Mechanistic Studies on Isopenicillin N Synthase

A Thesis submitted to the Board of the
Faculty of Physical Sciences
in partial fulfilment of the requirements for
the degree of

Doctor of Philosophy

in the

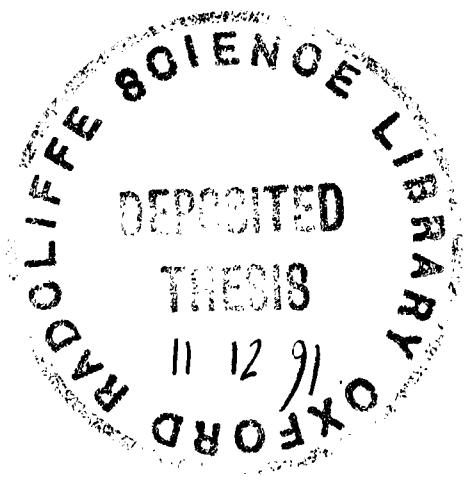
University of Oxford

by

Shaun Douglas Abbott

Wolfson College,
Oxford.

Dyson Perrins Laboratory,
Trinity Term 1991



ABSTRACT

Mechanistic Studies on Isopenicillin N Synthase

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The mechanism of penicillin formation through the action of the enzyme isopenicillin N synthase (IPNS) on the substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine and synthetic analogues, still raises many unresolved questions, despite many years' extensive investigation.

In order to investigate the stereochemistry of formation of the second ring in the product, *N*-δ-(L-α-aminoadipoyl)-3α-hydroxymethylpenicillin, resulting from incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine with IPNS, the deuterium labelled tripeptides δ-(L-α-aminoadipoyl)-L-cysteinyl-*E*-[3,4-$^2H_2$]-D-vinylglycine and δ-(L-α-aminoadipoyl)-L-cysteinyl-*Z*-[3,4-$^2H_2$]-D-vinylglycine were synthesised and incubated with recombinant IPNS. The pattern of deuterium labelling was retained in the penam products, indicating stereospecific ring closure. The unlabelled and labelled penicillins were converted to their corresponding γ-lactones, and the ^1H NMR coupling constants for the C(3)H–C(8)H_2 system used to assign the 8-Pro*R* and 8-Pro*S* resonances of the unlabelled lactone. Comparison with the labelled lactones indicated that the parent penicillins had been formed *via syn* addition of sulphur and oxygen across the double bonds of the respective tripeptides. This stereochemistry is consistent with a mechanism involving [$2\pi_s + 2\pi$] cycloaddition of an Fe=O species to the olefin.

^1H NMR spectroscopy was used to investigate the effect of addition of IPNS on the magnetic susceptibilities of solutions containing dioxygen, by observing the shift in frequency of resonances, due to the paramagnetism of dioxygen. A decrease in bulk magnetic susceptibilities of these solutions, by an amount proportional to the quantity of added IPNS, was observed. This provides supporting evidence for the binding of dioxygen to IPNS, in the absence of the substrate, to give a spin silent species.

Incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine with IPNS over a series of temperatures showed increasing bias towards the monooxygenase over the desaturase pathway for second ring closure with increasing temperature. Product ratios indicate a difference in activation energies between these two pathways of the order of 30kJ.mol^{-1}.

The use of cyanogen bromide to cleave recombinant IPNS (from *Cephalosporium acremonium*), has led to the identification of the fragment alanine-260 to threonine-338, containing histidine-261, which has been implicated as a key residue in substrate-mediated enzyme auto-inactivation.

To:

The Great Author
of all science

Everything that happens in this world happens at the time God chooses.
He sets the time for birth and the time for death,
The time for planting and the time for pulling up,
The time for killing and the time for healing,
The time for tearing down and the time for building.
He sets the time for mourning and the time for dancing,
The time for making love and the time for not making love,
The time for kissing and the time for not kissing.
He sets the time for finding and the time for losing,
The time for saving and the time for throwing away,
The time for tearing and the time for mending,
The time for silence and the time for talk.
He sets the time for love and the time for hate,
The time for war and the time for peace.

Ecclesiastes 3; 1-8

ACKNOWLEDGEMENTS

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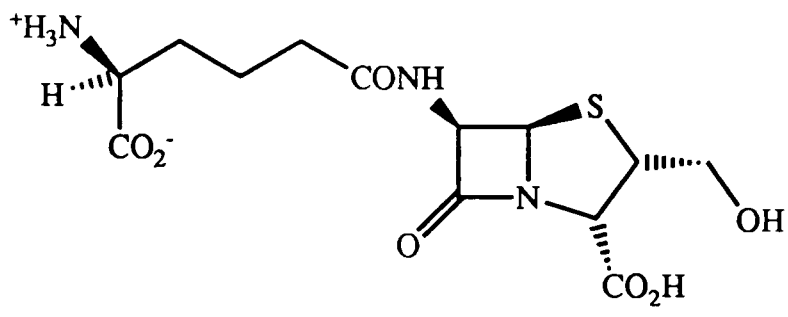
LIST OF ABBREVIATIONS

[A]	Represents the concentration of species A
L-α-AA	δ-(L-α-Aminoadipoyl)-
LL-AC	δ-(L-α-Aminoadipoyl)-L-cysteinyl-
LL-AC-D-Ag	δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-allylglycine
LLD-ACV	δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine
BOC-	*tert*-Butoxycarbonyl-
BOC-ON	2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile
DAC	Deacetylcephalosporin C
DAOC	Deacetoxycephalosporin C
DEPT	Distortionless Enhancement by Polarisation Transfer
DMF	*N*,*N*-Dimethylformamide
DSS	3-(Trimethylsilyl)-1-propanesulphonic acid, sodium salt [or 1,2-Dimethyl-2-Silapentane-5-Sulphonic acid, sodium salt]
DTT	Dithiothreitol
EEDQ	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
FID	Free induction decay
FMOC	(9-Fluorenylmethyl)oxycarbonyl
FPLC	Fast protein liquid chromatography
FT	Fourier transform
h	Hour(s)
HPLC	High performance liquid chromatography
IPN	Isopenicillin N
IPNS	Isopenicillin N synthase
IU	International unit

NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
ODS	Octadecasilyl coated silica (SiO_2)
PLC	Preparative thin layer chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate, polyacrylamide gel electrophoresis
TEMED	*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
tlc	Thin layer chromatography
TMS-	Trimethylsilyl-
TRIS	Tris(hydroxymethyl)aminomethane

All other unit abbreviations use SI standards

The name '3α-hydroxymethylpenicillin' is used to denote the structure [2*S*,3*S*,5*R*,6*R*]–6–[5–amino–5–carboxypentanamido]–3–hydroxymethyl–7-oxo-1-aza-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid.

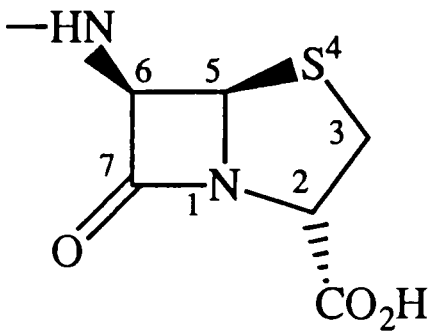


AMINO ACID NOMENCLATURE

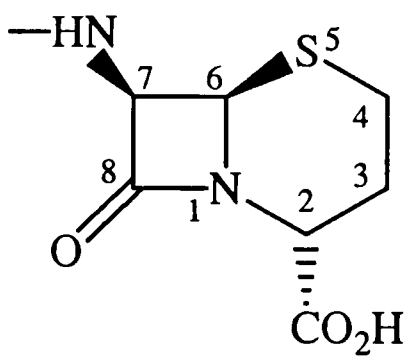
Amino Acid	Abbreviation	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenyl-alanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The homologue of an amino acid is shown by the prefix 'h', e.g. hSer indicates homoserine

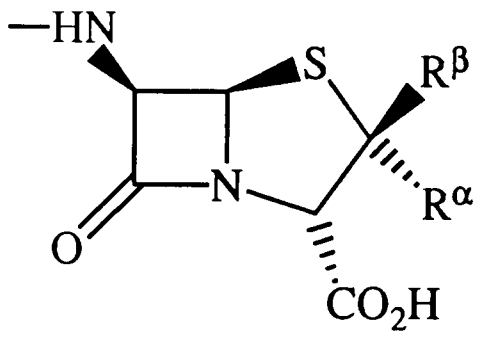
NOMENCLATURE



Penam



Cepham



β-Substituent

α-Substituent

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CHAPTER 1

BIOSYNTHESIS OF THE β-LACTAM RING:- THE HEART OF THE PENICILLIN AND CEPHALOSPORIN ANTIBIOTICS

1. BIOSYNTHESIS OF THE β-LACTAM RING:- THE HEART OF THE PENICILLIN AND CEPHALOSPORIN ANTIBIOTICS

1.1 Introduction

The aim of this chapter is to 'set the scene' for the research projects reported in this thesis. We shall start by looking at the fascinating historical story of the penicillin and cephalosporin antibiotics, followed by a brief look at the biosynthetic pathways involved in the production of these metabolites, before focusing on a single, but crucial, enzyme involved in these biosynthetic processes, namely isopenicillin N synthase (IPNS). Through a brief survey of key insights into the catalytic activity of IPNS, gained principally from the incubation of substrate analogues, it should be possible to place the work presented in this thesis into context.

1.2 The Beauty of Biosynthesis

It is no understatement to say that biosynthetic processes far surpass human efforts at organic synthesis. If we envisaged carrying out all our organic chemistry transformations in a predominantly aqueous environment, within a temperature range of a few dozen Kelvin, then any organic chemist would begin to consider the task to be beyond them. If, furthermore, we specified the necessity of a high selectivity between a range of possible starting materials, and then demanded high efficiency with a high degree of regio-,

stereo- and enantiocontrol, then even the world's foremost synthetic chemists would be left with a feeling of inadequacy.

Nevertheless, within these demanding restrictions, biological systems have developed just such organic transformations, the range and complexity of which can only leave us with a feeling of humility, as the feebleness of our best human efforts is highlighted.

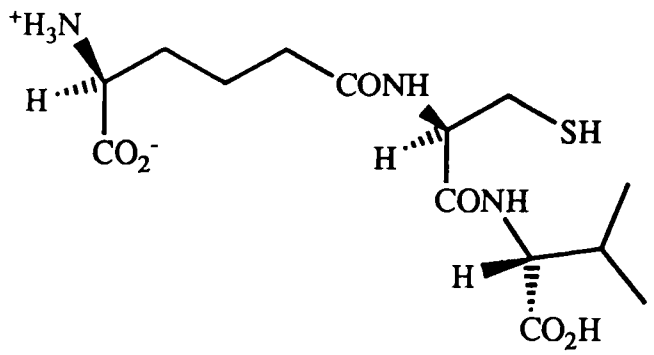
The key to biological synthesis lies in enzymic catalysis. For each step in a biosynthetic pathway there will usually be a specific enzyme which will act to lower the activation energy specifically for the one desired reaction pathway of a particular substrate. This molecular recognition, together with the provision of an environment within the enzyme active site which is far removed from the environment in 'free solution' (i.e. meaning away from the enzyme), are what serve to make enzymes such superlative catalysts, often controlling reactions which could not occur in free solution.

For these reasons it is with a sense of challenge and excitement that we come to the study of any biosynthetic pathway, and it is with that same sense of wonder as a child inspecting a lovingly and intricately created gift from its parent, that we attempt to unravel the secrets of the mechanism involved.

This work examines aspects of the mechanism of catalysis of one enzyme, isopenicillin N synthase (IPNS), involved in a single, yet fundamental step of one biosynthetic pathway of secondary metabolism; that of the cyclisation of the tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (LLD-ACV) (**1**) to the bicyclic system isopenicillin N (IPN) (**2**) (Scheme 1.1),[1] a vital step in the biosynthetic pathway to the penicillin and cephalosporin antibiotics.

(**1**)

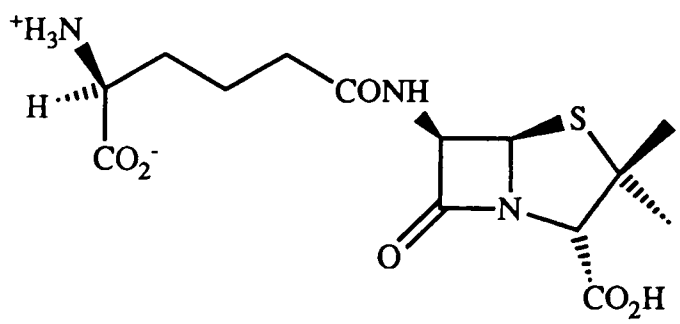
δ-L-α-Aminoadipoyl-
L-cysteinyl-D-valine



ISOPENICILLIN N
SYNTHASE/
O_2/cofactors

(**2**)

Isopenicillin N



SCHEME 1.1

1.3 The History of the Penicillin and Cephalosporin Antibiotics

1.3.1 Penicillins

The importance of penicillins and cephalosporins in nature arises from their rôle in providing their parent organisms with an advantage over competing micro-organisms in their environmental niche. Secreted into the environment, they are toxic to competing bacteria, and it is this antibacterial activity which attracts human interest. Nowadays we may well take our prescription of antibiotics from the doctor for granted, but it is sobering to consider that before the advent of antibiotics *ca.* 40% of all fatalities in the 25 to 35 age bracket were due to primary or secondary bacterial infections.[2]

The observation that certain micro-organisms were able to inhibit the growth of, or kill competing microbes had been made as long ago as the 1880's,[3] and was given the name antibiosis. However, this phenomenon was regarded as a curiosity at best, and indeed often as merely an inconvenience to the culturing of bacteria. The story of the discovery and development of the penicillin antibiotics marks the transformation of this phenomenon into a revolutionary medical breakthrough.

As in many key scientific discoveries, serendipity was to play a key rôle in the penicillin story. We owe the discovery of penicillin to a chance contamination of a petri dish containing a *Staphylococcus* species in the correct phase of growth,† belonging to one of probably only a few researchers of the time who would have recognised the significance of such an observation. Fleming himself was never able to repeat this event, despite attempts to mimic the original conditions![4]

The researcher in question, Alexander Fleming, had had an interest in antibiosis since his studies on antisepsis during World War I. During these earlier studies he had realised that Lister's antiseptics were of very limited therapeutic use, since they were at least as toxic (if not more so) to the patient as to the infecting organisms. Fleming realised the necessity of developing agents which would destroy bacteria without being toxic to the leukocytes of the host – the so called 'magic bullets' proposed by the German researcher Ehrlich.[5] In subsequent work he was to discover the antibacterial effects of the enzyme lysozyme,[6] which is widely found in nature and is one of the body's natural defences against bacteria. This discovery made him familiar with the significance of lysis in bacterial colonies.

† If the contaminating spore had fallen on an already incubated plate, then the effect would never have been observed.

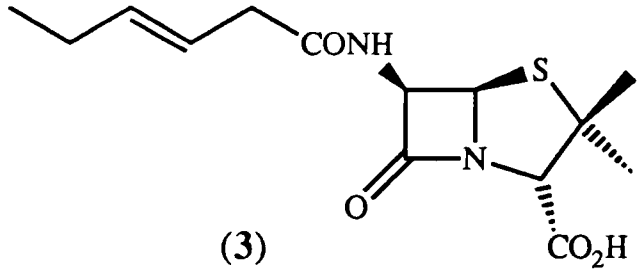
This all helped to set the scene for the first observation of the effect of the penicillin antibiotics. In September 1928 Fleming, whilst working in the laboratories of the Inoculation Department of St. Mary's Hospital, London, noted the antibiosis of *Staphylococcus* colonies, 'left aside on the laboratory bench', infected by a colony of mould from airborne contamination.[7] The infecting organism was identified as *Penicillium notatum*, and Fleming named the 'mould broth filtrate' from cultures of the mould 'penicillin', coining a word which has since become commonplace in our vocabulary. He made a brief study of the antibiotic properties of 'penicillin' on various micro-organisms, demonstrated that it was non-toxic to mice and rabbits, and showed that it caused no irritation in humans when applied to the skin or eyes. Fleming also carried out the vital experiments which demonstrated penicillin's non-toxicity to human leukocytes. In his original paper, Fleming made a note of its 'possible use in the treatment of bacterial infections',[7] but his own attempts to develop penicillin had to be abandoned through the inability, at that time, to purify the crude extracts. Indeed, this is no surprise, as it was not within Fleming's area of expertise as a bacteriologist. He did, however, retain a strong interest in penicillin throughout its later development.

Initial interest in penicillin was not as great as Fleming had hoped, with little development until the work was taken up by Howard Florey, Ernst B. Chain, Norman G. Heatley and Edward P. Abraham in Oxford in 1939; and simultaneously by at least three groups in the US. It is fortunate that penicillin was chosen for study at all since, at that time, there were over 200 known examples of one micro-organism inhibiting the growth of another.[8]

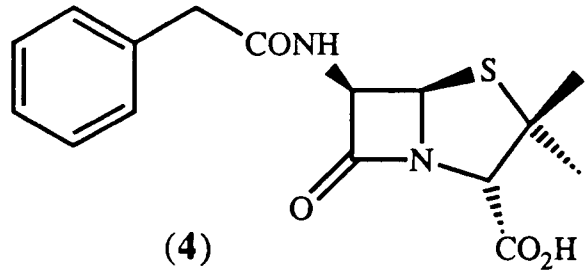
These groups had little more to start from than Fleming's original observations. Some research had been carried out in the interim, but with little

additional information being gleaned, although Dr C. G. Paine at Sheffield had successfully used crude penicillin extracts to treat eye infections in 1932.[9]

All the research workers in this field soon found that penicillin was not an easy problem to tackle. The active principle was difficult to purify from the crude extracts, and rapidly degraded on handling. Thus it is a considerable credit to the scientists on both sides of the Atlantic that pure samples of penicillin F (**3**) (in the UK) and penicillin G (**4**) (in the USA) were eventually obtained. However, it was perhaps typical of the nature of this difficult field of research that even this success provided some cause for consternation, by showing that the different groups were working with different penicillins (eventually traced to differences in the growth media used, which affected the final products of biosynthesis).[9]



(**3**)



(**4**)

Even with pure crystalline samples of these penicillins, debates over the structures raged, and were not finally settled until the conclusive proof of the 'novel' β-lactam structure was provided by Mozingo's desulphurisation experiments,[9] Jeff Webb's electrostatic titrations,[9] and, of course, Dorothy Hodgkin's X-ray structure determination.[9,10]

Synthesis of the penicillin structure proved difficult and eluded all attempts until 1957,† when Dr John Sheehan's team at the then Bristol Labora-

† Small quantities of Penicillin G had been produced as early as 1946 by du Vigneaud and others, but only by accident, in 0.008% yield as a side reaction in the attempted synthesis of an erroneous penicillin structure.[9]

tories succeeded in their synthesis of Penicillin V (which bears a $PhOCH_2CO$ side chain).[9] However, the scientific debate over the structure of penicillin, and the problems encountered by teams attempting to synthesise these metabolites did not prevent their use as therapeutic agents, with the development of penicillin from natural sources proceeding at a much more rapid pace.

The first, but relatively major obstacle to be overcome before the therapeutic action of penicillins could be systematically investigated, was the development of purification procedures which retained the antibiotic activity of the active principle. It was not necessary to obtain pure samples of penicillin for initial clinical trials, but certain impurities did have to be removed. In the first clinical trial in Oxford, on a woman dying of cancer, the penicillin extracts administered produced a severe reaction in the patient, which was traced to an impurity in the extracts used.[9] Successful clinical trials in Oxford began with the treatment of Albert Alexander, a 43 year old policeman suffering from a severe, and otherwise fatal, infection.[11] Penicillin rapidly aided his condition, but unfortunately Florey's team were unable to complete the treatment, since their supply of the drug ran out, even with recovery of unmetabolised penicillin from the patient's urine, so that they were unable to save him. They had been defeated by the small amounts of penicillin secreted by *P. notatum*, coupled with the difficult, time consuming nature of the necessary extraction and purification procedures. Undaunted, trials were continued, concentrating on children (who required smaller doses), and soon a string of successes on otherwise hopeless cases heralded penicillin's advent into the world of medicine.[11,†]

Encouraged by these successes, Florey and his team soon had an entire room turned over to the production of penicillin. Initially they employed any

† It should be noted that Oxford was not the only centre to be investigating the therapeutic action of penicillin extracts at this time, with several studies being carried out in the USA.[9]

suitably sized vessels, such as bed pans and biscuit tins for fermentation, though these were later replaced by fermentation vessels specifically designed by Heatley in 1941.[11]

With World War II proceeding none too well for the allies, penicillin soon caught the attention of military chiefs, and became one of the many projects of military concern. Despite having the status of a national secret, extensive interchange of information went on within the scientific community, allowing rapid progress. With government funding on both sides of the Atlantic, and work in both academic and pharmaceutical research laboratories, penicillin was brought to the front line medical units within a year, reaching military hospitals by July 1942.[12] By the time of the D-Day landings in 1944, many soldiers carried a supply of penicillin with them.[13]

This rapid development stemmed from a number of key factors, the most important of which were:-

i) The development of a growth media containing corn steep liquor (containing the important precursor phenylacetic acid), which produced greater quantities of penicillin in the form of penicillin G (**4**) rather than the less active penicillin F (**3**).[14]

ii) The isolation of a high yielding strain *P. chrysogenum*, discovered growing on a mouldy piece of cantaloupe melon by a researcher from the Northern Regional Research Laboratories in Peoria, Illinois.[15]

iii) Perhaps most importantly, the development of large scale, deep tank, fermentation processes by the US pharmaceutical companies.[16]

Today of course penicillins are just another part of modern life, often taken for granted, used routinely for the treatment of many 'minor' bacterial

infections, which used to be fatal. It is easy to forget the immense effort that went into their development.

1.3.2 Cephalosporins

As early as the late 1950's, the presence of penicillin-resistant staphylococci in hospitals began to cause serious clinical problems. At this juncture the culmination of many years' difficult research into a new β-lactam antibiotic, cephalosporin C, proved to be another vital step forward in the fight against disease. This antibiotic was resistant to many of the β-lactamase enzymes present in penicillin-resistant bacteria, and so active against the infections they caused.

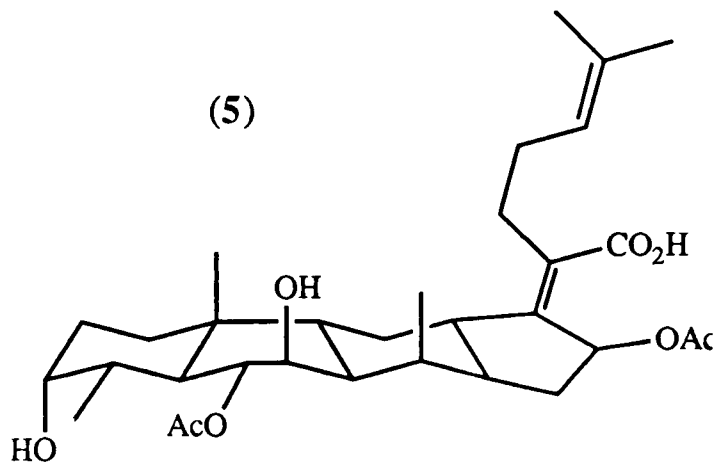
The history of cephalosporin C can be traced back (as far as human knowledge of it is concerned!) to 1948, in Sardinia. Dr Guiseppe Brotzu, searching for bacterial antagonists and, reasoning that the process of self-purification of water near sewage outlets might be due in part to such activity, was rewarded with the isolation of a wild strain of '*Cephalosporium*', which secreted materials which inhibited the growth of a broad spectrum of gram positive and gram negative bacteria.[17] (The name *C. acremonium* has often been taken up and mis-applied to this organism; it was originally denoted as *Cephalosporium* CMI† Nº 49137, but has since been reclassified to another fungal class and named *Acremonium chrysogenum*).

Through Brotzu's urging, the study of this organism and the antibiotics it secreted was taken up by researchers in Oxford. They discovered that three antibacterial agents were secreted by this '*Cephalosporium*' species.[18,19] The first of these agents, named cephalosporin P_1, (**5**), was readily extracted into organic solvents, and was characterised as an acidic steroid.[20,21] Cephalosporin P_1 had a limited antibacterial spectrum, affecting only a few

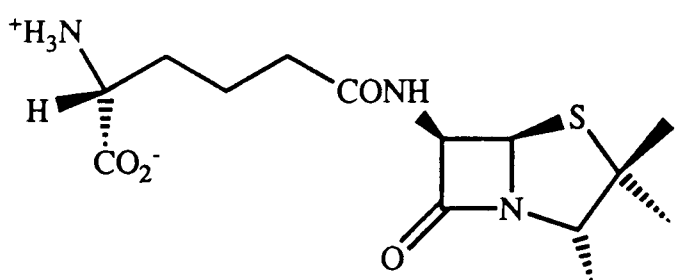
† Commonwealth Mycological Institute Kew.

gram positive bacteria, and therefore was not the the substance observed by Brotzu.

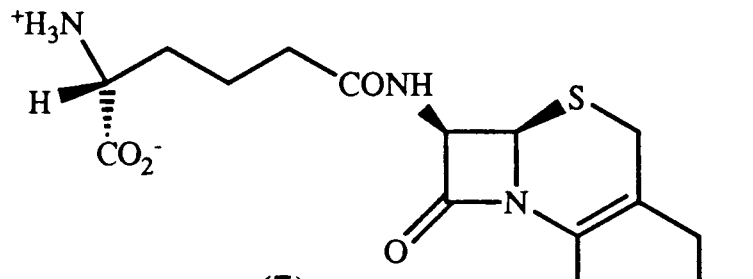
(5)



The two other antibiotics remained in the aqueous phase. The more abundant metabolite turned out to be another penicillin, possessing a δ-D-α-aminoadipoyl– side chain, (**6**), and with an antibacterial spectrum fitting that observed by Brotzu. This compound, named penicillin N, offered few therapeutic advantages over existing penicillins, as it too was ineffective against penicillin-resistant organisms. However, it did possess a greater activity against gram negative bacteria than existing penicillins, an observation which later led to the development of the first 'broad spectrum' antibiotic, ampicillin (bearing a D-$PhCH(NH_2)CO$– side chain), introduced in 1961.[12]



(6)



(7)

It was the last antibiotic, discovered as a minor component during the isolation of penicillin N, which proved the most valuable. This compound,

cephalosporin C, (**7**), whilst having only one tenth of the antibacterial activity of penicillin N (**6**) against bacteria destroyed by (**6**), was resistant to many β-lactamases, and so effective against the problematic penicillin-resistant bacteria.[18,22]

Cephalosporin C was only produced in minute quantities, and its activity was detectable only after concentration procedures used in the isolation of penicillin N. Problems were experienced due to a lack of material, but studies were initiated using material accumulated during penicillin N purification. These studies were accelerated when the Antibiotics Research Station at Clevedon succeeded in producing a mutant which produced much greater quantities of cephalosporin C than the wild strain.

Before long the future of cephalosporin C in medicine was assured, assisted by interest from pharmaceutical companies, such as Glaxo and Eli Lilly & Co., which stimulated further research. This research culminated in the successful introduction of the cephalosporin antibiotics into therapeutic practice.

1.4 The Biosynthetic Pathway to Penicillins and Cephalosporins

The ability to produce the penam and cepham ring systems is not very widespread in nature, but is possessed by taxonomically diverse micro-organisms.[23] Some are true fungi (eukaryotes) such as certain species of *Penicillium* (e.g. *P. notatum*, and *P. chrysogenum*), *Cephalosporium*, *Aspergillus* and one thermophilic fungus, *Malbranchea pulchella*; whilst others are gram-positive filamentous bacteria (prokaryotes) such as certain species of *Streptomyces* (e.g. *S. lipmanii*, and *S. clavuligeris*). Amongst these cephalosporin produc-

tion is more limited than that of penicillin, being confined to one species of *Cephalosporium* and the two species of *Streptomyces* noted above.

The biosynthetic pathways have been extensively studied,[24] and only a brief outline will be given here. Two distinct types of biosynthetic pathway have been observed in penicillin and cephalosporin producing organisms, branching from the formation of isopenicillin N (**2**), and manifested in the production of two different ranges of β-lactam metabolites. In the first, Path A, penicillins are the only β-lactams isolated. These bear a variety of acyl side chains (e.g hex-3-enoyl, 2-phenylacyl, and phenoxyacyl in penicillins F (**3**), G (**4**) and V (**8**) respectively), the type and proportions of which being profoundly influenced by the presence of appropriate carboxylic acids (or their derivatives) in the organism's growth medium. In the second, Path B, the only penicillin formed is penicillin N, its formation being unaffected by potential side chain precursors in the medium. In addition organisms utilising Path B may also produce cephalosporins. Paths A and B are identical until the formation of IPN (**2**), from which point they diverge. A generalised schematic representation of the complete pathway is shown in Scheme 1.2.

The part of the pathway common to both Path A and Path B can be considered to 'start' with the direct precursor to IPN (**2**), the tripeptide LLD-ACV (**1**). This is assembled from the three L-configured amino acids by a single enzyme ACV Synthetase (MW 422 486Da), with concomitant inversion of configuration of the valine component.[25,26] The unusual amino acid L-α-aminoadipic acid is naturally present as part of the lysine pathway of certain organisms. The next step is perhaps the most important single step in the pathway, the bicyclisation of LLD-ACV (**1**) to IPN (**2**), catalysed by IPNS (see Scheme 1.1),[1] details of which will be expounded in section 1.5. After IPN production Path A organisms have acyl transferase enzymes which allow the

exchange of the δ-L-α-aminoadipoyl side chain for other acyl type groups, leading to a diverse range of penicillins. Alternatively, organisms utilising Path B possess an epimerase enzyme which catalyses the conversion of the δ-L-α-aminoadipoyl side chain of IPN (**2**) to the δ-D-α-aminoadipoyl side chain, yielding penicillin N (**6**). Penicillin N is then acted upon by a ring expandase enzyme giving rise ultimately to cephalosporin C (**7**) in organisms such as *C. acremonium*, while it also provides access to other β-lactam structures such as the cephamycins produced by *S. clavuligerus* (see Scheme 1.2).

1.5 The Formation of Isopenicillin N (2)

The reaction catalysed by the enzyme IPNS is the key step in penicillin biogenesis; details of the reaction have therefore been extensively studied. The study of IPNS can be seen to have occurred in three phases:

i) Early experiments utilising whole cells, which suffered from the problems of transport across the cell wall, and from dilution of labelled probes with natural material.

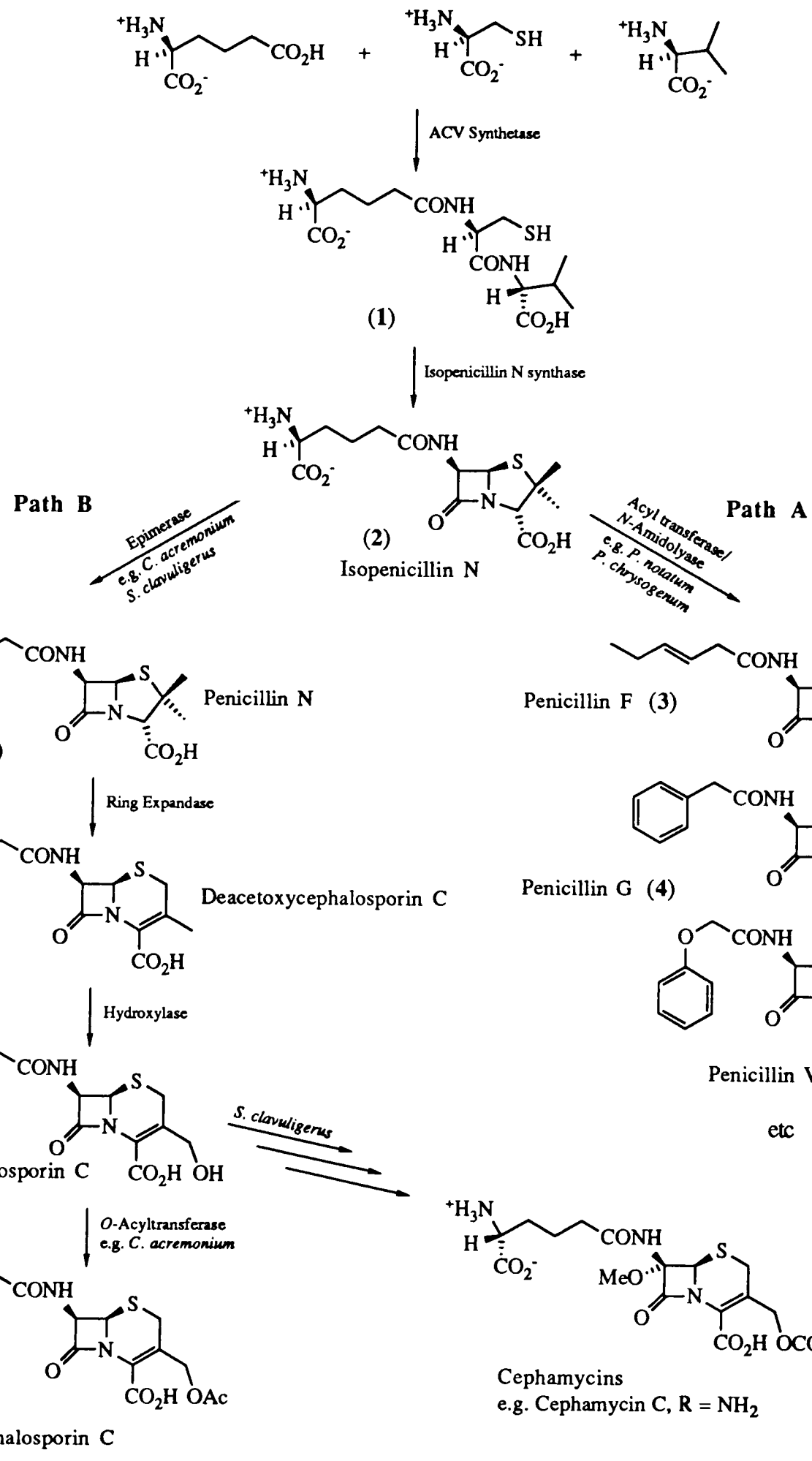
ii) The development of cell-free systems, which overcame many of these problems,[27,28] but which also suffered from low specific activity of the IPNS produced, and the presence of large quantities of undesired materials, also resulting from the lysis of the cells.

iii) The isolation, sequencing, cloning and over-expression (in *Escherichia coli*) of the IPNS gene from *Cephalosporium acremonium*,† which has allowed the formation and purification of large quantities of recombinant IPNS. This has resulted in much more facile studies of β-lactam formation catalysed by IPNS.[30,31]

† Recombinant IPNS has recently been prepared from several other sources, including *A. nidulans*.[29]

SCHEME 1.2

Outline of the penicillin and cephalosporin biosynthetic pathways[24]



It was not until the production of recombinant IPNS that it was shown conclusively that the formation of IPN from LLD-ACV was indeed catalysed by a single enzyme,[31] and not by a mixture of inseparable enzymes, as previously thought by some, in the early cell free systems.

Each development has opened up the possibility of conducting many more experiments on the IPNS reaction, and so has accelerated the growth of our knowledge of this biosynthetic step.

The enzymology of IPNS has been well documented elsewhere, but it should be noted that the IPNS enzyme exists in a number of organisms, and has been isolated and sequenced from at least seven sources.[30,32-37] These enzymes have a high degree of homology at the amino acid level (>50%), and generally similar molecular weights (37000–40000). The studies reported here are carried out on IPNS from *C. acremonium* expressed in *E.coli* (molecular weight 38301Da),¶ except where specifically stated otherwise. Little is known of the secondary or tertiary structure of IPNS. The two cysteine residues present in the protein (at positions 106 and 255) are close in space, being able to form a disulphide linkage, however this is not an essential structural feature of IPNS since it is the reduced (thiol) form of the enzyme which is most active.

1.5.1 Features of the Enzymic Reaction

IPNS is a non-heme iron-containing protein, requiring iron (II) as an essential requirement for activity. Studies on the apoenzyme, reloaded with increasing molar proportions of iron (II), have shown that a 1:1 stoichiometry of enzyme:iron (II) is required to restore full activity.[38] Iron (II) requirement is normally obtained from the growth medium during production of recombinant IPNS, so that active enzyme is recovered from the lysed cells. How-

¶ The complete amino acid sequence of IPNS from *C. acremonium* is given in Figure 5.1 of chapter 5.

ever, probably as a result of aerobic oxidation of iron (II) to iron (III), it has been found that the catalysis is optimised by further addition of iron (II) to the reaction medium (typically 100-200μM).[39]

Studies on the effects of various cofactors[40] have shown that optimal activity is achieved by the addition of a one-electron reducing agent, to maintain the iron in the 2^+ oxidation state (typically L-ascorbate, 1-2mM), and a disulphide reducing agent, to maintain the enzyme and substrate[§] in the more active, thiol form (typically dithiothreitol, 2-5mM).

The reaction catalysed by IPNS follows the simplest possible stoichiometry (Scheme 1.3) for the conversion of one mole of LLD-ACV to IPN. This necessitates the removal of four hydrogens, thus utilising both oxidising equivalents of one mole of the cosubstrate, dioxygen.[41] Detailed kinetic studies on the binding of these substrates have, however, not yet been carried out.



SCHEME 1.3

In terms of catalytic efficiency, IPNS is a relatively poor enzyme, and has been shown in cell free systems to undergo a form of substrate-mediated autoinactivation after only a few hundred turnovers.[40] The mechanism of inactivation is unknown, although results from the sequencing of inactivated

§ The disulphide form of LLD-ACV (**1**) is not a substrate for IPNS.

protein have revealed an unknown modification at His-261 (and possibly at His-64).[42]

1.5.2 Order and Stereochemistry of Ring Closures

i) Order of ring closures

Research carried out to date indicates that the most likely course for the IPNS catalysed formation of IPN (**2**) from LLD-ACV (**1**) is initial closure of the β-lactam ring, to give an intermediate which remains enzyme bound, followed by subsequent closure of the thiazolidine ring. Evidence for this has come from a number of experiments, which are discussed separately below.

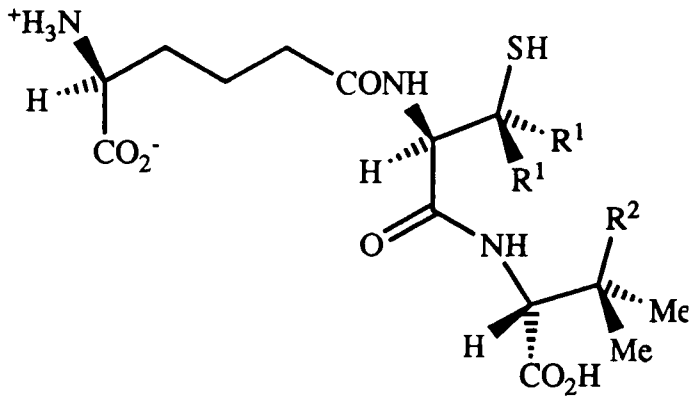
Some of the earliest experiments were kinetic studies involving deuterated isotopomers of the natural substrate LLD-ACV (**1**). These can be summarised as follows:

a) Separate competitive mixed label experiments, in which a 1:1 mixture¶ of unlabelled LLD-ACV and its isotopomer labelled with deuterium at either the 3-cysteinyl (**9**) or 3-valinyl (**10**) position was incubated with IPNS, were monitored (by mass spectral analysis) via the enrichment or otherwise of deuterated material in the unreacted pool. Observation of such enrichment indicated preferential turnover of the unlabelled over the labelled material, as shown schematically in Scheme 1.4. Enrichment was only observed for the 3-cysteinyl labelled material.[43] This is a V_{max}/K_m effect and this reflects events only up to the first irreversible step,† and so indicates the involvement of the 3-cysteinyl hydrogen in this event.

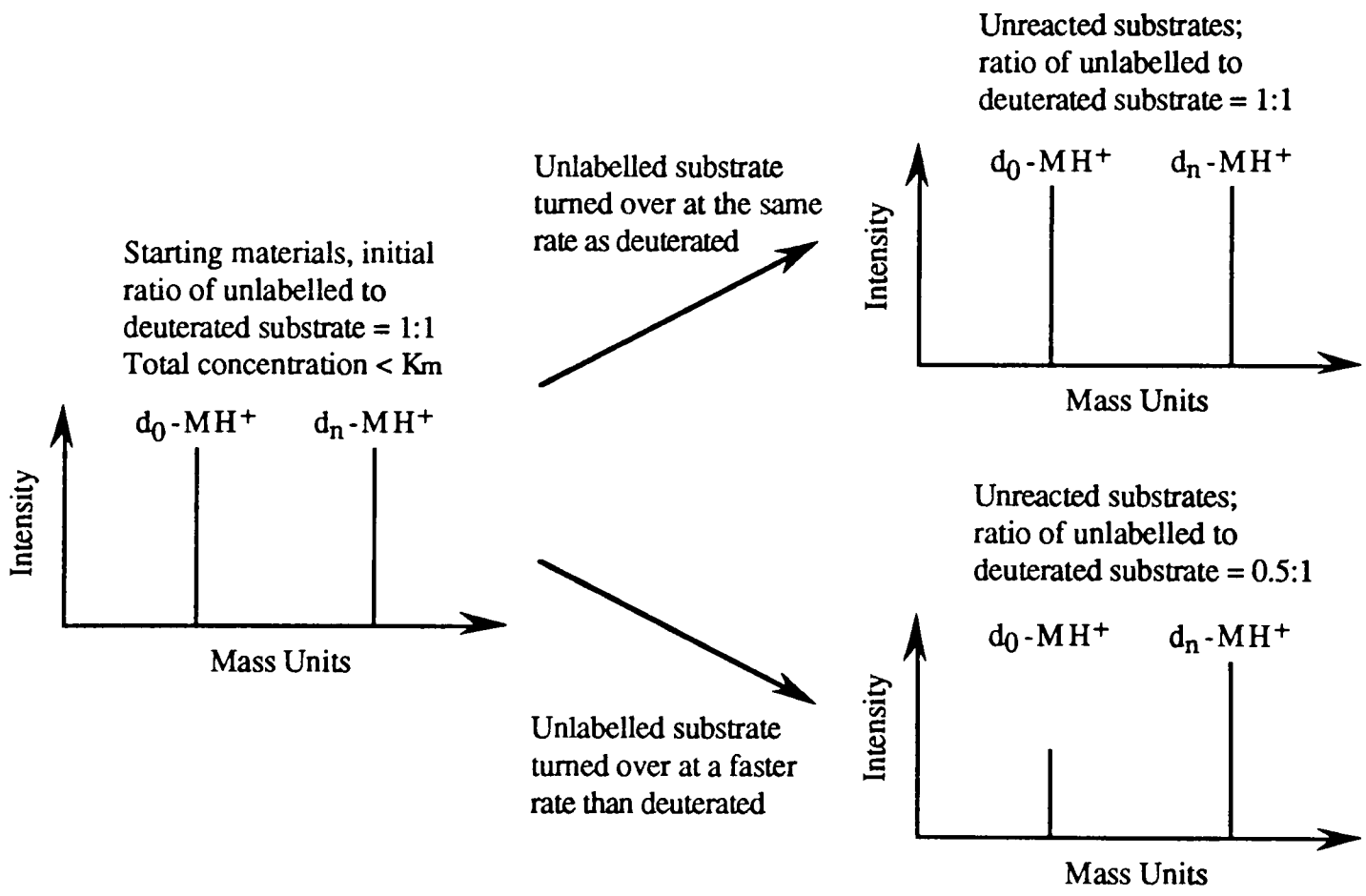
b) Both of the above labelled isotopomers, as well as the material labelled at both the 3-cysteinyl and 3-valinyl positions (**11**), showed significant V_{max}

¶ The concentration of the mixture of substrates was kept below the value of K_m.
† V_{max} is the maximum velocity of the reaction; K_m is the Michaelis constant.

isotope effects,[44,45] indicating that both sites are involved in steps which are at least partially rate limiting. This indicates that both steps may have similar activation energies.



(9) $R^1 = D, R^2 = H$
(10) $R^1 = H, R^2 = D$
(11) $R^1 = R^2 = D$



SCHEME 1.4

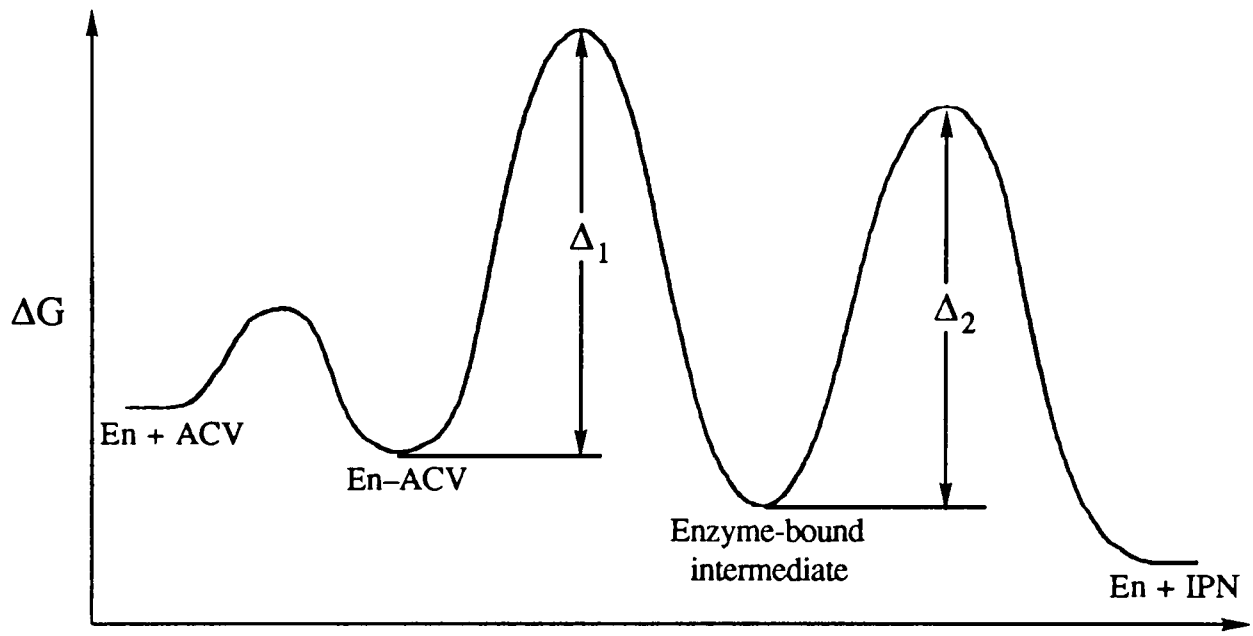
c) The above V_{max} isotope effects ($D_V = k_H/k_D$) follow the relationship,[44]

$$D_{V(1,2)} = D_{V(1)} + D_{V(2)} - 1,$$

which is that followed by a reaction where there is an enzyme-bound intermediate, and two sequential isotope effects.

These results can be summarised in terms of an energy diagram such as shown in Figure 1.1.

FIGURE 1.1
Proposed reaction energy profile for the
conversion of LLD-ACV (**1**) to IPN (**2**) with IPNS[43]

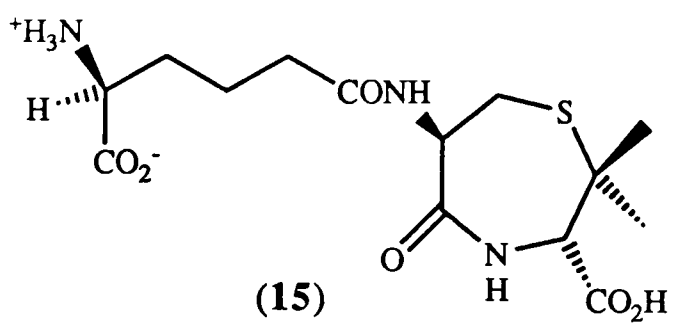


Unfortunately, the extreme difficulties in carrying out these kinetic measurements, coupled with the inherent ambiguities in interpreting kinetic data in terms of chemical changes, means that, taken in isolation, these results do not prove that an enzyme bound β-lactam is intermediate during penicillin formation with IPNS. However, this supposition is supported by all other evidence to date, and will be assumed to be the most likely explanation during the following discussions.

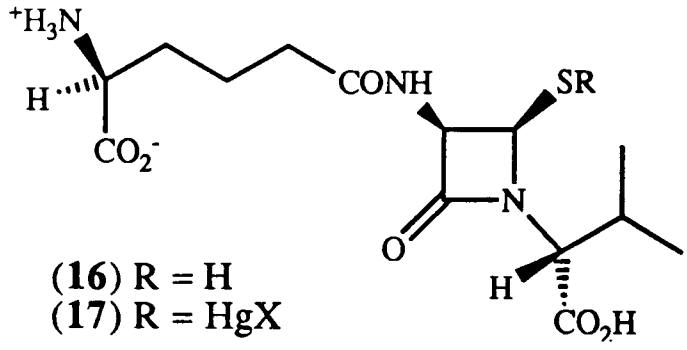
Strong evidence for an enzyme-bound β-lactam intermediate has also come from the identification of a 'shunt metabolite' pathway for the natural sub-

strate, LLD-ACV, with IPNS.[46] This pathway was revealed on incubation of isotopically labelled material bearing deuterium at the 3-valinyl position. A second label, in the form of ^{13}C at the 3-cysteinyl position (to give the labelled isotopomer (**12**) of LLD-ACV (**1**)), allowed facile identification of a new resonance at 89ppm in the ^{13}C NMR spectrum (distinct from the substrate, 39ppm, and IPN at 68ppm), which was shown by comparison with an authentic standard to arise from the 'shunt metabolite' (**13**). Subsequent examination of the ^{13}C NMR spectrum of the products from incubating LLD-ACV bearing only the ^{13}C label, confirmed the presence of the shunt metabolite under normal ring closure conditions. This alternative product (**13**) is thought to be formed from the enzyme-bound β-lactam intermediate (**14**) as shown in Scheme 1.5. The rate of cyclisation of (**14**) to IPN (**2**) is reduced by the deuterium label, enhancing the level of shunt metabolite relative to IPN (**2**).

A number of possible intermediates have been incubated with IPNS, such as the 7-membered ring intermediate (**15**),[47] but all with negative results. The free thiol form of the proposed enzyme-bound intermediate (**16**) was found,[48,49] after formation from the stable mercuric salt (**17**), to be spontaneously and rapidly hydrolysed at pH's well below† the enzyme's stability limit (~pH6) and so cannot be tested as a substrate. These results provide still more information supporting an intermediate which remains enzyme bound

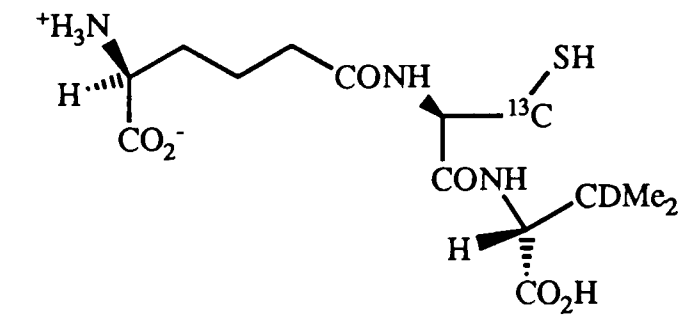


(**15**)



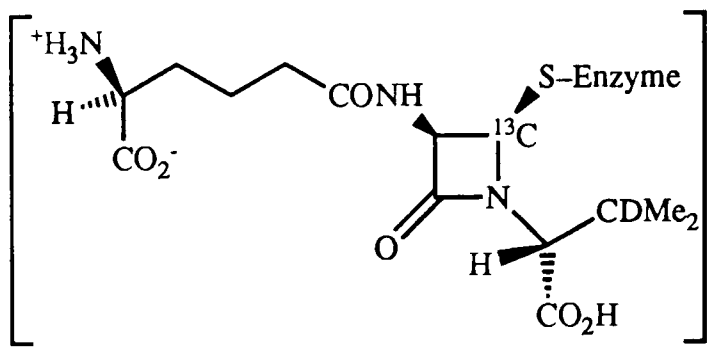
(**16**) R = H
(**17**) R = HgX

† $t_{½}$ ≈ 25min at 20°C and pH 1.5, decreasing rapidly as the pH increases towards neutrality.



(12)

IPNS

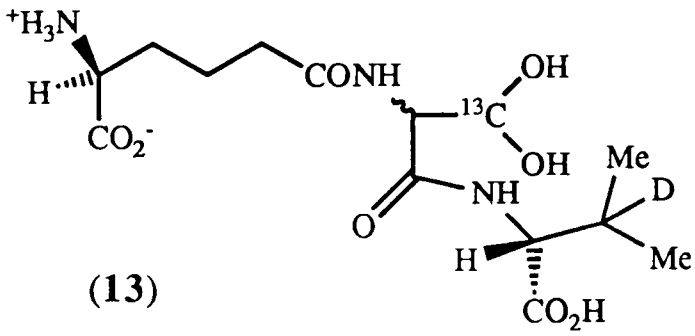


(14)

Normal pathway (major)

'Shunt metabolite' pathway (minor)

^{13}C-labelled Isopenicillin N (2)



(13)

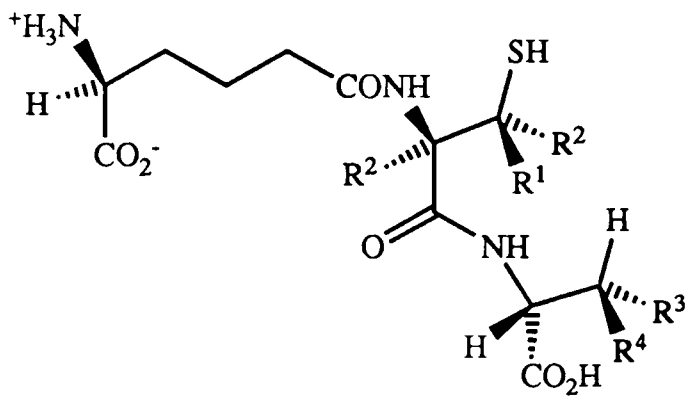
SCHEME 1.5

ii) Stereochemistry of ring closures

Isotopic labelling studies have allowed the stereochemical course of both the β-lactam and thiazolidine ring closures to be determined.

When the two tripeptides LLD-A-[3*S*-^{2}H]-CV (**18**) and LLD-A-[2,3*R*-$^{2}H_2$]-CV (**19**) were incubated with IPNS, both showed stereospecific loss of cysteinyl hydrogen, with complete loss of deuterium label (despite a primary

kinetic isotope effect) in the first case and complete retention of the label in the second.[50] These observations confirmed earlier studies involving the feeding of L-cysteines, chirally labelled with deuterium at C(3), to whole cells.[51] The stereospecificity of 3-cysteinyl hydrogen abstraction has also been observed in studies involving substrate analogues (see section 1.5.3). It has been shown that the 3-pro*R*-cysteinyl hydrogen may be replaced with certain groups to give analogues which are still substrates for IPNS, whereas in no case does replacement of the 3-pro*S* hydrogen give an active substrate.[45]



(18) R^1 = D, R^2 = H, R^3 = R^4 = Me
(19) R^1 = H, R^2 = D, R^3 = R^4 = Me
(20) R^1 = R^2 = H, R^3 = $^{13}CH_3$, R^4 = $^{12}CH_3$

Similarly, ^{13}C-labelling of the valinyl methyl groups has allowed investigation of the stereochemistry of the thiazolidine ring closure, showing that this proceeds with complete retention of configuration. The observations made during earlier work involving whole cells,[52,53] were confirmed by the synthesis and incubation with IPNS of the LLD-AC-(3*S*)-[4-^{13}C]-V (**20**) tripeptide, which gave IPN with the ^{13}C label exclusively in the α-position.[54]

1.5.3 Substrate Analogues of LLD-ACV

Some insight into the actual mechanism of the two ring closures catalysed by IPNS has been gained from the incubation of a large library of substrate analogues with IPNS. The introduction of a wide variety of substrate ana-

logues to IPNS has shown the enzyme to have a somewhat lax substrate specificity, and has resulted in the isolation of a large number of new biosynthetic products, which, with only a very few exceptions, share the common structural feature of a β-lactam ring[45] (in itself supportive evidence for a stepwise mechanism with initial formation of the β-lactam ring).

We shall examine the most important results gained from these analogue studies in the remainder of this chapter, by considering in turn variations of each of the three amino acid residues of LLD-ACV (**1**).

A) Variation of the δ-L-α-Aminoadipic Acid Residue

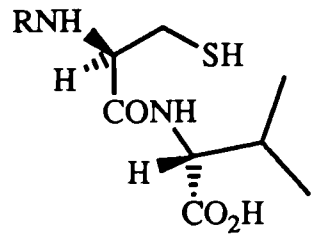
Incubation of substrate analogues of LLD-ACV in which the δ-L-α-aminoadipic acid residue has been modified has allowed the nature of the remote binding site, which anchors the side chain in the enzyme active site, to be investigated.

Such studies have shown that for an analogue to be readily acceptable to IPNS it requires a six atom chain terminating in a free carboxylic acid group.[55] Shorter and longer chains have been found to be unacceptable. There is apparently no interaction with the terminal amino group in the natural substrate, with both the δ-D-α-aminoadipoyl and hexandioyl variants being as acceptable as the natural substrate, but acceptability as a substrate is lost by the introduction of additional groups on nitrogen, perhaps due to steric factors.

Interestingly a number of apolar side chain containing analogues have also been found to be acceptable, if poor, substrates for IPNS,[56] perhaps indicating an apolar nature for the residues surrounding the carboxyl binding site in the cleft.

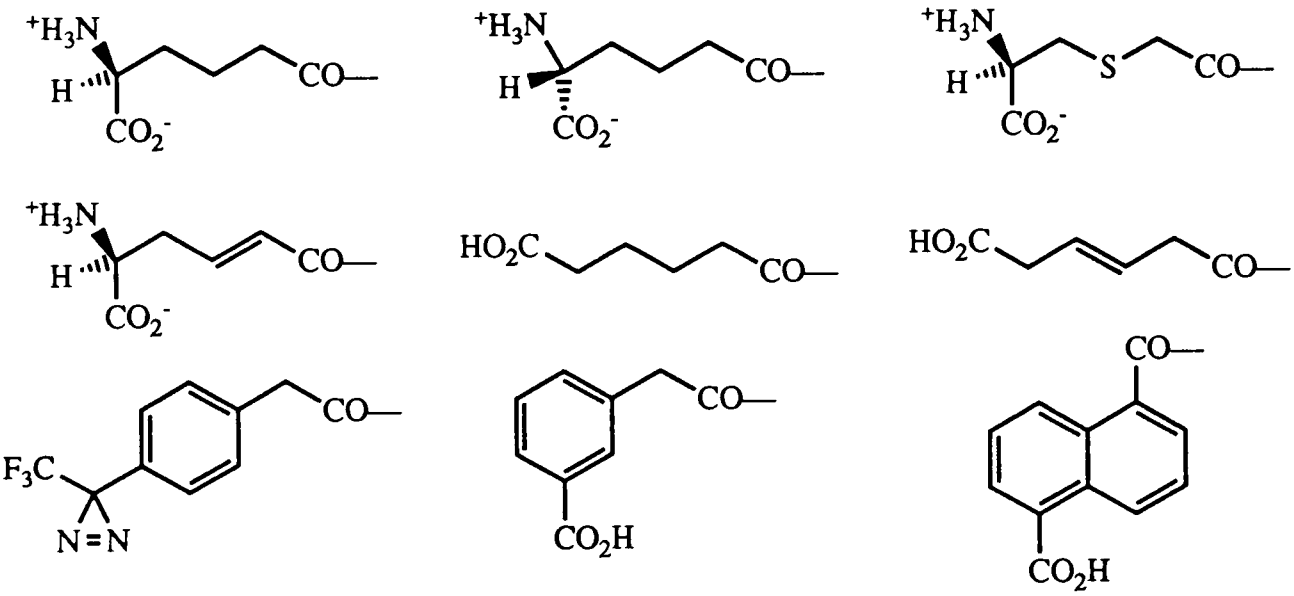
A range of acceptable and unacceptable analogues is shown in Table 1.1.

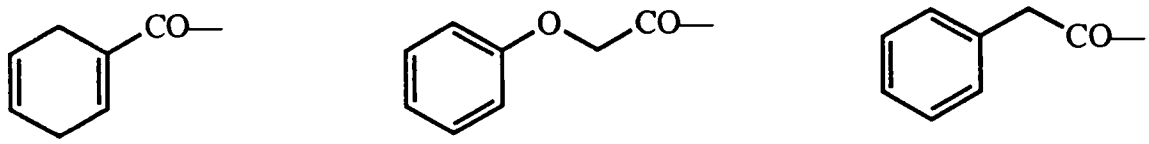
TABLE 1.1

Substrate Analogues of type 

Substrate Analogues Accepted by IPNS

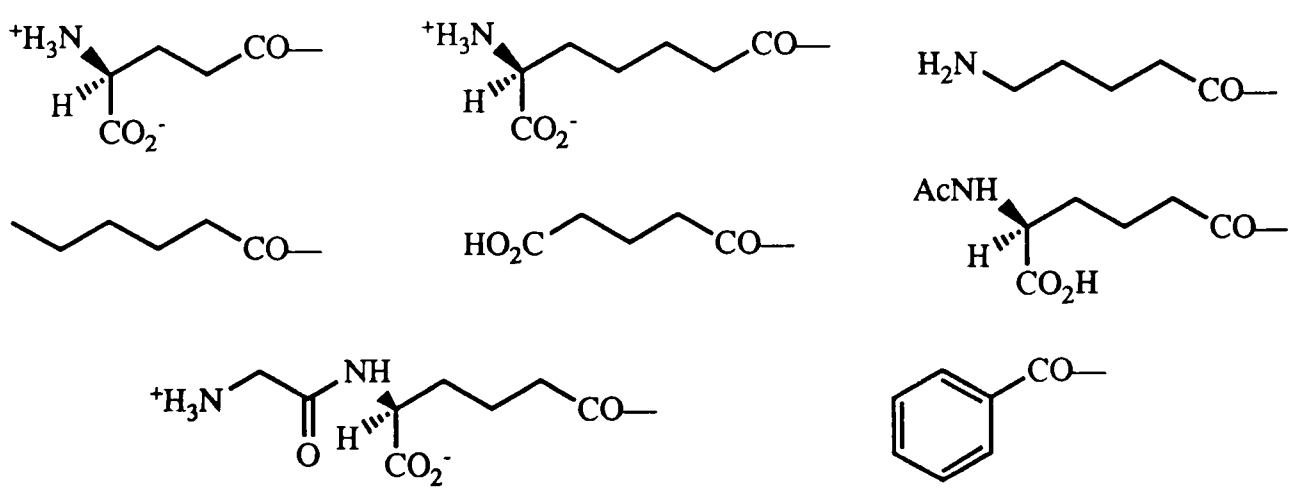
R– =





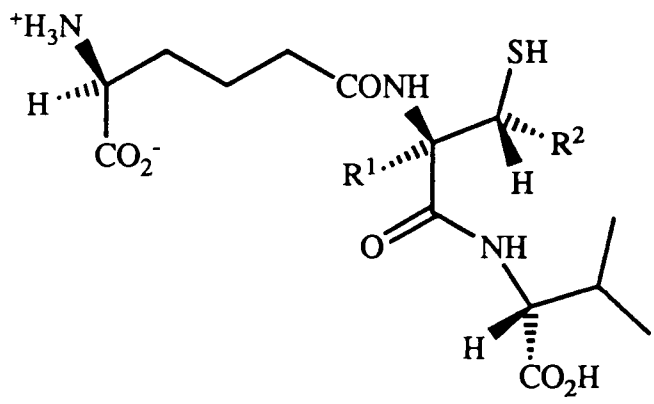
Substrate Analogues Not Accepted by IPNS

R— =



B) Variation of the Cysteine Residue

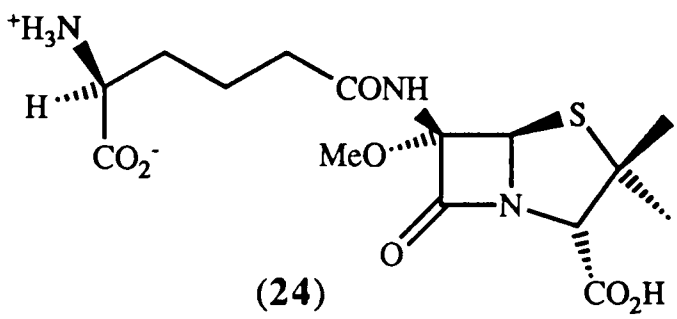
The cysteine moiety has proved to be the least variable component of the substrate LLD-ACV. Perhaps this is not unexpected if we consider the likelihood of a tightly bound transition state for the formation of the β-lactam ring. However, a few substituents have been successfully introduced which replace either of the 2 and 3-pro*R* hydrogens. As mentioned in section 1.5.2, no analogues containing substituents at the 3*S*-cysteinyl position have been found to be substrates for IPNS. This is not surprising considering that it is exclusively the 3-pro*S* hydrogen which is lost during ring closure.[50] In addition the steric bulk of substituents cannot be too great. Acceptable substrates incubated to date include the 2-Me (**21**),[57] 2-OMe (**22**)[58] and 3*R*-Me (**23**)[57] substituted analogues.



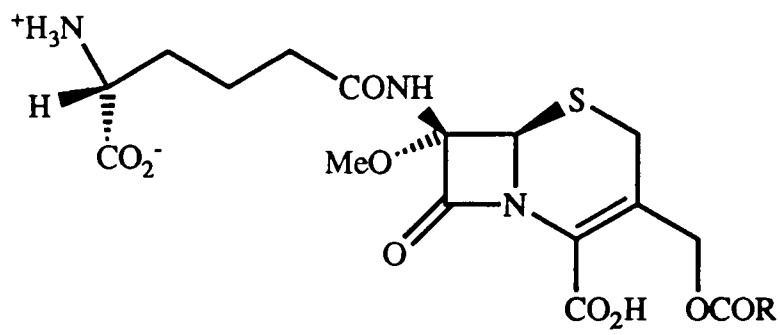
(**21**) R^1 = Me, R^2 = H
(**22**) R^1 = OMe, R^2 = H
(**23**) R^1 = H, R^2 = Me

The substrate analogue (**22**) is of particular interest owing to the similarity between the resulting penicillin product (**24**) and the cephamycin antibiotics (e.g. **25** and **26**).[59]

Replacement of the thiol functionality as in, for example, LLD-ASerV,[57] has not yet led to an acceptable substrate analogue; the selenocysteine variant remains to be synthesised.

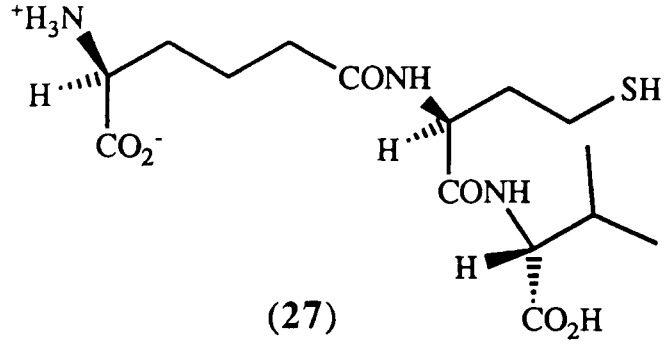


(24)

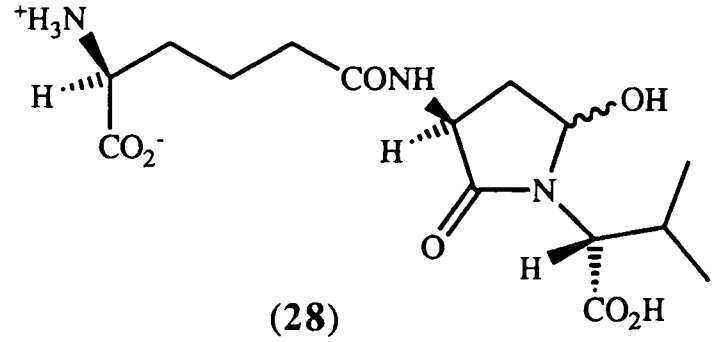


(25) R = Me 7-Methoxycephalosporin C
(26) R = NH_2 Deacetyl-3-*O*-carbamyl-7-methoxycephalosporin C

The one other variation of the cysteine residue which has been shown to be acceptable has been the homoanalogue, LLD-AhomoCV (**27**), which on incubation with IPNS yields the γ-lactam (**28**).[60]

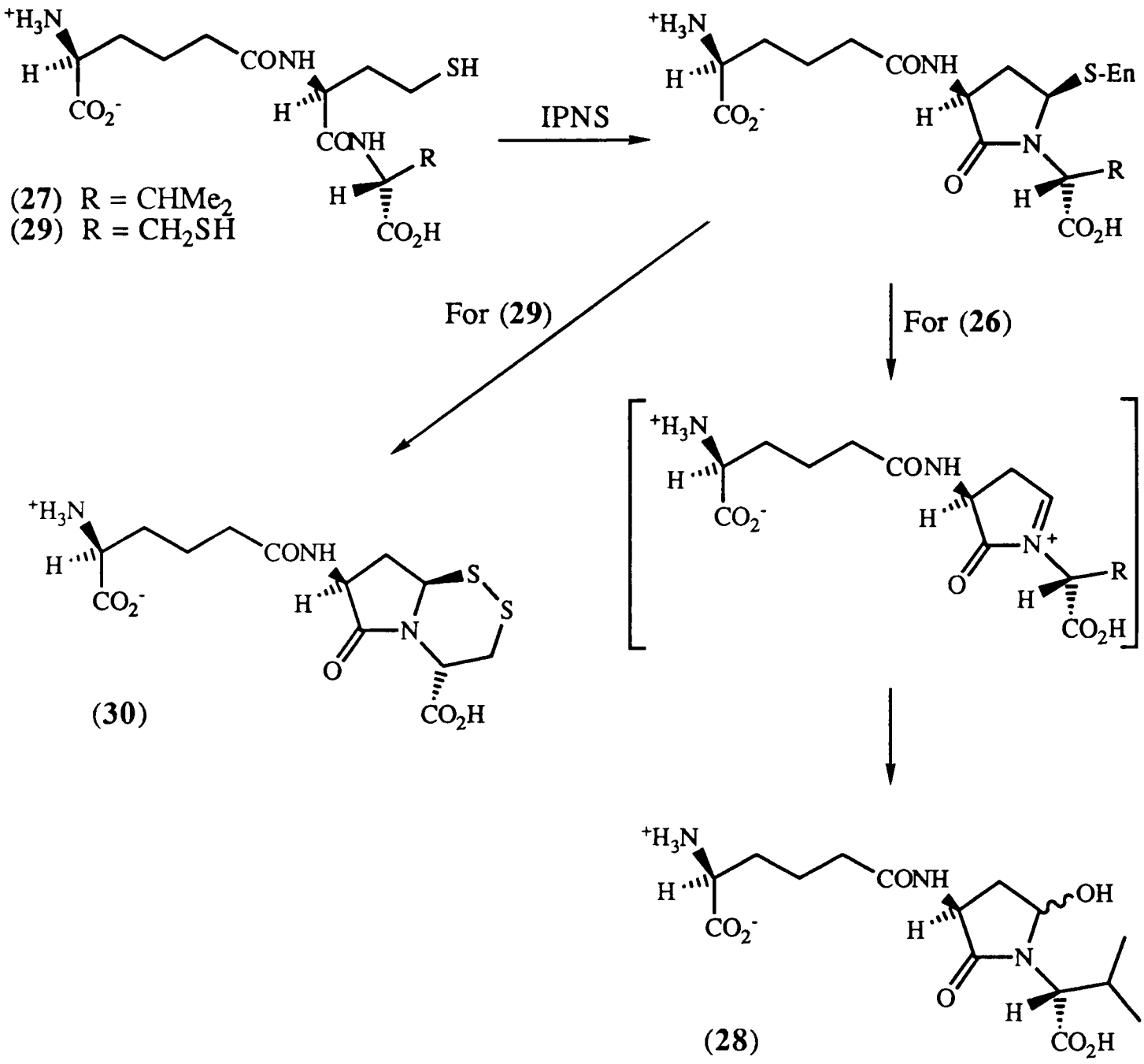


(27)

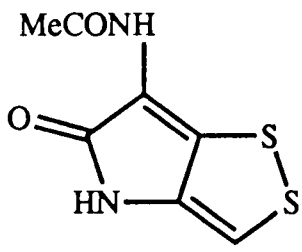


(28)

Incubation studies under an $^{18}O_2$ atmosphere have shown that the hydroxyl group in (**28**) derives, at least in part, from dioxygen.[60] It is thought that this product may arise from the shunt metabolite route described in section 1.5.2, an idea supported by disulphide trapping which occurs using the tripeptide LL-AhomoC-D-cysteine (**29**) (Scheme 1.6).[61] This product (**30**) is in itself of interest owing to its structural similarities to the naturally occurring antibiotic holomycin (**31**).[62]



SCHEME 1.6



(31)

C) Variation of the Valine Residue

It is in the area of substrate analogues in which the valine residue has been modified that the richest crop of successful substrate analogues has been reaped. IPNS has been challenged with scores of such analogues, many of which have been acceptable substrates for IPNS, giving rise to a plethora of new β-lactam products.

The results of such studies have not only allowed some inferences about the nature of the enzyme active site in the region of the second ring closure, to be drawn, but they have also provided some insight into the mechanism by which IPNS operates. Mechanistically it has been shown that there are a number of possible pathways, depending on substrate, which are open to the enzyme after formation of the putative enzyme-bound β-lactam intermediate. This suggests the presence of a highly reactive species in the enzyme active site, and a high oxidation state En–Fe=O species has been proposed.[45]

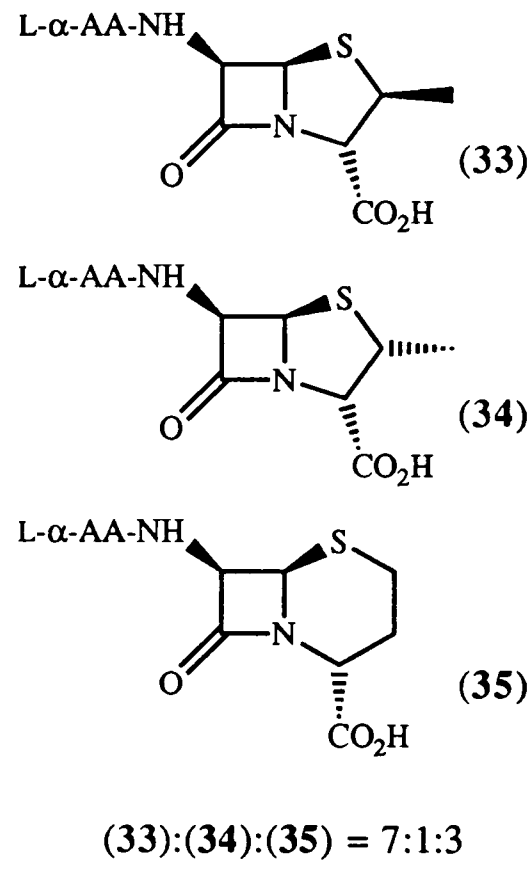
We shall look at the more important results obtained from incubation of these substrate analogues, first looking at results with implications for the enzyme's mechanism, grouping results under the broad classifications of **desaturase** and **mono-oxygenase** pathways for the closure of the second ring (with some analogues, products are seen which arise from each pathway), and then move on to examine results which point to aspects of the topography of this region of the IPNS active site.

i) Desaturase Pathway

This is the pathway which is seen to operate with the natural substrate LLD-ACV (**1**). The simplest mechanism for the formation of the second ring is one of abstraction of a hydrogen atom by some active site species, followed by trapping of the resulting radical by sulphur. The involvement of a radical

intermediate is supported by substrate analogue results, but other mechanisms can not yet be eliminated.

One of the first substrate analogues to be incubated with IPNS was LL-AC-D-2-aminobutyric acid (**32**), which was found to be an acceptable substrate for IPNS.[63,64] Incubation of (**32**) gave three β-lactam products, the 3β- and 3α-methylpenams (**33**, **34**) and the cepham (**35**) (Scheme 1.7). The formation of both cepham and penam products is consistent with a radical mechanism, as it reflects the smaller difference in activation energies for homolytic cleavage of a secondary vs. a primary C–H bond, as compared to the difference between a tertiary and a primary bond in LLD-ACV (**1**).†



SCHEME 1.7

Additionally the introduction of a specific deuterium label at the C(3) position of the 2-aminobutyric acid residue, to give the two tripeptides LL-AC-

† Bond dissociation energies at 298K (kJ.mol^{-1}): Me–H (435), Me_2CH–H (398), Me_3C–H (385).[65]

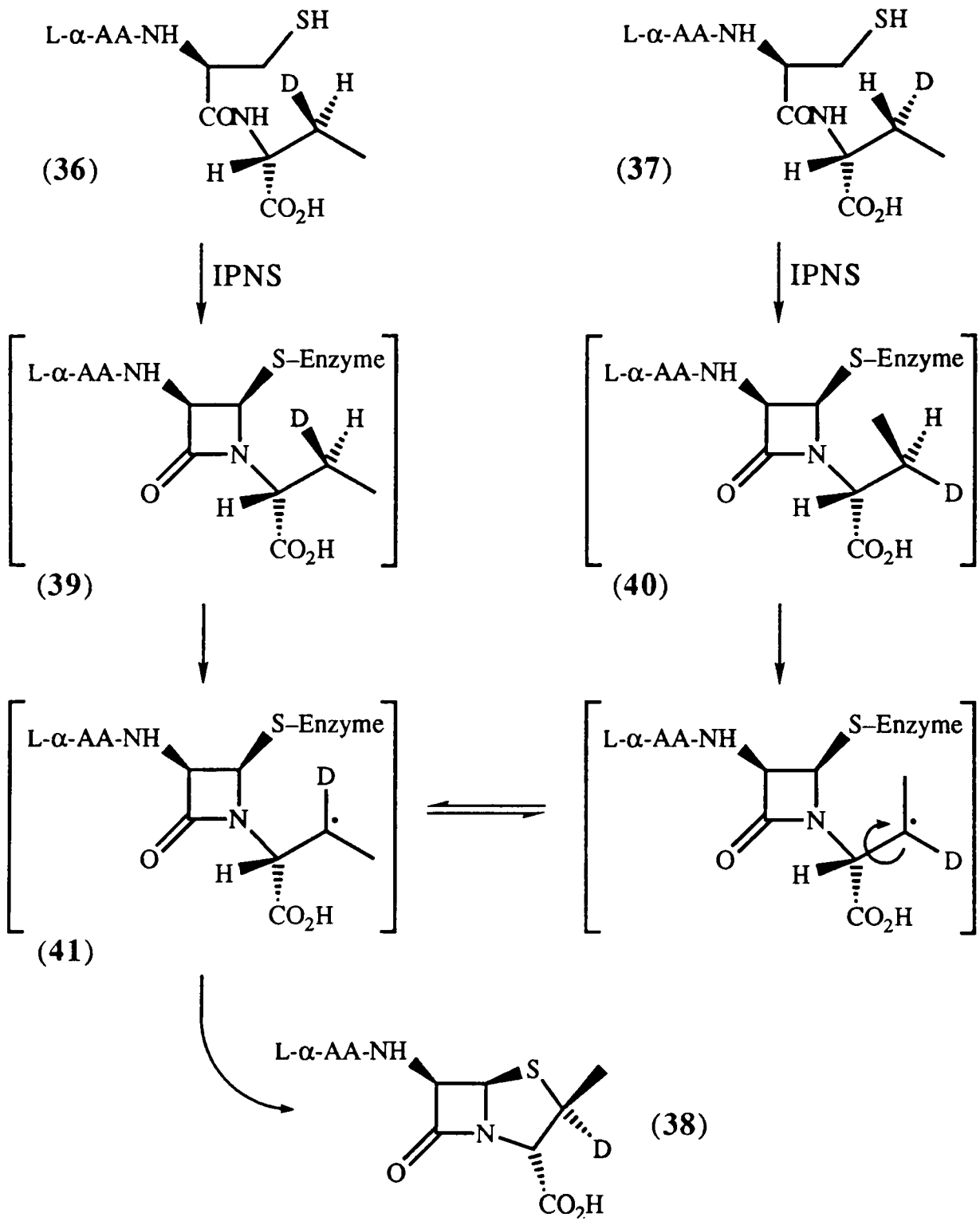
D-2-amino-[3*S*-^{2}H]-butyric acid (**36**) and LL-AC-D-2-amino-[3*R*-^{2}H]-butyric acid (**37**), gave analogues which on incubation with IPNS gave the same [3α-^{2}H]-3β-methylpenicillin (**38**).[66] This result is consistent with the participation of a radical intermediate. Such a rationalisation would predict a large primary kinetic isotope for radical formation, leading to hydrogen, rather than deuterium, atom abstraction from each of the proposed β-lactam intermediates (**39**, **40**), to give a common radical species (**41**). The formation of a common β-lactam product (**38**), indicates that the intermediate (**41**) must be sufficiently long lived, as compared to the time for rotation around the C(2)–C(3) bond, to give complete turnover to the preferred β-methyl conformation before closure of the second ring (Scheme 1.8).

The operation of a large primary kinetic isotope effect for the second ring closure has also been seen in the promotion of 'shunt metabolite formation (see section 1.5.2). A further example of this occurs with the substrate analogue LL-AC-D-2-aminopentanoic acid (**42**) and its [4,4,5,5-$^2H_4$]- analogue (**43**). The unlabelled tripeptide gives three products on incubation with IPNS,[67,68] the 4α-methylcepham (**44**) predominating over the 4β-methylcepham (**45**) and the 3β-ethylpenam (**46**) (ratio **44**:**45**:**46** = 7:1:1†) (Scheme 1.9). Incubation of the labelled material (**43**), however, showed complete reversal of product distribution, giving predominantly the deuterated penam (**47**) over the deuterated 4α-methylcepham (**48**) (ratio **47**:**48** = 4:1) (Scheme 1.9).[69]

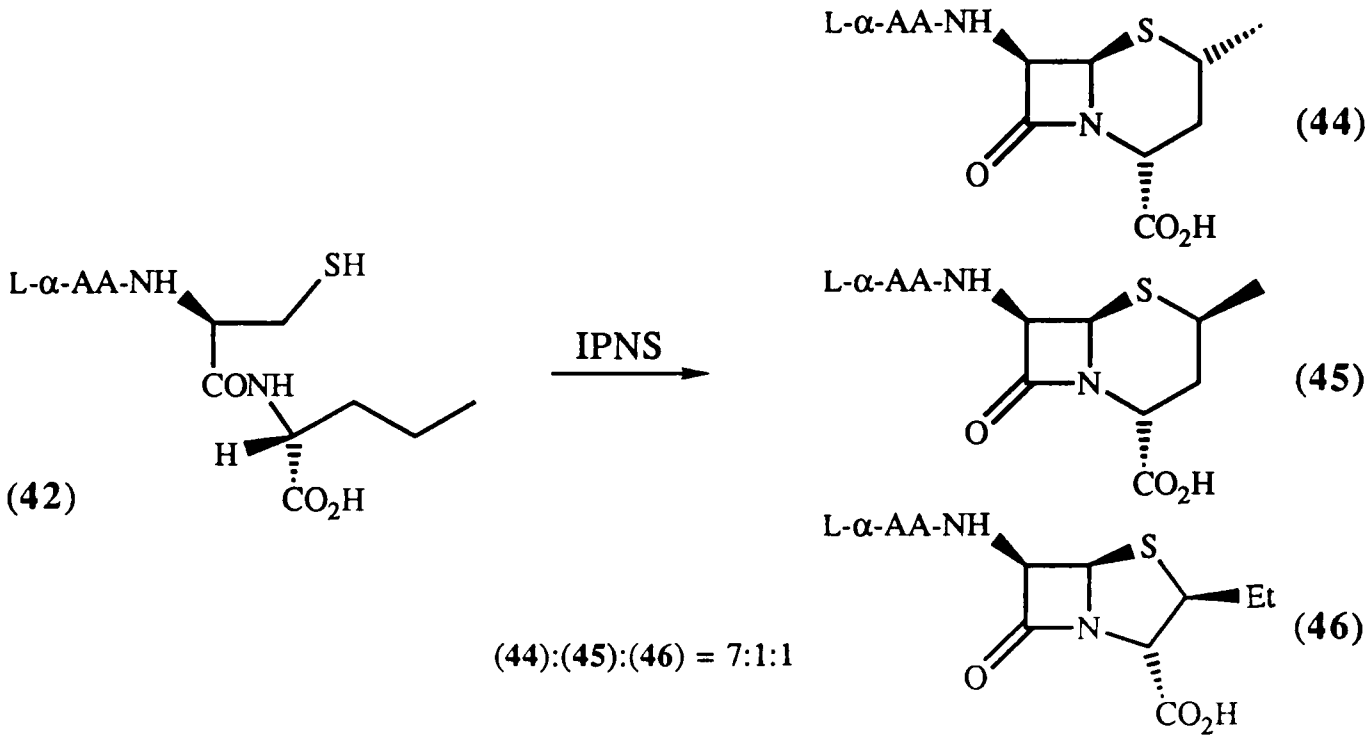
The desire to investigate the possibility of radical formation led to the synthesis of the analogue LL-AC-D-3-cyclopropylalanine (**49**). It was considered that abstraction of the C(3) hydrogen of the cyclopropylalanine residue would give a radical which, by analogy with the methylcyclopropyl radical,

† The penam was present in the original crude incubation mixtures, and its biological activity was detected in the original work. However its presence in the ^{1}H NMR spectrum was overlooked due to the unusual nature of the β-lactam AB quartet, which is collapsed virtually to a singlet. The ratio given here is derived from re-examination of the original spectra.

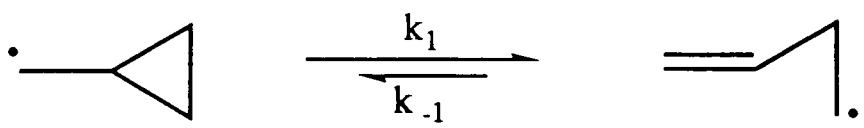
would be expected to undergo rapid ring opening (see Scheme 1.10),[70] allowing formation of the allylcarbinyl radical before subsequent closure of the second ring.



SCHEME 1.8



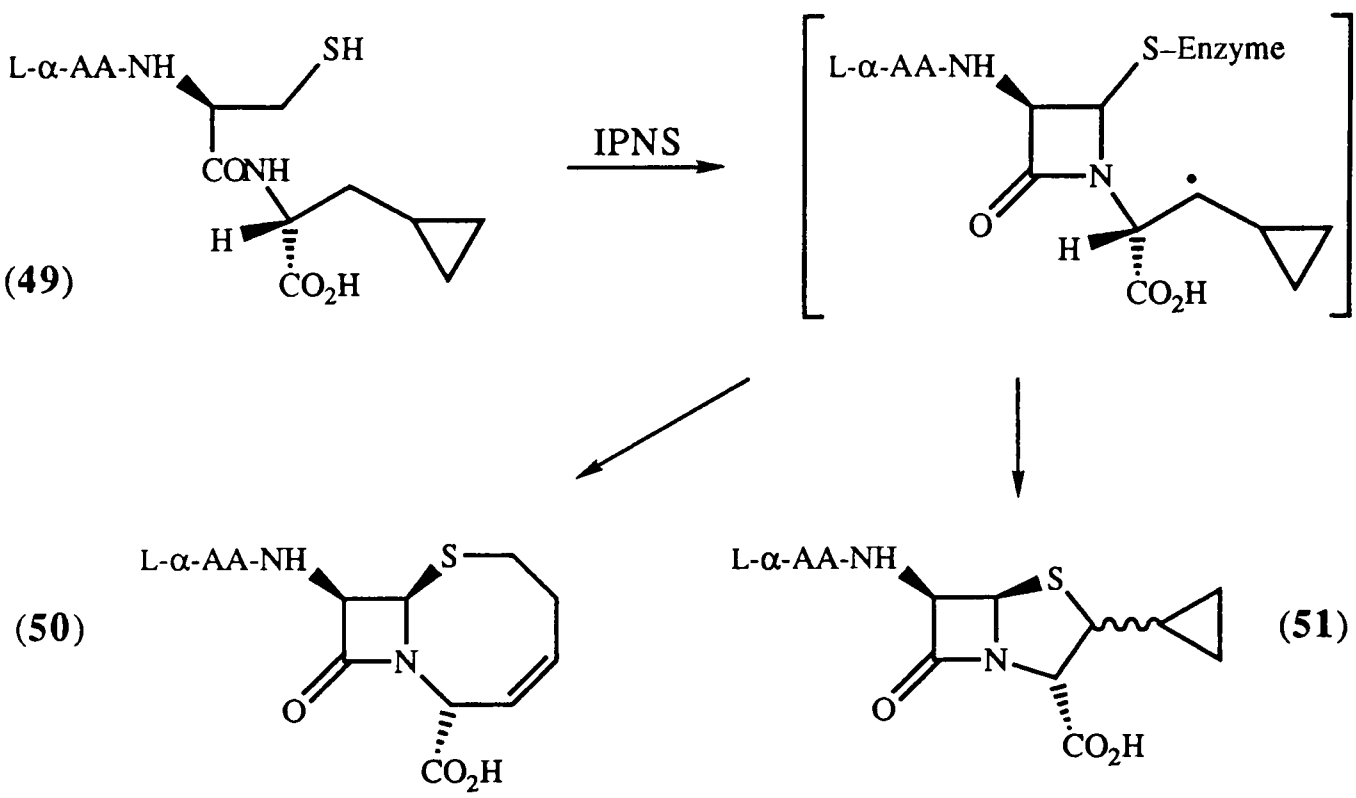
SCHEME 1.9



$k_1 = 10^8.s^{-1}$, $k_{-1} = 10^3.s^{-1}$

SCHEME 1.10

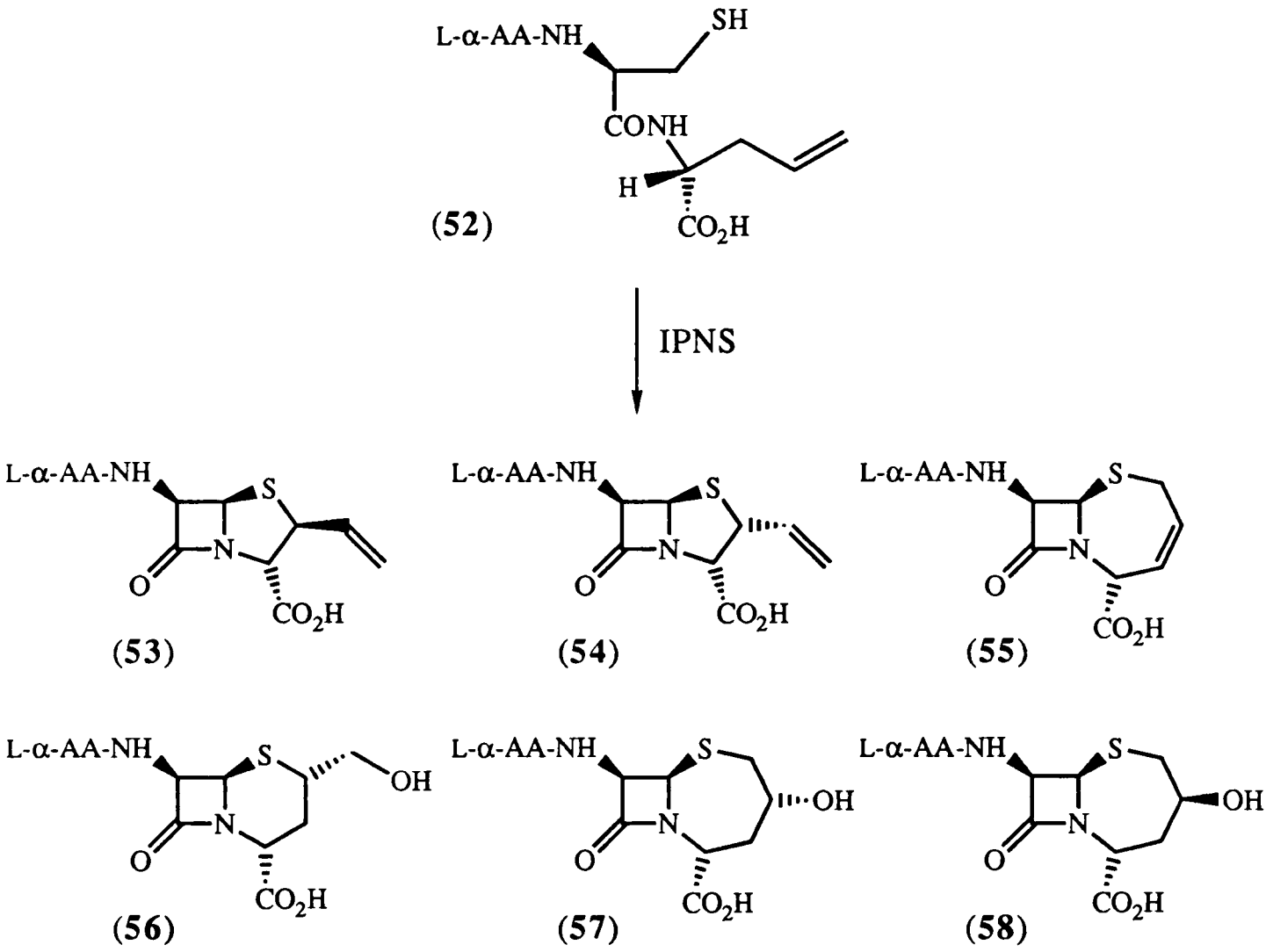
As expected, incubation of (**49**) with IPNS gave two products (**50**, **51**; ratio **50**:**51** = >3:1),[71] corresponding to ring closure onto the allylcarbinyl radical and cyclopropylmethylene radical respectively (Scheme 1.11).



SCHEME 1.11

The search for still further probes for radical character led to the synthesis and incubation of the tripeptide LL-AC-D-allylglycine (**52**). This analogue exceeded all expectations, being converted by IPNS to no fewer than six β-lactam products (**53** – **58**) (Scheme 1.12).[72,73]

Excitingly, three of these β-lactam products contained an additional oxygen atom, revealing for the first time the possibility of a mono-oxygenase pathway for closure of the second ring for IPNS, which will be discussed later. The three products from the desaturase pathway (**53**, **54** and **55**) are again consistent with radical formation, as they would result from trapping of an allylic radical at either the 3 or 5 position.

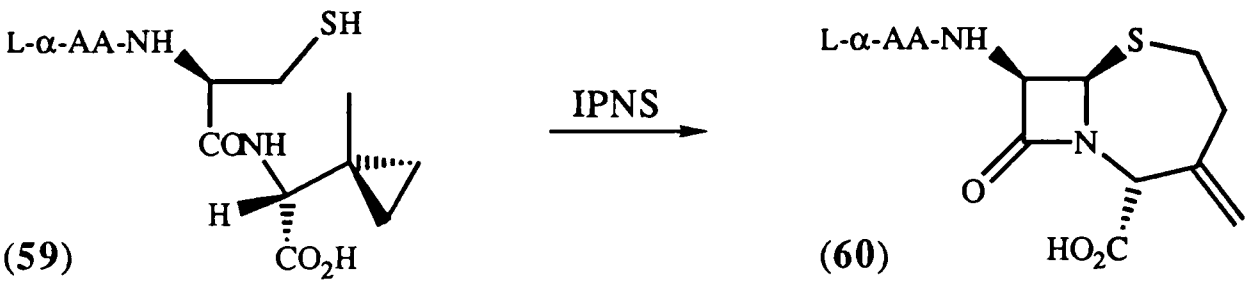


Product Ratio:

53:**54**:**55**:**56**:**57**:**58** = 4:1:10:3:10:trace

SCHEME 1.12

However, these last two cited examples[71,72,73] are not conclusive evidence for radical involvement, as in each case a concerted mechanism can be written to account for the rearranged products. This necessitated further investigation and so it was decided to synthesise isotopomers of the cyclopropyl containing tripeptide (**59**), an analogue which is converted by IPNS to a single β-lactam product (**60**) (Scheme 1.13).[71]



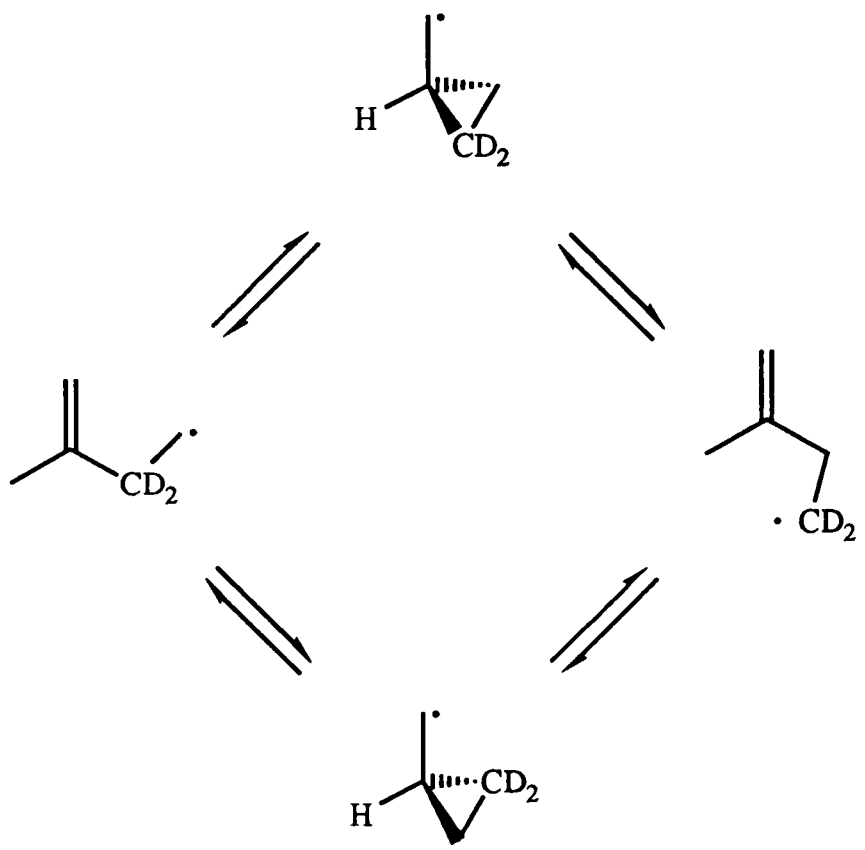
SCHEME 1.13

It was known from earlier experiments on LLD-ACV chirally labelled with $^{13}CH_3$ (**20**), that such branched systems are not free to rotate in the enzyme active site (see section 1.5.2). It was proposed that by specifically labelling each of the two cyclopropyl methylene groups of (**59**), to give the substrate analogues (**61**) and (**62**), it would be possible to test for the formation of a cyclopropyl radical. Such a system, by analogy with the cyclopropylmethylene radical, would be expected to rapidly rearrange, giving scrambling of the label in the incubation product (Scheme 1.14).

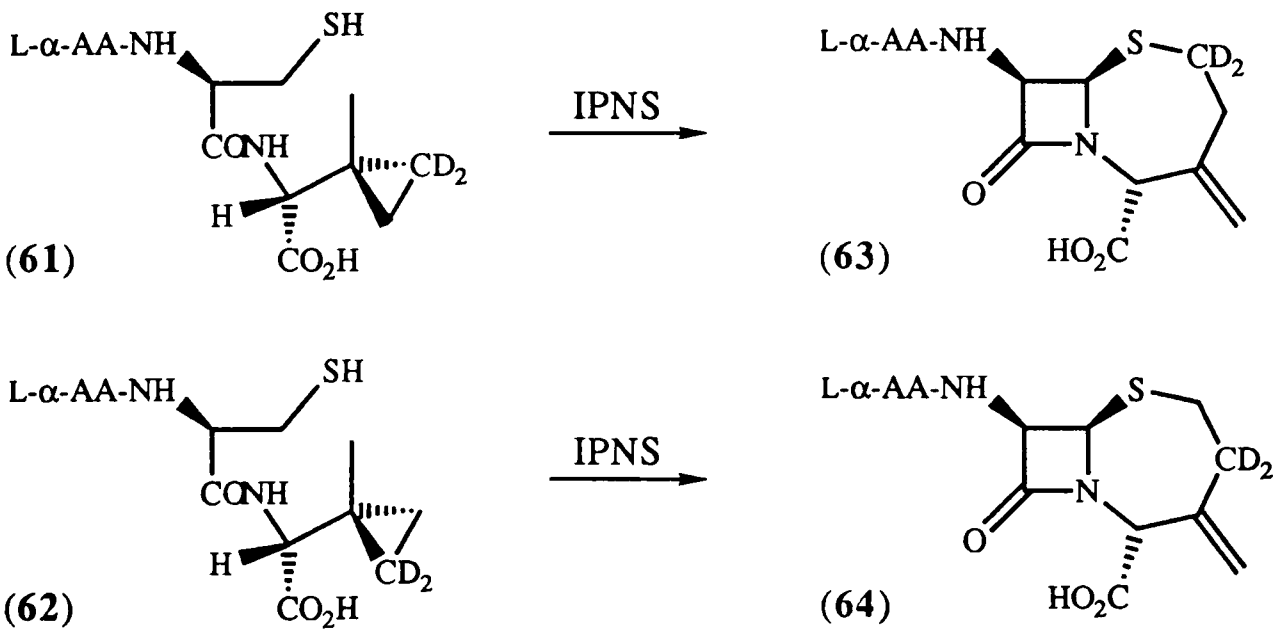
However, incubation of (**61**) and (**62**) with IPNS, gave the β-lactams (**63**) and (**64**) respectively, in which no scrambling of the label was seen (Scheme 1.15).[74,75]

Since a conformationally constrained system would also explain the lack of any scrambling of the deuterium label, a yet closer examination was necessary to differentiate between a concerted and a radical mechanism. A much faster 'radical clock' was examined through the introduction, in separate experiments of a chiral label into each of the cyclopropyl methylene groups, to give the tripeptides (**65**) and (**66**). In this case only a rotation around the C-CDH$^{\bullet}$ bond is required to give scrambling, a process which is faster than any conceivable ring closure reaction. Incubation of the analogue (**65**) with IPNS yielded the exomethylenehomocepham (**67**), in which complete scrambling of the deuterium label was observed (Scheme 1.16).[75] In contrast the

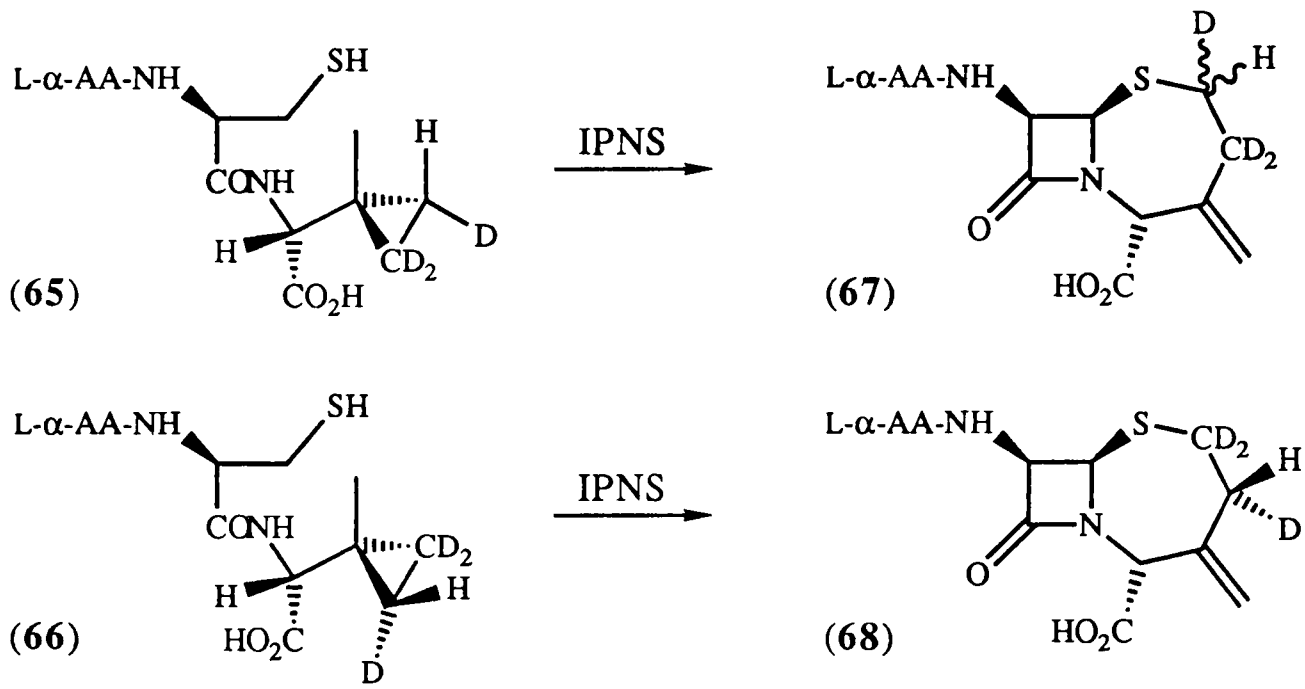
analogue (**66**) gave the product (**68**), in which the stereochemistry of the label had been conserved (Scheme 1.16).[76]



SCHEME 1.14



SCHEME 1.15

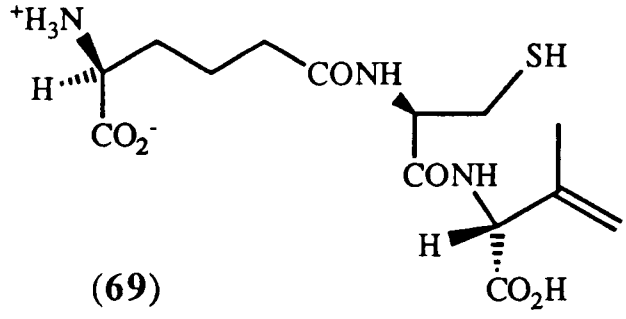


SCHEME 1.16

These results demonstrate the formation of a centre at the cyclopropyl-3*S*-methylene group in which the stereochemistry is lost (**65** → **67**), a result which is consistent with radical formation (however, other species can also be written to explain this observation). The second result, (**66**) → (**68**), in which no stereochemical information is lost, suggests that the putative radical formation follows a concerted rearrangement, since any formation of a radical of the cyclopropylmethylene type would be expected to also introduce radical character into the C(3)–3*R*-methylene bond (see Scheme 1.14).

Similar experiments on specifically labelled isotopomers of LL-AC-D-allylglycine (**52**) and LL-AC-D-3,4-dehydrovaline (**69**), giving a cepham (**70**), showing retention of stereochemistry,[77,78,79] are also indicative of an initial concerted type rearrangement, followed by hydrogen abstraction, although in these cases conformationally rigid allylic radicals cannot be ruled out.

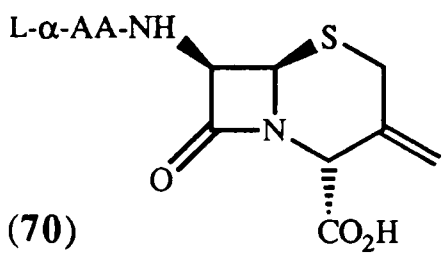
In summary, we have seen from these experiments that the desaturase pathway is sensitive to bond strengths, and subject to a strong primary kinetic isotope effect. The results are consistent with the involvement of radical intermediates, and in analogues containing unsaturated, or cyclopropyl groups, there is also evidence to support concerted rearrangement of the side chain prior to closure of the second ring.



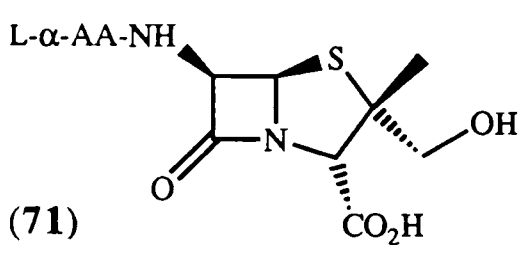
(69)

ii) **Mono-oxygenase Pathway**

We have seen earlier in this section that the incubation of LL-AC-D-allylglycine (**52**) with IPNS, gave six β-lactam products, including (**56**), (**57**) and (**58**), in which an additional oxygen atom is incorporated.[72,73] This mono-oxygenase pathway has since been seen to operate with other unsaturated substrate analogues; for example with LL-AC-D-3,4-dehydrovaline (**69**), which gives two β-lactam products with IPNS, (**70**) and (**71**), deriving from the desaturase and mono-oxygenase pathways respectively.[80]



(70)



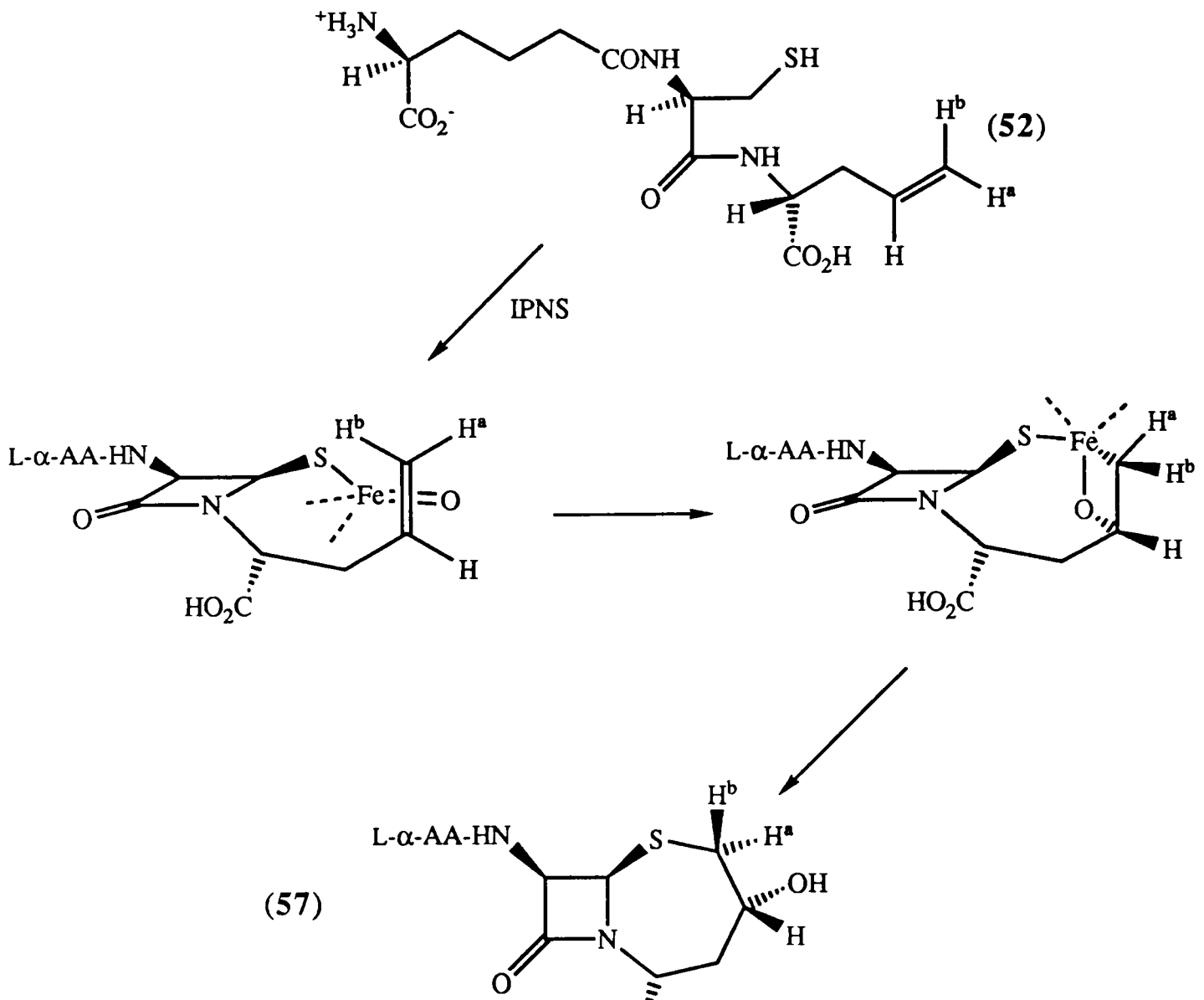
(71)

In the case of both (**52**) and (**69**), incubations under an $^{18}O_2$ atmosphere have shown that the additional oxygen atom is derived from the cosubstrate dioxygen.[81]

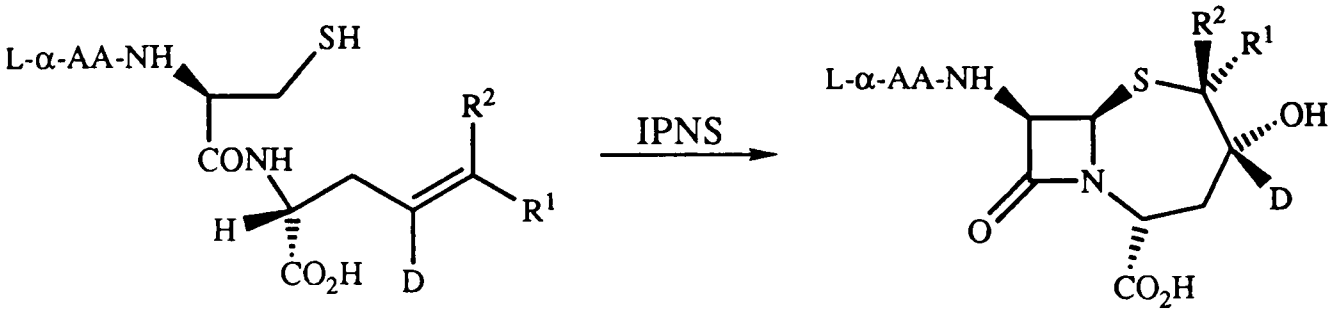
Results obtained to date have identified two distinct mechanisms for this type of addition across a double bond with IPNS, where addition of sulphur and oxygen occurs in either a net *syn* or *anti* fashion.

Syn addition is suggestive of a $[2\pi_s + 2\pi]$ cycloaddition of an iron-oxene species, followed by reductive elimination (Scheme 1.17). This stereochemical pathway has been shown to operate in the formation of the 4α-hydroxyhomocepham (**57**) from LL-AC-D-allylglycine (**52**) and of the 3α-hydroxymethyl-3β-methylpenam (**71**) from LL-AC-D-3,4-dehydrovaline (**69**), through the synthesis and incubation of specifically deuterated isotopomers. In these experiments the isotopomers (**72**) and (**73**) of (**52**) were converted stereospecifically to the 4α-hydroxyhomocephams (**74**) and (**75**) (Scheme 1.18),[77,78] and the isotopomers (**76**) and (**77**) of (**69**) to the 3α-hydroxymethyl-3β-methylpenams (**78**) and (**79**).[79] In the latter case stereochemistries were determined by NOE experiments on the corresponding lactones (**80**) and (**81**) (Scheme 1.19).

The second mono-oxygenase mechanism, that of overall *anti* addition, across a double bond has, to date, only been shown conclusively for one β-lactam product, the 4β-hydroxyhomocepham (**58**). This β-lactam product is the most minor component of the mixture of six β-lactams formed from the substrate analogue LLD-AC-allylglycine (**52**), and was only detected after enhancement of its level of production through the introduction of a dideutero label at the C(3) position of the allylglycine residue.[73] Further specific labelling at the olefinic terminus, to give the tripeptides (**82**) and (**83**), and



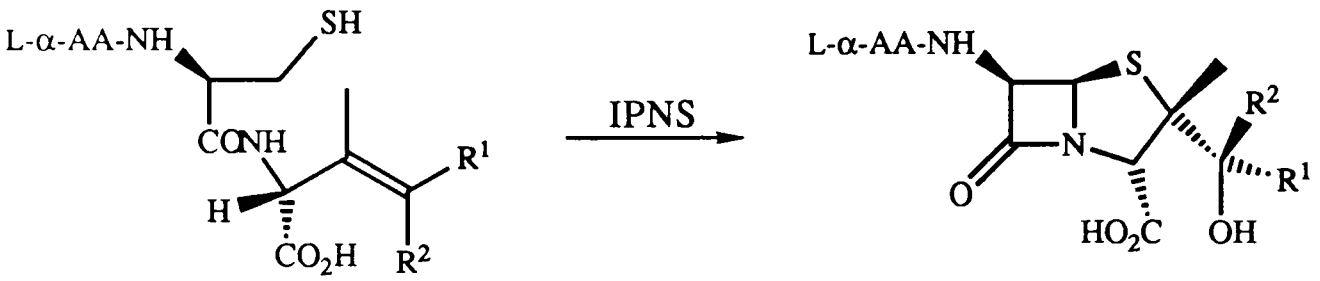
SCHEME 1.17



(**72**) R^1 = D, R^2 = H
(**73**) R^1 = H, R^2 = D

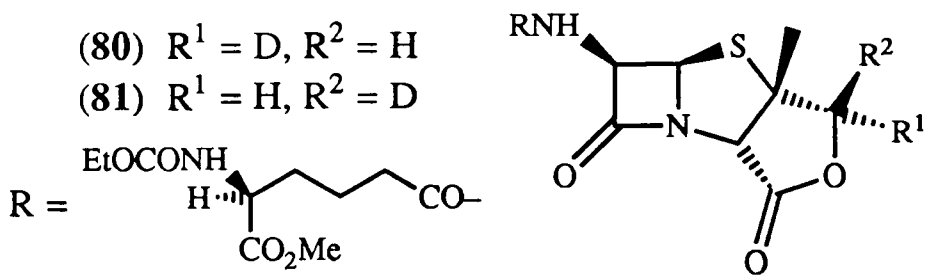
(**74**) R^1 = D, R^2 = H
(**75**) R^1 = H, R^2 = D

SCHEME 1.18

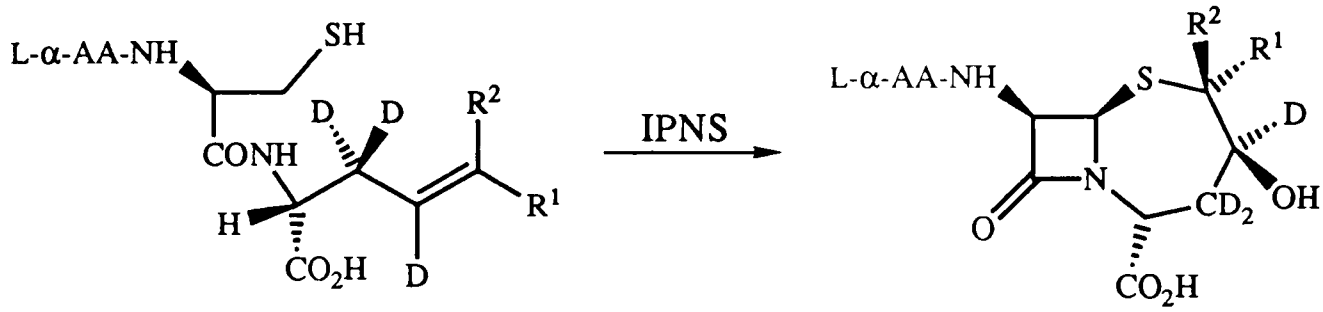


(**76**) R^1 = D, R^2 = H
(**77**) R^1 = H, R^2 = D

(**78**) R^1 = D, R^2 = H
(**79**) R^1 = H, R^2 = D



SCHEME 1.19



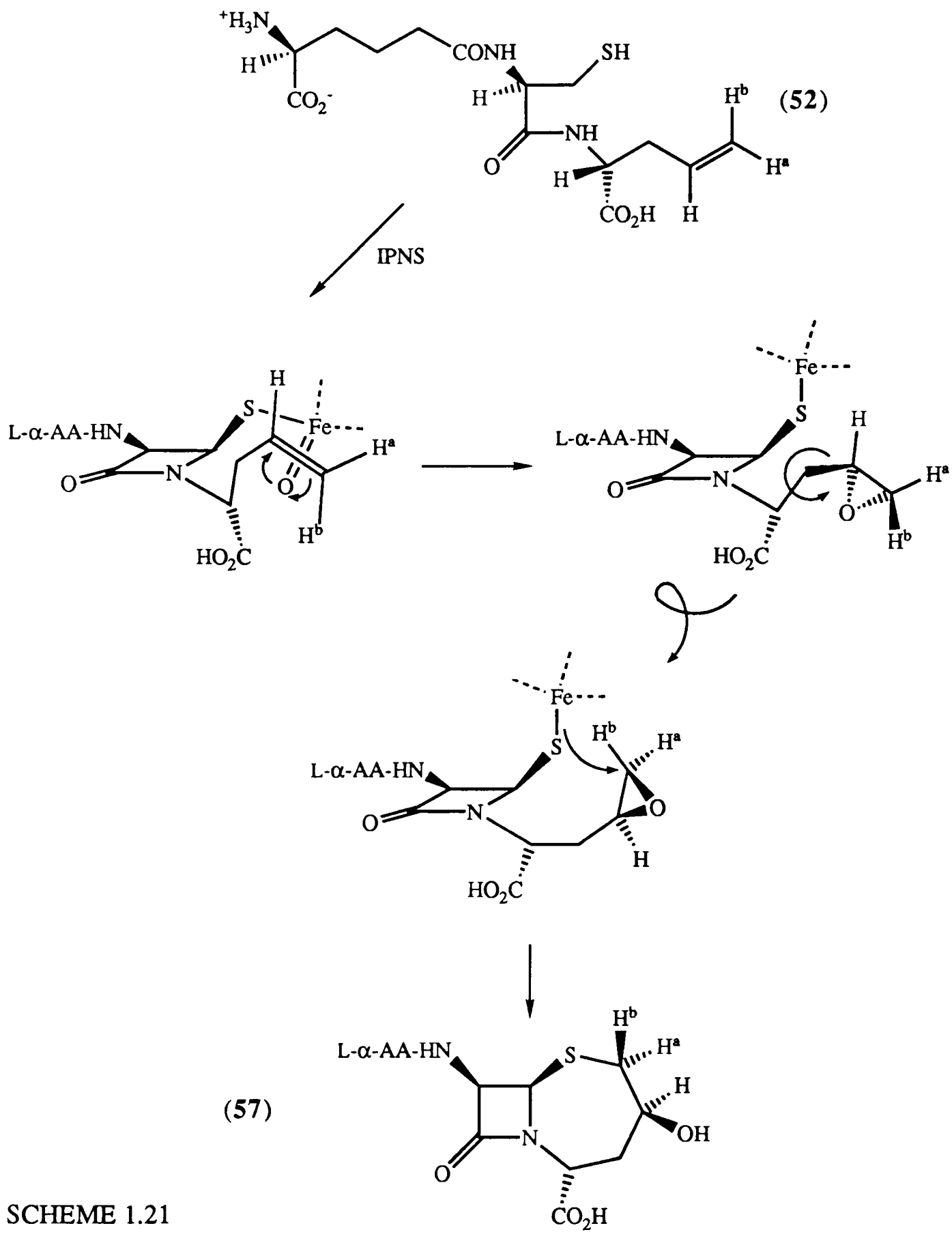
(**82**) R^1 = D, R^2 = H
(**83**) R^1 = H, R^2 = D

(**84**) R^1 = D, R^2 = H
(**85**) R^1 = H, R^2 = D

SCHEME 1.20

incubation of these analogues with IPNS, allowed the isolation of the labelled 4β-hydroxyhomocephams (**84**) and (**85**). NOE experiments on these β-lactams indicated that the stereochemistry of these products were consistent with *anti* addition across the double bonds of (**83**) and (**84**) (and hence by inference of (**52**)) during closure of the second ring (Scheme 1.20).[73] This is consistent

with a mechanism involving epoxide formation,† followed by ring opening at C(5) with inversion (Scheme 1.21).



SCHEME 1.21

† The enzymatic epoxidation of terminal olefins has good precedence, and has even been proposed as a synthetic tool.[82]

To summarise, we have seen that for many substrate analogues, in which the valinyl residue is replaced by an unsaturated amino acid, their is a facile monooxygenase pathway for second ring closure with IPNS. The predominant pathway appears from results gained so far, to involve *syn* addition across the double bond, and a mechanism has been proposed involving [$2\pi_s$ + 2π] addition of an iron-oxene species across the double bond, followed by reductive elimination of sulphur to form the sulphur-carbon bond with retention. A second, seemingly less facile, mechanism is also indicated, involving *anti* addition of sulphur and oxygen, which is consistent with a mechanism involving epoxide formation across the double bond, followed by opening of the epoxide by sulphur with inversion of configuration.

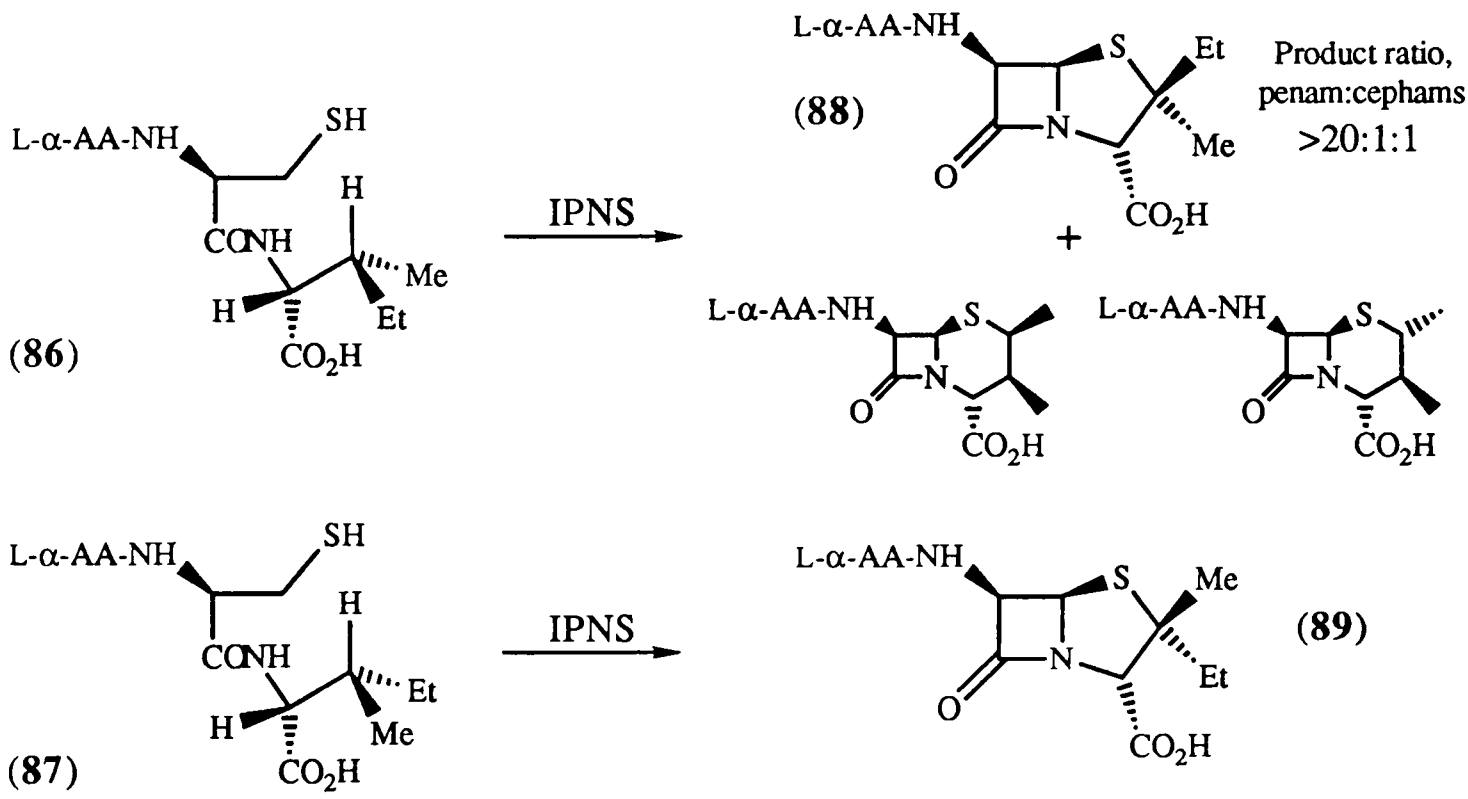
iii) Active site Topology and Environment

With attempts to crystallise IPNS for X-ray diffraction studies meeting with little success so far, most of our knowledge about the active site topology of IPNS comes from inferences made from the results of incubations of substrate analogues. This has already been discussed in relation to variations of the δ-(L-α-aminoadipoyl) and L-cysteinyl residues in parts A and B of this section. Similarly, variations of the valine component have allowed a fairly extensive, if not always unambiguous, mapping of the active site topology in the region of the second ring closure to be made.

All these inferences suffer from the lack of conclusiveness, not least in that by examining product ratios we are forced to make the unfounded assumption that all products are equally stable to the incubation and work up procedures. However, in the absence of a crystal structure, these results do provide us with valuable insights into the nature of the topography and make up of the IPNS active site.

Firstly, it has been found that only analogues of LLD-ACV (**1**) where valine is replaced by a D-configured amino acid are acceptable substrates for IPNS, indicating, as might be expected, the importance of the carboxyl group in substrate binding. Beyond this point there is a fair degree of conformational flexibility, and rings containing up to eight atoms have been formed.[45] We shall examine the restrictions on analogue variations imposed by the active site topology as we move successive atoms away from the valine carboxylic acid group.

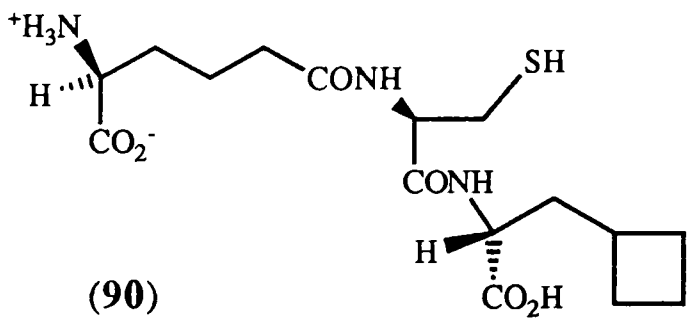
Results have shown that whilst rotation around the C(2)–C(3) bond is possible if there is only one C(3) substituent (as seen earlier with the deuterated aminobutyric acid containing tripeptides (**36**) and (**37**), Scheme 1.8), the system becomes conformationally restricted if a second substituent is introduced. This has been seen in (**20**), the isotopomer of LLD-ACV bearing a specific 3*R*-$^{13}CH_3$ group,[54] and is also observed, for example, in the 2*R*,3*R*-and 2*R*,3*S*-2-amino-3-methylpentanoic acid containing analogues (**86**) and (**87**), which yield (**88**) and (**89**) as the sole penam products (Scheme 1.22).[68]



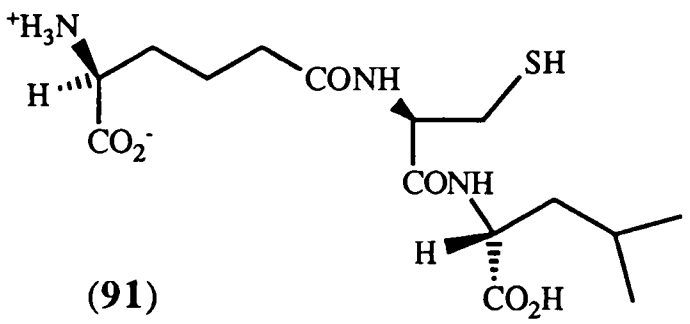
SCHEME 1.22

Branching of the chain at C(4) or beyond is unacceptable within the IPNS active site, with the exception of the conformationally restricted cyclopropyl system. Whereas the D-3-cyclopropylalanine containing tripeptide (**49**) is turned over by IPNS, though only poorly (~10% conversion),[71,83] the D-3-cyclobutylalanine and D-leucine containing tripeptides (**90** and **91**) are not acceptable substrates.[55,83,84]

If we examine the effect of increasing chain length on substrate acceptability, we find a difference depending on whether the replacement for the valine residue bears a saturated or unsaturated side chain. For saturated systems a chain length of five atoms (counting from the carboxylic acid carbon) is maximal, with LL-AC-D-2-aminopentanoic acid (**42**) being converted by IPNS,[68] and the D-2-aminohexanoic acid containing analogue (**92**) being unacceptable.[55] For unsaturated systems however, analogues bearing chains of six and even seven atoms have proved to be acceptable in the form of the analogues (**93**),[80] (**94**)[85] and (**95**).[85]

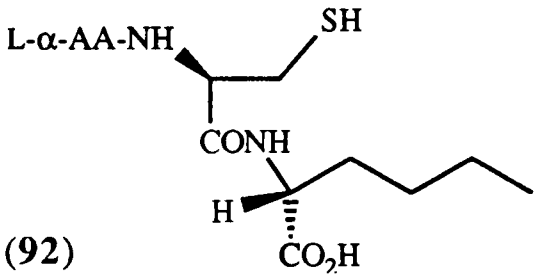


(**90**)

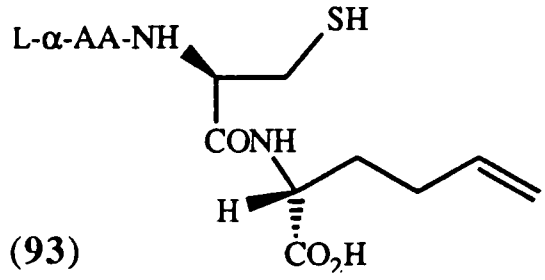


(**91**)

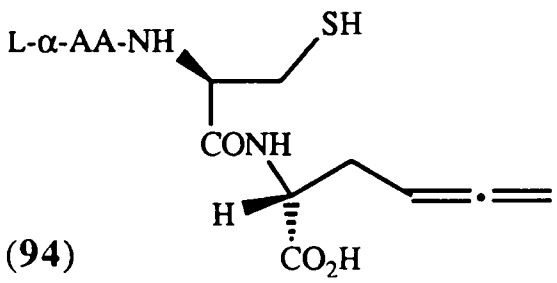
These results point to a fairly large cleft in the enzyme active site, able to accept substrate analogues with residues containing straight chains in place of the valine residue, but which is of restricted width, being unable to accommodate branching in the chain beyond the point found in the natural substrate LLD-ACV (**1**).



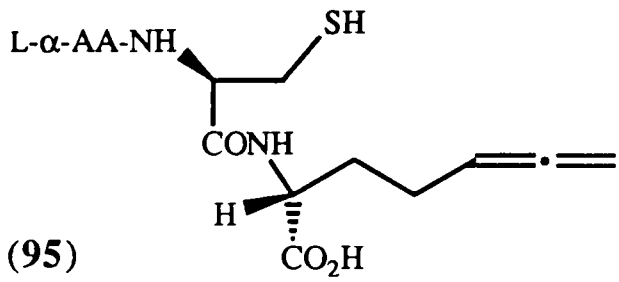
(92)



(93)

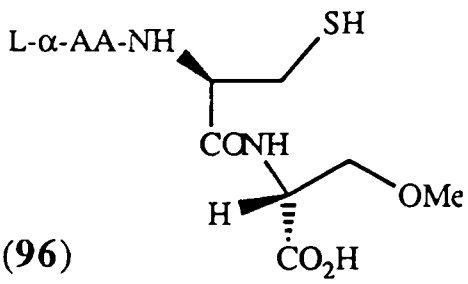


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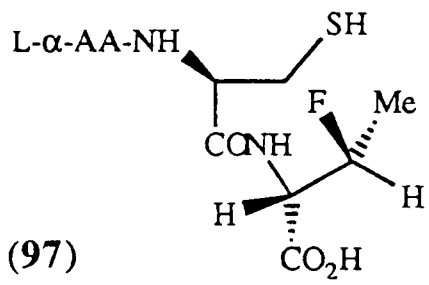


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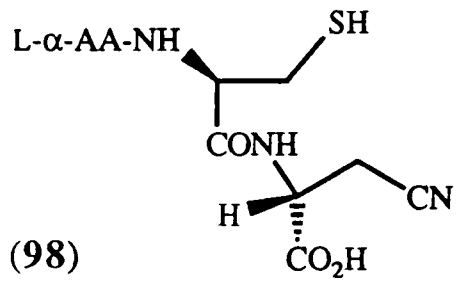
Finally, there are a number of results from analogue studies which point to the nature of the amino acid residues present in the region of the IPNS active site normally occupied by the valinyl residue. Analogue studies utilising residues with polar side chains have indicated that this region of the IPNS active site contains mainly hydrophobic residues. This conclusion arises from the observation that in general, analogues containing polar side chains are found to be unacceptable substrates for IPNS. Whilst there are certain exceptions, such as LL-AC-D-*O*-methylserine (**96**),[86] LL-AC-D-2-amino-3*R*-fluorobutyrate (**98**)[55,87] and LL-AC-D-3-cyanoalanine (**98**),[69,88] conversions of these analogues by IPNS have all been low (all <10%).



(96)



(97)

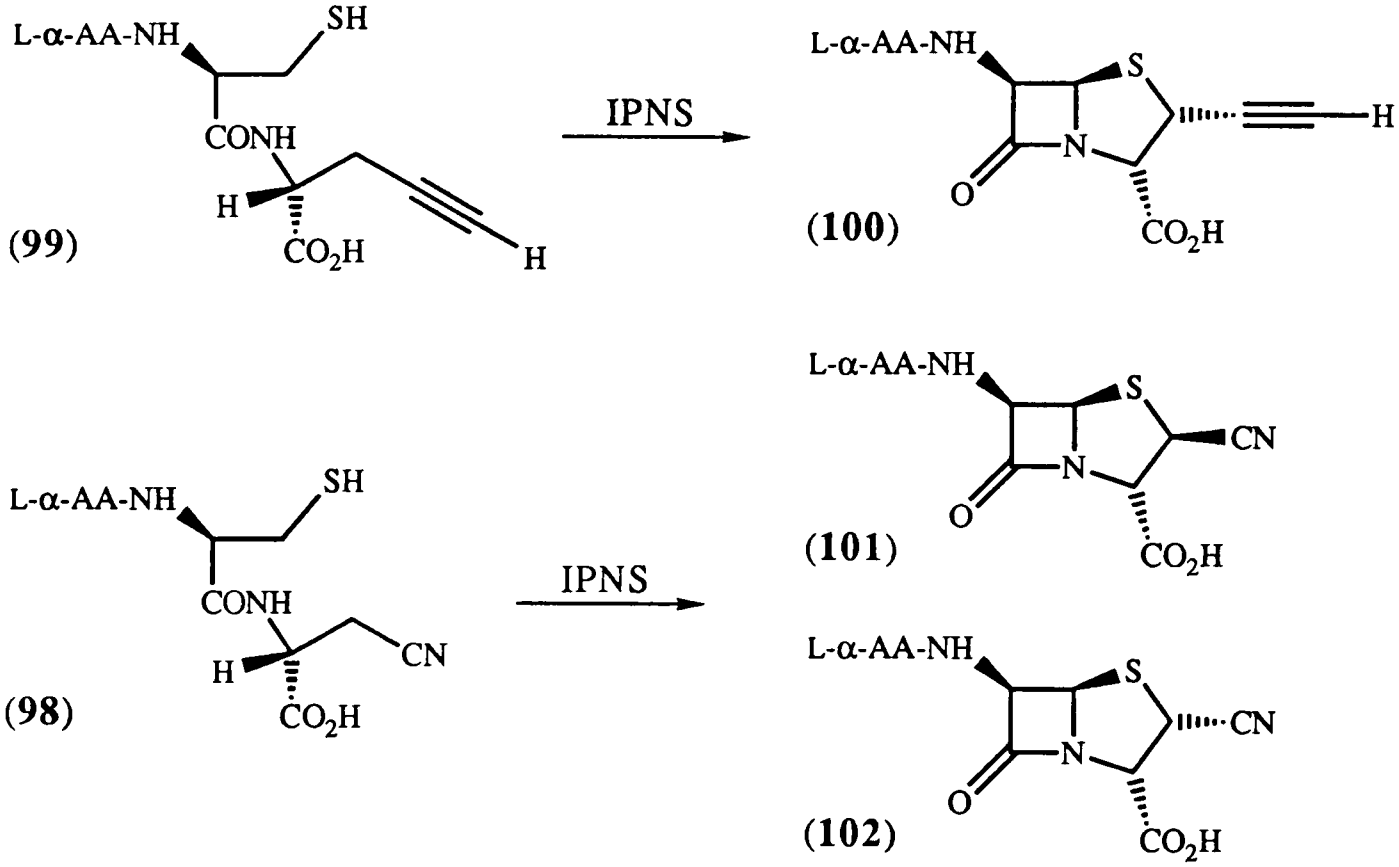


(98)

The analogue (**98**) is particularly interesting when compared to the acetylenic analogue (**99**). These tripeptides are both sterically and elec-

tronically similar, yet whereas (**99**) is converted by IPNS to a single penam product (**100**) with high conversion,[69,88] (**98**) is only a poor substrate, giving a 1:1 mixture of the 3-cyanopenams (**101**) and (**102**) (Scheme 1.23).[69,88] This difference was thought to arise from considerations of polarity, with the polarity of the nitrile functionality leading to unfavourable interactions in the IPNS active site.

The hydrophobicity of this region of the IPNS active site might be expected intuitively, both from the chemical nature of the valinyl terminus of the natural substrate, LLD-ACV (**1**), and from the possible formation of a high valence iron-oxene species during catalysis, which might be expected to be surrounded by residues bearing side chains of low reactivity, such as hydrocarbons.

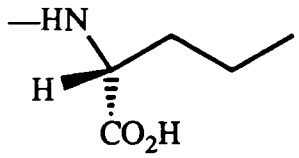
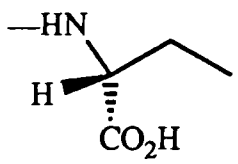
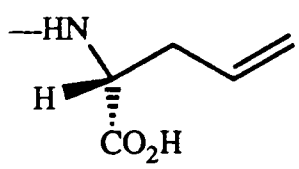
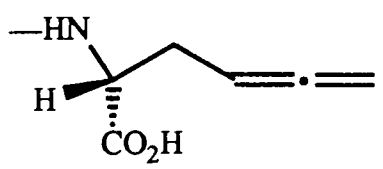
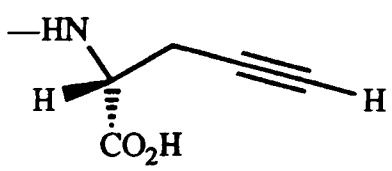


SCHEME 1.23

Comparison of the ratio of α- and β-penams bearing hydrocarbon side chains formed during various incubations, indicates preferential formation of 3β-penams for saturated side chains, and 3α-penams for unsaturated ones (Table 1.2). It is thought likely that this reflects the relative binding energies of such groups in the α- and β-positions of the transition states leading to ring closure of the penam system, and may be due to the presence of unsaturated protein residues (e.g. phenylalanine, tyrosine) close to where α-substituents sit in these transition states.

TABLE 1.2

The ratios of α- to β-penams formed from various substrate analogues

Tripeptide LLD-ACR R =		Penicillins Formed	(β:α ratio)	Reference
	**(48)**	Ethyl	β-product only	67,68
	**(32)**	Methyl	(**33**:**34** = 7:1)	63,64
	**(52)**	Vinyl	(**53**:**54** = 4:1)	72,73
	**(94)**	Allenyl	(1:2)	85
	**(99)**	Ethynyl	α-product only	69,88

In summary, these results indicate in a qualitative manner some aspects of the nature of the IPNS-active site, in the region probed by substrate analogues in which the valine residue has been replaced by another amino acid. The picture which begins to emerge gives us the following insights into the likely nature of this region:

a) Binding of the carboxylic acid group is essential for turnover of tripeptides with IPNS.

b) The chemical nature of this region is hydrophobic, with the possibility of aromatic amino acid residues being present in the area around the α-substituent.

c) The cleft has a topology such that branching beyond C(3) of the amino acid replacing the valine residue cannot be accommodated.

d) The enzyme will accommodate fairly long (see above) linear chains in the replacement amino acid, but it is not possible to say if these chains lie totally within the active site

1.6 Summary of Introduction

In this chapter we have seen something of the complexities underlying the transformation of LLD-ACV (**1**) to isopenicillin N (**2**) by IPNS.

After examination of the key position of the IPNS-catalysed reaction in the biosynthesis of the penicillin and cephalosporin antibiotics, the intimate stereochemical details of the reaction have been reviewed, establishing that IPNS catalyses the formation of the two rings of the penam nucleus in a stepwise manner, with control of stereochemistry at each step. Furthermore, it has been shown how the use of organic chemistry, through the synthesis of a wide variety of substrate analogues, has been a useful tool for eliciting information

about the details of the mechanisms involved in β-lactam formation, as well as providing evidence to support the formation of an enzyme bound monocyclic β-lactam intermediate. These studies have also indicated the possibility, with certain substrate analogues, of multiple pathways for formation of the second ring, and have provided some qualitative insight into the active site topology for IPNS from *C. acremonium*.

The work reported in this thesis continues these investigations, both through the 'traditional' substrate analogue approach, and through the involvement of techniques traditionally considered to belong to physical chemistry and biochemistry. A multidisciplinary approach has been found to be necessary to investigate a number of questions relating to the IPNS-catalysed reaction.

CHAPTER 2

ORDER OF SUBSTRATE BINDING TO IPNS

2. ORDER OF SUBSTRATE BINDING TO IPNS

2.1 Introduction

In converting LLD-ACV (**1**) to IPN (**2**), the enzyme IPNS binds both dioxygen and the tripeptide LLD-ACV (**1**). However, no studies have been carried out on the kinetics of binding of the two substrates. It was decided to approach the question from two angles: firstly through the use of an NMR experiment in an attempt to observe O_2 binding in the absence of LLD-ACV; and secondly through the use of initial rate kinetic studies to elucidate the mechanism of substrate binding.

2.2 NMR Studies

2.2.1 Theory

These experiments are based on the fact that the resonance frequency of a line in an NMR spectrum is in part dependent on the magnetic susceptibility of the solution used.[89] In the vast majority of NMR experiments the frequency shift resulting from the solvent is not detectable, as the spectrum is referenced internally to a line which will itself be shifted by the same magnitude. In addition, the magnetic susceptibility of a diamagnetic solution is of such a small magnitude that it will produce a frequency shift of only a fraction of a hertz.

The most commonly encountered paramagnetic species in NMR experiments is oxygen dissolved in solutions, a factor which is frequently overlooked in the acquisition of standard NMR spectra. The frequency of any NMR resonance will be in part dependent on the concentration of this dis-

solved oxygen and, by measuring the observed frequency shift it is possible to determine the concentration of oxygen in solution. The magnetic susceptibility,† χ, of oxygen is related to the frequency shift of an NMR resonance, $\Delta\delta$, according to equation 2.1,¶ and is in turn related to the effective magnetic moment, μ_{eff}, according to equation 2.2.¶ Since both these quantities are known for oxygen, we can calculate the concentration of oxygen in solution according to equation 2.3.

2.2.2 Development of Experimental Methodology

The requisite frequency shift was measured by observation of a ^1H NMR reference line, in this case the trimethylsilyl peak of 3-(trimethylsilyl)-1-propanesulphonic acid, sodium salt (DSS). A 3mm diameter reference tube, ending in a capillary, was introduced into a standard 5mm diameter NMR tube containing the sample, as shown in Figure 2.1. Both sample and reference were made up with 50m<u>M</u> TRIS buffer in D_2O solution at the same pD. Due to the volume ratio of the capillary to the external solution, it was found to be necessary to have different DSS concentrations between them (see Figure 2.2). For these experiments 0.2m<u>M</u> and 5.0m<u>M</u> DSS were used for the sample and reference solutions respectively.

The reference tube was made up as above, the solutions were then thoroughly degassed, followed by saturation with argon, before being sealed. Positioning of this tube inside a sample containing dissolved oxygen, allowed the frequency shift in the sample due to oxygen to be measured directly from the frequency difference between the two DSS resonances observed in the ^1H NMR spectrum, typically as shown in Figure 2.3.

† This is a dimensionless quantity, but whose value differs in different systems of units.
¶ The derivation of these equations is given in Appendix A.

$$\chi = -\frac{3\Delta\delta\rho''}{\nu c_m} + \chi_0 \qquad \text{Equation 2.1}$$

$$\mu_{eff}^2 = \frac{3kTM_m}{N_A\rho^o\mu_0\mu_B^2}\chi \qquad \text{Equation 2.2}$$

$$[O_2]/\underline{M} = \frac{3\Delta\delta\rho^{\ominus}}{1000\nu M_m}(\chi_0 - \chi) \qquad \text{Equation 2.3}$$

Where: $\rho'' = 1 kg.m^{-3}$ (concentration).

ν is the resonance frequency of the NMR spectrometer.

c_m is the mass concentration of the paramagnetic solute ($kg.m^{-3}$).

χ_0 is the mass susceptibility of the solvent (This has the value of -9.0×10^{-9}, in the SI system, for H_2O at 298K).[90]

k is the Boltzmann constant.

T is the absolute temperature.

M_m is the molar mass of the paramagnetic species (in $kg.mol^{-1}$).

$\rho^o = 1 kg.m^{-3}$ (density).

N_A is the Avogadro number.

μ_0 is the vacuum permeability, defined as $4\pi \times 10^{-7} N.A^{-2}$

μ_B is the Bohr magneton $= \frac{e\hbar}{2m_e} = 9.274\,078 \times 10^{-24} J.T^{-1}$

$\rho^{\ominus} = 1 kg.mol^{-1}$.

The mass susceptibility, χ, for O_2 at 293K $= 1.3544 \times 10^{-6}$.[91]

FIGURE 2.1

Diagram of the concentric NMR tubes used for measurement of the line shift $\Delta\delta$

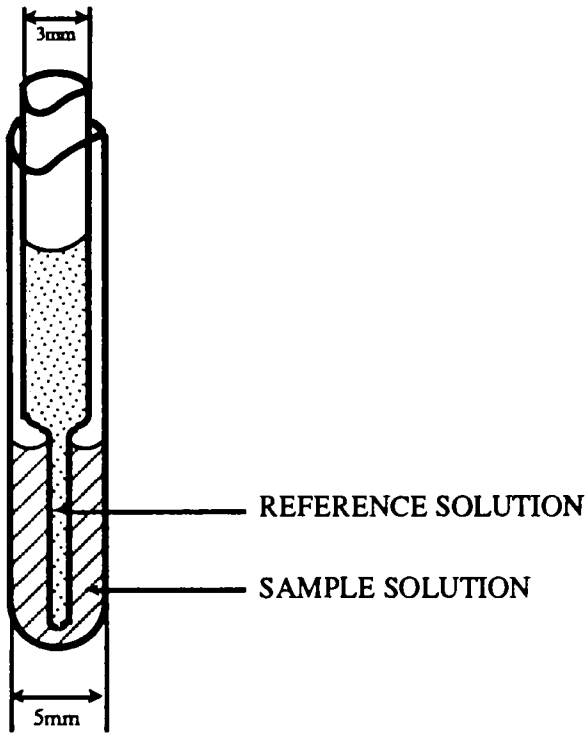


FIGURE 2.2

^1H NMR spectra showing the relative DSS peak heights for two different ratios of DSS concentrations between the inner and outer solutions

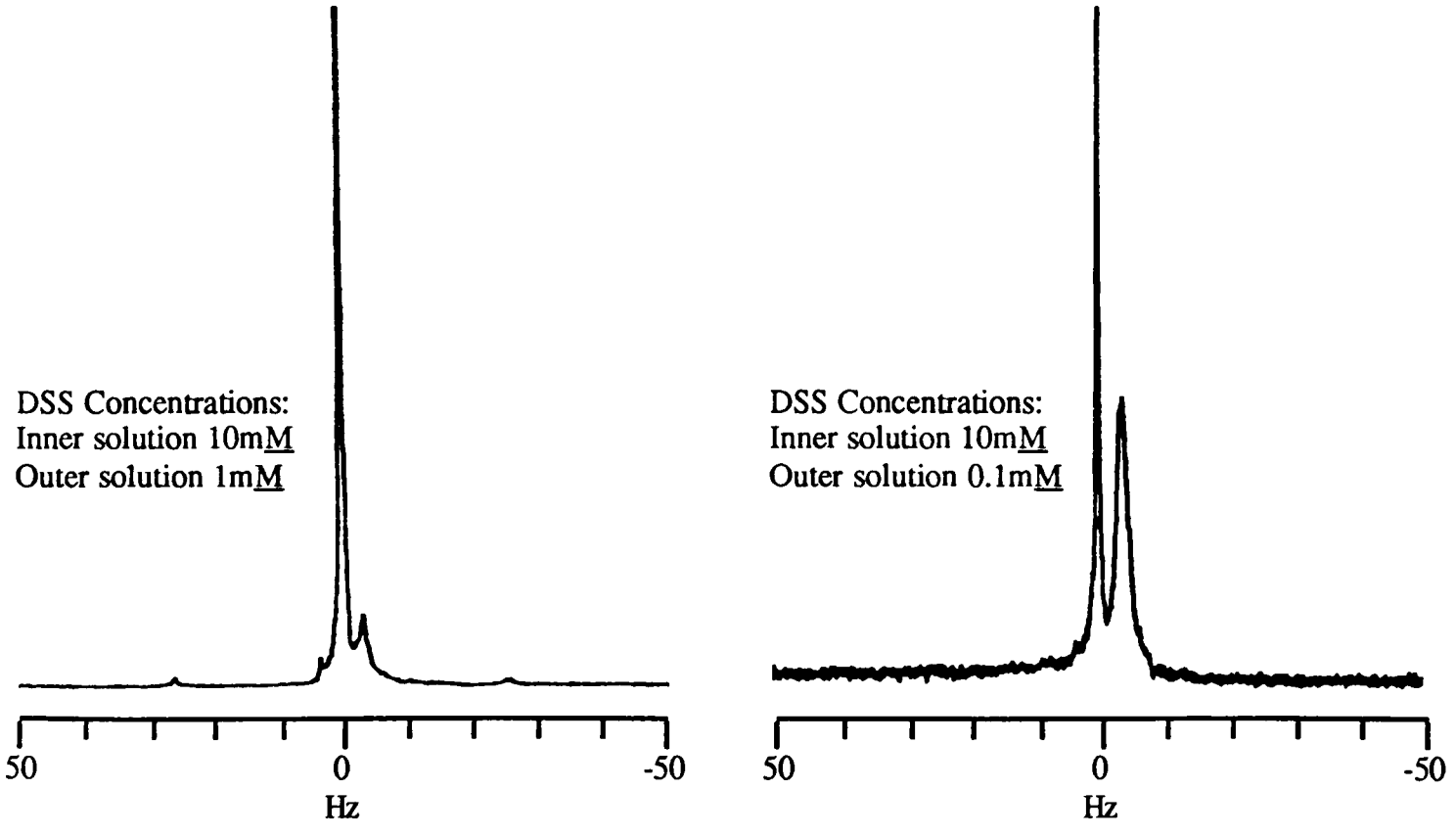
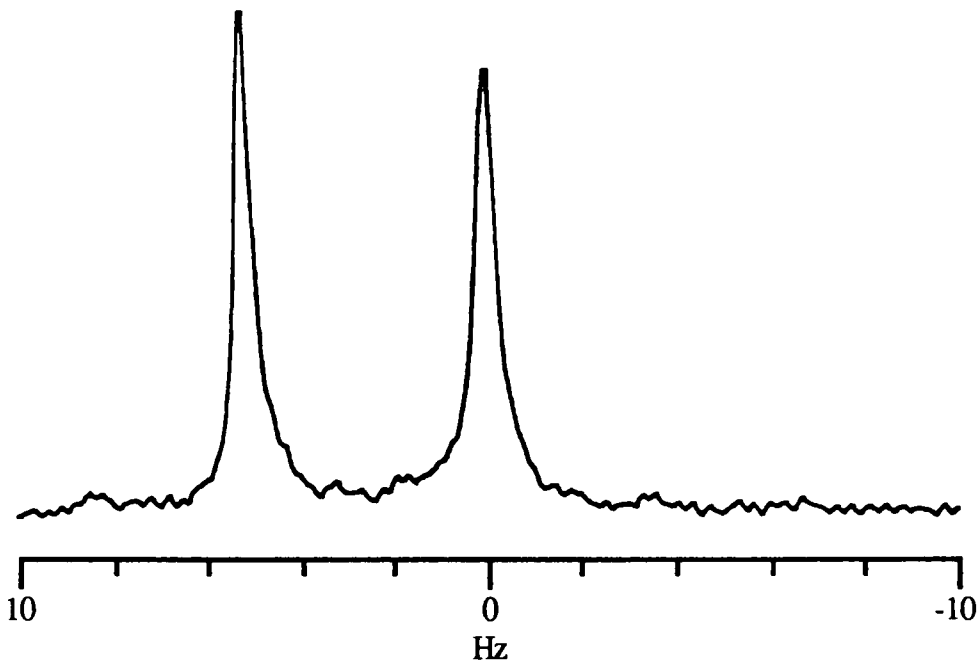


FIGURE 2.3

A typical ^1H NMR spectrum of the DSS resonances arising from the inner (right hand peak) and outer (left hand peak) solutions



The region from 1 to −1ppm was examined at 500MHz, collecting eight transients for each measurement of $\Delta\delta$. The Fourier transforms were generally carried out on unprocessed FID's except where the value of $\Delta\delta$ was very small, when Gaussian multiplication of the FID was carried out beforehand.

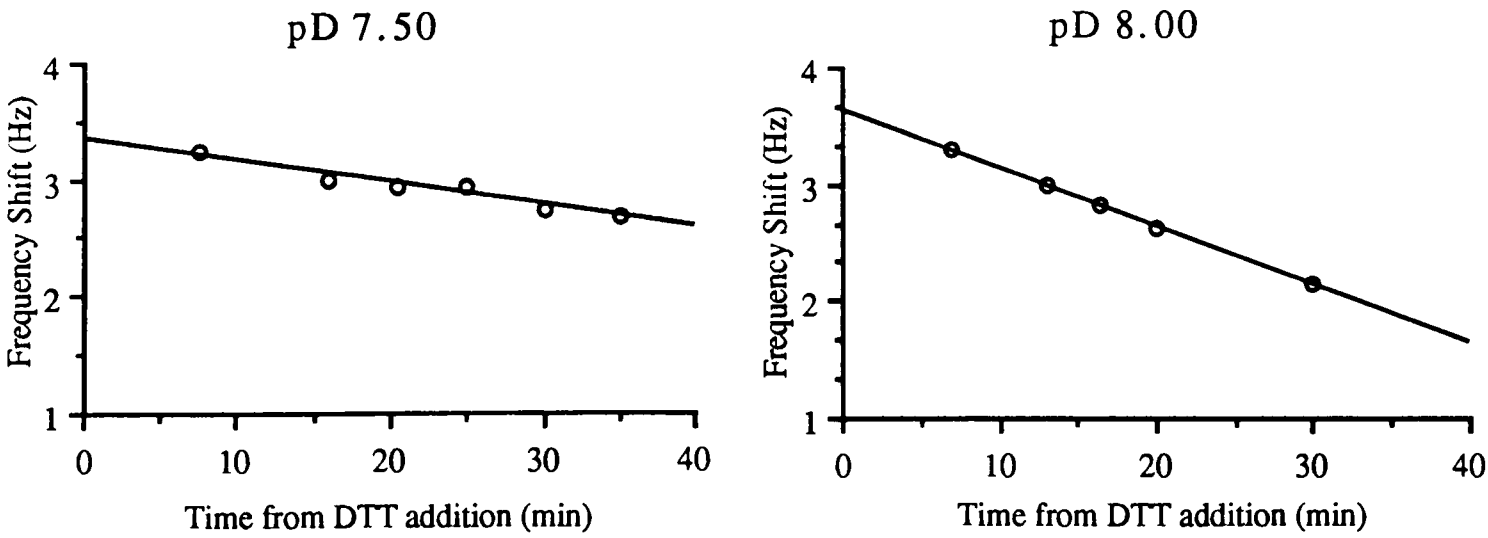
This system was used to measure frequency shifts with the sample solution being saturated with a 1:1 mixture of O_2:N_2 at atmospheric pressure and room temperature. This represents a concentration of *ca.* 600μM at one atmosphere and 298K/25°C.[92]

The experiments did, however, carry a further complication. The addition of IPNS to the sample solution before saturation of the sample with the O_2/N_2 mixture is precluded, as the enzyme is required in its active, dithiol form. Furthermore, this necessitates the presence of DTT to reverse the effects of aerobic oxidation of IPNS to the disulphide form. Since DTT itself reacts with oxygen we are forced to examine a time course, and to extrapolate back to 'time zero' (t_0) to obtain the required frequency shift.

The pD dependence of the reaction between DTT and oxygen was first investigated. 400µl of sample solutions at pD's of 7.00, 7.50 and 8.00 were each saturated with 50% O_2/N_2, and at t_0 were treated with DTT solution (130m<u>M</u> in D_2O; 12µl), to give a final concentration of 4m<u>M</u>. After shaking, the reference tube was introduced, the magnet shimmed, and the samples examined by 500MHz ^1H NMR (For experimental details see chapter 6). The results (Figure 2.4) showed a significant change in reaction rate with pD. From these results it was decided to carry out the experiment at pD 7.50, where the rate is not too rapid to prevent acquisition of the necessary data points, and where we are operating close to the optimum pH for IPNS of 7.8.†,93

FIGURE 2.4

The variation of the rate of oxygen depletion with the pD of the sample solution



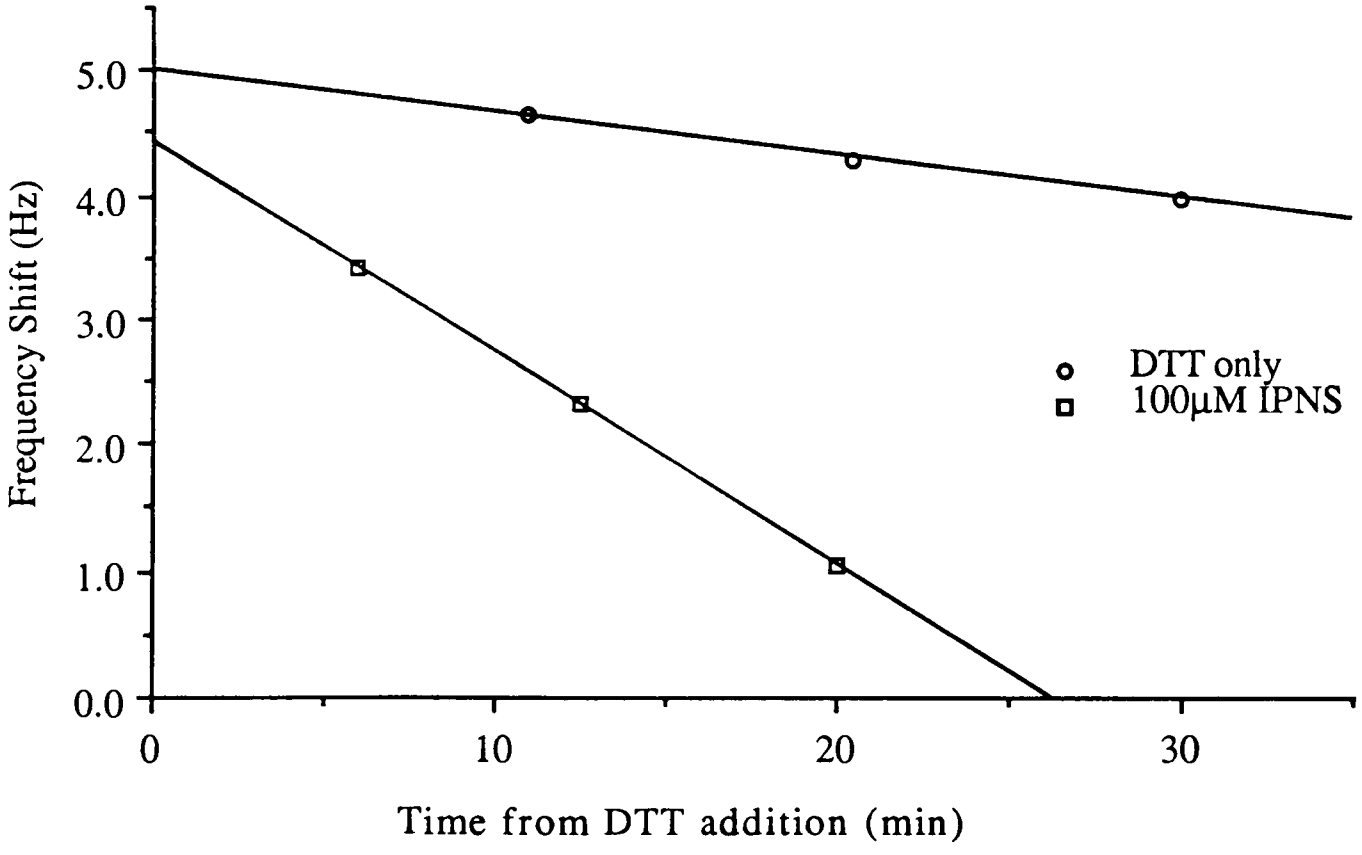
For pD 7.00 $\Delta\delta$ was invariant over 30min.

† pD and pH are related by the formula, pH = pD + 0.4.

For the experiments on IPNS itself, it was necessary to add the enzyme in a small volume of concentrated solution. Thus a quantity of IPNS was lyophilised, redissolved and filtered† to give a solution of active enzyme containing 153mg/ml protein.¶

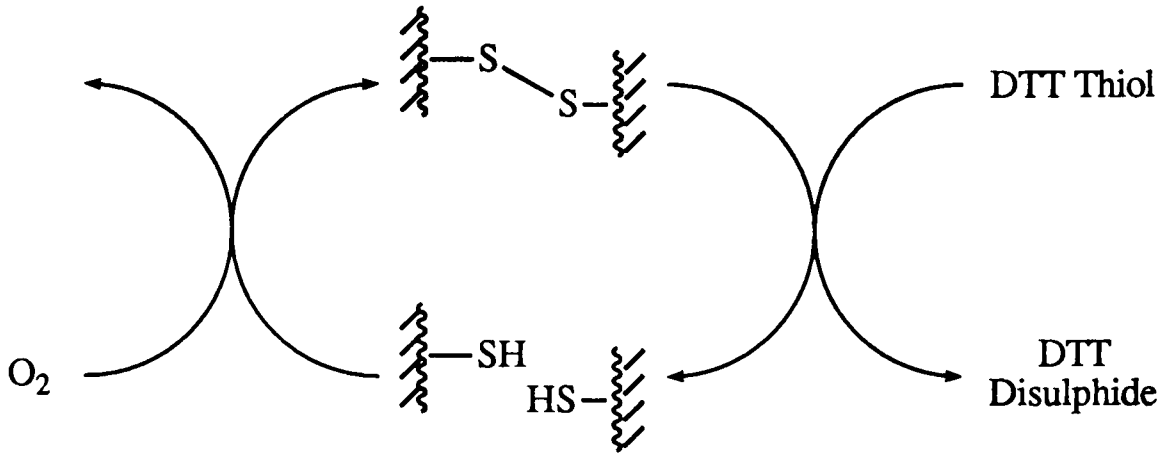
Addition of this solution (10μl), at t_0, to 400μl of sample solution saturated with 50% O_2/N_2, together with DTT solution (130m<u>M</u> in D_2O; 12μl) resulted in time courses such as shown in Graph 2.1. This showed, unexpectedly, a greatly increased rate of oxygen depletion compared to that observed with addition of DTT solution alone. It was considered that this might be due to a reaction cycle such as shown in Scheme 2.1.

GRAPH 2.1
Graph showing the variation of Δδ with
time in the presence and absence of IPNS



† Such procedures have been used successfully to obtain concentrated solutions of active enzyme for investigation of its NMR spectra.[94]

¶ To date there is no assay for determining the concentration of active IPNS in solution, we can merely show that solutions are active using the IPNS bioassay (see Chapter 6).

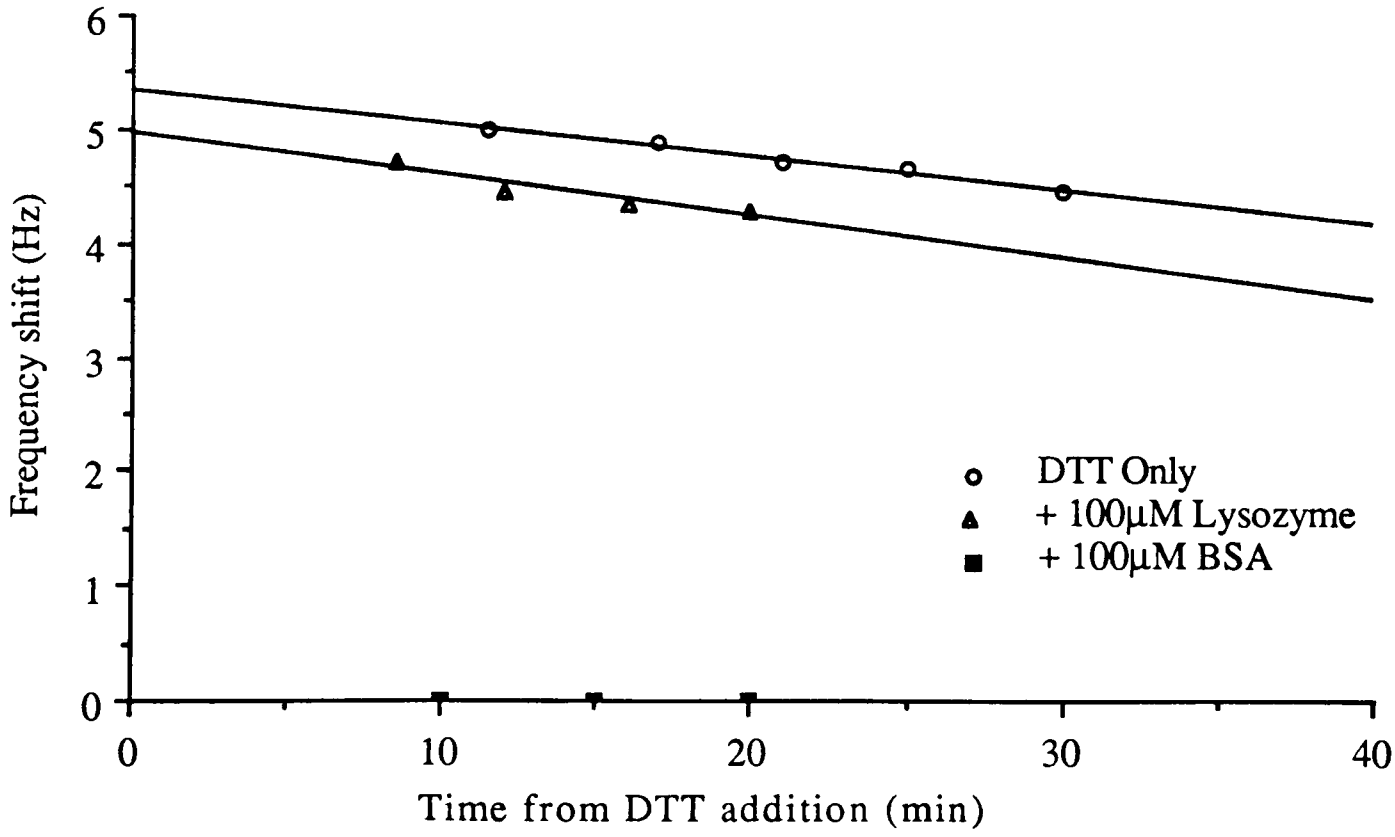


SCHEME 2.1

To test this proposal, the experiment was repeated, firstly using lysozyme, an enzyme which contains no accessible thiol/disulphide linkages which could participate in a reaction cycle as proposed above;[95] and secondly utilising bovine serum albumin (BSA), which contains many such groups.[95]

GRAPH 2.2

Graph showing the variation of $\Delta\delta$ with the addition of lysozyme and bovine serum albumin



The results of these controls (Graph 2.2) were in agreement with the surmise that the presence of free thiol groups capable of forming disulphide linkages increased the rate of depletion of oxygen in solution. The rate of depletion with added lysozyme followed that of DTT alone, whilst in the case of BSA all oxygen was depleted from solution before any transients could be acquired in the NMR spectrum.

2.2.3 Results

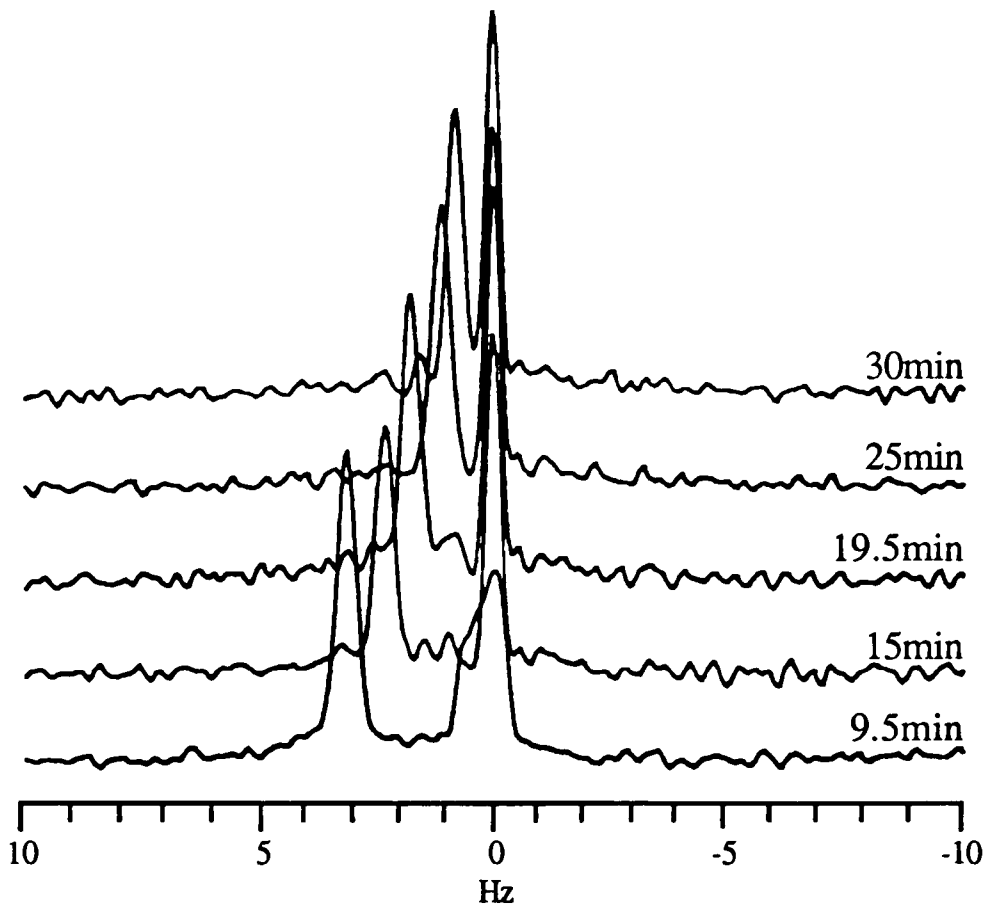
The experiment was carried out using concentrations of IPNS of 100 and 180μM, at oxygen saturations of 50 and 20%. A typical series of ^{1}H NMR spectra is shown in Figure 2.5. The mean results are given in Table 2.1, and a typical result is shown in Graph 2.3. These show a clear reduction in the t_0 oxygen concentration in the sample solution on addition of IPNS, which is linearly dependent on IPNS concentration (Graph 2.4).

These results provide evidence for initial binding of dioxygen to the active site of IPNS, as such binding to the non-heme iron (II) system would be expected to give a spin silent species such as (**103**), lowering the bulk magnetic susceptibility of the solution. This reduction in bulk magnetic susceptibility would be reflected in a lower value of observed frequency shift, $\Delta\delta$.

The supposition supported by these results, that oxygen will bind to IPNS in the absence of the cosubstrate LLD-ACV (**1**) places limits on the possible mechanisms of substrate binding and, in conjunction with initial rate studies, would allow the binding mechanism to be determined.

FIGURE 2.5

A typical series of ^1H NMR spectra showing depletion of oxygen with time



GRAPH 2.3

Run at 20% O_2 initial concentration

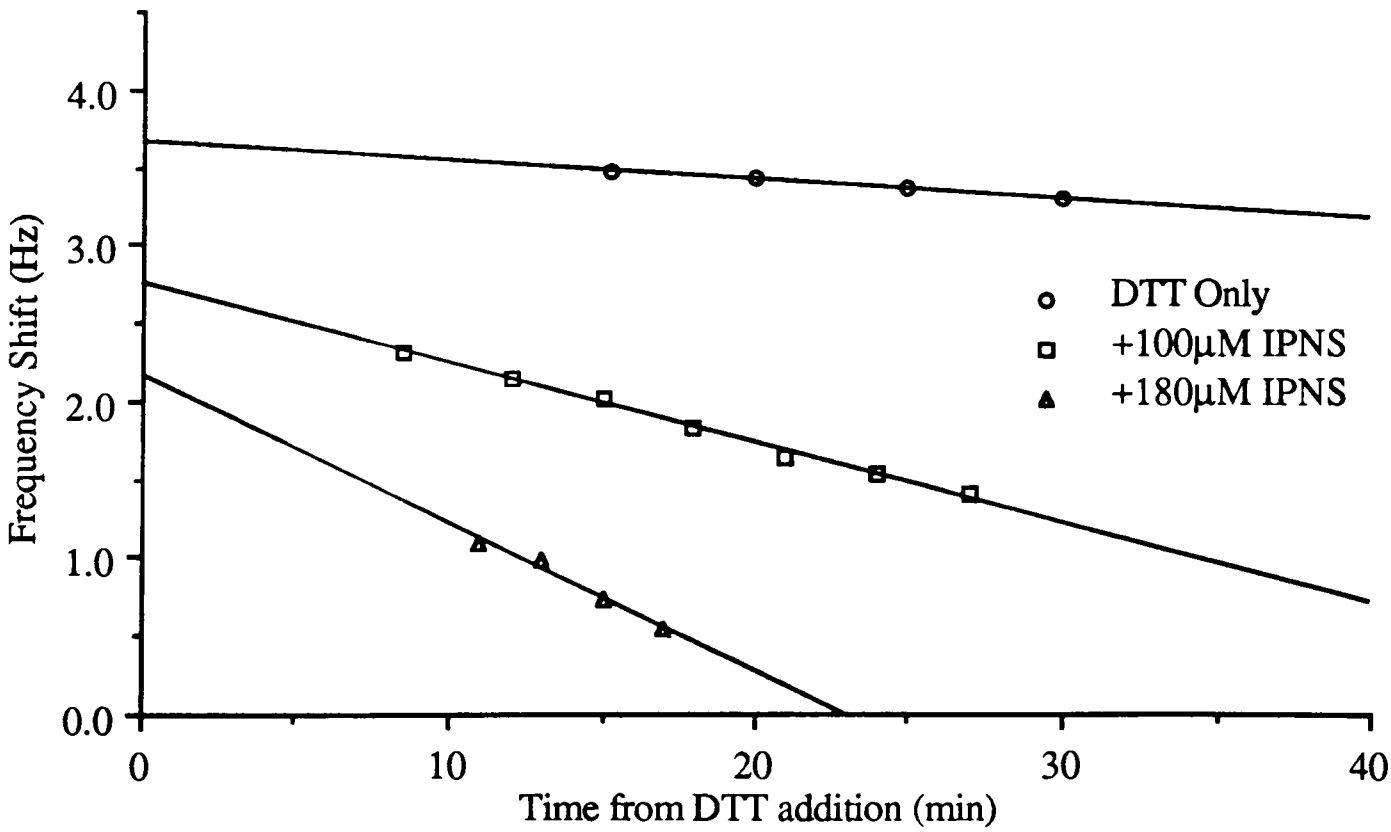


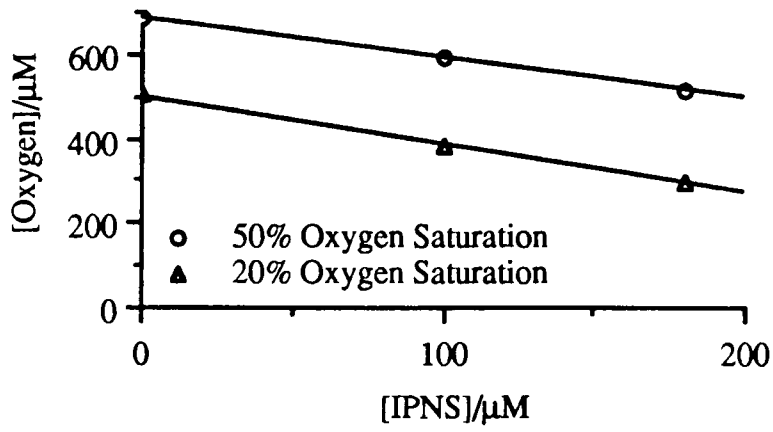
TABLE 2.1

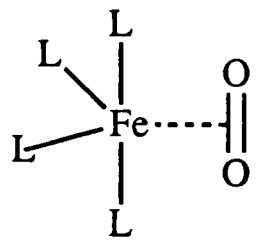
Showing the mean results for 'time zero' (t_0) oxygen concentrations for different IPNS concentrations at two levels of initial oxygen saturation.

INITIAL O_2 SATURATION	[IPNS]/μM	Mean Δδ/Hz (No. of runs)	t_0 [O_2] (μM)
50	0	4.95 (6)	681
50	100	4.27 (6)	588
50	180	3.75 (4)	516
20	0	3.66 (1)	504
20	100	2.75 (1)	378
20	180	2.17 (1)	298

GRAPH 2.4

Graph showing the relationship between t_0 oxygen concentration and the concentration of IPNS.





(103)

L represent ligands from the enzyme or from solution

Substrate binding for IPNS can be envisaged as taking place in either a random or an ordered process with O_2 or LLD-ACV (**1**) (thiol form) being the two substrates involved. These results show that in the absence of LLD-ACV (**1**), O_2 still has the ability to bind to the enzyme, complimenting the conclusions obtained by Chen *et al.*,[38] who used Mössbauer and ESR to probe the nature of the active site iron (II) species, and Fe-NO complexes. It was shown that in an anaerobic environment LLD-ACV (**1**) interacts with the iron (II) centre. Since O_2 can also bind to the iron (II) centre in the absence of LLD-ACV (**1**), one of the possible conclusions that can be drawn is that there is random binding of substrates. Such a possibility could be tested by a kinetic analysis utilising varying O_2 and LLD-ACV (**1**) concentrations, which should provide support for this conclusion.

2.3 Kinetic Studies

It is possible to determine the order of substrate binding for an enzyme catalysed reaction utilising two substrates through the use of initial rate studies.[96,97,98] An array of initial rate values are required, giving data for initial rate variations with [LLD-ACV] at a number of constant O_2 concentrations, and for the variation with [O_2] at a series of constant concentrations

of LLD-ACV (**1**). In most cases it is also necessary to run parallel experiments in the presence of a reversible inhibitor (one for each substrate), such as LL-AC-D-alanine and NO (as inhibitors for the LLD-ACV (**1**) and O_2 binding sites respectively), to allow the determination of the exact mechanism. Analysis of these results utilising double reciprocal plots of 1/(initial rate) against 1/[A] for different [B]'s (where A and B represent the two substrates for the enzyme) should give a series of straight lines,[96,97,98] the behaviour of which is indicative of the binding mechanism operating.[97]

The problem arising with the IPNS-catalysed reaction is how exactly to determine these initial rates. There are a number of difficulties: it is not possible to determine product formation by spectrophotometric or similar means; the use of bioassay to determine product formation,[1] is of low accuracy,[99] would not be possible for the use with an inhibitor such as NO, and because of the experimental manipulations necessary is unsuitable for assessing the first minutes of reaction, which are vital for initial rate studies.

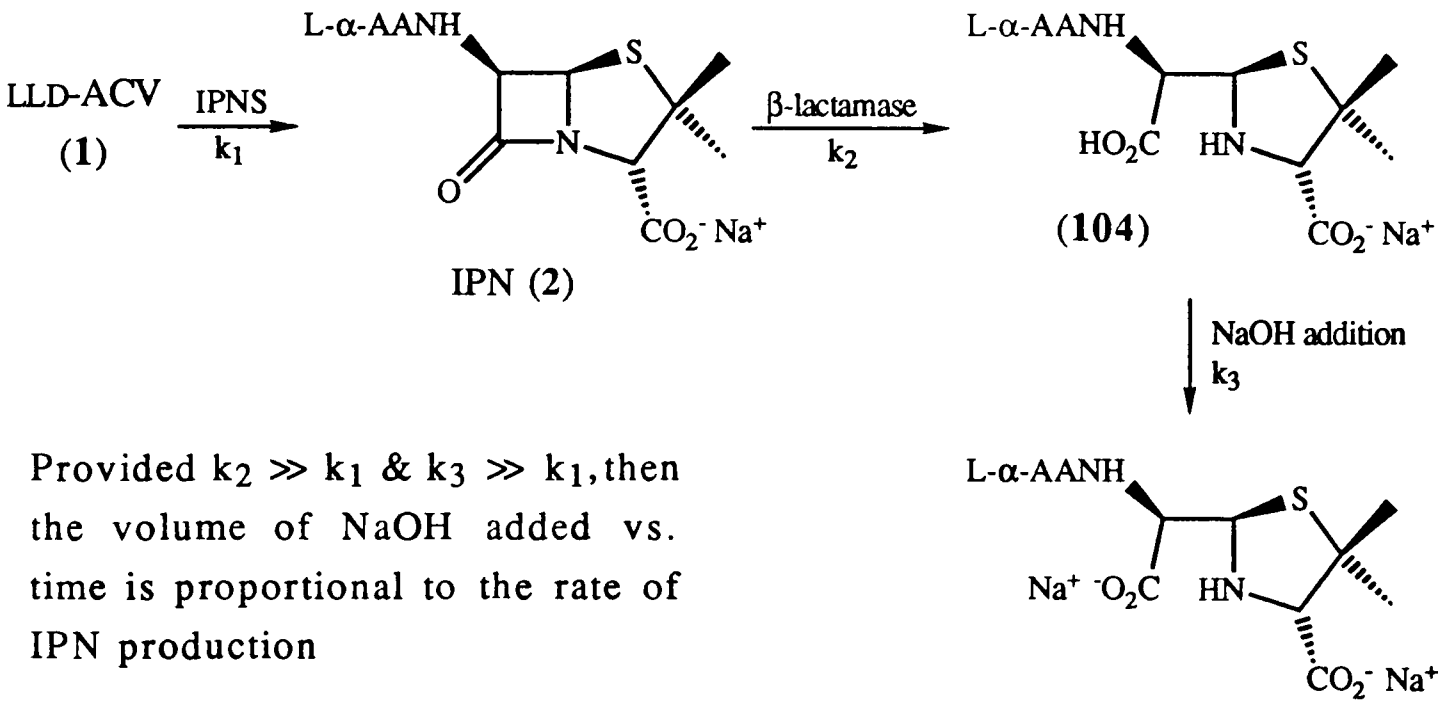
2.3.1 pH Stat Method

One method used previously in our laboratories, with some success,[44] involves coupling of product formation in the IPNS reaction to rapid degradation catalysed by the β-lactamase enzyme. The cleavage reaction of β-lactamase is essentially diffusion controlled, and so will not be rate limiting, which means that measurement of the rate of formation of penicillinic acid (**104**) produced would give directly the initial rate of isopenicillin N (**2**) formation. Penicillinic acid production was observed by use of a pH stat, a device which maintains a constant pH by addition of a monitored volume of a standard solution of alkali. In this manner, penicillinic acid (**104**) can be titrated with NaOH solution and provided, that neither the response time of

the pH stat, nor the rate of NaOH addition, k_3, were rate limiting, then the volume of NaOH added vs. time would be directly proportional to the rate of IPN (**2**) production by IPNS (Scheme 2.2). Output to a chart recorder would allow facile measurement of the initial rate.

Initial experiments, using this apparatus, were carried out using IPNS (from the same batch) over a range of oxygen and LLD-ACV (**1**) concentrations.

However, it proved impossible to obtain reproducible results from this system, with variation within repetitions being greater than those between the different data points. Similar results have been found on attempts to use this type of system by Eli Lilly & Co., who report that the manufacturers of the pH stat systems state its unsuitability for such kinetic experiments.[100] The discovery of this information led to the search for alternative methods of monitoring the initial rate.



SCHEME 2.2

2.3.2 Oxygen Electrode Method

The second approach, previously untried with the IPNS reaction, was to utilise an oxygen electrode to monitor the depletion of the cosubstrate, dioxygen, during LLD-ACV turnover. The apparatus used consisted of a thermostatically jacketed cell incorporating a Clarke type electrode in its base. The rapidly stirred sample solution was equilibrated with O_2/N_2, after which a stopper was introduced. Cofactors were introduced via a small hole, followed by IPNS solution to initiate the reaction. The electrode reading was displayed on a chart recorder from which initial rates could be determined. Since the IPNS catalysed reaction is stoichiometric in oxygen,[41] the rate of consumption of oxygen would equal the rate of IPN production.

Initial experiments with this apparatus seemed to indicate a rapid response, and reproducible traces were obtained. However, these trial experiments highlighted a problem which had not been revealed under the pH stat method, namely that addition of cofactors and LLD-ACV (**1**) caused an immediate depletion in oxygen concentration by an amount dependent on the quantities added. As it is vital to compare initial rates for identical oxygen concentrations, this would necessitate investigation of the variation in oxygen depletion with the amount of LLD-ACV (**1**) added, so that solutions could be equilibrated to differing oxygen concentrations to give the desired concentration of oxygen after cofactor and LLD-ACV (**1**) addition, immediately prior to IPNS addition. Due to time considerations these calibration experiments were not investigated.

2.4 Conclusions

Through the use of an unusual NMR experiment, it has been shown that addition of IPNS to solution causes a drop in the bulk magnetic susceptibility

† Although this method has not been used to obtain kinetic data, it has been used to determine the stoichiometry of O_2 uptake.[41]

of the solution, which is linearly dependent on the concentration of enzyme added. This result is suggestive of the formation of a spin-silent iron-dioxygen complex in the active site, which would reduce the effective concentration of dioxygen in solution. This binding of the substrate dioxygen in the absence of LLD-ACV (**1**), coupled with results from other workers which show binding of LLD-ACV (**1**) in anaerobic conditions,[38] is supporting evidence for an apparently random binding mechanism for the two substrates.

Investigation of methods for studying the initial rate of the IPNS reaction, has shown the unsuitability of methods utilising measurement of IPN (**2**) production through coupled, enzymatic, degradation. However, an alternative method involving direct measurement of the cosubstrate, dioxygen, using a Clarke type electrode, has produced encouraging initial results, showing good reproducibility. It is hoped that future investigation into this method by other workers will allow the details of the binding mechanism of the IPNS reaction to be revealed.

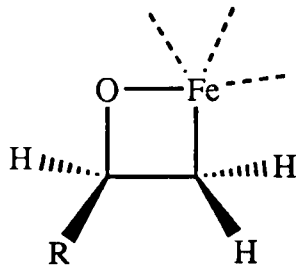
CHAPTER 3

MECHANISM OF OXYGENATION WITH THE SUBSTRATE ANALOGUE δ-L-α-AMINOADIPOYL-L-CYSTEINYL-D-VINYLGLYCINE

3. MECHANISM OF OXYGENATION WITH THE SUBSTRATE ANALOGUE δ-L-α-AMINOADIPOYL-L-CYSTEINYL-D-VINYLGLYCINE

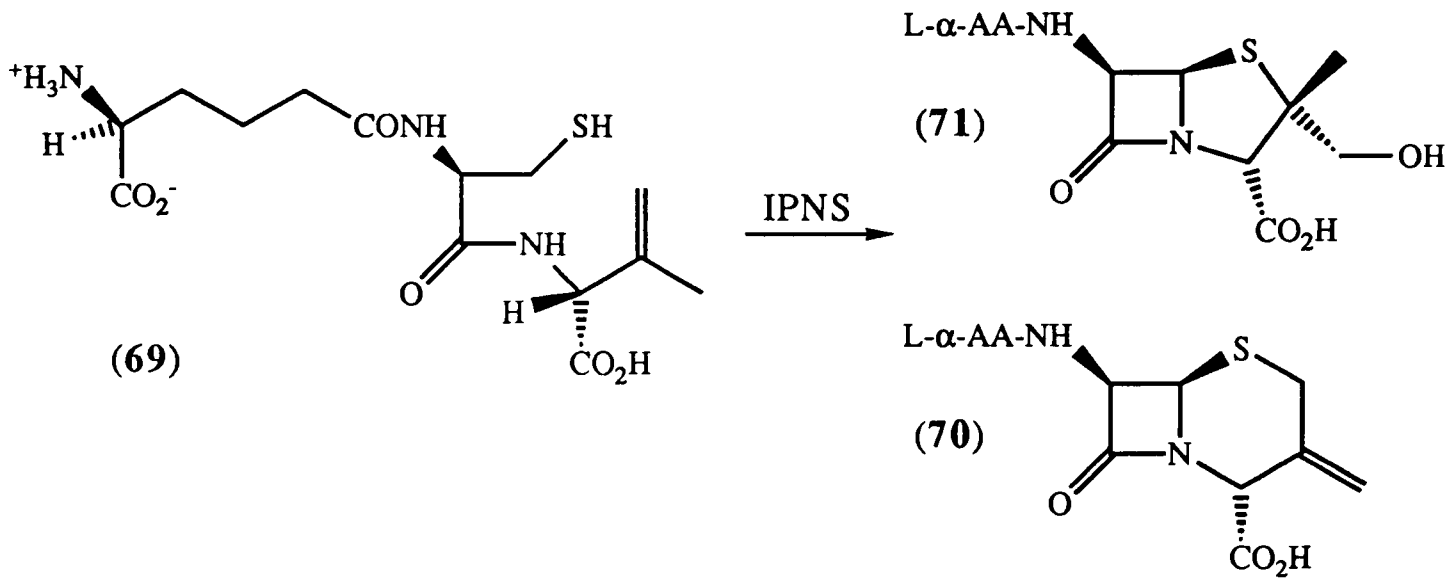
3.1 Introduction

We have seen (section 1.5.3) that in the monooxygenase pathway for the closure of the second ring, IPNS is able to catalyse this cyclisation and hydroxylation across a double bond in either a *syn* or an *anti* manner. With the substrate analogue LL-AC-D-allylglycine (**52**), the major monooxygenase pathway for this second ring closure proceeds with stereochemistry which is consistent with $[2\pi_s + 2\pi]$ cycloaddition of an En–Fe=O species, to give a four membered iron-oxetane ring (**105**), followed by reductive elimination to give addition of sulphur and oxygen in a *syn* manner (see Scheme 1.17, chapter 1).[55,77,78] The proposed mechanism of epoxide formation followed by attack of sulphur with inversion of configuration could account for the minor product, 4β-hydroxyhomocepham (**58**), which is formed with overall *trans* addition across the double bond (see Scheme 1.21, chapter 1).[55,73]



(**105**)

Similar addition across a double bond has also been seen in the formation of 3α-hydroxymethyl-3β-methylpenicillin (**71**), which is formed together with the exomethylenecepham (**70**), on incubation of the tripeptide LL-AC-D-3,4-dehydrovaline (**69**) with IPNS (Scheme 3.1).[80]

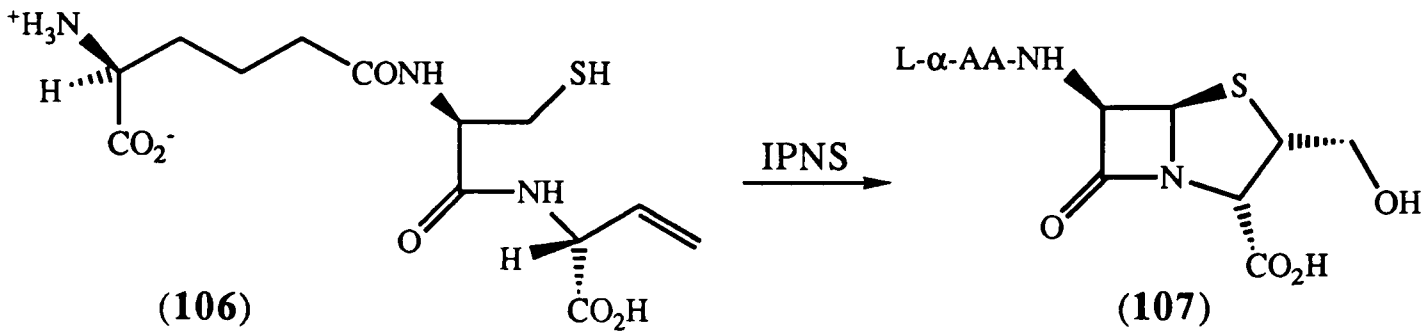


SCHEME 3.1

The stereochemistry of addition across the double bond of (**69**) has been investigated *via* the synthesis of tripeptides bearing stereospecifically labelled C=CHD groups (**76** and **77**). Incubation of these tripeptides and examination of the ^{1}H NMR spectra showed that the addition across the double bond occurred in a stereospecific manner.[101]

Further work succeeded in the lactonisation of the 3α-hydroxymethyl-3β-methylpenicillin products, allowing the determination of the stereochemistry of the methylene group through NOE studies. These results showed that the penicillin (**71**) was produced with stereochemistry arising from overall *syn* addition across the double bond, which is consistent with the proposed $[2\pi_s + 2\pi]$ cycloaddition/reductive elimination mechanism for second ring formation (see Scheme 1.19, chapter 1).[79]

Another substrate analogue which shows this type of oxygenation across a double bond is LL-AC-D-vinylglycine (**106**). Previous studies have shown this to give the 3α-hydroxymethylpenicillin (**107**) as the sole product on incubation with IPNS (Scheme 3.2).[80]



(**106**) (**107**)

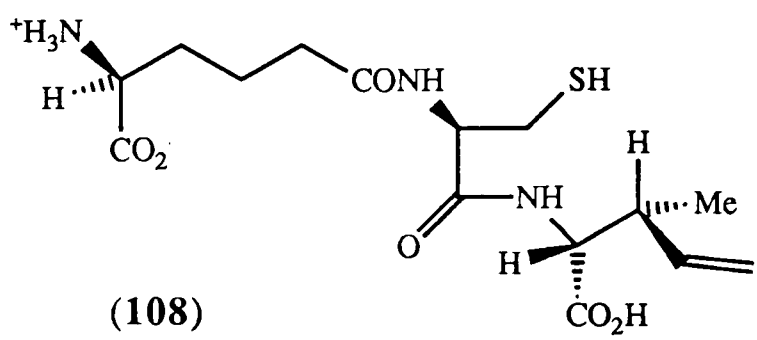
SCHEME 3.2

In a similar manner as for the formation of the 4α- and 4β-hydroxyhomocephams (**57** and **58**) from LL-AC-D-allylglycine (**52**) (See Schemes 1.17 and 1.21 in Chapter 1), it is possible to write both [$2\pi_s$ + 2π] cycloaddition and epoxide type mechanisms for the formation of the second ring in the penam (**107**) (Schemes 3.3 and 3.4) .

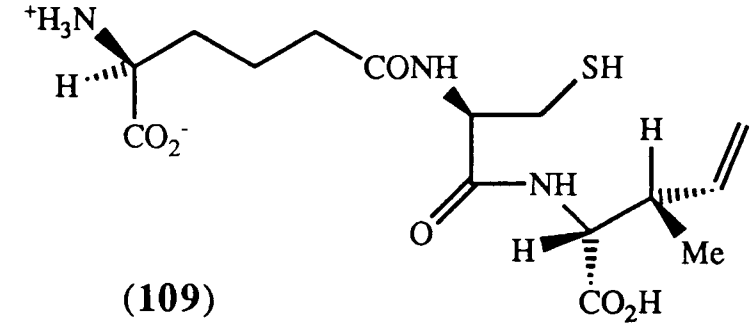
It is interesting to compare LL-AC-D-vinylglycine (**106**) with LL-AC-D-3,4-dehydrovaline (**69**), given the greater conformational freedom of the vinyl chain over the methylvinyl group. We know, from the studies carried out on deuterated analogues (**36** and **37**) of LL-AC-D-2-aminobutyric acid (**32**)[66] [see Section 1.5.3 C), Chapter 1], which is isosteric with (**106**), that side chains of this size are free to rotate within the enzyme active site during the course of events leading to closure of the second ring.

Furthermore, we know that the degree of conformational freedom available to the unsaturated side chain of a substrate analogue, is important to its turnover *via* the monooxygenase pathway. Restriction of conformational freedom greatly affects the ability of an analogue to be turned over along this pathway for closure of the second ring. For example, the addition of a 3-methyl group into LL-AC-D-Ag (**52**), to give substrate analogues (**108**) and (**109**) containing the epimeric amino acids 2*R*,3*R*-2-amino-3-methylpent-4-enoic acid and 2*R*,3*S*-2-amino-3-methylpent-4-enoic acid respectively, would, by analogy with other systems branched at C(3), prevent rotation around the C(2)-C(3)

bond during turnover. Incubation of (**108**) and (**109**) with IPNS indicated the effect of this restriction of conformational freedom on monooxygenase activity, with the analogue (**108**) giving two β-lactam products arising from the monooxygenase and desaturase pathways respectively, whilst the analogue (**109**) giving only a single β-lactam product, arising from the desaturase pathway.[102] This result showed that by restricting conformational freedom it proved possible to completely shut down the monooxygenase activity.



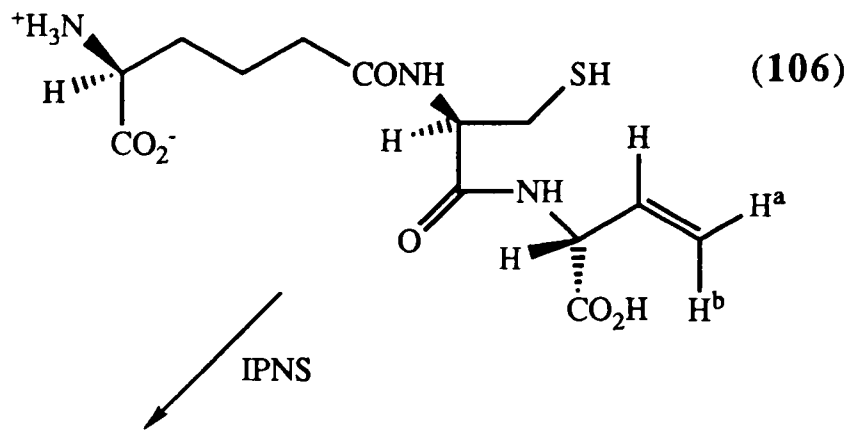
(108)

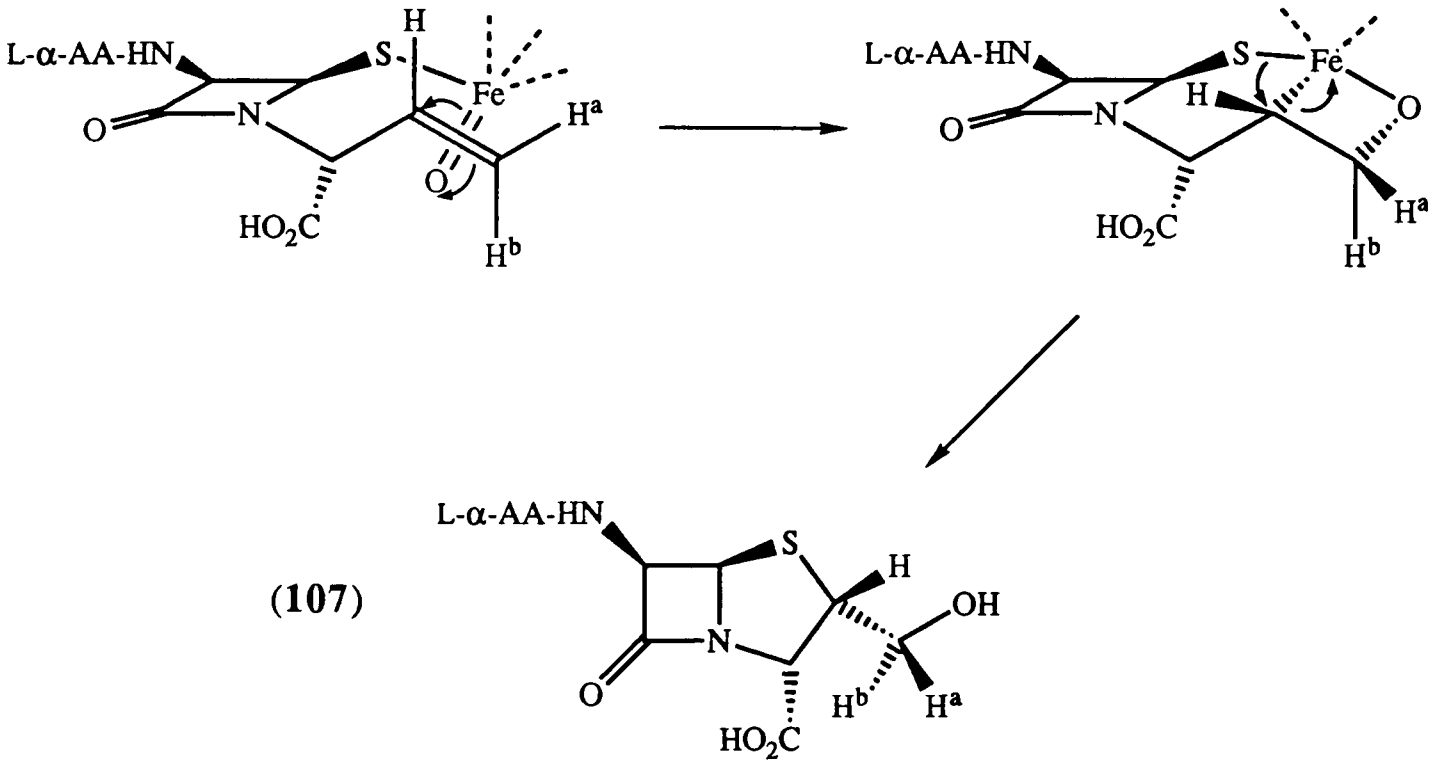


(109)

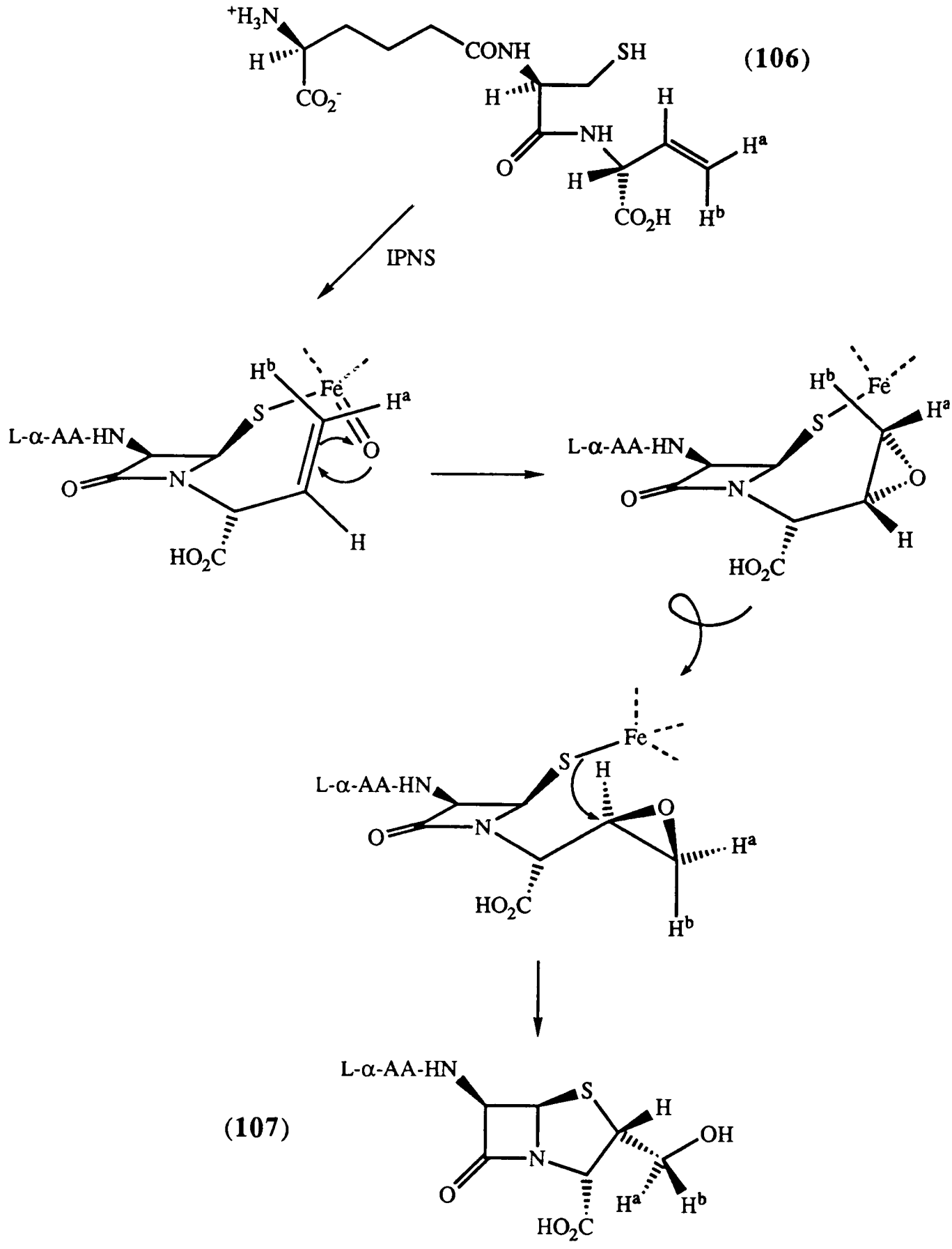
In contrast, the greater conformational freedom of the vinyl group in the analogue (**106**), may give more facile closure of the second ring by a monooxygenase mechanism. It is interesting to speculate as to whether the penicillin (**107**) will be formed by the same mechanism as for the formation of (**71**), or whether it is possible for both *syn* and *anti* addition mechanisms to operate.

In the same manner as the studies on 3α-hydroxymethyl-3β-methylpenicillin (**71**), incubation of specifically labelled forms of LL-AC-D-vinylglycine, (**106**), and examination of the penicillins produced by ^1H NMR spectroscopy would allow us to determine whether or not 3α-hydroxymethyl–penicillin (**107**) is formed from (**106**) with stereospecific or stereorandom addition across the double bond.





SCHEME 3.3



(106)

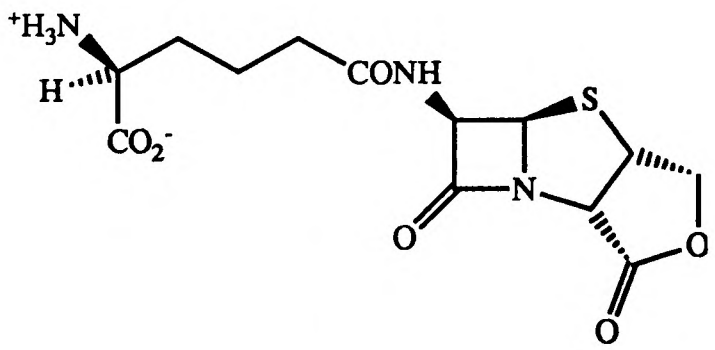
(107)

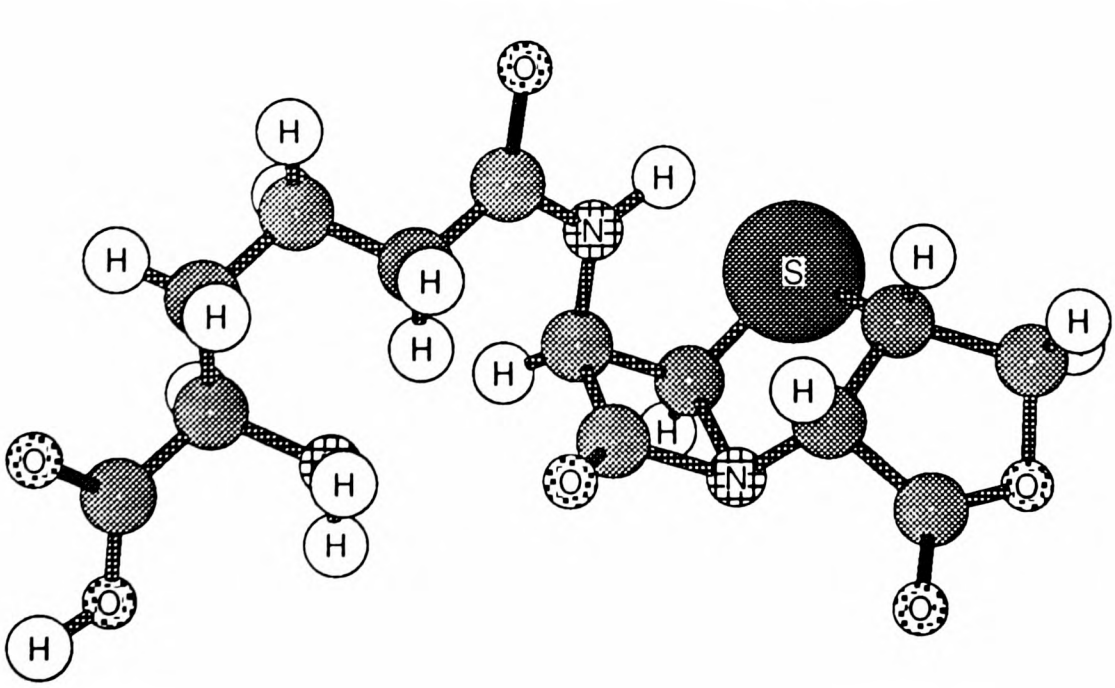
SCHEME 3.4

Furthermore, the lactonisation of the 3α-hydroxymethylpenicillin (**107**) to give a protected form of the lactone (**110**) (Figure 3.1) would allow determination of the stereochemistry of the hydroxymethyl group, through the coupling constants of the now conformationally restrained C(3)H–C(8)H_2 system (the different dihedral angles involved can be seen in Figure 3.1), and through the use of NOE experiments.

FIGURE 3.1

Line drawing and stereo view of the lactone (**110**).
The stereo view has been energy minimised in the ring system,
assuming standard bond parameters.

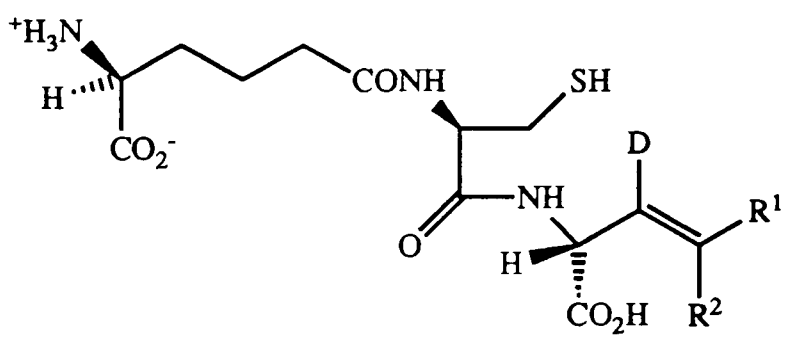




3.2 Synthetic Strategies

3.2.1 Methodology

To gain access to the stereochemical information contained in the hydroxymethyl group of 3α-hydroxymethylpenicillin (**107**) it was necessary to introduce a specific label into the vinyl group of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine (**106**). It was decided to synthesise the specifically deuterated tripeptides (**111**) and (**112**), which could be produced from the labelled vinylglycines (**113**) and (**114**) using the method of Sawada *et al.*[103]

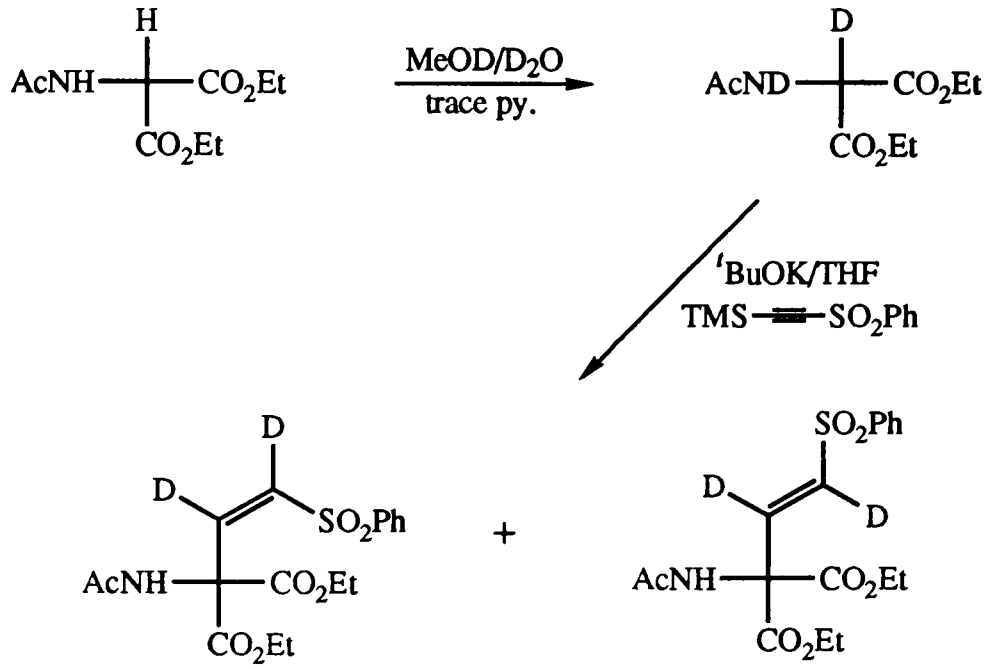


(**111**) R^1 = D, R^2 = H

(**112**) R^1 = H, R^2 = D

3.2.2 Synthesis of Labelled δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-vinylglycines (111) and (112)

The required deuterated vinylglycines, (**113**) and (**114**), were synthesised according to the method of Sawada *et al.*[103] (Scheme 3.5). Thus the *N*,2-dideutero form of diethylacetamidomalonate was formed by pyridine catalysed exchange in D_2O and MeOD, followed by coupling to phenyl-(2-trimethylsilyl)ethynyl sulphone. The mixture of *Z* and *E* sulphones, (**115**) and (**116**), thus formed were separated on silica and separately reduced, using aluminium amalgam, to produce the adducts (**117**) and (**118**) containing the desired specifically labelled vinyl groups.


H
AcNH
CO2Et
CO2Et
MeOD/D2O
trace py.
D
AcND
CO2Et
CO2Et
tBuOK/THF
TMS
SO2Ph
D
D
SO2Ph
AcNH
CO2Et
CO2Et
(115)
+
SO2Ph
D
D
AcNH
CO2Et
CO2Et
(116)
Separate geometric
isomers on SiO2
R1
D
R2
AcNH
CO2Et
CO2Et
(115) R1 = D, R2 = SO2Ph
(116) R1 = SO2Ph, R2 = D
Al/Hg/H2O
O
O
R1
D
R2
AcNH
CO2Et
CO2Et
(117) R1 = D, R2 = H
(118) R1 = H, R2 = D
6M HCl
D
Cl- +H3N
R1
R2
CO2H
(113) R1 = D, R2 = H
(114) R1 = H, R2 = D

SCHEME 3.5

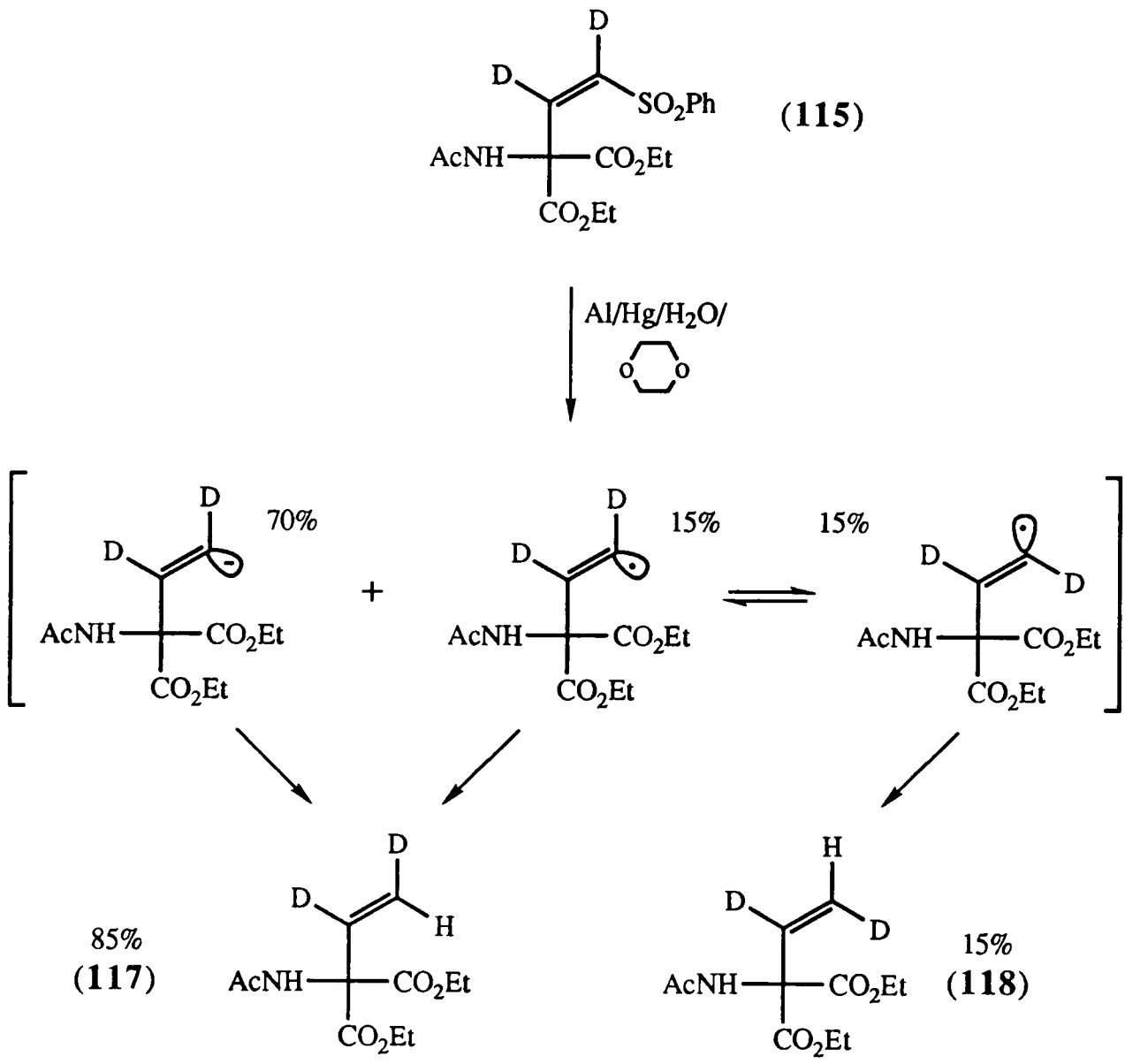
Although complete retention of stereochemistry was claimed for this reduction step in the literature,[103] it was consistently found that both the *Z* and *E* sulphones (**115** and **116**) gave *ca.* 15% reversal of stereochemistry. A similar ratio of isomers has also been found by other workers.[104,105] A query sent to the authors determined that the stereochemistry had been assigned by 60MHz ^{1}H NMR spectroscopy, which is of insufficient resolution to allow the separate resonances arising from the *E*- and *Z*- isomers (**117** and **118**) to be resolved [the separation is 20Hz (0.04ppm) at 500MHz]. This scrambling of the deuterium label, since it occurs to an identical extent for both the *Z* and *E* sulphones (**115** and **116**), probably occurs after cleavage of the sulphone group, and may be due to mixed ionic and radical pathways, as shown in Scheme 3.6.

It was decided that this degree of scrambling would not preclude analysis of the results of incubation with IPNS, and so the synthesis was continued using these materials.

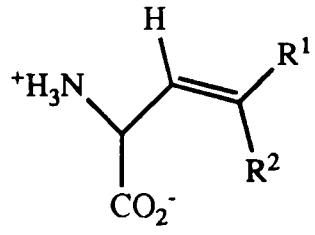
Hydrolysis of the diethylacetamidomalonate adducts, (**117**) and (**118**), in HCl (6M) gave the required *E* and *Z* [3,4-$^2H_2$]-vinylglycines, (**113**) and (**114**), respectively, in their racemic forms.

Synthesis of the mono-deuterated amino acids (**119**) and (**120**) was also attempted. These compounds would have facilitated NOE studies on the lactone derivative of the labelled hydroxymethyl penicillin formed upon incubation with IPNS, owing to the theoretically large NOE from H(3) to the hydroxymethyl group (the likely spatial proximity of these hydrogens can be seen in Figure 3.1). However, it was found that introduction of the deuterium label at the amalgam reduction step (aluminium amalgam was prepared from Al foil in a solution of $HgCl_2$ in D_2O) could only be achieved with 90% incor-

poration of the deuterium label from isomerically pure *Z*-diethylacetamido-[2-(phenylsulphonyl)vinyl]-malonate (ratio *E*-d_1:*Z*-d_1:d_0 = 75:15:10). In view of this result it was decided to proceed only with the doubly labelled vinylglycines.

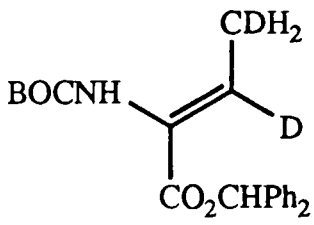


SCHEME 3.6

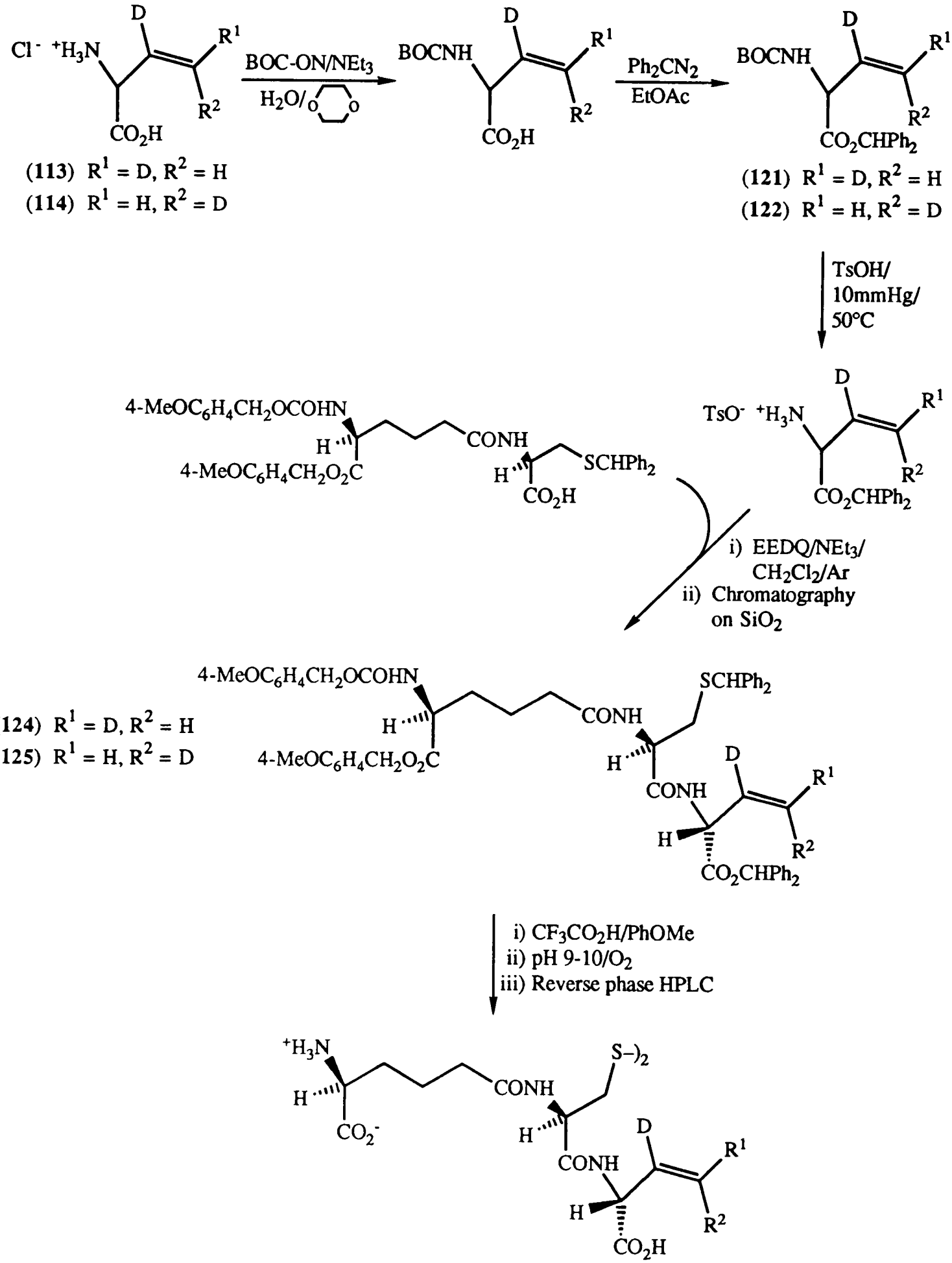


(119) R^1 = D, R^2 = H
(120) R^1 = H, R^2 = D

The amino acids, (**113**) and (**114**), synthesised according to Scheme 3.5 were incorporated into the disulphide forms of tripeptides (**111**) and (**112**) using standard techniques, as shown in Scheme 3.7. The amino acids from the crude hydrolysis mixtures were protected as the *N-tert*-butoxycarbonyl, benzhydryl ester derivatives (**121**) and (**122**) using BOC-ON in water/1,4-dioxane,[106] followed by treatment with diphenyldiazomethane[107] in EtOAc. These compounds proved to be base sensitive, rapidly isomerising to the αβ-unsaturated compounds (**123**) on treatment with, for example, NEt_3 so that care had to be taken to free the diphenyldiazomethane from basic decomposition products prior to use, and to avoid addition of excess NEt_3 during the EEDQ mediated coupling step. Separate treatment of (**121**) and (**122**) with one equivalent of toluene-4-sulphonic acid cleanly yielded the toluene-4-sulphonate salts of the free amines, which were coupled to a protected form of the δ-(L-α-aminoadipoyl)-L-cysteine dipeptide using EEDQ,[108] to give the protected tripeptides (**124**) and (**125**), along with their LLL-diastereoisomers. These diastereomeric protected tripeptides were separated by chromatography on silica, and the faster running LLD-diastereoisomers, (**124**) and (**125**), smoothly deprotected using trifluoroacetic acid and anisole.[109] The crude tripeptide thiols, (**111**) and (**112**), thus formed were oxidised at pH 9-10 with dioxygen and then purified by reverse phase HPLC. The 500MHz ^1H NMR spectra of the disulphide from of (**111**) and (**112**) are given in Figures 3.2 and 3.3 respectively.



(**123**)

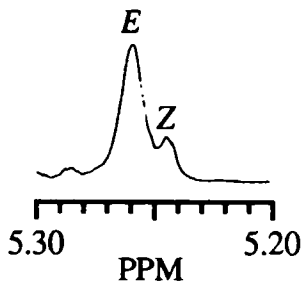


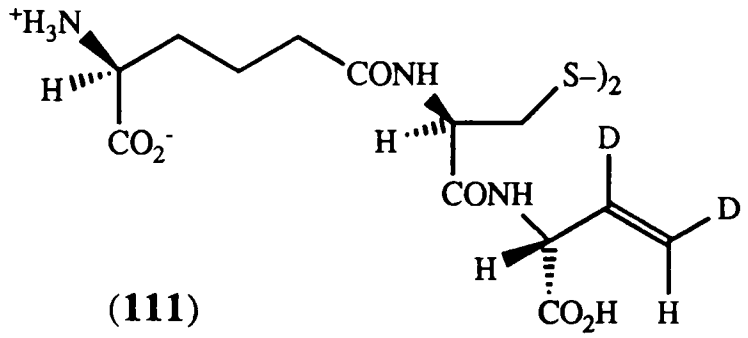
(113) R^1 = D, R^2 = H
(114) R^1 = H, R^2 = D

(121) R^1 = D, R^2 = H
(122) R^1 = H, R^2 = D

(124) R^1 = D, R^2 = H
(125) R^1 = H, R^2 = D

SCHEME 3.7





(**111**)

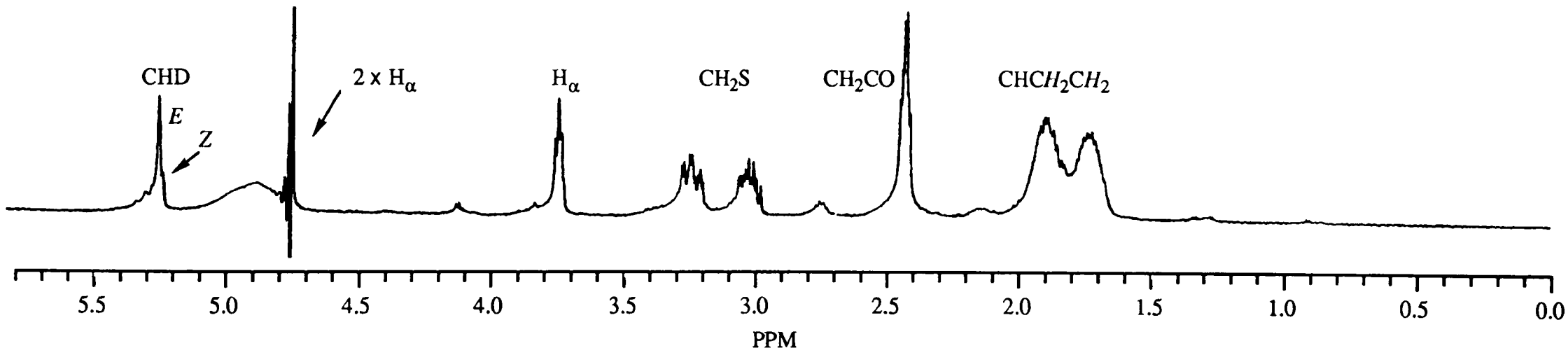
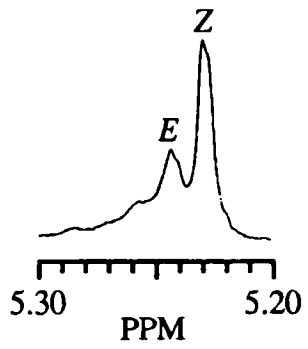
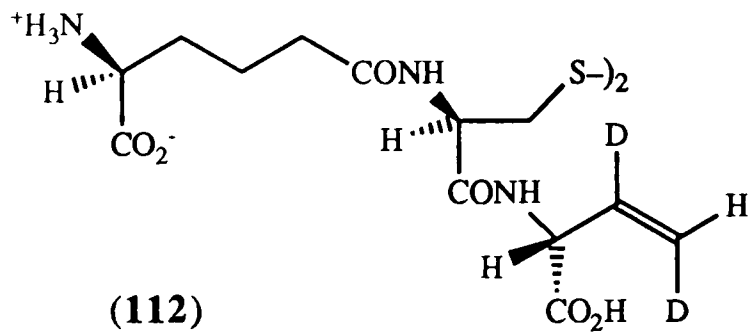


FIGURE 3.2
500MHz ^{1}H NMR spectrum of the *E*-d_2-tripeptide (**111**)





(**112**)

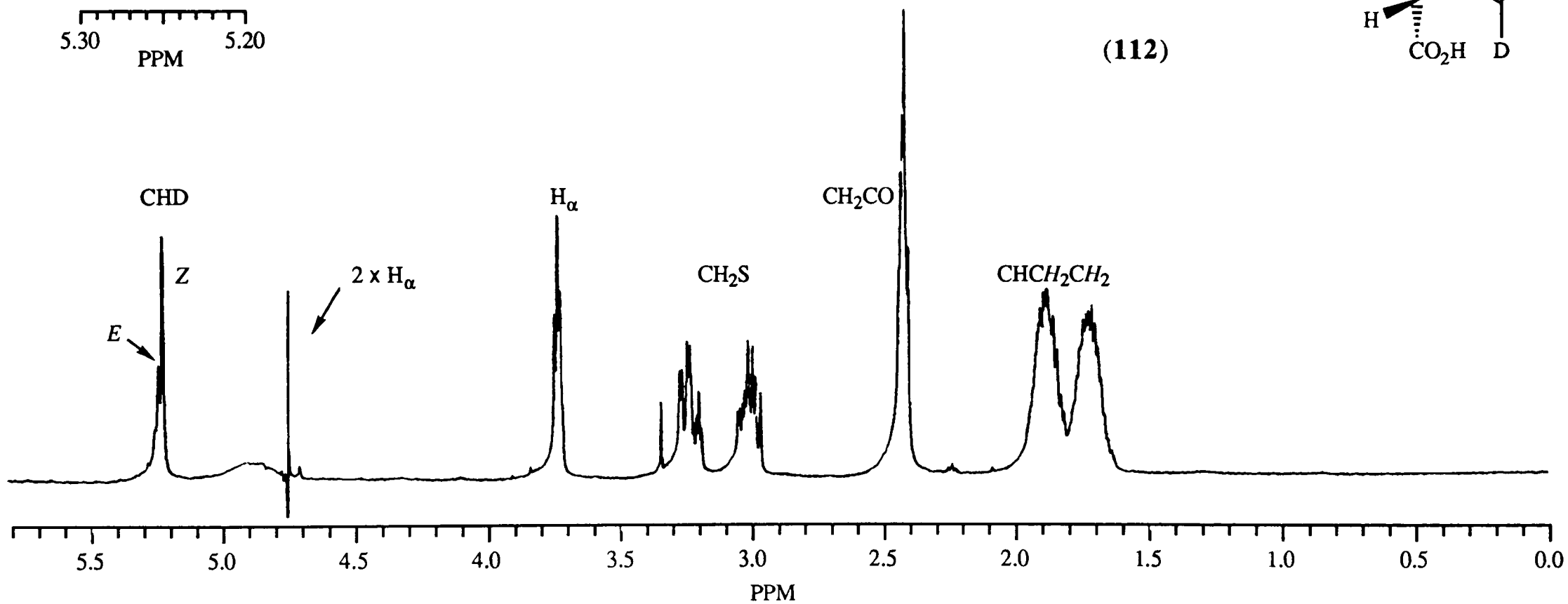
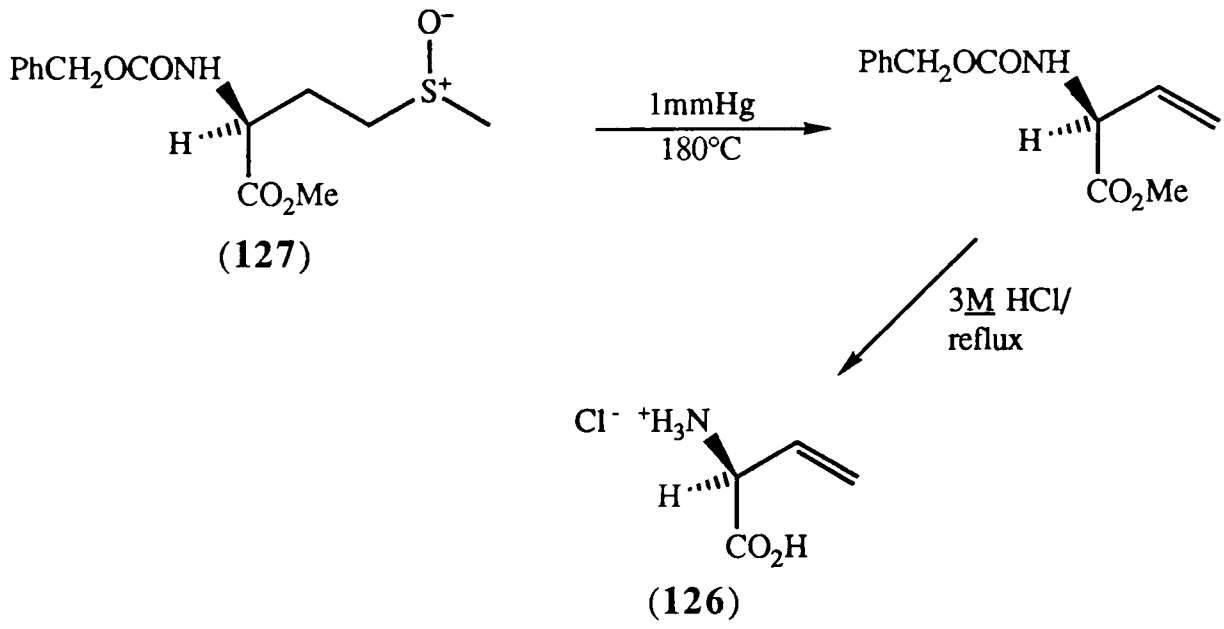


FIGURE 3.3
500MHz ^{1}H NMR spectrum of the *Z*-d_2-tripeptide (**112**)

3.2.3 Synthesis of Unlabelled δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-vinylglycine (106)

In order to investigate the purification and lactonisation of 3α-hydroxymethylpenicillin (**107**), a quantity of the unlabelled δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine tripeptide (**106**) was prepared. D-Vinylglycine (**126**) was prepared from *N*-benzyloxycarbonyl-D-methionine sulphoxide, methyl ester, (**127**), by vacuum pyrolysis, followed by hydrolysis of the protected vinylglycine thus formed (Scheme 3.8).[110]



SCHEME 3.8

This material was incorporated into the tripeptide in an analogous manner to that employed for the deuterated isotopomers (Scheme 3.7). The lability of the hydrogen at the C-2 position of vinylglycine was illustrated by the fact that EEDQ coupling of enantiomerically pure D-vinyl glycine, benzhydryl ester, toluene-4-sulphonate salt gave a mixture of LLL and LLD-protected tripeptides (indeed one batch of the LLD-tripeptide was prepared from L-configured vinylglycine).

3.3 Incubation Studies with IPNS - Unlabelled Materials

3.3.1 Incubation of δ-(L-α-Aminoadipoyl)-L-Cysteinyl-D-Vinylglycine (106) and Purification of 3α-Hydroxymethylpenicillin (107)

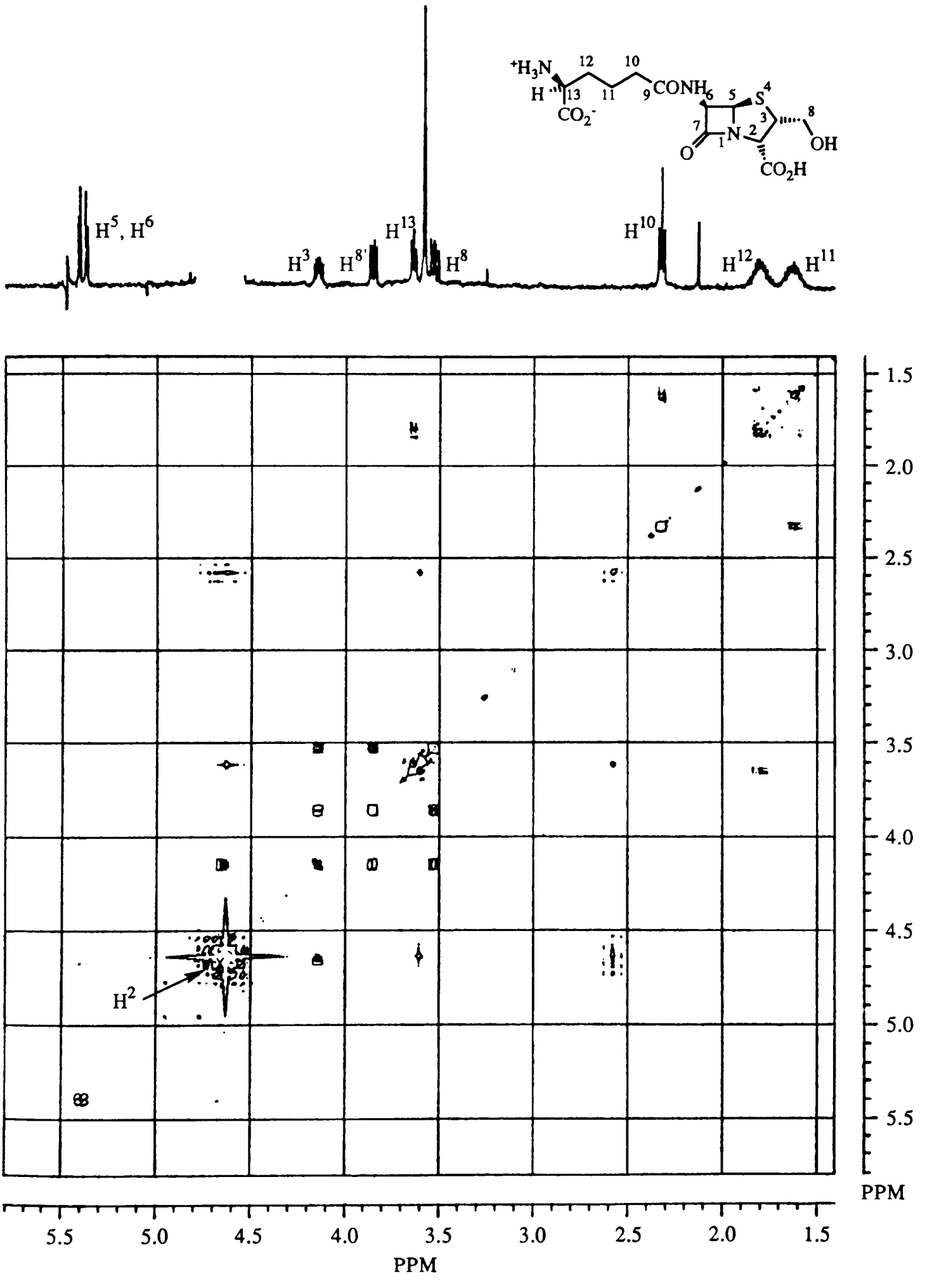
Incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine (**106**) with purified IPNS gave consistently 25-35% conversion† to the 3α-hydroxymethylpenicillin (**107**).

Purification of the 3α-hydroxymethylpenicillin was attempted by techniques used by previous workers,[111] namely isolation of the solvent front from reverse phase HPLC on Hypersil® ODS with mobile phase NH_4HCO_3 (20m<u>M</u>) and further purification of this using HCO_2H [0.025% (v/v)] as the mobile phase. However, this proved unsatisfactory, with large losses of material, and with the final purity being unsatisfactory. Investigations showed that much of the penicillin was lost during freeze drying of the HPLC fractions, presumably due to an increase in formic acid concentrations. This problem was overcome by neutralisation of fractions with NH_3 (66m<u>M</u>) solution (one tenth the volume of eluent; 0.025% HCO_2H being 6.6m<u>M</u>) immediately after elution from the column, and prior to freeze drying. Additionally it was found that if the first stage HPLC was omitted, and the flow rate was halved (to $0.5ml.min^{-1}$), then baseline separation of the penicillin containing components could be achieved (the sodium salt and free acid eluted separately using this weak buffer mobile phase), affording material of excellent purity, and with recoveries from 1mg incubations being greater than that from 10mg incubations under the previous purification protocol. 500MHz ^1H NMR and COSY spectra are shown in Figure 3.4.

† As determined by examination, in the 500MHz ^1H NMR spectra of the crude incubation mixtures, of the ratio of the integral of the CH=CH_2 resonance of unconverted vinylglycine to that of the β-lactam resonance of the penicillin product.

FIGURE 3.4

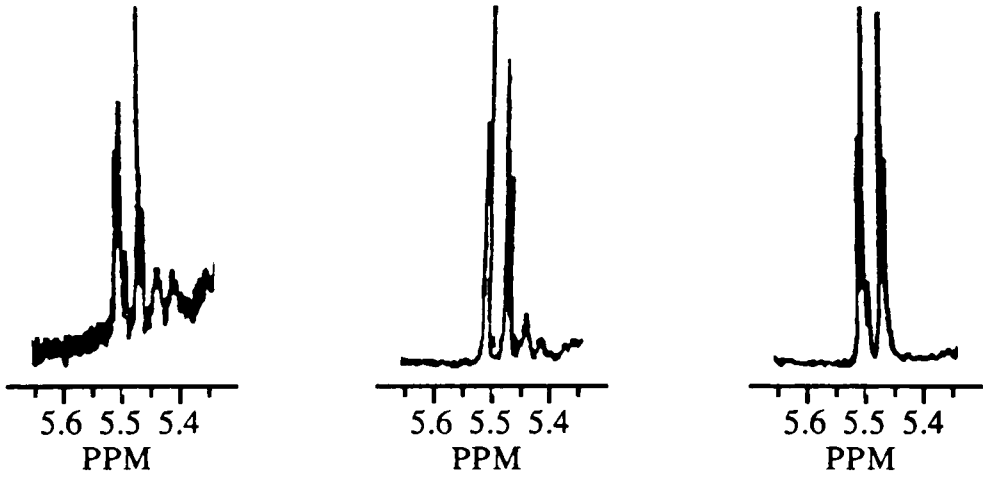
500MHz ^{1}H NMR and COSY spectra of 3α-hydroxymethylpenicillin (**107**)



3.3.2 Detection of a New β-Lactam Product

Large scale incubations of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine (**106**) revealed, on examination of the crude incubation mixtures by 500MHz ^{1}H NMR spectroscopy, a second set of β-lactam resonances. This observation was not reproducible, with some incubations containing this second set of β-lactam resonances, whilst others contained no trace of the resonances. Generally, where this second compound was detected, the ratio compared to the major product (**107**) was <1:10, although one incubation produced a ratio of 1:4. Expansions of the β-lactam region of the 500MHz ^{1}H NMR spectra of a selection of incubations are given in Figure 3.5.

FIGURE 3.5
Examples of the variation in the second set of ^{1}H NMR
β-lactam resonances seen in crude incubation mixtures



This second β-lactam product could only be partially purified by reverse phase HPLC, and was only isolated in minute quantities. Mass spectrometric analysis failed to yield a molecular ion, so that a definite assignment of the structure of this β-lactam was not possible.

3.3.3 $^{18}O_2$ Incorporation Experiment

The origin of the hydroxyl-oxygen atom in 3α-hydroxymethylpenicillin (**107**) was not determined during previous studies. With the improved purification protocol giving easy access to the purified penicillin, the origin of this oxygen atom was investigated by incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine (**106**) with IPNS under an atmosphere of $^{18}O_2$. The penicillin produced from this incubation was purified by reverse phase HPLC according to the procedure given in section 3.3.1, and compared by electrospray mass spectrometric analysis to a sample of the penicillin produced from an incubation in air. The mass spectrum showed an increase of two mass units in the MH^+ peak, consistent with incorporation of a single atom of ^{18}O, with the $[^{16}O_7]$-MH^+ peak not being observed above the background noise, indicating incorporation of 18-oxygen at a level of ≥90%. The ratio of the MH^+ peak to its ^{13}C satellite was identical to that of the penicillin from the incubation in air. The electrospray mass spectra of these samples of 3α-hydroxymethylpenicillin (**107**) are shown in Figure 3.6.

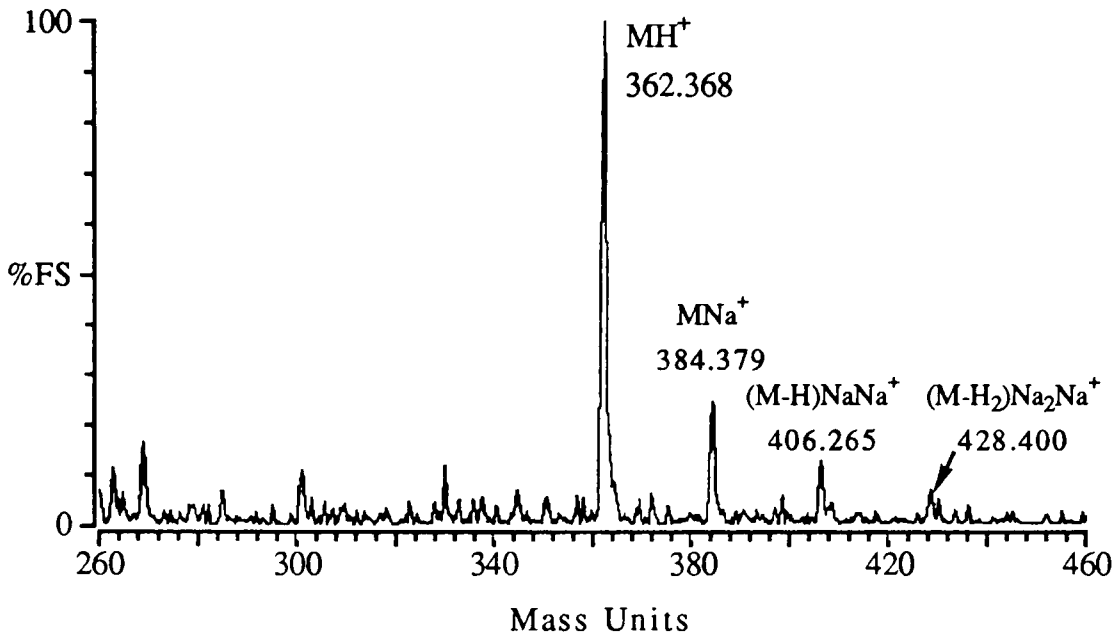
3.4 Incubation Studies with IPNS - Deuterium Labelled Materials

Both labelled tripeptides, δ-(L-α-aminoadipoyl)-L-cysteinyl-*E*-[3,4-$^2H_2$]-D-vinylglycine (**111**), and δ-(L-α-aminoadipoyl)-L-cysteinyl-*Z*-[3,4-$^2H_2$]-D-vinylglycine (**112**) were incubated with IPNS in the normal manner.[31] Both incubations showed conversion to a single β-lactam product (**128** and **129** respectively), as judged by analysis by ^1H NMR spectroscopy on the crude incubation mixtures, with β-lactam resonances identical to 3α-hydroxymethylpenicillin (**107**).

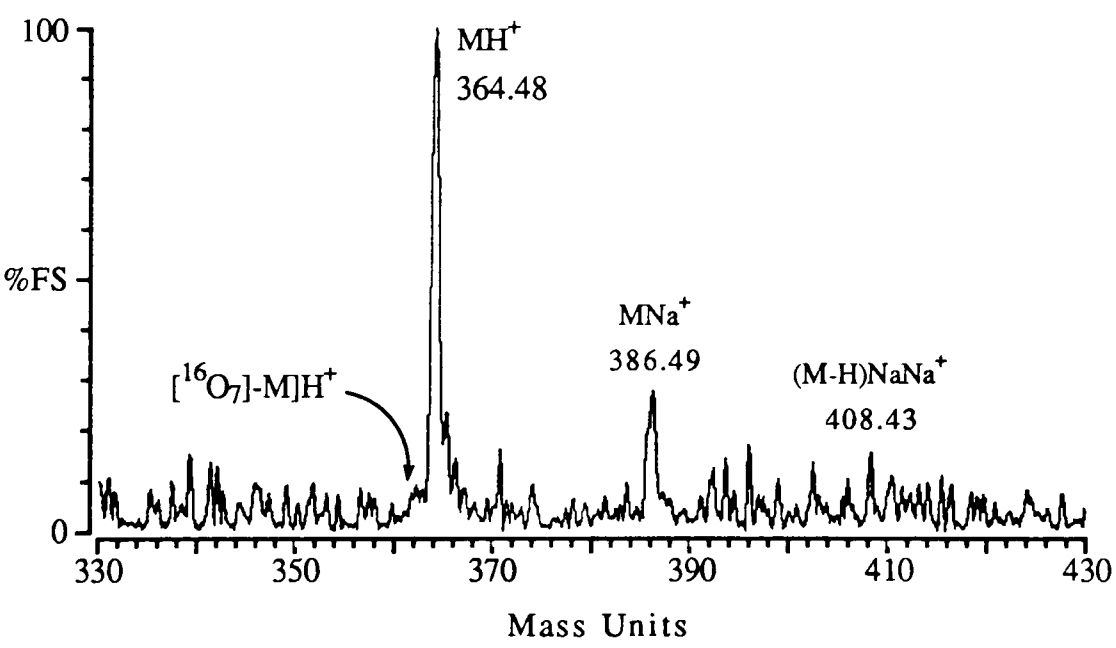
FIGURE 3.6

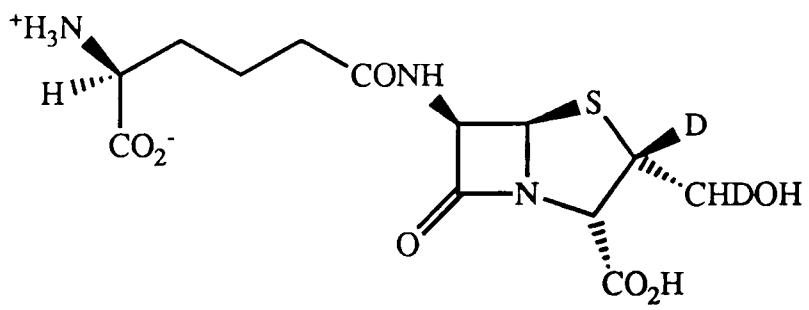
Mass spectra of 3α-hydroxymethylpenicillin (**107**) produced from incubation with IPNS; a) in air, b) under an $^{18}O_2$-atmosphere

a) Mass spectrum of [$^{16}O_7$]-(**107**)



b) Mass spectrum of [^{18}O, $^{16}O_6$]-(**107**)





(**128**) derives from the *E*-d_2-tripeptide (**111**)

(**129**) derives from the *Z*-d_2-tripeptide (**112**)

These products were purified according to the protocol developed in section 3.3.1, and from their retention times by reverse phase HPLC, analysis by ^{1}H NMR spectroscopy and mass spectrometric analysis were characterised as the deuterated 3α-hydroxymethylpenicillins (**128**) and (**129**) (the ^{1}H NMR spectra are shown in Figure 3.7).

Examination of the ^{1}H NMR resonances associated with the hydroxymethyl side chain showed complementary incorporation of the deuterium label into the two resonances (Figure 3.8). Furthermore, the level of the minor isotopomer present in each sample agreed precisely with the scrambling of the label in the parent tripeptides, indicating complete retention of the stereochemistry of the vinyl group during conversion to the penicillins (**128**) and (**129**).

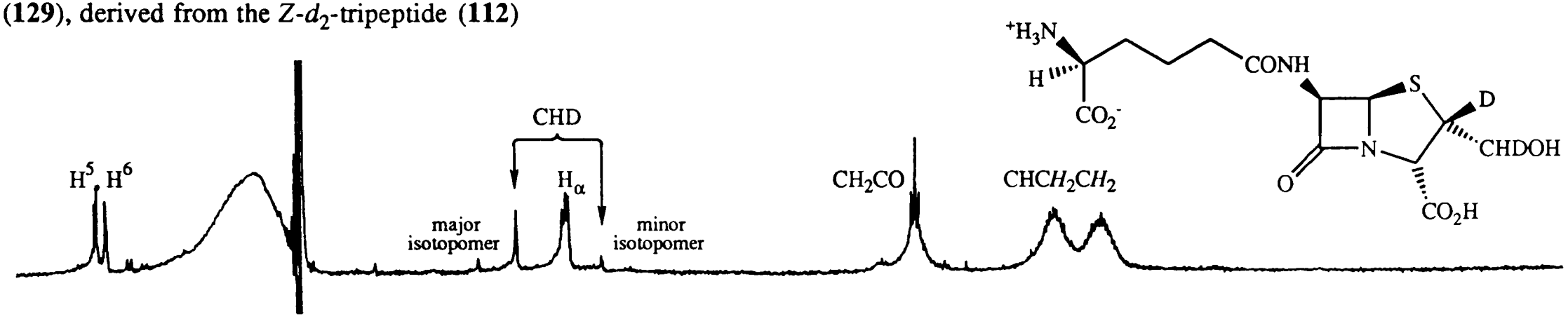
3.5 Lactonisation Studies on Unlabelled Materials

3.5.1 Previous Studies

Previous work by Baldwin *et al.*[79] on the lactonisation of the penicillin (**71**), which is very similar to (**107**), save only for an additional 3β-methyl group, achieved maximal results by *N*-protection with diethylpyrocarbonate, followed by acidification, extraction into organic solvent and treatment of the free acid with CH_2N_2 solution (Scheme 3.9). This afforded the lactone (**130**) in only 4% yield, but was sufficient for NOE studies.

Attempts to utilise these conditions with the 3α-hydroxymethylpenicillin (**107**) met with complete failure, yielding no β-lactam compounds as determined by analysis by ^{1}H NMR spectroscopy.

(**128**), derived from the *E*-d_2-tripeptide (**111**)
(**129**), derived from the *Z*-d_2-tripeptide (**112**)



(**129**), derived from the *Z*-d_2-tripeptide (**112**)

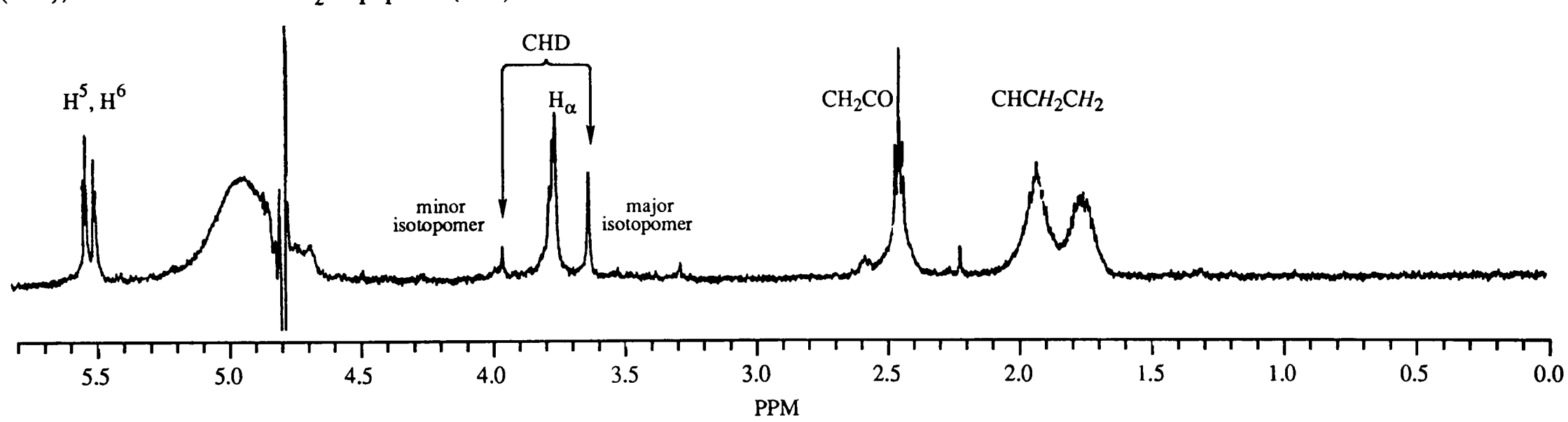
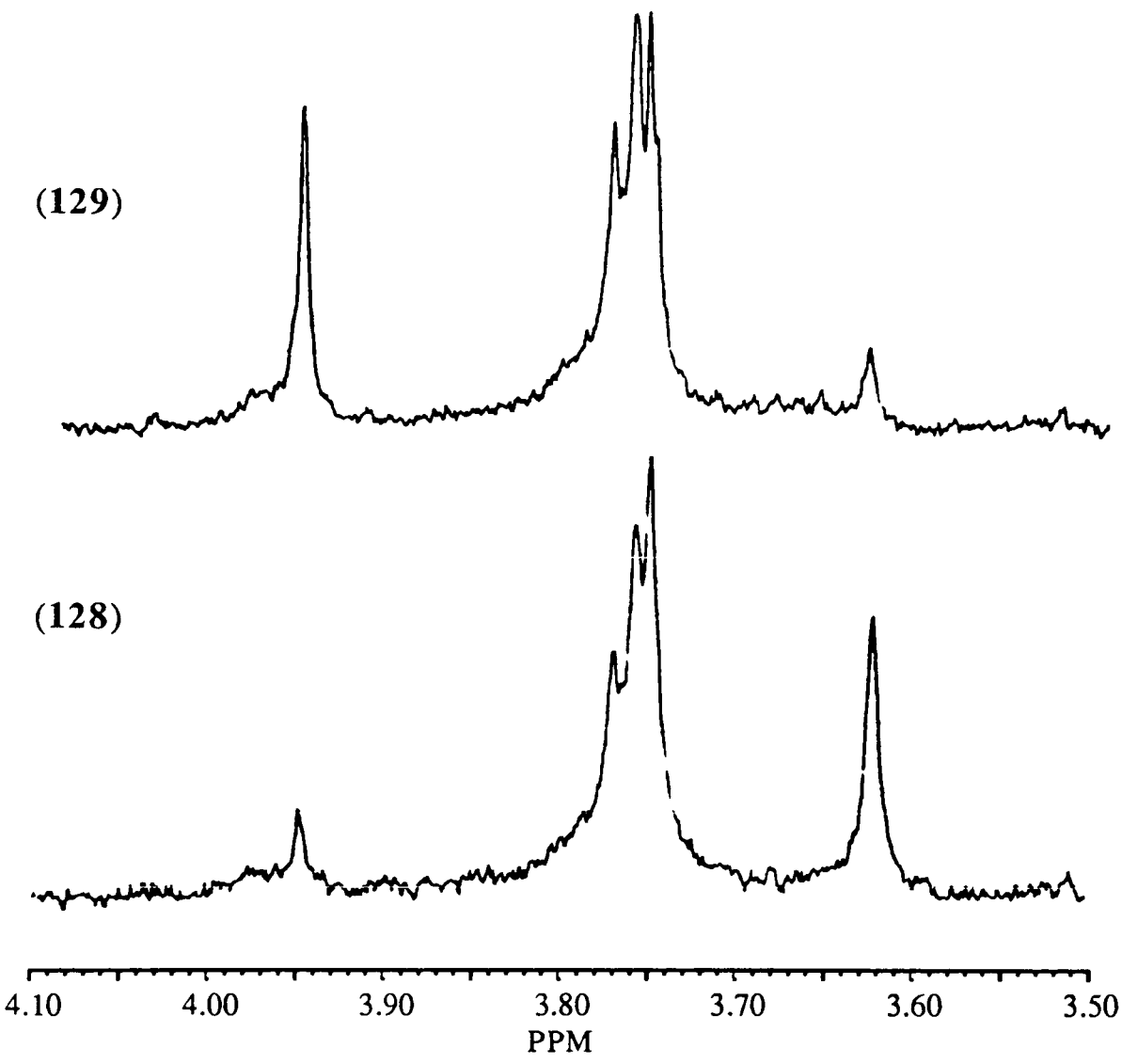


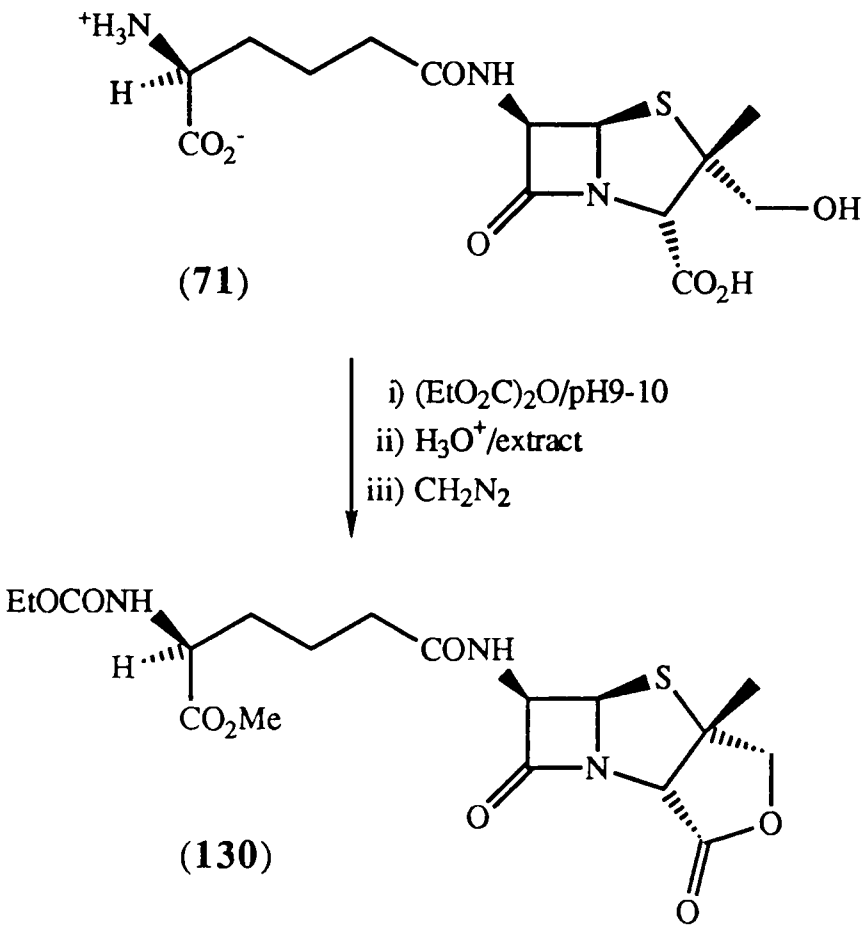
FIGURE 3.7

500MHz ^{1}H NMR spectra of the d_2-penicillins (**128**) and (**129**), derived from the *E*-d_2-tripeptide (**111**) and from the *Z*-d_2-tripeptide (**112**) respectively

FIGURE 3.8

^{1}H NMR resonances arising from the CHDOH groups in the d_2-penicillins (**128**) and (**129**).

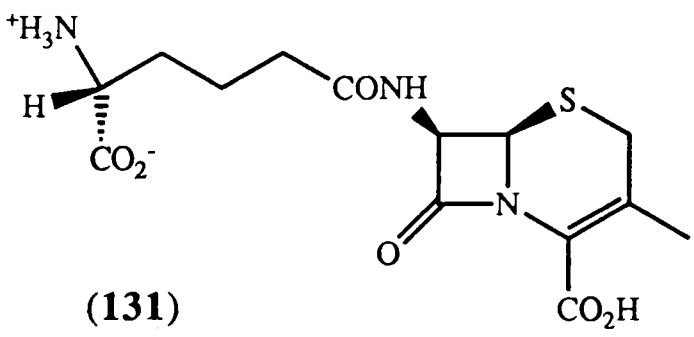




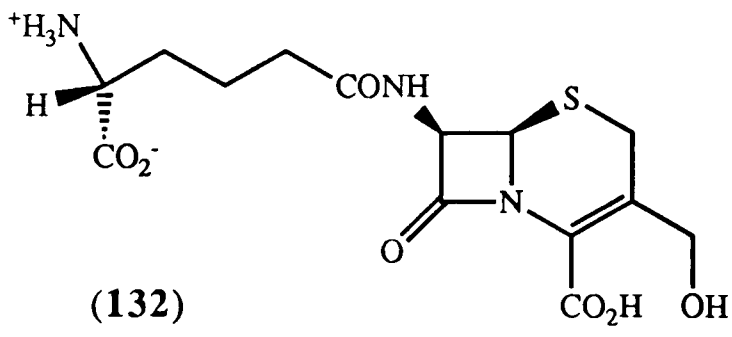
SCHEME 3.9

3.5.2 Model Studies

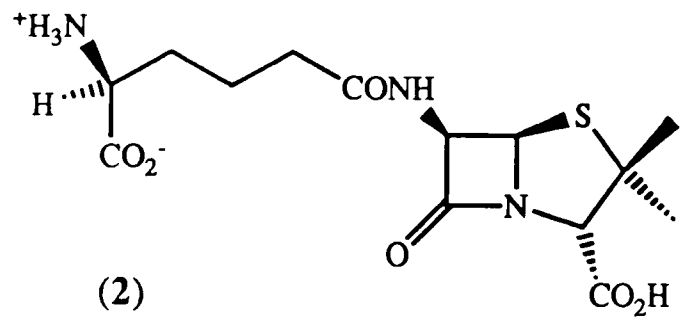
Rather than deplete supplies of 3α-hydroxymethylpenicillin (**107**), it was decided to first investigate the procedure using deacetoxycephalosporin C (DAOC) (**131**), deacetylcephalosporin C (DAC) (**132**) and isopenicillin N (**2**) as model compounds for the derivatisation reactions.



(131)



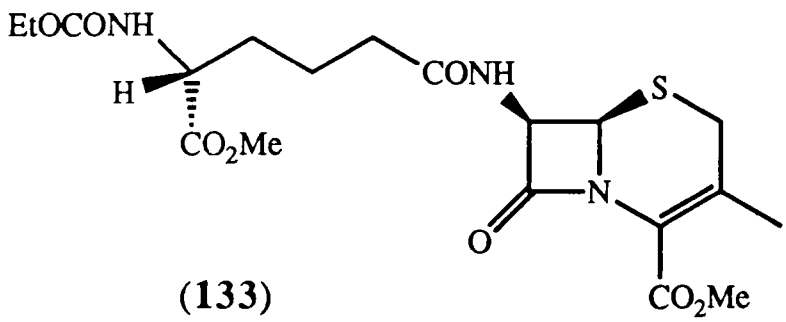
(132)



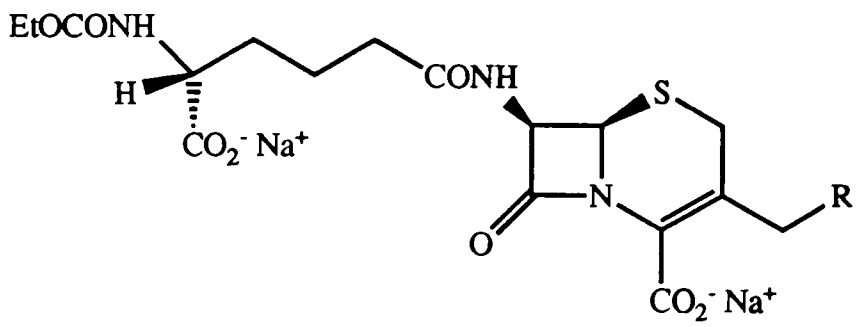
(2)

Derivatisation studies were first carried out on the cepham model, DAOC (**131**), using the procedure developed by Baldwin *et al.*[79,112] (as given in Scheme 3.9). It was found that the *N*-ethoxycarbonyl, dimethyl ester derivative (**133**) of DAOC was formed in ~30% yield on a large scale (5mg, 12μmol of DAOC), but that the same procedure carried out on a small scale (400 or 800μg, 0.9 or 1.8μmol) failed to yield any β-lactam products detectable by 500MHz ^{1}H NMR spectroscopy. These results necessitated the investigation of each step in the procedure individually.

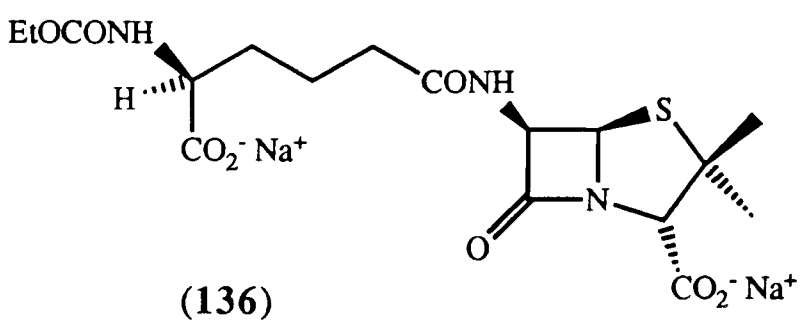
Investigation of the *N*-protection step showed the reaction to proceed virtually quantitatively in all cases, as determined by 500MHz ^{1}H NMR spectroscopy. The *N*-ethoxycarbonyl derivatives (**134**, **135** and **136**) of DAOC (**131**), DAC (**132**) and IPN (**2**) respectively could all be prepared routinely on all scales attempted (down to 400μg of each β-lactam model compound). Additionally, examination of the 500MHz ^{1}H NMR spectrum of (**135**) showed that the reaction proceeded with no detectable reaction at the hydroxyl group.



(**133**)



(134) R = H
(135) R = OH



(136)

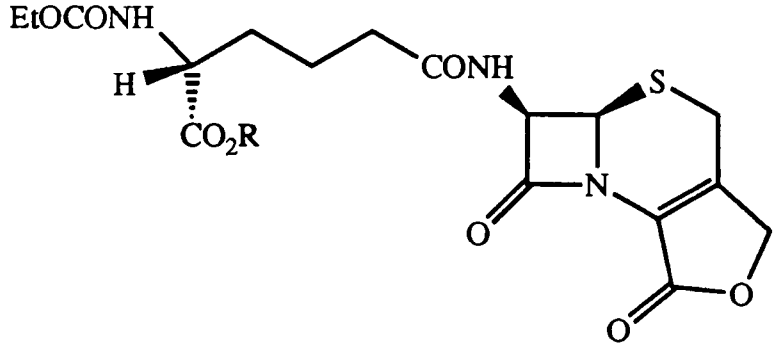
Solutions of these *N*-protected compounds were acidified and extracted with organic solvents according to the standard procedure. The results consistently showed this step to be of very low efficiency for small scale reactions. The extraction efficiency for 2-5mg scale reactions of *N*-ethoxycarbonyl-DAOC (**134**) was established through 500MHz ^{1}H NMR spectroscopy, and shown to be of the order of 30%, reflecting the overall yield found for the formation of the fully protected material (**133**). These extractions, when carried out on sub-milligram quantities of each *N*-ethoxycarbonyl derivative (**134**, **135** and **136**), failed to yield any β-lactams in the organic extracts. The yields of these extractions could not be improved by either the use of low volumes of aqueous solution (~100μl), or by the addition of saturated NaCl solution.

An alternative method of producing the free acid forms of the *N*-protected cepham derivatives (**134**) and (**135**) was developed which involved suspension of the solid derivatives in acetonitrile, addition of a little HCl (1M;

50-100μl), followed by drying of the solution (Na_2SO_4) and evaporation of solvent *in vacuo*. This method was used successfully on scales down to 400μg, producing the free diacid form of (**134**) in virtually quantitative yield, and converting the sodium salt of *N*-ethoxycarbonyl-DAC (**135**) to the corresponding lactone (**137**). The ready formation of the lactone (**137**) under these acidic conditions was thought to bode well for production of the desired protected form of the lactone, (**110**), of 3α-hydroxymethylpenicillin (**107**), owing to the structural analogy between this penicillin and DAC (**132**).

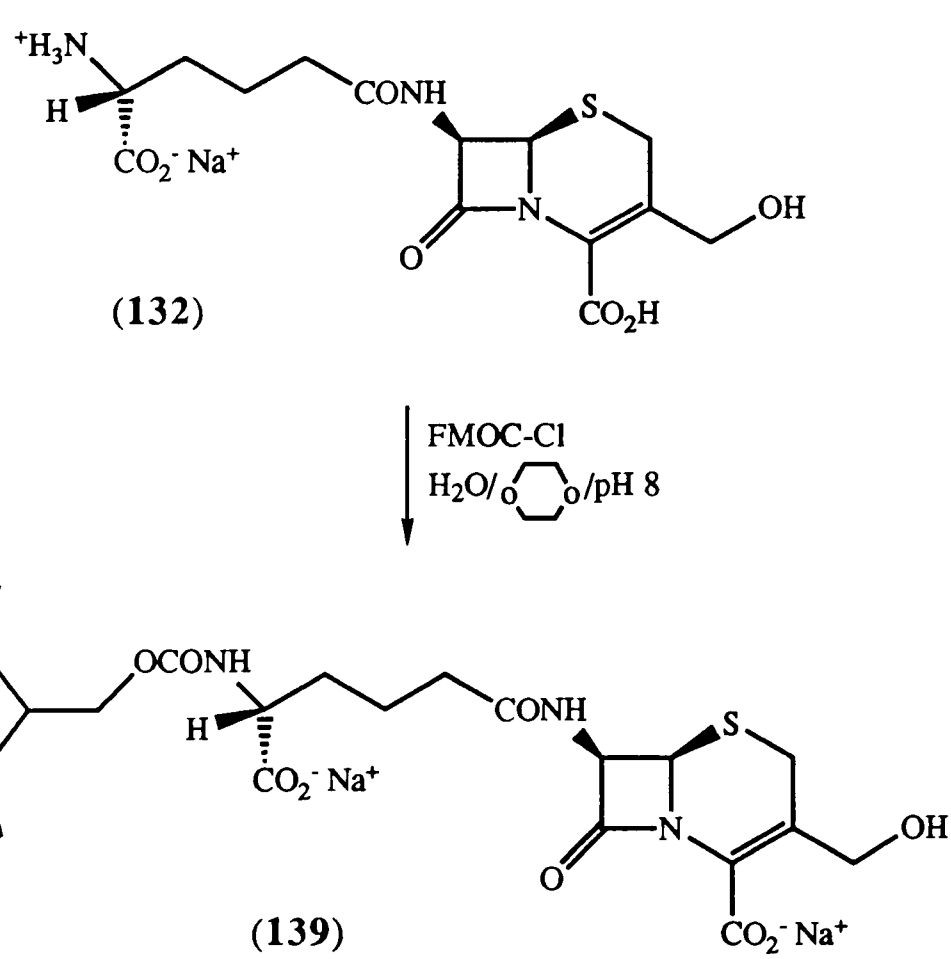
The acids formed in this manner could be quantitatively transformed into their methyl esters, (**133**) and (**138**), by addition of CH_2N_2 solution, confirming that there was no problem associated with the methylation step of the procedure.

(**137**) R = H
(**138**) R = Me



Having identified the extraction process as being the problematic step, and, having successfully developed an efficient alternative with the DAOC (**131**) and DAC (**132**) models, this protocol was applied to *N*-ethoxycarbonyl-isopenicillin N (**136**). Unfortunately, repeated attempts to produce the free diacid form of (**136**) all met with failure, with the greater sensitivity of the penicillin structure to acidic conditions leading to degradation of the β-lactam structure under the conditions employed.

A second approach to this problem was the utilisation of a much more hydrophobic *N*-protecting group, in the form of the (9-fluorenylmethyl)oxycarbonyl (FMOC) group,[113] in order to decrease the solubility of the free acids in aqueous media, and so hopefully increase the efficiency of the extraction process. The *N*-FMOC-protected form, (**139**), of DAC (**132**) was readily formed by reaction of DAC (**132**) with FMOC-chloride in alkaline aqueous solution (Scheme 3.10), but proved difficult to work with, having limited solubility in both aqueous and organic solvents (as either the disodium salt or free acid). Although this compound was readily soluble in mixed solvents (such as H_2O/acetone), this was of little use in the development of an extraction protocol.



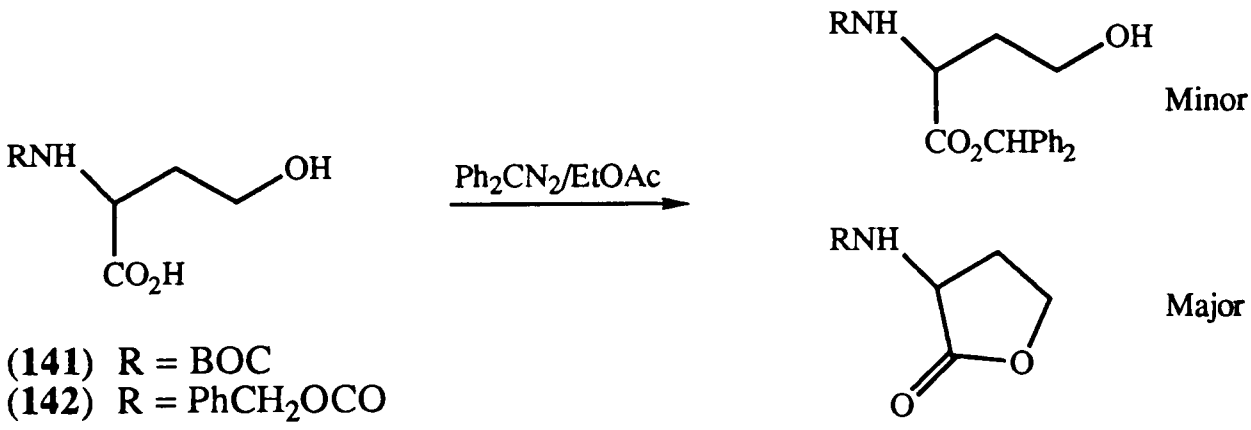
SCHEME 3.10

From the results of these studies with model compounds, it became clear that a different approach to the acidification/extraction process would need to be developed for the more acid sensitive 3α-hydroxymethylpenicillin (**107**). It also seemed that the DAC (**132**) model was not a good model because of its greater stability. Further studies were carried out on 3α-hydroxymethylpenicillin (**107**).

3.5.3 Derivatisation of 3α-Hydroxymethylpenicillin (107)

It was noted that in the mass spectrum of 3α-hydroxymethylpenicillin (**107**), there existed a weak peak consistant with the lactone (**110**). This could be formed in the solution used for sample injection, which contains HCO_2H. However, an attempt to lactonise 3α-hydroxymethylpenicillin (**107**) directly by treatment with HCO_2H failed to yield any β-lactam products.

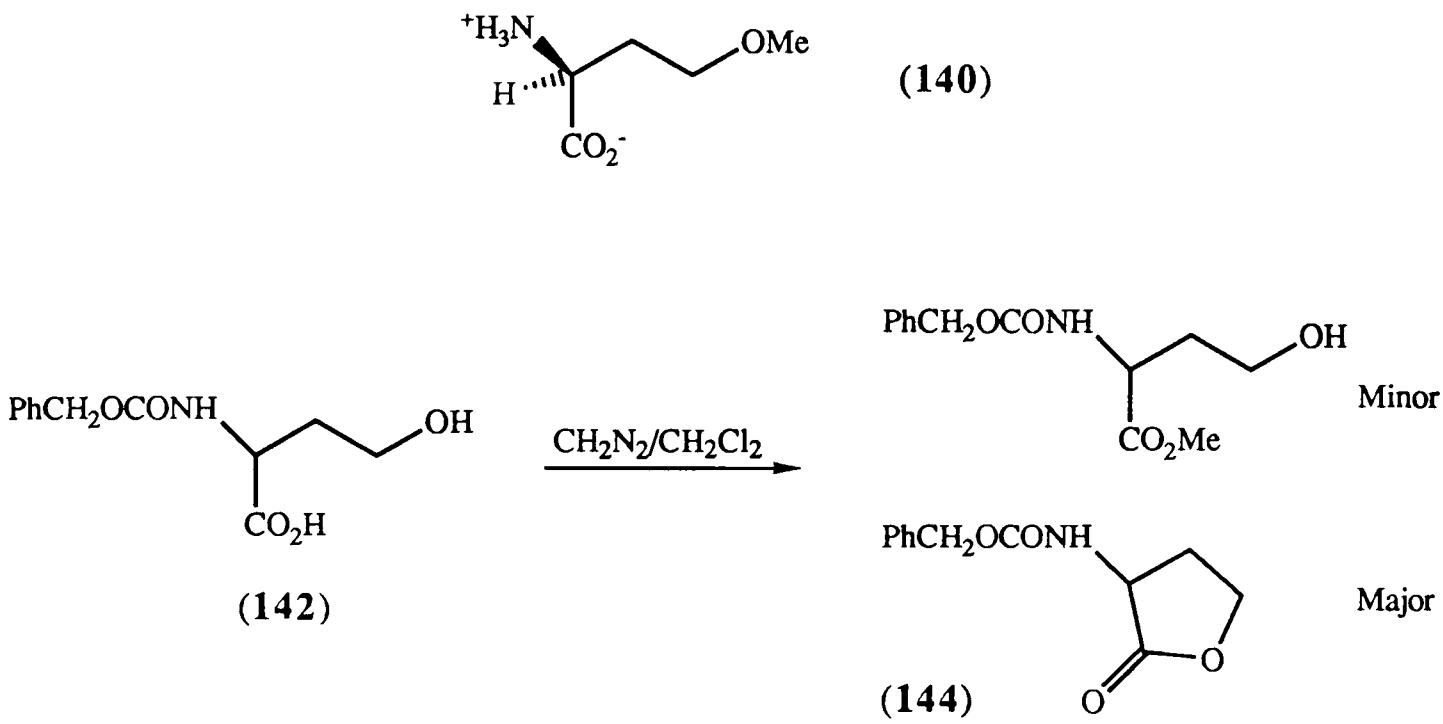
During the course of work on the synthesis of the amino acid 2*S*-amino-4-methoxybutyric acid (*O*-methyl-D-homoserine) (**140**) it was found that *N*-protected homoserine derivatives (**141** and **142**) would readily lactonise under conditions employed to protect the acid functionality (diphenyldiazomethane/EtOAc or CH_2N_2/CH_2Cl_2) to give the γ-lactones (**143**) and (**144**) (Schemes 3.11 and 3.12).



(**141**) R = BOC
(**142**) R = $PhCH_2OCO$

(**143**) R = BOC
(**144**) R = $PhCH_2OCO$

SCHEME 3.11



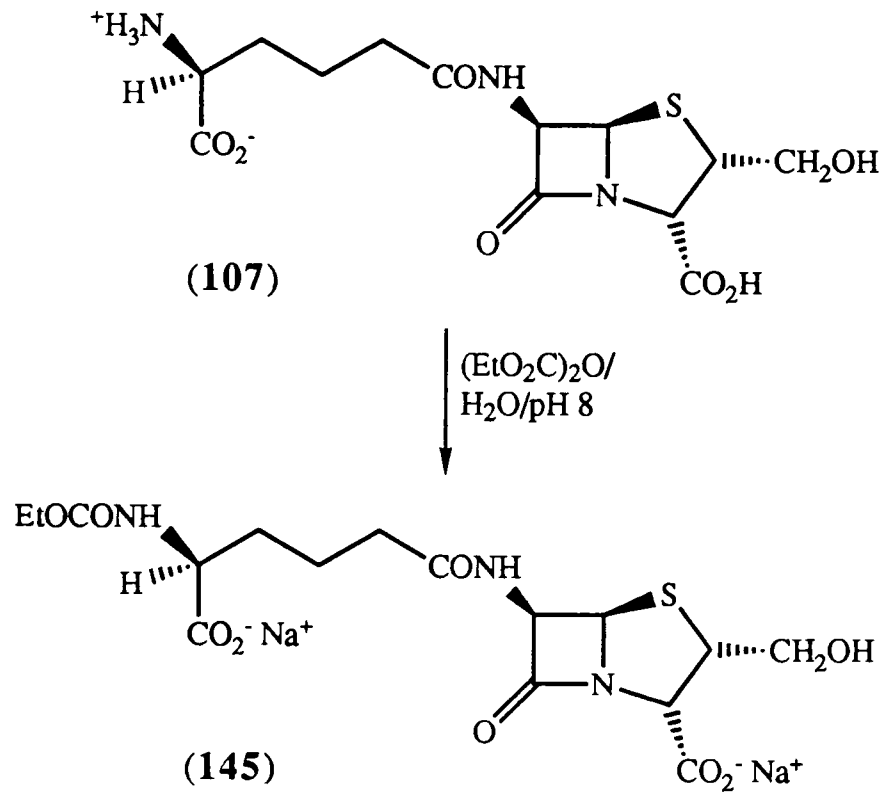
SCHEME 3.12

It was considered likely that this observation was due to facile lactonisation of the γ-hydroxy-ester functionality, especially in the case of the methyl ester (Scheme 3.12). It seemed unlikely that the acidification/extraction procedure would be successful, so it was decided to investigate this new line of approach with 3α-hydroxymethylpenicillin (**107**).

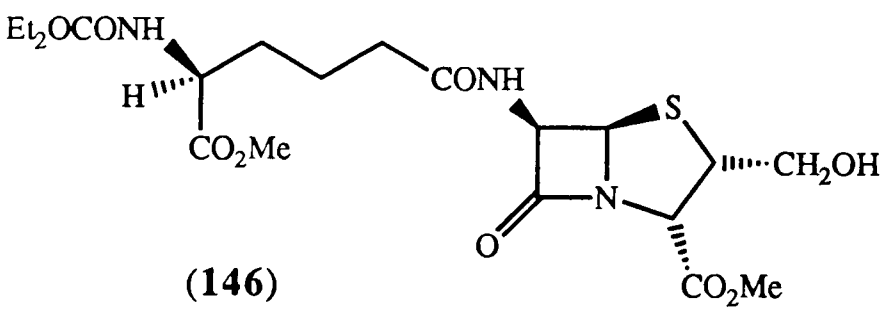
The *N*-ethoxycarbonyl derivative (**145**) of 3α-hydroxymethylpenicillin (**107**) was readily formed from purified samples of (**107**) by treatment with diethylpyrocarbonate in aqueous solution at pH 8 (Scheme 3.13). Analysis by ^{1}H NMR spectroscopy indicated no reaction of diethylpyrocarbonate at the hydroxyl group of (**107**), which was confirmed by mass spectrometric analysis.

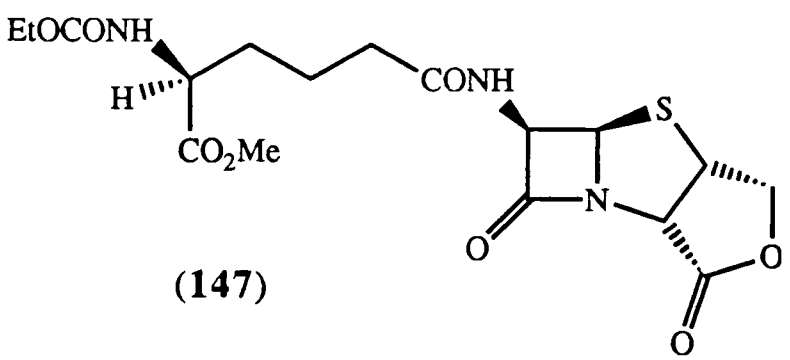
Reaction of the *N*-ethoxycarbonyl material (**145**) with MeI in DMF for 2h at room temperature produced a new β-lactam product, which was readily soluble in $CDCl_3$. Analysis by 500MHz ^{1}H NMR spectroscopy and mass spectrometric analysis characterised this product as the *N*-ethoxycarbonyl, dimethyl ester derivative (**146**) of 3α-hydroxymethylpenicillin (**107**) (Fig-

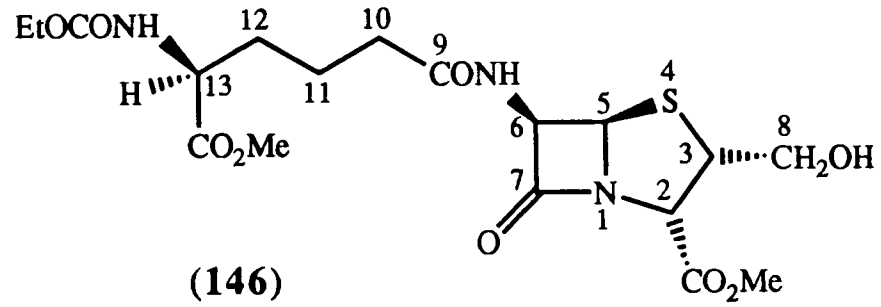
ure 3.9). In addition the mass spectrum of (**146**) confirmed that there had been no reaction at the hydroxyl group of (**107**) during the formation of the *N*-ethoxycarbonyl derivative (**145**), and showed a strong molecular ion peak for the protected lactone (**147**), indicating its ready formation from the dimethyl ester (**146**) under the conditions of mass spectrometric analysis.



SCHEME 3.13







(146)

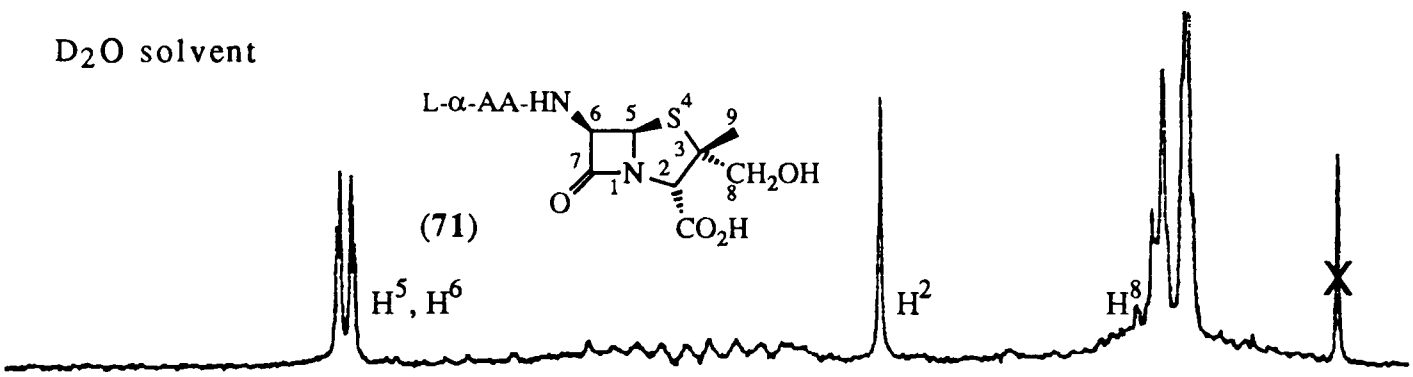
FIGURE 3.9

500MHz ^{1}H NMR spectrum of the *N*-ethoxycarbonyl, dimethyl ester derivative (**146**) of 3α-hydroxymethylpenicillin (**107**)

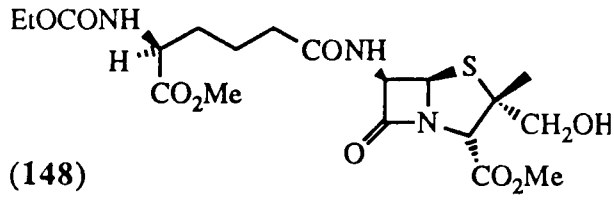
A closer examination of the 500MHz ^{1}H NMR spectrum of (**146**) revealed resonances which, by analogy with the lactone (**130**) formed from (**71**) (Figure 3.10),[114] were consistent with the presence of a small amount of the desired *N*-ethoxycarbonyl, methyl ester protected form (**147**) of the lactone (**110**) (Figure 3.11).

FIGURE 3.10[114]

Comparison between the ^{1}H NMR resonances in the penicillin (**71**) and the lactone (**130**), showing the changes in resonance frequencies.



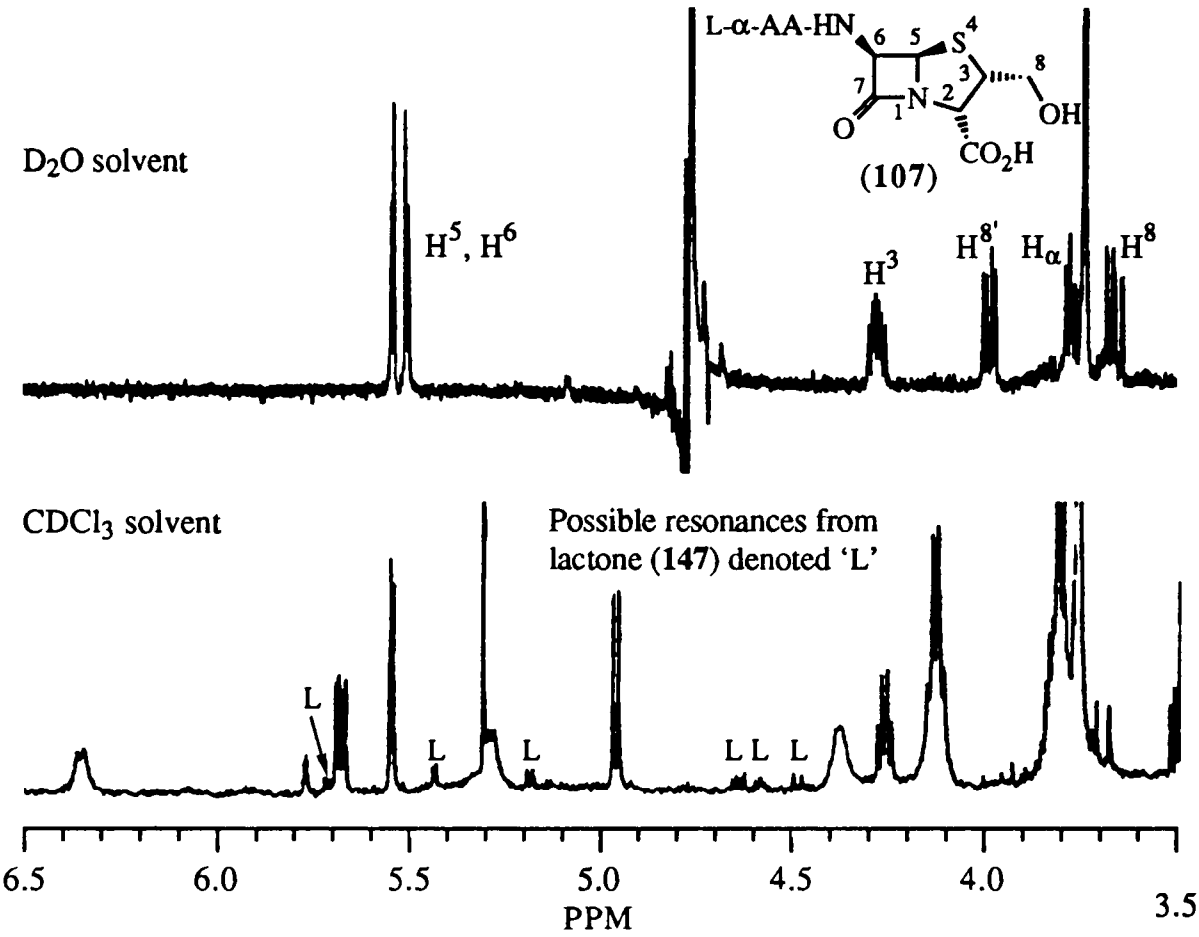
Resonances denoted 'a' arise from the dimethyl ester (**148**)



(**148**)

FIGURE 3.11

Comparison between the ^1H NMR resonances in the penicillin (**107**) with possible lactone (**147**) resonances in the spectrum of the derivative (**146**).



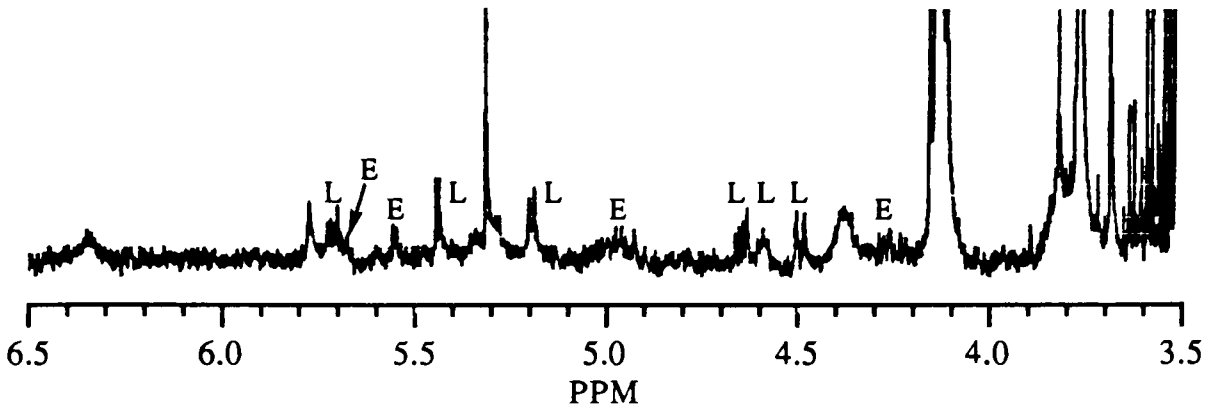
The reaction was repeated, with the *N*-ethoxycarbonylpenicillin (**145**) being stirred with MeI, in DMF, overnight to see if this would lead to lactone formation from the dimethyl ester (**146**). However, this procedure failed to give any enhancement of the putative lactone resonances in the ^1H NMR spectrum, and led to the degradation of most of the dimethyl ester (**146**) (Figure 3.12). Attempts to isolate the compound giving rise to the ^1H NMR resonances of interest by normal phase HPLC, met with no success.

The best yields of the lactone (**147**) were obtained for reaction times of 2-4h with MeI in d_7-DMF. The results were not reproducible, with the ratio of dimethyl ester (**146**) to lactone (**147**) produced in the reaction varying between 10:1 and 2:3.

FIGURE 3.12

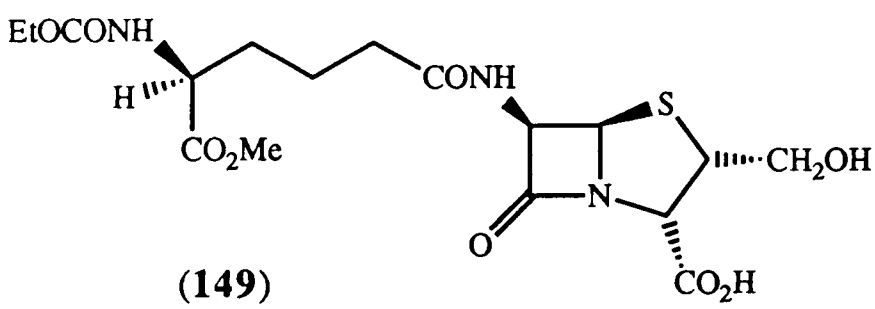
^{1}H NMR resonances after overnight reaction.

Residual resonances from the dimethyl ester (**146**) are denoted 'E', resonances which possibly arise from the lactone (**147**) are denoted 'L'.



In an attempt to lactonise the dimethyl ester (**146**), a sample of this material [prepared from 140μg of 3α-hydroxymethylpenicillin (**107**)] was suspended in toluene, and heated to 55°C for two hours. After evaporation of toluene *in vacuo*, examination of the residue by ^{1}H NMR spectroscopy revealed no $CDCl_3$-soluble β-lactams, but revealed the presence of a new D_2O soluble β-lactam, whose ^{1}H NMR spectrum was consistent with the γ-hydroxy-acid (**149**).

It was considered that, with this material having the amino acid functionality completely protected, it would have a greater likelihood of being extracted into organic solvents. However, it did not prove possible to protonate and extract this material into organic solvents, and furthermore, examination of the (neutralised) residual aqueous layer by ^{1}H NMR spectroscopy showed no remaining β-lactam signals.



(149)

In order to closely examine the course of the reaction of (**145**) in DMF, it was decided to carry out the reaction in deuterated solvent. Methylation of the *N*-protected derivative (**145**) was carried out in d_7-DMF, while the methylating agent was changed from MeI, to the non-volatile reagent dimethylsulphate. Examination of the ^{1}H NMR spectrum after 1.5 hours showed that, despite the change in methylating agent and the increase in solvent volume necessary to obtain the spectrum (to 400μl), formation of the expected dimethyl ester (**146**). Unfortunately, due to large resonances due to d_6-DMF, HOD, Me_2SO_4 and impurities, the majority of the ^{1}H NMR spectrum below 4.8ppm was obscured. This material was then heated to 40°C in the NMR probe for two hours, after which time all β-lactam signals were found to have disappeared from the ^{1}H NMR spectrum. Evaporation of solvent *in vacuo* yielded a residue which contained no β-lactam products detectable by 500MHz ^{1}H NMR spectroscopy in either $CDCl_3$ or D_2O.

A final attempt to generate the lactone (**147**) from the dimethyl ester (**146**) was made by addition of TsOH (~0.3 equiv) to a solution of (**146**) in $CDCl_3$. Analysis by ^{1}H NMR showed loss of the dimethyl ester (**146**) without enhancement of the lactone (**147**) resonances.

3.5.4 Characterisation of Protected Lactone (147)

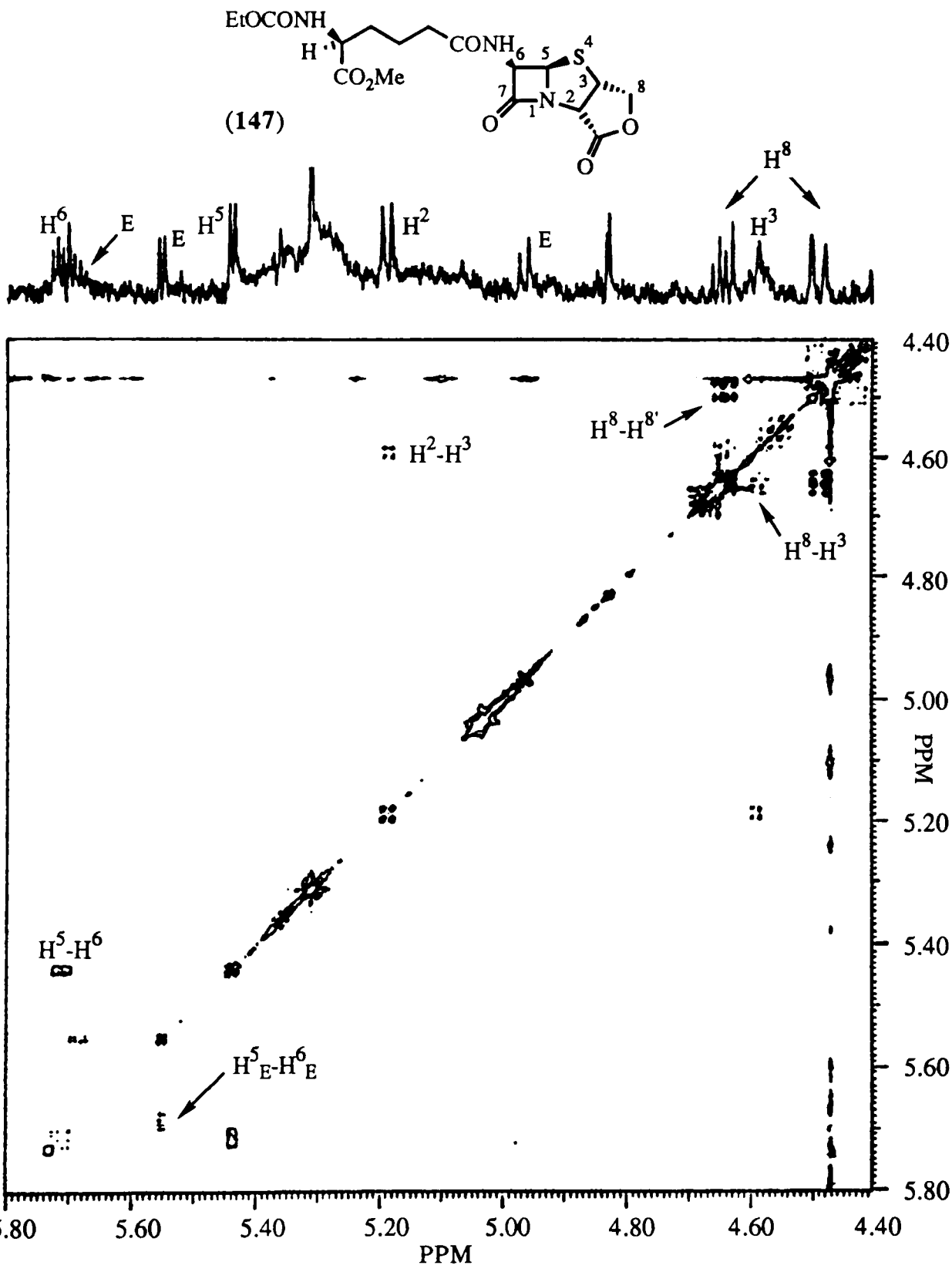
Characterisation of the protected lactone was carried out on a mixture of the dimethyl ester (**146**) and protected lactone (**147**) (*ca.* 1:1.5) produced by reaction of the *N*-protected penicillin (**145**) with MeI in d_7-DMF for 2h.

A 500MHz COSY spectrum confirmed the connectivity of the resonances assigned to the lactone (**147**) (Figure 3.13).

FIGURE 3.13

500MHz COSY spectrum of a mixture of (**146**) and (**147**)

Resonances from the dimethyl ester (**146**) are denoted 'E'



In an attempt to assign the the 8-Pro*R*- and 8-Pro*S*- H(8) resonances in this lactone, a series of NOE experiments were run on the sample, irradiating two resonances for each overnight experiment.

These experiments pushed the sensitivity of the spectrometer to its limits, with it only being possible to observe NOE's between the hydrogens in closest proximity, and these with a signal to noise ratio of two or less (no quantitative determinations were possible). Additionally, the difference spectra did not allow observation of any NOE's to resonances within 0.2ppm of the resonance irradiated, so that it was not possible to assess if any NOE's existed within the group of resonances at 4.45-4.65ppm (i.e. the region of greatest interest). The observed NOE's are given in Table 3.1.

TABLE 3.1
NOE enhancemants observed for lactone (**147**)

Resonance Irradiated	NOE Enhancement Observed
H(8) (4.49ppm)	none
H(3)	H(2)
H(2)	H(3)
H(5)	H(6)

A close examination of the coupling constants for the ABX system arising from C(3)H–C(8)H_2 (Table 3.2) however, provided strong evidence for the stereochemistry of the system. The value of the coupling constant between H(3) and H(8') (1.5Hz) indicates a dihedral angle of close to 90°, which is only possible for the H(8) hydrogen on the α-face of the fused ring system of the lactone (**147**), as shown in Figure 3.14.

TABLE 3.2

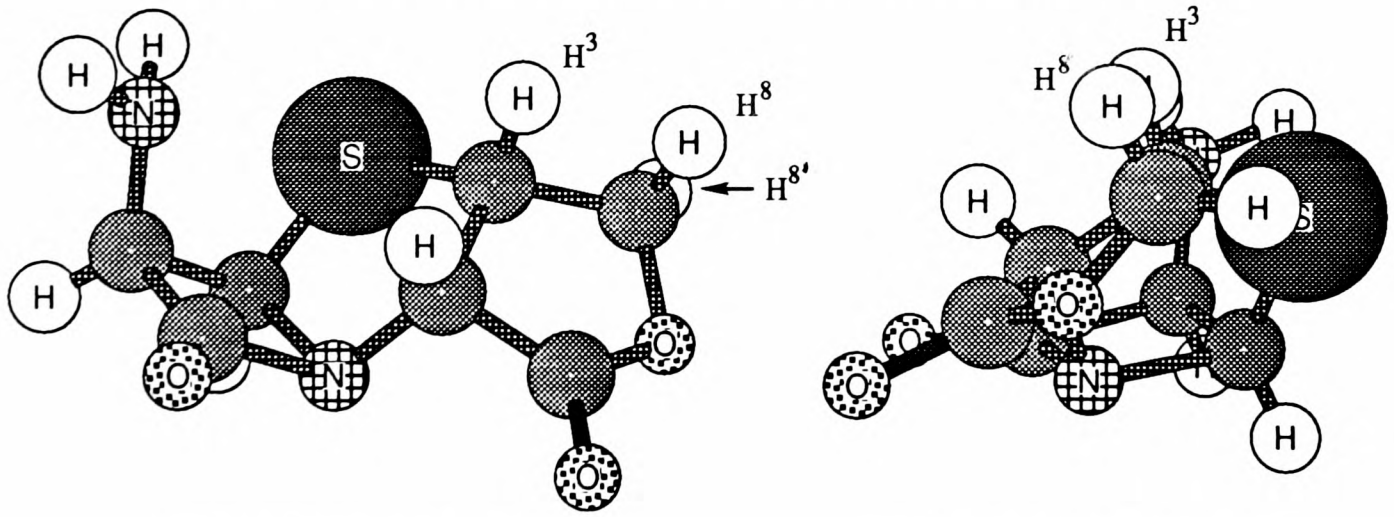
Coupling constants for hydrogens on the lactone ring of (**147**)

H(8) is taken as the resonance at 4.60ppm and H(8') as that at 4.49ppm

Coupling	Observed Value (Hz)
$J_{H(2)}$-$J_{H(3)}$	7.5
$J_{H(3)}$-$J_{H(8)}$	5.5
$J_{H(3)}$-$J_{H(8')}$	1.5
$J_{H(8)}$-$J_{H(8')}$	10.5

FIGURE 3.14

Stereochemistry of the C(3)H–C(8)H_2 atoms in the lactone (**147**) to explain the observed coupling constants



View of ring system in (147)

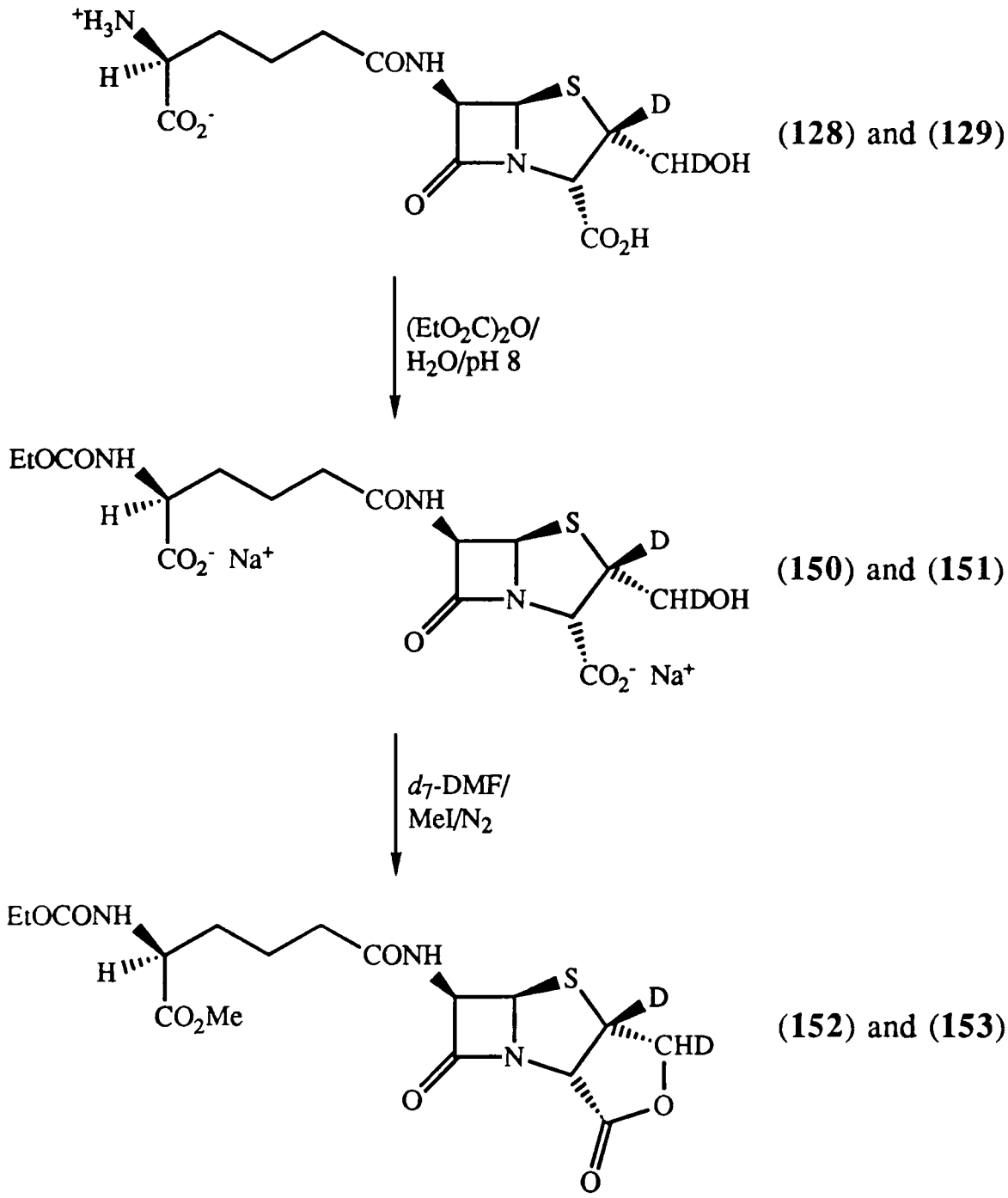
View along C(8)-C(3) bond

These coupling constants are good evidence therefore, for the assignment of H(3) (4.49ppm) as the 8-Pro*S*-hydrogen and H(8) (4.64ppm) as the 8-Pro*R*-hydrogen of the protected lactone (**147**).

3.6 Derivitisation of the Deuterated Penicillins (128) and (129)

3.6.1 Formation and Characterisation of Derivatives

The two [3β-^{2}H]-3α-[8-^{2}H]-hydroxymethylpenicillins (**128**) and (**129**) were protected according to the procedure developed for the unlabelled isotopomer (Scheme 3.14).



(**128**) and (**129**)

(**150**) and (**151**)

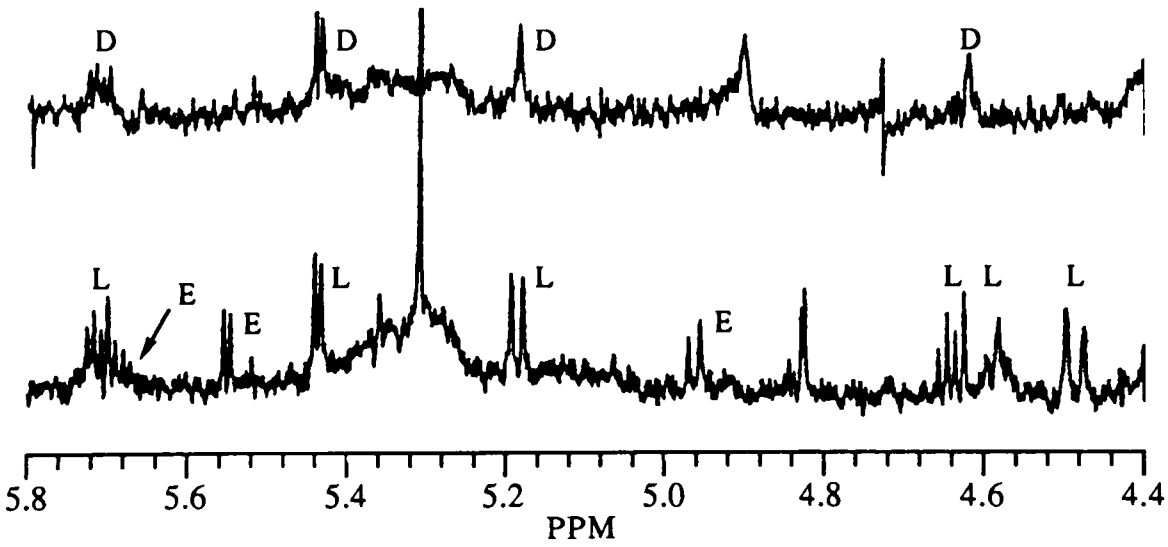
(**152**) and (**153**)

(**128**), (**150**) and (**152**) derive from the *E*-d_2-tripeptide (**111**)
(**129**), (**151**) and (**153**) derive from the *Z*-d_2-tripeptide (**112**)

SCHEME 3.14

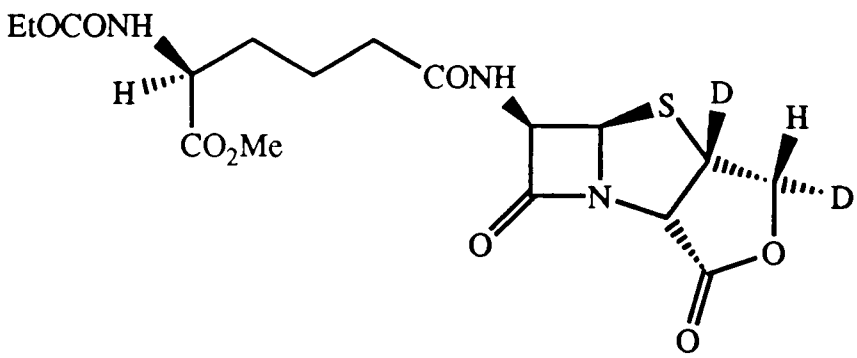
The *N*-protection reaction proceded in good yield to give the *N*-ethoxycarbonyl derivatives (**150**) and (**151**). These compounds were protected using MeI in d_7-DMF, employing a three hour reaction time. Analysis by 500MHz ^{1}H NMR spectroscopy of the product mixture from reaction of (**150**) indicated the presence of a single β-lactam product, which by comparison with the unlabelled lactone (**147**) and mass spectroscopic analysis, was characterised as the d_2-lactone (**152**). Comparison of the C(8)HD resonance with the C(8)H_2 resonances from (**147**) (Figure 3.15), taking the stereochemistry as assigned in section 3.5.4, identified the stereochemistry of the C(8) position of (**152**) as *S*.

FIGURE 3.15
Comparison of the ^{1}H NMR spectra of
the d_0- and d_2-lactones (**147**) and (**152**)
Key: 'D' = (**152**), 'L' = (**147**), 'E' = dimethyl ester (**146**)

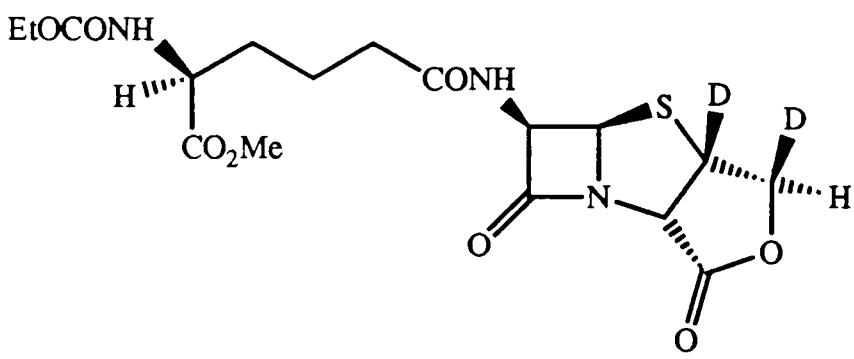


A similar analysis of the product mixture from reaction of (**151**) showed the presence of one, barely detectable, β-lactam product. It was not possible to carry out a similar characterisation of this compound, but by analogy with the formation of (**152**), and taking into account the complimentary deuterium

labelling patterns in the penicillins (**128**) and (**129**), it was assigned as the [8*R*-^{2}H]-lactone (**153**).



(**152**)



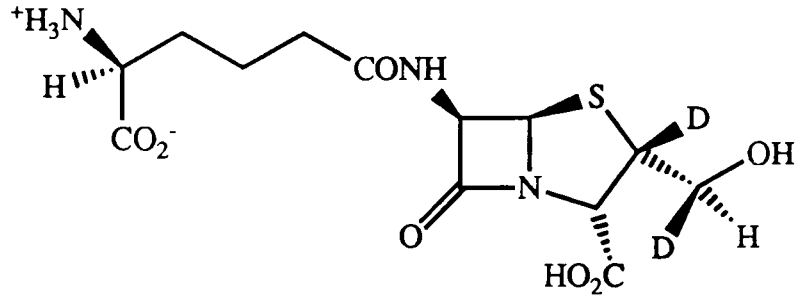
(**153**)

3.6.2 The Stereochemistry of Formation of 3α-hydroxymethylpenicillins (107, 128 and 129) with IPNS

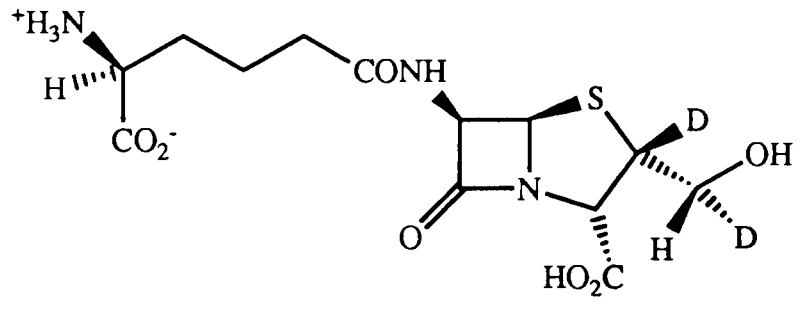
The production of the labelled penicillins (**128**) and (**129**) from the respective tripeptides (**111**) and (**112**), in which the label is retained in a defined stereochemistry, has shown that the addition of sulphur and oxygen across the double bond of LL-AC-D-vinylglycine (**106**) occurs in a stereo-specific manner.

The assignment of the stereochemistry of the labelled lactone (**152**), allows us to determine the overall stereochemistry of the formation of its parent penicillin (**128**) from incubation of the *E*-d_2-tripeptide (**111**) with IPNS. By analogy, this result also indicates the stereochemistry of formation of 3α-hydroxymethylpenicillin (**107**) and the labelled analogue (**129**) from their respective tripeptides (**106** and **112**) with IPNS. These results indicate

the stereochemistries of the labelled lactones (**128**) and (**129**) at the C(8) position to be *S* and *R* respectively, as shown below.



(**128**)



(**129**)

The 8*S*- stereochemistry indicated for the incubation product (**128**) from the *E*-d_2-tripeptide (**111**), arises from overall *syn* addition of sulphur and oxygen across the double bond. This stereochemistry is consistent with the proposed [$2\pi_s + 2\pi$] cycloaddition mechanism (See Scheme 3.3), in which iron and oxygen bond to the same face of the olefin, followed by insertion of sulphur into the iron-carbon bond.

3.7 Comparison Study with IPNS from *Aspergillus nidulans*[29,†]

The labelled tripeptides LLD-AC-*E*-[3,4-$^2H_2$]-vinylglycine (**111**) and LLD-AC-*Z*-[3,4-$^2H_2$]-vinylglycine (**112**) were each incubated with recombinant IPNS corresponding to the IPNS gene from the fungus *Aspergillus nidulans*.[29,†]

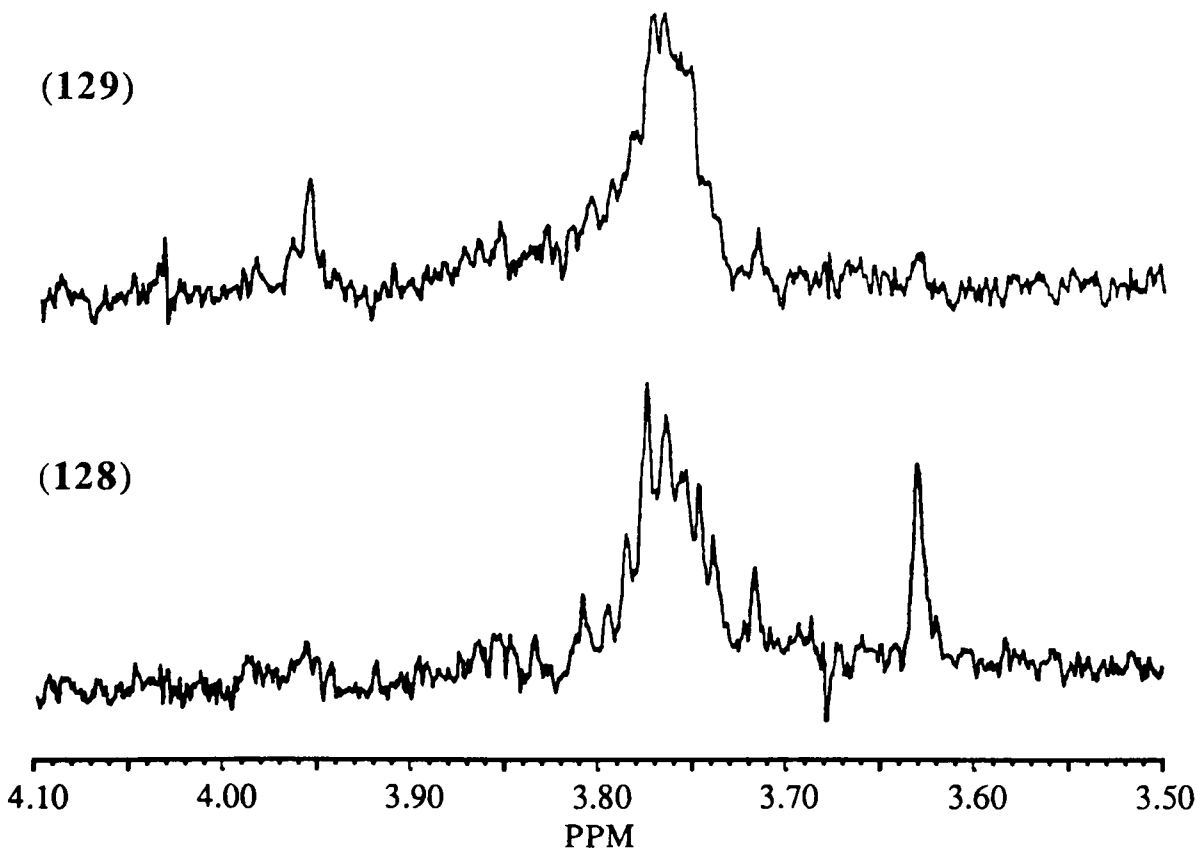
† The primary structure of this IPNS, and the homology with IPNS from *C. acremonium* are given in Appendix B.

Analysis by ^{1}H NMR spectroscopy of the crude incubation mixtures indicated that this new source of IPNS gave a single β-lactam product from each isotopomer of LLD-AC-vinylglycine (**111** and **112**), in the same manner as IPNS from *C. acremonium*, although with a much lower turnover (<10% *cf.* 25-35%). Furthermore, isolation and characterisation of these β-lactam products indicated that they possessed the identical structure and stereochemistry (**128** and **129** respectively) to the products formed with *C. acremonium* IPNS (to the limits of analysis by ^{1}H NMR spectroscopy). The C(8)HD resonances are shown in Figure 3.16.

FIGURE 3.16

(*cf.* Figure 3.8)

^{1}H NMR resonances arising from the CHDOH groups in the d_2-penicillins (**128**) and (**129**), from incubations with IPNS from *A. nidulans*.

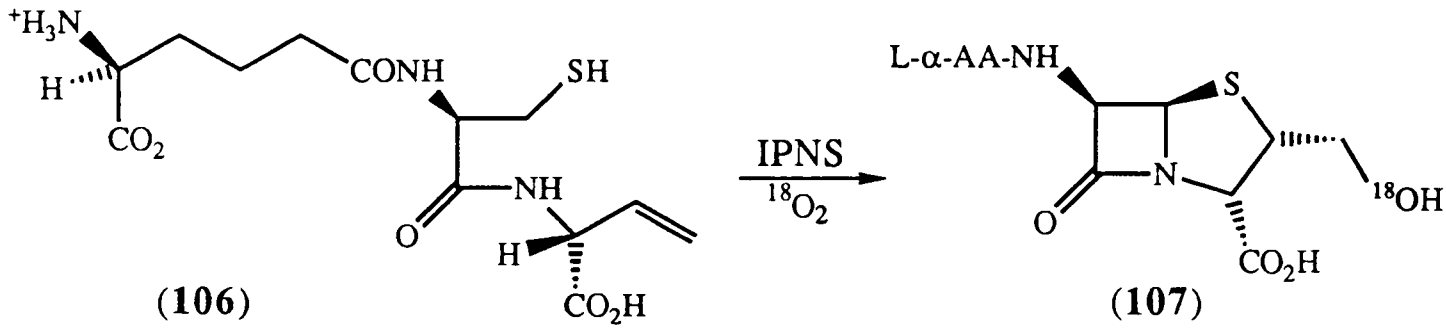


3.8 Conclusions

In this chapter we have intimately investigated the production of 3α-hydroxymethylpenicillin (**107**) from the substrate analogue LL-AC-D-vinylglycine (**106**).

The development of a successful purification protocol has allowed routine isolation of this penicillin from crude incubation mixtures with high purity and good recovery, which has been vital for the subsequent studies.

Through the use of ^{18}O-labelled oxygen gas, and mass spectrometric analysis of the purified product it has been shown that, to the limits of analysis, the hydroxyl oxygen in the product derives exclusively from molecular oxygen (Scheme 3.15).



(**106**) (**107**)

SCHEME 3.15

The incubation with IPNS, of specifically labelled isotopomers of (**106**), LL-AC-*E*-[3,4-$^2H_2$]-D-vinylglycine (**111**) and LL-AC-*Z*-[3,4-$^2H_2$]-D-vinylglycine (**112**) proceeded with no detectable scrambling of the deuterium label, indicating that the IPNS-catalysed reaction occurs in a stereospecific manner.

Derivatisation studies on 3α-hydroxymethylpenicillin (**107**) and model compounds succeded in producing a protected β-lactam (**147**) in which the hydroxymethyl group had been conformationally locked in the form of a γ-lactone. This derivative allowed strong evidence for the assignment of the ^1H

NMR resonances of the 8-Pro*R*- and 8-Pro*S*- hydrogens to be made through analysis of their coupling constants to H(3), and the necessary dihedral angles involved. Comparison by ^{1}H NMR spectroscopy of the analagous derivative, (**152**), of the labelled penicillin (**128**), formed from LL-AC-*E*-[3,4-$^2H_2$]-D-vinylglycine (**111**) with IPNS, provided strong evidence for the stereochemical course of ring closure onto the double bond of LL-AC-D-vinylglycine (**107**), indicating overall addition of sulphur and oxygen in a *syn* fashion.

This result would be consistant with a [$2\pi_s$ + 2π] cycloaddition of an Fe=O species across the double bond (see Scheme 3.3), and is the same stereochemistry as seen in the formation of 3α-hydroxymethyl-3β-methylpenicillin (**71**) from LL-AC-3,4-D-dehydrovaline (**69**)[79] (see Scheme 3.1), and is the major monooxygenase pathway for closure of the second ring during the incubation of LL-AC-D-allylglycine (**52**).[77,78] This result, for the mobile side chain in LL-AC-D-vinylglycine (**106**), would support the surmise that the *syn* addition monooxygenase pathway for second ring closure, is an inherantly more facile reaction for the reactive site species of IPNS than the *anti* addition pathway, and not merely due to conformational restraints in the bulkier 3,4-dehydrovaline and allylglycine systems.

In addition, the specifically deuterated LL-AC-D-vinylglycines (**111** and **112**) were incubated with recombinant IPNS from *A. nidulans*. This enzyme gave a lower conversion of the substrate analogues than IPNS from *C.acremonium* (<10% *cf.* 25-35%), but yielded the same β-lactam product, and with the same stereochemistry. These two IPNS enzymes have a 60% homology at the amino acid level,† and this experiment appeared to indicate that the differences in primary structure did not cause any change in the mechanism of conversion of the substrate analogue LL-AC-D-vinylglycine (**107**).

† For a diagram showing the homology between these two IPNS enzymes see Appendix B.

Final confirmation of the stereochemical assignments made here from analysis of coupling constants, would need to be made by NOE experiments on the unlabelled lactone (**147**). Clearly this would require either an improved method for forming the lactone (**147**), or else for incubation, purification and derivatisation to be carried out on a much larger scale.

CHAPTER 4

THE TEMPERATURE DEPENDENCE OF THE MECHANISM FOR SECOND RING CLOSURE WITH IPNS

4 THE TEMPERATURE DEPENDENCE OF THE MECHANISM FOR SECOND RING CLOSURE WITH IPNS

4.1 Introduction

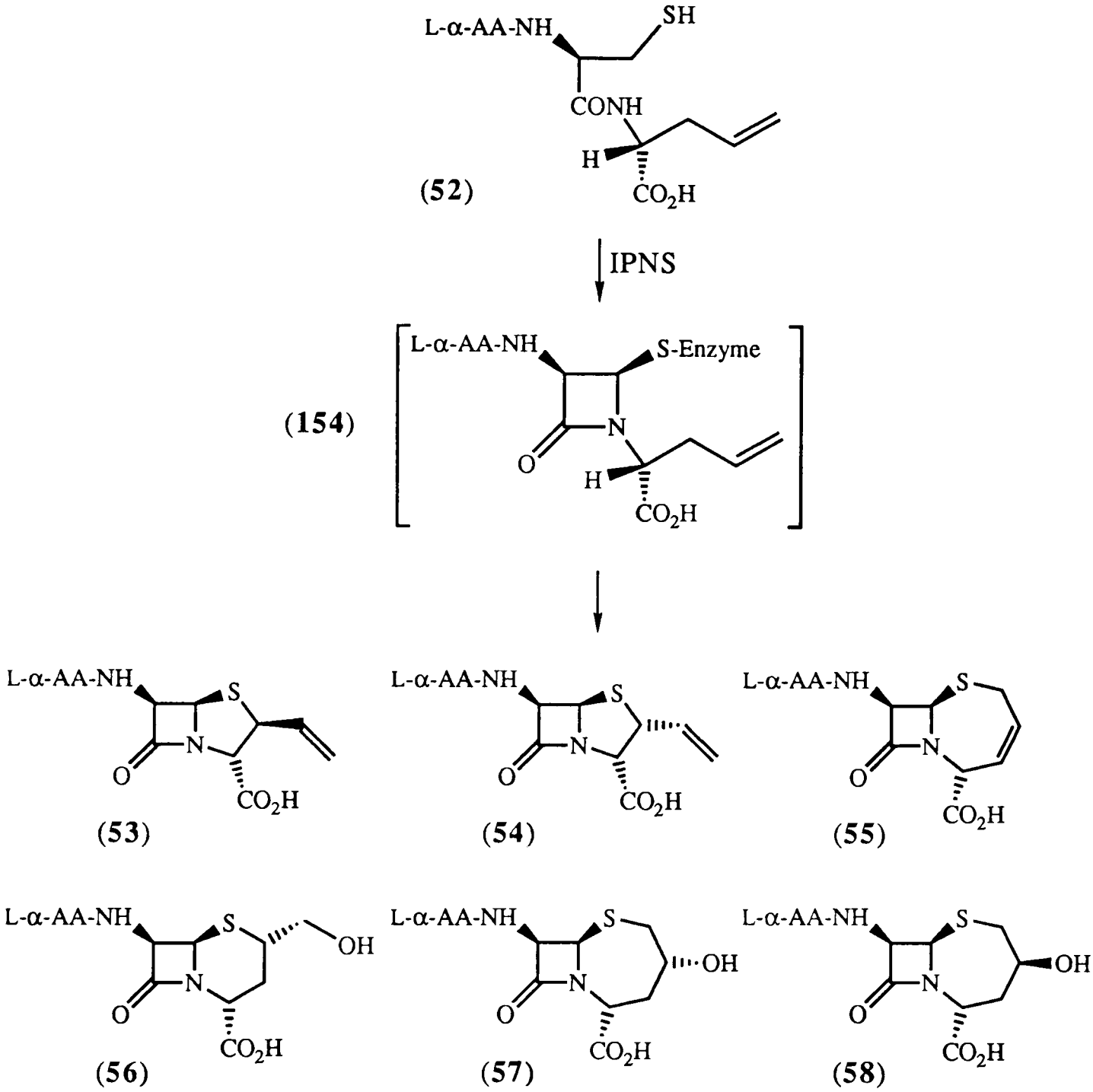
The conversion by IPNS of the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine (LL-AC-D-Ag) (**52**) to yield no fewer than six β-lactam products (**53** to **58**)[72,73] (Scheme 4.1) is the most striking example of the possibility of divergent pathways for second ring formation with IPNS, after formation of the putative enzyme bound β-lactam intermediate (**154**).[45]

In addition to providing evidence for a variety of mechanisms for closure of the second ring, this multitude of products also suggests the presence of a high energy species in the IPNS active site.† If such a high energy species did participate in the catalytic cycle, it would be expected to be in a position to proceed along a greater number of reaction pathways (given of course a suitable substrate) than a similar species of lower energy.

For any system where there are a number of possible products, arising from pathways with different activation energies, we would expect that a change in the temperature of the reaction, would be reflected in a change in the ratio of products formed, in a manner which would reflect the relative reaction energy profiles.

Despite the fact that for enzymatic systems, such as IPNS, the range of temperature available (basically from the freezing point of cofactor solution to the temperature at which the enzyme is denatured) is small compared to that for a typical organic chemistry transformation, such variation in incubation temperature could still prove informative.

† An iron-oxene species has been proposed for the IPNS system.[45]

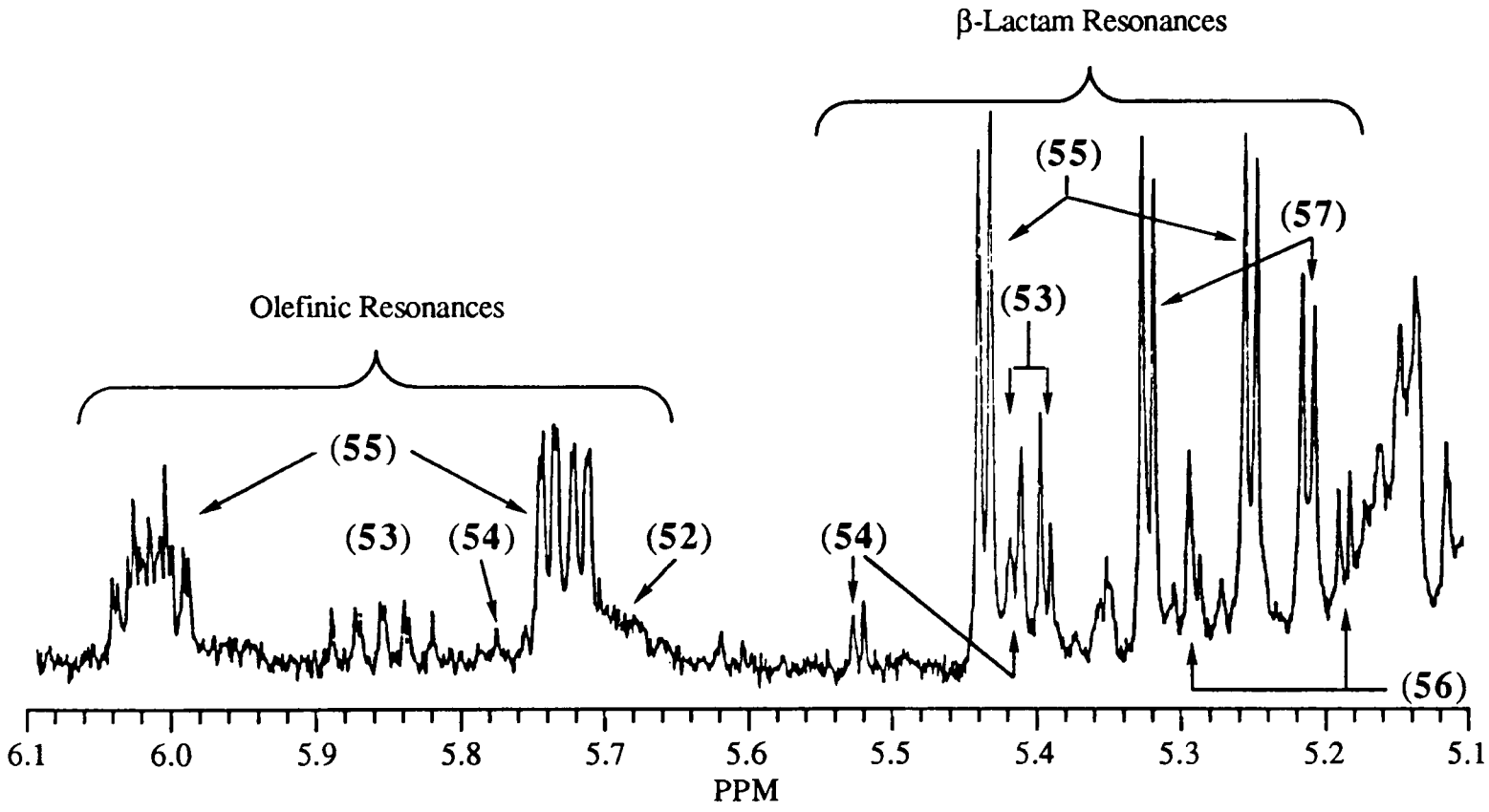


SCHEME 4.1

Furthermore, analysis of the product distribution at different temperatures would provide a convenient method for the determination of the differences in activation energies between the different pathways.

It was decided to investigate the conversion of LL-AC-D-Ag (**52**) by IPNS in this manner. The ratio of products formed could be determined by examination of the β-lactam region of the ^1H NMR spectra of the crude incubation mixtures, which have been identified by previous workers, as shown in Figure 4.1.[72,73]

FIGURE 4.1
The assignment of the ^1H NMR resonances in the β-lactam region of a standard (27°C) incubation of LL-AC-D-Ag (**52**) with IPNS



4.2 Theory

It is fairly easy to derive the relationship between the difference in the activation energies of two pathways branching from a common intermediate, and the ratio of products formed, provided that these reactions are irreversible, so that we are examining kinetic rather than thermodynamic effects.

The rate of any reaction, k_r, may be expressed in terms of the activation energy, E_A, by the relationship given in equation 4.1.[115]

$$k_r = \sigma N_A \left(\frac{8kT}{\pi\mu}\right)^{\frac{1}{2}} e^{-\left(\frac{E_A}{RT}\right)} \qquad \text{Equation 4.1}$$

Where: σ is a effective collision cross section.

N_A is the Avogadro number.

k is the Boltzmann constant.

T is the absolute temperature.

μ is the reduced mass of the system.

R is the molar gas constant, defined as $8.31441 J.mol^{-1}.K^{-1}$.

Consider a system in which two products, A and B, are formed from a common intermediate, with rate constants k_a and k_b (where $k_a > k_b$). If the reactions yielding these two products are irreversible, then the ratio of A to B formed in the reaction, q, will reflect kinetic factors, and will be equal to the ratio of the rate constants for the reactions. Substituting from equation 4.1, we obtain equation 4.2.

$$q = \frac{k_a}{k_b} = \left(\frac{\mu_b}{\mu_a}\right)^{\frac{1}{2}} \frac{\sigma_a e^{-\left(\frac{E_a}{RT}\right)}}{\sigma_b e^{-\left(\frac{E_b}{RT}\right)}} \qquad \text{Equation 4.2}$$

Where the subscripts a and b refer to the reactions yielding products A and B respectively.

If we group all the constants together (including any empirical correction factors for the reactions) in the term e^C, and take the natural logarithms of each side of the equation, we obtain equation 4.3.

$$\ln q = \frac{(E_b - E_a)}{RT} + C \qquad \text{Equation 4.3}$$

This equation indicates that a plot of lnq against 1/RT should give a straight line with a gradient equal to the difference between the activation energies for the two reactions.† Application of this method of analysis to the product ratios obtained from incubation of LL-AC-D-Ag (**52**), should yield information about the relative activation energies for the different pathways for closure of the second ring.

4.3 Incubations at Varying Temperature

4.3.1 Temperature Control

The temperature of incubations of LL-AC-D-Ag with IPNS was controlled in one of two ways. Incubations at above room temperature were carried out in a stirred constant-flow jacketed cell, connected to a constant temperature water bath; whereas incubations at below room temperature were carried out in glass vials suspended in a water bath maintained by a thermostatically controlled cold table.

4.3.2 Control Experiment

Before comparing the ratios of the β-lactam products formed in incubations of LL-AC-D-Ag (**52**) with IPNS at different temperatures, it was necess-

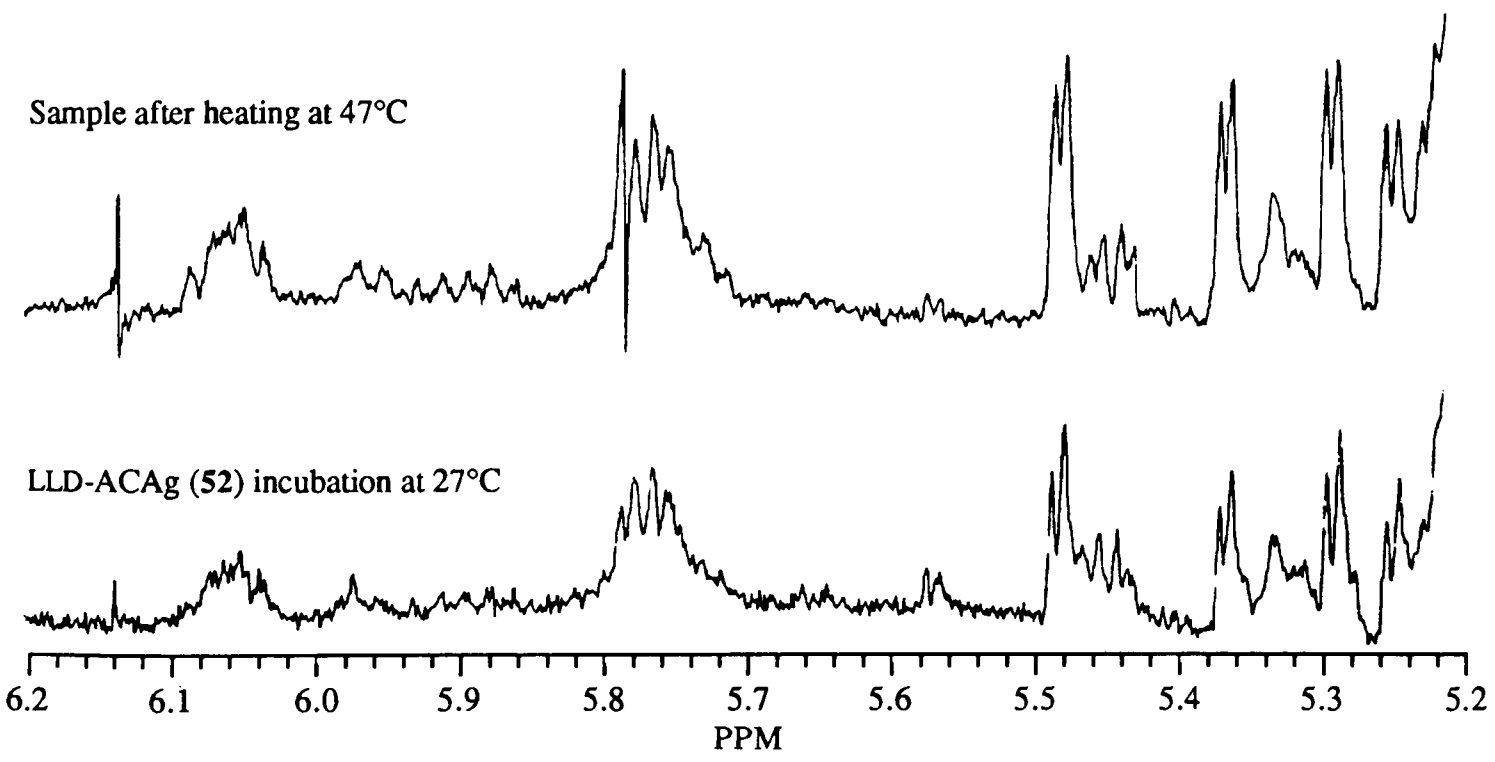
† In fact the gradient is actually equal to the difference in activation enthalpies, ΔH, between the reactions forming A and B, but the difference involved will be small unless the pathways are very different.

ary to determine the stability of these products within the temperature range to be investigated, in order to ensure that the ratios observed did in fact reflect the ratios in which the β-lactams were formed.

The crude incubation mixture from an incubation carried out at the optimum temperature of 27°C was examined by ^{1}H NMR spectroscopy, and the ratio of the β-lactam products compared after the sample had been warmed at room temperature for 24h, and then heated for 30min at 47°C. The spectra obtained showed there to be no detectable changes after 24h at room temperature, and whilst there was some loss of the β-lactam (**53**) at 47°C (Figure 4.2), the change in the ratio of the peaks was sufficiently small to be able to continue these investigations.

FIGURE 4.2

Comparison of the ^{1}H NMR resonances in
the β-lactam region after heating at 47°C for 30min



4.3.3 Incubations

Except for the temperature and duration of the incubations, identical conditions were employed for each incubation as far as practicably possibly.

Incubations were run for a series of temperatures at 10°C intervals above and below the optimum temperature for IPNS activity (27°C).[93] Incubation at 47°C gave virtually no turnover of LL-AC-D-Ag (**52**), marking the maximum incubation temperature beyond which the enzyme becomes totally inactive.

The 500MHz ^{1}H NMR spectra for the β-lactam region of each of the crude incubation mixtures are shown in Figure 4.3. The product ratios were determined by the relative integrals, with product assignment being made by reference to Figure 4.1. The results obtained are given in Table 4.1 and Figure 4.4.

TABLE 4.1

Product ratios, as determined by ^{1}H NMR spectroscopy, for incubations of LL-AC-D-Ag (**52**) with IPNS at different temperatures

Incubation Conditions		Degree of Conversion	(53)	(54)	(55)	(56)	(57)	(58)
Temp.	Duration							
7°C	160min	Good	2	1	10	2	3	–
17°C	80min	Good	3	2	10	5	7	–
27°C	40min	Average	2	3	10	6	8	–
37°C	30min	Poor	4	1	10	12	14	–
47°C	30min	V. Poor	–	✓	✓	–	✓	–
Lit Value (27°C)[72]			4	1	10	2	5	Trace

FIGURE 4.3

Comparison of the ^1H NMR resonances in the β-lactam region for incubations of LLD-ACAg (**52**) with IPNS at different tempeatures

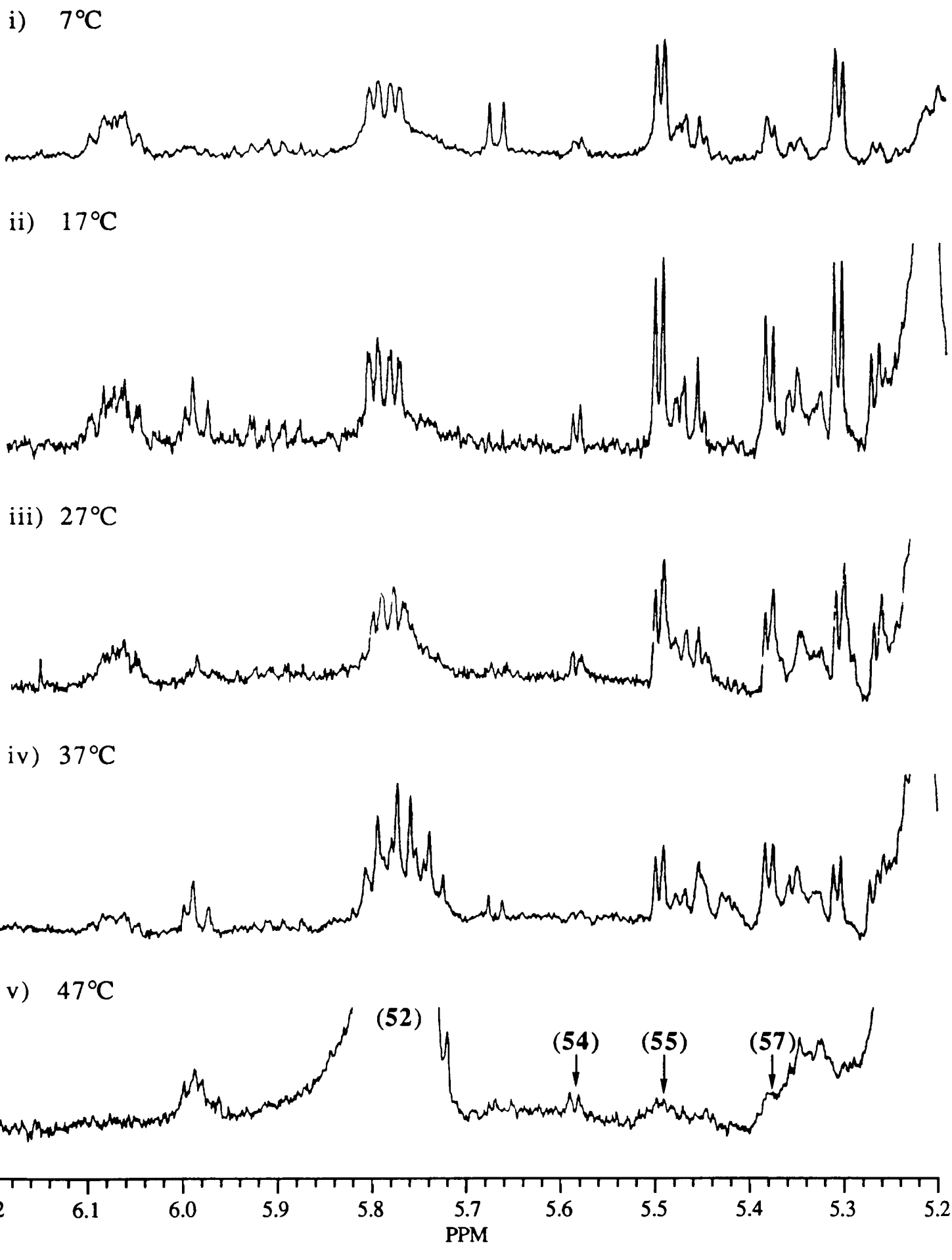
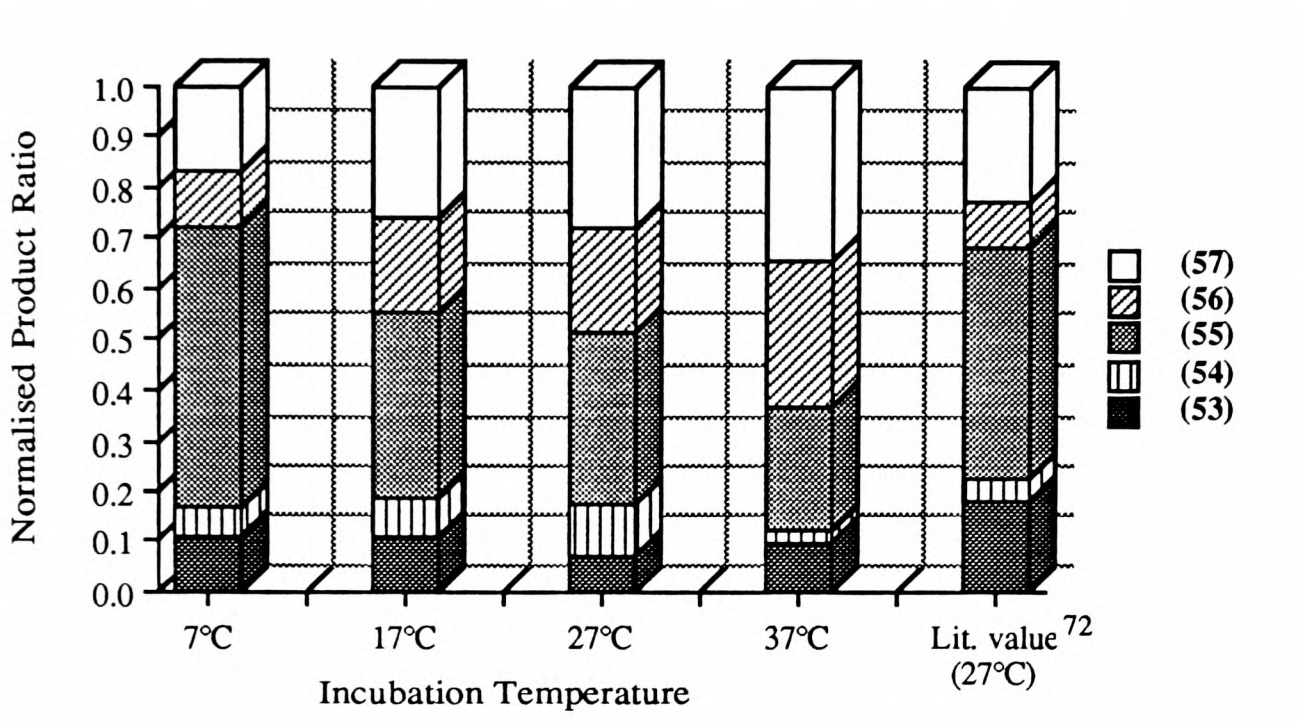


FIGURE 4.4

Stack plots showing the relative product ratios for incubations of LL-AC-D-Ag (52) with IPNS at different temperatures



4.4 Analysis of Results

Analysis of the product ratios obtained in these experiments indicates that both the ratios of the penams (**53** and **54**) to the homoceph-3-em (**55**), and of the 4α-hydroxymethylcepham (**56**) to the 3α-hydroxyhomocepham (**57**) are virtually invariant with temperature. If however, we examine the ratio of desaturase to monooxygenase products we find a four fold change in the product distribution over a 30°C temperature range (7-37°C). The ratios are given in Table 4.2, and the desaturase:monooxygenase ratios are depicted in Figure 4.5.

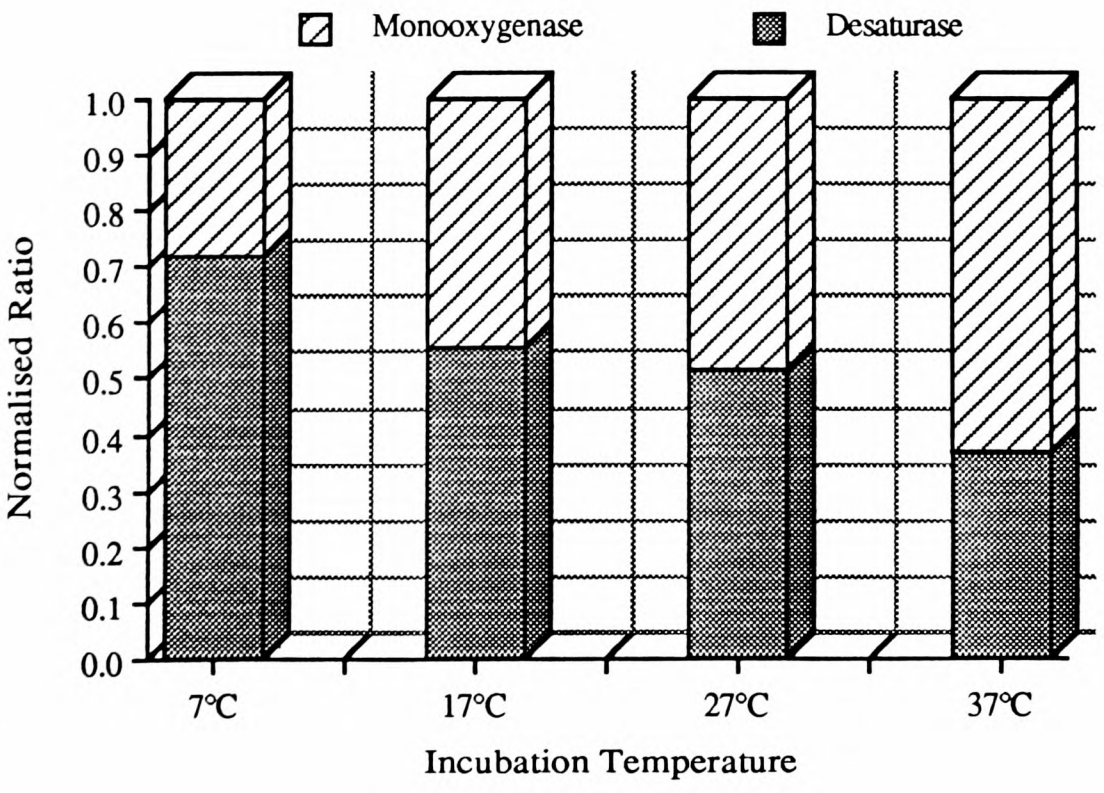
TABLE 4.2

Product ratios, as determined by ^{1}H NMR spectroscopy, for incubations of LL-AC-D-Ag (52) with IPNS at different temperatures

Incubation Temperature	Ratio desaturase:monooxygenase	Ratio penams:(55)	Ratio (56):(57)
7°C	15:6	3:10	7:10
17°C	15:12	5:10	7:10
27°C	15:14	5:10	8:10
37°C	15:26	5:10	9:10
Lit Value (27°C)[72]	15:7	5:10	4:10

FIGURE 4.5

Stack plots showing the variation of the desaturase to monooxygenase product distribution with temperature



The observation that only the ratio of desaturase to monooxygenase products varies with temperature, suggests that the products are formed from the putative enzyme bound intermediate (**157**) with initial steps which are irreversible. Furthermore, the constant ratio observed for the penams (**53** and **54**) to the homoceph-3-em (**55**), is consistent with a common intermediate, such as an allylic radical, being formed in the first step of desaturase-type closure of the second ring.

This biasing towards the monooxygenase pathway at higher temperature in consistent with a higher activation energy for this process than for the desaturase mechanism.

Application of equation 4.3, derived in section 4.2, gives us the results shown in Table 4.3, which are displayed in Graph 4.1. The gradient obtained from a root-mean-square line-fit routine gives a value for the difference in activation energies† between the desaturase and monooxygenase pathways for second ring closure of $30 kJ.mol^{-1}$.

4.5 Conclusions

The incubation of LL-AC-D-Ag (**52**) with IPNS over a range of temperatures has demonstrated the sensitivity of product distribution to the incubation temperature. The results highlight the difference between the desaturase and monooxygenase pathways for closure of the second ring, showing a four fold enhancement of the desaturase pathway in moving from 37℃ to 7℃, indicating a higher activation energy for the monooxygenase mechanism. Furthermore,

† As noted in section 4.2, this is more accurately the difference in activation enthalpies.

TABLE 4.3

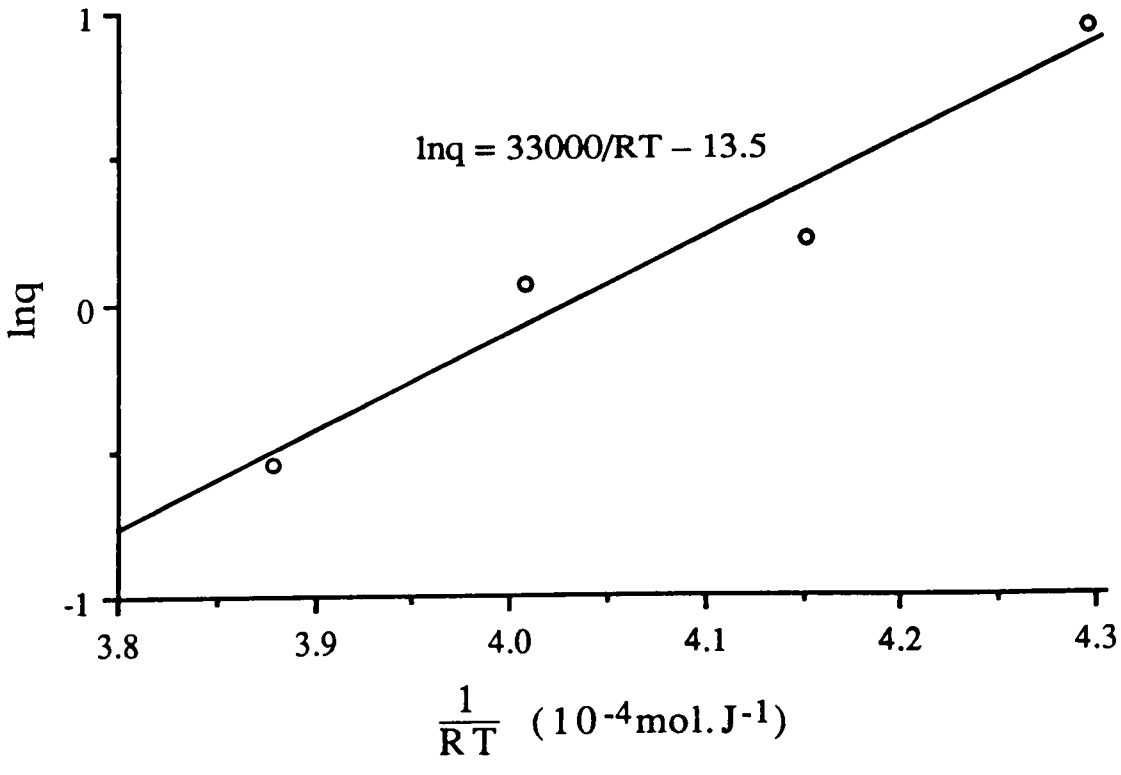
Data for the plot to determine the difference in activation energies between the desaturase and monooxygenase pathways

Incubation Temperature (K)	Ratio of monooxygenase to desaturase, q	lnq	$\frac{1}{RT}$ $J.mol^{-1} \times 10^4$
280	2.6	0.955	4.30
290	1.25	0.223	4.15
300	1.07	0.069	4.01
310	0.58	-0.550	3.88

GRAPH 4.1

Graph showing lnq (where q represents the ratio of monooxygenase to desaturase products) against 1/RT

The gradient of this graph is equal to the difference between the activation energies for the two pathways



results have indicated that the product distribution **within** each pathway is affected little by temperature, suggesting very similar reaction profiles for the different products formed (i.e. for **(53)**, **(54)** and **(55)**; and also for **(56)** *cf.* **(57)**).

The ratios obtained have been used to obtain a semi-quantitative figure for the difference in activation energies between the desaturase and monooxygenase of 30kJ.mol^{-1}.

Repetition of these experiments on a larger scale would allow a more precise figure to be obtained, through attainment of a better signal-to-noise ratio in the ^{1}H NMR spectra. However, a precise figure could not be obtained by this technique, owing to the inherent inaccuracy of assessment from ^{1}H NMR integrals arising from the different relaxation times for the nuclei involved.

CHAPTER 5

MODIFICATION OF THE HISTIDINE-261 RESIDUE OF IPNS DURING ENZYME AUTO-INACTIVATION

5. MODIFICATION OF THE HISTIDINE-261 RESIDUE OF IPNS DURING ENZYME AUTO-INACTIVATION

5.1 Introduction

In comparison with the majority of enzymes, IPNS does not possess a very high catalytic efficiency, undergoing some form of substrate-dependent auto-inactivation after only a few hundred turnovers.[40] Previous workers discovered a modification at the histidine-261 residue of the inactivated enzyme from *Cephalosporin acremonium* (the primary structure of IPNS from *C. acremonium* is given in Figure 5.1).[42]

It was further shown that the modification does not change the histidine residue to any known amino acid, such as aspartic acid[42] (one of the ultimate products resulting from oxidation of histidine).

The inactivation of proteins through the modification of a histidine residue has been observed in several systems, and this has in most cases been identified as being due to oxidation of the histidine ring system.[116,117] Such oxidation can occur either due to catalytic activity, as identified with IPNS, or due to the effects of metal ions and other species in the media.[118,119] The supposition that, in both cases, the modification is due to the presence of high valent metal species (at the active site or in solution), and has been modelled in solution through the use of metal ions in conjunction with a reagent such as DTT or L-ascorbic acid.[120,121]

FIGURE 5.1[122]

Primary structure of IPNS gene from *Cephalosporium acremonium*

MET	GLY	SER	VAL	PRO	VAL	PRO	VAL	ALA	ASN	10
VAL	PRO	ARG	ILE	ASP	VAL	SER	PRO	LEU	PHE	20
GLY	ASP	ASP	LYS	GLU	LYS	LYS	LEU	GLU	VAL	30
ALA	ARG	ALA	ILE	ASP	ALA	ALA	SER	ARG	ASP	40
THR	GLY	PHE	PHE	TYR	ALA	VAL	ASN	HIS	GLY	50
VAL	ASP	LEU	PRO	TRP	LEU	SER	ARG	GLU	THR	60
ASN	LYS	PHE	HIS	**MET**	SER	ILE	THR	ASP	GLU	70
GLU	LYS	TRP	GLN	LEU	ALA	ILE	ARG	ALA	TYR	80
ASN	LYS	GLU	HIS	GLU	SER	GLN	ILE	ARG	ALA	90
GLY	TYR	TYR	LEU	PRO	ILE	PRO	GLY	LYS	LYS	100
ALA	VAL	GLU	SER	PHE	CYS	TYR	LEU	ASN	PRO	110
SER	PHE	SER	PRO	ASP	HIS	PRO	ARG	ILE	LYS	120
GLU	PRO	THR	PRO	**MET**	HIS	GLU	VAL	ASN	VAL	130
TRP	PRO	ASP	GLU	ALA	LYS	HIS	PRO	GLY	PHE	140
ARG	ALA	PHE	ALA	GLU	LYS	TYR	TYR	TRP	ASP	150
VAL	PHE	GLY	LEU	SER	SER	ALA	VAL	LEU	ARG	160
GLY	TYR	ALA	LEU	ALA	LEU	GLY	ARG	ASP	GLU	170
ASP	PHE	PHE	THR	ARG	HIS	SER	ARG	ARG	ASP	180
THR	THR	LEU	SER	SER	VAL	VAL	LEU	ILE	ARG	190
TYR	PRO	TYR	LEU	ASP	PRO	TYR	PRO	GLU	PRO	200
ALA	ILE	LYS	THR	ALA	ASP	ASP	GLY	THR	LYS	210
LEU	SER	PHE	GLU	TRP	HIS	GLU	ASP	VAL	SER	220
LEU	ILE	THR	VAL	LEU	TYR	GLN	SER	ASP	VAL	230
GLN	ASN	LEU	GLN	VAL	LYS	THR	PRO	GLN	GLY	240
TRP	GLN	ASP	ILE	GLN	ALA	ASP	ASP	THR	GLY	250
PHE	LEU	ILE	ASN	CYS	GLY	SER	TYR	**MET**	ALA	260
HIS	ILE	THR	ASP	ASP	TYR	TYR	PRO	ALA	PRO	270
ILE	HIS	ARG	VAL	LYS	TRP	VAL	ASN	GLU	GLU	280
ARG	GLN	SER	LEU	PRO	PHE	PHE	VAL	ASN	LEU	290
GLY	TRP	GLU	ASP	THR	ILE	GLN	PRO	TRP	ASP	300
PRO	ALA	THR	ALA	LYS	ASP	GLY	ALA	LYS	ASP	310
ALA	ALA	LYS	ASP	LYS	PRO	ALA	ILE	SER	TYR	320
GLY	GLU	TYR	LEU	GLN	GLY	GLY	LEU	ARG	GLY	330
LEU	ILE	ASN	LYS	ASN	GLY	GLN	THR			

It was decided to investigate this phenomenon further in order to attempt to determine the nature of this modification, and to gain an insight into its effect on catalytic activity.

5.2 Methodology

In order to investigate the nature of the modification of the histidine-261 residue which occurs during auto-inactivation of IPNS, it was considered necessary to produce a smaller fragment of the protein containing this residue. This could be readily achieved, since numerous methods exist for cleaving peptides, both through the use of proteolytic enzymes and by chemical means.[123] Previous work in the group had successfully employed the proteolytic enzyme trypsin to cleave IPNS.[124] Tryptic, as with most other enzymatic digests, however, produce a great number of fragments (>50) which would necessitate a time consuming search for fragments containing the His-261 residue, after first separating them by reverse phase HPLC. Because of this problem with enzymatic digests, it was decided to investigate chemical cleavage using the reagent cyanogen bromide (CNBr). This reagent cleaves peptides specifically at methionine residues via the formation of homoserine lactone (the reaction is outlined in Scheme 5.1).[125]

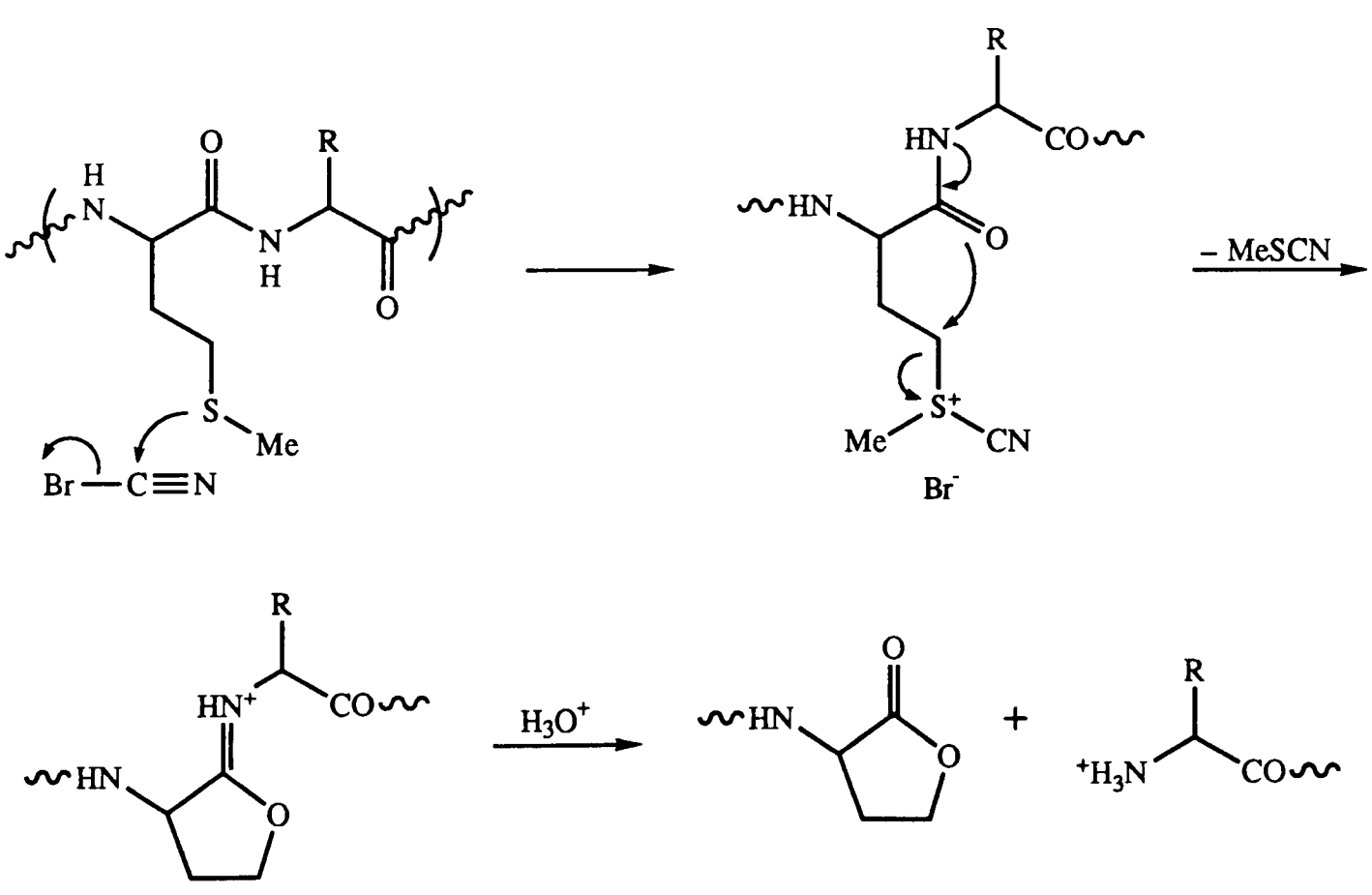
The reaction of CNBr with IPNS would be expected to yield four fragments, as shown in Figure 5.2, arising from cleavage at the four methionine residues (these are highlighted in bold type face in Figure 5.1).

It is immediately apparent that the fourth fragment (Ala-260 to Thr-338) would provide ready access to the His-261 residue for analysis by protein sequencing, providing of course that the fragment was not N-terminally blocked

(as can occur with CNBr digests).[126] It was also proposed to examine this fragment by electrospray mass spectrometric analysis, in order to determine the mass change associated with auto-inactivation.

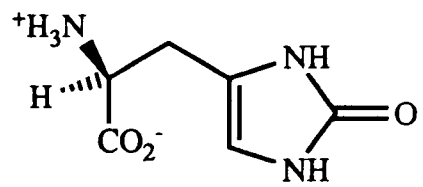
Figure 5.2
Peptide fragments expected from (perfect) CNBr cleavage of IPNS.
The numbering system refers to the N-terminal sequence of the whole protein.

Fragment 1	Gly-2	to	hSer-65	MW 7023
Fragment 2	Ser-66	to	hSer-125	MW 6923
Fragment 3	His-126	to	hSer-259	MW 15204
Fragment 4	Ala-260	to	Thr-338	MW 8715



SCHEME 5.1

It was considered possible that, after determination of the mass difference arising from histidine modification, the synthesis of likely amino acid products, such as the 2-oxoimidazolonyl containing species (**155**),† would allow comparison to the altered amino acid found in the IPNS fragment, by means of their retention times under peptide sequencing conditions.

(**155**) 

In addition it was considered possible that such a fragment from a CNBr digest of IPNS could be subjected to further, enzymatic digests, giving a far simpler range of product peptides than from the whole protein. Such short fragments containing the His-261 residue would facilitate study by mass spectrometric techniques.

5.3 Purification of IPNS

IPNS of approximately 70-90% purity is routinely prepared by the microbiology department of the Dyson Perrins laboratory, by: lysis of mutant *Escherichia coli* cells from an IPNS-producing fermentation with lysozyme; precipitation of DNA and RNA using protamine sulphate; centrifugation; and purification on a Pharmacia BioProcess® ion exchange column.

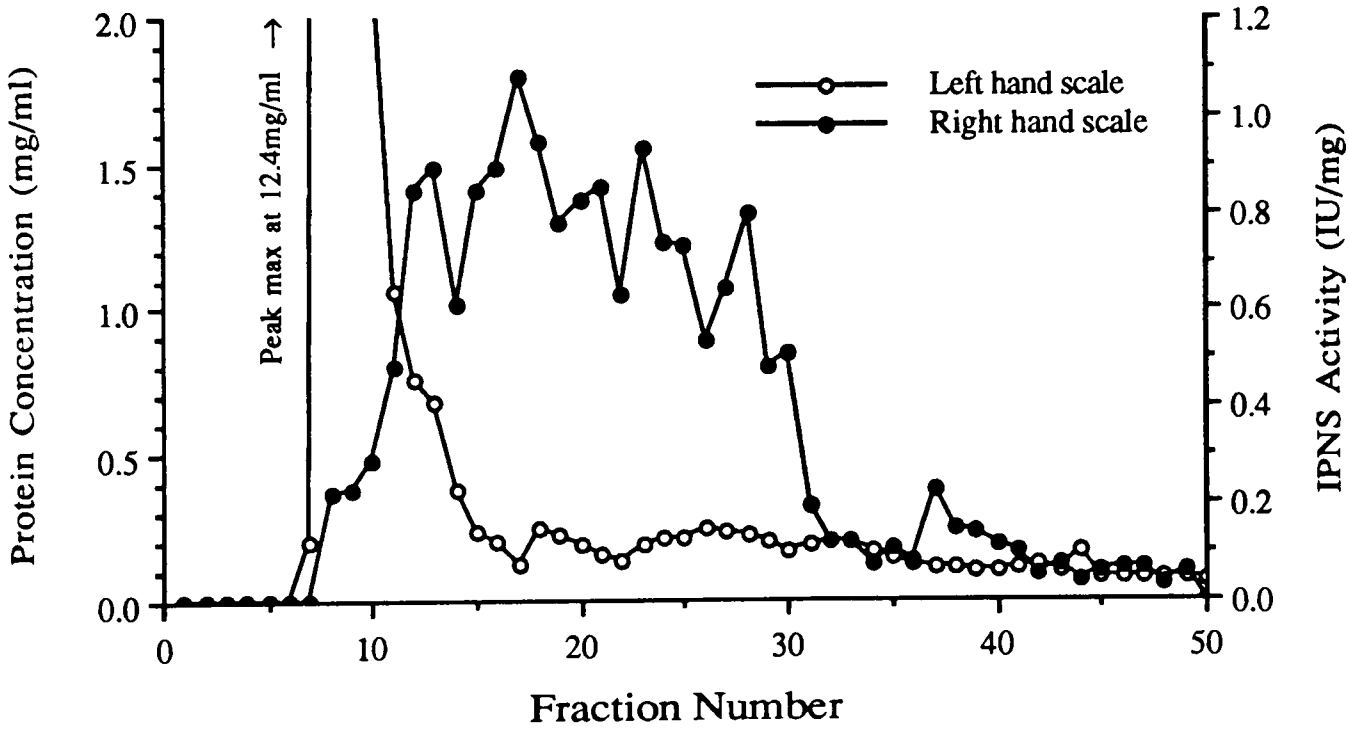
Further purification of this IPNS solution was carried out by FPLC, according to the procedure developed by Baldwin *et al.*[128] The solution was centrifuged and filtered, and the filtrate diluted and loaded onto a Mono Q

† This species has been produced in model systems,[127] and is consistent with studies carried out on histidine loss in some other proteins, which indicate the generation of a new carbonyl group.[116]

16/10 ion exchange column. The column was eluted with a NaCl gradient in TRIS buffer at pH 8, and 5ml fractions were collected.

Fractions were analysed for protein content (Bradford assay†), IPNS activity (bioassay†) and purity (SDS-PAGE†). The assay results are given in Table 6.3 of Chapter 6, and are depicted graphically in Graph 5.1. The SDS-PAGE gels are shown in Figure 5.3.

GRAPH 5.1
The protein content and activity of IPNS fractions
from ion exchange chromatography

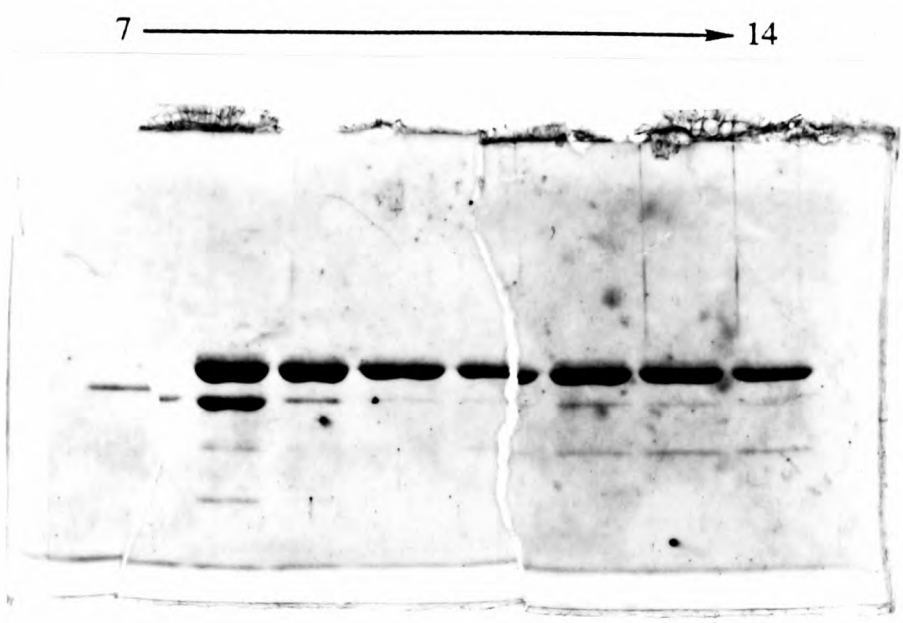


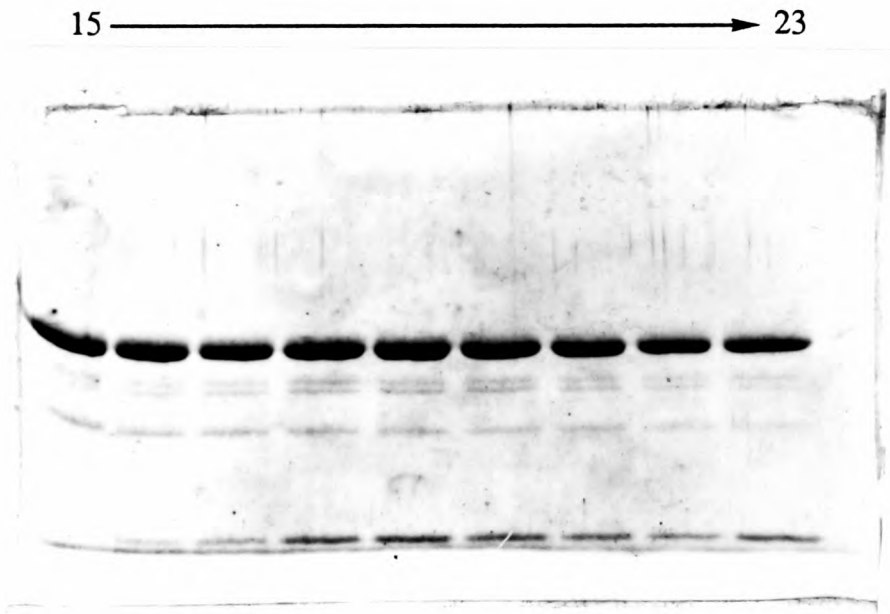
Combination of fractions 9-30 and concentration over a size exclusion membrane, yielded IPNS of greatly improved purity (Figure 5.4), and with good biological activity (18 $IU.ml^{-1}$, $9.2mg.ml^{-1}$). This protein solution was used in subsequent experiments.

† Details of these procedures are given in section 6.5 of Chapter 6

FIGURE 5.3

Showing SDS-PAGE gels (10%) of the FPLC fractions from the purification of IPNS





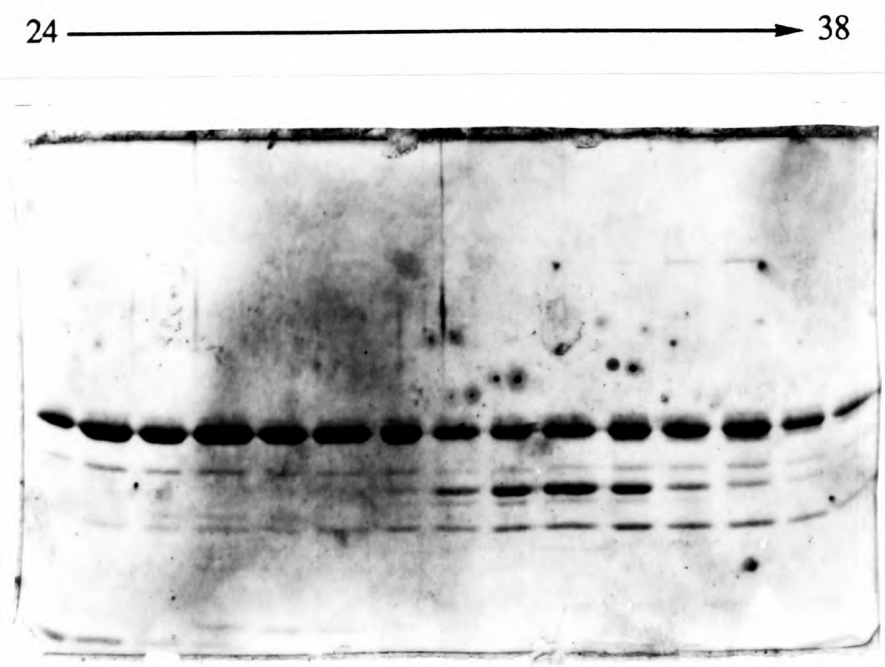
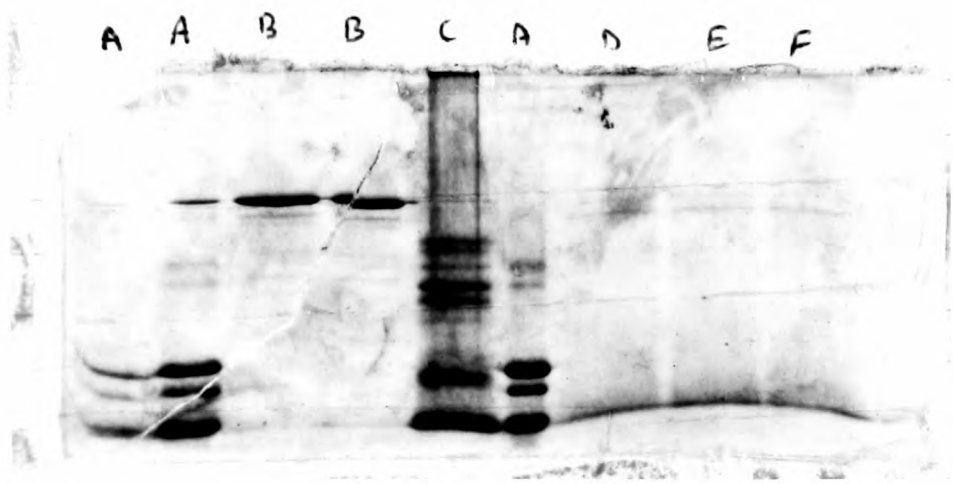


FIGURE 5.4

SDS-PAGE gel (15%) of purified IPNS

Key for lanes: A = BDH molecular weight markers (16949, 14405, 8159, 6214 and 2514Da); B = Purified IPNS; C = Trial CNBr digest; D, E and F = other FPLC fractions pooled)



5.4 Amino Acid Analysis of Whole Protein

Before investigating the fragments from CNBr digests of IPNS, the whole protein was examined by amino acid analysis, in order to determine whether this method would allow the detection of loss of a single histidine residue.

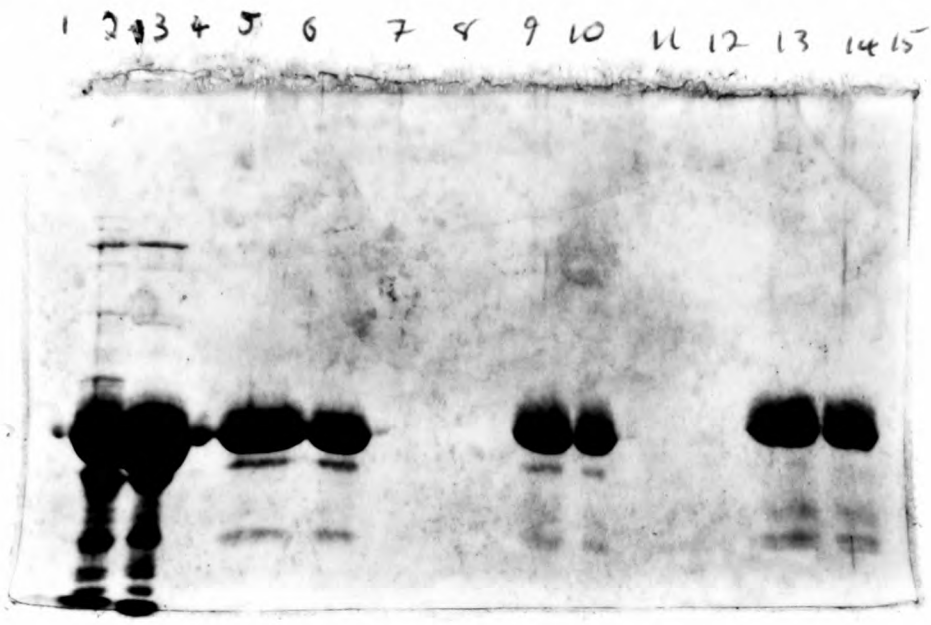
A sample of purified IPNS was compared to a sample which had been incubated in the presence of excess substrate, LLD-ACV (**1**), and to a control which had been incubated under identical conditions, save for the omission of substrate. Each sample was purified by reverse phase HPLC (Figure 5.5) prior to amino acid analysis.†

† This technique could not be employed for the purification of IPNS for use in these experiments, as the mobile phase employed ($MeCN/H_2O/CF_3CO_2H$) caused complete loss of activity.

FIGURE 5.5

SDS-PAGE gel (5%) of HPLC-purified samples for amino acid analysis

Key to lanes: 2, 3 = IPNS prior to HPLC; 5, 6 = un-incubated IPNS; 9, 10 = IPNS after incubation; 13, 14 = IPNS control



Unfortunately, the results of amino acid analysis (see Table 5.1) did not give sufficient precision for a minor change such as the loss of a single histidine residue to be determined, even with access to large quantities of each sample.

5.5 Cyanogen Bromide Digests of IPNS and Analysis of Fragments

5.5.1 Cyanogen Bromide Digests of IPNS

Digests of IPNS were carried out according to the method of Gross & Witkop,[129] by addition of solid CNBr to a solution of IPNS in aqueous HCO_2H [70% (v/v)] under argon, followed by stirring overnight at room temperature, under argon, in the absence of light.

TABLE 5.1

Amino acid analysis results for IPNS vs samples incubated in the presence and absence of substrate, LLD-ACV (**1**). Results are given in two forms, normalised to leucine or to alanine.

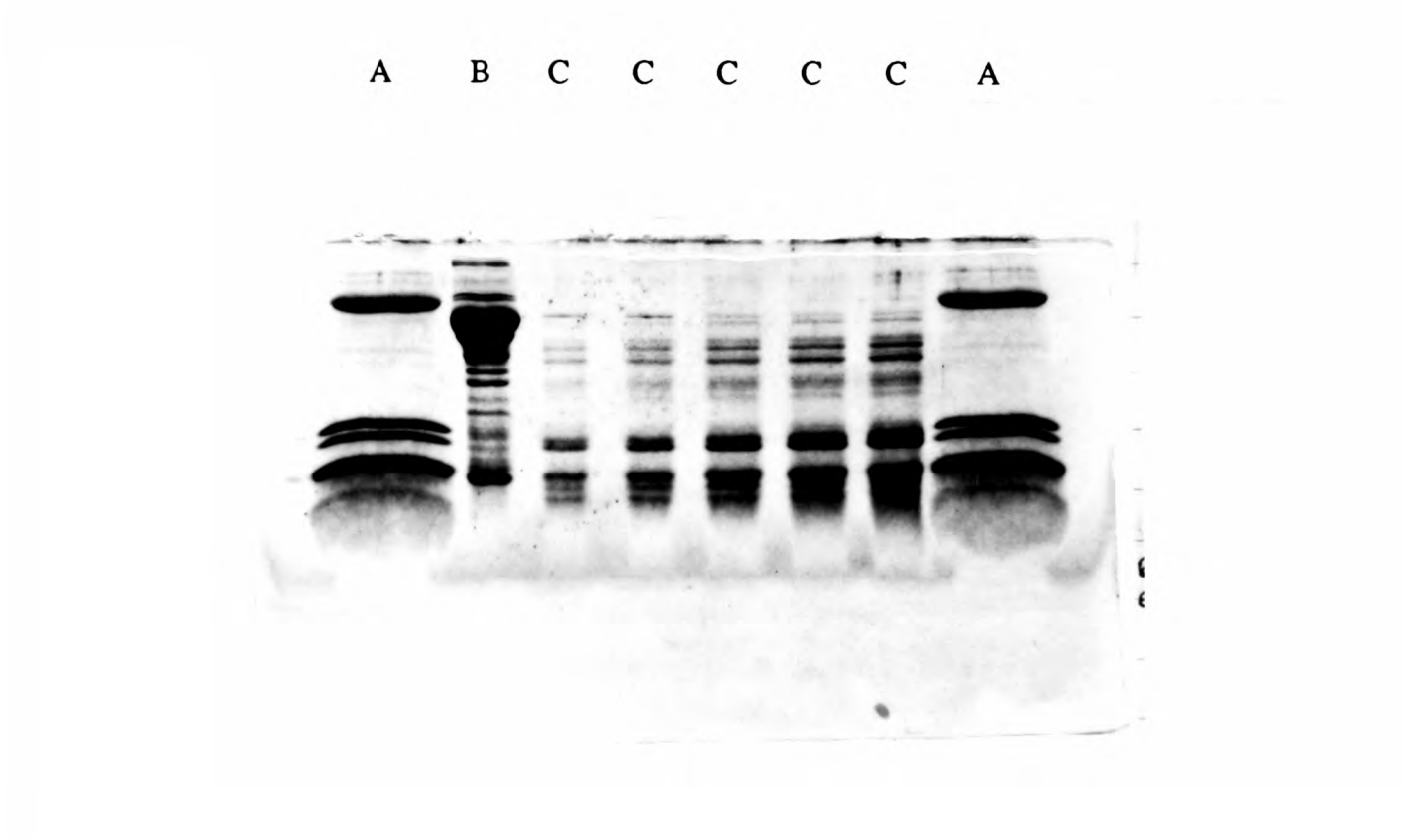
	Calc. for IPNS	IPNS Sample		Incubation		Control	
		Leu = 24	Ala = 27	Leu = 24	Ala = 27	Leu = 24	Ala = 27
Asp	28 (40)	19.6	18.1	18.3	22.2	13.1	16.0
Glu	20 (32)	12.5	11.5	13.6	16.5	11.4	13.9
Ser	20	17.2	15.9	18.9	22.9	18.1	22.1
Gly	21	19.4	18.0	23.7	28.7	21.0	25.6
His	10	8.9	8.2	9.1	11.0	8.9	10.8
Arg	17	17.3	16.0	18.2	22.0	17.1	20.9
Thr	15	15.5	14.3	16.0	19.4	15.4	18.7
Ala	27	29.2	**27**	22.3	**27**	22.1	**27**
Pro	25	18.3	16.9	19.6	23.8	18.6	22.7
Tyr	18	15.3	14.1	15.7	19.0	14.9	18.2
Val	22	21.8	20.1	22.7	27.5	21.7	26.5
Met	4	2.4	2.2	3.1	3.8	2.6	3.2
Cys	2	0.4	0.4	0.7	0.8	0.4	0.5
Ile	17	17.5	16.2	0.0	0.0	17.7	21.5
Leu	24	**24**	22.2	**24**	29.1	**24**	29.3
Phe	15	21.5	19.9	14.7	17.8	23.7	28.9
Lys	20	18.9	17.5	19.2	23.3	18.1	22.1
Trp	9	0.0	0.0	0.0	0.0	0.0	0.0
Asn	12	–	–	–	–	–	–
Gln	12	–	–	–	–	–	–

The mixtures of peptide fragments produced by these digests were analysed by SDS-PAGE, indicating virtually complete cleavage of IPNS to give primarily the desired lower molecular weight fragments. These results were readily reproducible, giving good yields of the desired fragments. An SDS-PAGE gel of a typical digest is shown in Figure 5.6.

FIGURE 5.6

SDS-PAGE gel (15%) of a typical CNBr digest of IPNS

Key to lanes : A = BDH molecular weight markers (16949, 14405, 8159, 6214 and 2514Da); B = IPNS prior to digest; C = different loadings of digest solution



5.5.2 Investigation of the Purification of CNBr Digest Fragments

i) Gel Filtration FPLC

Previous studies in this department had resulted in partial separation of fragments from a cyanogen bromide digest of IPNS through the use of gel

filtration FPLC (Superose® 12 column).[130] Although this previous work did not in itself produce fragments of sufficient purity, it was decided to investigate this method of purification with the aim of improving product resolution.

A portion of a CNBr digest was subjected to FPLC under identical conditions to those used in the previous studies, but repeated attempts failed to give any separation of the peptide fragments.

As this method seemed unsatisfactory, alternative FPLC systems for peptide separation were examined.

ii) Ion Exchange FPLC

A second method which was investigated for the purification of digest fragments, was the use of ion exchange FPLC.

Initial results were encouraging, with partial separation of at least eight peaks eluting at 100-170m<u>M</u> NaCl. However, analysis of these fractions showed their protein content to be below the detection limit for the Bradford assay, although the two largest peaks did show very faint bands at *ca.* 8000Da by SDS-PAGE.

It did not prove possible to improve upon these initial results, and so alternative purification procedures were investigated.

iii) Reverse Phase HPLC on ODS

A technique which is increasingly finding applications in peptide purification is reverse phase HPLC.

Reverse phase HPLC was first applied to the mixture of peptide fragments generated from CNBr digests of IPNS, using ODS with the mobile phase recommended for peptide purifications (acetonitrile/water containing 0.1% trifluoroacetic acid). Initial results were ambiguous, with several peaks being

eluted, but with extremely different elution characteristics. These fractions were isolated and examined by UV spectroscopy and SDS-PAGE techniques.

Analysis by UV spectroscopy in aqueous solution showed absorbances for each fraction at 220, 254 and 280nm, which were consistent with peptides containing aromatic residues. However analysis by SDS-PAGE showed the presence of only fast running (low molecular weight) bands, possibly arising from impurities present in the original IPNS sample.

Repeated attempts at the purification of digest samples by this technique all failed to yield any of the desired fragments, and so further advice was sought.

iv) Reverse Phase HPLC on C4 Support

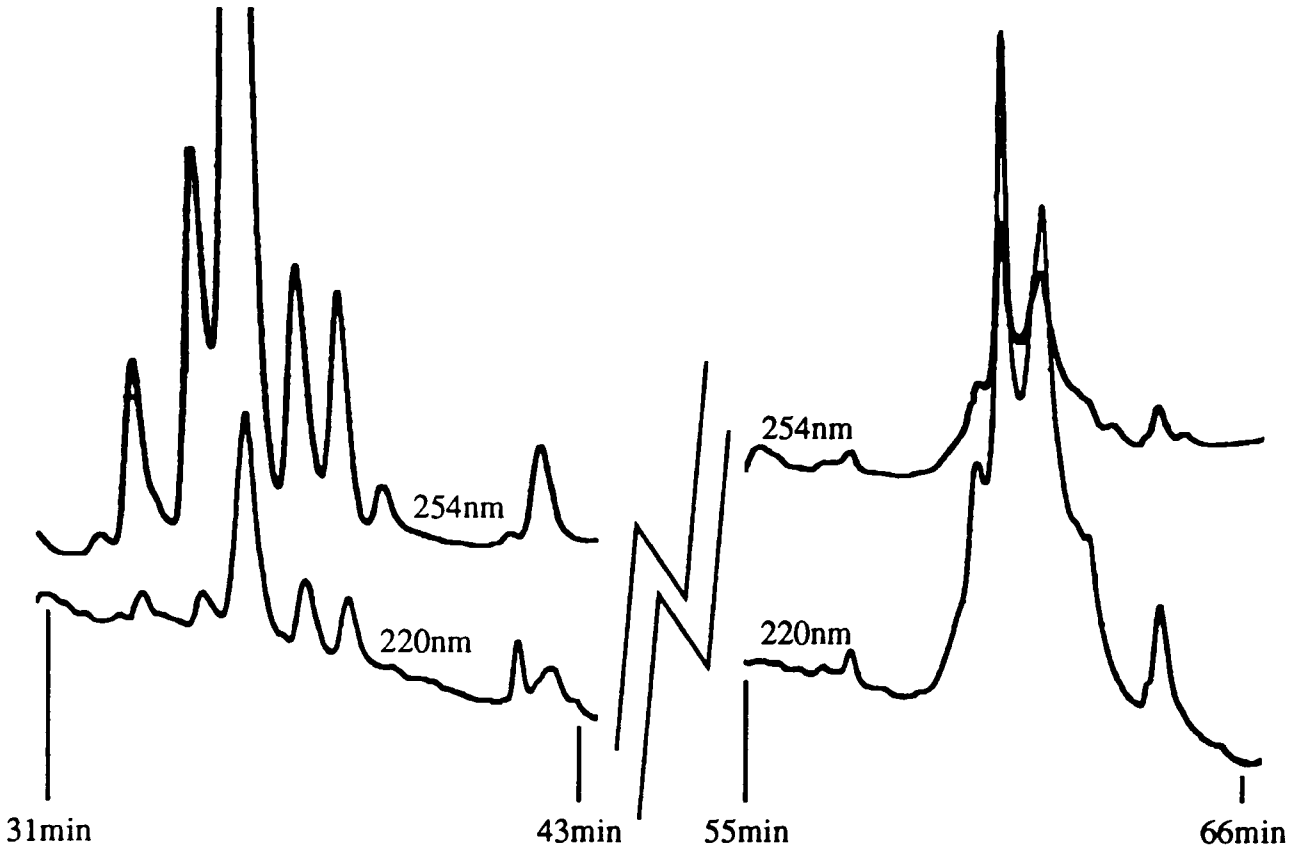
The failure of reverse phase HPLC on Hypersil® ODS may have been due to the failure of the peptides to enter the silica matrix, so that only the surface area of silica next to the column walls was utilised.[131] It was hoped that this problem could be overcome by use of a solid phase with a greater pore size. Additionally, the separation of peptide fragments of such a size as generated by CNBr digests is usually more facile on a C4 rather than a C18 solid phase.[131] This information led to the investigation of the purification of the digest fragments on a C4 300Å solid phase.

Investigation of this chromatography system proved highly encouraging, with good separation of a number of peaks (as detected by absorbance at 220 and 254nm), as shown in Figure 5.7, on the solvent gradient shown in Graph 5.2.

The fragments from a CNBr digest were separated using this HPLC system and solvent removed by freeze drying in soda glass vials. Attempts to

FIGURE 5.7

Section of HPLC trace showing peaks from CNBr digest solution

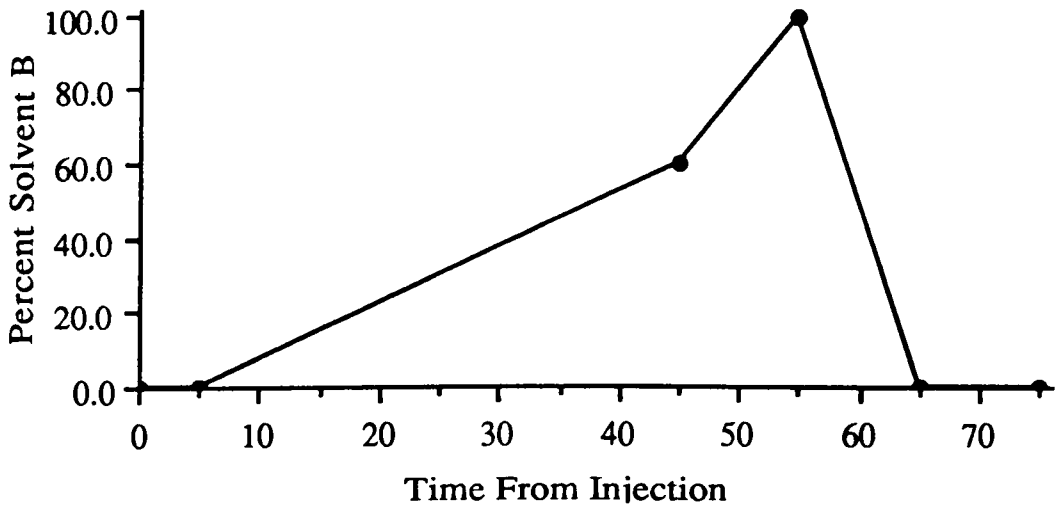


GRAPH 5.2

Gradient profile for HPLC trace shown in Figure 5.7

Flow rate 0.5ml.min^{-1}; Solvent A = H_2O containing 0.1% CF_3CO_2H;

Solvent B = 80% MeCN/20% H_2O/0.09% CF_3CO_2H; A + B = 100%



analyse these fractions by Bradford assay and SDS-PAGE found that no peptide material could be brought into solution in H_2O, ammonium bicarbonate buffer, saturated aqueous TRIS or by treating with formic acid and back diluting to 10% (v/v). Addition of Bradford's solution to the glass vials gave the distinctive blue protein stain on the surface of the glass. Repetition, but with freeze drying in polythene eppendorfs resulted in all peptide material being firmly bound to the plastic surfaces.

A number of glass vials were 'silanised' using dichlorodimethylsilane, and used to contain the HPLC fractions for freeze drying. This resulted in an insoluble residue, which could be suspended in solution through treatment with formic acid and back diluting with H_2O to 10% (v/v). However, all attempts to analyse these suspensions by SDS-PAGE met with failure, with no protein material entering the gel matrix, indicating that treatment with the sample buffer, pH adjustment and boiling had also failed to take any protein material into solution.

As an alternative to freeze drying, samples collected from HPLC were concentrated by the 'speedvac' technique (centrifugation in vacuum, with or without heating). This caused precipitation of all protein material (as indicated by Bradford staining of the precipitate and supernatant) as the sample volume reduced, and so did not lead to any solutions which would have allowed analysis of the HPLC fractions.

This HPLC protocol appeared to separate the fragments from CNBr digests of IPNS. It was hoped that further work would be able to overcome the problems encountered with product solubility, but there proved to be insufficient time for such investigations.

5.5.3 Electroblotting and Sequencing of Fragments

Although attempts to isolate the desired His-261 containing fragment had proved unsuccessful, sequencing information was still accessible through the use of electroblotting. This technique was carried out by Dr A. Willis and co-workers in the MRC Immunochemistry Unit, Oxford University. Analysis of a CNBr digest of IPNS in this way allowed the bands of interest ('a' to 'd' on Figure 5.8) to be successfully sequenced (Table 5.2).

FIGURE 5.8
SDS-PAGE gel (15%) of a CNBr digest of IPNS
showing the bands taken for sequence analysis
Key to lanes : 2-4 = digest solution; 6, 7 = BDH
molecular weight markers (16949, 14405, 8159, 6214 and 2514Da)

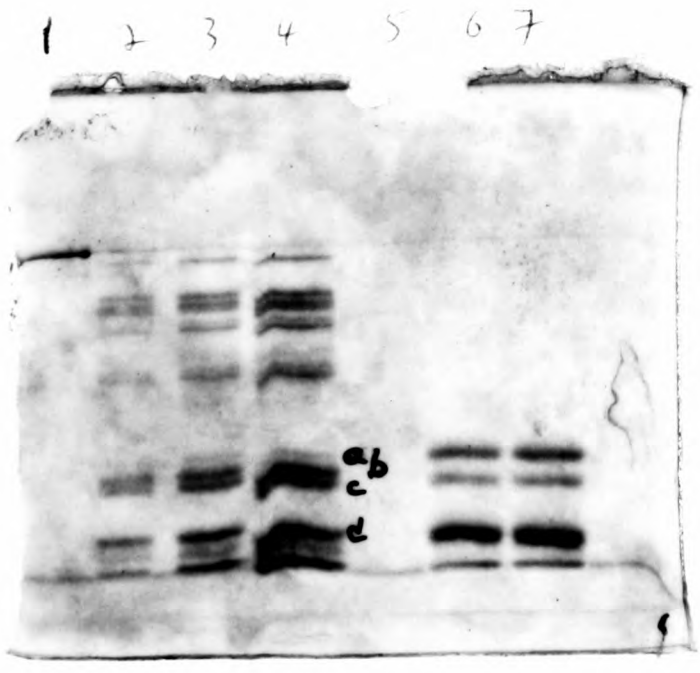


TABLE 5.2

Amino acid sequences for fragments 'a' to 'd'

No.	Band 'a'	Band 'b'	Band 'c'			Band 'd'
			Major	Minor	Minor	
1	His	His	Gly	His	Ser	Ala
2	Glu	Glu	Ser	Glu	Ile	His
3	Val	Val	Val	Val	Thr	Ile
4	Asn	Asn	Pro	Asn	Asp	Thr
5	Val	Val	Val	Val	Glu	Asp
6	Trp	Trp	Pro	Trp	Glu	Asp
7	Pro	Pro	Val	Pro	Lys	Tyr
8	Asp	Asp	Ala	Asp	–	Tyr
9	Glu	Glu	Asn	Glu	Gln	Pro
10	Ala	Ala	Val	Ala	Leu	Ala
11	Lys	Lys	Pro	Lys		Pro
12	His	His	Arg	His		Ile
13	Pro	Pro	Ile	Pro		His
14	Gly	Gly	Asp	Gly		Arg
15	Phe	Phe	Val	Phe		Val

Band 'd' also contains low levels (<10%) of Fragments 1, 2 and 3

Comparison of the sequences obtained from bands 'a' to 'd', with the sequences for IPNS (Figure 5.1) and for the expected CNBr digest fragments (Figure 5.2), allowed identification of these bands as given below:

Bands 'a' and 'b' correspond to Fragment 3 (His-126 to hSer-259).
Band 'c' corresponds to Fragment 1 (Gly-2 to hSer-65), contaminated with Fragments 2 (Ser-66 to hSer-125) and 3.
Band 'd' corresponds to the desired fragment, Fragment 4 (Ala-260 to Thr-338).

The sequence information obtained by these procedures was unequivocal, with the protocol employed providing ample protein for investigation of the *N*-terminal sequence of Fragment 4, and so would allow ready detection of any changes to the His-261 residue during auto-inactivation of IPNS.

5.5.4 Application of Electroblotting and Sequencing Techniques to Auto-Inactivated IPNS

A sample of auto-inactivated IPNS was prepared by repeated incubation of IPNS with LLD-ACV (**1**) under standard conditions, followed by gel filtration to remove molecules of low molecular weight, until no activity was seen in the protein solution, as determined by 'bioassay'. A control sample was prepared in an analogous manner except for the omission of substrate from the incubation medium.

These protein samples were concentrated by centrifugation over a protein impermeable membrane, and reacted with CNBr in HCO_2H solution (70% v/v) according to the method described in section 5.5.1.

During the time available, it did not prove possible to apply the electroblotting techniques to auto-inactivated IPNS prepared in this manner. Initial samples displayed anomalous behaviour under electrophoresis conditions, and it has not yet proved possible to obtain a gel with sufficient resolution of the desired bands. Their was insufficient time available for new samples to be prepared and analysed.

5.6 Conclusions

This work has shown that cleavage at the methionine residues of IPNS from *C. acremonium* with cyanogen bromide proceeds in good yield, producing predominantly the predicted fragments of molecular weight 7000-15000Da. It has been shown that these fragments are not *N*-terminally blocked, and so are open to analysis by amino acid sequencing techniques. Through the use of electroblotting from gels, the desired fragment (Ala-260 to Thr-338) containing the histidine-261 residue, which has been implicated in substrate-dependent auto-inactivation of IPNS, has been identified and sequenced.

Purification protocol for separation of the fragments from cyanogen bromide digests of IPNS were investigated, and although it proved possible to develop a method which appeared to give good separation of the fragments, problems with solubility prevented analysis of the results and identification of the desired fragment in the time available.

Samples of auto-inactivated IPNS were prepared and cleaved using cyanogen bromide, giving good yields of peptide fragments as determined by SDS-PAGE. However, sequence information on the histidine-261 containing fragments could not be obtained due to problems with resolution on the gels used for the electroblotting technique.

This work provides a ready basis from which the modification of this residue due to auto-inactivation of IPNS during turnover of the substrate LLD-ACV (**1**) can be investigated. Further work should be able to determine the problems with electroblotting of fragments from digests of auto-inactivated IPNS, and reveal any modification of the histidine-261 residue. It might also prove possible to investigate further the behaviour of the supposedly purified fragments from reverse phase HPLC on a 300Å C4 support, in order to solubilise these fragments for analysis by SDS-PAGE, sequencing and mass spectrometric analysis.

If the reported modification of the histidine-261 residue is confirmed, then characterisation of the amino acid produced, under the chromatography conditions employed for protein sequencing, and comparison with synthetic samples of likely structures would allow the exact nature of the change to be determined.

CHAPTER 6

EXPERIMENTAL PROCEDURES

6 EXPERIMENTAL PROCEDURES

6.1 Equipment and General Experimental Details

6.1.1 Monitoring and Purification

Analytical thin-layer chromatography (tlc) was carried out on aluminium-backed Merck 60 F_{254} plates bearing a 0.2mm thickness of SiO_2. Materials were imaged using a combination of UV fluorescence (254nm) and development using one of the following solutions, with heating where applicable:-

i) Molybdophosphoric acid (5% w/v) in EtOH.

ii) Ammonium molybdate (7% w/v) in H_2SO_4 (1M).

iii) Potassium permanganate (1% w/v) in a solution of K_2CO_3 (0.48M) containing NaOH (1.7% v/v of a 5% w/v solution).

Preparative thin layer chromatography (PLC) was performed on glass plates (0.2 x 0.2m) coated with Merck PF_{254} SiO_2 (1mm thickness).

Column chromatography was carried out using Merck Kieselgel 60 GF_{254}, 230-400 mesh SiO_2.

High performance liquid chromatography (HPLC) was performed on one of two systems. Purification of tripeptide disulphides was in general performed using a column packed with Zorbax® ODS (250 x 9.4mm internal diameter; 4ml.min^{-1}) supported by: Gilson gradient management 702 V1.2 software on an Apple II computer; two Gilson 303 pumps; a Rheodyne 7125 injector; and a Gilson HM holochrome detector set at 220nm. Purification of β-lactam products and smaller quantities (<5mg) of tripeptide disulphides was carried out using a column packed with Hypersil® 5μm ODS (250 x 4.6mm internal diameter; typically 1ml.min^{-1}) supported by: a Waters 510 gradient

controller; two Waters M-6000A pumps; a Rheodyne 7125 injector; and a Waters 441 absorbance detector set at 214nm.

Centrifugation was performed using a Beckman J2-21 centrifuge with a JA-20 rotor.

6.1.2 Characterisation

FT Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 200MHz on a Varian Gemini 200 spectrometer, at 300MHz on a Bruker WH-300 spectrometer, or at 500MHz on a Bruker AM-500 spectrometer. Non-aqueous samples were referenced internally to the residual solvent resonances (CD_2HCN, δ_H = 1.95ppm; d_5-acetone, δ_H = 2.05ppm; CD_2HOD, δ_H = 3.31ppm; $CHCl_3$, δ_H = 7.27ppm). Aqueous samples were referenced, only where stated, to DSS (TMS-resonance, δ_H = 0.00ppm) contained in a capillary tube inserted into the sample. The following abbreviations are used to describe multiplicities of individual resonances: s, singlet; d, doublet; t, triplet; t_D 1:1:1 triplet from H-D coupling; q, quartet; dd doublet of doublets; m, multiplet; b, broad signal. Coupling constants, *J*, are quoted in Hertz, and were measured to the nearest 0.5Hz. Coupling constants for ABX systems are given to first order approximations only. Where ambiguity exists in the sub-unit used to assign an individual resonance, then the source of the resonance is italicised.

FT Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 50.7MHz on a Varian Gemini 200 spectrometer, and are referenced relative to the solvent resonance ($CDCl_3$, δ_C = 77.0ppm). All spectra were fully ^{1}H decoupled, and peaks were assigned using DEPT editing. 1:1:1 triplets arising from C-D coupling are denoted t_D. Where ambiguity exists in the sub-unit

used to assign an individual resonance, then the source of the resonance is italicised.

FT Deuterium nuclear magnetic resonance (^{2}H NMR) spectra were recorded at 38.4MHz on a Bruker AM-250 spectrometer, and were referenced internally to added $CDCl_3$ ($CDCl_3$, δ_D = 7.27ppm).

Infra-red spectra were recorded on a Perkin-Elmer 1750 Fourier Transform spectrometer. The following abbreviations are used to describe relative absorption strengths: s, strong; m, medium; w, weak. Exceptionally broad absorptions are denoted 'b'. Only the most prominent peaks in the 'fingerprint' region (below 1500cm^{-1}) are recorded.

Mass spectrometric analyses were carried out on either a V.G. ZAB-1F or V.G. 20-250 Quadropole spectrometer or, for samples run under electrospray conditions, on a V.G. BIO-Q spectrometer. The following abbreviations are used to describe the techniques employed: MCA, multichannel analysis; DTT, dithiothreitol; DTE, dithioerythritol; Gly, glycerol; DCI, desorption chemical ionisation; CI, chemical ionisation.

Optical rotations were determined on a Perkin-Elmer 241 Polarimeter (path length 10cm), with $[\alpha]_D^{20}$ being determined using the standard formula:-

$$[\alpha]_D^{20} = \frac{100\alpha}{lc}$$

where:- α is the observed rotation.

l is the path length in units of 10^{-1}m (l = 1 for this polarimeter).

c is the concentration of the sample in units of g/100ml.

Melting points were determined on a Shandon Scientific Co./Reichert microscope and hot stage.

Microanalyses were performed by the microanalysis department of the Dyson Perrins Laboratory, on a Micros Carlo-Erba Strumentazone Elemental Analyser, model 1106.

Amino acid analyses, and electroblotting and sequencing of protein fragments were performed by courtesy of Dr A. Willis and colleagues in the MRC Immunochemistry Department, Oxford University.

6.1.3 Unit Definition

One International Unit (IU) of enzyme is that quantity which will form 1.0μmol of IPN (**2**) from LLD-ACV (**1**) per minute at pH 7.5 and 30°C with relevant cofactors present under standard conditions.

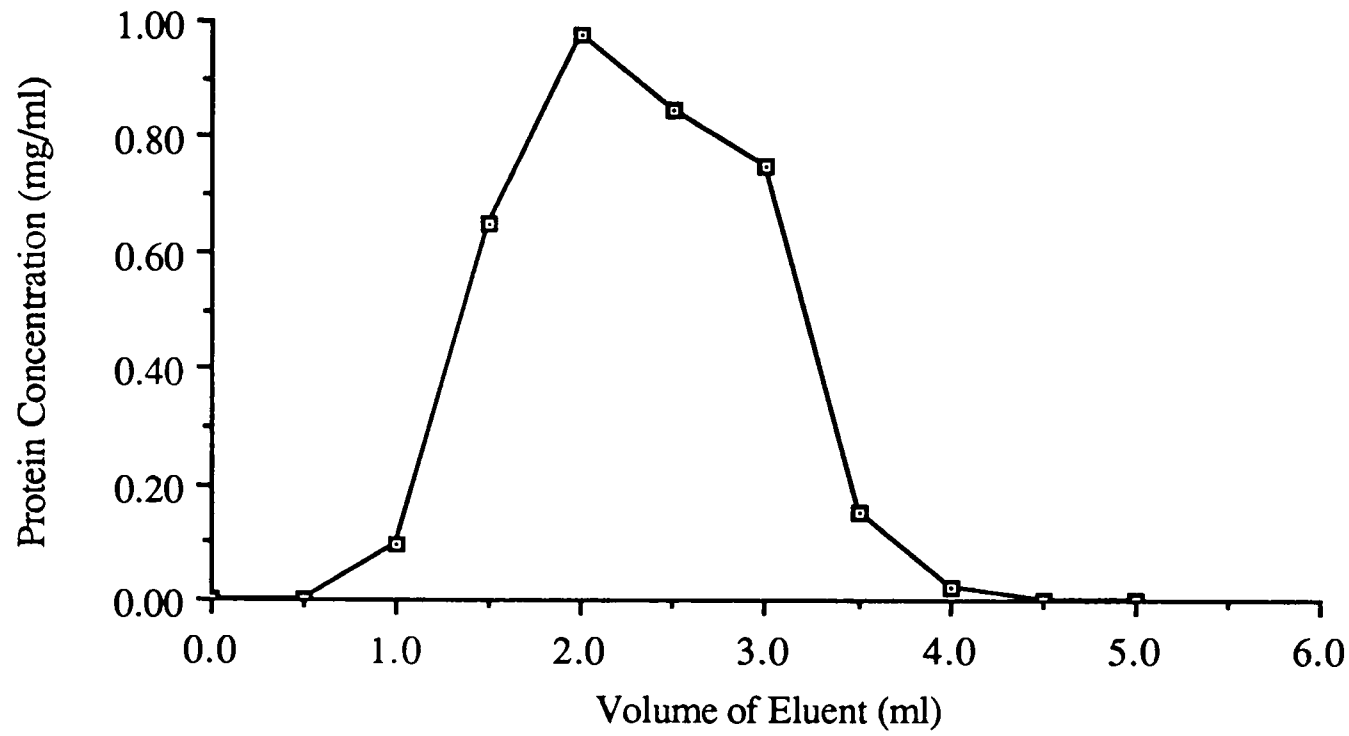
6.1.4 Determination of the Elution Profiles of Gel Filtration Columns

Pharmacia PD-10 and NAP-5 gel filtration columns were equilibrated with NH_4HCO_3 solution (25m<u>M</u>) and loaded with IPNS solution (~1.5mg.ml^{-1}; 2ml and 500μl respectively - i.e. the normal volumes used with these columns). The columns were eluted with NH_4HCO_3 solution (25m<u>M</u>), collecting 500μl and 100μl fractions respectively. These fractions were assessed for protein content by Bradford assay (see section 6.5.1). The resulting elution profiles are shown in Graphs 6.1 and 6.2.

From these elution profiles, where it was necessary to keep the solution volumes constant, protein was collected from 1.5 to 3.5ml of eluent with the PD-10 column, and from 300 to 800μl with the NAP-5.

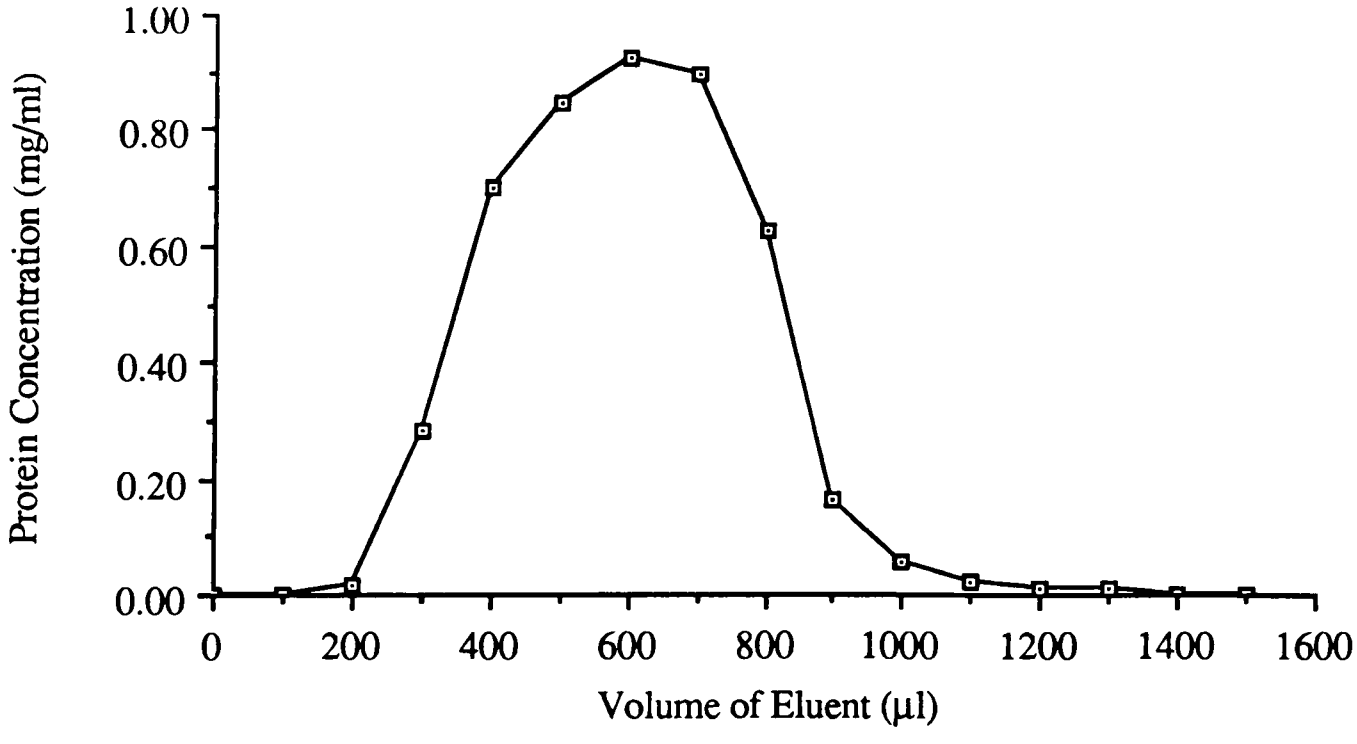
GRAPH 6.1

Elution profile for a Pharmacia PD-10 gel filtration column



GRAPH 6.2

Elution profile for a Pharmacia NAP-5 gel filtration column



6.1.5 General Procedure for Incubation of Tripeptides with IPNS[31]

A solution of isopenicillin N synthase was exchanged from TRIS.HCl buffer (50mM, pH 7.5) into aqueous NH_4HCO_3 (25mM) using a PD10 gel filtration column. Typically the column was pre-equilibrated with aqueous NH_4HCO_3 (25mM), and enzyme solution (2ml) was thawed from –80°C and loaded onto the column. The column was then eluted with aqueous NH_4HCO_3 (25mM), collecting eluent from 1.5 to 3.5ml (according to the PD-10 profile given in section 6.1.4).

The tripeptide disulphide (typically 1-3mg) was dissolved in NH_4HCO_3 (25mM, 1ml), pre-incubated with aqueous DTT solution (100mM; 100µl; final concentration 3mM) for 10min, and treated sequentially with aqueous L-ascorbate solution (50mM; 100µl; final concentration 1.5mM), aqueous iron (II) solution (5mM as sulphate; 100µl; final concentration 150µM), and the enzyme solution prepared above. The incubation mixture was split into two portions, and incubated in air at 27°C, 250rpm for 40min, with addition of fresh DTT solution (100mM; 50µl) and iron (II) solution (5mM as sulphate; 50µl), to each portion, after 20min. The two portions were combined and treated with acetone to *ca.* 70% (v/v), and the precipitated enzyme removed by centrifugation (12krpm, 10min). The supernatant was then evaporated *in vacuo* to yield the **crude incubation mixture**.

6.1.6 Preparation of Diazomethane Solution[132]

All apparatus used was especially designed for the preparation of diazomethane solution, having smooth glass joints throughout. All experiments carried out with diazomethane were carried out behind blast shields. The preparation may also be carried out with the substitution of Et_2O for CH_2Cl_2 throughout.

A solution of KOH (*ex.* M&B; 1.5g) in H_2O (2.5ml) was treated with 2-ethoxyethanol (*ex.* Aldrich; 8ml) and CH_2Cl_2 (20ml), and the mixture was heated at 70°C under distillation conditions. When distillation commenced, the mixture was treated dropwise with a solution of *N*-methyl-*N*-nitroso-4-toluene sulphonamide (Diazald®; *ex.* Aldrich; 5.35g, 25mmol) in CH_2Cl_2 (50ml) over 20min, and the solution of CH_2N_2 in CH_2Cl_2 which distilled from the reaction was collected at 0°C (the collection flask was treated with CH_2Cl_2 (10ml) prior to distillation). After distillation of CH_2Cl_2 had ceased further CH_2Cl_2 (10ml) was added and allowed to distil over before collection of CH_2N_2 was completed. The final solution is ~0.3M in CH_2N_2.

6.2 Experimental Procedures for Chapter Two

6.2.1 Magnetic Susceptibility Experiments

A) Preparation of Experiment

Reference Tube

A solution of DSS (5m<u>M</u>) in TRIS buffer (50m<u>M</u>) in D_2O was adjusted to pD 7.5 with a solution of DCl (1<u>M</u>) in D_2O. A 400µl aliquot of this solution was introduced into a 3mm diameter NMR tube terminating in a capillary tube. The solution in the tube was degassed at ~1mmHg, flushed with argon, degassed for a further 30min at ~1mmHg, flooded with argon and sealed.

Sample Solution

The sample solution was made up with DSS (0.2m<u>M</u>) and TRIS (50m<u>M</u>) in D_2O (10ml) and the pD was adjusted to 7.5 with DCl (1<u>M</u>) in D_2O. This solution was stored at 4°C prior to use.

Enzyme Solution

IPNS solution (batch CF25; 5.0 $IU.ml^{-1}$, 0.78 $IU.mg^{-1}$, 6.4$mg.ml^{-1}$; 4ml) was freeze dried, and the residue dissolved in D_2O (167µl; final concentration 153$mg.ml^{-1}$). This solution was stored at –80°C in 10 and 18µl aliquots prior to use.

DTT Solution

DTT solution (130m<u>M</u>) was freshly prepared for each experiment by dissolving DTT (5mg) in D_2O (250µl).

B) Sample Preparation

Oxygen and nitrogen gases (*ex.* BOC) were mixed using a system containing two Tube Cube™ flow meters, and the resulting gas mixture (20 or 50% O_2) was passed for 30min through the 'sample solution' (400µl) in a 5mm diameter NMR tube. After this time the tube was capped and treated sequentially with one of:-

i) D_2O (18µl); DTT solution (12µl).

ii) D_2O (8µl) and IPNS solution (10µl); DTT solution (12µl).

iii) IPNS solution (18µl); DTT solution (12µl).

and after capping and shaking the tube after each rapid addition, the reference tube was inserted into the sample tube and the system sealed. The addition of DTT solution was taken to mark the 'time zero' (t_0) for the experiment.

C) Acquisition of NMR Spectra

Experiments were performed at 500MHz on a Bruker AM-500 spectrometer. The concentric NMR tube system used was extensively shimmed using a sample prepared in an identical manner to usual save for the omission of enzyme and DTT solutions. This shim file was then used as a base for subsequent experiments.

A sample prepared as in section B) was inserted into the magnet, which was rapidly shimmed from the basic shim file (~10min), and a sample FID Fourier transformed. From the spectrum thus obtained frequency limits of +1 and −1ppm were selected and the sweep width set to 2000Hz. Other parameters were then set as follows: Pulse width set for 90° pulse; relaxation delay set for 4s; collecting 8K data points (0.6Hz per data point). Four dummy scans were run followed by the collection of eight transients. Fourier transform of the resultant FID allowed the value of the frequency shift ($\Delta\delta$) to

be measured directly from the separation of the two TMS- signals at ~0ppm. For cases where $\Delta\delta$ was small and the two TMS- resonances were partially overlapping, Gaussian multiplication of the FID was employed prior to Fourier transform to aid resolution.

Transients were collected every 2 to 10min as appropriate to the sample, over a period of 30 to 40min.

In cases where mixing or other effects did not allow good peak shapes to be obtained after ~10min of shimming, the experiments were abandoned.

The results obtained are given in Tables 6.1 and 6.2

6.2.2 pH Stat Experiments

A) Preparation of Solutions

The following aqueous solutions were used in the pH stat experiments:

i) LLD-ACV thiol (**1**): 10m<u>M</u>; pH adjusted to 7.50; stored in 0.5ml aliquots.

ii) L-Ascorbic acid: 44m<u>M</u>; pH adjusted to 7.70.

iii) DTT solution: 100m<u>M</u>; pH adjusted to 7.70.

iv) IPNS solution: batch CF 11b-14b; 5 $IU.ml^{-1}$; 0.4 $IU.mg^{-1}$; stored in 100µl aliquots.

v) Iron (II) solution: 5m<u>M</u> (as sulphate); unadjusted pH was 5.75.

vi) NaCl: 100m<u>M</u>.

vii) NaOH: 10m<u>M</u>.

viii) Penicillinase enzyme (*ex.* Difco Laboratories; 10 000 000 $IU.ml^{-1}$); freeze dried, then dissolved in one fifth the original volume.†

† 1µl of this solution was shown to cause complete loss of antibacterial activity from a solution of penicillin N (0.5$mg.ml^{-1}$; 90µl).

TABLE 6.1

Values of Δδ observed during NMR time courses

Times are quoted in min to the nearest 0.25min, Δδ values are quoted in Hz.

Initial	[IPNS]	Point 1		Point 2		Point 3		Point 4		Point 5	
O_2 sat.	(μM)	time	Δδ	time	Δδ	time	Δδ	time	Δδ	time	Δδ
50	0	11	4.64	20.5	4.27	30	3.97				
50	0	10	4.76	15	4.46	20	4.21	25	4.15	30	3.91
50	0	5.5	4.76	11	4.52	20	4.09	30	3.85		
50	0	8.25	4.70	15	4.52	20	4.33	25	4.21		
50	0	11.5	4.21	18.75	3.78	25	3.60	30	3.36		
50	0	9.5	4.66	15	4.47	20	4.29	25	4.11	30	3.93
50	100	6	3.42	12.5	2.32	20	1.04	30	0		
50	100	10	3.30	15	1.95	20	1.40	30	0.98		
50	100	9	2.20	15	0.92	17.5	0.61				
50	100	10.25	2.20	16.5	0.92	20	0.43				
50	100	7.25	3.36	10	3.17	15	2.69	20	2.01	25	1.28
Point 6 –>		30	0.67								
50	100	10	3.74	15	3.19	20	2.64	25	2.09	30	1.67
50	180	10	1.77	15	0.92	20	0				
50	180	7.5	1.83	10.5	1.10	13.5	0.49				
50	180	5.5	3.23	8.5	2.38	10	2.08	12	1.71	15	1.28
Points 6, 7 –>		17	0.85	19	0.37						
50	180	8	2.28	10	1.97	16	0.54	18	0.28		
20	0	10	3.60	15.25	3.48	20	3.42	25	3.36	30	3.30
20	100	8.5	2.32	12	2.14	15	2.01	18	1.83	21	1.65
Points 6, 7 –>		24	1.53	27	1.40						
20	180	11	1.10	13	0.98	17	0.55	19	0.49	21	0.55

TABLE 6.2

Showing the results for 'time zero' (t_0) $\Delta\delta$ values calculated from the timecourse data given in Table 6.1.

INITIAL O_2 SATURATION	[IPNS]/μM	Observed $\Delta\delta$/Hz
50	0	5.02
50	0	5.10
50	0	4.93
50	0	4.95
50	0	4.68
50	0	5.00
50	100	4.44
50	100	3.91
50	100	3.90
50	100	4.06
50	100	4.36
50	100	4.76
50	180	3.47
50	180	3.49
50	180	4.17
50	180	3.88
20	0	3.66
20	100	2.75
20	180	2.17

B) Apparatus

These experiments were performed using a Radiometer pH Stat system, consisting of a TTA80 Titration Assembly served by an ABU80 Autoburette, a TTT Titrator and a PHM82 pH meter. Results were plotted in real time on a REC80 Servograph chart recorder.

This system provided a sample cell in which were present; pH and reference electrodes, a microburette and a stirrer. In addition the gas mixture was introduced via a fine nozzle, and the cell was sat in a jacketed cell connected to a constant temperature water bath.

C) Procedure

NaCl solution (2.1ml) was placed in the reaction cell, and equilibrated with a mixture of O_2 and N_2 for 30min. This solution was treated sequentially with solutions of DTT (100µl), LLD-ACV (**1**) (volume varied as required), iron (II) (100µl) and penicillinase (20µl). The pH of the solution was brought to 7.70 with HCl solution (10mM), and when the pH was stable, the mixture was treated with IPNS solution (40µl). The pH was maintained at a constant value of 7.70 by addition of NaOH solution automatically through the burette system of the pH stat apparatus, and the volume added with time was output to a chart recorder.

The experiment was repeated with differing O_2/N_2 mixtures, and with the addition of differing volumes of LLD-ACV (**1**) solution, in order to obtain an array of initial rate values.

Initial rates were measured manually from the initial gradients for NaOH addition on the chart recorder output.

6.2.3 Oxygen Electrode Experiments

A) Preparation of Solutions

The following aqueous solutions were used in the oxygen electrode experiments:

i) LLD-ACV thiol (**1**): 50m<u>M</u>; stored at –80°C in 100µl aliquots.

ii) L-Ascorbic acid: 50m<u>M</u>; stored at –80°C in 500µl aliquots.

iii) Catalase enzyme: Diluted to one tenth of commercial supply (*ex*. Sigma); stored at 4°C.

iv) DTT solution: 100m<u>M</u>; stored at –80°C in 500µl aliquots.

v) IPNS solution: batch CF 25; in 50m<u>M</u> TRIS buffer at pH 7.7 containing 1m<u>M</u> DTT; stored stored at –80°C in 200µl aliquots.

vi) Iron (II) solution: 5m<u>M</u> (as sulphate); stored at –80°C in 500µl aliquots.

B) Apparatus

These experiments were performed using a Rank Brothers Ltd. Model 10 Digital Oxygen System, connected to a C. P. Goerz-Electro Servoscribe chart recorder.

This system consisted of a jacketed cell (connected to a constant temperature water bath), incorporating a Clarke type electrode in its base, and with magnetic stirring. The cell could be sealed from the atmosphere with a stopper containing two access holes for injection of materials and introduction of gas mixtures.

C) Procedure

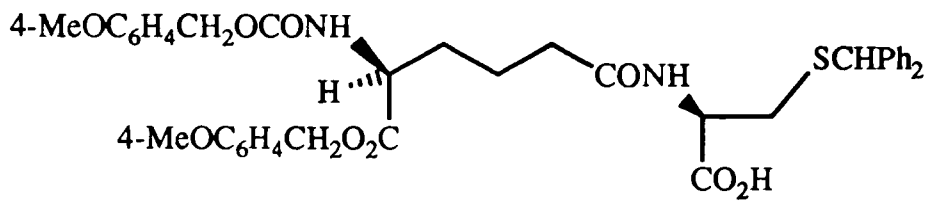
TRIS buffer (2.1ml) was placed in the cell, the stopper placed to leave room for gas flow over the surface of the solution, and O_2 gas was passed over the surface of the solution, with rapid stirring, until a constant reading was obtained from the Clarke electrode. The chart recorder was referenced to 100% using this value, and then the TRIS solution was treated with the desired O_2/N_2 mixture until a constant reading was obtained.

The solution in the reaction cell was treated sequentially with solutions of L-ascorbate (25µl), iron (II) (25µl), LLD-ACV (**1**) (volume varied as required), catalase (10µl) and DTT (50µl), after which the solution was re-equilibrated for a few minutes, the gas supply disconnected, and the stopper advanced to leave no gas space above the solution. The reaction was initiated with IPNS solution (40µl), and the addition holes sealed. The consumption of oxygen with time was measured on the chart recorder, from which traces initial rate measurements were made.

These experiments were repeated for different quantities of LLD-ACV solution and for different oxygen concentrations.

6.3 Experimental Procedures for Chapter Three

6.3.1 Synthesis of *N*–(4–Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteine



S-Benzhydryl-L-cysteine[133]

$C_{16}H_{17}NO_2S$; MW 287.4

A mixture of L-cysteine (*ex.* J.T. Baker Chemical Co.; 1.00g, 8.25mmol) and benzhydryl alcohol (*ex.* Koch Light; 1.52g, 8.25mmol) was treated with freshly distilled TFA (12ml, 156mmol) and the resultant yellow solution stirred at room temperature for 30min. Solvent was removed *in vacuo*, and the residue treated with Et_2O (90ml), and then with aqueous NaOAc (10% w/v) until the aqueous phase was at pH 6-7. The resulting mixture was filtered to collect the white suspension, and the residue washed with H_2O (250ml) and Et_2O (125ml), and dried *in vacuo* to constant mass (48h) to yield the title compound as a white powder (2.23g, 94%). δ_H (300MHz, 2M NaOD in D_2O) 2.14-2.21 and 2.24-2.30 (2H, AB part of ABX system, *J* 5.5, 6.5 and 13.5Hz, CH_2), 2.83-2.87 (1H, m, H_α), 4.86 (1H, s, C*H*Ph_2), 6.90-7.06 (10H, m, 2 × Ph); ν_{max} (KBr disk) 3450m (OH), 3300-3000bm (NH and CH), 1603s (CO), 1415m, 1391m, 1339m and 704s (Ph) cm^{-1}; *m/z* [DCI(NH_3)] 288 (34%, MH^+), 167 (100%, $^+CHPh_2$), 122 (36%), 90 (19%).

A small portion was recrystallised from H_2O:Acetone:NEt_3 (7:2:1) to yield white needles: $[\alpha]_D^{20}$ = +13.7° (*c* 0.99, H_2O:Acetone:NEt_3 [7:2:1]), m.p.

183-187°C (decomp) (*ex.* H_2O:Acetone:NEt_3 [7:2:1]) {Lit.,[134] $[\alpha]_D^{25}$ = +16.9° (*c* 2.9, 0.1M ethanolic HCl), m.p. 202-203°C (decomp) (*ex.* EtOH:5M HCl [15:1])}; $C_{16}H_{17}NO_2S$ requires C, 66.87; H, 5.96; N, 4.87; S, 11.16%: found C, 66.77; H, 6.14; N, 5.13; S, 10.86%.

4-Methoxybenzyl chloride

C_8H_9ClO; MW 156.6

4-Methoxybenzyl alcohol (*ex.* Aldrich; 2.0ml, 2.2g, 16mmol) was added to concentrated HCl [36% (w/v); 10ml], and the mixture stirred at room temperature for 1h. The inhomogeneous mixture was extracted with $CHCl_3$ (2 x 10ml), and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (10ml) and saturated aqueous NaCl (10ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield the title compound as a colourless mobile liquid (2.5g, 100%). δ_H (200MHz, $CDCl_3$) 3.84 (3H, s, MeO), 4.61 (2H, s, CH_2), 6.92 and 7.36 (4H, ABq, *J* 8.5Hz, Aromatic CH's).

N.B. As this compound may become explosively unstable on storage, excess material was destroyed within one week of synthesis.

***N*-(4-Methoxybenzyl)oxycarbonyl-L-α-aminoadipic acid**

$C_{15}H_{19}NO_7$; MW 325.3

A solution of L-α-aminoadipic acid (*ex.* Sigma; 2.00g, 12mmol) in aqueous $NaHCO_3$ (0.5M; 20ml) was adjusted to pH 9-10 with aqueous NaOH (2M), and subsequently treated with a solution of 4-methoxybenzyloxycarbonyl azide[135] (*ex.* Aldrich; 5.20g, 25mmol) in 1,4-dioxane (70ml). The reaction was stirred at room temperature for 8h, keeping the pH at 9-10 with aqueous NaOH (2M), and then at 40°C overnight. The reaction mixture was washed with Et_2O (2 x 60ml), acidified to pH 1 (HCl, 2M), and extracted with

EtOAc (3 x 60ml). The combined organic extracts were washed with saturated aqueous NaCl (50ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield the title compound as a white solid (3.50g, 87%). δ_H (200MHz, CD_3CN) 1.58-1.86 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.26-2.35 (2H, m, CH_2CO), 3.79 (3H, s, MeO), 4.09-4.17 (1H, m, H_α), 5.00 (2H, s, CH_2Ar), 5.91 (1H, d, *J* 8Hz, NH), 6.88-6.94 and 7.26-7.33 (4H, m, Aromatic CH's).

N-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-L-α-aminoadipic acid[136]

$C_{23}H_{27}NO_8$; MW 445.5

A solution of *N*-(4-methoxybenzyl)oxycarbonyl-L-α-aminoadipic acid (1.51g, 4.64mmol) in dry DMF (4ml) under argon, was treated sequentially with distilled NEt_3 (650µl, 4.6mmol), NaI (~10mg) and 4-methoxybenzyl chloride (630µl, 4.64mmol). The reaction was stirred under argon at 37°C overnight, treated with H_2O (30ml), and the resulting cloudy solution extracted with Et_2O (3 x 15ml). The combined organic extracts were washed with H_2O (4 x 10ml) and saturated aqueous NaCl (10ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield an orange oil (1.64g). This material was purified by column chromatography (SiO_2), with faster and slower running impurities eluting in CH_2Cl_2 and CH_2Cl_2:EtOAc (95:5), followed by eluting with 1% (v/v) AcOH in CH_2Cl_2:EtOAc (50:50) to yield the title compound as a pale yellow oil (1.31g, 63%). Tlc (SiO_2; EtOAc) R_f 0.5; δ_H (300MHz, $CDCl_3$) 1.59-1.88 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.30-2.37 (2H, m, CH_2CO), 3.80 (6H, s, 2 x MeO), 4.36-4.41 (1H, m, H_α), 5.03-5.11 (4H, m, 2 x CH_2Ar), 5.39 (1H, d, *J* 8Hz, NH), 6.86-6.89 and 7.26-7.29 (8H, m, Aromatic CH's), 10.37 (1H, bs, CO_2H); δ_C (50.7MHz, $CDCl_3$)

20.12, 31.67 and 33.11 ($(CH_2)_3$), 53.55 (C_α), 55.18 (2 x MeO), 66.87 and 67.12 (2 x CH_2Ar), 114.00-130.35 (Aromatic C's), 156.28-178.86 (3 x CO); *m/z* [FAB(MCA)] 468 (MNa^+).

N–(4–Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-S-benzhydryl-L-cysteine[136]

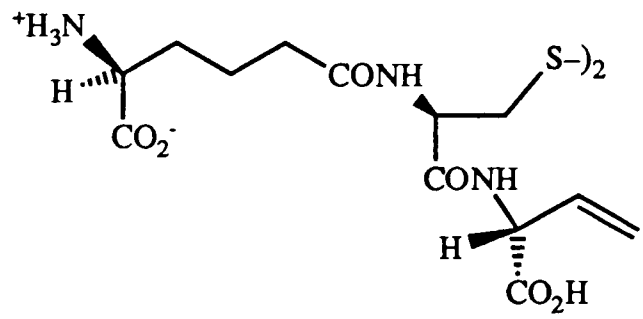
$C_{39}H_{42}N_2O_9S$; MW 714.8

A solution of *N*-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-L-α-aminoadipic acid (464mg, 1.0mmol) and distilled NEt_3 (145µl, 1.0mmol) in freshly distilled THF (10ml) under argon, was cooled to –15°C for 15min, and then treated with freshly distilled isobutylchloroformate (*ex.* Sigma; 135µl, 1.0mmol), followed by stirring at –15°C under argon for 1h. A solution (part suspension) of *S*-benzhydryl-L-cysteine (299mg, 1.0mmol) in H_2O (10ml) and NEt_3 (0.5ml) at 0°C was added in one portion to the mixed anhydride solution, and the temperature allowed to rise to room temperature over 1h. The resulting mixture was treated with H_2O (20ml), and washed with Et_2O (20ml, and 35ml). The aqueous layer was acidified to pH 1 (HCl, 1M), extracted with EtOAc (2 x 35ml), and the combined organic extracts were washed with saturated aqueous NaCl (35ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield an off-white foam (535mg). Purification by PLC (SiO_2; double elution with EtOAc:30-40°C petrol [60:40]) yielded recovered starting material as a pale yellow oil (43mg, 9%) and the title compound as a white solid (319mg, 47%). δ_H (500MHz, $CDCl_3$) 1.67-1.86 (4H, m, $CH_2CH_2CH_2CO$), 2.14-2.20 and 2.24-2.30 (2H, 2 x m, CH_2CO), 2.85-2.92 (2H, m, CH_2S), 3.80 (6H, s, 2 x MeO), 4.36-4.39 (1H, m, H_α), 4.62-4.66 (1H, m, H_α), 4.99 and 5.04 (2H, ABq, *J* 12Hz, CH_2Ar), 5.07-5.11 (2H, m, CH_2Ar), 5.21 (1H, s, $CHPh_2$), 5.41 (1H, d, *J* 7.5Hz, NH), 6.32 (1H, d, *J* 7Hz, NH), 6.86-6.89

and 7.22-7.40 (18H, 2 x m, Aromatic CH's), 9.25 (1H, bs, CO_2H); δ_C (50.7MHz, $CDCl_3$) 21.19, 31.57 and 33.80 ($(CH_2)_3$), 51.50, 53.46 and 54.23 (2 x C_α and $CHPh_2$), 55.19 (2 x MeO), 64.46 (CH_2S), 66.98 and 67.12 (2 x CH_2Ar), 114.00 and 127.47-130.35 (Aromatic CH's), 140.81-140.96 (Quaternary aromatic C's), 156.66-174.06 (4 x CO); ν_{max} (KBr disk) 3450m (NH), 2930bm (CH), 1718s (CO), 1615m, 1516s (CO), 1248s, 1174m, 1032m, 823m and 704s (Ph) cm^{-1}; *m/z* [FAB(DTT/DTE/Oxalic Acid)] 737 (19%, MNa^+), 167 (64%, $^+CHPh_2$), 121 (89%), 59 (79%), 45 (100%).

A portion was recrystallised from $CHCl_3/Et_2O$: $[\alpha]_D^{20}$ = +0.4° (*c* 1.01, $CHCl_3$); m.p. 50-53°C (*ex*. $CHCl_3/Et_2O$); $C_{39}H_{42}N_2O_9S$ requires C, 65.53; H, 5.92; N, 3.92%: found C, 65.62; H, 5.90; N, 4.21%.

6.3.2 Synthesis of bis-[L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine] (disulphide of 106)



***N*-Benzyloxycarbonyl-D-vinylglycine, methyl ester**[110]

$C_{13}H_{15}NO_4$; MW 249.3

N-Benzyloxycarbonyl-D-methionine sulphoxide, methyl ester (**127**) (*ex*. R. Freeman;[137] 2.56g, 8.17mmol) was placed in a preheated Kugelröhr oven at 180°C, and the vessel evacuated to 1mmHg. The compound was heated at

180°C, 1mmHg for 2h, after which time all the material had distilled into a room temperature collection flask to yield a foul smelling, light brown oil (1.88g). Purification by column chromatography (SiO_2; 40-60°C petrol:EtOAc [4:1]) yielded the title compound as a virtually colourless oil (1.16g, 57%). Tlc (SiO_2: 40-60°C petrol:EtOAc [4:1]) R_f 0.25; δ_H (200MHz, $CDCl_3$) 3.77 (3H, s, CO_2Me), 4.96-4.99 (1H, m, H_α), 5.14 (2H, s, CH_2Ph), 5.26-5.42 (2H, m, $C=CH_2$), 5.52-5.56 (1H, m, NH), 5.84-6.00 (1H, m, $CH=CH_2$), 7.37 (5H, s, Ph); δ_C (50.7MHz, $CDCl_3$) 52.28 (Me), 56.12 (C_α), 67.00 (CH_2O), 117.83 ($C=CH_2$), 128.13-128.25 (Aromatic CH's), 132.46 ($CH=CH_2$), 155.89 (OCON), 171.21 (CO_2Me); ν_{max} ($CHCl_3$) 3440m (NH), 2950m (CH), 2360w, 1730s (CO), 1520s (CO), 1340s, 1215s, 1055m, 995m, 930m, 755m, and 700m (Ar) cm^{-1}; *m/z* [CI(NH_3)] 267 (100%, MNH_4^+), 250 (95%, MH^+), 206 (59%), 108 (25%), and 91 (24%); $[\alpha]_D^{20} = -2.6°$ (*c* 1.20, $CHCl_3$); Calc. for $C_{13}H_{15}NO_4$: C, 62.64; H, 6.07; N, 5.62%: Found C, 62.84; H, 6.01; N, 5.85%.

D-Vinylglycine, hydrochloride salt (126)

$C_4H_7NO_2.HCl$; MW 137.61

N-Benzyloxycarbonyl-D-vinylglycine, methyl ester (1.16g, 4.65mmol) was heated at reflux in HCl (3M; 100ml) for 4.5h, at which point no oily droplets were visible. The solution was cooled to room temperature, washed with EtOAc (2 x 30ml) and solvent evaporated *in vacuo* to yield the title compound (**126**) as a cream coloured solid (685mg, 100%): δ_H (300MHz, D_2O) 4.42 (1H, d, *J* 7.5Hz, H_α), 5.32-5.38 (2H, m, CH_2), 5.70-5.82 (1H, m, $CH=CH_2$).

N-tert-Butoxycarbonyl-D-vinylglycine

$C_9H_{15}NO_4$; MW 201.2

A solution of D-vinylglycine, hydrochloride salt (38mg, 0.27mmol) in H_2O (0.5ml) and 1,4-dioxane (0.5ml), was treated with distilled NEt_3 (115µl; 3 equiv.) and BOC-ON[106] (*ex.* Aldrich; 81mg, 0.33mmol; 1.2 equiv.), and the solution stirred at room temperature overnight. The resulting clear yellow solution was partitioned between H_2O (10ml) and EtOAc (10ml), and the aqueous phase was treated with saturated aqueous NaCl (10ml), acidified to pH 1 (HCl, 1M), and extracted with EtOAc (2 × 10ml). The combined organic extracts were dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield the title compound as a colourless oil (54mg, 97%). δ_H (200MHz, $CDCl_3$) 1.45 (9H, s, Me_3C), 4.67-4.73 (1H, m, NH), 4.66-4.76 and 4.87-4.97 (1H, 2 × m, H_α), 5.26-5.44 (2H, m, CH_2), 5.84-6.02 (1H, m, $CH{=}CH_2$), 10.61 (1H, bs, CO_2H).

Diphenyldiazomethane[138]

$C_{13}H_{10}N_2$; MW 194.2

A solution of benzophenone hydrazone (*ex.* Aldrich; 10.0g, 51mmol), 1,1,3,3-tetramethylguanidine (*ex.* Koch Light; 6.4ml, 51mmol) and iodine (2.1ml of a 1% (w/v) solution in CH_2Cl_2) in CH_2Cl_2 (50ml) was cooled to 0°C, and 3-chloroperbenzoic acid (*ex.* Lancaster Synthesis; 8.80g, 51mmol) was added in portions over 1h. After addition was completed, the reaction was stirred at 0°C for 15min, and allowed to warm to room temperature overnight. The solution was then washed with H_2O (2 × 100ml), checking that the pH of the final washings was 6-7, dried (Na_2SO_4), filtered and solvent evaporated *in vacuo*. The residue was dissolved in a minimum amount of 40-60°C petrol at 4°C, filtered to remove insoluble benzophenone hydrazone, and

solvent evaporated *in vacuo* to yield the title compound as a dark purple solid (10g, 100%). δ_H (200MHz, $CDCl_3$) 7.21-7.49 (10H, m, 2 x Ph).

_N_-*tert*-Butoxycarbonyl-D-vinylglycine, benzhydryl ester

$C_{22}H_{25}NO_4$; MW 367.4

A solution of diphenyldiazomethane[107] (479mg, 2.47mmol; 1.2 equiv.) in EtOAc (5ml) was added to *N*-*tert*-butoxycarbonyl-D-vinylglycine (463mg, 2.30mmol) under argon, and the reaction stirred at room temperature under argon for 3.5h. Solvent was evaporated *in vacuo* to yield a yellow oil (843mg), which was purified by column chromatography (SiO_2; CH_2Cl_2) to yield the title compound as a white solid (584mg, 69%). Tlc (SiO_2; CH_2Cl_2) R_f 0.3; δ_H (200MHz, $CDCl_3$) 1.47 (9H, s, Me_3C), 4.99-5.07 (1H, m, H_α), 5.27-5.43 (3H, m, NH and $C{=}CH_2$), 5.89-6.03 (1H, m, $CH{=}CH_2$), 6.93 (1H, s, $CHPh_2$), 7.30-7.45 (10H, m, 2 x Ph); δ_C (50.7MHz, $CDCl_3$) 28.17 (3 x Me), 56.02 (C_α), 64.17 ($CHPh_2$), 78.22 ($CH{=}CH_2$), 80.15 (Me_3C), 117.95 (CH_2), 127.14-129.03 (Aromatic CH's), 139.70 and 139.77 (Quaternary aromatic C's), 155.41 (OCON), 170.15 (CO_2CH); ν_{max} (KBr disk) 3333s (NH), 3065m (CH), 1738s (CO), 1701s (CO), 1518s (CO), 1202s, 1162s and 698m (Ar) cm^{-1}; *m/z* [CI(NH_3)] 385 (57%, MNH_4^+), 368 (8%, MH^+), 327 (36%), 310 (81%), 271 (28%), 254 (30%), 167 (100%, $^+CHPh_2$).

A small portion was recrystallised from Et_2O/30-40°C petrol: m.p. 90.0-90.5°C (ex. Et_2O/30-40°C Petrol); $[\alpha]_D^{20}$ = +2.8° (*c* 1.00, $CHCl_3$); Calc. for $C_{22}H_{25}NO_4$: C, 71.91; H, 6.86; N, 3.81%: Found C, 71.62; H, 7.10; N, 3.78%.

D-Vinylglycine, benzhydryl ester, toluene-4-sulphonate salt

$C_{17}H_{17}NO_2.C_7H_8O_3S$; MW 439.5

A solution of *N*-*tert*-butoxycarbonyl-D-vinylglycine, benzhydryl ester (121mg, 0.33mmol) in Et_2O (10ml) and EtOH (10ml) was cooled to –10°C and treated with toluene-4-sulphonic acid (*ex.* Aldrich, recrystallised; 57mg, 0.33mmol). Upon dissolution, solvent was removed *in vacuo*, and the reaction heated at 40-60°C, 20mmHg for 2h to yield the title compound as a white solid (152mg, 100%). δ_H (200MHz, $CDCl_3$) 2.23 (3H, s, Me), 4.58-4.69 (1H, m, H_α), 5.17-5.38 (2H, m, $C{=}CH_2$), 5.66-5.83 (1H, m, $CH{=}CH_2$), 6.78 (1H, s, $CHPh_2$), 6.97-7.01 (2H, m, 2H of $MeC_6H_4SO_3^-$), 7.21-7.42 (10H, m, 2 × Ph), 7.58-7.62 (2H, m, 2H of $MeC_6H_4SO_3^-$), 8.31 (3H, bs, NH_3^+).

N–(4–Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-S-benzhydryl-L-cysteinyl-D-vinylglycine, benzhydryl ester

$C_{56}H_{57}N_3O_{10}S$; MW 964.1

A solution of D-vinylglycine, benzhydryl ester, toluene-4-sulphonate salt (114mg, 0.26mmol), *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteine (184mg, 0.26mmol) and EEDQ[108] (*ex.* Aldrich, recrystallised; 64mg, 0.26mmol) in CH_2Cl_2 (4ml) under argon, was treated with Na_2SO_4 (~25mg) and distilled NEt_3 (35µl, 0.26mmol). The reaction mixture was stirred at room temperature under argon overnight, and solvent evaporated *in vacuo*. The residue was partitioned between EtOAc (15ml) and saturated aqueous $NaHCO_3$ (15ml), and the organic layer washed with HCl (1M; 15ml) and saturated aqueous NaCl (15ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield a yellow oil (251mg). Purification by column chromatography (SiO_2; gradient elution CH_2Cl_2:EtOAc [(9:1)

(8:2)]) yielded the title compound as a colourless oil (149mg, 60%). Tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.5 (N.B. LLL-epimer R_f 0.4); δ_H (200MHz, $CDCl_3$) 1.60-1.82 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.09-2.19 (2H, m, CH_2CO), 2.73-2.80 (2H, m, SCH_2), 3.80 (6H, s, 2 x MeO), 4.33-4.39 (1H, m, H_α), 4.57-4.63 (1H, m, H_α), 5.02-5.37 (8H, m, 2 x CH_2Ar, $SCHPh_2$, H_α and $C=CH_2$), 5.50 (1H, d, *J* 8Hz, NH), 5.83-5.97 (1H, m, $CH=CH_2$), 6.32 (1H, d, *J* 8Hz, NH), 6.84-6.94 and 7.18-7.46 (30H, 2 x m, Aromatic CH's, NH and $OCHPh_2$); *m/z* [FAB(MCA)] 986 (100%, MNa^+), 964 (93%, MH^+).

Bis-[L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine] (disulphide of 106)

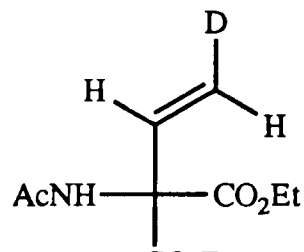
$C_{26}H_{40}N_6O_{12}S_2$; MW 692.8

A solution of *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteinyl-D-vinylglycine, benzhydryl ester (109mg, 0.11mmol) in freshly distilled TFA (2ml) and distilled anisole (400µl) was heated under reflux for 30min.[109] TFA was removed *in vacuo*, with the last traces being removed by azeotroping with toluene (2 x 1ml). The residual suspension in anisole was partitioned between H_2O (10ml) and EtOAc (10ml). The organic phase was extracted with H_2O (2 x 10ml) and the combined aqueous extracts freeze dried to yield the crude thiol form (**106**) of the title compound as a yellow solid (58mg): δ_H (500MHz, D_2O) 1.51-1.90 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.22-2.27 (2H, m, CH_2CO), 2.70-2.80 (2H, m, CH_2S), 3.75-3.78 (1H, m, H_α), 4.37-4.41 (1H, m, H_α), 4.76-4.79 (1H, m, H_α), 5.17-5.23 (2H, m, $C=CH_2$), 5.80-5.87 (1H, m, $CH=CH_2$).

The crude thiol was dissolved in H_2O (25ml) and the pH of the solution adjusted to 10 (NH_3 solution; 2M). Oxygen was bubbled through the solution for 3h and the solution was then freeze dried. Purification by reverse phase HPLC (Hypersil® ODS; mobile phase, gradient elution 0-15% [$1\%.min^{-1}$]

MeOH in 20m<u>M</u> NH_4HCO_3) yielded, after lyophilisation, the title compound (disulphide of **106**) as a white solid (retention time 10min). δ_H (500MHz, D_2O) (Number of H's for each resonance quoted for one half of disulphide only, i.e. for R of RS–SR) 1.48-1.70 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.17-2.22 (2H, m, CH_2CO), 2.76-2.84 and 2.99-3.06 (2H, 2 x m, CH_2S), 3.51-3.53 (1H, m, H_α), 4.45-4.48 (1H, m, H_α), 5.02-5.09 (2H, m, $C=CH_2$), 5.73-5.81 (1H, m, $CH=CH_2$), one H_α peak obscured by HOD signal; *m/z* [FAB(MCA)] 693 (MH^+).

6.3.3 Synthesis of *E*-Diethylacetamido-[2-^{2}H-vinyl]-malonate



Z–Diethylacetamido–[2–(phenylsulphonyl)vinyl]–malonate and *E*–diethylacetamido-[2-(phenylsulphonyl)vinyl]-malonate[103]

$C_{17}H_{21}NO_7S$; MW 383.4

Potassium *tert*-butoxide was purified by dissolving in freshly distilled THF, filtration to remove insoluble residue, followed by evaporation of solvent *in vacuo*.

A solution of diethylacetamidomalonate [*ex*. Sigma; 1.32g, 6.08mmol; δ_H (200MHz, $CDCl_3$) 1.30 (6H, t, *J* 7Hz, 2 x CH_3CH_2), 2.08 (3H, s, MeCO), 4.21-4.33 (4H, m, 2 x CH_3CH_2), 5.16 (1H, d, *J* 7Hz, CH), 6.56 (1H, d, *J* 7Hz, NH)] in freshly distilled THF (15ml) under argon, was treated with purified KO^{t}Bu (0.10g, 0.89mmol; 0.15 equiv.). The mixture was stirred at

room temperature for 1h, cooled to –10°C and then treated with a solution of phenyl-(2-trimethylsilyl)ethynyl sulphone (*ex.* Aldrich; 1.0g, 4.19mmol) in freshly distilled THF (4ml) over 10min. The reaction was stirred at –10°C under argon for 1h, then treated with H_2O (1ml) followed by stirring at room temperature under argon overnight. The solution was partitioned between H_2O (20ml) and Et_2O (20ml), and the aqueous phase extracted further with Et_2O (2 x 20ml). The combined organic extracts were dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield an orange oil (1.67g). Purification by column chromatography (SiO_2; gradient elution CH_2Cl_2;EtOAc [85:15 - 75:25]) yielded the mixture of title compounds free of impurities as a white foam (1.17g, 50%). Tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.4.

The geometric isomers were separated from a portion of this mixture (800mg) by column chromatography (SiO_2; Et_2O) to yield the faster eluting *Z*-isomer (118mg) and slower eluting *E*-isomer (203mg) as white solids, as well as fractions still containing a mixture of isomers (369mg, total recovery 86%).

i) Data for *Z*-isomer: tlc (SiO_2; Et_2O) R_f 0.3; δ_H (200MHz, $CDCl_3$) 1.28 (6H, t, *J* 7Hz, 2 x CH_3CH_2), 1.99 (3H, s, MeCO), 4.31 (4H, q, *J* 7Hz, 2 x CH_3CH_2), 6.31 (1H, d, *J* 12Hz, CHS), 7.47 (1H, d, *J* 12Hz, C*H*=CHS), 7.48 (1H, bs, NH), 7.44-7.66 and 7.81-7.8ɔ (5H, 2 x m, Ph); δ_C (50.7MHz, $CDCl_3$) 13.69 (2 x CH_3CH_2), 22.00 (CH_3CO), 63.57 (2 x CH_3CH_2), 64.28 ($C(CO_2Et)_2$), 127.07-134.02 (CHS and Aromatic CH's), 140.50 (Quaternary aromatic C, and CCH=C), 165.85-169.29 (2 x CO_2Et and CONH).

ii) Data for *E*-isomer: tlc (SiO_2; Et_2O) R_f 0.2; δ_H (200MHz, $CDCl_3$) 1.27 (6H, t, *J* 7Hz, 2 x CH_3CH_2), 2.02 (3H, s, MeCO), 4.28 (4H, q, *J* 7Hz, 2 x CH_3CH_2), 6.46 (1H, d, *J* 15Hz, CHS), 6.97 (1H, bs, NH), 7.51 (1H, d, *J* 15Hz, C*H*=CHS), 7.48-7.62 and 7.85-7.90 (5H, 2 x m, Ph).

Diethylacetamido-vinylmalonate[103]

$C_{11}H_{17}NO_5$; MW 243.3

Aluminium foil (830mg) was washed with 30-40°C petrol and cut into small (1-2cm^2) pieces. The pieces were placed in an 0.5% (w/v) aqueous solution of $HgCl_2$ (100ml) for 2min. The amalgam thus formed was collected by filtration under suction and added rapidly to a solution of *Z*- and *E*-diethyl-acetamido-[2-(phenylsulphonyl)vinyl]-malonates (371mg, 0.97mmol in total of isomers) in 1,4-dioxane (75ml) at 10°C under argon. The reaction was stirred at 10°C under argon for 2.5 days, at which point a small portion (*ca.* 2ml) was removed, filtered, solvent evaporated *in vacuo* and the residue examined by ^{1}H NMR spectroscopy. Comparison of the acyl integrals showed the extent of reaction to be *ca.* 50%.

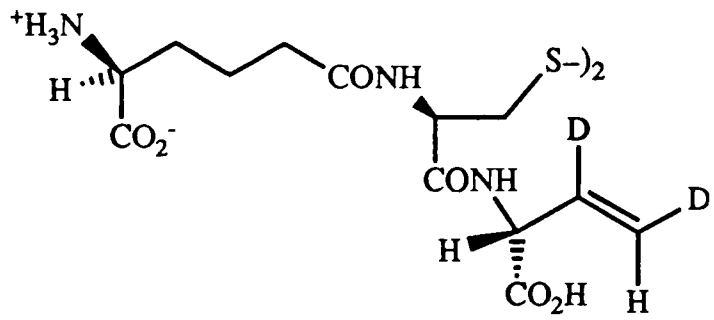
A further portion of aluminium amalgam (prepared as above from 830mg Al) was added, and the reaction stirred for a further 24h. Examination by ^{1}H NMR spectroscopy in an analogous manner to previously, showed the extent of reaction to be >90%. The reaction was filtered under suction through SiO_2, and the SiO_2 washed thoroughly with Et_2O (300ml). The filtrate and washings were combined and solvent evaporated *in vacuo* to yield a colourless oil (244mg). Purification by PLC (SiO_2; propan-2-ol:30-40°C petrol [15:85]) yielded the title compound as a colourless oil (99mg, 42%). Tlc (SiO_2; propan-2-ol:30-40°C petrol [15:85]) R_f 0.2; δ_H (200MHz, $CDCl_3$) 1.24, (6H, t, *J* 7Hz, 2 x CH_3CH_2), 2.06 (3H, s, MeCO), 4.24 (4H, q, *J* 7Hz, 2 x CH_3CH_2), 5.25 (1H, d, *J* 17Hz, CH=CH(*Z*)H(*E*)), 5.28 (1H, d, *J* 10.5Hz, CH=CH(*Z*)H(*E*)), 6.50 (1H, dd, *J* 10.5 and 17Hz, $CH=CH_2$), 6.97 (1H, bs, NH); δ_C (50.7MHz, $CDCl_3$) 13.69 (2 x CH_3CH_2), 22.57 (*Me*CO), 62.78 (2 x CH_3CH_2), 67.05 ($C(CO_2Et)_2$), 116.43 ($CH=CH_2$), 132.17 ($CH=CH_2$), 167.20 and 168.92 (3 x CO); ν_{max} ($CHCl_3$) 3420m (NH), 3000m (CH), 1740s (CO), 1685s (CO), 1495s, 1305m, 1280s, 1205s, 930w ($CH=CH_2$) cm^{-1}.

_E_-Diethylacetamido-[2-^{2}H-vinyl]-malonate[103]

$C_{11}H_{16}DNO_5$; MW 244.2

Aluminium foil (70mg) was washed with 30-40°C petrol and cut into small (1-2cm^2) pieces. The pieces were placed in a 5% (w/v) solution of $HgCl_2$ in D_2O (*ex.* Aldrich, 99.8 atom % D; 20ml) under argon for 2min. The amalgam thus formed was collected by filtration under suction in a nitrogen atmosphere and rapidly added to a solution of *E*-diethylacetamido-[2-(phenylsulphonyl)vinyl]-malonate (30mg, 78μmol) in 1,4-dioxane (5ml) under argon. The reaction mixture was stirred for 24h, treated with a further portion of aluminium amalgam (prepared as above from 70mg Al), and stirred for a further 5 days. The reaction was filtered through SiO_2, and the SiO_2 washed thoroughly with distilled EtOAc (~100ml). The combined filtrate and washings were evaporated *in vacuo* to yield a colourless oil (23mg). Purification by PLC (SiO_2; propan-2-ol:30-40°C petrol [15:85]) yielded the title compound as a colourless oil (13mg, 68%). Examination by 500MHz ^{1}H NMR spectroscopy and mass spectrometric analysis showed this material to be a mixture of the required *E*-isomer together with the *Z*-isomer and the d_0 isotopomer (ratio approx. 75:15:10 respectively). Tlc (SiO_2; propan-2-ol:30-40°C petrol [15:85]) R_f 0.2; δ_H (500MHz, $CDCl_3$) 1.27 (6H, t, *J* 7Hz, 2 x CH_3CH_2), 2.09 (3H, s, MeCO), 4.24-4.29 (4H, m, 2 x CH_3CH_2), 5.25, (0.75H, d, *J* 18Hz, *E*-RCH=C*H*D), 5.27 (0.10H, d, *J* 17Hz, RCH=C*H*(*Z*)H(*E*)), 5.29 (0.15H, d, *J* 10Hz, *Z*-RCH=C*H*D), 5.31 (0.10H, d, *J* 10Hz, RCH=CH(*Z*)*H*(*E*)), 5.50-5.54 (1H, m, RC*H*=C), 6.95 (1H, bs, NH); ν_{max} ($CHCl_3$) 3410m (NH), 3000m (CH), 1740s (CO), 1680s (CO), 1490m, 1270s, and 1200m cm^{-1}; *m/z* (Direct) 245 (100%, MH$^+$), 244 (10.7%, d_0-MH$^+$), 171 (19%), and 129 (16%).

6.3.4 Synthesis of bis-[L-α-aminoadipoyl)-L-cysteinyl-*E*-[2,3-$^2H_2$]-D-vinylglycine] (disulphide of 111)



[*N*,2-$^2H_2$]-Diethylacetamidomalonate[103]

$C_9H_{13}D_2NO_5$; MW 219.2

A solution of diethylacetamidomalonate (*ex.* Sigma; 10.0g, 46mmol) in CH_3OD (*ex.*, Aldrich; 99.5%+ atom % D; 40ml) and D_2O (*ex.* Aldrich; 99.8 atom % D; 10ml) was treated with dry distilled pyridine (2 drops, ~100µl) and stirred at room temperature for 2h. Solvent was evaporated *in vacuo* and the process repeated to yield the title compound as a white solid (10.0g, 100%). δ_H (250MHz, $CDCl_3$) 1.30 (6H, t, *J* 7Hz, 2 x CH_3CH_2), 2.08 (3H, s, MeCO), 4.27 (4H, q, *J* 7Hz, 2 x CH_3CH_2); δ_D (38.4MHz, $CHCl_3$) 5.13 (1D, s, CD), 6.63 (1D, s, ND); *m/z* (Field Desorption) 219 (100%, $M^{\cdot+}$).

Z-Diethylacetamido-[2-(phenylsulphonyl)-[1,2-$^2H_2$]-vinyl]-malonate (115) and E-diethylacetamido-[2-(phenylsulphonyl)-[1,2-$^2H_2$]-vinyl]-malonate (116)[103]

$C_{17}H_{19}D_2NO_7S$; MW 385.4

Potassium *tert*-butoxide was purified by dissolving in freshly distilled THF, filtering to remove insoluble residue, and evaporation of solvent *in vacuo*.

A solution of [*N*,2-$^2H_2$]-diethylacetamidomalonate (5.28g, 24.1mmol) in freshly distilled THF (60ml) under argon, was treated with purified KO^{t}Bu

(0.40g, 3.56mmol; 0.15 equiv.) at 0°C. The mixture was stirred at 0°C for 1h, then cooled to −10°C and treated with a solution of phenyl-(2-trimethylsilyl)ethynyl sulphone (*ex.* Aldrich; 4.48g, 18.8mmol) in freshly distilled THF (20ml) over 10min. The reaction was stirred at −10°C under argon for 2h, treated with D_2O (*ex.* Aldrich; 99.8 atom % D; 4ml) and stirred at room temperature under argon overnight. The solution was partitioned between H_2O (100ml) and Et_2O (100ml), and the aqueous phase extracted further with Et_2O (2 × 100ml). The combined organic extracts were dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield a viscous orange oil (8.15g). Purification by column chromatography (SiO_2; gradient elution CH_2Cl_2;EtOAc [85:15 - 60:40]) yielded the mixture of title compounds (**115** and **116**) free of impurities as a pale yellow solid (5.78g, 80%). Tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.4.

The geometric isomers were separated by column chromatography (SiO_2; Et_2O) to yield the faster eluting *Z*-isomer (**115**) (1.61g) and slower eluting *E*-isomer (**116**) (3.39g) as white solids, as well as fractions still containing a mixture of isomers (0.29g, total recovery 84%, overall yield 67%).

i) Data for *Z*-isomer (**115**): tlc (SiO_2; Et_2O) R_f 0.3; δ_H (300MHz, $CDCl_3$) 1.29 (6H, t, *J* 7Hz, 2 × CH_3CH_2), 1.98 (3H, s, MeCO), 4.30 (4H, q, *J* 7Hz, 2 × CH_3CH_2), 7.48 (1H, bs, NH), 7.51-7.68 and 7.81-7.85 (5H, 2 × m, Ph); δ_C (50.7MHz, $CDCl_3$) 13.69 (2 × CH_3CH_2), 22.03 (CH_3CO), 63.62 (2 × CH_3CH_2), 64.21 ($C(CO_2Et)_2$), 127.28, 129.51 and 134.02 (Aromatic CH's), 140.46 (Quaternary aromatic C), 165.86-169.40 (2 × CO_2Et and CONH); ν_{max} ($CHCl_3$) 3410m (NH), 3020m (CH), 1750s (CO), 1680s (CO), 1485m, 1310s (SO_2), 1270s, 1200s, and 1155s (SO_2) cm^{-1}; *m/z* [CI(NH_3)] 386 (100%, MH^+; d_1-peak <0.1%), 344 (13%), and 244 (22%).

A portion was recrystallised from Et_2O/30-40°C petrol to yield white crystals: m.p. 94.5-96.0°C (*ex* Et_2O/30-40°C petrol), [lit.,[103] for d_0 material 94-95°C]; $C_{17}H_{19}D_2NO_7S$ requires C, 52.86; H, 5.48; N, 3.63%: Found C, 53.03; H, 5.43; N, 3.42%.

ii) Data for *E*-isomer (**116**): tlc (SiO_2; Et_2O) R_f 0.2; δ_H (300MHz, $CDCl_3$) 1.28 (6H, t, *J* 7Hz, 2 × CH_3CH_2), 2.03 (3H, s, MeCO), 4.21-4.37 (4H, m, 2 × CH_3CH_2), 6.95 (1H, bs, NH), 7.51-7.65 and 7.82-7.90 (5H, 2 × m, Ph); δ_C (50.7MHz, $CDCl_3$) 13.65 (2 × CH_3CH_2), 22.33 (CH_3CO), 63.63 (2 × CH_3CH_2), 66.32 ($C(CO_2Et)_2$), 127.72, 128.47 and 133.81 (Aromatic CH's), 139.76 (Quaternary aromatic C), 165.32-169.43 (2 × CO_2Et and CONH); ν_{max} ($CHCl_3$) 3420m (NH), 3000m (CH), 2260w (CD), 1750s (CO), 1680s (CO), 1496m, 1310s (SO_2), 1270s, 1200s, 1160s (SO_2), and 650m (*E* R'CD=CDR) cm^{-1}; *m/z* [CI(NH_3)] 403 (100%, MNH_4^+), 386 (71%, MH^+), 344 (15%), 296 (15%), 244 (47%), and 125 (15%).

A portion was recrystallised from Et_2O/30-40°C petrol to yield white crystals: m.p. 103.5-104.5°C (ex Et_2O/30-40°C petrol), [lit.,[103] for d_0 material 108.5-109°C]; $C_{17}H_{19}D_2NO_7S$ requires C, 52.86; H, 5.48; N, 3.63%: Found C, 53.20; H, 5.55; N, 3.42%.

_E_-Diethylacetamido-[1,2-$^2H_2$-vinyl]-malonate (117)[103]

$C_{11}H_{15}D_2NO_5$; MW 245.2

Aluminium foil (4.2g) was washed with 30-40°C petrol and cut into small (2-4cm^2) pieces. The pieces were placed in a 0.5% (w/v) aqueous solution of $HgCl_2$ (100ml) for 2min. The amalgam thus formed was collected by filtration under suction and added rapidly to a solution of *Z*-diethylacetamido-[2-(phenylsulphonyl)-[1,2-$^2H_2$]-vinyl]-malonate (**115**) (1.87g, 4.85mmol) in

1,4-dioxane (160ml) at 0°C under argon. The temperature was allowed to rise to room temperature, and the mixture stirred at room temperature overnight. The mixture was filtered under suction through celite, and the residue washed on celite with 1,4-dioxane (~200ml) and EtOAc (~200ml). The combined filtrate and washings were evaporated *in vacuo* to yield a pale yellow oil (1.41g). Purification by column chromatography (SiO_2; propan-2-ol:40-60°C petrol [1:3]) yielded the title compound (**117**) as a colourless oil (977mg, 82%). Further elution of the column with propan-2-ol:40-60°C petrol [1:1] afforded some unreacted starting material (**115**) (154mg, 8%). The yield based on recovered starting material was 89%. Analysis by 500MHz NMR spectroscopy indicated that the product was a mixture of the *E* and *Z* isotopomers, predominating in the title isotopomer (85%). Tlc (SiO_2; propan-2-ol:30-40°C petrol [15:85]) R_f 0.2; δ_H (500MHz, $CDCl_3$) 1.28 (6H, t, *J* 7Hz, 2 × CH_3CH_2), 2.10 (3H, s, MeCO), 4.28-4.31 (4H, m, 2 × CH_3CH_2), 5.25 (0.85H, t_D, J_{HD} 2.5Hz, *E* RC*D*=C*H*D), 5.30 (0.15H, s, *Z* RCD=CHD), 6.94 (1H, bs, NH); δ_C (50.7MHz, $CDCl_3$) 13.67 (2 × CH_3CH_2), 22.53 (*Me*CO), 62.64 (2 × CH_3CH_2), 67.00 ($C(CO_2Et)_2$), 115.98 (t_D, J_{CD} 25Hz, CHD), 131.2-132.4 (m, *C*D=CDH), 167.17 and 168.93 (2 × CO_2Et and CONH); δ_D (38.4MHz, $CHCl_3$) 5.24 (1D, s, CHD), 6.47 (1D, s, C*D*=CDH), 7.39 (0.1D, s, residual ND); *m/z* [CI(NH_3)] 246 (100%, MH^+), 245 (7.8%, d_1-MH^+), 172 (14%), and 130 (15%).

***N*-*tert*-Butoxycarbonyl-*E*-[2,3-$^2H_2$]-DL-vinylglycine**

$C_9H_{13}D_2NO_4$; MW 203.2

A suspension of *E*-diethylacetamido-[1,2-$^2H_2$-vinyl]-malonate (**117**) (456mg, 1.86mmol) in HCl (6M; 5ml) was heated under reflux for 4.5h, and

solvent evaporated *in vacuo* to yield the intermediate *E*-[2,3-$^2H_2$]-DL-vinylglycine, hydrochloride salt as a yellow solid [δ_H (500MHz, D_2O) 4.50 (1H, s, H_α), 5.44 (1H, s, CHD)]. This material was dissolved in H_2O (4ml) and 1,4-dioxane (4ml), and the solution treated with distilled NEt_3 (775µl, 5.6mmol; 3 equiv.) and BOC-ON[106] (*ex.* Aldrich; 549mg, 2.23mmol; 1.2 equiv.). The reaction was stirred at room temperature overnight and then partitioned between H_2O (75ml) and EtOAc (75ml). The aqueous phase was treated with saturated aqueous NaCl (75ml), acidified to pH 1 (1M HCl) and further extracted with EtOAc (2 × 75ml). The combined organic extracts were dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield the crude title compound as an orange solid (198mg, 52%). δ_H (200MHz, $CDCl_3$) 1.48 (9H, s, Me_3C), 4.67-4.74 and 4.87-4.98 (1H, 2 × m, H_α), 5.33-5.43 (1H, m, CHD), 6.80 (1H, bs, NH), CO_2H signal too broad to be observed.

_N_-*tert*-Butoxycarbonyl-*E*-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester (121)

$C_{22}H_{23}D_2NO_4$; MW 369.4

Crude, solid _N_-*tert*-butoxycarbonyl-*E*-[2,3-$^2H_2$]-DL-vinylglycine (198mg, 0.97mmol), under argon, was treated with a solution of diphenyldiazomethane[138] (250mg, 1.28mmol; 1.3 equiv.) in EtOAc (4ml), and the resulting solution stirred at room temperature under argon overnight. Solvent was evaporated *in vacuo* to yield an orange oil (520mg) which was purified by column chromatography (SiO_2; CH_2Cl_2) to yield the title compound (**121**) as a pale yellow solid (173mg, 48%). Tlc (SiO_2; CH_2Cl_2) R_f 0.3; δ_H (500MHz, $CDCl_3$) 1.47 (9H, s, Me_3C), 5.03-5.06 (1H, s, H_α), 5.31 (1H, d, *J* 8Hz, NH), 5.35 (1H, t_D, J_{HD} 2Hz, CHD), 6.93 (1H, s, C*H*Ph_2), 7.33-7.35 (10H, m, 2 × Ph); δ_C (50.7MHz, $CDCl_3$) 28.18 (3 × Me), 55.81 (C_α), 78.22 (*C*HPh_2), 80.11 (Me_3C), 116.98-117.86 (m, CHD), 127.13-128.72 (Aromatic

CH's), 131-70-132.71 (m, *C*D=CHD), 139.66 (Quaternary aromatic C's), 155.21 (OCON), 170.10 (*C*O_2C); δ_D (38.4MHz, $CHCl_3$) 5.25-5.44 (1D, m, CHD), 6.00 (1D, s, C*D*=CDH); *m/z* [DCI(NH_3)] 387 (33%, MNH_4^+), 370 (27%, MH^+), 369 (4%, d_1-MH^+), 352 (30%), 167 (100%, $^+CHPh_2$); ν_{max} (KBr disk) 3333m (NH), 3982m (CH), 2355w (CD), 1737s (CO), 1702s (CO), 1518s (CO), 1367s, 1203s, 1161s, and 697s cm^{-1}

A portion of the title compound was recrystallised from Et_2O/30-40℃ petrol to yield white crystals: m.p. 89.5-90.5℃ (*ex.* Et_2O/30-40℃ petrol); Calc. for $C_{22}H_{23}D_2NO_4$: C, 71.53; H, 6.82; N, 3.79%: Found C, 71.69; H, 7.00; N, 3.64%.

_E_-[2,3-$^2H_2$]-DL-Vinylglycine, benzhydryl ester, toluene-4-sulphonate salt

$C_{17}H_{15}D_2NO_2.C_7H_8O_3S$; MW 441.5

A solution of *N-tert*-butoxycarbonyl-*E*-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester (**121**) (211mg, 0.57mmol) in Et_2O (15ml) and EtOH (15ml) was treated with toluene-4-sulphonic acid (*ex.* Aldrich, recrystallised; 98mg, 0.57mmol), and upon dissolution, solvent was removed *in vacuo*. The reaction mixture was then heated (~50℃) *in vacuo* (~20mmHg) for 1h to yield the title compound as a white solid. Analysis by tlc (SiO_2; CH_2Cl_2) indicated the absence of unreacted starting material. δ_H (200MHz, $CDCl_3$) 2.23 (3H, s, Me), 4.54-4.62 (1H, s, H_α), 5.27 (1H, s, CHD), 6.78 (1H, s, C*H*Ph_2), 6.81-6.90 (2H, m, 2H of C_6H_4), 7.18-7.29 (10H, m, 2 × Ph), 7.52-7.58 (2H, m, 2H of C_6H_4), 8.40 (3H, bs, NH_3^+).

_N_-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-_S_-benzhydryl-L-cysteinyl-_E_-[2,3-$^2H_2$]-D-vinylglycine, benzhydryl ester (124)

$C_{56}H_{55}D_2N_3O_{10}S$; MW 966.1

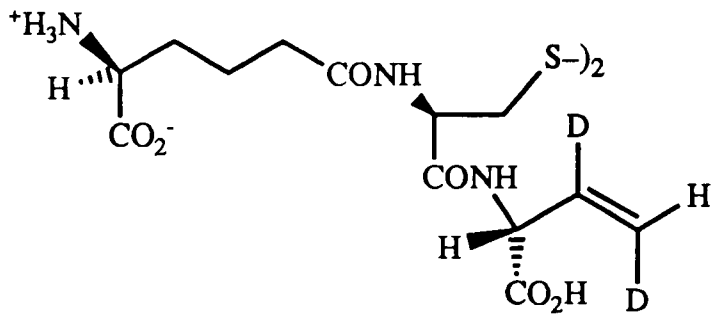
A solution of *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteine (490mg, 0.69mmol; 1.2 equiv.) in CH_2Cl_2 (2ml), containing a little Na_2SO_4 (~20mg), under argon was treated sequentially with EEDQ[108] (*ex*. Aldrich, recrystallised; 170mg, 0.69mmol; 1.2 equiv.), *E*-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester, toluene-4-sulphonate salt (252mg, 0.57mmol) and distilled NEt_3 (80μl, 0.57mmol), and the reaction stirred at room temperature under argon overnight. Solvent was evaporated *in vacuo* and the residue partitioned between saturated aqueous $NaHCO_3$ (30ml) and EtOAc (30ml). The organic phase was washed with HCl (1M; 30ml) and saturated aqueous NaCl (30ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield a pale yellow foam (625mg). Purification by column chromatography (SiO_2; gradient elution 30-40℃ petrol:EtOAc [6:4 - 1:1]) yielded the title compound (**124**) as a white foam (96mg, 17%), as well as affording the LLL-diastereomer [77mg, 14%; tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.4] and a mixed fraction (64mg, 12%). Data for the title compound (**124**): Tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.5; δ_H (500MHz, $CDCl_3$) 1.60-1.84 (4H, 2 × m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.05-2.21 (2H, m, CH_2CO), 2.68-2.73 and 2.77-2.81 (2H, 2 × m, CH_2S), 3.78 (6H, s, 2 × MeO), 4.32-4.38 (1H, m, H_α), 4.56 (1H, dd, *J* 6.5, 14Hz, vinylglycine H_α), 4.96-5.09 (6H, m, 2 × CH_2Ar, SCH and H_α), 5.17 (1H, d, *J* 7.5Hz, NH), 5.28 (1H, bs, CHD), 5.44 (1H, d, *J* 8Hz, NH), 6.28 (1H, d, *J* 8Hz, NH), 6.83-6.90 (5H, m, 4 × aromatic CH and OC*H*Ph$_2$), 7.23-7.42 (24H, m, aromatic CH); *m/z* [FAB(DTT/DTE/NaOAc)(MCA)] 1005 (11%, MK^+), 988 (100%, MNa^+).

Bis-[δ-(L-α-Aminoadipoyl)-L-cysteinyl-*E*-[2,3-$^2H_2$]-D-vinylglycine] (disulphide of 111)

$C_{26}H_{36}D_4N_6O_{12}S_2$; MW 696.7

A solution of *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteinyl-*E*-[2,3-$^2H_2$]-D-vinylglycine, benzhydryl ester (**124**) (171mg, 0.18mmol) in TFA (*ex.* Lancaster Synthesis; 2ml) and anisole (*ex.* Aldrich, distilled; 400μl) was heated at reflux for 30min.[109] TFA was removed *in vacuo*, final traces being removed by azeotroping with toluene (3 x 2ml). The residual suspension in anisole was partitioned between between H_2O (25ml) and EtOAc (25ml). The organic phase was extracted with H_2O (25ml), and the pH of the combined aqueous extracts adjusted to 9-10 (aqueous NH_3; 2M) and treated with oxygen gas overnight. The solution was freeze dried to yield a yellow solid (17mg). Purification by reverse phase HPLC (Hypersil® ODS; mobile phase 20mM NH_4HCO_3; 250 x 9.4mm internal diameter; 4ml.min^{-1}) yielded the title compound (disulphide of **111**) as a white solid (retention time 10min). δ_H (500MHz, D_2O; referenced to DSS) (Number of H's for each resonance quoted for one half of disulphide only, i.e. for R of RS–SR) 1.67-1.78 and 1.80-1.91 (4H, 2 x m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.41 (2H, t, *J* 7Hz, CH_2CO), 2.99 and 3.24 (2H, AB part of ABX system, *J* 5, 9 and 14Hz, CH_2S), 3.73 (1H, t, *J* 6Hz, H_α), 5.21-5.23 (1H, m, *E*-CHD and *Z*-CHD). 2 x H_α resonances obscured by the residual HOD resonance; *m/z* (Electrospray) 719 (17%, MNa^+), 697 (100%, MH^+), 679 (44%).

6.3.5 Synthesis of bis-[L-α-aminoadipoyl)-L-cysteinyl-*Z*-[2,3-$^2H_2$]-D-vinyl-glycine] (disulphide of 112)



Z-Diethylacetamido-[1,2-$^2H_2$-vinyl]-malonate (118)[103]

$C_{11}H_{15}D_2NO_5$; MW 245.2

Aluminium foil (6.3g) was washed with 30-40°C petrol and cut into small (2-4cm^2) pieces. The pieces were placed in a 0.5% (w/v) aqueous solution of $HgCl_2$ (100ml) for 2min. The amalgam thus formed was collected by filtration under suction and added rapidly to a solution of *E*-diethylacetamido-[2-(phenylsulphonyl)-[1,2-$^2H_2$]-vinyl]-malonate (**116**) (2.85g, 7.39mmol) in 1,4-dioxane (250ml) at 0°C under argon. The temperature of the reaction was allowed to rise to room temperature, and the mixture stirred at room temperature overnight. The mixture was filtered under suction through celite, the residue washed on celite with 1,4-dioxane (~300ml) and EtOAc (~300ml), and the combined filtrate and washings evaporated *in vacuo* to yield a pale yellow oil (2.0g). Purification by column chromatography (SiO_2; propan-2-ol:30-40°C petrol [15:85]) yielded the title compound (**118**) as a colourless oil (1.20g, 66%). Further elution of the column with propan-2-ol:30-40°C petrol [1:3] afforded some unreacted starting material (**116**) (943mg, 33%). The yield based on recovered starting material was 99%. Analysis by 500MHz NMR spectroscopy showed the product to be a mixture of the *Z* and *E* isotopomers, predominating in the title isotopomer (85%). Tlc (SiO_2; propan-2-ol:30-40°C petrol [15:85]) R_f 0.2; δ_H (500MHz, $CDCl_3$) 1.28 (6H, t, *J* 7Hz, 2

× CH_3CH_2), 2.09 (3H, s, MeCO), 4.27 (4H, q, *J* 7Hz, 2 × CH_3CH_2), 5.25 (0.15H, t_D, J_{HD} 2.5Hz, *E*-RC*D*=C*H*D), 5.29 (0.85H, s, *Z*-RC*D*=C*H*D), 6.93 (1H, bs, NH); δ_C (50.7MHz, $CDCl_3$) 13.61 (2 × CH_3CH_2), 22.42 (*Me*CO), 62.64 (2 × CH_3CH_2), 66.96 ($C(CO_2Et)_2$), 115.90 (t_D, J_{CD} 24Hz, CHD), 131.83 (t_D, J_{CD} 26Hz, *C*D=CHD), 167.11 and 169.96 (2 × CO_2Et and CONH); δ_D (38.4MHz, $CHCl_3$) 5.23 (1D, s, CHD), 6.46 (1D, s, C*D*=CDH); *m/z* [CI(NH_3)] 246 (100%, MH^+), 245 (12%, d_1-MH^+), 172 (15%), and 130 (14%).

Z-[2,3-$^2H_2$]-DL-Vinylglycine (114)[103]

$C_4H_5D_2NO_2$; MW 103.1

A suspension of *Z*-diethylacetamido-[1,2-$^2H_2$-vinyl]-malonate (**118**) (182mg, 0.74mmol) in HCl (6M; 1ml) and MeOH (1ml) was heated at reflux for 4h, and solvent then evaporated *in vacuo* to yield a yellow solid. Purification by ion exchange chromatography (neutral Dowex®50 400 mesh 1 × 8; eluting with 1% (v/v) aqueous pyridine) yielded the title compound (**114**) as a white solid (11mg, 14%). δ_H (300MHz, D_2O) 4.10 (1H, s, H_α), 5.29 (1H, t_D, J_{HD} 1.5Hz, C*D*=C*H*D); *m/z* [DCI(NH_3)] 104 (100%, MH^+), 103 (13%, d_1-MH^+), 58 (54%); ν_{max} (KBr disk) 2950bs (NH, CH), 2230m (CD), 1650s (CO), 1590s (CO), 1390s, and 1350m cm^{-1}.

***N-tert*-Butoxycarbonyl-*Z*-[2,3-$^2H_2$]-DL-vinylglycine**

$C_9H_{13}D_2NO_4$; MW 203.2

A suspension of *Z*-diethylacetamido-[1,2-$^2H_2$-vinyl]-malonate (**118**) (479mg, 1.95mmol) in HCl (6M; 5ml) was heated under reflux for 5h, and solvent then evaporated *in vacuo* to yield the intermediate *E*-[2,3-$^2H_2$]-DL-vinylglycine, hydrochloride salt as a yellow solid [δ_H (300MHz, D_2O) 4.10

(1H, s, H_α), 5.29 (1H, d, J_{HD} 1.5Hz, C*D*=C*H*D)]. This material was then dissolved in H_2O (4ml) and 1,4-dioxane (4ml), and the solution treated with distilled NEt_3 (820µl, 5.9mmol; 3 equiv.) and BOC-ON[106] (*ex.* Aldrich; 576mg, 2.34mmol; 1.2 equiv.). The reaction was stirred at room temperature overnight and then partitioned between H_2O (75ml) and EtOAc (75ml). The aqueous phase was treated with saturated aqueous NaCl (75ml), acidified to pH 1 (1M HCl) and extracted with EtOAc (2 x 75ml). The combined organic extracts were dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield the crude title compound as a yellow oil (232mg, 58%). δ_H (200MHz, $CDCl_3$) 1.45 (9H, s, Me_3C), 4.65-4.71 and 4.86-4.93 (1H, 2 x m, H_α), 5.26 (1H, s, CHD), 7.01-7.08 (1H, m, NH), 10.46 (1H, bs, CO_2H).

_N_-*tert*-Butoxycarbonyl-*Z*-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester (122)

$C_{22}H_{23}D_2NO_4$; MW 369.4

A solution of *N*-*tert*-butoxycarbonyl-*Z*-[2,3-$^2H_2$]-DL-vinylglycine (75mg, 0.37mmol) in EtOAc (2ml), under argon, was treated with diphenyldiazomethane[138] (86mg, 0.44mmol; 1.2 equiv.), and the resulting mixture stirred at room temperature under argon overnight. Solvent was evaporated *in vacuo* to yield an orange oil (151mg), which was purified by column chromatography (SiO_2; CH_2Cl_2) to yield the title compound (**122**) as a pale yellow solid (85mg, 62%). Tlc (SiO_2; CH_2Cl_2) R_f 0.3; δ_H (500MHz, $CDCl_3$) 1.47 (9H, s, Me_3C), 5.02-5.06 (1H, m, H_α), 5.27-5.30 (2H, m, CHD and NH), 6.94 (1H, s, C*H*Ph_2), 7.34-7.37 (10H, m, 2 x Ph); δ_C (50.7MHz, $CDCl_3$) 28.18 (3 x Me), 55.81 (C_α), 78.22 (*C*HPh_2), 80.11 (Me_3C), 116.98-117.86 (m, CHD), 127.13-128.72 (Aromatic CH's), 131-70-132.71 (m, *C*D=CHD), 139.66 (Quaternary aromatic C's), 155.21 (OCON), 170.10 (*C*O_2C); δ_D (38.4MHz, $CDCl_3$) 5.41 (1D, s, CHD), 5.99 (1D, s, C*D*=CDH); ν_{max} (KBr disk) 3333m (NH), 3982m (CH), 2355w (CD), 1737s (CO), 1702s (CO), 1518s (CO),

1367s, 1203s, 1161s, and 697s cm^{-1}; *m/z* [DCI(NH_3)] 387 (33%, MNH_4^+), 370 (27%, MH^+), 369 (4%, d_1-MH^+), 352 (30%), 167 (100%, $^+CHPh_2$).

A portion of the title compound was recrystallised from Et_2O/30-40℃ petrol to yield white crystals: m.p. 90.0-91.0℃ (*ex.* Et_2O/30-40℃ petrol); Calc. for $C_{22}H_{23}D_2NO_4$: C, 71.53; H, 6.82; N, 3.79%: Found C, 71.74; H, 6.99; N, 3.83%.

Z-[2,3-$^2H_2$]-DL-Vinylglycine, benzhydryl ester, toluene-4-sulphonate salt

$C_{17}H_{15}D_2NO_2.C_7H_8O_3S$; MW 441.5

A solution of *N-tert*-butoxycarbonyl-Z-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester (**122**) (89mg, 0.24mmol) in Et_2O (7ml) and EtOH (7ml) was treated with toluene-4-sulphonic acid (*ex.* Aldrich, recrystallised; 42mg, 0.24mmol), and upon dissolution, the solvent was removed *in vacuo*. The reaction mixture was heated (~50℃) *in vacuo* (~10mmHg) for 1h to yield the title compound as a white solid. Analysis by ^1H NMR spectroscopy indicated no unreacted starting material. δ_H (200MHz, $CDCl_3$) 2.25 (3H, s, Me), 4.55-4.63 (1H, s, H_α), 5.13 (0.85H, s, *Z*-CHD), 5.30 (0.15H, s, *E*-CHD), 6.78 (1H, s, C*H*Ph_2), 6.88-6.93 (2H, m, 2H of C_6H_4), 7.20-7.25 (10H, m, 2 x Ph), 7.54-7.58 (2H, m, 2H of C_6H_4), 8.41 (3H, bs, NH_3^+).

***N*-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteinyl-Z-[2,3-$^2H_2$]-D-vinylglycine, benzhydryl ester (125)**

$C_{56}H_{55}D_2N_3O_{10}S$; MW 966.1

A solution of *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteine (173mg, 0.24mmol) in CH_2Cl_2 (2ml), containing a little Na_2SO_4 (~20mg), under argon was treated sequentially with EEDQ[108] (*ex.* Aldrich, recrystallised; 60mg, 0.24mmol), *Z*-[2,3-$^2H_2$]-DL-vinylglycine, benzhydryl ester, toluene-4-sulphonate salt (107mg,

0.24mmol) and distilled NEt_3 (34µl, 0.24mmol), and the reaction stirred at room temperature under argon overnight. Solvent was evaporated *in vacuo* and the residue partitioned between saturated aqueous $NaHCO_3$ (15ml) and EtOAc (15ml). The organic phase was washed with HCl (1M; 15ml) and saturated aqueous NaCl (15ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield a pale yellow oil (278mg). Purification by column chromatography (SiO_2; gradient elution 30-40°C petrol:EtOAc [6:4 - 1:1]) yielded the title compound (**125**) as a white solid (120mg, 51%). Tlc (SiO_2; CH_2Cl_2:EtOAc [4:1]) R_f 0.5; δ_H (500MHz, $CDCl_3$) 1.58-1.86 (4H, m, $CH_2CH_2CH_2CO$), 2.05-2.20 (2H, m, CH_2CO), 2.68-2.73 and 2.78-2.82 (2H, 2 × m, CH_2S), 3.77-3.79 (6H, m, 2 × MeO), 4.32-4.37 (1H, m, H_α), 4.54-5.58 (1H, m, H_α), 4.97-5.09 (6H, m, 2 × CH_2Ar, SCH and H_α), 5.17 (1H, d, *J* 7.5Hz, NH), 5.23 (1H, bs, CHD), 5.43 (1H, d, *J* 8Hz, NH), 6.25 (1H, d, *J* 8Hz, NH), 6.80-6.86 (5H, m, 4 × aromatic CH and $OCHPh_2$), 7.20-7.42 (24H, m, aromatic CH); *m/z* [FAB(DTT/DTE/NaOAc) (MCA)] 1005 (30%, MK^+), 988 (100%, MNa^+).

Bis-[δ-(L-α-Aminoadipoyl)-L-cysteinyl-Z-[2,3-$^2H_2$]-D-vinylglycine] (disulphide of 112)

$C_{26}H_{36}D_4N_6O_{12}S_2$; MW 696.7

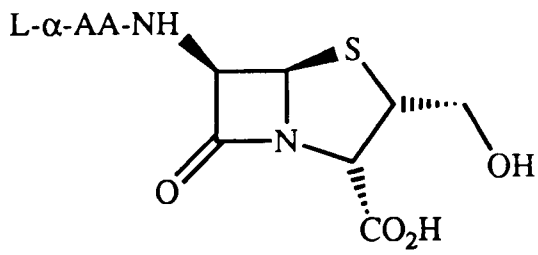
A solution of *N*-(4-methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteinyl-*Z*-[2,3-$^2H_2$]-D-vinylglycine, benzhydryl ester (**125**) (120mg, 0.12mmol) in TFA (*ex.* Lancaster Synthesis; 2ml) and anisole (*ex.* Aldrich, distilled; 400µl) was heated at reflux for 30min.[109] TFA was removed *in vacuo*, final traces being removed by azeotroping with toluene (3 × 2ml). The residual suspension in anisole was

partitioned between between H_2O (25ml) and EtOAc (25ml). The organic phase was extracted with H_2O (25ml), and the pH of the combined aqueous extracts adjusted to 9-10 (10% saturated aqueous NH_3 solution) and treated with oxygen gas overnight. The solution was freeze dried to yield a yellow solid (17mg). Purification by reverse phase HPLC (Hypersil® ODS; mobile phase 20m<u>M</u> NH_4HCO_3; 250 x 9.4mm internal diameter; 4ml.min^{-1}) yielded the title compound (disulphide of **112**) as a white solid (retention time 10min). δ_H (500MHz, D_2O; referenced to DSS) (Number of H's for each resonance quoted for one half of disulphide only, i.e. for R of RS–SR) 1.64-1.76 and 1.81-1.93 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.41 (2H, t, *J* 7Hz, CH_2CO), 2.96-3.04 and 3.18-3.26 (2H, 2 x m, CH_2S), 3.72 (1H, t, *J* 6Hz, H_α), 5.20-5.23 (1H, m, *Z*-CHD and *E*-CHD). 2 x H_α resonances obscured by the residual HOD resonance; *m/z* (Electrospray) 719 (9%, MNa^+), 697 (100%, MH^+).

6.3.6 Incubation of Tripeptides and Product Purification

Unlabelled 3α-Hydroxymethylpenicillin† (107)

$C_{13}H_{19}N_3O_7S$; MW 361.4



(107)

† The IUPAC name for this compound is [2*S*,3*S*,5*R*,6*R*]-6-[5-amino-5-carboxypentanamido]-3-hydroxymethyl-7-oxo-1-aza-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid.

δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-vinylglycine, disulphide (disulphide of **106**) (2mg) was incubated with IPNS solution (batch CF33 F7-9, 11.0mg.ml^{-1}, 0.5 IU.mg^{-1}; 1ml diluted to 2ml) according to the general incubation procedure. The crude incubation mixture was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 0.025% (v/v) [6.6m<u>M</u>] HCO_2H; 0.5ml.min^{-1}),† with all eluted fractions being treated with NH_3 solution (66m<u>M</u>, 10% by volume) prior to freeze drying, to yield the title compound (**107**). δ_H (500MHz, D_2O; referenced to DSS) 1.68-1.76 and 1.85-1.92 (4H, 2 × m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.41 (2H, t, *J* 7.5Hz, CH_2CO), 3.62 (1H, half of AB part of ABX system, *J* 9, 11.5Hz, 1H of C*H*$_2$OH), 3.73 (1H, t, *J* 6.5Hz, H_α), 3.94 (1H, half of AB part of ABX system, *J* 4.5, 11.5Hz, 1H of C*H*$_2$OH), 4.23 (1H, ddd, *J* 4.5, 7.5, 9Hz, C*H*CH$_2$OH), 5.46 and 5.50 (2H, ABq, *J* 4Hz, CHCHS). A COSY spectrum showed the H(2) resonance to be under the residual HOD resonance, which could be revealed by shifting the HOD resonance using CD_3CN. δ_H (500MHz, D_2O:CD_3CN [1:4]; referenced to DSS) 4.71 (1H, d, *J* 7Hz, H-2); *m/z* (Electrospray) 428 (7%, [M-2H+2Na]Na$^+$), 406 (13%, [M-H+Na]Na$^+$), 384 (24%, MNa$^+$), 362 (100%, MH$^+$), 344 (7%, LactoneH$^+$ [(**110**)H$^+$]), 269 (13%).

Second β-Lactam Product

A large scale incubation of δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-vinylglycine, disulphide (disulphide of **106**) (18mg) with IPNS solution (batch CF31 F5-7; 10.7mg.ml^{-1}, 2.07 IU.ml^{-1}, 0.2 IU.mg^{-1}; 10ml) according to the general incubation procedure (on five times the scale. N.B. 5 × PD10 columns) was carried out. Analysis of the ^{1}H NMR spectrum of the crude

† Using this weak buffer system (necessary to slow down the retention of the product) the major product peak is the disodium salt (see *m/z* data), and its retention time varies with the sample volume injected between *ca.* 10 and 16min (typical injection volume was 100μl, giving a retention time of 13.5min). The diacid is observed as a much smaller peak with a constant retention time of 17min.

incubation mixture indicated the presence of a second β-lactam product (ratio varies from one incubation to another, from <1:10 to 1:4 to the 3α-hydroxymethylpenicillin). This material (retention time 10min) was partially purified by reverse phase HPLC (Hypersil® ODS; mobile phase 0.025% (v/v) [6.6m<u>M</u>] HCO_2H; 0.5ml.min^{-1}), but was lost on further attempts at purification. δ_H (500MHz, D_2O) (sample too weak to allow integrals to be determined) 1.69-1.79 and 1.84-1.97 (2 × m, $CH_2CH_2CH_2CO$), 2.33-2.48 (m, CH_2CO), other peaks at 4.17-4.21 (m), 4.26-4.31 (m), 4.35-4.43 (m) and 5.41-5.43 and 5.46-5.47 (2 × m, β-lactam resonances). The region from 3.5-4.1ppm was obscured by impurities, and there is another resonance which may arise from the product at 4.58-4.62ppm (m).

Incubation of δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-vinylglycine (106) Under an $^{18}O_2$ Atmosphere

$C_{13}H_{19}N_3{}^{16}O_7{}^{18}OS$; MW 363.4

The head space of a glass vial of $^{18}O_2$ (*ex.* MSD Isotopes; 97 atom % ^{18}O; 100ml) was repeatedly evacuated and flushed with argon and then sealed. Cofactor (solutions as for standard incubation procedure), δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine, disulphide (disulphide of **106**) (4.2mg; in 1ml 25m<u>M</u> NH_4HCO_3 buffer), and IPNS solutions (batch CF33 F7-9; 0.5 IU.mg^{-1}, 11mg.ml^{-1}; 2ml) were all degassed and flushed with argon prior to use.

After breaking the neck seal of the septum sealed $^{18}O_2$ vial, the δ-(L-α-aminoadipoyl)-L-cysteinyl-D-vinylglycine solution was treated with degassed cofactors in the same manner as for the general incubation procedure, the mixture degassed and then introduced via syringe into the $^{18}O_2$ vial. Degassed

IPNS solution was then added in the same manner, and the sealed vial incubated at 27°C, 250rpm for 40min.

The reaction was quenched by addition of acetone via syringe to a final concentration of ~70% (v/v), the vial opened to the air and the incubation worked up according to the general incubation procedure.

The crude incubation mixture was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 0.025% (v/v) [6.6m<u>M</u>] HCO_2H; 0.5ml.min^{-1}),† with all eluted fractions being treated with NH_3 solution (66m<u>M</u>, 10% by volume) prior to freeze drying, to yield the title compound. Analysis by ^1H NMR gave an identical spectrum to the [$^{16}O_7$]-penicillin, whilst mass spectrometric analysis showed high (≥90%) incorporation of a single ^{18}O label, with no [$^{16}O_7$]-MH^+ peak being detectable above background noise (10% full scale). *m/z* (Electrospray) 408 (16%, [M-H+Na]Na^+), 386 (28%, MNa^+), 364 (100%, MH^+), 362 (<10%, [$^{16}O_7$]-MH^+).

Deuterium Labelled 3α-Hydroxymethylpenicillins (128 and 129)

$C_{13}H_{17}D_2N_3O_7S$; MW 363.4

i) From δ-(L-α-Aminoadipoyl)-L-cysteinyl-*E*-[3,4-$^2H_2$]D-vinylglycine (**111**): The *E*-d_2-tripeptide disulphide (3mg) was incubated with highly purified IPNS solution (*ex.* Y. Fujishima; 17.0mg.ml^{-1}, 0.5 IU.mg^{-1}; 1ml diluted to 2ml) according to the general incubation procedure. The crude incubation mixture was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 0.025% (v/v) [6.6m<u>M</u>] HCO_2H; 0.5ml.min^{-1}), with the eluted penicillin containing solution being treated with NH_3 solution (66m<u>M</u>, 10% by volume) prior to freeze

† Using this weak buffer system (necessary to slow down the retention of the product) the major product peak is the disodium salt (see *m/z* data), and its retention time varies with the sample volume injected between *ca.* 10 and 16min (typical injection volume was 100μl, giving a retention time of 13.5min). The diacid is observed as a much smaller peak with a constant retention time of 17min.

drying, to yield the first of the title compounds (**128**). δ_H (500MHz, D_2O; referenced to DSS) 1.67-1.79 and 1.82-1.95 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.40 (2H, t, *J* 7Hz, CH_2CO), 3.59 (0.85H, s, C*H*DOH), 3.71-3.73 (1H, m, H_α), 3.91 (0.15H, s, C*H*DOH), 5.45 and 5.49 (2H, ABq, *J* 4Hz, CHCHS). H(2) resonance obscured by the residual HOD resonance; *m/z* (Electrospray) 386 (14%, MNa^+), 364 (100%, MH^+).

ii) From δ-(L-α-Aminoadipoyl)-L-cysteinyl-*Z*-[3,4-$^2H_2$]D-vinylglycine (**112**): The *Z*-d_2-tripeptide disulphide (3mg) was incubated with highly purified IPNS solution (*ex.* Y. Fujishima; 17.0mg.ml^{-1}, 0.5 IU.mg^{-1}; 1ml diluted to 2ml) according to the general incubation procedure. The crude incubation mixture was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 0.025% (v/v) [6.6m<u>M</u>] HCO_2H; 0.5ml.min^{-1}), with the eluted penicillin containing solution being treated with NH_3 solution (66m<u>M</u>, 10% by volume) prior to freeze drying, to yield the second of the title compounds (**129**). δ_H (500MHz, D_2O; referenced to DSS) 1.70-1.92 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, t, *J* 7Hz, CH_2CO), 3.60 (0.15H, s, C*H*DOH), 3.73-3.75 (1H, m, H_α), 3.93 (0.85H, s, C*H*DOH), 5.46 and 5.50 (2H, ABq, *J* 4Hz, CHCHS). H(2) resonance obscured by the residual HOD resonance; *m/z* (Electrospray) 386 (17%, MNa^+), 381 (6%, MNH_4^+), 364 (100%, MH^+).

Close examination of the ^1H NMR spectrum indicated the presence of a second ABq in the β-lactam region of the spectrum [δ_H (500MHz, D_2O; referenced to DSS) 5.32 and 5.37 (0.15H *cf* major penicillin, ABq, *J* 8Hz)], which were not detectable in the ^1H NMR spectrum of the crude incubation mixture.

6.3.7 Derivatisation Studies on Model Compounds

Isopenicillin N (2)

$C_{14}H_{21}N_3O_6S$; MW 359.4

δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine, disulphide (disulphide of **1**) (*ex*. Eli Lilly and Co.; 21mg, 29μmol) was incubated according to the general incubation procedure (on double the scale) with IPNS solution (batch CF34 F4-8; 12mg.ml^{-1}, 30.6 IU.ml^{-1}, 2.7 IU.mg^{-1}; 4ml). The crude incubation mixture was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 20m<u>M</u> NH_4HCO_3, 4ml.min^{-1}) to yield the title compound (**2**) as a white solid (retention time 9min on 250 x 9.4mm internal diameter column). Analysis by ^{1}H NMR spectroscopy in D_2O containing a known quantity of DSS, gave the total yield as 10μmol (18%) (i.e the solid contained 39% (w/w) of product based on the free diacid). δ_H (500MHz, D_2O) 1.52 and 1.63 (6H, 2 x s, 2 x Me), 1.65-1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.40 (2H, t, *J* 7Hz, CH_2CO), 3.72-3.74 (1H, m, H_α), 4.23 (1H, s, $CHCMe_2$), 5.47 and 5.57 (2H, ABq, *J* 4Hz, CHCHS).

***N*-Ethoxycarbonyl-isopenicillin N, disodium salt (136)**[112]

$C_{17}H_{23}N_3Na_2O_8S$; MW 475.4

The pH of a solution of isopenicillin N (**2**) (39% (w/w) with inorganic salts; 1.2mg, 1.3μmol) in H_2O (1ml) was adjusted to 9 with $NaHCO_3$ (1<u>M</u>; 20μl), and the solution then treated with diethylpyrocarbonate (*ex*. Aldrich; 2μl, 14μmol). The reaction was stirred at room temperature for 6min, then washed with distilled Et_2O (2 x 2ml) and freeze dried to yield the title compound (**136**) as a white solid. Analysis by ^{1}H NMR spectroscopy in D_2O containing a known quantity of DSS, gave the approximate yield as 0.9-

1.3μmol (75-100%). δ_H (500MHz, D_2O) 1.17 (3H, t, *J* 7Hz, CH_3CH_2), 1.51 and 1.63 (6H, 2 x s, $SCMe_2$), 1.62-1.80 (4H, m, $CH_2CH_2CH_2CO$), 2.32-2.38 (2H, m, CH_2CO), 3.91-3.96 (1H, m, H_α), 4.09 (2H, q, *J* 7Hz, CH_3CH_2), 4.23 (1H, s, $CHCMe_2$), 5.43 and 5.55 (2H, ABq, *J* 4Hz, CHCHS).

N-Ethoxycarbonyl-deacetoxycephalosporin C, disodium salt (134)

$C_{17}H_{21}N_3Na_2O_8S$; MW 473.4

A solution of deacetoxycephalosporin C, dipotassium salt (**131**) [*ex.* Eli Lilly and Co.; 800μg, 1.8μmol; δ_H (500MHz, D_2O) 1.64-1.96 (4H, m, $CH_2CH_2CH_2CO$), 1.90 (3H, s, Me), 2.40 (2H, t, *J* 7Hz, CH_2CO), 3.22 and 3.58 (2H, ABq, *J* 18Hz, SCH_2), 3.72 (1H, t, *J* 6.5Hz, H_α), 5.07 and 5.55 (2H, ABq, *J* 4.5Hz, CHCHS)] in H_2O (1ml) was prepared by dilution of a standard solution (1.0mg.ml^{-1}; 800μl). The pH of this solution was adjusted to 9 with aqueous $NaHCO_3$ (1M; 10μl), and the solution then treated with diethylpyrocarbonate (*ex.* Aldrich; 2μl, 14μmol), and stirred at room temperature for 6min.[112] The reaction was washed with distilled Et_2O (2 x 2ml) and freeze dried to yield the title compound (**134**) as a white powder. δ_H (500MHz, D_2O) 1.21 (3H, t, *J* 7Hz, CH_3CH_2), 1.61-1.85 (4H, m, $CH_2CH_2CH_2CO$), 1.90 (3H, s, C=CMe), 2.30-2.39 (2H, m, CH_2CO), 3.21 and 3.57 (2H, ABq, *J* 18Hz, SCH_2), 3.90-3.92 (1H, m, H_α), 4.07 (2H, q, *J* 7Hz, CH_3CH_2), 5.06 and 5.53 (2H, ABq, *J* 4Hz, CHCHS); *m/z* [FAB(MCA)] 474 (66%, MH^+), 461 (54%), and 452 (100%, $[M-Na+H]H^+$).

N-Ethoxycarbonyl-deacetoxycephalosporin C

$C_{17}H_{23}N_3O_8S$; MW 429.5

A solution of deacetoxycephalosporin C, dipotassium salt (*ex.* Eli Lilly and Co.; 800μg, 1.8μmol) in H_2O (1ml) was prepared by dilution of a standard

solution (1.0mg.ml^{-1}; 800µl). The pH of this solution was adjusted to 9 with aqueous $NaHCO_3$ (1M, 20µl), and the solution treated with diethylpyrocarbonate (*ex.* Aldrich; 2µl, 14µmol), stirred at room temperature for 6min, and then washed with distilled Et_2O (2 x 2ml). Acidification to pH 2-3 or to pH 1 (1M HCl), extraction with EtOAc (3 x 2ml), drying (Na_2SO_4) and evaporation *in vacuo* of the combined extracts failed to yield any of the title compound. The crude title compound could however be obtained if, after washing with Et_2O, the solution was freeze dried to yield the disodium salt of the title compound as a white powder, followed by suspension of this material in CH_3CN (5ml), treatment with HCl (1M, 4 drops, ~100µl), drying (Na_2SO_4), filtration and evaporation *in vacuo* to yield the title compound as a white solid. Analysis by ^1H NMR spectroscopy in D_2O containing a known quantity of DSS, gave the approximate yield as 0.9-1.3µmol (47-70%). δ_H (500MHz, CD_3CN) 1.19-1.23 (3H, m, CH_3CH_2), 1.63-1.85 (4H, m, $CH_2CH_2CH_2CO$), 2.08 (3H, s, C=CMe), 3.29 and 3.56 (2H, ABq, *J* 18Hz, SCH_2), 4.04-4.13 (3H, m, CH_3CH_2 and H_α), 5.00 (1H, d, *J* 4.5Hz, CHS), 5.66 (1H, dd, *J* 4.5 and 8.5Hz, NHCHCH), 5.80 (1H, d, *J* 8Hz, NH), 7.09 (1H, d, *J* 8.5Hz, NH), CH_2CO resonance obscured by the residual HOD resonance.

N-Ethoxycarbonyl-deacetoxycephalosporin C, dimethyl ester (133)

$C_{19}H_{27}N_3O_8S$; MW 457.5

i) Large Scale.[112]

A solution of deacetoxycephalosporin C, dipotassium salt (**131**) (*ex.* Eli Lilly and Co.; 5.0mg, 12µmol) in H_2O (2ml) was treated with $NaHCO_3$ (1M; 60µl) and diethylpyrocarbonate (*ex.* Aldrich; 12µl, 84µmol), and the reaction stirred for 6min. The mixture was washed with Et_2O (2 x 2ml), treated with

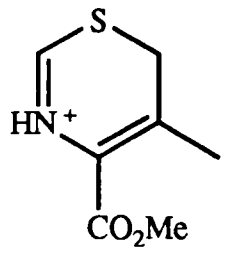
EtOAc (2ml) and acidified to pH 2-3 (1M HCl). The aqueous phase was extracted further with EtOAc (3 x 2ml), and each extract dried by passing through a short column of Na_2SO_4 (pasteur pipette). The combined dried extracts were cooled to 0°C and treated with a freshly prepared solution of CH_2N_2 in Et_2O (~0.3M; ~500µl). The reaction was stirred at 0°C for 10min, and solvent evaporated *in vacuo* to yield the title compound (**133**) as a colourless oil.

ii) Small Scale.

N-Ethoxycarbonyl-deacetoxycephalosporin C (**134**) was prepared from deacetoxycephalosporin C, dipotassium salt (**131**) (*ex.* Eli Lilly and Co.; 800µg, 1.8µmol) as described earlier. Extraction of the solution after washing with Et_2O, as carried out in i), followed by treatment with a freshly prepared solution of CH_2N_2 in Et_2O (~0.3M; ~500µl) failed to yield any β-lactam products. Alternatively the freeze dried material was suspended in CH_3CN (5ml), the suspension treated with HCl (1M; 4 drops, ~100µl), dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was dissolved in CH_3CN (1ml) and EtOAc (1ml) and treated with a freshly prepared solution of CH_2N_2 in Et_2O (~0.3M), dropwise until a visible yellow coloration persisted (~300µl). The reaction was stirred at room temperature for 10min, followed by evaporation of solvent *in vacuo* to yield the title compound (**133**) (quantity too small to be seen by eye).

Data for title compound (**133**): δ_H (500MHz, $CDCl_3$) 1.23-1.26 (3H, m, CH_3CH_2), 1.60-1.90 (4H, m, $CH_2CH_2CH_2CO$), 2.15 (3H, s, C=CMe), 2.26-2.41 (2H, m, CH_2CO), 3.22 and 3.53 (2H, ABq, *J* 19Hz, SCH_2), 3.76 and 3.85 (6H, 2 x s, 2 x CO_2Me), 4.11 (2H, q, *J* 7.5Hz, CH_3CH_2), 4.36-4.41 (1H, m, H_α), 4.98 (1H, d, *J* 4.5Hz, CHS), 5.30 (1H, d, *J* 8Hz, NH), 5.75 (1H, dd, *J* 4.5 and 8.5Hz, NHCHCH), 6.42 (1H, d, *J* 8Hz, NH); *m/z*

[FAB(DTT/DTE)] 458 (8%, MH^+), 287 (26%), 230 (18%), 172 (100%, **156**), 128 (26%), 98 (38%), and 59 (46%).

(156) 

***N*-Ethoxycarbonyl-deacetylcephalosporin C, disodium salt (135)**

$C_{17}H_{21}N_3Na_2O_9S$; MW 489.4

A solution of deacetylcephalosporin C (**132**) [*ex.* Eli Lilly and Co., 18% dipotassium salt, 82% free acid, mean MW 387.1; 22mg, 57μmol; δ_H (500MHz, D_2O) 1.73-1.85 and 1.89-1.99 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.47 (2H, t, *J* 7Hz, CH_2CO), 3.50 and 3.71 (2H, ABq, *J* 18Hz, SCH_2), 3.79 (1H, t, *J* 6Hz, H_α), 4.29 and 4.34 (2H, ABq, *J* 13Hz, CH_2OH), 5.17 and 5.67 (2H, ABq, *J* 4.5Hz, CHCHS)] in H_2O (8ml) was treated with aqueous $NaHCO_3$ (1M; 400μl) and diethylpyrocarbonate (*ex.* Aldrich; 50μl, 340μmol), and the reaction stirred vigorously for 10min,[112] washed with Et_2O (2 x 10ml), and freeze dried to yield the title compound (**135**) as a white solid (59mg, 100% allowing for added salts). No *O*-ethoxycarbonyl material was observable by 500MHz ^1H NMR spectroscopy. δ_H (500MHz, D_2O; referenced to DSS) 1.21 (3H, t, *J* 7.1Hz, CH_3CH_2), 1.65-1.81 (4H, m, $CH_2CH_2CH_2CO$), 2.34-2.38 (2H, m, CH_2CO), 3.43 and 3.64 (2H, ABq, *J* 18Hz, SCH_2), 3.91-3.94 (1H, m, H_α), 4.06-4.11 (2H, m, CH_3CH_2), 4.23 and 4.27 (2H, ABq, *J* 13Hz, CH_2OH), 5.10 and 5.60 (2H, ABq, *J* 4.5Hz, CHCHS).

_N_-Ethoxycarbonyl-deacetylcephalosporin C, lactone (137)

$C_{17}H_{21}N_3O_8S$; MW 427.4

The reaction was carried out on both large [i)] and small [ii)] scales. Samples of *N*-ethoxycarbonyl-deacetylcephalosporin C, disodium salt (**135**) [47% by mass with salts; i) 10.6mg, 10μmol; ii) 1.8mg, 1.7μmol] were each suspended in CH_3CN [i) 25ml; ii) 5ml] and treated with HCl [1M; i) ~400μl; ii) ~75μl]. The solutions were then dried (Na_2SO_4), filtered, and solvent evaporated *in vacuo* to yield, in both cases, the title compound (**137**) as a white solid. Confirmation of the lactone structure came from comparison (by 500MHz ^1H NMR spectroscopy) with *N*-ethoxycarbonyl-α-methyl-deacetylcephalosporin C lactone (**138**). Data for title compound (**137**): δ_H (500MHz, CD_3CN) 1.20-1.24 (3H, m, CH_3CH_2), 1.63-1.70 and 1.79-1.87 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.24-2.32 (2H, m, CH_2CO), 4.04-4.12 (2H, m, CH_3CH_2), 4.50-4.55 (1H, m, H_α), 4.76-4.83 (2H, m, CH_2O), 4.91-4.93 (1H, m, CHS), 5.75-5.93 (2H, bm, NH + CHCHS), 7.05-7.10 (1H, m, NH). The SCH_2 resonance was obscured by impurities.

_N_-Ethoxycarbonyl-α-(methyl ester)-deacetylcephalosporin C, lactone (138)[6ag]

$C_{18}H_{23}N_3O_8S$; MW 441.4

N-Ethoxycarbonyl-deacetylcephalosporin C, disodium salt (**135**) (1.8mg, 47% by mass with salts; 1.7μmol) was treated with CH_3CN (5ml) and HCl (1M; ~80μl). The mixture was shaken vigorously, dried (Na_2SO_4), filtered and solvent evaporated *in vacuo*. The residue was taken up in CH_3CN (1ml) and EtOAc (1ml) and the solution treated dropwise with a freshly prepared solution of CH_2N_2 in Et_2O (~0.3M) until a visible yellow coloration persisted. The reaction was stirred for 10min at room temperature and solvent removed *in vacuo* to yield the crude title compound (**138**) as a colourless oil. δ_H

(500MHz, $CDCl_3$) 1.20-1.26 (3H, m, CH_3CH_2), 1.70-1.79 and 1.87-1.91 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.28-2.42 (2H, m, CH_2CO), 4.10-4.15 (2H, m, CH_3CH_2), 4.37-4.41 (1H, m, H_α), 4.89 and 4.98 (2H, ABq, *J* 17Hz, CH_2OH), 5.05 (1H, d, *J* 5Hz, CHS), 5.30 (1H, d, *J* 9Hz, NH), 5.94 (1H, dd, *J* 5 and 8.5Hz, NHCHCH), 6.41 (1H, d, *J* 9Hz, NH). The SCH_2 resonance was obscured by impurities; the sample decomposed before mass spectrometric analysis could be completed.

Deacetylcephalosporin C, lactone

$C_{14}H_{17}N_3O_6S$; MW 355.4

The reaction was carried out on both large [i)] and small [ii)] scales. Deacetylcephalosporin C (**132**) [*ex.* Eli Lilly and Co.; 18% dipotassium salt, 82% free acid, mean MW 387.1; i) 5mg, 13μmol; ii) 500μg, 1.3μmol] was dissolved in HCO_2H [10μl, 265μmol for both i) and ii)] and immediately freeze dried to yield the title compound as a white solid. δ_H (500MHz, D_2O) 1.65-1.79 and 1.82-1.95 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.42 (2H, t, *J* 7Hz, CH_2CO), 3.71 and 3.88 (2H, ABq, *J* 14.5Hz, SCH_2), 3.72-3.75 (1H, m, H_α), 5.07 and 5.13 (2H, ABq, *J* 20Hz, CH_2O), 5.22 and 5.77 (2H, ABq, *J* 4.5Hz, CHCHS); ν_{max} (KBr disk) 3600-3100bs (OH and NH), 2928m (CH), 1768s (lactone CO), 1632s (CO), 1350m, and 1024m cm^{-1}; *m/z* [FAB(Gly/Oxalic Acid)] 356 (15%, MH^+), 223 (39%), 207 (24%), 131 (100%), 115 (38%) and 102 (36%).

***N*-(9-Fluorenylmethyl)carbonyl-deacetylcephalosporin C (139)**

$C_{29}H_{29}N_3O_9S$; MW 595.6

The pH of a solution of deacetylcephalosporin C (**131**) (*ex.* Eli Lilly and Co.; 18% dipotassium salt, 82% free acid, mean MW 387.1; 2mg, 5.2μmol) in

H_2O (1ml) and 1,4-dioxane (0.5ml) was adjusted to 8 with aqueous $NaHCO_3$ (1M), and the solution was treated dropwise with a solution of 9-fluorenylmethyl chloroformate[113] [*ex.* Sigma; (δ_H (500MHz, $CDCl_3$) 4.32 (1H, t, *J* 7.5Hz, C*H*CH_2), 4.57 (2H, d, *J* 7.5Hz, CHC*H*$_2$), 7.34-7.37, 7.44-7.46, 7.60-7.62 and 7.79-7.81 (8H, 4 x m, aromatic CH's); 6mg, 23μmol] in 1,4-dioxane (0.9ml). The mixture was stirred at room temperature for 1h, and solvent removed *in vacuo*. The residue was treated with H_2O (2ml), the solution washed with Et_2O (3 x 4ml), and the aqueous phase freeze dried to yield the title compound (**139**) as a white solid. δ_H (500MHz, D_2O:d_6-acetone [1:1]) 1.67-1.78 and 1.86-1.90 (4H, 2 x m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.36-2.43 (2H, m, C*H*$_2$CO), 3.42 and 3.61 (2H, ABq, *J* 16.5Hz, CH_2S), 3.94 (2H, d, *J* 7.5Hz, CH_2OCO), 4.02-4.06 (1H, m, H_α), 4.10 (1H, t, *J* 7.5Hz, C*H*CH_2OCO), 4.19 and 4.36 (2H, ABq, *J* 12Hz, C*H*$_2$OH), 5.09 and 5.68 (2H, ABq, *J* 4.5Hz, CHCHS), 7.33-7.50 and 7.67-7.80 (8H, 2 x m, aromatic CH's); *m/z* (Electrospray) 634 (100%, MK^+), 618 (56%, MNa^+).

6.3.8 Derivatisation of 3α-Hydroxymethylpenicillins

N-[N'-Ethoxycarbonyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin (145)

$C_{16}H_{23}N_3O_9S$; MW 433.4

The pH of a solution of 3α-hydroxymethylpenicillin (**107**) (typically 50-500μg, 0.12-1.2μmol) in H_2O (1ml) was adjusted to 8 with aqueous $NaHCO_3$ (1M), and the solution was treated with diethylpyrocarbonate (*ex.* Aldrich; 5μl, 34μmol).[112] The mixture was stirred rapidly for 5min, treated with H_2O (4ml), washed with Et_2O (3 x 5ml) and the aqueous solution freeze dried to yield the title compound (**145**) as a white solid. δ_H (500MHz, D_2O) 1.25

(3H, t, *J* 7.1Hz, CH_3CH_2), 1.66-1.82 (4H, m, $CH_2CH_2CH_2CO$), 2.40 (2H, t, *J* 7, CH_2CO), 3.65 (1H, 1H of AB part of ABX system, *J* 9, 11.5Hz, 1H of CH_2OH), 3.94-3.97 (2H, m, H_α and 1H of CH_2OH), 4.14 (2H, q, *J* 7Hz, CH_3CH_2), 4.24-4.28 (1H, m, H(3)), 5.48 and 5.52 (2H, ABq, *J* 4Hz, H(5) and H(6)). H(2) resonance obscured by HOD resonance; *m/z* (Electrospray) 456 (100%, MNa^+), 434 (79%, MH^+).

N-[N'-Ethoxycarbonyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin, dimethyl ester (146)

$C_{18}H_{27}N_3O_9S$; MW 461.5

The crude *N*-[*N'*-Ethoxycarbonyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin (**145**) (*ca.* 100μg) was suspended in d_7-DMF (*ex.* Aldrich; 99 atm % D; 400μl) under argon, and the mixture treated with dimethylsulphate (*ex.* Aldrich; 7.5μl, 80μmol). The mixture was stirred at 30℃ under argon for 2h and examined by 500MHz ^1H NMR spectroscopy to reveal a new β-lactam product. This material was characterised as the title compound (**146**) by comparison with samples of the title compound prepared below. (^1H NMR data quoted for next preparation).

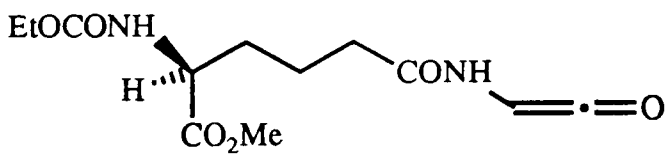
N-[N'-Ethoxycarbonyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin, dimethyl ester (146) and N-[N'-Ethoxycarbonyl-α-(methyl ester)-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin lactone (147)

$C_{18}H_{27}N_3O_9S$; MW 461.5 and $C_{17}H_{25}N_3O_9S$; MW 445.5 respectively

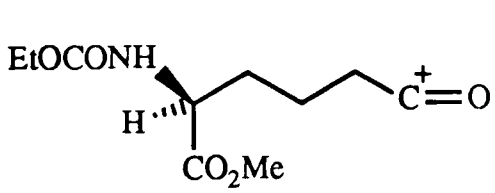
The crude *N*-[*N'*-Ethoxycarbonyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin (**145**) (*ca.* 100μg) was suspended in dry, distilled DMF (200μl) or d_7-DMF (*ex.* Aldrich; 99 atm % D; 200μl) under argon, and the mixture treated with methyl iodide (*ex.* John Ross Chemicals; 5μl, 80μmol).

The mixture was stirred for 1 to 24h, as discussed in chapter 3, and solvent removed *in vacuo*. Examination of the residue by ^1H NMR spectroscopy revealed the presence of the two title compounds, with a ratio which varied with the reaction time, and between repetitions, from 1:<0.1 (good yield) to <0.1:1 (poor yield). The title compounds were characterised by ^1H NMR spectroscopy and mass spectral analysis

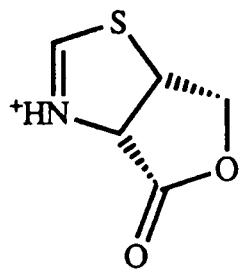
i) *m/z* for mixture [DCI(NH_3)], M^1 denotes the dimethyl ester (**146**), M^2 denotes the lactone (**147**); 462 (18%, M^1H^+), 430 (54%, M^2H^+), 287 (19%, (**157**)H^+), 247(100%,**158**NH_3), 230 (53%, **158**), 144 (31%, **159**). N.B. the relative peak heights for the lactone and dimethyl ester of course vary with the composition of the mixture, but always show a greater proportion of the lactone than present in the sample before exposure to mass spectrometric analysis conditions.



(**157**)



(**158**)



(**159**)

ii) Data for dimethyl ester (**146**): δ_H (500MHz, $CDCl_3$) 1.27 (3H, t, *J* 7Hz, CH_3CH_2), 1.68-1.92 (4H, m, $CH_2CH_2CH_2CO$), 2.26-2.42 (2H, m, CH_2CO), 3.76-3.84 (8H, m, 2 x CO_2Me and CH_2OH), 4.13 (2H, q, *J* 7Hz, CH_3CH_2), 4.24-4.28 (1H, m, H(3)), 4.36-4.40 (1H, m, H_α), 4.96 (1H, d, *J* 7.5Hz,

H(2)), 5.28 (1H, d, *J* 7.5Hz, NH), 5.55 (1H, d, *J* 4Hz, H(5)), 5.68 (1H, dd, *J* 4.1, 8.5Hz, H(6)), 6.35 (1H, d, *J* 8Hz, NH).

iii) Data for lactone (**147**): δ_H (500MHz, $CDCl_3$) 1.25-1.29 (3H, m, CH_3CH_2), 1.69-1.91 (4H, m, $CH_2CH_2CH_2CO$), 2.26-2.41 (2H, m, CH_2CO), 3.81 (3H, s, CO_2Me), 4.10-4.15 (2H, m, CH_3CH_2), 4.36-4.41 (1H, m, H_α), 4.49 (1H, 1H of AB part of ABX system, *J* 1.5, 10.5Hz, 1H of CH_2OH), 4.58-4.61 (1H, m, H(3)), 4.64 (1H, 1H of AB part of ABX system, *J* 5.5, 10.5Hz, 1H of CH_2OH), 5.18 (1H, d, *J* 7.5Hz, H(2)), 5.28-5.31 (1H, m, NH), 5.44 (1H, d, *J* 4Hz, H(5)), 5.71 (1H, dd, *J* 4, 9Hz, H(6)), 6.31-6.36 (1H, m, NH).

N-[N'-Ethoxycarbonyl-α-(methyl ester)-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin (149)

$C_{17}H_{25}N_3O_9S$; MW 447.5

A crude sample of *N*-[*N'*-Ethoxycarbonyl-α-methyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin, methyl ester (**146**) (*ca.* 100μg) was suspended in dry toluene (250μl), and heated at 55°C for 2h. Solvent was evaporated *in vacuo* and the residue examined by ^1H NMR spectroscopy. The residue was found to contain no β-lactam products soluble in $CDCl_3$, but was found to contain a new β-lactam soluble in D_2O and CD_3CN (sparingly). The ^1H NMR spectrum of this material was consistent with the structure of the title compound (**149**). δ_H (500MHz, $CDCl_3$) 1.22-1.27 (3H, m, CH_3CH_2), 1.55-1.90 (4H, m, $CH_2CH_2CH_2CO$), 2.37-2.41 (2H, m, CH_2CO), 3.53-3.57 (1H, m, 1H of CH_2OH), 3.76-3.84 (4H, m, CO_2Me and H_α), 3.91-3.98 (1H, m, 1H of CH_2OH), 4.09-4.14 (2H, m, CH_3CH_2), 4.22-4.27 (1H, m, H(3)),

5.47-5.48 and 5.50-5.51 (2H, 2m, H(5) and H(6)). The H(2) resonance was obscured by the residual HOD resonance..

A solution of this compound in saturated aqueous NaCl (0.5ml) was acidified to pH 2-3 (0.1M HCl) and extracted with EtOAc, (2 × 2ml). The combined organic extracts were dried (Na_2SO_4), filtered and evaporated *in vacuo*. The aqueous phase was neutralised with aqueous NH_3 (2M) and freeze dried. Analysis by ^1H NMR spectroscopy found there to be no β-lactam materials in the residues from either the organic or aqueous phases. (None of the original product remained for mass spectral analysis).

N–[N'–Ethoxycarbonyl-α-(methyl ester)-δ-(L-α-aminoadipoyl)]-[3β-^{2}H]-3α-hydroxy-[^{2}H]-methylpenicillin lactones (152 and 153)

$C_{17}H_{21}D_2N_3O_9S$; MW 447.5

The two d_2-penicillins (**128** and **129**; *ca.* 100μg of each) were each *N*-protected according to the procedure recorded for the unlabelled material, to yield the *N*-ethoxycarbonyl derivatives (**150** and **151** respectively) as white solids.

i) For *N*-protected d_2-penicillin (**150**) from *E*-d_2-tripeptide (**111**): δ_H (500MHz, D_2O) 1.20 (3H, t, *J* 7Hz, CH_3CH_2), 1.65-1.87 (4H, m, $CH_2CH_2CH_2CO$), 2.38-2.41 (2H, m, CH_2CO), 3.63 (0.85H, s, CHD), 3.92-3.98 (1.15H, m, H_α and CHD), 4.10-4.15 (2H, m, CH_3CH_2), 5.48 and 5.52 (2H, ABq, *J* 4Hz, H(5) and H(6)). H(2) resonance obscured by HOD resonance. The CHD integrals were not clear, and the values quoted are taken from the parent penicillin.

ii) For *N*-protected d_2-penicillin (**151**) from *Z*-d_2-tripeptide (**112**): δ_H (500MHz, D_2O) 1.20 (3H, t, *J* 7Hz, CH_3CH_2), 1.66-1.85 (4H, m, $CH_2CH_2CH_2CO$), 2.39-2.43 (2H, m, CH_2CO), 3.63 (0.15H, s, CHD), 3.93-

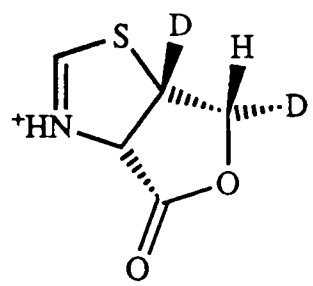
3.99 (1.85H, m, H_α and CHD), 4.11-4.16 (2H, m, CH_3CH_2), 5.48 and 5.53 (2H, ABq, *J* 4Hz, H(5) and H(6)). H(2) resonance obscured by HOD resonance. The CHD integrals were not clear, and the values quoted are taken from the parent penicillin.

The crude *N*-protected d_2-penicillins (**150** and **151**) were each treated with d_7-DMF (*ex.* Aldrich; 99 atom % D; 200µl) under N_2. The suspensions were each treated with MeI (*ex.* John Ross Chemicals; 5µl, 80µmol), and stirred at room temperature under N_2 for 3h. Solvent was evaporated *in vacuo*, and the residues examined by 500MHz ^{1}H NMR spectroscopy.

The residue from reaction of (**150**) showed only one β-lactam product, in very poor yield, which by comparison with the ^{1}H NMR spectrum of *N*-[*N'*-ethoxycarbonyl-α-methyl-δ-(L-α-aminoadipoyl)]-3α-hydroxymethylpenicillin lactone (**147**), was characterised as *N*-[*N'*-ethoxycarbonyl-α-methyl-δ-(L-α-aminoadipoyl)]-[3β-^{2}H]-3α-[8*S*-^{2}H]-hydroxymethylpenicillin lactone (**152**). The mass spectrum also indicated the presence of the *N*-[*N'*-Ethoxycarbonyl-α-methyl-δ-(L-α-aminoadipoyl)]-[3β-^{2}H]-3α-[8*S*-^{2}H]-hydroxymethylpenicillin, methyl ester. δ_H (500MHz, $CDCl_3$) 1.25-1.29 (3H, m, CH_3CH_2), 1.69-1.91 (4H, m, $CH_2CH_2CH_2CO$), 2.26-2.41 (2H, m, CH_2CO), 3.81 (3H, s, CO_2Me), 4.10-4.15 (2H, m, CH_3CH_2), 4.36-4.41 (1H, m, H_α), 4.61 (1H, s, CHD), 5.18 (1H, s, H(2)), 5.44 (1H, d, *J* 4Hz, H(5)), 5.71 (1H, dd, *J* 4, 8.6Hz, H(6)), 6.29-6.33 (1H, m, NH). One NH resonance obscured by impurities; *m/z* [DCI(NH_3)], M^1 denotes the dimethyl ester, M^2 denotes the lactone (**152**), 464 (4%, M^1H^+), 432 (19%, M^2H^+), 287 (28%, **157**H^+), 247 (56%, **158**NH_3), 230 (100%, **158**), 146 (21%, **160**).

The residue from (**151**) when analysed by ^{1}H NMR spectroscopy, was found to contain only a trace of one β-lactam product. This product appeared

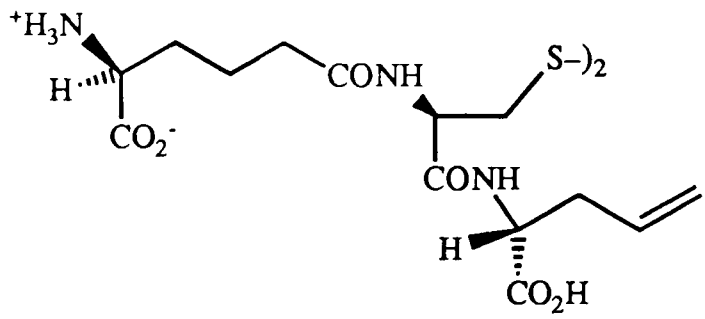
to be the lactone (**153**), but a reliable assignment could not be made from the ^1H NMR spectrum. Further evidence for the lactone (**153**) structure was made from the presence of the molecular ion in the mass spectrum of the reaction mixture. δ_H (500MHz, $CDCl_3$), only H(5) and H(6) resonances were visible above the background noise and impurity peaks, 5.44 (1H, d, *J* 4Hz, H(5)) and 5.70-5.72 (1H, m, H(6)); *m/z* relative to 158 (100%), 432 (3%, MH^+).



(160)

6.4 Experimental Procedures for Chapter Four

6.4.1 Synthesis of bis-[L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine] (disulphide of 52)[67]



D-Allylglycine, 4-methoxybenzyl ester, toluene-4-sulphonate salt

$C_{13}H_{17}NO_3.C_7H_8O_3S$; MW 407.51

A solution of *N-tert*-butoxycarbonyl-D-allylglycine, 4-methoxybenzyl ester (*ex*. C.A. Fletcher [δ_H (200MHz, $CDCl_3$) 1.43 (9H, s, Me_3C), 2.44-2.54 (2H, m, $C_\alpha CH_2$), 3.79 (3H, s, OMe), 4.34-4.44 (1H, m, H_α), 5.02-5.18 (5H, m, CO_2CH_2, $C{=}CH_2$ and NH), 5.54-5.71 (1H, m, $CH{=}CH_2$), 6.84-6.90 and 7.27-7.31 (4H, m, Aromatic CH's)]; 300mg, 0.89mmol) in Et_2O (25ml) and EtOH (25ml) was treated with toluene-4-sulphonic acid (*ex*. Aldrich, re-crystallised; 155mg, 0.90mmol). Upon dissolution, solvent was evaporated *in vacuo*, and the reaction heated at 40-50℃ *in vacuo* (~10mmHg) for 1h, to yield the title compound as a yellow solid (370mg, 100%). δ_H (200MHz, $CDCl_3$) 2.33 (3H, s, *Me*Ar), 2.54-2.60 (2H, m, $C_\alpha CH_2$), 3.78 (3H, s, OMe), 4.08-4.16 (1H, m, H_α), 4.90-5.13 (4H, m, CO_2CH_2 and $C{=}CH_2$), 5.48-5.69 (1H, m, $CH{=}CH_2$), 6.77-6.83 (2H, m, 2H of $MeOC_6H_4CH_2$), 7.09-7.13 (2H, m, 2H of $MeC_6H_4SO_3^-$), 7.18-7.22 (2H, m, 2H of $MeOC_6H_4CH_2$), 7.75-7.79 (2H, m, 2H of $MeC_6H_4SO_3^-$), 8.22 (3H, bs, NH_3^+).

N–(4–Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-S-benzhydryl-L-cysteinyl-D-allylglycine, 4-methoxybenzyl ester[67]

$C_{52}H_{57}N_3O_{11}S$; MW 932.1

A solution of D-Allylglycine, 4-methoxybenzyl ester, toluene-4-sulphonate salt (370mg, 0.89mmol), EEDQ (*ex*. Aldrich, recrystallised; 221mg, 0.89mmol) and *N*-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteine (639mg, 0.89mmol) in CH_2Cl_2 (5ml) under argon, with a little Na_2SO_4 (~20mg), was treated with distilled NEt_3 (125µl, 0.89mmol) and stirred at room temperature under argon overnight. Solvent was evaporated *in vacuo* and the residue partitioned between saturated aqueous $NaHCO_3$ (75ml) and EtOAc (75ml). The organic phase was washed with HCl (1M; 75ml) and saturated aqueous NaCl (75ml), dried (Na_2SO_4), filtered and solvent evaporated *in vacuo* to yield a yellow foam (810mg). Purification by column chromatography (SiO_2; EtOAc:CH_2Cl_2 [1:3]) yielded the title compound as a white foam (516mg, 62%). Tlc (SiO_2; CH_2Cl_2:EtOAc [3:1]) R_f 0.3; δ_H (500MHz, $CDCl_3$) 1.53-1.70 and 1.81-1.88 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.10-2.16 and 2.18-2.23 (2H, 2 × m, CH_2CO), 2.42-2.55 (2H, m, $C_\alpha CH_2CH$), 2.65-2.70 and 2.76-2.80 (2H, AB part of ABX system, *J* 6.5, 6.5 and 14Hz, SCH_2), 3.79-3.81 (9H, m, 3 × MeO), 4.33-4.39 (1H, m, H_α), 4.49-4.53 (1H, m, H_α), 4.56-4.60 (1H, m, H_α), 4.99-5.13 (8H, m, 3 × CH_2Ar and $C=CH_2$), 5.26 (1H, s, $CHPh_2$), 5.50-5.60 (2H, m, NH and $CH=CH_2$), 6.19 (1H, d, *J* 7Hz, NH), 6.62 (1H, d, *J* 7.5Hz, NH), 6.84-6.89 and 7.20-7.45 (22H, m, Aromatic CH's); *m/z* [FAB(MCA)] 954 (MNa^+), 932 (MH^+).

Bis-[δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine] (disulphide of 52)[67]

$C_{28}H_{44}N_6O_{12}S_2$; MW 720.8

A solution of *N*-(4-Methoxybenzyl)oxycarbonyl-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-benzhydryl-L-cysteinyl-D-allylglycine, 4-methoxybenzyl ester (216mg, 0.23mmol) in CH_2Cl_2 (~20ml) was dried (Na_2SO_4), filtered and solvent evaporated *in vacuo*. The dried residue was dissolved in freshly distilled TFA (2.5ml), and treated with distilled anisole (500μl), and the reaction heated at reflux for 30min. TFA was removed *in vacuo*, final traces being removed by azeotroping with toluene (3 × 2ml), with the residue and anisole being partitioned between H_2O (25ml) and EtOAc (25ml). The organic phase was extracted with H_2O (2 × 25ml) and the combined aqueous extracts freeze dried to yield the crude thiol (**52**) form of the title material as a yellow solid (111mg).

The crude thiol (**52**) was dissolved in H_2O (20ml) and pH of the solution adjusted to 9-10 with '0.880 NH_3' solution. Oxygen was bubbled through the solution for 3h, and the solution freeze dried to yield a yellow solid. This material was purified by reverse phase HPLC (Hypersil® ODS; mobile phase 10% MeOH in 20m<u>M</u> NH_4HCO_3) to yield the title compound (disulphide of **52**) as a white solid [retention times; 10min (250 × 9.4mm internal diameter column; 4ml.min^{-1})], or 7min (250 × 4.6mm internal diameter column; 1ml.min^{-1}). δ_H (500MHz, D_2O) (Number of H's for each resonance quoted for one half of disulphide only, i.e. for R of RS–SR) 1.69-1.82 and 1.84-2.01 (4H, 2 × m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.46 (2H, t, *J* 7.5Hz, CH$_2$CO), 2.48-2.54 and 2.61-2.66 (2H, 2 × m, C$_\alpha$C*H*$_2$CH), 3.01 and 3.25 (2H, AB part of ABX system, *J* 5, 9 and 14Hz, C$_\alpha$CH$_2$S), 3.80 (1H, t, *J* 6Hz, H$_\alpha$), 4.34 (1H, dd, *J* 5, 7.5Hz, H$_\alpha$), 5.18 (1H, d, *J* 10Hz, RC*H*=CH(*Z*)*H*(*E*)), 5.19 (1H, d, *J* 17Hz, RC*H*=C*H*(*Z*)H(*E*)), 5.81 (1H, ddd, *J* 7, 10, 17Hz, C*H*=CH$_2$). One H$_\alpha$

resonance was obscured by the residual HOD resonance; *m/z* [FAB(Gly/Oxalic Acid)] 721 (81%, MH^+), 362 (100%, Thiol-H^+), 247 (39%), 207 (41%), 144 (54%), 110 (68%) and 59 (81%).

6.4.2 Incubation Studies

All incubations of LL-AC-D-Ag (**52**) (1mg) were carried out according to the general incubation procedure, except for alterations in the duration and temperature of the incubations. Incubations at above room temperature (27℃, 37℃ and 47℃) were carried out in a stirred jacketed cell connected to a constant temperature water bath. Incubations at below room temperature (7℃ and 17℃) were carried out in a stirred glass vial held in a water bath on a thermostatically regulated cold table. The incubation temperatures and durations are given below.

	Temperature (with drift if applicable)	Incubation Duration
i)	6.6-7.0℃	160min.
ii)	16.1-16.8℃	80min.
iii)	26.9℃	40min.
iv)	36.9℃	30min.
v)	47.0℃	30min

After work up, the crude incubation mixtures were stored at –80℃ prior to analysis by ^1H NMR spectroscopy.

6.5 Experimental Procedures for Chapter Five

6.5.1 General Procedures

Bradford Assay for Determination of Protein Concentration[139]

The solution to be assayed (40µl, diluted from the sample if necessary) was treated with Bradford's reagent [Coomassie Brilliant Blue G 250 (100mg), EtOH (50ml) and aqueous H_3PO_4 (88% solution; 100ml); made up to 1000ml with H_2O] (2ml), and the solutions shaken and allowed to stand for 5min. The absorbances of the samples were examined at 595nm, with reference to a blank containing only Bradford's reagent. Protein concentrations were calculated from a curve made by calibration with protein solutions of known concentration.

Bioassay for Determination of IPNS Activity[1]

The solution to be assayed (300µl, diluted from the sample if necessary) was treated sequentially with aqueous solutions of LLD-ACV (**1**) (63m<u>M</u>; 7µl), DTT (100m<u>M</u>; 7µl), L-ascorbic acid (50m<u>M</u>; 7µl), and iron (II) (5m<u>M</u> as sulphate; 7µl), and the mixture incubated for 10min at 27°C, 250rpm. The incubation was then quenched by addition of acetone (0.7ml), which was then removed by passing a stream of air over the solution.

100µl of the resulting solution was placed in a 8mm diameter well in a petri dish containing agar growth medium infected with *Staphylococcus aureus* NCTC 6571, and the plate was incubated at 37°C for 15-18h.

The inhibition zones were measured by taking an average diameter of the area showing no growth of *S. aureus*, and from these the activity of IPN (**2**),

and hence the activity of IPNS in the the original solution, was calculated using a calibration curve for the batch of *S. aureus* plates used.†

SDS-PAGE Procedure[140]

A) Preparation of gels

SDS-PAGE gels were prepared and run using the procedure described by Laemmli.[140] 10 and 15% SDS-PAGE gel solutions were prepared by sequential addition of the solutions given in the recipe below, with gentle shaking after addition of SDS solution. Polymerisation was initiated with TEMED (5µl), the solutions were again shaken gently, and the gels were poured between glass plates, spaced 1mm apart, to a depth of ~5mm below the bottom of the comb to be used for production of the wells in the stacking gel. The gel surface was protected during setting by addition of distilled H_2O.

	Solution	10% gel	15% gel
i)	H_2O	0.75ml	–
ii)	Acrylamide [30% (w/v)]/ Bis-acrylamide [0.8% (w/v)]	2ml	3ml
iii)	TRIS (0.75M), pH 8.8	3ml	2.7ml
iv)	SDS [10% (w/v)]	60µl	60µl
v)	Ammonium persulphate [1% (w/v)]	0.25ml	0.3ml

The stacking gel was made up using the following recipe, shaking and initiating polymerisation with TEMED (5µl) as before.

† Calibration of each batch of bioassay plates was carried out using cephalosporin C, potassium salt. Specific activities against *S. aureus* NCTC 6571 are defined as 10 units.mg^{-1} for cephalosporin C, potassium salt; and 100 units.mg^{-1} for IPN (**2**).

	Solution	Stacking gel
i)	H_2O	2.32ml
ii)	Acrylamide [30% (w/v)]/	
	Bis-acrylamide [0.8% (w/v)]	0.32ml
iii)	TRIS (1M), pH 6.8	0.4ml
iv)	SDS [10% (w/v)]	32µl
v)	Ammonium persulphate [1% (w/v)]	0.16ml

When the gels were set, the protective H_2O layer was removed and the remaining space between the plates filled with stacking gel solution, inserting a 10 or 15 tooth comb to provide 10 and 15 well gels respectively.

B) Preparation of samples

Protein solutions (typically 50-100µl) were treated with one fourth their volume of '5× sample buffer' (TRIS, 7.57g; SDS, 10g; glycerol 50ml; bromophenol blue, 5mg; 2-mercaptoethanol, 5ml; made up to 100ml in H_2O and pH adjusted to 6.8), and boiled in sealed eppendorfs for 5-10min.

C) Running of Gels

The prepared samples were loaded into the wells in the stacking gel (aiming for 1µg per band; typically 10-20µg of sample per well in 2-20µl), and the gels were run in pairs at 40mA with 'running buffer' electrolyte solution (TRIS, 6g; glycine, 28.8; SDS, 1g; made up to 2l in H_2O), until the solvent front (blue line) reached the bottom of the plates.

D) Staining of Gels

The gels were separated from their stacking gels and from the glass plates and heated (30-40℃) in staining solution [Coomassie blue, 250mg; AcOH, 9.2ml; made up to 100ml in 50% (v/v) aqueous MeOH] for 10-30min. The staining solution not fixed to protein on the gels was removed by heating (30-40℃) in H_2O:AcOH:MeOH (875:50:75 by volume) for 1-4days, and the gels were fixed onto paper by heating for 30min on a Bio-Rad Gel Slab Dryer.

6.5.2 Purification of IPNS[128]

Purification of IPNS was carried out on a Pharmacia FPLC system, using a Mono Q 16/10 Ion exchange column (20ml column volume).

The column was prepared by running from aqueous EtOH [24% (v/v)] column storage solution to H_2O, flushing with a solution of NaCl (400m<u>M</u>) in aqueous TRIS (20m<u>M</u>) at pH 8.0, and equilibration with aqueous TRIS (20m<u>M</u>) at pH 8.0.

IPNS solution (batch CF18; 6.7mg.ml^{-1}; 32ml) was prepared by centrifugation (10krpm; 10min), filtration of the supernatant, and dilution to 100ml with aqueous TRIS (20m<u>M</u>) at pH 8.0, and then loaded onto the column. The column was eluted according to the gradient given in Graph 6.3, monitoring the eluent at 280nm, and with automated collection of 5ml fractions.

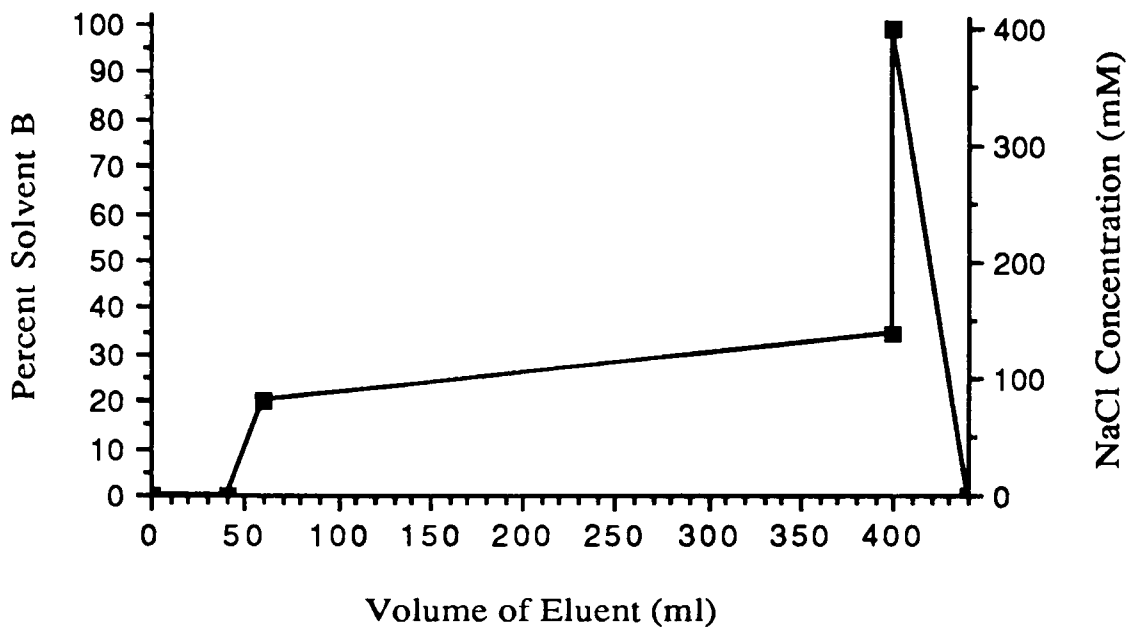
The FPLC fractions were analysed by Bradford assay (for protein content) and by Bioassay (for IPNS activity), giving the results shown in Table 6.3. fractions 7 to 38 were further examined by SDS-PAGE on 10% gels.

GRAPH 6.3

The gradient profile used for purification of IPNS

Flow rate = 2.5ml.min^{-1}; Solvent A = aqueous TRIS (20m<u>M</u>) at pH 8.0;

Solvent B = NaCl (400m<u>M</u>) in aqueous TRIS (20m<u>M</u>) at pH 8.0



Fractions 9-30 were combined, concentrated to 8ml by centrifugation through an Amicon membrane. Analysis by Bradford Assay and Bioassay showed this purified IPNS to be 9.2mg.ml^{-1} with an activity of 2.0 IU.mg^{-1}.

6.5.3 Preparation of Auto-inactivated IPNS

The pH of a solution of LLD-ACV (**1**) (*ex.* Eli Lilly & Co.; 30mg, 84μmol) in H_2O (1.2ml) and aqueous NaOH (2<u>M</u>; 80μl) was adjusted to 7.5 with aqueous HCl (1<u>M</u>)

Two samples ('incubation' and 'control') of the IPNS solution prepared in section 6.5.2 (9.2mg.ml^{-1}; each sample 400μl, 3.7mg of protein) were sequentially incubated at 30°C, 250rpm, with cofactor solutions as given below, except that for the 'control' sample aqueous NH_4HCO_3 (25m<u>M</u>) was substituted for the LLD-ACV (**1**) solution.

TABLE 6.3
Results of Bradford assay and Bioassay
on FPLC fractions from IPNS purification

Fraction	Protein Content ($mg.ml^{-1}$)	IPNS Activity ($IU.mg^{-1}$)	Fraction	Protein Content ($mg.ml^{-1}$)	IPNS Activity ($IU.mg^{-1}$)
1	0.00	–	26	0.25	0.54
2	0.00	–	27	0.24	0.65
3	0.00	–	28	0.22	0.80
4	0.00	–	29	0.21	0.49
5	0.00	–	30	0.17	0.51
6	0.01	–	31	0.19	0.19
7	0.20	0.00	32	0.20	0.12
8	12.42	0.22	33	0.20	0.12
9	5.97	0.23	34	0.16	0.07
10	2.36	0.29	35	0.14	0.11
11	1.06	0.49	36	0.13	0.08
12	0.76	0.51	37	0.11	0.23
13	0.68	0.57	38	0.11	0.15
14	0.38	0.81	39	0.10	0.14
15	0.24	0.85	40	0.10	0.11
16	0.20	0.89	41	0.11	0.10
17	0.13	1.08	42	0.12	0.06
18	0.25	0.95	43	0.10	0.07
19	0.22	0.78	44	0.17	0.04
20	0.19	0.82	45	0.08	0.06
21	0.16	0.85	46	0.07	0.07
22	0.14	0.63	47	0.08	0.06
23	0.19	0.93	48	0.08	0.03
24	0.21	0.74	49	0.08	0.08
25	0.21	0.73	50	0.06	0.00

After each incubation a sample of the incubation mixture (50μl) was removed, diluted to 100μl with H_2O, and placed in a 8mm diameter well in a petri dish containing growth medium infected with *S. aureus* NCTC 6571. The plates were incubated at 37°C for 15-18h, and the inhibition zones measured by taking an average diameter of the area showing no growth of *S. aureus*.

IPNS from the incubation mixture was purified for the next incubation by gel filtration on a NAP-5 or PD-10 column as described below.

Incubation 1

LLD-ACV (**1**) solution (200μl) was treated with aqueous DTT (100mM; 20μl) and after 6min the mixture was treated sequentially with solutions of L-ascorbic acid (50mM; 20μl), iron (II) (5mM as sulphate; 20μl), IPNS (as above), and DTT (100mM; 20μl).

After incubation as described above, the incubation mixture was loaded onto a NAP-5 column equilibrated with aqueous NH_4HCO_3 (25mM), and eluted with aqueous NH_4HCO_3 (25mM) collecting the fraction from 200-700μl.

The aliquot removed for testing with *S. aureus* as described above gave an inhibition zone of ~35mm.

Further incubations were carried out, and purified in an analogous manner, using the quantities given below:

	LLD-ACV Volume (µl)	Cofactor Volumes (µl)	Column Used	Fraction Collected	Inhibition Zone
Incubation 2	200	23	NAP-5	0.1-0.7ml	~30mm
Incubation 3	200	26	NAP-5	0-0.8ml	22mm
Incubation 4	400	40	PD-10	2.2-3.5ml	14mm
Incubation 5	400	65	PD-10	1.37-2.13ml	–
Incubation 6	400	100	2 x PD-10	1.74-3.50ml	11mm
After final Purification					0mm

The final samples of both the incubation and control were concentrated using a Centricon filtration system (5krpm, 1h) to ~500µl, and analysed by Bradford Assay, showing final protein concentrations of 0.17 and 0.09mg.ml^{-1} respectively (recoveries of 7 and 4%).

6.5.4 Preparation of Silanised Glass Vials

Soda glass vials were each in turn treated with Me_2SiCl_2 (*ex.* Aldrich; ~0.5ml), which was pipetted over all the internal surface before transferring the reagent to the next vial. The treated vials were washed with distilled H_2O and EtOH, and dried in air.

6.5.5 CNBr Digests of IPNS[125,129]

A solution of IPNS (typically 200µl) was treated with HCO_2H [460µl; final concentration 70% (v/v)], and the mixture placed under argon, treated with CNBr (*ex.* Aldrich; 20mg, 190µmol), and stirred in the dark, under argon, overnight. The mixture was treated with H_2O (6.5ml), and solvent and CNBr removed *in vacuo* (dry ice Büchi) to yield the digest mixture.

APPENDIX A

DERIVATION OF MAGNETIC SUSCEPTIBLITY EQUATIONS

APPENDIX A
DERIVATION OF MAGNETIC SUSCEPTIBLITY EQUATIONS

1. Derivation of the Relationship Between *Effective Magnetic Moment* and *Mass Magnetic Susceptibility.*

The magnetic susceptibility of a material may be expressed by two terms, the **volume magnetic susceptibility**, κ, and the **mass magnetic susceptibility**, χ. Both of these are dimensionless quantities and are related by the expression:[141]

$$\chi = \frac{\kappa \rho^{o}}{\rho} \qquad (1)$$

Where ρ is the density of the material in $kg.m^{-3}$; and $\rho^{o} = 1 kg.m^{-3}$.

For paramagnetic samples κ is positive (typically of the order of 10^{-3}), whereas for diamagnetic samples it has a negative value (typically of the order of 10^{-5}).

The permanent magnetic moment, m, for a species with a total spin quantum number, S, is given by:[141]

$$m^2 = g^2 S(S+1)\mu_B^2 \qquad (2)$$

Where: g is the g-value for the electron(s) in the species.

μ_B is the Bohr magneton $= \frac{e\hbar}{2m_e} = 9.274\,078 \times 10^{-24} J.T^{-1}$

The permanent magnetic moment is related to the magnetic susceptibility by the equation:[141]

$$\kappa = \mathcal{N}\mu_0\left(\xi + \frac{m^2}{3kT}\right) \qquad (3)$$

Where: $\mathcal{N}$ is the number of molecules per unit volume

μ_0 is the vacuum permeability, defined as $4\pi \times 10^{-7}N.A^{-2}$

ξ is the molecular magnetisability, i.e. it determines that part of the magnetic moment induced by an applied magnetic field.

k is the Boltzmann constant.

T is the absolute temperature.

If we assume the magnetisability term to be comparatively small, then omitting this term from the expression, and substituting for m^2 using equation **2**, we obtain:

$$\kappa = \frac{\mathcal{N}\mu_0 m^2}{3kT} = \frac{\mathcal{N}\mu_0\mu_B^2 g^2 S(S+1)}{3kT} \qquad (4)$$

Combining equations **4** and **1**, using $\rho = \frac{M_m \mathcal{N}}{N_A}$, we obtain:

$$\chi = \frac{N_A \rho^o \mu_0 \mu_B^2 g^2 S(S+1)}{3kTM_m} \qquad (5)$$

Where: N_A is the Avogadro number.

M_m is the molar mass (in $kg.mol^{-1}$).

Now, the **effective magnetic moment**, μ_{eff}, is related to the total spin quantum number of the system, S, by the relationship:[141]

$$\mu_{eff}^2 = g^2 S(S+1) \qquad (6)$$

So by combining equations **5** and **6** we obtain an expression for the effective magnetic moment of the molecule, μ_{eff}, in terms of its mass susceptibility, χ:

$$\mu_{eff}^2 = \frac{3kTM_m}{N_A \rho^o \mu_0 \mu_B^2} \chi \qquad (7)$$

2. Calculation of Mass Magnetic Susceptibility from ^{1}H NMR Line Shifts

In a ^{1}H NMR experiment, we can calculate the mass susceptibility, χ, of a paramagnetic solute from the shift it causes to the NMR resonance frequencies. The expression in CGS units is:[89]

$$\chi = -\frac{3\Delta\delta\rho'}{4\pi\nu c_m} + \chi_0 + \frac{\chi_0(d_0 - d_s)}{c_m} \qquad (8)$$

Where: $\Delta\delta$ is the frequency shift observed in a ^{1}H NMR reference line due to the solute (N.B. Shifts to higher δ are negative).

$\rho' = 1 g.ml^{-1}$.

ν is the resonance frequency of the NMR spectrometer.

c_m is the mass concentration of solute ($g.ml^{-1}$).

χ_0 is the mass susceptibility of the solvent.

d_0 and d_s are the densities of the solvent and solution respectively.

The final term in this expression is negligible for dilute solutions, and so we can approximate it to:

$$\chi = -\frac{3\Delta\delta\rho'}{4\pi\nu c_m} + \chi_0 \qquad (9)$$

The conversion factor for χ in the CGS system to χ in the SI system is:[141]

$$\chi \text{ (SI system)} = \frac{4\pi}{1000}\chi \text{ (CGS system)} \qquad (10)$$

Thus combining equations **9** and **10**, we obtain:

$$\boxed{\chi = -\frac{3\Delta\delta\rho''}{\nu c_m} + \chi_0} \qquad (11)$$

Where c_m is in $kg.m^{-3}$; $\rho'' = 1 kg.m^{-3}$.

For H_2O at 298K, $\chi_0 = -9.0 \times 10^{-9}$, in the SI system.[89]

APPENDIX B

THE PRIMARY STRUCTURE OF IPNS FROM *ASPERGILLUS NIDULANS* AND HOMOLOGY WITH IPNS FROM *CEPHALOSPORIUM ACREMONIUM*

Primary structure of IPNS gene from *Aspergillus nidulans*[142]

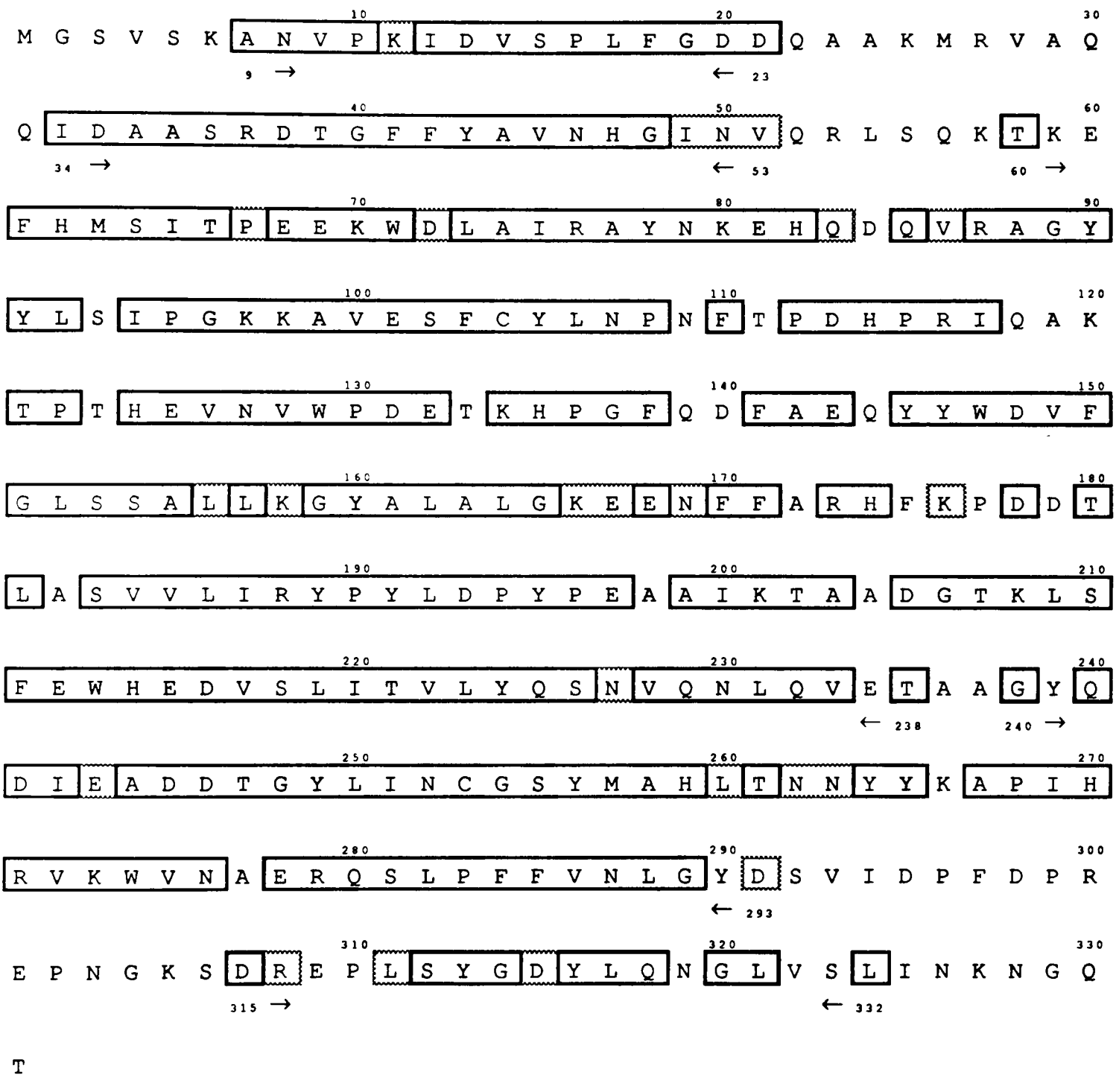
Met	Gly	Ser	Val	Ser	Lys	Ala	Asn	Val	Pro	10
Lys	Ile	Asp	Val	Ser	Pro	Leu	Phe	Gly	Asp	20
Asp	Gln	Ala	Ala	Lys	Met	Arg	Val	Ala	Gln	30
Gln	Ile	Asp	Ala	Ala	Ser	Arg	Asp	Thr	Gly	40
Phe	Phe	Tyr	Ala	Val	Asn	His	Gly	Ile	Asn	50
Val	Gln	Arg	Leu	Ser	Gln	Lys	Thr	Lys	Glu	60
Phe	His	Met	Ser	Ile	Thr	Pro	Glu	Glu	Lys	70
Trp	Asp	Leu	Ala	Ile	Arg	Ala	Tyr	Asn	Lys	80
Glu	His	Gln	Asp	Gln	Val	Arg	Ala	Gly	Tyr	90
Tyr	Leu	Ser	Ile	Pro	Gly	Lys	Lys	Ala	Val	100
Glu	Ser	Phe	Cys	Tyr	Leu	Asn	Pro	Asn	Phe	110
Thr	Pro	Asp	His	Pro	Arg	Ile	Gln	Ala	Lys	120
Thr	Pro	Thr	His	Glu	Val	Asn	Val	Trp	Pro	130
Asp	Glu	Thr	Lys	His	Pro	Gly	Phe	Gln	Asp	140
Phe	Ala	Glu	Gln	Tyr	Tyr	Trp	Asp	Val	Phe	150
Gly	Leu	Ser	Ser	Ala	Leu	Leu	Lys	Gly	Tyr	160
Ala	Leu	Ala	Leu	Gly	Lys	Glu	Glu	Asn	Phe	170
Phe	Ala	Arg	His	Phe	Lys	Pro	Asp	Asp	Thr	180
Leu	Ala	Ser	Val	Val	Leu	Ile	Arg	Tyr	Pro	190
Tyr	Leu	Asp	Pro	Tyr	Pro	Glu	Ala	Ala	Ile	200
Lys	Thr	Ala	Ala	Asp	Gly	Thr	Lys	Leu	Ser	210
Phe	Glu	Trp	His	Glu	Asp	Val	Ser	Leu	Ile	220
Thr	Val	Leu	Tyr	Gln	Ser	Asn	Val	Gln	Asn	230
Leu	Gln	Val	Glu	Thr	Ala	Ala	Gly	Tyr	Gln	240
Asp	Ile	Glu	Ala	Asp	Asp	Thr	Gly	Tyr	Leu	250
Ile	Asn	Cys	Gly	Ser	Tyr	Met	Ala	His	Leu	260
Thr	Asn	Asn	Tyr	Tyr	Lys	Ala	Pro	Ile	His	270
Arg	Val	Lys	Trp	Val	Asn	Ala	Glu	Arg	Gln	280
Ser	Leu	Pro	Phe	Phe	Val	Asn	Leu	Gly	Tyr	290
Asp	Ser	Val	Ile	Asp	Pro	Phe	Asp	Pro	Arg	300
Glu	Pro	Asn	Gly	Lys	Ser	Asp	Arg	Glu	Pro	310
Leu	Ser	Tyr	Gly	Asp	Tyr	Leu	Gln	Asn	Gly	320
Leu	Val	Ser	Leu	Ile	Asn	Lys	Asn	Gly	Gln	330
Thr										

Sequence homology between IPNS's from *A. nidulans* and *C. acremonium*[142]

The sequence of *A. nidulans* IPNS is shown.

Areas homologous with *C. acremonium* IPNS are boxed
('matches' of similar amino acids are shown in grey boxes)

The residue numbers for *A. nidulans* IPNS are shown above the sequence, and the corresponding figures for matched sequences from *C. acremonium* IPNS are marked below, at the start and end of aligned sequences.



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† Later editions do not contain the equation for converting magnetic susceptibilities from the cgs to the SI system.

May the Lord bless you and take care of you;
May the Lord be kind and gracious to you;
May the Lord look on you with favour
and give you peace.

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