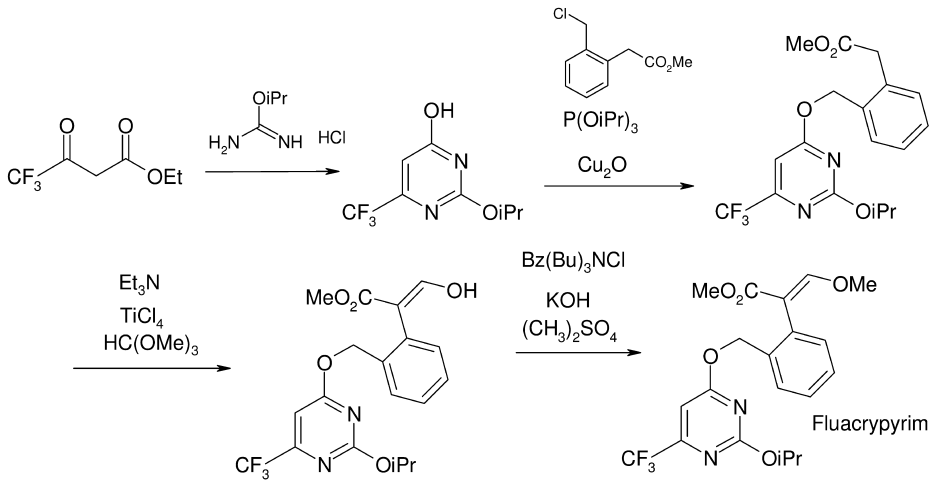
oxidation site (Q_0) of Complex III. Acequinocyl is a broad spectrum acaricide (Table 28.3.1) registered for use in pome and stone fruit, citrus, melons, fruiting vegetables and ornamentals [21].

Scheme 28.3.7 shows the synthesis of acequinocyl. 1,4-Naphthaquinone is epoxidized and acidified to give 2-hydroxy-1,4-naphthaquinone [77]. The dodecyl group is introduced through a condensation of 2-hydroxy-1,4-naphthaquinone with dodecanal in the presence of *n*-butylamine to give 3-(1-butylaminododecyl)-2-hydroxy-1,4-naphthaquinone, which is thermally eliminated to give the dodecenyl derivative [78]. The dodecenyl derivative is then hydrogenated and air oxidized to give 2-dodecyl-3-hydroxy-1,4-naphthaqiunone, which is acetylated to give acequinocyl [78].

28.3.3.2 **Fluacrypyrim**

The *β*-methoxyacrylates and strobilurins are well-known fungicides that act at Complex III of the MET chain [75, 79]. In addition to their fungicidal activity, many companies have patents that claim insecticidal activity as well as fungicidal activity for their strobilurin related chemistry. Fluacrypyrim (NA-83, Table 28.3.1), discovered by BASF and licensed to Nippon Soda [21], is the first methoxyacrylate derived compound to be marketed other than as a fungicide, in this case as an acaricide. Fluacrypyrim appears to be a MET-III inhibitor [4], in part, based on analogy with the strobilurins. Fluacrypyrim is targeted for mite control, especially spider mites (Table 28.3.1), on vegetables and tree fruit such as citrus, apples and pears [21].

Scheme 28.3.8 shows the synthesis of fluacrypyrim. The pyrimidine ring is formed through the condensation of trifluoroacetoacetate with *o*-isopropylisourea hydrochloride in the presence of base [80]. The product is then *O*-alkylated with methyl 2-(chloromethyl)phenylacetate in the presence of copper salts and to give



Scheme 28.3.8 Synthesis of fluacrypyrim.

the pyrimidyloxy derivative [81, 82]. The acrylic acid group is introduced in a two-step process wherein the pyrimidyloxy derivative is reacted with trimethylorthoformate in the presence of a Lewis acid and base to give the 3-hydroxyacrylic acid ester derivative [83], which is alkylated with dimethyl sulfate under phase-transfer conditions to give fluacrypyrim [84].

28.3.4 Metabolism

MET-I inhibitors are all composed of three sections: head, linker and tail. Most commercial MET-I acaricides possess a *t*-butyl, *t*-butyl ester or another long-chain moiety in the 4-position of the tail (Fig. 28.3.3). The *t*-butyl ester of fenpyroximate is rapidly cleaved via a monooxygenase mediated hydroxylation and ensuing transesterification (Fig. 28.3.3) [85, 86]. Other fenpyroximate-based metabolites also arise via monooxygenase activity and include oxidation of the 3-pyrazolomethyl group, *N*-demethylation, isomerization and cleavage of the oxime ether bond (Fig. 28.3.3) [15, 86]. Like fenpyroximate, rat metabolism studies with pyridaben also demonstrate the importance of monooxygenases in pyridaben metabolism. Metabolites include hydroxylation of either of the two *t*-butyl moieties, and cleavage of the thioether linkage (Fig. 28.3.3) [29]. As observed with the two prior acaricides, one of the primary metabolic pathways for fenazaquin in mammalian systems involves oxidation of the *t*-butyl moiety (Fig. 28.3.3) [87]. Additionally, other monooxygenase-mediated metabolic reactions include oxidation of the quinazoline ring and cleavage of ether linkage (Fig. 28.3.3) [87]. Fenazaquin is also rapidly metabolized in lepidopterous insects [88], with oxidation of the *t*-butyl moiety as the predominate metabolite [71]. Like the other MET-I acaricides, tebufenpyrad is also subject to oxidative metabolism at the *t*-butyl moiety (Fig. 28.3.3) [89]. Additionally, the ethyl substituent of the pyrazole head is also subject to oxidation [89], leading to the formation of the 1-hydroxy derivative. Unlike the above mentioned acaricides, tebufenpyrad also contains an amide moiety in the linker between the pyrazole head and the phenyl tail, with other significant metabolites involving amide cleavage (Fig. 28.3.3) [89]. The close structural similarity between tebufenpyrad and tolfenpyrad suggests that some of the metabolic pathways identified for tebufenpyrad (Fig. 28.3.3) are likely to apply to tolfenpyrad, and this appears to be the case; the primary metabolism appears to be oxidation of the 4-methyl group on the tolyl-tail coupled with hydroxylation of the ethyl moiety on the pyrazole head [90]. Public information on the metabolism of pyrimidifen and flufenerim appears to be lacking. By analogy with the other members of this class it is reasonable to assume that monooxygenase mediated metabolic pathways would predominate for both these compounds. Based on the studies with XR-100 [71], it seems likely that the metabolites and rate of metabolism of flufenerim may be altered by the presence of trifluoromethyl group on the tail and a 1-fluoroethyl moiety attached to the pyrimidinyl head. Clearly, studies specifically targeting the metabolism of these compounds will be needed to assess the above hypotheses.

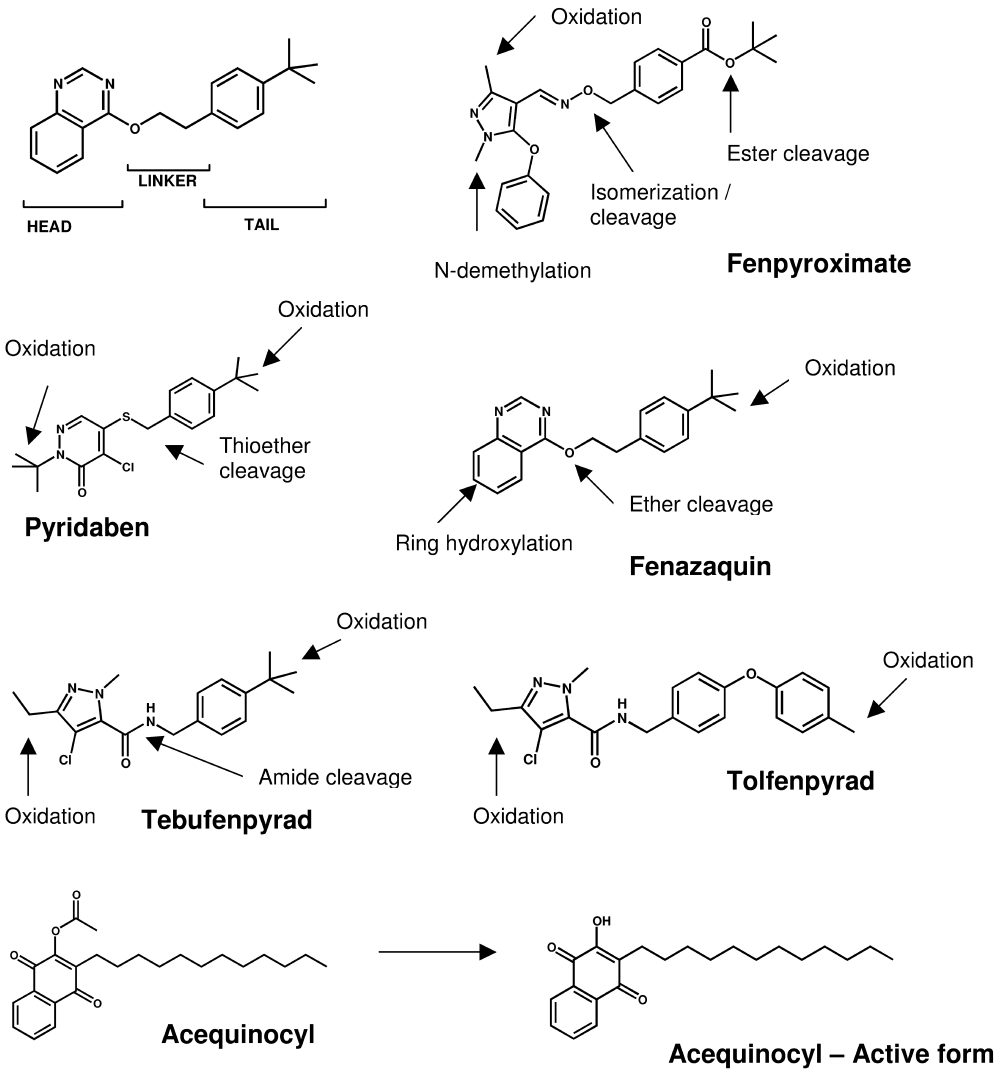


Fig. 28.3.3. Sites of metabolism for MET I & MET III acaricides and insecticides.

Unlike the MET-I acaricides and insecticides, the MET-III inhibitor acequinocyl is a pro-insecticide that requires biological activation for activity. Acequinocyl is hydrolyzed to the corresponding deacyl derivative, 2-hydroxy-3-*n*-dodecyl-1,4-naphthoquinone (DHN), in isolated mitochondria (Fig. 28.3.3) [76]. This is consistent with the observation of a time lag in MET inhibition with acequinocyl [76] and that acequinocyl itself does not directly inhibit MET activity [91].

28.3.5 Resistance and Resistance Mechanisms

As noted above, the MET inhibitor-based acaricides are, as a group, broadly active against a wide variety of mite species, including the two-spotted spider mite (TSSM), *Tetranychus urticae*. The TSSM is among the most prevalent pest mite species, with a long history of developing resistance to available acaricides, and is presently resistant to more insecticides/acaricides than any other mite or insect species [92]. One of the initial advantages of the MET-I acaricides was their efficacy against resistant mite species. However, it was quickly realized that with several of these new acaricides, all possessing the same mode of action, coming into the market place at about the same time, resistance could be a potential issue. IRAC (Insecticide Resistance Action Committee) had put an acaricide resistance management program in place in Europe [93] and the MET-I acaricides became part of this program as they entered the market place [94].

Mite strains highly resistant to hexathiazox (>1000-fold) show lower levels of cross-resistance to MET-I acaricides [95, 96]. Likewise, TSSM resistant to dicofol and clofentazine (100-fold) also show only low levels (1.9–7.2-fold) of cross-resistance to pyridaben, fenpyroximate and tebufenpyrad [97], while a clofentazine resistant strain of ERM (>2000-fold) showed virtually no cross-resistance to pyridaben [98]. In contrast, TSSM mite glasshouse strains resistant to the organophosphorus insecticides dimethoate (97–165-fold) and methamidophos (58–122-fold), also showed significant levels of cross-resistance to pyridaben (27–>100-fold) [99].

In the 15 years that the MET-I inhibitors have been in the market place, their high levels of efficacy on all mite stages, long residual, limited mobility, coupled with the rapid reproductive rate of mites, all contribute to the likelihood of resistance development. Thus, despite efforts attempting to minimize the chances for resistance [93, 94], it is not surprising that resistance to the MET-I inhibitors has developed in several mite species, including TSSM. High levels of resistance, in some cases beyond 1000-fold, have been observed to pyridaben and fenpyroximate in field strains that have received extensive MET-I acaricide use [95, 97, 100–103]. Lower levels of resistance have been observed for tebufenpyrad (6.7–97-fold) and fenazaquin (8–168-fold) [97, 101–105]. While cross-resistance between the MET-I inhibitors and the older acaricides is less common, cross-resistance within the MET-I acaricide family is more common. For example, TSSM resistant to fenpyroximate (252-fold) also exhibited some cross-resistance to pyridaben (38-fold) and tebufenpyrad (24-fold), but less so to fenazaquin (7.2-fold) [100]. Likewise, TSSMs resistant to tebufenpyrad (63-fold) were also cross-resistant to pyridaben (>210-fold) and fenpyroximate (24.6-fold) [101].

Consistent with the above mentioned trend of little MET-I cross-resistance from mites resistant to other types of acaricides, the MET-I resistant strains are, likewise, less likely to confer cross-resistance to other classes of acaricides and insecticides. MET-I acaricide-resistant TSSM strains exhibited little cross-resistance to dicofol, amitraz or chlorfenapyr [97, 103]. In the reverse case, a methidathion-resistant strain of the predatory mite *A. womersleyi* showed no cross-resistance to

pyridaben [106], nor did clofentezine (or clofentazine–dicofol) resistance strains of red mites and TSSM show any cross-resistance to MET-I acaricides such as pyridaben, tebufenpyrad or acequinocyl [98, 107]. An exception to these observations being TSSM strains resistant to dimethoate and methamidophos that are also exhibiting cross-resistance to pyridaben [99].

Because the MET-I acaricides share the same mode of action, a reasonable expectation would be that target site resistance may play a role in some of the cases where cross-resistance between MET-I acaricides has been observed. Perhaps, however, due to the complexity of the MET-I site with its many components originating from both mitochondrial and nuclear sources, target site-based resistance does not yet appear to be a primary resistance mechanism. Available information suggests that other resistance mechanisms such as metabolism predominate. As noted above, the MET-I acaricides are primarily metabolized by monooxygenases. A fenpyroximate-resistant strain of TSSM showed enhanced levels (ca. 2.5-fold) of monooxygenases and esterase activity, and the activity of fenpyroximate was highly synergized by the monooxygenase inhibitor piperonyl butoxide, but not by inhibitors of esterases or glutathione transferases [100]. Other studies have also demonstrated that MET-I acaricide resistance is associated with enhanced monooxygenases activity, and perhaps glutathione transferase [97, 108]. TSSM multi-resistant strains possessing higher titers of glutathione transferase activity, esterase activity, and, possibly, monooxygenase activity [109] also exhibited cross-resistance to pyridaben [99]. Likewise, studies with a hexathiazox-resistant strain of the CRM (citrus red mite) apparently lacking enhanced monooxygenases activity showed no cross-resistance to either fenpyroximate or pyridaben [101]. In apparent contrast, a field strain of TSSM highly resistant to clofentazine and dicofol, and possessing enhanced monooxygenase activity, showed no cross-resistance to either tebufenpyrad or acequinocyl [107]. However, since the monooxygenases are a large family of enzymes with varying substrate specificities [110, 111], the lack of cross-resistance to MET-I acaricides in a mite strain possessing enhanced monooxygenases activity is not inconsistent with results from the other studies.

Metabolically-based cross-resistance among the MET-I insecticides/acaricides should not be surprising. Despite the rather different chemistries involved, the MET-I compounds share similar molecular features, can assume a similar molecular shape [20], and the substituents on the ''tail'' region typically include a *t*-butyl or alkyl moiety. As noted in the above section on metabolism (Section 28.3.4), the *t*-butyl tail is also a common site for metabolism. Thus, any strain developing an enhanced metabolism to one of these compounds could reasonably be expected to exhibit some level of enhanced metabolism to the other members of this group, which is, in general, what has been observed.

28.3.6
The Future for MET Acaricides and Insecticides

Included in the key considerations for mite control are (a) a high degree of efficacy on several growth stages and (b) a lack of cross-resistance with acaricides

possessing other modes of action. Among others, the MET-I acaricides bring these very valuable attributes to the marketplace. The diverse chemistries of the MET-I acaricides and insecticides discovered to date suggests that other novel chemistries may yet be discovered that can also exploit these target sites. Although the first compounds were introduced 15 years ago, new compounds are still being developed. The ubiquitous nature of the MET target site holds the potential for the development of true broad spectrum (sucking and chewing) insect control agents. However, attempts to capitalize on the potential for broad spectrum insect control agents have thus far meet with limited success. The very similar, three-dimensional, whole molecule shape [20], and presence of the same substituents on many of the MET-I acaricides is likely to contribute to their similar metabolic profiles. Since many of the major lepidopteran pests possess potent metabolic systems, it is not surprising that the acaricidal MET-I inhibitors are poorly active on lepidopteran pests [71]. Elimination of these metabolically labile sites (e.g., *t*-butyl or *n*-alkyl moieties) by substitution of aromatics and/or haloalkyl substituents can lead to compounds with broader spectrum and efficacy, but also higher mammalian toxicity [71], since mammals use similar metabolic pathways. Tolfenpyrad was able to bring forward an expanded pest insect spectrum, compared with tebufenpyrad and the other MET-I acaricides, but mammalian selectivity appears reduced (Table 28.3.1).

Likewise attempts to improve upon fenazaquin also led to improved sucking and chewing insect activity, but with an associated increase in mammalian toxicity [71]. A pro-insecticidal approach also met with only limited success [73]. Thus, current chemistries have not yet been able to strike an optimal balance between spectrum, efficacy, mammalian and environmental selectivity, resulting in a true broad spectrum insect control agent possessing toxicological profiles comparable to some of the other newer chemistries (e.g., indoxacarb, spinosad).

Inhibitors acting on the MET-III system presents an interesting contrast to the MET-I inhibitors. Based on a very limited number of compounds and data, compounds in the MET-III group appear to exhibit, at least for acute rat oral toxicity, a far more favorable toxicological profile than the MET-I inhibitors discovered to date. While Complex III and the strobilurin motif have been widely exploited for the control for fungicide pests, thus far only fluacrypyrim exploits this target and chemistry for the control of mites or insects.

The need for new insect control agents utilizing novel modes of action is ever present. As such the MET chain remains an attractive target site.

Acknowledgments

The authors thank Drs. Joel Sheets, Mark Hertlein, and Frank Burroughs for their valuable suggestions and discussions, and Mike Delporte and Carol Freemen for assistance in obtaining the diverse reference materials used in the writing of this chapter. This work was supported by Dow AgroSciences.

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28.4
Inhibitors of Lipid Synthesis (Acetyl-CoA-carboxylase Inhibitors)

Thomas Bretschneider, Reiner Fischer, and Ralf Nauen

28.4.1
Introduction

Acetyl-CoA-carboxylase (ACCase) plays a fundamental role in fatty acid metabolism and is a biotinylated enzyme that catalyzes the carboxylation of acetyl-CoA.

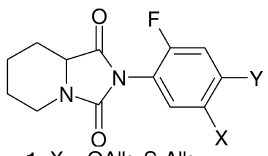
In eukaryotes and prokaryotes, ACCase is a key enzyme in fatty acid biosynthesis [1]. The reaction product, malonyl-CoA, is both an intermediate in the *de novo* synthesis of primary fatty acids and also a substrate in the formation of long-chain fatty acids and flavonoids in plants [2, 3]. Aryloxyphenoxypropionates (APPs) and cyclohexandiones (CHDs) are two chemical classes of molecules that selectively inhibit homomeric, chloroplastic ACCase from grasses [4, 5], which makes them post emergent herbicides used worldwide to control grassy weeds.

28.4.2
Discovery of the Cyclic Ketoenols, Spirodiclofen and Spiromesifen as a New Generation of ACCase Inhibitors

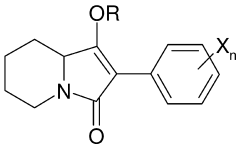
During a synthesis program in the field of protoporphyrinogen-IX-oxidase-inhibitors (PPO) we synthesized hydantoins of type **1** with strong activity against broad-leaved weeds (Fig. 28.4.1). Owing to of strong competitor activities in this field with overlapping patent applications we decided to substitute the central nitrogen atom by a carbon atom, leading to similar but not claimed C-aryl compounds, and so we synthesized the first tetramic acids **2** and **3** (Fig. 28.4.1).

Interestingly, the herbicidal activity of the 2,4-dichloro derivative **3** switched from the original activity of compound **1** to a weak activity against grassy weeds. Physiologically the symptoms were similar to aryloxyphenoxypropionic-acids (AAPs) and cyclohexandiones (CHDs). After intensive biochemical work based on a publication of Lichtenthaler et al. [6], it was shown that we had a new class of herbicides acting as ACCase inhibitors in our hands [7]. After some attempts at chemical optimization it was even more surprising that acylated derivatives, e.g., compound **4a** (Fig. 28.4.1), showed a weak acaricidal efficacy against the spider mite *Tetranychus urticae* (TETRUR). To improve this activity many different acylated compounds and aromatic substitution patterns were screened (Table 28.4.1).

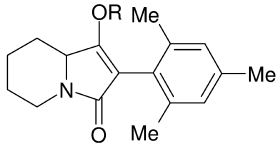
The 2,4,6-trimethylphenyl (mesityl) compound **4i** [8] showed an improved acaricidal potential against *Tetranychus urticae* (TETRUR) but were not satisfactory active under field conditions regarding another important mite species, *Panonychus ulmi* (PANOUL). To increase the efficacy against PANOUL a broad synthesis and screening of substituents on positions 1 and 5 of the lead structure was performed. The monocyclic 5,5-dimethyl tetramic acid derivatives **5a** and **5b** [9] (Fig.



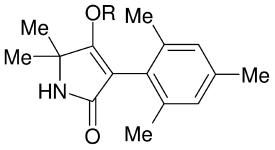
1 X = OAlk, S-Alk
Y = Me, CN



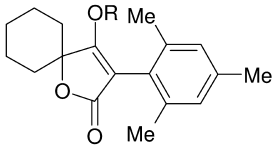
2 R = H; X_n = 4-Cl
3 R = H; X_n = 2,4-Cl_2
4a R = CO-CMe_3; X_n = 2,4-Cl_2



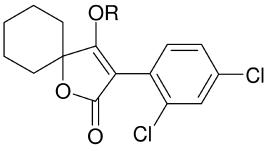
4i R = CO-tBu



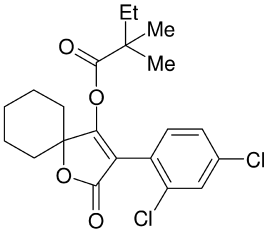
5a R = H
5b R = CO_2-CH(Me)Et



6a R = H
6b R = CO-tBu

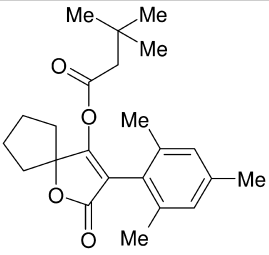


7a R = H



7f (Spirodiclofen)
3-(2,4-dichlorophenyl)-
2-oxo-1-oxaspiro[4.5]-
dec-3-en-4-yl-
2,2-dimethylbutyrate

$logP_{ow}$ 5.83
melt. point 95°C
water sol. 0.05 mg/l
LD_{50} (rat oral) >2500mg/kg

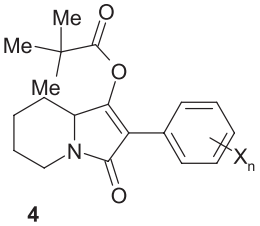


8a (Spiromesifen)
3-mesityl-2-oxo-1-
oxaspiro[4,4]non-3-en-
4-yl-3,3-dimethylbutanoate

$logP_{ow}$ 4.55
melt. point 98°C
water sol. 0.05 mg/l
LD50 (rat oral) >2500mg/kg

Fig. 28.4.1. Discovery of spirodiclofen **7f** and spiromesifen **8a**.

Table 28.4.1 Acaricidal efficacy of some pivaloyl substituted bicyclic tetramic acids **4** depending on the phenyl substitution pattern.



4

Entry	X_n	Efficacy against TETRUR
4a	2,4-Cl_2	+
4b	2-Cl, 6-F	++
4c	2,6-Cl_2	+
4d	2,4,6-Cl_3	+
4e	2-Cl,4-CF_3,6-F	++
4f	2,6-Cl_2,4-CF_3	++
4g	2,4-Me_2	++
4h	2-Me,4-*t*-Bu	+
4i	2,4,6-Me_3	++++
4j	2,4,5-Me_3	+
4k	2,3,4,6-Me_4	++

+ Weak; ++ moderate; +++ good; ++++ very good.

28.4.1) showed good and broad activity against a lot of important mite species under field conditions but caused some severe phytotoxic effects in some crops.

To overcome this drawback the core structure of the molecules was modified. We switched to other cyclic ketoenol subclasses, e.g., tetronic acids, pyrazolidin-3,5-diones, 4-hydroxy-pyrones and 6-hydroxy-thiazinones.

Only the synthesis of spirocyclic tetronic acid analogues **6a** and especially acylated compounds like the pivaloyl derivative **6b** [10] revealed excellent acaricidal performance with improved plant compatibility. In some sensitive crops, however, like stone fruits or grapes we still observed phytotoxic effects under special conditions. Therefore, in a ''back to the roots'' approach, we changed the mesityl substitution pattern back to the 2,4-dichloro-type examined earlier in the program. This led to the tetronic acid **7** as a template that combined good acaricidal activity against a lot of important mite species and good plant compatibility in all relevant crops. These properties were ''fine tuned'' by scanning a large set of different acylating reagents (Table 28.4.2) – the optimum was reached with the 2,2-dimethylbutyric acid derivative **7f**, which was selected for development under the common name spirodiclofen (BAJ2740, trade name: Envidor®) [11].

Table 28.4.2 SAR of different acylated compounds **7b–7h** of tetronic acid **7a** against TETRUR and PANOUL.

Entry	R	Efficacy against TETRUR	Efficacy against PANOUL
7a (enol)	H	++	+++
7b	CO-Me	+++	+++
7c	CO-*n*-Pr	++	++
7d	CO-*i*-Pr	+	+
7e	CO-*t*-Bu	++++	not tested
7f (Spirodiclofen)	CO-CMe_2-Et	+++++	+++++
7g	CO-CMe_2-*n*-Pr	++++	++++
7h	CO-CMe_2-*i*-Pr	+++	+++

+ Weak; ++ moderate; +++ good; ++++ very good; +++++ excellent.

During the acaricidal optimization process, surprisingly, a good efficacy against the white fly species *Bemisa tabaci* (BEMITA) was observed with **6b** in some field trials. During the optimization process it turned out that, especially, acylated 3-mesityl tetronic acids with spiro-cyclopentyl or spiro-cyclohexyl rings in position 5 were highly active against spider mites and showed at the same time an excellent performance against BEMITA.

The fine tuning process regarding activity, pest spectrum, toxicology and plant compatibility finally led to the 3,3-dimethyl-1-butyric acid derivative spiromesifen **8a** (BSN2060, trade name Oberon®) [12] (Table 28.4.3). Some physicochemical properties of the new products **7f** and **8a** are given in Fig. 28.4.1.

Table 28.4.3 SAR of acylated 5- and 6-membered spirocyclic mesityl tetronic acids against BEMITA.

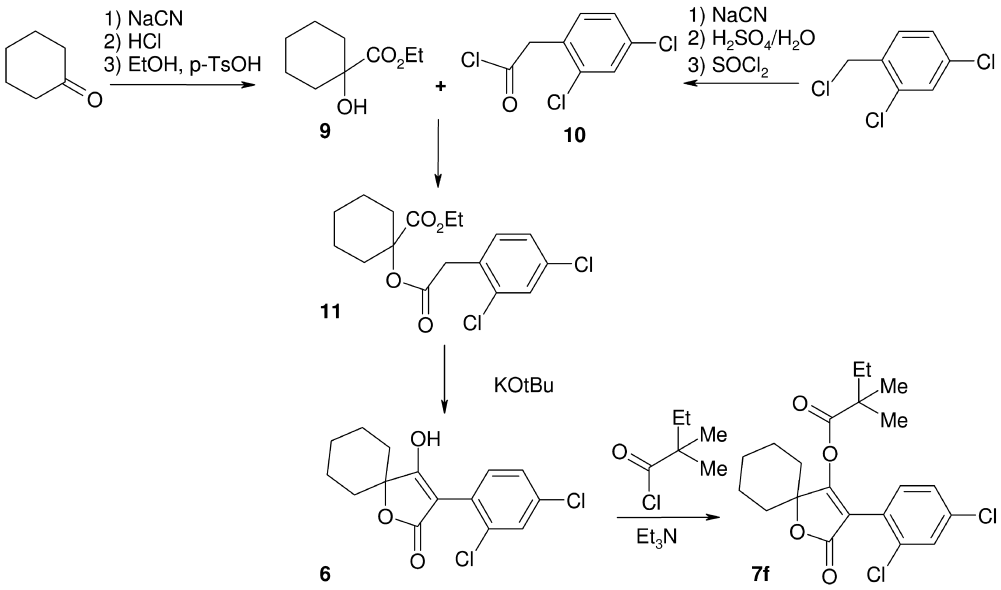
Entry	ring size	R	Efficacy against BEMITA
8a	5	*t*-Bu-CH_2	++++(+)
8b	5	*i*-Bu	++(+)
8c	5	*t*-Bu	+++
8d	5	*i*-Pr	+++(+)
6b	6	*t*-Bu	++++
6c	6	*i*-Pr	+++
6d	6	*t*-Bu-CH_2	+++(+)

+ weak; ++ moderate; +++ good; ++++ very good; +++++ excellent.

28.4.3
Synthesis of Spirodiclofen 7f and Spiromesifen 8a

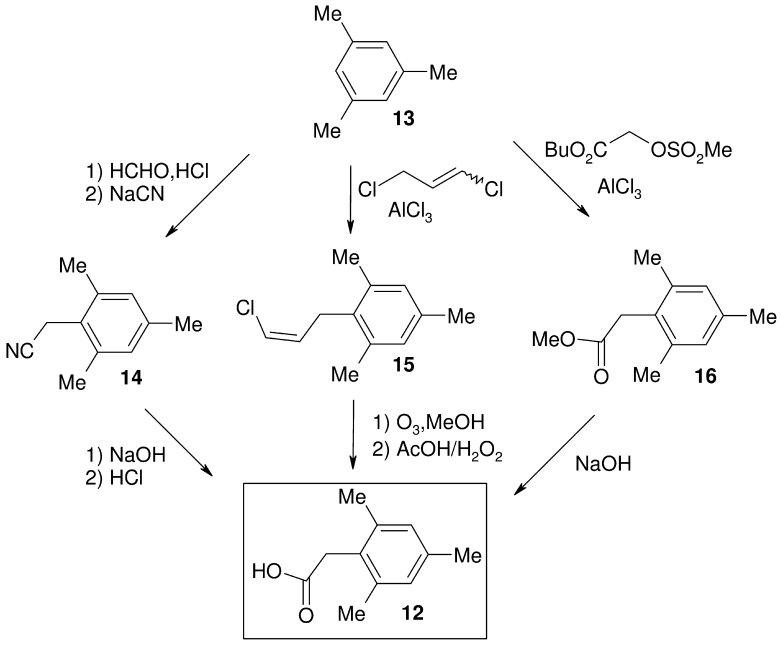
The first central intermediate of the spirodiclofen synthesis is ethyl 1-hydroxycyclohexanecarboxylate **9**, which is synthesized from cyclohexanone by hydrocyanic acid addition to the cyanohydrin, followed by saponification and esterification. The second central intermediate is 2,4-dichloro-phenylacetyl chloride **10**, which is synthesized from 2,4-dichlorobenzyl chloride by cyanide exchange, saponification and acid chloride preparation.

The combination of these two building blocks leads in a convergent way to the "diester" **11**, which is treated with a base, e.g., potassium-*tert*-butylate (KOtBu), to form the tetronic acid **6**. The final acylation with 2,2-dimethyl-butyryl chloride leads to spirodiclofen **7f** (Scheme 28.4.1).



Scheme 28.4.1. Synthesis of spirodiclofen **7f**.

Several possibilities for a large-scale synthesis of mesitylacetic acid **12**, a central building block in the synthesis of spiromesifen **8a**, were examined (Scheme 28.4.2). Using the classical standard route, mesitylene **13** is transferred into mesityl acetonitrile **14** via chloromethylation and cyanide exchange, which is then saponified to the aryl acetic acid. Another route examined is the Friedel–Crafts alkylation of mesitylene with 1,3-dichloro-propene to the adduct **15**, which is ozonolyzed to the corresponding aldehyde in the form of its dimethyl acetal and than further oxidized with hydrogen peroxide under acidic conditions to mesityl acetic

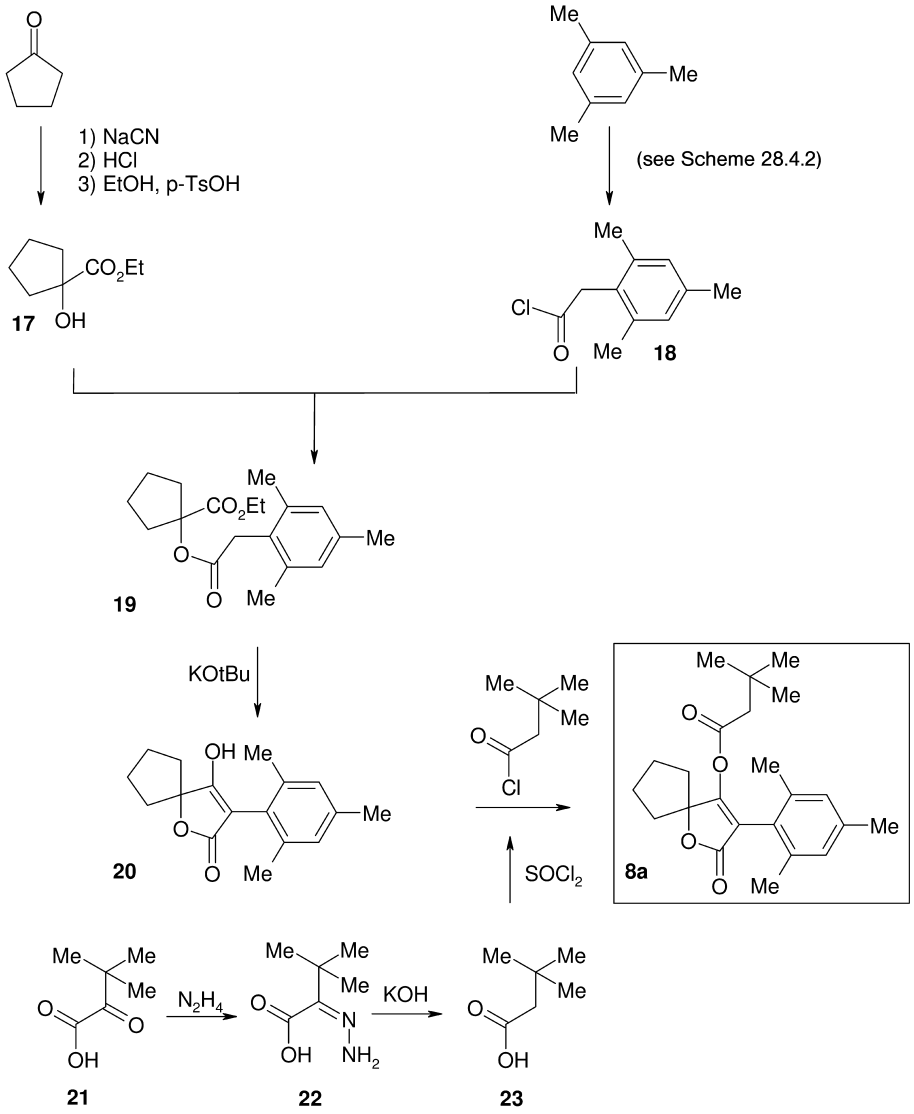


Scheme 28.4.2. Synthesis of mesityl acetic acid **12**.

acid **12** [13]. A straightforward route is the $AlCl_3$ mediated Friedel–Crafts alkylation of mesitylene with the C2-building block butyl [(methylsulfonyl)-oxy]acetate yielding **16**, which is then saponified to the free acid [14].

The further route to spiromesifen **8a** is similar to the above shown spirodiclofen **7f** synthesis (Scheme 28.4.3). Acylation of the cyclopentyl hydroxyester **17** (synthesized from cyclopentanone via the classical cyanohydrin route in three steps) with mesitylacetyl chloride **18** leads to the intermediate **19**, which is cyclized to the tetronic acid **20** using, e.g., potassium *tert*-butylate in *N*,*N*-dimethylformamide (DMF).

Several syntheses for the 3,3-dimethylbutyric acid **23**, (Scheme 28.4.3), used as acyl side chain in spiromesifen **8a**, were investigated. One interesting route starts from trimethylpyruvic acid **21**, which is transferred in a Wolff–Kishner reaction to the corresponding hydrazone **22** using hydrazine hydrate (optionally in a solvent, e.g., triethylene glycol) followed by a reductive cleavage with a base, e.g., potassium hydroxide, at elevated temperatures. The final acylation of the enol **20** with 3,3-dimethylbutyryl chloride leads to spiromesifen **8a**. The main process may also be conveniently done in a single-step/one-pot procedure starting from the intermediates **9** and **10** for spirodiclofen **7f**, and from **17** and **18** for spiromesifen **8a** [15].



Scheme 28.4.3. Synthesis of spiromesifen **8a**.

28.4.4
Biology and Mode of Action

Whiteflies (e.g., *Bemisia tabaci*) and spider mites (e.g., *Tetranychus urticae*) belong to the most serious sucking pests in many cropping systems. They have developed a high degree of resistance to many chemical classes of insecticides and

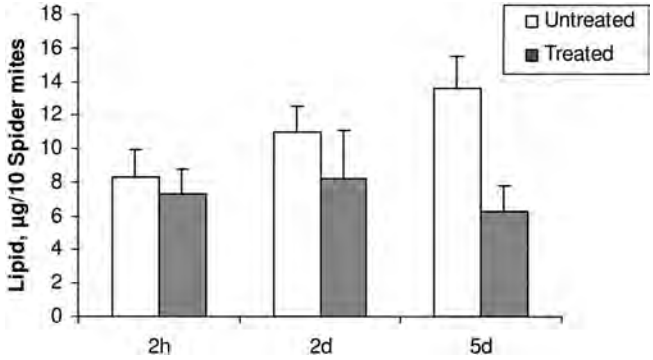


Fig. 28.4.2. Lipid decrease in spirodiclofen (**7f**) treated spidermites.

acaricides commercially available [16–18 and references cited therein]. Therefore new active ingredients with novel modes of action are needed to participate in resistance management programs to control these pests efficiently.

The symptomology of poisoning observed with the new tetronic acid derivatives indicated a new biochemical mode of action not yet observed with any commercially available acaricide or insecticide. They show no signs of neurotoxic activity, but act on mite and whitefly development. Spirodiclofen **7f** exhibited activity against all developmental stages of the mites, including the eggs, but does not kill adult mites. Additionally it reduces the fecundity of the female adults with the result that the number of laid eggs is strongly decreased. The eggs of females exposed to sublethal doses are not fertile. It was observed that the lipid content in treated female adults of *Tetranychus urticae* was significantly decreased, suggesting an interference with lipid biosynthesis (Fig. 28.4.2). This is in line with the slightly delayed onset of activity of the compounds. On the other hand, they show an excellent long-lasting effect and good plant compatibility under field conditions. The biological profile of spirodiclofen **7f** has recently been reviewed by Wachendorff-Neumann et al. [19]. Spirodiclofen **7f** and spiromesifen **8a** were extensively tested on several strains of *Tetranychus urticae*, collected worldwide, that showed a high level of resistance to established commercial acaricides. Both were shown to perform with outstanding activity [12, 16, 20].

Similarly to spirodiclofen **7f** the second compound in this class, spiromesifen **8a**, is also particularly active against juvenile stages. However, it also strongly affects fecundity of mite and whitefly adults in a dose-dependent manner by transovariole effects. It shows ovicidal effects in mites, whereas egg hatch in whiteflies was markedly reduced through transovariole effects upon pre-exposure of female adults. Spiromesifen **8a** was extremely effective against *Tetranychus* strains resistant to abamectin, pyridaben, fenpyroximate, hexythiazox and clofentezine (Table 28.4.4) and whiteflies resistant to pyrethroids, organophosphates, carbamates, cyclodienes and neonicotinoids [12].

Table 28.4.4 Resistance factors of several *Tetranychus* strains against commercial acaricides and spiromesifen **8a**.

T. strains	NL-00	Akita	UK-99	AU
Compound				
Abamectin	54	3	–	2
Pyridaben	22	2000	860	13
Fenpyroximate	–	1400	74	5
Hexythiazox	–	4	–	1100
Clofentezine	–	4	–	>770
Spiromesifen	4	1	1	3

Furthermore, field simulator studies revealed that spiromesifen **8a** is also a valuable tool to control pyriproxyfen resistant whiteflies (Fig. 28.4.3). In particular, the combination with neonicotinoid (chloronicotinyl) insecticides such as imidacloprid renders spiromesifen **8a** as a new valuable tool in resistance management strategies for whitefly control [12].

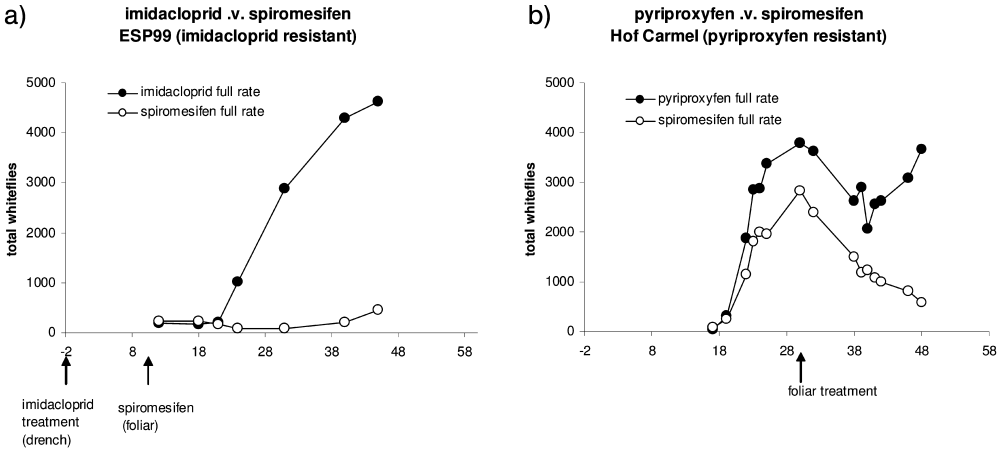


Fig. 28.4.3. Efficacy of spiromesifen **8a** against whitefly strains resistant to (a) imidacloprid and (b) pyriproxyfen (Taken from Guthrie et al., 2003.)

28.4.5
Development, Registration and IPM Suitability of Envidor® and Oberon®

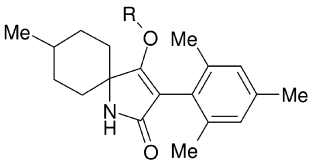
Envidor® with the active ingredient spirodiclofen **7f** is a new non-systemic foliar acaricide and provides excellent long-lasting efficacy and is effective in early to

late season applications. Spirodiclofen is being developed for worldwide use in pome fruit, stone fruit, citrus, grapes, almonds and nuts and showed good to excellent efficacy against all economical important mite species in these crops. Its performance is at least equal or superior to acaricidal standards of different chemical classes such as abamectin, pyradaben and hexythiazox. The recommended application rates are in the range 0.0048–0.0144% a.i., depending on crop and pest species. The product also controls some insect pests, e.g., *Psylla piri* and *Lepidosaphes ulmi*. Beside the worldwide trade name Envidor® other trade names such as Ecomite® (Japan, pome fruit), Daniemon® (Japan, citrus) and Sinawi® (Korea) are used. The first launch was in Korea 2002. Since then Envidor® has registrations in several important countries, e.g., Brazil, USA, Japan, Germany and Turkey.

Oberon® containing the active ingredient spiromesifen **8a** is a new foliar contact insecticide-acaricide and has been developed worldwide on vegetables, fruits, cotton, corn, beans, tea and some ornamentals. It provides good to excellent control of whiteflies (*Bemisia* spp., *Trialeurodes* spp.). In addition spiromesifen **8a** is highly efficacious against mites, including spider mites such as *Tetranychus* spp., *Tarsonemid mites* like broad mite and *Eriophyd mites* like tomato russet mite. In recent field trials carried out in the USA and Central America, Oberon® provided also excellent efficacy against tomato and pepper psyllids. The recommended application rates are in the range of 50–280 g-a.i. ha^{-1} or 0.0072–0.018% a.i., depending on crop and pest species. The new mode of action and the lack of cross-resistance to commercial products make spiromesifen **8a** a valuable tool for mite and whitefly resistance management. Oberon® was first registered in Indonesia 2003, followed by other important countries, e.g., USA and Mexico. Many studies against beneficial insects, predatory mites and spiders were conducted with both products. In conclusion, spirodiclofen **7f** and spiromesifen **8a** can be considered as very safe on beneficials according to the results obtained from laboratory and field tests. There were no permanent damaging effects on beneficial bugs, lacewings and parasitoids. This good selectivity offers possibilities of a combined use of these chemicals with beneficials. Thus, both compounds can be recommended for the use in Integrated Pest Management (IPM).

28.4.6
Discovery of Spirotetramat (32)

Parallel to the discovery of acaricidal active tetronic acid derivates we also tried to improve the acaricidal as well as the herbicidal efficacy in the subclass of tetramic acid derivatives. Starting with 1-amino-4-methyl-cyclohexanecarboxylic acid methyl ester, prepared by the Bucherer–Bergs reaction [21], we synthesized the tetramic acid **24a** and its acetyl derivative **24b** (Fig. 28.4.4). We found a significant improvement of herbicidal efficacy compared with unsubstituted spirocyclic analogues. We also registered an excellent acaricidal performance in the case of **24b** and, surprisingly, a moderate to good efficacy against the peach–potato aphid *Myzus persicae*, (MYZUPE) which was never noticed before.

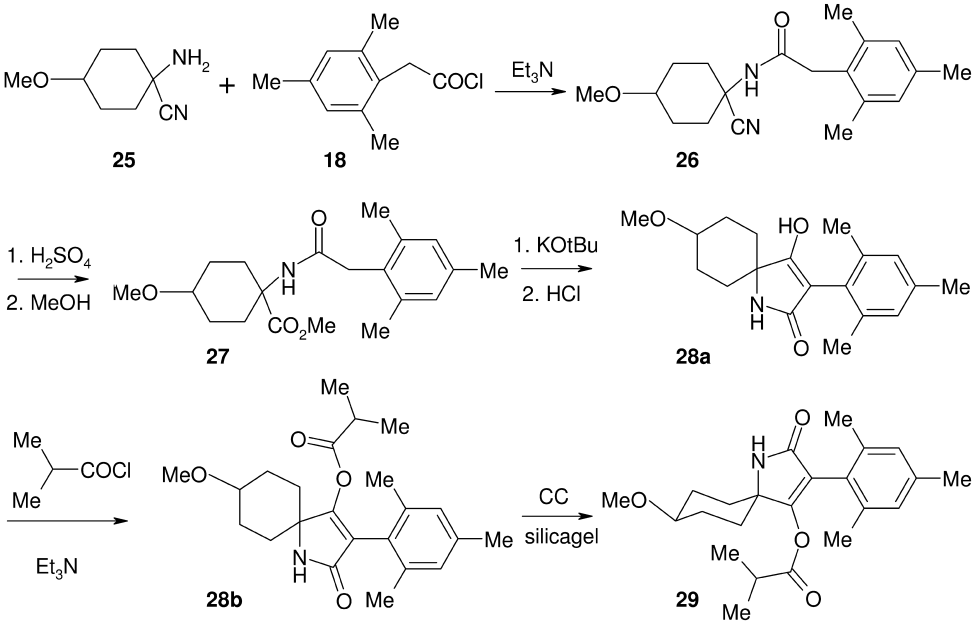


24 a R = H
24 b R = CO-Me

Fig. 28.4.4. Structure of **24a**, **b**.

Further evaluation led us to alkoxy-substituted spirocyclic tetramic acid derivatives. We took an alternative synthesis route starting from 4-methoxy-1-aminocyclohexanecarbonitrile **25**, which was prepared by a Strecker synthesis [21], to reduce the synthesis steps [22] and prepared compounds **28a** and **28b** (Scheme 28.4.4).

The shortage of **28b** in biology induced the repreparation of bigger amounts. During the workup we separated the pure isomers. The minor isomer was identified as the cis-compound **29** (Scheme 28.4.4) and showed a very good control of MYZUPE that was close to the efficacy of the best aphicidal standard imidacloprid. Our pleasure about these favorable results was destroyed rapidly by also improved herbicidal efficacy against crops.



Scheme 28.4.4. Discovery of substituted spirocyclic tetramic acid derivatives.

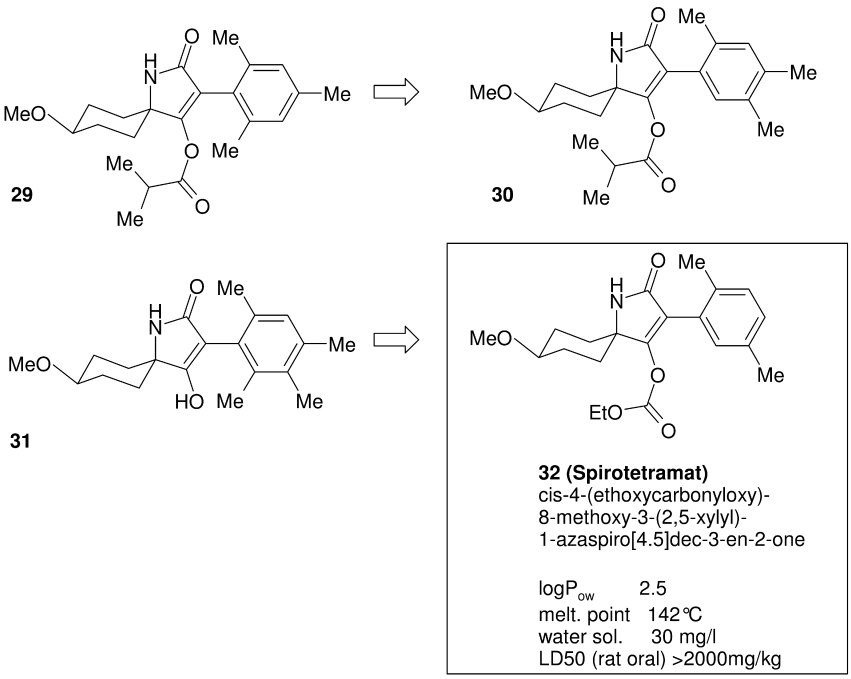


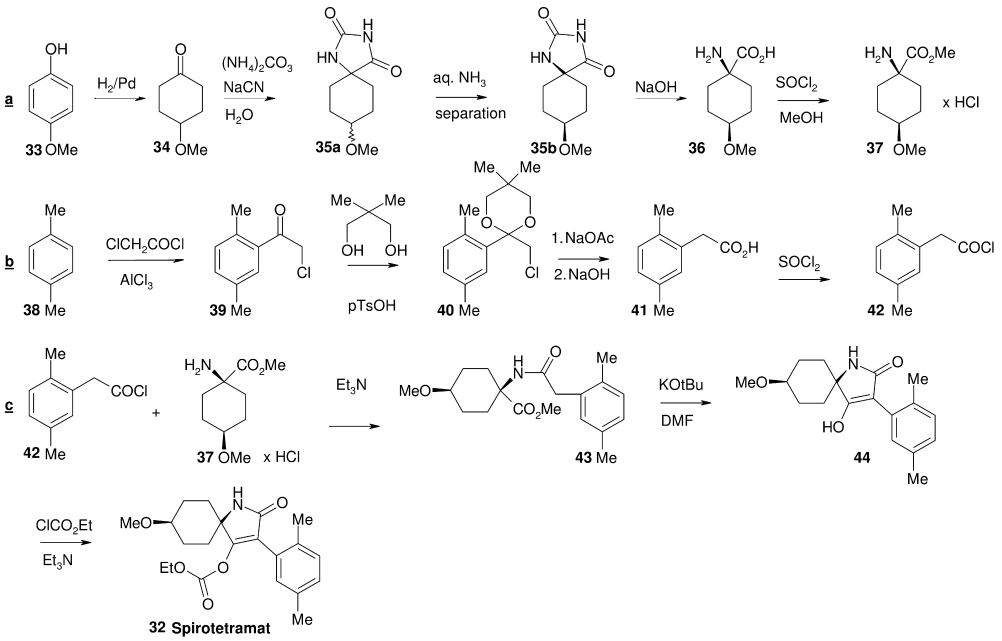
Fig. 28.4.5. Discovery of spirotetramat **32**.

Nevertheless, inspired by these results we reverted to the Bucherer–Bergs reaction for the synthesis of substituted 1-aminocyclohexanecarboxylic acids because it provides higher yields of the desired cis-isomer. We started an optimization process to retain the aphicidal efficacy and to improve the crop compatibility. After more than 3 years intensive work, also consulting computer-assisted calculations, there was no hint of separating the excellent aphicidal efficacy from the severe phytotoxic symptoms. Finally, the unconventional idea of combining the herbicidally most potent 4-methoxy-spirocyclic fragment with herbicidally weak phenyl moieties, resulting in compound **30** (Fig. 28.4.5), was successful [23]. The derivate **30** showed a good performance against the economically most important species *Myzus persicae* (MYZUPE) and *Aphis gossypii* (APHIGO). In comparison to **29** a significant improvement of crop compatibility in vegetables was observed. Additionally, good whitefly control was registered. With the enol **31** (Fig. 28.4.5) we found an improvement of aphicidal activity with preservation of the favorable plant compatibility [24]. At the end of a fine tuning process, lasting over a period of 4 years, compound **32** (Fig. 28.4.5) [25] was selected as a development candidate regarding physicochemical parameters, efficacy, pest spectrum, plant compatibility, toxicology, E-fate behavior and economy. A worldwide registration of compound **32** with the common name spirotetramat is planned.

28.4.7
Synthesis of Spirotetramat 32

Spirotetramat **32** can be synthesized in a twelve-step convergent synthesis. The first key intermediate is the *cis*-4-methoxy-1-aminocyclohexanecarboxylic acid methyl ester x HCl **37** which is synthesized in a five-step sequence (Scheme 28.4.5a). The first step is the hydrogenation of 4-hydroxy-anisol **33** to 4-methoxy-cyclohexanone **34** followed by a Bucherer–Bergs reaction to form the hydantoin **35a**. After separation of the cis-isomer **35b** the hydantoin is hydrolyzed to the amino acid **36**, which is esterified with thionyl chloride/methanol to the ester **37** [26]. The second key intermediate 2,5-dimethyl-phenylacetyl chloride **42** can be synthesized in a four-step straight forward route (Scheme 28.4.5b) starting from *p*-xylene **38**, which is acylated in a $AlCl_3$-mediated Friedel–Crafts to the chloroacetophenone **39** followed by ketalization with neopentylglycol to form ketal **40**. After sodium acetate catalyzed 1,2-arylshifting and saponification of the intermediate the resulting 2,5-dimethyl-phenylacetic acid **41** is transformed into the acid chloride **42** [27].

Acylation of **37** with **42** leads to the phenylacetylaminoester **43**. In a Dieckmann-cyclization with KO*t*Bu the tetramic acid **44** is formed, which is finally acylated with ethyl-chloroformate to spirotetramat **32**, (Scheme 28.4.5c).



Scheme 28.4.5. Synthesis of spirotetramat **32**.

28.4.8
Biology and Mode of Action of Spirotetramat 32

Spirotetramat (**32**) has the same mode of action as spirodiclofen **7f** and spiromesifen **8a**, i.e., inhibition of lipid biosynthesis [28]. Spirotetramat **32** strongly reduces the lipid content in aphids feeding on leaves treated with the compound (unpublished results), such as shown for spirodiclofen **7f** in spider mites (Fig. 28.4.2). More detailed investigations with ^{14}C-acetate as a radiolabeled precursor of fatty acids revealed a full inhibition of *de novo* synthesis of lipids in aphids [28]. Spirotetramat **32** showed excellent efficacy against insecticide resistant pests (including neonicotinoid resistant whiteflies) and will most likely be classified within group 23 of the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme, i.e., grouped together with spirodiclofen **7f** and spiromesifen **8a**. Spirotetramat **32** will be an invaluable new tool to manage insecticide resistance in many crops and pests worldwide.

Its physicochemical properties are quite different than those of spirodiclofen **7f** and spiromesifen **8a**, furthermore spirotetramat **32** effectively acts on a broader spectrum of pests, e.g., it shows an excellent efficacy against different aphid species, including *Myzus persicae*, *Aphis gossypii* and *Phorodon humuli* (Table 28.4.5).

Owing to its mode of action as lipid biosynthesis inhibitor, juvenile stages of aphids are particularly affected by spirotetramat **32**, whereas adults are strongly affected in their fecundity (unpublished results), which from an applied point of view will drastically reduce population development under field conditions. Spirotetramat **32** applied foliarly exhibited excellent systemic efficacy against aphids and whiteflies, whereas its contact efficacy against these pests is rather limited (Fig. 28.4.6).

The enol compound **44** also exhibited activity against aphids, but once penetrated into the plant it does not need a further conversion *in planta* such as spirotetramat **32**, which in leaves will be readily transformed to its enol form **44**. Spirotetramat **32** can be considered as a pro-insecticide and its above-mentioned systemic properties can be significantly improved by the co-application of an adjuvant, e.g., rape oil methyl-ester (Fig. 28.4.7). Figure 28.4.7 clearly shows that

Table 28.4.5 Physicochemical and biological properties of spirotetramat **32** compared with spiromesifen **8a** and spirodiclofen **7f**.

	Spirotetramat	Spiromesifen	Spirodiclofen
Log P_{OW}	2.5	4.6	5.8
Water solubility (mg L^{-1})	30	0.13	0.05
Melting point (°C)	142	98	95
Spider mites	+	+	+
Whiteflies	+	+	−
Aphids	+	−	−

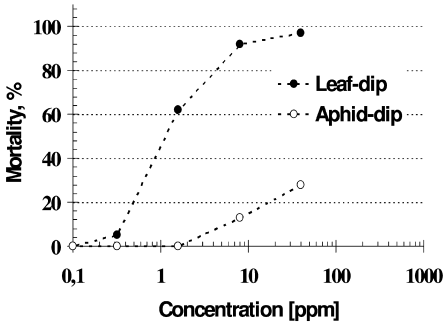


Fig. 28.4.6. Efficacy of spirotetramat **32** against 3–4 d old nymphs of *Myzus persicae* (72 h). Leaf-dip: Leaves were dipped (5 s) in serial dilutions of the compound and, after drying, aphids were transferred to the leaves. Aphid-dip: Aphids were dipped (5 s) in serial dilutions of the compound and then transferred to untreated leaves.

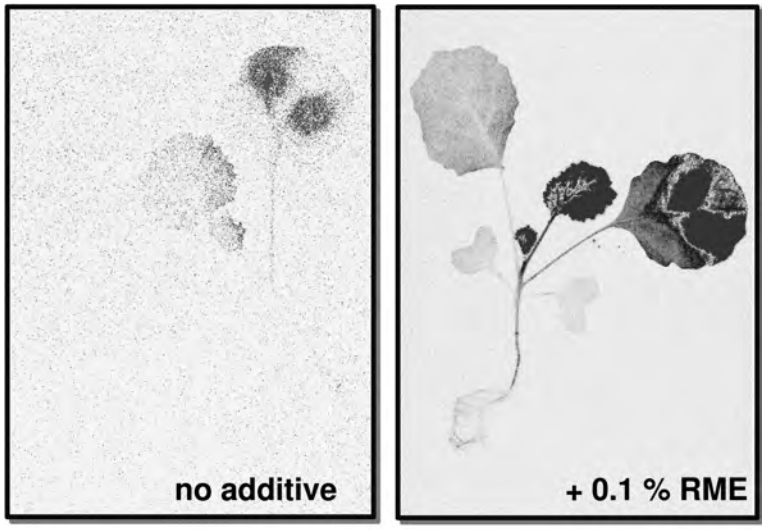


Fig. 28.4.7. Uptake and translocation of [^{14}C]spirotetramate **32** applied as SC 240 formulation to cabbage plants, either alone (no additive; picture 1) or in combination with rape oil methyl-ester (0.1% RME; picture 2). Two droplets of 5 µL (0.4 µg-a.i.) were applied onto the 1st true leaf 2 days before analysis.

once taken up by the leaf the radiolabel will be distributed in the whole plant, particularly protecting the younger leaves.

The spirotetramat-enol **44** shows a noticeable water solubility and is a weak acid (pK_a 4.9), rendering the compound mobile within the symplast (phloem) of the plant, according to the "weak acid hypothesis" [29]. Hence it can move acro- and basipetally, and even protects the roots when foliarly applied.

28.4.9
Development and IPM Suitability of Movento®

Movento® is the proposed brand name for the active ingredient spirotetramat **32** and is the first broad acting phloem mobile insecticide. Spirotetramat **32** is under development for worldwide use in pome fruits, stone fruits, citrus, grapes, almonds, nuts, hops, tea, vegetables, cotton and tropical fruits and performs well to excellent against a broad spectrum of sucking pests, including Aphididae (*Aphis* spp., *Myzus* spp., *Dysaphis* spp., *Toxoptera* spp., *Phorodon humuli*), Pemphigidae (*Eriosoma* spp., *Pemphigus* spp.), root aphids (*Phylloxera* spp.), Psyllids (*Psylla* spp., *Paratrioza cockerelli*), scales (*Ceroplastes* spp., *Pulvinaria* spp., *Aonidiella* spp., *Quadraspidiotus* spp., *Orthezia praelonga*), mealy bugs (*Pseudococcus* spp., *Planococcus* spp.) and whiteflies (*Bemisia* spp., *Trialeurodes vaporarium*). Spirotetramat has a slow initial but a very good long-lasting efficacy with excellent larvicidal activity. Also, new shoots are protected. Spirotetramat **32** has a very favorable ecotoxicology profile, which makes it interesting for use in Integrated Pest Management (IPM) programs. The first launch is scheduled for 2009.

28.4.10
Conclusion

The discovery process of the new chemical class of Cyclic Ketoenols started with a herbicidal spectrum shift from broad-leaved weeds to grassy weeds associated with a change in the mode of action (in our case from PPO to ACCase) followed by an indication shift from herbicidal to acaricidal activity. The careful follow up of the only weak acaricidal efficacy of the first hits in chemistry, an enthusiastic team in biological research, knowledge of the physiology and mode of action all played a fundamental role in the first part of the optimization and development process of spirodiclofen **7f** and spiromesifen **8a**. Another spectrum shift from acaricidal to aphicidal activity was the trigger for the discovery of aphicidal ketoenols. In the pre-development and development stages process chemistry was an important partner to find the most economic routes for a commercial large-scale production of the products. Many different synthesis routes for the key intermediates had to be examined, as shown in case of mesitylacetic acid **12**.

Physiological, biological and biochemical work revealed ACCase as the mode of action for the Cyclic Ketoenols, which is a novel target in acaricidal/insecticidal chemistry and, as a consequence, they showed high activity against pest populations resistant to conventional chemistry. Especially, this attribute of the new ketoenols combined with their excellent long-lasting efficacy, the favorable envi-

ronmental profile and the full systemic properties in the case of spirotetramat (**32**) make them a valuable tool for farmers worldwide.

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24 T. Bretschneider, C. Erdelen, R. Fischer, A. Graff, H. Hagemann, F. Lieb, M. Ruther, U. Schneider, U. Wachendorff-Neumann, A. Widdig (Bayer AG), Patent WO 97/36868.
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29
Nervous System

29.1
Nicotinic Acetylcholine Receptor Agonists, Target and Selectivity Aspects

Peter Jeschke and Ralf Nauen

29.1.1
Introduction

One of the insecticide molecular target sites of growing importance (market share 2003, 15.7% Elbert et al., Chapter 24.8 of this volume) is the nicotinic acetylcholine receptor (*n*AChR), which plays a central role in the mediation of fast excitatory synaptic transmission in the insect central nervous system (CNS). Despite the use of the alkaloid (*S*)-(−)-nicotine (**1**) as natural insecticide (aqueous tobacco extract) for a long time, the *n*AChR has been an underexploited biochemical target for modern insecticides, with an estimated total insecticide world market share of around 1.5% in 1987. Because of its high mammalian toxicity and relatively low level of insecticidal activity no major class could be established through taking **1** as lead structure. However, the *n*AChR has become an important target in crop protection with the discovery and commercialization of three classes of insecticides (Table 29.1.1):

- The very small group of so-called nereistoxin (**2**) analogues (*N*,*N*-dimethylamino-1,2-dithiolane-4-amines) such as the bis(thiocarbamate) proinsecticide cartap (**3**) [1], bensultap (**4**) [2, 3, 4] and thiocyclam (**5**) [5, 6].
- From the lead structure 2-nitromethylene-tetrahydro-1,3-thiazine (**6**, nithiazine) [7, 8], resulting neonicotinoids [9] such as the open-chain compounds, e.g., nitenpyram (**8**), acetamiprid (**9**), clothianidin (**12**), dinotefuran (**13**) (Chapter 29.2.1), the five-membered ring systems, e.g., imidacloprid (**7**), thiacloprid (**11**) (Chapter 29.2.2), and the six-membered ring systems, e.g., thiamethoxam (**10**), AKD 1022 (**14**) (Chapter 29.2.3).
- The spinosyns as a family of fermentation-derived insecticidal macrocyclic lactones, such as the bioinsecticide

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

Table 29.1.1 Evolution of nAChR agonists **1–15** used as insecticides.

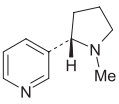
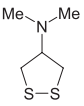
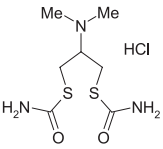
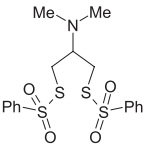
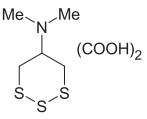
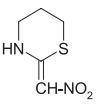
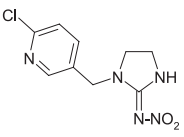
Compound[a]	Common name	Manufacturer (year introduced)	Agonist classes	Remarks
 **1**	(*S*)-(−)-Nicotine	1814	Nicotinoid	Natural product, extracts of tobacco
 **2**	[b]	[c]	Nereistoxin	Marine annelid
 **3**	Cartap hydrochloride	SumiTake[d] (1964)	Nereistoxin analogue	Prodrug of **2**
 **4**	Bensultap	SumiTake[d] (1968)	Nereistoxin analogue	Prodrug of **2**
 **5**	Thiocyclam	Sandoz (1979)	Nereistoxin analogue	Prodrug of **2**
 **6**	Nithiazine	Shell (1978)	Neonicotinoid	First lead structure for CNIs[b]
 **7**	Imidacloprid	Bayer CropScience (1991)	Neonicotinoid	First commercial CNI with highest turnover

Table 29.1.1 *(continued)*

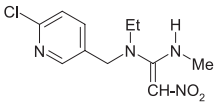
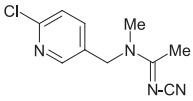
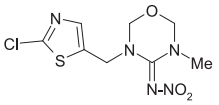
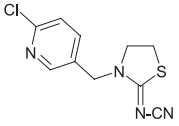
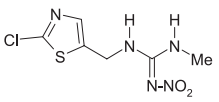
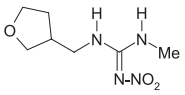
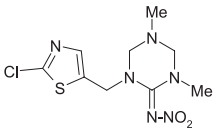
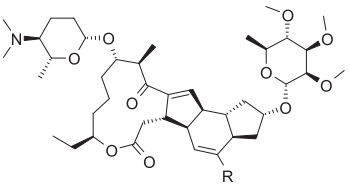
Compound[a]	Common name	Manufacturer (year introduced)	Agonist classes	Remarks
 **8**	Nitenpyram	SumiTake[d] (1995)	Neonicotinoid	Open-chain nitromethylene
 **9**	Acetamiprid	Nippon Soda (1995)	Neonicotinoid	Open-chain *N*-cyano-acetamidine
 **10**	Thiamethoxam	Syngenta (1998)	Neonicotinoid	Six-membered heterocyclic nitroguanidine
 **11**	Thiacloprid	Bayer CropScience (2000)	Neonicotinoid	Five-membered *N*-cyano-amidine
 **12**	Clothianidin	SumiTake/[d] Bayer CropScience (2000)	Neonicotinoid	Open chain *N*-nitroguanidine
 **13**	Dinotefuran	Mitsui Toatsu (2002)	Neonicotinoid	Racemic open-chain nitroguanidine
 **14**	AKD 1022	Agro Kanesho	Neonicotinoid	Prodrug of **12** not commercialized

Table 29.1.1 *(continued)*

Compound[a]	Common name	Manufacturer (year introduced)	Agonist classes	Remarks
 **15**	Spinosad [Principal components: spinosyns A (R = H) and D (R = CH_3)]	Dow AgroScience (1997)	Spinosyns and spinosoids	Natural product (different target site, IRAC group)

[a] Further details for each compound are given in subsequent parts of this chapter.
[b] No common name.
[c] Never commercialized for agricultural use.
[d] Sumitomo Chemical Takeda Agro Company Ltd.

spinosad (**15**) [10, 11], a naturally occurring mixture of two active components, spinosyn A (primary component) and spinosyn D (Chapter 29.3) [different binding site, different IRAC mode of action classification number (5)] (Chapter 24).

The *N*,*N*-dimethylamino-1,2-dithiolane-4-amines are based on the neurotoxic and insecticidally active natural occurring insect-paralyzing factor **2** isolated from the salivary glands of nereid annelid worm *Lumbriconercis heteropoda* Marenz [12, 13]. This natural product is active on cholinergic synapses [14]. Compounds **3**–**5** may be proinsecticides that are converted metabolically in the insect body into **2**, which then competes with ACh to block the *n*ACh-mediated signal [15, 16].

While nicotinoids are structurally similar to neonicotinoids, they primarily differ by containing an ionizable basic amine or imine substituent. Today, the seven commercial neonicotinoids **7**–**13** are the fastest growing and fourth major group of insecticides (behind OPs, methylcarbamates and pyrethroids), with widespread use against a broad spectrum of sucking and chewing pest insects by several modes of application [17, 18] in most countries and in many agronomic cropping systems. They act selectively on insect *n*AChRs and are used worldwide for insect pest management (IPM) [19, 20].

As the source for the family of novel tetracyclic macrolide polyketides, the spinosyns [21, 22] were found to be secondary metabolites of the soil bacterium actinomycete *Saccharopolyspora spinosa* [23, 24]. The spinosyn biosynthetic gene cluster has been cloned from *S. spinosa* and sequenced, and the results have been used to formulate a proposed biosynthetic pathway [25] (Chapter 29.3).

As is known, only minor structural variations of ligands can confer selectivity among the mammalian *n*AChR subtypes and between insects and mammals. De-

ployment of multidisciplinary approaches has led to an enhanced understanding of the molecular basis of this selectivity, but electrophysiology has been particularly instructive in identifying a rich diversity of actions of effectors on *n*AChRs [26]. These diverse actions depend on the chemical structure of the active ingredient as well as the subunit composition of *n*AChRs tested.

In contrast, important physicochemical parameters such as electrostatic interaction, H-bonding, π,π-stacking interaction, dipole–dipole interaction and van der Waals contact all act closely together with the insecticide action. Therefore, knowledge of the functional architecture and molecular aspects of insect versus mammalian *n*AChRs and their ligand-binding site is the basis for continued development of novel safe and effective active ingredients. Especially, neonicotinoids created a renaissance in the investigation of insect *n*AChRs [27]. The past decade has witnessed broadly increasing number of publications in the field of *n*AChRs, which reflects the importance of these receptors as a continuous source for rational design of novel insecticides [28] as well as medicinal drugs.

29.1.2
Structure of the Nicotinic Acetylcholine Receptors

The vertebrate *n*AChRs are agonist-gated ion channels responsible for rapid excitatory neurotransmission. *n*AChRs are well-characterized large pentameric transmembrane allosteric proteins (molecular weight ~290 kDa), involved in rapid gating of ions elicited by acetylcholine (ACh) at the vertebrate neuromuscular junction and in all animal central and peripheral nervous systems [29, 30]. As muscular *n*AChR it is assembled from a ring of five homologous subunits (α, γ, α, β, δ), each divided into three domains arranged around a central ion channel: (a) a large *N*-terminal extracellular ligand-binding domain, (b) a membrane-spanning pore, and (c) a smaller intracellular domain [31]. The *n*AChRs belongs to the ''Cys-loop'' superfamily of ligand-gated ion channels (LGICs) [32] that also includes ionotropic glutamate, glycine receptors [33] γ-aminobutyric acid type A and C ($GABA_A$ and $GABA_C$) receptors [34] and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors [35, 36] and has facilitated an impressive number of physiological, pharmacological and structural investigations [37, 38]. From studies on the invertebrate genetic models, *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (nematode), additional LGICs have been discovered, including GABA-gated cation channels [39], $5\text{-}HT_3$-gated chloride channels [40], glutamate-gated chloride channels [41], and histamine-gated chloride channels [42]. The *n*AChRs play important roles in neuronal and neuromuscular functions [43].

The *n*AChRs are homo- or heteromeric pentamers of structurally related subunits that encompass an extracellular *N*-terminal ligand binding domain, four transmembrane (TM)-spanning regions that form the cation-permeable channel [44], and an extended intracellular region between spans TM3 and TM4. The subunits are orientated around a central pore [45, 46] and the resulting transmembrane ion channel is formed by a pentameric rearrangement of the TM2 helical segments contributed by each of the five proteins [47]. They exist in four confor-

mational states with distinctive sensitivities to the nicotinic ligands that dictate channel gating and function: (a) basal or resting (closed, but rapidly activatable), (b) activated (open), and two (c) desensitized (closed) states [48]. The latter are refractory to activation on a time scale of milliseconds or minutes depending on the desensitized state but have high affinity (pM–nM) [49]. Ligand binding triggers conformational changes that are transmitted to the transmembrane-spanning region, leading to gating and changes in membrane potential.

At present, ten α (α_1–α_{10}), four β (β_1–β_4), δ and γ (replaced by ε in later stages of development) subunits have been identified. The skeletal muscle or electric ray (*Torpedo*) subtype is made up of two α1 subunits and one each of β1, γ, and δ (or ε in adult muscle) subunits. The human *n*AChR gene family consists of 16 subunits (α1–α7, α9, α10, β1–β4, δ, ε and γ) [50] whilst chicken possesses an additional subunit (α8). Analysis of the genome of the pufferfish, *Fugu rubripes*, has revealed the largest known set of vertebrate *n*AChR genes (16α and 12 non-α subunits) whose genesis is most likely through genome duplication [51]. In contrast, the animal nematode model *C. elegans* possesses the most-diverse *n*AChR gene family currently known, consisting of at least 27 subunits (20 α and 7 non-α) [52, 53].

To date, the smallest *n*AChR gene family is that of the *D. melanogaster*, consisting of seven α and three non-α-subunits [54, 55]. Seven of these subunits, Dα1 otherwise known as ALS (alpha-like subunit), Dα2 or SAD (second alpha-like subunit *Drosophila*) and Dα3–Dα7 are α subunits whilst Dβ1 or ARD (acetylcholine receptor *Drosophila*), Dβ2 or SBD (second beta-like subunit *Drosophila*) and Dβ3 are non-αs (Fig. 29.1.1).

As described, Dα5–Dα7 subunits are most closely related to vertebrate α7, sharing 45% peptide sequence identity. Dα1–Dα4 and Dβ1–Dβ2 are most closely related to each other (30–50% identity), reflecting 25–40% identity with vertebrate. Dα3 is the most distantly related, showing only 20% sequence identity with other *n*AChR subunits of both invertebrates and vertebrates. Dα2 shares the closest sequence identity with other insect α subunits. Additional diversity of *Drosophila* nAChRs arises from alternative splicing (4 of the 10 subunits), and RNA editing (first described for nAChRs in the fly) [56] serves to dramatically increase the number of possible subunit isoforms (over 30 000 Dα6 isoforms are theoretically possible).

These subunits form channels of a wide variety of multiple homo- or heteromeric neuronal *n*AChR subtypes [57].

The *n*AChR is composed of two ligand binding (α) and three non-α subunits (γ, δ or ε) or five α subunits [58]. The most common subunit stoichiometry has been determined to be $(\alpha X)_2(\beta Y)_3$ (X = 2–4; Y = 2–4) for heteromeric subtypes and $(\alpha Z)_5$ (Z = 7–10) for homomeric subtypes [59]. However, other more complex combinations have also been reported [60]. *n*AChR subtypes are found in different locations of central and peripheral nervous system and have been assigned different pharmacological functions (cf. α7, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha4\beta4$) [61, 62].

The *n*AChRs contain multiple binding domains that can accommodate different classes of endogenous and exogenous ligands. The *n*AChR ligand binding domain consists of seven loops (A–G) spaced on the protein chains of the α and

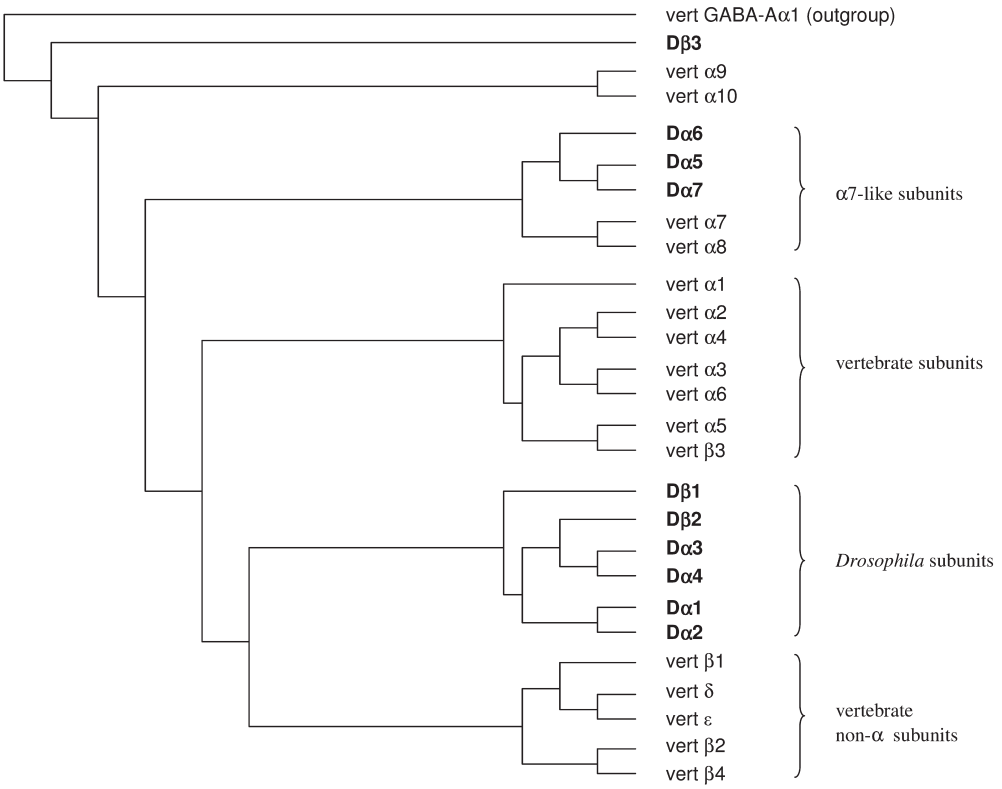


Fig. 29.1.1. Tree showing relationships of *Drosophila* nAChR subunits and vertebrate nAChR subunits. The tree was constructed using protein sequences aligned by the ClustalX program and displayed using the TreeView application (Adapted from D. B. Sattelle et al., 2005 [55]).

non-α subunits [63, 64]. Sequences for each *n*AChR subunit predict hydrophilic extracellular domains containing a binding site for cholinergic ligands and four transmembrane hydrophobic segments (TM1–4). The TM2 domain of the five subunits is considered to form the lumen of the cation-channel [65]. A large intracellular loop is between TM3 and TM4.

The agonistic ACh-binding site is located at the interface of two adjacent subunits (α and non-α) and is formed by six distinct regions (loops A–F) in the *N*-terminal extracellular domain, with each of the adjacent subunits contributing three loops. Subunits that have two adjacent Cys residues in loop C, which are essential for ACh binding [66], are referred to as α subunits, whereas subunits lacking this Cys doublet are referred to as non-α or *β*, *δ*, *ε* or *γ* subunits.

The ends of the internal lumen of *n*AChR are highly polar and negatively charged. This domain can be viewed as a cation selector in which noncompetitive inhibitors bearing a positive charge (e.g., amine moiety) are trapped and directed down the channel by an electrostatic gradient [67, 68].

Electron microscopical experiments on helical tubes grown from *Torpedo* postsynaptic membranes [69, 70], using a rapid sprayfreezing technique to mimic the synaptic release of ACh and trap the open-channel form, gave insight into the structural mechanism of gating. These experiments demonstrated that binding of ACh imitates two interconnected events in the ligand-binding domain.

29.1.2.1 **Agonist Binding Sites**

Several investigations have identified key interactions that lead to the binding of small molecules such as ACh, (*S*)-(−)-nicotine (**1**) and (−)-epibatidine (**16**) (cf. Fig. 29.1.2 below) at the agonist-binding site of *n*AChRs [71].

Agonist ligands acting at vertebrate neurotransmitter-gated ion channels are characteristically cationic nature and its binding induces a structural change, opening a pore in a channel that allows the passage of ions across the cell membrane. Early biochemical studies of the embryogenic muscle nAChR identified two agonist-binding sites localized to the α/δ and α/γ interfaces [72, 73, 74]. Unfortunately, as with many other integral membrane proteins, it has not been possible to obtain crystals of any *n*AChR of sufficient quality for high-resolution X-ray crystallography. However, both the crystal structure of a soluble homopentameric acetylcholine-binding protein (L-AChBP) from the fresh-water snail *Lymnaea stagnalis* [75, 76] and the refined model of the membrane-associated *Torpedo* AChR [77] based on the crystal structure of L-AChBP can support the understanding of the ligand–receptor interactions at the agonist binding site considerably. Using the L-AChBP structure as a template, several 3D models of ligand-binding domains of *n*AChR subtypes with ACh, **1** and **16** docked to the binding site have been published [78, 79]. A cationic center is contained in nearly all *n*AChR agonists such as ACh, **1** and **16**. As a recognition strategy of cations by biological molecules the cation–π interaction, stabilizing interaction between a cation and the electron-rich aromatic ring has been reported for several years [80, 81, 82]. Investigations of the muscle type *n*AChR using unnatural amino acid mutagenesis demonstrated that a key tryptophan (Trp α149) shows this potent cation–π interaction with ACh in the agonist binding site [83]. On the other hand, the L-AChBP confirmed a H-bonding interaction from the $^{+}$N–H of **1** to the backbone carbonyl in the same region of the agonist binding site [84]. In contrast, **16** achieves its high potency by taking advantage of both the cation–π interaction and the backbone H-bond. However, an important limitation of such modeling studies is the absence of the membrane-spanning helices and intracellular domain of *n*AChR, which could play an important role in receptor dynamics. In addition, the L-AChBP is not a neuroreceptor (only <25% sequence identity to its closest relative in the *n*AChR family, α7) and its crystal structure most likely represents the desensitizing state of the receptor. No conformational changes were observed in the L-AChBP that could explain receptor gating. Therefore, the functional significance of structural insights into *n*AChRs resulting from L-AChBP remains to be determined [85]. But, together with the more recent cryoelectron microscopy data of the membrane domains of *Torpedo n*AChRs [86], a model of the α7 *n*AChR was built to explore its gating mechanism [87].

The refined 4 Å resolution electron microscopy structure of the heteropentameric muscle-type, $(\alpha 1)_2\beta\gamma\delta$ *n*AChR has elegantly illustrated considerable structural similarity of L-AChBP with the *n*AChR ligand-binding domain. Therefore, L-AChBP is now considered a structural and functional surrogate of the *n*AChRs.

The crystal structure of A-AChBP (only 33% amino acid identity with L-AChBP) from the saltwater mollusc, *Aplysia californica*, in the apo form reveals a more open loop C and distinctive positions for other surface loops, compared with previous structures [88]. Analysis of *Aplysia* AChBP complexes with nicotinic ligands shows that loop C, which does not significantly change conformation upon binding of antagonists (e.g., methyllycaconitine), further opens to accommodate peptidic antagonists (e.g., α-conotoxin), but wraps around the agonists such as **16** [89]. The structures also reveal extended and non-overlapping interaction surfaces for the antagonists, outside the binding loci for agonists.

Very recently, the principal pathway that links agonist binding to channel gating by using Unwins atomic scale model of *Torpedo mamorata* in *n*AChRs at 4 Å resolution has been described [90]. The primary coupling pathway integrates contributions from several structural domains, with its distal limb likely representing the point at which the binding domain triggers opening of the channel.

29.1.3 Insect nAChRs

In insects, genes are identified encoding multiple *n*AChR subunits, suggesting the existence of diverse insect receptor subtypes. As an agonist-gated ion channel complex for rapid excitatory neurotransmission, the *n*AChR is widely distributed in insect CNS and constitutes a major target for insect action. However, the functional architecture and diversity of insect *n*AChRs are poorly understood compared with their vertebrate counterparts [91]. In general, insect *n*AChRs are diverse in structure, as are those from vertebrates.

Genes encoding the ligand-binding α and structural β *n*AChR subunits have been cloned in several insect species. Even though several genes encoding insect *n*AChR subunits have been isolated and the existence of further *n*AChRs subunits can be predicted from analysis of *D. melanogaster* genome (ten nAChR subunits have been identified by molecular cloning [92]), the functional architecture, diversity and three-dimensional (3D) structure of the insect native AChRs remains unknown. It is still difficult to express functional insect *n*AChRs not only in *Xenopus laevis* oocytes but also in several insect cell lines (see Chapter 6.1/3.1).

As in vertebrates, in insects *n*AChRs mediate fast synaptic transmission as an excitatory neurotransmitter-receptor complex widely distributed in the synaptic neutrophil regions of the CNS in the insect brain [93, 94].

Recently, the second complete set of insect *n*AChR gene family was identified from the genome of the malaria mosquito vector (*Anopheles gambiae*), revealing that the mosquito ortholog of Dβ2 is an α subunit (Agamα8) [95]. Interestingly, the loop C sequences for Dα2 and Agamα8 are very similar, with most changes

occurring within the vicinal Cys that define α subunits, leading to the suggestion that this represent a recent evolutionary transition between an α and a non-α subunit. It appears that insects have several types of *n*AChR subunits that could associate to form channels of disparate pharmacology, and this could explain some of the complex binding and electrophysiology seen with the insect cholinergic system. Seven *n*AChR subunits (four α-type, genomically nine α-types and three β-type, which exist only in *D. melanogaster*) have been cloned from fruit fly *D. melanogaster*.

Three further putative *n*AChR α subunits (Dα5–Dα7) with sequence similarity to the vertebrate α7 subunit have been identified from *Drosophila* genome sequence data but there have been no reports, as yet, of their characterization by heterologous expression [96].

Generally, insect *n*AChRs clearly vary with specificity of their interaction with neonicotinoid insecticides; however, the appropriate subunit is unclear so far. An investigation that supports the hypothesis that there is a conserved neonicotinoid special sensitive subtype of the *n*AChR binding site in different insects like *Musca domestica*, *D. melanogaster*, *Aphis craccivora*, *Myzus persicae* has been discussed [97].

Whenever it has not been possible to obtain crystals of any *n*AChR of sufficient quality for high-resolution X-ray crystallography, both the crystal structure of a soluble homopentameric AChBP and the refined model of the membrane-associated *Torpedo* AChR [98], based on the crystal structure of AChBP, can support the understanding of the ligand–receptor interactions considerably.

29.1.3.1 Consideration of AChBP versus nAChR α-Subunit

The AChBP has the same overall architecture as the extracellular portion of the *n*AChR [99], and the presence of the vicinal Cys pair characteristic of ligand-binding receptor subunits. Most of the key residues that have been shown to contribute to the agonist binding domain of the *n*AChRs were also conserved in AChBP. The AChBP is not an ion channel, but shows numerous *n*AChR properties, including binding of known *n*AChR agonists and competitive antagonists (e.g., ACh, **1**, *d*TC and α-Bgtx). Therefore, the ACh binding-site on the crystal structure of the AChBP can be used as an example of the *N*-terminal domain of an α-subunit of *n*AChRs as template for docking simulations of competitive ACh ligands such as agonists and antagonists [100] by modeling methods [101].

Recently, 3D models of the *N*-terminal part of *n*AChR were constructed and docked in the putative ligand-binding pocket. Ligand binding is driven by enthalpy and is accompanied by conformational changes in the ligand binding site. These hypothetical docking models offer a structural basis for rational design of drugs, such as neonicotinoids, differentially binding to resting and active (or desensitized) conformations of the *n*AChR site.

29.1.3.2 Interaction of Loop F of the α7 nAChR with Neonicotinoids

The use of site-directed mutagenesis combined with two-electrode voltage clamp electrophysiology revealed that G189D and G189E mutations markedly reduced

the responses of the α7 subunit of the *n*AChR to imidacloprid (**7**) and nitenpyram (**8**), whereas G189N and G189G mutations scarcely influenced the responses. In contrast, agonist action of des-*N*-nitro-**7** were strongly affected by the G189D and G189E mutations. This demonstrates that the reduction of the α7 *n*AChR responses to neonicotinoids can be attributed to the electrostatic repulsion between the negatively-charged oxygens of the [=N–NO_2]-group and the negatively-charged oxygens of the carboxylate group in the back-bone. In loop F of insect non-α subunits, aromatic residues are present at the position corresponding to isoleucine (Ile) 191 of the chicken α7 subunit, and Trp residues are most frequently observed. Based on mutagenesis studies on the α7 receptor, it is assumed that a Trp-residue in loop F may contribute to strengthening neonicotinoid–insect *n*AChR interactions.

29.1.3.3 Interaction of Loop D of the α7 nAChR with Neonicotinoids

As is known, AChBP can form a homo-pentamer like the α7 subunit. In its crystal structure, Y164 in loop F, corresponding to G189 of the α7 subunit, faces the agonist binding site. It was found that Q55, corresponding to Q79 in loop D of the α7 *n*AChR, is close to Y164 in loop F of the AChBP. Furthermore, the agonist responses of the α7 *n*AChR to **7** and **8** were markedly reduced by the Q79E mutation, whereas the responses were increased by mutations Q79K and Q79R. On the other hand, agonist actions of des-*N*-nitro-**7** were increased by the Q79E mutation, whereas responses were reduced by the Q79K and Q79R mutations. Therefore, it was postulated that a glutamine (Gln) residue Q79 in loop D (Table 29.1.2) and glycin (Gln) G189 in the loop F of the chicken α7 subunit can interact with the nitro group of neonicotinoids like **8** [=CH–NO_2], **7**, **10**, and **12–14** [each with =N–NO_2].

In most insect non-α subunits, amino acids residues corresponding Q79 of the α7 subunit are lysine (Lys) or arginine (Arg) moieties (Table 29.1.2). These basic residues may interact with the nitro group of neonicotinoids through electrostatic force, and H-bonding, strengthening the *n*ACh–insecticide interaction. Its substitution can result in the reduction of the insecticide sensitivity of *n*AChRs, but not in reduction of affinity.

29.1.4 Nicotinic Pharmacophore Models

Before the X-ray of the AChBP was described, the structure of the *n*AChRs binding site(s), the rational design of potent and selective *n*AChR ligands was facilitated by the identification of a specific 3D arrangement of essential chemical groups common to *n*AChR ligands, the so-called nicotinic pharmacophore. Designation of the nicotinic pharmacophore is the first essential step towards understanding the interaction between *n*AChR and the class of neonicotinoids (including the commercial products). Several early ''nicotinic pharmacophores'' were described, but these either did not consider specific binding data or were derived on the basis of pharmacological data from peripheral *n*AChR assays [102].

Table 29.1.2 Amino acid sequence in loop D of vertebrate and insect nAChR subunits. (Data taken from Shimomura et al., 2002.)

Proteins	Sequences
	↓**79**
Chicken α7	TNIWL **Q** MYWTD
AchBP	VVFWQ **Q** TTWSD
Chicken β2	TNVWL **T** QEWED
Chicken β4	TNVWL **N** QEWID
Human β2	TNVWL **T** QEWED
Human β4	TNVWL **K** QEWTD
Torpedo γ	TNVWI **E** IQWND
Torpedo δ	SNVWM **D** HAWYD
Drosophila ARD	SNVWL **R** LVWYD
Drosophila SBD	TNLWV **K** QRWFD
Drosophila β3	THCWL **N** LRWRD
Locusta β	SNVWL **R** LVWND
Myzus β1	SNVWL **R** LVWRD

The arrow points to Q79 in loop D of the α7 subunit and the corresponding amino acid residues of vertebrate and insect non-α subunits.

For example, in 1970 a useful nicotinic pharmacophore model was already described by Beers and Reich and subsequently improved by Sheridan et al. [103]. Starting from different models a distance from the onium group to a point on the van der Waals surface of the H-bond acceptor of 5.9 Å was common to several ligands. Up to now three binding models of neonicotinoids have been proposed (cf. Fig. 29.1.2) [104].

From SAR studies the first two models I and II (Fig. 29.1.2b) suggest a primary role for the nitrogen at the 1-position of the neonicotinoid, equating it to *N*-methyl-pyrrolidine nitrogen of nicotine (**1**). Yamamoto et al. [105] proposed that the nitrogen atom of the 6-chloro-pyrid-3-yl moiety and the nitrogen atom at the 1-position of the imidazolidine ring in **7** (X = N; E = NH; R^1-R^2 = CH_2CH_2) interact with the H-donating and electron-rich sites of *n*AChR, respectively, because the distance between these two nitrogen atoms is similar to that between the two nitrogen atoms on **1** (Fig. 29.1.2a). In an early study, the sp^2 pyridine nitrogen of **1** neutral form has been identified as the only H-bond acceptor site [106]. Later it was found that, in solution, both nitrogens of **1** are involved in the H-bond interactions, with 90% of these H-bonded complexes being formed on the pyridine nitrogen [107]. This result is in accordance with **1** and carbamoylcholine binding observed in AChBP [108].

On the other hand, Kagabu proposed that the nitrogen atom at the 1-position of the imidazolidine ring of **7** and one of the oxygen atoms of the nitro group within the [=N–NO_2]-pharmacophore (at the van der Waals surface) play an important role in the interaction with the binding sites on nAChR (Fig. 29.1.2b, cf. Model

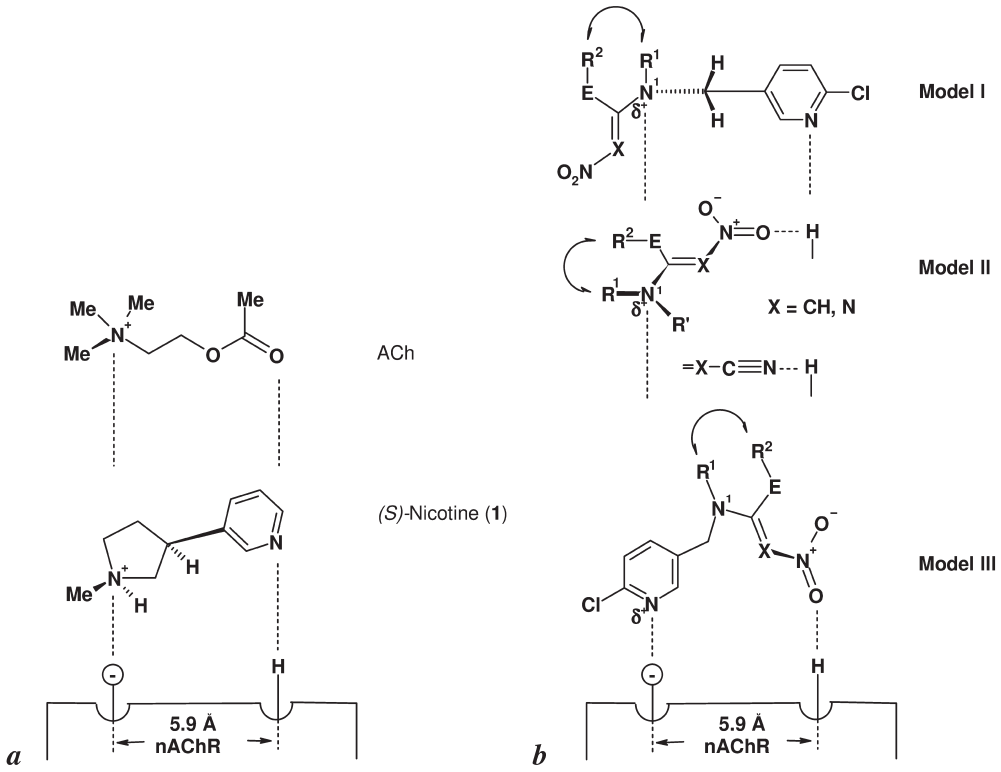


Fig. 29.1.2. Interaction of (a) ACh, (*S*)-nicotine (**1**) and (b) neonicotinoids (**7**–**14**) with the H-donating and electron rich sites of nAChR, exemplified by three models (I–III). (Adapted from Yamamoto et al., 1995 [105], and Tomizawa et al., 2003 [213].)

II). Thus the π-conjugated system composed of a *N*-nitro-imino- or *N*-cyano-imino group and the conjugated nitrogen in 1-position are considered essential moieties for the binding of neonicotinoids to the putative cationic subsite in insect *n*AChR.

The third and most recent model III (Fig. 29.1.2b) involves a crucial role for the *N*-nitro group within the [=X–NO_2]-pharmacophore, an important contribution from the 6-chloro-pyrid-3-yl ring nitrogen, and a supplemental role for the nitrogen in 1-position. A first confirmation of this model III is exemplified by interaction of **7** with the α7 *n*AChR, based on the AChBP [109].

29.1.5
Mode of Action in Insects

Efforts have been made to develop insecticides with high affinity to the *n*AChR, resulting in the development of nereistoxin analogues (e.g., **3**–**5**), neonicotinoids (e.g., **7**–**13**) and spinosyns (e.g., **15**).

29.1.5.1 **Nereistoxin and Analogues**

In contrast to *n*AChR agonists causing excitatory effects, **2** induces inhibitory neurotoxicity. Multiple actions of **2** have been reported with relatively high concentrations in vertebrate *n*AChRs such as (a) a potent blocker of the mechanosensory-giant interneurone cholinergic synapses in the cockroach (*P. americana*) terminal abdomal (TA) ganglion as well as of frog and rat muscle endplates [110], (b) a partial agonist [111], (c) an non-competitive or open-channel blocker (NCB) for the open status of the *n*AChR/channel from *Torpedo* electric organ. Dual actions are also proposed at the honeybee (*Apis mellifera*) *n*AChR because **2** binds to both the NCB and ACh sites with high and low affinities [112]. At low concentrations (0.1 μM), a voltage-dependent inhibitory effect of **2** on *n*AChRs was observed [113]. Furthermore, **2** inhibits the specific binding of [^{125}I]α-bungarotoxin (α-BgTx, a toxin from the elapid snake *Bungarus multicinctus*). But, the high concentration (0.17 mM) required for 50% inhibition of specific [^{125}I]αBtx binding suggests that the site of **2** blocking action may be distinct from the αBtx binding site.

Cartap [1,3-bis(carbamoylthio)-2-(*N*,*N*-dimethylamino)propane] (**3**) was the first commercial insecticide derived from **2** and acts in a similar manner at cercal afferent-interneurone synapses [114]. Indeed, the co-application of ACh and **3** (10 μM) induced the opening of the *n*AChR channel but for a shorter time, generating the appearance of burst [115].

Recent studies on recombinant chicken α7 and α4β2 receptors as well as *Drosophila*/chicken hybrid receptors Dα2β2 and Dα1β2 have shown that **2** is an effective blocker of native and expressed vertebrate *n*AChRs, acting as a NCB of the *n*AChR [116]. However, **2** was slightly more potent on recombinant *Drosophila*/chicken hybrid receptors than on chicken *n*AChRs [117].

29.1.5.2 **Neonicotinoids**

The biochemical MoA (mode of action) of neonicotinoids has been investigated and characterized in the past ten years. In insects, all neonicotinoids act selectively at the postsynaptic nAChRs at nanomolar level (**1** at micromolar level) and bind to the ACh binding site located on the hydrophilic extracellular domain of α-subunits. The neonicotinoid binding site in insects is the same or closely coupled to that of ACh, **1**, and α-BgTx. However, unlike **1**, which is hydrolyzed by AChEs, nicotinic agonists and antagonists lacking the ester linkage can generate, respectively, sustained activation or block of nAChRs.

Their ability to displace tritiated imidacloprid (**7**) ([^{3}H]-**7**) from its binding site correlates well with their insecticidal efficacy [118, 119]. The insecticidal activity of neonicotinoids is due to their action as insect *n*AChR agonists, causing channel opening. This was first demonstrated by electrophysiological and [^{125}I]α-BgTx binding studies with **6** and the cockroach nerve cord [120, 121]. This fact is supported by other studies which demonstrate a high correlation between nerve activity induced in cockroach preparations and their potential to control numerous target pests species [122, 123, 124]. It was verified with [^{3}H]-**7** or [^{125}I]α-BgTx using in binding studies with insect brain membranes [125, 126]. Two

α-BgTx-sensitive nAChR subtypes in cockroach neurons are identified as desensitizing (*n*AChRD), selectively inhibitable with 100 mM **7**, and non-desensitizing (*n*AChRN), selectively inhabitable with 100 pM methyllycaconitine. Although the desensitizing rate of *n*AChRD receptors is highly variable, pharmacology is largely independent and specifically measured in radiolabeled **7** binding assays [127].

More definitive confirmations were obtained with [^{3}H]-**7** by structure–activity correlations for displacement of binding potency with knockdown activity [128, 129] and electrophysiological responses. Agonistic action on *n*AChRs causes first hyperexcitation and then paralysis as shown with **7** on different insect species [130, 131, 132]. On the other hand, antagonistic ligands of mammalian *n*AChR were mostly less active as insecticides [133, 134].

Since the discovery of **7**, diverse imidacloprid-related insecticides referred to as neonicotinoids have been synthesized. Like **7**, all commercial neonicotinoids **8**–**13** bind with high affinity ($I_{50} \sim 1$ mM) to [^{3}H]-**7** binding sites on insect *n*AChRs.

One notable omission is the five-membered thiamethoxam (**10**) (Chapter 29.2.3), showing binding affinities up to 10 000-fold less than other neonicotinoids, using housefly head membrane preparations. This low affinity may be attributed to its proneonicotinoid structure, as it was shown to be activated to the open-chain clothianidin (**12**) (Chapter 29.2.1) in plants and insects [135]. The latter exhibits high activity as agonist on isolated neurons at concentrations as low as 30 mM.

Recently it was shown that cholinergic neurons express nAChRs that are highly sensitive to **7** and demonstrated a role for voltage-gated calcium channels in amplifying **7**-induced increase in intracellular calcium [136].

Several excellent reviews cover the MoA of neonicotinoid insecticides [137, 138, 139]. These reviews describe the current knowledge of the structure and function of insect *n*AChRs, characterized by receptor binding studies, phylogenetic considerations regarding receptor homologies between orthologs from different animal species, and electrophysiological investigations.

29.1.5.3 Spinosyns and Semi-synthetic Analogs (Spinosoids)

Spinosyns cause hyperexcitation, and ultimately disruption, of the insect CNS [140] by allosterically activating *n*AChRs and prolonging the responses of those receptors to agonists such as **1** and ACh [141]. It initially cause spontaneous involuntary muscle contraction, prostration with tremors by exciting motor neurons in the CNS [142], paralysis and finally death. Spinosyn A can directly excite the CNS when applied to isolated insect ganglia, indicating that, *in vivo*, the neuronal excitatory effect is directly due to **15** (Table 29.1.1) and not to a bioactivated metabolite. These effects are consistent with the activation of both *n*AChRs and also GABA receptor functions of neurons, which may increase their activity [143]. Insecticidal spinosyns and spinosoids disrupt GABA receptor function in small neurons from CNS of *Periplaneta americana*, while spinosyns lacking insecticidal activity do not [144]. However, spinosyns and spinosoids seems not to affect the binding site of either nicotinic or GABA receptor radioligands such as avermectins, fiproles or cyclodienes, suggesting that the macrolactones do not interact

directly with known binding sites for other nicotinic or GABAergic insecticides [145]. They suppressed the amplitude of GABA responses and activated a picrotoxin sensitive chloride current in small neurons from the CNS of *P. americana* [146].

Electrophysiological evidence indicates that spinosyns can alter nicotinic currents in neuronal cell bodies from CNS of the American cockroach [*P. americana* (L)]. The effect is correlated with toxicity to neonate *Heliothis virescens* larvae. It can be suggested that the spinosyns affect *n*AChR and GABA receptors through a so-called undetermined mechanism and differ from that of neonicotinoids [147]. Finally, it was shown, that the action of spinosyn A is different on both the desensitizing and non-desensitizing subtypes of α-Btx-sensitive nAChRs. Spinosyn A is a highly effective activator of non-desensitizing subtypes, whereas it blocked the desensitizing subtypes of *n*AChRs (for more details see Chapter 29.3).

29.1.6
Selectivity for Insect versus Vertebrate nAChRs

Neonicotinoid insecticides are more than 100-fold selective for insect *n*AChRs over vertebrate *n*AChRs, but little is known about the mechanism of selectivity [148]. Several research groups have described evidence related to the submolecular basis of this selectivity, based on the *n*AChR subunit composition and properties, as well as the steric charge distribution characteristics of neonicotinoids [149, 150, 151, 152]. Prolonged activation, modulation, or inhibition of LGICs such as *n*AChRs can result in toxicity. However, selective toxicity involving low hazard for vertebrates and high potency to insect pests is an essential requirement for identifying safe and effective insecticides for the future.

29.1.6.1 Neonicotinoids

Debnath and coworkers [153] have demonstrated, in a QSAR study performed using electrotopological state atom indices, that compounds with a [=N–NO_2] (e.g., **7**, **10**, **12**, **13** and **14**), [=CH–NO_2] (e.g., **8**) or [=N–CN]-pharmacophore (e.g., **9** and **11**) are more active, selectively, to *Drosophila n*AChR and safe for humans, whereas *N*-unsubstituted imines having affinity to mammalian receptor.

It has been shown that two important enzymes in metabolism of neonicotinoids, the liver microsomal CYP3A4 (mainly oxidation of imidazolidine moiety) [154] and cytosol aldehyde oxidase (AOX, reduction at the [=N–NO_2]-group) [155] result in either an increase or decrease of agonist potency, depending on the compound and specificity of the *n*AChR [156]. With the vertebrate $\alpha4\beta2$ *n*AChR, AOX enhances potency of **7** but CYP3A4 does not. The AOX system coupled with the *Drosophila* receptor strongly inactivates the neonicotinoids like **7**, **10**, **12** or **13**; with nitromethylenes **6** and **8** some inactivation was found.

In contrast to *S*-(−)-nicotine (**1**; rat oral LD_{50} = 50–60 mg a.i. kg^{-1}, high mammalian oral and dermal toxicity), neonicotinoid insecticides display excellent selectivity profiles that are largely attributable to specifity for insect versus vertebrate *n*AChRs. This is exemplified by the fact that the radioligand [^{3}H]-**7** serves

Table 29.1.3 Specificity of commercial neonicotinoids (**7–13**) for insect and vertebrate $\alpha 4\beta 2$ nAChRs. (According to M. Tomizawa and J. E. Casida, 2005 [169].)

Neonicotinoid	Insect[a]	IC_{50} (nM) vertebrate $\alpha 4\beta 2$[a,b]	Selectivity ratio
Imidacloprid (**7**)	4.6	2600	565
Nitenpyram (**8**)	14	49 000	3500
Acetamiprid (**9**)	8.3	700	84
Thiamethoxam (**10**)	5000	>100 000	>20
Thiacloprid (**11**)	2.7	860	319
Clothianidin (**12**)	2.2	3500	1591
(±)-Dinotefuran (**13**)	900	>100 000	>111

[a] IC_{50}s for displacing [^{3}H]-**7** binding to the house fly (*M. domestica*) **9**, aphid (*M. persicae*) **10**, and fruit fly (other neonicotinoids) receptor, and [^{3}H]-**1** binding to the vertebrate $\alpha 4\beta 2$ nAChR.
[b] IC_{50}s (μM) for the vertebrate $\alpha 7$ nAChR subtype (assayed by [^{125}I]α-BgTx binding) are **7**, 270; **8**, >300; **9**, 290; **10**, >300; **11**, 100; **12**, 190; **13**, >1000.

as an excellent probe for insect but not vertebrate *n*AChRs. On the other hand, [^{3}H]epibatidine (**16**) ([^{3}H]-**16**) [157] and [^{125}I]- or [^{3}H]α-BgTx [158] are important probes for characterizing the vertebrate $\alpha 4\beta 2$ and $\alpha 7$ *n*AChR subtypes, respectively. In native insect nAChRs, the [^{3}H]-**7** binding site in *Drosophila* is distinct from that of [^{3}H]α-BgTx [159]. Specific [^{3}H]-**16** binding has been found in some insects such as *P. americana* but not in others such as the housefly *M. domestica*.

Neonicotinoids have little or no effect on vertebrate peripheral *n*AChR $\alpha 1\gamma\alpha 1\delta\beta 1$ subtype [160, 161, 162, 163] or some neuronal subtypes [$\alpha 3\beta 2$ (and/or $\beta 4$) $\alpha 5$, $\alpha 4\beta 2$, and $\alpha 7$] [164, 165, 166, 167, 168]. Minor structural modifications of neonicotinoids confer differential subtype selectivity in vertebrate *n*AChRs [169]. Generally, nitromethylene analogues with strong insecticidal efficacy show comparable or higher affinity display comparable or higher affinity than that of **1** to the $\alpha 3\beta 2\beta 4\alpha 5$ or $\alpha 7$ subtype (Table 29.1.3).

Comparative binding studies indicate that imidacloprid (**7**) and related neonicotinoids have little or no affinity for several mammalian *n*AChRs. Electrophysiological measurements reported in numerous studies have revealed that *n*AChRs are widely expressed in the insect CNS on both post- and presynaptic nerve terminals, on the cell bodies of inter-, motor- and sensory neurons [170, 171, 172, 173, 174].

Electrophysiological and biochemical binding studies revealed that the primary target of the neonicotinoids were the *n*AChRs [175, 176, 177, 178, 179, 180, 181, 182]. Electrophysiological studies indicate that **7** acts as an agonist on two distinct *n*AChR subtypes on cultured cockroach dorsal unpaired motoneuron (DUM) neurons [183], an α-BgTx sensitive *n*AChR with "mixed" nicotinic/muscarinic

pharmacology and an α-BgTx intensive *n*AChR. The investigations were supported by binding studies with [^{3}H]-**7** in membrane preparations from *M. persicae*. These ligand competition studies revealed the presence of high and low-affinity *n*AChR binding sites for **7** in *M. persicae* [184]. The identification of multiple putative *n*AChR subunits by molecular cloning is consistent with a substantial diversity of insect *n*AChRs [185].

29.1.6.2 **Spinosyns**

Spinosad (**15**) as a reduced-risk insecticide with wider margins of safety for non-target organisms demonstrates also the different sensitivity of insect nAChRs versus mammalian nAChRs (see Chapter 29.3).

29.1.7 Insect Selectivity Found in Recombinant nAChRs

Pharmacological profiles of the recombinant hybrid insect α/vertebrate β *n*AChRs are poorly defined and the binding sites are not established for identified subunits versus native receptors. Functional expression of insect *n*AChRs of known subunit compositions facilitates understanding of the mechanism underlying these molecular interactions. However, it is difficult to heterologously express functionally robust *n*AChRs not only in *Xenopus oocytes* but also in *Drosophila* S2 cells [186]. Only a few functional receptors have been obtained after expression of different subunit combinations in *X. oocytes* or cell lines. Nevertheless, *Drosophila n*AChR α subunits can form homo-oligomeric functional nAChRs when co-expressed with a vertebrate β2 (non-α) subunit in *X. oocytes*. This has been demonstrated so far for:

- four locust nAChR subunits (three α-subunits: Lα1 from *Schistocerca gregaria* [187, 188], and Locα2 and Locα3 from *Locusta migratoria*; and one non-α-subunit: Locβ1 from *L. migratoria*) [189];
- six *D. melanogaster* nAChR subunits (four α-subunits: Dα1, Dα2 [190], Dα3 [191] and Dα4 [192]; and two non-α-subunits: ARD and SBD [193]);
- one *Manduca sexta* nAChR subunit [194];
- five *M. persicae* nAChR subunits (Mpα1–4 and Mpβ1) [195, 196].

However, the expression of these subunits was not very effective (low amplitude-current, 5–50 nA) following application of **1** or ACh. In addition, cDNAs of Locα1 and Locα4 from *L. migratoria* and Mpα5 from *M. persicae* have been cloned partially.

Alternatively, all three *Drosophila* α subunits (ALS, SAD, and Dα2) can form functional receptors in *X. oocytes* when co-expressed with a chicken neuronal α4β2 subunit [197, 198], suggesting that additional insect *n*AChR subunits remain to be cloned. Of the hybrid receptors, the *Drosophila* SAD-chicken β2 hybrid *n*AChR has been found to be highly sensitive at much lower concentrations to the

agonist actions of **7** and related neonicotinoids (more neonicotinoid sensitive than the $\alpha4\beta2$ receptor), suggesting that the *Drosophila* α subunits Dα1 and Dα2 have structural features favorable for selective interactions with neonicotinoids.

However, other studies using recombinant *n*AChRs stably expressed in cell lines have demonstrated that binding of **7** and methylcarbamoylcholine to the D$\alpha3/\beta2$ receptor was abolished by replacing the $\beta2$ with a vertebrate $\beta4$ subunit, suggesting that non-α subunits may also be important in determining sensitivity to neonicotinoids and other agonists.

Radioligand binding studies using several *M. persicae* α subunits co-expressed with a rat $\beta2$ subunit in the *Drosophila* S2 cell line also reflect pharmacological diversity in *M. persicae*.

Recently, the [^{3}H]-**7**, [^{3}H]-**16** and [^{3}H]α-BgTx binding sites in hybrid *n*AChR consisting of *D. melanogaster* or *M. persicae* $\alpha2$ co-assembled with rat $\beta2$ subunits (D$\alpha2$/R$\beta2$ and Mp$\alpha2$/R$\beta2$) in comparison with native insect and vertebrate $\alpha4\beta2$ nAChRs were studied [199]. The findings support the conclusion that the *n*AChR agonist binding site for neonicotinoids is located at the interface region between subunits in insects α/vertebrate β hybrids as well as native insect receptors. These binding studies demonstrate that imidacloprid (**7**) selective targets were formed by Mp$\alpha2$ and Mp$\alpha3$, but not Mp$\alpha1$ subunits.

On the other hand, a so-called "cleavage" of the imidazolidine 5-ring of **7** led to the efficacy of the open-chain **12**, which is higher than that of ACh or **7** on the SAD$\beta2$ hybrid nAChR expressed in *X. oocytes*. The super agonist action of **12** and related ligands may account for the more potent action of **12** than that of **7** on a wide range of insect pests [200].

By expressing hybrid nAChRs containing *Nilaparvata lugens* (susceptible laboratory strain of brown planthopper) α and rat $\beta2$ subunits, evidence was obtained that demonstrates that mutation Y151S is responsible for a substantial reduction in specific [^{3}H]-**7** binding [201]. It seems plausible that this mutation might cause an induced conformational change within the *n*AChR binding site region.

These examples indicate the complexity of insect *n*AChRs, which is difficult to understand. The considerable diversity of potential subunit combinations probably accounts for the multiplicity of distinctive pharmacological profiles in insect *n*AChRs. In that context, electrophysiology will play an essential role in determining the significance of certain subunit combinations in the MoA of neonicotinoid and further insecticidally active ligands.

29.1.8
Whole Cell Voltage Clamp of Native Neuron Preparations

The use of isolated neurons from insect CNS for electrophysiological studies is a suitable tool to investigate the MoA of new insecticidal compounds that act on a range of neuronal target sites. Therefore, primary neuronal cell cultures from *Heliothis virescens* larvae, one of the most important lepidopteran pest species, is one of these suitable tools.

H. virescens neurons respond to the application of ACh, with a fast inward current of up to 5 nA at a holding potential of –70 mV. The current reversed at a

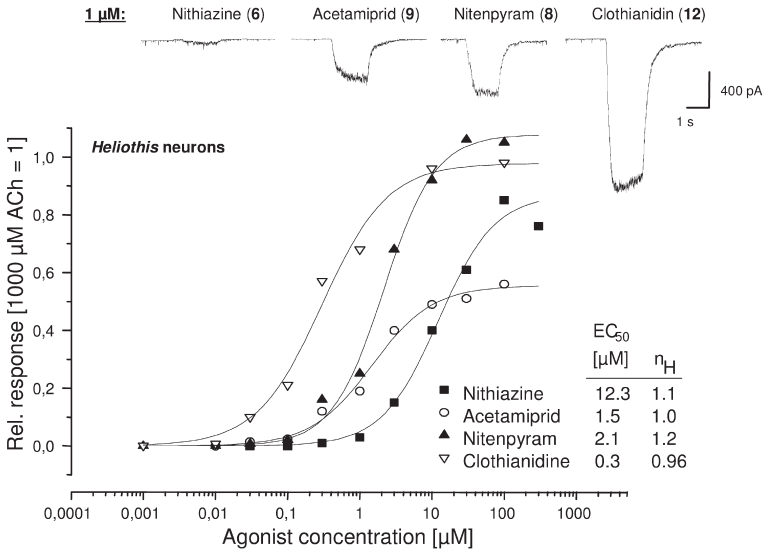


Fig. 29.1.3. Whole cell current responses of a neurone isolated from the CNS of *Heliothis virescens* after application of different neonicotinoids. The dose–response curve was fitted by the Hill equation. All currents were first normalized to mean amplitudes elicited by 10 µM ACh before and after each test concentration was applied and then normalized to the relative amplitude elicited by 1000 µM ACh. EC_{50}s given correspond to the half-maximal activation of nAChR by each agonist. The Hill coefficient (n_H) of all tested compounds was close to 1. Upper inset: corresponding responses for the neonicotinoids at 1 µM (holding potential −70 mV). All currents were obtained from the very same neurone (Nauen et al. 2001 [122]).

holding potential close to 0 mV, indicating the activation of nonspecific cation channels, i.e., *n*AChRs.

Figure 29.1.3 shows the whole cell currents elicited by application of 1 µM nithiazine (**6**) and the commercial open-chain neonicotinoids nitenpyram (**8**), acetamiprid (**9**) and clothianidin (**12**).

All of these act as agonists on the *n*AChR, but the potency and agonistic efficacy of each of these neonicotinoids were quite different. The five-membered imidacloprid (**7**) and the open-chain clothianidin (**12**) were the most potent neonicotinoids in this *Heliothis* preparation with an EC_{50} of 0.3 µM (Table 29.1.4).

With **7** there was a good agreement with electrophysiological measurements recorded from isolated cockroach neurons, where it exhibited an EC_{50} of 0.36 µM [202]. Neonicotinoids such as **8**, **9** and the natural toxin (±)-epibatidine (**16**) exhibited an EC_{50} of between 1 and 2 µM. Similar values were also observed for the cockroach preparation, with an EC_{50} between 0.5 and 0.7 µM for **16** and **9**, respectively. Nithiazine (**6**) had the lowest potency ($EC_{50} = 10$ µM). The commercial open-chain neonicotinoids **8** and **12** were full agonists, whereas **7**, **9** and **16** were partial agonists. On isolated cockroach and locust neurons [203] it was found that

Table 29.1.4 Comparison between electro-physiological and [^{3}H]-**7** displacement potencies for different neonicotinoids **6–9**, **13** and (±)-epibatidin (**16**) on insect nAChRs. Electrophysiological data [EC_{50} and relative (agonist) efficacy] were obtained from neuron cell bodies isolated from the CNS of *H. virescens*. EC_{50} and relative efficacy values represent the mean of separate experiments on different neurons. Inhibition of [^{3}H]-**7** binding to nAChR in housefly head membrane preparations by the compounds is expressed as pI_{50} (pI_{50} values (= −log *M*) correspond to the concentration of cold ligand displacing 50% of bound [^{3}H]-**7** from housefly head membranes). (Reproduced with permission from Ref. [122].)

Compound	*n*	EC_{50} (μM ± SD)	Relative efficacy (1 mM ACh = 1)	[^{3}H]IMI pI_{50}
6	4	9.60 ± 3.20	0.79 ± 0.06	6.8
7	4	0.31 ± 0.15	0.14 ± 0.02	9.3
8	3	1.66 ± 0.38	0.98 ± 0.07	8.6
9	3	1.07 ± 0.37	0.56 ± 0.05	8.7
12	3	0.33 ± 0.03	0.99 ± 0.08	9.2
(±)-**16**	3	1.69 ± 0.79	0.20 ± 0.05	6.2

7 acted as partial agonist on insect *n*AChRs. The partial agonistic action of **7** was also identified with chicken $\alpha4\beta2$ *n*AChRs, and on a hybrid *n*AChR formed by the co-expression of a *Drosophila* α-subunit (SAD) with chicken β2-subunit in *X.* oocytes. Imidacloprid (**7**) activates very small inward currents in clonal rat phaeochromocytoma (PC 12) cells, thus also indicating partial agonistic actions [204]. Single-cell analysis revealed that **7** activates predominantly a subconductance of approximately 10 pS, whereas ACh activated mostly the high conductance state with 25 pS. Multiple conductance states were also observed in an insect *n*AChR reconstituted into planar lipid bilayers [205] and on locust neurons [206].

29.1.8.1 Correlation Between Electrophysiology and Radioligand Binding Studies

A good correlation between electrophysiological measurements, using isolated *Heliothis* neurons, and radioligand binding studies on housefly head membranes regarding the affinity of ligand to *n*AChRs was found (Fig. 29.1.4).

This correlation for commercial neonicotinoids **8–13** may indicate that houseflies (binding data) and tobacco budworms (electrophysiology) have similar binding sites for **7** and related compounds.

The high-affinity [^{3}H]-**7** binding site is conserved in neonicotinoid sensitivity and specificity across a broad range of insects. Biochemical investigations using displacement of [^{3}H]-**7** as a radioligand in numerous of different insect membrane preparations, e.g., from *M. persicae*, *B. tabaci*, *N. cincticeps*, (Homoptera), *Manduca sexta*, *H. virescens*, (Lepidoptera), *Lucilia sericata*, *D. melanogaster*, (Diptera) *P. americana*, (Orthoptera), *Ctenocephalides felis*, (Siphonaptera), indicate

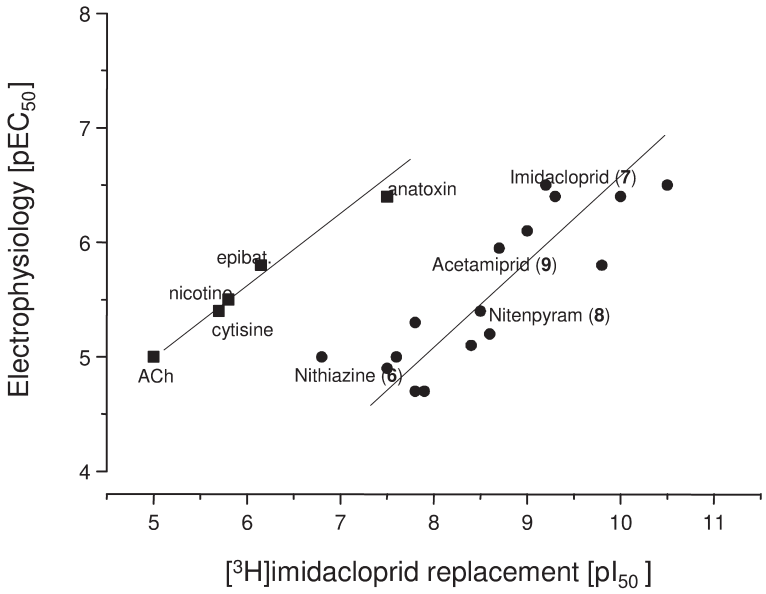


Fig. 29.1.4. Comparison between electrophysiological and binding potencies of different neonicotinoids (**6**–**9**) and nicotinoids. Electrophysiological data were obtained from neurone cell bodies isolated from the CNS of *H. virescens*. pEC_{50}s (= −log *M*) correspond to the half-maximal activation of nAChR by each agonist. Binding data: pI_{50}s (= −log *M*) correspond to the concentration of cold ligand displacing 50% of bound [^{3}H]imidacloprid from housefly head membranes (Nauen et al. 2001 [122]).

that many (if not all) insects have high specific imidacloprid (**7**) binding sites with K_d values of ∼1–10 mM.

Like **7**, all neonicotinoids bind with high affinity (I_{50}s ∼ 1 mM) to [^{3}H]-**7** binding sites on *n*AChRs (Table 29.1.5).

Whereas nithiazine (**6**), the open-chain compounds, **7** and **12**, and the five-membered compounds, **7** and **11**, have low I_{50}s, in the range 0.50–2.0 mM, *N*-methylimidacloprid (I_{50} = 1600 mM) and the six-membered compound thiamethoxam (**10**) (I_{50} = 5000 mM) show only weak activity in displacing [^{3}H]-**7** from its *n*AChR binding site in housefly head membrane preparations. This means that **10** is up to 10 000-fold less active than other neonicotinoids such as **11** (I_{50} = 0.50 mM). To clarify this difference, pharmacokinetic studies in different insect species were carried out. Recently, further investigations demonstrate that the six-membered compound **10** can be rapidly metabolized as a prodrug [207, 208] to open-chain clothianidin (**12**) (Chapter 29.2.1), which shows high affinity to *n*AChRs in both binding assays and whole cell voltage clamp studies. Kayser et al. have presented an alternative explanation for the obvious lack of **7** competition with all known tritiated *n*AChR ligands [209].

Table 29.1.5 Displacement of [^{3}H]-**7** by different neonicotinoids **7–12** from nAChR preparations from housefly head membranes, expressed as I_{50} in nM (this represents the concentration needed to displace half of the radioligand from its binding site).

Neonicotinoid	I_{50} (nM)
Imidacloprid (**7**)	0.79
Nitenpyram (**8**)	2.00
Acetamiprid (**9**)	1.26
Thiamethoxam (**10**)	5000.00
Thiacloprid (**11**)	0.50
Clothianidin (**12**)	0.60
N-Methylimidacloprid	1600.00

However, it was also found that only the homopteran species seems to have an additional very high affinity binding site. In general, the pI_{50}s obtained by displacement of specifically bound [^{3}H]-**7** from housefly head membrane were two to four orders of magnitude higher than the electrophysiologically determined pEC_{50} values obtained from isolated *Heliothis* neurons (cf. different vertebrate *n*AChRs) [210]. A possible explanation for that might be that each *n*AChR can exist in multiple stages, i.e., a resting state, an active (open) state, and one or more desensitized state(s), each of which has different affinities for ligands. The active state has a low affinity for ACh (K_d ranging from about 10 to 1000 μM), whereas the desensitized state(s) has a higher affinity (K_d ranging from about 10 mM to 1 μM) for nicotinic ligands [211].

The kinetics of the transitions between these states have been resolved for *Torpedo* nAChR *in vitro*. The rate of isomerization between the resting and active state lies in the μs to ms timescale, and within the desensitized state over a timeframe of seconds to minutes. Because binding studies are conducted over a timescale of minutes to hours they may reflect interaction with the desensitized state(s), whereas electrophysiological studies measure the interaction of ligands with the active state. Considering this, there is, surprisingly, a direct correlation between electrophysiological and biochemical binding studies for natural alkaloids such as **1**, cytosine, **16** and anatoxin (Fig. 29.1.4). For these compounds the pI_{50} and pEC_{50} values were in the same range, with good correlation. Generally, natural alkaloids such as **1** and **16** exhibit an agonistic potency in electrophysiological assay on isolated cockroach neurons and locust neurons. This potency is comparable to highly insecticidal neonicotinoids like **7**. Matsuda et al. 1998 [198], using a hybrid receptor formed by co-expression of the *Drosophila* α subunit SAD with the chicken β2, observed a comparable agonistic potency for both **1** and **7**. However, the agonistic potency of **16** was about two orders of magnitude grater. In contrast, all binding studies using [^{3}H]-**7** on housefly head membranes, whitefly preparations, and *Myzus* preparations indicate that imidacloprid (**7**) has

considerably higher potency in replacing specifically bound [^{3}H]-**7** than nicotine (**1**).

29.1.8.2 nAChR Agonists versus Antagonists

The advantage of electrophysiological measurements compared with biochemical binding assays is their ability to distinguish between agonists and antagonists of the *n*AChR. This functional difference in MoA of ligands with high specificity for the *n*AChR is very important for insecticidal potency. Electrophysiological measurements from isolated housefly neurons revealed that compounds acting agonistically on *n*AChR were in general insecticidal, as shown for **8**–**13** already introduced onto the market. In that context all neonicotinoids are part of a single MoA group as defined by the IRAC for resistance management purposes. It was found that nitenpyram (**8**) and clothianidin (**12**) act as full agonists, whereas acetamiprid (**9**) and imidacloprid (**7**) act as partial agonists, on the insect *n*AChR.

On the other hand, fully antagonistic neonicotinoids have a very limited insecticidal efficacy. This general observation is supported by other investigations that demonstrate the positive correlation between nerve activity induced in cockroach preparations and insecticidal activity of neonicotinoids against green rice leafhopper (*N. cincticeps*).

29.1.9 Molecular Features of a nAChR Agonists

The selectivity of neonicotinoids for insect over vertebrate *n*AChRs is likely to result from selective recognition by insect *n*AChRs of its structural features and *vice versa*. To elucidate the mechanism of selectivity, structural features of neonicotinoids and insect *n*AChRs contributing to this selectivity have been examined.

Both neonicotinoids **8**–**14** and nicotinoids such as **1** have common structural features, but different protonation states at physiological pH. All insect-selective neonicotinoids **8**–**14** are neither acids nor bases at pH 4–10 and the electronegative pharmacophore (bioisosteric electron-withdrawing groups **10**, **12**–**14** [=NNO_2]; **6**, **8**, [=$CHNO_2$]; **9**, **11**, [=NCN]) plays a crucial role in the high affinity and selectivity for insect *n*AChR. In contrast, the nitrogen in the *N*-methyl-pyrrolidine ring of **1** is mostly protonated, having a positive charge. Because of the fact that both nitrogens in the imidazoline ring of **7** are conjugated with the *N*-nitro group, the 2-*N*-nitro-imino-imidazolidine moiety of **7** has a planar structure, as demonstrated by X-ray crystallography. Semiempirical molecular-orbital calculations (method PM3 combined with the AMSOL program) have shown that the nitrogens in the imidazolidine ring of **7** are changed to positive once the *N*-nitro group oxygens forms a H-bond with the positively-charged ammonium cation. These results suggest that the basic residues in the *n*AChRs could play an important role in determining their neonicotinoid sensitivity. They may bind to a lysine (Lys) or arginine (Arg) residue in a cationic subsite of the insect nAChR [212], suggesting topological divergence of the agonist-binding subsites in insects and vertebrate nAChRs (see also Chapter 3; Fig. 29.1.5).

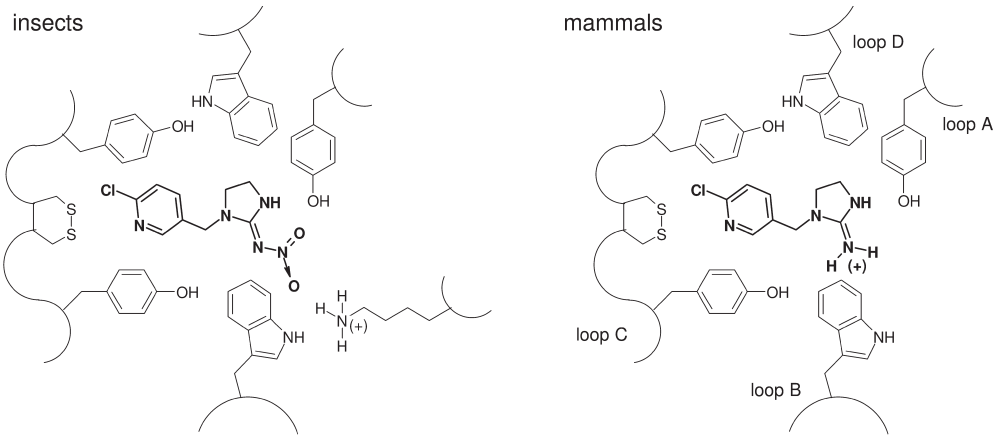


Fig. 29.1.5. Binding subsite specificity shown as hypothetical schematic models for the CNI imidacloprid (**7**) binding in the insect *n*AChR and nicotinoid *N-des*-nitro-**7** binding in the mammalian *n*AChR, each at the ACh agonist site (Adapted from Tomizawa and Casida, 2005 [169]).

The coplanar system between the electronegative pharmacophore and guanidine-amidine moiety extends the conjugation and facilitates negative charge flow toward the electron-withdrawing group, thereby enhancing interaction with the proposed cationic subsite such as both Lys and Arg in insect *n*AChRs.

Cation–π interaction is a prominent feature in the agonist recognition by the neurotransmitter-gated vertebrate *n*AChR [213]. Nicotinoids, including **1**, **16**, and the N-unsubstituted imine (des-N-nitro or des-N-cyano) analogues of neonicotinoids (cationic compounds), show a cationic character and can undergo this specific cation–π interaction with Trp at mammalian neuronal *n*AChR subsite at the $\alpha 4\beta 2$ interface. Thus, binding subsite specificity plays a major role in selective toxicity for neonicotinoids in insects and protonated nicotinoids in mammals.

29.1.10 Conclusions

To date, it is still not possible to create a perfect model of the insect native *n*AChR relative to structure and diversity. Identification and characterization of insect *n*AChR subtypes is still an important research field, and may open up a new era for subtype-selective insecticides. Neonicotinoids are very effective probes for structural investigations of insect *n*AChRs. The atomic determination of the AChBP, homolog to the amino-terminal extracellular domain of *n*AChRs, can be used to generate 3D models of the extracellular ligand-binding domain of ligand-gated ion channels. Therefore, the AChBP structure provides the theoretical basis for designing homology models of the corresponding receptor ligand binding domains within the *n*AChRs [214]. But, the models of *n*AChR extracellular domain

represent so-called frozen ''snapshots'' of a particular state constrained by the crystal structure of AChBP [215]. First insight into the 3D structure of the ion channel in the closed and open conformations gave the refined model of the membrane-associated *Torpedo* AChR at 4 Å resolution, which exemplified that all channels of the Cys-loop LGIC-superfamily are constructed around the same global principle. The AChBP resolves the various models of (*S*)-(–)-nicotine (**1**) and ACh binding in the past and provides novel explanations for important issues on both the nAChR and the ligand site by addressing topics such as the charge compensation and the inter-nitrogen distance in the nicotinoid or neonicotinoid pharmacophore.

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29.2
Chemical Structural Features of Commercialized Neonicotinoids

Peter Jeschke

Introduction

In general, all commercialized neonicotinoids can be divided into open-chain compounds (Chapter 29.2.1) and neonicotinoids having ring systems such as five-membered (Chapter 29.2.2) and six-membered compounds (Chapter 29.2.3) that differ in their molecular characteristics. The structural requirements for both neonicotinoids having open-chain structures and ring-system containing neonicotinoids consist of different segments listed below (Fig. 29.2.1, Tables 29.2.1 and 29.2.2) [1, 2].

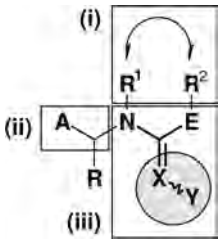
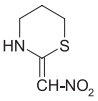
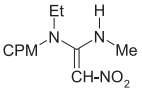
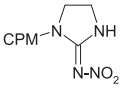
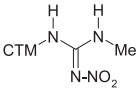
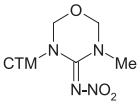
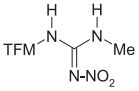
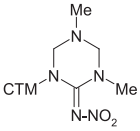
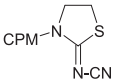
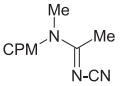


Fig. 29.2.1. Structural segments for neonicotinoids.

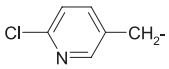
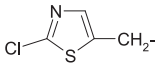
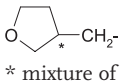
Table 29.2.1 Structure types of neonicotinoid insecticides, including commercialized products (**1**, **3–10**).

Pharmacophores [-N-C(E)=X-Y]	Five- and six-membered compounds (R^1-R^2, R^1-Z-R^2)	Open-chain compounds (R^1, R^2)
Nitromethylenes/nitroenamines [-N-C(E)=CH-NO_2], E = S, NH	 Nithiazine (**3**, $CH_2CH_2CH_2$)[a]	 Nitenpyram (**4**, Et, Me)
N-nitroimines/*N*-nitroguanidines [-N-C(E)=N-NO_2], E = NH, NMe	 Imidacloprid (**1**, CH_2CH_2)	 Clothianidin (**7**, H, Me)
	 Thiamethoxam (**5**, CH_2-O-CH_2)	 Dinotefuran (**8**, H, Me)
	 AKD 1022 (**6**, CH_2-NMe-CH_2)[b]	
N-Cyanoimines/*N*-cyanoamidines [-N-C(E)=N-CN], E = S, Me	 Thiacloprid (**9**, CH_2CH_2)	 Acetamiprid (**10**, Me, Me)

[a] Launched in U.S.A. for use in poultry (1997–1998, Quick strike®, Wellmark).
[b] Not commercially used.

(i) For open-chain compounds, the separate substituents (R^1, R^2) and for ring-systems the bridging fragment [R^1-R^2, R^1-Z-R^2; Z = O, NMe];
(ii) the heterocyclic or heteroalicyclic group A with a bridging chain [-CHR-; R = H; e.g., A-CH_2-: CPM, CTM, and TFM (see Table 29.2.2)], and

Table 29.2.2 Structural segments of commercialized neonicotinoids.

Structure of A-CHR-	Chemical name of this moiety	Abbreviation
	6-Chloro-pyrid-3-ylmethyl	CPM
	2-Chloro-1,3-thiazol-5-ylmethyl	CTM
 * mixture of (*R*)- and (*S*)-enantiomers	(±)-Tetrahydro-fur-3-ylmethyl	TFM

(iii) the functional group [=X-Y] as part of the different pharmacophore types [-N-C(E)=X-Y].

In all neonicotinoids launched so far, the methylene group (–CH_2–) is normally used as the bridging chain. Their pharmacophore (iii in Fig. 29.2.1) can be represented by the group [-N-C(E)=X-Y], where [=X-Y] is an electron-withdrawing group and E is NH, NMe, sulfur or methyl. Because the pharmacophore type influences the insecticidal activity of the neonicotinoids, commercialized open-chain compounds and neonicotinoids have differing ring systems regarding their pharmacophore types:

- Open-chain compounds: [-N-C(E)=CH-NO_2; E = NHMe] nitroenamines (nitro methylenes); [-N-C(E)=N-NO_2; E = NHMe] *N*-nitroguanidines; [-N-C(E)=N-CN; E = Me] *N*-cyanoamidines (Chapter 29.2.1).
- Five- and six-membered compounds: [-N-C(E)=N-NO_2; E = NH, NMe] *N*-nitroguanidines; [-N-C(E)=N-CN; E = S] *N*-cyanoamidines (Chapters 29.2.2 and 29.2.3, respectively).

Besides its influence on biological activity, the pharmacophore is also responsible for some specific physicochemical properties such as photolytic stability, degradation behavior in soil, metabolism in plants and insects, and toxicity to different animals as well [3, 4, 5, 6, 7, 8].

The Term Neonicotinoid

The term ''neonicotinoids'' [9] was originally proposed for imidacloprid (**1**) (Chapter 29.2.2) and related insecticidal compounds with structural similarity

to the insecticidal alkaloid (*S*)-(–)-nicotine (**2**), which has a similar mode of action (Chapter 29.1) [10, 11]. Up to now, various terms have been used in literature to subdivide these important commercial products based on their structural fragments such as:

1. Heterocyclic- or heteroalicyclic group A with bridging chain/A-CH_2-: chloronicotinyls (CNIs)/CPM [12], thianicotinyls/CTM [13] and furanicotinyls/TFM [14] or first generation (CMP), second generation (CTM vs. TFM) [15] and third generation (TFM).
2. Functional group [=X-Y] as part of the pharmacophore types [-N-C(E)=X-Y]: *N*-nitroimines [=N-NO_2] or *N*-nitroguanidines [-N-C(N)=N-NO_2], nitromethylenes [=CH-NO_2] or nitroenamines [-N-C(N)=CH-NO_2] and *N*-cyanoimines [=N-CN] or *N*-cyanoamidines [-N-C(E)=N-CN; E = S, Me].

The subdivision of commercialized neonicotinoids into different generations implies their ranking regarding novelty – a view not based on chemical or biochemical classification. The IRAC classification places all neonicotinoids in group 4A.

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29.2.1
Open-chain Compounds

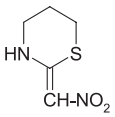
Peter Jeschke

29.2.1.1
Introduction

To date, four open-chain type neonicotinoids have been commercialized, mainly from Japanese companies. These open-chain type neonicotinoids can have, as separate substituents, (i) (R^1, R^2), e.g., R^1 = hydrogen or alkyl like ethyl (**1**, nitenpyram) and methyl (**2**, acetamiprid, E-R^2 = Me) and in the case of E = NH for the substituent R^2 an alkyl group such as methyl (**3**, clothianidin and **4**, dinotefuran) (Chapter 29.2, Table 29.2.1 and Fig. 29.2.1).

29.2.1.2
Nitenpyram

Starting from the cyclic nithiazine (**5**) [1, 2, 3, 4], nitenpyram (**1**, 1995, Takeda Chemical Industries Ltd., now Sumitomo Chemical Takeda Agro Company Ltd.) [5] was discovered during optimization of substituents of an open-chain nitroethene [6]. It was introduced to the Japanese market in 1995 under the trade name Bestguard®. In 1999, Novartis Animal Health introduced **1** as a systemic, fast-acting, adult flea control product in cats and dogs in veterinary medicine under the trade name Capstar® (oral tablet formulation) [7].



5

29.2.1.2.1 Chemical Classification and Physicochemical Properties

The nitromethylene **1** is characterized by its extremely high water solubility (840 g L^{-1}), low partition coefficient (−0.64) (Tables 29.2.1.1 and 29.2.1.2) [8] and a similar poor photostability as **5**.

The latter is responsible for the rapid decomposition in water under light and soil under aerobic conditions.

The outstanding characteristic of **1** is its insecticidal activity by translaminar action and translocation. Because of its high water solubility, **1** shows good systemic activity and no phytotoxic effects. Therefore, **1** can control pests by special soil treatment methods such as planting hole application, plant foot treatment before and after transplanting, and soil incorporation, e.g., application combined

Table 29.2.1.1 Chemical classification of nitenpyram (**1**).

Common name	Nitenpyram
Trade names	Bestguard®, Capstar®
Development codes	TI-304, CGA 246916

Table 29.2.1.2 Physicochemical properties of nitenpyram (**1**).

Melting point (°C)	83–84
Partition coefficient (log P_{OW} at 25 °C)	−0.64
Vapor pressure (mPa at 20 °C)	1.1×10^{-6}
Solubility in water (g L^{-1} at 20 °C, pH 7.0)	840
Solubility in organic solvents (g L^{-1} at 20 °C)	Methanol: 670, acetonitrile: 430, ethanol: 89, xylene: 4.5
Dissociation constant pK_a (at 20 °C)	3.1 and 11.5

with fertilizer using irrigation systems. With a half-life (DT_{50}) of 1–15 days the polar **1** has a relatively short persistence in soil, which probably offsets the relatively weak sorption that might otherwise lead to mobility through soil [9].

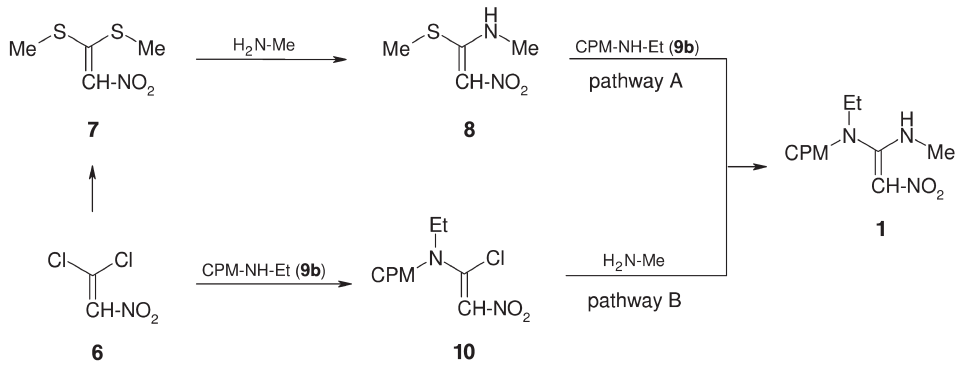
29.2.1.2.2 Chemistry

Compound **1** can be prepared by two synthetic pathways (A and B, Scheme 29.2.1.1).

According process A, 1,1-dichloro-2-nitroethene (**6**) [10] is transformed into 1,1-bis(methylthio)-2-nitro-ethene (**7**) [11], which reacts in the first step with *N*-methylamine to give 1-methylthio-1-(*N*-methyl)-amino-2-nitroethene (**8**), and in the second step with *N*-(6-chloro-pyrid-3-ylmethyl)-*N*-ethyl-amine (**9b**, CPMNHEt) [12, 13] to yield **1**. By process B, **6** is treated with (**9b**) to give 1-chloro-1-[*N*-(6-chloro-pyrid-3-ylmethyl)-*N*-ethyl]-amino-2-nitroethene (**10**) *in situ*, which reacts with *N*-methylamine to form **1** [14].

29.2.1.2.3 Efficacy on Target Pests and Application Rates

Nitenpyram (**1**) controls homopterous insect pests, such as leaf hoppers (e.g., *Empoasca* spp.), plant hoppers (e.g., *Nilaparvata lugens*) on rice, whiteflies (e.g., *Bemisia argentifolii*) and aphids on vegetables, and is also effective against thysanopter-



Scheme 29.2.1.1. Synthetic pathways for preparation of nitenpyram (**1**).

Table 29.2.1.3 Direction for use of nitenpyram (**1**) formulations in Japan – Bestguard® 1%G and 0.25% Dust. (Adapted from Y. Kashiwada, 1996.)

Bestguard® 1%G	**Crop**	**Insect pest**	**Dose**
	Rice	Planthoppers	30–40 kg ha^{-1}
		Green rice leafhopper	30–40 kg ha^{-1}
	Cucumber	Aphids	1–2 g per plant
		Melon thrips	2 g per plant
	Eggplant	Aphids	1–2 g per plant
		Melon thrips	1–2 g per plant
	Tomato	Aphids	2 g per plant
		Silver leaf whitefly	2 g per plant
	(Water)melon	Aphids	2 g per plant
Bestguard® 0.25%Dust	**Crop**	**Insect pest**	**Dose**
	Rice	Planthoppers	30–40 kg ha^{-1}
		Green rice leafhopper	30–40 kg ha^{-1}

ous insect pests on vegetable, fruit trees, tea and glasshouse crops. It is more active by ingestion than by contact. At low concentrations the product inhibits feeding. In addition, **1** also has ovicidal activity and shows no crop phytotoxicity. As direction for use in Japan the product is available [15]:

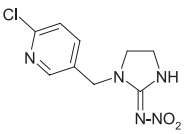
- As water soluble granule (WSG) Bestguard® 10% SP (**1** = 10%) for foliar spray (15–75 g-a.i. ha^{-1}) in rice and field crops,
- as granule Bestguard® 1% G (**1** = 1.0%) for rice, and
- as Bestguard® 0.25% Dust (**1** = 0.25%, 75–100 g-a.i. ha^{-1}) (Table 29.2.1.3).

Nitenpyram (**1**) applied at the rate of 75 g-a.i. ha^{-1} as Bestguard® 0.25% Dust formulation strongly suppressed brown planthopper density for over three weeks. However, with higher application rates such as 100 g-a.i. ha^{-1} a controlling of sting bugs (mixed population of *Nezara viridula*, *Leptocorisa chinensis*, and *Cletus punctiger*) in rice fields is described. Trials in Japan indicate that soil application of granular **1** formulations has good potential for the control of *Liriomyza trifolii* in tomatoes and chrysanthemums grown under greenhouse conditions.

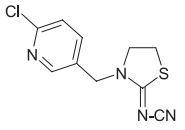
Combination products contain mixtures of **1** and the insecticide cartap hydrochloride, developed for the simultaneous control of these *Hemipterous* pests, and the fungicide validamycin, respectively.

29.2.1.3 Acetamiprid

The *N*-cyano-acetamidine acetamiprid (**2**, 1995, Nippon Soda Co., Ltd.) [16, 17, 18] contains, in analogy to **1** and the five-membered ring systems imidacloprid (**11**) and thiacloprid (**12**), the CPM moiety (see Chapter 29.2.2). It was discovered by Nippon Soda as part of its nitromethylene research program during optimization studies of special 2-*N*-cyanoimino compounds with an imidazoline



11



12

5-ring obtained from Nihon Bayer Agrochem K. K. [19]. As favorable substituent R^1 on the secondary amino group was methyl and ethyl identified, both leading to potent activity against the cotton aphid.

29.2.1.3.1 Chemical Classification and Physicochemical Properties

Because of its high water solubility (4.25 g L^{-1} at 25 °C), **2** shows a systemic and translaminar insecticidal efficacy (Tables 29.2.1.4 and 29.2.1.5).

Table 29.2.1.4 Chemical classification of acetamiprid (**2**).

Common name	Acetamiprid
Trade names	Adjust®, Assail®, Calex®, Chipco Tristar®, Conquest®, Epik®, Gazel®, Gazelle®, Intruder®, Manik®, Mospilan®, Molan®, Mothpiran®, Mothpyran®, Pristine®, Profil®, Profile®, Rescate®, Saurus®, Supreme®, Tata Manik®, Volley®
Development codes	NI-25

Acetamiprid (**2**) is stable in water at pH 4, 5 and 7 at all temperatures and at pH 9 at 22 °C, but is hydrolyzed at pH 9 at 35 and 45 °C, forming two major hydrolytic transformation products. Furthermore, **2** undergoes phototransformation at pH 7 with a half-life of 34 days.

In laboratory studies the systemic properties and the translaminar aphicidal activity of **2** was studied in comparison with imidacloprid (**11**) [20]. The translaminar residual activity of **11** on cabbage leaves was superior to that of **2**, whereas its translaminar efficacy against *Aphis gossypii* on cotton was inferior to that of **2**. However, efficacy of both against *Myzus persicae* and *A. gossypii* in oral ingestion bioassays using an artificial double membrane feeding system revealed no significant differences in their intrinsic activity.

Studies showed that **2** is a mobile, rapidly biodegradable CNI in most soils [21]. The DT_{50} values of **2** in clay loam or light clay soils were in range of 1–2 days. In soil from the field or that used in container studies, degradation of **2** varied widely (half-life 12 days).

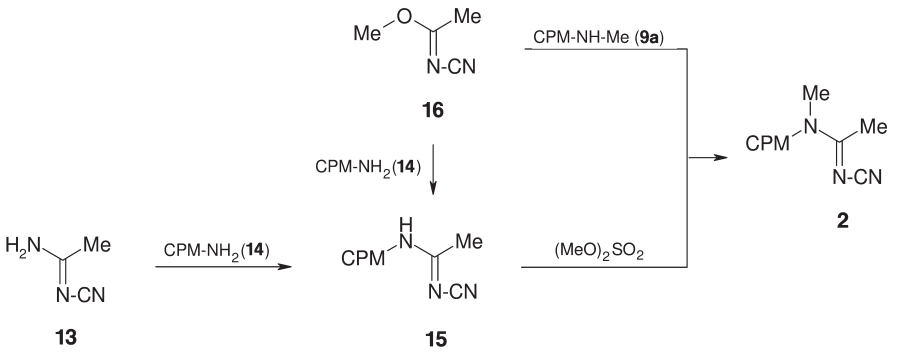
Table 29.2.1.5 Physicochemical properties of acetamiprid (**2**).

Melting point (°C)	98.9
Partition coefficient (log P_{OW} at 25 °C)	0.80
Vapor pressure (mPa at 25 °C)	$<1.0 \times 10^{-6}$
Solubility in water (g L^{-1} at 25 °C)	4.25 (distilled water) 2.95 (pH 7 puffer system)
Solubility in organic solvents (g L^{-1} at 20 °C)	Soluble in ethanol: >200, dichloromethane: >200, hexane: 0.00654
Dissociation constant pK_a (at 25 °C)	0.7 weak base

29.2.1.3.2 **Chemistry**

Scheme 29.2.1.2 outlines the synthetic routes to **2**.

Reaction of the key intermediate methyl *N*-cyano-acetimidate **13** [22] with *N*-(6-chloro-pyridin-3-ylmethyl)amine (**14**, CPM-NH_2) [23, 24, 25] in aqueous methanol leads to **15** (or by treatment of methyl *N*-cyano-acetimidate (**16**) [26] with **14**), which is further *N*-methylated by dimethyl sulfate and inorganic base, giving **2** in an excellent yield. Alternatively, **2** can be synthesized in one step by the reaction of **16** with *N*-(6-chloro-pyridin-3-ylmethyl)-*N*-methyl-amine (**9a**, CPM-NHMe) [27, 28].



Scheme 29.2.1.2. Synthetic pathways for preparation of acetamiprid (**2**).

29.2.1.3.3 **Efficacy on Target Pests and Application Rates**

Acetamiprid (**2**) is a broad-spectrum insecticide that is ovicidal and larvicidal against a wide range of sucking insects such as Hemiptera (especially aphids) or adults of Thysanoptera, Isoptera and Coleoptera pests. It has contact, stomach and systemic long-lasting action and is moderately activity against Lepidoptera such as the peach fruit moth (*Carposina niponensis*), the oriental fruit moth (*Grapholita molesta*), and the diamond back moth (*Plutella xylostella*) and has ovicidal effects on these species as well (Table 29.2.1.6) [29].

However, its penetration through the cuticle is low. Other hemipterous pests, such as sweet potato whitefly, *Bemisia tabaci*, and the citrus mealybug, *Planococcus citri*, or melon thrips, *Thrips palmi*, and the termite, *Reticulitermes speratus*, are also very susceptible to this CNI **2**.

Acetamiprid (**2**) is suitable for use on a wide range of crops, including cereals and sugar beet (Mospilan®), fruit and vegetables (Assail®), ornamentals (Chipco Tristar®), cotton (Intruder®, Assail®), tobacco (Epik®) and tea (Mospilan®). It can be applied to the soil as well as foliage and as seed treatment.

Translaminar efficacy against the green peach aphid (*M. persicae*) was found by the root-dipping method at low concentration [LC_{50} in ppm = 0.031 (eggplant);

Table 29.2.1.6 Spectrum of insecticidal activity (LC_{50} in ppm) of acetamiprid (**2**). (Adapted from Ref. [19].)

	Species	Developmental stage	LC_{50} (ppm)
Hemiptera	*Aphis craccivora*	Larvae and adults	0.91
	Aphis gossypii	1st Instar nymph	0.056
	Aphis spiraccola	1st Instar nymph	0.17
	Myzus persicae	1st Instar nymph	0.21
	Brevicoryne brassicae	1st Instar nymph	0.039
	Rhopalosiphum padi	Larvae and adults	0.032
	Bemisia tabaci	Eggs	4.8
	Planococcus citri	Larvae and adults	1.8
Lepidoptera	*Carposina niponensis*	Eggs	2.8
	Grapholita molesta	Eggs	3.1
	Mamestra brassicae	2nd Instar larvae	13
	Plutella xylostella	1st Instar larvae	4.4
	Spodoptera litura	1st Instar larvae	9.6
Thysanoptera	*Thrips palmi*	Adults	3.4
Isoptera	*Reticulitermes speratus*	Adults	0.16

0.023 (radish)]; activity against *Plutella xylostella* was around ten-fold lower in this test. For the diamond back moth it was demonstrated that **2** is active at different development stages. The LC_{50} against the 1st instar larvae is much smaller than values for the 3rd- and 4th instar larvae. This is important for the application timing of **2**. Furthermore, **2** exhibits high activity on insects OP and/or pyrethroid-resistant as well as susceptible strains.

As **2** is toxic to honeybees, it should not be applied when bees are present in the area being treated.

29.2.1.4 Clothianidin

During the optimization of open-chain neonicotinoids, Takeda researchers were able to demonstrate that compounds containing the *N*-nitroguanidine moiety, coupled with the CTM residue, have increased activity against some lepidopteran pests [30]. Clothianidin (**3**, 2002, Takeda Chemical Industries Ltd., now Sumitomo Chemical Takeda Agro Company Ltd., and Bayer CropScience) [31] emerged as the most promising derivative from this program. In this open-chain structure the *N*-nitro-guanidine pharmacophore is similar to the five-membered

Table 29.2.1.7 Chemical classification of clothianidin (**3**).

Common name	Clothianidin
Trade names	Arena®, Belay®, Celero®, Clutch®, Deter®, Dantotsu®, Focus®, Fullswing®, Poncho®, Prosper®
Development codes	TI-435

neonicotinoids imidacloprid (**11**) (Chapter 29.2.2), but the CPM group has been replaced by the CTM moiety, as also described in Chapter 29.2.3 for thiamethoxam (**17**) and AKD 1022 (**18**).

29.2.1.4.1 Chemical Classification and Physicochemical Properties

Because of no acidic or alkaline properties of **3** at the relevant pH [32], the pH of the aqueous system has no influence on its physicochemical properties. Clothianidin (**3**) is stable to hydrolysis in the pH range 4–9, but photolysis contributes significantly to its degradation in the environment, resulting in an elevated mineralization rate. Its degradation in water/sediment systems was observed to be significantly faster (factor 2–3) under anaerobic conditions than in aerobic conditions. The water solubility (0.327 g L^{-1} at 20 °C), vapor pressure (1.3×10^{-10} Pa at 25 °C) and volatility of **3** are relatively low compared with other neonicotinoids that have a *N*-nitroguanidine pharmacophore (Tables 29.2.1.7 and 29.2.1.8).

This is also reflected by the octanol–water partition coefficient, indicating a favorable absorption to soil (log $P_{OW} = 0.7$ at 25 °C). Plant uptake of **3** occurs via the cotyledons and roots of emerging seedlings and through the roots of established plants (see Chapter 26).

Table 29.2.1.8 Physicochemical properties of clothianidin (**3**).

Melting point (°C)	176.8
Partition coefficient (log P_{OW} at 25 °C)	0.7
Vapor pressure (Pa at 25 °C)	1.3×10^{-10} (extrapolated)
Solubility in water (g L^{-1} at 20 °C)	0.327
Solubility in organic solvents (g L^{-1} at 25 °C)	Methanol: 6.26, dichloromethane: 1.32, 1-octanol: 0.938, xylene: 0.0128, *n*-heptane: <0.00104
Dissociation constant pK_a (at 25 °C)	11.09

Translocation studies demonstrate that **3** moves in an acropetal and basipetal manner, e.g., the active ingredient taken up by the roots is transported rapidly to the leaves and the translaminar activity results in its efficient transport across the leaf tissues from one surface to the other.

On basis of these physicochemical properties no bioaccumulation is expected, nor any volatilization, and therefore no significant amounts of **3** are to be expected in the atmosphere.

29.2.1.4.2 **Chemistry**

In the laboratory, the active ingredient **3** can be obtained, for example, by treatment of *S*-methyl-*N*-nitro-*N′*-phthaloyl-isothiourea (**20**) [33], synthesized from *S*-methyl-*N*-nitro-isothiourea (**19**) [34] and phthaloyl chloride, with 2-chloro-5-(aminomethyl)-1,3-thiazole (**15**, CTM-NH_2) [35, 36, 37, 38, 39, 40, 41].

According to other synthesis methods, **3** can be prepared by coupling of the $N^{1,5}$-dialkylated 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine (**21**, R = alkyl, arylalkyl) with 2-chloro-5-(chloro-methyl)-1,3-thiazole (**22**, CCMT; Table 29.2.1.9) at the 3-position and subsequent ring cleavage reaction of the resulting bis-aminal structure within the six-membered system of **23** (Scheme 29.2.1.3) [42, 43, 44].

Alternatively, **19** is treated in the first step with **15** to give the *N*-alkylated *N′*-nitro-guanidine **24**, which is transformed into the $N^{3,5}$-dialkylated 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine (**25**). In the second step N^1-alkylation with methyl iodide leads to the $N^{1,3,5}$-trialkylated 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine (**23a**), which is cleavaged under acid conditions to form **3**.

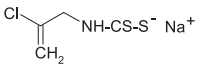
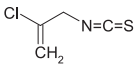
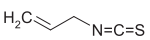
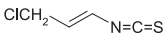
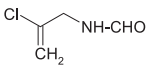
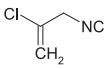
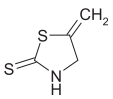
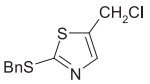
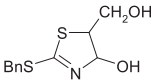
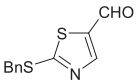
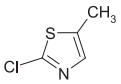
Numerous synthetic pathway for CCMT (**22**) have been studied by Bayer CropScience or Sumitomo Chemical Takeda Agro Company as well as other companies to develop practical and economic processes for this intermediate [45]. Therefore, several patent applications and publications have appeared in the literature for its technical synthesis [46]. As outlined in Table 29.2.1.9, various attractive synthetic routes are known, based on commercially available heterocyclic and open-chain starting materials.

Molecular modeling calculations (force-field methods, MMFF94s) at room temperature and NMR experiments have shown that the preferred orientation of the functional group [=N-NO_2] in **3** is in the *trans*-position; the (*Z*)-isomer with lowest energy is more than 2.6 kcal mol^{-1} above the optimal (*E*)-isomer [47]. Calculations as well as X-ray structure analysis have shown that the three C–N bonds involving atom C5 have some double bond character. The *N*-methyl group of **3** can flip easily from the *anti*- into the *syn*-position. The energies of its respective conformers, relative to the optional structure, are below 1.5 kcal mol^{-1}.

29.2.1.4.3 **Efficacy on Target Pests and Application Rates**

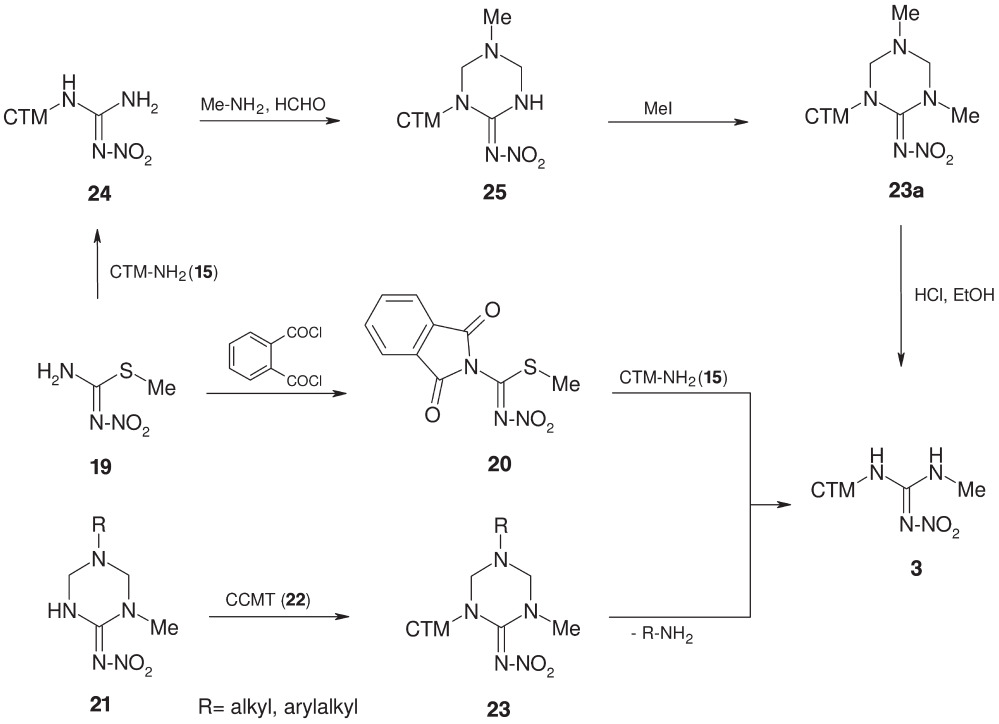
Clothianidin (**3**) has a broad spectrum of activity and acts as an acute contact and stomach poison, combining highly systemic properties with relatively low application rates, suitable for application to soil, foliage and seeds [48]. It is particularly active against sucking insects such as aphids, leaf hoppers, whiteflies and thrips. Furthermore, various species of beetles (e.g., *Atomaria* spp., *Agriotes lineatus*, *Dia-*

Table 29.2.1.9 Selection of synthetic pathways for preparation of the key intermediate CCMT (**22**).

Direct precursor	Reaction conditions	Ref.
	1. H_2O, KI, I_2 2. CH_2Cl_2, SO_2Cl_2	WO 97/23469 (**1997**) Ciba-Geigy AG
	SO_2, Cl_2, 50 °C Cl_2, MeCN, 10–15 °C	WO 20002016335 A2 (**2002**), Syngenta AG EP 1031566 A1 (**2000**), Bayer AG
	NCS, AlBN CH_2Cl_2, Cl_2, −15 to −10 °C (1 h)	WO 2001090089 (**2001**), DSM Fine Chem. DE 3631538 A1 (**1988**), Bayer AG
	SO_2Cl_2, 50–60 °C (3 h)	EP 794180 A1 (**1997**), Kuraray Co. Ltd.
	$SOCl_2$, SCl_2, reflux (24 h)	EP 794180 A1 (**1997**), Kuraray Co. Ltd.
	CCl_4, SCl_2, 40 °C (4 h)	WO 97/10226 (**1997**) Ciba-Geigy AG
	$CHCl_3$, Cl_2, −10 °C	EP 780384 A2 (**1997**), Bayer AG WO 9723469 A1 (**1997**), Novartis AG
	CH_2Cl_2 (2 h)	EP 780384 A2 (**1997**), Bayer AG
	$SOCl_2$, CH_2Cl_2 (3 h)	WO 98/27075 A1 (**1998**) Ciba-Geigy AG
	1. H_2, PtO_2, $FeCl_2{\cdot}4H_2O$, AcOEt (2 h) 2. $SOCl_2$, CH_2Cl_2 (3 h)	WO 98/27075 A1 (**1998**) Ciba-Geigy AG
	NCS, DBPO, CCl_4, reflux	WO 97/23469 (**1997**) Ciba-Geigy AG EP 775 700 A1 (**1997**), Kureha Chem. Ind. Co., Ltd.

Bn = benzyl, NCS = *N*-chloro-succinimide, AIBN = 2,2′-azobis(isobutyronitrile), DBPO = dibenzoyl peroxide.

brotica spp.) and some species of flies (e.g., *Oscinella frit* and *Pegomyia* spp.) and cut worm (e.g., *Agrotis* spp.) are effectively controlled. Because of its excellent root systemicity, **3** is very active against a broad spectrum of root-, stem- and leaf feeding pests as well as soil-inhabiting pests that dwell in the halo around the seed.



Scheme 29.2.1.3. Synthetic pathways for preparation of clothianidin (**3**).

This spectrum consists of Coleoptera, Lepidoptera, and Diptera, which covers most of the early and mid-season corn pests in the USA (Table 29.2.1.10) [49, 50].

Clothianidin (**3**) is marketed as insecticide: (a) for foliar application as Dantotsu® or Fullswing® (Sumitomo Chemical Takeda Agro Company); the latter was launched as a water dispersible granule on turf, providing control of beetle larvae and bluegrass worms; (b) for soil application as water soluble granule (cf. Dantotsu®) and as (c) flowable concentrate for seed treatment (FS) as 600 FS Poncho® (Bayer CropScience).

To control insects in different crops by Poncho® the following use rate of the active ingredient **3** is recommended: e.g., cereals (20–50 g-a.i. 100-kg^{-1}), corn (0.25–1.25 mg-a.i. seed^{-1}), sugar beet (10–60 g-a.i. per 100000 seeds) [51], oil seed rape (4–10 g-a.i. per kg of seeds) or sunflower (20–37.5 g-a.i. per 150000 seeds).

For rice, different formulations have been developed, including Dantotsu® 1 kg granule (1% a.i.), and various combination products such as: Dantotsupadan® (**3**+cartap), which provides additional control of stem borers and leaf rollers; nursery box formulations such as Dantotsu® Nursery Box Granule (1.5% a.i.),

Table 29.2.1.10 Spectrum of activity of clothianidin (**3**) seed treatment for corn root worm (CRW) and for secondary corn pests. (Adapted from Ref. [49]).

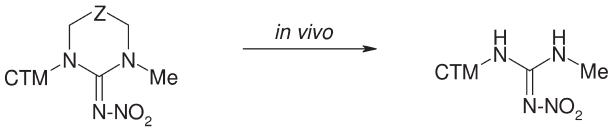
Coleoptera	Corn root worm	*Diabrotica* spp.
	Wireworm	*Melanotus* spp.
	Flea beetle	*Chaetocnema pulicania*
	Grape colaspis	*Colaspis brunnen*
	White grub	*Lachnosterna implicata*
Lepidoptera	Black cutworm	*Agrotis ypsilon*
Diptera	Seed corn maggot	*Hylemyia platura*
Homoptera	Corn leaf aphid	*Rhopalosiphum maidis*
Hemiptera	Chinch bug	*Blissus leucopterus*
	Stink bug	*Nezara viridula*
Hymenoptera	Imported fire ant	*Solenopsis* spp.

Windantotsu® (**3**+carpropamid) and Delausdantotsu® (**3**+diclocymet), providing additional control against rice blast; dust formulations for use in rice such as Dantotsu® Dust DL (0.15% a.i.), Dantotsupadanvalida® (**3**+cartap+validamycin) providing control of green rice caterpillars and sheath blight and Hustler® (**3**+cartap+validamycin+ferimzone+phthalide), which controls rice blast, panicle blight and *Curbularia* spp.

Further different combination products with insecticides and fungicides are known for seed treatment: e.g., Poncho® Beta (**3**+beta-cyfluthrin) in sugarbeets up to the 4th leaf stage (cf. Prosper® 400, containing a higher dosage of **3**).

Similar to imidacloprid (**11**) (see Chapter 29.2), **3** can control important vectors of virus diseases in sugar beet [52].

AKD 1022 (**18**) as well as thiamethoxam (**17**) (Chapter 29.2.3) can form the open-chain neonicotinoid **3** by ring cleavage, either by hydrolysis (see synthesis methods in this chapter) or metabolism (Scheme 29.2.1.4).



Thiamethoxam (**17**) (Z = O)
AKD 1022 (**18**) (Z = NMe)

3

Scheme 29.2.1.4. *In vivo* transformation of six-membered neonicotinoids into clothianidin (**3**).

Recently its use has been demonstrated for important and relevant insects, e.g., wireworm larvae *Agrotis segetum*, corn root worm *Diabrotica balteata* and Colorado potato beetle (CPB) *Leptinotarsa decemlineata*, in an *in vivo* assay (Scheme 29.2.1.4) [53]. This suggests a proinsecticidal MoA of **17** [54]. Differences in the binding site are suggested, in contrast to these findings by Wellmann and Kayser [55, 56]. Further pharmacokinetic investigations in CPB, one of the most relevant species targeted by neonicotinoid insecticides [57], revealed rapid conversion of **17** into **3** applied topically and orally. Additional evidence for **3** being the active principle of **17** was provided by considering neonicotinoid cross-resistance data. All neonicotinoids are classified in group 4A of the IRAC mode of action classification.

29.2.1.5 Dinotefuran

The discovery of the *N*-nitroguanidine dinotefuran (**4**, 2002, Mitsui Chemicals) [58, 59], resulted from the idea of incorporating an *N*-nitro-imino group into the ACh structure as lead compound [60]. After synthesis of neonicotinoids containing a *N*-(3-methoxy-propyl) moiety (hydrogen acceptor site) the investigation of cyclic ether groups led to the discovery of the novel THF moiety, which shows a more than ten-fold increase of insecticidal activity.

In contrast to other commercial neonicotinoids, **4** has an alicyclic and racemic (*RS*)-(±)-TFM moiety instead of the halogenated heteroaromatic CPM and CTM moieties (see Chapter 29.3). The non-aromatic oxygen atom of the TFM residue is situated in the position corresponding to that of the aromatic nitrogen atom of the other heterocyclic moieties of neonicotinoids – consequently the TFM structure can be taken as an isostere of the CPM and CTM moiety [61].

29.2.1.5.1 Chemical Classification and Physicochemical Properties

Dinotefuran (**4**) is characterized by high water solubility (54.3 g L^{-1}) and low partition coefficient (−0.644) (Tables 29.2.1.11 and 29.2.1.12).

Furthermore, **4** is stable in water at pH 4, 7 and 9 at 20 °C. In aerobic soil metabolism studies on loamy sand soil, the DT_{50}-value was 51.7 days. In a 320 day aquatic water-sediment system, the DT_{50}-value for **4** in the aerobic water

Table 29.2.1.11 Chemical classification of dinotefuran (**4**).

Common name	Dinotefuran
Trade names	Alubarin®, Alburin®, Daepo®, Oshin®, Phantom®, Safari®, Shuriken®, Starkle®, Starkul®, Venom®
Development codes	MTI-446

Table 29.2.1.12 Physicochemical properties of dinotefuran (**4**).

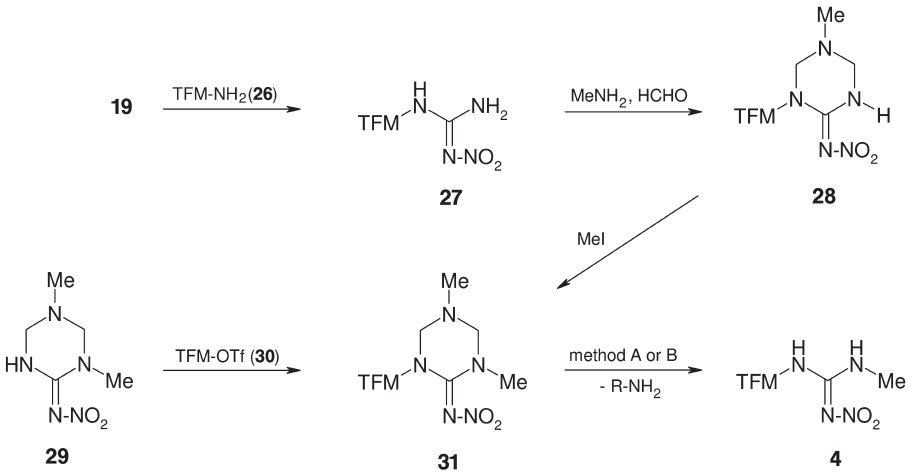
Melting point (°C)	94.5–101.5
Partition coefficient (log P_{OW} at 25 °C, pH 7)	−0.644
Vapor pressure (mPa at 25 °C)	n.d.[a]
Solubility in water (g L^{-1} at 20 °C)	54.3 ± 1.3 (purified water)
Solubility in organic solvents (g L^{-1} at 20 °C)	n.d.[a]
Dissociation constant pK_a (at 25 °C)	No dissociation in pH range 1.4–12.3

[a] n.d. = not described.

phase was 23–49 days and 45–128 days in largely anaerobic sediment layer. In addition, it has excellent systemic and translaminar action in many plants.

29.2.1.5.2 **Chemistry**

Generally, **4** can be synthesized, for example, by treatment of *N*-nitro-*N′*-[((*RS*)-(±)-tetrahydrofur-3-yl)methylamino]-guanidine (**27**) [62], synthesized from *S*-methyl-*N*-nitro-isothiourea (**19**) and (*RS*)-(±)-tetrahydrofur-3-ylmethylamine (**26**, TFM-NH_2) [63], with methylamine and formaldehyde. After *N′*-alkylation of the resulting *N*-alkylated 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine **28** with methyl iodide and subsequent ring cleavage reaction of the resulting bis-aminal structure **31** within the six-membered system, **4** can be obtained (Scheme 29.2.1.5).



Scheme 29.2.1.5. Synthetic pathways for preparation of dinotefuran (**4**).

Table 29.2.1.13 Biological activities of imidacloprid (**11**), clothianidin (**3**), (*RS*)-(±)-dinotefuran (**4**) and the separated enantiomers (*R*)-(−)-(**4**) and (*S*)-(+)-(**4**) against houseflies. (Adapted from K. Kiriyama et al., 2003) [67].

Entry	Insecticidal log(1/EC_{50}) (M) (observed)	Binding log(1/IC_{50}) (M) (radioligand [^{3}H]-11)
11	5.93 (±0.12) (3)	7.71 (±0.14) (2)
3	5.32 (±0.17) (2)	8.28 (±0.07) (2)
(*RS*)-(±)-**4**	5.02 (±0.19) (2)	6.67 (±0.09) (2)
(*S*)-(+)-**4**	5.14 (±0.03) (2)	6.82 (±0.01) (2)
(*R*)-(−)-**4**	3.93 (±0.34) (2)	5.73 (±0.30) (2)

Alternatively, **31** can be prepared from *N*-methyl 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine (**29**) by *N*-alkylation with the O-triflate **30** (TFM-OTf, Tf = SO_2CF_3) [64]. Subsequent ring cleavage is possible under basic (method A) or acidic (method B) conditions.

Structural modifications of **4** regarding the substitution pattern of the TFM moiety, modification of nitrogen substituents or variation of the pharmacophore have indicate that this results in drastic changes in insecticidal potency [65] and that the incorporation of structural fragments known from previous neonicotinoid insecticides does not necessarily lead to compounds retaining higher activity.

Similar to the other open-chain neonicotinoids, **4** has an agonistic action on nAChRs. The neural activities of racemic (*RS*)-(±)-**4**, its separated enantiomers and a competitive nAChR antagonist [^{125}I]α-BGTX in inhibiting [^{3}H]epibatidine (**32**) binding to the American cockroach, *Periplaneta americana* (L), nerve cord membranes were examined. It was found that the (*R*)-(−)-enantiomer of **4** was about two-fold less effective. In contrast the (*S*)-(+)-enantiomer of **4** was approximately 50-fold more insecticidally active than the (*R*)-(−)-enantiomer of **4** [66]. Recently, the insecticidal activity of (*RS*)-(±)-**4**, its enantiomers and the neonicotinoids clothianidin (**3**) and imidacloprid (**11**) against the housefly, *Musca domestica* (L), and their binding activity using housefly head membrane preparations were measured by using [^{3}H]-**11** as radioligand (Table 29.2.1.13) [67].

Dinotefuran (**4**) was less active than clothianidin (**3**) and imidacloprid (**11**) by a factor of 10 in molar concentrations. Finally, the enantiomer (*S*)-(+)-**4** was more potent than its counterpart (*R*)-(−)-**4**.

29.2.1.5.3 **Efficacy on Target Pests and Application Rates**

Dinotefuran (**4**) exhibits activity against numerous insects such as Hemiptera, Lepidoptera, Coleoptera, Diptera, Dictyoptera and Thysanoptera, as well as against some other important pests (e.g., stinkbugs, fruit moths, flea beetles, leaf miners)

Table 29.2.1.14 Spectrum of activity (LC_{50} in ppm a.i.) of dinotefuran (**4**) after foliar and leaf dipping application under laboratory conditions. (Adapted from K. Kodaka et al. 1998)

Insect	LC_{50} (ppm a.i.)	Application method
Sogatella furcifera	1–0.1	Foliar
Nilapavarta lugens	1–0.1	Foliar
Laodelphax striatellus	10–1	Foliar
Nephotettix cincticeps	1–0.1	Foliar
Myzus persicae	10–1	Foliar
Aphis gossypii	10–1	Foliar
Trialeurodes vaporariorum	10–1	Foliar
Bemisia tabaci	10–1	Foliar
Thrips palmi	10–1	Foliar
Phyllotreta striolata	100–10	Foliar
Plutella xylostella	100–10	Leaf dipping
Pieris rapae	100–10	Leaf dipping
Spodoptera litura	100–10	Leaf dipping
Liriomyza trifolii	100–10	Foliar

in various crops (e.g., sugar beet, fruit, vegetables, turf, cotton and ornamentals) at rates of 100–200 g-a.i. ha^{-1} via ingestion and contact, and 150–600 g-a.i. ha^{-1} by soil application, including root-systemic activity. It can be applied by foliar [112–224 g $(380\text{-L})^{-1}$, Safari®] or soil drench application [680 g $(380\text{-L})^{-1}$, Safari®].

The toxicological and environmental profile of **4** is favorable, which includes a low mammalian, avian and aquatic toxicity. The product is available in different formulations: (a) as 2% granule for use in paddy rice nursery boxes (Oshin®), (b) as 0.5% dust for foliar rice applications (Starkle®, Phantom®), (c) as 1% granule for soil incorporation in vegetables (Alubarin®), and (d) as 20% soluble granule for foliar applications to fruit and vegetables (Safari®).

29.2.1.6 Open-chain Compounds versus Ring Systems

There are numerous examples of isosterism between open-chain and ring systems among bioactive molecules [68]. In comparison to the corresponding five- and six-membered ring systems (Chapters 29.2.2 and 29.2.3, respectively), the open-chain compounds exhibit similar broad insecticidal activity, forming a so-called quasi-cyclic conformation when binding to the insect nAChR [69].

Generally, the open-chain neonicotinoids are less lipophilic than the corresponding neonicotinoids with a ring structure (Chapters 29.2.2 and 29.2.3). Based on CoMFA results, a binding model for imidacloprid (**11**) has been described.

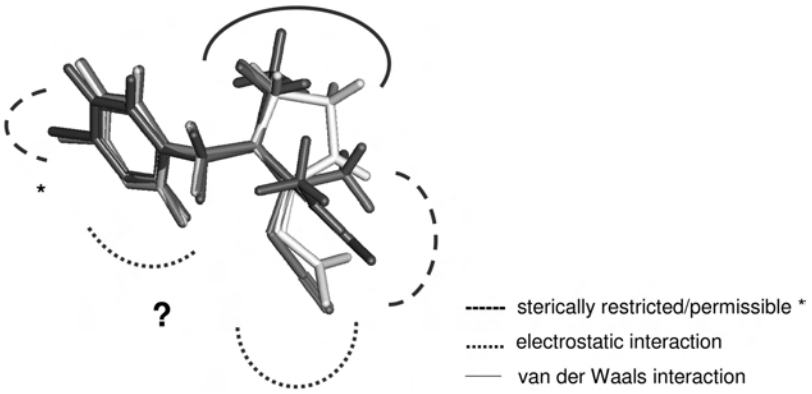


Fig. 29.2.1.1. Stable conformations of open-chain neonicotinoids (**1**, **2**), the five-membered ring system imidacloprid (**11**) and predicted properties of their binding site (Okazawa et al., 2000) [72].

This model clarified that the nitrogen of the CPM moiety interacts with a hydrogen-donating site of the nAChR, and that the nitrogen atom at the 1-position of the imidazolidine ring interacts with the negatively charged domain [70, 71]. The binding activity of open-chain structures (e.g., **1**, **2** and related compounds) to the nAChR of houseflies was measured and the results were analyzed using CoMFA. Superposition of stable conformations of open-chain neonicotinoids such as **1** and **2** as well as the five-membered neonicotinoid **11** showed that the preferred regions for negative electrostatic potentials are near the oxygen atoms of the *N*-nitro group (Fig. 29.2.1.1).

On the other hand, the sterically forbidden regions beyond the imidazolidine 3-nitrogen atom of **11** is important for binding [72]. The area around the 6-chloro atom of the CPM moiety was described as a sterically permissible region. Apparently, the steric interactions were more important for open-chain neonicotinoids than ring systems.

Generally, the nitrogen-containing hetarylmethyl group as *N*-substituent, such as CPM (**11**, **1**, **2** and **12**) and CTM (**3**, **17**, **18**), has a remarkably strong influence on the insecticidal activity of open-chain neonicotinoids as well as ring systems. In comparison with both groups, replacement by the isosteric TFM group (e.g., **4**) resulted in the in a markedly weaker H-bond acceptor. Atom-based alignments of open-chain neonicotinoids such as **2**–**4** as well as the ring system **11** demonstrate that the (*RS*)-(±)-tetrahydrofur-3-yl ring of **4** is more or less perpendicular to the heteroaromatic ring systems of the other neonicotinoids [73].

Finally, it was also shown that the open-chain neonicotinoids bind to the nAChR recognition site in a similar manner to ring structures like **11**, and that the electrostatic properties of the open-chain neonicotinoids and cyclic imidazolidine structures affected their binding affinity.

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29.2.2
Five-membered Compounds – Imidacloprid and Thiacloprid

Peter Jeschke and Koichi Moriya

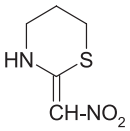
29.2.2.1
Introduction

Two commercial neonicotinoids containing five-membered ring systems belong to this group, imidacloprid (1) and thiacloprid (9).

29.2.2.2
Imidacloprid

In 1984, the discovery of the five-membered neonicotinoid imidacloprid (**1**) (see Scheme 29.2.2.1 below) (1991, Bayer CropScience) [1, 2, 3, 4, 5, 6, 7, 8] was a result of a search for improved activity by altering the structure of the originally announced six-membered nithiazine (**2**) [9, 10, 11, 12]. Because of the photolabile 2-nitromethylene group, **2** was never commercialized for broad agricultural use. In the early 1980s chemists at the subsidiary company of Bayer in Japan (Nihon Tokushu Noyaku Seizo K. K., now Bayer CropScience) started synthesis work on the basis of this lead structure **2** once again. By introducing an *N*-containing heteroaryl-methyl group (e.g., CPM) as substituent of the 2-nitromethylene-imidazolidine five-membered ring system (NTN32692, X-Y = CH-NO_2; see Fig. 29.2.1 in Chapter 29.2) [13], the insecticidal activity could be enhanced remarkably. After preparation of about 2000 compounds, **1** emerged from this program and it was selected for commercial use based on its insecticidal potential, photo-

stability and long-lasting effect in greenhouse and field conditions and its good systemic properties [14].



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As the first member of the CNI family it has become the most successful, highly effective and largest selling insecticide worldwide for agricultural use (registered in more than 120 countries worldwide and applied to over 140 crops) [15, 16, 17] and for application in non-agricultural field such as termite control (Hachikusan®, Japan; Premise®, cf. USA) [18, 19] as garden professional care product (Merit®, Provado®) [20] or in veterinary medicine as ectoparasiticide (Advantage®, K9 advantix®, Advantix®) [21].

29.2.2.2.1 Chemical Classification and Physicochemical Properties

Water solubility and a low partition coefficient in octanol–water are not influenced by pH values between 4 and 9, at 20 °C (Tables 29.2.2.1 and 29.2.2.2) [22].

The low partition coefficient of **1** indicates that it has no potential to accumulate in biological tissues and, therefore, also not in the food chain. The rapid uptake and translaminar transport of **1** is excellent, as observed in cabbage leaves [23] and in rice and cucumber [24]. Additionally, **1** has a considerable acropetal mobility in xylem of plants. In contrast, its penetration and translocation in cotton leaves was less pronounced, as shown by phosphor-imager autoradiography [25]. This xylem mobility makes **1** especially useful for seed treatment and soil application, but it is equally active for foliar application. Owing to its lack of any acidic hydrogen, the pK_a of **1** is >14 and, therefore, its transport within the

Table 29.2.2.1 Chemical classification of imidacloprid (**1**).

Common name	Imidacloprid
Trade names	Admire®, Akteur®, Alias®, Amigo®, Confidor®, Conidor®, Connect®, El Hombre®, Encore®, Escocet®, Evidence®, Faibel®, Gaucho®, Genesis®, Guang®, Hachikusan®, Imex®, Imicide®, Impower®, Intercept®, Legend®, Lizetan®, Marathon®, Merit®, Muralla®, Pasada®, Pre-Empt®, Premise®, Prescribe®, Provado®, Rapid®, Seed-one®, Tatamida®, Termex®, Trimax®, Trust®, Warrant®, Winner®, Yi Sha®, Yunta®, Zorro® FS 236,3
Development codes	NTN 33893

Table 29.2.2.2 Physicochemical properties of imidacloprid (**1**).

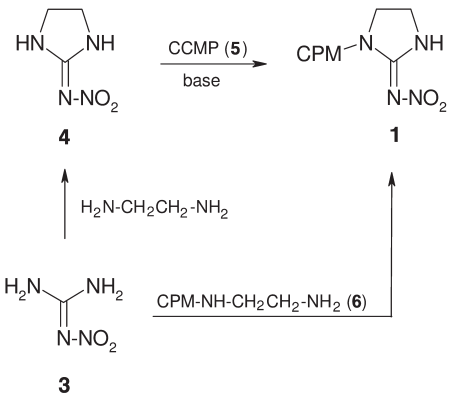
Melting point (°C)	144
Partition coefficient (log P_{OW} at 21 °C)	0.57
Vapor pressure (hPa at 20 °C)	4×10^{-10}
Solubility in water (g L^{-1} at 20 °C)	0.61, no influence of pH
Solubility in organic solvents (g L^{-1} at 20 °C)	Dichloromethane: 67, acetone: 50, methanol: 10, 2-propanol: 2.3, toluene: 0.68, *n*-hexane: <0.1
Dissociation constant pK_a (at 20 °C)	Not determined

phloem is unlikely [26, 27]. Its systemic properties have been examined using ^{14}C-labeled **1**.

The metabolism of **1** is strongly influenced by the method of application [28, 29, 30]. From the results of soil metabolism studies it was found that **1** is completely degradable to carbon dioxide and will not persist in soil. Under standard laboratory conditions the aerobic degradation of **1** is described with a half-life (DT_{50}) of 156 days.

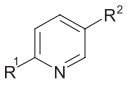
29.2.2.2.2 Chemistry

The first laboratory synthesis of (**1**) was carried out by *N*-alkylation of the 2-*N*-nitro-imidazolidine system (**4**) [31], obtained by cyclocondensation of *N*-nitro-guanidine (**3**) [32] and ethylenediamine, with 6-chloro-3-chloromethyl-pyridine (**5**, CCMP) (Scheme 29.2.2.1).



Scheme 29.2.2.1. Synthetic pathway for preparation of imidacloprid (**1**).

Table 29.2.2.3 Selection of synthetic pathways for preparation of the key intermediate CCMP (**5**).

Precursor R^1, R^2 	Reaction conditions	Ref.
Cl, CH_3	(a) AIBN, Cl_2; (b) *tert*-BuOCl, *hv*; (c) CCl_4, K_2CO_3	(a) WO 9841504 (**1998**), Bayer AG CN 1517338 A (**2004**), Tianjin Univ. DE 4016175 A1 (**1991**), Bayer AG; (b) L. Hu et al., *Huaxue Shijie* **1998**, 20, 313–316; (c) DE 3630046 A1 (**1988**), Bayer AG
SO_2-Ph, CH_3	AIBN, $SOCl_2$	WO 9626188 A1 (**1996**), Sagami Chem. Res. Center
Cl, CH_2NH_2	(a) NOCl, HCl; (b) aq. $NaNO_2$, HCl	(a) EP 632025 A1 (**1995**), Bayer AG; (b) JP 05178835 A2 (**1993**), Koei Chem. Co.
Cl, CH_2OH	(a) PCl_3, $POCl_3$; (b) $SOCl_2$	(a) EP 569947 A1 (**1993**), Lonza A. G.; (b) B. Latli, J. E. Casida, *J. Lab. Compd. Radiopharm.* **1992**, 31, 609–613
OCH_3, CH_2OCH_3	(a) $POCl_3$:PCl_5 (1:2)	EP 393453 A2 (**1990**), Bayer AG
Cl, COOH		I. Cabanal-Duvillard et al., *Heterocycl. Commun.* **1999**, 5, 257–262
Cl, Cl_3	(a) Zn powder or (b) Sn powder, aq. HCl	(a) JP 05320132 A2 (**1993**), Koei Chem. Co.; (b) EP 512436 A1 (**1992**), Ishihara Sangyo Kaisha, Ltd.
OH, CHO		EP 373463 A2 (**1990**), Bayer AG
O-alkyl, CHO		EP 373464 A2 (**1990**), Bayer AG

Numerous syntheses have been studied to develop practical and economical processes for the key intermediate **5** based on different commercially available starting materials as outlined in Table 29.2.2.3.

As an alternative technical process, **3** can be treated with *N*-(6-chloro-pyrid-3-ylmethyl)-ethylendiamine (**6**, PEDA) [33, 34, 35] to give **1** in good yield (Scheme 29.2.2.1).

Crystallographic analysis of **1** revealed a coplanar relationship of the five-membered imidazolidine ring to the *N*-nitroimino group at the 2-position [36, 37]. An intramolecular H-bond between ^{1}NH and O_2N-N=C^2 was confirmed by NMR techniques and the infrared (IR) spectrum (highly chelated ^{1}NH absorption) [38]. Investigations were also carried out using comparative molecular field analysis (CoMFA) [39, 40]. The deduced electron deficiency of the nitrogen atom

of **1** was proved explicitly by ^{15}N NMR spectroscopic measurements [41]. Tomizawa et al. [42] calculated by the MNDO method combined with the PM3 method (*semi*-empirical molecular orbital technique for calculating electronic structure) that the *N*-nitro group of **1** is much more important for binding at the receptor than the bridgehead nitrogen, which was only marginally positive. The important contribution of this *N*-nitro group and its H-bondable property for the insecticidal activity had already been predicted by Kagabu [43].

29.2.2.2.3 Efficacy on Target Pests and Application Rates

The neonicotinoid **1** is characterized by its extremely high intrinsic insecticidal potency, and excellent systemic properties. The uptake of the active ingredient via the roots is an important prerequisite for soil-directed application, e.g., via irrigation systems (drench), in-furrow-application, granular application or seed treatment [44, 45]. Therefore, imidacloprid (**1**) can be used as seed dressing (Gaucho®) [46] as well as foliar, soil treatment such as by irrigation, as granules (Admire®), e.g., seedling-box application in rice [47] or as plant rodlets (Provado®) or compacts (e.g., Confidor®, Admire®). Furthermore, plants or plant parts (e.g., stem) can be applied with **1** by spray, wettable powder (Admire®) pelleting, implantation, dipping, injection and painting. These methods have led to a more economic and environmentally friendly use of **1**.

Imidacloprid (**1**) has a broad spectrum of activity, a good long-lasting effect and plant compatibility. The main pest controlled by **1** (as Confidor®) are a wide range of sucking insects, e.g., aphids, whiteflies, plant- and leafhoppers (jassids), thrips (except certain *Frankliniella* spp.), scales, mealybugs, plant bugs and psyllids, including those already resistant to conventional insecticides (Tables 29.2.2.4 and 29.2.2.5) [48, 49].

Many of the sucking insects are known to be vectors of plant viruses, e.g., aphids [50], whiteflies, thrips and leafhoppers, or can transmit bacterial diseases (phytoplasma), e.g., leafhoppers and psyllids. Neonicotinoids such as **1** imidacloprid (1), **7** thiamethoxam (7) (Chapter 29.2.3) and **8** dothiomidin (8) (Chapter 29.2.1) can control important vectors of virus diseases, thereby impairing the secondary spread of viruses in various crops. Seed treatments provided highly efficient activity in controlling the barley yellow dwarf virus (BYDV) vectors *Rhopalosiphum padi* and *Sitobion avenae* and the subsequent infections [51, 52, 53]. Sugar beet seed pelleted with **1** [51] also protected especially against infections of beet mild yellow virus transmitted by the peach potato aphid (*M. persicae*) [52].

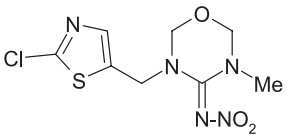


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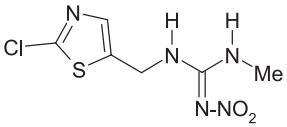
Table 29.2.2.4 Insecticidal efficacy of imidacloprid (**1**) after foliar application against Homoptera pest insects under laboratory conditions. (Adapted from Ref. [49].)

Pest species	Developmental stage	LC_{95}, rounded (ppm)
Homoptera		
Aphis fabae	Mixed	8
Aphis gossypii	Mixed	1.6
Aphis pomi	Mixed	8
Brevicoryne brassicae	Mixed	40
Myzus persicae	Mixed	1.6
Myzus persicae (tobacco)	Mixed	8
Phorodon humuli	Mixed	0.32
Laodelphax striatellus	3rd Instar	1.6
Nephotettix cinctiteps	3rd Instar	0.32
Nilapavarta lugens	3rd Instar	1.6
Sogatella furcifera	3rd Instar	1.6
Pseudococcus comstocki	Larvae	1.6
Bemisia tabaci	2nd Instar	8
Trialeurodes vaporariorum	Adult	40
Hecinothrips femoralis	Mixed	1.6

Table 29.2.2.5 Insecticidal efficacy of imidacloprid (**1**) after foliar application against Coleoptera and Lepidoptera pest insects under laboratory conditions. (Adapted from Ref. [49].)

Pest species	Developmental stage	LC_{95}, rounded (ppm)
Coleoptera		
Leptinotarsa decemlineata	2nd Instar	40
Lema oryzae	Adult	8
Lissorhoptrus oryzophilus	Adult	40
Phaedon cochleariae	2nd Instar	40
Lepidoptera		
Chilo suppressalis	1st Instar	8
Helicoverpa armigera	2nd Instar	200
Plutella xylostella	2nd Instar	200
Heliothis virescens	Eggs	40
Spodoptera frugiperda	2nd Instar	200



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Imidacloprid (**1**) provides additional control of *Coleopteran* spp. (e.g., rice water and tobacco weevil, Colorado potato beetle, rice leaf beetle, wireworms, grubs and other soil beetles) and *Dipterans* spp. (e.g., fruit fly, beet fly, bean and onion fly) and of selected micro-lepidopteran species (e.g., citrus, apple and potato leaf miner), ants (*Hymenoptera* spp.), termites (*Isoptera* spp.), cockroaches, grasshoppers and crickets (*Orthoptera* spp.) [53].

Several combination products of **1** with other insecticides and fungicides have been developed over the years for foliar and soil treatment on a wide range of crops: e.g., Confidor® Supra (100 EC, **1**+cyfluthrin), Leverage® (324 SC, **1**+cyfluthrin), Imprimo® or Montur® (**1**+tefluthrin), Chinook® (**1**+beta-cyfluthrin), Favilla® (23% WP, **1**+methiocarb), Monceren® Star (50 WP, **1**+pencycuron), Nemacur® Multi (246 SC, **1**+fenamiphos; mainly for greenhouse application at planting), WinAdmire® (6 GR, **1**+carpropamid), Camena® (4 GR, **1**+carpropamid), BeamAdmire® (6 GR, **1**+tricyclazole), Gaucho® Blé (**1**+bitertanol+anthraquinone) or Gaucho® Orange (**1**+tebuconazole+triazoxide).

Apart from the direct insecticidal activity of **1** it posses several sublethal side effects as well. The effects are sometimes dosage dependent and include repellency, reduction of cessation of feeding, reduction or cessation of reproductive activities, overall reduction of movement or activity, and increased susceptibility to biological control. The excellent anti-feeding effects of **1** results in less time for transmission and shorter xylem contact so that the number of infections per time units is reduced considerably.

Furthermore, **1** is harmless to many beneficial organisms, like predacious mites and spiders independent of the application method employed. Therefore, **1** is a suitable product for IPM (Integrated Pest Management).

The metabolism of **1** is strongly influenced by the method of application [54]. Depending on time and plant species **1** is degraded more or less completely, as comparative studies in many field crops have revealed [55].

29.2.2.3 Thiacloprid

In connection with the excellent biological performance and market acceptance of imidacloprid (**1**), a further, extensive research and development program led to the discovery and development of the five-membered neonicotinoid thiacloprid (**9**) (2000, Bayer CropScience) [56, 57], the second member of the CNI family.

Table 29.2.2.6 Chemical classification of thiacloprid (**9**).

Common name	Thiacloprid
Trade names	Alanto®, Bariard®, Biscaya®, CaLypso®, Monarca®
Development codes	YRC 2894

Similar to **1**, this neonicotinoid also contains the CPM residue attached to the cyclic 2-(*N*-cyanoimino)-thiazolidine (**11**, CIT) [58] moiety.

29.2.2.3.1 Chemical Classification and Physicochemical Properties

Once applied on leaves, thiacloprid (**9**) is stable towards hydrolysis even under conditions of heavy rain und sunlight, providing sufficient plant-uptake of the substance by a continuous penetration of the active ingredient into the leaf. The half-life in water at pH 5, 7 and 9 is over 500 hours (Tables 29.2.2.6 and 29.2.2.7).

Photolysis in water (buffered at pH 7) shows a half-life of >100 days. On oil surfaces **9** is also stable under sunlight irradiation. Because of the single peak maximum at 242 nm in the ultraviolet (UV) spectrum, **9** has better photostability than other neonicotinoids.

The penetration and translocation behavior of [^{14}C]-**9** [59] in cabbage is comparable that those reported for imidacloprid (**1**). Translaminar and acropetal aphicidal efficacy clearly confirmed that **9** can be systemically translocated. The

Table 29.2.2.7 Physicochemical properties of thiacloprid (**9**).

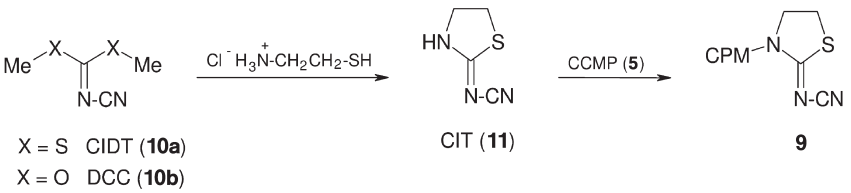
Melting point (°C)	136
Partition coefficient (log P_{OW} at 20 °C)	1.26
Vapor pressure (hPa at 20 °C)	3×10^{-12}
Solubility in water (g L^{-1} at 20 °C)	0.185 (not influenced by pH in the range pH 4–9)
Solubility in organic solvents (g L^{-1} at 20 °C)	Dichloromethane: 160, dimethyl sulfoxide: 150, acetone: 64, acetonitrile: 52, ethyl acetate: 9.4, propan-2-ol: 3.0, 1-octanol: 1.4, xylene: 0.3, *n*-heptane: <0.1
Dissociation constant pK_a (at 20 °C)	No acidic or basic properties in aqueous solutions; not possible to specify dissociation constants for water

visualization of the translocation pattern of [^{14}C]-**9** revealed xylem-mobility, i.e., translocation of **9** and in an upward direction, only one day after application to cabbage leaves.

The metabolic pathway of the systemic **9**, in quantitative and also qualitative terms, is similar in all crops (fruiting crops, cotton) investigated [60]. Its degradation in soil under aerobic conditions occurs rapidly with a mean half-life (DT_{50}) of approx. 16 days (9–27 days, depending on soil type). The anaerobic aquatic metabolism pathway is very similar, but slightly slower than the aerobic one. In a water-sediment system it is completely degraded to carbon dioxide [61]. Generally, hydrolysis, oxidation and conjugation are the main degradation steps of **9** [62].

29.2.2.3.2 **Chemistry**

Thiacloprid (**9**) can be synthesized by a simple, convergent one-step technical process starting from two key intermediates, CIT (**11**) and CCMP (**5**) [63, 64] according the Scheme 29.2.2.2.



Scheme 29.2.2.2. Synthetic pathway for preparation of thiacloprid (**9**).

The first building block, the commercially available **11**, is synthesized by a base-catalyzed cyclization reaction of *N*-cyano-dimethyldithioimidocarbonate (**10a**, CIDT) [65, 66] or *N*-cyano-dimethyl-imidocarbonate (**10b**, DCC) [67] with cysteamine hydrochloride. From the technical process for **1**, several attractive synthetic routes are described for the preparation of **5** (Table 29.2.2.3). Finally, the technical process is readily available by *N*-alkylation of **11** with **5**.

Thiacloprid (**9**) crystallizes in two different modifications depending on the solvent. The neonicotinoid crystallizes from dichloromethane as form I (mp = 136 °C) and from *iso*-propanol as form II (mp = 128.3 °C). From its physicochemical data, the technically active ingredient is form I. With regard to the configuration of the pharmacophore [-N-C(S)=N-CN], **9** exists only in the (*Z*)-configuration in both forms, I and II. In conjunction with X-ray analysis, **9** forms in solution exclusively the stable (*Z*)-configuration. Molecular modeling studies (force-field methods, MMFF94s) have shown that the (*Z*)-configured **9** is about 4 kcal mol^{-1} lower in energy than the (*E*)-isomer. The preference for the (*Z*)-configuration stems mainly from steric reasons. Quantum chemical calculations show that

the strong delocalization of the C=N double bond does not reduce the so-called ''double bond character'' significantly.

29.2.2.3.3 Efficacy on Target Pests and Application Rates

Thiacloprid (**9**) has been developed especially for foliar application and is applied in rates from 48 to 180 g-a.i. ha^{-1} up to three times per season depending on target crop. As standard formulation the suspension concentrate (480 SC CaLypso® or Alanto®) is used. In addition, water dispersible granules (WG 30 and 70 Bariard®) and an oil dispersion (240 OD, Bariard®) have been developed, which give a stable spray solution as well. The CNI **9** possesses a good systemic but also very stomach and good contact properties combined with relatively low rates of application, superior plant compatibility in different crops (e.g., canola, cereals, cotton, fruits, potatoes, rice, ornamentals and vegetables) and a favorable ecotoxicological profile [68]. The spectrum of activity of **9** covers three groups of target pests [69]:

1. The ''traditional'' insects from the CNI spectrum such as aphids, whiteflies, some thrips and beetles such as the rice water weevil from rice (*Lissoropterus oryzophilus*) and the apple weevil (*Anthonomus pomorum*) and micro-lepidopterans such as *Phyllocnistis citrella* in citrus.
2. The ''traditional'' insects from the CNI spectrum; however, with comparatively lower dosages than, i.e., **1** in the control of Colorado potato beetle (*Leptinotarsa decemlineata*) and some leafminers such as *Lithocolletis blancardella* and *Lyonetia clerkella*.
3. A completely new spectrum of control of the lepidopteran pests tortricides such as *Cydia pomonella* and *Cydia molesta* in pome and stone fruit as well as coleopterans such as *Anthonomus grandis* in cotton and *Mehligetes eaneus* in rape. Furthermore, **9** has an excellent efficacy in controlling *Diptera* spp. such as *Rhagolethis cerasi*, *Dacus oleae* and *Ceratitis capitata* in fruit crops like peaches and olives.

Table 29.2.2.8 summarizes the activity of **9** against important agricultural pests after leaf-dip application [70].

Thiacloprid (**9**) shows excellent performance against first and second instar larvae of codling moth (*C. pomonella*), which are the stages most likely to be exposed to spray coverage in a real field situation. The speed of its action against these most susceptible larval instars is remarkable. Even the lowest tested spray concentration of 8 ppm resulted in 100% affected larvae after 4 hours of exposure (cf. short period of hyperexcitation and total paralysis of the larvae), whereas more practical concentrations of 40 and 200 ppm resulted in 100% affected larvae after only 60 and 120 min, respectively. In addition, **9** (CaLypso®) controls freshly laid eggs; the optimal time for spray-application is between the beginning and the peak of egg-laying. Therefore, in comparison to IGRs, it has a wider application

Table 29.2.2.8 Activity of thiacloprid (**9**) against important agricultural pests after leaf-dip application.

Pest species	LC_{50} (mg-a.i. L^{-1})	Confidence limit 95%
Myzus persicae, mp[a]	1.5	1.4–1.7
Aphis fabae, mp	0.8	0.7–0.9
Aphis fabae, mp	≤0.6	–
Aphis gossypii, mp	0.8	0.7–0.9
Bemisia tabaci, mp	1.1	0.3–2.4
Nephotettix cincticeps, L2	0.6	0.5–0.7
Cydia pomonella, L2,3	1.1	0.8–1.4
Phaedon cochleariae, L2	18.5	15.9–21.6
Lissorhoptrus oryzophilus, ad	1.8	1.2–2.7

[a] Soil application, LD_{95}; mp = mixed population, L = larval stage, ad = adult.

window and allows more flexible application timing, which offers unique benefits in fruit farming. The ovicidal efficacy of **9** after spray application against eggs of *C. pomonella* is excellent (Table 29.2.2.9).

Another outstanding advantage is based on the fact that **9** has no effect on pollinating insects such as honey- and bumble bees or parasitic wasps, which allows its application not only before and after but also during the flowering period of fruit crops [71]. Consequently, as **9** does not disturb the predator–prey equilibrium, it is ideal for use in IPM programs as well (see also imidacloprid in Chapter 2.3) [72].

Several combination products of **9** have been developed for foliar treatment, e.g., the suspo-emulsion marketed as Monarca® (SE 112.5, **9**+beta-cyfluthrin) and Proteus® (**9**+deltamethrin). The latter is based on the new O-TEQ® formulation, which increased the penetration of the active ingredients remarkably.

Besides the formulations for foliar application, granules for rice seedling box application have been developed, 1.5 GR and 4.5 GR CaLypso®, and the combination product WinBariard® (5.5 GR, **9**+carpropamid).

Table 29.2.2.9 Ovicidal activity of thiacloprid (**9**) after spray application against eggs of codling moth (*C. pomonella*).

Concentration (ppm)	Number of eggs at day 2	Number of hatched larvae
40	41	0
200	80	0
Control	32	32

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29.2.3
Six-membered Heterocycles (Thiamethoxam, AKD 1022)

Peter Maienfisch

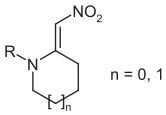
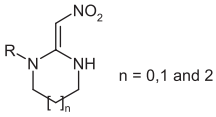
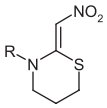
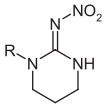
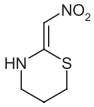
29.2.3.1
Introduction

Neonicotinoids are one of the more recent highlights in the area of insect control. This chapter reviews the discovery, chemistry and properties of six-membered neonicotinoids. The most prominent representatives of this subclass are nithiazine, AKD-1022 and thiamethoxam. Nithiazine has served as lead structure for the discovery of the neonicotinoid sales products and thiamethoxam is the only six-membered neonicotinoid entering the market-place.

29.2.3.2
History of Six-membered Neonicotinoids

Researchers at Shell discovered the chemical class of the neonicotinoids (see Chapters 29.1 and 29.2). The first representatives of this novel class of insecticides, including six-membered saturated nitromethylene heterocycles were described in 1973 [1]. Their patent application depicted the synthesis and insecticidal activity of 2-nitromethylene-piperidines (**1**, Table 29.2.3.1). Compounds of type **1** were claimed to possess good activity against houseflies, pea aphids, corn earworm, mosquito larva and cabbage loopers. A second patent application [2], filed on the same date, revealed the insecticidal activity of the corresponding five-membered ring analogues, the 2-nitromethylene pyrrolidines **2**, and indicated that five- and six-membered nitromethylenes possess similar insecticidal activity. Further inventions made by Shell cover 2-nitromethylene-1,3-diazacycloalkanes such as the imidazolidine, pyrimidine and diazepine nitromethylenes of type **3** [3], thiazines **4** [4], and most importantly also nitroguanidines of the general structure **5** [5].

Table 29.2.3.1 Inventions made by Shell in the early 1970s.

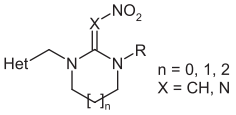
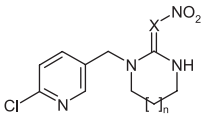
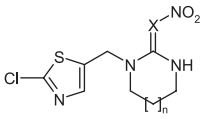
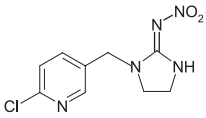
Compound type	General structure	Patent application	Publication year	Ref.
1 ($n = 1$)	 n = 0, 1	DE 2321523	1973	1
2 ($n = 0$)		DE 2321522	1973	2
3	 n = 0,1 and 2	DE 2445421	1975	3
4		FR 2270251	1975	4
5		US 4297496	1981	5
Development compound				
6 (Nithiazine)		FR 2270251	1975	4

Shell's pioneering work [6–8] in the 1970s on insecticidal nitromethylene heterocycles led to the invention of the nitroenamine and nitroguanidine pharmacophores and cumulated in the discovery of nithiazine (**6**), a compound that has never been commercialized as a crop protection agent but has been for animal health use. This was mainly due to rapid degradation under both hydrolytic and photochemical conditions [7] as well as its limited potency as an insecticide in crop protection.

However, this compound has served as a neonicotinoid lead structure. Thirteen years later Nihon Tokushu Nohyaku (a subsidiary of Bayer AG in Japan) achieved, with the synthesis of nitromethylene and nitroguanidine derivatives of imidazolidines, perhydro-pyrimidines, and diazepines of type **7** (Table 29.2.3.2), an important breakthrough in this chemistry [9, 10].

The extremely high insecticidal activity of neonicotinoids of the imidacloprid-type **7** (Chapter 29.2.1) triggered extensive research activities within several other companies; Ciba-Geigy (later Novartis, now Syngenta), Takeda, Nippon Soda,

Table 29.2.3.2 Inventions made by Nihon Tokushu Noyaku/BayerAG (only a selection of patent applications with regard to six-membered heterocycles).

Compound type	General structure	Patent application	Publication year	Ref.
7	 n = 0, 1, 2 X = CH, N	EP 163855 EP 192060	1986 1986	9 10
	Examples			
	 X = CH, N n = 0,1 and 2	 X = CH, N n = 0,1 and 2		
Sales product				
8 (Imidacloprid)		EP 192060	1986	10

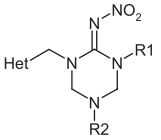
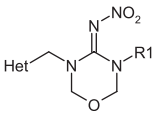
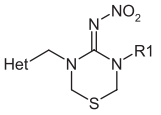
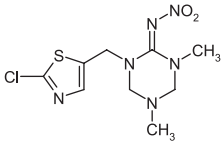
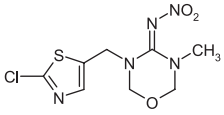
Agro Kanesho, Mitsui Chemicals, and others immediately entered this promising research area [11–13]. All these companies started to investigate some novel structural modifications. At that time, little was known on the influence of the nitroimino-heterocycle on the biological activity. Consequently, compounds possessing an additional heteroatom in the nitroimino-heterocycle were designed, synthesized and patent applications filed [14–21] (Table 29.2.3.3).

Research on six-membered neonicotinoids described above yielded three development compounds: nithiazine (**6**), AKD-1022 (**12**) and thiamethoxam (**13**), the latter being the only one to enter the market-place as an agricultural insecticide.

29.2.3.3 Biological Activity and Structure–Activity Relationship

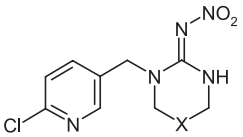
Compounds of types **9–11** provide good control of a broad range of commercially important pests, such as aphids, whiteflies, thrips, rice hoppers, Colorado potato beetle, flea beetles, wireworms, leaf miners as well as some lepidopterous species. The influence of the nitroimino-heterocycle on the biological activity has been studied in the greenhouse with compounds **14–16** [22]. These bioassays revealed

Table 29.2.3.3 Novel six-membered nitroimino-heterocycle with an additional heteroatom.

Compound type	General structure	Company	Patent application	Publication year	Ref.
Triazinane (hexahydro-1,3,5-triazines)					
9		Nihon Tokushu Noyaku/ Bayer	EP 386565	1990	14
		Nippon Soda	O 9101978	1991	15
		Agro-Kanesho	EP 428941	1991	16
		Ciba-Geigy	EP 483055	1992	17
		Ciba-Geigy	EP 483062	1992	18
Oxadiazinanes (hexahydro-1,3,5-oxadiazines)					
10		Ciba-Geigy	EP 580553	1994	19
		Nihon Tokushu Noyaku	JP 07224062	1995	20
		Mitsui Toatsu Chemicals	JP 08291171	1996	21
Thiadiazinanes (hexahydro-1,3,5-thiadiazines)					
11		Nihon Tokushu Noyaku	JP 07224062	1995	20
Development compound and sales product					
12 (AKD-1022)		Agro-Kanesho	EP 428941	1991	16
13 (Thiamethoxam)		Ciba-Geigy	EP 580553	1994	19

that among these compounds, the 4-nitroimino-1,3,5-oxadiazinane **15** exhibits clearly better insecticidal activity than the 2-nitroimino-1,3,5-triazinane **14** and the 4-nitroimino-1,3,5-thiadiazinane **16** and that its potency is close to imidacloprid (**8**), and its six-ring analogue **17** (Table 29.2.3.4).

Table 29.2.3.4 Insecticidal activity of six-membered neonicotinoids **14–16** compared with imidacloprid (**8**) and its six-ring analogue **17**.

Compound	Structure type	LC80 (mg-AI L^{-1})		
		***Aphis craccivora*** **m.p. Pea, foliar spray**	***Myzus persicae*** **m.p. Pea, into water**	***Diabrotica balteata*** **L2 Filter paper, spray**
14	Triazinane (X = NCH_3)	>200	3	200
15	Oxadiazinane (X = O)	50	0.05	3
16	Thiadiazinane (X = S)	200	0.8	12
17	Hexahydro-pyrimidine (X = CH_2)	12	0.2	3
8 (Imidacloprid)	Imidazolidine (X = bond)	12	0.05	0.8

29.2.3.3.1 **Structure–Activity Relationship**

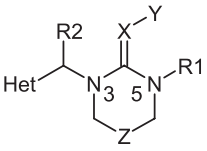
Table 29.2.3.5 shows the general structure–activity profile for six-membered neonicotinoids; the most favorable structural features are highlighted below:

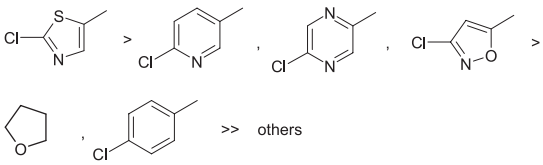
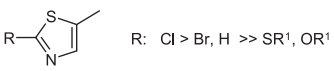
- The nitroguanidine pharmacophore (X-Y = NNO_2) leads to very high insecticidal activity, followed by the cyanoguanidine pharmacophore (X-Y = NCN).
- As pharmacophore "backbone", a 1,3,5-oxadiazinane ring (Z = O) is more favorable than other heterocyclic ring systems.
- The 2-chloro-5-thiazolyl moiety gives better overall insecticidal activity than the 6-chloro-3-pyridyl, which is present in the first generation neonicotinoids, and all the other heterocyclic groups investigated.
- In contrast to the structure–activity relationships in the imidacloprid series, the introduction of a methyl group at N(5) ($R^1 = CH_3$) led to a strong increase of the insecticidal activity.

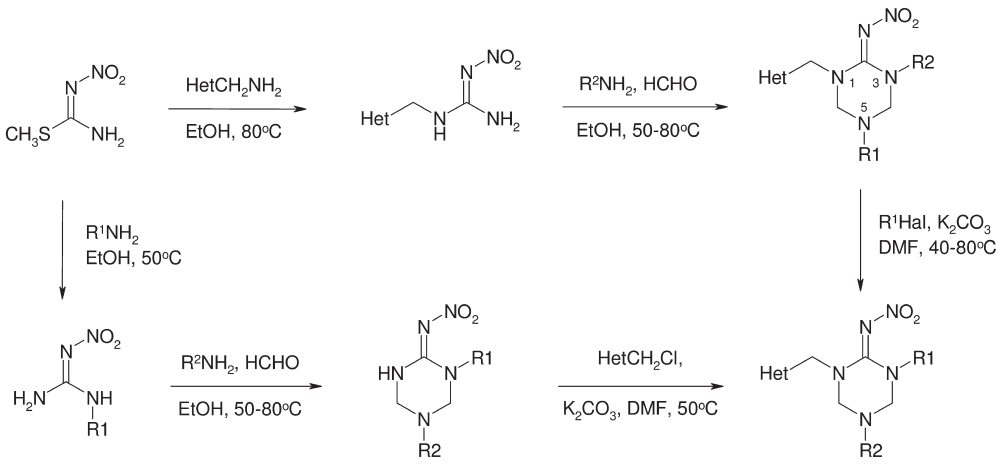
29.2.3.4
Synthesis

General synthetic methodologies involving Mannich-type cyclization reactions as the key step have been developed for the synthesis of the 2-nitroimino-1,3,5-triazinanes **9** [14–18, 22–26] and the 4-nitroimino-1,3,5-oxadiazinanes **10** [19–22, 26, 27] (Schemes 29.2.3.1 and 29.2.3.2). These methodologies allow the introduc-

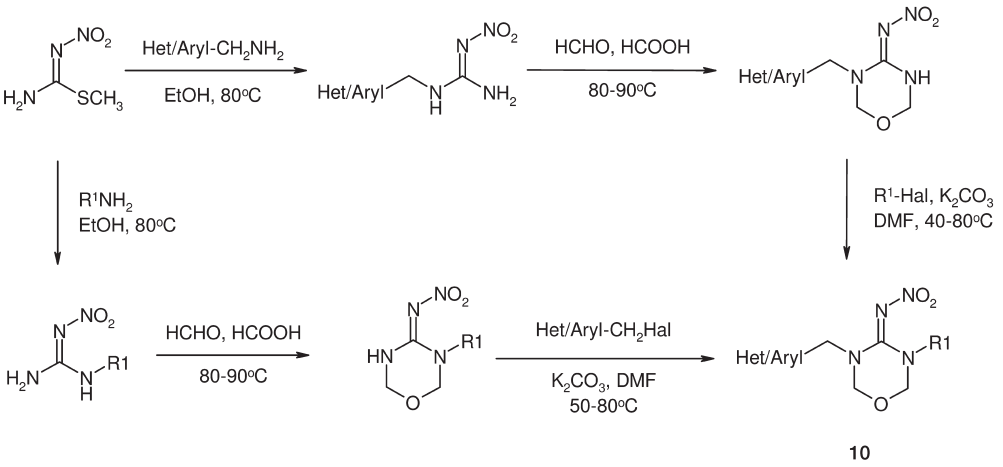
Table 29.2.3.5 Structure–activity profile for six-membered neonicotinoids.



Structural feature		Structure–activity relationship
Pharmacophore	X–Y	N-NO_2 > N-CN ≫ O, S, NH
Pharmacophore backbone	Z	O > N-CH_3, S, CH_2
Pharmacophore substituent	R^1	CH_3 > H > C_2H_5, n-Pr, allyl, propargyl ≫ larger substituents CH_3 > COR′, COOR′, CH_2OR'
Bridge substituent	R^2	H > CH_3 > larger groups
Heterocyclic group	Het	
Chlorothiazolyl as heterocyclic group	Het	



Scheme 29.2.3.1. Synthesis of 2-nitroimino-hexahydro-1,3,5-triazines **9**.

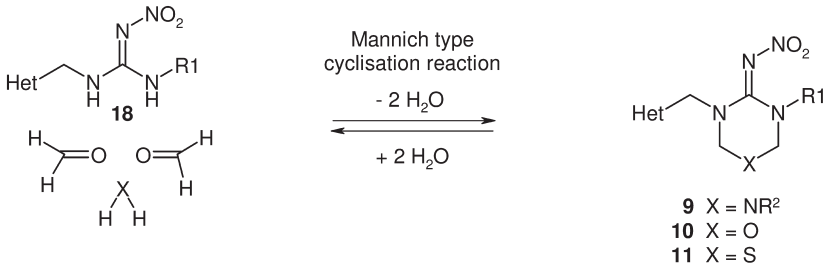


Scheme 29.2.3.2. Synthesis of 4-nitroimino-1,3,5-oxadiazinanes **10**.

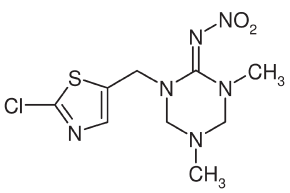
tion of the heterocyclylmethyl group either from a heterocyclylmethyl amine or a heterocyclylmethyl chloride, respectively. Thus, treatment of the readily available *S*-methyl-*N*-nitroisothiourea with amines affords the monosubstituted nitroguanidines in excellent yields, which can be converted into the monosubstituted nitroimino-triazanes or oxadiazinanes, respectively. Alkylation leads then in good to excellent yields to the target compounds **9** and **10**, respectively.

29.2.3.5
Hydrolytic Stability of the Six-membered Nitroimino-heterocycle

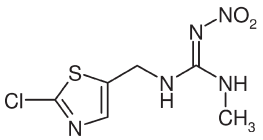
Six-membered nitroimino-heterocycles of type **9–11** are cyclic Mannich adducts containing a bis-aminal structure (Scheme 29.2.3.3). Generally, such Mannich adducts can cleave into their acyclic compounds, depending on the reaction conditions. The hydrolysis (ring cleavage) of **9–11** has been studied in detail [12, 17, 24, 25, 28–33]. Triazinanes **9** and thiadiazinanes **11** decay in a physiological salt solution (pH 7.6) at 25 °C to the corresponding acyclic compounds **18**, whereas the oxadiazinanes **10** are stable under these conditions [33]. In contrast to com-



Scheme 29.2.3.3. Hydrolytic degradation of nitroimino-heterocycles **9–11**.



AKD-1022
12



Clothianidin
19

Fig. 29.2.3.1. Structures of AKD-1022 (**12**) and clothianidin (**19**).

pounds of type **10** (X = O), the cleavage of **9** (X = NR^2) was also observed under acidic as well as basic aqueous conditions [31, 32]. Based on these findings it was concluded that triazinanes **9** could be regarded as hydrolytic proinsecticides of the corresponding acyclic nitroguanidine **18**, but that this prodrug concept does not apply to oxadiazinanes **10** [33].

29.2.3.6 AKD-1022

In the late 1980s Agro Kanesho announced the development of their own neonicotinoid insecticide AKD-1022 (**12** Fig. 29.2.3.1), a representative of the 2-nitroimino-1,3,5-triazinane subclass containing a 2-chloro-5-thiazolyl moiety as heterocyclic group [34]. However, this compound was never commercialized, possibly due the crowded patent situation (see Section 29.2.3.2) or the lack of hydrolytic stability (Section 29.2.3.5). AKD-1022 (**12**) has also been described [35] to possibly be a proinsecticide of the acyclic nitroguanidine clothianidin (**19**) (see also Chapter 29.2.1).

The synthesis of this compound was first described by Agro Kanesho [16]. Further preparations have been discussed in Section 29.2.3.4. As with all neonicotinoids, AKD-1022 (**12**) interacts with nicotinic acetylcholine receptors; however, it is much less potent than imidacloprid (**8**) and other commercial neonicotinoids. In particular, this has been demonstrated with *Myzus* and *Drosophila* membranes [23], as well as on American cockroaches [33]. It has been speculated that AKD-1022 (**12**), as a basic molecule, is ionized in the fluids of insects and, therefore, reaches the synapse only slowly through the lipophilic cuticles and the ion barriers. During retarded movement, the compound is prone to decompose, e.g., due to partial hydrolysis mediated enzymatically and/or non-enzymatically [33]. Therefore, acyclic nitroguanidines such as **19** may also contribute to the insecticidal activity observed in glasshouse and field studies.

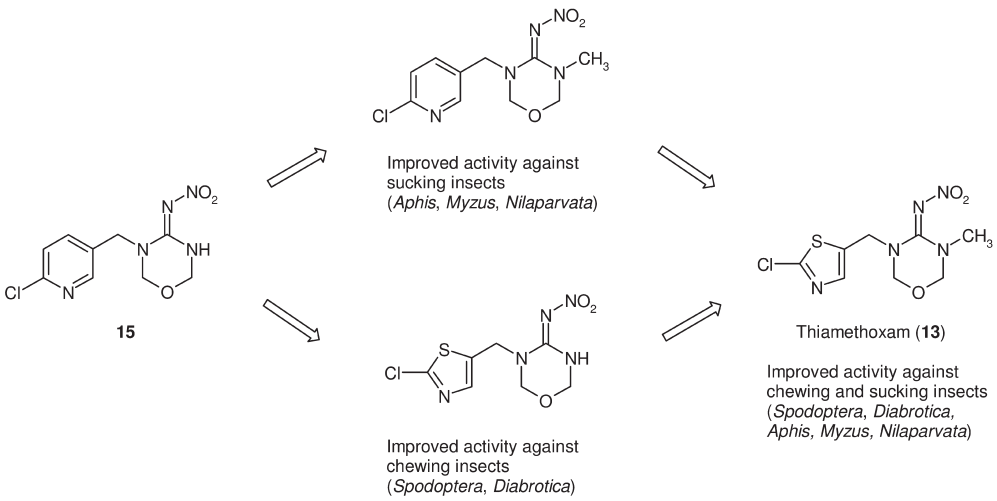
29.2.3.7 Thiamethoxam (CGA 293'343)

Ciba-Geigy (since 1996: Novartis; now: Syngenta) started a research program on neonicotinoids in 1985 that resulted in the discovery of thiamethoxam (**13**) [13,

22]. This compound is a second-generation neonicotinoid and belongs to the thianicotinyl subclass [13]. The combination of an oxadiazine ring with a N-methyl group as pharmacophore substituent is unique and seems to shape the biological properties of thiamethoxam (**13**, CGA 293'343). It was first synthesized in 1991 and has been marketed since 1998 under the trademarks Actara® for foliar and soil treatment and Cruiser® for seed treatment. In all its usages, thiamethoxam (**13**) provides excellent control of a broad range of commercially important pests, such as aphids, whiteflies, thrips, rice hoppers, Colorado potato beetle, flea beetles, wireworms, leaf miners as well as some lepidopterous species [13, 36, 37]. Low use rates, flexible application methods, excellent efficacy, and the favorable safety profile make this new insecticide well suited for modern integrated pest management programs in many cropping systems.

29.2.3.7.1 **Discovery**

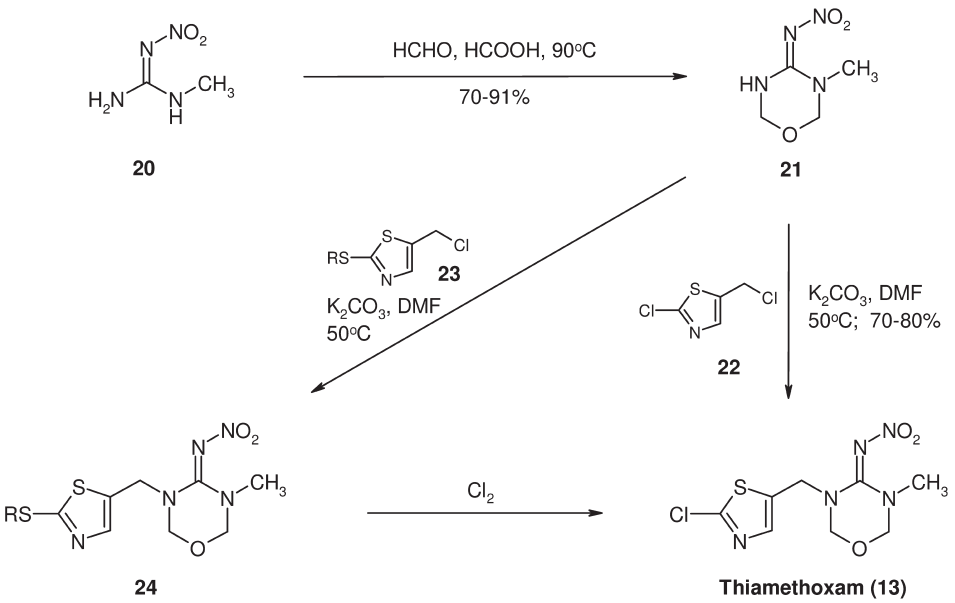
Ciba-Geigy's studies on the influence of the nitroimino-heterocycle on the biological activity led to the 4-nitroimino-1,3,5-oxadiazinane derivatives **10** [13, 22]. In a second optimization cycle the oxadiazinane lead structure **15** was further improved, resulting in the identification of thiamethoxam **13** as the optimum representative of this chemical subclass (Scheme 29.2.3.4).



Scheme 29.2.3.4 Optimization of 4-nitroimino-1,3,5-oxadiazinane lead structure **15**.

29.2.3.7.2 **Synthesis**

Thiamethoxam (**13**) was first synthesized in 1991 [19]. At that time, no practical methods for the preparation of 4-nitroimino-1,3,5-oxadiazinanes were available [22]. After much experimentation, optimized procedures [22, 32, 38–44] were developed (Scheme 29.2.3.5). The key step is the conversion of *N*-methyl-



Scheme 29.2.3.5. Syntheses of thiamethoxam (**13**).

nitroguanidine (**20**) into the oxadiazinane **21** by treatment with formaldehyde in the presence of formic acid [22, 32]. The subsequent alkylation with the thiazole **22** [38, 44–49] in dimethylformamide and potassium carbonate as a base afforded thiamethoxam (**13**) in good to excellent yields. Alternatively, the oxadiazinane **21** can be alkylated with a 2-mercapto-thiazol-5-ylmethyl chloride (**23**) to afford compound **24**, which can then be converted into thiamethoxam (**13**) by chlorination [50–52].

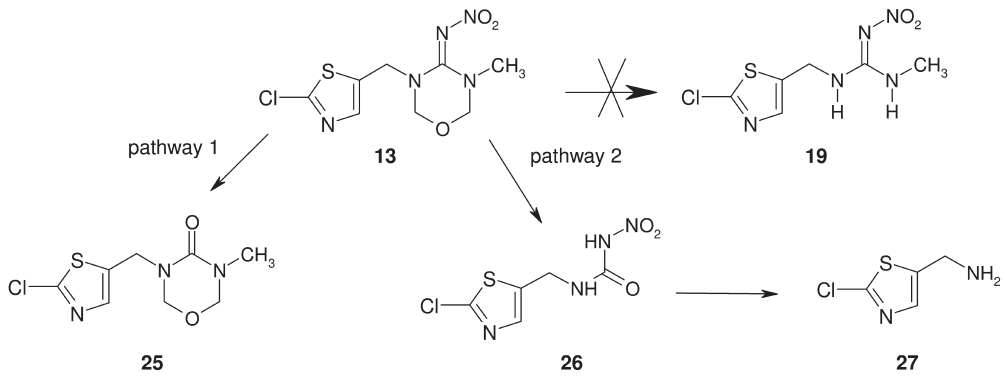
29.2.3.7.3 Chemical and Physical Properties

Table 29.2.3.6 shows the physicochemical properties of thiamethoxam (**13**). Its properties favor rapid and efficient uptake in plants and xylem transport [37, 53]. Through this systemic activity all plant parts situated acropetally from the application site can be protected.

Thiamethoxam (**13**) is hydrolytically very stable at pH 5 (half-life, >1 year at room temperature) and stable at pH 7 (estimated half-life at room temperature, approximately 200–300 days). The compound is, however, more labile at pH 9 (half-life, a few days). Two major degradation pathways were observed in the pH-range 5–9 [32, 54]. The first pathway led to the corresponding 1,3,5-oxadiazinan-4-one **25** and the second pathway to the ring-opened N-nitrourea **26** and then to 2-chloro-5-aminomethyl-thiazole (**27**) (Scheme 29.2.3.6). Hydrolytic cleavage of the 1,3,5-oxadiazinane ring to the corresponding acyclic nitroguanidine **19** (clothianidin) was not observed [32, 54].

Table 29.2.3.6 Chemical and physical properties of thiamethoxam (**13**).

Feature	Property
Melting point	139.1 °C
Vapor pressure at 25 °C	6.6×10^{-9} Pa
Water solubility at 25 °C	4.100 mg L^{-1}
pH	6.84 (saturated solution in water)
Partition coefficient [*n*-octanol/water at 25 °C (log P_{OW})]	−0.13
Dissociation constant	CGA 293'343 has no dissociation within the range pH 2–12
Hydrolysis (estimated half-life at 25 °C)	pH 5: 6990 days pH 7: 152 days pH 9: 6.1 days
Photodecomposition (half-life as droplet deposit on Teflon)	1 h



Scheme 29.2.3.6. Hydrolytic degradation pathways of thiamethoxam (**13**).

Thiamethoxam (**13**) is photolytically rapidly degraded (half-life ~1 h as a droplet deposit on Teflon). No decomposition was observed after storage of the active ingredient or formulations at 54 °C after 2 months. However, at temperatures above 150 °C, exothermic decomposition occurs [13, 37].

In laboratory soils, thiamethoxam (**13**) degrades at moderate to slow rates. The half-life ranges from 34 to 75 days under favorable conditions, but may increase by a factor of three under unfavorable conditions. Under field conditions, degra-

dation is generally faster, because field soils usually have higher microbial activity, and exposure to light is another important degradation pathway [13].

29.2.3.7.4 Mode of Action

Target Sites in the Nervous System Neonicotinoids target nicotinic acetylcholine receptors, as has mainly been demonstrated by studies with imidacloprid (**8**). Compared with imidacloprid and the other neonicotinoid sales products, thiamethoxam (**13**) binds in a different way, possibly to a different site of the receptor in aphids [55–58].

Saturation binding studies revealed the following data for affinity (K_d) and binding capacity (B_{max}) for thiamethoxam **13** and imidacloprid **8** with fresh membranes from *M. persicae* assayed at 2 °C:

Thiamethoxam (**13**): $K_d = 11.4$ nM; $B_{max} = 700$ fmol (mg-protein)$^{-1}$

Imidacloprid (**8**): $K_d = 2.5$ nM; $B_{max} = 1400$ fmol (mg-protein)$^{-1}$.

Non-specific binding was fairly low with both radioligands, typically around 10% with [^{3}H]thiamethoxam (**13**) and 5% with [^{3}H]imidacloprid (**8**).

These data showed that thiamethoxam (**13**), like imidacloprid (**8**) and the other neonicotinoids, binds with high affinity to nicotinic receptors [57]. However, there are clear differences to the other commercial neonicotinoids, as documented by a "kinetic" analysis of competition experiments [56]. While [^{3}H]thiamethoxam (**13**) binds to receptors with nanomolar affinity, micromolar concentrations are required to displace [^{3}H]imidacloprid (**8**). Further, the interaction between the two compounds is "non-competitive", meaning that binding of thiamethoxam (**13**) reduces the binding capacity of the receptor preparation for imidacloprid (**8**) but not its affinity. Thiamethoxam (**13**) shares this unusual mode of inhibition with other neonicotinoids (not commercialized) containing a N-methyl group as pharmacophore substituent [56, 58].

Furthermore, thiamethoxam (**13**) has been found to be highly potent for [^{3}H]epibatidine binding [59] and to act, at least in part, directly on the *Homolodisca* receptor [60]. Kagabu found high neuroblocking activity in American cockroach [33]. The stability, insecticidal and neuroblocking tests performed, revealed (in accordance with Syngenta results [58]) that bioactivation of thiamethoxam is not necessary. These findings do not support Nauen's conclusions [61–63] that thiamethoxam (**13**) is likely to be a neonicotinoid precursor for clothianidin (**19**) and is not active by itself.

In summary, varied and minor structural differences in neonicotinoid molecules may confer diversity in their binding modes, depending upon insect species and may explain the unique receptor binding behavior of thiamethoxam (**13**) [56–58] as well as of dinotefuran [64].

Biological Mode of Action Thiamethoxam (**13**) shows a very rapid action in target insects: Symptoms start 15 to 30 min after uptake in aphids and Colorado potato

Table 29.2.3.7 Recommended foliar and soil applications of thiamethoxam (**13**) (Actara®).

Crops	Target pests	Foliar spray	Soil application	Remarks
Citrus	Leafminers	X	X	Soil application
	Aphids	X	X	as nursery only
	Citrus whitefly	X		
	Mealybugs	X		
	Longicorn beetle	X		
	Soft scales	X		
	Cirtus Psylla	X		
	Brazilian Sharpshooter	X		
Coffee	Coffee leafminer		X	
	Cicades		X	
Cotton	Aphids	X		
	Jassids	X		
	Thrips	X		
	Whiteflies	X		
	Lygus bugs	X		
	Flea hoppers, mirids	X		
Lettuce	Aphids	X	X	
Mango	Mango Hoppers	X		
Pepper/eggplant	Aphids	X	X	
	Whiteflies	X	X	
	Jassids	X	X	
	Pepper weevil	X		
	Tomato bug	X		
	Colorado potato beetle	X	X	
	Thrips		X	
Pome fruits	Aphids	X		Soil application:
	Leafhoppers	X		trunk spray or
	Plum curculio	X		soil drench
	Apple sawfly	X		application
	Apple blossom weevil	X		
	Pear psylla	X		
	Comstock mealybug	X		
	Wooly apple aphid	X		
Potato	Aphids	X	X	
	Colorado potato beetle	X	X	
	Leafhoppers	X	X	
	Diabrotica	X	X	
	Wireworms		X	
	Potato psyllid	X	X	

Table 29.2.3.7 *(continued)*

Crops	Target pests	Foliar spray	Soil application	Remarks
Rice	Hoppers	X	X	Soil application:
	Rice stink bugs	X		seedling box or
	Rice leaf beetle		X	into water
	Rice water weevil		X	application
Soybean and beans	Stinkbugs	X		
	Whiteflies	X		
Sugarcane	Termites		X	
	Sugarcane froghoppers		X	
Tobacco	Aphids	X	X	Soil application:
	Brown tobacco leaf beetle	X	X	post planting
	Tobacco flea beetle	X		drench
	Wireworms		X	application
	Thrips		X	
Tomatoes	Aphids	X	X	
	Whiteflies	X	X	
	Jassids	X	X	

beetle and after 1 hour in whiteflies. Feeding ceases; the sucking insects withdraw their stylets, stretch their legs and move the antennae forwards. Even if the insects die only 24 hours later, the effects are comparable to those of knock-down compounds, since the feeding stop is irreversible and affected insects do not try to penetrate again [65].

29.2.3.7.5 Biological Activity and Use Recommendation

In laboratory tests and under field conditions, thiamethoxam (**13**) shows, after foliar, drench and seed treatment application at very low concentrations, good to excellent activity against Homopteran, Coleopteran and some Lepidopteran pests. Thysanopteran pests are best controlled after drench or seed treatment application [13, 22, 37].

Under field conditions thiamethoxam (**13**) is active at very low rates against many key pests in many crops. Table 29.2.3.7 documents the currently recommended uses after foliar spray and soil application of Actara®, formulated mainly as WG 25, SC 240 or GR 1. The soil applications have been optimized for each use and include soil surface, soil drench, soil drench surface, soil drench soil granule, trunk spray application, seedling box, into water and post transplanting drench application. Additionally, many other crops and non-crop uses are currently under evaluation. These include ornamentals, grapes, cocoa, pineapple, tea, hazelnuts, datepalms, papaya, durian, pecan, cereals, and non-crop uses

Table 29.2.3.8 Recommended seed treatment applications of thiamethoxam (**13**) (Cruiser®).

Crop	Target pests	
Beans	White flies	Leaf hoppers
	Stem borer	Bean leaf beetle
	Root worm	Seed corn maggot
Canola/oilseed rape	Aphids	Saw fly
	Flea beetle	
Cereals	Aphids	Soft scale
	Cereal ground beetle	Coleoptera
	Frit fly	Bugs
	Wireworms	
Corn/maize	Aphids	False wireworm
	Frit flies	White grubs
	Bugs	Root maggot
	Leaf hopper	Grasshoppers
	Black maize beetle	Corn flea beetle
	Cornstalk borer	Maize weevil
	Wireworms	
Cotton	Aphids	Thrips
	Cotton leaf worm	Wireworms
	Jassids	Cotton boll weevil
Peanuts	Thrips	Leaf hopper
Peas	Aphids	Thrips
	Pea weevils	Seed corn maggot
Potato	Aphids	Wireworms
	Colorado potato beetle	Cucumber beetles
	Potato leafhopper	Thrips
Rice	Stemborer	Reen leaf hoppers
	Grasshoppers	Termites
	Rice grain beetle	Cane borer
	Thrips	
Sorghum	Aphids	Grenn bug aphids
	Chinch bug	Wireworm
	Seed corn maggot	
Soybean	Soybean weevil	Bean leaf beetle
	Termites	Thrips
	Whiteflies	Corn stalk borer
Sugar beet	Aphids	Wireworm
	Mangold pygmy beetle	Weevils
	Flea beetle	Leaf hopper
	Maggot fly	

Table 29.2.3.8 *(continued)*

Crop	Target pests	
Sunflower	Aphids Jassida Ground weevil	Coleoptera (white grubs) Wireworm
Stored pest	Rice weevil Indian meal moth	Saw-tooth grain beetle

such as leafcutting ants, termites in pastures, and ants. The recommended rates of active ingredient (a.i.) per hectare vary from crops and pests. In most cases 10–100 g-a.i. ha^{-1} is sufficient to fully control the target pests [13, 36, 37, 66].

As a seed treatment, thiamethoxam (**13**) is highly active against a broad range of soil-dwelling insects. It also offers effective control of a wide range of early-season, leaf-feeding (Coleoptera and Lepidoptera) and sucking insects (Homoptera and Thysanoptera). Owing to its fast action on sucking insects, it also limits the transmission of plant pathogenic viruses (e.g., in cereals and sugarbeets). Table 29.2.3.8 shows the current seed treatment recommendations for Cruiser® in key crops. The rate of active ingredient (a.i.) per 100 kg seeds is generally in the rage 30–400 g-a.i. For potatoes, use rates of 5–7.5 g-a.i. per 100 kg seeds are sufficient [13, 36, 37, 67–70].

29.2.3.7.6 Safety Profile

Mammalian Toxicology (Table 29.2.3.9) Thiamethoxam (**13**) is rapidly and completely absorbed and readily eliminated predominantly as parent through the urine. It has low acute mammalian toxicity when applied to rats either orally ($LD_{50} = 1563$ mg kg^{-1}), dermally ($LD_{50} > 2000$ mg kg^{-1}) or by inhalation [LC_{50} (4 h) = >3720 mg m^{-3}], putting it into WHO hazard class III. Thiamethoxam (**13**) was found non-irritant to skin and eyes and devoid of a skin sensitizing potential [13, 36, 37].

In repeated dose studies in rodents and dogs, liver and kidneys (rat only) were the main target organs. In lifetime rodent studies, only mice showed increased incidences of liver tumors, which were found to be specific to this species [71–73]. They are regarded to be mediated by a non-genotoxic threshold mechanism and of no relevance to man in normal use. Thiamethoxam (**13**) has no mutagenic potential. Reproductive toxicity studies showed no evidence of developmental impairment or teratogenic potential [13, 37]. Applicator and consumer safety are very favorable for the label recommended uses.

Ecotoxicology (Table 29.2.3.10) Thiamethoxam (**13**) has a favorable ecological profile. It is practically non-toxic or only slightly harmful to water vertebrates

Table 29.2.3.9 Acute toxicity.

Acute toxicity test	Results	EPA toxicity category
$LD_{50\ \text{rat acute oral}}$	1563 mg kg^{-1}	III
$LD_{50\ \text{rat acute dermal}}$	>2000 mg kg^{-1}	III
$LC_{50\ \text{rat inhalation (4 h)}}$	>3720 mg m^{-3}	III
$\text{Skin irritation}_{\text{rabbit}}$	Non-irritant	IV
$\text{Eye irritation}_{\text{rabbit}}$	Non-irritant	IV
$\text{Skin sensitization}_{\text{guinea pig}}$	Non-sensitizing	IV
Genotoxicity	Non-genotoxic, non-mutagenic	
Reproduction	Neither developmental nor teratogenic potential	

Table 29.2.3.10 Ecological toxicology characteristics of thiamethoxam (**13**).

Acute toxicity test	Species	LD_{50}/LC_{50}	EPA toxicity category
Avian oral LD_{50}	Bobwhite quail Mallard duck	1552 mg kg^{-1} 576 mg kg^{-1}	Slightly toxic Slightly toxic
Avian dietary LC_{50}	Bobwhite quail Mallard duck	>5200 ppm >5200 ppm	Practically non-toxic Practically non-toxic
Freshwater fish LC_{50} (96 h)	Rainbow trout Bluegill	>125 mg L^{-1} >114 mg L^{-1}	Practically non-toxic Practically non-toxic
Marine fish LC_{50} (96 h)	Sheepshead minnow	>111 mg L^{-1}	Practically non-toxic
Freshwater invertebrate EC_{50} (48 h)	*Daphnia magna*	>100 mg L^{-1}	Practically non-toxic
Marine invertebrate EC_{50} (96 h)	Mysid shrimp Eastern oyster	6.9 mg L^{-1} >119 mg L^{-1}	Moderately toxic Practically non-toxic
Algae EC_{50} (72 h)	Green algae	>81.8 mg L^{-1}	None
Earthworm EC_{50} (14 d)	*Eisenia foetida*	>1000 mg $(\text{kg soil})^{-1}$	None
Bee contact LD_{50}	Honey bee	0.024 μg per bee	Highly toxic

and invertebrates, avians and soil invertebrates and beneficial arthropods with the exception of bees and bumble bees; **13** has to be considered toxic to bees and harmful to bumble bees. However, it showed no effects on bumble bees in tomatoes after drip irrigation according to label recommendations. Thiamethoxam (**13**) showed no bioaccumulation potential, it is moderately mobile in soil and degrades fast to moderate rates under field conditions [13, 37].

Effects on Beneficial Arthropods Thiamethoxam (**13**) is classified as slightly too moderately harmful to most beneficial insects, but safe to predatory mites in the field. This rating is quite similar to other neonicotinoid compounds [13].

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29.3
DE-175 (Spinetoram), a New Semi-synthetic Spinosyn in Development

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29.3.1
Introduction

Spinosyns (Fig. 29.3.1) are a class of fermentation-derived macrocyclic lactone bioinsecticides, produced by the actinomycete *Saccharopolyspora spinosa* [1]. They

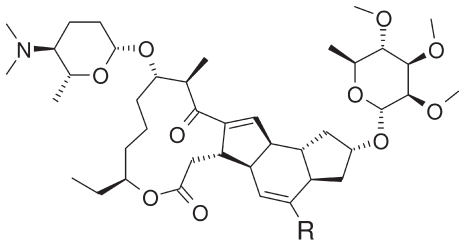


Fig. 29.3.1. The two principal components of spinosad are spinosyn A (**1**, R = H) and spinosyn D (**2**, R = CH_3).

are active against a broad range of insect pests, and exhibit activity through both oral (ingestion) and contact routes of administration. Their high level of efficacy, combined with a unique mode of action (Section 29.3.2) and favorable environmental and toxicological profile [2] have led to their rapid adoption in numerous agricultural settings where quick control of a broad range of chewing pests combined with a minimum impact on beneficial insects is required.

The unique chemistry and biological profile of the spinosyns has spurred a considerable amount of research since their commercial introduction in 1997. During the three-year period 2003–2005, over 250 studies on the chemistry, biology and biochemistry of spinosyns have been published or presented [3, 4]. This chapter will cover some of the newer aspects of the science and utility of this class of insect control agents.

29.3.2
Biological Activity and Primary Uses of Spinosad

Spinosad is currently registered for use in over 73 countries, and its labels include uses on over 250 crops. It is marketed under several trade names, which are formulated for different application conditions. Success® (240 g-a.i. per liter SC) is used primarily for vegetables and tree crops, Tracer® (480 g-a.i. per SC) is used primarily for field crops, and Conserve® (120 g-a.i. per SC) is used for control of insect pests of ornamental plants, turfgrass pests and tree farms, and of home and garden pests. An organic-certified bait formulation of spinosad, GF-120NF®, is used to control numerous destructive tephritid fruit fly species in tree fruits, nuts, vines, vegetables and ornamental crops [5–8]. Although the physical characteristics of the spinosyns are not conducive to most systemic applications, they have been reported to control pests through root uptake under conditions where soil binding is minimal [9], and to control insect pests of cabbage and cauliflower as a seed treatment [10].

Evaluation of spinosyns as a protectant for stored grains has also been reviewed [11]. Excellent control of Lesser grain borer (*Rhyzopertha dominica* (F.) [12–14], Indianmeal moth (*Plodia interpunctata*), and other grain beetle pests (e.g., *Tribolium*, *Cryptolestes*, *Sitophilus*, *Oryzaephilus*, *Prostephanus*) has been demonstrated

for numerous grain and seed commodities [12, 13], as well as for stored tobacco pests such as cigarette beetle, *Lasioderma serricorne* (F), and the tobacco moth, *Ephestia elutella* (Huebner) [15].

Spinosyns have also been found to control insect vectors [16, 17]. They have been shown to be effective in preventing breeding of mosquitoes in plastic containers at low concentrations for up to eight weeks [18], and have also been reported to control tsetse fly [19]. Recent studies have demonstrated the utility of spinosyns for use in control of parasitic pests in humans and other mammals. Spinosad is currently used for control of blowfly and lice in sheep in Australia [20], and also has been reported to control both ticks and fleas in cattle [21] and in companion animals [22]. It is also currently prescribed in use in humans for control of head lice [23].

Spinosad possesses highly favorable mammalian toxicity and environmental profiles. It was registered under the US EPA Reduced Risk Pesticide initiative in 1997 and received the US EPA Presidential Green Chemistry award in 1999. Spinosad is well known to present a relatively low risk to non-target insects compared with other broad-spectrum insecticide products. Extensive field experience indicates that the overall impact of spinosad on beneficial insects is generally limited and transitory, and it fits well into Integrated Pest Management (IPM) programs. Spinosad demonstrates large margins of safety to predacious insects such as lady beetles (Coccinelliedae), lacewings (Neuroptera), bigeyed bugs (*Geocoris* spp.), minute pirate bugs (*Orius* spp.) and others. Field studies on various crops using typical spinosad use rates have demonstrated that spinosad has low risk to adult honeybees and has little or no effect on hive activity and brood development [24].

29.3.3
Mode of Action of Spinosyns

Extensive studies on the mode of action (MOA) of spinosyns have been conducted by Dow scientists. Early studies of the spinosyn MOA were reliant on detailed characterization of the gross symptomological effects seen in insects exposed to spinosyn A [25]. For example, the physiological responses demonstrated by spinosyn A poisoned insects include a progression of symptoms, starting with muscle contractions, prostration and paralysis, which ultimately lead to death. These sequelae have been previously described in detail by Salgado [25]. Additionally, other symptoms such as distensions of the abdomen, terminalia and proboscises in several species, including cockroaches, flies and adult moths have been observed. These distensions can be so severe in some poisoned insects that a protrusion of the terminalia and the proboscis to the point of eventual disintegration of these structures is observed [26]. It is unclear how spinosyns produce this distension, but it is possible that there is some secondary perturbation of internal hydrostatic pressure in these affected insects. Another interesting symptom seen in spinosyn treated *Drosophila* is the very characteristic ''wings-down'' pattern that has been observed with various analogs of spinosyns. This effect seems

somewhat specific to spinosyns, but has also been observed with other neurally active agents. In total, these symptoms have helped guide an understanding of the actions of spinosyns in insects and continue to implicate a novel, neural MOA for these insecticides.

The symptomological studies indicating a neural MOA for spinosyns were further refined by utilizing electrophysiological tools to better characterize the physiological and neurophysiological effects of spinosyns [25, 27]. In addition, it was noted that the internal concentrations of spinosyn A within the neural tissues of poisoned insects were consistent with the concentrations required to elicit neurological symptomology [28]. As a result of these combined observations, it became apparent that the effects of spinosyns were primarily associated with the insect central nervous system (CNS) and did not appear to be involved with non-neural mechanisms such as insect growth regulation [26].

Once a neural mechanism was confirmed, attention was focused on using various biochemical and physiological methodologies to determine if the spinosyns were insecticidal due to interactions with any known insecticidal target sites. Thus, the effects of spinosyn A on the CNS of the American cockroach (*Periplaneta americana*) and housefly (*Musca domestica*) were characterized in greater detail. Of initial interest were the target sites for various insecticidal chemical classes, including pyrethroids, neonicotinoids, avermectins, organophosphates/carbamates, fiproles, and cyclodienes. Additionally, the target sites for metabolic and respiratory poisons as well as various other neural target sites, including the insect receptors for ryanodine, biogenic amines, and non-neonicotinoid acetylcholine receptors (nicotinic and muscarinic), were assessed for interactions with spinosyn A. Spinosyn A was also tested in several vertebrate neural receptor and enzyme based assays in an attempt to gain further insight into the nature of the spinosyn MOA [26].

Two physiologically relevant actions of spinosyns have been elucidated; these involve effects that appear to be nicotinic [25] and GABAergic [27] in nature. Both types of effects have been demonstrated in the insect CNS and both appear to be consistent with the observed biological consequences of spinosyn A intoxication in insects. To further explore whether the effects of spinosyn A were via an interaction at a known nicotinic receptor, various nicotinic radioligands (e.g., imidacloprid, thiamethoxam, epibatidine, alpha-bungarotoxin, methyllycaconitine, etc.) were assayed in receptor binding assays using insect CNS membranes. In all cases, spinosyn A did not significantly displace nor increase overall radioligand binding, suggesting that the nicotinic effects observed in physiological studies with spinosyns were through a novel binding site at nicotinic receptors [28]. Similarly, spinosyn A did not exhibit any direct interactions with known binding sites for GABAergic radioligands such as cyclodienes and fiproles [29]. Additionally, spinosyn A did not exhibit an interaction with the binding site for the GABAergic/glutamatergic insecticide avermectin [26]. These data suggest that both the nicotinic and GABAergic effects previously documented for spinosyn A are via novel mechanisms and not through a known insecticidal binding site.

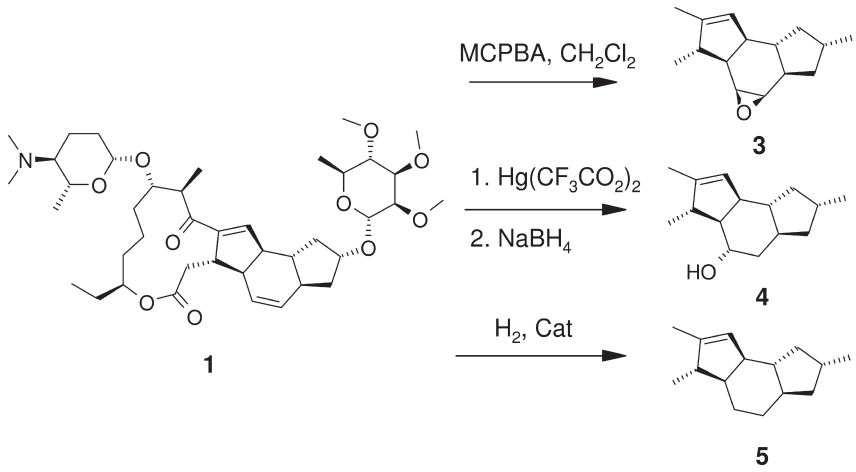
Recently, a Dow proprietary strain of spinosyn A resistant *Drosophila* has been extensively characterized and found to have significant resistance to spinosyns and related semi-synthetic analogs. Characterization of this RSN (resistance to spinosyn) fly strain indicates that the resistance is not due to penetration or metabolic factors, but is due to an alteration in the spinosad target site [26, 30]. This RSN strain, and several other strains with similar target site alterations, is currently being characterized using molecular genetic approaches to elucidate the molecular target site for spinosyns. Significant cross-resistance for the more recently discovered 21-butenyl spinosyn analogs in these RSN flies has also been demonstrated [30], suggesting that this new class of spinosyns acts at the same target site as spinosad. Molecular identification of the novel target site for spinosyns will provide an opportunity to better characterize the neurobiology of insects, since it will likely represent a novel CNS receptor. Further, the target protein for spinosyns, and the RSN flies, will likely provide important tools for the discovery of novel chemistries interacting at the spinosad target site.

29.3.4 Spinosyn Analogs

29.3.4.1 Core-modified Analogs (Aglycone)

As with most complex natural products, development of a systematic structure–activity relationship for spinosyns is impractical. Potential modifications are limited to existing reactive sites, and specific modifications of the spinosyns are limited by their susceptibility to acidic hydrolysis (loss of forosamine), basic hydrolysis (loss of forosamine and/or dehydration at C17), photolysis [31], and oxidation (formation of N-oxides and N-demethylation). Despite these limitations, a considerable amount of structural modification has been accomplished [3, 4]. As with many other complex natural products, most of the modification efforts have resulted in a decrease or complete elimination of biological activity. For example, loss of either or both sugar units, or disruption of the enone unit, results in analogs that retain $<10\%$ of the activity of the original natural product [32]. Similarly, removal of one or more methyl groups from the rhamnose sugar is highly detrimental to biological activity [3]. As a general rule, biological activity is closely related to the lipophilicity of the analog; modifications that increase the overall lipophilicity tend to improve biological activity, and the reverse holds for polar modifications.

A readily exploitable synthetic handle in spinosyn A is the isolated double bond at C5–C6. Electrophilic attack on the isolated double bond of spinosyn A proceeds with high π-diastereofacial selectivity (Scheme 29.3.1) [33]. Despite greater steric hindrance to electrophilic attack from the concave β face, epoxidation was found to favor the β epoxide (**3**) by a 5:1 ratio, an effect attributed to torsional steering. Similarly, oxymercuration/reduction resulted in a 5α-hydroxy derivative (**4**) with $>$30:1 selectivity. Hydrogenation using homogeneous catalysts (e.g., Wilkinson's catalyst) results in selective reduction of the 5,6-double bond. Although heteroge-



Scheme 29.3.1

neous catalysts such as Pd/C do show selectivity toward mono-reduction of the 5,6-double bond (**5**), careful monitoring of hydrogen uptake is required to avoid over-reduction of the C13–C14 bond as well. Analogs with a reduced 5,6-double bond show marginally improved biological as well as residual activity [4].

Among the spinosyn factors isolated from *S. spinosa*, only two different substitutions at C21 are found. With the exception of spinosyn E (**6**; Fig. 29.3.2), which has a methyl group at C21, all other factors are substituted with an ethyl group. This minor structural variation is critical to the biological activity, since **6** retains only about 10% of the activity of the corresponding ethyl derivative [3]. Lack of an appropriate synthetic handle had prevented further exploration of larger alkyl groups at C21. However, the recent discovery of a new spinosyn-producing organism, *Saccharopolyspora pogona*, has allowed more extensive exploration of this and other parts of the molecule [34, 35]. The most prevalent side-chain produced by *S. pogona* is a trans-2-butenyl group (**7**; Table 29.3.1), although other unsaturated and hydroxylated side-chains (**8**–**10**) are produced as well.

Other novel spinosyns have been created by genetic engineering of spinosyn biosynthetic genes [51]. The loading module from the avermectin PKS was

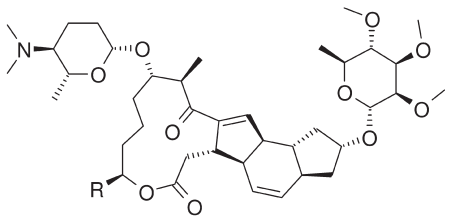


Fig. 29.3.2. C21 modified analogs.

Table 29.3.1 Spinosyns modified at C21 (Fig. 29.3.2).

Compound	R	Producing organism	H.V. LC_{50}[a]
1	-C_2H_5	*S. spinosa*	0.31
6	-CH_3	*S. spinosa*	4.6
7	*trans*-C_2H_5CH=CH-	*S. pogona*	0.29
8	*trans*-CH_3CH=CH-	*S. pogona*	–
9	*trans*-H_2C=CH-CH=CH-	*S. pogona*	–
10	*trans*-CH_3-CH(OH)CH=CH-	*S. pogona*	–
11	-*n*-C_3H_7	*S. spinosa*[b]	0.16
12	-*i*-C_3H_7	*S. spinosa*[b]	–
13	CH_3CH(OH)-	*Streptomyces* (from spinosyn A)	–

[a] LC_{50} of neonate *Heliothis virescens* larvae.
[b] Analogs were generated from a bioengineered strain of *S. spinosa*; see text.

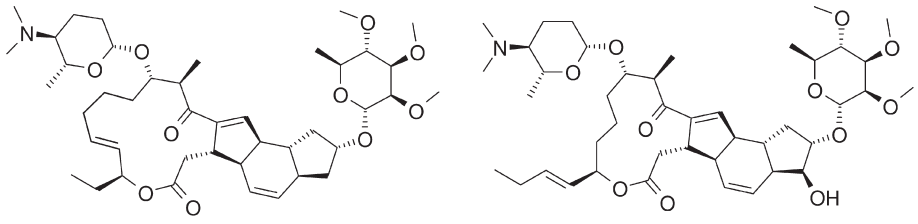
introduced into *S. spinosa* to form a hybrid PKS gene that initiated polyketides with branched chain carboxylic acids, yielding spinosyns with *n*-propyl (**11**) and *iso*-propyl (**12**) groups at C21. This engineered PKS could also initiate chains from fed cyclic carboxylic acids to produce C21-cyclobutyl and C21-cyclopropyl spinosyns.

The creation of a synthetic handle on the C21 side-chain through microbial oxidation has been reported [36]. Transformation of spinosyn A or its aglycone using a *Streptomyces* strain results in selective oxidation at C22, to generate **13** (Table 29.3.1). This functional group could then be used, in principle, to prepare various modifications, although no further analogs derived from this compound have been described.

Other novel core-modified factors have been isolated from *S. Pogona* fermentation broths as well, including analogs containing an expanded 14-membered lactone ring (**14**) and a hydroxyl group at C8 (**15**) (Fig. 29.3.3).

29.3.4.2 Modifications Involving the C17 Sugar

Most natural spinosyns have an amino sugar (generally, β-D-forosamine) attached at C17. Since forosamine is a 2-deoxy sugar, hydrolytic removal to form **16** can be accomplished selectively under mild conditions [37] (Scheme 29.3.2). Glycosylation to regenerate a β-linked sugar, however, is more problematic. Re-attachment of forosamine, using a 2-mercaptopyrimidinyl activating group, is accomplished in 17% yield and favors the α sugar by a 3:2 ratio [38]. The N-protected dihydro-



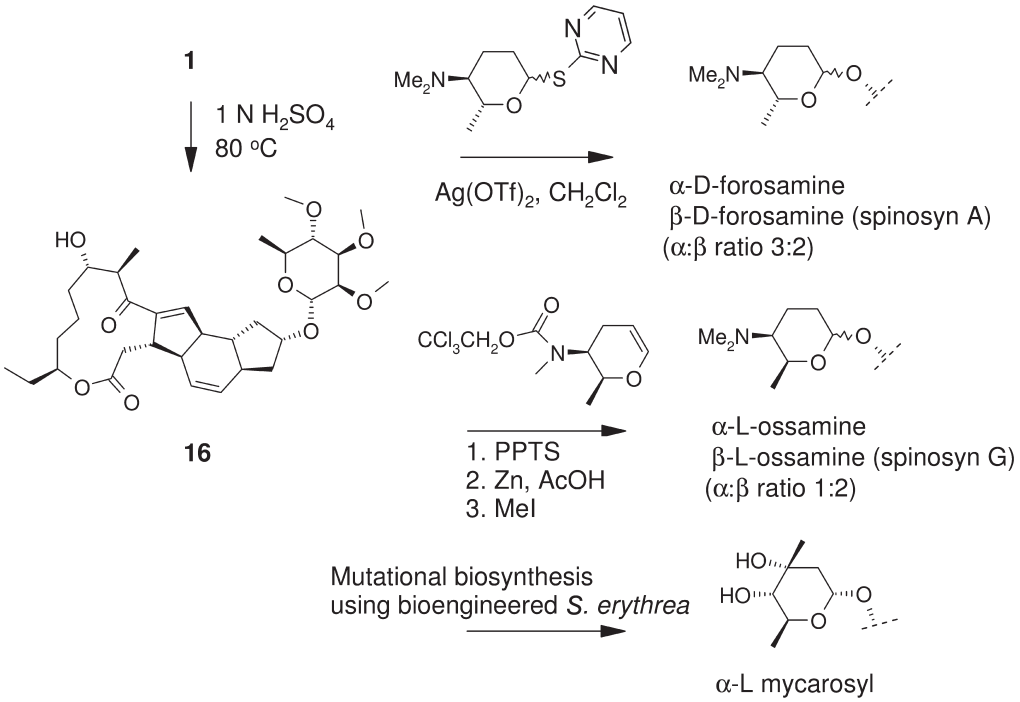
14 15

Fig. 29.3.3. Novel core-modified analogs from *Saccharopolyspora pogona*.

pyran used in the synthesis of spinosyn G, on the other hand, was attached in 36% yield (Scheme 29.3.2) [39]. In this case, the desired β anomer was favored by a 2:1 margin.

Novel sugar residues have also been incorporated at C17 through mutational biosynthesis. When **16** was fed to a strain of *Saccharopolyspora erythrea* engineered with the *spnP* gene, the product was found to have incorporated L-mycarose [40].

Spinosyns bearing novel sugars at C17 were also isolated from the butenyl-producing *S. Pogona* (Fig. 29.3.4) [34]. Besides D-forosamine, other sugars in-



Scheme 29.3.2

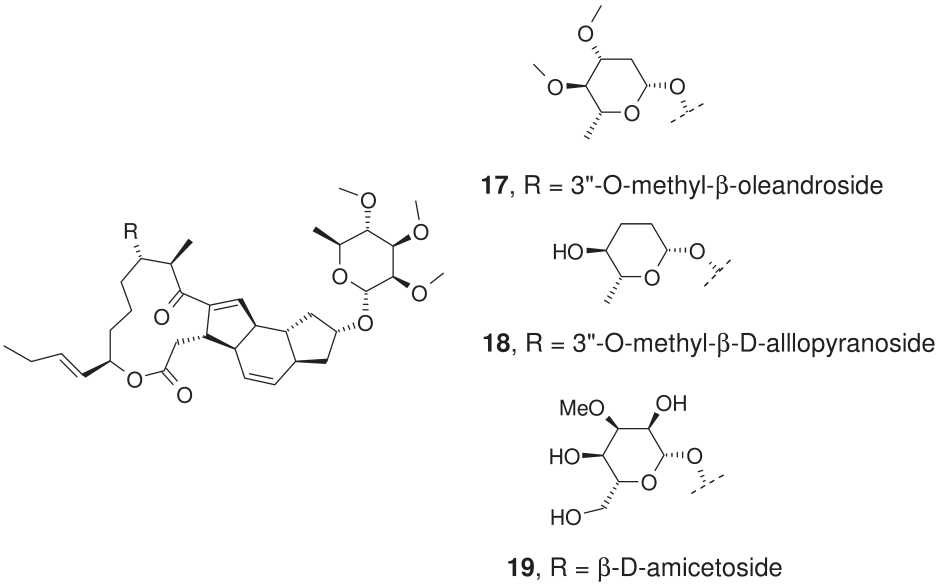


Fig. 29.3.4. Neutral C17-saccharides isolated from *Saccharopolyspora pogona*.

clude the neutral sugars 3″-*O*-methyl-*β*-D-oleandroside (**17**), 3″-*O*-methyl-*β*-D-allopyranoside (**18**), and *β*-D-amicetoside (**19**). The bioactivity and pest spectrum of these neutral sugar analogs is similar to the amino spinosyns [34].

29.3.4.3 Modifications Involving the C9 Sugar: Rhamnose Derivatives

Availability of spinosyns lacking one or more of the O-methyl groups from rhamnose (Fig. 29.3.5) has allowed for a wide variety of synthetic variation at this portion of the macrolide. This has also led to some of the most active analogs. Spinosyns are generally quite base-labile, and standard alkylating conditions (Williamson ether synthesis using NaH or K_2CO_3) led to extensive loss of forosamine. A non-aqueous, phase-transfer alkylation protocol involving powdered KOH and a quaternary salt such as $(n\text{-Bu})_4NI$ in a halocarbon solvent avoids these undesired reactions and leads to high yields of the corresponding alkylated derivatives **29**–

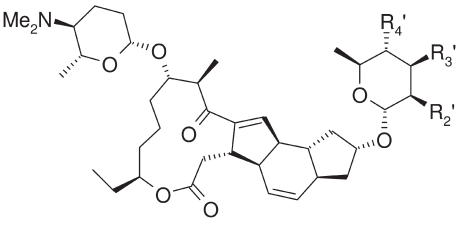


Fig. 29.3.5. Modified rhamnose analogs.

Table 29.3.2 Structure and insecticidal activity of rhamnose-modified spinosyn analogs (see Fig. 29.3.5).

Compound number	Position	Substituent[a]	Precursor	Reagent(s)	H.V. LC_{50}[b]
1	–	–	Natural factor		0.31
20	R_2'	–OH	Natural factor		3.2
21	R_3'	–OH	Natural factor		>64
22	R_4'	–OH	Natural factor		3.5
23	R_2'	=O	**20**		3.5
24	R_3'	=O	**21**	NCS, $(CH_3)_2S$, Py	10
25	R_4'	=O	**22**		–
26	R_2'	$-OCOCH_3$	**20**		1.2
27	R_3'	$-OCOCH_3$	**21**	Ac_2O, Py	33
28	R_4'	$-OCOCH_3$	**22**		1.3
29	R_2'	$-OC_2H_5$	**20**		0.11
30	R_3'	$-OC_2H_5$	**21**	C_2H_5Br, KOH (powdered), Bu_4NI	0.035
31	R_4'	$-OC_2H_5$	**22**		0.24
32	R_2'	H	**20**		0.23
33	R_3'	H	**21**	1. NaH, CS_2, MeI; 2. Bu_4SnH, AIBN	0.36
34	R_4'	H	**22**		4.1

[a] The two remaining rhamnose substituents groups are $-OCH_3$.
[b] LC_{50} of neonate *Heliothis virescens* larvae.

31 (Table 29.3.2) [4]. The corresponding acetate esters or ketones (**23–28**) show generally weaker activity than the corresponding ethers [41]. Deoxygenation of the free OH groups of spinosyns H, J, or K also resulted in active analogs; the 2′-deoxy analog **32** was more active than the parent spinosyn A [41].

Spinosyn analogs all exhibit largely similar pest spectrum, though with significant differences in potency. In general, the SAR of rhamnose analogs follows two rules: more lipophilic substituents are more active than less lipophilic ones, and modifications to the 3′ position are more impactful than are modifications to either the 2′- or 4′-positions [4]. Whereas the difference in activity between most polar (OH) and least polar substituent is approximately 10–30× for 2′ and 4′-positions, the difference is almost 2000× at the 3′-position.

29.3.5
DE-175, a New Semi-synthetic Spinosyn in Development

Efforts to improve the efficacy of natural spinosyns through chemical modification have led to the development of a new semi-synthetic analog based on the improved activity of analogs modified at the 3′-position on the rhamnose sugar (Table 29.3.2). Compound DE-175 (Fig. 29.3.6) is derived from spinosyns J and L, which, like spinosad, differ by the presence of a hydrogen or methyl group at

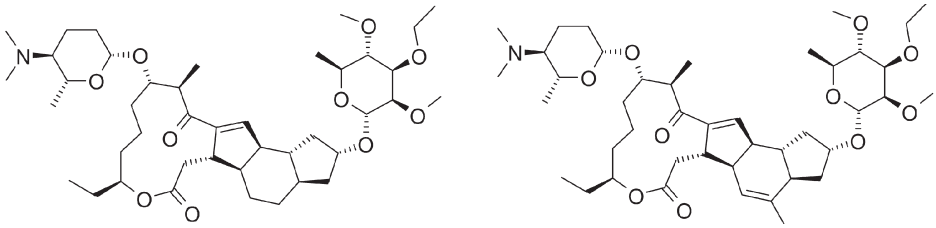


Fig. 29.3.6. Structure of DE-175, a mixture consisting of 3′-*O*-ethyl-5,6-dihydro spinosyn J (left-hand side) and 3′-*O*-ethyl spinosyn L.

C6. These factors are both *O*-ethylated at the 3′-position of the rhamnose, and the major factor (spinosyn J) is further reduced to the 5,6-dihydro derivative.

One effect of these chemical modifications is that DE-175 is intrinsically more active against pest insect species than spinosad. For example, in a laboratory bioassay of topical toxicity to beet armyworm (*Spodoptera exigua*) larvae, DE-175 is 48× more active than spinosad (Table 29.3.3). Similar results are observed in a bioassay of ingestion activity, where DE-175 is 58× more active on beet armyworm than spinosad. Improved potency of DE-175 is also observed against other lepidopterous insects, though to a lesser degree (Table 29.3.3).

Another result of the chemical modifications is improved residuality of DE-175 compared with spinosad. In a simulated field comparison, DE-175 shows significant enhancements in both residuality and potency against codling moth, *Cydia pomonella*, larvae (Fig. 29.3.7) [4]. Improved residuality and potency is also evident in a field trial against fall armyworm, *Spodoptera frugiperda*, infesting maize in Brazil (Fig. 29.3.8).

The non-target toxicological profile and environmental fate profile of DE-175 are similar to spinosad and are very favorable. Interestingly, although DE-175 is more active against pest insect species, under field conditions DE-175 has mini-

Table 29.3.3 Activity of spinosad (Fig. 29.3.1) and DE-175 (Fig. 29.3.6) against lepidopterous larvae in laboratory bioassays.

Species	Topical activity LD_{50} (μg per larva)		Ingestion activity LC_{50} (mg L^{-1} on diet)	
	DE-175	Spinosad	DE-175	Spinosad
Tobacco budworm (*Heliothis virescens*)	0.02	0.03	0.87	1.7
Beet armyworm (*Spodoptera exigua*)	0.013	0.63	0.1	5.8
Cabbage looper (*Trichoplusia ni*)	0.025	0.03	0.13	0.44

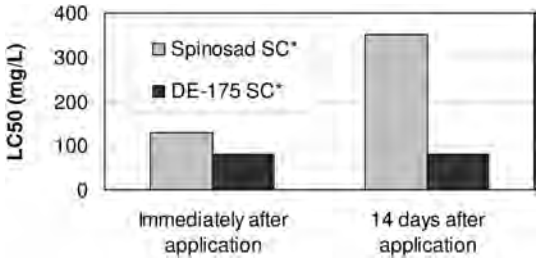


Fig. 29.3.7. Activity of DE-175 (Fig. 29.3.6) and spinosad (Fig. 29.3.1) against codling moth (*Cydia pomonella*) larvae on treated apples in a simulated field exposure study. * Suspension concentrate formulation.

mal impact on most beneficial arthropod populations. This is seen in the results of a field trial in Mississippi, USA that measured populations of beneficial insects and spiders in eggplant (Table 29.3.4). Cumulative numbers of beneficial arthropods in the DE-175-treated plots are equal to those in the spinosad-treated plots and greater than numbers counted in plots treated with the pyrethroid insecticide λ-cyhalothrin.

DE-175 was selected for development based on its greater efficacy and its longer residuality. In field trials around the world, DE-175 has demonstrated excellent, broad spectrum control of significant pests attacking tree fruit, tree nut, vine, and vegetable crops. It is particularly effective against lepidopterous larvae (including *Spodoptera* spp., codling moth (*Cydia pomonella*), oriental fruit moth (*Grapholita molesta*), and tortricid leafrollers), dipterous leafminers (*Agromyzidae*), and thrips (*Thysanoptera*).

In early 2006, DE-175 was accepted for expedited review under the US EPA Reduced Risk Pesticide Initiative. Registration and launch of products containing DE-175 are anticipated in late 2007 in the US, Canada, and Mexico.

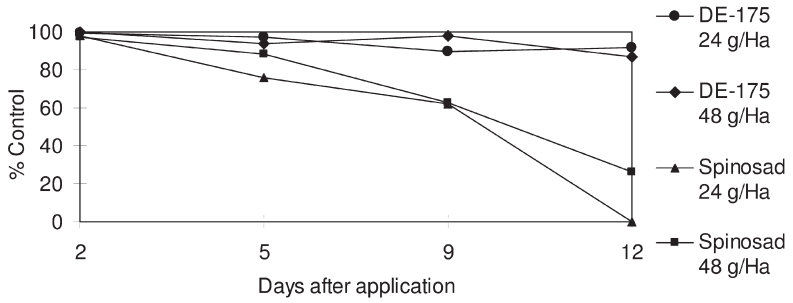


Fig. 29.3.8. Control of fall armyworm (*Spodoptera frugiperda*) in maize with DE-175 and spinosad, each applied at 24 and 48 g active ingredient per Ha.

Table 29.3.4 Effect of DE-175, spinosad, and λ-cyhalothrin applications on the total number of beneficial arthropods in eggplant.

Treatment	Rate (g-a.i. ha^{-1})	Total number of beneficial arthropods per 1.8 m of row[a]				
		1 DBA[b]	3 DAA[c]	7 DAA[c]	14 DAA[c]	Cumulative total
Untreated	–	9.8 m	11.1 m	7.3 m	7.6 m	26.1 m
Spinosad SC	70	7.8 m	12.8 m	5.9 m	8.1 m	26.6 m
Spinosad SC	88	8.8 m	11.1 m	5.3 m	7.6 m	24.0 m
DE-175 SC	44	8.8 m	11.5 m	8.1 m	8.1 m	27.3 m
DE-175 SC	70	6.5 m	8.9 m	6.8 m	6.8 m	23.8 m
λ-Cyhalothrin EC[d]	28	9.3 m	4.0 m	4.9 m	6.3 m	15.1 n

[a] Means within columns followed by the same letter are not significantly different (Duncan's New Multiple Range Test, $P = 0.10$).
[b] Days before application.
[c] Days after application.
[d] Emulsifiable concentrate formulation.

29.3.6
Biosynthesis and Genetics of the Spinosyns

Synthesis of the spinosyns, like other macrolides, is initiated by stepwise condensation of acylated carboxylic acids to form a linear polyketide. Each step involves the addition, by an acyltransferase (AT), of a malonyl or methylmalonyl residue to an acyl carrier protein (ACP). The acyl group is then transferred to the growing polyketide in a decarboxylative condensation catalyzed by a ketoacyl-ACP synthetase (KS). Following these obligatory steps there may be additional reactions that modify the preceding residue. The keto group may be reduced to a hydroxyl by a ketoacyl-ACP reductase (KR), changed by a KR and dehydratase (DH) to leave a double bond, or completely reduced to a backbone of saturated carbons by a KR, a DH and an enoyl reductase (ER). The full-length polyketide is cyclized by the action of a thioesterase (TE) to form a macrocyclic lactone. Each of these reactions is catalyzed by a unique domain in a large complex of multi-functional polypeptides called a Type I polyketide synthase (PKS). The carboxylic acid origin of spinosyns has been confirmed by incorporation studies with ^{13}C-labeled acetate and propionate [41]. The steps of polyketide synthesis were deduced from DNA sequence homologies to characterized PKS genes [42] and supported by the novel pentaketide structure produced from a truncated PKS expressed heterologously [43]. Unlike most macrolides, spinosyn polyketides are internally crosslinked by a process resembling an intramolecular cycloaddition. The resulting tetracyclic aglycone is the first detectable biosynthetic intermediate. The next intermediate is the pseudoaglycone (PSA), which contains a tri-*O*-methylated rhamnose at C9.

It is not known if the rhamnose is normally methylated before or after its addition to the aglycone, but *S. spinosa* cells can complete the methylation of fed pseudoaglycones lacking some rhamnose-*O*-methyl groups. The final step in spinosyn biosynthesis is the addition of forosamine at the C17 position. This glycosylation only occurs after rhamnose addition; it does not occur on the C17 of the aglycone. The forosamine must be di-*N*-methylated before incorporation because precursors lacking one or both of these methyl groups are not modified when fed to *S. spinosa* cultures. Unlike the C-methyl groups on the polyketide (which come from the carboxylic acid precursors), the sugar methyl groups are all derived from *S*-adenosyl-methionine [41].

The *spn* genes that encode the spinosyn biosynthetic enzymes have been cloned and sequenced. Most of them are clustered together in an 80 kb region of the *S. spinosa* chromosome. One half of the cluster consists of the five large PKS genes. They code for the five polypeptides containing the eleven modules (one for each carboxylic acid) that produce the macrocyclic lactone. Together these constitute a 2 MDa complex that catalyzes 50 different enzymatic reactions. The other half of the cluster contains 14 genes encoding the enzymes involved in modification of the polyketide and in the synthesis and addition of the deoxysugars. Polyketide crosslinking is rare, so it was difficult to identify the genes whose products contribute to this process. However, four candidates were tentatively assigned roles in aligning the polyketide chain and in C–C bridge formation based on limited sequence homology to genes of related function. A more detailed model of the crosslinking emerged from structural analysis of the novel monocyclic intermediate produced by a truncated PKS [43]. The five genes encoding the enzymes required to convert the common deoxysugar precursor NDP-4-keto-6-deoxy-D-glucose into forosamine were much easier to identify because their sequences were strikingly similar to the corresponding genes in other organisms. The role of three of these genes was confirmed by gene disruption leading to accumulation of PSA. Three *O*-methyltransferase genes and two glycosyltransferase genes were also easy to recognize by sequence homology. Disruption of these genes had pleiotropic effects that made it difficult to determine the specificity of the genes responsible for rhamnose methylation. However, precursor feeding provided clear evidence that rhamnose addition is encoded by *spnG* and forosamine addition by *spnP*. The four genes coding for the enzymes that generate NDP-4-keto-6-deoxy-D-glucose and then convert it into rhamnose are not present in the *spn* cluster. Rhamnose is an essential component of the cell wall as well as spinosyn, so its unique biosynthetic genes cannot be located in the region of chromosome containing the other *spn* genes because it is prone to deletion [44, 45].

S. spinosa produces a family of closely-related spinosyns that differ in the methylation patterns at C6, C16 or C21 of the polyketide nucleus, or on the sugars. These are either biosynthetic intermediates or shunt products derived from them that are generated by incomplete processing. They are generally present at low levels in the wild-type strain. Spinosyn D is an exception in that it is produced to about 20% of the level of the major product, spinosyn A. Spinosyn D is made when a methylmalonyl-CoA is incorporated instead of malonyl-CoA by the

AT domain of PKS module 8, resulting in a methyl group at C6. Some minor factors, such as spinosyn H (**19**), are accumulated to much higher levels in mutant strains generated by treatment of cells with *N*-nitrosoguanidine. In these strains a biosynthetic function has been lost completely due to a point mutation in one of the *spn* genes. Other strains derived in the same way accumulate similar intermediates, such as spinosyn K (**22**), that were undetectable in the wild-type [46]. The presence of a butenyl group in analogs isolated from *S. Pogona* is due to an extra module in the PKS that incorporates an additional carboxylic acid into the polyketide. Another variant, the 14-membered ring lactone (**14**), is presumably due to an altered cyclization pathway of the longer polyketide. Other minor factors carry neutral sugars such as amicetose, *O*-methyl glucose or *O*-methyl oleandrose instead of forosamine at C17 (See Fig. 29.3.4). These probably reflect the presence of a glycosyltransferase with a broad specificity, and sugar biosynthetic pathways that are not functional in *S. spinosa*. The unique hydroxylated spinosyns **10** and **15**, produced by *S. pogona*, could result from the incorporation of hydroxylated precursors by the PKS or by the action of unique P450 mono-oxygenases [47].

Another strain of *S. spinosa* was engineered to replace the AT domain of module 3 with AT domains that preferentially incorporate ethyl malonyl-CoA. Since this precursor is not normally synthesized by *S. spinosa*, a crotonyl-CoA reductase gene from *Streptomyces cinnamonensis* was introduced at the same time. The engineered strains produced C21-*n*-propyl and C6-ethyl spinosyns (rather than the targeted C16-ethyl spinosyn), presumably due to incorporation of ethyl malonyl-CoA by the native loading module or module 8, respectively [48, 49]. A spinosyn containing a novel sugar residue (L-mycarose) at C17 (Table 29.3.2) was generated by a strain of *Saccharopolyspora erythraea* engineered with the *spnP* gene and fed PSA. Clearly, the glycosyltransferase product of this gene has the ability to incorporate sugars other than forosamine [40].

29.3.7
Metabolism and Penetration of the Spinosyns

Spinosyn A, as a representative of the spinosyns in general, would appear to have quite a number of sites that would be targets for metabolism. Among these potential sites are N-demethylation of the forosamine, O-demethylation of one or more of the methoxy groups on the rhamnose, epoxidation of the 5,6 or 13,14-double bonds, opening of the macrocyclic lactone, hydroxylation of the tetracycle, or some combinations thereof, all leading to molecules that are less active than the parent. Indeed, studies of spinosyn A and D metabolism in rats showed that the parent molecules (spinosyn A and D) accounted for only a small fraction the material present 24 h post treatment in fecal extracts [50]. Thus, a substantial amount of metabolism had taken place, for both spinosyns A and D, with loss of a methyl group on the forosamine nitrogen (N-demethylation) and loss of a methoxy group from the rhamnose (O-demethylation) representing the primary pathways. These observations suggest that in rats the spinosyns are readily me-

tabolized. Likewise, studies of spinosyn metabolism in lactating goats detected the presence of eight metabolites for spinosyn A and five metabolites for spinosyn D. As observed in the rat metabolism studies, N-demethylation was observed as an important metabolic route. However, hydroxylation of the macrolide ring was also noted as an important metabolic route [51].

As noted for the mammalian studies, spinosyn metabolism in avian systems (poultry) also identified N-demethylation of the forosamine nitrogen and O-demethylation of the rhamnose as the primary metabolic pathways [52]. In these studies, O-demethylation of the rhamnose at the 2′- and 4′-positions, yielding spinosyns H and K, respectively, was favored over the 3′-position (spinosyn J formation). An additional secondary metabolic pathway involves the loss of the forosamine sugar to form the C17-pseudoaglycone [53]. As this study and the above mammalian studies clearly show, the spinosyns A and D are readily metabolized, with N-demethylation of the forsamine nitrogen as a predominant route in all three species [3]. N-Demethylation, coupled with O-demethylation of the rhamnose and macrolide ring hydroxylation, are consistent with oxidative metabolism via monooxygenases and/or the action of glutathione transferases [3].

In contrast to the mammalian and avian studies, the available information on the metabolism of the spinosyns by insects suggests that metabolism of the spinosyns (e.g., spinosyn A) is very limited. Studies of spinosyn A metabolism in tobacco budworm (*Heliothis virescens*) larvae show that the only component detected (within the limits of detection, up to 24 h post treatment) in larval homogenates of topically treated larvae was the parent, spinosyn A [53, 54]. In contrast, these same larvae readily metabolized the acaricide fenazaquin [3, 53], clearly demonstrating that *H. virescens* larvae have the capacity to metabolize xenobiotics. Further, studies with *H. virescens* larvae highly resistant to spinosad [55] also found no evidence for metabolism of spinosyn A [56, 57].

The apparent lack of spinosyn A sensitivity to metabolic processes in pest insects is further supported by studies showing a general lack of cross-resistance to spinosad in various insecticide resistant strains, many involving enhanced metabolism [3]. Likewise, synergist studies with house flies, using the monooxygenase inhibitor piperonyl butoxide (PBO), show PBO's ability to synergize the activity of permethrin (pyrethroid insecticide) but not the activity of spinosyn A [58]. Thus, within the limits of the available data, pest insects appear to have a limited capacity to metabolize spinosyns such as spinosyn A. Perhaps the above observations are best viewed when the very large molecular weight and unusual, complex structure of the spinosyns are taken into account, since metabolism systems show distinct substrate preferences.

The spinosyns are more active than most organophosphate and carbamate insecticides, and as active as many pyrethroids [3, 59, 60]. Compared with many of these same insecticides, the spinosyns are relatively slow to penetrate the insect cuticle [3, 53]. This slow penetration is offset, in part, by the limited ability of pest insects such as *H. virescens* to metabolize the spinosyns. This apparent balance between penetration and metabolism may be a key factor in the excellent insecticidal activity of the spinosyns by ensuring that the limited quantity of spi-

nosyn A that does penetrate remains intact for a comparatively long period of time, allowing the spinosyns to exert their effects.

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29.4
Sodium Channel Blocking Insecticides, Indoxacarb

Stephen F. McCann, Daniel Cordova, John T. Andaloro, and George P. Lahm

29.4.1
History and Discovery of the Sodium Channel Blockers

The pioneering work in pyrazoline insecticides by Kobus Wellinga and Rudolph Mulder provided the first leads toward the sodium channel blocking insecticides [1]. Figure 29.4.1 summarizes these early compounds. Pyrazoline PH 60-41 was found to have moderate activity on Lepidoptera, Coleoptera and Diptera. Optimization of PH 60-41 produced 5-phenyl derivatives with improved insecticidal activity across all three insect orders, with PH I-9 perhaps the most active of the group [2].

Further modification led to the discovery of the 4-aryl derivatives, such as PH 60-42 (Fig. 29.4.2), with excellent activity on a broad range of insects; these compounds demonstrated increased activity over the 5-aryl analogs by a factor of 3–100 [3]. Modification of the substituent groups on the pyrazoline, 3-aryl, 4-aryl and carbamoyl rings provided further information pertaining to structure–activity relationships [4]. Compounds containing a 4-trifluoromethoxyaniline substituent were first reported by researchers at Bayer [5]. This aniline substituent is perhaps the most active identified. Several significant issues associated with early pyrazolines likely contributed to their lack of commercialization. These problems

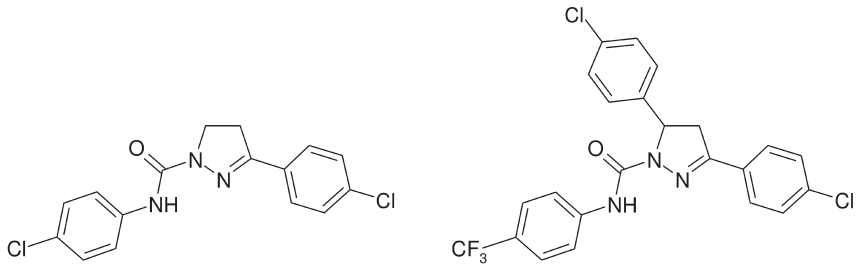


Fig. 29.4.1. Pyrazolines were the first insecticides in the field of sodium channel blockers.

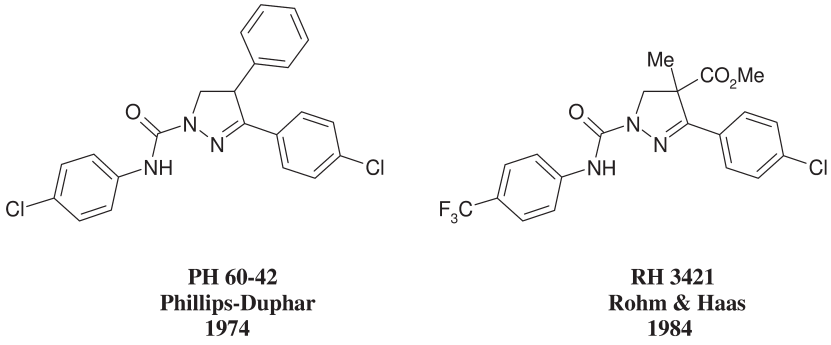
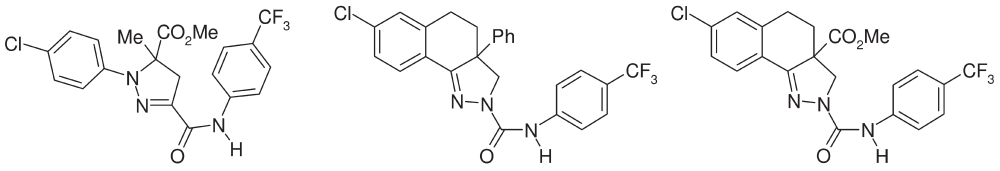


Fig. 29.4.2. Optimized versions of early pyrazoline sodium channel blockers.

included a perceived lack of photostability [6], environmental persistence [7], and problems associated with long-term toxicity and bioaccumulation [8]. The 4-methyl-4-carbomethoxypyrazolines discovered at Rohm and Haas made good progress toward a solution for these problems through prevention of photoaromatization and significant reduction in soil half-life [9].

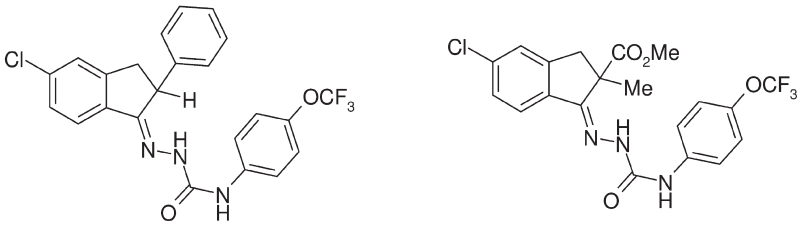
Modifications to the pyrazoline nucleus were first discovered at DuPont and provided information relevant to the orientation and spatial relationships of sodium channel blockers (Fig. 29.4.3). Carboxamides such as **1** gave new insight into the arrangement of atoms in the pyrazoline ring [10]. For the first time these ''inverse'' pyrazolines demonstrated that orientation of substituent groups around the ring was in fact the critical component for activity and not specific to the known N-carboxamide-3-aryl pyrazolines. Conformationally constrained pyrazolines, including indazoles such as **2** and **3**, provided structural insight into the spatial relationships of the aryl rings, carboxamide group and pyrazoline nucleus and suggested a planar arrangement of aryl rings was important [11].

Indazoles such as **2** provided the basis for design of semicarbazones [12] such as **3** and **5** owing to the observation that the indanone derived semicarbazones provided a good spatial match with the indazoles (Fig. 29.4.4). These compounds



1 **Carboxamides** **2** **Phenyl Indazoles** **3** **Carbomethoxy Indazoles**

Fig. 29.4.3. Discovery of 3-carboxamide pyrazolines and indazole insecticides.



4 **Phenyl Semicarbazones** **5** **Carbomethoxy Semicarbazones**

Fig. 29.4.4. Semicarbazone sodium channel blocking insecticides.

showed good insecticidal properties although, on balance, somewhat less than the better pyrazolines. Interestingly, they departed significantly from the developed structure–activity profile with 2-aryl compounds. Compounds containing a 2-phenyl group, such as **4**, showed the best activity while 2-carbomethoxy analogs, such as **5**, showed only weak activity.

Pyridazines [13] such as **6** were discovered from a combination of structural features derived from indazoles and semicarbazones such as **3** and **5** (Fig. 29.4.5). The pyridazines were some of the most potent analogs evaluated at DuPont. Activity in the laboratory was observed below 1 ppm, which was significantly better than insecticide standards. Compound **6** was in fact the most potent compound evaluated, with excellent field performance on Lepidoptera. Pyridazines, however, lacked acceptable soil residual properties. This problem was solved by introduction of the oxygen found in oxadiazines such as **7** (Fig. 29.4.6). This newest class of sodium channel blockers produced indoxacarb [14] following extensive optimization of chemical and biological attributes.

29.4.2 Discovery of Indoxacarb

The problems with slow breakdown in soils that were identified for **6** were due in part to the chemical robustness of the tricyclic pyridazine ring system. To attempt to solve this problem we envisaged that heteroatom substitutions into the core

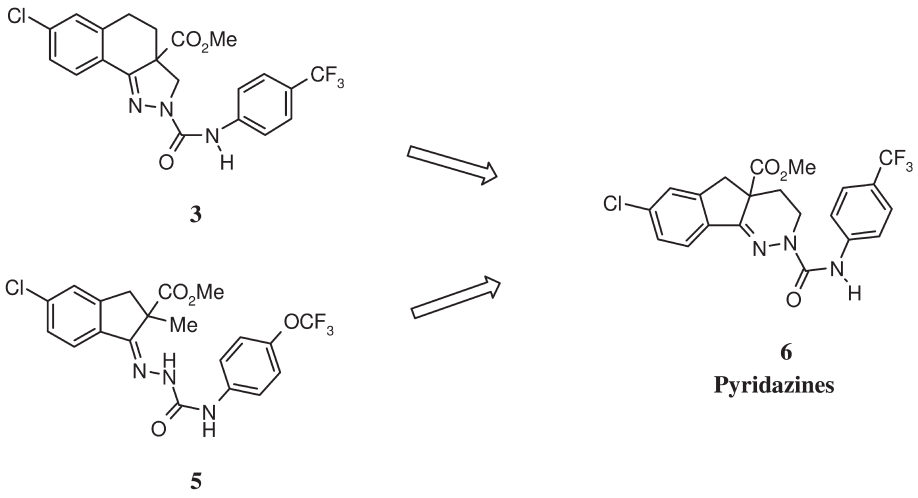


Fig. 29.4.5. Discovery of pyridazine insecticides.

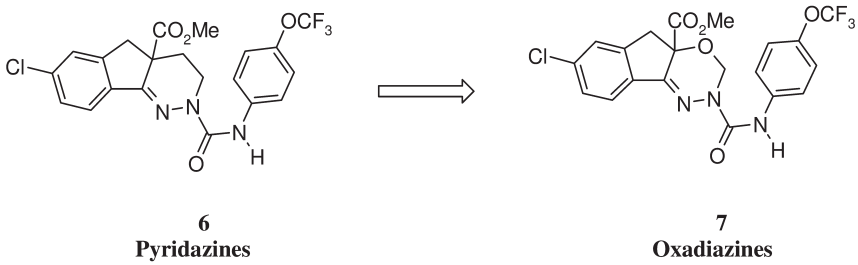


Fig. 29.4.6. Discovery of oxadiazine insecticides.

pyridazine would lead to more potential sites for chemical or metabolic breakdown. One possible target, the oxadiazine **7** [15], is a cyclic O,N acetal that could potentially ring-open under acidic conditions, exposing a product that could undergo further breakdown.

It was found that compound **7** did degrade rapidly in soil test systems, displaying a soil half-life ranging from approximately 1 to 4 weeks. This observation was made along with the finding that high insecticidal activity was maintained in the new, environmentally labile oxadiazine ring-system. Additionally, the oxadiazine ring was easier to prepare than the analogous pyridazine. Figures 29.4.7 and 29.4.8 compare the synthetic routes for pyridazines **6** and oxadiazines **7**. Of particular note is the low-yielding step that installs the ethylene bridge atoms of **10**. The reaction of salts of 2-carbomethoxy indanone anion **8** with 1,2-dibromoethane provided an undesirable 75:25 ratio of O-alkylated vs. C-alkylated products, **9** and **10**, respectively. Separation of **9** and **10** required tedious chromatography and the low yields (<25%) of the slower eluting **10** was problematic.

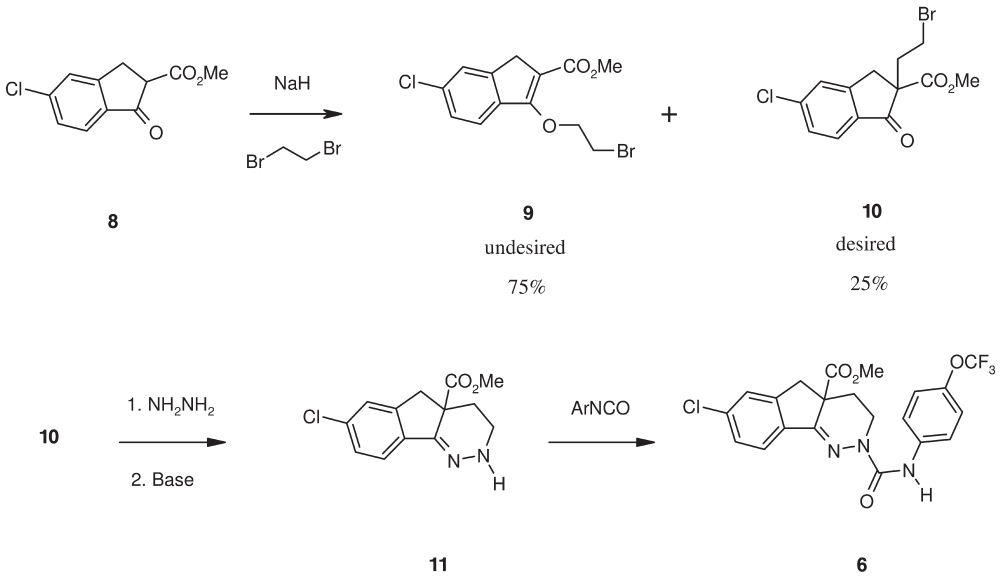


Fig. 29.4.7. Pyridazine synthesis.

Pyridazine **6** was prepared by subsequent treatment with hydrazine and aryl isocyanate.

The analogous ring-forming steps in the synthesis of **7** involves oxidation [16] of **8** to the hydroxy indanone **12** followed by formation of the hydrazone **13**, capping with an aryl isocyanate to form the semicarbazone [17] **14** and, finally, ring-closure with formaldehyde [18] or a formaldehyde equivalent to give the O,N-acetal **7** [19]. All steps are high-yielding and do not require chromatography.

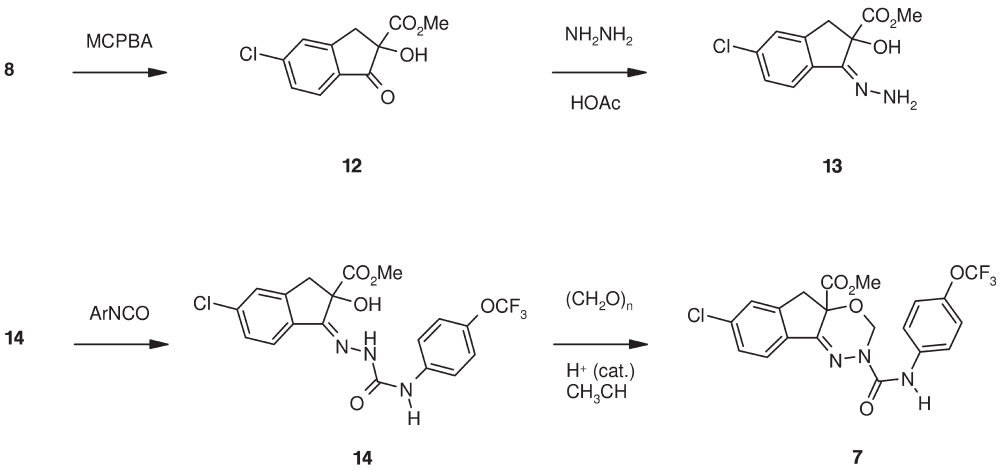


Fig. 29.4.8. Oxadiazine synthesis.

Structure–activity relationships for oxadiazines were found to be consistent with pyrazolines, indazoles and pyridazines. The wealth of data for pyrazoline-type insecticides allowed for the rapid preparation and identification of highly active analogs. Selection of candidate DPX-JW062 was based on a combination of observed high insecticidal efficacy, safety to non-target organisms, including predatory insects as well as fish, birds and mammals, and rapid dissipation in the environment [20].

Separation of the DPX-JW062 enantiomers using chiral HPLC and subsequent bioassay showed the (+)-enantiomer, DPX-KN128, to be approximately twice as active as the racemic material, while the (−)-enantiomer was inactive as an insecticide. This finding, consistent with that reported for analogous pyrazolines [21], prompted the investigation of chiral synthesis methods for the selective preparation of the (+)-enantiomer DPX-KN128. A wide variety of reagents and conditions were screened [14a, b] for the asymmetric hydroxylation of **8** to **15**. Use of the alkaloid cinchonine as a chiral basic catalyst in combination with *t*-butyl-hydroperoxide as the stoichiometric oxidant [22] provided optimum results, yielding **15** as a 75:25 mixture of enantiomers (50% e.e.). Compound **15** was then converted into the enantiomerically-enriched oxadiazine, DPX-MP062, consisting of a 75:25 mixture of (+)- and (−)-enantiomers (Fig. 29.4.9). Analysis of a deriva-

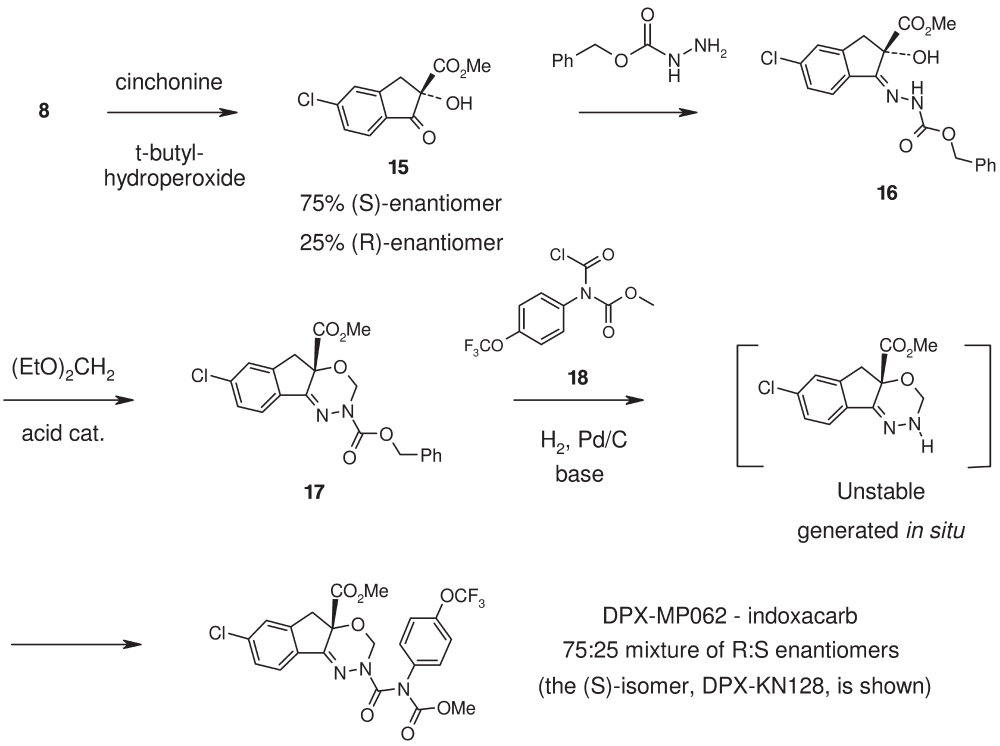


Fig. 29.4.9. Indoxacarb synthesis.

tive by X-ray diffraction showed the active (+)-enantiomer, DPX-KN128, to have the (S)-absolute configuration. DPX-MP062 is currently sold as a 75:25 mixture of enantiomers under the generic name indoxacarb. The commercial synthesis of indoxacarb is also shown in Fig. 29.4.9 [22].

29.4.3
Insecticidal Activity and Properties of Indoxacarb

Indoxacarb is currently registered in 84 countries and on over 100 crops. Registration was first acquired in Asian, Eastern European and West African countries in 1999. Physicochemical, toxicological, and environmental properties are described in Table 29.4.1 [23].

Table 29.4.2 summarizes DuPont's sodium channel blocking products, registered formulations, and content of the insecticidally active enantiomer.

Indoxacarb is a very effective crop protection product, with low toxicity to nontarget organisms [25], and short persistence in the environment. It is designated

Table 29.4.1 Indoxacarb properties.

Physical state (99% DPX-KN128)	Solid, powder
Formula weight	527.84
Melting point (DPX-KN128)	88.1 °C
Solubility (DPX-MP062)	Water: 0.20 ppm *n*-Heptane: 1.72 mg mL^{-1} 1-Octanol: 14.5 mg mL^{-1} Methanol: 103 mg mL^{-1} Xylene: 117 mg mL^{-1} Acetonitrile: 139 mg mL^{-1} Ethyl acetate: 160 mg mL^{-1} Dichloromethane: >250 g kg^{-1} Acetone: >250 g kg^{-1} Dimethylformamide: >250 g kg^{-1}
Partition coefficient Log K_{OW} (DPX-KN128)	4.65
Vapor pressure (DPX-KN128)	9.8×10^{-9} Pa (20 °C) 2.5×10^{-8} Pa (25 °C)
Acute toxicity (DPX-MP062)	Oral LC_{50}: 1730 mg kg^{-1} (rat male) Oral LC_{50}: 268 mg kg^{-1} (rat female) Dermal LD_{50}: >5000 mg kg^{-1} (rat) Inhalation LC_{50}, 4 h: >5.5 mg L^{-1} Dermal irritation: nonirritant Eye irritation: moderate eye irritant Ames test: negative

Table 29.4.2 Indoxacarb product summary.

Code	KN128 (%)	Formulation: products
JW062	50	SC: Tornado®
MP062	75	SC, WDG: Avaunt®, Steward®, Rumo®, Avatar®, Ammate®
KN128	100	EC: Steward®, Avaunt®

by the U.S. Environmental Protection Agency (EPA) as a ''reduced-risk'' pesticide, which is defined as having health and environmental advantages over existing products.

Though indoxacarb is a broad-spectrum lepidopteran insecticide it also has activity on additional pests from several insect orders [26]. Insects controlled by indoxacarb include most of the globally important lepidopteran pests such as species of *Heliothis* (bollworms), *Spodoptera* (armyworms), *Trichoplusia* (loopers), *Plutella* (diamondback moth), *Ostrinia* (borers), *Lobesia* (berry moths), *Cnaphalocrocis* (leaf folders), *Pandemis* (leafrollers), *Tuta* (pinworms), and *Agrotis* (cutworms). In addition, indoxacarb controls selected sucking insect pests, including leafhoppers, fleahoppers and plant bugs, as well as beetles, sawflies, leafminers, and apple maggot flies. Indoxacarb is also one of the most effective fire ant products for home, golf course, and public property uses [27]. It is used in a bait matrix to control numerous cockroach and ant species, with commercial activity observed on termites, fleas, mosquitoes, flies, and silverfish.

The primary route of entry into target insects is through ingestion, although the product can be absorbed through the cuticle. Though the larval lifestage is the major focus of control, indoxacarb is also a very effective ovilarvicide (kills developing larvae in egg and prevents hatching) as well as an adulticide. Indoxacarb causes very strong feeding inhibition even at sublethal rates. Insects exposed to a sublethal dose of indoxacarb eat much less, develop slower, and pupate and emerge later than untreated larvae. Inhibition of insect feeding occurs very rapidly, resulting in quick crop protection though live insects may be observed up to 24 hours. Typically these insects are partially paralyzed, smaller, desiccated and shrunken with no defense against environmental perils [28]. Other affected behaviors include reduced egg laying, mating disruption, inability to molt, difficulty to emerge from pupal case, inability to excavate soil for pupation, repellency, and uncoordinated F1 progeny. Unlike synthetic pyrethroids, high temperatures are positively correlated with indoxacarb control, thus hastening the decline of pest populations.

Indoxacarb typically provides excellent crop protection for 5–14 days, depending on rate and crop. It is highly lipophilic and absorbs into the waxy cuticle of leaves. This contributes to indoxacarb's residual control, aids in translaminar activity, and helps provide excellent rainfastness. The oil based SC and EC formulations can penetrate the leaf, resulting in control of various sucking insects. The

dry formulation is also translaminar but tank-mixing an oil-based surfactant can increase activity. Chemical stability of a pesticide in a spray tank is primarily dependent on the temperature and pH of the spray mix. Indoxacarb formulations exhibit excellent tank stability under a wide pH range (5–9). In addition, spray tank temperatures do not affect indoxacarb stability over the range 35–115 °F (−12.5–32 °C). Indoxacarb formulations have proven to be compatible with tank mix partners when added to the tank in the proper sequence. Application of indoxacarb, although typically by air and ground equipment, can also be made though center pivot or fixed sprinkler irrigation systems. All indoxacarb formulations are rainfast with excellent ultraviolet stability.

29.4.4 Indoxacarb Mode of Action

29.4.4.1 Overview of Insect Voltage-gated Sodium Channels

Indoxacarb is the most recent commercialized insecticide to target insect voltage-gated sodium channels (VGSCs). These channels play a critical role in the intercellular transmission of electrical impulses throughout the nervous system of vertebrates and invertebrates alike. As with vertebrate channels, insect VGSCs exists in three basic states: (1) a resting (closed) state where the channel is non-conductive; (2) an activated (open) state in which an inward flow of Na^+ occurs through the channel, depolarizing the cell and ultimately generating an action potential; and (3) an inactivated state (closed), in which the channel becomes non-conductive and unable to achieve activation (Fig. 29.4.10). Return of inactivated channels to the resting state is a voltage-dependent process, where cells remain in a refractory state until the cell membrane becomes re-polarized.

Various organisms such as spiders, scorpions, and carnivorous marine mollusks have developed highly selective neurotoxins that paralyze their prey through

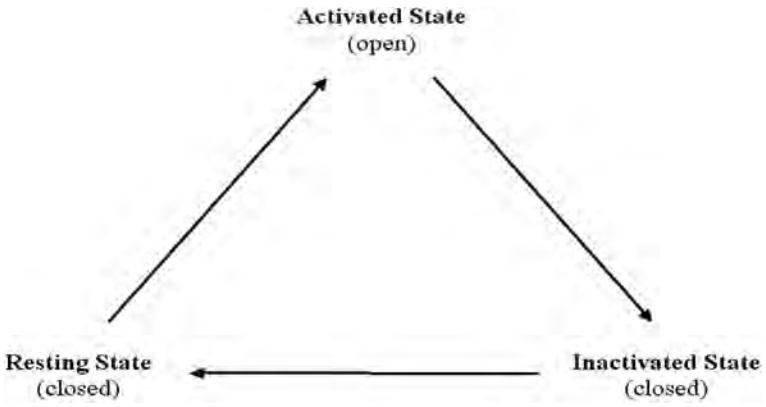


Fig. 29.4.10. Various voltage-gated sodium channel (VGSC) states and whether the channel is conductive (open) or non-conductive (closed).

Table 29.4.3 Known neurotoxin and insecticide binding sites on the VGSC.

Site	Neurotoxin/insecticide	Physiological effect
1	Tetrodotoxin, saxitoxin, μ-conotoxin	Inhibition of ion transport
2	Batrachatoxin, veratridine, aconitine, grayanotoxin, N-alkylamides	Persistent activation
3	α-Scorpion toxins, sea anemone II toxin	Enhancement of persistent activation
4	β-Scorpion toxins	Shift voltage dependence of activation
5	Brevetoxins, ciguatoxins	Shift voltage dependence of activation
6	δ-Conotoxins (δ-TxVIA)	Inhibition of activation
7	DDT-type chemistry, pyrethroids	Inhibition of activation
8	*Goniopora* coral toxin, *Conus striatus* toxin	Inhibition of activation
9	Local anesthetics, anticonvulsants, dihydropyrazoles	Inhibition of ion transport

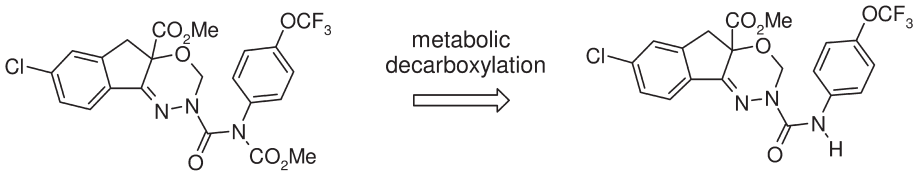
action on VGSCs (see reviews [29–32]). Nine distinct VGSC binding sites have been identified through the use of neurotoxins, synthetic insecticides, and local anesthetics (Table 29.4.3). Pyrethroids and DDT bind to Site 7, altering channel activation through a shift in voltage dependence [33]; N-alkylamides by contrast bind to Site 2 (batrachatoxin- and veratridine-binding site) where they stimulate persistent channel activation [34].

29.4.4.2 Pro-insecticide Action of Indoxacarb

Indoxacarb is a pro-insecticide, requiring bio-activation to confer potent insecticidal activity. In metabolism studies where pest insects were treated with ^{14}C-DPX-JW062, a 50:50 mixture of the active and inactive enantiomers of indoxacarb, rapid conversion into the N-decarbomethoxyllated metabolite, DCJW (**7**) was demonstrated (Fig. 29.4.11) [35]. This bio-activation is attributed to hydrolytic esterase and amidase metabolism in the midgut and fat bodies, with cytochrome P450 inhibitors having minimal impact on bio-activation. Using a preparation from the central nervous system of the Lepidoptera *Manduca sexta*, DCJW exhibited greater than 25-fold higher potency than DPX-JW062 in its ability to block nerve conduction [36]. Moreover, the insecticidal activity was attributed to the (S)-enantiomer given the 2× greater potency over the racemic mixture.

29.4.4.3 Block of VGSCs by Indoxacarb and Dihydropyrazoles

Lepidopteran larvae treated with indoxacarb produced neurotoxic symptoms, beginning with ataxia and feeding cessation followed by tremors, mild convulsions and progressing to a flaccid paralysis [36]. Such symptoms mirror those observed



Indoxacarb **7 DCJW**

Fig. 29.4.11. Indoxacarb is a pro-insecticide.

with the insecticidal dihydropyrazole RH-3421 [37]. Extracellular recording of nerve activity in the cockroach *P. americana* or lepidopteran larvae *M. sexta* poisoned with RH-3421, or indoxacarb, demonstrated a complete block in the spontaneous activity from sensory and central nervous system (Fig. 29.4.12).

Voltage clamp experiments using identified neurons, dorsal unpaired median (DUM) neurons from the terminal abdominal ganglion of *P. americana*, demonstrated that DCJW inhibited the peak Na^+ current with an IC_{50} of 28 nM [38]. DCJW (**7**) (100 nM) induced a hyperpolarization of DUM neurons associated with block of background Na^+ channels involved in maintenance of the resting potential. While the peak Na^+ current was inhibited, DCJW had no effect on either activation or inactivation kinetics (Fig. 29.4.13). Zhao et al. (2005) similarly

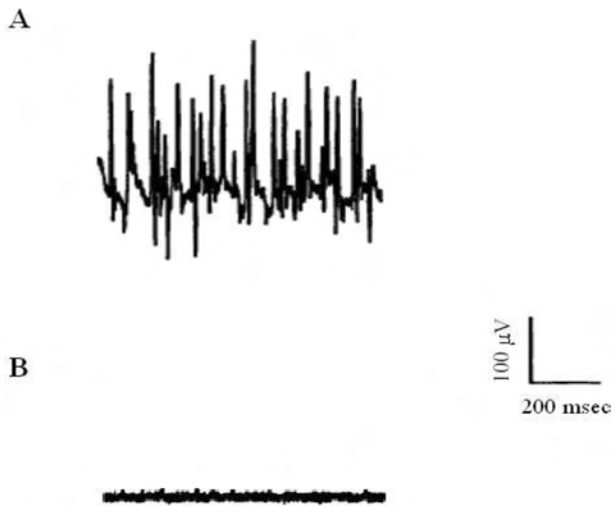


Fig. 29.4.12. Extracellular recordings of spontaneous CNS activity from nerve cords of 5th instar *M. sexta* injected with DMSO (A) or 10 μg g^{-1} DCJW (B). (Adapted from Wing et al., 1998 with permission from Wiley-Liss, Inc.) [36]

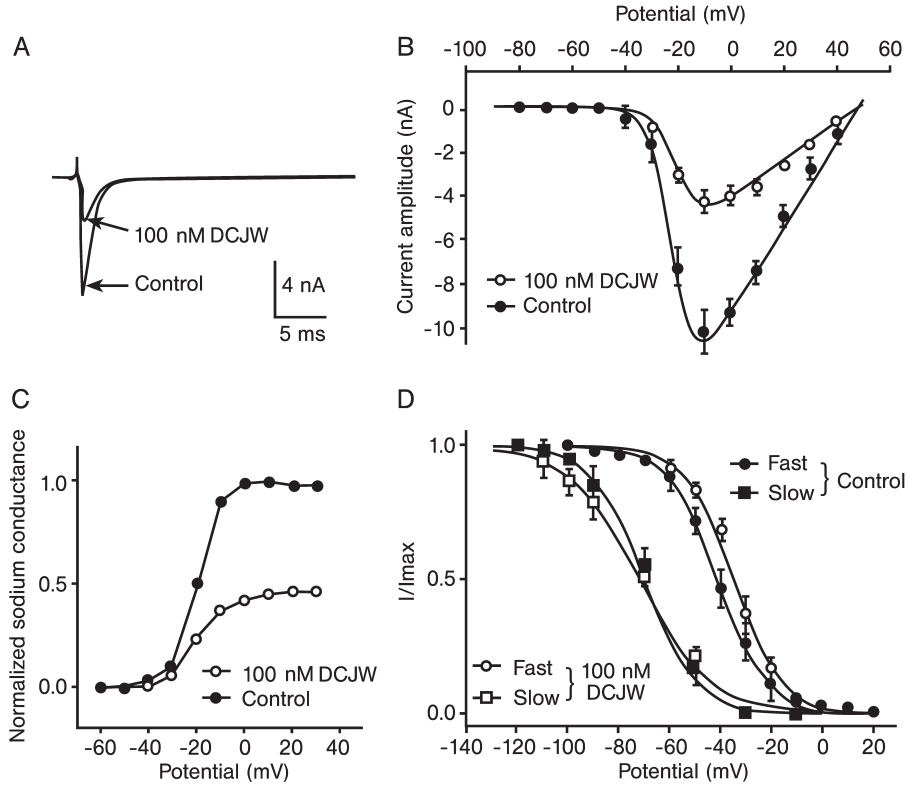


Fig. 29.4.13. Effects of DCJW (**7**) on the DUM neuron voltage-dependent inward sodium current. (A) Sodium inward current traces obtained by a 30-ms depolarizing pulse to −10 mV from a holding potential of −90 mV, in the absence and presence of 100 nM DCJW. (B) Effect of DCJW on the current–voltage relationship of the inward sodium current. The maximum peak current amplitude was plotted versus membrane potential before and after application of 100 nM DCJW. (C) Voltage dependence of the normalized sodium conductance of the inward current in normal saline before and after the application of 100 nM DCJW. (D) Superimposed voltage dependence of 100 nM DCJW. Data are means ± sem. (Reprinted by permission from MacMillan Publishers Ltd: [*Br. J. Pharmacol.*] Lapied et al., © 2001.) [38]

observed no effect on activation or inactivation kinetics [39]. However, in this study two distinct types of VGSCs were identified, labeled type I and type II, with DCJW and indoxacarb (1 μM) exhibiting differential activity on these currents. Though both compounds inhibit type I Na^+ channels at a membrane potential of −100 mV, less negative potentials (−60 to −40 mV) were required to observe a block in type II Na^+ channels. This difference was attributed to distinct inactivation kinetics between the two types of Na^+ currents, where type I Na^+ channels exhibited significant inactivation at −100 mV while type II Na^+ chan-

nels inactivated at less negative potentials. Furthermore, inhibition of type I Na^+ channels with DCJW was found to be irreversible, while inhibition by indoxacarb was fully reversed upon compound washout (Fig. 29.4.13). It was postulated that the higher insecticidal potency of DCJW, relative to indoxacarb, could be attributed to the irreversible nature of DCJW. However, given the higher potency (>250-fold) of DCJW for blocking compound action potential generation in *M. sexta* relative to indoxacarb [36], reversibility may have been less of a factor than inherent potency at the VGSC.

29.4.4.3.1 Oxadiazine & Dihydropyrazole Binding Site Identification

Radioligand binding studies with indoxacarb and other oxadiazine species have not been reported. However, studies conducted with dihydropyrazoles demonstrated that these molecules bind to a site distinct from DDT and pyrethroids; rather, dihydropyrazoles likely bind to Site 9 with allosteric interaction at Site 2 of the VGSC. As shown in Table 29.4.3, batrachatoxin binds to Site 2 of the VGSC. RH-3421 was found to inhibit binding of [^{3}H]-batrachatoxin-B ([^{3}H]-BTX-B) to mouse brain synaptosomes in a non-competitive manner [40]. RH-3421 decreased the number of available [^{3}H]-BTX-B binding sites without impacting binding affinity. Given the similar mode of action between dihydropyrazoles and local anesthetics, which bind to Site 9, Payne et al. (1998) investigated the combined effects of RH-3421 and dibucaine on [^{3}H]-BTX-B binding [41]. RH-3421 decreased dibucaine's potency as an inhibitor of [^{3}H]-BTX-B, which is consistent with binding to Site 9. Evidence that DCJW similarly binds to this site was supported by the ability of the local anesthetic, lidocaine, to reduce DCJW's suppression of the VGSC current in *P. americana* DUM neurons. Surprisingly, it was recently shown that, for a single isoform of the rat VGSC ($Na_v1.4$) expressed in *Xenopus laevis* oocytes, the current blocking efficacy of DCJW was reduced in the presence of indoxacarb while the RH-3421-induced current suppression was unaffected [42]. This finding suggested that the binding site for indoxacarb overlaps that of DCJW but is separate from the RH-3421 binding site.

29.4.4.4 Action of Indoxacarb on Mammalian VGSCs

To date, few studies have been conducted on the effect of indoxacarb and DCJW on mammalian VGSCs. Zhao et al. (2003) have investigated the ability of these oxadiazines to inhibit tetrodotoxin-sensitive (TTX-S) and resistant (TTX-R) sodium currents in rat dorsal ganglion (DRG) neurons [43]. As observed with insect channels, indoxacarb and DCJW inhibited sodium currents upon binding to the inactivated state. However, both oxadiazines exhibited much weaker potency against rat sodium channels over those of insects. Indoxacarb and DCJW (1 μM) irreversibly inhibited TTX-S sodium currents by 30% and 80%, respectively (Fig. 29.4.14B). In another study, indoxacarb was similarly found to be less potent (tenfold) than DCJW against TTX-R sodium currents in DRG neurons (Fig. 29.4.14A) [44]. In this study, however, the indoxacarb-induced sodium current suppression was partially reversible following compound washout whereas the DCJW-induced suppression was irreversible.

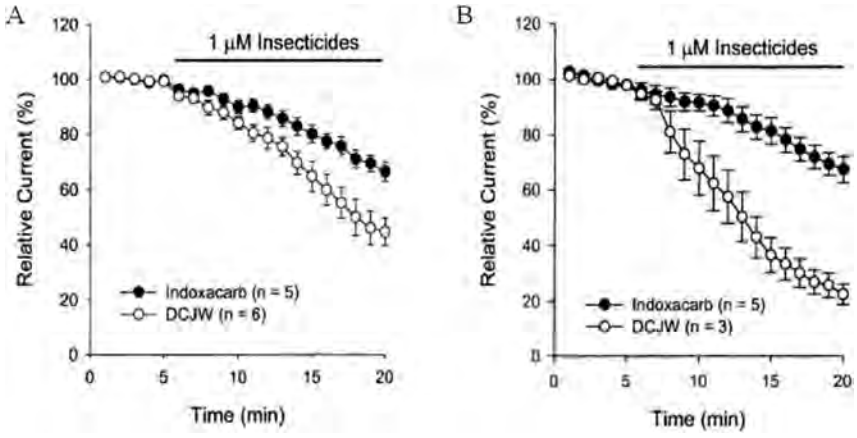


Fig. 29.4.14. Effect of indoxacarb (●) and DCJW (○) on TTX-R (A) and TTX-S (B) sodium currents of rat dorsal root ganglion neurons. Sodium currents were evoked by 10 ms step depolarization to 0 mV from a holding potential of −80 mV. (Adapted from Zhao et al., 2003, with permission from Elsevier.) [43]

More recently, effects of indoxacarb, DCJW, and the dihydropyrazole, RH-3421, were investigated on a single rat sodium channel isoform ($Na_v1.4$) expressed in *Xenopus laevis* oocytes [42]. As observed with DRG neurons, DCJW and RH-3421 (10 μM) irreversibly inhibited $Na_v1.4$ currents in a voltage-dependent manner. In contrast, indoxacarb (10 μM) failed to suppress $Na_v1.4$ currents.

Despite having activity against mammalian VGSCs, indoxacarb has excellent mammalian safety. Differential sodium channel affinity is a major factor contributing to its safety. In insects, DCJW is highly potent, with an IC_{50} value below 30 nM, as compared with the low μM IC_{50} value for rat VGSCs. Furthermore, indoxacarb, which has ten-fold lower potency than DCJW against rat VGSCs, is the predominant oxadiazine in mammals [23]. Minimal conversion of indoxacarb into DCJW occurs in mammals, while, as previously discussed, indoxacarb is rapidly metabolized into DCJW in insects [36].

29.4.4.5 **Indoxacarb Resistance**

Indoxacarb has proven to be an effective crop protection product in regions where insects have developed resistance to organophosphates, carbamates, synthetic pyrethroids, benzyl urea insect growth regulators, and organochlorines. Because indoxacarb is bioactivated via esterase and amidase enzymes, overproduction of esterases in insects resistant to organophosphates or pyrethroids could lead to faster liberation of the active toxin than in non-resistant insects. This suggests that certain resistant insects may in fact develop a negative cross-resistance to indoxacarb, as has been observed in laboratory strains [45] and field populations of *Helicoverpa armigera* [46]. However, after multiple years of repeated use on the

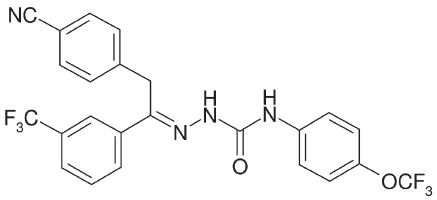


Fig. 29.4.15. Metaflumizone.

island of Oahu, Hawaii, where growers had access to very few insecticides, a population of diamondback moth (*Plutella xylostella*) developed resistance to indoxacarb [47]. The mechanism of resistance is believed to be enzymatic – however, this has yet to be verified. Certain geographical populations of oblique-banded leafroller, *Choristoneura rosaceana*, a pest of apples, are also known to be highly tolerant to Indoxacarb WG. Ahmad suggests that the resistance involves enhanced oxidative degradation through a mechanism originally selected by field exposure to azinphosmethyl [48]. Failure to activate the compound does not appear to be involved as a resistance mechanism.

29.4.5
Other Sodium Channel Blocking Insecticides

Indoxacarb is currently the only commercial product to work by blockade of the sodium channel. However, the semicarbazones provided the basis for the discovery of metaflumizone (Fig. 29.4.15), a new sodium channel blocker discovered by Nihon Nohyaku [49] and to be commercialized by BASF through a licensing agreement. BASF is currently completing registration for the control of caterpillars and beetles. Its toxicological profile and environmental behavior are reported as favorable. BASF expects U.S. and European registration by 2006/2007 [50].

29.4.6
Conclusion

Indoxacarb is characterized by attributes that offer a total plant protection package for cotton, vegetables, tree fruit, vines and other agricultural crops. It represents a new chemical class and a novel mode of action well suited for rotation in resistance management programs. Indoxacarb is extremely potent on its biochemical target, resulting in low field use rates and excellent safety to workers and consumers. Combined with general safety to predacious and parasitic arthropods, indoxacarb is an ideal fit in grower pest control programs and a good choice to alternate, replace, or complement existing insecticides. While the field of competitive sodium channel blocking insecticides is narrow we anticipate this will grow with time as new products such as metaflumizone move to market.

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29.5
Ligand-gated Chloride Channel Antagonists (Fiproles)

Vincent L. Salgado, Stefan Schnatterer, and Keith A. Holmes

29.5.1
Discovery and Development of Fipronil and other Fiproles

Fiprole insecticides belong to the chemical class of insecticidal phenylpyrazoles (arylpyrazoles) [1], discovered independently by Bayer AG [2] and by May&Baker, a subsidiary of Rhône-Poulenc [3–5], while studying herbicidal phenylpyrazoles (PPO-herbicides, Nipyraclofen) [6, 7].

Rhône-Poulenc Agrochimie (later Aventis CropScience) launched fipronil (**1**) as a broad spectrum crop insecticide in 1993 [8]. In the context of the acquisition of Aventis CropScience by Bayer CropScience AG in 2002, the fipronil business was sold to BASF Aktiengesellschaft in early 2003.

Fipronil is highly effective against a broad range of economically important insect pests, and has become a cornerstone in insect control programs for both crop and non-crop insect pests in many areas of the world. It is currently registered in over 70 countries for the control of insect pests in more than 100 crops, ranging from row crops, such as rice, corn, potatoes and small grains, to specialty crops, such as ornamentals, mangoes, and chili peppers. It is used in various formulations, either as a foliar spray, soil application or seed treatment, depending on the crop/pest situation. As it is not repellent, it is extremely effective in bait applications. Fipronil not only protects crops from insects, but in some cases can actively increase yields through incompletely understood plant health effects.

In the non-crop area, fipronil has rapidly grown to become the leading insecticide. It is the world's leading termiticide and is also a key component in urban pest control programs against cockroaches and ants. It is also used for control of

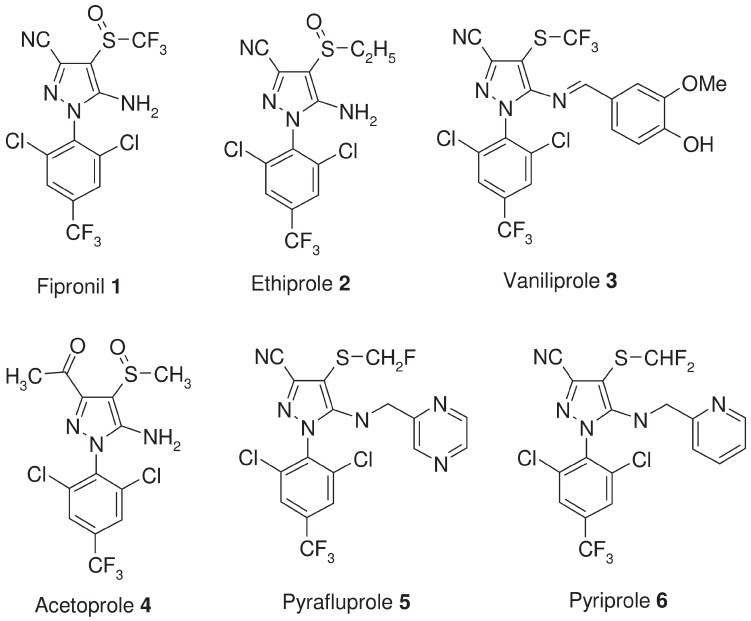


Fig. 29.5.1. Fiproles on the market (**1**, **2**) or in development (**3**–**6**).

mole crickets and fire ants in turf, and is one of the leading veterinary ectoparasiticides [9, 10].

Fipronil is highly effective against insects that are resistant to other insecticides, in part because of its unique mode of action. While it was known even before its launch that fipronil could block chloride channels gated by the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [11–13], it has recently been shown that fipronil and/or its predominant sulfone metabolite also potently block two types of glutamate-gated chloride channel in the insect central nervous system (CNS) [14]. Thus, fipronil acts at three target sites with high affinity.

Ethiprole (**2**) was launched as a crop insecticide by Bayer CropScience in 2005. Compared with fipronil, it has improved plant systemicity and controls a broader spectrum of sucking pests, but has much less activity on Lepidopteran insects. Four other phenylpyrazoles have reached development status as crop insecticides (Fig. 29.5.1).

29.5.2 Mode of Action

Fipronil and its predominant sulfone metabolite are unique among insecticides in that they have three known high-affinity target sites – the three ligand-gated chloride channels that mediate most inhibitory transmission in the insect nervous system: GABA receptors and the two subtypes of GluCls that have been de-

scribed in insects [14, 15]. This multiplicity of highly sensitive target sites reduces the potential of target-site resistance. Furthermore, fipronil and its sulfone are much more potent against insect than against mammalian GABA receptors. GABA receptors and GluCls mediate most fast inhibitory transmission in the insect nervous system. Inhibitory synapses are widespread in the CNS and are thought to be involved in the fine tuning of all types of behavior [16]. A certain level of inhibition is always present in the nervous system, and its disruption leads to hyper-excitation and convulsions. For this reason, GABA-gated chloride channel blockers are also referred to as convulsants. It is assumed that GluCls play a similar role to GABA receptors in inhibitory neurotransmission, but this has not been investigated. GABA receptors also mediate fast inhibitory transmission at insect nerve–muscle junctions. While it is well established that CNS effects are important in the convulsant actions of insecticides, it is not clear what role is played by muscle effects.

29.5.2.1 Discovery of the GABA Receptor as an Insecticide Target Site

From the mid-1940s to the late 1970s, more than three billion pounds of polychlorocycloalkane (PCCA) insecticides had been used [17] and resistance was widespread, representing 60% of all known cases of insecticide resistance [18]. Cross-resistance between all three classes of PCCAs – lindane, toxaphene and the cyclodienes (Fig. 29.5.2) – suggested early on that these compounds all had a common target site, and the observation [19] that several cyclodiene-resistant insect strains were cross-resistant to the botanical convulsant picrotoxin, long used in ointments to control lice [20] and known to be a non-competitive antagonist (NCA) of GABA receptors [21], fingered the GABA receptor as the likely PCCA target site. Action of PCCAs on GABA receptors was confirmed by their ability to inhibit GABA-induced chloride flux into cockroach muscle and the binding of [^{3}H]dihydropicrotoxinin to the NCA site in rat brain synaptosome GABA receptors [19]. Furthermore, the mammalian toxicity of PCCAs was closely correlated with displacement of [^{35}S]-TBPS, another ligand for the NCA site, from rat brain GABA receptors [22]. Block of GABA responses in cockroach neurons by lindane and the cyclodiene endrin was confirmed electrophysiologically [23].

[^{3}H]Dihydropicrotoxinin was the first successful radioligand for the NCA site, but newer ligands have improved properties [17]. [^{35}S]-TBPS is extensively used for the GABA receptor NCA site in mammalian brain, but does not measure a toxicologically relevant site in insects, which is consistent with the high mammalian toxicity and poor insecticidal activity of the bicyclophosphorous esters. However, the structurally-related bicycloorthocarboxylate esters, which are highly toxic to both insects and mammals, yielded [^{3}H]-EBOB, now the ligand of choice for the insect GABA receptor [17]. [^{3}H]-BIDN has also been used as a ligand for the NCA site [24].

Soon after the development of fipronil was announced in 1992 [8], its mode of action as an NCA of GABA-gated chloride channels was published. Fipronil and other insecticidal phenylpyrazoles were observed to cause symptoms in house flies and mice that were similar to those of the known GABA antagonists dieldrin

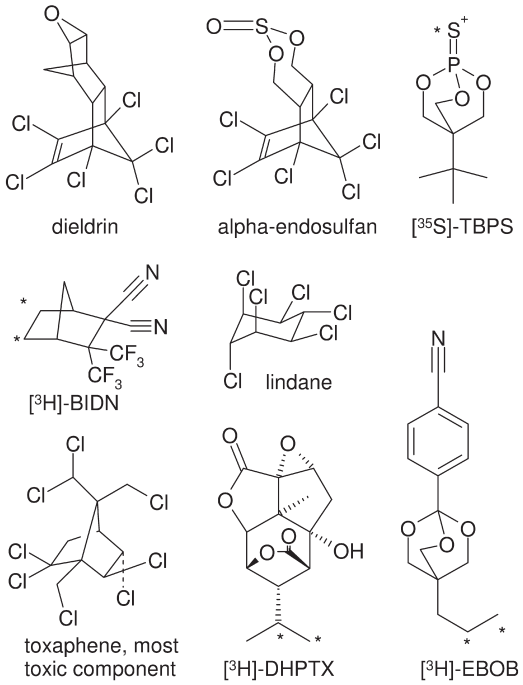


Fig. 29.5.2. Structures of the fiproles and other GABA antagonists discussed. Dieldrin and α-endosulfan are examples of cyclodienes. Labels in the radioligands are shown by asterisks.

and EBOB [11]. A dieldrin-resistant house fly strain was 20-fold cross-resistant to fipronil, and fipronil inhibited the specific binding of [^{3}H]-EBOB to GABA receptors in house fly head membranes with an IC_{50} of 2.3 nM. Furthermore, the potency among several phenylpyrazoles in inhibiting [^{3}H]-EBOB binding was correlated with insecticidal activity [11] and fipronil was shown to antagonize homomeric Rdl GABA receptors heterologously expressed in *Xenopus* oocytes [25–27].

29.5.2.2 Cloning the Insect GABA Receptor and Resistance Mutations

Ffrench-Constant and coworkers isolated and cloned the resistance to dieldrin (Rdl) gene [28] from a strain of *Drosophila melanogaster* that exhibited high levels of resistance [29] and nerve-insensitivity [30] to cyclodienes and picrotoxinin [28]. This single gene gave 4000-fold resistance to dieldrin and 22 000-fold resistance to picrotoxinin [31], and had high homology to vertebrate $GABA_A$ receptor subunits [28].

Rdl orthologs have since been cloned from many other insect species and, when heterologously expressed in the *Xenopus* oocyte expression system or in cell lines, give membrane receptors that behave like insect GABA receptors. Rdl

receptors are activated by GABA but not glutamate, and are blocked by picrotoxinin, dieldrin and fipronil, but not bicucculine. Rdl is widely expressed in adult *Drosophila* brain and thoracic ganglia, and occurs in regions that also stain for GABA and glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamate. Rdl is not expressed in muscle, although insect muscle is known to have GABA receptors [32, 33].

A single base-pair mutation leading to an alanine to serine (A-S) substitution near the cytoplasmic end of transmembrane domain II (designated the M2 segment) of Rdl at position 302, designated A302S, was found to be invariably present in many dieldrin-resistant strains of *D. melanogaster*, but not in susceptible strains. A mutation at this site was also invariably correlated with dieldrin resistance in *D. simulans*, but in this case the mutation was also sometimes to glycine (A302G) [34]. Elegant confirmation that this single base pair mutation in Rdl confers resistance was provided by using a susceptible Rdl allele to transform resistant individuals to susceptibility [28, 35].

A mutation in Rdl corresponding to A302S is associated with dieldrin resistance in many other species. Owing to variation in sequence length, a relative numbering system starting from the cytoplasmic end of the M2 segment is convenient when comparing different subunits. Thus, A302S becomes A2′S. Dieldrin-resistant aphids, *Nasonovia ribisnigri* with the A2′S mutation [36] and *Myzus persicae* with A2′G [37] have been described, as well as the mosquitoes *Aedes aegypti*, *Anopheles stephensi* [38], *Anopheles gambiae* and *Anopheles arabiensis* [39] with A2′S.

The functional consequence of the A2′S and A2′G mutations to the NCA site was established first with receptor binding studies. Both mutations reduced the affinity and density of [^{3}H]-EBOB binding sites, and also reduced the potency of eight NCAs in inhibiting [^{3}H]-EBOB binding. In *D. simulans*, A2′G was less effective than A2′S in protecting the chloride channel from the blockers, but equally effective in protecting the flies from their lethal effects [40]. In that study, *D. melanogaster* flies with the A2′S mutation were 73-fold resistant to fipronil, whereas *D. simulans* with A2′S and A2′G were 23- and 41-fold resistant, respectively. Similar levels of cross-resistance to fipronil in dieldrin-resistant house flies were seen [12], whereas fipronil resistance in dieldrin-resistant German cockroaches was only eight-fold, even though resistance to some fipronil analogs was much higher [41, 42]. Resistance levels are often highly structure-dependent when a mutation affects binding directly.

Rdl homomultimers containing the A2′S mutation, when heterologously-expressed in *Xenopus* oocytes, were highly resistant to dieldrin and picrotoxinin [43]. Careful measurement of the effect of fipronil on wild type *D. simulans* Rdl homomultimers showed the IC_{50} for block of the peak current decreased from 31 nM after 15 min of incubation to 3.6 nM after 30 min, in line with the binding data cited above. Because equilibration of fipronil with mutant receptors is almost complete after 15 min, the resistance ratio increased from only 3 at 15 min to 23 at 30 min [44]. Thus, the level of resistance to channel block by fipronil corresponds very well to the levels measured in bioassays, as discussed in the previous paragraph.

A second mutation in Rdl, T350M, was isolated from a *D. melanogaster* strain selected in the laboratory for high levels of resistance to fipronil. This mutation made the peak GABA-activated current five-fold resistant to fipronil. The A302S/T350M double mutant receptor was 50-fold resistant to fipronil. Nevertheless, even both of these mutations together could not account for the 20 000-fold resistance to fipronil in this strain. Other factors, such as metabolism, were not excluded [44].

29.5.2.3 **Ligand-gated Chloride Channel Structure and Classification**

The molecular biology and classification of GABA- and glutamate-gated chloride channels (GluCls) of insects has recently been reviewed [16, 45, 46]. GABA- and glutamate-gated chloride channels are members of the cys-loop family of ionotropic neurotransmitter receptors, which includes nicotinic acetylcholine receptors, 5-HT_3 (serotonin type 3) receptors and strychnine-sensitive glycine receptors of vertebrates, as well as 5-HT- and histamine-gated chloride channels of invertebrates. Cys-loop receptors are pentameric transmembrane proteins composed of up to four distinct but closely related subunits arranged symmetrically around an integral ion-conducting pore. Each subunit has four transmembrane regions, M1–M4, with a large intracellular loop containing phosphorylation sites between M3 and M4, and a long N-terminal extracellular region involved in ligand binding. The pore is formed largely from the M2 helices, with the large N-terminal regions forming two neurotransmitter binding sites per receptor.

The 2′ position in the M2 segment is now well established as a key residue in the NCA binding site for all ligand-gated chloride channels. Homomers of subunits containing 2′A are sensitive to NCAs, whereas those with S, T, M, or G in this position show reduced sensitivity. The NCA sensitivity of heteropentamers depends on their complement of 2′A-containing subunits (Section 29.5.2.5).

29.5.2.3.1 **GABA Receptors**

Vertebrate ionotropic GABA receptors are divided into $GABA_A$ and $GABA_C$ receptors, based on pharmacology and kinetics. $GABA_A$ receptors are complex allosteric proteins that desensitize, are antagonized by the alkaloid bicucculine and contain distinct binding sites for barbiturates, benzodiazepines, pregnane steroids, furosemide, loreclazole, picrotoxinin, zinc, lanthanum, volatile anesthetics and the anesthetic propofol. $GABA_C$ receptors are generally non-desensitizing and are insensitive to bicucculine and many of the modulators of $GABA_A$ receptors.

Nineteen different ionotropic GABA receptor subunits, named α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π and θ and ρ_{1-3}, have been cloned from vertebrates. Experiments using recombinant expression and immunoprecipitation indicate that native vertebrate $GABA_A$ receptors contain at least α, β and γ subunits, sometimes also with δ, ε, π or θ subunits, while $GABA_C$ receptors are believed to be composed only of ρ subunits.

$GABA_C$ receptors, found in the retina, cerebellum and spinal cord of mammals, are composed of three types of rho subunit, ρ_{1-3}. While the ρ_2 and ρ_3 sub-

units contain S in position 2′ and would be expected to be insensitive to many NCAs; the 2′P residue of ρ_1 is unique. The ρ_1 homomers bind lindane and EBOB quite well, but are highly resistant to fipronil [47]. Thus, while $GABA_C$ receptors may play a significant role in the mammalian toxicology of some insecticides, they are probably not significantly affected by fipronil.

Insect GABARs do not fit into the vertebrate classification, because most are simultaneously insensitive to the $GABA_A$-diagnostic antagonist bicucculine but sensitive the $GABA_A$-specific benzodiazepines and barbiturates. Bicucculine-sensitive receptors that are sensitive to allosteric modulators of $GABA_A$ receptors have also been observed in insects [26].

Two distinct types of ionotropic GABA receptor subunits have been cloned from insects. Rdl has been cloned from at least eight species, and ligand-gated chloride channel homolog 3 (LCCH3) has been cloned from *Drosophila*. A third subunit, glycine-receptor-like subunit of *Drosophila* (GRD) has 40–44% identity to vertebrate $GABA_A$ receptor α subunits and 40–41% identity to glycine receptor α subunits, but shows more similarity to ligand-gated cation channels in a region that determines ion selectivity. Accordingly, it coexpresses with LCCH3 to form a GABA-gated *cation* channel [48]. While this receptor is very sensitive to block by PTX, it is insensitive to dieldrin and lindane – its sensitivity to fipronil was not tested.

The Rdl polypeptide of insects has about 30–38% identity with vertebrate GABA receptor subunits, about the same amount as exists among the different types of vertebrate subunits, and is no more similar to the $GABA_A$ than $GABA_C$ types. In fact, it resembles vertebrate glycine receptors more than it does GABA receptors, but Rdl homomers do not respond to glycine. Rdl is expressed throughout the adult and embryonic insect nervous system, but not in muscle, and its pharmacology is like that of insect CNS GABA receptors and distinct from that of muscle GABA receptors. Homomeric Rdl receptors differ significantly from native GABA receptors in cockroach DUM neurons with respect to sensitivity to benzodiazepines [26], and single-channel studies show clear differences in conductance and gating between homomeric Rdl and native *Drosophila* GABA receptors in isolated neurons [49]. On the other hand, other native GABA receptor subtypes may exist that are not measured in the soma of isolated neurons.

LCCH3 has 47% identity to vertebrate GABA receptor β subunits, but is unlikely to be a subunit of native insect GABA receptors. Although it can form functional heteromultimers with Rdl subunits, these are unlike any known native insect GABA receptors in that they are bicucculine-sensitive, PTX-insensitive and undergo a slow desensitization [26, 49]. Furthermore, the distributions of LCCH3 and Rdl in the *Drosophila* nervous system do not overlap [50].

29.5.2.3.2 **Glutamate-gated Chloride Channels**

A single insect GluCl gene, DmGluClα, has been cloned from *Drosophila*, expression of which yields homomeric receptors with pharmacology that is distinct from native *Drosophila* GluCls, indicating that, like Rdl, it assembles with other subunits in native receptors [51–53]. One population of receptors containing

DmGluClα, but not Rdl, exists in the *Drosophila* nervous system and binds avermectin but not nodulisporic acid, both of which are allosteric activators of GluCls. Another population, assembled from both DmGluClα and Rdl subunits and binding both avermectin and nodulisporic acid, also exists in the *Drosophila* nervous system, but coexpression of DmGluClα and Rdl in the *Xenopus* oocyte expression system does not yield functional heteromultimers, indicating that other subunits may be needed [54].

The effect of NCAs on homomeric DmGluClα was not tested, but the presence of S at position 2′ suggests that these channels would have low sensitivity. In contrast, native insect GluCls are indeed sensitive to NCAs. Isolated cockroach neuronal somata contain two GluCl subtypes, desensitizing and non-desensitizing [15], which were blocked by fipronil with IC_{50}s of 800 and 10 nM, respectively. Fipronil sulfone, a major bioactive metabolite of fipronil [55], blocked these two receptors even better than fipronil, with IC_{50}s of 25 and 9 nM, respectively. By comparison, fipronil and its sulfone blocked GABA receptors in cockroach neurons with IC_{50}s of 27 and 20 nM, respectively [14]. The *C. elegans* chloride channel subunit GluClα, with 2′T, was almost insensitive to PTX, with an IC_{50} of 59 μM. GluClβ, however, with 2′A, was very sensitive, with an IC_{50} of 77 nM, and mutation to 2′S conferred more than 10 000-fold resistance [56]. GLC-3, a 2′T-containing GluCl from *C. elegans*, was insensitive to PTX but was blocked by BIDN, with an IC_{50} of 0.2 μM, and weakly by fipronil, with an IC_{50} of 11.5 μM [57].

In conclusion, fipronil and/or its sulfone potently block at least one subtype of GABA receptor and two subtypes of GluCl in insects. Since Rdl coassembles with DmGluClα *in vivo*, it may be a component of all fipronil-sensitive receptors, and dieldrin resistance may dampen the effect of fipronil on all of these receptors, which has not yet been tested. Two GABA (Rdl and LCCH3) subunits and a single GluCl (DmGluClα) subunit are known in insects. While LCCH3 can form heteromultimers with Rdl *in vitro*, they do not appear to occur together in native receptors.

29.5.2.3.3 Histamine-, Proton- and Glycine-gated Chloride Channels

Two histamine-gated chloride channel subunits (HisCl1 and HisCl2, Fig. 29.5.3) expressed in *Drosophila* eye both contain T at position 2′ and are insensitive to PTX and fipronil [58]. In addition, two novel proton-gated chloride channel subunits in this family (pHClA, B and C in Fig. 29.5.3), of unknown function, both contain M at position 2′ and are also insensitive to PTX and fipronil [59].

29.5.2.4 Mechanism of Block

The action of channel blockers can be dependent on the state of the channel. Ligand-gated ion channels are predominantly in the resting state in the absence of agonist, and can transition to activated, or open, states, and desensitized states when an agonist is applied. Activation of GABA and GluCl receptors enhances the blocking action of fipronil and its sulfone [14, 60, 61]. Furthermore, recovery of the desensitizing GluCl from block by either fipronil or its sulfone requires

```
                -2′  2′  6′ 9′
RN-GABA-α1    PARTVFGVTTVLTMTTLSIS
RN-GABA-α2    PARTVFGVTTVLTMTTLSIS
RN-GABA-α3    PARTVFGVTTVLTMTTLSIS
RN-GABA-α4    PARTVFGITTVLTMTTLSIS
RN-GABA-α5    PARTVFGVTTVLTMTTLSIS
RN-GABA-α6    PARTVFGITTVLTMTTLSIS

RN-GABA-β1    AARVALGITTVLTMTTISTH
RN-GABA-β2    AARVALGITTVLTMTTINTH
RN-GABA-β3    AARVALGITTVLTMTTINTH

RN-GABA-γ1    PARTSLGITTVLTMTTLSTI
RN-GABA-γ2    PARTSFGVTTVLTMTTLSTI
RN-GABA-γ3    PARTTLGITTVLTMTTLSTI

RN-GABA-ρ1    PARVPLGITTVLTMSTIITG
RN-GABA-ρ2    PARVSLGIMTVLTMSTIITG
RN-GABA-ρ3    PARVSLGITTVLTMSTIVTG

DM-Rdl        PARVALGVTTVLTMTTLMSS
HV-Rdl        PARVSLGVTTVLTMTTLMSS
DM-LCCH3      SARVALGITTVLTMTTISTG
DM-GRD        ADRVSLGITTVLTMTFLGLE

DM-GluClα     PARVSLGVTTLLTMATQTSG
CE-GLC-3      PARVTLGVTTLLTMTTQASG
CE-GluClα     PARVTLGVTTLLTMTAQSAG
CE-GluClα2    PARVTLGVTTLLTMTTQSSG
CE-GluClβ     AGRVALGVTTLLTMTTMQSA

DM-HisCl1     PARVTLGVTSLLTLATQNTQ
DM-HisCl2     PARVTLGVTSLLTLSTQHAK

DM-pHCl-A/B   PARSMIGVTTMLNFFTTSNG
DM-pHCl-C     PARVMIGVTTMLNFFTTSNG

RN-Gly-α3     PARVALGITTVLTMTTQSSG
RN-Gly-α1     PARVGLGITTVLTMTTQSSG
```

Fig. 29.5.3. Alignment of the cytoplasmic halves of the M2 segments of various ligand-gated chloride channel subunits. Species are given before the subunit name as RN (rat), DM (*Drosophila melanogaster*), HV (*Heliothis virescens*), and CE (*Caenorhabditis elegans*).

activation, whereas recovery of nondesensitizing GluCls does not [14, 61]. PTX is also an open channel blocker of GluCl-N and prevents access of fipronil to its binding site in the channel [61].

Single channel measurements show that BIDN and fipronil reduce mean open time and increase the mean closed time, which is consistent with an open-channel blocking mechanism [27, 62].

29.5.2.5 Structure of the Binding Site

While some earlier studies proposed multiple, partially overlapping NCA sites in the GABA receptor [63], it is now thought that all NCAs bind at a single site within the GABA receptor pore [64].

Because the A2′S mutation confers widely varying levels of resistance to various NCAs, it was recognized to directly affect their binding [65], thus locating the NCA site within the M2 domain. Systematic mutation of suspected channel-lining residues to cysteine, and showing that they were accessible to irreversible modification by charged sulfhydryl reagents in functional receptors, has been used to identify pore-lining residues [66]. This method has confirmed that A2′ lies within the pore, near its cytoplasmic mouth. T6′, just one turn deeper along the M2 helix, was also shown by this method to line the pore.

A different approach [64] led to the same conclusion, and also showed that residues A-1′, A2′, T6′ and L9′, which are consecutively aligned on one face of the M2 helix, line the pore. Furthermore, mutation of residues A2′, T6′ and L9′ dramatically reduced binding of the ligands [^{3}H]-EBOB and [^{3}H]-BIDN, indicating that these three residues contribute to binding interactions at the NCA site. Using a homology model of the $GABA_A$ receptor [64], various NCAs could be manually docked into the proposed binding site of a homopentameric mammalian $GABA_A$-β_3 receptor. Figure 29.5.4 shows the interactions of fipronil with, from the cytoplasmic end outward, A2′, T6′ and L9′, when docked in a similar model. Detailed study of homology models may prove useful for better understanding the QSAR of NCAs and the mechanism of resistance.

29.5.3 Chemistry

29.5.3.1 Chemistry and Synthesis of Fiproles and Intermediates

Table 29.5.1 summarizes the physicochemical properties of fipronil and ethiprole.

A remarkable ring closure reaction was elaborated for the synthesis of 3-cyano-1-(phenyl)pyrazoles [5]. 2,6-Dichloro-4-trifluoromethylaniline (**7**) is diazotized and then coupled with 2,3-dicyanopropionate to obtain phenyl-diazoester **8**, which is cyclized and decarboxylated to 5-amino-3-cyano-1-(2,6-dichloro-4-trichloromethyphenyl)pyrazole (**9**) [67] (Scheme 29.5.1).

The 4-trifluoromethylsulfinyl group can be introduced directly by treatment of **9** with trifluoromethyl*sulfinyl* chloride. Alternatively, the pyrazole-sulfide **10** can be synthesized by sulfenylation of **9** with trifluoromethyl*sulfenyl* chloride or with disulfur-dichloride to yield the dipyrazole-disulfide, which is then trifluoromethy-

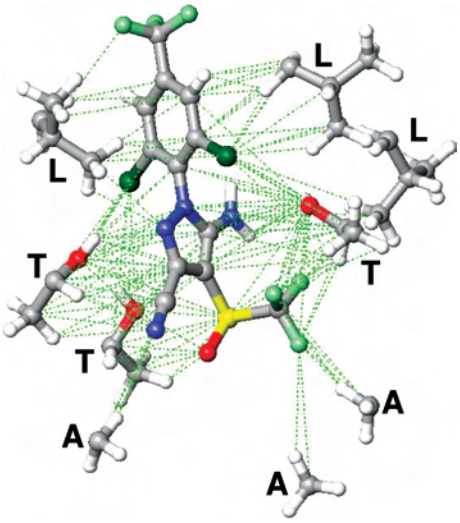
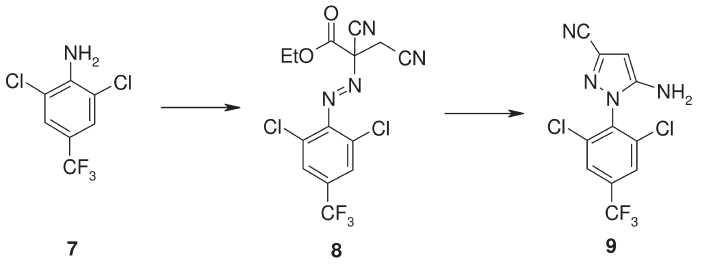


Fig. 29.5.4. Possible binding interactions of fipronil with side chains of residues A2′, T6′ and L9′ in the NCA site. Fipronil was docked into a model of the NCA binding site of a homopentameric mammalian $GABA_A$-β_3 receptor, after Ref. [64], using the Schrödinger Suite 2006 Induced Fit Docking protocol; Glide version 4.0, Schrödinger, LLC, New York, 2005; Prime version 1.5, Schrödinger, LLC, New York, 2005. (Figure courtesy Carsten Beyer, BASF AG.)

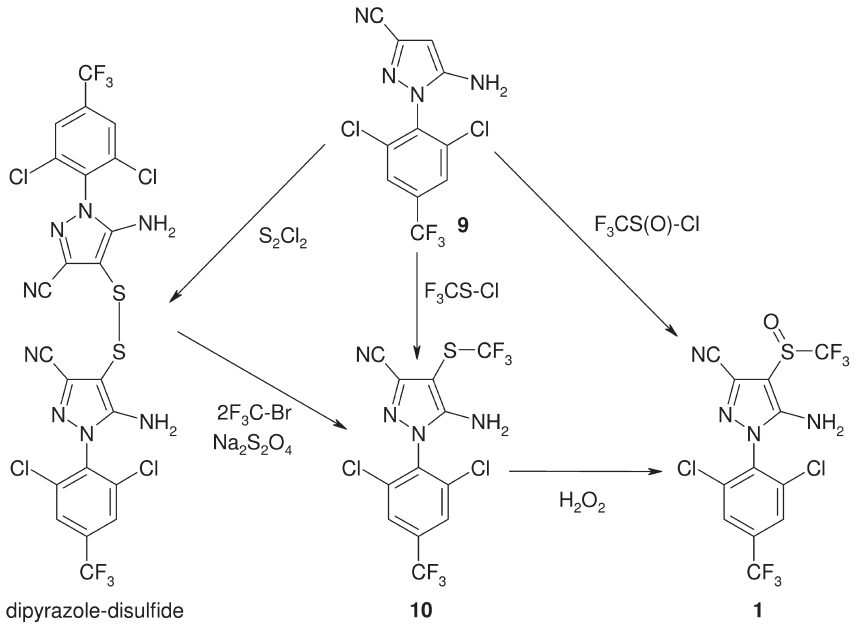
Table 29.5.1 Physicochemical properties [99, 140].

BSI common name	Fipronil (1)	Ethiprole (2)
Melting point (°C)	201	160–165
Vapor pressure (Pa)	3.7×10^{-7}	9.1×10^{-8}
Water solubility (20 °C) (mg L^{-1})	1.9–2.4	9
Log P_{OW} (20 °C)	4.00	2.9
Solubility in acetone (g L^{-1})	545.9	90.7
Patent	EP 295117	DE 19653417
Company	Rhône-Poulenc; Bayer CropScience, BASF	Rhône-Poulenc; Bayer CropScience, BASF



Scheme 29.5.1. Synthesis of 5-amino-3-cyano-pyrazole **9**.

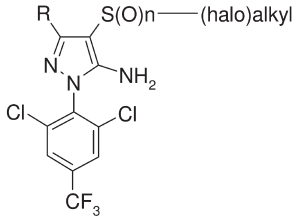
lated with bromotrifluoromethane in a radical-anion mediated reaction [5, 68, 79]. Oxidation of the pyrazole-sulfide **10** with peroxy agents leads to Fipronil (**1**) (Scheme 29.5.2).



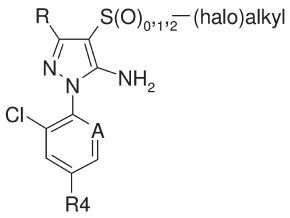
Scheme 29.5.2. Three routes for sulfenylation and trifluoromethylation of aminopyrazole **9**.

29.5.3.2 **Structure–Activity Relationships**

A broad program of chemical structure variation has been performed by several agrochemical, pharmaceutical and veterinary companies, as well as university groups. The 2,6-dichloro-4-(trifluoromethyl)phenyl group always gave the best insecticidal activity and became known as the ''Parnellophore'' or ''Magic Aryl'' group. Active phenyl variations are the 2-pyridyl analogues **12** [70, 71], the 2,6-dichloro-4-(pentafluorosulfenyl)phenyl group **13** [72], oxyfluormethylenes [73],



11 n=0 sulfide
n=1 sulfoxide
n=2 sulfone



12 A=N, R4= CF3 : Pyridyl analogues

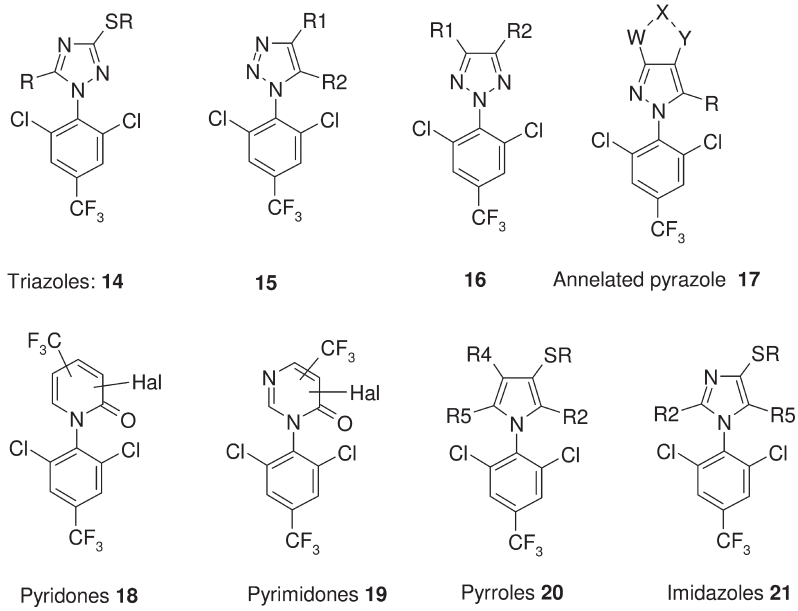
13 A= C-Cl, R4= SF5 : ICI-Fiproles

Fig. 29.5.5. Sulfur oxidation states and phenyl variations.

biphenyls [74] and other CF_3-substitutes [75]. All oxidation states of sulfur in the 4-(halo)alkylthio group are suitable for a good intrinsic activity, but sulfides, sulfoxides and sulfones **11** deliver different *in vivo* activity levels due to their polarity-related properties (Fig. 29.5.5).

The 2,6-dichloro-4-(trifluoromethyl)phenyl-heterocycle-4-(halo)alkylthio scaffold has been considered as the essential toxophore. The heterocyclic unit and the substituents at the 3- and 5-position on the heterocycle permit more variability and may serve as sites for prodrug attachment [76–79].

The heterocycles investigated include pyrazoles, triazoles [80–84], condensed pyrazoles [85], pyridones, pyrimidones [86], pyrroles [87, 88], imidazoles [89, 90] and indoles (**14–21**, Fig. 29.5.6), but pyrazoles are preferred [91].



Triazoles: **14** **15** **16** Annelated pyrazole **17**

Pyridones **18** Pyrimidones **19** Pyrroles **20** Imidazoles **21**

Fig. 29.5.6. Heterocycle variations.

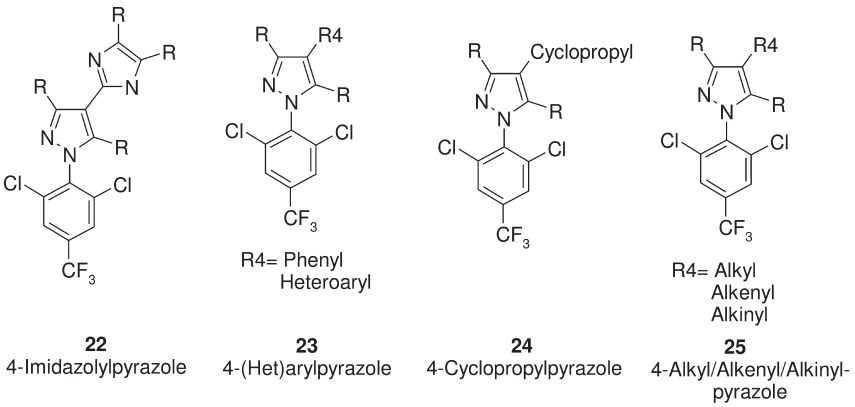


Fig. 29.5.7. Variations at the 4-position of the heterocycle.

Structural extensions to the essential toxophore concept include replacement of the 4-(halo)alkylthio group by imidazole [92] and other five-membered-heterocycles [93], by phenyl [94], by cyclopropyl and other cycloalkyls [95, 96], by alkyl, haloalkyl, alkenyl and alkinyl groups [75, 97] (**22**–**25**, Fig. 29.5.7).

The aim of the structural variation work was to improve pest spectrum, target receptor specificity, non-target safety, environmental behavior, degradation and photostability.

Despite the tremendous effort of so many research teams, none of the numerous variations have resulted in further development products to-date.

Although fipronil has a chiral, asymmetric sulfoxide group, studies have shown that there are no significant differences in activity between the two enantiomers on cotton stainer (*Dysdercus cingulatus*), grain weevil (*Sitophilus granarius*) or house fly (*Musca domestica*) [98].

29.5.4 Biological Properties

29.5.4.1 General

Fipronil has contact activity, but is particularly effective by ingestion. Because the target receptors are in the insect CNS, mortality may appear to be somewhat slow, but feeding cessation and other symptoms may be noted soon after treatment.

29.5.4.2 Biological Spectrum

Fipronil has extremely high activity on a wide range of insects and is registered for control of over 140 species on more than 100 crops across the globe. Insects in the orders Orthoptera (cockroaches, locusts), Isoptera (termites) and Diptera (flies) are generally highly sensitive and can be controlled at field use rates of from <1 to 25 g ha^{-1} (grams of active ingredient per hectare).

Insects in the orders Coleoptera (beetles and weevils), Thysanoptera (thrips), Hemiptera (true bugs) and some families within the Lepidoptera (Plutellidae,

Pyralidae) show high susceptibility to fipronil, and are generally controlled with field use rates of 25 to 75 g ha^{-1}.

Homopterans (aphids and whiteflies) and Lepidoptera, Noctuidae (bollworms, armyworms) show low susceptibility to fipronil, requiring use rates of more than 200 g ha^{-1} [8, 99].

29.5.4.3 Soil Applications

As a soil treatment, fipronil provides excellent control of a wide range of insect pests in numerous crops, at rates of 50–200 g ha^{-1}.

One of the largest uses of fipronil is the in-furrow, at-planting control of termites (e.g., *Heterotermes tenuis*, *Odontotermes takensis*) in sugarcane, oil palm and other plantation crops. At higher use rates (200–300 g ha^{-1}), a single treatment gives very good control in these long cycle crops. Good control of corn rootworm (*D. virgifera virgifera*), first generation European corn borer (*Ostrinia nubilalis*) and wireworm (*Agriotes* spp.) in maize is obtained with 100–150 g ha^{-1}, applied either as a granule or in-furrow spray at planting [99].

In paddy rice, granular application of fipronil at 25–75 g ha^{-1} provides control of virtually all major insect pests, including stem borers (*Chilo* spp., *Tryporyza* spp., *Rupela* spp., *Ostrinia* spp.), brown plant hopper (*Nilaparvata lugens*), rice water weevil (*Lissorhoptrus* spp.), and thrips (*Frankliniella* spp., *Stenchaetothrips* spp., *Thrips* spp., etc.).

Fipronil is used extensively for the control of insect pests in specialty crops. In bananas, good control of banana weevil (*Cosmopolites sordidus*) and some thrips species can be achieved with fipronil granules applied to the soil at the base of the mat at 0.1 to 0.2 g ha^{-1} [106]. In vegetable crops, in-furrow applications of both liquid and granular formulations give good control of root maggots and thrips [100, 101].

29.5.4.4 Seed Treatment

Fipronil may be applied to seeds using several application methods, from small-scale equipment to industrial-scale seed treatment stations.

Very good control of wireworm and white grubs in corn and of wireworm in sunflower and sugar beet has been demonstrated at the equivalent of 50 to 200 g ha^{-1} [99, 104]. A fipronil-based soybean seed treatment gives excellent control of white grubs and soybean stem weevil (*Sternechus subsignanthus*), and has become a standard treatment in this key crop in Brazil [99]. At 10–50 g ha^{-1}, good control of several species of thrips (Thysanoptera) is obtained from a seed treatment in cotton [105].

Fipronil's high intrinsic activity against Dipterans allows its successful use as a seed treatment in several crops for control of root maggots. In cereals, fipronil provides excellent control of wireworm and wheat bulb fly (*Delia coarctata*) at rates of 50 g per 100-kg seed [8]. In leeks, seeds film-coated with fipronil gave excellent control of onion fly (*Delia antiqua*) as well as thrips and onion moth [106, 107].

29.5.4.5 Use in Crop Baiting Systems

Fipronil-based baiting systems for agricultural and fruit fly pests have either been recently developed or are in development. If suitable attractants and/or feeding matrices are available, the non-repellency and high activity of fipronil should allow for the development of more of these insect control systems in the future.

Fipronil-based baits can control ant pests in crops at rates as low as a few grams, or even milligrams per hectare [99]. Sucrose-based liquid baits gave excellent control of Argentine ants (*Linepithema humile*) and may be a tool for controlling these attendant ants in California grapes [108]. A low-assay, protein-based bait gave excellent control of two pest species of *Iridomyrmex* in Australian citrus [109].

Tephritid fruit flies are extraordinarily susceptible to fipronil, which, combined with appropriate attractants, makes highly active bait formulations for use in control and eradication programs. Stations using Cue-lure as an attractant have been shown to attract and control melon fly (*Bactrocera cucurbitae*) for up to 77 days and oriental fruit fly (*Bactrocera dorsalis*) for up to 21 days [110]. Fipronil has good activity in baits that target the blueberry maggot (*Rhagoletis mendax*) when combined with olfactory and visual attractants [111, 112]. Several different fipronil-based systems have been commercialized or are in development for fruit fly control.

29.5.4.6 Urban Pest Control Applications

Outstanding activity against several urban insect pests, formulation flexibility (gel, liquid, granule, bait, etc.) and horizontal transfer in termite, ant, and cockroach populations, has quickly made fipronil one of the most successful urban pest control agents.

As a liquid termiticide, fipronil provides long-term (>10 years) control of many urban termite species [113]. It is not detected in the soil by termites [114, 115] and its relatively slow action against both subterranean (*Reticulitermes flavus*) and Formosan termites (*Coptotermes formosanus*) [116] allows transfer, through several routes, to other members of the colony, leading to colony elimination [117].

Fipronil is extraordinarily active against cockroaches, and shows no cross-resistance with currently available chemistry [118, 119]. Food-based gel bait formulations assist the transfer of active ingredient from exposed individuals to unexposed adults and nymphs. This has been shown to be significant through several different routes of exposure [120–122]. Fipronil-based gel bait formulations are highly palatable to cockroaches, which appears to enhance their effectiveness [123]. The high intrinsic activity and an excellent, palatable formulation have made gel baits containing fipronil a key component in cockroach control.

Fipronil is highly active against many nuisance ants. Exterior perimeter, or barrier, treatments have shown excellent results against most key species, including Pharaoh ants (*Monomorium pharaonis*) [124], Argentine ants (*Linepithema humile*) [125] and a mixed population of eight different species [126]. Fipronil was shown to be readily transferable among Argentine ants after crossing a treated sand barrier [126] and this may partly explain the success of this treatment method. The

use of fipronil-based exterior perimeter sprays has now become a key strategy for long-term control of ants infesting the interiors of structures. Fipronil gives long-term control of red imported fire ant (*Solenopsis invicta*) in baits at rates as low as 25.5 to 51 mg ha^{-1}, or with broadcast granular treatments [127] and these products are now one of the most widely used fire ant control tools in both the professional and the consumer arenas.

29.5.4.7 **Turf and Ornamental Applications**

In turf and ornamentals, fipronil is extremely effective as a granular formulation against larvae of black vine weevils (*Otiorhynchus sulcatus*) in containerized ornamentals [102] and against many Orthopteran insects, including mole crickets (*Scapteriscus* spp.) [103] in turfgrass. Fipronil gives excellent control of Japanese beetle (*Popillia japonica*) when applied as a soil drench or injection to field-grown ornamental trees.

29.5.4.8 **Animal and Human Health Uses**

Fipronil has high intrinsic activity against a wide range of animal health pests and has become the standard treatment for flea and tick control on domesticated animals. Cat fleas (*Ctenocephalides felis*) are extremely sensitive to fipronil [128] when applied as either a spray [129] or a ''spot-on'' [130] formulation to companion animals. Fipronil is also highly active on ticks [131, 132] and has been shown to reduce the transmission of the tick-borne causative agents of canine diseases [133, 134]. Fipronil has also been shown to control biting lice on both dogs and cats [135, 136]. Fipronil is applied as both a spray and a ''spot-on'' treatment as well as a combination product with s-methoprene.

A system for managing tick vectors of human Lyme disease is currently sold in the United States. Consisting of a plastic box that allows white-footed mice, hosts for the nymphal deer tick vector (*Ixodes scapularis*), to enter and receive a swipe of a liquid fipronil formulation on their backs, this system significantly reduces the number of adult and larval ticks on the mice, the infection rate of the spirochete (*Borrelia burgdorferi*) in the mice and the number of host-seeking nymphs on treated properties [137]. The system effectively interrupts the natural disease cycle and can lead to reduced numbers of human cases of Lyme disease when used properly.

29.5.4.9 **Resistance and Its Management**

While dieldrin target-site resistance (Section 29.5.2.2) can confer some level of resistance to fipronil, this cross-resistance has not led to failure of fipronil in the field. However, resistance due to other mechanisms was seen in southeast Asia as early as 1996 in diamondback moth (*Plutella xylostella*), within three years of the introduction of the product. The high intrinsic activity of fipronil and lack of alternatives in the mid-1990s led growers in countries such as Thailand to use it up to 40 times per year on cruciferous crops. By early 1997, many populations were resistant and field failures were widespread. At the same time, use of the

product against diamondback moth began to decrease as new, novel chemistries (indoxacarb, chlorfenapyr, spinosad) entered the Asian market.

By late 1998, the monitoring program in place since 1995 began to show a dramatic recovery of sensitivity to fipronil in *P. xylostella* populations from several locations in Thailand. In some areas, the LC_{50} returned to baseline levels. Subsequent laboratory studies indicated that resistant populations collected from Thailand showed a significant loss of fitness (Holmes, unpublished). Studies on cockroaches demonstrated a similar trend, with fipronil-resistant insects having lower fitness than susceptible insects [138]. Fipronil resistance in *Plutella* is incompletely recessive and controlled by a single locus [139], which, in combination with the high fitness penalty, makes rotation with other insecticides a very effective means of resistance management. As the resistance monitoring program showed in Thailand, when selection pressure was removed from the local populations of *P. xylostella*, the LC_{50} values returned almost to baseline levels.

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29.6
Chloride Channel Activators/New Natural Products (Avermectins and Milbemycins)

Thomas Pitterna

29.6.1
Introduction

To date, three compounds from this group of chloride channel activators (abamectin, emamectin benzoate, and milbemectin) have been commercialized in crop protection. In addition, a development compound has become known as lepimectin (provisionally approved ISO common name). In this introduction, the origin, synonyms and physicochemical properties of the marketed compounds [1] are summarized (see Tables 29.6.1 and 29.6.2). Further aspects will be discussed in detail in the following sections, such as their mode of action, discovery, chemistry, insecticidal activity, agronomic use, and safety. Several recent reviews cover

Table 29.6.1 Names and codes of market products.

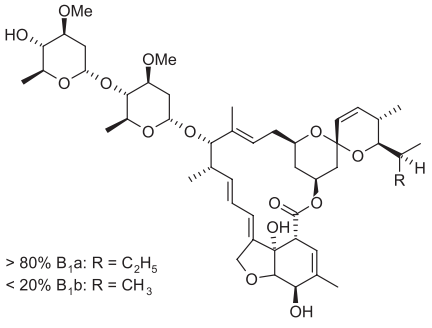
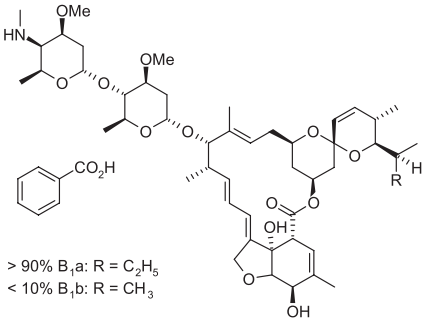
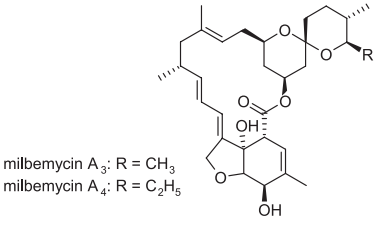
Compound name	Abamectin	Emamectin benzoate	Milbemectin
Structure			
Common names	Abamectin, abamectine	Emamectin, emamectine	Milbemectin
Other names	Avermectin B_1		
Composition	Mixture containing >80% avermectin B_1a and <20% avermectin B_1b	Mixture of emamectin B_1a (>90%) and emamectin B_1b (<10%), as their benzoate salts	Mixture of the homologues milbemycin A_3 (methyl) and milbemycin A_4 (ethyl) in the ratio 3:7
Selected product names	Agrimec®, Dynamec®, Vertimec®, Affirm®, Agri-Mek®, Avid®, Clinch®, Zephyr® (Syngenta)	Banlep®, Denim®, Proclaim® (Syngenta)	Milbeknock®, Ultiflora®, Koromite®, Matsuguard®, Mesa® (Sankyo Agro)

Table 29.6.2 Properties of market products.

Compound	Abamectin	Emamectin benzoate	Milbemectin
Melting point (°C)	161.8–169.4 (decomp.)	141–146	212–215
Solubility in water (mg L^{-1})	0.007–0.010 (20 °C)	24 (25 °C, pH 7)	7.20 (A_3, 20 °C) 0.88 (A_4, 20 °C)
Solubility org. solvents (g L^{-1})	Toluene: 350 (21 °C); cyclohexane: 6 (21 °C)	Toluene: 20 (25 °C); cyclohexane: 0.23 (25 °C)	Benzene: 143.1 (20 °C); *n*-hexane: 1.4 (20 °C)
Partition coefficient (log P_{OW})	4.4 ± 0.3 (pH 7.2)	3.0 (pH 5.1); 5.0 (pH 7.0); 5.9 (pH 9.0)	5.3 (A_3); 5.9 (A_4)
Vapor pressure (mPa)	$<3.7 \times 10^{-3}$ (25 °C)	4×10^{-3} (21 °C)	$<1.3 \times 10^{-5}$ (20 °C)
Dissociation constant	–	$pK_{a,1} = 4.18$ $pK_{a,2} = 8.71$	–

the family of avermectins and milbemycins, which has gained importance not only in crop protection but also in the field of animal health [2–7].

Abamectin is isolated from the fermentation of *Streptomyces avermitilis*, a naturally occurring soil Actinomycete (Table 29.6.2). It possesses strong anthelmintic, insecticidal, and acaricidal activity [8–13]. It was introduced as an acaricide and insecticide by Merck Sharp & Dohme Agvet (now Syngenta Crop Protection AG) in 1985.

Emamectin benzoate is produced by chemical synthesis from abamectin (Table 29.6.2) [14–17]. The extreme potency of this compound against Lepidoptera was discovered by Merck scientists [18]. Emamectin benzoate was introduced to the market by Novartis (now Syngenta Crop Protection AG) in 1997.

Milbemectin is isolated from the fermentation of *Streptomyces hygrocopicus*, another naturally occurring soil Actinomycete. It was introduced as an acaricide by Sankyo Co., Ltd. in 1990 (Table 29.6.2).

29.6.2 Mode of Action

The biochemical mode of action of avermectins and milbemycins has been discussed in several reviews [2–6]. All natural and semisynthetic avermectins and milbemycins interact with ligand-gated chloride channels, which are located in the nerve cells of their target organism. In particular, they act on invertebrate

glutamate-gated chloride channels and some vertebrate and invertebrate GABA receptors.

Binding of the neurotransmitter, such as glutamate and GABA, renders the channel transiently permeable to chloride ions. Avermectins and milbemycins exert their action by potentiating the effect of the neurotransmitter, thus increasing the influx of chloride ions into nerve cells. This results in the disruption of nerve impulses and cell function. As a consequence, invertebrates are rapidly paralyzed.

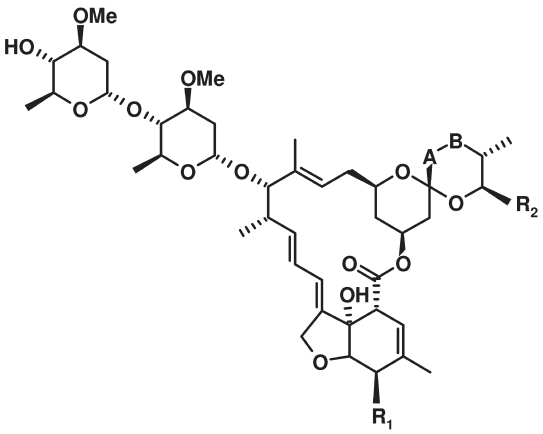
This chapter summarizes the key findings contributing to our current understanding of the pharmacological effects of avermectins and milbemycins on different target organisms. Fritz et al. [19] were first to find that avermectins act as chloride channel agonists and open chloride channels. It was shown that avermectins act at a site different to that of the cyclodiene insecticides [20]. Cassida and coworkers [21] demonstrated that avermectins bind to saturable, high-affinity binding sites in *Drosophila melanogaster*. They also showed, among several avermectin analogues, a correlation of binding (IC_{50}-values for displacement of radiolabeled avermectin) with insecticidal activity against flies. A binding site related to a glutamate-gated channel in *Drosophila melanogaster* has been identified [22, 23] and transcripts related to the same subunit (DrosGluCl-alpha) have been found in other insects, such as cat flea (*Ctenocephalides felis*), fall armyworm (*Spodoptera frugiperda*), and cotton bollworm (*Helicoverpa zea*). No such findings have been reported so far for spider mites.

Avermectins and milbemycins are taken up by insects and mites via contact and ingestion. Under field conditions, ingestion is the primary route of uptake [24]. Although the maximum mortality of affected insects may occur only after 2–4 days, feeding stops very soon because of irreversible paralysis. Thus, feeding damage on the crops is prevented.

29.6.3
Discovery and Chemistry of Avermectins

The naturally occurring avermectins are a group of 16-membered macrocyclic lactones, which are produced by fermentation from Actinomycetes from the genus *Streptomyces* (Fig. 29.6.1). The soil microorganism *Streptomyces avermitilis* MA-4860 (NRRL 8165) was first isolated at Merck Research Laboratories in 1976 from a soil sample of Japanese origin, collected by researchers of the Kitasato Institute [25]. From the fermentation, eight different avermectins were isolated, which consist of four pairs of homologues. Each pair contains a major component (the a-component) and a minor one (b-component), which are usually produced in a ratio between 80:20 and 90:10.

One of these pairs, avermectin B_1, i.e., the mixture of avermectins B_1a (>80%) and B_1b (<20%), is commonly referred to as abamectin (Fig. 29.6.2). It was found to be active against nematodes [8, 9], insects [10–12], and mites [13]. Subsequently, abamectin was selected for development in crop protection, and it was introduced to the market-place as an agricultural pesticide against a broad spectrum of phytophagous mites and insects in 1985. Merck scientists performed a



	R_1	A-B	R_2
A_1a	-OMe	-CH=CH-	*s*-butyl
A_1b	-OMe	-CH=CH-	*i*-propyl
A_2a	-OMe	$-CH_2$-CHOH-	*s*-butyl
A_2b	-OMe	$-CH_2$-CHOH-	*i*-propyl
B_1a	-OH	-CH=CH-	*s*-butyl
B_1b	-OH	-CH=CH-	*i*-propyl
B_2a	-OH	$-CH_2$-CHOH-	*s*-butyl
B_2b	-OH	$-CH_2$-CHOH-	*i*-propyl

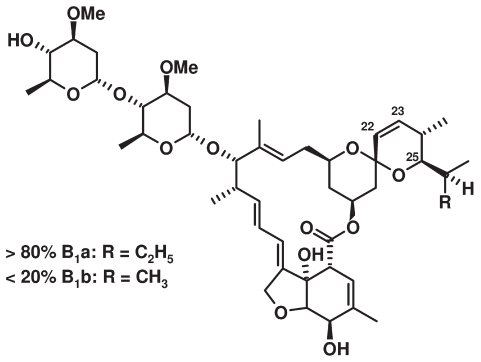
Fig. 29.6.1. Structures of naturally occurring avermectins.

targeted analoging program around abamectin, which was mainly focused on the identification of a compound active against a broad spectrum of Lepidoptera [26–29]. The program culminated in the discovery of emamectin [18], at first as its hydrochloride salt, which was labeled MK-243. The compound was developed as the benzoate salt (MK-244) for the control of Lepidoptera in crop protection. Emamectin benzoate was introduced to the market by Novartis (now Syngenta Crop Protection AG) in 1997 under the trade names Proclaim® and Affirm®.

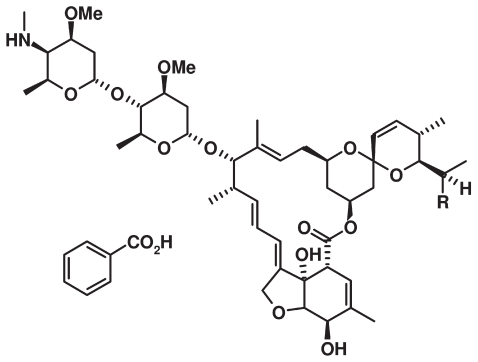
Emamectin is prepared from abamectin in four chemical steps [14–17]. Therefore, it is also a mixture of the two homologs B_1a and B_1b. The allylic hydroxy group at C5 of avermectins is the most reactive in the molecule. It has to be protected before reactions on the C4″ hydroxy group can be performed. Reaction with *t*-butyl-dimethylchlorosilane and imidazole in *N*,*N*-dimethylformamide gives the 5-O-*t*-butyldimethylsilyl ether (Scheme 29.6.1).

Alternatively, the C5 hydroxy group can be protected as 5-O-allyloxycarbonyl derivative instead (not shown in Scheme 29.6.1). This is done by reaction of abamectin with allylchloroformate and tetraethylendiamine in *t*-butyl methyl ether. In this case, the C5 hydroxy group can be deprotected in the last step by treatment with sodium borohydride in ethanol in the presence of catalytic amounts of tetrakis(triphenylphosphine)palladium.

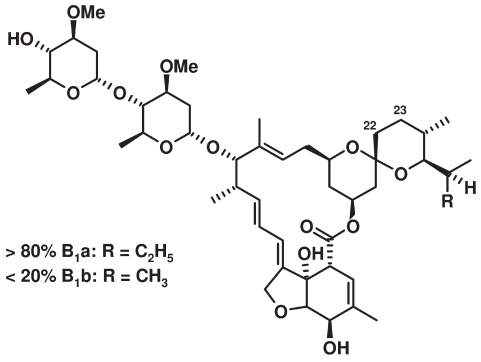
After protection, the C4″ hydroxy group is oxidized to the ketone, which can be achieved with dimethyl sulfoxide and phenyldichlorophosphate (or oxalyl chloride) in the presence of triethylamine. Subsequently, the reductive amination of the ketone can be performed with methylamine, acetic acid and sodium borohydride in methanol. Alternatively, the ketone is treated with heptamethyldisilazide and zinc chloride in *iso*-propyl acetate, followed by reduction of the intermediate imine with sodium borohydride in the presence of ethanol. This transformation leads to the (*R*)-configured 4″-desoxy-4″-epi-methylamino derivative as the predominant product, with only very small amounts of the 4″-(*S*)-isomer being



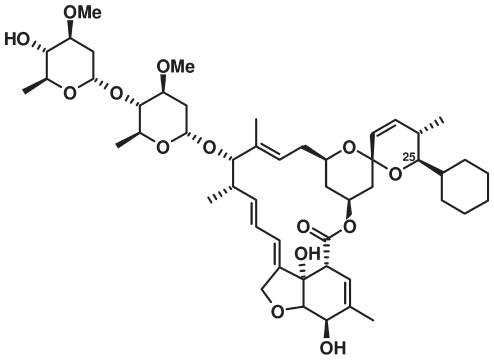
Abamectin (Vertimec®, Agrimec®)



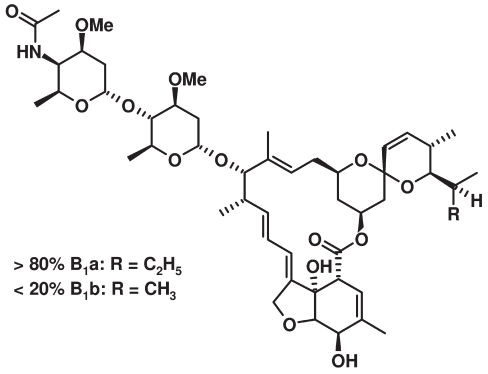
Emamectin Benzoate (Proclaim®, Affirm®)



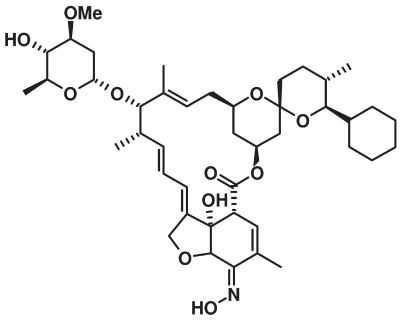
Ivermectin (Heartguard®, Ivomec®)



Doramectin (Dectomax®)

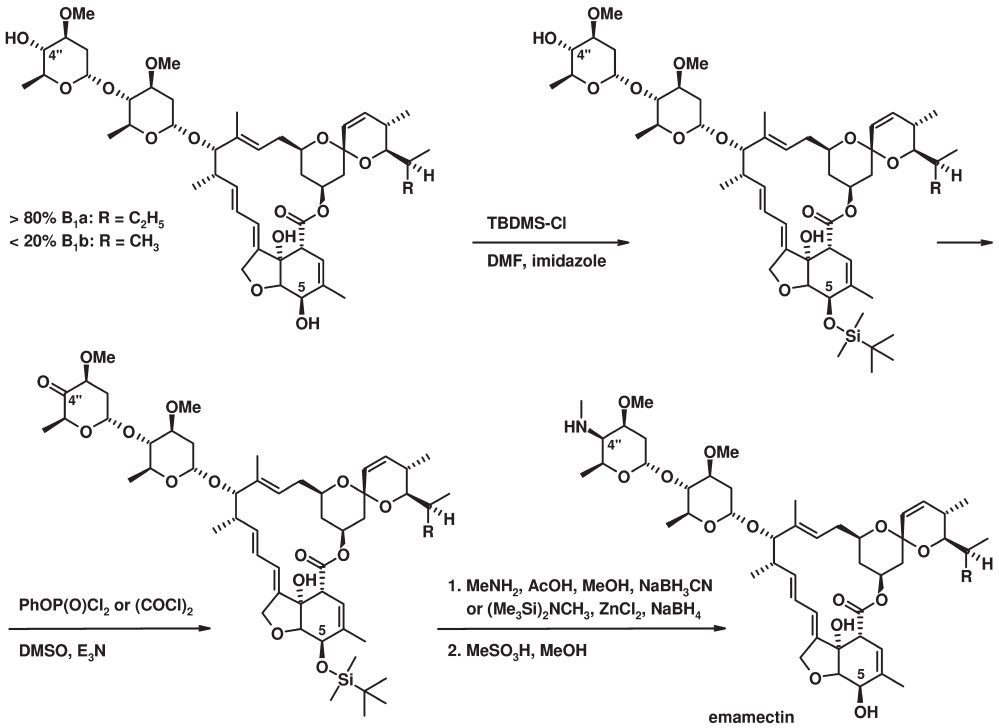


Eprinomectin



Selamectin

Fig. 29.6.2. Structures and names of avermectins.



Scheme 29.6.1. Synthesis of emamectin.

formed. Deprotection, e.g. with methanesulfonic acid in methanol, completes the synthesis of emamectin.

In addition to the use in crop protection, abamectin has been commercialized as an antiparasitic drug in animals. Further avermectin derivatives, ivermectin (Merck), doramectin (Pfizer), eprinomectin (Merck), and selamectin (Pfizer), were commercialized as endo- and ectoparasiticides (Fig. 29.6.2). As these four compounds are not used in crop protection, they will not be discussed in much detail here, except for the following. Ivermectin [30] is derived from avermectin B_1 via selective hydrogenation of the 22,23 double bond. As an anthelmintic in farm animals, it has been on the market since 1981. In dogs it is used for the prevention of heartworm infections. It has also found use in human medicine for the treatment of onchocerciasis, or river blindness [31]. Eprinomectin [32], 4″-desoxy-4″-acetylamino-avermectin B_1, is an advanced development product from Merck. It is a broad spectrum paraciticide for farm animals. In a quite similar manner as emamectin, eprinomectin is produced from avermectin B_1 by chemical synthesis. Doramectin [33, 34], another avermectin derivative for animal health applications, has been developed by Pfizer. The compound has the same structure as avermectin B_1a except that a cyclohexyl group replaces the *s*-butyl substituent at C25. Doramectin is produced by fermentation, using a mutant

Streptomyces strain capable of incorporating the cyclohexyl substituent at C25 from a source that is added to the fermentation medium. Selamectin [34, 35] is a new experimental antiparasitic drug introduced by Pfizer. It is a semisynthetic avermectin-monosaccharide derivative, and it is produced from doramectin by chemical synthesis.

29.6.4
Discovery and Chemistry of Milbemycins

The discovery of milbemycins was first reported by researchers from Sankyo in 1974 [36, 37]. The original producing strain SANK 60576 was designated *Streptomyces hygroscopicus* subsp. *aureolacrimosus* [38]. The fermentation products from this Actinomycete and its mutants are many. Thirteen milbemycins were isolated from the original strain, they were named α_1 to α_{10} and β_1 to β_3 [39]. Later, the α_1 component was named milbemycin A_3, the α_3 component milbemycin A_4. More derivatives were isolated from mutant strains [40, 41], among them milbemycin D (Fig. 29.6.3).

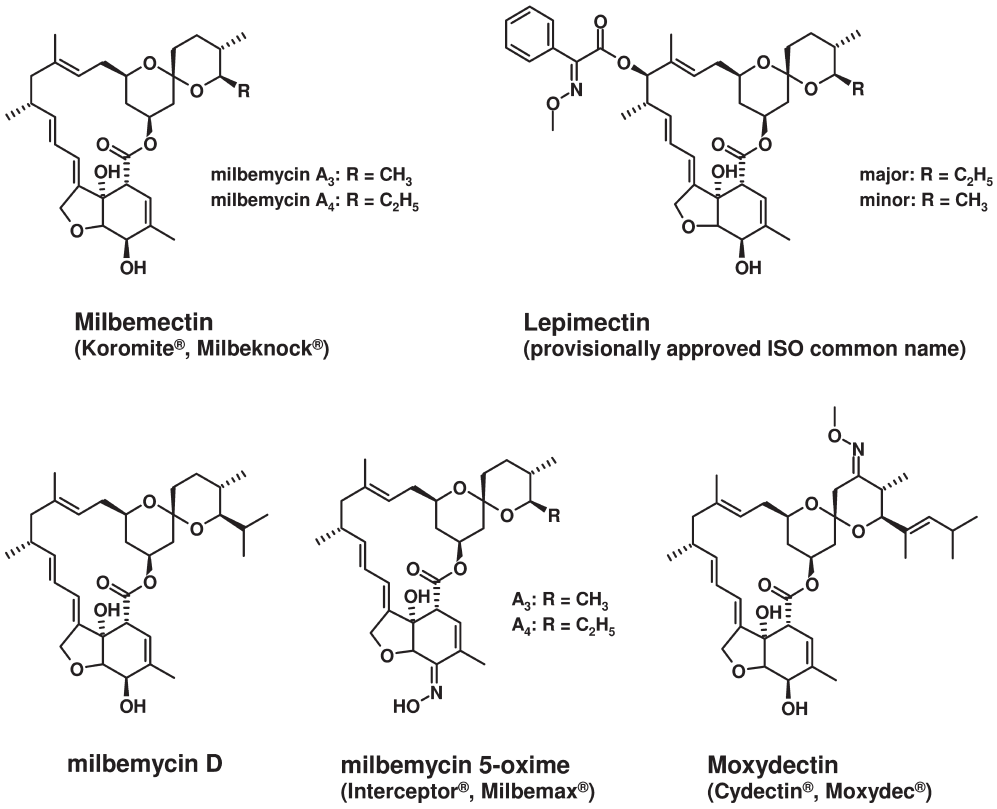
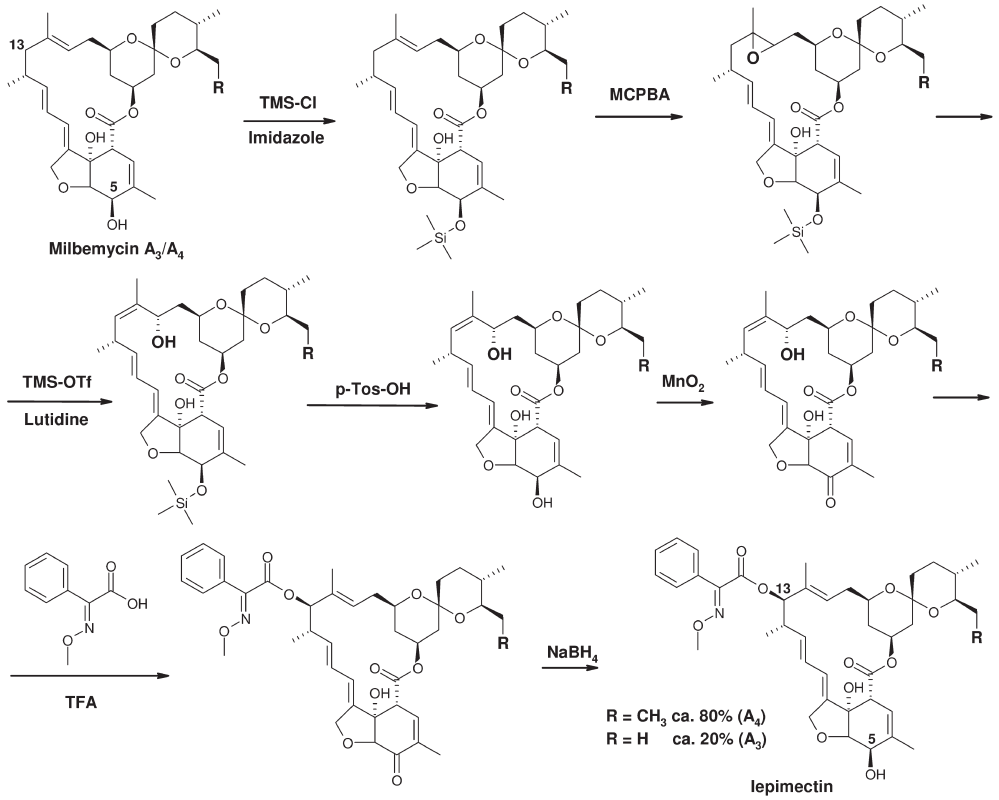


Fig. 29.6.3. Structures and names of milbemycins.

Milbemectin is the name for a mixture of milbemycins A_3 and A_4, also referred to as milbemycin A_3/A_4. It was launched as an acaricide under the trade name Milbeknock® by Sankyo in 1990. So far, it is the only milbemycin on the market for crop protection use. However, a second compound is expected to be launched by Sankyo, which became known as lepimectin (provisionally approved ISO common name) in 2004. Lepimectin is a semisynthetic derivative of milbemectin, thus containing an A_3 and an A_4 component, the latter being the major one.

The synthesis of lepimectin (Scheme 29.6.2) is described in the following, as it is published in the patent literature [42–44]. However, it may be assumed that this sequence will be modified in the actual industrial preparation. To introduce the required oxygen functionality at C13, milbemycin A_3/A_4 is first protected as 5-O-trimethylsilyl ether. Reaction with 3-chloroperbenzoic acid results in the epoxidation of the double bond between C14 and C15. The epoxide is rearranged by treatment with a mild Lewis acid (trimethylsilyl triflate), and the product is deprotected. To suitably protect the sensitive allylic C5 hydroxy group, it is oxidized to the ketone. The C13 ester substituent is introduced by an acid-mediated



Scheme 29.6.2. Synthesis of lepimectin.

substitution, accompanied with allylic rearrangement. Notably, the resulting stereochemical orientation of the C13 substituent is opposite to that found in avermectin derivatives. The sequence is completed by reduction of the C5 ketone with sodium borohydride, thereby restoring the natural stereochemistry of the C5 hydroxy group.

Some milbemycins found use in the animal health field as antihelmintics, the first among which was milbemycin D, launched by Sankyo in 1986 (Fig. 29.6.3). Later, milbemycin 5-oxime was introduced by Sankyo and Ciba-Geigy (the latter of which is now Novartis Animal Health) in 1990. Milbemycin 5-oxime is a semisynthetic derivative of milbemectin (milbemycin A_3/A_4). Another series of milbemycin analogues from *S. cyanogriseus* was found by scientists from American Cyanamide, and gave rise to the discovery and development of moxidectin (a synthetic derivative of F-28249α) as an animal health drug [45].

29.6.5
Acaricidal and Insecticidal Activity

The whole family of macrocyclic lactones, consisting of the closely related avermectins and milbemycins, displays unprecedented potency against mites, insects, and nematodes. LC_{90} values in greenhouse trials are often in the range 0.1–0.01 ppm, in some cases even lower. The structure–activity relationships of this chemical class have been the subject of many publications. The present section discusses selected key findings.

In 1981 Putter et al. [13] reported the activity of avermectin B_1a against several important agricultural pests. The activity of abamectin against a more complete list of mites and insects was described by Fisher [48] in 1989. These data (Table 29.6.3) show that abamectin is highly potent against most of the important mite species, although somewhat weaker against *Panonychus citri*. They also show very high activity against some Lepidoptera, whereas others are less sensitive to abamectin, in particular *Spodoptera* ssp.

First structure–activity relationships relating to crop protection targets were reported by Fisher in 1984 [47]. This study showed that avermectin B_1a was somewhat more active then milbemycin D against *Tetranychus urticae*, *Heliothis virescens*, and *Meloidogyne incognita*. As milbemycin D can be viewed as the 22,23-dihydro-13-desoxy derivative of avermectin B_1b, this comparison reflects the influence of the disaccharide portion of avermectins on their activity as pesticides.

More structure–activity information concerning the substituents on C13 of the avermectin aglycone are given by Fisher [48] and Mrozik et al. [49]. The most important conclusions are the following (Scheme 29.6.3). Against *Tetranychus urticae*, avermectin B_1 monosaccharide (**2**) is as active as avermectin B_1 (**1**). The avermectin B_1 aglycone (**3**) is 30× less active. Surprisingly, both 13-desoxy-avermectin B_1 aglycone (**4**) and 22,23-dihydro-13-desoxy-avermectin B_1 aglycone (**5**) are 3× more active than **1**. In contrast, 22,23-dihydro-avermectin B_1 (**6**) is 3× less active than abamectin. The monosaccharide **7** and the aglycone **8** are practically inactive against *Tetranychus urticae*. Furthermore, avermectin B_1 (**1**) is the

Table 29.6.3 Activity of abamectin against mites and insects.

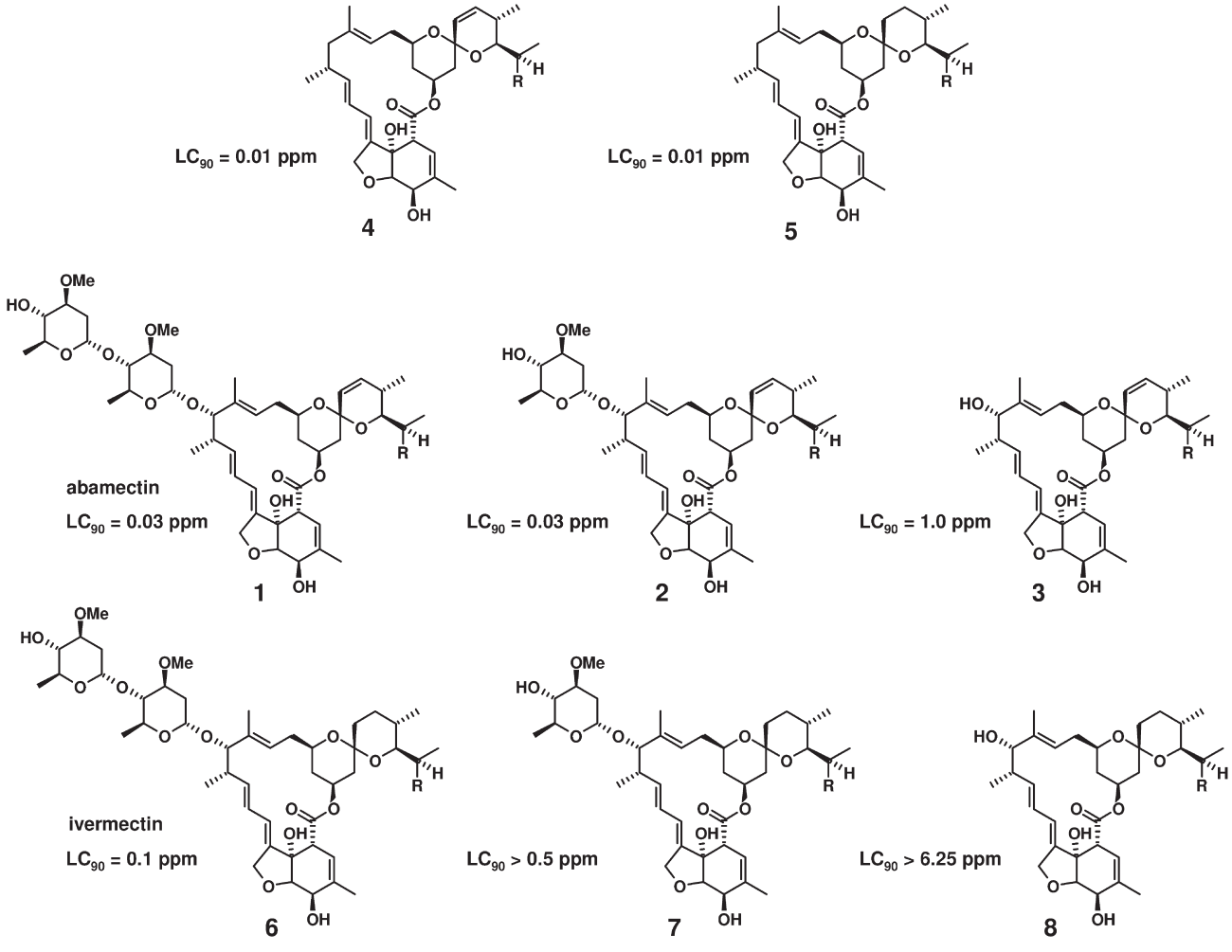
Mite species	LC_{90} (ppm)
Phyllocoptruta oleivora (citrus rust mite)	0.02
Tetranychus urticae (twospotted spider mite)	0.03
Panonychus ulmi (European red mite)	0.04
Polyphagotarsonemus latus (broad mite)	0.05
Panonychus citri (citrus red mite)	0.24
Insect species	**LC_{90} (ppm)**
Manduca sexta (tobacco hornworm)	0.02
Leptinotarsa decemlineata (Colorado potato beetle)	0.03
Heliothis virescens (tobacco budworm)	0.10
Epilachna varivestis (Mexican bean beetle)	0.40
Heliothis zea (cotton bollworm)	1.5
Spodoptera eridania (southern armyworm)	6.0
Spodoptera frugiperda (fall armyworm)	25.0

most active acaricide among the naturally occurring avermectins. Avermectins B_2, A_1, and A_2 are more than 10× weaker.

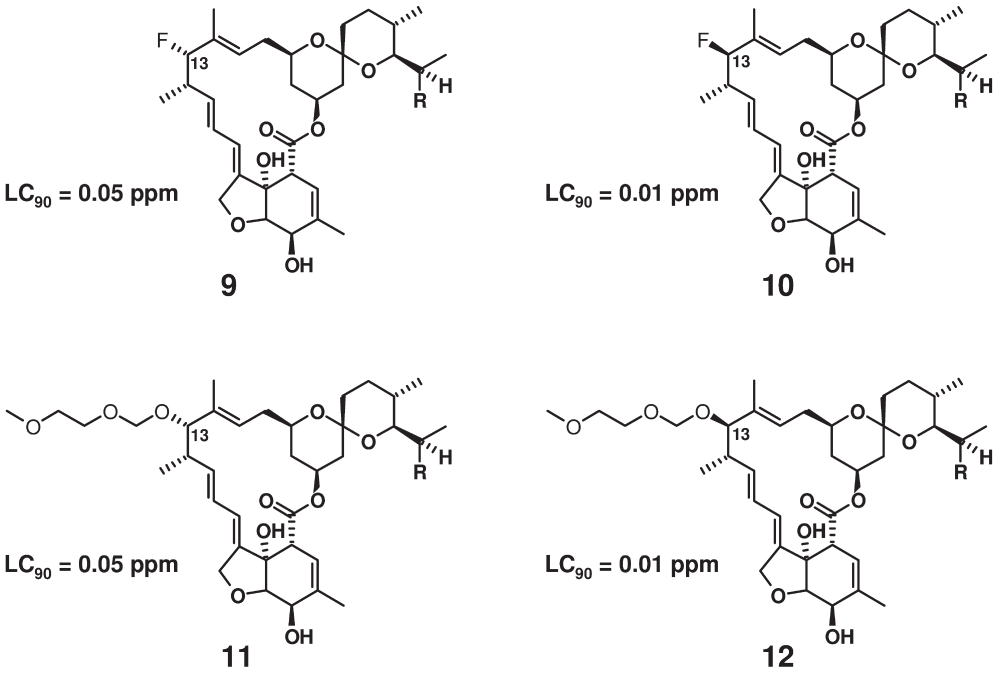
It is apparent from these studies [48, 49] that the disaccharide part is not essential for potent acaricidal activity. At the same time, no significant improvement of the activity against insects (*Spodoptera eridania*) was observed. Further examples of aglycone derivatives with high activity against *Tetranychus urticae* are the compounds shown in Scheme 29.6.4. While the fluorides **9** and **10** are structurally quite similar to **5**, the ether substituent of **11** and **12** appears to mimic the carbohydrate structure of a monosaccharide. Introducing this substituent confers activity to the otherwise inactive aglycone, and, interestingly, in this case the β-isomer at C13 is even more active than the α-isomer.

Table 29.6.4 shows the acaricidal and insecticidal activity of milbemectin, as described by Aoki et al. [50]. Except for effects against some insects (Thrips and some Lepidoptera), milbemectin is mainly an acaricide. This is consistent with the structure–activity relationships observed with avermectin derivatives [28]. In general, compounds with lipophilic substituents on C13 (or unsubstituted ones) are highly active, while polar substituents diminish the activity (cf. Schemes 29.6.3 and 29.6.4).

Given the relatively low toxicity of abamectin against *Spodoptera* ssp., Merck scientists embarked on a targeted screening program to improve the activity against Lepidoptera. In 1989, Mrozik et al. [28] reported the activity of 4″-desoxy-4″-epi-amino avermectins. In a test against neonate *Spodoptera eridania* larvae,



Scheme 29.6.3. Structure–activity relationships of avermectins against *Tetranychus urticae*; R = C_2H_5 (>80%) and CH_3 (<20%).



Scheme 29.6.4. Activity of avermectin aglycone derivatives against *Tetranychus urticae*; R = C_2H_5 (>80%) and CH_3 (<20%).

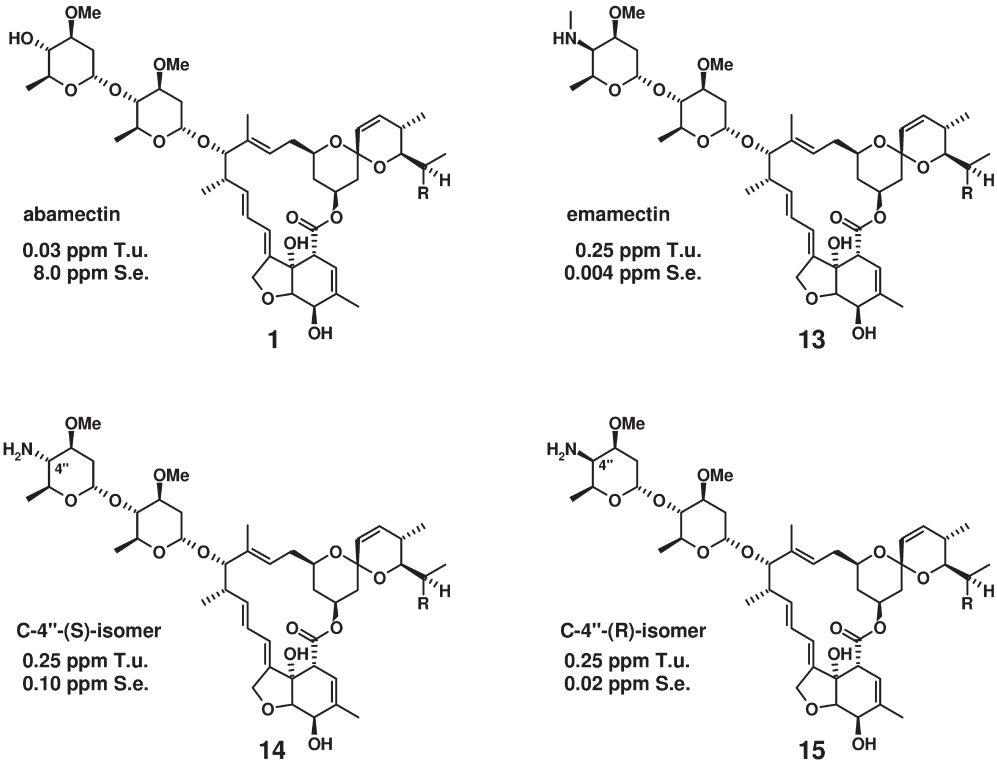
Table 29.6.4 Activity of milbemectin against mites and insects.

Species	Stage	LC_{50} (ppm)
Tetranychus kanzawai (Kanzawa spider mite)	Adults	0.7
	Eggs	1.5
Tetranychus urticae (twospotted spider mite)	Adults	4.7
	Eggs	3.3
Tetranychus cinnabarinus (carmine spider mite)	Adults	2.7
	Eggs	2.0
Panonychus citri (citrus red mite)	Adults	0.05
	Eggs	4.6
Myzus persicae (green peach aphid)		<10
Toxoptera aurantii (black citrus aphid)		<2.5
Spodoptera litura (common cutworm)		2.5
Caloptilia theivora (tea leafroller)		<10

Table 29.6.5 Activity of emamectin against mites and insects.

Species	LC_{90} (ppm)
Manduca sexta (tobacco hornworm)	0.003
Spodoptera exigua (beet armyworm)	0.005
Spodoptera frugiperda (fall armyworm)	0.010
Leptinotarsa decemlineata (Colorado potato beetle)	0.032
Tetranychus urticae (twospotted spider mite)	0.29
Aphis fabae (bean aphid)	19.9

4″-desoxy-4″-epi-methylamino avermectin B_1 (emamectin) displayed an LC_{50} of 0.004 ppm, and $LC_{90} < 0.02$ ppm. In the same test, abamectin was inactive at 0.1 ppm. Later, Fisher reported the activity of emamectin against several important pests [51, 52]. The data are shown in Table 29.6.5. Emamectin is very potent against Lepidoptera and significantly weaker against mites and aphids.



Scheme 29.6.5. Activity of 4″-amino-avermectin derivatives against *Tetranychus urticae* (T.u.) and *Spodoptera eridania* (S.e.), LC_{90} in ppm; R = C_2H_5 (>80%) and CH_3 (<20%).

Jansson and Dybas [5] have reported a summary of the comparative toxicity of abamectin and emamectin against 28 arthropod pests of agricultural importance. They show very clearly the complementary nature of these two compounds. Against all Lepidoptera, emamectin is stronger than abamectin. Against Choleoptera, both compounds perform equally well. Against Acarina, Diptera and Homoptera, abamectin is stronger than emamectin.

Fisher has also described the structure–activity relationships of several 4″-amino-avermectins against *Tetranychus urticae* and *Spodoptera eridania* [48]. Thus, it is reported that 4″-desoxy-4″-amino-avermectin B_1 (**14**) [i.e., the (*S*)-isomer at C4″; Scheme 29.6.5] is five times less active against *Spodoptera eridania* (S.e.) than 4″-desoxy-4″-epi-amino-avermectin B_1 (**15**) [i.e., the (*R*)-isomer at C4″]. Emamectin **13** has the highest activity against *S. eridania* among all 4″-amino derivatives. On the other hand, all 4″-amino derivatives are less active against *Tetranychus urticae* than abamectin (**1**).

29.6.6
Safety and Bioavailability

Table 29.6.6 summarizes selected toxicological and ecotoxicological data [1] of abamectin, emamectin benzoate, and milbemectin. The products are removed

Table 29.6.6 Safety and environment.

Compound	Abamectin	Emamectin benzoate	Milbemectin
Acute oral LD_{50} rats (mg kg^{-1})	18.4 (in sesame oil) 221 (in water)	76–89	762 (male) 456 (female)
Eye irritation rabbit	Slightly irritant	Severe irritant	Non-irritant
Skin irritation rabbit	Non-irritant	Non-irritant	Non-irritant
Acute oral LD_{50} birds (mg kg^{-1})	84.6 mallard duck >2000 bobwhite quail	46 mallard duck 264 bobwhite quail	650–660 chicken 968–1005 Japanese quail
LC_{50} (96 h) fish (μg L^{-1})	3.2 rainbow trout	174 rainbow trout	4.5 rainbow trout
EC_{50} (48 h) *Daphnia* (μg L^{-1})	0.34 *Daphnia magna*	0.99 *Daphnia magna*	–
LC_{50} earthworms (*Eisenia foetida*) (ppm)	28 (28 d)	>1000 (14 d)	61 (14 d)
Contact LD_{50} honey bee (μg per bee)	0.002 (48 h)	0.0039 (48 h)	0.025 (48 h)

rapidly from the environment after application. Photolysis on plant surfaces is fast, and they bind tightly to soil, where they are rapidly degraded by soil microorganisms [1]. No leaching or bioaccumulation occurs.

In general, all three products are safe for target crops [53]. Their impact on populations of predatory arthropods is lower than on target pests [46]. This has been the subject of many studies, in particular for abamectin [54–58], and for emamectin [59–61]. For example, Hoy and Cave [56] showed that abamectin is more toxic to *Tetranychus urticae* than to the predator *Metaseiulus occidentalis*. They also showed that field-aged residues of abamectin were safe for *Metaseiulus occidentalis* after 2 days.

The reason for this reduced impact on beneficial arthropods is the uptake and degradation behavior of the active ingredient, which makes the compound much less bioavailable to beneficials than to pests. Surface residues are subject to rapid photolysis, the half-life of the compounds is in the range 4–6 hours as thin films exposed to simulated sunlight [62], and less than a day on crops [63]. Despite the short half-life under sunlight, a sufficient amount of the active ingredient is taken up into the leaf tissue [24]. This is accompanied by translaminar distribution [62]. It appears that significant amounts are available in the parenchyma tissue, which acts as a reservoir, from which mites feed. Lacking true systemicity, the macrocyclic lactones are not distributed into the phloem and the xylem system after foliar application. This behavior explains also the lack of residual activity of abamectin against aphids, despite the good contact activity [64].

In summary, because of the rapid uptake into spayed foliage combined with fast degradation of surface residues, this family of compounds is safe to beneficials under field conditions.

29.6.7 Use in Agriculture

Abamectin is used worldwide in various crops – the most important are citrus, pome fruits, vegetables, cotton and ornamentals (Table 29.6.7). It is used to control most agronomically important mites, some Lepidoptera and dipterous leafminers. Typical application rates of abamectin are in the range 5.6–28 g-a.i. ha^{-1} for control of mites, and 11–22 g-a.i. ha^{-1} for leafminers.

Emamectin benzoate is used in many countries, mainly in all kinds of vegetable crops, such as brassicas, fruiting and leafy vegetables, but also in cotton (Table 29.6.8). Emamectin benzoate controls all agronomically important Lepidoptera in vegetables and cotton. Typical application rates for these uses are in the range 8.4–16.8 g-a.i. ha^{-1}. Some additional uses are the control of some pests in tea and of pine wood nematode in pine trees in Japan.

Milbemectin is mainly used as an acaricide against many important mites in tea and pome fruits, and also against pine wood nematode in Japan (Table 29.6.9). Application rates in the agricultural uses are in the range 5.6–28 g-a.i. ha^{-1}.

Table 29.6.7 Abamectin use in crop protection.

Pest species	Crops
Phyllocoptrura oleivora (citrus rust mite)	Citrus
Tetranychus cinnabarinus (carmine spider mite)	Cotton
Tetranychus pacificus (Pacific spider mite)	Cotton, deciduous tree nuts
Tetranychus urticae (twospotted spider mite)	Cotton, ornamentals, pome fruits, strawberry, vegetables
Liriomyza trifolii (serpentine leafminer)	Ornamentals, vegetables
Epitrimerus pyri (pear rust mite)	Pome fruits
Panonychus ulmi (European red mite)	Pome fruits
Psylla pyricola (pear psylla)	Pome fruits
Keiferia lycopersicella (tomato pinworm)	Vegetables
Plutella xylostella (diamondback moth)	Vegetables
Solenopsis invicta (red imported fire ant)	–

Table 29.6.8 Emamectin benzoate use in crop protection.

Pest species	Vegetables
Hellula rogatalis (cabbage webworm)	Brassicas
Mamestra brassicae (cabbage armyworm)	Brassicas
Pieris rapae (small white butterfly)	Brassicas
Plutella xylostella (diamondback moth)	Brassicas
Spodoptera litura (rice leafworm)	Brassicas
Trichoplusia ni (cabbage looper)	Brassicas, cotton, fruiting vegetables, leafy vegetables
Helicoverpa zea (corn earworm)	Brassicas, cotton, fruiting vegetables, leafy vegetables
Spodoptera exigua (beet armyworm)	Brassicas, cotton, fruiting vegetables, leafy vegetables
Spodoptera frugiperda (fall armyworm)	Brassicas, cotton, fruiting vegetables, leafy vegetables
Helicoverpa armigera (old world cotton bollworm)	Cotton

Table 29.6.8 (*continued*)

Pest species	Vegetables
Heliothis virescens (tobacco budworm)	Cotton, fruiting vegetables, leafy vegetables
Keiferia lycopersicella (tomato pinworm)	Fruiting vegetables
Liriomyza trifolii (serpentine leafminer)	Fruiting vegetables
Spodoptera eridania (southern armyworm)	Fruiting vegetables
Pest species	**Other crops**
Adoxophytes sp. (smaller tea tortrix)	Tea
Hormona magnamina (oriental tea tortrix)	Tea
Bursaphelenchus xylophilus (pine wood nematode)	Pine (trunk injection)

Table 29.6.9 Milbemectin use in crop protection.

Pest species	Crops
Tetranychus urticae (twospotted spider mite)	Apples, pears, strawberries, vegetables
Polyphagotarsonemus latus (broad mite)	Citrus fruits, vegetables
Tetranychus kanzawai (Kanzawa spider mite)	Pears, strawberries, tea, vegetables
Acaphylla theae (pink tea rust mite)	Tea
Calacarus carniatus (purple tea mite)	Tea
Bursaphelenchus xylophilus (pine wood nematode)	Pine (trunk injection)

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30
New Unknown Mode of Action

30.1
Selective Feeding Blockers (Pymetrozine, Flonicamid)

Peter Maienfisch

30.1.1
Introduction

The sucking pest market offers many commercial opportunities for innovative new products with novel modes of action, strong biological efficacy and low toxicity combined with high selectivity. This is mainly due to a high need for new products in IPM programs that show a high safety against beneficials, the development of resistance to current treatments, and customers demand to solve newly evolving pest problems as well as regulatory pressure on older products. Recently, the two new products pymetrozine and flonicamid, both acting as selective feeding blockers, have entered the market-place offering attractive alternatives to current sucking pest products such as carbamates, organophosphates, synthetic pyrethroids and neonicotinoids.

30.1.2
Pymetrozine

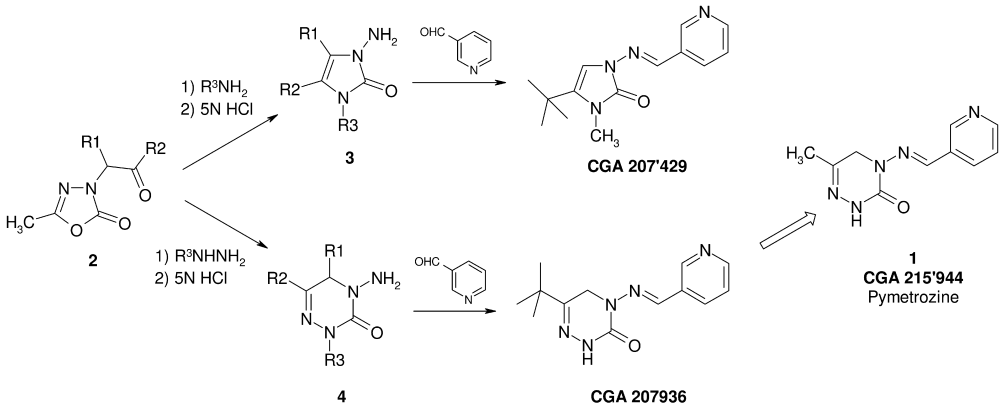
Pymetrozine (**1**; developmental code CGA 215'944) is an insecticide, highly active and specific against sucking pests. It is the only commercial representative of the chemical class of the pyridine azomethines. Pymetrozine is marketed by Syngenta under the trademarks of Chess™, Plenum™, Fulfill™, Relay™, and Sterling™ [1–4].

30.1.2.1 Discovery

Pymetrozine (**1**), a pyridine azomethine, was synthesized for the first time at the end of 1986 [3, 5]. The concept behind this discovery was primarily chemically directed with, however, a strong rational element [2]. In a first step ring transformations of 1,3,4 oxadiazolon-3-yl ketones **2** with N-nucleophiles were investigated, leading to numerous new five- and six-membered N-amino heterocycles,

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6



Scheme 30.1.1. Discovery of pymetrozine (**1**).

such as **3** and **4**, respectively (Scheme 30.1.1). In the second (intermediate) step many transformations and derivatizations of these N-amino heterocycles were made; among them the condensation with pyridine-3 aldehyde. The resulting compounds (e.g., CGA 207'429 and 207'936) turned out to possess good insecticidal activity. In the third (final) step a broad optimization program was started that resulted in the discovery of pymetrozine (**1**).

A first patent application [5] to protect compounds of the novel chemical class of the pyridine azomethines, such as **5**, was filed by Ciba-Geigy (later Novartis, now Syngenta) in 1989 and within a short time some further inventions (pyridine-3-carboxaldehyde *N*-oxides **6** [6], imidazoles **7** [7], 3-[(*N*-heterocyclyl) iminomethyl]pyridines **8** [8]) were made and patented (Table 30.1.1). In 1996 Nihon Nohyaku discovered the insecticidal activity of the corresponding aminoquinazolinones **9** and **10** [9–13] and has evaluated two compounds (NNI 0101 and NNI 9768) in official field tests in Japan. In 2006 Nihon Nohyaku has enquired a common name for NNI 0101 (proposed common name: pyrifluquinzon; IUPAC name: 1-acetyl-1,2,3,4-tetrahydro-3-[(3-pyridylmethylamino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one) suggesting that this compound is currently under development.

30.1.2.2 Pyridine Azomethines – Structure–Activity Relationship

Table 30.1.2 shows the general structure–activity profile for the pyridine azomethine insecticides.

30.1.2.3 Synthesis of Pymetrozine (1)

An economical route has been developed for the preparation of pymetrozine (**1**) (Scheme 30.1.2). The last step is quite remarkable: In a one-pot procedure pyridine-3-carbonitrile is converted into 3-pyridine aldehyde by a catalytic hydrogenation and subsequently condensed with 4-amino-6-methyl-4,5-dihydro-1,2,4-triazin-3(2*H*)-one to give pymetrozine in high yields [2, 5, 14–16].

Table 30.1.1 Key inventions related to the chemical class of the pyridine azomethines.

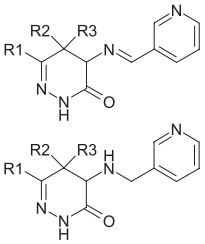
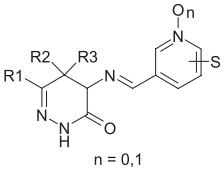
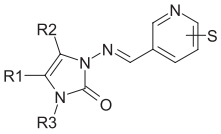
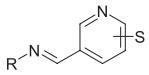
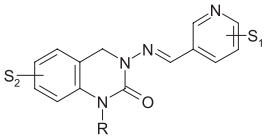
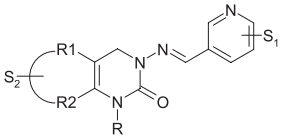
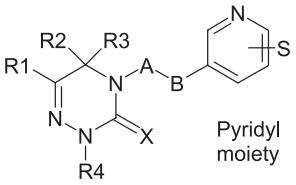
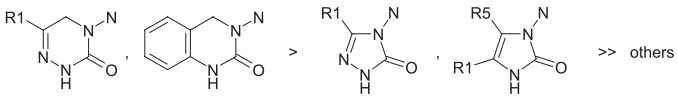
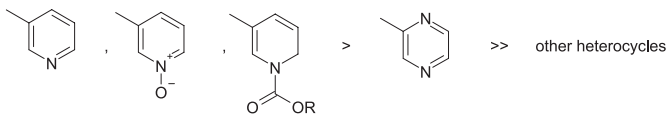
Compound type	General structure	Company	Patent application	Publication year	Ref.
5		Ciba-Geigy	EP 314615	1989	5
6	 n = 0,1	Ciba-Geigy	EP 391849	1990	6
7		Ciba-Geigy	EP 604365	1994	7
8	 R = N-attached heterocyclyl	Ciba-Geigy	WO 9518123	1997	8
9		Nihon Nohyaku	EP 735035 JP 11012254 EP 1097932 WO 2004099184	1996 1999 1999 2004	9 10 11 12
10	 R1, R2 = 5-6 membered heterocycle	Nihon Nohyaku	JP 11158180	1999	13

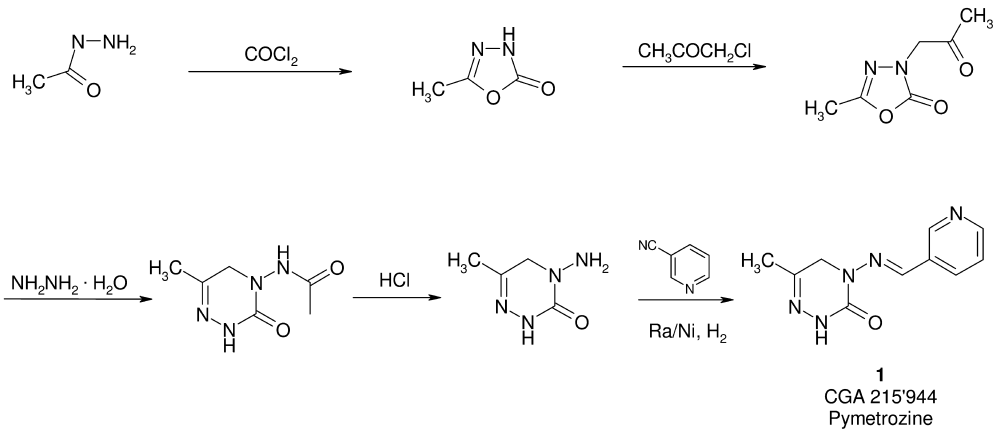
Table 30.1.2 Pyridine azomethines – structure–activity relationship.



Structural feature	Structure–activity relationship
Triazinone moiety	
Substituent R1	CH_3, *i*-Pr, *t*-Bu are most favorable; larger substituents decrease the insecticidal activity
Substituent R2, R3	H > larger groups
Substituent R4	H > CH_3, COR, COOR
Functional group C=X	C=O > C=S
Bond A–B	N=CH, NH-CH_2 ≫ N=C(alkyl), NH-CH(alkyl), others
Pyridyl moiety	
Substituent S	H is more favorable than all other substituents

30.1.2.4 **Physicochemical Properties of Pymetrozine (1)**

Table 30.1.3 shows the physicochemical properties of pymetrozine (**1**) [1, 3, 4]. Its properties favor an efficient uptake and translocation in plants [17, 18]. Its systemic behavior originates mainly from xylem but, to a minor extent, also from phloem mobility. The half-life in soil is only 2–29 days, indicating rapid degradation in the environment. Pymetrozine (**1**) and its major metabolites exhibit only low leaching potential and were generally found to remain in the upper soil layer, indicating low potential for groundwater contamination under recommended use conditions [4].



Scheme 30.1.2. Synthesis of pymetrozine (**1**).

30.1.2.5 **Mode of Action of Pymetrozine (1)**

In aphids, pymetrozine (**1**) inhibits feeding immediately after application, resulting in death by starvation without producing visible neurotoxic effects. No repellent or antifeedant effects were observed that could explain the inhibition of feeding.

Locusts, though not a plant-sucking insect, were revealed as a valid model to further study details of the underlying mechanisms. *Locusta migratoria* was found to respond to pymetrozine (**1**) by displaying unique symptoms, which were lifting

Table 30.1.3 Physicochemical properties of pymetrozine (**1**).

Feature	Property
Melting point (°C)	217
Vapor pressure at 25 °C (Pa)	$<4 \times 10^{-6}$
Water solubility at 25 °C (mg L^{-1})	290
pH	6.84 (saturated solution in water)
Partition coefficient [*n*-octanol/water at 25 °C (log P_{OW})]	−0.18
Soil mobility	Little mobility
Hydrolysis (estimated half-life at 25 °C)	pH 1: 2.8 h pH 5: 5–10 days pH 9: stable

and stretching of the hindlegs, in addition to the feeding inhibition. Pymetrozine (**1**) enhanced spontaneous spike discharge of the metathoracic and suboesophageal ganglia *in situ* at nanomolar concentrations. Similarly, **1** increased the spontaneous rhythmic contractions of the isolated foregut with maximal effects also in the nanomolar range. The actions of pymetrozine (**1**) were counteracted by biogenic amine receptor antagonists (such as mianserin, ketanserin, propranolol) and mimicked by serotonin, but not by dopamine and octopamine. Moreover, pymetrozine (**1**) and serotonin strongly potentiated the effects of each other. Pymetrozine (**1**) was inactive at all neurotransmitter receptors present on isolated locust neuronal somata, and at all other examined neuronal sites.

Similar effects were also observed in *Myzus persicae*: Electrical penetration graph experiments revealed that serotonin, like pymetrozine (**1**), inhibited stylet penetration, and strongly enhanced the action of pymetrozine (**1**). Biogenic amine receptor antagonists were not specifically active in the aphid. From these newest results it has been concluded that pymetrozine acts via a novel mechanism that is linked to the signaling pathway of serotonin [19–23].

Table 30.1.4 Current insecticidal spectrum of pymetrozine (**1**).

Target pests	Key crops
Acyrthosiphon pisum	Vegetables
Aphis citricola	Citrus
Aphis fabae	Vegetables, potato, cotton
Aphis frangulae	Potato
Aphis gossypii	Vegetables, potato, citrus, tobacco, cotton
Aphis nasturtii	Potato
Aphis nicotianae	Tobacco
Aulacorthum solani	Vegetables, potato
Bemisia tabaci	Vegetables, cotton
Bemisia trifolii	Vegetables
Brevicoryne brassicae	Vegetables
Idiocerus clypealis	Mango
Idiocerus niveosparsus	Mango
Laodelphax striatellus	Rice
Lipaphis erysimi	Vegetables
Macrosiphum euphorbiae	Vegetables, potato, tobacco, cotton
Myzus persicae	Vegetables, stone fruits, potato
Nasonovia ribisnigri	Vegetables
Nilaparvata lugens	Rice
Phorodon humili	Hop
Sogatella furcifera	Rice
Trialeurodes vaporariorum	Cotton
Toxoptera aurantii	Citrus

30.1.2.6 Biological Activity and Use Recommendation

Key targets for pymetrozine (**1**) are aphids in potatoes, vegetables and stone fruits. It is also active against hoppers in rice and controls the mobile stage of whiteflies nymphs (L1) and adults in various crops. Table 30.1.4 documents the currently recommended pest uses after foliar spray and seedling box application. The recommended rates of active ingredient (a.i.) vary according to crops and pests. In most cases 100–300 g-a.i. ha^{-1}, 10–30 g-a.i. hl^{-1} or 1–2 g-a.i. per seedling box are sufficient to fully control the target pests [1, 3, 4, 24–34].

30.1.2.7 Safety Profile

Table 30.1.5 shows the safety profile of pymetrozine (**1**); it has a low acute mammalian toxicity and an excellent safety profile for most non-target arthropods, birds, and fish. Pymetrozine (**1**) is of low risk to beneficial insects in the field and is therefore very well suited for use in IPM programs [1–4, 27, 35].

Table 30.1.5 Safety profile of pymetrozine (**1**).

Acute toxicity test	Species	Result
Oral LD_{50} (mg kg^{-1})	Rat	>5820
Dermal LD_{50} (24 h) (mg kg^{-1})	Rat	>2000
Inhalation LC_{50} (4 h) (mg m^{-3})	Rat	>1800
Skin irritation	Rabbit	Non-irritant
Eye irritation	Rabbit	Non-irritant
Avian oral LD_{50} (mg kg^{-1})	Mallard duck	>2000
Freshwater fish LC_{50} (96 h) (mg kg^{-1})	Rainbow trout	>100
Freshwater invertebrate EC_{50} (48 h) (mg kg^{-1})	*Daphnia magna*	>100
Algae EC_{50} (72 h) (mg L^{-1})	*Scenedesmus* sp. *Selenastrum* sp.	47.1 58
Earthworm EC_{50} (14 d) (mg per kg of soil)	*Eisenia foetida*	>1000
Bee oral LD_{50} (48 h) (µg per bee)	Honey bee	117
Bee contact LD_{50} (48 h) (µg per bee)		>200

30.1.3 Flonicamid

Flonicamid (**12**; developmental codes: IKI-220, F1785) is a selective systemic aphicide discovered by Ishihara Sangyo Kaisha, Ltd. This compound belongs to

the new trifluoromethylnicotinamide chemical class. In late 2001, FMC Corporation obtained the exclusive rights to develop, market and distribute flonicamid in North America, much of Latin America, UK, Spain and Portugal. In the rest of the European Union FMC and ISK jointly are developing this insecticide [36, 37].

30.1.3.1 Discovery of Flonicamid (12) and the Trifluoromethylnicotinamides Insecticides

The first trifluoromethylnicotinamides possessing aphicidal activity were described by Ishihara Sangyo Kaisha in 1994 [38]. This early patent application covered compounds of the general structure **11**, and flonicamid **12** was one of the compounds specifically mentioned (Table 30.1.6). The good aphicidal activity of trifluoromethylnicotinamides of the type **11** triggered research activities within several other companies such as Sumitomo, Hoechst/Aventis (now Bayer CropScience) and Syngenta. Some early key inventions made by these companies are highlighted in Table 30.1.6. Later, other companies such as Bayer CropScience and Sankyo Agro joint this research area. Currently some trifluoromethylnicotinamides of unknown structure are being evaluated in field tests.

30.1.3.2 Trifluoromethylnicotinamides – Structure–Activity Relationship

Table 30.1.7 shows the general structure–activity profile for trifluoromethylnicotinamides insecticides.

30.1.3.3 Synthesis of Flonicamid (12)

Flonicamid (**12**) was first synthesized in 1994 [38]. Starting from the commercially available 4-(trifluoromethyl)nicotinic acid (new synthetic methods for its production have recently been described [39, 48–51]) flonicamid (**12**) can be synthesized in only two steps [38] (Scheme 30.1.3). An alternative route involving 1,3,5-tricyanomethylhexyhydro-1,3,5-triazine (**19**) as source of amino-acetonitrile has also been used to prepare larger amounts of **12** [52].

30.1.3.4 Physicochemical Properties of Flonicamid (12)

The physicochemical properties of flonicamid (**12**) (Table 30.1.8) [36, 37] (e.g., low log *P*, high water solubility) favor systemic and translaminar activity. Flonicamid (**12**) has little tendency to persist due to its fast degradation, and its moderate soil mobility is negated by rapid metabolism and mineralization.

30.1.3.5 Mode of Action of Flonicamid (12)

30.1.3.5.1 Biological Mode of Action

After treatment with flonicamid (**12**) aphids completely stop feeding within 30 min. Furthermore, a concomitant reduction in aphid honeydew production and salivation with cessation in feeding is observed. Other behavioral changes noted in aphids following intoxication with flonicamid are pronounced sensitivity to light, random or irregular movement, altered righting response, and uncoordinated locomotion as well as creased and erratic antennal movement. These effects

Table 30.1.6 Early key inventions related to the chemical class of the trifluoromethylnicotinamides.

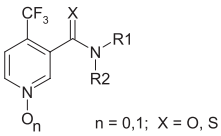
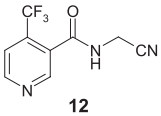
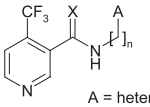
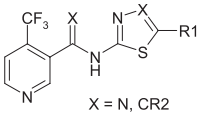
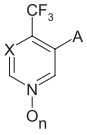
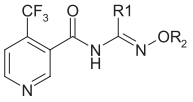
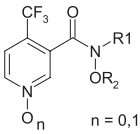
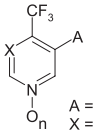
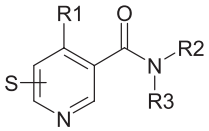
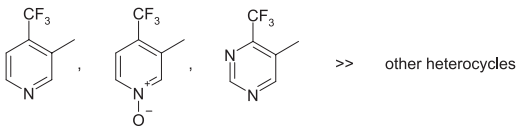
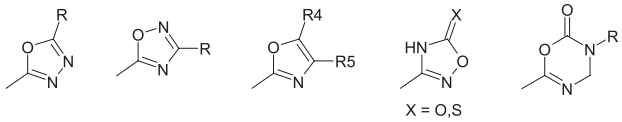
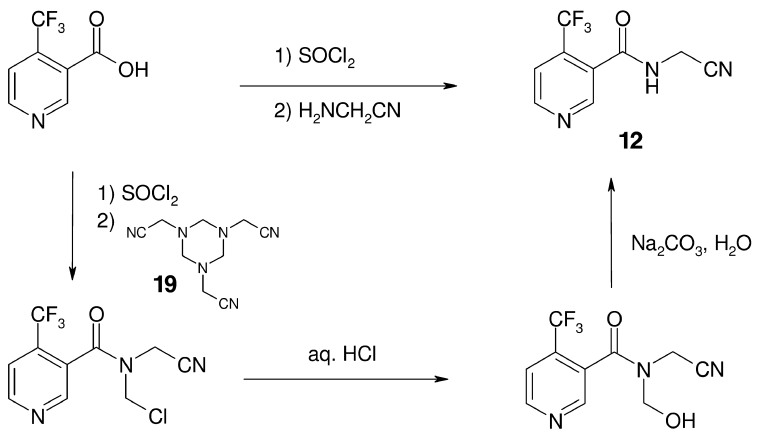
Compound type	General structure	Company	Patent application	Publication year	Ref.
11	 n = 0,1; X = O, S	Ishihara Sangyo Kaisha	EP 580374	1994	38
12	Example:  **12** Flonicamid (IKI-220, F1785)				
13	 A = heterocyclyl	Ishihara Sangyo Kaisha	JP 07010841 JP 07025853	1995 1995	39 40
14	 X = N, CR2	Sumitomo	JP 10195072	1998	41
15	 A = 5-membered heterocyclyl X = CH, N; n = 0,1	Hoechst Aventis Aventis	WO 9857969 WO 2000035912 WO 2000035913	1998	42 43 44
16		Sumitomo	JP 11180957	1999	45
17	 n = 0,1	Syngenta	WO 2001009104	2001	46
18	 A = 6-7-membered heterocyclyl X = CH, N; n = 0,1	Syngenta	WO 2001014373	2001	47

Table 30.1.7 Trifluoromethylnicotinamides – structure–activity relationship.



Pyridyl moiety | Amide moiety

Structural feature	Structure–activity relationship
Pyridyl moiety	 >> other heterocycles
Substituent R1	CF_3 is clearly more favorable than all other substituents
Substituent S	H is more favorable than all other substituents
Amide moiety	The CONR2R3 moiety can be replaced by certain five- or six-membered heterocycles, e.g.,  X = O,S
Substituents R2, R3	A broad range of substituents/groups is tolerated. R2, R3 may also both be hydrogen. Steric as well as electronic features seem not to play a very important role.



Scheme 30.1.3. Synthesis of flonicamid (**12**).

Table 30.1.8 Physicochemical properties of flonicamid (**12**).

Feature	Property
Melting point (°C)	157.5
Vapor pressure at 25 °C (kPa)	9.43×10^{-4}
Water solubility at 20 °C (g L^{-1})	5.2
Partition coefficient [*n*-octanol/water at 25 °C (log P_{OW})]	0.3
Photolysis DT_{50} (aqueous) (days)	267
DT_{50} (soil) (days)	22
Soil degradation DT_{50} (days)	<3
Soil mobility	Moderate
Hydrolysis: (estimated half-life at 25 °C)	pH 5: stable pH 7: stable pH 9: 204 days

are very different from those displayed by aphids treated with neonicotinoids [36, 37].

30.1.3.5.2 Target Sites

Although the structure of flonicamid (**12**) has some similarity to the neonicotinoids it does not bind to the nicotinic acetylcholine receptor as directly compared with nicotine and imidacloprid [37, 53]. However, flonicamid (**12**) is active on the A-type potassium channel currents. The current hypothesis is that flonicamid blockade of the A-type potassium channel in the presynaptic terminal underlies its lethal effect in insects. The loss of the A-type potassium rectifying current would lead to the disruption of controlled neurotransmitter release [53].

30.1.3.5.3 Cross-resistance

Laboratory studies as well as field observations have so far not shown any cross-resistance to OP- or carbamate-resistant populations of *Myzus persicae* [37].

30.1.3.6 Biological Activity and Use Recommendation

Flonicamid (**12**) exhibits excellent activity against numerous aphids of high agronomical importance (Table 30.1.9). The compound is reported to be highly active against both the larval and adult stages of aphids. In the field, the application rates of active ingredient (a.i.) required to provide commercially acceptable control typically range from 40 to 60 g-a.i. ha^{-1}; to achieve longer residual activity, and/or in situations with higher pest pressure, application rates of 60–80 g-a.i. ha^{-1} are recommended [36, 37, 54, 55].

Table 30.1.9 Current aphicidal spectrum of flonicamid (**12**).

Target pests	Target pests
Acyrthosiphon kondoi	*Hyalopterus pruni*
Acyrthosiphon pisum	*Lipaphis erysimi*
Aphis craccivora	*Macrosiphum euphorbiae*
Aphis fabae	*Macrosiphum rosae*
Aphis glycines	*Myzus persicae*
Aphis gossypii	*Myzus cerasi*
Aphis pomi	*Myzus nicotianae*
Aphis spiraecola	*Nasonovia ribisnigri*
Aulacorthum solani	*Phorodon humuli*
Anuraphis helichrysi	*Rhopalosiphum maidis*
Brevicoryne brassicae	*Sitobion avenae*
Diuraphis noxia	*Schizaphis graminum*
Dysaphis plantaginea	*Therioaphis maculata*
Eriosoma lanigerum	*Toxoptera citricidus*

Table 30.1.10 Safety profile of flonicamid (**12**).

Acute toxicity test	Species	Result
Oral LD_{50} (mg kg^{-1})	Rat	884 (male) 1768 (female)
Dermal LD_{50} (acute) (mg kg^{-1})	Rat	>5000
Inhalation LC_{50} (acute) (mg L^{-1})	Rat	>4.9
Skin irritation	Rabbit	Non-irritating
Eye irritation		Minimally irritating
Mutagenicity/genotoxicity		Negative
Avian oral LD_{50} (acute) (mg kg^{-1})	Mallard duck	1591
Freshwater fish LC_{50} (96 h) (mg kg^{-1})	Rainbow trout	>100
Freshwater invertebrate EC_{50} (48 h) (mg L^{-1})	*Daphnia magna*	>100
Algae EC_{50} (96 h) (mg L^{-1})		119

Good to excellent activity has also been observed against plant bugs (*Lygus* spp.), whitefly (*Trialeurodes vaporariorum*), thrips (*Thrips tabaci*) and Psylla (*Cacopsylla pyricola*).

30.1.3.7 Safety Profile of Flonicamid (12)

Like pymetrozine (**1**), flonicamid (**12**) has a very favorable toxicological, environmental and ecotoxic profile (Table 30.1.10) and has no major negative impact on beneficial insects and mites such as *Bombyx mori*, *Apis mellifera*, *Harmonia axyridis* and *Phytoseiulus persimilis* [36, 37].

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30.2
Neuroactive Miticides – Bifenazate

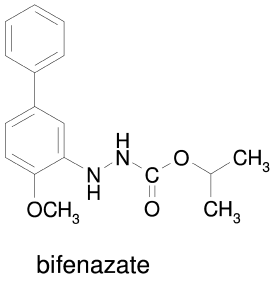
Mark A. Dekeyser

30.2.1
Introduction

Neurotoxic compounds have been traditionally the largest category of acaricides, with several representatives from the organophosphate, carbamate, and pyrethroid classes. Several new pyrethroid compounds such as acrinathrin, lubrocythrinate and halfenprox and an analog of the macrolide abamectin milbemectin were recently introduced as acaricides. A completely new acaricidal chemistry has been found with carbazate chemistry [1].

Serendipity in the traditional screening approaches is, up to now, one of the main resources for discovering acaricides with novel biochemical and physiological targets [2]. Based on the discovery that fungicidal phenylhydrazide compounds had some acaricidal activity, a synthesis program was initiated in Crompton Co. Research in 1990. From among several hundred carbazate derivatives synthesized and evaluated for acaricidal activity the methoxy-biphenyl-substituted carbazate (bifenazate, Fig. 30.2.1, Table 30.2.1) was selected for development [3]. In the USA, bifenazate was first approved and registered as a reduced-risk acaricide for the ornamental market (1999) and soon thereafter for crops such as apples, pears, peaches, plums, grapes, cotton, strawberries and hops. Bifenazate is now under worldwide development for the selective control of spider mites acting against eggs, larvae, nymphs and mites under the trade names Floramite®, Acramite®, and Mitekohne®.

In Europe, bifenazate received a unanimous positive vote by the EU legislative meeting for Annex I inclusion under Council Directive 91/414/EEC in 2005 [4]. Preliminary results from studies on the mode of action of bifenazate in insects indicate that, at high concentrations, bifenazate acts on the postsynaptic GABA receptor in the insect nervous system. This mode of action has not yet been con-



bifenazate

Fig. 30.2.1. Structure of bifenazate.

Table 30.2.1 Physicochemical data of bifenazate.

Common name	Bifenazate
IUPAC name	*N*′-(4-Methoxy-biphenyl-3-yl)hydrazine carboxylic acid isopropyl ester
Development code	D 2341, UCC – D 2341
Patent	US 5,367,093; 1994
Launch	1999
Melting point (°C)	120–124
Partition coefficient, log P_{OW} at pH 7	3.4 (25 °C)
Water solubility (mg L^{-1})	3.8 (20 °C)
Vapor pressure (Torr)	$<1 \times 10^{-7}$ (25 °C)
Hydrolysis	6.34 days at pH 4; 7.7 h at pH 7; 0.45 h at pH 9
Photolysis (hours)	16.20 (pH 5, 25 °C)
Aerobic soil metabolism (hours)	7.3

firmed in mites and GABA receptors in mites are not described up to now, but mites that have been sprayed with bifenazate will become hyperactive after approximately 3 h, and will no longer feed. After 3–4 days the maximal lethal effect on the population is reached [4].

The discovery, synthesis, structure–activity relationship and biology of this new class of carbazate acaricides and bifenazate is described in this chapter.

30.2.2 Discovery and Structure–Activity

Bifenazate belongs to the carbazate class, a new type of acaricide chemistry defined by the general formula shown in Fig. 30.2.2 (Table 30.2.2) [5–7].

After the discovery that ortho-biphenyl substituted hydrazide compounds [5] had acaricidal activity in the pesticide discovery screen (Table 30.2.3) several hundred – structurally diverse – biphenyl-substituted carbazate analogs were synthesized, and, in an optimization process using a bioassay with the two spotted spider mite (*Tetranychus urticae Koch*), isopropyl-2-(4-methoxy[1,1′-biphenyl]-3-yl) hydrazine carboxylate (bifenazate) [3, 8] was identified as the most advantageous compound.

As one can see from Table 30.2.3 compounds with the ester function consisting of a straight- or branched-chain alkyl group of three or four carbon atoms show the best biological activities in this series.

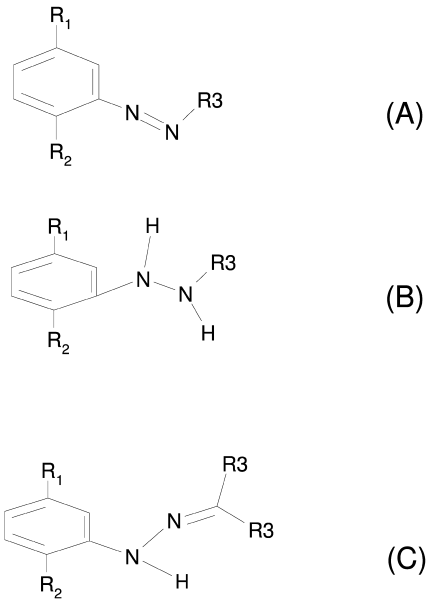
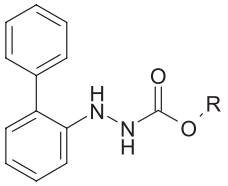


Fig. 30.2.2. General formula for carbazate acaricides.

Table 30.2.2 Definitions of general formula for carbazate acaricides (see Fig. 30.2.2).

Type	R_1	R_2	R_3	Patent
A	Phenyl	C1–C4 – alkoxy	COOR	US 5,438,123 US 5,367,093
A	Phenyl	C1–C4 – alkoxy	$PO(OR_3)_2$	US 5,543,404
A	Fluorenyl, thienyl, pyridyl, thiazolyl	C1–C4 – alkoxy	COOR	US 5,567,723
B	Phenyl, H	C1–C4 – alkoxy, phenyl	COOR	US 5,367,093 US 5,367,093
B	Fluorenyl, thienyl, pyridyl, thiazolyl	C1–C4 – alkoxy	COOR	US 5,567,723
B	Phenyl	C1–C4 – alkoxy	$PO(OR_3)_2$	US 5,543,404
C	Phenyl	C1–C4 – alkoxy	R_3 = aryl	US 6,706,895

Table 30.2.3 Structure–activity relations of ortho-biphenyl substituted hydrazide compounds [9, 10].



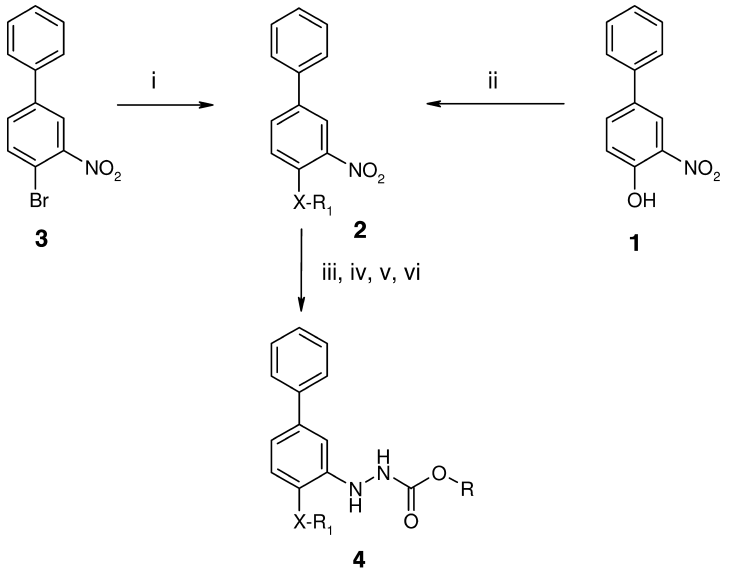
R	Mp (°C)	% Mortality *in vivo* at 5 days against *T. urticae* (25 ppm)
CH_3	97–100	19
C_2H_5	92–95	30/0[a]
$CH_2C_6H_5$	101–104	17
$CH(CH_3)_2$	104–106	42/additive, highest activity against *S. orizicola* (rice delphacids)
n-C_3H_7	88–90	80
n-C_4H_9	Oil	65
n-C_5H_{11}	Oil	22
CH_2-CH=CH_2	78–80	49
$C(CH_3)_3$	92–94	68/54[a]/additional high activity against *S. orizicola* (rice delphacids)
-$CH(CH_3)$-C_2H_5	80–83	72/79[a]/additional high activity against *S. orizicola* (rice delphacids)

[a] Data for N=N-COOR derivatives.

In contrast to the ortho-biphenyl-substituted carbazates the isomeric alkyl-meta-biphenyl-carbazates are available in a multistep synthesis, allowing one to introduce different substituents, like alkoxy, alkylthio, alkylsulfonyl, to the pendant phenyl group (Scheme 30.2.1) [12]:

Compound **1** was made from 4-hydroxybiphenyl, which was also an intermediate for an alternate synthesis of bifenazate and the hydroxy-analogs [11].

From these variations the structure–activity relationship for meta-substituted biphenyl carbazates can be summarized: The introduction of a substituent para to the pendant phenyl group improves the activity compared with the unsubstituted ortho- or meta-biphenyl carbazates. The most active carbazates are those with a methoxy, ethoxy, β-fluoro-ethoxy substituent para to the phenyl moiety in the meta-biphenyl carbazates (Fig. 30.2.3).

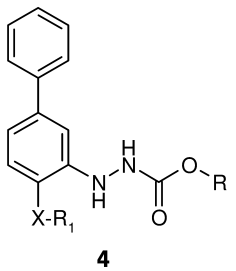


i: $NaXR_1$, methanol; ii: alkyl halide, K_2CO_3; iii: $SnCl_2$, ethyl acetate; iv: $NaNO_2$, HCl;

v: $SnCl_2$; vi: $ClCO_2R$, pyridine, ethyl acetate

R,R_1 = alkyl; X = O, S, SO_2

Scheme 30.2.1. Synthesis of meta-substituted biphenyl carbazates.



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$X = O > S > SO_2$

$R_1 = CH_3 > C_2H_5 > CH_2CH_2F > CH_2C_6H_5 > CH(CH_3)_2$

$R = CH(CH_3)_2 > CH(CH_3)C_2H_5 > C(CH_3)_3 > CH_3 > CH_2C_6H_5$

Fig. 30.2.3. Structure–activity relations of meta-biphenyl carbazates.

Table 30.2.4 LC_{50}s of bifenazate against different development stages of *T. urticae*.

Life stage	LC_{50} (ppm) at 5 days D 2341
Adults	0.3
Nymphs[a]	0.3
Larvae	0.3
Eggs	12

[a] Second, third and fourth stages.

30.2.3 Biology and Biochemistry

Bifenazate shows a broad activity spectrum on phytophagous mites such as *Tetranychus* spp. *Eutetranychus* spp., *Oligonychus* spp. and *Panonychus* spp. Compared with propargite the compound is 30–100-fold more active against adults, nymphs and larvae, e.g., of *Tetranychus urticae* (in cowpeas) [3] (Table 30.2.4).

The activity of bifenazate remains nearly constant over the temperature range 15–35 °C, allowing its use under a wide range of conditions.

The compound, formulated as a 50 WP formulation, allows the control of, for example, *P. ulmi* in apples or *P. citri*, with an application rate of 280 g-a.i. ha^{-1} for 20–40 days with a rapid knock down activity.

30.2.3.1 Ecobiology

The favorable ecobiological profile of bifenazate is remarkable [4, 13–15]. The compound does not affect beneficial insects, neither pollinating insects nor beneficial predatory mites nor wasps (Table 30.2.5).

Table 30.2.5 Ecobiological properties of bifenazate against beneficial insects.

Organism	Effect	Remarks
Honey bees	No adverse impact	[4]
Bumble bees	No adverse impact	[16]
Predatory mites	Harmless	[13–15]
Predatory bugs	Harmless	
Parasitic wasp	No effect on fecundity of surviving females (LC_{50} 752 g-a.i. ha^{-1} but use rate $<$ 600 g-a.i. ha^{-1})	
Lace wing	Harmless	With use rate 300 g-a.i. ha^{-1} only 3% mortality.

Table 30.2.6 Registration status of bifenazate (status 2004).

Country	Crop	Mite	Launch year	Marketing name
USA	Ornamentals	*Tetranychus* spp.	1999	Floramite®
USA	Apples, pears, grape vines, stone fruit, nectarines, cotton, strawberries, hops	*Panonychus* spp., *Tetranychus* spp.	2002	Acramite®
USA	Vegetables, almonds, tree nuts	*Panonychus* spp., *Tetranychus* spp.	2003	Acramite®
Japan	Vegetables, fruits, ornamentals	*Panonychus* spp., *Tetranychus* spp.	2000/2001	Mitokohne®
EU/Netherlands	Ornamentals, fruits, vegetables	*Panonychus* spp., *Tetranychus* spp.	2003	Floramite®
Australia	Apples, pears	*P. ulmi*, *T. urticae*	2003	Acramite®
Chile	Fruit trees	*Tetranychus* spp., *Panonychus* spp.	2004	Acramite®

Bifenazate is characterized as a compound with very low toxicity to beneficial arthropods at recommended application rates. Especially in uses under IPM situations and in combination with predatory mites the compound is very advantageous.

30.2.3.2 **Registration Status**

These ecobiological and also the toxicological properties, the rapid soil dissipation, the decreased application frequency and improved efficacy compared with existing compounds of bifenazate, together with the first introduction for use in ornamentals, led to the introduction of Floramite® in the United States under the reduced risk status (Table 30.2.6) [3, 17, 18].

The recommended application rate is 300–600 g-a.i. ha^{-1} in these cultures.

30.2.3.3 **Resistance Behavior**

Owing to its new mode of action bifenazate does not show cross-resistance with known miticides from the respiration inhibitor group, with other neuroactive miticides or with inhibitors of respiration in neither *T. urticae* [19–21] nor *P. ulmi* [22]. Investigations with resistant mites (*T. urticae*) against pyridaben, fenpyroximate, tebufenpyrad and Avermectin® from different investigators as well as with *P. ulmi* strains resistant to dicofol, organotins, clofentezine or hexathiazox support this claim.

30.2.4 Conclusions

With its carbazate chemistry, bifenazate, a structurally new miticide with favorable biological properties, has been identified and brought to the crop protection market.

Acknowledgments

I wish to thank the many Chemtura employees who had contributed to the success of bifenazate.

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30.3
Pyridalyl: Discovery, Insecticidal Activity, and Mode of Action

Shigeru Saito and Noriyasu Sakamoto

30.3.1
Introduction

In the early 1990s, synthetic pyrethroids, carbamates or organophosphorus insecticides were the major materials to control insect pests on agricultural crops. Although they had been useful materials because of their excellent efficacy for control of wide range of insect pests, reduction of their efficacy in various insect pests, due to the development of resistances, led to failure of crop protection from the pests, which became a serious worldwide problem. In addition, impacts of these synthetic pesticides on environment, including non-target organisms, as well as on potential risk for human health, were also of concern. Hence, efforts to reduce usage of these synthetic pesticides have been accelerated recently as a global trend.

Integrated Pest Management (IPM) is a way to minimize such resistance problems by introduction of different pest control methods other than the synthetic pesticides. However, synthetic pesticides should still have an important role in IPM programs to make them feasible for pest control. Since IPM programs need to be established under various field conditions – weather conditions, pest species and density, and growth condition of the crops – it is preferable for growers to have wide options in materials and methods for pest control. From the efficacy point of view, selective insecticidal activity against target pests would be a preferable characteristic of pesticides to be incorporated into IPM programs.

Taking such circumstances into consideration, we initiated a study to discover a new insecticide to control lepidopterous pests with excellent insecticidal activity, new mode of action, and lower impacts on non-target organisms. This was because lepidopterous pests, such as *Helicoverpa* spp., *Spodoptera* spp. and *Plutella xylostella*, were the major problems worldwide at that time. Thus, specific and efficient evaluation systems to detect insecticidal activity to lepidopterous insects were established, in parallel with the study of lead compound finding in the Agricultural Chemicals Research Laboratory of Sumitomo Chemical Co., Ltd. Then, several newly synthesized chemicals were evaluated, and the chemicals that showed high insecticidal activity were globally examined on their efficacy for control of lepidopterous pests in fields. We found several compounds with excellent efficacy through these studies, and pyridalyl (common name, experimental code: S-1812) was finally selected as an insecticide to be developed (Fig. 30.3.1). This compound shows excellent efficacy and its acute toxicity to mammals, avian and fishes is low (Table 30.3.1) [1].

We describe here the discovery of pyridalyl and its insecticidal activity, mode of action and information about commercial aspects.

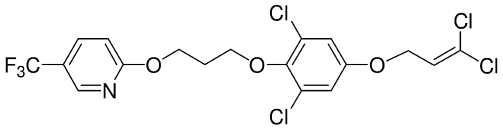

Fig. 30.3.1. Structure of pyridalyl.

Table 30.3.1 Toxicological profile of pyridalyl technical.

Subject	Animal	Toxicity
Acute oral	Rat	$LD_{50} > 5000$ mg per kg b.w.
Acute dermal	Rat	$LD_{50} > 2000$ mg per kg b.w.
Irritation (skin)	Rabbit	No irritation
Irritation (eye)	Rabbit	Slight irritation
Acute inhalation	Rat	$LC_{50} > 2.01$ mg L^{-1}
Skin sensitization	Guinea pig	Sensitizing
AMES		Negative
Avian toxicity	Bobwhite quail Mallard duck	$LD_{50} > 1133$ mg per kg diet $LC_{50} > 5620$ mg per kg diet
Fish toxicity	Carp Bluegill	96-h $LC_{50} > 10$ mg L^{-1} 96-h $LC_{50} > 24$ mg L^{-1}

30.3.2 Chemistry

30.3.2.1 Lead Generation

The discovery of a new insecticide begins with the generation of suitable lead compounds. They would be structurally modified to other compounds with higher insecticidal activity against target insects. Although various approaches may be used to create appropriate lead compounds, we should note that existing compounds were used as a lead compound in the case of pyridalyl.

Lepidopterous pests were important target pests, because they had already developed resistance to existing insecticides for cotton, vegetable and fruits. Among several compounds reported in journals to possess biological activity, some (**1**, **2**) were shown to be insect growth regulator, and had the dichloroallyl group as a

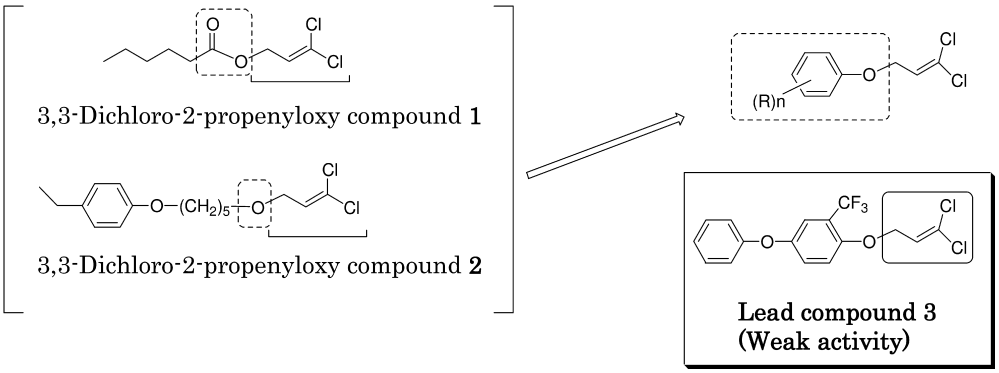

Fig. 30.3.2. Discovery of lead compound **3**.

common substituent, which prompted us to synthesize a series of compounds with the 3,3-dichloro-2-propenyl group to generate a new lead compound [2, 3].

The structure–activity relationship (SAR) of these compounds showed that the introduction of a phenyl ether linkage could improve their insecticidal activity. Some of these compounds were revealed to possess insecticidal activity against lepidoptera.

Furthermore, compound **3**, 4-phenoxy-2-(trifluoromethyl)phenyl 3,3-dichloro-2-propenyl ether, showed unique lethal symptoms as well as insecticidal activity against *S. litura* at 500 ppm, motivating us to use this **3** as the next lead compound (Fig. 30.3.2) [4, 5].

30.3.2.2 Optimization of the Lead Compound to Pyridalyl

The lead compound **3** was divided into three parts for its optimization. The SAR of the propenyl side chain revealed that the 3,3-dichloro-2-propenyl group was essential for insecticidal activity. Introduction of substituents at 3- and 5-positions of the benzene ring remarkably increased insecticidal activity. These substituent effects may indicate the importance of these positions to fix molecular conformations in their bioactive forms. Pyridine and phenyl rings were shown to be favorable substituents and gave highly active compounds. Their insecticidal activity was influenced by the linker between the two aromatic rings, depending on the length of the linkers. The linker with 1,3- or 1,4-alkylene dihydroxy group increased insecticidal activity to a large extent. Pyridalyl was successfully designed by taking these results into consideration (Fig. 30.3.3) [4, 5].

30.3.2.3 Physicochemical Properties

Table 30.3.2 depicts physicochemical properties of pyridalyl. Pyridalyl is an odorless liquid having a vapor pressure of 6.24×10^{-8} Pa (25 °C). Although soluble in most organic solvents, it is not readily soluble in water.

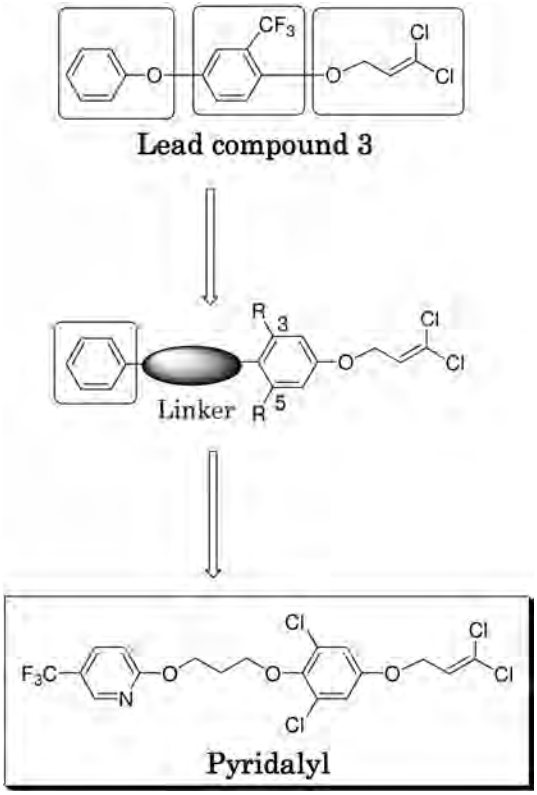

Fig. 30.3.3. Optimization of lead compound **3**.

Table 30.3.2 Physicochemical properties of pyridalyl technical.

ISO name	Pyridalyl
Code number	S-1812
Trade name	Pleo
Physical form	Liquid (20 °C)
Melting point	<-17 °C
Vapor pressure	6.24×10^{-8} Pa (25 °C)
Solubility	Water: 0.15 ppb (20 °C) Organic solvents: soluble in most

Table 30.3.3 Insecticidal activity of pyridalyl against lepidopterous pests.

Species	Stage[a]	Test method	Days after treatment	LC_{50} (mg-a.i. L^{-1})	LC_{90} (mg-a.i. L^{-1})
Cnaphalocrosis medinalis	L3	Foliar spray	5	1.80	4.95
Helicoverpa armigera	L3	Leaf dip	5	1.36	6.51
H. zea	L2	Leaf dip	5	3.23	6.06
Heliothis virescens	L2	Leaf dip	5	4.29	9.06
Mamestra brassicae	L3	Foliar spray	5	1.98	4.92
Spodoptera exigua	L3	Leaf dip	5	0.93	1.90
S. litura	L3	Foliar spray	5	0.77	1.53
Pieris rapae	L2	Foliar spray	5	3.02	6.10
Plutella xylostella	L3	Leaf dip	3	4.48	13.8

[a] L2 and L3 mean 2nd and 3rd instar larva, respectively.

30.3.3 Biological Aspects

30.3.3.1 Insecticidal Activity and Uses

The insecticidal activity of pyridalyl against larvae of several major lepidopterous pests have been determined and are expressed as the LC_{50} and the LC_{90} values in Table 30.3.3. The LC_{90}s of the compounds ranged between 1.53 and 13.8 mg-a.i. L^{-1}. Pyridalyl is also highly active against the resistant population of *P. xylostella*, which shows high resistance against conventional insecticides, such as synthetic pyrethroids, organophosphates or benzoylphenylureas (Table 30.3.4) [1]. It was also found that pyridalyl showed insecticidal activity against dipterous leafminer and thrips, such as *Liriomyza* spp., *Thrips palmi* and *Frankliniella occi-*

Table 30.3.4 Insecticidal activity of pyridalyl against insecticide resistant strain of *P. xylostella*.

Insecticide	Class	LC_{50} (mg-a.i. L^{-1})	
		Resistant strain	Susceptible strain
Pyridalyl		2.6	4.5
Cyfluthrin	Synthetic pyrethroid	>500	3.7
Pyrimifos methyl	Organic phosphate	>450	12.0
Chlorfluazuron	Benzoyl phenylurea	>25	3.4

Table 30.3.5 Beneficial arthropods not affected by pyridalyl at 100 mg-a.i. L^{-1}.

Scientific name	Beneficials	Stage[a]	Test method
Trichogramma japonicum	Parasitic wasp	Adult	Foliar spray
Chrysoperla carnea	Predatory Chrysopidae	L2-3	Insect dip
Harmonia axyridis	Predatory Coleoptera	L2-3	Foliar spray
Orius sauteri	Predatory Hemiptera	Adult/nymph	Foliar spray
Phytoseiulus persimilis	Predatory Acarina	Adult	Foliar spray
Apis mellifera	Pollinator	Worker	Direct spray
Bombus terrestris	Pollinator	Worker	Direct spray

[a] L2-3 means 2nd to 3rd instar larvae.

dentalis. Ovicidal activity of the compound is limited as it was observed only in *P. xylostella*.

In contrast to such strong insecticidal activity against lepidopterous insects, pyridalyl showed little insecticidal activity against species of Hemiptera, Coleoptera and Orthoptera. Such selectivity of the compound is reasonably regarded as a preferable characteristic for uses in IPM programs, because some natural enemy insects are included in those families. In fact, pyridalyl showed minimal impacts on various beneficial arthropods such as parasitic wasps, predatory insects and mite and pollinators (Table 30.3.5) [1, 6, 7].

Several field evaluations carried out to date have proved that pyridalyl at rates between 100 and 220 g-a.i. ha^{-1} or 10 g-a.i. hl^{-1} provides excellent control of lepidopterous pests on vegetables or cotton. In addition, pyridalyl is being developed for control of *Liriomyza* spp. and *Thrips palmi* in vegetables and ornamentals. The labeled or proposed crops in Japan or USA are expressed in Table 30.3.6. No crop injuries have been reported to date.

30.3.3.2 Mode of Action

Early on in the optimization study, it was found that the compounds produced a unique symptom that looked like barn scars in treated larvae. Such a symptom appeared in larvae that survived after treatment with pyridalyl but was not seen in the larvae died within several hours after treatment with higher (lethal) dosages. It was also similar in larvae of various lepidopterous insects treated with pyridalyl. Details of the appearance of the barn scars have been investigated using *S. litura* larvae [8]. The compound was topically applied onto the thoracic dorsum of larvae. Although there was no remarkable change one day after treatment with lower dosages of pyridalyl, the treated site turned darker 2 days after treatment and the barn scars appeared after ecdysis. In contrast, larvae died within several hours after treatment with lethal dosages of pyridalyl without any conspicuous symptoms, such as convulsion, spasm or vomiting. It was postulated that the

Table 30.3.6 Labeled or proposed crops and use patterns (January, 2006).

Country	Crop	Use rate (g-a.i. hL^{-1})
Japan		
	Cabbage	10
	Chinese cabbage	10
	Japanese radish	10
	Tomato	10
	Eggplant	10
	Pimento	10
	Lettuce	10
	Welsh onion, leek	10
	Strawberry	10
	Chrysanthemum	10
USA[a]		
	Cotton	0.1–0.15 lb-a.i. $acre^{-1}$
	Fruiting vegetables (EPA crop Group 8)	0.1–0.20 lb-a.i. $acre^{-1}$
	Brassica vegetables (EPA crop Group 5)	0.1–0.20 lb-a.i. $acre^{-1}$
	Leafy vegetables (EPA crop Group 4)	0.1–0.20 lb-a.i. $acre^{-1}$
	Ornamentals	60

[a] Proposed crops and use rates are expressed.

symptoms produced by both lethal and lower dosages of pyridalyl might relate to degeneration of cells in the larvae. Then, the effects of pyridalyl on cultured insect cell Sf9, which is established from ovary of *S. frugiperda* pupae, were investigated [9, 10]. Optical microscopic observation using the Trypan blue exclusion method showed that pyridalyl suppressed proliferation of the cells or reduced the number of cells. The ATP concentrations in Sf9 cells after treatment with pyridalyl (1.0–10 μM) were measured and it was demonstrated that it took 4–6 h after treatment to show the decrease of ATP concentration in treated cells. The effect of pyridalyl towards the Sf9 cells was thought to be related to its insecticidal activity, because the analogues of pyridalyl that did not show insecticidal activity did not show significant effects on the Sf9 cells. Furthermore, the effects of pyridalyl on the epidermal cells and Sf9 cells were observed by transmission electron microscope [11]. The ultrastructural changes in the epidermal cells were swellings of mitochondria, dilation of Golgi apparatus or endoplasmatic reticullum, disappearing of microvillus structure in the apical surface facing cuticle and shrinkages of nuclei. In the Sf9 cells, swelling of mitochondria and dispersing of polysomes appeared. Those symptoms are common in degenerating cells, but did not suggest that pyridalyl acted on any specific organelles. The change of ATP concentration and diagnosis of symptoms that appeared in the cells suggest that pyridalyl acts not by interference of cell division, inhibition of cytoskeleton, induc-

tion of apoptosis or inhibition of nucleic acid synthesis, but does act in a similar manner to mitochondrial respiration inhibitors. Consequently, the effect of pyridalyl on mitochondrial respiration was investigated by monitoring of oxygen consumption of suspension of mitochondria isolated from flight muscle of *S. litura* adults. However, no effects of pyridalyl were detected in isolated mitochondria [9].

Thus, the symptoms and diagnoses in insects or cells described above suggests that pyridalyl has a different biochemical mode of action from any existing insecticides. The lack of cross-resistance between any existing insecticides also supports such a presumption. Studies to clarify the biochemical mode of the action are on-going.

30.3.4 Development Status

Pyridalyl insecticides have been globally developed and introduced in the market in Japan, Korea and Thailand with the trade name "PLEO" as of February 2006. Furthermore, registration approvals are expected in several countries, including United States, South Africa and Australia within the next 2–3 years. Developments are on-going in some EU, Latin American and Asian countries.

Commercial or developmental formulations are 10% emulsion in water, 10% emulsible concentrate (EC), 35% wettable powder, 48% EC and 50% EC. In addition, some products of mixtures with other insecticides are also being developed.

30.3.5 Conclusion

In Japan, not a few studies to establish local IPM programs using natural enemies have been carried out over the last several years. This is because administrations, as well as consumers, expect to reduce the usage of synthetic pesticides. From the grower's point of view, IPM programs might reduce their work in pesticide applications. Under such circumstances, the selective insecticidal activity of pyridalyl was of interest to researchers and has been incorporated into such studies, mainly since 2002. Spray programs were then introduced and accepted well in some areas.

In other countries, although the problems of insecticide resistance in lepidopterous pests decreased after the introduction of Bt-gene expressed crops and some insecticides, including spinosad and indoxacarb, concerns are increasing again. Therefore, the commercialization of new insecticides with a mode of action that differs from existing insecticides is highly desirable.

Thus, pyridalyl is expected to take an important role in IPMs and insecticide-resistance management programs and to contribute to agricultural production worldwide.

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31
Insecticides Affecting Calcium Homeostasis – Flubendiamide

Hiroshi Hamaguchi and Takashi Hirooka

31.1
Introduction

A new insecticide attracts much attention in terms of resistant management, environmental friendliness, and high activity. The research and innovation of insecticides characterized by a new mode of action is undoubtedly highly ranked in the area of agrochemical research, especially because it is much more challenging than research based on patent busting.

A new insecticide, which is effective at very low dosage with a novel mode of action, is ideal for overcoming issues derived from resistance and ecobiological problems associated with older insecticides like pyrethroids and OPs [1, 2] and is set as a screening target in agrochemical companies worldwide.

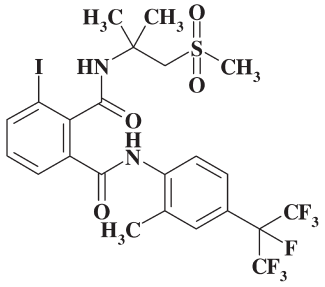
Research at Nihon Nohyaku Co., Ltd. (NNC) began with innovation of isoprothiolane [3] followed by buprofezin [4], the structures of which were quite new. Since then we have continued such research for new compounds characterized by a novel structure and new mode of action. The reason is quite simple. We consider that our research capacity is smaller but more compact than that of a big company. To compete with a leading company in the innovation of a new compound and its subsequent development, we believe that such a way of conducting research may be the best. In other words, competing with others by creating an original product is the only way to show its presence. During discovery screening at NNC, active observation by research workers is recognized as the most important factor. The observation of slight changes of symptoms etc. is carried out in addition to evaluation by the death or not judgment of target insects, and new chemicals are screened up from their overall process. These research characteristics have been inherited and have enabled us to discover a series of new products such as buprofezin [4] and fenpyroximate [5] for instance.

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

31.2
History of the Invention

Flubendiamide (Fig. 31.1) was discovered by NNC and is a novel insecticide for controlling lepidopterous insect pests from the novel chemical class of 1,2-benzendicarboxamides or phthalic acid diamides [6]. This achievement is the result of the accumulated research knowledge of NNC. The lead compound of benzenedicarboxamides was synthesized in 1993. Subsequently, about 2000 derivatives were synthesized, and the structure–activity relationships was examined [6, 7]. From the beginning, the research faced several difficulties: First there was the practical production because this compound has a complex, new chemical structure that was not similar to conventional insecticides. The second was the comprehensive approach to the whole profile of product safety, including an evaluation of the mode of action, that would justify safe future use under the appropriate regulations. The third problem was the seeking and understanding of a new mode of action; in addition, there was the characterization work of biological



Melting Point:	Partition coefficient	Vapour pressure
217.5-220.7 ^{0}C	Octanol/Water 25^{0}C	$<10^{-4}$ Pa at 25^{0}C
	log P = 4.2	
Solubility in water		29.9×10^{-6} g/l

Solubility in organic solvents

n-Heptane	83.5×10^{-5} g/l
p-Xylene	0.488 g/l
1,2-Dichlorthane	8.12 g/l
Methanol	26.0 g/l
Acetone	102 g/l
Ethyl acetate	29.4 g/l
Dimethyl sulfoxide	>1000 g/l

Fig. 31.1. Chemical structure of flubendiamide.

profiling against lepidopterous pests. Research continued to solve these issues until, finally, flubendiamide resulted from the collaboration of the chemistry, biology, and product safety evaluation.

The global developmental work has been accelerated by collaboration with Bayer CropScience (BCS) since 2001 and has now advanced up to the stage before launching. The earliest launch is expected in Japan, India, etc. in 2007, although it is subject to the approval of registration. To deepen knowledge about the compound, Professor Y. Mori at the Kyoto University has also supported NNC in the most advanced research of the mode of action. Part of the work done so far has been published in papers or was presented at academic meetings [8–14]. We describe here details of the invention of flubendiamide and its characteristics.

31.3 Mode of Action

The first reason why we define flubendiamide as a new generation of chemistry is a novel mode of action. This section summarizes research on the mode of action.

A novel mode of action was first suggested by the characteristic symptoms induced by flubendiamide, such as gradual contractions of insect body, thickening and shortening without convulsions (Fig. 31.2). Though the symptoms were obviously different from those of existing insecticides, similar symptoms were also seen in insects treated with the plant alkaloid ryanodine, a modulator of a

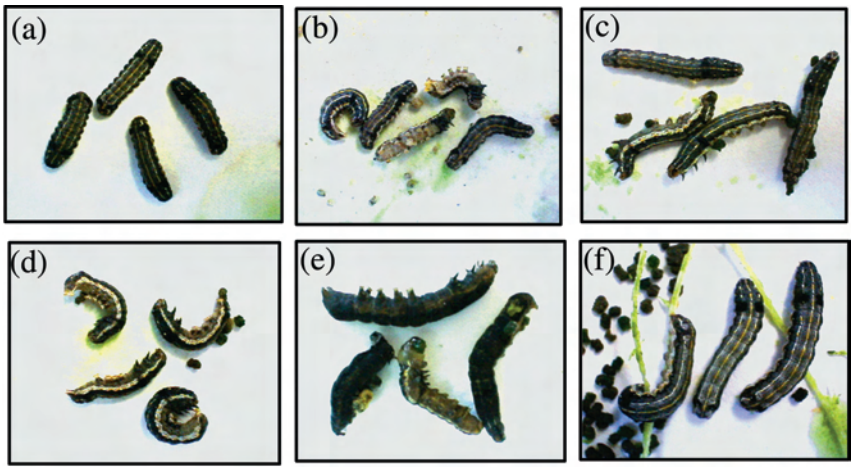


Fig. 31.2. Symptoms of fifth-instar larvae of *Spodoptera litura* treated by leaf dipping [8]. (a) Flubendiamide at 100 mg-a.i. L^{-1}, 24 h after application. (b) Cyhalothrin at 25 mg-a.i. L^{-1}, 24 h after application. (c) Emamectin-benzoate 5 mg-a.i. L^{-1}, 24 h after application. (d) Indoxacarb 50 mg-a.i. L^{-1}, 24 h after application. (e) Flufenoxuron 25 mg-a.i. L^{-1}, 72 h after application. (f) Untreated.

calcium release channel. Therefore, NNC researchers soon paid attention to muscular reaction, and have developed such mode of action research, assuming an influence on the calcium channel that seemed to be involved in muscle contraction.

31.3.1 Insecticides Affecting Calcium Homeostasis

Intracellular calcium is widely accepted as a pivotal regulator for specific cell functions. Versatile components are involved in the precise and dynamic control of intracellular calcium homeostasis. It is also recognized that functional modulations of these components have significant impacts on respective physiological functions. This knowledge implies that the components involved in intracellular calcium regulations should be possible targets for insecticides. Several researchers have indicated this possibility [15–18]. In fact, extracts from the tropical shrub *Ryania speciosa*, which affects calcium release channels, had been applied for pest controls in United States until EPA registration was voluntarily cancelled in 1997 [19]. However, a synthetic organic compound affecting intracellular calcium has never been commercially developed as a pesticide. In this sense, flubendiamide is the first compound possessing insecticidal activity via direct effect on the intracellular calcium homeostasis [10–12, 14]. After flubendiamide publication [6, 7] anthranilic diamides, which belong to a new chemical class with insecticidal spectrum, were published by Du Pont [20]. It was reported in 2005, coincidentally, that anthranilic diamides directly affected intracellular calcium concentrations although their structures are different from the benzenedicarboxamides [21].

31.3.2 Proposed Mode of Action

Figure 31.3 illustrates schematically the proposed mode of action of flubendiamide. In the resting state, intracellular calcium is stored in the sarcoendoplasmic

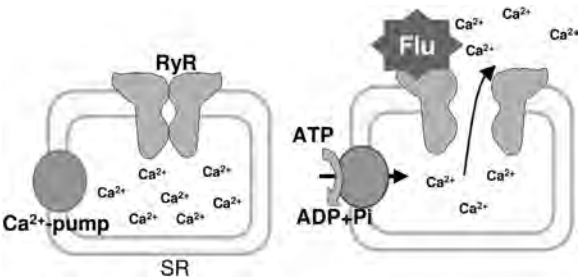


Fig. 31.3. Schematic illustration of the effect of flubendiamide on calcium intracellular homeostasis. RyR: Ryanodine sensitive calcium release channel; Flu: flubendiamide, benzendicarboxamide derivatives.

reticulum (SR). It can be mobilized by opening calcium release channels such as the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP_3R). Two experimental approaches employing calcium imaging in isolated neurons from the lepidopterous insect *Heliothis virescens*, and calcium release from the muscle membranes from the lepidopterous insect *Spodoptera litura*, clearly revealed that flubendiamide caused calcium mobilization from the internal stores [12, 14].

It is common knowledge that the ryanodine-sensitive calcium release channel (ryanodine receptors, RyRs) is an important component for calcium mobilizations in excitable cells. Ryanodine specifically suppressed calcium mobilization through functional modulations of this channel [22]. The calcium response induced by flubendiamide was suppressed by ryanodine, suggesting that flubendiamide acted on the RyR. This mode of action of flubendiamide is further demonstrated by evidence that flubendiamide induced calcium response in the transfected CHO cells expressing the ryanodine receptor from *Drosophila melanogaster* [12]. RyRs are homotetramers that consist of 450–550-kDa subunits [23, 24]. The putative ryanodine binding site located in the transmembrane channel pore region (Fig. 31.4) is sensitive to the channel conformation, which is reflected in the alteration of [^{3}H]ryanodine binding affinity [25–27]. Flubendiamide evidently potentiated the [^{3}H]ryanodine binding affinity to the muscle membrane of two lepidopterous insects without a significant effect on the receptor density (B_{max}), indicating that the compound shifted the conformational equilibrium of the RyR to the open state (Fig. 31.4) [12].

Interestingly, RyR activation by flubendiamide induced remarkable stimulation of the Ca^{2+}-pump activity of insects and showed a greater increase than those by

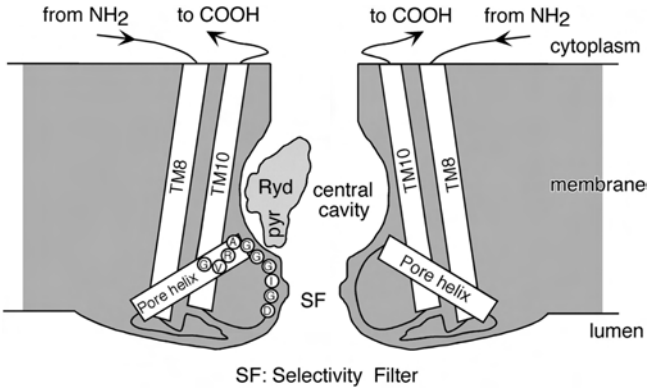


Fig. 31.4. Illustration of the interactions between ryanodine and the RyR conduction pathway [31]. Hypothetical model for ryanodine–RyR interactions. It is proposed that ryanodine (Ryd) binds to the putative central cavity of the RyR conduction pathway with its pyrrole group (pyr) anchoring at a specific site near the selectivity filter (SF) and its opposite end pointing toward the cytoplasmic mouth of the conduction pathway. Estimated dimensions of the ryanodine molecule and the putative selectivity filter are drawn to scale relative to the membrane bilayer.

the classical RyR modulators such as ryanodine and caffeine [14]. This characteristic property in the effect of flubendiamide is recognized as a consequence of specific interaction with the distinct binding site on the RyR. The binding of [^{3}H]flubendiamide to the muscle membranes of lepidopterous insect has a high affinity and is not competitive to the classical RyR modulators.

The distinct binding site of flubendiamide appears to contribute to the observed selectivity between insects and mammals. Namely, ryanodine, which binds to specific binding sites both on insect RyRs and on mammalian RyRs, provides high acute toxicity to mammals [28, 29]. In contrast, flubendiamide and its derivatives do not affect the mammalian skeletal muscle isoform RyR1 [12]. The diversity in primary structure of RyR should provide insight into the mechanism underlying the selective action of flubendiamide. The primary structure of RyR, which has been evaluated in various animal species, including insects, shows high homology among mammals but low homology between mammals and insects [18]. In agreement with evolutionary distance, the primary structure of RyR from a lepidopterous insect retains high levels of overall identity with *Drosophila* RyR, but relatively low levels of identity with mammalian RyR [18, 30, 31]. Even though most of the domain structures are highly conserved among RyRs, flubendiamide might discriminate an insect specific site of the channel. However, a greater understanding of the selectivity of flubendiamide requires clarification of the binding domain on RyR.

The mode of action of anthranilic diamides has been investigated in detail. It was reported that anthranilic diamides activate RyR, releasing stored calcium from the sarcoendoplasmic reticulum, and exhibit >500-fold differential selectivity toward insect, over mammalian, receptors [32]. Anthranilic diamides will be soon classified as RyR modulators within the IRAC classification [2]. The two chemical classes, benzenedicarboxamides and anthranilic diamides, could contribute to our understanding of the mechanism of calcium release channels in insects and mammals.

31.4 Chemistry

The second reason why we define flubendiamide as a new generation insecticide is its chemical structure compared with known insecticidal compound classes. This section summarizes the historical basis of improvement in chemical research.

NNC scientific researchers were greatly interested in diamide-type compound **1** (Fig. 31.5), which showed herbicidal activity [33], and has been investigated for herbicides [34]. During this research, an insecticidal activity for the benzenedicarboxamide derivative **2** was found. Although the level of activity was low, the compound attracted attention for two reasons, its novel insecticidal chemical structure and its intriguing insecticidal symptoms. As usual in this area of research, there were several points to be improved for a practical use: low insecticidal activity,

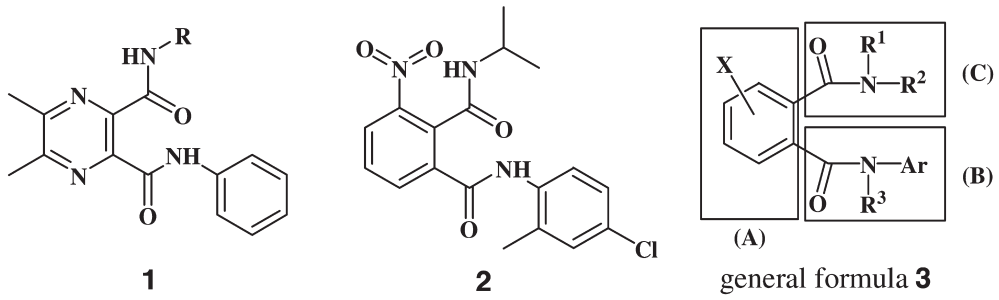


Fig. 31.5. Chemical structures of 1,2-benzendicarboxamide derivatives.

phytotoxicity to crops and instability of the compound. Benzenedicarboxamides are characterized by the following three parts of the chemical structure, as shown in the general formula **3** (Fig. 31.5): (A) phthaloyl moiety, (B) aromatic amide moiety and (C) aliphatic amide moiety.

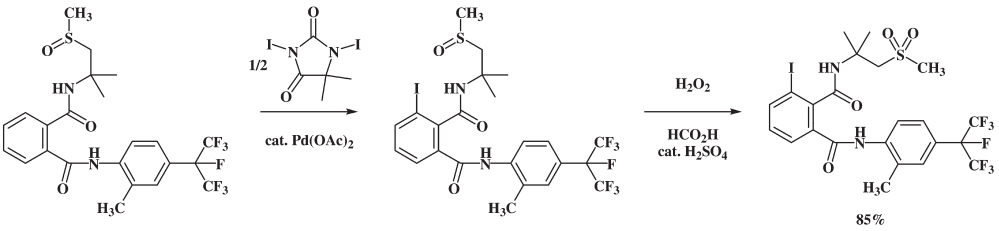
This complex and novel structure presented a challenge to researchers to design a facile synthetic method and to establish a practical and economical manufacturing method.

One problem was that three different groups had to be connected regioselectively to the 1,2- and 3-positions of the benzene ring in the phthaloyl moiety. An iodine atom was introduced selectively into the 3-position of the phthaloyl moiety by a palladium-catalyzed reaction in the presence of a specific substituent in the 2-position. On the basis of the structure–activity relationship, the introduction of lipophilic alkyl substituents, including fluorine atoms, seemed to increase the activity though a practical method of introduction was not available. The overcoming of such difficulties led to dramatic advances in terms of a more detailed study on the structure–activity relationship as well as the establishment of a facile synthetic method that provided various new derivatives.

31.4.1 Challenge of Chemistry

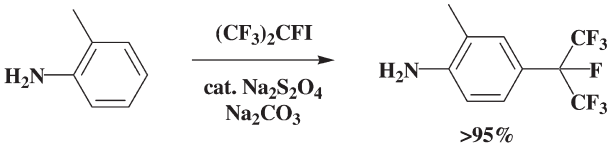
The first challenge was the regioselective introduction of an iodine atom into the benzene ring of the phthaloyl moiety. As a conventional synthetic method, the Sandmeyer reaction is well known. NNC, however, found a direct and facile substitution method of a hydrogen atom with an iodine atom [35]. This substitution method avoids the generation of waste materials as much as possible and the reaction may be classified as one of the best in green chemistry [36, 37]. The configuration of the palladium catalyst in connection with the sulfoxide of the aliphatic side chain may play a key role in introducing the iodine in a regiospecific manner though details of this reaction have to be clarified (Scheme 31.1).

Another challenge was the introduction of a bulky substituent into the benzene ring of the aromatic amide moiety. Insecticidal activity was improved significantly



Scheme 31.1

for those compounds where the pentafluoroethyl group or heptafluoroisopropyl group was introduced into the 4-position of the aniline ring, which is connected via the 1-position to the benzene ring of the phthaloyl moiety. Initially, metallic copper was applied in the coupling reaction [38] but there was no good synthetic method for this kind of aniline available. Later, it was synthesized by using the radical reaction described in the references [39, 40]. Notably, this radical reaction also improved significantly the practical conditions, and an excellent synthetic method in terms of yield and reaction conditions was established (Scheme 31.2).



Scheme 31.2

The above-mentioned two synthetic methods, namely the aniline synthesis and the iodine atom introduction, are new, potentially significant reactions in organic synthetic chemistry that could be applicable to synthesize similar compounds.

31.4.2 Structure–Activity Relationship

Details of structure–activity relationships for the three parts of benzenedicarboxamides (Fig. 31.5) used to select flubendiamide are quoted from the literature [8].

During the optimization process, changing the substituents of the phthaloyl moiety, the tendency could be seen that lipophilic and bulky substituents showed good activity. An iodine atom in the 3-position proved to be the best substituent, although there are very few commercial agrochemicals having an iodine atom in the structure.

For the aromatic amide moiety, the heptafluoroisopropyl group is very unusual since it has never been reported as a substituent in the chemical structure of conventional pesticides. After flubendiamide was found, the substituent of the 2-position on aniline was verified. As expected the methyl substituent gave the best

results, suggesting that a moderate bulky substituent is very suitable in this moiety.

Finally, for the aliphatic amide moiety, the isopropyl group was the most favorable one from simple alkyl groups. Introduction of a hetero atom, especially a sulfur atom, on the alkyl side chain markedly increased the insecticidal activity. This sulfonylalkylamine moiety also has a high novelty as an amine in pesticide chemistry.

The unique substituents described above account not only for the high activity of flubendiamide but also for its categorization as a totally new chemical structure.

31.4.3
X-Ray Structural Analysis

The structure of flubendiamide has been established by NMR spectroscopy and confirmed by single-crystal X-ray structure analysis. Flubendiamide possesses different and bulky substituents at the 1-, 2-, and 3-positions of the benzene ring of the phthaloyl moiety. The X-ray structural analysis also, interestingly, revealed a peculiar arrangement of substituents. In the case of benzamide, the carbonyl moiety and the benzene ring are coplanar. The most energetic stable three-dimensional structure of flubendiamide derived from molecular modeling calculations using MOPAC97 (AM1) is a conformer in which two carbonyl moieties face in opposite directions, while the conformer actually observed in the crystal structure has two carbonyl moieties facing in the same direction.

31.5
Biological Profiles

The third reason why we define flubendiamide as a new generation insecticide is the following biological profile, such as the high level of insecticidal activity, showing no cross-resistance to conventional insecticides due to the new mode of action, as mentioned previously, and the selectivity. This section gives a brief overview of the biological profile [8, 9, 13].

31.5.1
Activity against Lepidopterous Pests

Flubendiamide showed broad-spectrum activity against all lepidopterous pests, but is inactive against other insect species such as Coleoptera, Hemiptera and Acarina. It provides very high activity against all important lepidopterous insect pests shown in Table 31.1.

Although cyhalothrin, one of synthetic pyrethroids, shows activity on different developmental stages of lepidopterous pests, flubendiamide is most effective on larvae followed by adults, but it has no ovicidal activity and limited adulticidal ef-

Table 31.1 Insecticidal spectrum of flubendiamide on major insect pests in agriculture [13].

Scientific name	Common name	Tested stage[a]	DAT[b]	EC_{50} (mg-a.i. L^{-1})
Lepidoptera				
Plutella xylostella	Diamond-back moth	L3	4	0.004
Spodoptera litura	Tobacco cutworm	L3	4	0.19
Helicoverpa armigera	Old World bollworm	L3	4	0.24
Agrotis segetum	Turnip moth	L2-3	7	0.18
Autographa nigrisgna	Beet semi-looper	L3	4	0.02
Pieris rapae crucivora	Common cabbage worm	L2-3	4	0.03
Adoxophyes honmai	Smaller tea tortrix	L3	5	0.38
Homona magnanima	Oriental tea tortrix	L4	5	0.58
Hellula undalis	Cabbage webworm	L3	5	0.01
Chilo suppressalis	Rice stem borer	L3	7	0.01
Diaphania indica	Cotton caterpillar	L3	3	0.02
Coleoptera				
Sitophilus zeamais	Maize weevil	A	4	>1000
Hemiptera				
Nilaparvata lugens	Brown rice planthopper	L3	4	>1000
Myzus persicae	Green peach aphid	All stages	7	>1000
Pseudococcus comstocki	Comstock mealybug	L1	7	>100
Acarina				
Tetranychus urticae	Two-spotted spider mite	All stages	4	>100

[a] L2, L3, L4: second, third and fourth instar; A: adult.
[b] DAT: Day(s) after treatment.

fect. This shows that the main activity of flubendiamide is lavicidal. The symptoms of larvae affected by flubendiamide are quite unique, as mentioned in the preceding section, and the activity is mainly provided by ingestion.

Against the different larval stages of lepidopterous pests it is most effective on first instar larvae followed by third and fifth instar larvae (Table 31.2). It will be highly advantageous over organophosphates and spinosad, which are affected drastically in activity by larval size. Although even on fifth instar larvae flubendiamide provides very high activity relative to conventional insecticides, application to young stages of larvae is recommended to lead to more effective control in practical use [9, 13].

31.5.2 Fast-acting Activity and Persistence

The speed of appearance of symptoms on larvae treated with flubendiamide indicates its fast acting activity (Fig. 31.6). Symptoms caused by flubendiamide

Table 31.2 Insecticidal activity of flubendiamide on three different larval stages of *S. litura* [13].

Treatment	EC_{50} (mg-a.i. L^{-1}, 3 DAT)		
	1st instar	3rd instar	5th instar
Flubendiamide WDG	0.033	0.19	0.51
Cyhalothrin EC	0.08	0.36	0.72
Methomyl WP	13.8	17.3	15.4
Profenophos EC	1.38	17.3	54.8
Spinosad WDG	0.67	45.5	54.8

were observed within a few hours after exposure and were faster than those of indoxacarb, emamectin-benzoate and flufenoxuron, although it was slower than cyhalothrin.

Suppression of feeding damage on larvae at 24 h after exposure with flubendiamide also shows clearly its fast acting activity (Fig. 31.7). Flufenoxuron is a typ-

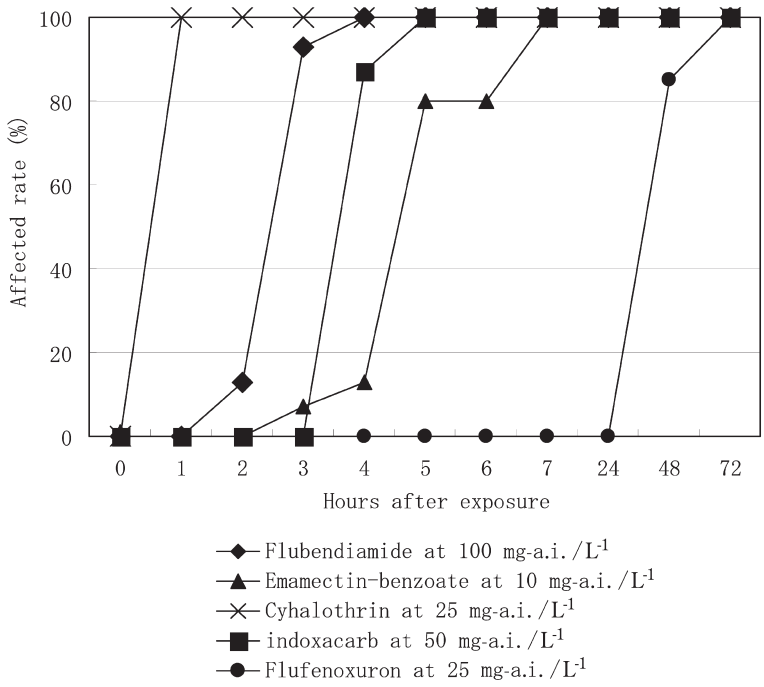


Fig. 31.6. Speed of appearance of symptoms on 5th instar larvae of *S. litura* after treatment by leaf dipping.

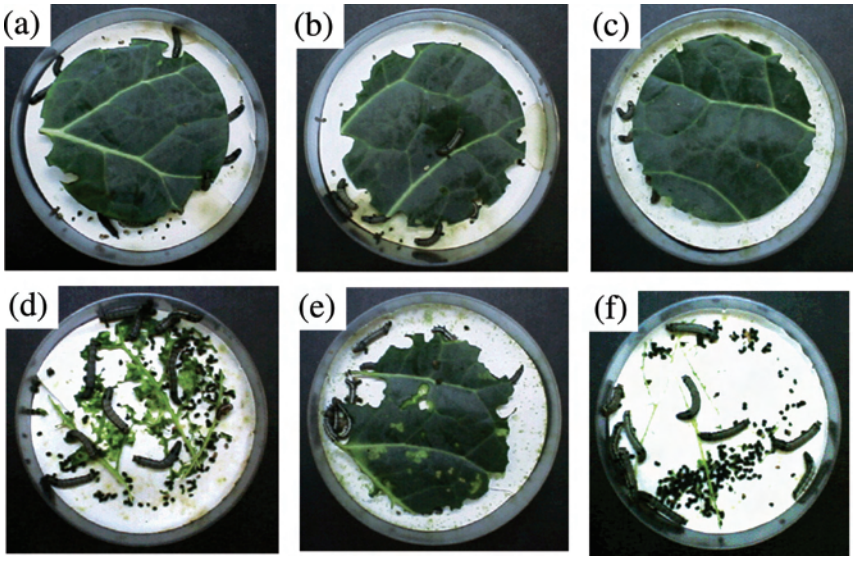


Fig. 31.7. Suppression of feeding damage on 5th instar larvae of *S. litura* 24 h after treatment by leaf dipping. (a) Flubendiamide at 100 mg-a.i. L^{-1}; (b) Flubendiamide at 25 mg-a.i. L^{-1}. (c) Cyhalothrin at 25 mg-a.i. L^{-1}. (d) Flufenoxuron at 25 mg-a.i. L^{-1}. (e) Emamectin-benzoate at 5 mg-a.i. L^{-1}. (f) Untreated.

ical slow-acting insecticide and the feeding damage on the treated plot with flufenoxuron was the same as on the non-treated plot. In contrast, flubendiamide showed suppression of feeding damage as quickly as cyhalothrin and emamectin-benzoate. Although the main symptom of flubendiamide is insect body contraction, its fast acting activity is demonstrated by the rapid cessation of feeding.

The long-lasting activity of flubendiamide on cabbage leaves has been investigated under glasshouse conditions (Fig. 31.8). Flubendiamide showed sufficient residual activity on treated leaves for more than 4 weeks. The activity was on the level of cyhalothrin, and superior to methomyl and emamectine-benzoate. It was also confirmed that the control efficacy of flubendiamide persists for about 2–3 weeks in many crops under field conditions at the recommended rate in Japan, 100 and 50 mg-a.i. L^{-1} [9, 13].

Field evaluations of flubendiamide have been conducted in many areas on various crops, including vegetables, top fruit and cotton. Flubendiamide showed excellent performance in controlling the major lepidopterous pests on each crop at the recommended doses and its efficacy was comparable to or better than those of standard insecticides [9, 13]. Flubendiamide (20% WDG) shows no phytotoxicity to any crop tested even though applied at double the recommended rates.

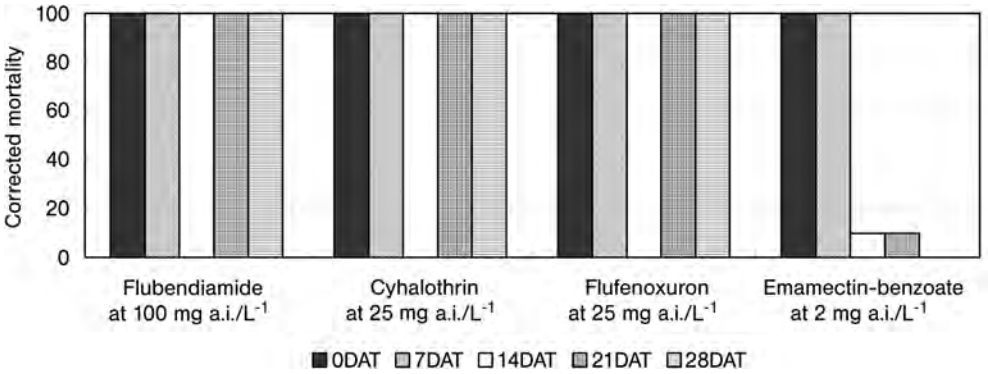


Fig. 31.8. Long-lasting activity of flubendiamide against 3rd instar larvae of *S. litura* on cabbage leaves under glasshouse conditions (DAT = days after treatment).

31.5.3
Cross-resistance

From its unique symptom and new mode of action flubendiamide would be expected not to show cross-resistance with conventional insecticides. Thus, when the activity of flubendiamide against larvae of *P. xylostella* resistant to synthetic pyrethroids, benzoylphenylureas, organophosphates and carbamates was evaluated, flubendiamide provided the same EC_{50} values against both the resistant and the susceptible strains [8, 13]. The absence of cross-resistance between flubendiamide and conventional insecticides is probably because of its new mode of action. This indicates that flubendiamide will fit well into insecticide resistance management (IRM) programs.

31.5.4
Toxicity to Beneficial Arthropods

Flubendiamide was inactive against beneficial arthropods tested at rates from 100 to 400 mg-a.i. L^{-1}, through the experiment of acute toxicity of flubendiamide to several species of beneficial arthropods (Table 31.3) [8, 13]. This indicates that flubendiamide would be compatible with Integrated Pest Management (IPM) programs.

31.6
Toxicological Properties

Table 31.4 shows some toxicological features of flubendiamide. Though flubendiamide is now under evaluation of registration after submission in Japan, India

Table 31.3 Toxicity of flubendiamide to natural enemies [8].

Common name	Scientific name	Test stage	Test method	EC_{30} (mg-a.i. L^{-1})
Lady beetle	*Harmonia axyridis*	Adult	Insect dipping	>200
	Coccinella septempunctata bruckii	Adult	Insect dipping	>200
Parasite wasp	*Encarsia formosa*	Adult	Dry film	>400
	Aphidius colemani	Adult	Dry film	>400
	Cotesia glomerata	Adult	Dry film	>100
Green lacewing	*Chrysoperla carnea*	Larva	Spraying on food & insect	>100
Predatory bug	*Orius strigicollis*	Adult	Spraying on food & insect	>100
Predatory midge	*Aphidoletes aphidimyza*	Larva	Spraying on food & insect	>100
Predatory mite	*Amblyseius cucumeris*	Adult	Spraying on food & insect	>200
	Phytoseiulus persimilis	Adult	Spraying on food & insect	>200
Spider	*Pardosa pseudoannulata*	Adult	Insect dipping	>100
	Misumenops tricuspidatus	Adult	Insect dipping	>200

Table 31.4 Toxicological profile [13].

Acute oral:	Rat LD_{50}	Male & female	>2000 mg kg^{-1}
Acute dermal:	Rat LD_{50}	Male & female	>2000 mg kg^{-1}
Eye irritation:			Slight
Skin irritation:			None
Mutagenicity:			Ames test: negative
Aquatic organism:		Carp LC_{50}	>546 μg L^{-1} (96 h)
Honeybee:		Oral/contact LD_{50}	>200 μg L^{-1} (48 h)
Bird (Bobwhite quail):		Oral LD_{50}	>2000 mg kg^{-1} body weight

and some other countries, it is suggested that flubendiamide is safe for mammals. The data that flubendiamide had almost no effect on mammalian RyR (type-1) also support this hypothesis [12].

31.7 Conclusions

Flubendiamide was discovered as a novel class insecticide having a unique chemical structure and is the first synthetic compound possessing insecticidal activity acting as a ryanodine receptor (RyR) modulator [2]. All data obtained indicate that flubendiamide can be classified as a new generation insecticide in view of its biochemical mode of action, its chemistry and its biology. Flubendiamide provides excellent activity against a broad spectrum of lepidopterous insect pests and shows no-cross resistance to conventional insecticides. In addition, it is much safer against natural enemies. With these properties it is demonstrated that flubendiamide will be very suitable for IRM and IPM programs.

Benzenedicarboxamide compounds, including flubendiamide, were found through original research at NNC. However, notably, anthranilic diamides, structurally very different from benzenedicarboxamides, were discovered by DuPont [20] to have the same mode of action [21] and a couple of companies are following with patent applications: Nissan Chemical [41] and Takeda Pharmaceutical Company [42] have applied for patents of the related compounds of benzenedicarboxamides, and Ishihara Sangyo Kaisha [43] has applied for patent of the related compounds of anthranilic diamides. The market entry of insecticides from this new generation could intensify competition with conventional insecticides in the future.

Acknowledgments

The authors as representatives of this research would like to thank sincerely all their distinguished colleagues in Nihon Nohyaku Co., Ltd. who have contributed to the research and development of flubendiamide. The authors also wish to acknowledge many scientists in Bayer CropScience AG and Professor Yasuo Mori at Kyoto University for their scientific discussion of the mode of action works especially.

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Part IV
New Research Methods

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

32
High Throughput Screening in Agrochemical Research

Mark Drewes, John C. W. Lohrenz, Klaus Stenzel, and Klaus Tietjen

32.1
Introduction

Efficient and economical agriculture is essential for sustainable food production to fulfill the demands for high-quality nutrition of the continuously growing population of the world. To ensure this, the control of weeds, fungal pathogens and insects, each posing a threat of yield-losses of about 13–15% before harvest, is a necessity (Fig. 32.1). Although a broad range of herbicides, fungicides and insecticides already exists, shifts in target organisms and populations and increasing requirements necessitate a steady innovation of crop protection compounds.

Evidently, weeds, fungal pathogens and insects belong to evolutionary fundamentally distinct organism groups (Fig. 32.2), which makes a single crop protection compound that solves all problems in one inconceivable. A closer look even reveals that insects, fungi and weeds are not a sufficiently correct depiction. Although the term insecticide sometimes is used for any chemical combating insects, spider mites or nematodes, the differences between these organisms are so significant that it is more precise to speak of insecticides, acaricides and nematocides. Among plant pathogenic fungi the evolutionary range is even broader, and oomycetes are not fungi at all, although oomyceticides are also commonly named fungicides. Hence, agrochemical screening of fungicides and insecticides requires a substantial range of diverse species. The situation for herbicide screening is, in some ways, the reverse, but it is not easier. The close genetic similarity between crop and weed plants generates challenges in regard to specificity of compounds, differentiating between crop and weed plants. This also results in the need to use a range of different crop and weed plants in screening.

Owing to these circumstances agrochemical screening has covered, in both the laboratory and glass-house trials, as many different pest species as could be afforded. The recent developments described in this chapter, however, have allowed ever higher throughput not only in glass-house tests on living organisms but they have also introduced biochemical (*in vitro*) target tests. Unsurprisingly, the implementation of molecular screening techniques and the ~omics technologies (func-

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

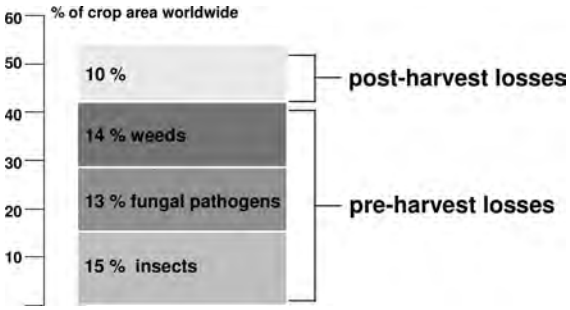

Fig. 32.1. Losses of potential agricultural harvest of major crops due to different pests, diseases and weeds [1, 2]. Non-treated, approximately 50% of harvest would be lost.

tional genomics, transcriptomics, proteomics, etc.) into agrochemical research was a big challenge due to the high diversity of the target organisms [6].

Molecular agrochemical research with biochemical high throughput target screening started with several model species, which have been chosen mainly because of easy genetic accessibility or specific academic interests. These first favorite model organisms of geneticists and molecular biologists were, mostly, not identical to the most important pest species in agriculture (Fig. 32.3).

Meanwhile the recent progress in genome sequencing has led to a steadily growing knowledge of agronomical relevant organisms (Table 32.1).

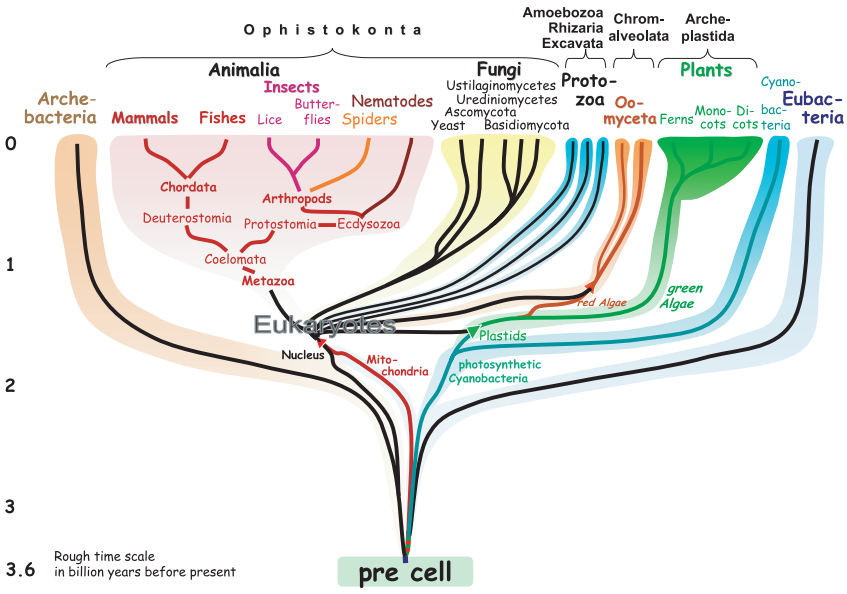

Fig. 32.2. Modern evolutionary tree of life. The view is based on Refs. [3] and [4]. For a more detailed view of fungi see Ref. [5].

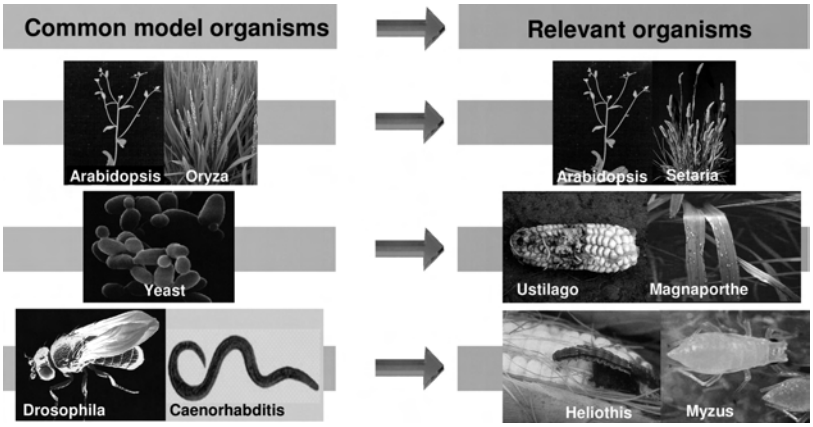

Fig. 32.3. Model organisms in molecular biology and agronomically relevant target species.

Table 32.1 Agronomically relevant organisms with completed or ongoing genome sequencing projects.[a]

Organisms		
Plants	**Fungi and oomycetes**	**Insects & nematodes**
Dicotyledonous plants	**Ascomycetes**	**Diptera**
Arabidopsis thaliana[b]	*Saccharomyces cerevisiae*[b]	*Drosophila melanogaster*[b]
Brassica oleracea	*Botrytis cinerea*[b]	**Aphids**
Glycine max	*Sclerotinia sclerotiorum*[b]	*Acyrthosiphon pisum*
Lotus corniculatus	*Fusarium graminearum*[b]	**Lepidoptera**
Solanum tuberosum[b]	*Magnaporthe grisea*[b]	*Bombyx mori*[b]
Monocotyledonous plants	*Aspergillus oryzae*[b]	**Coleoptera**
Oryza sativa[b]	*Neurospora crassa*[b]	*Tribolium castaneum*
Sorghum bicolor	*Alternaria brassicicola*	**Nematodes**
Triticum aestivum	**Ustilaginomycetae**	*Caenorhabditis elegans*[b]
Zea mays	*Ustilago maydis*[b]	
	Uredinomycetae	
	Phakopsora meibomiae	
	Phakopsora pachyrhizi	
	Basidiomycetes	
	Phanerochaete chrysosporium[b]	
	Zygomycota	
	Rhizopus oryzae[b]	
	Oomycetes	
	Phytophthora infestans[b]	

[a] Complete general overview: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj; fungi overview: Fungal genome Initiative: http://www.broad.mit.edu/annotation/fungi/fgi/index.html.
[b] Completed or close to completion, otherwise: in progress.

The situation is relatively simple for weeds, since all plants are closely related (Fig. 32.2). The first sequenced model plant, *Arabidopsis thaliana*, is genetically not very distinct from many dicotyledonous weeds, and the monocotyledonous crops are closely related to monocotyledonous weeds, which in fact only a few thousand years ago became the basis for breeding today's cereals species. The first sequenced insect genome of *Drosophila melanogaster*, a dipteran insect, was extensively exploited in genetic and molecular biological research. To better reflect relevant pest organisms like lepidoptera or aphids, *Heliothis virescens* and *Myzus persicae* have been investigated by the agrochemical industry, and *Bombyx mori*, *Acyrthosiphon pisum* and *Tribolium castaneum* have been sequenced in public projects (see Table 32.1). Baker's yeast, *Saccharomyces cerevisae*, has long been the commonly used model fungus. The ascomycete *Magnaporthe grisea* and the basidiomycete *Ustilago maydis* were the first sequenced relevant plant pathogens. Within the next few years, even the broad evolutionary range of the many different plant pathogenic fungi and oomycetes (Fig. 32.2) will be covered by genome projects.

32.2
Target-based High Throughput Screening

32.2.1
Targets

Progress in the molecular biology of agronomically relevant organisms has enabled the introduction of target-based biochemical HTS, significantly changing the approach of screening for agrochemicals during the past ten years. Target-based HTS is now a broadly utilized technology in the agrochemical industry to deliver active ingredients with defined modes of action [6].

Major research at agrochemical companies has established biochemical HTS, often supported by co-operation with companies having particular expertise in specific fields of biotechnology. The first wave of genomics – genome wide knock-out programs of model organisms – indicated that about a fourth of all genes are essential, i.e., they were lethal by knock-out [6, 7, 8]. The resulting high number of potential novel targets for agrochemicals has to be further investigated to clarify the genes functions (reverse genetics) and to better understand their role in the organism's lifecycle. Although the technology of genome wide knock-out itself was highly efficient and well established, it turned out that even knock-out of some known relevant targets was not lethal, either because of genetic or functional redundancy, counter-regulation, or because a knock-out does not perfectly mimic an agonistic drug effect on, for example, ion channels. Therefore, today, knock-out data are critically reviewed with respect to as many aspects as possible of the physiological roles of potential targets. Consequently, they are taken as just one argument for a gene to be an interesting potential target. In

addition, the clarification of a gene's function is a challenging and resource-consuming task and, therefore, today the focus is often on targets with a sound characterization of their physiological role.

The best proof for an interesting agrochemical target is "chemical validation" by biologically active compounds. This is true for all the established targets. However, chemical hit structures acting on such targets must have an advantage over the already known compounds. This may be chemical novelty, a novel binding site, increased performance, or overcoming resistance. From the standpoint of innovation and the chance to open new areas, novel targets are of particular interest, especially when active compounds are already known, e.g., natural products, such as, for example, the ryanodine receptor for insecticides. Most interesting are novel and proprietary targets that arise from genetics programs or from mode of action (MoA) discovery. Mode of action elucidation for biological hits has therefore gained much significance.

Modern analytical methods like high-performance liquid chromatography/mass spectrometry, electrophysiology, imaging and others form a gateway to today's novel target discovery. The benefit of electrophysiology for clarifying neurophysiological effects is obvious. Cellular imaging techniques complement electrophysiology and are, furthermore, general means for MoA studies. For metabolic targets, like those of sterol biosynthesis, direct target identification may be possible by metabolite analysis [9, 10]. For some compounds, gene expression profiling has also proved to be a valuable tool for MoA classification [11, 12]. Used as fingerprint methods, metabolite profiling and gene expression profiling allow at least a fast and reliable detection of known modes of action and a clear identification and classification of unknown modes of action. But, despite all the technological progress, mode of action elucidation of novel targets still is and will be in the near future a highly demanding challenge. Only the combination of all available methodologies, with emphasis on traditional careful physiological and biochemical examination, will reveal a clearly identified novel molecular target [13].

Identification of resistance mutations to pesticides has in recent decades been one of the most clear-cut ways of target clarification. The technological progress has fostered considerably throughput in screening for mutations with a certain phenotype, so-called "forward genetics" [14]. However, it does yet not seem to be a reliable source of novel targets.

Once a target has been envisaged, further criteria for a "good" target are applied. Obviously, most important is the druggability of a target, which means accessibility by agro-like chemicals (see below) [15]. It is no coincidence that the best druggable targets have preexisting binding niches, favoring ligands that comply with certain physicochemical properties. Furthermore, the target should be relevant during the damaging life phase of a pest and the destructive effect on a weed or pest under practical conditions should occur shortly after treatment.

Having passed all these hurdles an interesting target has to be assayable to be exploited, making assay technology capabilities a crucial asset. Overall, the number of promising targets remaining is at least two orders of magnitude lower than the number of potential targets found by gene knock-out [6]. Even after all these

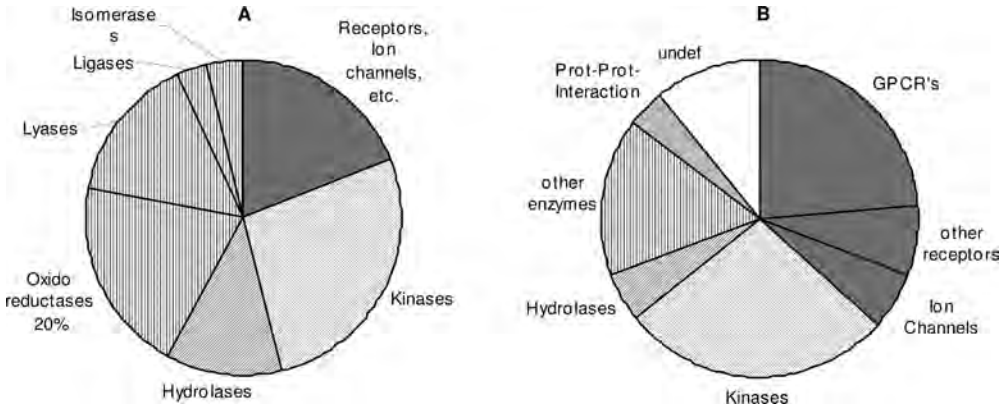

Fig. 32.4. Classification by function of agrochemical (A) and pharmaceutical (B) targets [21] for HTS.

efforts it still difficult to predict whether new active ingredients will be identified and whether a novel target will, finally, be competitive in the market.

Pharmaceutical research often systematically concentrates on particular target classes such as, for example, protein kinases in cancer research [16]. Thereby, know-how can be accumulated and specialized technology can be concentrated for higher productivity [17]. A successful target triggers attention on the next similar targets, leading to considerable understanding of, for example, the human kinome [18]. A similar approach in agrochemical research is of limited value since there are no such privileged target classes (Fig. 32.4). In fact, the common denominator of the diverse agrochemical targets often is the destructive character of the physiological consequences of interference with the target's function, sometimes even being a "side-effect" like the generation of reactive oxygen species [6]. Nevertheless there are exceptions. One is the class of protein kinases, which have been mentioned as a promising target class for fungicides [19, 20].

32.2.2 High Throughput Screening Techniques

In pharma research HTS has proven to be a major source of new lead structures [21], motivating agrochemical research to, at least in parts, introduce this approach into the drug discovery process as well. At Bayer AG, for example, the first HTS systems were set up in the late 1990s and the screening capacity expanded rapidly to more than 100 000 data points per day on a state-of-the-art technology platform, which includes fully automated 384-well screening systems, a sophisticated plate replication and storage concept, a streamlined assay validation and quality control workflow, an extension of the compound collection with the help of combinatorial chemistry and major investments in the development of a suitable data management and analysis system.

The concept allows the screening of large numbers of both compounds and newly identified targets yielding a corresponding number of hits. Simultaneously developed quality control techniques were able to separate valid hits from false-positives and/or uninteresting compounds due to various reasons (e.g., unspecific binding). Interestingly, several target assays deliver considerable numbers of *in vivo* active compounds while for some others the often remarkable target inhibition is not transferred into a corresponding *in vivo* activity. In some cases this can be attributed to insufficient target lethality of more speculative targets. As discussed earlier, the value of a thorough validation of (a) targets, (b) assays and (c) chemical hits becomes evident.

The extended target validation led to increased numbers of target screens with *in vivo* active compounds. Hence, even more time could be spent on hit validation, namely the introduction of control tests to eliminate, for example, readout interfering compounds (hits that were only found due to their optical properties or chemical interference with assay components).

At the same time, great efforts were made to increase the quality of the compound collections (see below) and of course quality checks of the compounds (e.g., LC/MS identity check of every single hit).

The process of continuous improvement has to date shifted to among others an extended characterization of hits with respect to reactivity, binding modes [22] (competitive/non-competitive, reversible/irreversible, etc. [23]), speed of action and erratic inhibition due to "promiscuous" behavior of the compound class [24] (Fig. 32.5 – Profiling). Concurrently, if feasible, the hits or hit classes are submitted to orthogonal assays like electrophysiology, in the case of neuronal targets, that help to further classify and validate the hits independently of the readout.

All these measures together have greatly increased the proportion of true hits so that, finally, the chemistry capacities are concentrated on fewer but well-characterized hit classes with a clearly increased likeliness of a successful hit-to-lead optimization (Fig. 32.5).

The huge amount of data and information generated during the various phases of HTS and subsequent validation triggered the development of sophisticated data analysis tools [25] that help biologists and chemists to select and prioritize the most promising hits or hit classes (cluster of similar compounds) (Fig. 32.6).

Biochemical *in vitro* screening may deliver compounds that despite a clear target activity cannot exhibit *in vivo* activity due to, for example, unfavorable physicochemical properties (lacking bioavailability), fast metabolization, insufficient stability, or poor distribution in the target organism. Nevertheless, these chemical classes are of interest to chemists, because these properties are characteristics of compounds that in principle can be overcome by chemical optimization. "Agro"-kinetics has identified pure *in vitro* hits as such and helped to elucidate the reasons for failure in an *in vivo* test, thus guiding the *in vitro* to *in vivo* transfer of hit classes.

Currently, two trends can be observed in the high throughput community: miniaturization into the nanoliter dispensing regime and new high content screening (HCS) techniques. Small volume screening (either on 1536-well plates or the re-

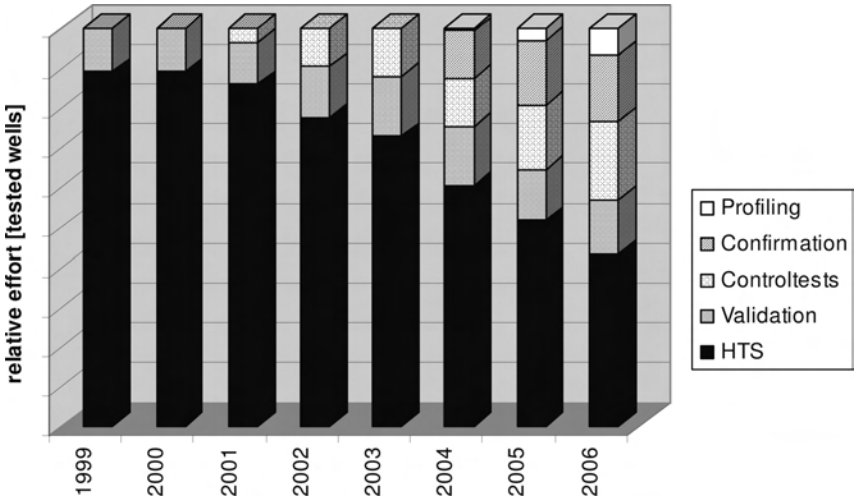

Fig. 32.5. Changes in the usage of the total screening capacity from primary HTS to in-depth hit characterization. HTS: high throughput screening. Confirmation: repetition of assay for hits with duplicates. Validation: IC_{50} determination. Control tests: tests for interference with the assay system. Profiling: tests to characterize the properties of the inhibitors. 2006 data predicted.

cently introduced low volume 384-well plates) clearly is of interest for agrochemical research as well, since enzymes and substrates of new target proteins are often difficult and costly to produce in larger quantities. Owing to the above-mentioned screening strategy this process is not so much driven by the need to

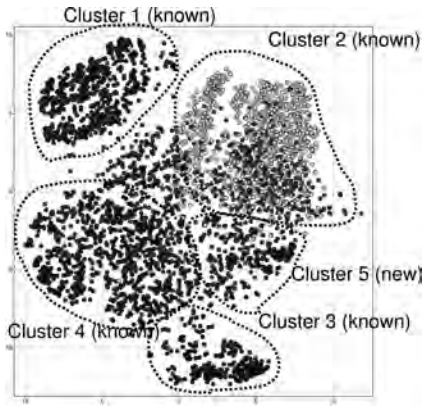

Fig. 32.6. Example of the visualization of the chemical space of hits and similar but inactive structures from a target assay; light grey: inactive; grey to black: decreasing IC_{50}.

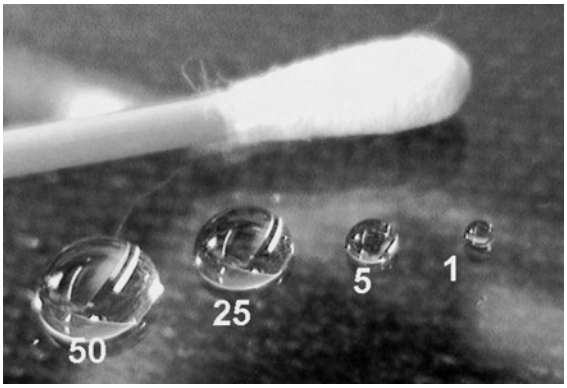

Fig. 32.7. Size comparison between water drops (50–1 μL) and a cosmetic tip.

further increase the capacity but solely by cost efficiency. At Bayer CropScience, for example, the standard reaction volume has decreased from more than 50 μL to 5–10 μL (Fig. 32.7). Further reductions are possible since new pipetting equipment has reached a robust quality with inaccuracies below 5% in the 1-μL range.

Very important for ion channel screening are the recently developed automated and, for now, medium throughput patch clamping systems that perfectly meet the increased demand for in-depth hit characterization. The future role of high content screening – fully automated confocal life cell microscopy imaging systems – is not as clear as it is in pharmaceutical research, where it is *the* validation and screening method development of the last few years [26]. The applicability of HCS for agrochemical research will have to be evaluated.

32.3 Other Screening Approaches

32.3.1 High Throughput Structure – Biology

Another important tool to support the drug discovery process is target structure determination. For various reasons (improved techniques, radiation sources, algorithms and increasing computing power) the number of published protein structures is exponentially increasing. By the end of 2005 the total number of structures deposited at PDB [27] reached 31 414 (Fig. 32.8).

In line with these technical advances the number of published structures that are interesting for agrochemical research is considerable and steadily increasing (Table 32.2). For many of the most interesting market established targets, structures were determined from relevant species. Even more structures are available

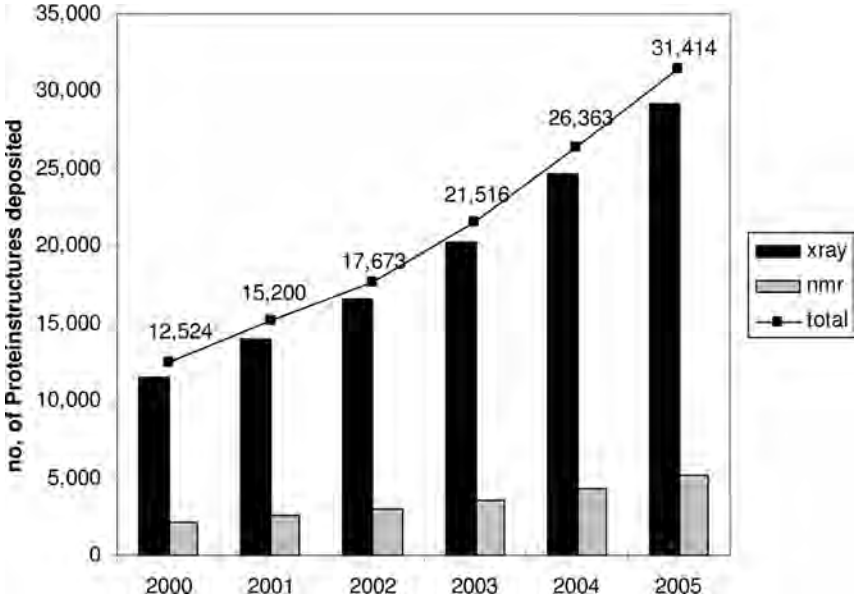

Fig. 32.8. Exponential growth of deposited protein structures at the Protein Data Base (PDB) until 2005-12-31 [27].

from other sources which are suitable for homology modeling based on related sequences [28].

Each structure determination method has is specific characteristics and advantages. NMR protein structure determination is feasible for relatively small (<40 kDa) and isotope labeled proteins. Newer methods and spectrometers with higher field strengths show promise to further increase this size limit up to 60 kDa [29]. As an interesting feature this method yields solution structures with information on dynamic behavior and does not depend on the derivation of crystallization conditions. However, NMR requires several weeks for data collection and processing, whereas crystallography, in the very best cases, can collect data using a synchrotron radiation source in less than one hour, with phasing information, and construct a 3D model in less than 1 week. There have been significant advances in recent years in the development and implementation of methods and instrumentation for macromolecular crystallography. Automation of the tedious optimization of crystallization conditions and recent developments in synchrotron radiation sources together with CCD detectors and cryo-crystallography have revolutionized protein crystallography, opening the door to high throughput protein structure determination.

The major obstacle with X-ray crystallography still is the need for sufficient amounts of soluble and very pure protein. As a consequence some protein classes like membrane bound proteins (e.g., receptors and ion channels) suffer from limited success rates [30, 31]. A true highlight in this context is certainly the award of

Table 32.2 List of most important agrochemical molecular targets with relevant entries in the protein data base. Bold entries: crystallized with agrochemical. Entries marked with an * indicate only related protein crystallized, allowing model building. In some cases more pdb entries exist.

Insecticide target	pdb	Herbicide Target	pdb	Fungicide target	pdb
Acetylcholine esterase	*: 1fss, 2ace, 1ea5, 1vot, 2dfp	Enolpyruvyl-shikimate phosphate synthase	**2aay**, **1g6s**, 1eps, 1g6t, 1xbt	Sterol C-14 demethylase	*: **1ea1**, **1e9x**, 1h5z, 1x8v
vgNa channel	*: 1j95, 1f6g, 2a79, 2ahy	Acetolactate synthase	1ybh, 1yhy, 1yhz, 1yi0, 1yi1, 1z8n	Cytochrome c reductase	1ezv, 1kb9, 1kyo, *: 1sqb, 1sqq, 1sqx, 1sqp, 1sqv, 1lol, 1ntk, 3bcc
Nicotinic acetylcholine receptor (nAChR)	*: 1i9b, 1uv6, **1uw6**, 1ux2, **2byq**, 2byr, 2bys, 2bjo, 2bg9	Photosystem II	1nze, 1izl, 1fe1	Tubulin	*: 1tub, 1z2b, 1sao, 1sa1, 1ffx, 1jff, 1tvk
$GABA_A$ receptor	*: as nAChR	Very long chain fatty acid elongase	*: 1bq6, 1cml, 1chw, 1cgk, 1cgz, 1uou, 1uow	Succinate dehydrogenase	*: **2fbw**, 1yq4, 1yq3

Table 32.2 *(continued)*

Insecticide target	pdb	Herbicide Target	pdb	Fungicide target	pdb
Glutamate gated chloride channel	*: as nAChR	Auxin binding protein 1	**1lrh**, 1lr5	Scyatalone dehydratase	2std, 3std, 4std, 5std, 6std, 7std, 1std
Ecdysone receptor	**1r20**, 1r1k, 1z5x	AcetylCoA carboxylase	*: **1uyr**, **1uys**, 1w93, 1w96, 1od2, 1w2x, 1uyt		
Succinate dehydrogenase	*: 2fbw, 1yq4, 1yq3	Tubulin	*: 1tub, 1z2b, 1sao, 1sa1, 1ffx, 1jff, 1tvk		
Chitin biosynthesis	./.	Protoporphyrinogen oxidase	1sez		
		4-Hydroxyphenylpyruvate dioxygenase	**1tg5**, **1tfz**, 1sqd, 1sqi, 1sp9, 1sp8		
		Glutamine synthetase	*: **1fpy**, 2bvc		
		Phytoene desaturase	./.		
		Cellulose biosynthesis	./.		

the Nobel prize for the successful structure determination of the photo-system by Huber [32]. Most insecticidal targets consist of neuronal targets and receptors that are currently technically too difficult to be routinely addressed by structural biological studies. Successful GPCR or ion channel crystallizations (e.g., the potassium channel was eligible for a Nobel prize [33]) will remain rare exceptions until new crystallization technologies (e.g., cubic phases [34]) can be established.

We now briefly summarize interesting applications of protein structures in the context of the drug discovery process – a more complete discussion is beyond the scope of this chapter.

An important field for good and reliable structural information is protein modeling: To understand binding motifs and optimize lead structures, rational drug design has to rely on high resolution structures. High throughput *virtual* screening with its flexible docking of virtual ligands into (real) proteins is bound to good structural information of the binding sites (see below).

A different field, not to be undervalued, is a better understanding of target resistance due to mutations at the binding site. With structural information at hand, it is possible (a) to identify the critical structural changes [35] and (b) to use guided design approaches to propose new chemical entities that are able to break resistance.

The above-mentioned advances in dataset collection and phasing algorithms enable several high throughput applications of X-ray crystallography: (a) target, (b) ligand and (c) fragment based screening. The first application is the most complex and demanding because here success is limited by the crystallization itself. Very pure protein batches are needed for each target, requiring individual optimization of expression and crystallization conditions. High throughput "co"-crystallization of various ligands with one target is either based on soaking of ready grown target crystals with the ligands or, alternatively, on crystallization of both, protein and ligand, together using minimally adapted protocols for the apo target structure crystallization. Hence, a multitude of ligand–protein structures are accessible in a short time frame, an essential piece of information to build up a true understanding of the structure–activity relationship (SAR) [36]. Finally, fragment based screening is a new approach to locate several small weakly binding molecules at different positions in the binding site with the aim of chemically combining several motifs (fragments) in the follow-up and, consequently, drastically enhancing the binding energy. This approach has to be based on very reliable biochemical, NMR, X-ray crystallography data or a combination of these measurements [37].

32.3.2 High Throughput Virtual Screening

Computational chemistry has become an inevitable partner in drug discovery in recent years. One of its contributions to high throughput methods is target-based virtual screening. Virtual screening [38] is often understood as any computational method that is applied to large sets of compound collections; some under consid-

eration of target structure information, some solely based on ligand similarity to various degrees of complexity. The more 3D information is incorporated, the more computationally demanding becomes the calculation, especially if even the flexibility of the target protein should be considered. Currently, massive screening with fully flexible models is not yet feasible, but the so-called flexible *docking* of huge, both real and virtual, compound collections into a rigid binding pocket has become routine [39]. The most obvious advantage of the latter method over the fast similarity searches is the fact that any compound that has binding site complementarity will be identified and that no similarity to a known ligand is needed. This stands in contrast to similarity based screening, where completely new scaffolds are rarely found.

To have reasonable hit enrichments by docking, computational chemistry has to start from high-resolution protein structures. If possible, more than one ligand co-crystal will be used for the construction of the binding domain. Some programs meanwhile are even able to handle a certain degree of target flexibility through *ensemble formations* of binding domains from various experimental structures [40]. Quality but also computational effort increases consequently.

Virtual target-based screening can be applied in many ways. The most obvious is the screening of huge libraries to prioritize the synthesis, acquisition and/or biochemical screening or to select reactants for combinatorial libraries that show highest hit likeliness. These applications do yield target-focused libraries and can be extended to families of targets, like, for example, kinases or GPCR's.

32.4 *In Vivo* High Throughput Screening

Since its beginning *in vivo* screening has been the basis of agrochemical research for the identification and characterization of active ingredients and the subsequent optimization process of interesting chemical classes. To ensure a sufficient number of starting points for optimization fulfilling the increasing demands in regard of activity, toxicological and economical characteristics, the number of compounds to be tested increased continuously. Since the mid-1990s, most major agrochemicals companies have established *in vivo* high throughput screening systems [15, 41, 42, 43, 44]. The numbers reported to be tested vary between 100 000 and 500 000 compounds per year. Less than 500 µg of substance is sufficient to produce relevant answers for a given set of different plants, insects, and fungi growing in 96-well or 384-well microtiter plates (MTPs) (Fig. 32.9). Inevitably, these high throughput systems are producing a large number of hits. To improve the quality of the hits additional dose rates and replicates have been implemented [15] to deliver highly validated hits to follow up in relevant screens.

All the above systems are based upon automation, miniaturization and often use model organisms or systems that are easy to handle. Such model systems using *Aedes aegypti*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Caenorhabditis elegans* [45] or cell growth based fungicide assays are successful in identifying

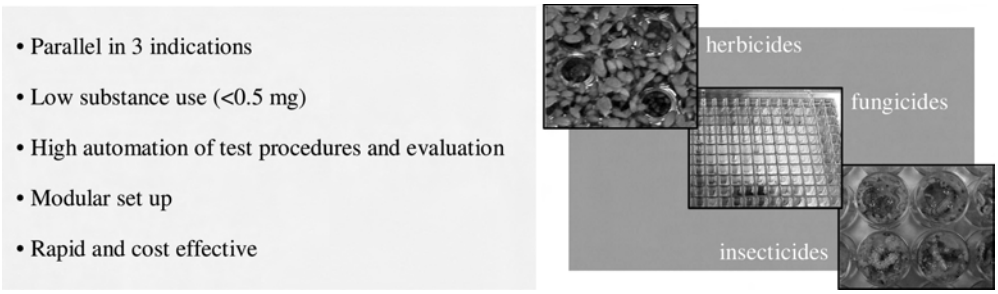

Fig. 32.9. Advantages of high throughput screening.

many hits. However, in follow up tests with relevant species the number of interesting substances decreases. Furthermore, high throughput tests with model organisms appear to miss relevant hits (Fig. 32.10).

The consequence thereof is the development of more relevant target systems, particularly for insecticides and fungicides. For instance, a test for sucking insects such as whitefly or aphids would increase the relevance of the initial screening process but has not yet been reported [15] in any high throughput screening. Within the fungicide process cell growth tests are also only covering part of the relevant target organisms; all the obligate pathogens such as the mildews or rusts are not tested. Additionally, such cell tests are not testing the relevant phases of the development of fungal pathogens on living plant tissues. This gap can be closed by using leaf discs [46, 47] or whole plants with relevant fungal species.

The development and further improvement of such relevant high throughput tests for insecticides and fungicides is a continuous challenge for the future. In most cases the tests are significantly more complex and the time and effort required to run target organism tests is by far higher than for previous model systems. This inevitably has to be considered in regard to throughput or dedicated resources for an *in vivo* high throughput screening. However, fewer but better

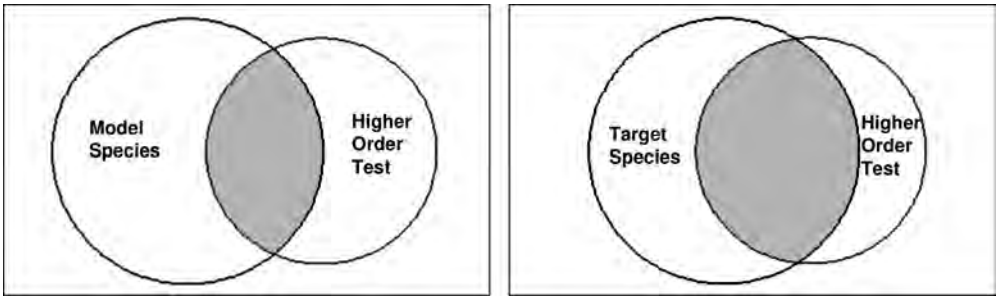

Fig. 32.10. Overlap of mutually active chemical hits found in model species tests versus target species tests.

characterized compounds with a relevant biological profile are certainly worth more than a high number of hits from model tests.

32.4.1 Chemical Compounds

To achieve the ambitious goals of high throughput screening, a large number of compounds is needed to satisfy the capacities of the tests. Many major chemical companies, pharmaceutical as well as agrochemical, started to buy off-the-shelf compounds [48] from so-called bulkers on a worldwide basis. Furthermore, the boom triggered by combinatorial chemistry also helped to quench the need for new substances. Many new companies such as ArQule, BioFocus or ChemBridge were founded. The substances initially purchased were predominantly driven by availability and chemical convenience. However, the number of new biologically active classes did not increase correspondingly. Soon it was recognized that not only for pharmaceutical compounds [but also for agrochemical substances] certain constraints were needed to obtain some biological activity (Fig. 32.11). These constraints and (sub-structural) fingerprints as descriptors [49] for molecular similarity were applied to choose chemical collections for agrochemical discovery.

A further refinement of the agro-like constraints and help from *in silico* screening has further improved the diversity [51] of the collections. However, in the case of combinatorial chemistry a major realignment is underway. The starting points needed for such libraries have changed from ''blue sky'' to scaffolds with a biological background [6]. Such considerations entail more intricate synthetic routes and cause the size of the libraries to decrease. The number of compounds that become available for the high throughput tests was reduced. However, the probability of obtaining better hit classes was far higher. This can be the only way forward in the early phase of lead finding. In future, the combination of agro-likeness tools and carefully chosen biological scaffolds will be the options that produce compounds (Fig. 32.12).

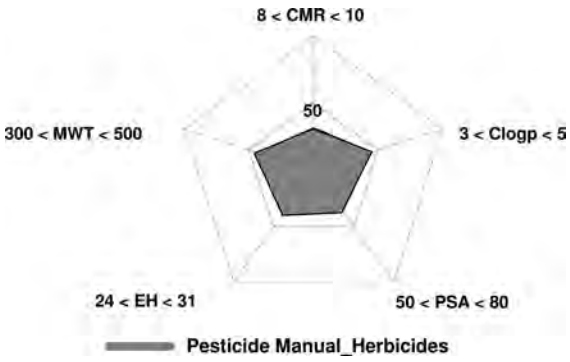

Fig. 32.11. Percentage of herbicides in the *Pesticide Manual* [50] within constraint range.

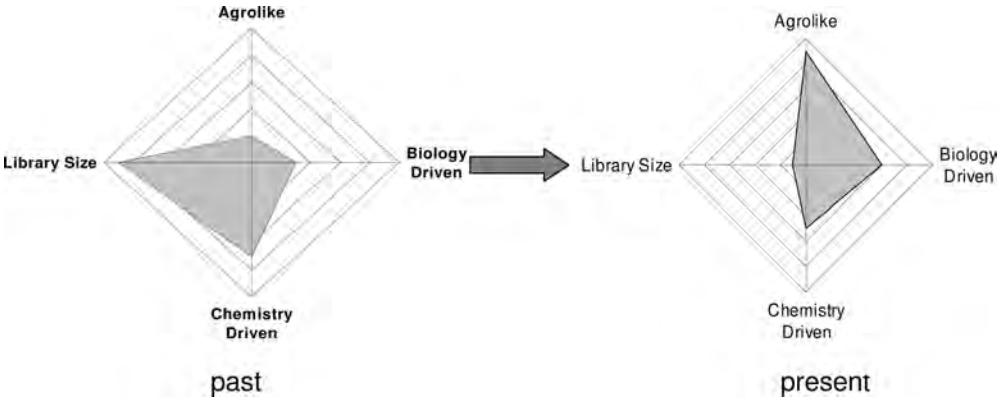

Fig. 32.12. Higher input of agro-likeness and biological input in combinatorial chemistry scaffolds.

32.5 Conclusions

During the last decade high throughput screening has been implemented in the agrochemical industry in the early research phase to tackle the challenges of decreasing success rates in the identification and development of new market compounds. Unlike the discovery approaches in the pharmaceutical industry, not target-based screening alone was implemented but HTS with target organisms – *in vivo* HTS – was developed on the basis and experience of classical and well-established biological screening. In agrochemical research the broad diversity of target organisms is presenting a specific and complex challenge that has to be carefully considered and solved in all screening systems. Fed by high throughput chemistry, functional genomic projects and significant progresses in robotic screening systems, procedures have successfully been established that allow the efficient testing of large numbers of compounds with a broad set of living organisms as well as against newly identified and well-established targets.

For efficient research it is essential to evaluate continuously and with increasing experience the chances and limitations of new and established technologies. Modern agrochemical research platforms are undergoing continuous and dynamic changes. Adjustments aim towards the integration of the most promising parts of the different approaches.

With the advancing implementation of new technologies into standard workflows in early and late research phases a broad knowledge has been gained that by far exceeds the specific high throughput screening approach alone. This is definitely leading to a new quality in agrochemical research. Finally, it is the expectation that innovative products from these new technologies will meet the needs of modern agriculture.

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33
Fast Identification of the Mode of Action of Herbicides by DNA Chips

Peter Eckes and Marco Busch

33.1
Introduction

Agrochemicals have played a major role in the large increase in agricultural productivity over the last 50 years. Nevertheless, still about 40% of the harvest is lost due to pests or weed infestation. The primary method for weed control, at least in industrialized countries, is the use of herbicides. Being by far the biggest segment in the crop protection market, herbicide sales have grown only moderately over the last 10 years because market dynamics were basically driven by replacement of established products, with new herbicides showing only slightly better properties. Higher demands on the efficiency and spectrum of new products, as well as increased regulatory hurdles, make it more and more difficult to bring new products on the market. Five from the six top selling herbicides in the year 2004 were launched between 30 and 60 years ago. These five products still comprise more than 30% of the world wide herbicide sales.

To be successful in the future a company has to develop novel solutions for weed control with superior agronomic properties; properties that would alter the market-landscape or which even would create new market segments. Compounds with novel herbicidal Modes of Action (MoA) would have the potential to fulfill these requirements. They would have the potential to open new segments and to trigger above average growth of the herbicide market.

Due to the high competitiveness of the crop protection market, the research process from synthesis of new chemicals to promotion of lead compounds to the project phase has to be as streamlined as possible. It is imperative not only to eliminate compounds with weak efficacy or phytotoxicity but also chemicals with non-desirable MoA as early as possible from further evaluation and to concentrate on a few promising candidates. Besides phenotypical inspection of treated plants, the target site of a compound is usually determined with specific enzyme assays in test tubes or microtiter plates, which is a time consuming, labor- and cost-intensive process. It requires either purification of the respective enzyme from plants or preparation of the proteins by heterologous expression in, for example,

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

bacteria or yeast. Additionally, for each enzyme a specific assay has to be developed, where the activity of that enzyme can be determined upon the presence and absence of the compound. To identify the MoA of several compounds, theoretically each compound has to be tested against each enzyme – and for most enzymes no test tube assays are available. Therefore, it would be desirable to have a method that could give a clue to the MoA of an herbicide in a single experiment.

33.2 Gene Expression Profiling – A Method to Measure Changes of the Complete Transcriptome

The functionality of an organism is determined by the information contained in its genes. Genes are transcribed into messenger RNA (mRNA), which is subsequently translated into the different proteins. As enzymes, these proteins are the ultimate effectors in the cell, converting one metabolite into another. The controlled action of these enzymes is necessary for the coordinated interaction of the metabolic pathways that maintain the functionality of the organism (Fig. 33.1). In this context, a key regulatory mechanism of living cells is the controlled expression of the respective genes. During development and differentiation, as well as by external perturbations, this network of expressed genes varies constantly to

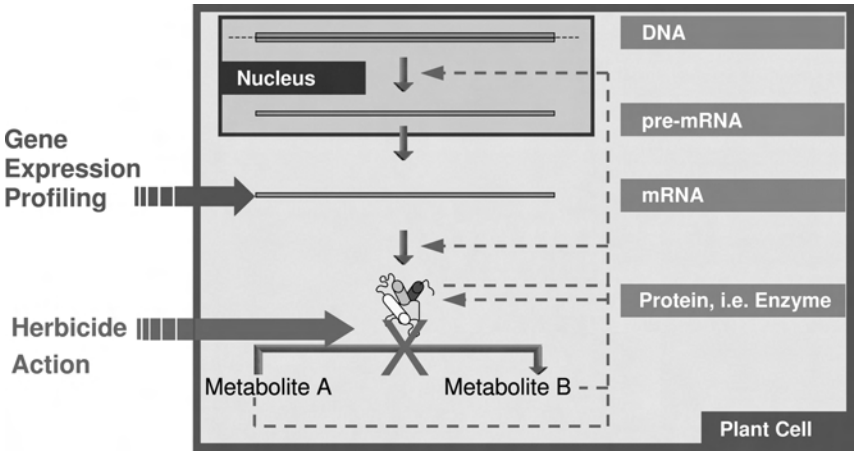


Fig. 33.1. Schematic representation of general cellular processes: DNA as the storage of genetic information is localized in the nucleus and is transcribed into messenger RNA (mRNA). The mRNA transports this information out of the nucleus into the cytoplasm of the cell and is translated into proteins there. The proteins can be enzymes that catalyze a reaction from metabolite A to metabolite B. An herbicide blocks this reaction by inhibition of the enzyme activity. Proteins and metabolites exercise regulation upon DNA transcription and RNA translation (thin dashed lines). Thus, effects on the cell processes exhibited by herbicides are reflected by changes in mRNA levels that can be analyzed by GEP.

adapt to changing environmental conditions. It is well established that measurement of mRNA expression is a valuable tool to assess the reactions of an organism to its environment although in the end the metabolic processes are mediated by the mRNA encoded proteins.

When a plant is treated with an herbicide, vital processes of that plant are affected, such as photosynthesis, cell wall formation or the biosynthesis of cellular components. This is reflected by changes in the transcriptome, the set of all mRNAs of a plant. The amount of mRNA of some genes increases, whereas the mRNA amount of other genes decreases. This snapshot of the transcriptional status of a plant is called the Gene Expression Profile (GEP).

The whole genome of *Arabidopsis thaliana* [1] and rice [2–4] has been sequenced. This information together with technical advances in automation, miniaturization and parallel synthesis of oligonucleotides has been used to develop full genome DNA microarrays for those plants [5, 6] which represent almost all genes of the respective species. In addition to the full genome plant microarrays there exist DNA chips for many different plants like, for example, corn, soybean, barley or tomato. These chips do not represent the complete genome but a large proportion of the expressed genes of the respective plants. Such DNA microarrays have been used to analyze the reaction of plants to biotic factors such as plant defense against pathogens [7], seed development [8], nitrate assimilation [9] and fruit ripening [10] or to abiotic factors like drought [11], cold [12], heat [13], etc. In this way it was possible to obtain new insights into molecular mechanisms regulating these processes.

The experiments described here use the *Arabidopsis* ATH1 GeneChip microarray, a full genome chip manufactured by Affymetrix (http://www.affymetrix.com/). It is about 1×1 cm in size and contains nucleic acid sequences of about 24.000 genes (Fig. 33.2). Short 25mer nucleotide sequences for each gene have been synthesized on specific spots on the chip. Each gene is represented by 11 different oligonucleotides (gene probes), scattered randomly over the chip. The multitude of oligonucleotides for each gene and their random distribution increase the significance of the statistical analysis of the expression results.

Because the ATH1 chip represents almost all *Arabidopsis* genes, it can also detect changes in the transcriptome caused by the circadian clock of the plant or by other environmental stimuli such as biotic or abiotic stresses. The effects of these stimuli on transcription can sometimes be much stronger than the changes caused by the action of herbicides. This would mask the expression pattern produced by the herbicide. Therefore, it is imperative to grow the plants under conditions as standardized as possible. Light-, temperature- and humidity controlled growth chambers are needed to grow the plants; all steps from sowing and watering of *Arabidopsis*, spraying of the compounds and harvest of the plants down to preparation of the mRNA have to be highly reproducible from experiment to experiment. By these means it is possible to compare expression profiles that have been produced over several years.

In a standard expression profiling experiment plants are harvested 24 h after treatment with a chemical. RNA from compound-treated plants and from control

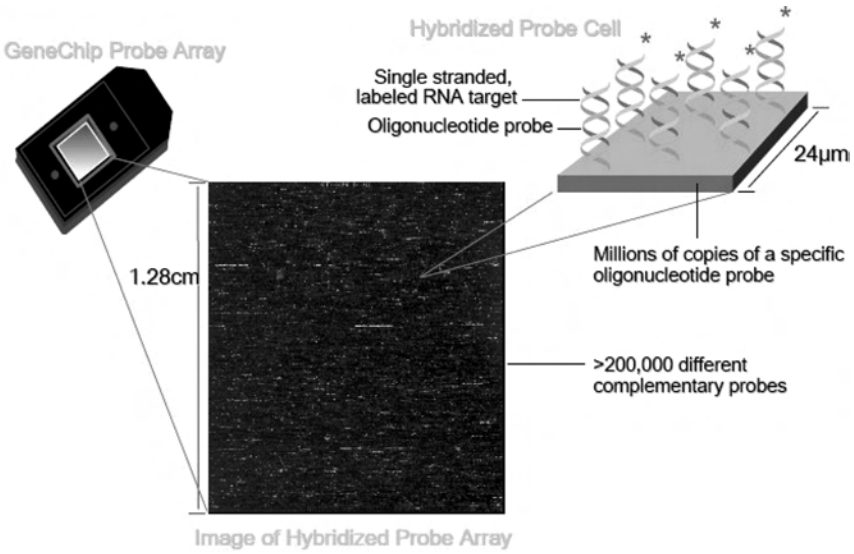


Fig. 33.2. The GeneChip system: RNAs isolated from herbicide treated plants and labeled with a fluorescent dye bind to their corresponding gene probes. Highly abundant RNAs produce bright signals, whereas rare RNAs produce only dim signals.

plants is isolated separately, labeled with a specific dye and then incubated with the nucleotide sequences on the chip. Because of sequence homology, the individual RNAs bind to their corresponding gene probes. Since the location of each *Arabidopsis* gene probe on the chip is known and since the RNA is labeled with a fluorescent dye, the amount of bound RNA for each gene probe can be measured individually in a scanner. Highly abundant RNAs will produce bright signals, whereas rare RNAs will produce only very dim signals. The difference in brightness between the samples determines whether the amount of RNA for a given gene has increased or decreased due to the herbicide treatment. Since all *Arabidopsis* genes are located on a single DNA chip it is possible to measure changes in RNA abundance for all genes in a single experiment. Each herbicide produces a distinctive gene expression pattern, a kind of fingerprint for that herbicide.

33.3 Classification of the Mode of Action of an Herbicide

Compounds that have the same MoA affect the same metabolic processes. Therefore, the expression profiles of plants treated with compounds having the same MoA should be very similar and clearly different from those of compounds with other MoAs. Under this assumption, a compendium of expression profiles from *Arabidopsis* plants treated with compounds/herbicides of known MoA was established. The compendium represents about 40 herbicides from eleven known

MoAs, such as, for example, acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO), photosystem I, photosystem II or 5-enolpyruvylshikimi-3-phosphate-synthase (EPSPS). All expression profiles in the compendium are derived from *Arabidopsis* plants sprayed with two different concentrations of the respective compounds and harvested 24 h after treatment. Analysis of the expression profiles by statistical methods like hierarchical clustering [14] revealed that the assumption was correct. The profiles of compounds representing the same MoA were much more similar to each other than to any profile derived from a compound with another MoA (Fig. 33.3).

The expression profiles are stored in a database, the GEP Compendium. Now, it is possible to classify compounds from the research pipeline with unknown

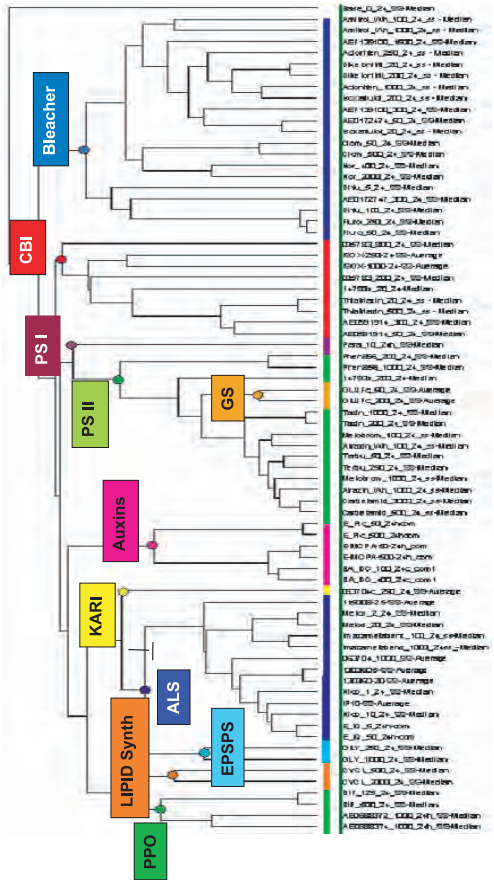


Fig. 33.3. Herbicide GEP Compendium: A hierarchical clustering of Gene Expression Profiles of 40 compounds from 11 different MoAs is shown. Individual profiling experiments are listed in the lower part. Experiments clustering in the individually colored branches belong to the same MoA.

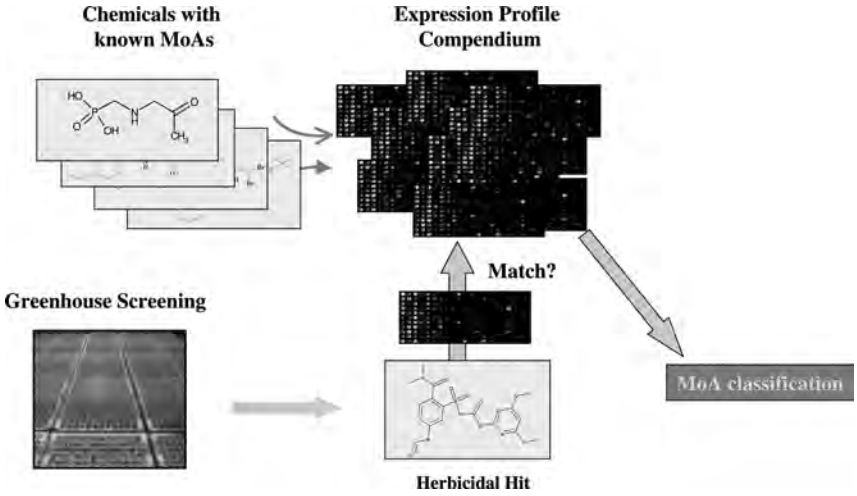


Fig. 33.4. Gene Expression Profile Compendium approach: A Gene Expression Profile of a compound identified as herbicidally active in the greenhouse is compared with existing profiles in the database. If a similar profile is present, the new compound most probably has the same MoA.

MoA into one of the known MoAs of the GEP Compendium with a single experiment. *Arabidopsis* plants are sprayed with the respective compound and the isolated and labeled RNA is analyzed on the *Arabidopsis* chip. Subsequently, the resulting expression profile is compared with those in the compendium. This comparison is done by supervised learning algorithms like Support Vector Machine (SVM) [15] or Analysis of Variance (ANOVA) [16]. When the new expression profile groups together with profiles of a specific MoA in the compendium there is an utmost probability that the corresponding compound has the same MoA (Fig. 33.4). If necessary, the MoA can be verified by classical methods such as enzyme assays or supplementation tests, if available.

Meanwhile we have also classified many different compounds, coming from the research pipeline, that had an unknown MoA. It was possible to eliminate compounds with an unwanted MoA very early from the research process and to concentrate on more promising substances. If compounds cannot be classified into an already existing MoA, the standard GEP Compendium approach can at least put them into specific unknown MoA groups.

33.4 Identification of Prodrugs by Gene Expression Profiling

An inherent problem in the MoA determination of herbicides by classical enzymatic assays is the evaluation of prodrugs. Prodrugs are compounds that are not

active per se, but have to be converted into an herbicidally active product inside the plant, e.g., by cytochrome P450 enzymes [17] or esterases [18]. In conventional enzyme assays the compounds are tested on purified target enzymes. Because the prodrug is not converted into the active form, the enzyme is not affected and the enzyme assay would not identify its MoA. Gene Expression Profiling is much closer to the "real situation", since whole plants are sprayed with a compound. There is enough time for a potential prodrug to be taken up by the plant, to be converted into the active form and to exert its effect on the target enzyme before the *Arabidopsis* plants are harvested for gene expression analysis. Therefore, Gene Expression Profiling can identify even the MoA of such prodrugs.

The active ingredient Compound A is an example of how Gene Expression Profiling can identify the MoA of a prodrug. Compound A has the ability to kill many different weeds. Unfortunately it was not possible to identify its MoA in a collection of very diverse classical enzymatic assays. In a Gene Expression Profiling experiment we were able to classify Compound A into the group of acetolactate synthase inhibitors (Fig. 33.5). The expression profile of Compound A treated *Arabidopsis* plants was much more similar to the profiles of plants treated with other ALS inhibitors than to the profiles of plants treated with compounds from other MoAs. More supporting evidence for ALS as MoA comes from the fact that Compound A induces the genes for alternative oxidase (data not shown). It is well

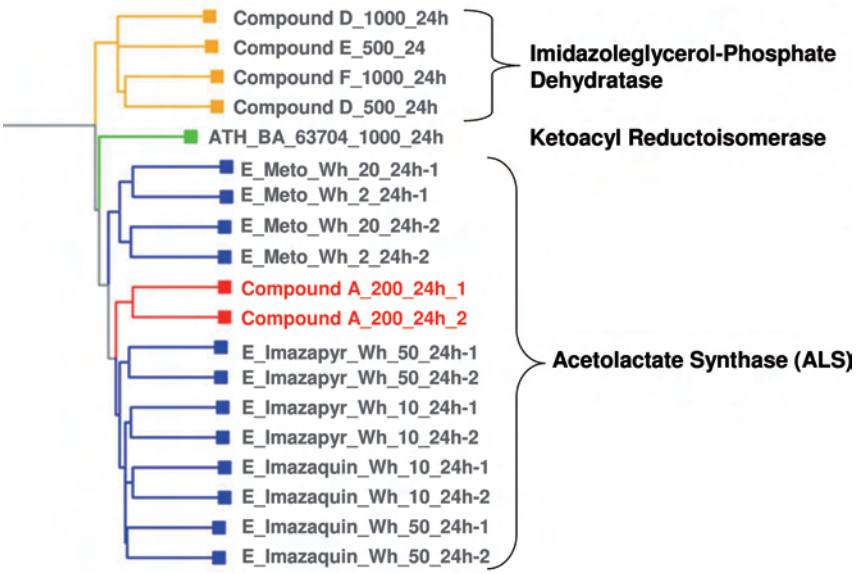


Fig. 33.5. Classification of an herbicide: The expression profile of Compound A clusters together with the profiles of known ALS inhibitors like Metosulam (Meto), Imazapyr or Imazaquin.

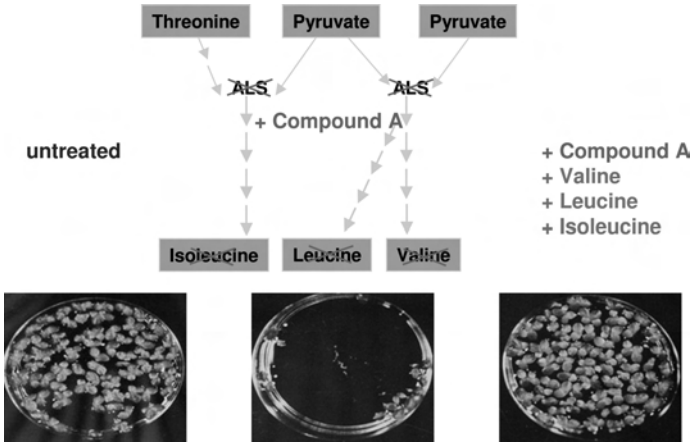


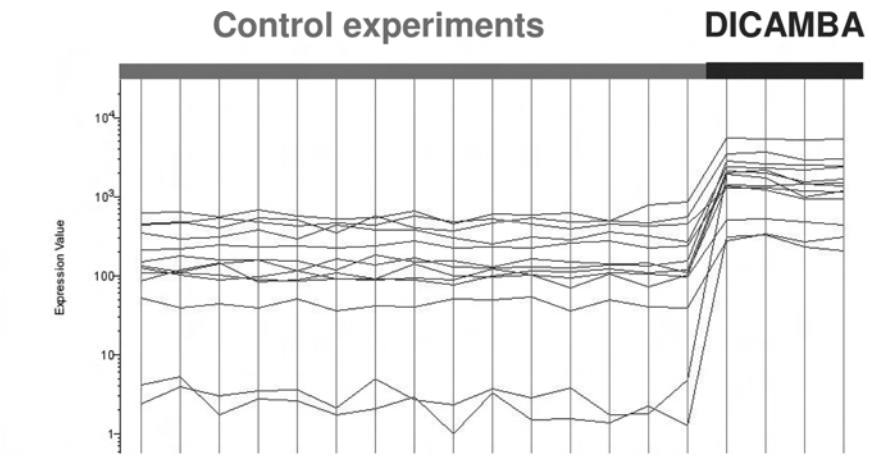
Fig. 33.6. Supplementation assay of *Lemna gibba* plants: Plants grow normally in water without Compound A (left). Addition of Compound A prevents plants from growing (middle). Addition of the three branched-chain amino acids valine (Val), leucine (Leu) and isoleucine (Ile) overcomes the herbicidal effect of Compound A and restores plant growth (right).

established that ALS inhibitors increase the level of α-ketoacids like pyruvate, which leads to an increase of the alternative oxidase protein [19]. Final proof that Compound A affects acetolactate synthase came from supplementation experiments with the small plant *Lemna gibba*. Acetolactate synthase is the first common enzyme in the parallel pathways for the synthesis of the branched-chain amino acids valine (Val), leucine (Leu) and isoleucine (Ile). Production of these amino acids is blocked by ALS inhibitors. The inhibition can be overcome by addition of micromolar concentrations of the branched-chain amino acids [20]. *Lemna gibba* plants treated with Compound A were only able to grow further when the growth medium was supplemented with Val, Leu and Ile. One branched-chain amino acid alone or any other of the 20 L-amino acids could not overcome the growth inhibitory effects of the compound (Fig. 33.6).

33.5 Analyzing the Affected Metabolic Pathways

In case the MoA of a compound could not be identified by the standard GEP Compendium approach, more detailed GEP studies, including several harvest time points and more compound concentrations, are performed. This further in-depth analysis can give some hints – which genes are consistently up-regulated or down-regulated. If these genes belong to one or a few specific metabolic pathways there is a good chance that these pathways are affected by the compound and that

the actual MoA can be assigned to an enzyme in this pathway. In a first attempt to validate this assumption we analyzed the genes that are up-regulated by the synthetic auxin DICAMBA. We expected that auxin responsive genes would be over-represented amongst the up-regulated genes. Indeed, we observed that 8 of the 13 highest up-regulated genes belong to the auxin responsive genes group (Fig. 33.7). Analysis of *Arabidopsis* genes, which are annotated as ''auxin related'', revealed that about $\frac{2}{3}$ of those genes are induced after treatment with DICAMBA. This is another clear indication that DICAMBA effects gene expres-



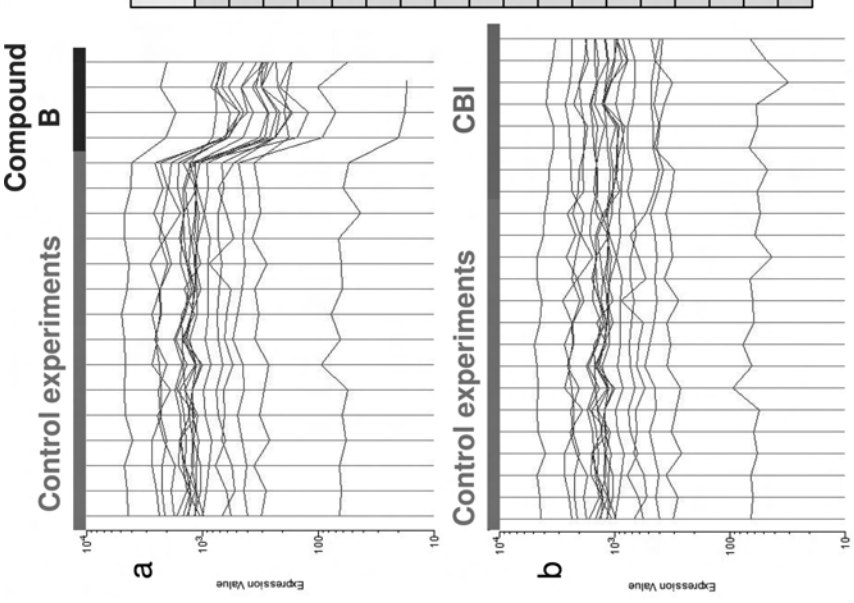
Probe Name	ORF Number	Gene Description
253908_at	At4g27260	auxin-responsive GH3 family protein
249306_at	At5g41400	zinc finger (C3HC4-type RING finger) family protein
262912_at	At1g59740	proton-dependent oligopeptide transport (POT) family protein
262099_s_at	At1g59500	auxin-responsive GH3 family protein
245397_at	At4g14560	auxin-responsive protein (IAA1)
266415_at	At2g38530	nonspecific lipid transfer protein 2 (LTP2)
261114_at	At1g75390	bZIP transcription factor family protein
261368_at	At1g53070	legume lectin family protein
253423_at	At4g32280	auxin-responsive AUX/IAA family protein
257766_at	At3g23030	auxin-responsive protein (IAA2)
248163_at	At5g54510	auxin-responsive GH3 protein, putative (DFL-1)
255177_at	At4g08040	1-aminocyclopropane-1-carboxylate synthase, putative
255788_at	At2g33310	auxin-responsive protein (IAA13)

Fig. 33.7. Mode of Action of DICAMBA: The gene expression of untreated control plants and DICAMBA treated plants are compared. The expression values (scaled logarithmically) represent the level of expression of the genes highly up-regulated by DICAMBA. The table lists the names of the genes. As expected for the action of a synthetic auxin, most of the genes are annotated as auxin responsive. Each vertical line represents one experiment.

sion as expected for an auxin herbicide. It is well known that auxins induce ethylene production in plants by triggering the expression of the genes for 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC 4.4.1.14) [21]. In our DICAMBA experiments this gene is amongst the 13 highest up-regulated genes. Some of the genes coding for the next enzyme in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (EC 1.14.17.4), are induced as well. A recent study of the change in expression of *Arabidopsis* genes after application of 2,4-dichlorophenoxyacetic acid, another synthetic auxin, describes similar observations [22]. Most of the other highly up-regulated genes encode stress related proteins like, for example, lipid transfer proteins, protein phosphatases 2C or transcription factors involved in general stress response. The data show that even without prior knowledge of the MoA of DICAMBA the changes in the transcriptome would clearly have pointed to an auxin effect of that compound.

Another example of how Gene Expression Profiling can identify the pathway that is affected by an herbicidal compound comes from the analysis of Compound B. About 60% of the photosynthesis related genes are down-regulated after treatment with the compound (data not shown). When we looked more specifically into the different metabolic pathways related to photosynthesis we observed that almost all genes responsible for the biosynthesis of chlorophyll are down-regulated (Fig. 33.8). This hints that the target of Compound B might be located in the chlorophyll biosynthesis pathway. A key enzyme in this pathway and well-known herbicidal target is protoporphyrinogen oxidase (PPO). In a PPO inhibition assay we could obtain a similar IC_{50} for Compound B as for Bifenox, a well-known inhibitor of PPO (Fig. 33.9). In contrast to Compound B the expression of chlorophyll biosynthetic genes remains unaffected after treatment of *Arabidopsis* with compounds inhibiting other herbicidal targets like, for example, cellulose biosynthesis (Fig. 33.8), acetyl-CoA carboxylase (ACCase) or hydroxyphenylpyruvate dioxygenase (4-HPPD) (data not shown). With Gene Expression Profiling we were able to identify the affected pathway (chlorophyll biosynthesis) and to exclude other pathways from the analysis. Even though PPO was among the down-regulated genes we were not able to pinpoint the actual target by GEP analysis alone. However, using GEP we were able to reduce the number of possible target sites from the complete enzyme universe to the few enzymes involved in the chlorophyll biosynthetic pathway. These examples demonstrate the capability of GEP to reduce the number of potential targets from the complete proteome to only a few promising candidates.

Fig. 33.8. Mode of Action of Compound B: The gene expression of untreated control plants and Compound B treated plants are compared (a). The expression values (scaled logarithmically) represent the level of expression of the highly down-regulated genes. The table lists the names of the genes. Genes involved in chlorophyll biosynthesis are down-regulated in plants treated with Compound B, but not in plants treated with a cellulose biosynthesis inhibitor (CBI) (b). Each vertical line represents one experiment.



Gene Name	Gene Description
At1g03475	coproporphyrinogen III oxidase
At1g03630	protochlorophyllide reductase C
At1g08520	magnesium-chelatase subunit chlD
At1g44446	chlorophyll a oxygenase (CAO) / chlorophyll b synthase
At1g58290	glutamyl-tRNA reductase 1
At2g26540	uroporphyrinogen-III synthase family protein
At2g30390	ferrochelatase II
At2g40490	uroporphyrinogen decarboxylase, putative
At3g48730	glutamate-1-semialdehyde aminotransferase 2 (GSA-AT 2)
At3g51820	chlorophyll synthetase, putative
At3g56940	magnesium-protoporphyrin IX monomethyl ester cyclase
At4g01690	protoporphyrinogen oxidase (PPOX)
At4g18480	magnesium-chelatase subunit chlI
At4g25080	magnesium-protoporphyrin IX methyltransferase, putative
At4g27440	protochlorophyllide reductase B
At5g08280	hydroxymethylbilane synthase
At5g45930	magnesium-chelatase subunit chlI
At5g54190	protochlorophyllide reductase A

Compound	IC50 (M) on PPO
Bifenox	1.8×10^{-08}
Compound B	2.5×10^{-08}

Fig. 33.9. *In vitro* PPO inhibition assay: The IC_{50} of Compound B is very similar to that of Bifenox, a known PPO inhibitor.

This provides a starting point for more detailed biochemical, cellular or molecular methods to identify the actual target [23].

33.6 Gene Expression Profiling – Part of a Toolbox for Mode of Action Determination

With Gene Expression Profiling it is possible to classify compounds into known MoAs or to identify pathway(s) affected by such compounds. But one has to keep in mind that not only RNA levels but also the amount and stability of expressed proteins (proteome) and the concentration of metabolites (metabolome) within a given cellular context determine gene activity (Fig. 33.1). This makes it difficult, if not impossible, to precisely identify a new target solely by Gene Expression Profiling. Recently, it has been possible to confirm the target of an herbicide by measuring the changes in the concentration of plant metabolites [29]. Further significant advances in the fields of proteomics and metabolomics [24–26] facilitated a thorough analysis of the changing pattern of proteins and metabolites of cells in a varying environment [27; 28], giving rise to the hope that these techniques can complement Gene Expression Profiling for MoA analysis in the near future. Systematic analysis of the symptoms produced by the different herbicidal compounds is another important tool in obtaining hints as to their MoA [30]. Finally, the target has to be eliminated from the cell by molecular methods like, for example, ''gene knock outs'' to unequivocally identify the target of a compound. This tool box of very diverse but complementary methods will help to get our hands on new MoAs that will serve as targets for herbicides with superior properties.

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34
Molecular Modeling in Agricultural Research

Klaus-Jürgen Schleifer

34.1
Introduction

The elixir of successful crop protection companies is innovative products and a multitude of new development candidates. The basis for this profitable portfolio is a continuously filled attractive pipeline of lead structures that nowadays may be efficiently searched for via combinatorial chemistry, parallel synthesis and high-throughput screening technologies. Simultaneous to this experimental progress computational approaches have been routinely introduced in the R&D process to reduce expensive laboratory capacities. Today, many *in silico* tools are applied to screen large compound libraries or to calculate physicochemical properties. To identify and optimize lead structures, molecular modeling software packages are applied to visualize, construct, compare and evaluate molecular structures on a three-dimensional level.

This contribution will give a general overview of current molecular modeling approaches for lead identification and lead optimization based on molecular structure information.

34.2
General Strategies

Two general screening strategies are followed to identify potential lead structures. First, chemicals are directly tested at harmful organisms (e.g., weeds) and relevant phenotype modifications are rated (e.g., bleaching). This *organism-based* approach indicates biological effects without knowledge of the addressed mode of action (MoA). Optimization strategies have to consider that more than one MoA may be involved and that the observed effect reflects a combination of target activity and bioavailability.

A second strategy, the so-called *mechanism-based* approach, allows target activity optimization. A fundamental condition for this procedure is availability of the

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

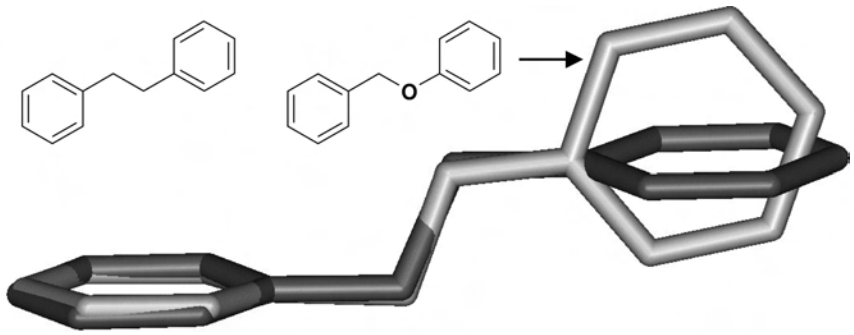

Fig. 34.1. Chemical structures and superimposed X-ray coordinates of 1,2-diphenylethane (dark, CSD-code DIBENZ04) and benzyloxybenzene (bright, CSD-code MUYDOZ) indicating the different orientation of one phenyl ring induced by substitution of methylene with an ether function.

molecular target protein and a suitable biochemical assay to study the protein function in the presence of screening compounds. In this case, transfer of activity from the biochemical assay to the biological system is the challenge.

This makes it clear that – independent of the strategy – screening hits rarely fulfill all necessary criteria for a new lead structure. Medicinal chemists have to analyze the screening results (usually structural formulas with corresponding biological or biochemical data) to derive a first structure–activity relationship (SAR) hypothesis.

Sometimes, 2D-analyses are not sufficient to clarify the *real* situation, which is in nature three-dimensional. Consequently, minor chemical variations may completely change the geometry of a molecule (Fig. 34.1) while even diverse substances (from a 2D-view) may bind to a common binding site (e.g., acetylcholinesterase inhibitors).

Nowadays, molecular modeling packages are applied to calculate relevant conformations of a molecule via an energy function (i.e., force fields [1]) that is adjusted to experimentally derived reference geometries (mostly X-ray structures). *Van der Waals* and Coulomb terms define steric and electrostatic features and each mismatch to reference values is penalized.

34.3 Ligand-based Approaches

To identify essential molecular features of hits in a common hit cluster all structures have to be superimposed to yield a pharmacophore model. Since this is done in 3D space, the relevant conformers of each ligand and critical molecular functions have to be determined. X-Ray structures of the relevant ligand (or of congeners) can be helpful to solve the conformational problem since they indicate

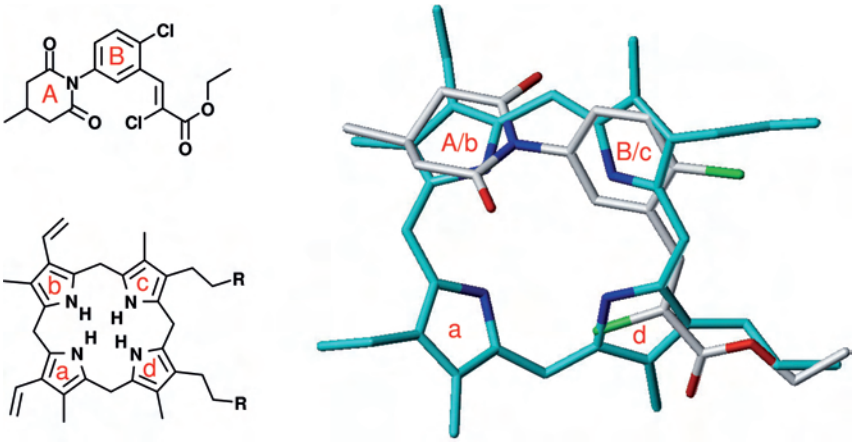

Fig. 34.2. Superposition of a pyridinedione-type Protox inhibitor on a calculated protoporphyrinogen-like template (cyan). For clarity, the corresponding ring systems are indicated and hydrogen atoms are omitted. Atoms are color coded as carbon, grey; nitrogen, blue; oxygen, red; sulfur, yellow; and chlorine, green.

at least one potential minimum conformation. Even more helpful may be the 3D structure of the physiological substrate or a postulated transition state of an enzyme reaction (Fig. 34.2).

Sometimes however, there is no experimental data at all. In this situation a theoretical exploration of relevant conformers has to be performed, taking into consideration all rotational degrees of freedom (e.g., a systematic conformational search). The yielded conformations are evaluated with respect to their potential energy. Corresponding to Boltzmann's equation, low energy values indicate higher chances of resembling reality. Very often, several distinct conformers are assessed to be energetically similar. In this case the most rigid highly active ligand serves as a template molecule to superimpose all other minimized ligands (i.e., the active analogue approach).

Identification of crucial functions – which should (at least in part) be present in all active ligands – takes place via a structure–activity relationship (SAR) analysis of all compounds of the cluster. Hypotheses derived from a SAR (Fig. 34.3) may be evaluated by tests of compounds that lack or optimize this pattern.

Several essential groups (e.g., carbonyl groups, aromatic rings, etc.) are chosen as fit points to superimpose energetically favorable conformers of each ligand. The yielded pharmacophore model characterizes the bioactive conformations by placing similar functional groups of all molecules in the same 3D space (Fig. 34.4). Lack of one or several of these functions is usually associated with a drop in activity.

Pharmacophore models may be used to derive ideas for the substitution of one group (e.g., hydroxyl) against another with similar features (e.g., amine group as

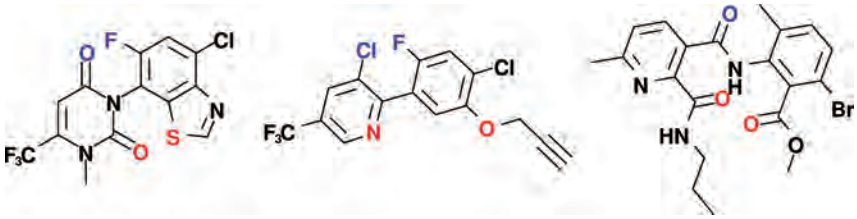

Fig. 34.3. Common interaction pattern of potent Protox inhibitors from uracil- (left) and pyridine-type. Each molecule consists of two ring systems and electron-rich functions on both sides of the linked rings (blue and red).

hydrogen-bond donor and acceptor). This is helpful to facilitate a planned synthesis strategy or a guided compound purchase. Modeling tools like CoMFA [2], CoMSIA [3] or PrGen [4] even allow estimation of effects on a quantitative level. These so-called 3D-QSAR studies (three-dimensional quantitative structure–activity relationships) require the pharmacophore model to determine significantly different interaction patterns that are directly associated with experimental data (e.g., activity). The statistics behind this is mainly based on principal component analyses (PCA) and partial least squares (PLS) regression. PCA transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called *principal components*. PLS regression is probably the least restric-

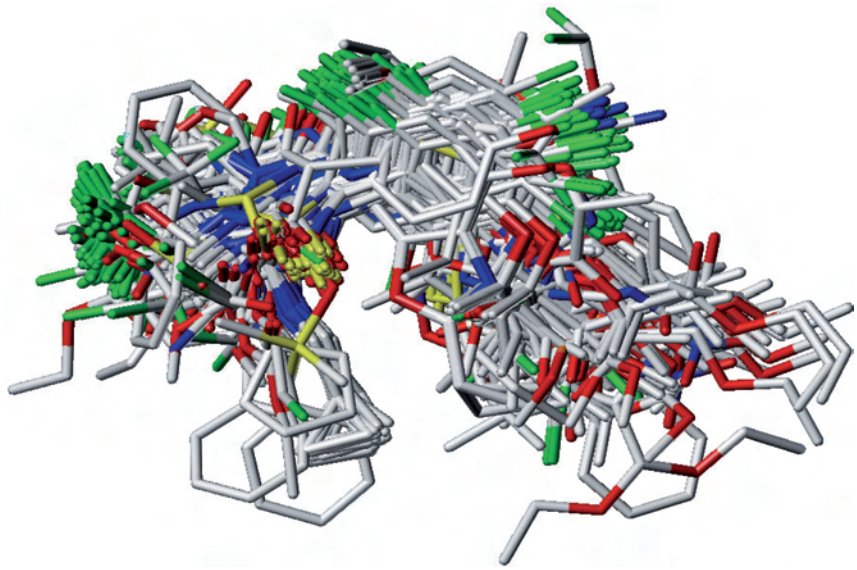

Fig. 34.4. Pharmacophore model of 318 Protox inhibitors (color code as indicated in Fig. 34.2).

tive of the various multivariate extensions of the multiple linear regression model. In its simplest form, a linear model specifies the (linear) relationship between a dependent (response) variable Y, and a set of predictor variables, the X's, so that

$$Y = b_0 + b_1X_1 + b_2X_2 + \cdots + b_pX_p \quad (1)$$

In Eq. (1), b_0 is the regression coefficient for the intercept and the b_i values are the regression coefficients (for variables 1 through p) computed from the data.

Correlation of experimental and calculated activities assesses the quality of 3D-QSAR models. The squared correlation coefficient (r^2) yielded by this statistics is a measure of the goodness of fit. The robustness of the model is tested via cross-validation techniques (leave-x%-out), indicating the goodness of prediction (q^2). Models with $q^2 > 0.4$–0.5 are considered to yield reasonable predictions for hypothetical or not yet tested molecules that are structurally comparable to those compounds used to establish the model (Fig. 34.5).

CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecular Similarity Indices Analysis) not only derive a mathematical equation but also generate a contour map (e.g., steric or electrostatic fields) that should or should not be occupied by new compounds with optimized characteristics (Fig. 34.6).

The pseudoreceptor modeling program PrGen [4] creates a pseudoreceptor model around the pharmacophore representing an image of the hypothetical binding site (Fig. 34.7). Ligand–pseudoreceptor site interactions, solvation and entropic energy terms are calculated to correlate experimental and computed free binding energies. Binding site construction may take into account experi-

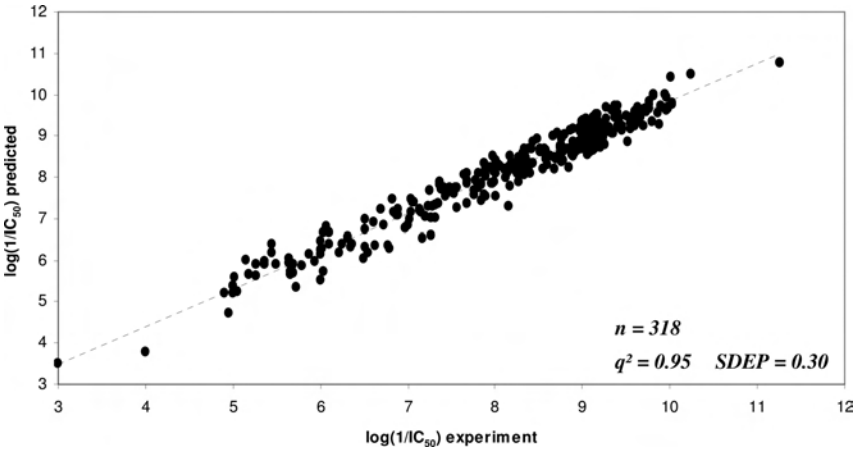

Fig. 34.5. Correlation of experimental and predicted IC_{50} values yielded by a "leave-one-out" cross-validation ($q^2 = 0.95$) for the pharmacophore model shown in Fig. 34.4.

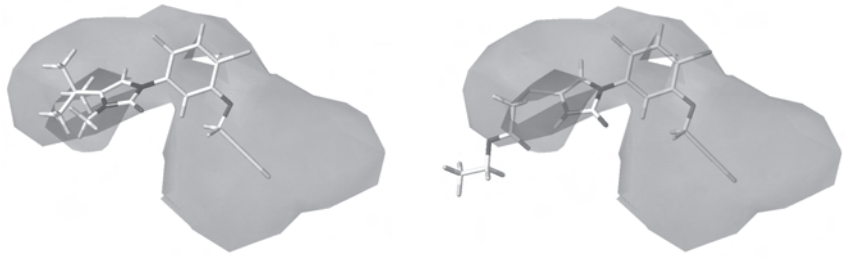

Fig. 34.6. Contour map derived by a 3D-QSAR study. Clouds indicate favorable space to be occupied by potent Protox inhibitors. While the highly active imidazolinone derivative (left) fits almost perfectly, the ethylcarboxylate residue of the weaker ligand protrudes from the preferred region (right).

mentally determined amino acid residues of the real binding site or just residues with complementary features to the ligands.

New hypothetical compounds may be introduced in the evaluated pseudoreceptor model to estimate free binding energies and, thus, to prioritize laboratory capacities.

A common drawback of ligand-based approaches is the fact that data derived from screening hits may only be interpolated to somehow similar compounds. If any structural information is not present in the training set compounds, transfer to totally new structures is generally not possible [5].

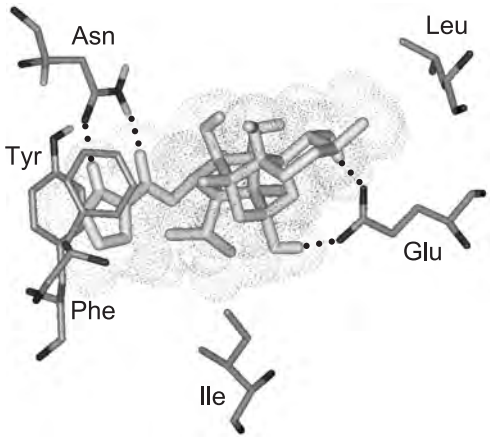

Fig. 34.7. Pseudoreceptor model for insecticidal ryanodine derivates constructed with the program PrGen [4]. The binding site model is composed of six amino acid residues and contains the structure of ryanodine [6]. Hydrogen bond interactions are indicated with dotted lines.

34.4 Structure-based Approaches

New scaffolds for active ingredients are classically obtained by an experimental random screening. Essential for this high-throughput experiment is a multitude of compounds that has to be purchased, synthesized and handled. For reasons of expense, it is desirable not to test all available compounds, but only those with a high chance of success. One helpful strategy to focus a compound library to a particular target is based on the molecular structure of the protein.

At present, highly sophisticated analytical methods like X-ray crystallography, NMR or cryo-electron microscopy are applied to solve 3D structures of enzymes, ion channels, G-protein-coupled receptors and other proteins. The coordinates for over 35 000 proteins are freely available at the Protein Data Base (PDB) [7]. In some cases even ligand–protein co-crystal structures are solved. Coordinates derived from co-crystals unambiguously localize the binding site and provide insight into the pose and binding mode of a bound ligand. This allows computational chemists to characterize specific interaction patterns that are crucial for tight binding.

With this information, the binding site may be used like a lock to find the best fitting key by virtually screening divers compound libraries (i.e., lead identification) or by increasing the specific fit of weak binders (lead optimization). Automation of this so-called (protein) *structure-based* approach [8, 9] is typically divided into a docking and a scoring step [10]. While docking yields the pose(s) of a ligand in the complex, scoring is necessary to discriminate good and bad binders by calculating free energies of binding for each generated conformation of a ligand.

In this context it is common to differentiate between *empirical* and *knowledge-based* scoring functions [11]. The term "empirical scoring function" stresses that these quality functions approximate the free energy of binding, $\Delta G_{\text{binding}}$, as a sum of weighted interactions that are described by simple geometrical functions, f_i, of the ligand and receptor coordinates r (Eq. 2). Most empirical scoring functions are calibrated with a set of experimental binding affinities obtained from protein–ligand complexes, i.e., the weights (coefficients) ΔG_i are determined by regression techniques in a supervised fashion. Such functions usually consider individual contributions from hydrogen bonds, ionic interactions, hydrophobic interactions, and binding entropy. As with many empirical approaches the difficulty with empirical scoring arises from inconsistent calibration data.

$$\Delta G_{\text{binding}} = \Sigma \Delta G_i, f_i(r_{\text{ligand}}, r_{\text{receptor}}) \tag{2}$$

Knowledge-based scoring functions have their foundation in the inverse formulation of the Boltzmann law, computing an energy function that is also referred to as a "potential of mean force" (PMF). The inverse Boltzmann technique can be applied to derive sets of atom-pair potentials (energy functions) favoring preferred contacts and penalizing repulsive interactions. The various approaches differ in the sets of protein–ligand complexes used to obtain these potentials, the form of

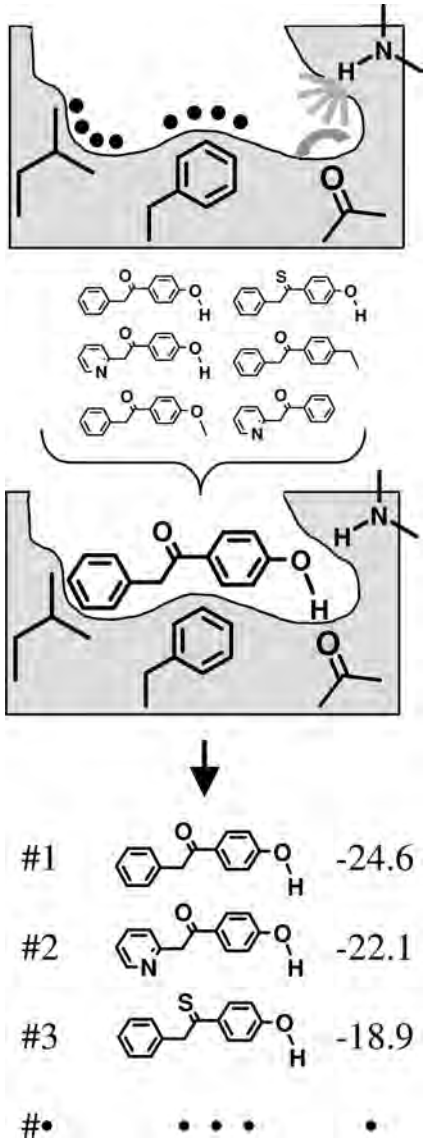

Fig. 34.8. Protocol of a classical docking and scoring procedure. The binding site cavity is characterized by, for example, hydrophobic (filled circles), hydrogen-bond donor (lines) and hydrogen-bond acceptor properties (circle segment). Each compound of a database (or real library) is flexibly docked into the binding site and its free binding energy is estimated by a mathematical scoring function. The output of this structure-based virtual screening is a ranked list indicating $\Delta G_{binding}$ for each pose of each ligand in a defined energy range.

the energy function, the definition of protein and ligand atom types, the definition of reference states, distance cutoffs, and several additional parameters [12].

An extension of docking procedures is *de novo* design [13] with BASF's archetype LUDI [14]. Here, molecular fragments are composed inside a given binding pocket to design a perfectly matched new molecule.

Both attempts rely on an accurate binding site characterization, an appropriate ligand/binding site complex generation and a reliable estimation of the free binding energies. Figure 34.8 illustrates the principle of a docking and scoring procedure.

To demonstrate a docking application, the crystal structure of mitochondrial protoporphyrinogen IX oxidase (Protox) from common tobacco complexed with an acidic phenylpyrazole inhibitor (INH) and a non-covalently bound FAD cofactor was chosen (PDB ID code 1SEZ [15]). A salt bridge primarily fixes the inhibitor from the carboxylate group to a highly conserved arginine (Arg98) at the entrance of the binding niche. Further stabilization is due to hydrophobic contacts to Leu356, Leu 372 and Phe392 in the core region.

In a first step, INH was extracted from the binding site cavity and a commercial docking program (FlexX [16]) was applied to control whether the original binding pose of the X-ray structure could be re-found. For this calculation not the complete protein but only a volume with a radius of 10 Å around the binding site was considered.

The program detects 98 favorable docking solutions within an energy range of 10.0 kJ mol^{-1} ($\Delta\Delta G$). Except for two poses all solutions strictly interact with their acidic function to the basic Arg98. However, only 20 of them are really located in the binding niche. The energetically most favorable proposals fix the guanidinium group of Arg98 from the solvent side (Fig. 34.9). A further 13 solutions are blocking the gorge to the binding site.

To rationalize the docking process and to circumvent non-realistic solutions (i.e., outside the known binding region), two pharmacophore-type constraints may be set. First, an interacting group in the receptor site may be specified (i.e., interaction constraint). During the simulation each docking solution is checked as to whether there is a contact between the ligand and this particular hot spot. If not, the solution is discarded. The second type is a spatial constraint. Here, a spherical volume in the active site and a specified atom or group of atoms from the ligand must lie within the sphere in the docking solution. FlexX-Pharm [17] offers both constraint types – they even may be combined.

Keeping in mind only the 20 accurate docking solutions, it must be stated that the original pose of INH is not perfectly found. Although most acidic groups interact with Arg98, the binding mode is different than for the experimentally solved X-ray structure. Only the more hydrophobic pyrazole ring matches (in some cases) its reference counterpart. Furthermore, two docking solutions are totally different. Their acid function interacts with the terminal amide group and the backbone NH of Asn67, which is opposite to Arg98 (Fig. 34.10).

Notably, the docking procedure used does not take into account flexibility of the binding site residues. Only the ligand is considered flexible in an energetically re-

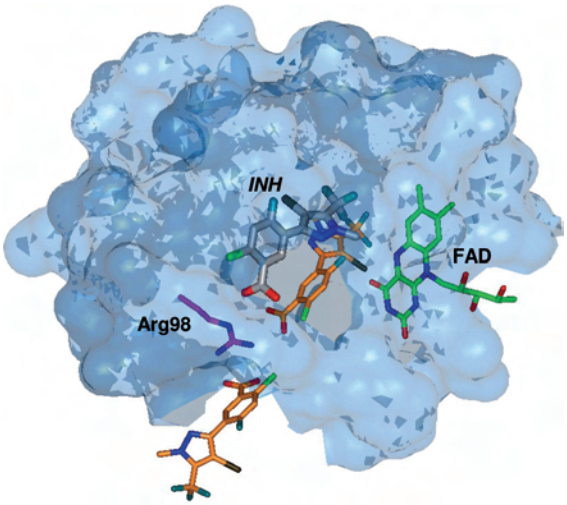

Fig. 34.9. X-Ray crystallographically determined binding site of Protox [15], including the co-crystallized inhibitor INH (for structural formula see Fig. 34.10) and a part of the co-factor FAD. Highlighted is Arg98 at the entrance of the binding site cavity, interacting with INH and almost all solutions of the FlexX approach via electrostatic and hydrogen bond interactions. Two docking poses representing a cluster of yielded solutions are indicated, one at the outside and one inside the binding site cavity (orange-colored carbon atoms).

stricted range. However, there are programs that allow concerted consideration of flexibility for ligand and binding site residues to simulate induced fit in its native way (e.g., GLIDE, FlexE).

In contrast to the charged INH inhibitor used for the co-crystallization experiment, all of BASF's in-house compounds presented in the above-mentioned 3D-QSAR study are uncharged. Therefore, a second docking study with a neutral uracil derivative (UBTZ, Fig. 34.11) should clarify how ligands without acid function bind to this target site.

Applying the default parameters, FlexX produced 122 solutions, predominantly located in the binding cavity. The energetically most favorable two solutions are compared with the original pose of INH (Fig. 34.11). Interestingly, each pose of UBTZ has a direct contact to Arg98. Once, a carbonyl oxygen of the uracil and a fluorine of the benzothiazole ring are involved. Alternatively, the nitrogen atom of the benzothiazole ring is directed to the positively charged Arg98. This docking solution shows a better total overlap with INH. Notably, although chemically diverse, both types of inhibitors (INH and UBTZ) obviously mimic similar physicochemical properties necessary for complex formation.

In a next step we tried to dock the physiological substrate, protoporphyrinogen IX, to the INH and Triton-X100 cleaned enzyme. This attempt failed, although the maximum overlap volume and the clash factors were modified in such a way that the narrow binding pocket was apparently relaxed and, subsequently, even the co-factor FAD was (non-physiologically) removed. Only the product of the

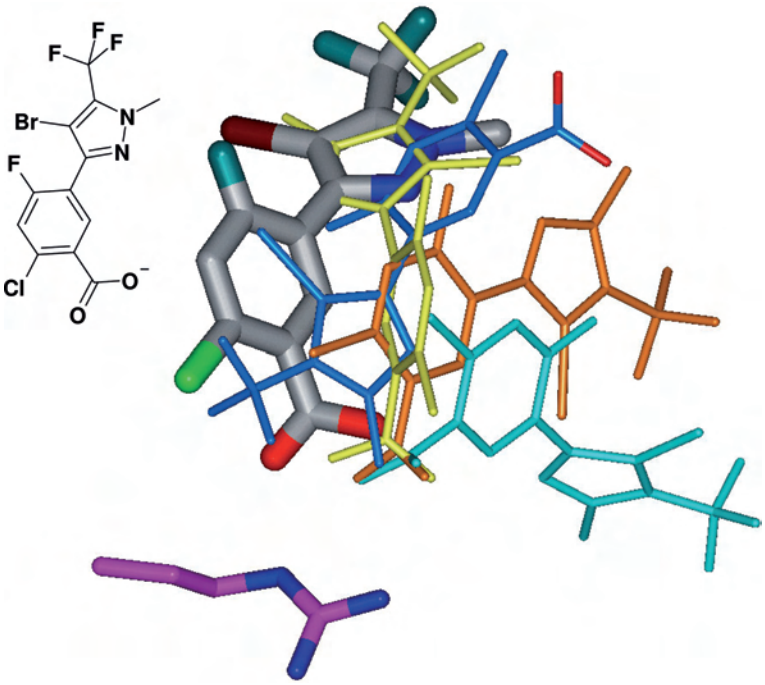

Fig. 34.10. Structural formula of INH and comparison of the poses derived from FlexX docking (single-colored) and crystallization experiment (thick bonds). Indicated is the crucial Arg98 that stabilizes all poses, with the exception of the blue colored solution, which interacts with the acid group (red-colored oxygen atoms) on the opposite side (i.e., Asn67).

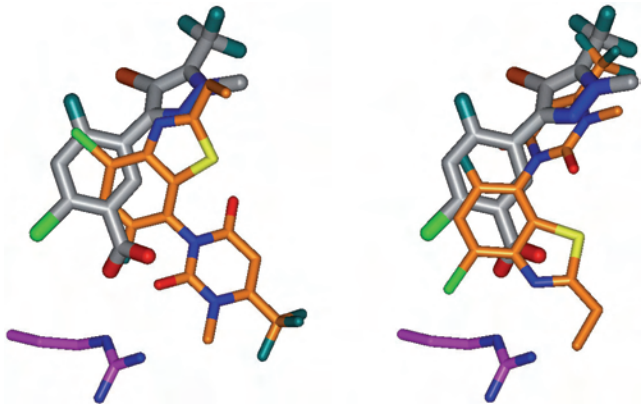

Fig. 34.11. Comparison of two docking solutions for BASF's uracil derivative UBTZ with the bound INH. UBTZ interacts with Arg98 over the carbonyl oxygen of uracil and a fluorine of the benzothiazole ring (left) or the nitrogen atom of the benzothiazole ring (right).

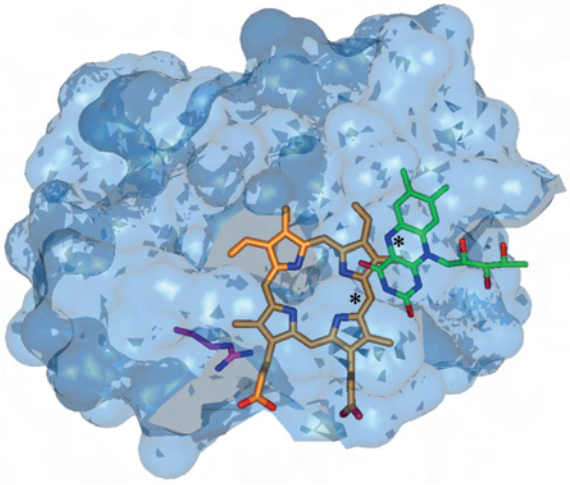

Fig. 34.12. Docking solution for protoporphyrin IX in the Protox binding site. One propionic acid is close to Arg98 but does not form an explicit hydrogen bond. Asterisks indicate the proposed reaction centers C20 of protoporphyrin IX and N5 of FAD (see text for details).

enzyme reaction, protoporphyrin IX, which is sterically not as demanding as the substrate, was inserted in the FAD-free binding site cavity (Fig. 34.12). The yielded solution interacts loosely with one propionate group to Arg98. The second acid group protrudes to the solvent region. Although the co-factor was not present during the calculation, the final pose indicates carbon atom C20 of protoporphyrin IX in close proximity to the electron-accepting nitrogen atom N5 of the flavin ring. This is in general agreement with the results obtained by Koch et al. [15] and Jordan [18] that propose the initial hydride transfer at C20, followed by hydrogen rearrangements in the whole ring system by enamine-imine tautomerizations.

One possible explanation for the failed docking of the tetrapyrrole derivatives under physiological conditions (i.e., in the presence of FAD) might be the reference topology of the binding site. During the co-crystallization experiment, the binding pocket perfectly encloses the small ligand INH. The obtained narrow cleft is not able to incorporate much larger ligands. Therefore, only the flattened protoporphyrin IX could be introduced, but not in the intuitively expected manner (i.e., completely buried in the binding site cavity with a tight contact to Arg98).

The presented example indicates typical challenges of structure-based approaches, starting from the need of a target structure, a multitude of yielded docking poses and problematic estimations of free binding energies. An enormous advantage of this technique is the unbiased use. Results obtained for a particular target site provide information for new chemical structures without prior

expert knowledge or selection. Technologically, there is ongoing improvement to gain more realistic docking solutions (e.g., interaction and spatial constraints or a post-processing step). Additionally, the quality of the energy estimation may be increased by tailor-made scoring functions. This requires much experimental data (e.g., co-crystal data and IC_{50} values) of a particular family of targets (e.g., kinases) for the calibration.

To summarize this topic, structure-based methods are extremely helpful in creating ideas for new scaffolds and further optimization strategies.

34.5 Conclusion

Ligand- and structure-based approaches are valuable tools for the identification and optimization of lead compounds. Each strategy needs special prerequisites and has strengths and weaknesses. In some cases only the strengths of both methods may be combined for a joint approach, called structure-based pharmacophore alignment. Here, the receptor site serves as a complement to build the pharmacophore model and sophisticated statistical methods from 3D-QSAR (PCA and PLS) are applied for the prediction of activity [19, 20].

Generally, computer-aided design of active ingredients is an emerging technology in the R&D process of agricultural companies. It benefits from increasing computer power and smart software solutions. The key, however, is joint project teams with experimentalists and computational chemists accepting the future challenges of the agricultural market.

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35
The Unique Role of Halogen Substituents in the Design of Modern Crop Protection Compounds

Peter Jeschke

35.1
Introduction

The past 30 years have witnessed a period of significant expansion in the use of halogenated compounds in the field of modern agrochemicals research and development [1]. Interestingly, there has been a significant rise in the number of commercial products containing ''mixed'' halogens, e.g., one or more fluorine, chlorine, bromine or iodine atom in addition to one or more further halogen atoms (Fig. 35.1) [2].

Extrapolation of the current trend indicates an expected definite growth in fluorine-substituted commercial products throughout the 21st century.

A survey of the new ISO provisionally approved active ingredients for modern crop protection available in the time frame 2000–2006 (February) shows that around 74% of them are halogen-substituted (Br, I < Cl, Cl/F < F). In this time, approximately four times more halogen-containing insecticides acaricides and fungicides, as well as two times more herbicides, were approved than non-halogenated active ingredients.

According to data from Phillips McDougall in each indication such as insecticides, fungicides and herbicides 11 halogen-containing products are among the 20 best selling compounds. These are the insecticides imidacloprid, chlorpyriphos-E, endosulfan and λ-cyhalothrin, the fungicides tebuconazole, pyraclostrobin and chlorothalonil, and the herbicides acetochlor, *S*-metolachlor, 2,4-D and clodinafop-P, all achieving sales between 165 and 315 Mio € in 2003.

Substituted aryl and hetaryl moieties are of great importance for active ingredients in modern crop protection because two-thirds of all known active ingredients for crop protection contain these molecular fragments. The correct selection and modification of appropriate substituents at the periphery of a molecule and their substitution pattern often play a decisive role in the achievement of excellent biological activity [3].

Within this context halogen and/or halogen-substituted key synthetic intermediates are important tools. Outstanding progress has been made, especially in

Modern Crop Protection Compounds. Edited by W. Krämer and U. Schirmer

ISBN: 978-3-527-31496-6

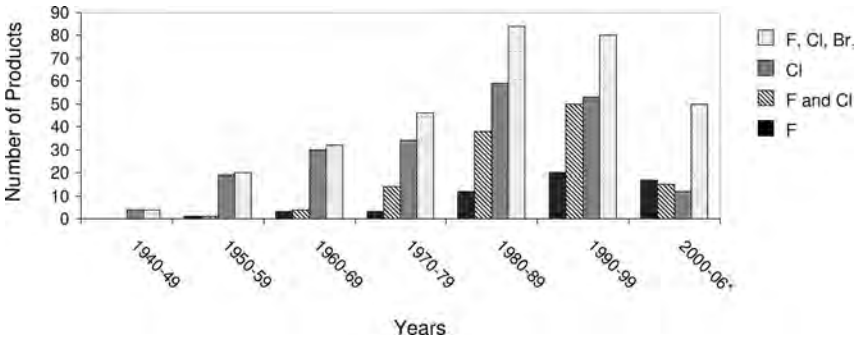


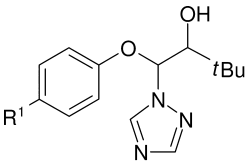
Fig. 35.1. Launch of halogenated commercial products in the time frame 1940–2006; * incl. ISO provisionally approved compounds till February 2006 (www.alanwood.net).

fluoroorganic chemistry [4], such as in the development of novel reagents and methodologies in asymmetric fluorination, trifluoromethylation, and perfluoroalkylation [5]. Electrophilic enantioselective fluorination is a very dynamic field that has experienced rapid growth in the past five years [6]. The combination with other core technologies (e.g., chlorination, catalytic hydrogenation, Cl/N- and Cl/O-exchange, Sandmeyer reactions, Suzuki cross-coupling and others) allows the synthesis of a broad variety of new fluorine-containing building blocks. These efforts also included the introduction of fluorinated aryl moieties, so-called ''fluoro-aromatics'', such as difluoromethoxy- or trifluoromethoxy-aryl fragments and other moieties into crop protection products. The basic raw material for such products, trifluoromethoxybenzene, is produced today on an industrial scale. Therefore, several agrochemicals from different indications produced from trifluoromethoxybenzene and its derivatives are known, such as indoxacarb (**28**; insecticide), triflumuron (**35**; insect growth regulator), thifluzamide (**66**; fungicide), flurprimidol (**79**; plant growth regulator) and flucarbazone-sodium (**104**; herbicide).

35.2 The Halogen Substituent Effect

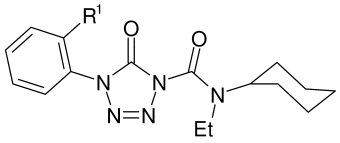
What is the rational behind using halogen atoms and/or halogen-containing substituents in the design of modern crop protection compounds? The influence of halogens on the efficacy of a biological active molecule can be exciting and remarkable. This can be demonstrated with examples coming from different agrochemical areas, e.g., from Bayer CropScience (Fig. 35.2).

- Whereas the unsubstituted triazole **1** shows only low fungicidal activity, incorporation of chlorine in the *para*-position of the phenyl moiety leads to the highly active cereal triazole fungicide triadimenol (**2**; 1980, Baytan®) [7].



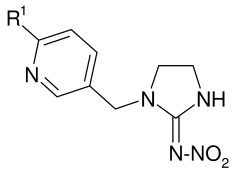
1 $R^1 = H$ << **2** $R^1 = Cl$

fungicidal activity



3 $R^1 = H$ << **4** $R^1 = Cl$

herbicidal activity



5 $R^1 = H$ << **6** $R^1 = Cl$

insecticidal activity

Fig. 35.2. Commercial products (**2**, **4**, **6**) obtained by incorporation of chlorine (R^1) into the aryl(hetaryl) moiety.

- Starting with **3** the herbicidal activity is strongly increased by introduction of chlorine in the *ortho*-position, giving the selective paddy amide herbicide fentrazamide (**4**; 2000, Lecs®) [8] with excellent crop compatibility, even on young seedlings.
- One of the most important structural requirements for active nAChR effectors (neonicotinoids, Chapter 29.1) such as imidachloprid (**6**; 1991, Gaucho®) [9] was the incorporation of chlorine in the 6-position of the pyridin-3-ylmethyl substituent of **5** [10].

The minimal variation of the chemical structure by incorporation of halogens such as chlorine into the aryl(hetaryl) moiety led to a commercial fungicide (**2**), herbicide (**4**) and insecticide (**6**).

The significant and increasingly important role of halogen atoms and/or halogen-containing substituents can be attributed to the well-known physicochemical effects arising from the introduction of fluorine, chlorine, bromine or iodine and/or halogenated substituents into biologically active molecules such in commercial products.

As expected for electronegative elements with accessible ion pairs, halogens can act as hydrogen-bond acceptors, but, in the 1950s, it became clear that halogens could also form complexes with hydrogen-bond acceptors [11, 12]. This behavior has been outlined based on molecular electrostatic potential surfaces [13]. Fluorine behaves like a ball of negative charge, therefore it can only act as a hydrogen-bond acceptor. The other halogens reflect a more positive region on the surface opposite to the C–halogen bond (Hal = Cl, Br, I) direction as well as an equatorial belt of negative potential. As result, they can act as hydrogen-bond donors or acceptors depending on the angle of approach. The magnitude and area of the zone of positive potential increases with the size of the appropriate halogen (F < Cl < Br < I). This means that iodine in particular makes relatively strong interactions with hydrogen-bond acceptors. On the other hand, the so-called ''fluorine-factor'' described in the literature several years ago stems from the unique combination of properties associated with the fluorine atom itself, namely, its high electronegativity and moderately small size, its three tightly-bound ion-pair electrons, and the excellent match between its 2s and 2p orbitals and those of carbon.

In the present chapter, a few examples are selected to illustrate how halogen substitution is used successfully in contemporary agrochemistry.

35.2.1 The Steric Effect

C–halogen bond lengths increase in the order C–F < C–Cl < C–Br < C–I (Table 35.1).

With a van der Waals radius of 1.47 Å [14], covalently bound fluorine occupies a smaller volume than a methyl, amino or hydroxy group (1.52 Å), but a substantially volume than a hydrogen atom (1.20 Å). Nevertheless, the substitution of a hydrogen atom by a fluorine atom is described as one of the most commonly applied bioisosteric replacements [15, 16].

For example, the fluorine atom was introduced [17] into the broad-spectrum fungicide flutriafol (**7**; 1984, Impact®, ICI/Zeneca, now Syngenta) [18] as a chemical isostere of the tertiary hydroxy group (–OH ~ –F, isoelectronic), which is essential for the fungicidal activity of the triazole (Fig. 35.3).

Notably, fluorine may also exert a substantial effect on the conformation of a molecule [19]. On the other hand, an excellent match is found for the carbonyl group (Table 35.1) [20, 21].

Table 35.1 Bond lengths, van der Waals radii and total size of carbon halogen bonds.

Bond	Length (Å)	van der Waals radius (Å)	Total size (Å)
C–F	1.35	1.47	2.82
C–Cl	1.77	1.80	3.57
C–Br	1.93	1.95	3.88
C–I	2.14	2.15	4.29
C–H	1.09	1.20	2.29
C=O	1.23	1.50	2.73
C–O–	1.43	1.52	2.95
O–H	0.96	1.20	2.16

The short C–F bond length is in the range of the C–O bond length, suggesting an isosteric behavior (mimic effect) of the hydroxy group in a bioactive compound with respect to steric requirements at receptor sites or enzyme substrate recognition [22]. Increasingly, these largely recognized aspects of fluorine substitution are used to enhance the binding affinity to the target protein.

On the other hand, the so-called Bondi [23] volumes ($cm^3\ mol^{-1}$) for halogen atoms attached directly to phenyl rings are: 5.8 (F) < 12.0 (Cl) < 15.12 (Br) < 19.64 (I).

This ranking of halogen atoms can be exemplified by the recently described SAR of halogenated phthalic acid diamides (F < Cl < Br < I) in the development product flubendiamide (**54**; ISO–proposed, Hal = I; Nihon Nohyaku Co., Ltd./ Bayer CropScience) [24], which activates selective ryanodine-sensitive intracellular Ca^{2+} release channels in insects as novel mode of action [25]. Introduction of a bulky and moderate lipophilic halogen such as iodine into 3-position of the phthalic acid aryl moiety increased the insecticidal activity considerably (Fig. 35.4) (for more details see Chapter 34).

Further examples of these steric halogen effects are the hydrolyses in soil degradation half-life (DT_{50}) of the herbicidal nitriles bromoxynil (**8**; 1962, Brominal®, May & Baker) [26], ioxynil (**9**; 1962, Actril®, May & Baker) [27], and diclobenil (**10**; 1960, Casoron®, Philips Duphar) [28] (Fig. 35.5a).

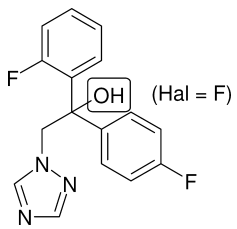


Fig. 35.3. Flutriafol (**7**) – replacement of the tertiary hydroxy group by fluorine.

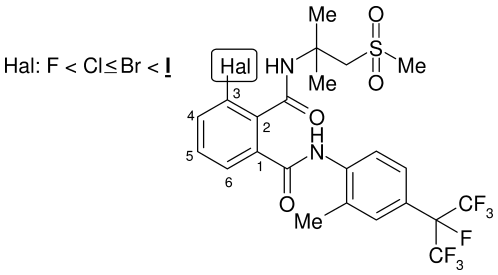


Fig. 35.4. SAR of halogen-containing phthalic acid diamides, flubendiamide (**54**; Hal = I).

Unhindered aryl nitriles are rapidly hydrolyzed via the amide to the carboxylic acid, but the hindered aryl nitriles are only transformed slowly into amides that are even more stable. Whereas **8** (Hal = Br) and **9** (Hal = I) form in soil, by hydrolysis and dehalogenation, less toxic substances such as 4-hydroxybenzoic acid [29], **10** undergoes degradation to the stable 2,6-dichlorobenzamide, which is slowly further hydrolyzed into the 2,6-dichlorobenzoic acid [30].

Similarly, the beneficial steric halogen effects of both the fluorine in the 2-position and that in the 6-position on the inhibition of insecticidal chitin syn-

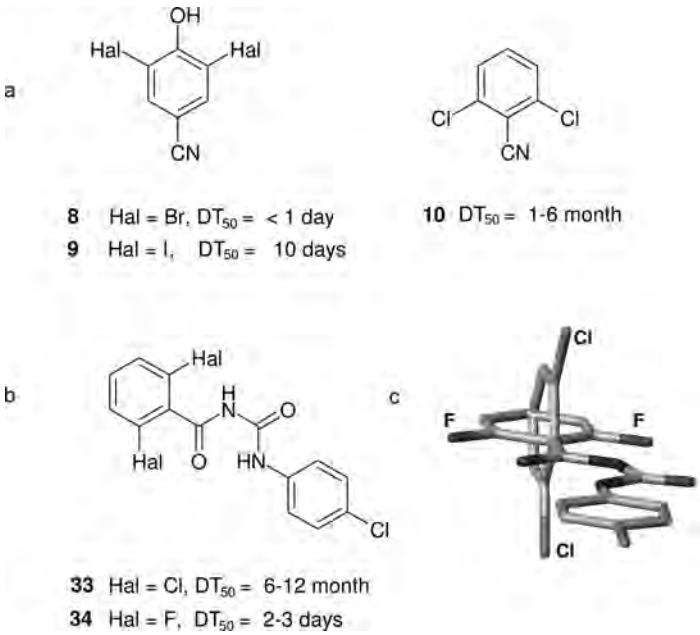


Fig. 35.5. Soil degradation (DT_{50}) of (a) benzonitriles (**8**–**10**) and (b) BPUs (**33**, **34**) – the effect of halogen substituents. (c) Relative orientations of the 2,6-dichloro- and 2,6-difluoro-benzoyl moieties in **33** and **34**, respectively.

thase [31], and the difference in soil degradation half-life caused by the presence of these atoms, have been discussed for diflubenzuron (**34**) [32] and its N-2,6-dichlorobenzoyl derivative **33** (Fig. 35.5b) [33, 34]. N-2,6-Difluorobenzoyl-N′-phenyl ureas like **34** are stable at acidic pH values but are hydrolyzed at pH 9–10 to give 2,6-difluorobenzoic acid and a N-4-chloro-phenylurea. In contrast to the conformation of the less active analogue **33**, which degrades in between six and twelve months, the 2,6-difluorobenzoyl moiety in **34** is in-plane with the whole urea structure (Fig. 35.5c). As a consequence, different metabolic pathways are observed for these two ureas.

The perfluoroalkyl group CF_3 has a relatively large van der Waals volume – larger than methyl, mono-fluoromethyl and di-fluoromethyl and between those of the *iso*-propyl and the *tert*-butyl groups [35]. The latter is comparable in size to the perfluoro-*iso*-propyl group.

35.2.2 The Electronic Effect

35.2.2.1 Effect of Halogens on Dipole Moment

Halogens connected to a carbon atom, such as chlorine and fluorine, withdraw electrons from other parts of the molecule and can create a large dipole moment (μ) of the C–halogen bond [36] (μ = C–Cl, 1.56; C–F, 1.51; C–Br, 1.48; C–I, 1.29 D), and overall reactivity and chemical inertness. The theoretical basis for using the dipole moment as a free energy related parameter in studying drug–receptor interaction and quantitative structure–activity relationship (QSAR) has been described for aromatic substituents of mono-substituted benzene derivatives [37].

The replacement of hydrogen by the most electronegative element fluorine (4.0 on the Pauling scale; cf. $> O = 3.5 > CF_3 = 3.3 > N \sim Cl = 3.0 > C_6F_5 \sim Br = 2.8 > C \sim S \sim I = 2.5 > P \sim H = 2.1$) affords bonds that possess a high ionic character and are strongly polarized $^{\delta+}C–F^{\delta-}$ [38]; this alters sterically and electronically the properties of the molecules, affecting the physicochemistry such as basicity or acidity of neighboring groups and strengthens all nearby bonds [39].

35.2.2.2 Effect of Halogens on pK_a

Depending on the position of the fluorine substituent relative to the acidic or basic group in the molecule, a pK_a shift of several log units can be observed, which can again improve absorption properties [40]. Quite often, a change in pK_a has a strong influence on both the pharmacokinetic properties of the molecule and its binding affinity. For instance, the pK_a of acids and alcohols are considerably reduced by several units when they bear a trifluoromethyl group. As a consequence, the hydrogen-bonding ability of fluoroalcohols is enhanced compared with that of non-fluorinated ones. This may result in enhanced intrinsic activity and, finally, may induce the reinforcement of the binding between active ingredient and biological target. Halogen bonds in active ingredients of modern agrochemicals clearly demonstrate the potential significance of this interaction in ligand binding and recognition [41].

Furthermore, the fluorine substituent with three tightly bound nonbonding electron pairs is associated with a set of electronic effects that encompass both "push" effects, like $+M$ or $+I\pi$ effects in aromatic systems and stabilization of α-carbocations ($=C^+-F \leftrightarrow =C=F^+$; relative stability: $^+CHF_2 > {}^+CH_2F > {}^+CF_3 > {}^+CH_3$), and "pull" effects, such as destabilization of β-carbocations and possibly negative (or anionic) hyperconjugation. The influence of fluorine regarding stabilization of tetrahedral transition states (e.g., CF_3 group) and prevention of decomposition through proteolysis, by forming a vicinal positive charge, has also been described [42]. The trifluoromethyl group has an electronegativity similar to that of oxygen [43] and a large hydrophobic parameter [44].

Unlike the hydroxy group, organic fluorine is a very poor hydrogen bond acceptor [45] and is not a hydrogen bond donor at all. The replacement of a hydroxy group by a fluorine atom totally perturbs the interaction pattern. However, fluorine can participate in hydrogen bonding interactions with H–C even if these bonds to C–F are definitely much weaker than those observed to oxygen or nitrogen [46]. Such C–F H–C interactions have been proposed as a design principle for crystal engineering [47]. Nevertheless, controversy remains over the existence of hydrogen bonds between the C–F group and –OH or –NH donors [48].

35.2.3
Improving Metabolic, Oxidative, and Thermal Stability with Halogens

Extensive surveys of structures in the Cambridge Structural Database [49] coupled with *ab initio* calculations have characterized the geometry of halogen bonds in small molecules and shown that the interaction is primarily electrostatic, with contributions from polarization, dispersion, and charge transfer. The stabilizing potential of halogen bonds is estimated to range from about half to slightly greater than that of an average hydrogen bond in directing the self-assembly of organic crystals [50, 51]. In comparison to C–H (416 kJ mol^{-1}), C–C (348 kJ mol^{-1}), C–N (305 kJ mol^{-1}), and other C–halogen bonds (C–Cl, 338; C–Br, 276; C–I, 238 kJ mol^{-1}), the high C–F bond energy of 485 kJ mol^{-1} [52] influences significantly metabolic degradation, oxidative and thermal stability. In mono-halogenoalkanes, the C–F bond is around 100 kJ mol^{-1} stronger than the C–Cl bond, and the difference in heterolytic bond dissociation energies is even greater ($\sim$130 kJ mol^{-1}). The high C–F bond strength, in connection with the poor nucleofugality of F^-, make alkyl mono-fluorides poor substrates in typical S_N1 solvolysis or S_N2 displacement reactions (alkyl chlorides are 10^2–10^6 times more reactive) [53].

Fluorination has little effect on C–F bonds, but significantly strengthens C–H bonds; for example, the C–H bond in $(CF_3)_2C\underline{H}$ is estimated to be at least 60 kJ mol^{-1} stronger than the tertiary C–H bond in $(CH_3)_2C\underline{H}$, which makes it stronger than C–H in methane.

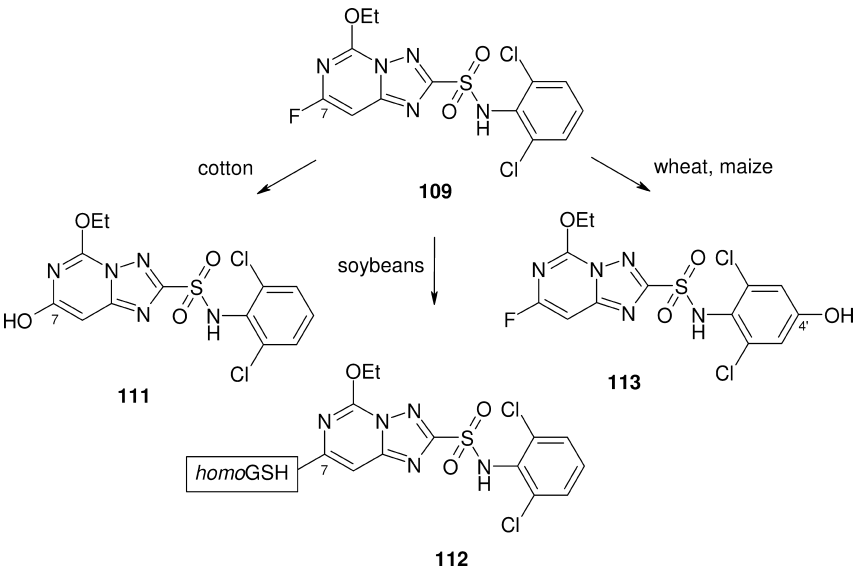
Oxidative metabolism of phenyl rings is a common problem, and fluorine substitution, usually at the 4-position, has become a wide-spread practice to increase stability in various substance classes. A plot of Hammett σ coefficients against

stability for various aromatic ring substituents shows that halogen atoms and halogen-containing substituents more strongly influence relative stability towards oxidation, hydrolysis, and/or soil degradation than the other residues. Electron-withdrawing, halogen-containing groups (e.g., CCl_3, CF_3, OCF_3, $OCHF_2$, $COCF_3$ or SO_2CF_3) can stabilize an aromatic ring system to oxidative (or electrophilic) attacks, but too many withdrawing groups may bring susceptibility to nucleophilic attack. On the other hand, halogens and halogen-containing groups such as CCl_3, CF_3 or OCF_3 are themselves very stable to attack. Therefore, an increased degradation stability is observed for biologically active molecules or fragments containing substituents from this special substitution pattern.

Metabolic stability is one of the key factors in determining the bioavailability of active ingredients. Rapid oxidative metabolism, e.g., by the P_{450} cytochrome enzymes can often lead to limited bioavailability. Therefore, a frequently employed strategy to overcome this problem is to block the reactive site by the introduction of halogen atoms. The replacement of hydrogen atoms on an oxidizable site by fluorine atoms protects from hydroxylation processes mediated by P_{450} cytochrome enzymes. The metabolic stability of the C–F bond can be exploited to make a proinsecticide, e.g., 29-fluorostigmasterol [54].

The different metabolic pathway of the triazolopyrimidine herbicide diclosulam (**109**; 1997, Spider®, Dow AgroScience) [55] are guided by the substituent at the 7-position on the triazolopyrimidine ring system. The predominance of one pathway is very crop specific (Scheme 35.1).

In cotton, **109** is metabolized by the displacement of the 7-flouro substituent on the triazolopyrimidine ring by a hydroxy group, forming **111**. Its soybean selectiv-



Scheme 35.1. Metabolism of diclosulam (**109**) in various crops.

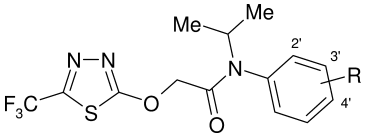


Fig. 35.6. Structure–activity-relationship (SAR) of oxyacetamides; flufenacet (**11**, R = 4′-F).

ity is attributed to facile conjugation with *homo*-glutathion (*homo*GSH), which displaces the 7-fluoro substituent (**112**). In maize and wheat, **109** is detoxified by hydroxylation at the 4′-position on the 2,6-dichloroaniline moiety (**113**) followed by subsequent glycosidation.

The special role of the substitution pattern has been demonstrated in the case of the oxyacetamide flufenacet (**11**; 1998, Axiom®, Bayer CropScience) [56], a selective inhibitor of cell growth and cell division (Fig. 35.6) (for more details see Chapter 8).

Whereas the unsubstituted phenyl moiety gives a good herbicidal activity against *Echinochloa crus galli*, the selectivity achieved is insufficient for soybeans and maize. By incorporating halogens like chlorine or fluorine, the selectivity of the oxyacetamides was significantly increased, but with chlorine this improvement correlated with reduced herbicidal activity. Only the 4-fluorophenyl-containing compound **11** showed good herbicidal efficacy and selectivity against grasses.

Furthermore, the stronger C–F bonds, compared with other C–halogen bonds such as the C–Cl, are the actual thermodynamic driving force for ''Halex reactions'' towards the ''fluoroaromatics'' [57]. The Halex reaction is a nucleophilic aromatic substitution (S_NAr) in which chlorine atoms activated by an electron-withdrawing group are displaced by fluorine upon reaction with a metal fluoride under polar aprotic conditions [58].

35.2.4
Effect of Halogens on Physicochemical Properties

35.2.4.1 Effect of Halogens on Molecular Lipophilicity

Lipophilicity is a key parameter that governs the absorption and transport *in vivo* and, hence, the bioavailability of active ingredients. The presence of halogen substituents in biologically active molecules enhances their lipophilicities, therefore these substituents can influence the pharmacokinetic behavior such as the uptake *in vivo*, e.g., by enhancing the passive diffusion of active ingredients across membranes and their transport *in vivo*. The incorporation of halogens, especially chlorine or fluorine, can be important for so-called fine-tuning of the positioning of bioactive substances between aqueous and fatty media. In this connection the poor polarizability of fluorine-substituted groups plays a crucial role in phase behavior. For example, numerous insecticides acting on the central nervous system (CNS) contain a fluorophenyl moiety or one of the most lipophilic functional

Table 35.2 Lipophilicity increments π as assessed for mono-substituted benzenes H_5C_6–X.

Substituent	π	Substituent	π
X = H	0.00	X = CH_3	0.56
X = F	0.14	X = CF_3	0.88
X = Cl	0.71	X = OCF_3	1.04
X = Br	0.86	X = SCF_3	1.44
X = I	1.12	X = SCF_5	1.23

groups such as trifluoromethyl and F_3C–X (X = O; S), which contributes to the overall pharmacological activity by enhancing CNS penetration [59]. What makes the trifluoromethoxy-aryl moiety attractive is the ability to improve the membrane permeability of the compound in which it is embedded.

While halogens such as chlorine, bromine and iodine as well as trifluoromethyl and trifluoromethoxy substituents invariably boost the lipophilicity, single fluorine atoms may alter this parameter in either direction (Table 35.2) [60].

If the halogen occupies a vicinal or homo-vicinal position with respect to hydroxy, alkoxy, or carbonyl oxygen atom it enhances the solvation energy in water more than in organic solvents and hence lowers the lipophilicity. Conversely, a fluorine atom placed near a basic nitrogen center will diminish the donor capacity of the latter and, as a consequence, cause a strong log *D* (log *P*) increase.

The increased lipophilicity (π), and a superior metabolic stability compared with the methyl analogue, often leads to an improved activity profile. Monofluorination and trifluorination of saturated aliphatic groups normally decrease lipophilicity, whereas higher fluoroalkyl groups (perfluoroalkyl groups) are introduced mainly to increase the lipophilicity [61], as recently shown for the 4′-perfluoro-*iso*-propyl-2-methyl-phenyl-amide fragment (4′-position: halogen < fluoroalkoxy < CF_3 < C_{2-4}-fluoroalkyl) in flubendiamide **54**; ISO–proposed; Nihon Nohyaku Co., Ltd./Bayer CropScience) (for more details see Chapter 31).

35.2.4.2 **Classification in the Disjoint Principle Space**

Systematic variation of substituents in a molecule has been the subject of various studies. Besides synthetic feasibility and economic considerations, properties such as polarity, size, and H-bonding capacity form the basis for choosing substituents like halogen atoms and/or halogen-containing substituents. The disjoint principle properties (DPP), derived from a large set of property descriptors for substituents including halogen atoms and/or halogen-containing substituents, can be used to make rational and effective choices [e.g., from the following similarities: (a) F ≈ SH, C≡CH; (b) Br, Cl, I ≈ CF_3, NCS; (c) SO_2CF_3 ≈ SO_2Me, SO_2NH_2; (d) OCF_3 ≈ COOMe, NHCOMe; (e) SCF_3 ≈ OPh, COPh]. Several excellent examples are described in Section 35.6.2, concerning sulfonylurea and triazolone herbicides; these examples include the successful exchange of the following:

- the 3-ethylsulfonyl group in the 2-pyridyl ring of rimsulfuron **95** ($R = SO_2Et$) with the 3-trifluoromethyl group to give flazasulfuron **97** ($R = CF_3$) (see Figs. 35.22 and 35.23 below);
- the 2-methoxycarbonyl group of propoxycarbazone-sodium **103** ($R^1 = COOMe$) with the 2-trifluoromethoxy group of flucarbazone-sodium **104** ($R^1 = OCF_3$) (see Fig. 35.25 below).

Therefore, the strong influence of halogen atoms and/or halogen-containing substituents can lead to biological superiority of halogenated active ingredients over their non-halogenated analogues. Various commercial products testify to the successful utilization of halogens in the design of active ingredients for modern agrochemicals, in particular insecticides/acaricides, fungicides, plant growth regulators, and herbicides. The biochemical targets are generally well-known, for example:

1. Voltage gated sodium channel; (vgSCh) γ-aminobutyric acid (GABA) receptor/chloride ionophore complex; chitin biosynthesis pathways; mitochondrial respiratory chain and ryanodine receptor for insecticides.
2. Sterol biosynthesis; mitochondrial respiratory chain; germination and hyphal growth; protein kinase for fungicides.
3. Gibberellin biosynthesis pathway for plant growth regulators.
4. Carotenoid biosynthesis, acetolactate synthase (ALS) and protoporphyrinogen IX oxidase (PPO) for herbicides.

35.3
Insecticides and Acaricides Containing Halogens

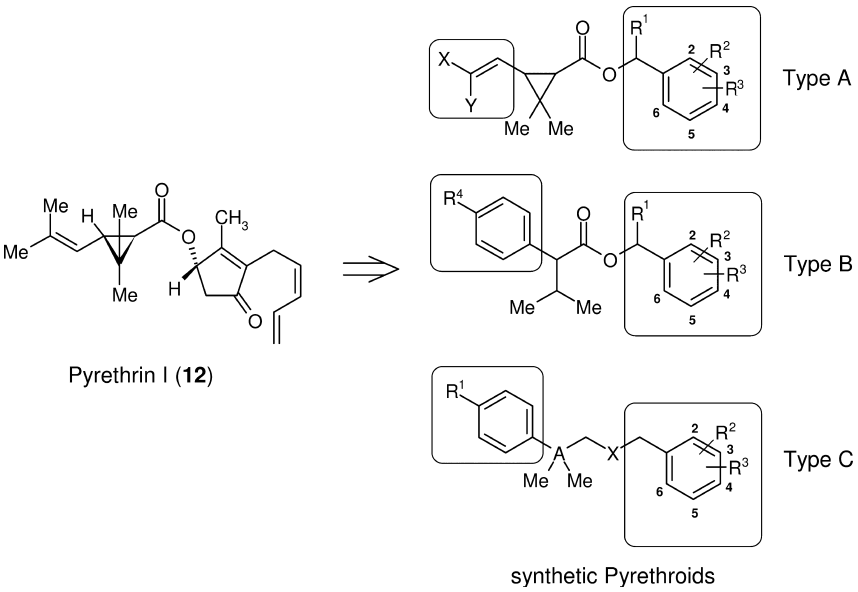
35.3.1
Voltage-gated Sodium Channel (vgSCh) Modulators

Synthetic pyrethroids block the nerve signal by prolonging the opening of the vgSCh. Beside natural pyrethrins, 37 ester (Type A and B) and two non-ester type pyrethroids (Type C) are currently registered worldwide (Scheme 35.2) [62].

From these different pyrethroid structures around 41% contain no halogen substituents, whereas around 59% are halogen substituted. Generally, 43% of pyrethroids containing fluorine/chlorine are the most important; 26% of pyrethroids contain only chlorine, 13% fluorine, around 9% bromine and around 2% are fluorine/bromine substituted.

35.3.1.1 **Type A Pyrethroids**

The development of synthetic pyrethroids provides a significant historical illustration of the introduction of halogens into active ingredients. More than 20 years after simplification of the pentadienyl side chain of pyrethrin (**12**) [63], replace-



Scheme 35.2. Structural evolution of synthetic pyrethroids (types A–C) from pyrethrin I (**12**).

ment of the cyclopentene alcohol group, insertion of an α-cyano substituent R^1 at the phenoxybenzyl alcohol and introduction of the di(halo)vinyl moiety resulted, for example, in permethrin (**13**) [64], cypermethrin (**14**) [65] and deltamethrin (**15**) [66] (Table 35.3).

In 1980 the first fluorine-containing pyrethroid, cyfluthrin (**16**) [67] was launched. This pyrethroid (**16**), containing the 4-fluoro-3-phenoxybenzyl substituent, was the remarkable result of a program directed at the synthesis of all possible isomers with fluorinated alcohol modifications [68]. Compared with cypermethrin (**14**), cyfluthrin (**16**) realized a more than threefold reduction in use rate for the control of cotton pests [69]. The more active form of **16** for interaction at receptor sites involves a conformation in which the 3-phenoxy substituent (R^2) is twisted because of the fluorine effect in the 4-position (Fig. 35.7).

In contrast, a different orientation of the 4-chloro-3-phenoxy-benzyl moiety and a lower insecticidal activity were observed. The launch of F_3C-containing pyrethroids started in the 1980s with λ-cyhalothrin (**17**) [70] from ICI/Zeneca (now Syngenta). That **17** represents the optimum choice of fluorine-containing substituents for activity has been shown by comparison with other fluorinated derivatives: $[F_3C(Cl)C=] > [F_3C(F)C=] = [Cl(Cl)C=] = [F(F)C=] \gg [F_3C(F_3C)C=]$.

Finally, the presence of the trifluoromethyl group in **17** has effects on phytophagous mites.

A comparison of the physical and chemical environment-related properties of structurally similar pyrethroids demonstrates the influence of both the fluorine atom at the phenyl moiety in the 4-position and the substitution on the vinyl

Table 35.3 Halogen-substituted pyrethroids of type A (**13**–**20**) (see Scheme 35.2 for basic structure).

Compound no.	Common name, trade name	Manufacturer (year introduced)	X	Y	R^1	R^2	R^3
13	Permethrin, Chinetrin®	ICI/Zeneca[a] (1977)	Cl	Cl	H	H	3-OPh
14	Cypermethrin, Viper®	ICI/Zeneca[a] (1978)	Cl	Cl	CN	H	3-OPh
15	Deltamethrin, Decis®	Roussel Uclaf[b] (1977)	Br	Br	CN	H	3-OPh
16	Cyfluthrin, Baythroid®	Bayer[b] (1980)	Cl	Cl	CN	F	3-OPh
17	λ-Cyhalothrin, Banish®	ICI/Zeneca[a] (1984)	CF_3	Cl	CN	H	3-OPh
18	Bifenthrin, Brigade®	FMC Corp. (1986)	CF_3	Cl	H	2-Me	3-Ph
19	Acrinathrin, Ardent®	Roussel Uclaf[b] (1991)	H	$COOCH(CF_3)_2$	CN	H	3-OPh
20	Tefluthrin, Attack®	ICI/Zeneca[a] (1988)	CF_3	Cl	H	2,3,5,6-F_4	4-Me

[a] Now Syngenta.
[b] Now Bayer CropScience.

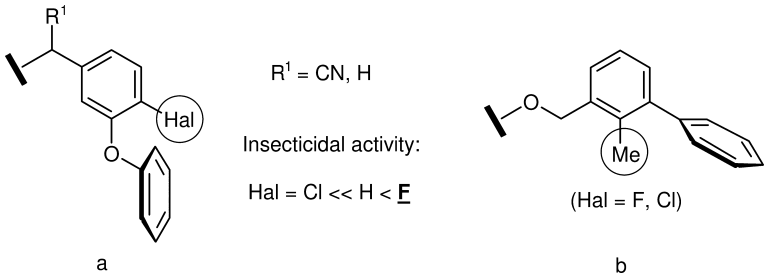


Fig. 35.7. Pyrethroid alcohol modification – conformation of preferred substituent pattern, e.g., in **16** (a) and **18** (b).

side chain such as hydrogen, halogen or the trifluoromethyl group [71]. Bifenthrin (**18**) [72] the single bridged biphenyl-type pyrethroid, is available in a partially resolved (*Z*)-(*1RS*)-*cis*-isomer mixture [73] and was marketed as one of the most important termiticides. In **18** the 3-phenyl substituent is twisted because of the effect of the methyl group in the 2-position (F, Cl ≪ Me; Fig. 35.5b). Replacement of the di(halo)vinyl moiety with a hexafluorine-containing isopropyl vinyl ester group led to acrinathrin (**19**) [74], which controls the larval and adult stages of a broad range of phytophagous mites as well as various sucking insects such as aphids, thrips and psyllids.

With extended exploitation of the acidic part of **17**, the tetrafluorobenzyl alcohol was selected specifically to obtain a soil-applicable insecticide such as the (*Z*)-(*1RS*)-*cis*-isomer of tefluthrin (**20**) [75], which is optimized in terms of stability, volatility, fast penetration, and water solubility.

35.3.1.2 Type B Pyrethroids

The pyrethroid racemate fenvalerate (**21**) [76], a non-systemic insecticide and acaricide with contact and stomach action, shows efficacy against chewing, sucking and boring insects such as lepidoptera (cotton: 30–150 g-a.i. ha^{-1}), coleoptera (potatoes: 100–200 g-a.i. ha^{-1}) and others (Table 35.4).

Today, so-called "chiral switches" [77], which exploit single enantiomers of existing racemic mixtures, are an important feature of active ingredient development portfolios. Within this context (2*S*,α*S*)-fenvalerate (**22**; esvenvalerate) [78] was introduced by Sumitomo Chem. Co., Ltd. into the market, which has enhanced insecticidal activity (cotton: 20–30 g-a.i. ha^{-1}) of **21** [79], respectively. Flucythrinate (**23**) [80], the difluoromethoxy derivative of **21**, is a highly active (cotton: 30–75 g-a.i. ha^{-1}), broad spectrum insecticide with excellent residual effi-

Table 35.4 Halogen-substituted pyrethroids of type B (**21**–**24**) (see Scheme 35.2 for basic structure).

Compound no.	Common name, trade name	Manufacturer (year introduced)	R^1	R^2	R^3	R^3
21	Fenvalerate, Belmark®	Sumitomo (1976)	CN	H	3-OPh	Cl
22	Esvenvalerate, Samurai®	Sumitomo (1986)	CN	H	3-OPh	Cl
23	Flucythrinate, Pay off®	ACC/BASF (1981)	CN	H	3-OPh	$OCHF_2$
24	Flubrocythrinate, Lubrocythrinate®	Shanghai Zhongxi (1992)	CN	H	3-O(4-Br-Ph)	$OCHF_2$

Table 35.5 Halogen-substituted pyrethroids of type C (**25**–**27**) (see Scheme 35.2 for basic structure).

Compound no.	Common name, trade name	Manufacturer (year introduced)	X	A	R^1	R^2	R^3
25	Etofenprox Fogger® Lenatop	Mitsui (1986)	O	C	OEt	H	H
26	Halfenprox, Prene EL®	Mitsui (1993)	O	C	OCF_2Br	H	3-OPh
27	Eflusilanate, Silonen®	Hoechst[a] (1991)	CH_2	Si	OEt	4-F	3-OPh

[a] Now Bayer CropScience.

cacy. Introduction of bromine into the para position of the phenoxy fragment of **23** leads to the lower toxic flubrocythrino (**24**) [81] [acute oral LD_{50} for rats: >1000 mg kg^{-1} (**24**); 67–81 mg kg^{-1} (**23**)]. The *para*-bromine derivative **24** shows additional activity against spider mites such as *Panonychus ulmi*, *Tetranychus telarius* and *T. veinnensis*. It is also active against eggs, larvae and has residual activity lasting more than three weeks.

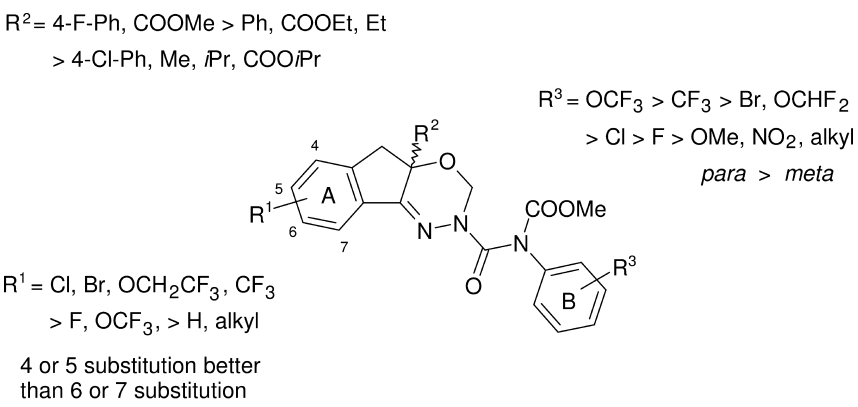
35.3.1.3 Type C Pyrethroids

The achiral etofenprox (**25**; X = O, A = carbon; R^1 = OEt) containing no halogen atom shows a non-ester exerting pyrethroid-like efficacy and is highly advantageous regarding the rice insecticide market (Table 35.5).

In this case, bromine and fluorine incorporation in R^1 leads to a shift in the spectrum of activity; the resulting halfenprox (**26**; $R^1 = OCBrF_2$) displays good acaricidal activity and shows a similar short environmental persistence in soil (DT_{50} = 10 days vs. ~6 days for **25**).

Finally, organosilicon pyrethroids such as eflusilanate [**27**; X = CH_2; A = silicon; R^1 = OEt)] are obtained by replacing the quaternary carbon atom (A) with the appropriate isosteric silicon atom and by replacing oxygen (X) with methylene. The latter has an extremely low fish and mammalian toxicity combined with insecticidal activity comparable to the parent compounds. Evolution of the insecticidal pyrazoline moiety (numerous halogenated pyrazolines have been described [82], which act by blocking vgSCh of neurons [83]; no commercial example) has led to the discovery of the proinsecticide indoxacarb (**28**; 1998, Steward®, DuPont) [84]/see Chapter 32, Section 32.4). Figure 35.8 outlines the SAR for 1,3,4-oxadiazines against fall armyworm, *Spodoptera frugiperda*.

Generally, halogens and/or halogen-containing substituents in the 4- or 5-position of the annellated benzo ring A, such as chlorine, bromine or trifluoroethoxy and trifluoromethyl, gave derivatives with the highest activity. The angular R^2-group was either 4-fluorophenyl or methoxycarbonyl in the most active analogues. Preferred R^3 substituents, such as trifluoromethoxy or trifluoromethyl groups, were best at the *para*-position of the phenyl ring B.



28 R^1 = Cl, R^2 = COOMe, R^3 = 4-OCF_3 *(S)* > *(R)* enantiomer

Fig. 35.8. Insecticidal activity (SAR) for 1,3,4-oxadiazines against *Spodoptera frugiperda*.

35.3.2
Inhibitors of the γ-Aminobutyric acid (GABA) Receptor/Chloride Ionophore Complex

The GABA receptor/chloride ionophore complex, which is located in the insect CNS and also in peripheral nerves, has been the focus of intense interest as both a target of insecticidal action and in its role in resistance [85].

One of the most important noncompetitive GABA agonists in insects belongs to the pyrazole insecticide class represented by the trifluoromethyl sulfoxide-containing fipronil (**29** $R^1 = H$, $R^2 = CF_3$, $n = 1$) (Fig. 35.9, Table 35.6) [86] (for more details see Chapter 29.5).

This N-2,6-Cl_2,4-CF_3-phenyl substituted 5-aminopyrazole ($R^1 = H$) either acts by interacting with an allosteric binding site or by irreversible binding [87] and

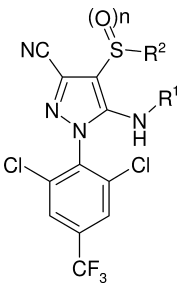
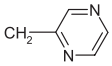
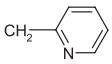


Fig. 35.9. Pyrazole insecticide class.

Table 35.6 N-2,6-Cl_2,4-CF_3-Phenyl-substituted 5-aminopyrazoles (**29**–**32**) (see Fig. 35.9 for basic structure).

Compound no.	Common name, trade name	Manufacturer (year introduced)	R^1	R^2	n	Use
29	Fipronil, Regent®	Rhone-Poulence (1993)	H	CF_3	1	Foliar, soil, rice seedling box
30	Ethiprole, Curbix®	Bayer CropScience (2005)	H	Me	1	Foliar, seed treatment
31	Pyrafluprole[a]	Nihon Nohyaku		CH_2F	0	n.d.
32	Pyriprole[a]	Nihon Nohyaku		CHF_2	0	n.d.

[a] ISO provisionally approved, development product; n.d. = not described.

has a wide margin of safety because it exhibits little activity at the corresponding mammalian channel [88]. Fipronil (**29**) is a broad spectrum insecticide that is systemic in plants and is highly active against lepidopterous larvae and numerous soil and foliar insects. It is also used as a household insecticide and for veterinary use [89].

The trifluoromethyl sulfoxide group at the 4-position ($n = 1$, $R^2 = CF_3$) of fipronil (**29**) can undergo cytochrome P_{450}-catalyzed oxidation in insects to give the corresponding trifluoromethyl sulfone metabolite ($n = 2$, $R^2 = CF_3$), which is slightly more toxic and 2–6-fold more active on the GABA receptor.

Consequently, this conversion of the pro–insecticide **29** could confer negative cross-resistance in insect strains having elevated cytochrome P_{450} detoxification activity. The trifluoromethyl sulfoxide group is a remarkable trigger for insecticidal activity, causing the indication switch. This can be demonstrated with the herbicidally active N-2,6-Cl_2,4-CF_3-phenyl-5-amino-4-nitro-pyrazole nipyraclofen (JKU 0422, Bayer CropScience) [90].

All new pyrazoles that are commercialized or in the developmental stage contain the essential N-2,6-Cl_2,4-CF_3-phenyl fragment. They differ mainly regarding their functional group at the 3-position, such as ethiprole (**30**; SO-Me) [91]; whereas mono- and di-fluoromethylthio-substituted pyrafluprole (**31**; S-CH_2F) and pyriprole (**32**; S-CHF_2) have an additional N-hetarylalkyl substituent at the 5-amino group (Table 35.6). The latter pyrazole is effective for control of coleopteran, hemipteran pests and exhibits fungicidal activity against *Pyricularia oryzae* as well.

35.3.3
Insect Growth Regulators (IGRs)

Over the past three decades, the N-benzoyl-N′-phenyl ureas (BPUs) have been developed and used as commercial IGRs acting by inhibiting chitin biosynthesis [92], thereby causing abnormal endocuticular deposition and abortive molting [93] (for more details see Chapter 26.1).

Up to now, eleven BPUs have been commercialized or are on late-stage development as chitin synthesis inhibitors that contain both fluorine (2–9) and chlorine (1–3) atoms. Early studies on structure–activity relationships (SAR) of BPUs reflected little scope for variation of substituents at the N-benzoyl moiety. Only derivatives with at least one ortho substituent retained insecticidal activity. Such an *ortho*-substituent (R^1) can be methyl, OCF_3, or OC_2F_5 and lead to active derivatives. However, all commercialized products have *ortho*-halogen substituents and the insecticidal or acaricidal activity generally follows in the order (Hal, R^1): 2,6-F_2 > 2-Cl, 6H > 2,6-Cl_2 > 2-F, 6H (Fig. 35.10, Table 35.7).

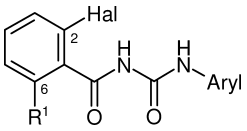


Fig. 35.10. N-Benzoyl-N′-phenyl ureas (BPUs).

The N′-arylamino moiety allows a broader variation. However, QSAR studies have shown that for optimum activity the N′-arylamino ring has to be substituted by electron-withdrawing groups such as halogen, halogenoalkyl, α-fluoroalkoxy or halogenated pyridin-2-yl. In this case, the *para*-position of the N′-arylamino moiety is preferable for high activity. Besides chlorine the N′-arylamine moiety of these ureas contains fluorine in most cases, sometimes together with various types of fluorinated substituents, such as F_3C, F_2HC-F_2C-O, F_3C-FHC-F_2C-O, giving a substitution pattern that often extended the pesticidal spectrum to include mites and ticks.

Starting with diflubenzuron (**34**; Hal, $R^1 = F$; Aryl= 4′-Cl-Ph) the intense search for potent BPUs provided further compounds containing chlorine and/or fluorine, such as teflubenzuron (**36**) [94] or flucycloxuron (**37**) [95], the first BPU that controlled rust mites. Chlorfluazuron (**38**) [96] controls chewing insects on cotton and *Plutella* spp., thrips and other on vegetables. It can be also used on fruit, potatoes, ornamentals and tea (2.5 g hl^{-1}). Flufenoxuron (**39**) [97] controls eggs, larvae and nymphs of spider mites and some insect pests. Bistrifluron (**44**) [98] has activity against whitefly and lepidopterous insects at 75–400 g-a.i. ha^{-1}. Hexaflumuron (**40**; 4-OCF_2CF_3) [99], lufenuron (**41**; 4-OCF_2CHFCF_3) [100], novaluron (**42**; 4-$OCF_2CHFOCF_3$) [101] and noviflumuron (**43**; 4-OCF_2CHFCF_3) [102], containing an α-fluoroalkoxy residue in the para position, are insecticides,

Table 35.7 Halogenated N-benzoyl-N′-phenylureas (BPUs) (**34**–**44**) (see Fig. 35.10 for basic structure).

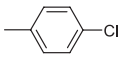
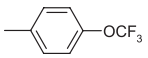
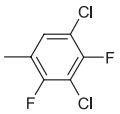
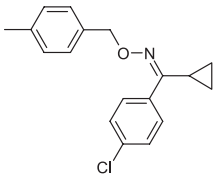
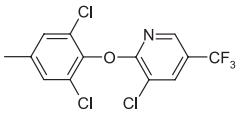
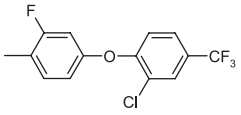
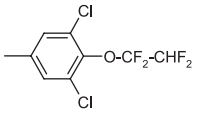
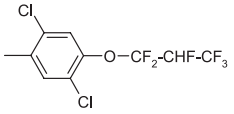
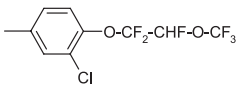
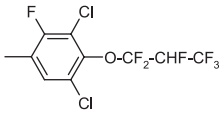
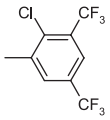
Compound no.	Common name, trade name	Manufacturer (year introduced)	R^1	Hal	Aryl	Application rate cotton (g-a.i. ha^{-1})
34	Diflubenzuron, Dimilin®	Philips Duphar (1975)	F	F		25–150
35	Triflumuron, Alsystin®	Bayer CropScience (1979)	H	Cl		100–200
36	Teflubenzuron, Nomolt®	Celamerck (1986)	F	F		15–75
37	Flucycloxuron, Andalin®	Philips Duphar (1988)	F	F		70–150
38	Chlorfluazuron, Aim®	Syngenta (1989)	F	F		25–200
39	Flufenoxuron, Cascade®	Shell/BASF (1989)	F	F		20–100
40	Hexaflumuron, Ridel®	Dow AgroScience (1989)	F	F		25–100
41	Lufenuron, Match®	Syngenta (1993)	F	F		10–15
42	Novaluron, Rimon®	Dow AgroScience (1998)	F	F		25–50

Table 35.7 *(continued)*

Compound no.	Common name, trade name	Manufacturer (year introduced)	R^1	Hal	Aryl	Application rate cotton (g-a.i. ha^{-1})
43	Noviflumuron, Recruit III®	Dow Agro Science	F	F		[b]
44	Bistrifluron[a]	Dongbu Hannong	F	F		75–400

[a] ISO provisionally approved, development product.
[b] Termiticide.

especially active against hymenoptera such as ants, cockroaches, fleas and termites (**43**) (Table 35.7). They are all more potent than diflubenzuron (**34**) against various agricultural pests [103].

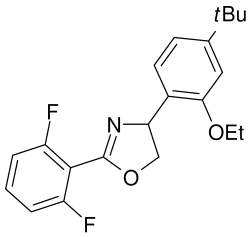
Among BPUs currently on the market, only the triflumuron (**35**; Hal = Cl; R^1 = H) [104] does not have the typical 2,6-difluoro substitution pattern (Hal, R^1 = F). During optimization of the N′-arylamino moiety, the pseudohalogenic trifluoromethoxy group in the 4-position was found to be beneficial for a broad insecticidal activity combined with a strong feeding and contact action against chewing pests like *Spodoptera frugiperda* and activity against coleopteran pests such as *Phaedon cochleariae* [105].

Because of their nontoxicity to vertebrates, the BPUs **35**, **40** and **41** are also used in veterinary medicine (**35**; Staricide®, **41**; Program®) and at home (**35**; Baycidal®, **43**; Recruit III®) against animal and human health pests such as fleas, ticks and cockroaches.

The oxazoline etoxazole (**45**; 1998, Baroque®, Yashima/Sumitomo) [106] is an acaricidal IGR (Chapter 26.2) and also possess the 2,6-difluorophenyl moiety like **46** and the BPU class (Fig. 35.11).

The mode of action (MoA) of **45** appears to be an inhibition of the molting process during mite development, similar to that of BPUs [107].

Closely related is the development product diflovidazin (**46**; 1996, Flumite®, Chinoin) [108], a 1,2,4,5-tetrazine acaricide that contains the 2,6-difluorophenyl group as common feature of BPUs (cf. acaricidally active **37** and **39**) and **46** (Fig. 35.11). The MoA of **46** involves inhibition of mite development at both the egg and chrysalis stages, but the mechanism by which this occurs has not been clarified [109]. The mite growth inhibitor clofentezine (**47**; 1983, Apollo®, Schering) [110] was launched as the first compound of the 1,2,4,5-tetrazine type. The design



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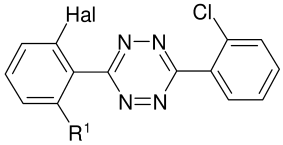


46 Hal, R^1 = F

47 Hal = Cl, R^1 = H

Fig. 35.11. Etoxazole (**45**) and 1,2,4,5-tetrazine acaricides (**46**, **47**).

of the 2,6-difluorophenyl group-containing heterocycles are described as mimics of the best conformation of the BPUs [111] based on a comparison of the X-ray structures of teflubenzuron (**36**) and **46**.

35.3.4 Mitochondrial Respiratory Chain

35.3.4.1 Inhibitors of Mitochondrial Electron Transport at Complex I

During the last few years, mitochondrial respiration is targeted by several new structurally diverse acaricides and insecticides [112] (for more details see Chapter 37.3). Beside non-halogenated compounds such as the pyrazole fenpyroximate (1991, Danitron®, Nihon Nohyaku) [113] or the quinazoline fenazaquin (1993, Magister®, Dow AgroScience) [114], mono-chlorinated heterocyclic inhibitors of the mitochondrial electron transport of complex I (NADH dehydrogenase) are described as so-called METI acaricides. The first example is pyridaben (**48**; 1991, Sunmite®, Nissan) [115] and the second, an acaricide from pyrazole chemistry, tebufenpyrad (**49**; 1993, Masai®, Mitsubishi) [116]. The pyrimidine amine insecticide pyrimidifen (**50**; 1995, Miteclan®, Sankyo/Ube Ind.) [117], is not only active against all stages of spider mites, as the former ones are, but also against the diamondback moth, *Plutella xylostella*. Activity against aphids and whitefly came the racemic development product flufenerim (**51**; Flumfen®, Ube Industries) (Fig. 35.12).

The 5-chloropyrimidine system of **51** contains a 6-α-fluoroethyl group (R^1 = F) as well as a novel 4-trifluoromethoxy-phenethylamino side chain (R^2) and is structurally closely related to the acaricide **50**.

35.3.4.2 Inhibitors of Q_o Site of Cytochrome bc1 – Complex III

Fluacrypyrim (**73**; 2002, Titaron®, Nippon Soda) [118] is the first strobilurin analogue to be marketed as an acaricide rather than a fungicide – it inhibits mitochondrial electron transport at complex III of the respiratory chain. It is active against all growth stages of spider mites and shows an acaricidal contact and

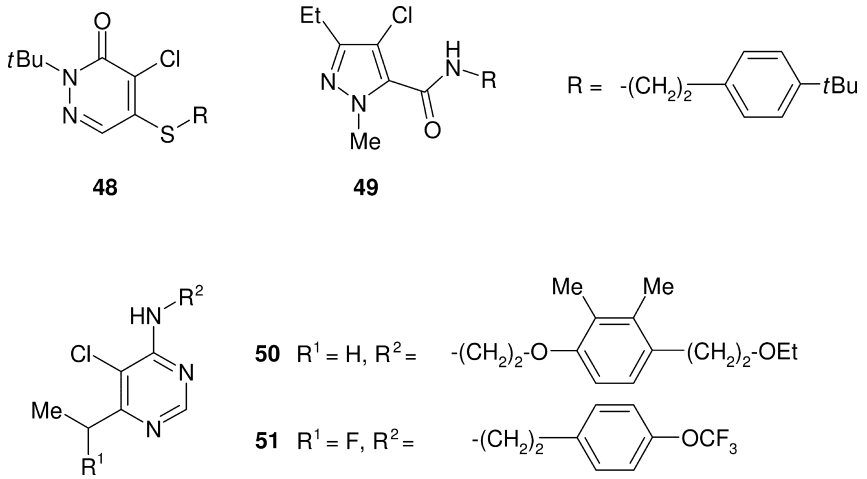
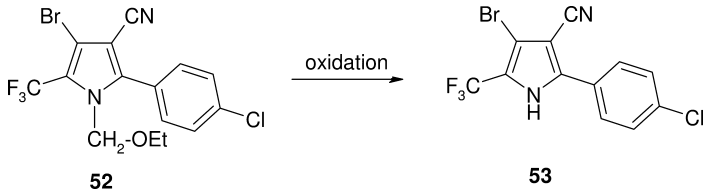


Fig. 35.12. Inhibitors of mitochondrial electron transport at complex I (**48**–**51**).

stomach action against *Panonychus ulmi* and *Tetranychus urticae* on citrus fruits and apples, as well as against spider mites on pears.

35.3.4.3 **Inhibitors of Mitochondrial Oxidative Phosphorylation**

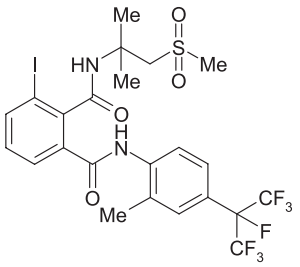
Chlorfenapyr (**52**; 1995, Pirate®, ACC/BASF) [119], a potent uncoupler of mitochondrial oxidative phosphorylation [120], is based on a trifluoromethyl substituted pyrrole (Scheme 35.3).



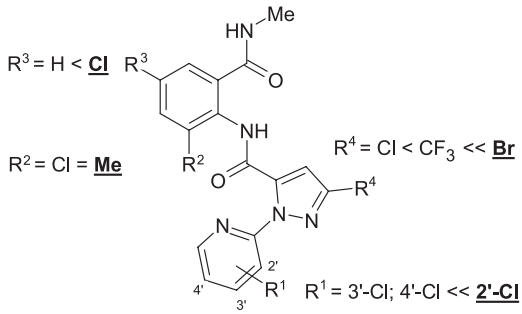
Scheme 35.3. Proinsecticide chlorfenapyr (**52**) and its N-dealkylated metabolite **53**.

It was modeled according to the fungicidal pyrrole natural product dioxapyrrolomycin [121] and contains three different halogens and/or halogenated groups in the molecule (CF_3, 4-Cl-Ph, Br).

As proinsecticide chlofenapyr (**52**) [122, 123] is activated by oxidative removal of the N-ethoxymethyl group, forming the N-dealkylated metabolite **53**, which is a potent uncoupler of mitochondrial oxidative phosphorylation [124] (for more details see Chapter 28.2).



54



55

Fig. 35.13. Phthalic acid and anthranilic acid diamides such as flubendiamide (**54**) and DP-23 (**55**).

35.3.5
Ryanodine Receptor (RyR) Effectors

Flubendiamide (**54**; ISO–proposed; Nihon Nohyaku Co., Ltd./Bayer CropScience) [125, 126, 127] (Fig. 35.13) with a heptafluoro-isopropyl moiety in the anilide part of the molecule (Chapter 34) induces ryanodine-sensitive cytosolic Ca^{2+} transients that were independent of extracellular Ca^{2+} concentration in isolated neurons from the pest insect *Heliothis virescens* as well as in transfected CHO cells expressing the RyR from *Drosophila melanogaster*. Binding studies on microsomal membranes from *H. virescens* flight muscle revealed that **54** interacts with a site distinct from the ryanodine binding site and disrupted the Ca^{2+} regulation of ryanodine binding by an allosteric mechanism.

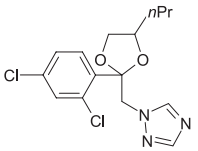
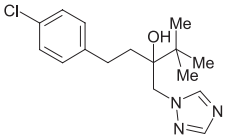
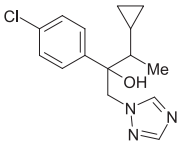
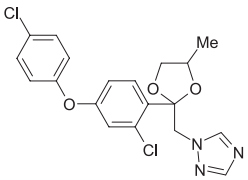
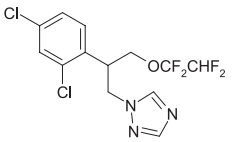
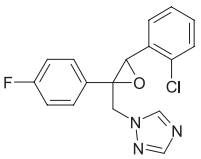
A second class of RyR effectors, the structurally different anthranilic amide derivative chlorantraniliprole (**55**; ISO–proposed; Rynaxypyr®; DuPont) was found to be also active against different species of lepidoptera such as *P. xylostella*, *S. frugiperda*, and *H. virescens*. The effect of different heterocyclic moieties and halogen atoms was investigated. Radioligand-binding studies with **55** and derivatives [128, 129] in *Periplaneta americana* skeletal muscle demonstrate a single saturable binding site, distinct from that of ryanodine as well (Fig. 35.13).

35.4
Fungicides containing Halogens

35.4.1
Sterol Biosynthesis Inhibitors (SBIs) and Demethylation Inhibitors (DMIs)

Conazole fungicides such as imidazoles and triazoles represent one of the most important chemical groups of widely used agrochemicals [130, 131]. Most so-

Table 35.8 Most important halogenated triazole fungicides (**56**–**61**).

Compound no.	Common name, trade name	Manufacturer (year introduced)	Structure
56	Propiconazole, Tilt®	Syngenta (1980)	
57	Tebuconazole, Folicur®	Bayer (1988)	
58	Cyproconazole, Sentinel®	(Sandoz) Bayer CropScience (1988)	
59	Difenoconazole, Score®	Syngenta (1989)	
60	Tetraconazole, Eminent®	Montedison Enichem (1991)	
61	Epoxiconazole, Opus®	BASF (1992)	

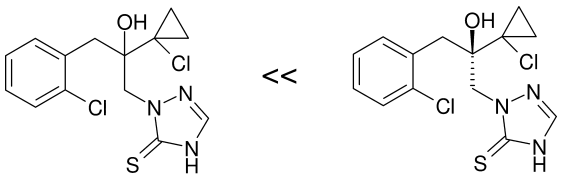
[a] Spray application; *Erysiphe* spp., *Septoria* and *Puccinia* spp.

called demethylation inhibitors (DMIs) [132] undergo systemic movement within plants (for details see Chapter 19). Among the azole derivatives almost 93% have halogen-containing substituents (F: ~11%, F/Cl: ~15%, Cl: ~70%, Cl/Br: ~4%); the chlorophenyl moiety is very common (~89%; preferred Ph substitution pattern: 2,4,6-Cl_3; 3-Cl ≪ 2,4-Cl_2 < 4-Cl), possibly because of the favorable physicochemical properties obtained through its use, such as an advantageous log *P*. Phenyl substituents, in most cases halogens, adjust the lipophilicity of the product to a suitable value for systemic movement in the plant.

Table 35.8 outlines the six well-known and most important halogenated DMIs, propiconazole (**56**) [133], tebuconazole (**57**) [134], cyproconazole (**58**) [135], difenoconazole (**59**) [136], tetraconazole (**60**) [137] and epoxiconazole (**61**) [138], which achieved sales between 27 and 271 Mio € in 2003.

Recently, a new fungicidal class of triazolinethiones was found by structural modification of the azole heterocycle [139]. The chlorine-containing prothiconazole (**62**; 2004, Proline®, Bayer CropScience) [140] was identified as an outstanding fungicide from this class (Fig. 35.14).

Prothiconazole (**62**) is a systemic fungicide (log $P_{OW} = 4.05$) with protective and curative properties (use rate: 200 g-a.i. ha^{-1}). It exists as 1:1 mixture of two enantiomers, from which the (*S*)-(−)-enantiomer **62** is significantly more active than the racemate [139].



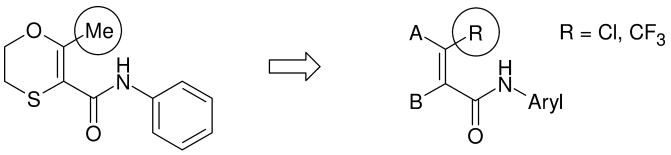
62 *S-(-)*-enantiomer **62**

Fig. 35.14. Prothioconazole (**62**) and its (*S*)-(−)-enantiomer.

35.4.2 Mitochondrial Respiratory Chain

35.4.2.1 Inhibitors of Succinate Dehydrogenase (SD) – Complex II

Succinate dehydrogenase is a membrane-bound enzyme that catalyzes the oxidation of succinic acid to fumaric acid. Following the introduction of the non-halogenated carboxin (**63**; 1966, Vitavax®, Uniroyal) [141], which has only particular activity against seedling diseases, a range of halogen and/or halogen-substituted carboxamides with an N-phenyl-2-butene amide structure (R = Cl, CF_3) has been described. Beside flutolanil (**64**) [142] more recent examples including the systemic trifluoromethyl-containing rice fungicide thifluzamide (**66**) [143], and the two chlorine-containing fungicides furametpyr (**65**) and boscalid



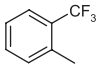
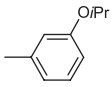
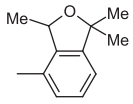
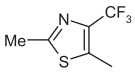
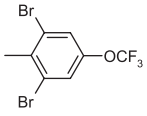
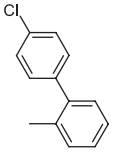
63 *N*-aryl-2-butene amide

Scheme 35.4. From carboxin (**63**) to halogenated inhibitors of succinate dehydrogenase complex II (**64**–**67**, see Table 35.9).

(**67**) (Scheme 35.4, Table 35.9). The specific chemistry of these new compounds broadens either the spectrum or improves the binding to the receptor compared with the halogen free precursors.

The physicochemical and biological properties and the chemistry of these fungicides are discussed specifically in Chapter 13.3.

Table 35.9 Halogenated inhibitors of succinate dehydrogenase (SD) – complex II (**64**–**67**) (see Scheme 35.4 for the basic structure).

Compound no.	Common name, trade name	Manufacturer (year introduced)	A, R / B	Aryl	Application rate[a] rice (g-a.i. ha^{-1})
64	Flutolanil, Flutranil®	Nihon Nohyaku (1985)			450–600
65	Furametpyr, Limber®	Sumitomo (1996)			150–200
66	Thifluzamide, Pulsor®	Monsanto (1997)			255–340
67	Boscalid, Cantus®	BASF (2003)			150–500[b]

[a] Seed treatment, systemic activity against *Rhizoctonia solani*.
[b] Broad spectrum systemic fungicide.

35.4.2.2 Inhibitors of Q_o Site of Cytochrome bc1 – Complex III

Strobilurin fungicides have been among the most commercially successful class of agricultural fungicides over the past seven years [144] (see Chapter 13.2). Like the most important lead structure strobilurin A [145], all strobilurins inhibit mitochondrial respiration by influencing the function of the so-called Q_o site of complex III (cytochrome bc_1 complex) [146, 147, 148] which is located in the inner mitochondrial membrane of fungi and other eucaryotes [149]. The binding site for the Q_o site inhibitors is distinct from the stigmastellin binding site within the membrane below the peripheral helix αcd1 [150].

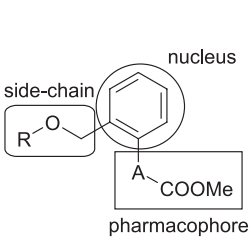
Despite being first introduced in 1996, the eight commercialized strobilurins are already the second largest group in the market, behind conazole fungicides (Section 35.4.1). Whereas the first broad-spectrum systemic strobilurins such as azoxystrobin (1996, Amistar®, Syngenta) [151] and kresoxim-methyl (1996, Stroby®, BASF) [152] are not halogen-substituted, the incorporation of halogen atoms or halogenated substituents into the side-chain began with trifloxystrobin (**68**; 1999, Flint®) [153], which contains a 3-trifluoromethylphenyl moiety in its oximether side-chain and belongs to a new generation of strobilurin fungicides. Crystallographic studies on **68** have shown that the 3-trifluoromethylphenyl moiety in the side-chain interacts with a hydrophobic domain in the binding pocket (Phe128, Ile146, Ala277, Leu294), which presents a higher amino acid variability among organisms and plays a role in species specificity towards β-methoxyacrylates. During the preparation of oximethers it had already been found that compounds with a fluorine-containing phenyl substituent such as trifluoromethyl showed particularly strong systemic activity. Like kresoxim-methyl (vapor pressure: 2.3×10^{-3} mPa at 20 °C), **68** delivers disease control in the plant by vapor action (Fig. 35.15, Table 35.10) [154].

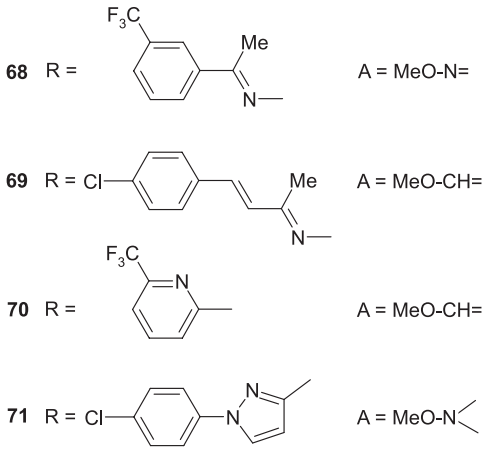
The low aqueous solubility (0.6 mg L^{-1}) and relatively high lipophilicity ($\log P_{OW} = 4.5$) contribute to a high affinity for the waxy layer on the surface of the plant leaf for a long time, which leads to the formation of a rain-resistant store of this active ingredient. High humidity after a short drying phase aids retention of the fungicide and increases redistribution. The special behavior of **68** on the surface of the plant, known as "mesosystemic activity", gives excellent control of apple scab because of its inhibitory effects on multiple stages of the life cycle of *Venturia inaequalis* [155].

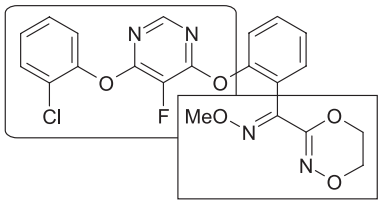
Recently, the new development product enestrobin (**69**; ISO–proposed, SYP-Z071, Shenyang Res. Inst. of Chem. Industry) [156], containing a 4-chlorophenyl unsaturated oximether side-chain, has been presented. Field trial results indicate that **69** is especially a fungicide active against crop diseases on cucumber such as downy mildew, powdery mildew and gray mold, which is useful in plastic sheet-covered cucumber fields.

Picoxystrobin (**70**; 2002, Acanto®; Syngenta) [157] has a 6-CF_3-pyridin-2-yl moiety in its arylalkyl ether side-chain and was developed initially for disease control in cereals and apples.

BASF's second strobilurin, the N-(4-Cl-phenyl)-1*H*-pyrazol-3-yloxy-containing pyraclostro–bin (**71**; 2002, F-500; BASF) [158], gets its broader spectrum from the introduction of the 4-chlorophenyl-1*H*-pyrazol-3-yloxy-moiety. One of Bayer's







72

Fig. 35.15. Commercialized strobilurin fungicides (**68**, **70**–**72**) and the development product **69**.

Table 35.10 Physical properties of halogen-containing compounds of strobilurin type (**68**–**73**).

Compound no.	Melting point (°C)	Vapor pressure (mPa at 20 °C)	Log P_{OW} (20 °C)	Solubility in water (mg L^{-1} at 20 °C)	Indication
68	72.8–72.9	3.4×10^{-3}[a]	4.5	0.6	Fungicide
69	n.d.	n.d.	n.d.	n.d.	Fungicide
70	75.0	5.5×10^{-3}	3.6	3.1	Fungicide
71	63.7–65.2	2.6×10^{-5}	3.99[b]	1.9	Fungicide
72	103–108	6.0×10^{-7}[c]	2.86	2.56[d], 2.29[e]	Fungicide
73	107.2–108.6	2.69×10^{-3}	4.51[f]	0.344	Acaricide

[a] At 25 °C.
[b] At 22 °C.
[c] Extrapolated.
[d] Unbuffered.
[e] At pH 7.
[f] At pH 6.8, 25 °C.
n.d. = not described.

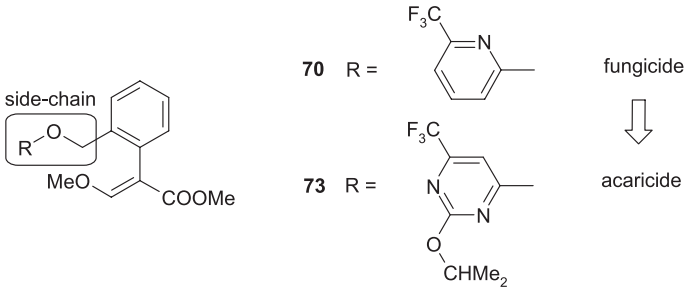


Fig. 35.16. Indication switch from fungicide to acaricide – picoxystrobin (**70**) and fluacrypyrim (**73**).

research programs focused on the variation of the toxophore moiety, which led to the incorporation of the carbocyclic acid moiety into a six-membered heterocycle.

The aryl ether structure of fluoxastrobin (**72**; 2005, HEC®; Bayer CropScience) [159] combines a methoximino 5,6-dihydro-1,4,2-dioxazin-2-yl toxophore with an optimally adjusted side-chain bearing a 6-(2-chlorophenoxy)-5-fluoro-pyrimidin-4-yl-oxy moiety as an essential element.

Under the provision that both **68** and **72** bind to their target in similar ways, it can be assumed that **72** has an advantage as no reorientation of the toxophore is necessary for binding to the target [160]. The excellent leaf systemicity is the basis for rapid uptake and even acropetal distribution of **72** in the leaf. SARs indicate that the fluorine atom has a beneficial effect on the phytotoxicity and leaf systemicity. Seed treatment with **72** provides both very good broad-spectrum control and long-lasting protection of young seedlings from seed and soil-borne pathogens.

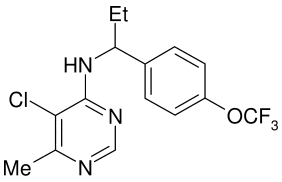
Combination with the chlorine-containing DMI-type fungicide prothioconazole (**62**; 2004, Fandango®, Bayer CropScience) [161] further increases the efficacy of **72**, serves as a built-in resistance management tool and further broadens the spectrum of activity against all important seed and soil-borne pathogens [162]. An indication switch from the fungicidally to acaricidally active strobilurin type with β-methoxyacrylate pharmacophore is achieved by exchange of the 6-CF_3-pyridin-2-yl moiety in the arylalkyl ether side-chain of **70** with a 2-*i*PrO-6-CF_3-pyrimidin-4-yl moiety to give fluacrypyrim (**73**) (Fig. 35.16).

This compound is more lipophilic (difference log $P_{OW} \sim 0.9$) and around a ten-fold lower water soluble than **70**.

35.4.2.3 NADH Inhibitors – Complex I

Agrochemical fungicides acting as NADH inhibitors with useful potency, spectrum and toxicological properties, interesting enough for commercialization are rare. Only one compound, diflumetorim (**74**; 1997, Pyricut®, Ube Ind.), was introduced into the market for use in ornamentals (Fig. 35.17) (see Chapter 15.5).

The compound possess a trifluoromethoxy group that acidifies the NH binding in the amid moiety to improve the inhibitory properties.

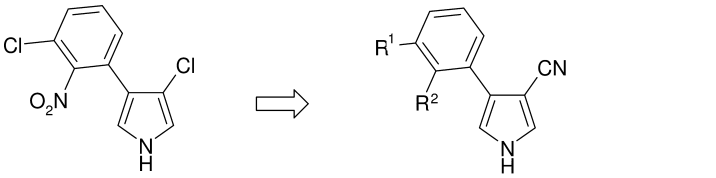


74

Fig. 35.17. Diflumetorim (**74**).

35.4.3
Fungicides acting on Signal Transduction

The nonsystemic pyrrole fungicides fenpiclonil (**76**; 1988, Beret®, Syngenta) [163] (R^1, $R^2 = Cl$) and its difluoromethylenedioxy analogue fludioxonil (**77**; 1993, Saphire®, Syngenta) [164] have been developed from the photo-unstable and chlorine-containing natural antibiotic pyrrolnitrin (**75**; Pyroace®, Fujisawa) that was first isolated from *Pseudomonas pyrrocinia* [165] (Fig. 35.18, Table 35.11).



75

76 $R^1, R^2 = Cl$

77 $R^1\text{-}R^2 = O\text{-}CF_2\text{-}O$

Fig. 35.18. From natural antibiotic pyrrolnitrin (**75**) to the pyrrole fungicides fenpiclonil (**76**) and fludioxonil (**77**).

Table 35.11 Physical properties of pyrrolnitrin (**75**) and synthetic pyrrole fungicides (**76**, **77**).

Compound no.	Melting point (°C)	Vapor pressure (mPa at 25 °C)	Log P_{OW} (at 25 °C)	Solubility in water (mg L^{-1} at 25 °C)	Light stability $t_{1/2}$ (h)
75	124.5	1.42×10^{-6}[a]	3.09	n.d.	n.d.
76	144.9–151.1	1.1×10^{-2}	3.86	4.8	48.0
77	199.8	3.9×10^{-4}	4.12	1.8	24.5

n.d. = not determined.
[a] Torr.

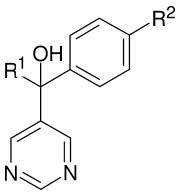
Especially, the introduction of the difluoromethylenedioxy moiety improved both biological activity and soil stability. Biochemical studies revealed that the pyrrole fungicides inhibit a protein kinase (PK-III) potentially involved in the osmosensing signal transduction pathway [166] (for more details see Chapter 15.2).

35.5
Plant Growth Regulators (PGRs) Containing Halogens

35.5.1
Reduction of Internode Elongation – Inhibition of Gibberellin Biosynthesis

Some of the triazoles, but especially their bioisosteric pyrimidine analogues such as the non-halogenated ancymidol (**78**; 1973, Arest®, Eli Lilly) [167], exhibit PGR activity in mono- and dicotyledonous species and act by reducing internodal elongation through interaction with the gibberellin biosynthesis pathway [168] (for more details see Chapter 11). Replacement of the methoxyphenyl group with a trifluoromethoxyphenyl moiety, and the cyclopropyl group with isopropyl leads to flurprimidol (**79**; 1989, Cutless®, Dow AgroScience) [169], which has several different physicochemical properties, e.g. log *P* and DT_{50} values or vapor pressure (Fig. 35.19, Table 35.12).

Whereas **78** only translocated in the phloem of plants, compound **79** is xylem and phloem mobile.



78 R^1 = *c*Pr, R^2 = OMe

79 R^1 = *i*Pr, R^2 = OCF_3

Fig. 35.19. Plant growth regulators ancymidol (**78**) and flurprimidol (**79**).

Table 35.12 Comparison of the physical properties of ancymidol (**78**) and flurprimidol (**79**).

Compound no.	Melting point (°C)	Vapor pressure (mPa at 25 °C)	Log P_{OW} (20 °C)	Solubility in water (mg L^{-1} at 25 °C)	DT_{50}
78	110–111	<0.13[a]	1.9[b]	~650	>30 days[c]
79	93.5–97	4.85×10^{-2}	3.34	114[d]	~3 h

[a] At 50 °C.
[b] pH 6.5 at 25 °C.
[c] pH 5–9, 25 °C.
[d] at 20 °C.

35.6
Herbicides containing Halogens

35.6.1
Inhibitors of Carotenoid Biosynthesis

Most commercial so-called bleaching herbicides inhibit the synthesis of carotenoids by interfering with carotenoid biosynthesis at the level of phytoene desaturase [170, 171, 172]. Enzyme kinetics with several inhibitors have revealed a reversible binding to the enzyme and non-competitive inhibition [173].

A common fragment in all commercial products (**80**–**86**) listed in Table 35.13 is the 3-trifluoromethylphenyl moiety (Fig. 35.20), e.g., norflurazon (**80**) [174], fluridone (**81**) [175], and fluorochloridone (**82**) [176].

Other commercial products are diflufenican (**83**) [177], flurtamone (**84**) [178], and picolinafen (**85**) [179], which contains a pyridine skeleton similar to that of **83**. On the other hand, the new selective herbicide for weed control in cereals, beflubutamid (**86**; 2003, Ube Ind.) [180] contains as an exemption 4-fluoro-3-trifluoromethylphenyl moiety ($R^1 = F$).

This moiety is a feature of either an arylanilide (X = N; log $P = 2.45, 3.36$; **80**, **82**, respectively), arylether (X = O; log $P = 4.90, 5.37, 4.28$; **83**, **85**, **86**, respectively) or of a substituted N-methyl-enaminone structure (X = C–C bond; log $P = 1.87$, n.d.; **81**, **84**, respectively) in a five- or six-membered heterocycle, respectively. It is assumed that the biochemical activity of these compounds is determined by the properties of the *meta*-trifluoromethylphenyl group, such as high lipophilicity (for X = O) and an electron-withdrawing nature. Furthermore, there are strict requirements for substitution at the five- or six-membered heterocycle of the inhibitor, especially at the position most distant from the carbonyl group (for more details see Chapter 4.1).

35.6.2
Inhibitors of Acetolactate Synthase (ALS)

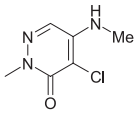
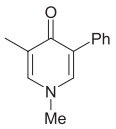
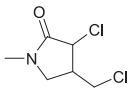
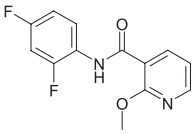
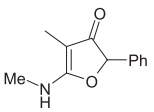
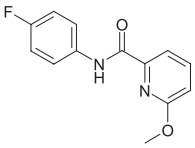
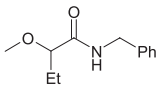
35.6.2.1 Sulfonylurea Herbicides

Sulfonylureas are generally extremely potent inhibitors of ALS [181] the key enzyme within the biosynthesis of branched amino acids such as leucine, isoleucine or valine [181, 182] (for more details see Chapter 2.2). Approximately 57.5% of commercialized sulfonylureas or development products are halogen free. The remaining 42.5% (halogens/subclass: a > b) launched so far contain halogen atoms such as fluorine (~24%), chlorine (~15%) or iodine (~3%) (Fig. 35.21).

Sulfonylureas (Fig. 35.22) can be further divided into two subclasses: (a) triazinylsulfonylurea herbicides (Y = N; Table 35.14) and (b) pyrimidinylsulfonylurea herbicides (Y = CH; Table 35.15).

Halogen-containing Triazinylsulfonylurea Herbicides Exchange of the *ortho*-chloro substituent (ring A, R^1) in the cereal-selective herbicide chlorsulfuron (**87**; 15–20

Table 35.13 Chemically different classes of phytoene desaturase inhibitors (**80**–**86**) (see Fig. 35.20 for the basic structure).

Compound no.	Common name; trade name(s)	Manufacturer (year introduced)	R^1	X	$-X-R^2$
80	Norflurazon; Zorial®, Telok®	Syngenta (1971)	H	N	
81	Fluridone; Brake®, Sonar®	Dow Agrow Science (1981)	H	C	
82	Flurochloridone; Rainbow®, Racer®	Makhteshim-Agan (1985)	H	N	
83	Diflufenican; Quartz®, Fenikan®	Bayer CropScience (1985)	H	O	
84	Flurtamone; Benchmark®, Bleacher®	Bayer CropScience (1997)	H	C	
85	Picolinafen; Pico®, Sniper®	BASF (2001)	H	O	
86	Beflubutamid Herbaflex®	Ube Ind. (2003)	F	O	

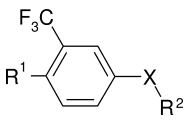


Fig. 35.20. The 3-trifluoromethylphenyl moiety common to commercial products **80**–**86**.

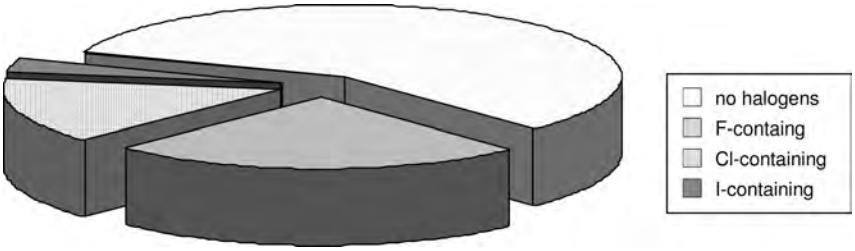
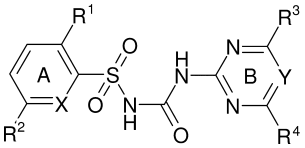


Fig. 35.21. Differentiation of commercialized sulfonylureas.



(i) Y = N Triazinylsulfonylurea herbicides

(ii) Y = CH Pyrimidinylsulfonylurea herbicides

Fig. 35.22. Sulfonyl ureas.

Table 35.14 Halogen-containing triazinylsulfonylurea herbicides (**87**–**93**) (see Fig. 35.22 for the basic structure).

Compound no.	Common name, trade name(s)	Manufacturer (year introduced)	R^1	R^2	X	R^3	R^4	Y
87	Chlorsulfuron, Glean®, Telar®	DuPont (1982)	Cl	H	CH	OMe	Me	N
88	Triasulfuron, Logran®, Amber®	Syngenta (1987)	$O(CH_2)_2Cl$	H	CH	OMe	Me	N
89	Triflusulfuron-methyl, Safari®, Debut®	DuPont (1992)	COOMe	H	CMe	NMe_2	OCH_2CF_3	N
90	Prosulfuron, Peak®, Scoop®	Syngenta (1994)	$(CH_2)_2CF_3$	H	CH	OMe	Me	N
91	Iodosulfuron-methyl[a] Husar®, Hussar®	Bayer CropScience (2000)	COOMe	I	CH	OMe	Me	N
92	Trifloxysulfuron[a] Envoke®	Syngenta (2001)	OCH_2CF_3	H	CH	OMe	OMe	N
93	Tritosulfuron[b], Biathlon®	BASF (2005)	CF_3	H	CH	OMe	CF_3	N

[a] Sodium salt.
[b] ISO provisionally approved, development product.

Table 35.15 Halogen-containing pyrimidinylsulfonylurea herbicides (**96**–**100**) (see Fig. 35.22 for the basic structure).

Compound no.	Common name, trade name(s)	Manufacturer (year introduced)	R^1	R^2	X	R^3	R^4	Y
96	Chlorimuron-ethyl, Classic®, Darban®	DuPont (1985)	COOEt	H	CH	OMe	Cl	CH
97	Flazasulfuron, Shibagen®	Ishihara (1989)	CF_3	H	N	OMe	OMe	CH
98	Flurpyrsulfuron-methyl[a] Lexus®, Oklar®	DuPont (1997)	COOMe	CF_3	N	OMe	OMe	CH
99	Primisulfuron-methyl, Beacon®, Tell®	Syngenta (1998)	COOMe	H	CH	$OCHF_2$	$OCHF_2$	CH
100	Flucetosulfuron[b]	LG Chem	CH(OR)–CHFMe[c]	H	N	OMe	OMe	CH

[a] Sodium salt.
[b] ISO provisionally approved, development product.
[c] R = CO–CH_2–OMe.

g-a.i. ha^{-1}) with *ortho*-2-chloroethyl or *ortho*-3,3,3-trifluoropropyl leads to triasulfuron (**88**; wheat and barley, 5–10 g-a.i. ha^{-1}) [183] and prosulfuron (**90**; maize, 10–40 g-a.i. ha^{-1}; winter wheat, 20–30 g-a.i. ha^{-1}) [184], respectively, which shows a selectivity shift – such changes facilitate patent applications. Maize-selective **90** is metabolized in maize by additional hydroxylation at the methyl group (ring B, R^4) of the triazine moiety (cf. Fig. 35.23).

A novel combination of substituents in ring B (R^3) and (R^4) is given in triflusulfuron-methyl (**89**; sugar beet, 10–30 g-a.i. ha^{-1}) [185], which contains the N,N-dimethylamino and 3,3,3-trifluoroethyoxy group. Iodosulfuron-methyl-sodium (**91**) [186], the iodine derivative (ring A, $R^2 = I$) of metsulfuron-methyl (**94**; 1984, Gropper®, DuPont) [187], has a ten-fold faster soil degradation ($DT_{50} = 1$–5 d) than the non-halogenated sulfonylurea (**94**; $DT_{50} = 52$ d). Trifloxysulfuron-sodium (**92**; cotton, 5–7.5 g-a.i. ha^{-1}) [188] and the development product tritosulfuron (**93**) demonstrate that the trifluoroethoxy or trifluoromethyl are useful substituents (R^1) in ring A; in addition the methyl (R^4) in ring B is exchangeable with the 3,3,3-trifluoroethyoxy (**89**) and trifluoromethyl group (**93**).

Halogen-containing Pyrimidinylsulfonylurea Herbicides The first halogenated member of this subclass is chlorimuron-ethyl (**96**; soya beans and peanuts, 9–13

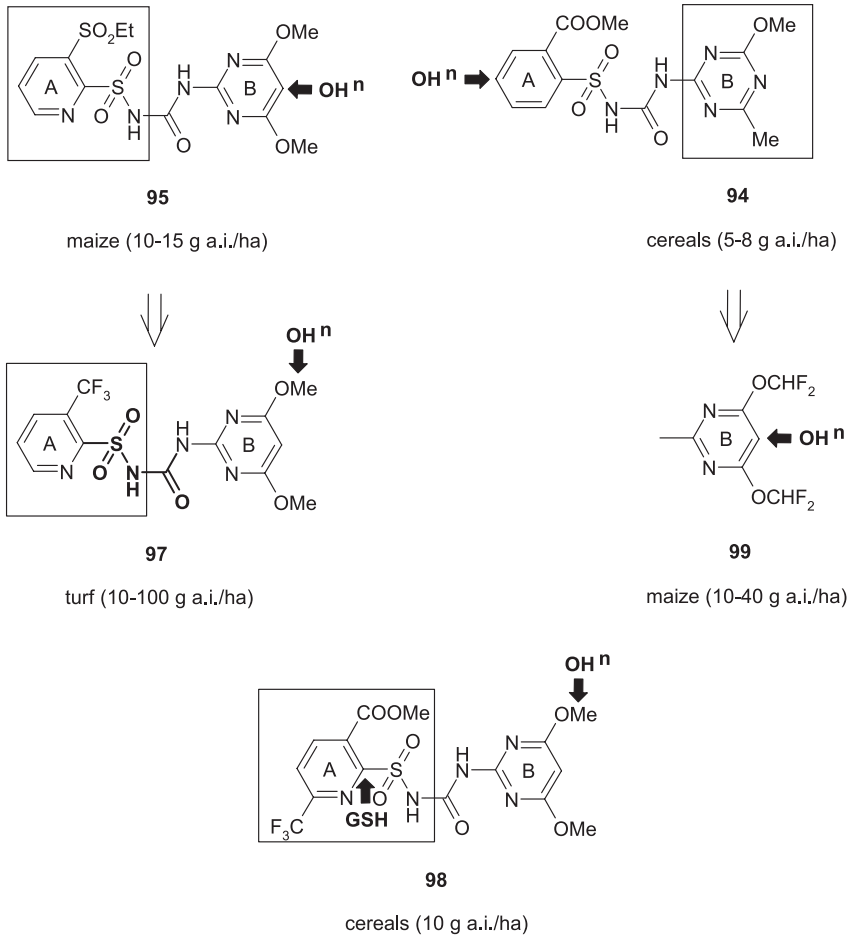


Fig. 35.23. Influence of fluorine-containing substituents on selectivity and metabolism.

g-a.i. ha^{-1}), which demonstrates the successful exchange of methoxy with chlorine in ring B (Fig. 35.22).

Comparison of rimsulfuron (**95**; 1991, Titus®, DuPont) [189] containing a 3-EtSO_2-pyridin-2-yl moiety with flazasulfuron (**97**) [190] shows that its 3-CF_3-pyridin-2-yl moiety has a marked impact on metabolism (Fig. 35.23). The key transformation on tolerant turf grass is an unusual rearrangement and contraction of the sulfonylurea bridge, followed by hydrolysis and O-demethylation of a pyrimidyl methoxy (R^3) group. In contrast to **97**, flurpyrsulfuron-methyl sodium (**98**) contains a 3-COOMe-6-CF_3-pyridin-2-yl moiety, which influences its metabolic pathway (Fig. 35.23). Beside glutathione conjugate formation (attack of GSH), O-demethylation is predominant in the detoxification of **98** in cereals (10 g-a.i. ha^{-1}).

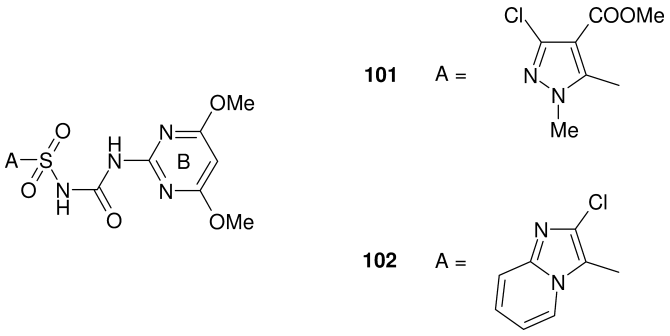


Fig. 35.24. Halogen-containing pyrimidinylsulfonyl herbicides halosulfuron-methyl (**101**) and imazosulfuron (**102**).

Primisulfuron-methyl (**99**) [191] is a maize-selective herbicide. Comparison with the unfluorinated triazine counterpart metsulfuron-methyl (**94**) indicates that crop safety for maize is achieved by replacement of the triazine methoxy (R^3) and methyl (R^4) group in ring B with two difluoromethoxy groups (R^3, R^4).

In addition, **99** is deactivated in maize by hydroxylation of the phenyl ring A and pyrimidyl moiety B followed by hydrolysis or further conjugation (Fig. 35.23).

Both systemic herbicides halosulfuron-methyl (**101**; maize, 18–35 g-a.i. ha^{-1}) [192] and imazosulfuron (**102**; paddy rice, 75–95 g-a.i. ha^{-1}) [193] demonstrate the structural variability of ring A, e.g., by incorporation of further halogenated heterocyclic systems such as 3-Cl,4-COOMe-1-CH_3-1*H*-pyrazol-5-yl (**101**) and 2-chloroimidazo[1,2-*a*]pyridine-3-yl (**102**) (Fig. 35.24).

35.6.2.2 **Triazolone Herbicides**

The exchange of the *ortho*-COOMe by the *ortho*-OCF_3 residue in the sulfonylaryl unit of propoxycarbazone sodium (**103**; 2001, Attribut®, Bayer CropScience) [194] led to the systemic herbicide flucarbazone sodium (**104**; 2000, Everest®, Bayer CropScience) [195] (Fig. 35.25) (for more details see Chapter 2.6).

During optimization of the sulfonyl component it was found that the sulfonylaryl moiety is more potent than the corresponding sulfonylmethylaryl structure. However, particularly good activity and cereal selectivity were identified for the halogenated substitution pattern in the *ortho*-position of the latter, such as trifluoromethyl and trifluoromethoxy.

35.6.2.3 **Triazolopyrimidine Herbicides**

The activity of the non-halogenated sulfonamide herbicide asulam (**105**; 1965, Asulox®, May & Baker; 1–10 kg-a.i. ha^{-1}) [196] was remarkable improved by replacing the 4-aminophenyl ring with a halogenated triazolopyrimidine moiety and/or by replacement the N-methoxycarbonyl group with a series of *ortho*-halogenated electron-deficient phenyl rings such as 2,6-difluoro-, 2,6-dichloro- or

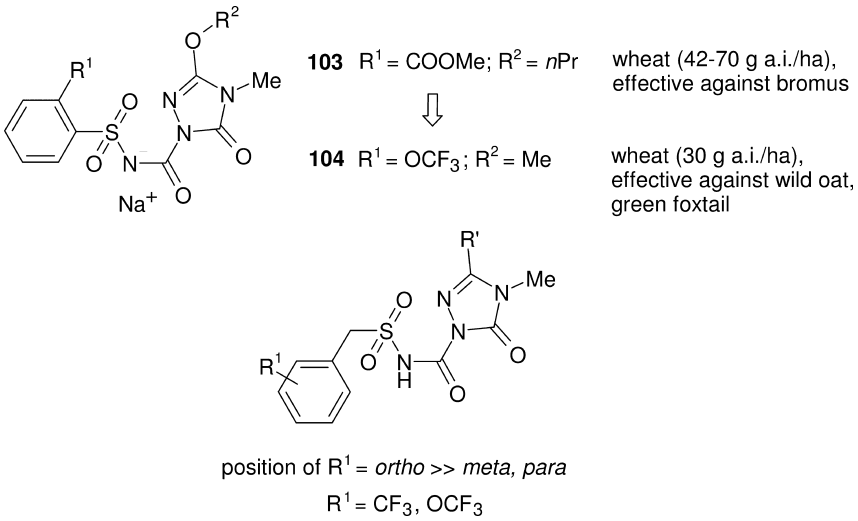


Fig. 35.25. Triazolone herbicides and optimization in their sulfonylmethylaryl moiety.

2-chloro-6-methoxycarbonyl-phenyl rings, forming the so-called ''sulam'' herbicides (Fig. 35.26, Table 35.16).

However, with the development product penoxsulam (**114**; 2004, Viper®, Dow AgroScience) [197], the 4-amino-phenyl ring in 105 was replaced by the 2-(2,2-difluoroethoxy)-6-trifluoromethyl-phenyl ring and the N-methoxycarbonyl group by a non-halogenated triazolopyrimidine moiety. All herbicides are active against broad-leaved weeds after pre- and/or post-emergence application with different application rates vs. crops: flumetsulam (**106**; 25–80 g-a.i. ha^{-1}, soya/

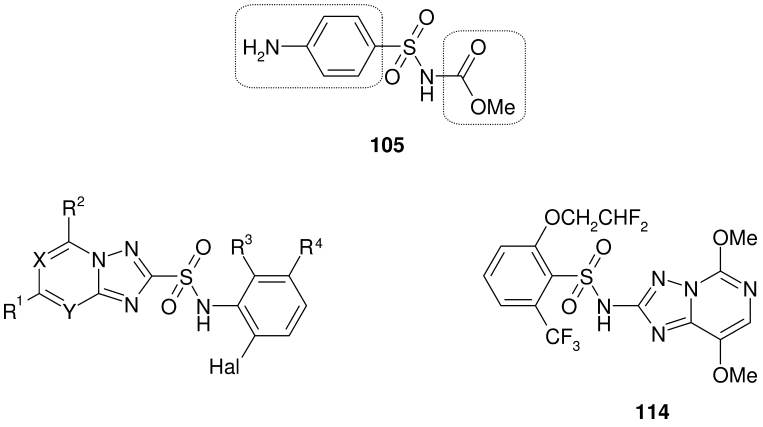


Fig. 35.26. Asulam (**105**) and triazolopyrimidine herbicides [**106**–**110** (Table 35.16), **114**].

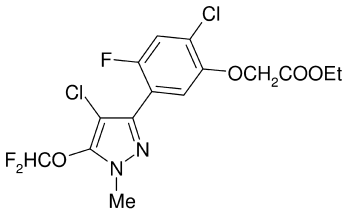
Table 35.16 Halogen-containing triazolopyrimidine herbicides (**106**–**110**) (see Fig. 35.26 for the basic structure).

Compound no.	Common name, trade name(s)	Manufacturer (year introduced)	R^1	R^2	X	Y	R^3	R^4	Hal
106	Flumetsulam, Broadstrike®, Preside®	Dow AgroScience (1992)	Me	H	CH	N	F	H	F
107	Metosulam, Eclipse®, Uptake®	Dow AgroScience (1993)	OMe	OMe	CH	N	Cl	Me	Cl
108	Chloransulam-methyl, Field Star®, First Rate®	Dow AgroScience (1997)	F	OEt	N	CH	COOMe	H	Cl
109	Diclosulam, Spider®, Strongarm®	Dow AgroScience (1997)	F	OEt	N	CH	Cl	H	Cl
110	Florasulam, Primus®, Boxer®	Dow AgroScience (1999)	H	OMe	N	CF	F	H	F

maize; systemic), metosulam (**107**; 5–30 g-a.i. ha^{-1}, maize) [198], cloransulam-methyl (**108**; 40–50 g-a.i. ha^{-1}, soya), diclosulam (**109**; 20–35 g-a.i. ha^{-1}, soya beans/peanuts), florasulam (**110**; 5–10 g-a.i. ha^{-1}, cereal/maize; systemic) [199], and penoxsulam (**114**; 25–40 g-a.i. ha^{-1}). Because of creation a different set of particular halogenated basic triazolopyrimidine moieties and the use of 2,6-dihalogenated anilines, a series of commercial valuable multi-outlet chemical intermediates was essential (see also Chapter 2.4).

35.6.3 Protoporphyrinogen IX Oxidase (PPO)

PPO inhibitors have a complex mechanism of action [200]. PPO, which is localized in the chloroplast and mitochondrial membranes, and catalyzes the conversion of protoporphorinogen IX into protoporphyrin IX. Many inhibitors mimic the hydrophobic region of protoporphorinogen IX. Over the past decade or so, different new PPO inhibitor classes containing halogen and/or halogen-substituted groups (for details see Chapter 3) with even higher mimicry to protoporphorinogen IX have been developed, such as (Fig. 35.27):



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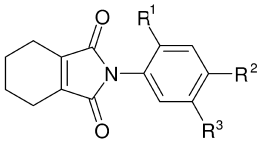
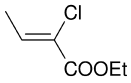
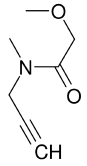


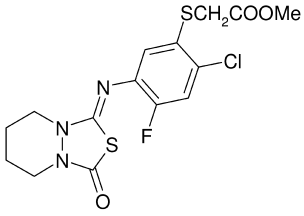
116 R^1 = H, R^2 = Cl, R^3 =



117 R^1 = F, R^2 = Cl, R^3 = $OCH_2COOnPen$

118 R^1 = F, R^2-R^3 =





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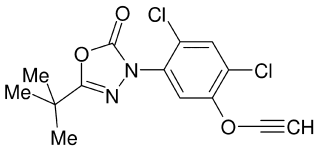

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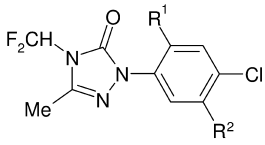
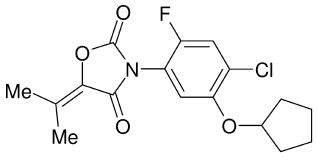


121 R^1 = Cl, R^2 = $NHSO_2Me$

122 R^1 = F, R^2 = $CH_2CHClCOOEt$



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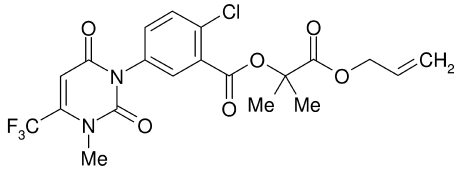

124

Fig. 35.27. Different classes of halogen-substituted PPO inhibitors (**115**–**124**).

1. *Phenylpyrazole herbicides*, e.g., the selective post-emergence cereal-selective herbicide pyraflufen-ethyl (**115**; 1999, Ecopart®, Nihon Nohyaku) [201].
2. *N-Phenylphthalimide herbicides*, e.g., the cereal-selective herbicide cinidon-ethyl (**116**; 1998, Lotus®, BASF) [202], the soya bean herbicides flumiclorac-pentyl (**117**; 1992, Resource®, Sumitomo) and flumioxazin (**118**; 1993, Sumisoya®, Sumitomo) [203].

3. *Thiadiazole herbicides*, e.g., the post-emergence maize-selective herbicide fluthiacet-methyl (**119**; 1999, Action®, Kumiai) [204].
4. *Oxadiazole herbicides*, e.g., the pre- and post-emergence rice herbicide oxadiargyl (**120**; 1996, Raft®, BayerCropScience) [205].
5. *Triazolone herbicides*, e.g., sulfentrazone (**121**; 1995, Authority®, FMC) [206] and carfentrazone-ethyl (**122**; 1997, Aim®, FMC) [207], which are adsorbed by roots (**121**) and by foliage with limited translocation in the phloem.
6. *Oxazolinedione herbicides*, e.g., pentoxazone (**123**; 1997, Wechser®, Kaken) [208].
7. *Pyrimidinedione herbicides*, e.g., butafenacil (**124**; 2000, Inspire®, Syngenta) [209] used in vineyards, citrus and non crop–land.

The various examples described above demonstrate that the introduction of halogens and/or halogen-substituted groups has had a dramatic effect on the metabolism of active ingredients through reaction at a location remote from the halogenated groups themselves. However, such effects cannot often be predicted as part of initial design of an active ingredient.

35.7 Summary and Outlook

In the search for an optimal product in modern crop protection in terms of efficacy, environmental safety, user friendliness, and economic viability, the substitution of active ingredients with halogen atoms or halogen-containing substituents is an important tool. However, the introduction of halogen atoms or halogen-containing substituents into a molecule can lead to an increase or a decrease in biological efficacy, depending on the mode of action, physicochemical properties, or target interaction of the compound. Generally, the metabolism of the active ingredient is influenced by the substitution pattern and by the soil stability and/or water solubility. Because of the complex SARs within active ingredients it is very difficult to predict sites where halogens or halogen-substituted substituents will increase biological efficacy. The technical availability of active ingredients containing halogens or halogen-substituted substituents has been improved by an increase in access to new intermediates.

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Index of Common Names

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b

C

d

e

f

g

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b

d

f

g

Wolfgang Krämer *gained his PhD in organic chemistry from the TU Stuttgart in 1968, after which he joined the Institute of Textile Chemistry at Stuttgart University, before moving to Bayer Plant Protection as lab leader in plant protection research in 1970. Between 1984 and 1990 he was Head of Global Chemistry Fungicides, and that of Insecticides until 2002. Retired since January 2005, Dr. Krämer has over 250 patent applications and publications to his name.*

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