THE MECHANISM OF THE ENZYMATIC RING EXPANSION

OF PENICILLIN N TO DEACETOXYCEPHALOSPORIN C.

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

Nicholas Paul Crouch



St. Johns College
Oxford
Trinity Term 1988

ABSTRACT

A thesis submitted in partial fulfilment of the requirements for the degree of D. Phil.

The Mechanism of the Enzymatic Ring Expansion of Penicillin N to Deacetoxycephalosporin C

Nicholas P Crouch

Trinity Term , 1988
St. Johns.

The order of events in the Deacetoxycephalosporin C /Deacetylcephalosporin C Synthetase (DAOC/DAC Synthetase) catalysed ring expansion of penicillin N to deacetoxycephalosporin C has been investigated by the use of labelled/unlabelled penicillin N mixed competitive kinetic isotope effect experiments, in which the labelled penicillin N substrates were either labelled in the pro R- and pro S -methyl groups or at C-3. In addition, to assisting in the determination of the position of the first irreversible event in this reaction, deuteration at C-3 gave rise to a bifurcation of the natural biosynthetic pathway which led to enhanced production of the shunt metabolite, (2R,3S,6R,7R)-1-aza-3-methyl-3-hydroxy-7-[(5R)-5-amino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylate.

The biosynthetic precursor to the 3S-hydroxycepham shunt metabolite has been investigated and the origin of the 3S-hydroxyl oxygen atom has been determined by the use of labelling studies with $^{18}O_2$ and shown to be derived from molecular oxygen.

^{13}C-labelling studies are described which indicate that the ring expansion process is stereospecific to within the limits of the detection system employed. These experiments confirm earlier investigations but, in addition to improving upon the assessment of the degree of stereospecificity, have shown that the 3S-hydroxycepham shunt metabolite is produced with the same stereospecificity as that observed for the usual biosynthetic products, DAOC and DAC.

Chapter 5 describes an investigation of the anomalous C-2 deuterium exchange detected in DAOC produced by incubation of di-($^2H_3$-methyl)-penicillin N with DAOC/DAC synthetase. The preliminary results from this study indicate that initially exchange occurs stereospecifically with the pro R C-2 deuterium atom being replaced by a hydrogen atom.

The origins of the unusual tripeptides L-α-aminoadipyl-L-serinyl-D-valine (L,L,D-ASV), α-aminoadipyl-serinyl-isodehydrovaline (ASdV) and α-aminoadipyl-cysteinyl-β-hydroxyvaline (AC-[β-OH]-V) isolated from *Penicillium chrysogenum* and *Cephalosporium acremonium*, have been examined by the use of variously ^{13}C-labelled L,L,D-α-aminoadipyl-cysteinyl-valine (L,L,D-ACV) and D,L,D-α-aminoadipyl-cysteinyl-valine (D,L,D-ACV) tripeptide isotopomers. The initial results obtained from this investigation may be considered as circumstantial evidence that ASdV is formed by the action of IPNS upon L,L,D-ACV.

Finally, various substrate analogues have been prepared and evaluated as substrates for the ring expansion and hydroxylation activities of the bifunctional DAOC/DAC synthetase enzyme.

'There is no such thing as a problem
without a gift for you in its' hands.

You seek problems because you
need their gifts.'

from 'Illusions'
by Richard Bach.

To my

Mother, Father

and

Nick.

ACKNOWLEDGEMENTS

I should like to express my sincere thanks and gratitude to the following people without whom the construction of this thesis would not have been possible.

To my supervisor, Professor J. E. Baldwin, for his enthusiasm and expert guidance during the course of the last three years.

To Dr. Robert Adlington, whose knowledge, patience and eye for detail make him a excellent scientist and useful ally in the pursuance of good scientific practice. A big thanks also to Dr. Chris. J. Schofield, particularly for help at the outset of this work and for useful advice towards the end.

To Drs. Jeff C. Cobb, Soo. Ko, Lionel King, and Nicholas J. Turner for their help and guidance and preparation of some samples.

To Dr Hong-Hoi Ting, John Keeping, Graham Knight, Wendy Sobey, Dr Janice Coates for the preparation of enzymes and to John Sutherland for the preparation of the genetic vectors which made recombinant enzymes available.

To Dr Robin T. Aplin and Robin Procter for providing endless mass spectra for the mixed competitive kinetic experiments and ^{18}O incorporation studies.

Thanks are also due to Dr. A. Derome, Miss T. Jackson and Mrs E. McGuiness for help in running n.m.r samples, to Valerie Lamburn (micro-analysis), and to Mrs. Shah (optical rotations).

Finally, I would like to thank Morag for her kind help, Rob, Mark, Chris Lowe and Chris Schofield for proof-reading this thesis and last but not least, the 'boys in room 30' for making my stay in the Dyson Perrins an enjoyable one.

CONTENTS

		Page
ABBREVIATIONS		1
NOMENCLATURE		2
CHAPTER 1	INTRODUCTION	
1.1	Development of Antibiotics	3
1.2	Biosynthetic Pathway to Penicillins	5
1.3	Biosynthetic Pathway to Cephalosporins	11
1.4	IPNS Substrate Analogue Studies	19
1.5	Development of New β-Lactam Antibiotics	21
CHAPTER 2	3β-HYDROXYCEPHAM	
2.1	Introduction	27
2.2	3β-Hydroxycepham - Shunt or Biosynthetic Intermediate?	31
2.3	Enhanced 3β-Hydroxycepham Formation via a Kinetic Isotope Effect	33
2.4	Origin of the Hydroxyl Oxygen Atom	37
2.5	Conclusions	43
CHAPTER 3	STEREOCHEMISTRY OF VALINE INCORPORATION INTO PENICILLIN N AND SUBSEQUENT RING EXPANSION	
3.1	Introduction	45
3.2	Design of Experiment and Synthesis of Substrate	48
3.3	Assignment of Chemical Shifts	49
3.4	Stereospecificity of Penicillin N formation from DLD-AC-(3S)-(2-^{2}H, 4-^{13}C)-V	50
3.5	Stereospecificity of DAOC and DAC formation from (2R)-(2'-^{13}C,3-^{2}H)-Penicillin N	52
3.6	Stereospecificity of 3β-Hydroxycepham Formation	54
3.7	Conclusions	55
CHAPTER 4	MIXED COMPETITIVE KINETIC ISOTOPE EXPERIMENTS	
4.1	Introduction	59
4.2	Design of Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effect Experiments for the Ring Expansion Process	62
4.3	Synthesis of Substrates	64

4.4 Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effect Experiments for Penicillin N C-3 Position 66
4.5 Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effect Experiments for Penicillin N β-methyl Position 69
4.6 Denatured Enzyme Control for the Penicillin N β-methyl Position 70
4.7 Discussions and Conclusions 71

CHAPTER 5 ANOMALOUS DEUTERIUM EXCHANGE

5.1 Introduction 75
5.2 C-2 Exchange Studies with Hexadeuteropenicillin N 77
5.3 Stereochemistry of Exchange 80
5.4 C-2 Exchange - Chemical or Enzymatic ? 81
5.5 Design of Experiments to Investigate Anomolous Exchange 82
5.6 Synthesis of Substrate 90
5.7 Incubation of DAOC with DAOC/DAC Synthetase in D_2O 95
5.8 Incubation of (3,-$^2H_3$-methyl)-DAOC with DAOC/DAC Synthetase in D_2O 96
5.9 Conclusions 97

CHAPTER 6 ANOMALOUS PEPTIDES FROM *P. CHRYSOGENUM* AND *CEPHALOSPORIUM SP.*

6.1 Introduction 99
6.2 Design of Experiments to Investigate the Biosynthetic Origin of α-Aminoadipoyl-serinyl-isodehydrovaline 103
6.3 Synthesis of Substrates 105
6.4 Synthesis of Authentic Standards 109
6.5 Incubation of LLD-AC-(2-^{2}H, 3-^{13}C)-Valine with IPNS 111
6.6 Control Experiment : Incubation of LLD-ACV with IPNS 113
6.7 (2-^{13}C, 3-^{2}H)-Isopenicillin N Control Experiment 115
6.8 Studies with Tri-13C-Labelled Tripeptides 117
6.8.1 Incubation of LLD-AC-(2-^{2}H, 3,4,4,-$^{13}C_3$)-Valine with IPNS
6.8.2 Incubation of LLD-A-Serinyl-(2-^{2}H, 3,4,4,-$^{13}C_3$)-Valine with IPNS 119
6.8.3 Studies with DLD-AC-(2-^{2}H, 3,4,4,-$^{13}C_3$)-Valine 121
6.9 Incubation of LLD-AC-β-Hydroxyvaline with IPNS 126
6.10 Conclusions 127

CHAPTER 7 SUBSTRATE ANALOGUE STUDIES WITH DAOC/DAC SYNTHETASE FROM *C. ACREMONIUM*.

7.1 Introduction 129
7.2.1 Ring Expansion Substrate Analogue Studies 133
7.2.2 Comparison of Wildtype and Recombinant DAOC/DAC Synthetase Substrate Specificity 134
7.2.3 Penicillin N β-Sulphoxide 135
7.2.4 β-Methoxymethyl Penicillin N 136
7.2.5 Exomethylene Penicillin N; α-Nor-methyl Penicillin N and β-Nor-methyl Penicillin N 137
7.3 Substrate Analogue Studies for the Hydroxylase Activity 139
7.3.1 Desmethyl Deacetoxycephalosporin C 139
7.3.2 Δ2-Deacetoxycephalosporin C 141
7.4 Conclusions 150

CHAPTER 8 EXPERIMENTAL

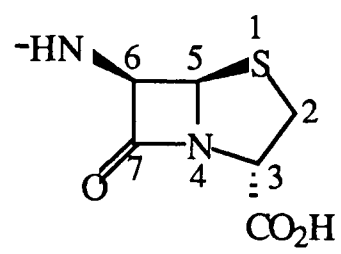
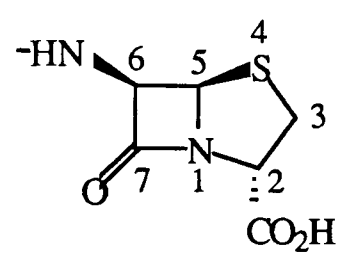
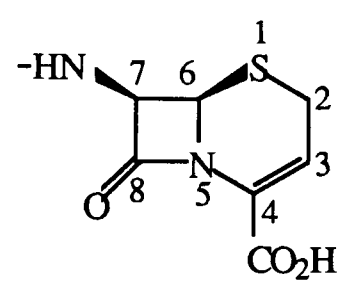
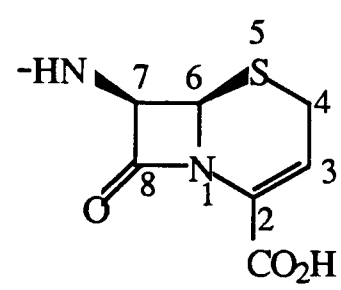
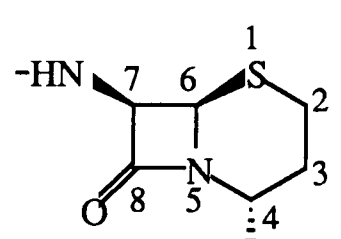
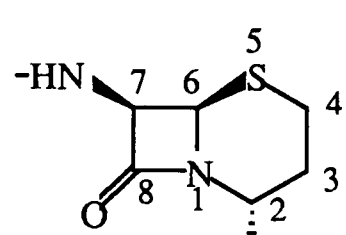
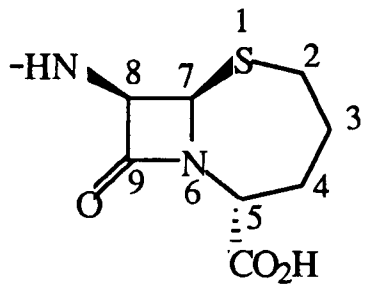
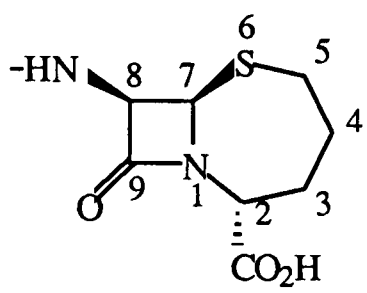
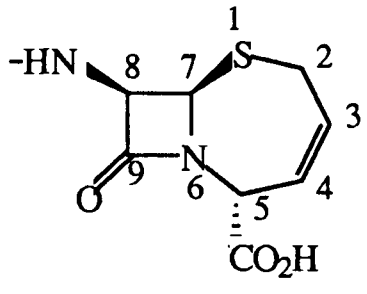
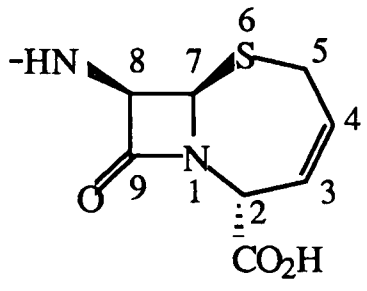
8.1 General Procedures 152
8.2 Experimental for Chapter 2 156
8.3 Experimental for Chapter 3 169
8.4 Experimental for Chapter 4 171
8.5 Experimental for Chapter 5 180
8.6 Experimental for Chapter 6 190
8.7 Experimental for Chapter 7 204

REFERENCES 214

ABBREVIATIONS

Me	methyl
Et	ethyl
^{t}Bu	*t*-butyl
Ph	phenyl
Ar	aryl
R	alkyl
Ts	tosyl
PMB	*para*-methoxybenzyl
PNB	*para*-nitrobenzyl
DTT	dithiothreitol
THF	tetrahydrofuran
TFA	trifluoroacetic acid
BzH	benzhydryl
Et_2O	diethyl ether
EtOH	ethanol
MCPBA	m-chloroperoxybenzoic acid
EEDQ	N-ethoxycarbonyl-2-ethoxydihydroquinoline
TMS	tetramethylsilane
TSP	sodium-2,2,3,3-tetradeutero-3-trimethylsilylpropanoate
D-αAA	D-α-aminoadipoyl
L-αAA	L-α-aminoadipoyl
TRIS-HCl	trishydroxymethylaminomethane hydrochloride
t.l.c	thin layer chromatography
p.l.c	preparative layer chromatography
h.p.l.c	high performance liquid chromatography
m.s.	mass spectrometry
DCI	desorption chemical ionisation
FAB	fast atom bombardment
FD	field desorption
TSP	thermaspray
EI	electron impact
n.m.r.	nuclear magnetic resonance
IPNS	isopenicillin N synthetase
DAOC/DAC	deacetoxycephalosporin C/deacetylcephalosporin C synthetase
DCM	dichloromethane
$CHCl_3$	trichloromethane
MeCN	acetonitrile
EtOAc	ethyl acetate
DMSO	dimethyl sulphoxide
DAOC	deacetoxycephalosporin C
DAC	deacetylcephalosporin C

NOMENCLATURE

NUCLEUS	CLASSICAL NUMBERING	IUPAC NUMBERING
PENAM		
CEPHEM		
CEPHAM		
HOMOCEPHAM		
HOMOCEPHEM		

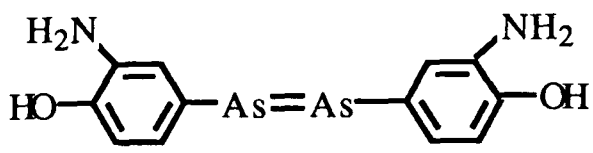
N.B. In the text (Chapters 1-7) the classical numbering system has been used for the sake of convenience, however, to describe fully the structures of the compounds occurring in the experimental section (Chapter 8) the IUPAC numbering system has been used alongside the classical description.

Chapter 1 : Biosynthetic Pathway to Penicillins and Cephalosporins.

1.1 Development of Antibiotics.

When micro-organisms were first discovered no one had sufficient scientific knowledge to even guess that they were responsible for many of the diseases prevalent at the time. It was to be a long time before the cause and effect relationship between micro-organisms and disease was recognised and still longer before suitable techniques were developed with which these organisms could be studied and the infections they caused controlled. The first documented attempts to do this were made by Lister, who used an aqueous spray of phenol to disinfect the area immediately adjacent to the surgical incision and in this way reduced the incidence of gangrene resulting from surgery.[1] Other substances such as formaldehyde and iodine were soon recognised to be effective as disinfectants but one problem with these chemicals was immediately apparent since not only did they kill bacteria but their reactivity and toxicity also led to tissue destruction. The necessity for less toxic but equally active antibacterial compounds was thus recognised.

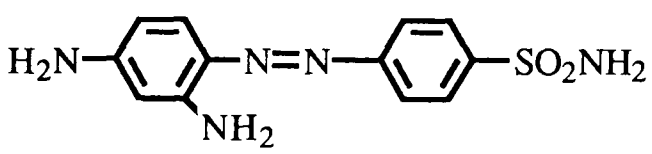
Although Arsphenamine (1) had been introduced by Ehrlich[2] in 1909, its use was limited principally to the treatment of syphilis. However, through the development of this and related compounds some direction was given to the search for antibiotic agents.



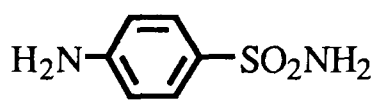
Arsphenamine (1)

It seems remarkable that some of the most important antibiotics now in routine use originate from micro-organisms, particularly as these substances were sought so that such organisms could be controlled. Despite the initial observations by Fleming[3] of the antibacterial properties of a mould of the fungus *Penicillium notatum* in 1928, no further progress was made in this field for several years. Indeed that Fleming lost interest in his discovery is readily apparent from what he later said : 'It [penicillin broth] was used in a few cases as a local antiseptic, but although it gave reasonably good results the trouble of making it seemed not worth while' [4]. Research into antibacterial agents was

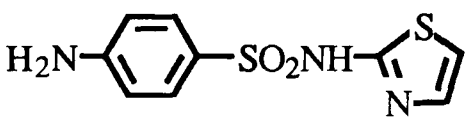
consequently focused not on penicillins but on other compounds. These included the sulphonamides such as Prontosil (2), Sulphanilamide (3), Sulphathiazole (4) and Sulphadiazine (5).



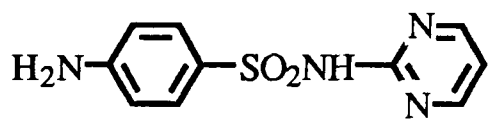
Prontosil (2)



Sulphanilamide (3)



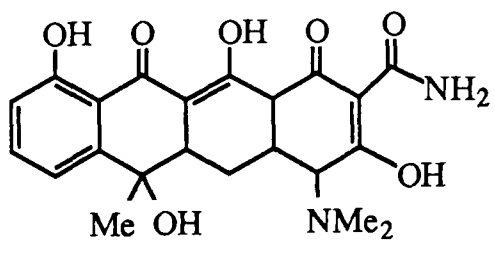
Sulphathiazole (4)



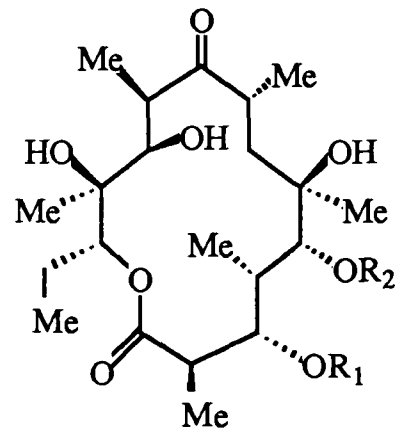
Sulphadiazine (5)

As a result of this effort, introduction of the first effective antibacterials occurred in the 1930's and 40's. With these drugs infections due to streptococci, gonococci and pneumonia as well as bacterial dysentery could be treated.

The eventual isolation of a crude sample of penicillin by Florey, Chain and co-workers[4] in Oxford together with its structural determination[5] a decade later, represent the first significant events in the comparatively short history of β-lactam antibiotics. Discovery and isolation of antibacterial agents produced by *Penicillium. notatum* inspired considerable effort to screen and identify other antibiotic producing organisms. Although the β-lactam antibiotics represent the most important class of anti-microbial fungal secondary metabolites so far known, other important compounds were also discovered in various micro-organisms. These metabolites include the tetracyclines *e.g.* (6), the macrocyclic antibacterials such as the erythromycins *e.g.* (7), streptomycins, polyacetylenes and the polypeptide antibiotics.



Oxytetracycline (6)



Erythromycin A (7)

[R_1 and R_2 = sugar residues]

Individually, these different classes of antibiotics are not as important as the β-lactams but due to the problems of bacterial resistance, represent essential alternatives in the treatment of bacterial infections.

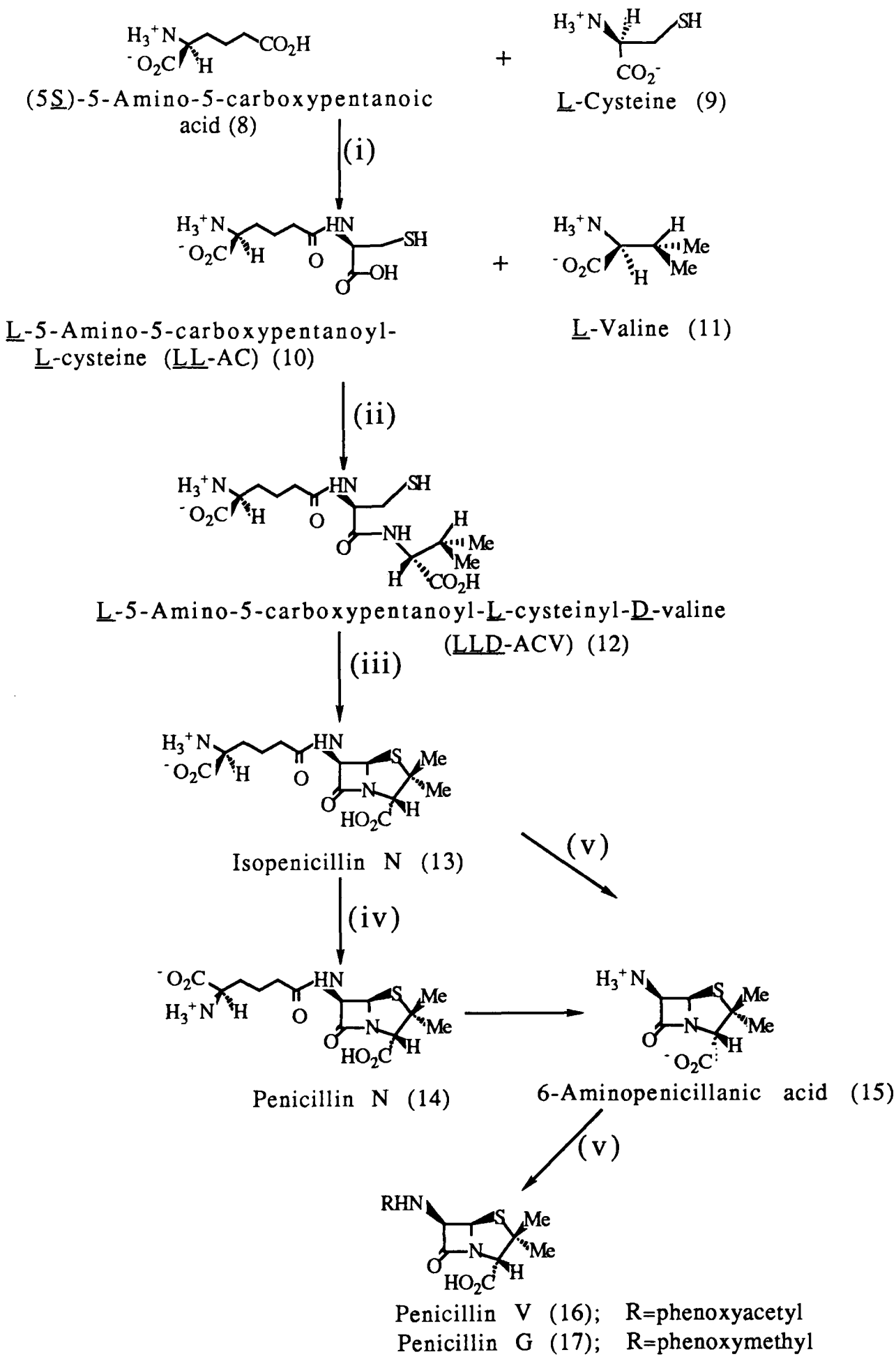
1.2. Biosynthesis of Penicillin N.

Many organisms are capable of producing penicillins and cephalosporins, a comprehensive survey of which has been reported[6], but the most important and frequently used, both industrially and in research laboratories are strains from *Penicillium* (penicillins only), *Cephalosporium* (penicillins and cephalosporins) and *Streptomyces* (penicillins, cephalosporins and cephamycins). The high producing strains used in commercial fermentation processes are generally highly selected strains of *Cephalosporium Sp.*

The full biosynthetic pathway to penicillin N (14) is shown below (Scheme 1.1.). Although the actual starting materials and products of each step are known, many of the mechanisms by which these transformations occur are not, and are consequently the subject of intensive investigation.

The first event in the biosynthetic pathway is the condensation of L-α-aminoadipic acid [(5S)-5-amino-5-carboxypentanoic acid] (8) with L-cysteine (9) to give a dipeptide (10),[7,8] a process catalysed by an enzyme which requires ATP. This process has been monitored by the release of inorganic phosphate but although downstream penicillin production was detected, no conclusive evidence that release of phosphate was associated with formation of the dipeptide was presented. It is however thought that the reaction proceeds by way of activation of the terminal δ-carboxyl group of L-α-aminoadipic acid (8) - a process believed to be analogous to that of the first stage in glutathione biosynthesis. Comparatively little is known about the enzyme itself and no molecular weight or sequence data is available.

The next enzyme in the pathway catalyses the addition of L-valine (11) to the dipeptide (10) to give the so-called Arnstein tripeptide, L L D-α-aminoadipoylcysteinyl valine (LLD-ACV) (12).[7,8] Although Arnstein[9] isolated this

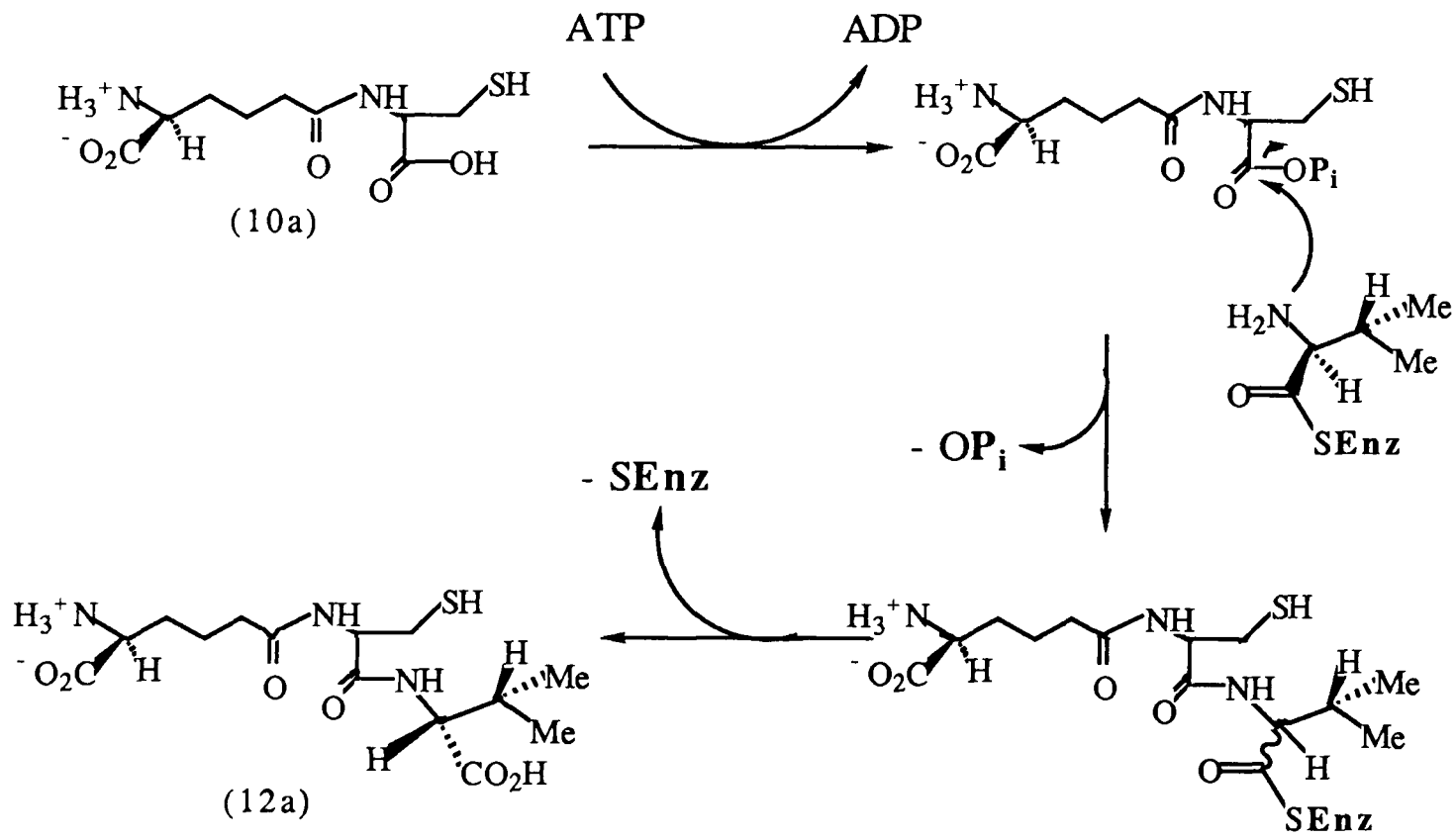


Key to Enzymes; (i) AC synthetase; (ii) ACV synthetase; (iii) Isopenicillin N Synthetase (IPNS); (iv) Epimerase; (v) Acylase/ Transacylase.

For 8-17 (a) = unlabelled compound,
(b) = specifically labelled compound.

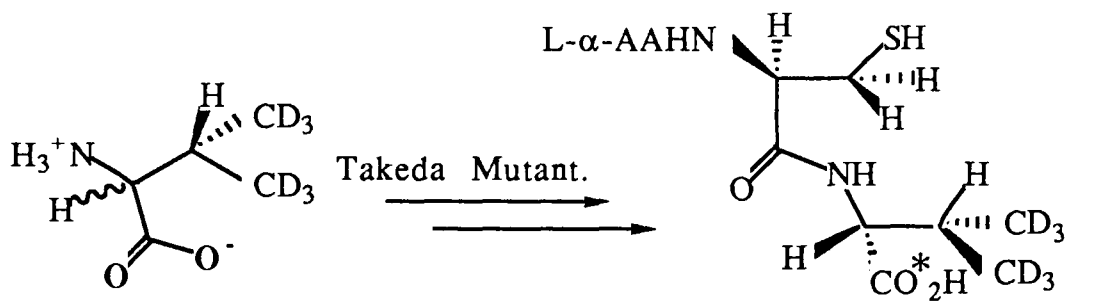
Scheme 1.1. Biosynthetic Pathway to Penicillin N (14).

tripeptide from the mycelium of *P. chrysogenum* and postulated that it was the immediate precursor of the penicillins, he was unable to assign the stereochemistry of the amino-acid residues or to prove conclusively that his prediction was correct. As for the previous enzyme, little physical data for the tripeptide synthetase enzyme has been determined. Evidence obtained from initial studies[10] in which ^{18}O-labelled valine was fed to intact cells of *P. chrysogenum*, was interpreted to imply that loss of one of the valinyl carboxyl oxygen atoms occurred during formation of LLD-ACV (12). This would seem to implicate formation of a thioester intermediate between (12) and the enzyme, which with subsequent inversion of configuration during product release would then give the observed D-valine containing tripeptide (12a) (Scheme 1.2.).



Scheme 1.2. Possible Mechanism of Tripeptide (12a) Formation.

These labelling studies have been repeated[11] but with a mutant which has the β-lactam pathway blocked at IPNS. Consequently LLD-ACV (12a) accumulates in the mycelium and fermentation broth and due to the absence of IPNS, is not consumed in the production of isopenicillin N (13a). Furthermore di-^{18}O labelled hexadeuterovaline (11b) was used so that endogenous LLD-ACV (12a) production could be distinguished from production with the labelled material (Scheme 1.3.).

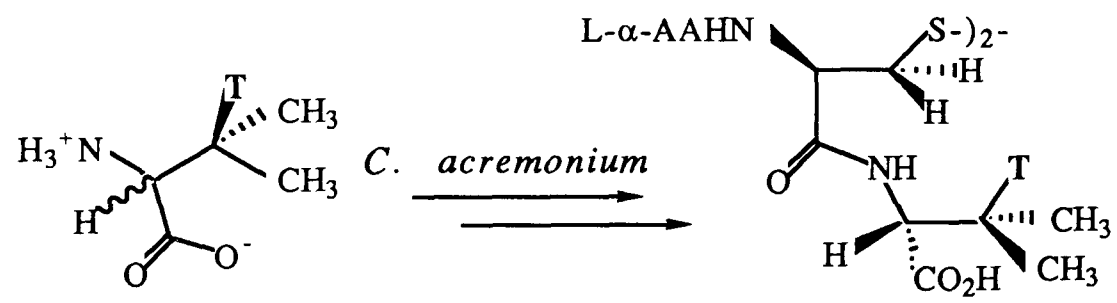


(11b, O = ^{18}O) (12b, O*= ^{16}O and/or ^{18}O)

Scheme 1.3.

These experiments have indicated that exchange or loss of label is not an essential step in the biosynthesis of (12a) since recovered (12b) showed loss of none, one, and in some cases both labelled oxygen atoms.

It has also been shown[12] that valine labelled with tritium in the β-postion (11c), retains its radioactivity when incorporated into LLD-ACV (12c) (Scheme 1.4.).



(11c) (12c)

Scheme 1.4.

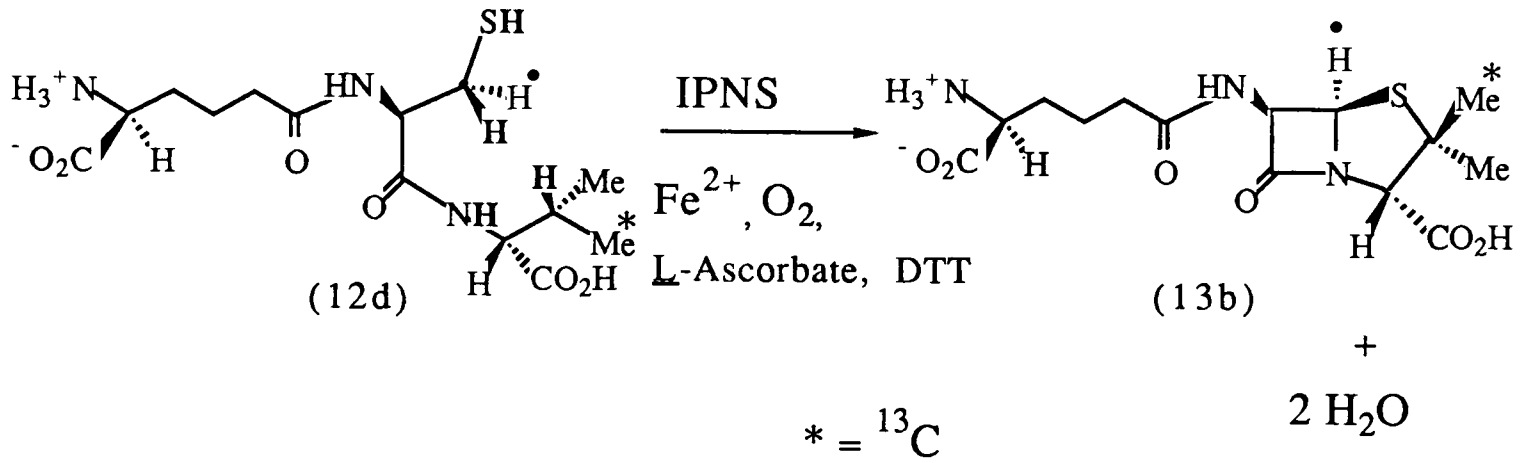
These observations tend to discount the involvement of a dehydrovaline intermediate such as had been proposed[11], either in the coupling to the dipeptide (10a) or during inversion of the configuration of L-valine (11a). Alternatively, these results imply that if a dehydrovaline intermediate is involved then replacement of the hydrogen or tritium atom removed during its formation must occur prior to release from the active site of the enzyme.

The seemingly trivial formation of LLD-ACV (12a) from its constituent amino-acids (8), (9) and (11a) still contains many difficult problems yet to solved.

Isopenicillin N synthetase (IPNS), the enzyme which converts the tripeptide (12a) to isopenicillin N (13a), has been the subject of intense investigation[13a,b]. This enzyme has been isolated from *C. acremonium* and the amino-acid sequence determined[14]. Once the sequence had been obtained, the protein was cloned into *E. coli* and expression obtained leading to production of recombinant IPNS.[15] The recombinant enzyme has a molecular weight of 38416 and contains 336 amino-acid residues. Experiments with recombinant enzyme

showed that it was identical to native wild type IPNS in terms of substrate specificity and product formation[16] (see Section 1.4).

IPNS is a dioxygenase enzyme catalysing the formation of the bicyclic fused β-lactam-thiazolidine ring system *via* oxidative removal of four hydrogen atoms (Scheme 1.5).



Scheme 1.5.

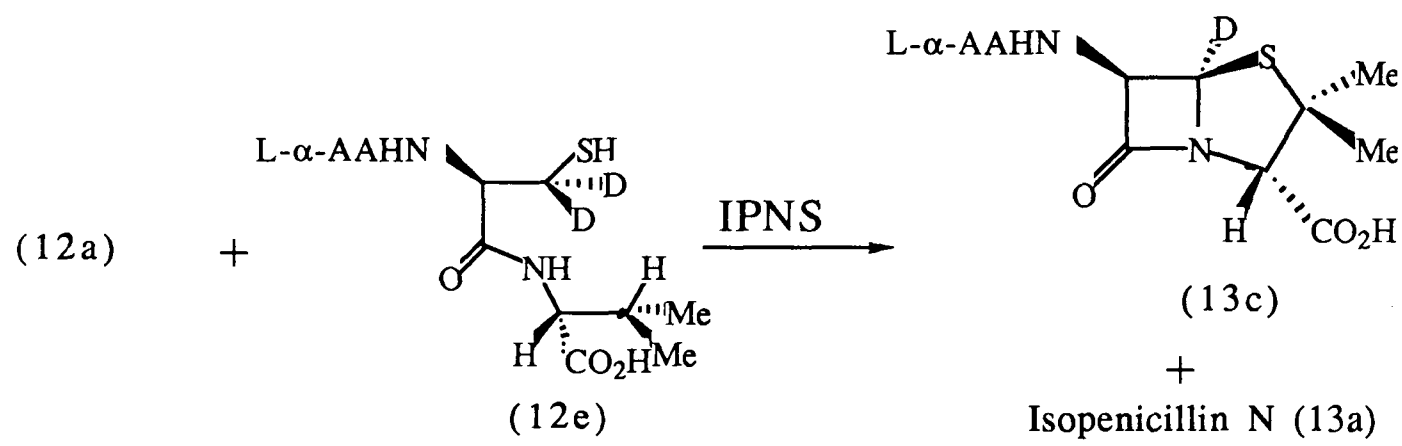
Molecular oxygen is required as a co-substrate[17,18], being consumed during the reaction to give ultimately water. Ferrous ions (Fe^{II}) and L-ascorbate are also needed as cofactors during the reaction[17,18]. For optimal activity, dithiothreitol (DTT) is required probably to maintain both the substrate (12a) and the enzyme itself in the active reduced form, with DTT being preferentially oxidized to its disulphide form. The stoichiometry of the cyclisation reaction has been examined with respect to substrate and molecular oxygen.[19] It was found that one mole of oxygen is consumed for every mole of ACV (12a) converted to isopenicillin N (13a). Since this enzyme doesn't contain a porphyrin ring system, as shown by cloning studies[15], classification into a group of non-haeme dioxygenases seems reasonable.

The *in vitro* turnover number has been estimated[20] as *ca* 400 - implying that on average every enzyme molecule catalyses the conversion of *ca* 400 substrate molecules before inactivation. This figure is much lower than the apparent *in vivo* turnover number and has led to speculation with regard the existence of a 'protector' protein, since the quantity of protein required by the cell to produce the levels of penicillins and cephalosporins observed with the *in vitro* turnover efficiency is much higher than the actual levels of protein found *in vivo*. Such a suggestion is not unreasonable particularly as a protector protein whose function is to prevent enzyme inactivation caused by a nonenzymatic Fe^{3+} / O_2/ thiol mixed function oxidase system has recently been reported[21].

Investigation of the stereochemical nature of bis-ring formation has shown that both rings are formed in a highly stereospecific manner[22,23]. In β-lactam

ring formation, the cysteinyl C-3 pro-S hydrogen atom is removed stereospecifically, as demonstrated[22] by isotope labelling studies with (12d) (Scheme 1.5). Furthermore, thiazolidine ring closure[23] proceeds with the pro-R methyl of the valinyl residue of LLD-ACV (12d) becoming the pro-S methyl of isopenicillin N (13b) (a more detailed account of this work is given in the introduction to Chapter 2).

Mixed competitive kinetic isotope experiments[24] with specifically deuterated LLD-ACVs (12e) and (12f) (see Scheme 1.6) which were either deuterated in the C-3 cysteinyl or C-3 valinyl positions in conjunction with unlabelled LLD-ACV (12a) gave results consistent with formation of the β-lactam ring prior to the thiazolidine ring closure.



Scheme 1.6. Mixed Labelled/Unlabelled Substrate Competitive Kinetic Isotope Experiments with IPNS.

Enzymatic discrimination between the labelled and unlabelled substrates will only occur in the case where the first irreversible event is subject to an isotope effect[25]. Since formation of isopenicillin N (13a) from (12f) exhibits no mixed competitive isotope effect, as demonstrated by a lack of discrimination between (12a) and (12f), this would suggest that the ring forming event at the valinyl C-3 position occurs subsequent to the event at C-3 of cysteine. This implies

that the ring closures take place in a non-concerted (stepwise) manner and proceeds *via* a monocyclic enzyme-bound intermediate. A more detailed account of competitive mixed labelled/unlabelled substrate kinetic isotope effect experiments will be given later (see Chapter 4).

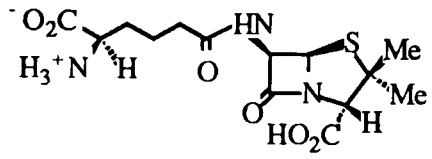
Before ring expansion of the penicillin nucleus to that of the cephems can proceed, the configuration of the L-α-aminoadipoyl side chain must be inverted to the D-configuration. The epimerase activity from *C. acremonium* responsible for this conversion was first detected in cell free preparations by Konomi *et al.*,[13b] and is seemingly very unstable - it has been reported[26,27] that crude extracts lose between 70-80% of the total activity present when stored overnight at -80°C. This instability has made characterisation of the enzyme very difficult and little is known about its cofactor requirements, molecular weight or amino-acid sequence. Further difficulties are encountered due to the lack of a satisfactory assay, the current procedure being based on differential bioassay, exploiting the observation that some bacterial strains are more sensitive to penicillin N (14) than isopenicillin N (13) and *vice versa* .

Work on this important enzyme has been concentrated on the more stable epimerase from *S. clavuligerus*. Initial reports[28,29] estimate that this enzyme is stabilised by pyridoxal phosphate but it is not known whether this compound is an essential cofactor. Other cofactor requirements have not yet been determined but the molecular weight has been estimated to be *ca* 60,000.

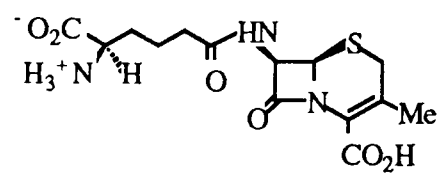
1.3. Biosynthetic Pathway to Cephalosporins and Cephamycins.

The deacetoxycephalosporin C / deacetylcephalosporin C synthetase enzyme (DAOC/DAC synthetase) catalyses the conversion of penicillin N (14) to deacetoxycephalosporin C (DAOC) (18) and thence to deacetylcephalosporin C (DAC) (19). and This enzyme requires α-ketoglutarate[30] and molecular oxygen as co-substrates and ferrous ions, and L-ascorbate[31] as cofactors (Scheme 1.7). For maximal activity DTT and ammonium sulphate appear to be benificial.

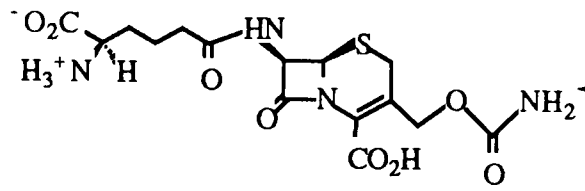
In *C. acremonium* the co-substrate and cofactor requirements essential for stimulation of hydroxylase activity are identical[32,33] to those required for ring expansion activity and thus led to speculation that one enzyme was responsible for both activities[34]. Such speculation was reinforced by the inability to separate the activities by ion exchange and fast protein liquid chromatography. In



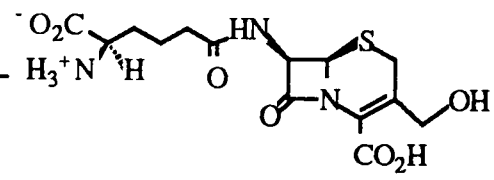
Penicillin N (14)



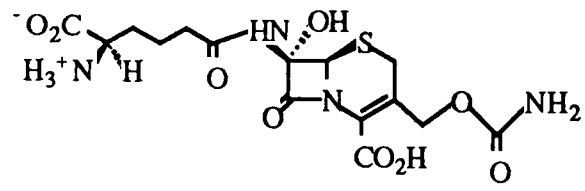
Deacetoxycephalosporin C (DAOC) (18)



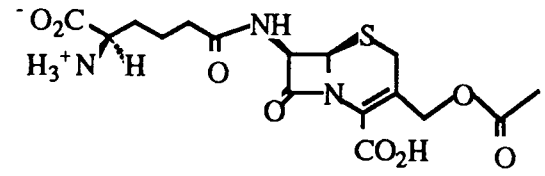
Q-carbamoyldeacetylcephalosporin C (21)



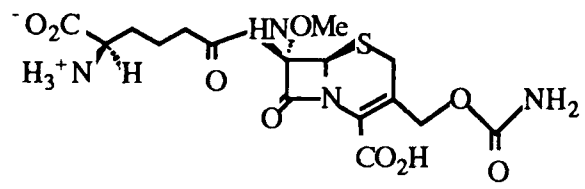
Deacetylcephalosporin C (DAC) (19)



7-Hydroxy-Q-carbamoylcephalosporin C (22)



Cephalosporin C (20)



Cephamycin C (23)

For 14-23 (a) = unlabelled compound,
(b) = specifically labelled compound.

Key to Enzymes; (i) DAOC/DAC synthetase, Fe^{2+} , O_2 , α-Ketoglutarate, L-Ascorbate , DTT ; (ii) Acetyltransferase; (iii) Carbamoyltransferase; (iv) Cephamycin hydroxylase; (v) Cephamycin methyltransferase.

Scheme 1.7. Biosynthetic Pathway to Cephalosporin C (20) and Cephamycin C (23).

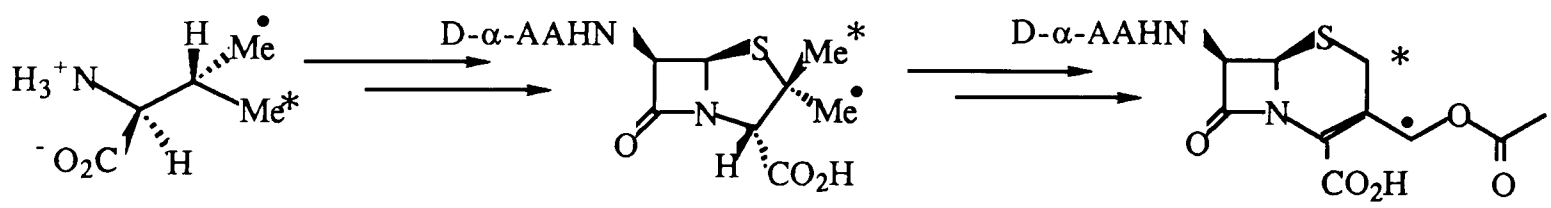
S. clavuligerus however, both activities were separated by ion exchange chromatography and thus shown to be due to two different proteins.[35]

Purification of the DAOC/DAC synthetase from *C. acremonium* to near homogeneity[36] and partial sequence determination was followed by cloning[37] and expression into *E. coli.* Sequence determination was hampered since the N-terminal appeared to be blocked and furthermore, expression of this protein in *E.coli* led to formation of cytoplasmic exclusion bodies. Formation of such granules

can occur with recombinant proteins particularly if the protein being expressed is cytotoxic. Although high levels of protein expression were observed, only small amounts of soluble protein could be obtained and unsuccessful attempts were made to solubilise the exclusion bodies with Triton X-100. It has since been shown that treatment of the exclusion bodies with 6M urea - conditions more usually associated with enzyme denaturation - leads to good recovery of soluble active protein.[38] Furthermore, the recovered protein (mol. wt. *ca.* 40,000), has been shown to possess both ring expansion and hydroxylation activities thus confirming that in *C. acremonium* a single bifunctional enzyme catalyses the conversion of penicillin N (14) to DAOC (18) and thence to DAC (19). Since *S. clavuligerus* is a prokaryotic organism and *C. acremonium* is eukaryotic, the combination of the proteins catalysing the ring expansion and hydroxylation in *S. clavuligerus* into a single protein such as that found in *C. acremonium*, may reflect an evolutionary event.[39]

Intensive research into the ring expansion enzyme has been undertaken over the past two decades in order to determine the mechanism by which the thiazolidine ring of penicillin N (14) is converted to the dihydrothiazine system of DAOC (18). The large effort so far expended reflects the commercial importance of cephalosporins as broad spectrum antibiotics (see Figure 1.1, Section 1.5).

Several stereochemical aspects of the ring expansion process have been studied from which mechanistic information and an indication as to the types of enzyme-bound intermediates present have been obtained. Initially, the stereochemistry of incorporation of the penicillin N (14a) methyl groups into cephalosporin C (20) was determined [40,41,42] by feeding stereospecifically labelled valines (11d) and (11e) to intact cells of *C. acremonium* (see Chapter 3) and was shown to proceed with retention of configuration (Scheme 1.8).

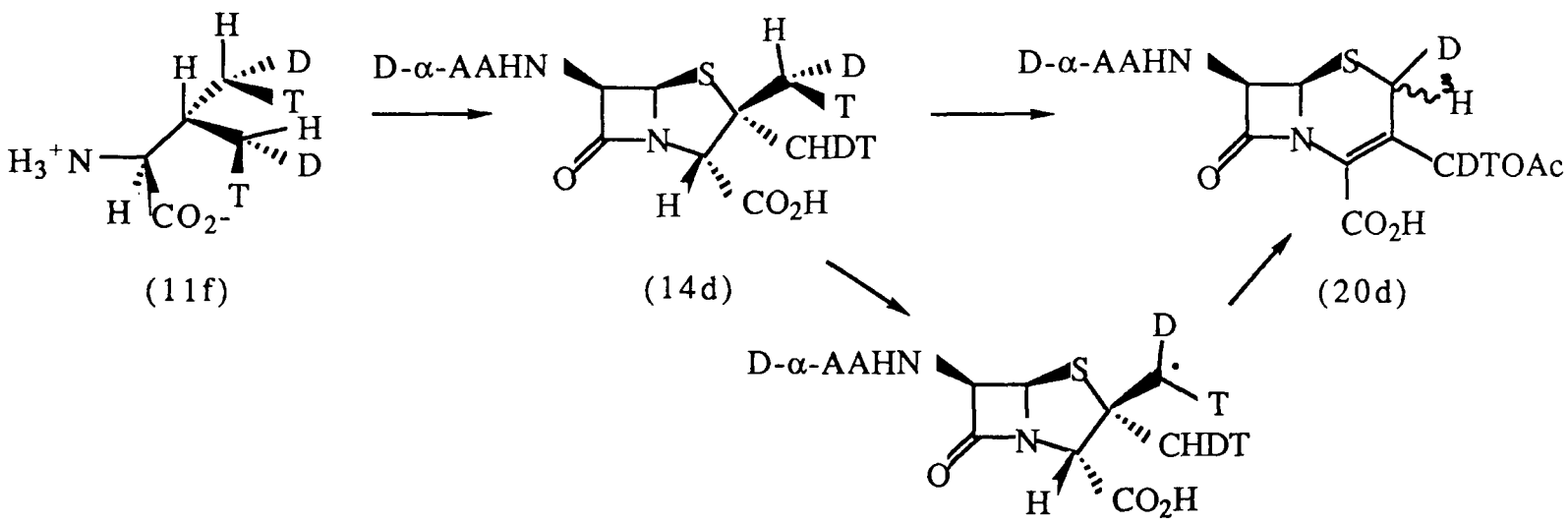


(11d, *= ^{13}C, e; • = ^{13}C)

(14b; * = ^{13}C, c; • = ^{13}C)

(20b; * = ^{13}C, c; • = ^{13}C)

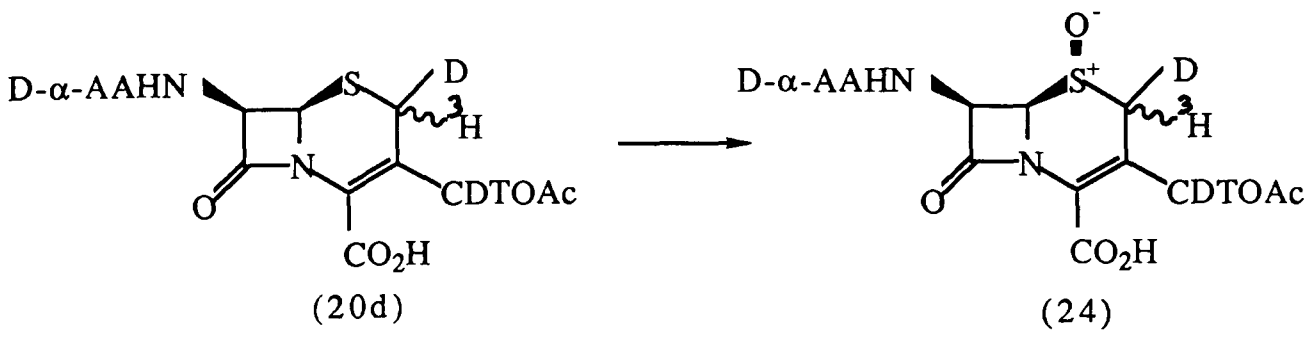
Scheme 1.8. Stereochemistry of Penicillin N (14) Methyl Group Incorporation into Cephalosporin C (20).



Scheme 1.9. Stereochemistry of Penicillin N (14d) β-Methyl Incorporation into Cephalosporin C (20d).

In addition the stereochemical course of the reaction at the β-methyl group was determined [43,44] by feeding the corresponding stereospecifically labelled valines to intact cells of *C. acremonium* and consequently leading to incorporation of a chiral methyl group into the penicillin N (14d) and ultimately cephalosporin C (20d) (Scheme 1.9).

The labelled cephalosporin C (20d) was isolated and the stereochemistry of incorporation determined in two different ways. One method used was to examine the ^{3}H-n.m.r spectrum of the cephalosporin C (20d).[43] Prior assignment of the C-2 methylene AB quartet enabled the position of tritium incorporation to be determined. The other method used was to oxidise the cephalosporin C (20d) to its sulphoxide (24) and to then correlate the release and retention of radioactivity with the distribution of tritium in the C-2 position (Scheme 1.10).[44] This was achieved on the predetermined basis that in the sulphoxide (24) the C-2 α-proton (pro-R) is ten times more acidic than the β-proton (pro-S) and hence will be exchanged approximately ten times faster.

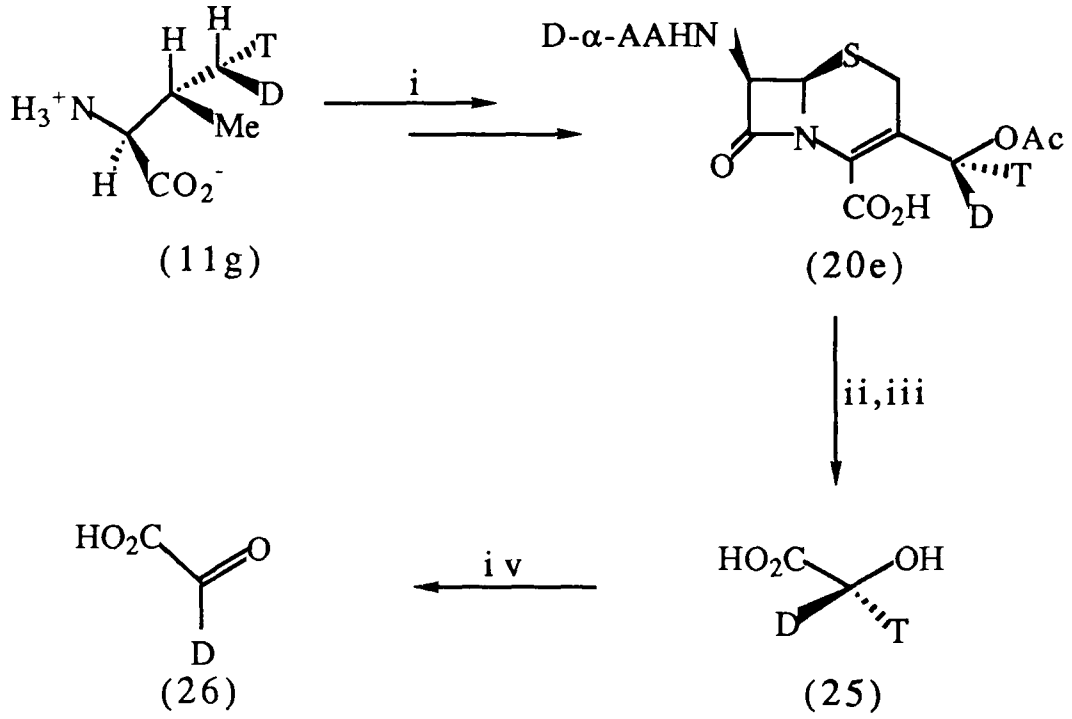


Scheme 1.10.

Thus by monitoring the rate of release of tritium, the approximate distribution in the pro-R and pro-S C-2 positions could be evaluated and the stereochemistry of incorporation assigned.

The stereochemistry of the hydroxylation of DAOC (18) to DAC (19) has also been investigated by feeding valine (11g), which contained a chirally labelled methyl group, to intact cells of *C. acremonium*.[45] Stereochemical analysis was achieved by degradation of the cephalosporin C (20e) initially by ozonolysis and saponification to glycolic acid (25) (Scheme 1.11) and then by enzymatic conversion of (25) to glyoxylic acid (26) with glycolate oxidase. The latter transformation is known to proceed with stereospecific loss of the pro-R proton.

Consequently by monitoring the loss of radioactivity to the medium, the stereochemistry of incorporation of the valine (11g) into (20e) was determined and was shown to have occurred with retention of configuration. This is an interesting result when considered in conjunction with the observed loss of

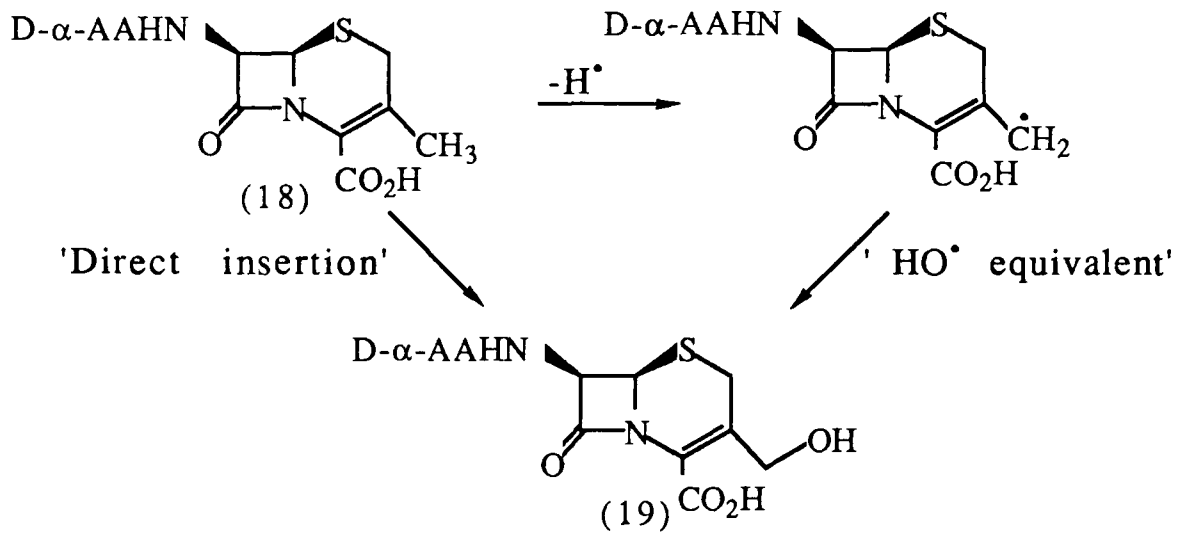


(11g) (20e) (26) (25)

Key: i) *C. acremonium* ; ii) O_3, H_2O ; iii) NaOH ; iv) glycolate oxidase, catalase.

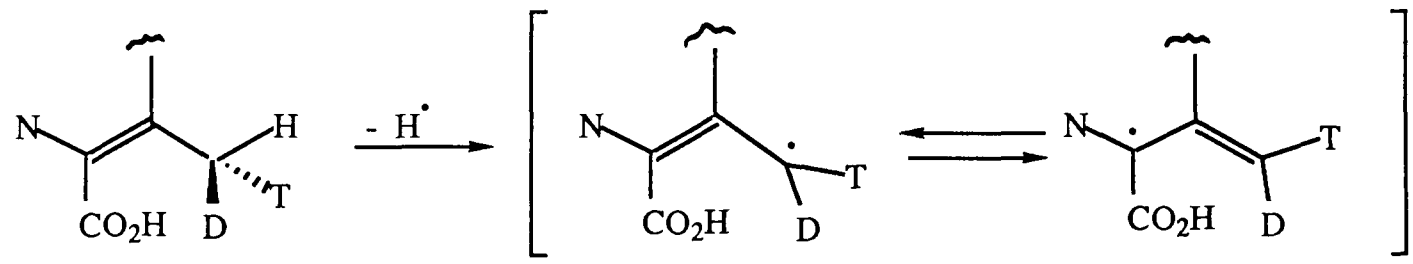
Scheme 1.11.

stereochemistry at the β-methyl position during ring expansion particularly since the same protein is responsible for both of these chemical transformations. However, these are two chemically distinct processes - one involving oxidase type activity and the other mono-oxidase activity - and thus the observed difference



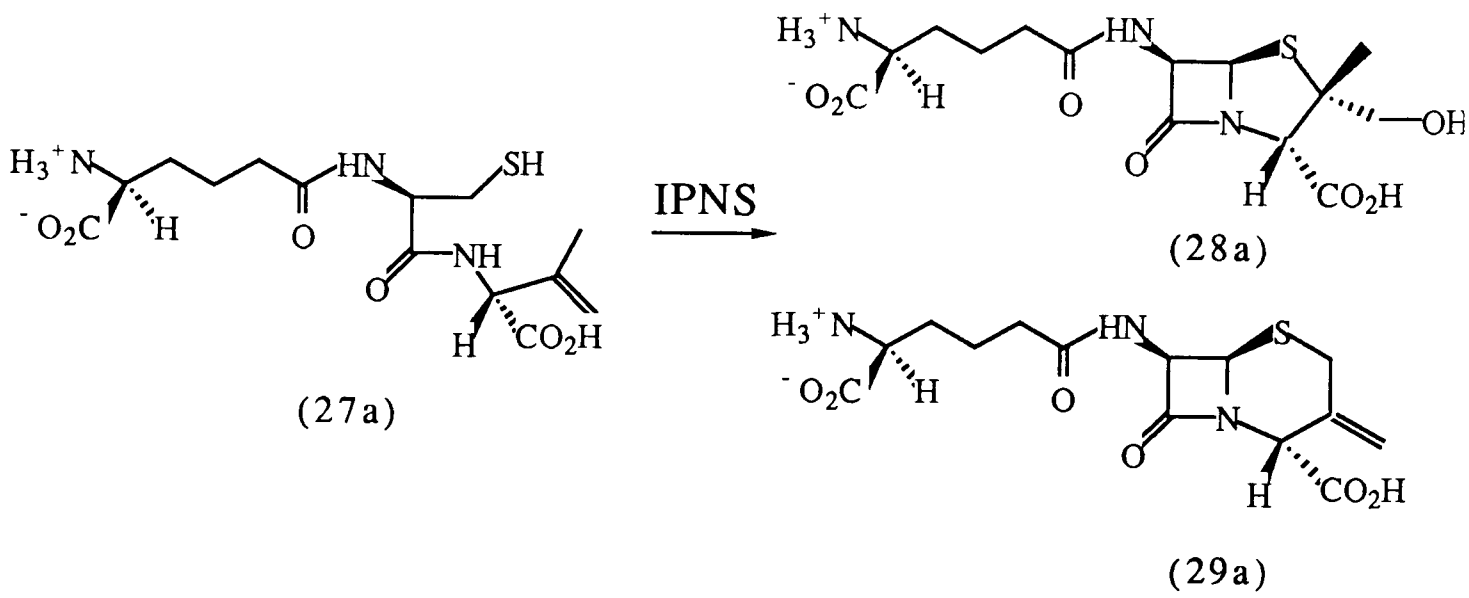
Scheme 1.12.

in stereochemistry is perhaps not so strange. To explain retention of configuration during hydroxylation one must consider that either direct insertion of oxygen into the C-H bond occurs or alternatively that a torsiometric radical is formed (Scheme 1.12). Such a radical could be stabilised by delocalisation (Scheme 1.13.) and/or the protein itself may restrict rotation thereby preventing loss of stereochemistry.



Scheme 1.13.

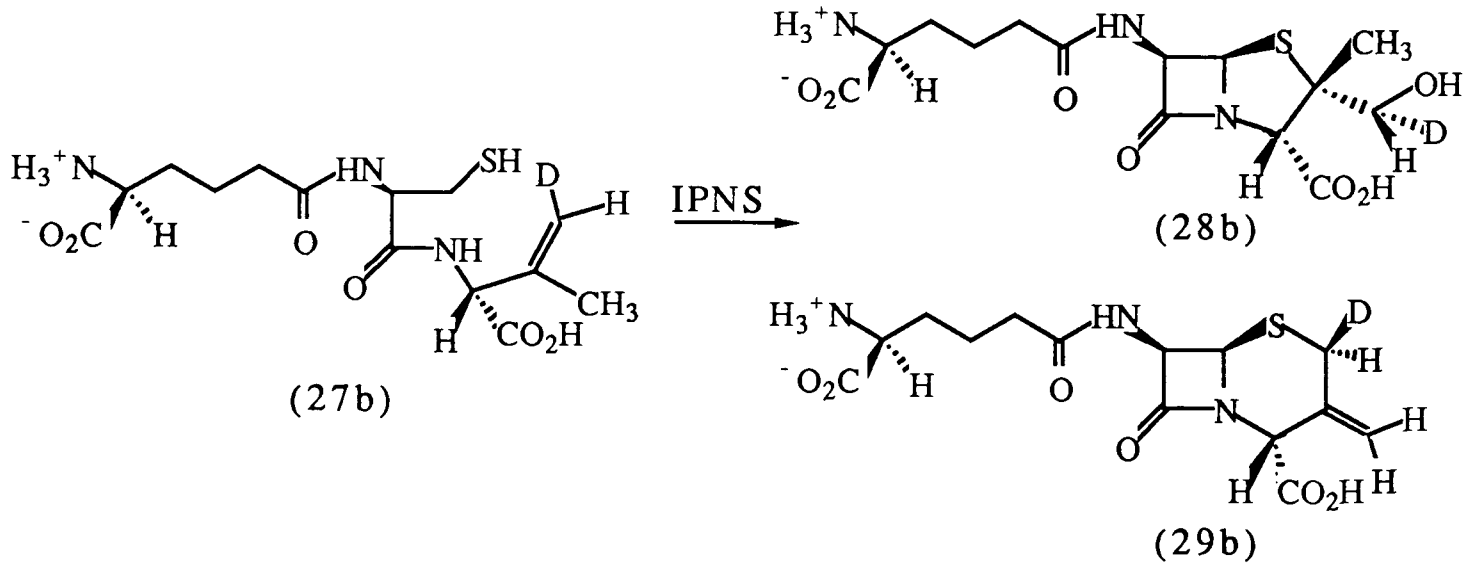
It is interesting to draw a comparison between the observed stereochemistry of hydroxylation by IPNS of the unnatural substrate, LLD-AC-isodehydrovaline tripeptide (27a)[46] (Scheme 1.14.) and the retention of configuration during hydroxylation of DAOC (18).



(27a) (28a) (29a)

Scheme 1.14.

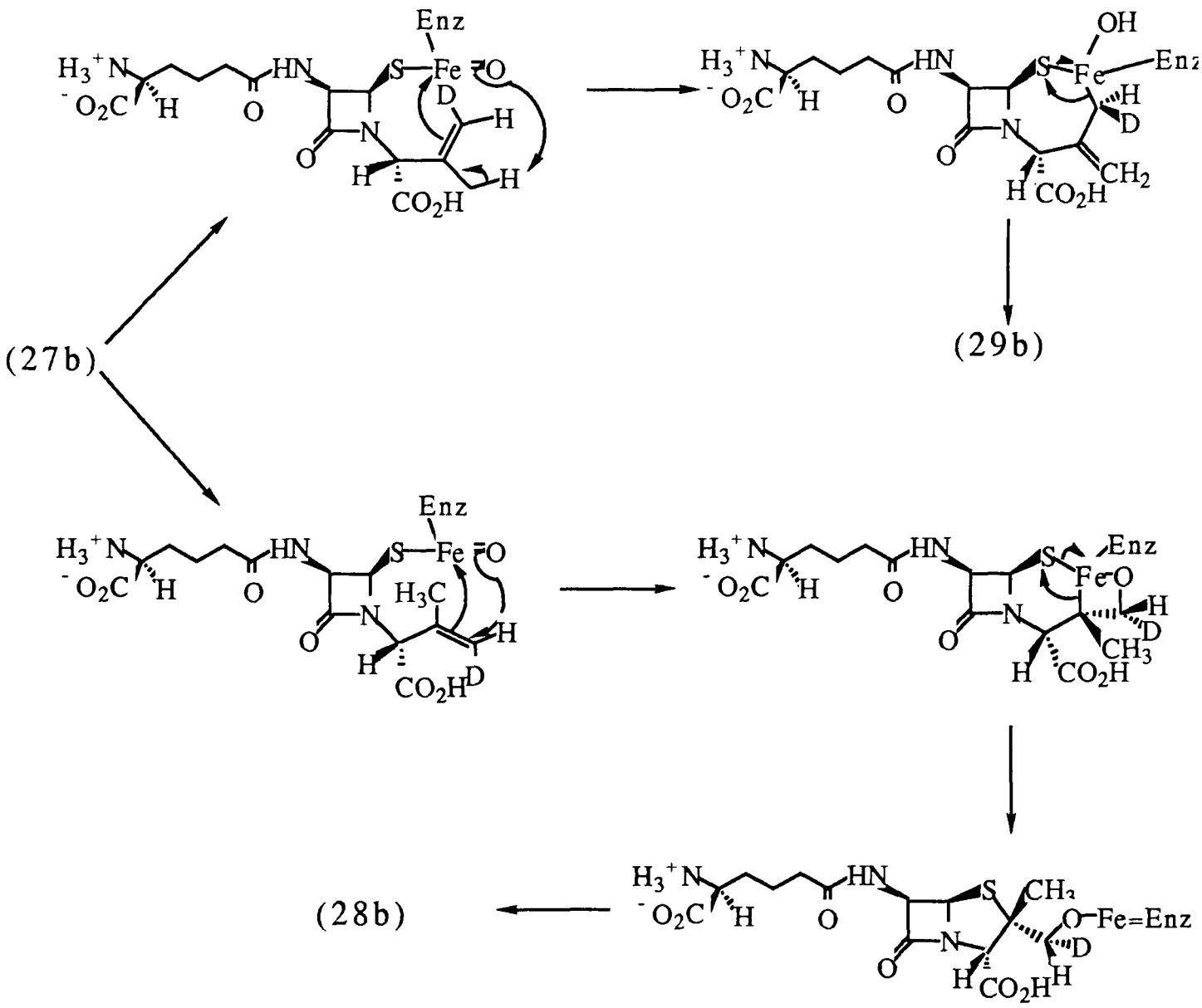
Recent labelling studies [47] with (27b) have shown that the hydroxylative pathway proceeds with greater than 95% retention of configuration (Scheme 1.15).



(27b) (28b) (29b)

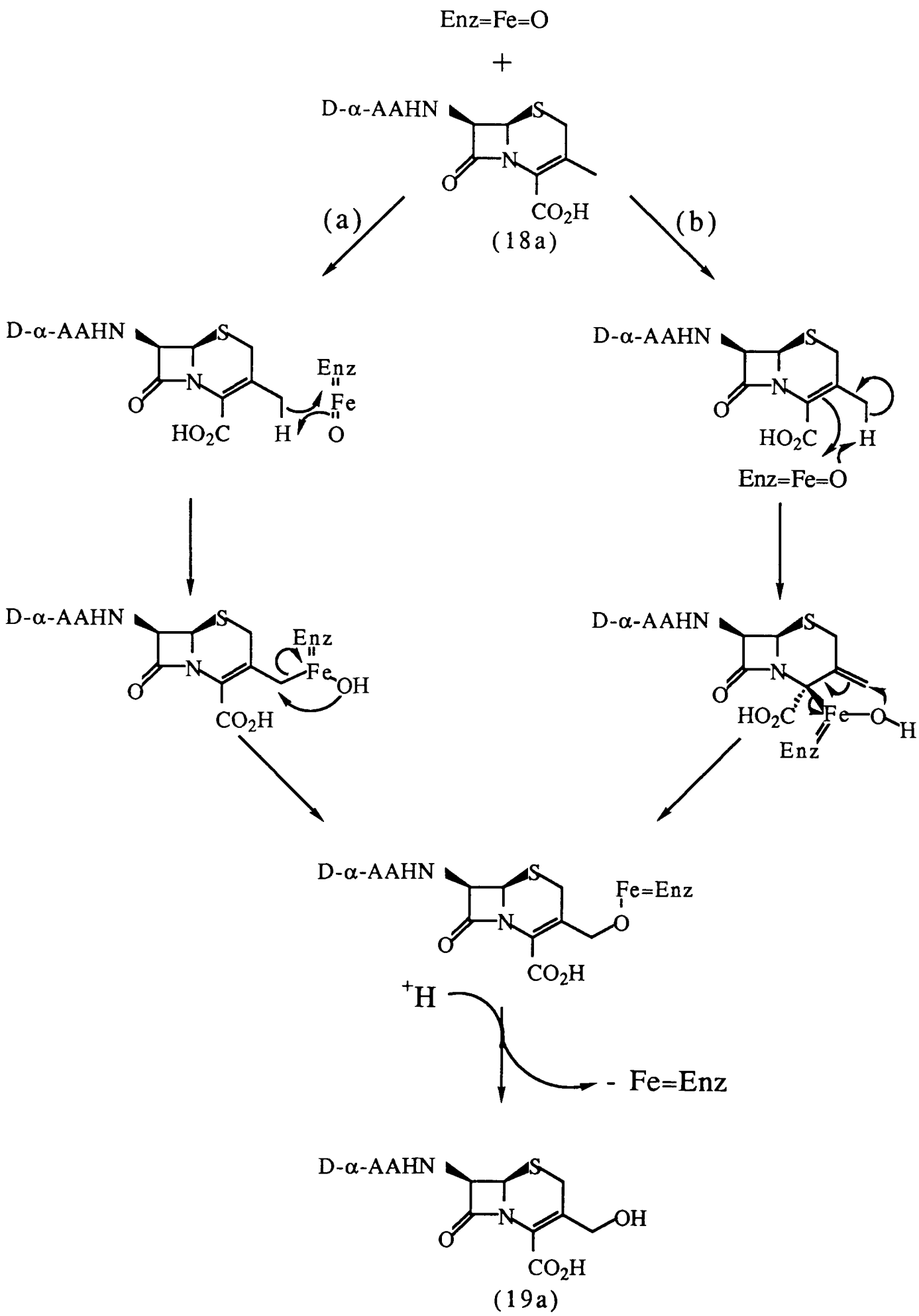
Scheme1.15.

This observation has been interpreted mechanistically to imply formation of a discrete carbon-iron bond (Scheme 1.16.), and supports the suggestion that an



Scheme 1.16.

analogous oxene type intermediate could be involved in the conversion of DAOC (18) to DAC (19) (Scheme 1.17.), *via* route (a) direct insertion and reductive elimination or route (b) ene type reaction followed by a (2,3) shift.



Scheme 1.17.

1.4. IPNS Substrate Analogue Studies.

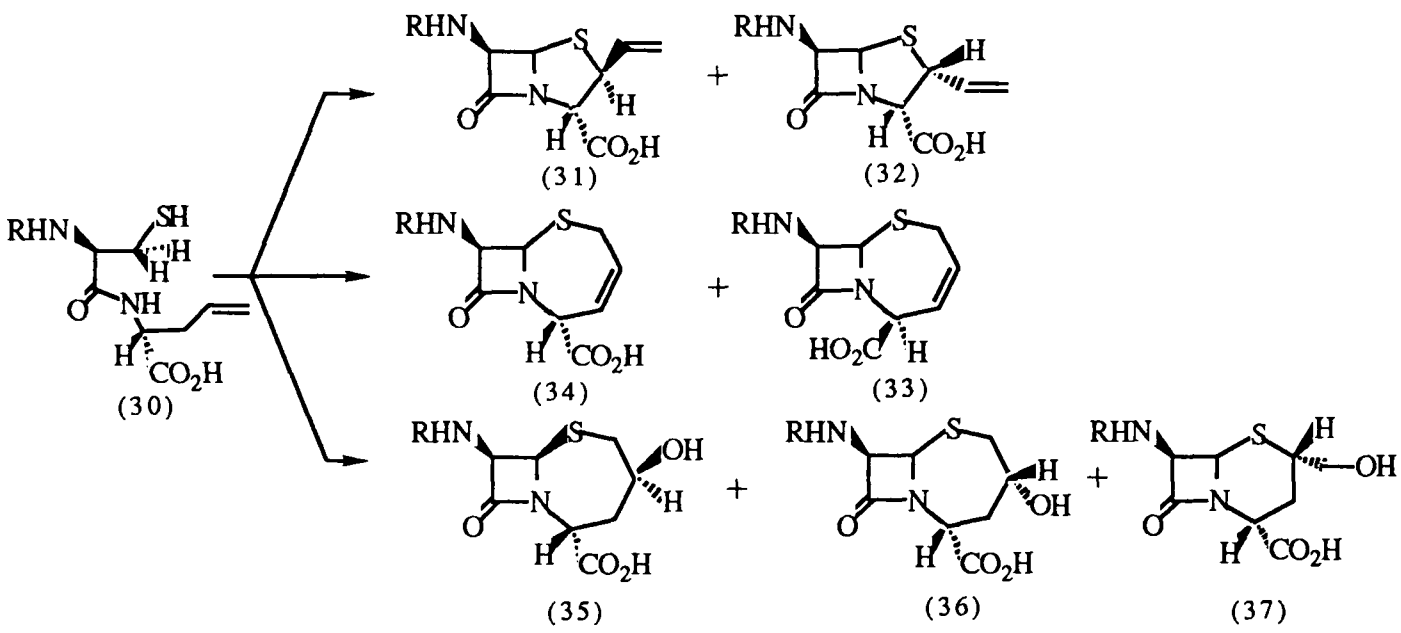
Extensive substrate analogue studies have been performed during the last decade and these have shown that IPNS is remarkably tolerant to substrate modification. Alteration or replacement of the valinyl residue has produced a

wide range of tripeptides, a considerable number of which are substrates for IPNS and give rise to a variety of novel products. The types of product formed can be classified into two groups :

i) those arising by overall desaturation (-4 H), and

ii) those from overall desaturase/mono-oxygenase type activity (-2 H, +1"O").

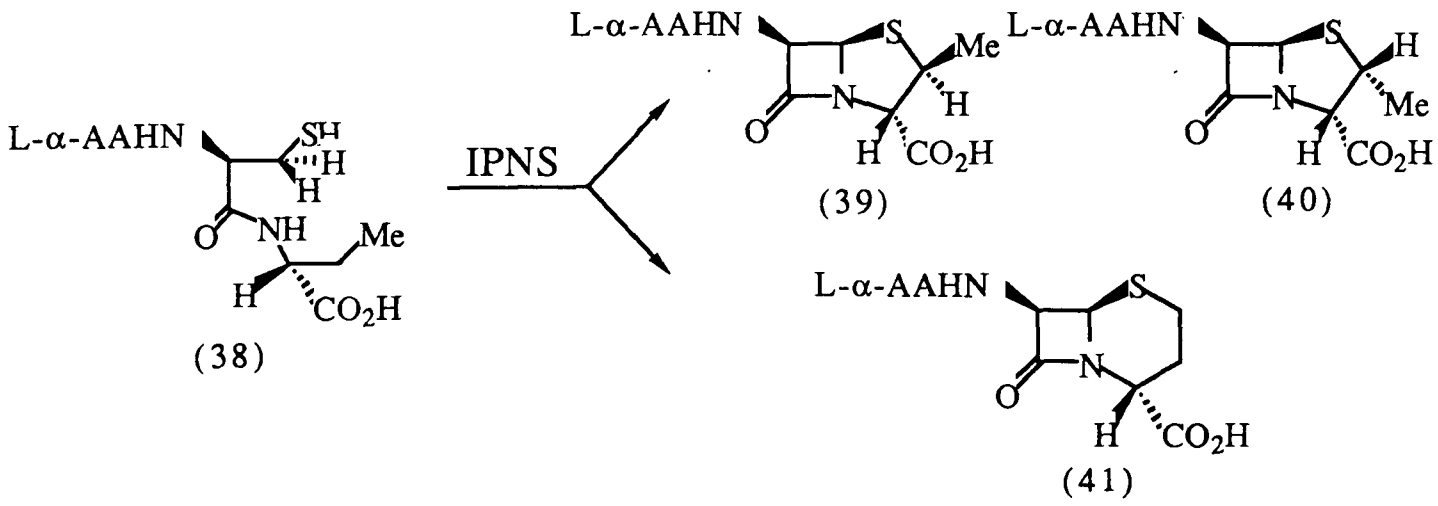
This is best illustrated with the D-allylglycine containing tripeptide (30)[48] (Scheme 1.18).



R = (5S)-5-Amino-5-carboxypentanoic Acid

Scheme 1.18.

The aminobutyrate containing tripeptide (38) is converted to a mixture of the penicillins (39) and (40) and to the cepham (41) (Scheme 1.19.), these products, (39), (40), and (41) being formed in a relative ratio of 7:1:3. [49]



Scheme 1.19.

With recombinant enzyme and the aminobutyrate peptide (38), the products (39), (40) and (41) were obtained in the same ratio as for the native enzyme, thus

suggesting that the two proteins (wildtype and recombinant IPNS) are identical in terms of substrate specificity and product formation.[16] Rationalisation of the results obtained from substrate analogue studies in mechanistic terms has led to a greater understanding of the process by which IPNS catalyses the conversion of LLD-ACV (12) to isopenicillin N (13). However, certain mechanistic aspects of this conversion still need to be clarified and in an attempt to resolve these areas of uncertainty, work is being undertaken to try and observe enzyme-bound intermediates, for example by the use of solid phase ^{13}C-n.m.r studies.

1.5. Development of β-Lactam New Antibiotics.

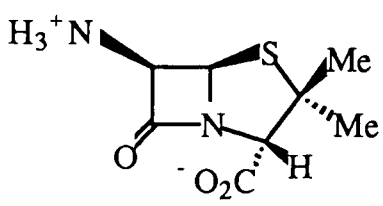
Cephalosporins are, in general terms, considered more important than penicillins due to the greater stability of the former to β-lactamases and acidic conditions. The observation that the cephalosporins are more stable to acidic conditions than penicillins is reflected in the availability of a variety of orally administered cephalosporins but comparatively few similar penicillin preparations. Consequently cephalosporins are of greater commercial value than penicillins or indeed any other individual class of antibiotics (see Figure 1.1).[50]

Antibiotic	Value 1985	Predicted Value 1990 (Millions $)
Injectable Antibacterials	**1388**	**1518**
Cephalosporins (Total)	765	888
1st generation	207	154
2nd generation	342	350
3rd generation	216	384
Gram positive drugs	180	160
Aminoglycosides	121	85
Extended Spectrum Penicillins	193	85
Monobactams	0	60
Penems and Carbapenems	0	95
Other	129	45
Oral Antibacterials	**1109**	**1509**
Cephalosporins	446	645
Tetracyclines	136	123
Erythromycins	144	162
Amoxycillin	59	75
Quinolones	0	155
Other	240	174
TOTAL	**2497**	**3027**

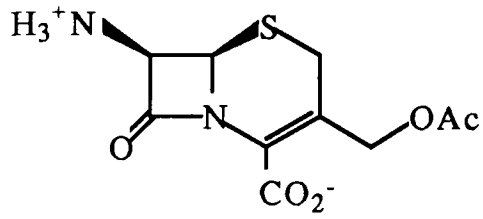
Figure 1.1. World Antibiotic Production.

Once the potential value of the β-lactam class of antibiotics had been recognised, intensive research into the biosynthesis of penicillins and cephalosporins was undertaken. Some of the results obtained from this research have already been discussed in the preceeding sections, but overall, this work has been directed towards the elucidation of the biosynthetic pathway and the subsequent optimisation of antibiotic production. Early on in this work it was discovered that by the introducion of various side chain precursors into the culture medium a selection of penicillins could be produced in preference to the normally formed benzyl- or 2-pentenylpenicillin products.[51] These precursors were in general mono-substituted acetic acids such as phenylacetic acid and phenoxyacetic acid.

From these efforts strains of the producing organisms have been selected and developed capable of producing very much higher yields of penicillins and cephalosporins. The efficiency of antibiotic production by fermentation with these high producing organisms has meant that chemical synthesis, as a source of these antibiotics, is commercially uncompetitive. However, second generation β-lactam antibiotics are produced *via* chemical modification of fermentation produced 6-aminopenicillanic (15) or 7-aminocephalosporanic acids (42).



(15)



(42)

Despite rapid development in the field of antibiotic technology problems still exist. The most important area of current concern is the rapid and widespread increase in bacterial resistance, a problem exaggerated by the ability of bacteria to exchange beneficial DNA fragments (plasmids) between each other. This, in conjunction with the often indiscriminate use of antibiotics has resulted in multiply resistant organisms - bacteria which are resistant to all but a few antibiotic agents. To understand bacterial β-lactam resistance, a basic knowledge of bacterial cell wall biosynthesis and the mode of action of penicillin is required.

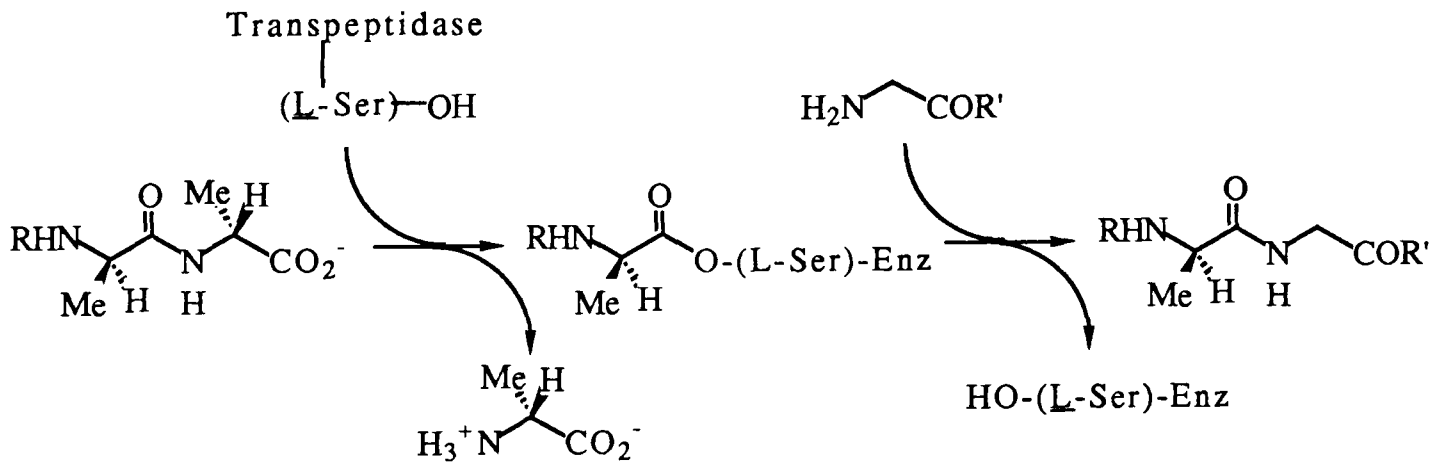
Bacteria are classified into two groups, gram-negative and gram-positive. This classification was initially an empirical distinction and made on the basis of whether the bacteria were stained by the violet dye used by Gram.[52a] The reason for the difference in staining is now understood.[52b,c] Gram-positive bacteria have two components to their cell envelopes - a peptidoglycan outer layer and an internal plasma membrane. The cell envelope of gram-negative bacteria are slightly more complex due to an additional membrane covering the peptidoglycan.

This outermost layer is approximately 80 Å thick and consists of protein, lipid and lipopolysaccharide.

The peptidoglycan consists of linear polysaccharide chains which are cross linked to one another by short peptides to give rise to a single enormous macromolecule. There are three repeating units found in the peptidoglycan which are :

i) a disaccharide of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in β-1,4-glycosidic linkage;
ii) a tetrapeptide of L-alanine, D-glutamate, L-lysine and D-alanine, and
iii) a pentaglycine bridge peptide.

In the intact peptidoglycan, NAG and NAM alternate in sequence to form a linear polysaccharide chain. The pentaglycine peptide then cross-links with NAM residues on different polysaccharide strands. The amino-group of $(Gly)_5$ forms a peptide bond with the carboxyl group of D-alanine and the carboxyl group of $(Gly)_5$ forms a peptide bond with the side-chain ε-amino group of L-lysine. Formation of the peptide bond between the amino-group of $(Gly)_5$ and the carboxyl group of a D-alanine residue on the polysaccharide chain, is catalysed by a glycopeptide transpeptidase, and results in the cleavage of the terminal D-alanine residue (Scheme 1.20.). It is this transpeptidation process which penicillin inhibits.

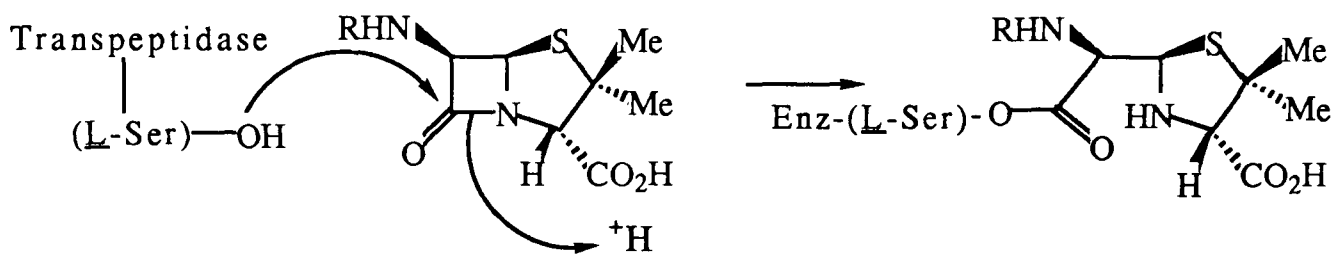


Key : R = peptidoglycan,
R'= pentaglycine bridge peptide

Scheme1.20. Transpeptidation Reaction in Bacterial Cell Wall Biosynthesis.

The transpeptidase normally forms an acyl intermediate with the penultimate D-alanine residue, which then reacts with the terminal glycyl amino-group of the peptide with which the cross-linkage is to be formed. Penicillin, which is believed to be structurally similar to the D-ala-D-ala terminus of the

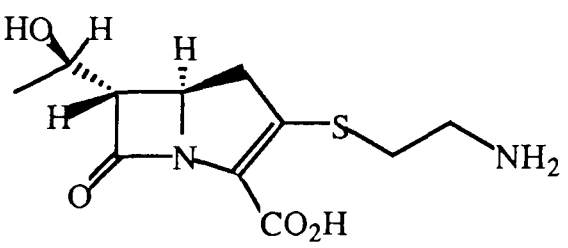
polysaccharide chain,[52b] inhibits this process by alkylating a serine residue at the active site of the transpeptidase enzyme (Scheme 1.21.). The resulting complex is not readily deacylated and consequently irreversibly inhibits the transpeptidase enzyme and hence cell wall biosynthesis. By inhibiting cell wall production in this way, the cell undergoes lysis and dies.



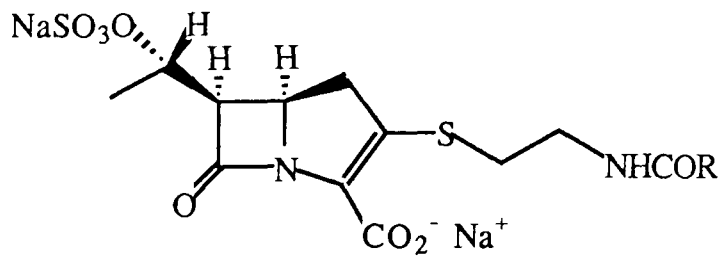
Scheme 1.21.

Resistance to β-lactam antibiotics is generally due to the production of non-chromosomally encoded β-lactamases. These are efficient enzymes, typically with turnover numbers of 10^3 sec^{-1}, capable of hydrolysing the β-lactam ring of the antibiotic before it causes inhibition of the bacterial transpeptidase enzyme. These enzymes are however sensitive to the nature of the penicillin side chain and consequently, it has been possible to overcome bacterial resistance simply by altering the C-6 substituent. However, exposure of the bacteria to non-fatal doses of the antibiotics in question, either as a result of the patient not following the prescribed dosage or by forgetting to take the correct dose at the correct time has aided the development of resistance. In addition, prescription of antibiotics has not been limited to cases of infection. Instead a 'prevention better than cure' attitude has been adopted, principally for commercial reasons, and for example farm livestock are now routinely administered antibiotics to prevent bacterial infection and increase agricultural and commercial productivity. This attitude has been short sighted to say the least.

As a direct consequence of the different factors described above, we find ourselves in a race to discover or develop new antibiotic agents to which resistance has not yet been induced. Once again fungal secondary metabolism has come to our aid with the discovery of new substances some of which possess both β-lactamase resistance and a degree of antibiotic activity *i.e.* thienamycin (43), olivanic acid (44), and clavulanic acid (45) which is a β-lactamase inhibitor.

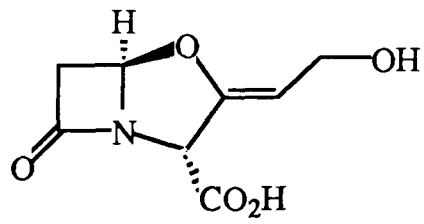


Thienamycin (43)



Olivanic acid (44)

Prescription of these β-lactamase inhibitors in conjunction with other β-lactam antibiotics has been reasonably successful in overcoming some resistant strains of bacteria.



Clavulanic acid (45)

Use of these new secondary metabolites represents only a short term solution to the problem of bacterial resistance and a more fundamental approach is needed if we are to maintain effective treatments for bacterial diseases. Until recently the most widely adopted approach used in the development of new β-lactam antibiotics has been based upon chemical modification of either the side chain or substituents on the nucleus itself. Much has been accomplished in this field, and compilation of structure activity relationships for modified antibiotics with a variety of different organisms has proved to be valuable in the development of increasingly potent antibiotics. However, the number of possible modifications to a particular compound is not infinite and thus a new, totally radical approach to the design and synthesis of new antibiotics is required to ensure that the essential supply of compounds to which resistance has not developed is maintained.

A recent approach adopted to solve this problem has been made possible by the rapid technological explosion in genetic engineering combined with a deeper understanding of the biosynthesis of the β-lactam antibiotics, in particular the enzymes involved and their mechanism of action. The approach is based upon modification of the isopenicillin N synthetase enzyme (IPNS) so that unnatural substrates may be converted in good yield directly to unnatural penicillins which, it is hoped, will possess antibacterial activity.[53] Modification of the IPNS enzyme is now possible due to preliminary work concerned with its isolation, sequence determination, cloning and expression into *E. coli* . Mutagenesis is then

performed by sequential degradation of the gene encoding the protein and re-annealing the altered strand of DNA with an unaltered one, so that misincorporations and hence mutations are induced. The proteins produced in this way but which have the ability to transform tripeptides to penicillins are then selected by a screening procedure which entails blotting substrate and cofactors onto the colonies of transformed bacteria. After incubation, the blot is then overlayed onto a plate of super sensitive *E. coli* , and the regions of antibiotic activity (as visualized by a zone of inhibition) related back to the original transformed colonies. Colonies identified in this way must be producing proteins capable of synthesising antibiotics from the tripeptide precursor and would then be grown in larger quantities to enable further investigation.

The extensive substrate analogue work performed in this laboratory with IPNS has shown that the wild-type enzyme is capable of tolerating extensive modification of the valine portion of the usual substrate, LLD-ACV (12). Furthermore mechanistic information has been obtained from this work which is of use in predicting the types of possible substrates which may be converted to penicillins and also the type (or types) of product expected.

In theory gene mutagenesis may also be applied to the next enzyme in the biosynthetic pathway, the DAOC/DAC synthetase enzyme. This enzyme has also recently been cloned [37] and expressed in *E. coli* and consquently the genetic information is available to allow similar manipulation of this protein as described for IPNS. If the studies on IPNS were to prove successful, then the penicillins so produced could be incubated with modified DAOC/DAC synthetase enzyme to provide new generations of cephalosporins.

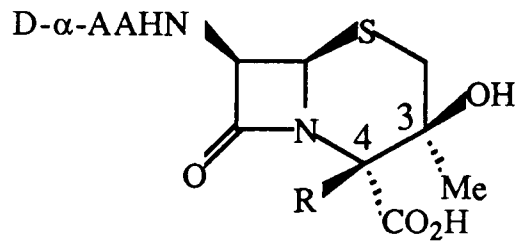
Genetic engineering of these enzymes and variation of their substrates may thus enable an enormous range of new penicillins and cephalosporins to be 'biosynthesised' and their usefulness as antibiotics evaluated. This approach arguably has the potential of becoming an important source of new antibiotic material in the forseeable future.

Although extensive investigations into the biosynthetic pathway to β-lactam compounds have permitted us to reach the exciting stage as outlined above, much is still unknown. As a result of the advances made in the field of biotechnology, some of the work described in this thesis has been undertaken with wildtype and recombinant IPNS and wildtype and recombinant DAOC/DAC synthetase purified or originally cloned from *Cephalosporium acremonium* .

Chapter 2 : 3β-Hydroxycepham (46).

2.1 Introduction.

Despite the substantial effort directed towards the elucidation of the biosynthetic origins of Cephalosporin C (20) in academic and industrial research laboratories, little progress was made until the 1970's. The main reason for this can be attributed to the inherent problems associated with intact cell studies - a situation in common with penicillin biosynthesis. Development of a cell free extract from *Cephalosporium acremonium*[54,55] which was capable of converting penicillin N (14) into a penicillinase resistant but cephalosporinase sensitive material enabled the biosynthetic relationship between penicillin N (14), deacetoxycephalosporin C (DAOC) (18) and deacetylcephalosporin C (DAC) (19) to be established.[55,56] Efforts were made to identify any other intermediates that might have been produced during the conversion of penicillin N (14) through to cephalosporin C (20) but none were detected. In 1981 the isolation and characterisation of the novel cepham metabolite 7β-[(5R)-5-amino-5-carboxypentanamido]-3β-hydroxy-3α-methyl-4α-carboxylic acid (46a) from the filtered broth of *C. acremonium* led to mechanistic speculation concerning the conversion of penicillin N (14) to DAOC (18).[57]



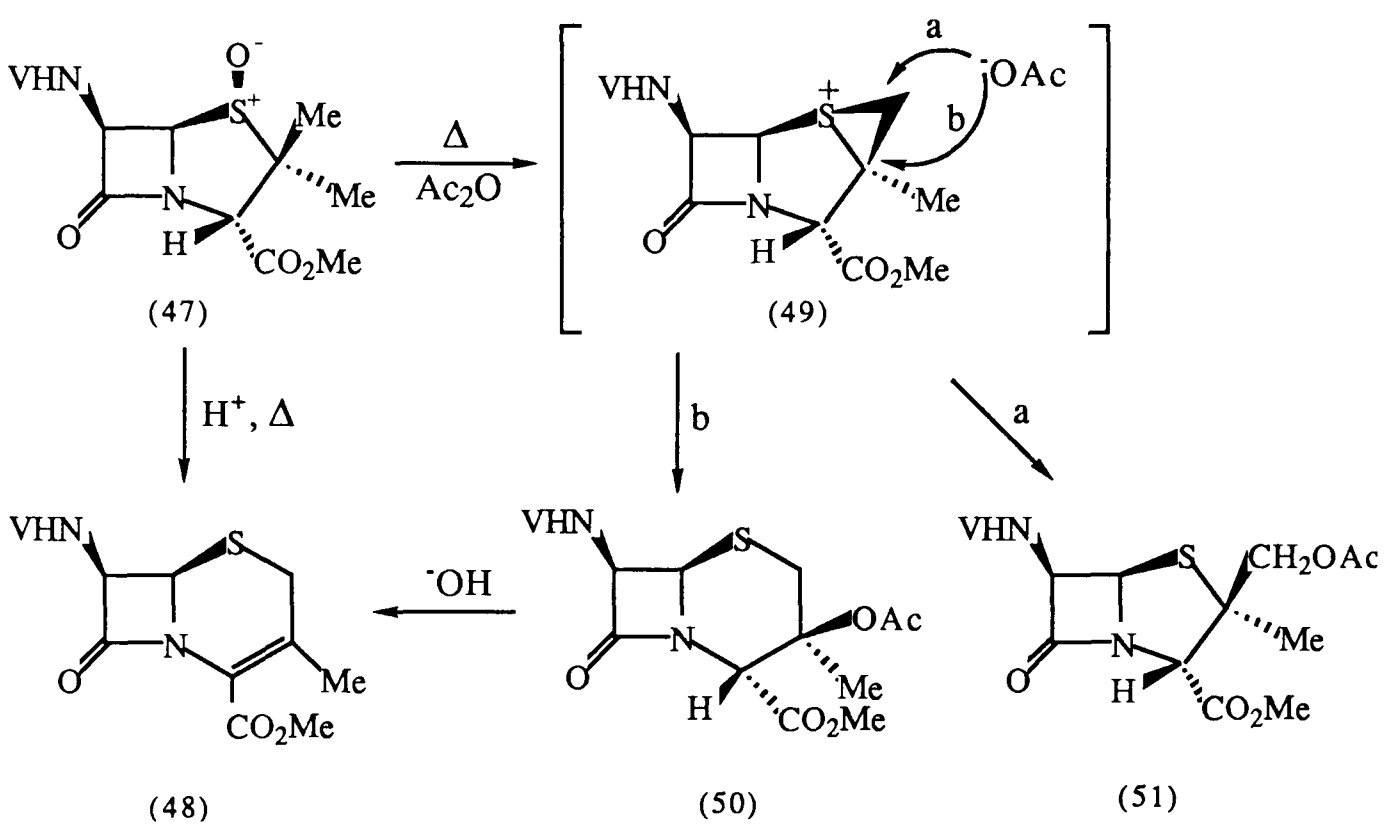
(46 a; R= H, b; R= D)

This novel cepham exhibited no antibacterial activity and was considered to be a shunt metabolite as distinct from a biosynthetic intermediate on the basis of the following :

i) incubation of (46a) with the crude cell free extracts available failed to produce any detectable quantity of DAOC (18) or DAC (19) by hplc assay, and

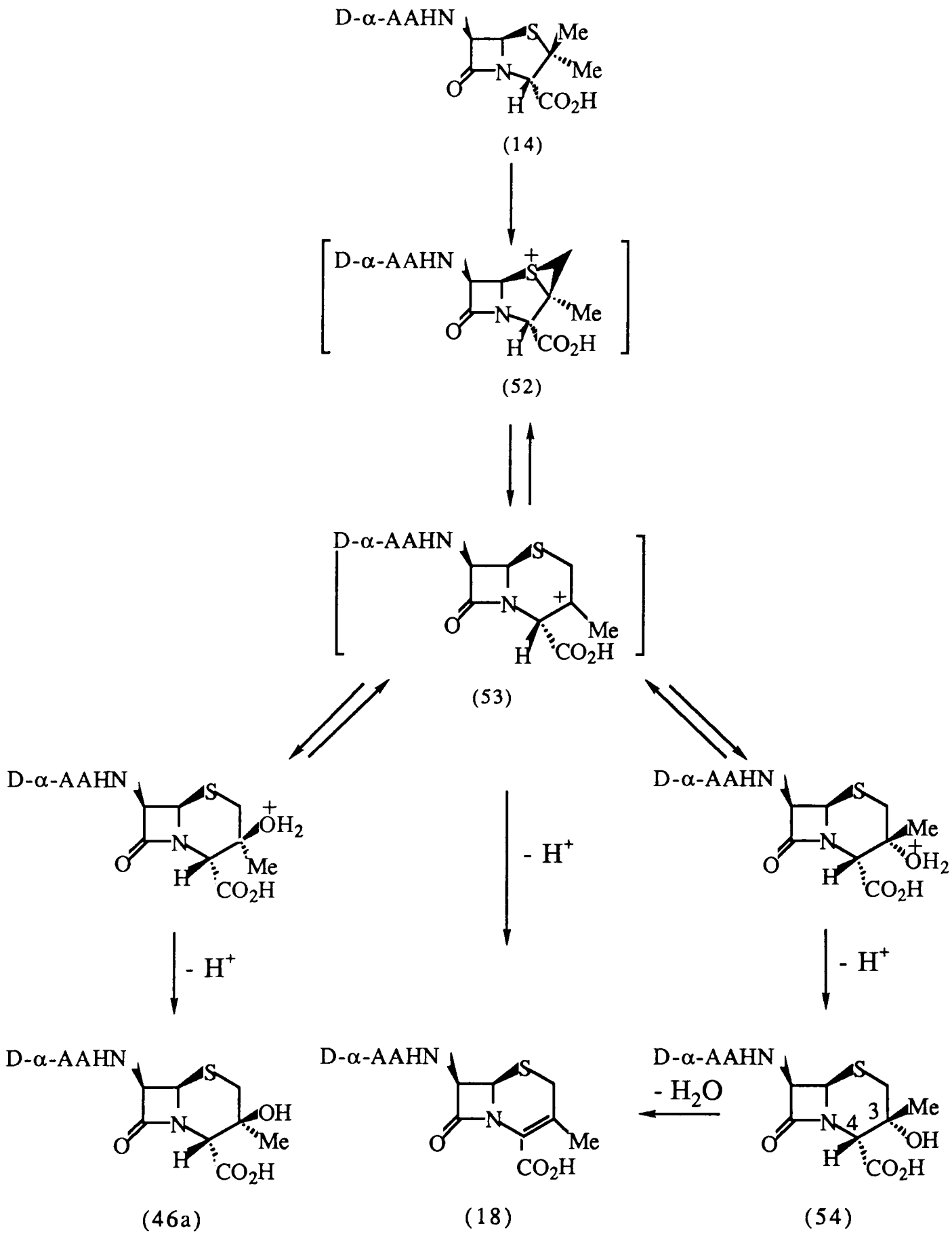
ii) DAOC/DAC synthetase was considered to belong to the class of enzymes known collectively as hydratases, which in general eliminate H and OH from substrates in which a trans relationship exists between the groups or elements to be expelled.[58,59]

Earlier chemical studies by Morin and co-workers [60] had shown that the conversion of penicillin V sulphoxide (47) to deacetoxycephalosporin V (48) also gave rise to an analogous product to the biosynthetic hydroxylated cepham (46a), namely (50). Formation of this product was believed to arise from interception of the proposed episulphonium ion intermediate (49) by acetate (Scheme 2.1.).



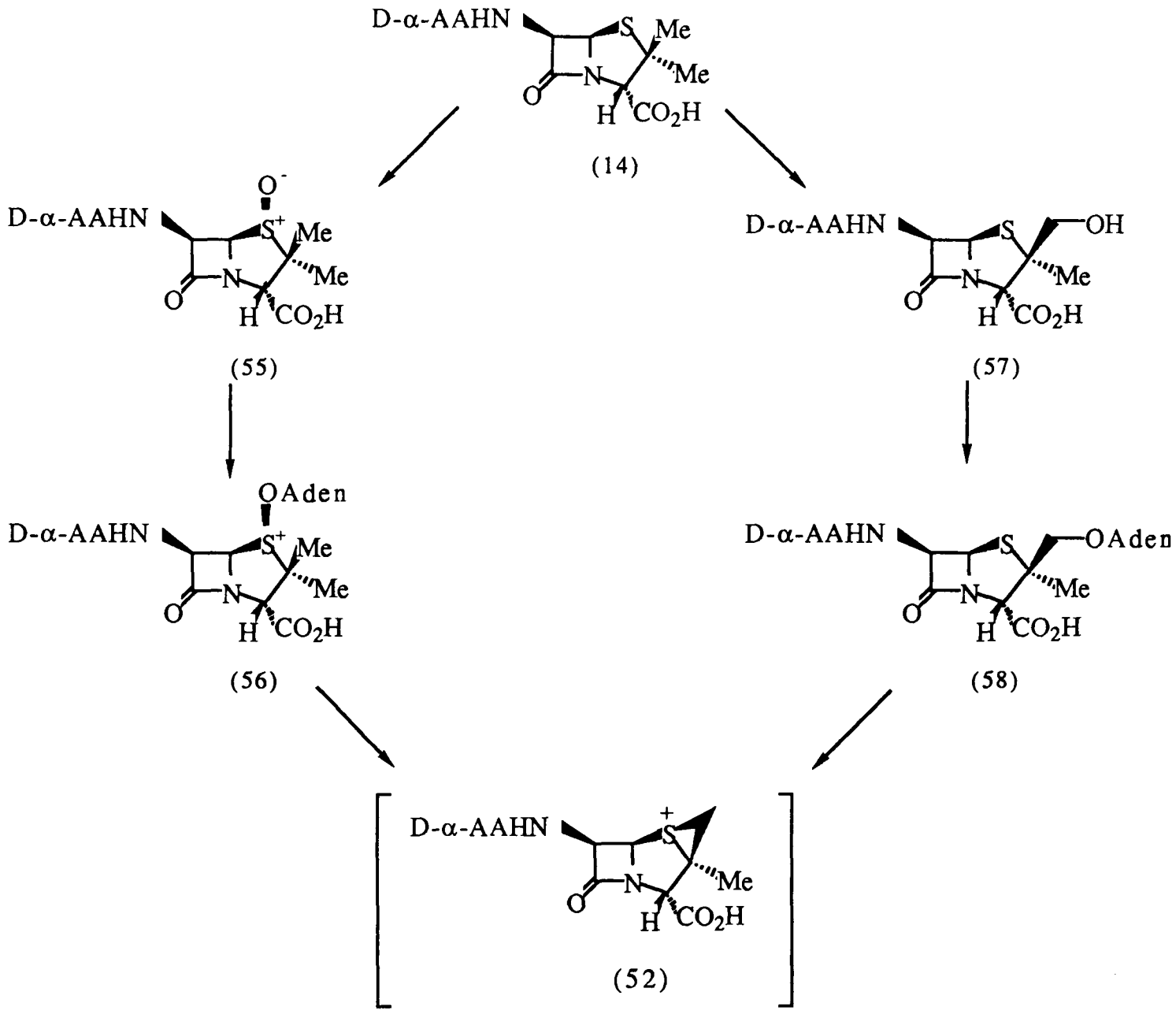
Scheme 2.1.

Taking these results into consideration, Kukolja *et al.*[57] suggested that the 3β-hydroxycepham (46a) was formed from the interception by water of either the proposed intermediate episulphonium ion (52) or the resulting carbocation (53) derived from ring opening of (52) (Scheme 2.2.). In addition, this carbocation could be trapped by water to give the 3α-hydroxycepham (54). If formed, the hitherto unisolated 3α-hydroxycepham (54) would be expected to rapidly dehydrate due to the trans relationship between the C-4 proton and C-3 hydroxyl group, to give DAOC (18). Alternatively, loss of a proton from (52) or (53) would result in DAOC (18) directly.



Scheme 2.2.

It was envisaged [57] that formation of the episulphonium ion could arise either from the adenylation of penicillin N sulphoxide (55) or β-hydroxymethyl penicillin N (57), thus leading to activated intermediates (56) and (58), which could then undergo elimination to give the episulphonium ion (52) (Scheme 2.3.).



Scheme 2.3.

In this way the misconception that adenosine triphosphate stimulated activity was satisfied and the generally accepted requirement for ferrous ions, α-ketoglutarate and molecular oxygen were given an oxidative role, analogous to other related systems.[62,63]

The involvement of both penicillin N sulphoxide (55) (see Chapter 7) and β-hydroxymethyl penicillin N (57) in the biosynthetic ring expansion process has been investigated[36] and both were shown not to be 'enzyme free intermediates'. (It is important to make a distinction between 'enzyme bound' and 'enzyme free' intermediates, since although a compound has been shown not to be an enzyme free intermediate, this does not necessarily mean that the compound is not a transient intermediate bound to the active site at some stage during the catalytic cycle).

Despite the initial interest in the 3β-hydroxycepham (46a) no further work was reported. Several points remained to be clarified, the most pertinent being

i) the identity of the immediate precursor of (46a), and

ii) the source of the hydroxyl oxygen atom.

2.2. 3β-Hydroxycepham - Shunt or Biosynthetic Intermediate?

The only direct evidence that the 3β-hydroxycepham (46a) was a shunt metabolite, was the inability of a crude cell free DAOC/DAC synthetase enzyme preparation from *C. acremonium* to convert (46a) to DAOC (18) or DAC (19).[57] Since the 'quality' of enzyme preparations available at the time was poor, re-investigation of this observation was undertaken. In addition, the identity of the immediate precursor to (46a) was hoped to be established, particularly as no conclusive experiments had been undertaken to determine from which of penicillin N (14), DAOC (18) or DAC (19) this cepham was formed. It was conceivable that (46a) was a product derived not from the ring expandase activity and penicillin N (14), but by hydroxylation of DAOC (18) by either the hydroxylase responsible for the conversion of DAOC (18) to DAC (19), or by an alternative enzyme present in the crude cell free prepartions. This confusion arose because whenever (46a) was isolated so too were DAOC (18) and DAC (19). An obvious experiment to clarify this situation was to incubate DAOC (18) with DAOC/DAC synthetase and to look for the production of the cepham (46a).

Initially however, the ratio of DAOC (18) to DAC (19) and 3β-hydroxycepham (46a) obtained from penicillin N (14) incubations with DAOC/DAC synthetase was determined. Thus synthetic penicillin N (14)[64] was incubated with DAOC/DAC synthetase and the ^{1}H-n.m.r spectrum of the total crude incubation mixture recorded. The β-lactam proton region (δ 5.5-4.9 ppm) indicated four sets of AB quartets, three of which were assigned to unconverted starting material (14) and to the cephem products (18) and (19). The fourth AB quartet was initially assigned to the cepham (46a) on the basis of comparison with previously reported ^{1}H-n.m.r data.[57] [This was later confirmed by synthesis of an authentic sample of the cepham (46a) by an analogous method to that previously reported,[61] and subsequent spectroscopic comparison]. Integration of this region gave an approximate ratio of DAOC (18), DAC (19) and 3β-hydroxycepham (46a) of 65 : 33 : 2, [(18): (19): (46a)].

Development of a suitable isolation procedure for all the β-lactam compounds discussed so far, was to prove an important advancement, not only for the work described in this chapter but for all the biosynthetic studies outlined in this thesis. Initially paper electrophoresis at 4 KV in a buffered solution at pH 3.5 gave promising results, but due to the relative instability of the 3β-

hydroxycepham (46a) and more particularly penicillin N (14) at this pH, an alternative procedure was required.

After considerable work, a suitable hplc system was developed using a reverse phase octadecylsilane stationary phase and 25 mM aqueous NH_4HCO_3 as the mobile phase. With this system all the β-lactam compounds could be efficiently separated from the crude incubation mixtures in one elution (see Diagram 2.1.).

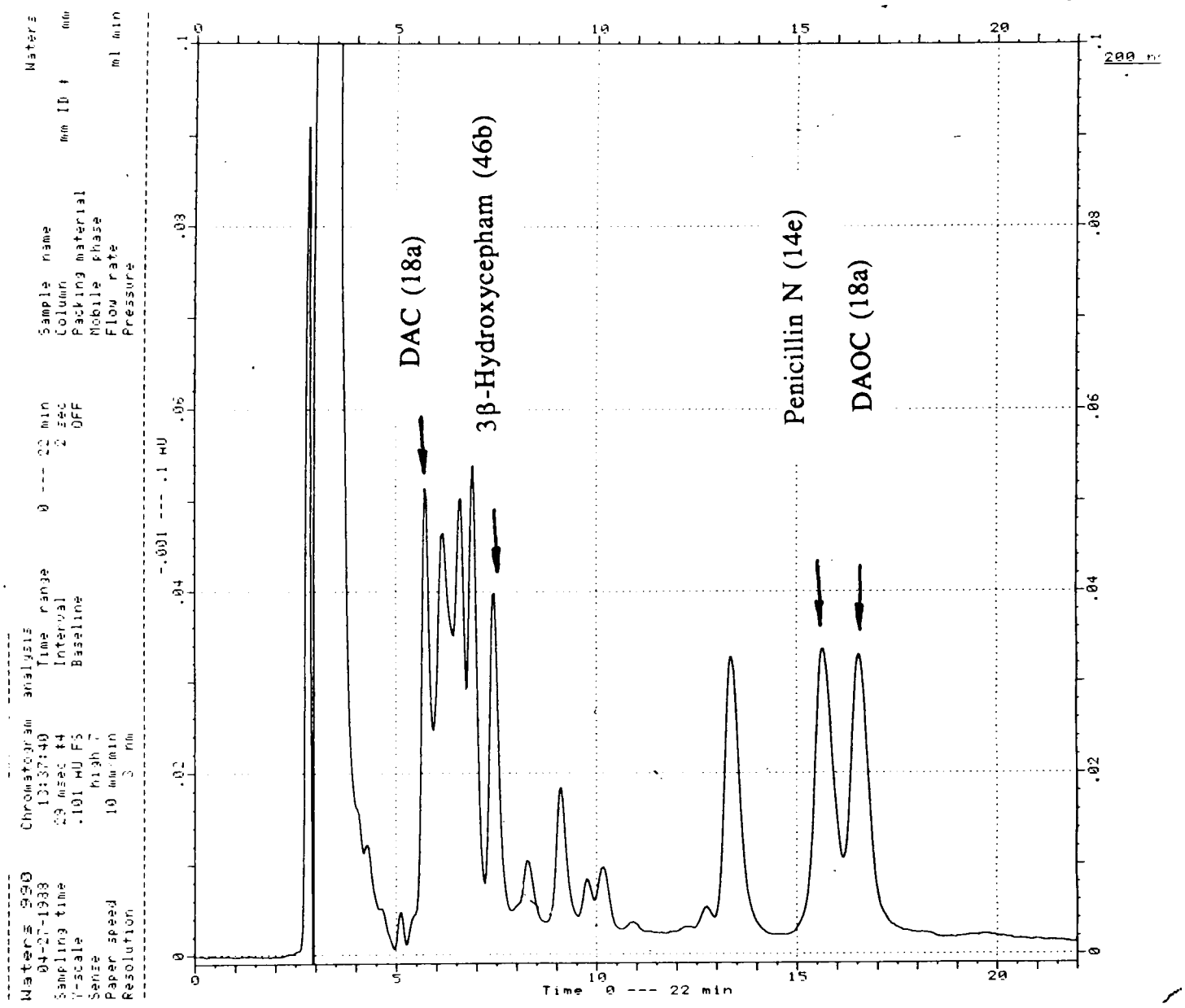


Diagram 2.1. Hplc Trace of (3-^{2}H)-Penicillin N (14e) Incubation with DAOC/DAC Synthetase.

The most striking feature observed during these chromatographic studies was the sensitivity of the retention times to the pH of the mobile phase. To assist in the purification of IPNS and DAOC/DAC synthetase incubation mixtures, alternative solvent systems such as 0.75% MeCN in 10mM aqueous NH_4HCO_3 and 0.05% HCO_2H in water were used. The former system was particularly suited to purification of the penicillin N (14) isotopomers after enzymatic synthesis from the respective tripeptides with IPNS.

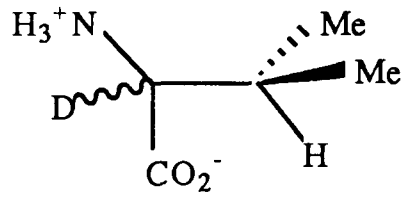
After the development of a suitable hplc assay to ensure maximum sensitivity, the origin of the 3β-hydroxycepham (46a) was investigated. Thus a

large scale (10mg) incubation of DAOC (18) with DAOC/DAC synthetase (*ca* 0.3 I.U) was performed and the crude incubation mixture examined by 500MHz ^{1}H-n.m.r. The spectrum indicated *ca* 10% conversion, with DAC (19) as the sole product - no resonances due to the 3β-hydroxycepham (46a) could be seen. In an attempt to increase the sensitivity of detection, the incubation mixture was purified by hplc [reverse phase with 0.75% MeCN in 10mM aqueous NH_4HCO_3] and the 'window' corresponding to the approximate elution position of (46a) (i.e. all fractions eluting between DAC (19) and DAOC (18), see Diagram 2.1) collected and re-examined by 500MHz ^{1}H-n.m.r. Once more, no evidence for the formation of (46a) from DAOC (18) was obtained.

Biosynthetic 3β-hydroxycepham (46b) was incubated with DAOC/DAC synthetase and the crude incubation mixture bio-assayed against *E. coli* ESS (+) and *S. aureus* at a concentration of 100μl ml^{-1}. Similarly a sample of 3β-hydroxycepham (46b) was tested for antibacterial activity at the same concentration and against the same organisms. In both cases no antibacterial activity was detected, indicating that no conversion of the cepham (46b) to the bio-active cephems (18) and (19) had occurred.

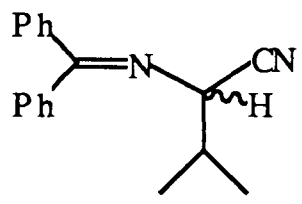
2.3 Enhanced 3β-Hydroxycepham (46b) Formation via a Kinetic Isotope Effect.

Investigation of the enzymatic ring expansion of penicillin N (14) to DAOC (18) by means of competitive kinetic isotope experiments with specifically deuterated penicillin Ns (see Chapter 4) required the synthesis of α-deuterovaline (11h).



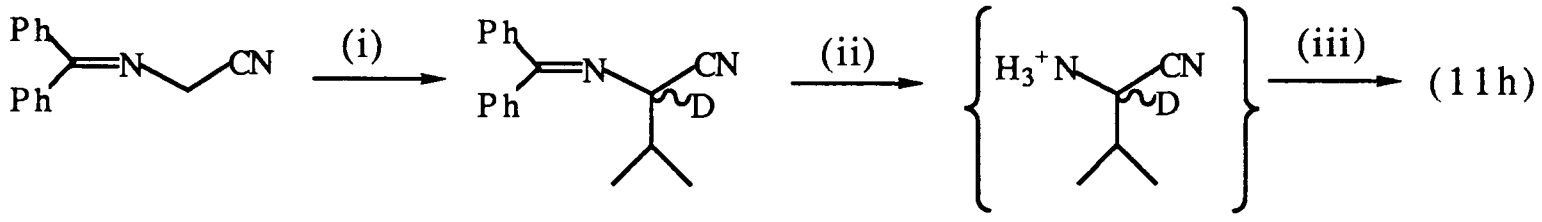
(11h)

The simplest racemic synthesis of this specifically deuterated amino acid (11h) was considered to be *via* alkylation and subsequent hydrogen/deuterium exchange at C-2 of the valinyl equivalent (60a) (Scheme 2.4).



(60a)

It was anticipated that both the alkylation and C-2 exchange could be achieved in one pot by using sodium deuteroxide as the base. Thus treatment of the imine [65] (59) under phase-transfer alkylation conditions with 2-bromopropane, in the presence of sodium deuteroxide and a catalytic amount of benzyltriethylammonium chloride (BTEAC) as phase transfer catalyst, gave after chromatography the alkylated imine (60b) in typically 60-70% yield. Hydrolysis of this imine with dilute acid (1N HCl) at room temperature gave the amino-nitrile (61b) which was further hydrolysed by reflux in 6N HCl to give the racemic valine hydrochloride salt (11h).



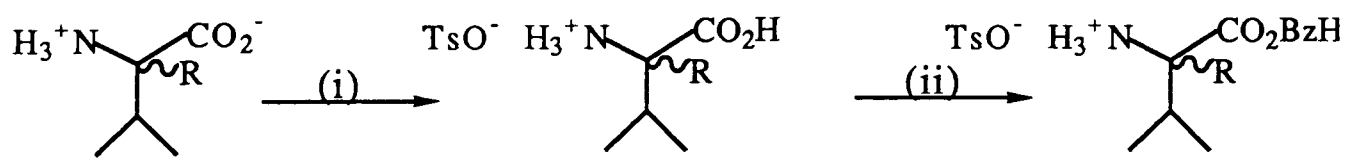
(59) (60b) (61b)

Reagents : (i) BTEAC, Toluene, 2-Bromopropane, 50% NaOD ;
(ii) 1N HCl, 23°C, 12 hours ;(iii) 6N HCl reflux, 12 hours.

Scheme 2.4.

Mass spectrometery and ^{1}H-n.m.r spectroscopy upon (11h) indicated that at least 98 atom % deuterium had been incorporated exclusively into the α-position. Purification by ion-exchange chromatography (Dowex-1x-8-400, acetate form) gave after lyophilization, the free amino-acid which was treated with one equivalent of *p*-toluenesulphonic acid in water and again lyophilized to give the ammonium *p*-toluenesulphonate salt (62b). Esterification with diphenyldiazomethane in MeCN gave the benzhydryl ester (63b) in near quantitative yield (Scheme 2.5.).

The racemic valine ester (63b) was converted to the free amine by treatment with aqueous sodium bicarbonate solution and then coupled to (5R)-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (64)[66] with EEDQ [67] to give the diastereomeric DLD- and

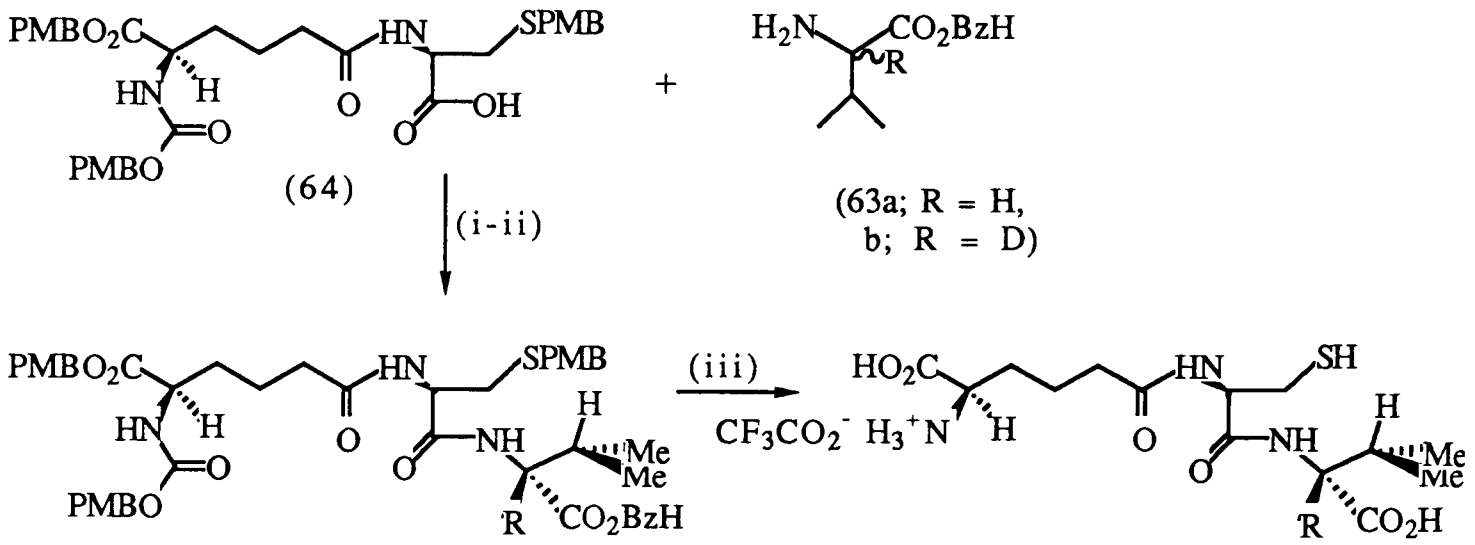


(11a; R=H, h; R=D) (62a; R=H, b; R=D) (63a; R=H, b; R=D)

Reagents : (i) *p*-Toluenesulphonic acid, H_2O/EtOH [1:1,v/v];
(ii) Ph_2CN_2, MeCN, 23°C.

Scheme 2.5.

DLL-tripeptides (Scheme 2.6.). Chromatographic comparison (SiO_2 thin layer) of this mixture against an authentic standard of similarly protected DLD-ACV (65a), prepared as described above but with optically pure D-valine benzhydryl ester, revealed that the required diastereomer was the less polar of the two. Separation by chromatography (flash SiO_2 follwed by preparative plate) gave the pure, specifically deuterated DLD- configured tripeptide (65b) , as shown by ^{1}H-n.m.r[68] and a second chromatographic comparison against (65a) (*i.e.* a co-spot of authentic tripeptide (65a) and deuterated tripeptide (65b) gave only one spot after elution). Deprotection with refluxing TFA containing 10% anisole[66] gave the required tripeptide (66b) of sufficient purity that further purification by hplc was unnecessary.



(65a; R = H,
b; R = D)

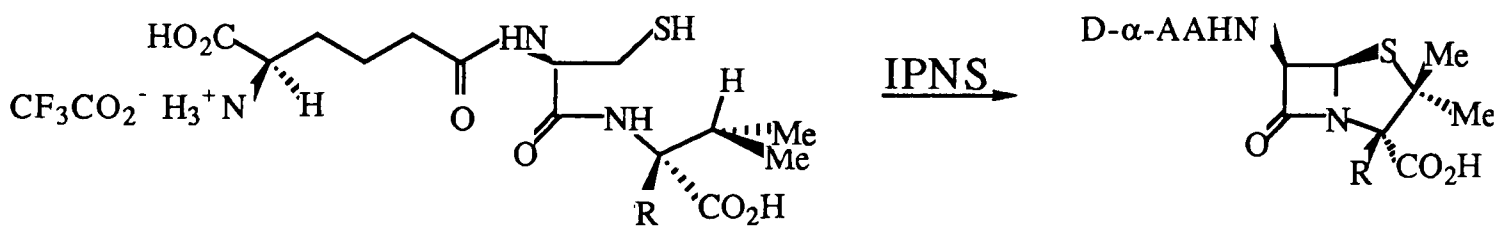
(66a; R = H,
b; R = D)

Reagents : (i) EEDQ, DCM, anhydrous Na_2SO_4; (ii) SiO_2 Chromatography
(iii) TFA/anisole [5:1, v/v], reflux 30 minutes.

Scheme 2.6.

Earlier work in this laboratory had previously shown that IPNS was capable of converting the unnatural tripeptide (5R)-5-amino-5-carboxypentanoyl-L-cysteinyl-D-valine (66a) directly into penicillin N (14) (Scheme 2.7).[69,70] Consequently enzymatic syntheses of (3-^{2}H)-penicillin N (14e) and other specifically deuterated penicillins from their respective tripeptides with IPNS could be achieved. This meant that difficult chemical syntheses could be avoided. Another advantage associated with this lack of substrate specificity by IPNS, was that an unattractive route *via* incubation of (5S)-5-amino-5-carboxypentanoyl-L-cysteinyl-D-valine (12a) with IPNS to give isopenicillin N (13) and subsequent epimerisation to penicillin N (14) could be avoided. This represented a significant advantage since the epimerase enzyme had not been isolated and purified to any

appreciable extent. Thus to perform an enzymatic epimerisation, a crude cell free preparation containing virtually all the metabolic machinery of the cell would have to be used so that exclusion of the epimerase activity would be advoided. Furthermore, the lability of the epimerase activity would have presented a severe problem, particularly as the freezing of crude extracts was reported to be sufficient to destroy virtually all epimerase activity.[27]



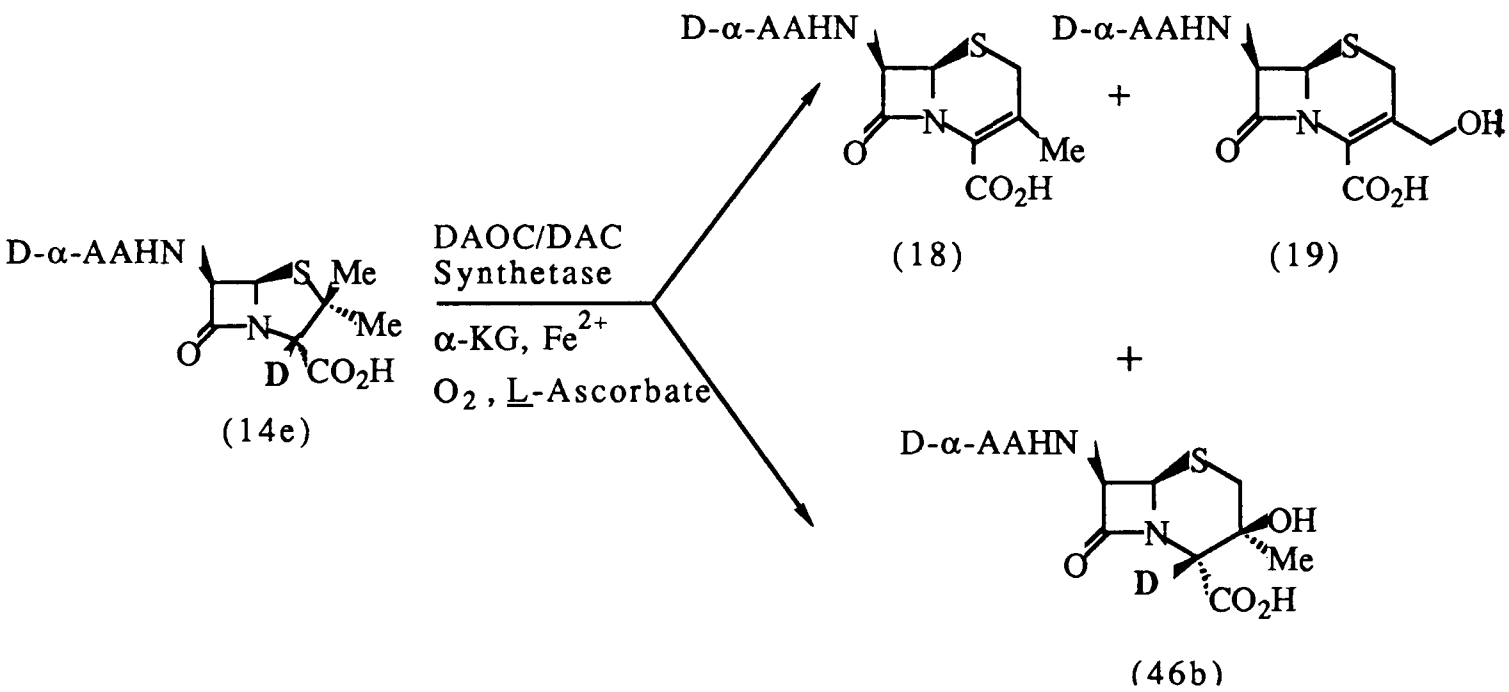
(66a; R = H,
b; R = D)

(14a; R = H,
e; R = D)

Scheme 2.7.

In summary, the "unnatural" enzymatic synthesis of labelled penicillins described provided a quick and comparatively efficient route to compounds otherwise inaccessible by the usual synthetic methodology.

Incubation of (5R)-5-amino-5-carboxypentanamido-L-cysteinyl-D-(2-^{2}H)-valine (66b) with IPNS in the presence of the usual co-factors gave (3-^{2}H)-penicillin N (14e) in good yield. Treatment of the crude IPNS incubation with DAOC/DAC synthetase from *C. acremonium* [36] in the presence of α-ketoglutarate, ascorbate, DTT, ferrous ions and molecular oxygen gave the cephems (18) and (19) and the cepham (46b) (Scheme 2.8.), as determined by 500MHz ^{1}H-n.m.r spectroscopy.



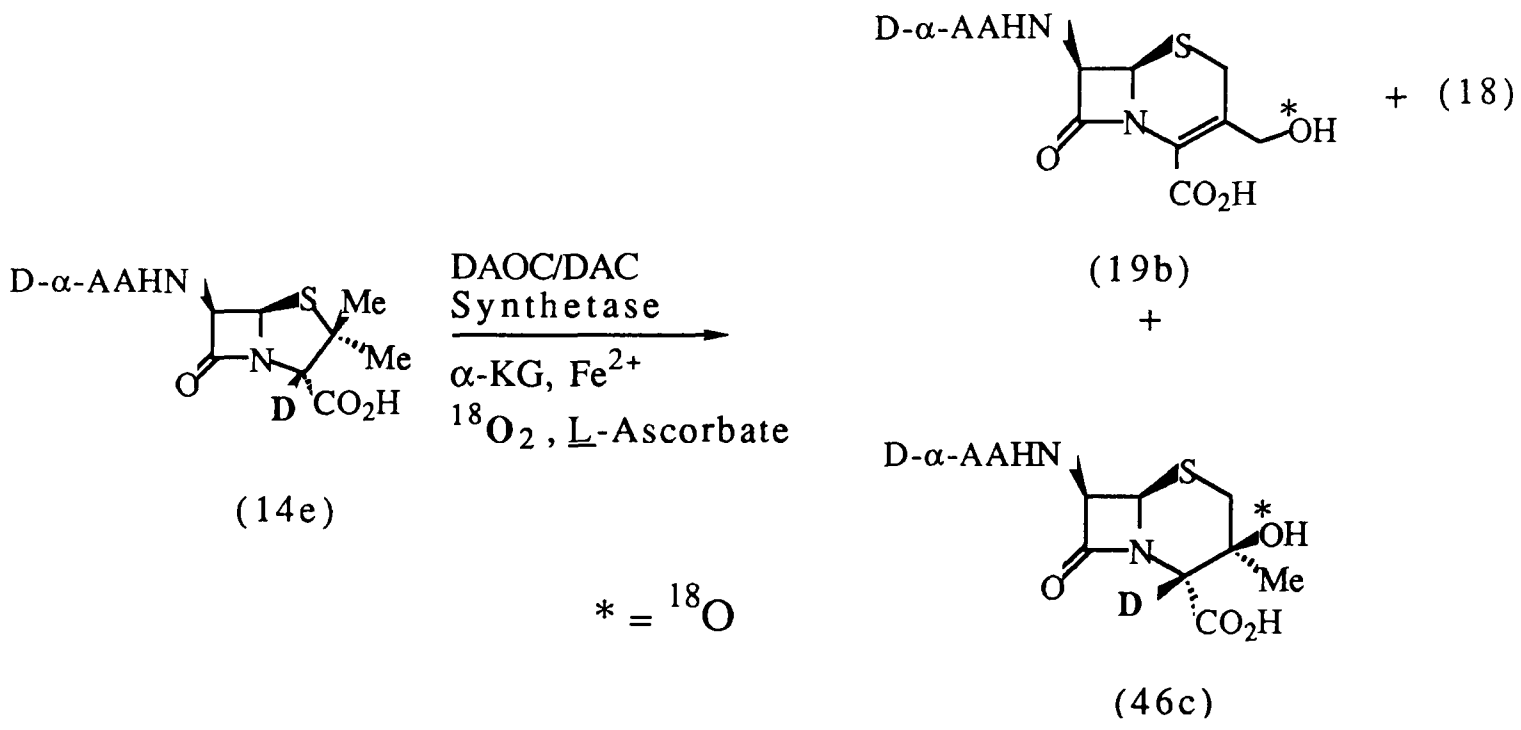
Scheme 2.8.

Integration of the β-lactam region (δ 5.5-4.9 ppm) indicated that the ratio DAOC (18): DAC (19) : 3β-hydroxycepham (46b) was approximately 40 : 25 : 35, *i.e.* the introduction of one deuterium atom had resulted in a 20-fold increase in the amount of 3β-hydroxycepham (46b) produced.[71] It is apparent from this observation that one of the enzyme bound intermediates formed during the ring expansion process must be particularly sensitive to the strength of the penicillin C-3 carbon-hydrogen bond. This observation also supports the conclusions of the previous section in which the origin of (46a) was assigned to penicillin N (14a), since it is an isotopic substitution in penicillin N (14e) that leads to enhanced production of the cepham (46b).

Initial purification and isolation of (46b) from the incubation mixture was achieved by hplc [reverse phase octadecylsilane with 25 mM aqueous NH_4HCO_3]. Characterisation by 500MHz ^{1}H-n.m.r and mass spectrometry (positive Ar FAB) indicated that this compound was identical to the previously isolated cepham (46a)[57] except for the absence of a resonance at δ 4.15 ppm, C-4H of (46a), and a protonated molecular ion of 377 (MH^+) - one mass unit greater than that reported for (46a) due to the presence of a deuterium atom at C-4 in (46b). Synthesis of an authentic sample of (46a) by an analogous method as that previously reported[61] and comparison by hplc retention time, mass spectrometry and 500MHz ^{1}H-n.m.r spectrometry indicated that the biosynthetic cepham (46b) was identical to the synthetic cepham (46a) except for the differences described above.

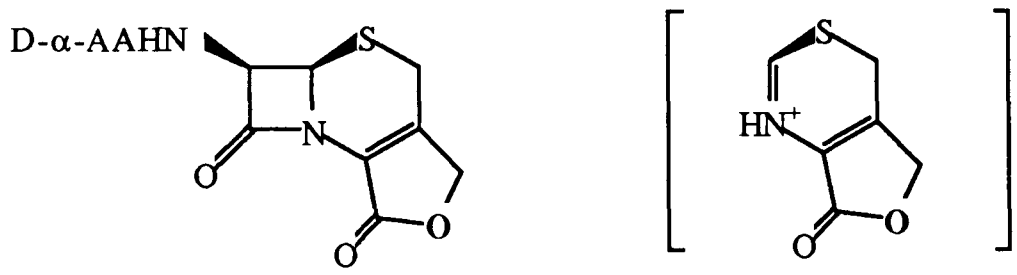
2.4 Origin of the Hydroxyl Oxygen Atom.

The increased production of the 3β-hydroxycepham (46) from (14e) *via* a kinetic isotope effect made the determination of the origin of the hydroxyl oxygen atom possible. Previously, any such attempt would have been considered impratical due to the low levels of (46a) obtained from unlabelled penicillin N (14a) incubations both from intact cells and partially purified DAOC/DAC synthetase preparations. Thus (3-^{2}H)-penicillin N (14e) was prepared from (66b) and IPNS, as previously described. The labelled product (14e) was isolated and purified from the IPNS incubation mixture by chromatography [reverse phase hplc with 0.75% MeCN in 10mM aqueous NH_4HCO_3]. Separate incubations of two samples of this penicillin N (14e) with DAOC/DAC synthetase under atmospheres of $^{18}O_2$ gas (96 atom% ^{18}O , supplied by Amersham International plc) [Experiments 1,2 Tables 2.1 and 2.2], both gave good conversions to the previously observed products, as judged by ^{1}H-n.m.r (Scheme 2.9.).



Scheme 2.9.

Isolation of the ring expanded products by hplc, as described before, and analysis by mass spectrometry (positive Ar FAB, from an oxalic acid/glycerol matrix) revealed significant incorporation of label into cepham (46c) and DAC (19b) (See Tables 2.1 and 2.2). Previous experience had shown that better mass spectral data for DAC (19) was obtainable after lactonisation to (67), a transformation readily accomplished by reaction of (19) with freshly distilled formic acid at room temperature for 15 minutes.[72] Under the mass spectral conditions fragmentation across the β-lactam ring occurred to give the fragment (68) for which data is included.



Expt.	*m / z*	(46c, MH^+)	*376*	*377*	*378*	*379*	*380*	*381*
1		Found (%)	29	80	53	100	38	8
2		Found (%)	-	69	19	100	23	15

Synthetic sample of (46a)							
	m / z	(MH^+)	*375*	*376*	*377*	*378*	*379*	*380*
	Found	(%)	7	100	22	10	1	-
	Calc.	(%)	-	100	18	7	1	-

Table 2.1. (3-^{2}H)-3ß-hydroxycepham (46c).

Expt.	*m / z* (67b, MH^+)	*355*	*356*	*357*	*358*	*359*	*360*
1	Found (%)	4	100	25	72	27	22
	Calc. (%)	-	100	18	7	1	-
	m/z (Fragment (68))	*155*	*156*	*157*	*158*	*159*	*160*
1	Found (%)	4	100	14	70	10	6
2	Found (%)	4	100	13	54	7	6
	Calc. (%)	-	100	8	5	-	-

Table 2.2. Deacetylcephalosporin C (19b).

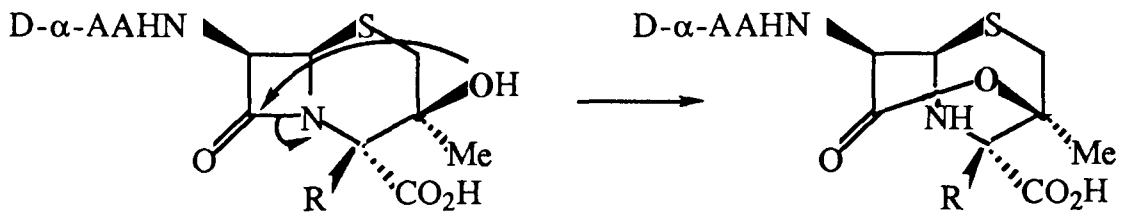
These results indicate that 50-60% incorporation of molecular oxygen into the 3β-hydroxycepham (46c) occurred, whereas only 30-40% incorporation into DAC (19b) was observed. A possible explanation for this is that label has been lost from the DAC lactone (67b) either during or after lactonisation. For this to happen, ring opening of the lactone (67b) by cleavage of the alkyl carbon-oxygen bond[73] must occur with effective transfer of the label into the carboxyl group and introduction of ^{16}O into the hydroxyl group of DAC (19b). Re-lactonisation would then result in partial loss of the label that had been transferred into the carboxyl group at a statistical rate of 50% for every lactonisation/ ring opening cycle. However, it has recently been demonstrated[74] that an approximate 1:1 mixture of (67a) and (67b), when stirred with formic acid does not result in loss of label from (67b).

Furthermore, leakage of labelled $^{18}O_2$ gas from the incubation vessel does not necessarily provide a reasonable explanation for the higher levels of incorporation into (46c) than than the downstream (19b), particularly as great care was taken to ensure that the system was gas tight. Assuming that leakage did occur which could give rise to an overall exchange of $^{18}O_2$ gas for $^{16}O_2$ gas, such an event would only perturb the degree of label incorporation into the products, (19b) and (46c) if these compounds were being produced at different rates. Since the 3β-hydroxycepham (46c) is formed by the ring expansion activity and DAC (19b) by the hydroxylase activity, a differential rate of formation does not seem unreasonable. However, consideration of the magnitude of the observed difference in label incorporation suggests that to account for the data obtained an unreasonably high degree of leakage and hence exchange is required.

Recent results suggest that the above observations are best explained by exchange of oxygen label from the active site of the enzyme prior to incorporation into the products (46c) and (19b)[74]. To account for the difference in degree of incorporation, the rate of exchange during the event responsible for

hydroxylation must be greater than that from for the event leading to ring expansion active site.

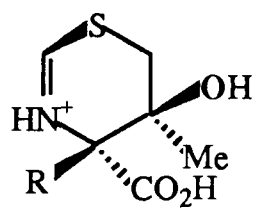
The stability of the 3β-hydroxycepham (46) was found to be pH dependent with decomposition occurring at acidic or basic pH to a compound whose ^{1}H-n.m.r and mass spectra are consistent with the lactone (69a).



(46a; R=H, O = ^{16}O,
b; R=D, O = ^{16}O,
c; R=D, O = ^{18}O)

(69a; R=H, O = ^{16}O,
b; R=D, O = ^{16}O,
c; R=D, O = ^{18}O)

Formation of this lactone and also the corresponding lactone with the V-side chain[75] is evidence that the hydroxyl group has the β-configuration since formation of these lactones from the cepham with the hydroxyl group on the alternative face (i.e α) is geometrically impossible. It may, however, be possible to form the lactone derived from the 3β-hydroxycepham (46) (and possibly the 3α-hydroxycepham) after initial cephalosporoate formation (*i.e.* after β-lactam ring opening), but since no evidence was obtained for cephalosporoate formation from (46) this suggestion cannot be substantiated. A consequence of lactonisation is that no ions due to β-lactam ring fragmentation (*i.e.* fragment (70)) are seen in the mass spectrum of (46).



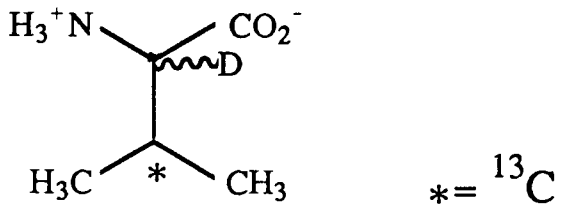
(70a; R=H, O = ^{16}O,
b; R=D, O = ^{16}O,
c; R=D, O = ^{18}O)

Incorporation of molecular oxygen into the hydroxyl group strongly contradicted the previously proposed mechanism of formation of (46) *via* interception of the intermediate episulphonium ion (52) or carbocation (53) by water [57] (Scheme 2.3). Despite the significant levels of incorporation of ^{18}O into (46c), this alternative pathway could not be totally dismissed.

The contribution of this alternative pathway could be determined by incubation of (3-^{2}H)-penicillin N (14e) with DAOC/DAC synthetase in $H_2{}^{18}O$. Practical considerations in setting up such an incubation would lead to at least a

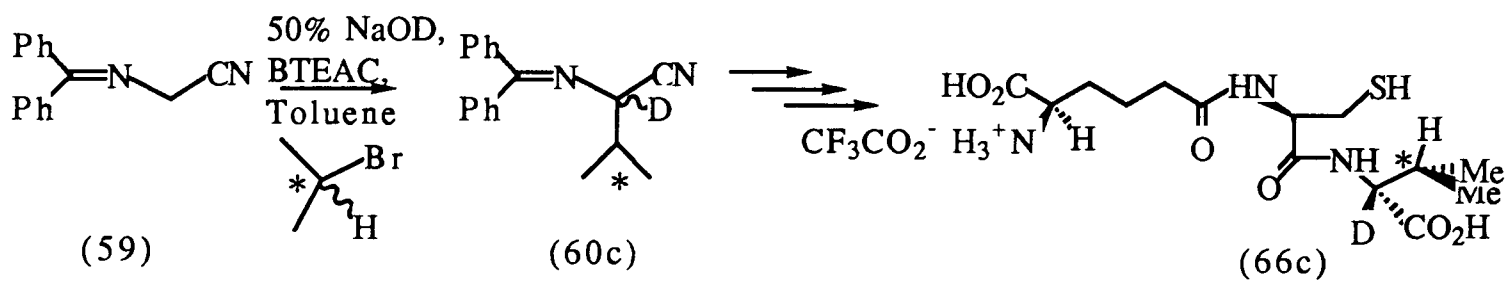
50% dilution of the labelled water. Thus for example if this pathway was responsible for 5% of total 3β-hydroxycepham (46) formed, only a 2.5% incorporation of label could be expected. Reliable detection, let alone quantification, of label incorporation at this level by mass spectrometry was anticipated to be impractical and an alternative analytical method was required. The approach chosen was to detect incorporation by observation of an ^{18}O-^{13}C isotope shift with ^{13}C-n.m.r spectroscopy. ^{18}O-^{13}C isotope shifts occur upfield to the corresponding ^{16}O-^{13}C resonance and the magnitude of the shift depends upon the structure of the molecule and type of functional group in which the label is located.[76] The best structural comparison possible for the expected labelled cepham was *t*-butyl alcohol for which an ^{18}O-^{13}C isotope shift of 0.035ppm has been reported.[77]

A synthesis of the α-deutero-β-^{13}C-labelled valine (11m) was thus required.



(11m)

This was accomplished using the same procedure as that described for the deuterated valine (11h), except that (2-^{13}C)-2-bromopropane (90 atom%^{13}C supplied by Merck, Sharpe and Dohme Isotopes Ltd) was substituted for 2-bromopropane in the alkylation stage (Scheme 2.10.).



(59) (60c) (66c)

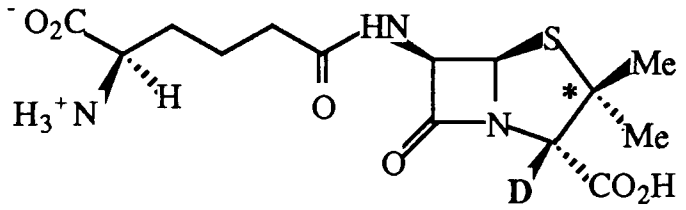
* = ^{13}C

Scheme 2.10.

Mass spectrometry revealed that the resulting valine (11m) contained at least 90 atom% ^{13}C and 98 atom% deuterium. The site and levels of labelling were also qualitatively confirmed by its ^{1}H-n.m.r spectrum (see Experimental). This amino-acid was then protected and coupled to acid labile protected DL-AC (64a) to give the DLD-and DLL-diastereomers which were separated as previously described and the DLD-isomer deprotected with refluxing TFA/anisole (4:1, v/v). Mass spectrometry at this stage indicated that no loss of label from the valine

residue (11m) had occurred during its synthetic incoroporation into the tripeptide (66c).

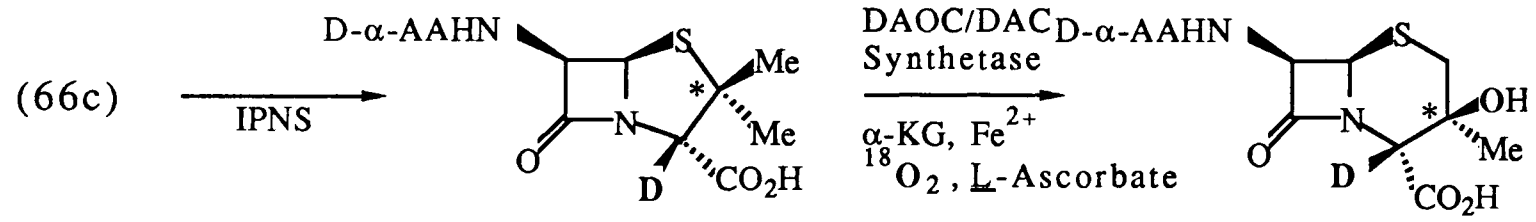
Enzymatic synthesis of the required (2-^{13}C, 3-^{2}H)-penicillin N (14f) was achieved in good yield from (66c) and IPNS in the presence of the usual cofactors.



(14f)

* = ^{13}C

After purification by hplc [Gilson system, 0.75% MeCN in 10 mM aqueous NH_4HCO_3] a sample of this labelled penicillin N (14f) (*ca* 1.5 mg) was incubated with DAOC/DAC synthetase (0.2 I.U.) under an atmosphere of $^{18}O_2$ gas (98.6 atom% ^{18}O , supplied by Amersham International plc) (Scheme 2.11). Examination of the crude incubation mixture by ^{1}H-n.m.r revealed that approximately 40% conversion of the penicillin N (14f) had occurred to the ring expanded products, (3-^{13}C)-DAOC, (3-^{13}C, 3'-^{18}O)-DAC and (3-^{13}C, 3-^{18}O)-3β-hydroxycepham (46d) as judged by integration of the β-lactam region (δ 5.6-4.9 ppm).



(14f)

(46d; O = ^{18}O
e; O = ^{16}O)

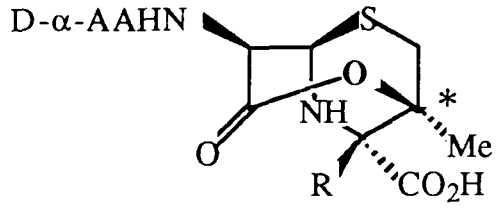
* = ^{13}C

Scheme 2.11.

Previous characterisation of synthetic 3β-hydroxycepham (46a) enabled assignment of the C-3 carbon atom to a resonance at *ca* δ 65.57 ppm and *ca* δ 81.51 ppm in the lactone (69). The crude incubation mixture was thus examined by ^{13}C-n.m.r at 125.77 MHz. After acquisition overnight, the spectrum was examined and shown to contain a weak peak at both δ 65.53 and 81.41 ppm.

To prove the existence of an ^{18}O-^{13}C isotope shift, comparison against natural abundance or labelled ^{16}O-^{13}C material was required. The introduction of a ^{13}C-label in (46d) gives rise to an observed increase in sensitivity in the ^{13}C-spectrum of 90 times that obtained with unlabelled cepham (46a). Thus to introduce sufficient ^{16}O-^{13}C labelled material in order to obtain a signal of

comparable intensity, 90 times the amount of labelled cepham (46d) that was present in the incubation would be required. From the known percentage conversion and the ratio of cepham (46b) to cephems (18) and (19), it can be calculated that approximately 18 mg of unlabelled (46a) would need to be added. This was obviously impractical and as an alternative a comparable amount of (3-^{2}H, 2-^{13}C)-penicillin N (14f) was incubated with DAOC/DAC synthetase in air, thereby producing ^{16}O-^{13}C labelled cepham (46e). The (^{16}O-^{13}C)-labelled cepham (46e) would need to be present at a comparable level as the already present (^{18}O-^{13}C)-labelled cepham (46d) in order to generate a signal of similar intensity. Thus the crude $^{18}O_2$ incubation mixture was re-examined by ^{1}H-n.m.r to ensure that the unconsumed labelled penicillin N (14f) had not decomposed to penicilloate, and then re-incubated with DAOC/DAC synthetase under an atmosphere of $^{16}O_2$ gas. Examination by ^{1}H-n.m.r of the resulting mixture indicated that now virtually all the labelled penicillin N (14f) had been converted to ring expanded products. Re-examination by ^{13}C-n.m.r as before, revealed a second peak at δ 81.45 ppm with a separation of approximately 0.04 ppm which was assigned to the ^{13}C-^{16}O labelled lactone (69e).



(69d; R=D, **O** =^{18}O, e; R=D, **O** =^{16}O) * = ^{13}C

Due to the scale of these conversions and loss of signal intensity due to partitioning between the 3β-hydroxycephams (46d) and (46e) and their coresponding lactones (69d) and (69e), poor sensitivity was obtained and no further attempt to investigate the incorporation of ^{18}O from an incubation in $H_2{}^{18}O$ has yet been made. However, the observation of an ^{18}O-^{13}C isotope shift in these experiments directly confirms that the previously detected incorporation of molecular oxygen is into the hydroxyl group of the 3β-hydroxycepham (46c) and shows that in principle, incorporation of ^{18}O from $H_2{}^{18}O$ into the cepham (46a) can be experimentally tested in this way.

2.5 Conclusions.

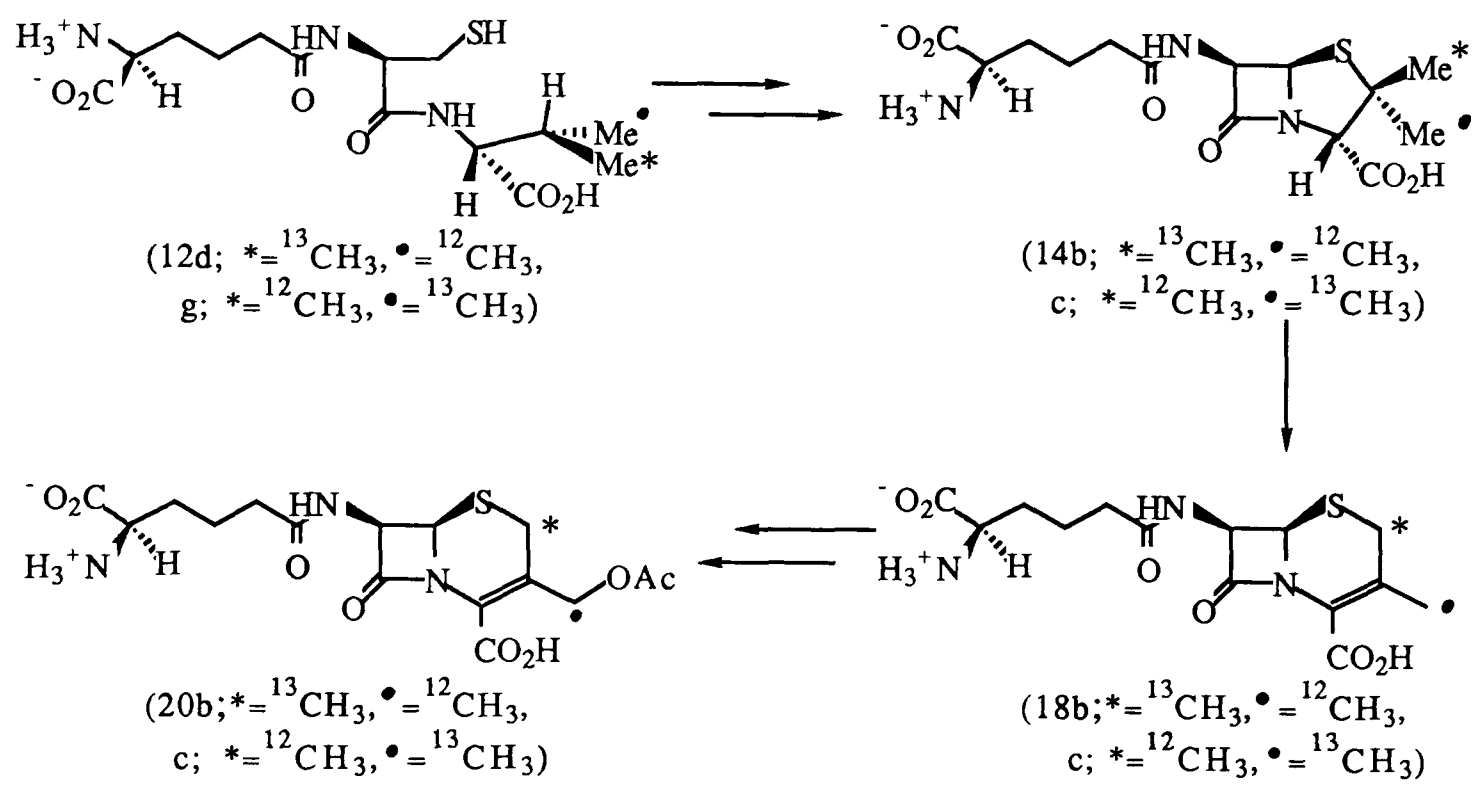
In this chapter the biosynthetic origin of the shunt metabolite (46) and its increased production by way of a primary isotope effect and the source of the hydroxyl group oxygen atom have been described. These observations have enabled an insight into the mechanism of enzymatic formation of this product, the details of which will be considered later (see Chapter 4, Section 4.7).

In addition, a preliminary investigation has been undertaken to test the feasibility of experimentally determining the contribution of water as a source for the 3β-hydroxycepham (46) hydroxyl oxygen atom. From the initial results obtained with the ^{13}C-^{18}O isotope shift studies, it would appear that this experimental method should allow for the contribution of this alternative pathway to be quantified. Problems do, however, still exist with regard to the sensitivity and hence the accuracy of this experimental technique and have yet to be overcome.

Chapter 3 : Stereochemistry of Valine Incorporation into Penicillin N (14) and Subsequent Ring Expansion.

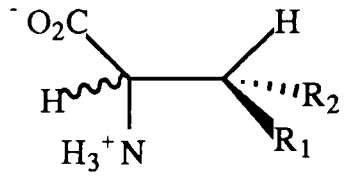
3.1 Introduction.

Although the isolation of LLD-ACV (12a) from the mycelium of *P. chrysogenum* by Arnstein and Morris[9] in 1960 represented good evidence that this compound was the immediate precursor of isopenicillin N (13a), it was not until the development of a cell free extract from *C. acremonium*[54] in 1976 that this was confirmed.[55,56] Previously, much attention had been given to the stereochemistry of incorporation of the diastereotopic methyl groups of valine. Particular points of interest were the stereochemistry of the thiazolidine ring closure of isopenicillin N (13a) and subsequent ring expansion of penicillin N (14a) to the dihydrothiazine system of the cephalosporins (Scheme 3.1.).



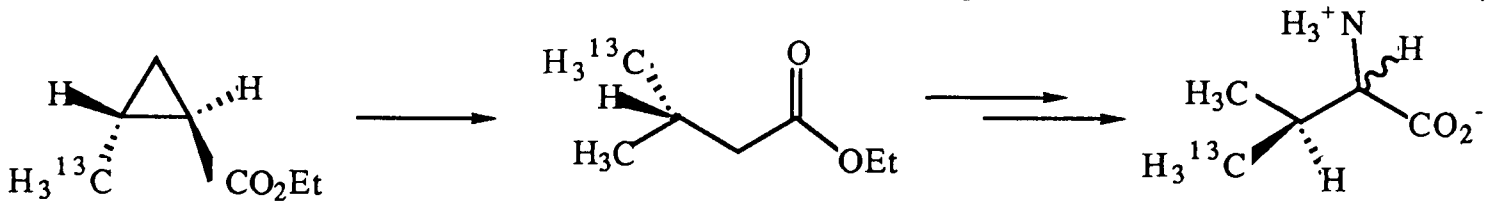
Scheme 3.1.

Studies were initiated to determine whether the thiazolidine ring closure was stereospecific and to identify which methyl group of penicillin N (14), either the pro-S or pro-R, became the endocyclic C-2 methylene and the exocyclic C-3' acetoxymethyl of cephalosporin C (20).[41,42] The obvious approach was to synthesize valines in which a ^{13}C-labelled methyl group had been stereospecifically introduced to give either the 3R or 3S configured valines (11d) or (11e) respectively.



(11d; R_1= $^{13}CH_3$, R_2= $^{12}CH_3$,
e; R_1= $^{12}CH_3$, R_2= $^{13}CH_3$)

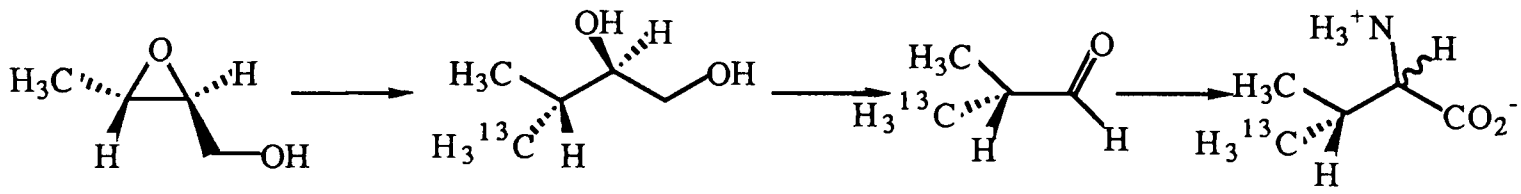
Four different syntheses of asymmetrically labelled valines were independently developed. Baldwin *et al.*[79] generated the chiral isopropyl group by reductive cleavage of the trans-(1S,2S)-([1-^{13}C]methyl)-2-cyclopropanecarboxylic acid (73) using lithium in liquid ammonia (Scheme 3.2.).



(73) (74) (11d)

Scheme 3.2.

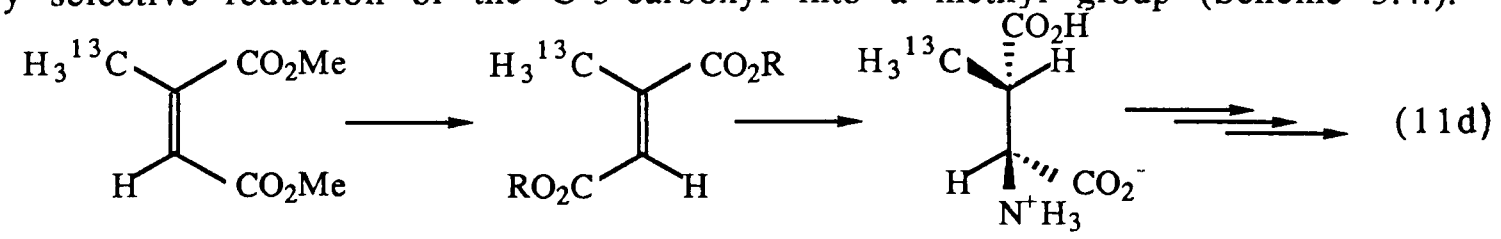
Aberhart *et al.*[80] chose to open the chiral epoxy alcohol (75) or its enantiomer with ^{13}C-labelled methyllithium to give diol (76). Transformation of (76) into aldehyde (77) was concluded with a Strecker type sequence to give the required valines (11d,11e) (Scheme 3.3.).



(75) (76) (77) (11e)

Scheme 3.3.

Sih *et al.*[40] used the β-methylaspartase catalysed interconversion of (^{13}C-methyl)-mesaconic acid (78) to (2S,3R)-3-[^{13}C]-methylaspartic acid (79), followed by selective reduction of the C-3-carboxyl into a methyl group (Scheme 3.4.).

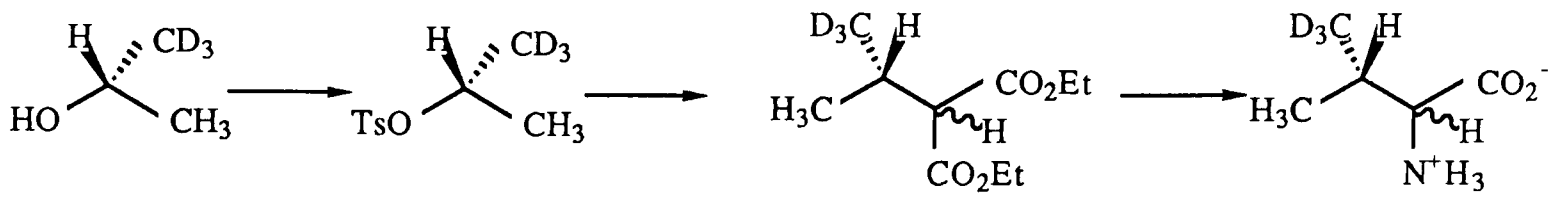


(78) (79) (80) (11d)

Scheme 3.4.

The final approach adopted by Hill *et al*[81] involved the synthesis of the chiral isopropyl alcohol (81)[82], formation of the tosylates (82) and subsequent

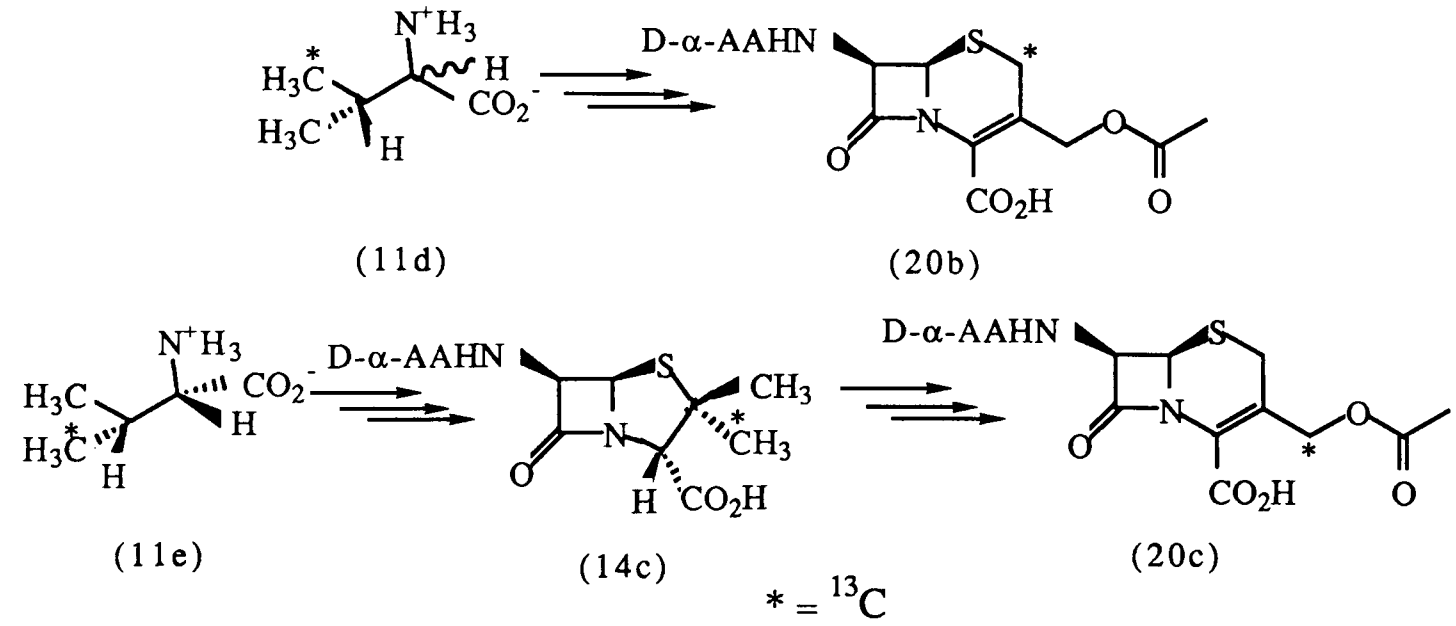
displacement with diethylmalonate to give the di-ester (83) which was transformed into (2R,3R)-(4,4,4-$^{2}H_3$)-valine (11i) (Scheme 3.5.).



(81) (82) (83) (11i)

Scheme 3.5.

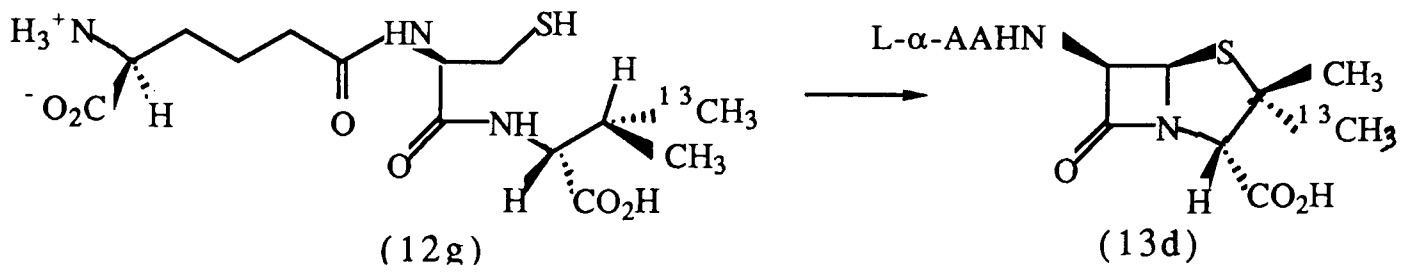
Intact cells of *C. acremonium* were then fed with (2RS,3R)-[4-^{13}C]-valine (11d)[42] and the cephalosporin C (20b) produced isolated. Comparison of the broadband ^{13}C-n.m.r spectrum of the labelled cephalosporin C (20b) with that of unlabelled (20a) indicated a five-fold enhancement in the intensity of the resonance corresponding to the C-2 methylene carbon atom. No enhancement of the acetoxymethyl resonance was observed (Scheme 3.6). In a complementary experiment, incorporation of (2S,3S)-[4-^{13}C]-valine (11e) into both penicillin N (14c) and cephalosporin C (20c) was studied.[40] Stereospecific incorporation of label into the α-methyl of penicillin N (14c) was observed by ^{13}C-n.m.r spectroscopy with approximately 20% incorporation of (11e) being obtained and indicating once more that the thiazolidine ring closure was stereospecific. Examination of the isolated cephalosporin C (20c) by ^{13}C-n.m.r revealed incorporation of label only into the exocyclic C-3' position.



Scheme 3.6.

The availability of partially purified IPNS prompted a re-examination of the stereochemistry of the conversion LLD-ACV (12) to isopenicillin N (13) to be

undertaken.[23] Thus, LLD-AC-[(3S)-(4-^{13}C)]-V (12g) was prepared and incubated with IPNS. Prior assignment of the isopenicillin N (13) methyl group resonances both in the ^{1}H-n.m.r spectrum by n.O.e experiments and subsequently in the ^{13}C-n.m.r spectrum, by selective heteronuclear decoupling (see Section 3.3), enabled the stereochemistry of incorporation to be shown to proceed with retention of configuration (Scheme 3.7.).

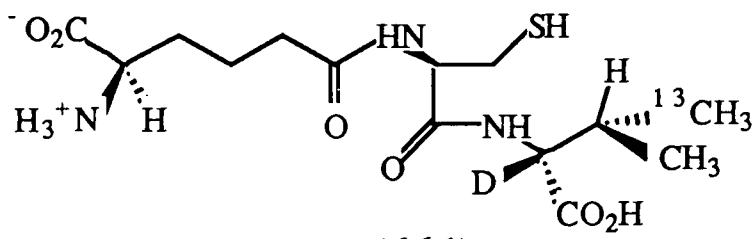


(12g) (13d)

Scheme 3.7.

3.2 Design of Experiment and Synthesis of Substrate.

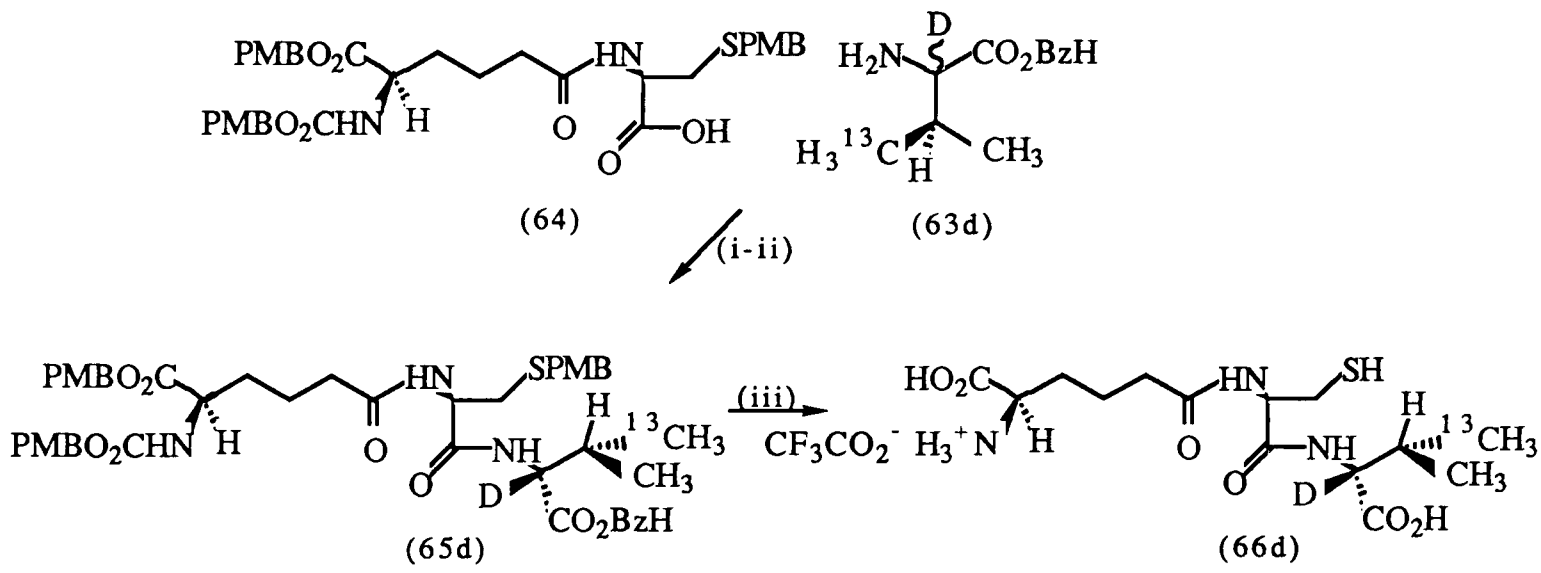
By the time that partially purified preparations of IPNS and DAOC/DAC synthetase had been developed, the investigations described in the previous section had been completed despite the limitations imposed by use of intact cells. Due to the low levels of incorporation obtained *ca* 8%[42] and 20%[40] the results reported were considered open to further scrutiny; particularly because at such low levels of incorporation added to the comparatively low sensitivity obtainable by ^{13}C-n.m.r spectroscopy at the time, non-stereospecific incorporation of label might not have been detected in these intact cell investigations. Besides improving upon these earlier experiments by examination of the incorporation of stereospecifically labelled penicillin N (14) into DAOC (18) and DAC (19), the hitherto unstudied conversion of DLD-AC-[(3S)-(2-^{2}H,4-^{13}C)]-V (66d) directly into penicillin N (14) and the subsequent formation of the 3β-hydroxycepham (46) could also be studied.



(66d)

It was with these objectives in mind as well as the obvious advantages provided by the use of cell free preparations of IPNS and DAOC/DAC synthetase and the development of higher field ^{1}H- and ^{13}C-n.m.r spectrometers, thereby increasing the assay sensitivity, that the tripeptide DLD-AC-[(3S)-(2-^{2}H,4-^{13}C)]-V

(66d)[83] was synthesised. Thus (2RS,3S)-[(2-^{2}H,4-^{13}C)]-valine benzhydryl ester (63d) and protected DL-AC (64) were coupled using the procedure described in Chapter 2 (Scheme 3.8) and deprotected with refluxing TFA containing anisole to give the tripeptide (66d). The stereochemical purity at C-3 of the valinyl residue of the deprotected tripeptide (66d) was shown to be greater than 99% by examination with ^{13}C-n.m.r spectroscopy - no signal due to incorporation of label into the other methyl group (i.e the 3R-labelled valinyl tripeptide) could be detected.



Reagents : (i) EEDQ, DCM, anhydrous Na_2SO_4; (ii) SiO_2 Chromatography
(iii) TFA/anisole [5:1, v/v], reflux 30 minutes.

Scheme 3.8.

3.3 Assignment of ^{13}C-Chemical Shifts.

To obtain maximum sensitivity in the final analysis of the degree of stereospecificity, for both thiazolidine and dihydrothiazine ring formations, it was important to assign all the carbon resonances where label incorporation could possibly occur. This data would then enable the regions in the ^{13}C-n.m.r spectra of the various β-lactam products associated with stereospecific and non-stereospecific incorporation of label to be scrutinized. Although such n.m.r data is available for some penicillins[84], DAC (19)[130] and cephalosporin C (20)[40,85], no unambiguous data was available for penicillin N (14), DAOC (18) and the 3β-hydroxycepham (46). Assignment of these resonances were made on the basis of selective ^{13}C-^{1}H decoupling experiments in the proton coupled ^{13}C-n.m.r specra.

Initial assignment of the α- and β-methyl groups of penicillin N (14a) were made on the basis of n.O.e experiments in a manner analogous to that employed for

the assignment of the methyl groups of isopenicillin N (13a)[23], by Dr. N. J. Turner.

In general, heteronuclear decoupling experiments were performed by initial determination of the chemical shift of the protons bonded to the carbon atom which was to be assigned. This was achieved by running a ^{1}H-n.m.r spectrum and direct measurement of proton shift (δ). Next a continuous wave (proton coupled) ^{13}C-n.m.r spectrum was recorded against which the selective decoupled spectrum would be compared. Then using low decoupling power, irradiation at the previously determined δ value was performed. In the case of the AB quartet corresponding to the C2 protons of the cepham (46a), irradiation at the δ values of both wings of the AB quartet were performed. Providing that the protons to be decoupled occurred in a region of the proton spectrum comparatively free of other resonances and that the decoupling power was not too high, only the ^{1}H-^{13}C coupling associated with the carbon atom to be identified collapsed. The results of the assignments made in this way are listed below (see Table 3.1.).

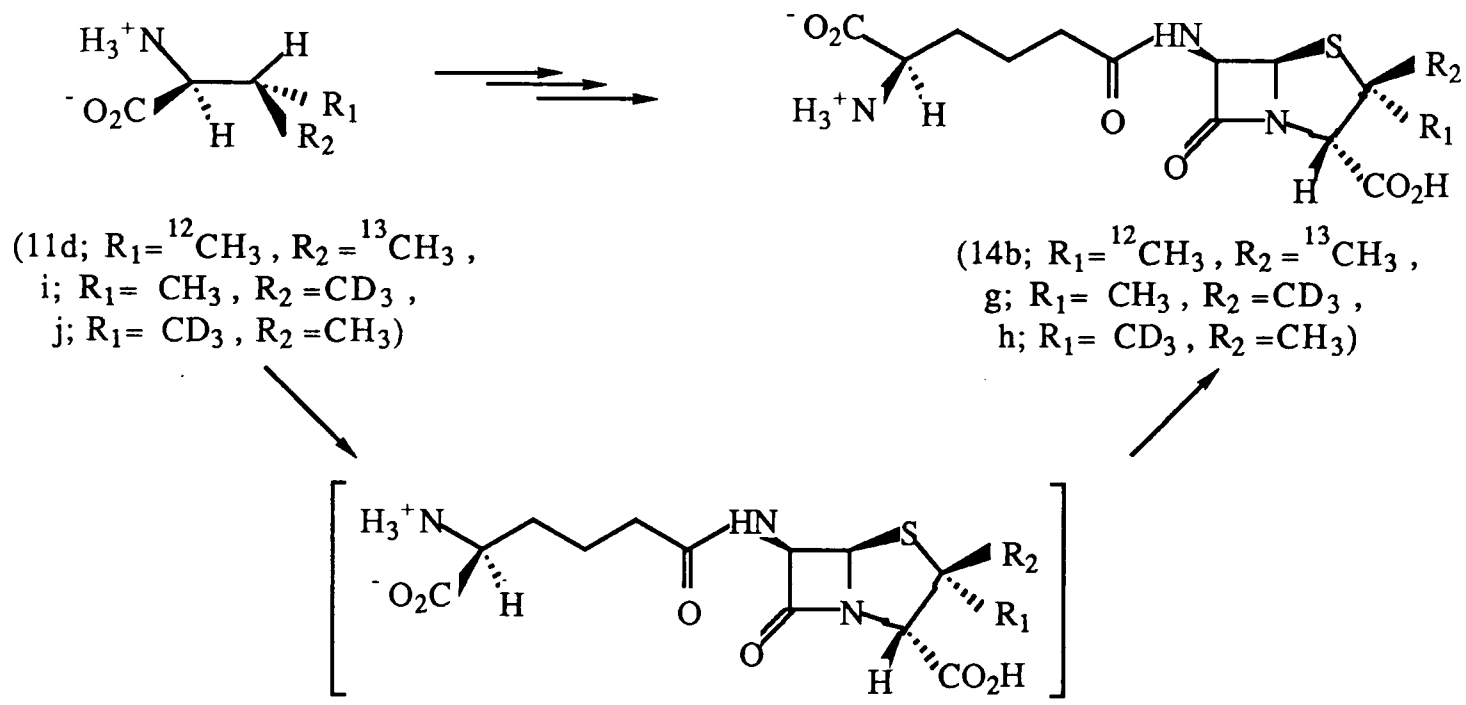
Table 3.1.

Compound	Resonance Assignment	Mutiplicity	^{1}H Chemical Shift	
	(δ_C ppm)		δ (ppm)	
Penicillin N (14)	27.22	q	1.52	α-methyl
	31.11	q	-	β-methyl
3β-hydroxycepham (46)	25.79	q	-	α-methyl
	34.89	t	2.66 and 3.56	C-2 CH_2
DAOC (18)	19.22	q	-	C-3' methyl
	29.17	t	3.38, 3.61	C-2 CH_2
DAC (19)	61.71	t	-	C-3' CH_2OH
	26.15	t	3.42, 3.60	C-2 CH_2

3.4 Stereospecificity of Penicillin N formation from DLD-AC-(3S)-[2-^{2}H,4-^{13}C]-V (66d).

Since all previous work[40,41] on the incorporation of chiral valines into penicillin N (14a) (Scheme 3.8) had been *via* intact cell studies and had

consequently proceeded through the usual biosynthetic pathway (Scheme 1.1.) to isopenicillin N (13a) and thence penicillin N (14a), the stereochemistry of the incorporation of the valinyl methyls directly into penicillin N (14a) by the action of IPNS *via* the unnatural substrate DLD-ACV (66) (Scheme 2.7) had not been examined. Only the stereochemistry of the direct conversion of the natural IPNS substrate, LLD-ACV (12) to isopenicillin N (13) had been investigated with a cell free preparation of partially purified IPNS, this not being possible until after the development of the cell free system due to the inability of tripeptides to penetrate the cell wall.[23]

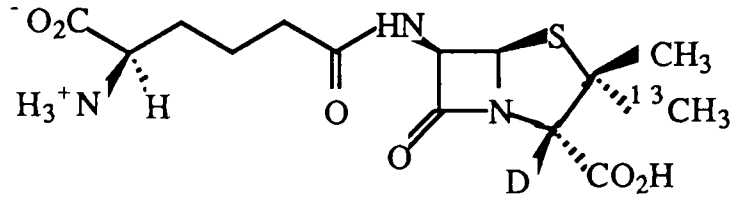


(13b; $R_1 = {}^{12}CH_3$, $R_2 = {}^{13}CH_3$,
d; $R_1 = CH_3$, $R_2 = CD_3$,
e; $R_1 = CD_3$, $R_2 = CH_3$)

Scheme 3.9.

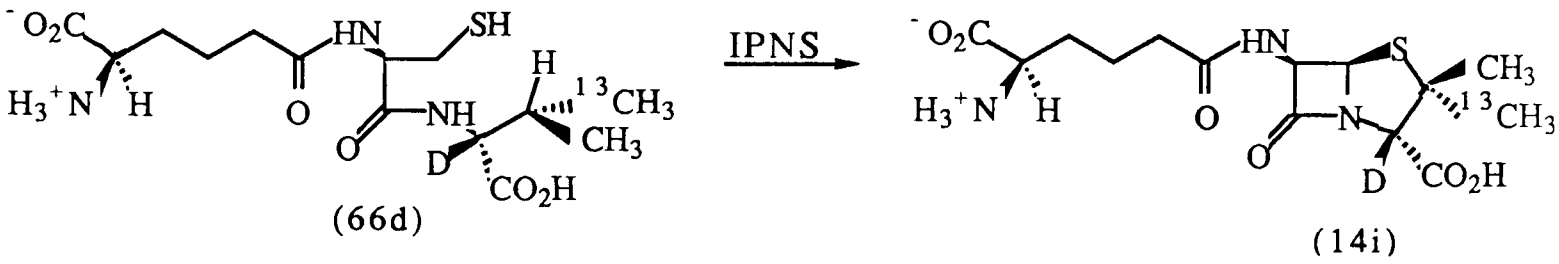
It was, however, considered unlikely that conversion of the unnatural IPNS substrate, DLD-ACV (66) would differ stereochemically from the stereospecific incorporation of valine observed with the natural biosynthetic pathway.

Incubation of (5R)-5-amino-5-carboxypentanoyl-L-cysteinyl-(2R,3S)-(2-^{2}H,4-^{13}C)-valine (66d) with IPNS and the usual cofactors, gave the labelled (2R)-(2'-^{13}C,3-^{2}H)-penicillin N (14i).



(14i)

Examination of the crude incubation mixture by ^{13}C-n.m.r (125.77MHz) revealed incorporation of label exclusively into the α-methyl group of (14i). No signal, due to incorpration into the β-methyl, at δ 31.11 ppm could be detected (Scheme 3.10.).



(66d) (14i)

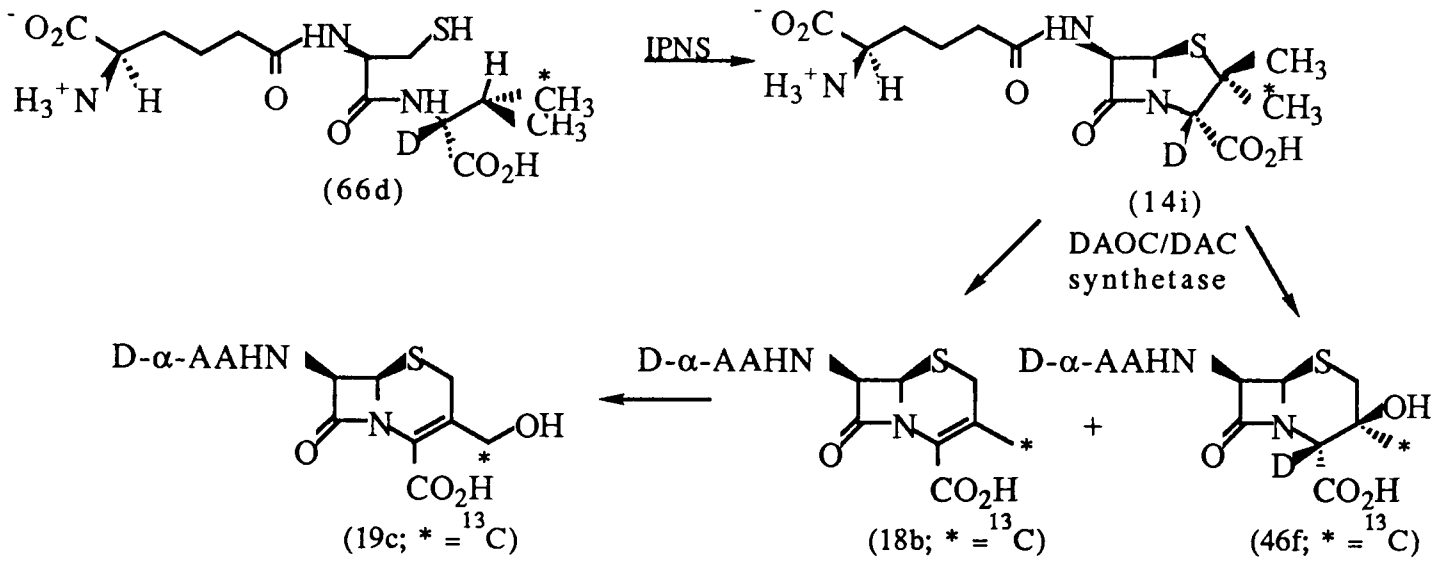
Scheme 3.10.

This result indicates that formation of penicillin N (14i) from the unnatural tripeptide precursor DLD-ACV (66d) is stereospecific within the detection limits of the assay used. Furthermore, from the signal to noise ratio obtained in this experiment, which was determined to be *ca* 43:1, the stereopecificity of this process can be estimated to be >97%.

3.5 Stereospecificity of DAOC (18) and DAC (19) formation from Penicillin N (14).

As discussed previously, the stereospecificity of DAOC (18) and DAC (19) formation from penicillin N (14) had not been studied directly but had been assumed on the basis of the observed stereochemical incorporation of specifically labelled valines into cephalosporin C (20).[40,42] The availability of a cell free DAOC/DAC synthetase preparation in addition to the specifically labelled penicillin N (14i) sample, enzymatically prepared from (5R)-5-amino-5-carboxypentanoyl-L-cysteinyl-(2R,3S)-[2-^{2}H,4-^{13}C]-valine (66d) as described in the previous section, prompted investigation of the stereospecificity of the ring expansion process.

Thus crude (2R)-[2'-^{13}C,3-^{2}H]-penicillin N (14i) was incubated with partially purified DAOC/DAC synthetase to give a mixture of DAOC (18b), DAC (19c) and 3β-hydroxycepham (46f) (Scheme 3.11) in a ratio of 20:45:35, [(18b): (19c): (46f)], as determined by integration of the β-lactam region in the ^{1}H-n.m.r spectrum of the incubation mixture.



Scheme 3.11.

The ^{13}C-n.m.r spectrum of this incubation mixture was compared with a ^{13}C-n.m.r spectrum of an identical incubation of (3-^{2}H)-penicillin N (14e). Particular attention was given to the regions of this spectrum in which signals associated with stereospecific and non-stereospecific incorporation of the ^{13}C-label were expected to occur. Detailed comparison in this way indicated that incorporation of label into the exocyclic methyl and hydroxymethyl positions of DAOC (18b), DAC (19c) and 3β-hydroxycepham (46f) had occurred. The degree of stereospecificity for the formation of the ring expanded products was estimated by analysis of the signal to noise ratio, as described for penicillin N (14i) formation. To increase sensitivity, the ring expanded products were isolated by hplc and re-examined individually by overnight ^{13}C-n.m.r spectroscopy thus resulting in an accumulation of approximately 45,000 transients. By this method a greater signal to noise ratio was obtained and hence a higher degree of stereospecificity could be assigned to the ring expansion process. Analysis of the signal to noise ratio was again performed and the results listed below (Table 3.2.).

Table 3.2 ^{13}C-NMR Signal : Noise Ratio

Compound	δC	Crude	Purified	% Stereospecificity
DAOC	19.12	8 :1	9:1	>90
DAC	61.74	18:1	30:1	>97

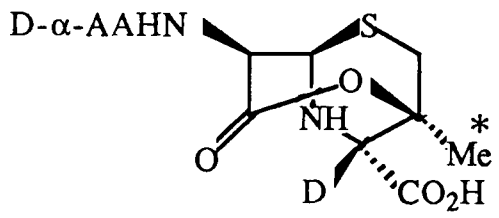
From these results it is possible to conclude that the incorporation of the penicillin N (14) methyl groups into the ring expanded products (18) and (19) occurs in a stereospecific manner, with the α-methyl group of (14) becoming the

C-3' carbon of the cephems (18) and (19). Furthermore, the results presented here are in accord with those obtained from the initial intact cell studies on the stereospecificity of the ring expansion process but represent considerable improvement over these earlier experiments. For example, the stereospecificity of DAOC (18) and DAC (19) formation has been examined directly thus avoiding the need to infer the stereospecificity from that determined for cephalosporin C (20). Furthermore, the sensitivity of this investigation has been increased substantially over that of the earlier intact cell experiments,[40,42] and this has enabled a higher degree of stereospecificity to be assigned to the ring expansion of penicillin N (14) than was previously possible.

3.6 Stereospecificity of 3β-Hydroxycepham (46) Formation.

Re-investigation of the stereospecificity of the ring expansion process could easily be adapted to include the formation of the 3β-hydroxycepham (46). As described previously (Chapter 2, Section 2.3), substitution of the α-proton of valine by a deuterium atom resulted in much higher levels of production of this shunt metabolite.[71] Thus, following the synthesis of (5R)-5-amino-5-carboxypentanoyl-L-cysteinyl-(2R,3S)-[2-^{2}H,4-^{13}C]-valine (66d) and its subsequent enzymatic conversion to (2R)-[2'-^{13}C,3-^{2}H]-penicillin N (14i), production of this cepham could be increased to sufficient levels such that investigation of the stereospecificity of its formation could be undertaken. In the previous section incubation of (14i) with DAOC/DAC synthetase to give DAOC (18b), DAC (19c) and (46f) was described. The signal to noise ratio in the ^{13}C-n.m.r spectrum of the crude (2R)-[2'-^{13}C,3-^{2}H]-penicillin N (14i) incubation mixture, for the 3α-methyl group of the 3β-hydroxycepham (46f) was determined to be *ca* 24:1. Isolation by hplc and re-examination by overnight ^{13}C-n.m.r gave a better signal to noise ratio. However, the situation was complicated by the interconversion of the cepham (46f) to the corresponding lactone (69f) - a process easily followed by comparison of the ^{13}C-n.m.r spectra obtained after 2,000, 10,000 and 47,000 transients. Approximately 70% lactonisation had occurred by the time acquisition was terminated at 47,000 scans, and thus a better signal to noise ratio could be obtained for the 3β-hydroxycepham lactone (69f) than for the unlactonised cepham (46f). Consequently, the stereospecificity of 3β-hydroxycepham (46f) formation was determined indirectly from that measured for the lactone (66f), for which a signal to noise ratio of *ca* 43:1 was obtained (Table 3.3.). It should be noted that no signal corresponding to incorporation of label into the C-2 methylene

position of either the cepham (46f) or the lactone (69f) could be discerned from the background spectral noise.



(69f; *=^{13}C)

Indirect determination of the stereospecificity is valid as no rearrangement of the carbon skeleton of the β-hydroxycepham (46f) occurs during lactonisation. Consequently there is no obvious process by which the stereospecifically incorporated label in the 3α–methyl group could be randomised.

Table 3.3. ^{13}C-NMR Signal : Noise Ratio

Compound	δC	Crude	Purified	%Stereospecificity
3β-Hydroxy-cepham (46f)	34.89	24:1	30:1	>97
Lactone (69f)	61.74	-	43:1	>98

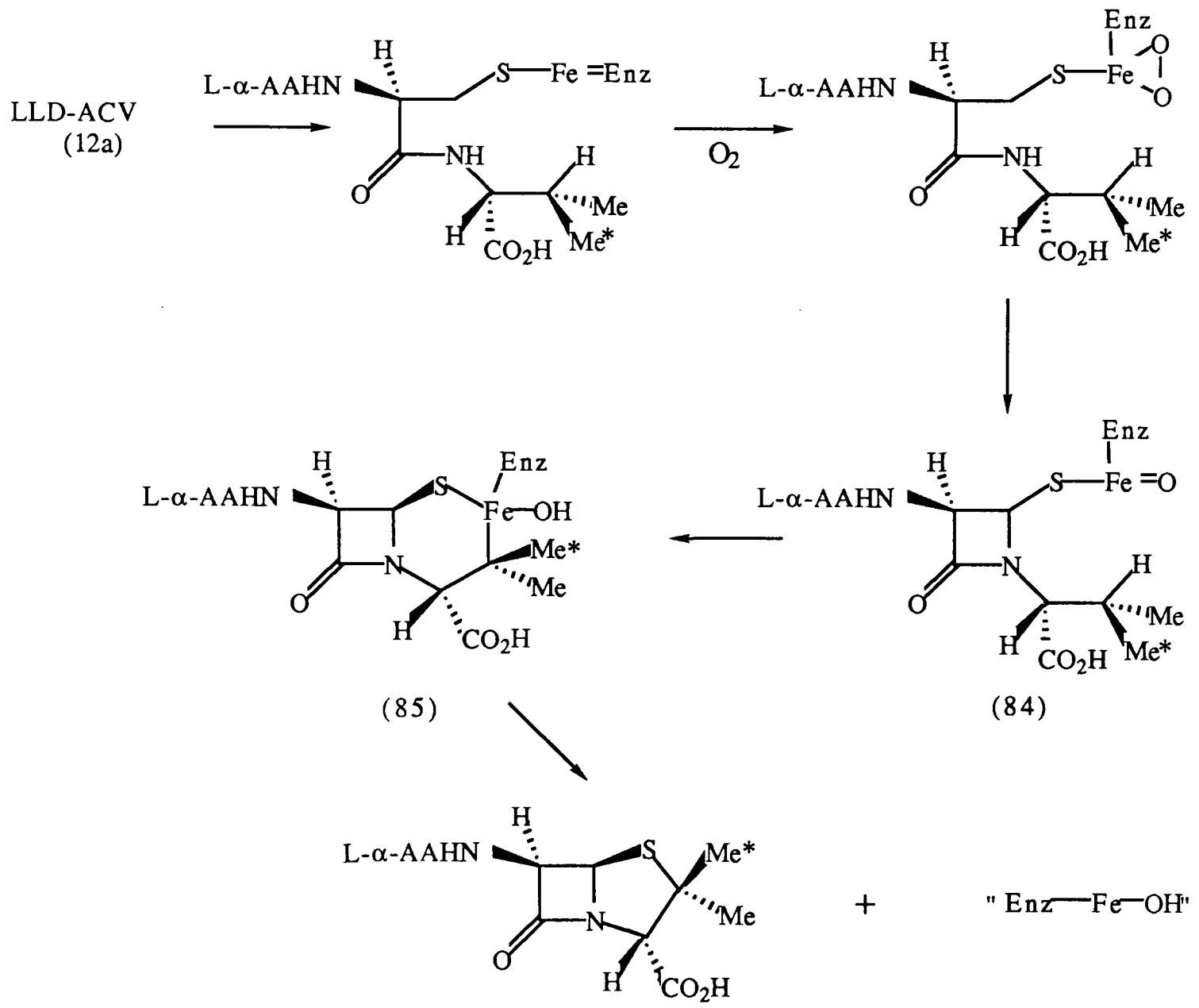
From these results, it can be concluded that incorporation of the penicillin N (14a) methyl groups into the shunt metabolite (46) proceeds in a stereospecific manner with the α-methyl group becoming the C-3' α-methyl of (46).

3.7 Conclusions.

The intact cell studies previously reported and the results described in this chapter have shown that the formation of penicillin N *via* the normal biosynthetic pathway and also from the unnatural substrate, DLD-ACV (66), by the action of IPNS are both stereospecific processes. Likewise the ring expansion process has been shown to be stereospecific but to a much higher degree of certainty than was previously achieved with intact cells. This has been made partly possible by advances in n.m.r technology, in particular the development of stronger super-conducting magnets leading to increased sensitivity, and also by the use of cell free enzyme preparations. The availability of partially purified IPNS and DAOC/DAC synthetase has enabled the problems associated with cell wall permeability that were experienced in the initial intact cell studies to be avoided. Having overcome these limitations, use of a more advanced biosynthetic

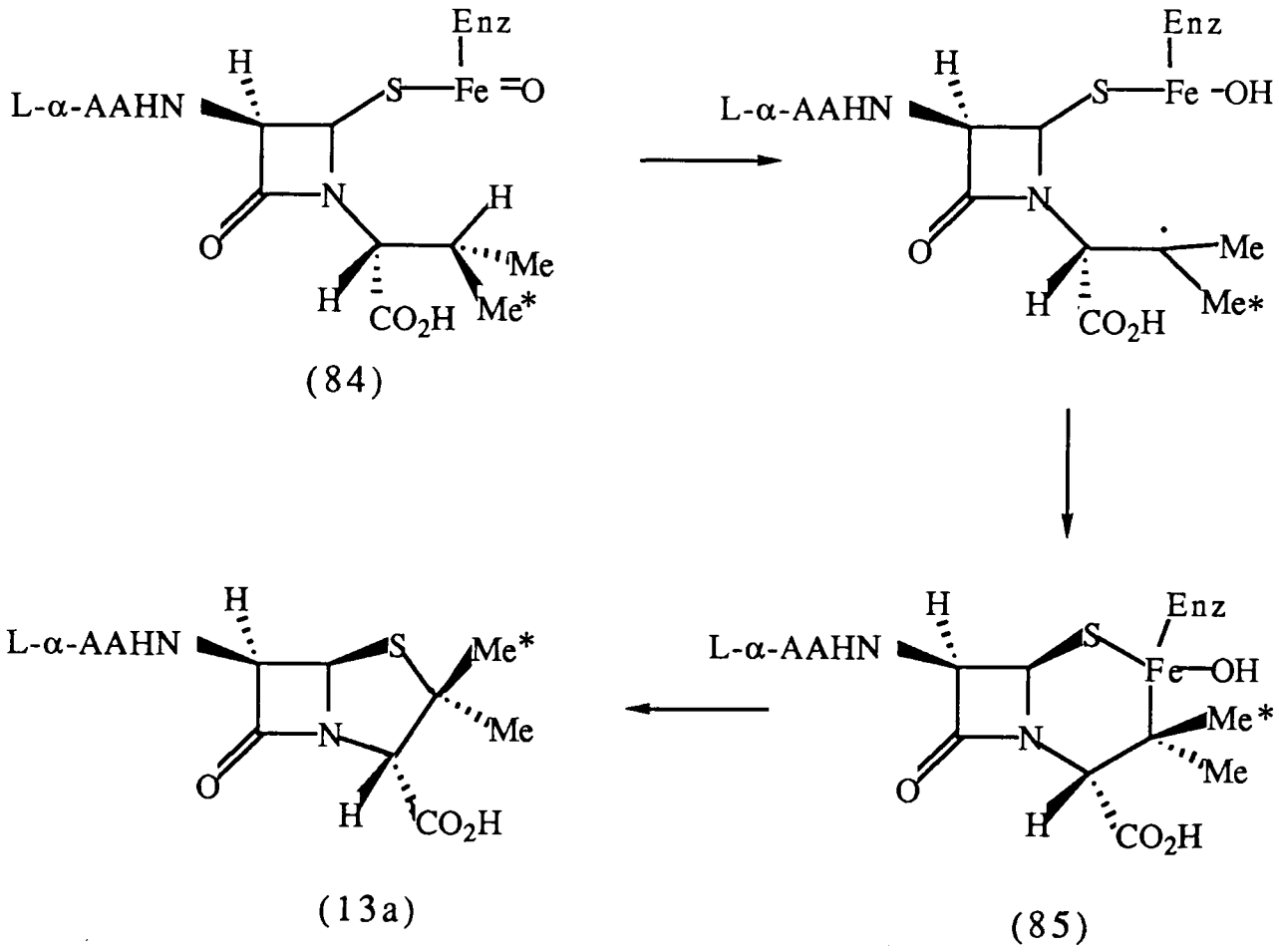
intermediate leading to virtually quantitative label incorporation has been achieved.

Mechanistically, the observation of stereospecific thiazolidine ring closure[23] from IPNS, involving removal of the β-hydrogen of the valinyl residue can be accounted for by the formation of either a short-lived radical species or a discrete Fe-C bonded species (Scheme 3.12.). Such an Fe-C bond would be expected to be weak, with a low energy of homolytic dissociation. The lifetime of any intermediate radical would need to be considerably shorter than the frequency of inversion.



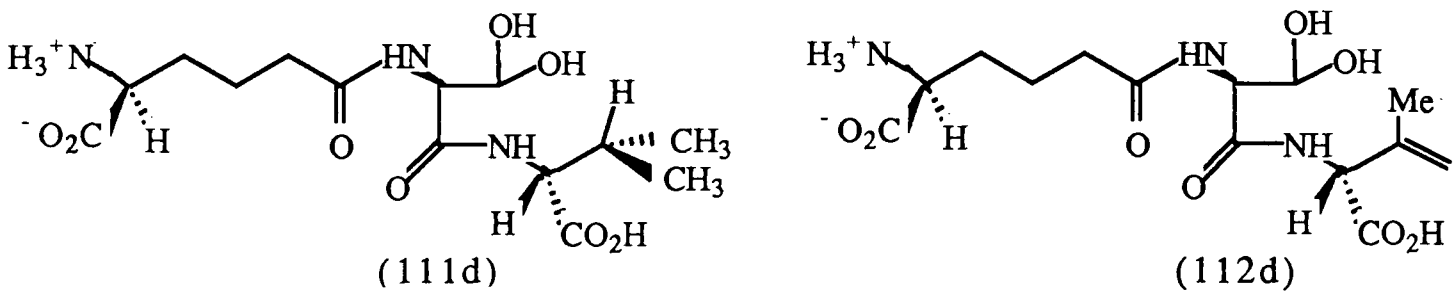
Scheme 3.12.

An alternative explanation to account for the observed stereospecificity is that the geometry of the radical is maintained by the topology of the active site (Scheme 3.13.).



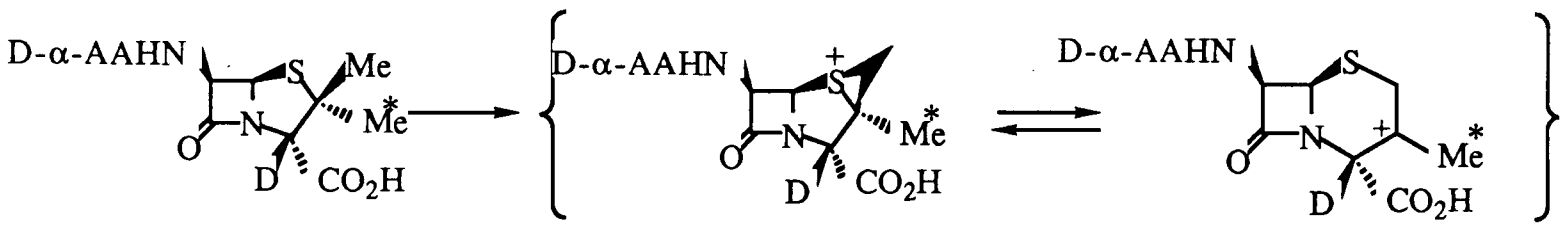
Scheme 3.13.

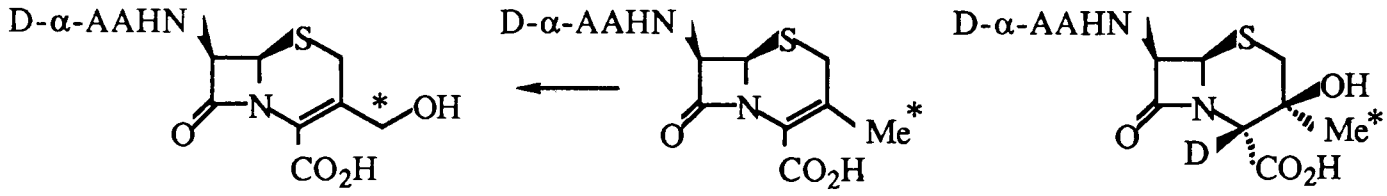
Recent unpublished work performed in this department has lent support to the formation of a monocyclic intermediate (84).[86] This work entails the isolation of a shunt metabolite, believed to be LLD-A-Serine aldehyde-V acetal (111d) or the corresponding isodehydrovaline tripeptide acetal (112d), produced by the action of IPNS upon LLD-ACV (13). This result has been most easily rationalised by proposing that (111d) is formed from (84) by replacement of the sulphur atom by a hydroxyl group and subsequent β-lactam ring opening to give an aldehyde which can then form the acetal (111d or 112d) (see Scheme 6.22.).



The results presented in this chapter on the stereospecific formation of DAOC (18) and DAC (19) from penicillin N (14) are in accord with those previously

published but have been achieved with a higher degree of sensitivity which permits quantification of the the degree of stereospecificity to a much higher level than previously obtained. Furthermore, the substitution of a deuterium atom at C-3 of penicillin N (14i) has enabled additional mechanistic information relating to the formation of 3β-hydroxycepham (46) to be obtained. Specifically, the observation that the methyl groups of penicillin N (14) are stereospecifically incorporated into the C-2 methylene and methyl of the 3β-hydroxycepham (46) with the same stereospecificity as for the usual biosynthetic cephems, is consistent with DAOC (18), DAC (19) and 3β-hydroxycepham (46) sharing a common enzyme-bound precursory intermediate (Scheme 3.14.). If so, it is also possible to infer that the formation of this key intermediate must also proceed in a stereospecific manner from penicillin N (14).





* = ^{13}C

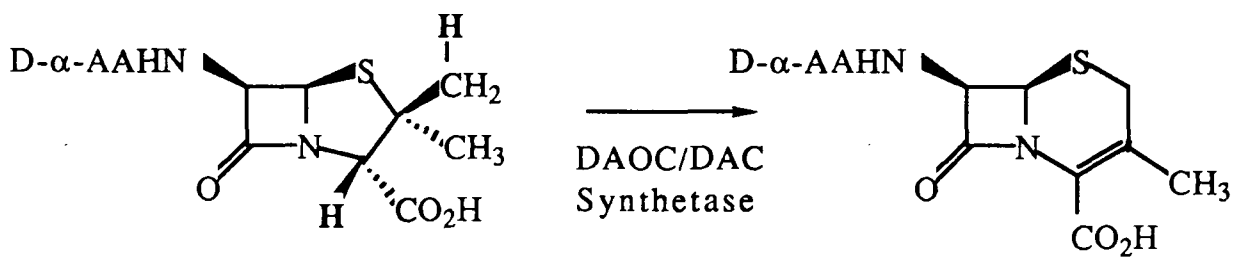
Scheme 3.14.

The structures of the proposed common enzyme-bound intermediates, from which formation of the cephems and cepham may occur will be considered in more detail later (see Chapter 4, Section 4.7.).

Chapter 4 : Mixed Competitive Kinetic Isotope Experiments.

4.1 Introduction.

In this chapter the use of isotope effects as a mechanistic probe will be discussed in relation to the ring expansion of penicillin N (14a) to DAOC (18a). Isotope effect data was expected to give information concerning two aspects of this process ; firstly the site of initial reaction between the enzyme and penicillin N (14a) and secondly, whether the removal of the two hydrogen atoms, one from the β-methyl and the other from C-3 of penicillin N (14a) (Scheme 4.1.) is a concerted or stepwise process.



(14a) (18a)

Scheme 4.1.

Kinetic isotope effects may be conveniently classified into primary and secondary effects. A primary effect is one in which the bond to the isotope is broken, whereas in a secondary effect the bond to the isotope is not broken but the reaction rate is affected due to the presence of the isotope adjacent to the bond being broken.

The use of isotope effect data as a mechanistic probe in the study of enzyme catalysed reactions[87] is based upon knowledge derived from investigations of these effects in non-enzymatic (chemical) systems (see for example references 88 and 89). However, the transition from the application of isotope effect data in non-enzymatic to enzymatic reactions is not a trivial one. For this reason a brief discussion of the similarities and differences encountered between these two systems is given below.

Generally, observation of a kinetic isotope effect identifies the slowest or rate determining step - even in multi-step processes such as those encountered with complicated chemical reactions or with the majority of enzyme catalysed processes. It is with regard to the magnitude of the observed isotope effect that the main differences between enzymatic and chemical reactions become apparent. In chemical systems, observation of a less than maximal effect for a single step

known to be rate determining may provide information relating to the transition state involved.[89] When considering enzyme catalysed reactions, however, it has been suggested that observation of a less than maximal effect is to be expected since enzymes have evolved to be efficient catalysts.[90] Catalysts work by lowering the activation energy of the reaction concerned; consequently modification of the overall energetics of the process by the enzyme reduces the dominance of any one particular step. If this is indeed true, then two reasons for the observation of a less than maximal effect may be suggested;

i) partial retention of the zero point energy of the bond concerned in the transition state, and

ii) the presence of other slow steps in the reaction sequence which prevent the isotopically sensitive step from becoming cleanly rate determining.

Northrop has argued that the labelling of a particular step in a multi-step enzymatic process as 'rate determining' be more appropriately referred to as 'rate limiting'.[91] This idea has been extended and refined so that a quantitative evaluation of the extent to which a particular step is defined as 'rate limiting' can be made. This has been achieved by the introduction of a sensitivity function which is applicable to each forward step of the process in question.[92] The rate limiting step is then defined as "the forward step for which the sensitivity function is maximal".

The expressions for the two fundamental kinetic parameters, V_{max} and V_{max}/K_m (where V_{max} is the maximum velocity of reaction and K_m is the Michaelis constant) for enzymatic processes contain different rate constants and are thus by definition, different. It is consequently important to make a clear distinction between any step identified as being rate limiting under the conditions associated with either of these parameters. For example, in many cases the difference in substrate concentrations between V_{max} and V_{max}/K_m systems means that different steps may be identified as rate limiting in a V_{max} experiment compared to a V_{max}/K_m experiment. This is generally because of differences in the partitioning of steady-state intermediate(s) between reactant and product.

Examination of the expressions for V_{max} and V_{max}/K_m reveals the main differences between the type of information available under V_{max} and V_{max}/K_m experimental conditions. To derive these expressions a slightly more complicated system must be considered than the one used to derive the simple Michaelis-Menten equation :

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} [EP] \xrightarrow{k_3} [E] + [P]$$

Scheme 4.2.

(It should be noted that the conversion of [ES] to [EP] in Scheme 4.2. represents the first irreversible event).

Solution of the steady-state expressions for the intermediates ES and EP and the rate equation (for a full treatment see ref. 88) gives the following expressions for v (the rate of reaction), K_m, V_{max}, and V_{max}/K_m ;

$$V = \frac{k_2 k_3 V_{max} [E]}{(k_2 + k_3)(K_m + [S])} \quad \text{and} \quad V/K_m = \frac{k_1 k_2 [E]}{(k_1 + k_2)}$$

$$\text{where} \quad K_m = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)} \quad \text{and} \quad V_{max} = \frac{k_2 k_3 [E]}{(k_2 + k_3)}$$

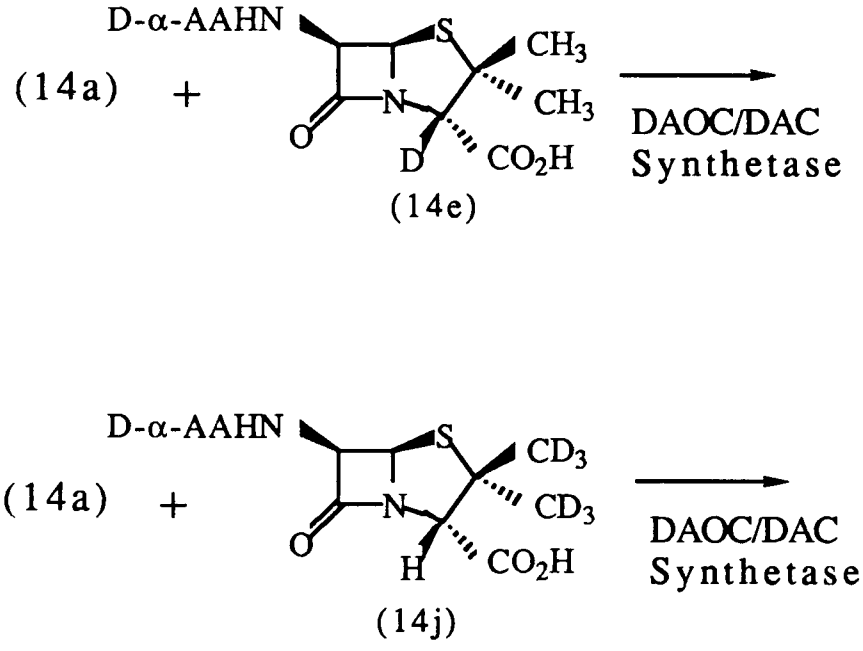
From these, it is clear that the expression for V_{max} contains no rate parameters for events up to and including the formation of the enzyme-substrate complex, [ES]. Furthermore, in the expression for V_{max}/K_m, no rate parameters relating to events occurring after the first irreversible event are included. Consequently V_{max} and V_{max}/K_m experiments give additional and generally complementary information about an enzyme catalysed process.

One other important point remains to be addressed; this is that V_{max}/K_m experiments apply to the case in which the enzyme 'selects' between substrates (*i.e.* reversible substrate binding). Since V_{max}/K_m isotope effects reflect events leading to the first irreversible event, it is possible to quantify them, assuming reversible binding of substrate to the enzyme, by using mixtures of unlabelled and labelled substrates. The substrates for these experiments may be totally different compounds or differ only in isotopic content, in which case they are refered to as competitive kinetic isotope effect experiments.

4.2 Design of Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effect Experiments for the Ring Expansion Process.

As summarised in the previous section, V_{max}/K_m experiments effectively examine the rate constants concerned with the formation and reaction of the enzyme-substrate complex but exclude those relating to steps occurring after the first irreversible event. Obviously this means that an isotope effect will only be observed in steps up to and including the first irreversible event. By exploiting this property of V_{max}/K_m systems, it should be possible to identify the site of initial reaction. For V_{max}/K_m experiments the first irreversible event is assumed to be a reaction occurring between the enzyme and substrate after formation of the enzyme-substrate complex *i.e.* after binding.

Two competitive mixed labelled/unlabelled substrate experiments were thus required to obtain V_{max}/K_m data for the ring expansion process. The two differently labelled substrates for this investigation needed to be specifically labelled in the sites of hydrogen abstraction during the ring expansion process *i.e.* β-methyl and C-3 of (14) (see Scheme 4.1.). These experiments are described schematically below (Scheme 4.3.).



Scheme 4.3.

The specifically deuterated penicillins (14e) and (14j) were accessible *via* the respective deuterated tripeptides (66b) and (66e) and IPNS. Hexadeutero-penicillin N (14j) was used in the mixed experiments since synthesis of stereospecifically labelled pro-R trideuteromethyl valine (11i), which is the isomer required to give β-trideuteromethyl penicillin N (14g), is considerably more complex than synthesis of di-trideuteromethyl valine (11k). Additionally,

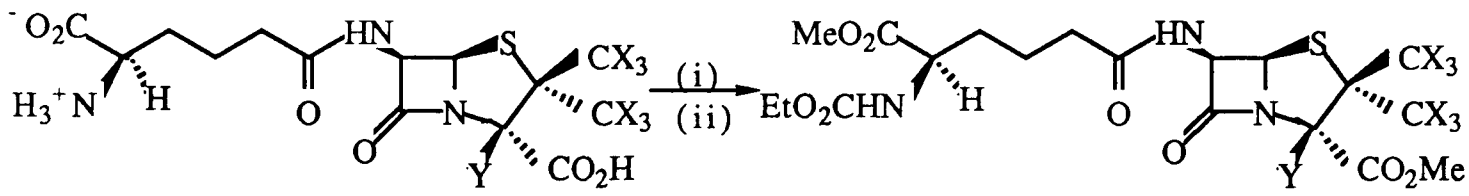
deuteration of the α-methyl of penicillin N (14j) was not expected to interfere with the V_{max}/K_m experiments concerned with the β-methyl position. However, deuteration at this site was expected to give rise to a primary isotope effect during the hydroxylation of DAOC (18) to DAC (19), as had previously been shown,[45] thus slowing down the conversion and leading to higher levels of DAOC (18) than usual. Again this was not expected to create problems providing that product inhibition was not so severe that it became impractical to obtain percentage conversions in the mixed experiments large enough for accurate measurement. Also, theoretically, factors affecting the rate of hydroxylation should not perturb the measured ratio of deuterated and undeuterated penicillin Ns, (14a) and either (14e) or (14j), which were not consumed during the ring expansion step, since the two processes are distinct from each other.

The final problem that needed to be addressed was the choice of an analytical method for the determination of any observed isotope effect. In many cases, isotopic analysis of both product and unconverted substrate can be used in V_{max}/K_m experiments, providing that the products derived from the isotopically labelled substrate are distinguishable from those derived from unlabelled substrate . However, product analysis in experiments concerned with the ring expansion process was considered too complex, not only because the usual ring expanded products from labelled and unlabelled substrate could not be distinguished from each other in the case of the mono-deuterated penicillin N (14e), but also since bifurcation of the pathway leading to the cepham (46b) occurred with (14e) as substrate.[71] In addition the main product from the ring expansion enzyme, DAOC (18), was further converted by the bifunctional DAOC/DAC synthetase enzyme to DAC (19) making product analysis more complicated. This is most pronounced in the case of the mixture of deuterated and undeuterated DAOCs from (14a) and (14j) since the sequential conversion of the deuterated DAOC to DAC would also be subject to an isotope effect which would alter the ratio of labelled to unlabelled DAOC products still further. Thus, the analysis of the ratio of labelled and unlabelled substrate remaining in the substrate pool after exposure to DAOC/DAC synthetase was essential; any results from product analysis could be used with caution as complementary evidence.

It was anticipated that the observation of an isotope effect up to and including the first irreversible event, at one position of the two possible positions, would result in enzymatic discrimination between the mixture of labelled and unlabelled substrates leading to a perturbation of the original ratio. For the alternative position at which cleavage could occur after the first

irreversible event, no such enzymatic discrimination would be seen and the ratio of labelled and unlabelled substrate would remain constant.

In the interest of obtaining the best possible isotope ratios and hence the most accurate determination of the ratio of labelled and unlabelled substrate, it was decided that derivatisation of the recovered penicillin N samples would be necessary. Thus, the penicillins were converted to their respective N-ethoxycarbonyl, dimethyl ester derivatives (86a-d) (Scheme 4.4.) by an analogous procedure to that used by Baldwin *et.al* to prepare the equivalent isopenicillin N derivative.[93]



(14a; X=Y=H, e; X=H, Y=D, j; X=D, Y=H, k; X=Y=D)

(86a; X=Y=H, b; X=H, Y=D, c; X=D, Y=H, d; X=Y=D)

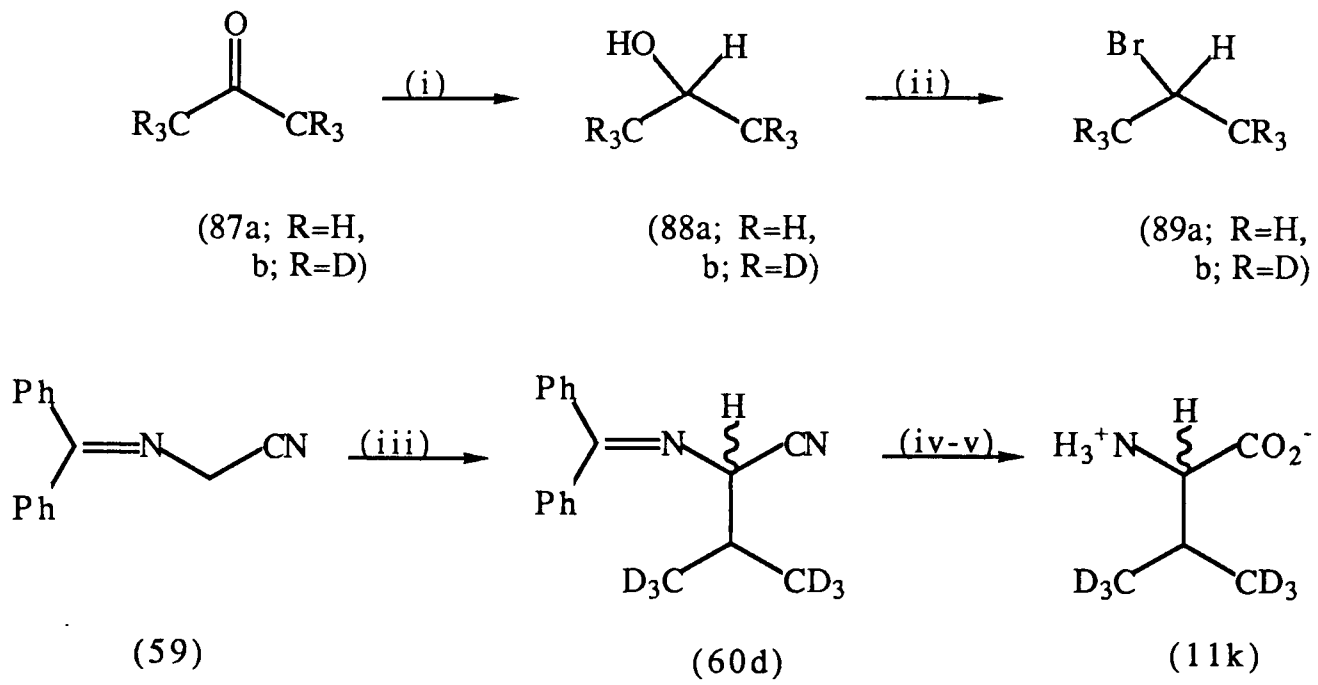
Reagents : i) diethyl pyrocarbonate, pH 8.5, 1 hour, 23°C,
ii) H^+, extract, then CH_2N_2, Et_2O.

Scheme 4.4.

4.3 Synthesis of Substrates.

As described above, the specifically deuterated penicillin Ns (14e) and (14j) were required for the V_{max}/K_m isotope experiments. These were synthesised as described earlier (Chapter 2) from the corresponding specifically deuterated tripeptides (66b) and (66e) by the action of IPNS. Synthesis of the α-deuterovaline tripeptide (66b) has already been described (Chapter 2) .

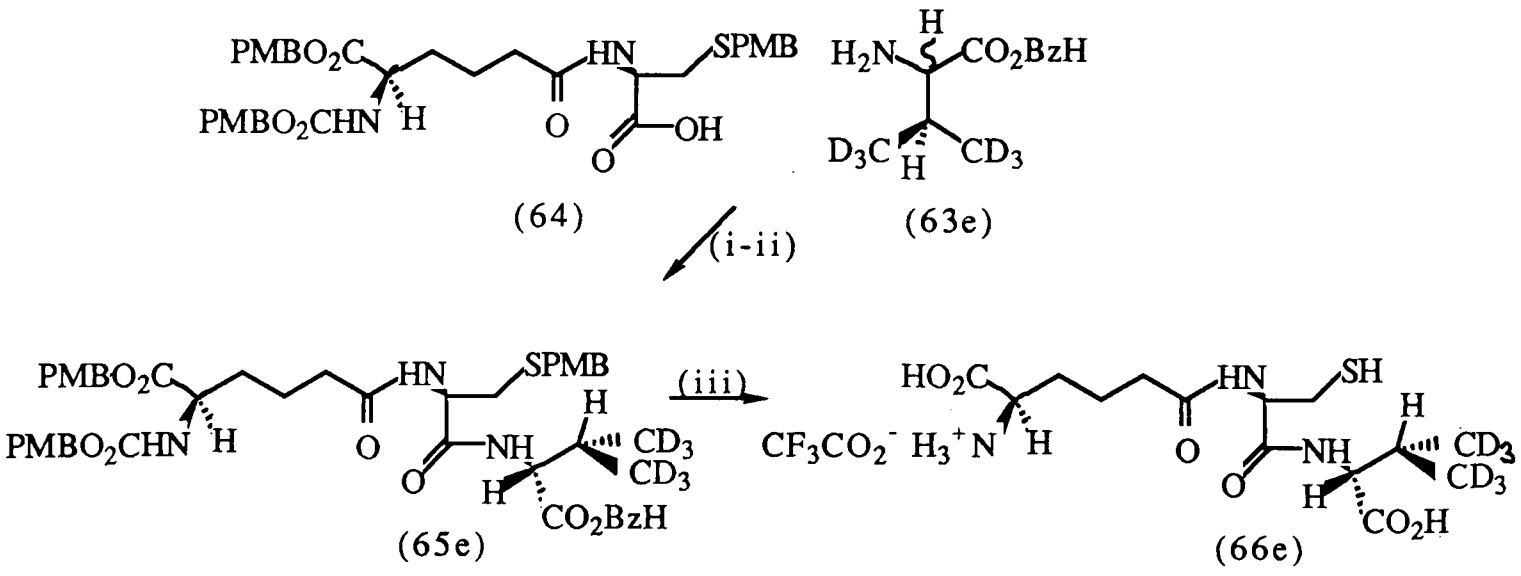
Hexadeuterovaline (11k) was synthesised in a manner analogous to that of the α-deuterovaline (11h), from N-(diphenylmethylene)aminoacetonitrile (59) and hexadeuteroisopropylbromide (89b) which in turn was prepared from hexadeuteroacetone. Thus hexadeuteroacetone (>99.96 atom % deuterium, supplied by Aldrich Chemical Co. Ltd.) was reduced to the alcohol (88b) with lithium aluminium hydride in THF at 0°C (Scheme 4.5).[94] The alcohol was distilled from the mixture after initial quenching with ethylene glycol. Treatment of the alcohol with phosporous tribromide gave the bromide (89b) which was again isolated by distillation. Subsequent alkylation of the imine (59) with this bromide gave (60d). Hydrolysis of the alkylated imine then gave the desired product (11k).



Reagents : i) $LiAlH_4$,diglyme, 0°C ; ii) PBr_3 ; iii) 50% NaOH, (89b), BTEAC, toluene ; iv) 1N HCl, 23°C, 12 hours ; v) 6N HCl, reflux, 12 hours.

Scheme 4.5.

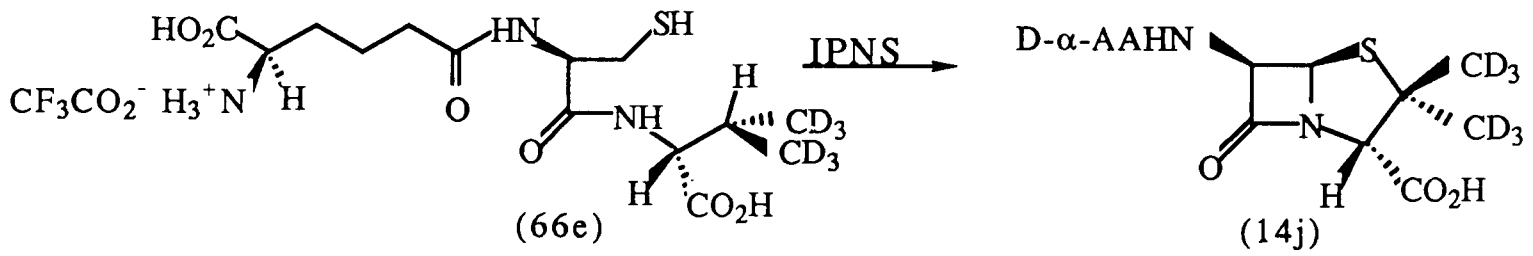
After protection as the benzhydryl ester (63e) and coupling with *p*-methoxybenzyl protected DL-AC (64) to give DLL- and DLD-AC-[4,4-($^2H_3$-Me)2]-V (65e), the diastereomers were separated by chromatography (Scheme 4.6). Deprotection with refluxing TFA/anisole (5:1, v/v) then afforded the tripeptide (66e) as the ammonium trifluoroacetate salt.



Reagents : (i) EEDQ, DCM, anhydrous Na_2SO_4; (ii) SiO_2 Chromatography
(iii) TFA/anisole (5:1, v/v), reflux 30 minutes.

Scheme 4.6.

Enzymatic conversion of this tripeptide into di-(trideutero)-methyl penicillin N (14j) was then accomplished by the action of IPNS in the presence of the usual co-substrates and co-factors, O_2, L-ascorbate, Fe^{2+} and DTT (Scheme 4.7). By this procedure the mono-deuterated penicillin N (14e) was also prepared.



Scheme 4.7.

Purification of the penicillins from the crude IPNS incubation mixtures by hplc [Gilson system, reverse phase with octadecylsilane and 0.75% MeCN in 10mM aqueous NH_4HCO_3 as the stationary and mobile phases respectively] gave approximately 30% yields of the pure, specifically deuterated penicillins (14e) and (14j). For the purposes of the mixed competitive kinetic experiments, a 1:1 mixture of the labelled and unlabelled penicillins was required. Preparation of these mixtures necessitated the determination of the amount of penicillin obtained from the IPNS incubations. In view of the small quantities involved, generally 1 to 3 mg and the presence of salts in the penicillin samples, this determination could not be accurately performed by weighing. Consequently all samples were calibrated by ^{1}H-n.m.r. Integration of both the β-lactam and α-aminoadipyl C-2 protons against the proton resonances due to a known amount of dioxan, enabled calculation of the molarity and hence mass of penicillin present in the sample.

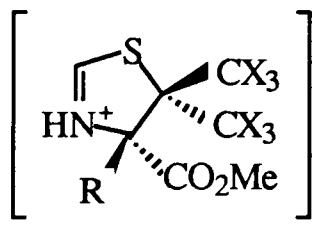
4.4 Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effects for Penicillin N C-3 Position.

A 1:1 mixture of penicillin Ns (14a) and (14e) was prepared using the procedures described above. A sample of this mixture was taken and the ratio (14a) to (14e) determined by ^{1}H-n.m.r integration. After derivatisation the ratio was measured by mass spectrometry to give a time zero reference, to which subsequent time points (or more conveniently in terms of % conversion, conversion points) would be compared. The remaining sample was then incubated with DAOC/DAC synthetase enzyme and portions were removed and quenched so as

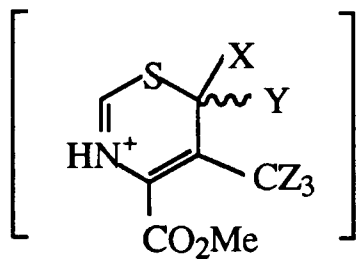
to give a selection of assay points corresponding to particular percentage conversions. Although the approximate rates of conversion of unlabelled and labelled penicillins (14a), (14e) and (14j), were determined in separate incubations, it was not possible to accurately obtain the assay points at will. However, this method of selection proved sufficiently reliable so that reasonably consistent percentage conversion points were obtained.

For each assay point, the percentage conversion of the penicillin N mixture to products was determined by ^{1}H-n.m.r integration and the unconverted penicillin N mixture isolated by hplc. Re-examination by ^{1}H-n.m.r integration gave an approximate value for the ratio of labelled to unlabelled substrate but analysis in this way was subject to an error of 10-20% due to integration of the penicillin N C-3H, which occurs close to the position of solvent suppression. These errors were increased still further when weak samples of penicillin Ns were examined, such as those from the 65 and 80 percentage conversions (see Table 4.1.). Derivatisation and analysis by mass spectrometry upon both the molecular ions (86a-c) and fragment ion (68c-e), gave accurate and generally more reliable values for the ratio of the labelled and unlabelled substrates remaining in the pool of unconverted starting material. Similarly, any DAOC (18) isolated from this and subsequent mixed labelled/ unlabelled experiments, was converted to its N-ethoxy, dimethyl ester derivative (90a-d)[93] (see Chapter 5, Section 5.2) and analysed by mass spectrometry both for the molecular ion and fragment ions (68f-i).

This procedure was repeated with a second sample of penicillins (14a) and (14e), and the results of both experiments are listed below (Table 4.1.).



(68c; R = X = H,
d; R = D, X = H,
e; R = H, X = D.)



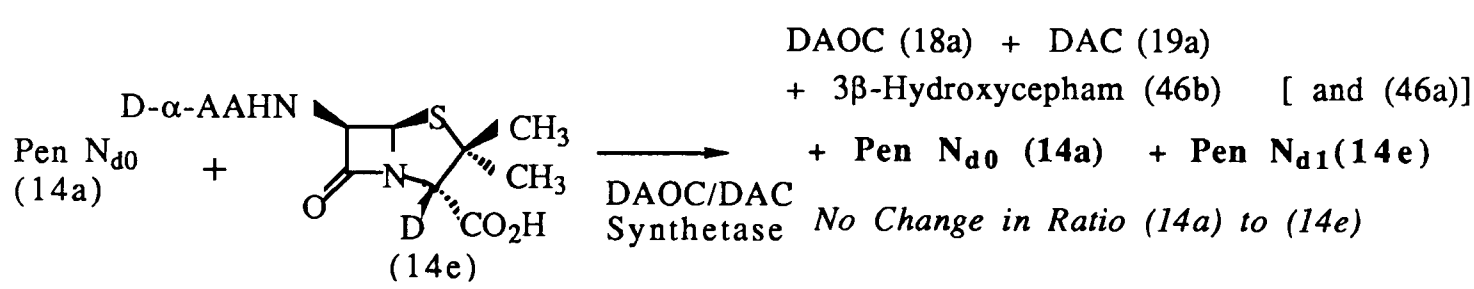
(68f ; X = Y = Z = H,
g; X = Y = Z = D,
h; X = H, Y = Z= D,
i; X = Y = H, Z = D)

Expt.	% Conversion	Ratio (14a) : (14e) determined by: ^{1}H-n.m.r integration[a]	Mass Spectrometry on Fragment ions (68c/d)	Molecular ions (86a/b)
1	0	1.0:1.0	0.99:1.00	0.95:1.00
	30	1.2:1.0	1.04:1.00	0.95:1.00
	65	1.3:1.0[b]	1.05:1.00	0.93:1.00
2	0	0.8:1.0	1.21:1.00	1.24:1.00
	30	1.2:1.0	1.22:1.00	1.22:1.00
	80	0.8:1.0[b]	1.19:1.00	1.20:1.00

Notes: [a] Subject to approximately 10-20% error due to integration of a signal close to solvent suppression, [b] Subject to an increased error due to weak sample.

Table 4.1.

These results show that the ratio of labelled to unlabelled penicillin remains essentially constant throughout the incubation and consequently that the enzyme is showing no isotopic discrimination of the substrates (Scheme 4.8.).



Scheme 4.8.

Comparison between the ratios of labelled and unlabelled substrates as determined by ^{1}H-n.m.r and mass spectrometry suggest that the determinations made by the latter appear more reliable. In addition, the good agreement between the ratio of (14a) to (14e) determined by measurements on the fragment and molecular ions, indicate a high degree of self-consistency for determination by mass spectrometry. The proton n.m.r integration method is valuable in terms of an alternative analytical procedure but does suffer from several sources of inaccuracy - specifically that integration of signals in the spectrum is only accurate to within 10% and secondly, that the smaller the total sample, the less reliable the integrals become especially so if the integral is close to the position of solvent suppression.

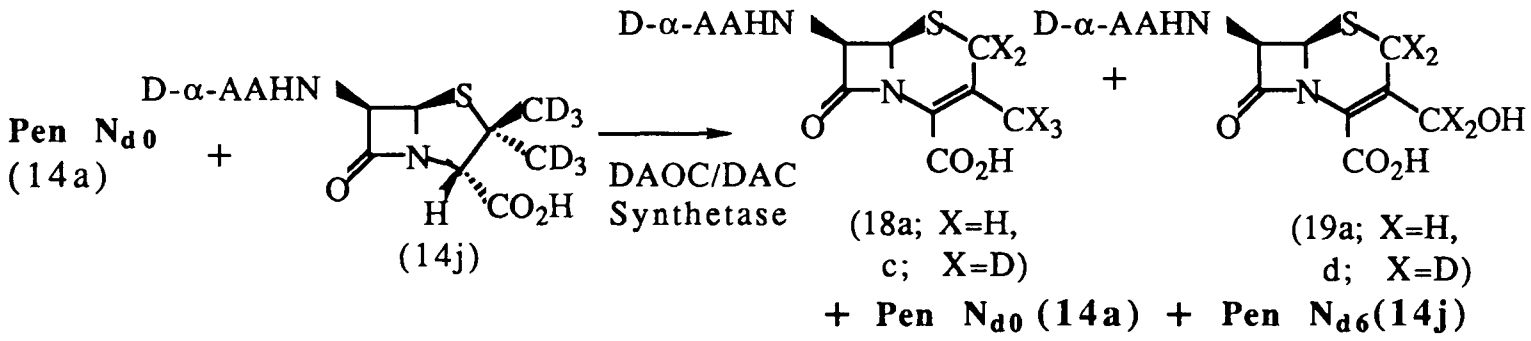
4.5 Mixed Labelled/Unlabelled Competitive Kinetic Isotope Effects for Penicillin N β-Methyl Position.

Determination of the competitive isotope effects for the β-methyl position of penicillin N (14a) was performed in an identical way to that described for the C-3 position above, except that di-(trideutero)-methyl penicillin N (14j) and unlabelled penicillin (14a) were used. The results are listed below (Table 4.2.).

Expt.	% Conversion	Ratio (14a) : (14j) determined by: ^{1}H-n.m.r integration	Mass Spectrometry on Fragment ions (68c/e)	Molecular ions (86a/c)
1	0	1.0:1.0	0.99:1.00	0.94:1.00
	30	0.6:1.0	0.50:1.00	0.50:1.00
2	0	0.9:1.0	0.94:1.00	1.00:1.00
	40	0.6:1.0	0.54:1.00	0.51:1.00
	60	0.5:1.0	0.47:1.00	0.41:1.00

Table 4.2.

These results clearly show that the ratio of unlabelled penicillin N (14a) to deuterated penicillin N (14j) is decreasing and consequently that (14a) is being converted at a higher rate than the labelled material (Scheme 4.9.).



Scheme 4.9.

Obviously the enzyme is exhibiting isotopic discrimination by processing the unlabelled substrate more rapidly than the hexadeuterated substrate (14j) thereby leading to an accumulation of the labelled substrate in the substrate pool. The full significance of these V_{max}/K_m experiments for both positions will be discussed later in this chapter.

4.6 Denatured Enzyme Control for the Penicillin N β-Methyl Position.

To confirm that the isotopic discrimination observed with the hexadeuteropenicillin (14j) and unlabelled penicillin (14a) was indeed the result of enzymatic discrimination and not due to an isotopically sensitive chemical process, a denatured enzyme control experiment was performed. Thus a 1:1 mixture of (14a) and (14j) (*ca* 1mg in total) was prepared as before and the precise ratio of labelled to unlabelled substrate determined by ^{1}H-n.m.r on the free penicillins and mass spectroscopy on the derivatives. The remaining sample was incubated with DAOC/DAC synthetase enzyme which had previously been heated at 100 °C for 2-3 minutes, cooled and then pre-incubated with co-factors as usual. The incubation was quenched after two hours - equivalent to the longest incubation time for the hexadeuteropenicillin N (14j)/ unlabelled penicilin N (14a) mixed substrate incubation - and then the crude incubation mixture examined by ^{1}H-n.m.r, which indicated that no activity had remained after denaturation, *i.e.* no conversion to ring expanded products had occurred. The penicillins were isolated by hplc and the ratio of labelled to unlabelled substrates determined as for the V_{max}/K_m experiments. The results are listed in Table 4.3.

	Ratio (14a) : (14j) determined by:		
Sample	^{1}H-n.m.r integration	Mass Spectrometry on Fragment ions (68c/e)	Molecular ions (86a/c)
Starting material	1.0:1.0[a]	0.96:1.00	1.03:1.00
'Incubated' material	0.9:1.0[a]	0.96:1.00	0.99:1.00

Notes: [a] Subject to approximately 10-20% error due to integration

Table 4.3.

The figures in Table 4.3 indicate that within experimental error, no isotopic discrimination has taken place. This strongly suggests that the discrimination between (14a) and (14j) observed when these substrates are incubated together with DAOC/DAC synthetase, is due to an enzymatic process and not a purely chemical event.

4.7 Discussion and Conclusions.

In this Chapter, the determination of the mixed substrate competitive kinetic isotope effects for the ring expansion process have been described.[95] The aim of this section is to present a mechanistic interpretation of these results, specifically in terms of the order of events at the penicillin N (14a) β-methyl and C-3 positions.

Elaboration of the basic kinetic scheme (Scheme 4.2.) used to derive the expressions for V_{max} and V_{max}/K_m , is possible so that expressions for the two isotopic species S and S' are included. This is achieved by considering Scheme 4.2 for the two separate isotopic species *i.e.*

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} [EP] \xrightarrow{k_3} [E] + [P]$$

$$[E] + [S'] \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} [ES'] \xrightarrow{k'_2} [EP'] \xrightarrow{k'_3} [E] + [P']$$

Construction of the rate expressions for the steady state intermediates [ES], [EP], [ES'] and [EP'] and then by combination, rearrangement and finally equating these expressions equal to zero, we obtain the following :

$$k_1\{[E] - [ES] - [ES'] - [EP] - [EP']\}\,[S] - (k_{-1} + k_2)[ES] = 0$$

and

$$k'_1\{[E] - [ES] - [ES'] - [EP] - [EP']\}\,[S'] - (k'_{-1} + k'_2)[ES'] = 0$$

These expressions appear not to be of any constructive use, until we appreciate that it is only the ratio of labelled and unlabelled substrates that is of interest to us in V_{max}/K_m experiments. Thus by rearrangement and substitution we obtain ;

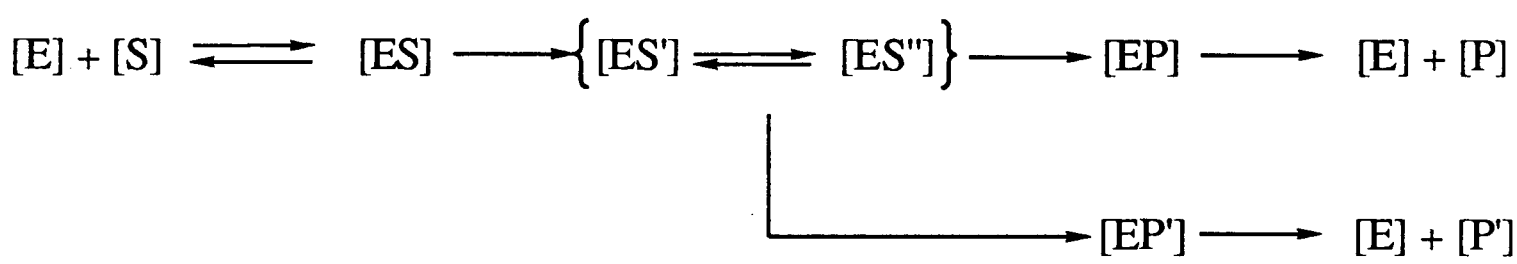
$$k/k' = (V_{max}/K_m)/\ (V'_{max}/K'_m)$$

[Where k and k' are the rate constants for conversion of unlabelled and labelled substrates, V_{max} and V'_{max} are the maximum velocities of the unlabelled and labelled conversions respectively, and K_m and K'_m are the Michaelis Constants for the unlabelled and labelled substrates respectively].

In many respects the deuterated substrate (14j) behaves as a competitive inhibitor, but unlike a true competitive inhibitor, reaction with the enzyme is still possible but obviously subject to a primary isotope effect and consequently a decrease in rate of conversion.

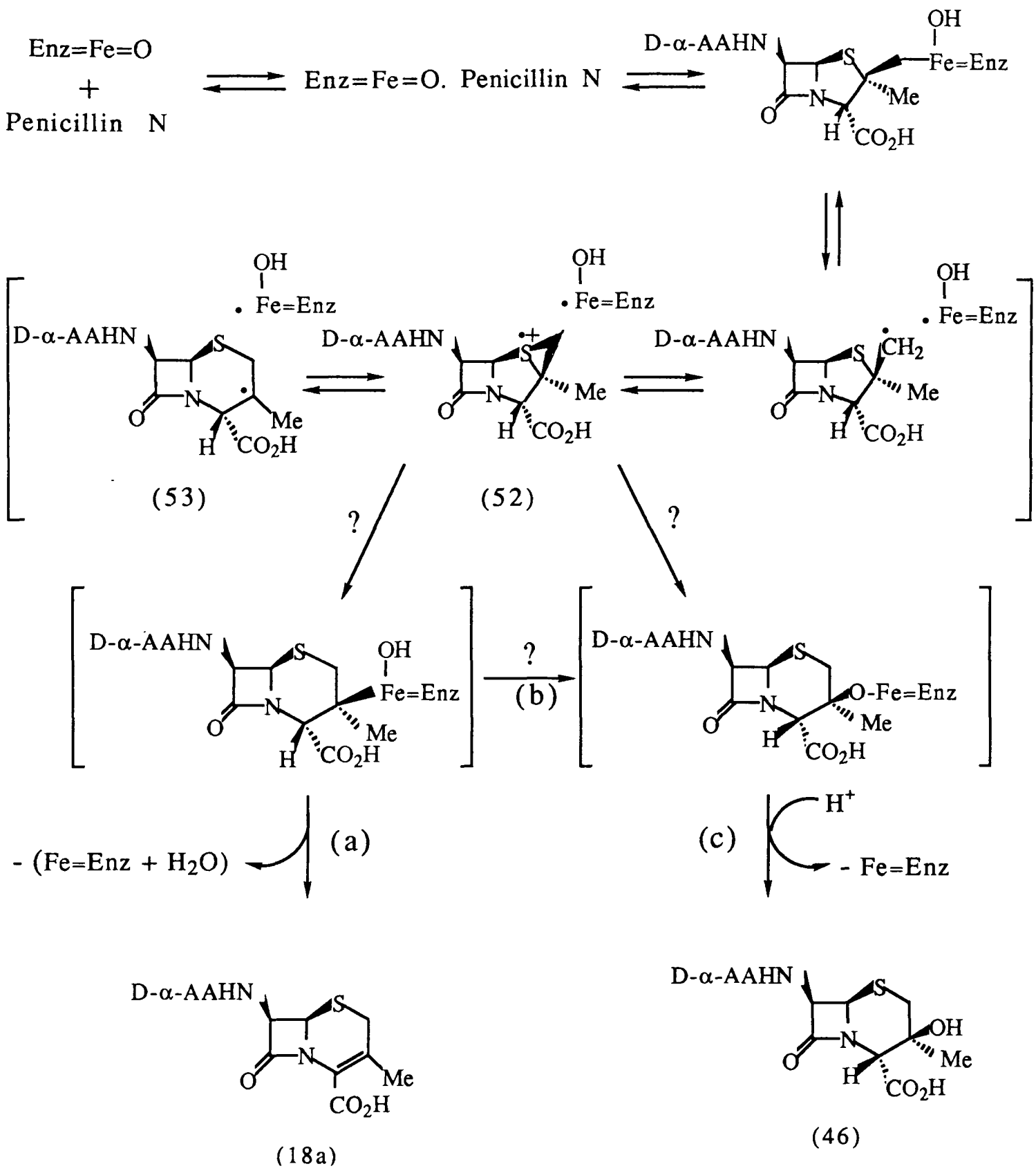
From the observation of enzymatic isotopic discrimination only for the substrate labelled in the methyl groups, *i.e.* hexadeutero-penicillin N (14j), and not for the (3-^{2}H)-penicillin N (14e), we can conclude that the first irreversible event most probably occurs at the β-methyl position. Furthermore, as a direct consequence of deuteration at C-3 of penicillin N (14e), bifurcation of the natural biosynthetic pathway occurs to give the deuterated 3β-hydroxycepham (46b). This divergence of the pathway arises due to operation of a primary isotope effect upon the reaction of one of the transient enzyme bound intermediates, but must occur after the first irreversible event otherwise enzymatic isotopic discrimination would have been observed in the mixed competitive experiment with labelled and unlabelled penicillins (14a) and (14e).

To account fully for these two important observations, we must propose that at least two enzyme-substrate intermediates and two enzyme-product complexes are involved in the ring expansion process (Scheme 4.10). This represents the minimum number of enzyme-substrate intermediates which are required to explain the non-concerted nature of the expansion process. Furthermore, a minimum of two enzyme-product complexes are required to account for the two products released from the active site: the usual DAOC (18) product and the 3β-hydroxycepham shunt metabolite (46b).[71]



Scheme 4.10.

In this scheme, the complex [ES'] may represent the proposed epi-sulphonium ion intermediate (52). It is possible that bifurcation of the pathway either occurs from this intermediate or from the ring opened form (53), which may represent the second enzyme-intermediate complex, [ES''] . This schematic representation of the ring expansion process may be presented in structural terms as shown below (Scheme 4.11.).



(18a)

(46)

Scheme 4.11.

The proposed intermediates (52) and (53) may equally be assigned radical or cationic character, as has been demonstrated by biomimetic studies (see Chapter 8).[96] This mechanistic scheme takes into consideration the observed increased formation of the 3β-hydroxycepham (46) and the results from the mixed competitive isotope effect experiments. Hence, the data obtained from the mixed competitive isotope effect experiments strongly suggests that the first irreversible event in the ring expansion process occurs at the β-methyl of penicillin N (14) to generate a radical, which is known to be capable of undergoing ring expansion to

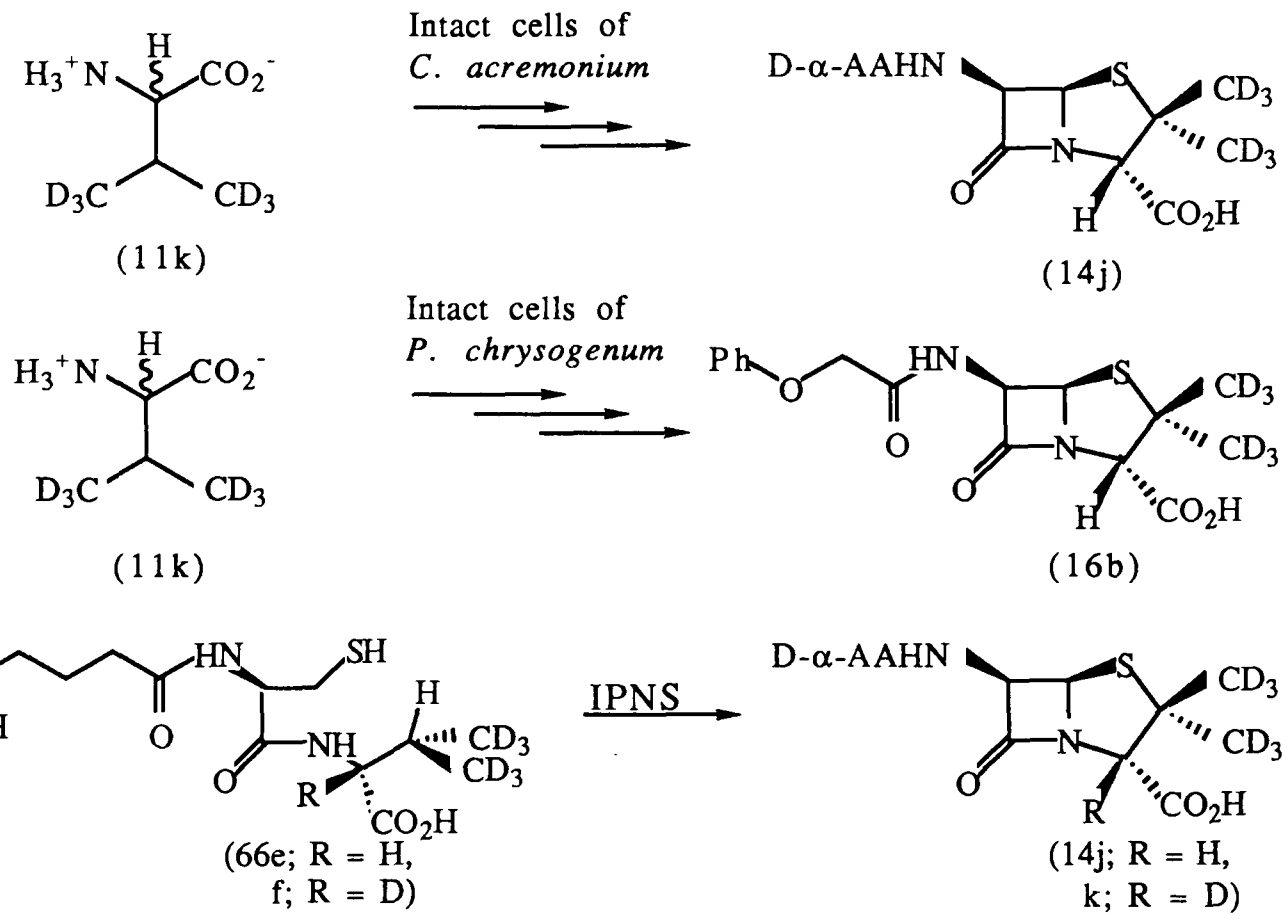
the cepham radical (53), as has been shown in the biomimetic studies performed in this laboratory. [96] However, it is not clear if the products formed *via* the ring expansion process are derived from the cepham radical (53) or the alternatively the proposed intermediate (52).

In the case of the 3β-hydroxycepham (46), if the step leading to formation of the DAOC (18) (Step a) is slowed down by the operation of a primary isotope effect (*i.e* deuteration at C-3 of penicillin N), then the alternative pathway (Step b), leading to the 3β-hydroxycepham (46), can compete. Such an effect will obviously lead to increased production of the 3β-hydroxycepham (46b), but it is quite possible that (46) could arise directly from the proposed epi-sulphonium ion intermediate (52) or cepham radical (53).

Chapter 5 : Anomalous Deuterium Exchange.

5.1 Introduction.

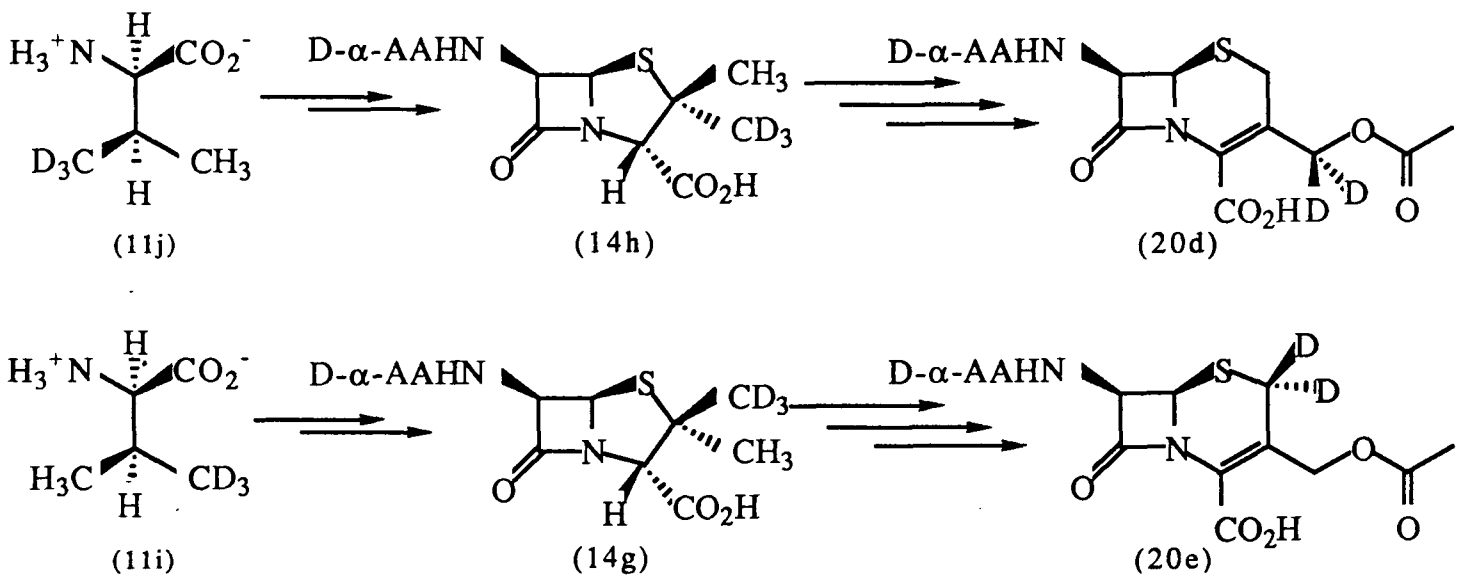
In the previous chapter mixed labelled/unlabelled competitive kinetic isotope effect experiments were described which required the synthesis of the specifically deuterated penicillins (14e) and (14j) respectively.[95] It has been shown, by mass spectrometry and ^{2}H-n.m.r, that retention of all the methyl deuterium atoms occurs during the biosynthesis of isopenicillin N (13), penicillin N (14j) and penicillin V (16b) from hexadeuterovaline (11k) with intact cells of *C. acremonium* and *P. chrysogenum* respectively.[41,97,98] Furthermore, our own results reveal that retention of all six deuterium atoms occurs during the formation of hexadeuteropenicillin N (14j) directly from DLD-AC-di-trideuteromethyl-valine (66e) by the action of IPNS (Scheme 5.1.), as shown by mass spectrometry.



Scheme 5.1.

Other studies on the stereochemistry of valinyl methyl group incorporation into LLD-ACV (12a), various penicillins, cephalosporin Cs (20d) and (20e) have been conducted by intact cell methods with chirally labelled trideuteromethyl-valines (11i) and (11j) and intact cells of *C. acremonium*.[41] Isotopic analysis of the recovered penicillins by mass spectrometry indicated that conversion had occured

with retention of the deuterium atoms (Scheme 5.2.). From these studies and the known sequence of valine incorporation initially into LLD-ACV (12) and thence isopenicillin N (13), penicillin N (14) and eventually cephalosporin C (20), it can be concluded that the (2R)-trideuteromethyl-penicillin N (14h) was converted to cephalosporin C (20d) with retention of both deuterium atoms (within the limits of detection) as anticipated.[41]



Scheme 5.2.

However, it may be concluded from their results that the downstream (2S)-2β-trideuteromethyl-penicillin N (14g) was converted to cephalosporin C (20e) with substantial loss of deuterium label at the C-2 position, as shown by mass spectrometry. In an attempt to resolve this unusual observation, hexadeuterovaline (11k) was converted to cephalosporin C (20)*via* the di-(trideuteromethyl)-penicillin N (14j), which from previous studies was known to retain all six deuterium atoms. Analysis of the recovered cephalosporin C (20) indicated that a range of labelled cephalosporin C (20) molecules had been formed which varied only in their deuterium content, the majority of molecules retaining only three of the expected four deuterium atoms (Table 5.1.).[41] No clear explanation was given for the apparent loss of deuterium label during the conversion of penicillin N (14j) to cephalosporin C (20) other than the suggestion that isotopic scrambling in the mass spectrometer may have occurred.

Loss of deuterium from deuterated DAOC (18) was also observed in the hexadeuteropenicillin N (14j) / unlabelled penicillin N (14a) mixed experiments, as shown by analysis of the derivatised recovered DAOC (90) by mass spectrometry

Valine precursor	Penicillin N							Cephalosporin C						
	d_0	d_1	d_2	d_3	d_4	d_5	d_6	d_0	d_1	d_2	d_3	d_4	d_5	d_6
(2S,3S)-[4,4,4-$^2H_3$]-valine (11i)[a]	56	0	1	43	0	0	0	63	4	30	3	0	0	0
(2S,3R)-[4,4,4-$^2H_3$]-valine (11j)[b]	60	1	8	31	0	0	0	78	20	2	0	0	0	0
(2RS)-[$^2H_6$]-valine (11k)[c]	71	0	0	0	0	0	29	72	10	7	10	1	0	0

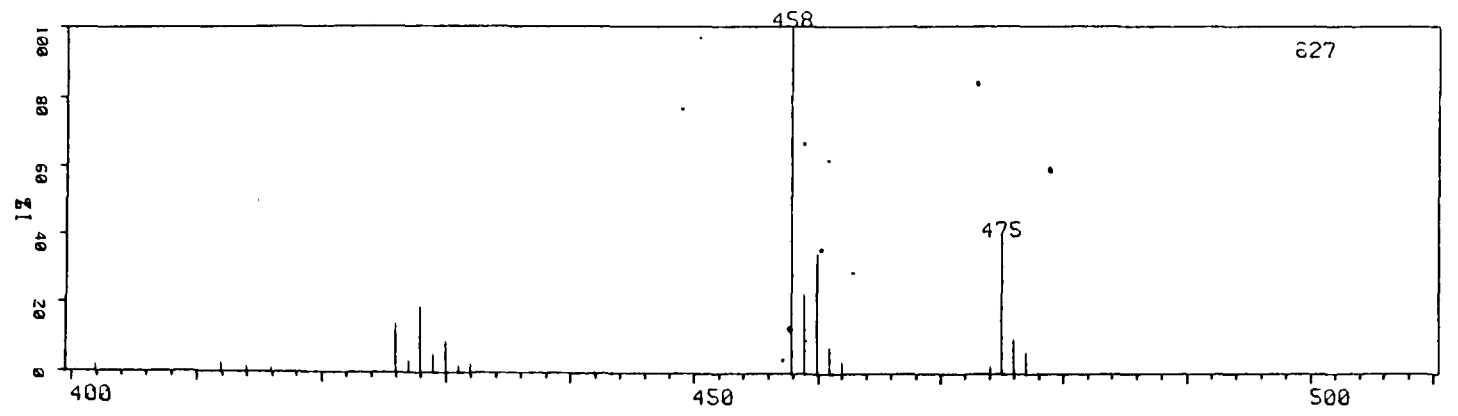
Notes: [a] The deuterium contents were 1% d_2 and 99% d_3.
[b] 3% d_0, 20% d_2, 77% d_3. [c] 95% d_6 and 5% d_5.

Table 5.1.

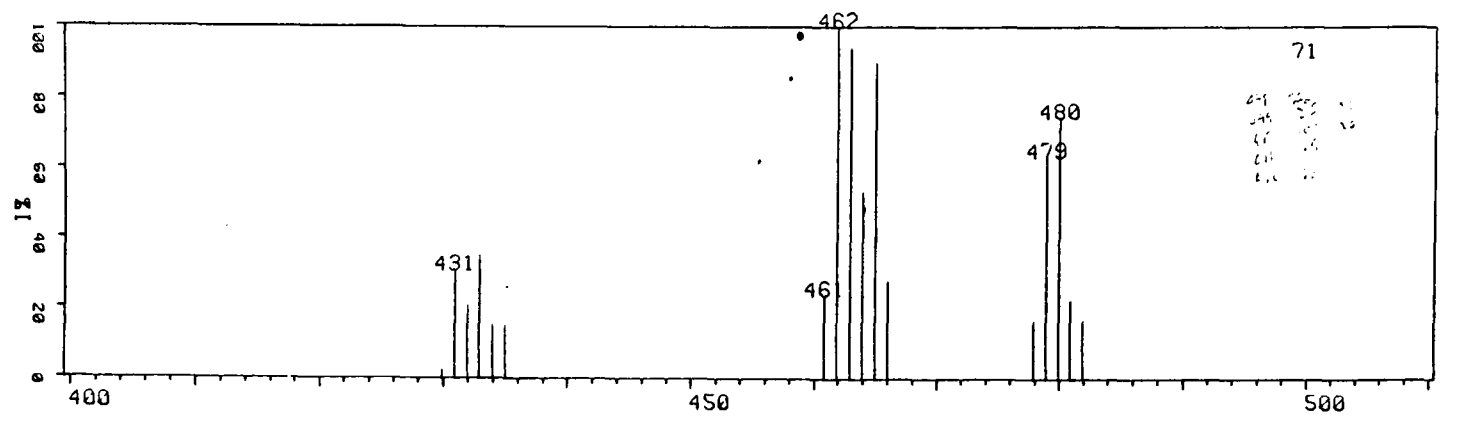
(NH_3, DCI) [see experimental for the mixed labelled / unlabelled substrate experiments with (14a) and (14j)]. This observation seemed to be in accordance with those already mentioned and implied that a process other than scrambling in the mass spectrometer was responsible, since the extent of exchange in our hands, depended upon the overall percentage conversion and hence on the amount of enzyme activity present. These unusual observations suggested that further investigation should be undertaken in an attempt to explain the loss of deuterium during the conversion of hexadeutero-penicillin N (14j) to the cephems.

5.2 C-2 Exchange Studies with Hexadeuteropenicillin N (14j).

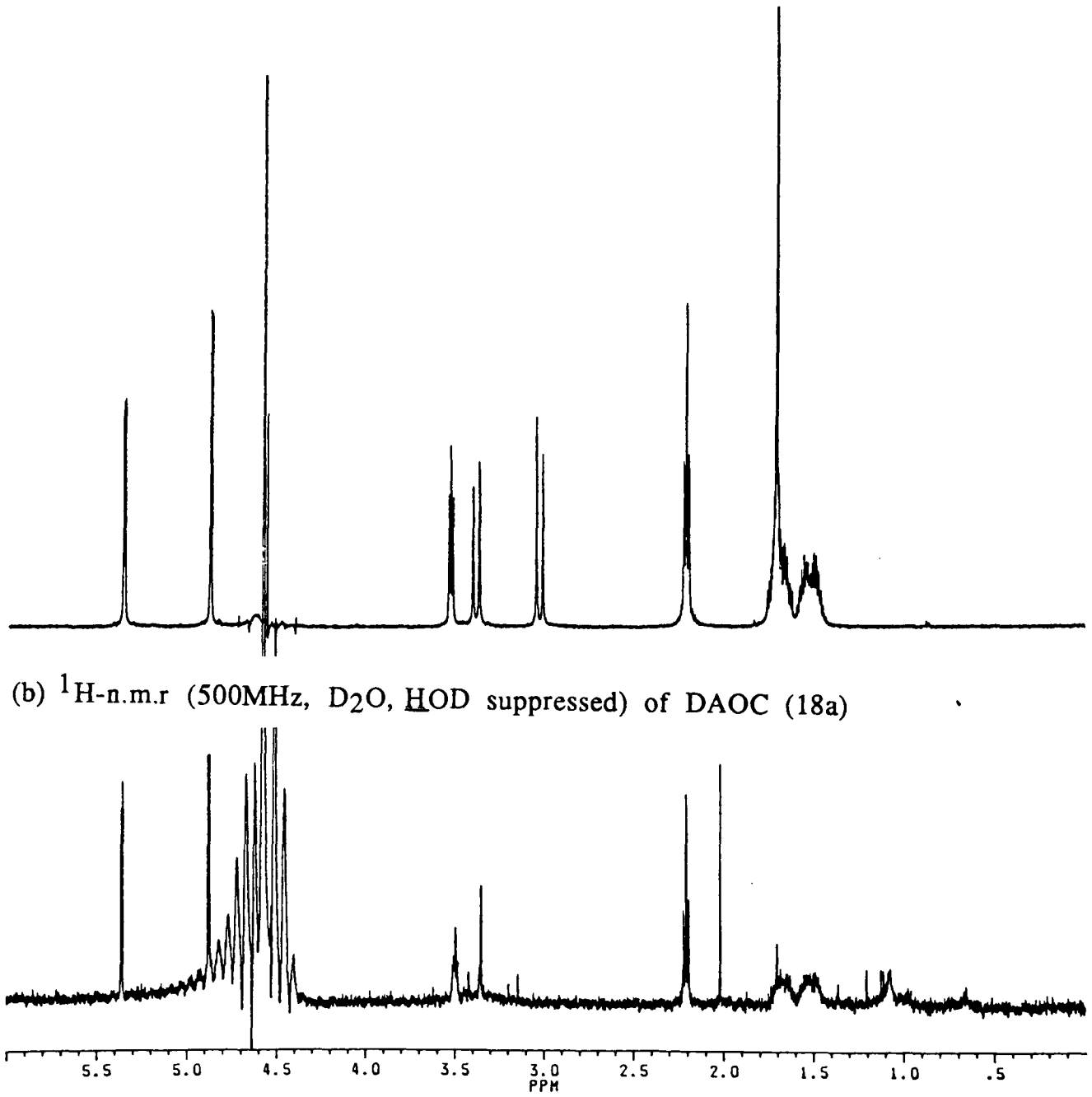
Hexadeuteropenicillin N (14j) was prepared as described earlier (Chapter 3) and then incubated with partially purified DAOC/DAC synthetase enzyme (*ca* 0.1 I.U.). The total crude incubation mixture was examined by 500 MHz ^1H-n.m.r spectroscopy and the β-lactam region (δ 5.5-4.9 ppm) integrated so that the percentage conversion of deuterated penicillin N (14j) to ring expanded products could be determined. Isolation of the starting material and cephem products was performed by hplc [Water's system, octadecylsilane with 25 mM aqueous NH_4HCO_3]. Examination of the purified DAOC (18) by 500 MHz ^1H-n.m.r spectroscopy indicated the presence of a singlet at δ3.**36** ppm - approximately bisecting the low field doublet of the C-2 methylene AB quartet (see Figure 5.1.). In addition, no proton signals could be detected in the vinyl trideuteromethyl region,*ca* δ1.76 ppm. The sample of DAOC (18) was derivatised to the N-ethoxycarbonyl, dimethyl ester (90)[93] so that the maximum sensitvity possible would be obtained from mass spectral analysis with the NH_3 DCI technique.



(d) Mass Spectrum of DAOC Derivative (90a).



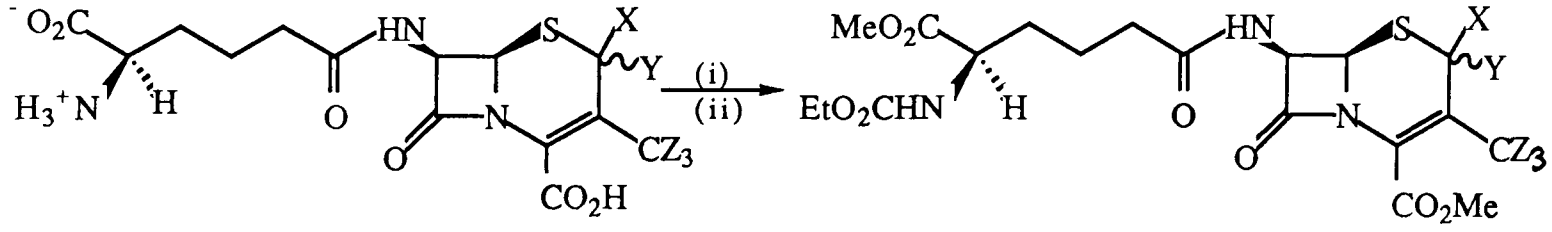
(c) Mass Spectrum of DAOC Derivative (90c) Isolated from Di-($^2H_3$-methyl)-penicillin N (14j) Incubation with DAOC/DAC Synthetase.



(b) ^1H-n.m.r (500MHz, D_2O, HOD suppressed) of DAOC (18a)

Figure 5.1.

(a) ^1H-n.m.r (500MHz, D_2O, HOD suppressed) of DAOC (18d) Isolated from Di-($^2H_3$-methyl)-penicillin N (14j) Incubation with DAOC/DAC Synthetase.



(18a; X=Y=Z=H,
c; X=Y=Z=D,
d; X=H,Y=Z=D,
e; X=Y=H, Z=D,
f; X=Y=D, Z=H)

(90a; X=Y=Z=H,
b; X=Y=Z=D,
c; X=H, Y=Z=D,
d; X=Y=H, Z=D)

Reagents : i) diethyl pyrocarbonate, pH 8.5, 1 hour, 23°C,
ii) H^+ , extract, then CH_2N_2, Et_2O .

Isotopic analysis revealed that principally penta- and tetradeuterated DAOC (90b) and (90c) had been produced, in addition to a small quantity of trideuterated material (90d) (Table 5.2.). This experiment was repeated with a second sample of hexadeuteropenicillin N (14j) (Expt.2, Table 5.2) to check the consistancy of the results. These experiments revealed two interesting points :-

i) significant exchange had occured as demonstrated by the high degree of conversion of pentadeutero DAOC (18d) to tetradeutero DAOC (18e), and

ii) the percentage exchange was seemingly related to the total percentage conversion of penicillin N (14j) to DAOC (18d-e) *i.e.* to the total amount of enzyme activity present.

Expt. No.	% Conversion (approximate)	Ratio $^2H_5$-DAOC (18c): $^2H_4$-DAOC (18d)
1	10	1.0 : 0.6
2	50	1.0 : 1.1

Table 5.2. Ratio of DAOC Isotopomers Produced from DAOC/DAC synthetase Incubations of Hexadeuteropenicillin N (14j).

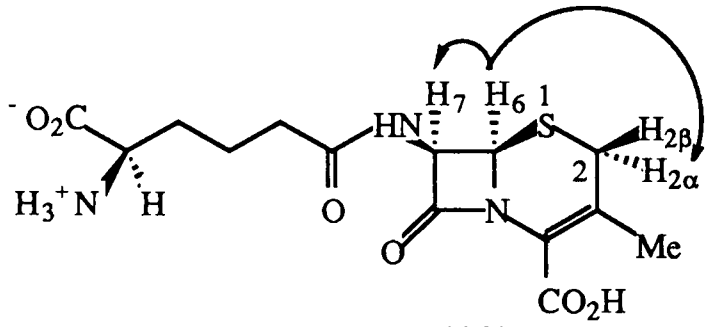
As in the first incubation of (14j), the 500 MHz ^{1}H-n.m.r spectrum of the second incubation revealed a singlet at δ3.58 ppm. This singlet was assigned to a proton in either the pro-R or pro-S positions of the C-2 methylene but closer examination of the ^{1}H-n.m.r spectra for both experiments indicated that the observed singlet did not exactly bisect the low field wing of the AB quartet. This

could reasonably be explained by the operation of an isotope shift since the presence of a deuterium atom adjacent to the proton in question would be expected to induce a deuterium isotope shift, moving the proton resonance upfield by a small amount.[99] Furthermore, the observation of a singlet which bisected only one of the two possible positions corresponding to the C-2 methylene ABq resonances, suggested that the exchange was initially occuring in a stereospecific manner.

5.3 Stereochemistry of Exchange:

As outlined above, the exchange process appeared to be stereospecific since exchange of a deuterium atom for a proton gave rise to only one singlet, in the ^1H-n.m.r spectra, corresponding to one wing of the C-2 methylene AB quartet. If assignment of the high and low field wings of this AB quartet could be made with respect to the pro-R and pro-S C2-methylene protons, then the stereochemistry of exchange could also be determined.

Assignment of the C-2 diastereotopic protons of DAOC (18) was made on the basis of n.O.e experiments, the details of which are given below (Table 5.3.).



(18)

Irradiation.	δ ppm	Enhancements (%)
H_6	5.14	H_7(18), $H_{2\alpha}$(3)
$H_{2\alpha}$	3.61	H_6(6), $H_{2\beta}$(25)
$H_{2\beta}$	3.38	$H_{2\alpha}$(27)
H_7	5.58	H_6(16)

Table 5.3.

In n.O.e experiments on cephalosporin compounds, irradiation of the β-lactam ring junction proton (H_6) leads to a positive n.O.e (*i.e* enhancement) for the ($H_{2\alpha}$) proton. With such an irradiation, no enhancement is observed for the

($H_{2\beta}$) proton. From the data above (Table 5.3.) it can be seen that irradiation of H_6 leads to a positive n.O.e to the low field wing of the C-2 methylene AB quartet and consequently this has been assigned to the 2-H_α proton.

In summary then, the observation of a singlet at *ca* δ3.60 and no signal at 3.38 ppm, when considered in conjunction with the assignment of the low field wing of the C-2 methylene AB quartet to the pro R proton implies that the exchange process is stereospecific and further that it is the C-2α deuteron which is exchanged.

5.4 C-2 Exchange - Chemical or Enzymatic?

Two pieces of evidence have been described which support the view that the deuteron-proton exchange is an enzyme mediated process. Firstly, the extent of exchange has been shown to be dependant upon the amount of enzyme activity present in the incubation mixture. Secondly, this process appears to be stereospecific, an observation in keeping with many other enzyme catalysed reactions.[100] However, it was considered possible that exchange might be occurring during the derivatisation of the DAOC (18a-e) samples prior to mass spectrometry. If this was the case then the exchange might be attributable to a chemically mediated process. Such speculation may seem unreasonable when considered against the evidence supporting the stereospecificity of exchange but it should be noted that the C-2α proton of cephalosporin C sulphoxide (24) is ten times more acidic than the C-2β proton.[44] This large difference in acidity could account for a chemical exchange process, in this compound, appearing to be stereospecific since obviously in a base catalysed exchange process the most acidic proton will be substituted most rapidly. To rule out the possibility that exchange occurs during derivatisation, a sample of undeuterated DAOC (18a) was derivatised in D_2O under the same conditions as those used for the derivatisation of the biosynthetic samples of deuterated DAOC (18), except that the scale of the derivatisation was approximately 2-5 times that of the biosynthetic experiments. This is a two step process, the first stage being base catalysed formation of a urethane followed by acidification, extraction and finally esterification with diazomethane to give the diester (90). Mass spectrometry (NH_3, DCI), indicated that limited incorporated of deuterium (*ca* 10-15%) had occurred, suggesting that under the conditions of derivatisation exchange could be taking place. However, there is a major difference between the derivatisation of unlabelled DAOC (18a) in D_2O and that of labelled material (18c) in H_2O. In the former case once introduction of a deuterium atom has occurred it is unfavourable to reverse this

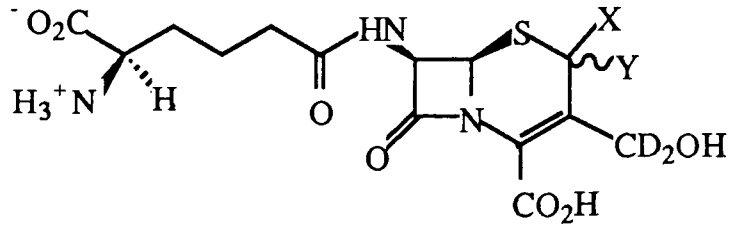
exchange due to the operation of an isotope effect. Thus the isotope effect favours the exchange process in this case. However, with labelled DAOC (18c), exchange is a disfavoured process since to exchange the C-2 deuterons the reaction must overcome the isotope effect and break a carbon-deuterium bond.

Any possible complications arising from derivatisation were completely dispelled in later experiments in which isotopic analysis was carried out by thermaspray mass spectrometry - a technique which does not require the derivatisation of samples.

Experiments in which base catalysed wash-in of deuterium is investigated are subject to another possible source of error since it is possible that any detected incorporation might not be due to exchange at C-2, but at one of the readily exchangeable amide or amino-protons. Furthermore, during the lyophilisation of crude incubation samples after n.m.r spectroscopy in D_2O, the concentration of buffer in these solutions increases and can lead to alkaline pH. At such high pH values, the rate of proton/deuteron exchange at readily exchangeable sites would be expected to increase. This could lead one to suppose that a process of this nature rather than an enzyme catalysed process is responsible for the loss of deuterium observed in the hexadeuteropenicillin N (14j) ring expansion experiment. However, it should be brought to attention that in the case of the hexadeuteropenicillin N (14j) incubations, it is loss of deuterium which is observed and although incorporation of deuterium into exchangeable sites would perturb the actual measured ratio of pentadeutero- to tetradeutero-DAOC, it cannot lead to dispute over the fact that overall deuterium loss has occurred. The effect of deuterium incorporation into non-specific, readily exchangeable positions appears to be insignificant when considering deuterium/protium substitution at C-2 of DAOC (18), as observed in the hexadeuteropenicillin N (14j) since it is loss of label that is observed in these incubations. However, such non-specific exchange becomes of major concern when dealing with experiments designed to examine incorporation of deuterium into the C-2 position because incorporation into such exchangeable sites could be confused with DAOC (18) C-2 incorporation.

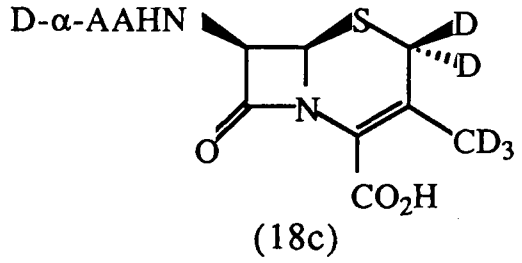
5.5 Design of Experiments to Investigate Anomalous C-2 Exchange.

The main disadvantage of studying the exchange process during DAOC/DAC synthetase catalysed conversion of the hexadeuteropenicillin N (14j) to DAOC (18c-d) and DAC (19d-f) is the difficulty associated with performing a comprehensive control experiment. A suitable control is essential in order that loss of deuterium from deuterated DAOC (18c) could be unambiguously assigned to the ring



(19d; X=Y=D,
e; X=H, Y=D,
f; X=Y=H)

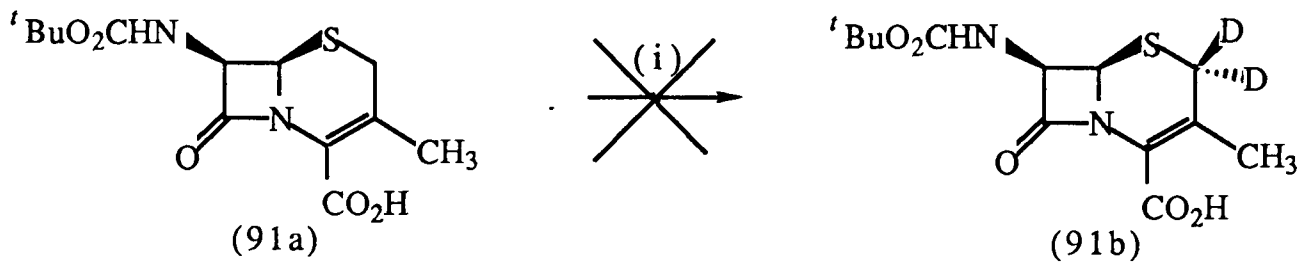
expandase or hydroxylase activities thereby ruling out a purely chemical process. The ideal experiment would be to incubate pentadeutero-DAOC (18c) with both active and denatured DAOC/DAC synthetase. If exchange is observed only with



(18c)

active enzyme then this will unambiguously confirm that an enzyme mediated process is involved. The experiment seems trivial but synthesis of the required substrate (18c) is not. Although this compound is formed when the hexadeuteropenicillin N (14j) is incubated with DAOC/DAC synthetase, it cannot be isolated without any tetradeutero-DAOC (18d), the presence of which in the control experiment would lead to unwanted complications. However, it was considered that providing the initial level of deuteration of DAOC (18c-d) in both the α and β C-2 positions was known, then material containing less than 100% deuterium at C-2 could be used for the control experiments. It was estimated that approximately one milligram of DAOC (18c-d) would be required for these experiments, and consequently attempts were made to produce sufficient biosynthetic DAOC (18c-d) from the incubation of hexadeuteropenicillin N (14j) with DAOC/DAC synthetase to enable the control experiments to be performed. Unfortunately, insufficient DAOC (18c-d) could be prepared in this way due to limitations on the availabillity of both IPNS, required to prepare the hexadeuteropenicillin (14j), and DAOC/DAC synthetase. It should be noted that the conversion of the hexadeuteropenicillin N (14j) to ring expanded products, by DAOC/DAC synthetase is considerably more difficult than the corresponding conversion of unlabelled penicillin N (14a). Typically three to five times the amount of enzyme is required to effect the same level of conversion with the deuterated substrate (14j) than with undeuterated penicillin N (14a).

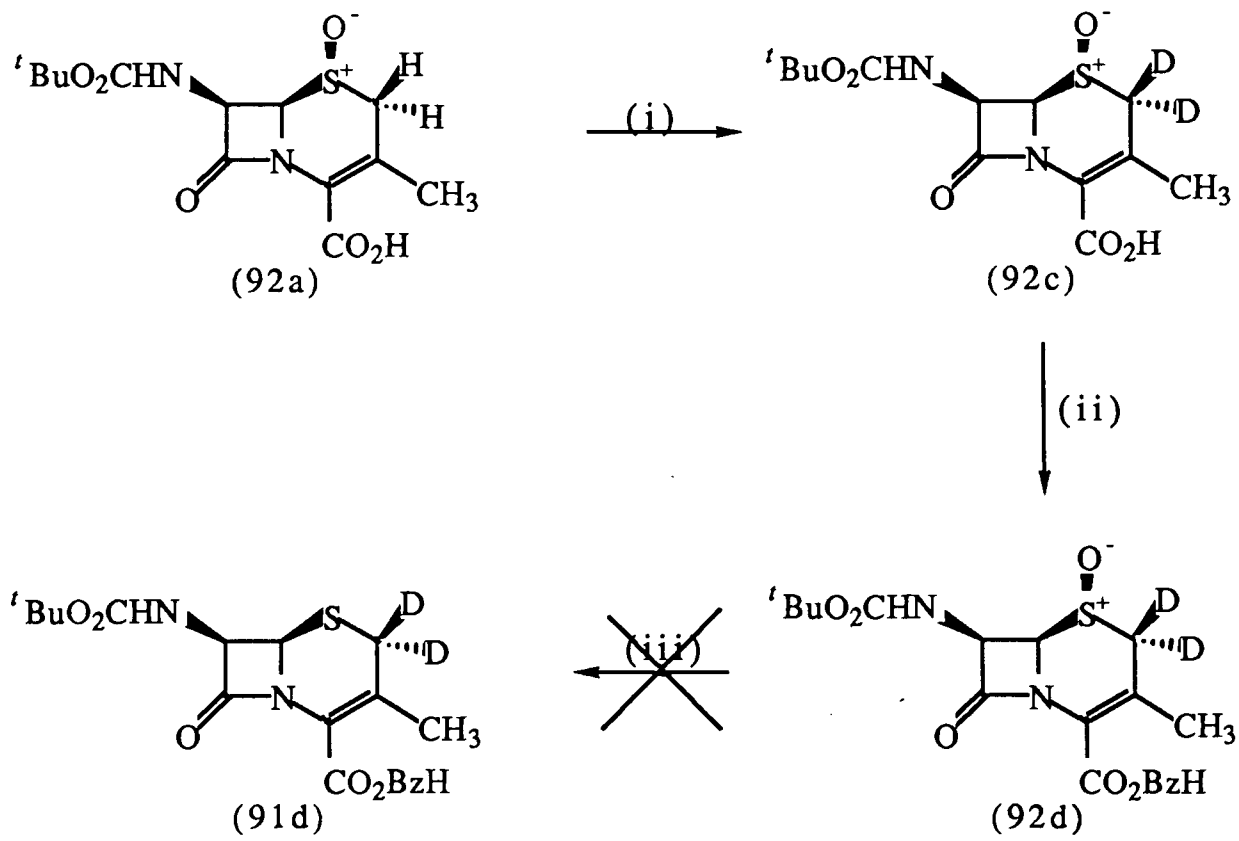
Work performed in this laboratory by Dr. C. J. Schofield, had shown that although it was not possible to exchange the C-2 protons of N-protected 7-β-aminodeacetoxycephalosporinate [7-(N-*t*-butyloxycarbonylamino)-DAOC] (91a) for deuterium atoms, it was possible to exchange the C-2 protons of N-protected 7-β-aminodeacetoxycephalosporinate sulphoxide [7-(N-*t*-butyloxycarbonylamino)-DAOC sulphoxide] (92a) and to obtain high levels of incorporation.[101]



Reagents : (i) D_2O, pH 8.5, 35°C, 24 hours.

Scheme 5.3.

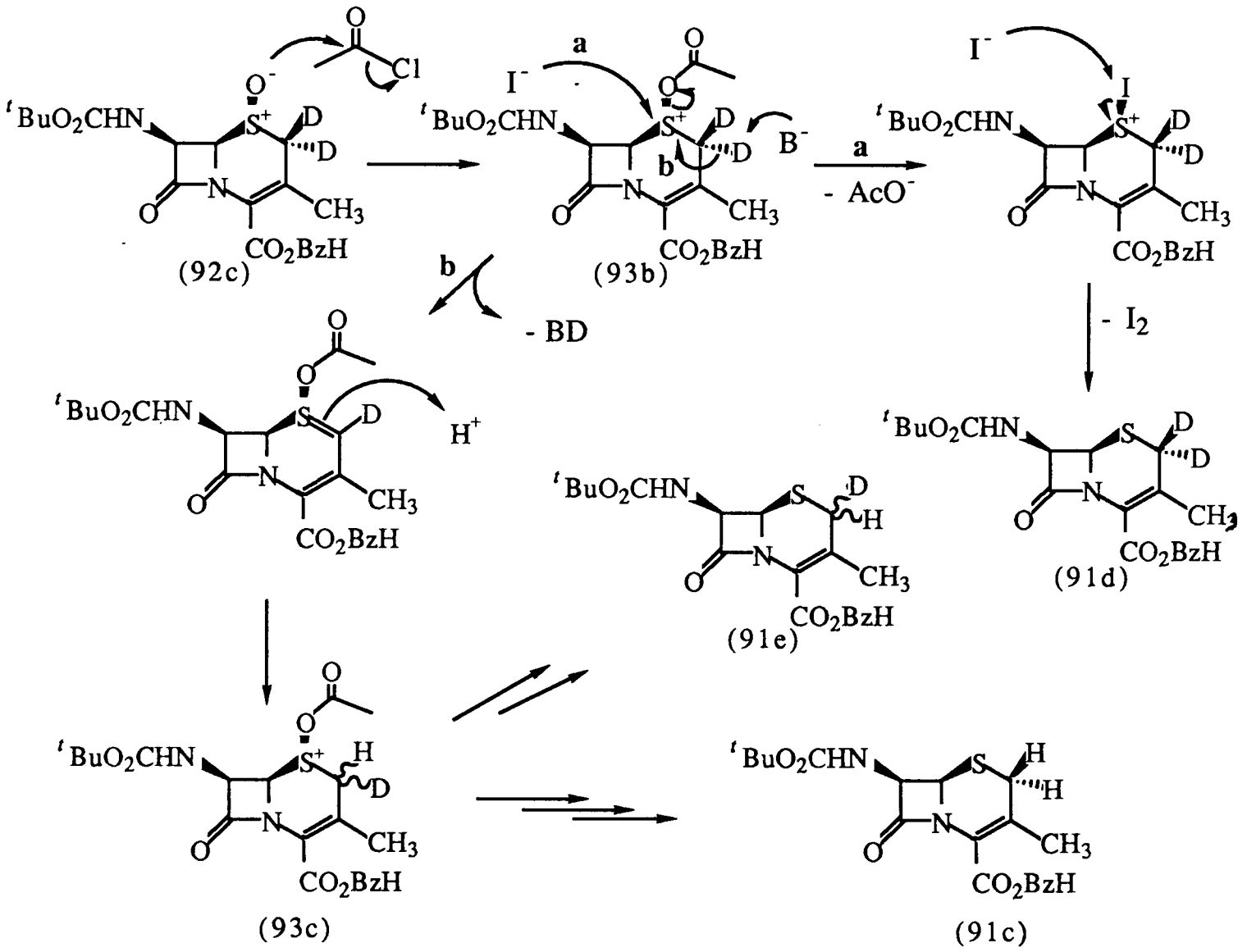
Unfortunately, it appeared that subsequent reduction of the sulphoxide back to protected 7-ADAOC (91a or b), by reaction with acetyl chloride and potassium iodide in DMF,[102] led to significant exchange of the newly introduced deuterium atoms (Scheme 5.4.).



Reagents : (i) D_2O, pH 8.5, 35°C, 24 hours, (ii) MeCN, Ph_2CN_2
(iii) KI, AcCl, DMF.

Scheme 5.4.

Loss of the incorporated deuterium from the sulphoxide (92d) during conversion back to the sulphide (91d) is not a totally unforeseeable possibility, since the mechanism of sulphoxide reduction involves initial formation of the S-acetyl compound (93) in which the protons adjacent to the suphur atom at C-2 have become appreciably acidic (Scheme 5.5.).[102]

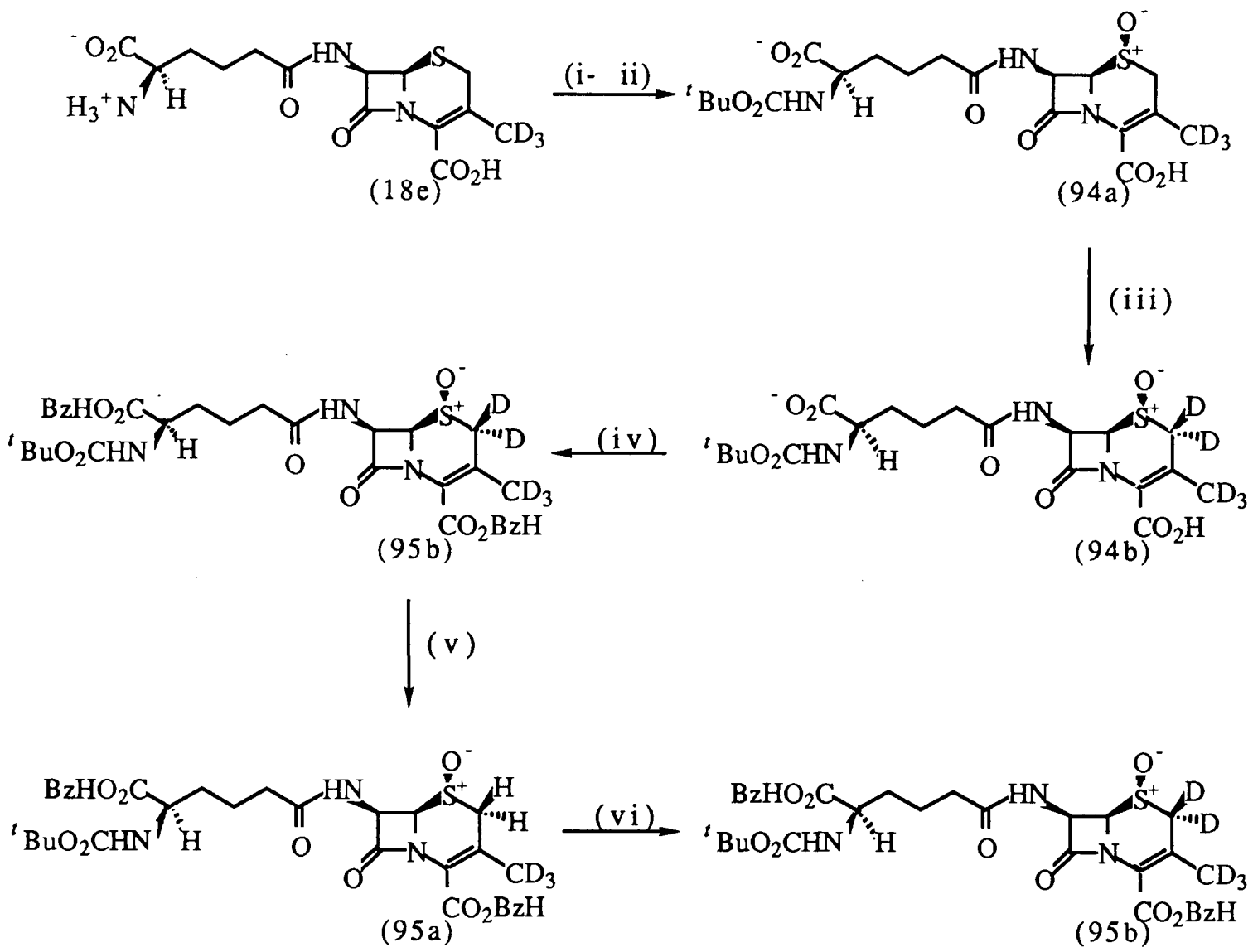


Scheme 5.5.

Exchange of these deuterium atoms would be most likely to arise at this stage providing that a proton source was available. However loss of label during this reduction could not be overcome by the use of totally deuterated acetyl chloride in the absence of any known proton source.[101] Thus, synthesis of dideutero-DAOC (18f) possessing a sufficiently high isotopic purity could not be achieved.

In order that the control experiments with pentadeutero-DAOC (18c) could be undertaken it was decided to re-investigate the earlier attempts at the synthesis of the dideutero-DAOC (18f) *via* C-2 exchange but to use the trideuteromethyl-DAOC (18e). Thus trideuteromethyl-DAOC (18e) (preparation of which will be described in Section 5.6) was protected as its N-*t*-Butyloxycarbonyl derivative by stirring overnight in saturated sodium bicarbonate solution / dioxan (1:1, v/v) with di-*t*-

butylpyrocarbonate followed by acidification and extraction into ethyl acetate. Oxidation with sodium periodate in water at pH 7.5 for two hours then gave principally the β-sulphoxide (94a) (Scheme 5.6.).[103]



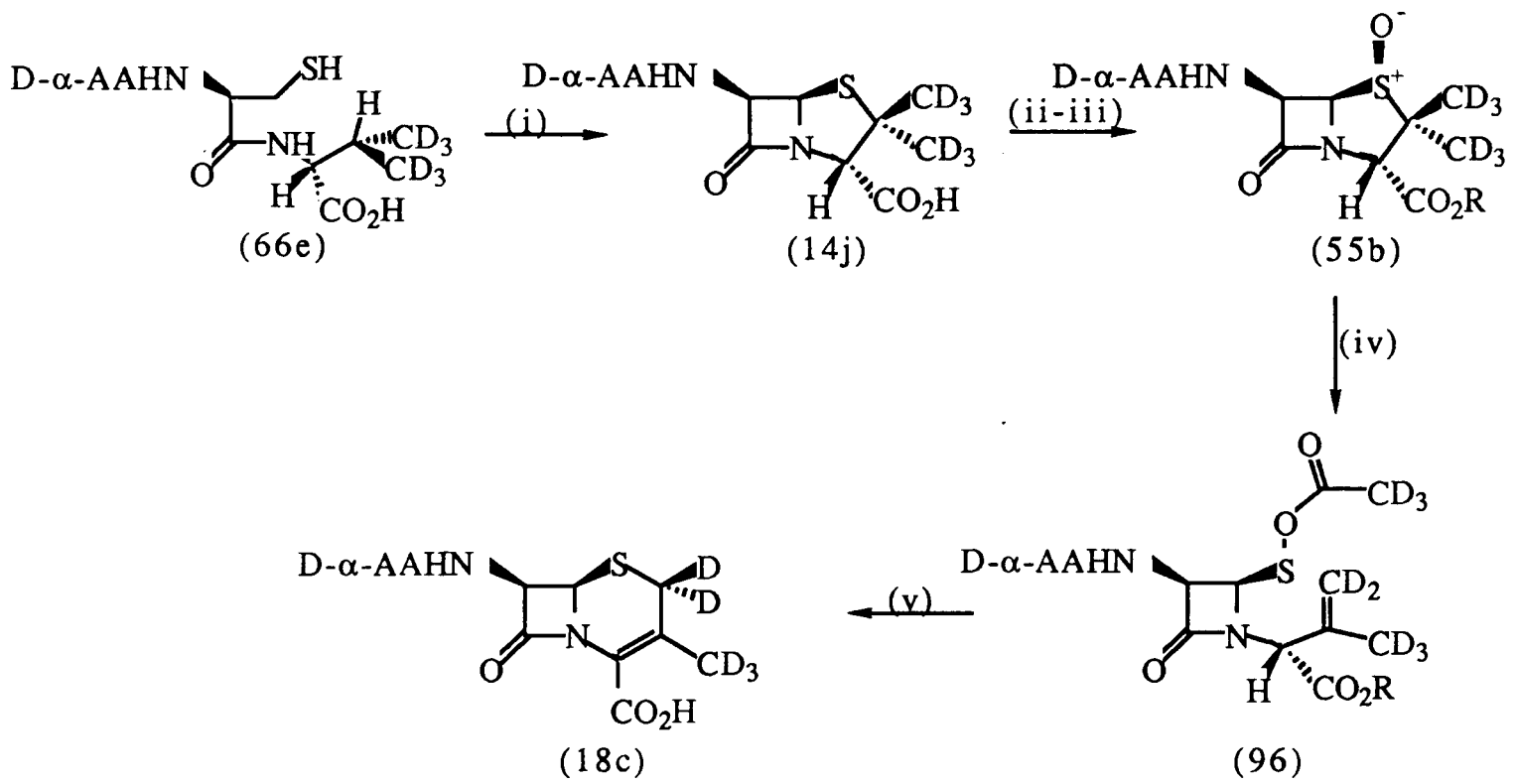
Reagents : (i) $(^tBuOCO)_2O$, sat. aq. $NaHCO_3$/Dioxan (1:1, v/v) ; (ii) $NaIO_4$, H_2O, pH 7.5 ; (iii) D_2O, K_2HPO_4 , K_3PO_4 , pH 8.5, 35°C, 24 hours; (iv) acidify, extract then Ph_2CN_2 , MeCN ; (v) SiO_2 chromatography ; (vi) K_2HPO_4 , K_3PO_4 , pH 8.5, 35°C, 24 hours,D_2O/THF (1:1,v/v).

Scheme 5.6.

The sulphoxide (94a) was then stirred overnight at 35°C in a phosphate buffer prepared in D_2O and adjusted to pH 8.5. Acidification and extraction into ethyl acetate gave the deuterated sulphoxide (94b) which was shown to be greater than 85 atom% pentadeutero-DAOC (95b). Esterification with diphenyldiazomethane in acetonitrile then gave the di-benzhydryl ester which was purified by chromatography [flash SiO_2, eluting with EtOAc/DCM (2:3, v/v)]. Examination of this ester by ^1H-n.m.r and mass spectrometry revealed that the deuterium introduced at C-2, had all been re-exchanged.

Exchange of the C-2 protons on samples of the sulphoxide diester (95a) was investigated under phase transfer (BTEAC catalyst with buffer/toluene) and homogeneous (THF/buffer) conditions with the deuterated phosphate buffer at pH 8.5-9.0. Both conditions gave high levels of incorporation as shown by ^{1}H-n.m.r, but the THF/buffer conditions ultimately gave a cleaner reaction mixture and was consequently used to exchange further sulphoxide di-ester (95a) to the deuterated sulphoxide di-ester (95b). Purification of the reaction mixture by chromatography as before then led to extensive re-exchange (Scheme 5.6.).

Synthesis of the pentadeutero-DAOC (18c) by general synthetic methods would also have been difficult, but one possible route, which might avoid the problems of deuterium loss from C-2 during synthesis, is shown below (Scheme 5.7.).[104]

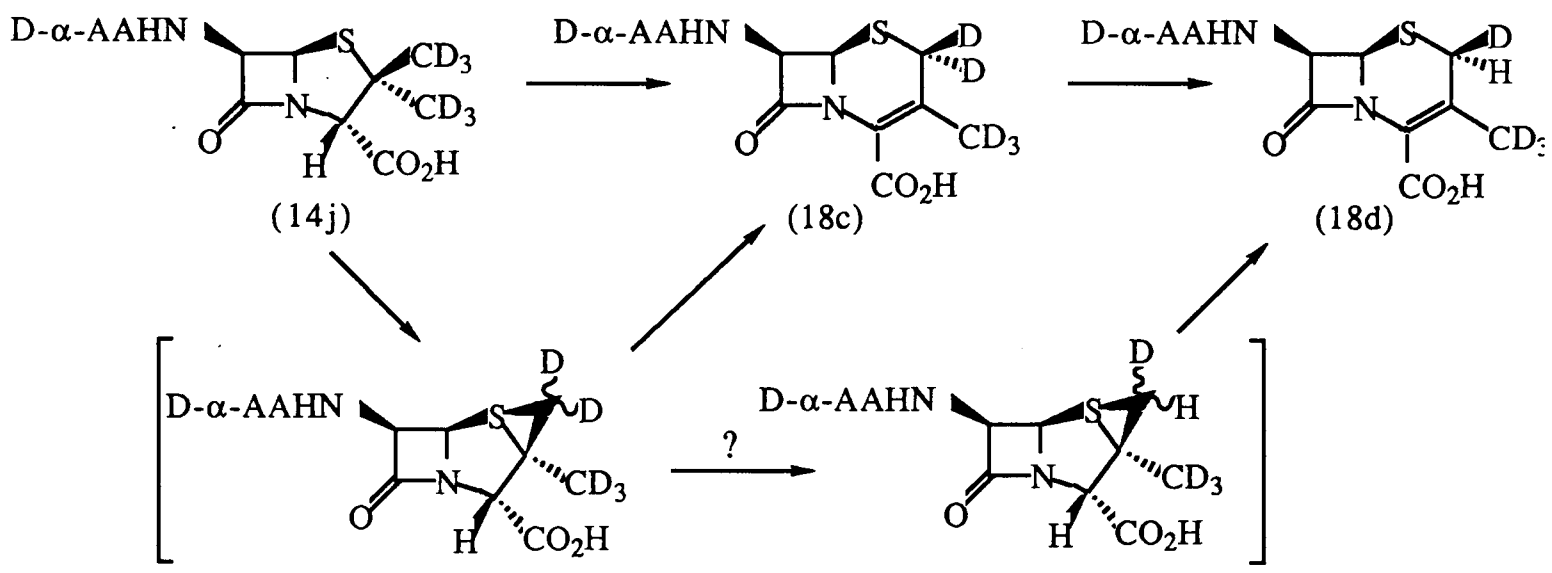


Scheme 5.7.

This synthetic route does however require the hexadeuteropenicillin N (14j) as the starting material. Since there are no easy chemical methods by which this compound can be synthesised, it would have to be enzymatically prepared from DLD-AC-(^{2}H$_6$)-V (66e) by the action of IPNS. The quantity of tripeptide and IPNS required to make a reasonable amount of starting material (14j) (*ca* 20mg) for the synthetic scheme, renders this approach to pentadeutero-DAOC (18c) impractical.

Alternative experiments to study this exchange process, based upon the enxymatic incorporation of deuterium during incubation of either penicillin N or DAOC with DAOC/DAC synthetase in D_2O were considered more feasible and will be described later.

The detection of fully deuterated DAOC (18c) from the incubation of hexadeuteropenicillin N (14j) with DAOC/DAC synthetase suggests either that exchange occurs principally <u>after</u> ring expansion and / or that exchange from a hitherto undetected enzyme free or enzyme bound intermediate may occur to a limited extent during this process (Scheme 5.8).



Scheme 5.8.

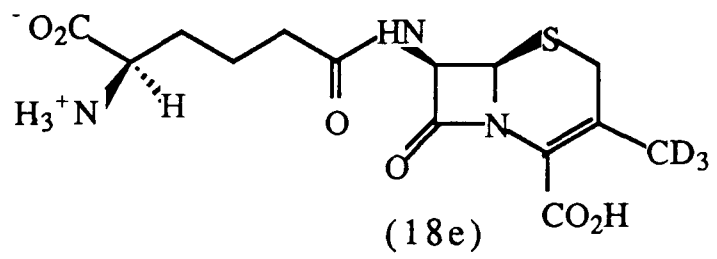
Assuming that exchange does not occur during the ring expansion reaction then either the fully deuterated DAOC (18c) returns to the active site responsible for ring expansion and undergoes exchange <u>or</u> exchange occurs after binding to the active site catalysing hydroxylation. In the introduction, details were given which substantiated evidence that the DAOC/DAC synthetase enzyme from *C.acremonium* is a single bifunctional protein; specifically both activities result from translation of a single piece of DNA, equivalent to a protein of approximately the same molecular weight as the DAOC/DAC synthetase enzyme.[37]

The question remains, however, as to whether the enzyme contains one or two active sites. Obviously for the enzymatic exchange to occur labelled DAOC (18) must return to the active site responsible for catalysing the exchange process. Hence, if the enzyme has two active sites, inhibition of only one of the two activities by DAOC (18) would implicate that activity as being the one responsible for exchange. Thus product inhibition of the ring expansion activity by DAOC (18) would imply that DAOC (18) was binding to the active site catalysing ring expansion and could support the view that the ring expansion activity was

responsible for the exchange phenomenon. Conversely, if the enzyme contained only one active site which catalysed both reactions then either activity could catalyse deuterium exchange from DAOC (18) or DAC (19) and furthermore, inhibition of activities would then be due to competition between penicillin N (14) and DAOC (18) for the active site.

During earlier investigations with the DAOC/DAC synthetase, penicillin N (14) conversions were obtained which were lower than anticipated. These observations prompted kinetic examination for product inhibition and the results obtained indicated that levels of DAOC (18) at a concentration of approximately 1mM did indeed inhibit the ring expansion of penicillin N (14), thus confirming product inhibition.[105] Until the number of active sites present on this enzyme is determined, the activity responsible for the exchange phenomenon cannot be identified with any degree of certainty.

Incubation of unlabelled DAOC (18) with both active and denatured DAOC/DAC synthetase in D_2O offered a possible means of investigating the exchange process. If incorporation was observed into either or both DAOC (18) and DAC (19) only in the incubation with active enzyme, then evidence that the exchange was an enzyme-mediated process would have been obtained. It was felt that this experiment could be improved in an attempt to identify which of the two activities associated with the DAOC/DAC synthetase were responsible for the exchange, by deuteration of the exocyclic methyl group of DAOC (18) to give (18e).



(18e)

Deuteration was expected to produce a primary isotope effect for the hydroxylation reaction. Such an effect had already been reported for the hydroxylation of DAOC (18) to DAC (19) during an investigation into the stereochemistry of this reaction (see Chapter 1, Section 1.3).[44] The operation of an isotope effect at this position might be anticipated to have two consequences for C-2 exchange in DAOC (18e) :-

i) If exchange was catalysed by the ring expansion activity upon DAOC (18e) then by reducing the rate of conversion of DAOC (18e) to DAC (19d-f), the level of deuterium incorporation into DAOC (18e) should be increased

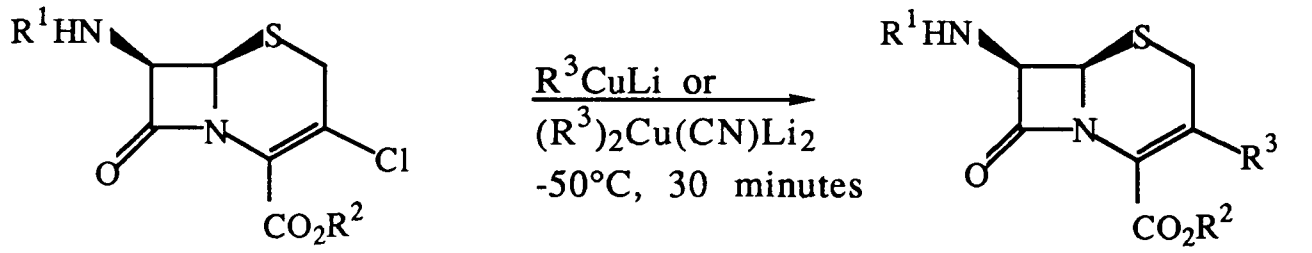
since the ratio of DAOC to enzyme would remain higher for a longer time, and

ii) If exchange was catalysed by the hydroxylase activity upon DAOC (18c) then by reducing the rate of hydroxylation, then the extent of deuterium incorporation into DAOC (18e) should increase since there would be more chance of redirecting the site of reaction from the C-3' methyl group to the C-2 methylene.

Examination of the exchange process by incubation of both unlabelled and trideutero-DAOC (18a) and (18e) respectively in D_2O with the DAOC/DAC synthetase was considered to be most advantageous since the results obtained were expected to be complimentary.

5.6 Synthesis of Substrate.

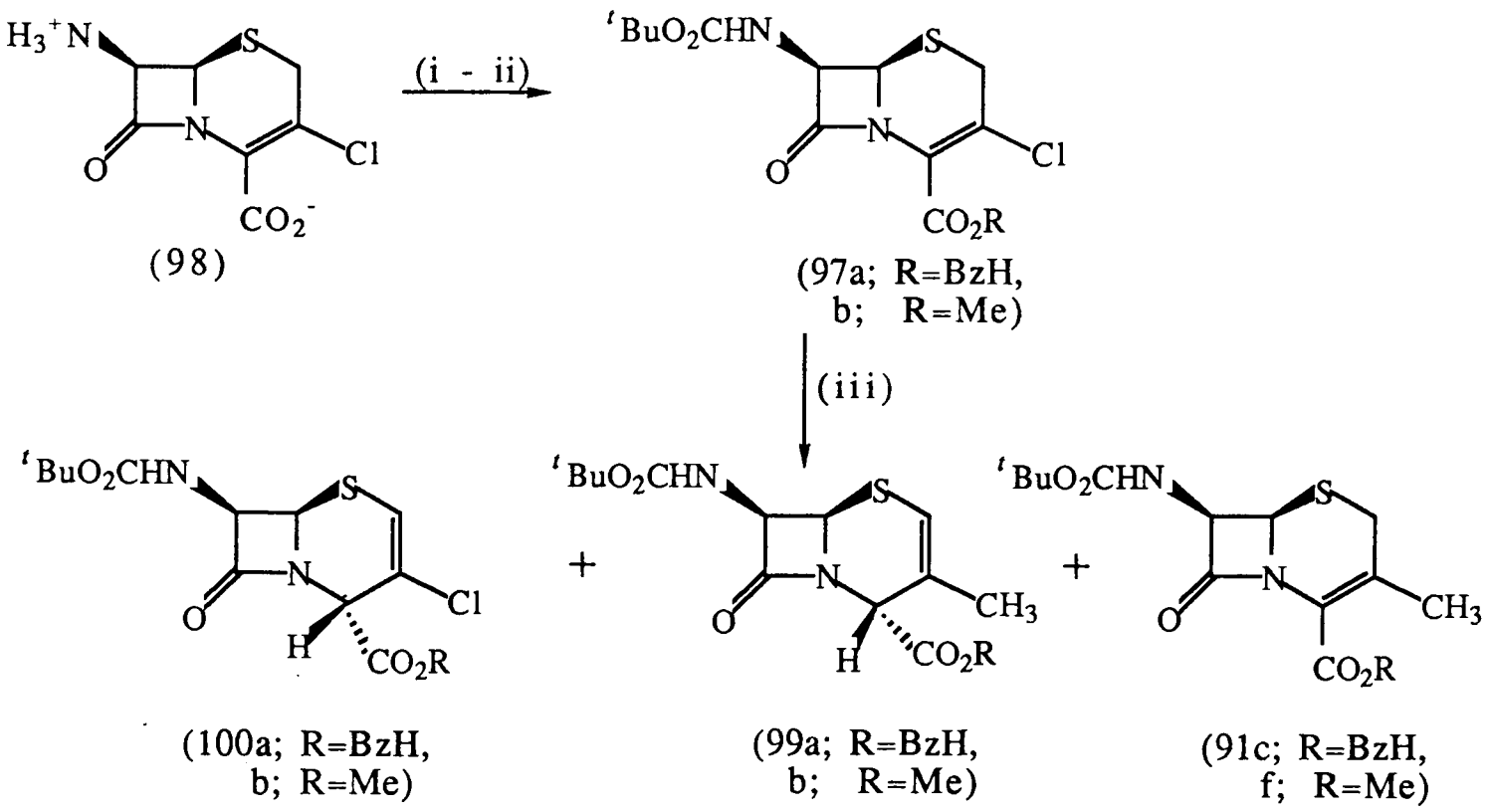
The most direct route to (18e) was considered to be by application of known cuprate chemistry upon the 3-chlorocephem (97) to generate 3-alkyl cephems;[106]



(97a; R^1=^{t}BOC, R^2=BzH,
b; R^1=^{t}BOC, R^2=Me)

R^1=^{t}BOC, R^2=BzH, R^3=alkyl,
R^1=^{t}BOC, R^2=Me, R^3=alkyl)

Thus a sample of 7-amino-3-chlorocephem carboxylate (98) (a gift from Eli Lilly & Co Ltd) was transformed into its N-*t*-butyloxycarbonyl derivative by treatment with di-*t*-butylpyrocarbonate in saturated sodium bicarbonate solution/ dioxan (1:1, v/v), acidified , extracted and then treated with diphenyldiazomethane to give the benzhydryl ester (97a) in good yield. However, treatment of this cephem with the dimethyl Lipschutz cuprate[107] under the literature described conditions gave, in low yield, a mixture of Δ2 starting material (100a) 13% yield from (97a), Δ2 DAOC (99a) 20% and a small quantity of the desired Δ3 DAOC (91c) 5% (Scheme 5.9.).



Reagents : (i) $(^tBuOCO)_2O$, sat. aq. $NaHCO_3$/Dioxan (1:1, v/v), (ii) Ph_2CN_2, MeCN, (iii) $Me_2Cu(CN)Li_2$, THF, -50°C, 30 minutes.

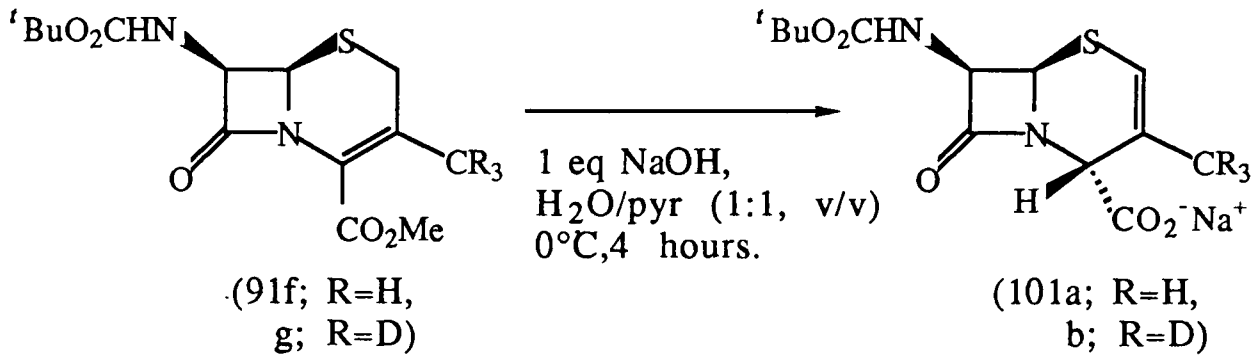
Scheme 5.9.

The relative yields of these unwanted products is probably controlled by the size of the ester protecting group - a bulky group blocking the approach of the cuprate and leading to deprotonation of the comparatively acidic C-2 methylene probably by basic contaminants present in the methyllithium used to prepare the cuprate. The presence of a bulky ester group slows down cuprate addition and thereby allows deprotonation to compete. Once formed, the isomerised starting material can no longer react with the Lipschutz cuprate.

In an attempt to overcome the problems generated by protection with a bulky ester group, synthesis of smaller aryl esters was undertaken. Problems were immediately encountered with the preparation of $\Delta 3$ esters without concomitant formation of the undesired $\Delta 2$ ester. The majority of esterification procedures which have basic characteristics give rise to isomerisation to a lesser or greater extent. Two methods were found, however, which enabled clean preparation of the $\Delta 3$ esters. These consisted of either treatment of the N-protected, $\Delta 3$ cephem acid with diazoalkanes or by reaction of the acid in DMF/dioxan (1:1,v/v) with one equivalent of sodium bicarbonate and an alkyl halide.[108] The latter method produced only low to moderate yields of the desired esters and and thus preparation with the diazoalkane route was utilised.

In the published examples of cuprate additions to the 3-chlorocephem nucleus, a methyl ester group was used.[106] Little consideration was paid to the use

of this ester since it was felt that any attempt at ester hydrolysis would lead to substantial opening of the β-lactam ring. However, hydrolysis of cephem methyl esters with one equivalent of sodium hydroxide in pyridine/water (1:1, v/v) has been reported to give cleanly the Δ2 cephem acids (101a) (Scheme 5.10.).[109]



(91f; R=H, g; R=D)

(101a; R=H, b; R=D)

Scheme 5.10.

Deprotection of these methyl esters without concomitant β-lactam hydrolysis can be attributed to the fact that isomerisation to give the Δ2 cephem ester occurs first. Since the Δ2 cephem β-lactam carbonyl groups are less reactive than their Δ3 analogues, the stability of the β-lactam ring to base hydrolysis is increased and is sufficiently stable to survive the conditions required for base catalysed methyl ester hydrolysis. Reduced reactivity may stem from the increased mesomeric interaction of the nitrogen lone pair with the carbonyl π-bond since, in the Δ3 cephems, this lone pair interacts with both the Δ3 double bond and the carbonyl π-bond (see Figure 5.2.) leading to a comparative weakening of the amide carbon-nitrogen bond and hence to a more reactive β-lactam ring.[110]

A complication associated with this method of deprotection is the subsequent need to transform the Δ2 cephem acids back to the Δ3 isomer. This transformation is easily achieved*via* re-esterification and oxidation to the sulphoxide, at which point the thermodynamically more stable βγ– unsaturated sulphoxide is formed. The sulphoxide is then reduced to give the Δ3 cephem ester.[102]

This synthetic approach was initially examined by carrying out the synthesis with unlabelled material. Thus, the dimethyl Lipschutz cuprate was

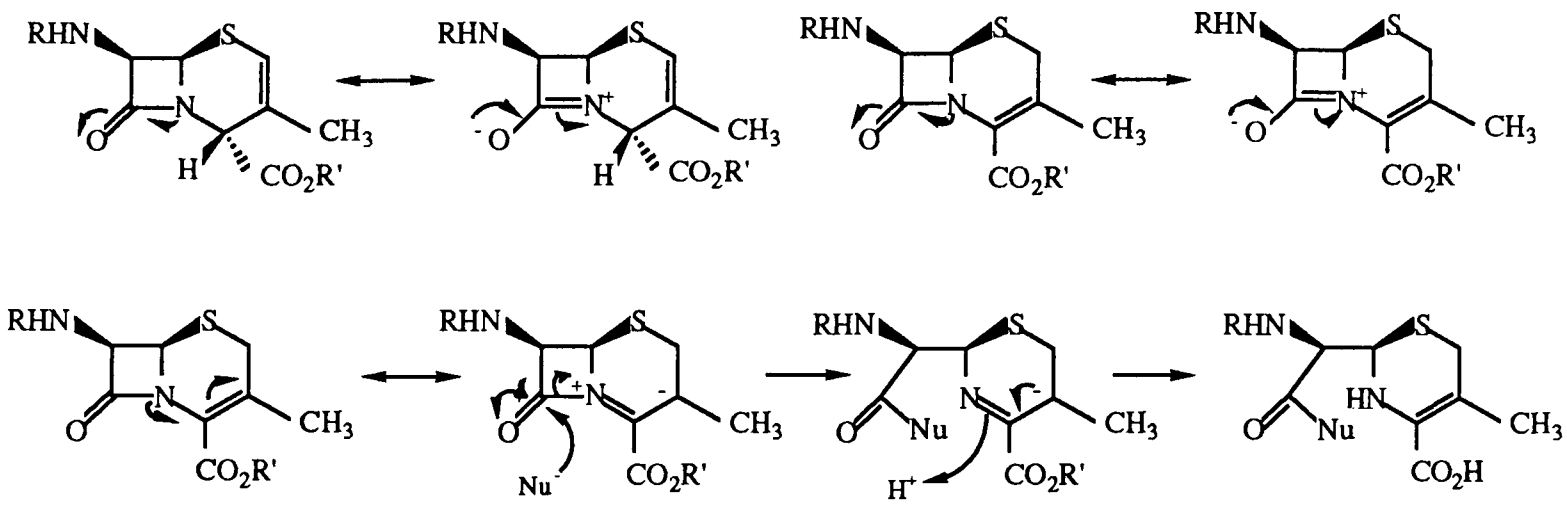
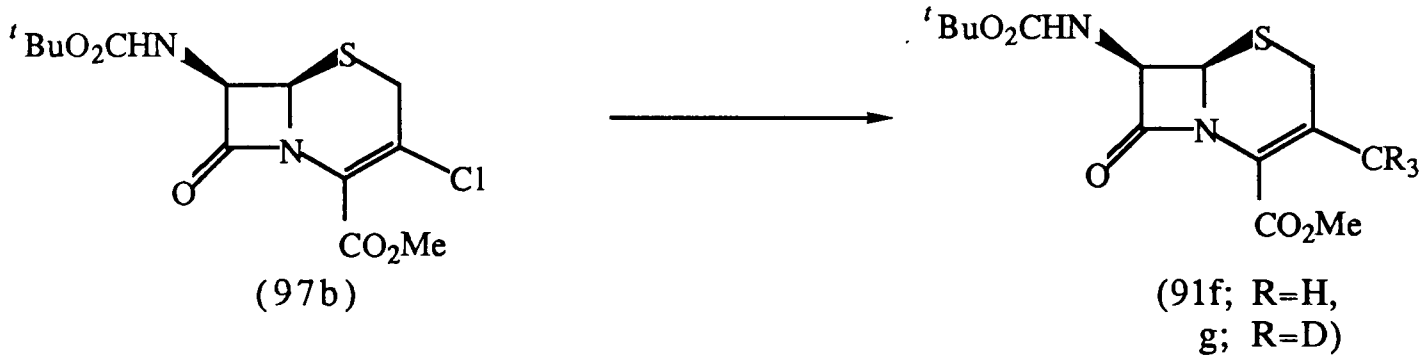


Figure 5.2.

prepared by the addition of methyllithium to cuprous cyanide suspended in THF initially at -70°C and then warmed to +5°C until a clear solution was obtained. To this cuprate solution at -70°C was then added the N-*t*-butyloxycarbonyl, 3-chlorocephem methyl ester (97b) (Scheme 5.11.) and the resulting solution warmed to -50°C and stirred for 30 minutes.



(97b)

(91f; R=H,
g; R=D)

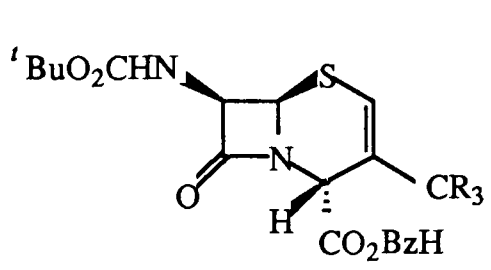
Reagents : $(CH_3)_2Cu(CN)Li_2$ or $(CD_3)_2Cu(CN)Li_2$
THF, -50°C, 30 minutes

Scheme 5.11.

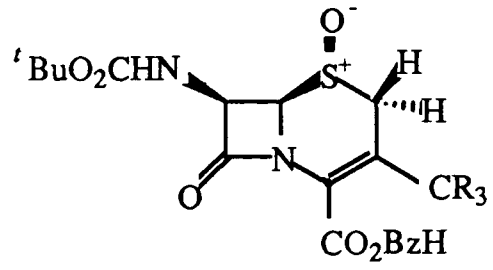
The resulting DAOC methyl ester (91f) was isolated by chromatography [flash SiO_2 eluting with ethyl acetate/petrol (1:4, v/v)] and then hydrolysed with one equivalent of sodium hydroxide in pyridine/water (1:1, v/v) at 0°C to give the $\Delta 2$ cephem acid (101a). Treatment of this acid with diphenyldiazomethane gave the benzhydryl ester (99a) which was then oxidised to the corresponding $\Delta 3$ cephem ester sulphoxide (92b) by reaction with MCPBA in DCM. Reduction of (92c) with acetyl chloride and potassium iodide in DMF gave the required protected 7-aminodeacetoxycephalosporinate benzhydryl ester (91c).

This sequence was then repeated with trideuteromethyllithium prepared by the addition of trideuteromethyliodide (99.5% atom D, supplied by Aldrich Chemical Co. Ltd.) to a suspension of lithium dispersion (containing 1% sodium) in diethyl ether.[111] This gave a solution of trideuteromethyllithium in diethyl ether, the molarity of which was determined by titration against diphenylacetonesulphonylhydrazone.[112] With this alkyllithium solution the di-trideuteromethyl Lipschutz cuprate was prepared by the addition of trideuteromethyllithium to cuprous cyanide suspended in THF. Repetition as described above (Scheme 5.11.) gave the protected trideutero-7-amino DAOC (91g) in 51% overall yield from 3-chlorocephem ester (97b).

This compound was then saponified,[109] as previously described for the undeuterated material, to the Δ2 cephem acid (101b), esterified with diphenyldiazomethane to give (99c), oxidised to (92e) and then reduced to give the

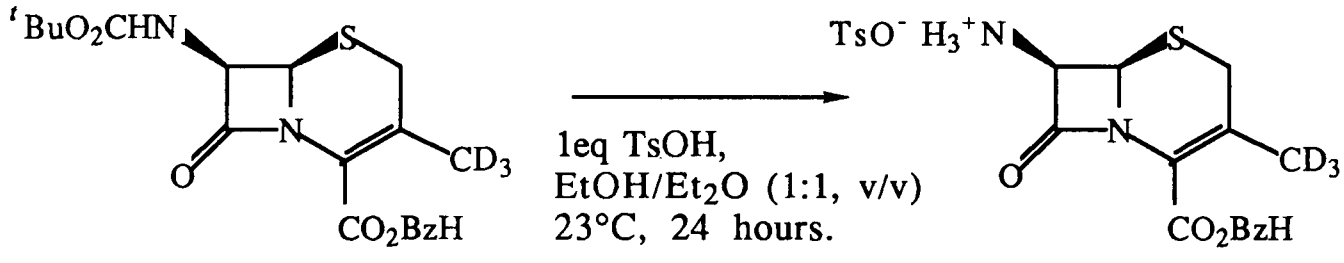


(99a; R = H,
c; R = D)



(92b; R = H,
e; R = D)

Δ3 cephem ester (91h). Conversion to the ammonium *p*-toluenesulphonate salt (102b) was achieved by stirring overnight at room temperature with one equivalent of *p*-toluenesulphonic acid in ethanol/diethyl ether (1:1, v/v) (Scheme 5.12.).

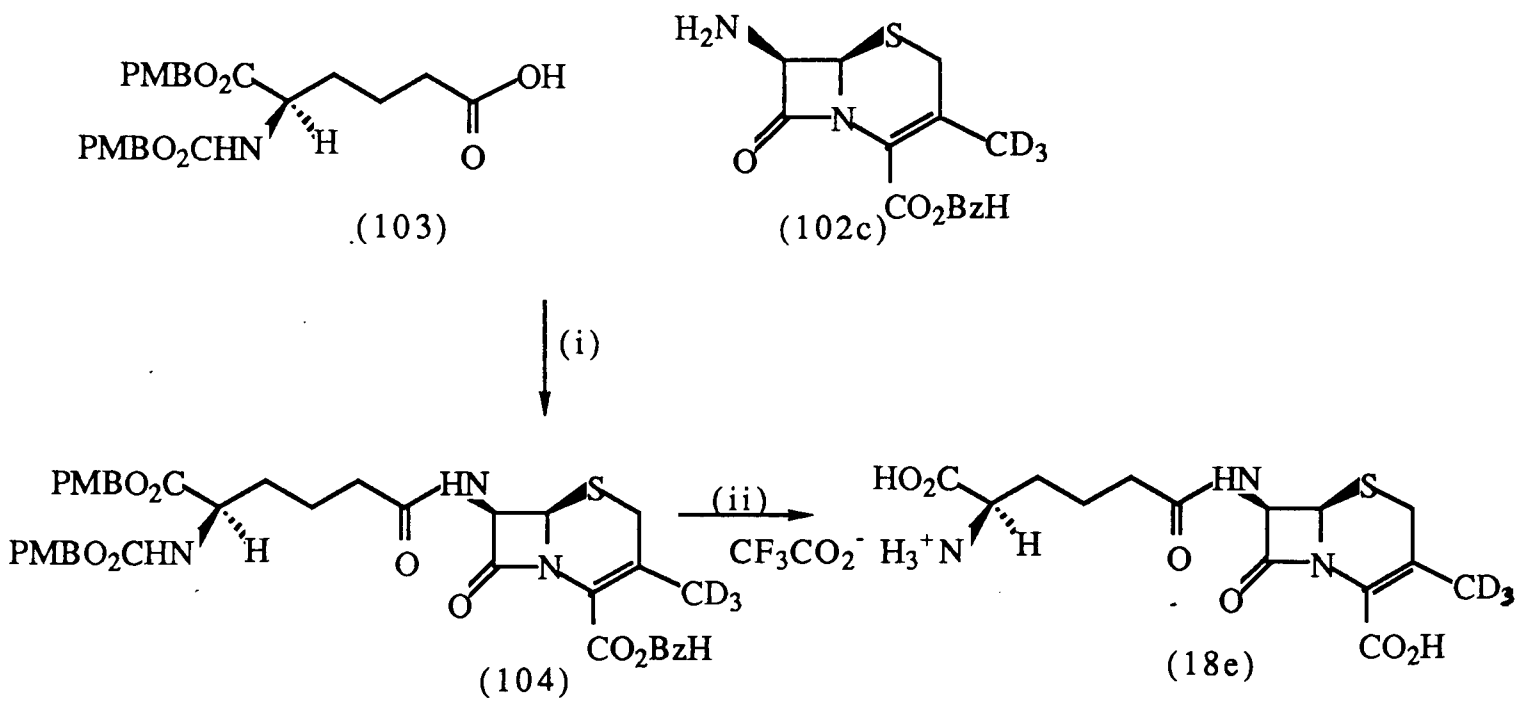


(91g) (102b)

Scheme 5.12.

Conversion to the free amine (102c) by ethyl acetate extraction from a saturated sodium bicarbonate solution, and coupling to acid labile protected (5R)-5-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid (103) with EEDQ[65] gave the protected trideutero DAOC (104). After purification by chromatography, all the protecting groups were removed by treatment with

TFA/anisole (4:1, v/v) in toluene at room temperature to give (18e) (Scheme 5.13). The deprotection of (104) in this way produced labelled DAOC (18e) of sufficient purity that further purification by hplc was not required, as judged by ^{1}H-n.m.r, 500 MHz.

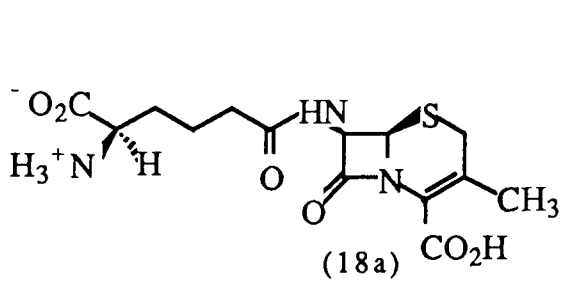


Reagents : (i) EEDQ, DCM, 23°C, 24 hours, (ii) TFA/anisole (5:1, v/v), reflux, 30 minutes.

Scheme 5.13.

5.7 Incubation of DAOC (18a) with DAOC/DAC Synthetase in D_2O.

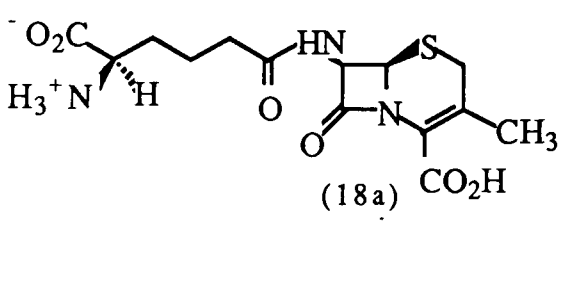
Partially purified DAOC/DAC synthetase enzyme was exchanged twice on a PD-10 sephadex ion exchange column into TRIS HCl buffer (50 mM, pH 7.5) prepared in D_2O. A sample of the resulting enzyme was denatured by heating at 100°C for 5 minutes. Both active and denatured enzyme samples were then pre-incubated with cofactors as usual, prior to incubation with unlabelled DAOC (18a) (Scheme 5.14.). After incubation, both mixtures were examined by 500 MHz ^{1}H-n.m.r., the spectra from which showed *ca* 30% conversion to DAC (19a) for the incubation with active enzyme and no conversion with the denatured enzyme. DAOC (18a), and DAC (19a) in the case of the active enzyme incubation, were then isolated from the incubation mixtures by hplc [Gilson system, 0.75% MeCN in 10 mM aqueous NH_4HCO_3] and examined for incorporation of deuterium by mass spectrometry (positive Ar, FAB, MCA). No incorporation of deuterium into DAOC (18a) could be detected (Table 5.4.).



(18a)

DAOC/DAC synthetase in D_2O,Fe^{2+}, O_2, L-Ascorbate, DTT. ⟶ *ca* 30% Conversion to DAC (19a)

No Incorporation of Deuterium into DAOC (18a)



(18a)

Denatured DAOC/DAC synthetase in D_2O,Fe^{2+}, O_2, L-Ascorbate, DTT. ⟶ No Conversion to DAC (19a)

No Incorporation of Deuterium into DAOC (18a).

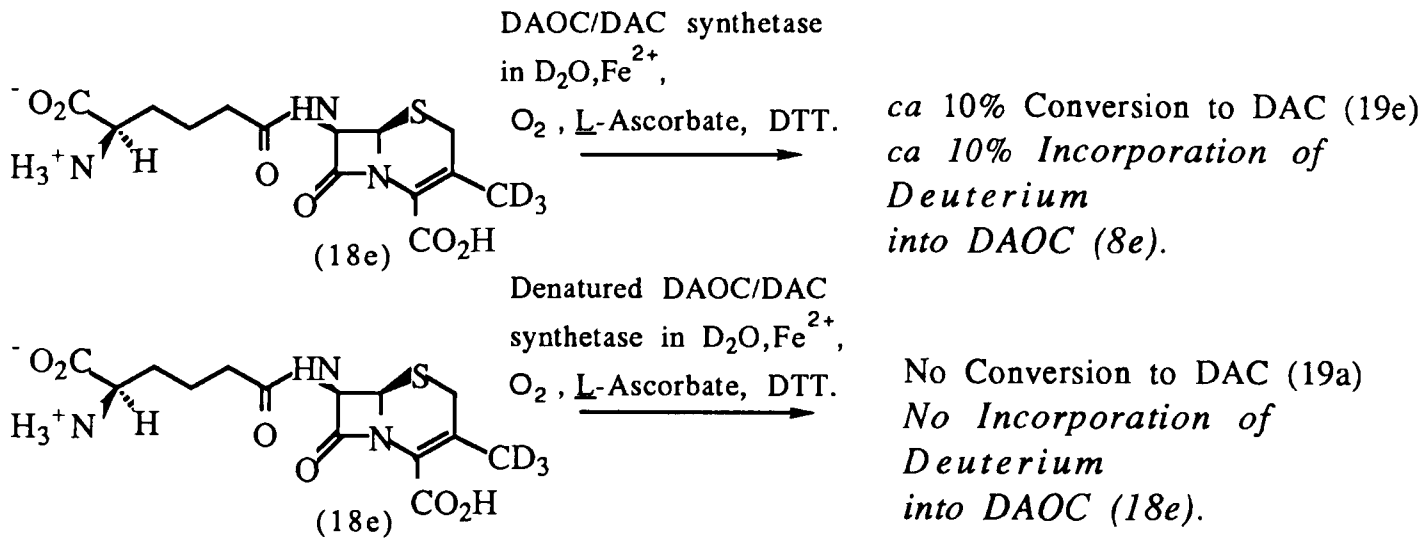
Scheme 5.14.

Experiment.	approximate % Conversion	*m/z* for DAOC (18a). *357*	*358*	*359*	*360*	*361*	*362*	*363*
Active Enzyme	30	5	100	22	9	6	2	2
Denatured Enzyme	0	5	100	19	8	8	3	4
Calculated	-	-	100	18	7	1	0	0

Table 5.4.

5.8 Incubation of (3'-$^2H_3$)-DAOC (18e) with DAOC/DAC synthetase in D_2O.

Incubations of (18e) in D_2O with active and denatured DAOC/DAC synthetase were performed in exactly the same way (Scheme 5.15.) and on the same scale as for those with unlabelled DAOC (Section 5.7.). Examination by 500 MHz ^1H-n.m.r indicated *ca* 10% conversion with the active enzyme and no conversion with the denatured enzyme. Mass spectrometry (positive Ar FAB, MCA) showed that in the case of the active enzyme incubation, *ca* 10% incorporation of deuterium into DAOC (18e) had occured (Table 5.5, Expt.1.). No incorporation of deuterium could be detected in the sample of DAOC (18e) from the denatured 'incubation'.



Scheme 5.15.

This experiment was repeated in order to obtain a higher percentage conversion for the active enzyme incubation. From this it was hoped that the effect of percentage conversion upon deuterium incorporation could be determined. Thus (18e) was incubated with active and denatured DAOC/DAC synthetase in D_2O as before and a conversion of *ca* 40% obtained. Analysis of deuterium incorporation into DAOC (18e) revealed approximately 20% incorporation (see Table 5.5, Expt.2).

	approximate	m/z for DAOC (18e)						
	% Conversion	*360*	*361*	*362*	*363*	*364*	*365*	*366*
Experiment.No.1								
Active Enzyme	10	6	100	28	14	12	3	2
Denatured Enzyme	0	4	100	17	10	5	4	3
Experiment.No.2								
Active Enzyme	40	11	100	49	22	8	6	4
Denatured Enzyme	0	12	100	26	16	6	6	-
Calculated		-	100	18	7	1	0	0

Table 5.5.

5.9 Conclusions.

The results presented in this chapter are in support of the earlier investigations upon the incorporation of hexadeuterovaline (11k) *via* intact cells of *C. acremonium* into cephalosporin C (20), in which loss of deuterium was shown

to have occurred during this transformation[41]. Furthermore, these results show that the loss of deuterium is most likely occurring after the ring expansion step and/or during the hydroxylation of DAOC (18) to DAC (19).

The investigations presented here have indicated that the position of exchange is the C-2 methylene of DAOC (18) and further that it is the C-2βD which is initially replaced by hydrogen.

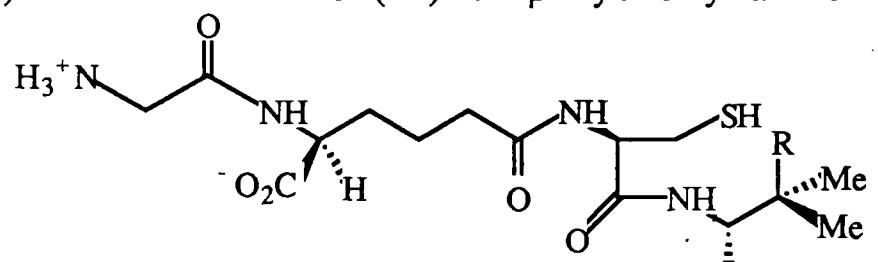
Experiments concerned with the incorporation of deuterium into DAOC (18) by incubation in D_2O suggest that the exchange process is related to the rate of hydroxylation, since significant incorporation of deuterium is only observed when the C-3' methyl of DAOC (18) or DAC (19) is replaced by a trideuteromethyl group. It is possible to conclude from these results that the operation of a primary isotope effect upon the hydroxylation of DAOC (18) to DAC (19) most likely permits exchange at C-2 of DAOC (18) to compete with hydroxylation.

Further work is required to investigate this process so that an understanding of the reasons for and mechanism of the unusual C-2 exchange may be obtained.

Chapter 6 : Anomalous Peptides from *P.chrysogenum* and *Cephalosporium Sp.*

6.1.Introduction.

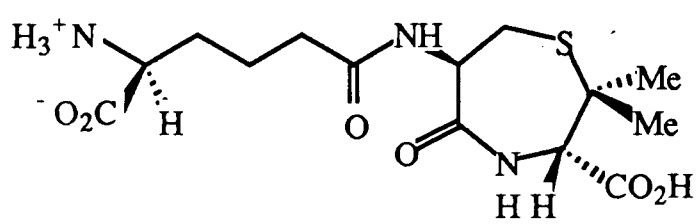
During an investigation into the biosynthetic origins of penicillins and cephalosporins a number of α-aminoadipoyl containing peptides were isolated from fermentation broths of *Penicillium*, *Cephalosporium*, *Streptomyces* and *Paecilomyces*,[113,114,143]. All of these species produce penicillins and cephalosporins except for *Penicillium* which can produce only penicillins[6]. Since the immediate biosynthetic precursor to the penicillins is LLD-ACV (12a),[12,13] it is of no surprise to find this substance produced in all of the species mentioned above. In addition, it has been found that *Cephalosporium. Sp* (C91) and *Paecilomyces. persicinus* (P-10) are also capable of producing tetrapeptides, the constituent amino acids of which were identified as α-aminoadipic acid (8), glycine, cysteine (9) and either valine (11) or β-hydroxyvaline[113,143].



(105a; R=H,
b; R=OH)

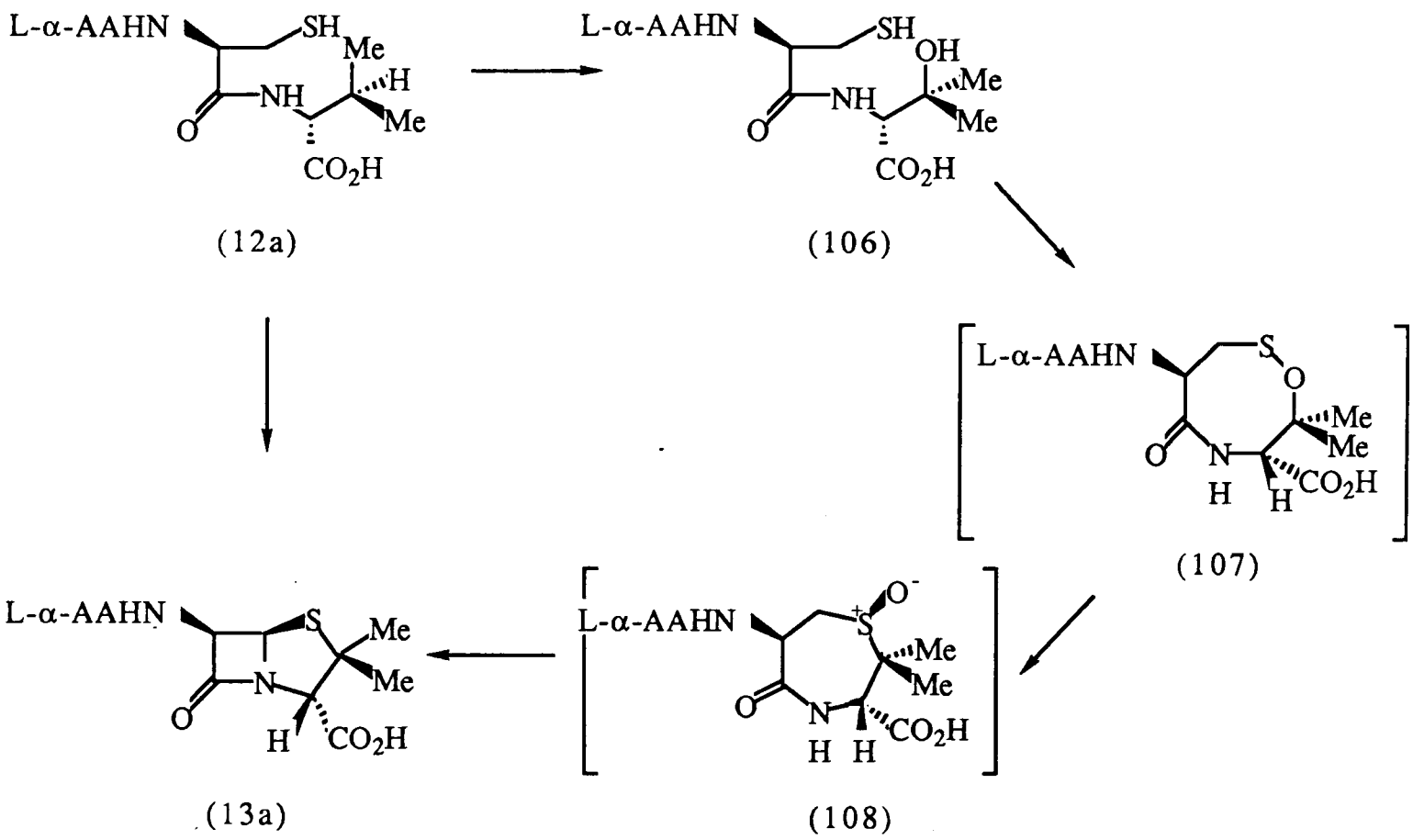
Isolation of the hydroxyvaline containing tetrapeptide (105b) led to biosynthetic and mechanistic speculation.[115] It was suggested that LLD-ACV (12a) was first converted to α-aminoadipoyl-cysteinyl-β-hydroxyvaline (106) prior to formation of isopenicillin N (13a) (Scheme 6.1.).

These ideas were put to the test by synthesis of α-aminoadipoyl cysteinyl-β-hydroxyvaline (106) and the two thiazepinone-containing peptides (108) and (109).[116] Incubation of these peptides with cell free preparations of IPNS resulted



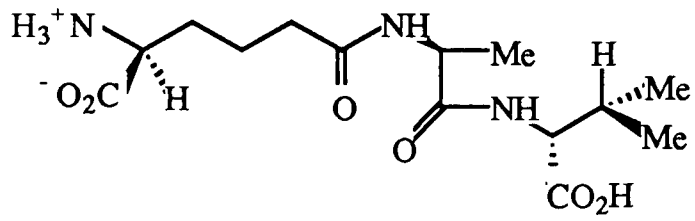
(109)

in no conversion to isopenicillin N (13a). These observations suggested that the biosynthetic and mechanistic speculation described in Scheme 6.1 were incorrect.

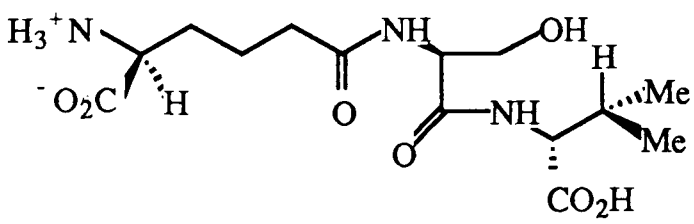


Scheme 6.1.

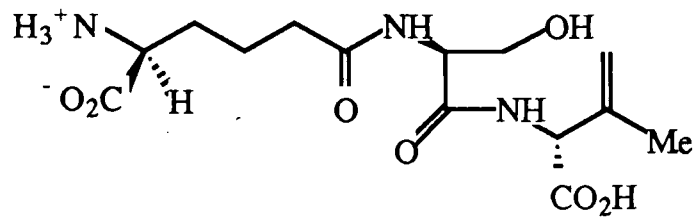
Perhaps the most interesting peptides so far isolated have come from *Penicillium chrysogenum*.[114] These peptides, which appear to have undergone modification at either or both the cysteine and valine residues, were found to include α-aminoadipylalanylvaline (110), α-aminoadipylserinylvaline (111) and α-aminoadipylserinylisodehydrovaline (112). Only the configurations of the amino



(110)



(111)



(112)

acids contained in the α-aminoadipylserinylvaline tripeptide (111) were determined and shown to be L-α-aminoadipyl-L-serinyl-D-valine.

The isolation of the isodehydrovaline-containing peptide (112) generated considerable interest in the possible involvement of this compound as an intermediate in penicillin biosynthesis, particularly as such a proposal had been made concerning similar dehydropeptides almost a decade earlier.[117] As a direct result of the isolation of (112) from *P. Chrysogenum*, synthesis of L-α-aminoadipoyl-L-cysteinyl-D-isodehydrovaline (LLD-ACdV) (27a) and L-α-aminoadipoyl-L-serinyl-D-isodehydrovaline (LLD-ASdV) (112) was undertaken.[118] Incubation of (27a) with IPNS at that time gave no conversion to isopenicillin N (13a) but it was shown that (27a) was a potent inhibitor of the natural conversion of LLD-ACV (12a) to isopenicillin N (13a). Later work revealed that this peptide (27a) was in fact a substrate for IPNS (see Chapter 1, Section 1.3).[46]

Interest in these peptides declined and consequently comparatively little work concerning their origin or biological importance has been reported. The dismissal of α-aminoadipoyl-cysteinyl-β-hydroxyvaline (106) as a biosynthetic intermediate[116] gave support to the view that these tripeptides and tetrapeptides resulted from lack of substrate specificity by the dipeptide synthetase or tripeptide synthetase enzymes. Such reasoning appears justifiable until consideration is given to the isodehydrovaline-containing tripeptide (112). Isodehydrovaline is not a natural product and as such would not be available in the cytoplasm for enzymatic incorporation into the tripeptide (112). Thus the most likely explanation for the production of this tripeptide is *via* enzymatic or chemical modification of either LLD-ACV (13a) or α-aminoadipylserinylvaline (111). Alternatively, formation by dehydration of α-aminoadipylcysteinyl-β-hydroxyvaline (106) (Scheme 6.2) could be responsible.

It should, however be brought to attention that the α-aminoadipyl-cysteinyl-β-hydroxyvaline tripeptide (106) has not been isolated from *Penicillium Sp.*, the organism from which the isodehydrovaline-containing peptide was obtained. Furthermore, no evidence for the production of (112) in organisms capable of cephalosporin biosynthesis, such as *Cephalosporium Sp.*, has been found.

Biosynthesis of the anomalous tripeptides from *P.chrysogenum* and *Paecilomyces* was considered worthy of further investigation in an attempt to resolve the following points :

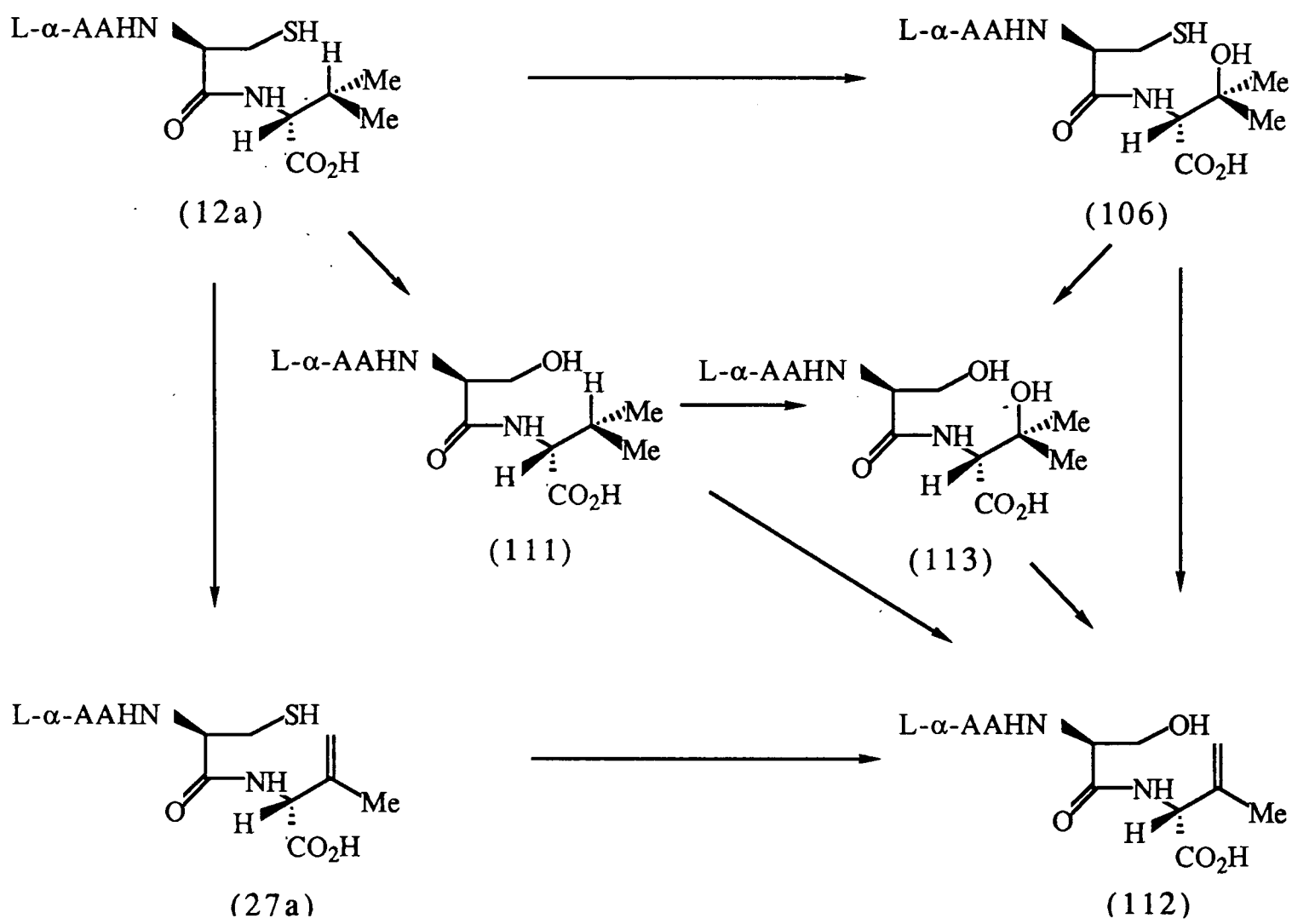
i) identification of the enzyme (or enzymes) associated with their biosynthesis,

ii) identification of the immediate biosynthetic precursors,

iii) investigation of the biosynthetic relationships, if any, between these peptides,

iv) are these tripeptides substrates for the enzymes responsible for β-lactam biosynthesis *i.e.* IPNS ? and

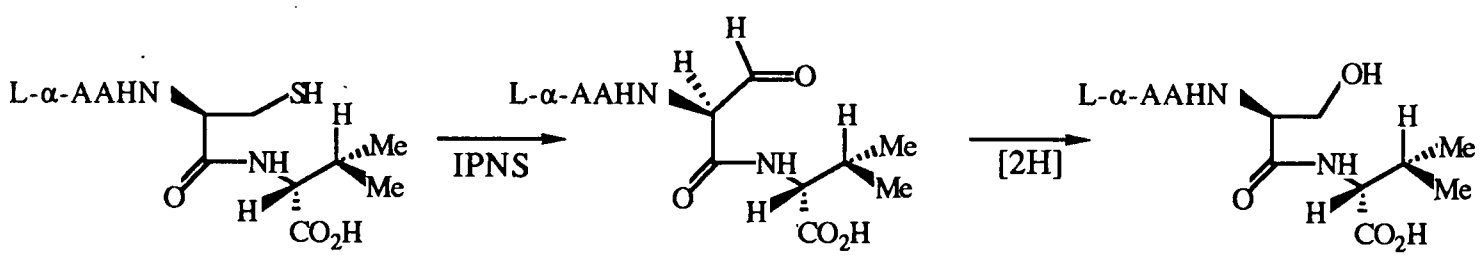
v) if these peptides are shunt metabolites or modified shunts, what mechanistic information can be obtained about the enzymes responsible for their formation?



Scheme 6.2.

No reports on the production of these peptides by cell free preparations have yet been published. A major problem which could lead to complications with the identification of peptides formed in studies with partially purified enzymes, becomes apparent if *in vivo* and *in vitro* conditions are compared. With experiments performed *in vivo*, all the enzymes responsible for primary and secondary metabolism are available since these would be intact and operational within the living cell. Consequently, the isolation of these compounds after excretion from intact cells (*i.e.* from the fermentation broths of the organisms described earlier) means that the initially formed peptide products would have been exposed to the variety of enzymes present within the cell. Thus the actual

peptides observed may not necessarily be the first formed products, since further modification prior to cellular excretion may have occurred. In the case of shunt metabolites, it is possible that the product isolated may have arisen from further modification of an initial enzyme shunt. In these cases identification of the enzyme producing the shunt and the first formed shunt metabolite, becomes more difficult. For example, IPNS may produce the α-aminoadipyl-serinylaldehyde-valine tripeptide (114) as a shunt product from LLD-ACV (12a) (Scheme 6.3.) (see also Section 6.9.). Subsequent reduction of this aldehyde by a NADH dependant reductase could then convert it to the reported α-aminoadipyl-serinyl-valine tripeptide (111).



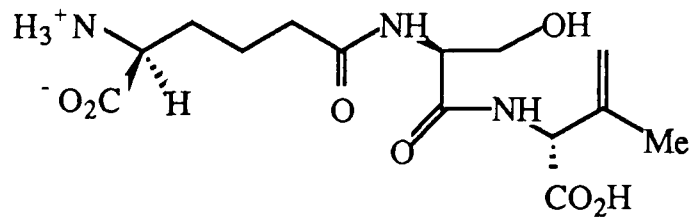
(12a) (114) (111)

Scheme 6.3.

6.2 Design of Experiments to Investigate the Biosynthetic Origin of α-Aminoadipyol-serinyl-isodehydrovaline (112).

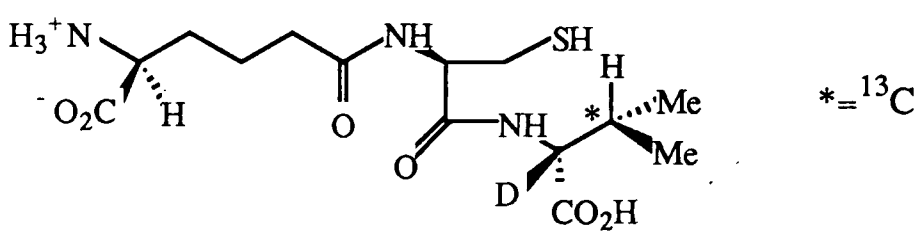
Investigation of the biosynthesis of minor secondary metabolites (*i.e* those which are produced in low yield) and of shunt metabolites, whose production may sometimes be at levels considerably lower than the usual enzymatic product, present problems associated with detection, isolation and characterisation due to the very small quantity of material involved. If such investigations are to be successful, attempts must be made to reduce these problems to a minimum. Direct observation of the substance in question *i.e* without prior purification stages, is perhaps the most efficient way to achieve this, since loss of material during isolation is avoided. Typically this has been achieved by observation of secondary metabolites in crude fermentation extracts or incubation mixtures by any one of a variety of spectroscopic techniques. Furthermore, the greater availability of stable isotopes and their use in conjunction with sophisticated spectroscopic techniques, has led to an increased understanding of many biosynthetic pathways.[119]

Of the tripeptides isolated, the one containing the isodehydrovaline residue, (112) was considered to be the most interesting due to the modifications that had occurred at the cysteine and valine residues.



(112)

In the article concerning the isolation and characterisation of this peptide from *P. chrysogenum*, it was reported that only 35 mg of pure material was isolated from 190 litres of fermentation broth.[114] Disregarding the very low level of production, investigation of this compound was thought preferable to the other peptides for two reasons. Firstly, use of the specifically labelled LLD-AC-(2-^{2}H,3-^{13}C)-valine tripeptide (12h) was applicable to investigation of this isodehydrovaline containing tripeptide and since a sample of (2-^{2}H, 3-^{13}C)-valine (11m) had already been prepared for other experimental work (see Chapter 2, Section 2.4), this was readily available. Secondly, the olefinic carbon atoms at C-3



*=^{13}C

(12h)

and C-4 of the isodehydrovaline residue provide a suitable marker with which easy spectroscopic detection could be obtained, particularly as it had been shown in the investigation concerned with the stereospecificity of the ring expansion process (Chapter 3), that the olefinic region of crude IPNS and DAOC/DAC synthetase incubation mixtures were free from interference by other spurious ^{13}C-resonances. On the basis of these two points it was decided that the introduction of a ^{13}C-label into the β-position of valine would permit conversion of LLD-ACV (12) to an isodehydrovaline-containing peptide able to be monitored by ^{13}C-n.m.r even at the low levels of (112) production that were expected.

Having focused attention onto (112), new objectives for the investigation were thought to be appropriate ;

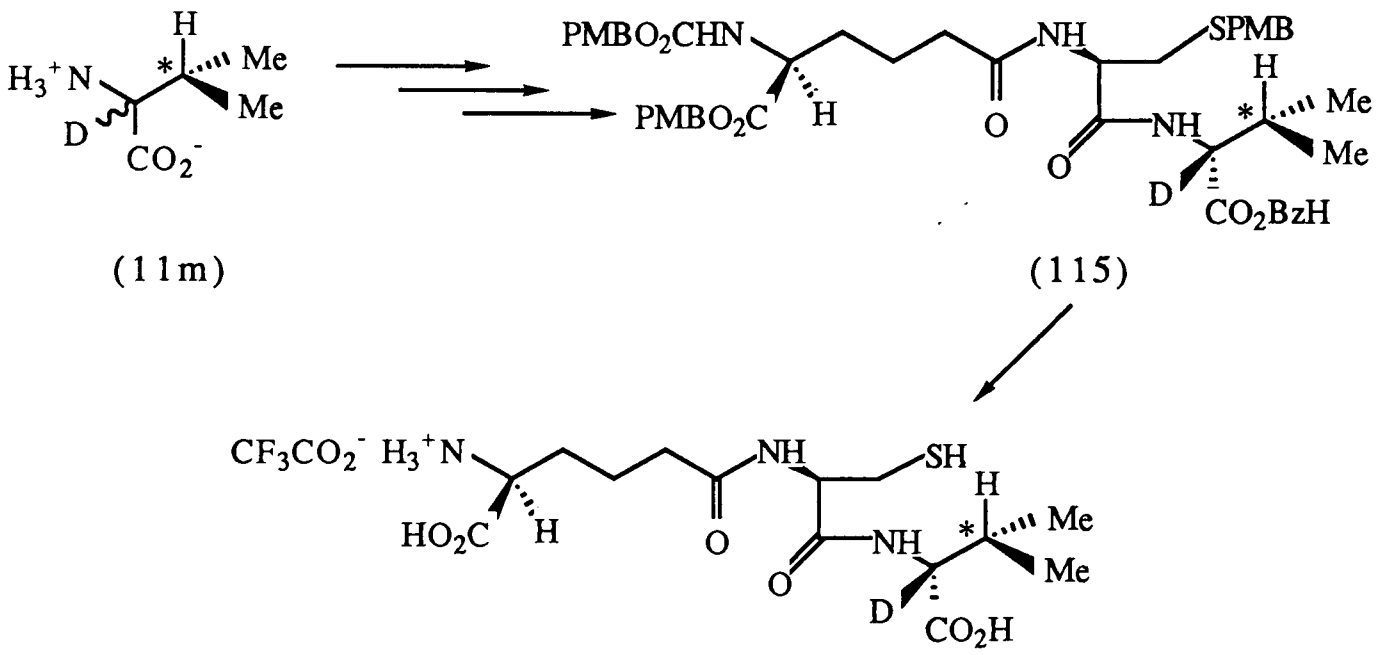
i) to investigate the possible role of LLD-ACV (12a) and LLD-ASV (110) as precursors to (112), and

ii) to determine whether IPNS and DAOC/DAC synthetase enzymes are involved in the biosynthesis of (112).

6.3 Synthesis of Substrates.

Synthesis of (2-^{2}H, 3-^{13}C)-valine (11m) has already been described (Chapter 2, Section 2.3). This racemic amino acid was protected and coupled to *p*-methoxybenzyl protected LL-AC (64b) with EEDQ[65] as previously described to give the protected LLD- and LLL-AC-(2-^{2}H, 3-^{13}C)-V diastereomers. Chromatographic purification of the desired LLD- isomer concurrent with the separation of the unwanted diastereomer and subsequent deprotection with refluxing TFA containing anisole (5:1, v/v) gave (12h) as the ammonium trifluoroacetate salt (Scheme 6.4.).

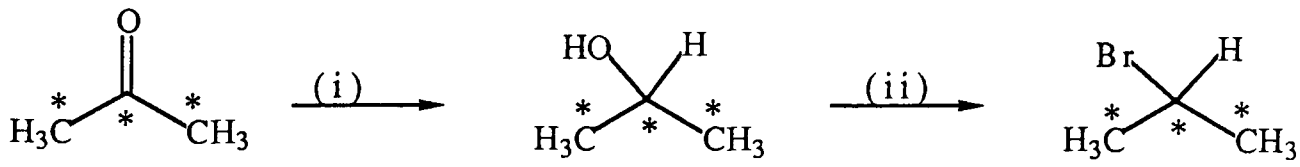
Promising results concerned with the enzymatic production of the isodehydrovaline-containing tripeptide (112) were obtained with (12h) (see Section 6.5) and in an attempt to confirm these inital findings, synthesis of the tri-^{13}C-labelled valine (11ℓ) was undertaken. This amino acid was prepared by lithium



* = ^{13}C

Scheme 6.4.

aluminium hydride reduction of the (1,2,3-$^{13}C_3$)-propanone (87c) (>99 atom% ^{13}C, supplied by Merck Sharpe and Dohme Isotopes Ltd). The resulting alcohol (88c) was separated from the reaction mixture by distillation and then treated with phosphorus tribromide to give (1,2,3-$^{13}C_3$)-2-bromopropane (89c) (see Figure 6.1.) which was again isolated by distillation (Scheme 6.5.).



(87c) (88c) (89c)

* = ^{13}C

Reagents : i) $LiAlH_4$, diglyme, 0°C ; ii) PBr_3 , 23°C, 24 hours

Scheme 6.5.

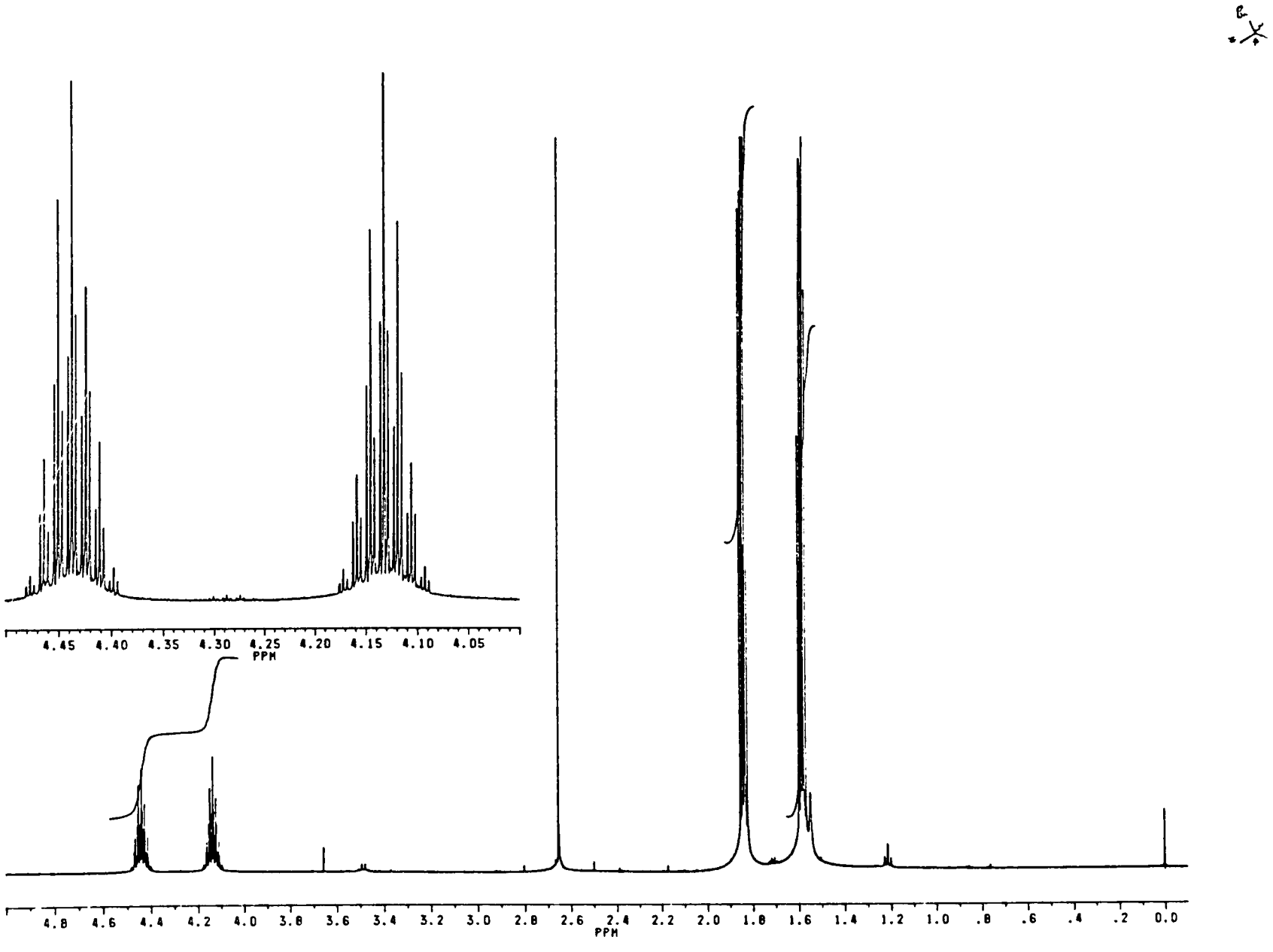
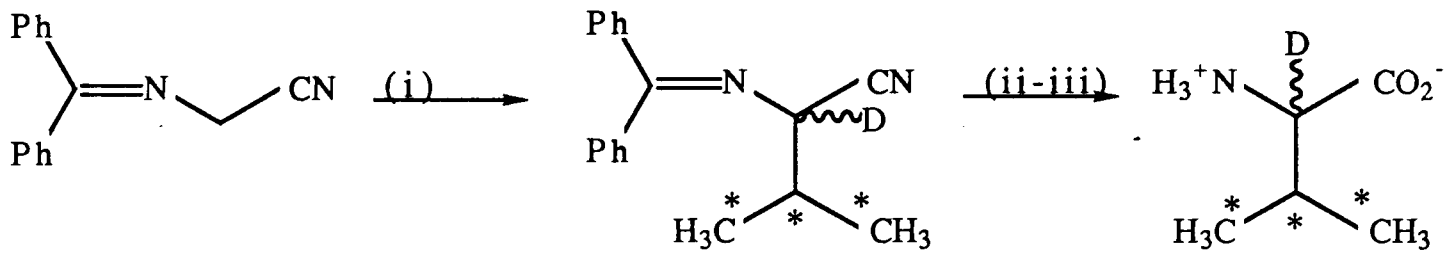


Figure 6.1. ^{1}H-n.m.r. (500MHz, $CDCl_3$) of 2-Bromo-(1,2,3-$^{13}C_3$)-propane (89c).

Although introduction of an α-deuterium atom was not essential for the studies on the isodehydrovaline tripeptide, deuteration at an early stage of the synthesis was trivial and would permit use of this amino acid in alternative biosynthetic investigations. In addition, deuteration at this position was not expected to interfere with the peptide studies (*i.e.* no isotope effect was anticipated for this position and hence formation of the isodehydrovaline-containing peptide (112) should not be retarded), and consequently was included in the synthetic route (Scheme 6.6). Alkylation of the imine (59) with the labelled bromide (89c) under the usual phase transfer conditions with sodium deuteroxide as the base, gave the valine equivalent (60e) (Scheme 6.6.). Initial hydrolysis of (60e) with dilute hydrochloric acid and then refluxing 6N HCl gave the labelled valine (11ℓ).



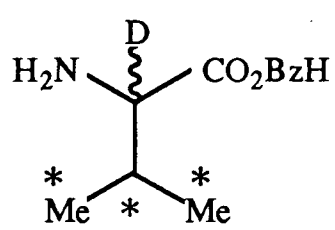
(59) (60e) (11ℓ)

* = ^{13}C

Reagents : i) 50% NaOD, (89c), BTEAC, Toluene; ii) 1N HCl, 23°C, 12 hours ; iii) 6N HCl, reflux, 12 hours.

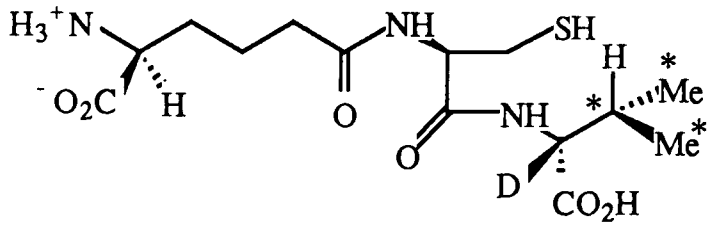
Scheme 6.6.

The isotopic content of this amino-acid was examined by mass spectrometry and ^{1}H-n.m.r.(see Experimental) and shown to contain at least 98 atom % ^{13}C and that greater than 98% deuterium incorporation had been achieved. Subsequent protection to give (2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (63f), as previously described and coupling to *p*-methoxybenzyl protected LL-AC (64b) in the usual manner gave a diastereomeric mixture of LLD- and LLL-tripeptides.



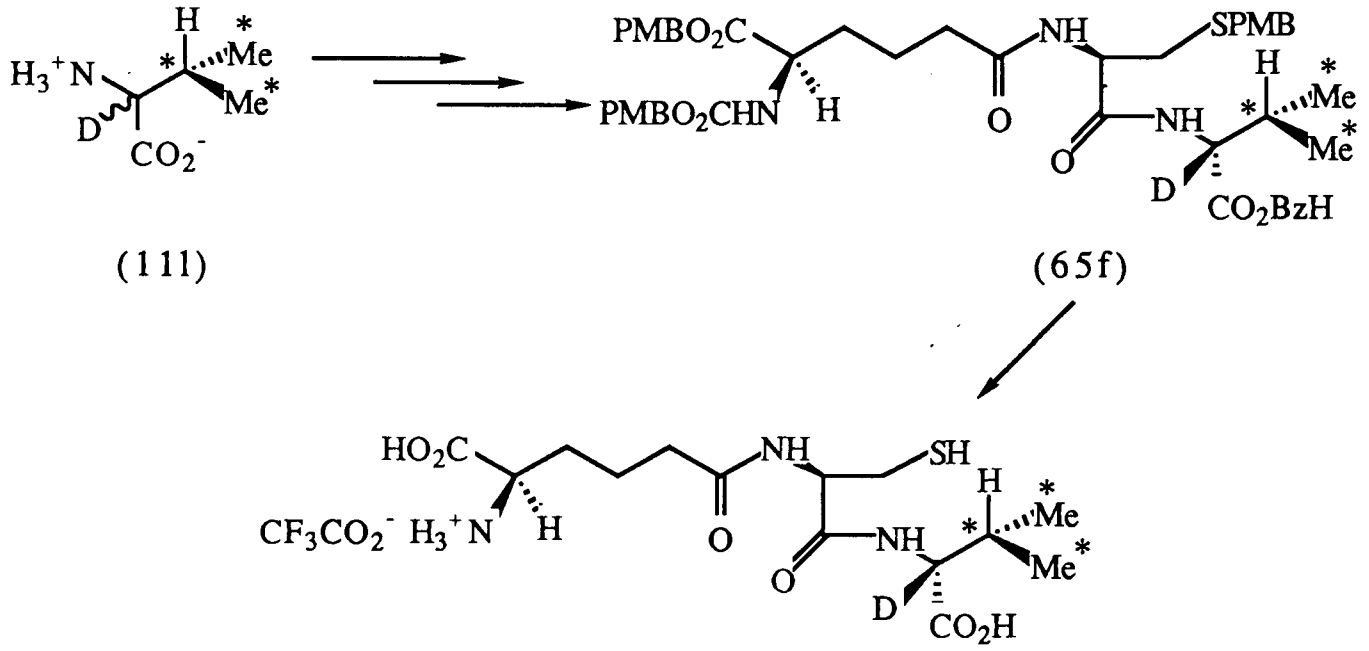
(63f)

Chromatographic purification of the desired LLD-isomer and concurrent separation of the unwanted diastereomer followed by deprotection with refluxing TFA containing 20% anisole, gave LLD-AC-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (12i) .



(12i) *=^{13}C

The corresponding DLD-AC (2-^{2}H, 3,4,4'-$^{13}C_3$)-V (66f) was also prepared as for the protected LLD-isomer (12i) but with acid labile protected DL-AC (64) (Scheme 6.7.).



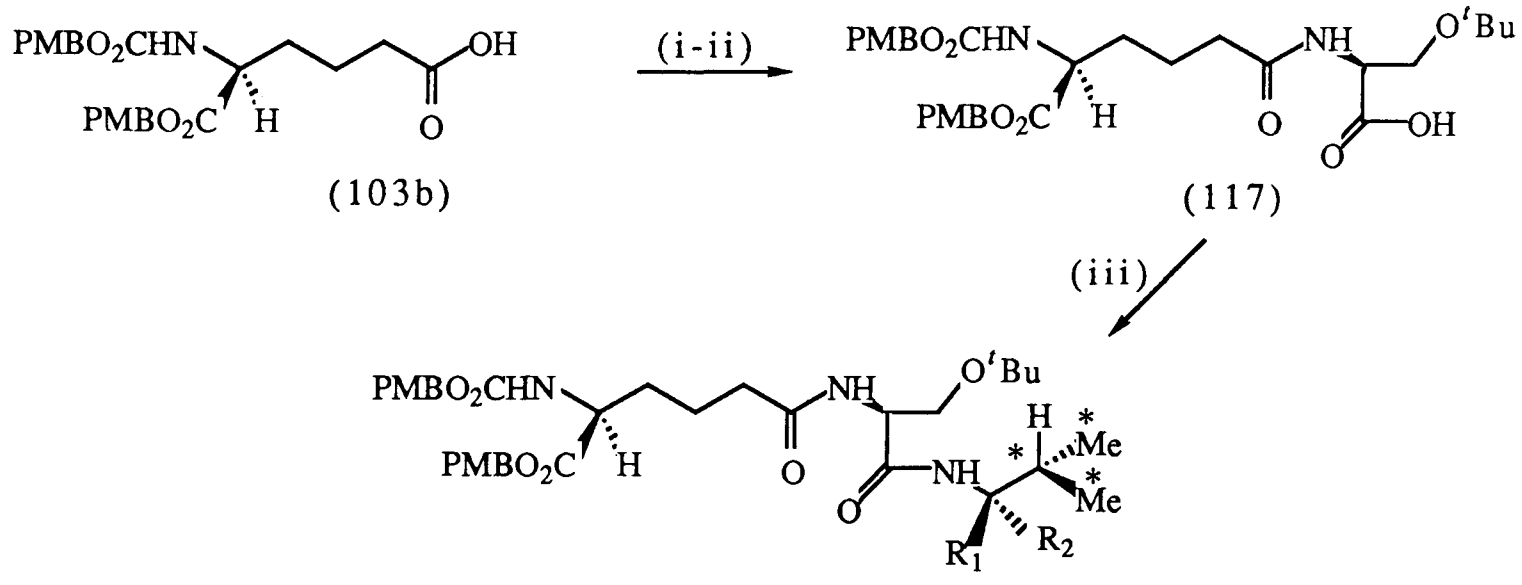
(111) (65f)

(66f)

* = ^{13}C

Scheme 6.7.

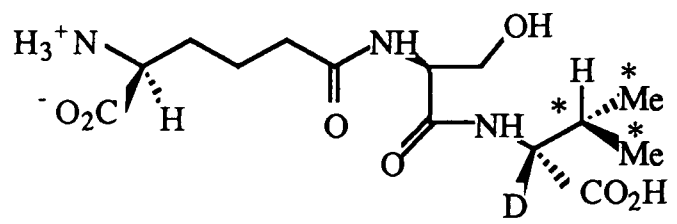
Preparation of LLD-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (111b) was undertaken to test the theory that this tripeptide was the precursor of the isodehydrovaline tripeptide (112). Thus, acid labile protected L-α-aminoadipic acid (103b) was coupled to serine-O-*t*-butyl ether *via* formation of the reactive mixed anhydride by treatment with isobutylchloroformate[120] and interception of this anhydride by the amino-group of serine-O-*t*-butyl ether. The resulting dipeptide (117) was then coupled with EEDQ to suitably protected (2-^{2}H, 3,4,4'-$^{13}C_3$)valine (111) to give protected LLD- and LLL-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (118b/c) (Scheme 6.8.).



Reagents : (i) Isobutylchloroformate, THF, Et_3N, 0°C ; (ii) L-Serine-O-*t*-butyl ether, Et_3N, 23°C ; (iii) (63f), EEDQ, Na_2SO_4, DCM, 23°C, 24 hours.

Scheme 6.8.

Attempted separation of these diastereomers by chromatography [flash column and preparative layer SiO_2] was unsuccessful. Loss of material occurred during this attempted purification which was attributed to the SiO_2 catalysed removal of the O-*t*-butyl ether group and consequently the mixture of diastereomers was deprotected with refluxing TFA/anisole (5:1, v/v) to give LLD-/LLL-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (111b/c) and separation by reverse phase hplc examined. Again separation was unsuccessful.

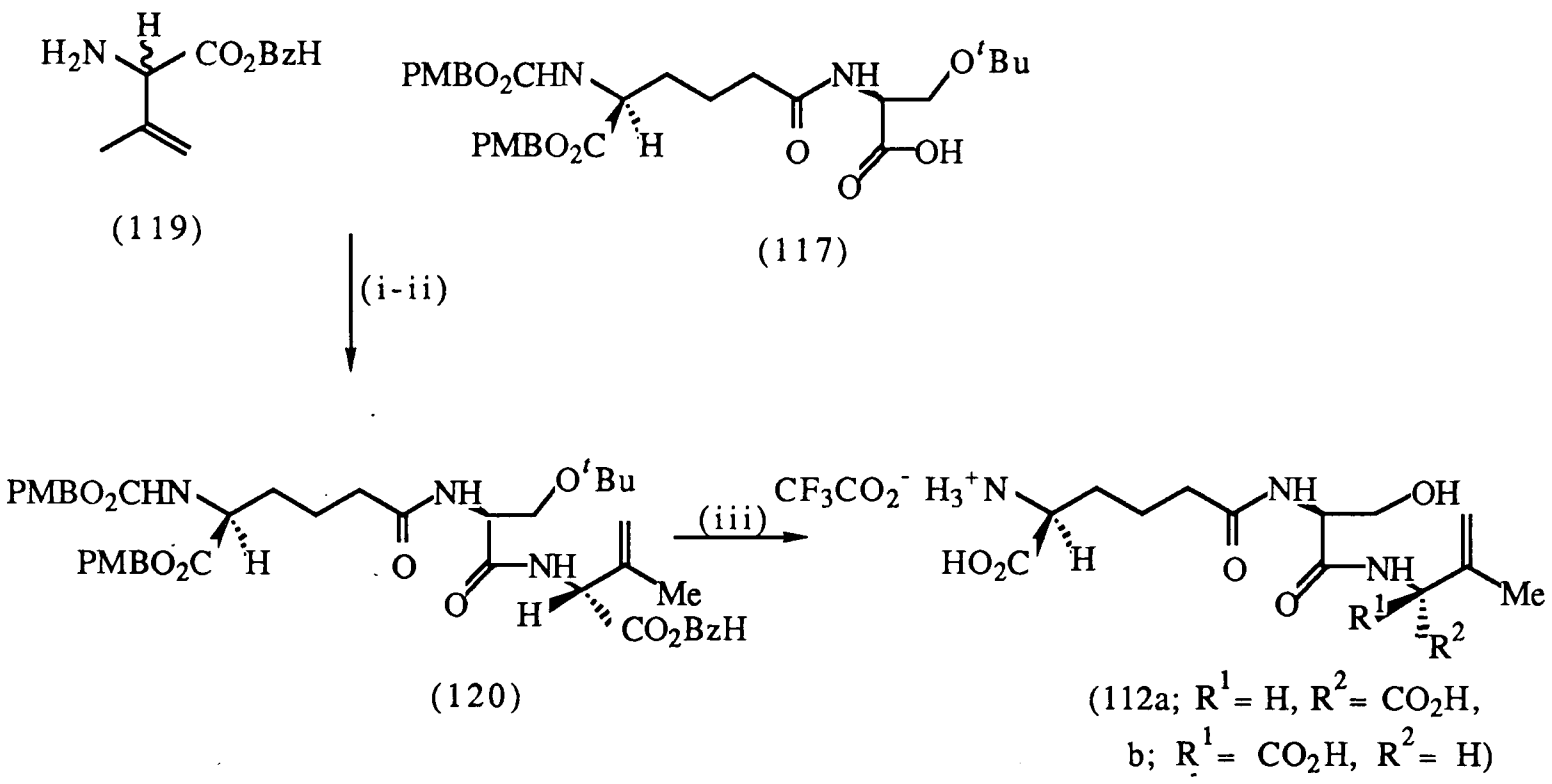


(111b)

6.4 Synthesis of Authentic Standards.

Syntheses of (112) and (106) were undertaken so that the spectral and physical properties, (*i.e* hplc retention time) of these compounds could be determined for use as authentic standards in this investigation. Thus, racemic isodehydrovaline[121] was protected as its ammonium tosylate, benzhydryl ester and the free amine (119) generated by ethyl acetate extraction from saturated sodium bicarbonate solution. The free amine was then coupled as previously described to

acid labile protected LL-AS (117) with EEDQ (Scheme 6.9.) to give the LLD- and LLL-diastereomers (112a) and (112b) respectively.



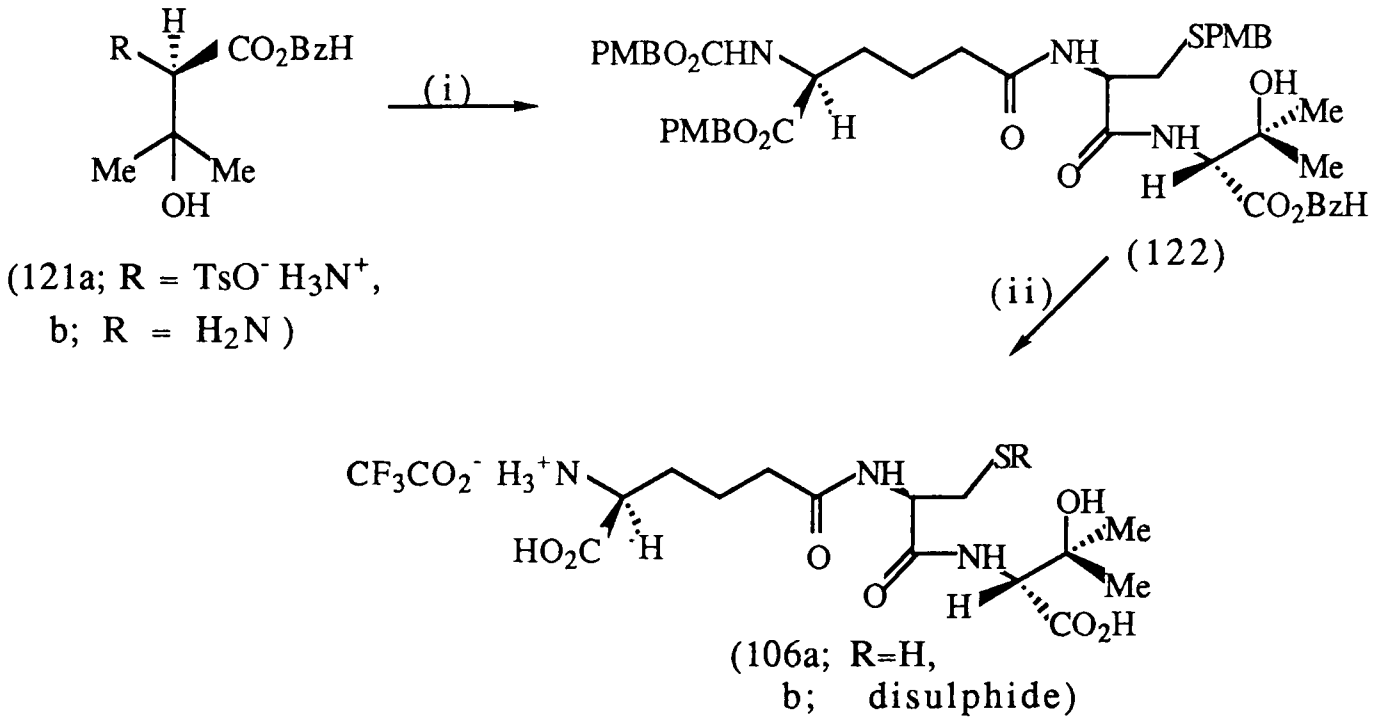
Reagents : (i) EEDQ, DCM, Na_2SO_4 , 23°C, 24 hours ; (ii) Chromatography (SiO_2); (iii) TFA/anisole (5:1, v/v), reflux, 30 minutes.

Scheme 6.9.

Chromatographic comparison of these diastereomers with an authentic sample of protected LLL-AS-isodehydrovaline (prepared as described above but with optically pure L-isodehydrovaline, benzhydryl ester) - both by co-spot and individual analysis - indicated that the less polar product was the required isomer. The diastereomers were then separated [preparative plate SiO_2] and the required tripeptide deprotected by the usual procedure (Scheme 6.10) to give (112) as the ammonium trifluoroacetate salt. As was noted with the other *t*-butyloxyserine-containing tripeptide (118), some loss of material occurred during chromatography on silica.

Examination of this tripeptide by ^{13}C-n.m.r spectroscopy indicated that the terminal and quaternary olefinic carbon atoms gave rise to signals at δ 115.3 and *ca* 142.0-143.0 ppm respectively, with the latter resonance showing an appreciable pH dependence.

Synthesis of the β-hydroxyvaline containing tripeptide (106) was as described for (66f) except that D-β-hydroxyvaline benzhydryl ester (121), prepared by the usual procedure from D-β-hydroxyvaline[122] , was used in place of valine (Scheme 6.10).



(121a; R = $TsO^- H_3N^+$,
b; R = H_2N)

(106a; R=H,
b; disulphide)

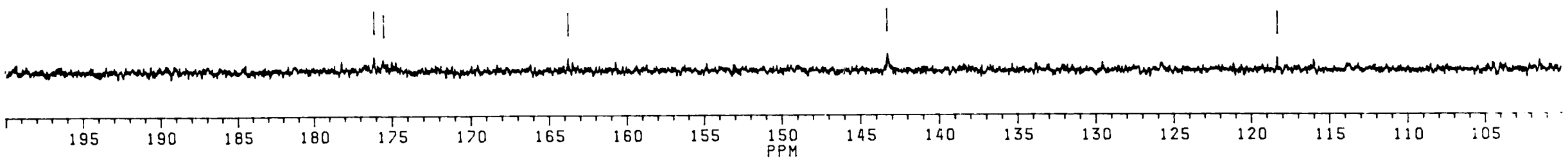
Reagents : (i) (64b), EEDQ, DCM, Na_2SO_4 , 23°C, 24 hours ;
(ii) TFA/anisole (5:1, v/v), reflux, 30 minutes.

Scheme 6.10.

Deprotection in the usual manner gave a mixture of the thiol and disulphide forms (106a/b) which were separated by hplc [Waters' system, 5% MeOH in 10 mM aqueous NH_4HCO_3]. ^{13}C-n.m.r spectroscopy indicated that the β-carbon resonance occurred at δ 72.70 and 72.59 ppm in the thiol and disulphide forms respectively.

6.5 Incubation of LLD-AC-(2-^{2}H, 3-^{13}C)-valine (12h) with IPNS.

Incubation of (12h) (*ca* 6 mg) with IPNS (*ca* 11 I.U.) was performed and the total crude incubation mixture examined by 500 MHz ^{1}H-n.m.r and 125.77 MHz ^{13}C-n.m.r. The proton n.m.r spectrum revealed good conversion to (2-^{13}C, 3-^{2}H)-isopenicillin N (13f) as judged by strong β-lactam resonances at δ 5.05 and 5.48 ppm (Scheme 6.11) but no olefinic signals in the region δ 6.0-4.8 ppm were apparent. Examination of the 125.77 MHz ^{13}C-n.m.r spectrum after acquisition overnight, revealed a weak signal at δ 143 ppm consistent with the quaternary olefinic carbon resonance of (112). Incubation of this substrate was repeated on a larger scale and again, examination by ^{13}C-n.m.r revealed a signal at δ 142 ppm (see Figure 6.2).



(b) ^{13}C-N.m.r Spectrum of LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) Incubation with IPNS.

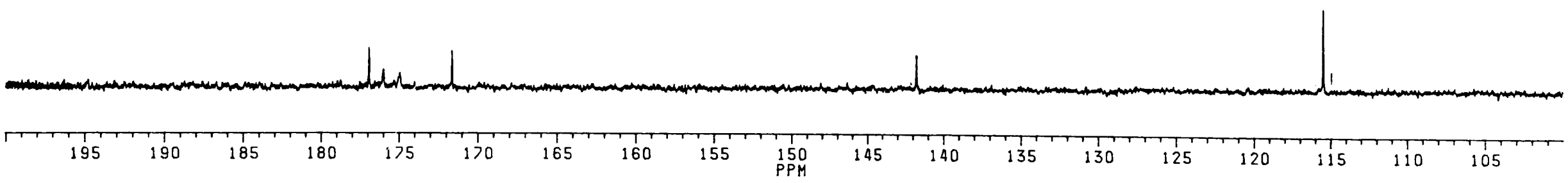
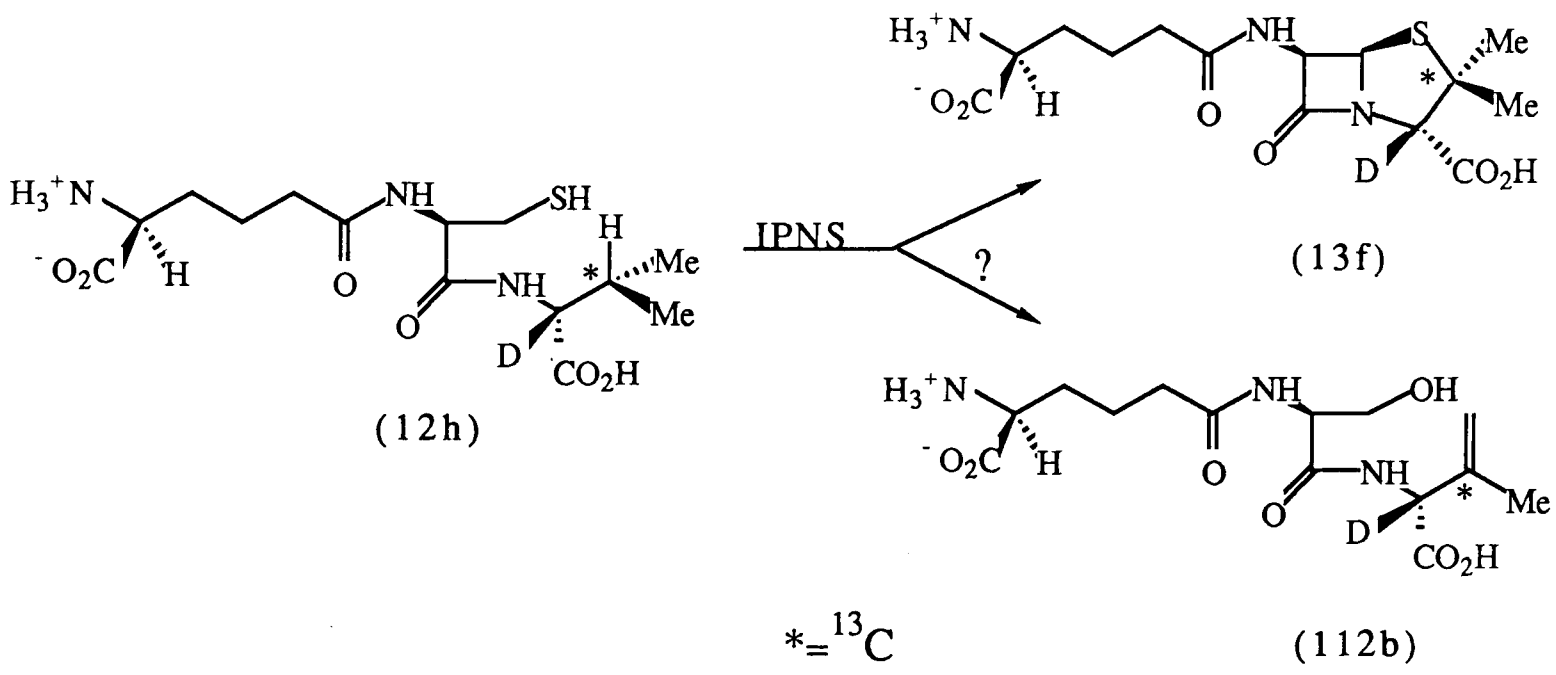


Figure 6.2. (a) Low field Region of ^{13}C-n.m.r Spectrum of Authentic LLD-ASdV (112a).



Scheme 6.11.

6.6 Control Experiment : Incubation of LLD-ACV (12a) with IPNS.

In an attempt to confirm that the δ 142 ppm signal observed in the ^{13}C-n.m.r spectrum of the LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) incubation was derived from the ^{13}C-label, incubation of unlabelled LLD-ACV (12a) was performed. To represent a reliable control, this incubation had to be performed under exactly the same conditions as those used in the first labelled substrate experiment. Thus exactly the same amount of substrate (6 mg) and the same quantity of cofactors were incubated with the same number of international units of the same enzyme batch. In addition, the same total incubation volume was used and the enzymatic conversion allowed to proceed for the same length of time at 27°C. The resulting crude incubation mixture was examined by ^{1}H-n.m.r and ^{13}C-n.m.r spectroscopy, which again showed good conversion to isopenicillin N (13a). No olefinic signals could be seen in the region δ 6.0-4.8 ppm of the proton spectrum. Examination of the ^{13}C-n.m.r spectrum (125.77 MHz), acquired overnight as before, revealed no resonances in the olefinic region δ 150-110 ppm (see Figure 6.3).

If conversion to the isodehydrovaline-containing peptide occurred with the unlabelled LLD-ACV (12a), no signal at δ 142 ppm should be observed since the low concentration of product in the incubation mixture would render observation by ^{13}C-n.m.r at 125.77 MHz impossible due to lack of sensitivity. However, observation at such a low concentration can be achieved by the introduction of a ^{13}C-label, since labelling effectively increases the sensitivity of detection by a factor of 90 (the natural abundance of the ^{13}C isotope being 1% that of the ^{12}C isotope and the level of label incorporation in this substrate was 90%, leading to a

90 fold increase in sensitivity). If the signal observed at δ 142 ppm in the labelled substrate incubation was due to a cofactor or an enzyme degradation product, then a similar resonance would be expected to be seen in the unlabelled substrate experiment. Since the two incubations differed only in the fact that one of the substrates contained a ^{13}C-label in the position expected to become the quaternary olefinic carbon atom of (112), the absence of an olefinic resonance in the control incubation supports the hypothesis that the isodehydrovaline containing peptide (112) arises from the action of IPNS upon LLD-ACV (12a).

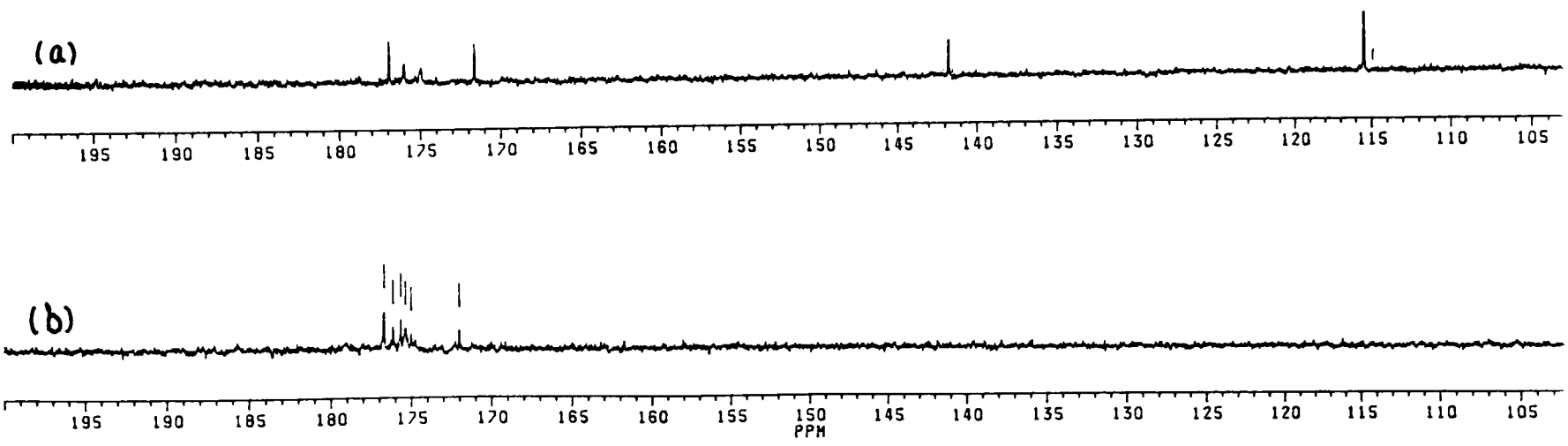


Figure 6.3. (a) Low field Region of ^{13}C-n.m.r Spectrum of Authentic LLD-ASdV (112a).
(b) ^{13}C-N.m.r Spectrum of LLD-ACV (12a) Incubation with IPNS.

The encouraging results obtained with LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) were not considered conclusive proof that LLD-ACV (12a) was converted to LLD-ASdV (112). Unambiguous proof could only be obtained by actual isolation of labelled LLD-AS-(2-^{2}H, 3-^{13}C)-dV(112b) from the crude IPNS incubation mixture. In an attempt to achieve this, the labelled LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) incubation mixture was subjected to analytical hplc [Waters' system] and all the fractions examined by overnight ^{13}C-n.m.r spectroscopy. The fraction containing the signal at δ142 ppm possessed a short retention time, *c a* 3.5 minutes. Unfortunately, a structural determination on the basis of the proton n.m.r spectrum of this sample proved inconclusive and consequently further purification by hplc was undertaken. However, no fraction was identified from this second hplc purification which gave a ^{13}C-signal at δ142 ppm. The most likely explanation for this was that too little material remained after mechanical loss to be detected by high field ^{13}C-n.m.r spectroscopy.

6.7 (2-^{13}C, 3-^{2}H)-Isopenicillin N (13f) Control Experiment.

The control experiment with unlabelled LLD-ACV (12a), described above did not rule out the possibility that the signal at δ 142 ppm observed in the overnight ^{13}C-n.m.r spectrum of the crude LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) incubation mixture was due to a minor decomposition product of the labelled isopenicillin N (13f). It was possible that due to low levels of formation, detection of such a decomposition product would only be observed when a ^{13}C-label was incorporated into it. Obviously, with the unlabelled control experiment, detection of a decomposition product might not be possible due to the 90 fold decrease in sensitivity caused by the absence of a label.

The only suitable control experiment which could take into consideration this possibility consisted of incubation of a sample of ^{13}C-labelled isopenicillin N, preferably on the same scale as the previous labelled and unlabelled control experiments (*i.e. ca* 6 mg), with an identical quantity of IPNS and co-factors as was used before.

Thus (2-^{13}C, 3-^{2}H)-isopenicillin N (13f) (*ca* 4 mg) (see Figure 6.4) which had

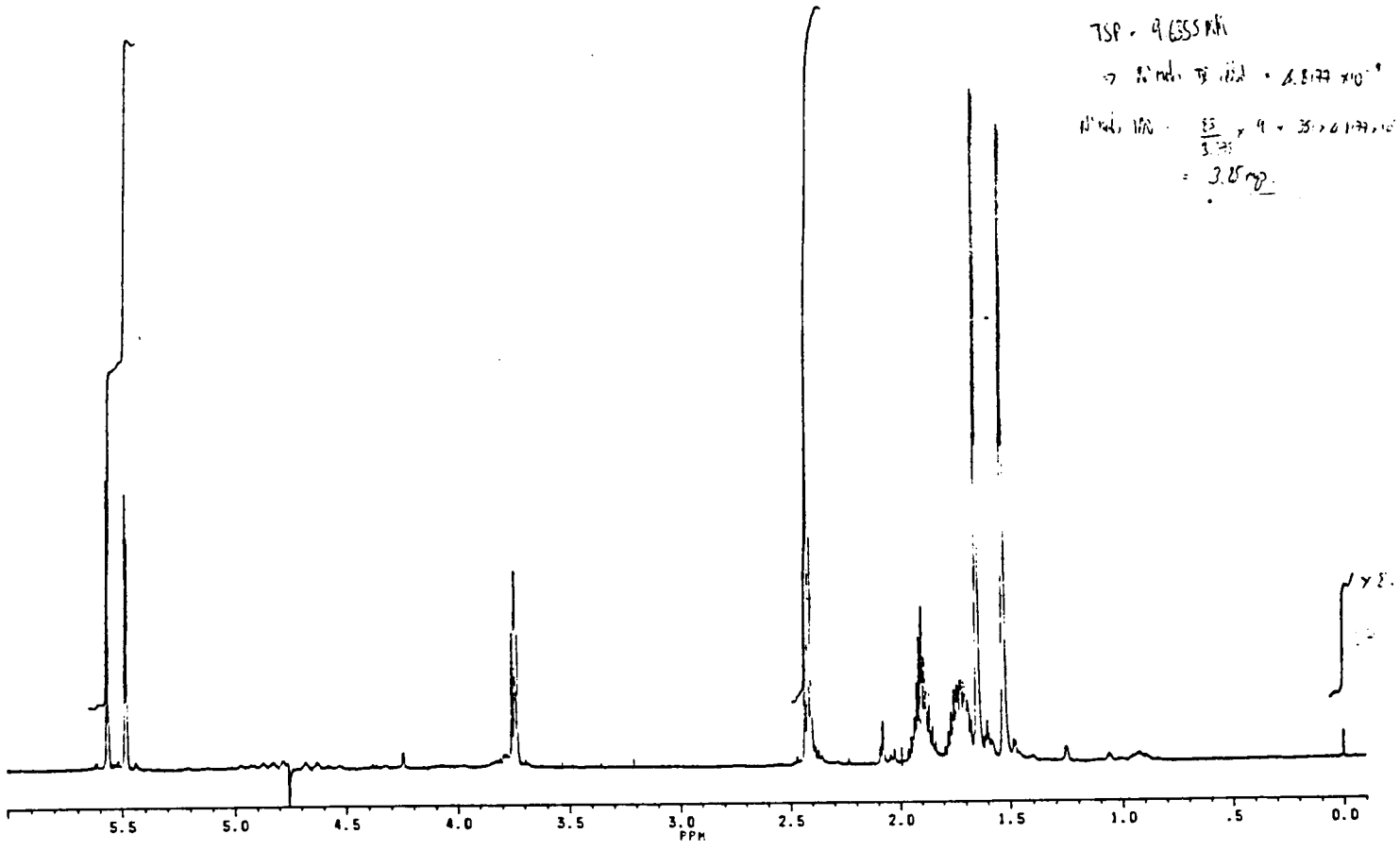
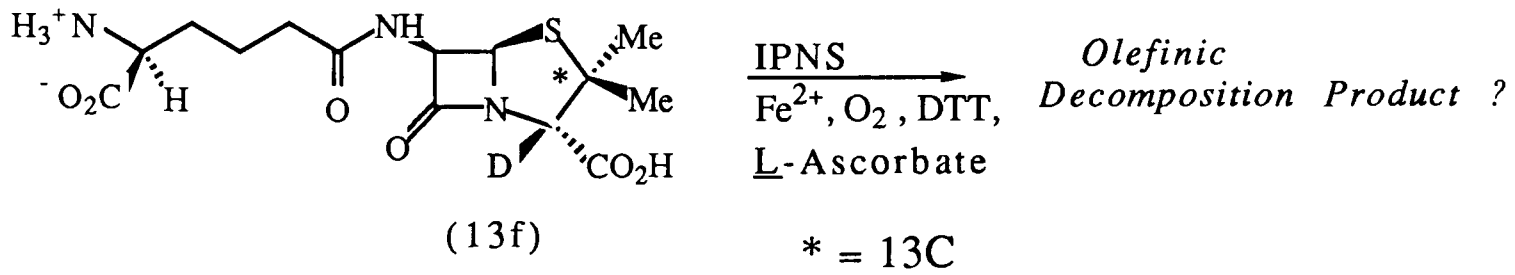


Figure 6.4. ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) of (2-^{13}C, 3-^{2}H)-Isopenicillin N (13f).

previously been isolated from an LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) IPNS incubation, was re-incubated with IPNS (*ca* 11 I.U.) in the presence of the usual co-factors

present at the same concentration that had been used in the previous control experiment (Scheme 6.12.).



(13f)

* = 13C

Scheme 6.12.

Examination of the crude incubation mixture by ^{1}H-n.m.r indicated that the (2-^{13}C, 3-^{2}H)-isopenicillin N (13f) had been degraded during the incubation. However, examination by overnight ^{13}C-n.m.r (125.77 MHz, D_2O) did not reveal any signals in the region δ 170-105 ppm (see Figure 6.5). The absence of ^{13}C-signals in

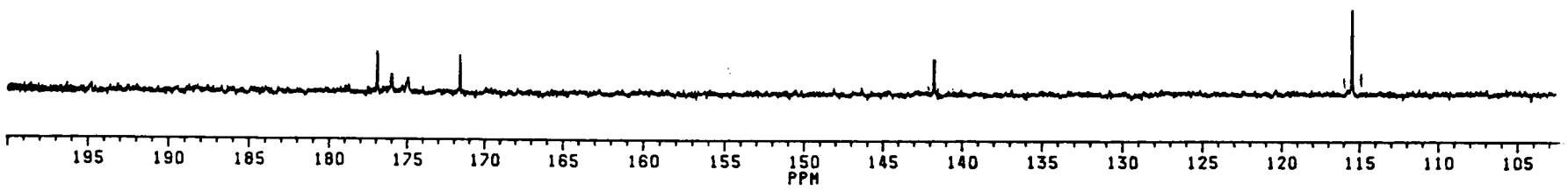
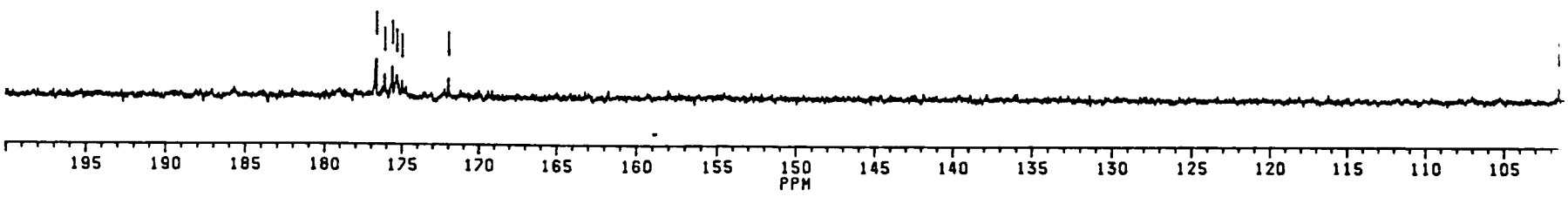


Figure 6.5. (a) Low field Region of ^{13}C-n.m.r Spectrum of Authentic LLD-ASdV (112a).



(b) ^{13}C-n.m.r. (125.77MHz, D_2O) of (2-^{13}C, 3-^{2}H)-Isopenicillin N (13f) Incubation with IPNS.

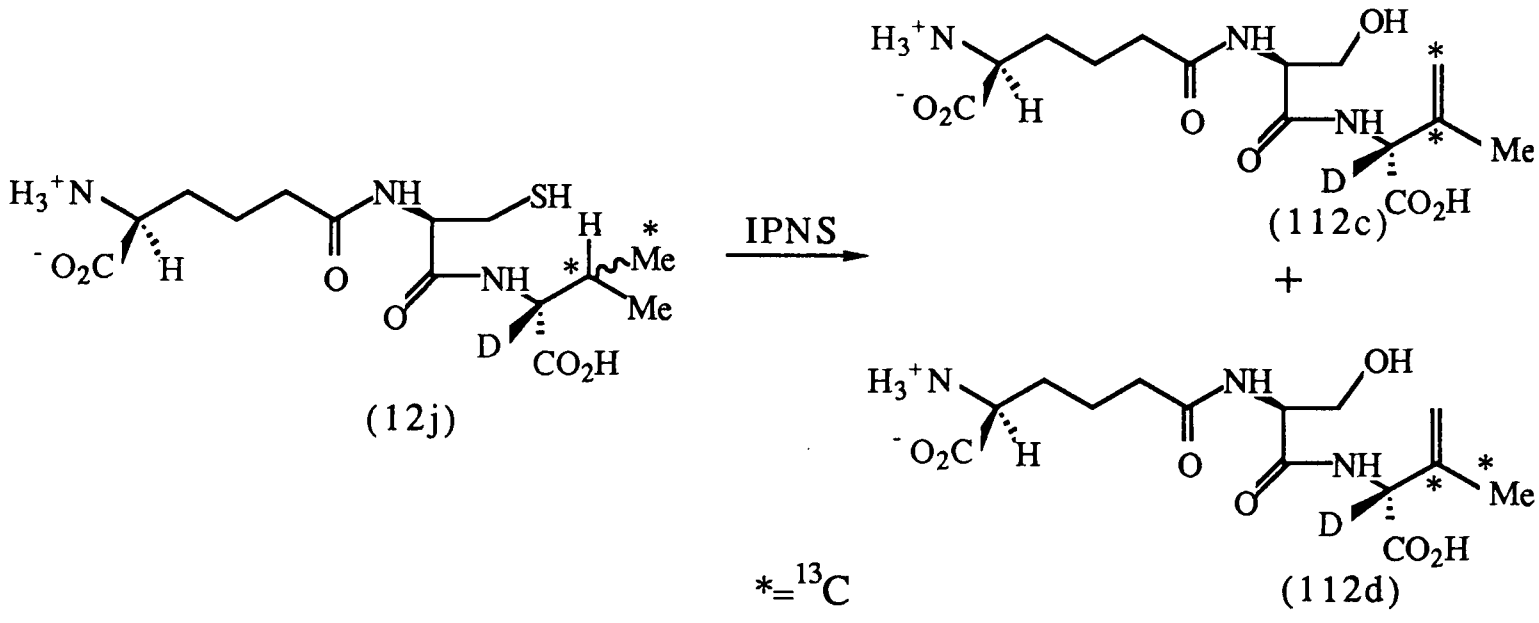
this region suggests that the resonance at δ 142 ppm is not due to an isopenicillin N (13) decomposition product, thus supporting the hypothesis that the isodehydrovaline containing peptide (112) arises from the action of IPNS upon LLD-ACV (12a).

6.8 Studies with Tri-^{13}C-labelled Tripeptides.

6.8.1 Incubation of LLD-AC-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (12i) with IPNS.

Isolation of the product responsible for the ^{13}C-signal at δ 142 ppm, as described above, was considered impractical and an alternative approach was needed to clarify the promising results obtained with (12h). Use of multiply ^{13}C-labelled valine was considered most appropriate since the ^{13}C - ^{13}C couplings expected for the olefinic ^{13}C-resonances of the isodehydrovaline-containing tripeptide (112) should be characteristic.[119] Observation of such coupling would confirm the formation of (112) from LLD-AC-(2-^{2}H, 3,4,4,-$^{13}C_3$)-V (12i).

The presence of three ^{13}C-atoms in (12i) represented both an advantage and a disadvantage. Three labels are required so that all the isodehydrovaline formed will be labelled in both olefinic positions. Had LLD-AC-[(3RS)-2-^{2}H, 3,4-$^{13}C_2$)]-V (12j) been used, then statistically only half the molecules converted to LLD-ASdV (112) would exhibit olefinic ^{13}C - ^{13}C coupling (*i.e* only compound (112c) will give rise to olefinic ^{13}C - ^{13}C coupling) (Scheme 6.13.), effectively reducing the sensitivity of the experiment by a factor of two. This was considered impractical because it was felt that the investigation was already close to the limits of detection available by ^{13}C-n.m.r at 125.77 MHz.

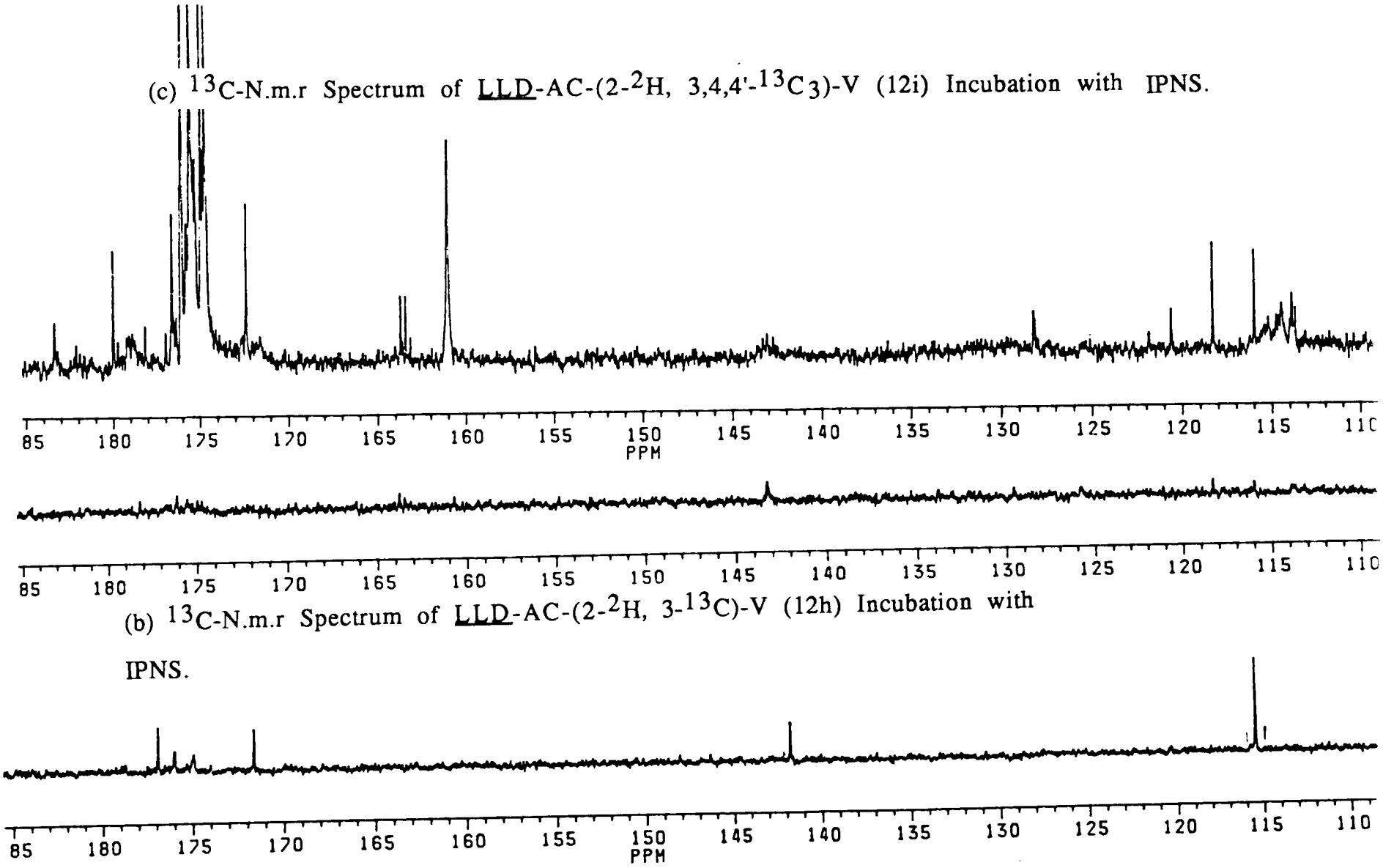


Scheme 6.13.

The ^{13}C - ^{13}C coupling, although essential to the investigation, leads to an effective decrease in intensity of the olefinic signals since the terminal and quaternary olefinic carbon resonances will now be split into a doublet and double-doublet respectively. From the ^{13}C-n.m.r spectrum of the authentic standard of (112), the ratio of the intensities of the terminal and quaternary resonances was

determined to be *ca* 2.5 :1. This means that an experiment with the tri-labelled tripeptide (12i), on the same scale as that used for the mono-labelled substrate (12h), should give rise to a doublet at δ 115.3 ppm, the intensity of the two signals being approximately the same as the singlet observed at δ 142 ppm in the initial experiment (Section 6.5.). Furthermore, by similar reasoning, the quaternary carbon at δ 142 ppm would become too diffuse to be observable in an ordinary proton decoupled ^{13}C-n.m.r experiment. However, the disadvantages associated with loss of intensity due to coupling could be overcome, in principle, by the use of selective ^{13}C - ^{13}C decouplings.

Large scale incubation of (12i) (*ca* 25 mg) with IPNS (*ca* 90 I.U.) gave good conversion to the corresponding tri-^{13}C-labelled isopenicillin N (13g) (Scheme 6.14.) as judged by ^{1}H-n.m.r. Examination of this incubation mixture by overnight ^{13}C-n.m.r at 125.77 MHz, suggested the presence of multiplets at both δ 142 and 114-115 ppm, an observation consistent with the formation of an isodehydrovaline containing peptide (see Figure 6.6.).

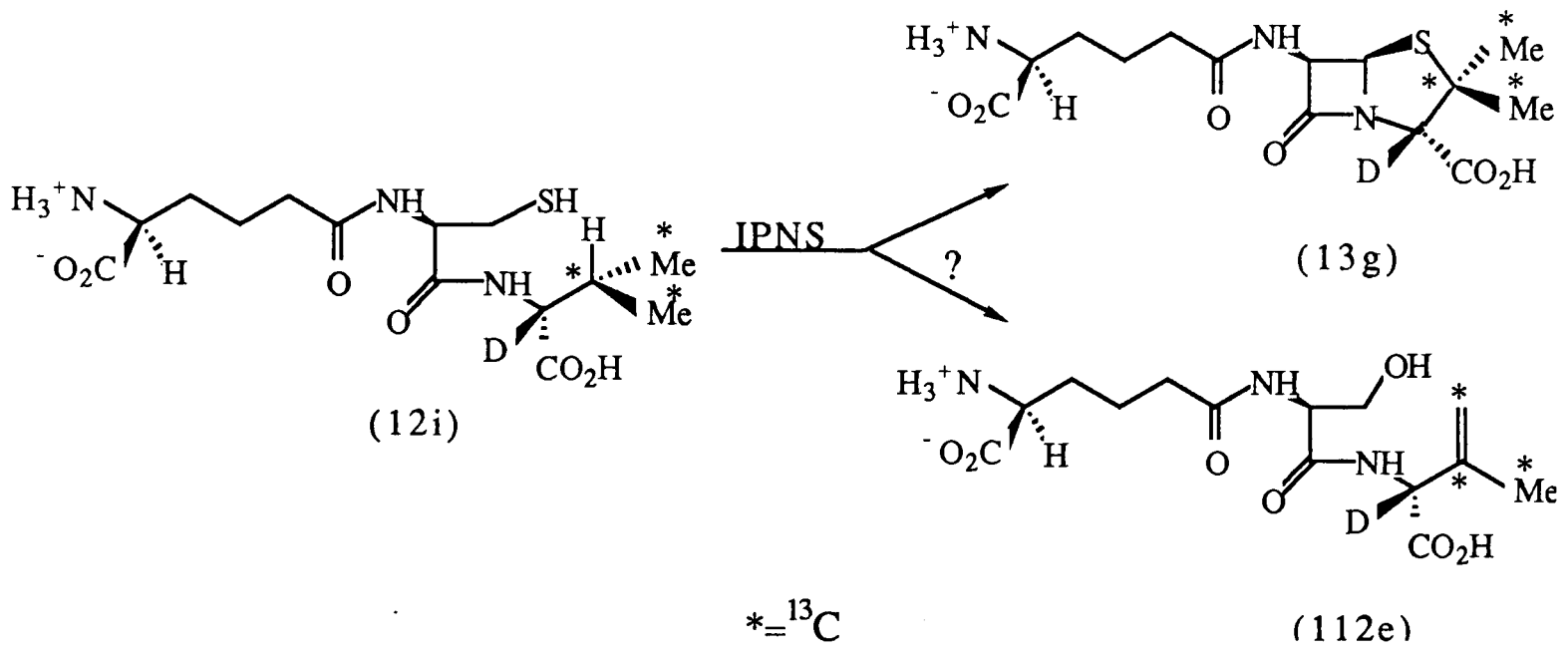


(c) ^{13}C-N.m.r Spectrum of LLD-AC-(2-^{2}H, 3,4,4'-^{13}C$_3$)-V (12i) Incubation with IPNS.

(b) ^{13}C-N.m.r Spectrum of LLD-AC-(2-^{2}H, 3-^{13}C)-V (12h) Incubation with IPNS.

(a) Low field Region of ^{13}C-n.m.r Spectrum of Authentic LLD-ASdV (112a).

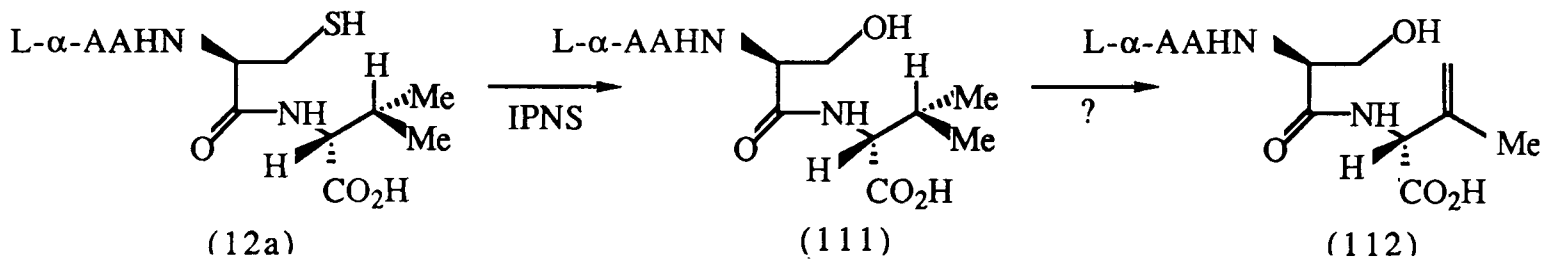
Figure 6.6.



Scheme 6.14.

6.8.2 Studies with LLD-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-Valine (111b) with IPNS.

At the outset of the work described in this section, the possibility that LLD-ACV (12a) was converted to LLD-ASdV (112) *via* the intermediacy of LLD-ASV (111), had not been investigated, although studies upon the conversion of LLD-ASV (111) by IPNS had shown that this compound was not converted to a β-lactam containing product.[123] Both of these compounds had been isolated from the fermentation broth of *P.chrysogenum*,[114] and it seemed logical that the biosynthesis of these peptides was possibly interrelated. If this was true then a scheme such as that below (Scheme 6.15) would conceivably represent the simplest possible relationship.

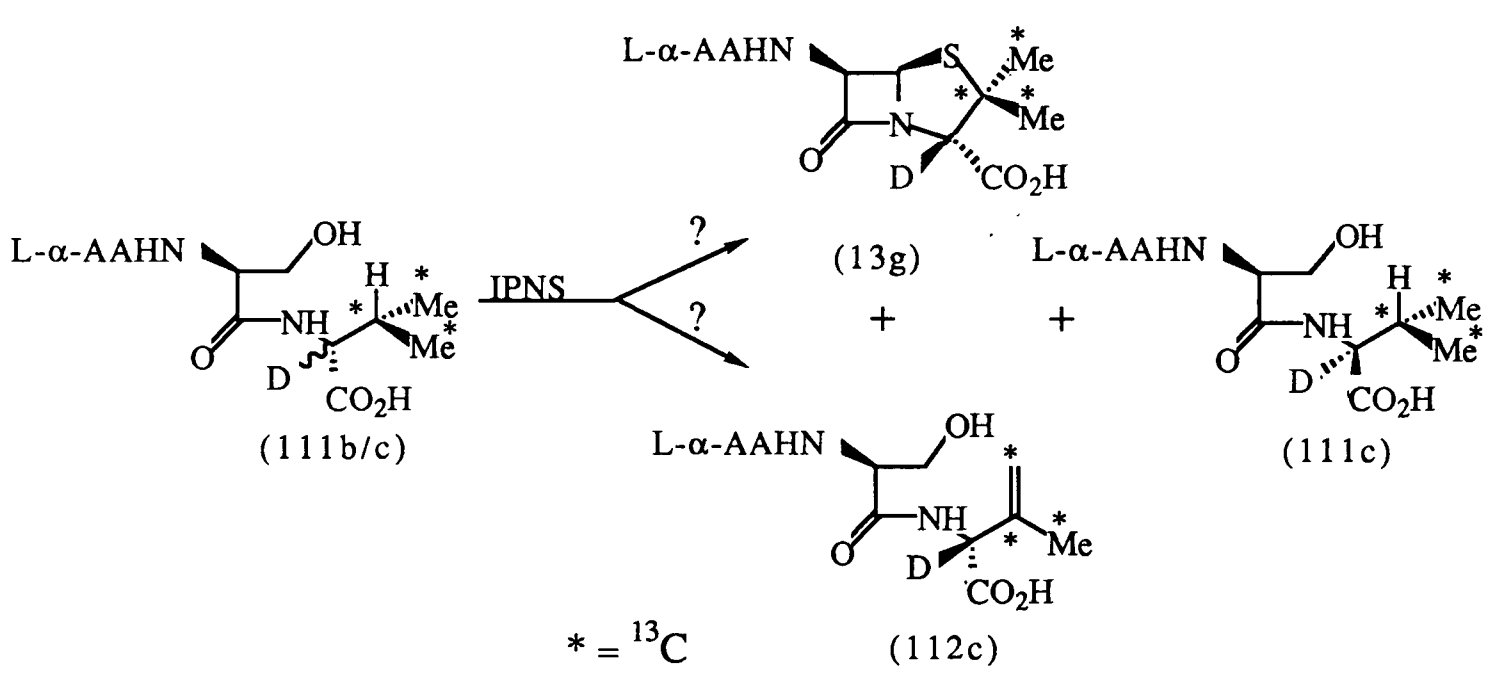


Scheme 6.15.

This hypothesis could most easily be tested by incubation of LLD-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (111b) with IPNS and examination of the crude incubation mixture for the presence of any LLD-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-dV (112c). Several advantages were thought to be associated with this experiment. Firstly, if the relationship described above was correct, then LLD-AS-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (111b) can be considered as a more advanced biosynthetic intermediate to (112). A better conversion of (111) to (112) might then be possibile since initial expenditure of

enzyme activity on the transformation of LLD-ACV (12) to LLD-ASV (111) would be unnecessary. Furthermore, the low levels of LLD-ASdV (112) isolated from the fermentation broths of *P.chrysogenum* could be explained by suggesting that formation of LLD-ASV (111) from (12) was the rate limiting process in the overall formation of LLD-ASdV (112) from LLD-ACV (12). Consequently by removing this step from the reaction sequence enhanced production of (112) might be anticipated.

As described in Section 6.3, racemic synthesis of the serinyl-tri-^{13}C labelled tripeptide (111b/c), could not be concluded with a satisfactory separation of the resulting LLD- and LLL- diastereomers. However, the presence of the other diastereomer in the incubation mixture was considered not to be a disadvantage; consequently a racemic mixture of (111b/c) (*ca* 8 mg) was incubated with IPNS (*ca* 18 I.U.) (Scheme 6.16). Examination of the resulting incubation mixture by ^{1}H-n.m.r. indicated no conversion to β-lactam containing compounds, as judged by the lack of double-doublets in the region δ 5.6-4.8 ppm. In addition, no olefinic

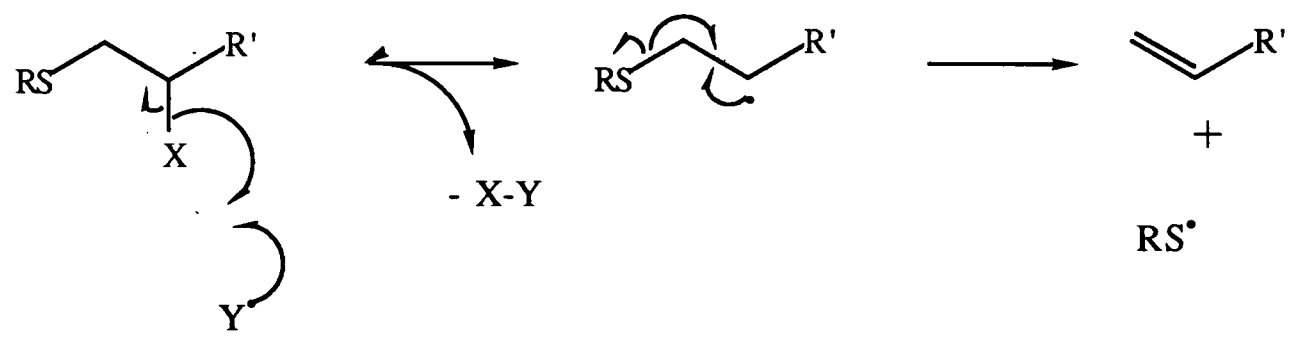


Scheme 6.16.

proton resonances could be identified in the ^{1}H-n.m.r spectrum of the crude incubation mixture. An absence of olefinic carbon resonances between δ 160-105 ppm was also noted in the ^{13}C-n.m.r spectrum of the crude incubation mixture obtained after acquisition overnight. From these results we cannot offer any evidence to support the conversion of LLD-ASV (111) into either a β-lactam containing compound or LLD-ASdV (112) by the action of IPNS.

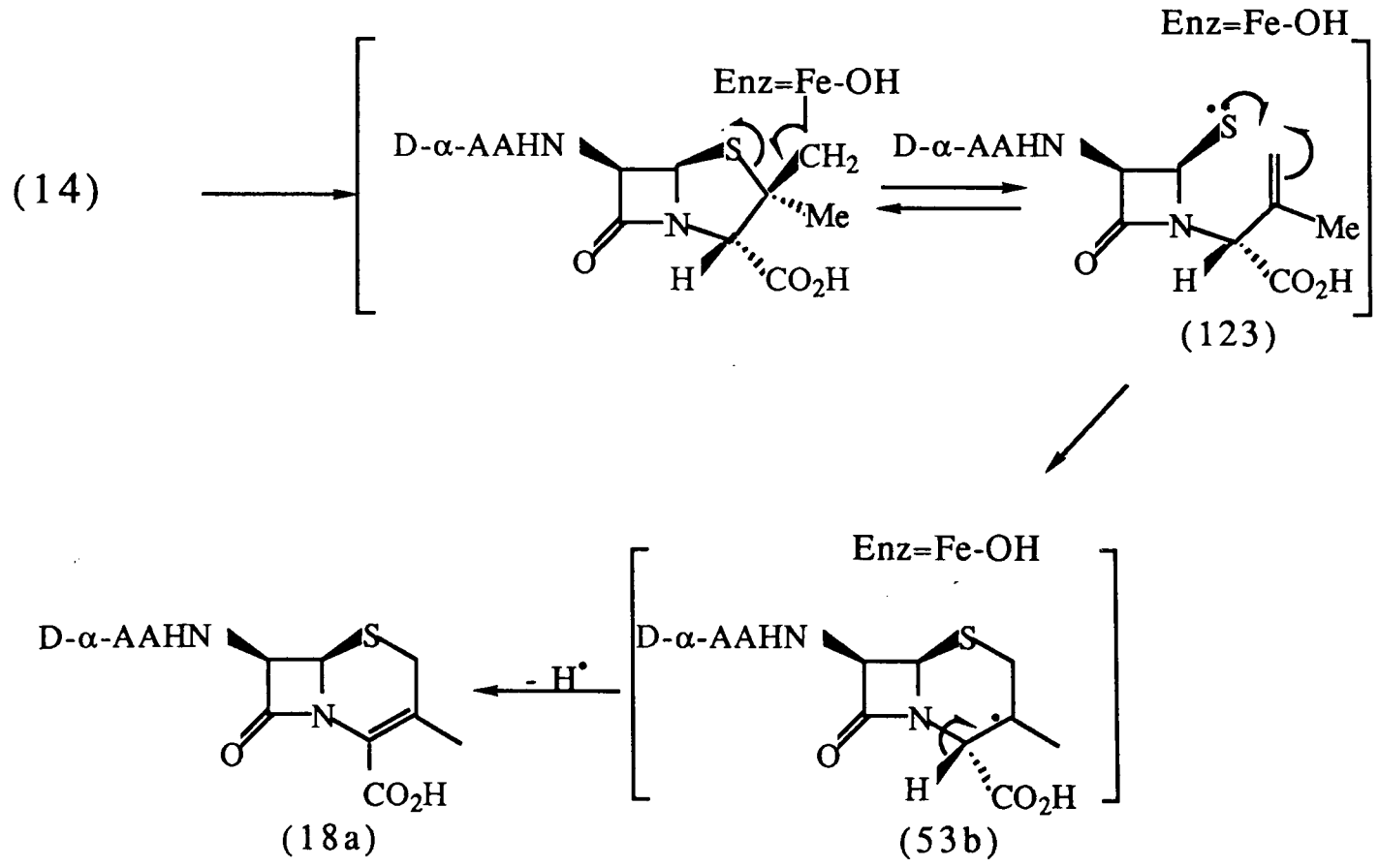
6.8.3 Studies with DLD-AC-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (66f).

Studies on the ring expansion of penicillin N (14) to cephalosporin C (20) with chirally labelled methyl groups had shown that loss of stereochemistry occurred during this transformation (see Chapter 1, Section 1.3).[43,44] This result implied that an intermediate possessing radical type character was involved. It has been reported that formation of a radical β to a sulphide leads to rapid cleavage of the sulphur-carbon bond (Scheme 6.17.).[124]



Scheme 6.17.

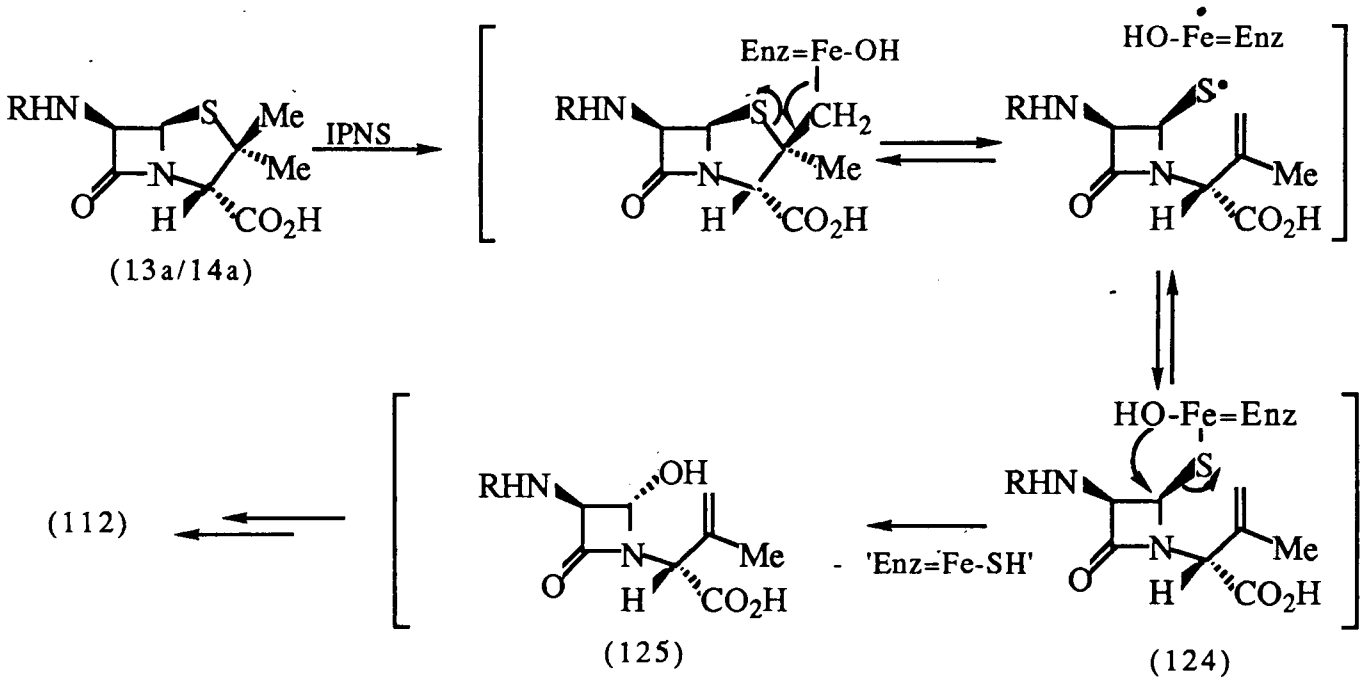
In the case of a strained cyclic sulphide, such as the thiazolidine ring of penicillin N (14), this may be anticipated to be an even more facile process. If correct, this assumption may have important implications for the ring expansion process since it could possibly imply the involvement of a ring opened intermediate such as (123) (Scheme 6.18.).



Scheme 6.18.

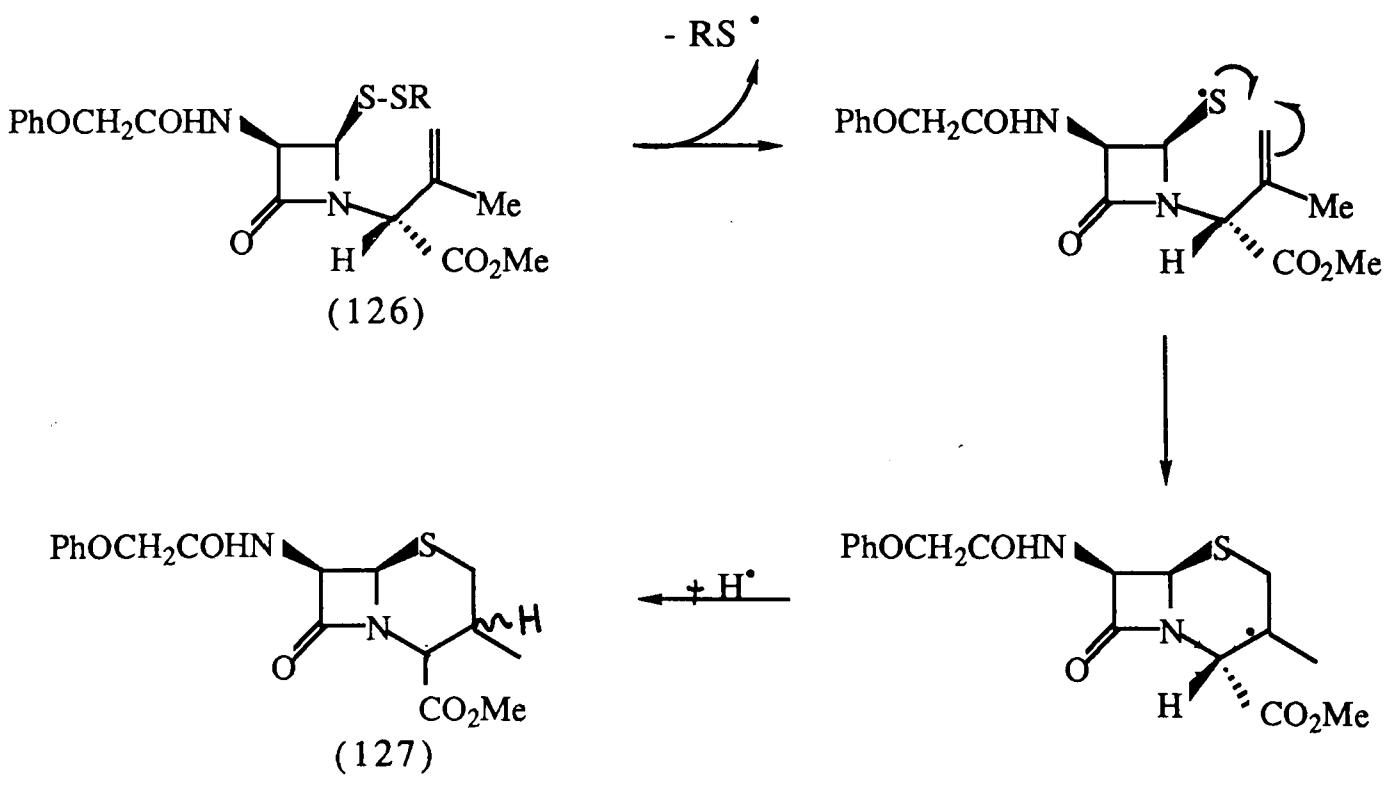
It is conceivable that formation of an isodehydrovaline containing shunt metabolite could occur from such an intermediate. If formation of DLD-ASdV (112e) was to occur in this way, ring opening of the β-lactam ring and subsequent reduction of the aldehyde so produced would be required (Scheme 6.19.).

This proposal may appear unduly speculative, but when considered in conjunction with other results obtained in this laboratory and discussed in the concluding section to this chapter, the intermediacy of (125) within this scheme becomes more conceivable.[86] Formation of the ring expanded product (Scheme 6.18.) or shunt (112) (Scheme 6.19), would depend upon the relative rates of steps



R = D-5-amino-5-carboxypentanoyl

Scheme 6.19.

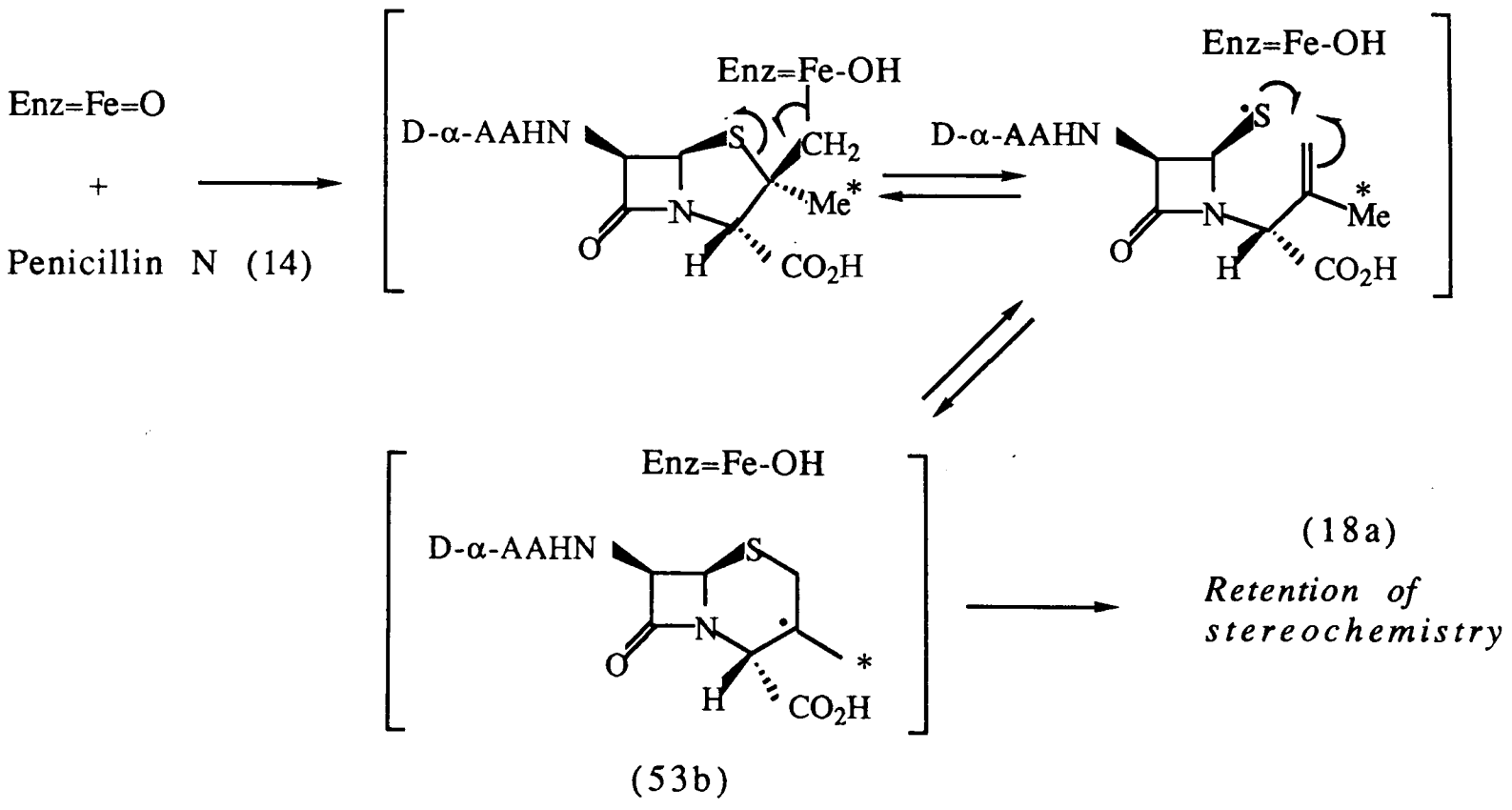


Scheme 6.20.

leading to their formation *i.e* conversion of (123) to (53b) and (124) to (125) respectively. It is of interest to note that production of ring expanded compounds from the isodehydrovaline containing the β-lactam compound (126) (Scheme 6.20) has been demonstrated in biomimetic studies within this department.[96]

Formation of the isodehydrovaline containing peptide (112) *via* a shunt pathway from the intermediate (125) may be influenced by the introduction of a deuterium atom at C-3 of penicillin N (14e), in a manner similar to that observed for the β-hydroxycepham (46b) (see Chapter 2, Section 2.3).[71] The likely effect of deuteration at this critical position would be to increase the lifetime of the intermediate (53b), thereby increasing the possibility of leakage down a shunt pathway. If the ring expansion process did indeed proceed according to the proposal in Scheme 6.18, then the observed stereospecificity of this transformation would need to be taken into account within any proposed mechanistic scheme.[40, 42] This doesn't necessarily present a problem, since the intermediate radical (123) would be expected to react only with the double bond which in turn is formed exclusively from the β-methyl of penicillin N (14). In this way the overall stereospecificity for this process would be assured (Scheme 6.21.).

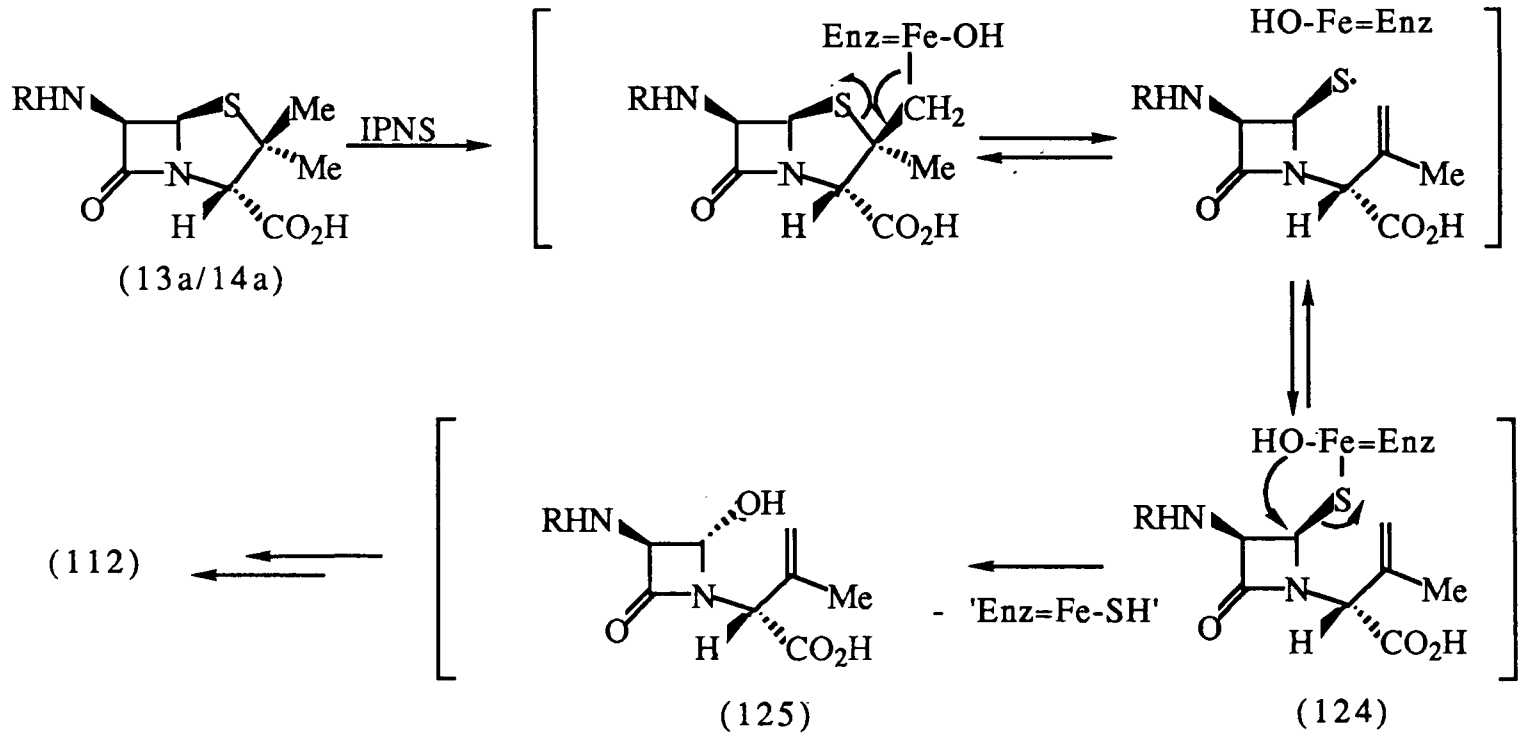
One piece of contradictory evidence existed which threw into doubt the formation of D L D-ASdV (112d) from penicillin N (14), and this was that the isodehydrovaline containing tripeptide (112), for which the configurations of the constituent amino acids had not been determined, had only been isolated from the non-cephalosporin producing organism *P.chrysogenum* - production of (112) by



Scheme 6.21.

cephalosporin producing organisms had never been observed. However, since no attempt to detect and isolate this tripeptide from *C.acremonium* or *Paecilomyces Sp.* has ever been reported, formation of (112) by these organisms cannot be discounted. It is therefore possible that cephalosporin producing organisms do produce (112) either in the same manner as *P.chrysogenum* or possibly as a shunt metabolite arising from ring expansion of penicillin N (14) as discussed above.

There is also an alternative scheme which could account for the formation of (112) from isopenicillin N (13a) or penicillin N (14a) but which differs from Scheme 6.18 in that β-methyl radical formation is not attributed to DAOC/DAC synthetase but to IPNS activity. Such a proposal requires that (13a) or (14a) returns to the IPNS active site and reacts either reversibly to give unchanged starting material or alternatively in a manner which leads to shunt formation. The possible involvement of IPNS in the production of (112) is outlined below (Scheme 6.22.)



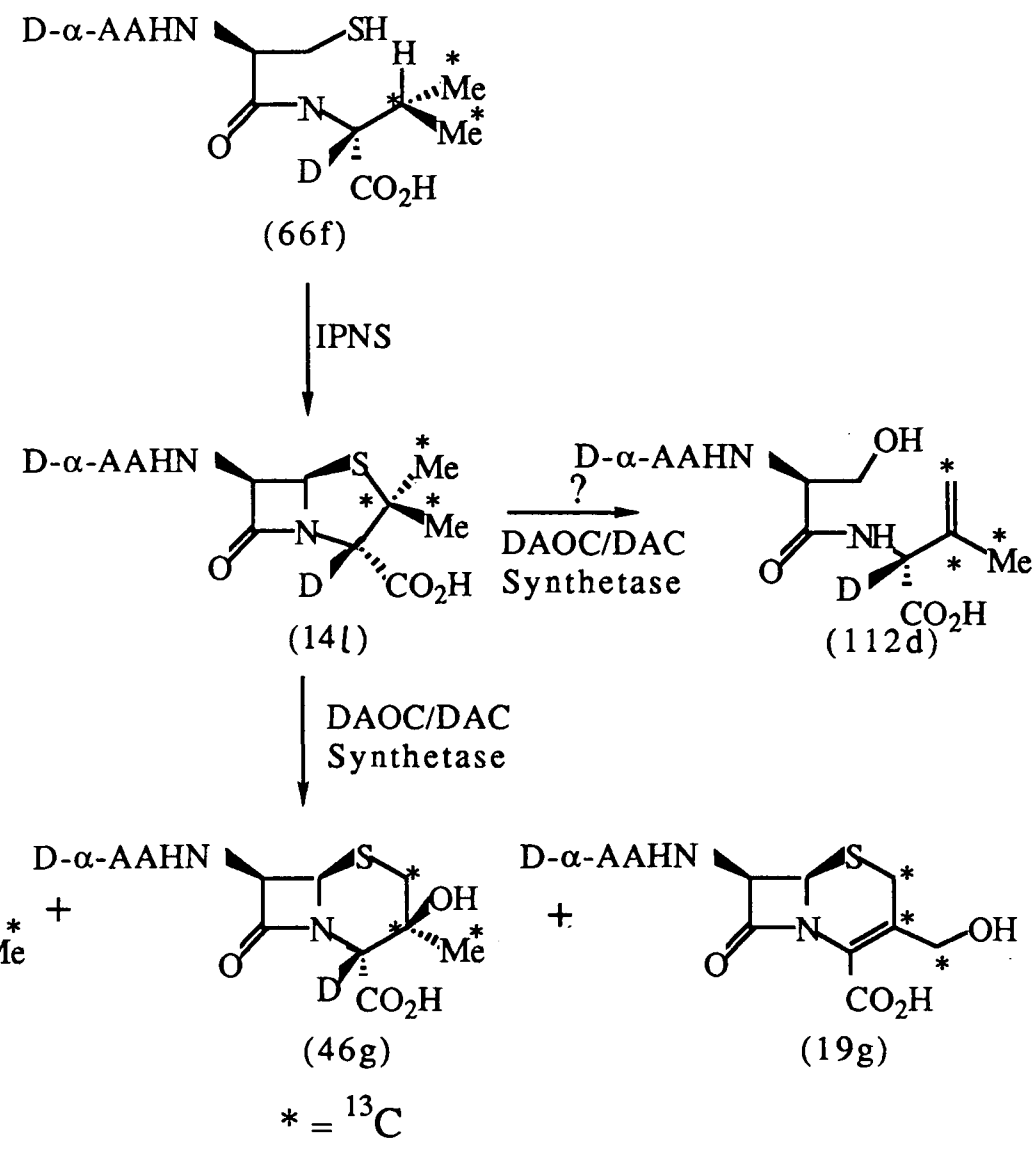
R = L- or D-5-amino-5-carboxypentanoyl

Scheme 6.22.

Several points should be raised about this proposed mechanism. Firstly, the same considerations on the behaviour of radicals β to sulphur, as were discussed for the scheme involving the DAOC/DAC synthetase activity, will apply here and secondly, if this scheme is representative of the actual situation, then product inhibition would be expected to be observed for IPNS incubations. Although this

type of inhibition has not been quantified during preparation of deuterated and ^{13}C-labelled penicillins from their respective tripeptides, required for the investigations detailed in this thesis, such inhibition has been reported for both isopenicillin N (13) and penicillin N (14).[70a,b]

Production of (112) by IPNS or the *C.acremonium* DAOC/DAC synthetase enzyme was considered worthy of further investigation in order that both the schemes considered above could be examined. Thus DLD-AC-(2-^{2}H, 3,4,4'-$^{13}C_3$)-V (66f) was synthesised as described in Section 6.3. Incubation of this tripeptide (*ca* 10 mg) with IPNS (*ca* 60 I.U.) gave good conversion to the tri-^{13}C-labelled penicillin N (14l) as determined by ^{1}H-n.m.r. Incubation of the crude IPNS incubation mixture with recombinant DAOC/DAC synthetase (*ca* 0.6 I.U.), derived originally from *C. acremonium*, and examination by ^{1}H-n.m.r indicated approximately 75% conversion to ring expanded products (Scheme 6.23.). This crude incubation mixture was then examined by ^{13}C-n.m.r (125.77 MHz, acquired overnight) and shown to be devoid of any resonances in the regions δ 143-2 and 115-114 ppm.



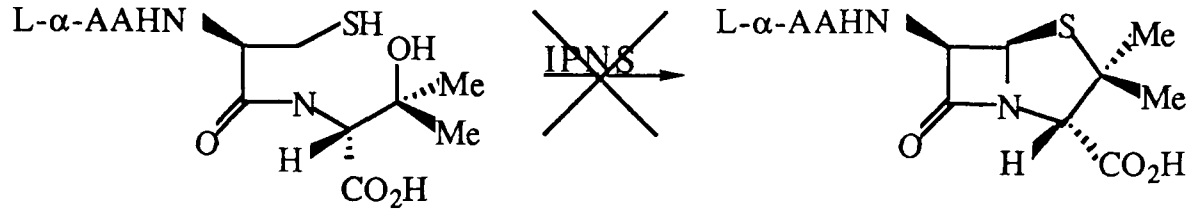
Scheme 6.23.

Although no evidence to support the formation of an isodehydrovaline containing tripeptide from penicillin N (140) was obtained, an intermediate derived from thiazolidine ring opening such as (123), could still be involved in the ring expansion process. From consideration of Schemes 6.18 and 6.19, it can be seen that shunt formation will depend upon the relative rates of the different pathways leading to the cepham radical (53b) and (125) respectively. Although the intermediate (123) or more importantly a shunt metabolite derived from it was not detected this could be due to a variety of reasons. Perhaps the most plausible explanation is that the lifetime of the proposed intermediate radical (123) is too short and hence leakage down a shunt pathway fails to occur at a significant level. This would seem reasonable since the critical event required for shunt formation is the replacement of the sulphur atom bound to the β-lactam ring by an oxygen atom, a process which could be expected to be slower than radical addition to the π - system of the double bond.

6.9 Incubation of LLD-AC-β-Hydroxyvaline (106) with IPNS.

The availability of recombinant IPNS of superior quality and greater quantity to that available previously, prompted the re-examination of (106) as a possible substrate for IPNS. Previous work had shown that this tripeptide was not an intermediate in isopenicillin N biosynthesis contradicting speculation at that time.[116] Dehydration of this tripeptide has been proposed as a possible biosynthetic route to the isodehydrovaline peptide (112) (see Section 6.1). Furthermore, no examination of this possibility has been reported in the literature. Although this proposal is unlikely because it requires kinetic βγ-dehydration as opposed to thermodynamic αβ- dehydration and also because IPNS generally acts as a desaturase, it was considered worthy of brief attention.

Thus (106) was prepared as previously described (Section 6.3.) and 1 mg incubated with IPNS (*ca* 15 I.U.) in the presence of the usual cofactors. Under these conditions no conversion to β-lactam containing products could be detected by 500 MHz ^{1}H-n.m.r examination of the β-lactam region δ 5.6-4.8 ppm or by bio-assay against *E. coli* [(-) ESS] and *S. aureus* 6571. In addition, no olefinic resonances could be observed in this region of the proton n.m.r spectrum suggesting that no conversion to an isodehydrovaline-containing product had occurred.



(106) (13a)

It would appear from these negative results that the β-hydroxyvaline tripeptide (106) is not a substrate for the IPNS enzyme and hence its early assignment as a possible biosynthetic intermediate in isopenicillin N (13) formation is again discounted. Furthermore, the production of the isodehydrovaline tripeptide (112) from the β-hydroxyvaline tripeptide (106) also seems improbable, although this result should perhaps be re-examined with a similar ^{13}C investigation, such as those described in the preceeding sections.

6.10 Conclusions.

In this chapter the origin of the unnatural tripeptide LLD-ASdV (112) has been investigated and evidence presented which suggests that the action of IPNS upon LLD-ACV (12) results in the formation of an isodehydrovaline containing compound. The exact identity of this isodehydrovaline containing compound has not been determined, principally due to formation of only very small quantities of this substance.

Calculation of the percentage formation of LLD-ASdV (112) in *P. chrysogenum* as compared to penicillin V (16) formation (calculated from the level of isolation[114] reported to be a crude total of 195 mg from 190 litres of spent fermentation broth from penicillin V (16) production, which gave a pure total of 35 mg, and the known level of penicillin V (16) production in commercial strains of this organism[142] of *ca* 5 gl^{-1}) indicates that if IPNS was responsible for the production of (112) and assuming that no other species were involved in the formation of (112) or derived from it, then it would appear that approximately one catalytic event in every 5000 leads to production of (112). It is likely however, that the excretion levels of tripeptides are far lower than those of penicillins and thus an additional quantity of the LLD-ASdV tripeptide would have remained in the fungal cells.

Comparison of this figure against the scale of the investigations outlined here suggests that detection of LLD-ASdV (112) formed from ACV and IPNS should be possible at a level of *ca* 10μg providing a ^{13}C-label is included in the substrate, since the sensitivity of ^{13}C-n.m.r. spectroscopy becomes equivalent to that of ^{1}H-

n.m.r. with ^{13}C-labelled substrates. Thus, performing the calculation for an incubation of 5 mg of ^{13}C-labelled ACV, approximately 10μg of LLD-ASdV would be required to be formed - a figure corresponding to formation of the shunt (112) in every 500 catalytic cycles.

From these considerations it becomes apparent that the inconclusive results are most likely due to lack of sensitivity and hence detection. Furthermore, it is possible to argue that the experimental procedures used here which entailed limited attempts at isolation prior to detection, provided the best opportunities for the observation of any LLD-ASdV (112) that may have been formed. In the case of reported isolation[114] approximately 3-4 chromatographic steps were employed each of which presumably led to some loss of material, combined with possibly high levels of tripeptide retained in the fungal cells. The figures presented here are thus likely to represent the lowest possible levels of formation from ACV (*i.e.* represent a conservative estimate).

No evidence was obtained for the formation of an isodehydrovaline containing compound from either isopenicillin N (13) or from penicillin N (14) and this observation has been discussed in terms of the mechanism of ring expansion of penicillin N (14) to DAOC (18). In addition, it has been argued that such lack of evidence suggests that if an isodehydrovaline containing intermediate is involved in the ring expansion process, that it is a short lived intermediate and hence shunt formation from it is improbable.

Re-investigation of both LLD-AC-β-hydroxyvaline (106) and LLD-ASV (111) as substrates for IPNS and examination of their proposed involvement in LLD-ASdV (112) biosynthesis has also been presented. No evidence supporting their conversion to β-lactam containing product or LLD-ASdV (112) was obtained and consequently, these results are in agreement with the previous reports which examined these peptides as substrates for IPNS.[116,123] Further investigation of the origins of these unnatural tripeptides should perhaps be concentrated on the use of IPNS derived directly from *P. chrysogenum* since any variation in shunt formation between IPNS from *P. chrysogenum* and *C. acremonium*, if any, would be avoided.

Chapter 7 : Substrate Analogue Studies with DAOC/DAC Synthetase from *C. acremonium.*

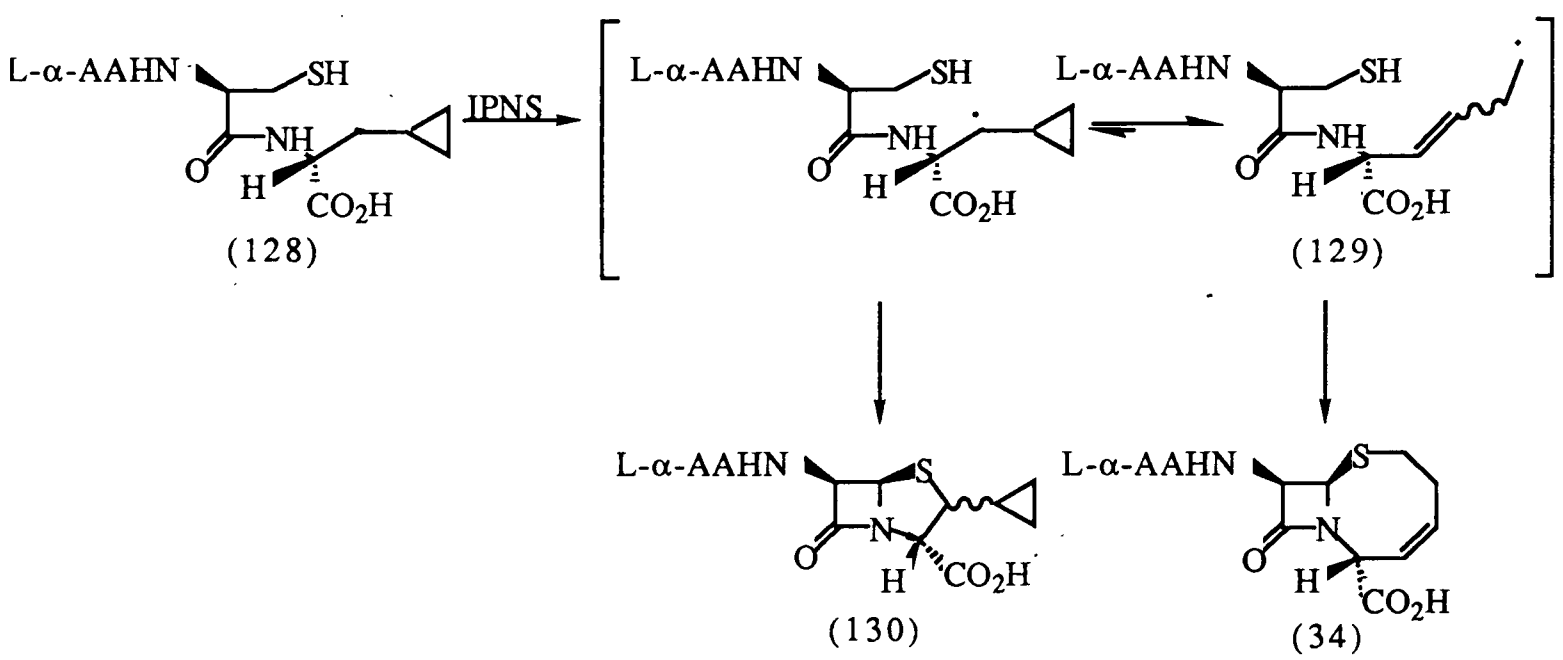
7.1 Introduction.

Investigations of enzymes responsible for secondary metabolism by use of substrate analogues can give information relating to several aspects and properties of the protein in question. Perhaps the most important information which can be obtained from this type of investigation comes from the interpretation of the results in mechanistic terms. In addition to mechanistic detail, substrate analogues can also be used to determine the functionalities present within the substrate which are essential for conversion to products. Such information is generally obtained by altering or completely removing the functional groups contained in the substrate. If the modified substrate is then incubated with the enzyme and conversion to a new product observed then obviously alteration of an essential feature of the substrate has not occurred. However, detection of a new product does not necessarily mean that the alteration in the substrate has not occurred at a critical position.

A true indicator of the overall effect of an alteration should be based upon assessment of the quantity of product formed and the relative rate of production *i.e.* kinetic data such as V_{max} and the Km values for the natural and modified substrates. Furthermore, although a particular substrate modification doesn't lead to product formation, evaluation of the altered substrate as an inhibitor should be undertaken. Valuable information can be obtained if the modified substrate is found to be an inhibitor since the alteration has prevented the enzymatic reaction from proceeding but not completely removed the ability of the substrate to bind to the active site. The type of inhibition exhibited by the substrate analogue can also provide important information. If the analogue displays non-competitive inhibition then either binding to the active site is very much stronger than that which occurs with the natural substrate or an irreversible reaction between the active site and modified substrate molecule has occurred. Consequently the active site is blocked thereby preventing further catalytic events. Competitive inhibition, on the other hand, implies that the modified substrate binds to the active site in a reversible manner and suggests that the modified substrate contains the features essential for enzyme recognition and binding but lacks those required for conversion to product.

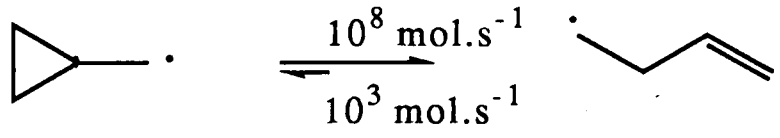
In summary then, alteration of a substrate may lead to modification of binding, of reaction and consequently product formation or indeed a combination of these. From a general knowledge of the mode of enzyme action and of the

particular type of chemical intermediates involved, it is possible to design a modified substrate so that only the product formed is altered. Such use of substrate analogues can lead to an increased understanding of the intermediates involved. A prime example of a modification of this kind is the use of the so called 'radical clock' - often applied to the investigation of intermediates suspected of possessing radical character.[125] Good use of this has been made in the study of the IPNS catalysed conversions of tripeptides to penicillins - a process believed to proceed via a radical type intermediate.[126] Thus the tripeptide (128) was synthesised and incubated with IPNS. Conversion to the expected penam (130) was observed and in addition the homocephem (34) was produced (Scheme 7.1.).



Scheme 7.1.

Formation of the homocephem (34) from the cyclopropyl containing tripeptide (128) is most readily explained by proposing initial formation of a radical α-to the cyclopropane ring. In such systems rapid ring opening to the allylic radical (129) occurs. Kinetic investigations of this process have enabled rate measurements to be made for the forward (ring opening) and backward (ring closure) reactions (Scheme 7.2).[125]

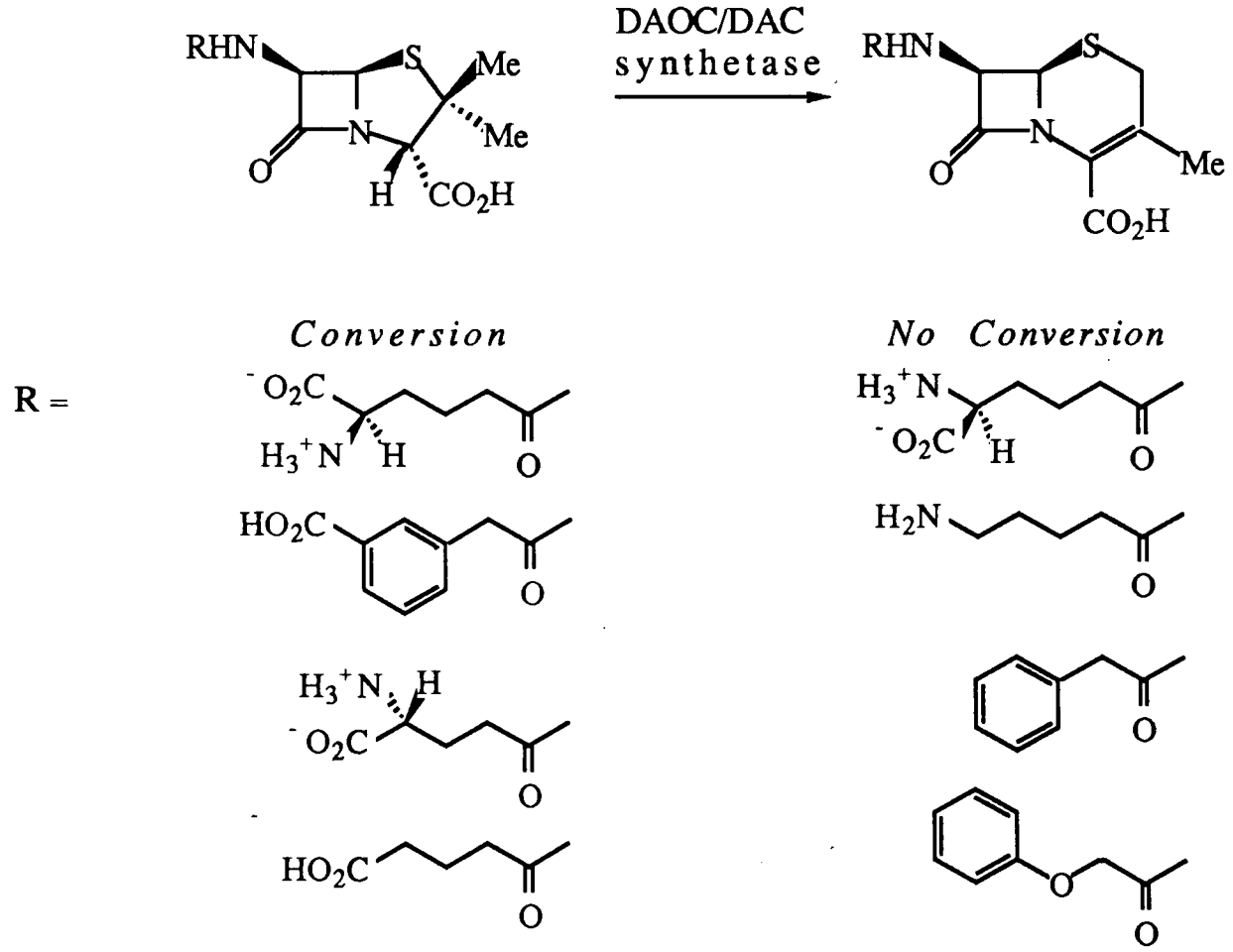


Scheme 7.2.

Many other examples of studies with substrate analogues exist in the field of penicillin biosynthesis.[127] Much attention has been paid to modification of the α-

aminoadipic acid and valine residues of LLD-ACV (12) with a view to active site mapping of the IPNS enzyme (see for example ref. 69). Some of the results of these studies were discussed earlier (see Chapter 1). Comparatively little substrate analogue work has been undertaken with DAOC/DAC synthetase. Investigations of this enzyme are best separated into two groups; those dealing with the expandase activity and those concerned with the hydroxylase activity. In the case of the ring expansion activity the observation that only a limited amount of work has been reported concerning substrate analogue studies may be attributed to the difficulty in preparation of the penicillins containing a modification to the penam nucleus. Too often, the long, complicated and technically difficult syntheses of these penicillins have given little useful information. More encouraging results have, however, been obtained with the hydroxylase activity.

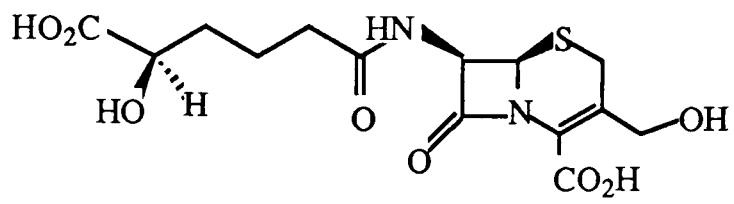
Investigation of the side chain specificity of DAOC/DAC synthetase has been undertaken in this laboratory.[128] Results from this study suggest that a six carbon chain terminating in a carboxyl group permitted efficient expansion of the thiazolidine ring to the dihydrothiazine ring of the cephems with the exception of the L-α-aminoadipoyl side chain (Scheme 7.3.).



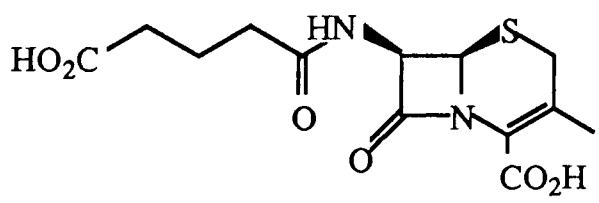
Scheme 7.3.

It is of interest to note that the penicillins with a five carbon chain are substrates for DAOC/DAC synthetase although they are poorer substrates than

those containing a six carbon chain. Isolation of cephalosporins with 5-hydroxy-5-carboxyvaleramido or glutaryl side chains (131) and (132) respectively, from *Cephalosporium Sp.* and *Streptomyces Sp.* , have been reported.[129,130]

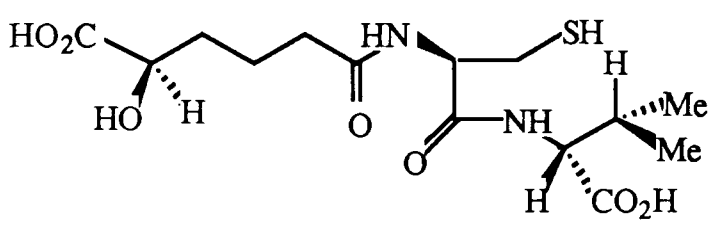


(131)

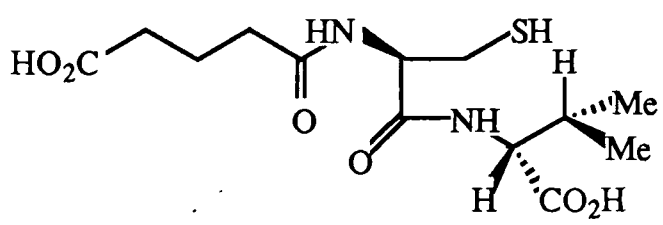


(132)

However it is known that one of the corresponding tripeptides (134) is not an effective substrate for IPNS[69] - no details concerning the other peptide (133) as a substrate for IPNS is known. It would thus appear that certainly the unusual cephalosporin (132) and most probably (131), are products derived by the action of an acylase or transacylase enzyme and a cephalosporin precursor, as has previously been proposed.

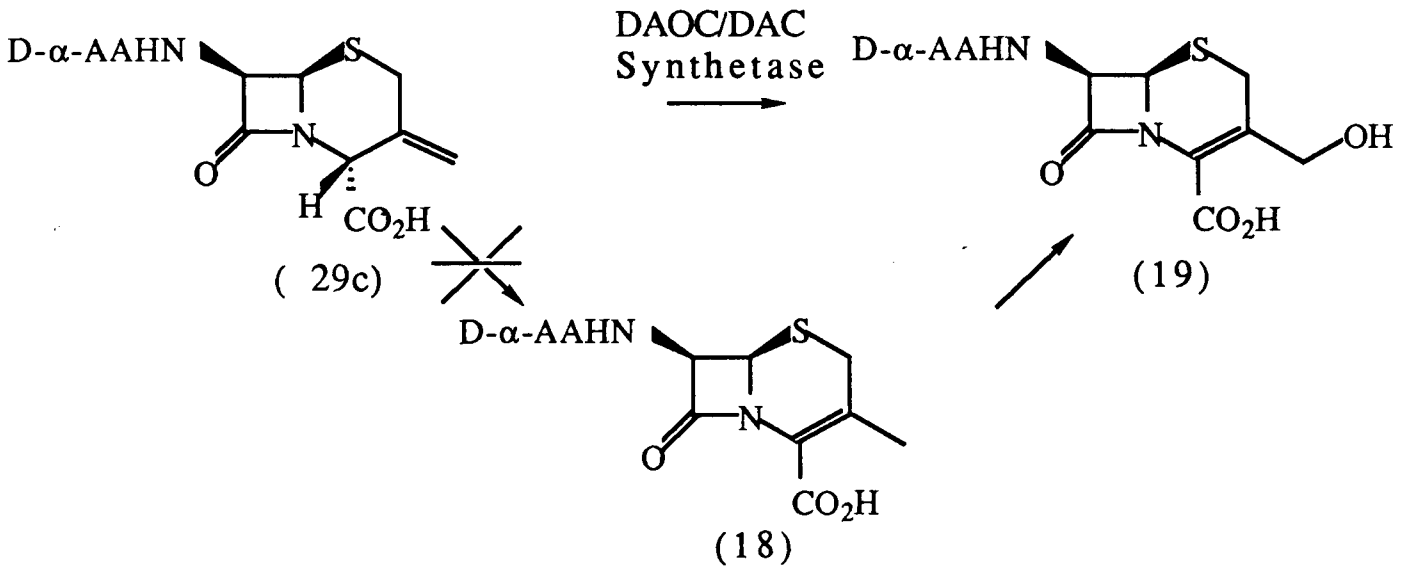


(133)



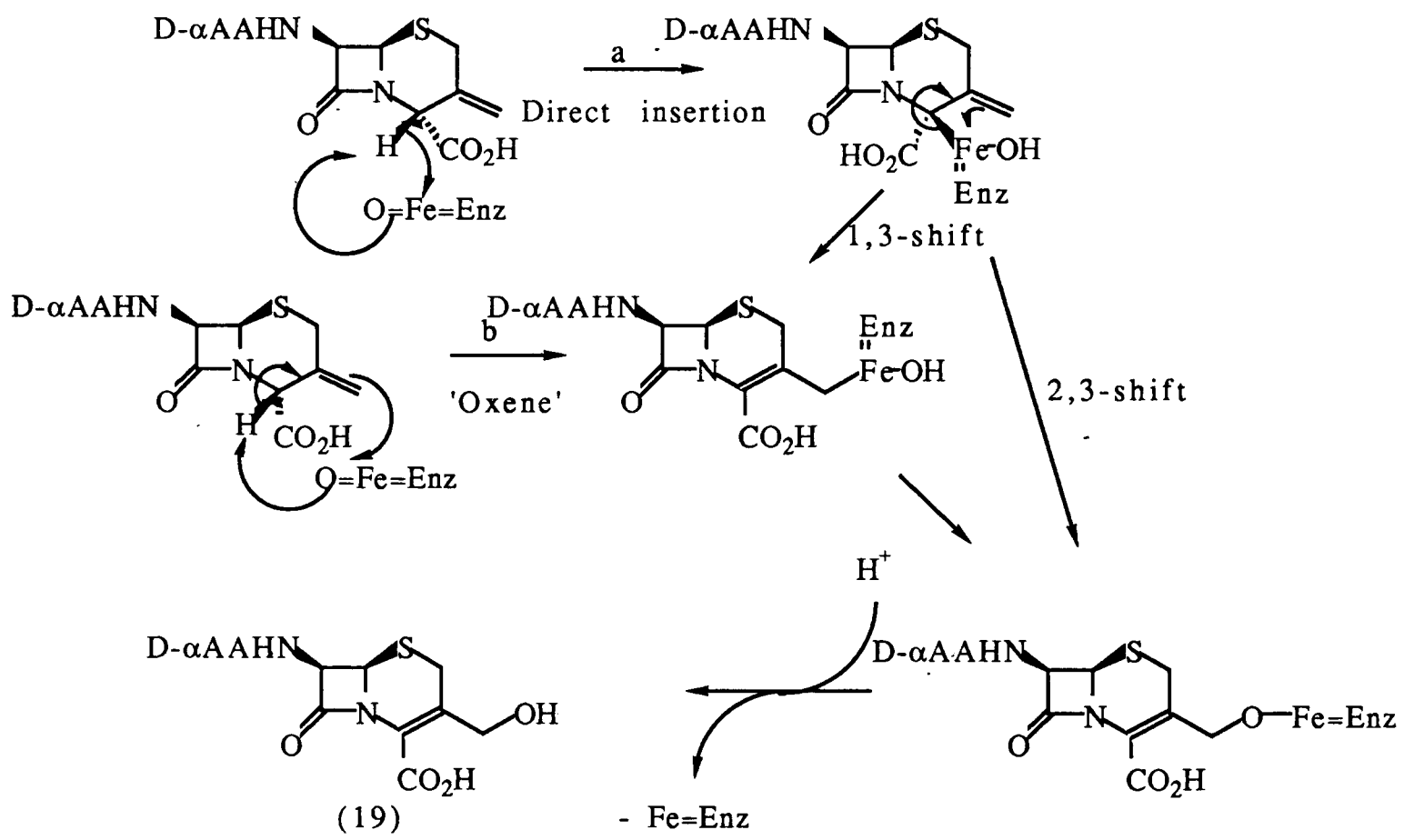
(134)

Perhaps the best studied substrate analogue for DAOC/DAC synthetase is the exomethylene cephalosporin C (29c). Incubation of this compound with DAOC/DAC synthetase leads directly to DAC (19) with no detectable formation of DAOC (18) (Scheme 7.4). [72, 131, 132]



Scheme 7.4.

Furthermore, the origin of the hydroxyl oxygen atom has been determined by incubation under an atmosphere of $^{18}O_2$ gas to obtain DAC (19) which exhibited approximately 50% incorporation of label as judged by mass spectroscopy.[131,132] Two mechanisms can be proposed to account for this observation - either a direct insertion into the carbon-hydrogen bond of the methyl group to be hydroxylated or alternatively an oxene type process (Scheme 7.5).



Scheme 7.5.

7.2.1 Ring Expansion Substrate Analogue Studies.

Despite the more challenging synthetic problems associated with penicillin substrate analogues, some investigations have been made. These have generally concentrated upon modification of the α- and β-methyl groups (Table 7.1.). From these results two general observations are apparent:-

i) modification of the β-methyl group leads to inactivity as a substrate, and

ii) modification of the α-methyl group sometimes results in activity as a substrate.

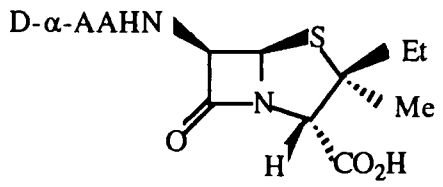
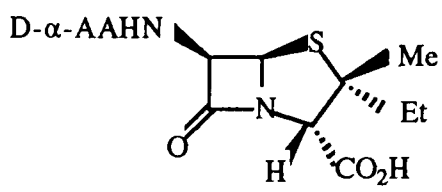
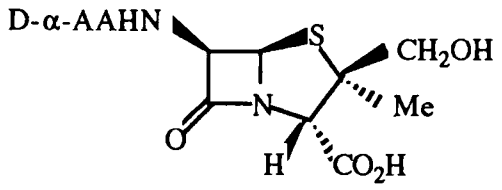
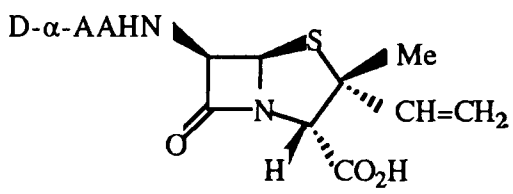
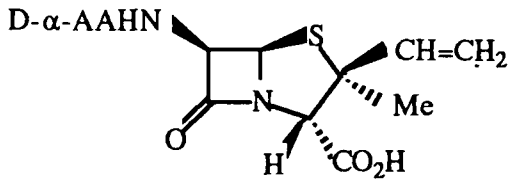
Not Converted	Converted
	
	
	

Table 7.1. Substrate Analogues for the Ring Expandase activity of DAOC/DAC Synthetase.

7.2.2 Comparison of Wildtype and Recombinant DAOC/DAC Synthetase Substrate Specificity.

Any biosynthetic study in which recombinant protein has been used in place of the wildtype enzyme, cannot be considered complete without a comparison of the protein from both sources. Such a comparison is important so that results obtained with recombinant material may be justifiably considered relevant to the wildtype enzyme. Without such data, it cannot be argued effectively that we are dealing with a single protein as opposed to two proteins of similar characteristics and properties.

To fulfil the above criteria, initially penicillin N (14a) was incubated with recombinant DAOC/DAC synthetase to give a mixture of DAOC (18) and DAC (19), in addition to the 3β-hydroxycepham (46a), obtained as a minor product, as judged by ^{1}H-n.m.r, (500 MHz, D_2O, HOD suppressed).[133] Furthermore, in a separate experiment DAOC (18) was converted by incubation with recombinant DAOC/DAC synthetase to give DAC (19) thus confirming the bifunctional nature of the cloned protein.

Incubation of the (3-^{2}H)-penicillin N (14e) with wildtype DAOC/DAC synthetase (see Chapter 2, Section 2.3) had previously been shown to produce in addition to DAOC (18) and DAC (19) the 3β-hydroxycepham (46b), but now as a major product [ratio of (18) + (19) : (46b) equal to 65:35].[71]

Hence incubation of (14e) with recombinant DAOC/DAC synthetase would provide a subtle kinetic comparison with the wildtype enzyme. Thus (14e) (*ca* 1 mg) was incubated with recombinant DAOC/DAC synthetase (*ca* 0.1 I.U.) in the

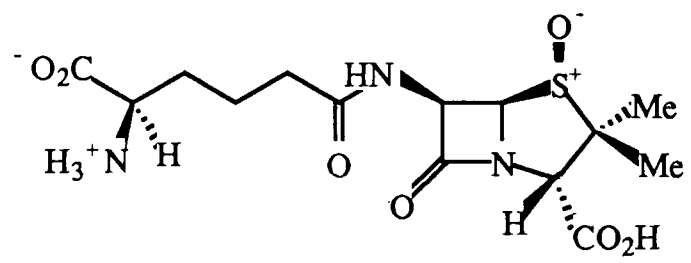
presence of the usual cofactors to give the same products, DAOC (18), DAC (19) and the 3β-hydroxycepham (46b), in a ratio of (18) + (19) : (46b) equal to 65:35. These results indicate within experimental error, essentially identical kinetic behaviour between recombinant and wildtype enzyme.

Furthermore, results obtained within this department indicated that the recombinant DAOC/DAC synthetase was capable of converting exomethylene cephalosporin C (29c) directly to DAC (19) without the detected intermediacy of DAOC (18), as had been previously reported with the fungal DAOC/DAC synthetase.[133]

From these results it would appear that the recombinant and wildtype enzymes are capable of processing substrates in an identical manner to each other and may thus so far be considered mechanistically equivalent in their ability to process substrates.

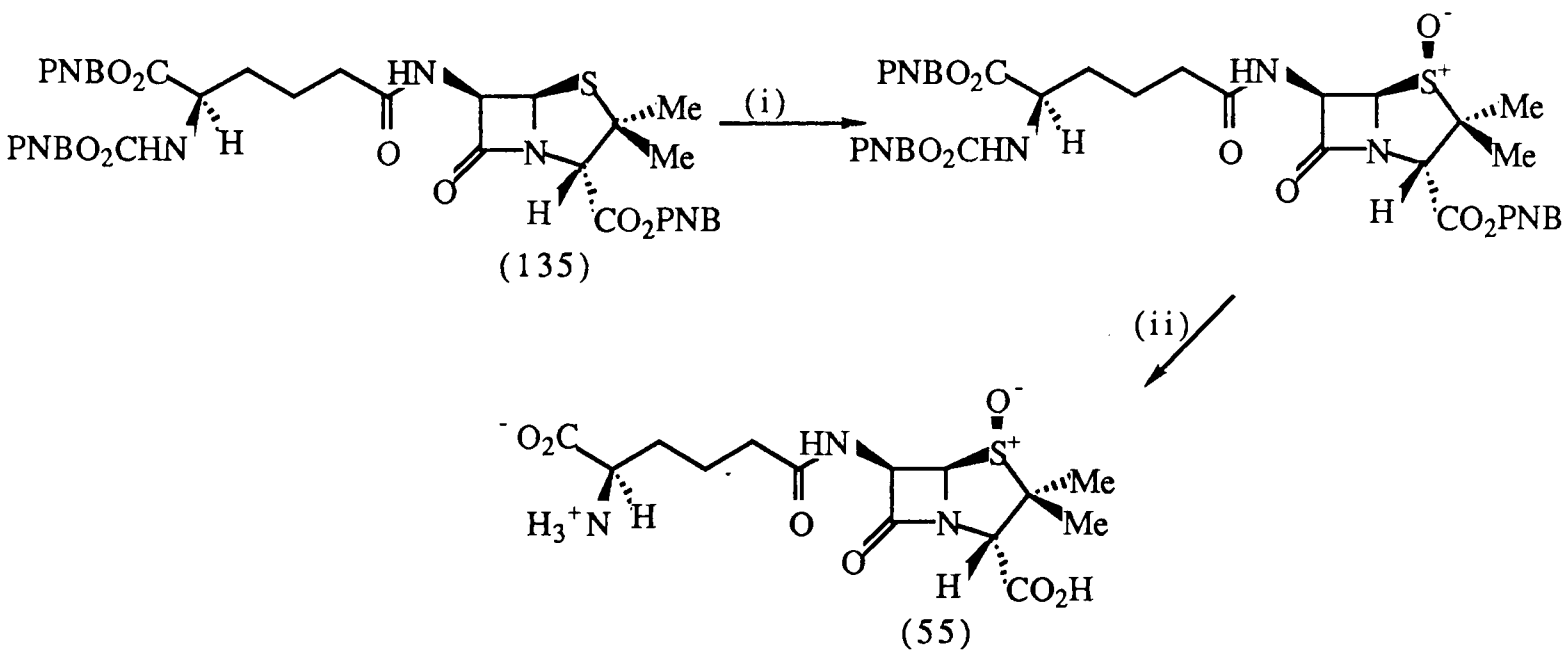
7.2.3 Penicillin N β-Sulphoxide (55).

From the table above it can be seen that the β-hydroxymethyl penicillin N (57) was found not to be a substrate for DAOC/DAC synthetase, as judged by 500 MHz ^{1}H-n.m.r.[36] This result is of interest when considered with the early mechanistic proposals concerned with formation of the episulphonium ion intermediate (52) (see Chapter 2, section 2.1). It was also suggested that penicillin N β-sulphoxide (55) was a potential biosynthetic intermediate in the conversion of penicllin N (14) to DAOC (18)[134a,b,c] and that formation of (52) might be possible from (55) (see Scheme 2.3.).[57]



(55)

Although this compound had already been examined as a substrate for DAOC/DAC synthetase, the availability of a greater quantity of partially purified enzyme preparations which possessed a higher specific activity than previously used, prompted re-examination of these previous reports.[135a,b] Consequently synthesis of this sulphoxide (55) was undertaken *via* oxidation of protected penicillin N (135) with MCPBA (Scheme 7.6)[149]. Oxidation with this reagent had previously been shown by n.O.e. experiments to produce exclusively the β-sulphoxide.[135]



Reagents : (i) MCPBA, DCM, ; (ii) H_2 , Pd/C, 3 hours.

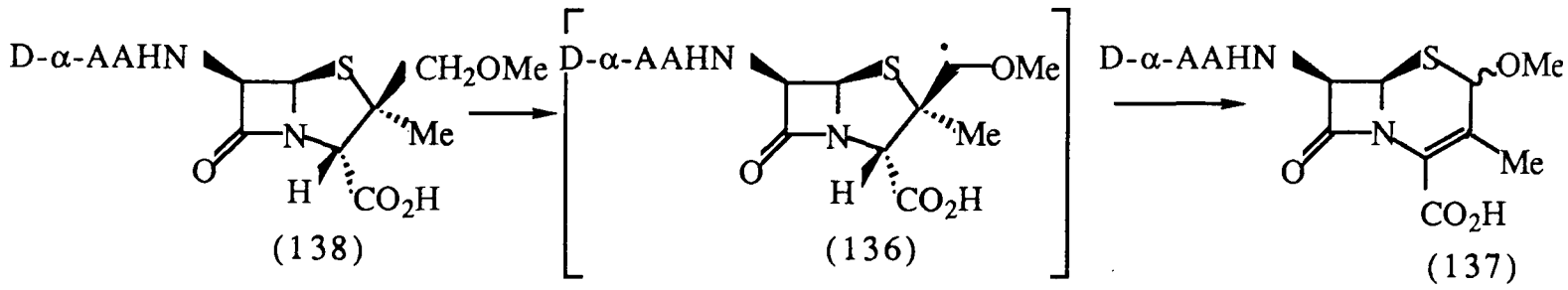
Scheme 7.6.

Deprotection of the protected sulphoxide by catalytic hydrogenation (H_2, 10% Pd/Charcoal) gave (55) which was further purified by hplc [Gilson system, 25 mM aqueous NH_4HCO_3]. Examination of this sulphoxide by 500 MHz ^{1}H-n.m.r and comparison of the spectrum obtained with previously published data revealed that assignment of (55) as the β-sulphoxide was correct.[135]

Incubation of (55) with partially purified DAOC/DAC synthetase and examination of the crude incubation mixture by 500 MHz ^{1}H-n.m.r. indicated that no conversion to a new β-lactam containing compound had occurred. To confirm this result, the incubation mixture was bio-assayed against *E. coli* in the presence of β-lactamase I. No zones of inhibition could be detected thus suggesting that conversion to a cephalosporin type compound had not occurred. These results are consequently in agreement with the observations previously published but give more confidence to the statement that the sulphoxide (55) is not an intermediate in the ring expansion process.

7.2.4 β-Methoxymethyl Penicillin N (138).

Our continuing interest in probing the mechanism of the ring expansion process prompted synthesis of the β-methoxymethyl penicillin N (138) by Dr. J. Cobb. It was felt that the favoured radical formation at the β-methyl might proceed to give the stabilised radical intermediate (136) (Scheme 7.7.).



Scheme 7.7.

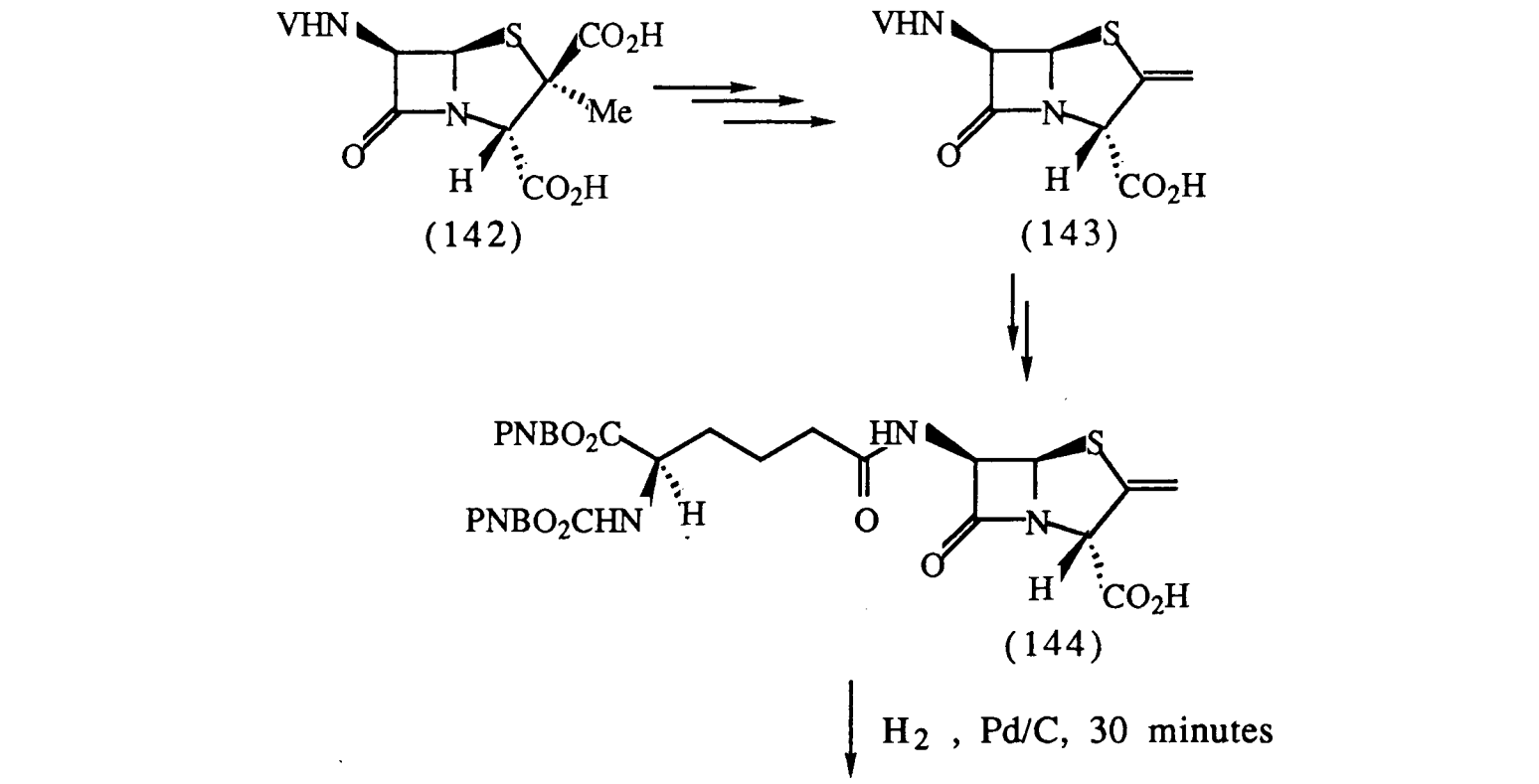
This could then undergo ring expansion to give 2-methoxy-cephalosporin (137). However incubation of this compound with partially purified DAOC/DAC synthetase gave no conversion to new β-lactam containing compounds as determined by 500 MHz ^{1}H-n.m.r and bio-assay against *E. coli* in the presence of β-lactamase I.

7.2.5 Exomethylene Penicillin N (139), α-Nor-methyl Penicillin N (140) and β-Nor-methyl Penicillin N (141).

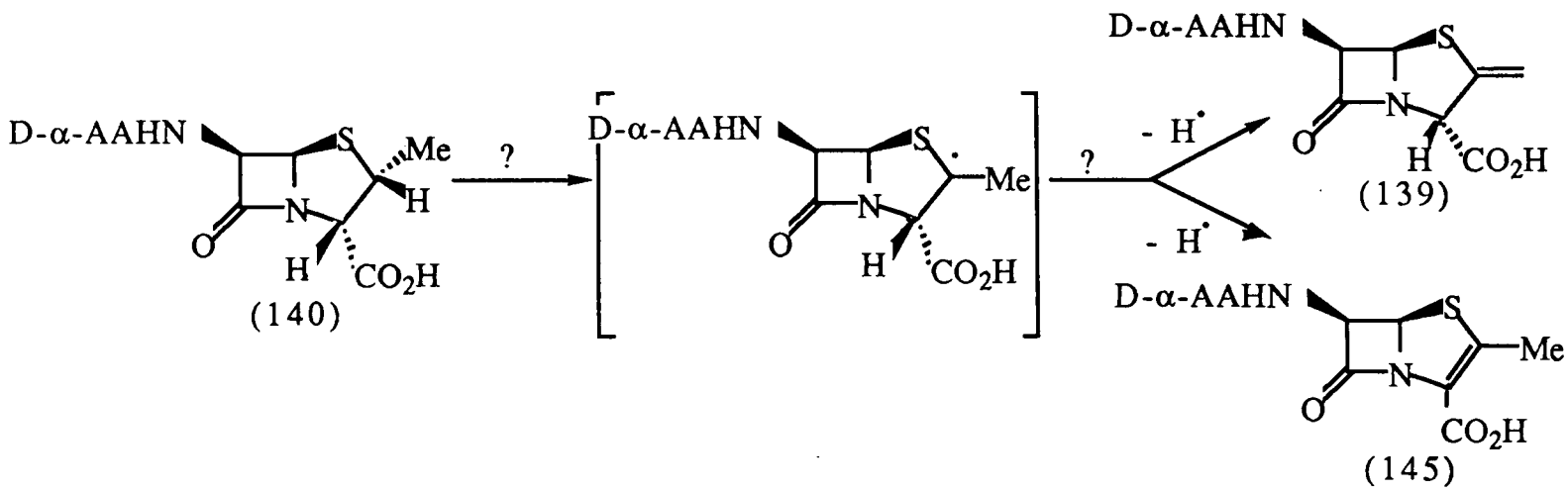
Chemical synthesis of the exomethylene penicillin N (139)[136] by Dr. S. Ko *via* removal of the phenoxyacetyl side chain from the exomethylene penicillin V[137] and replacement with the (5R)-5-amino-5-carboxypentanoyl group, enabled easy access to both the α- and β-nor-methyl penicillins (140) and (141) respectively (Scheme 7.8).

Incubation of the exomethylene penicillin N (139) with DAOC/DAC synthetase and examination of the resulting crude incubation mixture by ^{1}H-n.m.r, (500 MHz, D_2O, HOD suppressed) indicated that no conversion to ring expanded or modified penicillin products had occurred.

Since DAOC/DAC synthetase behaves as a desaturase by abstracting two hydrogen atoms during the ring expansion process, it was thought that desaturation and *via* hydrogen atom abstaction from the β-nor-methyl penicillin N (140) may occur to give either exomethylene penicillin N (139) or the penem (145) (Scheme 7.9.).



Scheme 7.8.



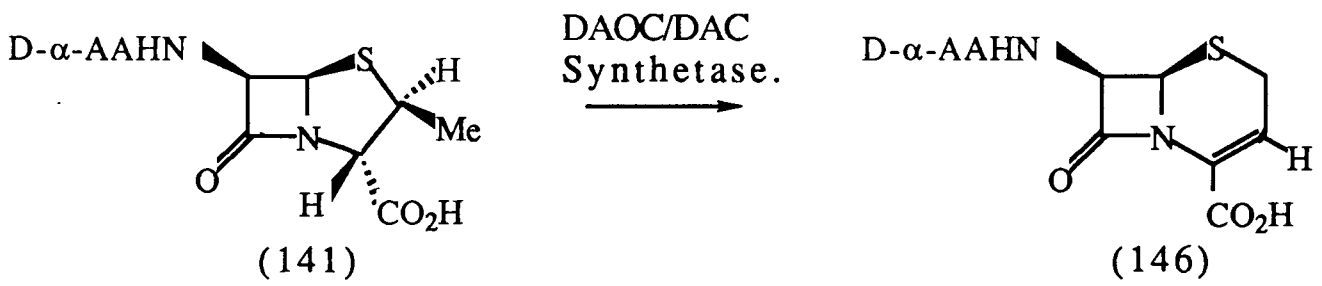
Scheme 7.9.

However, incubation of the β-nor-methyl penicillin N (140) with DAOC/DAC synthetase gave no conversion to new β-lactam containing products as judged by ^{1}H-n.m.r and bio-assay.

These results were not surprising since the first event in the ring expansion process involves formation of a radical at the β-methyl of penicillin N (14) (Scheme 1.9) as demonstrated by the studies with valine (11f) which contained chirally labelled methyl groups (see Chapter 1, Section 1.3).[43,44]

Obviously, these substrates do not contain an easily abstractable hydrogen atom in approximately the same spatial position as does the β-methyl group. The enzyme is thus unable to effect the ring expansion process. It is however, quite possible that the substrate analogues (139) and (140) would behave as inhibitors for the ring expansion process. Due to the limitations on the quantity of enzyme available inhibition studies were not undertaken with either of these substrates.

The α-nor-methyl penicillin N (141) was considered a more likely substrate analogue than either (139) or (140) with the expected product being the desmethyl DAOC (146) (scheme 7.10.).



(141) (146)

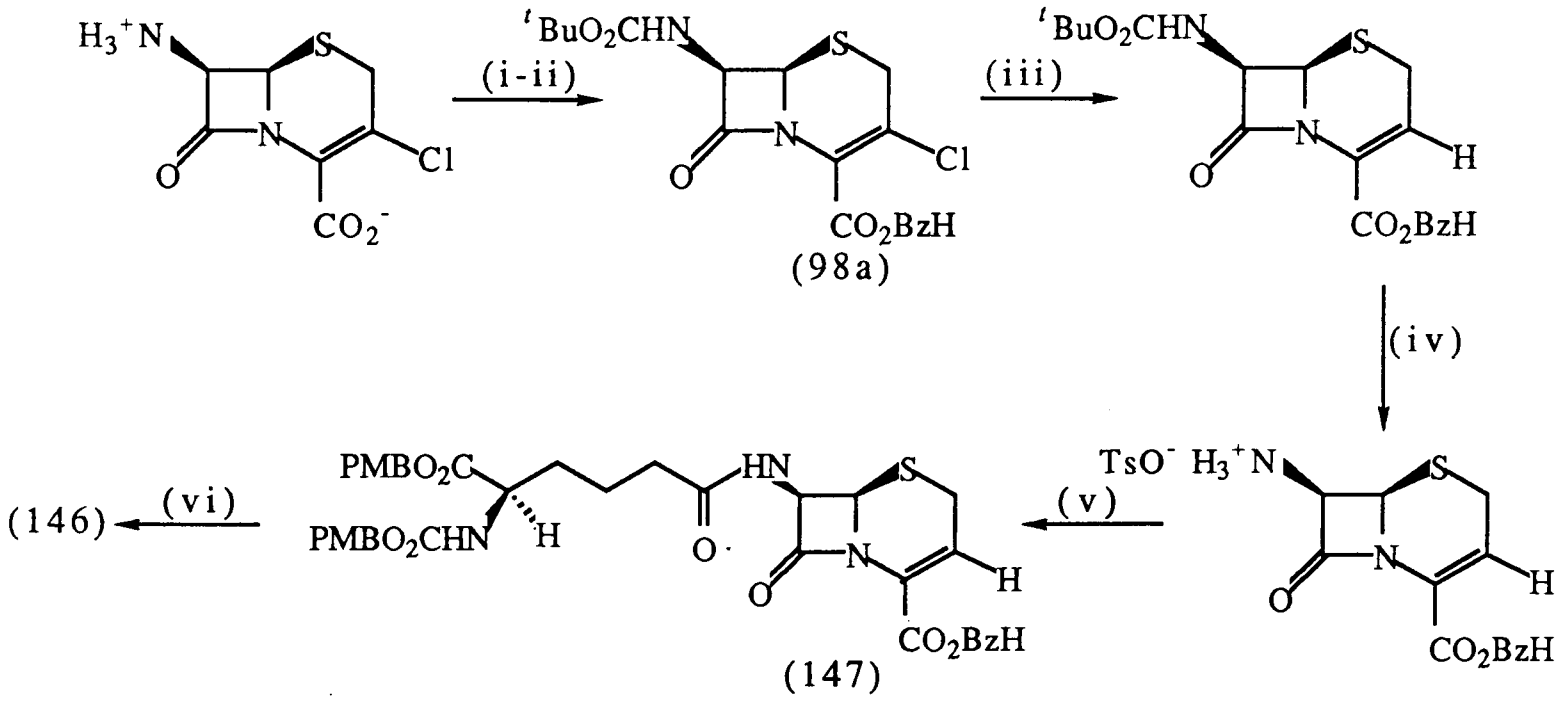
Scheme 7.10.

Due to the limited amounts of the α-nor-methyl penicillin N (141) available (hydrogenation of the exomethylene penicillin N (139) gives principally the β-nor-methyl penicillin N (140) and the α-nor-methyl penicillin N (141) as a minor product) incubation of this substrate with DAOC/DAC synthetase was not performed.

7.3 Substrate Analogue Studies for the Hydroxylase Activity.

7.3.1 Desmethyl Deacetoxycephalosporin C (146).

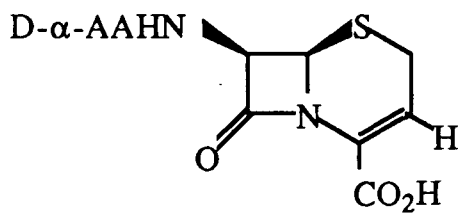
The expected product from conversion of the α-nor-methyl penicillin N (141) is the desmethyl DAOC (146). This compound was synthesised for use as a marker with which to examine the α-nor-methyl penicillin N (141) DAOC/DAC synthetase incubation mixture, in an attempt to facilitate identification and isolation of this anticipated product. This compound was prepared by the zinc and acetic acid reduction of the N-*t*-butyloxycarbonyl 3-chlorocephem benzhydryl ester (98a) (Scheme 7.11.).[138]



Reagents : (i) $(^tBuOCO)_2O$, sat. aq. $NaHCO_3$/Dioxan (1:1, v/v); (ii) Ph_2CN_2, MeCN, (iii) Zn, AcOH, THF, reflux 12 hours; (iv) TsOH, EtOH/Et_2O (1:1,v/v); (v) EEDQ, (103a), DCM, Na_2SO_4, 24 hours; (vi) TFA/ anisole (5:1, v/v), toluene, 23°C, 30 minutes.

Scheme 7.11.

Subsequent selective deprotection of the N-*t*-butyloxycarbonyl protecting group with *p*-toluenesulphonic acid gave the ammonium tosylate salt which was then treated with saturated sodium bicarbonate solution and extracted into ethyl acetate to give the free amine. This was immediately coupled to protected (5R)-5-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid (103) to give the protected cephem (147). Deprotection with TFA and anisole (5:1, v/v) in toluene at room temperature gave the desmethyl-DAOC (146) as its ammonium trifluoroacetate salt in 24% yield from (98a).



(146)

This compound was then examined as a substrate for the hydroxylase activity of DAOC/DAC synthetase. It was felt that although hydroxylation to a new product was unlikely, isomerisation of the double bond from the Δ-3 to Δ-2 position might occur, particularly as exchange - and hence presumably deprotonation - partly occurred at C-2 of DAOC (18c) when hydroxylation at the exocyclic methyl group was slowed down by deuteration (see Section 5.2.). By removing the possibility of hydroxylation completely, reaction at C-2 of the desmethyl DAOC

(146) might be induced. Thus (146) (*ca* 1 mg) was incubated with DAOC/DAC synthetase (*ca* 0.1 I.U.) and the crude incubation mixture examined by ^{1}H-n.m.r, (500 MHz, D_2O, HOD suppressed) in the region δ 5.6-4.8 ppm and by bio-assay against *E. coli* (+)(ESS). No isomerisation of the starting material or its conversion to any new β-lactam containing products was detected by either of the analytical techniques employed.

7.3.2 Δ2-Deacetoxycephalosporin C (148).

The ability of the DAOC/DAC synthetase enzyme to convert the unnatural substrate, exomethylene cephalosporin C (29c) directly to DAC (19) suggested that a certain amount of tolerance was acceptable in the relative spatial orientation of the C-4 α-carboxyl group. X-ray diffraction studies have shown that the spatial orientation of this carboxyl group varied remarkably little between penicillin N (14), exomethylene cephalosporin C (29c) and the Δ2 cephalosporins (Figure 7.1).[139]

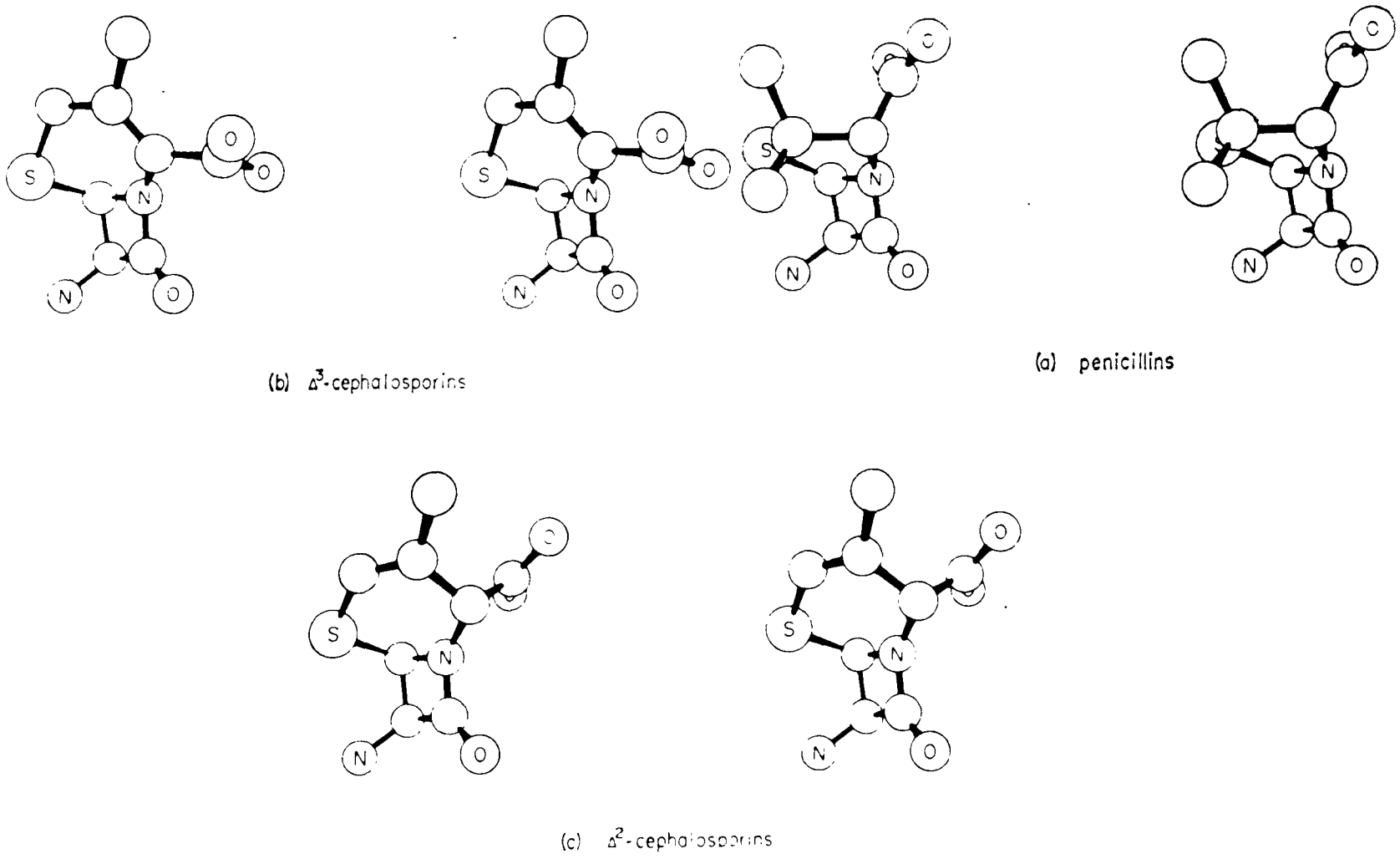
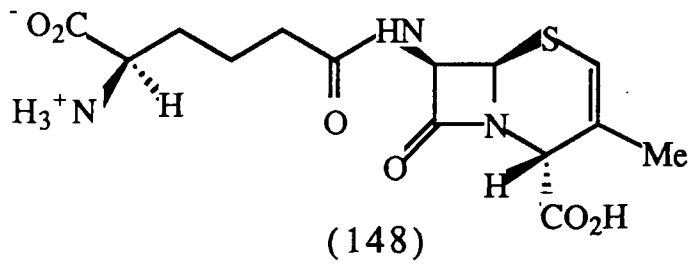


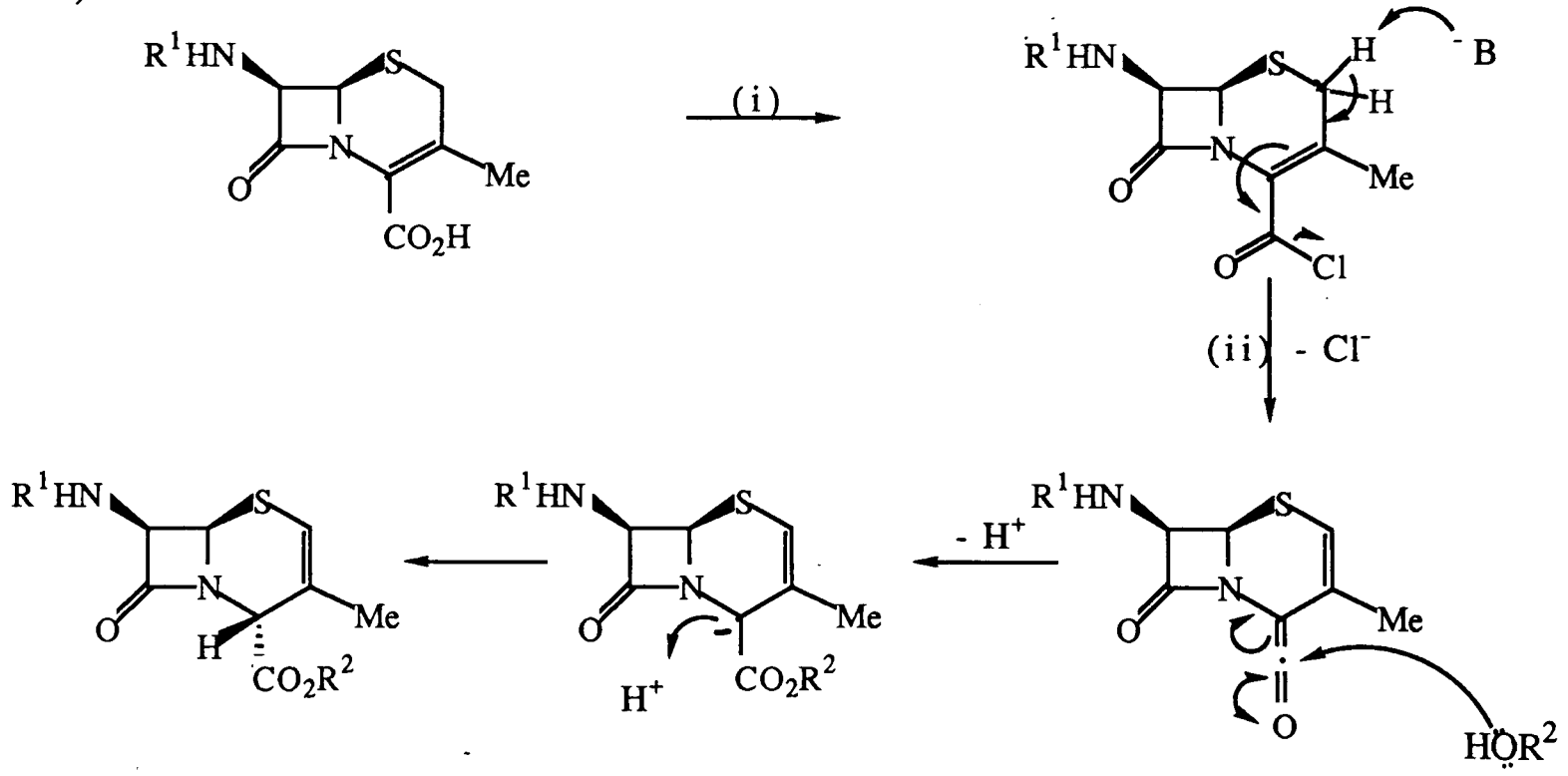
Figure 7.1.

In an attempt to further probe the mechanism of the C-2 exchange process (see Chapter 5), in which the involvement of a Δ2-type cephalosporin intermediate had not been discounted, synthesis of Δ2 deacetoxycephalosporin C (148) was undertaken.



(148)

Various methods exist for the isomerisation of Δ3-cephems to Δ2-cephems.[140,141] The ease of this transformation manifests itself in the production of Δ3-cephem esters (see Chapter 5, Section 5.6) in which any base present in the reaction mixture rapidly generates the isomerised C-4 anion after initial deprotonation α- to the sulphur atom.[141] The literature procedure for the preparation of Δ2 cephems involves treatment of Δ3 acid chlorides with base in the presence of an alcohol thus leading to the formation of the Δ2 ester (Scheme 7.12.).[140]



Reagents : (i) $(COCl)_2$, toluene, DMF (cat), 0°C ;
(ii) Et_3N, R^2OH, $CHCl_3$, -75 to -50°C.

Scheme 7.12.

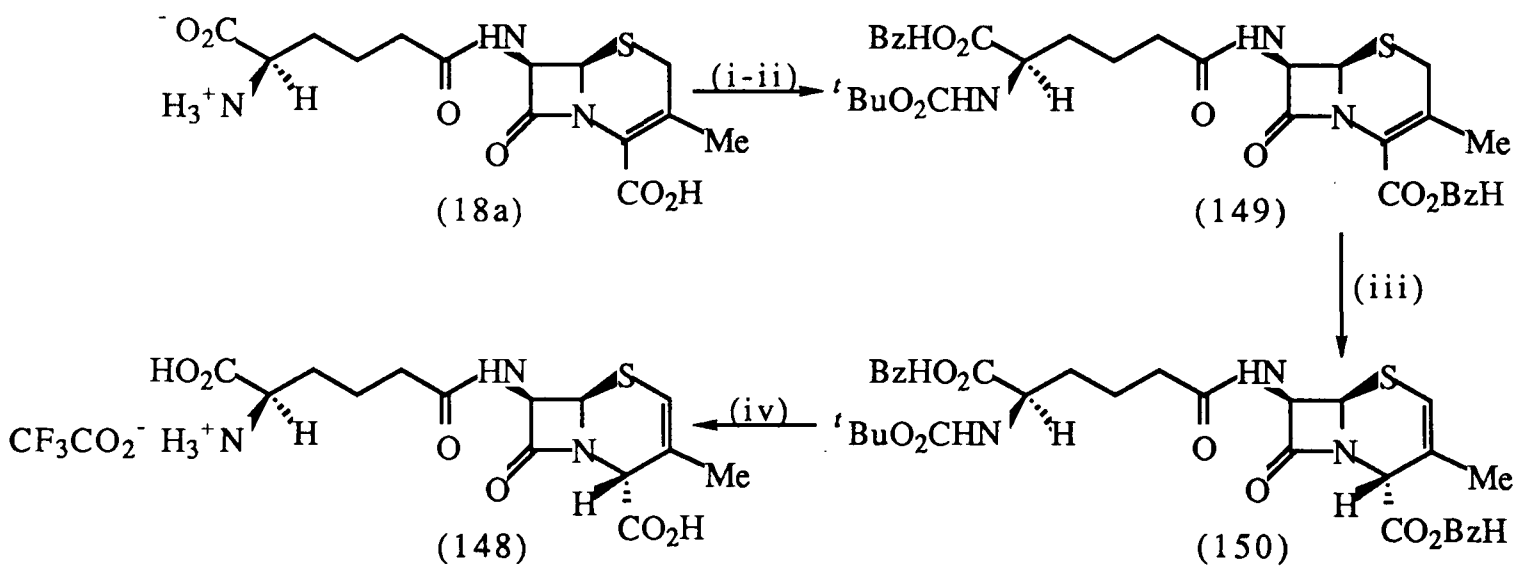
The apparent ease of base catalysed isomerisation and the availability of DAOC (18) suggested that the easiest synthetic route to (148) was *via* protection,

isomerisation and deprotection of DAOC (18) thus avoiding an unnecessary selective N-deprotection and subsequent coupling reaction of any isomerised cephem nucleus.

Thus DAOC (18) was protected as its N-*t*-butyloxycarbonyl, di-benzhydryl ester (149) and treated with three equivalents of LDA at -70°C in THF (Scheme 7.13.). The resulting solution was then quenched by the addition of three equivalents of acetic acid to give a mixture of protected Δ2- and Δ3-DAOC (148) and (149) respectively.

The desired Δ2-isomer was purified concurrently with separation of the unisomerised starting material by chromatography [flash silica, with EtOAc/petrol (1:2, v/v)]. Although separation of these protected isomers by chromatography on silica is difficult (the respective Rf's for (149) 0.38 and (150) 0.42 respectively [SiO_2 , EtOAc/petrol (1:1, v/v)]), separation of the deprotected isomers by reverse phase hplc is trivial as demonstrated by the large difference in retention times; (148) 4 minutes and (18) *ca* 12 minutes.

Deprotection of the protected Δ2-DAOC (150) by treatment at room temperature with a solution of TFA and anisole (5:1, v/v) in toluene gave Δ2-DAOC (148) [50 % yield from (18)]. No isomerisation back to the protected or deprotected Δ3-DAOC (149) and (18) respectively, was observed during the chromatography or deprotection.

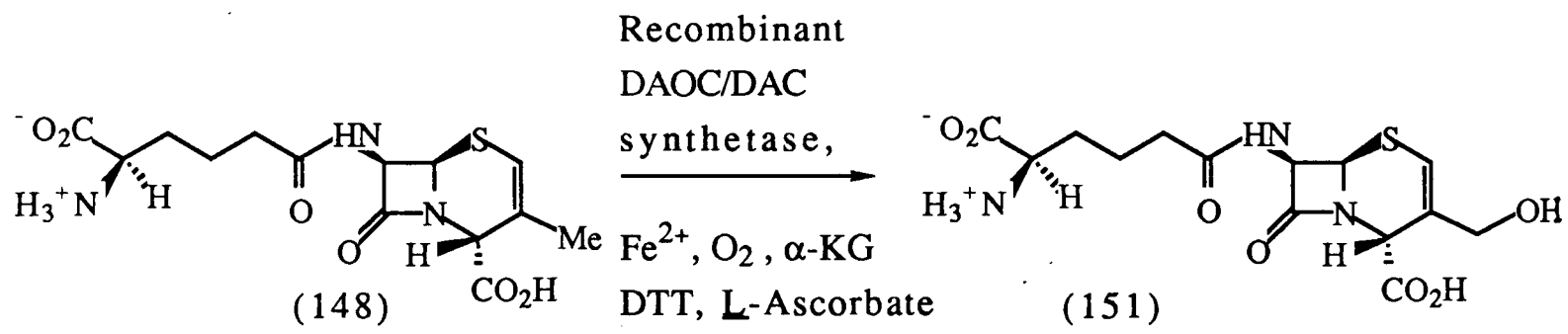


Reagents : (i) $(^tBuOCO)_2O$, sat. aq. $NaHCO_3$/Dioxan (1:1, v/v); (ii) Ph_2CN_2, MeCN, (iii) 3 eq. LDA, THF, - 70°C, 30 minutes; (iv) TFA/ anisole (5:1, v/v), toluene, 23°C, 30 minutes.

Scheme 7.13.

Thus Δ2-DAOC (148) (*ca* 1 mg) was incubated with recombinant DAOC/DAC synthetase (*ca* 0.2 I.U.) in the presence of the usual cofactors and co-substrates

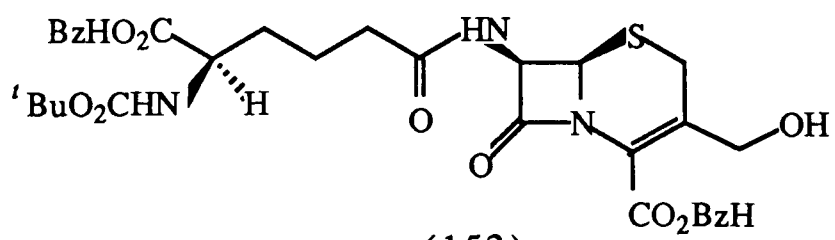
and under the usual conditions. Examination of the crude incubation mixture by ^{1}H-n.m.r. (500 MHz, D_2O, HOD suppressed) revealed a second set of β-lactam resonances in the region δ 5.5-5.0 ppm in addition to a second singlet in the region δ 6.0 - 6.4 ppm. Integration of the β-lactam signals and comparison with the ^{1}H-n.m.r (500 MHz, D_2O, HOD suppressed) spectrum of the starting material indicated that approximately 60% conversion to a new β-lactam containing compound had occurred. This new product was isolated by chromatography [reverse phase hplc, eluting with water, retention time *ca* 5 minutes] and re-examined by ^{1}H-n.m.r. (500 MHz, D_2O, HOD suppressed). In addition to the new set of β-lactam AB quartet resonances and olefinic proton singlet at δ 5.30, 5.39 and 6.34 ppm respectively a second AB quartet was apparent at δ 4.16 and 4.23 ppm. This second AB quartet was assigned to a hydroxymethyl group on the basis of position and magnitude of its coupling constant. The spectrum was thus consistent with hydroxylation of the Δ2-DAOC (148) to Δ2-DAC (151) (Scheme 7.14).



Scheme 7.14.

Mass spectrometry (positive Ar FAB, MCA) indicated that the protonated molecular ion of this new compound was 375 (MH^+, 100%) which corresponded to one mass unit higher than the expected molecular weight.

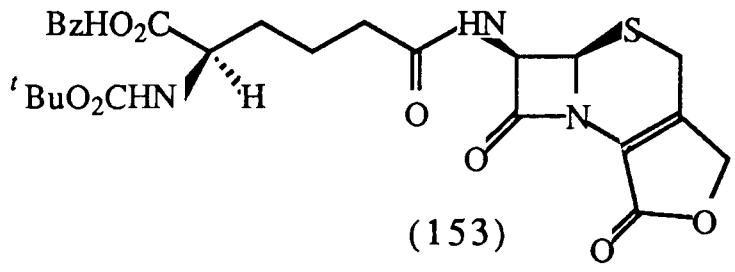
To resolve this discrepancy and to confirm that the incubation product was indeed Δ2-DAC (151), synthesis of an authentic sample was undertaken. Initial attempts concentrated on isomerisation of N-*t*-butyloxycarbonyl-Δ2-DAC, di-benzhydryl ester (152) by treatment with 4 equivalents of LDA.



(152)

These attempts were unsuccessful and consequently hydroxide saponification of this ester was examined - it is known that treatment of Δ3-cephem esters with one equivalent of sodium hydroxide in pyridine/ water solution (1:1, v/v) results in isomerisation and saponification (see Chapter 5, Section 5.6.)[109]. Again attempted isomerisation by this method was unsuccessful.

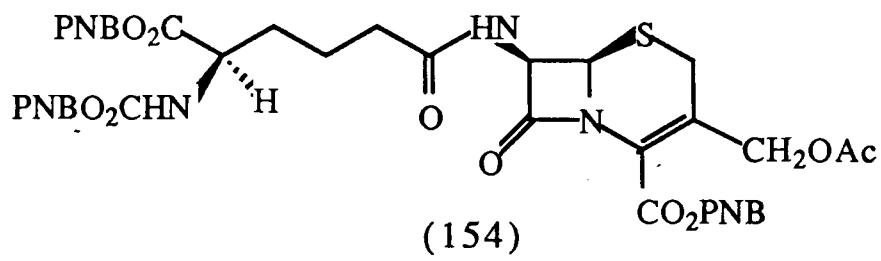
The synthetic approach to Δ2-DAC (151) was thus switched to attempts at isomerisation of the Δ3-DAC lactone (153). Treatment of this lactone with LDA followed



(153)

by quenching with acetic acid resulted only in the recovery of starting material - no evidence of isomerisation could be detected.

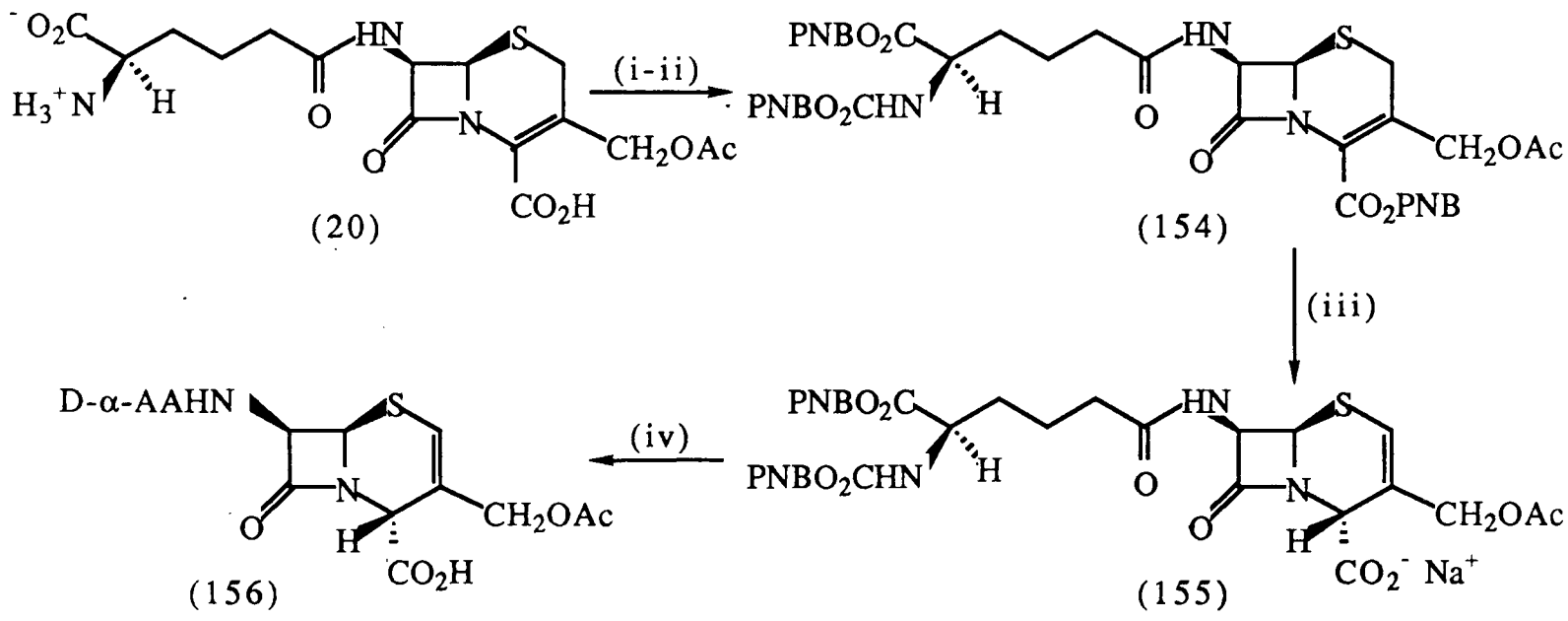
Synthesis from protected DAC (152) or (153) was abandoned and routes from cephalosporin C (20) examined. Thus cephalosporin C (20) was protected as its N-*t*-butyloxycarbonyl, di-benzhydryl ester resulting in a very insoluble compound. However, base catalysed isomerisation of this substance by treatment with sodium hydroxide in pyridine/water did appear to give a mixture of protected Δ2 cephems, which were believed to be protected Δ2-cephalosporin C and protected Δ2-DAC on the basis of ^{1}H-n.m.r. examination of the crude reaction mixture. This mixture was treated with a solution of TFA and anisole (5:1, v/v) in toluene at room temperature to effect deprotection prior to purification by reverse phase hplc. Unfortunately, deprotection in this way led only to extensive decomposition and consequently the protecting groups were changed. Cephalosporin C (20) was thus protected by initial reaction with *p*-nitrobenzylchloroformate followed by esterification with



(154)

p-nitrobenzylbromide in DMF in the presence of sodium bicarbonate to give N-*p*-nitrobenzyloxycarbonylcephalosporin C, di-*p*-nitrobenzyl ester (154). Purification of this compound was not practical due to severe insolubility and consequently the base catalysed isomerisation was performed on crude material.

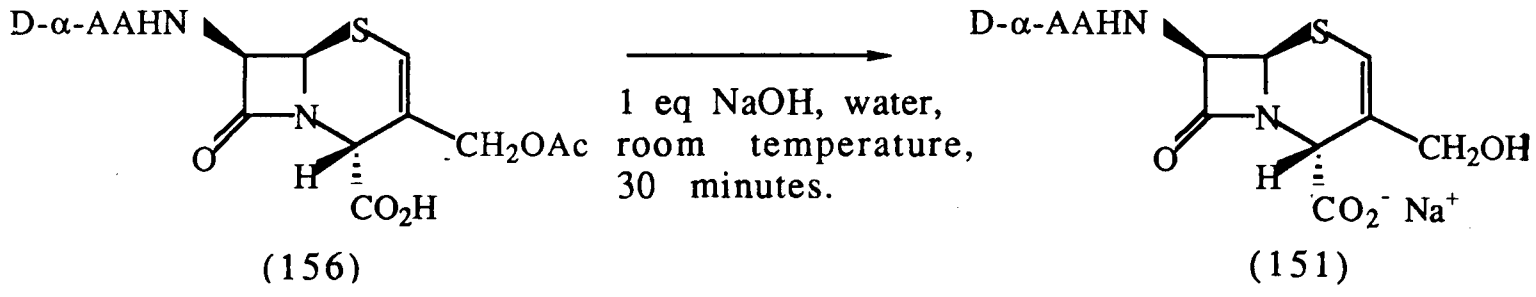
Thus, (154) was treated with one equivalent of sodium hydroxide in pyridine/water (1:1, v/v) solution to give a 10:1 mixture of N-*p*-nitrobenzyloxycarbonyl-Δ2-cephalosporin C, *p*-nitrobenzyl ester (155) and N-*p*-nitrobenzyloxycarbonyl-Δ2-deacetylcephalosporin C, *p*-nitrobenzyl ester which was then deprotected by catalytic hydrogenation under 1 atmosphere of H_2 and over 10% palladium on charcoal to give Δ2-cephalosporin C (156) (Scheme 7.15.).



Reagents : (i) $ClCO_2CH_2C_6H_4NO_2$, sat. aq. $NaHCO_3$/Dioxan (1:1, v/v); (ii) PNBBr, 2 eq $NaHCO_3$; (iii) 1 eq NaOH, Pyridine/water (3:1, v/v), 0°C, 4 hours ; (iv) acidify, extract and then H_2 , 10% Pd/C, THF/water (1:1, v/v), 2 hours.

Scheme 7.15.

Deacetylation of the resulting Δ2-cephalosporin C (156) was achieved by stirring at room temperature for 30 minutes in the presence of 1 equivalent of sodium hydroxide to give a mixture of Δ2-DAC (151) and some starting material (156) (Scheme 7.16.).

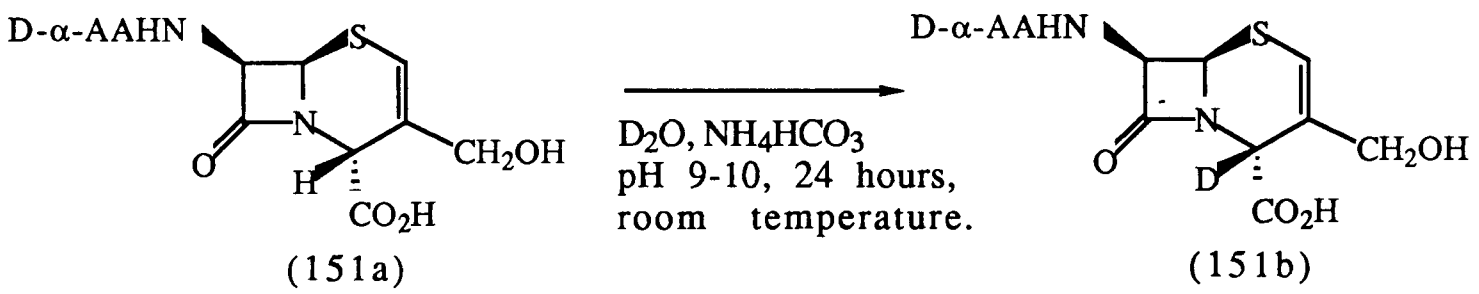


Scheme 7.16.

Purification and concomitant separation by chromatography [reverse phase hplc, eluting with 0.5% MeCN in water] gave pure Δ2-DAC (151). The ^1H-

n.m.r spectrum of this synthetic material was identical to the biosynthetic material except for the presence of a resonance at δ 4.85 ppm, assigned to the C-4 proton. The mass spectrum (positive Ar FAB, MCA) gave a protonated molecular ion of relative mass 375 (MH^+, 100%).

It thus appeared that the biosynthetic material had incorporated a single deuterium atom at some stage during its isolation from the incubation mixture. It was noted earlier (Chapter 5, Section 5.4.) that during lyophilisation of crude incubation mixtures after examination in D_2O by ^1H-n.m.r, the concentration of buffer can lead to the attainment of a high pH - it was thought that such a process was the most likely explanation for the observed deuterium incorporation into the biosynthetic Δ2-DAC (151). In an attempt to confirm that base catalysed exchange into Δ2-DAC (151) was possible, a sample was stirred overnight in D_2O at *ca* pH 9-10 (Scheme 7.17.).



Scheme 7.17.

Re-examination of this sample by mass spectrometry (positive Ar FAB, MCA) indicated that exchange had occurred (Table 7.2.).

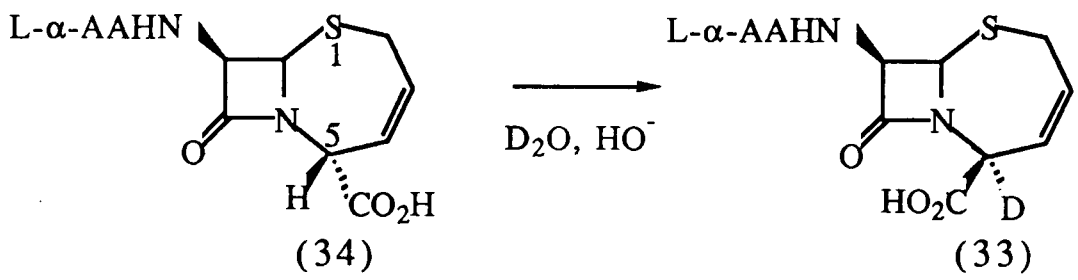
	m/z for Δ2-DAC (151)					
Origin of Δ2-DAC (151)	*373*	*374*	*375*	*376*	*377*	*378*
Biosynthetic	10	6	100	30	15	6
Synthetic	9	100	22	8	3	2
Exchanged Synthetic	18	100	93	26	12	6
Calculated	-	100	18	7	1	0

Table 7.2.

This experiment demonstrates that base catalysed incorporation of deuterium into synthetic (and hence presumably biosynthetic) Δ2-DAC (151) is possible and that it is the most likely explanation for the observed biosynthetic Δ2-DAC (151) molecular ion at 375 in the mass spectrum. It should be emphasised that the biosynthetic Δ2-DAC (151) was produced by incubation of Δ2-DAOC (148) with DAOC/DAC synthetase in water and only came into contact with D_2O during

examination by ^{1}H-n.m.r. spectroscopy and consequently, exchange into Δ2-DAC (151) has not occurred in this case as a result of enzyme activity.

Examination of this exchanged material by ^{1}H-n.m.r. (500MHz, $DMSO_{d6}$) revealed that incorporation had occurred exclusively into the C-4 position, as judged by integration. It is interesting to draw a comparison between the result described here and the observed C-5 epimerisation and deuterium incorporation observed with the homocephem (34)[66] (Scheme 7.18.).



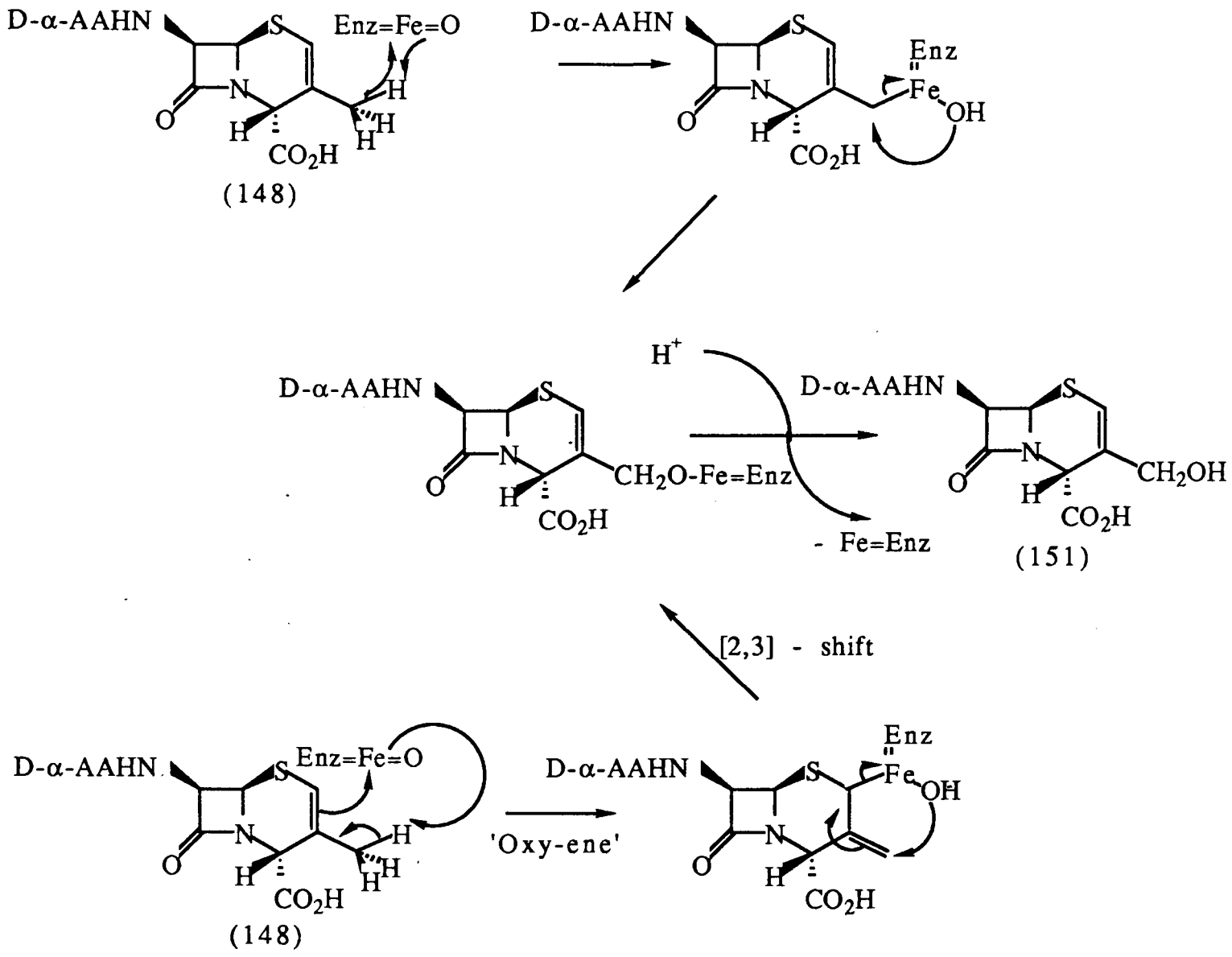
(34) (33)

Scheme 7.18.

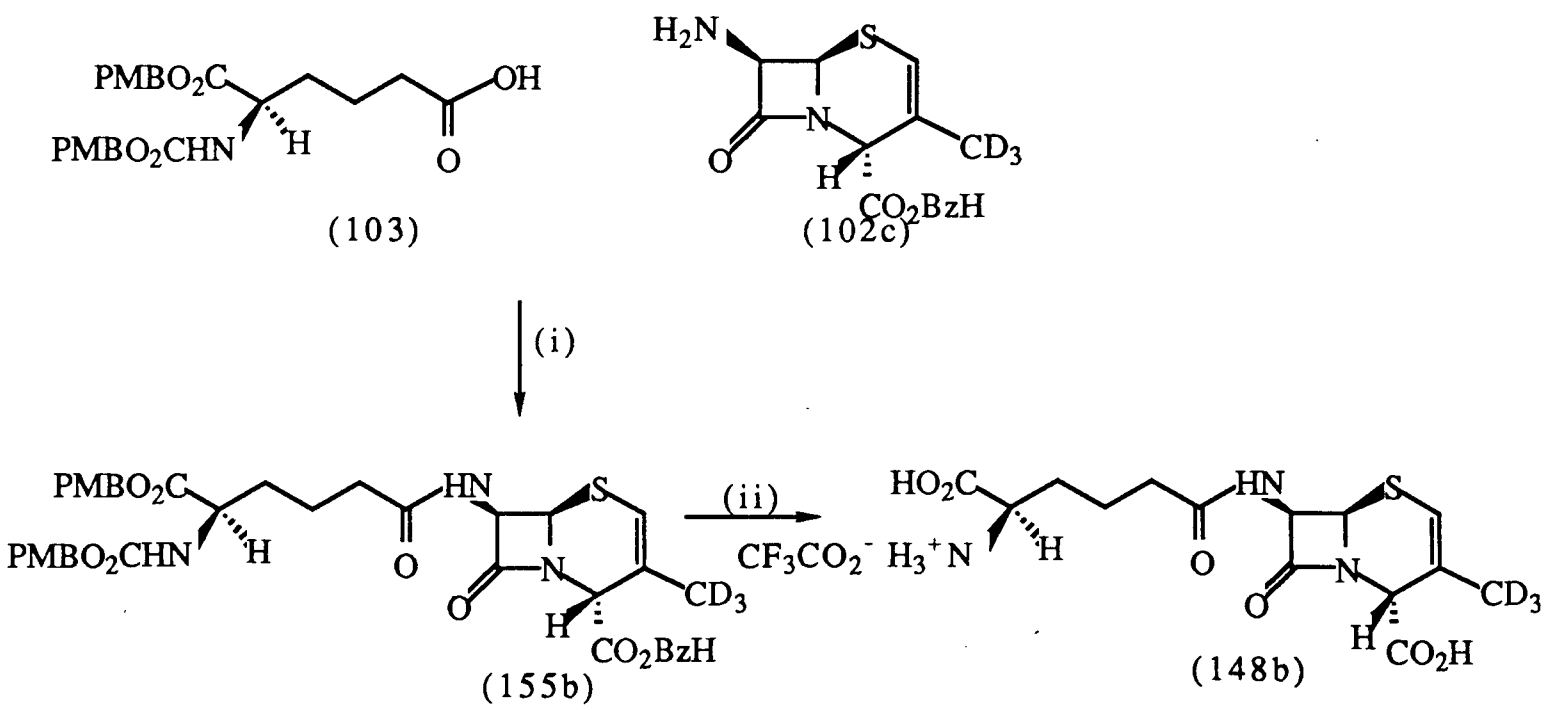
Furthermore, it is interesting to note that no <u>overall</u> isomerisation [(i.e. formation of Δ3-DAOC (18) or Δ3-DAC (19)] of the double bond occurred during the enzymatic conversion of Δ2-DAOC (148) to Δ2-DAC (151) as judged by ^{1}H-n.m.r (500 MHz, D_2O, <u>H</u>OD suppressed). It is, however, important not to rule out the transient existence of an enzyme bound Δ3-intermediate during the course of the hydroxylation process. A mechanism for hydroxylation of the Δ2-DAOC (148) can be written in an analogous manner to that proposed for the Δ3-DAOC (18) isomer (see Scheme 1.17.). Thus by suggesting the direct insertion of the ferryl-oxo species into the carbon-hydrogen bond of the C-3' methyl group (Scheme 7.19.) no involvement of the double bond need be proposed.

The absence of isomerisation does not completely discount the other possible oxene type mechanism (Scheme 7.19.).

In view of the observation of DAOC C-2 exchange (see Chapter 5) which was believed to be caused by a decrease in the rate of hydroxylation, which was in-turn due to a primary isotope effect, it was considered that isomerisation Δ2- to Δ3- might be induced in a similar manner. Thus the labelled (3'-$^2H_3$)-Δ2-DAOC (148b) was synthesised by coupling previously prepared 7β-amino-(3'-$^2H_3$)-Δ2-DAOC (102c) (Chapter 5, Section 5.6) to (5<u>R</u>)-5-<u>N</u>-*p*-methoxybenxyloxycarbonyl-5-*p*-methoxybenzylcarbonylpentanoic acid (103) with EEDQ by the usual procedure (Scheme 7.20.). Subsequent deprotection with TFA/ anisole (5:1. v/v) in toluene gave the desired (3'-$^2H_3$)-Δ2-DAOC (148b).



Scheme 7.19.



Reagents : (i) EEDQ, DCM, 23°C, 24 hours, (ii) TFA/anisole (5:1, v/v), reflux, 30 minutes.

Scheme 7.20.

Incubation of (148b) (*ca* 1 mg) with recombinant DAOC/DAC synthetase (*ca* 0.2 I.U.) gave approximately 5% conversion to what was assumed to be (3'-$^{2}H_2$)-Δ2-DAC (151c) as judged by ^{1}H-n.m.r. (500 MHz, D_2O, HOD suppressed) in the region δ 6.4-6.0 ppm. Isolation of the product on this scale was not attempted.

Operation of an isotope effect with the deuterated substrate (148b) is obvious when it is considered that the undeuterated substrate (148a) gave *ca* 50% conversion under identical conditions (i.e. same enzyme preparation and quantity of enzyme used with equivalent amount of cofactors). Furthermore, the low degree of conversion obtained with the deuterated substrate (148b) meant that it was not possible to unambiguously show that no isomerisation to give either Δ3-DAOC (18f) or direct conversion to Δ3-DAC (19f) had occurred.

7.5 Conclusion.

In this chapter the substrate specificity of recombinant DAOC/DAC synthetase has been examined and where possible a comparison made with the specificity demonstrated by the fungal wildtype enzyme. Furthermore, the substrate specificity of both the ring expandase and hydroxylase activities has been probed by the use of modified penicillins and cephalosporins.

From these studies it would appear that the ring expandase activity is much more selective in terms of acceptable substrates than is the hydroxylase activity. This is exemplified in the ability of the hydroxylase activity to convert the unnatural substrates, exomethylene cephalosporin C (29c) and Δ2-DAOC (148) to DAC (19) and Δ2-DAC (151) respectively.

The structural similarity in spatial arrangement of the penicillin N (14) C-3, exomethylene cephalosporin C (29c) and Δ2-DAOC (148) C-4, α-carboxyl groups may explain their conversion by DAOC/DAC synthetase. The obvious tolerance for variation in the position of this carboxyl group may be coincidental or, alternatively, may be explained if the active site for ring expansion of penicillin N (14) was the same as that responsible for the hydroxylation of DAOC (18).

Finally, it is worth stating that initial studies on the substrate specificity of the separate ring expandase and hydroxylase enzymes from *S. clavuligerus* have demonstrated that a much greater tolerance to unnatural substrates (*i.e.* substrates which have modifications to the penam or cephem nuclei) is apparent.[142] This decrease in substrate specificity as shown by the enzymes from *S. clavuligerus* compared to the DAOC/DAC synthetase from *C. acremonium*, may provide the possibility of developing new antibiotics by the conversion of unnatural substrates to new cephams and cephems.

Chapter 8 : Experimental.

8.1 General Experimental.

All solvents were distilled prior to use unless otherwise stated. THF was distilled from potassium benzophenone under nitrogen and both benzene and toluene were dried over sodium wire. Petrol refers to the fraction of petroleum ether boiling between 30 and 40°C unless otherwise indicated.

Flash chromatography was performed with Merck Kieselgel 60, 230-400 mesh. Preparative plate chromatography was performed with silica gel (HF254) coated onto glass plates. Thin layer chromatography was performed with Merck silica gel 60 F254 pre-coated onto aluminium plates and the plates developed with either a spray of 5% w/v dodecamolybdophosphoric acid in ethanol or 10% ammonium molybdate in 2N H_2SO_4 .

Infra-red spectra were recorded in either $CHCl_3$ or D_2O on a Perkin-Elmer 681 spectrometer or Perkin-Elmer 1750 fourier-transform spectrometer (absorbances measured as s strong, m medium, w weak, b broad).

Melting points were recorded on a Buchi 510 apparatus and are uncorrected.

^{1}H-n.m.r. spectra were either recorded at 200MHz on a Varian Gemini-200MHz spectrometer, 300MHz on a Bruker WH 300 NMR spectrometer or at 500MHz on a Bruker AM 500 NMR spectrometer and are referenced to either TMS (samples in $CDCl_3$ $\delta_{ref.}$ = 0.0 ppm) or TSP (samples in D_2O, $\delta_{ref.}$ = 0.0 ppm) unless otherwise indicated. ^{13}C-n.m.r. spectra were either recorded at 62.38 MHz on a Bruker AM 250 NMR spectrometer, at 50.31MHz on a Varian Gemini-200MHz spectrometer or at 125.77MHz on a Bruker AM 500MHz spectrometer and are referenced to either $CDCl_3$ ($\delta_{ref.}$ = 77.0ppm) or 1,4-dioxan (for samples in D_2O, $\delta_{ref.}$ = 67.3ppm).

Mass spectra in the electron-impact (EI) mode or chemical ionisation (CI) mode were recorded on a VG Micromass 30F spectrometer. Samples requiring field desorption chemical ionization (FD) or fast atom bombardment (FAB) were run on ZAB 1F or VG 20-250 spectrometers. Multichannel analysis was used for FAB samples run on the VG 20-250 spectrometer. Thermaspray (TSP) and hplc mass spectra were run on a VG 20-250 spectrometer.

High performance liquid chromatography (hplc) on crude incubation mixtures was performed with two Waters M-510 A pumps, a Rheodyne 7125 injector, a Waters 441 detector set at 220nm (unless otherwise stated) and columns packed with Hypersil 5 ODS (250 x 4.6mm internal diameter). Preparative scale hplc was performed using two Gilson 303 pumps, a Rheodyne 7125 injector, a

Gilson HM holochrome variable wavelength detector set at 220nm and columns packed with Zorbax ODS (250 x 9.4mm internal diameter).

Optical rotations were determined in either $CHCl_3$ or EtOH solution on a Perkin Elmer 241 polarimeter. Ultraviolet (UV) spectra were recorded on a Perkin Elmer 555 spectrophotometer with quartz cells and water as the solvent, unless otherwise indicated.

N.M.R. Calibration of Penicillin N Samples (General Method A).

The sample to be calibrated was dissolved in D_2O (1.0 ml) which contained 1,4-dioxan (0.10 $\mu l ml^{-1}$, 1.17 $\mu mol\ l^{-1}$). The ^1H-n.m.r. spectrum (500MHz, D_2O, HOD suppressed) was recorded over at least 40 transients and the resonances due to the dioxan, the CH_2CO of 5-amino-5-carboxypentanoic acid side chain and the β-lactam protons integrated. The concentration of the penicillin was then calculated from the equations :

$$\text{Penicillin Concentration} = \{ 1/2 \times \int CH_2CO / \int \text{Dioxan} \} \times 8 \times 1.17\ \mu mol\ l^{-1}$$

$$= \{ 1/2 \times \int \text{combined β-lactams} / \int \text{Dioxan} \} \times 8 \times 1.17\ \mu mol\ l^{-1}$$

Weight of Penicillin = Penicillin Concentration x molecular weight

General Procedure for Isopenicillin N Synthetase Incubations (General Method B).

Partially purified Isopenicillin N synthetase enzyme (2-3 ml, *ca* 1.0 International Units) in TRIS-HCl buffer (50 mMolar; pH 7.4) was exchanged into ammonium bicarbonate buffer (3.5 ml, 50 mMolar; pH 7.8) on a pre-equilibrated sephadex column (PD-10) in a cold-room at 4°C.

To an aqueous solution of the tripeptide (720 µl, *ca* 10 mMolar) was added dithiothreitol (80 µl, 2 mMolar), L-ascorbate (80 µl, 1 mMolar), ferrous sulphate (80 µl, 0.1 mMolar) and catalase (40 µl). The pH was adjusted to 7.8 by the addition of 1N NaOH solution and the enzyme (3.5 ml) added giving a total volume of 4.5 ml. This solution was divided into two aliquots, each of *ca* 2.3 ml and incubated at 27°C and 250 rpm for 10 minutes after which time more dithiothreitol (10 µl, 2 mMolar) was added to each aliquot. After a further 30 minutes the incubation was quenched by the addition of acetone to 70% (v/v). The precipitated protein was spun down by centrifugation (15 Krpm ; 2 minutes ; 0°C), the supernatant evaporated to dryness

and the residue dissolved in D_2O (0.5 ml). After filtering (ACRO LC13 disposable filter assembly, Product No. 4450 Gelman Sciences) the incubation mixture was examined by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed).

General Procedure for DAOC/ DAC synthetase Incubations (General Method C).

Partially purified DAOC/ DAC synthetase (2 ml, *ca* 0.1 International Units) in TRIS-HCl buffer (pH 7.4, 50 mMolar) was pre-incubated for 5 minutes at 27°C and 250 rpm with 200 µl of cofactor solution prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), iron (II) sulphate (1.4 mg) and ammonium sulphate (1.32 g) in distilled water (10 ml). The substrate (1 mg) in TRIS-HCl (1.8 ml, 50 mMolar, pH 7.4) was added and the pH adjusted to pH 7.4 by the addition of 1N NaOH solution. The resulting solution was incubated at 27°C and 250 rpm for 2 hours after which time the protein was precipitated by the addition of acetone to 70% (v/v). After centrifugation (15 Krpm, 2 minutes, 0°C) the supernatant was evaporated to dryness and the residue dissolved in D_2O (0.5 ml). After filtering (ACRO LC13 disposable filter assembly, Product No. 4450 Gelman Sciences) the crude incubation mixture was examined by ^{1}H-n.m.r. (500MHz; D_2O; HOD suppressed).

Derivatisation Procedure: (2R,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-N-Ethoxycarbonylamino-5-methyloxycarbonylpentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic Acid, Methyl Ester [N-Ethoxycarbonyl Penicillin N Dimethyl Ester] (86a-d) and (6R,7R)-1-Aza-3-methyl-7-amino-[(5R)-5-N-ethoxycarbonylamino-5-methyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid, Methyl ester [N-Ethoxycarbonyl Deacetoxycephalosporin C, Dimethyl Ester] (90a-d).
(General Method D).

The penicillin or DAOC sample to be derivatised was dissolved in water (2.5 ml) and the pH adjusted to pH 8-9 by the addition of saturated $NaHCO_3$ solution. Excess diethyl pyrocarbonate (*ca* 50-100µl) was added and the solution stirred at room temperature for 1 hour with occasional addition of saturated $NaHCO_3$ solution to maintain pH 8-9. The solution was then extracted with dichloromethane (3 x 10 ml) and acidified to *ca* pH 2-3 by the addition of 2N HCl. The acidic solution was

rapidly extracted with EtOAc (3 x 25 ml) and the combined organic layers dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo* to dryness.

The residue was dissolved in EtOAc (*ca* 2 ml) and cooled to 0°C in an ice bath set up in an efficient fume hood. An excess of freshly prepared diazomethane was then added and the resulting solution stirred stirred at room temperature for 15 minutes. The solvent and excess diazomethane were removed *in vacuo* in a fume hood and the resulting residue dissolved in the minimum volume of DCM. Hexane (*ca* 1 ml) was added and the product precipitated by removing the DCM with a stream of air. The remaining hexane was removed with a pasteur pipette, the residue dried *in vacuo* and then examined by ^{1}H-n.m.r. (500 MHz, $CDCl_3$) and mass spectrometry (NH_3, DCI).

(86a)[93] δ_H(500MHz, $CDCl_3$) 1.21 (3H, t, *J* 7Hz, C<u>H</u>$_3$CH$_2$O), 1.42 (3H, s, SCMe), 1.64-1.95 (4H, 2 x m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO and 3H, s, SCMe), 2.25-2.42 (2 x m, C<u>H</u>$_2$CO), 3.71 and 3.76 (2 x 3H, 2 x s, 2 x MeO$_2$C), 4.02-4.09 (2H, q, *J* 7Hz, CH$_3$C<u>H</u>$_2$O), 4.28-4.35 (1H, m, C<u>H</u>CH$_2$CH$_2$), 4.38 (1H, s, C<u>H</u>CMe$_2$), 5.19 (1H, d, *J* 8Hz, NH), 5.44 (1H, d, *J* 4Hz, CHC<u>H</u>S), 5.62 (1H, dd, *J* 4, 8Hz, NHC<u>H</u>CHS), 6.10 (1H, d, *J* 8Hz, NH). *m/z* (DCI, NH_3) 479 (2%), 478 (6), 477 ([MNH_4^+], 27), 463 (2), 462 (10), 461 (23), 460 (MH^+, 100), 459 (1). Fragment Ion (68c) 177 (2), 176 (10), 175 (12), 174 (MH^+, 100).

(90a)[93] M.p 172-5°C decomp. (from DCM/petrol). (Found C, 50.2; H, 6.1; N, 8.8%. $C_{19}H_{27}N_3SO_8$ requires C, 49.9 ; H, 6.0 ; N, 9.2%). V_{max} ($CHCl_3$) 1785 (s), 1725 (s), 1602 (m), 1505 (m), 1231 (s); δ_H(500MHz, $CDCl_3$) 1.22 (3H, t, *J* 7Hz, C<u>H</u>$_3$CH$_2$O), 1.64-1.95 (4H, 2 x m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.14 (3H, s, C=CMe), 2.25-2.42 (2 x m, C<u>H</u>$_2$CO), 3.22, 3.53 (2H, ABq, *J* 18Hz, SCH$_2$), 3.76 (3H, s, MeO$_2$C), 3.84 (3H, s, MeO$_2$C), 4.09-4.13 (2H, q, *J* 7Hz, CH$_3$C<u>H</u>$_2$O), 4.34-4.46 (1H, m, C<u>H</u>CH$_2$CH$_2$), 4.97 (1H, d, *J* 4Hz, CHC<u>H</u>S), 5.29 (1H, d, *J* 8Hz, NH), 5.74 (1H, dd, *J* 4 and 8Hz, NHC<u>H</u>CHS), 6.42 (1H, d, *J* 8Hz, NH); *m/z* (DCI, NH_3) 478 (2%), 477 (9), 476 (15), 475 ([MNH_4^+], 60), 474 (2), 462 (3), 461 (6), 460 (28), 459 (23), 458 (MH^+,100), 457 (1). Fragment Ion (68f) 176 (2), 175 (9), 174 (18), 173 (24), 172 (MH^+, 93).

Mixed Label Competitive Kinetic Experiments (General Method E).

An equal quantity of penicillin N (14a) (*ca* 1-3 mg) and the labelled penicillin N (14e) or (14j) (*ca* 1-3 mg) were mixed together and a sample (*ca* 250 μg) derivatised to the <u>N</u>-ethoxycarbonyl penicillin N dimethyl ester (86a/b) or (86a/c) by the usual procedure (General Method D).

The remaining penicillin N mixture was then incubated with DAOC / DAC synthetase (General Method B). Approximately one third of the incubation mixture was quenched by the addition of acetone to 70% (v/v) once it was considered that approximately 30% conversion had been achieved. The remaining incubation mixture was stopped at such a time as to give approximately 60-70% conversion.

After removal of the protein (precipitation, centrifugation), the unconverted penicillin N (14) in the incubation mixture was isolated by chromatography (hplc Waters' system, with 25 mMolar aqueous NH_4HCO_3 as the mobile phase). Each sample was examined by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) and the relative amounts of labelled and unlabelled penicillins present calculated by integration of either the penicillin N (14) C-2 methyl groups [for the mixture of (14a/j)] or C-3H [for the mixture (14a/e)] against the β-lactam or side chain CH_2CO protons. All penicillin samples from each 'conversion point' were derivatised (General Method D) and analysed by mass spectroscopy (NH_3, DCI).

General EEDQ Coupling Procedure (General Method F).

The free amine of the amino-acid benzhydryl ester (63a-f, 119 or 121) or 7-aminodeacetoxycephalosporin C benzhydryl ester (102a-c) or derivatives thereof were dissolved in DCM (*ca* 2 ml) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[67] (1.1eq), anhydrous Na_2SO_4 (ca 5-10 mg) and either (5R)- or (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyl-carbonylpentanamido]-S-*p*-methoxybenzyl-(L)-cysteine (64a or b)[66] (1 eq) respectively, was added. The resulting solution was stirred overnight under argon at room temperature and then evaporated to dryness *in vacuo*. The residue was redissolved in EtOAc (50 ml) and washed with water (25 ml), 2N HCl (25 ml), saturated $NaHCO_3$ solution (25 ml), brine (25 ml), dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo* to give the product, typically as a viscous oil.

8.2 Experimental for Chapter 2.

2-[N-(Diphenylmethylene)amino]-3-methylbutyronitrile (60a).[65]

N-(Diphenylmethylene)aminoacetonitrile (59) (1.00 g, 4.55 mmol), benzyltriethylammonium chloride (100 mg, 0.44 mmol), NaOH (1.10 g as a 50% aqueous solution) and toluene (1ml) were stirred together at 0°C in a round bottom flask sealed with a rubber septum. 2-Bromopropane (89a) (540 mg, 4.4 mmol) in toluene (1ml) was added *via* syringe over a period of 1-2 hours at 0°C. The solution was stirred at room temperature overnight and was then poured into a separating funnel containing dichloromethane (60ml) and water (80ml). The layers were separated and the aqueous layer extracted with DCM (3 x 30ml). The organic layers were combined, washed with water (20ml), brine (20ml), dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo*. Chromatography [flash silica, petrol/diethyl ether (9:1, v/v)] gave (60a) (800 mg, 3.0 mmol, 69% yield) as an oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25.; V_{max} ($CHCl_3$) 2948(s), 2177(w, CN), 1620(s), 1455(m), 1243(s); δ_H(500MHz, $CDCl_3$) 1.00 and 1.12 (2 x 3H, d, *J* 7.5 Hz, 2 x CH_3), 2.11-2.16 (1H, m, C<u>H</u>Me_2), 3.98 (1H, d, *J* 6Hz, C<u>H</u>CHMe_2), 7.20-7.82 (10H, m, Ar<u>H</u>); δ_C(125.77MHz, $CDCl_3$) 18.22 and 18.65 (2 x q, 2 x CH_3), 33.26 (d, <u>C</u>HMe_2), 59.18 (d, <u>C</u>HCHMe_2), 118.51(s, CN), 127.16-138.37 (ArC), 172.57 (s, Ph_2<u>C</u>=N); *m/z* (EI) 261 (5%), 262 (M^+, 8), 263 (MH^+, 12), 264 (3).

2-[N-(Diphenylmethylene)amino]-(2-^{2}H)-3-methylbutyronitrile (60b).

The above procedure was repeated with N-(diphenylmethylene)amino-acetonitrile (59) (887 mg, 4.03 mmol), benzyltriethylammonium chloride (100 mg, 0.44 mmol), NaOD (1.80 g as a 50% aqueous solution), toluene (1ml) and 2-bromopropane (89a) (500 mg, 4.03 mmol) in toluene (1ml). Chromatography [flash silica, petrol/diethyl ether (9:1, v/v)] gave (60b)[144] (675 mg, 2.55 mmol, 63% yield) as an oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25.; V_{max} ($CHCl_3$) 2950 (s), 2890 (m), 2177 (w, CN), 1605 (s), 1455 (m), 1307 (m), and 1243 (s); δ_H (500MHz, $CDCl_3$), 1.00 (3H, d, *J* 7Hz, CHC<u>H</u>$_3$), 1.12 (3H, d, *J* 7Hz, CHC<u>H</u>$_3$), 2.11-2.16 (1H, m, C<u>H</u>Me_2), 7.20-7.66 (10H, m, Ar<u>H</u>); *m/z* (EI) 263 (M^+, 10%), 264 (19), 265 (5), 266 (1).

D/L-Valine (11a).

To the imine (60a) (800 mg, 3.0 mmol) in diethyl ether (10 ml) was added 1N hydrochloric acid (25ml) and the solution vigorously stirred for 12 hours. The organic and aqueous layers were separated and the aqueous phase washed with diethyl ether (3 x 10ml). Concentrated hydrochloric acid (25 ml) was added and the resulting solution refluxed for 12 hours. The solution was evaporated and the amino-acid redissolved in a small quantity of water and purified by chromatography [ion-exchange, Dowex 80-400 acetate form (eluting with water)]. Column fractions were assayed with ninhydrin and the relevant fractions evaporated to dryness *in vacuo* to give racemic (11a)[65] (24 mg, 2.1 mmol, 67% yield) as a white solid. δ_H(500MHz, D_2O, HOD suppressed) 0.99 (3H, d, *J* 8 Hz, $CHCH_3$), 1.04 (3H, d, *J* 8 Hz, $CHCH_3$), 2.25-2.31 (1H, m, $CHCHCMe_2$), 3.60 (1H, d, *J* 4Hz, CHCH); *m/z* (CI, NH_3) 118 (MH^+, 100%), 119 (6), 120 (1).

D/L-(2-^{2}H)-Valine (11h).

Repetition of the above procedure with 2-N-(diphenylmethylene)amino-(2-^{2}H)-3-methylbutyronitrile (60b) (675 mg, 2.55 mmol) gave (2-^{2}H)-valine (11h)[144] (250 mg, 2.1 mmol, 80% yield). δ_H (500MHz, D_2O, HOD suppressed) 1.03 (3H, d, *J* 7Hz, $CHCH_3$), 1.05 (3H, d, *J* 7Hz, $CHCH_3$), 2.30-2.38 (1H, m, $CHMe_2$); *m/z* (CI, NH_3) 118 (4%), 119 (MH^+, 100), 120 (6), 121(1).

D/L-Valine Benzhydryl Ester, Ammonium Tosylate Salt.

To the racemic amino-acid (11a) (100 mg, 0.85 mmol) in water (10 ml) was added *p*-toluenesulphonic acid (163 mg, 0.85 mmol) dissolved in THF (10 ml). The resulting solution was evaporated to dryness *in vacuo* and the solid residue suspended in MeCN (20 ml). The suspension was stirred and titrated with Ph_2CN_2 (165-250 mg, 1-1.5 mmol) in MeCN (5 ml), until a pale pink colouration persisted. After 30 minutes the white precipitate was filtered off and washed with petrol (10 ml) to give the product (360 mg, 0.80 mmol, 94% yield)[145]. δ_H(500MHz, $CDCl_3$) 0.80-0.84 (6H, m, $CHMe_2$), 2.18-2.28 (1H, m, $CHCHMe_2$ and 3H, s, $ArCH_3$) 3.99 (1H, *ca* t, *J* 4Hz, $CHCHMe_2$), 6.85 (1H, s, $CHPh_2$), 6.98-7.65 (14H, m, ArH).

D/L-(2-^{2}H)Valine Benzhydryl Ester, Ammonium Tosylate Salt.

Repetition of the above procedure with the amino-acid (11h) (100 mg, 0.65 mmol) in water (10 ml), *p*-toluenesulphonic acid (124 mg, 0.65 mmol) and diphenyldiazomethane (1.0-1.5 eq) gave the title compound (186 mg, 0.41 mmol, 63% yield). M.p.169-70 °C (from EtOH/Et_2O/petrol). (Found C 65.60, H 6.62 and N 2.95. $C_{25}H_{28}DNSO_5$ requires C 65.77, H 6.40 and N 3.07%); δ_H (500MHz,$CDCl_3$) 0.82-0.85 (6H, m, CH$\underline{Me}_2$), 2.21-2.28 (1H, m, CDC$\underline{H}Me_2$ and 3H, s, ArC$\underline{H}_3$), 6.86 (1H, s, C$\underline{H}Ph_2$), 6.96-7.64 (14H, m, Ar$\underline{H}$); *m/z* (FAB) 285 (MH^+-TsO^-, 9%), 286 (2), 287 (1).

[(5$\underline{R}$)-5-$\underline{N}$-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-$\underline{S}$-*p*-methoxybenzyl-$\underline{L}$-cysteinyl-$\underline{D}$-valine Benzhydryl Ester (65a).

The ammonium tosylate salt of the $\underline{D}$-valine benzhydryl ester (77 mg, 0.17 mmol) was suspended in saturated aqueous $NaHCO_3$ (10 ml) and the free amine extracted into EtOAc (3 x 20 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and evaporated to dryness *in vacuo*.

To the free amine (63a) (46 mg, 0.17 mmol) in dichloromethane (2 ml) was added (5$\underline{R}$)-5-$\underline{N}$-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonyl-pentanamido-$\underline{S}$-*p*-methoxybenzyl-$\underline{L}$-cysteine (64a) (116 mg, 0.17 mmol), EEDQ (47 mg, 0.19 mmol) and anhydrous Na_2SO_4 (*ca* 5 mg). The resulting mixture was stirred at room temperature under argon for 24 hours.

The solution was evaporated to dryness *in vacuo* and the residue partitioned between EtOAc (50 ml) and water (50 ml) in a separating funnel. The layers were separated and the organic phase washed with 2$\underline{N}$ HCl (20 ml), saturated aqueous $NaHCO_3$ (20 ml) and brine (20 ml). The solution was dried (anhydrous Na_2SO_4), filtered and evaporated to dryness *in vacuo*. Chromatography [flash silica, with dichloromethane/ ethyl acetate (4:1 to 1:3, v/v)] gave (65a)[146] (125 mg, 0.14 mmol, 82% yield) as a white solid. T.l.c., [ethyl acetate/ petrol (1:1, v/v)] Rf 0.45; V_{max} ($CHCl_3$) 3020 (s), 1736 (s), 1682 (s) and 1515 (s); δ_H (500MHz, $CDCl_3$) 0.76 (3H, d, *J* 7Hz, CHC$\underline{H}_3$), 0.87 (3H, d, *J* 7Hz, CHC$\underline{H}_3$), 1.62-1.86 (4H, m, C$\underline{H}_2$C$\underline{H}_2$$CH_2$), 2.05-2.25 (3H, m, CH_2CO and C$\underline{H}(CH_3)_2$), 2.67, 2.82 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.70 (2H, s, SC$\underline{H}_2$Ar), 3.74 (3H, s, $SCH_2ArO\underline{Me}$), 3.78 (6H,s, 2 x $CH_2ArO\underline{Me}$), 4.28-4.35 (1H, m, C$\underline{H}CH_2CH_2$), 4.51 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, C$\underline{H}CH_2S$), 4.62-4.64 (1H, m, C$\underline{H}$CHMe$_2$), 5.00, 5.02 (2H, ABq, *J* 12Hz, OC$\underline{H}_2$Ar), 5.07 (2H, s, OC$\underline{H}_2$Ar), 5.45 and 6.29 (2H, 2 x d, *J* 8Hz, 2 x NH), 6.79-6.89 and 7.16-7.31 (23H, 2 x m, C$\underline{H}Ph_2$ and Ar$\underline{H}$); δ_C (125.77MHz, $CDCl_3$) 17.24 (q, CH$\underline{C}H_3$) 19.02 (q, CH$\underline{C}H_3$), 21.24 (t, $CH_2\underline{C}H_2CH_2$),

31.09 (d, CHMe2), 31.77 (t, CH2CH2CH2CO), 33.18, 35.27, 35.82 (3 x t, SCH2Ar, CH2S, and CH2CO), 52.14 and 53.59 (2 x d, NHCHCH2 and NHCHCHS), 55.17 (q, ArOMe), 57.31 (d, CHCHMe2), 66.74 and 66.92 (2 x t, CH2Ar), 77.93 (d, CHPh2), 113.86, 113.96 and 114.04 (3 x d, Ar), 126.89-139.57 (m, Ar), 156.09, 158.76, 159.52 and 159.73 (4 x s, ArO2CNH and C-4 of ArOMe), 170.24, 170.56, 172.06 and 172.48 (4 x C=O); *m/z* (FD) 933 (M^+).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-$^2H_1$)-valine Benzhydryl Ester (65b).

Similarly, repetition with ammonium tosylate salt of the D/L-(2-^{2}H)-valine benzhydryl ester (342 mg, 0.75 mmol) and (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (64)[66] (500 mg, 0.75 mmol) and EEDQ (207 mg, 0.83 mmol) gave (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoybenzyl-L-cysteinyl-D-(2-^{2}H)-valine benzhydryl ester (65b)[146] (248 mg, 0.27 mmol, 36% yield).T.l.c., [ethyl acetate/ petrol (1:1, v/v)] Rf 0.45; V_{max} ($CHCl_3$) 3020(s), 1725(s), 1670(m) and 1520(s); δ_H (500MHz, $CDCl_3$) 0.77 (3H, d, *J* 7Hz, CHCH3), 0.88 (3H, d, *J* 7Hz, CHCH3), 1.55-1.85 (4H, m, CH2CH2CH2), 2.0-2.26 (3H, m, CH2CO and CH(CH3)2), 2.65, 2.84 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH2S), 3.72 (2H, s, SCH2Ar), 3.76 (3H, s, SCH2ArOMe), 3.79 (6H,s, 2 x CH2ArOMe), 4.28-4.35 (1H, m, CHCH2CH2), 4.47 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, CHCH2S), 5.00, 5.02 (2H, ABq, *J* 12Hz, OCH2Ar), 5.46 and 6.29 (2H, 2 x d, *J* 8Hz, 2 x NH), 6.78-6.90 and 7.23-7.32 (23H, 2 x m, CHPh2 and ArH); δ_C (125.77MHz, $CDCl_3$) 17.25 (q, CHCH3) 18.75 (q, CHCH3), 21.20 (t, CH2CH2CH2), 30.81 (d, CHMe2), 31.51 (t, CH2CH2CH2CO), 33.27, 35.12, 35.68 (3 x t, SCH2Ar, CH2S, and CH2CO), 52.08 and 53.55 (2 x d, NHCHCH2 and NHCHCHS), 54.97 (q, ArOMe), 57.29 (br m, CDCH), 66.52 and 66.69 (2 x t, CH2Ar), 77.73 (d, CHPh2), 113.70, 113.80,and 113.84 (3 x d, Ar), 126.72-139.45 (m, Ar), 156.09, 58.57, 159.35 and 159.55 (4 x s, ArO2CNH and C-4 of ArOMe), 170.29, 170.46, 171.97, and 172.48 (4 x C=O); *m/z* (FAB) 934 (7%), 935 (MH^+, 100), 936 (62), 937 (29), 938 (13).

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-valine (66a).

To the dry fully protected tripeptide (65a) (70 mg, 0.08 mmol) under argon was added anisole (0.4 ml) and freshly distilled trifluoroacetic acid (2.0 ml). Immediately upon addition of the acid, the stirred solution turned pink and the solution was refluxed for 30 minutes. Evaporation of this mixture gave a residue which was redissolved in dry toluene and once more evaporated to dryness. The

solid material was partitioned between EtOAc (5 ml) and water (5 ml). After separation, the aqueous phase was washed with EtOAc (2 x 5 ml) and lyophilised to give the deprotected tripeptide as the ammonium trifluoroacetate salt (66a)[146] (26 mg, 0.07 mmol, 90% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.92-0.99 (6H, m, $CH(CH_3)_2$), 1.65-2.02 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.16-2.24 (1H, m, $CH(CH_3)_2$), 2.41 (2H, *ca* t, *J* 7Hz, CH_2CO), 2.88, 2.94 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.98 (1H, *ca*.t, *J* 6Hz, $CHCH_2S$), 4.28 (1H, d, *J* 6Hz, $CHCHMe_2$), 4.57 (1H, *ca*.t, *J* 7Hz, CH_2CHNH_3); δ_C(125.77MHz, D_2O); 18.02 (q, $CHCH_3$), 19.23 (q, $CHCH_3$), 21.45 (t, $CH_2CH_2CH_2CO$), 26.15 (t, $CH_2CH_2CH_2CO$), 30.00 (t, $CH_2CH_2CH_2CO$), 30.68 (d, $CH(CH_3)_2$), 35.29 (t, CH_2SH), 53.74 (d, $CHCH_2S$), 56.28 (d, $CHNH_3$), 59.18 (d, $CHCO_2H$), 172.64, 172.95, 175.62 and 176.37 (4 x s, 4 x C=O); *m/z* (FAB) 362 (6%), 363 (10),364 (MH^+,100), 365 (23), 366 (10).
Identical by ^{1}H-n.m.r. and mass spectroscopy to Lit. ref.[69]

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H)valine (66b).

The above procedure was repeated with (65b) (96 mg, 0.10 mmol) to give (66b)[146] (32 mg, 0.09 mmol, 90% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.92-0.99 (6H, *ca* t, *J* 7Hz, $CH(CH_3)_2$), 1.66-2.01 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.17-2.24 (1H, m, $CH(CH_3)_2$), 2.42 (2H, *ca*. t, *J* 7Hz, CH_2CO), 2.98, 2.95 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.97 (1H, *ca*. t, *J* 6Hz, $CHCH_2S$), 4.57 (1H, *ca*. t, *J* 7Hz, CH_2CHNH_3) ; δ_C(125.77MHz, D_2O) 17.96 (q, $CHCH_3$), 19.20 (q, $CHCH_3$), 21.45 (t, $CH_2CH_2CH_2CO$), 26.18 (t, $CH_2CH_2CH_2CO$), 30.00 (t, $CH_2CH_2CH_2CO$), 30.58 (d, $CH(CH_3)_2$), 35.26 (t, CH_2SH), 53.78 (d, $CHCH_2S$), 56.28 (d, $CHNH_3$), 58.91 (m, $CDCO_2H$), 172.73, 173.06, 175.66 and 176.34 (4 x s, 4 x C=O); *m/z* (FAB) 363 (2%), 364 (5), 365 (MH^+,100), 366 (18), 367 (10), 368 (2). [Identical to (66a) by ^{1}H-n.m.r. except for the absence of a doublet at δ4.28 ppm]

(2R,5R,6R)-1-Aza-(2-^{2}H)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14e). [(3-^{2}H)-Penicillin N]

Incubation of (5R)-5-amino-5-carboxypentanamido-L-cysteinyl-D-(2-^{2}H)-valine (66b) (10 mg) with partially purified isopenicillin N synthetase enzyme (General Method B) gave after chromatography [hplc Gilson system, eluting with 0.75% MeCN in 5mMolar aqueous NH_4HCO_3, retention time 8 minutes], (2R,5R,6R)-1-aza-(2-^{2}H)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thia-

bicyclo[3.2.0]heptane-2-carboxylate (14e)[153] (2.1 mg, *ca* 20% yield). δ_H (500MHz, D_2O, HOD suppressed) 1.52 (3H, s, CH_3), 1.63 (3H, s, CH_3), 1.65-1.96 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.77 (1H, *ca* t, *J* 6Hz, $CHCH_2CH_2$), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, CHCHS). Identical to authentic penicillin N (14a) except for the absence of a singlet at δ4.34 ppm.

Incubation of (2R,5R,6R)-1-aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14a) with DAOC/DAC Synthetase Enzyme.

(2R,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14a) (*ca* 1mg) was incubated with partially purified DAOC/DAC synthetase according to the usual procedure (General Method C). Examination of the crude incubation mixture by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ 4.9-5.5 ppm indicated that three β-lactam compounds had been formed in addition to unconverted penicillin N (14a). These ring expanded products were identified as deacetoxycephalosporin C (18a), deacetylcephalosporin C (19a) and the 3β-hydroxycepham (46a) by comparison to authentic samples. Integration of this region indicated that the ratio of these products was *ca* 80:1 [(18a) + (19a):(46a)].

Incubation of (2R,5R,6R)-1-aza-(2-^{2}H)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14e) with DAOC/DAC Synthetase Enzyme.

(2R,5R,6R)-1-Aza-(2-^{2}H)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14e) (*ca* 1mg) was incubated with partially purified DAOC/DAC synthetase in the usual manner (General Method C). Examination of the crude incubation mixture by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ4.9-5.5 ppm indicated that deacetoxycephalosporin C (18a), deacetylcephalosporin C (19a) and the (4-^{2}H)-3β-hydroxycepham (46b) had been formed as judged by comparison with authentic samples. Integration of this region indicated that the ratio of these products was *ca*.65:35 [(18a) + (19a):(46b)]. Purification [hplc, Waters' system, 25 mMolar aqueous NH_4HCO_3] gave (18a), (19a) and (46b).
(46b); δ_H (500MHz, D_2O, HOD suppressed) 1.36 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca*.t, *J* 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, *J* 14Hz, SCH_2), 3.75

(1H, m, C<u>H</u>CH$_2$CH$_2$), 5.29, 5.46 (2H, ABq, *J* 4Hz, C<u>H</u>C<u>H</u>S). Identical to authentic 3β-hydroxycepham (46a) except for the absence of a singlet at δ4.16 ppm.
; *m/z* (FAB) 377 (MH^+)

Incubation of (2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-aza-(2-^{2}H)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14e) with DAOC/DAC Synthetase Enzyme under an Atmosphere of $^{18}O_2$ Gas.

A solution of 50 mMolar TRIS-HCl, pH7.4 (12ml) in a 2-necked 100ml round bottom flask was thoroughly degassed after which the vessel was returned to atmospheric pressure with argon. This procedure was then repeated a further three times. A sample of $^{18}O_2$ gas (99.8 atom%^{18}O, 40ml) was transferred to the incubation vessel *via* gas tight syringe and introduced into the TRIS-HCl solution through a rubber septum. The solution was vigorously stirred by means of an efficient magnetic stirrer for 3 hours.

The DAOC/DAC synthetase enzyme (*ca* 1 I.U.) in TRIS-HCl (4ml) was injected into the incubation vessel and stirred for 5 minutes. Cofactors [160 µl containing α-ketoglutarate (14.6 mg), <u>L</u>-ascorbate (17.6 mg), iron (II) sulphate (1.4 mg) and dithiothreitol (30.8 mg) in a previously withdrawn sample of the oxygenated TRIS-HCl solution (2ml)] were added and the vessel briefly degassed and returned to atmospheric pressure with argon. $^{18}O_2$ gas (40ml) was injected into the enzyme solution which was then stirred for 2 minutes after which time (2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-aza-(2-^{2}H)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo [3.2.0]heptane-2-carboxylate (14e) (*ca* 3mg) in degassed TRIS-HCl (1ml) was added *via* syringe. The resulting solution was then shaken at 250 rpm, 27°C for 2 hours afterwhich the protein was precipitated by the injection of acetone to 70% (v/v) and the incubation worked up as in the general procedure.

Purification as before gave the β-lactam compounds (14e), (18a), (19b) and (46c). Mass spectral analysis upon derivatised (14e) and (18a) (see General Method D) indicated no incorporation of ^{18}O. Conversely, analysis of (19b) as its lactone (67b)[131] [prepared by stirring (19b) in freshly distilled formic acid and purification by hplc (Gilson system, eluting with 25 mMolar aqueous NH_4HCO_3)] indicated *ca* 50% incorporation of label (see Expt.1 in Tables 8.1-8.4 below)[71].

This procedure was repeated with a further sample of (2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-aza-(2-^{2}H)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo [3.2.0]heptane-2-carboxylate (14e) (*ca* 3.6mg) and the products isolated and analysed as above (see Expt.2 in Tables 8.1-8.4).

Expt.	*m / z* (MH$^+$)	*376*	*377*	*378*	*379*	*380*	*381*
1	Found (%)	29	80	53	100	38	8
2	Found (%)	-	69	19	100	23	15
Synthetic							
3β-hydroxy-	*m / z* (MH$^+$)	*375*	*376*	*377*	*378*	*379*	*380*
cepham (46a)[147]	Found (%)	7	100	22	10	1	-
	Calc. (%)	-	100	18	7	1	-

Table. 8.1; (4-^{2}H)-3β-Hydroxycepham(46c).

Expt.	*m / z* (MH$^+$)	*355*	*356*	*357*	*358*	*359*	*360*
1	Found (%)	4	100	25	72	27	22
	m/z						
	(Fragment (68a/b))	*155*	*156*	*157*	*158*	*159*	*160*
1	Found (%)	4	100	14	70	10	6
2	Found (%)	4	100	13	54	7	6
	Calculated	-	100	8	5	-	-

Table 8.2; Deacetylcephalosporin C (19a/b)

Expt.							
	m/z (Fragment (68d))	*174*	*175*	*176*	*177*	*178*	*179*
1	Found (%)	20	100	18	15	12	8

Table 8.3; (3-^{2}H)-Penicillin N derivative (86c)

Expt.	*m/z* deriv. (90a)(MH$^+$)	*457*	*458*	*459*	*460*	*461*	*462*
1	Found (%)	3	100	26	52	14	10
2	*m / z* (MH$^+$)	*357*	*358*	*359*	*360*	*361*	*362*
	Found (%)	6	100	34	9	7	5
	m/z (Fragment (86f))	*157*	*158*	*159*	*160*	*161*	*162*
	Found (%)	1	100	10	10	16	3

Table 8.4; Deacetoxycephalosporin C (18a)

(2R,3S,6R,7R)-1-Aza-3-methyl-[3-chloro-2-oxo-1-oxapropyl]-7-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitrobenzylcarbonyl-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic Acid Benzyl Ester.

(2R,3S,6R,7R)-1-Aza-3-methyl-[3-chloro-2-oxo-1-oxapropyl]-7-amino-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester[148] (175 mg, 0.44 mmol), EEDQ (122 mg, 0.48 mmol) and (5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-

p-nitrobenzylcarbonylpentanoic acid[149] (210 mg, 0.44 mmol) were coupled by the usual procedure (General Method F). Chromatography [flash silica, EtOAc/petrol (1:1, v/v)] gave (2R,3S,6R,7R)-1-aza-3-methyl-[3-chloro-2-oxo-1-oxapropyl]-7-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitrobenzylcarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester (270 mg, 0.32 mmol, 72% yield)[147]. T.l.c., [EtOAc/petrol (1:1, v/v)] Rf 0.1; V_{max} ($CHCl_3$) 3020 (s), 1741 (s), 1526(s), 1349 (s); $[\alpha]_D^{20}$ +14.6 [c = 6.2, $CHCl_3$]; δ_H (500MHz, $CDCl_3$) 1.51 (3H, s, CH_3), 1.61-1.80 (4H, m, $CH_2CH_2CH_2CO$), 2.26-2.39 (2H, m, CH_2CO), 3.42 (2H, ABq, *J* 14Hz, SCH_2), 4.05 (2H, ABq, *J* 14Hz, $COCH_2Cl$), 4.45 (1H, m, $CHCH_2CH_2$), 4.81 (1H, s, $CHCO_2H$), 5.19-5.30 (7H, m, CHCHS, OCH_2Ph and 2 x CH_2ArNO_2), 5.48 (1H, d, *J* 9Hz, NH), 5.57 (1H, dd, *J* 4, 9Hz, $NHCHCH_2S$), 6.30 (1H, d, *J* 9Hz, NH), 7.37 (5H, m, CH_2Ph), 7.50, 8.22 (8H, 2 x m, ArH).

(2R,3S,6R,7R)-1-Aza-3-methyl-3-hydroxy-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylate (46a).

Thiourea (16 mg, 0.21 mmol) and (2R,3S,6R,7R)-1-aza-3-methyl-[3-chloro-2-oxo-1-oxapropyl]-7-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitrobenzylcarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester (100 mg, 0.12 mmol) were disolved in freshly distilled EtOH (2 ml) and the resulting solution stirred at 60°C under argon for 1 hour. The solvent was evaporated and the residue resuspended in EtOAc (50 ml), washed with water (3 x 20 ml), brine (20 ml), dried (anhydrous Na_2SO_4), and filtered. The solvent was evaporated *in vacuo* to give the crude alcohol which was then dissolved in THF/water [1:1, v/v (20 ml)] and hydrogenated with 10% Pd/C under an atmosphere of H_2 for 5 hours, followed by filtration through celite, washing with EtOAc (3 x 5 ml) and freeze-drying to give the deprotected 3β-hydroxycepham (46a). Chromatography [hplc, Gilson system, flow rate 4 $ml.min^{-1}$, with 5mM aqueous NH_4HCO_3/MeCN, (99.75 : 0.25, v/v)] gave (46a) (13 mg, 0.03mmol, 25% yield) Rt 4.5 minutes and the lactone (69a) (8 mg, 0.02mmol, 15% yield) Rt 9 minutes.

(46a). δ_H (500MHz, D_2O, HOD suppressed) 1.39 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2$), 2.42 (2H, *ca*.t, *J* 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, *J* 14Hz, SCH_2), 3.75 (1H, m, $CHCH_2CH_2$), 4.16 (1H, s, $CHCO_2H$), 5.29, 5.45 (2H, ABq, *J* 4Hz, CHCHS); δ_C (125.77MHz, D_2O); 21.67 (t, $CH_2CH_2CH_2$), 26.52 (q, CH_3), 30.57 (t, $CHCH_2CH_2$), 34.89 (t, SCH_2), 35.63 (t, CH_2CO), 55.08 , 55.27 (2 x d, CHCHS), 59.39 (d, $CHCH_2CH_2$), 64.42 (d, $CHCO_2H$), 65.57 (s, COH), 167.79, 174.97, 172.09 and 177.30 (C=O); m/z (FAB) 375 (7%),

376 (MH^+, 100), 377 (22), 378 (10), 379 (1). Identical by ^{1}H-n.m.r. and mass spectroscopy to previously reported data.[61]

3β-Hydroxycepham Lactone (69a); δ_H (500MHz, D_2O, HOD suppressed) 1.49 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.39-2.45 (2H, m, CH_2CO), 3.03, 3.34 (2H, ABq, *J* 13Hz, SCH_2), 3.74 (1H, m, $CHCH_2CH_2$), 4.12 (1H, s, $CHCO_2H$), 4.32 (1H, s, CHCHS), 5.44 (1H, s, CHCHS); δ_C (125.77MHz, D_2O); 21.56 (t, $CH_2CH_2CH_2$), 25.82 (q, CH_3), 30.53 (t, $CHCH_2CH_2$), 30.63 (t, SCH_2), 35.35 (t, CH_2CO), 55.09 , 59.41, 61.73 and 64.44 (4 x d, $CHCH_2CH_2$, $CHCO_2H$ and CHCHS), 81.52 (s, COH), 167.79, 172.02, 175.84 and 177.30 (4 x C=O).

Incubation of 7β-[(5R)-5-Amino-5-carboxypentanamido]-3β-hydroxy-3-methyl-[4-^{2}H]-cepham-4-carboxylic Acid (46b) with Partially Purified DAOC/DAC Synthetase Enzyme.

The title compound (46b) (*ca* 60μg) was incubated with partially purified DAOC/DAC synthetase enzyme using the general incubation procedure (General Method C). The incubation mixture was bio-assayed against *E.coli* ESS(+) and *S. aureus* at a concentration of 100μg of (46b) or derived product ml^{-1}. No antibacterial activity was observed. Similarly pure (46a) was assayed against the same organisms at the same concentration (100μg ml^{-1}) and no antibacterial activity observed.

2-[N-(Diphenylmethylene)amino]-(2-^{2}H,3-^{13}C)-3-methylbutyronitrile (60c).

The reaction was carried out as for compound (60a) except that N-(diphenylmethylene)aminoacetonitrile (59) (887 mg, 4.03 mmol), benzyltriethylammonium chloride (100 mg, 0.4 mmol), NaOH (1.8 g as a 50% aqueous solution), toluene (1ml) and 2-(2-^{13}C)-bromopropane (500 mg, 4.03 mmol, 92.2 atom % ^{13}C) in toluene (1ml) were used. Chromatography [flash silica, petrol/diethyl ether (9:1, v/v)] gave (60c)[144] (840 mg, 3.17 mmol, 79% yield) as a pale green oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25.; δ_H (500MHz, $CDCl_3$) 0.86-0.94 and 1.03-1.05 (6H, 2 x m, $J_{^{13}C\text{-}1H}$ 85Hz, $^{13}CH(CH_3)_2$), 1.85-1.94, 2.04-2.08 and 2.15-2.22 (1H, 3 x m, $J_{^{13}C\text{-}1H}$ 131Hz, $^{13}CH(CH_3)_2$), 7.11-7.85 (10H, m, ArH); δ_C(125.77MHz, $CDCl_3$) 18.50 and 18.79 (2 x dq , *J* 53Hz $^{13}CH(CH_3)_2$), 33.41 (d, $J_{^{13}C\text{-}1H}$ 72Hz,$^{13}CHMe_2$), 118.81 (s, Ph_2C=N), 127.43-138.62 (Ph), 172.85 (s, CN).

(2-^{2}H, 3-^{13}C)-Valine (11m).

Procedure as for compound (11a) except that 2-[N-(diphenylmethylene)amino]-(2-^{2}H,3-^{13}C)-3-methylbutyronitrile (60c) (840 mg, 3.17 mmol) were used to give (2-^{2}H, 3-^{13}C)-valine (11m)[144] (374 mg, 3.14 mmol, 94% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.99 and 1.04 (2 x 3H, 2 x m, $^{13}CH(CH_3)_2$), 2.14, 2.27 and 2.40 (1H, 3 x m, $J_{^{13}C-^{1}H}$ 67Hz, $^{13}CH(CH_3)_2$ and $^{12}CH(CH_3)_2$); δ_C(125.77MHz, D_2O) 17.34 and 18.62 (dq, $J_{^{13}C-^{13}C}$ 34Hz, $^{13}CH(CH_3)_2$), 29.67 (d, $J_{^{13}C-^{1}H}$ 72Hz, ^{13}CH); *m/z* (FAB) 118 (2%), 119 (17), 120 (MH^+, 100), 121 (5), 122 (1).

D/L-(2-^{2}H, 3-^{13}C)-Valine Benzhydryl Ester, Ammonium Tosylate Salt.

The reaction was carried out as for D/L-valine benzhydryl ester ammonium tosylate salt except that (2-^{2}H, 3-^{13}C)-valine (11m) (200 mg, 1.7 mmol) , *p*-toluenesulphonic acid (335 mg, 1.75 mmol) and diphenyldiazomethane (330 mg, 1.75 mmol) were used to give the title compound (709 mg, 1.6 mmol, 90% yield)[145]. M.p 173-4°C; (Found C 65.46, H 6.55, N 2.95%. $C_{24}{}^{13}CH_{28}DSNO_5$ requires C 65.64, H 6.39 and N 3.06%). δ_H (500MHz, $CDCl_3$) 0.82-0.85 (6H, m, CHMe$_2$), 2.06-2.12, 2.21-2.28 and 2.30-2.48 (4H, 3 x m, CD^{13}CHMe$_2$ and 3H, s, ArCH$_3$), 6.86 (1H, s, CHPh$_2$), 6.96-7.64 (14H, m, ArH).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H,3-^{13}C)-valine Benzhydryl Ester (65c).

Procedure as for compound (65a) except that D/L-(2-^{2}H,3-^{13}C)-valine benzhydryl ester (150 mg, 0.53 mmol), [(5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteine (64a) (352 mg, 0.53 mmol), EEDQ (146 mg, 0.58 mmol) and anhydrous Na_2SO_4 (*ca* 10 mg) were used. Chromatography [preparative plate, EtOAc/Hexane (1:1, v/v)] gave (65c)[146] (175 mg, 0.19 mmol, 36% yield) and the diastereomer (170 mg, 0.18 mmol, 35% yield). V_{max} ($CHCl_3$) 3020(s), 1725(s), 1670(m) and 1520(s); δ_H (500MHz, $CDCl_3$) 0.74-0.77 and 0.86-0.89 (6H, 2 x m, $J_{^{13}C-^{1}H}$ 57Hz, $^{13}CH(CH_3)_2$), 1.55-1.85 (4H, m, $CH_2CH_2CH_2CO$), 2.04-2.26 (3H, m, CH_2CO and $^{13}CH(CH_3)_2$), 2.65, 2.84 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7, J_{BX} 6Hz, CH_2S), 3.72 (2H, s, SCH_2Ar), 3.76 (3H, s, SCH_2ArOMe), 3.79 (6H,s, 2 x CH_2ArOMe), 4.34 (1H, br s, $CHCH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7, J_{BX} 6Hz, $CHCH_2S$), 5.00, 5.02 (2H, ABq, J 12Hz, OCH_2Ar), 5.44 (1H, br.s, NH),

6.23 (1H, br s, NH), 6.78-6.90 and 7.23-7.32 (23H, 2 x m, C<u>H</u>Ph2 and ArH); *m/z* (FAB) 934 (7%), 935 (MH^+, 100), 936 (62), 937 (29), 938 (13).

[(5<u>R</u>)-5-Amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H,3-^{13}C)-valine (66c).

Procedure as for (66a) except that [(5<u>R</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonyl-amino-5-*p*-methoxybenzyloxycarbonylpentanamido]-<u>S</u>-*p*-methoxybenzyl-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H, 3-^{13}C)-valine benzhydryl ester (65c)[146] (115 mg, 0.12 mmol) was used to give [(5<u>R</u>)-5-amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H, 3-^{13}C)-valine (66c) as the ammonium trifluoroacetate salt (47 mg, 0.10mmol, 82% yield). δ_H (500MHz, D_2O, <u>H</u>OD suppressed) 0.90-0.97 (6H, m, ^{13}CH(C<u>H</u>3)2), 1.64-2.02 (4H, 2 x m, C<u>H</u>2C<u>H</u>2CH2CO), 2.05, 2.19, 2.34 (1H, 3 x m, $J_{^{13}C\text{-}1H}$ 130Hz, ^{13}CHMe2), 2.42 (2H, *ca* t, 7 Hz, CH2CO), 2.87, 2.95 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7, J_{BX} 6Hz, CH2S), 3.96 (1H, *ca* t, *J* 6Hz, C<u>H</u>CH2S), 4.56 (1H, *ca* t, *J* 7Hz, CH2C<u>H</u>NH3); *m/z* (FAB) 365 (MH^+ for $^{12}C_{14}H_{23}DN_3O_5$, 22%), 366 (MH^+ for $^{13}C^{12}C_{13}H_{23}DN_3O_5$, 100), 367 (21), 368 (1).

(2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f). [(2-^{13}C, 3-^{2}H)-Penicillin N]

[(5<u>R</u>)-5-Amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H,3-^{13}C)-valine (66c) (14 mg) was incubated with IPNS (*ca* 30 I.U.) according to the usual procedure (General Method B). Chromatography [hplc Gilson system, 0.75% MeCN in 5mMolar aqueous NH_4HCO_3, retention time 8 minutes], gave (2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f)[153] (2.8 mg, *ca* 20% yield). δ_H (500MHz, D_2O, <u>H</u>OD suppressed) 1.51 (3H, d, $J_{^{13}C\text{-}1H}$ 4 Hz,^{13}CHC<u>H</u>3), 1.63 (3H, d, $J_{^{13}C\text{-}1H}$ 4 Hz,^{13}CHC<u>H</u>3), 1.65-1.95 (4H, 2 x m, C<u>H</u>2C<u>H</u>2CH2CO), 2.40 (2H, *ca* t, *J* 7Hz, CH2CO), 3.77 (1H, *ca* t, *J* 6Hz, C<u>H</u>CH2CH2), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, C<u>H</u>C<u>H</u>S). Identical by ^{1}H-n.m.r. to authentic penicillin N (14a) except for the presence of additional ^{13}C-coupling to the C-3 methyl groups and the absence of a singlet at δ4.34 ppm.

Incubation of (2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5<u>R</u>)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f) with DAOC/DAC Synthetase Enzyme under an Atmosphere of $^{18}O_2$ Gas.

A solution of 10 mMolar TRIS-HCl (12ml, pH7.4) in a 2-necked 50ml round bottom flask was thoroughly degassed after which the vessel was returned to atmospheric pressure with argon. This procedure was then repeated a further three times. Partially purified DAOC/DAC synthetase (4 ml, *ca* 0.4 I.U) in TRIS-HCl (10 mM) was briefly degassed by bubbling argon through the enzyme solution and then transferred *via* syringe to a 100 ml flask containing $^{18}O_2$ gas (98.6 atom % ^{18}O , supplied by Amersham International plc). To this solution was added, *via* syringe, the usual cofactors and co-substrates which had been prepared in the degassed TRIS-HCl buffer. After 5 minutes, (2R,5R,6R)-1-aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f) (*ca* 1.5 mg) in degassed water (1 ml) was added *via* syringe and the resulting mixture shaken at 250 r.p.m for 2 hours at 27°C. The incubation mixture was worked up as usual (General Method C) and then examined by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) which indicated approximately 40% conversion to the usual cephalosporin products in addition to the 3β-hydroxycepham (46d). Examination of the crude incubation mixture by overnight ^{13}C-n.m.r (125.77 MHz, D_2O) revealed a signal at δ 81.41 ppm [^{13}C-^{18}O of 3β-hydroxycepham lactone (69d)].

Incubation of (2R,5R,6R)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f) with DAOC/DAC Synthetase under an Atmosphere of $^{16}O_2$ Gas.

The crude, [partially converted to (46d), DAOC and DAC], (2R,5R,6R)-1-aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f) from the previous incubation under an atmosphere of $^{18}O_2$ gas, was re-incubated with partially purified DAOC/DAC synthetase (*ca* 0.5 I.U.) under an atmosphere of $^{16}O_2$ gas according to the usual procedure (General Method C). Examination of the crude incubation mixture by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) indicated that approximately 10% of the (2R,5R,6R)-1-aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14f) remained. Examination of the total crude incubation mixture by overnight ^{13}C-n.m.r (125.77 MHz, D_2O) revealed a second signal at δ 81.38 ppm [^{13}C-^{16}O of 3β-hydroxycepham lactone (69e)] in addition to the ^{13}C-^{18}O signal at δ 81.41 ppm.

8.3 Experimental for Chapter 3.

(2R,3R,5R,6R)-1-Aza-(2-^{2}H)-3-methyl-3-(^{13}C-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14i). [(2R)-(2'-^{13}C, 3-^{2}H)-penicillin N].

[(5R)-5-Amino-5-carboxypentanoyl]-L-cysteinyl-D-(3S)-(2-^{2}H,4-^{13}C)-valine (66d)[83] (*ca* 1.5mg) was incubated with partially purified IPNS enzyme according to the usual procedure (General Method B). The incubation mixture was worked up as usual and the total crude sample examined by ^{1}H-n m.r (500MHz, D_2O, HOD suppressed) in the region δ5.0-5.5ppm (ABq, *J* 4.5Hz, β-lactam protons). Partial δ_C (125.77MHz, D_2O) 27.22 ([2S] -^{13}CH$_3$, signal : noise ratio, *ca* 43 : 1), no signal at 31.11 (SCH$_2$).

Incubation of [(2S,3R)-(2'-^{13}C,3-^{2}H)]-Penicillin N (14i) with DAOC/DAC Synthetase.

Crude [(2S,3R)-(2'-^{13}C,3-^{2}H)]-penicillin N (14i) (*ca* 1-1.5mg) was incubated with partially purified DAOC/DAC Synthetase in accordance with the general procedure (General Method C). After work up, examination by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ4.9-5.5 ppm indicated good conversion to the products (46f), (18b) and (19c). The total crude incubation mixture was then examined by ^{13}C-n.m.r (125.77MHz, D_2O, *ca* 40-50 thousand transients) and the unconverted [(2S,3R)-(2'-^{13}C,3-^{2}H)]-Penicillin N (14i) and ring expanded products isolated by chromatography (reverse phase hplc, Waters' system eluting with 25mMolar aqueous NH_4HCO_3).

After examination by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed), the individual β-lactam compounds were examined by ^{13}C-n.m.r (125.77MHz, D_2O, *ca* 40-50 thousand transients). No signals corresponding to incorporation of ^{13}C-label into the endocyclic C-2 position of the cephalosporin products were observed (see Table 3.1.).

Penicillin N (14a); δ_H(500MHz, D_2O, HOD suppressed) 1.52 (3H, s, CH_3), 1.63-1.96 (3H, s, CH_3 and 4H, 2 x m, CH$_2$CH$_2$CH$_2$CO), 2.40 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.77 (1H, *ca* t, *J* 6Hz, CHCH$_2$CH$_2$), 4.42 (1H, s, $CHCMe_2$), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, CHCHS); δ_C (125.77MHz, D_2O) 21.67 (t, $CH_2CH_2CH_2$), 27.22 (q, α-CH_3), 30.80 (t, $CHCH_2CH_2$), 31.11 (q, β-CH3), 32.27 (t, CH_2CO), 55.28 , 57.43 (2 x d, CHCHS), 58.74 (d, $H_3^+NCHCO_2^-$), 73.91

(d, CHCO$_2$H), 161.05, 175.21, 175.58 and 176.63 (4 x s, C=O). Selective irradiation at O2=7391.1 Hz (^{1}H-frequency of α-methyl group as determined by n.O.e expt.)[23] in the c.w. ^{13}C-n.m.r, with minimum decoupling power (30H) collapses the 27.22 ppm quartet to a broad singlet.

[(2S,3R)-(2'-^{13}C,3-^{2}H)]-Penicillin N (14i) Partial δ_C (125.77MHz, D$_2$O) 27.22 ([2S] -^{13}CH$_3$, signal : noise ratio, *ca* 43 : 1), no signal at 31.11 (β-CH$_3$).

DAOC (18a); δ_H(500MHz, D$_2$O, HOD suppressed) 1.67-2.03 (4H, 2 x m, CH$_2$CH$_2$CH$_2$CO), 1.94 (3H, s, CH$_3$), 2.44 (2H, *ca* t, CH$_2$CO), 3.38 and 3.61 (2 x 1H, 2 x d, *J* 18Hz, SCH$_2$), 4.02 (1H, *ca* t, CHNH$_3$), 5.14 and 5.58 (2H, ABq, *J* 4Hz, CHCHS); δ_C(125.77MHz, D$_2$O) 19.22 (q, CH$_3$), 21.70 (t, CH$_2$CH$_2$CH$_2$CO), 29.17 (t, SCH$_2$), 30.61 (t, CHCH$_2$CH$_2$), 35.40 (t, CH$_2$CO), 55.23 , 57.53 (2 x d, CHCHS), 59.44 (d, H$_3^+$NCHCO$_2^-$), 122.90 and 127.39 (2 x s, C=CMe), 165.16, 170.66, 175.07 and 177.29 (4 x s, C=O). Selective irradiation at O2=8273.4 Hz (^{1}H-frequency of the high-field doublet of C2 methylene ABq) in the c.w. ^{13}C-n.m.r, with minimum decoupling power (30H) collapses the 29.17 ppm triplet to a doublet.

3'-^{13}C-DAOC (18b); Partial δ_C (125.77MHz, D$_2$O) 19.12 (^{13}CH$_3$, signal : noise ratio, *ca* 9 : 1), no signal at 29.17 (SCH$_2$).

DAC (19a); δ_H (250MHz, D$_2$O, HOD suppressed) 1.64-1.94 (4H, 2 x m, CH$_2$CH$_2$CH$_2$CO), 2.44 (2H, *ca* t, *J* 7Hz, CH$_2$CO), 3.42 and 3.60 (2H, ABq, *J* 17Hz, SCH$_2$), 3.70 (1H, *ca* t, *J* 6Hz, CHNH$_3$), 4.79 (2H, s, CH$_2$OH), 5.07 and 5.58 (2H, ABq, *J* 4Hz, CHCHS); δ_C(125.77MHz, D$_2$O) 21.67 (t, CH$_2$CH$_2$CH$_2$CO), 26.15 (t, SCH$_2$), 30.60 (t, CHCH$_2$CH$_2$), 35.38 (t, CH$_2$CO), 55.21 , 57.97 (2 x d, CHCHS), 59.66 (d, H$_3^+$NCHCO$_2^-$), 61.71 (t, CH$_2$OH), 122.01 and 130.20 (2 x s, C=CMe), 165.66, 169.72, 175.11 and 172.27 (4 x s, C=O). Selective irradiation at O2=8367.7 Hz (^{1}H-frequency of the high-field doublet of C-2 methylene ABq) in the c.w. ^{13}C-n.m.r, with minimum decoupling power (30H) collapses the 26.15 ppm triplet to a doublet.

3'-^{13}C-DAC (19c); Partial δ_C (125.77MHz, D$_2$O); 61.74 (^{13}CH$_2$OH, signal : noise ratio, *ca*. 30 : 1), no signal at 26.15 ppm (SCH$_2$).

3β-Hydroxycepham (46a); δ_H(500MHz, D$_2$O, HOD suppressed) 1.39 (3H, s, CH$_3$), 1.63-1.94 (4H, m, CH$_2$CH$_2$CH$_2$CO), 2.42 (2H, *ca* t, *J* 7Hz, CH$_2$CO), 2.66, 3.56 (2H, ABq, *J* 14Hz,

SCH_2), 3.75 (1H, m, C<u>H</u>CH_2CH_2), 4.16 (1H, s, C<u>H</u>CO_2H), 5.29, 5.45 (2H, ABq, *J* 4Hz, C<u>H</u>C<u>H</u>S); δ_C(125.77MHz, D_2O) 21.67 (t, $CH_2\underline{C}H_2CH_2CO$), 26.52 (q, CH_3), 30.57 (t, $CH\underline{C}H_2CH_2$), 34.89 (t, SCH_2), 35.63 (t, $\underline{C}H_2CO$), 55.08, 55.27 (2 x d, <u>C</u>HCHS and CH<u>C</u>HS), 59.39 (d, <u>C</u>HCH_2CH_2), 64.42 (d, <u>C</u>HCO_2H), 167.79 , 174.97 and 177.30 (3 x s, <u>C</u>=O). Individual selective irradiations at O2=8000 and 8440 Hz (^{1}H-frequency of the high-field and low-field doublets of C-2 methylene ABq) in the c.w. ^{13}C-n.m.r, with low decoupling power (30H) collapses the 34.89 ppm triplet to a doublet.

<u>(3'-^{13}C)-3β-Hydroxycepham (46f);</u> δ_H(500MHz, D_2O, <u>H</u>OD suppressed, referenced to <u>H</u>OD δ4.64 ppm) 1.26, 1.39 and 1.51 (3H, 3 x s, *J* ^{13}C-^{1}H 125Hz, $^{13}CH_3$), 1.63-1.94 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2$), 2.42 (2H, *ca.* t, *J* 7Hz, $C\underline{H}_2CO$), 2.66, 3.56 (2H, ABq, *J* 14Hz, $SC\underline{H}_2$), 3.75 (1H, m, C<u>H</u>CH_2CH_2), 5.29, 5.45 (2H, ABq, *J* 4Hz, C<u>H</u>C<u>H</u>S); Partial δ_C(125.77MHz, D_2O) 26.49 ($^{13}\underline{C}H_3$, signal : noise ratio, *ca.* 42 : 1), 25.79 ($^{13}\underline{C}H_3$ of (3'-^{13}C)-3β-Hydroxycepham lactone (69f)), no signal at 34.89 ($S\underline{C}H_2$).

8.4 Experimental for Chapter 4.

Propan-2-ol (88a).

To a stirred suspension of $LiAlH_4$ (166 mg, 4.3 mmol) in diglyme (10 ml, dried over CaH_2) under argon at 0°C, was slowly added acetone (87a) (500 mg, 8.6 mmol). The resulting solution was stirred at 0°C for 1hour and then ethylene glycol (15 ml) slowly added. The product was distilled from the reaction mixture and the fraction distilling between 79-89°C collected to give (88a)[151] as a colourless liquid (585 mg, contained solvent) which was used without further purification.

(1-$^2H_3$,3-$^2H_3$)-Propan-2-ol (88b).

(1-$^2H_3$,3-$^2H_3$)-Propan-2-ol was prepared as for (88a) except that $^2H_6$-acetone (87b) (99.96% atom D, 10.0 g, 156 mmol) and $LiAlH_4$ (2.5 g, 65 mmol) in diglyme (50 ml, dried over CaH_2) were used. The product was distilled from the reaction mixture and the fraction boiling between 79-105°C collected to give (88b)[151] (10.0 g,151 mmol, 97% yield) which was used without further purification.

2-Bromopropane (89a).

To a stirred solution of (88a) (585 mg, mmol) in toluene (at -10°C was added dropwise phosphorus tribromide (2.33 g, 8.6 mmol). The resulting solution was stirred at room temperature overnight and the product (89a)[151] isolated by distillation; the fraction boiling between 59-62°C was collected (1.169 g, contained solvent). δ_H (500MHz,$CDCl_3$) 1.70 (6H, d, *J* 7Hz, 2 x C<u>H</u>$_3$), 4.29 (1H, m, C<u>H</u>Me_2).

(1-$^2H_3$,3-$^2H_3$)-2-Bromopropane(89b).

As for (89a) except (1-$^2H_3$,3-$^2H_3$)-propan-2-ol (88b) (9.5 g, 144 mmol) and phosphorus tribromide (24.9 g, 92 mmol) were used. The fraction boiling between 50-62 °C was collected to give (89b)[151] (14.0 g, 109 mmol, 76% yield). δ_H (500MHz,$CDCl_3$) 4.25 (1H, s, C<u>H</u>$(CD_3)_2$)

2-[<u>N</u>-(Diphenylmethylene)amino]-3-($^2H_3$)-methyl,4-($^2H_3$)-butyronitrile (60d).

As for compound (60a) except that <u>N</u>-(diphenylmethylene)amino-acetonitrile (59) (9.17 g, 41.7 mmol), benzyltriethylammonium chloride (840 mg, 3.7 mmol), NaOH (5.18 g as a 50% aqueous solution), toluene (10ml) and (1-$^2H_3$, 3-$^2H_3$)-2-bromopropane (89b) (6.45 g, 50.0 mmol) in toluene (10ml) were used. Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave (60d)[144] (5.3 g, 20 mmol, 40% yield) as a pale yellow oil. T.l.c., [petrol/diethyl ether (9:1,v/v)] Rf 0.5.; V_{max} ($CHCl_3$) 2228(w), 1619(s), 1458(m); δ_H(300MHz, $CDCl_3$) 2.11 (1H, d, *J* 6, C<u>H</u>$(CD_3)_2$), 3.99 (1H, d, *J* 6, C<u>H</u>CH$(CD_3)_2$), 7.11-7.58 (10H, m, 10 x ArH); δ_C (125.77MHz, $CDCl_3$) 17.22-18.23 (m, 2 x <u>C</u>D_3), 32.93 (d, <u>C</u>H$(CD_3)_2$), 59.23 (d, <u>C</u>HCH$(CD_3)_2$), 118.66 (s, <u>C</u>N), 127.29-138.49 (m, <u>Ph</u>), 172.67 (s, Ph_2<u>C</u>=N); *m/z* (DCI, NH_3), 269 (MH^+, 100%), 270 (34), 271 (6), 272 (1).

<u>D</u>/<u>L</u>-Di-($^2H_3$-methyl)-Valine (11k).

As for (11a) except that 2-[<u>N</u>-(diphenylmethylene)amino]-3-($^2H_3$)-methyl-4-($^2H_3$)-butyronitrile (60d) (5.03 g, 18.8 mmol) gave di-($^2H_3$-methyl)-valine (11k)[144] (1.375 g, 11.1 mmol, 59% yield). δ_H (500MHz, D_2O, <u>H</u>OD suppressed), 2.31 (1H, br.s, C<u>H</u>CD_3) 3.92 (1H, d, *J* 4Hz,C<u>H</u>CHCD_3); δ_C (62.5 MHz, D_2O) 18.11 (sept, 2 x <u>C</u>D_3), 29.36 (d, <u>C</u>HCD_3), 61.07 (d, <u>C</u>HNH_3), 174.87 (s, <u>C</u>O_2); *m/z* (CI, NH_3) 122 (1%), 123 (6), 124 (MH^+, 100), 125 (6), 126 (1).

D/L-di-(^{2}H$_3$-methyl)-Valine Benzhydryl Ester, Ammonium tosylate salt.

As for D/L-valine benzhydryl ester, ammonium tosylate salt except that (11k) (0.5 g, 4.1 mmol), in water (10 ml), *p*-toluenesulphonic acid (779 mg, 4.1 mmol) and diphenyldiazomethane (1.0-1.5 eq) were used to give the title compound (1.68 g, 3.69 mmol, 90% yield)[145]. M.p.174-5 °C (From EtOH/Et_2O/Petrol). (Found C 64.88, H 6.30 and N 2.87%. $C_{25}H_2D_6NSO_5$ requires C 65.05, H 6.33 and N 3.03%); δ_H (500MHz,$CDCl_3$) 2.20 (1H, br.d, *J* 4Hz, CHCH$(CD_3)_2$), 2.28 (3H, s, ArCH_3), 3.95 (1H, *ca*.t, *J* 4Hz, CHCH), 6.85 (1H, s, CHPh_2), 6.97-7.64 (14H, m, ArH).

(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyl-carbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-[di-(^{2}H$_3$-methyl)]-valine Benzhydryl Ester (65e).

As for compound (65a) except that the ammonium tosylate salt of di-(^{2}H$_3$-methyl)-valine benzhydryl ester (507 mg, 1.10 mmol) was used to give the free amine (63e) which was then coupled with (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonyl-S-*p*-methoxybenzyl-L-cysteine (64a)[66] (735 mg, 1.10 mmol) with EEDQ (306 mg, 1.22 mmol) to give (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyl-carbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-[di-(^{2}H$_3$-methyl)]-valine benzhydryl ester (65e)[146] (393 mg, 0.42 mmol, 76% yield) as a viscous oil. V_{max} ($CHCl_3$) 2959(w), 1734(m), 1718(m), 1515(s) and 1223(s); δ_H (500MHz, $CDCl_3$) 1.26-1.81 (4H, m, CH_2CH_2CH_2CO), 2.05-2.20 (3H, m, CH_2CO and CH$(CD_3)_2$), 2.65, 2.82 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7, J_{BX} 6Hz, CH_2S), 3.70 (2H, s, SCH_2Ar), 3.77 (3H, s, SCH_2ArOMe), 3.78 (6H,s, 2 x CH_2ArOMe), 4.28-4.34 (1H, m, CHCH_2CH_2), 4.50 (1H, X of ABX , J_{AX} 7, J_{BX} 6Hz, CHCH_2S), 4.62 (1H, dd, *J* 4, 7Hz, CHCHMe$_2$), 5.00, 5.03 (2H, ABq, *J* 12Hz, OCH_2Ar), 5.07 (2H, s, OCH_2Ar), 5.44 (1H,d, *J* 8Hz, NH), 6.24 (1H,d,*J* 8Hz, NH), 6.77-6.90 and 7.16-7.32 (23H, 2 x m, CHPh_2 and ArH); δ_C (125.77MHz, $CDCl_3$) 16.20 and 18.10 (2 x br m, CH(CD_3)$_2$), 21.21 (t, CH_2CH_2CO), 30.51 (d, CH$(CD_3)_2$), 31.63 (t, CHCH_2CH_2), 33.27, 35.20 and 35.74 (3 x t, CH_2CO, CH_2SAr and SCH_2Ar), 52.10 and 53.50 (2 x d, NHCHCH_2S and CHCH_2CH_2), 55.05 (q, ArOMe), 57.36 (d, CHCH$(CD_3)_2$), 66.61 and 66.79 (2 x t, CH_2Ar), 77.81 (d, CHPh_2), 113.76, 113.86,and 113.91 (3 x d, Ar), 126.79-139.51 (10 x s, Ar), 156.10, 158.65, 159.45 and 159.64 (4 x s, ArO_2CNH and Ar C-O of ArOMe), 169.62, 170.43, 172.01 and 172.31 (4 x s, C=O); *m/z* (FAB) 940 (MH^+).

(5R)-5-Amino-5-carboxypentanamido-L-cysteinyl-D-[di-(^{2}H$_3$-methyl)]-valine (66e).

Preparation as for compound (66a) except that (65e) (34.4 mg, 0.037 mmol) was used to give (66e)[146] (10.1mg, 0.03 mmol, 74% yield); δ_H (500MHz, D_2O, HOD suppressed) 1.65-2.01 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.20 (1H, m, $CH(CH_3)_2$), 2.42 (2H, *ca* t, *J* 7Hz, CH_2CO), 2.98, 2.95 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.97 (1H, *ca*. t, *J* 6Hz, $CHCH_2S$), 4.57 (1H, *ca* t, *J* 7Hz, $CH_2CHN^+H_3$); δ_C(125.77MHz, D_2O) 17.20 and 18.60 (2 x br. m, 2 x CD_3), 21.42 (t, $CH_2CH_2CH_2CO$), 26.19 (t, $CH_2CH_2CH_2CO$), 29.92 (t, $CH_2CH_2CH_2CO$), 30.24 (d, $CHCH(CD_3)_2$), 35.23 (t, CH_2SH), 53.58 (d, $CHCH_2S$), 56.28 (d, $CHCH(CD_3)_2$), 59.07 (d, $CHCH_2CH_2$), 172.69, 172.74, 175.60 and 176.34 (4 x s, C=O); *m/z* (FAB) 368 (5%), 369 (7), 370 (MH^+, 100), 371 (20), 372 (8), 373 (2).

(2R,5R,6R)-1-aza-3,3-di-(^{2}H$_3$-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14j). [Di-(^{2}H$_3$-methyl)-penicillin N]

Procedure as for (14e) except that (5R)-5-amino-5-carboxypentanamido-L-cysteinyl-D-[di-(^{2}H$_3$-methyl)]-valine (66e) (*ca* 10 mg) was used to give, after chromatography [hplc, Gilson system, eluting with 0.75% MeCN in 5mMolar aqueous NH_4HCO_3, retention time 8 minutes], di-(^{2}H$_3$-methyl)-penicillin N (14j)[153] (3.5 mg) as determined by ^{1}H-n.m.r. calibration (General Method C). δ_H (500MHz, D_2O, HOD suppressed) 1.67-2.02 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.43 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.81 (1H, *ca* t, *J* 6Hz, $CHCH_2CH_2$), 4.34 (1H, s, $CHCO_2H$), 5.50, 5.59 (2H, ABq, *J* 4Hz, CHCHS).

(2R,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14a) [Penicillin N] and (2R,5R,6R)-1-Aza-(2-^{2}H)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo [3.2.0]heptane-2-carboxylate (14e) [(3-^{2}H)-Penicillin N]; Competitive Kinetic Isotope Incubation.

Penicillin N (14a)[64] (3 mg) and (3-^{2}H)-penicillin N (14e) (3 mg) were mixed together and incubated with partially purified DAOC/DAC synthetase enzyme according to the general method for the competitive mixed label experiments (General Method E). Three samples of the incubation mixture were taken coresponding to 0, 30, and 65% conversions. ^{1}H-n.m.r. examination (500MHz, D_2O, HOD suppressed) and comparison of the β-lactam and C3-H proton integrals, in

addition to mass spectral analysis (NH_3 D.C.I.) of the derivatised penicillins from the three conversion points indicated no enzymatic isotopic discrimination (see Expt.1 in tables 8.5, 8.6 and 8.7 below).

The above procedure was repeated with (14a) (3.2 mg) and (14e) (3.2 mg) to give penicillin N samples corresponding to 0, 30 and 80% conversions. Analysis by ^{1}H-n.m.r and mass spectroscopy again indicated no enzymatic isotopic discrimination between the labelled and unlabelled penicillins (see Expt.2 in Tables 8.5, 8.6 and 8.7 below).

Expt.	% Conversion of Penicillin Ns	C3-H integral (mm)	Lowfield β-lactam integral (mm)	Ratio (14a):(14e)[a]
1	0	34	67	0.97:1.00
	30	34	75	1.21:1.00
	65	29	66	1.28:1.00
2	0	45	82	0.82:1.00
	30	27	59	0.84:1.00
	80	21	37	0.76:1.00

Note: [a] in original incubation mixture.

Table 8.5. Determination of the Ratio of Penicillin N (14a) to (2-^{2}H)-Penicillin N (14e) by ^{1}H-n.m.r. Integration.

		Relative intensities of the fragment ions (68c) / (68d) m/z								
Expt.	% Conversion of Penicillin Ns	173	174	175	176	177	178	179	180	Ratio (14a):(14e)
1	0	4	92	100	15	8	1	2	1	0.99:1.00
	30	2	86	100	14	6	1	1	1	1.04:1.00
	65	3	88	100	14	7	1	1	1	1.05:1.00
2	0	3	100	91	15	7	1	3	1	1.21:1.00
	30	7	100	95	33	20	9	17	12	1.22:1.00
	80	17	100	95	34	23	16	18	44	1.19:1.00

Table 8.6. Determination of the Ratio of Penicillin N (14a) to (2-^{2}H)-Penicillin N (14e) by Mass Spectrometry on the Fragment Ions (68c and d).

Expt.	% Conversion of Penicillin Ns	Relative intensities of molecular ions (MH+) for (86a) / (86c) m/z 459	460	461	462	463	464	465	Ratio (14a):(14e)
1	0	1	78	100	27	9	2	0	0.95:1.00
	30	3	78	100	27	10	2	0	0.95:1.00
	65	3	76	100	27	10	2	0	0.93:1.00
2	0	1	96	100	28	10	2	0	1.24:1.00
	30	-	94	100	28	9	0	-	1.22:1.00
	80	6	93	100	29	11	2	-	1.20:1.00

Table 8.7. Determination of the Ratio of Penicillin N (14a) to (2-^{2}H)-Penicillin N (14e) by Mass Spectrometry on the Molecular Ions (86a and c).

(2R,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14a) [Penicillin N] and (2R,5R,6R)-1-Aza-3,3-di-(^{2}H$_3$-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14j) [Di-(^{2}H$_3$-methyl)-penicillin N] ; Competitive Kinetic Isotope Incubation.

Penicillin N (14a) (0.9 mg) and di-(^{2}H$_3$-methyl)-penicillin N (14j) (0.9 mg) were mixed together and incubated according to the usual method (General Method E). Two samples were taken which corresponded to 0 and 30% conversions. Isolation of the penicillin Ns and analysis by ^{1}H-n.m.r and mass spectroscopy indicated isostopic discrimination had occurred (see below, Expt. 1 Tables 8.8, 8.9 and 8.10).

This was repeated with (14a) (1.0 mg) and (14j) (1.0mg) to give samples corresponding to 0, 40 and 60% conversions. Similar analysis again revealed isostopic discrimination (see below, Expt. 2 Tables 8.8, 8.9 and 8.10).

The cephalosporin products were isolated from both of these mixed experiments by chromatography [reverse phase hplc Waters' system, eluting with 25 mMolar aqueous NH_4HCO_3]. The samples of DAOC were then derivatised to give the N-ethoxycarbonyl, dimethyl ester derivatives (90a)/(90b-d)[93] and the isolated DAC (19a/d-e) transformed to the lactone (67). The ratio of labelled and unlabelled molecules was determined either by DCI or FAB mass spectrometry (see Expts.1 and 2 below, Tables 8.11 and 8.12).

Expt.	% Conversion of Penicillin Ns	2/3 x highfield methyl integral (mm)	Lowfield β-lactam integral (mm)	Ratio (14a):(14j)[a]
1	0	70	74	0.95:1.00
	30	32	52	0.61:1.00
2	0	26	26	0.65:1.00
	60	31	56	0.55:1.00

Note: [a] in original incubation mixture.

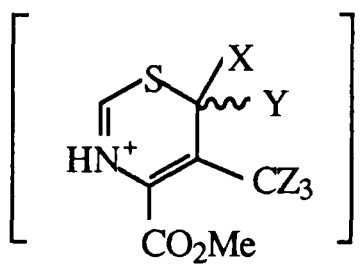
Table 8.8. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by ^{1}H-n.m.r. Integration.

		Relative intensities of the fragment ions (68c) / (68e) m/z											
Expt.	% Conversion of Penicillin Ns	173	174	175	176	177	178	179	180	181	182	183	Ratio (14a):(14j)
1	0	2	94	10	6	1	1	3	100	10	7	1	0.99:1.00
	30	2	50	6	4	2	1	3	100	10	7	2	0.50:1.00
2	0	2	94	13	8	2	1	4	100	14	9	2	0.94:1.00
	40	2	54	7	4	1	1	3	100	11	7	2	0.54:1.00
	60	17	47	19	45	28	13	14	100	28	28	28	0.47:1.00

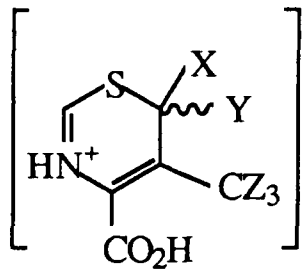
Table 8.9. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by Mass Spectrometry on Derivative (68c/e) Fragment Ions.

		Relative intensities of molecular ions (MH^+) for (86a) / (86c) m/z											
Expt.	% Conversion of Penicillin Ns	459	460	461	462	463	464	465	466	467	468	469	Ratio of (14a):(14j)
1	0	1	94	23	9	2	0	2	100	25	10	2	0.94:1.00
	30	1	50	12	5	2	0	2	100	25	10	2	0.50:1.00
2	0	2	100	26	10	6	2	3	100	26	11	3	1.00:1.00
	40	0	51	14	7	4	3	2	100	28	10	1	0.51:1.00
	60	8	41	16	8	7	7	8	100	30	20	10	0.41:1.00

Table 8.10. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by Mass Spectrometry of Derivative (86a/c) Molecular Ions.



(68f ; X = Y = Z = H,
g; X = Y = Z = D,
h; X = H, Y = Z= D,
i; X = Y = H, Z = D)



(68j ; X = Y = Z = H,
k; X = Y= Z = D,
l ; X = H, Y= Z =D,
m; X = Y = H, Z = D)

Expt.	% Conversion of Penicillin Ns	Relative intensities of the fragment ions (68f) / (68g-i) m/z 171	172	173	174	175	176	177	178	179	180	Ratio of (18a):(18c-e)
1	0	N/A										
	30	3	100	14	7	8	27	36	6	3	1	1.0 : 0.6[b]

		Relative intensities of the fragment ions (68j) / (68k-m) m/z 157	158	159	160	161	162	163	164	165	166	
2	0	N/A										
	40	5	100	15	15	16	21	50	11	10	3	1.0 : 0.7[a, c]
	60	-	100	13	16	20	29	83	14	15	11	1.0 : 1.1[a, c]

Notes: [a] ratio in original incubation mixture; [b]determined for derivative fragment ions (68f-i) by DCI *m/z*; [c] determined for fragment ions (68j-m) by TSP *m/z*.

Table 8.11. Determination of the Ratio of DAOC (18a) to Deuterated DAOCs (18c-e) by Mass Spectrometry of Derivative (90a-d) Fragment Ions (68f-i)[b](Expt.1) and DAOC (18a, c-e) Fragment Ions (68j-m)[c] (Expt.2).

Expt.	% Conversion of Penicillin Ns	Relative intensities of molecular ions (MH+) for the fragment ion (68a) m/z 155	156	157	158	159	160	161	162	163	Ratio of (19a): (19d-f)
1		None Isolated									
2	0	N/A									
	40	2	100	11	6	2	1	1	1	1	1.0 : 0.0
	60	2	100	16	13	8	3	4	1	2	1.0 : 0.1

Table 8.12. Determination of the Ratio of DAC (19a) to Deuterated DACs (19d-f) by Mass Spectrometry of DAC Lactone (67) Fragment Ions.

(2R,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14a) [Penicillin N] and (2R,5R,6R)-1-Aza-3,3-di-($^2H_3$-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14j) [Di-($^2H_3$-methyl)-penicillin N] ; Competitive Kinetic Isotope Control Experiment.

Penicillin N (14a) (0.5 mg) and di-($^2H_3$-methyl)-penicillin N (14j) (0.5 mg) were mixed and a sample (*ca* 0.5 mg) derivatised (General Method D).

Partially purified DAOC/DAC Synthetase enzyme (*ca*. 0.5 I.U in 50mM Tris.HCl buffer) was heated at 100°C for 2-3 minutes and then pre-incubated with cofactor solution (200µl) (see General Method B). The remaining penicillin N sample was then incubated as usual with this denatured enzyme for 2 hours and the penicillin N re-isolated. Examination by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) and mass spectroscopy (positive argon FAB) revealed no change in the ratio (14):(14j) (see Tables 8.13, 8.14 and 8.15 below).

	% Conversion of Penicillin Ns	2/3 x highfield methyl integral (mm)	Lowfield ß-lactam integral (mm)	Ratio of (14a):(14j)[a]
Starting Material	0	66	66	1.00:1.00
Incubated Material	0	57	52	0.91:1.00

Note: [a] in original incubation mixture.

Table 8.13. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by ^{1}H-n.m.r. Integration.

	% Conversion of Penicillin Ns	Relative intensities of the fragment ions (68c) / (68e) m/z 173	174	175	176	177	178	179	180	181	182	183	Ratio (14a):(14j)
Starting Material	0	0	96	17	8	2	1	2	100	18	9	2	0.96:1.00
Incubated Material	0	0	96	13	8	1	1	1	100	14	9	2	0.96:1.00

Table 8.14. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by Mass Spectrometry on Derivative (68c/e) Fragment Ions.

	% Conversion of Penicillin	Ns	Relative intensities of molecular ions (MH+) for (86a) / (86c) m/z 459	460	461	462	463	464	465	466	467	468	469	Ratio (14a): (14j)
S.M	0	-	100	32	12	3	1	2	97	33	12	3		1.03:1.00
I.M	0		1	99	28	10	2	1	2	100	29	11	2	0.99:1.00

Table 8.15. Determination of the Ratio of Penicillin N (14a) to Di-($^2H_3$-methyl)-Penicillin N (14j) by Mass Spectrometry of Derivative (86a/c) Molecular Ions.

8.5 Experimental for Chapter 5.

Incubation of (2R,5R,6R)-1-Aza-3,3-di-($^2H_3$-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14j) [Di-($^2H_3$-methyl)-penicillin N] with DAOC/DAC Synthetase.

(2R,5R,6R)-1-Aza-3,3-di-($^2H_3$-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (14j) (*ca* 1 mg) was incubated with DAOC/DAC synthetase (*ca* .05 I.U) according to the general procedure (General Method C). Examination of the crude incubation mixture by ^1H-n.m.r. (500MHz, D_2O, HOD suppressed) indicated approximately 10% conversion to ring expanded products had occurred as judged by integration of the β-lactam region δ5.6-4.9 ppm. The products were isolated by chromatography [reverse phase hplc, Waters' system, with 25mMolar aqueous NH_4HCO_3] to give DAOC (18), DAC (19) and recovered (14j). After re-examination of the isolated products by ^1H-n.m.r. (500MHz, D_2O, HOD suppressed), the di-($^2H_3$-methyl)-penicillin N (14j) and DAOC (18) were derivatised (General Method D) to their N-ethoxycarbonyl, dimethyl esters (86c) and (90) respectively, and examined by mass spectrometry (DCI, NH_3). This experiment was then repeated with a second sample of (14j) (*ca* 1 mg) but with a greater quantity of DAOC/DAC synthetase (*ca* 0.1 I.U.) to obtain ca 50% conversion to DAOC (18) and DAC (19) (Expt.2, in Table 8.16 below).

(14j) δ_H(500MHz, D_2O, HOD suppressed) 1.40-1.71 (4H, $CHCH_2CH_2CH_2$), 2.16 (2H, m, CH_2CO), 3.48 (m, $CHCH_2CH_2$), 3.99 (1H, s, $CHCMe_2$), 5.23 and 5.41 (2H, ABq, *J* 4Hz, CHCHS).

Expt.	% Conversion of Penicillin Ns	Relative intensities of the fragment ion (68e) m/z (%) 178	179	180	181	182	183
1	10	-	8	100	11	13	6
2	50	-	1	100	11	5	-
m/z Calculated for (14j)		-	-	100	9	1	-

Table 8.16. Determination of the Deuterium Content of Unconverted Di-($^2H_3$-methyl)-Penicillin N (14j) Re-isolated from DAOC/DAC Synthetase Incubations.

(18c/d) from Expt. 2 (50% Conversion); δ_H(500MHz, D_2O, HOD suppressed) 1.45-1.74 (4H, $CHC\underline{H}_2C\underline{H}_2CH_2$), 2.21 (2H, *ca* t, *J* 7 Hz, $C\underline{H}_2CO$), 3.36 (*ca* 1/2H, s, $SC\underline{H}$/D), 3.51 (1H, *ca* t, $C\underline{H}CH_2CH_2$), 4.87 and 5.36 (2H, ABq, *J* 4Hz, $C\underline{H}C\underline{H}S$).

Expt.	% Conversion of Penicillin Ns	Relative intensities of the fragment ions (68g-i) *m/z* 174	175	176	177	178	179	180	Ratio of (18c):(18d)
1	10	Not Measured							
2	50	7	19	78	100	20	16	7	1.0 : 0.6

Expt.	% Conversion of Penicillin Ns	Relative intensities of the Molecular Ions (MNH_4^+) (90b-d) *m/z* 477	478	479	480	481	482	183	Ratio of (18c):(18d)
1	10	-	4	49	100	47	36	4	1.0 : 0.5
2	50	-	22	85	100	29	22	-	1.0 : 0.9

Table 8.17. Determination of the Ratio of Deuterated DAOCs (18c-e) by Mass Spectrometry on Derivative (90a-d) and Fragment Ions (68j-m) (Expt.2).

(6R,7R)-1-Aza-3-chloro-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Methyl Ester (97b).

To the chlorocephem (98) (2.0 g, 9.0 mmol) dissolved in saturated aqueous $NaHCO_3$ (15 ml) and dioxan (15 ml) was added di-*t*-butylpyrocarbonate (2.9 g, 13.5 mmol) in one portion. The resulting solution was stirred overnight at room temperature. After washing with DCM (3 x 50 ml) the solution was acidified to *ca* pH 2 by the addition of 2N HCl and then rapidly extracted with EtOAc (3 x 50 ml). The EtOAc layers were combined, dried (anhydrous Na_2SO_4), filtered and evaporated to dryness *in vacuo* to give the N-*t*-BOC acid (2.1 g, 6.2 mmol, 69% yield).

The acid was redissolved in EtOAc (20 ml) stirred at 0°C and treated with excess diazomethane. Chromatography [flash silica, DCM / EtOAc (3:2, v/v)] gave (97b) (1.74 g , 5.0 mmol, 55% yield). T.l.c., [DCM/ EtOAc (3:2, v/v)] R_f 0.7. M.p 151-152°C (from DCM/petrol) ; V_{max} ($CHCl_3$) 3440 (w), 1793 (s), 1723 (s), 1506 (s), 1370 (m); δ_H(500MHz, $CDCl_3$) 1.45 (9H, s, C(C<u>H</u>$_3$)$_3$), 3.51 and 3.82 (2 x 1H, 2 x d, *J* 18Hz, C<u>H</u>$_2$S), 3.89 (3H, s, $CO_2C\underline{H}_3$), 5.02 (1H, d , *J* 4.5Hz, C<u>H</u>S), 5.21 (1H, d, *J* 9Hz, N<u>H</u>), 5.61 (1H, dd, *J* 9, 4.5Hz, NHC<u>H</u>CH); *m/z* (DCI, NH_3) 366 (MNH_4^+, 100%), 367 (23), 368 (47), 369 (9), 370 (3).

(6R,7R)-1-Aza-3-chloro-7-amino-(<u>N</u>-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Benzhydryl Ester (97a).

As for compound (97b) except that chlorocephem (98) (1.0 g, 4.5 mmol) and *t*-butylpyrocarbonate (1.5 g, 6.8 mmol) were used to give the <u>N</u>-*t*-butyloxycarbonyl acid (1.2 g, 3.6 mmol) which was dissolved in acetonitrile (10ml) and esterified with diphenyldiazomethane (0.7 g, 3.6 mmol). The resulting solution was evaporated *in vacuo* and purified by chromatography [flash silica, EtOAc/ petrol (3:7, v/v)] gave (97a) (1.2 g , 2.4 mmol, 67% yield). T.l.c., [EtOAc/ petrol (3:7, v/v)] R_f 0.50. M.p 145-6°C (from DCM/petrol). (Found C 62.00, H 4.97 and N 5.70%. $C_{25}H_{25}N_2ClSO_5$ requires C 61.91, H 5.20 and N 5.78%). $[\alpha]_D^{20}$ + 25.1 [c = 0.5, $CHCl_3$]; V_{max} ($CHCl_3$) 3435 (w), 1790 (s), 1720 (s), 1505 (s), 1370 (m); δ_H(500MHz, $CDCl_3$) 1.45 (9H, s, C(C<u>H</u>$_3$)$_3$), 3.52 and 3.82 (2 x 1H, 2 x d, *J* 18Hz, C<u>H</u>$_2$S), 5.03 (1H, d , *J* 4.5Hz, C<u>H</u>S), 5.21 (1H, d, *J* 9Hz, N<u>H</u>), 5.63 (1H, dd, *J* 9 and 4.5Hz, NHC<u>H</u>CHS), 7.01 (1H, s, C<u>H</u>Ph$_2$), 7.27-7.45 (10H, m, CH<u>Ph</u>$_2$); δ_C(125.77MHz, $CDCl_3$) 28.12 (q, C(<u>C</u>H$_3$)$_3$), 31.08 (t, S<u>C</u>H$_2$), 57.67 and 60.79 (2 x d, <u>C</u>H<u>C</u>HS), 79.80 (d, <u>C</u>HPh$_2$), 81.21 (s, <u>C</u>(CH$_3$)$_3$), 124.48 and 125.49 (2 x s, <u>C</u>=CCl and C=<u>C</u>Cl), 127.09-139.08 (8 x Ar<u>C</u>), 154.49, 159.50 and 164.74 (3 x s, C=O).

Methyllithium.

Into a two necked flask equiped with a stirrer bar, reflux condensor and dropping funnel was put lithium metal containing 1% sodium (2.3 g, 83.0 mmol, 2.4 eq, as a 50% dispersion in oil) which was washed with dry diethyl ether (2 x 10 ml) and then suspended in diethyl ether (20ml). Methyliodide (5.0 g, 34.5 mmol) was added at such a rate as to maintain gentle reflux. The resulting solution was stirred overnight at room temperature and then filtered *via* cannula into a dry flask to

give methyllithium (20.0 ml, 0.83 Molar [as determined by calibration against diphenylacetonetosylhydrazone] [112] 16.7 mmol, 54% yield)[111].

$^{2}H_3$-Methyllithium.

The previous experiment was repeated with $^{2}H_3$-methyliodide (5.0 g, 34.5 mmol , 99.5 atom % D) and lithium metal containing 1% sodium (2.3 g 83 mmol, 2.4 eq as a 50% dispersion in oil) suspended in diethyl ether (10ml) to give $^{2}H_3$-methyllithium (16.0 ml, 0.91 Molar [as determined by calibration against diphenylacetonetosylhydrazone] [112] 14.5 mmol, 42% yield)[154].

(6R,7R)-1-Aza-3-methyl-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Methyl Ester (91f).

Copper cyanide (40 mg, 4.5 mmol, 3eq) was azeotroped with toluene (3 x 2 ml) on a high-vacuum line, each time equilibrating under an atmosphere of argon. The cyanide was suspended in THF (20 ml) and cooled with stirring to -78°C for 5 minutes after which time methyllithium (7.3 ml, 1.24 M, 9.0 mmol, 6eq) was added dropwise . This mixture was stirred at 5°C for 20 minutes to give a clear pale brown solution. Upon cooling back to -78°C, a heavy precipitate again developed. To this suspension was added dropwise a degassed solution of (97b) (520 mg, 1.5 mmol) in THF (10 ml) and the solution then warmed to -50°C for 30 minutes. The reaction was quenched by the addition of saturated aqueous NH_4Cl (20 ml) and EtOAc (150 ml) and then filtered.

The organic layer was separated, washed with water (3 x 30 ml), brine (30 ml), dried (anhydrous Na_2SO_4), filtered and then evaporated *in vacuo* to dryness. Chromatography [flash silica, EtOAc / DCM (1:4, v/v)] gave (91f)[106] and the $\Delta 2$-isomer (99b) as a mixture (265 mg, 0.81 mmol, 54% yield). T.l.c., [EtOAc / DCM (1:2.8, v/v)] Rf 0.65. M.p 142-3°C (from DCM/Et_2O/petrol). (Found C 50.98, H 6.16 and N 8.21%. $C_{14}H_{20}N_2SO_5$ requires C 51.21, H 6.14 and N 8.53%); ν_{max} ($CHCl_3$) 3440 (w), 3020 (m), 1780 (s), 1720 (s), 1505 (s), and 1370 (m); *m/z* (DCI, NH_3) 329 (MH^+, 28%), 330 (6), 331 (7); (91f) δ_H (500MHz, $CDCl_3$) 1.46 (9H, s, $C(C\underline{H}_3)_3$), 2.14 (3H, s, C=CMe), 3.22 and 3.51 (2 x 1H, 2 x d, *J* 18Hz, $C\underline{H}_2S$), 3.84 (3H, s, $CO_2C\underline{H}_3$), 4.95 (1H, d, *J* 4.5Hz, $C\underline{H}S$), 5.00 (1H, d, *J* 13Hz, $N\underline{H}$), 5.56 (1H, dd, *J* 13 and 4.5Hz, $NHC\underline{H}CH$); (99b) δ_H (500MHz, $CDCl_3$) 1.47 (9H, s, $C(C\underline{H}_3)_3$), 1.89 (3H, s, SC=CMe), 3.80 (3H, s, $CO_2C\underline{H}_3$), 4.76 (1H, s, SC=CCH), 5.21 (1H, d, *J* 13Hz, $N\underline{H}$), 5.42-5.47 (1H, br m, $NHC\underline{H}CH$), 5.96 (1H, s, SCH=C).

(6R,7R)-1-Aza-3-[($^2H_3$)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Methyl Ester (91g).

The above procedure was repeated with the Lipshutz cuprate[107] formed from copper cyanide (630 mg, 7.0 mmol), and $^2H_3$-methyllithium (15.4 ml, 0.91 M, 14.0 mmol, 2.0 eq)[155] with concomitant formation of a heavy white precipitate, presumed to be lithium iodide. To this solution was added (97b) (810 mg, 2.3 mmol). Chromatography [flash silica, EtOAc/DCM (1:4 , v/v)] gave only (91g)[156] (40 mg, 1.2 mmol, 52% yield). T.l.c., [EtOAc / DCM (1: 2.8, v/v)] R_f 0.65. M.p. 109-112°C (from DCM/petrol); $V_{max}(CHCl_3)$ 1780 (m), 1720 (m), and 1560 (m); δ_H (500MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>$_3$)$_3$), 3.22 and 3.51 (2 x 1H, 2 x d, *J* 18Hz, C<u>H</u>$_2$S), 3.84 (3H, s, CO_2C<u>H</u>$_3$), 4.95 (1H, d, *J* 4.5Hz, C<u>H</u>S), 5.21 (1H, d, *J* 13Hz, N<u>H</u>), 5.56 (1H, dd, *J* 13 and 4.5Hz, NHC<u>H</u>CH); *m/z* (DCI, NH_3) 332 (MH^+, 39%), 333 (6), 334 (4), 335 (1).

(2R,6R,7R)-1-Aza-3-methyl-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (101a).

Procedure as described by E. M. Van Heyningen, and L. K. Ahern [109] except that to a stirred solution of (91f) (200 mg, 0.61 mmol) in water / pyridine (1:1, v/v, 10ml) at 0°C was added 1<u>N</u> NaOH (630 µl, 0.63 mmol, 1.03 eq). The resulting solution was stirred at 0°C for a further 4 hours and then washed with DCM (2 x 20 ml), acidified to *ca* pH 2 with 2<u>N</u> HCl and rapidly extracted with EtOAc (3 x 30 ml). The combined EtOAc layers were dried (anhydrous Na_2SO_4), filtered, and evaporated to dryness *in vacuo* to give (101a) (187 mg, 0.61 mmol, *ca* 100% yield) as a white solid which was used without further purification. M.p. 160-5°C decomp. (from Et_2O); $V_{max}(CHCl_3)$ 3400 (w), 3020 (s), 1760 (w), 1710 (s), 1420 (m), and 1360 (m); δ_H(200MHz, $^2H_6$-acetone) 1.36 (9H, s, C(C<u>H</u>$_3$)$_3$), 1.87 (3H, s, CH_3), 4.63 (1H, s, C<u>H</u>CO_2H), 5.16 (1H, d, *J* 3Hz, C<u>H</u>S), 5.29 (1H, br s, NHC<u>H</u>CH), 6.05 (1H, s, SCH=C), 6.71 (1H, br s, NH); *m/z* (DCI, NH_3) 332 (MNH_4^+, 100%), 333 (18), 334 (15), 335 (3).

(2R,6R,7R)-1-Aza-3-($^2H_3$-methyl)-7-amino-(N-*t* -butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (101b).

The above experiment was repeated with (91g) (202 mg, 0.61 mmol) to give (101b)[157] (160 mg, 0.53 mmol, 86% yield) as a white solid which was used without

further purification. M.p. 168-171°C decomp. (from Et_2O); $V_{max}(CHCl_3)$ 3020 (s), 1760 (w), 1710 (s), and 1360 (m); δ_H(200MHz, $^2H_6$-acetone) 1.36 (9H, s , C(C<u>H</u>$_3$)$_3$), 4.67 (1H, s, C<u>H</u>CO_2H), 5.21 (1H, d, *J* 3Hz, C<u>H</u>S), 5.41 (1H, br.s, NHC<u>H</u>CH), 6.11 (1H, s, SC<u>H</u>=C), 6.72 (1H, brs, NH); *m/z* (DCI, NH_3) 335 (MNH_4^+, 100%), 336 (19), 337 (11), 338 (2).

(2<u>R</u>,6<u>R</u>,7<u>R</u>)-1-Aza-3-methyl-7-amino-(<u>N</u>-*t* -butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester (99a).

The free acid (101a) (185 mg, 0.61 mmol) was dissolved in MeCN (10 ml) and stirred at room temperature. Diphenyldiazomethane (119 mg, 0.61 mmol, 1.0 eq) was added and the resulting solution stirred until the esterification was complete. The solvent was removed *in vacuo* to give (99a) (287 mg, 0.60 mmol, 98% yield) as a white solid, which was used without further purification. M.p 161-4°C (from DCM/petrol); V_{max} ($CHCl_3$) 3020 (s), 1776 (m), 1743 (m), 1720 (m), and 1507 (m); δ_H (200MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>$_3$)$_3$), 4.86 (1H, d, *J* 1.5Hz, C<u>H</u>CO_2H), 5.22 (1H, d, *J* 3.5Hz, C<u>H</u>S), 5.38-5.45 (1H, br.m, NHC<u>H</u>CH), 5.95 (1H, d, *J* 1.5Hz, SC<u>H</u>=C), 6.91 (1H, s, C<u>H</u>Ar_2), 7.34 (10H, s, ArH); *m/z* (DCI, NH_3) 498 (MNH_4^+, 100%), 499 (32), 500 (10), 501 (3).

(2<u>R</u>,6<u>R</u>,7<u>R</u>)-1-Aza-3-(^{2}H$_3$-methyl)-7-amino-(<u>N</u>-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester (99c).

The above procedure was repeated with (101b) (150 mg, 0.49 mmol) and diphenyldiazomethane (95 mg, 0.49 mmol) to give (99c) (236 mg, 0.48 mmol, 99% yield). M.p 124-6°C (from DCM/petrol); $[\alpha]_D^{20}$ + 316 [c = 1.1, $CHCl_3$]; (Found C 64.58, H 5.90 and N 5.79. $C_{26}H_{25}D_3N_2O_5S$ requires C 64.42, H 6.29, N 5.76%). $V_{max}(CHCl_3)$ 3021 (m), 1776 (s), 1720 (s), and 1507 (s); δ_H (200MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>$_3$)$_3$), 4.86 (1H, d, *J* 1.5Hz, C<u>H</u>CO_2H), 5.22 (1H, d, *J* 3.5Hz, C<u>H</u>S), 5.38-5.45 (1H, br m, NHC<u>H</u>CH), 5.95 (1H, d, *J* 1.5Hz, SC<u>H</u>=C), 6.91 (1H, s, C<u>H</u>Ar_2), 7.34 (10H, s, Ar<u>H</u>); δ_C (50.3MHz, $CDCl_3$) 28.08 (C(<u>C</u>H$_3$)$_3$), 53.04 and 53.12 (2 x d, <u>C</u>H<u>C</u>HS), 61.74 (d, <u>C</u>HCCD$_3$), 78.86 (d, <u>C</u>HPh_2), 81.30 (s, <u>C</u>(CH$_3$)$_3$), 114. 31 (d, <u>C</u>H=CCD$_3$), 120.19 (s, CH=<u>C</u>CD$_3$), 126.88-139.22 (8 x ArC), 154.90, 165.54 and 166.77 (3 x s, C=O); *m/z* (DCI, NH_3) 501 (MNH_4^+, 100%), 502 (31), 503 (11), 504 (3).

(2R,5R,6R,7R)-1-Aza-3-methyl-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid-5-oxide, Benzhydryl Ester (92b).

To a stirred solution of the Δ2-cephem (99a) (250 mg, 0.5 mmol) in DCM (6 ml) at 0°C, was added dropwise MCPBA (140 mg, 0.81 mmol) in DCM (4 ml). The resulting solution was stirred at 0°C for 3 hours and then transfered to a separating funnel and washed with aqueous sodium thiosulphate (20 ml, 10%), saturated aqueous $NaHCO_3$ (20 ml), brine (20 ml) and then dried (anhydrous Na_2SO_4). This solution was filtered and the solvent evaporated *in vacuo* to give after chromatography [flash silica, EtOAc/DCM (2:3, v/v)] (92b)[158] (185 mg, 0.375 mmol, 72% yield). T.l.c., [EtOAc/DCM (1:4, v/v)] Rf 0.5. M.p 79-82°C (from Et_2O/petrol); $V_{max}(CHCl_3)$ 3420 (w), 3020 (s), 1795 (s), 1720 (s), 1505 (s), 1456 (m), and 1225 (s); δ_H (500 MHz, $CDCl_3$) 1.46 (9H, s, $C(C\underline{H}_3)_3$), 2.16 (3H, s, $C\underline{H}_3$), 3.24 and 3.62 (2H, ABq, *J* 19Hz, $SC\underline{H}_2$), 4.47 (1H, d, *J* 3Hz, CHC<u>H</u>SO), 5.75-5.80 (1H, br m, NHC<u>H</u>CHS), 6.94 (1H, s, $C\underline{H}Ph_2$), 7.26-7.50 (10H, m, ArH); *m/z* (DCI, NH_3) 514 (MNH_4^+, 100%), 515 (33), 516 (14), 517 (4).

(2R,5R,6R,7R)-1-Aza-3-($^2H_3$-methyl)-7-amino-(N-*t*-butyloxy-carbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid-5-oxide, Benzhydryl Ester (92e).

The experiment above was repeated with (99b) (243 mg, 0.50 mmol) and MCPBA (147 mg, 0.85 mmol) to give (92e)[158] (270 mg) which was used without further purification. T.l.c.[EtOAc/DCM (1:4, v/v)] Rf 0.5, M.p 78-80°C (from Et_2O/petrol); $V_{max}(CHCl_3)$ 3420 (w), 3020 (s), 1800 (s), 1720 (s), 1654 (w), 1456 (m), and 1230 (s); δ_H (200 MHz, $CDCl_3$) 1.46 (9H, s, $C(C\underline{H}_3)_3$), 3.24 and 3.61 (2H, ABq, *J* 19Hz, $SC\underline{H}_2$), 4.45 (1H, d, *J* 3Hz, CHC<u>H</u>SO), 5.75-5.80 (1H, br m, NHC<u>H</u>CHS), 6.94 (1H, s, $C\underline{H}Ph_2$), 7.24-7.51 (10H, m, ArH); *m/z* (DCI, NH_3) 517 (MNH_4^+, 100%), 518 (33), 519 (15), 520 (1).

(6R,7R)-1-Aza-3-methyl-7-amino-(N-*t*-butyloxy-carbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Benzhydryl Ester (91c).

To a stirred solution of the sulphoxide (92a) (180 mg, 0.37 mmol) in dry DMF (4 ml) at 0°C was added potassium iodide (1.2 g) and acetyl chloride (freshly distilled from quinoline)[159] (0.2 ml). The resulting solution was stirred at 5°C for 1 hour and then saturated aqueous sodium metabisulphite (8 ml) was added. This

mixture was extracted with EtOAc (3 x 10 ml) and the combined organic layers washed with water (20 ml), saturated aqueous $NaHCO_3$ (20 ml), brine (20 ml) and dried (anhydrous Na_2SO_4). After filtering, the solvent was evaporated *in vacuo* to give (91c)[158] (170 mg, 0.354 mmol, 97%) as a pale yellow foam. T.l.c., [EtOAc/DCM (2:3, v/v)] Rf 0.8. ; δ_H (500 MHz, $CDCl_3$) 1.46 (9H, s, $C(C\underline{H}_3)_3$), 2.11 (3H, s, $C\underline{H}_3$), 3.21 and 3.48 (2H, ABq, *J* 18Hz, $SC\underline{H}_2$), 4.95 (1H, d, *J* 4Hz, C<u>H</u>S), 5.30 (1H, d, *J* 9Hz, N<u>H</u>CH), 5.58 (1H, dd, *J* 4 and 9Hz, NHC<u>H</u>CHS), 6.92 (1H, s, $C\underline{H}Ph_2$), 7.24-7.46 (10H, m, $CH\underline{Ph}_2$).

(6<u>R</u>,7<u>R</u>)-1-Aza-3-($^2H_3$-methyl)-7-amino-(<u>N</u>-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Benzhydryl Ester (91h).

The previous procedure was repeated with (92e) (250 mg, 0.51 mmol) to give after chromatography [EtOAc/DCM (1:4, v/v)] (91d) (120 mg, 0.25 mmol, 50%). T.l.c.,[EtOAc/DCM (2:3, v/v)] Rf 0.8. M.p. 90-92°C (from DCM/petrol); V_{max}($CHCl_3$) 3435 (w), 3020 (w), 1785 (s), 1720 (s), 1505 (s), and 1371 (m); δ_H(500 MHz, $CDCl_3$) 1.46 (9H, s, $C(C\underline{H}_3)_3$), 3.21 and 3.48 (2H, ABq, *J* 18Hz, $SC\underline{H}_2$), 4.95 (1H, d, *J* 4Hz, C<u>H</u>S), 5.30 (1H, d, *J* 9Hz, N<u>H</u>CH), 5.59 (1H, dd, *J* 4 and 9Hz, NHC<u>H</u>CHS), 6.92 (1H, s, $C\underline{H}Ph_2$), 7.24-7.46 (10H, m, $CH\underline{Ph}_2$); δ_C(125.77 MHz, $CDCl_3$) 18.8-19.5 (br.m, CD_3), 28.04 (q, $C(\underline{C}H_3)_3$), 55.47 (t, $S\underline{C}H_2$), 60.44 (d, CH<u>C</u>H), 67.01 (d, NH<u>C</u>H), 79.46 (d, $\underline{C}HAr_2$), 80.84 (s, $\underline{C}(CH_3)_3$), 122.31 and 125.82 (2 x s, C=<u>C</u>CD_3 and <u>C</u>=CCD_3), 127-139 (Ar<u>C</u>), 154.55, 160.28 and 164.88 (3 x s, <u>C</u>=O); *m/z* (DCI, NH_3) 501 (MNH_4^+, 100%), 502 (31), 503 (12), 504 (3).

(6<u>R</u>,7<u>R</u>)-1-Aza-2,3-3-[($^2H_3$)-methyl]-7-amino-[(5<u>R</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Benzhydryl Ester (104).

The *t*-BOC-cepham (91d) (100 mg, 0.21 mmol) was dissolved in EtOH/Et_2O [1:1, v/v (2 ml)] and *p*-toluenesulphonic acid (40 mg, 0.21 mmol) added. The resulting solution was stirred at room temperature for 48 hours and the solvent evaporated to give (102b) (115 mg, 0.21 mmol, 100% yield). The crude tosylate salt was dissolved in saturated aqueous $NaHCO_3$ (10 ml) and the resulting solution then extracted with EtOAc (3 x 15 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo* to give the free amine (79 mg, 0.21 mmol). The amine was then coupled with (5<u>R</u>)-5-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid (103)[146] (92 mg, 0.21 mmol) by the usual procedure (General Method F) to give

after chromatography [flash silica, EtOAc/petrol (1:1, v/v)] (104) (135 mg, 0.17 mmol, 81% yield). T.l.c.,[EtOAc] Rf 0.2. M.p 68-9°C (from DCM/Et2O/petrol). δ_H(200MHz, $CDCl_3$) 1.68 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2$), 2.25 (2H, m, $C\underline{H}_2CO$), 3.09 and 3.60 (2H, ABq, *J* 19Hz, $SC\underline{H}_2$), 3.79 (6H, s, $ArOC\underline{H}_3$), 4.32-4.44 (1H, m, $C\underline{H}NHCO$), 4.91 (1H, d, *J* 4Hz, CHS), 5.02 and 5.11 (2 x 2H, 2 x s, 2 x $C\underline{H}_2Ar$), 5.62 (1H, d, *J* 8Hz, $N\underline{H}CH$), 5.78 (1H, dd, *J* 4 and 8Hz, $NHC\underline{H}CH$), 6.81- 6.97 and 7.25-7.48 (18H, 2 x m, ArH); δ_C(50.31MHz, $CDCl_3$) 21.28 (t, $CH_2\underline{C}H_2CH_2$), 30.12 (t, $\underline{C}H_2CH_2CH_2$), 31.81 (t, $\underline{C}H_2CO$), 34.88 (t, SCH_2), 53.44, 55.19, and 57. 40 (3 x d, NHCH, $CH\underline{C}HS$ and $NH\underline{C}HCH_2$), 58.97 (q, $\underline{C}H_3OAr$), 66.85 and 67.05 (2 x t, $\underline{C}H_2Ar$), 79.02 (d, $\underline{C}HPh_2$), 114.03 and 114.11 (2 x d, ArC), 122.73 (s, $C=\underline{C}CD_3$), 127.06-130.31 (7 x s, ArC), 133.93 (s, $\underline{C}=CCD_3$), 139.72, 139.87 (2 x s, ArC), 154.53, 156.61, 159.79 and 159.99 (4 x s, $MeO\underline{C}$ of ArOMe and $ArO_2\underline{C}NH$), 161.67, 165.32, 172.46, and 173.26 (5 x s, C=O); *m/z* (FAB) 811 (MH^+, 100%), 812 (39), 813 (17), 814 (7).

(6R,7R)-1-Aza-3-($^2H_3$-methyl)-7-amino-[(5R)-5-amino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (18e). [(3'-$^2H_3$-methyl)-DAOC]

To protected (104) (120 mg, 0.15 mmol) was added anisole and TFA [1:4, v/v (2 ml)] and toluene (4 ml) and this solution stirred at room temperature for 30 minutes. The solvent was then evaporated and the residue partitioned between water (5 ml) and EtOAc (5 ml). The aqueous phase was extracted with EtOAc (3 x 5 ml) and freeze-dried to give (18e) as the ammonium trifluoroacetate salt (58.5 mg, 0.13 mmmol, 88% yield)[146] which was used without further purification.

λ_{max} (H_2O) 195 and 256 nm; δ_H(500 MHz, D_2O, $\underline{H}OD$ suppressed); 1.67-2.03 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.44 (2H, *ca* t, CH_2CO), 3.38 and 3.61 (2 x 1H, 2 x d, *J* 18Hz, $SC\underline{H}_2$), 4.02 (1H, *ca* t, $C\underline{H}NH_3$), 5.14 and 5.58 (2H, ABq, *J* 4Hz, $C\underline{H}C\underline{H}S$); *m/z* (FAB) 360 (4%), 361 (MH^+, 100), 362 (18), 363 (13), 364 (2). [Identical to authentic DAOC (18a) by ^{1}H-n.m.r. except for the absence of the vinyl methyl group at δ 1.94 ppm].

Incubation of DAOC (18a) with Partially Purified DAOC/DAC synthetase.

DAOC (18a) (10 mg) was incubated with partially purified DAOC/DAC synthase enzyme (*ca* 1.2 I.U.) in Tris HCl buffer (6 mls, 10 mM) according to the usual procedure (General Method C). Examination of the crude incubation mixtures by ^{1}H-n.m.r (500 MHz, D_2O, $\underline{H}OD$ suppressed) indicated *ca* 12% conversion to DAC (19) as judged by examination and integration of the region δ5.8-4.8 ppm.

No signals due to the β-lactam resonances of the 3β-hydroxycepham (46a) could be detected in this region. The incubation mixture was purified by chromatography [reverse phase hplc, Gilson system, eluting with 0.75% MeCN in 10 mM aqueous NH_4HCO_3,] and the fractions eluting between DAC (19) and DAOC (18) (*i.e.* the region in which the 3β-hydroxycepham (46a) elutes) were collected and pooled. After lyophilisation, the pooled fraction was examined by ^{1}H-n.m.r (500 MHz, D_2O, HOD suppressed) particular attention being paid to the β-lactam resonance region δ 5.8-4.8 ppm. Again no signals corresponding to the 3β-hydroxycepham (46a) β-lactam resonances could be detected.

Incubation of DAOC (18a) with Partially Purified DAOC/DAC synthetase in D_2O.

Partially purified DAOC/DAC synthetase enzyme (*ca* 2.5 I.U.) in Tris HCl buffer (12 mls, 10 mM) was diluted with Tris HCl buffer (100 mls, 10 mM, pH 7.4 prepared in D_2O 99.7 atom % deuterium, supplied by the Aldrich Chemical Company Ltd.). After equilibration at 4°C for 15 minutes, the enzyme solution was concentrated to a volume of 12 mls (Amicon 8050, 50 ml ultrafiltration cell Product No. 5122). A sample of this enzyme solution (2 mls, *ca* 0.4 I.U.) was taken and pre-incubated with co-factors and co-substrates as usual (General Method C) except that the co-factors had been prepared in D_2O. To this pre-incubated solution was then added DAOC (18a) (*ca* 0.5 mg) in D_2O (500ml) and the resulting solution incubated at 27°C, 250 r.p.m for 2 hours.

An additional sample of the enzyme (2 ml, *ca* 0.4 I.U.) was taken and heated at 100°C for 5 minutes and then allowed to cool to room temperature. This sample of denatured protein was then treated in exactly the same manner as described above for the active enzyme.

Both incubations were worked-up according to the usual procedure. Examination of the crude incubation mixtures by ^{1}H-n.m.r (500 MHz, D_2O, HO D suppressed) indicated *ca* 30% conversion to DAC (19) in the case of the active enzyme incubation and no conversion with the denatured enzyme, as judged by examination and integration of the region δ 5.8-4.9 ppm. Chromatography [reverse phase Hplc, Gilson system eluting with 0.75% MeCN in 10 mM aqueous NH_4HCO_3] on both incubation samples gave purified unconverted DAOC (18) and DAC (19) from the active enzyme incubation and unconverted DAOC (18) from the denatured 'incubation'. Both DAOC samples were analysed by ^{1}H-n.m.r (500 MHz, D_2O, HOD suppressed) and by mass spectrometry (FAB);

Experiment.	approximate % Conversion	*m/z* for DAOC (18a). *357*	*358*	*359*	*360*	*361*	*362*	*363*
Active Enzyme	30	5	100	22	9	6	2	2
Denatured Enzyme	0	5	100	19	8	8	3	4
m/z Calculated for $C_{14}H_{19}N_3SO_6$		-	100	18	7	1	0	0

Incubation of Trideuteromethyl-DAOC (18e) with Partially Purified DAOC/DAC synthetase in D_2O.

As above except that trideuteromethyl DAOC (18e) was used. Examination of the crude incubation mixtures by ^{1}H-n.m.r (500 MHz, D_2O, HOD suppressed) indicated *ca* 10% conversion to DAC (19f) in the case of the active enzyme incubation and no conversion with the denatured enzyme, as judged by examination and integration of the region δ 5.8-4.9 ppm. Chromatography as before gave, as a mixture, purified DAOC (18d) and (18e) and DAC (19f) from the active enzyme incubation and DAOC (18e) from the denatured 'incubation'. Both DAOC samples were analysed by ^{1}H-n.m.r (500 MHz, D_2O, HOD suppressed) and by mass spectrometry (FAB);

	approximate % Conversion	*m/z* for DAOC (18e) *360*	*361*	*362*	*363*	*364*	*365*	*366*
Experiment.No.1								
Active Enzyme	10	6	100	28	14	12	3	2
Denatured Enzyme	0	4	100	17	10	5	4	3
Experiment.No.2								
Active Enzyme	40	11	100	49	22	8	6	4
Denatured Enzyme	0	12	100	26	16	6	6	-
m/z Calculated for $C_{14}H_{16}D_3N_3SO_6$		-	100	18	7	1	0	0

8.6 Experimental for Chapter 6.

D-3-Hydroxyvaline Benzhydryl Ester, Ammonium Tosylate Salt (121a).

Procedure as for compound D/L-valine benzhydryl ester, ammonium tosylate salt except that D-3-hydroxyvaline (278 mg, 2.10 mmol)[122] was used to give (121a)[145] (875 mg, 1.86 mmol, 89%) a white solid which was recrystalised from

ethanol/ ether/ petrol. M.p 174-6°C (from EtOH/Et_2O/petrol). (Found C 63.50, H 6.44, N 2.44%. $C_{25}H_{29}NO_6S$ requires C 63.68, H 6.20, N 2.97%); V_{max} ($CHCl_3$) 3020 (s), 1745 (m), 1600 (m), 1495 (m); δ_H (500MHz, $CDCl_3$) 0.89 (3H, s, CH_3), 1.35 (3H, s, CH_3), 2.27 (3H, s, $ArCH_3$), 4.16 (1H, s, C<u>H</u>CO_2BzH), 6.81 (1H, s, C<u>H</u>BzH), 7.02-7.63 (14H, m, ArH).

[(5<u>S</u>)-5-<u>N</u>-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyl-carbonylpentanamido]-(<u>L</u>)-<u>S</u>-*p*-methoxybenzyl-cysteinyl-(<u>D</u>)-3-hydroxyvaline Benzhydryl Ester (122).

As for the general coupling procedure (General Method F) except that [(5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentan-amido]-<u>S</u>-*p*-methoxybenzyl-(<u>L</u>)-cysteine (64b)[66] (412 mg, 0.62 mmol), EEDQ (170 mg, 0.68 mmol) and <u>D</u>-3-hydroxyvaline benzhydryl ester, ammonium tosylate salt (320 mg, 0.68 mmol) were used. Chromatography [flash silica, EtOAc/DCM (1:4, v/v)] gave (122)[146] (480 mg, 0.51 mmol, 81%) as a white foam. Rf [EtOAc/DCM (3:7, v/v)] 0.30. M.p. 52-56°C (from DCM/petrol); $[\alpha]_D^{20}$ + 4.2 [c = 5.6, $CHCl_3$]; (Found C 66.02, H 6.64, N 4.05%. $C_{52}H_{59}N_3SO_{12}$ requires C 65.81, H 6.61, N 4.42%); V_{max} ($CHCl_3$) 3020(m), 1719(m), 1676(m), 1515(s) and 1249(s); δ_H (500MHz, $CDCl_3$) 1.12 (3H, s, CH_3), 1.18 (3H, s, CH_3), 1.60-1.67 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$C<u>H</u>$_2$), 2.11 (2H, m, CH_2CO), 2.68, 2.84 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7, J_{BX} 6Hz, CH_2S), 3.63 (2H, s, SC<u>H</u>$_2$Ar), 3.77 (3H, s, SC<u>H</u>$_2$ArOMe), 3.79 (6H, s, 2 x ArO<u>Me</u>), 4.34-4.37 (1H, m, C<u>H</u>CH_2CH_2), 4.51 (1H, X of ABX, C<u>H</u>CH_2S), 4.55 (1H, d, *J* 8Hz, C<u>H</u>COH), 5.00, 5.02 (2H, ABq, *J* 10Hz, CO_2C<u>H</u>$_2$Ar), 5.08 (2H, s, C<u>H</u>$_2$Ar), 5.53 (1H, d, *J* 8Hz, NH), 6.16 (1H, d, *J* 7Hz, NH), 6.81-6.89 (6H, m, ArH), 7.04 (1H, d, *J* 8Hz, NH), 7.20-7.30 (16H, m, ArH); *m/z* (FAB) 949 (6%), 950 (MH^+,100), 951 (62), 952 (18), 953 (7).

[(5<u>S</u>)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-3-hydroxyvaline (106).

Procedure as for compound (66a) except that [(5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-<u>S</u>-*p*-methoxybenzyl-(<u>L</u>)-cysteinyl-(<u>D</u>)-3-hydroxyvaline benzhydryl ester (122) (140 mg, 0.15 mmol) was used to give a mixture of [(5<u>S</u>)-5-amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-3-hydroxyvaline (106)[146,116] (57 mg, 0.12 mmol, 82%) in the thiol (106a) and di-sulphide (106b) forms. Separation by chromatography [reverse phase hplc, Gilson system, 95% 10mM aqueous NH_4HCO_3, 5% MeOH, flow rate 4 ml min^{-1}] Retention times; (106a thiol) 6 minutes, (106b di-sulphide) 12 minutes. (106a [thiol form]) δ_H (500MHz, D_2O, <u>H</u>OD suppressed) 1.25

and 1.31 (2 x 3H, 2 x s, COH(CH3)2), 1.65-1.79 and 1.80-1.94 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2$), 2.41 (2H, m, CH_2CO), 3.04, 3.22 (2H, AB part of ABX, J_{AB} 14, J_{AX} 9, J_{BX} 5Hz, $CHC\underline{H}_2S$), 3.78-3.80 (1H, m, $C\underline{H}CH_2CH_2$), 4.33 (1H, s, $C\underline{H}COH$). [$C\underline{H}CH_2S$ assumed to be under water suppression]; δ_C (125.77MHz, D_2O) 21.69 (t, $CH_2\underline{C}H_2CH_2CO$), 26.28 (q, CH_3), 26.44 (q, CH_3), 30.67 (t, $CH\underline{C}H_2CH_2$), 35.58 (t, CH_2S), 39.54 (t, $\underline{C}H_2CO$), 53.67, 55.26, and 63.10 (3 x d, $N\underline{C}HCOH$, $HN\underline{C}HCH_2$ and $\underline{C}HCH_2SH$), 72.70 (s, COH), 172.11, 175.09, 176.74 and 176.78 (4 x s, $\underline{C}$=O); *m/z* (FAB) 379 (21%), 380 (MH^+, 100), 381 (38), 382 (21), 383 (14), 384 (11).

(106b di-sulphide) δ_H (500MHz, D_2O, $\underline{H}OD$ suppressed) 1.21 (3H, s, CH_3), 1.26 (3H, s, CH_3), 1.69-1.91 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 3.00, 3.25 (2H, AB part of ABX, J_{AB} 14, J_{AX} 9, J_{BX} 5Hz, $CHC\underline{H}_2S$), 3.74 (1H, *ca*.t, *J* 7Hz, $C\underline{H}CH_2CH_2$), 4.21 (1H, s, $C\underline{H}CO_2H$); δ_C (125.77MHz, D_2O); 21.65 (t, $CH_2\underline{C}H_2CH_2CO$), 26.22 (q, CH_3), 26.53 (q, CH_3), 30.56 (t, $CH\underline{C}H_2CH_2$), 35.55 (t, CH_2S), 39.32 (t, $\underline{C}H_2CO$), 53.45, 55.18, and 63.16 (3 x d, 3 x CH), 72.59 (s, COH), 172.08, 175.08, 176.74 and 176.77 (4 x s, $\underline{C}$=O); *m/z* (FAB) 756 (3%), 757 (MH^+, 100%), 758 (45), 759 (18), 760 (6), 761 (3).

[(5$\underline{S}$)-5-$\underline{N}$-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-($\underline{L}$)-$\underline{O}$-*t*-butyl-serine (117).

To a solution of (5$\underline{S}$)-5-$\underline{N}$-p-methoxybenzyloxycarbonylamino-5-p-methoxybenzylcarbonylpentanoic acid (103b) (250 mg, 0.56 mmol) and Et_3N (57 mg, 0.56 mmol) in THF (10 ml) at -15°C was added isobutyl chloroformate (77 mg, 0.56 mmol). The resulting solution was stirred for a further 30 minutes at -15 to -10°C afterwhich time a solution of $\underline{O}$-*t*-butyl-$\underline{L}$-serine (90 mg, 0.56 mmol)[160] in water (10 ml) containing Et_3N (62 mg, 0.62 mmol, 1.1 eq) was added. The mixture was vigarously shaken for 2 minutes and then stirred at room temperature for a further 50 minutes. Water (20 ml) was added and the aqueous layer transfered to a separating funnel, washed with diethyl ether (2 x 20 ml), acidified to *ca* pH 2 by the addition of 2$\underline{N}$ HCl and then extracted with EtOAc (3 x 50 ml). The combined EtOAc layers were dried (anhydrous Na_2SO_4), filtered and evaporated *in vacuo* to give [(5$\underline{S}$)-5-$\underline{N}$-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-$\underline{O}$-*t*-butyl-(L)-serine (117)[146] (337 mg, 0.5 mmol, 87%), which was used without further purification. V_{max} ($CHCl_3$) 3020(s), 1715(s), 1600(s), 1515 (s) and 1250(s); δ_H (500MHz, $CDCl_3$) 1.09 (9H, s, $C(CH_3)_3$), 1.54-1.96 (4H, 2 x br m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.07-2.25 (2H, br.m, CH_2CO), 3.49-3.76 (8H, br m, 2 x ArOMe and $CHC\underline{H}_2O$), 4.23-4.44 (2H, br m, $C\underline{H}CH_2CH_2$ and $C\underline{H}CH_2O$), 4.91-5.02 (4H, br m, 2 x

C<u>H</u>$_2$Ar), 6.76-6.87 and 7.12-7.27 (9H, 2 x m, ArH and NH); *m/z* (FAB) 588 (3%), 589 (MH+, 100), 590 (31), 591 (7), 592 (2).

<u>D</u>/<u>L</u>-3,4-Dehydrovaline Benzhydryl Ester, Ammonium Tosylate Salt.

As for compound (63a) except that racemic 3,4-dehydrovaline (150 mg, 1.3 mmol)[150], was used to give the <u>D</u>/<u>L</u>-3,4-dehydrovaline benzhydryl ester, ammonium tosylate salt (119)[145] (532 mg, 1.2 mmol, 90%). V_{max} ($CHCl_3$) 3020(s), 1750(s), 1600(m), 1500(m) and 1230(s); δ_H (500MHz, $CDCl_3$) 1.57 (3H, s, CH_3), 2.28 (3H, s, ArC<u>H</u>$_3$), 4.57 (1H, br d. *J* 5Hz, C<u>H</u>CO_2BzH), 4.98, 5.10 (2 x 1H, 2 x s, C=CH_2), 6.82 (1H, s, C<u>H</u>Ph_2), 6.96-7.61 (14H, m, ArH), 8.45 (3H, br s, N^+H_3).

[(5<u>S</u>)-5-<u>N</u>-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-<u>O</u>-*t*-butyl-(<u>L</u>)-serinyl-(<u>D</u>)-3,4-dehydrovaline Benzhydryl Ester (120a).

As for general coupling procedure (General Method F) except that [(5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-<u>O</u>-*t*-butyl-(<u>L</u>)-serine (117)[146] (150 mg, 0.22 mmol), <u>D</u>/<u>L</u>-3,4-dehydrovaline benzhydryl ester ammonium tosylate salt (119) (100 mg, 0.22 mmol) and EEDQ (60 mg, 0.24 mmol) were used. Chromatography [Preparative silica plates, with EtOAc/hexane (1:1, v/v)] gave [(5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-<u>O</u>-*t*-butyl-(<u>L</u>)-serinyl-(<u>D</u>)-3,4-dehydrovaline benzhydryl ester (120a) (55 mg, 0.06 mmol, 26%) and the LLL-diastereoisomer (43 mg, 0.05 mmol, 23%). Diastereomerically pure (5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-(<u>L</u>)-<u>O</u>-*t*-butyl-serinyl-(<u>L</u>)-3,4-dehydrovaline benzhydryl ester (120b) was prepared from (<u>L</u>)-3,4-dehydrovaline benzhydryl ester ammonium tosylate salt[151] and used as a T.l.c. reference; (LLL-diastereomer, 120b) [EtOAc/hexane (2:1, v/v)] Rf 0.38, (LLD-diastereomer, 120a) [EtOAc/hexane (2:1, v/v)] Rf 0.40.; V_{max} ($CHCl_3$) 3425 (w), 3020(s), 1740 (m), 1665 (m), 1614 (m), 1515 (s) and 1250(s); δ_H (500MHz, $CDCl_3$) 1.17 (9H, s, $C(CH_3)_3$), 1.61-1.89 (4H, br m, C<u>H</u>$_2$C<u>H</u>$_2$CH_2CO and 3H, s, CH_2=C<u>Me</u>), 2.11-2.24 (2H, br m, CH_2CO), 3.24-3.28 (2H, m, CHC<u>H</u>$_2$O), 3.79 and 3.82 (6H, 2 x s, ArO<u>Me</u>), 4.33-4.38 (1H, br m, C<u>H</u>CH_2CH_2), 4.40-4.44 (1H, br m, NHC<u>H</u>CH_2O), 4.98-5.06 and 5.08-5.13 (6H, 2 x m, CO_2C<u>H</u>$_2$Ar, C<u>H</u>$_2$Ar and MeC=C<u>H</u>$_2$), 5.39 and 6.40 (2H, 2 x d, *J* 8, 7Hz, 2 x NH), 6.85-6.91 and 7.24-7.45 (19H, 2 x m, ArH), 7.57 (1H, d, *J* 8Hz, NH).

[(5S)-5-amino-5-carboxypentanamido]-(L)-serinyl-(D)-3,4-dehydrovaline (112a).

Procedure as for compound (66a) except that [(5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarboxypentanamido]-(L)-O-*t*-butylserinyl-(D)-3,4-dehydrovaline benzhydryl ester (120a) (37 mg, 0.04 mmol) was used to give [(5S)-5-amino-5-carboxypentanamido]-(L)-serinyl-(D)-3,4-dehydrovaline (112a)[146] as its ammonium trifluoroacetate salt (10 mg, 0.02 mmol, 50%). Chromatography [reverse phase hplc Waters system, 0.0125% HCO_2H, 4.00% MeOH in water, Rt 8.5 minutes]. ; δ_H (500MHz, D_2O, HOD suppressed) 1.70 (3H, s, CH_3), 1.73-1.99 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7Hz, CH_2CO) 3.72-3.76 (1H, m, $CHCH_2CH_2$), 3.85 (2H, d, *J* 5Hz, $CHCH_2OH$), 4.47 (1H, *ca* t, *J* 5Hz, $CHCH_2OH$), 4.80 (1H, s, CHC=Me), 5.03 (2H, s, $C=CH_2$); δ_C (125.77MHz, D_2O) 18.96 (q, CH_3), 21.60 (t, $CH_2CH_2CH_2CO$), 30.48 (t, $CH_2CH_2CH_2CO$), 35.45 (t, CH_2CO) 49.63, 55.10 and 61.39 (3 x d, $NHCHCH_2$, $CHCH_2OH$ and $CHC=CH_2$), 61.87 (t, $CHCH_2OH$), 115.46 (d, $C=CH_2$), 141.76 (s, $C=CH_2$), 171.62, 174.93, 175.98 and 176.90 (4 x s, C=O); *m/z* (FAB) 345 (3%), 346 (MH+, 100), 347 (20), 348 (9), 349 (1) [*m/z* calculated for $C_{14}H_{23}N_3O_7$ 346 (MH^+, 100%), 347 (18), 348 (3)].

[(5S)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-S-p-methoxybenzyl-(L)-cysteinyl-(D)-(2-^{2}H,3-^{13}C)-valine Benzhydryl Ester (115).

Procedure as for (65a) (General Method F) except that (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (64b) (235 mg, 0.35 mmol), D/L-(2-2H, 3-^{13}C)-valine benzhydryl ester (95 mg, 0.35 mmol), EEDQ (95 mg, 0.38 mmol) and anhydrous Na_2SO_4 (*ca* 10 mg) were used. Chromatography [flash silica, EtOAc/hexane (2:3,v/v)] gave (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H,3-^{13}C)-valine benzhydryl ester (115)[146] (110 mg, 0.12 mmol, 33% yield). T.l.c., [ethyl acetate/petrol (1:1, v/v)] Rf 0.15 (LLL-diastereomer, Rf 0.10). δ_H (500MHz, $CDCl_3$) 0.75-0.77 (3H, m, $^{13}CHCH_3$), 0.86-0.88 (3H, m, $^{13}CHCH_3$), 1.54-1.86 (4H, m, $CH_2CH_2CH_2$), 2.23-2.48 (3H, m, CH_2CO and $^{13}CH(CH_3)_2$), 2.63, 2.82 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.71 (2H, s, SCH_2Ar), 3.75 (3H, s, SCH_2ArOMe), 3.78 (6H,s, 2 x CH_2ArOMe), 4.28-4.35 (1H, m, $CHCH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7, J_{BX} 6Hz, $CHCH_2S$), 4.99 and 5.02 (2H, ABq, *J* 12Hz, OCH_2Ar), 5.08 (2H, s, OCH_2Ar), 5.43 (1H, d, *J*

8Hz, NH), 6.24 (1H, d, *J* 7Hz, NH), 6.74 (1H, s, C<u>H</u>Ph$_2$), 6.81-6.88 and 7.23-7.36 (22H, 2 x m, Ar<u>H</u>).

[(5<u>S</u>)-5-Amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H,3-^{13}C)-valine (12h).

Procedure as for compound (66a) except that [(5<u>S</u>)-5-<u>N</u>-*p*-methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzylcarbonylpentanamido]-(<u>L</u>)-<u>S</u>-*p*-methoxy-benzyl-cysteinyl-<u>D</u>-(2-^{2}H, 3-^{13}C)-valine benzhydryl ester (115)[146] (65 mg, 0.07 mmol) was used to give [(5<u>S</u>)-5-amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H, 3,4,4-$^{13}C_3$)-valine (12h) as the ammonium trifluoroacetate salt (35 mg, 0.07 mmol, 100% yield). δ_H (500MHz, D_2O, <u>H</u>OD suppressed) 0.93-0.99 (6H, m, ^{13}CH(C<u>H</u>$_3$)$_2$), 1.65-1.96 (4H, 2 x m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.02-2.09, 2.13-2.22 and 2.28-2.36 (1H, 3 x m, J ^{13}C-^{1}H 130Hz, ^{13}CHMe$_2$), 2.42 (2H, ca t, 7 Hz, CH$_2$CO), 2.88, 2.93 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH$_2$S), 3.85-3.88 (1H, *m*, C<u>H</u>CH$_2$S), 4.56 (1H, *ca* t, J 6Hz, CH$_2$C<u>H</u>NH$_3$); δ_C(125.77MHz, D_2O) 17.95 (m, ^{13}CH<u>C</u>H$_3$), 19.16 (m, ^{13}CH<u>C</u>H$_3$), 21.50 (t, CH$_2$<u>C</u>H$_2$CH$_2$CO), 26.16 (t, <u>C</u>H$_2$CH$_2$CH$_2$CO), 30.17 (t, <u>C</u>H$_2$CO), 30.59 (d, ^{13}<u>C</u>H(CH$_3$)$_2$), 35.28 (t, CH$_2$SH), 54.23 and 56.28 (2 x d, <u>C</u>HCH$_2$S and CHN^{+}H$_3$), 172.82, 173.90, 174.91 and 176.51 (4 x s, 4 x <u>C</u>=O); m/z (FAB) 364 (MH^{+} for $^{12}C_{14}H_{24}N_3O_6$, 9%), 365 (MH^{+} for $^{12}C_{14}H_{23}DN_3O_6$, 15), 366 (MH^{+} for $^{13}C^{12}C_{13}H_{23}DN_3O_6$, 100), 367 (23), 368 (3). [Identical to authentic LLD-ACV (12a)[161] by ^{1}H-n.m.r. except for the presence of two additional multiplets at δ2.02-2.09, and 2.28-2.36 ppm due to valinyl C-3, ^{13}C-^{1}H coupling and the absence of a valinyl C-2H doublet at δ4.28 ppm].

(1,2,3-$^{13}C_3$)-Propan-2-ol (88c).

As for (88a) except that (1,2,3-$^{13}C_3$)-propanone (87c) (500 mg, 8.2 mmol, 99.2 atom %^{13}C, supplied by Merck Sharpe and Dhome Isotopes Ltd) was used. The product was distilled from the reaction mixture and the fraction distilling between 60-120°C collected to give (88c)[151] (780 mg, *ca* 60% pure by ^{1}H-n.m.r) which was used without further purification. δ_H (500MHz, $CDCl_3$) 1.06-1.09 and 1.31-1.34 (2 x 3H, 2 x m, J^{13}C-^{1}H 130 Hz, ^{13}CH(^{13}C<u>H</u>$_3$)$_2$, 1.84 (1H, br.s, ^{13}CHO<u>H</u>), 3.85-3.90 and 4.14-4.18 (1H, 2 x m, J 13C-1H 142 Hz, ^{13}C<u>H</u>(^{13}CH$_3$)$_2$); δ_C (125.77MHz, $CDCl_3$) 25.25 (dq, J^{13}C-^{13}C 38Hz and J^{13}C-^{1}H 78Hz, ^{13}CH(^{13}<u>C</u>H$_3$)$_2$), 64.29 (dt, J_{13C-1H} 108Hz and J^{13}C-^{13}C 38Hz, ^{13}<u>C</u>H(^{13}CH$_3$)$_2$).

2-Bromo-(1,2,3-$^{13}C_3$)-propane (89c).

Procedure as for compound (89a) except that crude (1,2,3-$^{13}C_3$)-propan-2-ol (88c) (780 mg, *ca* 60% pure by ^{1}H-n.m.r) and phosphorus tribromide (2.23 g, 8.6 mmol) were used. The product was distilled from the reaction mixture, the fraction distilling between 40-85°C being collected, to give (89c)[151] (835 mg, 6.63 mmol, 81% from (1,2,3-$^{13}C_3$)-propanone (87c)) which was shown to be pure by ^{1}H-n.m.r. δ_H (500MHz, $CDCl_3$) 1.49-1.60 and 1.82-1.85 (2 x 3H, 2 x m, $J^{13}C\text{-}^1H$ 130 Hz, $^{13}CH(^{13}C\underline{H}_3)_2$, 4.11-4.17 and 4.41-4.47 (1H, 2 x m, $J^{13}C\text{-}^1H$ 153 Hz, $^{13}CHBr$); δ_C (125.77MHz, $CDCl_3$) 28.47 (dq, $J^{13}C\text{-}^{13}C$ 37Hz and $J^{13}C\text{-}^1H$ 83Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 45.55 (dt, $J^{13}C\text{-}^1H$ 118Hz and $J^{13}C\text{-}^{13}C$ 37Hz, $^{13}\underline{C}H(^{13}CH_3)_2$). [Identical to authentic 2-bromopropane (89a)[161] by ^{1}H-n.m.r. except for the presence of $^{13}C\text{-}^1H$ coupling].

2-[<u>N</u>-(Diphenylmethylene)amino]-2-(^{2}H), 3-^{13}C, 4'-(^{13}C methyl),4-^{13}C-butyronitrile (60e).

Procedure as for (60a) except that <u>N</u>-(diphenylmethylene)aminoaceto-nitrile (59) (1.46 g, 6.6 mmol), benzyltriethylammonium chloride (150 mg, 0.7 mmol), NaOD (2mls of *ca* 50% solution), toluene (10ml) and 2-bromo-(1,2,3-$^{13}C_3$)-propane (89c) (835 mg, 6.6 mmol) in toluene (10ml). Chromatography [flash silica, petrol/diethyl ether (9:1, v/v)] gave (60e)[144] (665 mg, 2.5 mmol, 38% yield) as a pale yellow oil. T.l.c., [petrol/diethyl ether (9:1,v/v)] Rf 0.25. V_{max} ($CHCl_3$) 2228(w), 1619(s), 1458(m); δ_H(500MHz, $CDCl_3$); 0.86-1.26 (6H, 4 x m, $^{13}CH(^{13}C\underline{H}_3)_2$), 1.96-2.05 and 2.23-2.31 (1H, 2 x m, $^{13}C\underline{H}(^{13}CH_3)_2$), 7.20-7.66 (10H, m,10 x Ar<u>H</u>); δ_C (125.77MHz, $CDCl_3$) 18.54 and 18.83 (2 x dq, $J^{13}C\text{-}^1H$ 95Hz and $J^{13}C\text{-}^{13}C$ 35Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 33.43 (dt, $J^{13}C\text{-}^1H$ 90Hz and $J^{13}C\text{-}^{13}C$ 35Hz, $^{13}\underline{C}H(^{13}CH_3)_2$); *m/z* (DCI, NH_3) 265 (4%), 266 (MH^+ for $^{12}C_{15}{}^{13}C_3H_{18}N_2$, 16), 267 ($MH^+$ for $^{12}C_{15}{}^{13}C_3H_{17}DN_2$, 100), 268 (17), 269 (2).

<u>D</u>/<u>L</u>-(2-^{2}H, 3,4,4'-^{13}C)-Valine (11).

Procedure as for compound (11a) except that 2-[N-(diphenylmethylene)amino]-(2-^{2}H, 3-^{13}C), 3'-(^{13}C methyl)-4-^{13}C-butyronitrile (60e) (665 mg, 2.5 mmol) was used. Chromatography [ion exchange with Dowex 1 x 8-400 acetate form, eluting with water] gave a white solid, (11)[144] as the ammonium acetate salt (400 mg, 2.2 mmol, 88% yield). δ_H (500MHz, D_2O, <u>H</u>O D

suppressed) 0.87-0.94 and 1.12-1.19 (2 x 3H, 2 x m, $J^{13}C\text{-}^{1}H$ 127 Hz, $^{13}CH(^{13}C\underline{H}_3)_2$), 2.15-2.22 and 2.42-2.48 (1H, 2 x m, $J^{13}C\text{-}^{1}H$ 132 Hz, $^{13}C\underline{H}(^{13}CH_3)_2$); δC (125.77MHz, $CDCl_3$)[of enriched carbons only] 17.45 and 18.28 (2 x dq, $J^{13}C\text{-}^{1}H$ 109Hz and $J^{13}C\text{-}^{13}C$ 35Hz , $^{13}CH(^{13}\underline{C}H_3)_2$), 29.66 (dt, $J^{13}C\text{-}^{1}H$ 89Hz and $J^{13}C\text{-}^{13}C$ 35Hz, $^{13}\underline{C}H(^{13}CH_3)_2$); *m/z* (FAB) 121 ($MH^+$ for $^{12}C_2{}^{13}C_3H_{11}NO_2$, 2%), 122 ($MH^+$ for $^{12}C_2{}^{13}C_3H_{10}DNO_2$, 100). [Identical to authentic valine (11a)[65] by ^{1}H-n.m.r. except for the presence of additional $^{13}C\text{-}^{1}H$ coupling and the absence of a doublet at δ3.60 ppm].

Incubation of [(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-3-hydroxyvaline (106) with Partially Purified IPNS Enzyme.[116]

[(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-3-hydroxyvaline (106) (*ca* 1 mg) was incubated with partially purified IPNS enzyme (*ca* 15 I.U.) according to the usual procedure (General Method B). Examination of the crude incubation mixture by ^{1}H-n.m.r (500MHz, D_2O, $\underline{H}OD$ suppressed) indicated no conversion to a β-lactam containing compound as judged by the absence of resonances in the region δ5.8-4.9 ppm. In addition, bio-assay against (-) ESS *E. coli.* and *S. aureus.* at a concentration of 100μg ml^{-1} gave no zones of inhibition and hence indicated that conversion to an antibiotic substance *e.g* isopenicillin N (13a), had not occurred.

Incubation of [(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3-^{13}C)-valine (12h) with Partially Purified IPNS Enzyme.

[(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3-^{13}C)-valine (12h) (*ca* 4 mg) was incubated with partially purified IPNS enzyme (*ca* 11 I.U.) according to the general procedure (General Method B). After the usual work-up, the total crude incubation mixture was examined by ^{1}H-n.m.r (500MHz, D_2O, $\underline{H}OD$ suppressed) which indicated greater than 80% conversion to isopenicillin N (13f) as judged by the absence of tripeptide resonances in the region δ1.0-0.9 ppm and the appearance of β-lactam resonances in the region δ5.8-4.9 ppm. The incubation mixture was then examined overnight by ^{13}C-n.m.r 125.77 MHz, D_2O) which indicated an intense signal at δ30.87 ppm ($S^{13}\underline{C}Me_2$ of isopenicillin N (13f)) and a weak signal at δ142.2 ppm.

This incubation was repeated with two additional samples of [(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3-^{13}C)-valine (12h) (6 and 15 mg

respectively). To assist spectroscopic analysis the latter crude incubation mixture was purified by chromatography [reverse phase hplc Gilson system with 0.25% MeCN in 5 mMolar aqueous NH_4HCO_3] gave (2-^{13}C, 3-^{2}H)-isopenicillin N (13f) [retention time 7.5 minutes]. All fractions eluting before (13f) were combined and lyophilised. In both cases, analysis of the partially purified incubation mixture [fractions eluting between 1-7.5 minutes] by overnight ^{13}C-n.m.r. (125.77MHz, D_2O) revealed a weak signal at *ca* δ142 ppm. (13f); δ_H(500MHz, D_2O, HO D suppressed); 1.53 (3H, d, *J* ^{13}C-^{1}H 4 Hz, ^{13}CHC<u>H</u>$_3$), 1.63 (3H, d, *J* ^{13}C-^{1}H 4 Hz, ^{13}CHC<u>H</u>$_3$), 1.65-1.95 (4H, 2 x m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.41 (2H, *ca* t, *J* 7Hz, CH$_2$CO), 3.77 (1H, *ca* t, *J* 6Hz, C<u>H</u>CH$_2$CH$_2$), 5.48, 5.57 (2H, ABq, *J* 4.5Hz, C<u>H</u>C<u>H</u>S).

Control Incubation of [(5<u>S</u>)-5-Amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-valine (12a) with Partially Purified IPNS Enzyme.

Unlabelled [(5<u>S</u>)-5-amino-5-carboxypentanamido]-<u>L</u>-cysteinyl-<u>D</u>-valine (12a) (*ca* 4 mg) was incubated with partially purified IPNS enzyme (*ca* 11 I.U.) according to the general procedure (General Method B). Examination of the crude incubation mixture as above indicated a similar percentage conversion to unlabelled isopenicillin N (13a) as that obtained with the labelled tripeptide (12h). Overnight examination by ^{13}C-n.m.r 125.77 MHz, D_2O) indicated a moderately intense signal at δ30.77 ppm [S<u>C</u>Me$_2$ of isopenicillin N (13a)] but no signals in the region δ170-105 ppm could be observed.

(2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5<u>S</u>)-5-amino-5-carbonyplentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (13f) [(2-^{13}C,3-^{2}H)-isopenicillin N] Control Incubation with Partially Purified IPNS Enzyme.

(2<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5<u>S</u>)-5-amino-5-carboxy-pentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (13f) (*ca* 4 mg), was incubated with partially purified IPNS enzyme (*ca* 11 I.U.) according to the general procedure (General Method B). Examination of the crude incubation mixture by ^{1}H-n.m.r. (500MHz, D_2O), <u>H</u>OD suppressed) indicated that (13f) had been degraded as judged by the absence of β-lactam resonances in the region δ5.6-5.4 ppm. Examination of this crude incubation mixture by overnight ^{13}C-n.m.r. (125.77 MHz, D_2O) revealed two intense signals at δ59.9 and 60.6 ppm [assigned to S^{13}<u>C</u>Me$_2$ of penicilloate] and a complete absence of signals in the region δ175-105 ppm.

[(5S)-N-*p*-Methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzyl-carbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine Benzhydryl Ester (115b).

Procedure as for (65a) (General Method F) except that (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (64b) (240 mg, 0.36 mmol), D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (63f)[146] (95 mg, 0.33 mmol) and EEDQ (90 mg, 0.36 mmol) were used. Chromatography [flash silica, EtOAc/hexane (2:3, v/v)] gave (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (115b) (87 mg, 0.10 mmol, 30% yield). T.l.c., [ethyl acetate/ petrol (1:1, v/v)] Rf 0.15 (LLL-diastereomer, Rf 0.10). $[\alpha]_D^{20}$ - 3.2 [c = 0.6, $CHCl_3$]; V_{max} ($CHCl_3$) 3020 (s), 2960 (m), 1720 (s), 1610 (m), 1465 (s), 1250 (s); δ_H (500MHz, $CDCl_3$) 0.62-0.65, 0.73-0.76, 0.86-0.90 and 0.98-1.01 (6H, 4 x m, $^{13}CH(^{13}CH_3)_2$), 1.56-1.86 (4H, m, $CH_2CH_2CH_2CO$), 2.07-2.36 (3H, m, CH_2CO and $^{13}CH(^{13}CH_3)_2$), 2.63, 2.82 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.71 (2H, s, SCH_2Ar), 3.75 (3H, s, SCH_2ArOMe), 3.78 (6H,s, 2 x CH_2ArOMe), 4.28-4.35 (1H, m, $CHCH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $CHCH_2S$), 4.99 and 5.03 (2H, ABq, J 12Hz, OCH_2Ar), 5.08 (2H, s, OCH_2Ar), 5.43 (1H, d, J 8Hz, NH), 6.24 (1H, d, J 7Hz, NH), 6.73 (1H, s, $CHPh_2$), 6.82-6.88 and 7.21-7.34 (22H, 2 x m, ArH) [Identical to (5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-valine benzhydryl ester[66] by ^{1}H-n.m.r. except for the presence of additional multiplets at δ0.62-0.65, 0.73-0.76, 0.86-0.90 and 0.98-1.01 ppm and 2.07-2.36 ppm due to ^{13}C-^{1}H coupling caused by ^{13}C labels at valinyl C-3, C-4, and C-4' and the absence of a valinyl C-2H multiplet at δ4.64 ppm].

[(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (12i).

Procedure as for compound (12a) except that [(5S)-5-N-*p*-methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzylcarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (115b) (100 mg, 0.10 mmol) was used to give [(5S)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine as the ammonium trifluoroacetate salt (12i)[146] (30 mg, 0.06 mmol, 60% yield). δ_H (500MHz, D_2O, HOD suppressed, [referenced to HOD = 4.64

ppm]) 0.65-0.72 (3H, m, $^{13}CH^{13}C\underline{H}_3$), 0.90-0.97 (3H, m, $^{13}CH^{13}C\underline{H}_3$), 1.65-1.96 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 1.96-2.34 (1H, 3 x m, J ^{13}C-^{1}H 130Hz, $^{13}C\underline{H}(^{13}CH_3)_2$), 2.42 (2H, *ca* t, 7 Hz, CH_2CO), 2.88, 2.93 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.83-3.85 (1H, *m*, $C\underline{H}CH_2S$), 4.56 (1H, *ca* t, J 6Hz, $CH_2C\underline{H}NH_3$); δ_C(125.77MHz, D_2O) 17.93 (dq, J ^{13}C-^{13}C 35 Hz and J ^{13}C-^{1}H 75Hz, $^{13}CH^{13}\underline{C}H_3$), 19.15 (dq, J ^{13}C-^{13}C 35 Hz and J ^{13}C-^{1}H 75Hz, $^{13}CH^{13}\underline{C}H_3$), 30.54 (dt, J ^{13}C-^{1}H 93Hz and J ^{13}C-^{13}C 35 Hz, $^{13}\underline{C}H(CH_3)_2$); *m/z* (FAB) 366 (1%), 367 (2), 368 (MH^+ for $^{12}C_{11}{}^{13}C_3H_{23}DN_3O_6$, 9), 369 (4), 370 (2). Disulphide form; *m/z* (FAB) 732 (21%), 733 (MH^+, 100), 734 (77), 375 (54), 376 (26), 377 (13). [Identical to authentic LLD-ACV (12a)[161] by ^{1}H-n.m.r. except for the presence multiplets at δ0.65-0.72, 0.90-0.97, and 1.96-2.34 ppm due to ^{13}C-^{1}H coupling caused by ^{13}C labels at valinyl C-3, C-4, and C-4' and the absence of a valinyl C-2H doublet at δ4.12 ppm].

Incubation of [(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (12i) with Partially Purified IPNS.

[(5S)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (12i) (*ca* 25 mg) was incubated with partially purified IPNS enzyme (*ca* 90 I.U.) according to the general procedure (General Method B). After the usual work-up, the total crude incubation mixture was examined by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) which indicated good conversion (*ca* 70%) to (2R,5R,6R)-1-aza-(3-^{13}C)-3,3-di-(^{13}C-methyl)-6-[(5S)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate (13f) [(2,2',2'-$^{13}C_3$)-isopenicillin N] as judged by the absence of tripeptide resonances in the region δ1.0-0.9 ppm and the appearance of β-lactam resonances in the region δ5.6-5.4 ppm. The incubation mixture was then examined overnight by ^{13}C-n.m.r 125.77 MHz, D_2O) which indicated an intense signals in the region δ30-15 ppm [$S^{13}\underline{C}(^{13}\underline{C}H_3)_2$ of isopenicillin N (13g)], a weak multiplet at δ143 ppm and a broad multiplet at 116-113 ppm.

[(5R)-N-*p*-Methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzyl-carbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine Benzhydryl Ester (65f).

Procedure as for (65a) (General Method F) except that (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (64a)[66] (136 mg, 0.20 mmol), D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (63f) (58 mg, 0.20 mmol) and EEDQ (56 mg, 0.20 mmol) were

used. Chromatography [flash silica, EtOAc/hexane (2:3, v/v)] gave (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (65f)[146] (45 mg, 0.05 mmol, 25% yield). T.l.c., [ethyl acetate/ petrol (3:2, v/v)] Rf 0.30 (DLL-diastereomer, Rf 0.25). $[\alpha_D^{20}]$ = - 5.5 [c = 0.33, $CHCl_3$]; V_{max} ($CHCl_3$) 3020 (s), 2960 (m), 1720 (s), 1515 (m), 1448 (s), 1250 (s); δ_H (500MHz, $CDCl_3$) 0.62-0.65, 0.73-0.76, 0.86-0.90 and 0.98-1.01 (6H, 4 x m, $^{13}CH(^{13}CH_3)_2$), 1.57-1.88 (4H, m, $CH_2CH_2CH_2CO$), 2.04-2.38 (3H, m, CH_2CO and $^{13}CH(^{13}CH_3)_2$), 2.63-2.67, 2.82-2.85 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.71 (2H, s, SCH_2Ar), 3.75 (3H, s, SCH_2ArOMe), 3.78 (6H,s, 2 x CH_2ArOMe), 4.29-4.35 (1H, m, $CHCH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $CHCH_2S$), 5.00 and 5.03 (2H, ABq, J 12Hz, OCH_2Ar), 5.08 (2H, s, OCH_2Ar), 5.43 (1H, d, J 8Hz, NH), 6.23 (1H, d, J 7Hz, NH), 6.77 (1H, s, $CHPh_2$), 6.81-6.90 and 7.22-7.32 (22H, 2 x m, ArH).

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (66f).

Preparation as for compound (66a) except that [(5R)-5-N-*p*-methoxybenzyl-oxycarbonyl-5-amino-5-*p*-methoxybenzylcarbonylpentanamido]-S-*p*-methoxy-benzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (65f) (30 mg, 0.03 mmol) was used to give [(5R)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine as the ammonium trifluoroacetate salt (66f)[146] (15 mg, 0.03 mmol, 97% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.78-0.85 and 1.04-1.11 (6H, 2 x m, J ^{13}C-^{1}H 126Hz, $^{13}CH(^{13}CH_3)_2$), 1.54-1.98 (4H, 2 x m, $CH_2CH_2CH_2CO$), 1.96-2.34 (1H, 3 x m, J ^{13}C-^{1}H 126Hz, $^{13}CH(^{13}CH_3)_2$), 2.42 (2H, *ca* t, 7 Hz, CH_2CO), 2.88, 2.93 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.85-3.87 (1H, *m*, $CHCH_2S$), 4.56 (1H, *ca*. t, J 6Hz, $CH_2CHN^+H_3$); Partial δ_C(125.77MHz, D_2O) 17.93 (dq, J ^{13}C-^{13}C 35 Hz and J ^{13}C-^{1}H 75Hz,$^{13}CH^{13}CH_3$), 19.15 (dq, J ^{13}C-^{13}C 35 Hz and J ^{13}C-^{1}H 75Hz, $^{13}CH^{13}CH_3$), 30.54 (dt, J ^{13}C-^{1}H 93Hz and J ^{13}C-^{13}C 35 Hz, $^{13}CH(CH_3)_2$); *m/z* (FAB) 366 (8%), 367 (22), 368 (MH^+ for $^{12}C_{11}{}^{13}C_3H_{23}DN_3O_6$, 100), 369 (54), 370 (13), 371 (4).

Incubation of [(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (66f) with Partially Purified IPNS.

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-(D)-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (66f) (*ca* 10 mg) was incubated with IPNS (*ca* 60 I.U.) according to the general procedure (General Method B). Examination of the resulting crude incubation mixture by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) indicated good

conversion to (2R,5R,6R)-1-aza-(2-^{2}H, 3-^{13}C)-3,3-di-^{13}C-methyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylate [(2,2',2'-^{13}C, 3-^{2}H)-penicillin N] (14) as judged by the presence of strong β-lactam resonances at δ5.48 and 5.57ppm, which was used without further purification.

Incubation of (2R,5R,6R)-1-aza-(2-^{2}H, 3-^{13}C)-3,3-di-(^{13}C-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0] heptane-2-carboxylate (14) [(2,2',2'-$^{13}C_3$, 3-^{2}H)-penicillin N] with Partially Purified DAOC/DAC synthetase.

Crude (2,2',2'-^{13}C, 3-^{2}H)-penicillin N (14) was incubated with DAOC/DAC synthetase (*ca* 0.7 I.U.) according to the usual procedure (General Method C). Examination of the resulting crude incubation mixture by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) indicated approximately 65% conversion to (2,3,3'-$^{13}C_3$)-DAOC (18g), (2,3,3'-$^{13}C_3$)-DAC (19g) and (2,3,3'-$^{13}C_3$)-3β-hydroxycepham (46g). Examination by overnight ^{13}C-n.m.r. (125.77MHz, D_2O) revealed intense signals in the region δ32-17 ppm due to α- and β-^{13}C-methyl groups of (2,2',2'-^{13}C, 3-^{2}H)-penicillin N (14), S$^{13}CH_2$C of (2,3,3'-$^{13}C_3$)-DAOC (18g) and S$^{13}CH_2$C of (2,3,3'-$^{13}C_3$)-DAC (19g), 35.0 [d, ^{13}C(OH)^{13}CH$_3$ of (2,3,3'-$^{13}C_3$)-3β-hydroxycepham (46g)], 60.2 [d, ^{13}C^{13}CH$_2$OH of (2,3,3'-$^{13}C_3$)-DAC (19g)], 65.0 [t, ^{13}C(^{13}CH$_3$)$_2$ of (14)], 65.6 (^{13}CH$_2$^{13}C(OH)^{13}CH$_3$ of (46g)], 122.6 [t, ^{13}CH$_2$^{13}C^{13}CH$_2$OH of (19g)] and 123.8 [t, ^{13}CH$_2$^{13}C^{13}CH$_3$ of (18g)] . No signals in the region δ170-125 ppm could be detected.

[(5S)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyl-carboxypentanamido]-(L)-O-*t*-butyl-serinyl-(D)-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine Benzhydryl Ester (118).

As for general coupling procedure (General Method F) except that [(5S)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido] -(L)-O-*t*-butyl-serine (117) (50 mg, 0.07 mmol), D/L-(2-^{2}H,3,4,4'-$^{13}C_3$)-valine benzhydryl ester ammonium tosylate salt (63f) (35 mg, 0.07 mmol), EEDQ (20 mg, 0.08 mmol) and anhydrous Na_2SO_4 (*ca* 5 mg) were used. Attempted separation of the diastereomers by chromatography [preparative plate silica, with EtOAc/hexane (3:2, v/v)] was unsuccessful and the mixture of diastereomers (118b/c) (20 mg, 0.02 mmol, 30% yield)[146] was used without further purification.

T.l.c [EtOAc/hexane (3:2, v/v)] LLD- and LLL-diastereomers (118b) and (118c) respectively, Rf 0.2. V_{max} ($CHCl_3$) 3020 (s), 1730 (m), 1665 (m), 1516 (s), 1250 (m).

[(5S)-5-Amino-5-carboxypentanamido]-L-serinyl-D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (111b/c).

As for compound (66a) except that [(5S)-5-N-*p*-methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzylcarbonylpentanamido]-Q-*t*-butyl-L-serinyl-(D/L)-(2-^{2}H, 3,4,4-$^{13}C_3$)-valine benzhydryl ester (118b/c) (20 mg, 0.02 mmol) was used to give [(5S)-5-amino-5-carboxypentanamido]-L-serinyl-(D/L)-(2-^{2}H, 3,4,4-$^{13}C_3$)-valine as the ammonium trifluoroacetate salt (111b/c)[146] (8 mg, 0.02 mmol, 86% yield). Separation of the diastereomers was attempted by chromatography [hplc, Waters' system, reverse phase with 10 mMolar aqueous NH_4HCO_3] gave no separation as judged by ^{1}H-n.m.r. (111b/c) δ_H (500MHz, D_2O, HOD suppressed) 0.73-1.12 (6H, 6 x m, $J^{13}C-^1H$ 126Hz, $^{13}CH(^{13}CH_3)_2$), 1.64-1.86 (2 x m, $CHCH_2CH_2$), 1.87-2.02, 2.08-2.16, 2.21-2.27 and 2.34-2.46 (3H, 4 x m, $^{13}CH(^{13}CH_3)_2$, CH_2CO), 3.74 (1H, ca t, *J* 6Hz, $CHCH_2CH_2$), 3.85-3.89 (2H, m, $CHCH_2OH$), 4.50 (1H, *ca* t, *J* 5Hz, $CHCH_2OH$); m/z (FAB) 351 (10%), 352 (MH^+, 100), 353 (20), 354 (4), 355 (1).

Incubation of [(5S)-5-Amino-5-carboxypentanamido]-L-serinyl-D-(2-^{2}H, 3,4,4-$^{13}C_3$)-valine (111b/c) with Partially Purified IPNS.

[(5S)-5-Amino-5-carboxypentanamido]-L-serinyl-(D/L)-(2-^{2}H, 3,4,4-$^{13}C_3$)-valine (111b/c) (*ca* 6 mg) was incubated with IPNS (*ca* 18 I.U.) according to the general procedure (General Method B). Examination of the resulting crude incubation mixture by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) indicated no conversion to a β-lactam containing compound as judged by the absence of AB quartets in the region δ5.6-5.0 ppm. Examination of the incubation mixture by overnight ^{13}C-n.m.r. (125.77MHz, D_2O) revealed intense resonances in the region δ32-29 [m, $^{13}CH(^{13}CH_3)_2$] and 20-17 ppm [m, $^{13}CH(^{13}CH_3)_2$] assumed to be associated with the starting material (11b/c) and a complete absence of signals in the region δ170-105ppm.

8.7 Experimental for Chapter 7.

(6R,7R)-1-Aza-3-methyl-7-amino-[(5R)-5-N-*t*-butyloxycarbonylamino-5-diphenylmethyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo [4.2.0]oct-2-ene-2-carboxylic Acid Benzhydryl Ester (149).

Deacetoxycephalosporin C (18a) (500 mg, 1.4 mmol) was dissolved in saturated aqueous $NaHCO_3$ / dioxan (40 ml, 1:1, v/v) and di-*t*-butylpyrocarbonate (458 mg, 2.1 mmol, 1.5 eq) added in one portion. The resulting solution was stirred at room temperature overnight, extracted with DCM (3 x 30 ml), acidified to *ca* pH 2 with 2N HCl and extracted with EtOAc (3 x 50 ml). The combined EtOAc layers were dried (anhydrous Na_2SO_4), filtered and evaporated to give the N-*t*-BOC-di-acid (672 mg, 1.4 mmol, 100% yield). This was re-dissolved in MeCN (10 ml) and diphenyldiazomethane (535 mg, 2.8 mmol) in MeCN (5 ml) added. After stirring overnight, the solvent was evaporated and the residue taken-up in EtOAc (50 ml), washed with 1N HCl (20 ml), saturated aqueous $NaHCO_3$ (20 ml), brine (20 ml), and dried (anhydrous Na_2SO_4). The solution was filtered and evaporated *in vacuo* to give, after chromatography [flash silica, with EtOAc/DCM (3:1, v/v)], (149) (1.127g, 1.4 mmol, 100% yield). T.l.c [EtOAc/hexane (1:1, v/v)]Rf 0.38. M.p 104-106°C (from DCM/petrol); $[\alpha]_D^{22}$ + 28.6 [c = 1.7, $CHCl_3$] ; (Found C 68.31, H 6.08 and N 5.38%. $C_{43}H_{45}N_3O_{10}S$ requires C 68.42, H 6.00, N 5.32%). V_{max} ($CHCl_3$) 3430 (w), 3020 (s), 1785 (m), 1720 (s), 1500 (s), 1284 (m); δ_H(500MHz, $CDCl_3$) 1.41 (9H, s, C(C<u>H</u>$_3$)$_3$), 1.57-1.68 (4H, br.m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$), 2.11 (3H, s, C<u>H</u>$_3$), 2.15-2.27 (2H, br m, C<u>H</u>$_2$CO), 3.18 and 3.46 (2H, ABq , *J* 18Hz, SC<u>H</u>$_2$), 4.92-4.95 (1H, m , C<u>H</u>NHCO), 4.95 (1H, d, *J* 4Hz, C<u>H</u>S), 5.13 (1H, d, *J* 8Hz, N<u>H</u>CH), 5.74 (1H, dd, *J* 4, 8Hz, NHC<u>H</u>CHS), 6.89 and 6.93 (2 x 1H, 2 x s, 2 x C<u>H</u>Ph$_2$), 7.24-7.81 (20H, m, 2 x C<u>H</u>Ph$_2$).

(2R,6R,7R)-1-Aza-3-methyl-7-amino-[(5R)-5-N-*t*-butyloxycarbonyl-amino-5-diphenylmethyloxycarbonylpentanamido]-8-oxo-5-thia-bicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester (150).

Protected deacetoxycephalosporin C (149) (500 mg, 0.635 mmol) in THF (10 ml) was stirred and cooled to -78°C and LDA (1.90 mmol) in THF (10 ml) was added dropwise *via* a syringe. The resulting solution was stirred at 0°C for 20 minutes and then quenched by the addition of glacial acetic acid (270 mg, 4.45 mmol). The solvent was evaporated and the residue re-dissolved in EtOAc (50 ml), washed with water (20 ml), dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo*. Chromatography [flash silica, EtOAc/hexane (1:3 to 1:1, v/v)] gave (150)

(310 mg, 0.39 mmol, 62% yield). T.l.c. [EtOAc/hexane (1:1, v/v)] Rf 0.41. M.p. 78-81 °C (from DCM/petrol), $[\alpha]_D^{22}$ + 261 [c = 0.8, $CHCl_3$] ; (Found C 68.76, H 6.29 and N 5.47% $C_{43}H_{45}N_3O_{10}S$ requires C 68.42, H 6.00, N 5.32%). V_{max} ($CHCl_3$) 3435 (w), 3020 (s), 1780 (s), 1742 (s), 1710 (s), 1500 (s); δ_H(500MHz, $CDCl_3$); 1.46 (9H, s, C(C<u>H</u>$_3$)$_3$), 1.62-1.78 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH_2CO), 1.81 (3H, s, C<u>H</u>$_3$), 2.14-2.35 (2H, m, C<u>H</u>$_2$CO), 4.41-4.44 (1H, m, C<u>H</u>NHCO), 4.83 (1H, s, C<u>H</u>CO_2BzH), 5.13 (1H, d , *J* 8Hz, N<u>H</u>CH), 5.21 (1H, d, *J* 4Hz, CHC<u>H</u>S), 5.55 (1H, dd, *J* 4 and 8Hz, NHC<u>H</u>CH), 5.91 (1H, s, SC<u>H</u>=C), 6.25 (1H, d, *J* 8Hz, N<u>H</u>CO_2), 6.89 (2H, s, 2 x C<u>H</u>Ph_2), 7.25-7.37 (20H, m, 2 x CH<u>Ph</u>$_2$); δ_C(125.77MHz, $CDCl_3$); 20.96 (t, CHCH_2<u>C</u>H_2), 21.87 (q, <u>C</u>H_3), 28.12 (q, C(<u>C</u>H_3)$_3$), 31.55 (t, CH<u>C</u>H_2CH_2), 34.76 (t, <u>C</u>H_2CO), 52.72, 53.03 and 60.13 (3 x d, NH<u>C</u>HCHS, NHCH<u>C</u>HS, <u>C</u>HCH_2CH_2 and <u>C</u>HC=CH), 78.67 (d, <u>C</u>HPh_2), 79.64 (s, <u>C</u>$(CH_3)_3$) 114.16 (d, S<u>C</u>H=CMe), 119.84 (s, CH=<u>C</u>Me), 126.53-139.49 (m, <u>Ar</u> and CHC=<u>C</u>H), 155.40, 164.59, 166.20, 171.44 and 172.41 (5 x s, <u>C</u>=O).

(6<u>R</u>,7<u>R</u>)-1-Aza-3-methyl-7-amino-[(5<u>R</u>)-5-amino-5-carboxypentan-amido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (148).

The cephem (150) (14 mg, 0.018 mmol), was dissolved in toluene/TFA/anisole [20:3:1, v/v (5 ml)] and stirred at room temperature for 30 minutes. The solvent was evaporated and the residue partitioned betweeen water (5 ml) and EtOAc (5 ml). The aqueous phase was separated, extracted with EtOAc (3 x 5 ml) and freeze-dried. Chromatography [hplc ,Water's system, flow rate = 1.0 $ml.min^{-1}$, with 25 mmol aqueous NH_4HCO_3 as solvent, Rt 5.5 minutes] gave (148)[146] (8 mg, 0.017 mmol, 95% yield). $_{max}$ (H_2O) 195nm; δ_H(500MHz, D_2O, <u>H</u>OD suppressed) 1.70-2.11 (7H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH_2CO and CH=C<u>Me</u>), 2.43 (2H, *ca* t, *J* 7Hz, C<u>H</u>$_2$CO), 4.03 (1H, *ca* t, *J* 6Hz, C<u>H</u>CH_2CH_2), 4.85 (1H, s, NHC<u>H</u>C=CH), 5.30 and 5.41 (2 x 1H, ABq, *J* 4Hz, C<u>H</u>C<u>H</u>S), 6.08 (1H, s, SC<u>H</u>=CMe); δ_C(500MHz, D_2O) 21.38 (t, CHCH_2<u>C</u>H_2), 22.03 (q, CH=C<u>Me</u>), 29.91 (t, CH<u>C</u>H_2CH_2), 35.13 (t, <u>C</u>H_2CO), 53.10, 53.51, 54.20 and 60.63 (4 x d, NH<u>C</u>HCHS, NHCH<u>C</u>HS, <u>C</u>HCH_2CH_2 and <u>C</u>HC=CHS), 113.86 (d, C=<u>C</u>HS), 121.97 (s, Me<u>C</u>=CHS), 166.48, 171.86, 172.70 and 176.71 (4 x s, C=O); *m/z* (FAB) 357 (15%), 358 (MH^+, 100), 359 (35), 360 (16), 361 (15), 362 (7).

(2<u>R</u>,6<u>R</u>,7<u>R</u>)-1-Aza-3-($^2H_3$-methyl)-7-amino-[(5<u>R</u>)-5-<u>N</u>-*p*-methoxy-benzyloxycarbonylamino-5-*p*-methoxylcarbonyl-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester (155b).

(2<u>R</u>,5<u>R</u>,6<u>R</u>,7<u>R</u>)-1-Aza-3-($^2H_3$-methyl)-7-amino-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic acid, benzhydryl ester, ammonium tosylate salt (102c) (555 mg,

0.21 mmol) was shaken with saturated aqueous sodium bicarbonate solution and then extracted into EtOAc (3 x 25 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and evaporated *in vacuo* to give the free amine. The amine was then coupled (General Procedure F) to (5R)-5-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid (103)[146] (95 mg, 0.21 mmol) with EEDQ (60 mg, 0.23 mmol, 1.1 eq) in the presence of anhydrous Na_2SO_4 (*ca* 5 mg). Chromatography [flash silica, EtOAc/petrol (3:2, v/v)] gave (155b)[146] (115 mg, 1.42 mmol, 68% yield) as a white solid. T.l.c., [EtOAc] R_f 0.65. M.p 68-9°C (from DCM/petrol). (Found C 64.96, H 5.91 and N 4.83%. $C_{44}H_{42}D_3N_3SO_{10}$ requires C 65.17, H 5.59 and N 5.18%). V_{max} ($CHCl_3$) 3430 (w), 3020 (s), 1775 (s), 1740 (s), 1720 (s), 1615 (m), 1515 (s), 1250 (s); δ_H(500MHz, $CDCl_3$) 1.62-1.87 (4H, br m, $CH_2CH_2CH_2CO$), 2.14-2.35 (2H, m, CH_2CO), 3.80 (6H, s, 2 x ArOMe), 4.36-4.39 (1H, m, CHNHCO), 4.83 (1H, s, $CHCO_2BzH$), 5.00 and 5.05 (2H, ABq, *J* 12Hz, CO_2CH_2Ar), 5.10 (2H, s, CH_2Ar), 5.21 (1H, d, *J* 4Hz, CHCHS), 5.35 (1H, d, *J* 8Hz, NH), 5.56 (1H, dd, *J* 4, 8Hz, NHCHCHS), 5.89 (1H, s, SCH=C), 6.33 (1H, d, *J* 8Hz, NH), 6.87-6.89 and 7.23-7.37 (19H, 2 x m, $CHPh_2$, 2 x ArOMe and $CHPh_2$); δ_C(125.77MHz, $CDCl_3$) 21.06 (t, $CHCH_2CH_2$), 31.80 (t, $CHCH_2CH_2$), 34.85 (t, CH_2CO), 52.77, 53.07, 53.35 (3 x d, NHCHCHS, NHCHCHS, $CHCH_2CH_2$), 55.09 (q, ArOMe), 60.13 (d, CHC=CH), 66.69 and 66.91 (2 x t, CH_2Ar), 78.76 (d, $CHPh_2$), 113.78 and 113.87 (2 x d, Ar), 114.12 (d, SCH=CMe), 119.93 (s, CH=CMe), 126.61-138.95 (m, Ar and CHC=CH), 156.05 (s, ArO_2CNH), 159.44 and 159.65 (2 x s, MeOC of ArOMe), 164.73, 166.23, 171.98 and 172.42 (4 x s, C=O).

(6R,7R)-1-Aza-3-($^2H_3$-methyl)-7-amino-[(5R)-5-amino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (148b).

Preparation as for compound (148a) except that (2R,6R,7R)-1-aza-3-($^2H_3$-methyl)-7-amino-[(5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxylcarbonyl-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic acid benzhydryl ester (155b) (84 mg, 0.01 mmol) was used to give (148b)[146] as the ammonium trifluoroacetate salt (40 mg, 0.08 mmol, 80% yield). $_{max}$ (H_2O) 194nm; δ_H(500MHz, D_2O, HOD suppressed) 1.70-2.11 (4H, m, $CH_2CH_2CH_2$), 2.43 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.99 (1H, *ca* t, *J* 6Hz, $CHCH_2CH_2$), 4.76 (1H, s, NHCHC=CH), 5.30 and 5.41 (2 x 1H, ABq, *J* 4Hz, CHCHS), 6.07 (1H, s, SCH=CMe); δ_C(500MHz, D_2O) 21.42 (t, $CHCH_2CH_2$), 30.02 (t, $CHCH_2CH_2$), 35.18 (t, CH_2CO), 53.13, 53.79, 54.52 and 60.59 (4 x d, NHCHCHS, NHCHCHS, $CHCH_2CH_2$ and CHC=CHS), 113.60 (d, C=CHS), 122.18 (s, MeC=CHS), 166.49, 172.24, 173.09 and 176.78 (4 x s, C=O); *m/z* (FAB) 360 (4%), 361 (MH^+, 100), 362 (18), 363 (7), 364 (2).

(6R,7R)-1-Aza-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester.

3-Chlorocephem ester (98a) (340 mg, 0.70 mmol) was dissolved in THF (10 ml) and acetic acid and water [9:1, v/v (8 ml)] added. Activated zinc dust (1.0 g) was added and the mixture heated to reflux with stirring. Additional activated zinc dust (3.0 g) was added over a 6 hour period and the reflux continued for a further 6 hours after the addition was complete. The mixture was cooled and filtered through a scinter funnel and the filtrate partitoned between water (30 ml) and DCM (50 ml). The organic layer was dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo*. Chromatography [flash silica, EtOAc/ petrol (1:4, v/v)] gave the title compound (185 mg, 0.40 mmol, 58% yield)[152]. T.l.c., [EtOAc/ petrol (1:2, v/v)] Rf 0.55. $[\alpha]_D^{22}$ +39.3 [c = 0.8, $CHCl_3$] ; $V_{max.}$ ($CHCl_3$) 3430 (w), 3020 (w), 1792 (s), 1718 (s), 1500 (s), 1220 (s); δ_H (500 MHz, $CDCl_3$) 1.47 (9H, s, $C(CH_3)_3$), 3.41 and 3.59 (2 x 1H, AB of ABX, J_{AB} 20, J_{AX} 6 and J_{BX} 3Hz, SCH_2), 4.93 (1H, d, J 5Hz, CHS), 5.29 (1H, d, J 10Hz, NHCH), 5.69 (1H, dd, J 5 and 10Hz, NHCHCH), 6.65 (1H, X of ABX, J_{AX} 6 and J_{BX} 3Hz, SCH_2CH), 6.96 (1H, s, $CHPh_2$), 7.25-7.47 (10H, m, $CHPh_2$); δ_C(125.77MHz, $CDCl_3$) 24.10 (t, SCH_2), 28.19 (q, $C(CH_3)_3$), 57.35 and 61.35 (2 x d, CHCHS), 79.09 (d, $CHPh_2$), 81.11 (s, $C(CH_3)_3$), 120.67 (d, C=CH), 127.09-139.63 (8 x m, C=CH and Ar), 154.61, 160.65 and 165.11 (3 x s, C=O); *m/z* (DCI, NH_3) 484 (MNH_4^+, 3%), 167 (100%).

(6R,7R)-1-Aza-7-amino-[(5R)-5-N-*p*-methoxybenzyloxycarbonyl-amino-5-*p*-methoxybenzyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid Benzhydryl Ester (147).

The cephem ester (64.5 mg, 0.14 mmol) and *p*-toluenesulphonic acid (24 mg, 0.14 mmol) were dissolved in EtOH/Et_2O [1:1, v/v (2 ml)] and the resulting solution stirred at room temperature overnight. The solvent was evaporated to give the crude tosylate salt (72 mg) a sample of which, (52 mg, 0.10 mmol) was then dissolved/ suspended in saturated $NaHCO_3$ (10 ml) and this solution extracted with EtOAc (3 x 15 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo* to give the free amine (37 mg, 0.10 mmol) which was coupled to (103a) (45 mg, 0.10 mmol) by the usual method (General Method F). Chromatography [preparative plate, EtOAc/DCM (1:1, v/v)] gave (147)[146] (33 mg, 0.04 mmol, 42% yield). T.l.c., [EtOAc/DCM (1:1, v/v)] Rf 0.80. V_{max} ($CHCl_3$) 3430 (w), 3019 (s), 1790 (m), 1720 (s), 1515 (s), 1220 (s); δ_H(500MHz,

$CDCl_3$) 1.56-1.62 (4H, br m, $C\underline{H}_2C\underline{H}_2CH_2$), 2.09-2.28 (2H, br.m, $C\underline{H}_2CO$), 3.25 and 3.47 (2 x 1H, AB of ABX, J_{AB} 20,J_{AX} 6, J_{BX} 3Hz, $SC\underline{H}_2$), 3.71 (6H, s, $ArOC\underline{H}_3$), 4.30 (1H, br.m, $C\underline{H}NHCO$), 4.85 (1H, d, J 5Hz, $C\underline{H}S$), 4.94 (2H, dd, J 3 and 10Hz, $ArC\underline{H}_2$), 5.03 (2H, s, $ArC\underline{H}_2O_2CNH$), 5.32 (1H, d, J 10Hz, $N\underline{H}CH$),5.78 (1H, dd, J 5 and 10Hz, $NHC\underline{H}CH$), 6.40 (1H, d, J 10Hz ,$ArOCON\underline{H}$), 6.53 (1H, X of ABX, J_{AX} 6 and J_{BX} 3Hz, $SCH_2C\underline{H}$), 6.89 (1H, s, $C\underline{H}Ph_2$), 6.77-6.81 and 7.17-7.38 (18H, 2 x m, ArH).

(6R,7R)-1-Aza-7-amino-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (146).

Procedure as for compound (148a) except that (6R,7R)-1-aza-7-amino-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester (147) (30 mg, 0.04 mmol) was used to give (146)[146] as the ammonium trifluoroacetate salt (15 mg, 0.03 mmol, 75% yield). Purification by chromatography [reverse phase hplc, Waters' system with ODS C_{18} as the stationary phase and 25 mM aqueous NH_4HCO_3 as elutant, Rt. 5.5 minutes] gave pure (146) as the ammonium salt. δ_H(500MHz, D_2O, $\underline{H}OD$ suppressed) 1.70-1.94 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.43 (2H, *ca* t, 7 Hz, CH_2CO), 3.49, 3.68 (2H, ABX, J_{AB} 19Hz, J_{AX} 6, J_{BX} 3Hz, $SC\underline{H}_2CH$), 3.73 (1H, *ca* t, J 6Hz, $C\underline{H}CH_2CH_2$), 5.12, 5.69 (2H, ABq, $NHC\underline{H}C\underline{H}S$), 6.31 (1H, X of ABX, J_{AX} 6, J_{BX} 3Hz, $SCH_2C\underline{H}$); m/z (FAB) 344 (MH^+,100%), 345 (37), 346 (19).

Incubation of (6R,7R)-1-Aza-7-amino-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (146) with Partially Purified DAOC/DAC synthetase.

Desmethyl DAOC (146) (1 mg) was incubated with with partially purified DAOC/DAC synthetase (*ca* 0.05 I.U.) in the usual way (General Method C). Examination of the ^{1}H-n.m.r spectrum in the region δ 4.9-5.5 ppm, indicated the presence of no additional β-lactam resonances. Similarly bio-assay against *(+) E. coli* (ESS 21/30) gave no zones of inhibition.

Penicillin N β-sulphoxide (55)[36].

To a solution of (2S, 5R, 6R)-1-aza-3,3'-dimethyl-6-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitrobenzyloxycarbonylpentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid *p*-nitrobenzyl ester β-sulphoxide (71 mg, 0.10 mmol)36 in THF (10 ml) were added water (10 ml) and sodium bicarbonate

(7.8 mg, 1 eq). The resulting solution was hydrogenated at 0.1 MPa (1atm gauge) over 10% Pd on charcoal (100 mg) for 3 hours. This solution was then filtered through celite, washing with water (10 ml) and the filtrate extracted with EtOAc (2 x 10 ml). The aqueous layer was then lyophilized to give the crude sulphoxide (55) which was purified by chromatography [reverse phase Hplc, Gilson system with ODS C_{18} as the stationary phase and 25 mM aqueous NH_4HCO_3 as elutant, Rt. 20 minutes] to give pure (55) as the ammonium salt. V_{max} (D_2O); 1775 (m) and 1630 (m); δ_H (500 MHz, D_2O, HOD suppressed) 1.11 (3H, s, α -Me), 1.50 (3H, s, β -Me), 1.45-1.59 and 1.63-1.77 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.19-2.23 (2H, m, CH_2CO), 3.60-3.63 (1H, m, $NCHCH_2$), 4.31 (1H, s, $NCHCO_2H$), 5.18 and 5.67 (2H, ABq, *J* 4.5 Hz, β -lactam); *m/z* (FAB) 376 (MH^+, 100%), 377 (22), 378 (15), 379 (5).

Incubation of Penicillin N β-sulphoxide (55) with Partially Purified DAOC/DAC synthetase.[36]

Penicillin N β-sulphoxide (55)[36] (2 mg) was incubated with partially purified DAOC/DAC synthetase in the usual way (General Method C). Examination of the ^{1}H-n.m.r spectrum in the region δ 4.9-5.5 ppm, indicated the presence of no additional β-lactam resonances. Similarly bio-assay against *(+) E. coli* (ESS 21/30) gave no zones of inhibition, thereby indicating that no conversion to penicillinase resistant antibiotics had occurred.

Incubation of (2S,3R,5R,6R)-1-Aza-6-[(5R)-5-carboxypentanamido]-3-methoxymethyl-3-methyl-7-oxo-4-thiabicyclo[3.2.0]-heptane-2-carboxylic acid (138)[148] **with Partially Purified DAOC/DAC synthetase.**

β-Methoxymethyl penicillin N (138) (1.5 mg) was incubated with with partially purified DAOC/DAC synthetase in the usual way (General Method C). Examination of the ^{1}H-n.m.r spectrum in the region δ 4.9-5.5 ppm, indicated the presence of no additional β-lactam resonances. Similarly bio-assay against *(+) E. coli* (ESS 21/30) gave no zones of inhibition, thereby indicating that no conversion to penicillinase resistant antibiotics had occurred.

Incubation of Δ2-DAOC (148a) with Partially Purified DAOC/DAC synthetase.

Δ2-DAOC (148) (*ca* 1mg) was incubated with partially purified DAOC/DAC synthase according to the usual procedure (General Method C). Examination of the

crude incubation mixture by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) indicated that approximately 60% conversion to a new β-lactam containing compound had occurred, as judged by the appearance of a new set β-lactam resonances in the region δ 5.5-5.0 ppm and a new olefinic signal at δ 6.38 ppm.

Chromatography [reverse phase hplc, Water's System eluting with 0.015% HCO_2H in methanol/ water (24 : 1, v/v), retention time 3.5 minutes] gave Δ2-DAC (151), the ^{1}H-n.m.r spectrum (500MHz, D_2O, HOD suppressed) of which was identical to that of an authentic synthetic sample of Δ2-DAC (151) (prepared as outlined below). λ_{max} (H_2O) 195nm; δ_H (500MHz, D_2O, HOD suppressed) 1.65-1.82 and 1.84-1.98 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7 Hz, CH_2CH_2CO), 3.74 (1H, *ca* t, *J* 6 Hz, H_2NCHCO_2H), 4.16 and 4.23 (2H, ABq, *J* 14 Hz, CH_2OH), 5.30 and 5.39 (2H, ABq, *J* 4Hz, β-lactams), 6.34 (1H, s, SCH=C) (the $NCHCO_2H$ resonance was assumed to be under the suppressed solvent peak). The mass spectrum gave the protonated molecular ion as 375. *m/z* (FAB); 374 (MH^+ for $C_{14}H_{19}N_3SO_7$, 5%), 375 (MH^+ for $C_{14}H_{18}DN_3SO_7$, 100), 376 (30), 377 (15).

Incubation of Δ2-[3'-(^{2}H$_3$-methyl)]-DAOC (148b) with Partially Purified DAOC/DAC synthetase.

As for unlabelled Δ2-DAOC DAOC/DAC synthetase incubation except that Δ2-[3'-(^{2}H$_3$-methyl)]-DAOC (148b) (*ca* 1 mg) was incubated according to the usual procedure (General Method C). Examination of the crude incubation mixture by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed) indicated ca 5% to a new β-lactam containing compound, assumed to be the Δ2-[3'-($^2H_2$-hydroxymethyl)]-DAC (151c), as judged by the appearance of a new set β-lactam resonances in the region δ 5.5-5.0 ppm and a new olefinic signal at δ 6.38 ppm.

(6R,7R)-1-Aza-3-acetoxymethyl-7-amino-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitrobenzylylcarbonyl-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Di-*p*-nitrobenzyl Ester (154). (N-*p*-Nitrobenzyloxycarbonyl-Δ2-Cephalosporin C, Di-p-nitrobenzyl Ester)

Cephalosporin C (20) (500 mg, 1.59 mmol) was dissolved in water (10 ml) and saturated $NaHCO_3$ (5 ml). To this was added *p*-nitrobenzylchloroformate (400 mg, 1.9 mmol) in dioxan (10 ml) and the solution stirred at room temperature for 1 hour. The reaction mixture was extracted with DCM (3 x 20 ml), acidified to *ca* pH 2 and then rapidly extracted with EtOAc (3 x 50 ml). The combined EtOAc layers were

dried (anhydrous Na_2SO_4), filtered and evaporated *in vacuo* to give the N-protected cephalosporin C as a white solid which was used without further purification.

The N-protected di-acid was dissolved in dry DMF (20 ml) and $NaHCO_3$ (290 mg, 2.2 eq) added. *p*-Nitrobenzylbromide (760 mg, 3.5 mmol) was added and the resulting solution stirred for 48 hours at room temperature. The reaction mixture was diluted with EtOAc (80 ml) and washed with water (2 x 50 ml), NaOH (2N, 20 ml), brine (50 ml), dried (anhydrous Na_2SO_4) filtered and evaporated *in vacuo*. The product, (6R,7R)-1-aza-3-acetoxymethyl-7-amino-[(5R)-5-N-*t*-butyloxycarbonyl-amino-5-diphenylmethyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (Δ3-Cephalosporin C) (154) (680 mg, 0.79 mmol, 50% from (20)) was used without further purification due to extreme insolubility. M.p 62-64°C (from EtOAc).

(6R,7R)-1-Aza-3-acetoxymethyl-7-amino-[(5R)-5-amino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid (156). (Δ2-Cephalosporin C)

(6R,7R)-1-Aza-3-acetoxymethyl-7-amino-[(5R)-5-N-*t*-butyloxycarbonyl-amino-5-diphenylmethyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (154) (110 mg, 0.13 mmol) was dissolved in pyridine (10 ml) and water (5 ml) added. The resulting solution was stirred and cooled to 0°C in an ice bath and NaOH (130µl of 1.0 N solution, 1 eq) added. Stirring at 0°C was continued for a further 3 hours and the reaction mixture then washed with DCM (20 ml), acidified to *ca* pH 2 by the addition of 2N HCl and rapidly extracted with EtOAc (3 x 50 ml). The combined EtOAc layers were dried (anhydrous Na_2SO_4) filtered and evaporated *in vacuo* to give a crude mixture of protected Δ2-cephalosporin C (156) and protected Δ2-DAC (65 mg total), in a ratio of approximately 10:1 as judged by ^{1}H-n.m.r (500 MHz, $^2H_6$-DMSO).

This crude reaction mixture was hydrogenated under 1 atmosphere of H_2 gas with 10% Pd on charcoal (100 mg) as the catalyst, for 1.5 hours. The resulting solution was then filtered through celite, washing with water (10 ml) and the filtrate extracted with EtOAc (3 x 20 ml). The aqueous layer was then lyophilized to give the crude Δ2-cephalosporin C (156) and Δ2-DAC (151) (38 mg total) which was used without further purification. λ_{max} (H_2O) 195nm;

(6R,7R)-1-Aza-3-hydroxymethyl-7-amino-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (151). (Δ2-DAC)

Δ2-Cephalosporin C (156) and Δ2-DAC (151) (38 mg total, *ca* 0.13 mmol) were dissolved in water (1ml) and 1.0 N NaOH (130μl, 0.13 mmol, 1 eq) was added. The resulting orange solution was stirred at room temperature for 30 minutes and the pH then adjusted to *ca* pH 7 by the addition of 2N HCl. The resulting neutral solution was lyophilised and the residue purified by chromatography [reverse phase hplc, Gilson system with 0.55% MeCN in water] to give unconverted Δ2-Cephalosporin C (156) (15 mg, 0.04 mmol) and Δ2-DAC (151) (10 mg, 0.03 mmol) as judged by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed).

(151). λ_{max} (H_2O) 195nm; δ_H(500MHz, D_2O, HOD suppressed) 1.65-1.94 and 1.84-1.98 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7 Hz, CH_2CH_2CO), 3.74 (1H, *ca* t, *J* 6 Hz, H_2NCHCO_2H), 4.16 and 4.23 (2H, ABq, *J* 14 Hz, CH_2OH), 4.85 (1H, s, NCHC=CH), 5.30 and 5.39 (2H, ABq, *J* 4Hz, β -lactams), 6.34 (1H, s, SCH=C); δ_C(125.77MHz, D_2O) 21.62 (t, $CH_2CH_2CH_2CO$), 30.60 (t, $CH_2CH_2CH_2CO$), 35.42 (t, CH_2CO), 53.82, 54.11 and 55.27 (3 x d, NHCHCHS, NHCHCHS, $NHCHCH_2CH_2$), 60.42 (d, NCHC=CH), 64.66 (t, CH_2OH), 116.64 (d, SCH=C), 126.97 (s, SCH=C), 166.36, 174.33, 175.07 and 177.06 (4 x s, C=O); *m/z* (FAB) 374 (MH^+, 100%), 375 (32), 376 (15), 377 (4); *m/z* calculated for $C_{14}H_{19}N_3SO_7$ 374 (MH+, 100%), 375 (18), 376 (7), 377 (1).

(156). λ_{max} (H_2O) 195nm; δ_H(500MHz, D_2O, HOD suppressed) 1.65-1.95 and 1.84-1.98 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.11 (3H, s, OAc), 2.42 (2H, *ca* t, *J* 7 Hz, CH_2CH_2CO), 3.75 (1H, *ca* t, *J* 6 Hz, H_2NCHCO_2H), 4.85 (1H, s, NCHC=CH), 5.31 and 5.42 (2H, ABq, *J* 4Hz, β -lactams), 6.52 (1H, s, SCH=C) Vinyl hydroxymethyl group obscured by water suppression ; δ_C(125.77MHz, D_2O) 21.10 (q, O_2CCH_3), 21.61 (t, $CH_2CH_2CH_2CO$), 30.60 (t, $CH_2CH_2CH_2CO$), 35.40 (t, CH_2CO), 53.74, 54.43 and 55.27 (3 x d, NHCHCHS, NHCHCHS, $NHCHCH_2CH_2$), 60.43 (d, NCHC=CH), 67.62 (t, CH_2OH), 120.77 (d, SCH=C), 122.39 (s, SCH=C), 166.23 174.05, 174.79 and 177.08 (4 x s, C=O).

Incorporation of Deuterium into Δ2-DAC (151).

Synthetic Δ2-DAC (151) (*ca* 5 mg) was dissolved in D_2O and the pH adjusted to *ca* pH 9-10 by the addition of ammonium bicarbonate. The resulting solution was stirred at room temperature overnight and then lyophilised. δ_H(500MHz, $DMSO_{d6}$) 1.56-1.74 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.17 (2H, m, CH_2CO), 3.15 (1H, m, $CHCH_2CH_2$), 3.97 and 4.03 (2H, ABq, *J* 13Hz, CH_2OH), 4.42 (2/3H, s, CH(D)C=CH), 5.13 (1H, d, J 4Hz, CHCHS), 5.20 (1H, dd, J 4 and 8Hz, NHCHCHS), 6.06 (1H, SCH=C)8.76 (1H, d, *J* 8Hz, NH); *m/z* (FAB) 374 (MH^+ for $C_{14}H_{19}N_3SO_7$, 100%), 375 (MH^+ for $C_{14}H_{18}DN_3SO_7$, 49), 376 (23), 377 (3); *m/z* (Calc.) 375 (MH^+, 100%), 376 (18), 377 (7), 378 (1).

REFERENCES

1. T. S. Work, and E. Work in *The Basis of Chemopherapy*, p.6, 1948, Oliver and Boyd, Lond.
2. B. Ehrlich, *U. S. Pat.* 986,148 (1911 to Hoechst).
3. A. Fleming, *Br. J. Exp. Pathol.*, 1929, **10**, 226.
4. For a review on the early history of penicillin see E. P. Abraham, *Nat. Prod. Rep.* , 1987, 41.
5. D. Hodgkin, *Advancement of Sci.* 1949, **6**, 85.
6. S. E. Jensen, CRC Critical Reviews in Biotechnology, 1986, **3**, 277.
7. P. B. Loder, and E. P. Abraham, *Biochem. J.*, 1981, **123**, 477.
8. J. E. Baldwin, R. M. Adlington, M. Lopez-Nieto, J. A. Murphy, and N. Patel, *Biochem. J.*, 1983, **213**, 573.
9. H. R. V. Arnstein and D. Morris, *Biochem. J.*, 1960, **76**, 375.
10. J. S. Delderfield, E. Mtetwa, R. Thomas and T. E. Tyobeka, *J. Chem. Soc., Chem. Commun.*, 1981, 650.
11. J. E. Baldwin, R. M. Adlington and J. Bird, unpublished results, 1988.
12. J. E. Baldwin and T. S. Wan, *Tetrahedron*, 1981, **37**, 1589.
13. a J. O'Sullivan, J. C. Bleaney, J. A. Huddlestone and E. P. Abraham, *Biochem. J.*, 1979, **184**, 421.
    b T. Konomi, S. Herchen, J. E. Baldwin, M. Yoshida, N. A. Hunt and A. L. Demain, *Biochem. J.*, 1979, **184**, 427.
14. C.-P. Pang, B. Chakravarti, R. M. Adlington, H.-H. Ting, R. L. White, G. S. Jayatilake, J. E. Baldwin and E. P. Abraham, *Biochem. J.*, 1984, **222**, 789.
15. S. M. Samson, R. Belagaje, D. T. Blankenship, J. L. Chapman, D. Perry, P. L. Skatrud, R. M. VanFrank, E. P. Abraham, J. E. Baldwin, S. W. Queener and T. D. Ingolia, Nature (London), 1985, **318**, 191.
16. J. E. Baldwin, S. J. Killin, A. J. Pratt, J. D. Sutherland, N. J. Turner, M. J. C. Crabbe, E. P. Abraham and A. C. Willis, *J. Antibiot.*, 1987, **40**, 652.
17. Y. Sawada, J. E. Baldwin, P. D. Singh, N. A. Solomon and A. L. Demain, *Antimicrob. Agents and Chemother.*, 1980, **18**, 465.
18. E. P. Abraham, J. A. Huddlestone, G. S. Jayatilake, J. O'Sullivan and R. L. White, in 'Recent Advances in the Chemistry of β-lactam antibiotics', ed. G. I. Gregory, Royal Society of Chemistry, London, 1981, 125.
19. R. L. White, E. M. John, J. E. Baldwin and E. P. Abraham, *Biochem. J.*, 1982, **203**, 791.
20. J. E. Baldwin and H.-H. Ting, unpublished results, 1985.
21. K. Kim, I. H. Kim, K.-Y. Lee, S. G. Rhee and E. R. Stadtman, *J. Biol. Chem.*, 1988, **263**, 4704.
22. J. E. Baldwin, R. M. Adlington, N. G. Robinson, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 409.
23. J. E. Baldwin, R. M. Adlington, B. P. Domayne-Hayman, H.-H. Ting, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1986, 110.
24. J. E. Baldwin, R. M. Adlington, S. E. Moroney, L. D. Field, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1984, 984.
25. H. Simon, and D. Palm, *Angew. Chem., Int. Ed. Engl.*, 1966, **5**, 920.
26. G. S. Jayatilake, J. A. Huddlestone, and E. P. Abraham, *Biochem. J.*, 1981, **194**, 645.
27. J. E. Baldwin, J. W. Keeping, P. D. Singh, and C. A. Vallejo, *Biochem. J.*, 1981, **194**, 649.
28. S. E. Jensen, D. W. S. Westlake, and S. Wolfe, *J. Antibiot.*, 1982, **35**, 483.
29. S. E. Jensen, D. W. S. Westlake, and S. Wolfe, *Can. J. Microbiol.*, 1983, **29**, 1526.
30. J. Kupka, Y.-Q. Shen, S. Wolfe, and A. L. Demain, *FEMS Microbiol. Lett.*, 1983, **16**, 1.
31. D. J. Hook, L. T. Chang, R. P. Elander, and R. B. Morin, *Biochem. Biophys. Res. Commun.*, 1979, **87**, 258.
32. S. J. Brewer, J. E. Farthing and M. K. Turner, *Biochem. Soc. Trans.*, 1977, **5**, 1024.
33. M. K. Turner, J. E. Farthing and S. J. Brewer, *Biochem. J.*, 1978, **173**, 839.

34. A. Scheidegger, M. T. Kuenzi and J. Nuesch, *J. Antibiot.*, 1984, **37**, 322.
35. S. E. Jensen, D. W. S. Westlake and S. Wolfe, *J. Antibiot.*, 1985, **38**, 263.
36. J. E. Baldwin, R. M. Adlington, J. B. Coates, M. J. C. Crabbe, N. P. Crouch, J. W. Keeping, G. C. Knight, C. J. Schofield, H.-H.Ting, C. A. Vallejo, M. Thorniley, and E. P. Abraham, *Biochem. J.*, 1987, **245**, 831.
37. S. M. Samson, J. E. Dotzlaf, M. L. Slisz, G. W. Becker, R. M. VanFrank, L. E. Veal, N.-K. Yeh, J. R. Miller, S. W. Queener and T. D. Ingolia, *Biotechnology*, 1987, **5**, 1207.
38. Eli. Lilly & Co. Ltd., private communication.
39. L. G. Carr, P. L. Skatrud, M. E. Scheetz, S. W. Queener and T. D. Ingolia, *Gene*, 1986, **48**, 257.
40. H. Kluender, C. H. Bradley, C. J. Sih, P. A. Fawcett, and E. P. Abraham, *J. Am. Chem. Soc.*, 1973, **95**, 6149.
41. H. Kluender, F.-C. Huang, A. Fritzberg, H. Schnoes, C. J. Sih, P. A. Fawcett, and E. P. Abraham, *J. Am. Chem. Soc.*, 1974, **96**, 4054.
42. N. Neuss, C. H. Nash, J. E. Baldwin, P. A. Lemke, and J. B. Grutzner, *J. Am. Chem. Soc.*, 1973, **95**, 3797.
43. E. P. Abraham, C.-P. Pang, R. L. White, D. H. G. Crout, M. Lustorff, P. J. Morgan and A. E. Derome, *J. Chem. Soc., Chem. Commun.*, 1983, 723.
44. C. A. Townsend, A. B. Theis, A. S. Neese, E. B. Barrabee, and D. Poland, *J. Am. Chem. Soc.*, 1985, **107**, 4760.
45. C. A. Townsend and E. B. Barrabee, *J. Chem. Soc., Chem. Commun.*, 1984, 1586.
46. J. E. Baldwin, R. M. Adlington, A. Basak, S. L. Flitsch, A. K. Forrest, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 273.
47. J. E. Baldwin, M. Parishi, and L. King, in press, 1988.
48. J. E. Baldwin, R. M. Adlington, A. E. Derome, H.-H. Ting, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1986, 1305.

49a. J. E. Baldwin, E. P. Abraham, R. M. Adlington, B. Chakravarti, A. E. Derome, J. A. Murphy, L. D. Field, N. B. Green, H.-H. Ting and J. J. Usher, *J. Chem. Soc., Chem. Commun.*, 1983, 1317

b. S. L. Flitsch, D. Phil. Thesis, Oxford Unversity, 1986.

50. S. C. Stinson, *Chemical and Engineering News*, 1986, **64**, 33.
51. O. K. Behrens, J. Corse, J. P. Edwards, L. Garrison, R. G. Jones, Q. P. Soper, F. R. van Abeele and C. W. Whitehead, *J. Biol. Chem.*, 1948, **175**, 793.

52.a C. Gram, *Fortschr. Med.*, 1884, **2**, 185.

b L. Stryer, *Biochemistry*, Ch. 32, p. 773, 2nd. Ed. Freeman, N.Y., 1981, and references cited therein.

c H. Henry, and M. Stacey, Nature, Lond., 1943, **151**, 671.

53. J. E. Baldwin and J. D. Sutherland, unpublished results, 1988.
54. M. Kohsaka, and A. L. Demain, *Biochem. Biophys. Res. Commun.*, 1976, **70**, 465.
55. M. Yoshida, T. Konomi, M. Kohsaka, J. E. Baldwin, S. Herchen, P. D. Singh, N. A. Hunt, and A. L. Demain, *Proc. Nat. Acad. Sci. USA.*, 1978, **75**, 6253.
56. J. E. Baldwin, P. D. Singh, M. Yoshida, Y. Sawada, and A. L. Demain, *Biochem. J.* 1980, **186**, 889.
57. R. D. Miller, L. L. Huckstep, J. P. McDermott, S. W. Queener, S. Kukolja, D. O. Spry, T. K. Elzey, S. M. Lawrence, and N. Neuss, *J. Antibiot.*, 1981, **34**, 984.
58. M. Dixon and E. C. Wells, in *Enzymes*, p. 250, Academic Press, New York (1964).
59. H. R. Mahler, and E. H. Cordes, in *Biological Chemistry*, pp. 615, Harper and Row, New York (1966).

60a. R. B. Morin , B. G. Jackson, R. A. Mueller, E. R. Lavagnino, W. B. Scanlon, and S. L. Andrews, *J. Am. Chem. Soc.*, 1963, **85**, 1896.

b R. B. Morin , B. G. Jackson, R. A. Mueller, E. R. Lavagnino, W. B. Scanlon, and S. L. Andrews, *ibid* 1969, **91**, 1401.

61. D. O. Spry, R. D. Miller, L. L. Huckstep, N. Neuss, and S. Kukolja, *J. Antibiot.*, 1981, **34**, 1078.
62. M. T. Abbott, A. L. Tappel, and S. Undenfriend, *Arch. Biochem. Biophys.*, 1967, **118**, 231.
63. M. T. Abbott, E. K. Schandel, R. F. Lee, T. S. Parker, and R. J. Midgett, *Biochem. Biophys. Acta.*, 1967, **132**, 525.
64. J. E. Baldwin, S. R. Herchen and P. D. Singh, *Biochem. J.*, 1980, **186**, 881.
65. M. J. O'Donnell, and T. M. Eckrich, *Tetrahedron. Lett.*, 1978, (47), 4625.
66. N. J. Turner, D. Phil. Thesis, Oxford Unversity, 1986, and references cited therein.
67. B. Belleau, and G. Malek, *J. Am. Chem. Soc.*, 1968, **90**, 1651.
68. S. E. Moroney, D. Phil. Thesis, Oxford Unversity, 1985, p.61 and references cited therein.
69. J. E. Baldwin, E. P. Abraham, R. M. Adlington, G. A. Bahadur, B. Chakravarti, B. P. Domayne-Haymen, L. D. Field, S. L. Flitsch, G. S. Jayatilake, A. Spakovskis, H.-H. Ting, N. J. Turner, R. L. White, and J. J. Usher, *J. Chem. Soc., Chem. Commun.*, 1984, 1225.
70a. Y.-Q. Shen, S. Wolfe, and A. L. Demain, *J. Antibiot.*, 1984, **37**, 1044.
b A. L. Demain, S. Wolfe, and Y.-Q. Shen, *J. Antibiot.*, 1986, **39**, 1007.
71. J. E. Baldwin, R. M. Adlington, R. T. Aplin, N. P. Crouch, G. Knight, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 1651.
72. J. E. Baldwin, R. M. Adlington, B. Chakravarti, M. Jung, S. E. Moroney, J. A. Murphy, P. D. Singh, J. J. Usher, and C. Vallejo, *J. Chem. Soc., Chem. Commun.*, 1983, 153.
73 W. A. Cowdrey, E. D. Hughes, C. K. Ingold, S. Masterman, and A. D. Scott, *J. Chem. Soc.*, 1937, 1264.
74. J. E. Baldwin, R. M. Adlington, and M. E. Wood, unpublished results, 1988.
75. G. E. Gutowski, C. M. Daniels, and R. D. Cooper, *Tetrahedron. Lett.*, 1971, (37), 3429.
76. For a review see J. C. Vederas, *Can. J. Chem.*, 1982, **60**, 1637.
77. J. M. Risley, and R. L. Van Etten, *J. Am. Chem. Soc.*, 1980, **102**, 4609.
78. P. A. Fawcett, J. J. Usher, J. A. Huddlestone, R. C. Bleaney, J. J. Nisbet, and E. P. Abraham, *Biochem. J.*, 1976, **157**, 651.
79. J. E. Baldwin, J. Loliger, W. Rastetter, N. Neuss, L. L. Huckstep, and N. De La Higuera, *J. Am. Chem. Soc.*, 1973, **95**, 3796 and 6511.
80. D. J. Aberhart, and L. J. Lin, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2320.
81. R. K. Hill, S. Yan, and S. M. Arfin, *J. Am. Chem. Soc.*, 1973, **95**, 7857.
82. K. Mislow, R. E. O'Brien, and H. Schaefer, *J. Am. Chem. Soc.*, 1962, **84**, 1940.
83. Prepared by Dr. N. J. Turner, Dyson Perrins Laboratory, 1986.
84. R. A. Archer, R. D. G. Cooper, P. V. Demarco, and L. R. F. Johnson, *J. Chem. Soc., Chem. Commun.*, 1970, 1291.
85. N. Neuss, C. H. Nash, P. A. Lemke, and J. B. Grutzner, *J. Am. Chem. Soc.*, 1971, **93**, 2337.
86. J. E. Baldwin, R. M. Adlington, M. Bradley, W. J. Norris, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1988, 1125.
87. For a comprehensive introduction to enzyme kinetics see A. Fersht, *Enzyme Structure and Mechanism*, Freeman, New York, 1985.
88. L. Melander, *Isotope Effects on Reaction Rates*, Roland Press, New York, 1960.
89. F. H. Westheimer, *Chem. Rev.*, 1961, **61**, 265.
90. J. R. Knowles, and W. J. Albery, *Acc. Chem. Res.*, 1977, **10**, 105.
91. D. B. Northrop, *Biochemistry*, 1981, **20**, 4056.
92. W. J. Ray, *Biochemistry*, 1983, **22**, 4625.
93. Analogous to derivatisation procedure for isopenicillin N see R. M. Adlington, R. T. Aplin, J. E. Baldwin, B. Chakravarti, L. D. Field, and E-M. M. John, *Tetrahedron*, 1983, **39**(7), 1061.
94. M.Jung , D. Phil. Thesis, Oxford University, 1980.

95. J. E. Baldwin, R. M. Adlington, R. T. Aplin, N. P. Crouch, C. J. Schofield, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1654.
96. J. E. Baldwin, R. M. Adlington, T. W. Kang, E. Lee, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 104.
97. D. J. Aberhart, J. Y.-R. Chu, N. Neuss, C. H. Nash, J. Occolowitz, L. L. Huckstep and N. De La Higuera, *J. Chem. Soc., Chem. Commun.*, 1974, 564.
98. J. E. Baldwin, M. Jung, J. J. Usher, E. P. Abraham, J. A. Huddlestone, and R. L. White, *J. Chem. Soc., Chem. Commun.*, 1981, 246.
99. See for example F. A. Bovey, *Nuclear Magnetic Spectroscopy*, p. 87, Academic Press, N.Y., 1969 .
100. See for example ref 87, Chp. 8, and references cited there in.
101. J. E. Baldwin, and C. J. Schofield, unpublished results, 1987.
102. G. V. Kaiser, R. D. G. Cooper, R. E. Kochler, C. F. Murphy, J. A. Webber and E. M. Van Heyningen, *J. Org. Chem.*, 1970, 35, 2430.
103. J. M. Essery, K. Dadabo, W. J. Gottstein, A. Hallstrand, and L. C. Cheney, *J. Org. Chem.*, 1965, **30**, 4388.
104. S. Kukolja, S. R. Lammert, M. R. B. Gleissner, and A. I. Ellis, *J. Am. Chem. Soc.*, 1976, **98**, 5040.
105. J. E. Baldwin, M. J. C. Crabbe, and N. P. Crouch, unpublished results, 1987.
106. D. O. Spry and A. R. Bhala, *Heterocycles*, 1985, **23**, 1901.
107. B. H. Lipshutz, R. S. Wilhelm, J. A. Kozlowski and D. Parker, *J. Org. Chem.*, 1984, **49**, 3928.
108. S. Mobashery and M. Johnston, *J. Org. Chem.*, 1986, **51**, 4723.
109. E. M. Van Heyningen, and L. K. Ahern, *J. Med. Chem.*, 1968, **11**, 933.
110. R. B. Woodward in *Recent Advances in the Chemistry of β-Lactam Antibiotics*, Ed. J. Elks, Royal Society of Chemistry, Special Publication No. 28, 1976.
111. U. Schollkopf, J. Paust, and M. R. Patsch, *Org. Synth.* Coll. Vol. V, 860, Note 4.
112. M. F. Lipton, C. M. Sorensen, A. C. Sadler, and R. H. Shapiro, *J. Organometal. Chem.*,1980, **186**, 155.
113. L. A. Eriquez and M. A. Pisano, *Antimicrobial Agents and Chemotherapy*, 1979, **16**, 392.
114. N. Neuss, R. D. Miller, W. Nakatsukasa, J. Mabe, L. L. Huckstep, N. De La Higuera, A. H. Hunt, J. L. Occolowitz and J. H. Gilliam, *Helv. Chim. Acta.*, 1980, **63**, 1119.
115. S. Wolfe, R. J. Bowers, S. K. Hasan and P. M. Kazmaier, *Can. J. Chem.*, 1981, **59**, 406.
116. G. Bahadur, J. E. Baldwin, T. Wan, M. Jung, E. P. Abraham, J. A. Huddlestone and R. L. White, *J. Chem. Soc., Chem. Commun.*, 1981, 1146.
117. J. E. Baldwin, S. B. Haber, and J. Kitchin, *J. Chem. Soc., Chem. Commun.*, 1973, 790
118. J. E. Baldwin, B. Chakravarti, L. D. Field, J. A. Murphy, and K. R. Whitten, *Tetrahedron*, 1982, **38**, 2773.
119. For a recent review see J. C. Vederas, *Nat. Prod. Rep.*, 1987, 277.
120. M. Fieser, and L. F. Fieser, *Reagents for Organic Synthesis*, **6**, 307.
121. J. E. Baldwin, S. B. Haber, C. Hoskins and L. I. Kruse, *J. Org. Chem.*, 1977, **42**, 1239.
122a. H. C. Beyerman, L. Maat, D. de. Rijke, and J. P. Visser, *Rec. Trav. Chim. Pays-Bas*, 1967, **86**, 1057.
b Prepared by T. Wan, 1981.
123. E. P. Abraham, Proceedings of the 2nd. International Symposium, 1980, on Recent Advances in the Chemistry of β-Lactam Antibiotics, ed. G. I. Gregory, The Royal Society of Chemistry, 1980, p. 125.
124. M. Tada, and S. Totoki, *Chemistry and Industry*, 1986, 790.
125. B. Maillard, D. Forrest, and K. U. Ingold, *J. Am. Chem. Soc.*, 1976, 98, 7024.
126. J. E. Baldwin, R. M. Adlington, B. P. Domayne-Hayman, G. Knight, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1661.
127. For a review see J. E. Baldwin, *Nat. Prod. Rep.*, 1988, 129.

128. J. E. Baldwin, R. M. Adlington, J. M. C. Crabbe, G. Knight, T. Nomoto, C. J. Schofield and H.-H. Ting, *Tetrahedron*, 1987, 43, 3009.
129. K. Kitano, Y. Fujisawa, K. Katamoto, K. Nara and Y. Nakao, *J. Ferment. Technol.*, 1976, 54, 712.
130. J. Shoji, R. Sakazaki, K. Matsumoto, T. Tanimoto, Y. Terui, S. Kozuki, and E. Kondo, *J. Antibiot.*, 1983, 36, 167.
131. J. E. Baldwin, R. M. Adlington, C. J. Schofield, N. P. Crouch, and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1556.
132. J. E. Baldwin, R. M. Adlington, C. J. Schofield, and N. P. Crouch, *Tetrahedron*, 1988, **44**, 643.
133. J. E. Baldwin, R. M. Adlington, N. P. Crouch, J. B. Coates, J. W. Keeping, C. J. Schofield, W. A. Shuttleworth, and J. D. Sutherland, in-press, 1988.
134a E. P. Abraham, and G. G. F. Newton , *Advances in Chemotherapy*, 1965, **2**, 23.
b H. Troonen, P. Rochants, and B. Boon, *J. Antibiot.*, 1976, 29, 1258.
c J. E. Baldwin, S. R. Herchen, J. C. Clardy, K. Hirotsu, and T. S. Chou, *J. Org. Chem.*, 1978, **43**, 1342.
135a J. E. Baldwin, M. Jung, P. D. Singh, T. Wan, S. Haber, S. R. Herchen, J. Kitchin, A. L. Demain, N. A. Hunt, M. Kokshake, T. Konowi and M. Yoshida, *Philos. Trans. R. Soc. London B.*, 1980, **289**, 169.
b S. Kukolja, S. W. Queener, R. D. Miller, D. C. Duckworth, D. E. Dorman, L. L. Huckstep, and N. Neuss, *Helv. Chim. Acta.*, 1984, **67**, 876.
136. J. E. Baldwin, and S. Ko, unpublished results, 1987.
137. J. E. Baldwin, A. K. Forrest, S. Ko and L. N. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1987, 81.
138. R. Bucourt, Proceedings of the 2nd. International Symposium, 1980, on Recent Advances in the Chemistry of β-Lactam Antibiotics, ed. G. I. Gregory, The Royal Society of Chemistry, 1980, p. 20.
139. R. M. Sweet, and L. F. Dahl, *J. Am. Chem. Soc.*, 1970, **92**, 5489.
140. E. M. Van Heyningen, and L. K. Ahern, *J. Org. Chem.*, 1968,
141. C. F. Murphy, and R. E. Koehler, *J. Org. Chem.*, 1970, **35**, 2429.
142. Eli Lilly Co.& Ltd., confidential communication.
143. P. B. Loder and E. P. Abraham, *Biochem. J.*, 1971, **123**, 471.
144. Prepared by an analogous method as those described in ref. 65.
145. R. M. Adlington, private comunication, 1986.
146. Prepared by an analogous method as those described in ref. 66.
147. Prepared by an analogous method as those described in ref. 61
148. Prepared by Dr. J. Cobb, 1987.
149. Prepared by Dr. C. J. Schofield.
150. Prepared by L. Kruse, .
151. Prepared by an analogous method as those described in ref.94.
152. For a synthesis of the analogue (6R,7R)-1-aza-7-amino-(N-benzyloxy-carbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic Acid benzhydryl ester, see R. Scartazzini, and H. Bickel, *Helv. Chim. Acta.*, 1974, 1919
153. Prepared by an analogous method as those described in ref.69.
154. Prepared by an analogous method as those described in ref.111.
155. Prepared by an analogous method as those described in ref.107.
156. Prepared by an analogous method as those described in ref.106.
157. Prepared by an analogous method as those described in ref.108.
158. Prepared by an analogous method as those described in ref.102.
159. D. D. Perrin, W. L. F. Armarego, and D. R. Perrin *Purification of Laboratory Chemicals*, p. 83, 2nd. ed. 1980, Pergamon Press.
160. Supplied by B A Chem, Bubendorf, Switzerland.
161 J. E. Baldwin, S. R. Herchen, B. L. Johnson, M. Jung, J. J. Usher, and T. Wan., *J. Chem. Soc., Perkin I.*, 1981, 2253.