The degradation of benzylpenicillin in aqueous solution

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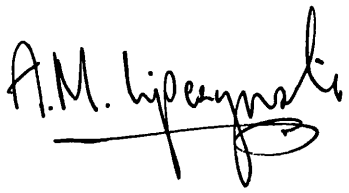
THE DEGRADATION OF BENZYLPENICILLIN IN AQUEOUS SOLUTION

submitted by Andrew Martin Lipczynski

for the degree of PhD

of the University of Bath

1988



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Dedicated with love to Rosemary

and our daughter Alice

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

'Little Gidding' from *Four Quartets* by T. S. Elliot.

ACKNOWLEDGEMENTS

I am especially grateful to Dr Alan Casy, my academic supervisor, for his great enthusiasm and encouragement throughout these studies. I wish to thank all colleagues and friends in the School of Pharmacy and Pharmacology at the University of Bath for making my three years such a pleasant time. In particular I am indebted to Dr Sarah Branch for her invaluable help and advice with the NMR studies, Dr Terry Jefferies for the benefit of his experiences in HPLC, Dr Paul Christie for aid with the computing and to Harry Hartell, Diane Belk, Kevin Smith and Richard Sadler for their skilled technical assistance.

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Last, and by no means least, thanks to my wife Rosemary for her help in typing this thesis and for her patience and understanding.

THE DEGRADATION OF BENZYLPENICILLIN IN AQUEOUS SOLUTION

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SUMMARY

The degradation of benzylpenicillin 5 in aqueous solution is complex and incompletely understood. A number of previously reported degradation products were therefore prepared in order to quantitatively investigate the kinetics and mechanisms of the individual steps in the degradation sequence.

The prepared degradation products were characterised by ^{1}H and ^{13}C NMR (2D-^{1}H,^{13}C COSY NMR permitted the unambiguous assignment of the ^{1}H NMR spectrum of benzylpenicillin). A comparison of ion-pair and 'buffered' reversed-phase HPLC methods indicated that the latter was the preferred technique for the separation of benzylpenicillin from its degradation products.

Quantitative, specific and stability-indicating HPLC methods were developed and, together with UV and ^{1}H NMR spectroscopy, used to monitor the breakdown of benzylpenicillin and its degradation products in aqueous solution.

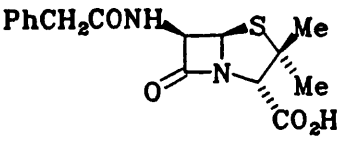
Benzylpenilloic acid 43 was found to epimerise rapidly at C-5 and, in acid, degrade to D-penicillamine 10 and benzylpenilloaldehyde 53. The dominant breakdown product of benzylpenillic acid 42 was found to be (3S,5R,6R)-benzylpenicilloic acid 41A (and to a lesser extent benzylisopenillic acid 56). This is good evidence for benzylpenillic acid 42 possessing (3S,5R,6R) stereochemistry, its absolute configuration not having been reported previously.

Benzylpenicilloic acid 41 in acidic solution was found to exist in complex equilibria with its C-5 and C-6 stereoisomers, benzylpenillic acid 42 and trace amounts of benzylpenamaldic acid 44. The dominant degradation pathways were epimerisation at C-5 and stereoselective decarboxylation to benzylpenilloic acid 43.

Benzylpenicillenic acid 28 was found to be highly unstable in acid solution. The dominant breakdown products were benzylpenillic acid 42, the four possible (3S)-benzylpenicilloic acid stereoisomers 41 and benzylpenamaldic acid 44. The yields were very sensitive to pH.

Benzylpenicillin 5 degraded in acidic solution to benzylpenillic acid 42, (3S,5R,6R)-benzylpenicilloic acid 41A and benzylpenicillenic acid 28. Empirical and kinetic approaches were used for the analysis of the entire degradation sequence.

In basic solution benzylpenicillin 5 was hydrolysed to (3S,5R,6R)-benzylpenicilloic acid 41A and also epimerised at C-6 to 6-epibenzylpenicillin 5A, which in turn was hydrolysed to (3S,5R,6S)-benzylpenicilloic acid 41C. The kinetics governing the system are proposed.



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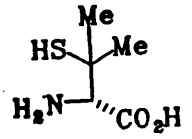

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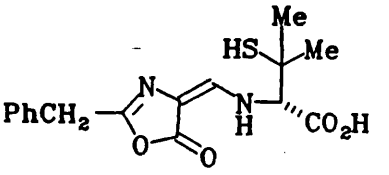

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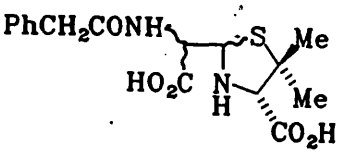

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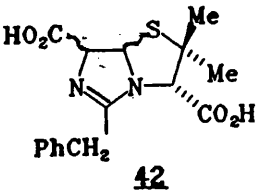

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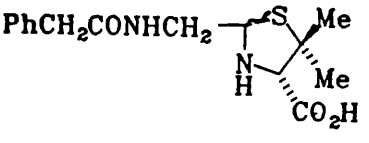

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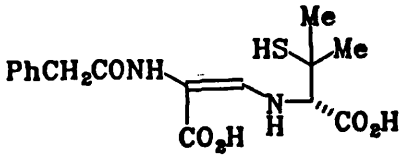

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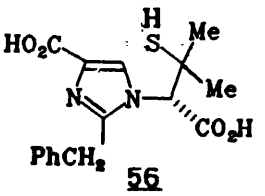
PhCH$_2$CONHCH$_2$CHO

53



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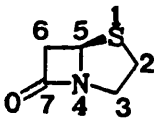
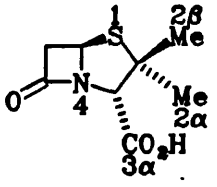
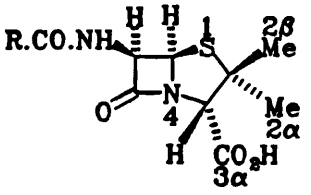
1 NOMENCLATURE

The fused thiazolidine-azetidinone (β-lactam) ring system 1 which comprises the framework of *penicillins* is denoted by the trivial name *penam* or systematically 7-oxo-1-thia-4-azabicyclo[3.2.0]heptane. When this structure bears methyl groups at position 2 and a carboxylic acid at position 3 it is called *penicillanic acid* 2 (2,2-dimethyl-7-oxo-1-thia-4-azabicyclo[3.2.0]heptane-3-carboxylic acid). The methyl group above the plane of the penam ring is termed 2β and the one below 2α.







1 2 3

Penicillin was originally the trivial name given to a mixture of naturally occuring compounds having the structure 3 and differing only in the nature of R at position 6. There are at least six naturally occuring penicillins (Table 1).

Table 1 Naturally occuring penicillins.

Chemical Name	Trivial Name	3 R=	
Pent-2-enylpenicillin	Penicillin I or F	$-CH_2CH{:}CHCH_2CH_3$	4
Benzylpenicillin	Penicillin II or G	$-CH_2C_6H_5$	5
p-Hydroxybenzylpenicillin	Penicillin III or X	$-CH_2C_6H_4OH$	6
n-Heptylpenicillin	Penicillin IV or K	$-(CH_2)_6CH_3$	7
n-Amylpenicillin	Dihydro-F-penicillin	$-(CH_2)_4CH_3$	8
Phenoxymethylpenicillin	Penicillin V	$-CH_2OC_6H_5$	9

There are six possible stereoisomers for penicillin. The absolute configuration for naturally occuring penicillins is 3S,5R,6R referring to the stereochemistry of protons at positions 3, 5 and 6. Therefore naturally-occuring benzylpenicillin **5**, sometimes called benzyl-D-penicillin, is systematically (3S,5R,6R)-2,2-dimethyl-7-oxo-6-[(phenyl acetyl)amino]-1-thia-4-azabicyclo[3.2.0]heptane-3-carboxylic acid.

The author was aware of an alternative ring numbering system for penams, used by Chemical Abstracts, in which priorty is given to the nitrogen atom in the fused ring system and naturally occuring benzylpenicillin is termed [2S-(2α,5α,6β)]-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid. However the aforementioned numbering system was used for the ease of comparison with most of the work published in this field.

In this thesis naturally occuring benzylpenicillin was called benzylpenicillin or (3S,5R,6R)-benzylpenicillin. The absolute configuration of epimeric penicillins was defined and abbreviated in a logical manner e.g. (3S,5R,6S)-benzylpenicillin would be abbreviated to 6-*epi*-benzylpenicillin.

Degradation products derived from benzylpenicillin were termed by the trivial names in common use. To avoid confusion, the penam numbering system was also used for the degradation products retaining the 3-carboxy-2,2-dimethylthiazolidine ring.

2 INTRODUCTION

2.1 The discovery of Penicillin

Penicillin was discovered by Alexander Fleming in 1928.[1] Apocryphally, as the result of accidently sneezing upon an open culture dish sometime in 1922, Fleming made the brilliant and deceptively simple observation...

> "While working with staphylococcus variants a number of culture plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis. Subcultures of this mould were made and experiments conducted with a view to ascertaining something of the properties of the bacteriolytic substance which had evidently been formed in the mould culture and which had diffused into the surrounding medium. It was found that broth in which the mould had been grown at room temperature for one or two weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the common pathogenic bacteria."

Charles Thom at the US Department of Agriculture positively identified Fleming's strain as *Penicillium notatum* (Westling) a relatively rare *Penicillium* belonging to the group of which *Penicillium chrysogenum* is the type species.[2]

The inhibition of one microbe by another was a common occurence. The genius of Fleming was to recognise a new significance i.e. its clinical potential for treating bacterial infections in man. Fleming was encouraged by, and fortunate that a crude extract had no apparent

toxicity when injected into animals. He summarised his findings thus:

"The toxicity to animals of powerful antibacterial mould broth filtrates appears to be very low, Twenty c,c, injected intravenously into a rabbit were not more toxic than the same quantity of broth, Half a c,c, injected intraperitoneally into a mouse weighing about 20 gm, induced no toxic symptoms, Constant irrigation of large infected surfaces in man was not accompanied by any toxic symptoms, while irrigation of the human conjunctiva in a dilution of 1 in 600 does not interfere with leukocyte function to a greater extent than does ordinary broth."

Attempts to concentrate and purify the active antibiotic were hampered by a lack of resources and time, He concluded...

"Penicillin is easily destroyed, and to all intents and purposes we failed, We were bacteriologists - not chemists - and our relatively simple processes were unavailing"

The acidic nature of penicillin was indicated by the studies of Raistrick *et al* [3] who found that very little of the antibiotic activity was extracted into ether from neutral aqueous solution, whereas at pH 2, extraction was almost complete. It was also noted that solutions of penicillin were moderately stable at pH 5 to 6 and normal temperature, but very labile towards acid, alkali, oxidants and heat.

The instability of penicillin continued to frustrate attempts at isolation until Ernest Chain, a biochemist, and Howard Florey, a pathologist, applying both extractive and the then novel chromatographic procedures, prepared a penicillin salt in solid, though impure, form and demonstrated its effectiveness against various pathogens in experimental animals [4] and in man.[5]

The rapid development of the fermentation process for the bulk

production of penicillin owed much to the advances initiated at the Northern Regional Laboratories, Peoria, Illinois, [6] i.e. the replacement of surface-culture of the mould by deep fermentation, the selection of high-yielding mould strains and the use of corn steep liquor in the nutrient media, an ingredient of which, phenylacetic acid, favoured the production of the effective benzylpenicillin.

These incisive studies led to the isolation, chemical characterisation and clinical use of penicillin. Fleming, Chain and Florey shared the Nobel Prize in 1945 "for the discovery of penicillin and its curative effects in various infectious diseases". Partisan arguments about the relative contributions of the three men continue to this day. Excellent accounts of the early history of penicillin have been written by Florey *et al* [7], Sheehan [8] and Sneader.[9]

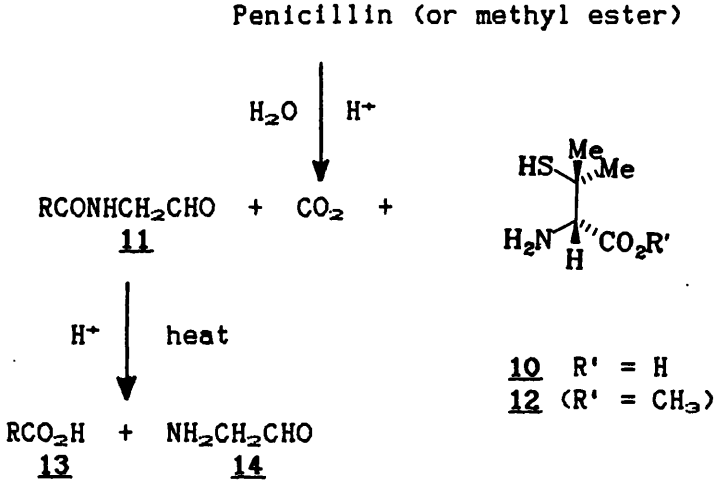
2.2 The structural elucidation of Penicillin.

The chemistry and structure of penicillin was the subject of tremendous scientific effort in the UK and USA (second only perhaps to the "Manhattan Project" - the development of the atomic bomb) and is summarised in a massive monograph.[10] Therein is described the elegant combination of extensive chemical (degradative), physical and physicochemical studies which provided an unequivocal demonstration of the β-lactam-thiazolidine structure of penicillin.

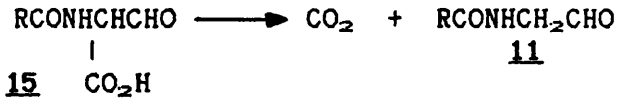
Depending on the strain of mould used and the composition of the fermentation media, various penicillins differing only with respect to a single acyl side chain (R) are produced. The remainder of the molecule is the same in all penicillins.

The penicillins were all found to be strong monocarboxylic acids

without basic properties. Upon hydrolysis by hot dilute inorganic acids; one carbon atom was eliminated as carbon dioxide and two products were obtained in equimolecular amounts, one being an amine, *D-penicillamine* 10, and the other an aldehyde, *penilloaldehyde* 11. All penicillins gave the same amine but different aldehydes, differing in respect to (R). When the methyl ester of penicillin was similarly degraded, the methyl ester of D-penicillamine 12 was formed and hence the carboxy group in penicillamine was the carboxy group of penicillin itself. On vigorous hydrolysis all the penilloaldehydes gave a substituted acetic acid 13 and aminoacetaldehyde 14. Thus the penilloaldehydes were acylated derivatives of aminoacetaldehyde.

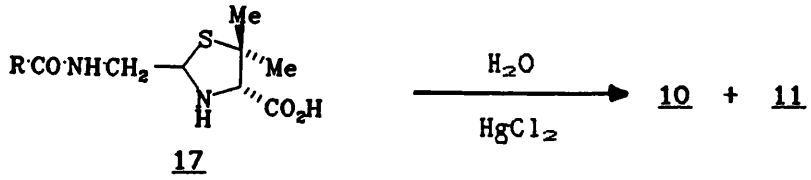


The formation of CO_2 was explained as being due to the ready decarboxylation of an unstable acid e.g. a β-keto-acid, *penaldic acid* 15



The mild alkaline hydrolysis of a penicillin produced a dicarboxylic acid, *penicilloic acid* 16 which readily decarboxylated to form *penilloic acid* 17 . This suggested that a carboxyl group was in the β-position with respect to an electrophillic group.

Penilloic acid, on hydrolysis with aqueous mercuric chloride, gave penicillamine and penilloaldehyde. This was characteristic of compounds containing a thiazolidine ring, e.g.



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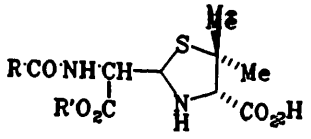
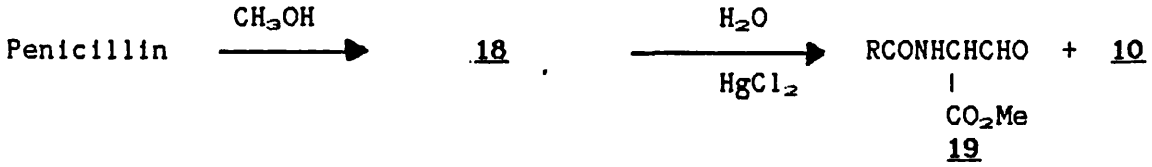
and hence penicilloic acid would be:



16 R' = H
18 R' = CH_3

This structure was supported by the fact that treatment of penicillin with methanol gave *methyl penicilloate* 18 which, on hydrolysis with aqueous mercuric chloride, gave methyl penaldate 19 & D-penicillamine 10.



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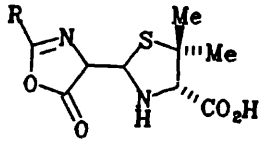
Penicillin was obviously a dehydration product of penicilloic acid 16 and of many possible structures considered, the choice lay between

the oxazolone-thiazolidine structure 20 or the fused β-lactam-thiazolidine structure 3.



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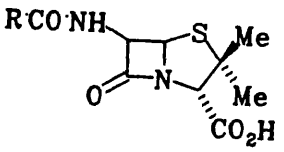
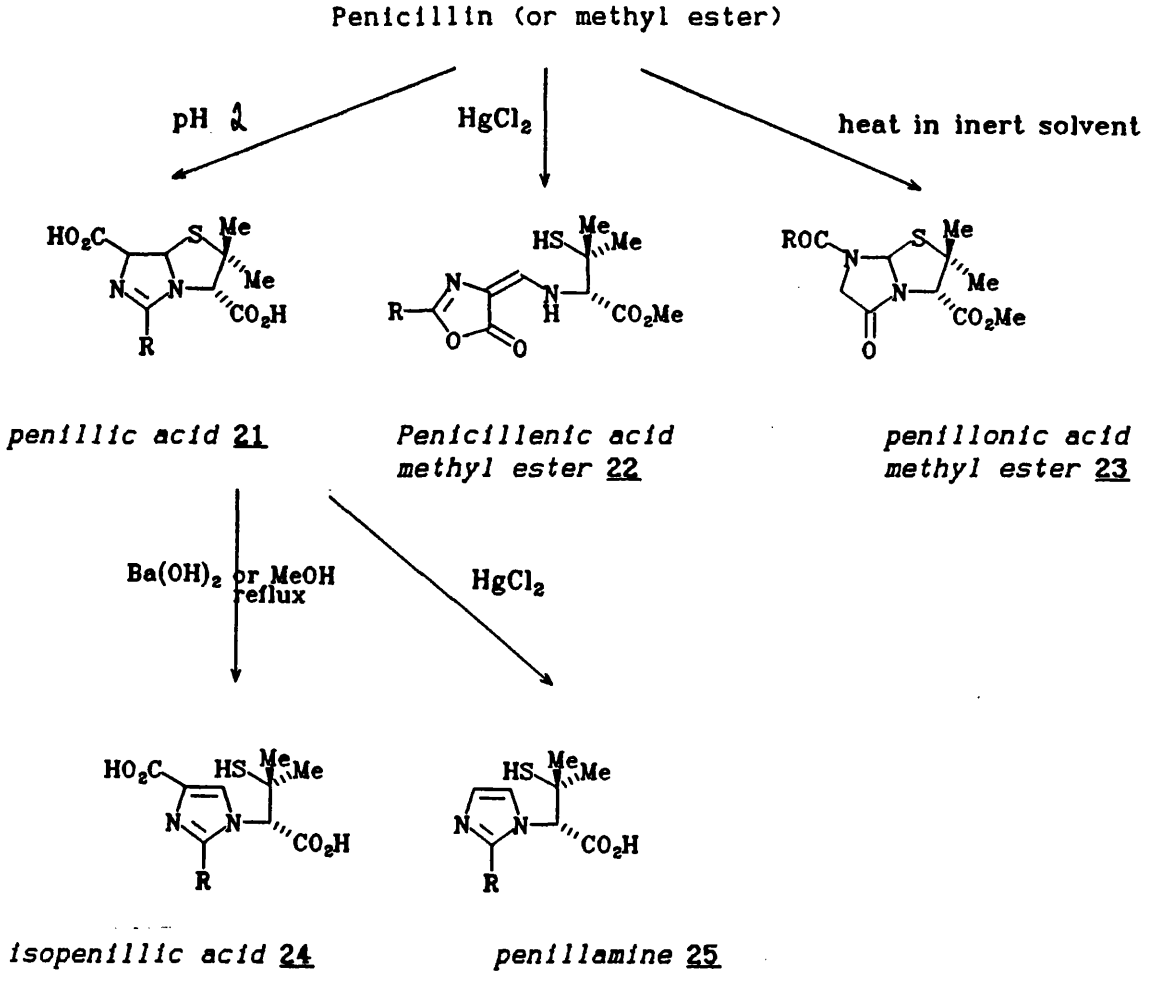
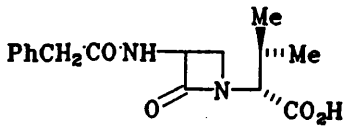


3

In the course of the intensive chemical studies many other degradation/rearrangement products had been identified and are summarised below:



However the definitive chemical experiment by Mozingo *et al* [11] which provided evidence in support of the β-lactam structure, was the desulphurisation of *benzylpenicillin* 5 in hot aqueous solution over fresh Raney nickel to give *desthiobenzylpenicillin* 26 in which the β-lactam ring is preserved intact.



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Conclusive proof of the β-lactam structure was provided by potentiometric titration experiments [12] which showed that penicillin lacked a basic group (and hence penicillin could not have structure 20), by infrared spectroscopy [13] (see below) and finally by X-ray crystallography of penicillin salts [14] by which the structure and stereochemistry were conclusively defined.

The infrared evidence may be illustrated with the methyl ester and sodium salt of benzylpenicillin, which showed the following maxima [15] (characteristic of all the penicillins in these regions)

Methyl ester : 3333 1770 1748 1684 1506 cm^{-1}

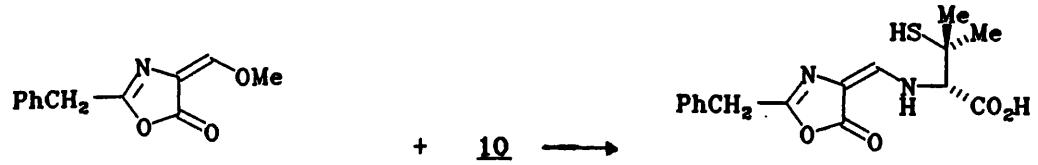
Sodium salt : 3333 1770 1613 1681 1515 cm^{-1}

The band at 3333 cm^{-1} in both compounds was assigned to the NH group (str.) and the 1748 cm^{-1} band of the ester and the 1613 cm^{-1} band of the salt were assigned to the carbonyl function (str.) in the carboxyl group (as ester or salt). The results of studies of simple oxazolones were inconclusive. However studies of simple primary, secondary, and tertiary amides suggested that penicillins had a

secondary amide structure and account for the bands at 1684/1681 cm^{-1} and 1506/1515 cm^{-1}. Finally a number of simple β-lactams and fused (β-lactam-thiazolidines) were studied. The former did not show a band at 1770 cm^{-1}, but all the latter did. This accounted for the fifth band and so it followed that penicillin had the fused thiazolidine-β-lactam structure 3.

2.3 The "synthetic" Penicillins

Attempts at the total chemical synthesis of penicillins actually began before the structure was completely elucidated. Livermore *et al* [16] condensed D-penicillamine 10 with 2-benzyl-4-methoxymethyleneoxazol-5-(4H)-one 27 to yield benzylpenicillenic acid 28 which when heated in pyridine/pyridinium hydrochloride gave traces of antibacterial activity. The mechanism is unclear but 28 may undergo intramolecular rearrangement via the benzyloxazolone-thiazolidine 29 to give traces of benzylpenicillin 5.



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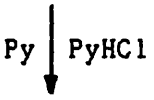
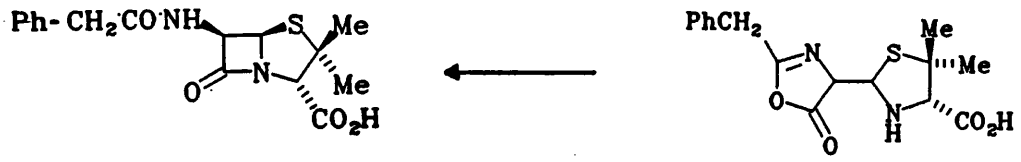
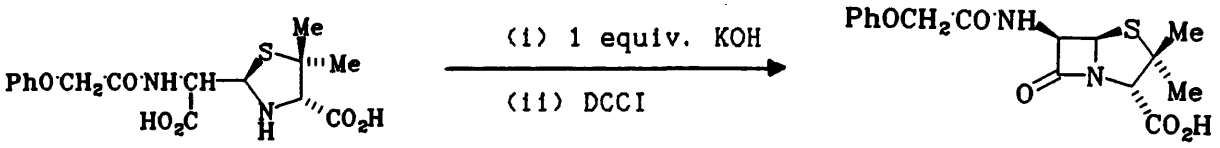




5 29

More rational attempts culminated in the cyclisation of phenoxymethylpenicilloic acid 30 using NN'-dicyclohexylcarbodimide (DCCI) to yield phenoxymethylpenicillin 9 in useful yield. [17,18]



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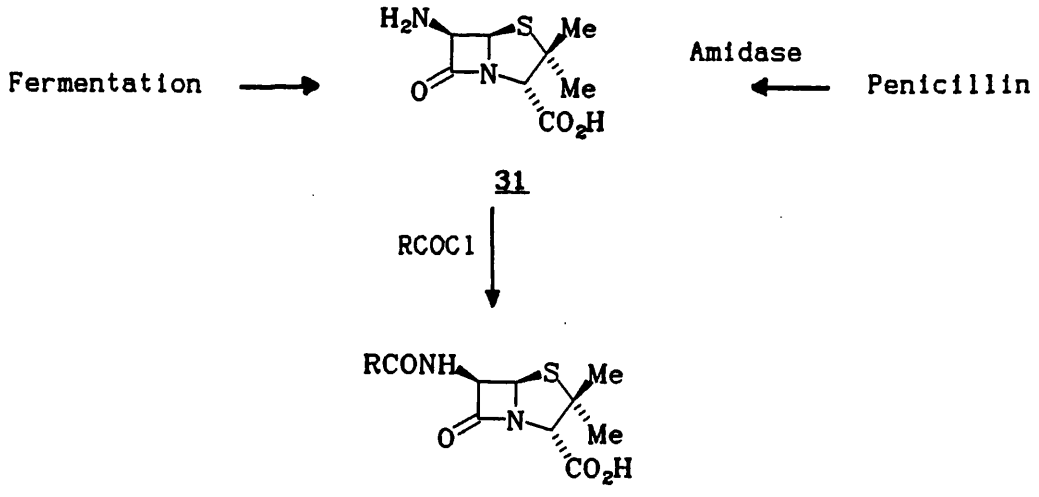
Although of great academic interest all such approaches were overshadowed by the introduction of the key intermediate 6-aminopenicillanic acid 31 made available principally by fermentation [19] but also by enzymatic degradation of penicillins.[20]



31

3

6-Aminopenicillanic acid was now used as the starting point of many new semi-synthetic penicillins (see Table 2) by acylation of the amine

(usually by using the appropriate acid chloride in an anhydrous solvent). One penicillin made thus on a large scale [21] was methicillin 32.

The early semi-synthetic penicillins suffered from one or more of the following limitations in clinical use. They were.....

(1) Only active against Gram-positive bacteria

(2) Acid-unstable and hence could not be taken orally

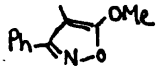
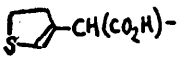
(3) Sensitive to penicillin β-lactamases (an enzyme secreted by some bacteria that cleaves the amide bond in the β-lactam ring to form the penicilloic acid)

(4) Poorly absorbed or highly serum bound.

These limitations were overcome by the development of two important series of broad spectrum penicillins. The first series represented by ampicillin 34 and later amoxycillin 35 (and their associated "pro-drugs" or active esters) were the first orally-active penicillins to become widely used in general practice.

The second series represented by carbenicillin 36 and later by ticarcillin 37 were the first injectable penicillins effective in the treatment of Gram-negative organisms.

Table 2 Some representative semi-synthetic and natural penicillins; their activities and properties.

Trivial name	Chemical name	3 R=		Activity & Properties
Penicillin G	Benzylpenicillin	$PhCH_2$-	5	G(+)-effective acid unstable β-lactamase sensitive
Methicillin	2,6-Dimethoxyphenyl-penicillin	$(MeO)_2C_6H_4$-	32	as benzylpenicillin
Penicillin V	Phenoxymethyl-penicillin	$PhOCH_2$-	9	G(+)-effective, acid stable, well absorbed β-lactamase sensitive
Oxacillin	3-Phenyl-5-methyl-4-isooxazolylpenicillin		33	G(+)-effective β-lactamase resistant highly serum bound orally absorbed
Ampicillin	D-α-(-)-Aminobenzyl-penicillin	[R] $PhCH(NH_2)$-	34	Broad spectrum very acid stable well absorbed β-lactamase sensitive
Amoxycillin	D-α-(-)-Amino-*para*-hydroxybenzyl-penicillin	[R] $HOC_6H_4CH(NH_2)$-	35	as ampi cillin
Carbenicillin	α-carboxy-benzyl-penicillin	[R] $PhCH(CO_2H)$-	36	Broad spectrum and *Ps aeruginosa* effective acid unstable
Ticarcillin	α-carboxy-(3-methyl-thiophene)-penicillin		37	as carbenicillin

2.4 The biological activity of Penicillins

In 1957 Lederberg [22] showed that bacteria ordinarily susceptible to penicillin could be grown in its presence if a hypertonic medium was used. The organisms obtained in this way, called *protoplasts*, are devoid of a cell wall. Thus it was inferred that penicillin interfered with bacterial cell wall synthesis. In 1965, Strominger [23] and Park [24] independently deduced that penicillin blocks the last step in cell-

wall biosynthesis i.e. a transpeptidase reaction, that cross-links different peptidoglycan strands, in which a D-alanine unit is removed from a N-acyl-D-Ala-D-Ala-CO_2H peptide (see Figure 1).

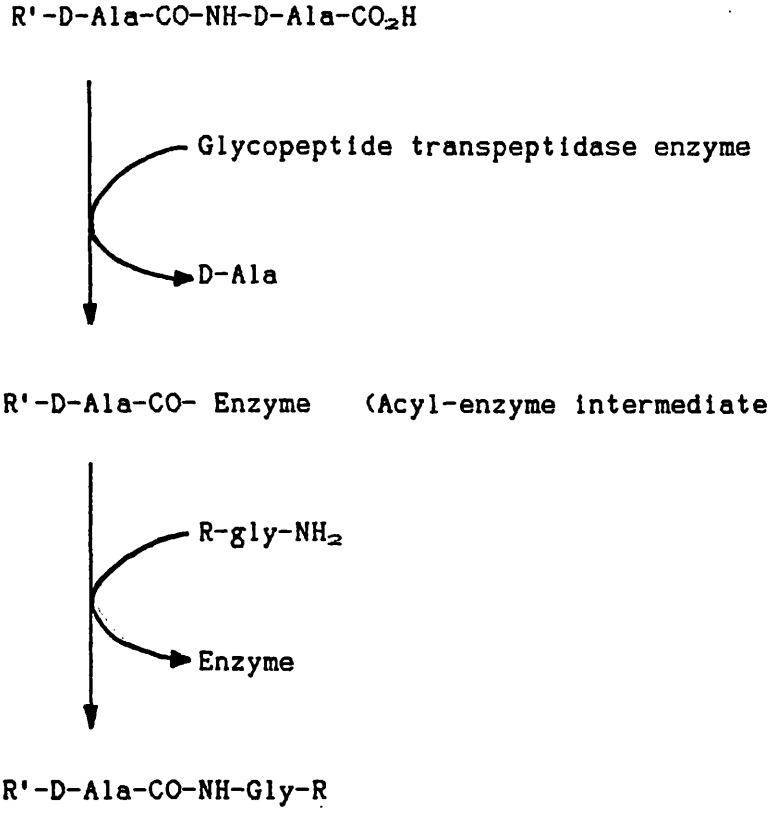


Figure 1

It seems likely that penicillin inhibits the enzyme by forming a covalent bond with a residue at the active site. This inactive penicillinoyl-enzyme complex does not undergo deacylation and hence the enzyme is irreversibly inhibited.

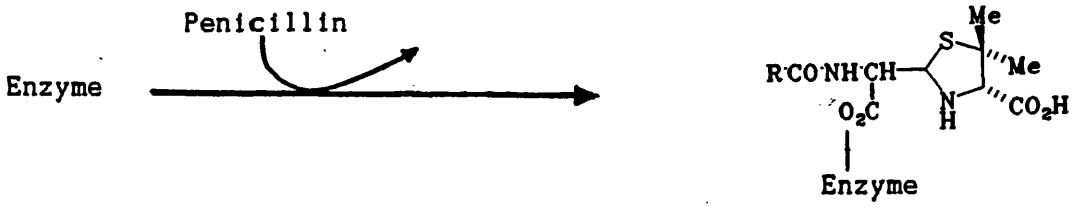
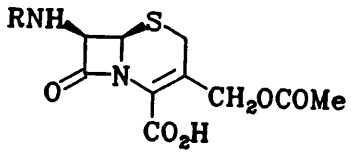


Figure 2

The specificity of the penicillin for the enzyme has been taken to imply the existence of a structural relationship. Molecular models have revealed [23] that penicillin resembles acyl-D-Ala-D-Ala, one of the substrates of this enzyme, and probably has the structure of a transition state of the normal substrate.

2.5 The Cephalosporins.

Following the clinical introduction of the penicillins, Guiseppe Brotzu isolated the mould *Cephalosporium acremonium* from sea-water near a sewerage outflow in Sardinia. Sub-cultures of the mould had a wider spectrum of antibacterial action than had penicillin, being active against Gram-negative and Gram-positive bacteria. Penicillin N was solvent-extracted from the mould and shown [25] to be D-4-amino-4-carboxybutyl-penicillin (3 R = $-(CH_2)_3(NH_2)CH.CO_2H$). One of the other antibiotic compounds produced by this mould was isolated and named Cephalosporin C.[26] The structure was elucidated by Abraham *et al* [27] and shown to be 38.



38 R = $-CO(CH_2)_3(NH_2)CHCO_2H$ 39 R = -H

Cephalosporin C retains the β-lactam but the fused thiazolidine ring, in penicillins, is replaced by the fused dihydrothiazine ring. It was found to be resistant against penicillinases. The structural similarity between the two β-lactam classes of antibiotics and the fact that both are produced in the cephalosporin fermentation suggest

that there might be a common intermediate in the biosynthetic pathways. One possibility is that cephalosporin C is a metabolic transformation product of penicillin N. Morin *et al* [28] have synthetically transformed phenoxymethylpenicllin sulphoxide into the analogous cephalosporin.

When subjected to mild acid hydrolysis, the side chain of 38 is removed to give 7-aminocephalosporanic acid 39 and in a similar fashion described for 6-aminopenicillanic acid 31, semi-synthetic cephalosporins are readily available.

So far only two types of β-lactam antibiotics have been found in nature, the penicillins and cephalosporins. The latest edition of Martindale's Extra Pharmacopoeia lists 43 penicillins and 18 cephalosporins in clinical use, together with several in the supplement of very recent introduction. Over the last forty years they have made a major contribution towards the control and treatment of infectious diseases worldwide.

A discussion of the biosynthesis of β-lactam antibiotics was not considered necessary to this present work. The chemistry of the degradation of cephalosporins is sufficiently dissimilar to that of the penicillins, so that further discussion is beyond the scope of this thesis. These subjects have been adequately reviewed.[29,30,31]

For recent reviews of the advances into the chemistry and biology of the β-lactam antibiotics, the reader is directed to reference 32.

2.6 The mechanisms and products of degradation of benzylpenicillin in aqueous solution.

The majority of the bonds in the fused thiazolidine-β-lactam ring system of penicillin are potentially activated for cleavage and, in many cases, the activation energies for bond-cleavage are similar. Consequently, small changes in structure or in experimental conditions can dramatically change the course of reaction. Indeed the penicillins have been described [33] as "diabolic concatenations of reactive groupings". The rearrangements of penicillanic acid derivatives have been recently reviewed.[34] The remainder of this introduction will be devoted to the degradation of benzylpenicillin in aqueous solution.

In aqueous solution, the penicillins are not only degraded by acid and base, but also by metal ions, penicillinase enzymes, oxidising agents, alcohols, amines and a wide range of other nucleophiles and electrophiles.[10,31] In what follows only inactivation processes caused by water and its ions are considered in detail.

The degradation of benzylpenicillin has been found to be first-order with respect to benzylpenicillin in acidic, neutral and basic aqueous solutions.[35,36] The overall rate of degradation depends on hydrogen ion concentration, $[H^+]$. In alkaline solution, the rate is inversely proportional to $[H^+]$ at constant salt concentration.[36] In acid solution, the rate increases with increasing $[H^+]$ but there is no strict linear relationship. A curvature in the pH-rate profile is observed at ca. pH 2·8.[36] This corresponds to the pK_A of benzylpenicillin (2·73 ± 0·05) [37] and implies that the free acid and the anion are inactivated at different rates. The pH-rate profile has a minimum at ca. pH 6·5 [36] and therefore to obtain optimum stability of

penicillin solutions it is necessary to buffer at this pH.

There are many reports in the literature of the effect of buffers on the rate of degradation. It has been found [38] that dihydrogen citrate, monohydrogen phosphate and borate are catalytic with respect to benzylpenicillinate , while acetic acid catalyses the degradation of undissociated benzyl-penicillin. There is a positive salt effect in weakly alkaline solution.[38] Recently it has been shown [39] that micellar, i.e. > 0.25M, benzylpenicillin is 2.5 times as stable as non-micellar solutions under the conditions of constant pH and ionic strength, over the pH range 5.0 - 9.5.

The overall rate of degradation of benzylpenicillin in aqueous solutions is inversely proportional to temperature and found [36] to obey the Arrhenius expression.

2.6.1. Acid hydrolysis of benzylpenicillin

The instability of benzylpenicillin in acidic aqueous solution is well known [10] and there have been many studies of its degradation for two main reasons. First, an understanding of the mechanism of degradation would help in the design of orally-active penicillins, stable in the acidic conditions prevailing in the stomach.[40] Second, allergic responses to penicillin are thought to be due to the formation of complexes between proteins and penicillin degradation products. These subjects have been reviewed.[31,41]

The biological activity and chemical reactivity of the penicillins has been attributed to the β-lactam ring, an unique feature rarely found in nature. In penicillins the nitrogen atom in the fused ring system is 0.4Å out of the plane defined by its substituents.[42] The

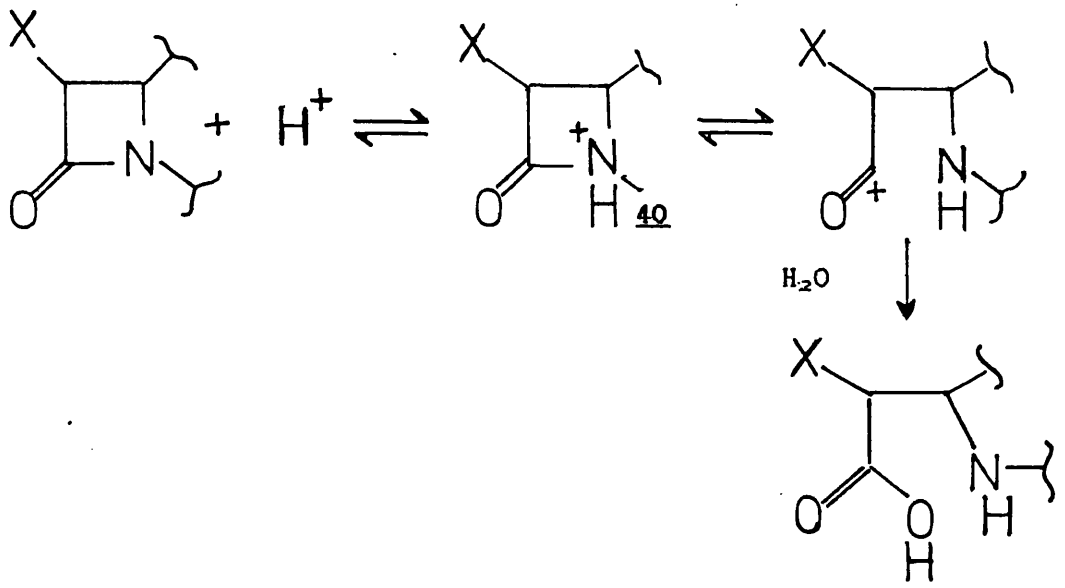
ease of nucleophilic attack upon the penicillin β-lactam carbonyl is usually ascribed to a reduction in the usual amide resonance, due to the non-planarity of the ring system,[12] or to a relief of ring strain upon opening the β-lactam ring [43] although evidence to support these concepts is ambiguous.

Amides are less reactive toward nucleophiles than are ketones because of the resonance stabilisation energy resulting from delocalisation of the lone pair of electrons on nitrogen over the carbonyl group. It is estimated [44] that amide resonance is ca. 18 Kcal mol^{-1} and therefore a reaction which involved the loss of this resonance could proceed up to 10^{13}-fold faster than the analogous stabilised system.

The strain energy of a four-membered ring is 26-29 Kcal mol^{-1} [45] and therefore a reaction involving ring-opening could occur up to ca. 10^{20}-fold faster than the analogous acyclic system. Therefore the total possible rate enhancement of a resonance-inhibited β-lactam, as proposed for penicillin, is ca. 10^{33}-fold. It has been shown [46] that simple β-lactam hydrolyse ca. 100-fold faster than the amide/anilide analogues and that fusing the β-lactam ring to make a bicyclic [3.2.0] system increases the rate of hydroxide-ion catalysis by another 100-fold for an amine of given basicity. Although this is a significant rate enhancement it is well below that predicted for the release of strain in opening a four-membered ring or from an amide resonance-inhibited system.

If amide resonance in penicillins is inhibited, the β-lactam nitrogen should be more basic than the normal amide nitrogen. It has been demonstrated [47] that in acidic solution N-protonation takes place

but the nitrogen is insufficiently basic for complete or partial conversion to the N-protonated amide. In addition, N-protonation is not the result of reduced amide resonance but is simply an intrinsic property of the β-lactam ring.[47] A uni-molecular mechanism for acid-catalysed hydrolysis of the β-lactam ring with protonation occuring on nitrogen has been proposed [47], see Scheme 1.



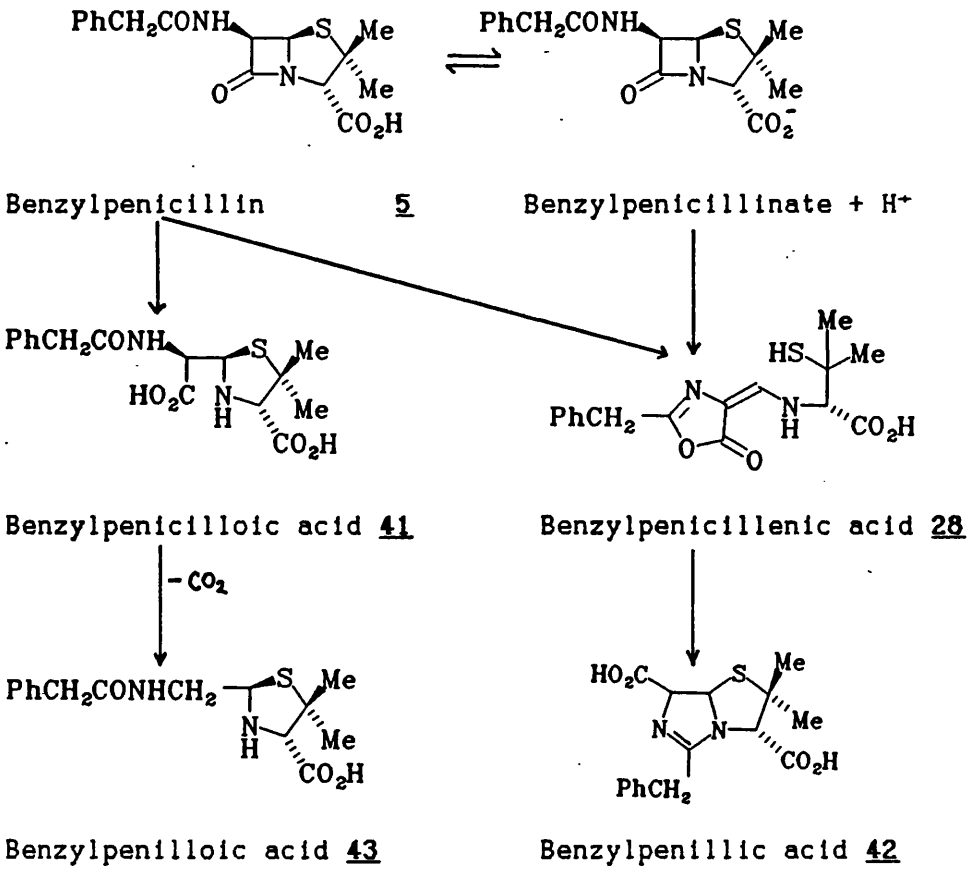
Scheme 1

i.e. the intermediate acylium ion **40** is trapped by water. By analogy, benzylpenicillin should give by such a mechanism benzylpenicilloic acid **41**. However, it is well known [10] that the major degradation product of benzylpenicillin, in acid solution, is benzylpenillic acid **42**. In addition, benzylpenicillenic acid **28**, detected by its intense u.v. absorption at 322 nm, was observed in degradation studies.[48,12]

Later it was proposed [49] that benzylpenicilloic acid, which in turn readily decarboxylates in acidic solution to yield benzylpenilloic acid **43** , are also important degradation products.

Krejci [50] determined rate constants for both the overall rate of degradation of benzylpenicillin and the rate of formation of benzyl-

penicillenic acid in acidic solution using polarography. The overall rate constants were resolved by Schwartz [51] into rate constants for; (1) acid catalysed hydrolysis of undissociated benzylpenicillin to form benzylpenilloic acid via the decarboxylation of benzylpenicilloic acid. (2) the rearrangement of benzylpenicillin anion following attack by proton or (3) the kinetically equivalent uncatalysed reaction of undissociated benzylpenicillin to form benzylpenicillenic acid, which then rearranges to benzylpenillic acid. (see Scheme 2)

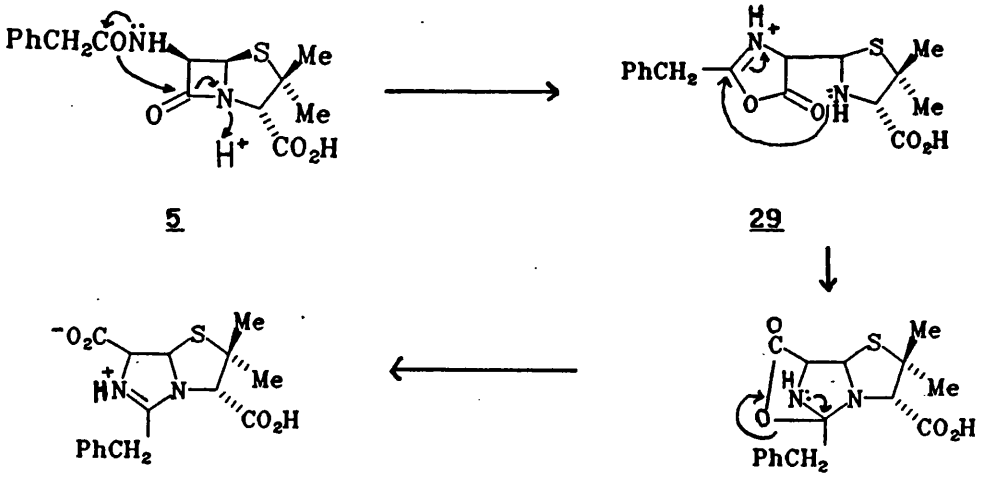


Scheme 2 [51]

Krecji [50] originally proposed that benzylpenillic acid formed from the acid catalysed reaction of undissociated benzylpenicillin, however

Schwartz contended [51] that if this was so, the yield of benzylpenillic acid would not show a maximum as it does at pH 2 - 3.[49] The similarity of the mechanisms proposed [12] for the formation of benzylpenicillenic and benzylpenillic acids was cited [51] as additional support.

As the first step, Woodward *et al* [12] proposed an attack by the amide carbonyl oxygen to produce the oxazolone-thiazolidine intermediate **29**, first suggested as the possible structure of benzylpenicillin (see section 2.2). Followed by attack of the thiazolidine nitrogen to give a new intermediate which via a simple electron shift gives benzylpenillic acid (here drawn as the zwitterion).



5 **29** **42**

Alternatively the oxazolone-thiazolidine intermediate would be expected to isomerise readily to benzylpenicillenic acid.

The conversion of benzylpenicillenic acid to benzylpenillic acid is supported by the observation [16] that when benzylpenicillenic acid is allowed to stand in 95% ethanol at room temperature for one hour, a 25% yield of benzylpenillic acid is obtained. Further evidence for the presence of the oxazolone-thiazolidine intermediate in the

isomerisation of benzylpenicillin is provided by kinetic studies [52] of the acid degradation of benzylpenicillin and on theoretical grounds.[53]

Bundgaard [54] studied the degradation of benzylpenicillin and its methyl ester and of their transformations into the corresponding benzypenicillenic acid and methyl ester respectively, in acidic aqueous solution. As before [36] a curvature in the overall rate of degradation of benzylpenicillin-pH profile was noted at ca. pH 2-3. The shape of the pH-rate profile was accounted for by specific acid-catalysed reactions of penicillin free acid (BP) and of ionised penicillin (BP^-) :

$$BP + H^+ \xrightarrow{k_H} \text{products} \qquad (1)$$

$$BP^- + H^+ \xrightarrow{k'_H} \text{products} \qquad (2)$$

or the kinetically equivalent reaction to (2) - spontaneous or water catalysed degradation of the free acid:

$$BP \xrightarrow{k_o} \text{products} \qquad (3)$$

Other investigators favoured reaction (3) in view of the lack of appreciable ionic strength effect on the rate of degradation of benzylpenicillin in the pH 4-6 region [38 55] and because of an observed solvent isotope effect (K^{H_2O}/K^{D_2O}) of 1.5 at pH 6.[56] Assuming that reactions (1) and (2) predominate, the overall rate of benzyl-penicillin degradation K was described [54] by the following expression:

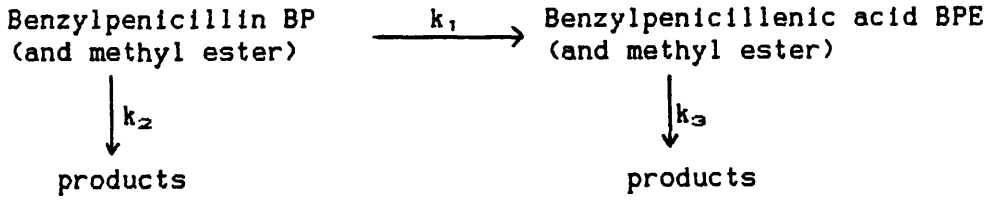
$$K[BP]_T = k_H a_H [BP] + k_o [BP] \qquad [1]$$

Where $[BP]_T$ represents total penicillin concentration ($[BP]+[BP^-]$) and a_H is the hydrogen ion activity. k_H and k_o, the rate constants for specific acid-catalysed and spontaneous degradation respectively were

calculated [54] to be 5.8 M^{-1} min^{-1} and 0.042 min^{-1} respectively.

The pH-rate profile for the overall degradation of the methyl ester shows a straight line and k_H = 5.2 M^{-1} min^{-1}; k_o = 0.002 min^{-1}.[54] As expected, k_H for the free acid and methyl ester are similar. However the large difference between the values of K_o casts doubt on the preference of reaction (3) over reaction (2). The possibility of intramolecular participation of the free carboxy group may be discounted [47] because of the similarity of the rate constants for the degradation of benzylpenicillin and its methyl ester.

It was also demonstrated [54] that the rate of formation of benzylpenicillenic acid (and the methyl ester) showed a first-order dependency on $[H^+]$ and became independent of pH at higher acidities. The experimental data fitted Scheme 3 which assumes that the degradation of benzylpenicillin (and the methyl ester) occurs by one or more simultaneous pseudo-first-order processes.



Scheme 3 [54]

In Scheme 3, benzylpenicillin and benzylpenicillenic acid have a time dependence given by the following expressions:

$$[BP]_t = [BP]_o \, e^{-Kt} \qquad [2]$$

$$[BPE]_t = \frac{k_1 [BP]_o}{K - k_3} (e^{-k_3 t} - e^{-Kt}) \qquad [3]$$

Where $[BP]_o$ is the initial molar concentration of benzylpenicillin, $[BP]_t$ and $[BPE]_t$ represent the molar concetrations of benzylpenicillin

and benzylpenicillenic acid, respectively, at time t. K is the overall rate of degradation of benzylpenicillin and is equal to $k_1 + k_2$. k_2 may represent one or more degradation processes.

By adopting experimental conditions in which benzylpenicillenic acid is stabilised while k_1 and k_2 remain unaffected, the reaction represented by k_3 may be neglected and equation [3] is simplified to:

$$[BPE]_t = \frac{k_1 [BP]_o}{K} (1 - e^{-Kt}) \qquad [4]$$

Further manipulation of equation [4] enabled values of k_1 and K to be determined. The percentage of benzylpenicillin converted to penicillenic acid is given by:

$$\% \text{ BPE} = k_1/K \times 100 \qquad [5]$$

The % formation of benzylpenicillenic acid (and ester) as a function of pH [54] is given in Table 3 (included in which is the earlier data of Krecji [50] & Schwartz [51]).

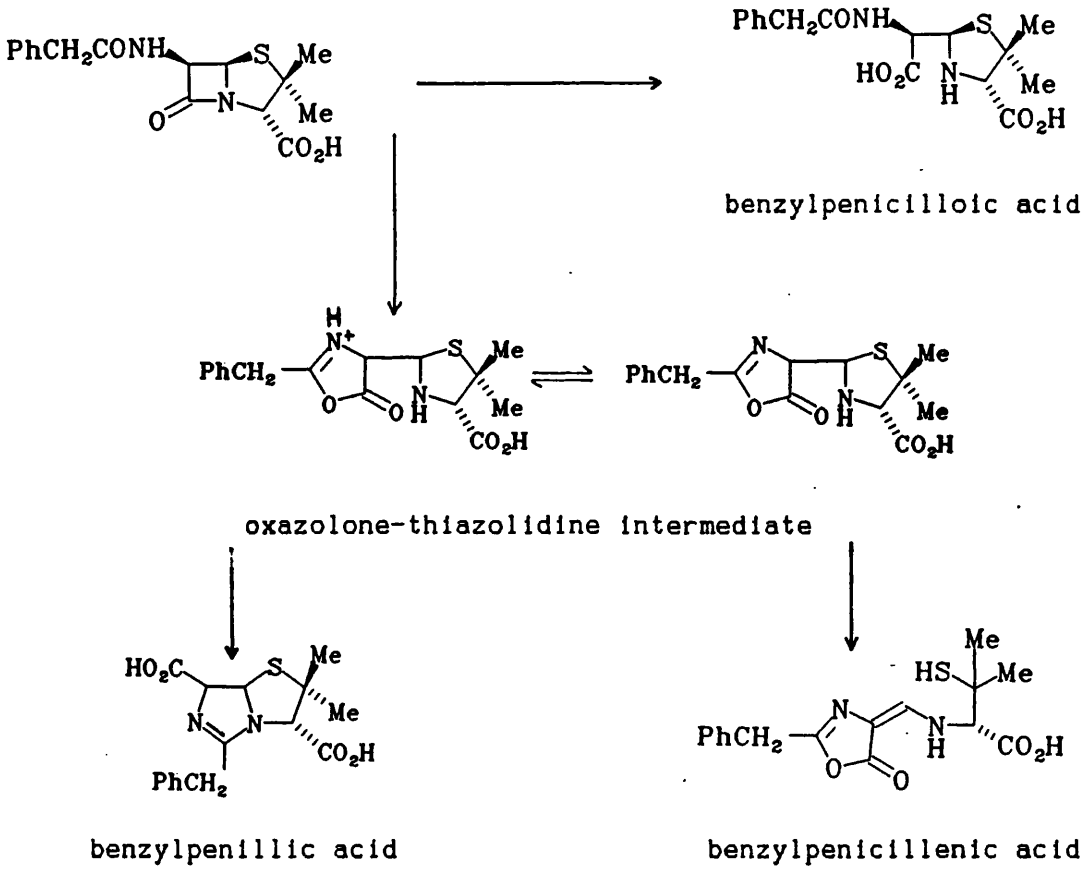
Table 3 Percentage formation of benzylpenicillenic acid (and ester) as a function of pH

	pH	1,28	1,32	1,47	1,52	1,60	1,82	1,85	2,03	2,10	2,27
% BPE (acid)[54]		nd	-	-	17,6	-	-	30,0	-	43,8	56,4
% BPE (acid)[50,51]		-	15,4	18,4	-	19,1	25,2	-	41,3	-	-
% BPE (ester)[54]		5,8	-	-	10,6	-	-	19,7	-	27,9	37,0
	pH	2,28	2,46	2,49	2,72	2,76	2,85	3,04	3,16	3,32	4,02
% BPE (acid)[54]		-	62,5	-	65,5	-	69,6	66,7	75,8	76,3	72,7
% BPE (acid)[50,51]		42,0	-	44,9	-	43,7	-				
% BPE (ester)[54]		-	43,3	-	47,3	-	nd	50,9	nd	47,2	nd

Note; ref.54, temperature 30°C; ref.50 & 51, temperature 25°C

The log k_1-pH profiles for benzylpenicillin and its methyl ester are similar in behaviour in contrast to the K - pH profiles. This indicates that similar mechanisms operate in the rearrangement of benzylpenicillin and its ester to the corresponding penicillenates.

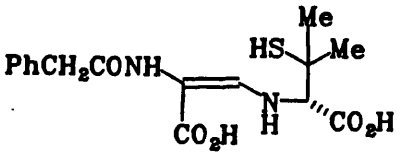
Kinetic evaluation of the data led to the proposed [54] scheme 4 which involves the presence of the oxazolone-thiazolidine structure as a meta-stable intermediate capable of only transitory existence.[12]



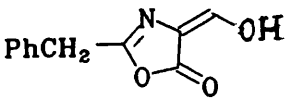
Scheme 4 [54]

There is still considerable uncertainty about the details of the degradation of benzylpenicillin and the inter-relationships between the various degradation products.

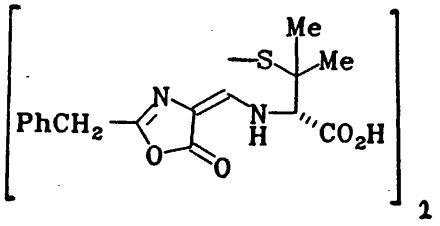
Various studies indicate that benzylpenicillenic acid is highly unstable. Under a variety of reaction conditions benzylpenicillenic acid has been shown to rapidly hydrolyse not only to benzylpenillic acid [16] but also to benzylpenamaldic acid **44** [12,31], benzylpenicilloic acids [57] and 2-benzyl-4-hydroxymethyleneoxazol-5(4H)-one **45**.[14] In addition benzylpenicillenic acid is oxidised to the more stable disulphide **46** [57] which has a similar uv spectrum.



44 (enamine tautomer)

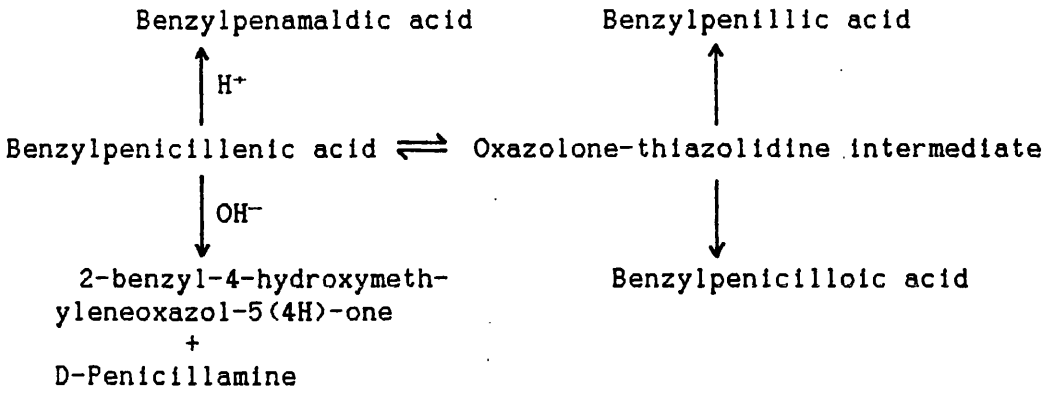


45



46

Benzylpenicillenic acid shows a complex rate of hydrolysis - pH profile. Over the pH range 0.5 - 14, six changes in acidity dependence were observed [58] and analysed in terms of the dissociation constants and relative reactivities of the tautomeric species present. A reaction scheme (scheme 5) was proposed.



Scheme 5. Scheme for the hydrolysis of benzylpenicillenic acid [58]

The half-life (t½) of benzylpenicillenic acid was estimated from data in reference 58 and together with the determined yields of the four degradation products, given in Table 4.

Table 4 Variation of t½ and the percentage product yields from benzylpenicillenic acid as a function of pH.[58]

pH	Penamaldic acid	Penillic acid	Penicilloic acid	2B4HMO +DPAM	t½ @ 25°C minutes
0	94 ± 5				-
1.5	83 ± 5	17 ± 5			0.7
2.0	56 ± 5	44 ± 5			2.2
2.9	26 ± 5	74 ± 5			6.9
3.9		66 ± 5	34 ± 7		21.9
6.0			100 ± 10*		43.7
11.2			96 ± 8		2.2
13.0			87 ± 5	13 ± 5	0.7
14.0			2 ± 5	77 ± 5	0.1

* = corrected for benzylpenicillenic acid disuphide formation.
2B4MO = 2-Benzyl-4-hydroxymethyleneoxazol-5(4H)-one
DPAM = D-Penicillamine

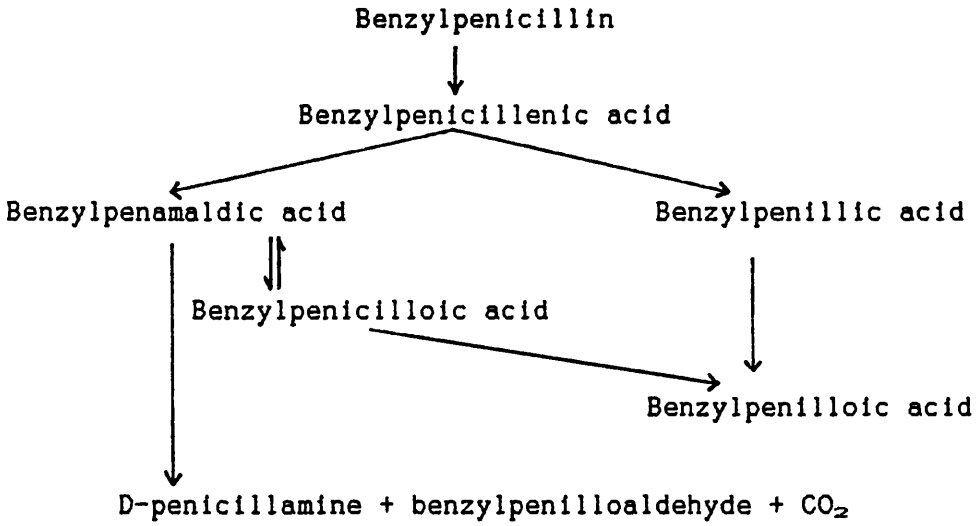
In highly acid solution the over-riding reaction is hydrolysis of the oxazolone ring to yield benzylpenamaldic acid. As the pH rises the amount of benzylpenillic acid increases. The oxazolone-thiazolidine intermediate is produced by intramolecular nucleophilic attack of the

thiol group. This conclusion is supported by the observation that the disulphide, which cannot undergo such a reaction, is much more stable and has properties similar to other oxazolones studied.[59]

The oxazolone-thiazolidine intermediate may then rearrange to benzylpenillic acid or the oxazolone ring is hydrolysed to produce benzylpenicilloic acid. At neutral pH, the latter reaction predominates together with disulphide formation. In alkaline solution hydrolysis of the enamine bond in benzylpenicillenic acid results in the formation of 2-benzyl-4-hydroxymethyleneoxazol-5(4H)-one and D-penicillamine.

Blaha [60] studied the degradation of benzylpenicillin at pH 2.7 by HPLC. The effects of temperature, buffer concentration and ionic strength were examined. A degradation scheme was proposed (Scheme 6) based solely upon the order of appearance of the degradation products.

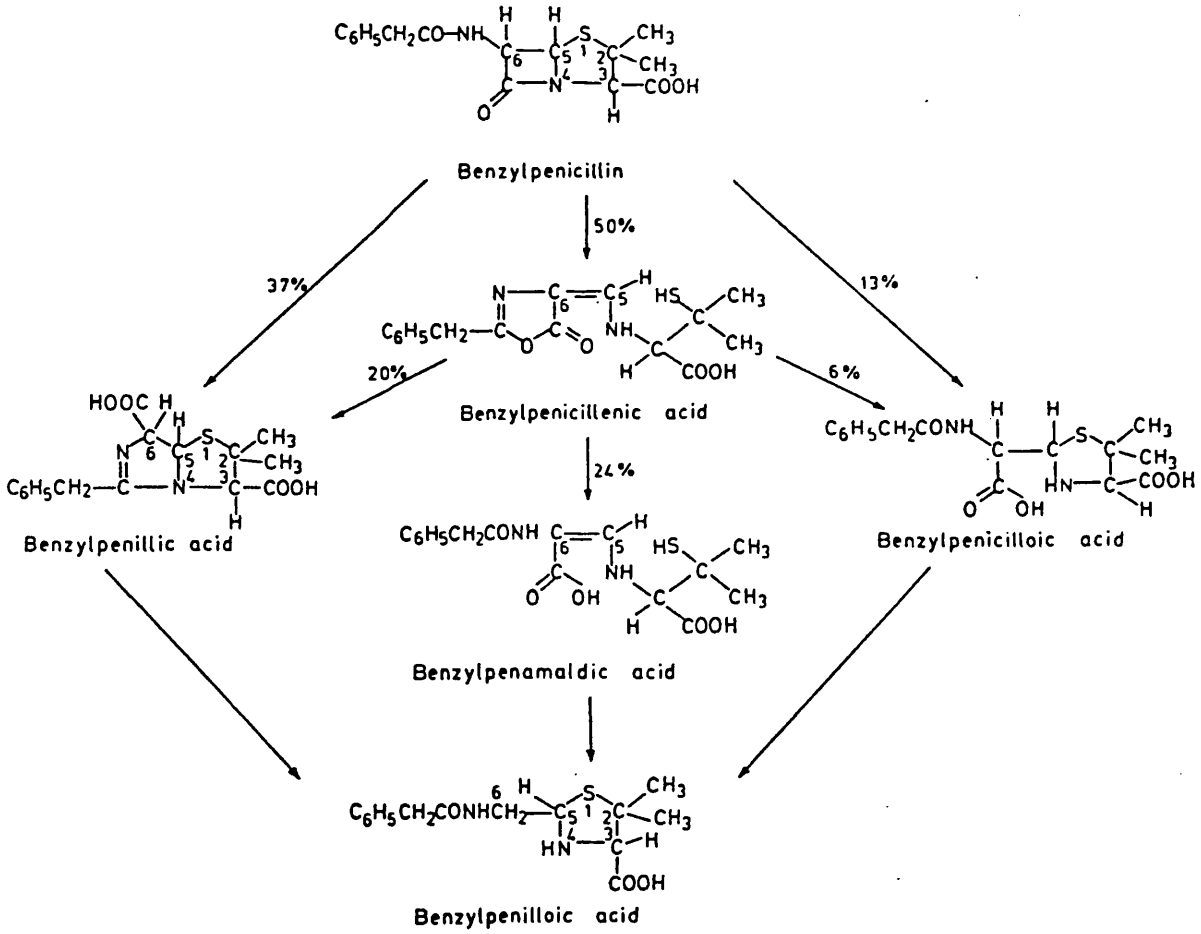
Scheme 6 Proposed degradation scheme for benzylpenicillin in acidic solution [60]



Surprisingly, benzylpenicilloic acid was not detected in this study and this was attributed to its existence in equilibrium with

benzylpenamaldic acid and its rapid decarboxylation to produce benzylpenilloic acid. The proportions of benzylpenicillenic acid reacting to produce benzylpenillic and benzylpenamaldic acids varied slightly with changes in experimental conditions, the mean ratio being ca. 2.5 : 1.

^{1}H NMR studies [61] of the degradation of benzylpenicillin at pH 2.5 and 37°C showed that while benzylpenicillenic acid is an intermediate in the formation of some benzylpenillic and benzylpenicilloic acids, it is not on their major formation pathways.



Scheme 7. Proposed scheme for the degradation of benzylpenicillin at pH 2.5 and 37°C.[61]

The modified degradation scheme (Scheme 7) was proposed.[61] In the early stages of reaction, NMR signals were detected from benzylpenillic, penicilloic and penamaldic acids. When all the penicillin had degraded, the relative amounts of the 3 products were 57%, 19% and 24%, respectively. Although not detected by NMR, due to its low concentration, the prescence of benzylpenicillenic acid in the early stages of degradation was confirmed by its uv absorption at 322nm.

After 1 day benzylpenilloic acid was observed, mainly from the decarboxylation of penicilloic acid, and also from the slower degradation of penillic and penamaldic acids. The 5R,6R diastereoisomer of penicilloic acid was formed first but after 100 minutes a mixture of the 5R,6R and 5S,6R epimers was observed. Benzylpenilloic acid was observed as a similar mixture of epimers but neither D-penicillamine nor benzylpenilloaldehyde were detected.

The exact location of benzylpenicillenic acid in the degradation scheme was defined in the following manner. Benzylpenicillenic acid has a double bond between C-5 and C-6 and no hydrogen at C-6. As the degradation was carried out in DCl-D_2O, products possessing a single bond between C-5 and C-6 and which have been formed from benzylpenicillenic acid will bear deuterium at C-6. This deuterium was detected by examining the H-5 and H-6 resonances of the products. For instance, in the spectra of benzylpenillic and penicilloic acids one observes well-resolved doublets for H-5 and H-6. If the compounds were deuterated at C-6, the H-6 doublet would be absent and the H-5 doublet collapse to a broad singlet (small triplet splitting $J_{HD} = 0.15 \times J_{HH}$) see Figure 3.

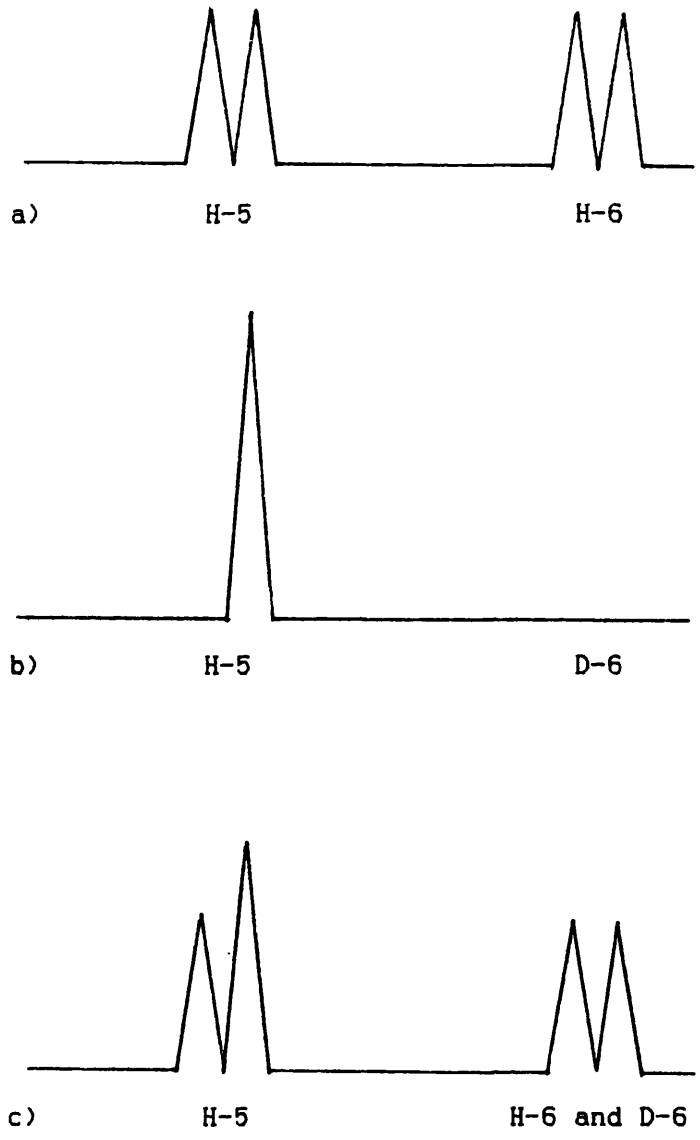


Figure 3 Illustration of the effect upon the nmr resonances of H-5 and H-6 by partial deuteration at C-6.

Examination of the spectra of benzylpenillic acid and the benzylpenicilloic acid epimers revealed that, in addition to the H-5 and H-6 doublets, there were additional singlet resonances superimposed on the

high field component of the H-5 doublets. The intensity of the H-6 doublets were substantially less than the intensity of the H-5 doublets. Both observations support deuteration at C-6 and no deuteration at C-5.

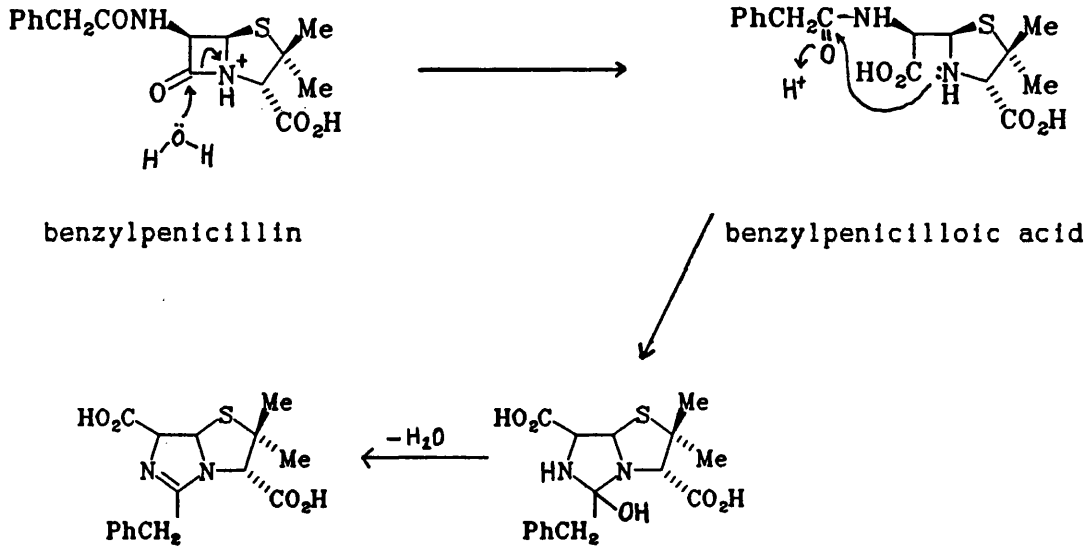
Hence by measurement of the relative intensities of the H-5 and H-6 resonances (for benzylpenillic acid) or the relative intensities of the singlets and doublets of the H-5 resonance (for benzylpenicilloic acids) it was possible to estimate the proportion deuterated at C-6. This was found to be 35% for penillic acid and 30% for the penicilloic acids. Therefore 35% of the 57% (20%) of penillic acid and 30% of the 19% (6%) of penicilloic acids had an intermediate related to benzylpenicillenic acid on their formation pathway. The remainders being formed directly from benzylpenicillin (see scheme 7).

Similar procedures applied to the spectra of the benzylpenilloic acids confirmed these results and consequently indicated that neither penillic nor penicilloic acids degrade to penilloic acid via a penicillenic acid-type intermediate. No evidence was found that penicilloic and penillic acids are in reversible equilibrium with each other. As penicilloic acid degrades to penilloic acid much more rapidly than does penillic acid it remains uncertain whether penillic acid degrades directly to penilloic acid or via penicilloic acid. No detailed mechanisms were described to support this proposed degradation scheme.

An examination [62] of the fit of the theoretical predictions to the experimental data revealed that the concentrations predicted by Scheme 7 did not coincide with the n.m.r. data [61] for benzylpenicillin or any of the degradation products.

In addition the presence of D-penicillamine in aged acidic solution of benzylpenicillin was confirmed [62] by HPLC and differential pulse polarography. These workers [63] found it necessary to substantially change the proportion of benzylpenicillin degrading through benzylpenicillenic, benzylpenillic and benzylpenicilloic acids in order for Scheme 7 to fit the n.m.r. data.

The rate constants best describing the n.m.r. data have benzylpenillic acid as the major degradation product of benzylpenicillin and benzylpenicillenic acid degrading exclusively via benzylpenamaldic acid [63]. Mechanistic considerations indicate that the direct conversion of benzylpenicillin to benzylpenillic acid is not possible and that a likely intermediate is benzylpenicilloic acid. This hypothesis was supported by the observation that the degradation of benzylpenicilloic acid at pH 2.5 occurs with the formation of benzylpenillic acid [63] i.e.



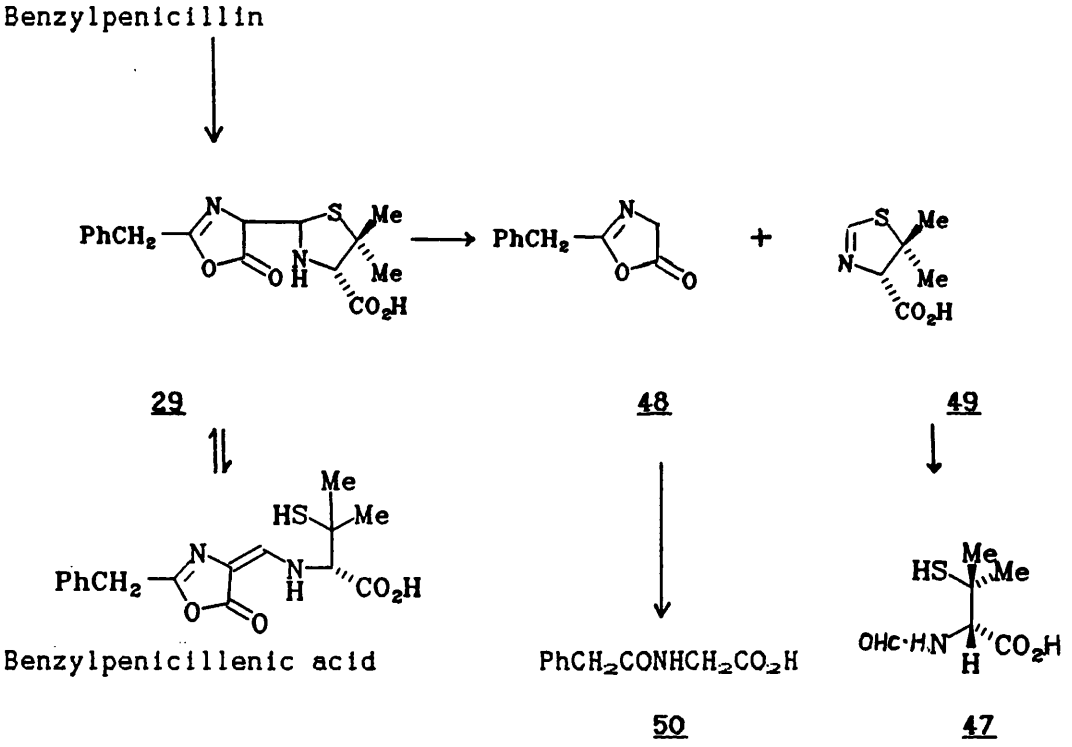
benzylpenicillin

benzylpenicilloic acid

benzylpenillic acid

The proposed mechanism [63] involves initially, acid-catalysed hydrolysis of the β-lactam bond to produce benzylpenicilloic acid, followed by nucleophillic attack by the thiazolidine nitrogen on the amide carbonyl to form the fused ring system and finally loss of water to yield benzylpenillic acid e.g

Recently it has been shown [64] that N-formylpenicillamine **47** is produced from benzylpenicillin (and other penicillins) at pH values between 2.5 and 7.0 with a maximum yield of 30% at pH 5 (see Scheme 8)



Scheme 8 [64]

N-formylpenicillamine is formed by the acid degradation of benzylpenicillenic acid [64,65] and has been observed [66] in the acid degradation of methicillin (this was attributed to degradation of the penicillenic acid derived from methicillin).

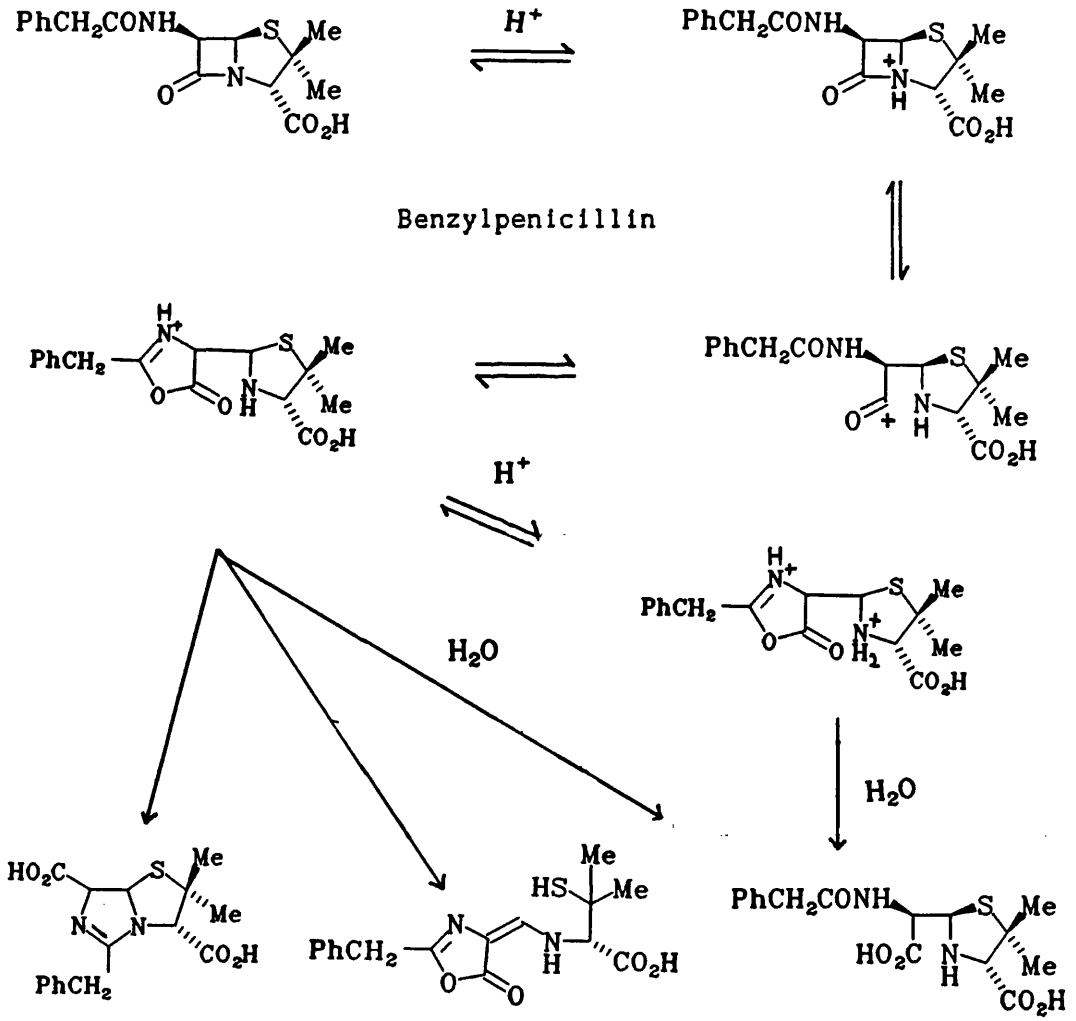
Both 48 and 49 are known to undergo rapid hydrolysis in aqueous solution to give respectively N-phenylacetylglycine (phenaceturic acid) 50 [53] and N-formyl-D-penicillamine [67] and hence the oxazolone-thiazolidine 29 is a likely intermediate in the formation of N-formyl-D-penicillamine.

D-penicillamine was also detected [64] in acid degraded samples of benzylpenicillin, but to a far lesser extent (e.g. 0.8% at pH 2.5).[61]

It has been shown [47] that the overall rate of acid hydrolysis of benzylpenicillin is ca. 10^3-fold faster than that for penicillanic acid 2. This rate enhancement indicates neighbouring group participation by the acylamido ($PhCH_2CONH-$) side chain. Although the exact mechanism remains unclear, the oxazolone-thiazolidine metastable intermediate has been suggested [47] as being formed by the acylium ion 40 (see Scheme 1), being trapped by the intramolecular group, rather than by water.

The reason why benzylpenicillenic acid is not formed significantly below pH 1 and is the major product at higher pH [50,51,52] was explained [47] in the following manner. The protonated oxazolone nitrogen (in 29) has a pK_a of ca. 0 [58]. There is no obvious reason why protonation of the imine should inhibit elimination across C5-C6 to give benzylpenicillenic acid. The pK_a of the protonated thiazolidine nitrogen is ca. 3 [68]. Protonation of this nitrogen would inhibit formation of both penicillenic and penillic acids. Therefore the kinetically important ionisation has been attributed [47] to thiazolidine nitrogen. On the basis of these conclusions Scheme 9 was proposed [47] for the degradation of benzylpenicillin in acidic aqueous solution.

i.e. Hydrolysis of benzylpenicillin in acid solution occurs via the N-protonated β-lactam and then, depending on the ionisation state, the oxazolone-thiazolidine intermediate can react with water to give benzylpenicilloic acid, undergo intramolecular nucleophilic attack of the thiazolidine nitrogen on the carbon of the protonated oxazolone to give benzylpenicillenic acid or eliminate across C-5 - C-6 to give benzylpenicillenic acid. The inter-relationship between the various degradation products remains ambiguous.



Scheme 9 [47]

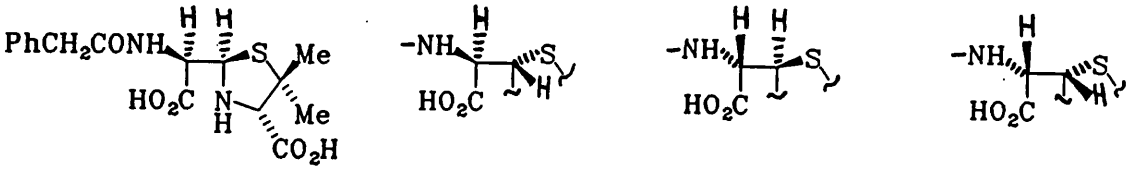
2.6.2 Base hydrolysis of benzylpenicillin

It was shown [56] that the rate of inactivation of benzylpenicillin in aqueous alkaline solution is first-order with respect to penicillin concentration and is inversely proportional to $[H^+]$ or proportional to $[OH^-]$, at constant salt concentration and temperature. These two possibilities involve either a negative catalytic effect (inhibition) of H^+ or more likely, a positive catalytic effect of OH^-. An observed [56] deuterium solvent isotope effect (K^{H_2O}/K^{D_2O}) of 0.59 for the hydroxide-ion-catalysed degradation of benzylpenicillin in the pH (pD) range of 7 - 10 indicates that the mechanism is of nucleophilic attack of OH^- on the β-lactam ring.

It has been shown [47,71] that penicillins are ca. 100-fold more reactive to base hydrolysis than β-lactams of amines of similar basicity and that the rate-limiting step is the formation of a tetrahedral intermediate. The magnitude of the rate-enhancement is much lower than that predicted for a system in which amide resonance is inhibited or from the release in strain in opening a four-membered ring. There is no evidence that the 3-carboxylate group facilitates the hydrolysis and electron withdrawing groups at C-6 increase the rate by a purely inductive effect.[47]

Early in the investigation of the structure of benzylpenicillin it was found [69] that the alkali-inactivation product was benzylpenicilloic acid **41**. Since benzylpenicilloic acid has three asymmetric centres (at C-3, C-5 and C-6), 8 stereoisomers are possible. Since it was shown [10] that the absolute configuration of penicillamine is D, (3S-) and since the degradation of penicillin does

not change the configuration at this point, it is possible to know the absolute configuration of this centre at all times. Therefore there are only 4 possible stereoisomers of 'natural' D-benzylpenicilloic acids i.e.



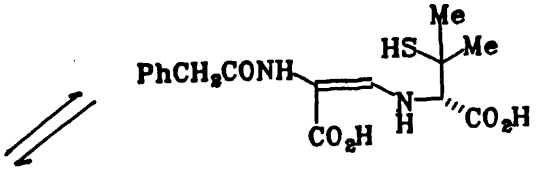
41A (3S,5R,6R) **41B** (3S,5S,6R) **41C** (3S,5R,6S) **41D** (3S,5S,6S)

Indeed, four benzylpenicilloates were obtained and designated [69] by the letters α, β, γ, and δ. Their configuration, except that of the α-isomer which corresponds to the natural penicillin (3S,5R,6R), was unknown. The configuration of the four dimethyl D-benzylpenicilloates was determined [70] using a series of transformations and physicochemical techniques. The compounds designated β, γ and δ isomers of "Chemistry of Penicillin" were found to have the 5S,6R, 5R,6S and 5S,6S configuration respectively.

Benzylpenicilloic acid has been reported [31,69,72] to exist as a mixture of isomers in alkaline and neutral aqueous solutions. It has been observed that the solutions of 5R,6R-benzylpenicilloic acid undergo a mutarotation [69,70] parallelled by a decrease in the pK of the protonated thiazolidine nitrogen from 5.3 to 4.7 [37]. This has been attributed [37] to the transformation of benzylpenicilloic acid to benzylpenamaldic acid (enamine tautomer **44A**). Other workers [31] have proposed that the two compounds exist in equilibrium. This transformation seems unlikely as no strong absorption at 280 nm,

characteristic of penamaldate, was observed [70]. When a solution of (3S,5R,6R)-benzylpenicilloic acid was kept until a constant $[\alpha]_D$ was obtained, ^1H NMR analysis revealed that all four isomers were present but the 5S,6R-isomer was the main component (ca. 70%) [70].

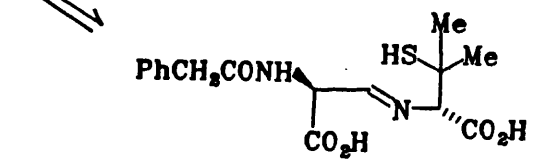
Ghebre-Sellassie *et al* [73] used a combination of HPLC, polarography, uv spectroscopy and ^1H NMR to separate the (3S,5R,6R)- and (3S,5S,6R)-benzylpenicilloic acid epimers, monitor the epimerisation process and examine the epimerisation mechanism. It was found [73] that (3S,5R,6R)-benzylpenicilloic acid, in aqueous solution at room temperature, gradually epimerises to the 5S,6R epimer. At equilibrium the 5S,6R-epimer was the favoured product (80% by ^1H NMR, 83% by HPLC). Based on mechanistic considerations, two pathways were proposed [73] for the epimerisation process.



(enamine tautomer **44A**)

5R,6R-benzyl-penicilloic acid **41A**

5S,6R-benzyl-penicilloic acid **41B**



(imine tautomer **44B**)

benzylpenamaldic acid **44**

The imine tautomer **44B** was suggested [73] as the probable intermediate on the basis of the following observations;

(1) Lack of strong absorption in the 280 nm region of the uv spectrum

(as mentioned previously).

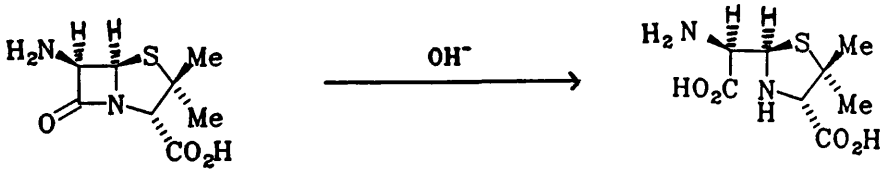
(2) The epimerisation process proceeds without deuterium incorporation and/or exchange at C-6 when carried out in D_2O solution.

(3) No epimerisation at C-6 was noted.

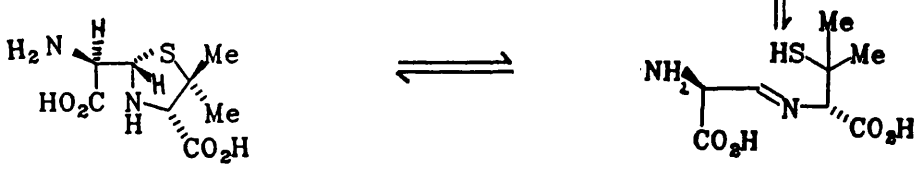
(4) The presence of a C=N group was confirmed by differential pulse polarography (the enamine intermediate does not contain this group).

Kinetic evaluation [73] of the ^1H NMR data gave the forward rate constant k_f as 0.074 hr^{-1} and the reverse rate constant k_r as 0.018 hr^{-1}. Other workers [74] have shown that benzylpenicilloic acid epimerises at C-5 via the intermediate iminium ion formed by unimolecular ring opening, whereas the C-7 methyl ester of benzylpenicilloic acid ring opens to give the enamine intermediate by a base-catalysed elimination process involving C-6-H.

Also in general agreement are the observations of Carroll *et al* [75] who found that the base hydrolysis of 6-aminopenicillanic acid yields first the expected (5R,6R)-penicic acid **51A** which epimerises at C-5, via an imine intermediate **52**, to form the thermodynamically stable (5S,6R)-penicic acid epimer **51B**.



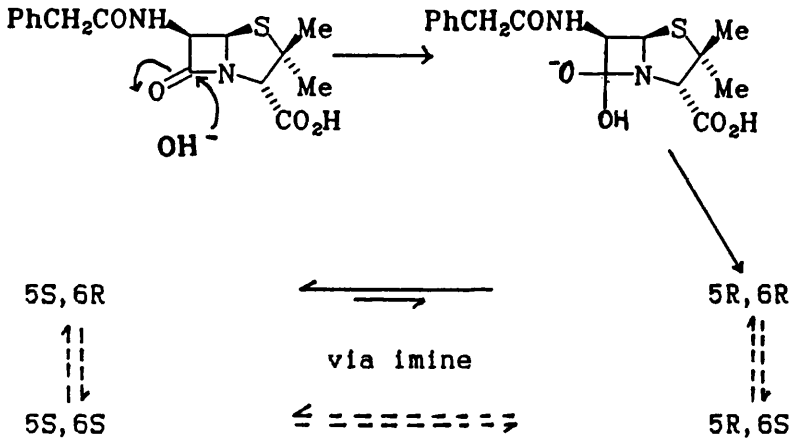
6-Aminopenicillanic acid **51A**



51B **52**

Recently the HPLC separation of the 4 stereoisomers of 'natural' benzylpenicilloic acid and the two stereoisomers of 'natural' benzylpenilloic acid has been reported.[76] Studies of the epimerisation of benzylpenicilloic acid concluded that epimerisation at C-5 was the major feature but it is apparent from the reported data that epimerisation at C-6 is also possible, i.e. when any of the benzylpenicilloic acid epimers were left in basic solution, small amounts of the 6-*epi* compounds were observed. The authors did not attach any significance to these results.

All the observations discussed may be summarised in a scheme (scheme 10) for hydroxyl-ion-catalysed hydrolysis of benzylpenicillin.



benzylpenicilloic acid epimers

Scheme 10

i.e. alkaline hydrolysis proceeds via the rate-limiting formation of a tetrahedral intermediate [47] followed by cleavage of the β-lactam bond to produce (5R,6R)-benzylpenicilloic acid which in turn epimerises at C-5, via an imine intermediate, to give the 5S,6R epimer. The possibility of epimerisation at C-6 is included in the scheme.

3.OBJECTS AND AIMS OF THE THESIS.

The degradation of benzylpenicillin 5 in acidic aqueous solutions is complex and incompletely understood. The degradation mechanisms and breakdown schemes so far proposed, do not fully describe experimental observations.

In particular, the role of the benzyloxazolone-thiazolidine 29 and benzylpenicillenic acid 28 intermediates, the relationship between benzylpenicilloic acid 41 and benzylpenillic acid 42 (the stereochemistry of which has not been reported), and other inconsistencies highlighted in the Introduction require clarification.

In basic aqueous solution the pres ence of the 6-*epi*-benzylpenicilloic acids may be envisaged as arising from the epimerisation at position 6 of benzylpenicilloic acid or intact benzylpenicillin or from both processes simultaneously. Neither process has been reported in the literature to date.

Therefore the aim of this thesis is to obtain or prepare the known degradation products of benzylpenicillin and to develop and optimise quantitative HPLC and NMR analytical methods capable of studying the decomposition of benzylpenicillin and its degradation products in acidic and basic aqueous solutions.

Using such methods, the nature of the reaction products and the kinetics (i.e. the rate, stoichiometry and order) of the individual degradation steps can be determined.

If these objectives are met, a kinetic study of the entire degradation process will be attempted and reaction mechanisms, consistent with the experimental results, tentatively proposed.

4 SYNTHESIS OF BENZYLPENICILLIN DEGRADATION PRODUCTS AND RELATED COMPOUNDS.

4.1 Introduction

The following compounds were synthesised by standard methods or modifications thereof. The original reference is given in the relevant section. The other compounds used in these studies were made available with the permission of the original researchers or were obtained from commercial sources.

4.2 Experimental

Melting points were determined on a Gallenkamp electrically heated melting point apparatus and are uncorrected. Kieselgel 60 F_{254} (silica gel) plates were used for t.l.c. and were visualised using u.v. light or an iodine vapour chamber. Solvent evaporation under reduced pressure was carried out using a Buchi Rotavapour R110 rotary evaporator. Lyophilisation was carried out using an Edwards Modulyo freeze-drier operating at ca. -40°C/3 mbar.

^{1}H and ^{13}C NMR were recorded on Jeol JNM PMX60 S1, FX 90Q and JNM-GX-270 instruments (full operating details are given in Section 6) using ca. 5% w/v D_2O and DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate) as internal standard at δ 0.00ppm. Unless otherwise stated assignments were established by comparison with published data and/or on the basis of expected chemical shift, signal multiplicity and the use of DEPT and INEPT ^{13}C NMR pulse sequences.

4.3 Compounds prepared

4.3.1 Benzylpenillic acid 42

Benzylpenicillin sodium 5 (5.0g, 0.014 Moles) was dissolved in double distilled water (1000ml) and the solution was adjusted to pH 2.5 by the addition of 1M HCl and allowed to stand at room temperature for three days. The resulting white crystalline precipitate was isolated by filtration, washed with cold water and dried *in vacuo* over P_2O_5 giving benzylpenillic acid 42 (1.9g, 41%), m.p. 176-177°C (lit.,[77] 188-189°C).

See Section 6.3.5 for ^1H and ^{13}C NMR data.

4.3.2 Disodium (3S,5R,6R)- 41A and (3S,5S,6R)-benzylpenicilloates. 41B

Following the procedures of Knevel *et al* [73] benzylpenicillin sodium 5 (8.27g, 0.023moles) and (3.57g, 0.010moles) gave disodium (3S,5R,6R)-benzylpenicilloate 41A (10.0g, crude yield 110%, m.p. 195-198°C dec.) and disodium (3S,5S,6R)-benzylpenicilloate 41B (4.09g, crude yield 103%, m.p. 195-198°C dec.) respectively.

See Section 6.3.4 for ^1H and ^{13}C NMR data.

4.3.3 (3S,5R)- 43A and (3S,5S)-Benzylpenilloic acids 43B

Following the procedure of Woroniecki *et al* [78] benzylpenicillin sodium 5 (2.0g, 0.0056 moles) was dissolved in cold double-distilled water (10ml) and the solution was adjusted to ca. pH 13 by the addition of 10M NaOH (0.5ml). After two hours ethanol (10ml) was added and the solution warmed to 75°C. The solution was adjusted to, and mantained at, pH 4 by the addition of 5M HCl (total volume 3ml).

After 40 minutes effervescence ceased and the solution was rapidly cooled in ice-water. Approximately half the ethanol was removed under reduced pressure and the solution was left overnight at 2°C. The product was isolated as the free acid by filtration and dried *in vacuo* over P_2O_5 (1.19g, 69%). The sample was recrystallised from water/ethanol, 50/50 v/v (0.80g, 46%). T.l.c., 8:1:1 EtOAc-H_2O-AcH, 2 spots; major Rf 0.42, minor Rf 0.37, m.p. 93-95°C.

See Section 6.3.8 for ^1H and ^{13}C NMR data.

4.3.4 Benzylpenilloaldehyde 53

Following the published procedures of Cook [79] the following modifications are noted:

a) Synthesis of Phenylacetylaminoacetaldehyde diethylacetal **54**

Freshly distilled phenylacetylchloride (b.p. 90°C/9mm Hg) (13.9g, 0.090 moles) and aminoacetaldehyde diethylacetal (10g, 0.075 moles) gave phenylacetylaminoacetaldehyde diethylacetal **54** (17.3g, 92%) as a low melting point solid. 16.3g of the sample was transferred to a three-bulb Kugelrohr apparatus and the product was distilled at ca. 140°C 10.2 mm Hg. Yield 16.2g, m.p. 35°C (lit.[79], 36.4 - 37.7°C corr.)

60 MHz ^1H NMR ($CDCl_3$, TMS) : δppm 7.20 (5H, s, C_6H_5), 5.53 (1H, broad s, NH), 4.35 (1H, t, J = 6 Hz, CH), 3.51 (2H, s, Ph-CH_2), 3.45 (4H, dt, J = 7 Hz and ca. $2H_2$, $2CH_2$-Me), 3.35 (2H, d, J = 6 Hz, CH_2) and 1.10 (6H, t, J = 7 Hz, 2 CH_3)

22.5 MHz ^{13}C NMR ($CDCl_3$, TMS) : δppm 171.0 (CO, amide), 135.0, 129.4, 128.9 and 127.3 (phenyl), 100.7 (CH), 62.8 (CH_2, ethyl), 43.8 and 42.0 (2 × CH_2) and 15.2 (CH_3, ethyl)

b) Acid hydrolysis of Phenylacetylaminoacetaldehyde diethylacetal 54

The acetal (5g, 0.02 moles) gave benzylpenilloaldehyde hemihydrate, after drying (1.17g) m.p. 92-95°C. Dehydration and recrystallisation from chloroform/petroleum ether 40-60 gave benzylpenilloaldehyde 53 (0.57g, 16%) m.p. 106-107°C (lit. [79], 111-114°C).

See section 6.3.14 for ^{1}H and ^{13}C NMR data.

4.3.5 Dimethylbenzylpenillate 55

Benzylpenillic acid 42 (51.3mg, 1.5×10^{-4} moles) was suspended in diethylether (1ml) at 0°C. Freshly prepared ethereal diazomethane was added until evolution of nitrogen ceased, yellow colour persisted and the sample had completely dissolved. After allowing to warm to room temperature the ether was removed by gentle evaporation (crude yield 66mg). The sample was recrystallised from cyclohexane/petroleum ether 60-80 to yield dimethylbenzylpenillate 55, Principally one spot by t.l.c. 1:1 CH_2Cl_2-EtOAc v/v, Rf = 0.70, m.p. 104°C.

See Section 6.3.6 for ^{1}H and ^{13}C NMR data.

4.3.6 Benzylisopenillic acid 56

Following the published procedure,[80] benzylpenillic acid 55 (50mg, 1.5×10^{-4} moles) was refluxed in methanol (30ml) for 22 hours. The reaction was monitored by t.l.c. 8:1:1 EtOAc-H_2O-AcH (Benzylpenillic acid Rf ca. 0, benzylisopenillic acid Rf 0.13). The solution was filtered and methanol removed by distillation under reduced pressure. The residue was recrystallised from EtOH-H_2O 50:50 v/v, yielding benzylisopenillic acid 56 (20mg, 40%) containing ca. 10% benzylpenillic acid 55. See Section 6.3.7 for ^{1}H and ^{13}C NMR data.

4.3.7 (3S,5R,6R)-Benzylpenicillin-1S-sulphoxide 57

Adapting the method of Morin *et al* [28] sodium metaperiodate (1.5g, 0.0070 moles) was added in one portion with stirring to a solution of benzylpenicillin sodium (2.5g, 0.0070 moles) in H_2O (50ml) at room temperature. After 1 hour the starch-iodide test became negative, the solution was filtered and immediately lyophilised. The crude product (~ 8% benzylpenicillin by HPLC) was used without further purification. See Section 6.3.3 for ^1H and ^{13}C NMR data.

4.3.8 Sodium 2-benzyl-4-hydroxymethylene-5(4H)-oxazolone 45

NaOH (3M, 0.30ml) was added to benzylpenicillenic acid 28 (72.5mg, 2.17×10^{-4} moles) and the solution left standing at 2°C for four days. The precipitate was suspended in ethylacetate (3ml), filtered and washed with ethylacetate (2 × 1ml), diethylether (1ml) and dried *in vacuo* over silica gel, yielding a white crystalline solid (22mg, crude yield 45%). The material was used without further purification.

4.3.9 3S-Benzylpenamaldic acid 44

Benzylpenicillenic acid 28 (5.5mg) was dissolved in absolute ethanol (500µl) and added to HCl (0.01M, 25ml) at 37°C. After twenty minutes the solution was neutralised by the addition of NaOH (0.2M), frozen and immediately lyophilised. After drying the sample was redissolved in D_2O (1ml) and analysed by HPLC and ^1H NMR (see Section 6.3.9.)

4.4 Other materials and reagents used.

(3S,5R,6R)-benzylpenicillin sodium **5** was generously donated by Glaxo Group Research Limited, Greenford, Middlesex. A 50g sample of Batch URH 0003 8755 sampled on 19.12.1983 was received 17.3.1984. The certificate of analysis accompanying the sample is reproduced (see Appendix I). All chromatographic and spectroscopic studies indicate that the sample is of high purity and it was assumed to be 100% pure (a slight loss of weight, 0.2%, was noted on drying at 105°C). The sample was stored cold and dry *in vacuo* and no degradation was noted throughout the studies.

(3S,5R,6S)-benzylpenicillin potassium **5A** was a kind gift from Professor H. Vanderhaeghe, Katholieke Universiteit Leuven, Belgium. It was prepared by the base-catalysed epimerisation of the trimethylsilyl derivative of benzylpenicillin benzyl ester in the presence of DBN.[81]

(5R/S)-aminomethyl-2,2-dimethylthiazolidine-3S-carboxylic acids **58A/B** were prepared by Susan Jacobs, University of Bath.[82] 8-hxdroxypenillic acid **59** and (3S,5R/S)-penicic acids **60A/B** were prepared by Justin Speight, University of Bath.[83]

(3S,5R,6R)-6-aminopenicillanic acid **31**, benzylpenicillenic acid **28**, N-formyl-D-(-)-penicillamine **47**, D-(-)-penicillamine **10**, D-(-)-penicillamine disulphide **61**, N-phenylacetylglycine **50**, phenylacetic acid **62** and hippuric acid **63** were obtained from commercial sources and were all used without further purification.

All other chemicals used were of analytical-reagent grade.

5 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (RPHPLC) ANALYSIS OF BENZYLPENICILLIN AND ITS ACID/BASE DEGRADATION PRODUCTS

5.1 Introduction

A wide variety of physical, chemical and microbiological methods have been utilised for the assay of penicillins. The chemical and physical methods may be sub-divided, arbitrarily, into titrimetric, spectroscopic (uv-visible, infrared, nuclear magnetic resonance and mass specrometry), calorimetric, fluorometric, polarimetric (optical rotary dispersion and circular dichroism), electrophoretic and chromatographic (TLC, LC and HPLC, GLC) methods of analysis. The analytical methods used in the early days of penicillin research and development have been reviewed [84] and more recently by Hughes *et al* [85] [86] and Fairbrother.[87] [88]

Alcino [89] found that benzylpenicillin **5** did not react with iodine, but that iodine was absorbed by products of inactivation with alkali ie benzylpenicilloic acids **41**. He devised an accurate and specific method of analysis based on the base hydrolysis of penicillin followed by reaction with iodine and back titration of the excess with thiosulphate. Use of a blank containing penicillin which had not been inactivated allowed for analysis in the presence of iodine-absorbing contaminants, including precursors and degradation products. The mechanism of reaction with iodine is incompletely understood and numerous modifications have been proposed.[86] Nevertheless the utility of the method is reflected in its wide adoption for several penicillins (including the Na and K salts of benzylpenicillin) and cephalosporins as the official quantitative assay method in the USP.[90]

In addition to the potency assay requirements, the compendia may require other analytical tests such as optical rotation, pH, colour tests, uv-spectrophometric methods and microbiological assay.

Another chemical method, similar in its principle to the iodometric method, is the determination of penicillins with mercury (II) solution .[91] It has replaced the iodometric assay method in the British [92] and European Pharmacopoeia [93] for some penicillins. However the most suitable methods available for the compound-specific and stability-indicating analyses of benzylpenicillin and its acid/base degradation products are HPLC and to a lesser extent ^{1}H NMR. The remainder of this section is devoted to the development of liquid chromatographic methods. ^{1}H NMR methods are treated in section 6.

5.1.1 Liquid chromatographic (LC) methods for the analysis of β-lactam antibiotics

It is interesting to note that the refinement of LC methods parallels somewhat the development of penicillin antibiotics. The purification of crude penicillin was greatly aided by chromatographic adsorption on alumina and silica-gel columns (numerous references [10]). Fisbach *et al* [94] successfully separated and quantitatively determined penicillin K (heptylpenicillin **7**) in the presence of the other natural penicillins by adopting the novel liquid-liquid partition chromatographic techniques of Martin and Synge [95 96], for which Martin was awarded a Nobel Prize in 1952.

It was not until the early 1970's that the technology existed for the routine LC analysis of β-lactam antibiotics. The commercial development of robust pumping systems, UV detectors and small-diameter

and high-efficiency column-packing materials permitted good separations within a relatively short time with sensitive detection.

At first, anion [97] [98] [99] and cation [100] ion-exchange materials were used as stationary phases and then the development of reversed-phase materials brought substantial improvements and were rapidly adopted as the chosen technique. White *et al* [101] clearly demonstrated that reversed-phase RPHPLC techniques were superior to ion-exchange LC and other chemical assays, for the separation and detection of penicillins, cephalosporins, tetracyclines and other miscellaneous antibiotics. Wilson and Hughes [102] reported the use of RPHPLC to separate and analyse a wide range of β-lactam and other antibiotics. Improved RPHPLC methods for the separation and quantitation of antibiotics using high efficiency small particle column packing materials (5 - 10μ) [103] [104] and ion-pairing techniques [104] have been reported. Such techniques have been widely used to separate and simultaneously analyse a wide range of penicillins and cephalosporins using isocratic [105] [106] [107] and gradient elution.[108] The selectivity of 8 brands of C_8 and C_{18} reversed-phase packing materials in the separation of 16 cephalosporins has been reported.[109] Salto [110] has reported the use of RPHPLC to separate penicillin and cephalosporin diastereoisomers, and the use of chiral stationary phases permit the separation of β-lactam enantiomers.[111]

The great success of RPHPLC is reflected in the proliferation of such methods for the analysis of penicillins and cephalosporins in biological fluids and pharmaceutical/veterinary formulations. The reader is referred to several recent reviews.[112] [113] [114] Recently an in-depth study of the routine HPLC analysis of 15 β-lactam antibiotics

in body fluids has been reported [115] and the results highlight the advantages in terms of precision, accuracy and speed of analysis.

As yet the regulatory authorities have been hesitant in adopting HPLC methods as the official assay, presumably as HPLC methodological guide-lines have yet to be finalised.[116]

Little literature exists about the comparison of HPLC with the other chemical methods. Roksvaag [99] and Nachtmann [117] have compared the HPLC quantitative determination of benzylpenicillin with the iodometric, mercury titration and microbiological assays. Excellent correlations were found but the only method able to produce accurate results in all tested cases i.e. in the presence of degradation products and other penicillins, was HPLC. In addition, advantages in terms of speed, accuracy and precision are clearly demonstrated. Bird [118] has compared the HPLC, imidazole and mercurimetric assays of some 6-substituted penicillins. HPLC has also found application for the analysis of penicillins in fermentation media [119] and in the large scale purification of non-extractable β-lactam antibiotics.[120]

In all the HPLC methods so far described, detection by UV is commonly employed. For those β-lactam antibiotics which lack an aromatic chromophore, greater sensitivity is normally achieved by monitoring of wavelengths below 230 nm. However, at these wavelengths selectivity is poor, resulting in high background interference when trace analyses are required for samples such as complex fermentation media or in clinical analysis. Pre- and post-column derivatisation techniques enhance selectivity and sensitivity. Westerlund *et al* [121] used post-column derivatisation involving formation of the mercuric mercaptide of penicillenic acid. A variety of pre- and post-column

techni ques have recently been compared.[122] Although not in routine use, the "Thermospray" LC-MS analysis of ampicillin 34 has been briefly reported.[123]

5.1.2 HPLC analysis of benzylpenicillin and its acid/base degradation products

A detailed study of the break-down pathways of benzylpenicillin in acidic and basic aqueous solutions is best achieved by the use of experimental methods capable of the quantitative analysis of benzylpenicillin and subsequent degradation products. Ideally methodology should be aimed at the simultaneous analysis of all compounds and therefore avoid inter-experimental sources of error.

HPLC is such an analytical technique particuarly suited to the task. The first step must be the development and optimisation of robust and compound-specific HPLC methods capable of separating, identifying and quantitatively analysing all possible degradation products.

Blaha *et al* [97] employed anion-exchange chromatography for the separation and quantification of benzylpenicillin and five of its decomposition products i.e. benzylpenillic 42, benzylpenamaldic 44, benzylpenicilloic 41, benzylpenilloic acids 43 and D-penicillamine 10. The technique was later used [60] to study the degradation of benzylpenicillin at pH 2.7 and 25°, 37° and 45°C. Adriaens *et al* [100] utilised cation-exchange chromatography to separate a comprehensive series of decomposition products derived from double-labelled benzylpenicillin. Several authors [62 124 125] have used bonded-phase packing materials to separate benzylpenicillin from its major

degradation products. Ressler *et al* [76] described similar HPLC methods capable of the almost complete resolution of all four possible natural benzylpenicilloic acid epimers and the two benzylpenilloic acid epimers. RPHPLC has also been used to monitor the epimerisation of benzylpenicilloic acid in alkaline media.[73] Ueno *et al* [126] used RPHPLC to separate polymers formed when concentrated solutions of benzylpenicillin were kept standing in the dark at room temperature for 14 days.

Other workers [127] examined the influence of pH, ion-pair reagent and buffer concentrations and acetonitrile content upon the HPLC separation of benzylpenicillin, benzylpenicilloic, benzylpenillic and benzylpenilloic acids. An optimised ion-pair system was used [63] to study the degradation of benzylpenicillin at pH 2.5 and 37°C.

The RPHPLC separation of nonpolar, nonionic samples is usually simply achieved by choosing the correct mobile phase composition consisting of water and an organic solvent (modifier). However the RP-HPLC separation of ionic compounds is not so straightforward. The key to successful separation is the careful optimisation of pH, buffer concentration, organic modifier type/content and temperature with the aim of retaining the ionic analytes. For example, benzylpenicillin and its degradation products, with the exception of benzylpenilloaldehyde 53, contain at least one carboxylic acid functional group. Table 5 includes limited pK_A data (for the carboxylic acids of the compounds to be studied) readily available from the literature. The data is incomplete and no reliable data exists for the benzylpenilloic acids. However the carboxyl groups of the thiazolidine-3-carboxylic acid and its 2,2-*gem*-dimethyl derivative have pK_A values of 1.6 - 1.7 [12] and

owe their high acidity mainly to the positive charge located on the thiazolidine amino group.

Therefore by buffering the mobile phase to a pH below that of the pK_A, retention of the analyte may be increased. This technique is termed "ionic suppression".[128] Alternatively if the pH is between 6 and 8, weak acids may be retained with the addition of an "ion-pairing" reagent. This technique is commonly termed "ion-pair chromatography".[129] If ionic suppression techniques are employed the mobile phase must be buffered to about pH 1. Under these conditions the column has only a limited life-time and more importantly the marked instability of benzylpenicillin and especially benzylpenicillenic acid **28** [58] at low pH renders this technique unsuitable for degradation monitoring. In addition the instability of silica gel based column packing materials enforces a limit of the mobile phase pH to below pH 7 - 8. Therefore ion-pair and a technique personally nominated as "buffered" RPHPLC (i.e. buffering the mobile phase between pH 3 and 7) modes of separation were compared. The exact retention mechanisms of such techniques are not clearly understood. Bidlingmeyer [132] has recently compared and proposed some theoretical models.

The work described in the remainder of this section compares the analysis of benzylpenicillin and its degradation products using ion-pair and buffered RPHPLC separation modes. The effects of pH, buffer concentration, organic modifier type and content, column type and temperature on sample capacity with the ultimate aim of studying the decomposition of benzylpenicillin in acidic and basic aqueous solutions in a greater detail than hitherto attempted.

Table 5 Key to symbols and abbreviations used for test compounds

Symbol		Compound	pK_A†	Ref.
o	BP	(3S,5R,6R)-Benzylpenicillin 5	2.71-2.73	37
	BPAL	Benzylpenilloaldehyde 53		
	BPE	(3S)-Benzylpenicillenic acid 28		
	BPEDS	(3S)-Benzylpenicillenic acid disulphide 46		
	BPENAM	(3S)-Benzylpenamaldic acid 44		
Δ	RRBPC	(3S,5R,6R)-Benzylpenicilloic acid 41A		
▲	SRBPC	(3S,5S,6R)-Benzylpenicilloic acid 41B		
	RSBPC	(3S,5R,6S)-Benzylpenicilloic acid 41C		
	SSBPC	(3S,5S,6S)-Benzylpenicilloic acid 41D		
□	BPI	(3S)-Benzylpenillic acid 42	2.0 & 2.5-2.6	131
	BIPI	(3S)-Benzylisopenillic acid 56		
▽	RBPO	(3S,5R)-Benzylpenilloic acid 43A	1.35 & 5	12
▼	SBPO	(3S,5S)-Benzylpenilloic acid 43B		
	BPSO	(3S,5R,6R)-Benzylpenicillin-S-sulphoxide 57		
	DPAM	D-Penicillamine 10	1.8 (thiol)	130
	DPAMDS	D-Penicillamine disulphide 61		
	NFDPAM	N-Formyl-D-penicillamine 47		
	NPAG	N-Phenylacetylglycine 50		
●	PAA	Phenylacetic acid 62	4.25	130
	6APA	(3S,5R,6R0-6-Aminopenicillanic acid 31		

Notes: † Dissociation constant (carboxylic acid) in aqueous solution at ca.25°C.

5.2 EXPERIMENTAL

5.2.1 Materials and reagents

Deionised, glass distilled water was used throughout. Methanol, acetonitrile and tetrahydrofuran (THF) were all HPLC grade (Fisons, Loughborough, Leics.). Tetrabutylammonium bromide, TBAB, was obtained from Fisons and all chemicals used were AR grade. HPLC solvents were filtered and helium-degassed immediately prior to use. The preparation and s ources of the analytical reference standards of benzylpenicillin Na and its degradation products are described in section 4 and listed, together with the symbols and abbreviations used in the text, in Table 5. Although not reported as degradation products, benzylpenicillin-sulphoxide 57 and 6-aminopenicillanic acid 31 were included in this study.

5.2.2 Apparatus

All studies were conducted using a Constametric IIIG HPLC metering pump and Spectromonitor III variable wavelength spectrophotometric detector, equipped with a 12µl, 1cm path-length flow cell. (Laboratory Data Control/Milton Roy, Riviera Beach, Florida, US). Column temperature was maintained using a model 7930 column block heater (Jones Chromatography, Hengoed, Mid Glamorgan, UK).

Sample injection was performed by a model 7125 valve injector (Rheodyne, Cotati, California, US) equipped with a 20µl sample loop. Retention times and peak area integration were measured on a model 3390A computing integrator (Hewlett-Packard, Avondale, Pennsylvania, US). The detector signal was also recorded on a Servogor SE120 chart

recorder (BBC Goerz Metrawatt, Wien, Austria).

Two 250 x 4.9mm i.d. stainless steel HPLC columns were used:
Column 1; Spherisorb S5ODS (5µ, C_{18}, 7% carbon-loading).
Column 2; Spherisorb S5ODS2 (5µ, C_{18}, 12% carbon-loading, end-capped).
(Phase Separations, Queensferry, Clwyd, UK packed by Hichrom Ltd., Reading, Berkshire, UK)

5.2.3 Conditions.

All samples were freshly prepared prior to analysis, by dissolving the compound in the HPLC mobile phase, to give a final concentration of ca. 1mg/ml. The dissolution of the free acids was aided by the addition of the minimal amount of sodium bicarbonate.

20µl injections were performed in duplicate and the mean retention time and peak shape noted. The mobile phase compositions are described in the text. The flow rate was kept constant at 1 ml/min. The detector wavelength was usually set at 230nm except for the analysis of benzylpenicillenic acid (322nm) and bezylpenamaldic acid (280nm). All measurements were performed at 25 ± 1°C except where noted.

5.2.4 Column Testing

The columns were periodically tested throughout the course of the studies and a summary of the results are given in Table 6. From time to time, it was necessary to repair the columns by replacing the top few millimetres with fresh column packing material (applied in a acetone slurry). As may be seen from the results the columns maintained near-new efficiency throughout the studies despite their less than gentle use.

Table 6 Number of theoretical plates N and retention time t_r (in minutes) for test solutes on columns 1 & 2. Columns tested before and after studies.

Column number	Pressure (psig)	Benzophenone N	Benzophenone t_r	Biphenyl N	Biphenyl t_r
1 before	1100	9800	6.09	11800	8.07
1 after	960	11000	5.78	13500	7.58
2 before	1100	21000	6.15	22500	9.55
2 after	1050	18900	6.12	18400	9.27

Conditions: Acetonitrile:Water 70:30 (v/v) at 1ml/min., UV 254nm.

5.3 Results and discussion

5.3.1 Ion-pair reversed-phase HPLC (IPRPHPLC)

5.3.1.1 Effect of organic modifier type and content

The effect of acetonitrile organic modifier content, mobile phase pH, buffer concentration and ion-pair reagent (tetrabutylammonium chloride TBACl) concentration upon the retention of benzylpenicillin, benzylpenicilloic (presumably the 3S,5R,6R-epimer), benzylpenillic and (3S,5R/S)-benzylpenilloic acids has been described [49]. The optimised mobile phase consisted of acetronitrile : (0.006 M phosphate buffer, pH 7.5, containing 0.008 M TBACl) 30:70 v/v. The effect of the other organic modifiers was not described. This deficiency was remedied by studies comparing the effects of acetonitrile and methanol (Table 7)

It is clear that acetonitrile has superior elutropic strength. 40% v/v methanol is equivalent to ca. 26 ± 2% v/v acetonitrile. Selectivity differences are also apparent. In solvent systems containing methanol, the (3S,5R/S)-benzylpenilloic acid epimers co-elute, while the (3S,5R/S,6R)-benzylpenicilloic acid epimers are almost completely resolved (α = 1.12 @ 40% v/v MeOH). With acetonitrile, separation is reduced (α = 1.01 @ 25% v/v MeCN) and the elution order is reversed. while the separation of the benzylpenilloic acid epimers is much improved (α = 1.09 @ 25% v/v MeCN).

All analytes, with the exception of benzylpenilloaldehyde, show an almost exponential increase in retention as organic modifier content decreases. The anomalous behaviour of the aldehyde is possibly due to the absence of a carboxylic group present in all other compounds.

Table 7. Capacity factors of benzylpenicillin degradation products on column 1 using acetonitrile or methanol : 0.006M Na_2HPO_4 pH 7 containing 0.008M TBAB (* 0.016M TBAB) at 1 ml/min and 25°C.

	% v/v Acetonitrile				% v/v Methanol	
Compound	35	30	25	30*	50	40
BP	1.69	3.40	10.4	4.52	1.92	7.38
BPE	5.46	12.3	R	16.8	6.69	R
BPI	0.55t	1.06t	2.92t	1.63	0.75	2.85
RRBPC	0.92	1.90	6.25	3.10	0.68	3.58
SRBPC	0.94	1.88	6.17	3.10	0.71	4.00
RBPO	1.55t	2.71t	7.58t	3.58	1.85	6.44
SBPO	1.55t	2.92t	8.29t	3.81	1.85	6.44
BPAL	0.60	0.75	1.21	0.79	1.15	1.77
NPAG	0.63	1.13	2.08	1.44	0.60	1.71
BPENAM	1.21	2.13	5.75	2.88	1.02	3.75
NFDPAM	0.40	0.63	1.25	0.82	nd	0.98
BIPI	1.29t	3.06t	nd	4.83t	1.29t	9.83t
BPSO	1.10	2.10	nd	2.79	1.00	3.38
6APA	0.29	0.46	0.92	0.60	nd	0.75

Notes: R = compound irreversibly bound/retained on-column.
nd = not determined. t = peak shows significant tailing.

$$\text{Capacity factor} = k' = (t_r - t_o)/t_o$$

Where t_r is the mean retention time in minutes and t_o is the column dead volume estimated by the injection of neat organic modifier and timing the first baseline perturbation

Benzylpenicillenic acid is relatively strongly retained in both solvent systems. This is possibly due to the hydrophobic nature of the highly conjugated benzyloxazolone part of the molecule.

It is fortunate that all other degradation products are more polar than benzylpenicillin and hence benzylpenicillin elutes last. This aids method development considerably.

In acetonitrile mobile phases, benzylpenillic, benzylpenilloic and benzylisopenillic acids gave tailing peaks. Substitution of acetonitrile with methanol improved peak shape except for benzylisopenillic acid. In both solvent systems benzylpenilloaldehyde eluted as a broad yet symmetrical peak (N = 250 theoretical plates, K' = 1.2, @ 25% v/v acetonitrile) in contrast to benzylpenicillin (N = 1700 theoretical plates, K' = 10.4). Possible causes for the poor elution characteristics of benzylpenilloaldehyde are discussed later. More importantly is the observation that D-penicillamine and its disulphide elute in very broad and tailing peaks (hence no retention data is included in Table 7). The incompatability of penicillamine (thiol and disulphide) in ion-pair reversed-phase HPLC systems is clearly due to the presence of a primary amine group, as N-formyl-D-penicillamine gave a sharp and symmetrical peak.

5.3.1.2 Effect of doubling ion-pairing reagent content.

The effect of doubling the concentration of TBAB to 0.016 M in the acetonitrile : (0.006 M phosphate buffer, pH 7) 30 : 70 (v/v) mobile phase system is included in Table 7. The retention of all the dicarboxylic acid solutes is increased by ca. 60% while for analytes containing one carboxylic acid group, the increase is ca. 30%. This is equivalent to a small decrease in acetonitrile content. The independence of benzylpenilloaldehyde retention with respect to ion-pairing reagent concentration is to be expected. An improvement in the peak shapes of benzylpenillic and benzylpenilloic acids was observed at the higher ion-pair concentration.

5.3.1.3 Effect of omitting the ion-pairing reagent

As the poor peak shape of the penicillamines could interfere with the quantification of other analytes, the effect of omitting the ion-pair reagent was investigated. Retention data for the analytes in Methanol : 0.01 M Na_2HPO_4 pH 7 mobile phases are given in Table 8.

Omission of TBAB led to the expected decrease in retention all analytes except benzylpenilloaldehyde which remained unchanged. Satisfactory peak shapes were obtained for all analytes including the penicillamines (although they were barely retained) but excepting benzylpenilloaldehyde. No gross changes in selectivity was noted. One can conclude that the presence of an ion-pairing reagent offers few advantages in the RPHPLC analysis of benzylpenicillin degradation products.

Table 8 Capacity factors of benzylpenicillin degradation products on column 2 using MeOH : 0.01M Na_2HPO_4 pH 7 at 1 ml/min. and 25°C

	% v/v Methanol					% v/v Methanol			
Compound	60	40	30	20	Compound	60	40	30	20
BP	0.20	2.00	6.52	R	BPAL	0.65	2.02	4.00	8.27
BPE	1.33	8.16	40.0	R	NPAG	nd	0.29	0.60	1.35
BPI	0.01	0.31	0.92	3.06	PAA	nd	0.31	0.54	1.04
RRBPC	0.01	0.08	0.27	0.96	BPENAM	0.35	0.63	1.69	nd
SRBPC	0.01	0.08	0.33	1.20	DPAM	0.02	0.04	0.04	0.20
SSBPC	nd	nd	nd	1.94	DPAMDS	0.04	0.13	0.23	nd
RSBPC	nd	nd	nd	1.41	NFDPAM	0.02	0.13	0.23	nd
RBPO	0.19	1.48	4.73	16.9	BIPI	0.01	0.67	3.17	R
SBPO	0.19	1.48	5.13	19.2	BPSO	0.08	0.73	2.15	8.31
6APA	0.01	0.08	0.17	0.33					

Notes: R = retained on column, nd = not determined.

5.3.2 Buffered reversed-phase HPLC (BRPHPLC)

It has been shown in the previous section that the presence of an ion-pairing reagent is not necessary for the satisfactory separation of benzylpenicillin degradation products. Therefore the effects of variables governing separation and retention of analytes in a "buffered" reversed-phase BRPHPLC separation mode were studied.

5.3.2.1 Effect of temperature, pH and buffer concentration

Using mobile phases consisting of acetonitrile and phosphate buffers with column 1, the effect of variables governing retention and separation of benzylpenicillin, benzylpenilloic, benzylpenicilloic, benzylpenillic and phenylacetic acids were studied.

The retention of all analytes increased as pH decreased (Figure 4) and is due primarily to a change in the degree of ionisation of the carboxylic acid groups. If the studies had been expanded the typical retention - pH sigmoidal profile would be shown.

As expected the retention of all analytes changed almost exponentially with temperature (Figure 5).

All analytes show an increase in retention with increasing buffer concentration to a limiting value of about 0.05 M (Figure 6). Changes in buffer concentration and changes in ion-pair concentration [49] have analogous effects on retention behaviour. In ion-pair chromatography, increasing ion-pairing reagent concentration may enrich the population of coulombic-complexes (ion-pairs). In buffered RPHPLC an increase in buffer concentration increases the buffering capacity within the

chromatographic band to a limiting value where the buffer capacity exceeds the ability of the analyte to modify local pH. Further increases have little additional effect. In either case the overall result is an increase in the population of lipophillic species available for retention by the stationary phase.

Figure 5 Buffered RPHPLC; effect of temperature on capacity factor (k').
Column 1, eluent: MeCN : 0.05M KH_2PO_4 (pH3.8) 14 : 86 v/v
Symbols as in Table 5.

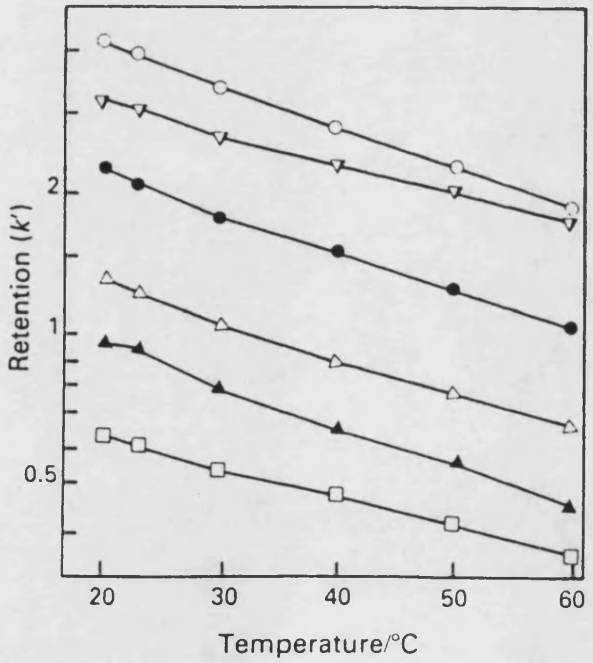


Figure 4 Buffered RPHPLC; effect of buffer pH on capacity factor. Conditions as in figure 4.

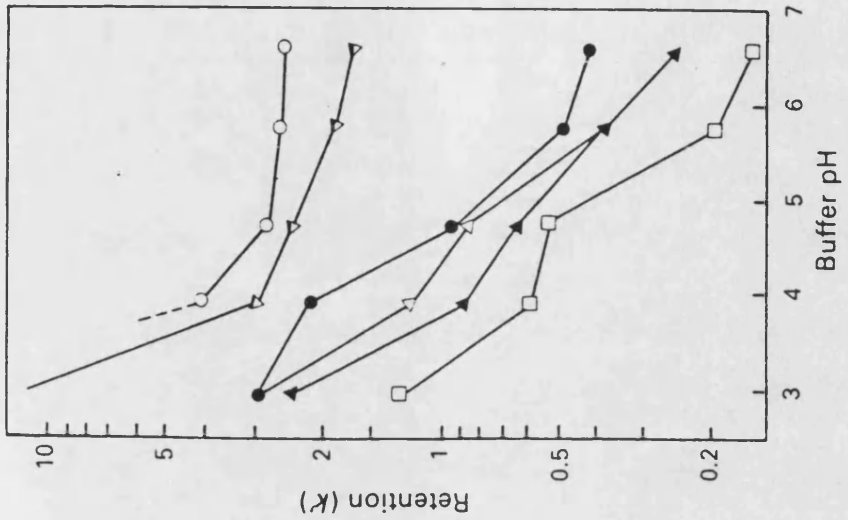
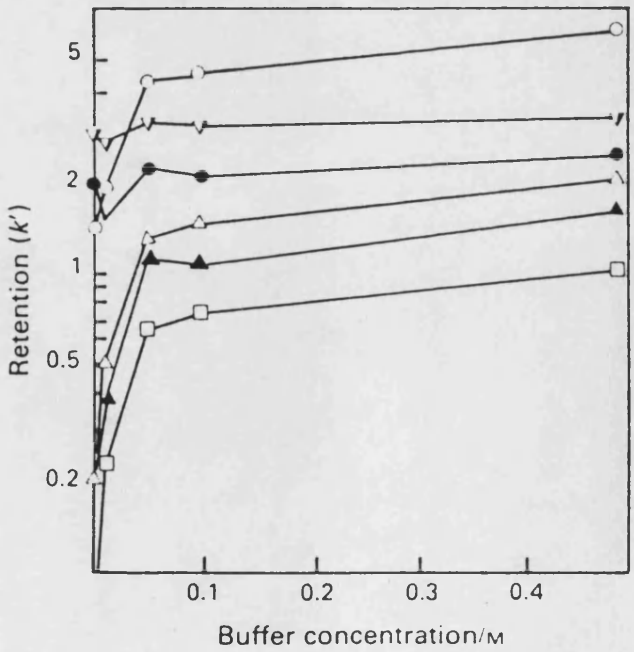


Figure 6 Buffered RPHPLC; effect of buffer concentration on capacity factor. Conditions as in figure 4



5.3.2.2 Effect of organic modifier type and content

Using column 2 and 0.05 M phosphate buffer at pH 3.8, acetonitrile, methanol and tetrahydrofuran organic modifiers were compared (Table 9). It is clear from the data, that the order of solvent elutropic strength is tetrahydrofuran > acetonitrile > methanol. Although a wide range of solutes are involved, one can estimate that 35 - 30% v/v MeOH is equivalent in eluotropic strength to ca. 15% MeCN which is equivalent to ca. 7.5% v/v THF, best illustrated in Figures 7a-c. A similar elutropic strength relationship has been observed [133] for the RPHPLC separation of ten corticosteroid hormones i.e. 35% MeOH ≡ 20% MeCN ≡ 12% THF. This general order of elutropic strength is now well accepted, however the quantitative relationships are ambiguous. The various methods used for the prediction of eluotropic strengths of organic modifiers have recently been compared.[134] As the empirical and theoretical rules so far developed are best limited to non-polar, non-ionic solutes, further discussion was beyond the scope of the thesis.

Selectivity differences are best illustrated by comparing the separation of the 4 benzylpenicilloic acid epimers and the two benzylpenilloic acid epimers. All three organic modifiers tested are capable of the almost complete resolution of the 4 benzylpenicilloic acid epimers (see Figures 7a-c). Of the organic modifiers studied only THF was capable of the almost complete separation of the benzyl-penilloic acid epimers in a reasonably short time (Table 9). However at low concentrations of THF the two epimers coelute. The epimers may also be separated by adjusting the methanol or acetonitrile content, however the total analysis time is longer. Methanol is clearly superior to acetonitrile in this respect (cf. Figures 7a and 7b).

Table 9 Capacity factors of benzylpenicillin degradation products using acetonitrile, methanol or tetrahydrofuran : 0.05M KH_2PO_4 pH 3.8 at 1 ml/min and 25°C

	% v/v acetonitrile						% v/v methanol				
Compound	75	50	25	20	17	14	60	40	35	30	25
BP	0	0,05	1,64	4,68	11,0	19,1	0,50	1,18	7,36	13,2	25,7
BPE	0,03	0,11	6,18	22,9	R	R	1,26	17,1	40,8	R	R
BPEDS	nd	nd	nd	nd	nd	nd	nd	1,18	nd	3,32	5,72
BPI	nd	nd	0,14	0,39	0,68	1,22	0,16	0,71	1,24	1,92	3,28
RRBPC	nd	nd	0,40	1,05	2,09	3,91	0,24	1,60	2,88	4,63	8,42
SRBPC	nd	nd	0,27	0,68	1,55	2,75	0,24	1,24	2,28	3,65	6,77
SSBPC	nd	nd	nd	nd	nd	4,43	0,24	1,60	2,88	4,63	8,81
RSBPC	nd	nd	nd	nd	nd	3,30	0,24	1,47	2,62	4,40	7,58
RBPO	nd	nd	1,36	3,41	6,45	11,9	0,48	3,93	6,76	11,1	19,8
SBPO	nd	nd	1,36	3,41	6,45	12,1	0,48	3,83	6,48	10,6	18,6
BPAL	nd	nd	0,77	1,05	2,23	3,23	0,50	nc	nc	nc	nc
NPAG	nd	nd	0,55	1,09	1,89	2,50	0,21	0,80	nd	1,62	2,34
PAA	nd	nd	3,25	6,05	9,27	12,0	0,78	3,04	4,60	6,24	9,12
BPENAM	0	nd	0,63	1,50	2,98	5,14	0,21	1,09	1,80	2,87	4,94
DPAM	0,11	0,05	0,14	0,20	0,25	0,30	nd	nd	nd	0,30	0,60
DPAMDS	0,02	nd	0,05	0,08	0,10	0,14	nd	nd	nd	0,20	0,20
NFDPAM	0,02	nd	0,18	0,43	0,60	0,75	nd	nd	nd	0,68	0,90
BIPI	nd	nd	nd	nd	2,98	nd	nd	2,00	nd	6,16	12,0
BPSO	nd	nd	nd	nd	3,22	nd	nd	1,22	3,18	3,61	6,57
6APA	0	0	0,10	nd	nd	0,20	nd	nd	nd	nd	0,24

(continued overleaf)

Table 9 (continued)

	% v/v tetrahydrofuran							
Compound	40	30	25	20	15	10	5	0 ***
BP	0,40	1,03	1,76	3,20	4,80	9,64	22,6	R
BPE	0,86	3,07	6,26	15,3	R	R	R	R
BPI	nd	0,02	0,10	0,18	0,28	0,61	1,64	21,1
RRBPC	nd	0,25	0,41	0,80	1,14	2,15	5,24	R
SRBPC	nd	0,25	0,41	0,63	0,92	1,80	4,53	R
RBPO	0,52	1,04	1,49	2,24	3,09	5,32	12,2	R
SBPO	0,42	0,90	1,34	2,10	2,97	5,32	12,2	R
BPAL	nd	0,58	0,79	1,10	1,36	1,96	3,14	R
NPAG	nd	0,42	0,65	0,87	1,07	1,52	2,45	26,1
PAA	1,64	3,23	4,42	6,22	7,12	8,85	11,7	42,4
BPENAM	nd	0,37	0,50	0,61	0,87	1,57	3,61	R
DPAM	nd	nd	nd	0,14	0,16	0,20	0,28	1,14
DPAMDS	nd	nd	nd	0,04	0,05	0,06	0,10	1,92
NFDPAM	nd	nd	nd	0,38	0,53	0,63	0,92	4,76
BIPI	nd	0,20	0,38	0,71	1,10	2,54	7,24	R
BPSO	nd	0,24	0,45	0,81	1,22	2,31	5,24	R
6APA	nd	nd	nd	0,10	0,10	0,11	0,16	1,14

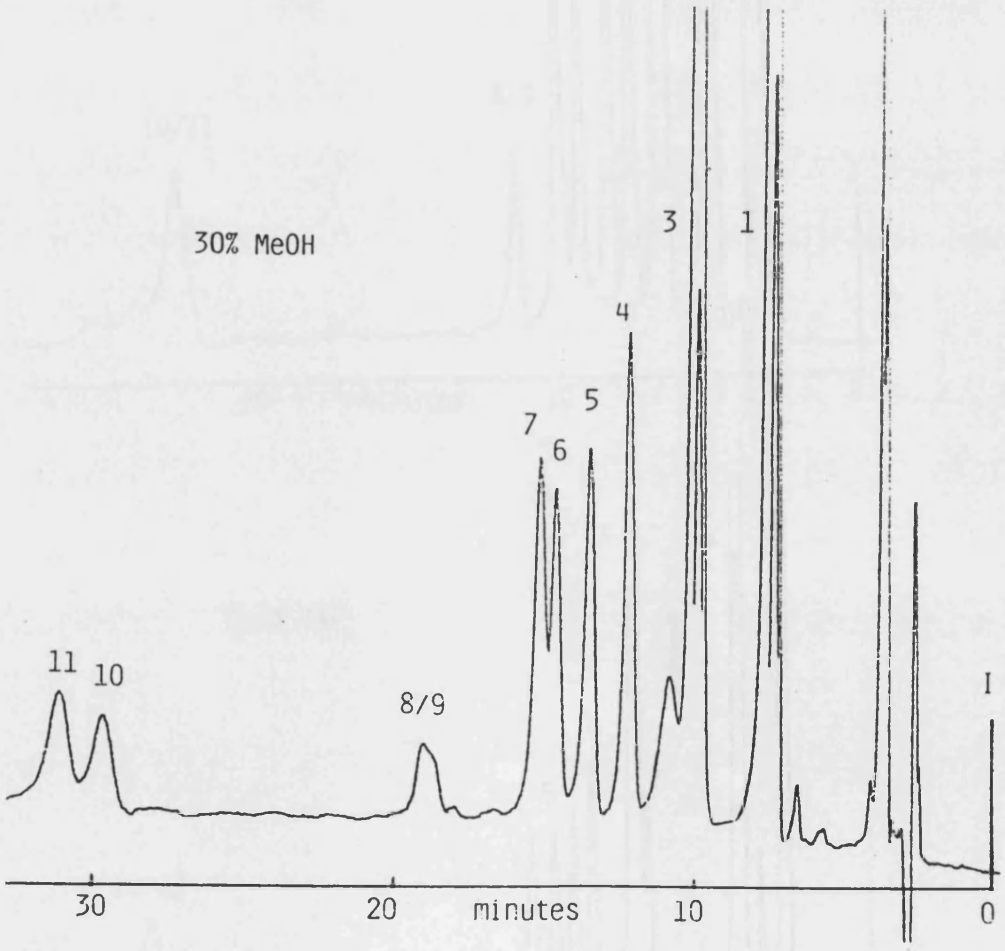
Notes; R = retained on column nd = not determined nc = not calculated, not compatible with this mobile phase,

*** i,e, 100% phosphate buffer pH 3,8

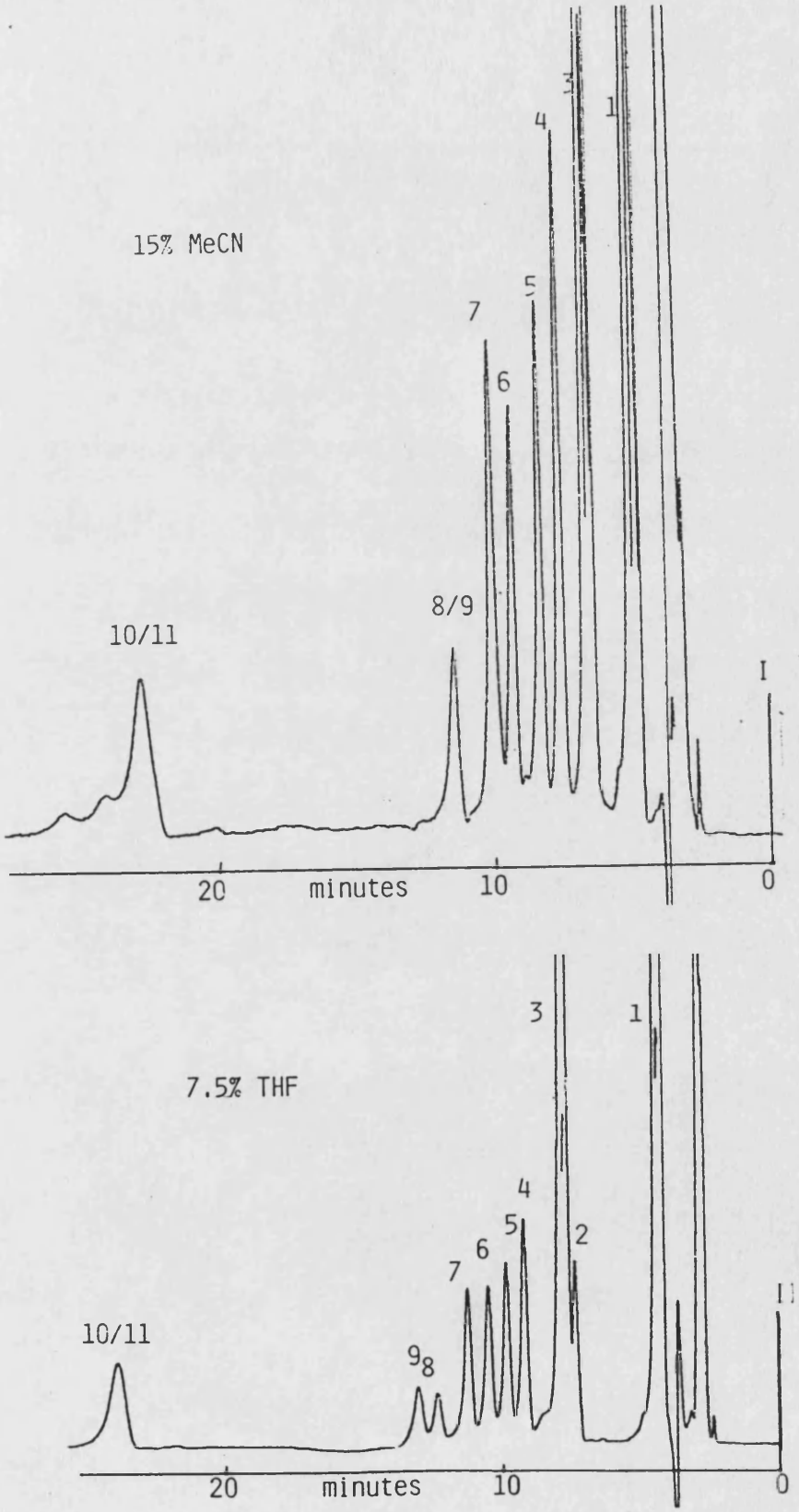
Figure 7 Chromatograms illustrating the effect of organic modifier type and content upon separation. The mobile phases chosen are approximately isoeluotropic. Conditions: Column 2, 0.05M phosphate buffer (pH 3.8) and a) MeOH 30% v/v, b) MeCN 15% v/v and c) THF 7.5% v/v. Flow rate 1ml/min, temperature 25°C and uv detection at 230nm.

Key: 1=BPI, 2=BPAL, 3=BPENAM, 4=SRBPC, 5=RSBPC, 6=RRBPC, 7=SSBPC, 8=BPEDS, 9=BIPI, 10=SBPO, 11=RBPO, I=Injection.

Figure 7a.



Figures 7b and 7c



D-penicillamine/disulphide are only retained, to any appreciable extent, in 100% aqueous phase.

The chromatographic elution peak of benzylpenilloaldehyde was distorted in all modifiers tested. In mobile phases containing methanol the peak elution profile (Figure 8a) is typical of irreversible or slow reversible reactions occuring on-column.[135] In acetonitrile and tetrahydrofuran mobile phase (Figures 8b and 8c respectively) the elution peak of benzlpenilloaldehyde is broadened, more So in acetonitrile. These phenomena may be rationalised in the following manner.

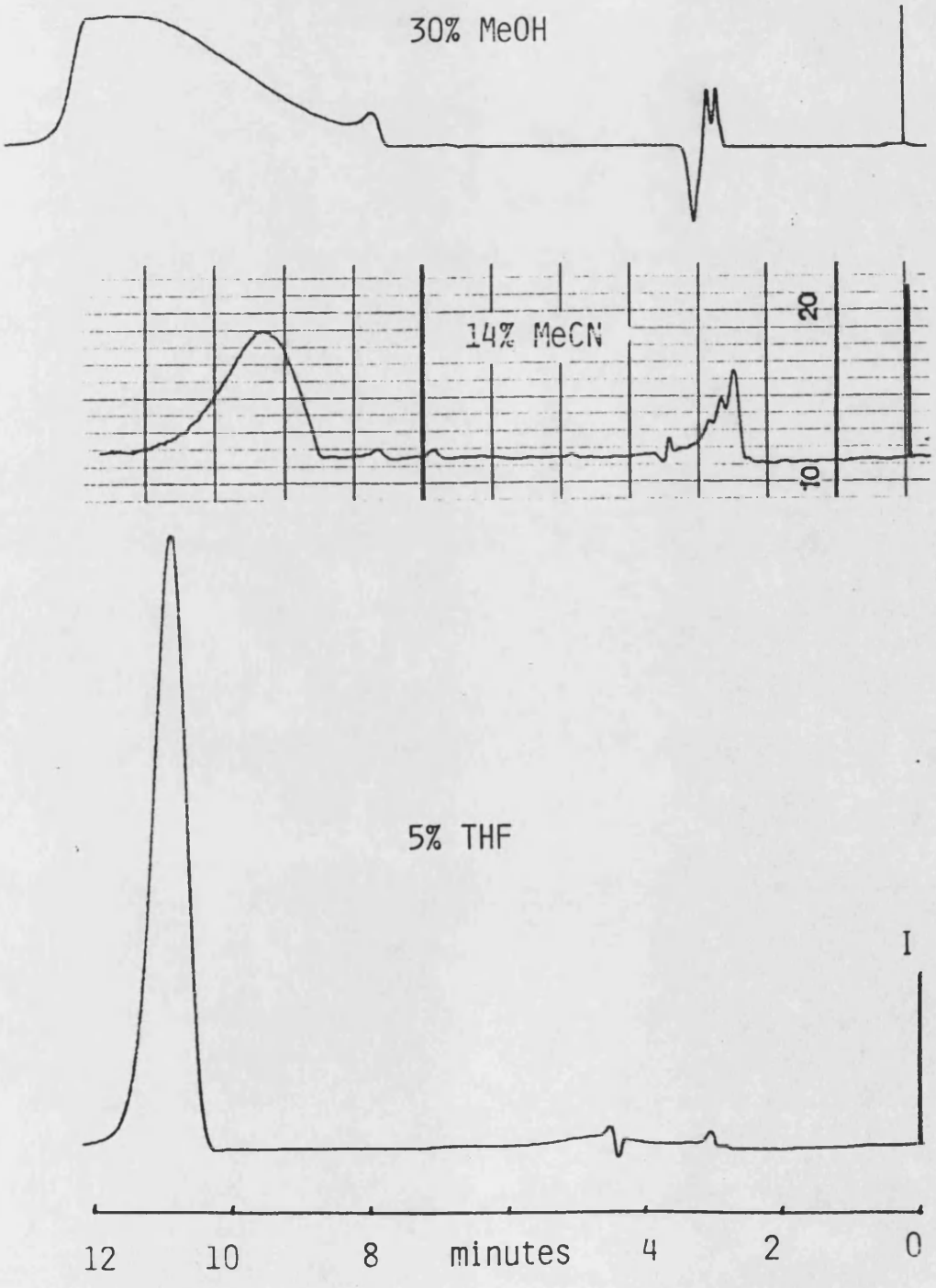
^{1}H NMR evidence (Section 6.3.14) indicates that in aqueous solution benzylpenilloaldehyde exists predominantly as the *gem*-diol product. In acidic aqueous solutions containing methanol the *gem*-diol addition product is probably in equilibrium with the hemiacetal and acetal addition products i.e.

$$\underset{}{\text{R-CHO}} \overset{H_2O}{\rightleftharpoons} \underset{\textit{gem}\text{-diol}}{\text{R-CH(OH)}_2} \overset{CH_3OH}{\rightleftharpoons} \underset{\text{hemiacetal}}{\text{R-CH(OH)OCH}_3} \overset{CH_3OH}{\rightleftharpoons} \underset{\text{acetal}}{\text{R-CH(OCH}_3)_2}$$

(R = $PhCH_2CONHCH_2$-)

Hence the distorted elution profile reflects these transformations occuring on-column. In acetonitrile or tetrahydrofuran, acetal formation is not possible and the broad peaks are probably due to strong intra-molecular hydrogen bonding. It is difficult to see why the peak profile is improved in mobile phases containing tetrahydrofuran rather than acetonitrile.

Figure 8 Distortion of the chromatographic elution band of benzylpenilloaldehyde as an effect of organic modifier type. Conditions: column 2, eluent 0.05M phosphate buffer pH 3.8 and a) MeOH 30% v/v, b) MeCN 14% v/v and c) THF 5% v/v. Flow rate 1 ml/min. Detection uv 230nm. Temperature 25°C.



5.3.2.3 Effect of HPLC column type

The degree and order of retention for a wide range of penicillin degradation products on S5ODS and S5ODS2 columns were similar. The higher carbon loaded and end-capped S5ODS2 column was preferred as peak tailing was reduced, however benzylpenilloaldehyde gave poor peak shape on either column.

5.3.2.4 Initial calibration and sample loading studies

Benzylpenicillin calibration studies performed on column 1 with acetonitrile : 0.05 M phosphate buffer pH 3.8 (14:86 v/v) show linear peak area responses in the concentration range 1×10^{-6} to 1×10^{-3} M, with a limit of detection of approximately 1×10^{-7} M.

It was noted that concentrated solutions of benzylpenicillin degradation products led to a marked reduction in retention with increased tailing. This is probably due to the analytes exceeding local buffer capacity. Dilution studies performed on the same system with an equimolar solution of benzylpenicillin, benzylpenillic, benzylpenilloic and phenylacetic acids show that a 1×10^{-3}M solution could be analysed without any significant loss in retention.

5.4 Conclusion and recommendations

An examination of the differences between ion-pair and buffered RP-HPLC separation modes indicated that the latter is the preferred technique for the separation and quantification of a wide range of benzylpenicillin degradation products. The main features of this work have been recently reported.[136] Although no one isocratic system was capable of the base-line resolution of all analytes examined, in a reasonable time, the optimum separation system would consist of an end-capped octadecylsilane packing material and a 0.05 M phosphate buffer (pH 3.8) mobile phase. The choice of organic modifier is somewhat subjective, however tetrahydrofuran was chosen for the following reasons:

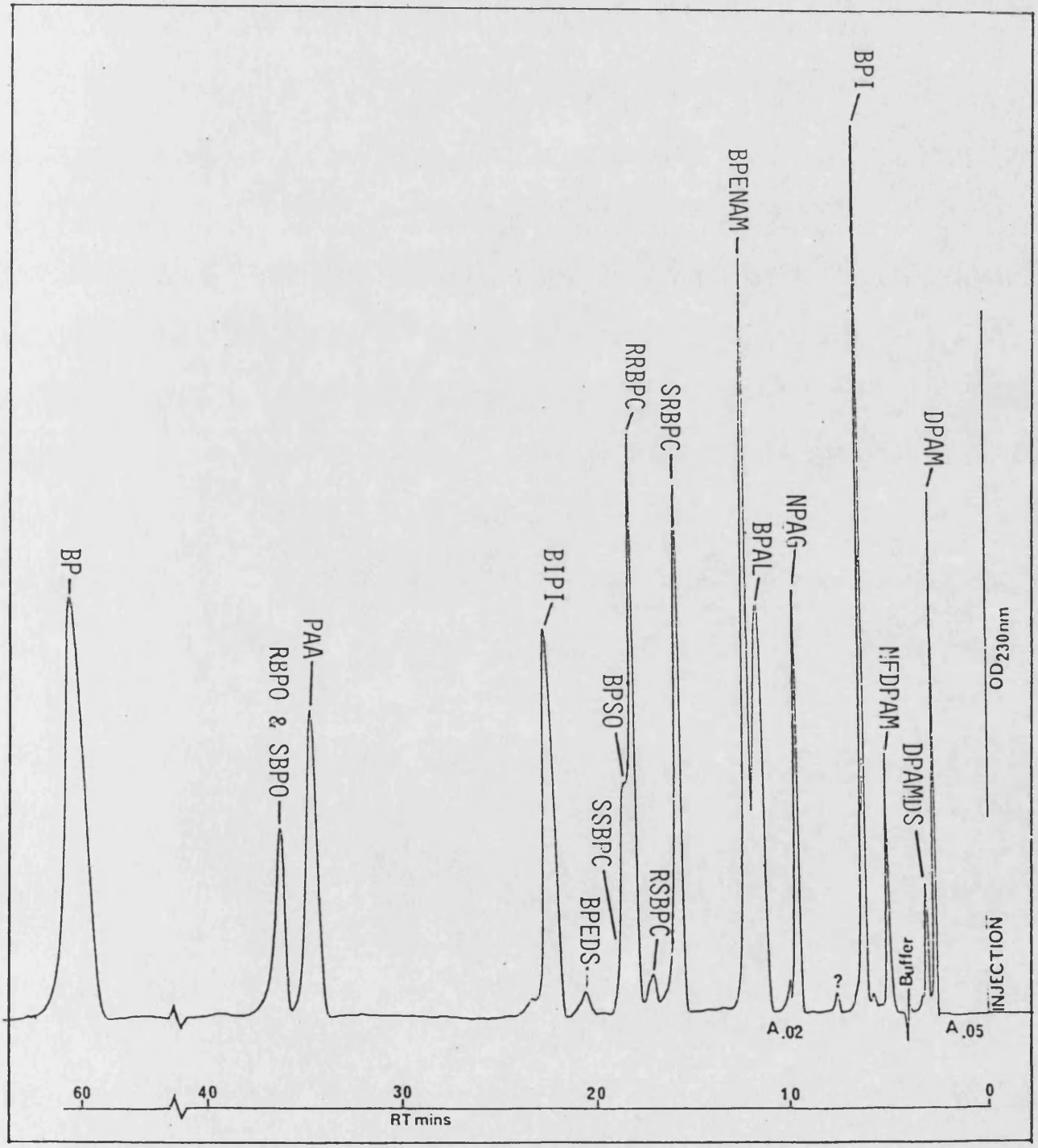
The elution profile of benzylpenilloaldehyde is optimal in mobile phases containing tetrahydrofuran, otherwise it would interfere in the quantification of degradation products with similar retention times.
A consequence of this statement is that separate analytical methods must be used for:

(a) monitoring of the epimerisation of benzylpenilloic acids which are only separated with relatively high levels of THF

(b) for the analysis of benzylpenicillenic acid which was irreversibly bound using low concentrations of THF.

The use of gradient elution could remedy the latter deficiency however there is a price to be paid in re-equilibration of the column with initial mobile phase, before the next sample may be analysed. Mobile phases containing more than one organic modifier were not studied. A typical chromatogram of the benzylpenicillin degradation products developed under optimum conditions is given in Figure 9.

Figure 9 Chromatogram of benzylpenicillin degradation products by buffered RPHPLC. Each analyte, approximately 2 µg on-column except BPI and BIPI (200 ng each).Conditions: column 2, eluted with THF : 0.05M phosphate buffer (pH 3.9) 5 : 95 v/v at 1 ml/min. Injection volume 20 µl, temperature 25°C and uv detection @ 230nm (0.05 and 0.02 AUFS).



6 ^{1}H AND ^{13}C NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPIC ANALYSIS OF BENZYLPENICILLIN, ITS DEGRADATION PRODUCTS AND RELATED COMPOUNDS.

6.1 Introduction

The ^{1}H and ^{13}C NMR characteristics of most of the penicillin and cephalosporin antibiotics in world-wide clinical use are exhaustively discussed in recent reviews.[137] [138] The influence of solvent and ionisation state upon spectral parameters and the potential of NMR to study degradation, stereochemistry, biological structure-activity relationships, association and protein binding of β-lactams is described. Data on the degradation products of benzylpenicillin obtained during the course of investigations described in this thesis were included and acknowledged.

6.1.1 ^{1}H NMR characteristics of benzylpenicillin

The absolute stereochemistry and ring-numbering system for benzylpenicillin 5 are given in Figure 10.

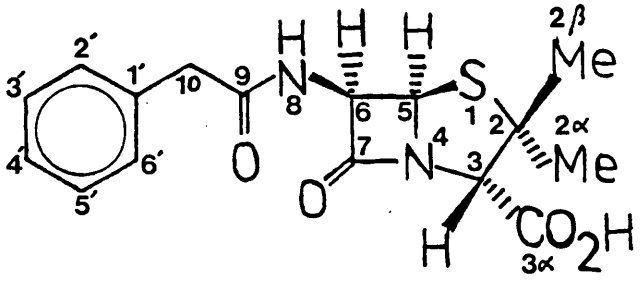


Figure 10 (3S,5R,6R)-Benzylpenicillin 5

Collations of the ^1H NMR features of penicillins and cephalosporins have been previously reported.[139] [140] The 270 MHz ^1H FT NMR spectrum of benzylpenicillin sodium in D_2O was recorded and is shown in Figure 11.

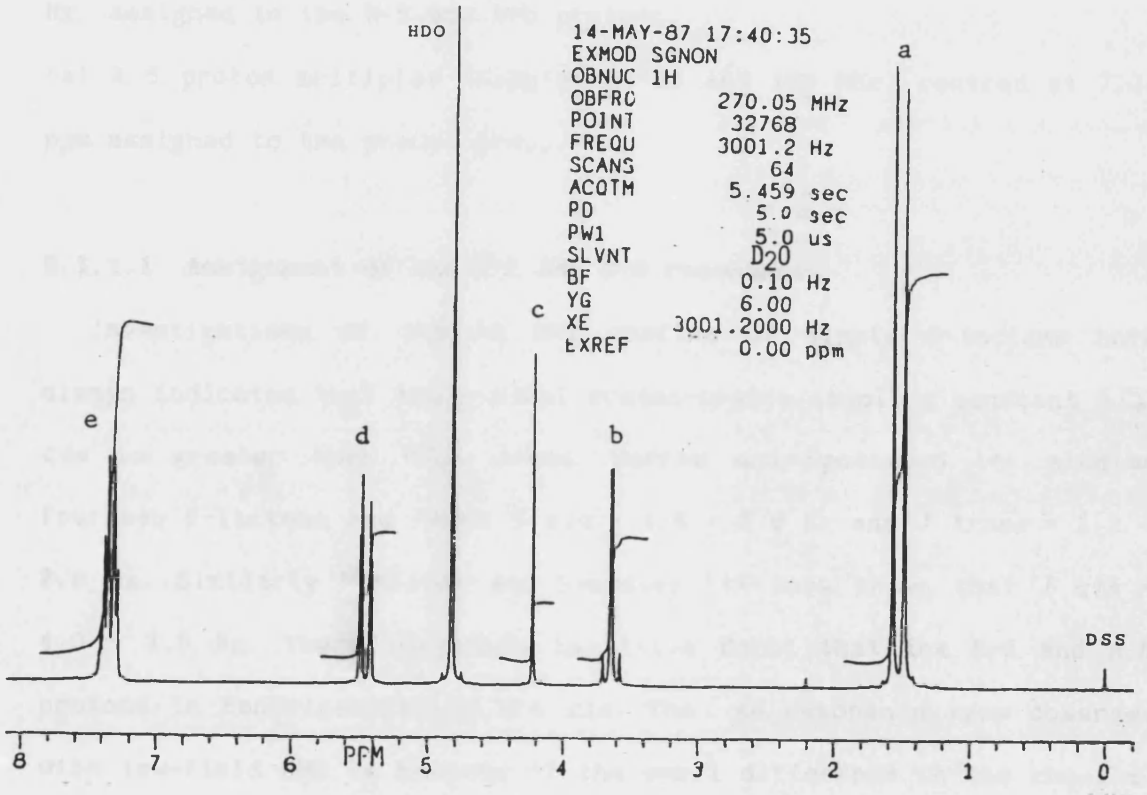


Figure 11 270 MHz ^1H NMR spectrum of benzylpenicillin Na in D_2O (experimental conditions as in section 6.2.1)

The main features are:

(a) Two 3 proton singlets at 1.48 ppm and 1.57 ppm, assigned to the 2α-and 2β- *gem*-dimethyl groups.

(b) A 2 proton 1:7:7:1 AB quartet (singlet at 60 and 100 MHz) centred at 3.64 ppm with a coupling constant of 15.0 Hz, assigned to the non-equivalent benzylic CH_2 protons.

(c) A 1 proton singlet at 4.23 ppm due to the H-3 resonance.

(d) Two 1 proton doublets (a doublet of doublets at 60 and 100 MHz) centred at 5.42 ppm and 5.49 ppm, both with coupling constant of 3.85 Hz, assigned to the H-5 and H-6 protons.

(e) A 5 proton multiplet (singlet at 60 and 100 MHz) centred at 7.34 ppm assigned to the phenyl group.

6.1.1.1 Assignment of the H-5 AND H-6 resonances

Investigations of the ^1H NMR spectra of simple β-lactams have always indicated that the vicinal proton-proton coupling constant $^3J_{HH}$ *cis* is greater than $^3J_{HH}$ *trans*. Barrow and Spotswood [141] studied fourteen β-lactams and found *J cis* = 4.9 - 5.9 Hz and *J trans* = 2.2 - 2.8 Hz. Similarly McMillan and Stoodley [142] have shown that *J cis* = 4.0 - 4.5 Hz. Therefore there is little doubt that the H-5 and H-6 protons in benzylpenicillin are *cis*. The AB resonance type observed with low-field NMR is because of the small difference in the chemical shift of H-5 and H-6, and this has hindered the unambiguous assignment of these protons for benzylpenicillin in D_2O.

Green *et al* [139 143] assigned the high field resonance at 5.43 ppm to the H-5 proton and the low field resonance at 5.54 ppm to the H-6 proton (presumably by comparison of the ^1H NMR spectra of penicillins in organic solvents, in which coupling between H-6 and the non-ionised amide proton is observed). However evidence from studies on the biosynthesis of deuterated benzylpenicillin reversed this assignment.

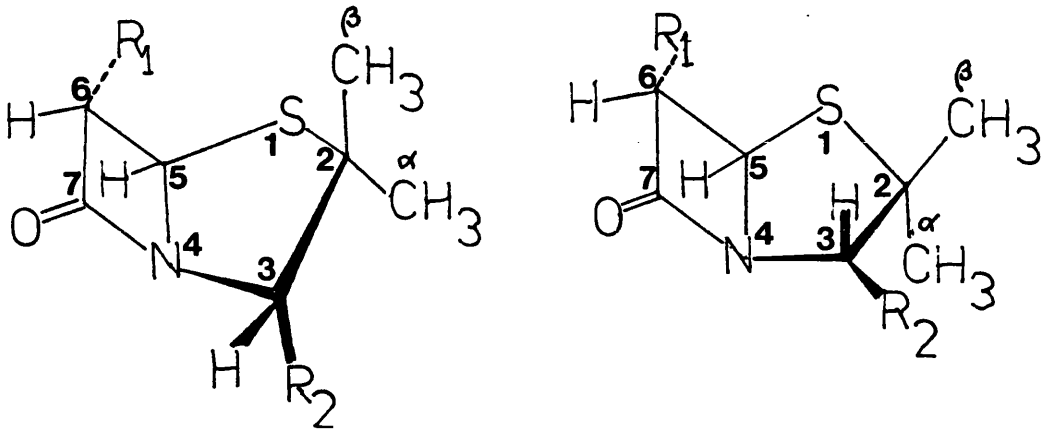
Carlstedt *et al* [144] noted that in the ^1H NMR spectrum of benzylpenicillin potassium deuterated at C-6, the high field doublet is absent and the doublet at 5.53 ppm is replaced by a singlet at 5.50 ppm. Later work [145] showed that in the spectrum of the N-ethylpiperidyl ester of benzylpenicillin (deuterated at C-6) in $CDCl_3$, a lack of splitting is observed for the amide proton.

The assignment of the H-5 and H-6 protons is complicated by studies suggesting non-uniform concentration [146] dependence observed for the β-lactam protons (the H-5, H-6 proton resonances coalesce at 1 M concentration) and the effects of solvents. [147] It is reasonable to assume a pH dependence, although no studies have been reported.

6.1.1.2 Assignment of the 2α- and 2β-methyl resonances

The stereochemistry of the thiazolidine ring and the assignment of the 2α- and 2β-methyl signals of phenoxymethylpenicillin methyl ester have been established by a combination of nuclear Overhauser enhancement (NOE) effects and chemical shift data. [148] The two possible conformations of the thiazolidine ring of the penicillin nucleus are shown in Figure 12 (see overleaf).

First, the amide H-8 and ring protons H-3, H-5 and H-6 were assigned from their expected splitting patterns (in CCl_4 and C_6D_6). In (a) the 2α and 2β-methyl groups lie close to H-3, whereas in (b) only the 2β-methyl protons are in close proximity to H-3. Thus the β-methyl protons in either conformation should contribute to the intramolecular relaxation of H-3, while the α-methyl protons should relax H-3 in conformation (a) only.



(a) (b)

Figure 12 (R_1 = $PhOCH_2CONH$- R_2 = $MeCO_2$-)

In addition H-5 should be relaxed by the 2α-methyl in conformation (b) but not in (a). Results from the NOE experiments show that penicillin prefers conformation (a) i.e. H-3 signal relaxed by both α and β methyls, H-5 influenced by neither. A further conclusion is that the low-field methyl signal is due to the β-group which is *cis* to H-3, while the high-field signal is due to the α-group *trans* to H-3 and *cis* to H-5.

Evidence from paramagnetic shift studies (section 6.1.3) confirm this assignment for benzylpenicillin in D_2O solution. It should be noted that paramagnetic shift (section 6.1.3) and solid-state ^{13}C NMR studies (section 6.1.4) both indicate that in aqueous solution, the thiazolidine ring puckers of benzylpenicillin are of similar energy and are rapidly interconverting.

6.1.2 ^{13}C NMR characteristics of benzylpenicillin

The 67.8 MHz ^{13}C FT NMR proton noise decoupled and DEPT spectra of benzylpenicillin sodium in D_2O are given in Figure 13.

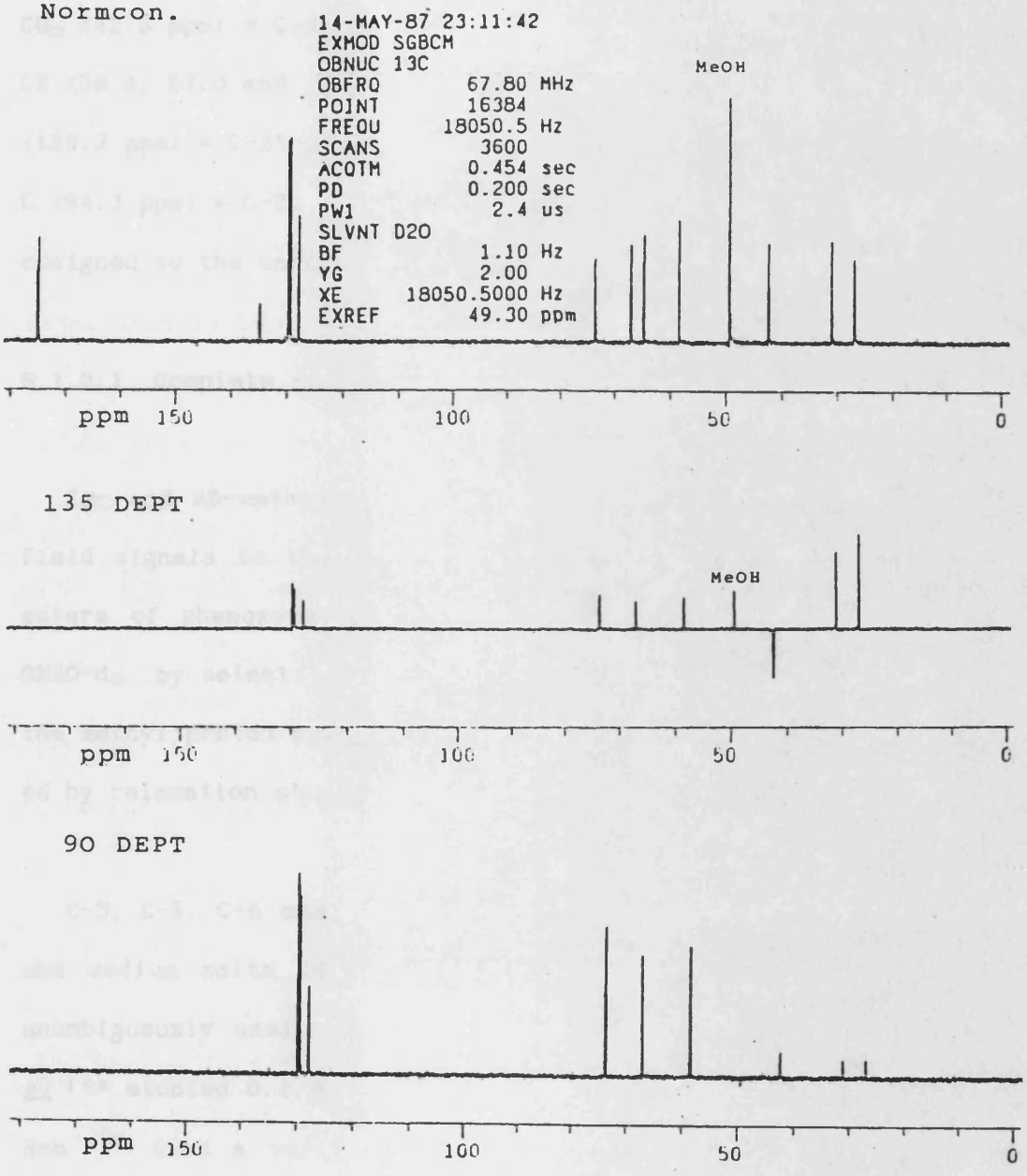


Figure 13 The 67.8 MHz ^{13}C FT NMR proton noise decoupled (NORMCON) and DEPT spectra of benzylpenicillin sodium in D_2O (experimental conditions as in section 6.2.1)

The use of DEPT pulse sequences allows one to readily distinguish between methyl, methylene, methine and quarternary carbons.

Preliminary assignments are straightforward, as follows;

CH_3 (26.8 and 30.9 ppm) = C-2α and C-2β.

CH_2 (42.3 ppm) = C-10.

CH (58.4, 67.0 and 73.5 ppm) = C-3, C-5 and C-6, (127.9 ppm) = C-4', (129.3 ppm) = C-3' and C-5', (129.7 ppm) = C-2' and C-6'.

C (64.7 ppm) = C-2, (134.8 ppm) = C-1' and (173.9, 174.8 & 175.0 ppm) assigned to the carbonyls C-3α, C-7 and C-9.

6.1.2.1 Complete assignment of the ^{13}C NMR spectrum

2α- and 2β-methyls; Archer *et al* [149] assigned the high- and low-field signals to the 2α- and 2β-methyls, respectively, in the methyl esters of phenoxymethylpenicillin **9** and its 6-acetomido analogue in DMSO-d_6 by selective proton irradiation based on prior assignments of the methyl proton resonances. [148] These assignments have been confirmed by relaxation studies (section 6.1.3) of benzylpenicillin in D_2O.

C-3, C-5, C-6 and carbonyls; the ^{13}C NMR spectrum of the potassium and sodium salts of benzylpenicillin in D_2O and DMSO-d_6 have been unambiguously assigned (apart from the C-2α, β-methyls). Mondelli *et al* [150] studied 0.2 M solutions of the sodium salt in D_2O and Chang and Hem [151] used a variety of techniques and stronger solutions of the potassium salt in D_2O and DMSO-d_6.

Using low power selective (single frequency) proton decoupling the carbonyls assignment are straightforward. The C-3α carboxylate signal

appears as a doublet because of two-bond coupling with H-3. The C-7 β-lactam carbonyl gives a three doublet splitting pattern due to the couplings with H-3, H-5 and H-6. The amide carbonyl C-9 gives a triplet doublet in DMSO-d_6 and a broad triplet in D_2O due to coupling with C-10 methylene protons and long distance coupling with H-6.

Because of the uncertainty in the assignment of H-5 and H-6 direct measurements of the ^{13}C-^{1}H coupling were obtained by proton coupled spectra in 'gated' decoupling mode. Based on the electronegativity of adjacent groups it is reasonable to predict relative coupling constant magnitudes in the order $^{1}J_{C5-H5}$ (C-5 adjacent to sulphur and nitrogen) > $^{1}J_{C6-H6}$ > $^{1}J_{C3-H3}$. This was seen in practice and the method provides unambiguous assignment of C-3, C-5 and C-6.

The ^{13}C NMR data has been summarised in Table 10 (see next page)

Table 10, ^{13}C NMR chemical shifts (p.p.m.) of (3S,5R,6R)-Benzylpenicillin 5 in solution and solid-state

Salt	Conc,	Solvent Operating	Reference frequency	C-2	C-2α	C-2β	C-3	C-3α	C-5	C-6	C-7	C-9	C-10	C-1'	C-2' C-6'	C-3' C-5'	C-4'	Ref.
Na	0,02M	D_2O 67,8 MHz	Methanol 49,3ppm	64,7	26,8	30,9	73,5	174,8	67,0	58,4	175,0	173,9	42,3	134,8	129,7	129,3	127,9	This work
Na	0,20M	D_2O 25,18MHz	Dioxan 67,4ppm	65,2	27,3 (130)	31,7 (130)	73,9 (145)	174,7* [4,0] H-3	67,4 (178)	58,9 (153)	175,3 [16] H-5+H-6 (5,0) H-3	174,1* [6,5] H-10 (2,0) H-6	48,2 (130)	135,3	130,0	129,7	128,2	150
K	2,0M	D_2O	Methanol 49,0ppm	64,6 m	26,9 bq (127,8)	31,1 bq (128,0)	73,5 bd (146,7)	174,1 d [4,3] H-3	66,9 bd (178,2)	58,1 bd (153,8)	174,7 ddd [5,1] H-6 (7,0)H-5,(9,2)	173,1 bt [6,7] H-10 H-3	42,3 t (129,7)	134,7 bs [4,9] H-3' H-5'	129,5 bd (158,1)	128,9 dd (160,2) [6,6] H-3',5'	127,3 bd (161,1)	151
K	1,0M	DMSO-d^6	DMSO 39,6ppm	64,4 m	27,5 qm (127,6)	31,8 qm (127,6)	74,0 dm (140,4)	169,8 d [4,2] H-3	66,9 dt (175,4) [3,8] H-3,H-6	57,9 dd (151,0) [1,5] H-5	172,7 ddd [4,9] H-6 (7,0)H-5,	170,4 td [6,4] H-10 (9,8)H-3	41,6 bt (129,4)	135,8 m	129,8 dm (157,3)	129,0 dd (159,9) [4,9] H-3',5'	126,4 dm (163,0)	151
K + 0,3M La^{III}	0,05M	D_2O,pH4,4	Dioxan 67,4ppm	65,1	26,8	31,4	73,6	-	67,4	59,1	-	-	42,6		(Aromatics ~128,4) (Carbonyls ~172,4)			153
Na	Solid		Adamantane 29,23ppm	63,9	27,0	35,7	73,9 (65)	170,7	68,9 (55)	60,3 [115]	175,3 (60)	166,1 (85)	43,9	136,9	129,2 130,0	131,0	127,4	154
K	Solid		Adamantane 29,23ppm	64,6	26,4	37,0	74,4 (70)	171,7	67,7 (65)	60,1 [105]	overlap		42,6	135,8	b	b	127,9	154

Notes; b = broad resonance; d = doublet, t = triplet, q = quartet, m = multiplet resonances
(one bond), [two bond], (three bond) ^{13}C-^{1}H coupling constants in Hz, (one bond), [two bond] ^{13}C-^{14}N coupling constants in Hz,
* Measured at 0,8M as the shifts were equivalent at 0,2M

6.1.3 Paramagnetic shift studies of benzylpenicillin

In the presence of certain paramagnetic ions the observed spin-spin transverse relaxation times (T_2) provide information on molecular structure. Protons closest to the metal ion are preferentially broadened due to efficient relaxation by a dipolar mechanism and indicate the site of co-ordination.

Studies of the interaction between Mn^{2+} and Cu^{2+} with benzylpenicillin in D_2O (0.1M, pH 5.5, 25°C) by ^1H NMR, [152] indicate that the metal ion complexes through the ring nitrogen and the carboxy group, rather than involving the side chain (i.e. H-3 broadened and no appreciable effect on benzylic CH_2). A comparison of measured and experimentally determined proton-metal distances indicated:

(a) The conformation of benzylpenicillin in D_2O slightly favours that in Figure 12 (b).

(b) In either conformation the high- and low-field methyl resonances can be assigned to 2α-CH_3 and 2β-CH_3 respectively, thus confirming studies previously mentioned.

(c) The high-field doublet can be assigned to H-5, the low-field to H-6. Using the reverse assignment no meaningful site of co-ordination was apparent.

^1H and ^{13}C NMR studies of benzylpenicillin potassium in D_2O (50 mmol, pH 4.4, 25°C) using lanthanide shift reagents [153] indicated that the two possible conformations of the thiazolidine ring are similar in energy. In addition ^{13}C NMR assignments mentioned previously were confirmed (Table 10). ^1H NMR experiments assigned the high- and low-field methyl resonances (1.48 and 1.57 ppm) to the 2α- and 2β-methyls

respectively. However the high- and low-field doublet resonances (5.42 and 5.49 ppm) were assigned to H-6 and H-5 respectively. The carboxylate group was found to be the binding group for the lanthanides. NOE experiments did not permit assignment of 2α- and 2β-CH_3 nor H-5 and H-6. It is obvious that the assignments of H-5 and H-6 remain ambiguous.

6.1.4 Solid-state ^{13}C NMR studies

The 50.32 MHz ^{13}C cross-polarization-magic angle spinning (MAS) spectra for crystalline penicillins have been reported recently. [154] The assignment of resonances in the spectra of benzylpenicillin Na and K salts was first made by comparison with previously reported solution ^{13}C NMR data [150] (see Table 10). The similarity of chemical shifts permitted the immediate assignment of the well-resolved C-2, C-3, C-5, C-6, C-2α-Me and C-2β-Me resonances. The use of non-quaternary suppression (NQS) pulse sequences, (in which protonated carbon resonances, with the exception of the rapidly spinning methyl groups, are suppressed) permitted assignment of C-1'. Of the three carbonyl resonances (C-3α, C-7 and C-9), two are apparent doublets due to incompletely suppressed ^{13}C-^{14}N dipolar interactions and the third is a sharp singlet. Hence the singlet can be directly assigned to C-3α.

Distinction between C-7 and C-9 was achieved by comparison with the spectrum of 6-aminopenicillanic acid 31, (C-7 @ 167·1ppm, ^{13}C-^{14}N splitting 60 Hz). The magnitude of the splitting for a wide range of penicillins was found to between 55 and 70 Hz and is diagnostic of the β-lactam carbonyl.

Two aspects of interest emerged from the work. First, dynamic behaviour of the benzyl side-chain was evident in the spectra and shown to depend critically on the crystalline enviroment of the penicillin molecule. Second, by correlating chemical shifts, in particular the 2β-Me, it was possible to predict features in the solid - state conformations of unknown crystal structure. Further, on comparison with solution spectra, the existence of rapidly interconverting thiazolidine ring puckers in solution was suggested and their relative populations were estimated for benzylpenicillin Na. as 68% (Fig.12a) and 32% (Fig.12b).

6.1.5 Miscellaneous NMR studies of benzylpenicillin

The interaction of benzylpenicillin with guanosine in DMSO [155] and the binding of benzylpenicillin with bovine serum albumin [156] has been reported. The ^{15}N resonances of benzylpenicillin methyl ester [157] have been detected at natural abundance level and structure-chemical shift correlations ascertained.

6.1.6 NMR studies of the degradation of benzylpenicillin

There are numerous references to the use of NMR to identify and characterise penicillin degradation products. The ^{1}H NMR features of penicilloic 16 and penilloic acids 17, [78] [158] derived from a number of penicillins and the ^{1}H and ^{13}C NMR features of benzylisopenillic acid 56 [159] have been reported. Hem *et al* [73] have used ^{1}H and ^{13}C NMR to follow the epimerisation of benzylpenicilloic acid 41 in basic media.

Mitsumori *et al* [160] have monitored the spontaneous degradation of benzylpenicillin in aqueous solution by ^1H NMR using a correlation technique. Feeney *et al* [61] followed the degradation of benzylpenicillin (at pH 2.5 and 37°C) by 270 MHz ^1H FT NMR. The chemical shifts of benzylpenillic **42**, benzylpenamaldic **44**, (3S,5R,6R)- **41A** and (3S,5S,6R)-benzylpenicilloic **41B** and benzylpenilloic acids **43** are reported. Their conclusions are discussed in detail in the Introduction (pp 30 -33).

6.2 Experimental

^1H and ^{13}C NMR spectra were recorded on a variety of instruments (full experimental details are listed below). Samples were prepared in 5mm o.d. tubes as approximately 5% w/v solutions in D_2O or $CDCl_3$. For the ^1H NMR spectra, D_2O solutions were referenced to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate, δ_H = 0.00 ppm). $CDCl_3$ solutions were referenced to TMS (tetramethylsilane, δ_H = 0.00 ppm).

For the ^{13}C NMR spectra, D_2O solutions were referenced externally to a fine TMS capillary (δ_C = 0.00 ppm), or for more accurate measurements referenced internally to methanol (δ_C = 49.3 ppm). For $CDCl_3$ solutions TMS was used as reference (δ_C = 0.00 ppm). External TMS was not used as a reference for ^1H spectra as the errors involved in their use are comparatively much greater than for ^{13}C studies. Comparison of data in Table 11 shows that a TMS capillary results in values approximately 1 ppm down-field compared to a true internal reference.

For Fourier Transform (FT) NMR experiments the deuterium of the solvent provided the lock signal.

Assignments were established by comparison with published data and/or on the basis of expected chemical shifts and signal multiplicity. ^1H assignment was aided by spin decoupling experiments where appropriate. ^{13}C assignment was aided by the use of insensitive nuclei enhanced by polarisation transfer (INEPT) and distortionless enhancement by polarisation transfer (DEPT) pulse sequences, allowing the distinction between ^{13}C atomic groups. A ^{13}C-^1H 2D correlated spectroscpy (COSY) experiment was conducted to aid the assignments of the

spectra of benzylpenicillin sodium. These techniques have been recently reviewed. [161]

Alkali metal salts of the compounds were freely soluble in D_2O, while solutions of the free acid were promoted by the addition of the minimum amount of sodium hydrogen carbonate. 6-Aminopenicillanic acid was dissolved in D_2O by the addition of the minimum amount of dilute DCl and analysed immediately (the use of bicarbonate resulted in an as yet unexplained reaction).

6.2.1 Experimental procedures

60 MHz ^1H NMR Continuous Wave (CW) spectra were recorded on a Jeol JNM-PMX 60 SI instrument at ambient temperature using a sweep width of 600 Hz (sweep time 250 seconds) and a 20 Hz filter.

22.5 MHz ^{13}C FT NMR spectra were recorded on a Jeol FX 90Q instrument. Proton noise-decoupled spectra with Nuclear Overhauser Enhancement NOE were recorded with 8 K data points at a probe temperature of 23°C. For an average spectral width of 5000 Hz, a 4 μs pulse corresponding to a tilt angle of 30 degrees was employed with a 1.8192 second interval (acquisition time plus 1 second pulse delay) between pulses. The normal spectra was supplemented by INEPT pulse sequences (90 degree pulse, 500 Hz) set to measure CH, CH_3 and CH_2 (inverted) [PRD = $\frac{1}{4}J \simeq 5.1 - 6.0$ ms] or CH alone [PRD = $\frac{1}{8}J \simeq 3.4 - 4.0$ ms]

67.8 MHz ^{13}C FT NMR spectra were recorded on a Jeol JNM-GX-270 instrument. Proton noise-decoupled spectra with NOE were recorded with

16K data points, zero filled to 32K, at a probe temperature of 22°C. For an average spectral width of 18000Hz, a 2.4μs pulse corresponding to a tilt angle of 30° was employed with a 0.654 second interval (acquisition time plus 0.2 second pulse delay) between pulses. The normal spectra were supplemented by DEPT pulse sequences set to measure CH, CH_3 and CH_2 (inverted) [pulse angle = 135° DEPT] or CH alone [pulse angle = 90° DEPT].

270 MHz ^1H FT NMR spectra were recorded on a Jeol JNM-GX-270 instrument. 32K data points were used in acquiring spectra with widths of 3001.2 Hz at a probe temperature of 22°C. A 5 μs pulse corresponding to a tilt angle of 30° was employed with a 5.459 second interval (acquisition time plus 0.541 second pulse delay) between pulses. A filter of 3000 Hz was employed and spectral resolution was .18 Hz. Some experiments were carried out using pulse delays of 5 seconds to give more accurate measurements. The ^{13}C-^1H 2D COSY experiment was performed by Dr Sarah Branch, Department of Pharmacy & Pharmacology, University of Bath.

400 MHz ^1H FT NMR spectra were recorded on a Jeol-GX400 instrument (see section 12.3.3/4 for full experimental details). The 400 MHz ^1H FT NMR spectra of benzylpenilloic acid were recorded by the SERC WH-400 NMR spectroscopic service of the Department of Chemistry, University of Warwick, using a Bruker Spectrospin WH-400 instrument.

6.3 Results and discussion

The ^{1}H NMR and ^{13}C NMR data for benzylpenicillin, its degradation products and related compounds are listed in Tables 11 and 12 respectively, located at the end of the NMR section (pp 111 - 117).

6.3.1 (3S,5R,6R)-Benzylpenicillin 5 : $^{1}H,^{13}C$ COSY experiment

The 2D-$^{13}C,^{1}H$ correlation diagram of benzylpenicillin sodium in D_2O is given in Figure 14. The complete and unambiguous assignment of the ^{13}C spectrum obtained using a variety of techniques was described in section 6.1.2. The 2D spectrum (see Table 13) confirms this assignment and now allows the unambiguous assignment of all ^{1}H signals.

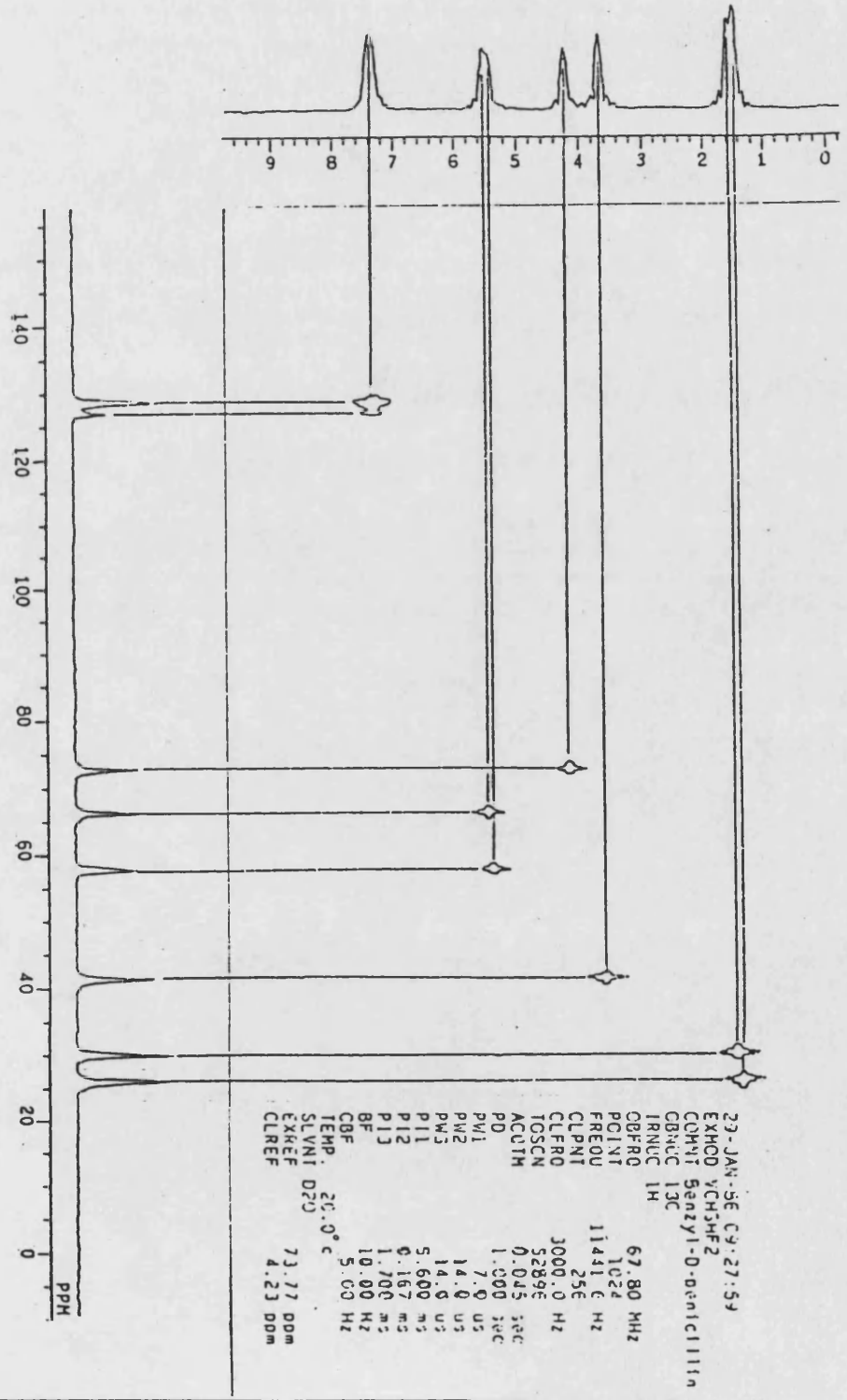
Table 13 ^{1}H-^{13}C chemical shift correlations for benzylpenicillin

δ_C ppm	δ_H ppm	Proton Assignment
26	1.5 (high field singlet)	H_3-2α
30	1.5 (low field singlet)	H_3-2β
41.5	3.6	H_{2-10}
57.5	5.5 (high field doublet)	H-6
66	5.5 (low field doublet)	H-5
72.5	4.2	H-3
126-130	7.2-7.5	* Aromatics

* The digital resolution used for the 2D spectrum led to a loss of the ^{1}H multiplet structure and the fine detail of the ^{13}C-phenyl signals.

Figure 14 2D-^{13}C,^{1}H correlation spectrum of benzylpenicillin Na in D_2O

The abscissa shows the δ_C values and the ordinate, the δ_H values. The 1D-^{1}H and the ^{1}H-decoupled 1D-^{13}C NMR spectra are plotted along the respective frequency axes.

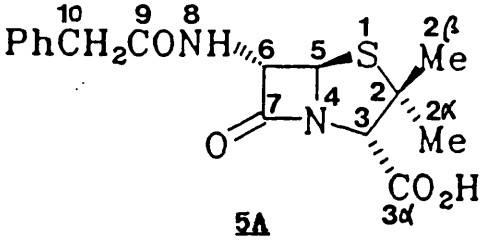


The ^1H assignment of the 2α-methyl to the high-field singlet and the 2β-methyl to the low field singlet validate the assignments made by analogy to NOE studies.[148]

The assignment of the low-field doublet to H-5 and the high field doublet to H-6 confirm the results published from biosynthesis [144] [145] and lanthanide shift reagent [153] studies.

Some preliminary NOE studies were attempted but proved inconclusive, presumably because in D_2O the thiazolidine ring puckers are rapidly interconverting [154] and any signal-enhancement is time-averaged out.

6.3.2 (3S,5R,6S)-Benzylpenicillin 5A



5A

The preparation and ^1H NMR characteristics of 6-*epi*-benzylpenicillin potassium in D_2O has been described.[81] The doublets at 4.79 ppm and 5.24 ppm were assigned to H-6 and H-5 respectively. No expla nation was offered and in the same paper H-5 and H-6 of benzylpenicillin are now known to be mis-assigned.

The ^1H and ^{13}C spectra were recorded using material kindly supplied by Professor Vanderhaeghe, however not enough material was available for 2D-^1H,^{13}C COSY experiments, therefore the unambiguous assignments of CH-6 and CH-5 remains in doubt.

6-*epi*-Benzylpenicillin shows the expected decrease in the ^3J value of H-5 to H-6 coupling to 1.65 Hz, characteristic of *trans* protons in

β-lactams. The specific assignments of the 2α and 2β-methyls are made by analogy to benzylpenicillin. It is noteworthy that the benzylic CH_2 protons of benzylpenicillin are non-equivalent and produce an AB quartet (^1J = 15Hz) when examined by 270 MHz ^{1}H NMR. Under similar conditions the CH_2 of 6-*epi*-benzylpenicillin give a singlet. No explanation for this observation is offered.

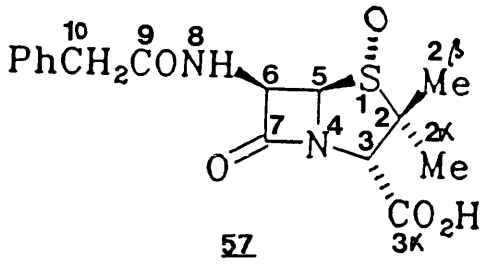
The C-3α, C-7 and C-9 carbonyl resonances are assigned by analogy to benzylpenicillin. Literature values for the ^{1}H NMR characteristics of the 4 possible epimers of (3S)-benzylpenicillin are given in Table 14. No data for the 3S,5S,6S epimer (as a salt) has been reported.

Table 14 ^{1}H NMR data for epimeric 3S-Benzylpenicillins and methyl esters

Configuration	CH_3-2α	CH_3-2β	H-3	H-5	H-6	NHCO	$PhCH_2$	Ph	OCH_3	Reference
5R,6R Na salt	1,48s	1,57s	4,23s	5,49d (3,85)	5,42d (3,85)	-	3,64q (15)	7,29-7,39m	-	this study
Me ester	1,45s	?	4,37s	5,48d (4,0)	5,64dd (4,0,8)	6,35d (8)	3,61s	7,30s	3,73s	81
5R,6S K salt	1,49s	1,56s	4,30s	[4,79d & 5,24d] (1,6)	(1,6)	-	3,62s	7,34s	-	81
Me ester	1,42s	1,57s	4,44s	5,12d (1,8)	5,01dd (1,8,8)	6,92d (8)	3,57s	7,27s	3,70s	81
5S,6S Me ester	1,30s	1,58s	3,78s	5,21d (4,0)	5,51dd (4,0,9)	6,40d (9)	3,61s	7,35s	3,74s	70
5S,6R Na salt	1,44s	1,57s	3,63s	[4,82d & 5,10d] (2,0)	(2,0)	-	3,63s	7,31s	-	162
Me ester	1,38s	1,55s	3,69s	5,03d (2,0)	4,78dd (2,0,7)	7,20br	3,50s	7,20s	3,71s	162

Notes; Methyl esters ($CDCl_3$, TMS), salts (D_2O), d = doublet, q = quartet, br = broad resonance

6.3.3 (3S,5R,6R)-Benzylpenicillin-1S-sulphoxide 57



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The ^1H NMR spectra and configuration of phenoxymethylpenicillin-sulphoxide methyl ester (formed by metaperiodate oxidation of the parent sulphide) in organic solvents has been established by a combination of NOE and chemical shift data. [148] The low and high field methyl signals were assigned to 2β- and 2α-methyl groups respectively.

Systematic differences in chemical shift values occurred between a given penicillin and the sulphoxide derivative i.e. 2α-methyl and H-5 protons are shielded (upfield shift) and 2β-methyl, H-3 and H-6 protons deshielded (downfield shift) by a syn-axial effect in the sulphoxide relative to the corresponding chemical shifts of the sulphide.

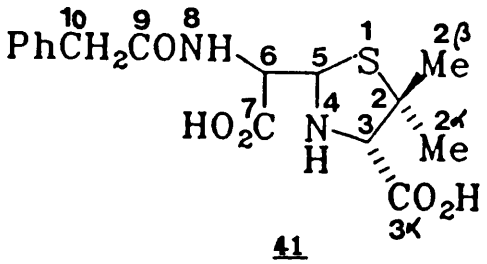
Similar effects in the spectra of phenoxymethylpenicillin and its sulphoxide in aqueous solution have been observed.[153] Aromatic solvent -induced shifts, hydrogen-bonding studies and X-ray crystallography [148,163] indicate the sulphoxide configuration to be S (*cis* to H-3). The assignment was confirmed by other workers [164] and the described chemical shift differences are not observed to the same extent where sulphoxide configuration is R. In addition strong intramolecular hydrogen-bonding between the amide proton and the oxygen of the (S)-sulphoxide (leading to large downfield-shift of the NH proton) is not observed for the (R)-sulphoxide where the distance between the two

groups would be too great for the existence of a hydrogen-bond of more than nominal strength.[165]

Comparison of the ^1H NMR chemical shift data for benzylpenicillin sodium and its sulphoxide in D_2O indicate that the configuration of the sulphoxide is (S) and assignment of C-2α, C-2β-methyls, H-5 and H-6 is made by analogy.

The assignment of the ^{13}C NMR spectrum is established by comparison with published ^{13}C characteristics of some penicillins and related sulphoxides.[149] It is evident that C-2 and C-5 (carbons which flank sulphur) resonances more downfield and both 2α and 2β-methyl resonances move upfield relative to the shifts in the parent sulphide (see Table 11 for details).

6.3.4 (3S,5R/S,6R/S)-Benzylpenicilloic acids 41A-D



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Samples of (3S,5R,6R)- and (3S,5S,6R)-benzylpenicilloic acids were obtained by the base hydrolysis of benzylpenicillin. Although isomerically pure samples could not be obtained, samples in which the 5R,6R and 5S,6R epimers preponderated allowed assignment of all resonances and the ^{13}C and ^1H nmr data are close to those reported by Ghebre-Sellassie *et al*.[73]

The ^1H and ^{13}C spectra of the 5R,6S and 5S,6S epimers (obtained by base hydrolysis of 6-*epi*-benzylpenicillin in D_2O-NaOD solution) were

recorded and assignments made in a similar fashion although the resonances for C-6 and CH_2 were not observed due to deuteration.

Care should be exercised in the interpretation and comparison of the ^1H NMR data, especially of ionic compounds in which pH, ionic strength and concentration are not the same. Fortunately during base hydrolysis of benzylpenicillin, benzylpenicillin, 6-*epi*-benzylpenicillin and all four 3S-benzylpenicilloic acid epimers are present (see section 12) allowing valid comparison of the ^1H NMR data (see Table 15). The data in Tables 11 and 15 are similar and discrepancies lie within experimental error

Cleavage of the β-lactam ring results in systematic chemical shift changes. The assignment of the 2α and 2β-methyls is made by analogy to intact benzylpenicillin. Both methyl signals experience an upfield shift and the separation of the signals is enhanced. The H-3, H-5 and H-6 resonances move upfield. The magnitude of the vicinal $^3J_{H5-H6}$ couplings of the benzylpenicilloic acids are in the order 5R,6R > 5R,6S ≈ 5S,6S > 5S,6R and confirm *cis* coupling for the 5S,6R epimer (6 Hz) and *trans* coupling for the 5S,6R epimer (3 Hz). However, due to free rotation between the C-5 and C-6 carbons the ^3J couplings of the 5R,6S and 5S,6S epimers have the intermediate magnitude of about 4.5 Hz. The chemical shifts of the benzylic group are unaffected. Similar effects are observed in the ^1H literature data [70] for the dimethylbenzylpenicilloates (literature data included in Table 15).

Cleavage of the β-lactam ring results in little ^{13}C chemical shift changes. The separation of the methyl resonances is reduced. Assignment of the 2α and 2β-methyls is made by analogy to benzylpenicillin and this is assumed to be valid for all other degradation products.

Table 15. ^{1}H NMR characteristics of (3S,5R,6R)-, (3S,5R,6S)-benzylpenicillin and the (3S)-benzylpenicilloic acid epimers

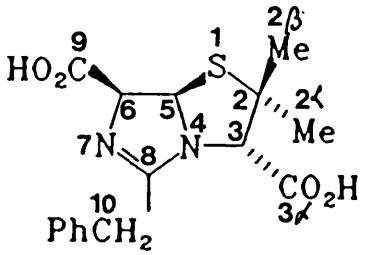
COMPOUND	CH_3-2α	CH_3-2β	H-3	H-5	$^3J_{H-5,H-6}$ (Hz,)	H-6	Ph-CH_2	Ph
Benzylpenicillins and benzylpenicilloic acids (Operating frequency 400 MHz, solvent D_2O, reference HOD at δ_H 0,00 ppm)								
(3S,5R,6R)-Benzylpenicillin 5	1,48s	1,56s	4,23s	5,49d	(3,9)	5,41d	3,65q (^1J 15 Hz)	(7,3 - 7,6)m
(3S,5R,6S)-Benzylpenicillin 5A	1,45s	1,53s	4,28s	(5,23d	(1,7)	4,82d	3,70q (^1J 15 Hz)	"
(3S,5R,6R)-Benzylpenicilloic acid 41A	1,20s	1,48s	3,40s	5,03d	(6,1±0,2)	4,23d	3,65q (^1J 15 Hz)	"
(3S,5S,6R)-Benzylpenicilloic acid 41B	1,01s	1,54s	3,39s	5,04d	(3,0±0,4)	4,76d	3,77q (^1J 15 Hz)	"
(3S,5R,6S)-Benzylpenicilloic acid 41C	1,17s	1,39s	3,27s	5,18d	(4,5±0,2)	4,50d	ca 3,74 (^1J 15 Hz)	"
(3S,5S,6S)-Benzylpenicilloic acid 41D	1,16s	1,56s	3,35s	4,87d	(4,4)	4,56d	ca 3,72 (^1J 15 Hz)	"

Benzylpenicilloic acids dimethyl esters (solvent $CDCl_3$, reference TMS δ_H 0,00 ppm)								Other features			
							7β	NH	CONH	2x CO_2Me
(3S,5R,6R)-	1,12s	1,43s	3,31s	5,09d	(4,0)	4,61dd(9,0)	3,62s	7,34s	2,94b	6,20d(9)	3,71s
(3S,5S,6R)-	0,83s	1,54s	3,49s	5,05d	(2,5)	5,15dd(9,5)	3,63s	7,30s	-	6,32d(9,5)	3,68s,3,70s
(3S,5R,6S)-	1,12s	1,30s	3,43s	5,10d	(4,3)	4,86dd(9,0)	3,61s	7,30s	3,2b	6,25d(9)	3,66s,3,70s
(3S,5S,6S)-	1,11s	1,59s	3,53s	4,95m	(nd,)	4,95m (nd,)	3,61s	7,28s	3,2b	6,5m(nd,)	3,74s,3,75s

Table 15

Notes; s = singlet, d = doublet, m = multiplet, b = broad resonance, nd = not determined,
Operating frequency not reported in reference 70.

6.3.5 (3S,5R,6R)-Benzylpenillic acid 42



42

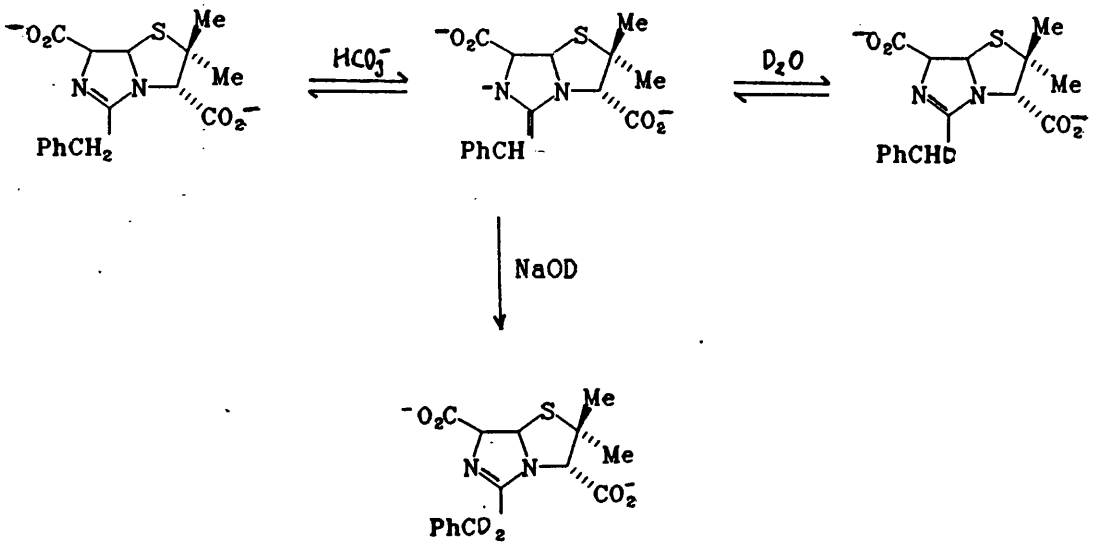
The configuration of benzylpenillic acid has not been reported, however degradation studies (see section 9.4 p.152) indicate that its formation from benzylpenicillin occurs without inversion of either H-5 or H-6. The ^{1}H NMR data has been reported [61] and results from this study are in close agreement.

When benzylpenillic acid was dissolved in D_2O-$NaHCO_3$ and analysed immediately the expected ^{1}H spectrum was obtained. The 2α- and 2β-CH_3 resonances are just resolved at 400 MHz. The H-3 and H-6 doublets moved upfield and the H-5 doublet moved downfield compared to benzylpenicillin. The $^3J_{H5-H6}$ coupling of 4.8Hz may indicate a *cis* configuration and support the proposed 5R,6R stereochemistry.

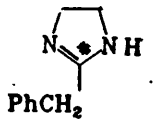
After 24 hours the CH_2 signal was absent due to deuteration and the H-5 and H-6 resonances appear as poorly resolved sextets. Addition of a little NaOD cause the H-5 and H-6 resonances to appear as doublets again with a slightly reduced coupling of 4.2Hz.

These observations may be explained as follows. The addition of $NaHCO_3$ causes slow deuteration of the CH_2 group via charge-delocalisation. The transition states are in slow equilibrium, permitting the intermediates to be present for a finite time, hence the complexity observed for the H-5 and H-6 resonances. Addition of

more alkali forces the equilibrium to give complete ionisation of the imidazoline nitrogen and hence the H-5 and H-6 doublets reappear i.e.



The exchange of the benzylic methylene group has been observed in similar 2-benzylimidazoline derivatives.[166] e.g. tolazoline HCl 64 exchanges within 24 hours in DMSO/D_2O at room temperature.



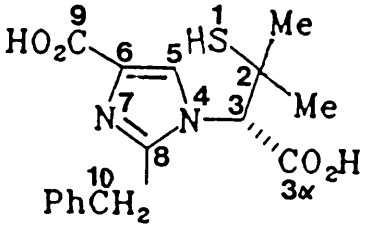
64

The benzylic CH_2 is absent in the ^{13}C spectrum. The methine carbons C-3, C-5 and C-6 have been assigned from proton-coupled experiments.[138] The assignment of the C-8 ring carbon (170.2 ppm) is made by assuming that the C-3α and C-7 carboxylate groups will have a very similar chemical shift (174.3 ppm and 175.6 ppm). The analagous carbon to C-8 in tolazoline* has a chemical shift of 165.6 ppm.[166]

6.3.6 (3S,5R,6R)-Benzylpenillic.dimethyl ester 55

The ^1H data is as expected, the benzylic CH_2 resonance is present at 35.5 ppm. The methine and carbonyl carbon resonances have not been unambiguously assigned.

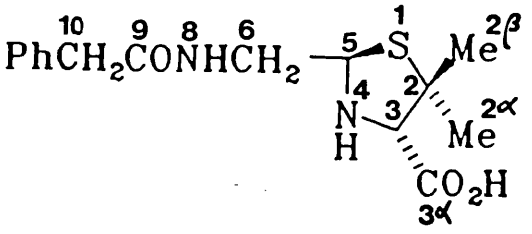
6.3.7 3S-Benzylisopenillic acid 56



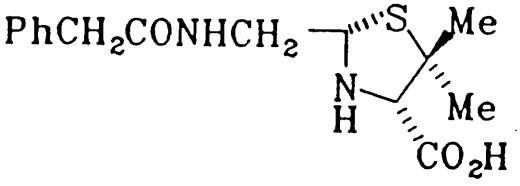
56

The ^1H and ^{13}C data are in close agreement with previously published data.[159] In imidazole each carbon and nitrogen may be considered as sp^2 hybridised and hence the observed pseudo-aromatic chemical shifts of C-5, C-6 and C-8. The aromatic nature of imidazole prevents deuterium exchange at the benzylic CH_2 group under the mild conditions employed.

6.3.8 (3S,5R)- 43A and (3S,5S)-Benzylpenilloic acids 43B



43A



43B

^1H NMR indicated that the sample of benzylpenilloic acid examined consisted of a mixture of two *epi*mers in the ratio 3:1 (1:1 after 48 hours in $D_2O/NaHCO_3$ solution).

The spectrum is complicated due to the duplication of all resonances and the non-equivalent protons at C-6. Analysis of the 400 MHz spectrum revealed the H_2-6 signals were both composed of 8 lines as required by the ABX system (CH_2CH) and the geminal and vicinal coupling constants were measured. Assignment of resonances for each conformer was made by examination of the relative intensities.

The assignment of the ^{13}C spectrum was made likewise. The C-6 resonance is moved upfield and is easily distinguished using an INEPT pulse sequence. The ^{13}C chemical shift differences between the two conformers are small.

Benzylpenilloic acid was prepared by the base hydrolysis of benzylpenicillin to give (3S,5R,6R)-benzylpenicilloic acid followed immediately by decarboxylation. Assuming that the penicilloic acid did not have the opportunity to epimerise at C-5 to any great extent, that decarboxylation proceeds with retention of configuration at C-5 and C-3 and the penilloic acid was isolated before it could epimerise at C-5, then the configuration of the major conformer will be 3S,5R.

McMillan and Stoodley [167] indicate the possibility of assigning the absolute stereochemistry at C-5 of 2,2-dimethylthiazolidine-3S-carboxylic acids by ^1H NMR. If both stereoisomers at C-5 are available, the isomer in which the C-3 proton appears at lowest field posseses the *trans* configuration relative to the C-5 proton.

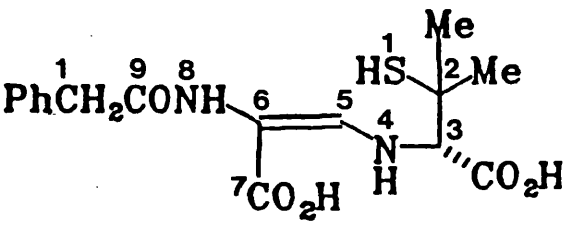
The H-3 resonances of benzylpenilloic acids designated 3S,5R (*trans*) and 3S,5S (*cis*) are 3.48 and 3.40 ppm, respectively i.e the empirical rule is followed. The rule also holds for penicic acid isomers **51A/B** and (3S,5R,6R)- and (3S,5S,6R)-benzylpenicilloic acids (see data in Table 11).

However the reverse pattern is observed for (3S,5R,6S)- and (3S,5S,6S)-benzylpenicilloic acids, the 4 dimethyl benzylpenicilloates and the 5-aminomethyl-2,2-dimethylthiazolidine-3S-carboxylic acids **58A/B** (configuration assigned by comparison of $^{2}J_{H6}$ and $^{3}J_{H5-H6}$ coupling constants with benzylpenilloic acids). This breakdown of the general rule has been observed by other workers. [168]

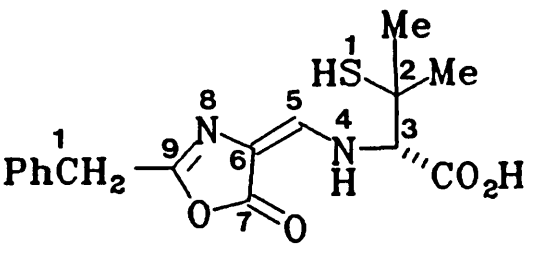
Additional support for the proposed assignment of the configuration of benzylpenilloic acid epimers as being correct is given by unpublished crystallography studies (Z. Ruzic-Toros in reference 158). It is possible that unambiguous assignment of absolute stereochemistry at C-5 may be obtained from NOE experiments.

6.3.9 3S-Benzylpenamaldic acid (enamine tautomer) 44A

The 270 MHz ^{1}H NMR characteristics of benzylpenamaldic acid, obtained from the degradation of benzylpenicillin at pH 2.5 and 37°C, have been reported.[61] Benzylpenamaldic acid contaminated with mainly benzylpenillic and benzylpenicilloic acids (see Section 12.4.5) was examined by 270 MHz ^{1}H NMR and spectral assignments made by comparison of the relative intensities and known chemical shifts of the aforementioned degradation products. The H-5 resonance (now vinylic) is partially obscured by the phenyl multiple resonances. The results are in fair agreement with literature data.



44A



28

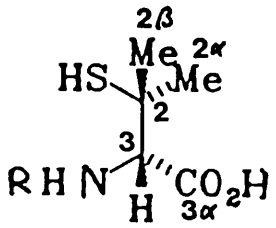
6.3.10 3S-Benzylpenicillenic acid 28

Benzylpenicillenic acid was found to be unstable in all solvents tried. However the ^1H characteristics can be obtained, in either deuterated methanol or chloroform, if the spectra are recorded immediately after the solutions are prepared.

The 2α- and 2β-methyl resonances are equivalent in CD_3OD and marginally resolved in $CDCl_3$. The H-3 and benzylic CH_2 resonances appear as singlets in CD_3OD, while in $CDCl_3$ the H-3 resonance is a doublet (J = 9.5 Hz, presumably due to coupling with NH).

The methylene protons are non-equivalent (AB quartet ^2J = 15.0 Hz). In either solvent the vinylic H-5 resonance is just downfield of the phenyl multiplet. The spectrum of benzylpenicillenic acid in $CDCl_3$ allows the tentative assignment of the C-3α carboxylic acid, the thiol and amino protons. Both samples appeared considerably degraded upon re-examination.

6.3.11 D-Penicillamine 10 and N-Formyl-D-penicillamine 47

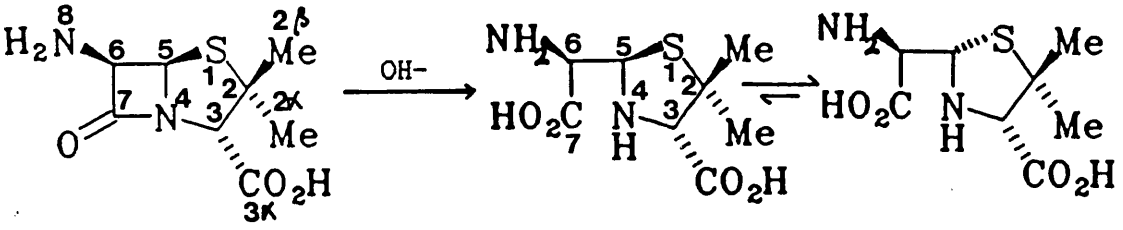


10 R = -H, **47** R = -CHO

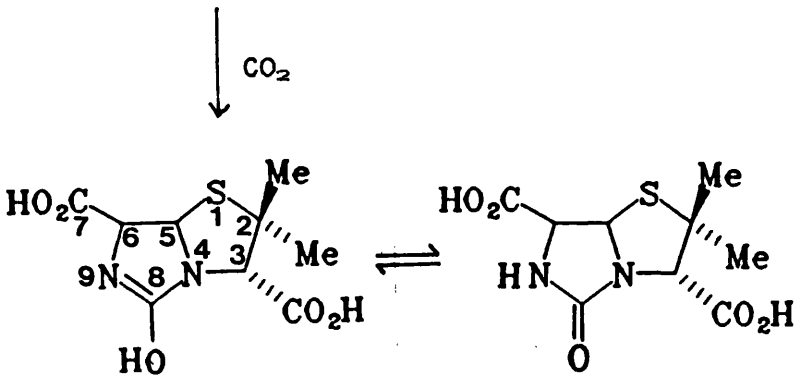
The ^1H and ^{13}C NMR characteristics of D-penicillamine and N-formyl-D-penicillamine are straightforward. The aldehyde resonance of N-formyl-D-penicillamine in D_2O is at higher field (δ_C = 164.9 ppm, δ_H = 8.14 ppm) than expected for a free aldehyde and may indicate hydration.

6.3.12 (3S,5R,6R)-6-Aminopenicillanic acid 31, (3S,5R/S,6R)-Penicic acids 60A/B and 8-Hydroxypenillic acid 59

6-aminopenicillanic acid (6-APA) in its protonated amine form gives the anticipated ^{1}H and ^{13}C spectra. The doublets of the H-5 and H-6 resonances have a greater chemical shift difference than that of benzylpenicillin and the $^{3}J_{H5-H6}$ coupling constant of 4.2 Hz indicates that the protons are *cis* supporting 5R,6R stereochemistry.



31 60A 60B



tautomer I 59 tautomer II

Base hydrolysis of 6-aminopenicillanic acid gives initially (3S,5R,6R,)-penicic acid which slowly epimerises to the more stable 5S-epimer.

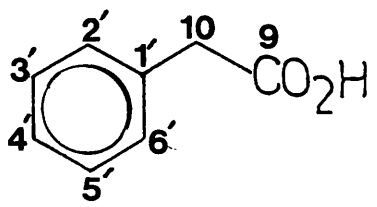
The ^{1}H and ^{13}C data of the penicic acid epimers reported by Carroll *et al* [75] have been included in Tables 11 and 12 respectively. The

spectral changes observed during the base hydrolysis of 6-APA are analogous to the base hydrolysis of benzylpenicillin to give (3S,5R/S,6R)-benzylpenicilloic acids. The $^3J_{H5-H6}$ magnitude for (3S,5R)-penicic acid (7.0 Hz) is greater than that of (3S,5S)-penicic acid (4.0 Hz) indicating that the protons in the former are *cis*, and *trans* in the latter.

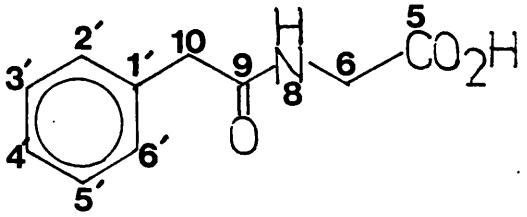
8-Hydroxypenillic acid, formed by the reaction of 6-APA with CO_2 , has similar ^1H and ^{13}C spectral characteristics to benzylpenillic acid, however the $^3J_{H5-H6}$ magnitude of 2.1 Hz for 8-hydroxypenillic acid may indicate that H-5 and H-6 are *trans* implying that 8-hydroxypenillic acid is formed with inversion of stereochemistry at H-5 or H-6.

The ^{13}C assignments for C-3, C-6 and C-3α, C-8 are tentative and may be reversed. The high field signal assigned to C-8 at 164.4 ppm indicates that 8-hydroxypenillic acid in D_2O solution exist predominately as tautomer II

6.3.13 Phenylacetic acid 62 and N-Phenylacetylglycine 50



62



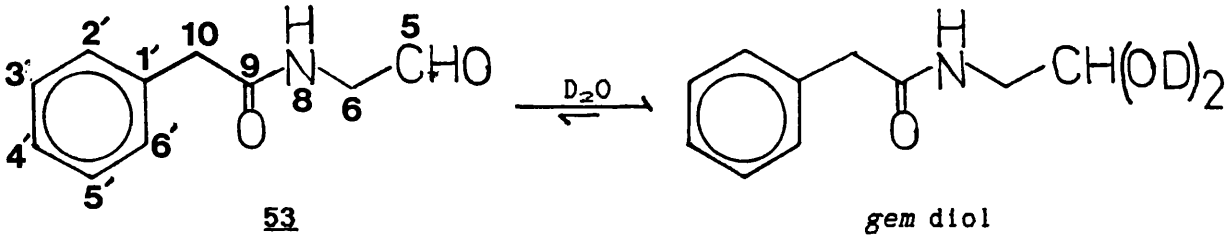
50

The ^1H and ^{13}C assignments of phenylacetic acid and N-phenylacetylglycine are trivial. The assignment of the CH_2-10 group in N-phenylacetylglycine is made by comparison with phenylacetic acid.

6.3.14 Benzylpenilloaldehyde 53

In the ^1H spectra of benzylpenilloaldehyde in $CDCl_3$ solution, the 2 proton singlet (3.64 ppm), the 2 proton doublet (4.15 ppm, J = 5.13 Hz), the 1 proton broad singlet (6.16 ppm) and the low field 1 proton singlet (9.60 ppm) were assigned H_2-10, H_2-6 ,NH and CHO protons respectively. These assignments were based on the observation that spin-decoupling at 6.16 ppm led to the collapse of the doublet to a singlet. No coupling of the aldehyde proton was observed. The ^{13}C spectrum is straightforward, the methylene resonances at 43.4 and 50.3 ppm, being assigned to C-10 and C-6 respectively.

In D_2O solution the ^1H spectrum shows the low field aldehyde resonance greatly reduced in intensity and a new triplet at 5.06 ppm (J = 5.31 Hz). The H_2-6 doublet is shifted to higher field (3.29 ppm, J = 5.31 Hz). In the ^{13}C spectrum, the aldehyde resonance is absent and is replaced by a signal at 89.1 ppm. Both observations provide evidence that benzylpenilloaldehyde in D_2O solution exists predominantly as the *gem* diol addition product.



53 *gem* diol

The magnitude of equilibrium constant K, can be calculated easily:

$$K = \frac{[gem\text{-diol}]}{[aldehyde]} = \frac{integral\ (\delta_H = 5.06ppm)}{integral\ (\delta_H = 9.50ppm)} = \frac{33}{3} = 11$$

Table 11. ^{1}H NMR characteristics of benzylpenicillin, degradation products and related compounds (continued overleaf)

COMPOUND	Solvent Operating	Reference frequency	CH_3-2α	CH_3-2β	H-3	H-5	$^1J_{H-5,H-6}$ (Hz,)	H-6	Ph-CH_2	Ph	Notes
(3S,5R,6R)-Benzyl-penicillin Na 5	D_2O 270 MHz	DSS 0,00ppm	1,48s	1,57s	4,23s	5,49d	(3,85)	5,42d	3,64q (^1J 15,0Hz)	(7,29-7,39)m	
(3S,5R,6S)-Benzyl-penicillin K 5A	D_2O 270 MHz	DSS 0,00ppm	1,47s	1,55s	4,30s	(5,24d	(1,65)	4,88d)	3,67s	(7,31-7,44)m	1
(3S,5R,6R)-Benzyl--1S-sulphoxide 57	D_2O 270 MHz	DSS 0,00ppm	1,24s	1,63s	4,35s	5,26d	(4,5)	5,87d	3,68s	(7,30-7,44)m	
(3S,5R,6R)-Benzyl-penicilloic acid 2Na 41A	D_2O 60 MHz	DSS 0,00ppm	1,23s	1,47s	3,40s	5,04d	(5,4)	4,23d	3,67s	7,35s	
(3S,5S,6R)-Benzyl-penicilloic acid 2Na 41B	D_2O 60 MHz	DSS 0,00ppm	1,01s	1,54s	3,35s	4,97d	(3,0)	4,71d	3,72s	7,31s	
(3S,5R,6S)-Benzyl-penicilloic acid 2Na 41C	D_2O 270 MHz	HDO 4,80ppm	1,14s	1,35s	3,23s	5,13d	(~4,6)	4,46d	Not determined	(7,30-7,40)m	
(3S,5S,6S)-Benzyl-penicilloic acid 2Na 41D	D_2O 270 MHz	HDO 4,80ppm	1,13s	1,53s	3,32s	4,89d	(nd)	4,52d	Not determined	(7,30-7,40)m	
(3S,5R,6R)-Benzyl-penillic acid 42	D_2O $NaHCO_3$ 270 MHz	DSS 0,00ppm	1,49s	1,51s	4,16s	5,75d	(4,8)	4,66d	3,91q (^1J 18 Hz)	(7,30-7,45)m	2
"	"	"	1,49s	1,50s	4,15s	5,73m	(nd)	4,66m	absent	"	3
"	"	"	1,48s	1,49s	4,13s	5,67d	(4,2)	4,60d	absent	"	4

Notes: (1) Assignement of H-5 and H-6 may be reversed, Impurities at 1,90 and 8,44ppm (2) Analysed immediately after preparation (3) Spectrum recorded 24 Hrs after preparation (4) Plus 1 drop dilute NaOD nd = not determined.

Table 11 (continued). ^{1}H NMR characteristics of benzylpenicillin, degradation products and related compounds (continued overleaf)

COMPOUND	Solvent Operating frequency	Reference	CH_3-2α	CH_3-2β	H-3	H-5	$^1J_{H-5\ H-6}$ (Hz,)	H-6	Ph-CH_2	Ph	Notes
(3S,5R,6R)-Benzyl-penillic acid dimethyl ester 55	$CDCl_3$ 60 MHz	TMS 0,00ppm	1,37s		4,17s	5,70d	(3,6)	4,75d	3,73s	7,15s	5
(3S)-Benzylisopenillic acid 56	D_2O $NaHCO_3$ 270 MHz	DSS 0,00ppm	1,16s	1,52s	4,61s	8,16s		-	4,21q (^1J 16,7Hz)	(7,27-7,39)m	
(3S,5R)-Benzylpenilloic acid 43A	D_2O $NaHCO_3$ 400 MHz	TSP 0,00ppm	1,21s	1,56s	3,48s	4,77t(7,16)		H_2-6 3,33‡	3,60s	(7,31-7,43)m	6
(3S,5S)- " 43B	"	"	1,18s	1,58s	3,40s	4,67t(4,90)		3,58‡	3,64s	"	7
(5R/S)-Aminomethyl-2,2-dimethylthiazolidine-3S-carboxylic acids 58A/B	D_2O $NaHCO_3$ 270 MHz	DSS 0,00ppm	1,52s 1,47s	1,75s 1,74s	4,48s 4,43s	5,27t(5,86) 5,19t(7,04)		3,67‡ 3,51‡	- -	- -	8 9
(3S)-Benzylpenamaldic acid 44	D_2O 270 MHz	DSS 0,00ppm	1,38s	1,62s	3,70s	7,34s		-	3,74s	7,4m	
(3S)-Benzylpenicillenic acid 28	CD_3OH 270 MHz	TMS 0,00ppm	1,46s		4,15s	7,39s		-	3,90s	(7,21-7,33)m	10
(3S)-Benzylpenicillenic acid 28	$CDCl_3$ 270 MHz	" "	1,49s	1,51s	3,92d (9,5Hz)	7,36s		-	4,01q (^1J 15,0Hz)	(7,21-7,34)m	11

Notes; (5) Additional signals at 3,53s and 3,73s ($2xCO_2Me$)
8-line double AB signals centre at ‡ (6) 7,2,13,6Hz (7) 5,3,14,5Hz (8) 5,86,15,1Hz (9) 7,02,13,4Hz
(10) Tentative assignments (11) Additional signals at 9,04bs (CO_2H), 2,06s (SH) and 3,60bs (NH)

Table 11 (continued) ^{1}H NMR characteristics of benzylpenicillin degradation products and related compounds

COMPOUND	Solvent Operating frequency	Reference	CH_3-2α	CH_3-2β	H-3	H-5	$^2J_{H-5\ H-6}$ (Hz,)	H-6	Ph-CH_2	Ph	Notes
D-Penicillamine 10	D_2O 270 MHz	DSS 0,00ppm	1,48s	1,56s	3,69s	-		-	-	-	
N-Formyl-D-penicillamine 47	D_2O $NaHCO_3$ 270 MHz	DSS 0,00ppm	1,41s	1,48s	4,33s	-		-	-	-	12
(3S,5R,6R)-6-Amino-penicillanic acid 31	D_2O DCl 270 MHz	DSS 0,00ppm	1,58s	1,71s	4,67s	5,68d	(4,21)	5,10d	-	-	
8-Hydroxypenillic acid 59	D_2O 270 MHz	DSS 0,00ppm	1,50s	1,56s	4,23s	5,53d	(2,02)	4,21d	-	-	
(3S,5R,6R)-Penicic acid 60A	D_2O pH8,5 60 MHz	"	1,33s	1,75s	3,62s	5,06d	(7,0)	3,80d	-	-	13
(3S,5S,6R)-Penicic acid 60B	"	"	1,26s	1,60s	3,45s	4,93d	(4,0)	3,58d	-	-	13
Phenylacetic acid 62	D_2O $NaHCO_3$ 270 MHz	DSS 0,00ppm	-	-	-	-		-	3,56s	(7,28-7,40)m	
N-Phenylacetylglycine 50	"	"	-	-	-	-		H_2-6 3,73s	3,65s	(7,32-7,40)m	14
Benzylpenilloaldehyde 53	$CDCl_3$ 270 MHz	TMS 0,00ppm	-	-	-	-		4,15d (5,13Hz)	3,64s	(7,27-7,41)m	15
Benzylpenilloaldehyde 53	D_2O 270 MHz	DSS 0,00ppm	-	-	-	-		3,29d (5,31Hz)	3,62s	(7,30-7,41)m	16

Notes; (12) Additional signal 8,14s (CHO) (13) Data taken from ref () (14) H_2-6 and Ph-CH_2 assignments may be reversed (15) Additional signals 9,60s (CHO) 6,16bs (NH) and 1,75bs (impurity) (16) Additional signals 9,50s (trace CHO) and 5,06t 5,31Hz ($CH(OD)_2$)

Abbreviations; s singlet, d doublet, t triplet, q quartet, m multiplet, bs broad singlet.

Table 12. ^{13}C NMR characteristics of benzylpenicillin degradation products and related compounds (continued overleaf)

COMPOUND	Solvent Operating	Reference frequency	C-2	C-2α	C-2β	C-3	C-3α	C-5	C-6	C-7	C-9	C-10	C-3α C-7 C-9	C-1'	C-2' C-6'	C-3' C-5'	C-4'	Note
(3S,5R,6R)-Benzyl-penicillin Na 5	D_2O 67,8 MHz	CH_3OH 49,3ppm	64,7	26,8	30,9	73,5	174,8	67,0	58,4	175,0	173,9	42,3	-	134,8	129,7	129,3	127,9	
(3S,5R,6R)-Benzyl-penicillin Na 5	"	TMS capillary 0,00ppm	65,7	27,8	32,1	74,4	175,4	68,0	59,2	175,9	174,5	43,3	-	135,7	130,6	130,0	128,4	
(3S,5R,6S)-Benzyl-penicillin K 5A	D_2O 67,8 MHz	C-1' 134,8ppm	64,4	25,9	32,3	72,2	174,7	[65,1,69,1]		175,3	172,7	42,4	-	134,8	129,6	129,4	127,8	1
(3S,5R,6R)-Benzyl-1S-sulphoxide 57	D_2O 22,5 MHz	TMS capillary 0,00ppm	76,7	19,2	20,5	76,9	176,3	69,2	56,5	?	?	43,3	- 174,6 175,5	135,3	130,7	130,3	128,9	2
(3S,5R,6R)-Benzyl-penicilloic acid 2Na 41A	D_2O 22,5 MHz	TMS capillary 0,00ppm	59,7	27,7	28,3	76,7	?	67,5	60,7	?	?	43,9	175,2 176,7 177,2	136,1	130,8	130,3	128,7	3
(3S,5S,6R)-Benzyl-penicilloic acid 2Na 41B	D_2O 22,5 MHz	TMS capillary 0,00ppm	59,7	29,2	29,4	77,1	?	68,6	56,9	?	?	44,0	175,9	135,5	130,9	130,5	128,9	3
(3S,5R,6S)-Benzyl-penicilloic acid 2Na 41C	D_2O NaOD 67,8 MHz	C-1' 136,0ppm	60,1	27,5	27,7	76,8	?	67,6	*	?	?	*	176,4 182,7	136,0	130,7	130,4	128,8	4
(3S,5S,6S)-Benzyl-penicilloic acid 2Na 41D	D_2O NaOD 67,8 MHz	C-1' 136,0ppm	59,2	28,9	29,4	77,2	?	68,1	*	?	?	*	169,6 175,5	136,0	130,5	130,2	128,5	4

Notes; (1)Assignments of carbonyl resonances may be reversed, (2)Assignment of C-7 and C-9 not possible, (3)Carbonyls not assigned, (4)C-6 and C-10 absent, due to deuteration *.

Table 12 (continued) ^{13}C NMR characteristics of benzylpenicillin degradation products and related compounds (continued overleaf)

COMPOUND	Solvent / Operating frequency	Reference	C-2	C-2α	C-2β	C-3	C-3α	C-5	C-6	C-7	C-9	C-10	C-3α / C-7 / C-9	C-1'	C-2' / C-6'	C-3' / C-5'	C-4'	Note
(3S,5R,6R)-Benzyl-penillic acid 42	D_2O $NaHCO_3$ 22,5 MHz	TMS capillary 0,00ppm	58,9	27,1	30,0	69,3	?	73,4	74,1	?	C-8 170,2	*	174,3 175,6	132,6	130,7	130,5	129,5	5
(3S,5R,6R)-Benzylpenill-ic acid dimethyl ester 55	$CDCl_3$ 22,5 MHz	TMS capillary 0,00ppm	57,1	27,1	30,9	71,9	?	72,5	74,6	?	C-8 166,4	35,5	171,3 169,0	134,5	129,0	128,8	127,2	6
3S-Benzylisopenillic acid 56	D_2O $NaHCO_3$ 22,5 MHz	TMS capillary 0,00ppm	47,3	31,2	32,9	71,4	174,2	125,7	136,5	161,7	C-8 149,9	34,3	-	138,1	130,3	129,8	128,3	7
3S-Benzylisopenillic acid 56	" 67,8 MHz	CH_3OH 49,3ppm	46,5	30,1	31,9	70,5	173,2	124,8	135,4	160,7	C-8 148,9	33,4	-	137,1	129,4	128,9	127,4	8
(3S,5R)-Benzylpenilloic acid 43A	D_2O $NaHCO_3$ 22,5 MHz	TMS capillary 0,00ppm	60,0	28,0	28,4	77,4	?	65,0	46,8	-	?	43,6	176,0 176,1	136,1	130,3	130,1	128,5	9
(3S,5S)-Benzylpenilloic acid 43B	"	"	59,7	29,0	29,4	75,1	?	66,2	43,2	-	?	43,6	175,9 176,2	136,0	130,5	130,1	128,5	9
(5R/S)-Aminomethyl-2,2-dimethylthiazolidide-(3S)-carboxylic acids 58A/B	D_2O 67,8 MHz	TMS capillary 0,00ppm	54,1	26,6	28,5	71,9	168,3	58,6	40,7	-	-	-	-	-	-	-	-	
			55,1	26,2	27,3	70,3	168,8	58,6	42,2	-	-	-	-	-	-	-	-	
D-Penicillamine 10	D_2O 22,5 MHz	TMS capillary 0,00ppm	45,2	29,1	31,6	65,9	172,6	-	-	-	-	-	-	-	-	-	-	
N-Formyl-D-Penicillamine 47	D_2O $NaHCO_3$ 22,5 MHz	" "	46,6	30,9	31,1	64,3	176,6	164,9	-	-	-	-	-	-	-	-	-	

Notes; (5) C-10 absent due to deuteration * (6) Methyl esters 52,0 and 52,6ppm, C-3, C-5 and C-6 assignments may be reversed
(7) Additional signal at 172,2ppm (8) Additional signal at 171,1ppm (9) C-3α and C-9 assignments may be reversed

Table 12 (continued) ^{13}C NMR characteristics of benzylpenicillin degradation products and related compounds

COMPOUND	Solvent Operating	Reference frequency	C-2	C-2α	C-2β	C-3	C-3α	C-5	C-6	C-7	C-9	C-10	C-3α C-7 C-9	C-1'	C-2' C-6'	C-3' C-5'	C-4'	Note
(3S,5R,6R)-6-Amino-penicillanic acid 31	D_2O DCl 67,8 MHz	TMS capillary 0,00ppm	65,6	26,1	30,2	70,4	171,0	63,7	56,9	169,0	-	-	-	-	-	-	-	
8-Hydroxypenillic acid 59	D_2O 67,8 MHz	TMS capillary 0,00ppm	57,8	25,9	31,5	73,6	177,5	59,4	69,4	176,0	C-8 164,4	-	-	-	-	-	-	10
(3S,5R,6R)-Penicic acid 60A	D_2O 25 MHz	Dioxan 67,4ppm	64,7	27,6	31,8	73,5	176,7	69,5	61,3	176,0	-	-	-	-	-	-	-	11
(3S,5S,6R)-Penicic acid 60B	"	"	60,5	27,2	27,2	75,4	175,7	67,4	60,5	173,4	-	-	-	-	-	-	-	11
Phenylacetic acid 62	$CDCl_3$ 22,5 MHz	TMS 0,00ppm									178,3	41,0		133,2	129,3	128,6	127,3	
N-Phenylacetylglycine 50	D_2O $NaHCO_3$ 22,5 MHz	TMS capillary 0,00ppm						175,5	44,8		177,8	43,5		136,2	130,5	130,1	128,5	
Benzylpenilloaldehyde 53	$CDCl_3$ 22,5 MHz	TMS 0,00ppm						196,2	50,3		171,3	43,4		134,5	129,4	129,0	127,5	
Benzylpenilloaldehyde 53	D_2O 67,8 MHz	TMS capillary 0,00ppm						89,1	46,0		175,9	43,1		135,8	130,0	129,8	128,2	

Notes; (10) Tentative assignments for C-3 C-6 and for C-3α C-7 (11) Data taken from ref.75

7 GENERAL INTRODUCTION TO THE DEGRADATION STUDIES

7.1 Materials and reagents

The preparation and sources of all compounds used in the degradation studies have been described in Section 4. The identities of the compounds were confirmed by ^{1}H and ^{13}C NMR (see Section 6.3). All compounds are deemed pure unless specifically noted. All other chemicals used were of at least reagent grade. De-ionised glass-distilled water was used throughout.

7.2 Buffers

Buffer solutions were prepared as described by Perrin *et al* [169] with the exception of the pH 2.5 phosphate buffer. All buffer solutions had an ionic strength (μ) of 0.1 M.

(a) HCl/KCl buffer (25°C), μ = 0.1 M, pH 2.20; 42 ml 0.2 M HCl (17.16 ml conc. HCl, (S.G. 1.18, about 36% HCl, $HgCl_2$ free) to 1 litre with water) and 458 ml 0.20 M KCl (14.911 g KCl dissolved to 1 litre with water) diluted to 1 litre with water. Final pH = 2.20 ± 0.05.

(b) Phosphate buffer (25°C), $\mu \simeq$ 0.1 M, pH = 2.50; 1 litre 0.10 M NaH_2PO_4 (15.601 g $NaH_2PO_4.2H_2O$ dissolved to 1 litre with water) adjusted to pH 2.5 by the addition of ~ 3 ml conc. H_3PO_4 (SG 1.75, about 88% H_3PO_4). Final pH 2.5 ± 0.05.

(c) Phosphate buffer (25°C), μ = 0.1 M, pH = 7.00; 0.0198 M $H_2PO_4^-$ (3.0890 g $NaH_2PO_4.H_2O$) and 0.0268 M HPO_4^- (9.5982 g $Na_2HPO_4.12H_2O$) dissolved to 1 litre with water. Final pH = 7.00 ± 0.05.

(d) NaOH/KCl buffer (25°C), μ = 0.1 M, pH = 12.0; 60 ml 0.20 M NaOH and 250 ml 0.20 M KCl diluted to 1 litre with water. (final pH = 12.0 ± 0.1)

7.3 Apparatus

7.3.1 High performance liquid chromatography

The HPLC apparatus used was essentially as described in Section 5.2.2. The columns and eluents used, and any modifications of, or additions to the basic apparatus are described in the individual experimental sections.

7.3.2 Nuclear magnetic resonance

Described in the individual experimental sections

7.3.3 Ultraviolet spectroscopy

Absorbancy measurements were performed with a Perkin-Elmer 550S UV-Vis Spectrophotometer and a Cecil Instruments CE 595 Double Beam Digital UV Spectrophometer (equipped with a thermostatted sample and reference cell compartments and a CE 500 Control-Record Module) using 1 cm quartz cuvettes. The Perkin-Elmer spectrophometer was calibrated with 0.006 % w/v potassium dichromate.[170] The results are given in Appendix II.

7.3.4 Measurement of temperature and pH

Temperature measurements were made with a mercury in glass thermometer (-5° to 50°C, 0.1°C divisions) with reference to a mercury in glass thermometer calibrated against a NPL certificated platinum resistance thermometer (Department of Chemistry, University of Bath). The corrected temperature readings are used throughout.

The pH measurements were made with Philips PW 9410 and 9421 digital pH meters calibrated in accordance with the manufacturers specifications i.e. 0.050 M potassium hydrogen phthalate, pH 4.01

0.025 M phosphate, pH 6.86

0.050 M sodium borate, pH 9.18

...all at 25°C. All pH measurements at 37°C were made using automatic temperature compensation.

7.3.5 Volumetric apparatus and weighings

Class A volumetric flasks and pipettes were used throughout. The transfer of small volumes (<1 ml) of liquids were made with a variable volume air displacement pipette with disposable tips (Gilson Pipetman P200; range 50 to 200 µl, quoted accuracy ± 1.0%, standard deviation < 0.4%). Weighings were performed on Oertling NA 164 and Sartorius Analytic balances accurate to 0.1 mg.

7.3.6 Temperature control

Samples were maintained at constant temperature by immersing the containers in a water bath heated by Grant Instruments SU 5 electrical heaters at 25 ± 0.5°C or 37 ± 0.1°C.

7.3.7 Glassware

All glassware was cleaned by immersing in chromic acid for at least 1 hour followed by thorough rinsing with tap water and finally deionised distilled water. The glassware was dried in a hot air oven after rinsing with acetone. This procedure was repeated periodically.

7.4 Data analysis

A BBC Master B Series microcomputer and a general purpose statistics package (INSTAT, Statistical Services Centre, University of Reading 1986) were used for interactive analysis of kinetic data

Rate constants were calculated using a simple linear regression model (least squares method) which treated concentration versus time data in the regression model.

Upon regression, the results consisted of the analysis of variance for the regression and the value of the 'coefficient of determination' or 'correlation coefficient' CC. In addition the regression estimates of the slope and intercepts of the linear relationship, and the standard errors of the estimates are provided. The calculations of first-order rate constants are described in Appendix III. The error in Kinetic results was usually expressed as the standard deviation σ and was obtained either from regression analysis or INSTAT simulation.

Propagation of error for a computed result was obtained by standard methods.[171]

8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES OF THE DEGRADATION AND EPIMERISATION OF (3S,5R)- AND (3S,5S)-BENZYLPENILLOIC ACIDS

8.1 Introduction

The sample of benzylpenilloic acid (BPO) **43** used in these studies consisted of the two epimers 3S,5R **43A** and 3S,5S **43B** in the ratio of 3:1. The major epimer was assumed to possess the 3S,5R stereochemistry (see section 6.3.8). HPLC studies were conducted to monitor the degradation and epimerisation of benzylpenilloic acid at an initial concentration of ca. 5×10^{-4} M at 25°C (pH 2.5, 7 and 12) and 37°C (pH 2.2 and 2.5).

8.2 Experimental

8.2.1 Instrumentation

The HPLC instumentation has been described previously in section 5.2.2. Separations were performed on a 250 × 4.9 mm i.d. Spherisorb S5ODS2 column. The mobile phase consisted of tetrahydrofuran - 0.05 M phosphate buffer (0.05 M KH_2PO_4 1 litre and 1 M H_3PO_4 1 ml, pH 3.9) (25:75 v/v) at a flow rate of 1 ml/min. The detector wavelength was 230 nm. The amount of sample injected was 20 µl in all cases. Separations were carried out at ambient temperature.

8.2.2 Materials and reagents

The buffer solutions used have been described in section 7.2.

8.2.3 Standard calibration solutions

Stock solutions of benzylpenilloic acid were prepared by dissolving

the required weight of compound in water and analysing immediately by HPLC. The calibration studies were conducted using five concentrations of benzylpenilloic acid in the range 4.0 x 10^{-4} M to 5.5 x 10^{-4} M i.e. (0.13 - 0.18 mg/ml).

8.2.4 Kinetic studies

Degradation and epimerisation studies were carried out by weighing out accurately about 1.5 mg of benzylpenilloic acid and dissolving in 10 ml of the appropriate buffer, prewarmed to either 25°C or 37°C. The dissolution of samples in acidic buffers was aided by vigorous shaking and ultrasonification

Aliquots of acid and neutral solutions were analysed at timed intervals directly by HPLC. 100 µl aliquots of pH 12 solutions were first diluted 1:1 with HPLC eluent immediately prior to analysis. This procedure was followed to minimise damage to the HPLC column packing material. HPLC analysis was assumed to quench further reaction. The degradation and epimerisation of benzylpenilloic acid was monitored by HPLC. The pH of all solutions was checked before and after each experiment and if it changed by more than 0.05 pH units, the experiment was rejected.

The order and magnitude of the overall rate of degradation of benzylpenilloic acid were obtained by regression analysis of change in benzylpenilloic acid HPLC peak area with time.

The kinetic transformation of (3S,5R)-benzylpenilloic acid to its 3S,5S epimer was determined qualitatively by HPLC. Assuming that the intermediate attains a steady state concentration during the epimerisation process, that the reaction rates are first-order and

that the degree of degradation has no effect on the rates of epimerisation, the rate constants for both the forward, kf and reverse reactions, kr, were computed using the following equations.[73]

$$3S,5R \underset{kr}{\overset{kf}{\rightleftharpoons}} 3S,5S \qquad (1)$$

$$\log \frac{R_o - R_{eq}}{R - R_{eq}} = \frac{(kf + kr)\, t}{2.303} \qquad (2)$$

$$K = \frac{kf}{kr} = \frac{S_{eq}}{R_{eq}} \qquad (3)$$

where:

R_o = initial % of (3S,5R)-benzylpenilloic acid

R = % of (3S,5R)-BPO at time t

R_{eq} = equlibrium % (3S,5R)-BPO

S = % of (3S,5S)-BPO at time t

S_{eq} = equlibrium % (3S,5S)-BPO

and K = equilibrium constant of the reaction

for example:

$$R_o = \frac{\text{initial peak area of (3S,5R)-BPO}}{\text{sum of peak areas of (3S,5R)- and (3S,5S)-BPO}} \times 100\%$$

as determined by HPLC.

Therefore by plotting $\log \frac{R_o - R_{eq}}{R - R_{eq}}$ against time t

$$kf + kr = \text{slope} \times 2.303 \qquad (4)$$

kf and kr are determined by dividing equation (4) by kr and substituting K for kf/kr i.e.

$$\frac{kf + kr}{kr} = \frac{(\text{slope} \times 2.303)}{kr} = K + 1$$

$$\text{therefore } kr = \frac{(\text{slope} \times 2.303)}{(K + 1)} \qquad (5)$$

and hence kf by substituting the value of kr in equation (4).

8.2.5 HPLC separation.

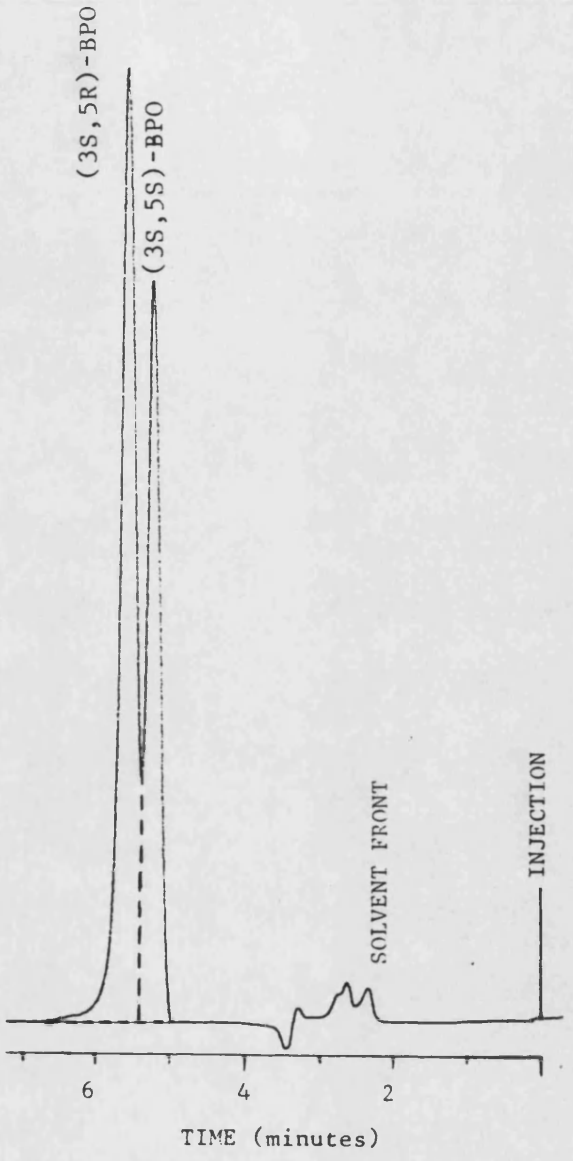


Figure 15 HPLC separation of benzylpenilloic acid epimers. Conditions: Column 250 x 4.9 mm id S5ODS2, eluent THF : phosphate buffer (pH 3.9), 25 : 75 v/v, flow 1ml/min., detection uv λ 230 nm.

Figure 15 shows a representative chromatogram of the separation of benzylpenilloic acid epimers. It is obvious that the two epimers are not completely resolved. Studies of the chromatographic behaviour of

the benzylpenilloic acids indicated that the percentage of THF in the mobile phase was critical and optimum separations, in terms of total analysis time, resolution and separation from solvent front, were obtained using 25:75 v/v THF-phosphate buffer (see Table 16).

Table 16 Effect of THF concentration upon the retention and separation of benzylpenilloic acid epimers

	% v/v Tetrahydrofuran*						
Parameter	40	30	25	20	15	10	5
α	1.00	1.15	1.11	1.07	1.04	1.00	1.00
Analysis time (mins)	4	5	6	8	11	17	35

α = separation factor = k'_2/k'_1, the capacity factors of the epimers.
Analysis time = time after last epimer elutes from the column.
* other chromatographic conditions as in Figure 15.

8.2.6 Determination of epimer ratio

The epimer ratio was estimated by calculating the peak area ratio. This was obtained by partitioning the merged peaks by a vertical drop-line from the valley point to a baseline defined by the beginning and end points of the cluster of merged peaks (as illustrated in Figure 15). The procedure was performed automatically by the integrator and assumed that the contribution to the peak areas of peaks by neighbouring peaks occured to equal extents. This technique was validated by comparing HPLC and ^1H NMR results.

For instance, the equilibrium ratio of benzylpenilloic acid epimers in pH 7 buffer at 25°C was found to be about 55 : 45 (the 3S,5S epimer being the major component) by HPLC. A similar mixture was analysed by 270 MHz ^{1}H NMR and the epimer ratio was calculated by comparing the relative heights of the CH_3-2α (55.6 : 44.4), CH_3-2β (55.5 : 44.5) and H-3 (55.4 : 44.6) resonances. The agreement between HPLC and ^{1}H NMR results was within experimental error.

8.2.7 Qualitative analysis of benzylpenilloic acids

The total concentration of benzylpenilloic acids was obtained by summing the integrated peak areas of the two epimers. The peak area response was linear in the concentration range examined, see Table 17.

Table 17 Regression data for calibration of benzylpenilloic acids

[BPO]	Total peak area (mean of duplicate assays)
4.05×10^{-4} M	5.29×10^6
4.22×10^{-4} M	5.41×10^6
4.22×10^{-4} M	5.59×10^6
5.52×10^{-4} M	7.06×10^6
5.52×10^{-4} M	7.35×10^6

Notes: [BPO] = benzylpenilloic acid concentration.
HPLC peak area in arbitary units

Correlation coeff. (n=6) = 0.9983

Gradient = 1.305×10^{10} $\sigma = 2.66 \times 10^8$

Intercept = -1.269×10^3 $\sigma = 1.15 \times 10^6$

8.3 Results

8.3.1 Degradation studies

No appreciable degradation at pH 7 or pH 12 (both at 25°C) was observed over five days. Degradation does occur in acidic solution and the overall rate of degradation k was found to be first-order with respect to benzylpenilloic acid. First-order plots for the degradation of benzylpenilloic acid in pH 2.2 and 2.5 buffers at 37°C are drawn in Figure 16. The regression data and calculated rate constants are given in Table 18. Degradation in pH 2.5 buffer at 25°C was too slow to be measured accurately.

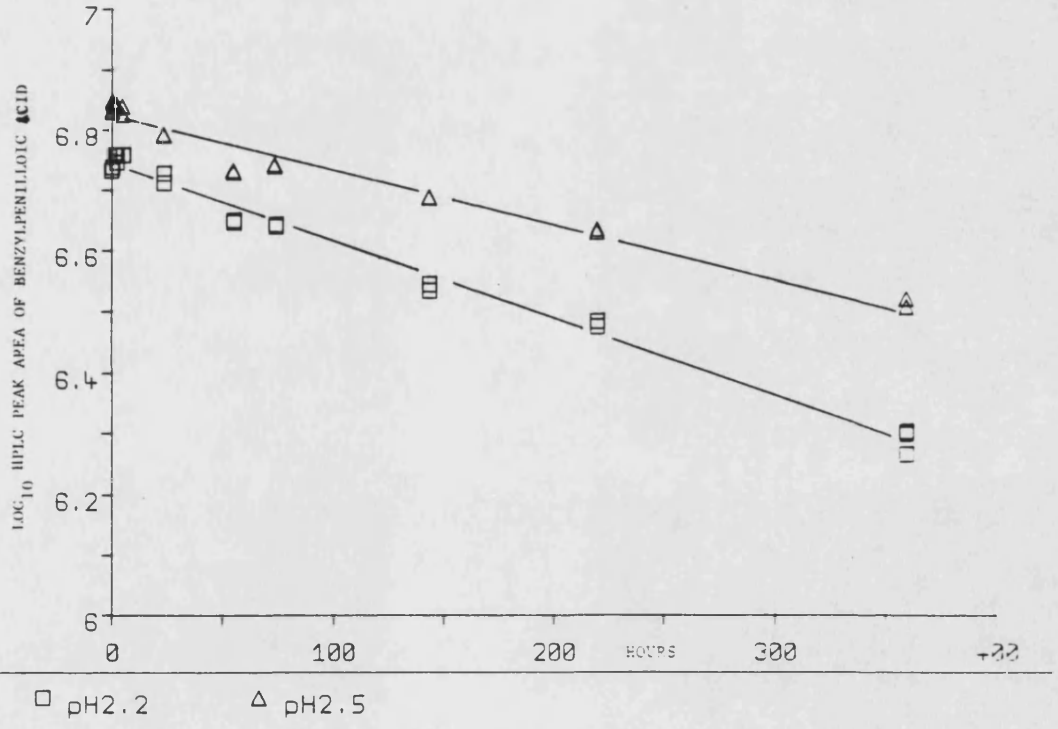


Figure 16 First-order plots for the degradation of benzylpenilloic acid at pH 2.2 and 2.5 at 37°C

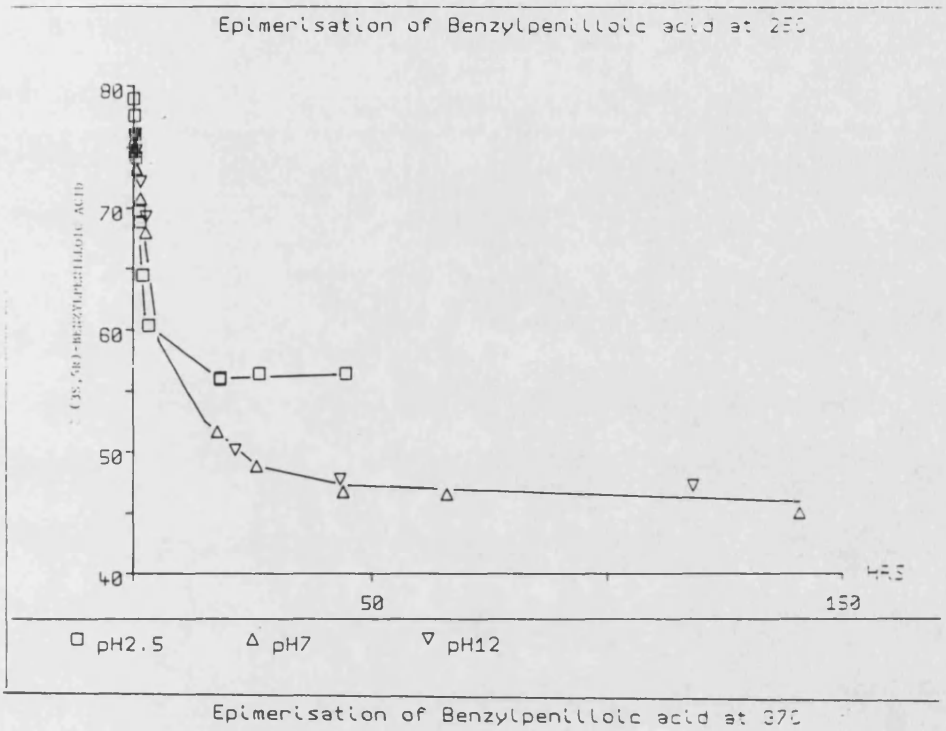
Table 18 Summary of regression data and rate constants for the degradation and epimerisation of benzylpenilloic acid.

Conditions	pH 2,5 25°C	pH 7 25°C	pH 12 25°C	pH 2,2 37°C	pH 2,5 37°C
[BPO]initial	$4,22x10^{-4}$ M	$5,52x10^{-4}$ M	$8,10x10^{-4}$ M	$4,22x10^{-4}$ M	$5,52x10^{-4}$ M
			DEGRADATION DATA		
Observations	-	-	-	22	20
Corr. Coeff.				0,99463	0,98171
k hrs^{-1}				$2,91x10^{-3}$	$2,04x10^{-3}$
σ hrs^{-1}				$6,76x10^{-5}$	$9,31x10^{-5}$
t½ days				9,93	14,2
			EPIMERISATION DATA		
Observations	6	6	6	5	5
Corr. Coeff.	0,99911	0,99414	0,99999	0,99996	0,99968
K	0,78	1,22	1,12	0,78	0,78
σ	0,02	0,02	0,02	0,02	0,02
kf hrs^{-1}	0,186	0,0577	0,0567	1,26	0,858
σ hrs^{-1}	0,00652	0,00331	0,00115	0,0358	0,0270
kr hrs^{-1}	0,238	0,0473	0,0506	1,62	1,10
σ hrs^{-1}	0,00568	0,00260	0,000486	0,0199	0,0201

8.3.2 Epimerisations studies

Figures 17 and 18 show the epimerisation of (3S,5R)- to (3S,5S)-benzylpenilloic acid, in pH 2.5, 7 and 12 solutions at 25°C and pH 2.2 and 2.5 solutions at 37°C respectively, as measured by HPLC.

Figure 17 Epimerisation of (3S,5R)- to (3S,5S)-benzylpenilloic acid in pH 2.5, 7 and 12 solutions at 25°C.



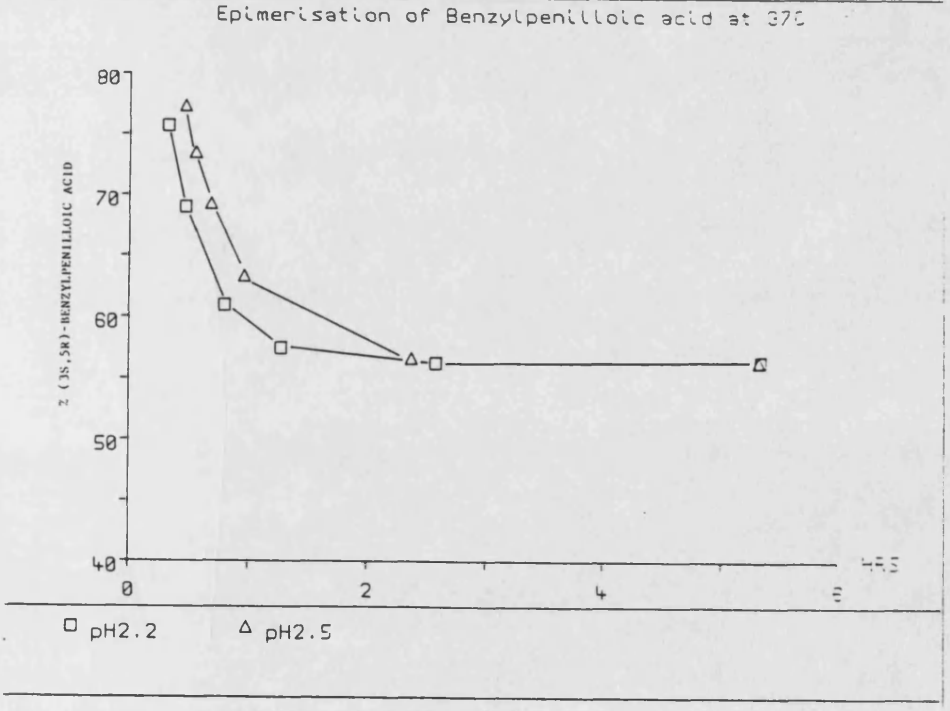


Figure 18 Epimerisation of (3S,5R)- to (3S,5S)-benzylpenilloic acid in pH 2.2 and 2.5 solutions at 37°C

In neutral and basic solutions, at equilibrium, the 3S,5S epimer was the favoured product (≃ 46 : 54, 5R : 5S).

In acidic solution, equilibrium was reached faster and the 3S,5R epimer was favoured (≃ 56 : 44, 5R : 5S). At 37°C acidic solutions attained equilibrium within 2 to 3 hours. The equilibrium constant, **K** and forward kf and reverse kr rate constants are given in Table 18.

8.3.3 Identification of the acid-catalysed degradation products of benzylpenilloic acid

Typical chromatograms obtained in degradation/epimerisation studies of benzylpenilloic acid in acid solution are shown in Figure 19(a) to (f). After a few hours a new peak, eluting close to the solvent front, was observed which increased in size as the study continued.

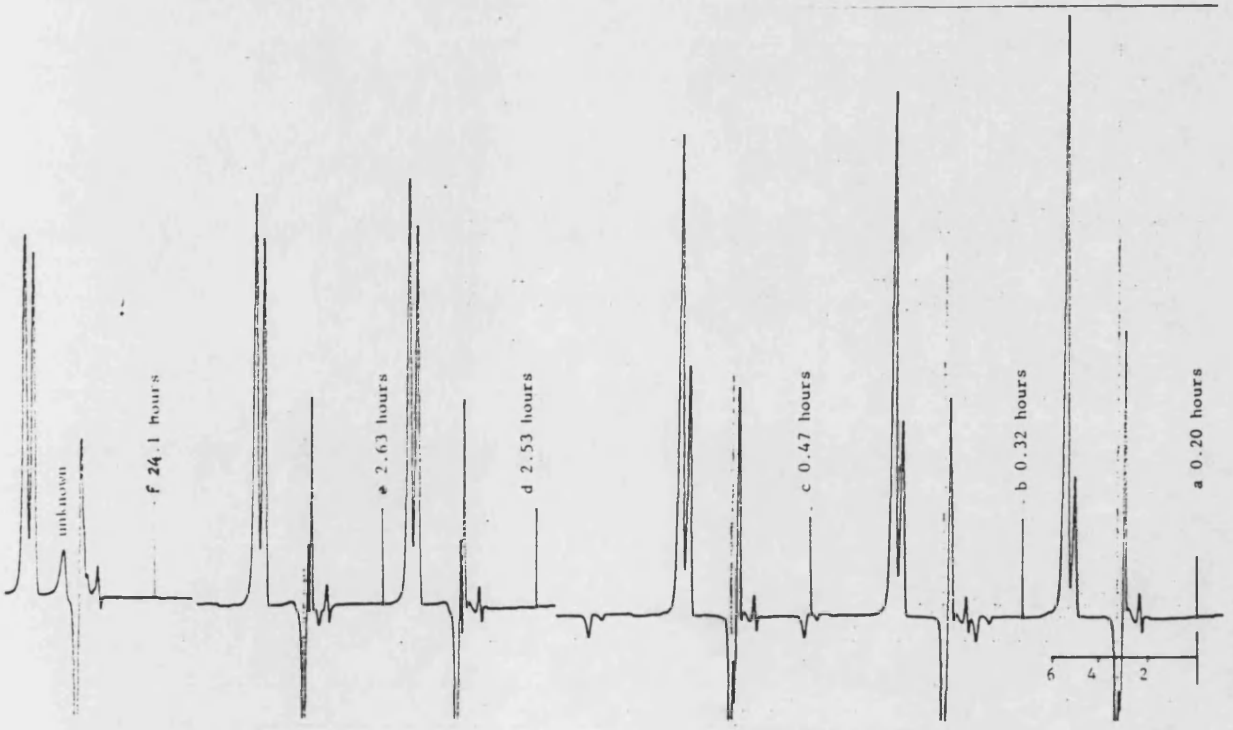


Figure 19 Chromatograms following the degradation and epimerisation of benzylpenilloic acid (4.22×10^{-4} M) at pH 2.2 and 37°C. Conditions as in Fig. 15.

The HPLC solvent system was modified to increase the separation of the degradation product from the solvent front and so that it could be then identified by comparison with authentic samples of possible degradation products. Figure 20 is the chromatogram obtained when the solvent system was changed to THF : 0.05 M phosphate buffer (5:95 v/v). All other operating parameters are unchanged. In this solvent system the benzylpenilloic acid epimers were more retained and co-elute. The retention of the degradation product was also increased and a new degradation product was found.

Spiking experiments revealed that the degradation products were benzylpenilloaldehyde **53** and D-penicillamine **10**

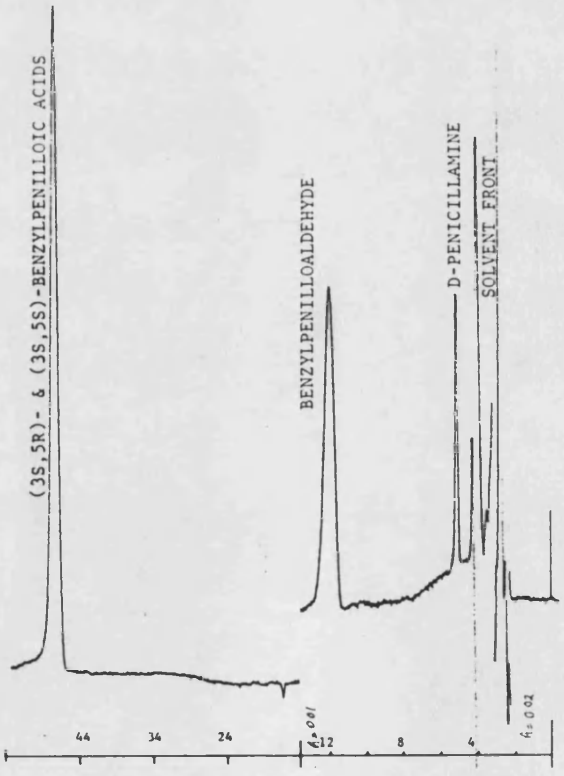
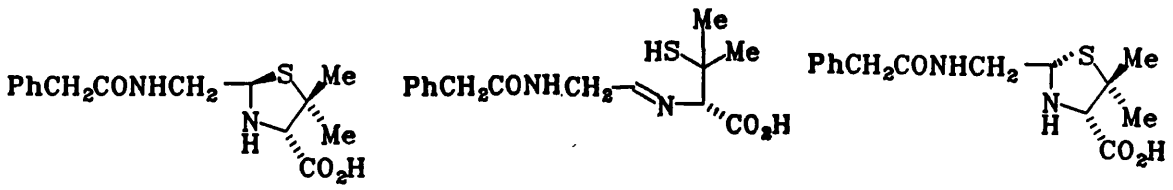


Figure 20 Chromatogram of benzylpenilloic acid aged for 6 days in pH 2.5 buffer at 25°C. Conditions as in Fig. 15 except eluent was THF : phosphate (pH3.9) 5:95 v/v.

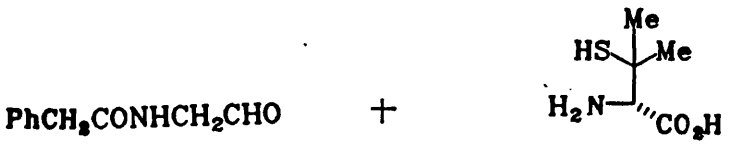
8.4 Discussion

Benzylpenilloic acid was found to readily epimerises at C-5 in aqueous solution. The forward and reverse rates and the equilibrium constants are pH and temperature dependent. Benzylpenilloic acid was found to be stable in neutral and basic solution, while in acidic solution degrades, albeit slowly, to benzylpenilloaldehyde and D-penicillamine (Scheme 11).



(3S,5R)-BPO ⇌ imine intermediate ⇌ (3S,5S)-BPO

H^+



benzylpenilloaldehyde D-penicillamine

Scheme 11

There are numerous reports of the C-5 epimerisation of benzylpenilloic acid [61 69 76 158 172], thiazolidine-3-carboxylic acids [173 174] and more closely related 5-substituted thiazolidine-3-carboxylic

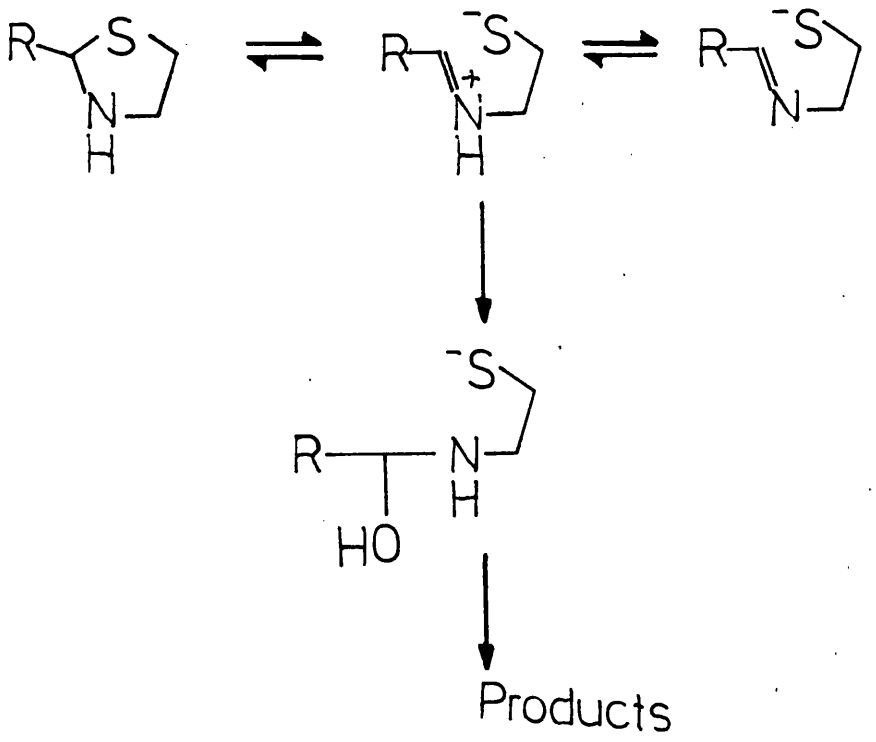
acids [175] [176] e.g. benzylpenicilloic acid 41 (see list in ref. 76) and penicic acid 60 [75] (the base hydrolysis product of 6-APA 31)

The involvement of an imine intermediate in the epimerisation of benzylpenilloic acid has been confirmed by differential pulse polarography [82] and by ^{1}H NMR studies in which deuteration at H-5 and H-6 was not observed, see section 6.3.8). Similar evidence for imine intermediates in the epimerisation of benzylpenicilloic and penicic acids was discussed in section 2.6.2.

The hydrolysis of imines occurs readily in water and involves the addition of water and elimination of the nitrogen moiety to give amine and aldehyde. The observed stability of benzylpenilloic acid in neutral and basic solutions may be due to ring closure of the imine occuring faster than hydrolysis, or that the degradation is reversible Previous studies [60-63] of the degradation of benzylpenicillin and its decomposition products in acidic solution have assumed the stability of benzylpenilloic acid. Our results indicate this assumption to be invalid.

Literature studies [175] [176] have shown that thiazolidines exist in equilibrium with their carbonyl and amino-thiol precursors and involve the imine and other open-chain tautomers. Thiazolidine-3-carboxylic acids is reported [175] to be stable in acidic, basic and neutral solutions. No changes in the ^{1}H NMR spectrum of 5- substituted aliphatic 2,2-dimethylthiazolidine-3-carboxylic acids in acidic (up to 8 M HCl) and neutral solutions were observed. However derivatives with aromatic substituents at C-5 degrade to a mercaptal if there is no steric hindrance. In strongly basic solution (>3 M NaOH) thiazolidines readily decompose to penicillamine and aldehyde. The kinetics of base

hydrolysis of 5-aryl substituted thiazolidines has been measured [176] in 1 x 10^{-3}M to 1 M NaOH at 25°C and show rate saturation at high base concentration. The proposed [176] mechanism has two equilibrium steps. First the thiazolidine ring opens between C-5 and S-1 to form a zwitterionic intermediate which then undergoes base-dependent proton loss, from nitrogen, to give a Schiff base, which is then hydrolysed to the aryl aldehyde and aminoethanethiol



9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES OF THE DEGRADATION OF BENZYLPENILLIC ACID

9.1 Introduction

HPLC studies were conducted to monitor the degradation of benzylpenillic acid BPI 42 at an initial concentration of about 5×10^{-4} M in pH 2.2, 2.5, 7 and 12 buffers at 37°C. The decomposition products were identified and quantified.

9.2 Experimental

The HPLC instrumentation has been described in section 5.2.2. Separations were performed on a 250 × 4.9 mm i.d. Spherisorb S5ODS2 column. The mobile phase consisted of THF : 0.05 M phosphate buffer (0.05 M KH_2PO_4 1 litre and 1 M H_3PO_4 1 ml) (7.5 : 92.5 v/v) at a flow rate of 1 ml/min. The amount of sample injected was 20 µl. Separations were carried out at ambient temperature. The detector wavelength was 230 nm.

9.2.1 Materials and reagents

The buffer solutions used have been described in section 7.2.

9.2.2 Calibration studies of reference standards

Stock solutions of benzylpenillic acid and possible decomposition products (benzylpenilloic BPO 43, benzylpenicilloic BPC 41, benzylisopenillic BIPI 56 acids and benzylpenilloaldehyde BPAL 53) were prepared by dissolving the required weight of compound in water and immediately analysing by HPLC. The dissolution of the free acids was

aided by vigorous shaking and ultrasonification. The purity of the analytical standards used in these studies is discussed:

a) Benzylpenillic acid BPI.

The sample of benzylpenillic acid used in these studies (preparation described in section 4.3.1) was assumed to be pure. Only one peak was present in all HPLC systems used and no impurities were obvious in the ^{1}H and ^{13}C spectra. The calibration studies were carried out using a solution of benzylpenillic acid (19.5 mg in 1.5 ml water = 3.89×10^{-2} M). Dilutions in the range 1.82×10^{-5} M to 5.83×10^{-4} M were assayed in duplicate.

b) Benzylpenilloic acid BPO

The sample of benzylpenilloic acid used in these studies (preparation described in section 4.3.3) was deemed to be 100% pure. No impurities were obvious by HPLC, ^{1}H and ^{13}C NMR. The calibration studies were carried out using a solution of benzylpenilloic acid (17.0 mg in 1.5 ml water = 3.67×10^{-2} M). Dilutions in the range 1.72×10^{-5} M to 5.51×10^{-4} M were analysed in duplicate. The 3S,5S and 3S,5R epimers co-elute in the HPLC conditions employed.

c) Benzylpenicilloic acid BPC

The sample of disodium benzylpenicilloate used in these studies (preparation described in section 4.3.2) consisted mainly of the 3S,5R,6R (RR-BPC)and 3S,5S,6R (SR-BPC) epimers with trace amounts of the 6S epimers. The sample was probably a hydrate of unknown stoichiometry, contaminated with appreciable amounts of sodium chloride. No further purification steps were undertaken.

The purity was assessed by comparing the standard, by HPLC, with benzylpenicilloic acids obtained by the complete and quantitative base

hydrolysis of benzylpenicillin i.e.

Disodium benzylpenicilloate (18.1 mg) was dissolved in 10 ml water (theoretical concentration = 4.57×10^{-3} M benzylpenicilloic acid). A 75 µl aliquot was diluted to 1 ml and analysed by HPLC. The results of duplicate analyses are given in Table 19.

Table 19 HPLC analysis of benzylpenicilloic acids BPC standard

	Peak areas		
3S,5S,6R-BPC	3S,5R,6R-BPC	3S,5R/S,6S-BPC	∑ BPC
5.30×10^5 (13.1%)	3.48×10^6 (86.0%)	3.67×10^4 (0.9%)	4.05×10^6
5.17×10^5 (13.3%)	3.35×10^6 (86.0%)	3.00×10^4 (0.8%)	3.90×10^6
		Mean =	3.98×10^6

Benzylpenicillin sodium (19.5 mg) was dissolved in 10 ml pH 12 buffer to give a 5.47×10^{-3} M solution. 75 µl aliquots were taken at timed intervals, diluted to 1 ml and analysed by HPLC. Upon complete hydrolyis of the benzylpenicillin the mean maximum peak area for the benzylpenicilloic acid epimers produced was 5.40×10^6.

Therefore the purity of the disodium benzypenicilloate was:

$$\frac{3.98 \times 10^6}{5.40 \times 10^6} \times \frac{5.47 \times 10^{-3}}{4.57 \times 10^{-3}} \times 100\%$$

= 88 % as benzylpenicilloic acid

The calibration studies were carried out using a solution of disodium benzylpenicilloate (19.9 mg in 10 ml water; 5.02×10^{-3} M uncorrected). Dilutions in the range 1.57×10^{-5} M to 2.51×10^{-3} M (uncorrected) were analysed in duplicate.

d) Benzylisopenillic acid BIPI

The sample of benzylisopenillic acid used in these studies (preparation described in section 4.3.6) was contaminated with benzylpenillic acid (≃ 8% by HPLC, ≃ 10% by ^{1}H NMR) and a small amount of an unknown compound. No further purification steps were taken and the purity was estimated at 90%. Calibration studies were conducted using a solution of benzylisopenillic acid (10.6 mg in 10 ml water = 3.17×10^{-3} M, uncorrected). Dilutions in the range 9.91×10^{-6} to 1.58×10^{-3} M (uncorrected) were analysed in duplicate.

e) Benzylpenilloaldehyde BPAL

The sample of benzylpenilloaldehyde used in these studies (preparation described in section 4.3.4) was essentially pure by HPLC and ^{1}H NMR. Calibration studies were conducted using a solution of benzylpenilloaldehyde (0.6 mg in 10 ml water = 4.85×10^{-3} M). Dilutions in the range 1.52×10^{-5} M to 2.43×10^{-3} M were analysed in duplicate.

N.B. The sample elutes from the HPLC column as, possibly, the *gem*-diol addition product.

The results of the calibration studies are given in Table 20. The peak area responses for all compounds examined were found to be linear within the concentration ranges studied.

Table 20 Results of HPLC calibration studies.

HPLC data

benzyl-penillic acid			benzyl-penilloic acid			benzyl-penicilloic acid			benzyl-isopenillic acid			benzyl-penilloaldehyde		
Conc x10^5	RT mins	PA x10^{-6}	Conc x10^5	RT mins	PA x10^{-6}	Conc x10^5	RT mins	∑PA x10^{-6}	Conc x10^5	RT mins	PA x10^{-6}	Conc x10^5	RT mins	PA x10^{-6}
58,3	4,80	46,0	55,1	30,1	57,8	221	#	26,8	143	13,1	242	243	8,82	17,8
29,2	4,80	23,1	27,6	30,1	29,3	22,1		2,76	14,3	13,2	nd	24,3	8,82	1,90
14,6	4,82	11,6	13,8	30,3	14,5	11,0		1,27	7,13	13,7	12,2	12,1	8,87	,933
7,28	4,81	5,39	6,89	30,2	7,21	5,52		,706	3,57	13,9	5,90	6,07	8,88	,481
3,64	4,82	2,66	3,44	30,2	3,31	2,76		,332	1,78	13,7	2,80	3,03	8,88	,228
1,82	4,88	1,38	1,72	30,7	1,70	1,38		,138	,892	13,7	1,28	1,52	8,91	,139

Regression analysis data

N	7	7	7	6	6
CC	0,99996	0,99995	0,99999	1,00000	0,99998
Slope	7,93x10^{10}	1,05x10^{10}	1,21x10^{10}	1,70x10^{11}	7,32x10^{9}
σ	3,76x10^{8}	5,08x10^{7}	2,60x10^{7}	1,13x10^{8}	2,20x10^{7}
Icept	-1,42x10^{5}	-7,91x10^{3}	1,14x10^{3}	-1,06x10^{5}	4,30x10^{4}
σ	1,03x10^{5}	1,32x10^{4}	2,20x10^{4}	6,58x10^{4}	2,20x10^{4}

Notes; Conc = corrected concentration i,e, penicilloic acids (88% pure) & isopenillic acid (90%)

PA = HPLC peak area,(arbitary units) mean of duplicate assays

∑PA = sum of peak areas of the penicilloic acid epimers,

RT = retention time in minutes,

Mean RT for (5R,6R)-BPC = 10,0 mins,, (5S,6R)-BPC = 11,3 mins,

N = number of observations including 0,0 CC = correlation coefficient, Icept = intercept

N,B, The (3S,5R/S)-benzylpenilloic acid epimers coeluted under these chromatographic conditions,

9.2.3 Kinetic studies

Studies of the degradation of benzylpenillic acid were carried out by dissolving about 20 mg of benzylpenillic acid in dilute sodium hydrogen carbonate aqueous solution. A 100 µl aliquot was added to 9.9 ml of the appropiate buffer prewarmed to 37°C. Aliquots of acidic and neutral solutions were analysed directly by HPLC at timed intervals. 100 µl aliquots of pH 12 solututions were first diluted 1:1 with HPLC eluent immediately prior to analysis. Studies at pH 2.2 and 2.5 were performed in duplicate. The pH of all solutions was checked before and after each experiment and if it had changed by more than 0.05 pH units the experiment was rejected.

The order and magnitude of the overall rate of degradation of benzylpenillic acid were obtained by regression analysis of the change in benzylpenillic acid HPLC peak area with time.

9.2.4 Quantitative analysis of the degradation products

The concentration of benzylpenillic acid and compounds formed during degradation were obtained by dividing the peak area of the analyte by the appropiate HPLC response factor (RF). The RFs are equivalent to the slope of the appropiate calibration lines (Table 20).

For example, the concentration of benzylpenillic acid at time t, $[BPI]_t$ is given by:

$$[BPI]_t = PA_{BPI} / RF_{BPI} \text{ moles}$$

where PA_{BPI} is the area of the HPLC peak corresponding to benzylpenillic acid and RF_{BPI} is the HPLC response factor obtained from the calibration studies. This quantitative technique is commonly known as *external calibration* (or *standardisation*)

The concentrations of identified products formed during degradation may be obtained in a similar fashion.

As a check on the data the mass balance (MB) was calculated for each assay i.e.

$$MB = \frac{\sum [\text{all compounds}]}{[BPI]_i} \times 100\%$$

where $\sum$ [all compounds] is the sum of the concentrations of all compounds quantitated and $[BPI]_i$ is the initial concentration of benzylpenillic acid. A mass balance of 100% would therefore indicate that the total reaction mixture had been accounted for.

The percentage of the reaction mixture present as benzylpenillic acid, at time t, is simply:

$$\% BPI_t = \frac{[BPI]_t}{[BPI]_i} \times 100\%$$

The percentage of other components present are obtained in a similar fashion.

The identity of the decomposition products formed during degradation was confirmed by comparison of their retention times with the retention times of the authentic reference standards.

9.3 Results

9.3.1 Degradation studies

The rate of degradation of benzylpenillic acid in acidic, neutral and basic solutions at 37°C was found to be first-order. The first-order plots are given in Figure 21. The regression data and calculated rate constants are given in Table 21. The rate constants obtained for degradation in acidic solution agree closely with the limited literature data available for comparison (see Table 22).

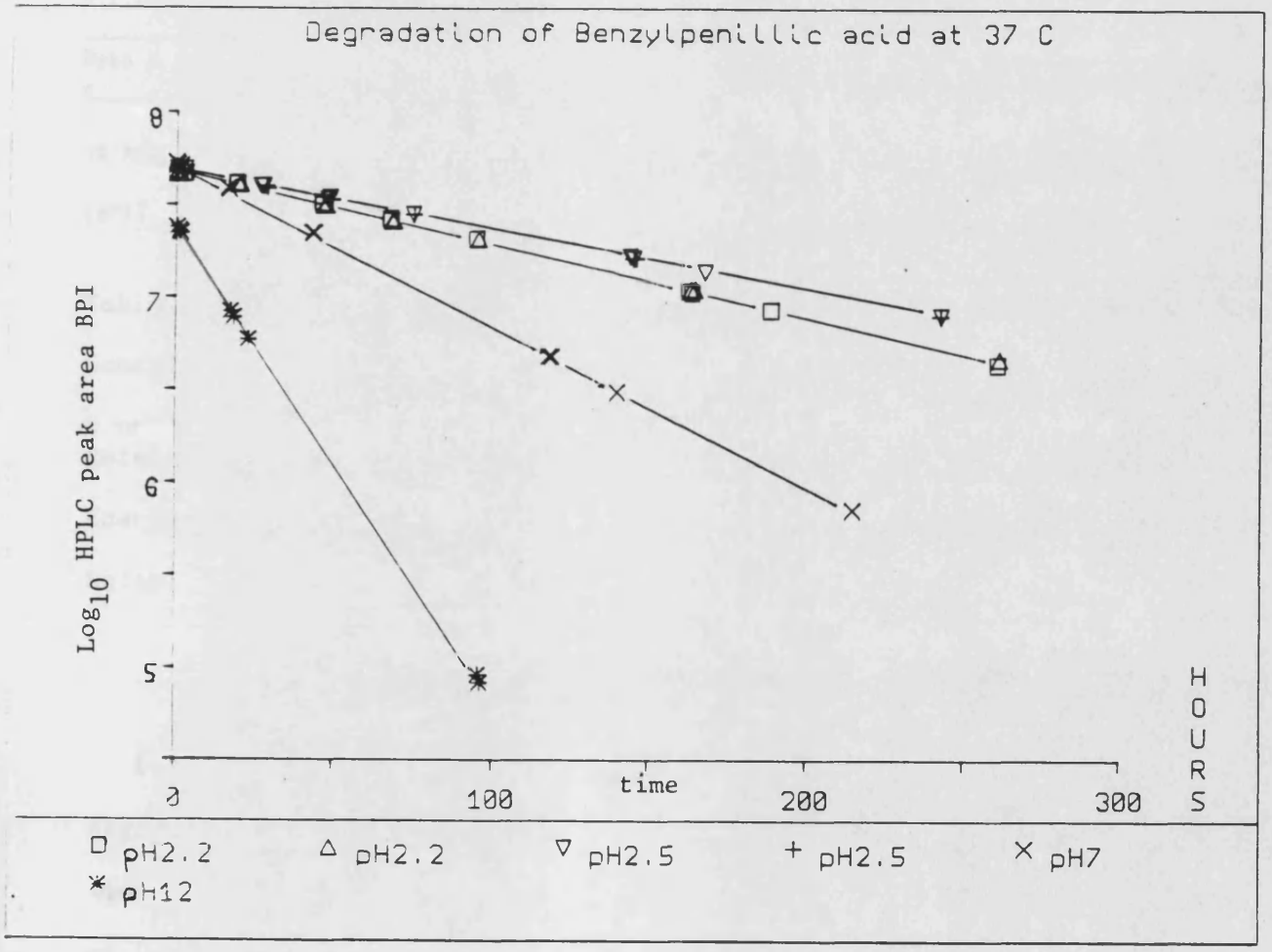


Figure 21 First-order plots for the degradation of benzylpenillic acid at pH 2.2, 2.5, 7 and 12 at 37°C

Table 21 Summary of regression data and rate constants for the degradation of benzylpenillic acid at 37°C

Conditions	pH 2,2 37°C Sample 1	pH 2,2 37°C Sample 2	pH 2,5 37°C Sample 1	pH 2,5 37°C Sample 2	pH 7 37°C	pH 12 37°C
N	15	12	11	10	11	9
CC	0,9994	0,9997	0,9997	0,9997	0,9999	0,9998
Slope x 10^3	-3,99	-3,85	-3,28	-3,26	-8,78	-25,4
σ x 10^5	2,62	2,08	1,98	2,00	3,14	14,7
Intercept	7,69	7,68	7,69	7,69	7,73	7,38
σ x 10^3	2,88	2,30	2,23	2,13	2,97	7,31
K hr^{-1} x 10^3	9,19	8,87	7,55	7,51	20,1	58,5
σ x 10^5	6,03	4,79	4,56	4,61	7,23	33,9
Mean K hr^{-1}	9,03 x 10^{-3}		7,53 x 10^{-3}		2,01 x 10^{-2}	5,85 x 10^{-2}
σ	6,03 x 10^{-5}		4,61 x 10^{-5}		7,23 x 10^{-5}	3,39 x 10^{-4}
t½ hours	76,7		92,0		34,5	11,8
$[BPI]_i$ x 10^4 M	6,18	6,04	6,18	6,18	6,77	6,05

Table 22 Literature data for degradation of benzylpenillic acid.

Conditions	pH 2.7 25°C †	37°C †	37°C ‡	45°C †	pH 2.5 37°C #
K hr^{-1}	2.94×10^{-3}	7.20×10^{-3}	1.20×10^{-2}	1.26×10^{-2}	3.42×10^{-3}
Reference	60	60	60	60	61

Energy of Activation E_A [60] = 13.8 Kcal mol^{-1} deg^{-1}

Notes: † 0.03 M citric acid - 0.0067 M disodium phosphate and NaCl to adjust ionic strength to 0.195
‡ 0.11 M citric acid - phosphate buffer (NaCl to μ = 0.195)
# D_2O - DCl (pH uncorrected for deuterium isotope effects)

Both sets of rate constants cited were obtained from studies of the degradation of benzylpenillic acid formed *in situ* by the acid degradation of benzylpenicillin. The rate constants associated with the work of Degelaen *et al* [61], although not explicitly stated in the reference, are easy to deduce and were reported by Kessler.[62] The original rate constants were in units of min^{-1}.

9.3.2 Quantitative analyses of degradation products

9.3.2.1 Degradation of benzylpenillic acid at pH 12 and 37°C

The relative concentrations of the various products formed during reaction are given in Figure 22 and Table 23.

Table 23 Relative concentrations of products formed during degradation of benzylpenillic acid at pH 12 and 37°C

Time hours	Concentration (% total) BPI	5S,6R-BPC	5R,6R-BPC	BIPI	% Mass balance
0	100	0	0	0	100,0
0.6	93.1	0	6.2	0.6	100.0
1.1	89.2	1.2	7.9	0.8	99.1
1.7	86.9	1.9	9.8	0.9	99.6
17.7	32.3	50.1	13.5	4.4	100.3
18.6	30.2	51.9	13.1	4.3	99.4
23.4	22.6	60.0	12.9	4.9	100.4
95.9	0.4	79.1	10.4	4.0	93.8
96.4	0.3	79.8	10.3	3.8	94.2

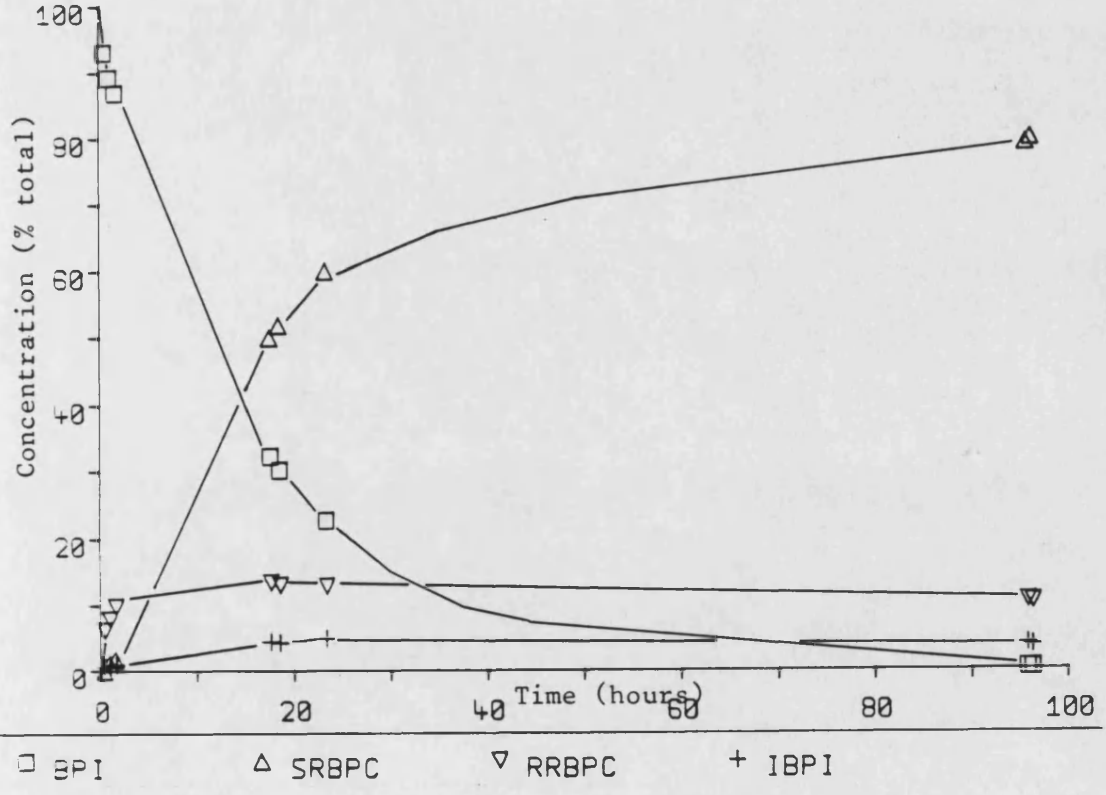


Figure 22 Plot of the concentrations of the degradation products of benzylpenillic acid at pH 12 and 37°C v time

The first products to appear in the chromatogram (see Figure 23 a) were (3S,5R,6R)-benzylpenicilloic acid (RR-BPC) and benzylisopenillic acid (BIPI). After 1.1 hours appreciable amounts of (3S,5S,6R)-benzylpenicilloic acid (SR-BPC) were observed (see Figure 23 b). After 23.4 hours ca. 22% benzylpenillic acid (BPI) remained and the relative amounts of products at this stage were 73% BPC (RR:SR = 18:82) and 5% BIPI (see Figure 23 c). Up to this stage the mass balance was close to 100% i.e. all the sample could be accounted for.

After 96 hours only 0.3% of the initial amount of BPI remained and the products were 90% BPC (RR : SR = 11.5 : 88.5) and 4% BIPI. The remaining 6% of the sample was small amounts of (3S,5R,6S)- and (3S,5S,6S)-benzylpenicilloic acids and several unknown peaks (see Figure 23 d). These remain unidentified and probably were decomposition products of benzylisopenillic acid.

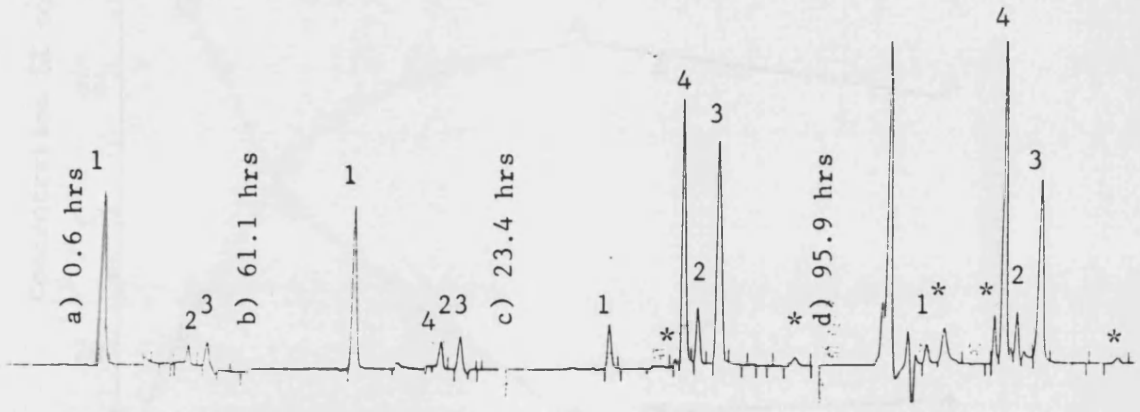


Figure 23 Chromatograms of the degradation of benzylpenillic acid at pH 12 and 37°C. (Conditions as in Section 9.2)

Key: 1 = BPI, 2 = RR-BPC, 3 = BIPI, 4 = SR-BPC and * = unknowns.

9.3.2.2 Degradation of benzylpenillic acid at pH 7 and 37°C

The relative concentrations of products found during degradation are given in Figure 24 and Table 24.

Table 24 Relative concentrations of products formed during degradation of benzylpenillic acid at pH 7 and 37°C

Time hours	Concentration (% total) BPI	5S,6R-BPC	5R,6R-BPC	5R,6S-BPC	BIPI	BPO	% Mass balance
0	100.0	0	0	0	0	0	100.0
0.02	98.9	0	1.0	0	0.3	0	100.2
0.5	97.8	0	2.0	0	0.4	0	100.0
1.3	96.3	0	2.6	0	0.4	0	99.3
16.4	71.7	20.8	7.1	0	0.9	0	100.5
16.9	71.0	22.2	7.6	0	0.9	0	101.7
43.2	41.9	47.6	9.0	0	1.8	1.4	101.7
43.8	41.5	48.1	9.0	0	1.7	1.5	101.8
118	9.0	68.7	9.8	2.5	2.2	6.1	98.3
119	8.8	68.7	9.9	2.5	2.1	5.9	98.0
140	5.7	62.5	7.9	1.6	2.3	10.6	90.6
215	1.3	60.5	8.2	4.0	2.0	13.1	89.1

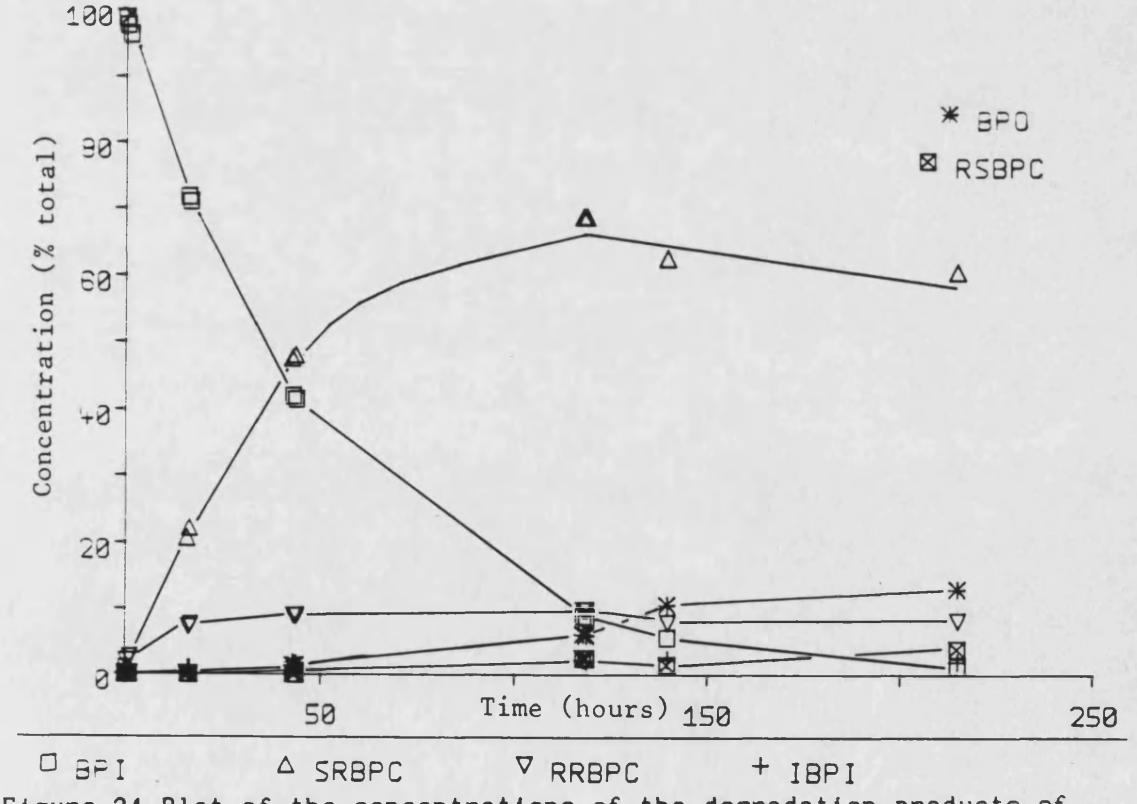


Figure 24 Plot of the concentrations of the degradation products of benzylpenillic acid at pH 7 and 37°C v time

The degradation product profile was similar to that observed at pH 12. Improved peak recognition and inte_gration routines allowed the quantitation of (3S,5R,6S)-benzylpenicilloic acid (RS-BPC). The major difference between studies at pH 7 and pH 12 was the appearance of (3S,5R/S)-benzylpenilloic acids (BPO) in aged solutions at pH 7, presumably due to decarboxylation of benzylpenicilloic acid.

Representative chromatograms are included in Figure 25. The calculated mass balance was about 100% for all but the last two data sets. The discrepancies were probably due an to unknown peak, indicated in the chromatogram (Figure 25 c), plus the accumulated experimental errors inherent in quantifying a large number of analytes simultaneously.

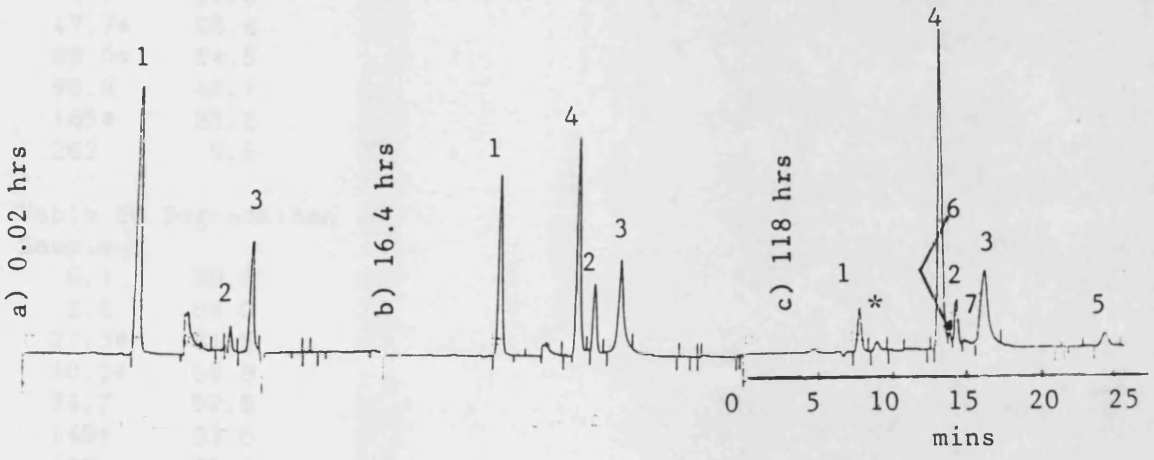


Figure 25 Chromatograms of the degradation of benzylpenillic acid at pH 7 and 37°C. (Conditions as in Section 9.2)
Key: 1 = BPI, 2 = RR-BPC, 3 = BIPI, 4 = SR-BPC, 5 = R&S-BPO, 6 = SR-BPC, 7 = SS-BPC and * = unknowns.

9.3.2.3 Degradation of benzylpenillic acid at pH 2.2 and 2.5, 37°C

The relative concentrations of products observed during degradation at pH 2.2 and pH 2.5 are given in Tables 25 and 26, respectively.

Table 25 Relative concentrations of products formed during degradation of benzylpenillic acid at pH 2.2 and 37°C.

Time hours	Concentration (% total) BPI	5S,6R-BPC	5R,6R-BPC	BIPI	BPO	BPAL	% Mass balance
Sample 1							
0	100.0	0	not	0	0	0	100.0
0.1	97.6	0.8	observed	0.4	0	0	98.8
1.1	98.5	1.3		0.4	0	0	100.2
2.4	97.4	0.9		0.5	0.7	0	99.5
3.0	96.4	2.2		0.5	1.9	0.5	101.4
19.2*	84.9	3.0		1.0	12.1	1.3	101.8
46.4*	65.8	2.5		1.8	26.8	4.8	101.7
67.9*	54.9	2.3		2.3	34.4	8.6	102.5
94.7	43.3	0		2.6	41.9	13.2	101.0
164*	22.4	0		2.4	46.6	28.0	99.4
189	17.7	0		1.8	45.2	29.3	93.9
261	9.1	0.6		1.6	40.2	45.2	96.6
Sample 2							
0.6	97.6	1.3		0.4	0	0	99.3
1.7	97.0	2.0		0.5	0	0	99.4
47.7*	65.6	1.1		2.1	27.5	4.9	101.2
69.0*	54.5	1.6		2.6	35.7	8.7	102.9
95.9	42.7	0		3.1	43.8	12.5	102.1
165*	23.2	0		3.6	53.5	23.2	103.5
262	9.6	0.5		3.3	53.5	32.9	99.8

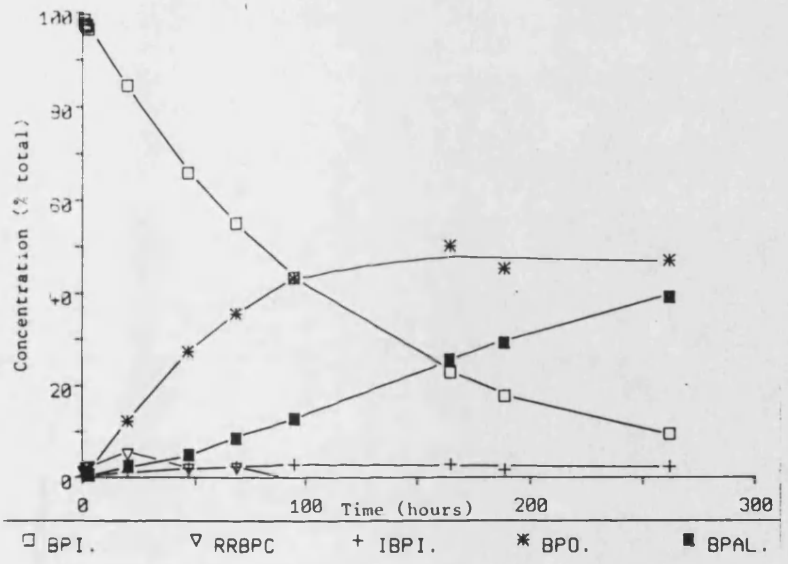
Table 26 Degradation of benzylpenillic acid at pH 2.5 and 37°C.

Sample 1							
0.1	99.8	1.4	0	0.4	0	0	101.5
2.8	99.0	2.2	0	0.5	0	0	101.7
27.3*	81.5	1.1	0.5	2.4	13.7	1.9	101.1
48.3*	69.9	1.0	0.3	3.6	22.5	5.4	102.7
74.7	57.5	0.8	0	5.0	31.3	10.7	105.2
145*	33.6	0	0	7.1	46.5	18.4	105.5
168	28.4	0	0	7.4	48.7	20.5	104.9
243	16.2	1.3	0	8.0	53.8	26.8	106.1
Sample 2							
0.6	99.6	3.2	0	0.4	0	0	103.2
1.3	98.4	2.0	0	0.5	0	0	100.8
28.9*	80.4	1.2	0.4	2.5	14.2	1.9	100.6
49.2*	68.5	0.9	0.3	3.6	22.9	5.5	101.8
75.3	56.9	0.8	0	5.0	31.4	10.3	104.5
146*	33.2	0.2	0	7.2	46.5	17.8	104.8
243	16.0	0	0	8.4	55.6	24.5	104.6

* Mean of duplicate assays.

It may be seen that there was good agreement between duplicates (apart from the latter data points of the pH 2.2 studies) and that the degradation profiles observed for pH 2.2 and pH 2.5 studies were similar. For the sake of clarity, the means of the duplicate experiments have been plotted with respect to time in Figures 26 and 27 for pH 2.2 and pH 2.5, respectively.

Figure 26 Plot of the concentrations of the degradation products of benzylpenillic acid at pH 2.2 and 37°C v time



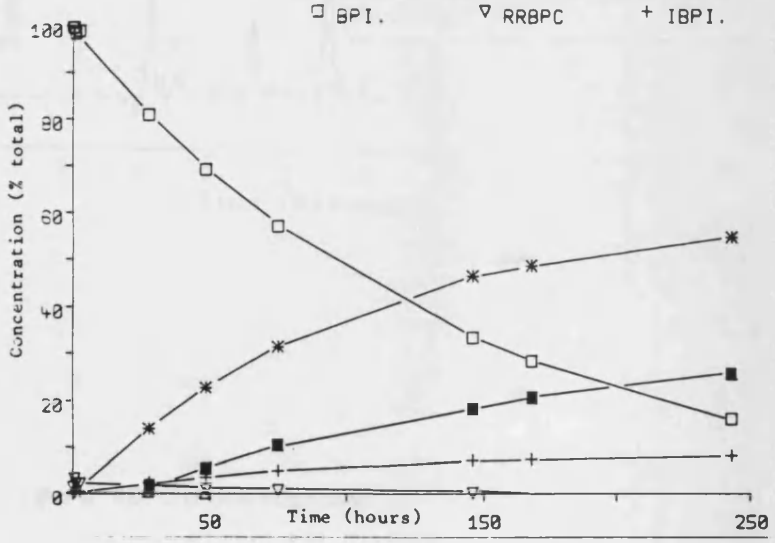


Figure 27 Plot of the concentrations of the degradation products of benzylpenillic acid at pH 2.5 and 37°C v time

In common with studies in neutral and basic solution the initial products in acidic solution were BIPI and RR-BPC. SR-BPC was observed in small amounts in pH 2.5 studies. The benzylpenicilloic acids decarboxylate to benzylpenilloic acid (BPO) which in turn degraded to benzylpenilloaldehyde (BPAL) and presumably D-penicillamine (DPAM), as described in Section 8, which co-eluted with the solvent front in this HPLC system. Representative chromatograms of the final analyses are shown in Figures 28 a and b.

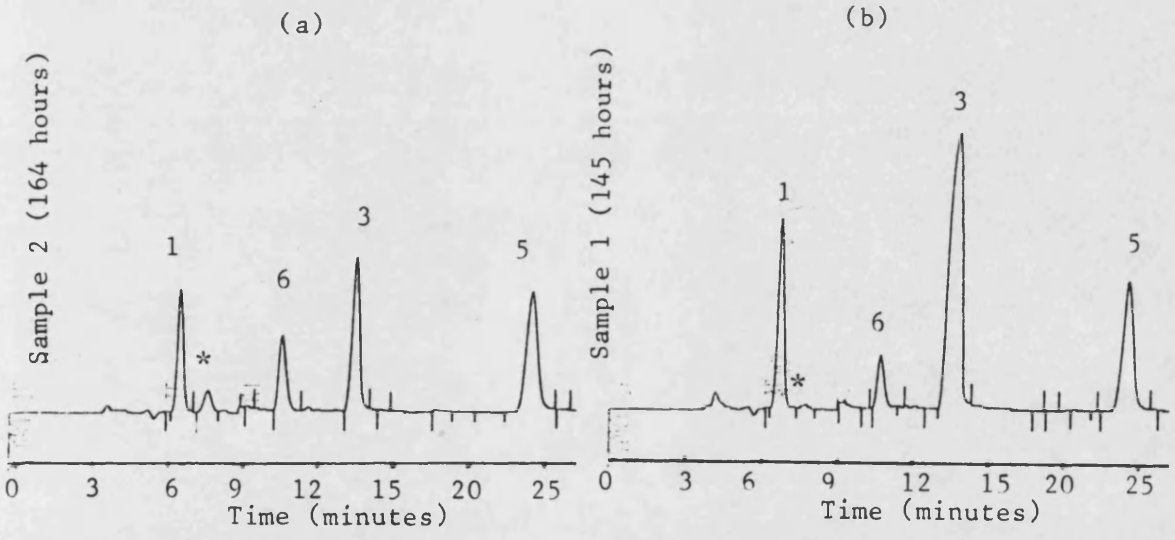


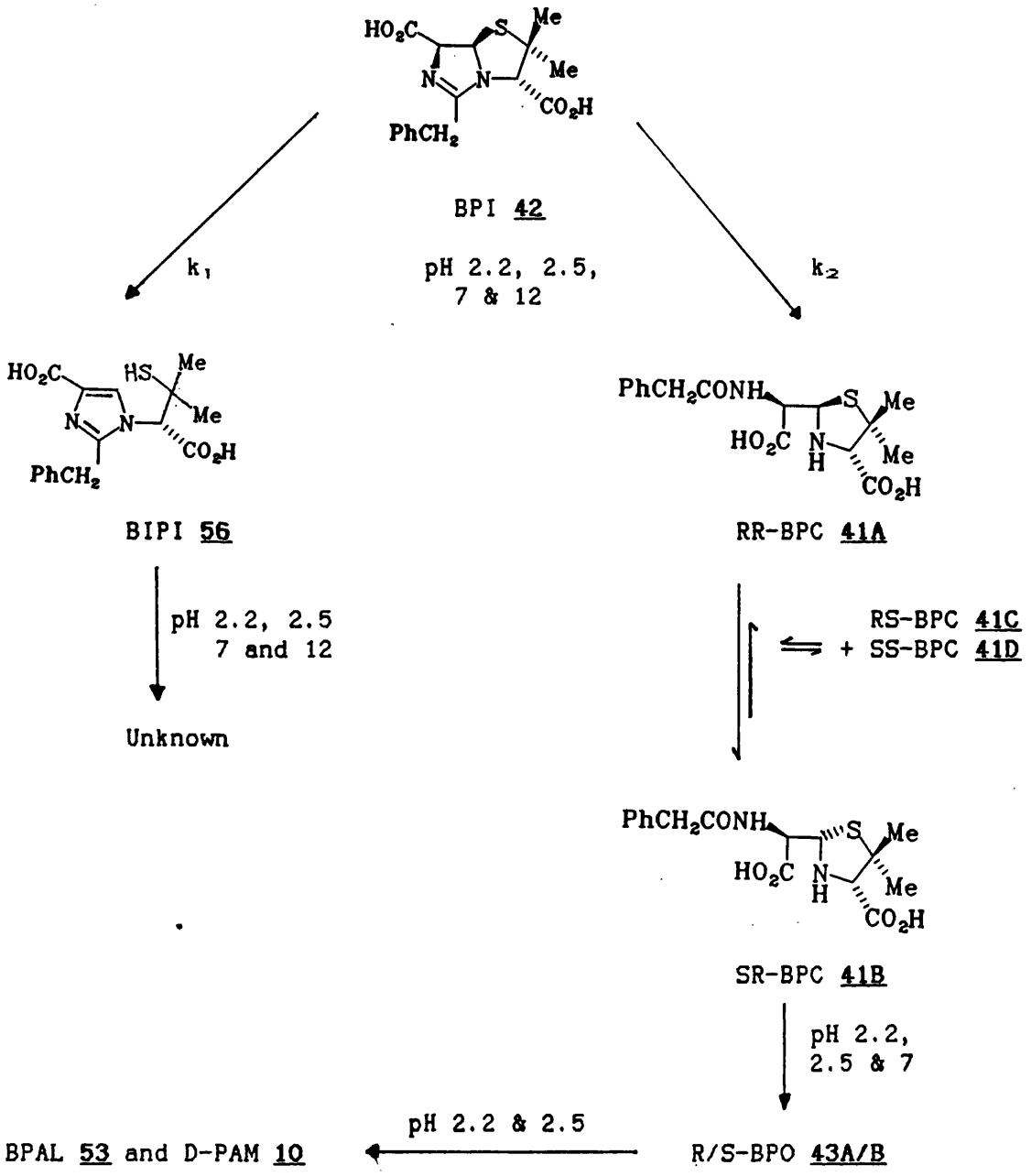
Figure 28 Chromatograms of the degradation of benzylpenillic acid at a) pH 2.2 and b) pH 2.5, 37°C. (Conditions as in Section 9.2)

Key: 1 = BPI, 3 = BIPI, 5 = R&S-BPO, 6 = BPAL and * = unknowns.

9.4 Discussion

9.4.1 Proposed degradation scheme and mechanisms

The proposed scheme for the degradation of benzylpenillic acid at pH 2.2, 2.5, 7 and 12 is given in Scheme 12.



Scheme 12 Proposed scheme for the degradation of benzylpenillic acid at pH 2.2, 2.5, 7 and 12 at 37°C.

This scheme describes the initial step, at all pHs, as the simultaneous formation of (3S,5R,6R)-benzylpenicilloic acid RR-BPC and benzylisopenillic acid BIPI. It was obvious that in all conditions studied, RR-BPC epimerised at C-5, the 3S,5S,6R epimer SR-BPC being kinetically favoured. The presence of small amounts of 3S,5R,6S and 3S,5S,6S-benzylpenicilloic acids (RS- & SS-BPC) in the reaction mixtures indicated that epimerisation at C-6 also occured, although to a much lesser extent.

In neutral and acidic solution benzylpenicilloic acids decarboxylated producing (3S,5R)- and (3S,5S)-benzylpenilloic acids BPO, which in acidic solution degraded further to benzylpenilloaldehyde BPAL and D-penicillamine DPAM.

If the formation of BIPI and RR-BPC follow first-order kinetics, then the overall rate of degradation of BPI, K is given by:

$$K = k_1 + k_2$$

where k_1 is the rate of formation of BIPI and k_2 the rate of formation of RR-BPC. It follows that at time t:

$$k_1 : k_2 = [BIPI]_t : [RR\text{-}BPC]_t$$

However, RR-BPC rapidly epimerised in aqueous solution and decarboxylated in acidic and neutral solutions to BPO which in turn broke down to BPAL and D-PAM. Therefore:

$$k_1 : k_2 \simeq [BIPI]_t : (\sum[BPC])_t + [BPO]_t + [BPAL]_t$$

As there was evidence that BIPI was degraded to unknown compounds, [BIPI]t was estimated as:

$$[BIPI]_t \simeq [BPI]_i - ([BPI]_t + (\sum[BPC])_t + [BPO]_t + [BPAL]_t)$$

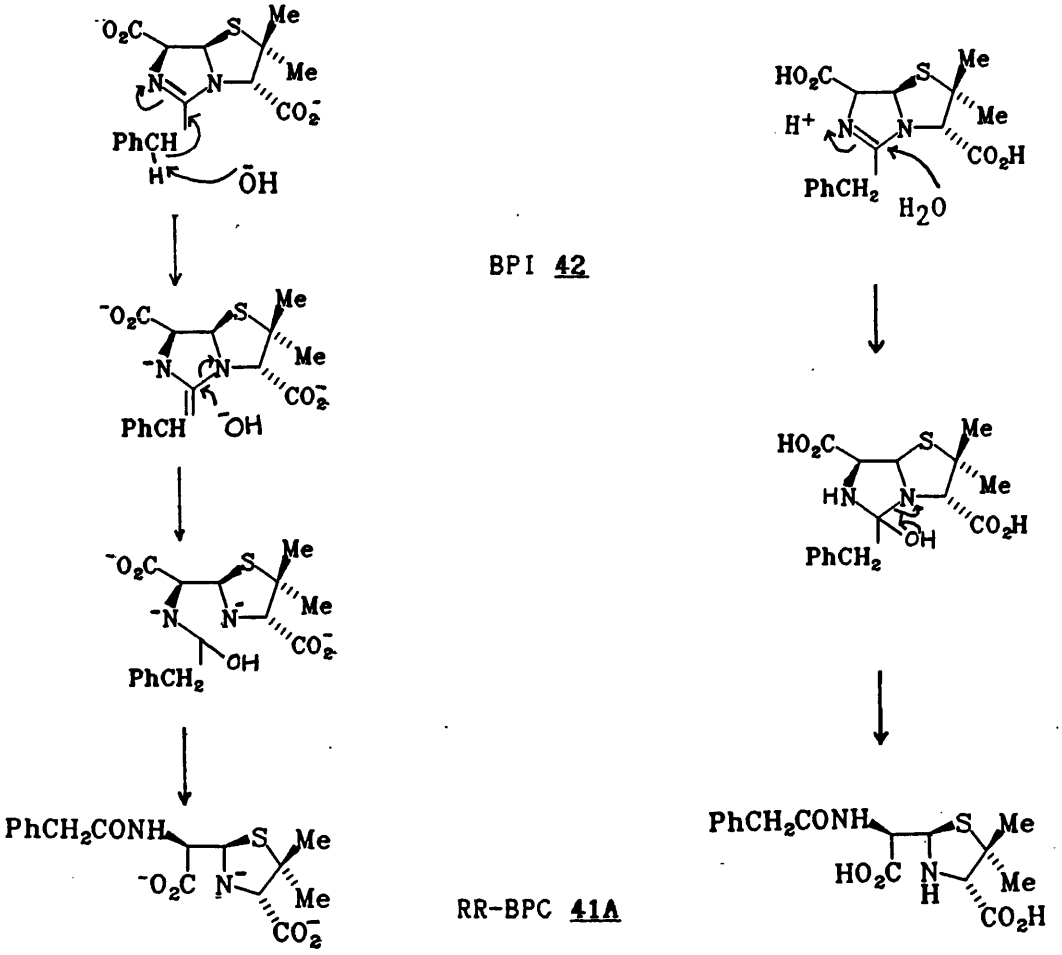
where $[BPI]_i$ is the initial concentration of benzylpenillic acid.

From Tables 23, 24, 25 & 26, k_1 : k_2 was estimated as 1 : 9 at pH 12, 1.5 : 8.5 (pH 7), 1 : 10 (pH 2.5) and ca. 1 : 40(±15) at pH 2.2. As K has already been determined (see Table 21), the absolute values of k_1 and k_2 are easily calculated.

It was obvious that the dominant degradation product was (3S,5R,6R)-benzylpenicilloic acid. This was additional evidence that benzylpenillic acid possesses 3S,5R,6R stereochemistry. The absolute stereochemistry of benzylpenillic acid has not been reported, however the $J_{H-5,H-6}$ value of 4.8 Hz (see Section 6.3.5) would seem to suggest that H-5 and H-6 are *cis* (cf β-lactams).

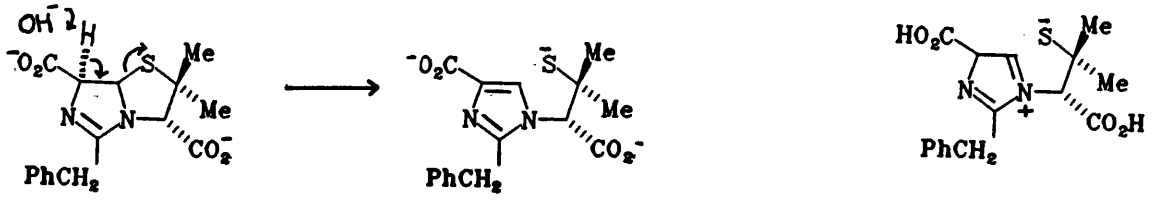
Previously proposed degradation schemes [60] [61] [62] assumed that benzylpenillic acid in acidic solution degraded exclusively and directly to benzylpenilloic acid. The formation of benzylisopenillic acid is well known [80] (small amounts produced at pH 11.6, higher yields obtained by heating BPI in refluxing methanol, or at room temperature in methanolic KOH or Baryta), although it was not observed in acidic solution. Our results indicate that benzylisopenillic acid is produced in acidic solution, albeit at low levels (1.6 to 3.3% at pH 2.2, 8.0 to 8.4% at pH 2.5). Fortunately the comparatively high uv absorbance of BIPI renders the HPLC method particularly sensitive.

The mechanisms of formation of (3S,5R,6R)-benzylpenicilloic acid from benzylpenillic acid in acidic, neutral and basic solutions are shown in Scheme 13. In basic solution reaction commences by nucleophilic attack by hydroxyl ion at C-10. In acidic solution the initial stage may be protonation of N-7 followed by net addition of water at C-8. Subsequent ring cleavage gives (3S,5R,6R)-benzylpenicilloic acid. Both mechanisms allow for the retention of configuration at C-5 & C-6.



Scheme 13

The driving force for the formation of benzylisopenillic acid is aromatisation upon formation of the imidazole. In basic solution, the proposed initial step is either loss of the basic proton α to the C-6α carboxylic group (see Scheme 14) or may involve the acidic protons of the benzyl side chain. In acid solution an imine intermediate 65 (cf. C-5 epimerisation of thiazolidines) may be involved.



Scheme 14 65

10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES OF THE EPIMERISATION AND DEGRADATION OF (3S,5R,6R)-BENZYLPENICILLOIC ACID

10.1 Introduction

HPLC studies were conducted to monitor the degradation and epimerisation of (3S,5R,6R)-benzylpenicilloic acid **41A** (initial concentration ca. 5×10^{-3} M) at pH 2.2, pH 2.5 (at 25° and 37°C) and pH 7 at 37°C. (studies of epimerisation in basic solution are treated in Section 12). The stereoisomers and decomposition products were separated, identified and their concentrations monitored during the degradation process.

10.2 Experimental

The HPLC instrumentation has been described in section 5.2.2. Separations were performed on a 250 × 4.9 mm i.d. Spherisorb S5ODS 2 column. The mobile phase consisted of THF : 0.05 M phosphate buffer (0.05 M KH_2PO_4 1 litre and 1 M H_3PO_4 1 ml, pH 3.9) (7.5:92.5 v/v) at a flow rate of 1 ml/min. The amount of sample injected was 20 µl. Separations were carried out at ambient temperature. The detector wavelength was 230 nm.

10.2.1 Materials and reagents

The buffer solutions used have been described in section 7.2.

10.2.2 Calibration studies of reference standards

The calibration of the HPLC system used, with respect to benzylpenicilloic, benzylpenilloic, benzylpenillic, benzylisopenillic acids and benzylpenilloaldehyde has been described in Section 9.2.2.

Some additional calibration studies were undertaken i.e.

a) Benzylpenillic acid BPI in very dilute solution.

6.7 mg. of benzylpenillic acid was dissolved in 10 ml of water with the addition of a minimum amount of $NaHCO_3$ to give a final concentration of 2.00×10^{-3} M). Three solutions (2.00×10^{-6}, 1.00×10^{-6} and 2.00×10^{-7} M) were prepared by dilution and assayed in triplicate by HPLC. The results are given in Table 27.

b) N-Formyl-D-penicillamine NFDPAM

A reference sample of N-formyl-D-penicillamine was analysed by HPLC and estimated to be ca. 90% pure by peak area normalisation at 230 nm.

5.9 mg of N-formyl-D-penicillamine was dissolved in 10 ml of water to give a final concentration of 3.34×10^{-3} M (3.01×10^{-3} M corrected). Two solutions (3.01×10^{-4} and 3.01×10^{-5} M) were prepared by dilution, and assayed in triplicate by HPLC. The results are given in Table 27.

c) Benzylpenamaldic acid BPENAM

Benzylpenamaldic acid could not be isolated in pure form. The HPLC response factor was estimated in section 11.4. as $(3.88 \pm 0.1) \times 10^{10}$ M per peak area unit. Note the degree of uncertainty in this estimate.

d)(3S,5R,6R)-Benzylpenicilloic acid RR-BPC

The sample of (3S,5R,6R)-benzylpenicilloate disodium salt has been previously estimated in section 9.2.2. to be 88% pure (as benzylpenicilloic acid). The stereoisomeric purity was determined by ^1H NMR and HPLC, see section 10.3.2. for results.

Table 27 Raw data and regression analyses for benzylpenillic acid and N-formyl-D-penicillamine HPLC calibration studies.

Benzylpenillic acid			N-formyl-D-penicillamine		
Conc.	RT mins*	PA*	Conc.	RT mins*	PA*
2.00×10^{-6}	4.41	1.60×10^{5}	3.01×10^{-3}	5.48	1.48×10^{7}
1.00×10^{-6}	4.41	7.94×10^{4}	3.01×10^{-4}	5.27	1.14×10^{6}
2.00×10^{-7}	4.44	1.71×10^{4}	3.01×10^{-5}	5.24	1.16×10^{5}
		* = mean of three assays			
CC	0.9959	(n = 10)	1.0000	(n = 10)	
Slope	7.96×10^{10}	($\sigma = 1.82 \times 10^{9}$)	4.91×10^{9}	($\sigma = 1.18 \times 10^{7}$)	
Intercept	5.60×10^{2}	($\sigma = 2.23 \times 10^{2}$)	-3.24×10^{4}	($\sigma = 1.78 \times 10^{4}$)	

10.2.3 Kinetic studies.

Studies of the degradation of benzylpenicilloic acid were carried out by dissolving ca. 20 mg, accurately weighed, of (3S,5R,6R)-benzylpenicilloic acid in the appropriate pre-warmed buffer. Aliquots of the solutions were analysed directly by HPLC at timed intervals. The pH of all solutions was checked before and after each experiment and if it changed by more than 0.05 pH units the experiment was rejected. The order and magnitude of the overall rate of degradation were obtained by regression analysis of the change in the summed HPLC peak areas of the benzylpenicilloic acid stereoisomers with time.

10.2.4 Quantitative analysis of the degradation products.

The concentrations of benzylpenicilloic acid stereoisomers and compounds formed during the degradation of benzylpenicilloic acid were obtained by dividing the peak area of the compound by the appropriate HPLC response factor, in the manner described in section 9.2.4. The HPLC response factors are summarised in Table 28.

Table 28 HPLC response factors RFs of reference compounds.

Compound	HPLC RF	σ	RSD	Source
Benzylpenicilloic acids	1.21×10^{10}	2.60×10^{7}	0.2 %	Table 20
Benzylpenillic acid	7.93×10^{10}	3.76×10^{8}	0.5 %	Table 20
	7.96×10^{10}	4.91×10^{9}	6.2 %	Table 27
Benzylpenilloic acids	1.05×10^{10}	5.08×10^{7}	0.5 %	Table 20
Benzylpenamaldic acid	3.88×10^{10}	1.00×10^{9}	2.6 %	Sect. 11
Benzylpenilloaldehyde	7.32×10^{9}	2.20×10^{7}	0.3 %	Table 20
N-Formyl-D-penicillamine	4.91×10^{9}	1.18×10^{7}	0.2 %	Table 27
Benzylisopenillic acid	1.70×10^{11}	1.13×10^{8}	0.1 %	Table 20

Notes: RSD = relative standard deviation about the mean.

= (100 σ)/mean %

HPLC RFs in peak area units per mole.

The RFs for benzylpenillic acid were similar. The value derived from calibration studies in section 9.2.2. (Table 20) was used as the RSD was much lower. The HPLC response factors for stereoisomers were assumed to be the same.

10.3 Results

10.3.1 Degradation studies

The apparent overall rates of degradation of benzylpenicilloic acid in acid and neutral solutions were found to be first-order. The first-order plots are given in Figures 29 a, b and c. The regression data and calculated rate constants are given in Table 29.

Table 29 Summary of regression data and rate constants for the degradation of benzylpenicilloic acid.

Conditions	pH 2,2 25°C	pH 2,5 25°C	pH 2,2 37°C Sample 1	pH 2,2 37°C Sample 2	pH 2,5 37°C Sample 1	pH 2,5 37°C Sample 2	pH 7 37°C
[BPC]i x 10^3	3,37	3,59	4,65	3,26	3,63	3,46	3,51
weighed	3,46	3,60	4,63	3,42	3,62	3,40	3,42
Observations	15	10	9	7	7	8	19
CC	0,9952	0,9968	0,9969	0,9982	0,9988	0,9986	0,9899
Slope x 10^4	-1,85	-1,78	-13,4	-12,8	12,5	13,4	-0,0938
σ x 10^4	3,57	3,54	28,0	24,6	19,0	20,2	0,50
Intercept	7,61	7,64	7,76	7,60	7,64	7,62	7,62
σ x 10^3	10,5	9,39	4,99	18,8	12,7	4,74	2,74
k min^{-1} x 10^4	4,26	4,10	30,9	29,5	28,8	30,9	0,216
σ x 10^4	8,22	8,15	64,5	56,7	43,8	46,5	1,15
K hr^{-1}	0,0256	0,0246	mean = 0,181		mean = 0,179		0,00130
σ	4.93×10^{-4}	4.89×10^{-4}	4.20×10^{-3}		6.00×10^{-3}		6.90×10^{-5}
t½ hours	27,1	28,2	3,82		3,87		22,3 days

Notes; Slope in units of min^{-1}.

The rates of degradation in acidic solution were found to be greater than previously reported in the literature (see Table 30).

Table 30 Literature data for degradation of benzylpenicilloic acid.

Conditions	pH 2.7 25°C †	37°C †	37°C ‡	45°C †	pH 2.5 25°C #
K hr^{-1} ref	0.0108 [60]	0.0300	0.0510	0.0486 [60]	0.0396 [61]

For key see Table 22, page 144.

There were differences in the pH and buffer composition, however the similarity of our results obtained from experiments conducted at pH 2.2 (KCl/HCl) and pH 2.5 (phosphate), would seem to indicate that previous studies have over-estimated the stability of benzylpenicilloic acid in acidic solution. It was also found that an increase in temperature had a more pronounced effect upon the rate of degradation than reported by other workers. Blaha *et al* [60] reported the energy of activation to be 14.6 KCal mol^{-1} deg^{-1}. From our limited experimental data the energy of activation over the range 25°C (298 K) to 37°C (310 K) for the degradation of benzylpenicilloic acid at pH 2.2 or pH 2.5 was estimated as ca, 30 KCal mol^{-1} deg^{-1}.

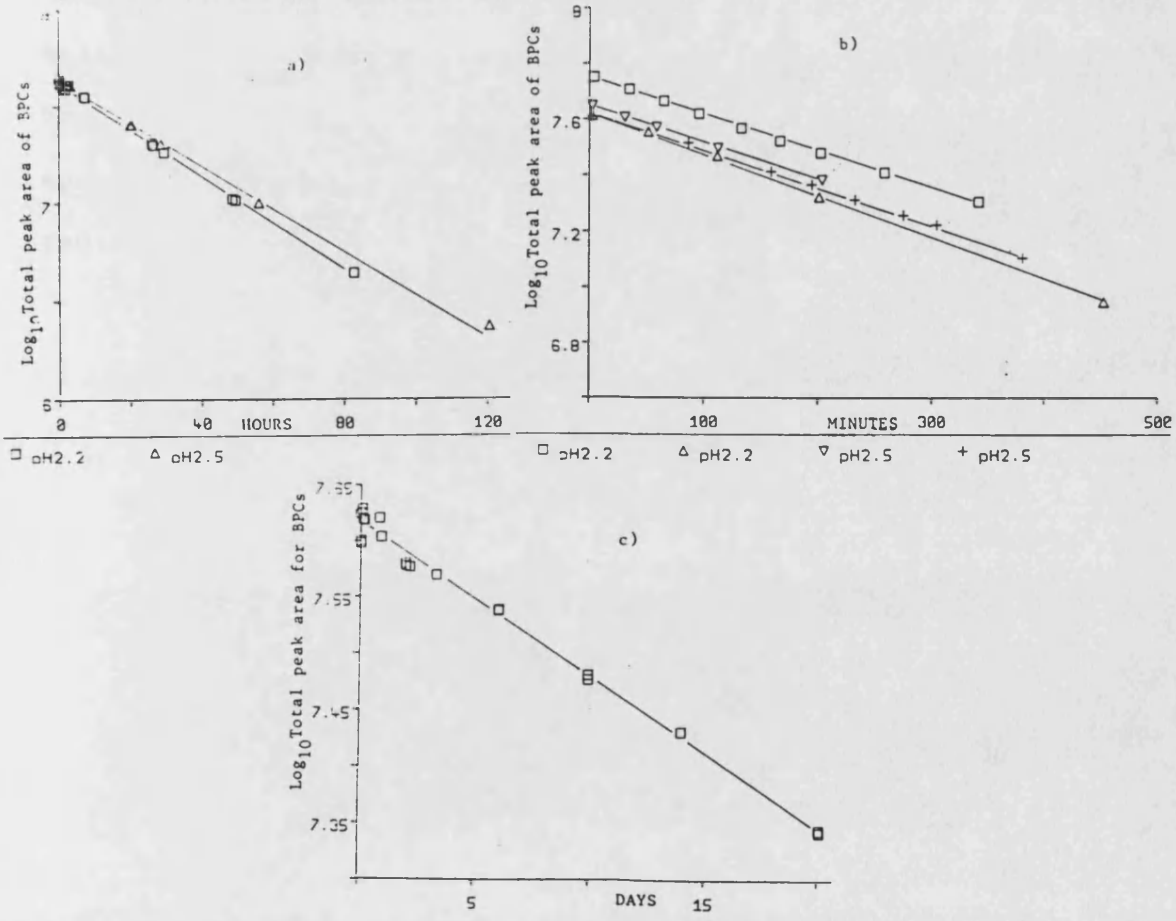


Figure 29 First-order plots for the degradation of benzylpenicilloic acid at pH 2.2 & 2.5 at a) 25°C, b) 37°C and c) pH 7 and 37°C

10.3.2 Epimerisation and degradation of benzylpenicilloic acid

The sample of (3S,5R,6R)-disodium benzylpenicilloate RR-BPC used in these studies was prepared by the base hydrolysis of (3S,5R,6R)-benzylpenicillin. ^{1}H NMR analysis indicated that the sample contained about 15% (3S,5S,6R)-benzylpenicilloic acid SR-BPC. HPLC analysis of the sample revealed two other impurities (Figure 30) which were found to be (3S,5R,6S)- RS-BPC and (3S,5S,6S)-benzylpenicilloic acids SS-BPC The identity of the peaks is discussed in Section 12. An accurate measurement of the initial stereoisomer ratios was difficult due to rapid epimerisation at C-5 (and possibly C-6) and inherent sources of error in the ^{1}H NMR and HPLC assays. However the initial ratios are estimated to be 12.5 : 2.5 : 85.0 : trace, for 5S,6R : 5R,6S : 5R,6R : 5S,6S, respectively. The presence of the 6S diastereoisomers implied epimerisation at C-6 and/or epimerisation at C-6 of *intact* benzylpenicillin, prior to base hydrolysis.

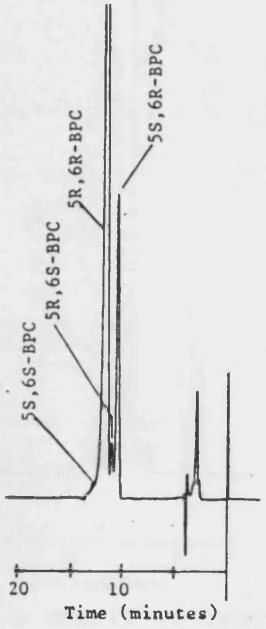


Figure 30 HPLC chromatogram of a 'fresh' sample of benzylpenicilloic acid (conditions described in section 10.2)

The relative concentrations of the four 3S-benzylpenicilloic acid stereoisomers and degradation products formed during ageing in acidic and neutral solutions are given in Tables 31 - 35 and Figures 31 - 35. The mass balance (MB) for most of the data was within 100 ± 5%. The discrepancies were assumed to be due to analytical error and consequently the data has been normalised to 100%.

Under all conditions examined, the primary degradation products were (3S,5R)- and (3S,5S)-benzylpenilloic acid (R-BPO and S-BPO), which then decomposed to benzylpenilloaldehyde BPAL and D-penicillamine DPAM. Very low levels of benzylpenillic acid BPI, in acidic and neutral solutions, and benzylpenamaldic acid BPENAM, in acidic solutions only, were observed throughout the respective experiments. Significant amounts of N-formyl-D-penicillamine NFDPAM were seen in acid solutions. A representative chromatogram is given in Figure 36.

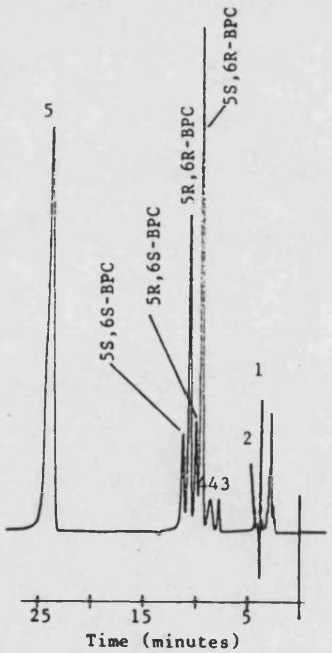


Figure 36 Chromatogram of a sample of benzylpenicilloic acid after 28.9 hours at pH 2.5 and 25°C. Conditions in section 10.2.

Key: 1 = NFDPAM, 2 = BPI, 3 = BPENAM, 4 = BPAL, 5 = R-BPO & S-BPO.

Table 31 Percentage concentration of products, normalised to 100%, formed during the degradation of benzylpenicilloic acid at pH 2.2 and 37°C. (BPC stereoisomer ratios included).

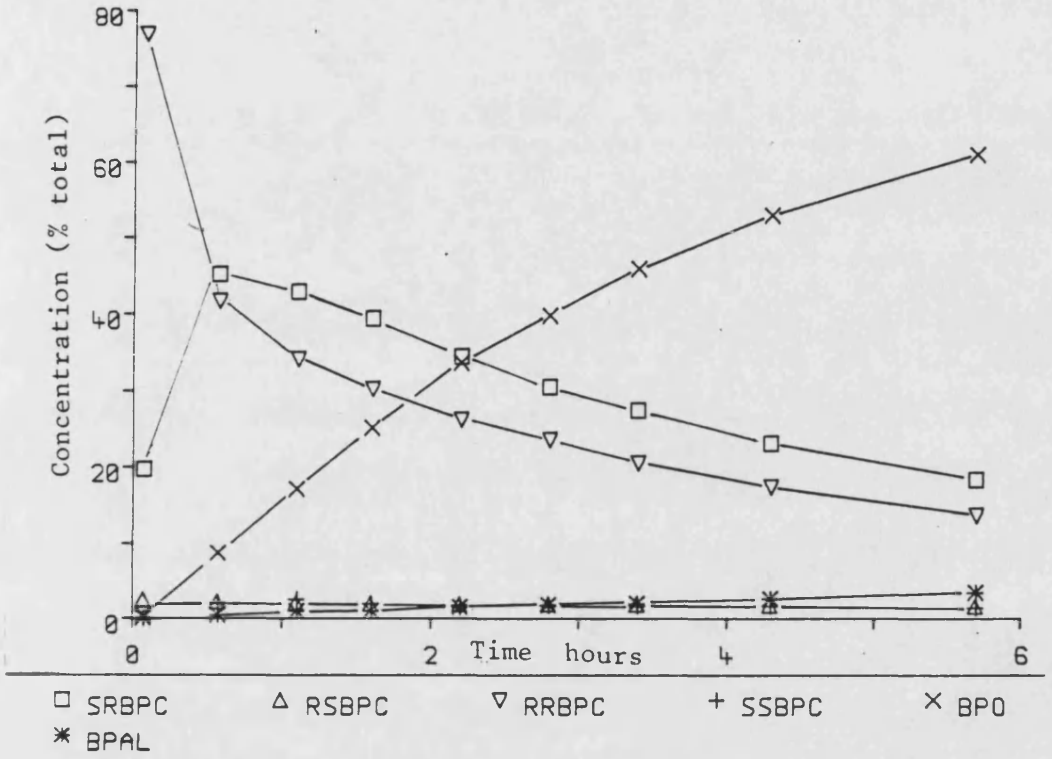
Initial concentration = 0.00465 M

TIME (hrs)	BPI	5S,6R BPC	5R,6S BPC	5R,6R BPC	5S,6S BPC	R/S BPO	BPAL	BPENAM	MB %
0,08	0,01	19,8 19,9	2,6 2,6	76,9 77,5	trace 0	0,5	0,2	-	101,2
0,58	0,04	45,2 49,5	2,3 2,5	41,8 45,6	2,1 2,4	8,7	0,5	0,02	99,4
1,09	"	42,9 52,4	2,2 2,7	34,1 41,8	2,6 3,1	17,2	1,0	0,04	100,9
1,61	"	39,4 53,3	2,1 2,8	30,3 41,1	2,0 2,7	25,2	1,1	"	100,0
2,22	"	34,5 53,4	2,0 3,1	26,3 40,6	1,9 2,9	33,7	1,6	0,06	101,5
2,80	0,05	30,6 53,1	2,0 3,4	23,7 40,1	1,8 3,2	39,8	1,9	"	102,6
3,38	"	27,5 52,8	2,0 3,8	20,7 39,9	1,8 3,5	46,0	2,1	"	102,4
4,32	"	23,2 52,4	1,9 4,3	17,5 39,4	1,7 3,9	52,9	2,7	0,08	101,7
5,70	"	18,5 52,1	1,7 4,8	13,8 38,8	1,5 4,3	61,0	3,5	"	100,9
21,60	"	1,9 42,7	0,6 13,6	1,4 31,1	0,5 12,7	87,0	9,1	-	97,4
22,20	"	1,7 42,1	0,6 14,4	1,2 30,6	0,5 12,9	87,1	8,9	-	99,8

Table 31 a) 5S,6R-BPC : 5R,6R-BPC epimer ratios during reaction.

Time (hrs) 0.08, 20.4 : 79.6 ; 0.58, 52.1 : 47.9 ; 1.09, 55.6 : 44.4 ;
1.61, 55.5 : 44.5 ; 2.22, 56.8 : 43.2 ; 2.80, 57.0 : 43.0 ;
3.38, 57.0 : 43.0 ; 21.6, 57.9 : 42.1 ; 22.2, 57.9 : 42.1

Figure 31 Plot of the concentrations of the degradation products of (5R,6R)-benzylpenicilloic acid at pH 2.2 and 37°C v time.



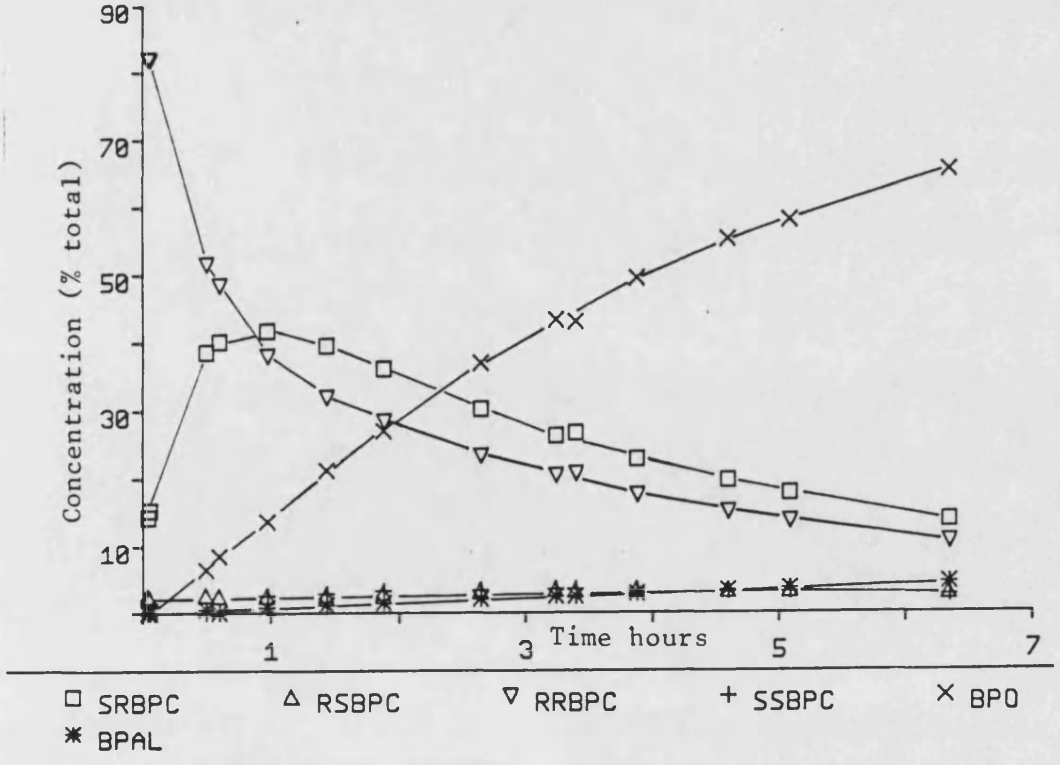


Figure 32 Plot of the concentrations of the degradation products of (5R,6R)-benzylpenicilloic acid at pH 2.5 and 37°C v time.

Table 32 Percentage concentrations of products (normalised to 100%) formed during the degradation of benzylpenicilloic acid at pH 2.5 and 37°C. (BPC stereoisomer ratios included)

Initial concentrations Sample 1, 0.00363 M; Sample 2. 0.00346 M.

Sample	Time (hrs)	BPI	5S,6R BPC	5R,6S BPC	5R,6R BPC	5S,6S BPC	BPO	BPAL	BPENAM	MB
1	0,05	0,01	14,2	2,7	82,1	trace	1,0	0	0	102,3
			14,3	2,7	82,9	-				
2	0,06	"	15,3	2,2	(81,9	-)	0,5	0	0	97,4
			15,4	2,2	(82,4	-)				
1	0,51	0,03	38,7	2,8	(51,6	-)	6,4	0,5	0,06	98,9
			41,6	3,0	(55,4	-)				
2	0,61	0,04	40,3	2,7	(48,5	-)	8,5	0	0,08	89,6
			44,0	3,0	(53,0	-)				
1	0,98	"	41,9	2,7	38,2	2,6	13,7	0,9	0,10	99,5
			49,1	3,2	44,7	3,0				
2	1,4	"	39,7	2,8	32,1	2,9	21,2	1,3	0,14	100,6
			51,2	3,6	41,4	3,7				
1	1,8	"	36,3	3,1	28,5	3,3	27,1	1,6	0,16	99,7
			51,0	4,4	40,0	4,6				
2	2,7	"	30,3	3,5	23,4	3,5	37,1	2,1	0,2	101,7
			49,9	5,8	38,6	5,8				
2	3,3	"	26,4	3,6	20,5	3,5	43,3	2,6	"	102,3
			48,9	6,7	38,0	6,5				
1	3,4	"	26,8	3,4	20,6	3,6	43,0	2,5	"	101,3
			49,3	6,3	37,9	6,6				
2	3,9	"	22,9	3,6	17,6	3,5	49,4	3,0	"	102,6
			48,1	7,6	37,0	7,4				
2	4,6	0,05	19,7	3,4	15,0	3,2	55,0	3,5	"	104,0
			47,8	8,2	36,3	7,7				
2	5,1	0,06	17,8	3,4	13,7	3,2	57,9	3,8	"	104,0
			46,7	8,9	36,0	8,4				
2	6,4	0,05	13,7	3,1	10,5	2,6	65,3	4,5	0,4	101,0
			45,8	10,4	35,1	8,7				
1	20,0	"	0,7	0,9	0,6	0,7	86,2	10,8	0	100,1
			24,1	31,0	20,7	24,1				
1	20,5	0,04	0,7	1,0	0,7	0,8	85,5	11,3	0	101,6
			21,9	31,3	21,9	25,0				

Table 32 a) 5S,6R-BPC : 5R,6R-BPC epimer ratios during reaction.

Time (hrs) 0.98, 52.3 : 47.7 ; 1.40, 55.3 : 44.7 ; 1.80, 56.0 : 44.0 ;
2.70, 56.4 : 43.6 ; 3.30, 56.3 : 43.7 ; 3.40, 56.5 : 43.5 ;
3.90, 56.5 : 43.5 ; 4.60, 56.8 : 43.2 ; 5.10, 56.5 : 43.5 :
6.40, 56.6 : 43.4 ; 20.5, 50.0 : 50.0

Table 33 Percentage concentrations (normalised to 100%) of products formed during the degradation of benzylpenicilloic acids at pH 7 and 37°C. (BPC stereoisomer ratios included).

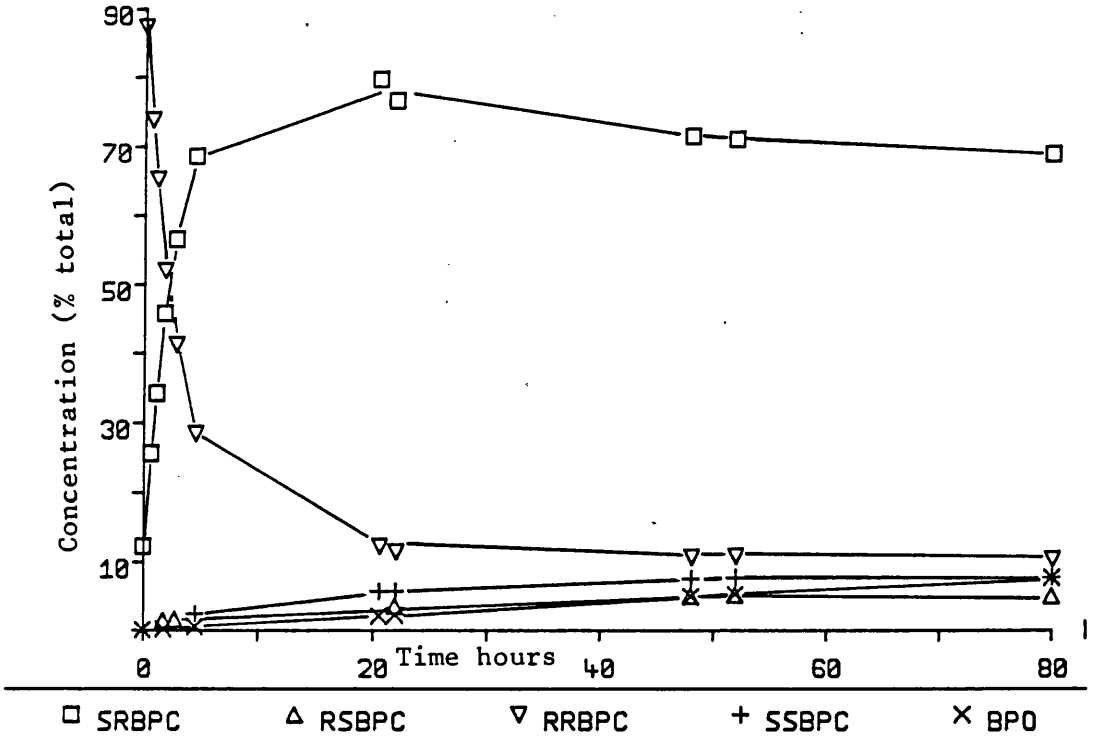
Initial concentration 0.00351 M

TIME hrs	5R,6R BPI	5S,6R BPC	5R,6S BPC	5R,6R BPC	5S,6S BPC	5R/S BPO	BPAL	MB
0,06	-	(12,3	-)	87,5	trace	0,2	0	102,2
		12,3		87,7				
0,7		(25,7	-)	(73,9	-)	0,2	0	96,7
		25,8		74,2				
1,2		(34,4	-)	(65,3	-)	0,3	0	96,2
		34,5		65,5				
1,9		45,9	1,6	(52,0	-)	0,4	0	103,2
		46,1	1,6	52,3				
2,9		56,6	1,7	(41,3	-)	0,4	0	101,2
		56,8	1,7	41,5				
4,6		(68,6	-)	28,5	2,5	0,6	0	101,2
		67,1		28,6	2,5			
20,6		(79,8	-)	12,3	5,7	2,1	0	103,0
		81,6		12,6	5,8			
22,0	4×10^{-3}	76,6	3,6	11,5	5,8	2,3	0,1	99,7
		78,6	3,7	11,8	5,9			
48,1	5×10^{-3}	71,5	5,1	10,7	7,5	4,9	0,2	96,8
		75,4	5,4	11,3	7,9			
52,0		71,1	5,2	10,8	7,6	5,2	0,3	96,5
		75,1	5,5,	11,4	8,0			
80,5	5×10^{-3}	68,9	5,1	10,4	7,8	7,6	0,3	97,5
		74,7	5,5	11,3	8,5			
145		62,9	5,7	9,4	8,6	13,3	0,2	96,8
		72,6	6,6	10,9	9,9			
(last 3 data sets not normalised due to large errors in the mass balances)								
239	4×10^{-3}	51,2	5,6	7,7	8,5	18,2	0,2	91,5
		70,1	7,7	10,5	11,6			
336	2×10^{-2}	44,7	5,2	6,8	8,5	23,1	-	88,3
		68,6	8,0	10,4	17,8			
483	1×10^{-2}	35,6	4,6	5,4	7,6	28,5	-	81,7
		66,9	8,6	10,2	14,3			

Table 33 a) 5S,6R-BPC : 5R,6R-BPC epimer ratios during reaction.

Time (hrs) 22.0, 86.9 : 13.1 ; 48.1, 87.0 : 13.0 ; 52.0, 86.8 : 13.2 ;
80.5, 86.9 : 13.1 ; 145 , 87.0 : 13.0 ; 239 , 87.0 : 13.0 ;
336 , 86.8 : 13.2 ; 483 , 86.8 : 13.2

Figure 33 Plot of the concentrations of the degradation products of (5R,6R)-benzylpenicilloic acid at pH 7 and 37°C v time.



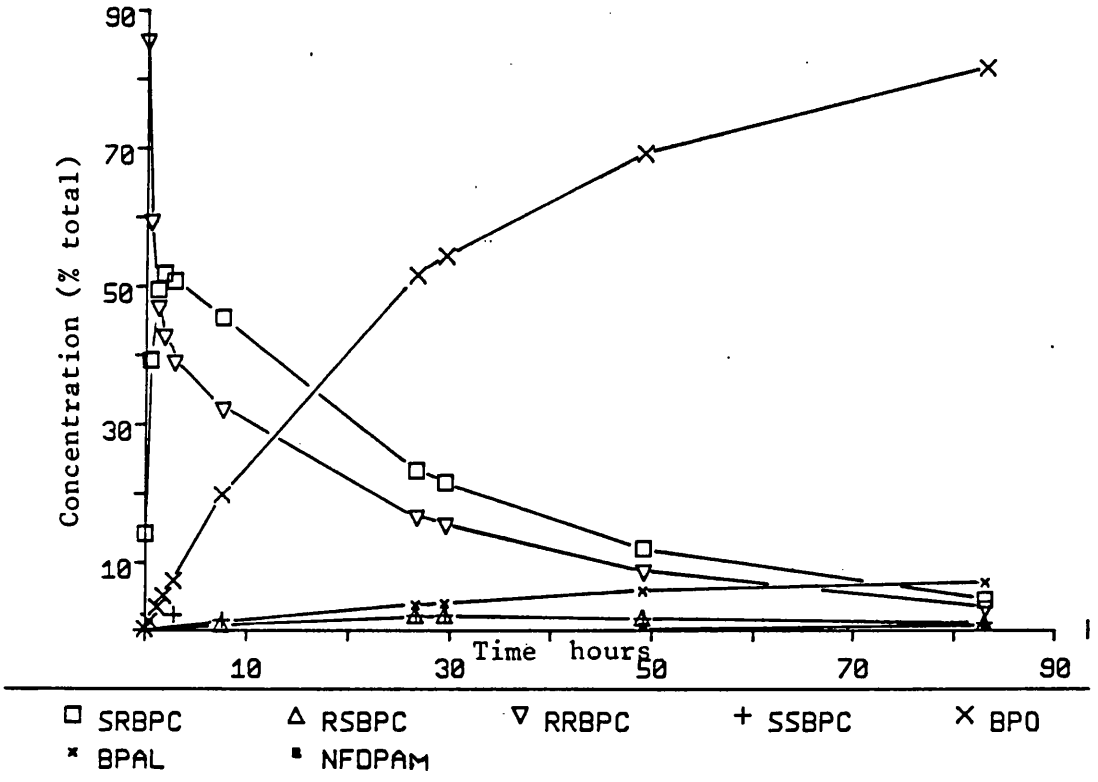


Figure 34 Plot of the concentrations of the degradation products of (5R,6R)-benzylpenicilloic acid at pH 2.2 and 25°C v time.

Table 34 Percentage concentrations (normalised to 100%) of products formed during the degradation of benzylpenicilloic acid at pH 2.2 and 25°C. (BPC stereoisomer ratios included).

Initial concentration 0.00347 M

TIME hrs	5R,6R BPI	5S,6R BPC	5R,6S BPC	5R,6R BPC	5S,6S BPC	5R/S BPO	BPAL	BPENAM	NFDPAM	MB
0,04	0	14,1	-	85,5	trace	0,4	0	0	0	101,1
		14,2		85,8						
0,5	0,02	39,4	-	59,1	-	1,4	0	0	0	99,4
		40,0		60,0						
1,3	0,03	50,0	-	46,9	-	3,5	0	0,006	0	98,3
		51,6		48,4						
1,8	0,03	51,9	-	42,7	-	5,1	0,3	0,02	0	98,0
		54,9		45,1						
2,3	0,04	51,4	-	41,8	-	6,0	0,8	0,08	0	104,1
		55,2		44,8						
2,8	0,04	50,7	-	38,8	2,4	7,3	0,8	0,06	0	106,5
		55,2		42,2	2,6					
7,1	0,03	46,0	1,2	32,3	1,8	18,7	-	0,06	0	104,9
		56,6	1,5	39,7	2,2					
7,6	0,03	45,5	1,0	32,0	1,6	19,9	-	0,06	0	104,5
		56,8	1,2	40,0	2,0					
26,2	0,05	24,4	2,4	16,6	2,3	50,5	3,8	0,12	0	106,9
		53,4	5,3	36,3	5,0					
26,7	0,05	23,4	2,4	16,5	2,3	51,6	3,8	0,14	0	106,1
		52,5	5,4	37,0	5,2					
30,0	0,04	21,6	2,4	15,3	2,3	54,3	4,0	0,14	0	104,9
		51,9	5,8	36,8	5,5					
49,1	0,05	12,0	1,9	8,5	1,8	69,2	5,8	0,16	0,6	103,4
		49,6	7,9	35,1	7,4					
49,9	0,05	11,7	1,9	8,2	1,9	69,8	5,8	0,16	0,6	104,2
		49,4	8,0	34,6	8,0					
83,1	0,04	4,5	1,2	3,1	1,2	81,7	6,9	0,16	1,1	104,1
		45,0	12,0	31,0	12,0					
147	0,05	0,9	0,5	0,6	0,4	87,0	8,5	0,14	2,0	110,1
		37,5	20,8	25,0	16,7					

Table 34 a) 5S,6R-BPC : 5R,6R-BPC epimer ratios during reaction.

Time (hrs) 7.10, 58.8 : 41.2 ; 7.60, 58.7 : 41.3 ; 26.2, 59.5 : 40.5 ; 26.7, 58.7 : 41.3 ; 30.0, 58.5 : 41.5 ; 49.1, 58.6 : 41.4 ; 49.9, 58.8 : 41.2 ; 83.1, 59.2 : 40.8 ; 147 , 60.0 : 40.0.

Table 35 Percentage concentration (normalised to 100%) of products formed during degradation of benzylpenicilloic acid at pH 2.5 and 25°C. (BPC stereoisomer ratios included)

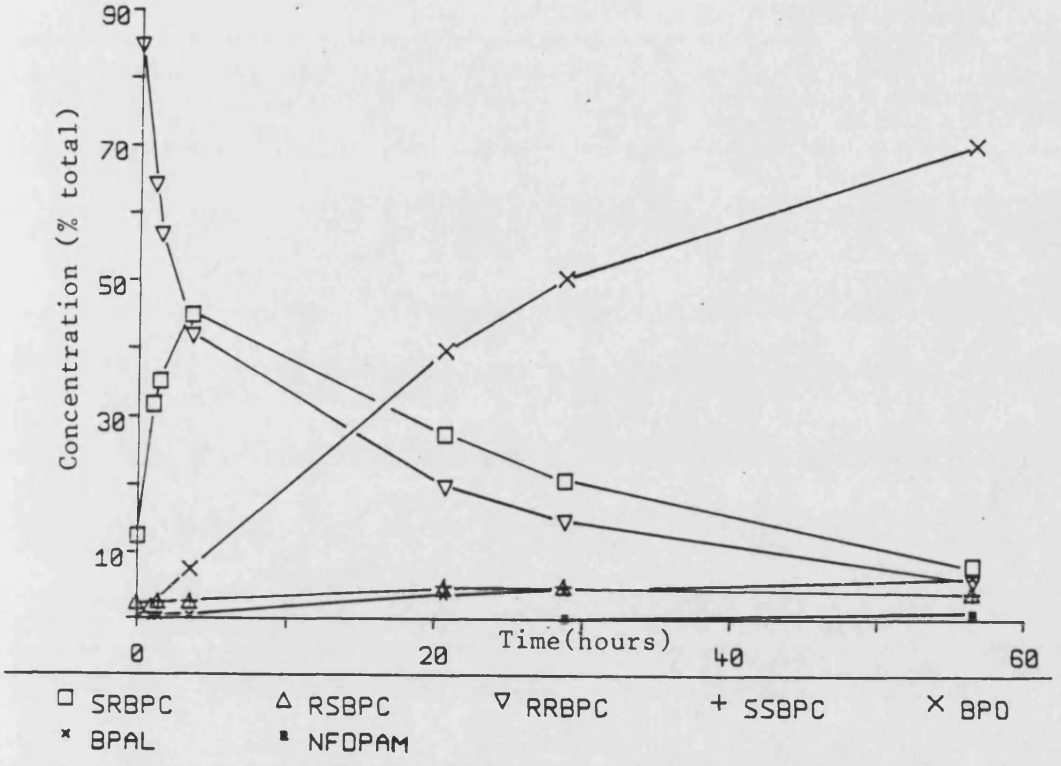
Initial concentration 0.00360 M

TIME hrs	5R,6R BPI	5S,6R BPC	5R,6S BPC	5R,6R BPC	5S,6S BPC	5R/S BPO	BPAL	3S-BPENAM	NF-DPAM	MB
0,04	0	12,5 12,5	2,5 2,5	(84,7 85,0	-)	0,3	0	0	0	105,7
1,0	0,02	31,8 32,5	2,0 2,0	(64,1 65,5	-)	1,9	0,2	0,07	0	97,5
1,5	0,03	35,1 36,2	2,6 2,7	56,7 58,5	2,5 2,6	2,6	0,4	0,07	0	105,2
3,0	0,03	43,5 46,6	2,6 2,8	44,8 48,0	2,5 2,7	6,1	0,4	0,08	0	102,4
3,6	0,03	45,0 48,9	2,6 2,8	41,8 45,4	2,6 2,8	7,4	0,4	0,08	0	101,6
4,1	0,04	45,5 50,1	2,7 3,0	39,9 43,9	2,8 3,1	8,5	0,5	0,1	0	101,4
20,3	0,04	27,1 46,9	6,4 11,1	19,4 33,6	4,9 8,5	38,6	3,3	0,4	0	105,0
20,7	0,04	27,4 52,6	4,6 8,8	19,6 37,6	5,0 10,0	39,6	3,5	0,4	0	102,9
28,9	0,04	20,7 45,9	4,9 10,9	14,6 32,4	4,9 10,9	50,3	4,2	0,4	0,2	102,6
56,5	0,05	8,2 37,1	4,1 18,6	5,8 26,2	4,0 18,1	70,2	6,5	0,4	1,0	104,1
121	0,05	1,2 23,5	1,6 31,4	0,9 17,6	1,4 27,5	83,7	8,6	0	2,6	107,8

Table 35 a) 5S,6R-BPC : 5R,6R-BPC epimer ratios during reaction.

Time (hrs) 1.50, 38.2 : 61.8 ; 3.00, 49.3 : 50.7 ; 3.60, 51.9 : 48.1 ;
4.10, 53.3 : 46.7 ; 20.3, 58.3 : 41.7 ; 20.7, 58.3 : 41.7 ;
28.9, 58.6 : 41.4 ; 56.5, 58.6 : 41.4 ; 121 , 57.2 : 42.8

Figure 35 Plot of the concentrations of the degradation products of (5R,6R)-benzylpenicilloic acid at pH 2.5 and 25°C v time.



10.4 Discussion.

10.4.1 Epimerisation of benzylpenicilloic acids.

The results indicated that the four (3S)-benzylpenicilloic acid stereoisomers were in complex equilibrium. Therefore, no attempt was made to derive the kinetic expressions governing the system, but two observations were noteworthy:

a) By examining the 5R,6R : 5S,6R and 5R,6S : 5S,6S epimer ratios during ageing, it was found that steady states were attained. (Table 36).

Table 36 3S-Benzylpenicilloic acid steady state epimer ratios

(abstracted from data in Tables 31 to 35)

Conditions pH	Temp°C	5S,6R : 5R,6R	Time taken to equilibrium	5R,6S : 5S,6S	Time taken to equilibrium
2.2	25	59 : 41	7 hours	51 : 49	*
2.2	37	57 : 43	2 - 3 hours	52 : 48	*
2.5	25	58.5 : 41.5	< 20 hours	51 : 49	*
2.5	37	56.5 : 43.5	2 - 3 hours	56 : 44	*
7	37	87 : 13	≃ 20 hours	40 : 60	*

Notes: * Equilibrium state attained rapidly.

b) The relative amounts of the 6S epimers to the 6R epimers increased with time. This implied that the 6S epimers are thermodynamically favoured and/or that they were more slowly degraded.

The C-5 epimerisation of benzylpenicilloic acid is well known and has been discussed previously (see section 2.6.2 pp 40 - 42). Similarly, the epimerisation of benzylpenilloic acid and other 5-substituted thiazolidine-3-carboxylic acids was discussed in section 8.4, pp 133 - 135. In all cases, in alkaline and neutral aqueous solutions, it was the 5S epimer that was thermodynamically favoured. This is in accord with the findings of McMillan and Stoodley [167] i.e. thiazolidines substituted at C-3 and C-5 (penicillin numbering) favour *cis* substitiuents.

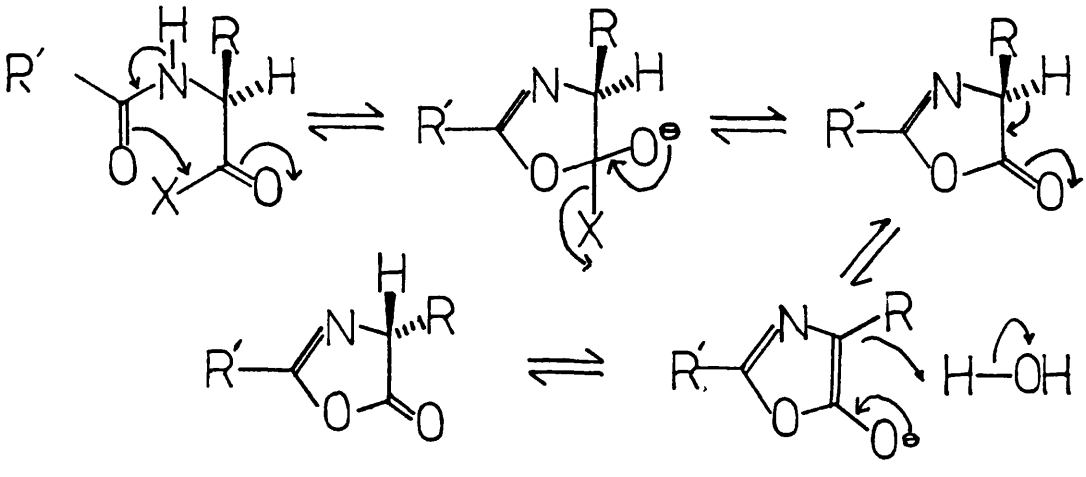
In acidic solution this general rule may no longer apply. At pH 2.2 and pH 2.5 the equilibrium epimer ratio for (3S,5S) and (3S,5R)-benzylpenilloic acids was 44 : 56 (see section 8.3.2), for (3S,5S,6R) : (3S,5R,6R) and for (3S,5S,6S) : (3S,5R,6S)-benzylpenicilloic acids 58 : 42 and ca. 48 : 52, respectively. An explanation may be the

effect of ionisation state of the thiazolidine nitrogen, fully protonated in acid solution at pH 2.2 - 2.5,[37] on ring conformation.

The involvement of an imine intermediate **44B** in the C-5 epimerisation of benzylpenicilloic acid is well known, however no peak in the HPLC chromatogram, which could be assigned to the imine, was observed. Hence, either the imine has only a transitory existence or its concentration in solution is below the HPLC limit of detection.

Very low amounts of the enamine tautomer of benzylpenamaldic acid **44A** were present in acid solutions of benzylpenicilloic acid. The involvement of the enamine in the epimerisation process is thought unlikely for reasons outlined previously and also because we have found that it is relatively stable in acid solution (see Section 12).

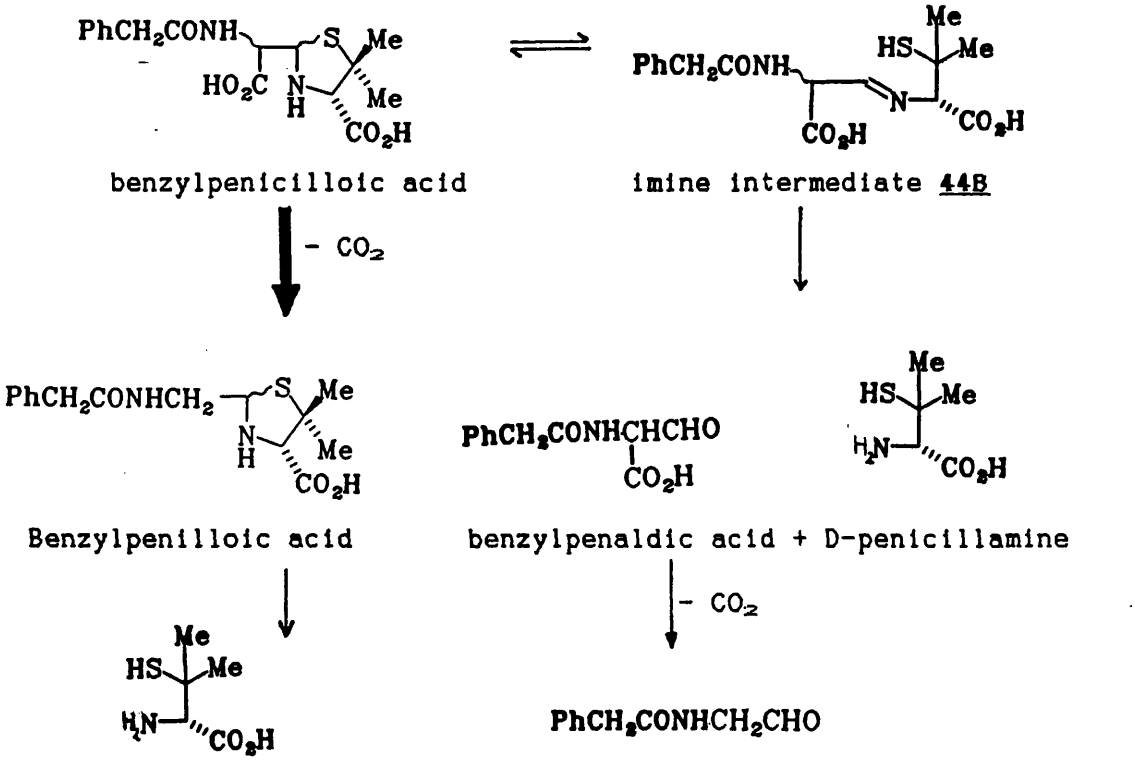
The possibility of C-6 epimerisation of benzylpenicilloic acid, first suggested in section 2.6.2., has been confirmed by our results however it is much slower than epimerisation at C-5. The racemisation of simple N-acylated α-amino acids is well known [177] and is thought to proceed via an oxazolone intermediate (see Scheme 15). The rate of epimerisation is dependent upon the nature of R, R' and X. It is conceivable that a similar mechanism may arise in benzylpenicilloic acid.



Scheme 15

10.4.2. The degradation of benzylpenicilloic acids

Benzylpenicilloic acids are known to be stable in alkaline solution [73] but degrade in acidic or neutral solution. Two pathways are possible (Scheme 16). Either decarboxylation to give benzylpenilloic acid which in turn decomposes to benzylpenilloaldehyde BPAL and D-penicillamine DPAM and/or hydrolysis of the imine intermediate 44B to give DPAM and benzylpenaldic acid 66 which spontaneously decarboxylates [178] to BPAL. Therefore the presence of BPAL, first observed in the very early stages of acid degradation, may arise from either route. However the increasing concentration of benzylpenilloic acid indicated that direct decarboxylation of benzylpenicilloic acid was the dominant degradative route. No benzylpenaldic acid was observed by HPLC.



Scheme 16 Proposed pathways for the degradation of benzylpenicilloic acid in acidic aqueous solution.

In carboxylic acids electron-withdrawing groups in the α- position facilitate decarboxylation.[179] [180] The ease of decarboxylation of benzylpenicilloic acid in acidic and neutral solutions was probably due to the inductive (-I) effect of the α-amido group. It is not clear whether decarboxylation proceeds via an oxazolone intermediate as in Scheme 15 or via a cyclic 6-centred transition state, involving the benzylamido group or thiazolidine nitrogen and the C-6 carboxylic acid in a manner analogous to the facile decarboxylation of malonic acid and other β-keto acids.

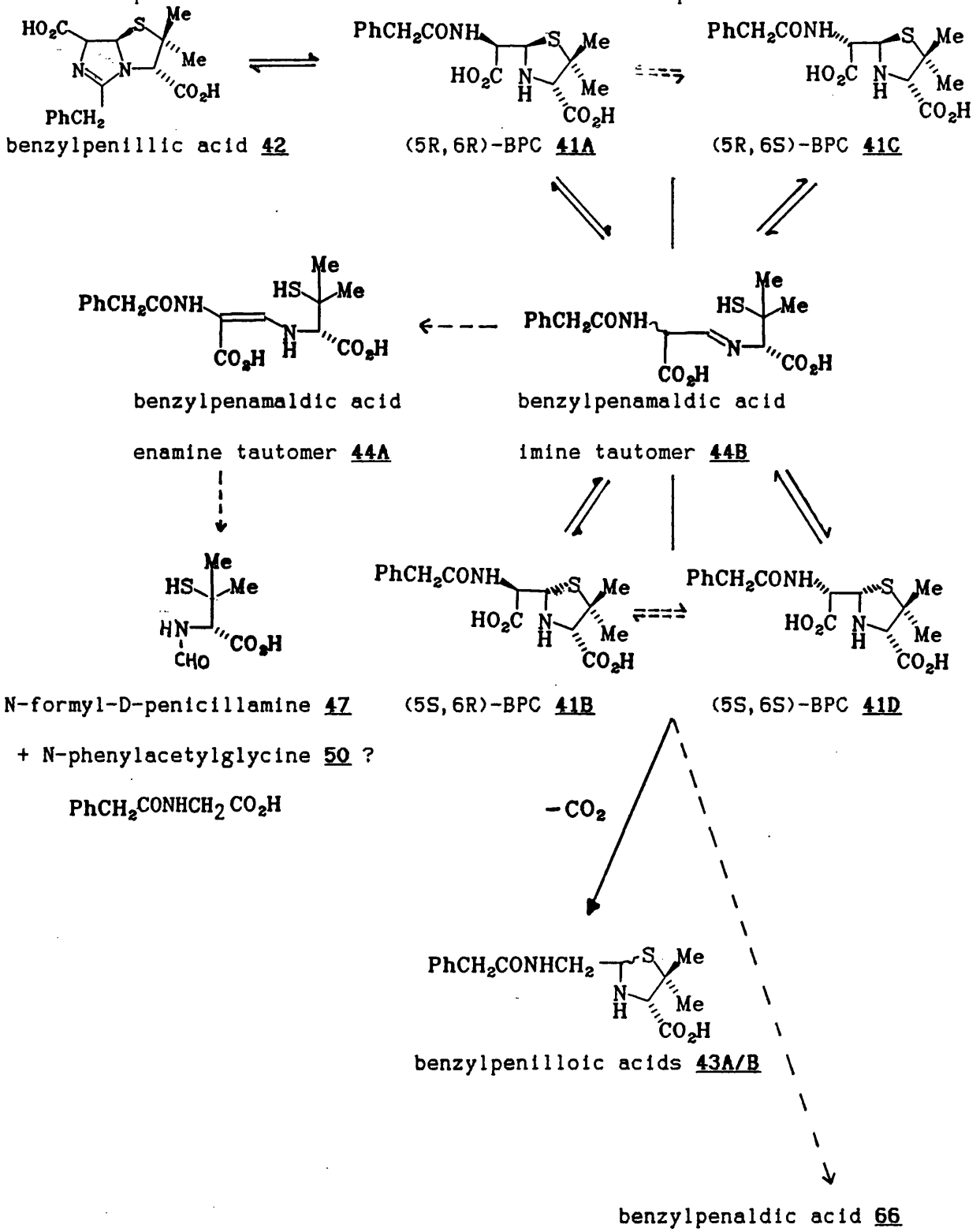
Our results suggested that the 6S-benzylpenicilloic acid epimers may be more stable than the 6R epimers to acid-catalysed decarboxylation. Stereoselective decarboxylation of peptides of C-substituted aminomalonic acids ($PhCH_2O_2CNHCHRCONHC(CO_2H)_2$) has been reported.[181]

From previous studies ,see Section 9.3.2, we know that (3S,5R,6R)-benzylpenicilloic acid was the major degradation product of benzylpenillic acid. The presence of small amounts of benzylpenillic acid observed in acidic and neutral solutions of benzylpenicilloic acid suggested that the conversion of benzylpenillic to benzylpenicilloic acid was reversible, to a small extent.

The presence of N-formyl-D-penicillamine in aged acidic solutions of benzylpenicilloic acids was thought to arise from the degradation of the enamine tautomer of benzylpenamaldic acid **44A**. The obvious by-product N-phenylacetylglycine **50** was not observed.

The complex epimerisation and degradation pathways of benzylpenicilloic acids suggested by our studies have been summarised in Scheme 17 (see overleaf).

Scheme 17 Pathways for the epimerisation and degradation of benzylpenicilloic acid BPC in acid and neutral aqueous solutions.



11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC AND ULTRAVIOLET SPECTROSCOPIC STUDIES OF THE DEGRADATION OF BENZYLPENICILLENIC ACID

11.1 Introduction

The purity of commercially available benzylpenicillenic acid 28 was determined by uv spectroscopy. The degradation of benzylpenicillenic acid was monitored by uv (230 - 400 nm) spectroscopic analysis. The overall rate of degradation was determined and products formed during degradation were separated, identified and quantified by HPLC.

11.2 Experimental

11.2.1 Materials and reagents

General chemicals, reagents and buffers solutions used have been described in section 7.2. Ethanol was HPLC Grade (Fisons, Loughborough Leics.). The internal standard used in some experiments was 3,4,5-trihydroxybenzoic acid (BDH Laboratory Reagents) and was pure by HPLC and ^{1}H NMR. The buffers used have been described in section 7.2.

11.2.2 Apparatus

UV studies were performed using the Perkin-Elmer model 550S double-beam spectrophotometer described in section 7.3.3. The HPLC apparatus has been described in section 5.2.2. The Spectromonitor III G variable wavelength detector was set at 230 or 322 nm. Samples were injected using a Rheodyne model 7125 valve injector equipped with a 20 µl loop. Separations were carried out using three systems:

System 1 Column: 50 × 2.1 mm i.d. Brownlee 5 μ RP-8.

Eluent: 0.05 M phosphate buffer pH 3.9 : MeOH (44 : 55 v/v)

Flow rate: 0.5 ml/min, at ambient temperature.

Detector wavelength: 322 nm.

System 2 Column: 250 × 4.9 mm i.d. Spherisorb S5ODS2

Eluent: 0.05 M phosphate buffer pH 3.9 : MeOH (40 : 60 v/v)

Flow rate: 1.0 ml/min, at ambient temperature.

Detector wavelength: 322 nm.

System 3 Column: 250 × 4.9 mm i.d. Spherisorb S5ODS2

Eluent: 0.05 M phosphate buffer pH 3.9 : THF (95.2 : 4.8 v/v)

Flow rate: 1.0 ml/min at 23°C.

Detector wavelength: 230 nm

Some studies were performed using a rapid scanning linear photo-diode array UV-Vis HPLC detector (LKB Bromma model 2140) equipped with an 8 μl flow cell of 10 mm path length. Data acquisition, manipulation and storage were performed on an Olivetti PC M24 micro-computer equipped with a Canon PJ-1080A colour printer. The detector scanned from 190 - 370 nm per second at 1 nm band-widths.

11.2.3 Determination of purity of benzylpenicillenic acid by uv.

Benzylpenicillenic acid (Sigma Chemicals) was stored cold and dry *in vacuo* in an amber glass bottle (as supplied). Immediately prior to calibration and degradation experiments, the sample was allowed to warm to room temperature and about 5 mg was accurately weighed and dissolved in 50 ml ethanol. A 1 ml aliquot was further diluted to 10 ml with ethanol to provide a solution containing about 0.01 mg per ml benzylpenicillenic acid (2.99×10^{-5} M uncorrected). 3 ml of the final

solution were rapidly transferred to a quartz cuvette of 1 cm path length, the absorbance at 322 nm (A_{322}) measured and the spectrum from 230 - 400 nm recorded. The reference cell contained pure ethanol. Assays were performed in duplicate. The concentration (c) is given by;

$$c = A_{322} / \varepsilon_{max} \text{ moles}$$

where ε_{max} is the molar extinction coefficient at 322 nm *

The % purity of benzylpenicillenic acid was therefore;

$$\% \text{ purity} = (c / c') \times 100\%$$

where c' is the concentration of benzylpenicillenic acid calculated from the weighings. The molecular weight was taken as 334.4 amu calculated for $C_{16}H_{18}O_4N_2S$. The sample was assumed to be anhydrous.

* There is some discrepancy in values of ε_{max} obtained from the literature. Livermore [16] calculated ε_{max} as 26 600 for a 0.01 mg per ml ethanolic solution of recrystallised material. Longridge [58] gives a value of log ε_{max} = 4.36 (ε_{max} = 22 909). No experimental conditions were given. Both workers quote λ_{max} as 322 nm. All values of the purity of benzylpenicillenic acid were calculated using ε_{max} = 26 600. This assumption was supported by results from the HPLC analysis of benzylpenicillenic acid, see Results section.

11.2.4 Determination of purity of benzylpenicillenic acid by HPLC

About 5 mg of benzylpenicillenic acid was accurately weighed and dissolved in 1 ml ethanol. A 200 µl aliquot was immediately added to 4.8 ml cold distilled water and then immediately analysed by HPLC using system 3.

11.2.5 Monitoring the degradation of benzylpenicillenic acid by uv spectroscopy

To 3 ml of the appropriate buffer solution at room temperature was added a 60 µl aliquot of a 0.48 mg per ml ethanolic solution of benzylpenicillenic acid. Final concentration was 9.42×10^{-3} mg per ml (2.81×10^{-5} M, uncorrected). The untreated buffer solution was used as reference. The uv spectrum (230 - 400 nm) was recorded at a scan speed of 120 nm per min. at timed intervals.

11.2.6 HPLC analysis of the degradation of benzylpenicillenic acid.

A rapid and sensitive HPLC stability-indicating method (System 1) utilising a short and narrow bore HPLC cartridge column, was developed to monitor the rapid degradation of dilute solutions of benzylpenicillenic acid. A typical chromatogram is shown in Figure 37

Studies at higher concentration exceeded the limit of linear detector response on System 1 and hence these studies were conducted using System 2 obviating the need for dilution prior to analysis.

A Initial concentration 2.5×10^{-5} M (uncorrected)

To 10 ml of the appropriate buffer solution prewarmed to 25° or 37°C was added a 200 µl aliquot of a 10.8 mg per 25 ml methanolic solution of benzylpenicillenic acid. Final concentration 8.47×10^{-3} mg per ml (2.53×10^{-5} M, uncorrected). 20 µl samples of acidic and neutral solutions were analysed directly by HPLC using System 1, at timed intervals. To prevent damage to the HPLC column pH 12 solutions were first neutralised by adding a 100 µl aliquot to 100 µl of pH 2.2 buffer. 20 µl of the neutralised solution was then immediately assayed

by HPLC. The peak areas were measured by an inte grator. As benzylpenicillenic acid was highly unstable, insufficient data were obtained from single experiments. Hence the experiments were performed in duplicate, at least, and the data combined for kinetic analysis. The methanolic solution of benzylpenicillenic acid was stored in an ice-bath during experiments and no appreciable degradation was noted during storage.

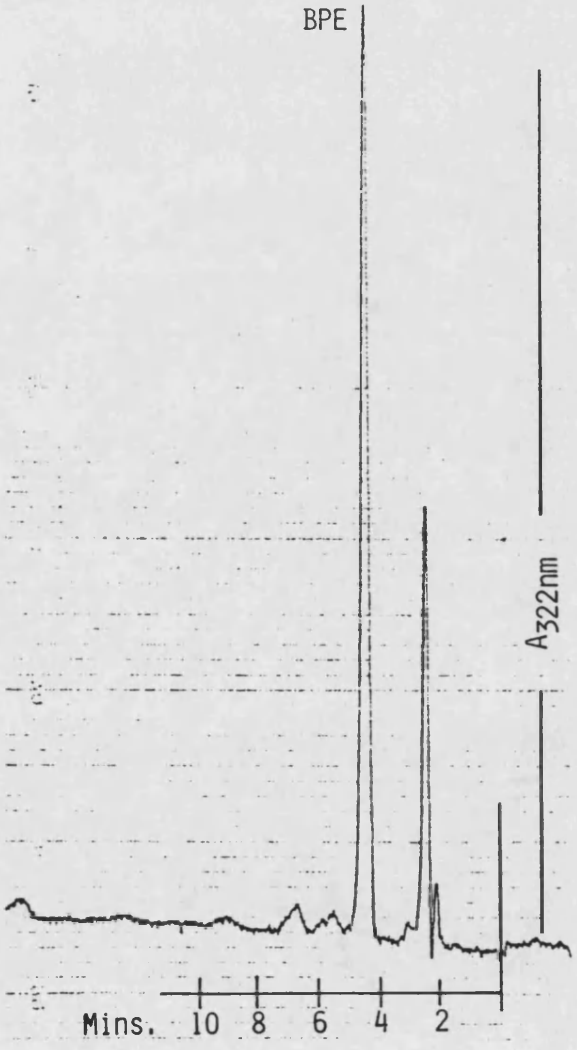


Figure 37 HPLC analysis of benzylpenicillenic acid using System 1.

Chromatogram represents ca. 12 nanograms on-column.

B Initial concentration 2.3×10^{-4} M (uncorrected)

The degradation of benzylpenicillenic acid in acidic solutions at 37°C was too rapid to monitor accurately using an initial concentration of 2.5×10^{-5} M. Hence degradation studies were conducted at 2.3×10^{-4} M. To 9.8 ml of the appropriate buffer solution pre-warmed to 25° or 37°C was added a 200 µl aliquot of a 3.84 mg per ml ethanolic solution of benzylpenicillenic acid. The final concentration was 0.0768 mg per ml (2.3×10^{-4} M, uncorrected). Solutions were analysed directly by HPLC using System 2. The remaining procedure was identical to that described above.

11.2.7. External calibration of HPLC Systems 1 and 2.

A System 1

5.8 mg of benzylpenicillenic acid was dissolved in 50 ml ethanol. A 1 ml aliquot was diluted to 10 ml with ethanol (1.16×10^{-2} mg per ml, 3.47×10^{-5} M, uncorrected). The uv spectrum from 230 - 400 nm was recorded (Figure 38) and at λ_{max} 322 nm, A_{322} = 0.665 AU. Therefore concentration = 0.665 / 26 600 = 2.5×10^{-5} M. Hence the % purity was (2.5×10^{-5} / 3.47×10^{-5}) x 100% = 72 %. Following the same procedure 2.6 mg of benzylpenicillenic acid was found to have a purity of 74 %. Therefore the mean purity was calculated as 73 ± 1 %

15.8 mg benzylpenicillenic acid was dissolved in 25 ml methanol (0.632 mg per ml). A series of dilutions over the range 1.26×10^{-3} to 5.06×10^{-1} mg per ml were made. Aliquots of the dilutions were further diluted to 5 ml with distilled water to give a final concentration range of 1.26×10^{-4} to 2.53×10^{-2} mg per ml and immediately analysed by HPLC (see Table 37 a).

The experiment was repeated using 10.8 mg benzylpenicillenic acid in 25 ml methanol (Table 37 b). The response of chromatographic System 1 was shown to be linear over the concentration range studied. The limit of quantifiable detection was about 4×10^{-7} M (uncorrected). The mean molar HPLC response was $4.44 \times 10^{11} \pm 7 \times 10^{9}$, assuming 100% purity. However uv studies described above indicated that the actual purity was 73.5 %. Hence the molar HPLC response factor was corrected to $6.04 \times 10^{11} \pm 9.5 \times 10^{9}$ Moles per arbituary peak area unit.

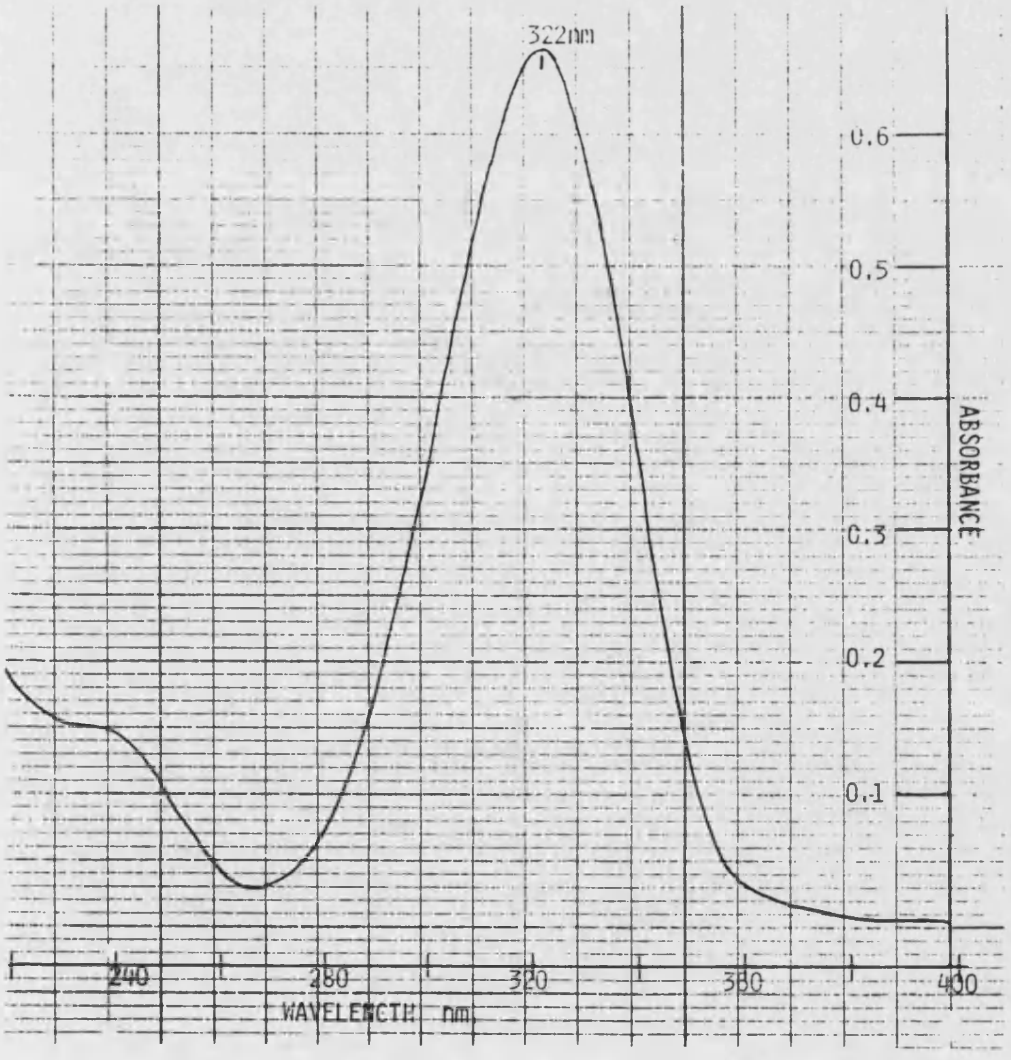


Figure 38 UV spectrum of benzylpenicillenic acid in ethanol

Concentration = 3.47x10-5 M

Table 37. External calibration data for the HPLC analysis of benzyl-penicillenic acid, using system 1

Concentration mgs/ml	Concentration M (uncorrected)	Amount injected on-column per 20µl nanograms	Mean peak area arbitary units (n=2)
		37 a	
0.0001264	3.78×10^{-7}	2.53	1.470×10^{6}
0.001264	3.78×10^{-6}	25.3	1.634×10^{6}
0.002528	7.56×10^{-6}	50.6	3.163×10^{6}
0.005056	1.51×10^{-5}	101	6.120×10^{6}
0.007584	2.27 "	152	9.568×10^{6}
0.01011	3.02 "	202	1.322×10^{7}
0.01264	3.78 "	253	1.548 "
0.02528	7.56 "	506	3.371 "

N = 9 (inc. 0,0) CC = 0.99916

Slope = $(4.42 \pm 0.07) \times 10^{11}$ Intercept = $-(2.66 \pm 2.16) \times 10^{5}$

		37 b	
0.00173	5.18×10^{-6}	34.6	2.105×10^{6}
0.00432	1.29×10^{-5}	86.5	5.754×10^{6}
0.00864	2.58×10^{-5}	173	1.141×10^{7}

N = 4 (inc. 0,0) CC = 0.99982

Slope = $(4.45 \pm 0.06) \times 10^{11}$ Intercept = $-(6.51 \pm 8.64) \times 10^{4}$

B SYSTEM 2

A similar procedure was used to calibrate HPLC System 2, the results are given in Table 38. The response of the chromatographic System 2 was found to be linear over the concentration range studied.

TABLE 38. External calibration data for the HPLC analysis of benzyl-penicillenic acid, using system 2

Concentration mgs/ml	Concentration M (uncorrected)	Amount injected on-column per 20µl nanograms	Mean peak area arbitary units (n=3)
0.00100	2.99×10^{-6}	20.0	5.527×10^{6}
0.0100	2.99×10^{-5}	200	5.921×10^{6}
0.1000	2.99×10^{-4}	2000	5.917×10^{7}

N = 4 (inc. 0,0) CC = 1.0000

Slope = $(1.979 \pm 0.001) \times 10^{11}$ Intercept = $-(1.26 \pm 1.40) \times 10^{4}$

The molar HPLC response was $1.98 \times 10^{11} \pm 1.00 \times 10^{8}$, assuming 100% purity. However uv studies indicated that the benzylpenicillenic acid used in these studies was only 69.5 %. Hence the molar HPLC response factor was corrected to $2.85 \times 10^{11} \pm 1.4 \times 10^{8}$ Moles per arbitrary peak area unit.

11.2.8 HPLC quantitative analysis of products formed during the degradation of benzylpenicillenic acid.

A Degradation in pH 7 and pH 12 solutions at 25°C and 37°C.

17.4 mg benzylpenicillenic acid (89.6 % pure by uv) was dissolved in 5 ml ethanol. A 200 µl aliquot of the ethanolic solution was added to 4.8 ml of the appropriate buffer pre-warmed to 25°C or 37°C (final concentration 0.1392 mg per ml ≡ 4.16×10^{-4} M uncorrected) and the degradation monitored by HPLC using System 3.

B Degradation in pH 2.2 and pH 2.5 solutions at 25°C and 37°C

Ca. 5 mg benzylpenicillenic acid (89.6 % pure by UV) was accurately weighed and dissolved in 1 ml ethanol containing 3,4,5-trihydroxy-benzoic acid (3.5 mg in 50 ml ethanol) as internal standard. 200 µl aliquots of the ethanolic solution of benzylpenicillenic acid were added to 4.8 ml of the appropriate buffer solution, pre-warmed to 25° or 37°C (final conc. 0.20 mg per ml ≡ 5.98×10^{-4} M, uncorrected).

After a period equivalent to 5 half-lives (equivalent to ca. 97 % degradation) had elapsed the sample was immediately analysed by HPLC, using System 3. A blank sample was prepared by adding 200 µl of the internal standard solution to the appropriate buffer and then analysed by HPLC. A control sample (to be used as initial) was prepared by adding 200 µl of the ethanolic benzylpenicillenic acid solution

(containing the internal standard) to 4.8 ml cold distilled water which was then immediately analysed by HPLC.

C Degradation at pH 5 and 37°C

4.7 mg benzylpenicillenic acid (89.6 % pure by UV) was dissolved in 1 ml ethanol. A 200 µl aliquot was added to 4.8 ml pH 5.0 buffer solution * at 37°C (final concentration 0.188 mg per ml ≡ 5.62×10^{-4} M uncorrected) and the degradation monitored by HPLC using System 3.

[* pH 5 buffer (0.1 M sodium acetate adjusted to ph 5 by dropwise addition of glacial acetic acid).]

11.2.9 Choice of internal standard

A number of compounds were analysed by HPLC using System 3 with a view to use as internal standard (see Table 39). 3,4,5-Trihydroxybenzoic acid was chosen as it eluted within a *retention window* in the chromatogram of a simulated test mixture of degradation products (Fig. 39). 3,4,5-Trihydroxybenzoic acid was found to be stable in aqueous solution and did not effect degradation rates nor interfere with the degradation products of benzylpenicillenic acid.

TABLE 39 Retention times t_r of possible internal standards

Compound tested	Retention time mins	Impurity t_r mins	Comments
Hippuric acid	7.87	-	Elutes near benzylpenillic acid
p-hydroxyphenyl-acetic acid	19.1	8.46	Co-elutes with benzylpenicilloic acid
Phthallic acid	7.37	-	Elutes near benzylpenillic acid
Iso-phthallic acid	15.3	-	Co-elutes with buffer peak
Tere-phthallic acid	24.1	7.3 & 15.3	Impure
3,4,5-Trihydroxy-benzoic acid	8.45	-	Pure, no interference with any analyte

A variety of other compounds were tested e.g. 2,5- and 2,6-dihydroxybenzoic acids, benzoic acid, 3- and 4-hydroxybenzoic acids, 3- and 4-toluic acids but all had retention times in excess of 40 minutes.

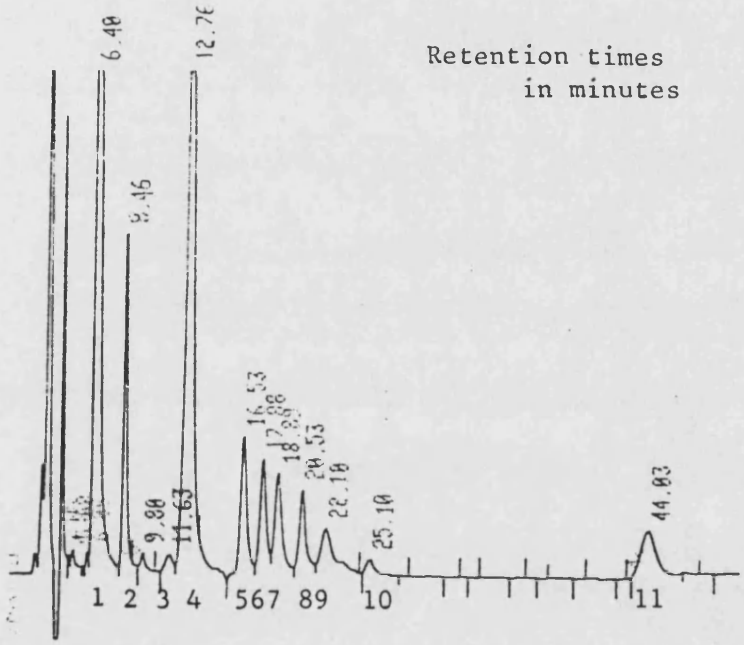


Figure 39 Chromatogram of degradation product test mixture including 3,4,5-trihydroxybenzoic acid as internal standard IS.

Key: 1 penillic acid , 2 IS, 3 penilloaldehde, 4 penamaldic acid, 5 5S,6R, 6 5R,6S, 7 5R,6R, 8 5S,6S-penicilloic acids, 9 isopenillic acid & penicillenic acid disulphide,10 buffer peak and 11 = 5R & 5S-penilloic acid.

11.2.10 External calibration of HPLC System 3 for benzylpenillic acid and benzylpenicilloic acids

Benzylpenillic acid (8.1 mg) and (3S,5R,6R)-benzylpenicilloic acid (11.3 mg, @ 88 % pure) were dissolved in 25 ml distilled water. Three dilutions were prepared in duplicate ie.(× 10, × 100 and × 1000) and assayed by HPLC (Table 40).

TABLE 40 Calibration data for benzylpenillic and penicilloic acids

Benzylpenillic acid conc. M	Mean peak area arbitary units	Benzylpenicilloic acid conc. M (corrected)	Mean peak area arbitary units
9.69×10^{-7}	8.43×10^{4}	9.92×10^{-7}	not detected
9.69×10^{-6}	7.92×10^{5}	9.92×10^{-6}	1.32×10^{5}
9.69×10^{-5}	7.63×10^{6}	9.92×10^{-5}	1.45×10^{6}
9.69×10^{-4}	7.76×10^{7}	9.92×10^{-4}	1.44×10^{7}
N = 5 (inc. 0,0)		N = 4 (inc. 0,0)	
CC = 1.0000		CC = 1.0000	
Slope = (8.01 ± 0.01) x 10^{10}		Slope = (1.45 ± 0.00) x 10^{10}	

The peak area responses of HPLC System 3 were shown to be linear over the concentration ranges studied. The HPLC molar response factors RF's are equal to the slopes in Table 40. As the RFs were very similar to those obtained earlier (see section 10.2.4 Table 28) it was not considered necessary to repeat additional calibration studies.

11.2.11 Calculation of concentrations of products formed during the degradation of benzylpenicillenic acid.

a) Degradation of benzylpenicillenic acid at pH 5, 7 and 12

The concentrations of products formed during the degradation of benzylpenicillenic acid were calculated by dividing the HPLC peak area by the appropriate molar HPLC response factor, less the amount of product, if any, initially present in the impure sample of benzylpenicillenic acid. The mass balance was calculated in the usual manner.

b) Degradation of benzylpenicillenic acid at pH 2.2 and 2.5

A similar procedure as that described above was used. In addition an internal standard was used to correct for any dilution errors in the working procedure. The method was best illustrated by the worked example given in the Results section 11.3.7, pages 203 to 204.

11.2.12 Comparison of the HPLC and ^1H NMR analyses of degradation of benzylpenicillenic acid in 0.01 M HCl at 37°C

5.5 mg benzylpenicillenic acid (89.6 % pure by uv) was dissolved in 0.5 ml ethanol. The entire sample was added to 25 ml 0.01 M HCl at 37°C. After six minutes, during which time the pH remained constant at 1.92, the solution was rapidly cooled, neutralised to pH 7 by the addition of 0.02 M NaOH and lyophilised. The freeze-dried product was reconstituted in 1 ml D_2O, 40 µl of which was diluted to 1 ml with distilled water and analysed by HPLC using System 3, the remainder was analysed by 270 MHz ^1H NMR.

11.3 Results

11.3.1 Monitoring of the degradation of benzylpenicillenic acid by uv spectroscopy

The benzylpenicillenic acid used in these studies was 73 ± 1 % pure by uv. The effective concentration was 2.2×10^{-5} M. Upon addition of ethanolic benzylpenicillenic acid to pH 7 buffer the spectrum was recorded immediately and revealed (see Figure 40) λ_{max} 310 nm (ε_{max} = 8 200) and 243 nm (ε = 2 050). The intensity of the 310 nm absorption band decreased with time and the band at 243 nm was replaced by a broad band (245 - 260 nm). The results indicated that benzylpenicillenic acid shows a 12 nm hypsochromic shift, when pH 7 buffer replaced ethanol as a solvent, and the molar extinction coefficient was reduced by a factor of about 3.

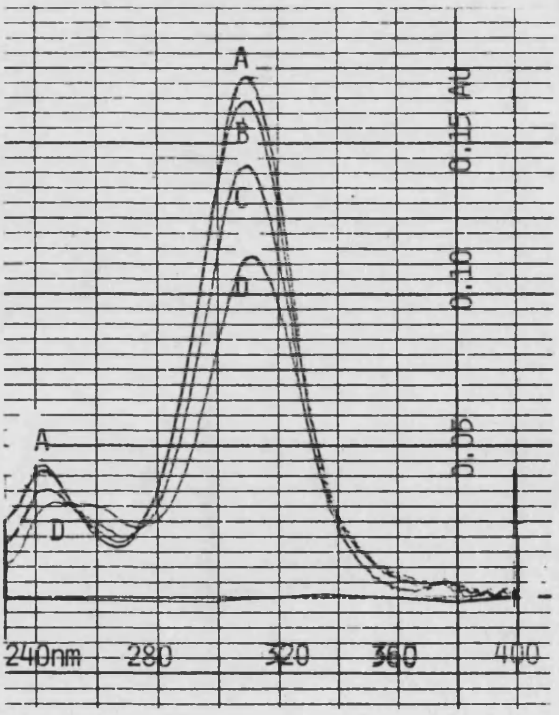


Figure 40 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in pH 7 buffer at 24 ± 1°C.

Key: A initial, B 5, C 10 and D 30 minutes after addition to buffer.

In pH 12 buffer (Figure 41) there was a rapid decrease in absorbance (λ_{max} 310 nm) and broadening of the main absorption band at lower wavelength (290 - 300 nm) was noted during degradation. Degradation was completed within 10 minutes.

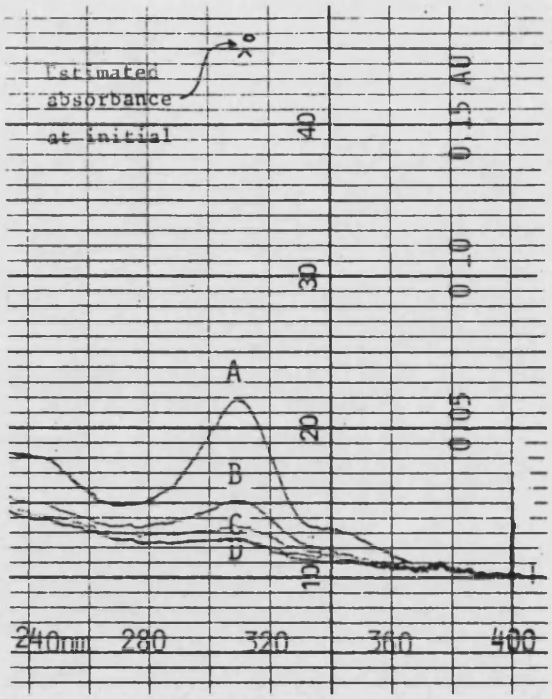


Figure 41 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in pH 12 buffer at 23.5 °C.

Key: A 0.25, B 2.25, C 4.25 and D 10.25 minutes after addition.

The uv studies of benzylpenicillenic acid in pH 2.2 (Figure 42) and pH 2.5 (Figure 43) buffers were similar. In both cases there was a rapid decrease in absorbance at λ_{max} 321 nm (it was difficult to calculate the initial ε_{max} due to such rapid change, however it was estimated as being close to ε_{max} in ethanol) and a new band at 278 nm was observed. The intensity of the 278 nm band was higher in pH 2.2 buffer (pH 2.2 ε_{278} = 7 400, pH 2.5 ε_{278} = 3 900).

Figure 42 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in pH 2.2 buffer at 22°C.

Key: A 0.17, B 2.17, C 4.5, D 8.0, E 12.5 and F 17 mins after addition

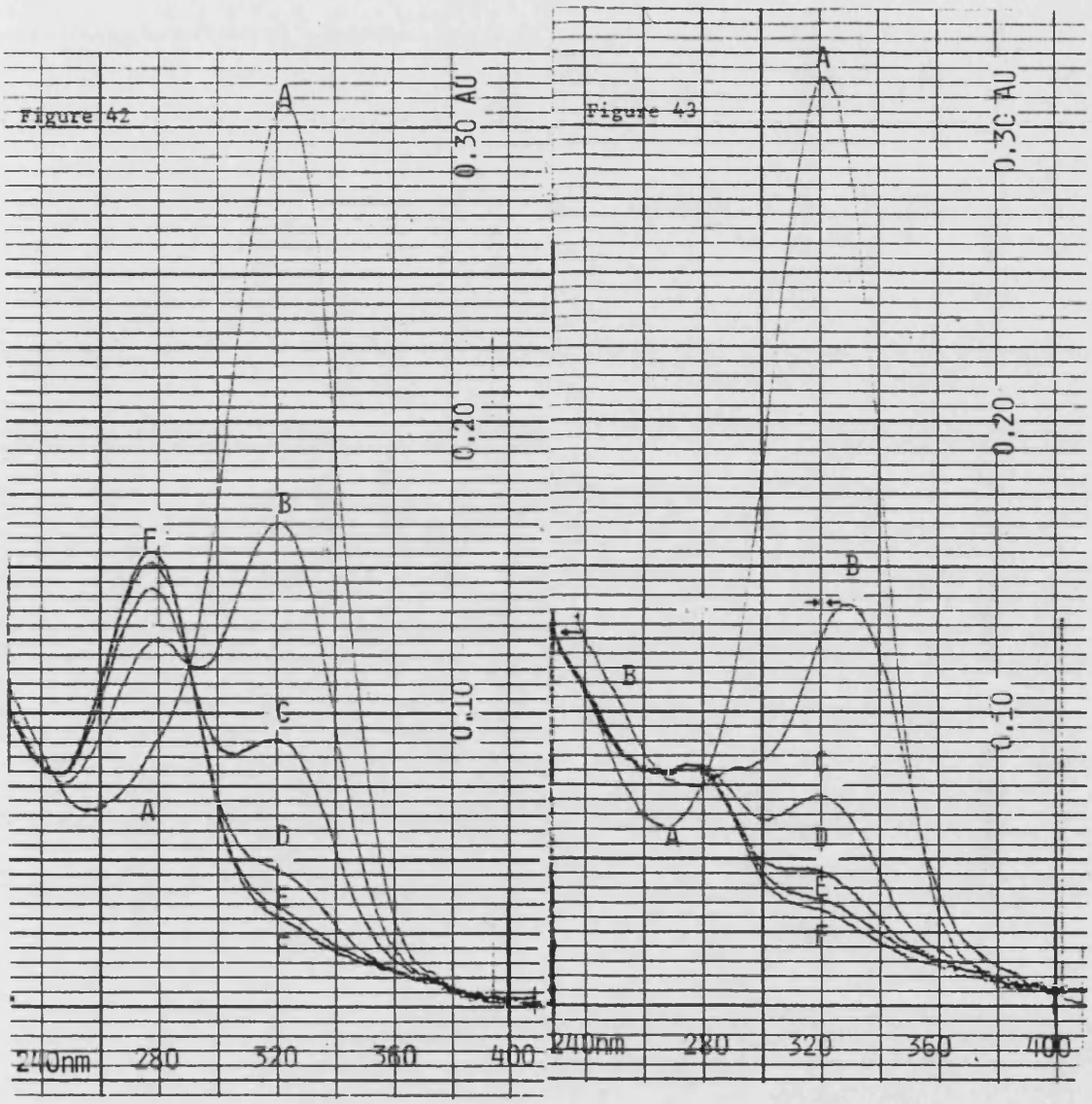


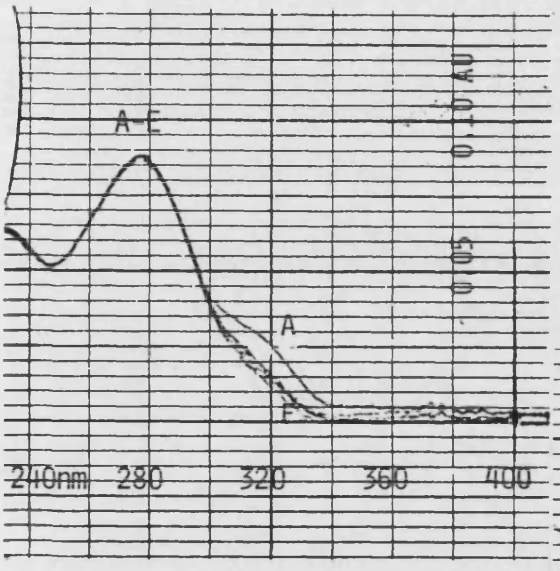
Figure 43 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in pH 2.5 buffer at 23°C.

Key: A 0.25, B 2.67, C 5.25, D 8, E 13 and F 20 mins after addition.

In 0.2 M HCl (Figure 44) there was a very rapid decrease in absorbance at ca. 320 nm. This was replaced by a new band centred at 278 nm (ε_{278} = 4 150). In 0.2 M NaOH (Figure 45) there was a rapid decrease in absorption, λ_{max} 322 nm, being replaced by a new band at about 294 nm (ε_{294} = 2 050).

Figure 44 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in 0.2 M HCl at 17°C.

Key: A 0.25 mins, B to E 2.67 to 22 minutes after addition.



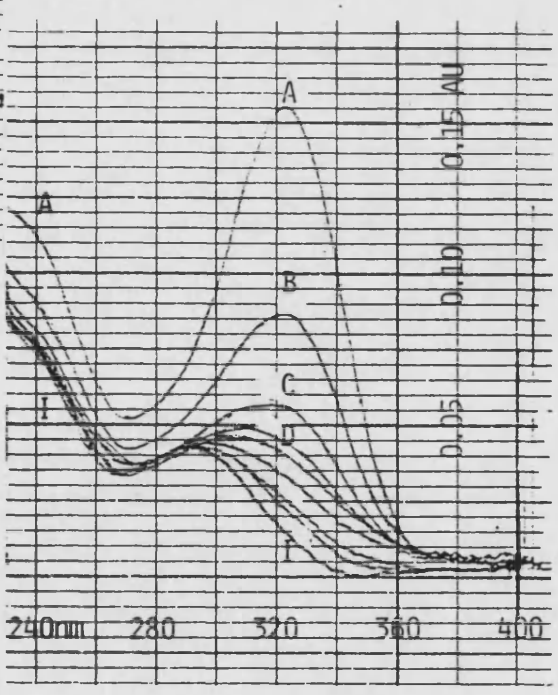
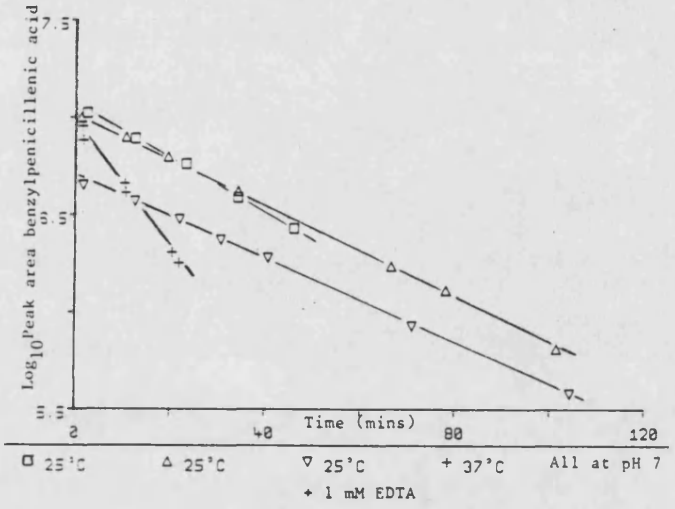


Figure 45 UV spectra of benzylpenicillenic acid (concentration = 2.1 x 10^{-5} M, corrected) in 0.2 M NaOH at 20°C.

Key: A 0.25, B 2.5, C 5, D 7.5, E 10, F 20, G 40, H 62 and I 104 mins after addition. (Spectra E to H not labelled for sake of clarity)

11.3.2 HPLC studies. Kinetic results

The apparent overall rates of degradation of benzylpenicillenic acid in acidic, neutral and basic solutions at 25° and 37°C were found to be first-order. The first-order plots are drawn in Figs 46 a, b & c



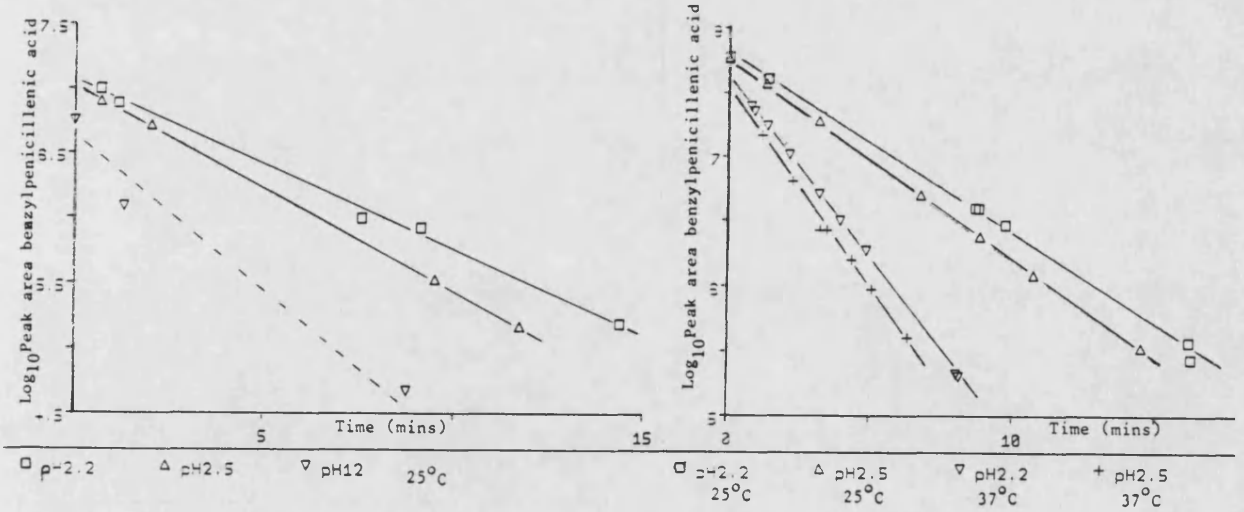


Figure 46 First-order plots for the degradation of benzylpenicillenic acid. See individual keys for experimental details.

The regression data and calculated rate constants, K_{obs}, are given in Table 40. The rate of degradation at pH 12 and 25°C was too rapid to be measured accurately. The values of K_{obs} were independent of initial concentration, as expected for a 1st-order reaction, and are in broad agreement with data published in the literature (Table 41).

Table 40 Regression data and calculated rate constants for the degradation of benzylpenicillenic acid in aqueous solution

Initial concentration 2,5 x 10^{-5} M (uncorrected)

Conditions	pH 2,2		pH 2,5		pH 7				pH 12
Temperature	25°C	37°C	25°C	37°C	25°C	25°C	25°C*	37°C	25°C
Experiments	2	nd	2	nd	1	1	1	2	2
Data points	5	-	4	-	5	7	7	6	3
CC	0,9972	-	0,9999	-	0,9992	0,9996	0,9992	0,9957	0,9673
-Slope min^{-1}	0,130	-	0,157	-	0,0136	0,0118	0,0106	0,0325	0,220
σ	0,00509		0,000839		0,000324	0,000102	0,000189	0,00151	0,0405
Intercept	7,0573	-	7,0287	-	7,0654	7,0261	6,6961	6,9746	6,579
σ	0,0426		0,00642		0,00919	0,00578	0,00991	0,0207	0,208
K_{obs} min^{-1}	0,300	-	0,363	-	0,0314	0,0272	0,0244	0,0749	0,507
σ	0,0117		0,00193		0,000747	0,000235	0,000436	0,00348	0,0933

Notes; * pH 7 buffer plus 1mM EDTA, nd = not determined as degradation too rapid,

Initial concentration 2,3 x 10^{-4} M (uncorrected)

Conditions	pH 2,2		pH 2,5	
Temperature	25°C	37°C	25°C	37°C
Experiments	3	7	3	7
Data points	8	9	7	7
CC	0,9991	0,9998	0,9999	0,9968
-Slope min^{-1}	0,140	0,284	0,155	0,297
σ	0,00248	0,00195	0,00119	0,0107
Intercept	7,7986	7,6182	7,7672	7,4714
σ	0,0244	0,00889	0,0097	0,0433
K_{obs} min^{-1}	0,322	0,654	0,357	0,684
σ	0,00571	0,00449	0,00274	0,0246

SUMMARY

Mean rate constants min^{-1}

	pH 2,2		pH 2,5	
	25°C	37°C	25°C	37°C
	0,311	0,654	0,360	0,684
σ	0,011	0,0045	0,0030	0,025

Half life in minutes

	2,23	1,06	1,93	1,01

TABLE 41 Literature data, degradation rates.

Experimental conditions			K_{obs} min^{-1}	t½ mins	Reference
pH 2.0,	25°C	(dilute NaCl adjusted to)	0.45†	1.5	58
pH 2.5,	"	(the required pH using a)	0.25†	2.8	58
pH 7.0,	"	(pH-stat.)	0.018†	38.5	58
pH 12,	"	ibid	0.35†	2.0	58
pH 7.5	25°C	(0.1 M phosphate buffer)	0.032‡	21.5	57
pH 7.5	37°C	(ibid, plus 1mg/ml EDTA)	0.11‡	6.5	57

Notes: † estimated from a figure of K_{obs} v pH given in reference 58
‡ estimated from half-life data given in reference 57.

11.3.3 HPLC results. Analysis of benzylpenicillenic acid

Benzylpenicillenic acid (89.6 % [@ ε_{max} = 26 600] or 96.3 % [@ ε_{max} = 22 909] pure by uv) was analysed by HPLC. A number of impurities were observed of which benzylpenillic, benzylpenamaldic acids and the 4 benzylpenicilloic acid stereoisomers could be positively identified. Only benzylpenillic and benzylpenicilloic acids could be reliably quantified i.e. at 0.8 % and 5.4 %, respectively (mean of 3 assays). These results suggested that ε_{max} 26 600 was the more reliable measure of assessing the purity of benzylpenicillenic acid.

11.3.4 HPLC results. Degradation at pH 7 and 25°C

The degradation of benzylpenicillenic acid was monitored by HPLC. The results are summarised in Table 42. About 20 % of the reaction mixture, after degradation was complete, was a mixture of all four 3S-benzylpenicilloic acid stereoisomers, N-formyl-D-penicillamine, N-phenylacetylglycine and trace amounts of benzylpenillic acid. A number of unknown degradation products were observed and have been nominated "unknowns 1, 2 and 3". The principal unknown product, 3, was collected

directly upon elution from the HPLC column, transferred to a micro-cuvette and the uv spectrum from 230 - 400 nm recorded (Figure 47).

The unknown exhibited the typical benzylpenicillenic acid - type chromophore (λ_{max} 324 nm and 259.5 nm) and was assumed to be benzylpenicillenic acid disulphide BPEDS 46. The other unknowns in the reaction mixture remain unidentified. A typical chromatogram is shown in Figure 48 a.

Table 42 Relative concentration of products formed during the degradation of benzylpenicillenic acid (initial conc. 3.73 x 10^{-4} M, corrected) at pH 7 & 25°C

Compound	NFDPAM	BPI	NPAG	Unknown 1	Unknown 2	BPC	Unknown 3	
tr mins	5,39	6,52	10,4	11,6	15,7	*	24,2	
T, mins	% Conc‡	% Conc	P,A,	P,A,	P,A,	% Conc	P,A, (% Conc)†	MB (MB)
29,8	15,2	0,1	1,54x10^5	2,8x10^3	-	-	3,54x10^4 (17,0)	15,3 (32,3)
58,7	25,3	0,1	3,58x10^5	5,2x10^4	2,35x10^5	9,2	5,41x10^4 (25,5)	34,6 (60,1)
97 ,8	27,6	0,2	5,13x10^5	1,00x10^5	1,08x10^5	7,4	6,73x10^4 (31,8)	35,2 (67,0)
131	26,2	0,2	6,22x10^5	1,33x10^5	1,03x10^5	8,3	7,18x10^4 (33,9)	34,7 (68,6)
251	20,7	0,2	8,12x10^5	-	-	22,2*	7,56x10^4 (35,7)	43,1 (78,8)

Notes; Conc = concentration, P,A, = HPLC peak area, tr = retention time and T = reaction time,

All conc/peak area data was corrected for amount present initially as impurity

(MB) Mass balances including estimated concentration of benzylpenicillenic acid disulphide / unknown 3,

† Conc, calculated using estimated HPLC molar response factor of 5,68 x 10^{10}
‡ Conc, calculated using estimated HPLC molar response factor of 4,91 x 10^9
(see section 12,)

* 5S,6R ; 5R,6S ; 5R,6R ; 5S,6S = 53,5 ; 14,0 ; 12,0 ; 20,5,
Retention times were 17,7, 18,9, 19,9 and 21,5 minutes, respectively

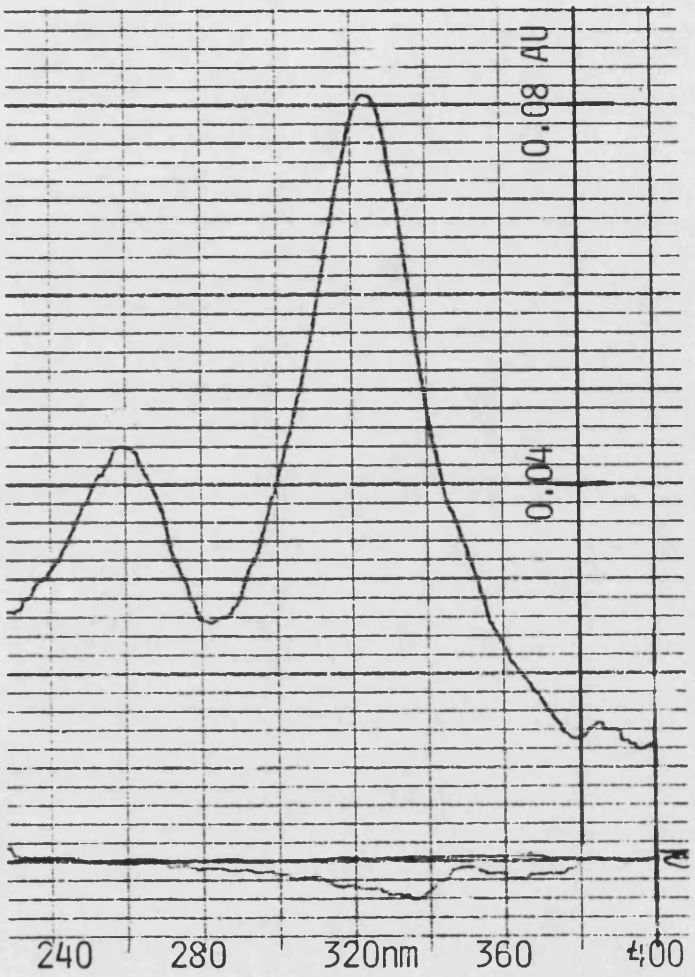


Figure 47 UV spectrum of unknown 3. Lower trace HPLC solvent blank.

11.3.5 HPLC results. Degradation at pH 5 and 37°C

The degradation of benzylpenicillenic acid was monitored by HPLC. Upon completion of degradation the main products were identified as N-formyl-D-penicillamine, benzylpenillic acid, all 4 3S-benzylpenicilloic acids, N-phenylacetylglycine and unknown 3, viz benzylpenicillenic acid disulphide. The results are summarised in Table 43. A typical chromatogram is given in Figure 48 b.

Table 43 Relative concentration of products formed during the degradation of benzylpenicillenic acid (initial conc. 5.06 × 10^{-4} M corrected) at pH 5 and 37°C

Compound	NFDPAM	BPI	NPAG	Unknown 1	Unknown 4	BPC	Unknown 3	
tr mins	5,00	6,36	9,70	11,0	12,0	*	22,0	
T, mins	% Conc‡	% Conc	P.A.	P.A.	P.A.	% Conc	P.A. (% Conc)†	MB (MB)
				Sample 1				
29,9	20,1	9,5	1,04x10^6	3,13x10^5	6,25x10^5	4,1	7,87x10^4 (27,4)	33,7 (61,1)
58,9	29,1	10,7	1,25 "	3,86 "	6,70 "	4,7	8,54 " (29,7)	44,5 (74,2)
161,5	29,9	11,3	1,27 "	4,17 "	7,40 "	5,5*	8,05 " (28,0)	46,7 (74,7)
				Sample 2				
102,4	32,4	12,6	1,41x10^6	6,17x10^5	8,40x10^5	7,3*	9,22x10^4 (31,5)	52,3 (83,8)

Notes; See Table 42.
* 5S,6R ; 5R,6S ; 5R,6R ; 5S,6S = 52,5 ; 22 ; 18 ; 7,5 (mean of duplicate assays)
Retention times were 16,4, 17,9, 18,9 and 20,5 minutes, respectively.

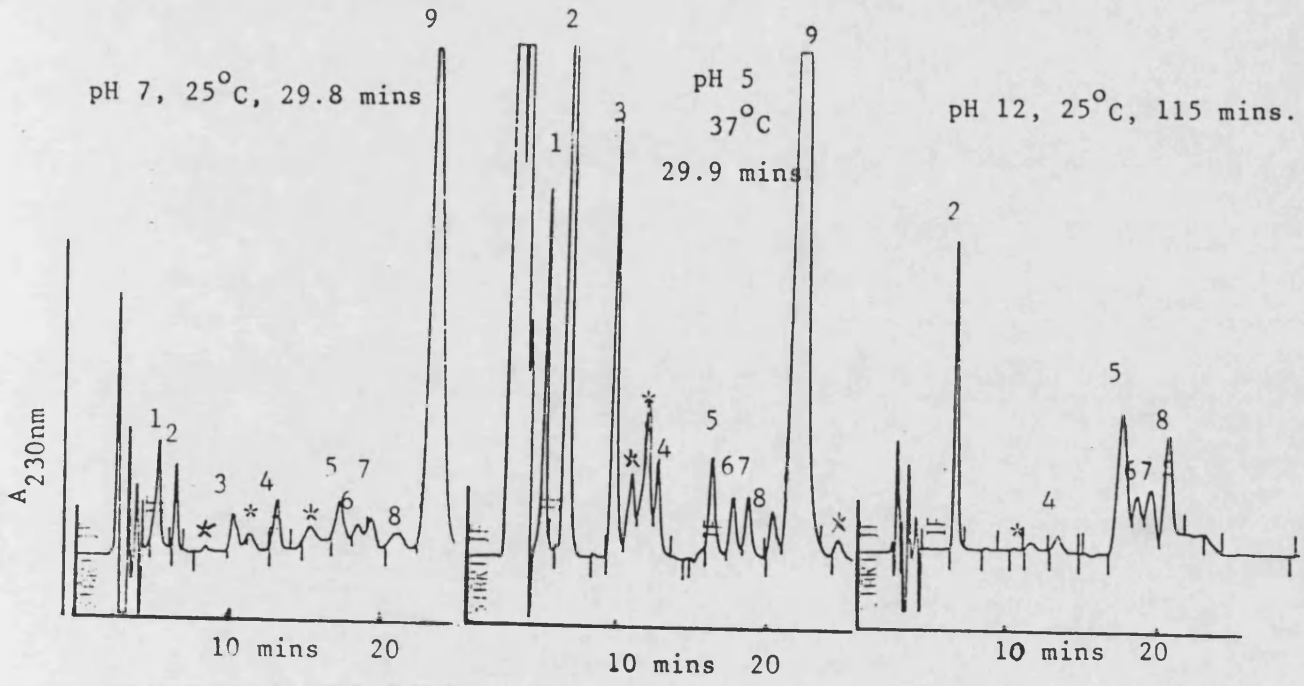


Figure 48 Chromatograms of degradation products, Key 1 NFDPAM, 2 BPI, 3 NPAG, 4 BPENAM, 5 SR-BPC, 6 RS-BPC, 7 RR-BPC, 8 SS-BPC, 9 BPEDS / Unknown 3 and * unknowns.

11.3.6 HPLC results. Degradation at pH 12 and 25°C

The degradation of benzylpenicillenic acid was monitored by HPLC. Upon complete degradation i.e. after about 6 minutes, the only degradation products observed were benzylpenillic acid (3.6 % of total) and all four 3S-benzylpenicilloic acid stereoisomers (38.4 % of total).

The stereoisomer ratios are included in Table 44. Only 42 % of the initial amount of benzylpenicillenic acid could be accounted for indicating that the majority of the sample degraded in a manner hidden from the HPLC analytical method. A typical chromatogram is given in Figure 48 c (see previous page).

Table 44 Relative concentration of products formed during the degradation of benzylpenicillenic acid (initial conc. 3.73×10^{-4} M corrected) at pH 12 and 25°C.

Compound	BPI	5S,6R-BPC	5R,6S-BPC	5R,6R-BPC	5S,6S-BPC	MB
tr mins	6.50	17.7	18.8	19.7	20.9	
T. mins			% Concentration			% TOTAL
6.2	3.6	20.0	4.2	3.9	10.3	42.0
		(52.1	10.9	10.2	26.8)	
52.3	3.4	24.8	6.6	6.8	14.8	56.4
		(46.8	12.5	12.8	27.9)	
115	3.3	25.7	7.4	8.7	14.8	59.9
		(45.4	13.1	15.4	26.1)	
246	3.2	27.3	9.9	5.7	12.9	59.0
		(48.9	17.7	10.2	23.1)	

Concs. corrected for the amounts initially present as impurities

11.3.7 HPLC results. Degradation at pH 2.2, 2.5, 25°C and 37°C.

The HPLC analyses of products present in acidic solutions of benzylpenicillenic acid aged for a time equivalent to five half-lives (calculated from the values of K_{obs} given in Table 40) are summarised in Table 45. A representative chromatogram is given in Figure 49.

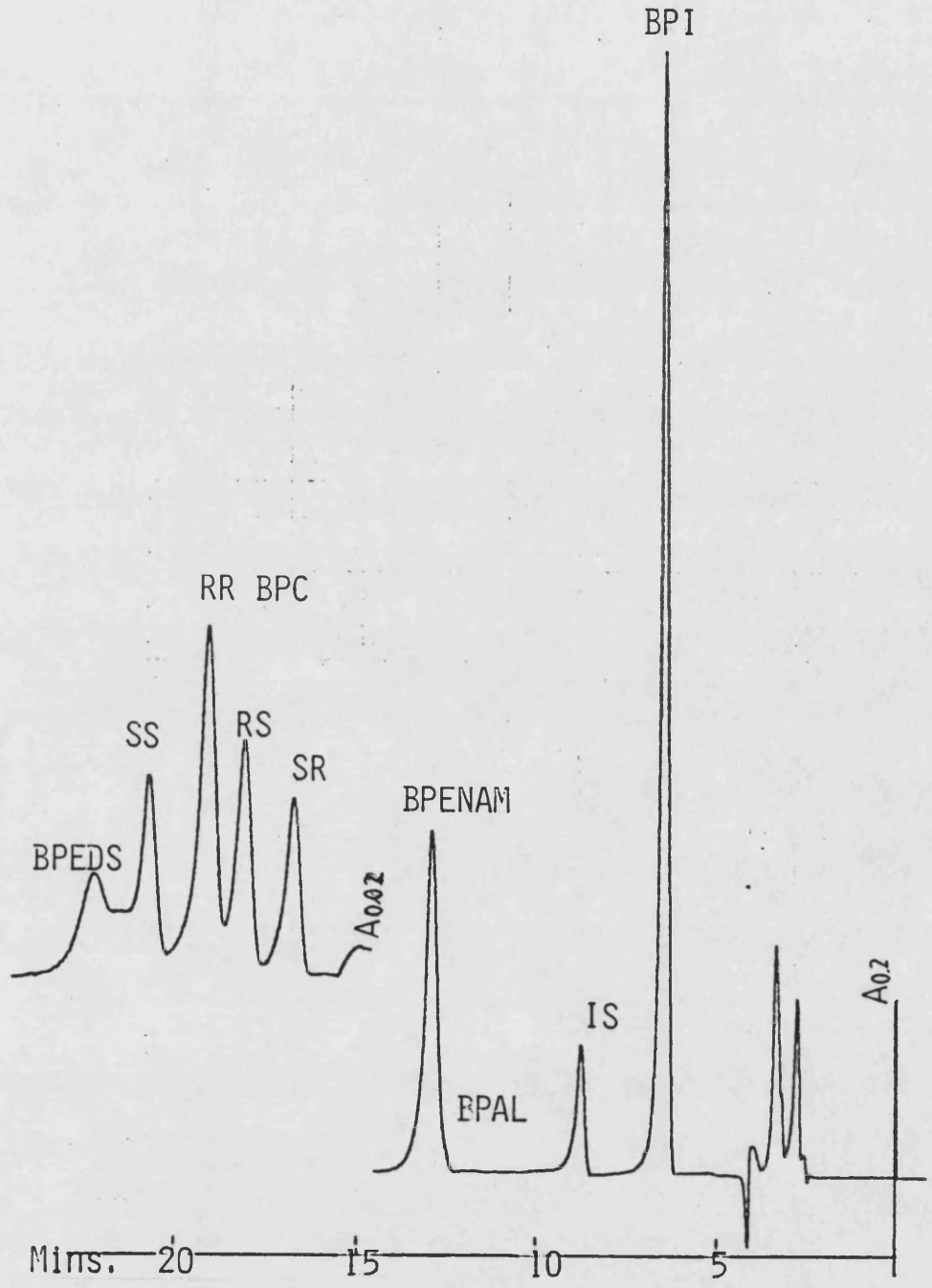
Table 45 HPLC data and calculated relative concentration of products formed after the complete degradation of benzylpenicillenic acid at pH 2.2 and pH 2.5, 25°C and 37°C

Conditions		pH 2,2, 25°C	pH 2,2, 37°C	pH 2,5, 25°C	pH 2,5,37°C
Experiments		4	6	4	4
BPE initial conc, x 10^4 M		5,26 ± 0,16	5,70 ± 0,14	5,43 ± 0,08	5,82 ± 0,05
Mean PA penillic acid x 10^{-6}		5,11 ± 0,11	5,31 ± 0,14	13,0 ± 0,10	14,0 ± 0,10
Mean PA penicilloic acids x 10^{-6}		1,32 ± 0,14	1,38 ± 0,19	2,08 ± 0,22	2,01 ± 0,18
Mean PA penamaldic acid x 10^{-6}		18,9 ± 0,00	18,8 ± 0,10	7,03 ± 0,07	6,36 ± 0,11
COMPOUND		CONCENTRATION (% TOTAL)			
Benzylpenillic acid		12,2 ± 0,3	11,6 ± 0,1	29,9 ± 0,3	30,1 ± 0,2
Benzylpenicilloic acid (total)		17,5 ± 1,5	16,7 ± 2,2	26,4 ± 2,6	23,9 ± 2,2
stereo isomer percentages	3S,5S,6R	21,6 ± 1,5	24,3 ± 2,8	18,3 ± 1,2	19,6 ± 1,1
	3S,5R,6S	16,5 ± 1,0	19,3 ± 2,4	25,2 ± 0,8	24,9 ± 1,8
	3S,5R,6R	37,1 ± 4,5	35,0 ± 2,0	40,6 ± 2,5	38,7 ± 3,4
	3S,5S,6S	24,9 ± 3,9	21,5 ± 4,9	15,9 ± 3,0	16,8 ± 3,0
Benzylpenamaldic acid (enamine)		69,1 ± 2,2	69,8 ± 2,6	42,4 ± 2,5	43,6 ± 2,8
Benzylpenilloaldehyde		1,1 ± 0,7	0,8 ± 0,4	1,4 ± 0,3	0,7 ± 0,2
Benzylpenicillenic acid disulphide		0,2 ± 0,4	1,1 ± 0,2	0	1,8 ± 0,8

Notes; PA = HPLC Peak area

Mean peak areas of the minor degradation products, benzylpenilloaldehyde and benzylpenicillenic acid disulphide not included in this table

Figure 49 Chromatogram of benzylpenicillenic acid degradation products @ pH 2.5 and 25°C. Conditions: System 3 see page 178.



Column: 25 x .49 cm Spherisorb S5 ODS2
Eluent: .05M Phosphate pH 3.9 : THF (95:5 v/v)
Flow: 1 ml/min.
Detector: UV 230 nm 0.2 and 0.02 AUFS
Sample: 20 microlitres

The data in Table 45 were arrived at in the following manner: Following the procedure described on page 185, the mean peak area of the internal standard in the blank solution was (1.79 ± 0.05) × 10^6 (mean of duplicate assays of three tests, n = 6). The initial concentration of benzylpenicillenic acid was ca. 5.49 × 10^{-4} M, corrected.

A summary of the HPLC data for benzylpenicillenic acid in water, analysed immediately, i.e. the control sample X and in pH 2.2 buffer at 37°C after five half-lives had elapsed Y, are given in Table 46.

Table 46 Worked example of pH 2.2, 37°C data in Table 45

			HPLC PEAK AREAS						
Test	BPI	IS	BPAL	BPENAM	SR-BPC	RS-BPC	RR-BPC	SS-BPC	BPEDS
X	6,22x10^5	1,27x10^6	3,00x10^6	2,52x10^5	9,64x10^6	9,05x10^6	1,41x10^5	9,53x10^6	1,03x10^5
Y	6,09x10^6	1,81x10^6	8,94x10^6	1,93x10^7	4,60x10^5	3,61x10^5	6,87x10^5	5,47x10^5	5,57x10^5
X'	8,86x10^5	1,81x10^6	4,28x10^6	3,59x10^5	1,37x10^5	1,29x10^5	2,01x10^5	1,36x10^5	1,47x10^5
Y-X'	5,20x10^6	0	4,66x10^6	1,89x10^7	3,23x10^5	2,32x10^5	4,86x10^5	4,11x10^5	4,10x10^5
Conc, M x10^5	6,49		0,598	(37,7)	2,23	1,60	3,35	2,83	0,722
%TOTAL	11,7		1,1	67,9	4,01	2,88	6,03	5,09	1,3

Notes; X' = X x 181 / 127 IS = Internal standard 3,4,5-trihydroxybenzoic acid.

The concentration of each degradation product was obtained by first subtracting the peak area of compound present initially as an impurity from the peak area of compound measured after the complete degradation of benzylpenicillenic acid. The remainder was then divided by the appropriate HPLC response factor.

However, as a reference sample of benzylpenamaldic acid was not available, the HPLC reponse factor could not be obtained directly. Therefore the concentration of benzylpenamaldic acid was calculated by

subtracting the sum of the concentrations of quantifiable degradation products from the initial concentration of benzylpenicillenic acid

For example:

The actual initial concentration of benzylpenicillenic acid was;

$$5.49 \times 10^{-4} \times 181 / 179 = 5.55 \times 10^{-4} \text{ M}$$

The concentration of benzylpenamaldic acid present was therefore;

$$5.55 \times 10^{-4} - (6.49 \times 10^{-5} + 5.98 \times 10^{-5} + 2.23 \times 10^{-5} + 1.6 \times 10^{-5} + 3.35 \times 10^{-5} + 7.22 \times 10^{-6})$$

$$= 5.55 \times 10^{-4} - 1.78 \times 10^{-4} = 3.77 \times 10^{-4} \text{ M}$$

Therefore benzylpenicillenic acid upon complete degradation at pH 2.2 and 37°C gave benzylpenillic acid (11.7 %), benzylpenilloaldehyde (1.1 %), benzylpenamaldic acid (67.9 %), benzylpenicillenic acid disulphide (1.3%) and the 4 3S-benzylpenicilloic acid stereoisomers (18 %) (5S,6R : 5R,6S : 5R,6R : 5S,6S = 22.3 : 16.0 : 33.5 : 28.3). This procedure was repeated three times and the mean results are presented in Table 45.

The molar HPLC response factor for benzylpenamaldic acid could now be estimated (by statistical correlation of the HPLC peak areas of benzylpenamaldic acid v calculated concentrations) as (3.88 ± 0.094) x 10^{10} peak area units per mol. The relative standard deviation was about 2.5 %.

11.3.8 Comparison of HPLC and ^{1}H NMR analyses of benzylpenicillenic acid degraded in 0.01 M HCl and 37°C (pH 1.92)

The entire procedure described in section 11.3.7 is built upon the assumption that no major degradation product was 'hidden' from the HPLC method.

To test the validity of the assumption a sample of benzylpenicillenic acid was degraded in 0.01 M HCl at 37°C and analysed by HPLC. The percentage concentrations of the products present were calculated, in the manner described in the previous section, as being; benzylpenillic acid (13 %), benzylpenilloaldehyde (3.5 %), benzylpenicillenic acid disulphide (1.5 %) and 3S-benzylpenicilloic acid stereoisomers (29.5 %); 5S,6R : 5R,6S : 5R,6R : 5S,6S = 25.5 : 19.5 : 33.5 : 23.5.

The same sample was analysed by ^{1}H NMR and the concentrations of the products present were estimated by measuring the heights of the 2α and β methyl resonances in the δ 1.0 - 1.7 ppm region (see Table 47 and Figure 50). Assignments were based on data described in Section 6.

Table 47 ^{1}H NMR results and comparison with HPLC data.

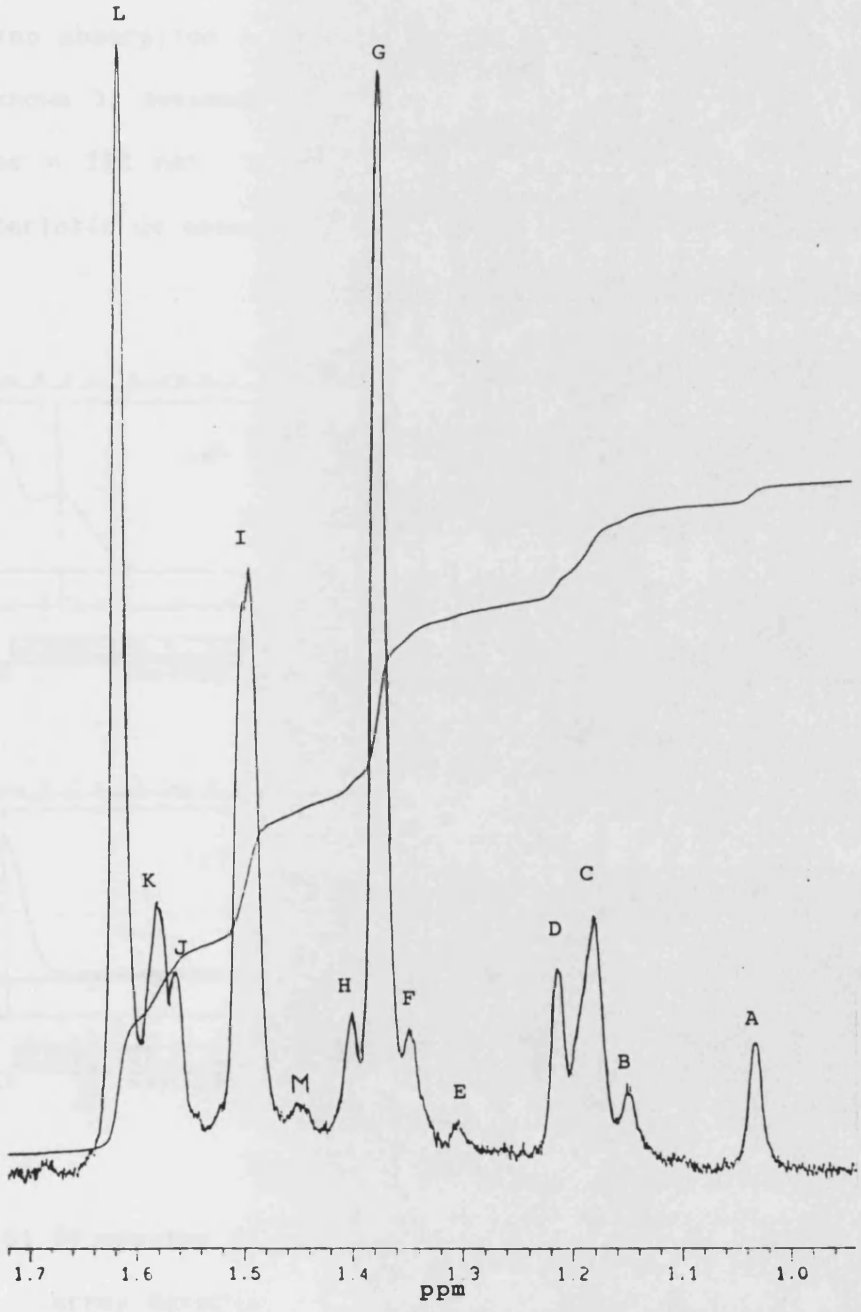
Peak	δ ppm	Height mm	Tentative assignment of Me resonances	Ref.ppm
A	1.030	20	α 5S,6R-benzylpenicilloic acid	1.01
B	1.15	10	?	
C	1.181	37	α 5R,6S & 5S,6S-benzylpenilloic acids	1.16 1.17
D	1.213	25	α 5R,6R-benzylpenicilloic acid	1.20
E	1.31	3	?	
F	1.35	15	?	
G	1.375	167	α benzylpenamaldic acid	1.38
H	1.40	17	β 5R,6S-benzylpenicilloic acid	1.39
I	1.495	88	α and β benzylpenillic acid	1.49 1.51
J	1.56	20	β 5S,6R-benzylpenicilloic acid	1.54
K	1.581	35	β 5S,6S-benzylpenicilloic acid	1.56
L	1.616	170	β benzylpenamaldic acid	1.62
M	1.45	3	?	

COMPOUND	% TOTAL by NMR	by HPLC†	difference
Benzylpenillic acid	14.4	13.5	- 0.9
Benzylpenamaldic acid	55.3	54.4	- 0.9
Benzylpenicilloic acids plus others ‡	30.3	32.1	+ 1.8

Notes: † Data excluding benzylpenilloaldehyde (no methyls) normalised to 100 %.
‡ e.g. benzylpenicillenic acid disulphide.

The good agreement found between the HPLC and NMR results supported the assumption made in the HPLC procedure.

Figure 50 270 MHz ^{1}H NMR spectrum of benzylpenicillenic acid degradation products. For key, see Table 47.



11.3.9 HPLC results. Use of photo-diode array uv detector

A sample of benzylpenicillenic acid degraded in acidic solution was analysed by HPLC using a diode array detector in place of the single wavelength spectrophotometer normally used. Figure 51 shows the spectra of (a) benzylpenillic acid, (λ_{max} observed = 238 nm), (b) benzylpenamaldic acid, (λ_{max} observed = 275 nm), (c) Benzylpenicilloic acid, (no absorption > 240 nm, all 4 stereoisomers identical) and (d) the unknown 3, assumed to be benzylpenicillenic acid disulphide (λ_{max} observed = 322 nm). The other degradation products studied gave no characteristic uv absorption above 230 nm.

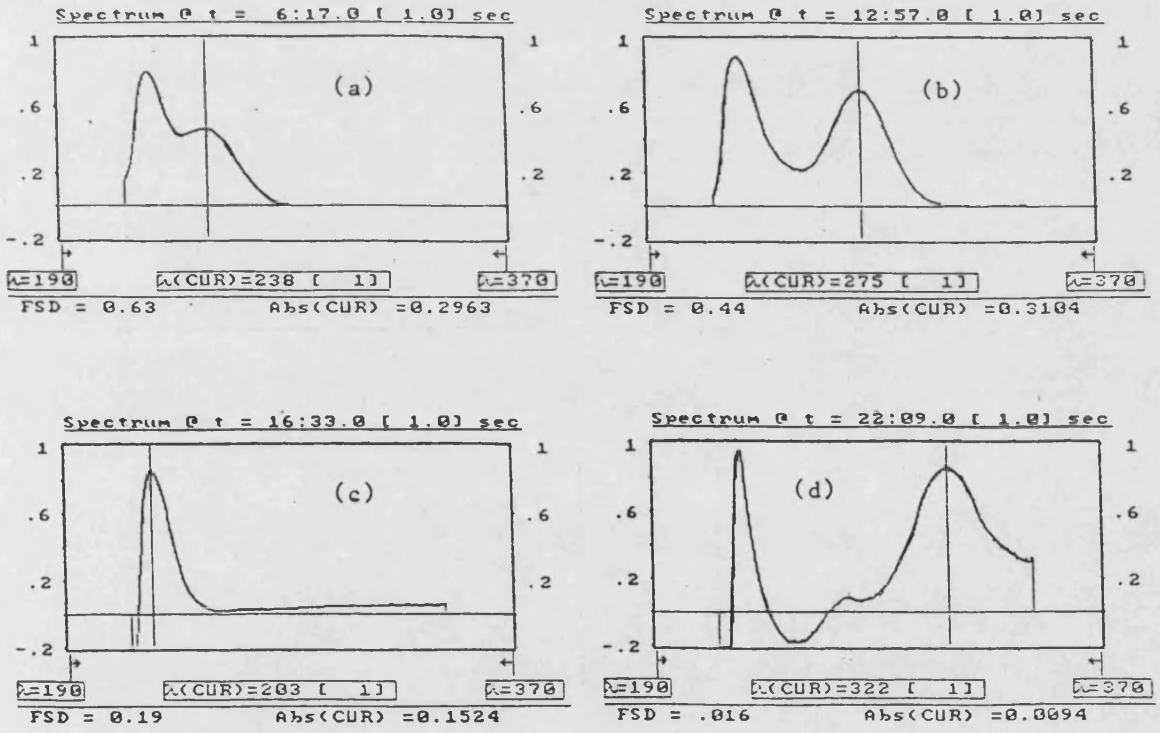


Figure 51 UV spectra of degradation products obtained using HPLC diode array detector.

11.4 Discussion

11.4.1 Degradation at pH 5, 7 and 12

The nature and relative concentration of compounds formed during the degradation of benzylpenicillenic acid were strongly dependent upon pH.

At pH 12 the major degradation products observed were the benzylpenicilloic acid stereoisomers, however 58 % of the sample could not be accounted for. Livermore [16] prepared the sodium salt of 2-benzyl-4-hydroxymethyleneoxazol-5(4H)-one **45** (λ_{max} 298 nm, ε_{max} 17 650) and D-penicillamine by the degradation of benzylpenicillenic acid in concentrated alkali. Longridge [58 59] also noted that the oxazolone was the major degradation product formed in solutions above pH 12.

Our uv studies indicated a transitory absorption band at ca. 300 nm in pH 12 buffer (Fig. 41) while in 0.2 M NaOH, benzylpenicillenic acid degrades rapidly to give a new band at 294 nm (Fig. 45). During HPLC studies, no peak with the retention time of the oxazolone **45** was seen. These observations indicated that the oxazolone was only a transitory intermediate in the degradation of benzylpenicillenic acid at pH 12.

At pH 7 the main products observed were benzylpenicilloic acids, N-formyl-D-penicillamine, N-phenylacetylglycine and an unknown compound assumed, on the basis of uv information, to be benzylpenicillenic acid disulphide **46**. This assumption was confirmed by the findings of Levine [57] who noted that the residual stable absorption band at 322 nm was due to the oxidised disulphide form of benzylpenicillenic acid.

The formation of N-formyl-D-penicillamine in solutions of benzylpenicillin has been reported.[64 65 159] Hitomi [65] proposed that this

was due to the hydrolysis of benzylpenicillenic acid to give N-formyl-D-penicillamine and N-phenylacetylglycine (phenaceturic acid). Bird [64] states that N-formyl-D-penicillamine was produced from benzylpenicillin at pH values between 2.5 and 7 with a maximum yield of 30 % at pH 5. He also reported that benzylpenicillenic acid at pH 5 gave a 20 % yield of N-formyl-D-penicillamine. This observation was in agreement with our studies at pH 5, whence the primary degradation products were N-phenylacetylglycine and N-formyl-D-penicillamine (31 %) together with benzylpenillic and benzylpenicilloic acids and benzylpenicillenic acid disulphide.

At all pHs the principal benzylpenicilloic acid isomers formed had 5S stereochemistry. The limited studies described in section 10 indicated that 5S epimers are thermodynamically favoured also.

11.4.2 Degradation at pH 2.2 and 2.5

In acidic solution the primary degradation products observed were benzylpenillic and benzylpenamaldic acids, the four benzylpenicilloic acid stereoisomers together with small amounts of benzylpenicillenic acid disulphide and benzylpenilloaldehyde. The relative amounts of the three major degradation products were exquisitely sensitive to either the small difference in buffer pH or the buffer composition (see Table 45, page 201). In contrast, the effect of temperature was relatively insignificant.

Longridge [58] reported that benzylpenicilloic acid was formed in solutions above pH 3 only. Degalaen [61] noted 12 % benzylpenicillenic acid degraded to benzylpenicilloic acids at pH 2.5 and 37°C. This was probably an underestimate as only the 5S,6R and 5R,6R epimers were

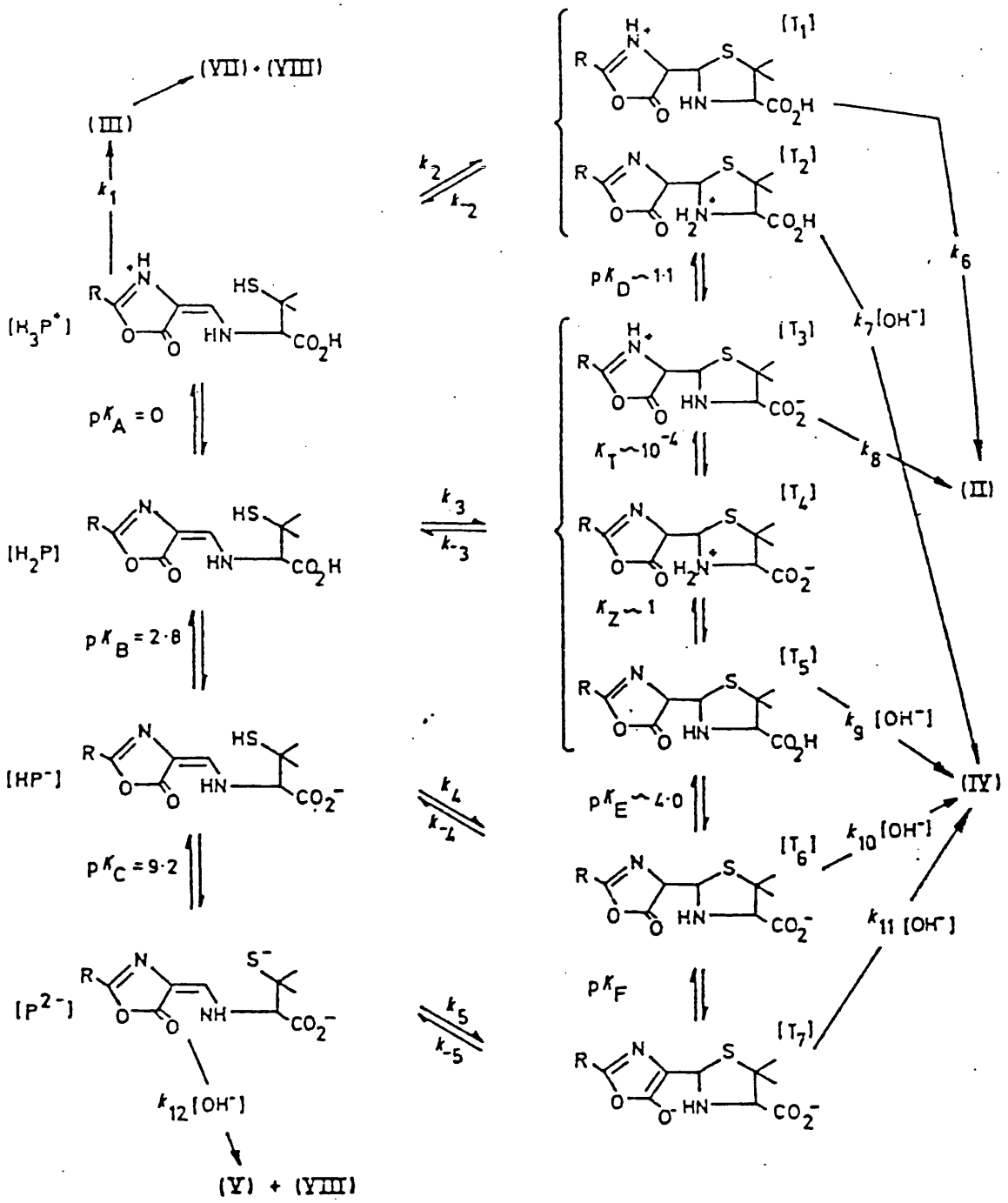
considered to be formed. Our studies showed that all four benzylpenicilloic acids were present, regardless of pH and temperature.

Longridge [58] has proposed a detailed reaction scheme (Scheme 18) to explain the complex dependence of the rate of degradation and nature of degradation products upon pH. The formation of the benzyloxazolone-thiazolidine intermediate 29 was attributed to intramolecular nucleophilic attack of the thiol group of benzylpenicillenic acid. In contrast, benzylpenicillenic acid disulphide cannot undergo such a reaction and is relatively stable.

Benzylpenicillenic acid and the benzyloxazolone-thiazolidine intermediate can exist in a complex equilibrium of several anionic, cationic and tautomeric forms capable of reactions at different rates via differing mechanisms. Either compound can react with water to give benzylpenicilloic and benzylpenamaldic acids or rearrange to give benzylpenillic acid.

It has been suggested [64] that the benzyloxazolone-thiazolidine intermediate may also fragment to give N-formyl-D-penicillamine and N-phenylacetylglycine (see Introduction, pages 35 - 36).

The observed presence of benzylpenilloaldehyde may be due to the hydrolysis of benzylpenicillenic acid to give D-penicillamine and benzylpenaldic acid which then spontaneously decarboxylated to produce benzylpenilloaldehyde. The facile benzylpenicillenic acid thiol-disulphide redox system had an important role in conditions where benzylpenicillenic acid was relatively stable i.e. neutral pH and low temperature. The yield of disulphide may depend [183] on the presence of mild oxidising agents, metal ions etc. in the system. (The presence of disulphide will interfere in uv analyses of benzylpenicillenic acid.)



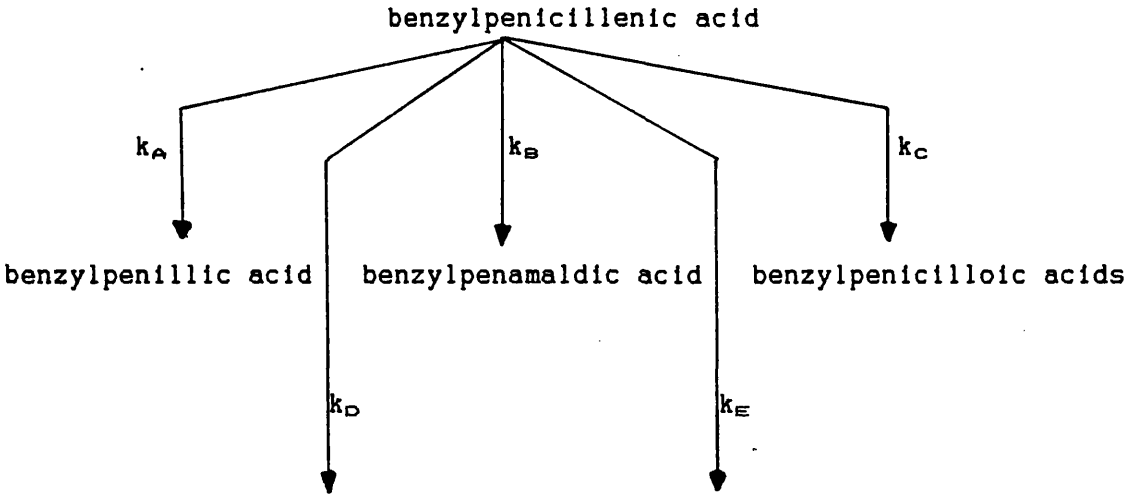
Scheme 18 Proposed [58] reaction scheme for the hydrolysis of benzylpenicillenic acid

Key: H_2P penicillenic acid, T_1 - T_7 oxazolone-thiazolidine tautomers, II penillic acid II penamaldic acid, IV penicilloic acid, V 4-hydroxymethyleneoxazol-5(4H)-one, VII penilloaldehyde, VIII D-penicillamine, R = Benzyl-

11.4.3 Kinetic studies of the degradation of benzylpenicillenic acid at pH 2.2 and pH 2.5, 25°C and 37°C

A kinetic study of the reaction pathways drawn in Scheme 18 and the complex relationships that exist between the degradation products was beyond the capabilities of the author. Fortunately, the degradation of benzylpenicillenic acid in acidic solutions can be simplified (see Scheme 19) by assuming that the benzyloxazolone-thiazolidine intermediate, if present, had only a transitory role in the reaction scheme.

Scheme 19 Simplified scheme for the degradation of benzylpenicillenic acid in acidic aqueous solution.



This scheme describes a system in which a single compound, benzylpenicillenic acid, degrades to at least five products simultaneously and in parallel. Our studies show that the overall rate of degradation K_{obs} is first order. Assuming that the individual reactions follow

first-order kinetics, it follows [184] that the overall rate of degradation is the sum of the individual rate constants i.e. :

$$K_{obs} = k_A + k_B + k_C + k_D + k_E$$

and for such a system;

$$[BPI] : [BPENAM] : [BPC] : [BPAL] : [BPEDS] = k_A : k_B : k_C : k_D : k_E$$

i.e. the concentration of products are in a constant ratio, independent of the initial concentration of benzylpenicillenic acid and reaction time, if the products are not degraded further.

From our studies of the degradation of benzylpenillic and benzylpenicilloic acids one can safely assume that this was the case during the relatively short time taken to completely degrade penicillenic acid. However this assumption may not be valid for benzylpenamaldic acid. Therefore its stability was measured by noting the change in concentration of benzylpenamaldic acid (produced *in situ* by the degradation of benzylpenicillenic acid) with time.

By plotting $\log_{10}$ (HPLC peak area of benzylpenamaldic acid) v time, the loss of benzylpenamaldic acid was found to be first order. The calculated rate constants are given in Table 48.

Benzylpenamaldic acid was found to be relatively stable and hence the individual rate constants could now be estimated with a fair degree of confidence. The values of K_{obs} have been taken from Table 40 page 195 and the percentage concentrations of degradation products taken from Table 45 page 201. The results are summarised in Table 49.

For example:

$$k_A = \frac{K_{obs} \times [BPI]}{100}$$

Table 48 Regression data and calulated rate constants K for the degradation of benzylpenamaldic acid at pH 2.2 & 2.5, 25° and 37°C

Conditions	N	CC	K $mins^{-1}$ x 10^4	σ x 10^6	t½ hours
pH 2.2 25°C	13	0.9985	0.745	2.5	155
	12	0.9964	0.734	3.9	157
			Mean = (7.40 ± 0.40) x 10^{-5}		156
pH 2.2 37°C	6	0.9997	2.68	7.0	43.2
	6	0.9999	2.79	4.0	41.4
			Mean = (2.74 ± 0.10) x 10^{-4}		42.3
pH 2.5 25°C	8	0.9997	2.13	4.0	54.3
	6	0.9998	2.18	4.0	52.9
			Mean = (2.16 ± 0.04) x 10^{-4}		53.6
pH 2.5 37°C	5	0.9920	9.44	139	12.2
	4	0.9975	9.65	96	12.0
			Mean = (9.55 ± 1.39) x 10^{-4}		12.1

Table 49 Calculated rate constants, min^{-1}, for the degradation of benzylpenicillenic acid, K_{obs}, and for the formation of products as described by Scheme 19.

Conditions	K_{obs}	k_A	k_B	k_C	k_D	k_E
pH 2.2, 25°C	0.311	0.0379	0.215	0.0544	0.00342	0.00062
pH 2.2, 37°C	0.654	0.0759	0.456	0.109	0.00523	0.00719
pH 2.5, 25°C	0.360	0.108	0.153	0.0950	0.00504	-
pH 2.5, 37°C	0.684	0.206	0.298	0.163	0.00479	0.0123

12 HPLC AND ^{1}H NMR STUDIES OF THE DEGRADATION OF BENZYLPENICILLIN IN ACIDIC AND BASIC SOLUTIONS

12.1 Introduction

The rate of degradation of (3S,5R,6R)-benzylpenicillin BP 5 in pH 2.2 and 2.5 solutions at 25° and 37°C and in pH 12 solution at 25°C was determined by HPLC. The products formed during degradation were separated, identified and quantified using HPLC and ^{1}H NMR.

The degradation of (3S,5R,6S)-benzylpenicillin 5A in pH 12 solution at 25°C was monitored by HPLC and ^{1}H NMR.

12.2 Experimental

12.2.1 Materials and reagents

The buffer solutions used have been described in section 7.2. The reference sample of benzylpenicillin sodium (Glaxo) was of high purity, see Appendix I, and considered to be 100 % pure.

12.2.2 Apparatus

The HPLC instrumentation used has been described in section 5.2.2 Separations were carried out using four systems:

System 1 Column: 50 × 2.1 mm i.d. Brownlee 5 µ RP-8

Eluent: 0.05 M phosphate buffer pH 3.9 : MeCN (82 : 18 v/v)

Flow rate: 1 ml min^{-1}, at ambient temperature.

Pressure: 1000 psig

Detector: uv @ 230 nm (unattenuated signal to integrator)

Sample: 20 µl

System 2 as System 1 except:

Eluent: 0.05 M phosphate buffer, pH 3.9 : MeOH (45 : 55 v/v)

Detector: uv @ 322 nm

System 3 Column: 250 × 4.9 mm Spherisorb S5 ODS 2

Eluent: 0.05 M phosphate buffer pH 3.9 : THF (93 : 7 v/v)

Flow rate: 1 ml min^{-1}

Detector: uv @ 230 nm (unattenuated signal to integrator)

Sample: 20 µl

Temperature: 25 ± 0.5°C

System 4 as System 1 except:

Eluent: 0.05 M phosphate buffer pH 3.9 : MeCN (87 : 13 v/v)

Detector: uv @ 230 nm.

12.3 Experimental procedures

12.3.1 Degradation of benzylpenicillin (2.5×10^{-5} M) at pH 2.2, 2.5 and 12 at 25°C and 37°C

(i) Calibration studies

During initial studies the peak area response of HPLC System 1 was shown to be linear over the concentration range 4.81×10^{-7} M to 9.61×10^{-2} M (CC = 0.9999, n = 14) with a limit of detection of about 1×10^{-7} M. However examination of the peak shape of penicillin at concentrations above 1×10^{-3} M showed asymmetry indicative of overloading. Therefore the working concentration range of the column was limited to 1×10^{-6} M to 5×10^{-4} M.

Benzylpenicillin sodium (18.0 mg) was dissolved to 100 ml with double-deionised distilled water and a series of dilutions over the

concentration range 1.97×10^{-6} M to 5.05×10^{-4} M prepared and assayed by HPLC System 1. The results are given in Table 50. The response of the chromatographic system was shown to be linear over the concentration range 1.97×10^{-6} to 5.05×10^{-4} M (n = 10, CC = 0.9997). The HPLC molar response factor was found to be 3.28×10^{10} peak area units per mol. The system was demonstrated to be *stability-indicating* i.e. free from interferences by the degradation products.

Table 50 Calibration data and regression analysis of concentration of benzylpenicillin sodium versus peak area by HPLC (System 1).

Concentration Molar	Amount on column per 20 µl nanograms	tr minutes (mean of 3)	Peak area (mean of 3) $\times 10^{-5}$	Peak height mm (mean of 3)
1.974×10^{-6}	14.3	5.45	1.365	8.5
3.948×10^{-6}	28.5	5.43	1.677	10.8
7.895×10^{-6}	57	5.42	2.903	18.3
1.579×10^{-5}	113	5.42	6.152	39.5
3.158×10^{-5}	225	5.43	10.15	66.3
6.315×10^{-5}	450	5.40	20.24	132.3
1.263×10^{-4}	900	5.40	40.65	267.5
2.526×10^{-4}	1800	5.40	80.75	537
5.051×10^{-4}	3600	5.44	166.9	1068

Regression analysis (peak area v concentration)

No. of observations = 10 (inc, 0,0)
CC = 0.9997

Intercept $\pm \sigma = 1.81 \times 10^{3} \pm 3.89 \times 10^{3}$
Slope $\pm \sigma = 3.28 \times 10^{10} \pm 2.11 \times 10^{8}$

(ii) Kinetic studies.

Studies of the degradation of benzylpenicillin were carried out by dissolving benzylpenicillin sodium (88.1 mg) to 100 ml with double-deionised distilled water. An 100 µl aliquot was added to 9.9 ml of

appropriate buffer pre-warmed to 25° or 37°C (initial concentration 2.47×10^{-5} M). 20 µl aliquots of the solution were analysed directly (even the pH 12 solutions) by HPLC System 1, at timed intervals *.
The pH of all solutions was checked before and after each experiment and no appreciable change was noted. The order and magnitude of the overall rate of degradation was obtained by regression analysis of the change in HPLC peak area versus time.

* The mean peak area of benzylpenicillin at time 0 was determined by multiple assays of samples prepared by diluting 100 µl of the stock solution with 9.9 ml cold distilled water and analysing immediately.

12.3.2 HPLC and ^{1}H NMR analyses of products formed during degradation of benzylpenicillin in acid solutions.

As no one isocratic HPLC analytical system was found capable of the resolution of all degradation products in a reasonable short time, see Section 5.4, and as the concentration of benzylpenicillenic acid during the degradation of benzylpenicillin was very low with respect to other products, it was decided to develop two HPLC methods which together would monitor the concentration of all the degradation products.

12.3.2.1 HPLC quantitative analysis of benzylpenicillenic acid

a) External calibration of benzylpenicillenic acid

Benzylpenicillenic acid (10.1 mg; 73% pure by UV) was dissolved to 25 ml with methanol. A series of dilutions over the concentration range 4.04×10^{-4} to 8.08×10^{-3} mg ml^{-1} (1.208×10^{-6} to 2.416×10^{-5} M, uncorrected) were analysed using HPLC System 2. Results in Table 51

Table 51 Calibration of benzylpenicillenic acid

Concentration M uncorrected	Peak area (mean of 3 analyses)
1.208×10^{-6}	4.896×10^{5}
7.249×10^{-6}	3.116×10^{6}
1.208×10^{-5}	5.047×10^{6}
2.416×10^{-5}	9.963×10^{6}

No of data points = 5 (inc 0,0) CC = 0.9998
Slope ± σ = $4.125 \times 10^{11} \pm 3.45 \times 10^{9}$
Intercept ± σ = $3.547 \times 10^{4} \pm 4.319 \times 10^{4}$

The peak area response of HPLC System 2 was shown to be linear over the concentration range studied. The molar HPLC response factor for benzylpenicillenic acid was calculated, for 73% purity, as $5.65 \times 10^{11} \pm 4.73 \times 10^{9}$ mols per peak area unit.

b) Kinetic studies

Benzylpenicillin sodium (23.5 mg) was dissolved to 25 ml with double-deionised distilled water. A 200 µl aliquot was added to 9.8 ml pre-warmed pH 2.2, 2.5, 7 and 12 buffer solutions at 25°C to give an initial concentration of 5.28×10^{-5} M. The sample was assayed at timed intervals for benzylpenicillenic acid using HPLC System 2. Each experiment was carried out in duplicate.

A similar series of experiments were carried out at 37°C using an initial benzylpenicillin concentration of 5.79×10^{-5} M.

12.3.2.2 HPLC quantitative analysis of the other products formed during the degradation of benzylpenicillin in acid solution

a) External calibration of benzylpenicillin and degradation products.

HPLC system 3 with slight modifications to the eluent had been used previously for the quantitative analysis of the degradation products

of benzylpenicillenic (Section 11.2), benzylpenicilloic (10.2) and benzylpenillic acids (9.2). No internal standard was used for the benzylpenicillin degradation experiments. New calibration experiments were undertaken to determine the HPLC response factor of penicillin and to ensure that RFs for the degradation products had not changed.

Three fresh solutions of benzylpenicillin Na were prepared and assayed immediately and in duplicate using HPLC System 3. Similar studies were conducted for benzylpenillic and benzylpenicilloic acids.

The peak area responses of HPLC System 3 were found to be linear over the concentration ranges studied and were within experimental error of those obtained previously. The RFs used in these studies have been summarised in Table 52.

Table 52 Molar HPLC peak area response factors for benzylpenicillin and possible acid degradation products

Compound	HPLC peak area response factor‡ mol per peak area unit ± σ	Source
Benzylpenicillin	$3.72 \times 10^{10} \pm 1.20 \times 10^{9}$	†
Benzylpenillic acid	$8.01 \times 10^{10} \pm 8.30 \times 10^{7}$	Tab 40, p188
Benzylpenicilloic acid	$1.45 \times 10^{10} \pm 1.30 \times 10^{7}$	Tab 40, p188
Benzylpenamaldic acid	$3.88 \times 10^{10} \pm 9.40 \times 10^{8}$	estimated p204
Benzylpenilloic acid	$1.05 \times 10^{10} \pm 5.08 \times 10^{8}$	Tab 20, p140
Benzylisopenillic acid	$1.70 \times 10^{11} \pm 1.13 \times 10^{8}$	Tab 20, p104
Benzylpenicillenic acid disulphide	$5.68 \times 10^{10} \pm 5.00 \times 10^{9}$	estimated p197
Benzylpenilloaldehyde	$7.32 \times 10^{9} \pm 2.20 \times 10^{7}$	Tab 20, p104
N-formyl-D-penicillamine	$4.91 \times 10^{9} \pm 1.18 \times 10^{7}$	Tab 27, p158

Notes: † Calculated over the concentration range 5×10^{-4} - 1×10^{-3} M
n = 6, CC = 0.9990

‡ All RFs were corrected for any impurities present in reference materials.

b) HPLC analysis and experimental procedure.

About 44.6 mg of benzylpenicillin Na was accurately weighed and dissolved in 1 ml water. A 200 µl aliquot was transferred to 24.8 ml of the appropriate buffer solution, pre-warmed to 25°C or 37°C. The initial concentration of penicillin was about 1.00×10^{-3} M. 1 ml aliquots of the aged solutions were taken at timed intervals and immediately frozen over solid CO_2. When time permitted the frozen samples were rapidly thawed and analysed directly by HPLC using System 3. Peak areas of the analytes were measured by computing integrator and concentrations obtained by using the appropriate response factor. The mass balance for each analysis was obtained in the usual manner. Each experiment was carried out in duplicate.

As solutions were analysed directly without any pre-treatment or dilution step, use of an internal standard was considered redundant. There was no evidence to indicate that the freeze-thaw step in the experimental procedure affected the overall result in any way.

12.3.3 Comparison of HPLC and ^{1}H NMR analyses of the degradation of benzylpenicillin in DCl/D_2O at pH 2.5 and 25°C

a) HPLC method

The method has been described above. However in this experiment the % concentration of benzylpenamaldic acid was estimated by subtracting the sum of the concentrations of quantifiable degradation products from the initial concentration of benzylpenicillin rather than the molar response factor estimated in section 11.

b) 400 MHz ^{1}H NMR method

400 MHz spectra were recorded on a Jeol-GX400 spectrometer at the

Department of Chemistry, University of Bristol. Samples were prepared in 5 mm o.d. tubes. DSS was used as an internal chemical shift reference. 32 K data points were used in acquiring spectra with widths of 4000 Hz (-0.4 to 9.6 ppm). For most samples 64 transients were accumulated at a pulse repetition rate of 4.6 seconds (pulse delay 0.5 s plus acquisition time 4.1 s) at a pulse angle of 45°. Later samples were analysed using a pulse delay of 5 seconds (pulse repitition 9.1 s) and 256 transients were accumulated. In addition a later sample was analysed with and without a solvent (D_2O) elimination pulse sequence (signal homogated, D_2O resonance irradiated). The probe temperature was 23°C. Semi-quantitative analysis was achieved by comparison of the peak heights of the 2α- and 2β-methyl resonances of benzylpenicillin and its degradation products (see Discussion section).

c) Experimental procedure

Benzylpenicillin Na (89.7 mg) was dissolved in 50 ml D_2O (initial concentration 5.03×10^{-3} M) pre-warmed to 25°C. The pH was rapidly adjusted to and maintained at 2.5 by the addition of 1 M DCl in D_2O (total volume added about 150 µl). The pH never deviated by more than 0.02 units from the initial value and pH values are all meter readings uncorrected for deuterium isotope effects.

At timed intervals 1 ml and 200 µl aliquots were withdrawn. The 1 ml aliquots were frozen immediately for later analysis by NMR. (Prior to analysis a few drops of dilute DSS solution were added for reference purposes). The 200 µl aliquots were diluted and neutralised with 800 µl pH 7 buffer and were either analysed immediately by HPLC using System 3 or frozen and then analysed later the same day. The dilution step in the HPLC analysis was necessary to prevent column overload.

N.B. Trial experiments indicated that an initial concentration of 5×10^{-3} M was the maximum amount above which precipitation of benzylpenillic acid was observed either during the reaction or during the freeze-thaw processes.

12.3.4 HPLC and ^{1}H NMR analyses of products formed during degradation of (3S,5R,6R)- and (3S,5R,6S)-benzylpenicillin at pH 12 & 25°C

12.3.4.1 HPLC method

HPLC System 4 was used. As previous calibration studies (Section 5.3.2.4) had shown this system to have a linear peak area response, it was considered unnecessary to re-calibrate.

(a) Experimental procedures.

1. (3S,5R,6R)-Benzylpenicillin Na (45.1 mg) was dissolved in 1 ml water. A 100 µl aliquot was added to 24.9 ml pH 12 buffer pre-warmed to 25°C (initial concentration of penicillin 5.06×10^{-4} M). At timed intervals 200µl aliquots were neutralised with 200 µl 0.01 M HCl (final pH 6.8 ± 0.1) and analysed immediately by HPLC. A control was prepared by adding 200 µl pH 12 buffer to 200 µl 0.01 M HCl and assaying by HPLC. The procedure was repeated using 45.2 mg (5.07×10^{-4} M) (3S,5R,6R)-benzylpenicillin.

2. (3S,5R,6S)-Benzylpenicillin K (4.0 mg) was dissolved in 300 µl water. The purity of the 6-epi-penicillin was checked by adding a 50 µl aliquot to 9.95 ml water and analysed immediately by HPLC.

A 100 µl aliquot was added to 9.9 ml pH 12 buffer pre-warmed to 25°C (initial conc. 3.8×10^{-4} M). At timed intervals 200 µl aliquots were neutralised with 200 µl 0.01 M HCl and analysed immediately by HPLC.

12.3.4.2 ^{1}H NMR analysis of the degradation of (3S,5R,6R)-benzylpenicillin in NaOH/H_2O at ambient temperature.

Benzylpenicillin Na (47.3 mg; 1.33 x 10^{-4} mols) was dissolved in 0.2 M NaOH (662 µl; 1.32 x 10^{-4} mols), left for 30 minutes at room temperature and then freeze-dried. When dry the sample was reconstituted with 2 ml D_2O and analysed by HPLC and 400 MHz ^{1}H NMR

12.3.4.3 ^{1}H NMR analysis of the four 3S-Benzylpenicilloic acid stereoisomers

Benzylpenicillin Na (19.5 mg) was dissolved in 10 ml pH 12 buffer and left for four days at room temperature after which the sample was freeze-dried, reconstituted with 800 µl D_2O, filtered and analysed by HPLC and 400 MHz ^{1}H NMR.

12.3.4.4 ^{1}H NMR analysis of the degradation of (3S,5R,6R)-benzylpenicillin in NaOD/D_2O

Benzylpenicillin Na (10.0 mg; 2.81 x 10^{-5} Mols) was dissolved in 1.5 ml dilute NaOD/D_2O (ca. 60 µl 40% w/w NaOD diluted to 50 ml D_2O; ca. 2 x 10^{-5} Mols) and after 30 minutes at ambient temperature, analysed by 270 MHz ^{1}H NMR.

12.4 Degradation of benzylpenicillin in acidic solution. Results and Discussion

12.4.1 Overall rates of degradation of (3S,5R,6R)-benzylpenicillin at pH 2.2 and 2.5 at 25°C and 37°C

The apparent overall rates of degradation (K_{obs}) of benzylpenicillin were measured at 25° and 37°C at pH 2.2 and pH 2.5 and were found to be first-order. The regression data and results are given in Table 53. The degradation was followed for about five half-lives. The first-order plots are drawn in Figure 52.

Table 53 Regression data and calculated first-order rate constants K_{obs} for the degradation of (3S,5R,6R)-benzylpenicillin

Conditions	pH 2,2				pH 2,5			
	25°C		37°C		25°C		37°C	
Data points	15	15	7	7	11	9	10	10
CC	0,9997	0,9961	0,9945	0,9997	0,9996	0,9992	0,9956	0,9991
-Slope	0,01397	0,01379	0,04670	0,04603	0,00869	0,00865	0,02649	0,02619
σ	0,00007	0,00024	0,00155	0,00037	0,00006	0,00009	0,00063	0,00028
Intercept	5,9202	5,9042	5,9115	5,9083	5,9191	5,9176	5,9158	5,8972
σ	0,0044	0,0137	0,0308	0,00652	0,00371	0,00425	0,0203	0,00968
Conc x10^5†	2,54	2,45	2,49	2,47	2,53	2,52	2,51	2,41
Kobs min^{-1}	0,03217	0,03176	0,1076	0,1060	0,02001	0,01993	0,0610	0,06032
σ	0,00017	0,00055	0,00357	0,00084	0,00014	0,00022	0,00144	0,00065
Mean K_{obs} min^{-1}	0,0320		0,107		0,02		0,0607	
t½ minutes	21.7		6.49		34.7		11.4	

Notes;† Initial concentration of benzylpenicillin = [antilog(intercept) /3,28 x 10^{10}] M

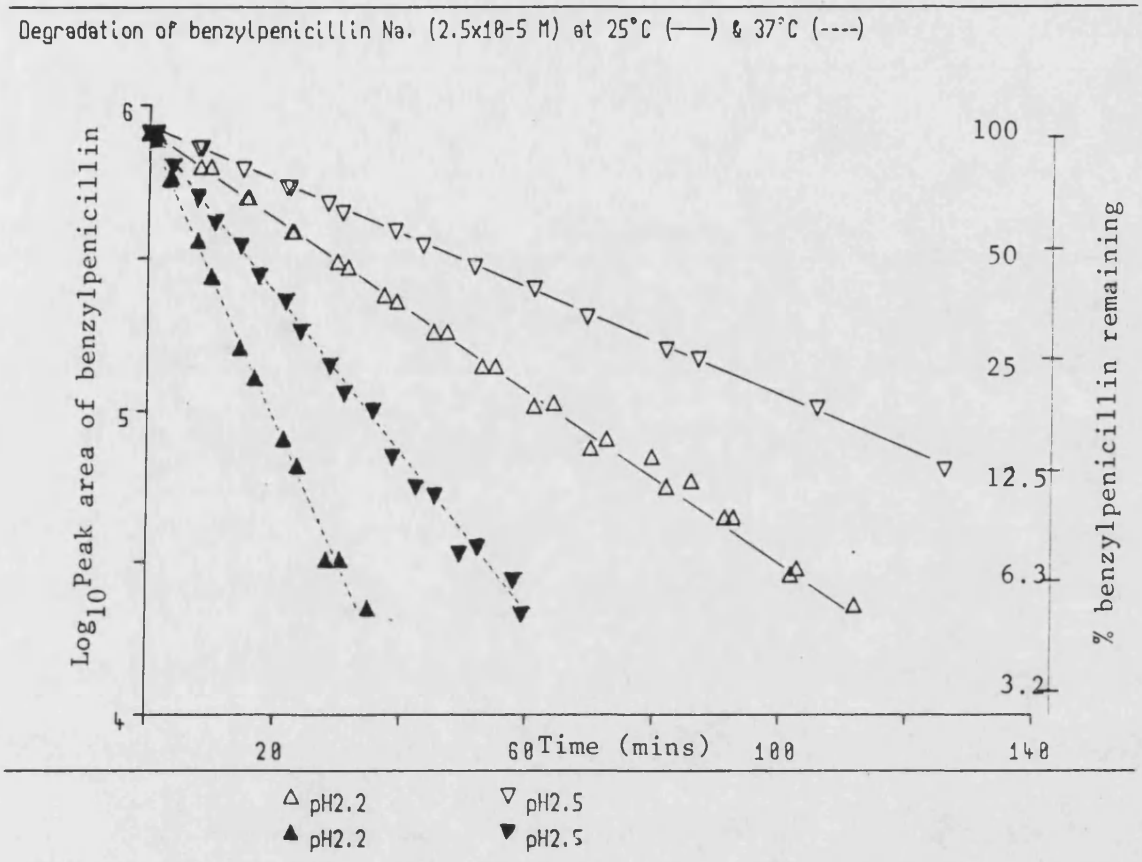


Figure 52 1st-order plots for the degradation of benzylpenicillin

12.4.2 Concentration of benzylpenicillenic acid during the degradation of benzylpenicillin at pH 2.2 and 2.5, 25° and 37°C

In all conditions studied the concentration of benzylpenicillenic acid increased rapidly from nil present to a maximum concentration equivalent to ca. 1.5 % of the initial concentration of benzylpenicillin at pH 2.2 and 25°C, 2.7 % (pH 2.5, 25°C), 2.0 % (pH 2.2, 37°C) and 3.0 % (pH 2.5, 37°C), followed by an exponential decrease in concentration with time. The plots are drawn in Figures 53 and 54. The insets show a plot of log (concentration of benzylpenicillenic acid) v time after the initial increase. First-order plots were obtained and the regression data are given in Table 54.

Table 54 Regression data for 1st-order plots derived from Figs 53 & 54

Conditions	pH 2,2, 25°C		pH 2,5, 25°C		pH 2,2, 37°C	pH 2,5, 37°C	
Experiment	1	2	1	2	1 & 2	1	2
Data points	8	8	12	10	10	7	9
CC	0,9991	0,9989	0,9996	0,9976	0,9972	0,9929	0,9955
-Slope min-1	0,01616	0,01523	0,009163	0,008782	0,04711	0,02642	0,02588
σ	0,00020	0,00020	0,000055	0,000153	0,00088	0,00100	0,00066
Intercept	-5,920	-5,947	-5,783	-5,793	-5,708	-5,569	-5,587
σ	0,0093	0,0084	0,0042	0,0105	0,0207	0,0342	0,0258
[BPE]max Mt	$7,6\times10^{-7}$	$8,0\times10^{-7}$	$1,4\times10^{-6}$	$1,3\times10^{-6}$	$1,2\times10^{-6}$	$1,7\times10^{-6}$	$1,7\times10^{-6}$

Notes; † Estimated maximum observed concentration of benzylpenicillenic acid

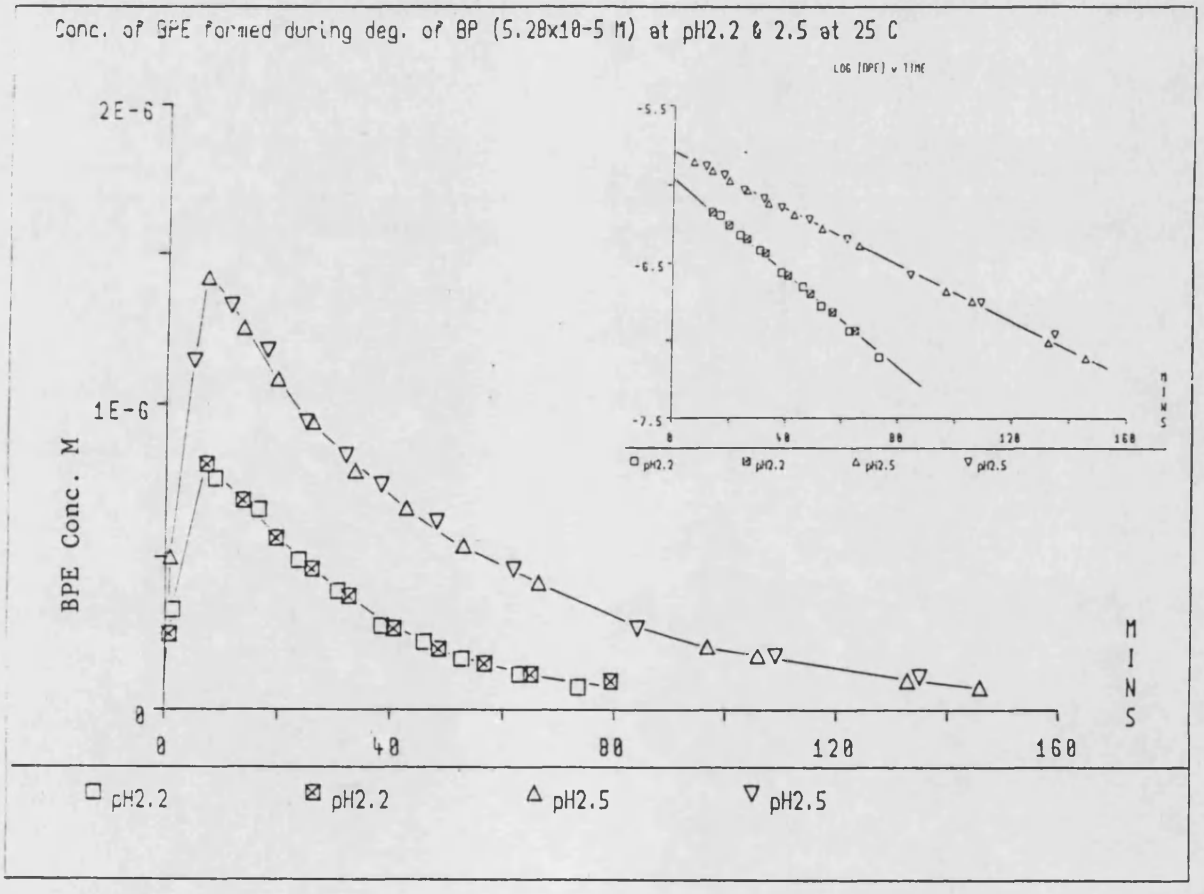


Figure 53 Conc. of benzylpenicillenic acid during degradation of benzylpenicillin (5.28×10^{-5} M) at pH 2.2 and 2.5 at 25°C.

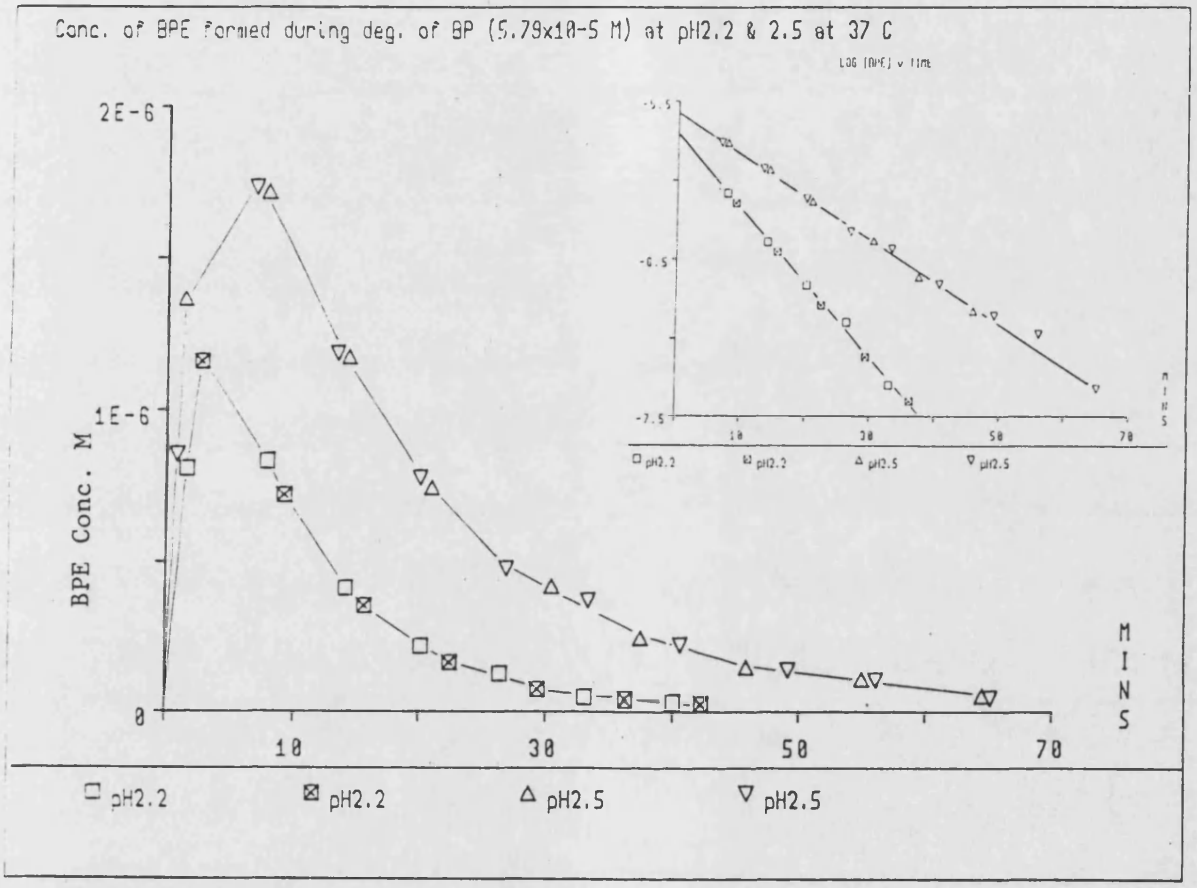
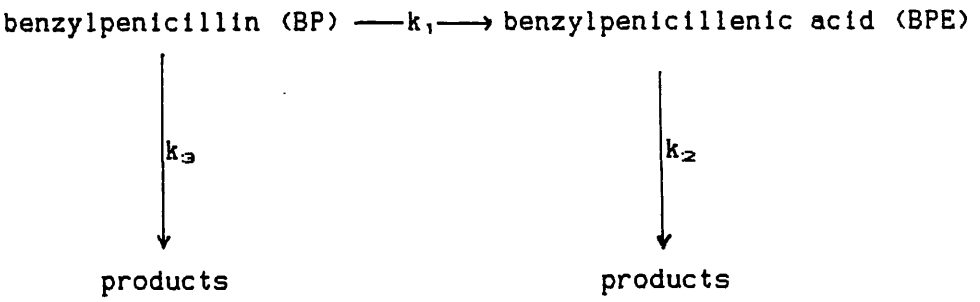


Figure 54 Conc. of benzylpenicillenic acid during degradation of benzylpenicillin (5.79×10^{-5} M) at pH 2.2 and 2.5 at 37°C.

From previous studies we have shown that the overall rates of degradation of benzylpenicillin (see this section) and benzylpenicillenic acid (Section 11.3.2) are first-order.

Assuming that the degradation of benzylpenicillin occurs by two or more simultaneous processes the following degradation scheme may be proposed (Scheme 20):



Scheme 20

In this reaction scheme compounds BP and BPE have a time dependence given by the following expressions: [54]

$$[BP]_t = [BP]_o \, e^{-Kt} \qquad (1)$$

$$[BPE]_t = \frac{k_1[BP]_o}{K - k_2} \left(e^{-k_2 t} - e^{-Kt}\right) \qquad (2)$$

where $[BP]_o$ is the initial molar concentration of benzylpenicillin, $[BP]_t$ and $[BPE]_t$ represent the molar concentration of benzylpenicillin and benzylpenicillenic acid respectively at time t, K is the overall rate of degradation of benzylpenicillin ($= k_1 + k_3$) and k_2 is the overall rate of degradation of benzylpenicillenic acid. k_1 is the rate of formation of benzylpenicillenic acid from benzylpenicillin.

Rearranging equation (2) for k_1 gives:

$$k_1 = \frac{[BPE]_t \, (K - k_2)}{[BP]_o \left(e^{-k_2 t} - e^{-Kt}\right)} \qquad (3)$$

K and k_2 have been determined independently in previous experiments. By substituting the values of K, k_2, $[BPE]_t$ and t into equation 3 values of k_1 may be obtained. However an examination of the values of K and k_2 will show that k_2 was always greater than K.

Rearranging equation (2) gives:

$$[BPE]_t = \frac{k_1[BP]_o}{k_2 - K} (e^{-Kt} - e^{-k_2 t}) \qquad (4)$$

as $k_2 > K$, the term e^{-k2t} approximates to zero with time, therefore:

$$[BPE]_t = \frac{k_1[BP]_o}{k_2 - K} e^{-Kt} \qquad (5)$$

$$\log [BPE]_t = \log \frac{k_1[BP]_o}{k_2 - K} - \frac{Kt}{2.303} \qquad (6)$$

Hence from the plots of log $[BPE]_t$ versus time t;

$K = \frac{-\text{slope}}{2.303}$ and k_1 = Antilog (intercept) x $\frac{k_2 - K}{[BP]_o}$

The values of K, k_1 and (k_1 / K x 100 %), i.e. the percentage of benzylpenicillin degraded exclusively to benzylpenicillenic acid, are given in Table 55.

Table 55 Values of the overall rate of degradation of benzylpenicillin (K) and rate of formation of benzylpenicillenic acid (k_1).

Conditions	pH 2,2, 25°C		pH 2,5, 25°C		pH 2,2, 37°C	pH 2,2, 37°C	
$[BP]_o$ M	5,28 x 10^{-4}		5,28 x 10^{-4}		5,79 x 10^{-4}	5,79 x 10^{-4}	
Experiment	1	2	1	2	1 & 2	1	2
K min^{-1}	0,0372	0,0351	0,0211	0,0202	0,108	0,0608	0,0596
σ	0,00046	0,00046	0,00013	0,00035	0,002	0,0023	0,0015
k_1 min^{-1}	0,00622	0,00590	0,0106	0,0104	0,0185	0,0291	0,0279
σ	0,000262	0,000243	0,000152	0,000264	0,000936	0,00284	0,00193
100 k_1/K %	16,7	16,8	50,2	51,3	17,1	47,8	46,9
σ	0,8	0,7	0,8	1,6	0,8	4,8	3,2
			MEAN VALUES				
K min^{-1}	0,0362 ± 0,0005		0,0207 ± 0,0004		0,108 ± 0,002	0,0602 ± 0,0023	
k_1 min^{-1}	0,00606 ± 0,00026		0,0105 ± 0,0003		0,0185 ± 0,00094	0,0285 ± 0,0028	
100 k_1/K %	16,7 ± 0,8		50,7 ± 1,6		17,1 ± 0,8	47,3 ± 4,8	

Note: σ (standard deviations about the mean) calculated by INSTAT simulation.

The percentage of benzylpenicillin degraded to benzylpenicillenic acid was found to be very sensitive to pH but the values obtained at 25° and 37°C were similar within experimental error.

The values of K obtained from these studies were within experimental error of those described previously and summarised in Table 53 (this agreement would seem to validate the approxiamation made in the kinetic analysis of reaction scheme 20).

The values of K, k_1 and k_2 at pH 2.5, 25° and 37°C are close to data previously reported in the literature and described in the Introduction section 2.6 pp 21 - 26. Degalean *et al* [61] reported that 50 % of benzylpenicillin was converted to benzylpenicillenic acid at pH 2.5 and 37°C, in close agreement with our studies.

It was surprising that in our studies at pH 2.2, only about 17 % benzylpenicillin was converted to benzylpenicillenic acid.

12.4.3 HPLC analysis of other products formed during the degradation of benzylpenicillin in acidic solution.

In the early stages of the degradation of benzylpenicillin at pH 2.2 and pH 2.5 at 25° and 37°C, the initial simultaneous formation of benzylpenillic (BPI), benzylpenamaldic (BPENAM) and benzylpenicilloic acids (BPC) was noted, together with trace amounts of N-phenylacetylglycine (NPAG), N-formyl-D-penicillamine (NFDPAM), benzylpenicillenic acid disulphide (BPEDS). A little later when some benzylpenicillin was still present small amounts of benzylpenilloic acid (BPO) were seen.

The relative concentrations of the various products at different stages of the reaction were measured from the HPLC peak area divided by the appropriate response factor. The total calculated concentration

was then compared with the initial concentration of benzylpenicillin to obtain the mass balance (MB). It was therefore possible to follow the changes in concentration of benzylpenicillin and the degradation products with time and obtain the kinetic data plotted in Figures 55 to 58. These figures include all data from duplicate analyses. All data are normalised to 100 % as the mass balance of all analyses was consistently 100 ± 10 %. The standard deviation was deemed satisfactory for the multi-analyte nature of this quantitative analyses and is in the order predicted by the summed accumulation / propagation of all the indeterminate errors involved.

The composition of the reaction mixtures when all or nearly all the benzylpenicillin had degraded and the maximum observed peak area of the degradation products during reaction are given in Table 56.

Figure 55 Plot of the concentrations of the degradation products of (3S,5R,6R)-benzylpenicillin (0.992 mM) at pH 2.2 and 25°C

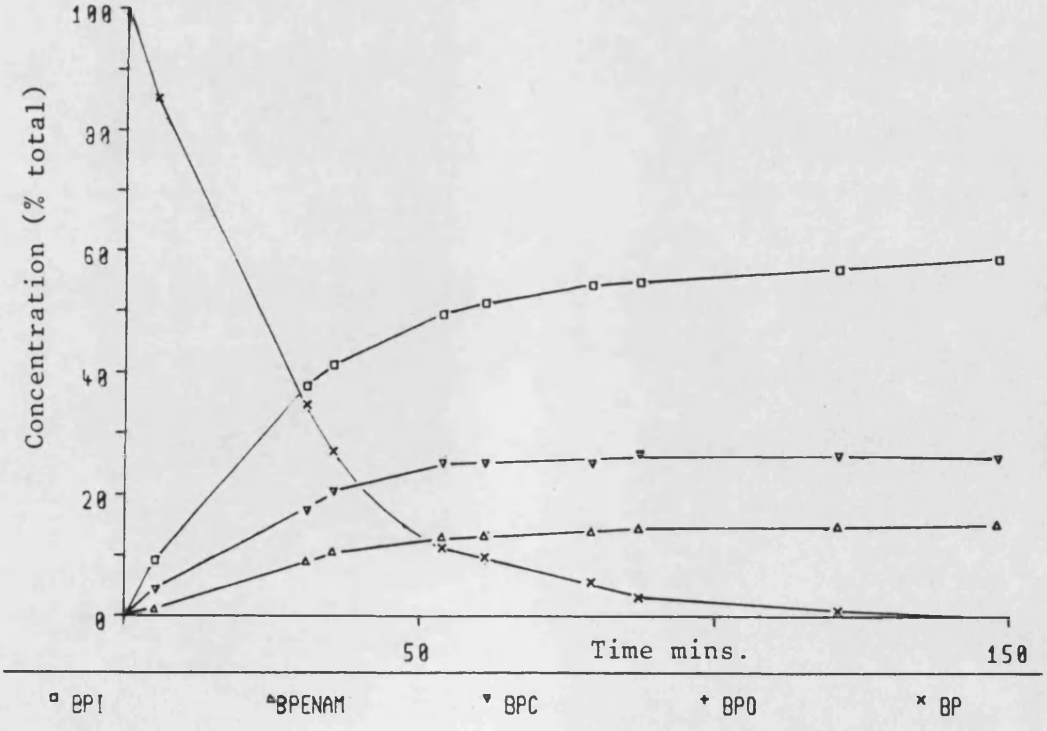
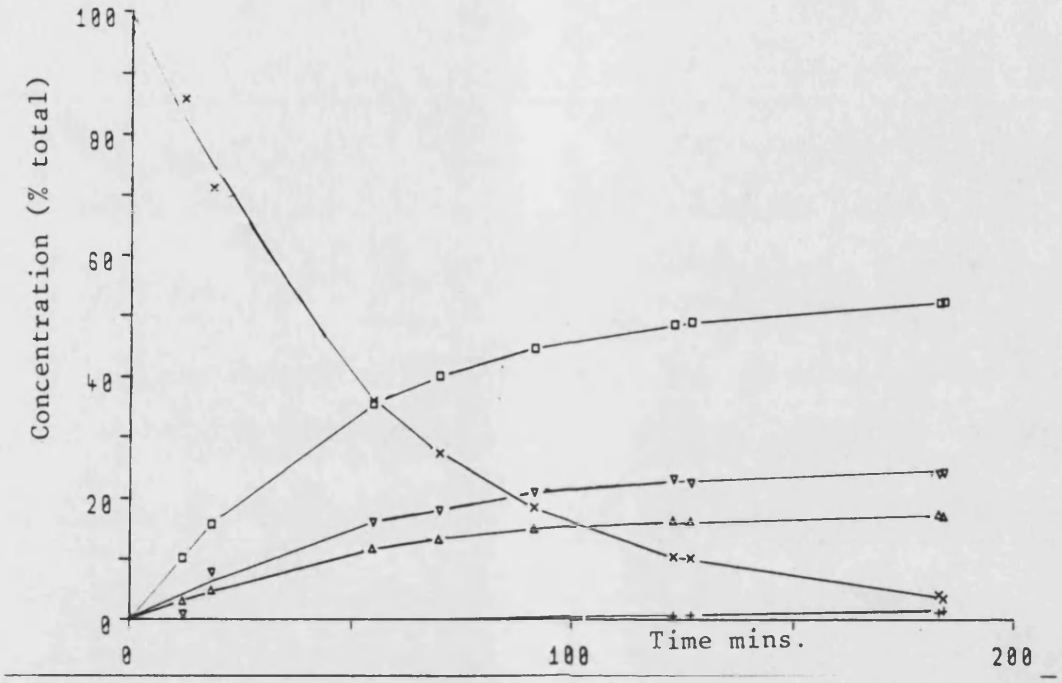


Figure 56 Plot of the concentrations of the degradation products of (3S,5R,6R)-benzylpenicillin (0.992 mM) at pH 2.5 and 25°C



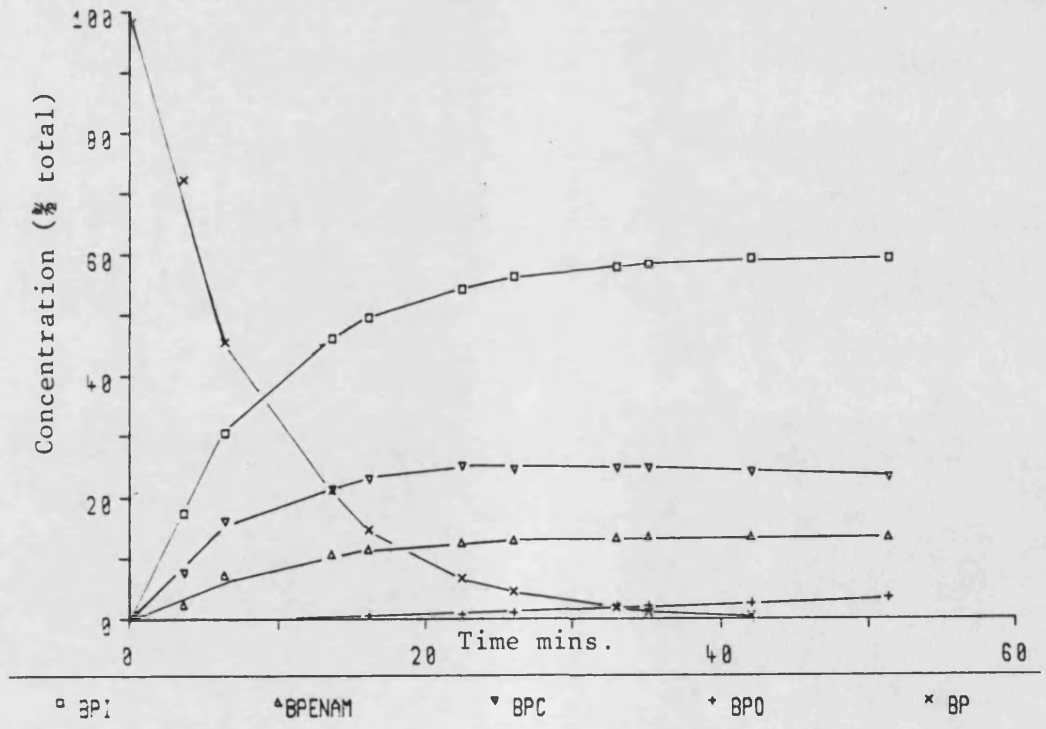


Figure 57 Plot of the concentrations of the degradation products of (3S,5R,6R)-benzylpenicillin (0.996 mM) at pH 2.2 and 37°C

Figure 58 Plot of the concentrations of the degradation products of (3S,5R,6R)-benzylpenicillin (0.997mM) at pH 2.5 and 37°C

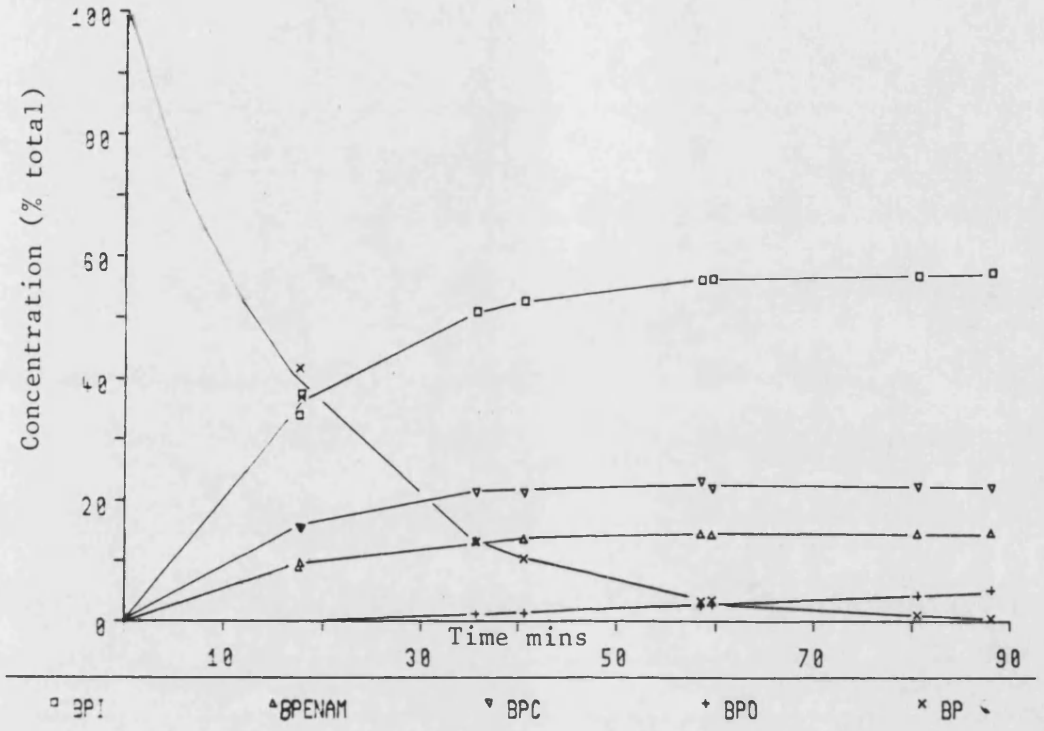


Table 56 Percentage concentration (and maximum peak area) of products formed upon complete degradation of benzylpenicillin.

Conditions	pH 2,2, 25°C	pH 2,5, 25°C	pH 2,2, 37°C	pH 2,5, 37°C
Compound	mean (n = 2) concentration, normalised to 100 %			
Benzylpenicillin BP	0	0	0	0,9 ± 0,3
Benzylpenillic acid	57,6 ± 0,9	54,7 ± 0,3	59,3 ± 0,1	57,2 ± 0,3
Benzylpenamaldic acid	15,0 ± 0,2	18,0 ± 0,1	13,6 ± 0,1	14,6 ± 0,1
Benzylpenicilloic acids	26,0 ± 0,1	24,5 ± 0,2	22,6 ± 0,6	22,2 ± 0,1
5S,6R;5R,6S;5R,6R;5S,6S =	39;3;58;trace	35,5;14;40,5;9	49;3;48;trace	38,5;15;35;11,5
Benzylpenilloic acid	0,7 ± 0,7	1,8 ± 0,2	4,3 ± 0,8	4,7 ± 0,4
NFDPAM and NPAG	1,0 ± 0,4	1,2 ± 0,1	0,3 ± 0,3	0,5 ± 0,5
Penicillenic acid disul,	trace	trace	trace	trace
Mass balance MB	108 ± 4	102,5 ± 1,0	106 ± 2	101 ± 2

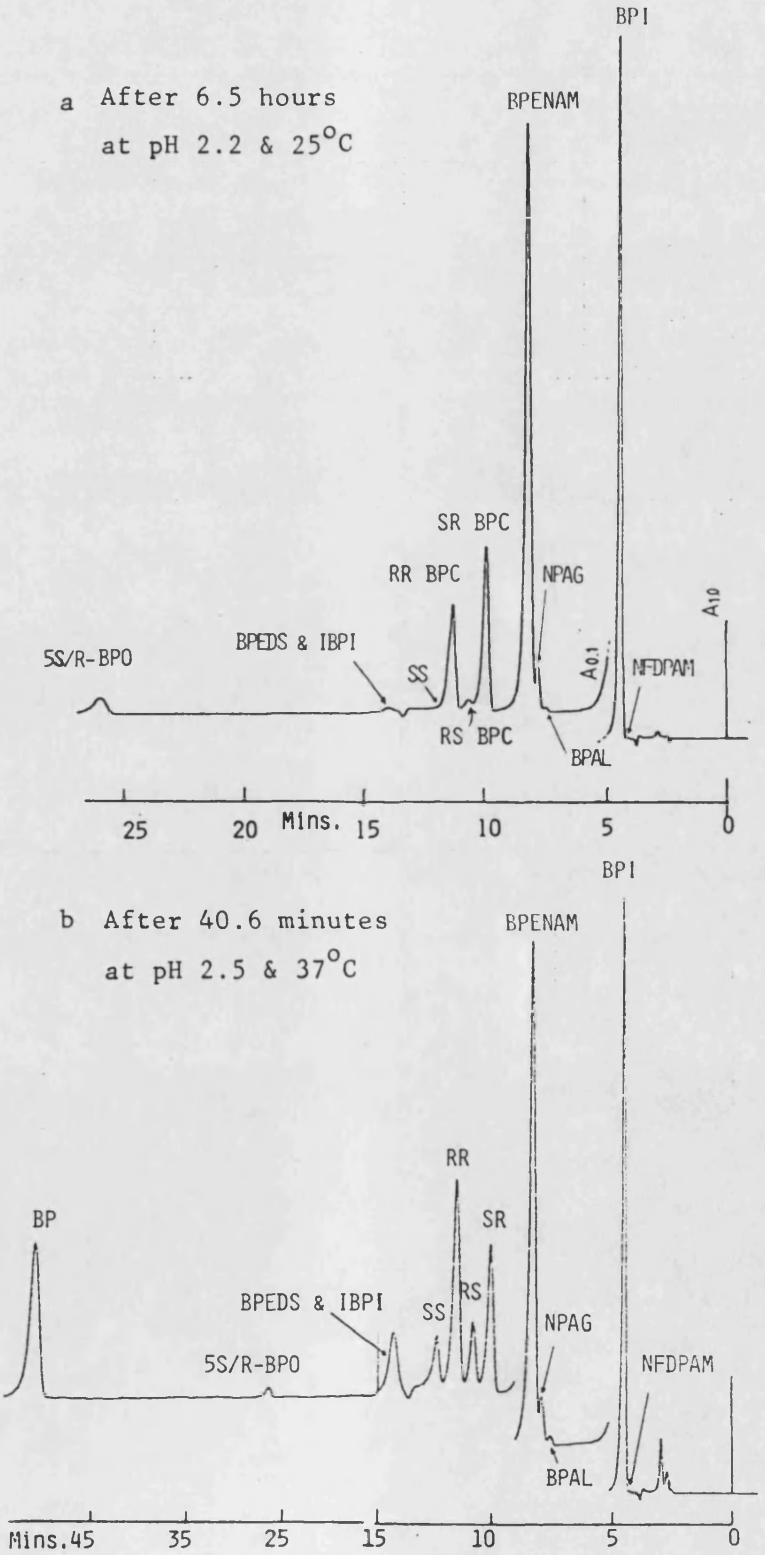
maximum observed peak areas

Experiment	1	2	1	2	1	2	1	2
Initial conc BP mM	0,994	0,990	0,992		0,992	1,000	0,994	0,999
Benzylpenillic acid $x10^{-7}$	5,05	4,85	4,53	4,45	5,12	4,96	4,64	4,64
B,penicilloic acids $x10^{-6}$	4,18	3,86	3,67	3,65	3,92	3,75	3,34	3,26
Benzylpenamaldic acid $x10^{-6}$	6,34	6,02	7,15	7,03	5,67	5,48	5,72	5,73

Typical chromatograms are shown in Figures 59 a and b

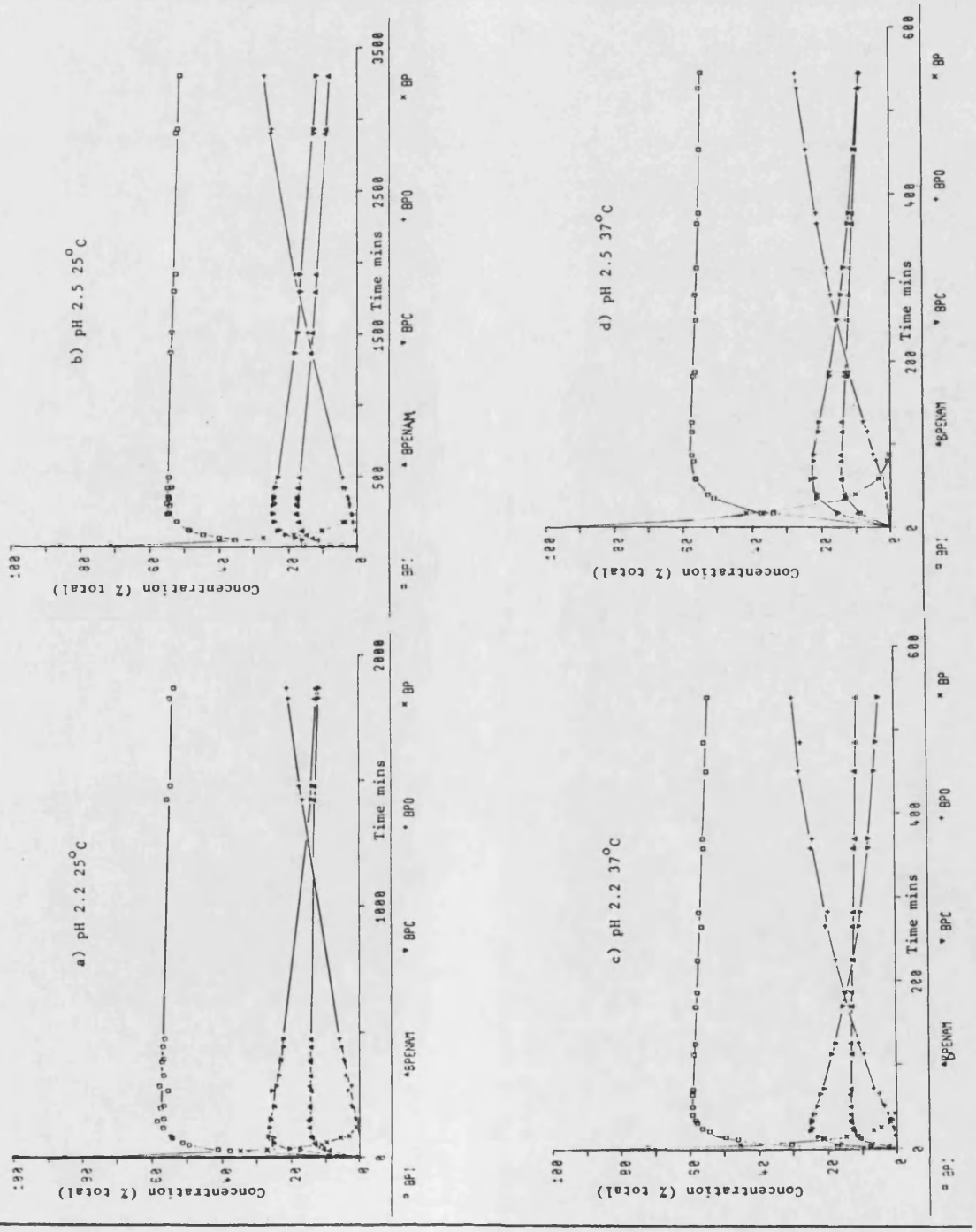
Figure 59 Chromatograms of degradation products of benzylpenicillin

HPLC conditions: system 3, see page 216.



Samples were analysed by HPLC long after all the benzylpenicillin had degraded (Figures 60a - d). As expected, the concentration of the benzylpenicilloic acids decreased rapidly relative to benzylpenamaldic acid. The concentration of benzylpenillic acid decreased very slowly.

Figure 60 Plots of the concentrations of degradation products versus time. a pH 2.2, 25°C; b pH 2.5, 25°C; c pH 2.2, 37°C; d pH 2.5, 37°C



The concentration of the next degradation products to appear, the benzylpenilloic acid epimers, increased with time and eventually these breakdown and trace amounts of benzylpenilloaldehyde (and presumably D-penicillamine, masked by the solvent front) are observed. In addition trace amounts of benzylisopenillic acid, the minor degradation product of benzylpenillic acid, were observed in aged samples.

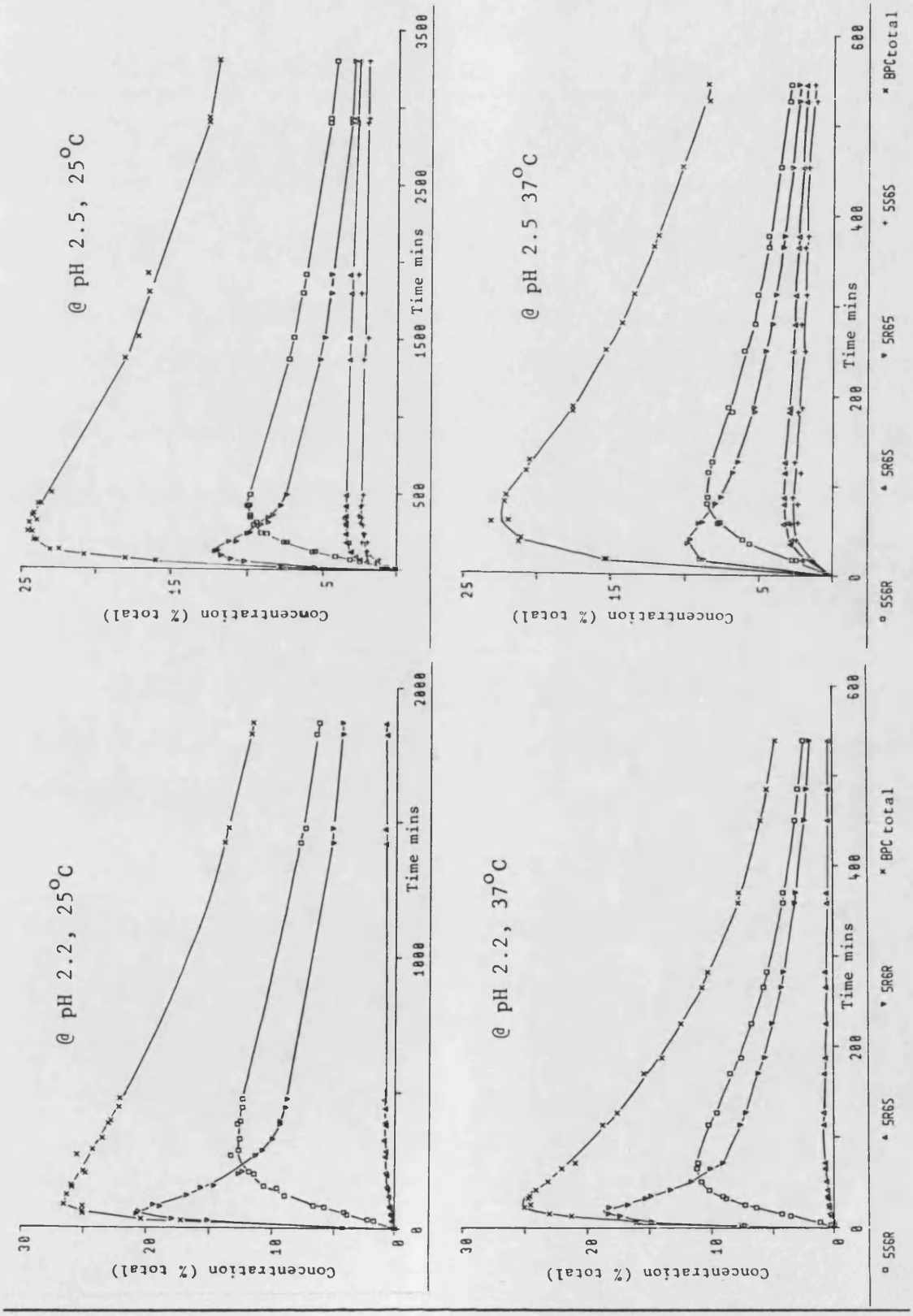
The yields and profiles of the main degradation products obtained at pH 2.2 and pH 2.5 appeared at first glance to be very similar apart from the obvious differences in time scale. However the HPLC analysis not only provided data on the total concentration of the four benzylpenicilloic acid stereoisomers but also on the concentration of each individual stereoisomer.

Figures 61 a - d are plots of the data obtained. In all cases 5R,6R was the first isomer to appear and this rapidly epimerised to give the thermodynamically-favoured 5S,6R-epimer. Close scrutiny of the experimental results revealed that at pH 2.5 the amounts of the 6S-epimers (5R,6S and 5S,6S) relative to the 6R-epimers (5R,6R and 5R,6S) were lower than those observed at pH 2.2. This difference may be best illustrated by comparison of the chromatograms in Figure 59 a and b.

As benzylpenicilloic acids derived from benzylpenicillenic acid have been shown to possess all four possible conformations, while those derived from benzylpenicillin directly are assumed to possess 6R stereochemistry only, it appears that at pH 2.5 very little benzylpenicilloic acid was formed via benzylpenicillenic acid.

In all the HPLC studies carried out, every peak could be positively identified. There was no evidence of the benzyloxazolone-thiazolidine intermediate.

Figure 61a - d Plots of the concentrations of benzylpenicilloic acid stereoisomers versus time.



12.4.4 Overall rates of degradation of benzylpenicillin, benzyl - penillic, penamaldic and penicilloic acids.

From the data plotted in Figures 60a to d and 61a to d it was possible to calculate the overall rates of degradation of benzylpenicillin and its breakdown products. From the HPLC data one could obtain not only the overall rate of degradation of the summed benzylpenicilloic acids but also the rates of degradation of the 6R and 6S stereoisomers by assuming that epimerisation at C-6 was negligible. All rates were found to be first-order and a brief data summary is given in Table 57.

Table 57 Rate constants for the degradation of benzylpenicillin, benzylpenillic, penamaldic and penicilloic acids

Conditions	pH 2,2, 25°C		pH 2,5, 25°C		pH 2,2, 37°C		pH 2,2, 37°C	
Experiment	1	2	1	2	1	2	1	2
Init. conc BP mM	0,994	0,990	0,992	0,992	0,992	1,000	0,994	0,999
				Benzylpenicillin				
CC	0,9961	0,9974	0,9982	0,9995	0,9995	0,9928	0,9997	0,9999
K min^{-1}	0,0400	0,0362	0,0170	0,0178	0,125	0,128	0,0601	0,0555
Mean K ± σ min^{-1}	0,0381 ± 0,002		0,0174 ± 0,0004		0,127 ± 0,002		0,0578 ± 0,0023	
				Benzylpenillic acid				
CC	0,9631	0,9972	0,9685	0,8975	0,9916	0,9932	0,9988	0,9991
K min^{-1} x 10^4	0,441	0,364	0,283	0,246	1,65	1,39	1,14	1,13
Mean K ± σ min^{-1}	(4,03±0,39)x10^{-5}		(2,65±0,19)x10^{-5}		(1,52±0,13)x10^{-4}		(1,14±0,01)x10^{-4}	
				Benzylpenamaldic acid				
CC	0,9919	0,9909	0,9988	0,9990	0,9916	0,9918	0,9973	0,9956
K min^{-1} x 10^4	0,970	0,836	2,56	2,50	3,45	3,47	10,4	10,2
Mean K ± σ min^{-1}	(9,03±0,67)x10^{-5}		(2,53±0,03)x10^{-4}		(3,46±0,01)x10^{-4}		(1,03±0,01)x10^{-3}	
				Benzylpenicilloic acids (Total)				
CC	0,9932	0,9986	0,9920	0,9990	0,9985	0,9967	0,9969	0,9986
K min^{-1} x 10^4	4,83	4,71	2,54	2,69	32,6	32,2	20,4	21,7
Mean K ± σ min^{-1}	(4,77±0,06)x10^{-4}		(2,62±0,08)x10^{-4}		(3,24±0,02)x10^{-3}		(2,11x0,08)x10^{-3}	
				Benzylpenicilloic acids (6R epimers)				
CC	not determined		0,9958	0,9994	not determined		0,9948	0,9997
K_R min^{-1} x 10^4			3,29	3,48			23,9	25,3
Mean K ± σ min^{-1}			(3,39 ± 0,10) x 10^{-4}				(2,46 ± 0,07) x 10^{-3}	
				Benzylpenicilloic acids (6S epimers)				
CC	not determined		0,8006	0,9146			0,9910	0,9976
K_S min^{-1} x 10^4			0,920	0,954			13,5	13,9
Mean K ± σ min^{-1}			(9,37 ± 0,17) x 10^{-5}				(1,37 ± 0,02) x 10^{-3}	

The overall degradation rates of benzylpenicillin, benzylpenillic benzylpenamaldic and benzylpenilloic acids were found to be similar in magnitude to those obtained on single components in previous studies. Hence we can assume that any co-catalytic effects by products present in the reaction mixture upon the overall rates of degradation are negligible. There was no evidence of side-reactions occuring between degradation products.

Comparison of the overall rates of degradation of the 6R-benzylpenicilloic acid epimers (i.e. 5R,6R- and 5S,6R-) with the 6S- epimers (i.e. 5R,6S- and 5S,6S-) at pH 2.5 indicated that $K_R > K_S$ (i.e. 3.6 × greater at 25°C and 1.8 × greater at 37°C). Data for studies at pH 2.2 was not calculated due to the very low levels Of the 6S-epimers.

This observation would support the suggestion first made in section 10.4.2 that the decarboxylation of benzylpenicilloic acid has a stereoselective preference and that decarboxylation of the 6R- epimer to be relatively facile.

12.4.5 Comparison of HPLC and ^1H NMR analyses of the degradation of benzylpenicillin in DCl/D_2O

Comparison of the ^1H NMR spectra with the data of Degalaen *et al* [61] enabled the complete assignment of all resonances observed (Table 58).

The ^1H NMR spectra of the degradation mixtures were analysed on a semi-quantitative basis by measurement of the peak heights of the 2α- and 2β- methyl resonances of the products. This procedure was adopted for the following reasons:

(a) The methyl resonances are singlets, reasonably well resolved and the assignments are unambiguous.

Table 58 Complete assignment of the ^1H NMR spectra of benzylpenicillin degraded in DCl/D_2O

Line No.	δ obs	δ ref [61]	assignment		$(^{\prime}J)$obs Hz
1	8.25s	-	BPEDS or BIPI	H-5	
2	7.82s	7.78	Benzylpenamaldic acid	H-5	
3	5.93d	5.94	Benzylpenillic acid	H-5	(5.9)
4	5.55d	5.56	Benzylpenicillin	H-5	(3.9
5	5.46d	5.45	Benzylpenicillin	H-6	(3.9)
6	5.40d	5.38	(5R,6R)-Benzylpenicilloic acid	H-5	(5.1)
7	5.35d	5.33	(5S,6R)-Benzylpenicilloic acid	H-5	(5.7)
8	5.06t	5.01	(5S)-Benzylpenilloic acid	H-5	(5.1)
9	5.00t	4.95	(5R)-Benzylpenilloic acid	H-5	(4.8)
10	4.88d	4.84	Benzylpenillic acid	H-6	(ca. 5.6)
11	4.37s	-	Benzylpenicillin	H-3	
12	4.32s	4.32	Benzylpenillic acid	H-3	
13	4.10s	4.09	Penillic H_2-9 or (5S,6R)-penicilloic acid	H-3	
14	4.06s	4.10	(5S)-Benzylpenilloic acid	H-3	
15	4.02s	4.03	(5R)-Benzylpenilloic acid	H-3	
16	3.98s	3.98	(5R,6R)-Benzylpenicilloic acid	H-3	
17	3.90s	3.88	Benzylpenamaldic acid	H-3	
18	3.75s	3.72	Benzylpenamaldic acid	H_2-9	
19	3.73s	3.69	(5R,6R)-Benzylpenicilloic acid	H_2-9	
20	3.71s	3.69	(5S,6R)-Benzylpenicilloic acid	H_2-9	
21	3.69q	3.75	Benzylpenicillin	H_2-9	(15)
			Methyl resonances		
22	1.65s	1.67	(5R)-Benzylpenilloic acid	2β	
23	1.64s	1.64	Benzylpenamaldic acid	2β	
24	1.63s	1.64	(5S,6R)-Benzylpenicilloic acid	2β	
25	1.61s	1.60	(5S)-Benzylpenilloic acid	2β	
26	1.59s	1.57	Benzylpenicillin	2β	
27	1.57s	1.57	(5R,6R)-Benzylpenicilloic acid	2β	
28	1.55s	1.54	Benzylpenillic acid	2β	
29	1.53s	1.52	Benzylpenillic acid	2α	
30	1.50s	1.52	Benzylpenicillin	2α	
31	1.47s	1.47	Benzylpenamaldic acid	2α	
32	1.41s	1.40	(5R,6R)-Benzylpenicilloic acid	2α	
33	1.40s	1.42	(5S)-Benzylpenilloic acid	2α	
34	1.34s	1.35	(5R)-Benzylpenilloic acid	2α	
35	1.28s	1.26	(5S,6R)-Benzylpenicilloic acid	2α	
36	0	0	DSS reference		

Notes: s = singlet, d = doublet, t = triplet and q = quartet
H_2-9 refers to benzylic CH_2 protons.
Resonances for (5R,6S)- & (5S,6S)-benzylpenicilloic acids not seen
Benzylpenilloic acid H_2-6 octets present at ca. 3.6 to 3.8 ppm but not measured. 4.8 to 4.9 ppm region of the spectrum obscured by HDO resonance and its spinning side bands. Aromatics 7.3 to 7.7 ppm.

(b) The relative intensities of the methyl resonances are high and hence yield favourable signal-to-noise ratios.

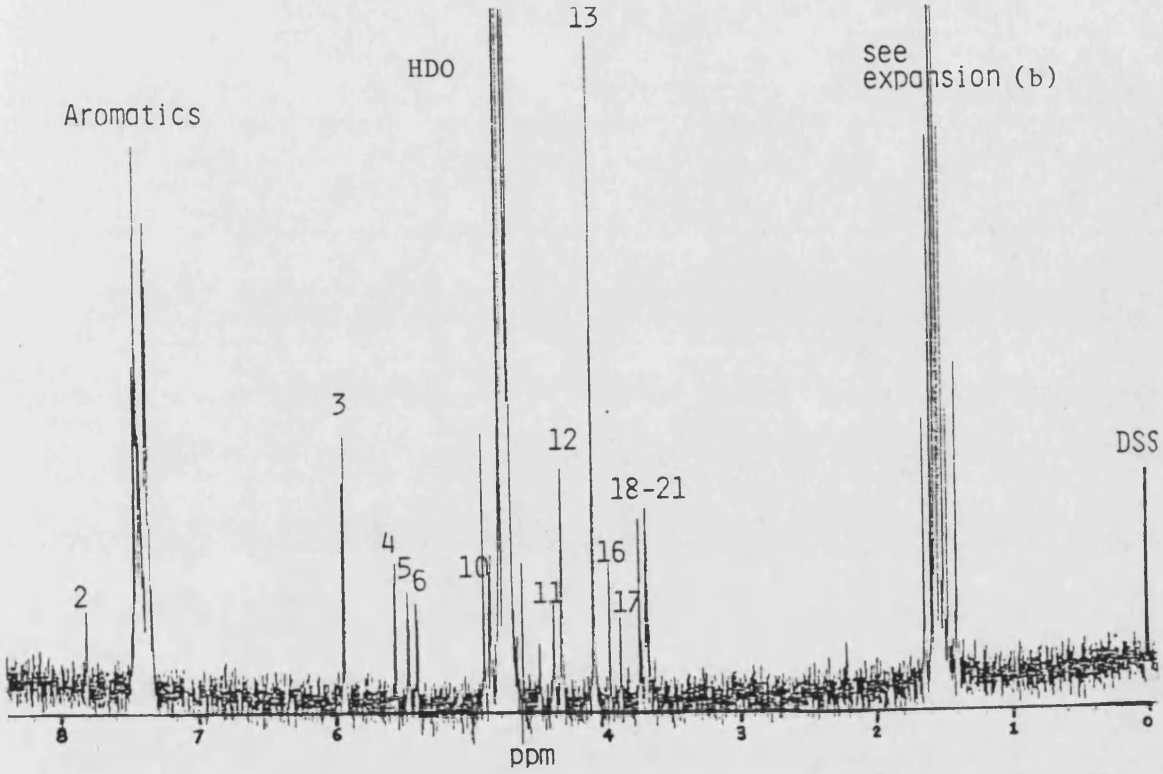
(c) There are no complications due to deuteration or solvent peaks.

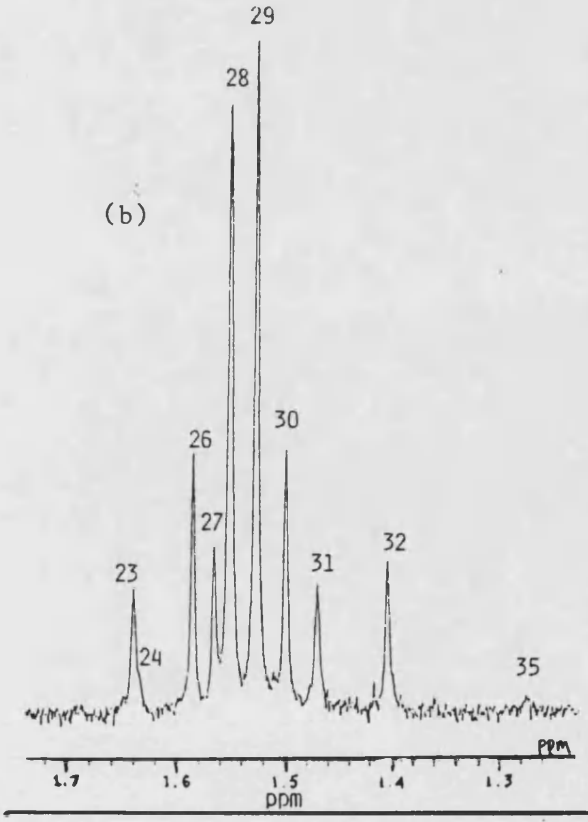
The quantitative aspects of ^1H NMR are well understood and have been described.[185] Calculations of the spin-lattice relaxation time constants (T_1) of benzylpenicillin Na by the inversion recovery method were made available.[186] The T_1 values of the 2α- and 2β- methyl resonances (for a 0.126 M solution of benzylpenicillin Na in D_2O) were less than 250 milliseconds.

Assuming that T_2, the spin-spin relaxation time constant, ≃ T_1, then the relaxation time $1/T_2$ for the methyl resonances is ca. 4 seconds. Therefore a pulse repitition rate of 20 seconds, equivalent to 5 half-lifes, should be used to allow the excited protons to return to the lower energy spin state and avoid signal saturation. However this delay would increase analysis time beyond optimum. Therefore it was assumed, that as all the degradation products studied retain the *gem*-dimethylthiazolidine nucleus, then the T_2 values will be of a similar magnitude, hence permitting the use of faster signal accumulations. This procedure, at best, was only semi-quantitative.

Figure 62a shows the 400 MHz ^1H NMR spectrum of the reaction mixture at pH 2.5 and 25°C and was recorded between 73 to 78 minutes after the start of the experiment. Peak height analysis of the expansion of the high-field region of the spectrum containing the methyl resonances (Figure 62b) gave the composition as benzylpenicillin (21.5 %), benzylpenillic acid (55.0 %), benzylpenamaldic acid (10.5 %), and benzylpenilloic acids (13.0 %; 5R,6R-, 12.3 % and 5S,6R-, 0.7 %).

Figures 62 a - c. 400 MHz ^{1}H NMR spectra of the degradation of benzylpenicillin. See text for details and Table 58 for key.





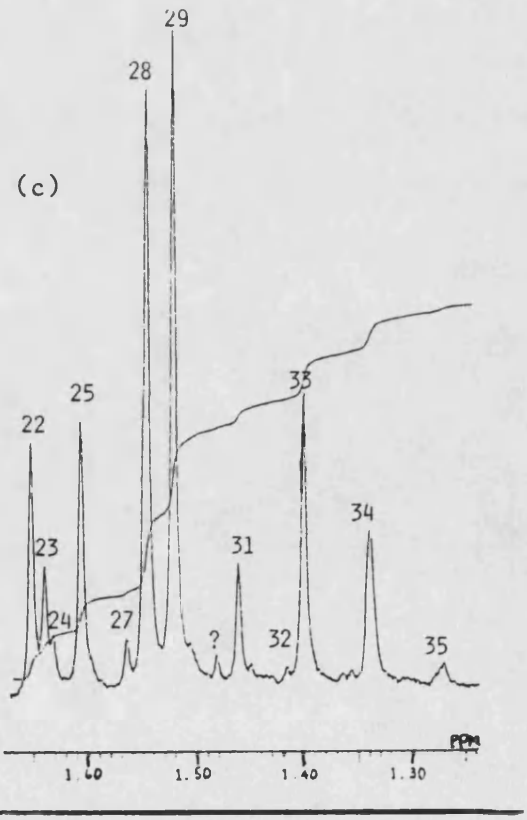
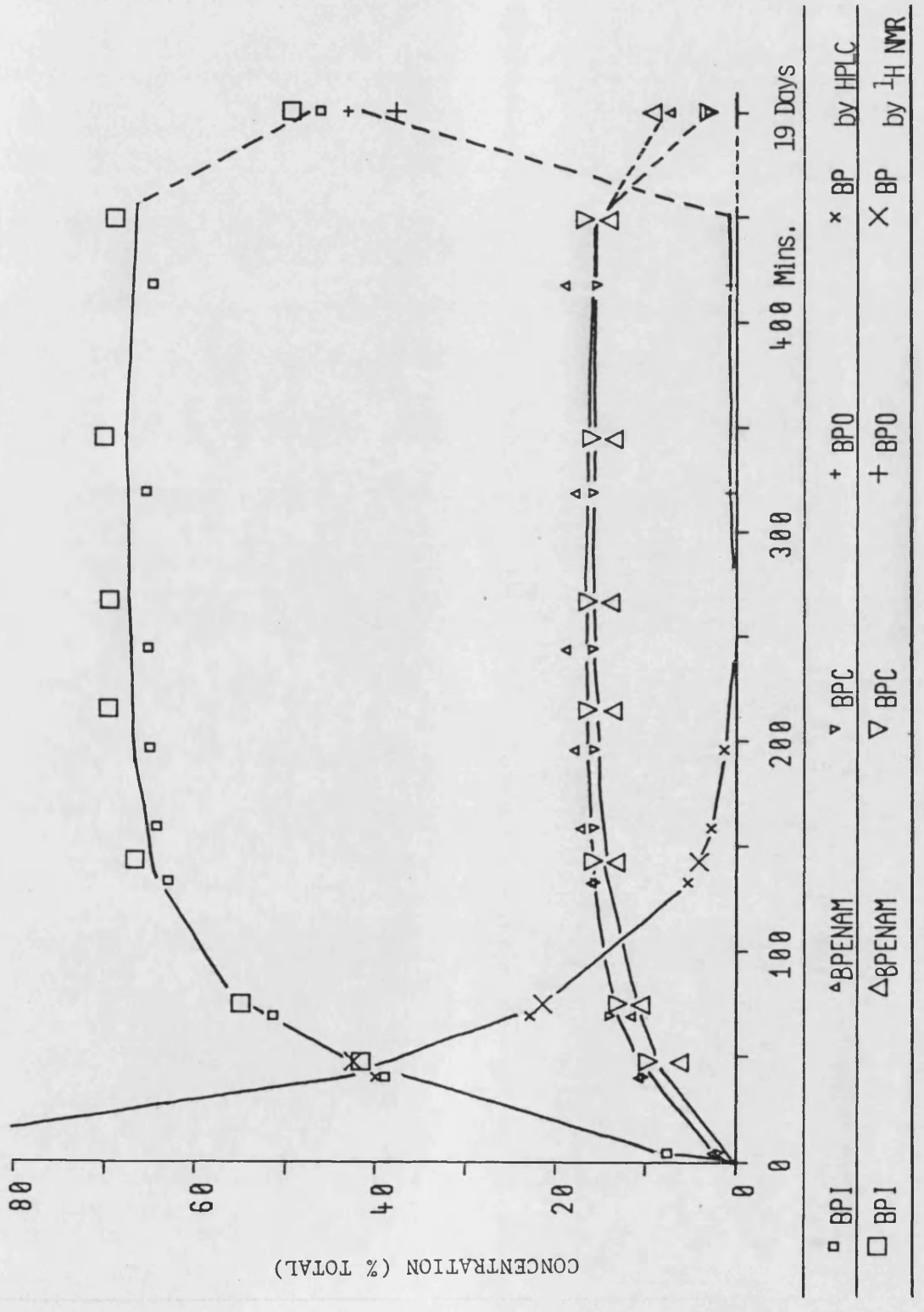


Figure 62c is the expansion of the spectrum obtained 19 days after the start of the experiment and the composition of the reaction mixture calculated as benzylpenillic acid (49.6 %), benzylpenamaldic acid (9.3 %), benzylpenicilloic acids (3.2 %; 5R,6R-, 1.6 % and 5S,6R-, (1.6 %) and benzylpenilloic acids (37.9 %; 5S : 5R = 57 : 43). The benzylpenilloic acid epimer ratio was as expected from our studies of its epimerisation in acidic solution (section 8).

The compositions of the reaction mixtures were similar irrespective of whether the results were obtained using a pulse repitition rate of 4.6 or 9.1 seconds. Experiments using a signal homogated pulse sequence for solvent elimination also gave similar results. However no matter how the irradiation strength was varied either the HDO peak was not completely suppressed or the intensity of resonances close to HDO was reduced. This was primarily due to the low concentrations of the analytes. For the same reason and also because the intensities of the components of doublet resonances showed an appreciable departure from the first-order-approximations it was not possible to estimate the proportion of benzylpenillic and benzylpenicilloic acids derived from either benzylpenicillin directly or via benzylpenicillenic acid using the methods of Degalaen *et al*.[61]

The data from the HPLC and NMR analyses were in close agreement (see Figure 63) and would seem to justify the analytical approaches adopted.

Figure 63 Comparison of the HPLC and ^1H NMR analyses of benzylpenicillin degradation in DCl/D_2O at pH 2.5 and 25°C



12.4.6 Empirical analyses of the degradation of benzylpenicillin in acidic solution.

a) Benzylpenamaldic acid

It is generally accepted that benzylpenamaldic acid is formed from benzylpenicillenic acid directly. It was a relatively trivial exercise to test this assumption. For example:

From kinetic evaluation of benzylpenicillenic acid concentration during the degradation of benzylpenicillin at pH 2.2 and 25°C (Section 12.4.2) it was estimated that 16.7 ± 0.8 % benzylpenicillin was converted to benzylpenicillenic acid. In identical experimental conditions it was found (Section 11.3.7) that 69.1 ± 2.2 % penicillenic acid was converted to penamaldic acid. From this it follows that 11.5 ± 0.7 % benzylpenicillin must be converted via penicillenic acid to penamaldic acid. In fact a 15 ± 0.1 % yield of penamaldic acid was observed. Similar discrepancies were noted for the other experiments.

These differences lay outside the limits set by experimental errors and reflected the high degree of uncertainty in the value of the HPLC response factor of penamaldic acid used, plus the sum of the errors in the response factors of all other analytes. These errors were avoided in the following fashion:

For instance at pH 2.2 and 25°C, 5.26×10^{-4} moles of penicillenic acid gave upon complete degradation penamaldic acid with a HPLC peak area of 1.89×10^{7} units (Table 45), equivalent to 3.59×10^{10} units per mole.

Under identical experimental and analytical conditions 9.94×10^{-4} moles of penicillin gave, upon complete degradation, penamaldic acid

with a maximum observed HPLC peak area of 6.34×10^6 units (Table 56) equivalent to 6.38×10^9 units per mole. Assuming that all the penamaldic acid was formed from penicillenic acid only, the percentage of penicillin converted to penicillenic acid will be:

$$\frac{6.38 \times 10^9}{3.59 \times 10^{10}} \times 100\ \% = 17.8\ \%$$

This calculation was repeated for all the experiments and a summary of the results are given in Table 59

Table 59 Comparison of estimates of the percentage of benzylpenicillin converted to benzylpenicillenic acid.

Conditions	pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
BPE studies	16.7 ± 0.8	50.7 ± 1.6	17.1 ± 0.8	47.3 ± 4.8
BP studies †	17.4 ± 0.5	55.5 ± 0.5	17.3 ± 0.0	52.8 ± 0.1

Notes:† mean of duplicate experiments.

The results from the pH 2.2 studies were in excellent agreement while those at pH 2.5 showed a slight divergence.

From these results it was considered safe to assume that benzylpenamaldic acid was formed exclusively from benzylpenicillenic acid.

b) Benzylpenillic acid

The proportions of benzylpenillic acid derived from either benzylpenicillin directly or via benzylpenicillenic acid were estimated in the following fashion. For instance, at pH 2.2 and 25°C, 9.94×10^{-4} moles of penicillin upon complete degradation gave penillic acid with a maximum observed HPLC peak area of 5.05×10^7 arbitary units (Table 56) equivalent to 5.08×10^{10} units per mole.

Under identical experimental and analytical conditions, 5.26×10^{-4} mols of penicillenic acid upon complete degradation gave 5.11×10^{6} units of penillic acid (Table 45) i.e. 9.71×10^{9} units per mole.

We know that 16.7 % penicillin was converted to penicillenic acid i.e. 9.94×10^{-4} mols of penicillin produces 1.66×10^{-4} mols of penicillenic acid. Therefore the amount of penillic acid derived via penicillenic acid was $9.71 \times 10^{9} \times 1.66 \times 10^{-4} = 1.61 \times 10^{6}$ units.

Therefore the amount of penillic acid formed exclusively from penicillin was $5.05 \times 10^{7} - 1.61 \times 10^{6} = 4.89 \times 10^{7}$ units, and hence the proportion of penillic acid formed from penicillin, to that from penicillenic acid was $4.89 \times 10^{7} : 1.61 \times 10^{6} = 96.8\ \% : 3.2\ \%$, respectively. The results (means of 2 expts.) are given in Table 60.

Table 60 Percentage of benzylpenillic acid formed from either benzylpenicillin BP directly or via benzylpenicillenic acid BPE.

Conditions	pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
% from BP	96.8 ± 0.1	73.3 ± 0.3	96.9 ± 0.1	75.5 ± 0.1
% via BPE	3.2 ± 0.1	26.7 ± 0.3	3.1 ± 0.1	24.5 ± 0.1

c) Benzylpenicilloic acids.

A similar approach to that used above was used to estimate the proportion of benzylpenicilloic acid derived from either benzylpenicillin directly or via benzylpenicillenic acid.

However, as the degradation of penicilloic acid was relatively fast the maximum *observed* peak area of penicilloic acid will be lower than the true maximum. Fortunately, as the degradation of penicilloic acid obeyed first order kinetics, the true maximum may be estimated by

extrapolating the penicilloic acid concentration versus time data to time zero.

For instance, at pH 2.2 and 25°C, the maximum observed peak area was 4.18×10^6 (Table 56) while the extrapolated value was calculated as 4.43×10^6. The differences, as expected, were even greater at 37°C. The results are summarised in Table 61a.

Similar results were estimated by comparison of the penicilloic acid stereoisomer ratios when all penicillin has degraded (Table 56) with the ratios from penicillenic acid degradation experiments (Table 45). We know that penicillenic acid decomposed to give all four possible stereoisomers, whereas penicilloic acids formed from penicillin directly will possess either 5R,6R or 5S,6R stereochemistry only.

Assuming that C-6 epimerisation of penicilloic acid was negligible and that the stereoselective nature of the decarboxylation of penicilloic acids will have no significant effect upon the stereoisomer ratios (during the relatively brief period taken to decompose all penicillin and any penicillenic acid present), then examination of the 6R : 6S epimer ratio may indicate the parent of penicilloic acid.

i) pH 2.5;

From the penicillenic acid experiments the 6R : 6S ratio (5S,6R + 5R,6R : 5R,6S + 5S,6S) was 58.9 : 41.1 at 25°C and 58.3 : 41.7 at 37°C

From the penicillin experiments the 6R : 6S ratio when all the penicillin had decomposed was 76.6 : 23.4 at 25°C and 73 : 27 at 37°C. Therefore the % of 6R epimers formed via penicillenic acid was:

$$(23.4 \times 58.6) / 41.4 = 33.1\ \%\ \text{at } 25°C \text{ and}$$

$$(27.1 \times 58.6) / 41.4 = 38.4\ \%\ \text{at } 37°C.$$

Therefore the % of penicilloic acids formed directly from penicil-

lin was 76.6 - 33.1 = 43.5 % at 25°C and 73.1 - 38.4 = 34.6 at 37°C.

ii) pH 2.2

These experiments were further complicated because when all the penicillin had degraded, the 5S,6S epimer was present at levels too low for accurate measurements. This itself suggests that most of the penicilloic acid was produced directly from penicillin. However the actual amount may be estimated by comparing the 5R,6S : (5S,6R + 5R,6R + 5S,6S) ratios in a similar fashion to that described above.

The results agree well with those obtained from the "extrapolation" method and are summarised in Table 61b.

Table 61 Percentage of benzylpenicilloic acid formed from either benzylpenicillin directly or via benzylpenicillenic acid

Conditions	pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
		61a		
% from BP	90.4 ± 0.2	50.9 ± 0.1	90.0 ± 0.2	57.7 ± 0.7
% via BPE	9.6 ± 0.2	49.1 ± 0.1	10.0 ± 0.2	42.3 ± 0.7
		61b		
% from BP	87.9 ± 1.4	43.5 ± 1.0	84.5 ± 2.7	34.6 ± 2.4
% via BPE	12.1 ± 1.4	56.5 ± 1.0	15.5 ± 2.7	65.4 ± 2.4

d) Benzylpenicillin

Based on the results above it was now possible to estimate the percentage of penicillin degrading to penillic and penicilloic acids.

For example, at pH 2.2 and 25°C we know that:

1. The % of penicillin converted to penicillenic acid was 16.7 ± 0.8 %
2. The proportions of penillic acid formed directly from penicillin or

via penicillenic acid were 96.8 ± 0.1 : 3.2 ± 0.1 % respectively.

3. The proportions of penicilloic acid formed directly from penicillin or via penicillenic acid were 90.4 ± 0.2 : 9.6 ± 0.2 (87.9 ± 1.4 : 12.1 ± 1.4) respectively.

4. Penicillenic acid upon complete degradation gave penillic (12.2 ± 0.3 %), penamaldic (69.1 ± 2.2 %) and penicilloic acid (17.5 ± 1.5 %).

5.Therefore, 16.7 ± 0.8% penicillin degraded via penicillenic acid to;

penillic acid 16.7 ± 0.8 × 12.2 ± 0.3 / 100 = 2.0 ± 1.0 %,

penicilloic acids 16.7 ± 0.8 × 17.5 ± 1.5 / 100 = 2.9 ± 0.3 %

and penamaldic acid 16.7 ± 0.8 × 6.91 ± 2.2 / 100 = 11.5 ± 0.7 %

6. Hence the % of penicillin converted directly to penillic acid was;

2.0 ± 0.1 × 96.8 ± 0.1 / 3.2 ± 0.1 = 60.5 ± 3.6 %

7.The % of penicillin converted directly to penicilloic acids was

a) 2.9 ± 0.3 × 90.4 ± 0.2 / 9.6 ± 0.2 = 27.3 ± 2.9 % or

b) 2.9 ± 0.3 × 87.9 ± 1.4 / 12.1 ± 1.4 = 26.6 ± 4.2 %

Mean = 27.0 ± 0.4 %

The complete results, normalised to 100 %, are given in Table 62.

Table 62 Estimated product yields from benzylpenicillin directly.

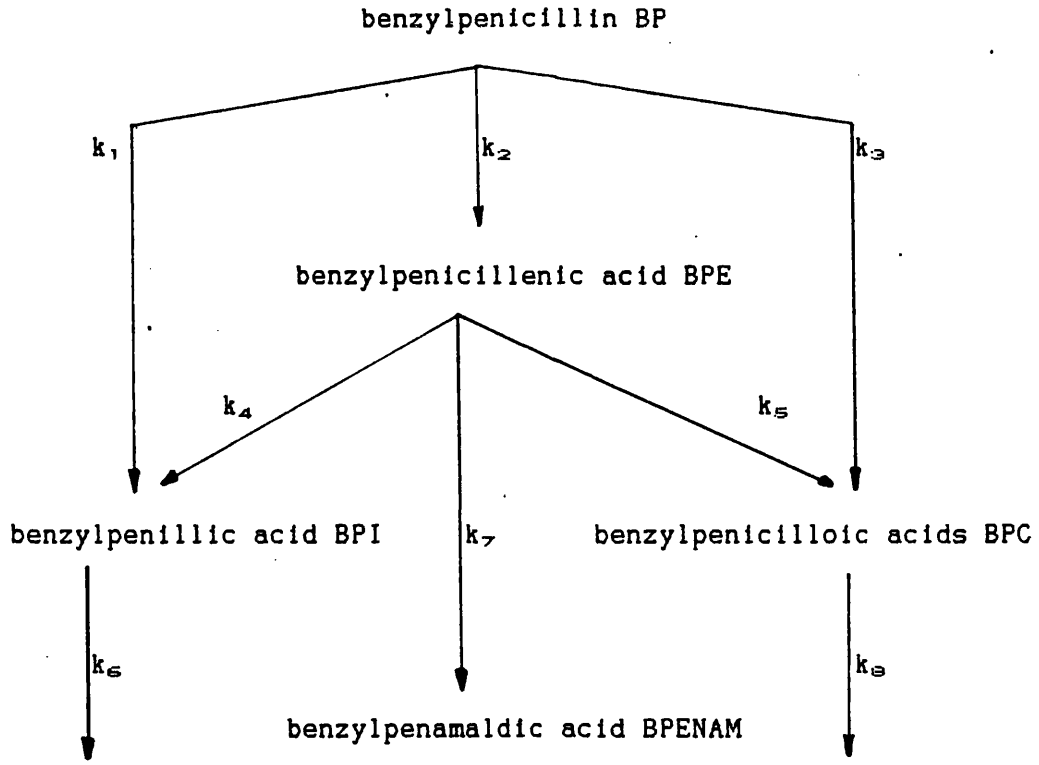
Conditions	pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
BPE	16.0 ± 0.8	48.5 ± 1.5	17.0 ± 0.8	46.5 ± 4.7 [50%]
BPI	58.0 ± 3.5	40.0 ± 1.4	62.0 ± 3.7	43.0 ± 0.3 [37%]
BPC	26.0 ± 0.4	11.5 ± 1.7	21.0 ± 5.0	10.5 ± 4.6 [13%]
Actual totals	104.2 ± 4.8	104.5 ± 4.9	100.6 ± 9.5	101.8 ± 9.8

Notes: Figures in [ ] refer to the data of Degalaen [61] (pH 2.5 DCl/D_2O at 37°C)

12.4.7 Kinetic analysis of the degradation of benzylpenicillin in acidic solution.

Studies of the degradation of benzylpenicillenic acid and benzylpenicillin, described previously, have highlighted the complex nature of their relationships with each other, the oxazolone-thiazolidine intermediate and other breakdown products. Full kinetic evaluation of such a complex system would require highly sophisticated numerical analysis far beyond the scope of this thesis.

Fortunately from knowledge of the principal degradation pathways and by assuming that the oxazolone-thiazolidine intermediate had only a transitory existence the following simplified scheme may be proposed:



Scheme 21 Simplified scheme for the degradation of benzylpenicillin in acidic aqueous solution

Let K = overall rate of degradation of penicillin = $k_1 + k_2 + k_3$ and K'= overall rate of degradation of penicillenic acid = $k_4 + k_5 + k_7$. k_6 and k_8 are the overall rates of degradation of penillic and penicilloic acids respectively. k_1 and k_3 are the unknowns. Assuming that all the individual rates are first-order;

$$\frac{\delta[BPI]}{\delta t} = k_1[BPI] + k_4[BPE] - k_6[BPI] \quad (1)$$

however k_6 was relatively very small, therefore

$$\frac{\delta[BPI]}{\delta t} = k_1[BP] + k_4[BPE] \quad (2)$$

The kinetic expression for benzylpenicillin is;

$$[BP] = [BP]_o e^{-Kt} \quad (3)$$

and from previous studies, see page 229;

$$[BPE] = \frac{k_2[BP]_o}{K' - K} (e^{-Kt} - e^{-K't}) \quad (4)$$

where $[BP]_o$ is the concentration of benzylpenicillin at time t = 0 and $[BPE]_o$ = 0. Substituting equations (3) and (4) into (2) gives

$$\frac{\delta[BPI]}{\delta t} = k_1[BP]_o e^{-Kt} + \frac{k_4 k_2[BP]_o}{K'-K} (e^{-Kt} - e^{-K't}) \quad (5)$$

however as K' > K equation (5) reduces with time to;

$$\frac{\delta[BPI]}{\delta t} = k_1[BP]_o e^{-Kt} + \frac{k_4 k_2[BP]_o\, e^{-Kt}}{K'-K} \quad (6)$$

$$= \frac{\delta[BPI]}{\delta t} = [BP]_o(k_1 + \frac{k_4 k_2}{K'-K}) e^{-Kt} \quad (7)$$

intergrating (7) with respect to time t gives:

$$\int_{[BPI]_o}^{[BPI]_t} \delta[BPI] = [BP]_o(k_1 + \frac{k_4 k_2}{K'-K}) \int_{t=0}^{t=t} e^{-Kt}\, \delta t \quad (8)$$

$$= [BPI]_t - [BPI]_o = [BP]_o (k_1 + \frac{k_4 k_2}{K'-K}) (\frac{e^{-K_o}}{K} - \frac{e^{-K_t}}{K}) \quad (9)$$

as the concentration of benzylpenillic acid at t=0 was zero

$$K[BPI]_t = [BP]_o\ (k_1 + \frac{k_4k_2}{K'-K})\ (1 - e^{-Kt}) \quad (10)$$

as time increases the term $(1 - e^{-Kt})$ tends to 1 and $[BPI]_t = [BPI]_s$ where $[BPI]_s$ was the steady state concentration of benzylpenillic acid, assuming that no degradation occurs. Therefore;

$$K[BPI]_s = [BP]_o(k_1 + \frac{k_4k_2}{K'-K}) \quad (11)$$

rearranging equation (11) for k_1 gives

$$k1 = \frac{K[BPI]_s}{[BP]_o} - \frac{k_4k_2}{K'-K} \quad (12)$$

Equation (12) may be simplified further in a manner reducing inter-experimental errors:

let C_1 = the proportion of penicillin converted to penicillenic acid

i.e. $C_1 = k_2/K$, therefore $k_2 = KC_1$ (13)

and C_2 = proportion of penicillenic acid converted to penillic acid

i.e. $C_2 = k_4/K'$, therefore $k_4 = K'C_2$ (14)

substituting for k_2 and k_4 in equation (12) gives;

$$k_1 = \frac{K[BPI]_s}{[BP]_o} - \frac{K'C_2KC_1}{K'-K} \quad (15)$$

Hence the percentage of benzylpenicillin converted directly to benzylpenillic acid is

$$k_1/K \times 100\ \% \quad (16)$$

and the percentage of benzylpenicillin converted directly to benzylpenicilloic acid is

$$100 - (\frac{100k_1}{K} + 100C_1)\ \% \quad (17)$$

The raw data are given in Table 63 and the final results in Table 64 in comparison with results obtained by the empirical approaches described in the previous section.

Table 63 Kinetic analysis; Raw data (means ± σ of duplicate analyses)

Conditions	pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
$[BP]_o \times 10^{-4}$ M	9.92 ± 0.02	9.92 ± 0.01	9.96 ± 0.04	9.97 ± 0.03
$[BPI]_s \times 10^{-4}$M †	6.18 ± 0.10	5.61 ± 0.05	6.29 ± 0.14	5.79 ± 0.02
K min^{-1} x 100 ‡	3.81 ± 0.22	1.74 ± 0.05	12.7 ± 0.08	5.78 ± 0.07
K'min^{-1} x 10 #	3.11 ± 0.11	3.60 ± 0.03	6.54 ± 0.04	6.84 ± 0.25
C_1 x 100 *	16.7 ± 0.80	50.7 ± 1.60	17.1 ± 0.80	47.3 ± 4.80
C_2 x 100 *	12.2 ± 0.30	29.9 ± 0.30	11.6 ± 0.10	30.1 ± 0.20
k_1 min^{-1} x 100 §	2.30 ± 0.14	0.72 ± 0.24	7.96 ± 0.52	2.53 ± 0.10

Notes : † Obtained from Table 56 p234 (Max observed peak area of BPI divided by HPLC response factor, 8.01 x 10^{10})
‡ From Table 57 p239, # from Table 40 p195, * from Table 55 p230
§ Calculated from equation 15, σ by INSTAT simulation.

Table 64 Comparison of the results obtained from Empirical (E) and Kinetic (K) analyses of the degradation of benzylpenicillin

Conditions		pH 2.2, 25°C	pH 2.5, 25°C	pH 2.2, 37°C	pH 2.5, 37°C
		Percentage of benzylpenicillin converted directly to:			
BPE	E	16.0 ± 0.8	48.5 ± 1.5	17.0 ± 0.8	46.5 ± 4.7
	K	16.7 ± 0.8	50.7 ± 1.6	17.1 ± 0.8	47.3 ± 4.8 [50]
BPI	E	58.0 ± 3.5	40.0 ± 1.4	62.0 ± 3.7	43.0 ± 0.3
	K	60.3 ± 5.1	41.4 ± 1.8	62.7 ± 5.7	43.8 ± 1.8 [37]
BPC	E	26.0 ± 0.4	11.5 ± 1.7	21.0 ± 5.0	10.5 ± 4.6
	K	23.0 ± 5.9	7.9 ± 3.4	20.2 ± 6.5	8.9 ± 6.6 [13]

BPI Benzylpenillic, BPE benzylpenicillenic and BPC (3S,5R,6R)-benzylpenicilloic acids. Data in [] refer to the work of Degalaen *et al*.[61]

N.B. It should be noted that ca. 1 % benzylpenicillin was converted to N-phenylacetylglycine and N-formyl-D-penicillamine at pH 2.2 & 2.5 at 25°C, and ca.0.5 % at 37°C

Close agreement was obtained between the results from the empirical and kinetic approaches adopted. The results agreed surprisingly well with the data from the ^{1}H NMR work of Degalaen [61].

The kinetic scheme (Scheme 21), first proposed by Degalaen [61], would appear to offer a simple explanation to the complex degradation of benzylpenicillin in acidic aqueous solution even though a number of assumptions and approximations had to be used.

The manner in which benzylpenicillin degrades in acid solution would appear to be extremely sensitive to pH and/or the buffer components present. The relative insensitivity of degradation product composition to temperature, over the narrow range examined, would suggest that the activation energies for bond rearrangement are very similar.

Our studies indicated that the benzylpenillic and benzylpenicilloic acids formed possessed 3S,5R,6R stereochemistry. Hence the rearrangements of benzylpenicillin in acidic solution preserve stereochemistry at all three chiral centres. It is interesting to speculate on whether the 5S stereoisomer of benzylpenicillin would degrade in acid to produce the 5S stereoisomer of benzylpenillic acid.

The author will not attempt futher discussion of the mechanisms involved. An obvious area for further research on this subject would be a more detailed look at the effects of buffer type/concentration and pH upon degradation product composition.

12.5 Degradation of (3S,5R,6R)- and (3S,5R,6S)-benzylpenicillin at pH 12 and 25°C. Results.

12.5.1 Overall rates of degradation of benzylpenicillin and 6-epibenzylpenicillin

Regression analyses of HPLC data on the degradation of (3S,5R,6R)-benzylpenicillin, hereinafter referred to as benzylpenicillin BP, and (3S,5R,6S)-benzylpenicillin, 6-epibenzylpenicillin, showed the overall rates of degradation to be first-order (see Table 65).

Table 65 Regression data and overall degradation rates, K of benzylpenicillin and 6-epi-benzylpenicillin at pH 12 and 25°C

Compound	benzylpenicillin		6-epibenzylpenicillin
Init. conc.M	5.06×10^{-4}	5.07×10^{-4}	ca. 3.8×10^{-4}
Data points	6	6	8
CC	0.9999	0.9999	1.0000
K min^{-1}	0.065 ± 0.0003	0.0650 ± 0.0002	0.0311 ± 0.0001
Intercept I	6.948 ± 0.07	6.949 ± 0.005	6.807 ± 0.002
Antilog I	8.87×10^{6}	8.89×10^{6}	6.42×10^{6}

The overall rate of degradation of benzylpenicillin was about twice that found for 6-epibenzylpenicillin.

12.5.2 HPLC analysis of products formed during the degradation of benzylpenicillin and 6-epibenzylpenicillin at pH 12 and 25°C

The percentage concentrations of benzylpenicillins and benzylpenicilloic acids were calculated in the following manner:

The % concentration of the benzylpenicillins at time t is given by:

$$[BP]_t = \frac{(PA_{BP})_t}{(PA_{BP})_0} \times 100\ \%$$

where $(PA_{BP})_t$ was the HPLC peak area of penicillin at time t and $(PA_{BP})_o$ the HPLC peak area of penicillin at time zero and was equal to the antilog of the intercept calculated from the regression analysis (see Table 65). The percent concentration of all the benzylpenicilloic acids at time t was given by:

$$[\sum BPC]_t = \frac{(\sum PA_{BPC})_t}{(\sum PA_{BPC})_{max}} \times 100\ \%$$

where $(\sum PA_{BPC})_t$ was the sum of the HPLC peak areas of (5R,6R)-, (5S,6R)-, (5R,6S)- and (5S,6S)-benzylpenicilloic acids present at time t and $(\sum PA_{BPC})_{max}$ was the sum of the HPLC peak areas of all the penicilloic acids present when all the penicillins had degraded. The % concentration of each penicilloic acid stereoisomer was given by:

$$[BPC]_t = \frac{(PA_{BPC})_t}{(\sum PA_{BPC})_t} \times [\sum BPC]_t$$

The mass balance MB, at time t, was given by:

$$MB_t = [BP]_t + [\sum BPC]_t$$

and should equal 100 % ± experimental errors.

12.5.3 Degradation of benzylpenicillin

A typical HPLC chromatogram of partially degraded benzylpenicillin is given in Figure 64a. Figure 65a shows the combined HPLC data from the duplicate experiments. The mass balances of the experiments were consistently within 101 ± 1%.

Benzylpenicillin rapidly degraded in alkaline solution to produce primarily (3S,5R,6R)-benzylpenicilloic acid RR-BPC (which in turn epimerised at C-5 to give the 5S,6R epimer SR-BPC) and trace levels of a novel product. Attempts to isolate this product by semi-preparative HPLC were unsuccessful. Examination of the degradation mixture by HPLC

at 230, 254, 280 and 322 nm suggested that the unknown product possessed a benzylpenicillin-type uv chromophore. The compound was identified as 6-epibenzylpenicillin by the following observations.

a) The compound had the same HPLC retention time as an authentic sample of 6-epibenzylpenicillin. No other degradation product examined had a similar retention time.

b) From the ^1H NMR analysis of the degradation mixture (sect. 12.5.5).

Figure 64 Chromatograms, using system 4, of a) benzylpenicillin after 18.1 minutes and 6-epibenzylpenicillin b) fresh and c) after 16.2 mins at pH 12 and 25°C

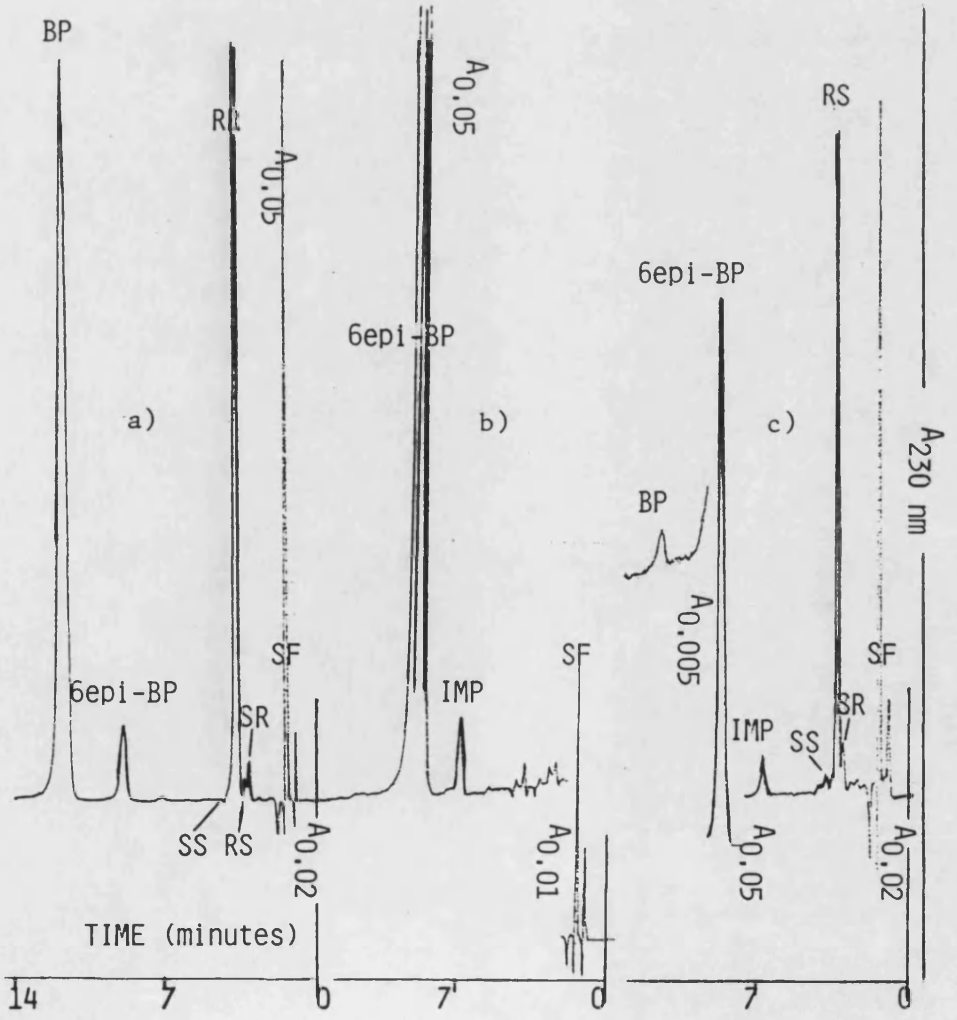
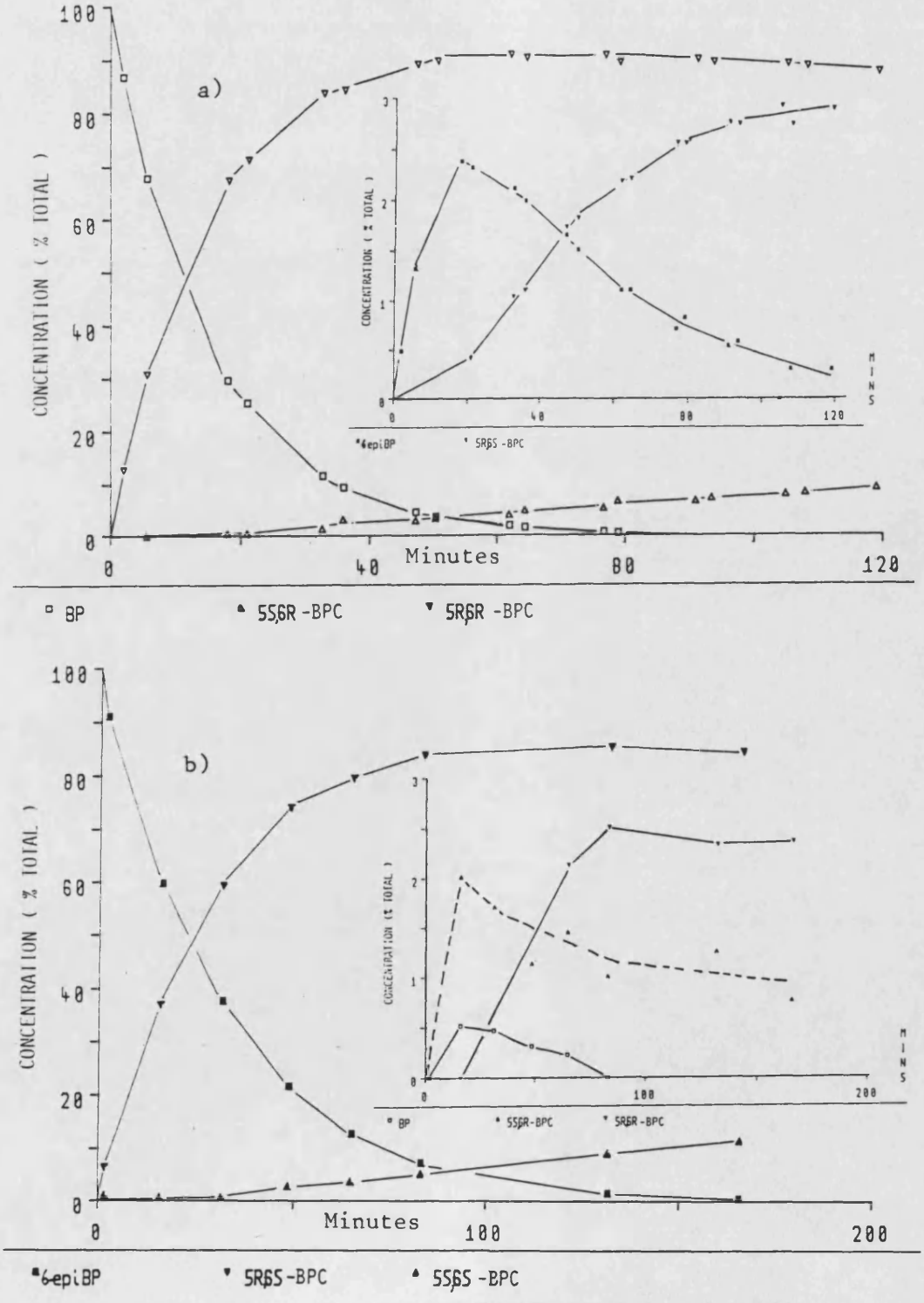


Figure 65 Plot of the concentrations of the degradation products of a) benzylpenicillin (0.507 mM) and b) 6-epibenzylpenicillin (ca. 0.38 mM) at pH 12 & 25°C. Insets show low level components.



The maximum concentration of 6-epibenzylpenicillin observed during the degradation of benzylpenicillin was about 2.5 % of the total.

6-Epibenzylpenicillin slowly degraded to give (3S,5R,6S)-benzylpenicilloic acid RS-BPC which in turn epimerised at C-5 to give the 3S,5S,6S epimer SS-BPC. The mean % composition of the mixture after all the penicillins had disappeared was 10.6 : 2.9 : 86.5 : trace for 5S,6R : 5R,6S : 5R,6R : 5S,6S-benzylpenicilloic acids, respectively.

This indicated that about 3 % benzylpenicillin epimerised at C-6 prior to complete degradation, assuming that the C-6 epimerisation of the benzylpenicilloic acids was very slow.

12.5.4 Degradation of 6-epibenzylpenicillin

A sample of authentic 6-epibenzylpenicillin was dissolved in water and immediately analysed by HPLC. It was found to be ≥ 98 % pure (by peak area normalisation at 230 nm) contaminated with trace amounts of (5R,6R)- and (5R,6S)-benzylpenicilloic acids and an unknown impurity (at ca. 1.2 %). No (3S,5R,6R)-benzylpenicillin was present. The actual chromatogram is reproduced as Figure 64b.

6-Epibenzylpenicillin degraded in alkaline solution at about half the rate observed for benzylpenicillin and produced RS-BPC (which in turn epimerised at C-5 to SS-BPC) and trace quantities of benzylpenicillin. The maximum concentration of benzylpenicillin observed was about 0.5 % of the total. This in turn rapidly degraded to give RR-BPC as discussed previously. The identity of benzylpenicillin was confirmed by the HPLC retention time.

The HPLC data is presented in Figure 65b. The mass balance was consistently within 103 ± 2 %. A typical chromatogram of a partially

degraded sample of 6-epibenzylpenicillin is shown in Figure 64c.

The % composition of the mixture after all the penicillins had disappeared was 0.8 : 85.3 : 2.4 : 11.5 for SR : RS : RR : SS benzylpenicilloic acids, respectively. This indicated that ca. 3.2 % of 6-epibenzylpenicillin epimerised at C-6 prior to complete degradation and that the epimerisation process was reversible.

The HPLC peak area of the unknown impurity remained unchanged throughout the experiment i.e. it was stable to alkali and did not react with the penicillins and penicilloic acids. No further studies with the aim of identifying this impurity were conducted.

12.5.5 HPLC and 400 MHz ^{1}H NMR analyses of the degradation of benzylpenicillin, partially degraded in dilute NaOH/H_2O

The D_2O reconstituted sample of partially degraded benzylpenicillin was analysed by HPLC immediately before and after the ^{1}H NMR spectrum was recorded. The results (Table 66) were in good agreement and indicated that apart from an increase in the % concentration of (5S,6R)-benzylpenicilloic acid, at the expense of the 5R,6R epimer, the composition remained unaltered.

Table 66 Comparison of HPLC and ^{1}H NMR analyses.

compound	concentration (% total)		
	before HPLC	after	^{1}H NMR
Benzylpenicillin	6.3	6.4	6.0
6-Epibenzylpenicillin	1.7	2.0	1.5
(5R,6R)-Benzylpenicilloic acid	84.0	72.8	75.5
(5S,6R)- " " "	6.4	17.7	15.5
(5R,6S)- " " "	1.6	1.1	1.5
(5S,6S)- " " "	trace	trace	not observed

The complete assignment of the 400 MHz ^{1}H NMR spectrum (Figure 66, expansions a - e & Table 67) was made by comparison with the ^{1}H NMR

data of the pure compounds and from the expected signal multiplicity.

Table 67 Assignments of the 400 MHz ^1H NMR spectrum of a partially degraded sample of benzylpenicillin.

Line No.	δ obs. ppm†	J obs. Hz	δ ref. ppm‡	J ref. Hz‡	Assignment	
1	(7.60-7.25)m	-	-		Aromatics	
2	5.49d	3.91	5.49d	3.85	(5R,6R)-Benzylpenicillin	H-5
3	5.41d	3.91	5.42d	3.85	(5R,6R)-Benzylpenicillin	H-6
4	5.23d	1.71	5.24d	1.65	(5R,6S)-Benzylpenicillin	H-5
5	5.18d	4.64	5.13d	~4.6	(5R,6S)-Benzylpenicilloic acid	H-5
6	5.04d	~2.4	4.97d	3.0	(5S,6R)-Benzylpenicilloic acid	H-5
7	5.00d	~6.1	5.04d	5.4	(5R,6R)-Benzylpenicilloic acid	H-5
8	4.83d¶	nd	4.88d	1.65	(5R,6S)-Benzylpenicillin	H-6
9	4.80bs	-	-	-	HDO reference	
10	4.76d	2.93	4.71d	3.0	(5S,6R)-Benzylpenicilloic acid	H-6
11	4.51d	4.64	4.46d	~4.6	(5R,6S)-Benzylpenicilloic acid	H-6
12	4.28s	-	4.30s	-	(5R,6S)-Benzylpenicillin	H-3
13	4.22s	-	4.23s	-	(5R,6R)-Benzylpenicillin	H-3
14	4.19d	~4.2	4.23d	5.4	(5R,6R)-Benzylpenicilloic acid	H-6
15	3.77q	15	3.72s	-	(5S,6R)-Benzylpenicilloic acid	H_2
16	3.67q	15	3.67s	-	(5R,6R)-Benzylpenicilloic acid	H_2
17	(3.7-3.6)	nd	3.67s	-	(5R,6S)-Benzylpenicillin	H_2
	" §	nd	3.64q	15	(5R,6R)-Benzylpenicillin	H_2
18	3.41s	-	3.40s	-	(5R,6R)-Benzylpenicilloic acid	H-3
19	3.39s	-	3.35s	-	(5S,6R)-Benzylpenicilloic acid	H-3
20	3.28s	-	3.23s	-	(5R,6S)-Benzylpenicilloic acid	H-3
21	1.56s	-	1.57s	-	(5R,6R)-Benzylpenicillin	H_3-2β
22	1.54s	-	1.54s	-	(5S,6R)-Benzylpenicilloic acid	"
23	1.53s	-	1.55s	-	(5R,6S)-Benzylpenicillin	"
24	1.48s	-	1.47s	-	(5R,6R)-Benzylpenicilloic acid	"
25	1.47s	-	1.48s	-	(5R,6R)-Benzylpenicillin	H_3-2α
26	1.45s	-	1.47s	-	(5R,6R)-Benzylpenicillin	"
27	1.39s	-	1.35s	-	(5R,6S)-Benzylpenicilloic acid	H_3-2β
28	1.20s	-	1.23s	-	(5R,6R)-Benzylpenicilloic acid	H_3-2α
29	1.18s	-	1.14s	-	(5R,6S)-Benzylpenicilloic acid	"
30	1.17s*	-	"	-	"	"
31	1.01s	-	1.01s	-	(5S,6R)-Benzylpenicilloic acid	"

Key: b = broad resonance, d = doublet, m = multiplet, s = singlet
† Signals referenced to HDO at 4.80 ppm
‡ See Table 11, section 6.
¶ High field component of doublet obscured by HDO
§ Benzyl H_2 signal of (5R,6S)-Benzylpenicilloic acid obscured
* Either line 29 or 30 = (5R,6S)-Benzylpenicilloic acid H_3-2α

Signals for (5S,6S)-Benzylpenicilloic acid not observed.

Figure 66 400 MHz ^1H NMR spectrum of benzylpenicillin degraded at pH 12 and 25°C. See Table 67 for key.

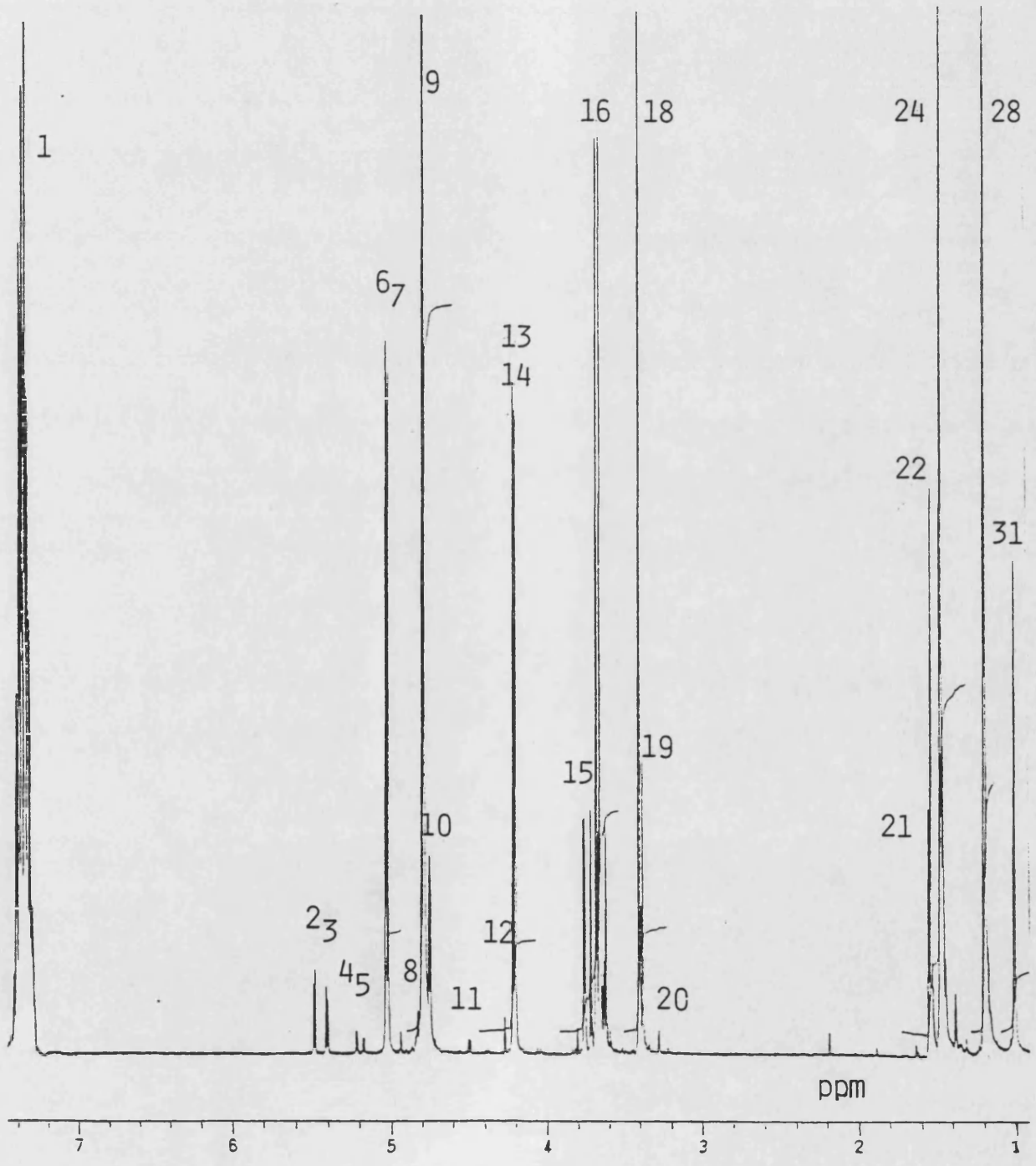


Figure 66a to e. Expansions of spectrum in figure 66.

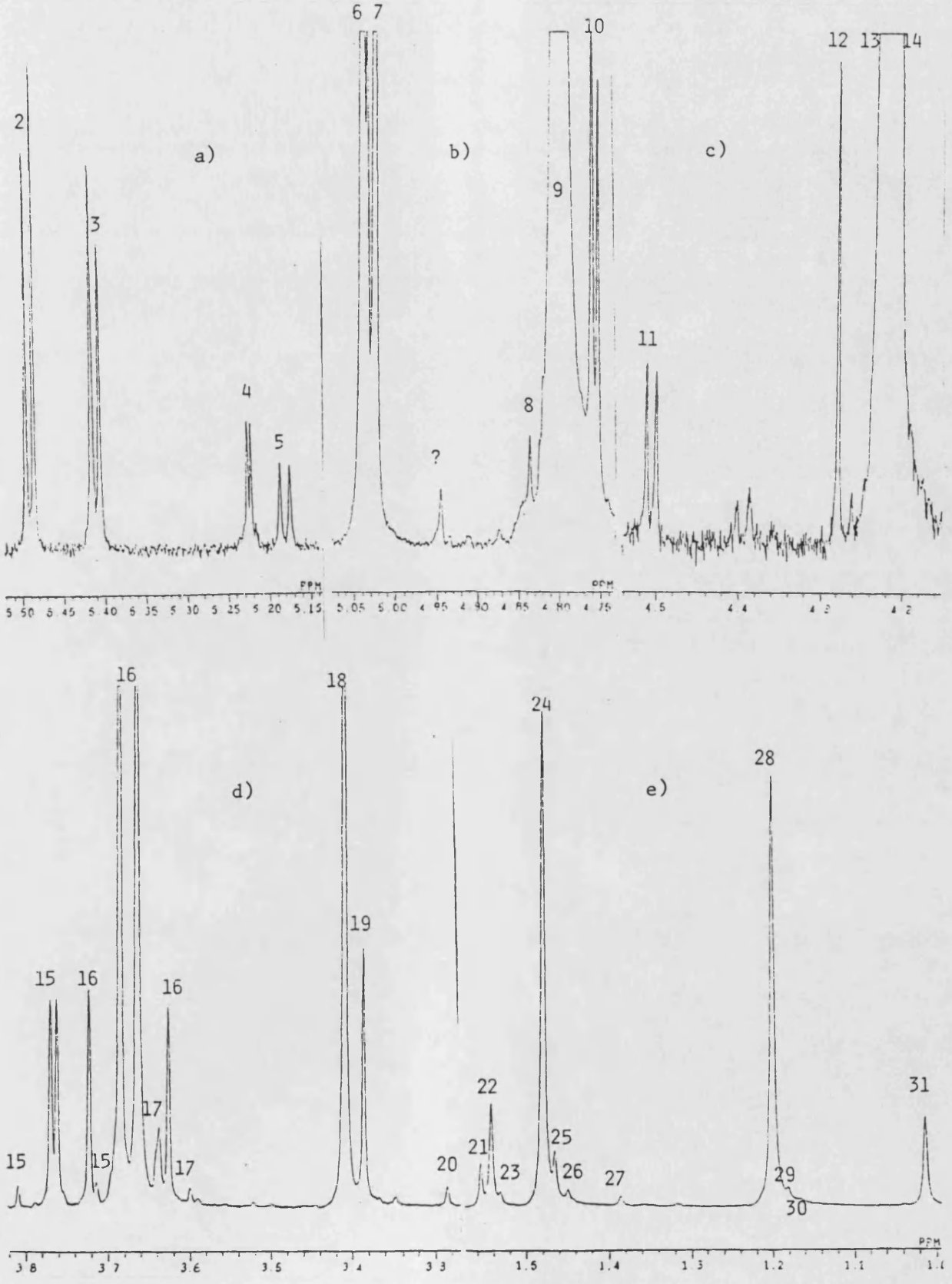


Figure 66a clearly shows that the H-5 and H-6 doublets of benzylpenicillin with the vicinal coupling constant of 3.91 Hz, the H-5 doublet (separation 1.71 Hz) of 6-epibenzylpenicillin and the H-5 doublet (4.64 Hz) of (5R,6S)-benzylpenicilloic acid. The composition of the sample was determined by semi-quantitative analysis of the 2α and 2β-methyl resonances (Fig.66e) in the manner described previously in section 12.4.5.

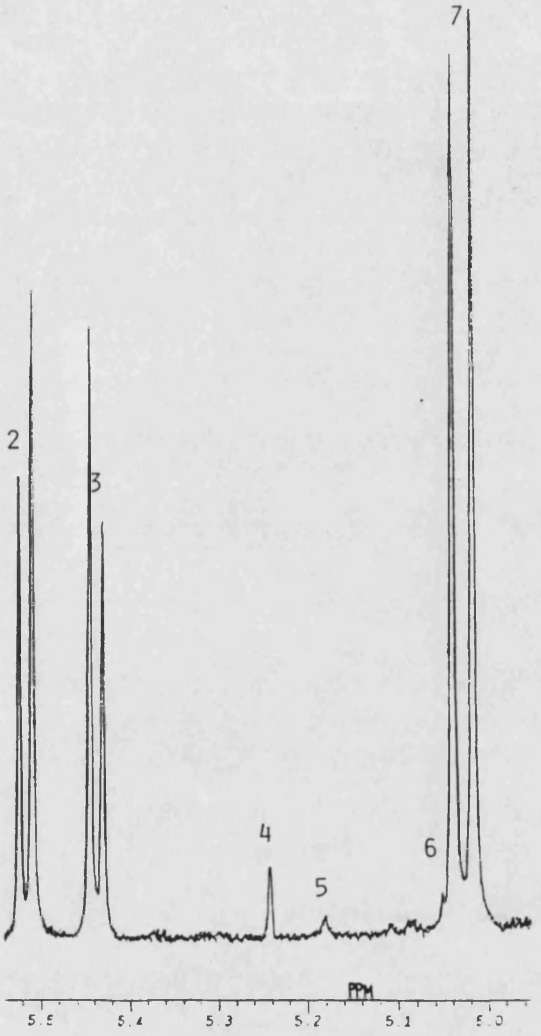
No (5S,6S)-benzylpenicilloic acid was observed in the NMR spectrum and the high-field component of the H-6 doublet of 6-epibenzylpenicillin was obscured by the HDO signal. Attempts to eliminate the solvent signal using a homogated pulse sequence were unsuccessful.

12.5.6 270 MHz ^{1}H NMR analysis of benzylpenicillin, partially degraded in dilute NaOD/D_2O

Semi-quantitative analysis of the 2α and β-methyl resonances gave the composition of the sample as: benzylpenicillin 34.5 %, 6-epibenzylpenicillin 1.5%, (5S,6R)-, (5S,6R)-, (5R,6S) -benzylpenicilloic acids as 62.5%, 1.5% and trace, respectively. No (5S,6S)-benzylpenicilloic acid was observed.

Examination of part of the expanded spectrum (Figure 67) showed that the H-5 resonances of 6-epibenzylpenicillin and (5R,6S)-benzylpenicilloic acid had collapsed to singlets and the H-6 signals were absent. This implied that the C-6 epimerisation of benzylpenicillin in D_2O solution occurred with deuterium incorporation at C-6.

Figure 67 Expansion of 270 MHz NMR spectrum of benzylpenicillin partially degraded in $NaOD/D_2O$



12.5.7 400 MHz ^1H NMR analysis of the benzylpenicilloic acids

The sample of benzylpenicillin (degraded in dilute $NaOH/H_2O$ lyophilised and reconstituted in D_2O) was analysed by HPLC immediately prior to analysis by NMR. With the aid of the NMR data described previously (Table 67) and knowing the relative proportions of the four benzylpenicilloic acid epimers the ^1H NMR characteristics of the 5S,6S-epimer could be determined by elimination, see Table 68. Again the HPLC and NMR results were in good agreement (Table 69).

Table 68 400MHz ^{1}H NMR characteristics of (5S,6S)-benzylpenicilloic acid

δ obs. ppm†	J obs. Hz	δ ref. ppm‡	J ref. Hz‡	assignment
(7.60-7.28)m	-	-	-	aromatics
4.87d	4.4	4.89d	nd	H-5
4.56d	4.4	4.52d	nd	H-6
(3.69-3.67)	nd	nd	nd	H_2 benzylic
3.35s	-	3.32s	-	H-3
1.56s	-	1.53s	-	H_3-2β
1.16s	-	1.13s	-	H_3-2α

Notes: nd = not determined, s = singlet, d = doublet, m = mutiplet

† signals referenced to H_3-2α resonance of (5S,6R)-BPC at 1.01ppm (actually 1.07ppm at HDO 4.80ppm).

‡ see Table 11 in NMR section 6.

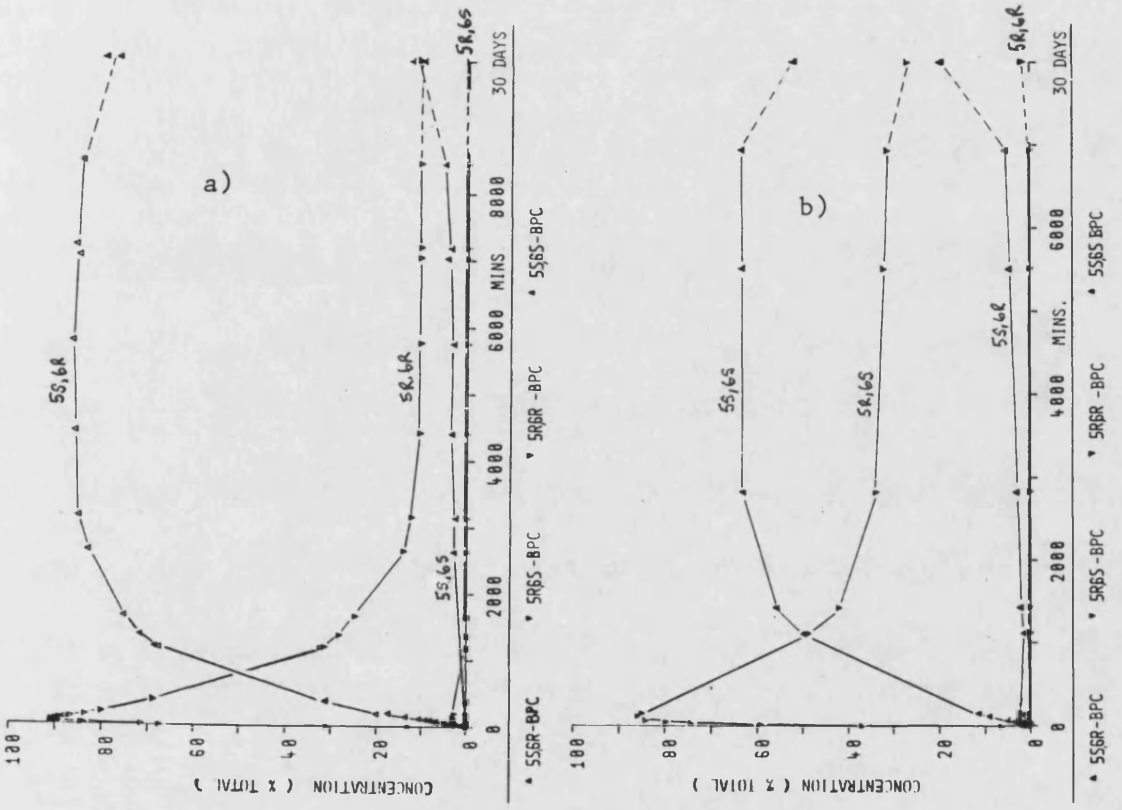
Table 69 Comparison of HPLC and 400MHz ^{1}H NMR analyses.

Compound	Concentrations (% total) HPLC	NMR
(5R,6R)-benzylpenicilloic acid	10	9.5
(5S,6R)-benzylpenicilloic acid	80	78.5
(5R,6S)-benzylpenicilloic acid	2.5	4
(5S,6S)-benzylpenicilloic acid	6.5	8

12.5.8 Epimerisation at C-5 and C-6 of benzylpenicilloic acid at pH 12 and 25°C

The HPLC analyses of the benzylpenicilloic acids produced during base hydrolysis of benzylpenicillin and 6-epibenzylpenicillin were continued for 30 days after all the penicillins had degraded. The changes in concentration with time of the four benzylpenicilloic acids stereoisomers are shown in Figures 68a and b and the chromatograms obtained after thirty days at pH 12 and 25°C in Figures 69a and b.

Figure 68 Plots of the concentrations of the benzylpenicilloic acids derived from a) benzylpenicillin & b) 6-epibenzylpenicillin at pH 12 and 25°C, measured with time.



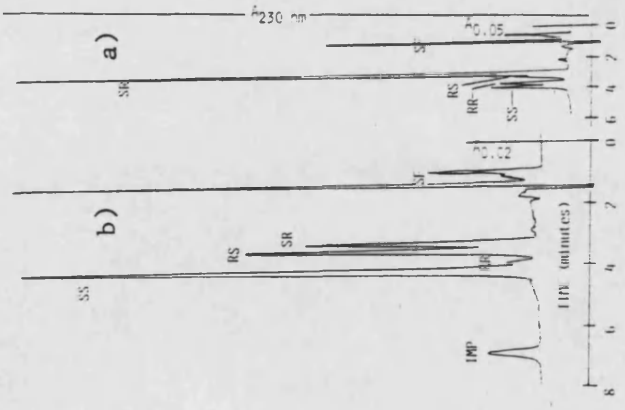


Figure 69 Chromatograms of benzylpenicilloic acids from a) benzylpenicillins and b) 6-epibenzylpenicillin, after 30 days at pH 12 & 25°C.

From the results the following points may be made:

(a) Benzylpenicilloic acids were very stable in dilute alkali at room temperature, no degradation products were observed.

(b) Epimerisation at C-5 occurred relatively rapidly. After 5 days the 5S,6R : 5R,6R epimer ratio became constant at 89:11, the 5R,6S : 5S,6S epimer ratio was 33:67. In both cases the 5S-epimers are thermodynamically favoured.

The kinetic transformations of the 5R,6R/S-penicilloic acids to the 5S,6R/S-epimers were determined from the HPLC data, presented in Figures 68a and b, in a similar fashion to that described in section 8.2.4. This method assumed that the reaction rates were first-order and that epimerisation at C-6 during the first 5 days of the experiments was negligible. The results are summarised in Table 70.

Table 70 Results for the kinetic transformations of a) 5R,6R to 5S,6R- and b) 5R,6S to 5S,6S-benzylpenicilloic acid at pH 12 & 25°C.

Equilibrium ratio	K †	Equil. time	N	CC‡	(kf + kr) min^{-1}	kf min^{-1}	kr min^{-1}
				(a)			
89;11 5S,6R;5R,6R	8,1 ± 0,1	~5 days	16	0,9982	1,22 ± 0,01 x 10^{-5}	1,08 ± 0,01 x 10^{-3}	1,33 ± 0,02 x 10^{-4}
89;11 5S,6R;5R,6R	8,1 ± 0,1	~5 days	12	0,9992	1,27 ± 0,01 x 10^{-5}	1,13 ± 0,01 x 10^{-3}	1,40 ± 0,02 x 10^{-4}
89;11 5S,6R;5R,6R	8,1 ± 0,1	5 days	- MEAN -		1,25 ± 0,03 x 10^{-5}	1,11 ± 0,03 x 10^{-3}	1,37 ± 0,04 x 10^{-4}
				(b)			
33;67 5R,6S;5S,6S	2,0 ± 0,1	~5 days	12	0,9994	1,27 ± 0,04 x 10^{-3}	8,46 ± 0,01 x 10^{-4}	4,23 ± 0,02 x 10^{-4}

Notes; † K = kf/kr = Seq/Req, ‡ Correlation coefficient for plot of $\log((Ro - Req)/(Rt - Req)) = (kf + kr)t/2,303$

where; K =equilibrium constant, kf and kr = forward & reverse rate constants
Ro=initial concentration of 5R,6R- (or 5R,6S-) benzylpenicilloic acid
Req=equilibrium " " " " "
Seq= " " " 5S,6R- (or 5S,6S-) "
and Rt=concentration of 5R,6R- (or 5R,6S-) benzylpenicilloic acid at time t

The kinetic transformations of 5R,6R- to 5S,6R-benzylpenicilloic acid in alkaline media (exact conditions not apparent) have been determined previously [73] as kf = 1.23 x 10^{-3}, kr = 3.00 x 10^{-4} min^{-1} and hence K = 4.1.

(c) Epimerisation at C-6 occurred relatively slowly. The composition of the benzylpenicilloic acid mixtures after all the penicillins had degraded and after 30 days are given in Table 71

Table 71 Percentage Concentrations of the four (3S)-benzylpenicilloic acid stereoisomers at pH12 and 25°C

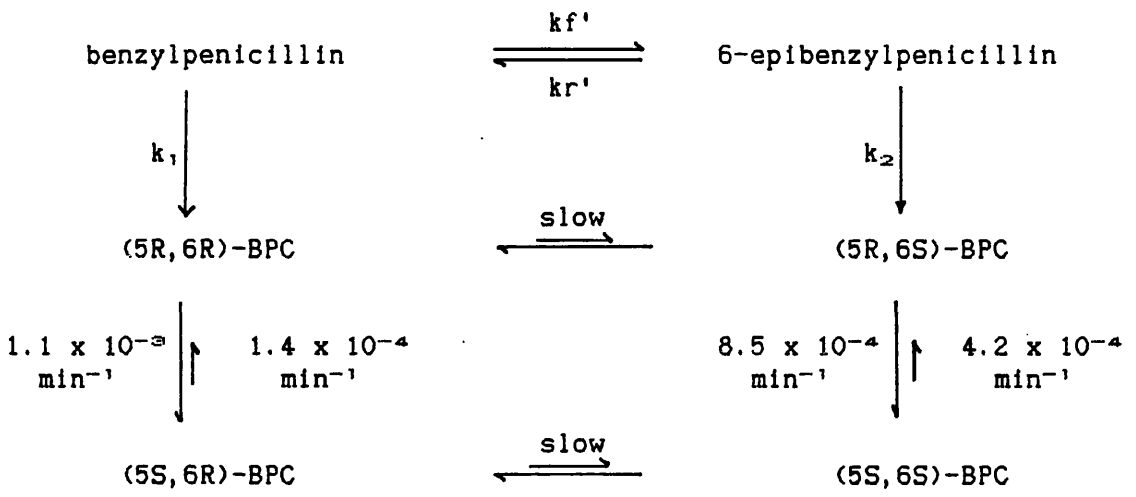
Parent penicillin	Time	5S,6R	5R,6S	5R,6R	5S,6S
3S,5R,6R	132 mins.	10.6	2.9	86.5	trace
"	30 days	78.1±1.6	trace	10.5±0.2	11.4±1.4
3S,5R,6S	166 mins.	0.8	85.3	2.4	11.5
"	30 days	19.6±0.2	26.7	1.8±0.2	52.0±0.3

The proportion of the 6S-benzylpenicilloic acid epimers derived from benzylpenicillin had increased from ca. 3 % to 11.4 % after 30 days. The proportion of the 6S-benzylpenicilloic acid epimers derived from 6-epibenzylpenicillin had increased from ca. 3 % to 21.4 %.

As there was no evidence that the system had attained equilibrium, a kinetic analysis of the results was not possible, however the results suggested that the 6R-benzylpenicilloic acid epimers are thermodynamically favoured.

12.6 Discussion of the overall scheme for the degradation of benzylpenicillin at pH 12 and 25°C.

From the results described above an overall scheme (Scheme 22) for the degradation of benzylpenicillin at pH 12 & 25°C may be proposed:



BPC = benzylpenicilloic acid.

Scheme 22 Kinetic scheme for the degradation of benzylpenicillin at pH 12 and 25°C.

For reasons described previously, the forward and reverse rates controlling the C-6 epimerisation of benzylpenicilloic acid could not be estimated. Estimates of kf' and kr' were made in the following manner:

For example, the differential equation for the change in concentration of benzylpenicillin BP, assuming all rates follow first-order kinetics, is;

$$\partial[BP]/\partial t = kr'[6\text{-epiBP}] - (kf' + k_1)[BP] \qquad 1$$

however the concentration of 6-epibenzylpenicillin [6-epiBP] was relatively very small and hence equation 1 reduces to:

$$\partial[BP]/\partial t = -(kf' + k_1)[BP] \qquad 2$$

i.e. the system can be considered as simple parallel first-order, the overall rate of degradation of benzylpenicillin is equal to $kf' + k_1$ and $kf' : k_1 = [6\text{-epiBP}] : [5R,6R\text{-BPC}]$.

However, due to the rapid hydrolysis of 6-epibenzylpenicillin and the C-5 epimerisation of benzylpenicilloic acids, $kf' : k_1$ was estimated, (assuming that the epimerisation at C-6 of the penicilloic acids was minimal during the short time taken to hydrolyse all the penicillins) as:

$$\frac{kf'}{k_1} = \frac{([5R,6S\text{-BPC}] + [5S,6S\text{-BPC}])}{([5R,6R\text{-BPC}] + [5S,6R\text{-BPC}])}$$

From Table 71; $kf' / k_1 = 2.9 / 87.1$

and from Table 65 $(k_1 + kf') = 6.50 \times 10^{-2}$ min^{-1}

Therefore: $k_1 = 6.3 \times 10^{-2}$ min^{-1} and $kf' = 2.2 \times 10^{-3}$ min^{-1}

The degradation of 6-epibenzylpenicillin was treated similarly:

From Table 71 $kr' / k_2 = 3.2 / 86.8$

and from Table 65 $(k_2 + kr') = 3.1 \times 10^{-2}$ min^{-1}

Therefore: $k_2 = 3.0 \times 10^{-2}$ min^{-1} and $kr' = 1.1 \times 10^{-3}$ min^{-1}

From the calculated values of kf' and kr' one can say that the epimerisation at C-6 of intact benzylpenicillin thermodynamically favours the 6S-epimer. Kinetic analysis of the complete system without making the approximations mentioned was beyond the scope of the thesis

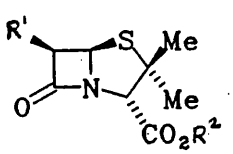
The penam nucleus contains acidic protons at positions 3, 5 and 6. Deprotonation-reprotonation processes may therefore result in a change of configuration at these sites. Although there is evidence that the H-3 atom can be removed under basic conditions, the derived anion undergoes β-elimination more rapidly than reprotonation. [34]

5-epibenzylpenicillin methyl and benzyl esters have been obtained [162] by replacement of the phthalimido side chain of the corresponding 5-epiphthalimidopenicillins which were prepared by the method of Kukolja [187] i.e. chlorinolysis of the S-C5 bond of penicillin followed

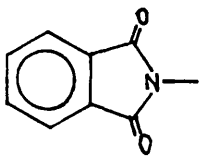
by recyclisation with $SnCl_2$

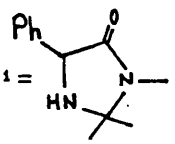
From our studies, there was no evidence to suggest that the H-5 atom of benzylpenicillin was sufficiently acidic to be removed in the mild conditions employed (i.e. pH 12 and 25°C).

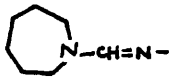
Deprotonation-reprotonation may occur at position 6 resulting in epimerisation at this centre and was first reported for methyl-6-phthalimidopenicillanate ester **67ax** [188] and subsequently in hetacillin **67by** [189] and mecillinam **67cy**.[190]



67

a R^1 = 

b R^1 = 

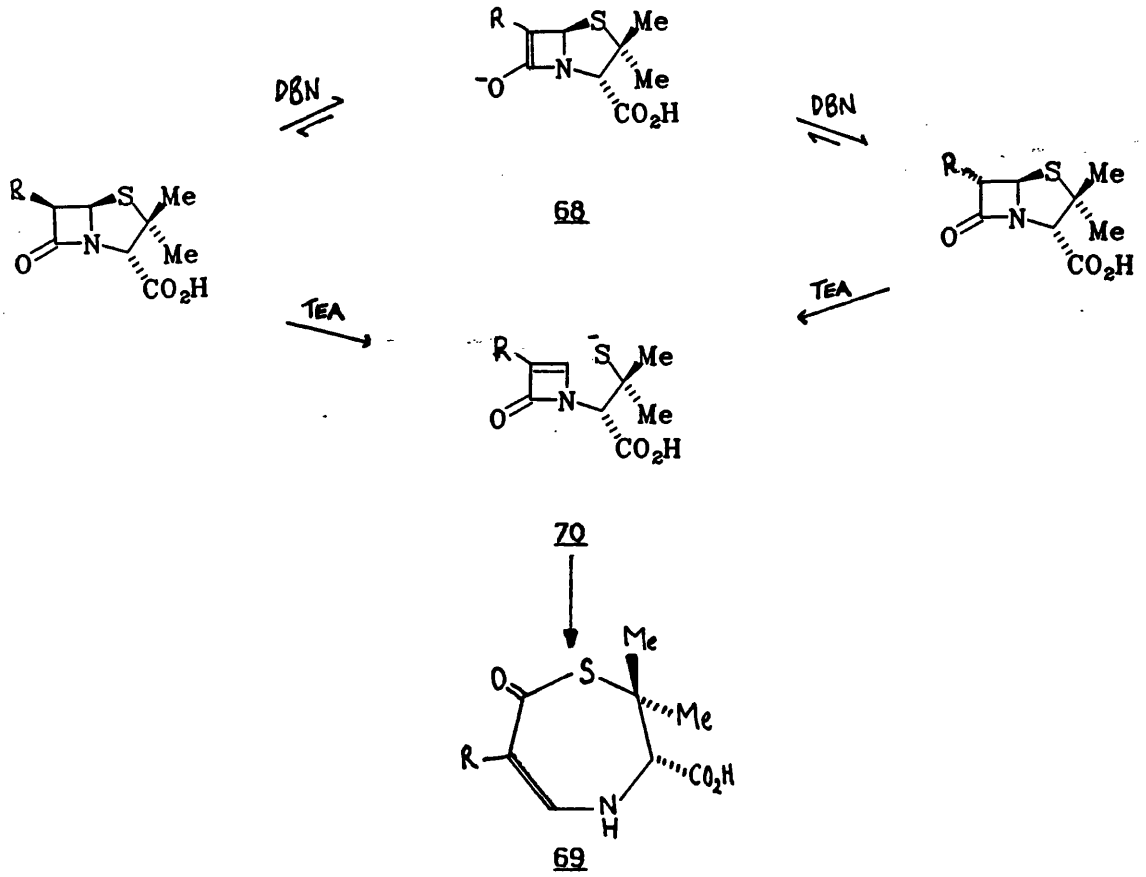
c R^1 = 

x R^2 = CH_3- **y** R^2 = H-

It is now clear that when a bulky group is present at position 6, epimerisation occurs readily in mild basic conditions and there is an overwhelming thermodynamic preference for the 6S-epimer. The higher free energy of the 6R-substituent has been ascribed [34] to a compressional interaction between the 6-substituent and the 2β-methyl group. A reduction in the bulkiness of the 6-substituent increases the equilibrium concentration of the 6R-epimer and as much as 47 % of the 6R-epimer can be present at equilibrium.[34]

Base-catalysed epimerisation at position 6 of benzylpenicillin has not been previously reported, the failure being ascribed [191] to the proximity of the negative charge of the secondary amide function (resulting from removal of the amide proton by base) preventing the loss of a second proton at position 6.

The epimerisation at position 6 of benzylpenicillin with protected secondary amide side-chain has been reported.[81 192] The method is based on the N-silyation of the amide group with N,O-bis(trimethylsilyl)acetamide BSA followed by treatment with the organic base 1,5-diazabicyclo[4.3.0]-non-5-ene DBN to give a 1:3 ratio of normal to 6-epibenzylpenicillin, via a proposed intermediate enolate 68. When a weaker base, triethylamine TEA, was used a 1,4-thiazepine 69 was obtained via a proposed azetinonethiolate intermediate 70. If 70 was the intermediate during epimerisation at position 6, reprotonation must exclusively regenerate the original configuration at position 5.



It is likely that the rate determining step in either route is the formation of 68 which undergoes competitive protonation i.e. epimerisation at position 6, or β-elimination to give 70.

Our results indicated that in dilute aqueous alkaline solution both benzylpenicillins and 6-epibenzylpenicillins were in reversible equilibrium, that the epimerisation process involved deuterium incorporation /exchange at position 6 and the 6S:6R epimer equilibrium ratio was ca. 2:1. However as the rates of base hydrolysis were ca. 30x fold greater than the rates of epimerisation, the epimerisation process had been previously overlooked. It follows that the ionisation of the secondary amide group on the side chain of benzylpenicillin does not prevent the loss of a proton at C-6.

It was the development and use of HPLC systems capable of the separation of the four benzylpenicilloic acid stereoisomers that first hinted that C-6 epimerisation of benzylpenicillin had occured prior to base hydrolysis.

APPENDIX I Manufacturers Certificate of Analysis for benzylpenicillin (Sodium Penicillin G).

Telephone 01 493 4060
Telex 25456
Cables Glaxogroup London W1

Protocol/Certificate of Analysis

SODIUM PENICILLIN G (STERILE)

Batch no.	URH00038755
Date of manufacture	July, 1983
Expiration date	July, 1988
Appearance	Fine white powder
Identity	Complies with the tests
Clarity and colour of solution	Clear colourless solution substantially free from extraneous matter
pH (10% w/v aqueous solution)	7.1
Light Absorption at 280nm A(0.180%, 1cm) at 264nm	0.008 0.840
Specific optical rotation $(a)_D^{20}$ (2% w/v solution)	+302°
Loss at 105° C - % w/w	0.2
Total penicillins as Sodium Penicillin G - % w/w	100
Sterility	Sterile
Pyrogens	Complies with test

C.O.

Control No.

Customer Order No.

Date 26.5.87

RH 3

Signature .....

M. D. Breese, M.I.Biol.
Position Qualified Person
Location Barnard Castle

APPENDIX II Calibration of Perkin-Elmer 550S uv spectrophotometer

Sample: 0.006 % w/v aqueous potassium dichromate

Reference: distilled-deionised water

1 cm quartz cuvettes, temperature 22.5°C

Results

Literature values [170]		Experimental values*		% difference
λ max	A^1_1	λ max	A^1_1	
257 nm	145.2	255 nm	144.8	- 0.3%
350 nm	106.7	348 nm	107.3	+ 0.6%
λ min.		λ min.		
235 nm	124.8	232 nm	125.5	+ 0.6%
313 nm	48.7	311 nm	48.3	- 0.8%

* Mean of two experiments

A consistent wavelength error of - 2 nm was observed. All wavelength data noted in the thesis were corrected for this error.

APPENDIX III. First-order reactions

$$A \xrightarrow{K} B$$

The rate of a first-order reaction is proportional to the first power of the concentration of only one reactant i.e. the amount $\partial[A]$, which undergoes chemical change in the short time interval ∂t, depends only on the amount of A present at that instant, assuming that there is no change in any other factor (i.e. volume, temperature, pH, ionic strength etc) that may affect the reaction. The rate expression which describes a first-order reaction is:

$$-\frac{\partial[A]}{\partial t} = K[A] \qquad (1)$$

Equation (1) can be rearranged and upon integrating between limits (with the resultant elimination of the integration constant I) equation (5) is obtained i.e.:

$$-\int_{[A]_o}^{[A]} \partial[A]/[A] = K \int_{t_1}^{t_2} \partial t \qquad (2)$$

$$= \quad (-\ln [A] = Kt_2 + I) - (-\ln [A]_o = Kt_1 + I) \qquad (3)$$

$$\Rightarrow \quad -\ln [A] + \ln [A]_o = K\,(t_2 - t_1) \qquad (4)$$

$$\Rightarrow \quad \ln [A] = \ln [A]_o - K\,(t_2 - t_1) \qquad (5)$$

The exponential form of this equation is:

$$[A] = [A]_o\, e^{-K\,(t_2 - t_1)} \qquad (6)$$

Converting (5) to the decadic logarithm, and if $t_1 = 0$ and $[A]_o$ is the initial concentration, equation (7) is obtained:

$$\log [A] = \log [A]_o - \frac{Kt}{2.303} \qquad (7)$$

and in exponential form:

$$[A] = [A]_o\, 10^{-Kt/2.303} \qquad (8)$$

The average rate constant K for the reaction is obtained by plotting log [A] against time t. The linear expression in equation (7) shows that the slope of the line is log $[A]_o$ and the antilog of the intercept equal to the initial amount of A.

A straight line indicates that the reaction is first-order with respect to A. Equation (7) can be rearranged to

$$K = \frac{2.303}{t} \log\frac{[A]_o}{[A]} \qquad (9)$$

$$\Rightarrow \quad t = \frac{2.303}{K} \log\frac{[A]_o}{[A]} \qquad (10)$$

The period of time required for A to decompose to one half the initial concentration $[A]_o$ is the half-life t½ therefore:

$$t_{½} = \frac{2.303}{K} \log \frac{[A]_o}{½[A]_o} \qquad (11)$$

$$t_{½} = \frac{2.303}{K} \log 2 \qquad (12)$$

$$\Rightarrow \quad t_{½} = \frac{0.693}{K} \qquad (13)$$

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