Studies on
Deacetoxy/deacetylcephalosporin C Synthase

A thesis submitted to the
Board of the Faculty of Physical Sciences
in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy
in the
University of Oxford

by

Inês Antunes Cardoso Pereira

Wolfson College
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The Dyson Perrins Laboratory
Hilary Term 1993

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THESIS

Abstract

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Inês A. C. Pereira
Wolfson College
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This thesis describes an investigation of the mechanism of the bifunctional, α-ketoglutarate dependent dioxygenase, deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS), which catalyses the ring-expansion of penicillin N to deacetoxycephalosporin C (DAOC) and the hydroxylation of this to deacetylcephalosporin C (DAC).

The conversion of the unnatural substrate 3-exomethylene cephalosporin C by DAOC/DACS has been investigated in detail. A new metabolite was isolated from incubations of the deuterated [4-^{2}H]-3-exomethylene cephalosporin C, and was identified as the 3β-spiroepoxide cepham, (2R,3R,6R,7R)-1-aza-[2-^{2}H]-3-spiroepoxy-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid. The results obtained indicate that this metabolite is a shunt product whose formation is enhanced by the operation of a deuterium kinetic isotope effect on an enzyme-bound intermediate. It has also been found that this 3β-spiroepoxide cepham is further converted by DAOC/DACS to 3-formyl cephalosporoate products.

The mechanism of oxygenation of DAOC/DACS was investigated through ^{18}O-labelling studies. Incubations of [2-^{13}C,3-^{2}H]penicillin N and [4-^{2}H]-3-exomethylene cephalosporin C with DAOC/DACS were carried out under $^{18}O_2$ or in $H_2{}^{18}O$. Incorporation of ^{18}O-label into the products [3-^{13}C]DAC, [3-^{13}C,4-^{2}H]-3β-hydroxycepham and 3β-spiroepoxide cepham was observed from both sources. The results suggest that intermediates capable of oxygen-exchange are formed during the enzymatic reactions.

Two substrate analogues, the 5-epipenicillin N and the 2β-difluoromethyl penicillin N, have been synthesised in order to probe the substrate specificity of DAOC/DACS with respect to the ring-expansion activity. The 5-epipenicillin N was not accepted as a substrate by DAOC/DACS, and the observations made indicate that it was unstable under the incubation conditions. No product was either observed from incubations of the 2β-difluoromethyl penicillin N with DAOC/DACS, although bioassay tests suggested a cephem product had been formed in very small amounts.

Finally, the results of a substrate specificity comparison between the soluble recombinant enzymes deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) from *Cephalosporium acremonium* and deacetoxycephalosporin C synthase (DAOCS) from *Streptomyces clavuligerus* are described.

If one does not expect the unexpected one will not find it out,
since it is not to be searched out, and is difficult to compass.

Heraclitus of Ephesus

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Corrections

Page	Line	For	Read
11	12	synthase	synthetase
21	5	complementary	complementarilly
23	6	immediatly	immediately
31	5	were	was
31	7	hydrogens	hydrogen
35	6	give	gives
49	2	formylcephem	formylcephalosporoate
61	6	chanelled	channelled
68	6	stericaly	sterically
75	4	occured	occurred
84	7	appearence	appearance
100	14	muti	multi
114	6	pratically	practically
124	last	the	then
131	9	byciclic	bicyclic
154	24	difficultating	hindering
157	12	pratical	practical
163	5	strucural	structural
172	9	dissapeared	disappeared
190	20	tittle	title
210	23	diference	difference
218	8	pentacloride	pentachloride
237	4	envolving	involving

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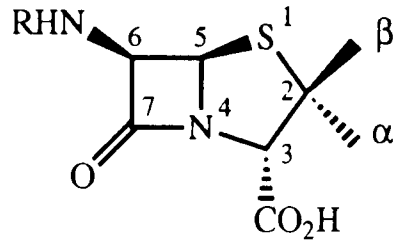
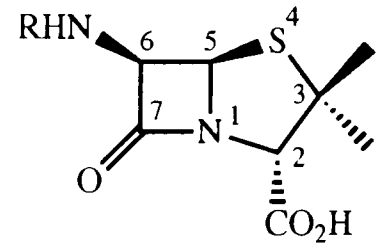
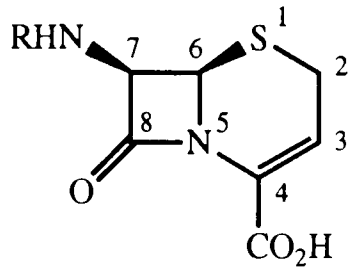
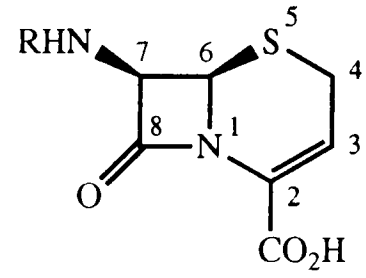
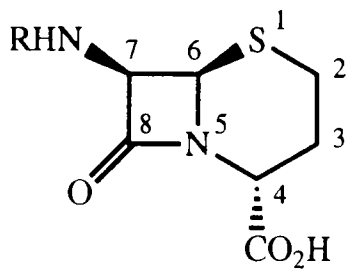
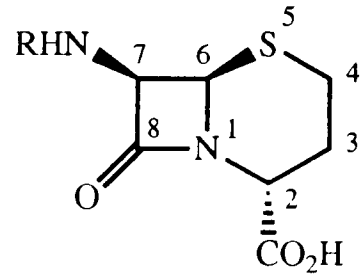
Abbreviations

Å	Angstrom
Ac	Acetyl group
AC	Aminoadipoyl-cysteine dipeptide
AcOH	Acetic acid
ACV	Aminoadipoyl-cysteinyl-valine tripeptide
ACVS	ACV synthase
6-APA	6-Aminopenicillanic acid
Ar	Aryl group
ATP	Adenosine triphosphate
Bn	Benzyl group (CH_2Ph)
BTEAC	Benzyltriethylammonium chloride
n-BuOH	*n*-Butanol
BzH	Benzhydryl group ($CHPh_2$)
$CDCl_3$	Deuterated chloroform
CoA	Coenzyme A
COSY	Correlation Spectroscopy
CV	Cysteinyl-valine dipeptide
CyOC	Cyano-*t*-butyloxycarbonyl group
Δ	Heat
D	Deuterium
Da	Dalton
D-AA	D-α-aminoadipoyl group
DAC	Deacetylcephalosporin C
DACS	Deacetylcephalosporin C synthase
DAOC	Deacetoxycephalosporin C
DAOC/DACS	Deacetoxy/deacetylcephalosporin C synthase
DAOCS	Deacetoxycephalosporin C synthase
DAST	Diethylaminosulphur trifluoride
DCI	Desorption Chemical Ionisation
DCM	Dichloromethane
DEPT	Distortionless enhancement of insensitive nuclei by polarisation transfer
DMA	N,N-Dimethylaniline
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid

D_2O	Deuterated water
DTT	Dithiothreitol
EEDQ	N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
EPR	Electron paramagnetic resonance
eq	Equivalent
ESMS	Electrospray mass spectrometry
Et	Ethyl group
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB	Fast atom bombardment
FMOC	9-Fluorenylmethoxycarbonyl group
g	Grams
Gly	Glycine
h	Hour
HPLC	High performance liquid chromatography
Hz	Hertz
IPN	Isopenicillin N
IPNS	Isopenicillin N synthase
IU	International unit
J	Coupling constant
α-KG	α-Ketoglutarate
K_m	Michaelis constant
L-AA	L-α-Aminoadipoyl group
M	Molar
MCPBA	*m*-Chloroperoxybenzoic acid
Me	Methyl group
MeCN	Acetonitrile
MeOH	Methanol
mg	Milligrams
MHz	Megahertz
min	Minutes
ml	Millilitres
mm	Millimetres
mM	Millimolar
M.p.	Melting point
MW	Molecular weight
m/z	Weight/charge ratio

µl	Microlitres
µmol	Micromolar
N	Normal equivalent
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NEt_3	Triethylamine
nm	Nanometres
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser effect
pen N	Penicillin N
Ph	Phenyl group
P.l.c.	Preparative layer chromatography
PMB	*p*-Methoxybenzyl group
PNB	*p*-Nitrobenzyl group
ppm	Parts per million
Pro	Proline
R	Alkyl group
Rf	Retention factor
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
T.l.c.	Thin layer chromatography
TMS	Tetramethylsilane
TRIS	Tris(hydroxymethyl)aminomethane
Ts	Tosyl group
TsOH	*p*-Toluenesulfonic acid (tosic acid)
TSP	3-(Trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt
UV	Ultra-violet
v/v	Volume/volume ratio
V_{max}	Maximal velocity
w/v	Weight/volume ratio
w/w	Weight/weight ratio
Z	Benzyloxycarbonyl group

Nomenclature

Nucleus	Classical numbering	IUPAC numbering
Penam		
Cephem		
Cepham		

In the text (Chapters 1-5) the classical numbering system has been used for the sake of convenience, but in the experimental section (Chapter 6) the IUPAC numbering system was used to describe fully the structures of the compounds.

CHAPTER 1



The Biosynthesis of Penicillins and Cephalosporins

Chapter 1

The Biosynthesis of Penicillins and Cephalosporins

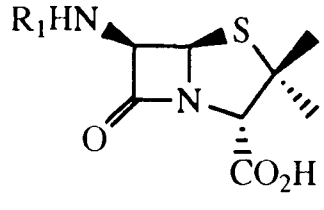
1.1 Introduction

The family of natural products comprises a fascinating variety of chemical structures. In these small molecules nature has explored a great range of chemical functions to create amazing levels of diversity and complexity. The enzymatic machinery by which such diversity is produced is in itself quite extraordinary. Indeed, the beauty and elegance with which some of the biosynthetic transformations are achieved is not matched by even the best attempts of synthetic organic chemistry. This is one of the reasons why the study of metabolism and biosynthetic pathways has been – and will continue to be – a most interesting and stimulating problem to be involved with.

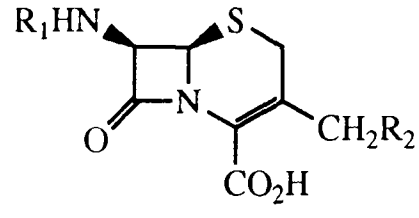
If primary metabolism is striking for its efficiency, and in some respects simplicity, secondary metabolism has to be recognised for its variety of products, and also of reactions. One of the factors that may contribute to this diversity is the fact that the enzymes of secondary metabolism appear to have much less stringent substrate specificities than their counterparts from primary metabolism. As a result they may be capable of transforming several molecules presented to them, leading to the creation of new products and the opening up of new pathways. One particular group of enzymes where such a situation is apparent is the group of oxygenases. These play an important role in metabolism, particularly in increasing the functionality of hydrophobic residues in some compounds, making them more hydrophilic in nature and thus more biologically active and/or more susceptible to further biosynthetic transformation.

In the secondary metabolism of animals, plants and microorganisms, a whole array of compounds are formed having numerous biological functions, many of which have not

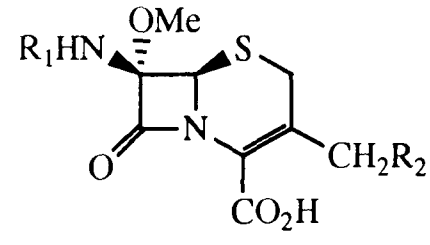
yet even been fully recognised. In many cases, these compounds give one species a decisive advantage over others, providing a clear example of how evolution through selection can operate at the chemical level. One such case is found in the family of penicillins and cephalosporins. Penicillins and related compounds (cephalosporins and cephamycins) give the organisms that are able to produce them the ability to kill many of their competitors, providing them with a crucial tool for survival.



Penicillins



Cephalosporins

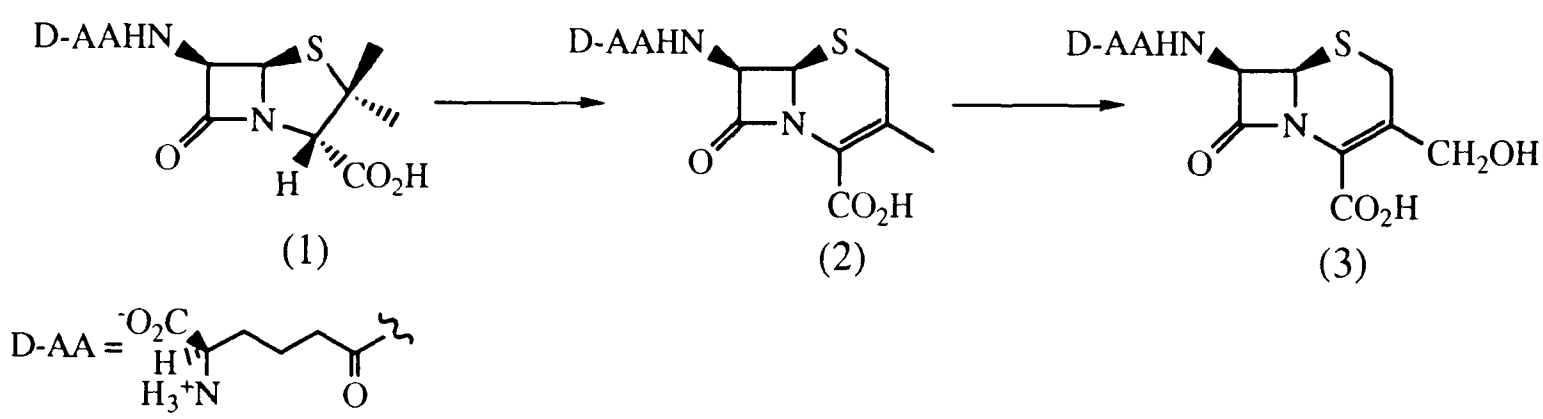


Cephamycins

The biosynthesis of penicillins and cephalosporins has received widespread interest, and has been the target of intensive research over the years. This is due not only to the obvious medicinal - and as a consequence, commercial - importance of this class of compounds, but also to its interest from a chemical point of view. These molecules have a relatively simple structure, and yet are chemically quite difficult to assemble. The enzymes that carry out this task have for a long time attracted the curiosity of chemists and biochemists alike, but despite this their isolation and characterisation has proven a difficult problem. As in the case of many other biosynthetic pathways, it was not until recently that with the help of molecular biology (through the cloning and expression of their genes, leading to the over-production of recombinant enzymes) significant progress in this field has occurred.

Cephalosporins are biosynthetically derived from penicillins, but have the advantage of greater stability to β-lactamases (the enzymes responsible for inactivation of penicillins through hydrolysis of their β-lactam ring) and acidic conditions. They therefore show bioactivity against a range of microorganisms that are resistant to penicillins, and on the other hand are suitable for oral administration, thus being more important as antibiotics than penicillins. The work described in this thesis studied an important dioxygenase enzyme

involved in the biosynthesis of cephalosporins in the fungus *Cephalosporium acremonium* called deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS), which carries out the conversion of penicillin N (1) to the first of the cephalosporins, deacetoxycephalosporin C (DAOC) (2), and also the hydroxylation of this to deacetylcephalosporin C (DAC) (3) (Scheme 1.1). In this chapter, a brief overview of the biosynthetic pathway to penicillins and cephalosporins will be given, followed by a review of investigations into DAOC/DACS and other enzymes of the same class (α-ketoglutarate dependent dioxygenases).



Scheme 1.1

1.2 The Biosynthetic Pathway to Penicillins, Cephalosporins and Cephamycins

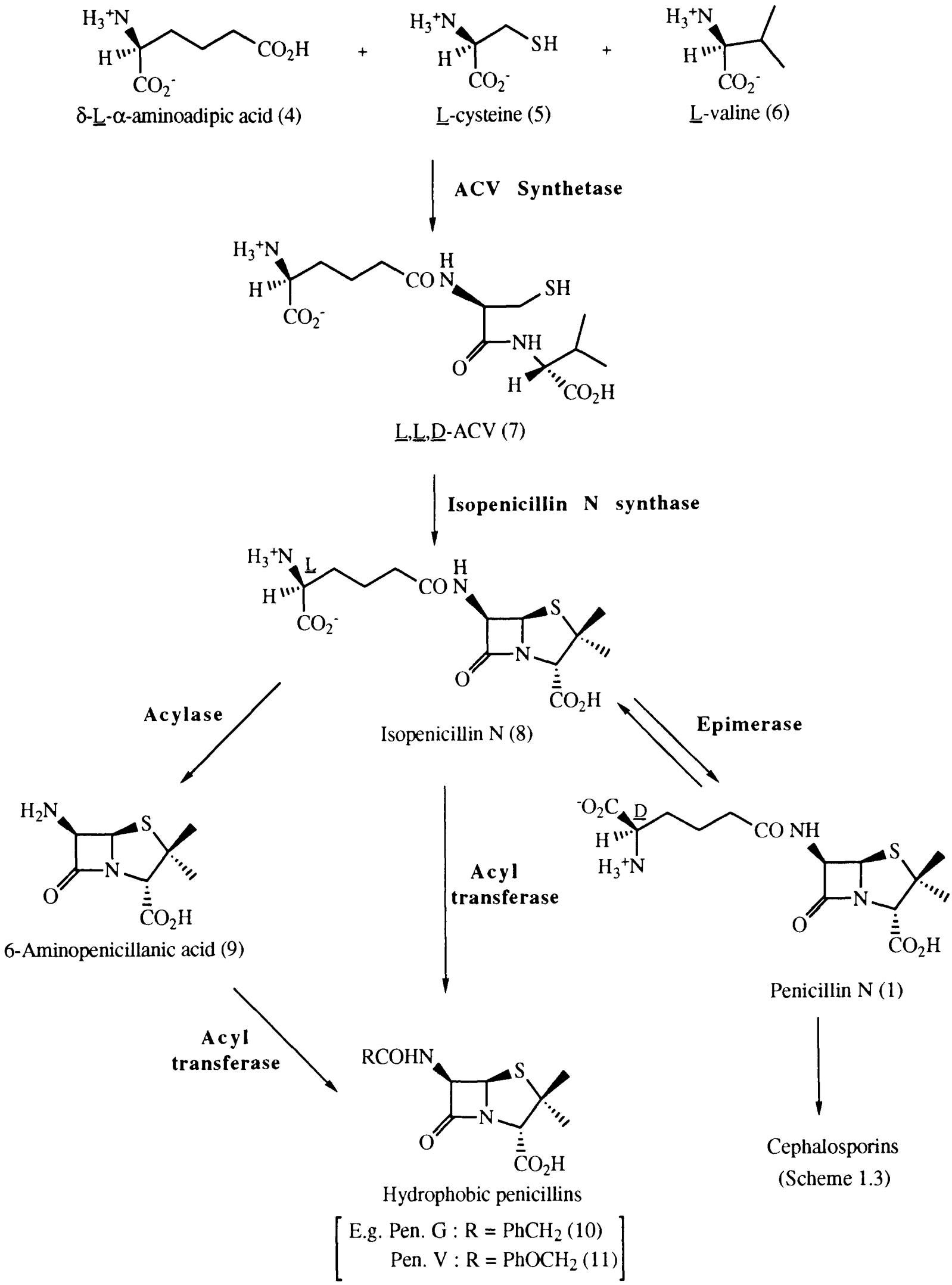
The ability to produce β-lactam compounds is not very widespread amongst microorganisms, being found in some fungi (eukaryots) (e.g. species of *Penicillium*, *Cephalosporium* and *Aspergillus*), and also in some filamentous bacteria (prokaryots) (e.g. several species of *Streptomyces*) (Table 1.1).

Producing organisms*	Products
Aspergillus (Emericella, Eurotium); Epidermophyton (Gymnoascus); Malbranchea; Microsporum (Nanizza); Penicillium (Eupenicillium, Talaromyces, Carpenteles); Polypaecilium (Thermoascus); Sartorya; Trichophyton (Gymnoascus, Arthroderma)	Penicillins
Anixiopsis; Arachnomyces; Cephalosporium (Emericellopsis); Diheterospora; Paecilomyces (Byssochlamys); Scopulariopsis; Spiroidium	Penicillins and cephalosporins
Streptomyces sp. including *S. lipmanii; S. griseus; S. lactamdurans;* and others	Penicillins and cephamycins
Streptomyces sp. including *S. clavuligerus; S. jumonjinensis;* and *S. katsurahamanus*	Penicillins, cephamycins, and oxypenams
Streptomyces sp. including *S. olivaceus; S. cattleya;* and others	Penicillins, cephamycins, carbapenams and carbapenems
Nocardia	Penicillins and nocardicins

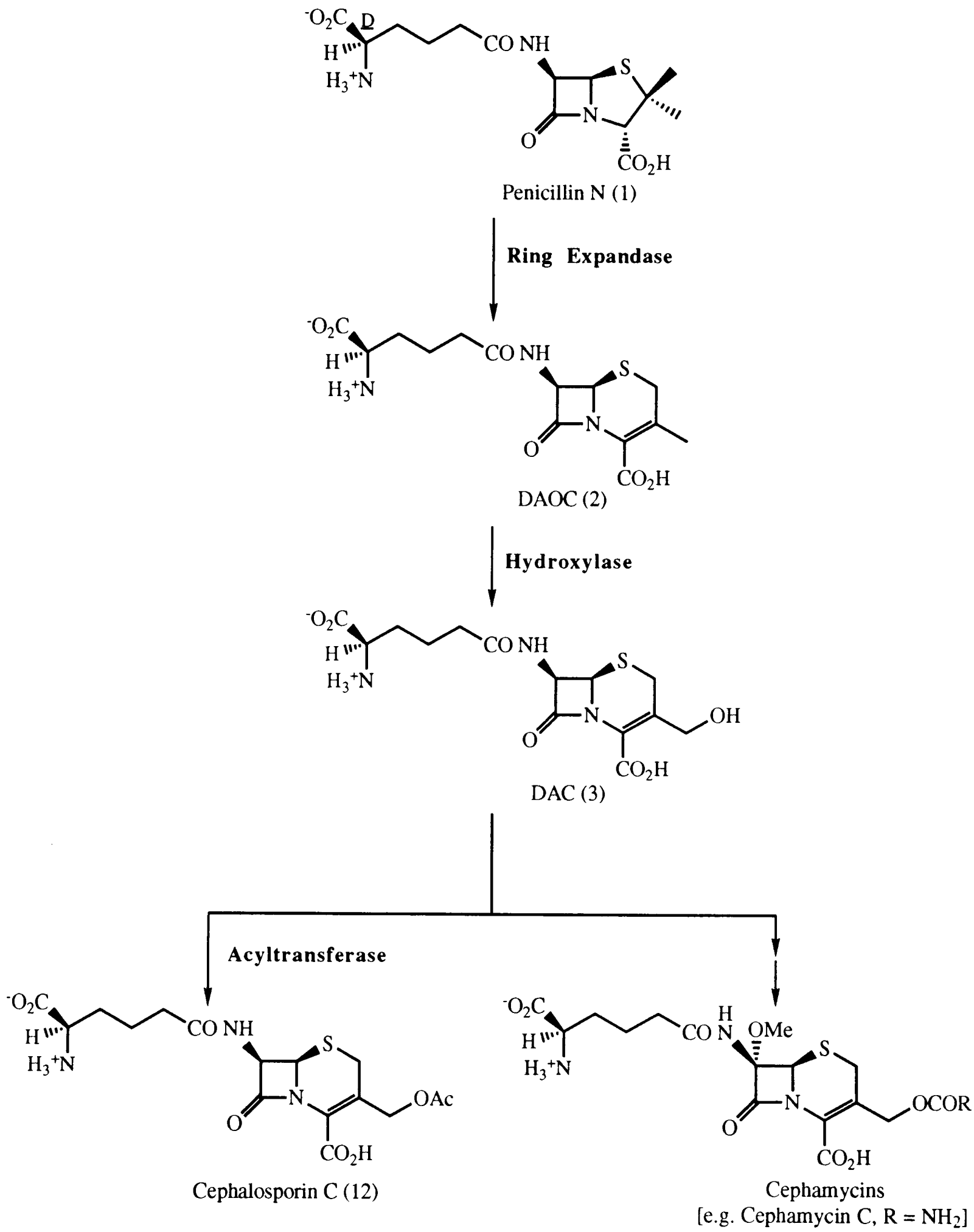
* Names in parentheses represent related sexual forms

Table 1.1 : Microorganisms that produce β-lactam compounds[1]

The capacity to produce cephalosporins and cephamycins is much less common than the capacity to produce penicillins, and it is found that all organisms which produce the former compounds are also able to produce penicillins. This is a result of the now well established facts that penicillins are the direct biosynthetic precursors of cephalosporins, and that these, in turn, are precursors of cephamycins (Schemes 1.2 and 1.3).



Scheme 1.2 : The biosynthetic pathway to penicillins

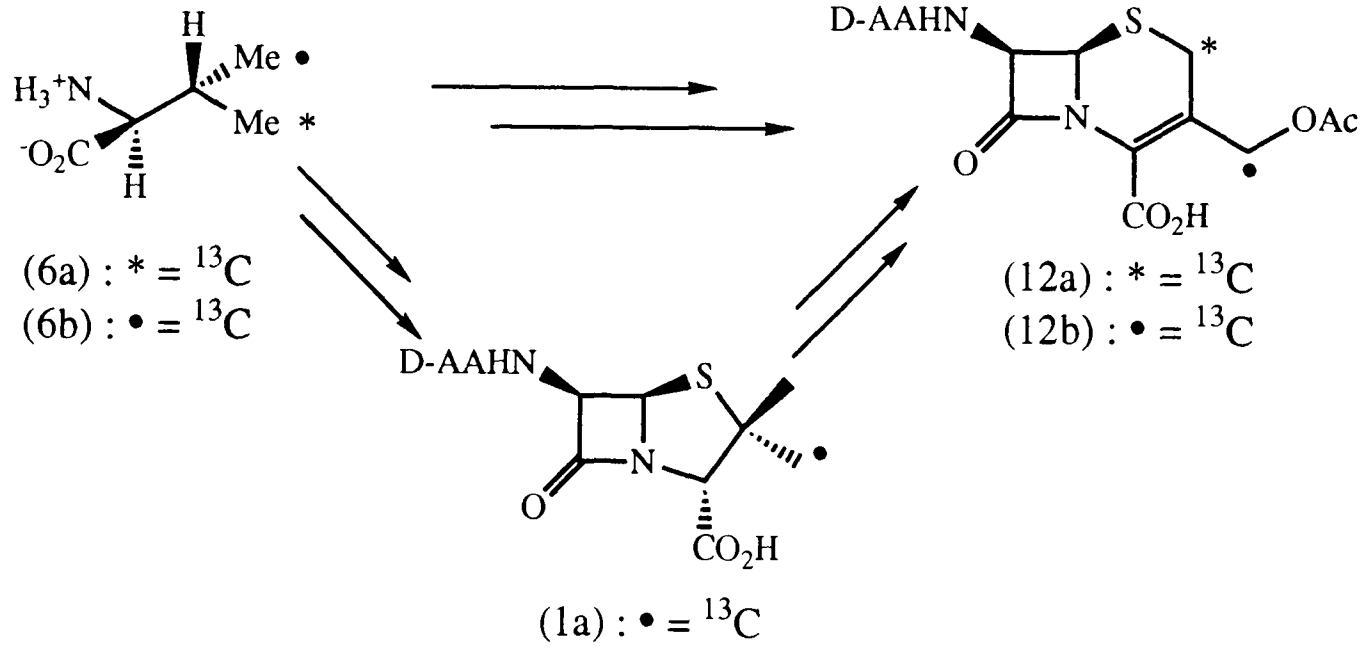


Scheme 1.3 : The biosynthetic pathways to cephalosporins

1.2.1 Whole Cell Studies

The first biosynthetic studies on the origin of penicillins and cephalosporins were carried out using intact cells. Feeding experiments with labelled L-cysteine and valine showed that these aminoacids were incorporated into the ring-systems of both penicillins and cephalosporins.[2,3,4] In 1960, Arnstein and Morris[5] isolated a peptide from *P. chrysogenum* [δ-(α-aminoadipoyl)- cysteinylvaline] that looked like a possible intermediate in penicillin biosynthesis. A similar peptide was isolated from *C. acremonium* and shown to be δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine [L,L,D-ACV (7)].[6] However, because ACV is not transported inside mycelial cells, its precursory role could not be demonstrated until the development of cell-free extracts.

Using intact cells of *C. acremonium* it was also possible to show that the ^{13}C of (2RS, 3R)-[4-^{13}C]-valine (6a) was incorporated exclusively into the endocyclic methylene group of cephalosporin C (12a) (Scheme 1.4).[7] Likewise, the ^{13}C of (2S,3S)-[4-^{13}C]-valine (6b) was shown to be incorporated exclusively at the α-methyl group of penicillin N (1a), and the exocyclic methylene group of cephalosporin C (12b).[8] Similar studies using (2S,3S)-[4,4,4-$^{2}H_3$]-valine and (2S,3R)-[4,4,4-$^{2}H_3$]-valine[9] confirmed that the methyl group of valine which became the 2α-methyl group of penicillins also became the exoxyclic methylene group of cephalosporin C, and that the 2β-methyl group of penicillins became the endocyclic methylene (C-2) of cephalosporin C.



Scheme 1.4

1.2.2 Studies with Cell-Free Extracts

The first successful cell-free extracts were obtained from *C. acremonium* by formation and gentle osmotic lysis of protoplasts.[10] Using this system it was shown that isotopically labelled L,L,D-ACV (7) was converted to isopenicillin N (IPN) (8).[11,12] Crude extracts of *P. chrysogenum* were found to catalyse the formation of penicillin G (10) from both 6-aminopenicillanic acid (6-APA) (9) and IPN (8), in the presence of phenylacetyl-coenzyme A.[13,14,15] The extracts also catalysed the hydrolysis of IPN (8), as well as of penicillin V (11), to 6-APA (9).[16]

In species of both *Cephalosporium* and *Streptomyces*, but not in those of *Penicillium*, an epimerase was found to convert IPN (8) into penicillin N (pen N) (1).[12,17] Pen N (1) had for a long time been thought of as a precursor of cephalosporin C (12), but it was not until 1976 that its conversion into a cephalosporin-like substance was demonstrated in a protoplast lysate of *C. acremonium*.[18] The immediate product of the reaction was subsequently shown to be deacetoxycephalosporin C (DAOC) (2).[19,20]

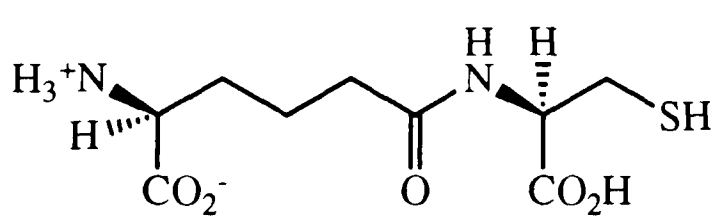
The use of cell-free extracts provided crucial insight into the order of reactions in the biosynthetic pathway. It became evident that the biosynthesis of penicillins and cephalosporins from amino acids followed the same course up to the formation of IPN (8), and that this was the first branching point of the pathway. In species of *Penicillium* and *Aspergillus*, IPN would give origin to 6-APA (9) and hydrophobic penicillins [e.g. (10) and (11)], whereas in species of *Cephalosporium* and *Streptomyces* it would be epimerised to pen N (1), which was further converted into several cephalosporins.

Considerable progress has occurred since the days of cell-free extracts.[21,22,23] Most of the enzymes in the pathway have been purified to homogeneity, and in some cases their genes have been cloned and over-expressed in different organisms. This has enabled detailed mechanistic studies to be carried out for some of the key reactions, which seem to have little parallel in other fields of biochemistry [e.g. formation of IPN (8) from L,L,D-ACV (7), and ring expansion of pen N (1) to DAOC (2)]. A small description of each of the enzymes involved in the pathway will be presented, followed by a more detailed account of the studies into deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS).

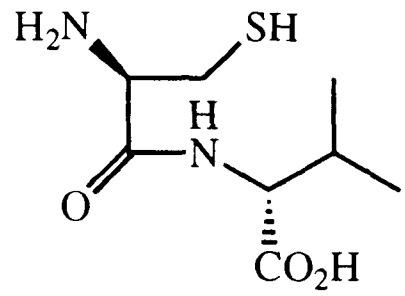
1.2.3 ACV Synthetase

The enzyme ACV synthetase (ACVS) carries out the conversion of δ-L-α-aminoadipic acid (4), L-cysteine (5) and L-valine (6) into the tripeptide L,L,D-ACV (7). It has been purified from *C. acremonium*[24,25], *Streptomyces clavuligerus*[24,26] and *Aspergillus nidulans*[27]. Optimum activity for ACVS was observed at pH 7.5, and it was found to require ATP and Mn^{2+} or Mg^{2+}.

Traces of the dipeptide δ-(L-α-aminoadipoyl)-L-cysteine (AC) (13) were isolated from cultures of *C. acremonium*[28], and it was subsequently shown that cell-free extracts of the same organism could convert a mixture of L,L-AC (13) and L-valine (6) into ACV (7), but not a mixture of δ-L-α-aminoadipic acid (4) and L-cysteinyl-D-valine (CV) (14) into ACV[29]. However, synthesis of ACV (7) from the three individual amino acids was later described to be far more rapid than tripeptide formation from L,L-AC (13) plus valine (6).[30] This implies that a single enzyme carries out the conversion, contrary to what had previously been suggested, and that this enzyme needs to have the three amino acids bound for maximum activity, although the AC bond is presumably formed before the CV bond. It was also found that D-valine was not accepted as a substrate[30], which suggests that the inversion of configuration occurs at an enzyme bound stage.



L,L-AC (13)



L,D-CV (14)

All these results are in agreement with ACVS being a member of the non-ribosomal peptide synthetase class of enzymes. These are multi-enzyme complexes which have been proposed to operate *via* a thiol-template mechanism with a pantotheine "arm", which transfers activated amino acids to sites on the enzyme capable of catalysing peptide bond formation.[31] Amino acid activation is thought to involve a two step process, with the initial

formation of an aminoacyl adenylate, followed by its transfer onto the enzyme, probably *via* a thioester linkage to a cysteine residue.[27,31] This theory was supported by the fact that pantothenic acid was shown to be associated with purified ACVS (in the form of phosphopantotenate) from both *C. acremonium* and *S. clavuligerus*.[24,32] In addition, a single ^{18}O-oxygen was incorporated into ACV, from incubation with D,L-[$^{18}O_2$]-valine[33], which provides evidence for the formation of a covalent bond between the valine carboxyl group and ACVS, presumably during activation of the amino acid as an aminoacyl adenylate.

The ACVS gene has been cloned in *Penicillium chrysogenum*[34,35], and also in *C. acremonium*[36]. Parts of this last gene showed significant homology to the gramicidin S synthetase gene, providing some more evidence that ACVS belongs to the group of non-ribosomal peptide synthases.

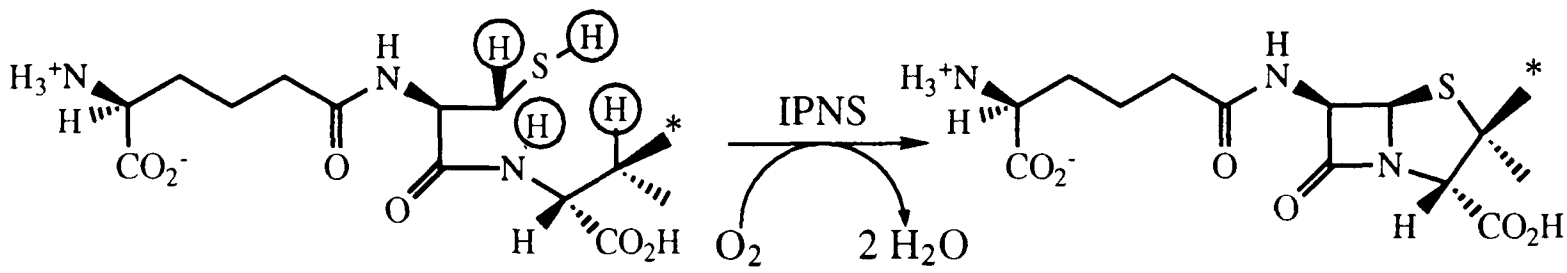
1.2.4 Isopenicillin N Synthase

Isopenicillin N synthase (IPNS) is by far the most well studied enzyme of the pathway, reflecting the importance of the key β-lactam forming step, as well as the interest in the unusual oxidative cyclisation it performs. IPNS has been purified from several fungal and bacterial sources [*C. acremonium*[37,38], *P. chrysogenum*[28], *S. clavuligerus*[39], *S. lactamdurans*[40], *S. lipmanii*[41], *Aspergillus nidulans*[41] and *Flavobacterium* sp.[42]], and its gene was first cloned from *C. acremonium* and over-expressed in *E. coli* in 1985[43]. Since then, the IPNS gene has been sequenced from several other fungal[41,44,45] as well as bacterial sources[41,46], all showing a high amino acid sequence homology and similar molecular weights (37,000-40,000).

IPNS is a non-heme iron(II) containing enzyme which requires Fe^{II}, O_2, dithiothreitol (DTT) and L-ascorbate for activity.[47,48] Of these, ferrous ion is essential for activity, dioxygen is a cosubstrate, and L-ascorbate and DTT are not essential for catalysis but are required for maximal activity. DTT is needed to keep ACV as a monomer which is

the true substrate, and the enzyme in its active reduced form, whereas ascorbate is thought to maintain the iron in the ferrous oxidation state.[49]

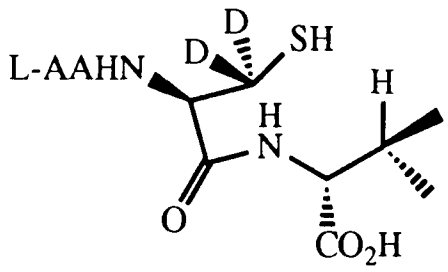
IPNS catalyses the removal of four hydrogens from ACV (7), with the concomitant reduction of one molecule of dioxygen to water (Scheme 1.5).[50] The two new bonds are formed with complete retention of configuration.[51,52] Only the four marked hydrogens are lost[53], and there is no loss or exchange of the oxygen and sulphur atoms[54,55].



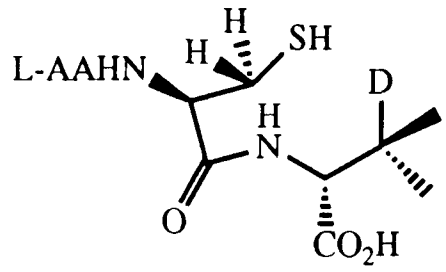
(7) (8)

Scheme 1.5

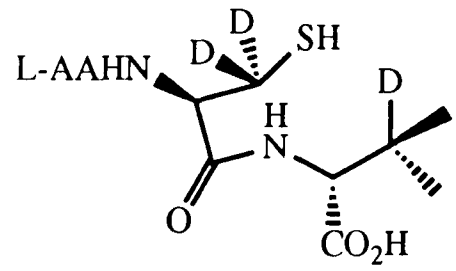
Several possible intermediates for this reaction were synthesised, but none of them were accepted by the enzyme.[56] Attempts to observe directly an enzyme-free intermediate also proved unsuccessful. Elucidation of the order of hydrogen abstraction on ring-formation was achieved through the study of deuterium kinetic isotope effects.[57] For these studies, specifically labelled tripeptides at the C-3 cysteine residue (7a), C-3 valine residue (7b), and at both positions (7c), were synthesised. In competitive mixed label experiments with (7a) and (7b) (V_{max}/K_m experiments–for a discussion see appendix A) it was found that IPNS discriminated between deuterated and protiated substrate *only* in the case of (7a). This indicates that abstraction of the cysteinyl hydrogen occurs before or during the first irreversible step, and that abstraction of the valinyl hydrogen occurs after this step. However, non-competitive experiments showed that both (7a) and (7b) [as well as the doubly labelled (7c)] exhibited significant isotope effects on V_{max}[56,58], which suggests that both sites are involved in steps which are at least partially rate limiting, and have similar activation energies.



(7a)

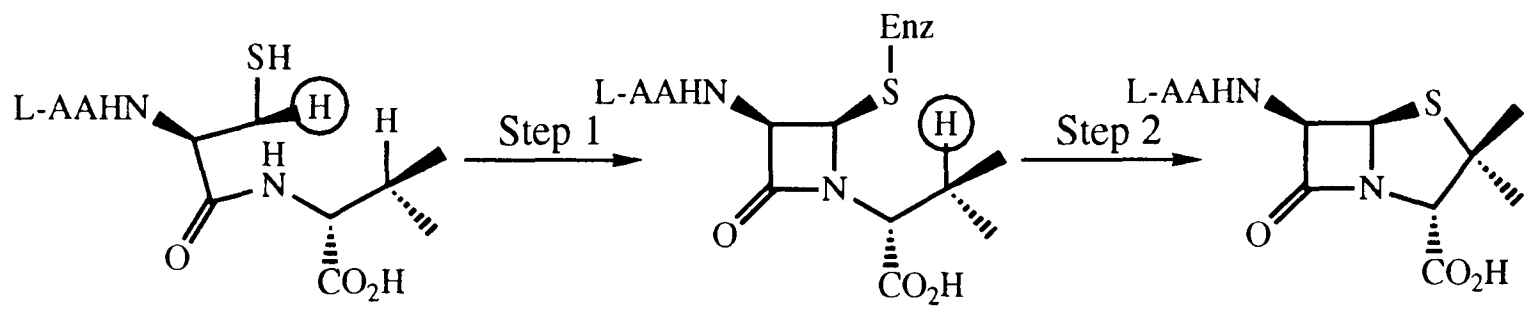


(7b)



(7c)

From these experiments it was concluded that the cyclisation of ACV (7) to IPN (8) is a stepwise and not a concerted process, and that the C-3 hydrogen of the cysteine residue is the first to be removed, leading to the formation of an enzyme-bound intermediate which is thought to be the monocyclic β-lactam (15) (Scheme 1.6). Other mechanistic experiments gave results consistent with the existence of this β-lactam intermediate.[59]

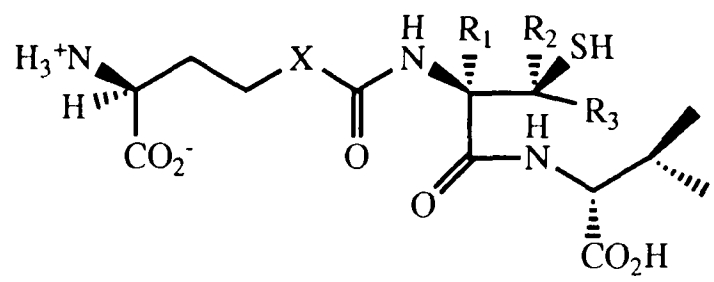


(7) (15) (8)

Scheme 1.6

Extensive investigations into the mechanism of cyclisation have been carried out through the systematic modification of the natural substrate three amino acid residues. Variations of the L-α-aminoadipic acid residue showed that the minimal structural requirement for this part of the tripeptide is a six carbon or equivalent chain, terminating in a carboxyl group.[59,60] This suggests the possibility of a binding pocket for the aminoadipoyl side-chain carboxyl group. The amino group has apparently little influence, as IPNS is also capable of converting a tripeptide with an adipic acid side-chain, as well as one substituted with a D-α-aminoadipic acid. This fact has enabled the synthesis of a range of labelled penicillin N's for the study of DAOC/DACS, by using IPNS to convert labelled D,L,D-ACV's.[61]

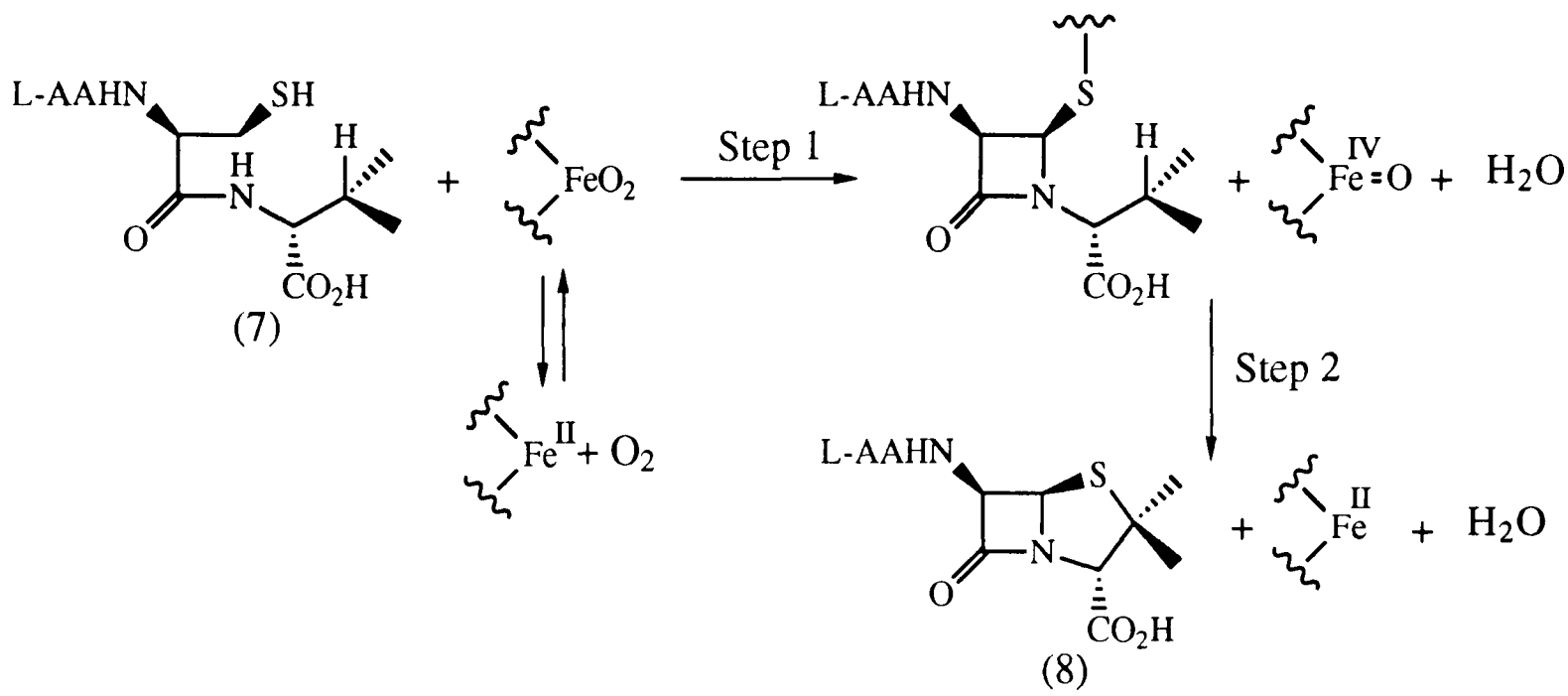
The cysteinyl residue of ACV is the one where least variation is acceptable, which is not surprising considering this residue will probably be involved in a strained "transition structure" on formation of the β-lactam ring. The methylated tripeptides (16) and (17) were accepted as substrates by IPNS, whereas (18) was not accepted in accordance with the stereospecificity observed for the natural conversion.[62] The tripeptide (19) was converted to the corresponding penicillin (c.f. cephamycins).[63]



(16) R_1=Me; R_2=H; R_3=H; X=CH_2
(17) R_1=H; R_2=Me; R_3=H; X=CH_2
(18) R_1=H; R_2=H; R_3=Me; X=CH_2
(19) R_1=OMe; R_2=H; R_3=H; X=O

A surprisingly large degree of variability is accepted by IPNS with regard to the valinyl residue of ACV, the only major constraint being the D-configuration of the carboxyl group. IPNS has been challenged with a great number of distinct analogues[59] giving rise to a family of new products, all of which conserve the β-lactam ring but which may differ in the size of the second ring, as well as in the level of oxidation. These studies have allowed significant mechanistic insight as they have unravelled new modes of reactivity for IPNS, and have led to the proposal of a unifying mechanism for the catalytic cycle.[23]

The cyclisation of the substrate involves a four-electron oxidation, with concomitant reduction of one molecule of dioxygen to two molecules of water. Since this was found to be a stepwise process, a simple assumption is that the iron mediates two separate, two-electron reductions of iron-bound dioxygen by the substrate. Based on this, a formal mechanism was proposed (Scheme 1.7). In this mechanism the first two-electron oxidation of the substrate leads to the formation of a monocyclic β-lactam intermediate, as well as a high-energy iron(IV)-oxene which is capable of inserting into non-activated carbon-hydrogen bonds and mediates the second ring-closure.[59]

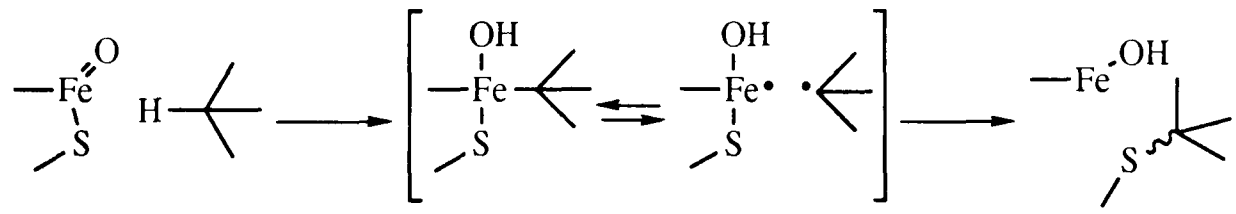


Scheme 1.7

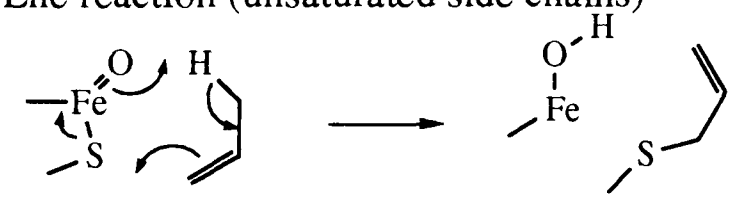
Once the ferryl species is generated at the active site, the enzyme-bound intermediate has to be converted forward due to the high chemical potential of this group. This fact may explain why a large number of variations of the valinyl residue are accepted by IPNS, as well as the different types of reactivity observed in the second ring-closure.

Four modes of reactivity have been recognised for the ferryl species[23,59] :

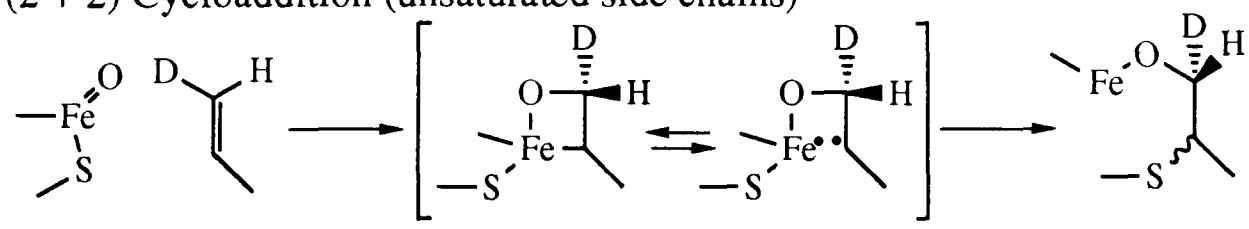
1) Homolysis / recombination (saturated side chains)



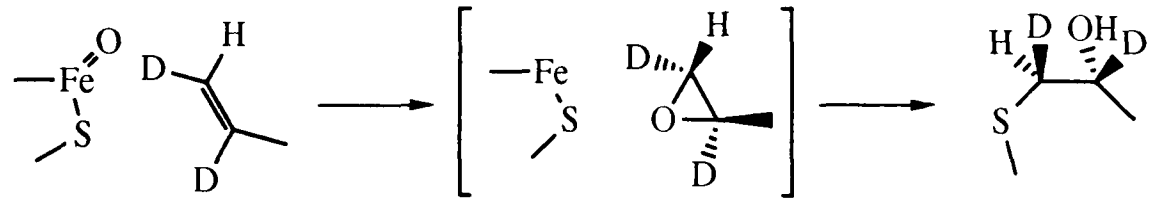
2) Ene reaction (unsaturated side chains)



3) (2 + 2) Cycloaddition (unsaturated side chains)

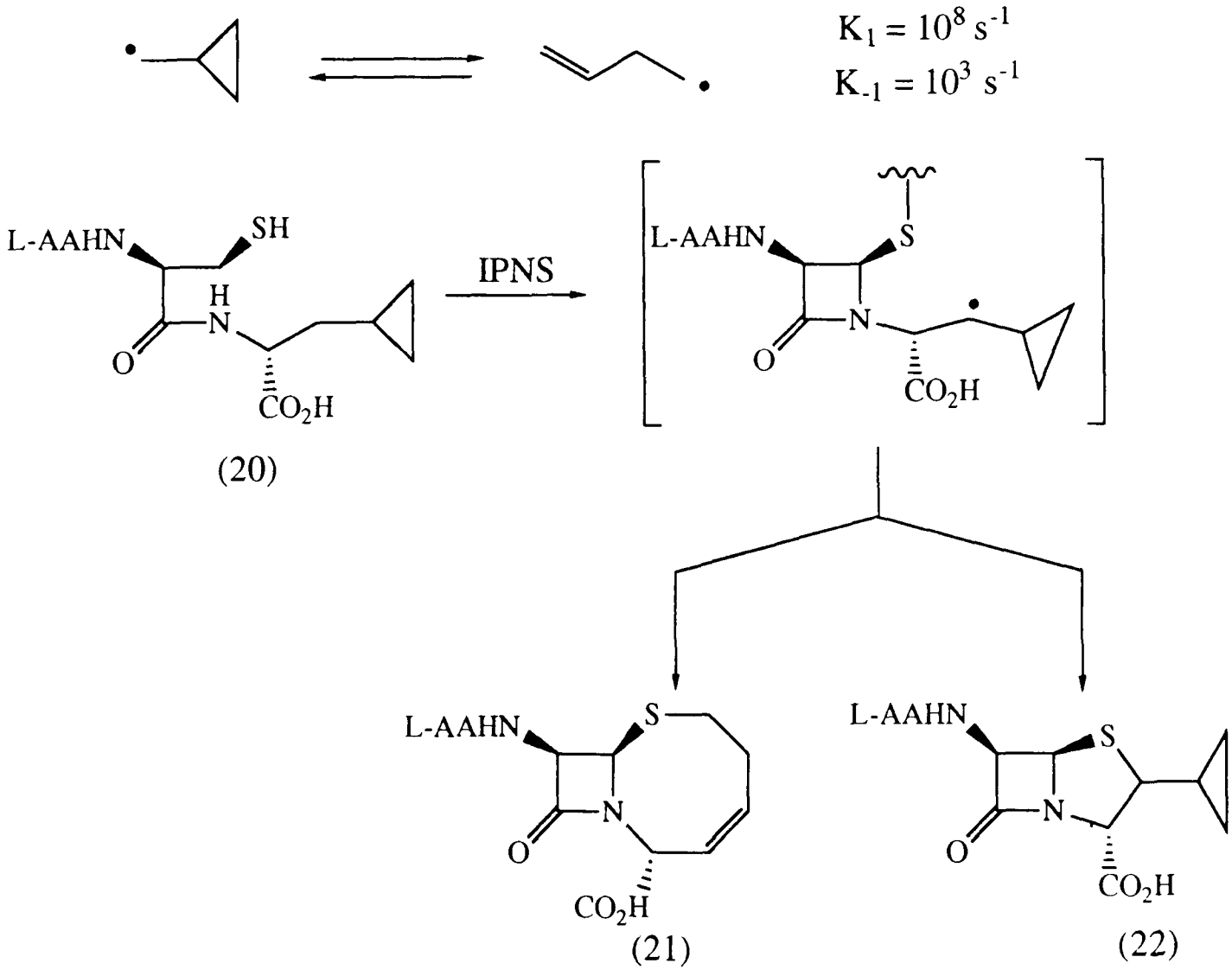


4) Epoxidation / inverting displacement (unsaturated side chains)



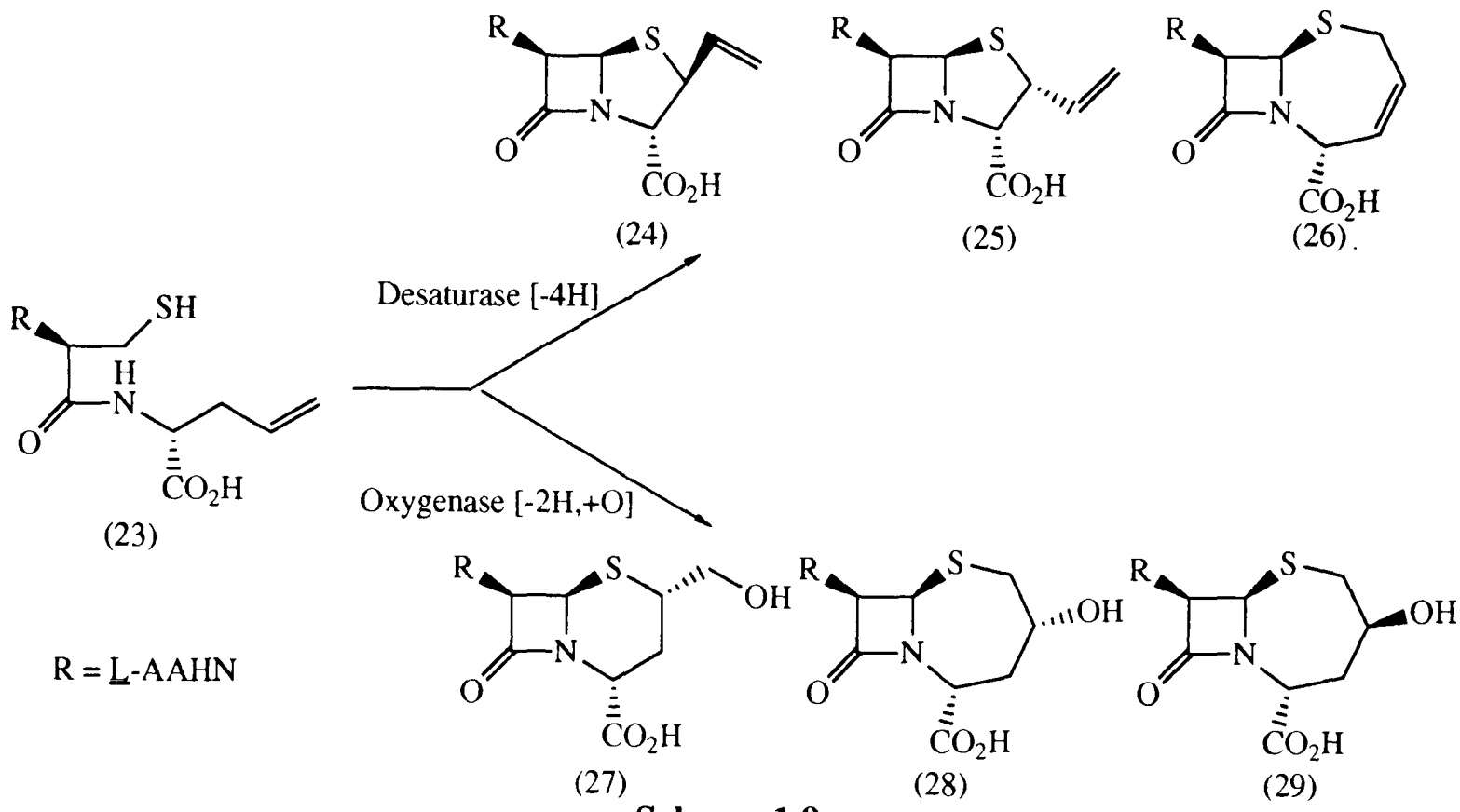
The first two modes of reactivity are examples of *desaturase* activity by IPNS, and the second two are examples of *monoxygenase* activity. This interesting ability of IPNS to perform different types of activity is not very common amongst other oxygenases, but it is observed for another enzyme belonging to the cephalosporin pathway, deacetoxy/deacetyl-cephalosporin C synthase (DAOC/DACS)[64], for which an iron(IV)-oxene intermediate has also been proposed.

The homolysis/recombination mechanism is the one by which the natural substrate is thought to be converted. To test the hypothesis of radical involvement, a substrate with a cyclopropyl group (20) was synthesised. Cyclopropylcarbinyl radicals are well known to rearrange to the allylcarbinyl system in a reversible manner. This rearrangement is so fast that it was hoped that formation of a cyclopropylcarbinyl radical in a substrate like tripeptide (20) would lead to ring opening relatively quickly with respect to radical trapping. In fact, when (20) was incubated with IPNS, two products (21) and (22) were isolated suggesting such a process had indeed occurred (Scheme 1.8).[65]



Scheme 1.8

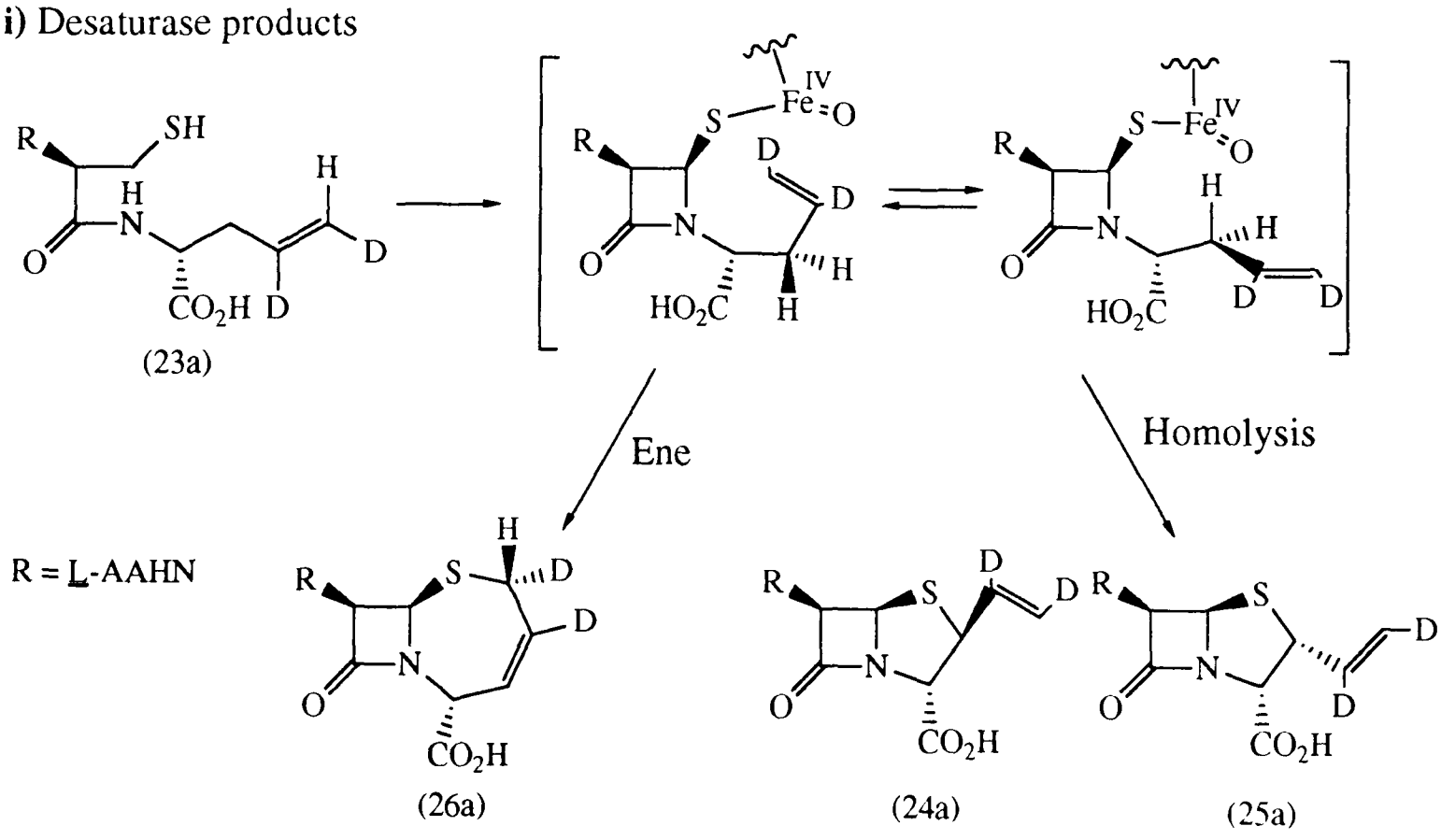
The allylglycine analogue of ACV (23) was unprecedented in that its incubation with IPNS gave rise to six products, providing evidence of all four types of mechanism (Scheme 1.9).[66,67] The proposed mechanisms for these transformations are outlined for the deuterium labelled substrate (23a) (Schemes 1.10A and 1.10B). It is apparent that the selection between the different modes of reaction depends upon the steric relationship between the ferryl species and the modified valine residue.



(23) (24) (25) (26) (27) (28) (29)

Scheme 1.9

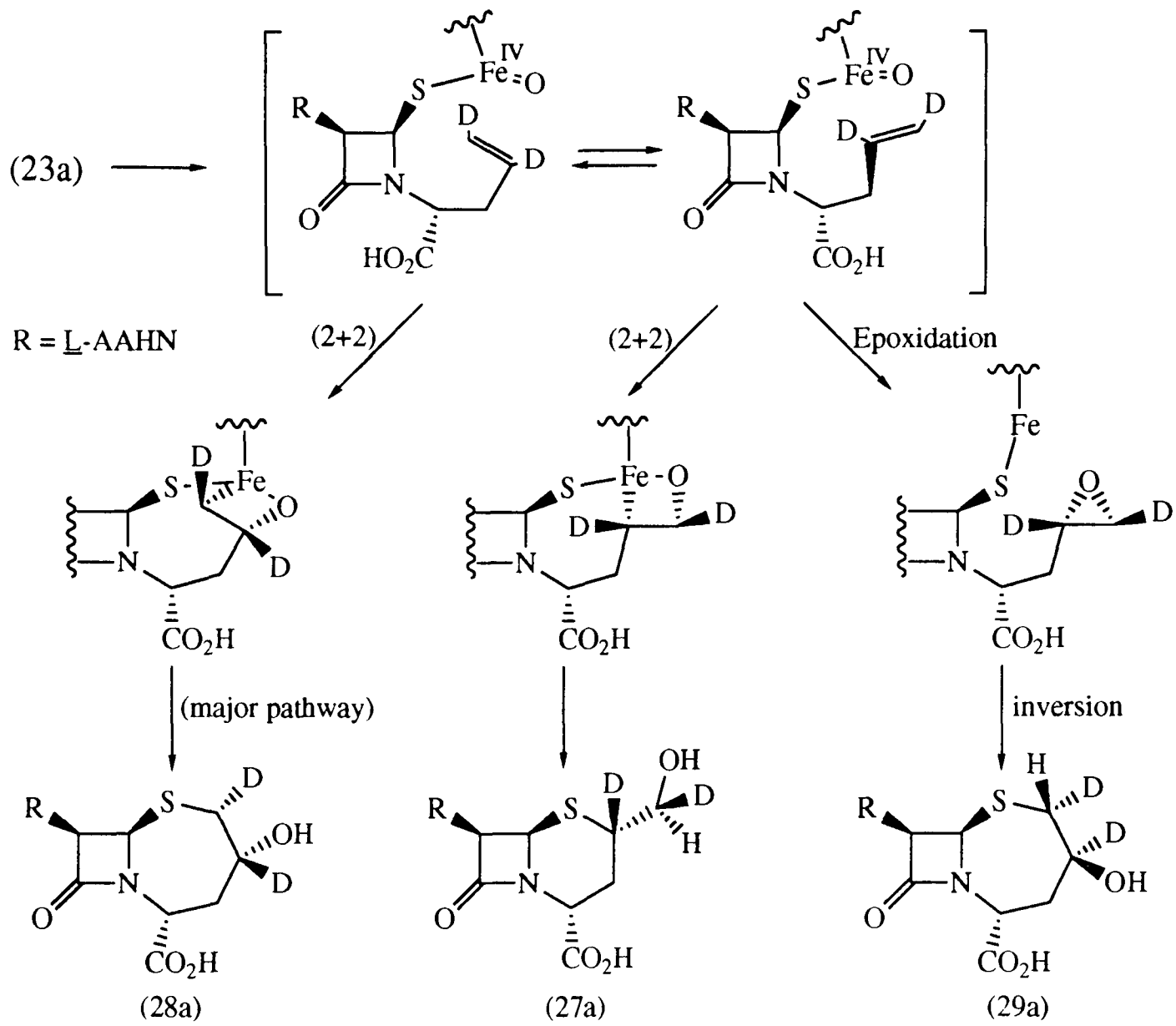
i) Desaturase products



(23a) (26a) (24a) (25a)

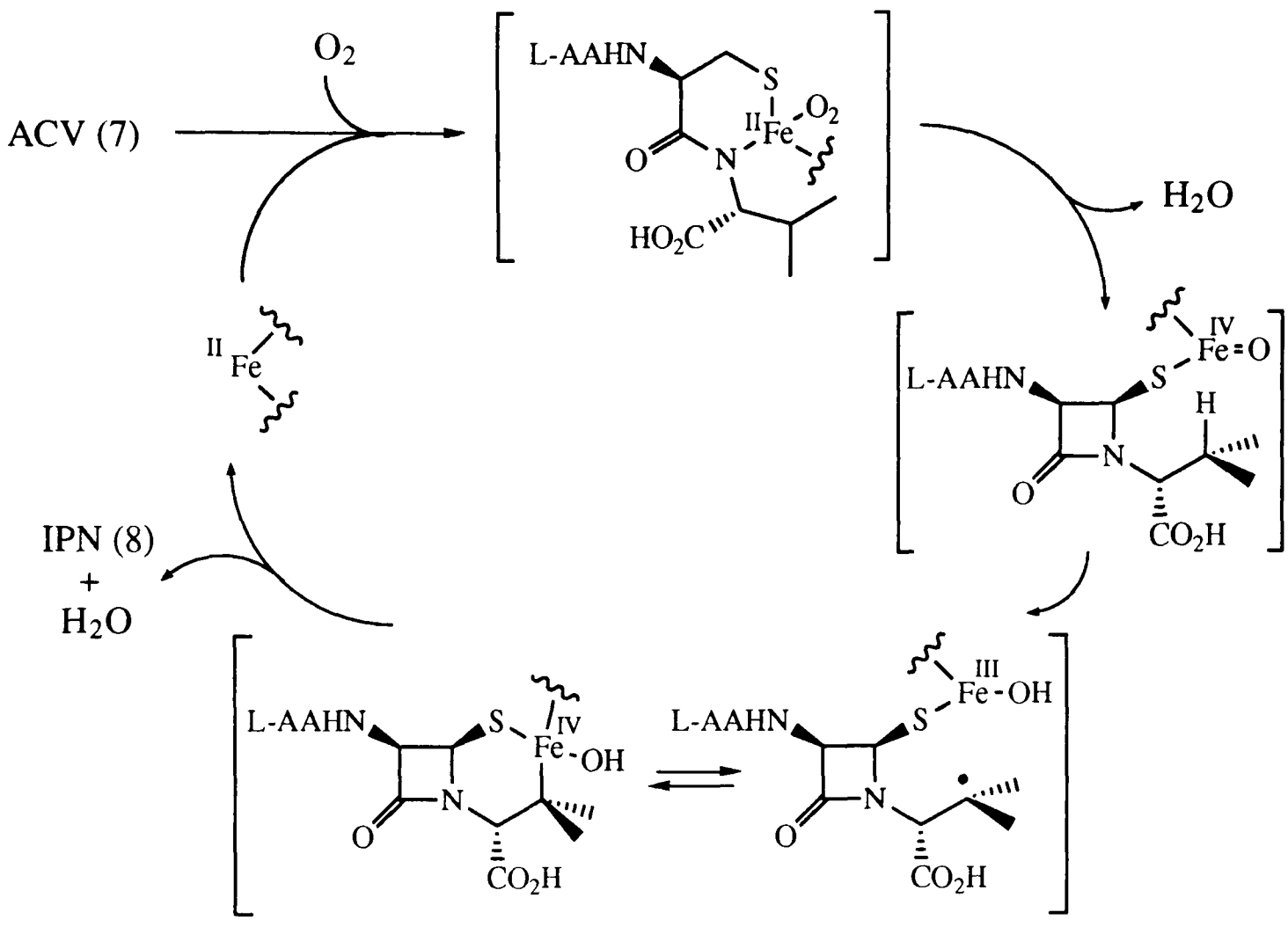
Scheme 1.10 A

ii) Monoxygenase products



Scheme 1.10 B

Many other ACV analogues not described here have been tested with IPNS, and the collective results from this study led to a proposal for its catalytic cycle (Scheme 1.11).[23] The process by which the β-lactam ring is formed is still unclear, but it is probable that the sulphur acts as a connection between the 3-cysteinyl CH bond and the dioxygen molecule, permitting through the iron, the oxidation of the former and the reduction of the latter with concomitant formation of the iron-oxene. This iron-oxene is then capable of abstracting the valinyl hydrogen with formation of an intermediate with radical character. Once this intermediate is formed the iron has two ligands (sulphur and hydroxyl) which can be transfered onto the carbon radical. In the case of saturated substrates sulphur seems to be the ligand preferentially transfered, and in the case of unsaturated substrates both ligands can be transfered onto the product.

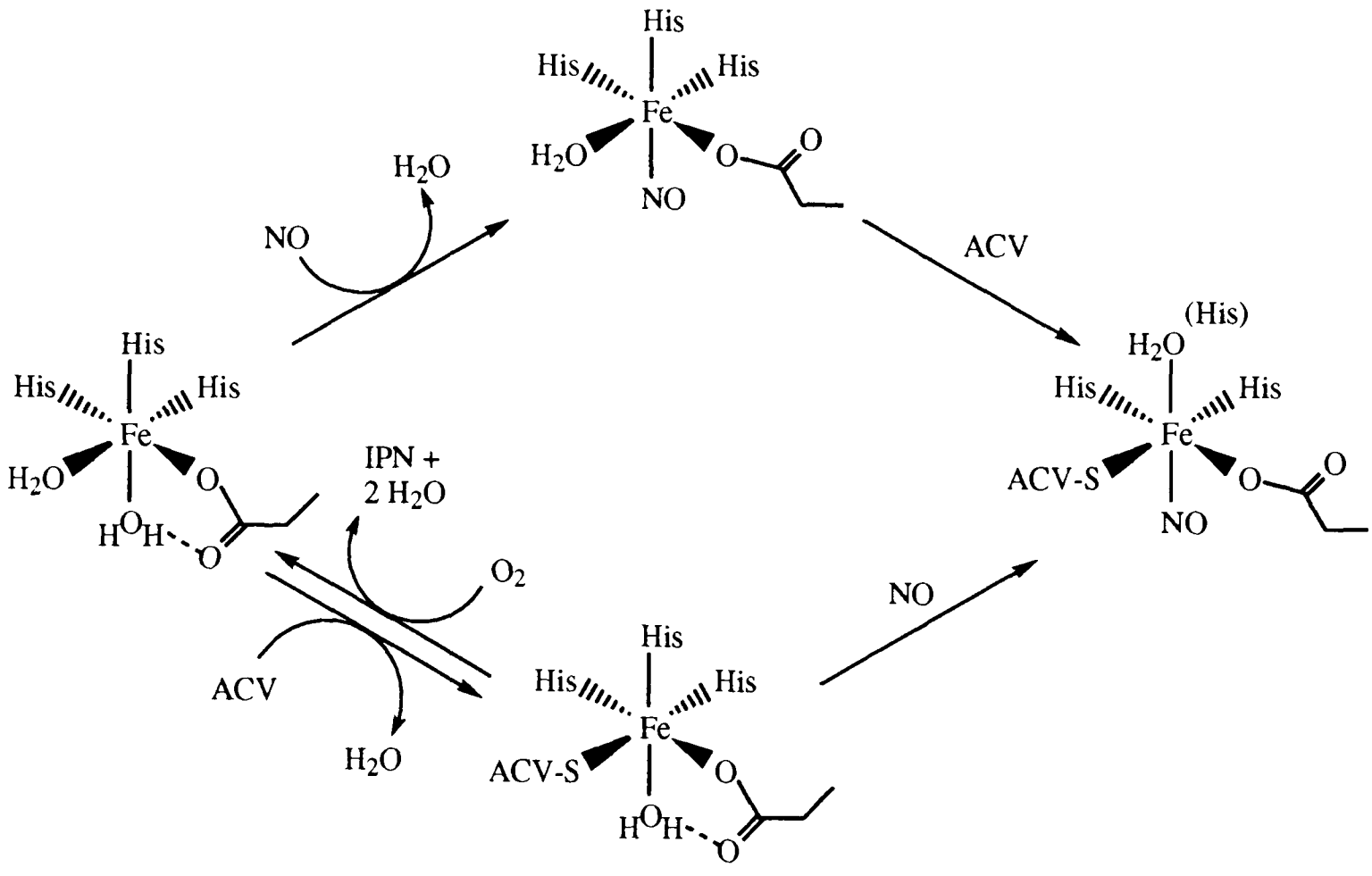


Scheme 1.11

Modelling studies with synthetic iron complexes bearing heteroatom ligands in which an iron-oxene was generated, showed that this species could abstract a hydrogen to form a carbon radical and that there was preferential transfer of the heteroatom ligand (X = Cl, Br, N_3) over the hydroxyl ligand (perhaps due to the lower oxidation potential of the X group), leading to halogenation rather than hydroxylation.[68] These experiments provide some chemical precedent for the enzymatic formation of isopenicillin N (8), where the sulphur ligand is transferred preferentially over the hydroxyl ligand. Thus, heteroatom ligation to the iron (for IPNS, X=S) and preferential transfer of this ligand is a possible explanation for the different mode of reactivity of the ferryl species in IPNS (oxidative cyclisation), as opposed to the one usually observed in other oxidative systems (hydroxylation).

Physical studies with recombinant IPNS have reinforced the idea that the ACV thiol is directly coordinated to the iron[69,70], and this was recently confirmed by X-ray absorption studies[71].

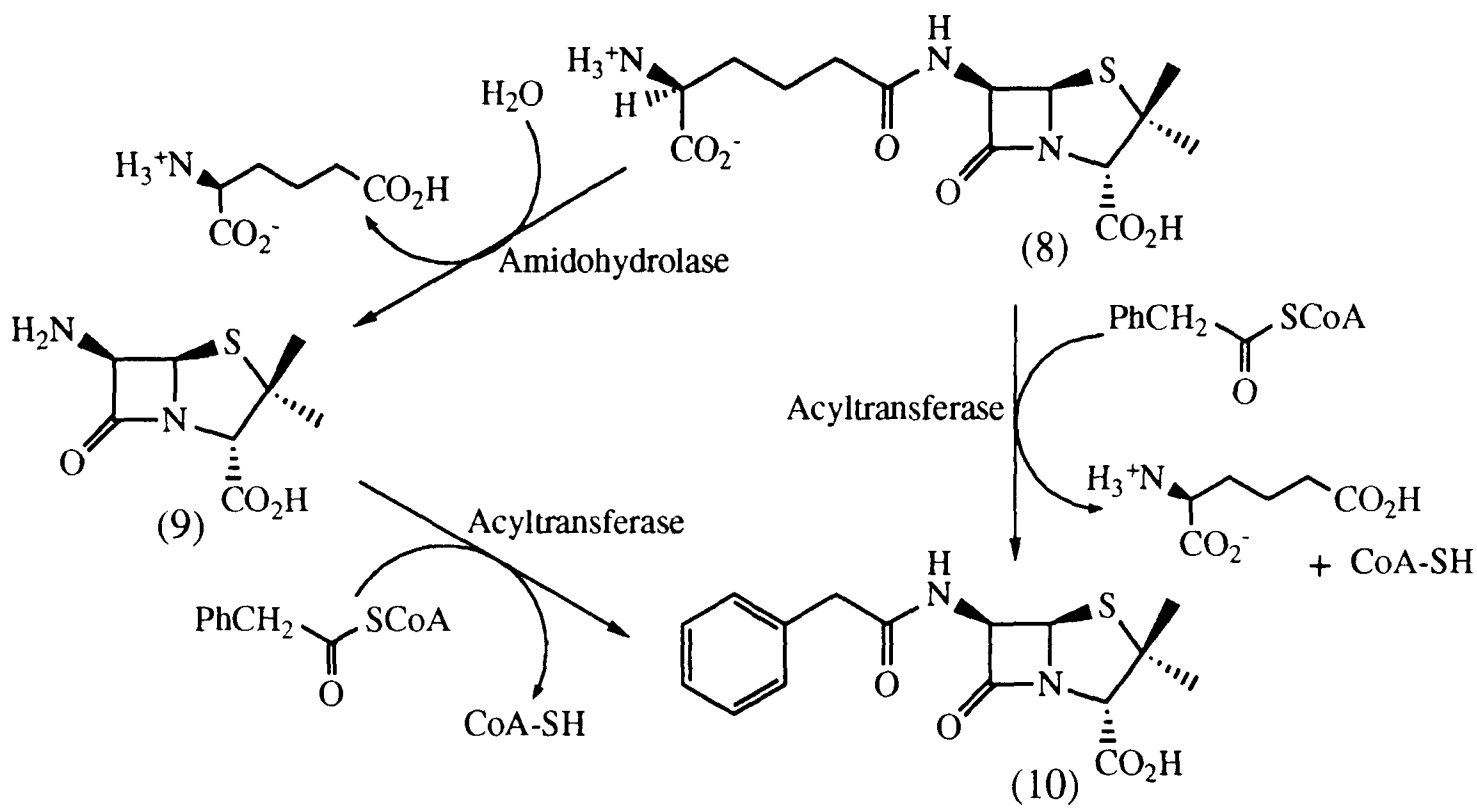
The active site of IPNS has been investigated by the use of spectroscopic techniques[69,70,72] including Mössbauer, EPR, NMR and electronic absorption. The results led to the proposal of an active site containing high-spin Fe^{II} with four endogenous ligands (3 histidines and one aspartate), and two solvent ligands (H_2O). Binding of ACV (through the sulphur atom) and NO (used as an analogue for dioxygen) apparently displaces one water molecule and one of the endogenous ligands (probably His) (Scheme 1.12).[72]



Scheme 1.12

1.2.5 Acyltransferase

The last step in the biosynthesis of hydrophobic penicillins [e.g. penicillin G (10)], is the exchange of the L-α-aminoadipoyl side-chain of isopenicillin N (8) (Scheme 1.13).



Scheme 1.13

In 1972, Loder[73] showed that extracts of *P. chrysogenum* catalysed the incorporation of ^{14}C from [^{14}C]-phenylacetyl-coenzyme A into penicillin G (10) in the presence of IPN (8). Complementary, Abraham *et al.*[15,74] showed that similar extracts converted IPN (8) labelled with ^{3}H in the 3β-methyl group to [^{3}H]penicillin G in the presence of phenylacetyl-coenzyme A. It was later shown that if IPN (8) was incubated in the absence of phenylacetyl-coenzyme A, 6-aminopenicillanic acid (6-APA) (9) was produced.[75]

Recently, Whiteman *et al.* have reported the purification to apparent homogeneity of acyltransferases from both *P. chrysogenum* and *A. nidulans*.[76] The enzymes exhibited phenylacetyl-CoA:6-APA acyltransferase activity, together with IPN amidohydrolase and phenylacetyl-CoA:IPN acyltransferase activities. The three activities could not be separated by ion-exchange, gel filtration, hydrophobic interaction or affinity column chromatography.

It was found that each of the enzymes could be separated into two fragments (about 30 and 10 KDa each) on SDS-PAGE. Neither of the individual fragments displayed bioactivity, but this could be restored on recombination of the separated proteins which implies that the acyltransferase is a heterodimer and requires both subunits for optimal activity.

The acyltransferase gene encoding a 40 KDa protein was cloned from *P. chrysogenum*, and was shown to code for both the 30 and 10 KDa subunits.[77,78] This means that a single polypeptide is produced, which subsequently undergoes post-translational cleavage to give a heterodimer. The mechanisms of the acyltransferase and amidohydrolase reactions remain to be investigated in detail.

1.2.6 Isopenicillin N/Penicillin N Epimerase

The enzyme IPN/Pen N epimerase catalyses the inversion of configuration of the L-α-aminoadipoyl side-chain of isopenicillin N (8) to the D-α-aminoadipoyl side-chain of penicillin N (1). This enzyme represents the branching point between the penicillin and cephalosporin biosynthetic pathways, and as such is not found in those organisms which produce only penicillins.

Konomi *et al.*[12] were the first to suggest that pen N (1) was produced by epimerisation of IPN (8), a fact that was later confirmed by direct evidence of a highly labile epimerase in extracts of *C. acremonium*[20,79]. However, this enzyme is so unstable that its purification has not yet been achieved.

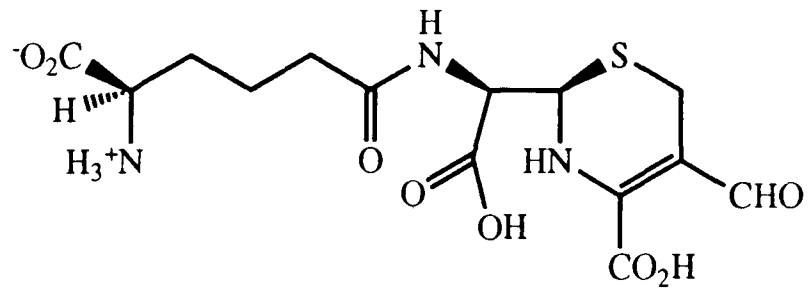
The bacterial epimerases are apparently more stable, and have been purified from *S. clavuligerus*[80,81] and *S. lactamdurans*[82]. The epimerase from *S. clavuligerus* was purified to homogeneity by Usui *et al.* [81] to give a protein with a mass of 47-50 KDa which catalysed the epimerisations of both IPN (8) to pen N (1), and of pen N to IPN. The K_m for IPN (0.30 mM) was found to be lower than that of pen N (0.78 mM), which indicates that when the concentration of IPN is low, the penicillin N produced will be consumed by ring expansion activity before being epimerised back to IPN. Homogeneous epimerase was

found to exhibit absorption maxima at 280 and 420 nm, and to contain one equivalent of pyridoxal-5'-phosphate. This cofactor could be removed by treatment with hydroxylamine followed by gel filtration to give an apo-protein with no absorption maxima at 420 nm. Thus, isopenicillin N/penicillin N epimerase appears to be similar to other bacterial amino acid racemases. The epimerase gene from *S. clavuligerus* has been sequenced and expressed in *E. coli*.[83] This gene was found to be located immediatly upstream of the deacetoxycephalosporin C synthase gene.

1.2.7 Deacetylcephalosporin C Acyltransferase

The acetylation of deacetylcephalosporin C (DAC) (3) to cephalosporin C (12) is the last step in the biosynthesis of cephalosporins, and is carried out by an acyltransferase enzyme.[84] Extracts of *C. acremonium*[85] were shown to convert DAC (3) to cephalosporin C (12) in the presence of acetyl-CoA and Mg^{2+}. The optimal pH of the enzyme was 7.0-7.5. Other divalent cations also supported activity (Zn^{2+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Cu^{2+}), although at lower levels than those observed for Mg^{2+}.

A mutant of *C. acremonium* blocked in the conversion of DAC (3) to cephalosporin C (12) accumulated a new compound, the 3-formylcephalosporoate (30), in which the hydroxyl group of DAC has been oxidised to an aldehyde and the β-lactam ring hydrolysed as a result of its increased reactivity.[86] This suggests that *in vivo*, acetylation of DAC (3) may have evolved to preserve the integrity of the β-lactam ring.



(30)

1.3 Deacetoxycephalosporin C / Deacetylcephalosporin C Synthase

The expansion of the thiazolidine ring of penicillin N (1) to the dihydrothiazine ring of deacetoxycephalosporin C (DAOC) (2) is the crucial step in the biosynthesis of cephalosporins (Scheme 1.3). The C-3 methyl group of DAOC (2) is subsequently hydroxylated to give deacetylcephalosporin C (DAC) (3), which constitutes another branching point in the β-lactam pathway. In some species (e.g. *C. acremonium*) DAC will be acetylated to give cephalosporin C (12) (see section 1.2.7), whereas in others (e.g. some *Streptomyces* species) it will be converted to cephamycins (through carbamoylation of the hydroxyl group[87], followed by hydroxylation and methylation to introduce the 7-α-methoxy group[88]).

1.3.1 Enzymology

Evidence for the ring expansion of pen N (1) was first observed with cell-free extracts of *C. acremonium*[18,19] These extracts displayed very low levels of activity until Hook *et al.* demonstrated the importance of ferrous iron, α-ketoglutarate (α-KG), and ascorbic acid as the cofactors necessary for optimising catalysis.[89] The same cofactor requirements had previously been shown for the hydroxylase activity [(2) → (3)] in extracts of *C. acremonium*[90,91] and *S. clavuligerus*[91]. These cofactor requirements are characteristic of a class of dioxygenases which usually catalyse hydroxylations[92], and use α-ketoglutarate as a cosubstrate converting it to succinate and CO_2 (for a more detailed discussion see section 1.3.4).

The expandase activity was first partially purified from *C. acremonium* by Kupka *et al.* [93], who confirmed the cosubstrate role of α-KG by using [1-^{14}C]-α-ketoglutarate in the assay system and following the release of $^{14}CO_2$. Later work revealed that the expandase

and hydroxylase activities from *C. acremonium* could not be separated by gel filtration, anion exchange chromatography, chromatofocusing or isoelectricfocusing, and remained associated with an unstable protein of molecular weight 33,000.[94] It was thus proposed that a single bifunctional enzyme catalysed both reactions. In contrast, Jensen *et al.*[95,96] showed that in *S. clavuligerus* the two activities could be clearly separated by anion exchange chromatography. The partially purified proteins from this organism had estimated molecular weights of 29,500 (expandase) and 26,000 (hydroxylase), and both showed the same cofactor requirements as the bifunctional enzyme from *C. acremonium*, but each appeared to have greater stability. The expandase from *S. lactamdurans* was subsequently also purified and characterised.[97]

The enzyme from *C. acremonium* deacetoxycephalosporin C/deacetylcephalosporin C synthase (DAOC/DACS) has been purified to near homogeneity.[64,98] The purified protein retains expandase and hydroxylase activities. It has a molecular weight of 40,000, and an absolute requirement for Fe^{II}, dioxygen and α-KG, but maximum activity also requires the addition of dithiothreitol (DTT) and ascorbate. ATP, which had been reported to stimulate activity, is found to have only a marginal effect[64,94] (the enzyme should thus be called synthase and not synthetase). The requirement for α-KG seems to be quite specific, with α-ketoadipate, pyruvate, glutamate and citrate only displaying 5-10% of the activity showed with α-KG.[99]

1.3.2 Recombinant DAOC/DACS

Direct amino acid sequencing of purified DAOC/DACS was not successful, presumably due to an amino terminal blockage. However, a partial amino acid sequence was obtained by proteolitic fragmentation of the molecule and this was used to obtain probes and clone the DAOC/DACS gene.[100] This gene was expressed in *E. coli* in high yield and the resultant purified protein shown to catalyse both expandase and hydroxylase activities, and to be indistinguishable from the wild-type enzyme by SDS gel

electrophoresis. This evidence confirms the proposal that DAOC/DACS is a bifunctional enzyme, which raises the question of whether there are one or two active sites in the enzyme. This question remains to be answered.

The predicted amino acid sequence for the DAOC/DACS gene showed similarities to that of the IPNS gene. Although the overall homology was only slightly greater than 11%, certain areas showed higher homologies. In particular, a decapeptide of DAOC/DACS containing a cysteine residue at position 100, showed 50% homology to the corresponding region containing the cysteine residue at position 106 of IPNS[100], which appears to be involved in substrate binding[101].

The bifunctionality of DAOC/DACS in *C. acremonium versus* the existence of two separate enzymes in species of *Streptomyces*, represents the first significant difference observed between the prokaryotic and eukaryotic biosynthetic pathways to cephalosporins. It has been suggested that the DAOC/DACS polypeptide could have evolved from the fusion of two contiguous separate genes[100], which is in accord with an earlier suggestion that cephalosporin biosynthesis evolved first in prokaryotic organisms (e.g. *Streptomyces*), and that truncated forms of more complex prokaryotic pathways subsequently spread to eukaryotes (e.g. *Cephalosporium*) by horizontal gene transfer[44].

The genes for deacetoxycephalosporin C synthase (DAOCS) (expandase) and deacetylcephalosporin C synthase (DACS) (hydroxylase) from *S. clavuligerus* have been cloned and over-expressed in *E. coli*.[102,103,104] A small amount of expandase activity was observed for recombinant DACS[103], and of hydroxylase activity for recombinant DAOCS[102], so both enzymes are also partially bifunctional. This suggests that the two proteins have a common evolutionary origin, which is confirmed by the fact that the DAOCS and DACS genes have sequence homologies of 59 and 71% respectively at the amino acid and DNA levels.[103] The sequence homology between the DAOCS and DAOC/DACS genes was as high as 57 and 67% at the amino acid and DNA levels, and between DACS and DAOC/DACS it was 54% at the amino acid level. A comparison of the kinetic and catalytic properties of the three enzymes is given in Table 1.2.[105]

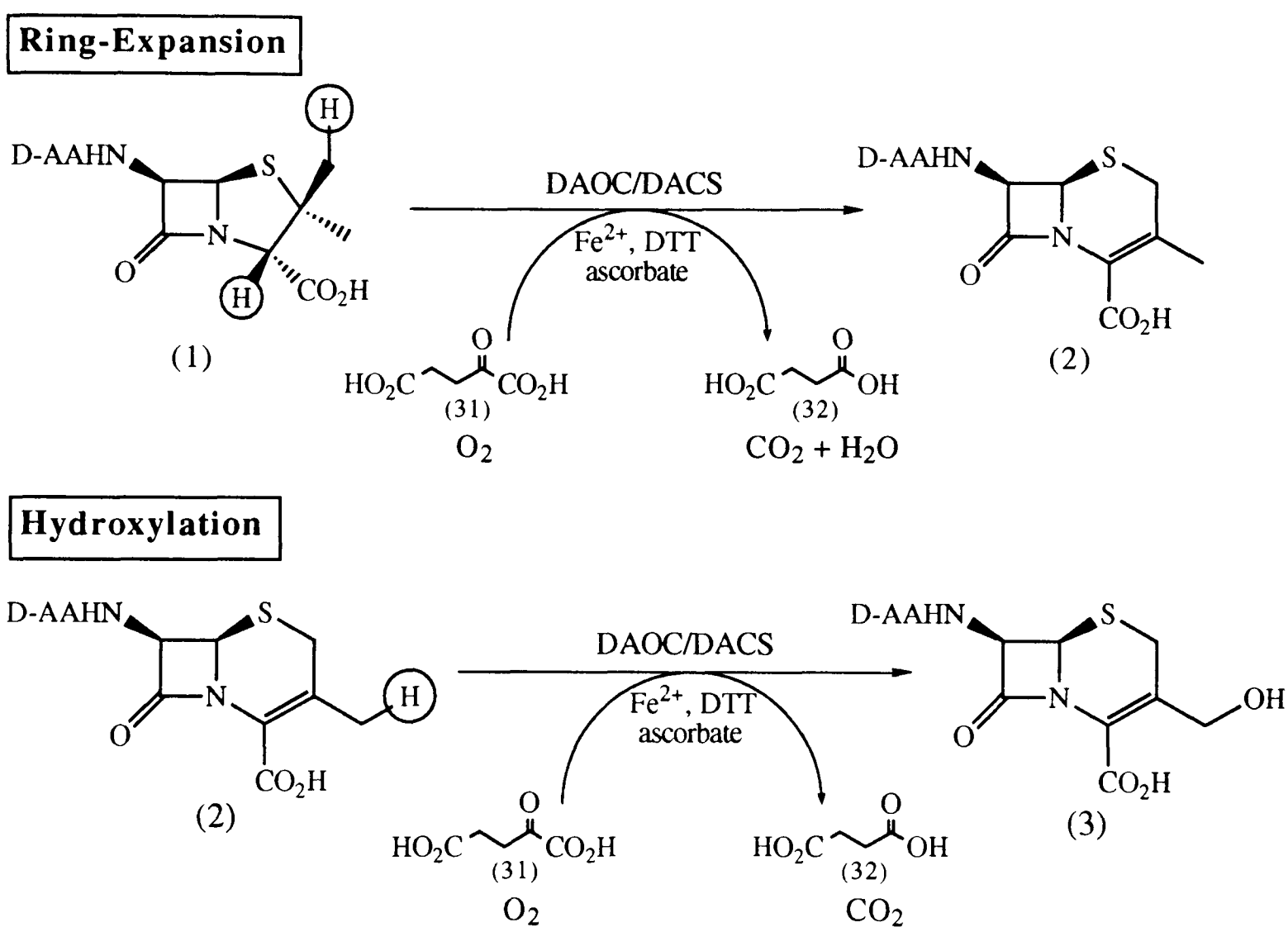
Two systems have been developed for the expression of recombinant DAOC/DACS. One uses a λP_L promoter[100], and the expressed enzyme is produced in the form of inclusion bodies which have to be treated with 6M urea for solubilising the protein, followed by elimination of the urea *via* slow dialysis to permit refolding. This process leads to quite substantial loss of activity.[106] Recently, a new expression system has been developed under the control of the *trc* promoter, which leads to the production of soluble highly active DAOC/DACS needing no urea treatment.[107] The substrate specificities of both recombinant enzymes have been tested and shown to be identical to the wild-type enzyme.[108,109] The availability of recombinant enzymes has greatly enhanced the possibility of detailed mechanistic studies, and all the work described in this thesis was done with recombinant DAOC/DACS produced in *E. coli*.

	C. acremonium		***S. clavuligerus***	
Property	Expandase activity	Hydroxylase activity	Expandase	Hydroxylase
Optimal pH	7.5-7.8	7.0-7.5	7.4	7.0-7.4
Optimal temp. (°C)	26-34	34-38	36	28-31
Requirement for Fe^{2+}, α-KG and O_2	YES	YES	YES	YES
Stimulation by				
DTT	Great	Great	Great	Great
Ascorbate	Great	Great	Great	Slight
ATP	Moderate	Slight	None	Moderate
K_m (μM) for				
Pen N (1)	29	—	35	—
DAOC (2)	—	18	—	25
α-KG	22	20	22	14
K_a (μM) for Fe^{2+}	6	13	4	30
V_{max} (μmol/min/mg protein)	0.4-0.8	0.1-0.3	0.4	0.5

Table 1.2 : Catalytic and kinetic properties of DAOC/DACS from *C. acremonium*, and DAOCS and DACS from *S. clavuligerus* (determined with native and/or recombinant enzymes).[105]

1.3.3 Mechanistic Studies

DAOC/DACS catalyses the sequential ring expansion of pen N (1) to deacetoxycephalosporin C (DAOC) (2) and its subsequent hydroxylation to deacetylcephalosporin C (DAC) (3). Each step requires stoichiometric amounts of dioxygen and α-ketoglutarate (31) and is energetically driven by the oxidative decarboxylation of α-KG (31) to succinate (32) and CO_2[93,110], which provides two of the four electrons needed for the complete reduction of dioxygen (Scheme 1.14).



Scheme 1.14

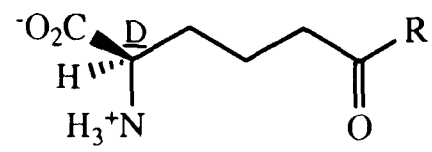
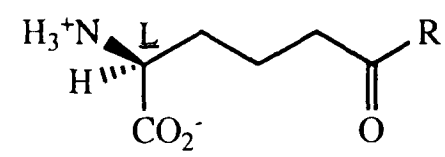
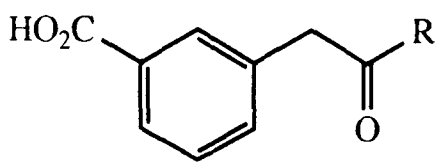
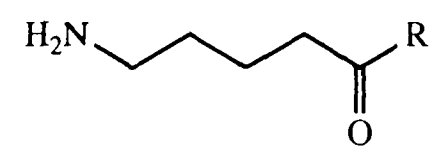
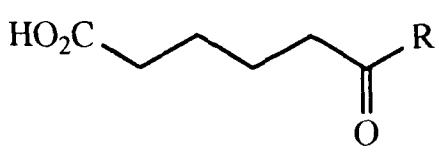
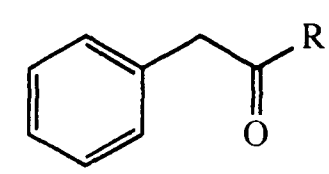
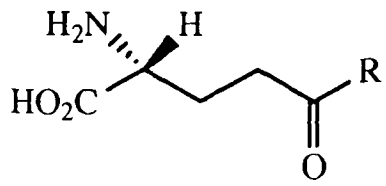
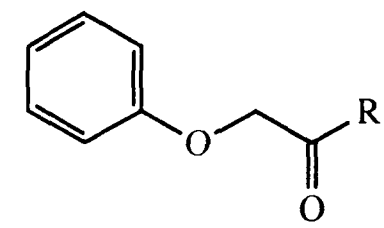
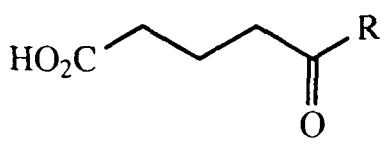
Most of the other known α-ketoglutarate dependent oxygenases (see section 1.3.4) catalyse direct hydroxylations of the substrates (other exceptions being clavaminic acid synthase[111] which is also involved in β-lactam biosynthesis, and flavone I synthase[112]).

It is interesting to note that in the case of DAOC/DACS two distinct reactions occur. In the ring expansion step two hydrogens are removed from the substrate and molecular oxygen is reduced to water, with no incorporation of oxygen into the product DAOC (2). On the other hand, in the hydroxylation reaction a classical hydroxylation of a C-H bond occurs (this duality will be discussed in more detail in the next section).

1.3.3.1 Mechanistic Studies on the Ring Expansion

The side-chain specificity of DAOC/DACS was investigated by the use of penicillins with modified side-chains (Table 1.3).[113,114] It was found that penicillins with *m*-carboxyphenylacetyl (33) or adipoyl (34) side-chains underwent ring expansion at about half the rate of pen N (1). A much lower rate was observed in the case of the other side-chains. These results indicate that a six carbon N-acyl side-chain terminating in a carboxyl group permits reasonable conversion by DAOC/DACS. This is similar to the side-chain requirement of IPNS (see section 1.2.4), and also suggests the existence of a binding pocket for the aminoadipoyl residue at the enzyme active site. However, contrary to IPNS, DAOC/DACS does not accept the L configuration of the δ-(α-aminoadipoyl) side-chain.

Studies with intact cells of *C. acremonium* revealed that the methyl group of valine which became the 2α-methyl group of penicillin N, also became the exocyclic methylene group of cephalosporin C (12), and the one which became the 2β-methyl group of pen N also became the endocyclic methylene (C-2) of cephalosporin C (see Scheme 1.4). The stereospecificity of the ring expansion was investigated by feeding a stereospecifically labelled valine (6c) to cells of *C. acremonium*, and it was found to occur with complete loss of stereochemistry (Scheme 1.15).[115,116] This result contrasts with the stereospecificity of hydroxylation observed in the conversion of DAOC (2) to DAC (3) by DAOC/DACS (see section 1.3.3.2), and also in conversions catalysed by other α-ketoglutarate dependent dioxygenases.

Conversion	Initial velocity ($\mu mol.m^{-1}x10^4$)	% conversion	No conversion
 (1)	3.88	100	 (8)
 (33)	1.93	50	 (37)
 (34)	1.56	40	 (10)
 (35)	0.54	14	 (11)
 (36)	0.13	3	H-R (9)

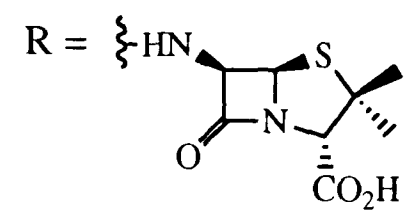
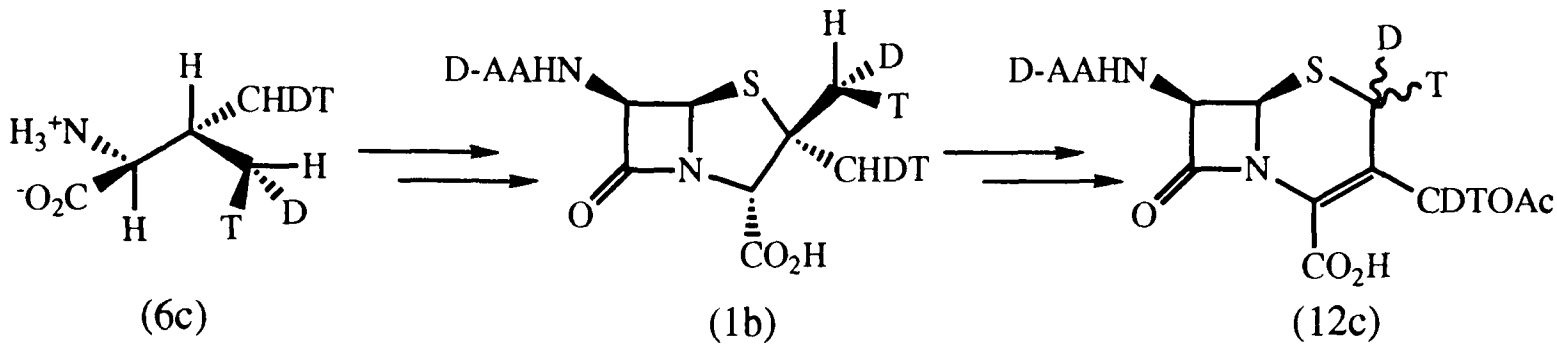
R = 

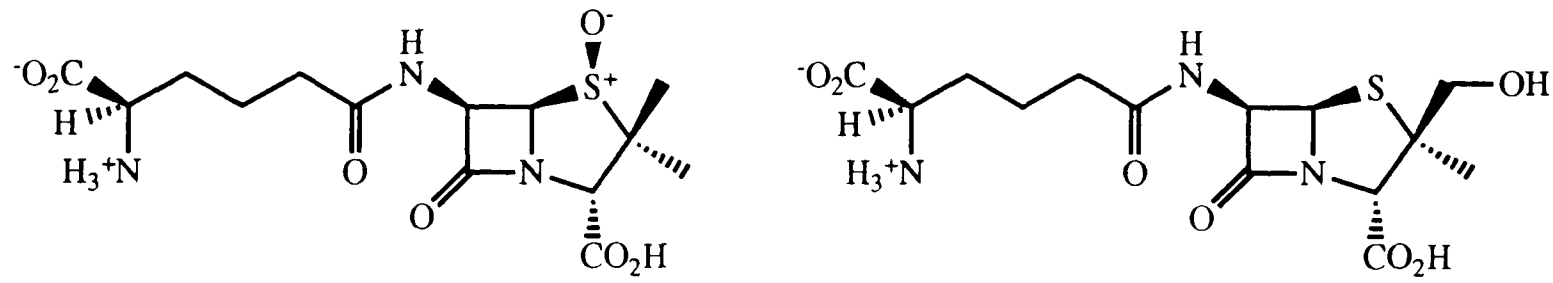
Table 1.3



(6c) (1b) (12c)

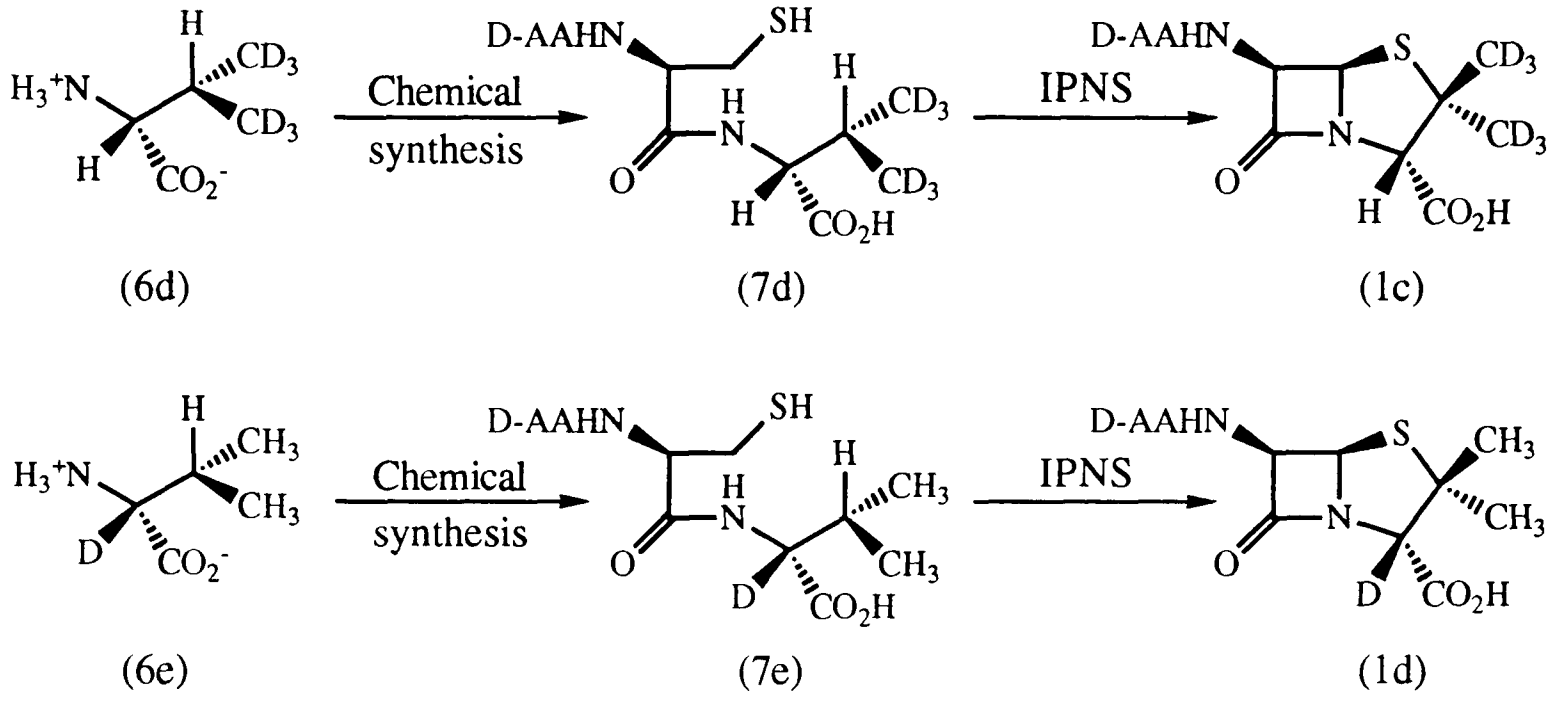
Scheme 1.15

By analogy with known non-enzymatic conversions of penicillins into cephalosporins, and also given the fact that α-ketoglutarate dependent dioxygenases usually insert one oxygen atom into the product, it was proposed that the β-sulphoxide (38) or the β-hydroxymethyl penicillin (39) could be intermediates in the ring expansion reaction. However, neither of these compounds were converted to cephalosporins when incubated with DAOC/DACS[64] proving that they are not enzyme-free intermediates.



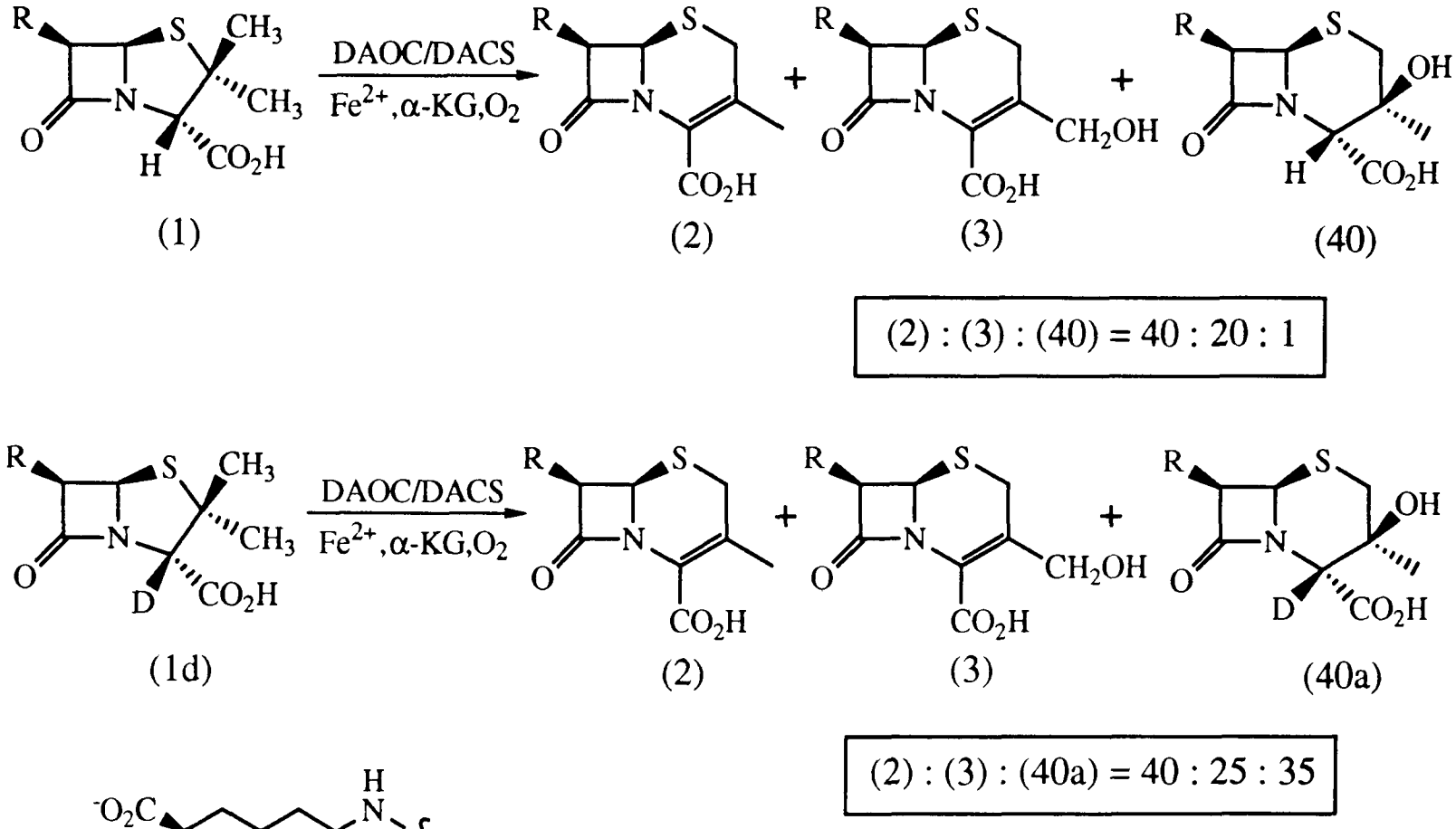
(38) (39)

The ring expansion of pen N (1) involves the abstraction of two hydrogens atoms, one from the β-methyl group and the other from the C-3 (Scheme 1.14). To investigate whether these two events occurred in a concerted or stepwise manner, it was decided to conduct deuterium isotope effect studies, which had been so informative in the case of IPNS. For this study, two specifically labelled penicillin N isotopomers, (1c) and (1d), were synthesised from the corresponding tripeptides using IPNS, since it had previously been shown that this enzyme could accept the D-α-aminoadipoyl side chain[59,60] (Scheme 1.16).



Scheme 1.16

Initially, a careful incubation of unlabelled pen N (1) revealed that beside the two expected products (2) and (3), a third minor product (40) could be detected in a ratio of (2):(3):(40) = 40:20:1 (Scheme 1.17).[117] The 3β-hydroxycepham (40) had also been previously isolated from a filtered broth of *C. acremonium.*[118] When the monodeuterated penicillin N (1d) was incubated with DAOC/DACS the same products were isolated, but in a substantially different ratio [(2):(3):(40a) = 40:25:35].[117,119] These results suggested that a primary kinetic isotope effect was leading to a change in product ratio. The products (40) and (40a) were shown not to be substrates for DAOC/DACS, and not to have antibacterial activity against *Staphylococcus aureus* NCTC 6571, or *Escherichia coli* ESS, at a concentration of 100 $\mu g.ml^{-1}$.[119] In addition, an incubation of monodeuterated penicillin N (1d) under an atmosphere of $^{18}O_2$ revealed there was incorporation of ^{18}O-label both into DAC (3) (40%) and into the 3β-hydroxycepham (40a) (50-60%), indicating that the origin of both hydroxyl groups was molecular oxygen.[117,119]



Scheme 1.17

The two deuterated penicillins (1c) and (1d) were next used in competitive kinetic isotope experiments. In these experiments an approximately 1 : 1 mixture of labelled and unlabelled substrate was incubated with the enzyme, and aliquots of the incubation mixture taken at several conversion points. The unconverted starting material was then recovered from these aliquots and the ratio of labelled to unlabelled substrate determined (in this case, by mass spectrometry), to look for enzyme discrimination between the two. The results obtained in these experiments are summarised in Tables 1.4 and 1.5.[120] They show that DAOC/DACS discriminates between (1) and (1c), but not between (1) and (1d), that is, it converts (1) faster than (1c), but (1d) at the same rate as (1).

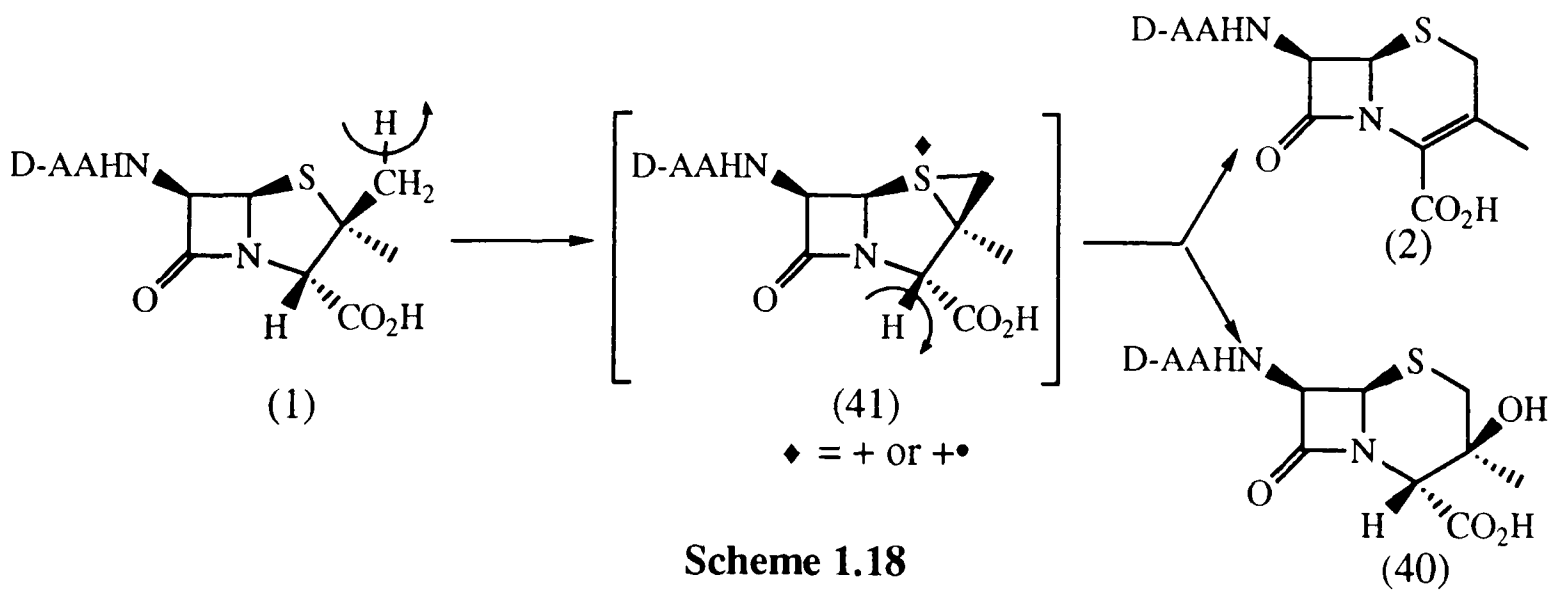
Expt.	**% Conversion**	**Ratio (1) : (1c)**
1	0	0.94 : 1
	30	0.50 : 1
2	0	1.00 : 1
	40	0.51 : 1
	60	0.41 : 1

Table 1.4 : Enzymatic conversion of a mixture of (1) and (1c)

Expt.	% Conversion	Ratio (1) : (1d)
1	0	0.95 : 1
	30	0.95 : 1
	65	0.93 : 1
2	0	1.24 : 1
	30	1.22 : 1
	80	1.20 : 1

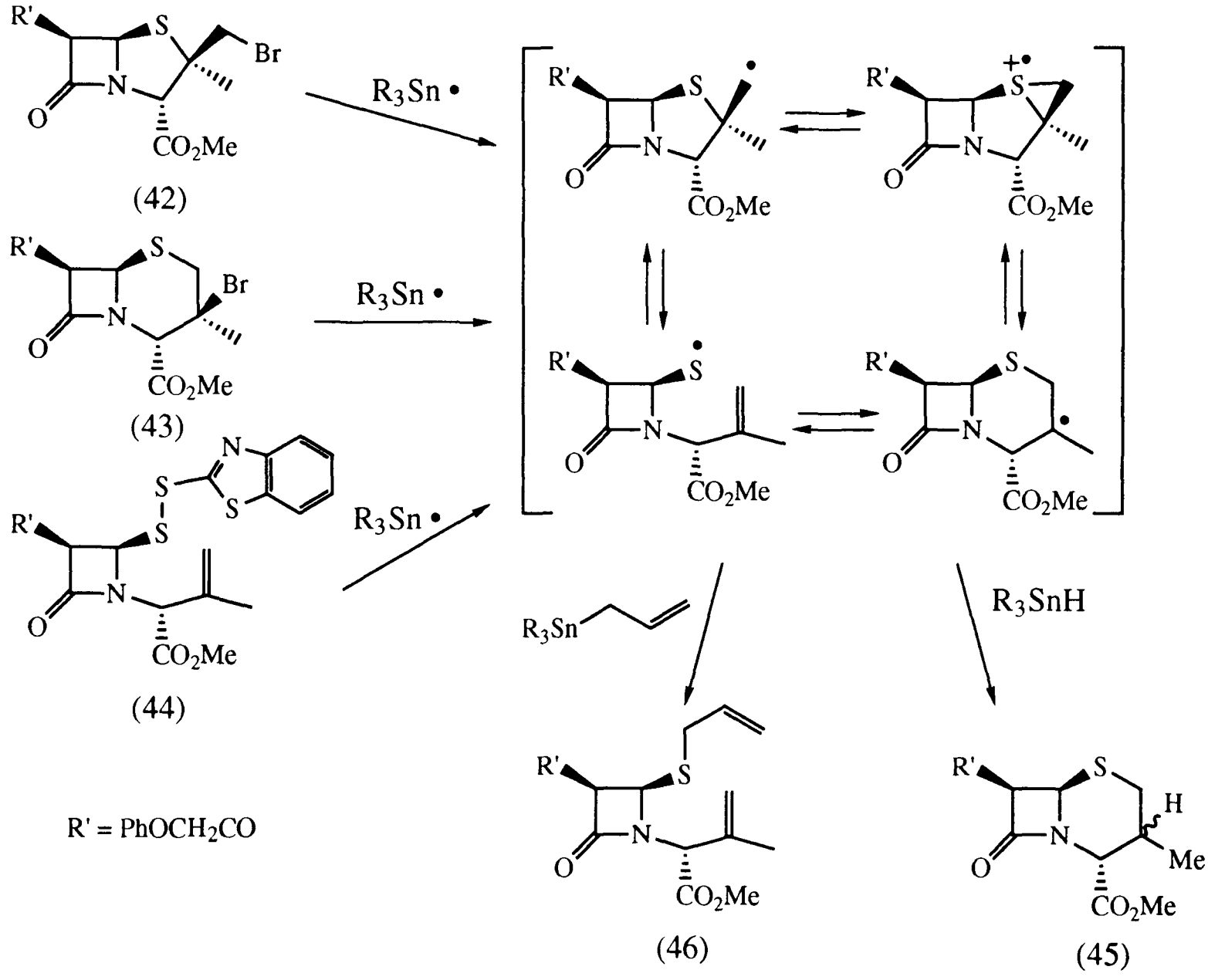
Table 1.5 : Enzymatic conversion of a mixture of (1) and (1d)

Competitive mixed label experiments express differences in the parameter V_{max}/K_m between the labelled and unlabelled substrate. This parameter reflects events which occur only up to, and including the first irreversible step (see appendix A for a detailed discussion of competitive kinetic isotope experiments). The enzymatic discrimination in the case of (1c), and its absence in the case of (1d) indicates that the removal of the two hydrogens is a stepwise and not a concerted process. The observation of a V_{max}/K_m effect only for (1c) further indicates that the hydrogen from the 3β-methyl group is removed in events up to or including the first irreversible event, whereas the removal of the C-3 hydrogen occurs after this event. This type of sequential mechanism points to the existence of at least one enzyme-bound intermediate (41), formed on abstraction of the first hydrogen (Scheme 1.18). The existence of such an intermediate may also explain the difference in product ratio between (1) and (1d); the operation of a primary isotope effect on the abstraction of the C3-H makes the addition of a hydroxyl group more competitive with regard to desaturation (normally being only a minor branching pathway), and this leads to increased formation of the shunt product (40) (Scheme 1.18).



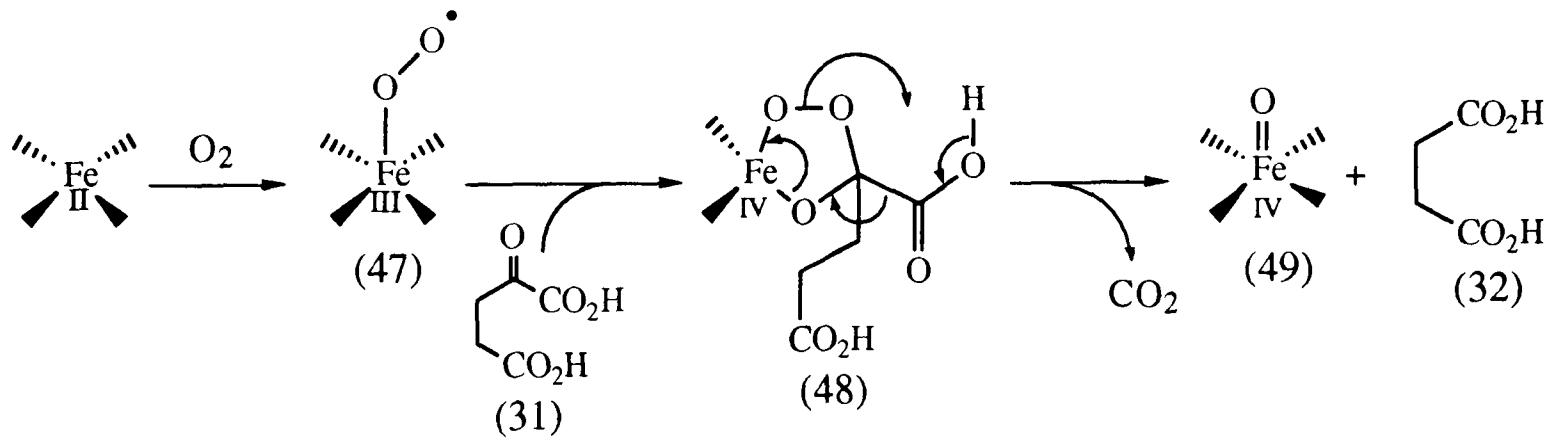
Scheme 1.18

The nature of the enzyme bound intermediate (41) can be envisaged to be either a cation or a radical. The chemical validity of the involvement of radical intermediates in the ring expansion reaction was confirmed through the generation of a penicillin β-methyl radical, which was shown to undergo ring expansion (Scheme 1.19).[121,122] Radical generation from either the bromides (42) or (43), or the disulphide (44), generates an equilibrating pool of radicals, which if trapped with tin hydride give only the cepham (45), or if trapped with allyl tin, the secopenam (46).



Scheme 1.19

The intermediacy of carbon radical species has also been proposed for IPNS (see section 1.2.4), for other α-ketoglutarate dependent dioxygenases (e.g. prolyl hydroxylase[123,124] and γ-butyrobetaine hydroxylase[125]), and also for P450 enzymes[126,127]. In accordance with these oxidative systems, it was envisaged that in DAOC/DACS the entity capable of homolysis of a non-activated carbon-hydrogen bond could be a ferryl species [Fe(IV)=O].[120] This enzyme-bound iron-oxene (49) is possibly generated at the active site by the oxidative decarboxylation of α-KG (31), mediated by the Fe^{II} and dioxygen. Scheme 1.20 depicts a proposed mechanism for the formation of the ferryl species (49).



Scheme 1.20

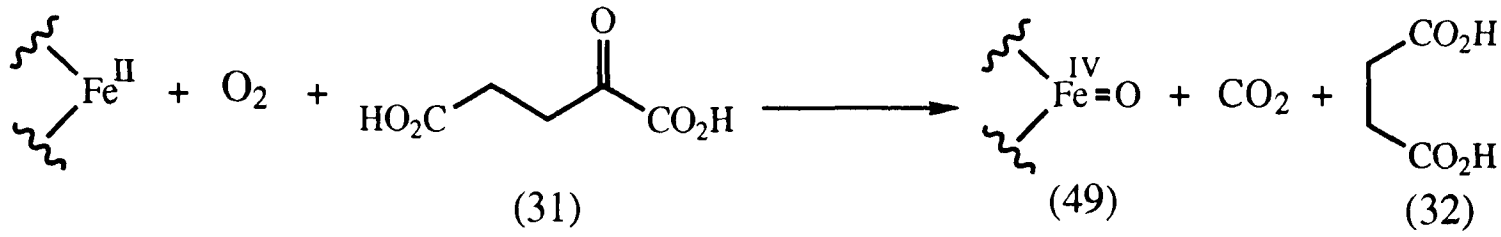
It is proposed that the enzyme bound Fe^{II} binds dioxygen with concomitant electron transfer and oxidation to Fe^{III} to give (47), in a process analogous to the conversion of haemoglobin to oxyhaemoglobin[128] [in the case of prolyl 4-hydroxylase it has been shown that addition of Fe^{II} and α-KG (31) to the enzyme in the absence of ascorbate, leads to the formation of EPR-active Fe^{III} in the active site[129]]. A second electron transfer will produce Fe^{IV} with a peroxide ligand, a species which may be capable of effecting the decarboxylation of α-KG (31), with formation of Fe^{IV}=O (49), succinate (32) and CO_2.

The iron in the iron-oxene (49) is considered to be in the 4+ oxidation state because of a convention of inorganic chemistry which dictates that the electron pair forming a coordination bond is deemed to reside entirely on the ligand, and is not shared with the metal. It is not known whether α-KG (31) is also a ligand for the iron (as has been proposed to be the case in prolyl 4-hydroxylase[124]), or whether it binds to the enzyme before or after dioxygen (for other α-ketoglutarate dioxygenases binding of α-KG appears to occur before binding of O_2 and substrate - see section 1.3.4). The formation of such an iron-oxene intermediate permits the coupling of the exothermic decarboxylation of α-KG to the oxidation of an aliphatic carbon, which has a high endothermic activation barrier.

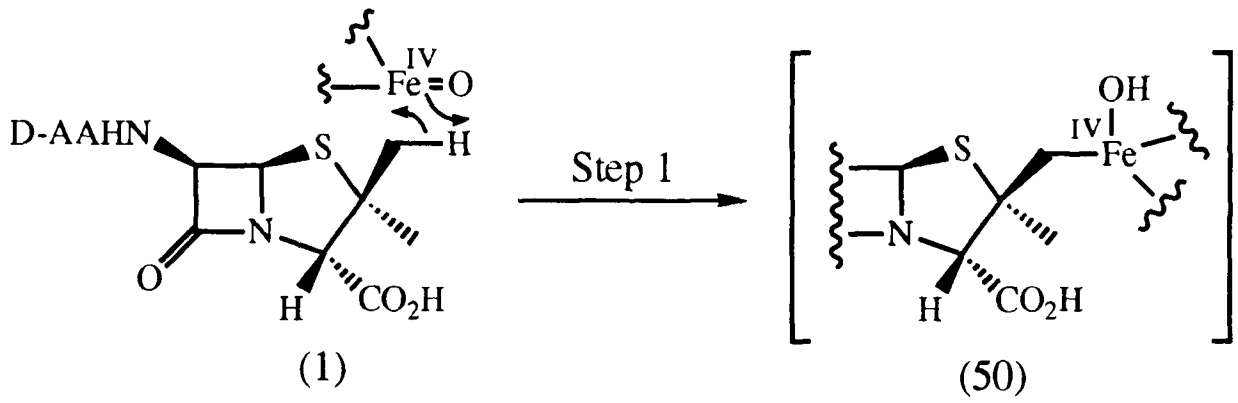
Based on the results described so far, a mechanism was proposed for the ring expansion of penicillin N (1) (Scheme 1.21).[23,120] The first step in the mechanism is probably the irreversible formation of the enzyme reactive species, Fe^{IV}=O (49). It should be noted that for an isotope effect to be observed in the β-lactam substrate after this necessarily irreversible step, it is required that the binding of that substrate is a reversible event even after this step occurs (this reversible binding may occur before and throughout formation of (49), or only after it). If binding of the substrate was irreversible once the iron-oxene was formed, then its formation would be the first irreversible event with respect to the β-lactam substrate as well, and no isotope effect would be observable.

In the proposed mechanism, the first irreversible event with respect to the β-lactam substrate is considered to be the insertion of (49) into a C-H bond of the β-methyl group of pen N (1) (Step 1). It is not known if the enzyme-bound intermediate formed in this step involves a direct C-Fe bond [as in (50)], or if it has a free radical character [as in (51)] (sometimes throughout this thesis only a C-Fe bond will be drawn for convenience). Once the substrate undergoes rearrangement to a ring expanded intermediate, it can either lose a hydrogen (Step 2) with formation of DAOC (2), H_2O and regeneration of Fe^{II}, or it can be hydroxylated (Step 3) with formation of the 3β-hydroxycepham (40) and regeneration of Fe^{II}. Step 3 is normally only a minor pathway, but a deuterium isotope effect expressed on intermediate (51) may cause the rate of the normal pathway (Step 2) to decrease, resulting in increased formation of (40a).

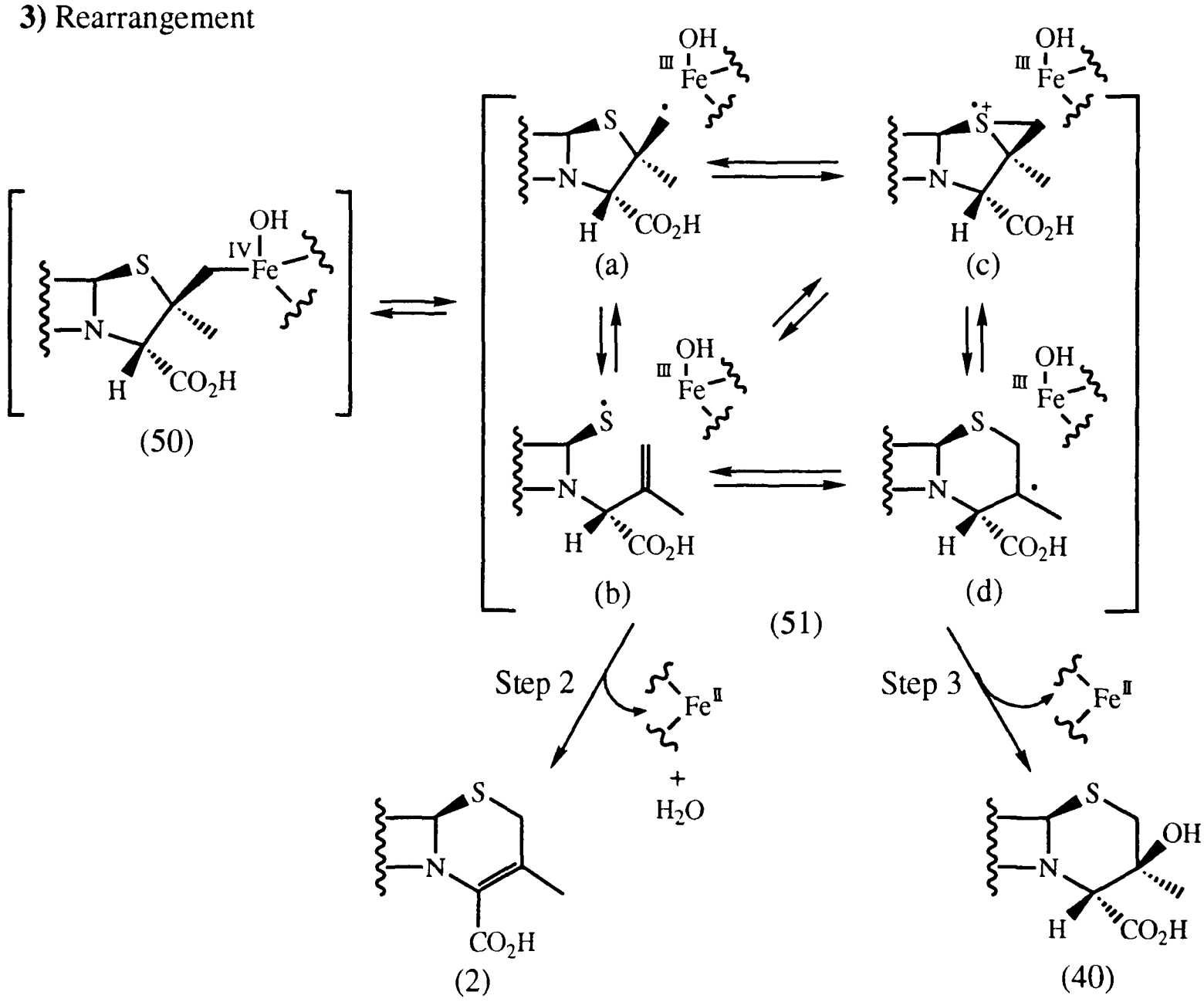
1) Iron-oxene formation



2) Insertion into carbon-hydrogen bond

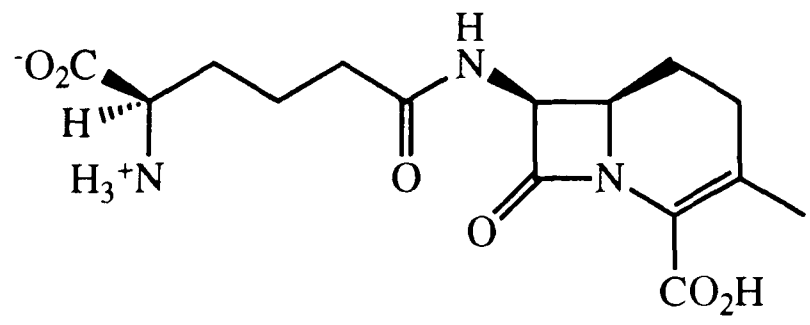


3) Rearrangement



Scheme 1.21

It is interesting to note that the ring expansion results in the loss of stereochemistry at the C-2 position of the cephem ring[115,116], compared to the observed retention of stereochemistry in the hydroxylation of DAOC (2) by DAOC/DACS, as well as hydroxylations catalysed by several other α-ketoglutarate dioxygenases (see section 1.3.4). The loss of stereochemistry at C-2, together with the fact that the major product of ring expansion does not involve addition of an oxygen atom, are the two major differences between DAOC/DACS and most other α-ketoglutarate dioxygenases. It is possible that both these facts are the result of direct involvement of the sulphur in the mechanism of ring expansion. As has been discussed for IPNS (see section 1.2.4), sulphur coordination to the iron possibly results in its preferential transfer to a carbon radical (over the hydroxyl ligand) leading to oxidative cyclisation rather than simple hydroxylation. A similar process may be envisaged to occur in the ring expansion of pen N (1), which would point to the existence of an intermediate like (51b), similar to the one proposed for IPNS. Alternatively, the role of sulphur may be to stabilise the neighbouring β-methyl radical [(51a/c)] (increasing its lifetime thus facilitating racemisation), and to promote rearrangement to the more stable ring expanded radical (51d). This can easily lose the labile hydrogen at C-4 to give a very stable, conjugated product (2). It is thus suggested that it is the involvement of sulphur in the ring expansion reaction that causes the divergence of DAOC/DACS from a simple hydroxylative mechanism to one of oxidative cyclisation (an analogous proposal has been made for the enzyme clavaminic acid synthase[130]). In support of this argument is the fact that the sulphur atom is not involved in the stereospecific hydroxylation of DAOC (2), as would be expected, and was demonstrated by incubation of the carba-DAOC (52) with DAOC/DACS, which resulted in hydroxylation of its C3 methyl group.[131] It would be interesting to test the corresponding carba-penicillin N.



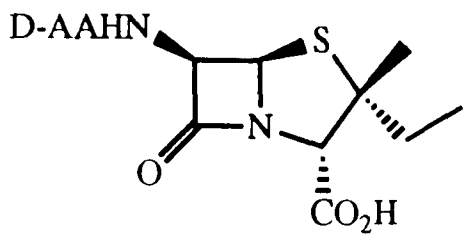
(52)

Another possible explanation for the diversion from hydroxylase to desaturase activity is that it is simply caused by the spatial arrangement between the substrate and the ferryl species, making abstraction of the C-4 acidic proton preferable to hydroxylation once the β-methyl radical has undergone ring expansion (a process that is thermodynamically favourable). It should be noted that DAOC/DACS does form the hydroxylated product (40), but this is usually only a minor product suggesting it is a less favourable pathway than desaturation. However, its formation can be increased through a deuterium isotope effect which makes the hydrogen abstraction step energetically more demanding.

It should be borne in mind that it is not known whether DAOC/DACS has one or two active sites responsible for each reaction (ring expansion and hydroxylation). Structural studies on DAOC/DACS, which have been hampered by its instability, should provide interesting information such as whether there is one or two iron atoms per protein molecule, the general stucture of the active site(s), and how, or if, each of the substrates is coordinated to the iron during catalysis.

Relatively few substrate specificity studies have been carried out for the ring expansion reaction, reflecting the difficulty of chemically modifying the penam nucleus. The penicillin analogues so far prepared contain principally modifications to the methyl groups.[64,132,133,134] These studies have led to the conclusion that modification of the β-methyl group of pen N (1) leads to inactivity, whereas modification of the α-methyl group permits some degree of conversion (see Table 1.6).

Accepted as substrates	Not accepted as substrates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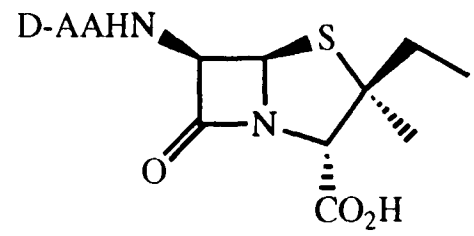
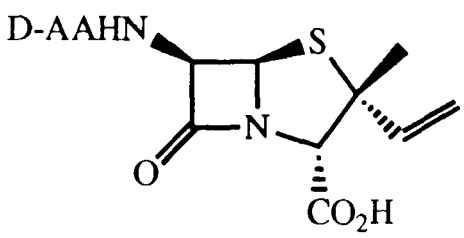

(54)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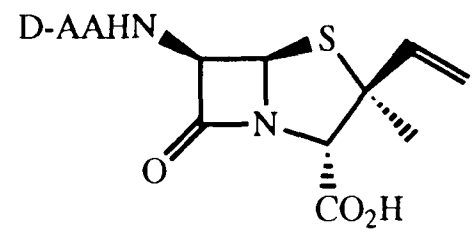
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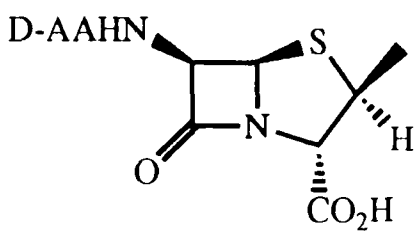
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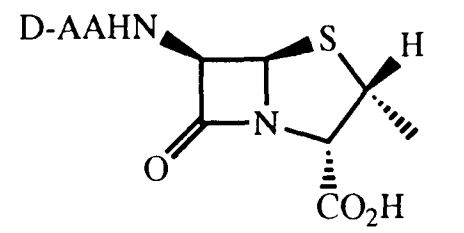
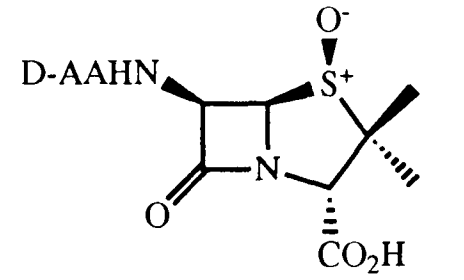
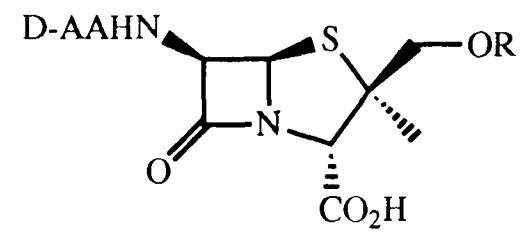
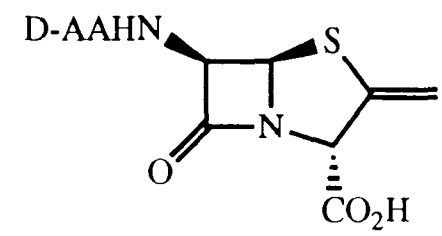

(58)


(38)


(39) : R = H
(59) : R = Me


(60)

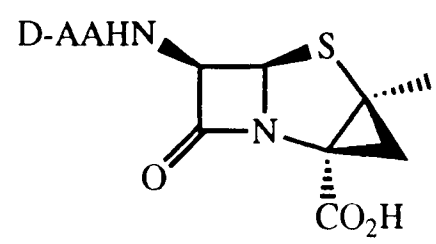
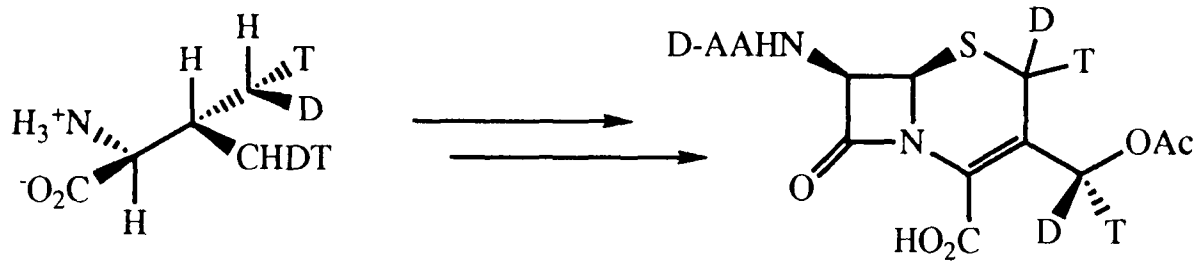

(61)

Table 1.6

1.3.3.2 Mechanistic Studies on the Hydroxylation

In the hydroxylation of DAOC (2) to DAC (3) (Scheme 1.14), DAOC/DACS carries out a simple hydroxylation as is typical of most α-ketoglutarate dioxygenases. This reaction has been shown to be stereospecific by feeding the chirally labelled valine (6f) to intact cells of *C. acremonium* (Scheme 1.22), and thus to occur with complete retention of configuration.[115,135]

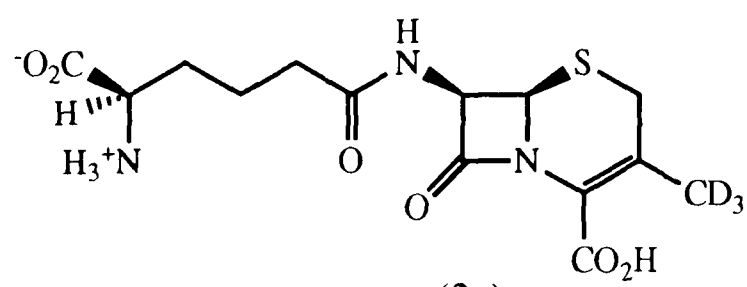


(6f) (12d)

Scheme 1.22

As previously discussed, this result is in agreement with observations for other α-ketoglutarate dioxygenases (see section 1.3.4), but in contrast with the loss of stereochemistry at the C-2 position on the ring expansion of pen N (1). This retention of stereochemistry does not rule out the involvement of a carbon radical at the methyl group, but suggests such a radical would be short lived, recombining quickly with a hydroxyl radical to give (3). Another possible explanation for the observed retention of stereochemistry of such a carbon radical is that steric constraints of the active site may prevent its rotation which would result in racemisation.

Competitive kinetic isotope experiments have also been carried out for the hydroxylation reaction.[136] For these experiments the trideuterated DAOC (2a) was synthesised and incubated with DAOC/DACS in an approximately 1 : 1 mixture with unlabelled DAOC (2).



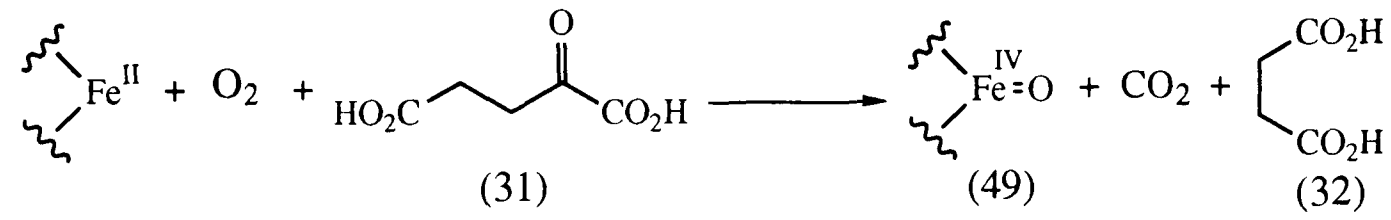
(2a)

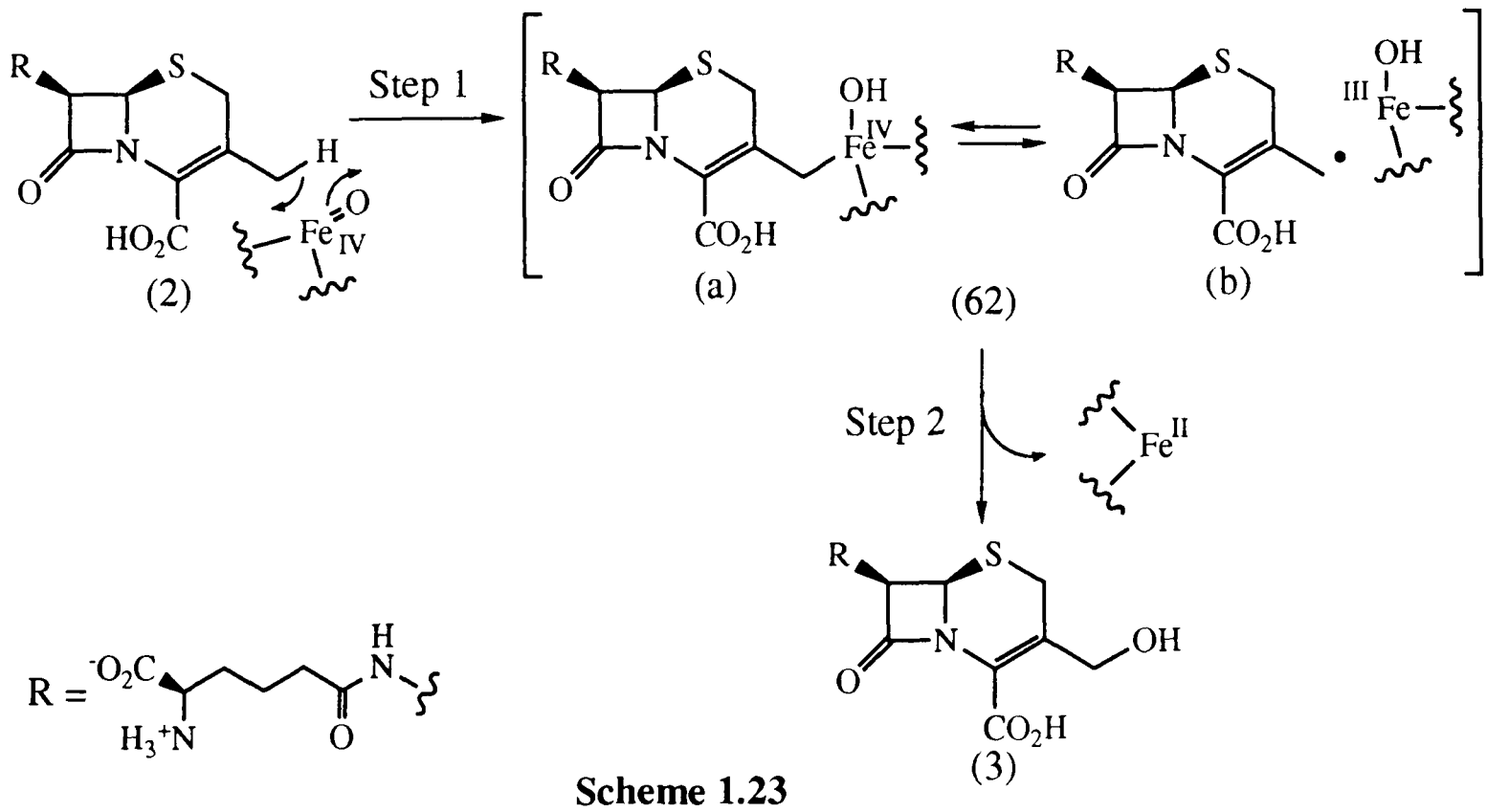
The unconverted starting material was recovered from aliquots of the incubation mixture taken at different times, and the ratio of (2) to (2a) determined by mass spectrometry (Table 1.7).

Expt.	% Conversion	Ratio (2) : (2a)
1	0	1.02 : 1
	55	0.71 : 1
2	0	0.84 : 1
	20	0.77 : 1
	35	0.70 : 1
	60	0.52 : 1

Table 1.7 : Enzymatic conversion of a mixture of (2) and (2a)

The results show that there is a V_{max}/K_m isotope effect for the hydroxylation, which means that DAOC/DACS discriminates between labelled (2a) and unlabelled DAOC (2). A control experiment with denatured enzyme showed no conversion of either (2) or (2a). These results indicate that the first irreversible event in the hydroxylation is abstraction of a hydrogen from the methyl group, and led to the proposal of a mechanism for this reaction (Scheme 1.23).

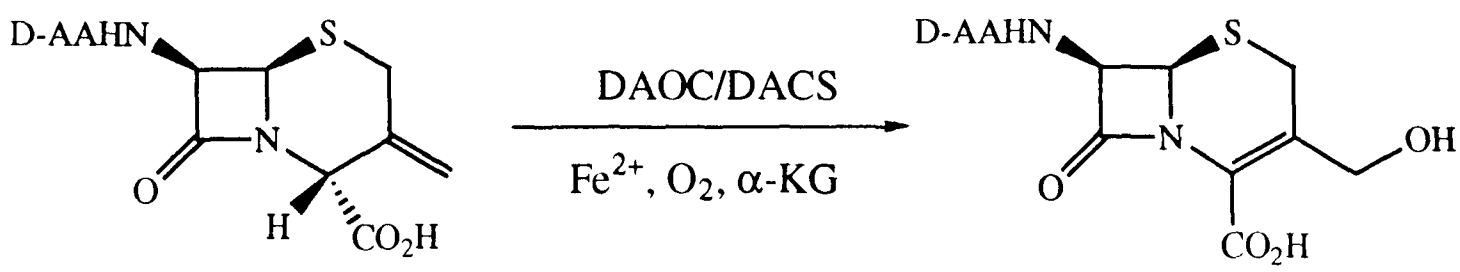




Scheme 1.23

As in the case of the ring expansion, the substrate DAOC (2) must be in reversible binding throughout formation of the ferryl species (49), or the isotope effect at the C3 methyl group of DAOC would not be observed. The first irreversible event with respect to the substrate is thus proposed to be the insertion of (49) into a C-H bond of the methyl group of (2) (Step 1). In this step an intermediate is formed which can be thought of as being a free radical (62b), or as involving a direct C-Fe bond (62a). Reductive elimination from (62) regenerates Fe^{II} and gives the product DAC (3) (Step 2). If the intermediate (62) is indeed a free radical [as (62b)] it must be prevented from rotation, a process which would destroy the stereospecificity observed for this reaction.

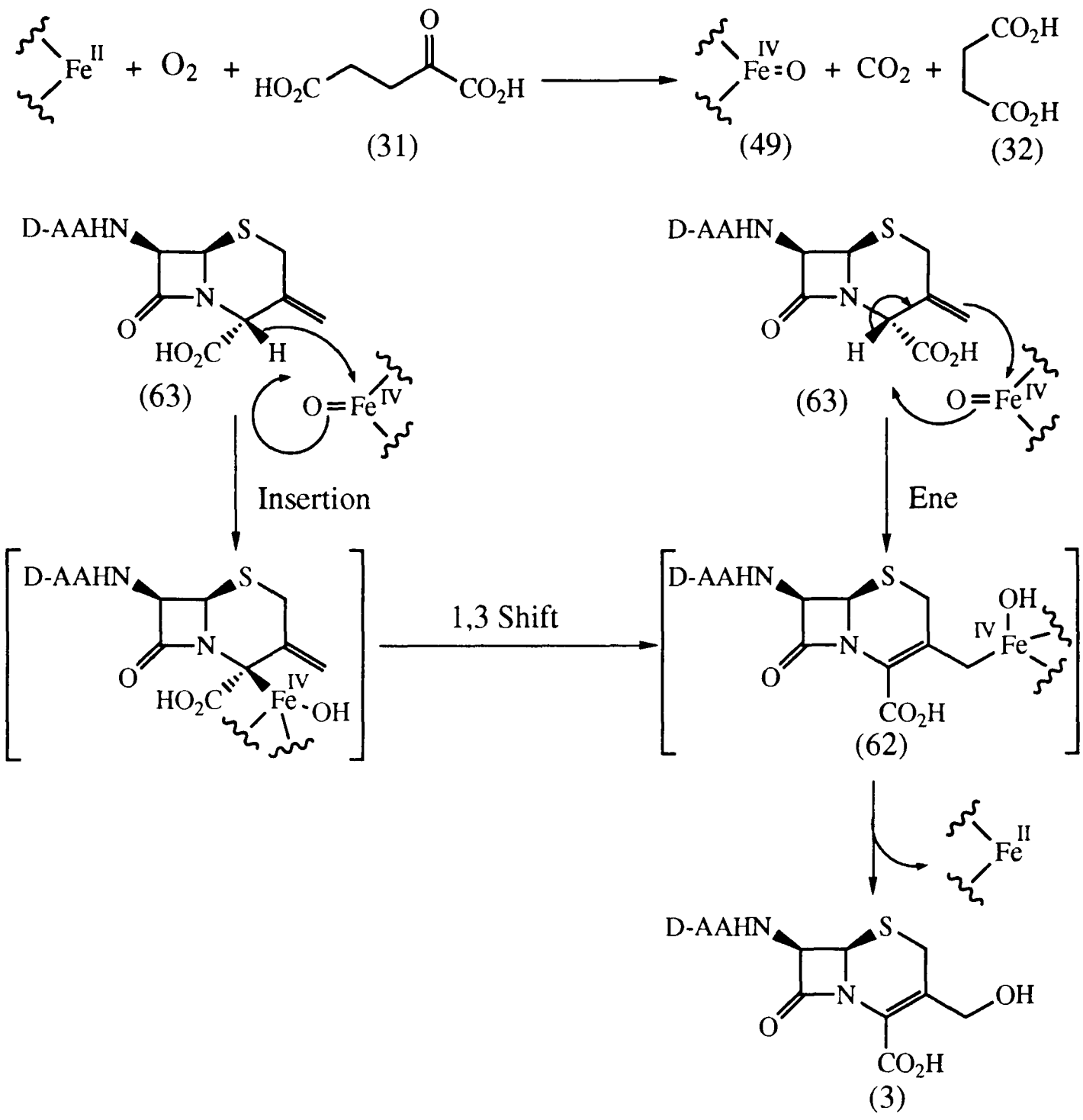
Several DAOC analogues have been prepared to study the substrate specificity of DAOC/DACS with respect to the hydroxylation reaction. Of these, the most significant is the exomethylene cephalosporin C (63), which was initially proposed[137] to be a possible intermediate in the ring expansion of pen N (1) to DAOC (2). However, it was found that (63) was not converted to DAOC (2), and furthermore, that it was a powerful inhibitor of the pen N(1) to DAOC (2) conversion.[138] It was later observed that exomethylene cephalosporin C (63), in contrast to exomethylene penicillin N (60) (Table 1.6), did serve as a substrate for DAOC/DACS, but that it was converted directly to DAC (3) (Scheme 1.24).[139,140] Again, no traces of DAOC (3) were found in this reaction, and so the possibility that (63) was first isomerised to DAOC (2) followed by conversion to DAC (3), was dismissed.



(63) (3)

Scheme 1.24

Two alternative mechanisms have been proposed for the conversion of exomethylene (63) into DAC (3) (Scheme 1.25).[141]

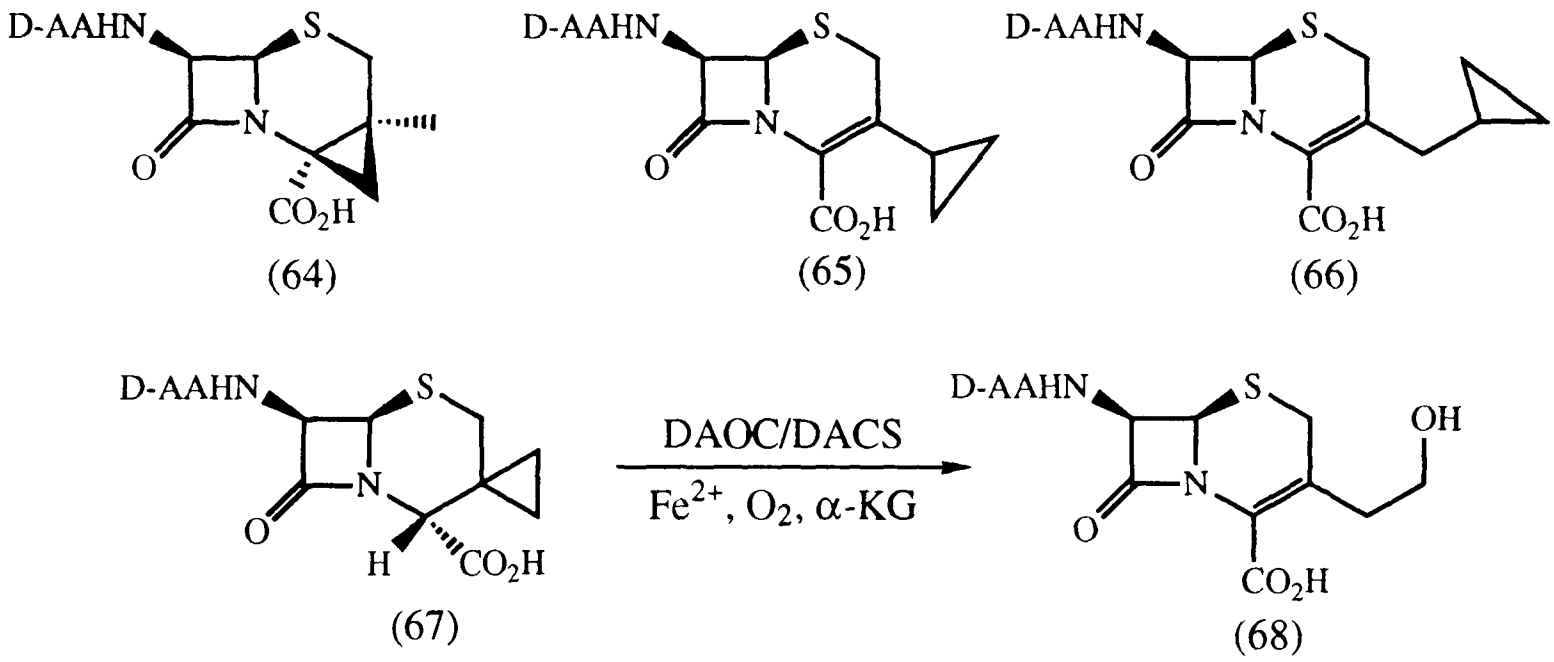


Scheme 1.25

One possible mechanism involves insertion of the iron-oxene (49) into the C4-H bond, followed by rearrangement to give intermediate (62) which is also proposed to be involved in the hydroxylation of DAOC (2). An alternative mechanism is an ene-type reaction leading directly to (62) from which the product DAC (3) is formed by reductive elimination, regenerating Fe^{II}.

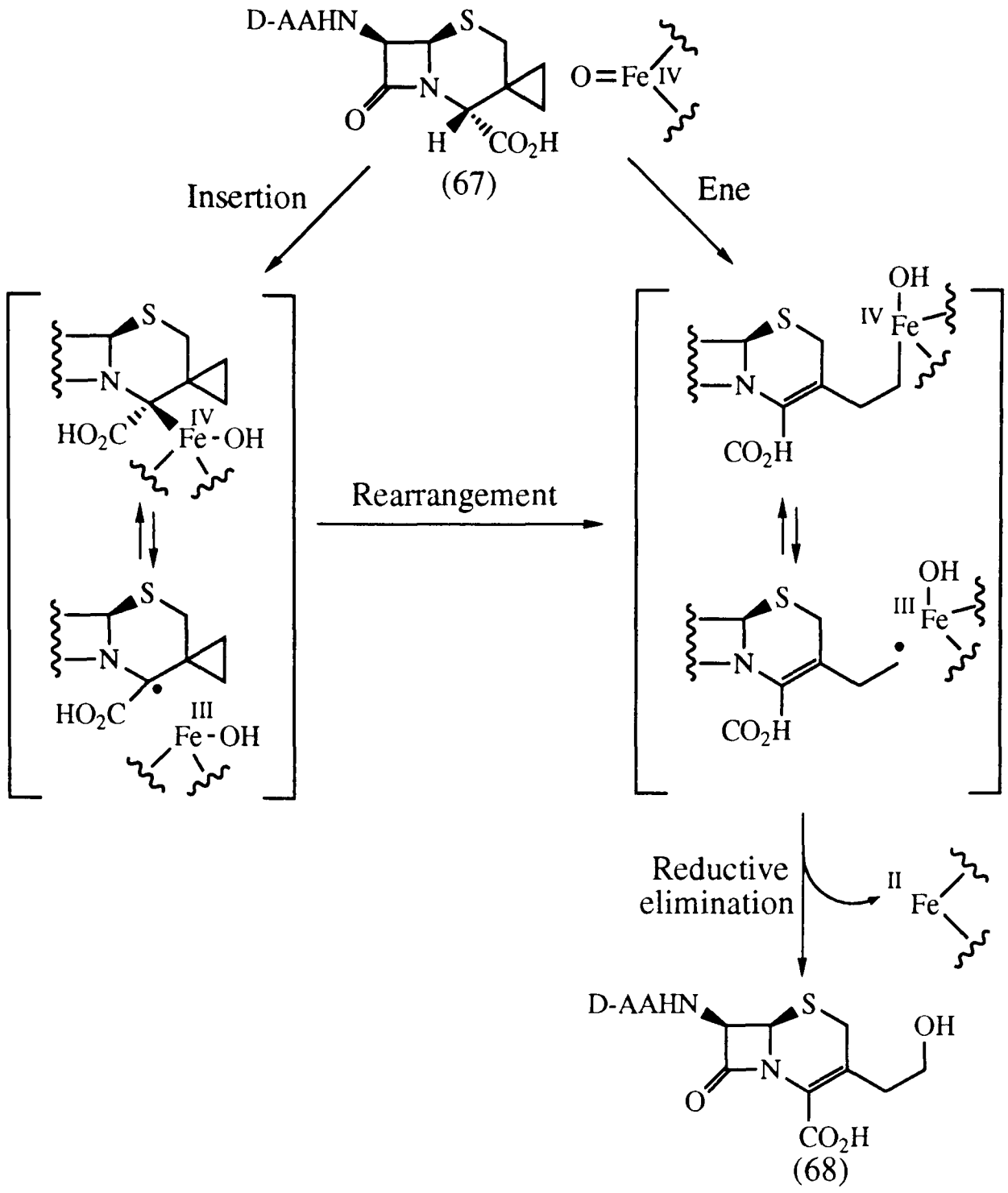
The fact that exomethylene (63) is hydroxylated to DAC (3) and is also a powerful inhibitor of the ring expansion of pen N (1), seems to suggest that DAOC/DACS has only one active site capable of both ring expansion and hydroxylation. Competitive inhibition studies of the effects of pen N (1) on hydroxylation, and of DAOC (2) on ring expansion should provide some more information in this respect. However, the possibility of two active sites capable of binding both substrates but only of converting one of them would not be possible to rule out.

To test the hypothesis of involvement of a free radical intermediate in the hydroxylation of DAOC (2), several cyclopropyl analogues [(64) to (67)] have been synthesised.[142,143] Of these, only compound (67) was accepted by DAOC/DACS and converted to the hydroxylated product (68) (Scheme 1.26).



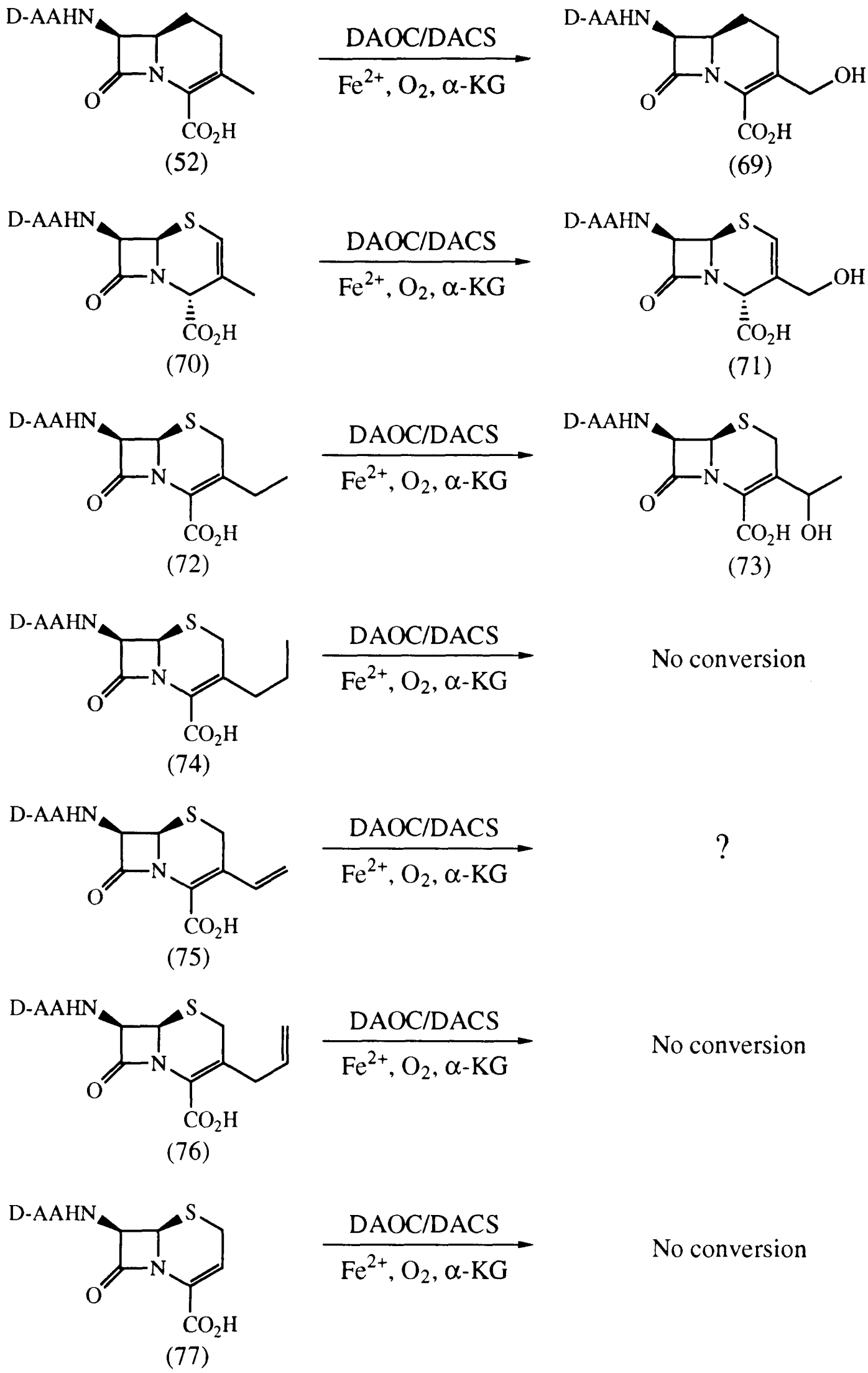
Scheme 1.26

Compounds (64) to (66) may not have been accepted as substrates because of steric constraints imposed by the active site. Two mechanisms have been proposed for the conversion of (67) to (68) (Scheme 1.27).[142]



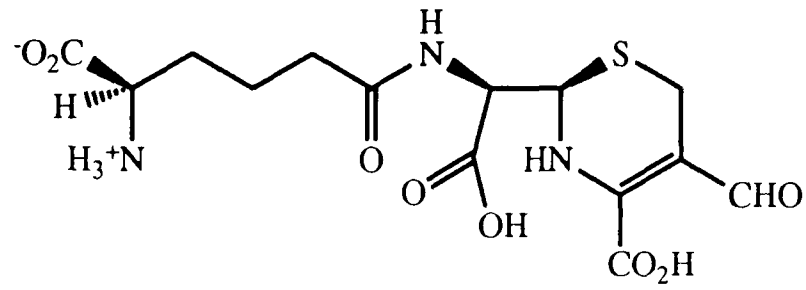
Scheme 1.27

Several other analogues have been tested with DAOC/DACS (Scheme 1.28). The carba-DAOC (52) was hydroxylated to (69)[131], showing that the sulphur atom is not involved in the hydroxylation process. The Δ2-cephem (70) was also hydroxylated to (71)[108,133], and the 3-ethylcephem (72) to (73)[144], providing evidence for some tolerance in steric variations. However, the 3-*n*-propyl-cephem (74) was not accepted as a substrate.[143] The 3-vinylcephem (75) is apparently oxidised by DAOC/DACS, but the product has not yet been identified.[144] Finally, the compounds (76)[143] and (77)[133] were not accepted as substrates by DAOC/DACS.


D-AAHN
DAOC/DACS
Fe2+, O2, α-KG
OH
CO2H
(52)
(69)
(70)
(71)
(72)
(73)
(74)
(75)
(76)
(77)
No conversion
?

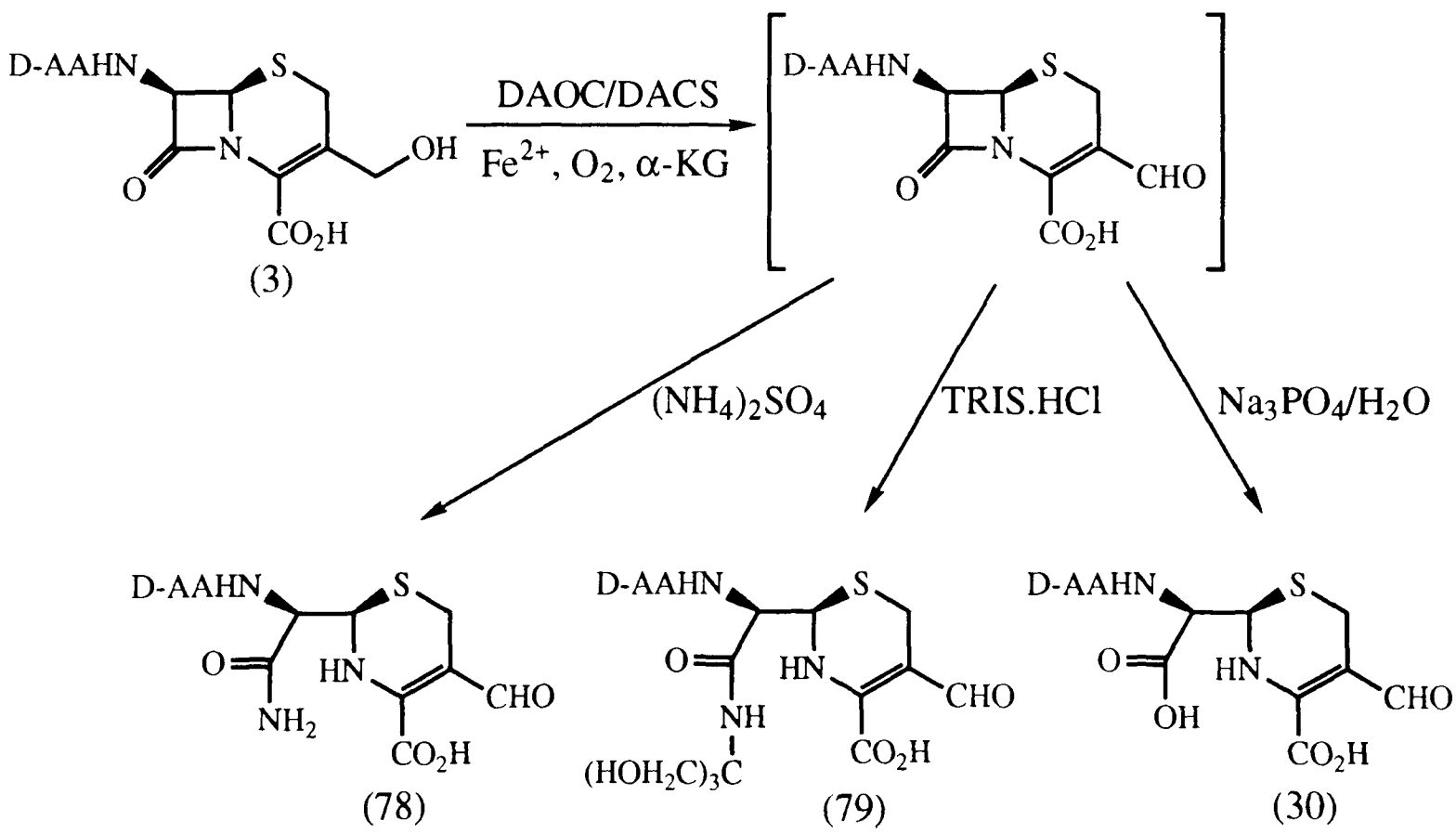
Scheme 1.28

Following reports that a mutant of *C. acremonium* blocked in the acetylation of DAC (3) accumulated the formylcephem (30)[86], it was envisaged that DAOC/DACS might be responsible for the oxidation of the hydroxyl group of DAC (3) to an aldehyde, which would greatly increase the reactivity of the β-lactam ring making it very susceptible to hydrolysis.



(30)

It has recently been reported that indeed DAOC/DACS oxidises the hydroxyl group of DAC (3) to an aldehyde.[145] The isolated product again has a ring opened β-lactam but the use of different buffers in the incubation mixture results in β-lactam cleavage by different nucleophiles (Scheme 1.29). This result indicates that DAOC/DACS is capable of oxidising a hydroxyl group to an aldehyde, a characteristic only observed for one other α-ketoglutarate dependent dioxygenase, thymine 7-hydroxylase[146].



Scheme 1.29

1.3.4 α-Ketoglutarate Dependent Dioxygenases

The class of α-ketoglutarate dependent dioxygenases has been recognised only relatively recently, and so far includes a limited number of enzymes. This fact is probably due to the difficulty in detecting and isolating these proteins, which is caused by their apparent instability, need of multiple cofactors for activity, and lack of a tracer group such as a coloured heme. However, an increasing number of these dioxygenases have been recognised in the primary and secondary metabolism of animals, plants and microorganisms, suggesting they are more important and widespread than was originally suspected.

It was considered of interest to include in this thesis a short description of the most relevant work published on α-ketoglutarate dioxygenases, with a view to a better understanding of their general mode of catalysis, as well as to put the results obtained with DAOC/DACS into context.

All α-ketoglutarate dioxygenases need Fe^{II}, α-ketoglutarate (α-KG) (31) and dioxygen for activity, and catalyse the oxidation of the substrate with concomitant decarboxylation of α-KG (31) to succinate (32) and CO_2. They are classified as "intermolecular dioxygenases" as one atom of oxygen from O_2 is inserted into each of the substrates.[147] Some of the known α-ketoglutarate dioxygenases include :

i) Prolyl 4-hydroxylase[92,148], prolyl 3-hydroxylase[149] and lysyl hydroxylase[92,150], all of which are involved in the hydroxylation of selected proline or lysine residues in nascent chains of procollagen.

ii) γ-Butyrobetaine hydroxylase[151,152] and ε-N-trimethyllysine hydroxylase[153], both of which are involved in the biosynthesis of carnitine, a quaternary amino acid with important biological functions in lipid metabolism.

iii) Thymine 7-hydroxylase[146], pyrimidine deoxyribonucleoside 2'-hydroxylase[154] and deoxyuridine 1'-hydroxylase[155], which are involved in pyrimidine and nucleoside metabolism.

iv) Aspartyl β-hydroxylase[156] which is responsible for the hydroxylation of specific aspartate residues in several vitamin-K dependent proteins.

v) Clavaminic acid synthase[111,157] which is involved in the biosynthesis of clavulanic acid, a bicyclic β-lactam compound that is a potent inhibitor of β-lactamases.

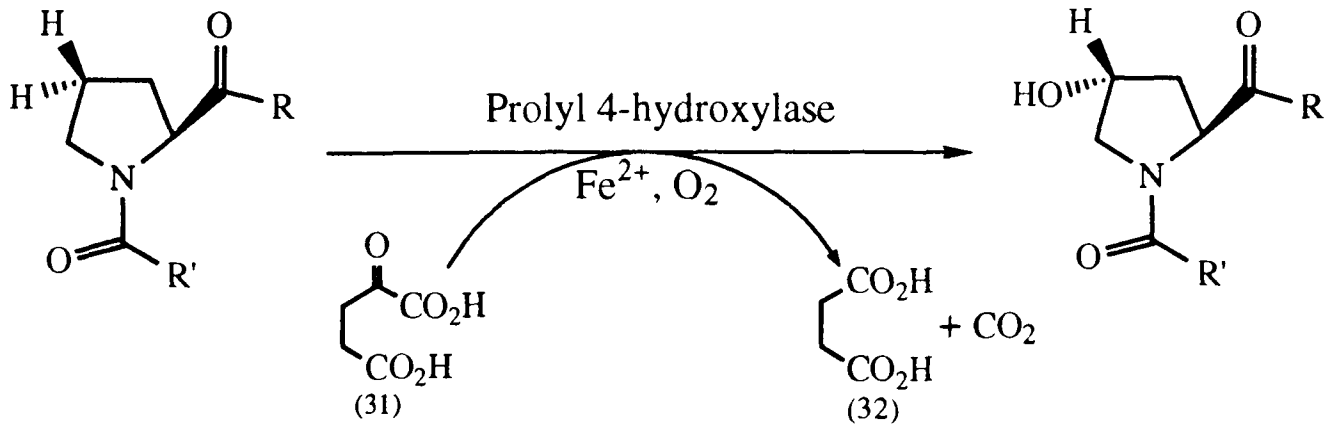
vi) Flavone synthase I[112] and (2S)-flavanone 3-hydroxylase[158], which are involved in the biosynthesis of flavonoids.

vii) Hyoscyamine 6β-hydroxylase[159] involved in the biosynthesis of tropane alkaloids.

viii) Several enzymes involved in the biosynthesis of gibberellins.[160,161]

Of these enzymes, the ones that have been studied in most detail are prolyl 4-hydroxylase, γ-butyrobetaine hydroxylase, thymine 7-hydroxylase and clavaminic acid synthase.

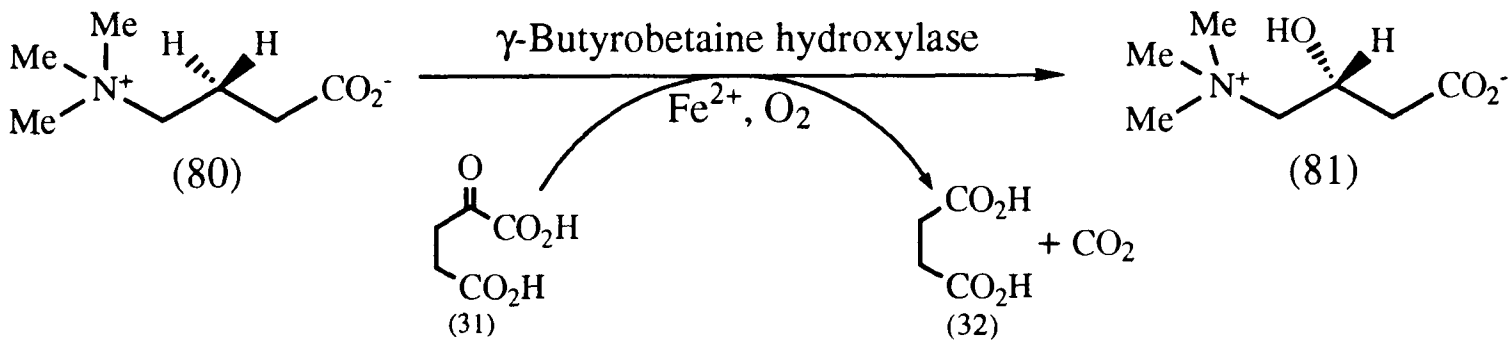
Prolyl 4-hydroxylase is a very important enzyme involved in the biosynthesis of collagen, whose excess formation is typical of fibrotic diseases. Collageneous proteins are characterised by the non-covalent association of three chains in a rod-like triple helix. These triple helical rods are resistant to proteolysis, making the body unable to degrade excessively deposited collagen. The stability of the triple helices is known to be dependent on the extent of hydroxylation of specific proline residues by prolyl 4-hydroxylase (Scheme 1.30). Insufficiently hydroxylated chains do not form triple-helical domains, remaining gelatinous rather than collageneous proteins, and are therefore susceptible to normal catabolism. This central role of prolyl 4-hydroxylase makes it a potential target for therapeutic modulation of excessive collagen formation, and has prompted extensive studies on the enzyme (for reviews see refs. 124, 148 and 150).



Scheme 1.30

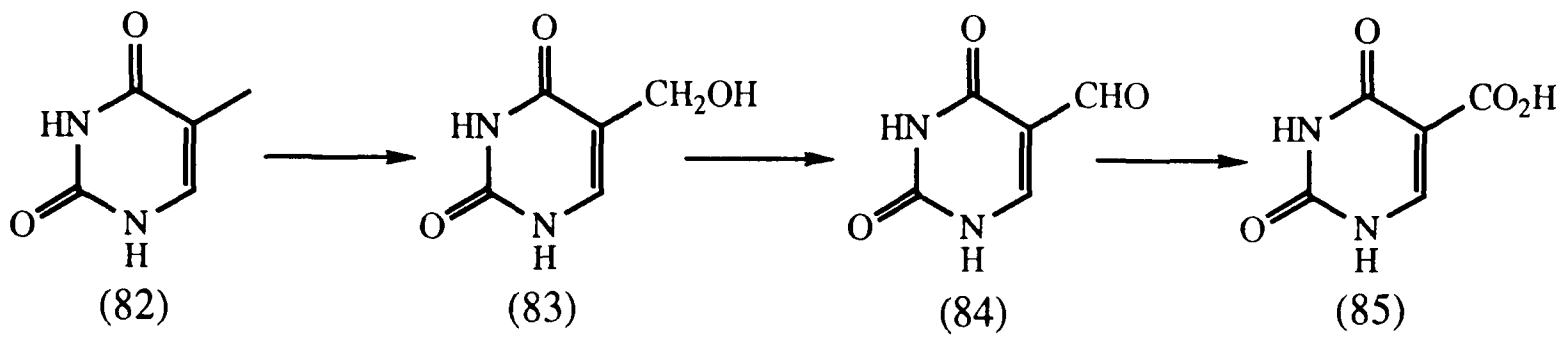
Prolyl 4-hydroxylase is a tetramer ($\alpha_2\beta_2$) with a molecular weight of about 240,000, and consists of two different types of inactive monomers with molecular weights of about 64,000 (α unit) and 60,000 (β unit). The tetramer protein appears to have two active sites as it contains two Fe^{II} atoms and two peptide and α-KG (31) binding sites. This enzyme has been purified to homogeneity from several animal and plant tissues. The minimum sequence requirement for hydroxylation is fulfilled by an X-Pro-Gly sequence, but the enzyme has higher affinity for long chain peptides.

γ-Butyrobetaine hydroxylase catalyses the hydroxylation of γ-butyrobetaine (80) to carnitine (81) (Scheme 1.31), a vitamin that functions as a carrier molecule in the transport of medium and long chain fatty acids across the inner mitochondrial membrane. This enzyme has been purified from several animal tissues, and from a species of *Pseudomonas.*[162] Homogeneous γ-butyrobetaine hydroxylase from calf-liver is a homodimer with two 46,000 subunits, and the enzyme from *Pseudomonas* is a heterodimer with subunit molecular weights of 37,000 and 39,000.



Scheme 1.31

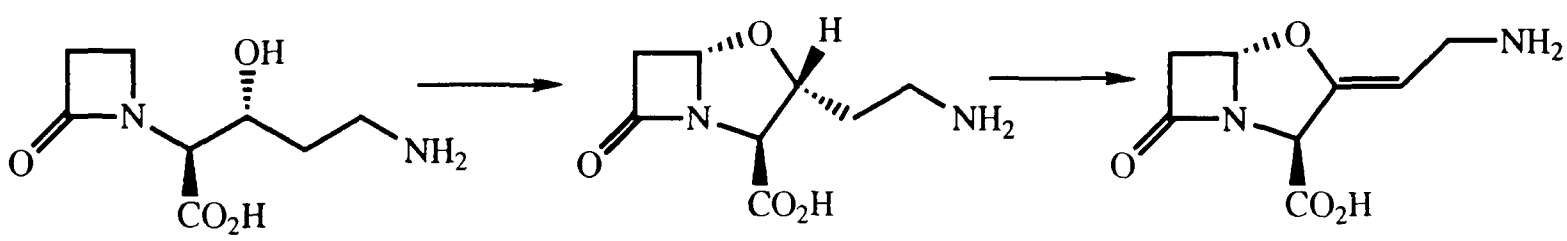
Thymine 7-hydroxylase is a rather unusual α-ketoglutarate dioxygenase, in that it carries out three sequential oxidations. It is a trifunctional enzyme that hydroxylates thymine (82) to 5-hydroxymethyluracil (83), oxidises this to 5-formyluracil (84), and thence to uracil-5-carboxylic acid (85) (Scheme 1.32).[146] All three steps have a specific requirement for Fe^{II}, α-KG (31) and O_2. Thymine 7-hydroxylase has been purified from several fungal microorganisms. The enzyme from *Rhodotorula glutinis* is apparently a monomer with molecular weight 42,700.[163]



(82) (83) (84) (85)

Scheme 1.32

Clavaminic acid synthase carries out two sequential oxidations converting proclavaminic acid (86) to clavaminic acid (88), through dihydroclavaminic acid (87) (Scheme 1.33), with each step requiring Fe^{II}, α-KG (31) and O_2.[164] This enzyme has been purified to homogeneity from *Streptomyces clavuligerus*, and shown to be a monomer of molecular weight 47,000.[157]



(86) (87) (88)

Scheme 1.33

As can be seen from the four enzymes described, the class of α-ketoglutarate dioxygenases comprises proteins that are apparently quite different (at least in terms of molecular weight and quaternary structure). However, many of these enzymes have similar characteristics, which suggests that they operate through analogous mechanisms.

α-Ketoglutarate dioxygenases are non-hemic ferrous enzymes. Fe^{III} does not permit activity unless a reductant is included, which is capable of converting Fe^{III} to Fe^{II}. All the enzymes have a specific requirement for the addition of Fe^{II}, which means the proteins do not incorporate enough iron for activity and suggests loose binding for the metal. Studies with prolyl 4-hydroxylase indicated that the binding of Fe^{II} was in thermodynamic equilibrium.[165]

All the enzymes use stoichiometric amounts of α-KG (31) and O_2, and decarboxylate α-KG (31) with formation of succinate (32) and CO_2. The need for α-KG is very specific. These enzymes also require ascorbate for activity although this is not used in stoichiometric amounts. The need for ascorbate does not seem to be completely specific as in some cases other reductants have been shown to support activity, although at reduced levels. In the cases of prolyl 4-hydroxylase[166] and lysyl hydroxylase[167], hydroxylation in the absence of ascorbate was observed at a high rate for a few seconds followed by inactivation of the enzyme, but interestingly the activity could be restored upon addition of ascorbate. In separate studies it was shown that addition of α-KG (31) to prolyl 4-hydroxylase loaded with Fe^{II} led to the oxidation of the iron to EPR-active Fe^{III}, with concomitant loss of activity by the enzyme.[129] Again, activity could be restored by addition of ascorbate. These studies indicate that the most probable role of ascorbate is to keep the iron in an active ferrous form.

Dithiothreitol (DTT) and catalase have also been reported to stimulate activity. For prolyl 4-hydroxylase thiol groups have been implicated at the active site as sulfhydryl specific reagents led to inactivation of the enzyme[168,169], which could be prevented by binding of α-KG (31) or substrate[170]. Sulfhydryl reagents have also been shown to inhibit γ-butyrobetaine hydroxylase.[171] It seems probable that the role of DTT is to keep these thiol groups in an active reduced form, and in the case of prolyl 4-hydroxylase DTT could indeed reverse the inhibition caused by the sulfhydryl reagents[169].

An interesting characteristic of α-ketoglutarate dioxygenases is that they are capable of decarboxylating α-KG (31) in the absence of the substrate, or in the presence of substrate analogues that can not be hydroxylated. For prolyl 4-hydroxylase this uncoupled turnover occurred in the absence of substrate at a rate of about 1/80 of that observe in its presence.[172] However, when competitive inhibitors were present the ratio of rates increased to about 10%.[173] Uncoupled decarboxylation in the absence of substrate, or non-hydroxylatable substrate has also been observed for lysyl hydroxylase[167], γ-butyrobetaine hydroxylase[174], thymine 7-hydroxylase[175], DAOC/DACS[144] and clavaminic acid synthase[157]. Increased

levels of uncoupled decarboxylation in the presence of substrate inhibitors have also been reported for γ-butyrobetaine hydroxylase[174] and thymine 7-hydroxylase[176]. These results suggest that Fe^{II}, α-KG (31) and O_2 can bind at the enzyme active site in the absence of substrate permitting decarboxylation of α-KG (31), but that this process is apparently facilitated by a possible conformational change induced by the occupancy of the substrate binding site.

Experiments with prolyl 4-hydroxylase and lysyl hydroxylase indicated that the uncoupled turnover of α-KG (31) occurred with stoichiometric consumption of ascorbate.[177] This suggests that the uncoupled turnover occurs with oxidation of the iron which needs to be reduced back in order to restore activity. It is possible that some degree of uncoupled decarboxylation also occurs in the presence of the substrate, and this would explain the less than stoichiometric need for ascorbate, as the coupled reaction regenerates Fe^{II} and should not require a reductant. Although in the case of most of the enzymes the production of CO_2 is almost completely coupled to hydroxylation, it has been shown for γ-butyrobetaine hydroxylase from rat liver that 100% coupling only occurred in the presence of K^+ ions, and that in its absence decarboxylation of α-KG (31) (in the presence of substrate) exceeded hydroxylation by a factor of 3.[178] In addition, when deuterated [2,2,3,3,4,4,-$^2H_6$]-γ-butyrobetaine was used as a substrate, the ratio of decarboxylation to hydroxylation rose to 7.5.[179] These results seem to support the idea that uncoupled decarboxylation can occur in the presence of the substrate.

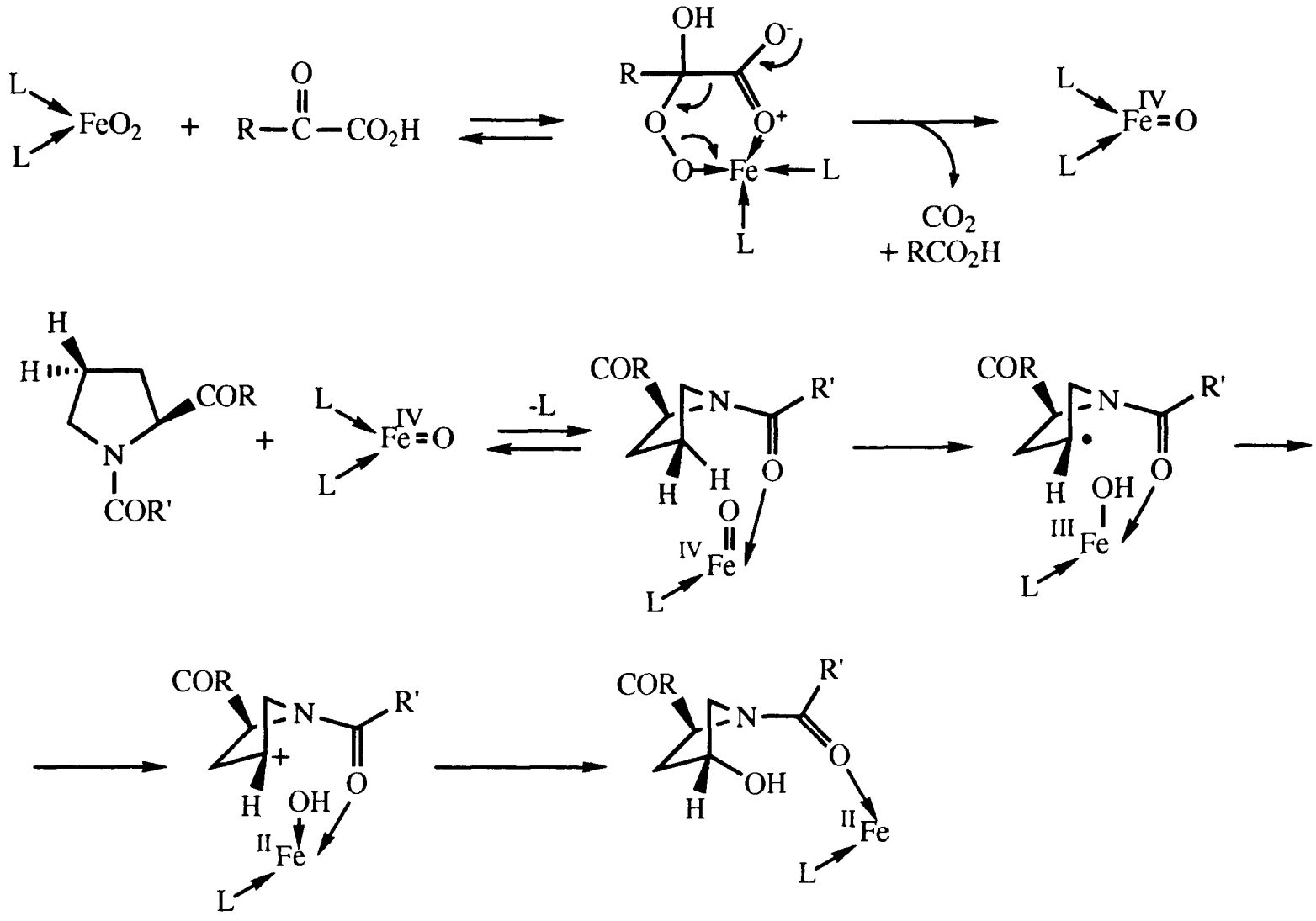
Competitive inhibition studies carried out with prolyl 4-hydroxylase suggest the enzyme has different binding sites for Fe^{II}, α-KG (31) and the substrate.[172] In addition, kinetic studies with prolyl 4-hydroxylase[164], thymine 7-hydroxylase[176,180], lysyl hydroxylase[181] and clavaminic acid synthase[157], have indicated that these enzymes follow a sequential mechanism in which all reactants must bind before any products are released. It is not clear if this sequential mechanism is completely, or only partially ordered, and it is possible that this varies from one enzyme to another. The apparent order of binding in these enzymes is Fe^{II}, followed by α-KG (31), O_2 and substrate (or substrate and O_2). Results with γ-butyrobetaine hydroxylase also showed that α-KG (31) binds before

γ-butyrobetaine.[125] In the case of prolyl 4-hydroxylase, it was found that the iron cannot dissociate after binding of α-KG (31). The binding order of ascorbate is not clear, but probably occurs after one or more products have been released. Product release also appears to be ordered.

A sequential type mechanism has been supported by observations that several of these enzymes can form inactive dead-end complexes with either substrate or α-KG (31), when either of these are added to the enzyme before the other reactants. Both substrates are non-competitive inhibitors of several of these enzymes suggesting that once α-KG (31) is bound, Fe^{II} cannot associate with the enzyme, and similarly that if the substrate binds before Fe^{II} and α-KG (31), one or both of them are prevented from entering the active site.

Most of the oxidations performed by α-ketoglutarate dependent dioxygenases occur at non-activated carbons, and reactive intermediates must be involved in these processes. The first mechanism for these reactions was proposed for prolyl 4-hydroxylase, by Lindstedt *et al.*[182], and Cardinale *et al.*[183], who suggested that a carbanion of the prolyl substrate would react with dioxygen to give a peroxide anion intermediate, which would then react with α-KG (31) to give the hydroxylated substrate, succinate and CO_2. This mechanism is not in accordance with the observed uncoupled decarboxylation of α-KG (31), and was criticised by Hamilton[184] who questioned how a hydroperoxide derivative of an unreactive aliphatic substrate could be initially formed. He proposed a sequential mechanism in which decarboxylation of α-KG (31) by molecular oxygen would lead to the formation of peroxysuccinic acid, which would be responsible for hydroxylation. However, peroxysuccinic acid was shown not to be able to replace α-KG (31)[183], and furthermore aliphatic hydrocarbons are usually stable to oxidation by such mild peracids.

Siegel[123] was the first to propose that in prolyl 4-hydroxylase, a high energy species capable of reacting with a non-activated substrate would be an iron(IV)-oxene complex, formed on decarboxylation of α-KG (31) mediated by Fe^{II} and O_2 (Scheme 1.34). The iron-oxene species would abstract a hydrogen from the substrate forming a secondary carbon radical and a Fe^{III} hydroxide complex. Electron transfer from the substrate to the iron would generate a carbonium ion and Fe^{II}, and direct hydroxide transfer onto the cation would produce the product.



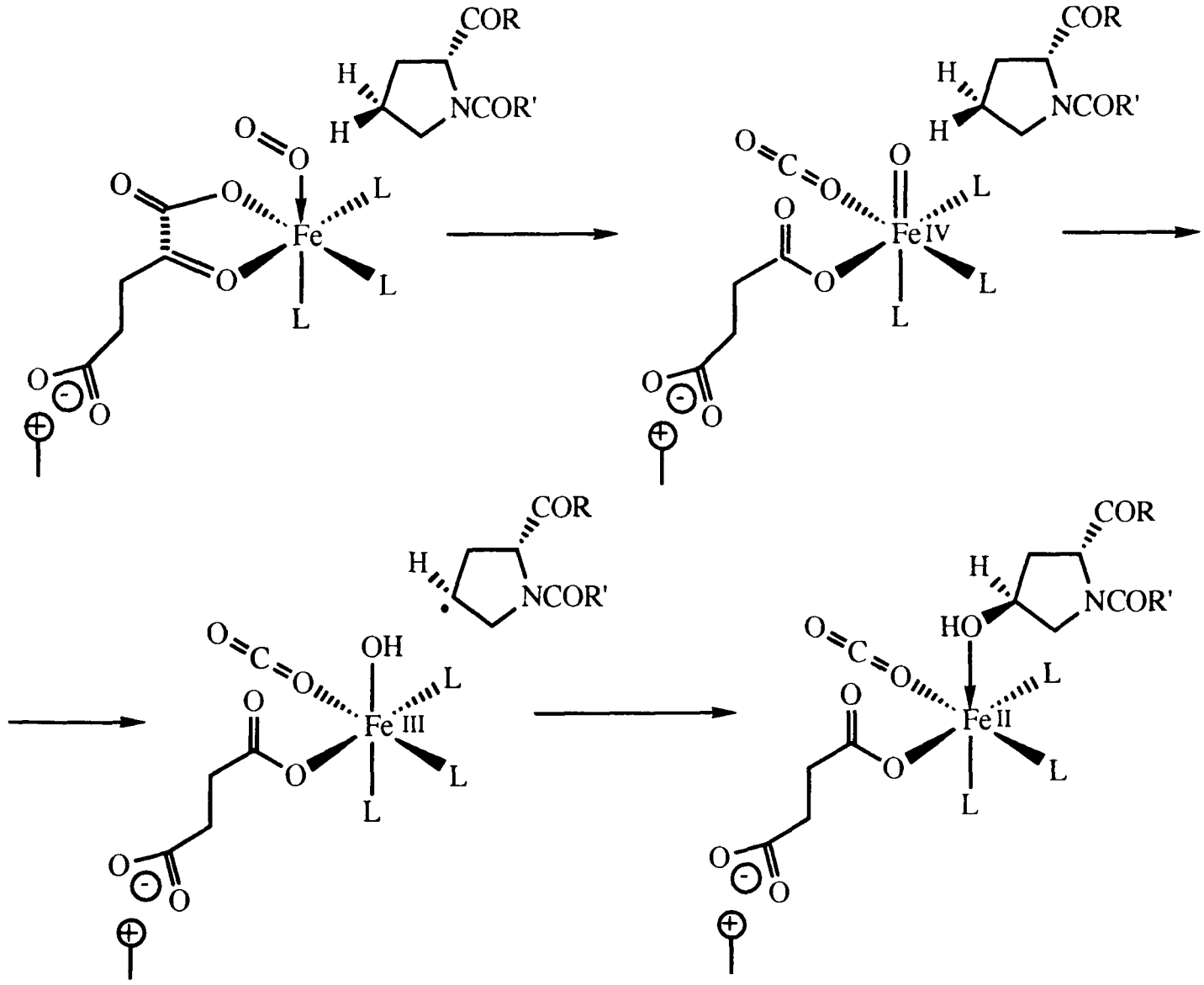
Scheme 1.34

An iron(IV)-oxene had previously been proposed to explain the observed stereoselectivity of the oxidation of cyclohexanol to *cis*-1,3-cyclohexanediol by Fenton's reagent (Fe^{2+}/H_2O_2).[185] In addition, iron-oxene intermediates are generally accepted as being involved in the reactions of P-450 enzymes, peroxidases and other oxygenases.[128]

The mechanism proposed by Siegel for prolyl 4-hydroxylase was later revised and expanded by Günzler *et al.* who ruled out carbocation involvement in the reaction.[124] Günzler proposed a detailed model of the active site and mechanism of prolyl 4-hydroxylase that could explain all the experimental observations, and is still used today as a working model for further investigation[186]. This mechanism proposes there are separate binding sites for Fe^{II}, α-KG (31) and substrate (Scheme 1.35). The iron has an octahedral sphere of coordination with three ligands being supplied by amino acid residues of the protein. α-KG (31) is bound through the C-5 carboxyl group to a positively charged residue on the enzyme and is coordinated to the iron through the C-1 carboxyl group and C-2 carbonyl group. The dioxygen molecule is also bound to the iron through one of the

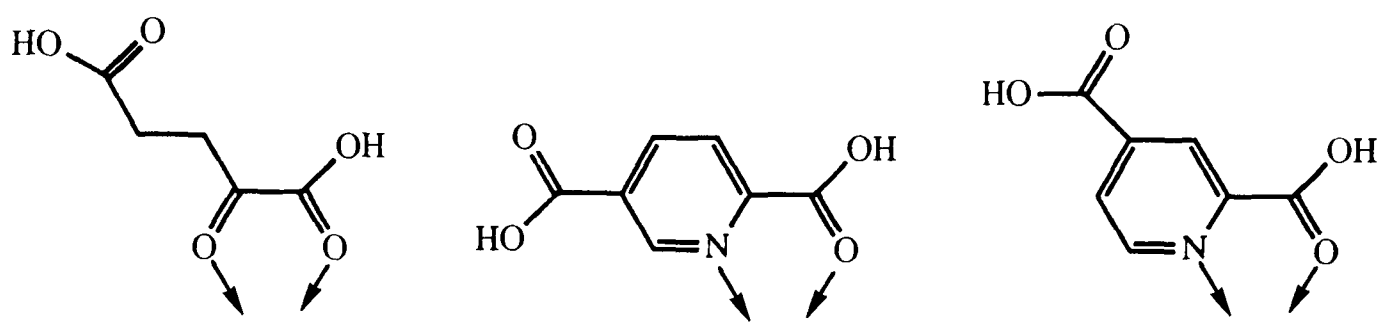
oxygen atoms, at an angle of 120-135°. The O_2 molecule is restricted from movement by the topology of the active site and is orientated so that the iron-bound oxygen atom is close to the substrate, and the other is located above the α-KG carbonyl group. The substrate is not coordinated to the iron and binds to the enzyme at a subsite near the catalytic centre, but closer to the enzyme surface. The spatial accessibility imposed by the protein determines the temporal sequence of coordination to the iron : α-KG (31) is the first to bind in a pocket remote from the enzyme surface, followed by O_2 and the substrate.

The coordination of α-KG (31) to the iron reduces the electron density of the carbonyl C2, and facilitates its decarboxylation by the oxygen molecule. The decarboxylation generates succinate and CO_2 which remain enzyme-bound, and an iron-oxene species. This species then carries out the homolytic abstraction of a hydrogen from the substrate, followed by rapid recombination of the hydroxyl and carbon radicals thereby regenerating Fe^{II}. Dissociation of the hydroxyl product opens the enzyme pocket and allows release of CO_2 and succinate.



Scheme 1.35

This mechanistic hypothesis led to the successful prediction that α-KG analogues which retained the characteristics necessary for binding (the possibility of forming bidentate coordination to the iron and a negative group orientated to the carboxyl binding site) but were unable to decarboxylate should be competitive inhibitors of prolyl 4-hydroxylase. The best inhibitors of this type are pyridine 2,5-dicarboxylate (89), pyridine 2,4-dicarboxylate (90)[187], and 3,4-dihydroxybenzoate (91)[188] (Scheme 1.36). These compounds are also known to inhibit other α-ketoglutarate dioxygenases like γ-butyrobetaine hydroxylase[189], DAOC/DACS[190], hyoscyamine 6β-hydroxylase[159], flavone synthase I[112] and (2S)-flavanone 3-hydroxylase[158].



The arrows indicate the possible iron coordination points

Scheme 1.36

A mechanism involving an iron(IV)-oxene species is now generally accepted as operating for all α-ketoglutarate dioxygenases. The formation of this species can explain many of the results obtained with these enzymes, including the uncoupled decarboxylation of α-KG (31). It is thought that this uncoupled decarboxylation leads to the normal generation of a ferryl species which in the absence of the substrate requires another

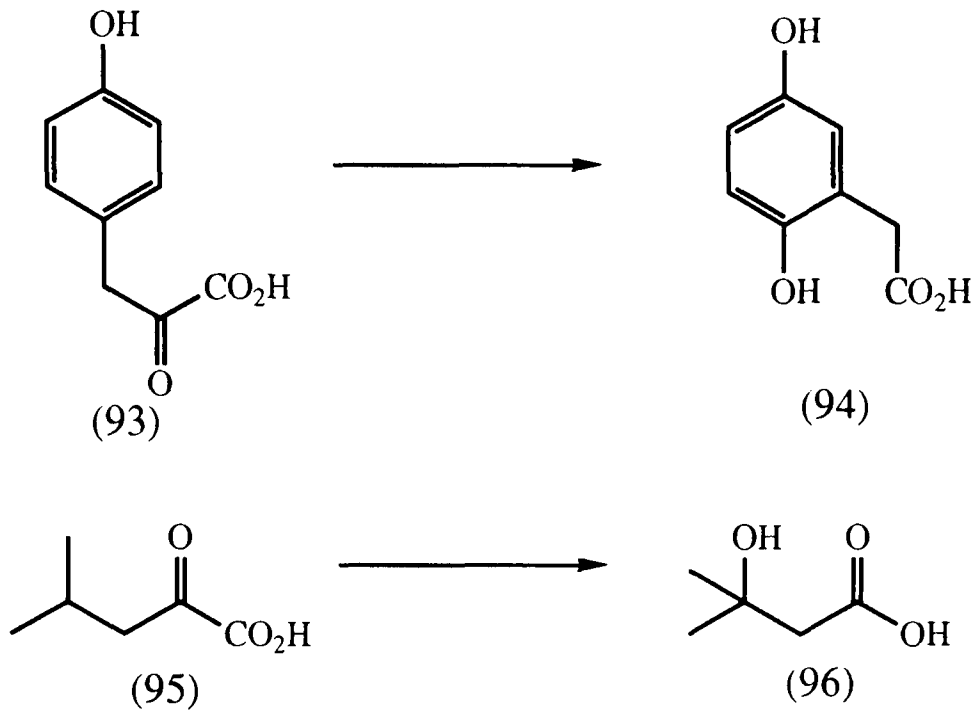
reductant to convert the iron back to Fe^{II}, itself needed to continue the catalytic cycle. This role is fulfilled by ascorbate (92) which probably binds to the iron at the same coordination site as α-KG (31) (Scheme 1.36).

The involvement of carbon radicals in the reactions performed by this class of enzymes was supported by the fact that the hydroxylation of γ-butyrobetaine (80) by γ-butyrobetaine hydroxylase exhibits both primary and secondary isotope effects[125], indicating that the carbon involved is completely sp^2 hybridised in the transition state. This result is consistent with the formation of a carbon radical or a carbonium ion, but enzymological precedents favour a homolytic reaction, and in addition carbocation generation has been ruled out in ferryl mediated hydroxylations of aliphatic carbons[127]. Isotope effects have also been observed for DAOC/DACS (see section 1.3.3) and for clavaminic acid synthase[191]. As was previously discussed for DAOC/DACS, the fact that an isotope effect is observed after the irreversible formation of the iron-oxene, indicates that binding of the substrate is reversible throughout these events and up until abstraction of the first hydrogen from the substrate occurs.

The reactions catalysed by prolyl 4-hydroxylase[192], γ-butyrobetaine hydroxylase[193], clavaminic acid synthase[157] and the hydroxylation of DAOC by DAOC/DACS[115,135], all occur with complete retention of stereochemistry. This has not been interpreted as being contrary to the formation of a carbon radical, but rather as indicating that this radical is not free to epimerise and reacts rapidly to form the product.

Two other enzymes should be mentioned as they are thought to operate by a similar mechanism to α-ketoglutarate dioxygenases. They are *p*-hydroxyphenylpyruvate dioxygenase[92,194] which converts *p*-hydroxyphenylpyruvate (93) to homogentisate (94) (Scheme 1.37), and α-ketoisocaproate dioxygenase[195,196] which converts α-ketoisocaproate (95) to β-hydroxyisovalerate (96). Both these enzymes require Fe^{II}, O_2, ascorbate, and a thiol reagent (e.g. DTT) for catalysis. They are the only two examples so

far known of α-keto acid dependent dioxygenases for which a single substrate incorporates both the α-keto acid from which the iron(IV)-oxene is probably generated, and the carbon to be hydroxylated.



Scheme 1.37

On a more general note, it has been suggested that formation of iron-oxene intermediates is a common attribute of all iron-containing oxygenase enzymes, and that this reactive species can be chanelled to perform several types of difficult transformations.[128] If this is the case, α-ketoglutarate dioxygenases may just be considered as another member of a larger family, and a metabolic alternative to better studied systems like P-450 enzymes.

This thesis describes work aimed towards an investigation of the mechanism of deacetoxycephalosporin C/deacetylcephalosporin C synthase (DAOC/DACS), which is a very interesting α-ketoglutarate dioxygenase that provides evidence for different modes of reactivity of the iron-oxene species.

CHAPTER 2

The Conversion of 3-Exomethylene Cephalosporin C

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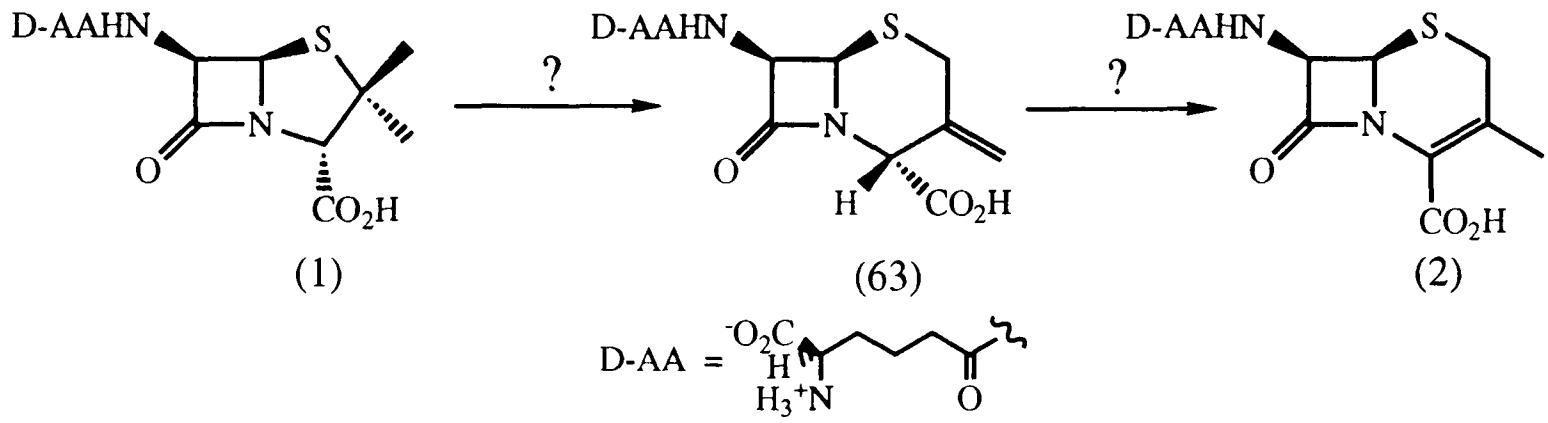
The Conversion of 3-Exomethylene Cephalosporin C

2.1 Introduction

In 1980 Baldwin *et al.* proposed the 3-exomethylene cephalosporin C (63) as a possible intermediate in the conversion of penicillin N (1) to deacetoxycephalosporin C (DAOC) (2) (Scheme 2.1).[137]



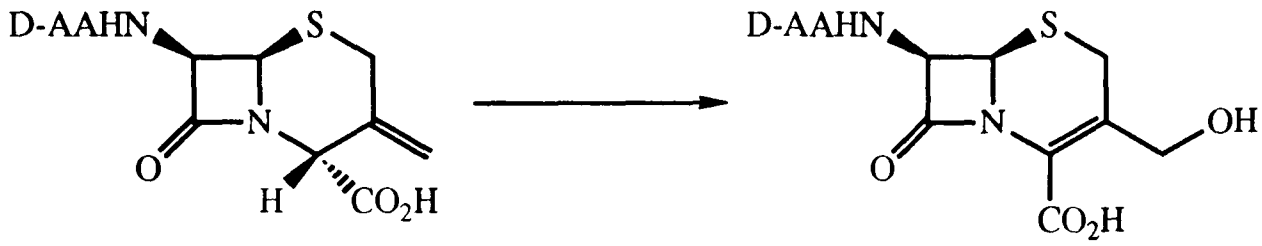
(1) (63) (2)

Scheme 2.1

This hypothesis was subsequently tested by the synthesis of (63) and its incubation with a cell-free extract of *C. acremonium.*[138] It was found that not only was the exomethylene (63) not converted to DAOC (2), but also that it acted as a powerful inhibitor of the conversion of (1) to (2), and so the possible intermediacy of (63) was ruled out.

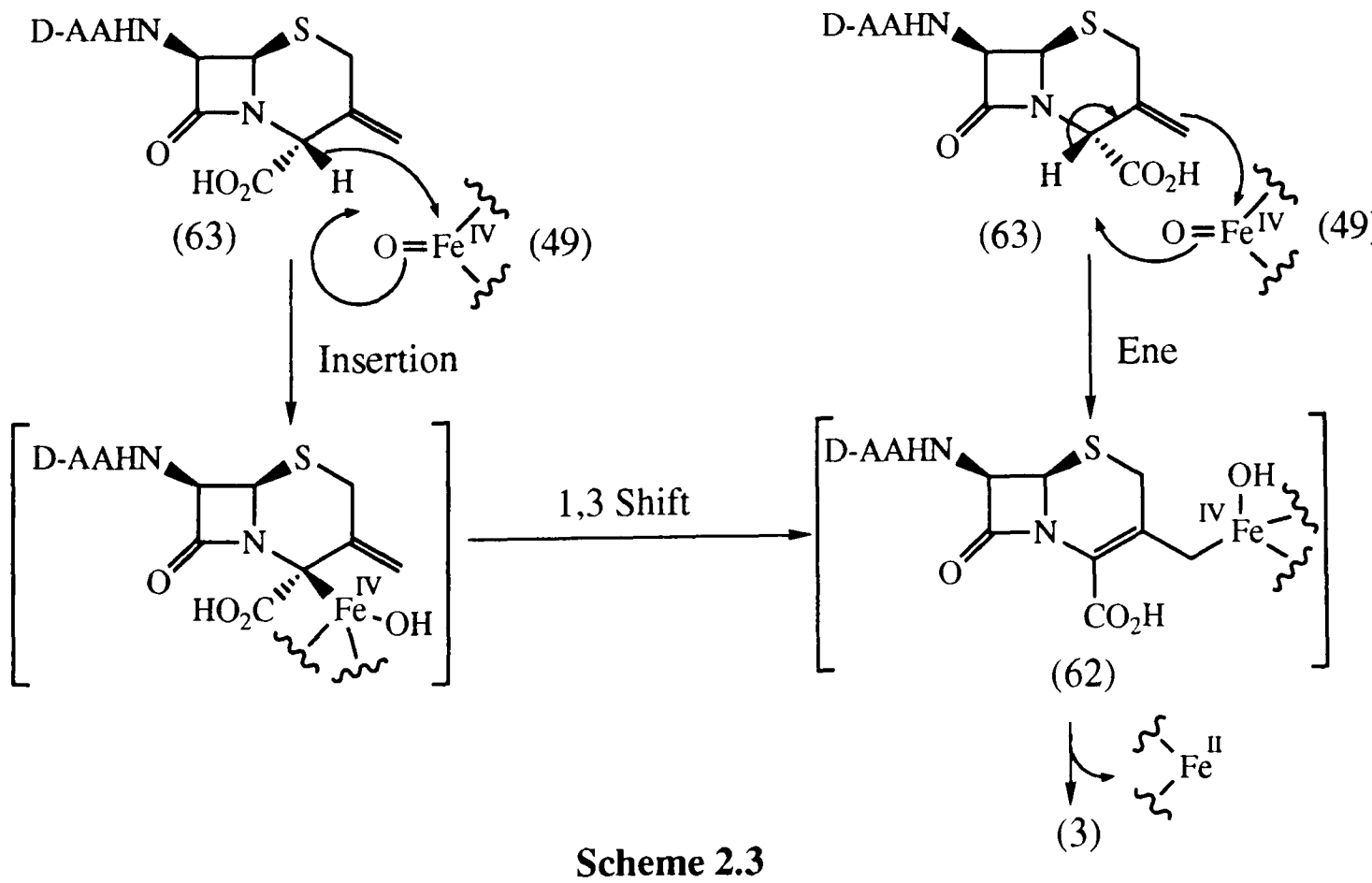
However, it was later reported that similar cell-free extracts of *C. acremonium* were capable of converting (63) directly to deacetylcephalosporin C (DAC) (3) (Scheme 2.2).[139] As it had previously been shown that (63) was not converted to DAOC (2), and its presence was not detected in the incubation mixtures, it was concluded that the conversion of (63) to (3) was a direct enzymatic process which did not involve the intermediacy of (2). When purified DAOC/DACS became available it was shown that this was the enzyme responsible

for the conversion of exomethylene (63) to DAC (3), and also that the new oxygen atom in (3) originated at least in part from dioxygen.[140,141]



Scheme 2.2

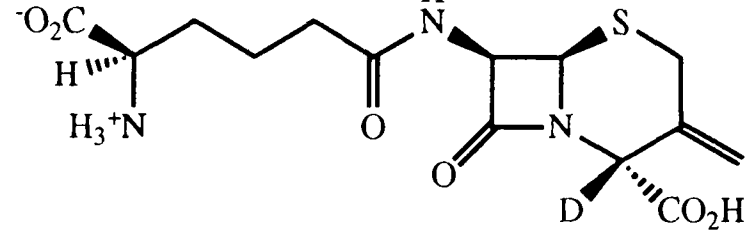
This interesting reaction provided the first example of hydroxylation of an unsaturated substrate by DAOC/DACS. Two mechanisms were proposed by which the ferryl intermediate (49) could mediate the conversion of (63) to (3) (Scheme 2.3).[141] One such mechanism proposed the abstraction of the C4 hydrogen as the first irreversible step, with formation of an intermediate which could rearrange by a 1,3-shift to the conjugated species (62). The intermediate (62) is also thought to be involved in the hydroxylation of DAOC (2) to DAC (3) (see section 1.3.3.2), and can undergo reductive elimination of ferrous ion to give the product (3). The alternative mechanism proposed that hydrogen abstraction and rearrangement occurred concurrently in an oxy-ene reaction to give intermediate (62) directly.



Scheme 2.3

Both these mechanisms predicted that cleavage of the C4-H bond would occur in the first irreversible step with respect to the β-lactam substrate. As such, it was envisaged that an isotope effect for the cleavage of the C4-H bond would be observed, and it was decided to carry out competitive kinetic isotope experiments (see Appendix A) in order to substantiate the proposed mechanisms.

For these experiments the deuterated [4-^{2}H]-3-exomethylene cephalosporin C (63a) was prepared, and incubations of approximately equimolar amounts of (63) and (63a) were carried out with DAOC/DACS. The unconverted starting material was recovered from these incubations and the ratio of (63) to (63a) determined by mass spectrometry (Table 2.1).[197] A faster rate of conversion of substrate (63) compared to (63a) would indicate isotopic discrimination during the C4-H/D cleavage event, and would be consistent with C4-H/D cleavage as the first irreversible step in the conversion of the exomethylene (63).



(63a)

Expt.	% Conversion of (63)/(63a) mixture	Relative intensities of molecular ions (MH^+) for recovered (63)/(63a) *m/z* 356	357	358	359	360	361	362	Ratio (63) : (63a)
1	0	1	8	92	100	18	7	1	1 : 0.92
	15	1	2	96	100	16	6	-	1 : 0.86
	35	1	4	93	100	18	9	2	1 : 0.90
	60	3	6	88	100	15	10	3	1 : 0.95
2	0	3	8	92	100	18	7	1	1 : 0.92
	10	2	2	97	100	24	6	1	1 : 0.85
	25	3	4	94	100	17	8	2	1 : 0.88
	40	1	3	94	100	14	5	1	1 : 0.88

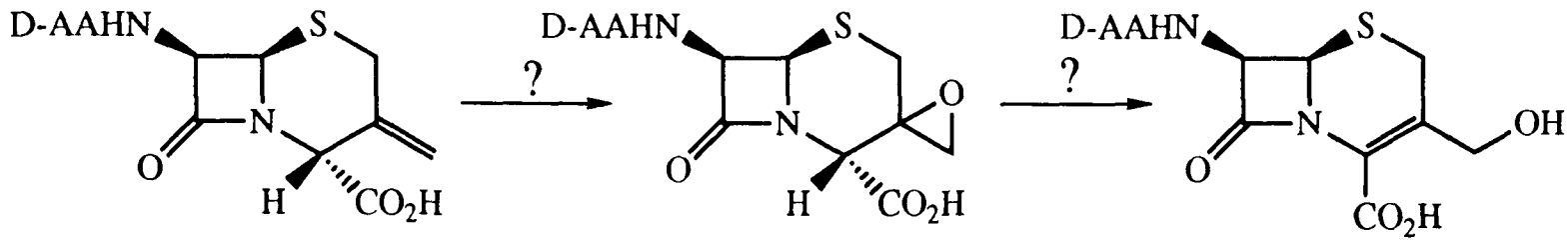
Table 2.1

Contrary to the expectations, it was found that there was no enrichment of (63a) in the starting material pool, indicating that both substrates were being converted at the same rate by the enzyme. The fact that no isotope effect was observed for the conversion of (63) and (63a) meant that the two compounds had identical V_{max}/K_m parameters, which express the mechanistic events up to and including the first irreversible step. As cleavage of the C4-H bond has to occur at some stage in the conversion of (63) to (3), the absence of a V_{max}/K_m effect indicated that an irreversible step involving the β-lactam substrate was occurring before cleavage of this bond, and thus preventing the observation of the isotope effect.

It was assumed that the formation of the iron-oxene (49) did not represent the first irreversible step with respect to the β-lactam substrate, in analogy to the conversions of pen N (1) to DAOC (2), and DAOC (2) to DAC (3) for both of which V_{max}/K_m isotope effects were observed for cleavage of the relevant carbon-hydrogen bond (see section 1.3.3). The observation of such V_{max}/K_m isotope effects indicated that binding of both substrates (1) and (2) was still reversible after the proposed formation of the iron-oxene species, and so it appeared logical to assume that reversible binding was also occurring in the case of the 3-exomethylene cephalosporin C (63).

In addition, a careful examination of an incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) revealed the presence of small amounts of a second β-lactam product, apart from DAC (3), which was not observed in incubations of unlabelled (63).[197] Attempts to isolate the new β-lactam metabolite by HPLC proved unsuccessful, as it co-eluted with the main product DAC (3) on all the systems that were tried.

The observation of a new metabolite in the conversion of (63a), coupled with the fact that an irreversible step preceded the cleavage of the C4-H bond, strongly suggested that an enzyme-free intermediate was involved in the conversion of (63) to (3), and that this intermediate was accumulating in the case of (63a) as a result of a deuterium isotope effect. It was envisaged that a possible intermediate in this conversion could be a spiro-epoxide cepham (formed by addition of the ferryl species oxygen to the double bond), which would subsequently be opened to give the product DAC (3) (Scheme 2.4).



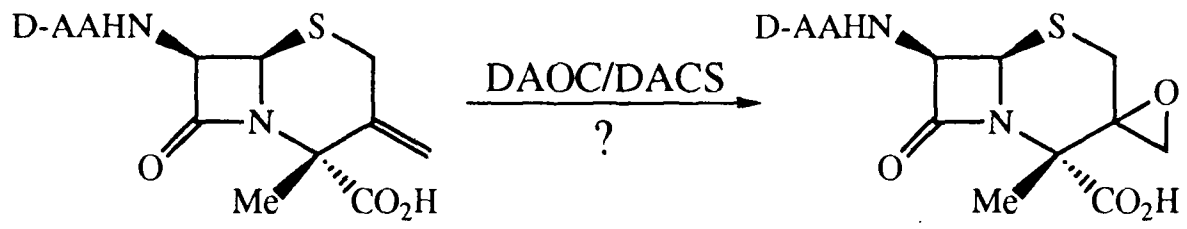
(63) (3)

Scheme 2.4

The aims of the work described in this chapter were to isolate and characterise the new metabolite observed on incubations of (63a), and to elucidate its role as a possible intermediate in the conversion of (63a) to (3). This would require separation of the unknown metabolite from the main product DAC (3), and its re-incubation with DAOC/DACS.

2.2 4-Methyl-3-Exomethylene Cephalosporin C

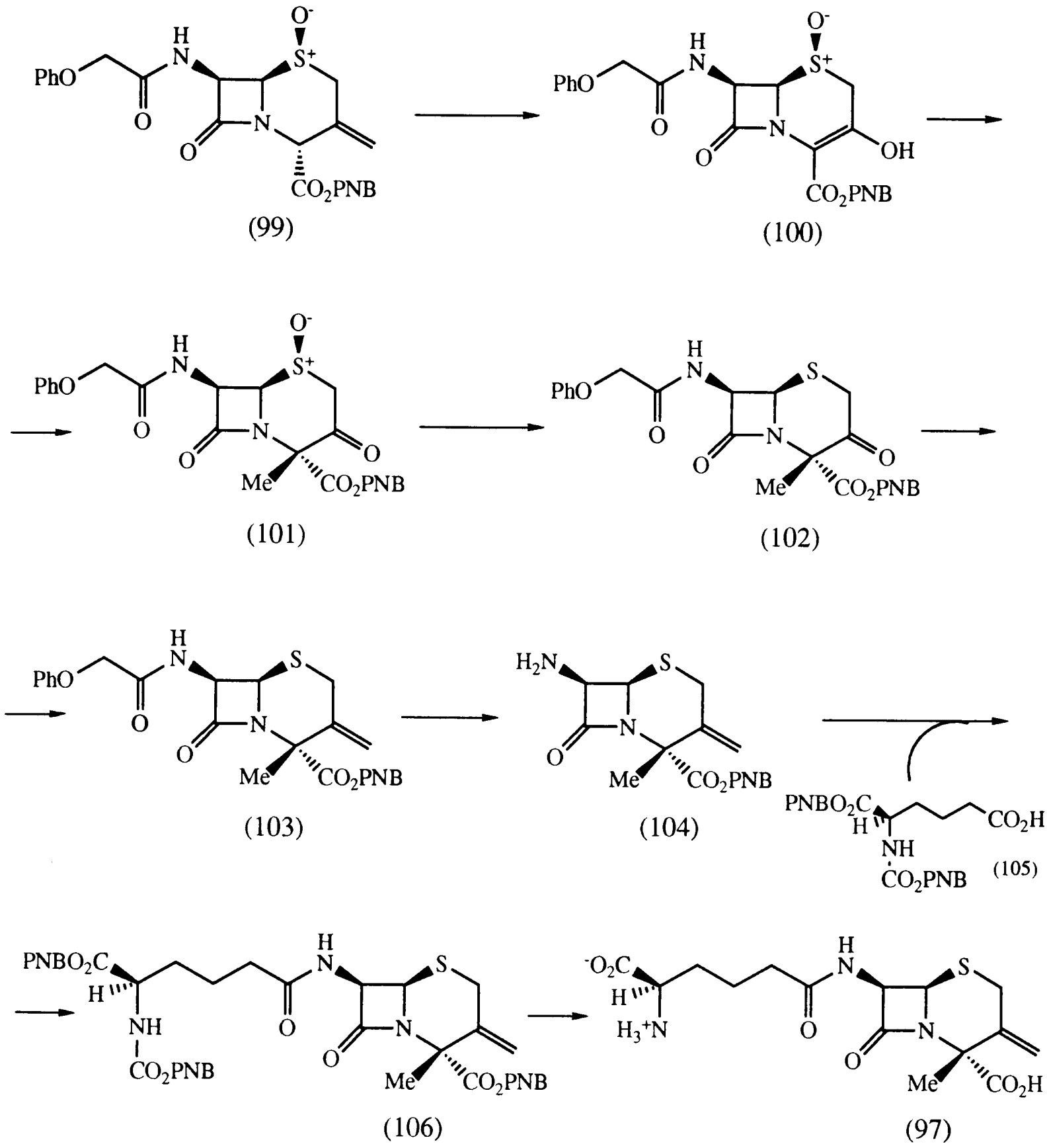
Initially, it was thought that if DAOC/DACS was capable of epoxidising the double bond of 3-exomethylene cephalosporin C (63) with formation of an enzyme-free intermediate which could accumulate if breakage of the C4-H bond was slowed down, then a way of forcing this intermediate to accumulate further and avoid formation of DAC (3), would be to replace the C4 hydrogen with a methyl group. It was hoped that this methyl group would not stericaly impede attack of the ferryl species on the double bond, and that the substrate analogue 4-methyl-3-exomethylene cephalosporin C (97) would be accepted as a substrate by DAOC/DACS with possible conversion to the 4-methyl-3-epoxide cepham (98) (Scheme 2.5).



(97) (98)

Scheme 2.5

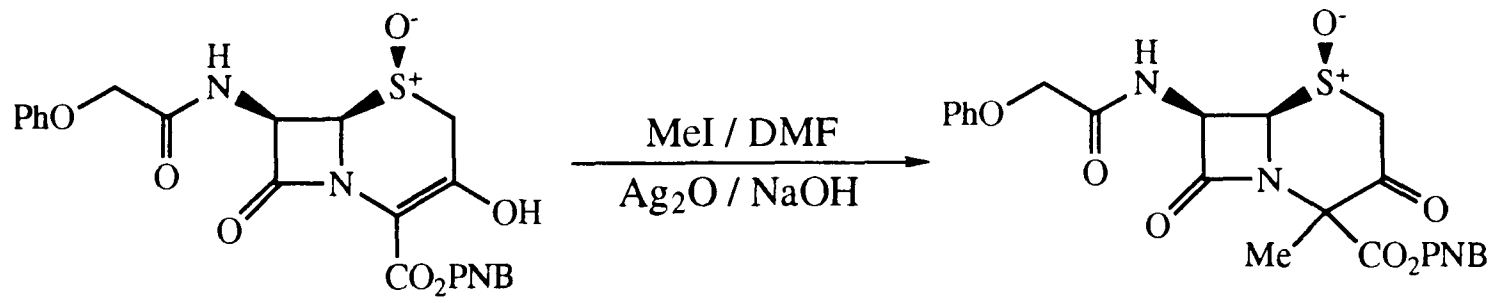
It was thus necessary to prepare (97) and the following synthesis was proposed (Scheme 2.6) : Ozonolysis of the available *p*-nitrobenzyl ester of phenoxyacetyl exomethylene sulphoxide[198] (99) would provide the corresponding ketone which is known to exist mainly as the enol (100). C-methylation of the enol (100) would give the ketone (101), at which stage the sulphoxide could be reduced down to the sulphide (102). A Wittig reaction on (102) would re-form the exomethylene at the 3 position to give (103), concluding the construction of the bicyclic nucleus of (97). Cleavage of the phenoxyacetyl side chain to the amine (104), followed by coupling with protected D-α-aminoadipic acid (105) (see section 4.2.1.2) would give (106) which could be converted to (97) by hydrogenolysis of the protecting groups.


PhO
H
N
O
O
O-
S+
N
CO2PNB
(99)
OH
(100)
Me
(101)
S
(102)
H2N
(103)
(104)
PNBO2C
CO2H
NH
(105)
-O2C
H3+N
(106)
(97)

Scheme 2.6

3-Hydroxy-3-cephem compounds such as (100) were first prepared by ozonolysis of 3-exomethylene cephams.[199,200] They are well studied compounds, being key intermediates in the synthesis of cephem antibiotics bearing electronegative substituents in the 3 position (these substituents increase the reactivity of the β-lactam ring thus enhancing bioactivity).[201,202] Although the double bond of 3-exomethylene cephams is more reactive towards ozone than the sulphur atom[203], it is often observed that oxidation of the sulphide group also occurs during ozonolysis leading to a mixture of products. As such, it was thought that by using the available exomethylene sulphoxide[198] (99) this problem could be avoided, and that the reduction to the sulphide could easily be accomplished at a later stage in the synthesis. The ozonolysis of (99) in MeOH/DCM at -60°C resulted as expected in the clean formation of the enol (100). In accordance to what is usually observed for 3-hydroxy-3-cephems, (100) was found to exist largely in the enol form depicted as no signal for a proton at C4 was observed in the ^{1}H-NMR spectrum.

Alkylations of 3-hydroxy-3-cephems have typically been directed at the production of 3-alkoxy-3-cephems as possible bioactive compounds. However, it was reported that use of Williamson type ether synthesis led instead to alkylation at C4, starting from either 3-hydroxycephem sulphides or sulphoxides.[204] In particular, reaction of sulphoxide (100) with methyl iodide in DMF in the presence of sodium hydroxide and silver oxide was reported to yield the desired 4-methyl cepham (101) (Scheme 2.7).[205]

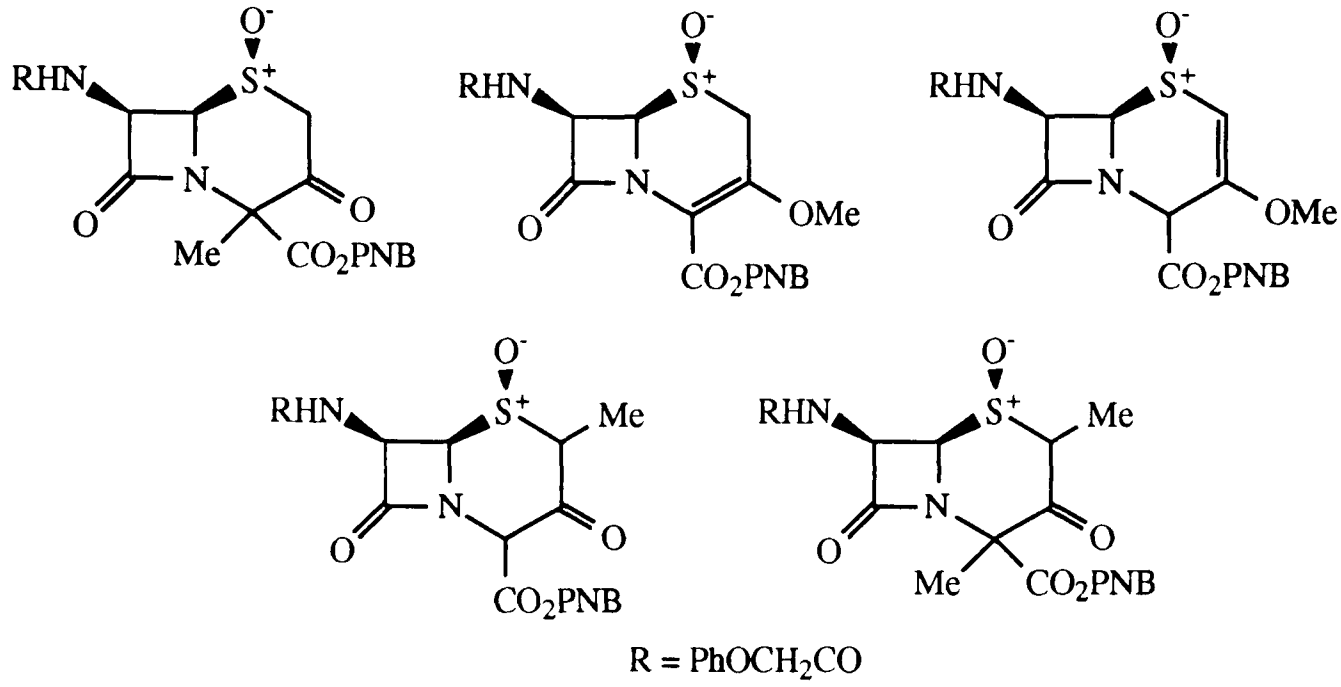


(100) (101)

Scheme 2.7

However, when this reaction was attempted a complex mixture of products was obtained. ^{1}H-NMR analysis suggested that alkylation had also occurred on the oxygen, as

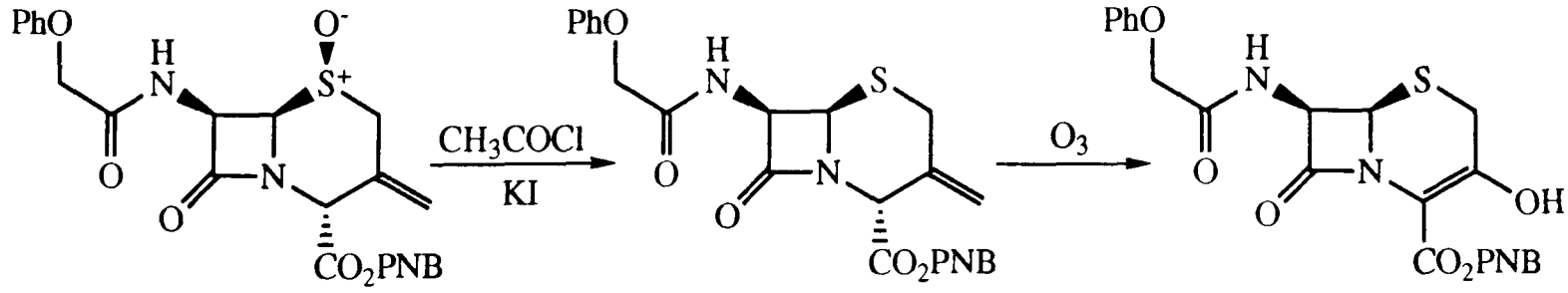
well as on the C2 carbon after isomerisation of the enol (100). The structures of possible products formed are depicted in Scheme 2.8.



Scheme 2.8

Another procedure using methyl iodide and potassium carbonate in acetone had also been reported to produce C4 alkylation on a 3-hydroxycepham sulphide in 32% yield.[206] However, when this reaction was tried on the sulphoxide (100) a similar mixture of products was again observed.

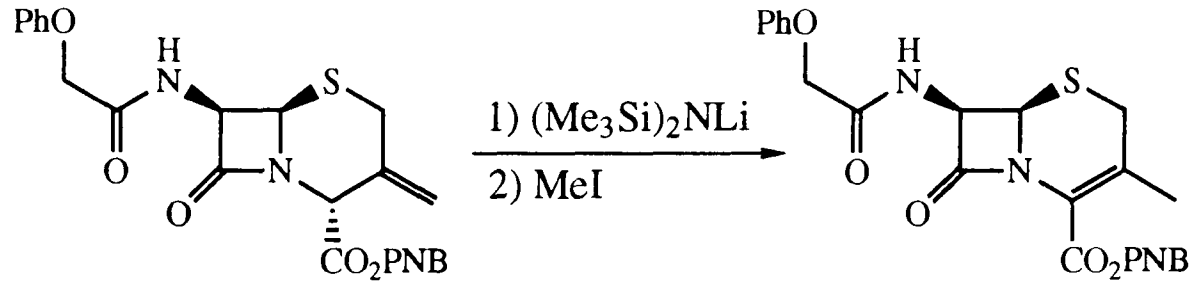
It was then decided that it would probably be better to try the alkylation reaction on the sulphide, as the sulphoxide obviously promoted alkylation at C2 by increasing the acidity of the C2 hydrogens. Reduction of the 3-exomethylene sulphoxide cepham (99) with acetyl chloride and potassium iodide[207] gave the corresponding sulphide (107)[208] in good yield (Scheme 2.9). Ozonolysis of (107) was, as expected, a less clean reaction than the corresponding one with (99) but the 3-hydroxycephem (108)[209] could be isolated in 64% yield after chromatography (Scheme 2.9).



(99) (107) (108)

Scheme 2.9

The alkylation conditions described previously were again attempted with the 3-hydroxycephem (108). Unfortunately, these also led to mixtures of several products. It was then envisaged that it might be possible to alkylate the exomethylene (107) directly, and that this might be a cleaner reaction. As such, (107) was treated with lithium bis(trimethylsilyl)amide and reacted with methyl iodide, but the only product isolated (109) resulted from isomerisation (Scheme 2.10). No product resulting from alkylation was observed.



(107) (109)

Scheme 2.10

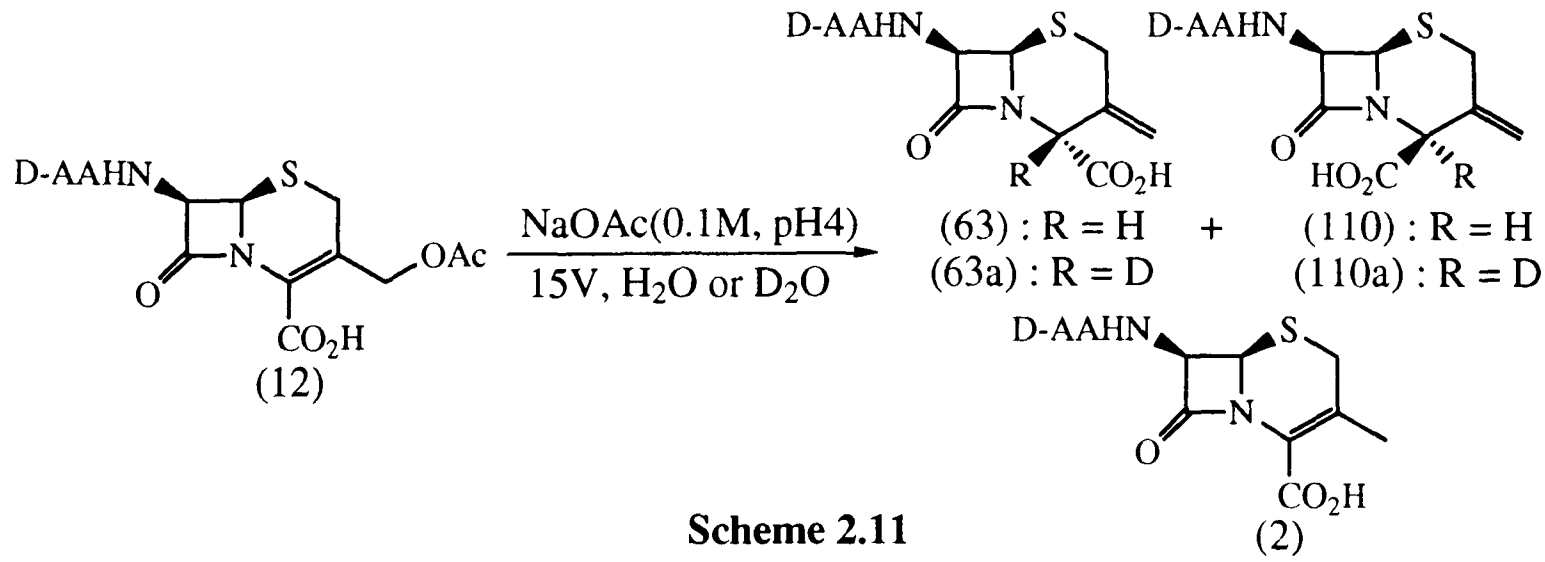
At this point, it became apparent that the approach of using a 4-methyl-3-exomethylene analogue for DAOC/DACS was proving too problematical. The alkylation reaction was obviously not going to be a simple problem to solve, and because of the competing reactions the yield was likely to be too low for such an early step in the synthesis. On the other hand, even if the synthesis of (97) was successfully accomplished, there was still the problem that it might not be accepted as a substrate by DAOC/DACS. Furthermore, even if (97) was converted it would still be necessary to isolate the compound observed in the incubation of (63a) to determine its structure unambiguously, and to re-incubate it with DAOC/DACS in order to elucidate its role as a possible intermediate. For all these reasons it seemed that our efforts should be concentrated in isolating the new metabolite formed on incubation of (63a) with DAOC/DACS.

2.3 The Conversion of [4-^{2}H]-3-Exomethylene Cephalosporin C

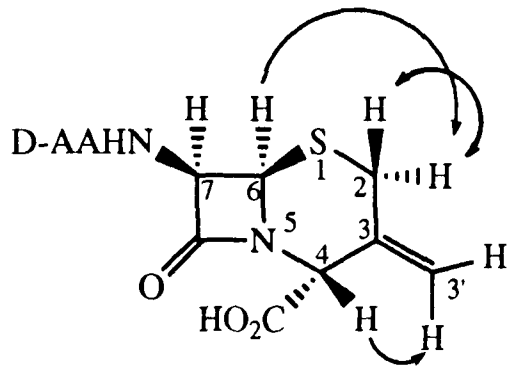
To start with, it was necessary to prepare the unlabelled and labelled 3-exomethylene cephalosporin C (63) and (63a) and to repeat their incubations with DAOC/DACS in order to confirm the results previously observed[197].

2.3.1 Synthesis of Protiated and Deuterated 3-Exomethylene Cephalosporin C

Several syntheses of 3-exomethylene cephams have been described.[202] From these, a very convenient direct method – the electrochemical reduction of cephalosporanic acids[210,211] – was chosen. Cephalosporin C (12) was electrolysed in a sodium acetate buffer (0.1M, pH 4) using a mercury pool cathode and a platinum sheet anode separated by a Nafion membrane. The reaction was complete after 5h and the crude product was purified by HPLC to give 3-exomethylene cephalosporin C (63), along with some DAOC (2) and a third β-lactam compound in a ratio of 2:1:1. This β-lactam compound gave the same mass spectrum as (63) and its ^{1}H-NMR spectrum also showed the same number of hydrogens, although with different chemical shifts for the C4, C6, C7 and methylene protons. It was thought that this compound was the C4-epimer of (63) with structure (110) (Scheme 2.11). This was confirmed by nOe studies which revealed that the C4 proton of (110) displayed an nOe to the C6 proton, an effect that could not be observed in the case of (63) (Scheme 2.12).

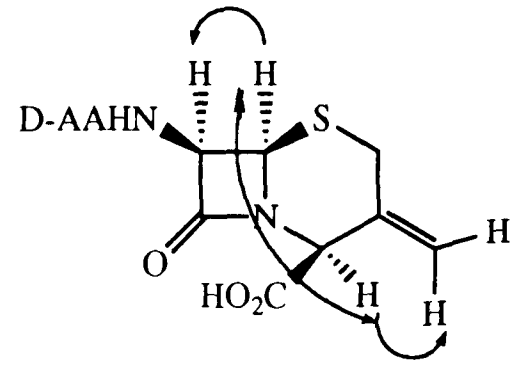


Scheme 2.11



(63)

Irradiation	nOe
C2α-H (3.61)	C2β-H (30%)
C2β-H (3.88)	C2α-H (17%)
C4-H (4.97)	C3'-H (5.24;5%)
C6-H (5.40)	C2α-H (5%)



(110)

Irradiation	nOe
C4-H (4.68)	C6-H (5%) C3'-H (5.33;7%)
C6-H (5.11)	C4-H (2%) C7-H (5.23;17%)

Scheme 2.12

Interestingly, when the electrolysis reaction is carried out in phosphate buffer at pH 7 only (63) is obtained.[212] Formation of the two other analogous isomers has also been reported in the electrochemical reduction of cephalotin [7-(thiophene-2-acetamido)-cephalosporin C], with the relative amounts of each product being dependant upon the pH and electrode potential.[213,214]

The electrolysis reaction was repeated using a sodium acetate buffer prepared in deuterium oxide. HPLC purification gave the deuterated products (63a) and (110a), and also some (2). The ratio of products obtained in this case [(63a):(2):(110a) = 6:1:1] was different, probably reflecting variations in the electrolysis potential which tended to oscillate with time. Analysis by ^{1}H-NMR and mass spectroscopy revealed that the deuterium incorporation into (63a) and (110a) was 95%.

2.3.2 Incubations with DAOC/DACS

The 3-exomethylene cephalosporin C (63) was incubated with DAOC/DACS, and examination of the crude incubation mixture by ^{1}H-NMR revealed that a clean conversion to a single product had occured. This mixture was purified by HPLC and the product isolated was identified as DAC (3).

The C4-epimer (110) provided another test for the substrate specificity of DAOC/DACS. Thus (110) was incubated with DAOC/DACS under similar conditions to those used for converting (63). Examination of the crude incubation mixture by 500 MHz ^{1}H-NMR revealed that the starting material was still intact and no new β-lactam products could be observed. The incubation mixture was purified by HPLC, but again only (110) was isolated.

The possible formation of a cephem product in minute quantities was also inspected by bioassay tests. Bioassay studies can be a very sensitive test to detect the formation of a cephalosporin by DAOC/DACS. Bioassay of an incubation mixture against *E. coli* with, and without β-lactamase can reveal the presence of a cephem compound as this is not usually affected by the β-lactamase enzyme. The 3-exomethylene cephams (63) and (110) showed no bioactivity against *E. coli* X580 (with or without β-lactamase), or against *Staphilococcus aureus*. Incubation mixtures of the two compounds with DAOC/DACS were bioassayed against *E. coli* (-) and *E. coli* (+). The incubation mixture of (63) showed bioactivity in both cases as was expected, but with the incubation from (110) no activity could be observed in either case. It was thus concluded that the C4-epimer (110) was not accepted as a substrate by DAOC/DACS, a result which has further defined its substrate specificity.

An incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) was then carried out with DAOC/DACS. Analysis by ^{1}H-NMR revealed that as had previously been described two new β-lactam resonances could be observed at δ 5.37 and 5.50 ppm with a coupling constant of 4 Hz, suggesting that a second β-lactam product had been formed in addition to

DAC (3) (Figure 2.1). Purification of the crude incubation mixture by HPLC again revealed that DAC (3) and the new product had co-eluted as a single peak. A second HPLC run did not succeed in separating the two compounds.

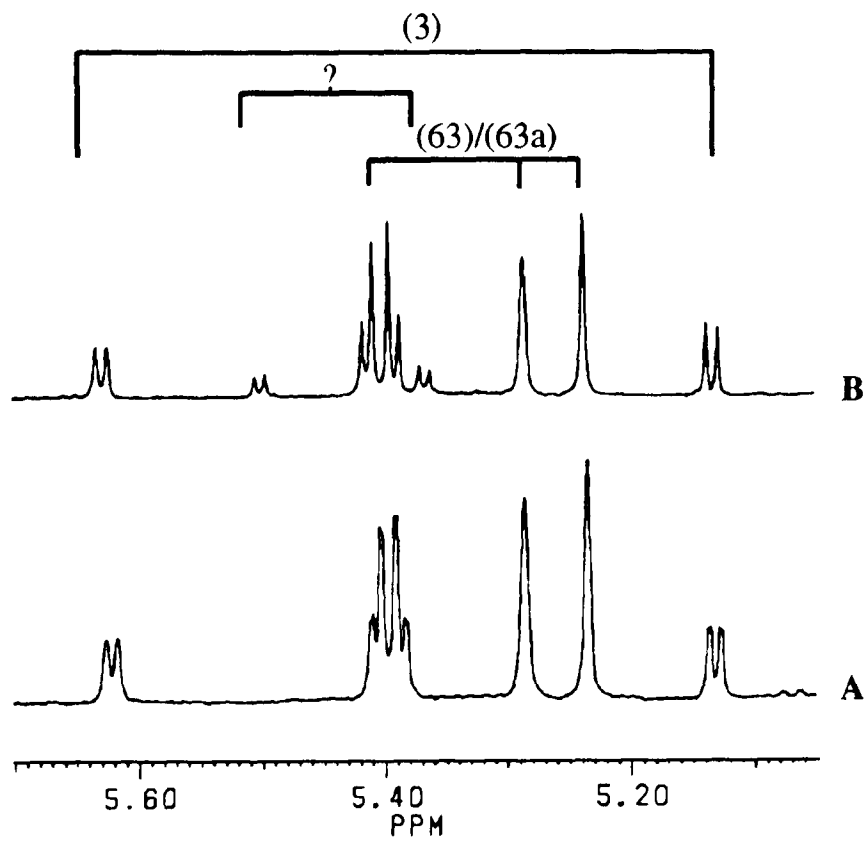


Figure 2.1 : The ^{1}H-NMR β-lactam absorbing region of incubations with (63) (**A**), and (63a) (**B**)

In order to investigate the formation of this new metabolite several parallel incubations of (63) and (63a) were carried out with DAOC/DACS, using varying amounts of enzyme and/or substrate with the aim of obtaining a range of percent conversions of the substrates. The crude incubation mixtures thus obtained were analysed by 500 MHz ^{1}H-NMR in order to determine the extent of conversion and ratio of products. The new compound could not be detected in incubations of (63), with DAC (3) being the only β-lactam product ever observed. On the other hand, incubations of the deuterated (63a) revealed that the ratio of the main product DAC (3) to the new metabolite varied with the overall degree of conversion. Larger amounts of the unknown product were observed at low conversions, with its ratio to DAC (3) decreasing as conversion increased, until its presence could not be detected when conversion was complete (Table 2.2). The ratio of DAC (3) to the new compound was sometimes slightly different for different incubations with the same percentage of conversion, but the overall trend was consistent.

Approximate % conversion of (63a)	Ratio of DAC(3) to unknown
25	1 : 0.4
50	1 : 0.25
75	1 : 0.17
92	1 : 0.1
100	1 : 0

Table 2.2

It was found that the maximum amount of the new compound was obtained when the extent of conversion was about 40%, which typically gave a ratio of products of between 3:1 to 4:1. At lower conversions both products had still been formed in small amounts, and it was difficult to separate them from the large excess of starting material by HPLC due to close retention times. At higher conversions, the amount of the new metabolite started to decrease, and the ratio of the two products was too high complicating attempts to separate them. As such, the incubations of (63a) with DAOC/DACS carried out through the course of this work were performed with the amounts of enzyme and substrate that would approximately result in a 40% conversion.

The results described gave further support to the idea that the new metabolite was an enzyme-free intermediate in the conversion of (63a), whose amounts decreased as conversion increased because it was being converted through to DAC (3).

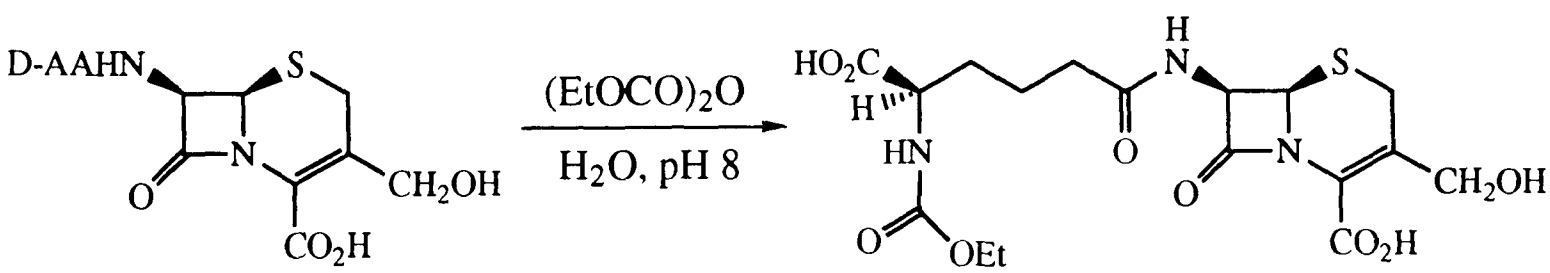
2.4 Isolation of the New Metabolite from Incubations of [4-^{2}H]-3-Exomethylene Cephalosporin C

As it was found that DAC (3) and the second product co-eluted on HPLC, some procedure had to be developed which would enable separation of the two compounds. On the assumption that the new metabolite was an epoxide, it was considered that this procedure could not involve exposure to acidic conditions as this would probably result in the destruction of the epoxide functionality.

2.4.1 N-Derivatisation Experiments

It was envisaged that protecting the amino group of both products thus eliminating their zwitterionic character, would make the polarity of the two compounds sufficiently different for their separation on HPLC to be possible. Furthermore, this procedure would increase their retention time on reverse phase HPLC allowing for a more effective separation.

The first N-protecting group to be tried was the ethoxycarbonyl group. Initially, DAC (3) was derivatised with diethylpyrocarbonate[6] at pH 8 as a model reaction, which gave the N-protected DAC (111) quantitatively (Scheme 2.13). This reaction was repeated on the small scale in which the incubation products are usually isolated (< 1 mg) and again resulted in clean formation of the product (111).

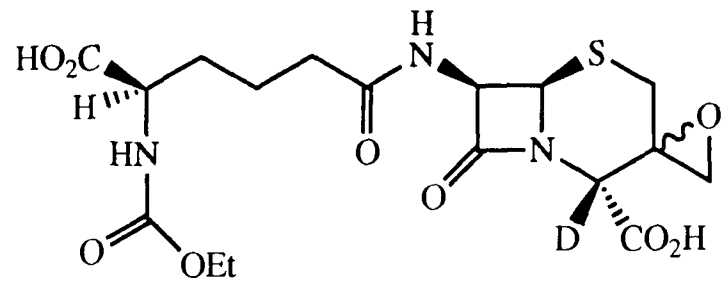


Scheme 2.13

In order to try this procedure with the enzymatic products, a large scale incubation of (63a) with DAOC/DACS was carried out. After HPLC purification a mixture of the two

products was obtained with a ratio of about 4:1. The ^{1}H-NMR spectrum of this mixture (Figure 2.2 A) showed two doublets at δ 3.28 and 3.40 ppm with a coupling constant of 4 Hz, strongly suggesting the new metabolite was indeed a spiro-epoxide.

This mixture was N-derivatised with diethylpyrocarbonate at pH 8 and then lyophilised. ^{1}H-NMR analysis confirmed that the reaction had been quantitative and that the ratio of the two products had remained constant. The crude reaction mixture was then purified by HPLC. Unfortunately, there was still no clear separation of the products but two partially co-eluting peaks could be observed which were collected as two separate fractions. The first fraction contained only (111), whereas the second one contained a mixture of the two products in a ratio of almost 1:1 (Figure 2.2 B). This second fraction was subject to another HPLC purification and the analogous fraction obtained in this case contained the two products in a ratio of 1:2, with the new metabolite now being the major compound (Figure 2.2 C). Spectroscopic data (^{1}H-NMR, COSY and mass) on this partially purified compound were consistent with its having the structure of the expected spiro-epoxide (112).[109]



(112)

Thus, the approach of N-derivatisation had yielded at least some partial success. However, the N-ethoxycarbonyl group could not be removed from (112) for re-incubation with DAOC/DACS, and so a different more easily removed N-protecting group was necessary in order to obtain the epoxide cepham in a free form.

The choice of protecting groups was limited to one which could be removed under neutral or mildly basic conditions. The first such group to be tried was the Z-group (benzyloxycarbonyl group) which could be removed by catalytic hydrogenation. DAC (3) was reacted with benzylchloroformate with formation of Z-DAC (113) (Scheme 2.14), a reaction that could also be repeated on a small scale.

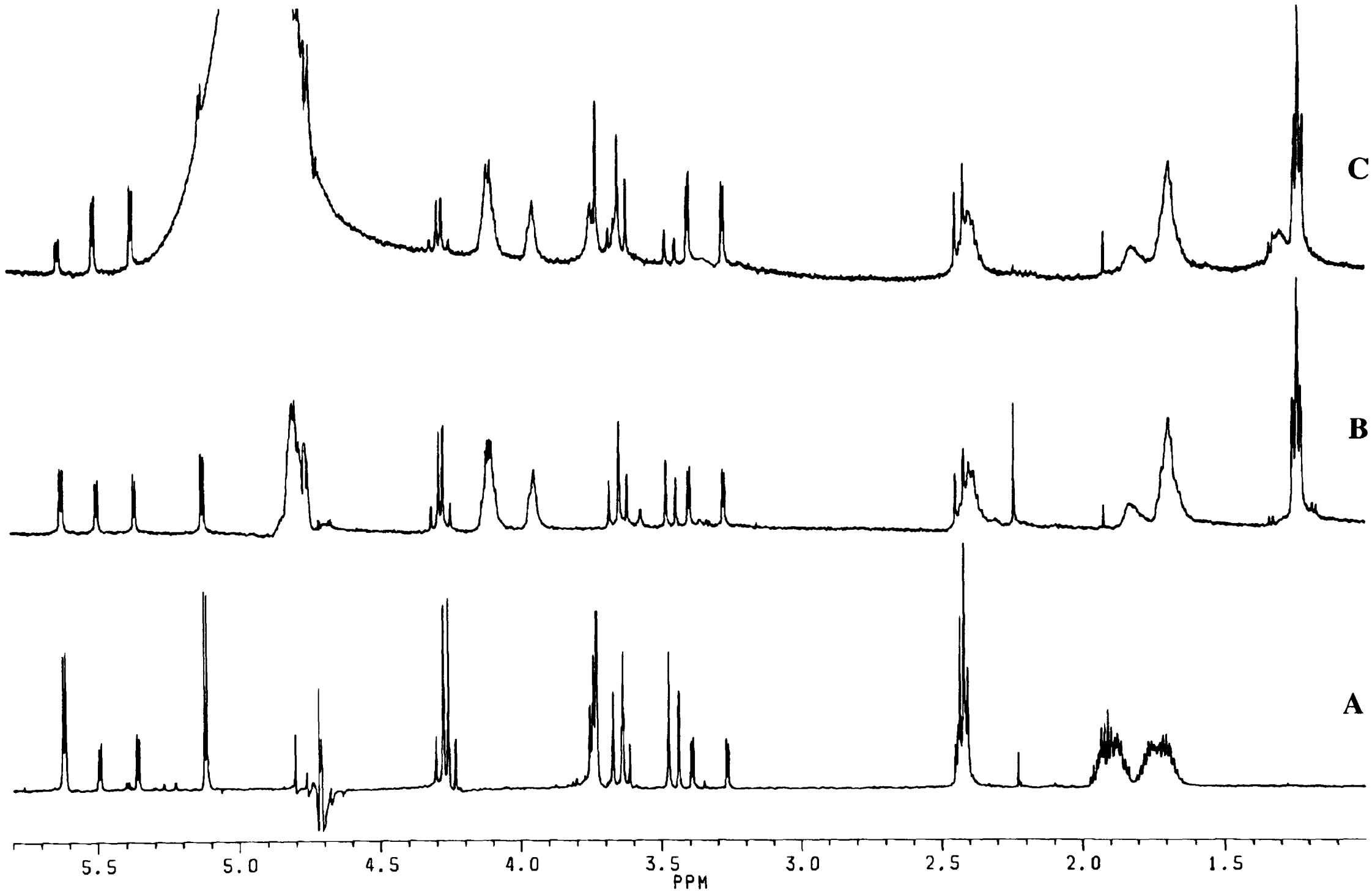
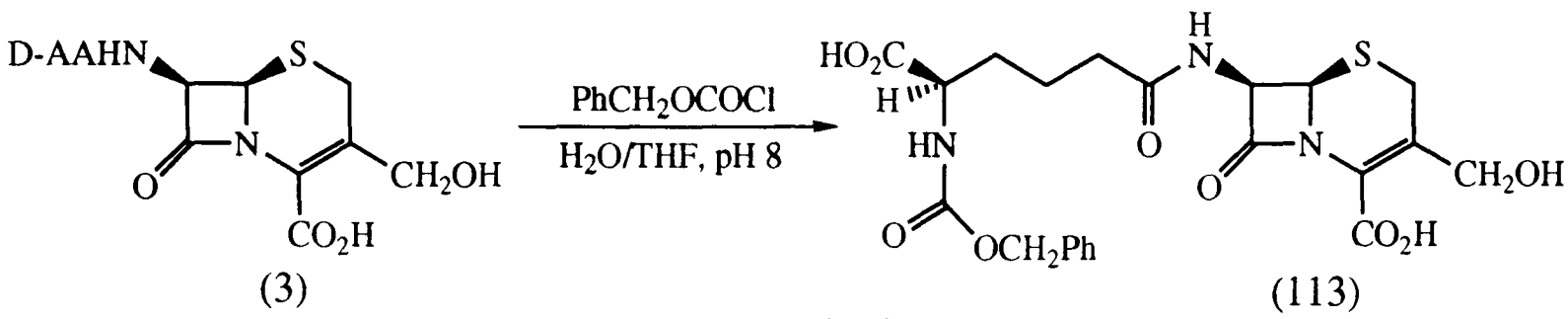


Figure 2.2

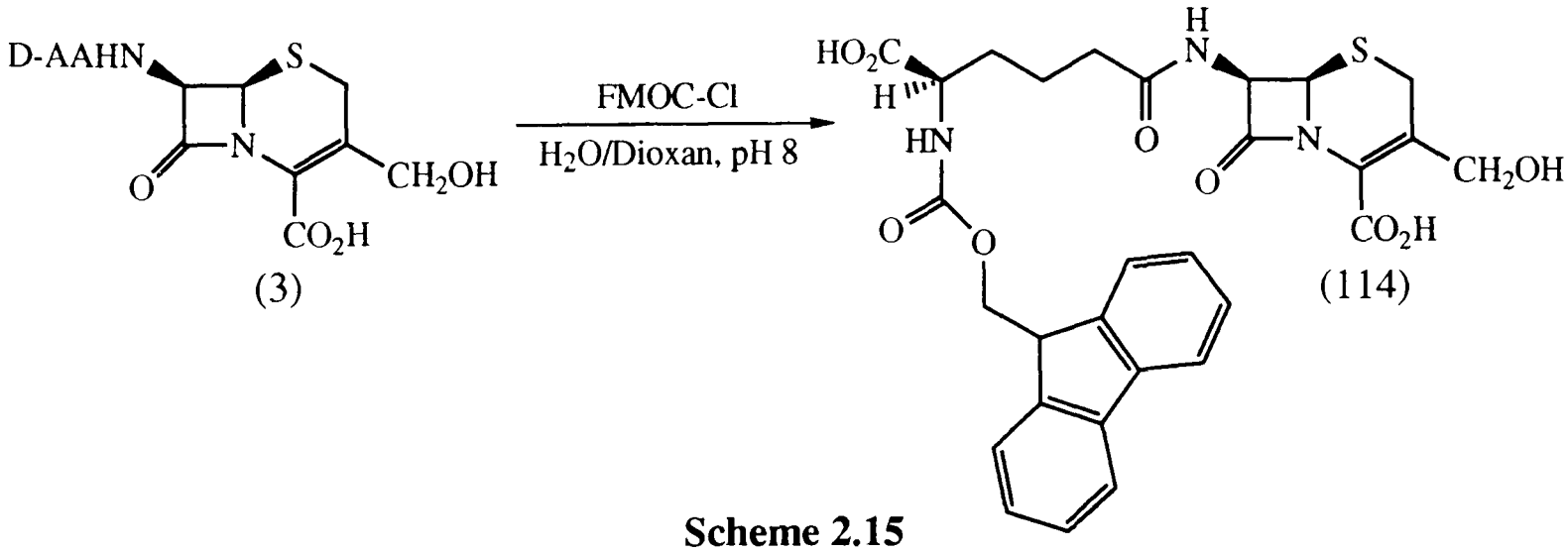


Scheme 2.14

This reaction was repeated with a mixture of DAC (3) and the epoxide metabolite isolated from an incubation of (63a) with DAOC/DACS. Purification of the reaction mixture by HPLC revealed an identical behaviour to the previous case where some separation of the two products could be achieved.

Before further purifying the Z-epoxide compound it was decided to attempt the removal of the N-protecting group by catalytic hydrogenation with palladium on carbon, using Z-DAC (113) as a model. Unfortunately, all the conditions under which this reaction was tried resulted in decomposition and formation of several products, possibly resulting from sulphur poisoning of the palladium catalyst. A problematic reaction was obviously unsuitable for the minute amounts of the Z-epoxide that would be obtained, and so it was necessary to find an alternative protecting group.

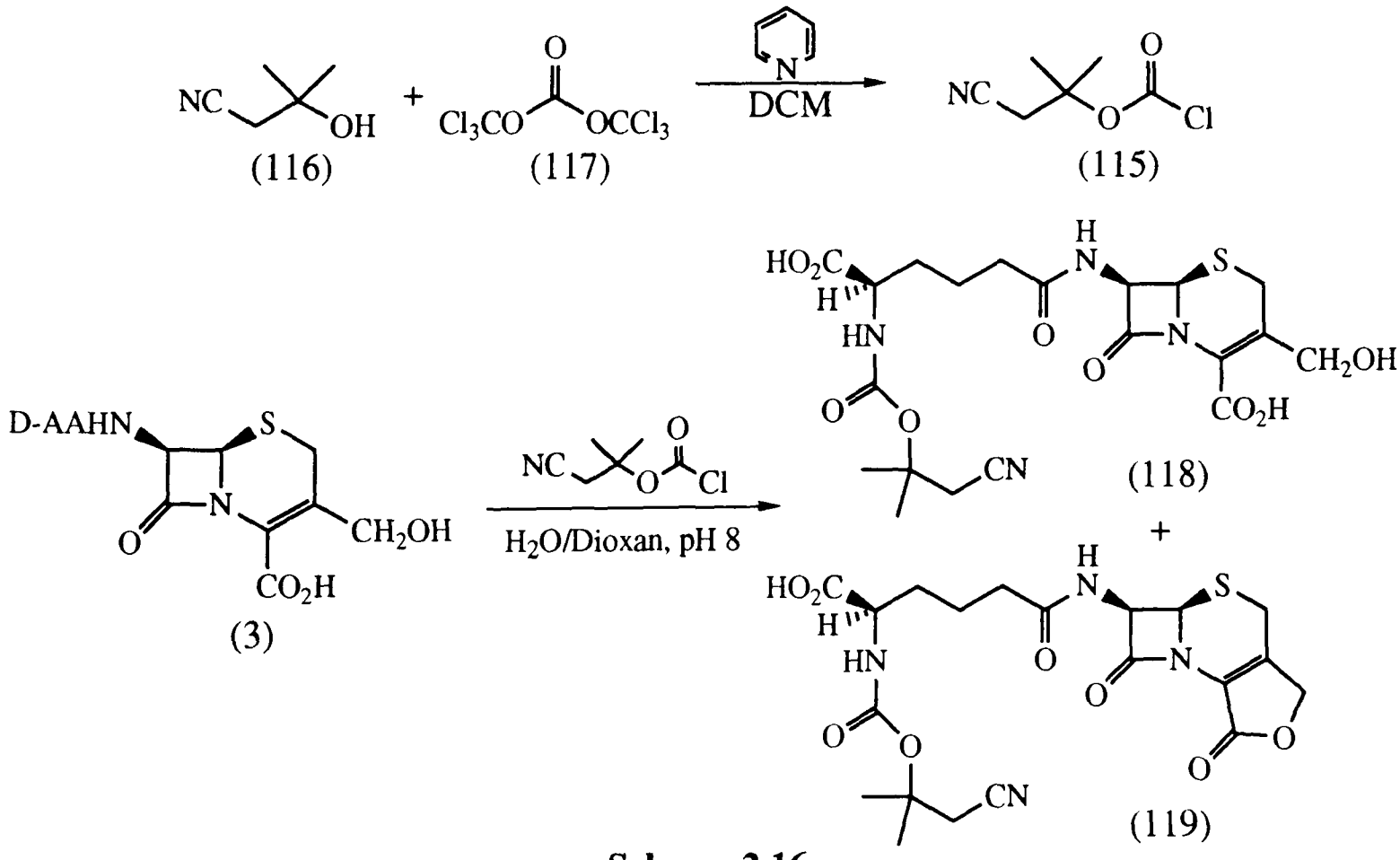
We next turned our attention to the FMOC group[215] (9-fluorenylmethoxycarbonyl group), widely used in peptide synthesis[216], which can be removed by treatment with a secondary amine. Derivatisation of DAC (3) with 9-fluorenylmethyl chloroformate gave the corresponding FMOC-DAC (114), but only in 58% yield (Scheme 2.15). Due to the large hydrophobic FMOC group, (114) was not soluble in pure water but required the use of an organic co-solvent.



Scheme 2.15

Removal of the FMOC group from (114) was attempted using piperidine in H_2O/THF, and after HPLC purification the product DAC (3) was obtained in 67% yield. Although the FMOC group could be put in place and be removed by relatively mildly basic conditions in the case of DAC (3), it was decided that it would not be suitable for isolating the epoxide metabolite as it would almost certainly result in loss of too much of the material. The relatively low yields for the protection and deprotection reactions, together with the solubility problem which would certainly pose difficulties with the HPLC, indicated that with the amounts of epoxide available at that time (< 1 mg) not enough material would be recovered for re-incubation with DAOC/DACS.

A smaller and more polar protecting group which was also reported to be cleaved by treatment with a mild basic solution was the cyano-*t*-butyloxycarbonyl group (CyOC).[217] The 1,1-dimethyl-2-cyanoethyl chloroformate (115) necessary for N-protecting DAC (3) was prepared by treatment of 3-hydroxy-3-methylbutyronitrile (116) with triphosgene[218] (117) in the presence of pyridine (Scheme 2.16). The crude (115) obtained was used to derivatise DAC (3) in the normal way. This time, two products were formed in the derivatisation reaction : the expected CyOC-DAC (118) and the corresponding lactone (119) (Scheme 2.16).



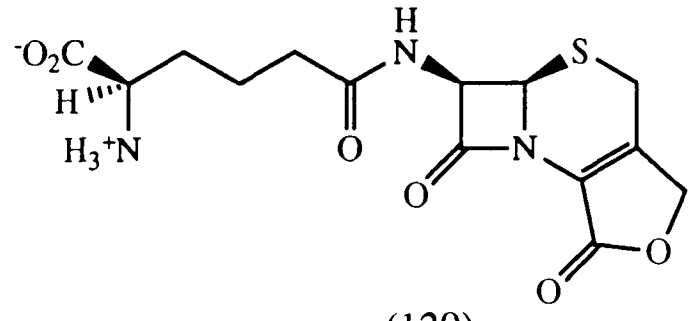
Scheme 2.16

Removal of the CyOC group in (118) was attempted using the reported conditions.[217] Unfortunately, treatment with aqueous potassium carbonate, sodium bicarbonate or triethylamine were all unsuccessful in removing the protecting group, with some degradation of (118) being observed.

2.4.2 Acid Treatment

In the course of investigations into the behaviour of the epoxide metabolite it was decided to analyse its response to acid conditions. It was expected that these would lead to opening up of the epoxide ring with formation of DAC (3). To test this, a mixture of DAC (3) and the epoxide compound was dissolved in D_2O and small amounts of DCl were added, following the reaction by ^{1}H-NMR.

Much to our surprise, it was observed that after addition of enough acid, DAC (3) had lactonised to (120) and the epoxide compound had suffered relatively little decomposition. At this point the pH of the solution was down to 1. This unexpected result showed that the epoxide could survive acid treatment much better than previously thought, and this provided a possible way of isolating it, as the lactone (120) would probably have a different retention time on HPLC.



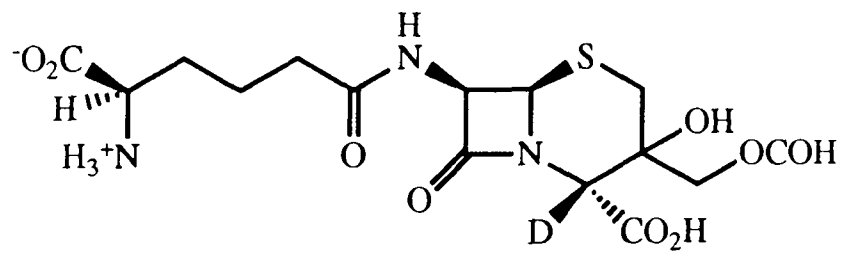
(120)

The usual procedure for lactonising DAC (3) is to treat it with formic acid[141], and it was thought preferable to use that method for the mixture of DAC (3) and the epoxide, as the absence of water would possibly minimise its decomposition. A suitable HPLC system for isolating the lactone (120) was also investigated. It was found that using a buffer like ammonium bicarbonate led to some hydrolysis of the lactone back to DAC (3), a process

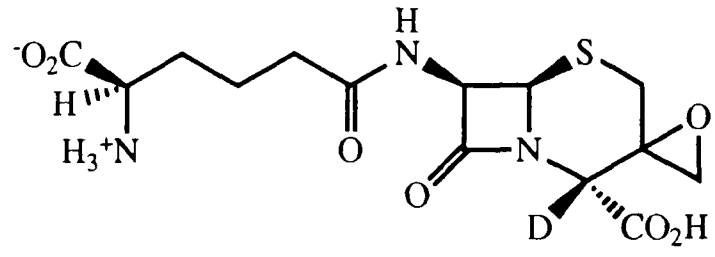
which was obviously undesirable. However, using a 5% solution of CH_3CN in water it was possible to recover intact (120) with no hydrolysis to DAC (3) being detected.

A large scale incubation of (63a) was then carried out resulting in about 40% conversion to DAC (3) and the epoxide (Figure 2.3 A). HPLC purification of the crude incubation gave the usual mixture of DAC (3) and the epoxide metabolite in a ratio of about 4:1 (Figure 2.3 B). This mixture was treated with formic acid which resulted in formation of the lactone (120) and a little decomposition of the epoxide evidenced by the appearence of two new small β-lactam peaks at δ 5.33 and 5.46 ppm in the ^{1}H-NMR spectrum (Figure 2.3 C). These peaks are not observed when pure DAC (3) is lactonised to (120), so they must originate from the epoxide.

The mixture obtained on treatment with formic acid was purified by HPLC using the CH_3CN/H_2O system. This succeeded in completely separating the lactone (120), but unfortunately the other compounds co-eluted with the solvent front. The resulting mixture contained mainly the epoxide and the other unknown β-lactam compound (Figure 2.3 D). A mass spectrum on this fraction showed peaks at 375 for the epoxide (MH^+) and at 421 (MH^+), suggesting that the second β-lactam compound was the hydroxy formate ester (121) formed by formate attack on the epoxide (122). A second HPLC run using an ammonium bicarbonate buffer enabled the separation of these two compounds, finally achieving the isolation of the epoxide metabolite (122) in a pure form (Figure 2.3 E).



(121)



(122)

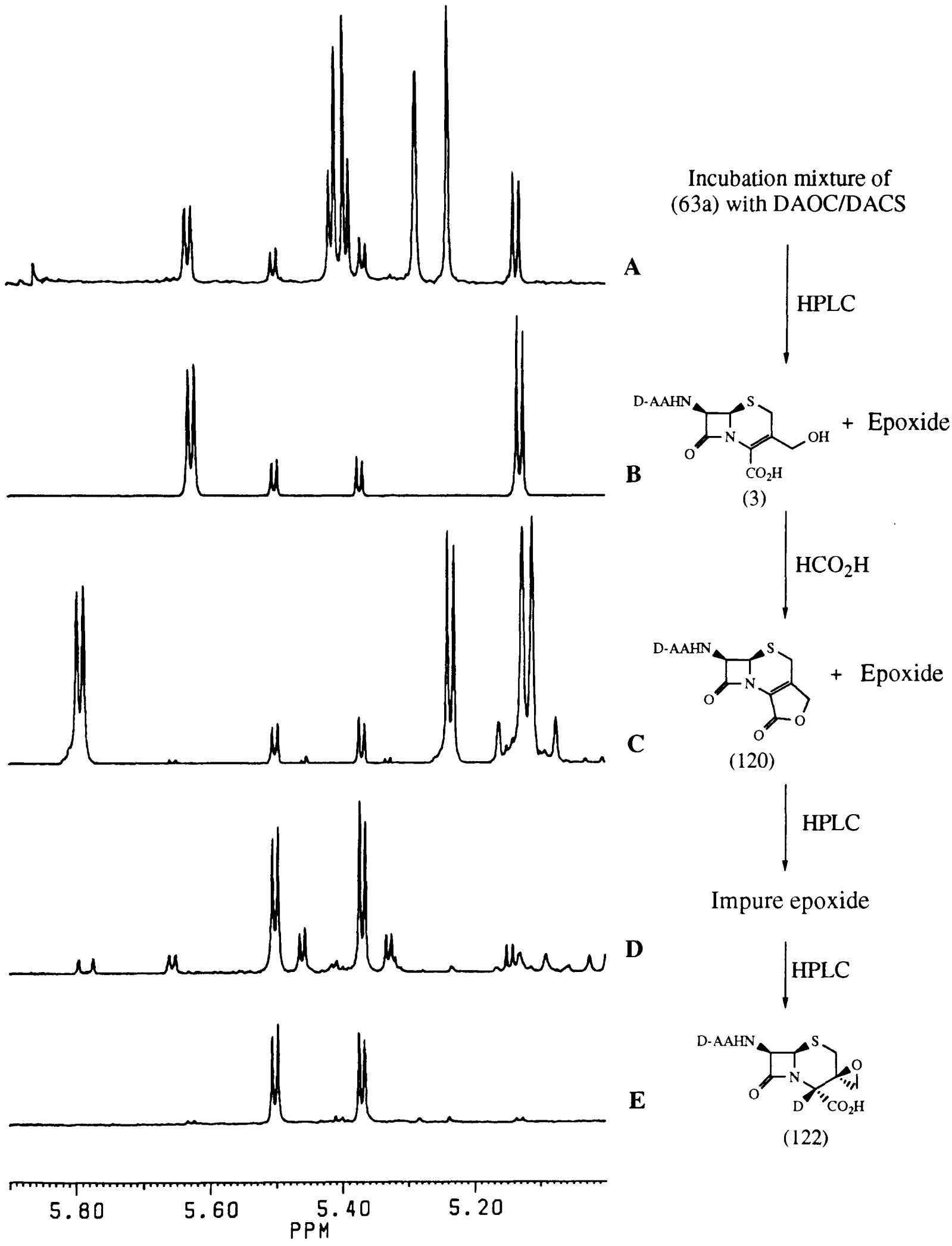
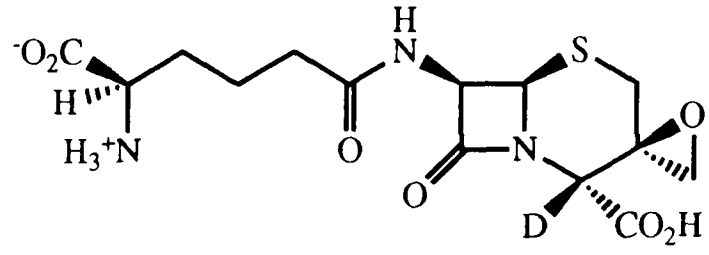


Figure 2.3 : The ^{1}H-NMR β-lactam region during the several stages in the purification of (122)

2.5 The Spiro-Epoxide Cepham

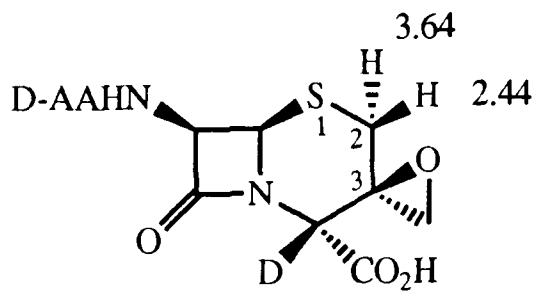
2.5.1 Structure

The physical data next described confirmed that the isolated metabolite was a spiro-epoxide as predicted, and furthermore gave strong evidence that the epoxide ring in (122) had a β-configuration.



(122)

The ^{1}H-NMR spectrum of (122) is shown in Figure 2.4 (the assignment of the protons was confirmed by a COSY experiment). The feature that first attracted our attention in this spectrum was the splitting of the C2-H resonances. This splitting was very similar to that observed for the shunt metabolite 3β-hydroxycepham (40), but distinct from what is usually found for other cephams [e.g. (63)]. In both cases it was observed that one of the C2 hydrogens was considerably shifted upfield relative to the other systems (Scheme 2.17). This was a strong indication that (122) and (40) had identical stereochemistry at C3.



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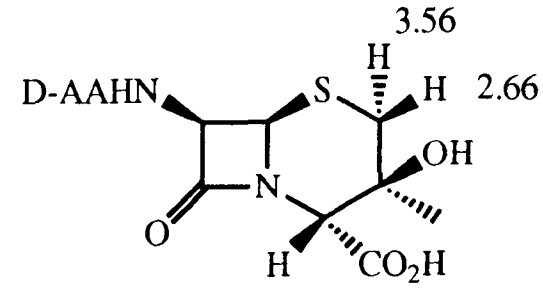
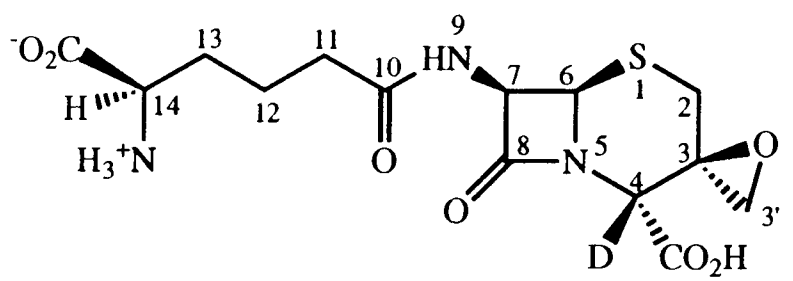


(40)

Scheme 2.17

The electrospray mass spectrum of (122) run under normal acidic conditions revealed that some loss of deuterium was occuring in the mass spectrometer presumably by ring-opening to DAC (3) (Figure 2.5 A). This could be avoided by running the mass spectrum in the absence of acid, which produced a clean spectrum for (122) (Figure 2.5 B).



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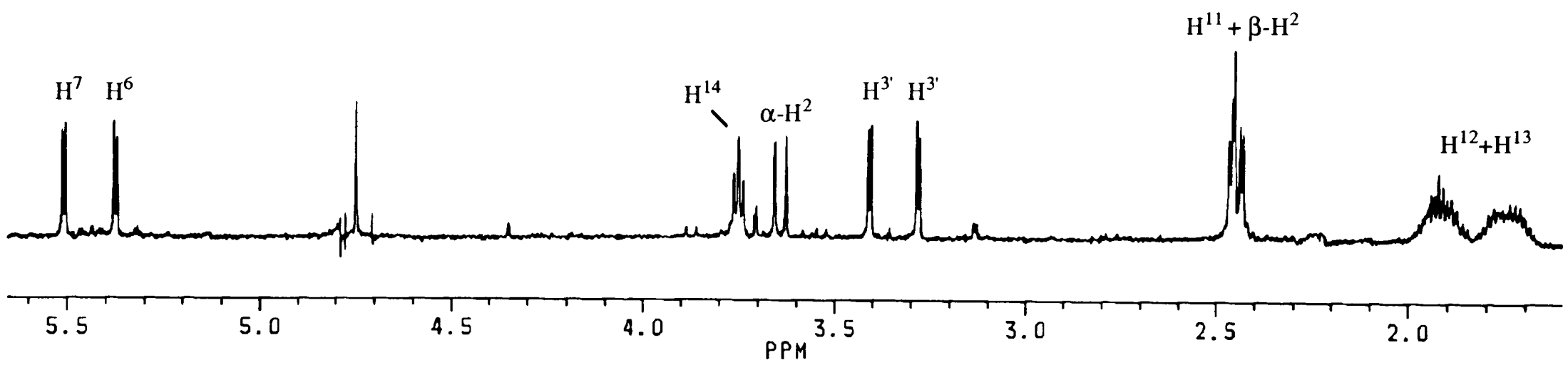
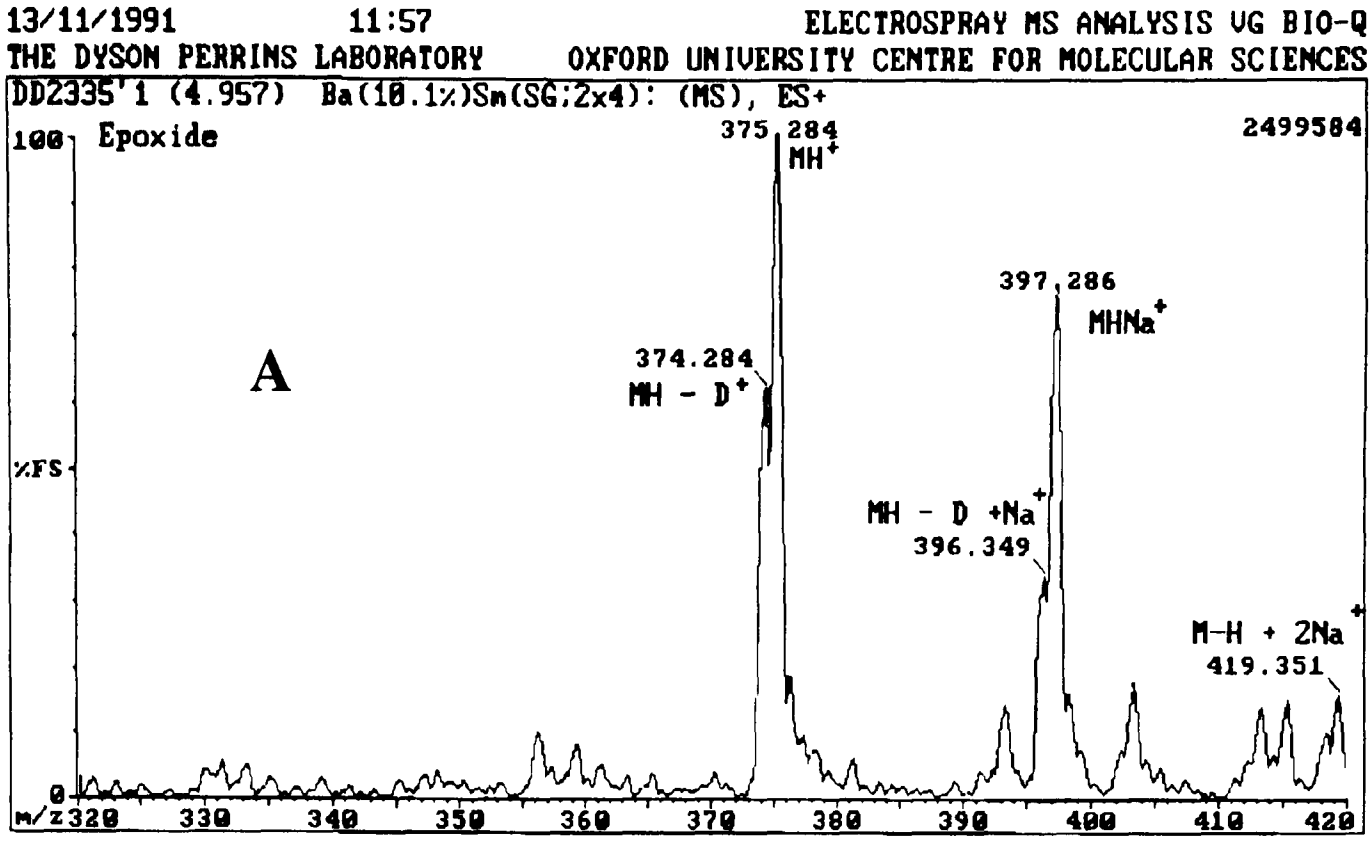


Figure 2.4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THE DYSON PERRINS LABORATORY OXFORD UNIVERSITY CENTRE FOR MOLECULAR SCIENCES
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100
Epoxide
375.284
MH+
2499584
397.286
MHNa+
A
374.284
MH - D+
%FS
MH - D +Na+
396.349
M-H + 2Na+
419.351
0
m/z 320 330 340 350 360 370 380 390 400 410 420
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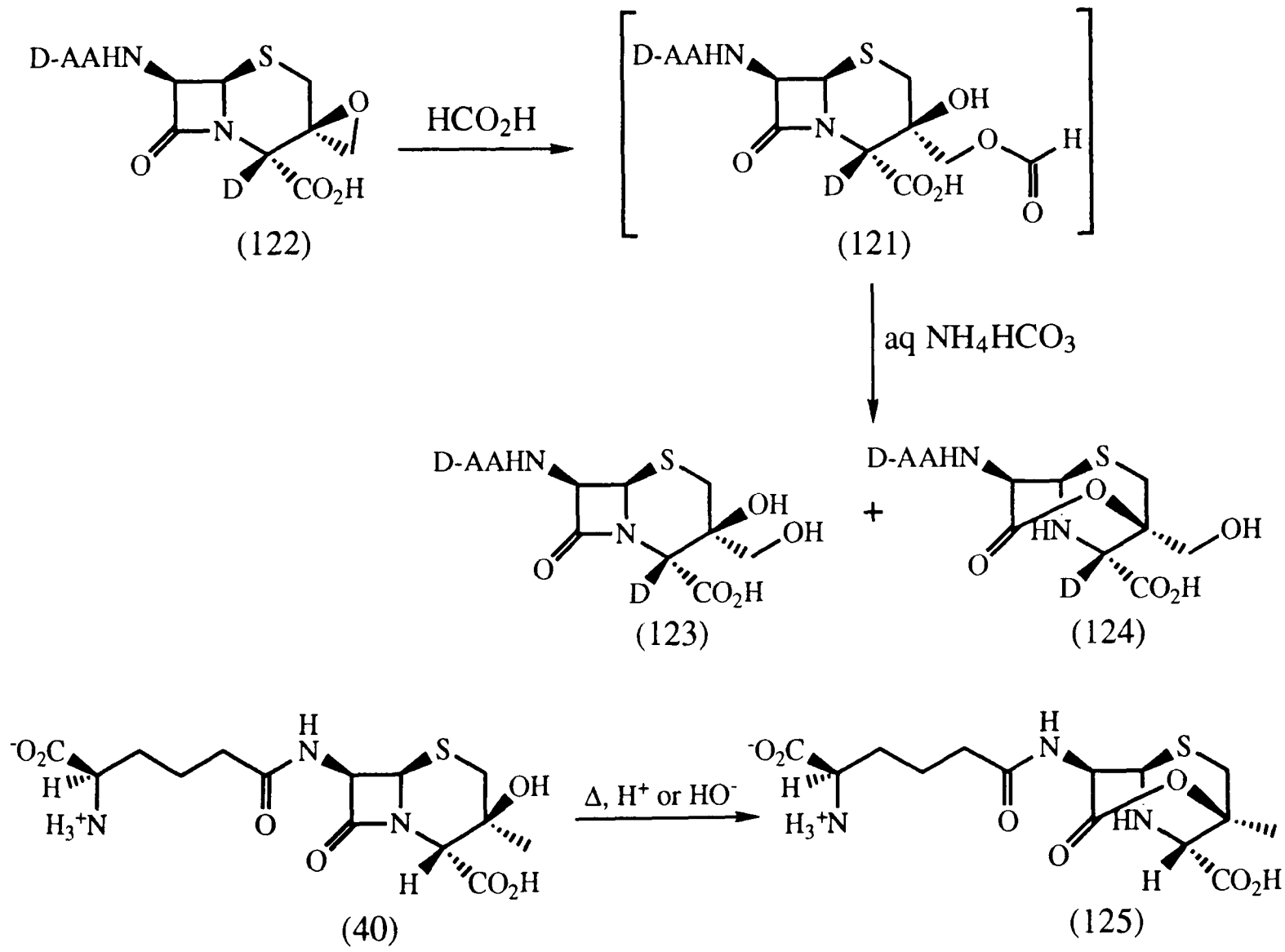
m/z 340 350 360 370 380 390 400 410 420

Figure 2.5

On the HPLC purification run that yielded the pure epoxide (122) a mixture of two other compounds was also isolated co-eluting as one peak. These two compounds were consistent with the diol (123) and lactone (124) (Scheme 2.18) by mass spectroscopy, and by comparison of the ^{1}H-NMR spectrum with that of a similar mixture of the 3β-hydroxycepham (40) with the corresponding lactone (125)[133,219] (Figure 2.6). The compounds (123) and (124) presumably resulted from hydrolysis of the hydroxyformate (121) under the basic HPLC conditions.

It is well known that the 3β-hydroxycephams like (40) give the corresponding lactones very easily by exposure to acid, base or heat[133,219], and the two compounds (40) and (125) also co-run under the HPLC conditions with which (123) and (124) were isolated as a single peak.



Scheme 2.18

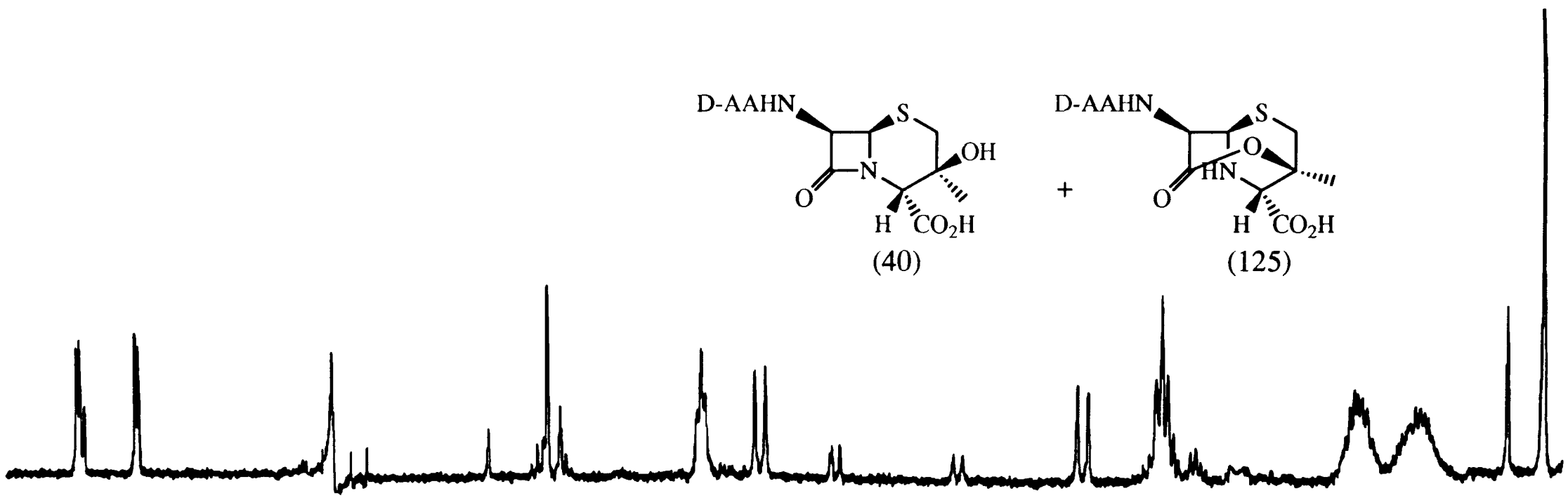
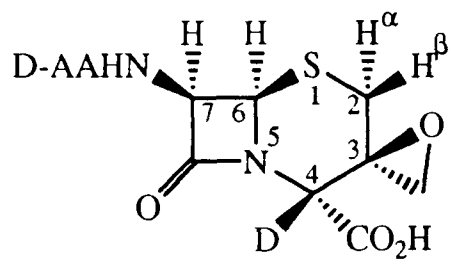

D-AAHN
S
OH
N
O
H
CO_2H
(40)
+
D-AAHN
S
O
HN
O
H
CO_2H
(125)
D-AAHN
S
OH
N
OH
O
D
CO_2H
(123)
+
D-AAHN
S
O
HN
OH
O
D
CO_2H
(124)
5.5
5.0
4.5
4.0
3.5
PPM
3.0
2.5
2.0
1.5

Figure 2.6

The formation of (123) and (124) further supported a 3β stereochemistry for the epoxide (122), as opening of the β-lactam ring is only allowed geometrically with a 3β-hydroxy group. Steric constraints would prevent a 3α-hydroxy group from attacking the β-lactam ring.

To further confirm this stereochemistry, the study of nOe effects on (122) was undertaken (Table 2.3). It was hoped that an nOe effect between the epoxide and the β-lactam hydrogen atoms would be present, but although this effect could be observed its magnitude was too small to ascertain whether or not it was genuine. Somewhat surprising was the relationship observed between the epoxide hydrogens and the C2β-H, as it was assumed they would be closer to the C2α-H. However, a computer model for the 3β-epoxide (122) confirmed that these hydrogens were indeed closer to the C2β-H than to the C2α-H.[197]



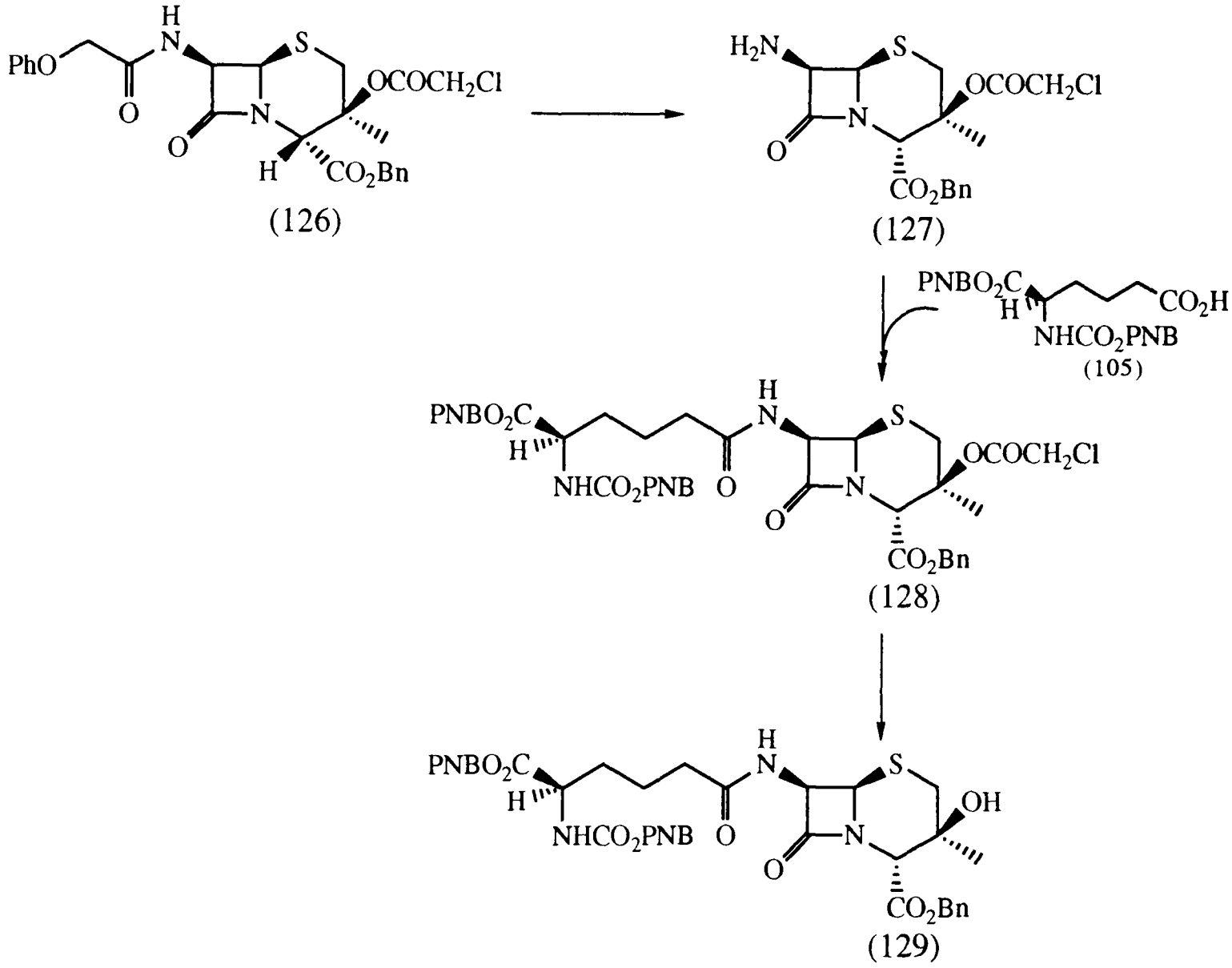
(122)

Proton irradiated	nOe Effect: 2β-H (2.44 ppm)	Epoxide H (3.28ppm)	Epoxide H (3.40 ppm)	2α-H (3.64 ppm)	6H (5.37 ppm)
2α-H (3.64 ppm)	12	–	–	–	3
2β-H (2.44 ppm)	–	5	3	5	–
Epoxide H (3.28ppm)	7	–	30	–	–
Epoxide H (3.40 ppm)	3	28	–	–	–
6H (5.37 ppm)	–	s	s	4	–

Table 2.3 : nOe effects on (122) (in %; s = too small to quantify).

It was thought that the nOe effects for (122) should be compared to those in the 3β-hydroxycepham (40) to see whether the same steric relationships were observed, and it was thus necessary to synthesise (40).

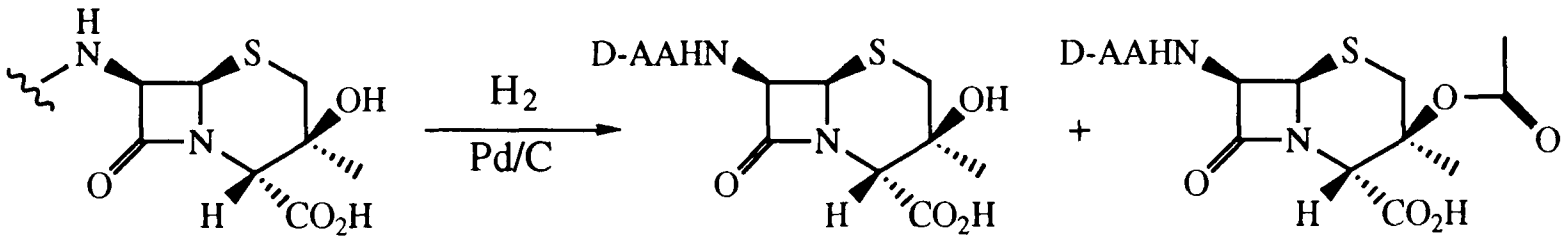
Cleavage of the side-chain of (126) (see section 4.3.1.1) using the PCl_5 reaction described in Chapter 4 (see section 4.2.1.2) gave the corresponding amine (127) in good yield, which was coupled with protected D-α-aminoadipic acid (105) using EEDQ[220] to give (128) (Scheme 2.19). Treatment of (128) with thiourea in hot ethanol (see section 4.3.1.1) gave the corresponding alcohol (129), which was not purified due to its susceptibility to attack of the hydroxyl group on the β-lactam ring.



Scheme 2.19

Hydrogenolysis of (129) over palladium on carbon proved to be a problematic reaction due to poisoning of the catalyst by thiourea present in crude (129). However, the

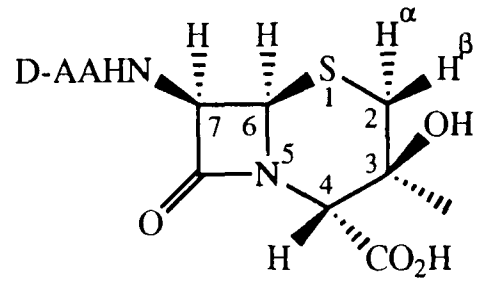
product (40) could be isolated by HPLC, along with some 3β-acetate cepham (130) (Scheme 2.20) which probably originated from reduction of a compound formed by incomplete reaction of (128) with thiourea. Some decomposition of (40) also occurred and it was isolated as a mixture with the lactone (125).



(129) (40) (130)

Scheme 2.20

NOe studies on the 3β-hydroxycepham (40) revealed similar steric relationships to those observed for the epoxide (122), namely that the α-methyl group at C3 was closer to the β-C2 hydrogen than to the α-C2 one (Table 2.4), which further supported the stereochemical assignment made for (122).



(40)

		nOe Effect			
		Me (1.39 ppm)	2β-H (2.66 ppm)	2α-H (3.56 ppm)	6H (5.29 ppm)
H irradiated	2α-H (3.56 ppm)	2	10	–	4
	2β-H (2.66 ppm)	6	–	19	–

Table 2.4 : nOe results on (40) (in %).

2.5.2 Incubations with DAOC/DACS

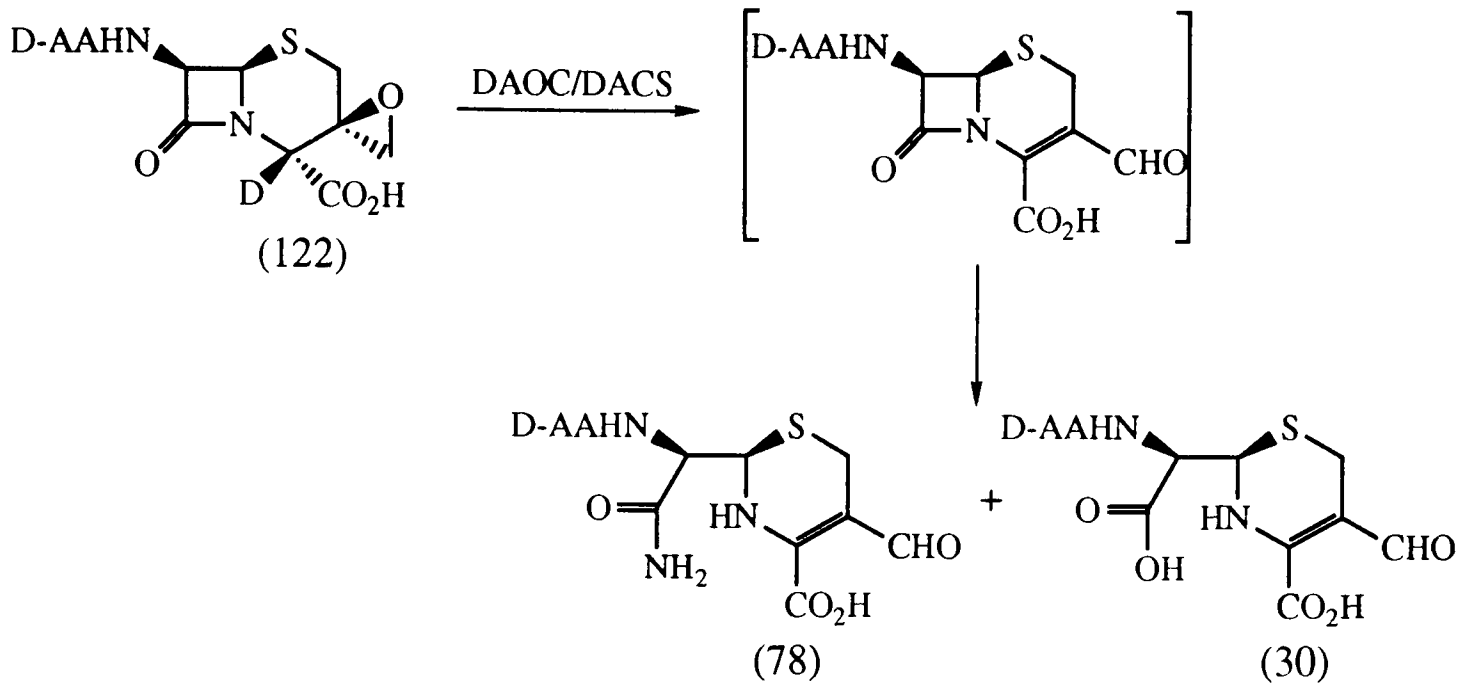
Having finally isolated the second product in the incubation of (63a) with DAOC/DACS and identified it as the epoxide (122), it was then necessary to re-incubate it with the enzyme to elucidate its possible role as an intermediate. It was expected that (122) would be an enzyme-free intermediate in the conversion of (63a) to DAC (3), and that it would be converted through to DAC (3) by DAOC/DACS.

However, when the epoxide (122) was incubated with DAOC/DACS it was observed that the main product of conversion was an aldehyde cephalosporoate [as is obtained in the enzymatic oxidation of DAC (3)], with DAC (3) being detected in only trace amounts. The same result was obtained in several incubations displaying different degrees of conversion of the epoxide (122).

Two separate incubations of (122) were carried out as control experiments. In one of them the enzyme was pre-inactivated by heat, and in the other active enzyme was used but in the absence of the co-substrate α-ketoglutarate (31). Both these incubations displayed no conversion at all, with the epoxide (122) being recovered intact. This confirmed that the conversion of (122) was mediated by DAOC/DACS, and furthermore that it was dependent on the formation of the iron(IV)-oxene (49) through decarboxylation of α-ketoglutarate.

The crude incubations of (122) showed formation of apparently three aldehyde products by ^{1}H-NMR analysis. One of these incubation mixtures was purified by HPLC and two products (78) and (30) were isolated (Scheme 2.21), with (78) being the major one. These products probably arise from a single enzymatically formed aldehyde which was not isolated due to the high reactivity of its β-lactam ring. Attack by different nucleophiles leads to the formation of different products as was observed in a separate study on the oxidation of DAC.[145] Although only the two products (78) and (30) were isolated in sufficient amounts to be characterised, other peaks were observed in the HPLC run absorbing at λ 300 nm (absorption characteristic of the vinyl formyl system) suggesting that

other aldehyde products had been formed (as indicated by the ^{1}H-NMR spectrum of the crude incubation mixtures which showed three aldehyde peaks). Multiple product formation was also observed in the oxidation of DAC (3) by DAOC/DACS.[221]



Scheme 2.21

These surprising results indicated that, contrary to what was expected, the epoxide (122) was not an enzyme-free intermediate but a shunt product whose formation was obviously being enhanced by the operation of a deuterium isotope effect. The possibility that (122) was being converted to DAC (3) which was then oxidised straight to the aldehyde was ruled out, as that would imply that the rate of oxidation of DAC (3) was higher than the rate of epoxide conversion to DAC . If this was the case, no DAC (3) would have been observed in the incubations of (63a) as it would be oxidised rapidly to the aldehyde after having been formed. We were thus led to the conclusion that the epoxide (122) was oxidised directly to the aldehyde.

In the previous incubations of [4-^{2}H]-3-exomethylene cephalosporin C (63a) the formation of cephalosporoate aldehyde products was not expected, so their presence had gone undetected. Some further incubations of (63a) were carried out and these confirmed that aldehydic products were being formed as well as DAC (3) and the epoxide (122). This meant that in the incubations of (63a) two of the products formed [DAC (3) and the epoxide

(122)] were also substrates for DAOC/DACS, and so there were three metabolites present competing for the enzyme active site.

In order to try to study this process in more detail, parallel incubations of protiated and deuterated 3-exomethylene cephalosporin C (63) and (63a) were carried out under the same conditions. It was observed that in incubations of (63) the presence of aldehydic products could not be detected until conversion of (63) was practically complete. On the other hand, in incubations of (63a) aldehydic products were always detected for conversions higher than 20%. These observations enabled the following conclusions to be drawn :

- The aldehydic products in incubations of (63a) arise from conversion of the epoxide (122) and not from DAC (3).
- The 3-exomethylene (63) is a better substrate for DAOC/DACS than DAC (3) (higher V_{max} and/or lower K_m), as this is not oxidised until practically all (63) has been converted.
- The epoxide (122) is a better substrate for oxidation than DAC (3) but a similar one to (63a), as its conversion is also observed as a competing process during the conversion of (63a).

It is not surprising that the oxidation of DAC (3) should have a low specificity constant (K_{cat}/K_m, where $K_{cat}=V_{max}/[E_o]$) as this is a biologically undesirable reaction, resulting in destruction of the β-lactam ring.

It was expected that formation of the aldehydic products in the incubations of (63a) would increase linearly with the decrease in amounts of the epoxide (122) present. However a simple direct relationship was not observed, although higher amounts of aldehydes were usually observed when the amounts of epoxide were decreasing. One possible explanation for the fact that no direct relationship was observed is the difficulty in quantifying the amounts of aldehyde products formed (due to their multiplicity). Another possibility is that these aldehydes are further oxidised to carboxylic acids by DAOC/DACS (presumably before their β-lactam ring is hydrolysed) in a reaction analogous to that performed by thymine 7-hydroxylase[146] (see section 1.3.4).

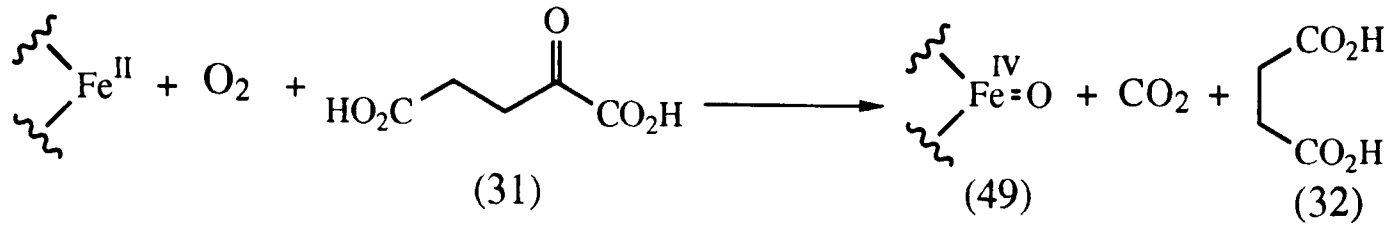
2.6 Conclusions

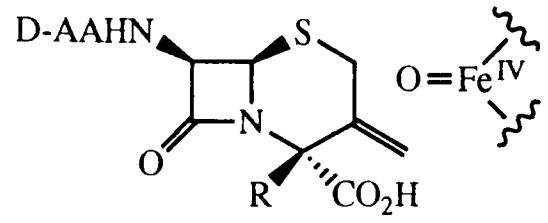
From the work that has been described the following conclusions could be drawn with respect to the conversion of 3-exomethylene cephalosporin C (63) by DAOC/DACS :

- The protiated and deuterated 3-exomethylene cephalosporin C (63) and (63a) have identical V_{max}/K_m parameters, which indicates that the first irreversible step in the conversion of (63) does not involve cleavage of the C4-H bond.
- A second product is formed in the conversion of (63a) [the epoxide (122)], which is not observed in incubations of (63).
- This epoxide metabolite (122) is not an intermediate in the conversion of (63a) to DAC(3), but a shunt metabolic product which is also a substrate for DAOC/DACS being oxidised to an aldehyde.

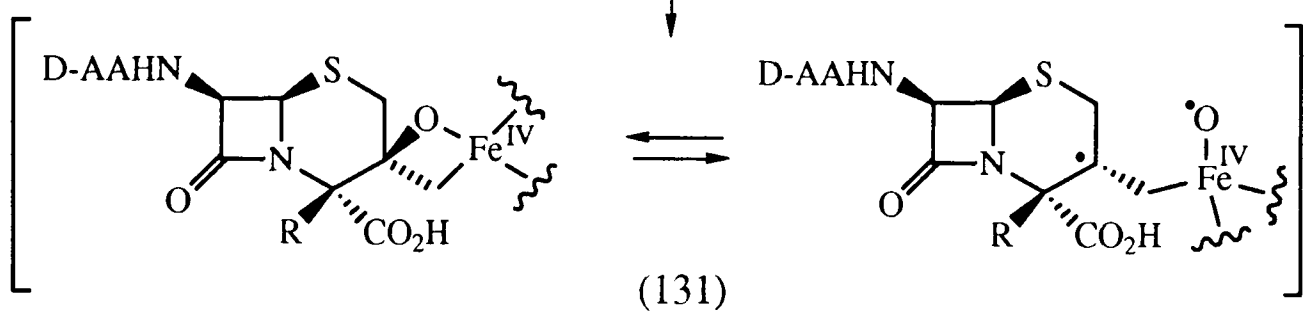
Taking account of all these observations the following mechanism is proposed to be operating in the conversion of (63)/(63a)[197] (Scheme 2.22) : After formation of the iron-oxene (49), the first irreversible step with respect to the β-lactam substrate is the addition of the ferryl species (49) to the double bond with formation of the enzyme-bound intermediate (131). In the case of (63) (R = H), cleavage of the C4-H bond to give intermediate (62) is the next step (pathway A) followed by reductive elimination of Fe^{II} to give the product DAC (3). In the case of (63a) (R = D) the rate of pathway A is slowed down due to the operation of a deuterium isotope effect, which leads to an increase in the concentration of the intermediate (131), and thus to an increase in the rate of pathway B in which direct reductive elimination from (131) generates the epoxide (122).

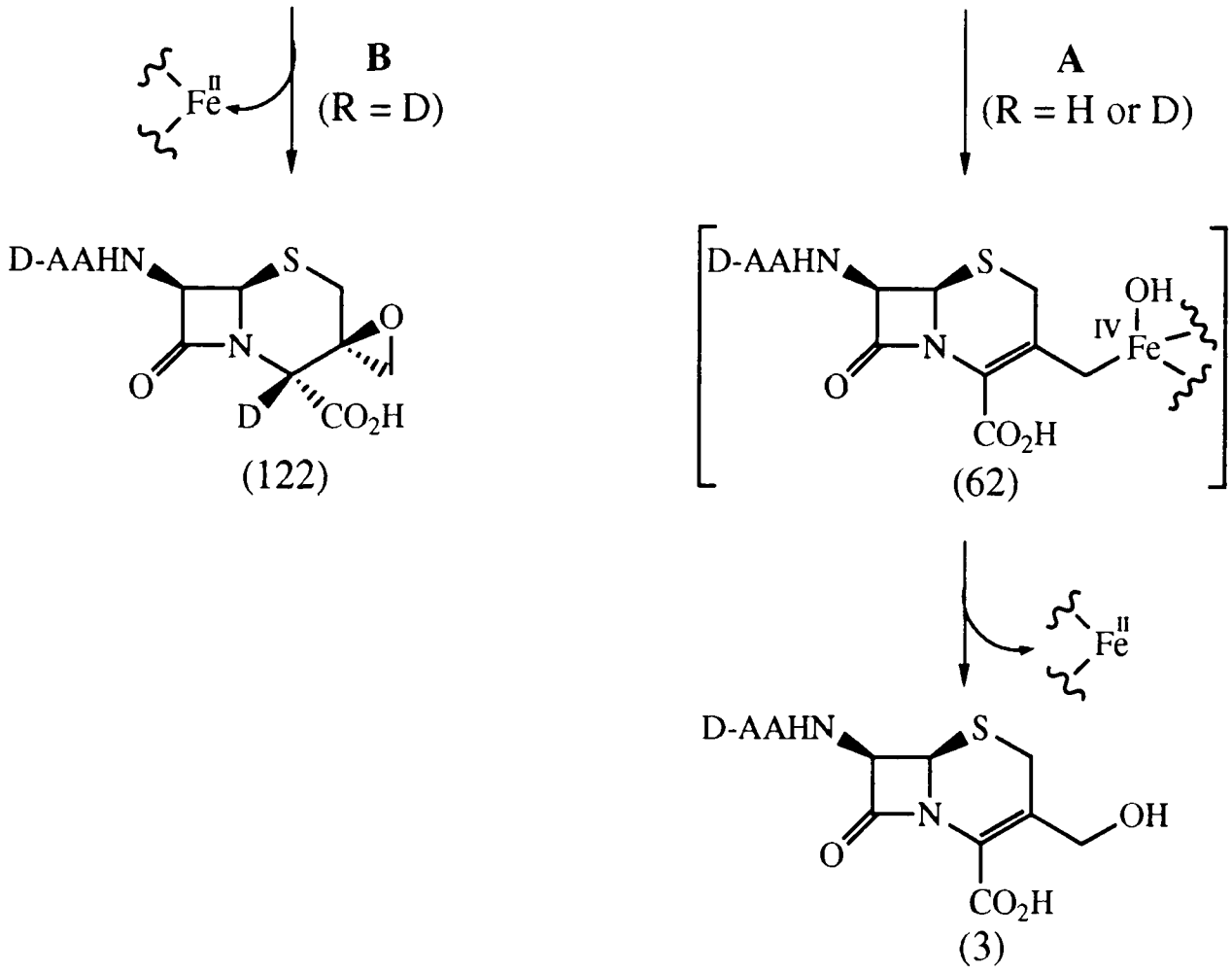
It is believed that in the conversion of (63) (R = H) both pathways are possible but only the more rapid pathway A is observed. In the case of (63a), it is the operation of a deuterium isotope effect on the enzyme-bound intermediate (131) that causes branching of the pathway with formation of the shunt metabolite (122). An analogous isotope-induced branching to form a shunt metabolic product has also been proposed to account for the increased formation of the 3β-hydroxycepham (40a) in the incubation of deuterated penicillin N (1d) (see section 1.3.3.1).[117,120]





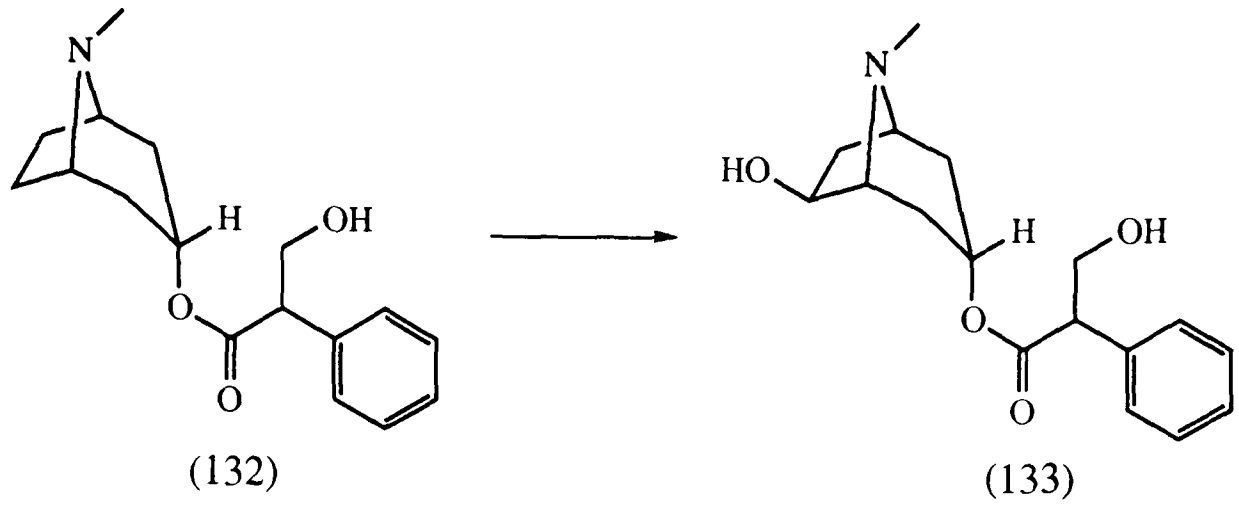
(63) R = H
(63a) R = D



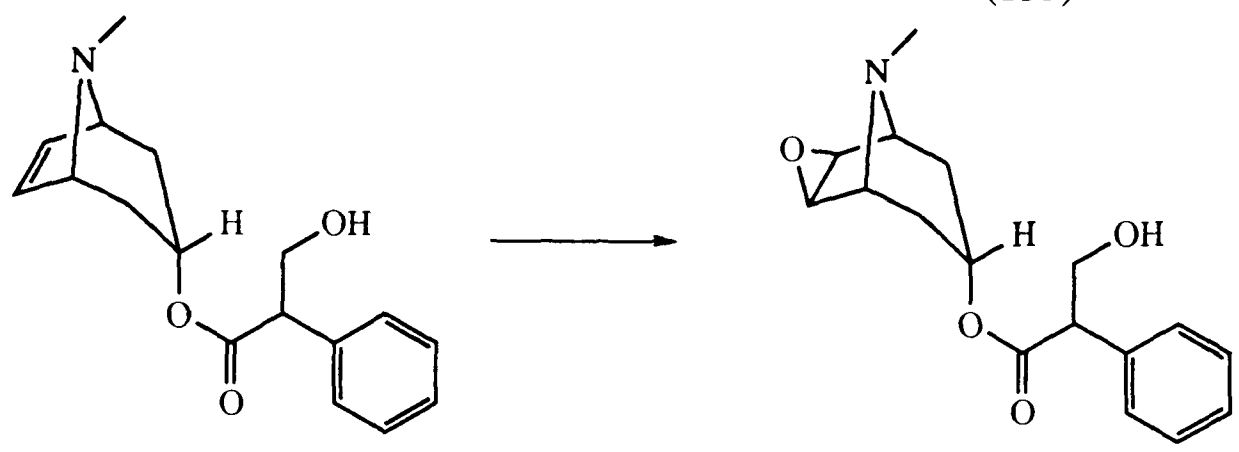


Scheme 2.22

The formation of (122) represents the first example of epoxidase activity so far detected with DAOC/DACS. This type of activity has only been observed for two other α-ketoglutarate dependent dioxygenases. In the case of hyoscyamine 6β-hydroxylase which converts hyoscyamine (132) to 6β-hydroxyhyoscyamine (133) it was found that the enzyme also epoxidated 6,7-dehydrohyoscyamine (134) to scopolamine (135) (Scheme 2.23).[159]



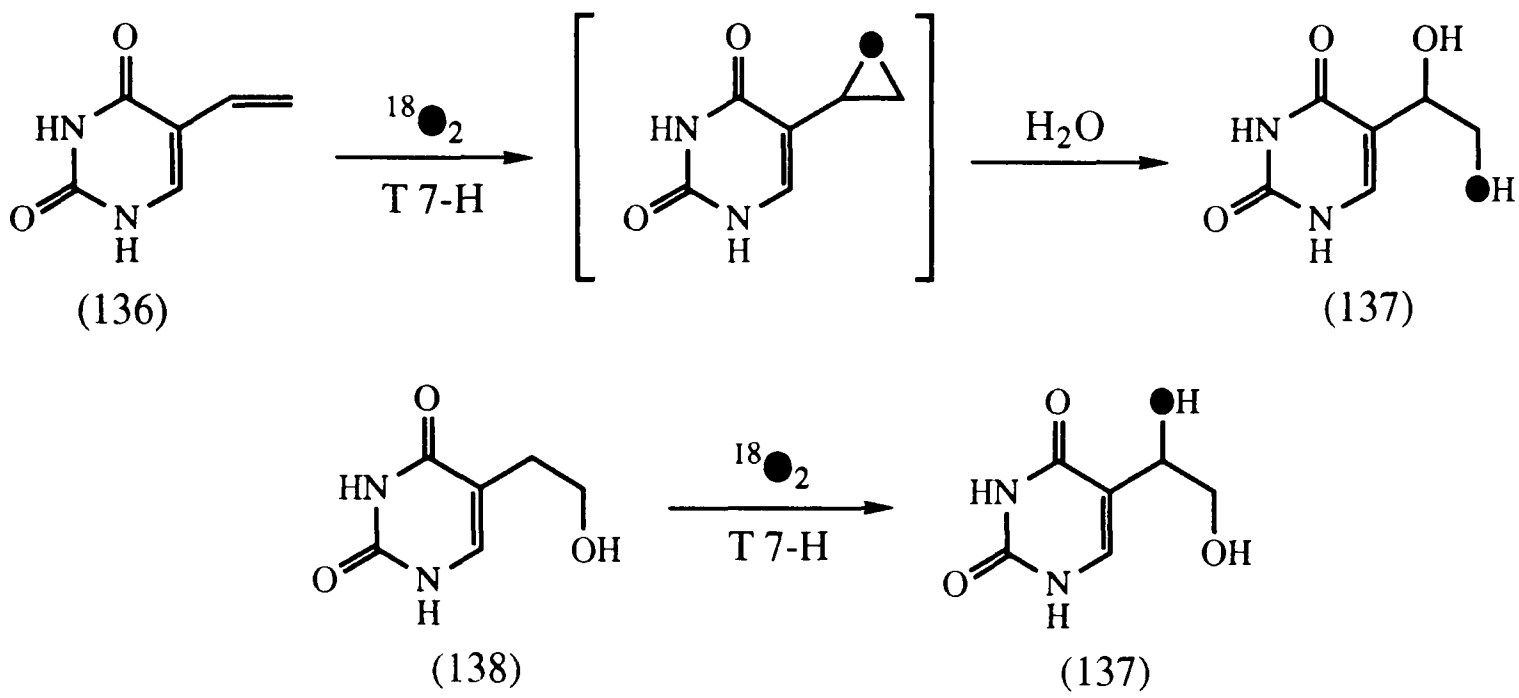
(132) (133)



(134) (135)

Scheme 2.23

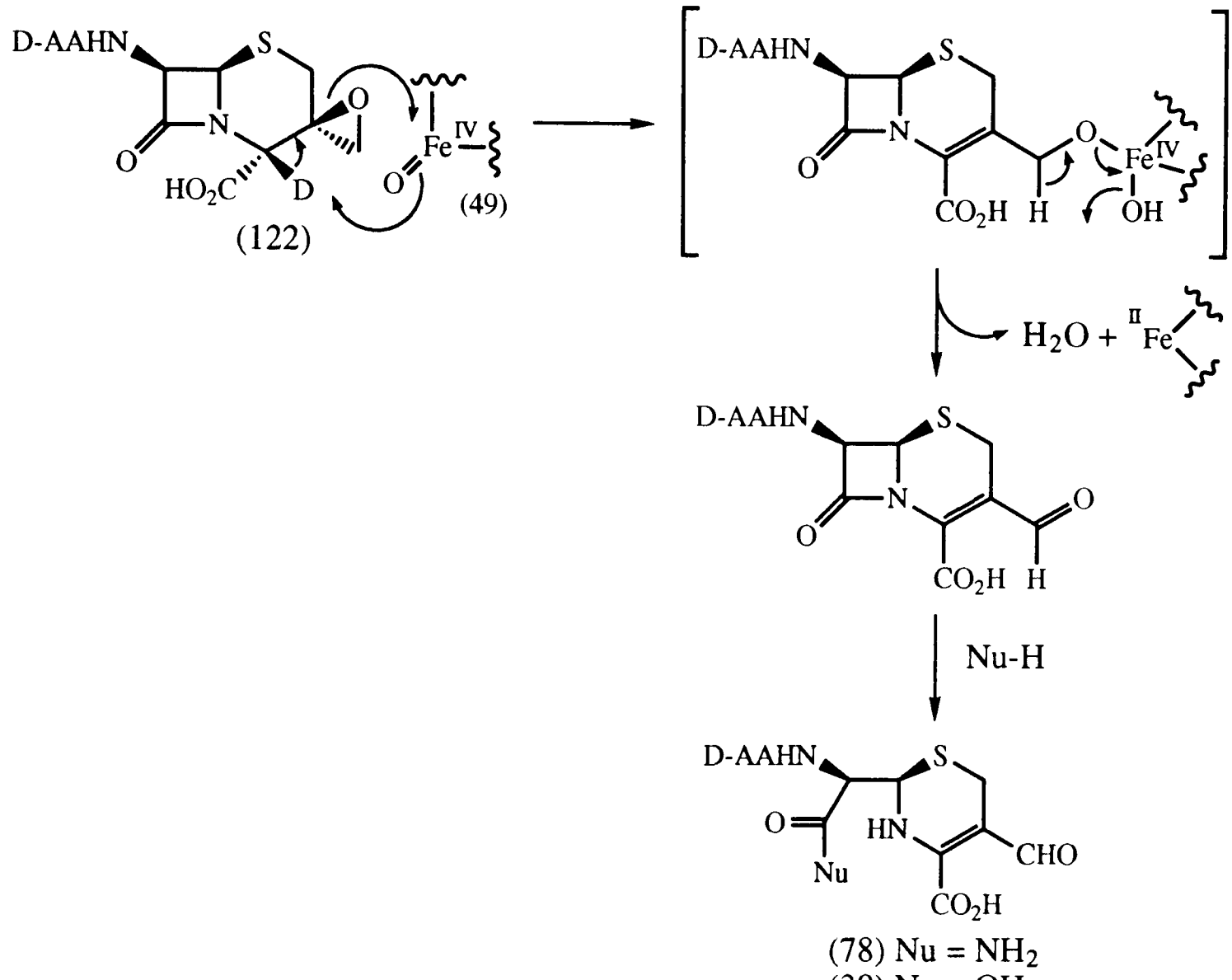
Evidence for epoxide formation was also obtained in the case of thymine 7-hydroxylase (T 7-H), the trifunctional enzyme that carries out three successive oxidations of the methyl group of thymine (82) to uracil-5-carboxylic acid (85) (see section 1.3.4). It was found that incubation of 5-vinyluracil (136) resulted in the isolation of 5-(1,2-dihydroxyethyl)uracil (137), identical to the product obtained by conversion of 5-(2-hydroxyethyl)uracil (138) (Scheme 2.24).[222] It was thought that (137) resulted from enzyme-catalysed epoxidation of (136) followed by ring opening (assisted by the N-1 position of the uracil ring) and Michael addition by the solvent. This proposal was supported by incubations with $^{18}O_2$.



Scheme 2.24

In the present case, the further oxidation of the epoxide (122) to an aldehyde by DAOC/DACS is an unprecedented reaction. It is proposed that this reaction occurs by initial ferryl-mediated isomerisation of (122) to enzyme-bound DAC (3) (Scheme 2.25). This process does not lead to a change in the oxidation level of either the substrate or the ferryl moiety, and thus permits the rapid oxidation of the bound DAC to an aldehyde, which then suffers attack on the β-lactam ring to produce the aldehyde cephalosporoates isolated [e.g. (78) and (30)]. Minor leakage of isomerised epoxide from the active site would also account for the very low levels of DAC (3) observed in incubation mixtures of (122) with DAOC/DACS [although these may also be the result of chemical isomerisation of (122)].

In conclusion, a new mode of reactivity has been identified for DAOC/DACS which led to the isolation of a previously unknown metabolite, the 3β-spiroepoxide cepham (122). These studies have contributed to our understanding of the *modus operandum* of this important enzyme, further demonstrating its versatility in terms of the reactions it can catalyse. This muti-reactivity, which is shared with some other α-ketoglutarate dioxygenases, is possibly a result of the high chemical potential and consequently low selectivity of the proposed iron(IV)-oxene intermediate (49).


D-AAHN
S
N
O
HO2C
D
(122)
Fe IV
(49)
H2O + II Fe
CO2H
H
OH
Nu-H
HN
CHO
Nu
(78) Nu = NH2
(30) Nu = OH

Scheme 2.25

CHAPTER 3



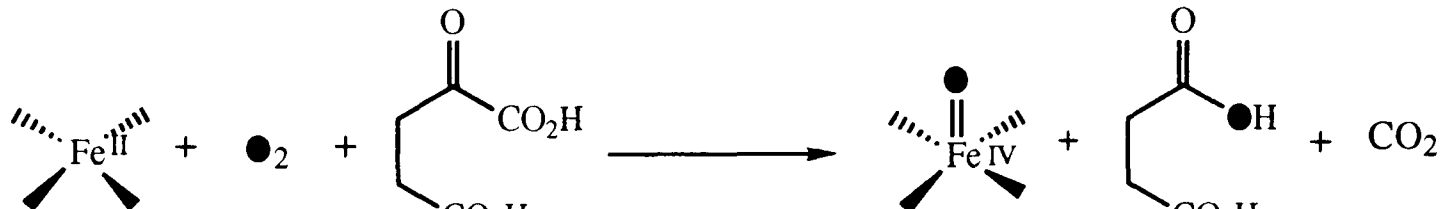
^{18}O-Oxygen Studies with DAOC/DACS

Chapter 3

^{18}O-Oxygen Studies with DAOC/DACS

3.1 Introduction

The formation of the reactive intermediate iron(IV)-oxene (49) through oxidative decarboxylation of α-ketoglutarate (α-KG) (31) is central to the mechanism of all α-ketoglutarate dependent dioxygenases (Scheme 3.1).



(31) (49) (32)

Scheme 3.1

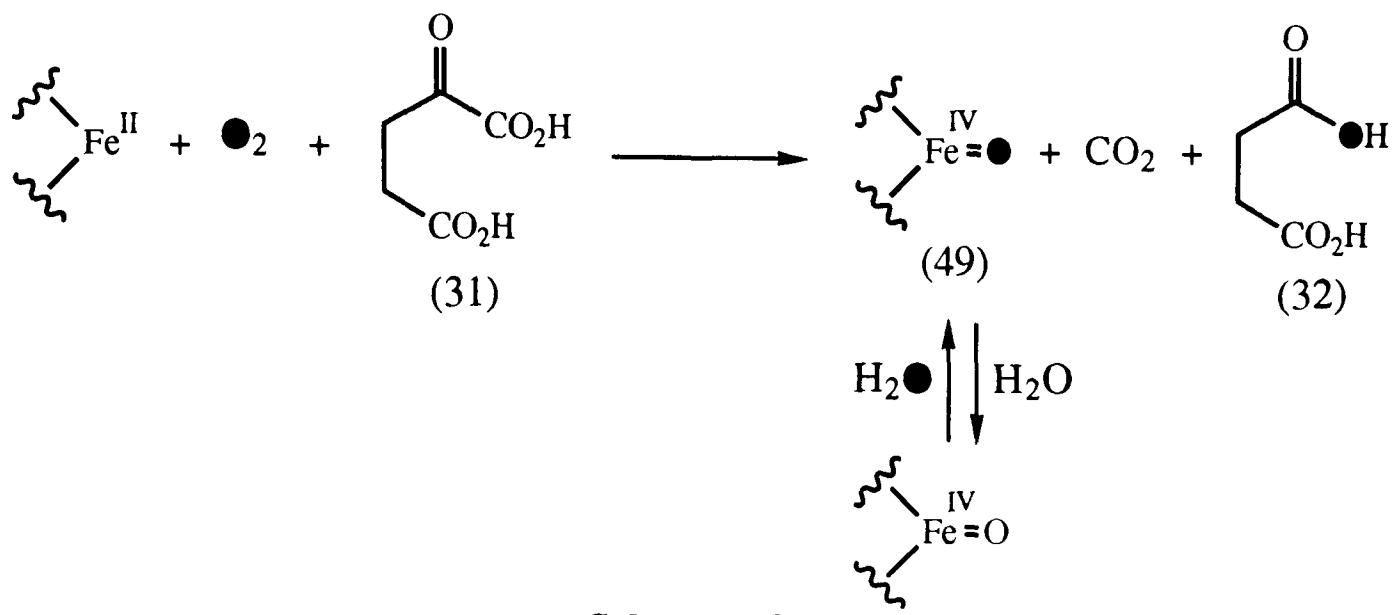
Evidence and characterisation of iron-oxene species in which an electron-deficient iron is coordinated to a single oxygen atom, is well documented in several oxidative enzymatic[128,223] and synthetic systems[224]. In the case of α-ketoglutarate dependent dioxygenases the decarboxylation of α-KG (31) is thought to be coupled to the breakage of the O-O bond in dioxygen, so that one of the oxygen atoms from O_2 appears in succinate (32) and the other one forms the Fe^{IV}=O (49) (and ultimately appears in the product or is reduced to water). Labelling studies using ^{18}O-oxygen provide a good experimental test to this proposal, and some of these studies have been carried out for this type of enzyme.

Very early studies into the hydroxylation of proline residues using intact cells revealed that there was incorporation of ^{18}O into hydroxyproline resulting from incubations under $^{18}O_2$ [225], but no apparent incorporation of label from $H_2{}^{18}O$ [226,227]. An incubation

with γ-butyrobetaine hydroxylase under an $^{18}O_2$ atmosphere led to the isolation of ^{18}O-succinate containing about 75% label incorporation.[182] In the case of thymine 7-hydroxylase, labelling studies with $^{18}O_2$ showed that high incorporation of label was observed in both the product and succinate for all the three steps it catalyses [except for the oxidation of 5-hydroxymethyluracil (83) to 5-formyluracil (84), where incorporation of label was only analysed in succinate due to expected label wash-out in the product (84) by exchange with water].[228] These results gave strong support to the proposed mechanism of oxygenation of α-ketoglutarate dioxygenases.

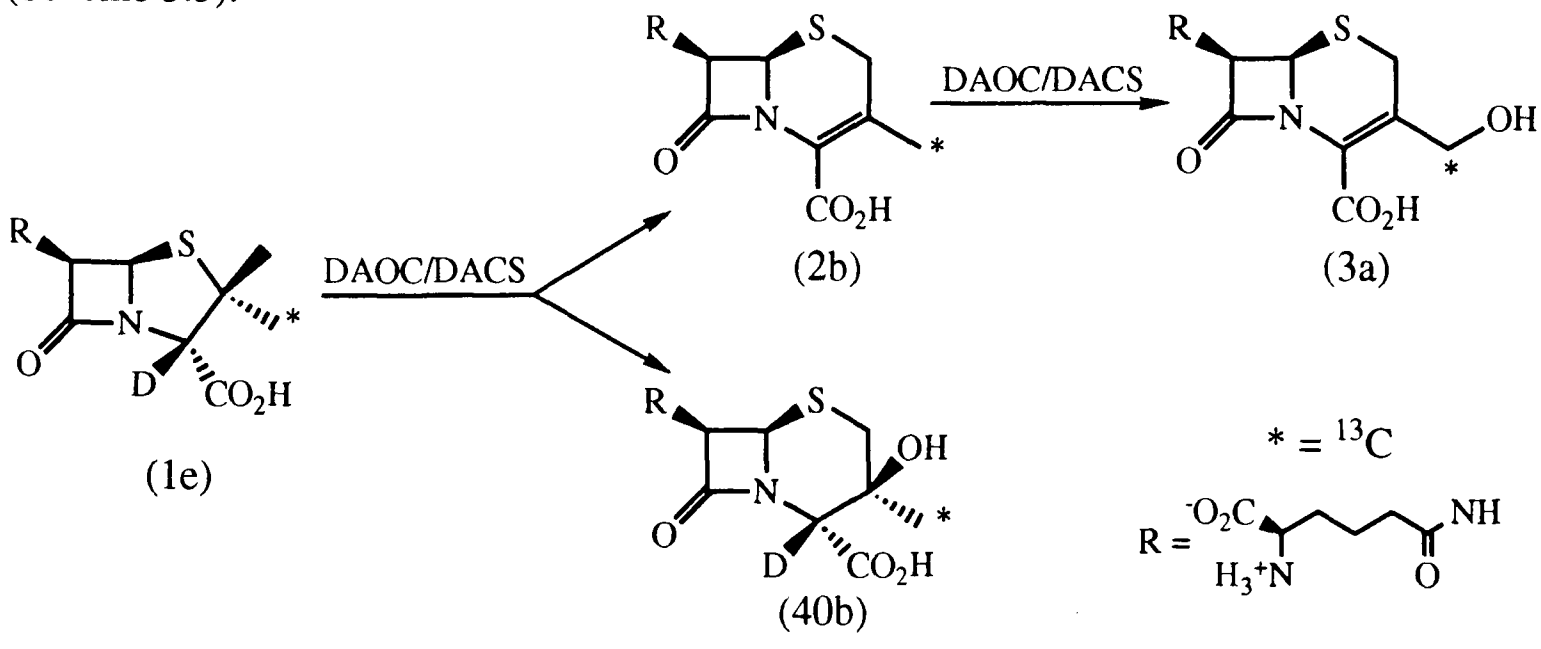
Some ^{18}O-labelling studies have also been carried out with DAOC/DACS. Incubations of [3-^{2}H]penicillin N (1d) (see Scheme 1.17) under an $^{18}O_2$ atmosphere (99 atom % ^{18}O) led to 30-40% incorporation of label into DAC (3) and 50-60% incorporation into the 3β-hydroxycepham (40a).[117,119] It was considered somewhat intriguing why the incorporation values were so low, and also why they were different from one product to the other. When this experiment was repeated for the conversion of 3-exomethylene cephalosporin C (63), a level of 30-40% ^{18}O-incorporation was again observed into the product DAC (3).[140,141]

Further labelling studies with DAOC/DACS were carried out to investigate the incorporation of oxygen into succinate (32). Surprisingly, it was found that after incubating DAOC (2) with DAOC/DACS under an atmosphere of $^{18}O_2$ (96 atom % ^{18}O) the isolated DAC (3) retained about 50% ^{18}O label, whereas the incorporation into succinate was over 90%.[229] This result seemed to suggest that exchange of oxygen was occurring after formation of the iron(IV)-oxene and before product release from the enzyme. It was then proposed that this exchange occurred between the $Fe^{IV}=O$ (49) and water from the medium, resulting in wash-out of label (Scheme 3.2). This would explain the low levels of ^{18}O incorporation into the products (40a) and (3), and also the difference of levels between the two as different rates of product formation would affect the extent of oxygen exchange.



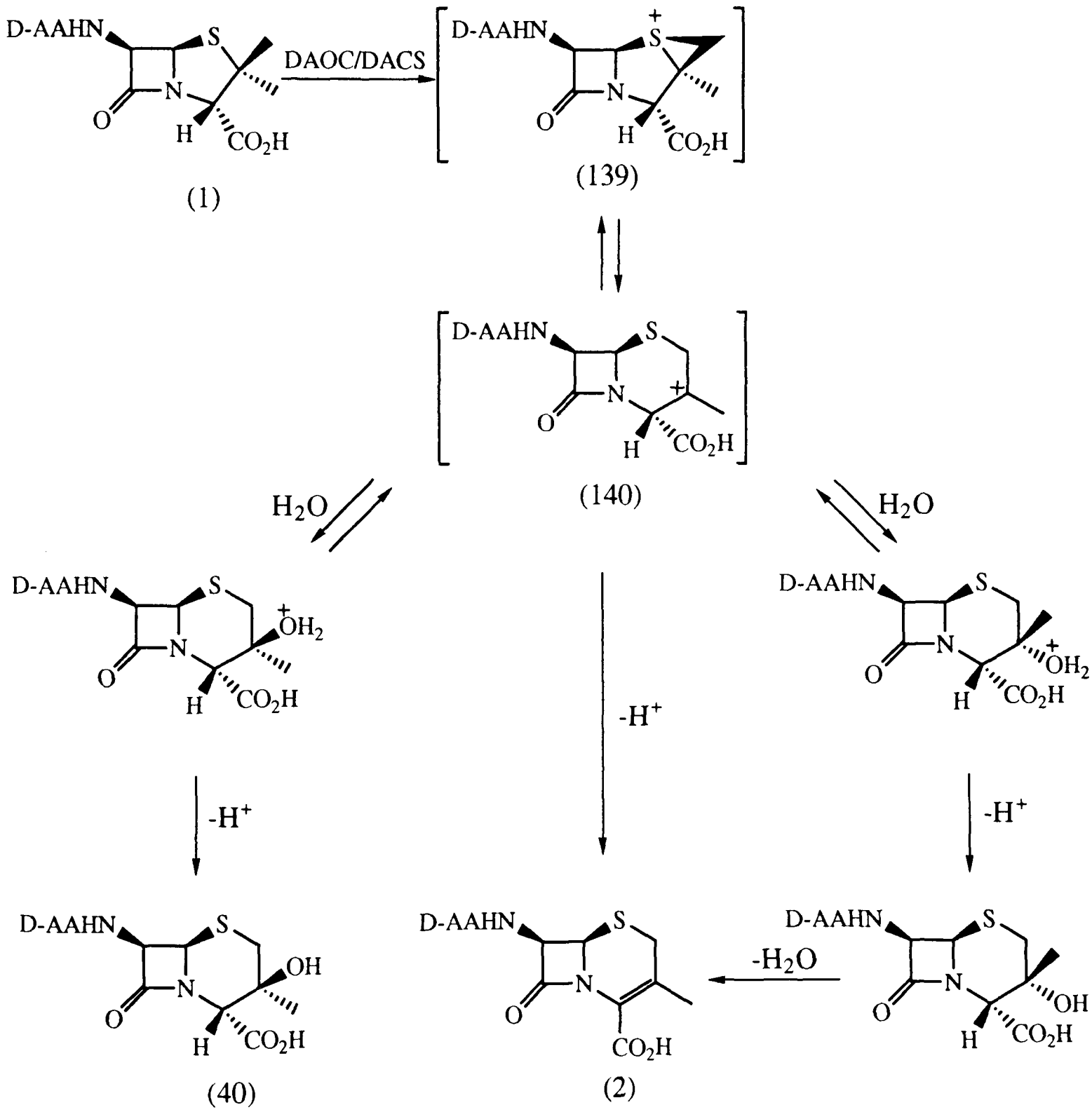
Scheme 3.2

It was envisaged that such a proposal could be tested by carrying out incubations in $H_2{}^{18}O$. Due to the technical difficulties in obtaining a high $H_2{}^{18}O$ enrichment of the incubation medium, it was feared that detection of ^{18}O incorporation would present a problem, as the mass spectroscopy technique (FAB) available for this type of samples at the outset of this work was not particularly sensitive. It was thus decided to use ^{13}C-labelled substrates as it is known that an ^{18}O-oxygen causes an upfield shift in the ^{13}C-NMR resonance of the carbon to which it is bonded[230]. The magnitude of such shift depends upon the structure of the molecule and the functional group in which the label is located.[231] The observation of an ^{18}O-^{13}C isotope shift in the ^{13}C-NMR of the products would provide a complementary method for detecting ^{18}O-incorporation to the usual mass spectrometry, and would also confirm the expected position of label incorporation. Previous ^{13}C-labelling studies with [2*-^{13}C,3-^{2}H]penicillin N (1e) and DAOC/DACS had demonstrated the stereospecificity of the incorporation of ^{13}C-label into the products (2b), (40b) and (3a) (Scheme 3.3).[119,232]



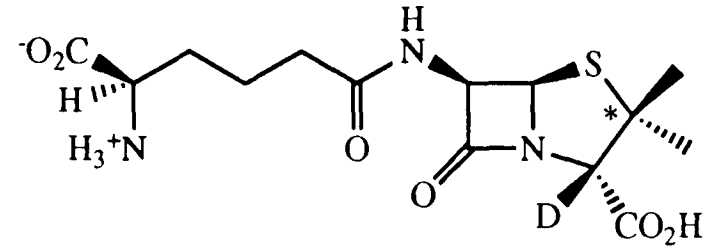
Scheme 3.3

It was decided to place the ^{13}C-label in the C2 of penicillin N (1), which would ultimately become the C3 of the products and would lead to an observable ^{18}O-^{13}C shift in the 3β-hydroxycepham (40) [but not in DAC (3)]. It was considered of special interest to detect $H_2{}^{18}O$ incorporation into the 3β-hydroxycepham (40) in order to clarify earlier mechanistic speculations[118] which suggested that this compound was formed by water interception of the episulphonium (139) or carbocation (140) (Scheme 3.4).



Scheme 3.4

The $H_2{}^{18}O$ incubations had to be carried out with a C3 deuterated penicillin N in order to increase formation of the shunt product 3β-hydroxycepham (40). It was thus necessary to synthesise the [2-^{13}C,3-^{2}H]penicillin N (1f).



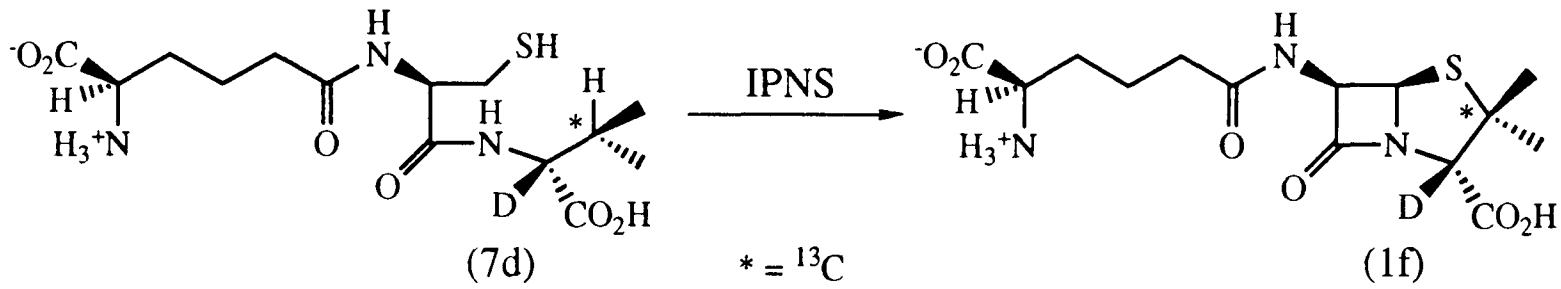
(1f)

* = ^{13}C

In addition, it was proposed to study the ^{18}O incorporation into the new epoxide metabolite (122) (see section 2.5) by carrying out incubations of [4-^{2}H]-3-exomethylene cephalosporin C (63a) with DAOC/DACS in $H_2{}^{18}O$ or under $^{18}O_2$.

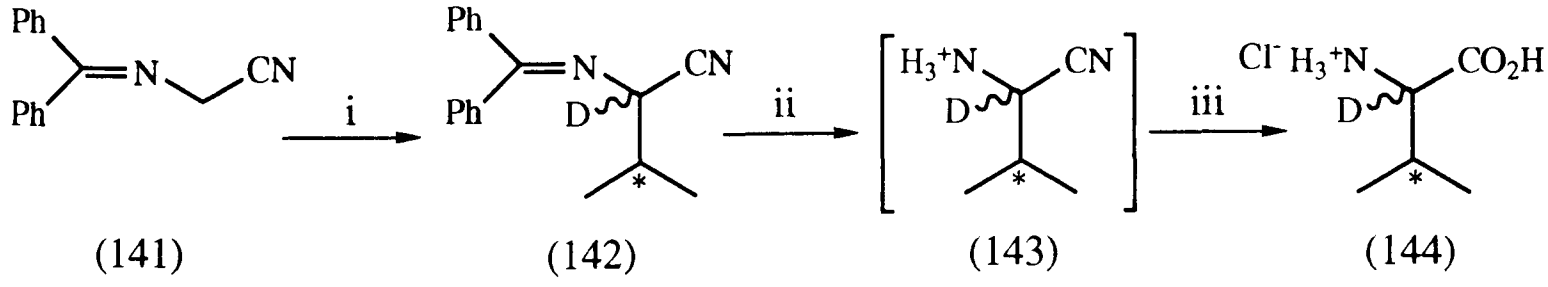
3.2 Synthesis and Incubation of [2-^{13}C,3-^{2}H]Penicillin N

The synthesis of the labelled penicillin (1f) could be achieved by the usual enzymatic cyclisation of the corresponding tripeptide (7d) using IPNS (Scheme 3.5). The tripeptide (7d) could be synthesised in a protected form through coupling of the corresponding protected amino acids. Acid labile protecting groups are usually used in the synthesis of tripeptides, as these can be easily removed at the end of the synthesis by refluxing in TFA/anisole.



Scheme 3.5

It was thus necessary to prepare the [^{13}C,^{2}H]-labelled valine (6f)[61]. This was accomplished in the following way[233] (Scheme 3.6) : Treatment of the imine (141) under phase transfer alkylation conditions with (2-^{13}C)-bromopropane (90 atom % ^{13}C), in the presence of sodium deuteroxide and a catalytic amount of benzyltriethylammonium chloride (BTEAC) as phase transfer catalyst, gave the alkylated imine (142). Hydrolysis of this imine with dilute acid (1N HCl) at room temperature gave the amino-nitrile (143) which was further hydrolysed by reflux in 6N HCl to give the racemic valine hydrochloride salt (144).

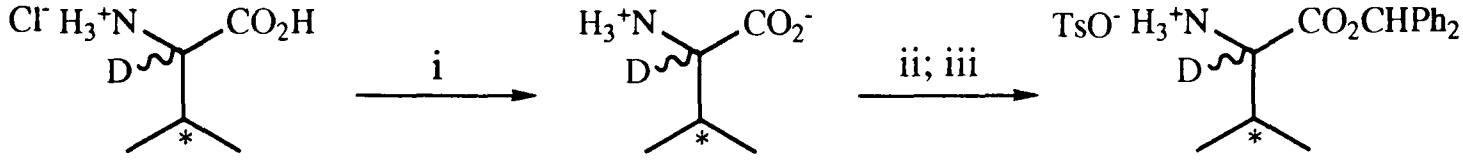


Reagents : i) BTEAC, Toluene, (2-^{13}C)-2-bromopropane, 50% NaOD
ii) 1N HCl, 23°C, 12h
iii) 6N HCl, reflux, 12h

* = ^{13}C

Scheme 3.6

Purification of (144) by ion-exchange chromatography (Dowex-1X-8-400, acetate form) gave after lyophilisation the free labelled valine (6f) (Scheme 3.7). Mass spectrometry revealed that 98 atom % deuterium had been incorporated into (6f). Protection of (6f) with *p*-toluenesulphonic acid and diphenyldiazomethane gave the protected labelled valine (145) which retained the same level of labelling as (6f).



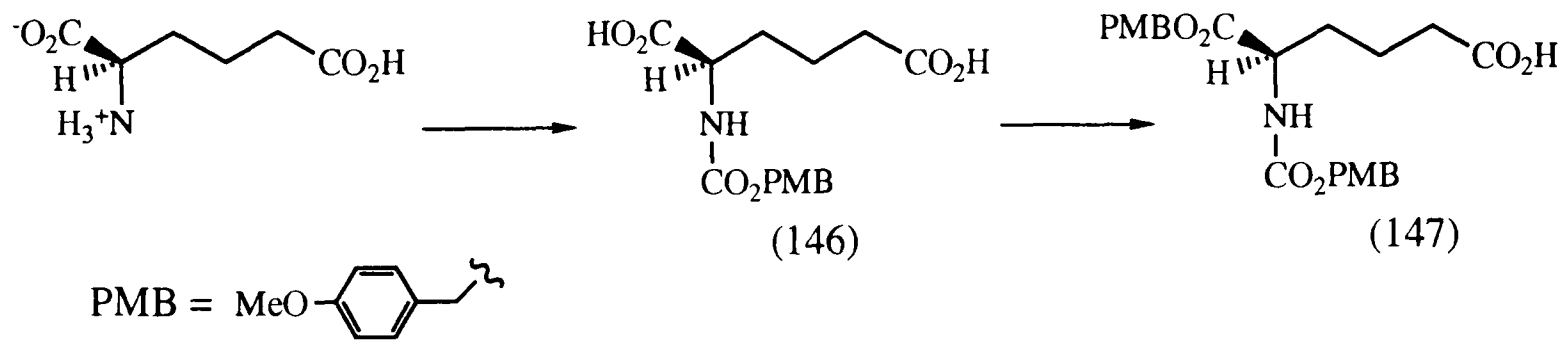
(144) (6f) (145)

Reagents : i) Ion exchange chromatography
ii) TsOH, H_2O/EtOH
iii) Ph_2CN_2, CH_3CN

* = ^{13}C

Scheme 3.7

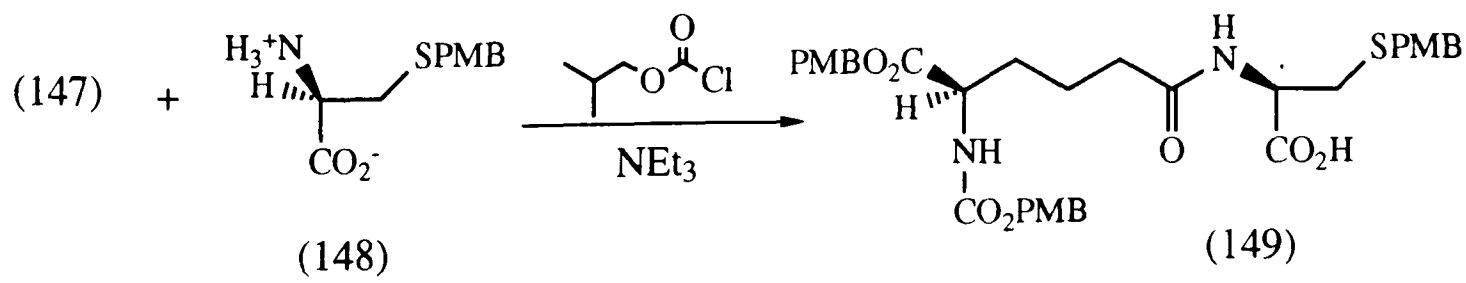
The protected form of δ-D-α-aminoadipic acid was obtained by treating it with *p*-methoxybenzyloxycarbonylazide to give (146), followed by reaction with *p*-methoxybenzylchloride to give the desired di-protected form (147) (Scheme 3.8).



(146) (147)

Scheme 3.8

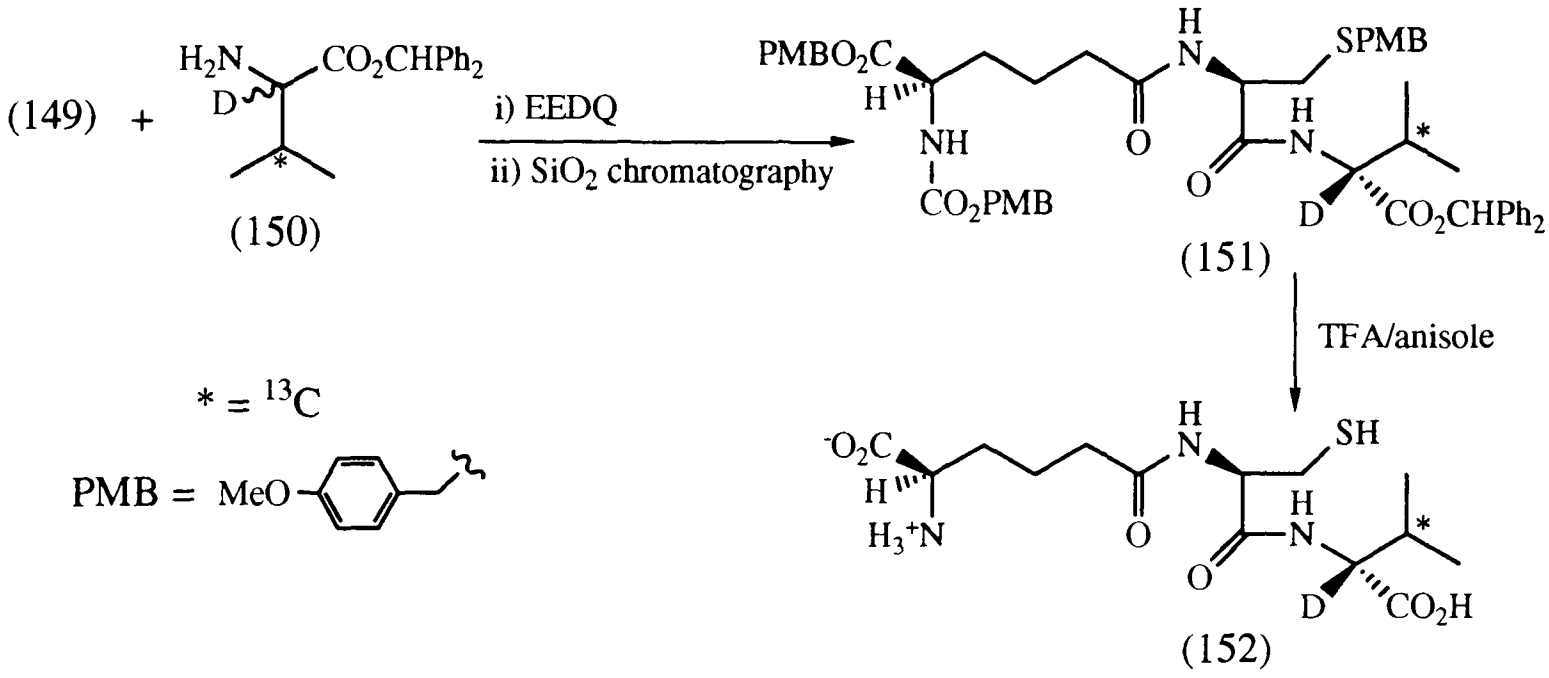
The protected D-α-aminoadipic acid (147) was coupled with S-*p*-methoxybenzyl-L-cysteine (148) using isobutylchloroformate and triethylamine to give the protected dipeptide D,L-AC (149) (Scheme 3.9).



(148) (149)

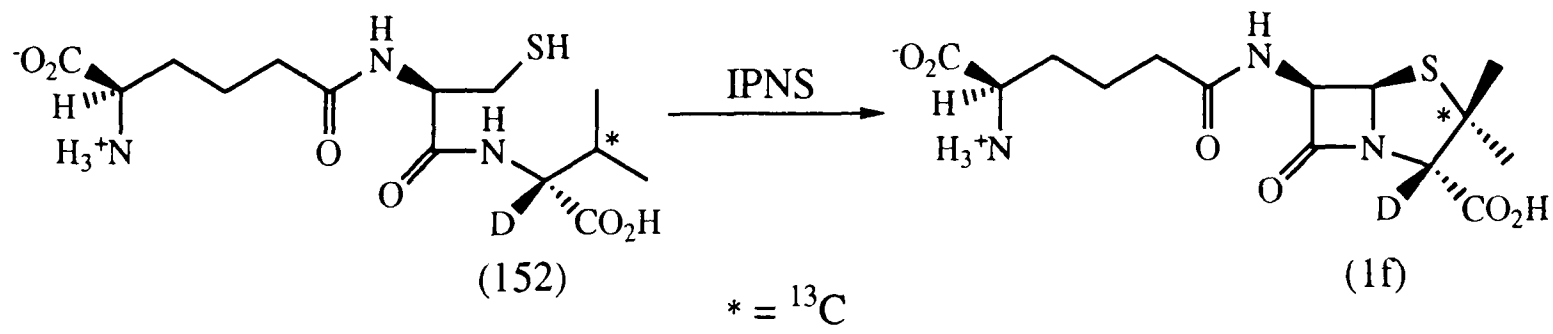
Scheme 3.9

D,L-AC (149) was then coupled to the racemic protected [2-^{2}H,3-^{13}C]valine (150) using EEDQ[220], to give a mixture of the two diastereomeric D,L,D- and D,L,L-tripeptides which could be separated by chromatography on silica gel yielding the desired D,L,D-tripeptide (151) (which is the less polar of the two isomers[133]) (Scheme 3.10). The tripeptide (151) was deprotected by refluxing in trifluoroacetic acid containing 20% anisole[234] to give the labelled D,L,D-ACV (152) of sufficient purity that further purification by HPLC was unnecessary.



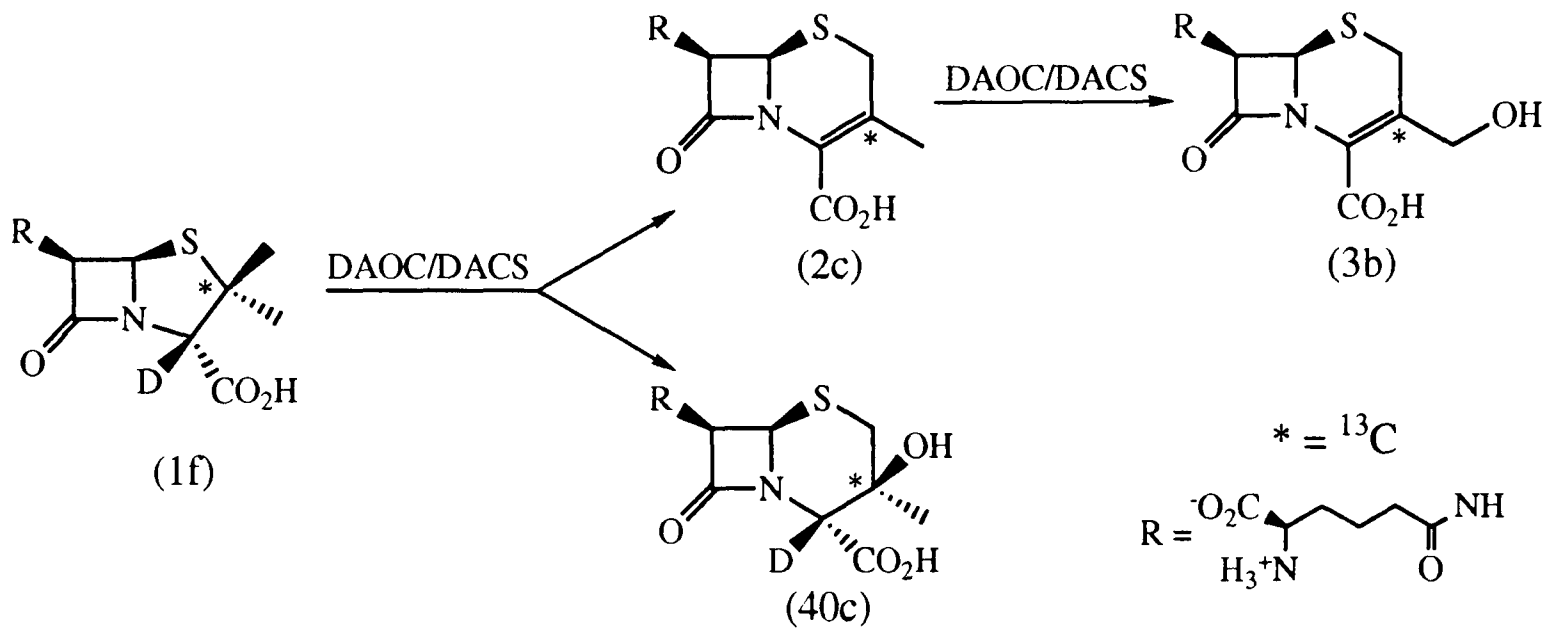
Scheme 3.10

The tripeptide (152) was then incubated with IPNS in the presence of the usual cofactors to give after HPLC purification the desired [2-^{13}C,3-^{2}H]penicillin N (1f) (Scheme 3.11).



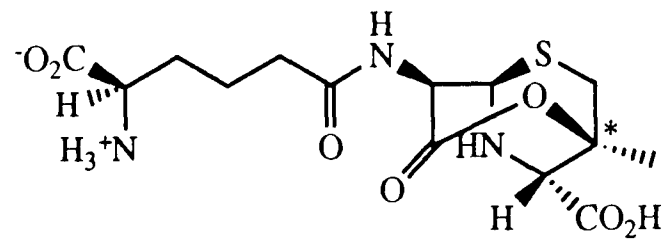
Scheme 3.11

Before carrying out any ^{18}O-labelling studies, it was decided to incubate the penicillin (1f) with DAOC/DACS under a normal atmosphere as a control experiment. Examination of the crude incubation mixture by ^{1}H-NMR revealed that about 85% conversion of (1f) to the expected products had occurred. Purification by HPLC led to the isolation of the labelled products [3-^{13}C]DAOC (2c), [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40c) and [3-^{13}C]DAC (3b) (Scheme 3.12).



Scheme 3.12

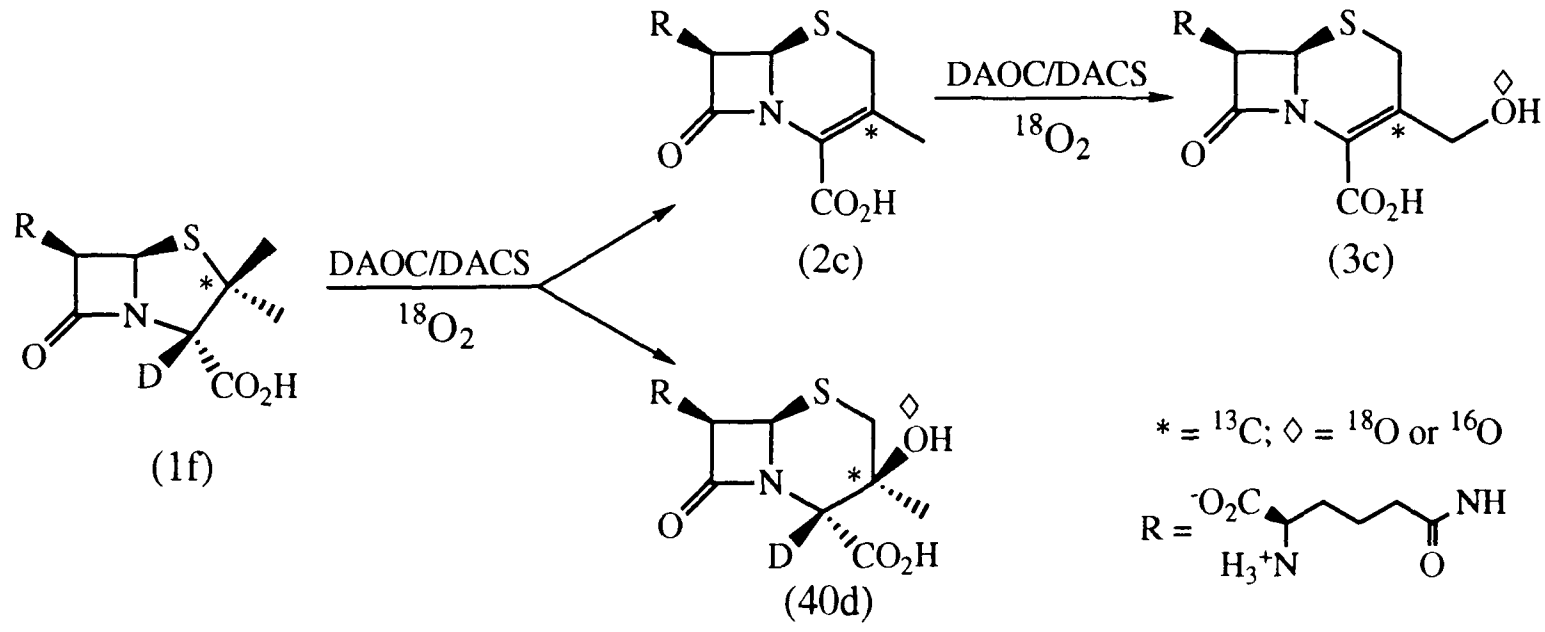
The ^{13}C-NMR of (40c) was acquired overnight. Unfortunately this led to some decomposition of (40c) to the corresponding lactone (125a), so that two ^{13}C resonances could be observed in the spectrum - one at δ 65.46 ppm for the C3 of (40c) and one at δ 81.37 ppm for the C3 of the lactone (125a) [based on previous assignment of the ^{13}C resonances of compounds (40) and (125)[133]] [see Figure 3.1 A for (125a)].



(125a)

3.3 Studies with $^{18}O_2$

An incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) was then carried out under an atmosphere of $^{18}O_2$ (98 atom % ^{18}O, 100 ml, supplied by MSD Isotopes Ltd). The substrate and cofactor solution was thoroughly degassed before incubation, but the enzyme solution was only briefly degassed due to danger of inactivation. Both these solutions were carefully inserted into a septum sealed $^{18}O_2$ vial, and this was incubated as usual. After 2h acetone was added to quench the reaction, the vial was opened to air, and the usual work-up was carried out. Examination of the crude incubation mixture by 500 MHz ^{1}H-NMR revealed about 87% conversion of the substrate (1f) to the products (2c), (40d) and (3c) (Scheme 3.13), in a ratio of (2c):(40d):(3c) = 20:53:27. These products were isolated by HPLC and analysed by electrospray mass spectrometry (ESMS). The results showed no incorporation of label into (2c) as expected, and 71% ^{18}O-label incorporation into the 3β-hydroxycepham (40d) and 52% into DAC (3c)[235] (Table 3.1, Experiment 1).



Scheme 3.13

[3-^{13}C]DAOC (2c)

	Conditions							**% ^{18}O**
		m/z (MH^+)	*358*	*359*	*360*	*361*	*362*	
	$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–
	$^{16}O_2/H_2{}^{16}O$	Found (%)	14	100	20	9	4	–
Exp. 1	$^{18}O_2/H_2{}^{16}O$	Found (%)	12	100	20	9	3	–
		m/z (MNa^+)	*380*	*381*	*382*	*383*	*384*	
Exp. 2	$^{18}O_2/H_2{}^{16}O$	Found (%)	20	100	21	15	4	–

[3-^{13}C, 4-^{2}H]-3β-Hydroxycepham (40d)

	Conditions								**% ^{18}O**
		m/z (MH^+)	*377*	*378*	*379*	*380*	*381*	*382*	
	$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–	–
	$^{16}O_2/H_2{}^{16}O$	Found (%)	15	100	26	10	2	–	–
Exp. 1	$^{18}O_2/H_2{}^{16}O$	Found (%)	9	42	29	100	21	9	71
		m/z (MNa^+)	*399*	*400*	*401*	*402*	*403*	*404*	
Exp. 2	$^{18}O_2/H_2{}^{16}O$	Found (%)	11	47	31	100	20	9	69

[3-^{13}C]DAC (3c)

	Conditions								**% ^{18}O**
		m/z (MH^+)	*374*	*375*	*376*	*377*	*378*	*379*	
	$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–	–
	$^{16}O_2/H_2{}^{16}O$	Found (%)	14	100	20	11	3	–	–
Exp. 1	$^{18}O_2/H_2{}^{16}O$	Found (%)	5	90	34	100	25	13	52
Exp. 2	$^{18}O_2/H_2{}^{16}O$	Found (%)	10	74	21	100	20	15	57

Table 3.1 : ^{18}O-oxygen incorporations[235] into the products (2c), (40d) and (3c) obtained from incubations of (1f) under $^{18}O_2$.

† calculated using oxygen at natural abundance.

This experiment was repeated under the same conditions, resulting in about 75% conversion of (1f) to the expected products in a ratio of (2c):(40d):(3c) = 26:52:22. These products were again isolated by HPLC and analysed by mass spectrometry. The results showed a level of ^{18}O-label incorporation of 69% into (40d) and 57% into (3c)[235] (Table

3.1, Experiment 2). Again no incorporation was observed into (2c). The levels of ^{18}O incorporation into (40d) and (3c) observed in the two experiments are in good agreement with each other (to within experimental error), and also to the levels previously reported[117,119].

The ^{13}C-NMR of the 3β-hydroxycepham (40d) obtained on the second $^{18}O_2$ incubation was acquired overnight. Again pratically complete decomposition of (40d) to its lactone (125b) had occurred, and so the signals for (40d) were quite weak. However, an ^{18}O-^{13}C shift could be observed for both compounds [(40d) and (125b)] with a magnitude of 0.03 ppm in the case of (40d) and of 0.04 ppm in the case of (125b) [see Figure 3.1-B for the shift in (125b)]. In both cases the intensity of the ^{16}O-^{13}C resonance was about 30% of the ^{18}O-^{13}C resonance, confirming the mass spectral results for the ^{18}O-label incorporation. The mixture of (40d) and (125b) was doped with the mixture of (40c) and (125a) previously obtained. This led to the expected increase in the intensity of the ^{16}O-^{13}C resonance for both compounds [see Figure 3.1-C for (125b)].

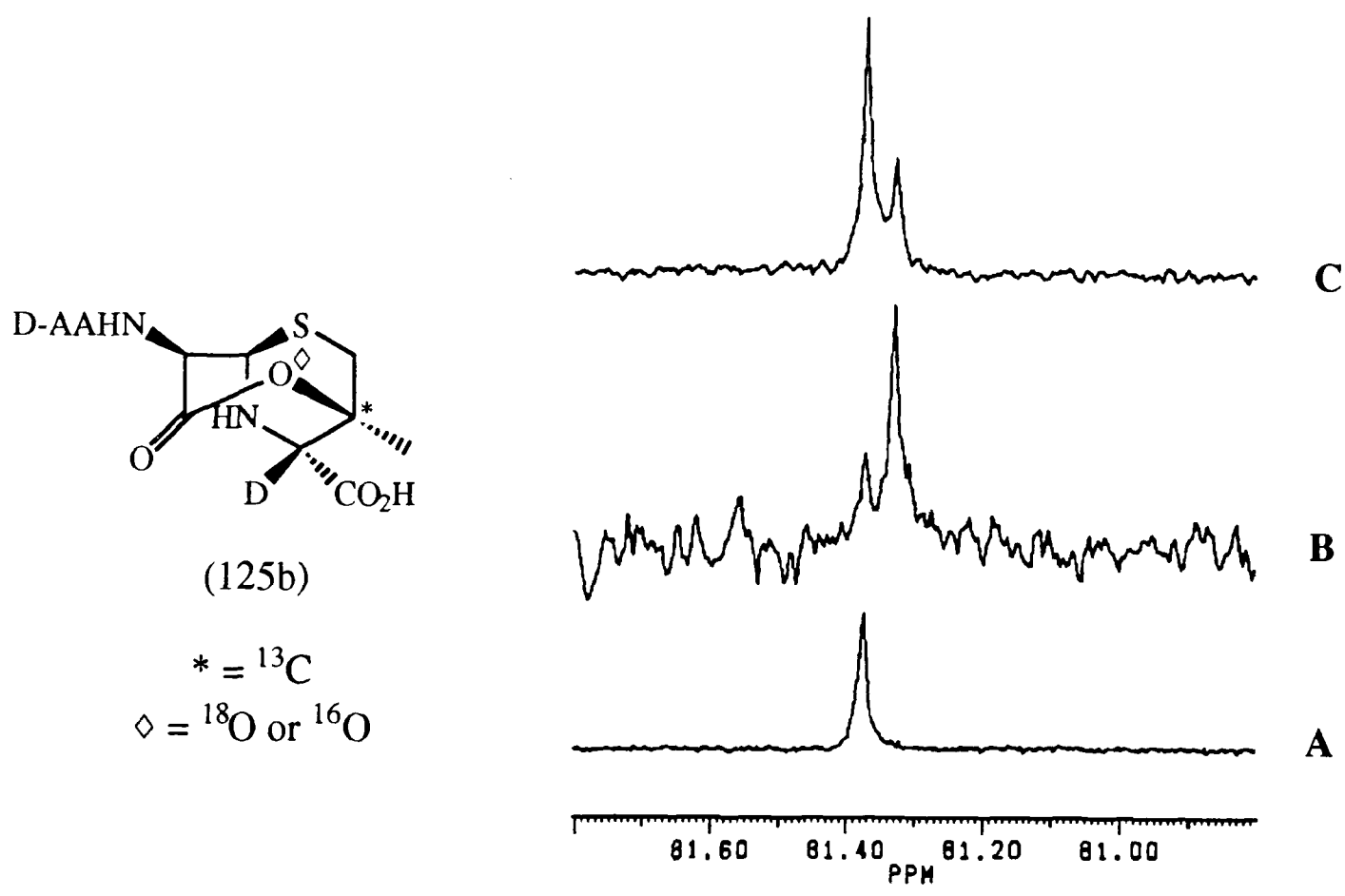
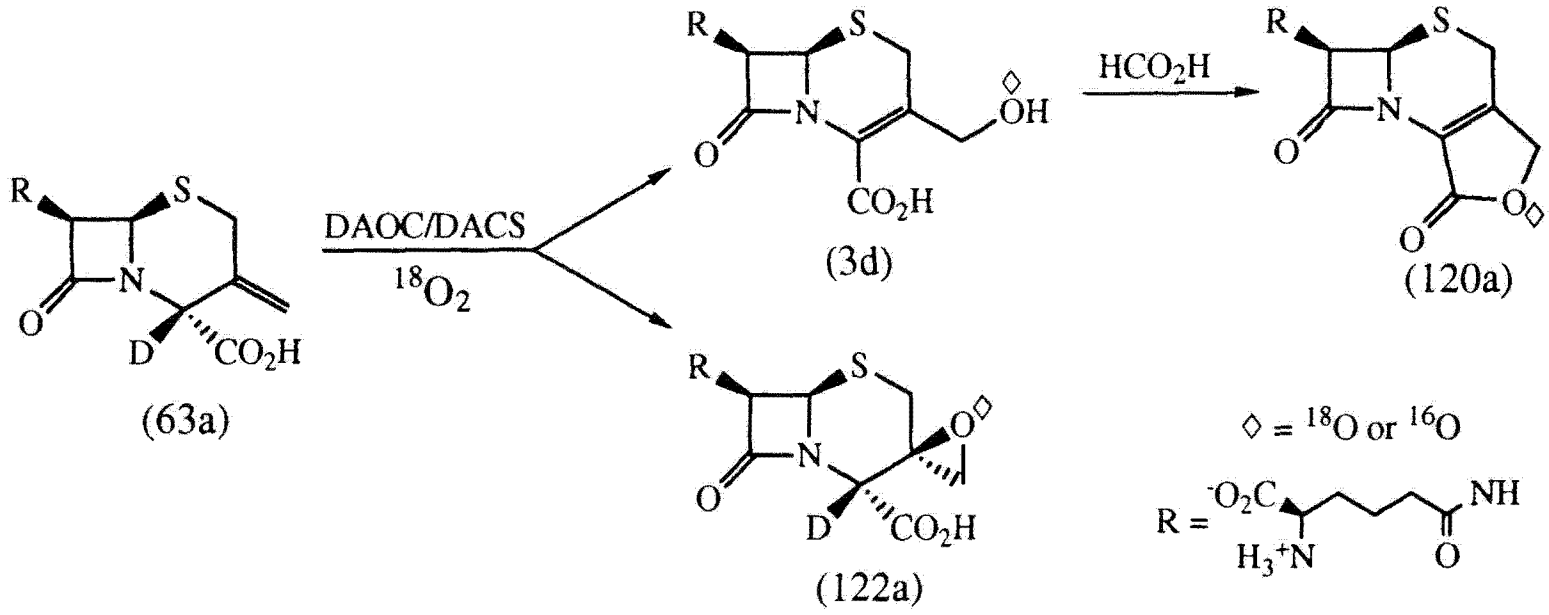


Figure 3.1 : The ^{13}C resonance of (125b) from **A** - incubation of (1f) under $^{16}O_2$
B - incubation of (1f) under $^{18}O_2$
C - **B** doped with **A**

Due to the very high sensitivity of electrospray mass spectrometry it was felt that it was not necessary to have a ^{13}C-label in [4-^{2}H]-3-exomethylene cephalosporin C (63a) when carrying out the ^{18}O studies of its incubations with DAOC/DACS.

Initially, a normal incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) under $^{16}O_2$ was performed, and the procedure described in section 2.4.2 was used to isolate the products DAC (3) [as its lactone (120)] and the epoxide (122). These compounds were then analysed by mass spectrometry (ESMS). This incubation was repeated under an $^{18}O_2$ atmosphere (98 atom % ^{18}O, 100 ml, supplied by MSD Isotopes Ltd.) in a procedure similar to that carried out for (1f), with conversion to the products (3d) and (122a).[236] The usual procedure for separation of the products gave the DAC lactone (120a) and the epoxide (122a) (Scheme 3.14).



Scheme 3.14

Mass spectral analysis of (120a) and (122a) revealed the level of ^{18}O-incorporation to be 46% in the lactone (120a) and 94% in the epoxide (122a)[235] (Table 3.2 - Experiment 1). This indicated that the epoxide oxygen in (122) was derived from molecular oxygen, as anticipated. Loss of label between (3d) and (120a) was ruled out as it had previously been shown that no exchange of oxygen occurred on lactonisation of DAC (3) to the lactone (120).[229]

DAC lactone (120a)

	Conditions	*m/z* (MH^+)	*356*	*357*	*358*	*359*	*360*	*361*	% ^{18}O
	$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	100	18	7	1	–	–	–
	$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	–	–	–
Exp. 1	$^{18}O_2/H_2{}^{16}O$	Found (%)	100	24	88	22	7	2	46
Exp. 2	$^{18}O_2/H_2{}^{16}O$	Found (%)	77	22	100	19	7	1	56

[4-^{2}H]-3β-Epoxide cepham (122a)

	Conditions	*m/z* (MH^+)	*375*	*376*	*377*	*378*	*379*	*380*	% ^{18}O
	$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	100	18	7	1	–	–	–
	$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	–	–	–
Exp. 1	$^{18}O_2/H_2{}^{16}O$	Found (%)	9	12	100	19	8	2	94
Exp. 2	$^{18}O_2/H_2{}^{16}O$	Found (%)	7	9	100	20	8	2	95

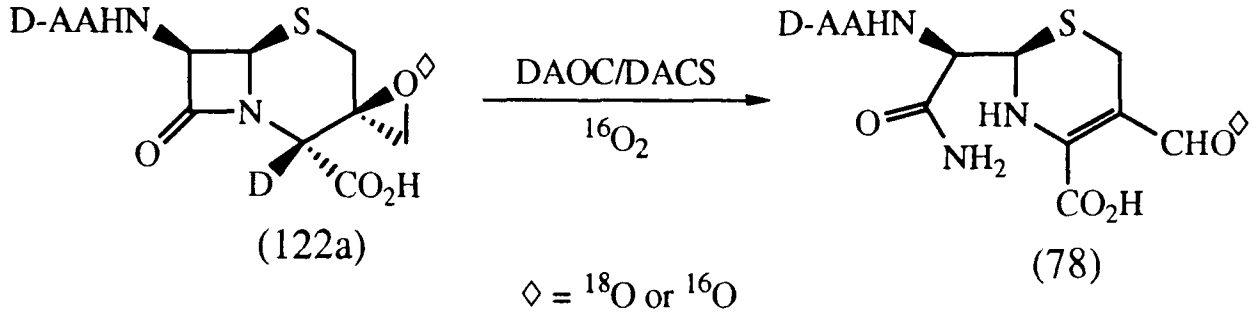
Table 3.2 : ^{18}O-oxygen incorporations[235] into the products (120a) and (122a) obtained from incubations of (63a) under $^{18}O_2$.

†calculated using oxygen at natural abundance.

A second incubation of (63a) with DAOC/DACS under $^{18}O_2$ was carried out, and the products (120a) and (122a) were isolated as before. Mass spectral analysis gave an ^{18}O-label incorporation of 56% into the lactone (120a) and 95% into the epoxide (122a)[235] (Table 3.2 - Experiment 2). These results indicated a consistent incorporation of label between the two experiments in the case of (122a), but less consistency in the case of (120a), which suggested some factor was affecting the oxygen incorporation into each of these compounds differently.

Since the level of ^{18}O-oxygen incorporation into the epoxide (122a) was so high, it was considered of interest to re-incubate it with DAOC/DACS. The proposed mechanism for the conversion of (122a) (see Scheme 2.25) would predict that the oxygen in the epoxide functionality would be retained in the aldehyde product, provided no chemical exchange occurred after its formation. As such, (122a) was incubated with DAOC/DACS under a

normal atmosphere, with complete conversion being observed. The product (78) (Scheme 3.15) was isolated by HPLC, but mass spectral analysis revealed that it retained only about 14% ^{18}O label (see Table 3.3).



(122a) (78)

◊ = ^{18}O or ^{16}O

Scheme 3.15

3-Aldehyde cephalosporoate (78)

Conditions							**% ^{18}O**
	m/z (MH^+)	*388*	*389*	*390*	*391*	*392*	
$^{16}O_2/H_2{}^{16}O$	Calculated† (%)	–	100	19	8	1	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	9	100	19	24	11	14

Table 3.3 : ^{18}O-oxygen incorporation into the product (78) obtained from an incubation of (122a) under $^{16}O_2$.

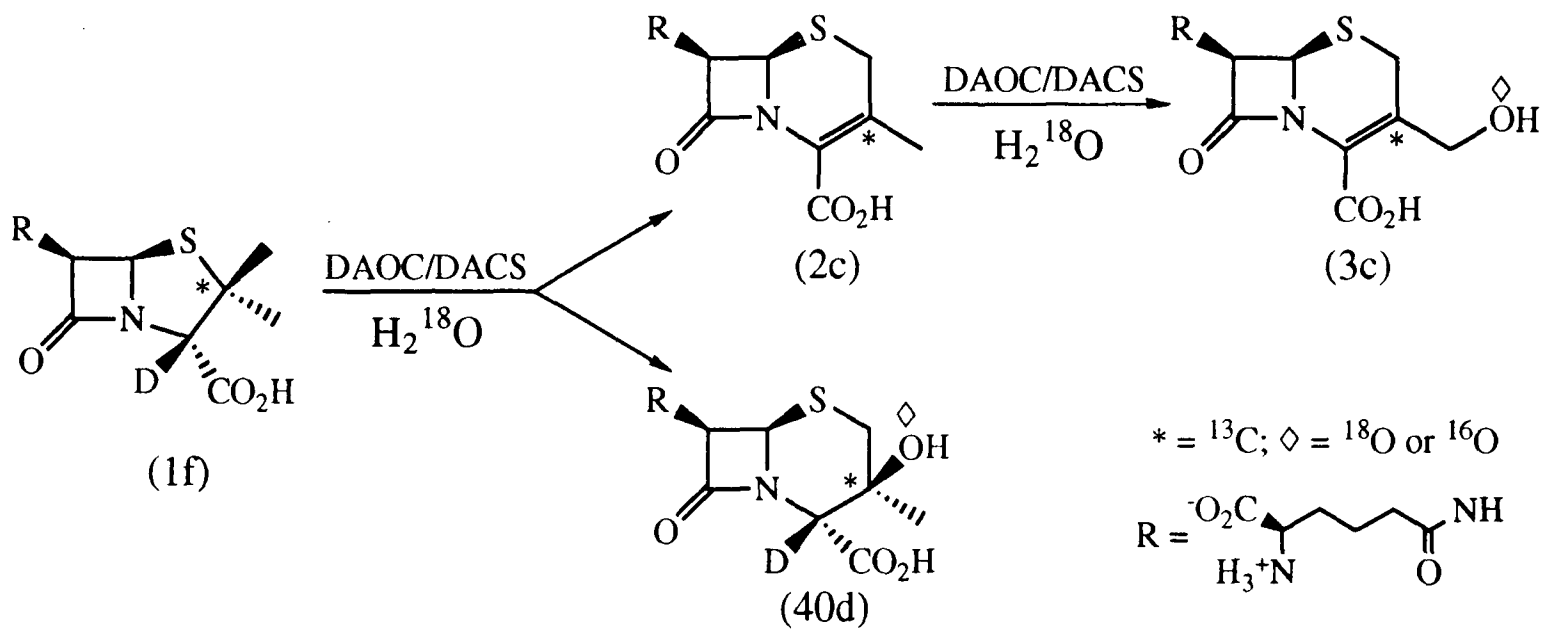
†calculated using oxygen at natural abundance.

However, this result was considered not to be conclusive with regard to the mechanism of the enzymatic oxidation of (122a), because wash-out of label by oxygen exchange of the aldehyde functionality (either before or after opening of the β-lactam) could not be ruled out. Exchange could be tested for the compound (78) by dissolving it in $H_2{}^{18}O$, but as exchange in the corresponding aldehyde with an intact β-lactam ring could not be tested or ruled out, no control experiment was performed.

3.4 Studies with $H_2{}^{18}O$

The enzyme DAOC/DACS is not stable to lyophilisation, and as such had to be used in a $H_2{}^{16}O$ solution. It was envisaged that in order to obtain an incubation mixture with a high level of $H_2{}^{18}O$, a very concentrated enzyme solution should be used and diluted with a solution of substrate and cofactors in $H_2{}^{18}O$. The viability of this procedure was initially tested by concentrating DAOC/DACS and carrying out an incubation with [3-^{2}H]penicillin N (1d) with $H_2{}^{16}O$, which resulted in a good level of conversion.

For the actual $H_2{}^{18}O$ incubation with [2-^{13}C,3-^{2}H]penicillin N (1f) the right amounts of substrate and cofactors had previously been lyophilised. These were dissolved in $H_2{}^{18}O$ (2 ml, 95 atom % ^{18}O, supplied by Aldrich Chemical Co.) and added to the concentrated DAOC/DACS (0.5 ml) in $H_2{}^{16}O$, resulting in a final $H_2{}^{18}O$ enrichment of the incubation mixture of *ca.* 76%. Incubation by the standard procedure resulted in 59% conversion to the products [3-^{13}C]DAOC (2c), [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40d) and [3-^{13}C]DAC (3c) in a ratio of (2c):(40d):(3c) = 21:44:35 (Scheme 3.16).[236]



Scheme 3.16

After HPLC purification the products were analysed by mass spectrometry (ESMS) which showed there had been incorporation of ^{18}O-label into both (3c) and (40d), but not into (2c). This time the level of label incorporation was higher into (3c) (50%) than into

(40d) (16%). The corrected values of incorporation taking into account the $H_2{}^{18}O$ dilution of the incubation mixture were 66% into (3c) and 21% into (40d) (Table 3.4).

[3-^{13}C]DAOC (2c)

Conditions	*m/z* (MH^+)	*358*	*359*	*360*	*361*	*362*	**% ^{18}O**
$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	14	100	20	9	4	–
$^{16}O_2/H_2{}^{18}O$	Found (%)	14	100	22	9	2	–

[3-^{13}C, 4-^{2}H]-3β-Hydroxycepham (40d)

Conditions	*m/z* (MH^+)	*377*	*378*	*379*	*380*	*381*	*382*	**% ^{18}O**
$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	15	100	26	10	2	–	–
$^{16}O_2/H_2{}^{18}O$	Found (%)	18	100	23	26	7	3	21

[3-^{13}C]DAC (3c)

Conditions	*m/z* (MH^+)	*374*	*375*	*376*	*377*	*378*	*379*	**% ^{18}O**
$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	–	100	17	7	1	–	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	14	100	20	11	3	–	–
$^{16}O_2/H_2{}^{18}O$	Found (%)	17	92	37	100	21	10	66

Table 3.4 : ^{18}O-oxygen incorporations[235] into the products (2c), (40d) and (3c) obtained from an incubation of (1f) in $H_2{}^{18}O$.

†calculated using oxygen at natural abundance.

The [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40d) was analysed by ^{13}C-NMR overnight. An ^{18}C-^{13}C isotope shift of 0.03 ppm could be clearly observed for the 3-^{13}C resonance of (40d) [which this time had not decomposed to the lactone (125b)], and the ^{18}O-^{13}C peak had an intensity of about 17% of that of the ^{16}O-^{13}C peak, in good agreement with the incorporation values obtained by mass spectrometry (see Figure 3.2).

To determine whether some chemical process was leading to exchange of label during the incubations, the ^{18}O-labelled products (40d) and (3c) obtained in the $H_2{}^{18}O$ incubation were re-incubated with denatured DAOC/DACS and cofactors in $H_2{}^{16}O$. The two products were re-isolated and again analysed by mass spectrometry, which showed no loss of label had occurred to within experimental error.

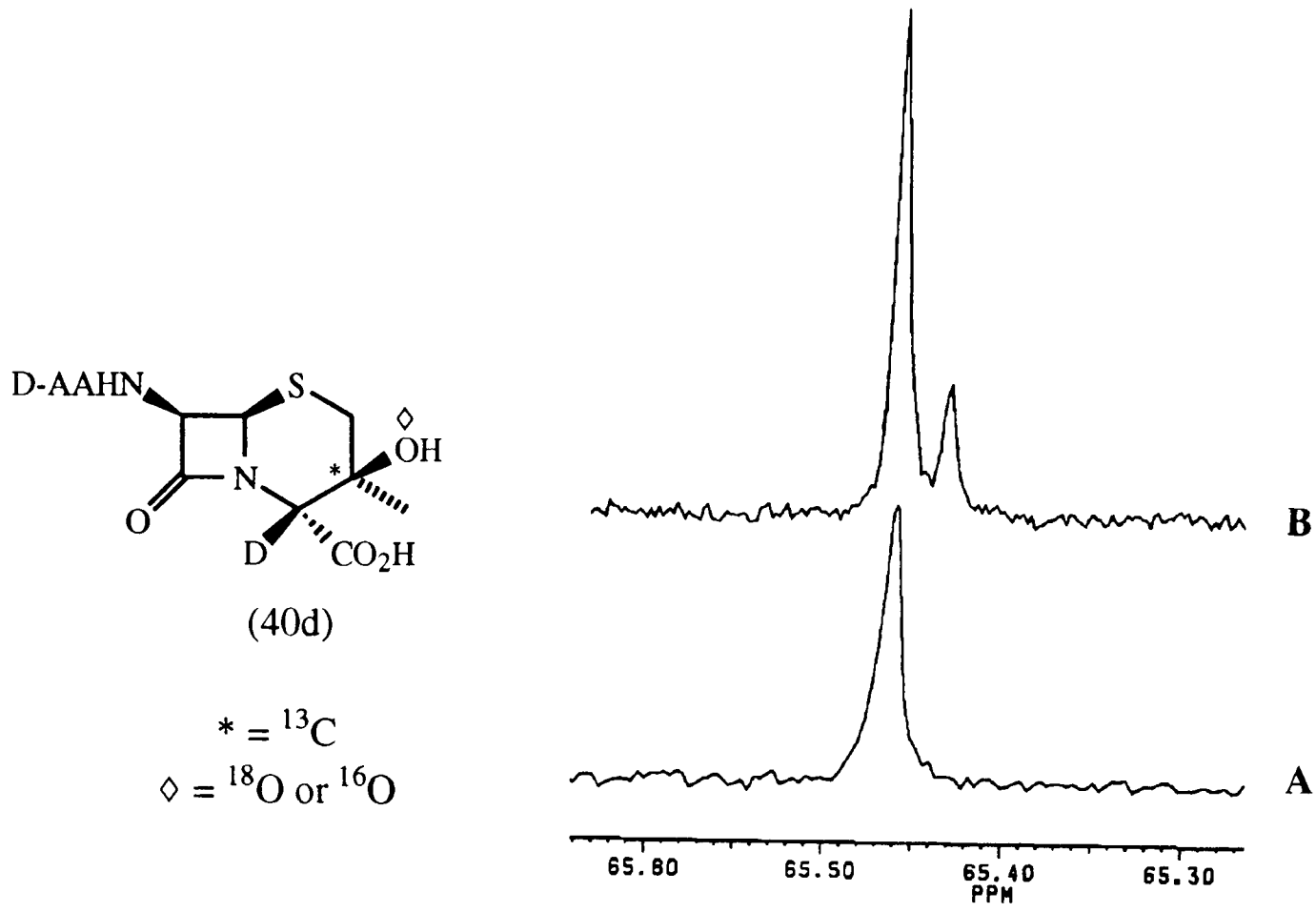
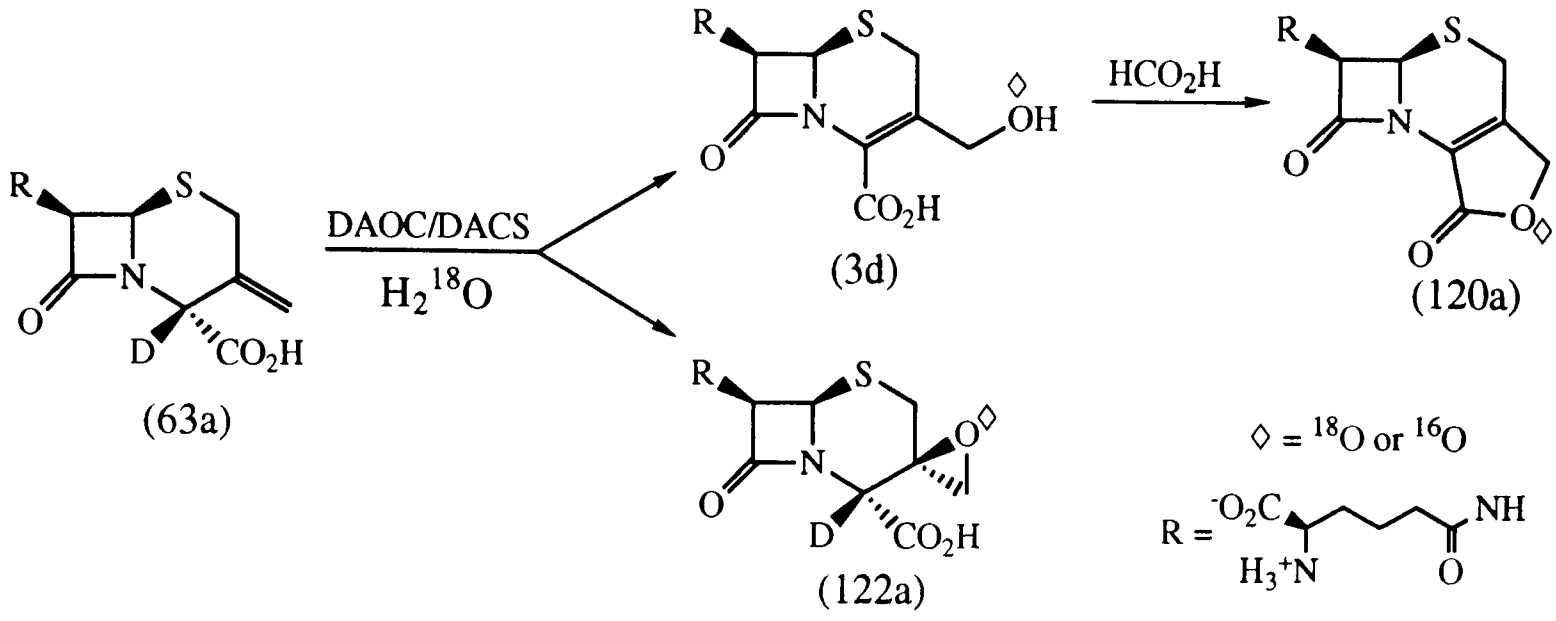


Figure 3.2 : The ^{13}C resonance of (40d) from **A** - incubation of (1f) in $H_2{}^{16}O$
B - incubation of (1f) in $H_2{}^{18}O$

A similar incubation in $H_2{}^{18}O$ was carried out with the substrate [4-^{2}H]-3-exomethylene cephalosporin C (63a). After separation of the products, the epoxide (122a) and DAC lactone (120a) were obtained (Scheme 3.17). Analysis by mass spectrometry revealed that (122a) contained 13% ^{18}O-label, and (120a) contained 30% ^{18}O-label. Considering the $H_2{}^{18}O$ enrichment of the incubation mixture, the corrected ^{18}O-incorporation values are 17% into (122a) and 40% into (120a) (Table 3.5).



Scheme 3.17

DAC lactone (120a)

Conditions								**% ^{18}O**
	m/z (MH^+)	*356*	*357*	*358*	*359*	*360*	*361*	
$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	100	18	7	1	–	–	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	–	–	–
$^{16}O_2/H_2{}^{18}O$	Found (%)	100	25	50	11	4	1	40

[4-^{2}H]-3β-Epoxide cepham (122a)

Conditions								**% ^{18}O**
	m/z (MH^+)	*375*	*376*	*377*	*378*	*379*	*380*	
$^{16}O_2/H_2{}^{16}O$	Calcul.† (%)	100	18	7	1	–	–	–
$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	–	–	–
$^{16}O_2/H_2{}^{18}O$	Found (%)	100	20	22	5	2	1	17

Table 3.5 : ^{18}O-oxygen incorporations[235] into the products (120a) and (122a) obtained from an incubation of (63a) in $H_2{}^{18}O$.

†calculated using oxygen at natural abundance.

As a control experiment some unlabelled epoxide (122) was incubated with denatured DAOC/DACS in $H_2{}^{18}O$. Mass spectrometry analysis on the recovered (122) showed no incorporation of label had occurred.

3.5 Conclusions

The results described (Sections 3.3 and 3.4) clearly indicate that there is incorporation of ^{18}O-label from either $^{18}O_2$ or $H_2{}^{18}O$ into the products (40d) and (3c) [from incubations of (1f) with DAOC/DACS], and into the products (3d) and (122a) [from incubations of (63a) with DAOC/DACS]. The corrected incorporation values for these products are described in Table 3.6.[236]

		% ^{18}O incorporation	
Conditions	**(2c)**	**(40d)**	**(3c)**
$^{18}O_2/H_2{}^{16}O$	–	71	52
$^{18}O_2/H_2{}^{16}O$	–	69	57
$^{16}O_2/H_2{}^{18}O$	–	21	66

	% ^{18}O incorporation	
Conditions	**(120a)**	**(122a)**
$^{18}O_2/H_2{}^{16}O$	46	94
$^{18}O_2/H_2{}^{16}O$	56	95
$^{16}O_2/H_2{}^{18}O$	40	17

Table 3.6 : ^{18}O-label incorporation values[235] into the products (2c), (40d) and (3c) from incubations of (1f), and (120a) and (122a) from incubations of (63a).

It is believed that the ^{18}O-incorporation values into the individual products from incubations with $^{18}O_2$ and $H_2{}^{18}O$ are complementary to each other, to within experimental error. This error is about ± 10%, which is not too high considering there are several possible factors which may affect the extent of label exchange [such as temperature, rate of shaking (aeration) and concentration and activity of the enzyme], and which were not necessarily the same from one incubation to the other. This error would obviously be

decreased if it was possible to repeat the experiments several times in order to obtain a mean value of incorporation for each compound, but this is not economically feasible. However, it is considered that the most significant result from these experiments is the observation that $H_2{}^{18}O$ is indeed incorporated into the products in significant amounts.

The fact that the activity of DAOC/DACS is completely dependent on dioxygen, together with the observation that the succinate generated on an incubation under $^{18}O_2$ incorporated over 90% of label into a single oxygen atom, strongly suggest that the formed iron(IV)-oxene intermediate (49) has also a fully labelled oxygen when generated from $^{18}O_2$ (see Scheme 3.1). The observation that oxygen from $H_2{}^{18}O$ is incorporated into the products gives strong support to the idea that some exchange process occurs between formation of the iron(IV)-oxene (49) and release of products from the enzyme. It can be envisaged that this exchange process occurs directly between the ferryl species (49) and water, or between another intermediate subsequently formed and water. It has been shown that metal oxenes in synthetic complexes exchange their oxygen readily with water[237,238], and the same was observed in the particular case of an iron-oxene generated in an iron-porphyrin complex[239].

Exchange between an iron-oxene and water has also been proposed to explain $H_2{}^{18}O$ incorporation catalysed by some oxygenase enzymes; in the case of liver michrosomal cytochrome P-450, a relatively low 8.6% ^{18}O incorporation was observed into cyclohexanol derived from cyclohexane, when the incubation was performed in the presence of $H_2{}^{18}O$[240]; in the case of toluene dioxygenase[241] the oxygen incorporation values in the oxidation of indan to 1-indanol were 31% from $^{18}O_2$ and 68% from $H_2{}^{18}O$.

A more detailed labelling study with cytochrome P-450 revealed that oxygen incorporation from $H_2{}^{18}O$ into the product was complete if the iron-oxene was generated from iodosobenzene, but could not be observed if this species was generated from the physiological NADPH, O_2 system.[242,243] This result was interpreted as indicating that in the case of iodosobenzene the iron-oxene was formed in the absence of the substrate and was free to exchange with water, whereas in the physiological case the iron-oxene is known

to be formed only after binding of the substrate which possibly prevents the exchange process from occurring.

Some later evidence obtained for horseradish peroxidase indicated that exchange of oxygen between the iron-oxene and water in this system occurred only in the presence of a proton source, *i.e.* when the iron-oxene was hydrogen-bonded to a protonated histidine residue of the active site.[244] At basic pH, this histidine residue was not protonated and the exchange process could not be observed.

In the present case, the difference in ^{18}O-incorporation values between the several products of DAOC/DACS was most informative for the particular case of the two products (3d) and (122a), derived from the [4-^{2}H]-3-exomethylene cephalosporin C (63a). As previously discussed (see section 2.6) these two products originate from a common enzyme-bound intermediate formed in the first irreversible step of the reaction, which is addition of the iron-oxene (49) to the double bond of (63a). The fact that such different ^{18}O-incorporation values are obtained in the two products seems to rule out the possibility that the exchange process occurs only between the ferryl species (49) and water. If this was the case, identical incorporation values should be observed between (3d) and (122a).

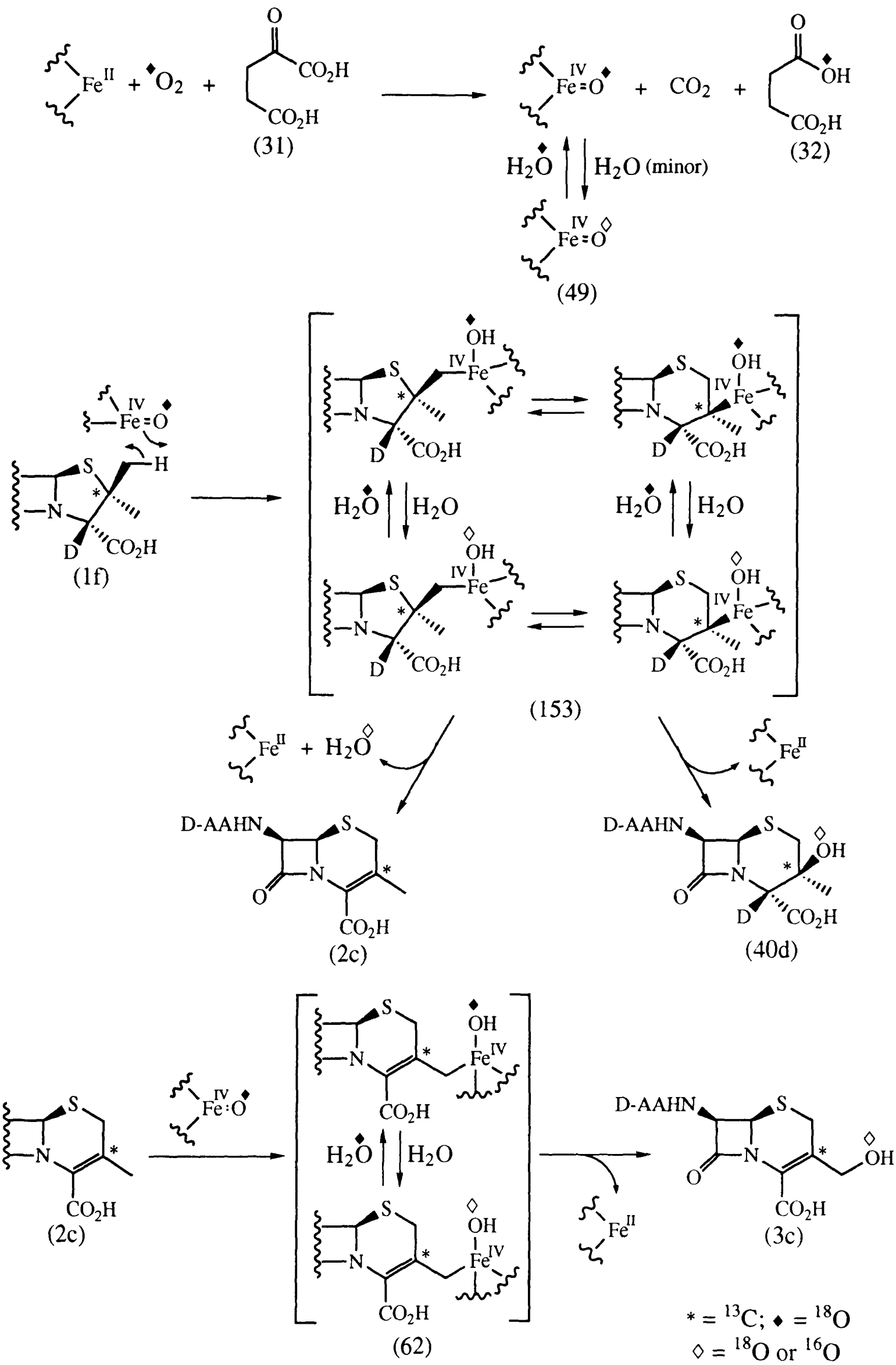
It is proposed that for DAOC/DACS exchange at the ferryl oxidation state probably constitutes a minor pathway, and that the majority of exchange occurs after reaction with the substrate, between an enzyme-bound intermediate and water. It is thought that this intermediate is probably one where a hydroxyl group is coordinated to the iron, as in that case the iron-bound oxygen is already protonated and should no longer require a proton source, thus facilitating the exchange process. Such an intermediate is not thought to be involved in the formation of the epoxide (122a), which would explain the very high retention of ^{18}O-label from $^{18}O_2$ into (122a).

The following mechanisms are thus proposed (Schemes 3.18 and 3.19). In the conversion of [2-^{13}C,3-^{2}H]penicillin N (1f) (Scheme 3.18), the intermediate (153) from which the products (2c) and (40d) are formed, is thought to undergo significant exchange with water, a process which is responsible for the loss of label from $^{18}O_2$ in (40d). The product (2c) is the further converted to (3c) through the intermediate (62) which is also

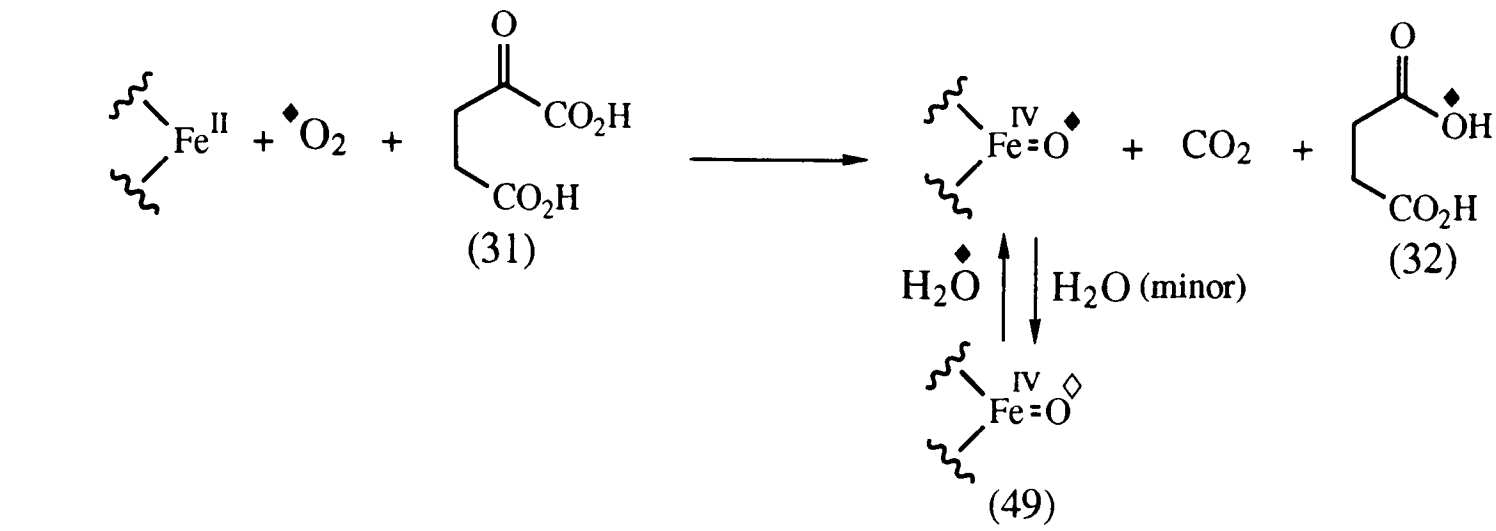
believed to be involved in a similar exchange process. The different incorporation values into (40d) and (3c) probably reflect different rates of conversion of the intermediates (153) and (62) to the respective products, allowing for more or less exchange to occur. Given the relatively low ^{18}O-incorporation into (40d) from $H_2{}^{18}O$ (20%), it is thought that this product does not originate from water interception of the episulphonium (139) or the carbocation (140) (see Scheme 3.4) as previously suggested[118].

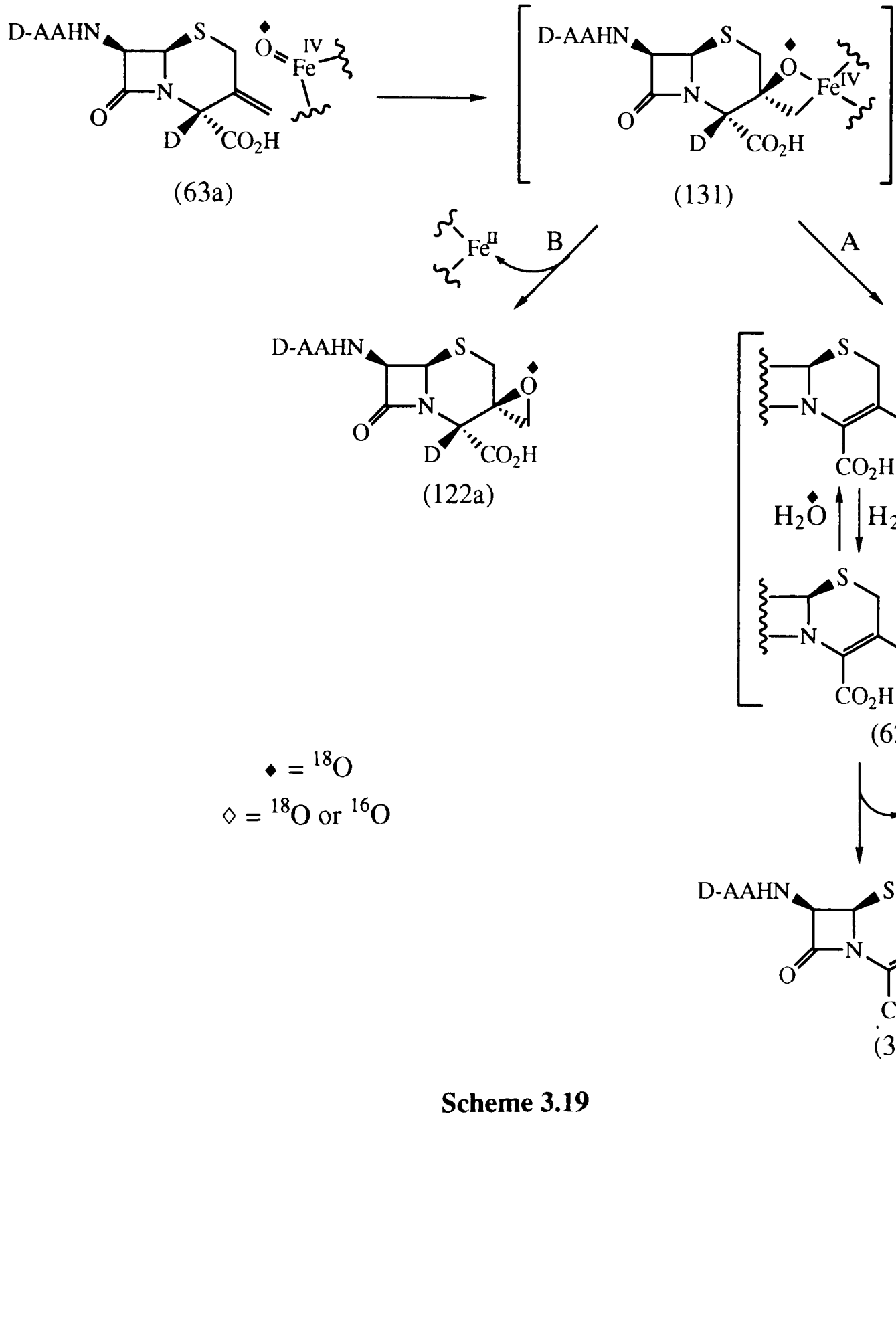
In the conversion of [4-^{2}H]-3-exomethylene cephalosporin C (63a) (Scheme 3.19) it is thought that the enzyme-bound intermediate (131) formed in the first irreversible step does not exchange oxygen with water, and thus the epoxide (122a) formed directly from it (pathway B) retains a high level of ^{18}O-label from $^{18}O_2$. The intermediate (131) is also converted to intermediate (62) (pathway A) which is thought to undergo exchange with water before reductive elimination of Fe^{II} to give the product DAC (3d). The fact that similar ^{18}O-incorporation values are observed in the product DAC (3c or 3d) formed from either (2c) or (63a) supports the idea that the same intermediate (62) is responsible for oxygen exchange in both cases. Direct exchange between the ferryl species (49) and water is thought to be only a minor competing pathway but nevertheless to occur, being responsible for the low ^{18}O-incorporation from $H_2{}^{18}O$ into the epoxide (122a).

In conclusion, it is believed this is the first evidence for water exchange of intermediates in an α-ketoglutarate dependent dioxygenase. In the case of the two α-ketoacid dependent oxygenases, *p*-hydroxyphenylpyruvate oxygenase[194] and α-ketoisocaproate oxygenase[245] similar ^{18}O-incorporation values from $H_2{}^{18}O$ were also reported. It is possible that an analogous exchange process to that proposed here for DAOC/DACS is also operating for these two oxygenases.



Scheme 3.18


Fe^II
O2
CO2H
CO2H
(31)
Fe^IV=O
CO2
OH
CO2H
(32)
H2O
H2O (minor)
Fe^IV=O
(49)


D-AAHN
S
N
O
D
CO2H
Fe^IV
(63a)
(131)
Fe^II
B
A
OH
(122a)
(62)
(3d)
◆ = 18O
◇ = 18O or 16O

Scheme 3.19

CHAPTER 4



Two Substrate Analogues for the Ring Expansion Activity

Chapter 4

Two Substrate Analogues for the Ring Expansion Activity

4.1 Introduction

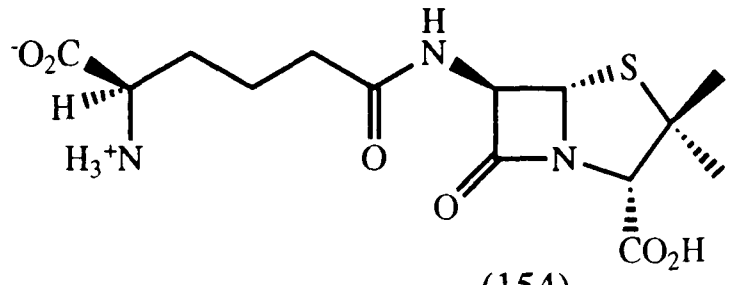
The use of substrate analogues is a valuable tool for the investigation of the catalytic properties and mechanism of an enzyme. Substrate analogues usually have specific alterations in relation to the natural substrate, and can serve as probes at the active site, providing information about the mode of binding and/or the reaction mechanism.

The characteristics of binding are usually studied through the alteration or removal of specific functional groups in the substrate, to determine which ones are essential for conversion. In the case of mechanistic studies, the analogues are normally designed with basis on a proposed mechanism, so that a modification of reaction is provoked. Analysis of the new products formed can provide crucial information about the intermediates involved.

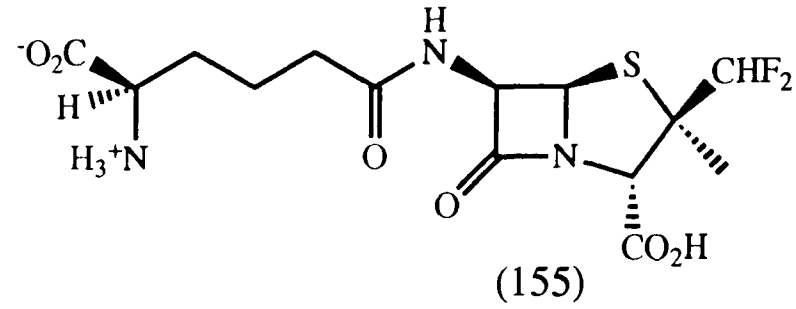
Evaluation of a substrate analogue should be made by comparison with the natural substrate, in terms of extent of conversion and relative rate of product formation (through the kinetic parameters V_{max} and K_m). In the case where the analogue is not converted by the enzyme, its role as an inhibitor should be studied, as well as the type of inhibition observed. Competitive inhibition by a modified substrate indicates it has the features necessary for reversible binding to the enzyme, but not those needed for conversion to product. Non-competitive inhibition suggests either irreversible binding without conversion, or a permanent modification of the active site rendering it incapable of catalysis.

The extensive use of substrate analogues for the study of IPNS has provided invaluable insight into its mechanism (see section 1.2.4). A comparatively small number of studies have been undertaken with DAOC/DACS, particularly with respect to the ring

expansion reaction, a fact that can be attributed in part to the difficulty in the synthesis of modified penicillins. The investigations carried out previously concentrated mainly on modifications to the methyl groups of the penam system (see section 1.3.3.1). This chapter describes the synthesis and evaluation of two substrate analogues for the ring expansion, the 5-epipenicillin N (154) and the 2β-difluoromethyl penicillin N (155).



(154)



(155)

4.2 5-Epipenicillin N

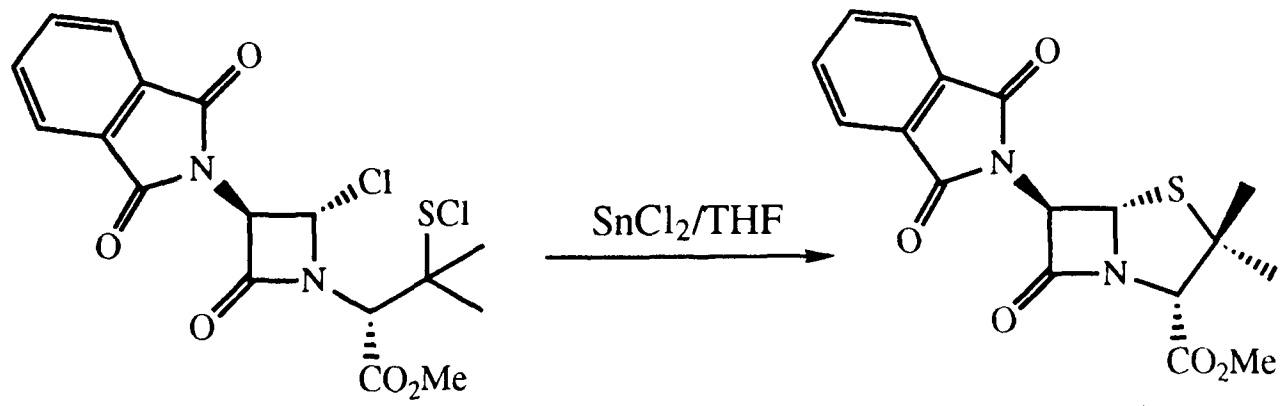
It is thought that the sulphur atom of penicillin N plays an important role in the ring expansion reaction, due to its proximity to the β-methyl group, which is the initial site of reaction (see section 1.3.3.1). As such, it was envisaged that the 5-epipenicillin N (154) would provide an interesting test of the importance of the sulphur spatial arrangement with respect to the β-methyl group and the β-lactam ring.

4.2.1 Synthesis of 5-Epipenicillin N

4.2.1.1 Construction of the ring system

The byciclic ring system of penicillins is characterised by three chiral centres with a (3S, 5R, 6R) configuration. Epimers with a 6S configuration are reported to be accessible through epimerisation of penicillin esters by base treatment.[246] The 5S epimers are however somewhat more difficult to obtain, and require cleavage of the C5-S bond, when starting from natural penicillins.

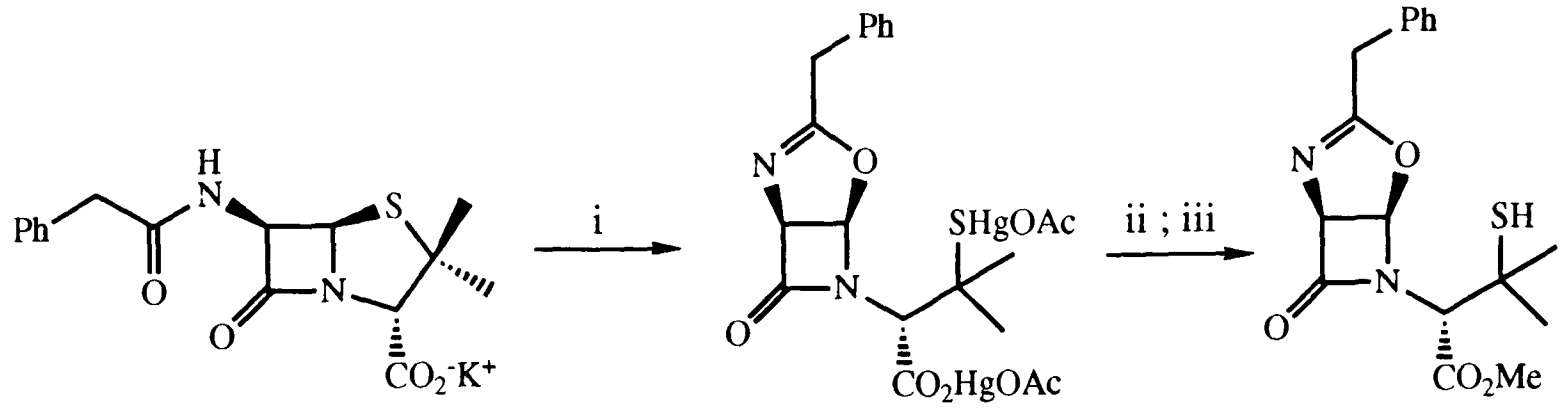
The first synthesis of a 5-epipenicillin was described by Kukolja in 1971.[247] He discovered that treatment of penicillin esters with halogen electrophiles resulted in the opening of the C5-S bond without disruption of the azetidinone ring.[248] Using chlorine as the electrophile he was able to prepare the azetidinone sulphenyl derivative (156). Treatment of (156) with anhydrous stannous chloride in THF, resulted in a stereospecific reductive cyclisation to the 5-epipenicillin ester (157) (Scheme 4.1).



(156) (157)

Scheme 4.1

Later Stoodley *et al.* reported that the degradation of penicillin esters using mercury(II) acetate provided another way of opening the thiazolidine ring at the C5-S bond without disrupting the β-lactam ring.[249,250] The initial product of this reaction is the dimercury salt (158), which can be converted into the mercaptobutanoate ester (159a) by treatment with hydrogen sulphide followed by esterification with diazomethane (Scheme 4.2).[251]

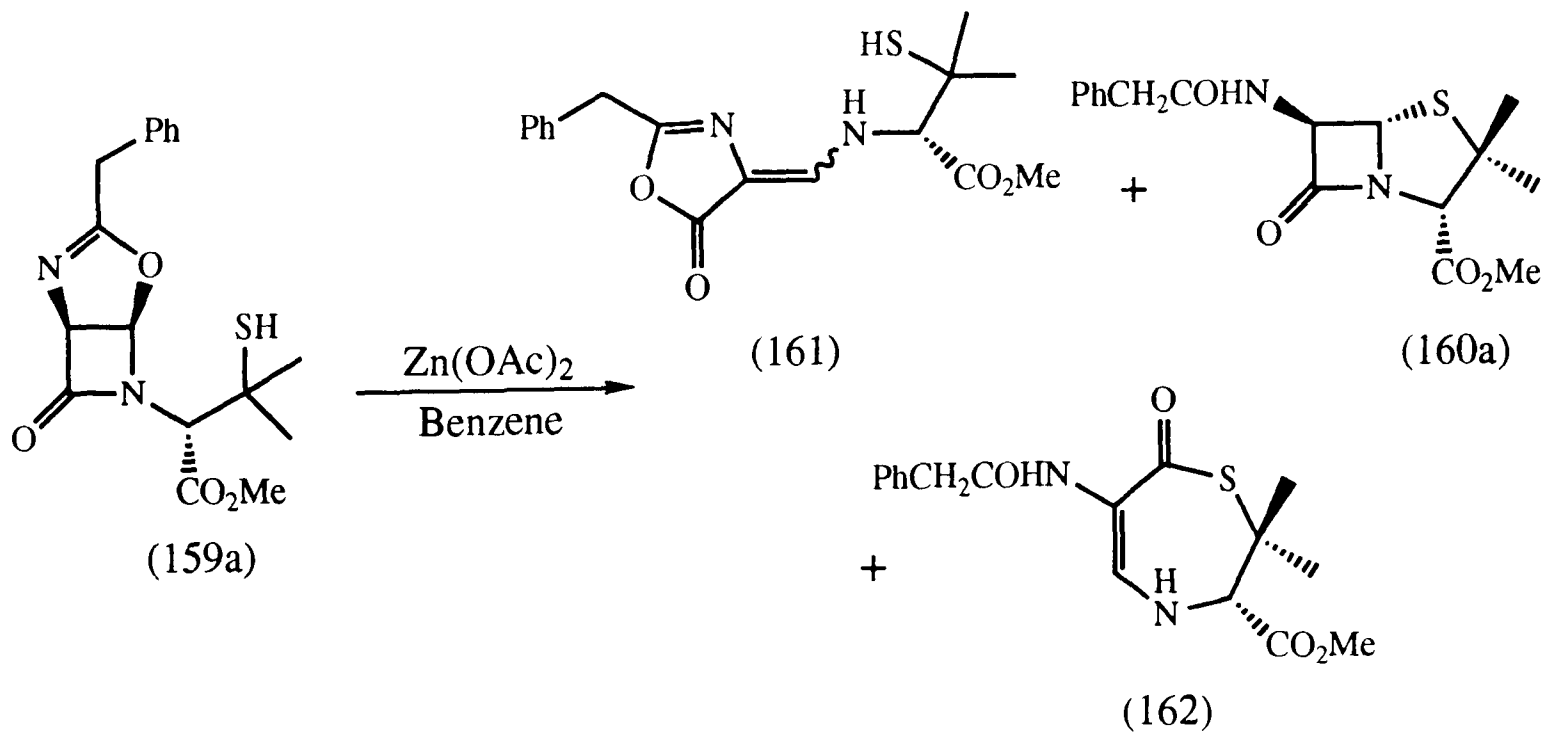


(10a) (158) (159a)

Reagents : i) $Hg(OAc)_2$/HOAc ; ii) H_2S ; iii) CH_2N_2

Scheme 4.2

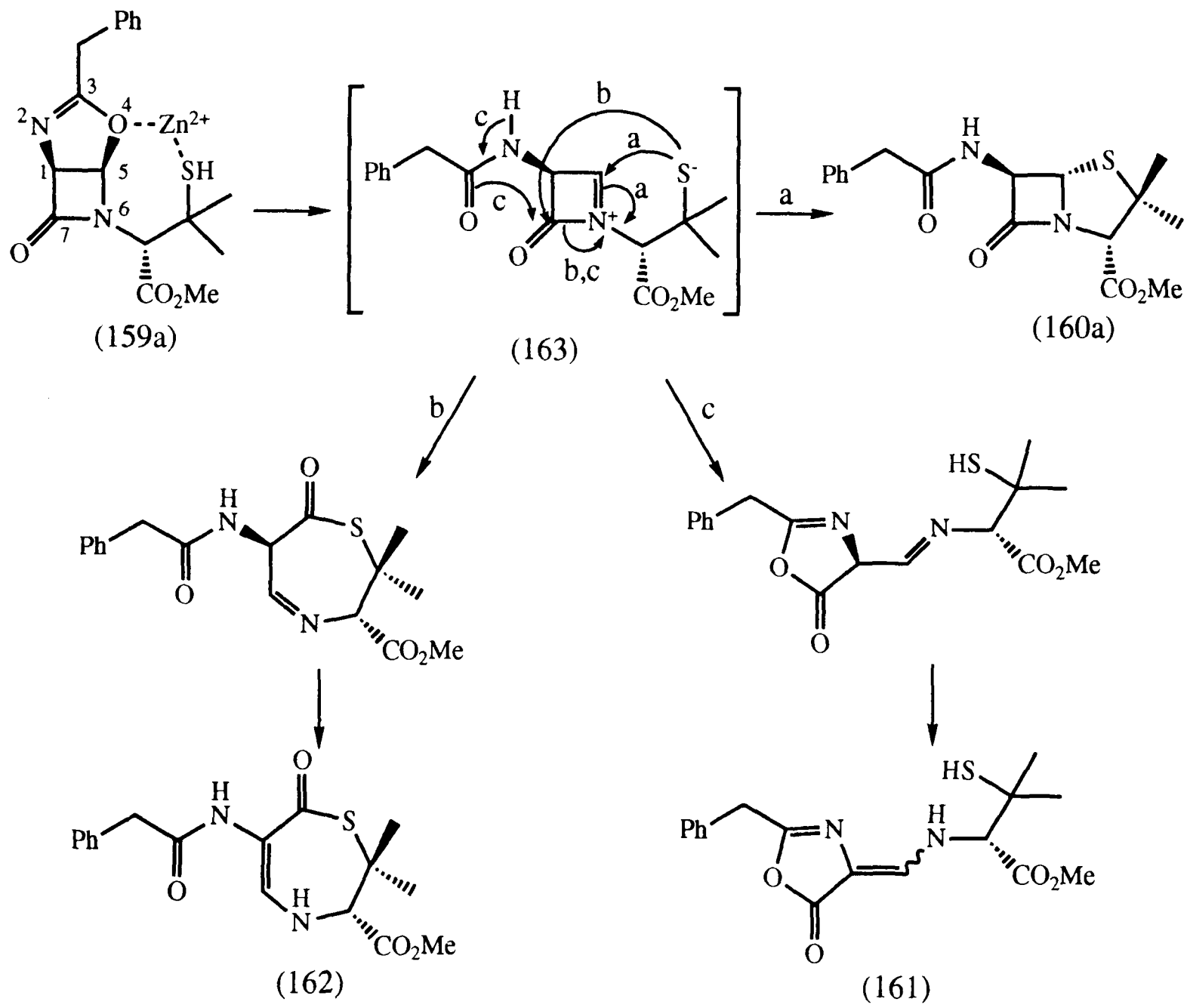
Stoodley *et al.* envisaged that (159a) could be recyclised to give a penicillin nucleus. This was indeed achieved by treatment of the mercaptobutanoate (159a) with zinc acetate in benzene with the formation of three products, one of which was the 5-epipenicillin (160a) (Scheme 4.3).[252,253]



(159a) (161) (160a) (162)

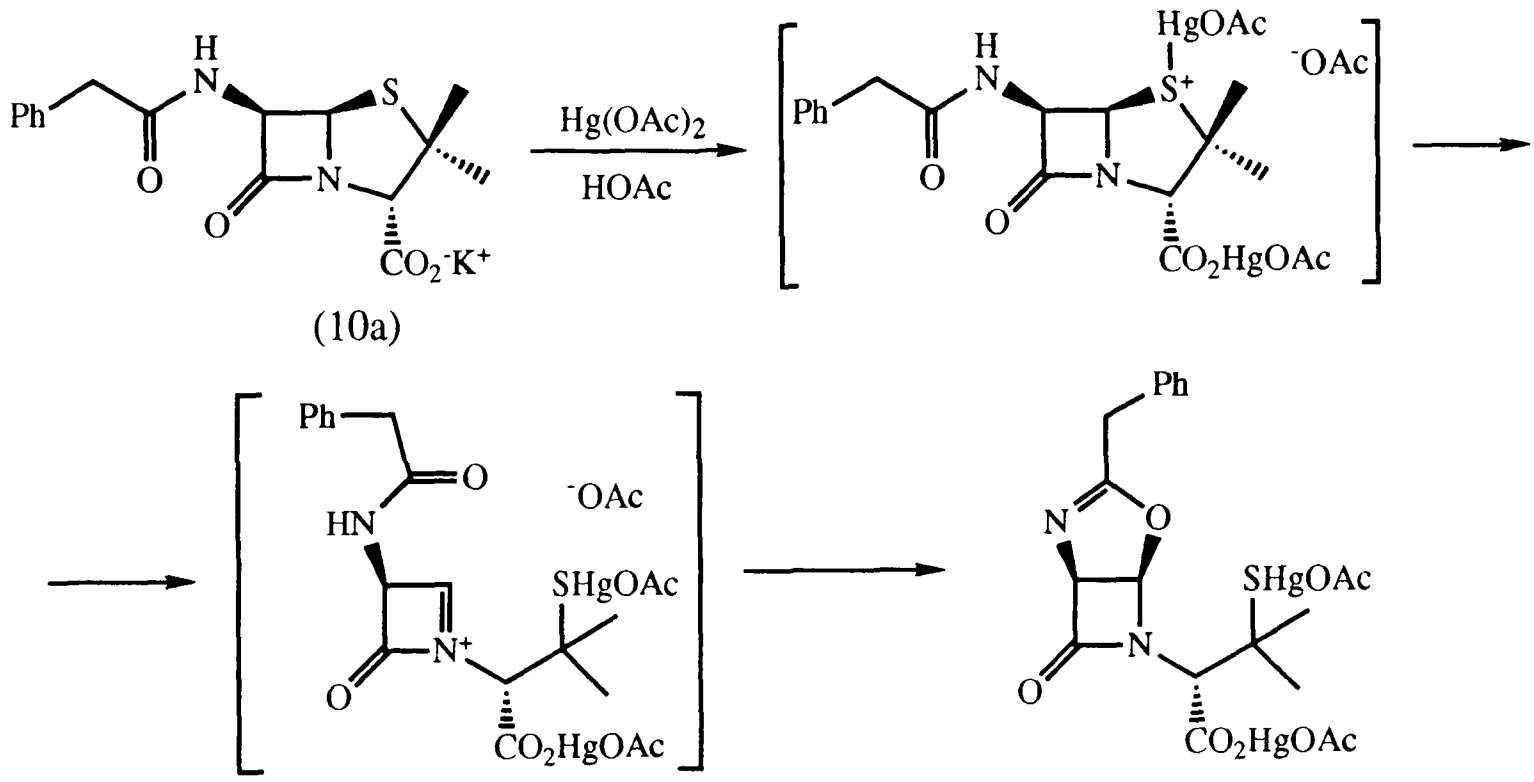
Scheme 4.3

The major product of the reaction was the thiazepinone (162) (*ca.* 50%), followed by the 5-epipenicillin (160a) (*ca.* 20%) and the penicillenate (161) (*ca.* 15%). However, when the reaction was carried out with the *p*-nitrobenzyl ester no penicillenate was formed, and the two isolated products were the corresponding thiazepinone (*ca.* 60%) and the 5-epipenicillin (*ca.* 30%).[253] This Lewis acid catalysed cyclisation probably involves the intermediacy of the azetidinium cation (163), which can be neutralised by thiolate attack on C5 to give (160a) (route a), or on C7 to give (162) after isomerisation (route b), or by recyclisation of the side chain onto the β-lactam C7 to give (161) (after isomerisation) (route c) (Scheme 4.4).



Scheme 4.4

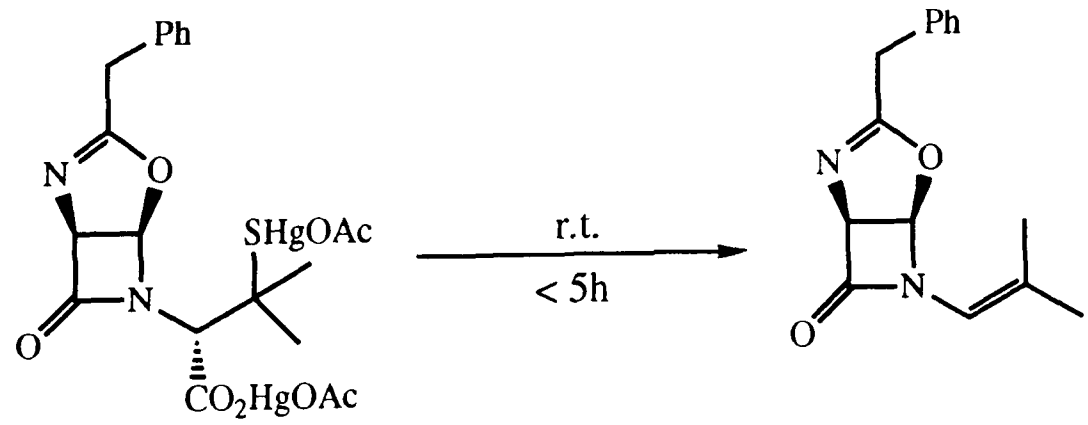
Due to problems associated with the removal of the phthalimide side-chain it was decided to follow this second route to obtain the 5-epipenicillin nucleus. Thus, penicillin G potassium salt (10a) was added to a solution of mercury(II) acetate in acetic acid, upon which the dimercury salt (158) precipitated and was filtered off. This reaction probably proceeds with electrophilic attack of mercury on the sulphur atom, followed by cleavage of the C5-S bond assisted by the β-lactam nitrogen, with possible formation of the azetidinium cation (164). This cation is then neutralised by cyclisation of the side chain onto the azetidine ring with formation of (158) (Scheme 4.5).



(10a) (164) (158)

Scheme 4.5

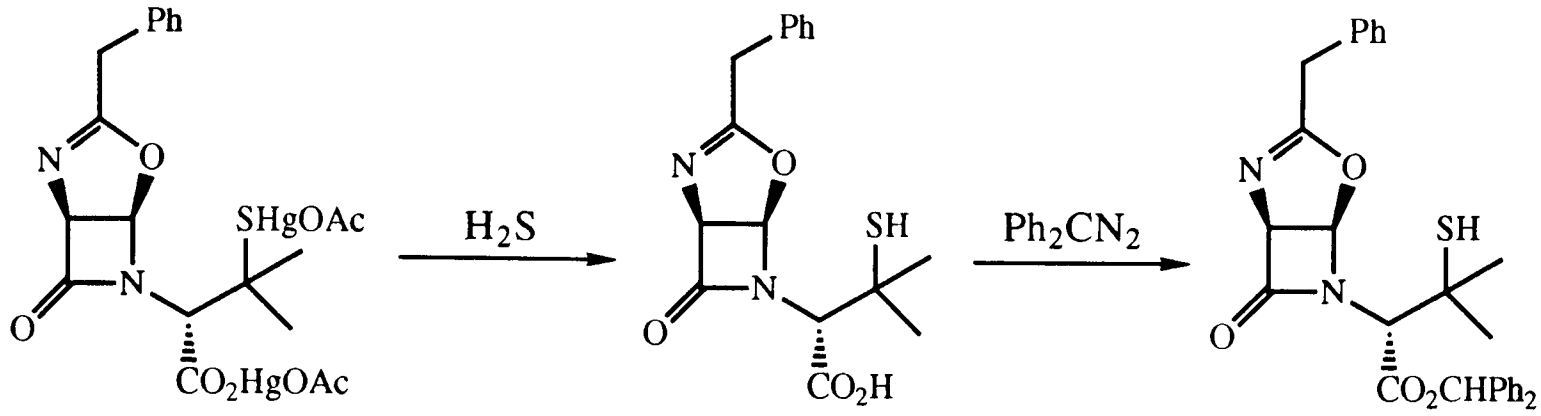
The mercury salt (158) is known to be unstable at room temperature undergoing decarboxylation and elimination to the oxazoline (165) in less than 5h (Scheme 4.6).[250]



(158) (165)

Scheme 4.6

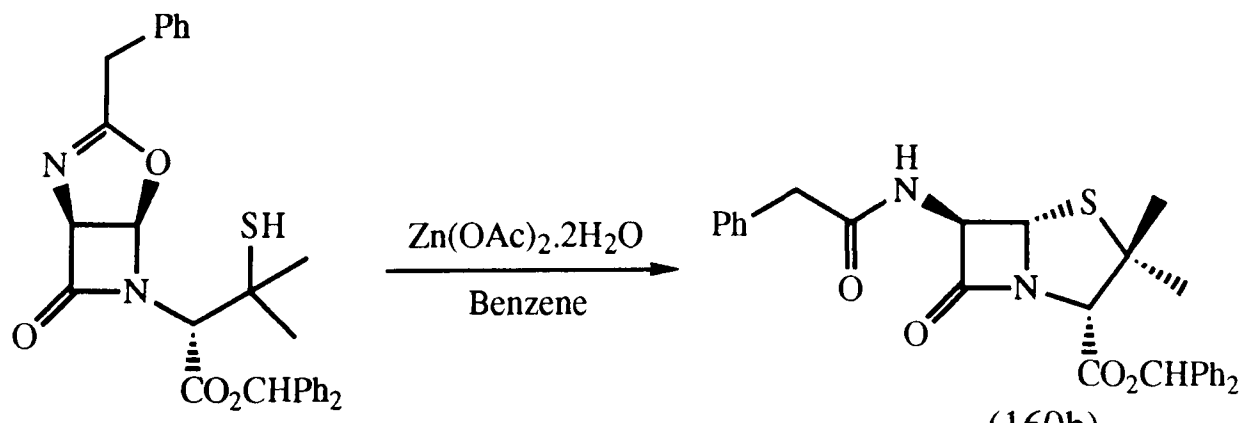
It was thus necessary to use (158) immediately after its formation. Treatment of (158) with hydrogen sulphide effected the removal of mercury to give the mercaptobutanoic acid (166) (Scheme 4.7), which is also an unstable compound decomposing to give several products.[251] As a consequence (166) was not isolated, being esterified straight away with diphenyldiazomethane to give the thiol (159b), which was purified by column chromatography. The benzhydryl ester was chosen as a protecting group to enable its eventual removal by catalytic hydrogenation to give the free acid.



(158) (166) (159b)

Scheme 4.7

In the next step, the cyclisation of the thiol (159b) was effected by treatment with zinc acetate in refluxing benzene. The reaction was followed to completion by T.l.c. with the observed formation of two products. Purification by chromatography proved the less polar product to be the benzhydryl 5-epipenicillin (160b) isolated in 41% yield (Scheme 4.8).



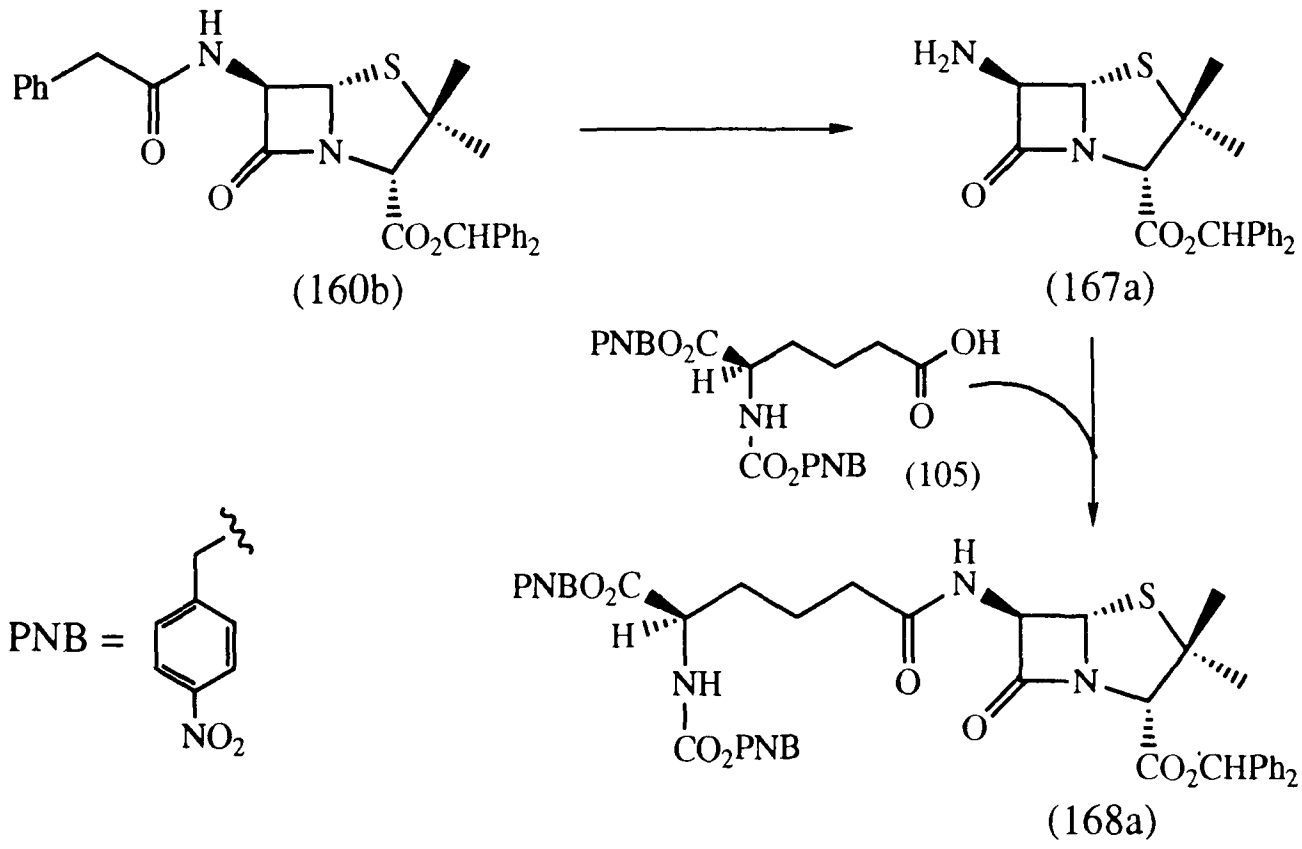
(159b) (160b)

Scheme 4.8

The proton NMR of (160b) is characteristic of the 5-epipenicillin ring system[254], where the C6-H resonates at a higher field than the C5-H (contrary to the natural isomer), and the C3-H is also significantly shielded. The smaller coupling constant of the C5-H and C6-H (J 2 Hz) compared to the natural isomer (J 4 Hz) is characteristic of a trans arrangement of the β-lactam protons, and is also observed in the case of the 6-epipenicillins.[252]

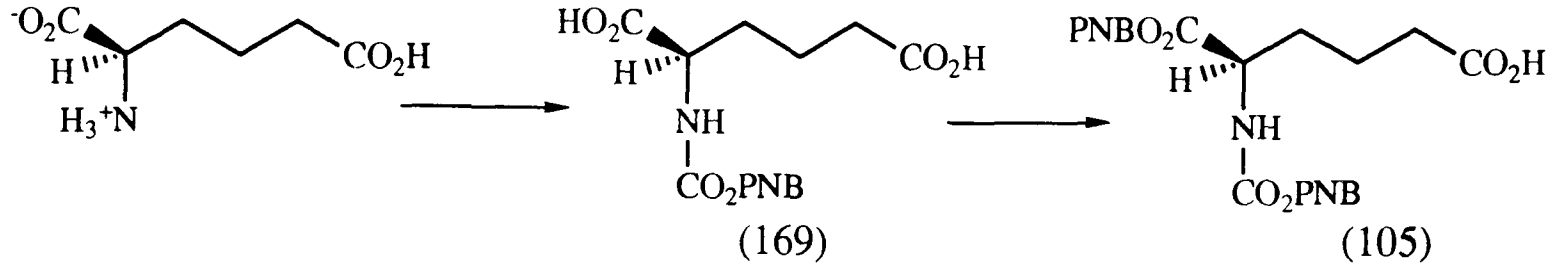
4.2.1.2 Construction of the side chain

Having constructed the 5-epipenicillin nucleus, it was next necessary to replace the benzyl side chain of (160b) with the D-α-aminoadipoyl side chain of 5-epipenicillin N (154). This required cleavage of the (160b) side chain and formation of the 6-amino-5-epipenicillanic acid benzhydryl ester (167a). Coupling of this with diprotected D-α-aminoadipic acid (105) would provide (168a), the protected version of 5-epipenicillin N (154) (Scheme 4.9).



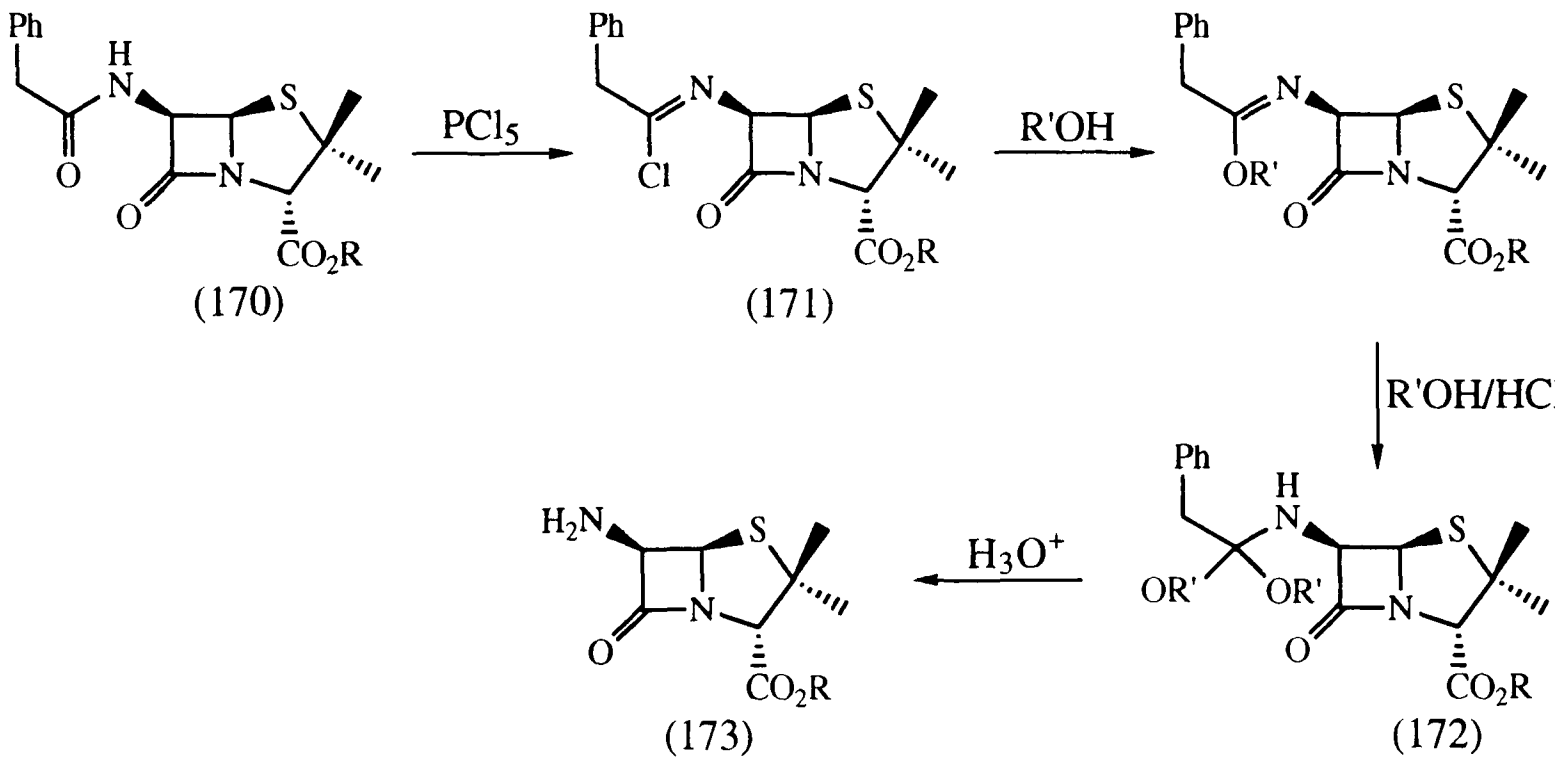
Scheme 4.9

The *p*-nitrobenzyl protected D-α-aminoadipic acid (105) was obtained using standard procedures by treatment of D-α-aminoadipic acid with *p*-nitrobenzyl-chloroformate to give the monoprotected acid (169), followed by reaction with *p*-nitrobenzylbromide to give (105) (Scheme 4.10).



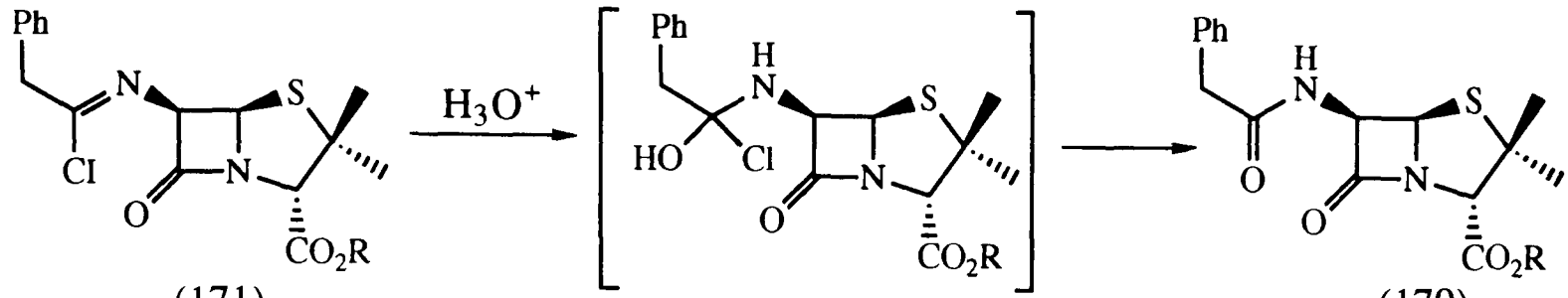
Scheme 4.10

The chemical cleavage of a penicillin side chain is known to be a difficult reaction, due to the general sensitivity of the penicillin nucleus to chemical manipulation. One of the procedures available, first developed for cephalosporins[255] but later applied to penicillins[74,256], involves treatment with phosphorus pentachloride and a base to give the imino chloride (171), followed by reaction with an alcohol to form (172), which on acid work up hydrolyses to give the free amine (173) (Scheme 4.11).



Scheme 4.11

It is important that the reaction should be carried out in an anhydrous medium as interception of the imino chloride (171) by water leads to regeneration of starting material (Scheme 4.12).

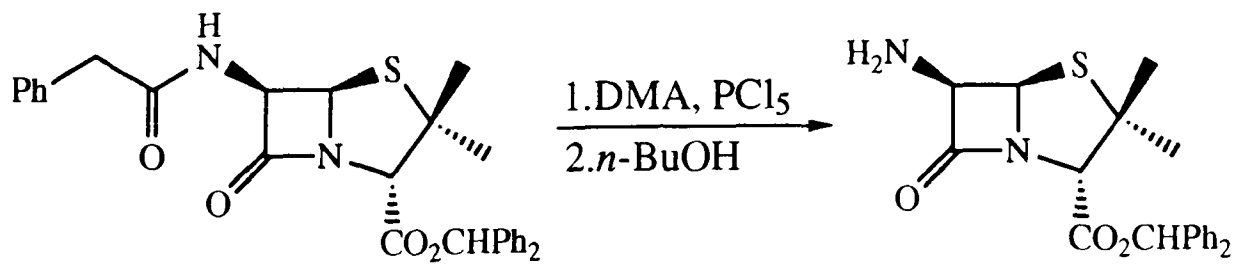


(171) (170)

Scheme 4.12

This reaction poses no problems in the case of cephalosporins, but is known to be very problematic when applied to penicillins.[257,258] It was decided, however, to attempt this procedure for the formation of (167a), as the alternative method for removal of the side-chain of (160b) – an enzymatic hydrolysis using an acylase – was considered less favourable, since deprotection and later reprotection of the acid function would be required. In addition, this enzymatic hydrolysis is usually carried out only on small scales.

In the first attempted reaction a solution of (160b) and N,N-dimethylaniline (DMA) in dry DCM was cooled to -78°C, and PCl_5 was added. After 1h at -40°C, the reaction mixture was recooled to -78°C, an excess of isobutanol was added and the reaction was left to warm up to 0°C over 1h. After another 40 min at 0°C, the reaction was quenched by addition of water and the pH was adjusted to 10. The solid was extracted into an organic phase (EtOAc) which was dried and evaporated to a yellow oil. Purification by flash chromatography yielded several impure products. After further purification by P.l.c. the amine (167a) was finally obtained in a disappointing 12% yield. Not having been very successful, it was decided to study the reaction with the more readily available benzhydryl ester of penicillin G (170a) (Scheme 4.13).

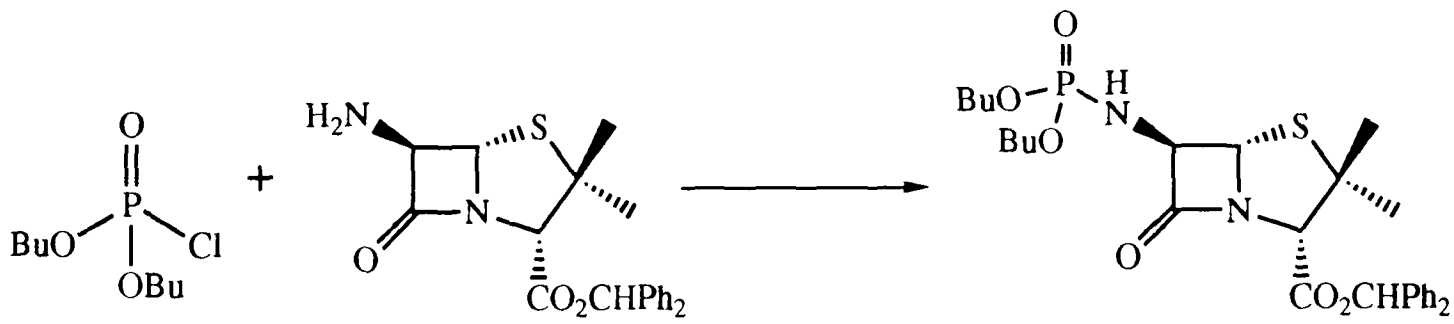


(170a) (173a)

Scheme 4.13

The PCl_5 reaction was repeated for (170a) as before, but using *n*-BuOH instead of isobutanol. It was found that on work up, basification to pH 8 was sufficient to enable extraction of the amine into the organic phase. Again, purification by chromatography gave a mixture, revealing that some of the products had very similar Rf values. The amine (173a) was finally obtained in 10% yield after further purification by P.l.c.. Reaction with (170a) was once again repeated with longer times being allowed for hydrolysis, although this was found to be complete by T.l.c. after 5 min. This time, chromatography gave the pure amine (173a) in an improved yield of 30%.

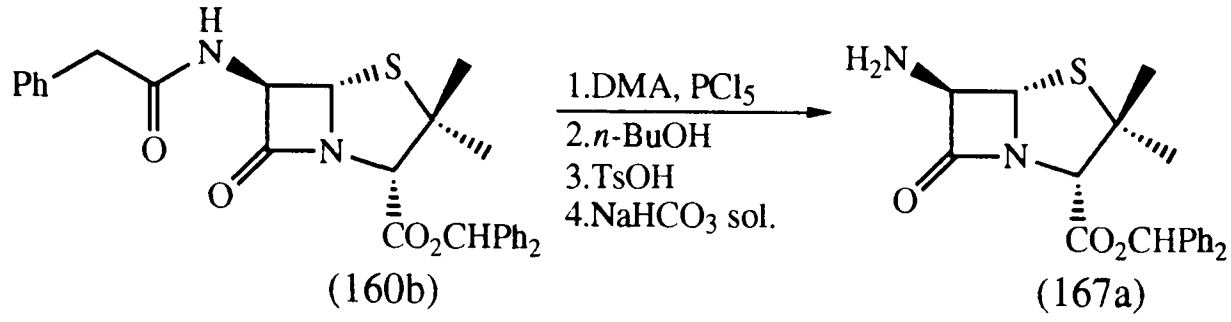
The side-chain removal was again attempted with the 5-epipenicillin (160b). The solution obtained on work up showed by T.l.c. that the amine (167a) was the major product by careful comparison with authentic material. However, after solvent evaporation and flash chromatography the major product was not the amine, although it had a very similar Rf. After acid washing to remove whatever amine was present, this major product was obtained pure and identified as the phosphoramide (174). An analogous phosphoramide had previously been observed in an identical reaction with benzyl penicillin V.[258] As only the amine (167a) was present right after work up, it is probable that formation of (174) occurred on solvent evaporation or on chromatography, by reaction of the amine (167a) with some chlorophosphate (175) present in the mixture (Scheme 4.14).



Scheme 4.14

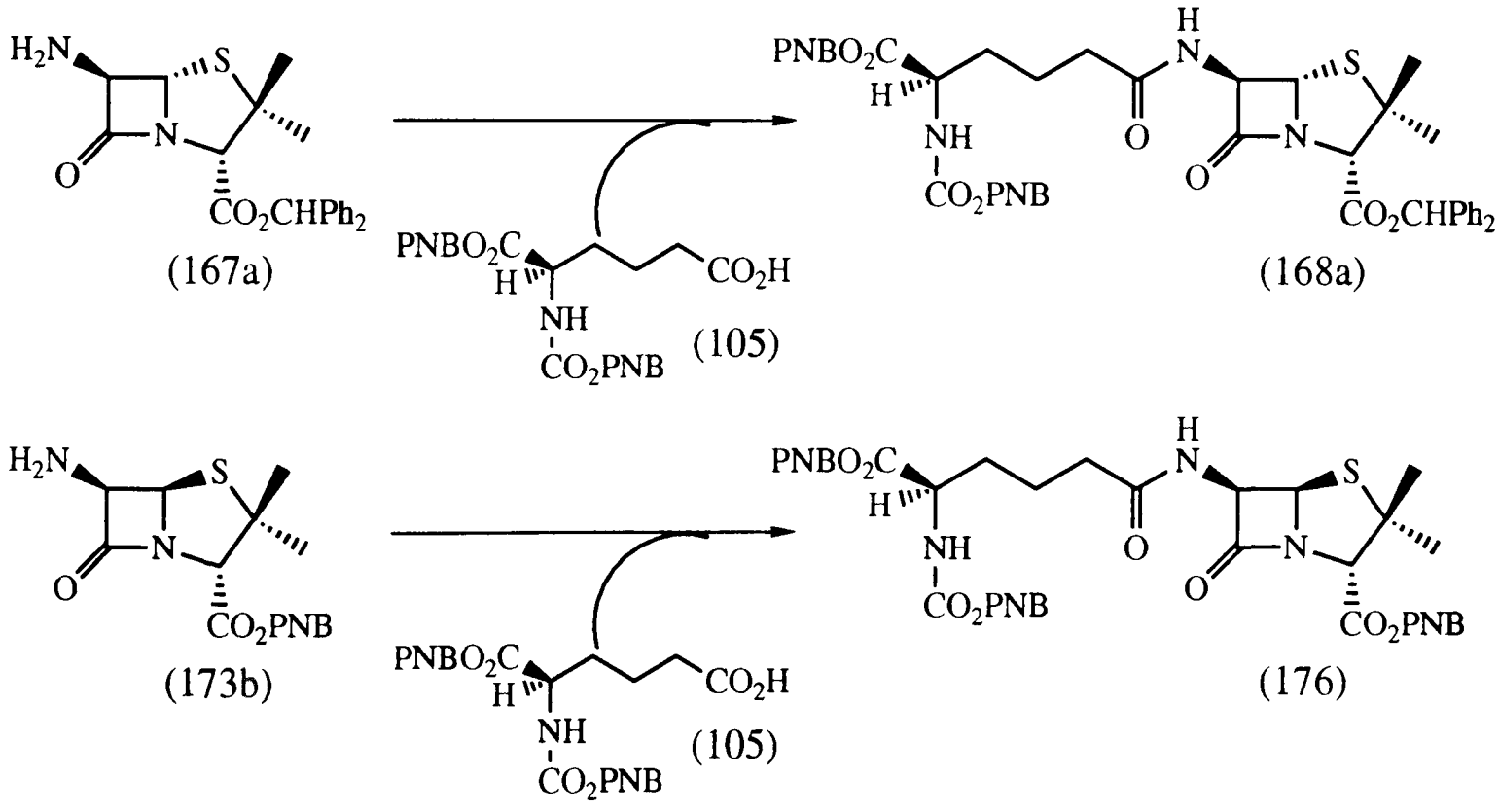
As such, it was envisaged that if the amine was protected before concentration and chromatography, formation of (174) could possibly be avoided. The reaction was then repeated for (160b), with *p*-toluenesulfonic acid being added to the organic solution

obtained on work up. Chromatography of the crude product gave a mixture of tosic salts of the amine (167a) and N,N-dimethylaniline. Treatment with base to release the amines, followed by quick flash chromatography to separate the two, finally provided the amine (167a) in an acceptable yield of 55% from (160b) (Scheme 4.15). This procedure was later repeated for this and other penicillins, and has proven to be reproducible giving reasonable yields of amine (*ca.* 60%), and avoiding formation of the phosphoramides.



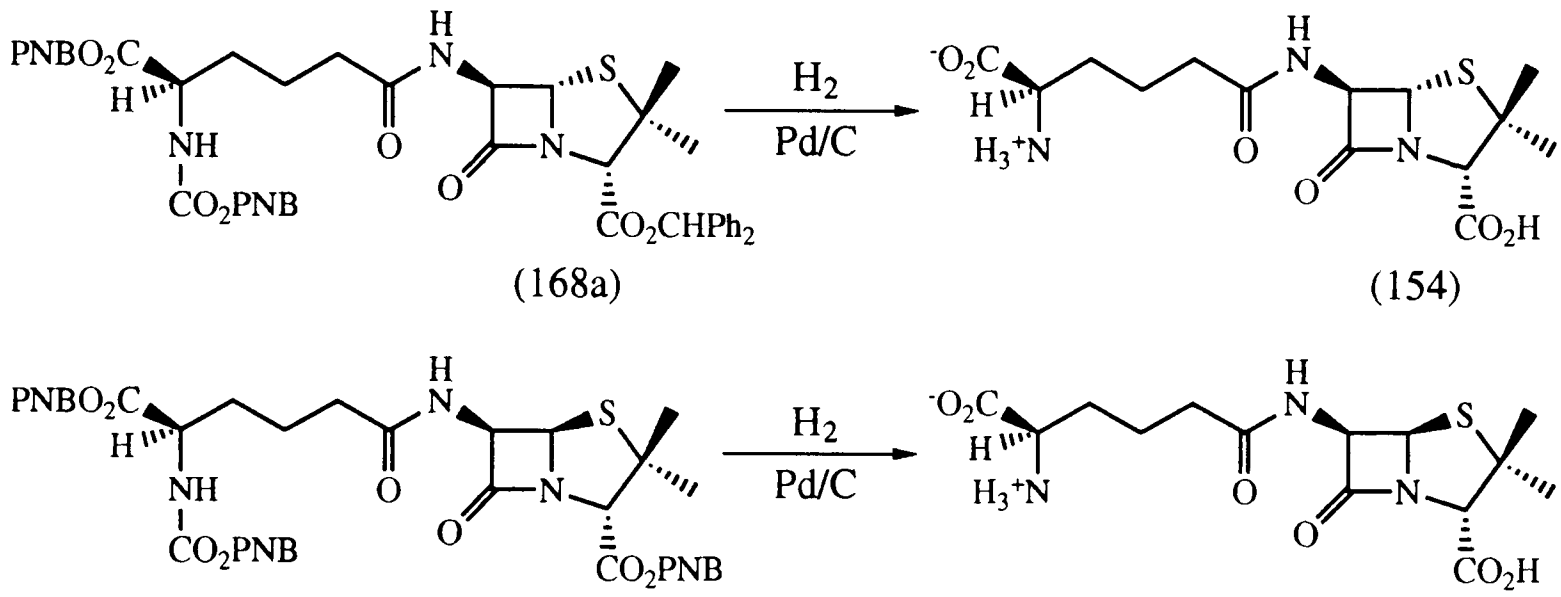
Scheme 4.15

In the next step, the amine (167a) was coupled to the *p*-nitrobenzyl protected D-α-aminoadipic acid (105) using EEDQ[220], to give the fully protected 5-epipenicillin N (168a) (Scheme 4.16). The same procedure was repeated with available *p*-nitrobenzyl protected 6-aminopenicillanic acid (173b) to obtain the protected form of penicillin N (176).



Scheme 4.16

The removal of the protecting groups in (168a) to give the 5-epipenicillin N (154) was expected to be a staightforward step, easily accomplished by catalytic reduction with hydrogen over palladium on carbon (Scheme 4.17). However, when this reaction was carried out on (168a) the crude product appeared to be a complex mixture as judged by NMR, and the product (154) was isolated in only 2% yield after purification by HPLC. The organic washings of the crude reaction showed no evidence of starting material. When the same reaction was repeated with (176), penicillin N (1) was obtained cleanly in 64% yield.



(168a) (154)

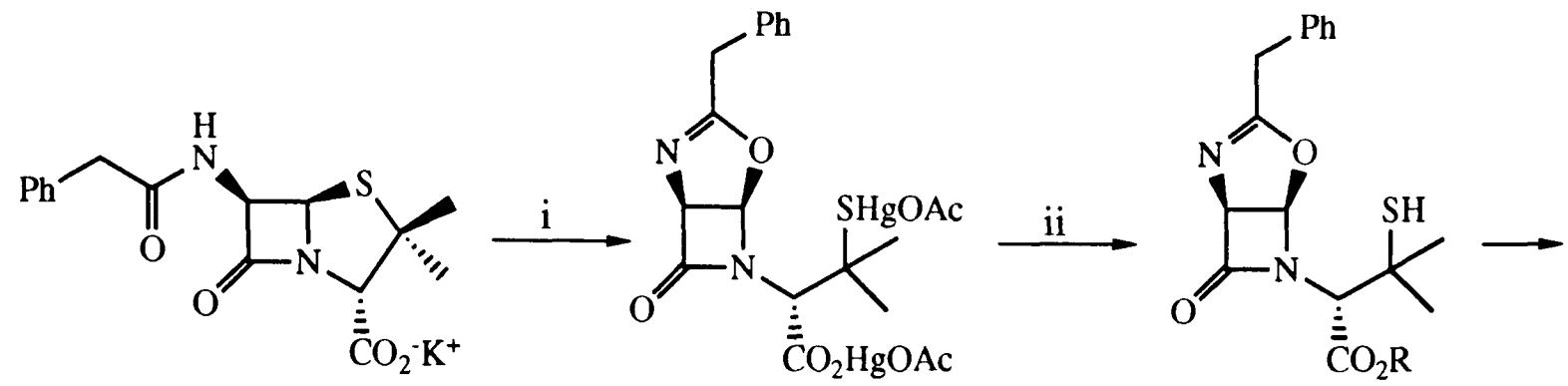
(176) (1)

Scheme 4.17

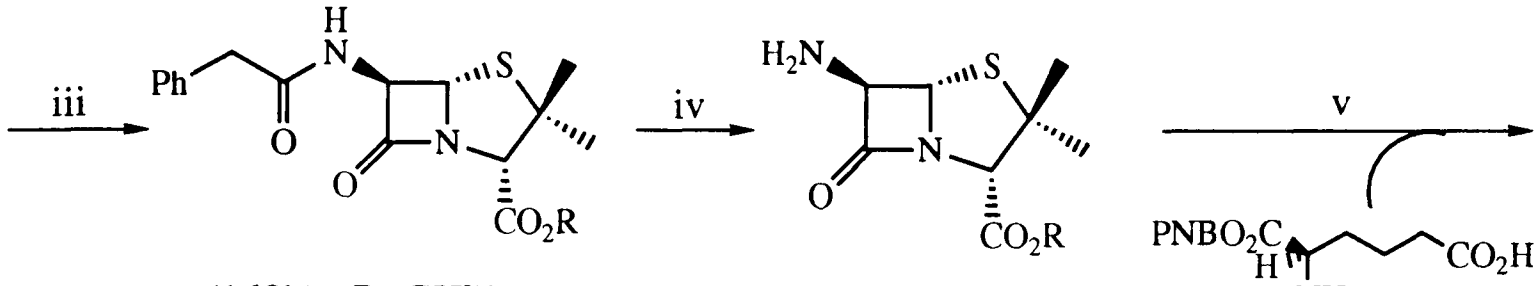
As there were no reports of particular instability for the 5-epipenicillin nucleus, it was thought that maybe the benzhydryl group was more difficult to remove than the *p*-nitrobenzyl one, thus resulting in only partial deprotection of (168a). In an attempt to avoid this, the whole synthesis was repeated replacing diphenyldiazomethane with *p*-nitrophenyldiazomethane[259] in order to obtain the *p*-nitrobenzyl ester (168b) (Scheme 4.18).

However, hydrogenolysis of fully *p*-nitrobenzyl protected 5-epipenicillin N (168b) again gave an unclean mixture of products from which (154) was isolated in 6% yield, with

no starting material recovered. It is apparent that some intrinsic property of the 5-epipenicillin nucleus is promoting its decomposition on hydrogenolysis, as there are no major problems in the preparation of penicillin N (1). It is possible that the sulphur atom in (168b) is less hindered than in (176), and is capable of poisoning the palladium catalyst thereby inhibiting the hydrogenolysis reaction. Stoodley *et al.* have also reported that hydrogenolysis of the *p*-nitrobenzyl ester of 5-epipenicillin G gave no β-lactam products, whereas the *p*-nitrobenzyl ester of 5-epipenicillin V gave the corresponding acid in 6% yield.[253] In contrast, Busson *et al.* have reported that hydrogenolysis of the benzyl ester of 5-epipenicillin G gave the corresponding acid in 60% yield.[254]



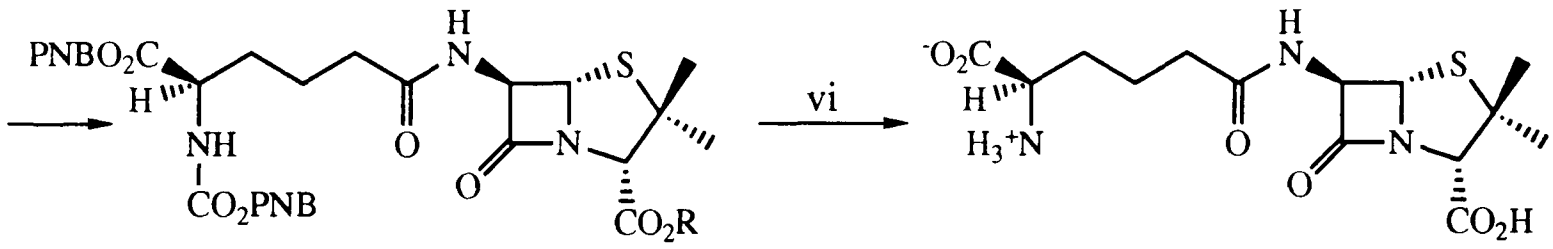
(10a) (158) (159b) : R=$CHPh_2$
(159c) : R=PNB



(160b) : R=$CHPh_2$
(160c) : R=PNB

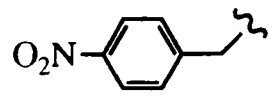
(167a) : R=$CHPh_2$
(167b) : R=PNB

(105)



(168a) : R=$CHPh_2$
(168b) : R=PNB

(154)

PNB = 

Reagents : i) $Hg(OAc)_2$,HOAc; ii) H_2S,DCM then Ph_2CN_2 or O_2NArCN_2
iii) $Zn(OAc)_2$,Benzene; iv) PCl_5,DMA,DCM then *n*-BuOH then TsOH;
iv) EEDQ,DCM,Na_2SO_4; vi) H_2,Pd/C

Scheme 4.18

4.2.2 Incubations of 5-Epipenicillin N with DAOC/DACS

The 5-epipenicillin N (154) was incubated with DAOC/DACS and cofactors according to the normal procedure. After work up and lyophilisation, the crude incubation mixture was analysed by 500 MHz ^{1}H-NMR. This showed that the starting material (154) was still present and that no new β-lactam products had been formed, and hence that no conversion had occurred. The crude incubation mixture was lyophilised once again, and another ^{1}H-NMR was run on the following day. This showed that, surprisingly, (154) was no longer present in the mixture. Two multiplets (δ 5.16 and 5.25 ppm) could be observed in the β-lactam absorbing region suggesting that the β-lactam ring had been destroyed.

To determine whether the 5-epipenicillin N was inherently unstable, a solution of (154) in D_2O was left at room temperature and monitored by 500 MHz ^{1}H-NMR. After 25h no decomposition of (154) could be observed, indicating that it is not particularly unstable on its own.

As a control for the catalytic activity of the enzyme a 1:1 mixture of 5-epipenicillin N (154) and penicillin N (1) was incubated with DAOC/DACS. Analysis of the crude incubation mixture by 500 MHz ^{1}H-NMR revealed about 80% conversion of pen N (1) to DAOC (2) and DAC (3), but no new products from (154) could be observed. This was present in a considerably diminished amount, suggesting that decomposition had once again occurred.

Bioassay tests against *E. coli* X580 (-) (without β-lactamase), *E. coli* X580 (+) (with β-lactamase) and *Staphilococcus aureus* NCTC 6571 were carried out with the 5-epipenicillin N (154) and penicillin N (1) as isolated compounds, and after incubation with active DAOC/DACS and with denatured enzyme. These tests showed that (154) is not bioactive against the organisms tested in accord with previous observations[252,253], and that it is not converted by DAOC/DACS into a cephalosporin-like β-lactamase resistant compound. Penicillin N (1) showed bioactivity against *E. coli* (-) and *S. aureus*, and not

against *E. coli* (+), but was converted by DAOC/DACS to a compound [DAOC (2) and/or DAC (3)] exhibiting activity against *E. coli* (-) and (+).

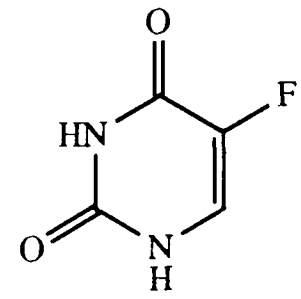
These results led us to the conclusion that 5-epipenicillin N (154) is not accepted as a substrate by DAOC/DACS, which indicates that the 5R configuration of the penicillin nucleus is crucial for conversion by DAOC/DACS. Inhibition studies were not carried out due to the limited amounts of (154) available by synthesis and also to its apparent instability to the incubation conditions.

The absence of bioactivity for the 5S and 6S epimers of penicillins indicates that a *cis* 5R,6R configuration for the β-lactam ring is a requisite for the antibiotic activity of penicillins.

4.3 2β-Difluoromethyl Penicillin N

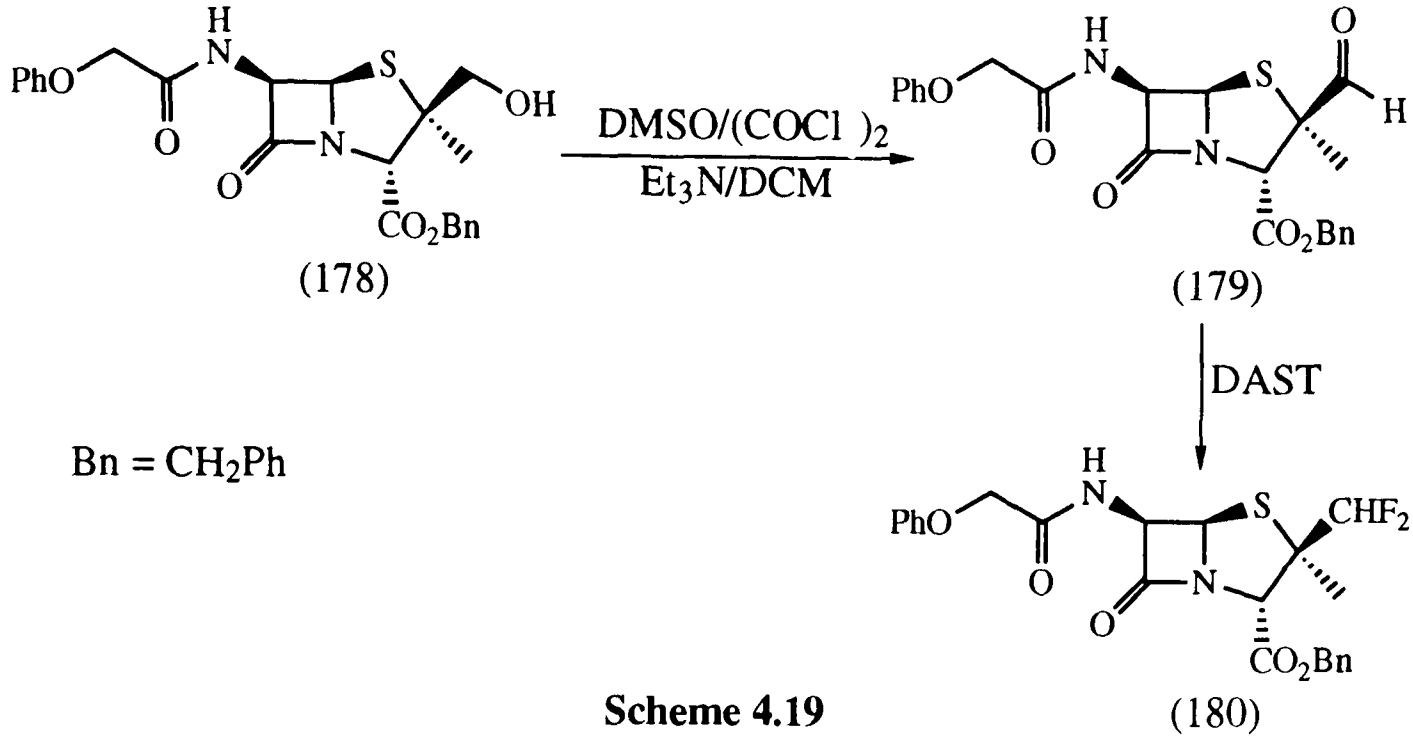
Fluorine is the second smallest element of the periodic table in terms of size, as its Van der Waals radius ($r_F \approx 1.35$ Å) closely resembles that of hydrogen ($r_H \approx 1.10$ Å). As a result of this fluorine can replace hydrogen without significant steric consequences. In contrast, the two atoms have considerably different reactivities, as the high density of positive charge in the nucleus and the tendency to complete its valence shell render fluorine strongly electronegative. This similarity of size and difference in reactivity has prompted the synthesis of many fluorinated analogues for the study of metabolic conversions.[260]

A good example of the drastic effect replacement of hydrogen by fluorine can have, is provided by 5-fluorouracil (177)[261], which is used in cancer chemotherapy. This compound is metabolised to 5-fluoro-2'-deoxyuridine monophosphate which is a very strong inhibitor of thymidylate synthetase – an enzyme involved in the synthesis of thymine, an essential component of DNA. Administration of 5-fluorouracil to cancerous cells causes the replication of DNA to breakdown, and prevents further cell division.[262]



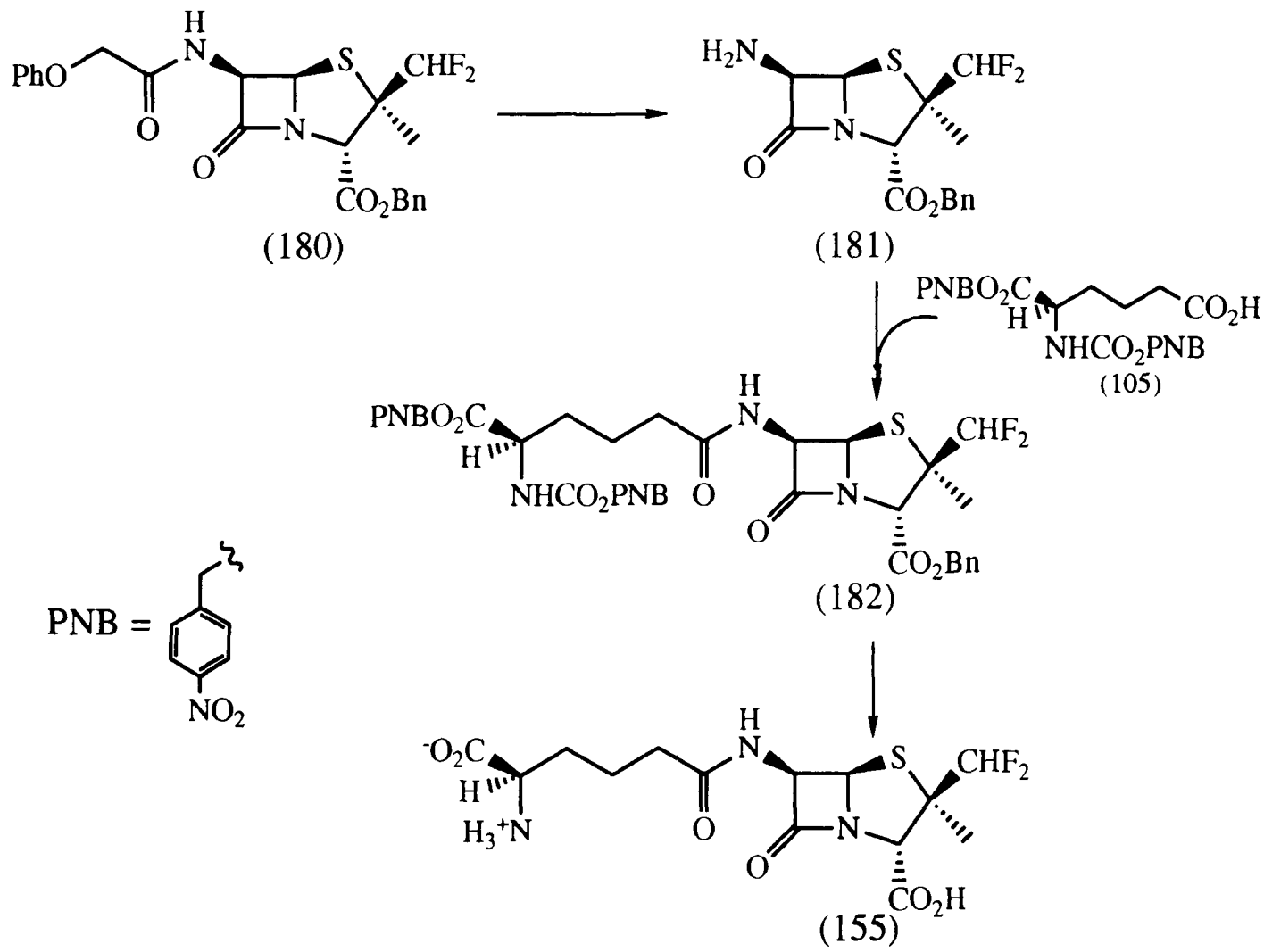
(177)

In the sequence of investigations towards the synthesis of 2β-functionalised penicillins, it was found that Swern oxidation of the 2β-hydroxymethyl penicillin (178) afforded the aldehyde (179), which could be fluorinated with diethylaminosulphur trifluoride (DAST) to the 2β-difluoromethyl penicillin (180) (Scheme 4.19).[263]



Scheme 4.19

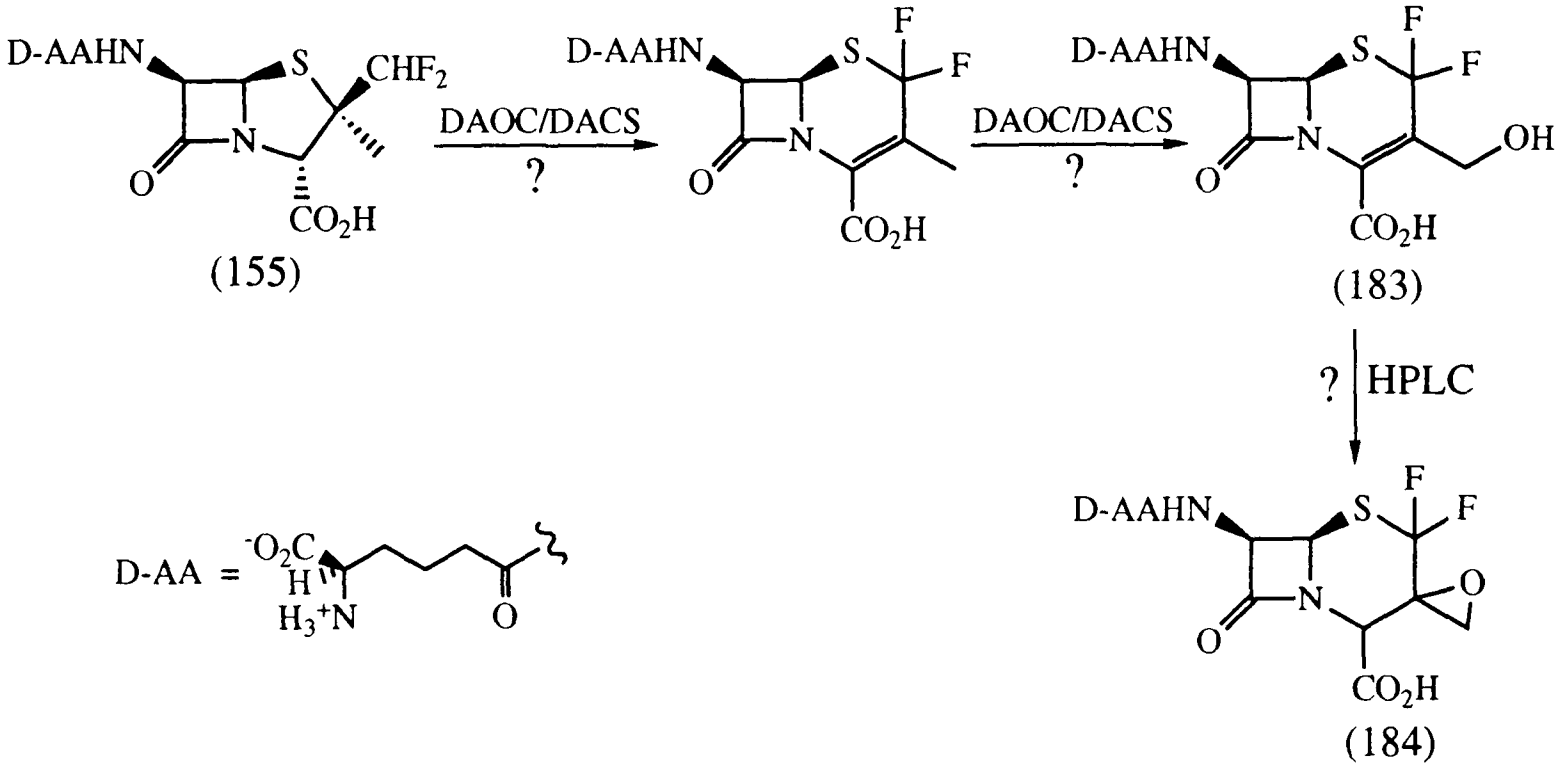
The availability of (180) prompted the synthesis of 2β-difluoromethyl penicillin N (155), to be used as a substrate analogue for DAOC/DACS. This was achieved by cleavage of the phenoxyacetyl side chain of (180) to give the amine (181), followed by coupling with *p*-nitrobenzyl protected D-α-aminoadipic acid (105) to give (182). Subsequent hydrogenolysis of (182) provided the desired 2β-difluoromethyl penicillin N (155) (Scheme 4.20).[258,264]



Scheme 4.20

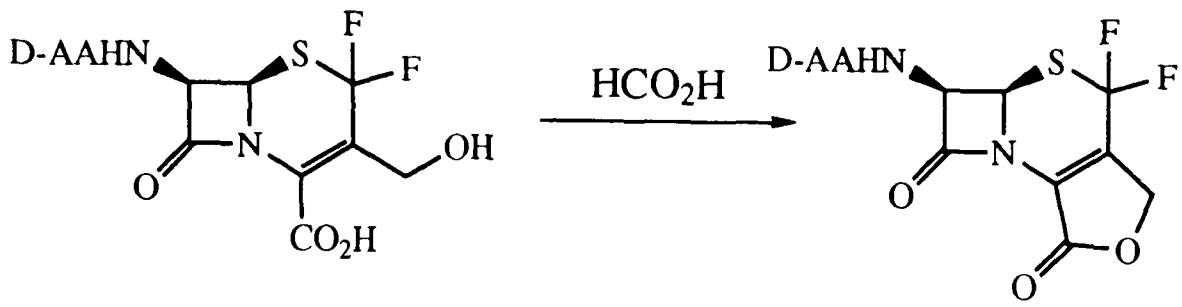
Incubations of (155) with DAOC/DACS resulted in the formation of a new β-lactam product as judged by 500 MHz ^{1}H-NMR.[258,264] This compound showed bioactivity against *S. aureus* NCTC 6571 and *E. coli* X580, and diminished activity against the latter in the presence of β-lactamase, which suggested the presence of a cephem product.

Attempted HPLC purification of this product resulted in considerable loss of material, and apparent conversion to a new less polar β-lactam product.[264] Mass spectroscopy revealed that this new product had a mass increase of 14 relative to (155), which corresponds to the loss of two hydrogens and the gain of one oxygen. The observed loss of material indicated that the product of the DAOC/DACS incubation with (155) was unstable. It was suggested that the new β-lactam product observed after HPLC purification could be the epoxide (184), formed from the expected product of DAOC/DACS, the difluoro-DAC (183) (Scheme 4.21).



Scheme 4.21

In an attempt to stabilise the possible product (183), lactonisation with formic acid was attempted prior to purification (Scheme 4.22).[258] After addition of formic acid to the crude incubation product of (155) with DAOC/DACS, survival of the β-lactam compounds was confirmed by ^{1}H-NMR. However, after HPLC purification no β-lactam products were isolated apart from the starting material (155).



(183) (185)

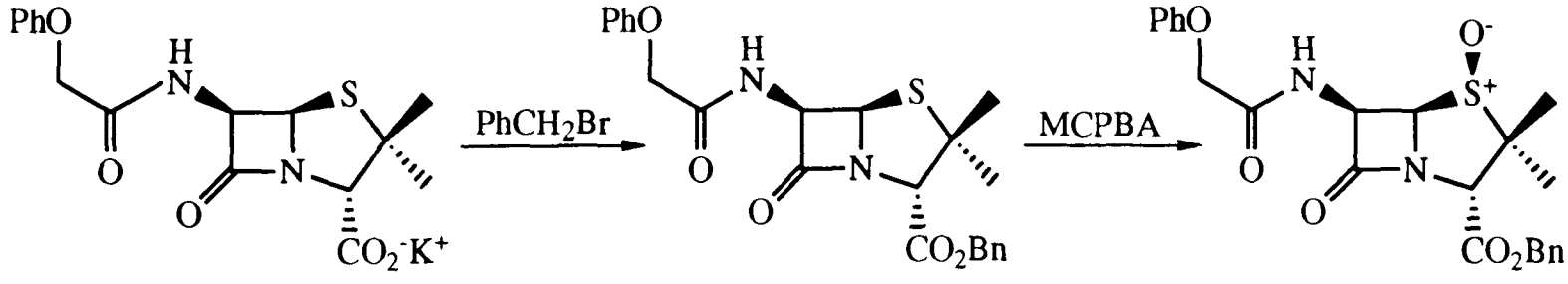
Scheme 4.22

In view of the availability of the new soluble recombinant DAOC/DACS possessing considerably higher activity, it was suggested that the synthesis and incubation of the 2β-difluoromethyl penicillin N (155) be repeated, in the hope that the new enzyme would provide larger amounts of the unstable incubation product, thereby enabling its isolation and characterisation.

4.3.1 Synthesis of 2β-Difluoromethyl Penicillin N

4.3.1.1 Construction of the ring system

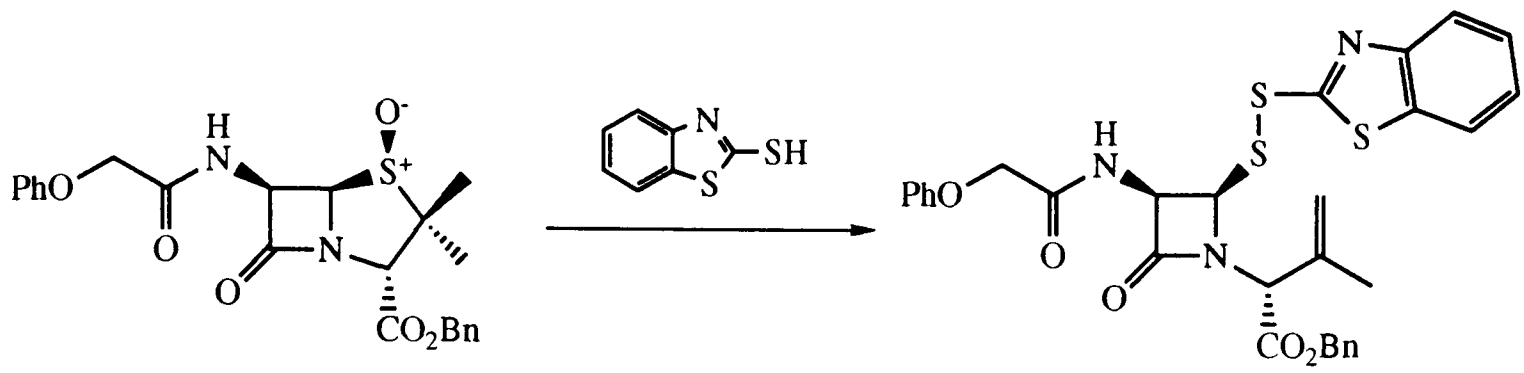
Synthesis of the 2β-difluoromethyl penicillin nucleus required the 2β-hydroxymethyl penicillin as starting material. Its preparation was achieved according to reported procedures[265,266] in the following way : esterification of penicillin V potassium salt (11a) with benzyl bromide provided the corresponding benzyl ester (186). This was oxidised to the β-sulfoxide (187) using *m*-chloroperoxybenzoic acid (MCPBA) (Scheme 4.23). Only the β-sulfoxide is formed in this reaction because the β-amido group at C6 forms a hydrogen bond with the peracid, directing the stereochemical outcome of the reaction[267].



(11a) (186) (187)

Scheme 4.23

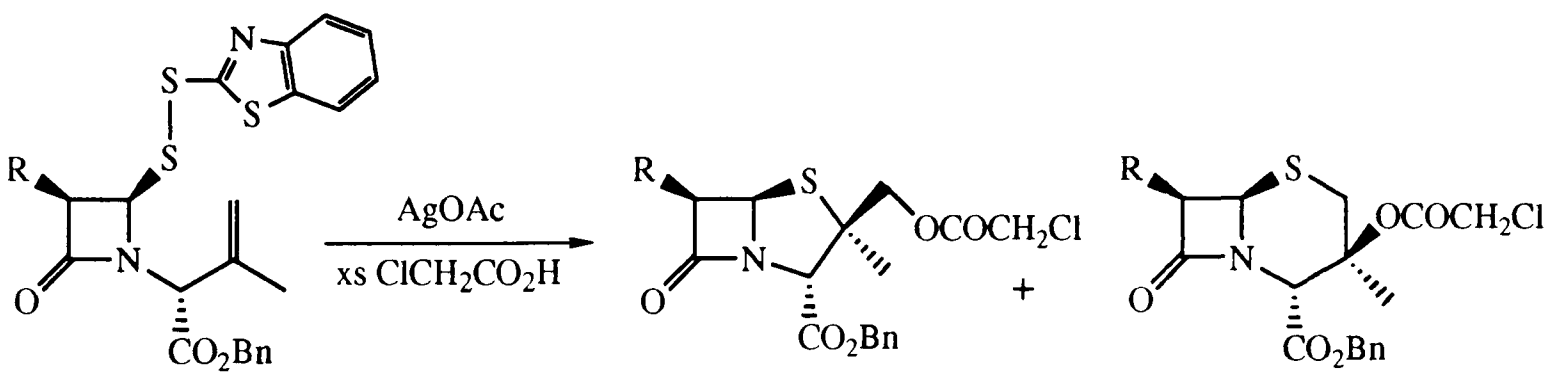
Condensation of the sulfoxide (187) with mercaptobenzothiazole by refluxing in toluene using a Dean-Stark apparatus, provided Kamiya's disulphide (188)[268] (Scheme 4.24) :



(187) (188)

Scheme 4.24

Treatment of disulphide (188) with silver acetate in the presence of an excess of chloroacetic acid resulted in the formation of a mixture of 2β-chloroacetoxymethyl penicillin (189) and 3-chloroacetoxy cepham (126) in 42% and 30% yield respectively (Scheme 4.25). Although these two products have very similar Rf values, they could be separated by careful flash chromatography.

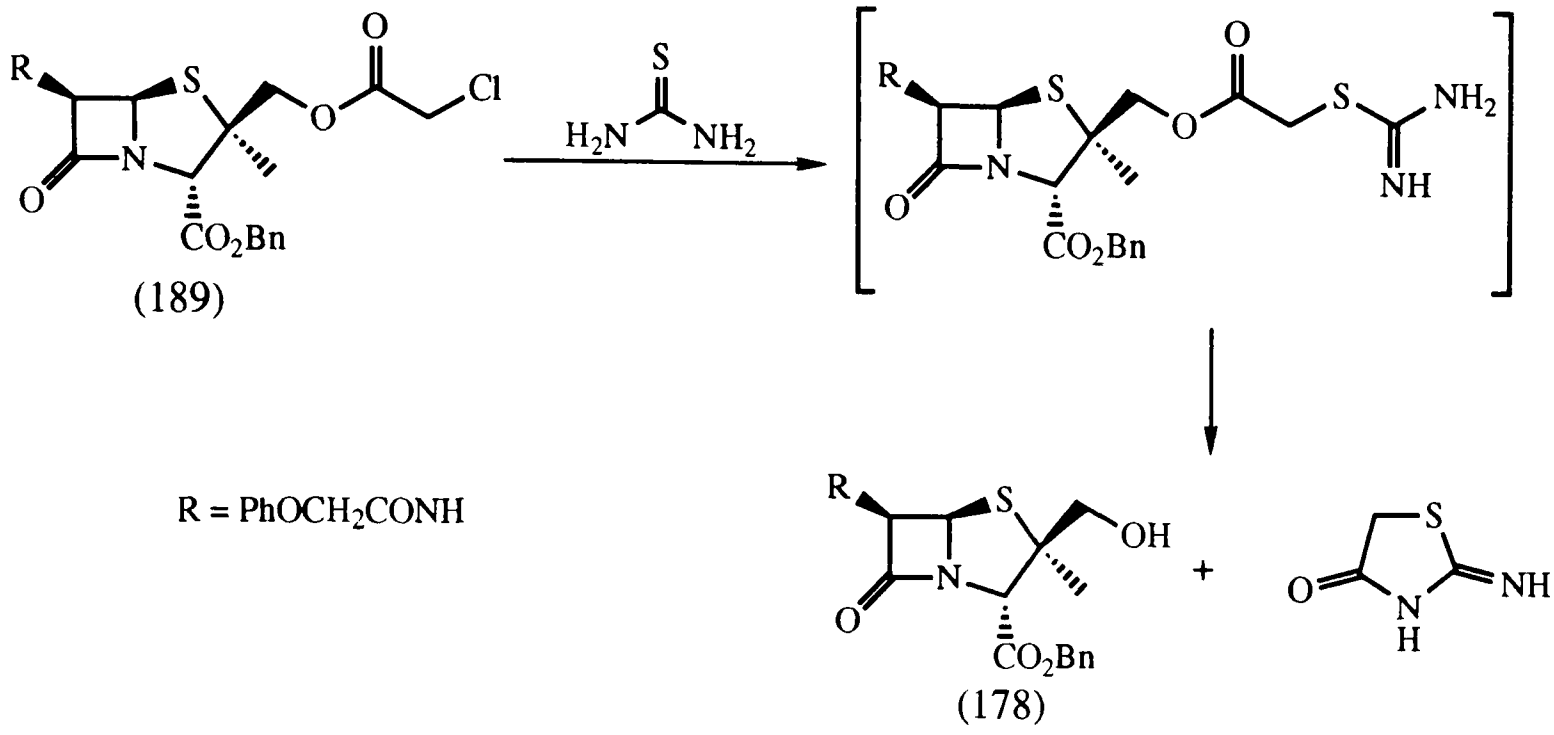


(188) (189) (126)

R = $PhOCH_2CONH$

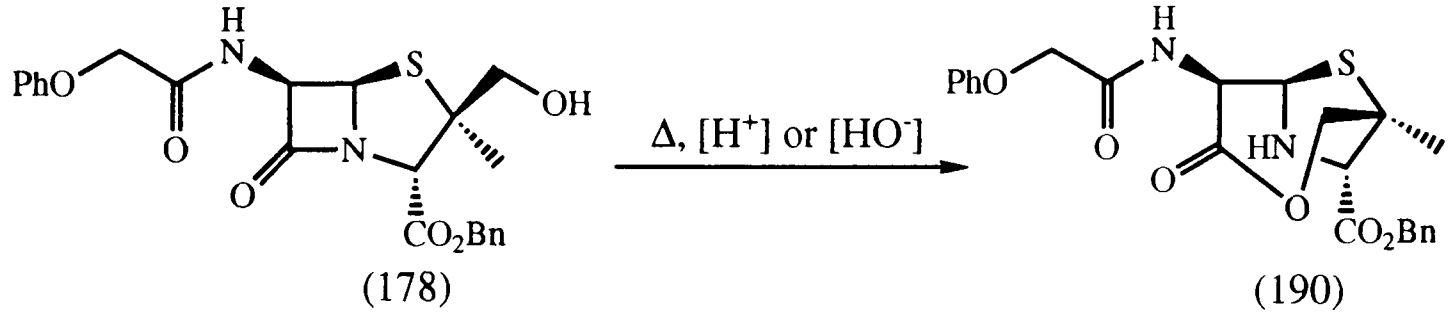
Scheme 4.25

Reaction of the 2β-chloroacetoxymethyl penicillin (189) with thiourea in hot ethanol provided the desired 2β-hydroxymethyl penicillin (178) (Scheme 4.26).



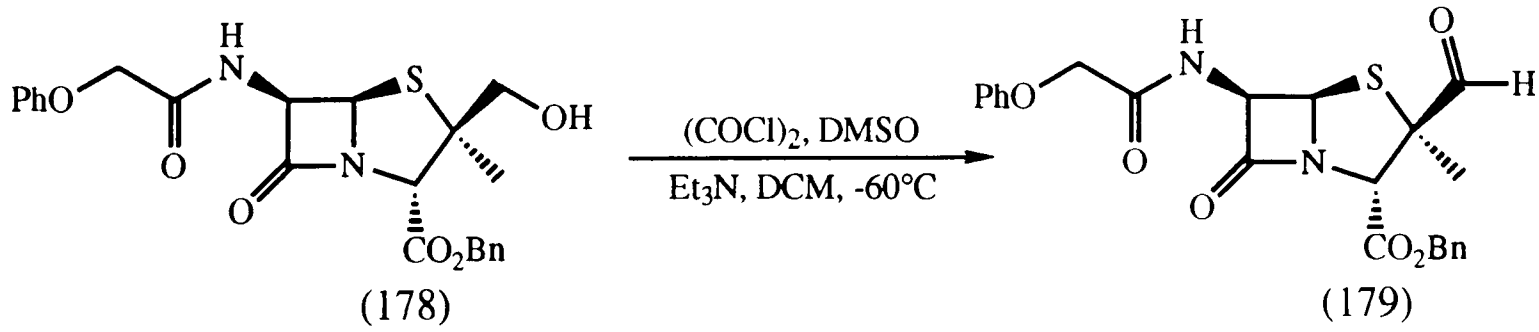
Scheme 4.26

The penicillin alcohol (178) is very unstable requiring little provocation to convert it to the lactone (190) (e.g. silica gel or heat) (Scheme 4.27).[263,265] For this reason the product (178) was not purified but used crude in the next step.



Scheme 4.27

Swern oxidation[269] of the alcohol (178) gave the aldehyde (179) in good yield (Scheme 4.28). This again could not be purified by silica chromatography due to instability, and was used crude in the next reaction.



Scheme 4.28

Treatment of the aldehyde (179) with diethylaminosulphur trifluoride (DAST)[270] in DCM provided the 2β-difluoromethyl penicillin (180) which was purified by flash chromatography (Scheme 4.29). The presence of the CHF_2 group was easily recognised in the ^{1}H-NMR by a triplet at 5.63 ppm with a proton-fluorine coupling constant of 56 Hz.

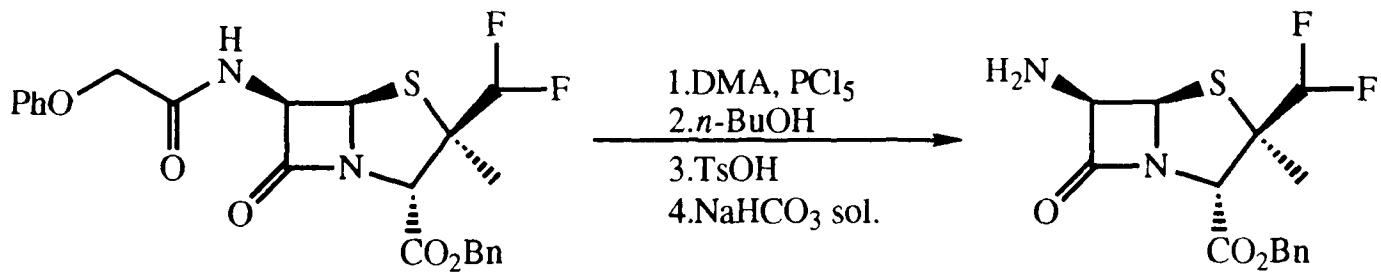


(179) (180)

Scheme 4.29

4.3.1.2 Construction of the side chain

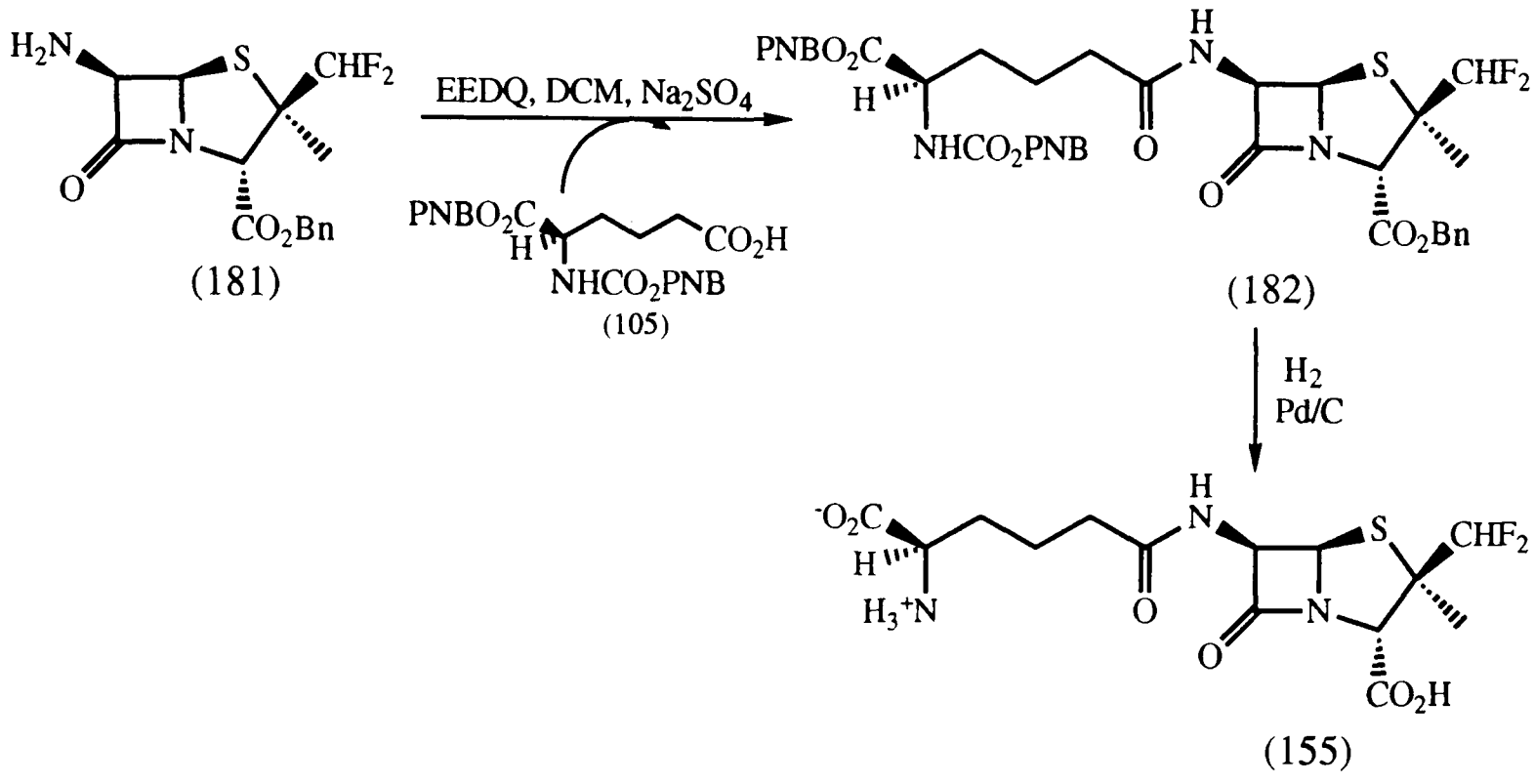
The phenoxyacetyl side chain of (180) was cleaved using the procedure developed for the preparation of the 5-epipenicillamine (167a), which provided the 2β-difluoromethyl penicillamine (181) in a very good 68% yield (Scheme 4.30).



(180) (181)

Scheme 4.30

Coupling of the amine (181) with N-*p*-nitrobenzyloxycarbonyl-D-α-aminoadipic acid-α-nitrobenzyl ester (105) using EEDQ[220] provided the fully protected 2β-difluoromethyl penicillin (182). Hydrogenolysis of (182) over palladium on carbon yielded the desired 2β-difluoromethyl penicillin N (155) (Scheme 4.31).



Scheme 4.31

4.3.2 Incubations of 2β-Difluoromethyl Penicillin N with DAOC/DACS

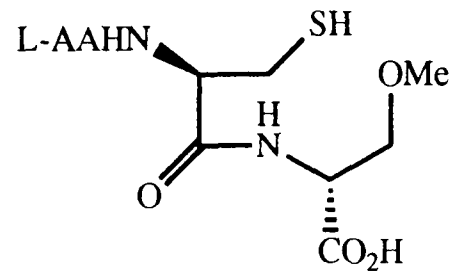
Several incubations of (155) with active recombinant DAOC/DACS were carried out using different amounts of enzyme and/or substrate, and also different batches of enzyme. ^{1}H-NMR analysis usually showed that practically no conversion, if any, had occurred. The starting material could still be observed, together with some other very weak peaks in the β-lactam region, but a clear single product could not be identified. HPLC purification of several of the crude incubation mixtures resulted only in the isolation of starting material.

The 2β-difluoromethyl penicillin (155) showed the expected bioactivity against *S. aureus* and *E. coli* , but not against the latter in the presence of β-lactamase. One of the crude incubation mixtures was bioassayed against these organisms, and displayed activity even against *E. coli* in the presence of β-lactamase. This indicates that a cephem product was present in the incubation mixture in very small amounts, albeit in not enough quantity to be detected by ^{1}H-NMR.

In a control experiment the penicillin (155) was incubated with denatured DAOC/DACS. Analysis by ^{1}H-NMR revealed that the starting material was the only β-lactam present. This experiment indicated that the very weak signals sometimes observed in the β-lactam region of incubations with active DAOC/DACS, resulted from some reaction between the enzyme and the substrate, and not just from degradation of the starting material.

A sample of 2β-difluoromethyl penicillin N (155) was supplied to scientists of Eli Lilly & Co. who were also unable to observe its conversion by DAOC/DACS, but found that it acted as a competitive inhibitor in the conversion of penicillin N (1) to DAOC (2), thus demonstrating that (155) can at least reversibly bind to the active site.

The inability to observe the product previously reported to be formed in incubations of (155) with wild-type DAOC/DACS[258,264], may be due to the fact that recombinant enzyme, which may have a slightly different reactivity compared to the natural enzyme, was used. A similar difference in activity was observed in the conversion of the unnatural substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-(D-O-methyl serine) (191) by wild-type and recombinant IPNS.[271]



(191)

In view of the observed difficulty in the conversion of (155) by DAOC/DACS even with very active enzyme, and also to the reported instability of the product formed, it was decided not to expend any further efforts on this problem. It is possible that the reason why the 2β-difluoromethyl penicillin (155) is a poor substrate for DAOC/DACS is that the initial hydrogen abstraction at the β-methyl group (with formation of a carbon radical) is inhibited by the presence of the two electron-withdrawing fluorine atoms. In the case of prolyl 4-hydroxylase it has been found that *cis*-fluoroprolyl is an inhibitor for the enzyme (presumably because of difficultating radical formation), whereas *trans*-fluoroprolyl is hydroxylated with loss of the fluorine atom.[124]

CHAPTER 5



A Substrate Specificity Comparison of Recombinant DAOC/DACS and DAOCS

Chapter 5

A Substrate Specificity Comparison of Recombinant DAOC/DACS and DAOCS

5.1 Introduction

The purification of the eukaryotic enzyme deacetoxy/deacetylcephalosporin C synthase[64,98] (DAOC/DACS; expandase/hydroxylase) from *C. acremonium*, and of the prokaryotic enzymes deacetoxycephalosporin C synthase[272,273] (DAOCS; expandase) and deacetylcephalosporin C synthase[103] (DACS; hydroxylase) from *S. clavuligerus* has enabled the cloning and sequencing of their corresponding genes. These genes have been expressed in *E. coli* under the control of the λP_L promoter[100,102,104], but unfortunately the proteins expressed under this system are produced as insoluble granules which require treatment with urea for solubilisation, and this process results in quite substantial loss of activity[106]. Recently, a new expression system using a *trc* promoter has been developed which has enabled the expression of DAOC/DACS[107] and of DAOCS[274] in a soluble form. This system permits the preparation of large quantities of highly active recombinant enzymes in both cases.

The genes for the three enzymes DAOC/DACS, DAOCS and DACS all display high sequence homologies between them, which led to the early suggestion that the DAOC/DACS gene evolved by a horizontal fusion-truncation mechanism from the two separate genes for prokaryotic DAOCS and DACS.[100] However, this theory has been contradicted by recent observations that the three enzymes have similar molecular weights, and that both DAOCS and DACS are also partially bifunctional. It was found that DAOCS was capable of low levels of hydroxylation of both DAOC (2) and 3-exomethylene cephalosporin C (63)[273], and that DACS was capable of a low level ring-expansion of

pen N (1)[103]. In addition, kinetic studies indicated that both these enzymes possessed one common active site responsible for both activities. Thus, it became apparent that the three enzymes did not have totally different activities, but were rather characterised by the different relative efficiencies with which they carried out the ring-expansion and hydroxylation of the three β-lactam substrates. In view of these results, it was suggested that the three enzymes represented divergent evolutionary products from a common ancestral gene.[103]

It would be interesting to understand which characteristics are responsible for the difference in activities of these very similar enzymes, and also what factors drove natural selection for a fungal bifunctional enzyme in contrast to two independent bacterial enzymes. A detailed understanding of the mode of action of these three enzymes is also of great pratical importance. Of particular interest is the possibility of "designing" an expandase enzyme which is capable of ring expanding penicillin G (10) or V (11), as this would provide a way of improving industrial manufacturing processes for clinical cephalosporin antibiotics. A second possibility of improving these industrial processes would be to increase the biosynthetic formation of DAOC (2), which can be enzymatically deacylated to 7-aminodeacetoxycephalosporin C[275], itself an important intermediate in the manufacturing of oral cephalosporin antibiotics. This possibility could be accomplished by the specific inactivation of the hydroxylase activity of DAOC/DACS in *C. acremonium* or of DACS in *S. clavuligerus*, allowing for the accumulation of DAOC (2) to occur *in vivo*.

The availability of large quantities of recombinant DAOC/DACS and DAOCS will enable detailed comparative studies to be performed. It was considered that to start with, a more detailed analysis of the substrate specificities of the two enzymes should be carried out, and that study is described in this chapter.

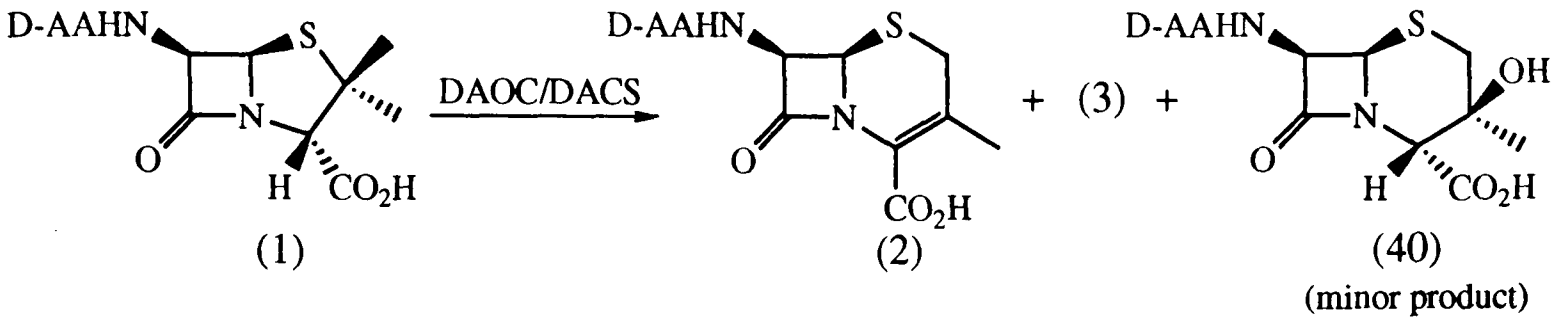
5.2 The Substrate Specificity of DAOC/DACS

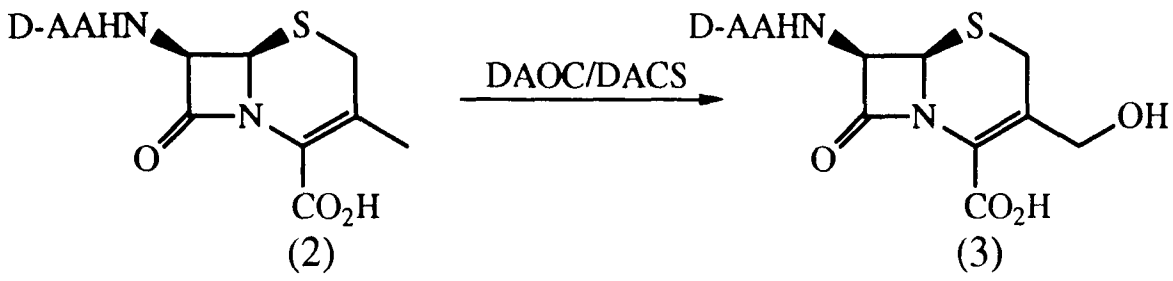
The reactions catalysed by recombinant DAOC/DACS have been described throughout this thesis, and are essentially identical to those performed by the wild-type enzyme[109] (Scheme 5.1). The recombinant enzyme readily carries out the two physiological reactions, which are the ring-expansion of penicillin N (1) to deacetoxycephalosporin C (DAOC) (2), and the hydroxylation of DAOC (2) to deacetylcephalosporin C (DAC) (3). It is also observed that in the ring-expansion step a very small amount of a second product is formed, the 3β-hydroxycepham (40). In the presence of the deuterated substrate [3-^{2}H]penicillin N (1d), recombinant DAOC/DACS forms significantly increased amounts of the shunt product [4-^{2}H]-3β-hydroxycepham (40a)[109], which has been interpreted as being the result of a deuterium kinetic isotope effect expressed on an enzyme-bound intermediate[117,119].

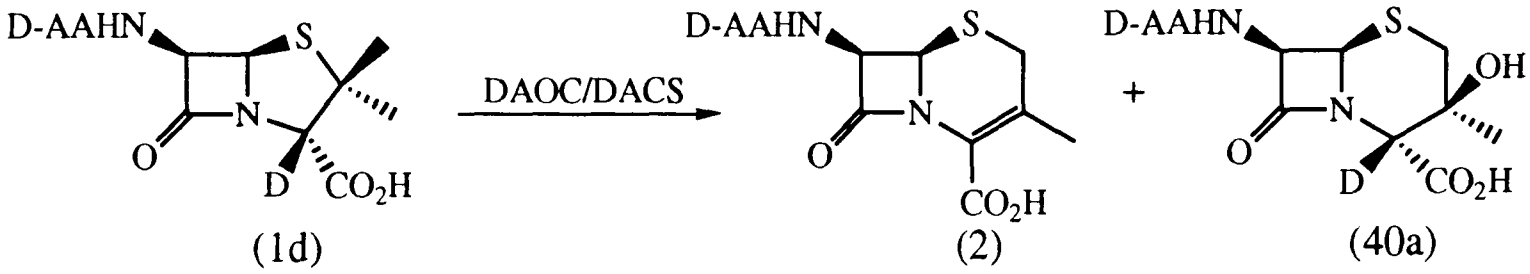
It has also been observed that DAOC/DACS is capable of further oxidising the hydroxyl group of DAC (3) to an aldehyde[145], a process which brings about the hydrolysis of the β-lactam ring due to its increased reactivity.

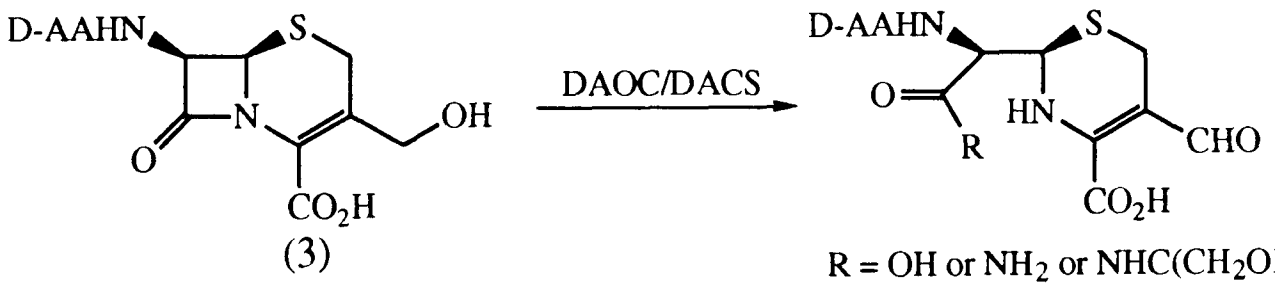
In terms of substrate analogues, 3-exomethylene cephalosporin C (63) is readily accepted as a substrate by DAOC/DACS, being directly converted to DAC (3). As previously described in this thesis (see Chapter 2), it was also found that on conversion of the deuterated [4-^{2}H]-3-exomethylene cephalosporin C (63a), a second product was formed by epoxidation of the double bond of (63a) to give the [4-^{2}H]-3β-spiroepoxide cepham (122).[197] As in the case of the [4-^{2}H]-3β-hydroxycepham (40a), experimental evidence indicates that (122) is a shunt metabolite whose formation is increased due to the operation of a deuterium kinetic isotope effect on an enzyme-bound intermediate. In addition, it was found that DAOC/DACS is also capable of further converting the epoxide (122) to identical 3-formyl cephalosporoate products as those obtained in the oxidation of DAC (3).

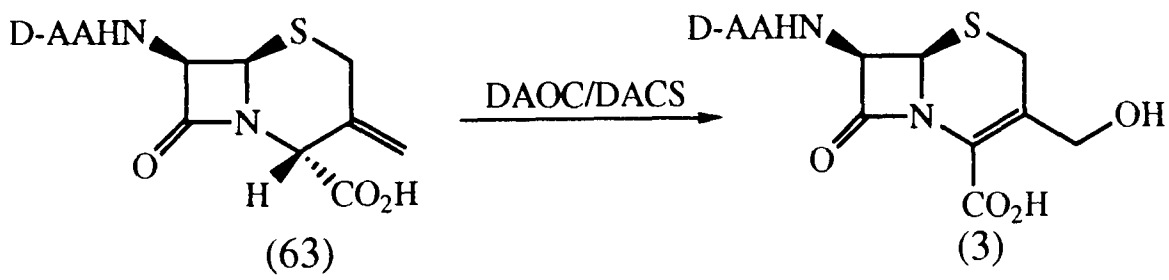
The reactions catalysed by DAOC/DACS indicate that it has a fairly relaxed substrate specificity in terms of the nature and orientation of the group which is oxidised, and also that it is capable of several modes of reactivity, namely desaturation, hydroxylation, epoxidation and hydroxyl oxidation to an aldehyde. The capacity to catalyse all these reactions possibly reflects the involvement of the high-energy intermediate iron-oxene $Fe^{IV}=O$ (49). It is still not clear whether all these reactions are catalysed at a single active site.

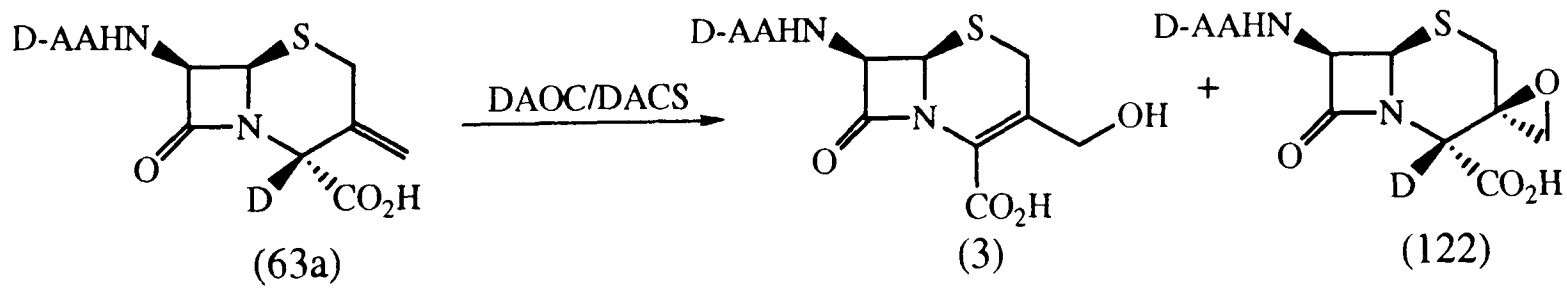




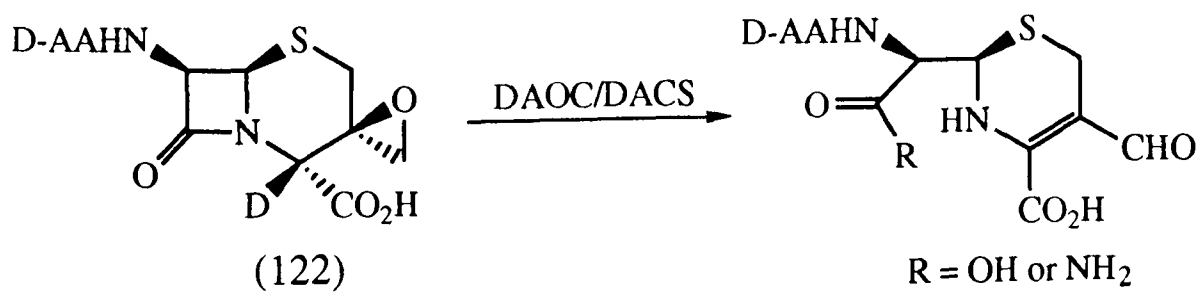








+ 3-formyl cephalosporoate products



Scheme 5.1

5.3 The Substrate Specificity of DAOCS

Given the apparently more restricted reactivity of the bacterial expandase enzyme DAOCS, it was obviously of interest to investigate which of these reactions it was capable of performing. In these studies, partially purified but highly active DAOCS was used, and incubations were carried out in a similar procedure to that performed for incubations of DAOC/DACS. Several substrates were prepared as previously described, and were each incubated with DAOCS under identical conditions. Analysis by 500 MHz ^{1}H-NMR of the crude incubation mixtures revealed the extent of conversion as well as the nature of the products formed.

It was found that DAOCS readily catalysed the ring-expansion of pen N (1), but with formation of a single major product DAOC (2) and only trace amounts of DAC (3) (Scheme 5.2). When DAOC (2) was incubated with DAOCS, a very low level of hydroxylation to DAC (3) was observed (< 5%), in agreement with previous observations[273].

It was expected that incubation of [3-^{2}H]penicillin N (1d) with DAOCS would result in increased formation of the shunt [4-^{2}H]-3β-hydroxycepham (40a), as observed in the ring-expansion of (1d) by DAOC/DACS. However, it was found that conversion of (1d) by DAOCS resulted only in formation of DAOC (2), with (40a) being observed in only trace amounts (< 2%).

DAC (3) was then incubated with DAOCS to investigate whether the enzyme was capable of further oxidation of the hydroxyl group to an aldehyde group. It was found that this oxidation could not be observed at all, with DAC (3) remaining intact in the incubation mixture.

Given this lack of hydroxylase activity, it was somewhat surprising to observe that DAOCS converted the 3-exomethylene cephalosporin C (63) to its usual product DAC (3), with approximately 20% of the efficiency with which it converted pen N (1) to DAOC (2).

On incubation of the deuterated [4-^{2}H]-3-exomethylene cephalosporin C (63a) the same level of conversion was observed, with formation of not only DAC (3), but also of the 3β-spiroepoxide cepham (122) as observed in the conversion of (63a) by DAOC/DACS. In addition, some 3-formyl cephalosporoate products could be detected in the crude incubations of (63a) with DAOCS. As it was previously found that this enzyme was not capable of oxidising DAC (3), it was concluded that these aldehydic products probably resulted from further oxidation of the epoxide (122) as observed for DAOC/DACS. However, due to the limited amounts of (122) available, this was not confirmed by incubation of isolated (122) with DAOCS.

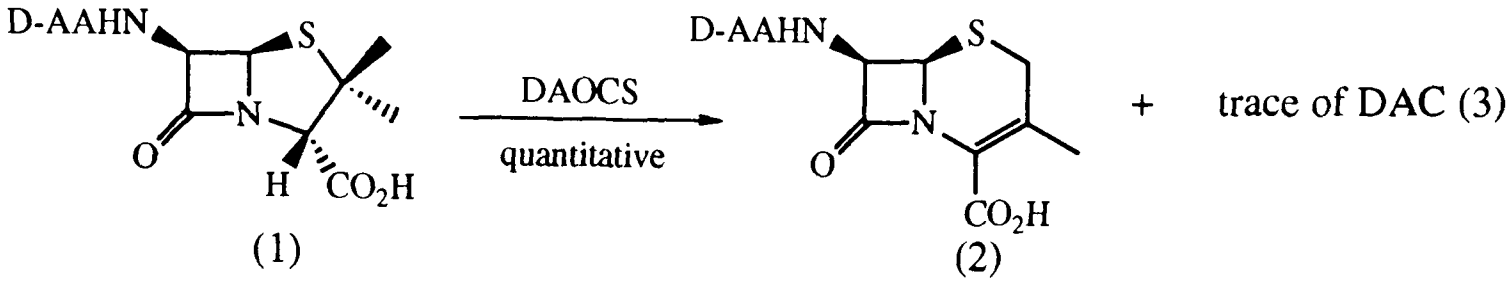
These results show that the substrate specificity of DAOCS is different from that of DAOC/DACS, although perhaps by not as much as previously expected. To start with, it is quite interesting that the [4-^{2}H]-3β-hydroxycepham (40a) itself a product of the ring-expansion activity of DAOC/DACS, is not formed in the conversion of [3-^{2}H]penicillin N (1d) by DAOCS. This indicates that DAOCS is not capable of the pathway-branching that leads to the formation of (40a) in DAOC/DACS, and so in this case the deuterium isotope effect is probably just expressed as a lower rate of conversion of (1d) to (2) (lower V_{max}).

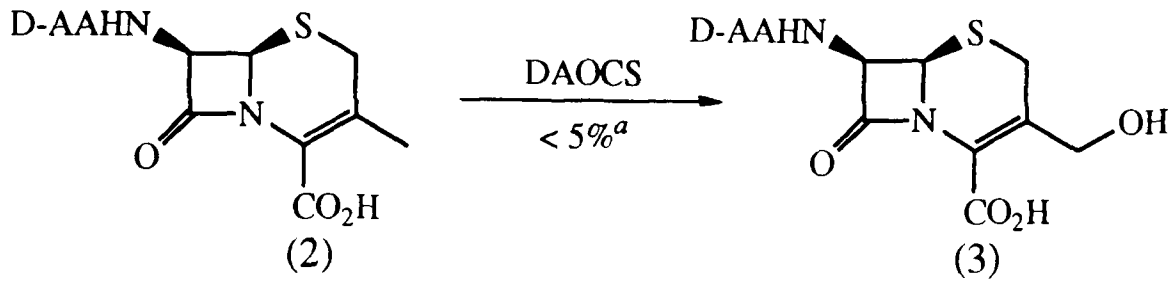
Despite not converting DAOC (2), DAOCS is capable of producing DAC (3) through the conversion of 3-exomethylene cephalosporin C (63). It is very interesting to observe that DAOCS is also capable of epoxidation activity, and of oxidation of the epoxide (122) to aldehydic products, although it can not oxidise DAC (3) to these products.

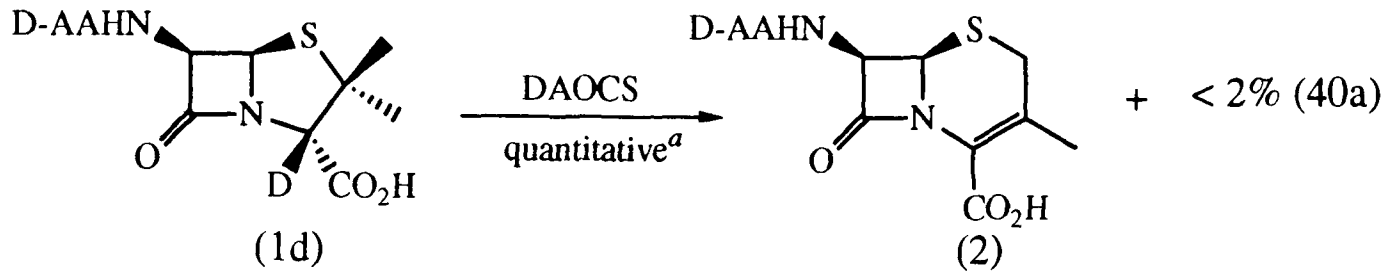
All these observations reveal that the activities of DAOC/DACS and of DAOCS are indeed more similar than once thought, and reinforce the idea that the differences between them are expressed mainly in terms of the efficiency with which each reaction is performed. As the cofactor requirements (i.e. Fe^{2+}, O_2 and α-ketoglutarate) are identical for the two enzymes it is envisaged that in both cases the reactions proceed *via* the involvement of an iron-oxene Fe^{IV}=O (49). As previously discussed, the high chemical potential of this group is probably responsible for the diversity of reactions catalysed by DAOC/DACS. If a ferryl species is also involved in the reactions of DAOCS, then it might be considered that its more limited substrate specificity is caused not by an intrinsic lack of capacity to catalyse

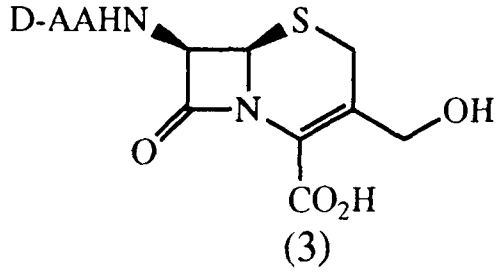
the reactions, but maybe by steric constraints of the active site. It seems possible that the difference in activities between DAOC/DACS and DAOCS is the result of a few different amino acid residues at the active site of each of them, resulting in differential steric arrangement between enzyme and substrate. In the case of DAOCS, such effects may limit the efficiency with which it catalyses some of the reactions. Strucural studies on both enzymes should provide interesting information in this respect. Once the specific residues directly involved in activity are identified and the mechanism elucidated, it is envisaged that site-directed mutagenesis will be capable of modifying at will the activities of each of these proteins, with possible interesting and important results.

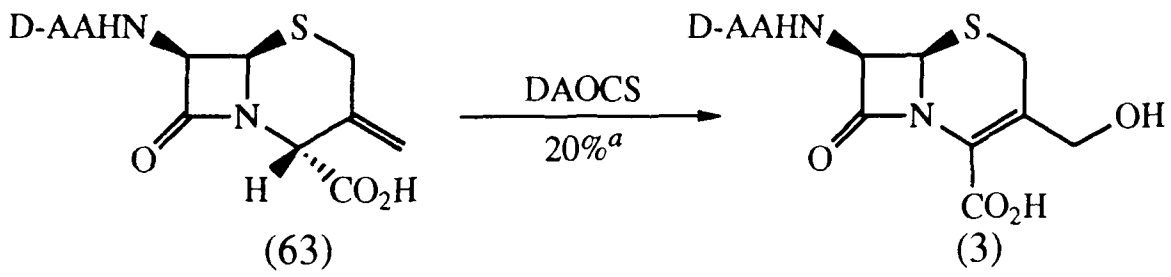
Soluble expression of the bacterial hydroxylase enzyme DACS is presently under investigation in this laboratory. Investigation of its substrate specificity will provide complementary information to that which has been described here.

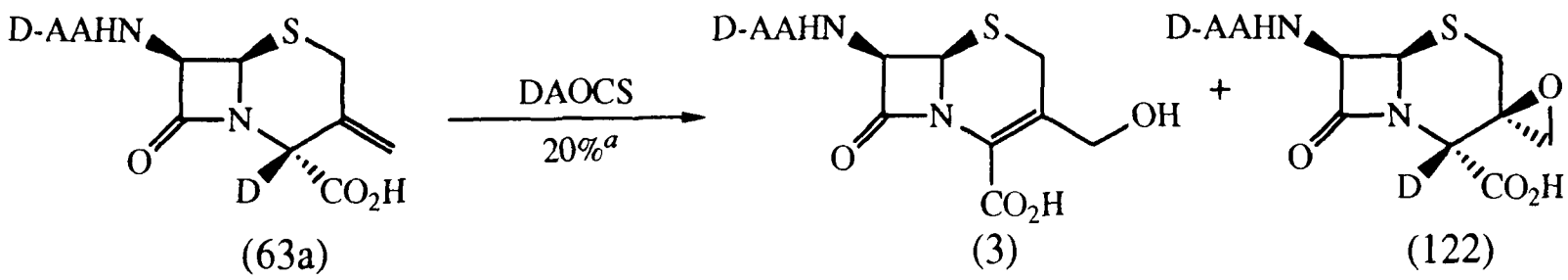












[a]Approximate conversion levels observed under the conditions used to fully convert pen N (1)

Scheme 5.2

CHAPTER 6



Experimental Procedures

Chapter 6

Experimental Procedures

6.1 General Experimental Procedures

All **solvents** were distilled before use. Petrol refers to the fraction of light petroleum ether boiling between 30-40°C. Dichloromethane was distilled from CaH_2 under argon. N,N-dimethylformamide was stirred over CaH_2 under argon for 24h, distilled under reduced pressure and stored over 4Å molecular sieves under argon. Tetrahydrofuran was distilled from sodium/benzophenone under argon. Toluene and benzene were dried over sodium wire. Triethylamine was distilled from CaH_2 and stored over sodium hydroxide under argon. N,N-dimethylaniline was distilled from potassium hydroxide under argon.

Reaction times were recorded in minutes (min) or hours (h). Reactions were followed by T.l.c. unless otherwise stated, and solvents were evaporated under reduced pressure on a Büchi RE111 Rotavapor. High-boiling solvents were evaporated on a Büchi R110 Rotavapor fitted with a dry ice condenser at < 2 mmHg.

Flash column chromatography was performed using Merck Kieselgel 60 F_{254} 230-400 mesh. Preparative layer chromatography (P.l.c.) was performed on glass backed silica plates (60 F_{254} , 200×200×1 mm layer). Thin layer chromatography (T.l.c.) was performed using Merck aluminium foil backed plates pre-coated with Kieselgel 60 F_{254} , and were visualised using UV fluorescence (254 nm) and developed with either 5% (w/v) dodecamolybdophosphoric acid in ethanol and heating, or 10% ammonium molybdate in 2N H_2SO_4 and heating.

Melting points (M.p.) were determined on a Büchi 510 capillary apparatus and are uncorrected.

Infra-red spectra were recorded on a Perkin-Elmer 1750 fourier transform spectrometer. Only selected absorbances (ν_{max}) are reported [in wavenumbers (cm^{-1})]

with relative absorption strengths described as (s) strong, (m) medium, (w) weak and (br) broad.

^{1}H-NMR spectra were recorded either at 200 MHz on a Varian Gemini 200 spectrometer, or at 500 MHz on a Bruker AM-500 spectrometer, and are internally referenced to either TMS (for samples in $CDCl_3$; δ_{ref} = 0.0 ppm), or TSP (for samples in D_2O; δ_{ref} = 0.0 ppm), or in the case of another solvent to the residual non-deuterated solvent peak. Chemical shifts (δ_H) are quoted in parts per million (ppm), and the following abbreviations are used : s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets and br.s, broad singlet. Coupling constants (J) are quoted in Hertz, and are reported to the nearest 0.5 Hz (except in the case of some β-lactam protons).

^{13}C-NMR spectra were either recorded at 50.3 MHz on a Varian Gemini 200 spectrometer, or at 125.8 MHz on a Bruker AM-500 spectrometer. Chemical shifts (δ_C) are quoted in parts per million (ppm), and are internally referenced to either $CDCl_3$ (δ_{ref} = 77.0 ppm) or to 1,4-dioxan (for samples in D_2O; δ_{ref} = 67.3 ppm). All spectra were fully ^{1}H decoupled, and peaks were assigned using DEPT editing.

Low resolution mass spectra (*m/z*) in the desorption chemical ionisation (DCI) mode were run either on a VG Micromass 30F or a ZAB 1F spectrometer. Samples requiring fast atom bombardment (FAB) were run on a VG Micromass ZAB 1F, and samples run under electrospray conditions (ESMS) on a VG BIO-Q spectrometer. Peak intensities are quoted as percentages of the base peak.

High performance liquid chromatography (HPLC) was performed on one of two systems : i) two Gilson 303 pumps, a Rheodyne 7125 injector, a Gilson holochrome variable wavelength detector set at 220 nm, and a column packed with Hypersil ODS (250 × 10 mm diameter); or ii) a Waters 600E Multisolvent Delivery System, a Rheodyne 7125 injector, a Waters 991 Photodiode Array Detector, and a column packed with Hypersil ODS (250 × 7 mm diameter).

Incubations were performed on a New Brunswick Scientific G24 environmental incubation shaker.

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

Bioassays were carried out using the "holed-plate" method[276] with *Escherichia coli* X580 plates with (+) or without (-) β-lactamase I (from *Bacillus cereus*), and *Staphilococcus aureus* NCTC 6571 plates. The samples for bioassay were applied in aqueous solutions (100 µl) on to the agar plates and incubated at 37°C for > 15h.

NMR calibration of aqueous samples : The sample to be calibrated was dissolved in D_2O (0.5 - 1 ml) containing TSP (0.29 mM). The ^{1}H-NMR spectrum (500 MHz, HOD suppressed) was recorded over at least 40 transients and the resonances due to TSP and the β-lactam protons integrated. The concentration of the β-lactam compound was then calculated from the equation :

$$\text{β-lactam compound concentration} = \frac{1/2 \times \int \text{β-lactam protons}}{1/9 \times \int \text{TSP protons}} \times 0.29 \text{ mM}$$

One International Unit (IU) of enzyme is defined as the amount that converts 1 µmol of penicillin N to products in 1 minute, under the standard incubation conditions. These activity values were estimated for DAOC/DACS using a bioassay calibration method to determine the amount of product formed. This method is subject to several sources of experimental error, and so the values obtained are only a rough guideline for the enzyme activity.

General procedure for DAOC/DACS incubations : A cofactor solution was prepared with iron (II) sulphate (1.5 mg, 1 mM), α-ketoglutarate (21 mg, 14.4 mM), L-ascorbate (17.6 mg, 10 mM), dithiothreitol (30.8 mg, 20 mM) and ammonium sulphate (0.33 g, 0.25 M) in distilled water (10 ml), and pH adjusted to 7.5 with 1M NaOH. A solution of DAOC/DACS (2 ml, *ca.* 0.14 International Units) in TRIS-HCl buffer (pH 7.4, 50 mM) was pre-incubated with cofactor solution (200 µl) for 5 min at 27° C and 250 rpm. The substrate (1 mg) in water (800 µl) was added, and the resulting solution was incubated at 27° C and 250 rpm for 2h, after which time the protein was precipitated by the addition of acetone to 70% (v/v). After centrifugation (15 Krpm, 5 min, 0°C) the supernatant was

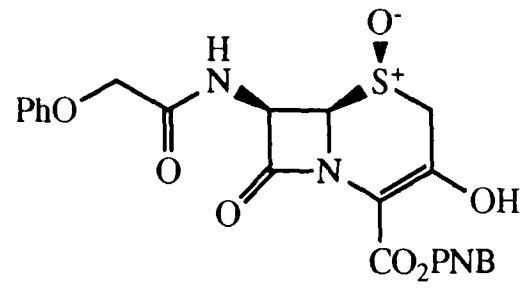
evaporated to dryness, and the residue dissolved in D_2O (0.5 ml) for examination by ^1H-NMR (500 MHz, HOD suppressed). Varying degrees of conversion were obtained by varying the amount of substrate or the incubation time. Each batch of enzyme would differ in terms of concentration and activity and was always tested with penicillin N to determine its activity under the conditions mentioned above, and the amount of enzyme to use for conversion of the unnatural substrates.

6.2 Experimental for Chapter 2

6.2.1 Experimental for Section 2.2

(6R,7R)-1-Aza-3-hydroxy-7-phenoxyacetamido-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *p*-nitrobenzyl ester β-sulfoxide (100)

$C_{22}H_{19}N_3O_9S$; MW 501



A solution of exomethylene sulfoxide (99) (5g, 10 mmol) in MeOH/DCM [250 ml, 1:4 (v/v)] was cooled to -60°C and ozone bubbled through. After 90 min the reaction mixture showed a pale blue colour and there was no evidence of starting material by T.l.c.. Oxygen was bubbled through until the blue colour disappeared. Sodium hydrogen sulfite (23g, 0.22 mol, 22 eq) was added and the mixture stirred at 0°C for 1h. The supernatant solution was decanted and washed with 1N HCl and brine. The organic phase was dried (Na_2SO_4) and evaporated to a white solid. The solid was washed with ether and filtered off to give (100) (4.5 g, 9 mmol, 90% yield).

T.l.c. [MeOH/$CHCl_3$ (10%,v/v)] Rf 0.33

ν_{max} ($CHCl_3$) : 3386 (m), 1801 (s, β-lactam C=O), 1700 (s), 1602 (m), 1525 (s, NO_2), 1495 (m), 1440 (m), 1350 (s, NO_2), 1048 (m), 758 (m), 726 (m)

δ_H (200 MHz, $CDCl_3$) : 3.53 and 3.82 (2H, ABq, J 18 Hz, C$\underline{H}_2$S), 4.57 (2H, s, C$\underline{H}_2$OPh), 4.64 (1H, d, J 4.5 Hz, NHCHC$\underline{H}$S), 5.32 and 5.55 (2H, ABq, J 13 Hz, $CO_2C\underline{H}_2ArNO_2$), 6.07 (1H, dd, J 10 and 4.5 Hz, NHC$\underline{H}$CHS), 6.88-8.30 (9H, m, aromatic C$\underline{H}$), 7.87 (1H, d, J 10 Hz, N$\underline{H}$), 11.54 (1H, br. s, O$\underline{H}$)

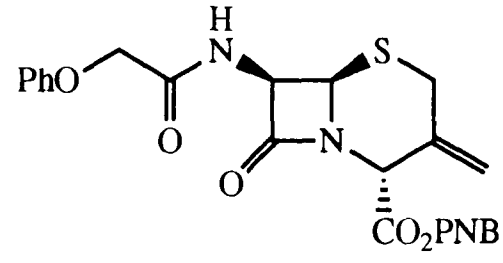
δ_C (125.8 MHz, $CDCl_3$) : 46.17 (t, $\underline{C}H_2$S), 57.21 and 67.44 (2 × d, NH$\underline{C}$H$\underline{C}$HS), 66.33 and 67.16 (2 × t, $\underline{C}H_2$OPh and $\underline{C}H_2ArNO_2$), 104.40 (s, $\underline{C}$=COH), 114.91, 122.38, 123.82, 128.55 and 129.76 (5 × d, aromatic $\underline{C}$H), 141.63 (s, aromatic C1 of $ArNO_2$), 148.04 (s,

aromatic C4 of $ArNO_2$), 155.76 (s, aromatic C1 of CH_2OPh), 157.07 (s, C=<u>C</u>OH), 164.19, 166.45 and 168.90 (3 × s, 3 × <u>C</u>=O)

m/z (FAB) : 502 ([MH^+], 37%), 371 (20%), 129 (58%), 69 (85%), 55 (100%)

(2<u>R</u>,6<u>R</u>,7<u>R</u>)-1-Aza-3-methylene-7-phenoxyacetamido-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid *p*-nitrobenzyl ester (107)[208]

$C_{23}H_{21}N_3O_7S$; MW 483



To a solution of the exomethylene sulfoxide (99) (3g, 6 mmol) in dry DMF (75 ml) at 0°C was added potassium iodide (19.8 g, 0.12 mol, 20 eq) and acetyl chloride[207] (3.29 ml, 46 mmol, 7.7 eq). The resulting solution was stirred for 1h at 0°C, after which time T.l.c. showed complete consumption of starting material. A solution of saturated sodium metabisulfite (100 ml) was added and the resulting mixture extracted with EtOAc (3 × 50 ml). The organic phase was washed with water (3 × 50 ml), sat. $NaHCO_3$ (2 × 50 ml) and brine (50 ml). The organic phase was dried (Na_2SO_4) and evaporated to give the crude product as a foam. This was purified by chromatography [flash silica, EtOAc/DCM (30%, v/v)] to give (107) (2.6 g, 5.4 mmol, 90% yield).

T.l.c. [EtOAc/DCM (1:1, v/v)] Rf 0.65

ν_{max} ($CHCl_3$) : 1776 (s, β-lactam C=O), 1750 (s, ester C=O), 1694 (s, amide C=O),1602 (m), 1525 (s, NO_2), 1496 (m), 1350 (s, NO_2), 1323 (m), 1176 (m)

δ_H (200 MHz, $CDCl_3$) : 3.29 and 3.64 (2H, ABq, J14 Hz, C<u>H</u>$_2$S), 4.55 (2H, s, C<u>H</u>$_2$OPh), 5.23 (1H, s, C<u>H</u>CO_2PNB), 5.25-5.36 (4H, m, $CO_2C\underline{H}_2ArNO_2$ and C=C<u>H</u>$_2$), 5.44 (1H, d, J 4.5 Hz, NHCHC<u>H</u>S), 5.77 (1H, dd, J 10 and 4.5 Hz, NHC<u>H</u>CHS), 6.90-7.56 and 8.23-8.34 (10H, m, aromatic C<u>H</u> and N<u>H</u>)

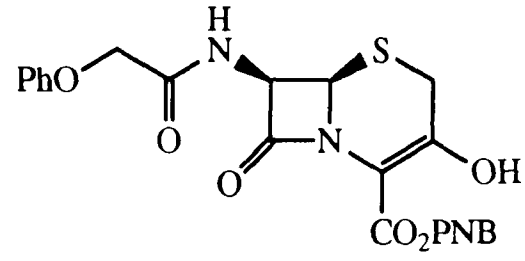
δ_C (125.8 MHz, $CDCl_3$) : 29.27 (t, <u>C</u>H_2S), 56.31, 56.43 and 58.86 (3 × d, NH<u>C</u>H<u>C</u>HS and <u>C</u>HCO_2PNB), 66.08 and 67.32 (2 × t, <u>C</u>H_2OPh and <u>C</u>H_2ArNO_2), 117.44 (t, C=<u>C</u>H_2), 114.90, 122.35, 123.92, 128.45 and 129.73 (5 × d, aromatic <u>C</u>H), 133.43 (s, <u>C</u>=CH_2), 141.79 (s, aromatic C1 of $ArNO_2$), 148.09 (s, aromatic C4 of $ArNO_2$), 157.06 (s, aromatic

C1 of CH_2OPh), 165.22, 167.45 and 168.41 (3 × s, 3 × <u>C</u>=O)

m/z [DCI (NH_3)] : 501 ([MNH_4^+], 4%), 484 ([MH^+], 5%), 293 (100%), 192 (45%), 107 (45%), 94 (55%)

(6<u>R</u>,7<u>R</u>)-1-Aza-3-hydroxy-7-phenoxyacetamido-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *p*-nitrobenzyl ester (108)[209]

$C_{22}H_{19}N_3O_8S$; MW 485



A solution of the exomethylene (107) (1g, 2 mmol) in MeOH/DCM [100 ml, 1:4 (v/v)] was cooled to -78°C and ozone bubbled through. After 25 min a slight blue colour appeared and oxygen was then bubbled through until this colour dissapeared. Sodium hydrogen sulfite (4.8 g, 46 mmol, 23 eq) was added and the mixture stirred at 0°C for 1h. The supernatant solution was washed with 1N HCl and brine. The organic phase was dried (Na_2SO_4) and evaporated to a white foam. This was purified by chromatography [flash silica, EtOAc/DCM (1:1, v/v)] to give (108) (0.64 g, 1.3 mmol, 64% yield).

T.l.c. [MeOH/$CHCl_3$ (10%,v/v)] Rf 0.48

ν_{max} ($CHCl_3$) : 3409 (m), 1785 (s, β-lactam C=O), 1695 (s), 1602 (m), 1595 (s, NO_2), 1496 (m), 1350 (s, NO_2), 1240 (m)

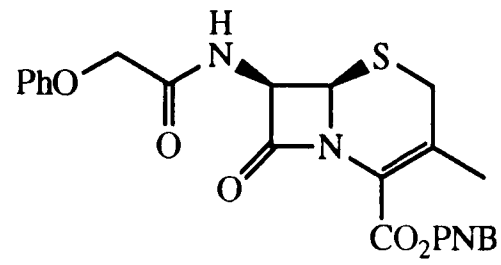
δ_H (500 MHz, $CDCl_3$) : 3.34 and 3.50 (2H, ABq, J17 Hz, C<u>H</u>$_2$S), 4.59 (2H, s, C<u>H</u>$_2$OPh), 5.09 (1H, d, J 4 Hz, NHCHC<u>H</u>S), 5.29 and 5.49 (2H, ABq, J 13 Hz, $CO_2C\underline{H}_2ArNO_2$), 5.71 (1H, dd, J 9 and 4 Hz, NHC<u>H</u>CHS), 6.90-7.67 and 8.21-8.29 (10H, m, aromatic C<u>H</u> and N<u>H</u>), 11.53 (1H, br. s, O<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 26.37 (t, <u>C</u>H_2S), 58.53 and 58.71 (2 × d, NH<u>C</u>H<u>C</u>HS), 65.71 and 67.34 (2 × t, <u>C</u>H_2OPh and <u>C</u>H_2ArNO_2), 103.37 (s, <u>C</u>=COH), 114.88, 122.43, 123.75, 128.32 and 129.77 (5 × d, aromatic <u>C</u>H), 141.99 (s, aromatic C1 of $ArNO_2$), 147.95 (s, aromatic C4 of $ArNO_2$), 157.07 (s, aromatic C1 of CH_2OPh), 165.28, 165.93, 167.21 and 168.78 (4 × s, 3 × <u>C</u>=O and C=<u>C</u>OH)

m/z [DCI (NH_3)] : 307 (35%), 295 (5%), 192 (40%), 116 (100%)

(6R,7R)-1-Aza-3-methyl-7-phenoxyacetamido-8-oxo-5-thiabicyclo[4.2.0]octa-2-ene-2-carboxylic acid *p*-nitrobenzyl ester (109)

$C_{23}H_{21}N_3O_7S$; MW 483



A solution of the exomethylene (107) (0.1 g, 0.2 mmol) in dry THF (10 ml) was cooled to -70°C under argon, and a solution of lithium bis(trimethylsilyl)amide (1M in THF, 207 µl, 0.2 mmol, 1 eq) was added dropwise. The solution was stirred at -70°C for 10 min and methyl iodide added (65 µl, 1 mmol, 5 eq). The solution was left to warm up to room temperature and stirred for 1h. The reaction mixture was partitioned between EtOAc and water, the organic phase dried (Na_2SO_4) and evaporated to a yellow oil. This was purified by chromatography [flash silica, EtOAc/DCM (25%, v/v)] to give (109) (56 mg, 0.12 mmol, 56% yield).

T.l.c. [EtOAc/DCM (1:1, v/v)] Rf 0.76

ν_{max} ($CHCl_3$) : 1785 (s, β-lactam C=O), 1731 (s, ester C=O), 1697 (s, amide C=O),1601 (m), 1525 (s, NO_2), 1496 (s), 1441(m), 1350 (s, NO_2), 1236 (s), 1109 (m)

δ_H (200 MHz, $CDCl_3$) : 2.17 (3H, s, C<u>H</u>$_3$), 3.24 and 3.53 (2H, ABq, J 18 Hz, C<u>H</u>$_2$S), 4.58 (2H, s, C<u>H</u>$_2$OPh), 5.02 (1H, d, J 5 Hz, NHCHC<u>H</u>S), 5.29 and 5.39 (2H, ABq, J 13 Hz, $CO_2C\underline{H}_2ArNO_2$), 5.87 (1H, dd, J 9 and 5 Hz, NHC<u>H</u>CHS), 6.89-7.68 (10H, m, aromatic C<u>H</u> and N<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 19.98 (q, <u>C</u>H$_3$), 30.24 (t, <u>C</u>H$_2$S), 56.88 and 58.38 (2 × d, NH<u>C</u>H<u>C</u>HS), 65.88 and 67.10 (2 × t, <u>C</u>H$_2$OPh and $\underline{C}H_2ArNO_2$), 114.80, 121.90, 123.64, 128.76 and 129.71 (5 × d, aromatic <u>C</u>H), 122.35 and 133.98 (2 × s, <u>C</u>=<u>C</u>), 142.31 (s, aromatic C1 of $ArNO_2$), 147.80 (s, aromatic C4 of $ArNO_2$), 156.91 (s, aromatic C1 of CH_2OPh), 161.49, 164.11 and 169.38 (3 × s, 3 × <u>C</u>=O)

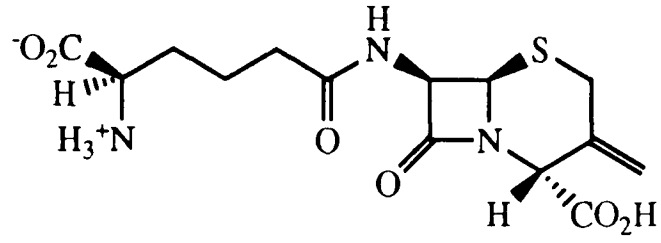
m/z (FAB) : 506 ([MNa^+], 14%), 484 ([MH^+], 10%), 293 (100%)

Identical to an authentic sample.

6.2.2 Experimental for Section 2.3

(2R,6R,7R)-1-Aza-3-methylene-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (63)

$C_{14}H_{19}N_3O_6S$; MW 357



A solution of cephalosporin C (12) (0.25 g, 0.602 mmol) in a sodium acetate buffer (0.1 M, pH 4) was electrolysed[210,211] at 15V using a mercury pool cathode and a platinum sheet anode separated by a Nafion membrane, and the reaction was followed by ^{1}H-NMR. After 5h there was no evidence of starting material, and the reaction mixture was lyophilized. The crude mixture was purified by chromatography (HPLC, Gilson system, solvent 0.75% MeCN in 10mM aqueous NH_4HCO_3, flow rate 4 ml/min) to give 3-exomethylene cephalosporin C (63) (retention time 4.5 min, *ca.* 48 mg by NMR calibration, 24%), DAOC (2) (retention time 6.5 min,*ca.* 23 mg by NMR calibration, 11%), and the 3-exomethylene cephalosporin C epimer (110) (retention time 8 min, *ca.* 24 mg by NMR calibration, 12%).

Data for (63) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.64-1.92 (4H, m, $CHCH_2CH_2$), 2.40 (2H, t, J 7 Hz, CH_2CO), 3.38 and 3.61 (2H, ABq, J 14 Hz, CH_2S), 3.71 (1H, t, J 6Hz, $H_3N^+CHCO_2^-$), 4.97 (1H, s, $CHC=CH_2$), 5.24 and 5.28 (2H, 2 × s, $C=CH_2$), 5.40 and 5.41 (2H, ABq, J 4 Hz, HNCHCHS)

δ_C (125.8 MHz, D_2O) : 22.60 (t, $CHCH_2CH_2$), 30.34 (t, CH_2S), 33.16 (t, $CHCH_2$), 35.87 (t, CH_2CO), 57.25, 57.55, 59.74 and 60.61 (4 × d, NHCHCHS, $H_3N^+CHCO_2^-$ and $CHC=CH_2$), 116.46 (t, $C=CH_2$), 137.97 (s, $C=CH_2$), 166.73, 174.26, 177.69 and 182.00 (4 × s, 4 × C=O)

m/z (ESMS) : 380 ([MNa^+], 100%), 381 (19%), 382 (8%)

Data for (2) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.63-1.95 (4H, m, $CHCH_2CH_2$), 1.93 (3H, s, CH_3), 2.42 (2H, t, J 7 Hz, CH_2CO), 3.26 and 3.60 (2H, ABq, J 18 Hz, CH_2S), 3.72-3.76 (1H, m, $H_3N^+CHCO_2^-$), 5.09 and 5.57 (2H, 2 × d, J 4 Hz, HNCHCHS)

δ_C (125.8 MHz, D_2O) : 19.15 (q, $\underline{C}H_3$), 22.65 (t, $CHCH_2\underline{C}H_2$), 29.26 (t, $\underline{C}H_2S$) 33.14 (t, $CH\underline{C}H_2$), 35.92 (t, $\underline{C}H_2CO$), 57.50, 57.67 and 59.50 (3 × d,$NH\underline{C}H\underline{C}HS$ and $H_3N^+\underline{C}HCO_2^-$), 123.35 and 127.66 (2 × s, $\underline{C}=\underline{C}$), 165.30, 170.54, 178.03 and 182.02 (4 × s, 4 × $\underline{C}$=O)

m/z (ESMS) : 358 ([MH^+], 100%), 359 (19%), 360 (8%)

Identical to an authentic sample.

Data for (110) :

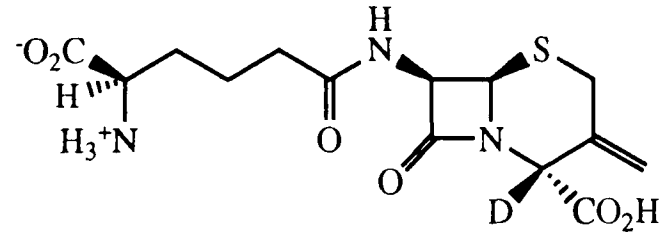
δ_H (500 MHz, D_2O, $\underline{H}OD$ suppressed) : 1.64-1.97 (4H, m, $CHC\underline{H}_2C\underline{H}_2$), 2.45 (2H, t, J 7 Hz, $C\underline{H}_2CO$), 3.46 and 3.67 (2H, ABq, J 12 Hz, $C\underline{H}_2S$), 3.72 (1H, t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$), 4.68 (1H, s, $C\underline{H}C=CH_2$), 5.11 and 5.23 (2H, ABq, J 4 Hz, $HNC\underline{H}C\underline{H}S$), 5.33 and 5.37 (2H, 2 × s, $C=C\underline{H}_2$)

δ_C (125.8 MHz, D_2O) : 22.06 (t, $CHCH_2\underline{C}H_2$), 29.04 (t, $\underline{C}H_2S$), 32.09 (t, $CH\underline{C}H_2$), 35.93 (t, $\underline{C}H_2CO$), 55.83, 55.92, 60.07 and 64.81 (4 × d, $NH\underline{C}H\underline{C}HS$, $H_3N^+\underline{C}HCO_2^-$ and $\underline{C}HC=CH_2$), 118.28 (t, $C=\underline{C}H_2$), 137.76 (s, $\underline{C}=CH_2$), 165.78, 173.07, 177.47 and 182.26 (4 × s, 4 × $\underline{C}$=O)

m/z (ESMS) : 358 ([MH^+], 100%), 359 (20%), 360 (7%), 361 (2%)

(2R,6R,7R)-1-Aza-(2-^{2}H)-3-methylene-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5- thiabicyclo[4.2.0]octane-2-carboxylic acid (63a)

$C_{14}H_{19}DN_3O_6S$; MW 358



A solution of cephalosporin C (0.5 g, 1.14 mmol) in a sodium acetate buffer prepared in deuterium oxide (0.1 M, pH 4) was electrolysed for 7h. The same purification procedure as for (63) gave (63a) (retention time 5 min, *ca.* 140 mg by NMR calibration, 34%), DAOC (2) (*ca.* 28 mg by NMR calibration, 7%), and (110a) (retention time 7.5 min, *ca.* 24 mg by NMR calibration, 6%).

Data for (63a) : ^{1}H-NMR identical to (63) except for the absence of the C4-H resonance at δ 4.97 ppm

m/z (ESMS) : 380 (6%), 381 ([MNa^+], 100%), 382 (18%), 383 (8%)

Data for (110a) : ^{1}H-NMR identical to (110) except for the absence of the C4-H resonance at δ 4.68 ppm

m/z (ESMS) : 358 (5%), 359 ([MH^+], 100%), 360 (18%), 361 (8%), 362 (2%)

Incubation of (63) with DAOC/DACS

Exomethylene cephalosporin C (63) was incubated with DAOC/DACS according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR revealed conversion to a single β-lactam product. Purification by HPLC (Waters system, solvent 10 mM aqueous NH_4HCO_3, flow rate 1 ml/min) gave DAC (3) (retention time 9 min), identical to an authentic sample by ^{1}H-NMR.

δ_H (500 MHz, D_2O, HOD suppressed) : 1.65-1.97 (4H, m, $CHCH_2CH_2$), 2.43 (2H, t, J 7 Hz, CH_2CO), 3.47 and 3.67 (2H, ABq, J 18 Hz, SCH_2), 3.74 (1H, t, J 6 Hz, $H_3N^+CHCO_2^-$), 4.26 and 4.30 (2H, ABq, J 13 Hz, CH_2OH), 5.13 and 5.63 (2H, 2 × d, J 4.6 Hz, HNCHCHS)

Bioassay tests with (63) and (110)

The following solutions were tested according to the general bioassay procedure :

A)
- 4R-3-exomethylene (63) - 25 µg/ml (*E. coli*) or 1 mg/ml (*S. aureus*)
- 4S-3-exomethylene (110) - 25 µg/ml (*E. coli*) or 1 mg/ml (*S. aureus*)
- penicillin N (1) - 25 µg/ml
- cephalosporin C (12) - 20 µg/ml (*E. coli*) or 1 mg/ml (*S. aureus*)

B)
- Denatured enzyme incubation mixtures prepared with :
  - 30 µl DAOC/DACS denatured with 300 µl MeOH
  - 90 µl 200 mM TRIS.HCl pH 8
  - 30 µl cofactor solution
  - 150 µl of a 100 µg/ml solution of substrate [(63), (110) or (1)]

C)
- Active enzyme incubation mixtures prepared with :
  - 30 µl DAOC/DACS
  - 90 µl 200 mM TRIS.HCl pH 8
  - 30 µl cofactor solution
  - 150 µl of a 100 µg/ml solution of substrate [(63), (110) or (1)]

- the above mixture was incubated for 40 min at 27°C, after which 300 µl MeOH were added

The following inhibition zones (in mm) were obtained :

A) Isolated compounds

	E.coli (-)	*E. coli* (+)	*S. aureus*
(63)	0	0	0
(110)	0	0	0
(1)	25	0	8
(12)	26	26	20

B) Incubation mixtures with denatured enzyme

	E.coli (-)	*E. coli* (+)
(63)	0	0
(110)	0	0
(1)	25	0

C) Incubation mixtures with active enzyme

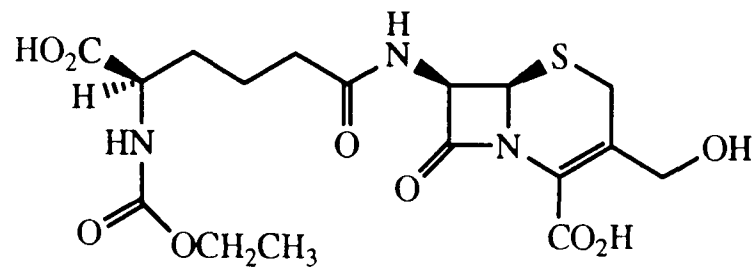
	E.coli (-)	*E. coli* (+)
(63)	22	23
(110)	0	0
(1)	25	24

Incubation of (63a) with DAOC/DACS

[4-^{2}H]-3-Exomethylene cephalosporin C (63a) (5 mg) was incubated with DAOC/DACS (10 ml, 0.7 IU) and cofactor solution (1 ml) according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR showed *ca.* 65% conversion to DAC (3) and a second β-lactam product (β-lactam resonances at δ 5.37 and 5.50 ppm). On purification of the crude incubation mixture by HPLC (Gilson system, 10 mM aqueous NH_4HCO_3, 2 ml/min) the two products eluted as one single peak (retention time 10 min) (*ca.* 3 mg DAC by NMR calibration; ratio DAC:unknown compound = 4 : 1). Several other incubations of (63a) were carried out using varying amounts of substrate and/or enzyme. The extent of conversion and ratio of products formed was determined by 500 MHz ^{1}H-NMR analysis.

6.2.3 Experimental for Section 2.4

(6R,7R)-1-Aza-3-hydroxymethyl-7-[(5R)-5-N-ethoxycarbonylamino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (111)
$C_{17}H_{23}N_3O_9S$; MW 445



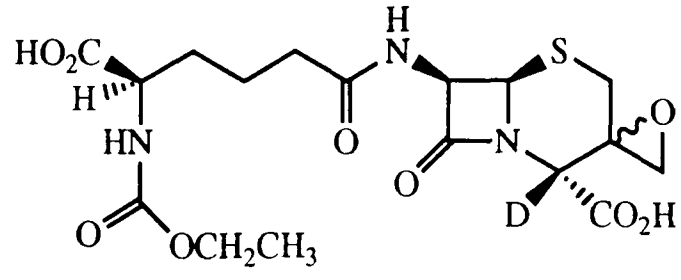
To a solution of DAC (3) (22 mg, 0.06 mmol) in distilled H_2O (8 ml) was added 1N $NaHCO_3$ (400 µl) to adjust pH to 8, and diethylpyrocarbonate[6] (50 µl, 0.34 mmol, 7 eq). The resulting solution was stirred for 10 min at room temperature, washed with ether and the aqueous layer evaporated to give (111) as a clean product (*ca.* 26 mg by NMR calibration; 0.06 mmol; 100% yield).

δ_H (500 MHz, D_2O, HOD suppressed) : 1.24 (3H, t, J 7 Hz, CH_2CH_3), 1.64-1.88 (4H, m, $CHCH_2CH_2$), 2.38-2.45 (2H, m, CH_2CO), 3.46 and 3.66 (2H, ABq, J 18 Hz, CH_2S), 3.92-3.98 (1H, m, $HNCHCO_2H$), 4.08-4.14 (2H, m, CH_2CH_3), 4.26 and 4.30 (2H, ABq, J 13 Hz, CH_2OH), 5.12 and 5.62 (2H, 2 × d, J 4.5 Hz, $HNCHCHS$)

δ_C (125.8 MHz, D_2O) : 14.60 (q, CH_3), 22.65 (t, $CHCH_2CH_2$), 26.19 (t, CH_2S) 32.20 (t, $CHCH_2$), 35.65 (t, CH_2CO), 56.93, 58.09 and 59.75 (3 × d, $NHCHCHS$ and $HNCHCO_2H$), 61.82 (t, CH_2OH), 62.57 (t, CH_2CH_3), 122.26 and 130.47 (2 × s, C=C), 158.97, 165.67, 169.65, 177.93 and 180.19 (5 × s, 5 × C=O)

m/z (ESMS) : 468 ([MNa^+], 100%), 469 (30%), 470 (13%), 471 (3%)

(2R,6R,7R)-1-Aza-(2-^{2}H)-3-spiroepoxy-7-[(5R)-5-N-ethoxycarbonylamino-5-carboxy pentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (112)
$C_{17}H_{22}DN_3O_9S$; MW 446



A mixture of DAC (3) (*ca.* 4.5 mg) and the new metabolite (ratio 4.5 : 1) was dissolved in distilled water (2 ml). The pH was adjusted to 8 with a sat. $NaHCO_3$ solution

(100 µl) and diethylpyrocarbonate (13 µl, 0.09 mmol, *ca.* 6 eq) was added. The reaction mixture was stirred at room temperature for 20 min, washed twice with ether and lyophilized. Analysis by ^{1}H-NMR showed reaction was quantitative and the ratio of the two products was still the same. Purification of the crude mixture by HPLC (Waters system, solvent 8% MeOH in aqueous 10 mM NH_4HCO_3, flow rate 1 ml/min) revealed two partially coeluting peaks which were collected as two fractions (fr.1:15-17 min; fr.2:17-19 min). Fr.1 consisted of N-ethoxycarbonyl-DAC (111) (*ca.* 2.1 mg by NMR calibration). Fr.2 had the two products in a ratio of 1.3:1 (*ca.* 490 µg of N-ethoxycarbonyl-DAC by NMR calibration) and was again purified by HPLC using the same procedure as before. The second fraction of this run contained the N-ethoxycarbonyl derivatives of DAC and the unknown in a ratio of 1 : 2, and the latter could be identified as the spiro-epoxide (112).

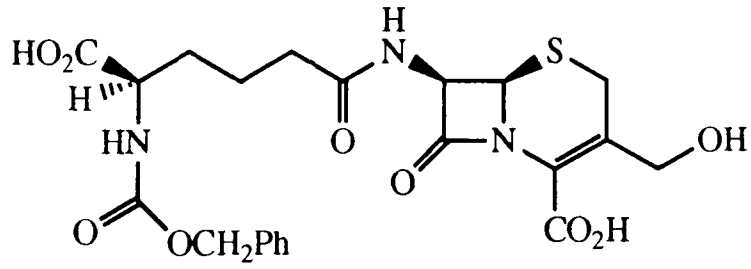
Data for (112) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.24 (3H, t, J 7 Hz, CH_2CH_3), 1.65-1.86 (4H, m, $CHCH_2CH_2$), 2.34-2.45 (2H, m, CH_2CO), 2.43 and 3.63 (2H, ABq, J 15 Hz, CH_2S), 3.28 and 3.40 (2H, 2 × d, J 3.7 Hz, epoxide H's), 3.92-3.98 (1H, m, $HNCHCO_2H$), 4.07-4.15 (2H, m, CH_2CH_3), 5.36 and 5.49 (2H, 2 × d, J 4 Hz, $HNCHCHS$) (the couplings were confirmed by a COSY experiment)

m/z (ESMS) : 447 ([MH^+], 20%), 469 ([MNa^+], 100%), 470 (21%), 471 (11%), 472 (3%)

(6R,7R)-1-Aza-3-hydroxymethyl-7-[(5R)-5-N-benzyloxycarbonylamino-5-carboxy-pentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (113)

$C_{22}H_{25}N_3O_9S$; MW 507



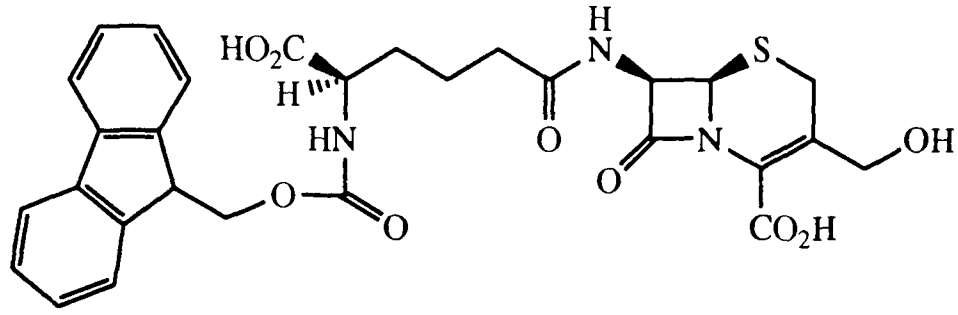
To a solution of DAC (3) (22 mg, 0.06 mmol) in distilled water (3 ml) was added 1N $NaHCO_3$ (150 µl) to adjust pH to 8, and THF (3 ml) was added slowly with stirring. Benzylchloroformate (50 µl, 0.35 mmol, 6 eq) in THF (500 µl) was added dropwise, and the solution stirred for 30 min at room temperature. The THF was evaporated and the aqueous solution washed twice with DCM and lyophilized to give the product (113) (21 mg, 0.04 mmol, 70% yield).

δ_H (500 MHz, D_2O, HOD suppressed) : 1.63-1.87 (4H, m, $CHCH_2CH_2$), 2.34-2.43 (2H, m, CH_2CO), 3.39 and 3.62 (2H, ABq, J 18 Hz, CH_2S), 3.93-3.99 (1H, m, $HNCHCO_2H$), 4.24 and 4.28 (2H, ABq, J 13 Hz, CH_2OH), 5.08-5.21 (3H, m, CH_2Ph and β-lactam CH) 5.60 (1H, d, J 4 Hz, β-lactam CH), 7.38-7.56 (5H, m, aromatic CH)

δ_C (125.8 MHz, D_2O) : 22.64 (t, $CHCH_2CH_2$), 26.14 (t, CH_2S) 32.07 (t, $CHCH_2$), 35.55 (t, CH_2CO), 56.67, 58.03 and 59.66 (3 × d, NHCHCHS and $HNCHCO_2H$), 61.77 (t, CH_2OH), 67.57 (t, CH_2Ph), 122.27 and 130.32 (2 × s, C=C), 128.30, 129.02 and 129.52 (3 × d, aromatic CH), 158.50, 165.65, 169.65, 177.84 and 179.76 (5 × s, 5 × C=O)

m/z (ESMS) : 530 ([MNa^+], 100%), 531 (30%), 532 (12%), 533 (3%), 552 ([di-Na salt + H^+], 40%)

(6R,7R)-1-Aza-3-hydroxymethyl-7-[(5R)-5-N-(9-fluorenylmethoxycarbonylamino)-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (114)
$C_{29}H_{29}N_3O_9S$; MW 595



To a solution of DAC (3) (20 mg, 0.054 mmol) in water (2 ml) was added 1N $NaHCO_3$ (100 µl) to adjust pH to 8, and 1,4-dioxan (2.5 ml). To this stirred solution was added 9-fluorenylmethyl chloroformate[215] (63 mg, 0.24 mmol, 4.4 eq) in dioxan (2 ml). The mixture was stirred at room temperature for 2h and evaporated to dryness. The residue was taken up in H_2O/DCM, the aqueous phase washed again with DCM and lyophilized to give (114) (*ca.* 18 mg by NMR calibration, 0.03 mmol, 58% yield).

δ_H (500 MHz, D_2O/d_6-Acetone (1:1, v/v), HOD suppressed) : 1.63-1.95 (4H, m, $CHCH_2CH_2$), 2.36-2.45 (2H, m, CH_2CO), 3.41 and 3.60 (2H, ABq, J 18 Hz, CH_2S), 3.93 (2H, d, J 7 Hz, $CHCH_2OCO$), 4.00-4.06 (1H, m, $HNCHCO_2H$), 4.10 (1H, t, J 7 Hz, $CHCH_2OCO$), 4.18 and 4.35 (2H, ABq, J 12 Hz, CH_2OH), 5.09 and 5.67 (2H, 2 × d, J 4 Hz, NHCHCHS), 7.35-7.88 (8H, m, aromatic CH)

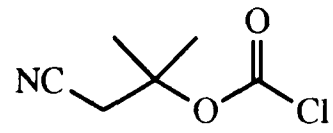
δ_C (125.8 MHz, D_2O) : 22.46 (t, $CHCH_2\underline{C}H_2$), 26.16 (t, $\underline{C}H_2S$), 32.34 (t, $CH\underline{C}H_2$), 35.57 (t, $\underline{C}H_2CO$), 47.89 (d, $\underline{C}HCH_2OCO$), 49.71 (t, $\underline{C}H_2OCO$), 56.64, 58.02 and 59.62 (3 × d, $NH\underline{C}H\underline{C}HS$ and $HN\underline{C}HCO_2H$), 61.76 (t, $\underline{C}H_2OH$), 120.84, 125.72, 128.16 and 128.71 (4 × d, aromatic $\underline{C}H$), 122.46 and 130.67 (2 × s, $\underline{C}=\underline{C}$), 141.74 and 144.75 (2 × s, aromatic $\underline{C}$), 158.20, 165.68, 169.56, 177.63 and 179.71 (5 × s, 5 × $\underline{C}=O$)

m/z (ESMS) : 618 ([MNa^+], 70%), 640 ([di-Na salt + H^+], 100%), 641 (30%), 642 (12%), 643 (4%), 662 ([di-Na salt + Na^+], 50%)

Deprotection of FMOC-DAC (114)

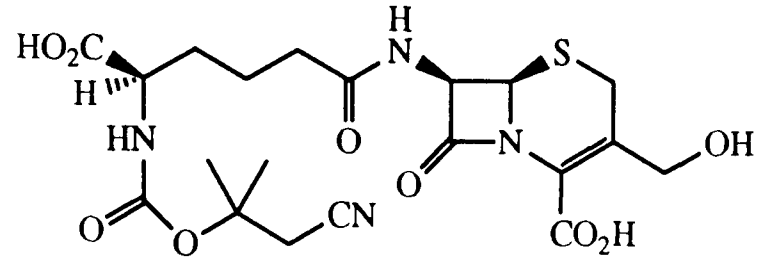
FMOC-DAC (114) (1.3 mg; 2.1 μmol) was suspended in water (0.5 ml) and a solution of 5% piperidine in THF (0.5 ml) was added. The resulting solution was stirred for 30 min after which the THF was evaporated and the aqueous phase was washed with ether and lyophilised. HPLC purification of the crude product (Waters system, solvent 10 mM aqueous NH_4HCO_3, flow rate 1 ml/min) resulted in the isolation of DAC (3) (*ca.* 530 μg by NMR calibration, 67% yield, retention time 10.5 min).

1,1-Dimethyl-2-cyanoethyl chloroformate (115)[217]
$C_6ClH_8NO_2$; MW 161



To a solution of 3-hydroxy-3-methylbutyronitrile (116) (524 μl, 5 mmol) in DCM (5 ml) at -70°C was added pyridine (408 μl, 5 mmol, 1 eq), followed by triphosgene[218] (117) (0.5 g, 1.68 mmol, 0.33 eq). The reaction was stirred for 4h at room temperature, then diluted with DCM, washed with ice cold 1N HCl and ice cold water, dried (Na_2SO_4) and evaporated to give the crude product as an oil which was used without purification in the next reaction. δ_H (200 MHz, $CDCl_3$) : 1.63 (6H, s, 2 × $C\underline{H}_3$), 2.89 (2H, s, $C\underline{H}_2CN$)

(6R,7R)-1-Aza-3-hydroxymethyl-7-[(5R)-5-N-(cyano-*t*-butoxycarbonylamino)-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (118)
$C_{20}H_{26}N_4O_9S$; MW 498



To a solution of DAC (3) (22 mg, 0.06 mmol) in water (4 ml) was added 1N $NaHCO_3$ (pH 8). A solution of crude (115) (100 mg, in excess of 8 eq) in dioxan (2 ml) was added dropwise. The reaction was stirred for 90 min, the solvent evaporated and the residue taken in water/ether. The aqueous phase was separated, washed again with ether and lyophilised. ^{1}H-NMR analysis showed formation of two products which were separated by HPLC (Waters system, solvent 20% MeOH in aqueous 10mM NH_4HCO_3, flow rate 1 ml/min) to give (118) (retention time 6 min, *ca.* 9.6 mg by NMR calibration) and (119) (retention time 12 min, *ca.* 9.8 mg by NMR calibration).

Data for (118) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.57 (6H, s, 2 × C$\underline{H}_3$), 1.60-1.92 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.36-2.45 (2H, m, C$\underline{H}_2$CO), 3.03 and 3.11 (2H, ABq, J 16 Hz, C$\underline{H}_2$CN), 3.47 and 3.66 (2H, ABq, J 18 Hz, C$\underline{H}_2$S), 3.90-3.96 (1H, m, HNC$\underline{H}$CO$_2$H), 4.25 and 4.30 (2H, ABq, J 13 Hz, C$\underline{H}_2$OH), 5.12 and 5.62 (2H, 2 × d, J 4.5 Hz, NHC$\underline{H}$C$\underline{H}$S)

δ_C (125.8 MHz, D_2O) : 22.62 (t, CHCH$_2$$\underline{C}H_2$), 26.17 (t, $\underline{C}$H$_2$S), 27.13 and 27.42 (2 × q, 2 × $\underline{C}$H$_3$), 32.00 (t, CH$\underline{C}$H$_2$), 35.58 (t, $\underline{C}$H$_2$CO), 39.02 (t, $\underline{C}$H$_2$CN), 56.28, 58.06 and 59.71 (3 × d, NH$\underline{C}$H$\underline{C}$HS and HN$\underline{C}$HCO$_2$H), 61.76 (t, $\underline{C}$H$_2$OH), 122.28 and 142.15 (2 × s, $\underline{C}$=$\underline{C}$), 126.23 (s, $\underline{C}$N), 157.19, 165.65, 169.51, 177.81 and 179.64 (5 × s, 5 × $\underline{C}$=O)

m/z (ESMS) : 521 ([MNa$^+$], 100%), 522 (28%), 523 (15%), 524 (4%), 543 ([Na salt + Na$^+$], 55%)

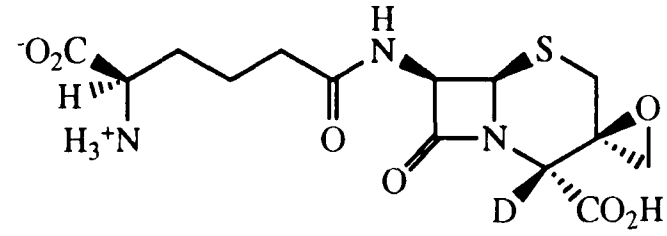
Data for (119) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.55 (6H, s, 2 × C$\underline{H}_3$), 1.62-1.88 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.33-2.45 (2H, m, C$\underline{H}_2$CO), 3.02 and 3.09 (2H, ABq, J 17 Hz, C$\underline{H}_2$CN), 3.72 and 3.89 (2H, ABq, J 19 Hz, C$\underline{H}_2$S), 3.94-3.99 (1H, m, HNC$\underline{H}$CO$_2$H), 5.08 and 5.13 (2H, ABq, J 18 Hz, C$\underline{H}_2$OCO), 5.22 and 5.77 (2H, 2 × d, J 5 Hz, NHC$\underline{H}$C$\underline{H}$S)

m/z (ESMS) : 481 ([MH$^+$], 100%), 482 (29%), 483 (12%), 484 (3%), 503 ([MNa$^+$], 80%)

(2R,3R,6R,7R)-1-Aza-(2-^{2}H)-3-spiroepoxy-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (122)

$C_{14}H_{18}DN_3O_7S$; MW 374



A mixture of DAC (3) (*ca.* 7.6 mg) and the epoxide metabolite (ratio 4 : 1) was dissolved in distilled formic acid (9 ml) and left to stand at room temperature for 45 min. The formic acid was evaporated under vacuum with no heating. ^{1}H-NMR analysis showed complete lactonisation of DAC to the lactone (120) and the presence of a third β-lactam product in trace amounts indicating that some decomposition of the epoxide had occurred. The crude mixture was purified by HPLC (Waters system, solvent 4.5% MeCN/H_2O, flow rate 1 ml/min) to give a mixture of the epoxide and the third compound coeluting as one peak (retention time 4.5 min), and the lactone (120)[141] (retention time 15 min). A mass spectrum (ESMS) on the fraction containing the epoxide and the third compound revealed peaks at 375 ([MH$^+$], epoxide) and 421 ([MH$^+$], hydroxyformate ester (121)). This fraction was further purified by HPLC (Waters system, solvent 10 mM aqueous NH_4HCO_3, flow rate 1 ml/min) to give the pure spiro-epoxide (122) (*ca.* 960 μg by NMR calibration, retention time 8.5 min), and the diol (123) (*ca.* 38 μg by NMR calibration) and lactone (124) (*ca.* 100 μg by NMR calibration) which coeluted as one peak (retention time 13.2 min). The epoxide (122) showed no bioactivity against *E. coli* X580.

Data for (120) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.68-1.96 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.44 (2H, t, J 7 Hz, C$\underline{H}_2$CO), 3.74 and 3.91 (2H, ABq, J 18 Hz, C$\underline{H}_2$S), 3.74 (1H, t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$), 5.10 and 5.15 (2H, ABq, J 18 Hz, C$\underline{H}_2$OCO), 5.24 and 5.80 (2H, 2 × d, J 5 Hz, HNC$\underline{H}$C$\underline{H}$S)

δ_C (125.8 MHz, D_2O) : 21.62 (t, CHCH$_2$C$\underline{H}_2$), 23.41 (t, C$\underline{H}_2$S) 30.61 (t, CH$\underline{C}H_2$), 35.32 (t, $\underline{C}H_2$CO), 55.31 and 57.96 and 60.57 (3 × d, NH$\underline{C}$H$\underline{C}$HS and $H_3N^+\underline{C}HCO_2^-$), 73.49 (t,

$\underline{C}H_2OCO$) 123.75 and 144.59 (2 × s, $\underline{C}$=$\underline{C}$), 165.69, 170.19, 174.91 and 177.15 (4 × s, 4 × $\underline{C}$=O)

m/z (ESMS) : 356 ([MH^+], 100%), 357 (20%), 358 (8%), 359 (2%)

Data for (122) :

δ_H (500 MHz, D_2O, $\underline{H}$OD suppressed) : 1.66-1.95 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.44 (2H, t, J 7 Hz, C$\underline{H}_2$CO), 2.44 and 3.64 (2H, ABq, J 15 Hz, C$\underline{H}_2$S), 3.28 and 3.40 (2H, 2 × d, J 4 Hz, epoxide H's), 3.74 (1H, *ca.* t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$), 5.37 and 5.50 (2H, 2 × d, J 4 Hz, HNC$\underline{H}$C$\underline{H}$S) (the couplings were confirmed by a COSY experiment)

m/z (ESMS) : 375 ([MH^+], 100%), 376 (20%), 377 (8%), 378 (2%), 397 ([MNa^+], 44%)

Data for (123) and (124) :

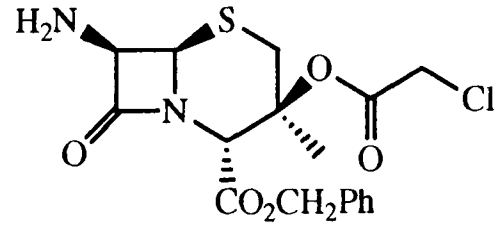
δ_H (500 MHz, D_2O, $\underline{H}$OD suppressed) : 1.68-1.95 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$ of (123) and (124)), 2.38-2.48 (2H, m, C$\underline{H}_2$CO of (123) and (124)), 2.77 and 3.37 (2H, ABq, J 14 Hz, C$\underline{H}_2$S of (123)), 3.11 and 3.15 (2H, ABq, J 13 Hz, C$\underline{H}_2$S of (124)), 3.53 and 3.87 (2H, ABq, J 13 Hz, C$\underline{H}_2$OH of (124)), 3.57 and 3.78 (2H, ABq, J 12 Hz, C$\underline{H}_2$OH of (123)) 3.75 (1H, *ca.* t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$ of (123) and (124)), 4.35 and 5.43 (2H, 2 × s, HNC$\underline{H}$C$\underline{H}$S of (124)), 5.32 and 5.46 (2H, 2 × d, J 4 Hz, HNC$\underline{H}$C$\underline{H}$S of (123)) (assignments based on relative intensities of the peaks and a COSY experiment)

m/z (ESMS) : 393 ([MH^+], 100%), 394 (19%), 395 (8%), 396 (2%)

6.2.4 Experimental for Section 2.5

(2$\underline{R}$,3$\underline{S}$,6$\underline{R}$,7$\underline{R}$)-1-Aza-3-chloroacetoxy-3-methyl-7-amine-8-oxo-5-thiabicyclo[4.2.0] octane-2-carboxylic acid benzyl ester (127)

$C_{17}ClH_{19}N_2O_5S$; MW 398.5



To a solution of the 3-chloroacetoxy cepham (126) (see section 6.4.2) (350 mg, 0.66 mmol) in dry DCM (3 ml) under argon at -78°C, was added dry $\underline{N}$,$\underline{N}$-dimethylaniline (234

μl, 1.84 mmol, 2.8 eq) and phosphorus pentachloride (178 mg, 0.86 mmol, 1.3 eq) (see section 4.2.1.2 for a discussion on this reaction). After stirring at -78°C for 5 min, the reaction was stirred for 1h at -40°C, then recooled to -78°C and *n*-butanol (1 ml) added dropwise. After 5 min the temperature was changed to -30°C and the reaction was left to warm up to 0°C over 40 min. The reaction mixture was poured into water (15 ml) and stirred vigorously for 10 min. The pH was adjusted to 8 with 1N $NaHCO_3$ and the suspension extracted with DCM (3 × 30 ml). To the dried (Na_2SO_4) combined organic layers was added *p*-toluenesulfonic acid monohydrate (476 mg, 2.5 mmol, 3.8 eq). The resulting solution was evaporated *in vacuo* with no heating to give a yellow oil. This was purified by flash chromatography using a small amount of silica (20 g) eluting with 30% EtOAc/DCM (200 ml), EtOAc (100 ml) and 30% MeOH/EtOAc (200 ml) (v/v). On elution with 30% MeOH/EtOAc the tosic salts of the product and of DMA were recovered. Evaporation of the solvent gave a yellow solid which was dissolved in DCM/$NaHCO_3$ 1N [100 ml, 1:1 (v/v)]. The organic phase was washed with H_2O (50 ml), dried (Na_2SO_4) and evaporated to a yellow oil. This was purified by chromatography [flash silica, EtOAc/DCM (10% to 20%, v/v)] to give the amine (127) (213 mg, 0.54 mmol, 81% yield) as a white foam.

T.l.c. [EtOAc/DCM (30%,v/v)] Rf 0.25

$\nu_{max}(CHCl_3)$: 3039 (m), 1767 (s, β-lactam C=O), 1743 (s, ester C=O), 1152 (m), 728 (m)

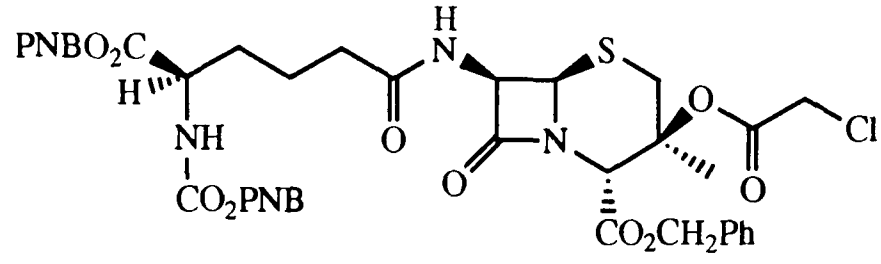
δ_H (500 MHz, $CDCl_3$) : 1.51 (3H, s, C$\underline{H}_3$), 3.39 and 3.46 (2H, ABq, J 15 Hz, SC$\underline{H}_2$), 4.03 and 4.07 (2H, ABq, J 15 Hz, C$\underline{H}_2$Cl), 4.47 (1H, d, J 4 Hz, β-lactam C$\underline{H}$), 4.80 (1H, s, C$\underline{H}CO_2Bn$), 5.16 and 5.21 (2H, ABq, J 12 Hz, PhC$\underline{H}_2$), 5.22 (1H, d, J 4 Hz, β-lactam C$\underline{H}$), 7.33-7.40 (5H, m, aromatic C$\underline{H}$)

δ_C (125.8 MHz, $CDCl_3$) : 21.73 (q, $\underline{C}H_3$), 30.22 (t, S$\underline{C}H_2$), 41.26 (t, $\underline{C}H_2$Cl), 53.30, 56.03 and 63.91 (3 × d, NH$\underline{C}H\underline{C}$HS and $\underline{C}HCO_2Bn$), 67.84 (t, $\underline{C}H_2Ph$), 75.50 (s, $\underline{C}CH_3$), 128.62, 128.82, 128.46, and 128.94 (3 × d, aromatic $\underline{C}$H), 134.58 (s, aromatic C1 of Ph), 165.90, 166.84 and 170.55 (3 × s, 3 × $\underline{C}$=O)

m/z (FAB) : 399 ([MH^+], 12%), 314 (44%), 91 (100%)

(2R,3S,6R,7R)-1-Aza-3-chloroacetoxy-3-methyl-7-[(5R)-5-N-*p*-nitrobenzyloxycarbonyl amino-5-*p*-nitrobenzyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester (128)[133]

$C_{38}ClH_{38}N_5O_{14}S$; MW 855



To a solution of (2R,3S,6R,7R)-1-aza-3-chloroacetoxy-3-methyl-7-amine-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester (127) (213 mg, 0.54 mmol) in dry DCM (3 ml), was added N-*p*-nitrobenzyloxycarbonyl-D-α-aminoadipic acid-α-*p*-nitrobenzyl ester (105) (254 mg, 0.54 mmol, 1 eq), EEDQ[220] (146 mg, 0.59 mmol, 1.1eq) and anhydrous Na_2SO_4 (*ca.* 30 mg). The reaction was left stirring under argon for 20h, after which time the solvent was evaporated to dryness and the residue partitioned between EtOAc/H_2O [100 ml, 1:1 (v/v)]. The organic phase was washed with 2N HCl (30 ml), sat. $NaHCO_3$ (30 ml), brine (30 ml), dried (Na_2SO_4) and evaporated to give the crude product. This was purified by chromatography [flash silica, EtOAc/DCM (10%, v/v)] to give (128) (340 mg, 0.4 mmol, 74% yield) as a white foam.

T.l.c. [EtOAc/DCM (30%,v/v)] Rf 0.40

ν_{max} ($CHCl_3$) : 3022 (s), 1777 (s), 1741 (s), 1525 (s), 1349 (s), 1236 (s), 1071 (m), 910 (m), 807 (m)

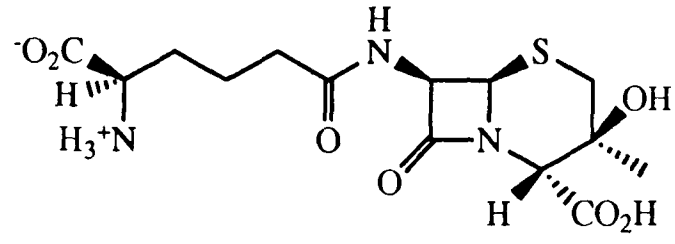
δ_H (500 MHz, $CDCl_3$) : 1.51 (3H, s, C<u>H</u>$_3$), 1.68-1.96 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.24-2.32 (2H, m, C<u>H</u>$_2$CO), 3.36 and 3.46 (2H, ABq, J 15 Hz, SC<u>H</u>$_2$), 4.05 and 4.17 (2H, ABq, J 15 Hz, C<u>H</u>$_2$Cl), 4.40-4.48 (1H, m, NHC<u>H</u>CH$_2$), 4.78 (1H, s, C<u>H</u>CO$_2$Bn), 5.18-5.33 (7H, m, NHCHC<u>H</u>S, PhC<u>H</u>$_2$ and 2 × C<u>H</u>$_2$ArNO$_2$), 5.54 (1H, dd, J 4 Hz and 9 Hz, NHC<u>H</u>CHS), 5.85 (1H, d, J 8 Hz, N<u>H</u>), 6.78 (1H, d, J 9 Hz, N<u>H</u>), 7.36-7.53 and 8.17-8.28 (13H, m, aromatic C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 20.70 (t, CHCH$_2$<u>C</u>H$_2$), 21.38 (q, <u>C</u>H$_3$), 30.16 (t, S<u>C</u>H$_2$), 31.42 (t, CH<u>C</u>H$_2$CH$_2$), 34.82 (t, <u>C</u>H$_2$CO), 41.31 (t, <u>C</u>H$_2$Cl), 53.88, 54.41, 57.74 and 59.15 (4 × d, NH<u>C</u>H<u>C</u>HS, <u>C</u>HCO$_2$Bn and NH<u>C</u>HCH$_2$), 65.43 and 65.56 (2 × t, 2 × <u>C</u>H$_2$ArNO$_2$), 67.97 (t,

$\underline{C}H_2Ph$), 75.21 (s, $\underline{C}CH_3$), 123.58, 123.70, 127.95, 128.35, 128.56, 128.71 and 128.89 (7 × d, aromatic $\underline{C}H$), 134.29 (s, aromatic C1 of Ph), 142.41 and 143.63 (2 × s, aromatic C1 of $ArNO_2$), 147.76 and 147.96 (2 × s, aromatic C4 of $ArNO_2$), 155.52 (s, $HN\underline{C}O_2PNB$), 165.56, 166.18, 166.32, 171.60 and 171.94 (5 × s, 5 × $\underline{C}$=O)

(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-carboxylic acid (40)

$C_{14}H_{21}N_3O_7S$; MW 375



To a suspension of (128) (13 mg, 15.2 μmol) in absolute ethanol (2 ml) was added thiourea (2 mg, 26.3 μmol, 1.7 eq) and the solution stirred at 60°C for 1h under argon. The solvent was evaporated with no heat, and the residue suspended in EtOAc (30 ml), washed with water (3 × 30 ml), brine (30 ml), dried (Na_2SO_4) and filtered. The solvent was evaporated *in vacuo* with no heat to give the crude alcohol (129), which was then dissolved in THF/H_2O [5 ml, 3:2 (v/v)] and hydrogenated with 10% Pd/C wet (200 mg, 50% water) under an atmosphere of H_2 for 5h. The suspension was filtered through a pad of celite, the THF evaporated off, and the aqueous phase washed with EtOAc (3 × 20 ml). Lyophilisation gave the crude product which was purified by HPLC [Gilson system, solvent 2.5% MeCN in 5 mM aqueous NH_4HCO_3 (v/v), flow rate 4 ml/min] to give the 3β-hydroxycepham (40) and the lactone (125) coeluting as one peak [retention time 5 min, 0.47 mg, 1.3 μmol, 9% yield], and the 3β-acetate cepham (130) [retention time 15 min, 1.3 mg, 3.1 μmol, 20% yield].

Data for (40) and (125) :

δ_H (500 MHz, D_2O, $\underline{H}OD$ suppressed) : 1.39 (3H, s, $C\underline{H}_3$ of (40)), 1.49 (3H, s, $C\underline{H}_3$ of (125)), 1.67-1.95 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.42-2.48 (2H, m, $C\underline{H}_2CO$), 2.66 and 3.56 (2H, ABq, J 14 Hz, $SC\underline{H}_2$ of (40)), 3.03 and 3.35 (2H, ABq, J 11 Hz, $SC\underline{H}_2$ of (125)), 3.71-3.75 (1H, m, $H_3N^+C\underline{H}CO_2^-$), 4.12 (1H, s, $C\underline{H}CO_2H$ of (125)), 4.15 (1H, s, $C\underline{H}CO_2H$ of

(40)), 4.32 and 5.44 (2H, 2 × s, NHC<u>H</u>C<u>H</u>S of (125)), 5.29 and 5.45 (2H, 2 × d, J 4 Hz, NHC<u>H</u>C<u>H</u>S of (40))

m/z (ESMS) : 376 ([MH^+], 100%), 377 (20%), 378 (9%), 379 (2%)

Identical by ^{1}H-NMR and mass spectra to lit. reference.[133]

Data for (130) :

δ_H (500 MHz, D_2O, <u>H</u>OD suppressed) : 1.64 (3H, s, C<u>H</u>$_3$), 1.66-1.95 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.12 (3H, s, C<u>H</u>$_3$), 2.44 (2H, t, J 7 Hz, C<u>H</u>$_2$CO), 3.42 and 3.55 (2H, ABq, J 15 Hz, SC<u>H</u>$_2$), 3.70 (1H, t, J 6 Hz, H_3N^+C<u>H</u>CO_2^-), 4.61 (1H, s, C<u>H</u>CO_2H), 5.36 and 5.50 (2H, 2 × d, J 4 Hz, NHC<u>H</u>C<u>H</u>S)

m/z (ESMS) : 418 ([MH^+], 100%), 419 (23%), 420 (9%), 421 (3%)

Incubation of (122) with DAOC/DACS

The epoxide (122) (*ca.* 0.5 mg) was incubated with DAOC/DACS (1 ml) and cofactor solution (100 μl). Examination of the crude incubation mixture by ^{1}H-NMR showed complete conversion of (122). Purification of the mixture by HPLC (Waters system, solvent 0.1% HCO_2H/H_2O, flow rate 2 ml/min, monitoring at 300 nm) enabled isolation of the aldehyde products (78) (retention time 8 min) and (30) (retention time 9.5 min).

Data for (78) :

λ_{max} (H_2O) : 300 nm

δ_H (500 MHz, D_2O, <u>H</u>OD suppressed) : 1.60-1.90 (4H, m, CHC<u>H</u>$_2$C<u>H</u>$_2$), 2.43 (2H, t, J 7 Hz, C<u>H</u>$_2$CO), 3.46 and 3.62 (2H, ABq, J 16 Hz, SC<u>H</u>$_2$), 3.73-3.80 (1H, m, H_3N^+C<u>H</u>CO_2^-), (C6-H resonance obscured by <u>H</u>OD peak), 4.95 (1H, d, J 7.5 Hz, 7H), 9.19 (1H, s, C<u>H</u>O)

m/z (ESMS) : 389 ([MH^+], 100%)

Data for (30) :

λ_{max} (H_2O) : 300 nm

δ_H (500 MHz, D_2O, <u>H</u>OD suppressed) : 1.65-1.93 (4H, m, CHC<u>H</u>$_2$C<u>H</u>$_2$), 2.38 (2H, t, J 7 Hz, C<u>H</u>$_2$CO), 3.50 and 3.57 (2H, ABq, J 16 Hz, SC<u>H</u>$_2$), 3.65-3.30 (1H, m, H_3N^+C<u>H</u>CO_2^-),

(C6-H resonance obscured by HOD peak), 4.90 (1H, d, J 6 Hz, 7H), 9.15 (1H, s, CHO)

m/z (ESMS) : 390 ([MH^+], 100%)

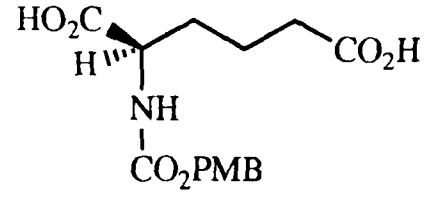
Both products gave data identical to literature reference.[145]

6.3 Experimental for Chapter 3

6.3.1 Experimental for Section 3.2

N-*p*-Methoxybenzyloxycarbonyl-D-α-aminoadipic acid (146)

$C_{15}H_{19}NO_7$; MW 325

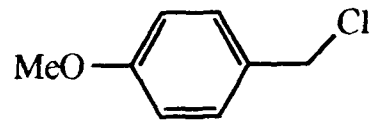


A solution of D-α-aminoadipic acid (2 g, 12 mmol) in aqueous $NaHCO_3$ (0.5 M, 18 ml) was adjusted to pH 9.5 with 2M NaOH. *p*-Methoxybenzyloxycarbonylazide (5.14 g, 25 mmol, 2 eq) in dioxan (40 ml) was added. The reaction was stirred at room temperature for 3h, with addition of 2M NaOH to maintain the pH at 9-10, and then at 35°C overnight. The reaction mixture was washed with DCM (2 × 40 ml), acidified to pH 2 (conc. HCl) and extracted with EtOAc (3 × 40 ml). The combined EtOAc extracts were dried (Na_2SO_4) and evaporated to give the product (146) as a white solid (3.80 g, 11.7 mmol, 94% yield).

δ_H (200 MHz, d_6-acetone) : 1.60-1.94 (4H, m, $CH_2CH_2CH_2$), 2.25-2.40 (2H, m, CH_2CO), 3.77 (3H, s, OCH_3), 4.15-4.28 (1H, m, $CHCH_2CH_2$), 4.99 (2H, s, CH_2Ar), 6.53 (1H, d, J 8 Hz, NH), 6.82-6.95 and 7.26-7.36 (4H, m, aromatic CH)

***p*-Methoxybenzylchloride**

C_8H_9ClO; MW 156.5

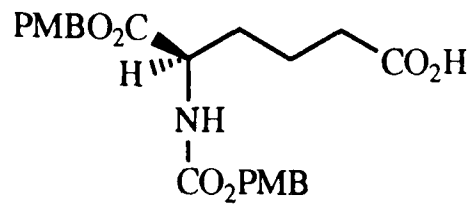


p-Methoxybenzylalcohol (10 ml, 11.08 g, 80 mmol) was added to concentrated HCl [36%(w/v), 10 ml] and the mixture stirred for 1h at room temperature. The two phases were separated and the organic phase was dried ($CaCl_2$) and distilled under reduced pressure to give the tittle compound as a colourless liquid (11.2 g, 71.5 mmol, 89% yield).

δ_H (200 MHz, $CDCl_3$) : 3.84 (3H, s, OCH_3), 4.60 (2H, s, CH_2), 6.92 and 7.36 (4H, ABq, J 9 Hz, aromatic CH)

N–*p*–Methoxybenzyloxycarbonyl–D–α-aminoadipic acid–α–*p*–methoxybenzyl ester (147)

$C_{23}H_{27}NO_8$; MW 445



To a solution of N-*p*-methoxybenzyloxycarbonyl-D-α-aminoadipic acid (146) (4 g, 12 mmol) in dry DMF (20 ml) under argon, was added dry NEt_3 (1.73 ml, 12 mmol, 1 eq) and *p*-methoxybenzylchloride (1.68 ml, 12 mmol, 1 eq). The reaction was stirred overnight at 35°C. EtOAc (100 ml) was added and the precipitate filtered off. The organic solution was washed with H_2O (3 × 50 ml), dried (Na_2SO_4) and evaporated to give a yellow oil. This was purified by chromatography [flash silica, MeOH/$CHCl_3$ (2%, v/v)] to give (147) (3.00 g, 6.7 mmol, 56 % yield).

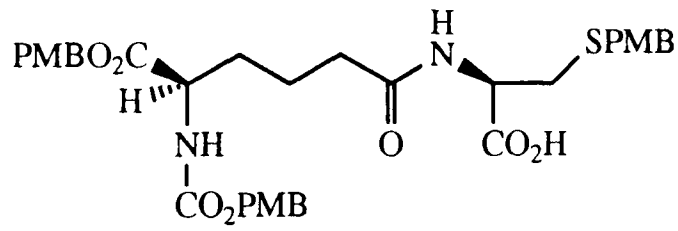
T.l.c. [MeOH/$CHCl_3$ (10%,v/v)] Rf 0.5

δ_H (200 MHz, $CDCl_3$) : 1.57-1.94 (4H, m, $CH_2CH_2CH_2$), 2.26-2.40 (2H, m, CH_2CO), 3.80 (6H, s, 2 × OCH_3), 4.34-4.46 (1H, m, $CHCH_2CH_2$), 5.02-5.17 (4H, m, 2 × CH_2Ar), 5.52 (1H, d, J 8 Hz, NH), 6.83-6.94 and 7.23-7.39 (4H, m, aromatic CH)

m/z (FAB) : 468 ([MNa^+], 100%)

D-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (149)

$C_{34}H_{40}N_2O_{10}S$; MW 668



A solution of N-*p*-methoxybenzyloxycarbonyl-D-α-aminoadipic acid-α-*p*-methoxy benzyl ester (536 mg, 1.2 mmol) (147) and dry NEt_3 (168 µl, 1.2 mmol, 1 eq) in dry THF (17 ml) under argon was cooled to -15°C. After stirring for 15 min, freshly distilled isobutylchloroformate (156 µl, 1.2 mmol, 1 eq) was added, and the reaction stirred for another 30 min. At 0°C, a solution of S-*p*-methoxybenzyl-L-cysteine (148) (334 mg, 1.2 mmol, 1 eq) and NEt_3 (370 µl, 2.6 mmol, 2.2 eq) in H_2O (14 ml) was added. The reaction

mixture was shaken vigorously for 2 min, and then stirred at room temperature for 50 min. The THF was removed under vacuum and H_2O (20 ml) added. The solution was washed with ether (2 × 20 ml), acidified to pH 2 with 2N HCl, and extracted with EtOAc (3 × 30 ml). The combined EtOAc layers were dried (Na_2SO_4) and evaporated to give (149) as a colourless oil (716 mg, 1.07 mmol, 89% yield).

T.l.c. [MeOH/$CHCl_3$ (10%,v/v)] Rf 0.18

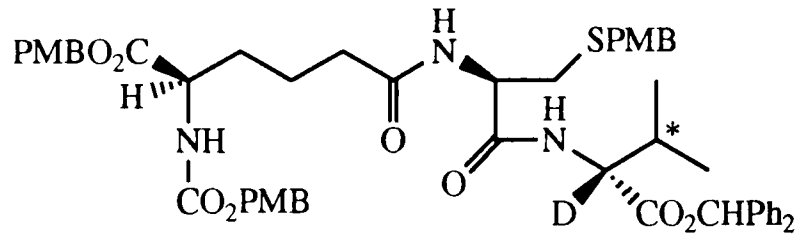
ν_{max} ($CHCl_3$) : 3430 (m), 2938 (m), 1718 (s, C=O), 1674 (m), 1613 (s), 1521 (s), 1256 (s), 1176 (s), 823 (m), 704 (s)

δ_H (200 MHz, $CDCl_3$) : 1.58-1.92 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.15-2.38 (2H, m, C<u>H</u>$_2$CO), 2.84-2.93 (2H, m, C<u>H</u>$_2$S), 3.79 (9H, s, 3 × OC<u>H</u>$_3$), 4.32-4.45 (1H, m, C<u>H</u>CH$_2$CH$_2$), 4.69-4.80 (1H, m, α-H), 4.98-5.14 (6H, m, 3 × C<u>H</u>$_2$Ar), 5.61 (1H, d, J 8 Hz, N<u>H</u>), 6.59 (1H, d, J 7 Hz, N<u>H</u>), 6.80-6.92 and 7.16-7.32 (2 × 6H, 2 × m, aromatic C<u>H</u>)

m/z (FAB) : 713 ([MNa salt + Na^+], 23%), 691 ([MNa^+], 50%), 121 (100%)

<u>D</u>-5-<u>N</u>-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido-<u>S</u>-*p*-methoxybenzyl-<u>L</u>-cysteinyl-<u>D</u>-(2-^{2}H,3-^{13}C)-valine benzhydryl ester (151)

$C_{51}{}^{13}CH_{58}DN_3O_{11}S$; MW 935



* = ^{13}C

The ammonium tosylate salt of <u>D/L</u>-(2-^{2}H, 3-^{13}C)-valine benzhydryl ester[61,133] (145) (125 mg, 0.27 mmol) was suspended in aqueous $NaHCO_3$ (1N, 10 ml) and the free amine (150) extracted into EtOAc (2 × 20 ml). The combined organic phases were dried (Na_2SO_4) and evaporated. To the free amine (150) (77 mg, 0.27 mmol) in dry DCM (2 ml) was added <u>D</u>-5-<u>N</u>-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonyl pentanamido-<u>S</u>-*p*-methoxybenzyl-<u>L</u>-cysteine (149) (185 mg, 0.28 mmol, 1 eq), EEDQ[220] (75 mg, 0.30 mmol, 1.1 eq) and anhydrous Na_2SO_4 (*ca.* 10 mg). The resulting mixture was stirred at room temperature under argon for 15h. The solution was evaporated to dryness

and the residue was partitioned between EtOAc (30 ml) and water (30 ml). The two phases were separated and the organic phase washed with 2N HCl (20 ml), sat. $NaHCO_3$ (20 ml) and brine (20 ml), dried (Na_2SO_4) and evaporated. Purification of the crude product by P.l.c. [EtOAc/Hexane (1:1, v/v)] gave (151) (96 mg, 0.1 mmol, 37% yield).

T.l.c. [Petrol/EtOAc (2:3, v/v)] Rf 0.58

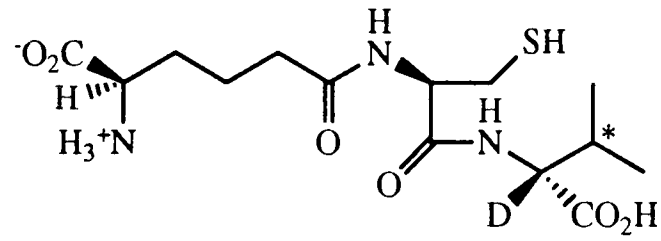
ν_{max} ($CHCl_3$) : 3428 (m), 3037 (s), 1734 (s), 1680 (m), 1515 (s), 1240 (s), 1175 (m), 1035 (m), 829 (m)

δ_H (500 MHz, $CDCl_3$) : 0.73-0.78 and 0.85-0.90 (2 × 3H, 2 × m, $^{13}CH(C\underline{H}_3)_2$), 1.60-1.85 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.02-2.10 (2H, m, $C\underline{H}_2CO$), 2.12-2.20 and 2.33-2.38 (1H, 2 × m, $^{13}C\underline{H}(CH_3)_2$), 2.65 and 2.83 (2H, AB part of ABX system, J_{AB} 14 Hz, J_{AX} 7 Hz, J_{BX} 6 Hz, $CHC\underline{H}_2S$), 3.72 (2H, s, $SC\underline{H}_2Ar$), 3.75 (3H, s, $SCH_2ArOC\underline{H}_3$), 3.79 (6H, s, 2 × $CH_2ArOC\underline{H}_3$), 4.32-4.38 (1H, m, $C\underline{H}CH_2CH_2$), 4.51 (1H, X of ABX system, J_{AX} 7 Hz, J_{BX} 6 Hz, $C\underline{H}CH_2S$), 4.99-5.08 (4H, m, 2 × $OC\underline{H}_2Ar$), 5.48 (1H, d, J 8 Hz, N$\underline{H}$), 6.26 (1H, d, J 7 Hz, N$\underline{H}$), 6.79-6.90 and 7.22-7.36 (23H, m, $C\underline{H}Ph_2$ and aromatic C$\underline{H}$)

m/z (FAB) : 957 (15%), 958 ([MNa^+], 100%), 959 (53%), 960 (25%), 961 (7%)

D-5-Amino-5-carboxypentanamido-L-cysteinyl-D-[2-^{2}H, 3-^{13}C]valine (152)

$C_{13}{}^{13}CH_{24}DN_3O_6S$; MW 365



* = ^{13}C

To the fully protected tripeptide (151) (46 mg, 0.05 mmol) under argon was added anisole (0.4 ml) and freshly distilled trifluoroacetic acid (2 ml)[234]. The resulting solution was refluxed for 30 min. Evaporation of this mixture gave a residue which was redissolved in toluene and again evaporated to dryness. The resulting residue was partitioned between EtOAc (10 ml) and water (10 ml), and the aqueous phase washed again with EtOAc (2 × 10 ml) and lyophilised to give (152) (12 mg, 0.03 mmol, 67% yield).

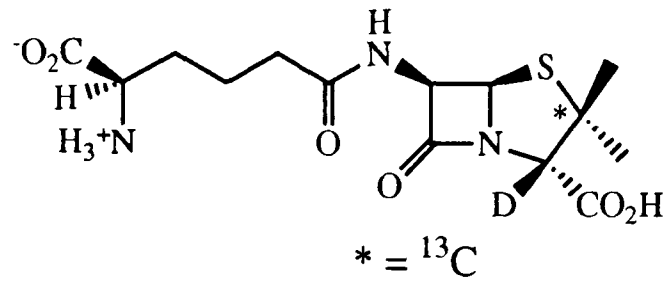
δ_H (500 MHz, D_2O, $\underline{H}OD$ suppressed) : 0.90-0.98 (6H, m, $^{13}CH(C\underline{H}_3)_2$), 1.68-2.00 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.05-2.08 and 2.30-2.36 (1H, 2 × m, $^{13}C\underline{H}(CH_3)_2$), 2.43 (2H, t, J 7 Hz,

C<u>H</u>$_2$CO), 2.88 and 2.93 (2H, AB part of ABX system, J_{AB} 14 Hz, J_{AX} 7 Hz, J_{BX} 6 Hz, CHC<u>H</u>$_2$S), 3.98 (1H, X of ABX system, J_{AX} 7 Hz, J_{BX} 6 Hz, C<u>H</u>CH$_2$S), 4.57 (1H, t, J 7 Hz, C<u>H</u>CH$_2$CH$_2$)

m/z (ESMS) : 365 (17%), 366 ([MH$^+$], 100%), 367 (18%), 368 (7%), 369 (2%)

(2<u>S</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-carboxy pentanamido] -7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (1f)

$C_{13}{}^{13}CH_{20}DN_3O_6S$; MW 361



* = ^{13}C

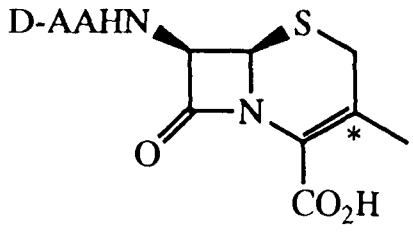
To [^{2}H,^{13}C]<u>D</u>,<u>L</u>,<u>D</u>-ACV (152) (5 mg, 14 μmol) in H_2O (1 ml) was added dithiothreitol (100 mM, 100 μl), and the resulting solution incubated for 10 min at 27°C and 250 rpm. Partially purified Isopenicillin N Synthase enzyme (2.5 ml, 22 IU) in TRIS-HCl buffer (50 mM, pH 7.4) was exchanged into ammonium bicarbonate buffer (3.5 ml, 25 mM, pH 7.8) on a pre-equilibrated sephadex column (Pharmacia, PD-10). To the solution of the tripeptide was added <u>L</u>-ascorbate (50 mM, 100 μl) and $FeSO_4$ (5 mM, 100 μl), the pH adjusted to 7.8 with 1N NaOH, and the enzyme solution added. The resulting solution was divided into two aliquots (*ca.* 2.4 ml each) and incubated at 27°C and 250 rpm. After 20 min some more $FeSO_4$ (50 μl) and dithiothreitol (50 μl) were added. After a further 20 min the incubation was quenched by the addition of acetone to 70% (v/v). The precipitated protein was spun down by centrifugation (17 Krpm, 2 min, 0°C) and the supernatant lyophilised. Purification of the crude incubation mixture by HPLC (Gilson system, solvent 0.75% MeCN in 10 mM aqueous NH_4HCO_3, flow rate 4.5 ml/min) gave [2-^{13}C,3-^{2}H]-penicillin N (1f) (retention time 6.5 min, *ca.* 2.2 mg by NMR calibration, 6.1 μmol, 44% yield).

δ_H (500 MHz, D_2O, <u>H</u>OD suppressed) : 1.53 (3H, d, J_{13C-H} 4 Hz, ^{13}CC<u>H</u>$_3$), 1.64 (3H, d, J_{13C-H} 4 Hz, ^{13}CC<u>H</u>$_3$), 1.62-1.95 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$CO), 2.41 (2H, *ca.* t, J 7 Hz,

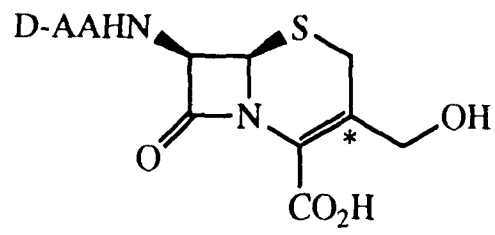
C$\underline{H}_2$CO), 3.72-3.78 (1H, m, $H_3^+NC\underline{H}CO_2^-$), 5.49 and 5.57 (2H, 2 × d, J 4 Hz, NHC$\underline{H}$C$\underline{H}$S)

m/z (ESMS) : 361 (17%), 362 ([MH$^+$], 100%), 363 (21%), 364 (8%), 365 (2%)

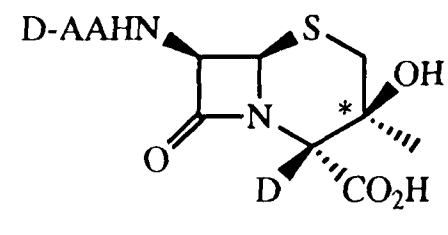
Incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) with DAOC/DACS under $^{16}O_2$



(2c)



(3b)



(40c)

* = ^{13}C

[2-^{13}C,3-^{2}H]Penicillin N (1f) (4 mg) was incubated with DAOC/DACS (4 ml, 0.74 IU) and cofactor solution (400 µl) according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR showed *ca.* 85% conversion to [3-^{13}C]DAOC (2c), [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40c) and [3-^{13}C]DAC (3b). Integration of the β-lactam region showed the ratio of the products to be (2c):(40c):(3b) = 11:46:43. Purification of the crude incubation mixture by HPLC (Waters system, solvent 10 mM aqueous NH_4HCO_3, flow rate 2 ml/min) gave (3b) (retention time 4.5 min, *ca.* 550 µg by NMR calibration), (40c) (retention time 5.8 min, *ca.* 765 µg by NMR calibration) and (2c) (retention time 10.4 min, *ca.* 174 µg by NMR calibration). The [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40c) obtained from this experiment was analysed by ^{13}C-NMR (overnight acquisition, 20,395 transients) and two peaks were observed, one for the C3 of (40c) (65.46 ppm, $^{13}\underline{C}$Me) and one for the C3 of the corresponding lactone (125a) (81.37, $^{13}\underline{C}$Me).

Data for [3-^{13}C]DAOC (2c) :

δ_H (500 MHz, D_2O, $\underline{H}$OD suppressed) : 1.67-1.95 (4H, 2 × m, C$\underline{H}_2$C$\underline{H}_2$CH_2CO), 1.94 (3H, d, $J_{^{13}C\text{-}H}$ 7 Hz, ^{13}CC$\underline{H}_3$), 2.41 (2H, t, J 7 Hz, C$\underline{H}_2$CO), 3.25 and 3.59 (2H, AB part of ABX system (X=^{13}C), J_{AB} 18 Hz, J_{AX} 7 Hz, J_{BX} 5 Hz, ^{13}CC$\underline{H}_2$S), 3.75 (1H, t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$), 5.08 and 5.56 (2H, 2 × d, J 4 Hz, NHC$\underline{H}$C$\underline{H}$S)

Partial δ_C (125.8 MHz, D_2O) : 122.80 (s, C=$^{13}\underline{C}$)

m/z (ESMS) : 358 (14%), 359 ([MH$^+$], 100%), 360 (20%), 361 (9%), 362 (4%)

Data for [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40c) :

δ_H (500 MHz, D_2O, HOD suppressed) : 1.38 (3H, d, J_{13C-H} 4 Hz, $^{13}CC\underline{H}_3$), 1.70-1.97 (4H, 2 × m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.43 (2H, t, J 7 Hz, $C\underline{H}_2CO$), 2.64 (1H, dd, J_{H-H} 14 Hz, J_{H-13C} 5 Hz, $^{13}CC\underline{H}HS$), 3.55 (1H, d, J_{H-H} 14 Hz, $^{13}CCH\underline{H}S$), 3.73-3.77 (1H, m, $H_3N^+C\underline{H}CO_2^-$), 5.28 and 5.44 (2H, 2 × d, J 4 Hz, NHC$\underline{H}$C$\underline{H}$S)

Partial δ_C (125.8 MHz, D_2O) : 65.46 (s, $^{13}\underline{C}CH_3$)

m/z (ESMS) : 377 (15%), 378 ([MH^+], 100%), 379 (26%), 380 (10%), 381 (2%)

Data for [3-^{13}C]DAC (3b) :

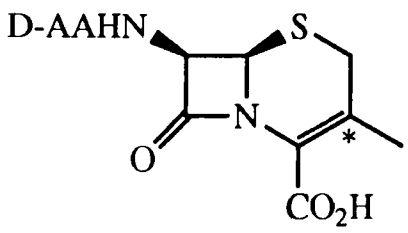
δ_H (500 MHz, D_2O, HOD suppressed) : 1.70-1.96 (4H, 2 × m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.43 (2H, t, J 7 Hz, $C\underline{H}_2CO$), 3.47 and 3.66 (2H, AB part of ABX system (X=^{13}C), J_{AB} 18 Hz, J_{AX} 7 Hz, J_{BX} 6 Hz, $^{13}CC\underline{H}_2S$), 3.74 (1H, t, J 6 Hz, $H_3N^+C\underline{H}CO_2^-$), 4.26 and 4.30 (2H, AB part of ABX system (X=^{13}C), J_{AB} 13 Hz, J_{AX} 4 Hz, J_{BX} 3 Hz, $^{13}CC\underline{H}_2OH$) 5.13 and 5.63 (2H, 2 × d, J 5 Hz, NHC$\underline{H}$C$\underline{H}$S)

Partial δ_C (125.8 MHz, D_2O) : 121.81 (s, C=$^{13}\underline{C}$)

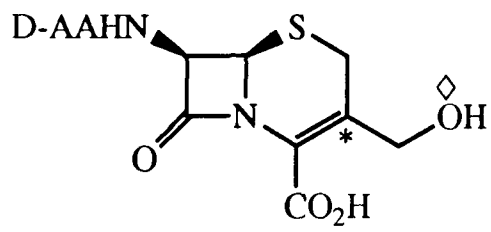
m/z (ESMS) : 374 (14%), 375 ([MH^+], 100%), 376 (20%), 377 (11%), 378 (3%)

6.3.2 Experimental for Section 3.3

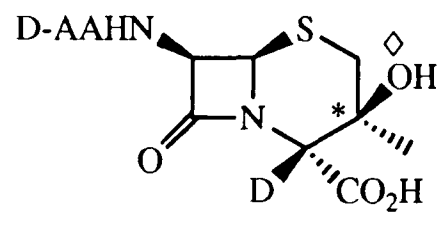
Incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) with DAOC/DACS under $^{18}O_2$



(2c)



(3c)



(40d)

* = ^{13}C; ◇ = ^{18}O or ^{16}O

The head space of an intact glass vial of $^{18}O_2$ (MSD Isotopes, 98 atom % ^{18}O, 100 ml) was repeatedly evacuated and flushed with argon, and then septum sealed. A solution of [2-^{13}C,3-^{2}H]penicillin N (1f) (1.75 mg) in water (1.65 ml) was degassed and flushed with argon, after addition of the standard cofactor solution (350 µl). DAOC/DACS (5 ml, 0.02 IU) was also briefly degassed and flushed with argon. After breaking the glass neck

seal of the septum sealed $^{18}O_2$ vial, the enzyme was introduced *via* syringe, followed by the solution of the substrate and cofactors. The sealed vial was incubated for 2h at 27°C and 250 rpm. Acetone was added *via* syringe to a final concentration of 70% (v/v), the vial opened to air, and the incubation worked up according to the general procedure. Examination by ^{1}H-NMR showed *ca.* 87% conversion to [3-^{13}C]DAOC (2c), [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40d) and [3-^{13}C]DAC (3c). Integration of the β-lactam region revealed the ratio of products to be (2c):(40d):(3c) = 20:53:27. Purification of the crude incubation mixture according to the standard procedure gave (3c) (*ca.* 128 μg by NMR calibration), (40d) (*ca.* 185 μg by NMR calibration) and (2c) (*ca.* 143 μg by NMR calibration), identified by ^{1}H-NMR and HPLC retention times. Mass spectral analysis of the products indicated no ^{18}O incorporation into (2c), 71% incorporation of ^{18}O into (40d) and 52% incorporation into (3c).[235]

This experiment was repeated with [2-^{13}C,3-^{2}H]penicillin N (1f) (2.3 mg), cofactor solution (300 μl) and DAOC/DACS (3.7 ml, 0.07 IU). ^{1}H-NMR analysis of the crude incubation mixture showed *ca.* 75% conversion to (2c), (40d) and (3c) [ratio (2c):(40d):(3c) = 26:52:22]. Purification by HPLC gave (3c) (*ca.* 170 μg by NMR calibration), (40d) (*ca.* 420 μg by NMR calibration) and (2c) (*ca.* 213 μg by NMR calibration), identified by ^{1}H-NMR and HPLC retention times. Mass spectral analysis of the products showed no ^{18}O incorporation into (2c), 69% incorporation of ^{18}O into (40d) and 57% incorporation into (3c).[235] The [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40d) obtained on this second experiment was analysed by ^{13}C-NMR (overnight acquisition, 31,925 transients), and an ^{18}O-^{13}C shift was observed for both (40d) and the lactone (125b). The ^{16}O-^{13}C peak for both (40d) and (125b) had an intensity of about 30% of that of the ^{18}O-^{13}C peak, confirming the mass spectral results for ^{18}O incorporation.

Mass spectrum of [3-^{13}C]DAOC (2c) (ESMS) :

	m/z (MH^+)	*358*	*359*	*360*	*361*	*362*
Experiment 1	Found (%)	12	100	20	9	3
	m/z (MNa^+)	*380*	*381*	*382*	*383*	*384*
Experiment 2	Found (%)	20	100	21	15	4

Mass spectrum of [3-^{13}C,4-^{2}H]-3-β-Hydroxycepham (40d) (ESMS) :

	m/z (MH^+)	*377*	*378*	*379*	*380*	*381*	*382*
Experiment 1	Found (%)	9	42	29	100	21	9
	m/z (MNa^+)	*399*	*400*	*401*	*402*	*403*	*404*
Experiment 2	Found (%)	11	47	31	100	20	9

Mass spectrum of [3-^{13}C]DAC (3c) (ESMS) :

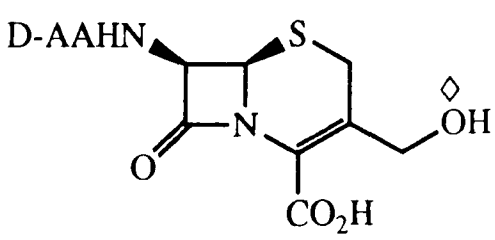
	m/z (MH^+)	*374*	*375*	*376*	*377*	*378*	*379*
Experiment 1	Found (%)	5	90	34	100	25	13
Experiment 2	Found (%)	10	74	21	100	20	15

^{13}C-NMR analysis (125.8 MHz, D_2O) of [3-^{13}C-4,^{2}H]-3β-Hydroxycepham (40d) and of the lactone (125b) :

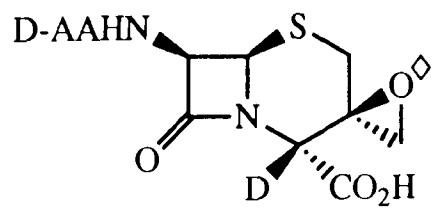
(40d)	$^{13}\underline{C}$-^{16}OH	$^{13}\underline{C}$-^{18}OH	Δδ
δ(ppm)	65.45(7)	65.42(7)	0.03(0)

(125b)	$^{13}\underline{C}$-^{16}OH	$^{13}\underline{C}$-^{18}OH	Δδ
δ(ppm)	81.37(3)	81.41(6)	0.04(3)

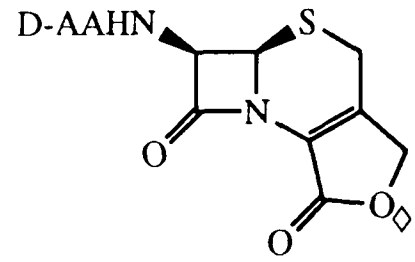
Incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) with DAOC/DACS under $^{18}O_2$



(3d)



(122a)



(120a)

◊ = ^{18}O or ^{16}O

DAOC/DACS (2 ml, 1.3 IU) in TRIS-HCl buffer (50 mM, pH 7.4) was exchanged into ammonium hydrogen carbonate buffer (7 ml, 10 mM, pH 7.8, 2 mM DTT) on a pre-

equilibrated Sephadex column (PD-10), and this solution was rapidly evacuated and flushed with argon. The head space of an intact glass vial of $^{18}O_2$ (MSD Isotopes, 98 atom % ^{18}O, 100 ml) was repeatedly evacuated and flushed with argon, and then septum sealed. To a solution of [4-^{2}H]-3-exomethylene cephalosporin C (63a) (8 mg) in water (1 ml) was added the cofactor solution (800 µl), and the resulting solution was also repeatedly evacuated and flushed with argon. After breaking the glass neck seal of the septum sealed $^{18}O_2$ vial, the enzyme was introduced *via* syringe, followed by the solution of the substrate and cofactors. The sealed vial was incubated for 2h at 27°C and 250 rpm. Acetone was added *via* syringe to a final concentration of 70% (v/v), the vial opened to air and the incubation worked up according to the general procedure. Examination by ^{1}H-NMR showed *ca.* 4% conversion to DAC (3d) and the epoxide (122a). Integration of the β-lactam region revealed the ratio of products to be (3d):(122a) = 2.5:1. Purification of the crude incubation mixture by HPLC (Gilson system, solvent 10 mM aqueous NH_4HCO_3, flow rate 2 ml/min) gave a mixture of DAC (3d) (*ca.* 210 µg by NMR calibration) and epoxide (122a) (*ca.* 80 µg by NMR calibration) coeluting as one peak (retention time 9.7 min). This mixture was treated with formic acid as previously described (section 6.2.3) to give after HPLC purification the DAC lactone (120a) (*ca.* 105 µg by ^{1}H-NMR analysis) and the epoxide (122a) (*ca.* 60 µg by NMR calibration). Mass spectral analysis of the two products showed 46% incorporation of ^{18}O into the DAC lactone (120a) and 94% incorporation into the epoxide (122a).[235]

This experiment was repeated with [4-^{2}H]-3-exomethylene cephalosporin C (63a) (8 mg), cofactor solution (700 µl), and DAOC/DACS (5 ml, 1.2 IU) (the enzyme was used in the TRIS-HCl buffer). ^{1}H-NMR analysis of the crude incubation mixture showed *ca.* 10% conversion to (3d) and (122a) [ratio (3d):(122a) = 2:1]. Purification by HPLC gave a mixture of (3d) (*ca.* 442 µg by NMR calibration) and (122a) (*ca.* 244 µg by NMR calibration) coeluting as one peak. This mixture was treated with formic acid to give after HPLC purification the DAC lactone (120a) (*ca.* 228 µg by ^{1}H-NMR calibration) and the epoxide (122a) (*ca.* 134 µg by ^{1}H-NMR calibration). Mass spectral analysis of the two

products showed 56% incorporation of ^{18}O into the lactone (120a) and 95% into the epoxide (122a).[235]

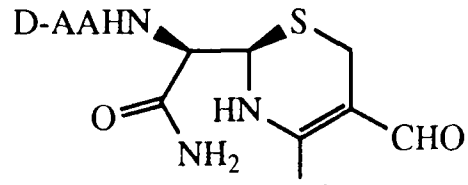
Mass spectrum of DAC-lactone (120a) (ESMS) :

	m/z (MH^+)	*356*	*357*	*358*	*359*	*360*	*361*
Experiment 1	Found (%)	100	24	88	22	7	2
Experiment 2	Found (%)	77	22	100	19	7	1

Mass spectrum of the epoxide (122a) (ESMS) :

	m/z (MH^+)	*375*	*376*	*377*	*378*	*379*	*380*
Experiment 1	Found (%)	9	12	100	19	8	2
Experiment 2	Found (%)	7	9	100	20	8	2

Incubation of ^{18}O-labelled epoxide cepham (122a) with DAOC/DACS



The ^{18}O-labelled epoxide (122a) (*ca.* 200 μg, 94% ^{18}O label) obtained on the incubations of [4-^{2}H]-3-exomethylene cephalosporin C (63a) under $^{18}O_2$ was incubated with DAOC/DACS (0.5 ml) according to the general procedure. ^{1}H-NMR analysis showed complete conversion to aldehyde products. Purification by HPLC (Waters system, solvent 0.1% HCO_2H in water, flow rate 2 ml/min, monitoring at λ 300 nm) gave the aldehyde cephalosporoate (78) (retention time 6.6 min). Mass spectral analysis showed practically complete loss of ^{18}O label (only 14% ^{18}O-label left).

Mass spectrum of aldehyde (78) (ESMS) :

m/z (MH^+)	*388*	*389*	*390*	*391*	*392*
Found (%)	9	100	19	24	11

6.3.3 Experimental for Section 3.4

Incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) with DAOC/DACS in $H_2{}^{18}O$

DAOC/DACS (2 ml) was concentrated down to 0.5 ml on a centrifuge, using a Centricon 10 microconcentrator (6 Krpm, 25 min, 0°C), and 0.5 ml of $H_2{}^{18}O$ (Aldrich Chemical Co., 95 atom % ^{18}O) was added. A cofactor solution (300 µl) that had previously been lyophilised was dissolved in 0.5 ml $H_2{}^{18}O$ and added to the enzyme solution, which was then pre-incubated for 5 min at 27°C and 250 rpm, after which [2-^{13}C,3-^{2}H]penicillin N (1f) (2 mg) was dissolved in 1 ml $H_2{}^{18}O$ and was added. The resulting solution was incubated for 2h at 27°C and 250 rpm, after which time the protein was precipitated by the addition of acetone to 70% (v/v), and the incubation worked up as in the general procedure. Analysis by ^{1}H-NMR showed *ca.* 59% conversion to (2c), (40d) and (3c) [ratio (2c):(40d):(3c) = 21:44:35]. Purification by HPLC (Gilson system, solvent 10 mM aqueous NH_4HCO_3, flow rate 4 ml/min) gave [3-^{13}C]DAC (3c) (retention time 5 min, *ca.* 174 µg by NMR calibration), [3-^{13}C,4-^{2}H]-3β-hydroxycepham (40d) (retention time 6.3 min, *ca.* 402 µg by NMR calibration) and [3-^{13}C]DAOC (2c) (retention time 13 min, *ca.* 354 µg by NMR calibration). Mass spectral analysis of the products indicated no ^{18}O incorporation into (2c), 16% incorporation of ^{18}O into (40d) and 50% incorporation into (3c). Considering the $H_2{}^{16}O$ dilution, the ^{18}O enrichment of the incubation solution was 76% which means the corrected ^{18}O incorporation into (40d) is 21%, and into (3c) 66%. Analysis of (40d) by ^{13}C-NMR (overnight acquisition, 29,437 transients) showed a peak for the ^{18}O-$^{13}\underline{C}$ resonance with an intensity of about 17% of the peak for the ^{16}O-$^{13}\underline{C}$ resonance, in accordance with mass spectral results.

Mass spectrum of [3-^{13}C]DAOC (2c) (ESMS) :

m/z (MH^+)	*358*	*359*	*360*	*361*	*362*
Found (%)	14	100	22	9	2

Mass spectrum of [3-^{13}C,4-^{2}H]-3β-Hydroxycepham (40d) (ESMS) :

m/z (MH^+)	*377*	*378*	*379*	*380*	*381*	*382*
Found (%)	18	100	23	26	7	3

Mass spectrum of [3-^{13}C]DAC (3c) (ESMS) :

m/z (MH^+)	*374*	*375*	*376*	*377*	*378*	*379*
Found (%)	17	92	37	100	21	10

^{13}C-NMR analysis (125.8 MHz, D_2O) of [3-^{13}C,4-^{2}H]-3-β-Hydroxycepham (40d) :

(40d)	$^{13}\underline{C}$-^{16}OH	$^{13}\underline{C}$-^{18}OH	Δδ
δ(ppm)	65.46(1)	65.43(4)	0.03

Control incubations of (2c) and (40d) with DAOC/DACS

The products (2c) (*ca.* 100 μg) and (40c) (*ca.* 400 μg) obtained on the incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) in $H_2{}^{18}O$ were independently incubated with denatured DAOC/DACS (1 ml) (pre-inactivated by heat) and cofactors according to the general procedure. ^{1}H-NMR analysis of the crude incubation mixtures showed the two products were still intact, and these were isolated using the usual HPLC system. Analysis by electrospray mass spectrometry of the recovered (2c) and (40d) showed no loss of label had occurred to within experimental error.

Mass spectrum of [3-^{13}C,4-^{2}H]-3β-Hydroxycepham (40d) (ESMS) :

	m/z (MH^+)	*377*	*378*	*379*	*380*	*381*	*382*
Before control inc.	Found (%)	18	100	23	26	7	3
After control inc.	Found (%)	21	100	31	34	8	2

Mass spectrum of [3-^{13}C]DAC (3c) (ESMS) :

	m/z (MH^+)	*374*	*375*	*376*	*377*	*378*	*379*
Before control inc.	Found (%)	17	92	37	100	21	10
After control inc.	Found (%)	22	80	33	100	17	15

Incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) with DAOC/DACS in $H_2{}^{18}O$

DAOC/DACS (1.5 ml, 0.3 IU) was concentrated down to 0.5 ml as described for the incubation of [2-^{13}C,3-^{2}H]penicillin N (1f) in $H_2{}^{18}O$. Exactly the same procedure was followed using [4-^{2}H]-3-exomethylene cephalosporin C (63a) (2.7 mg) and lyophilised cofactor solution (400 µl). Analysis by ^{1}H-NMR showed 18% conversion to DAC (3d) and the epoxide (122a) [ratio (3d):(122a) = 3:1]. Isolation of the products as described before gave DAC lactone (120a) (*ca.* 96 µg by ^{1}H-NMR calibration) and the epoxide (122a) (*ca.* 25 µg by ^{1}H-NMR calibration). Mass spectral analysis of the products showed 30% ^{18}O incorporation into (120a) and 13% incorporation into the epoxide (122a). Considering the $H_2{}^{16}O$ dilution, the ^{18}O enrichment of the incubation solution was 76%, which means the corrected ^{18}O incorporation into (120a) is 40%, and into (122a) 17%.

Mass spectrum of DAC-lactone (120a) (ESMS) :

m/z (MH^+)	*356*	*357*	*358*	*359*	*360*	*361*
Found (%)	100	25	50	11	4	1

Mass spectrum of epoxide (122a) (ESMS) :

m/z (MH^+)	*375*	*376*	*377*	*378*	*379*	*380*
Found (%)	100	20	22	5	2	1

Control incubation of (122) with DAOC/DACS

The unlabelled epoxide (122) [*ca.* 200 µg; obtained from an incubation of [4-^{2}H]-3-exomethylene cephalosporin C (63a) under standard conditions] was dissolved in $H_2{}^{18}O$ (0.5 ml), and a previously lyophilised cofactor solution (200 µl) was also dissolved in $H_2{}^{18}O$ (0.5 ml). These two solutions were added to heat-denatured DAOC/DACS (250 µl) and the incubation performed as usual. ^{1}H-NMR analysis of the crude incubation mixture showed no conversion of the epoxide (122) which was recovered by HPLC purification

(Waters system, solvent 10 mM aqueous NH_4HCO_3, flow rate 1 ml/min). Analysis by mass spectrometry showed no incorporation of ^{18}O-label into (122) had occurred.

Mass spectrum of epoxide (122) (ESMS) :

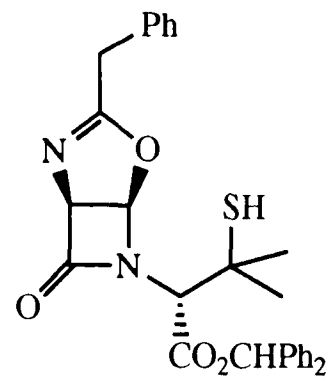
	m/z (MH^+)	*375*	*376*	*377*	*378*	*379*	*380*
Before control inc.	Found (%)	100	20	8	2	–	–
After control inc.	Found (%)	100	16	11	3	1	–

6.4 Experimental for Chapter 4

6.4.1 Experimental for Section 4.2

(2S)-2-[(1S,5R)-3-Benzyl-7-oxo-4-oxa-2,6-diazabicyclo[3.2.0]hept-2-en-6-yl]-3-mercapto-3-methyl-butanoic acid benzhydryl ester (159b)

$C_{29}H_{28}N_2O_4S$; MW 500



Penicillin G potassium salt (10a) (1.86 g, 5 mmol) was added to a stirred solution of mercury(II) acetate (3.18 g, 10 mmol, 2 eq) in glacial acetic acid (80 ml) at room temperature.[249,250] A gel rapidly formed and the solution was diluted with acetone (80 ml). The insoluble material was filtered and washed with acetone followed by ether to give the dimercury salt (158) (2.72 g, 3.2 mmol, 64% yield). The dimercury salt (158) (1.80 g, 2.1 mmol) was suspended in DCM (50 ml) and hydrogen sulphide bubbled through.[251] The black precipitate was filtered off using celite, and the filtrate was washed with 1N HCl (20 ml), H_2O (20 ml), brine (20 ml) and dried (anhydrous Na_2SO_4). To the filtered solution was added dropwise, over one hour, a solution of diphenyldiazomethane (0.61 g, 3.15 mmol, 1.5 eq) in MeCN (10 ml) until a light pink colour persisted. The solution was evaporated *in vacuo* and purification of the crude product by chromatography [flash silica, EtOAc/petrol (30% to 50%, v/v)] gave (159b) [0.80 g, 1.6 mmol, 76% yield from (158)].

T.l.c. [EtOAc/DCM (10%, v/v)] Rf 0.60

ν_{max} ($CHCl_3$) : 1781 (s, β-lactam C=O), 1740 (s, ester C=O), 1650 (m, C=N), 1196 (s), 785 (s), 749 (m), 730 (m), 700 (m)

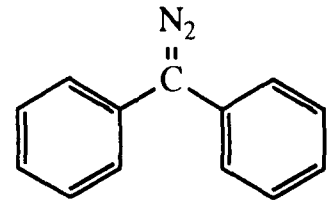
δ_H (200 MHz, $CDCl_3$) : 1.11 (3H, s, C<u>H</u>$_3$), 1.26 (3H, s, C<u>H</u>$_3$), 1.89 (1H, s, S<u>H</u>), 3.60 and 3.72 (2 × 1H, ABq, J 15 Hz, C<u>H</u>$_2$Ph), 4.55 (1H, s, C<u>H</u>CO_2BzH), 5.09 and 6.06 (2 × 1H, 2 × d, J 3 Hz, NC<u>H</u>C<u>H</u>O), 6.90 (1H, s, C<u>H</u>Ph_2), 7.24-7.44 (15H, m, aromatic C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 29.58 (q, $\underline{C}H_3$), 29.91 (q, $\underline{C}H_3$), 35.74 (t, $\underline{C}H_2Ph$), 44.61 (s, $\underline{C}Me_2$), 64.62 (d, $\underline{C}HCMe_2$), 78.94 (d, $\underline{C}HPh_2$), 81.70 and 87.34 (2 × d, N$\underline{C}H\underline{C}$HO), 127.05, 127.50, 128.41, 128.68, 128.81 and 129.40 (6 × d, aromatic $\underline{C}$H), 133.61, 139.01 and 139.13 (3 × s, aromatic C1), 166.95, 167.80 and 169.34 (3 × s, 2 × $\underline{C}$=O and $\underline{C}$=N)

m/z (FAB) : 501 ([MH^+], 100%)

Diphenyldiazomethane[277]

$C_{13}H_{10}N_2$; MW 194

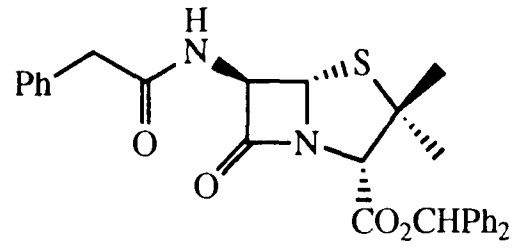


To a stirred suspension of benzophenone hydrazone (26 g, 133 mmol), anhydrous Na_2SO_4 (30 g, 211 mmol, 1.6 eq) and mercury oxide (70 g, 323 mmol, 2.4 eq) in Et_2O (400 ml) was added a saturated solution of KOH in EtOH (10 ml). After stirring for 90 min, the suspension was filtered and the solvent evaporated *in vacuo* . The residue was dissolved in the minimum amount of 30-40°C petrol, filtered to remove insoluble hydrazone, and solvent evaporated *in vacuo* to yield the title compound as an oil which crystallized upon cooling (23 g, 90% yield).

δ_H (200 MHz, $CDCl_3$) : 7.21-7.49 (10H, m, 2 × Ph)

(2$\underline{S}$,5$\underline{S}$,6$\underline{R}$)-1-Aza-3,3-dimethyl-6-phenylacetamido-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (160b)

$C_{29}H_{28}N_2O_4S$; MW 500



To a solution of (159b) (1.45 g, 2.9 mmol) in benzene (100 ml) was added zinc acetate dihydrate (80 mg, 0.44 mmol, 0.15 eq).[253] The reaction mixture was refluxed for 3.5 h, after which time there was no evidence of starting material by T.l.c.. The solvent was evaporated *in vacuo* and the residue redissolved in EtOAc (50 ml). This was washed with

sat. $NaHCO_3$ (20 ml), H_2O (20 ml), brine (20 ml), dried (Na_2SO_4) and the solvent evaporated. Chromatography [flash silica, EtOAc/Petrol (30%, v/v)] gave (160b) (600 mg, 1.2 mmol, 41% yield).

T.l.c. [EtOAc/DCM (10%, v/v)] Rf 0.69

M.p.= 143-144°C (from EtOAc/petrol)

Found : C 69.42%, H 5.59%, N 5.99%. $C_{29}H_{28}N_2O_4S$ requires C 69.58%, H 5.64%, N 5.60%

ν_{max} (KBr) : 1780 (s, β-lactam C=O), 1750 (s, ester C=O), 1672 (s, amide C=O), 1525 (m), 1173 (s), 1159 (s), 741 (m), 705 (m), 695 (m)

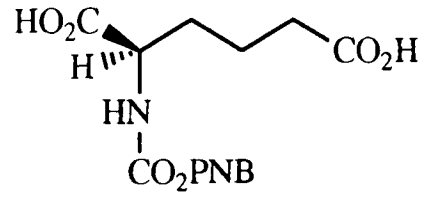
δ_H (500 MHz, $CDCl_3$) : 1.20 (3H, s, C<u>H</u>$_3$), 1.58 (3H, s, C<u>H</u>$_3$), 3.62 (2H, s, C<u>H</u>$_2$Ph), 3.84 (1H, s, C<u>H</u>CO_2BzH), 4.86 (1H, dd, J 2 and 7 Hz, NHC<u>H</u>CHS), 5.05 (1H, d, J 2 Hz, NHCHC<u>H</u>S), 5.98 (1H, d, J 7 Hz, N<u>H</u>), 6.96 (1H, s, C<u>H</u>Ph_2), 7.22-7.38 (15H, m, aromatic C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 25.07 (q, <u>C</u>H_3), 30.76 (q, <u>C</u>H_3), 43.28 (t, <u>C</u>H_2Ph), 64.12, 67.48 and 70.59 (3 × d, NH<u>C</u>H<u>C</u>HS and <u>C</u>HCMe_2), 65.28 (s, <u>C</u>Me_2), 79.14 (d, <u>C</u>HPh_2), 127.12, 127.61, 128.03, 128.44, 128.52, 129.15 and 129.48 (7 × d, aromatic <u>C</u>H), 134.03 and 139.17 (2 × s, aromatic C1), 165.68, 167.32 and 171.02 (3 × s, 3 × <u>C</u>=O)

m/z (FAB) : 501 ([MH^+], 100%); (DCI, NH_3) : 492 (30%), 352 (17%), 326 (53%), 176 (67%), 167 (100%), 91 (10%)

<u>N</u>-*p*-Nitrobenzyloxycarbonyl-<u>D</u>-α-aminoadipic acid (169)

$C_{14}H_{16}N_2O_8$; MW 340



<u>D</u>-α-aminoadipic acid (2.00 g, 12.4 mmol) was dissolved in a sat. $NaHCO_3$ solution (80 ml). *p*-Nitrobenzylchloroformate (3.16 g, 14.6 mmol, 1.2 eq) in dioxan (40 ml) was added dropwise at 0°C, maintaining the pH between 9 and 10 by the addition of 2N NaOH. The reaction mixture was allowed to warm up to room temperature, and was left stirring overnight, after which it was neutralised by the addition of conc. HCl, and washed with

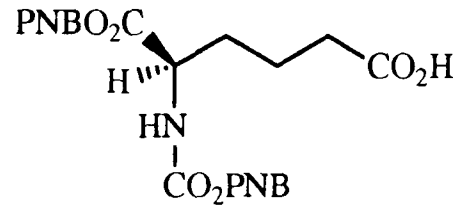
EtOAc (2 × 80 ml). The aqueous layer was acidified to pH 2 with 2N HCl, and extracted with EtOAc (3 × 60 ml). The combined last three organic layers were washed with water, dried (Na_2SO_4), and evaporated *in vacuo* to give (169) (4.22 g, 12.4 mmol, 100% yield).

δ_H (200 MHz, $(CD_3)_2CO$) : 1.69-2.35 (6H, m, $(C\underline{H}_2)_3$), 4.21-4.28 (1H, m, $CH_2C\underline{H}$), 5.23 (2H, s, $C\underline{H}_2Ar$), 6.75 (1H, d, N<u>H</u>), 7.55-7.65 and 8.13-8.25 (2 × 2H, m, aromatic C<u>H</u>)

m/z (FAB) : 341 ([MH^+], 40%), 136 (45%), 115 (100%)

<u>N</u>-*p*-Nitrobenzyloxycarbonyl-<u>D</u>-α-aminoadipic acid-α-*p*-nitrobenzyl ester (105)

$C_{21}H_{21}N_3O_{10}$; MW 475



The acid (169) (4.22 g, 12.4 mmol) was dissolved in dry DMF (100 ml) under argon, and dry triethylamine (1.74 ml, 12.4 mmol, 1 eq) was added. After addition of *p*-nitrobenzylbromide (2.69 g, 12.4 mmol, 1 eq) in dry DMF (15 ml), the resulting solution was stirred overnight under argon at 35°C. EtOAc (300 ml) was added and the white precipitate filtered off. The filtrate was washed with water (3 x 100 ml), dried (Na_2SO_4) and evaporated *in vacuo* to a yellow oil. This was purified by chromatography [flash silica, $MeOH/CHCl_3$ (1% to 3%, v/v)] to give (105) (4.5 g, 9.5 mmol, 76% yield).

T.l.c. [$MeOH/CHCl_3$ (10%, v/v)] Rf 0.31

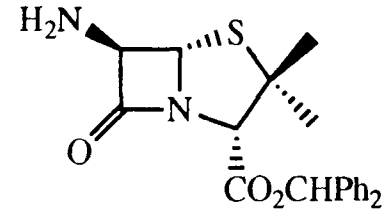
δ_H (200 MHz, $CDCl_3$) : 1.60-2.00 (4H, m, $CH(C\underline{H}_2)_2$), 2.34-2.40 (2H, m, $CH(CH_2)_2C\underline{H}_2$), 4.40-4.52 (1H, m, $NHC\underline{H}CH_2$), 5.20-5.30 (4H, m, 2 × $C\underline{H}_2Ar$), 5.60 (1H, d, N<u>H</u>), 7.40-7.55 and 8.10-8.22 (2 × 4H, m, aromatic C<u>H</u>)

m/z (FAB) : 498 ([MNa^+], 100%), 520 ([Na salt + Na^+], 50%)

• PCl_5 reaction for cleavage of the side chain

(2S,5S,6R)-1-Aza-3,3-dimethyl-6-amino-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (167a)

$C_{21}H_{22}N_2O_3S$; MW 382



a) Reaction of (160b) with Isobutanol

To a solution of (160b) (0.20 g, 0.4 mmol) and dry N, N-dimethylaniline (143 ml, 1.13 mmol, 2.8 eq) in dry DCM (2 ml) stirred at -78°C under argon, was quickly added phosphorus pentachloride (109 mg, 0.52 mmol, 1.3 eq). The resulting suspension was stirred for 5 min at -78°C, warmed to -40°C and stirred at that temperature for one hour. After re-cooling to -78°C, isobutanol (750 ml) was added dropwise and the solution was left to warm to 0°C (*ca.* 1h). After 40 min at 0°C the reaction was quenched by addition of H_2O (10 ml). The pH was adjusted to 10 with 2N NaOH, and the resulting milky suspension was extracted with EtOAc (3 × 30 ml). The combined organic layers were dried (Na_2SO_4) and evaporated *in vacuo* to a yellow oil (550 mg). Attempted purification by chromatography [flash silica, EtOAc/DCM (20% to 100%, v/v)] gave no pure product. The fractions which seemed to contain some amine were evaporated *in vacuo* (170 mg) and purified by P.l.c. (SiO_2, EtOAc). The amine (167a) (18 mg, 0.05 mmol, 12% yield) was obtained as an oil.

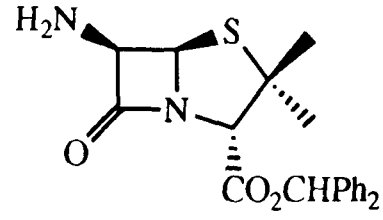
T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.35

δ_H (200 MHz, $CDCl_3$) : 1.20 (3H, s, C<u>H</u>$_3$), 1.62 (3H, s, C<u>H</u>$_3$), 3.82 (1H, s, C<u>H</u>CO_2BzH), 4.34 and 4.87 (2 × 1H, 2 × d, J 2 Hz, H_2NC<u>H</u>C<u>H</u>S), 6.98 (1H, s, C<u>H</u>Ph_2), 7.22-7.46 (10H, m, aromatic C<u>H</u>)

m/z [DCI (NH_3)] : 400 ([MNH_4^+], 4%), 383 ([MH^+], 13%), 355 (10%), 326 (37%), 167 (100%)

b) Reaction of (170a) with *n*-Butanol

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-amino-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (173a)



The same procedure as in a) was followed using (170a) (400 mg, 0.8 mmol) and equivalent amounts of the other reagents, but with *n*-butanol instead of isobutanol. The reaction was quenched by pouring the solution into H_2O (20 ml). The pH was adjusted to 8 with 2N NaOH and the mixture extracted with EtOAc. The aqueous phase was separated, the pH adjusted to 10 and re-extracted with EtOAc. No compound was extracted into the organic phase this time. The combined organic layers from the first extraction were dried (Na_2SO_4), and evaporated *in vacuo* to a yellow oil (900 mg). This was purified by chromatography [flash silica, EtOAc/DCM (10% to 30%, v/v)] but the product was still not pure. Fractions containing the amine were evaporated (122 mg) and purified again by P.l.c. [SiO_2, EtOAc/DCM (30%,v/v)] to give (173a) (30 mg, 0.08 mmol, 10% yield).

T.l.c. [EtOAc/DCM (20%,v/v)] Rf 0.35

δ_H (200 MHz, $CDCl_3$) : 1.27 (3H, s, C<u>H</u>$_3$), 1.62 (1H, s, C<u>H</u>$_3$), 4.51 (1H, s, C<u>H</u>CMe$_2$), 4.56 and 5.53 (2 × 1H, 2 × d, J 4 Hz, H_2NC<u>H</u>C<u>H</u>S), 6.95 (1H, s, C<u>H</u>Ph$_2$), 7.25-7.40 (10H, m, aromatic C<u>H</u>)

c) Reaction of (170a) with *n*-butanol (longer hydrolysis time)

The same procedure as in b) was followed. When the reaction mixture was poured in water (30 ml) it was left stirring, and 10 ml aliquots were taken at 5, 15 and 30 min. Each aliquot was added to a 1N $NaHCO_3$ solution (20 ml) and the pH adjusted to 8 with more 1N $NaHCO_3$. All three milky suspensions were extracted with EtOAc, the organic phases dried (Na_2SO_4) and evaporated *in vacuo*. There was no diference between the three cases by ^{1}H-NMR or T.l.c. (which showed amine quite clean as the major product), so they were

purified together. Chromatography [flash silica,EtOAc/DCM (20% , v/v)] gave (173a) (90 mg, 0.24 mmol, 30% yield).

d) Reaction of (160b) with *n*-butanol

The same procedure as in b) was followed using (160b) (200 mg, 0.4 mmol) and the equivalent amounts of the other reagents. Reaction mixture was quenched by pouring it into H_2O (10 ml). After stirring for 15 min 1N $NaHCO_3$ (10 ml) was added. The pH was adjusted to 8 (1N $NaHCO_3$) and the mixture extracted with EtOAc (3 x 20 ml). The combined organic layers were dried (Na_2SO_4) and T.l.c. showed amine was present as the major product by comparison with an authentic sample. Evaporation *in vacuo* gave an oil (600 mg) which was purified [flash silica, EtOAc/DCM (30%,v/v)]. NMR of the fractions which seemed to contain the amine (by T.l.c.) showed major compound present was not the amine. Closer examination by T.l.c. (EtOAc/DCM, 30% v/v, double elution) revealed major compound has having a higher Rf (0.57) than the amine (0.43). This mixture was dissolved in EtOAc and washed with 1N HCl. T.l.c. of the dried organic phase showed no amine, but the major compound was still present. Evaporation of the solvent *in vacuo* gave the phosphoramide (174) (85 mg, 0.15 mmol, 38% yield).

T.l.c. [EtOAc/DCM (40%,v/v)] Rf 0.43

δ_H (200 MHz, $CDCl_3$) : 0.86-0.98, (6H, m, 2 × $CH_2CH_2C\underline{H}_3$), 1.18 (3H, s, $C\underline{H}_3$), 1.30-1.75 (8H, m, 2 × $CH_2C\underline{H}_2C\underline{H}_2CH_3$), 1.64 (3H, s, $C\underline{H}_3$), 3.82 (1H, s, $C\underline{H}CMe_2$), 3.92-4.10 (4H, m, 2 × $OC\underline{H}_2CH_2$), 4.52 (1H, ddd, J 12 Hz, 11 Hz, 2 Hz, NHC$\underline{H}$), 4.98 (1H, d, J 2 Hz, NHCHC$\underline{H}$), 6.97 (1H, s, $C\underline{H}Ph_2$), 7.20-7.40 (10H, m, aromatic C$\underline{H}$)

m/z [DCI (NH_3)] : 575 (MH^+, 6%), 547 (23%), 326 (14%), 167 (100%)

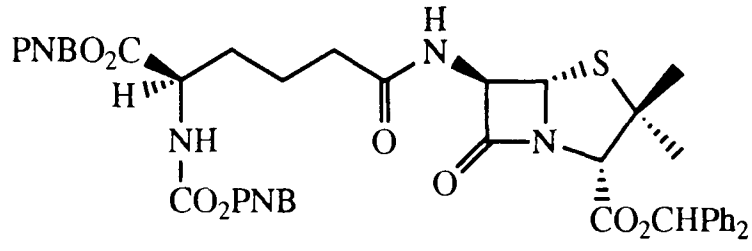
e) Reaction of (160b) adding TsOH

The same procedure as in d) was followed using (160b) (200 mg, 0.4 mmol). To the EtOAc solution obtained on work-up was added *p*-toluenesulfonic acid monohydrate (289 mg, 1.52 mmol, 3.8 eq). The resulting solution was evaporated under high vacuum at 0°C

to give an oil (0.93 g). This was purified by chromatography [flash silica, EtOAc/DCM (20%,v/v) to neat EtOAc to MeOH/EtOAc (35%, v/v)]. The fractions where the tosic salts of N,N-dimethylaniline and (167a) were present, were evaporated and to the residue added EtOAc (25 ml) and 1N $NaHCO_3$ (25 ml). The aqueous phase was extracted with EtOAc (2 x 20 ml) and the combined organic layers dried (Na_2SO_4) and evaporated to an oil. This was purified once again by chromatography [flash silica, EtOAc/DCM (20% to 40%, v/v)] to give (167a) (84 mg, 0.22 mmol, 55% yield).

(2S,5S,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitro benzyloxycarbonylpentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (168a)

$C_{42}H_{41}N_5O_{12}S$; MW 839



To a solution of (167a) (44 mg, 0.115 mmol) in dry DCM (2 ml) was added EEDQ[220] (31 mg, 0.13 mmol, 1.1 eq), N-*p*-nitrobenzyloxycarbonyl-D-α-aminoadipic acid-α-*p*-nitrobenzyl ester (105) (55 mg, 0.115 mmol, 1 eq) and anhydrous Na_2SO_4 (5-10 mg). The reaction mixture 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 (20 ml), 2N HCl (20 ml), sat. $NaHCO_3$ (20 ml), brine (20 ml), dried (Na_2SO_4) and evaporated to an oil (122 mg). This was purified by P.l.c. [silica, EtOAc/DCM (35%, v/v)] to give (168a) (71 mg, 0.085 mmol, 74% yield).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.63

$\nu_{max}(CHCl_3)$: 1785 (s, β-lactam C=O), 1732 (s, ester C=O), 1605(m), 1525 (s, NO_2), 1349 (s, NO_2), 1240 (s), 1063 (m), 854 (m)

δ_H (200 MHz, $CDCl_3$) : 1.19 (3H, s, CH_3), 1.60 (3H, s, CH_3), 1.62-2.05 (4H, m, $CHCH_2CH_2CH_2$), 2.12-2.36 (2H, m, CH_2CH_2CO), 3.90 (1H, s, $CHCO_2BzH$), 4.30-4.47 (1H, m, $CHCH_2CH_2$), 4.90 (1H, dd, J 7 Hz and 2 Hz, NHCHCHS), 5.10 (1H, d, J 2 Hz, NHCHCHS), 5.19 and 5.27 (2 × 2H, 2 × s, CO_2CH_2Ar), 5.86 (1H, d, J 8 Hz, NH), 6.77 (1H,

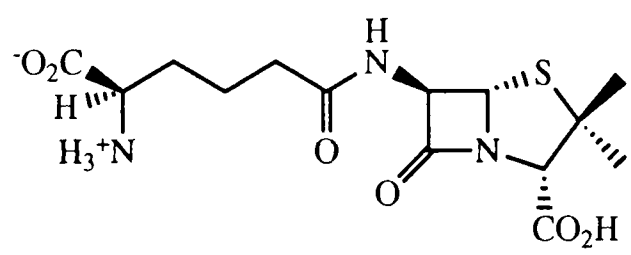
d, J 7 Hz, NH), 6.98 (1H, s, CHPh$_2$), 7.22-7.58 (14H, m, CHPh$_2$ and ArNO$_2$), 8.12-8.30 (4H, m, ArNO$_2$)

δ_C (125.8 MHz, $CDCl_3$) : 20.84 (t, $CHCH_2CH_2$), 24.81 (q, CH_3), 30.91 (t, $CHCH_2CH_2$), 31.31 (q, CH_3), 34.51 (t, CH_2CO), 53.80 (d, $CHCH_2CH_2$), 63.54, 67.57 and 70.29 (3 × d, NHCHCHS and $CHCMe_2$), 65.44 and 65.68 (2 × t, 2 × CH_2Ar), 67.32 (s, CMe_2), 79.04 (d, $CHPh_2$), 123.70, 123.83, 126.84, 127.98, 128.04, 128.44 and 128.52 (7 × d, aromatic CH), 138.99 (s, aromatic C1 of Ph), 142.37 and 143.61 (2 × s, aromatic C1 of $ArNO_2$), 147.50 and 147.81 (2 × s, aromatic C4 of $ArNO_2$), 155.70 (s, $NHCO_2Ar$), 165.78, 168.13, 171.78 and 172.60 (4 × s, 4 x C=O)

m/z (FAB) : 862 ([MNa^+], 100%), 846 (25%), 840 ([MH^+], 23%)

(2S,5S,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (154)

$C_{14}H_{21}N_3O_6S$; MW 359



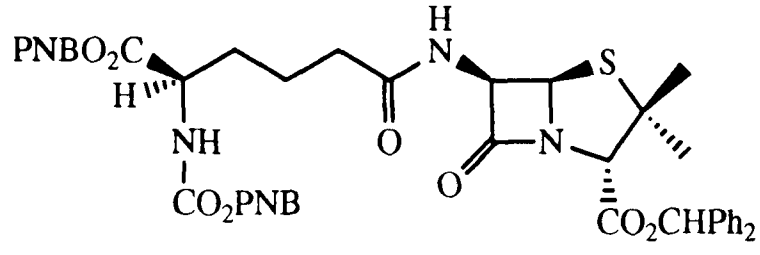
To a solution of (168a) (33 mg, 0.04 mmol) in THF/water [5 ml, 50% (v/v)], was added $NaHCO_3$ (3.5 mg, 0.04 mmol, 1 eq) and Pd/C 10% (40 mg). The reaction mixture was evacuated and purged with hydrogen five times, and then stirred vigorously under one atmosphere of hydrogen for 4 h. After this time it was filtered through a pad of celite, washed with water, the THF removed under vacuum and the filtrate extracted with EtOAc (2 x 20 ml). The aqueous layer was lyophilised, and the crude product was purified by chromatography [HPLC, Waters system, solvent 2.5% MeOH in 10 mM aqueous NH_4HCO_3, flow rate 2 ml/min] to give (154) (retention time 14 min, *ca.* 290 µg by NMR calibration, 2% yield).

δ_H (500 MHz, D_2O, HOD suppressed) : 1.46 (3H, s, CH_3), 1.63 (3H, s, CH_3), 1.60-1.85 (4H, m, $CHCH_2CH_2CH_2$), 2.37 (2H, t, J 6 Hz, CH_2CH_2CO), 3.52-3.60 (1H, m, $H_3N^+CHCO_2^-$), 3.69 (1H, s, $CHCMe_2$), 4.83 and 5.25 (2 × 1H, 2 × d, J 1.6 Hz, NHCHCHS)

m/z (FAB) : 360 ([MH^+], 100%)

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-N-*p*-nitrobenzyloxycarbonylamino-5-*p*-nitro benzyloxycarbonylpentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (176)

$C_{36}H_{36}N_6O_{14}S$; MW 808



The tosylate salt of 6-aminopenicillanic acid *p*-nitrobenzyl ester (1.20 g, 2.1mmol) was taken up in sat. $NaHCO_3$ (20 ml) and extracted with EtOAc (3 × 25 ml). The combined organic layers were dried (Na_2SO_4) and the solvent evaporated to give the free amine (173b) (0.85 g, 2.1 mmol). The amine was dissolved in dry DCM (3 ml) and added to a solution of N-*p*-nitrobenzyloxycarbonyl-D-α-aminoadipic acid-α-*p*-nitrobenzyl ester (105) (1.00 g, 2.1 mmol), EEDQ[220] (0.57 g, 2.32 mmol, 1.1 eq) and Na_2SO_4 (*ca.* 20 mg) in dry DCM (3 ml) under argon, *via* syringe. The resulting solution was stirred under argon at room temperature for 24h, then evaporated to dryness and the residue partitioned between water (20 ml) and EtOAc (20 ml). The organic layer was separated, washed with 2N HCl (15 ml), sat. $NaHCO_3$ (15 ml), brine (15 ml), dried (Na_2SO_4) and evaporated to a white foam (2.2 g). This was purified by chromatography [flash silica, EtOAc/DCM (15% to 40%, v/v)] to give (176) (1.25 g, 1.55 mmol, 74% yield).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.56

$\nu_{max}(CHCl_3)$: 1789 (s, β-lactam C=O), 1747 (s, ester C=O), 1692 (s, amide C=O), 1609 (m), 1536 (s, NO_2), 1458 (m), 1349 (s, NO_2), 1156 (m), 1067 (m), 860 (m)

δ_H (500 MHz, $CDCl_3$) : 1.44 (3H, s, C$\underline{H}_3$), 1.63 (3H, s, C$\underline{H}_3$), 1.60-2.00 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$CH$_2$), 2.20-2.35 (2H, m, C$\underline{H}_2$CO), 4.40-4.48 (1H, m, NHC$\underline{H}$CH$_2$), 4.48 (1H, s, C$\underline{H}$CMe$_2$), 5.20-5.33 (6H, m, 3 × C$\underline{H}_2$ArNO$_2$), 5.53 (1H, d, J 4 Hz, NHCHC$\underline{H}$S), 5.69 (1H, dd, J 4 Hz and 9 Hz, NHC$\underline{H}$CHS), 5.80 (1H, d, J 8 Hz, N$\underline{H}$), 6.27 (1H, d, J 9Hz, N$\underline{H}$), 7.49-7.56 and 8.18-8.25 (12H, 2 × m, aromatic C$\underline{H}$)

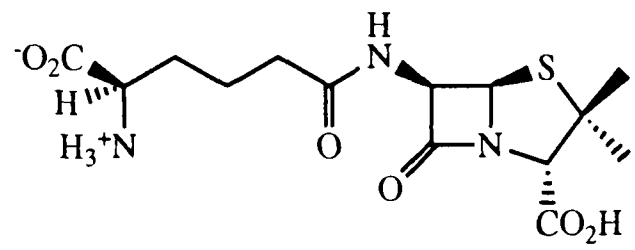
δ_C (125.8 MHz, $CDCl_3$) : 20.93 (t, CHCH$_2$$\underline{C}H_2$), 26.90 (q, $\underline{C}$H$_3$), 31.45 (t, CH$\underline{C}$H$_2$CH$_2$), 31.61 (q, $\underline{C}$H$_3$), 34.79 (t, CH$_2$$\underline{C}H_2$CO), 53.86 58.82 and 68.03 (3 × d, $\underline{C}$HCH$_2$CH$_2$ and NH$\underline{C}$H$\underline{C}$HS), 64.84 (s, $\underline{C}$Me$_2$), 65.46, 65.61 and 65.83 (3 × t, 3 × $\underline{C}$H$_2$Ar), 70.45 (d,

$\underline{C}HCMe_2$), 123.61, 123.73, 123.83, 127.98, 128.38 and 128.87 (6 × d, aromatic $\underline{C}H$), 141.73, 142.38 and 143.61 (3 × s, aromatic C1), 147.74, 147.94 and 148.13 (3 × s, aromatic C4), 155.55 (s, $NH\underline{C}O_2Ar$), 167.12, 171.46, 171.60 and 173.48 (4 × s, $\underline{C}$=O)

m/z (FAB) : 831 ([MNa^+], 25%), 809 ([MH^+], 16%), 515 (34%), 295 (32%), 85 (100%)

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (1)

$C_{14}H_{21}N_3O_6S$; MW 359



The same procedure as for (154) was followed using (176) (100 mg, 0.117 mmol), $NaHCO_3$ (10 mg, 1 eq) and 10% Pd/C (121 mg). Lyophilisation of the aqueous solution obtained in the work-up produced the product (1) (*ca.* 30 mg, 0.08 mmol, 64% yield).

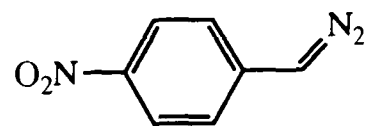
δ_H (500 MHz, D_2O, $\underline{H}OD$ suppressed) : 1.53 (3H, s, $C\underline{H}_3$), 1.65 (3H, s, $C\underline{H}_3$), 1.60-1.95 (4H, m, $CHC\underline{H}_2C\underline{H}_2$), 2.42 (2H, t, J 7Hz, $CH_2C\underline{H}_2CO$), 3.73 (1H, t, J 6Hz, $H_3N^+C\underline{H}CO_2^-$), 4.25 (1H, s, $C\underline{H}CMe_2$), 5.48 and 5.57 (2 × 1H, ABq, J 4 Hz, $NHC\underline{H}C\underline{H}S$)

m/z (ESMS) : 360 ([MH^+], 100%), 361 (19%), 362 (8%), 363 (2%)

Identical by ^{1}H-NMR and mass spectra to an authentic sample.

***p*-Nitrophenyldiazomethane**[259]

$C_7H_5N_3O_2$; MW 163



p-Nitrobenzaldehyde hydrazone (0.50 g, 3 mmol) was dissolved in DCM (20 ml) and a solution of iodine in DCM [1% (w/v),124 ml] added, followed by 1,1,3,3-tetramethyl-guanidine (380 µl, 1 eq). The stirred solution was cooled to 0°C and MCPBA (0.52 g, 3 mmol, 1 eq) added in portions over one hour. The orange solution was left stirring at room temperature for another hour, after which time it was washed with H_2O, until the pH of the aqueous phase was 6-7. The organic phase was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was dissolved in ether, and the insoluble material (hydrazone) filtered off.

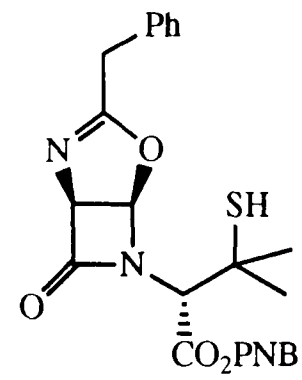
Evaporation of the solvent gave the title compound (450 mg, 2.8 mmol, 92% yield) as an orange solid.

ν_{max} (KBr) : 2077 (s, diazo), 1588 (s, NO_2), 1498 (m), 1328 (s, NO_2), 1110 (m), 836 (m), 746 (m)

δ_H (200 MHz, $CDCl_3$) : 5.13 (1H, s, C<u>H</u>N_2), 6.96-7.03 (2H, m, aryl C<u>H</u>), 8.14-8.20 (2H, m, aromatic C<u>H</u>)

(2<u>S</u>)-2-[(1<u>S</u>,5<u>R</u>)-3-Benzyl-7-oxo-4-oxa-2,6-diazabicyclo[3.2.0]hept-2-en-6-yl]-3-mercapto-3-methyl-butanoic acid *p*-nitrobenzyl ester (159c)

$C_{23}H_{23}N_3O_6S$; MW 469



The same procedure as for (159b) was followed, with penicillin G potassium salt (10a) (2g, 5.4 mmol) and mercury(II) acetate (3.43 g, 10.8 mmol, 2 eq) to give (158) (3.21 g, 3.8 mmol, 70% yield). This was treated as before with H_2S and *p*-nitrophenyl-diazomethane (0.93 g, 5.7 mmol, 1.5 eq), to give after purification by chromatography [flash silica, EtOAc/DCM (10% to 20%, v/v)] (159c) (1.23 g, 2.62 mmol, 70% yield from (158)).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.63

M.p. = 104-105°C (from EtOAc/petrol)

Found : C 59.10 %, H 4.83 %, 9.15 %. $C_{23}H_{23}N_3O_6S$ requires C 58.84 %, H 4.94 %, N 8.95%

ν_{max} (KBr) : 1763 (s, β-lactam C=O), 1727 (s, ester C=O), 1647 (m, C=N), 1606 (m), 1520 (s, NO_2), 1348 (s, NO_2), 1222 (m), 1197 (m), 1161 (m), 982 (m), 849 (m), 720 (m)

δ_H (200 MHz, $CDCl_3$) : 1.13 (3H, s, C<u>H</u>$_3$),1.29 (3H, s, C<u>H</u>$_3$), 1.96 (1H, s, S<u>H</u>), 3.63 and 3.74 (2 x 1H, ABq, J 15 Hz, C<u>H</u>$_2$Ph), 4.49 (1H, s, C<u>H</u>CMe_2), 5.19 and 6.25 (2 × 1H, 2 × d,

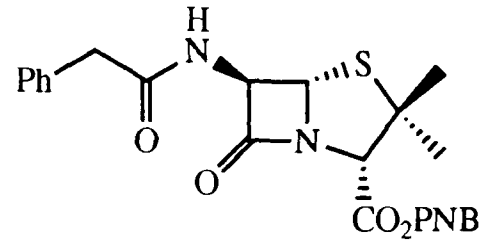
J 3 Hz, NC<u>H</u>C<u>H</u>O), 5.26 (2H, s, $CO_2C\underline{H}_2Ar$), 7.30 (5H, s, phenyl C<u>H</u>), 7.53 and 8.24 (2 × 2H, 2 × d, J 9 Hz, aromatic C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 29.46 (q, $\underline{C}H_3$), 29.85 (q, $\underline{C}H_3$), 35.55 (t, $\underline{C}H_2Ph$), 44.58 (s, $\underline{C}Me_2$), 64.27 (d, $\underline{C}HCMe_2$), 65.87 (t, $\underline{C}H_2ArNO_2$), 81.57 and 87.36 (2 × d, N<u>C</u>H<u>C</u>HO), 123.77, 127.39, 128.76 and 129.25 (4 × d, aromatic <u>C</u>H), 133.37 (aromatic C1 of CH_2Ph), 141.70 (aromatic C1 of $ArNO_2$) 147.94 (s, aromatic C4 of $ArNO_2$), 167.43, 167.54 and 169.29 (3 × s, 2 × <u>C</u>=O and <u>C</u>=N)

m/z [DCI (NH_3)] : 470 ([MH^+], 11%), 436 (18%), 295 (100%), 176 (96%)

(2<u>S</u>,5<u>S</u>,6<u>R</u>)-1-Aza-3,3-dimethyl-6-phenylacetamido-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid *p*-nitrobenzyl ester (160c)

$C_{23}H_{23}N_3O_6S$; MW 469



The same procedure as for (160b) was followed, using (159c) (1.23 g, 2.62 mmol) and zinc acetate dihydrate (72 mg, 0.39 mmol, 0.15 eq). Purification of the crude product by chromatography [flash silica, EtOAc/DCM (10% to 20%, v/v)] gave (160c) (550 mg, 1.17 mmol, 45% yield).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.72

ν_{max} ($CHCl_3$) : 1791 (s, β-lactam C=O), 1751 (s, ester C=O), 1682 (s, amide C=O), 1609 (m), 1510 (s, NO_2), 1351 (s, NO_2), 1246 (s), 1185 (s), 1078 (m), 1016 (m), 851 (m)

δ_H (200 MHz, $CDCl_3$) : 1.31 (3H, s, $C\underline{H}_3$), 1.54 (3H, s, $C\underline{H}_3$), 3.53 (2H, s, $C\underline{H}_2Ph$), 3.75 (1H, s, $C\underline{H}CO_2PNB$), 4.72 (1H, dd, J 2 Hz and 7 Hz, NHC<u>H</u>CHS), 5.13 (1H, d, J 2 Hz, NHCHC<u>H</u>S), 5.15 and 5.23 (2 × 1H, ABq, J 13 Hz, $C\underline{H}_2ArNO_2$), 6.28 (1H, d, J 7 Hz, N<u>H</u>), 7.17-7.32 (5H, m, phenyl C<u>H</u>), 7.49 and 8.14 (2 × 2H, 2 × d, J 9 Hz, aryl C<u>H</u>)

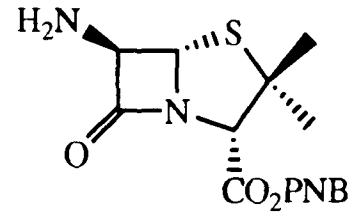
δ_C (125.8 MHz, $CDCl_3$) : 24.56 (q, $\underline{C}H_3$), 30.66 (q, $\underline{C}H_3$), 42.61 (t, $\underline{C}H_2Ph$), 63.54 and 67.33 (2 × d, NH<u>C</u>H<u>C</u>HS), 65.13 (t, $\underline{C}H_2ArNO_2$), 65.85 (s, $\underline{C}Me_2$), 69.65 (d, $\underline{C}HCMe_2$), 123.77, 127.41, 128.92 and 129.42 (4 × d, aromatic <u>C</u>H), 134.11 (aromatic C1 of CH_2Ph),

142.00 (aromatic C1 of $ArNO_2$) 147.80 (s, aromatic C4 of $ArNO_2$), 166.47, 168.29 and 171.78 (3 × s, 3 × C=O)

m/z (FAB) : 492 ([MNa^+], 100%)

(2S,5S,6R)-1-Aza-3,3-dimethyl-6-amino-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid *p*-nitrobenzyl ester (167b)

$C_{15}H_{17}N_3O_5S$; MW 351



The procedure e) for formation of (167a) was followed using (160c) (200 mg, 0.43 mmol), N,N-dimethylaniline (151 ml, 1.19 mmol, 2.8 eq), phosphorus pentacloride (116 mg, 0.56 mmol, 1.3 eq), *n*-butanol (750 ml) and *p*-toluenesulfonic acid monohydrate (311 mg, 1.63 mmol, 3.8 eq). Purification of the final crude product by chromatography [flash silica, EtOAc/DCM (20% to 40%, v/v)] gave (167b) (75 mg, 0.21 mmol, 50% yield).

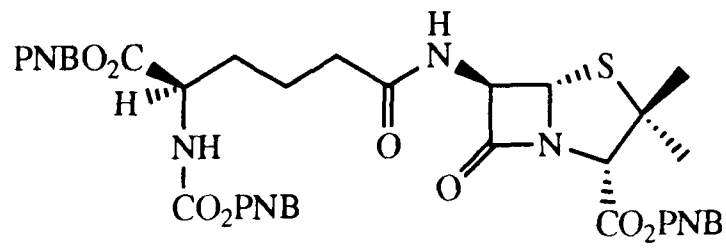
T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.23

δ_H (500 MHz, $CDCl_3$) : 1.41 (3H, s, CH_3), 1.64 (3H, s, CH_3), 3.79 (1H, s, $CHCO_2PNB$), 4.32 and 4.89 (2 × 1H, 2 × d, J 2 Hz, NHCHCHS), 5.26 and 5.36 (2 × 1H, ABq, J 13 Hz, CH_2ArNO_2), 7.59 and 8.23 (2 × 2H, 2 × d, J 9 Hz, aromatic CH)

m/z (FAB) : 352 ([MH^+], 9%), 295 (100%)

(2S,5S,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 (168b)

$C_{36}H_{36}N_6O_{14}S$; MW 808



The same procedure as for (168a) was followed using (167b) (72 mg, 0.21 mmol), EEDQ[220] (56 mg, 0.23 mmol, 1.1 eq), N-*p*-nitrobenzyloxycarbonyl-D-α-aminoadipic acid-α-*p*-nitrobenzyl ester (105) (97 mg, 0.21 mmol, 1 eq), and Na_2SO_4 (*ca.* 10 mg).

Purification of the crude product by P.l.c. [silica, EtOAc/DCM (40%, v/v)] gave (168b) (143 mg, 0.18 mmol, 86% yield).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.55

M.p. = 79-80°C (from EtOAc/petrol)

Found : C 53.55%, H 4.57 %, N 10.70 %. $C_{36}H_{36}N_6O_{14}S$ requires C 53.46 %, H 4.49 %, N 10.39 %

ν_{max} ($CHCl_3$) : 1783 (s, β-lactam C=O), 1747 (s, ester C=O), 1702 (s, amide C=O), 1609 (m), 1527 (s, NO_2), 1349 (s, NO_2), 1181 (s), 1073 (m), 852 (m)

δ_H (500 MHz, $CDCl_3$) : 1.41 (3H, s, $C\underline{H}_3$), 1.64 (3H, s, $C\underline{H}_3$), 1.60-2.05 (4H, m, $CHC\underline{H}_2C\underline{H}_2CH_2$), 2.23-2.37 (2H, m, $CH_2C\underline{H}_2CO$), 3.87 (1H, s, $C\underline{H}CMe_2$), 4.38-4.47 (1H, m, $C\underline{H}CH_2CH_2$), 4.83 (1H, dd, J 2 Hz and 7 Hz, $NHC\underline{H}CHS$), 5.12-5.38 (7H, m, $CHC\underline{H}S$ and 3 × $CO_2C\underline{H}_2Ar$), 5.65 (1H, d, J 8 Hz, $N\underline{H}$), 6.37 (1H, d, J 7 Hz, $N\underline{H}$), 7.44-7.60 and 8.14-8.28 (2 × 6H, 2 × m, aromatic $C\underline{H}$)

δ_C (50.3 MHz, $(CD_3)_2CO$) : 22.13 (t, $CHCH_2\underline{C}H_2$), 25.20 (q, $\underline{C}H_3$), 30.96 (t, $CH\underline{C}H_2CH_2$), 31.44 (q, $\underline{C}H_3$), 35.17 (t, $CH_2\underline{C}H_2CO$), 55.04 (d, $\underline{C}HCH_2CH_2$), 64.81 and 68.40 (2 × d, $NH\underline{C}H\underline{C}HS$), 65.61, 65.87 and 66.58 (3 × t, 3 × $\underline{C}H_2Ar$), 70.53 (d, $\underline{C}HCMe_2$), 124.53, 129.04, 129.41 and 130.13 (4 × d, aromatic $\underline{C}H$), 144.11, 144.86 and 146.06 (3 × s, aromatic C1 and C4), 157.19 (s, $NH\underline{C}O_2Ar$), 167.59, 169.67, 173.05 and 173.57 (4 × s, 4 × $\underline{C}$=O)

m/z (FAB) : 831 ([MNa^+], 100%), 809 ([MH^+], 7%)

Hydrogenation of (168b)

The same procedure as for hydrogenation of (168a) was followed using (168b) (90 mg, 0.1 mmol), $NaHCO_3$ (8 mg, 1 eq) and Pd/C 10% (119 mg). Purification of the crude product by chromatography [HPLC, Gilson system, solvent 0.75% MeCN in aqueous 10 mM NH_4HCO_3, flow rate 4 ml/min] gave (154) (retention time 9 min, *ca.* 2.5 mg by NMR calibration, 6% yield).

Incubation of (154) with DAOC/DACS

5-Epipenicillin N (154) (1 mg) was incubated with DAOC/DACS (4 ml, *ca.* 0.06 IU) according to the general procedure. Examination of the crude incubation mixture by 500 MHz ^{1}H-NMR (D_2O, HOD suppressed, TSP referenced) showed (154) was still present, and no other β-lactam products could be observed. The sample was freeze-dried and another 500 MHz ^{1}H-NMR was run on the following day. This revealed that (154) was no longer present, and two multiplets (δ 5.16 and 5.25 ppm) were observed, suggesting the β-lactam ring had decomposed.

Incubation of a mixture of (154) and (1) with DAOC/DACS

A mixture of penicillin N (1) (0.5 mg) and 5-epipenicillin N (154) (0.5 mg) was incubated with DAOC/DACS (3.5 ml, 0.06 IU), according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR (D_2O, HOD suppressed, TSP referenced) showed about 80% conversion of (1) to DAOC (2) and DAC (3), a trace amount of (154) was still present, but no new β-lactam products could be observed.

Bioassay tests with (154) and (1)

The following solutions were tested according to the general bioassay procedure :

A)
- 5-epipenicillin N (154) 25 µg/ml
- penicillin N (1) 25 µg/ml

B)
- Denatured enzyme incubation mixtures prepared with :
  - 30 µl DAOC/DACS denatured with 300 µl MeOH
  - 90 µl 200 mM TRIS.HCl pH 8
  - 30 µl cofactor solution
  - 150 µl of a 100 µg/ml solution of substrate [(154) or (1)]

C)
- Active enzyme incubation mixtures prepared with :
  - 30 µl DAOC/DACS
  - 90 µl 200 mM TRIS.HCl pH 8

- 30 µl cofactor solution
- 150 µl of a 100 µg/ml solution of substrate [(154) or (1)]
- the above mixture was incubated for 40 min at 27°C, after which 300 µl MeOH were added

The following inhibition zones (in mm) were obtained :

A) Isolated compounds

	E.coli (-)	*E. coli* (+)	*S. aureus*
(154)	0	0	0
(1)	28	0	18

B) Incubation mixtures with denatured enzyme

	E.coli (-)	*E. coli* (+)	*S. aureus*
(154)	0	0	0
(1)	27	0	18

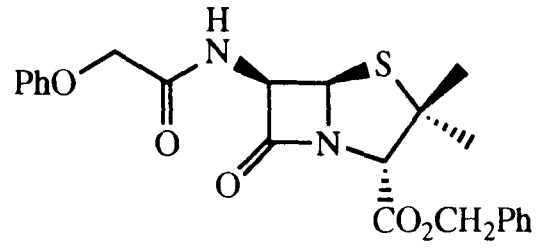
C) Incubation mixtures with active enzyme

	E.coli (-)	*E. coli* (+)
(154)	0	0
(1)	33	34

6.4.2 Experimental for Section 4.3

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo[3.2.0] heptane-2-carboxylic acid benzyl ester (186)

$C_{23}H_{24}N_2O_5S$; MW 440



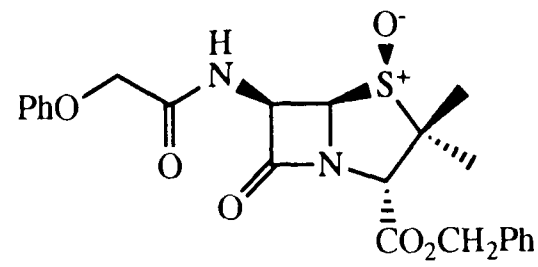
Penicillin V potassium salt (11a) (10 g, 25.8 mmol) and a catalytic amount of sodium iodide (194 mg, 1.3 mmol, 0.05 eq) were suspended in dry DMF (175 ml) under argon. Benzyl bromide (3.37 ml, 28 mmol, 1.1 eq) was added *via* syringe. The reaction mixture was stirred overnight at 30°C under argon. The solvent was evaporated *in vacuo*, and the residue partitioned between H_2O (100 ml) and EtOAc (100 ml). The organic phase was washed with 1N $NaHCO_3$ (100 ml), brine (75 ml), dried (Na_2SO_4) and evaporated to give the product (186) as a yellow oil (11 g, 25 mmol, 97% yield).

T.l.c. [EtOAc/petrol (2:3,v/v)] Rf 0.60

δ_H (200 MHz, $CDCl_3$) : 1.40 (3H, s, C<u>H</u>$_3$), 1.55 (3H, s, C<u>H</u>$_3$), 4.49 (1H, s, C<u>H</u>CO$_2$Bn), 4.53 (2H, s, PhOC<u>H</u>$_2$), 5.18 (2H, s, CO$_2$C<u>H</u>$_2$Ph), 5.57 (1H, d, J 4 Hz, NHCHC<u>H</u>S), 5.72 (1H, dd, J 4 and 9 Hz, NHC<u>H</u>CHS), 6.90-7.45 (11H, m, N<u>H</u> and aromatic C<u>H</u>)

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo[3.2.0] heptane-2-carboxylic acid benzyl ester β-sulfoxide (187)

$C_{23}H_{24}N_2O_6S$; MW 456



To a solution of penicillin V benzyl ester (186) (11.0 g, 25 mmol) in DCM (100 ml) was added MCPBA (50-60%) (8.6 g, 30 mmol, 1.2 eq) in small portions, following reaction by T.l.c. [EtOAc/DCM, 1:1 (v/v)]. When there was no evidence of starting material the reaction mixture was washed with a saturated solution of sodium thiosulphate (2 × 50 ml),

sat. $NaHCO_3$ (2 × 50 ml), water (75 ml) and brine (75 ml). The organic phase was dried (Na_2SO_4) and the solvent evaporated *in vacuo* to give (187) as a solid (10.8 g, 23.8 mmol, 95% yield).

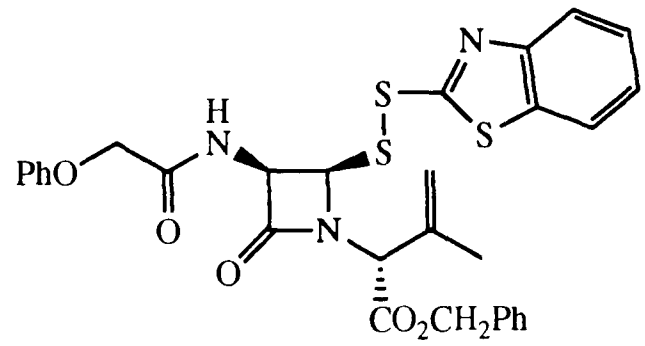
T.l.c. [EtOAc/DCM (20%,v/v)] Rf 0.35

δ_H (200 MHz, $CDCl_3$) : 1.08 (3H, s, C<u>H</u>$_3$), 1.68 (3H, s, C<u>H</u>$_3$), 4.54 (2H, s, PhOC<u>H</u>$_2$), 4.71 (1H, s, C<u>H</u>CO_2Bn), 5.03 (1H, d, J 5 Hz, NHCHC<u>H</u>S), 5.17 and 5.31 (2H, ABq, J 12 Hz, C<u>H</u>$_2$Ph), 6.11 (1H, dd, J 5 and 10 Hz, NHC<u>H</u>CHS), 6.92-7.43 (10H, m, aromatic C<u>H</u>), 8.27 (1H, d, J 10 Hz, N<u>H</u>)

m/z [DCI (NH_3)] : 474 ([MNH_4^+], 5%), 457 ([MH^+], 46 %)

(2<u>R</u>,3<u>R</u>)-2-(Benzothiazole-2-dithio)-1-[(1<u>R</u>)-1-isoprop-1-enylacetic acid benzyl ester] -3-phenoxyacetamido-1-azetidin-4-one (188)

$C_{30}H_{27}N_3O_5S_3$; MW 605



A solution of penicillin V benzyl ester β-sulfoxide (187) (4.20 g, 9.2 mmol) and mercaptobenzothiazole (1.54 g, 9.2 mmol, 1 eq) in toluene (150 ml) was refluxed using a Dean-Stark apparatus for 3h.[268] The solvent was removed *in vacuo* to give the crude product as a yellow foam. This was purified by chromatography [flash silica, EtOAc/DCM (10%, v/v)] to give the product (188) (4.75 g, 7.85 mmol, 85% yield).

T.l.c. [EtOAc/DCM (20%,v/v)] Rf 0.55

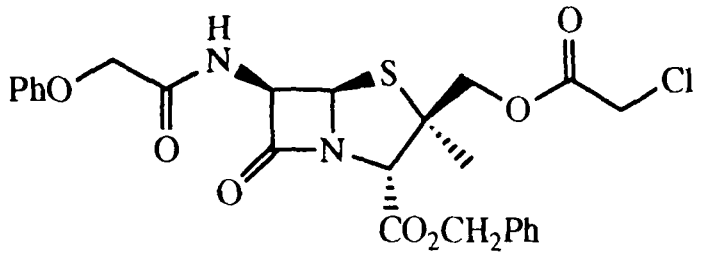
δ_H (200 MHz, $CDCl_3$) : 1.97 (3H, s, C<u>H</u>$_3$), 4.52 and 4.63 (2H, ABq, J 15 Hz, PhOC<u>H</u>$_2$), 5.01-5.25 (5H, m, C<u>H</u>CO_2C<u>H</u>$_2$Ph and C=C<u>H</u>$_2$), 5.47 (1H, dd, J 5 and 8 Hz, NHC<u>H</u>CHS), 5.62 (1H, d, J 5 Hz, NHCHC<u>H</u>S), 6.90-7.86 (15 H, m, N<u>H</u> and aromatic C<u>H</u>)

m/z [DCI (NH_3)] : 623 ([MNH_4^+], 6%), 606 ([MH^+], 10 %), 440 (100%)

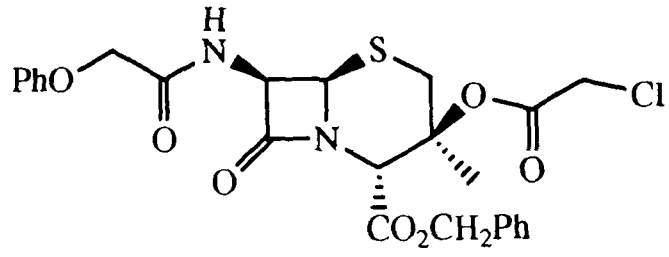
(2S,3R,5R,6R)-1-Aza-3-chloroacetoxy-3-methyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzyl ester (189)

(2R,3S,6R,7R)-1-Aza-3-chloroacetoxy-3-methyl-7-phenoxyacetamido-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid benzyl ester (126)

$C_{25}ClH_{25}N_2O_7S$; MW 532



(189)



(126)

To a solution of silver acetate (5.06 g, 30.32 mmol, 2 eq) and chloroacetic acid (60.74 g, 643 mmol, 42.5 eq) in DCM (250 ml) was added (188) (9.16g, 15.14 mmol).[263] The resulting suspension was protected from light, stirred for 3.5 h and then filtered through a pad of silica gel (40 g) washing with DCM (400 ml). The filtrate was neutralized with 1N $NaHCO_3$ (2 × 300 ml), washed with brine, dried (Na_2SO_4) and the solvent evaporated to a yellow oil. Purification by chromatography [flash silica, EtOAc/petrol (1:3, v/v)] gave the penam (189) (3.40 g, 6.39 mmol, 42% yield) and the cepham (126) (2.42 g, 4.55 mmol, 30% yield).

a) Data for (189) :

T.l.c. [EtOAc/petrol (1:2,v/v)] Rf 0.41

ν_{max}(KBr) : 1789 (s, β-lactam C=O), 1745 (s, ester C=O), 1692 (s, amide C=O), 1599 (m), 1520 (m), 1495 (m), 1305 (m), 1242 (m), 1206 (m), 1162 (m), 755 (m), 693 (m)

δ_H (200 MHz, $CDCl_3$) : 1.41 (3H, s, C$\underline{H}_3$), 4.02 and 4.08 (2H, ABq, J 15 Hz,C$\underline{H}_2$Cl), 3.93 and 4.39 (2H, ABq, J 12 Hz, C$\underline{H}_2$OCO), 4.57 (2H, s, PhOC$\underline{H}_2$), 4.73 (1H, s, C$\underline{H}$CO$_2$Bn), 5.21 (2H, s, C$\underline{H}_2$Ph), 5.66 (1H, d, J 4 Hz, NHCHC$\underline{H}$S), 5.74 (1H, dd, J 4 and 9 Hz, NHC$\underline{H}$CHS), 6.91-7.45 (11H, m, phenyl C$\underline{H}$ and N$\underline{H}$)

δ_C (50.3 MHz, $CDCl_3$) : 21.60 (q, $\underline{C}H_3$), 40.31 (t, CO$\underline{C}H_2$Cl), 58.79 and 65.22 (2 × d, NH$\underline{C}$H$\underline{C}$HS), 66.87 (s, $\underline{C}CH_3$), 66.96 and 67.53 (2 × t, $\underline{C}H_2$Ph and $\underline{C}H_2$OPh), 68.22 (d, $\underline{C}$HCO$_2$Bn), 71.38 (t, $\underline{C}H_2$OCO), 114.65, 122.31, 128.69, 128.86 and 129.81 (5 × d, aromatic $\underline{C}$H), 134.39 (s, aromatic C1 of CH_2Ph), 150.96 (s, aromatic C1 of CH_2OPh), 166.86, 167.01, 167.99 and 171.52 (4 × s, 4 × $\underline{C}$=O)

m/z [DCI (NH_3)] : 550 ([MNH_4^+], 18%), 533 ([MH^+], 23%), 342 (100%), 192 (62%)

Identical by IR, ^{1}H-NMR and mass spectroscopy to literature reference.[263]

b) Data for (126)

T.l.c. [EtOAc/petrol (1:2,v/v)] Rf 0.35

ν_{max} ($CHCl_3$) : 1783 (s, β-lactam C=O), 1750 (s, ester C=O), 1698 (s, amide C=O), 1605 (m), 1590 (m), 1522 (s), 1498 (s), 1440 (m), 1150 (m), 702 (m), 690 (m)

δ_H (200 MHz, $CDCl_3$) : 1.51 (3H, s, C<u>H</u>$_3$), 3.38 and 3.46 (2H, ABq, J 15 Hz, SC<u>H</u>$_2$), 3.94 and 4.00 (2H, ABq, J 15 Hz, C<u>H</u>$_2$Cl), 4.54 (2H, s, PhOC<u>H</u>$_2$), 4.81 (1H, s, C<u>H</u>CO_2Bn), 5.18 and 5.22 (2H, ABq, J 12 Hz, PhC<u>H</u>$_2$), 5.33 (1H, d, J 4 Hz, NHCHC<u>H</u>S), 5.67 (1H, dd, J 4 and 10 Hz, NHC<u>H</u>CHS), 6.90-7.44 (11H, m, phenyl C<u>H</u> and N<u>H</u>)

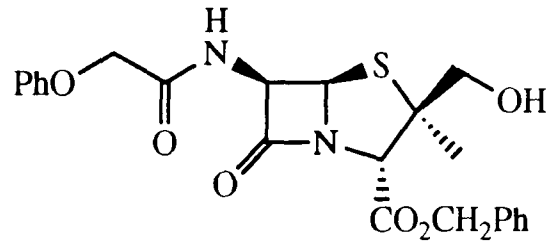
δ_C (125.8 MHz, $CDCl_3$) : 21.26 (q, <u>C</u>H_3), 29.88 (t, S<u>C</u>H_2), 41.00 (t, <u>C</u>H_2Cl), 53.98, 57.54 and 58.20 (3 × d, NH<u>C</u>H<u>C</u>HS and <u>C</u>HCO_2Bn), 67.00 and 67.80 (2 × t, <u>C</u>H_2Ph and <u>C</u>H_2OPh), 74.92 (s, <u>C</u>CH_3), 114.61, 122.13, 128.46, 128.61, 128.79 and 129.58 (6 × d, aromatic <u>C</u>H), 134.12 (s, aromatic C1 of CH_2Ph), 156.86 (s, aromatic C1 of CH_2OPh), 165.52, 165.74, 166.11 and 168.23 (4 × s, 4 × <u>C</u>=O)

m/z (FAB) : 555 ([MNa^+], 52%), 533 ([MH^+], 13%), 342 (27%), 149 (52%), 91 (100%)

Identical by IR, ^{1}H-NMR and mass spectroscopy to literature reference.[263]

(2<u>S</u>,3<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-3-hydroxymethyl-3-methyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzyl ester (178)

$C_{23}H_{24}N_2O_6S$; MW 456



A solution of chloroacetate penam (189) (2.84 g, 5.34 mmol) and thiourea (2.04 g, 26.8 mmol, 5 eq) in absolute ethanol (100 ml) was stirred at 70°C for 10 min, and for a further 30 min at 50°C.[263] The solution was evaporated to dryness *in vacuo* with no heating. The residue was dissolved in EtOAc/H_2O [100 ml, 1:1 (v/v)] and the organic phase washed with water (50 ml), dried (Na_2SO_4) and evaporated *in vacuo* with no heating to give the crude product (178) (2.50 g) as a white foam.

ν_{max} (KBr) : 3412 (m), 3336 (m), 1788 (s, β-lactam C=O), 1745 (s, ester C=O), 1680 (s, amide C=O), 1599 (m), 1532 (s), 1496 (s), 1431 (m), 1295 (m), 1246 (m), 1196 (s), 755 (m)

δ_H (200 MHz, $CDCl_3$) : 1.32 (3H, s, C$\underline{H}_3$), 2.10 (1H, br.s, O$\underline{H}$), 3.44 and 3.67 (2H, ABq, J 11 Hz, C$\underline{H}_2$OH), 4.54 (2H, s, C$\underline{H}_2$OPh), 4.73 (1H, s, C$\underline{H}$CO$_2$Bn), 5.20 (2H, s, PhC$\underline{H}_2$), 5.61-5.66 (2H, m, NHC$\underline{H}$C$\underline{H}$S), 6.90-7.38 (10H, m, phenyl C$\underline{H}$), 7.82 (1H, d, J 12 Hz, N$\underline{H}$)

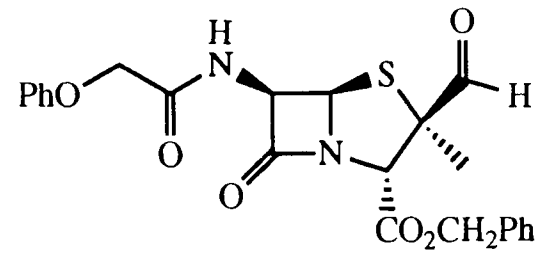
δ_C (50.3 MHz, CD_2Cl_2) : 20.54 (q, $\underline{C}H_3$), 59.12 and 65.97 (2 × d, NH$\underline{C}$H$\underline{C}$HS), 67.09 and 67.37 (2 × t, $\underline{C}H_2$Ph and $\underline{C}H_2$OPh), 68.44 (d, $\underline{C}$HCO$_2$Bn), 68.63 (s, $\underline{C}$CH$_2$OH), 70.97 (t, $\underline{C}H_2$OH), 114.85, 122.13, 128.77 and 129.84 (4 × d, aromatic $\underline{C}$H), 134.08 (s, aromatic C1 of CH_2Ph), 157.47 (s, aromatic C1 of CH_2OPh), 167.94, 168.20 and 172.45 (3 × s, 3 × $\underline{C}$=O)

m/z (FAB) : 457 ([MH$^+$], 34%), 266 (78%), 91 (100%)

Identical by IR, ^{1}H-NMR and mass spectroscopy to lit. reference.[263]

(2S,3R,5R,6R)-1-Aza-3-formyl-3-methyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo [3.2.0]heptane-2-carboxylic acid benzyl ester (179)

$C_{23}H_{22}N_2O_6S$; MW 454



To a solution of oxalyl chloride (40 µl, 0.42 mmol, 1.5 eq) in dry DCM (15 ml) stirring at -60°C under argon, was added DMSO (34.4 µl, 0.48 mmol, 1.7 eq).[269] Ten minutes later a solution of (178) (130 mg, 0.28 mmol) in dry DCM (3 ml) was added dropwise, and the reaction stirred for one hour at -60°C. Dry triethylamine (148 µl, 1.06 mmol, 3.8 eq) was added and after 5 min the reaction was warmed to room temperature and then poured into 0.2N HCl (50 ml). After separation, the aqueous layer was extracted with DCM (2 x 50 ml), and the combined organic layers washed with water (50 ml), dried (Na_2SO_4) and evaporated to give the aldehyde (179) as a crude product (120 mg, 93% crude yield).

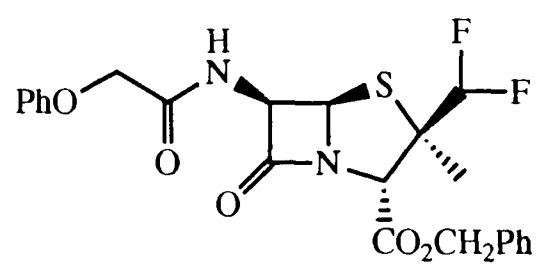
$\nu_{max}(CHCl_3)$: 1798 (s, β-lactam C=O), 1750 (s, ester C=O), 1726 (s, aldehyde C=O), 1690 (s, amide C=O), 1598 (m), 1520 (s), 1495 (s), 1295 (m), 1065 (m), 705 (m)

δ_H (200 MHz, $CDCl_3$) : 1.43 (3H, s, C<u>H</u>$_3$), 4.55 (2H, s, PhOC<u>H</u>$_2$), 5.17 (1H, s, C<u>H</u>CO_2Bn), 5.23 (2H, s, C<u>H</u>$_2$Ph), 5.57 (1H, dd, J 4 and 8 Hz, NHC<u>H</u>CHS), 5.68 (1H, d, J 4 Hz, NHCHC<u>H</u>S), 6.95-7.40 (11H, m, phenyl C<u>H</u> and N<u>H</u>), 9.13 (1H, s, C<u>H</u>O)

Identical by IR and ^{1}H-NMR to lit. reference.[263]

(2<u>S</u>,3<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-3-difluoromethyl-3-methyl-6-phenoxyacetamido-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid benzyl ester (180)

$C_{23}F_2H_{22}N_2O_5S$; MW 476



To a solution of the aldehyde (179) (1.58 g, 3.48 mmol) in dry DCM (20 ml) was added dropwise a solution of diethylaminosulphur trifluoride[270] (643 µl, 4.87 mmol, 1.4 eq) in dry DCM (15 ml). The resulting solution was stirred at room temperature for 30 min, diluted with DCM (100 ml), washed with 1N $NaHCO_3$ (150 ml), H_2O (100 ml), dried (Na_2SO_4) and evaporated to give an oil. This was purified by chromatography [flash silica, EtOAc/petrol (30%, v/v)] to give the product as a white foam (0.91 g, 1.89 mmol, 54 % yield).

T.l.c. [EtOAc/DCM (40%,v/v)] Rf 0.50

ν_{max} (KBr) : 3410 (m), 1794 (s, β-lactam C=O), 1747 (s, ester C=O), 1697 (s, amide C=O), 1600 (m), 1523 (s), 1496 (s), 1457 (m), 1303 (s), 1243 (s), 1209 (s), 1174 (s), 1083 (s), 1053 (m), 755 (m), 693 (m)

δ_H (200 MHz, $CDCl_3$) : 1.38 (3H, s, C<u>H</u>$_3$), 4.53 (2H, s, PhOC<u>H</u>$_2$), 4.90 (1H, s, C<u>H</u>CO_2Bn), 5.21 (2H, s, C<u>H</u>$_2$Ph), 5.63 (1H, t, J_{H-F} 56 Hz, C<u>H</u>F_2), 5.64-5.68 (2H, m, NHC<u>H</u>C<u>H</u>S), 6.90-7.41 (11H, m, phenyl C<u>H</u> and N<u>H</u>)

δ_C (50.3 MHz, $CDCl_3$) : 18.01 (q, <u>C</u>H_3), 59.27 and 62.66 (2 × d, NH<u>C</u>H<u>C</u>HS), 66.75 (t, <u>C</u>H_2OPh), 67.72 (t, <u>C</u>H_2Ph), 68.09 (s, <u>C</u>CH_3), 68.63 (d, <u>C</u>HCO_2Bn), 116.39 (dt, J_{C-F} 250 Hz, <u>C</u>HF_2), 114.64, 122.25, 128.78, 128.95 and 129.78 (5 × d, aromatic <u>C</u>H), 134.31 (s,

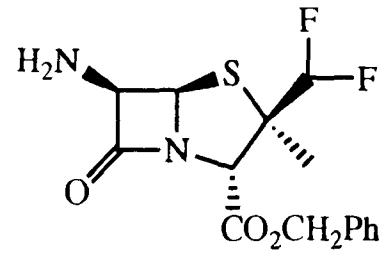
aromatic C1 of CH_2Ph), 156.97 (s, aromatic C1 of CH_2OPh), 166.39, 167.92 and 170.13 (3 × s, 3 × C=O)

m/z [DCI (NH_3)] : 477 ([MH^+], 7%), 286 (100%), 91 (75%)

Identical by IR, ^{1}H-NMR and mass spectroscopy to lit. reference.[263]

(2S,3R,5R,6R)-1-Aza-3-difluoromethyl-3-methyl-6-amino-7-oxo-4-thiabicyclo[3.2.0] heptane-2-carboxylic acid benzyl ester (181)

$C_{15}F_2H_{16}N_2O_3S$; MW 342



To a solution of (180) (466 mg, 0.98 mmol) in dry DCM (4 ml) under argon was added N,N-dimethylaniline (360 µl, 2.84 mmol, 2.9 eq) at -60°C. To the stirred solution was rapidly added phosphorus pentachloride (264 mg, 1.27 mmol, 1.3 eq). After stirring for 5 min at -60°C the temperature was raised to -40°C, the reaction mixture stirred for one hour, and then re-cooled to -60°C. *n*-Butanol (1.5 ml) was added dropwise over 5 min, the temperature raised to -30°C, and the reaction then left to warm up to 0°C over 40 min.

The reaction mixture was poured into H_2O (20 ml), the resulting emulsion stirred vigorously and the pH adjusted to 8 with 1N $NaHCO_3$. The suspension was extracted with DCM (3 × 50 ml) and to the dried (Na_2SO_4) organic phase was added *p*-toluenesulfonic acid monohydrate (727 mg, 3.82 mmol, 3.9 eq). The resulting solution was evaporated *in vacuo* with no heating to give a yellow oil. This was purified by flash chromatography using a small amount of silica (20 g) eluting with 30% EtOAc/DCM (100 ml), 60% EtOAc/DCM (100 ml), EtOAc (100 ml) and 20% MeOH/EtOAc (100 ml) (v/v). On elution with 20% MeOH/EtOAc the tosic salts of the product and of DMA were recovered. Evaporation of the solvent gave a yellow solid which was dissolved in DCM/1N $NaHCO_3$ [100 ml, 1:1 (v/v)]. The organic phase was washed with H_2O (50 ml), dried (Na_2SO_4) and evaporated to a yellow oil. This was purified by chromatography [flash silica, EtOAc/DCM

(10% to 30%, v/v)] to give the amine (181) (228 mg, 0.67 mmol, 68% yield) as a white foam.

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.22

ν_{max} ($CHCl_3$) : 1790 (s, β-lactam C=O), 1747 (s, ester C=O), 1687 (m), 1604 (m), 1500 (m), 1456 (m), 1303 (s), 1196 (s), 1090 (s), 1055 (s), 955 (m), 798 (m)

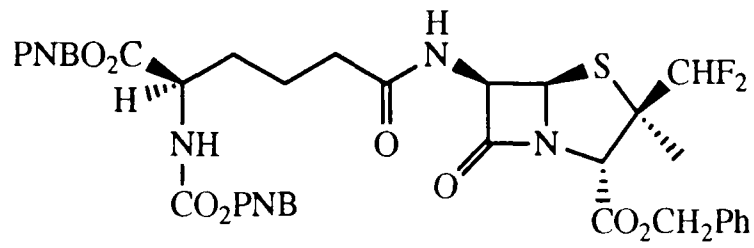
δ_H (200 MHz, $CDCl_3$) : 1.42 (3H, s, C<u>H</u>$_3$), 1.82 (2H, br.s, N<u>H</u>$_2$), 4.54 (1H, d, J 4 Hz, H_2NCHC<u>H</u>S), 4.86 (1H, s, C<u>H</u>CO_2Bn), 5.22 (2H, s, C<u>H</u>$_2$Ph), 5.62 (1H, dd, J 2 and 4 Hz, H_2NC<u>H</u>CHS), 5.66 (1H, t, J_{H-F} 56 Hz, C<u>H</u>F_2), 7.39 (5H, s, aromatic C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 18.10 (q, <u>C</u>H$_3$), 62.61 and 64.11 (2 × d, H_2N<u>C</u>H<u>C</u>HS), 67.72 (t, <u>C</u>H_2Ph), 70.84 (d, <u>C</u>HCO_2Bn), 116.04 (dt, J_{C-F} 249 Hz, <u>C</u>HF_2), 128.73 (d, aromatic <u>C</u>H), 134.15 (s, aromatic C1 of CH_2Ph), 166.52 and 174.48 (2 × s, 2 × <u>C</u>=O)

m/z [DCI (NH_3)] : 343 ([MH^+], 100 %), 315 (90%), 286 (41%), 91 (47%)

(2<u>S</u>,3<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-3-difluoromethyl-3-methyl-6-[(5<u>R</u>)-5-<u>N</u>-*p*-nitrobenzyloxy-carbonylamino-5-*p*-nitrobenzyloxycarbonylpentanamido]-7-oxo-4-thiabicyclo[3.2.0] heptane-2-carboxylic acid benzyl ester (182)

$C_{36}F_2H_{35}N_5O_{12}S$; MW 799



To a solution of (181) (106 mg, 0.31 mmol) in dry DCM (4 ml) was added EEDQ[220] (84 mg, 0.34 mmol, 1.1 eq), <u>N</u>-4-nitrobenzyloxycarbonyl-<u>D</u>-α-aminoadipic acid-α-nitrobenzyl ester (105) (147 mg, 0.31 mmol, 1 eq) and anhydrous Na_2SO_4 (20 mg). The resulting suspension was stirred under argon for 39 h. The solvent was evaporated to dryness, and the residue taken up in EtOAc/H_2O [100 ml, 1:1 (v/v)]. The organic phase was washed with 2N HCl (50 ml), saturated $NaHCO_3$ (50 ml), brine (50 ml), dried (Na_2SO_4) and evaporated to give the product (240 mg, 0.30 mmol, 97% yield).

T.l.c. [EtOAc/DCM (50%,v/v)] Rf 0.57

ν_{max} (KBr) : 3373 (m), 1794 (s, β-lactam C=O), 1746 (s, ester C=O), 1608 (m), 1522 (s, NO_2), 1455 (m), 1348 (s, NO_2), 1248 (m), 1210 (m), 1174 (m), 1069 (m), 737 (m)

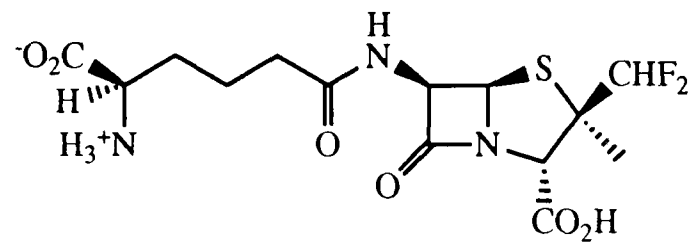
δ_H (500 MHz, $CDCl_3$) : 1.39 (3H, s, C<u>H</u>$_3$), 1.70-2.04 (4H, m, CHC<u>H</u>$_2$C<u>H</u>$_2$), 2.30-2.45 (2H, m, CH_2C<u>H</u>$_2$CO), 4.42-4.51 (1H, m, C<u>H</u>CH_2CH_2), 4.89 (1H, s, C<u>H</u>CO_2Bn), 5.18-5.33 (6H, m, 2 × C<u>H</u>$_2$ArNO$_2$ and C<u>H</u>$_2$Ph), 5.59 (1H, dd, J 4 and 8 Hz, NHC<u>H</u>CHS), 5.66 (1H, d, J 4 Hz, NHCHC<u>H</u>S), 5.75 (1H, t, J_{H-F} 56 Hz, C<u>H</u>F_2), 6.10 (1H, d, J 8 Hz, N<u>H</u>), 6.81 (1H, d, J 8 Hz, N<u>H</u>), 7.32-7.57 (9H, m, aromatic C<u>H</u>), 8.09-8.20 (4H, m, aryl C<u>H</u>)

δ_C (125.8 MHz, $CDCl_3$) : 17.68 (q, <u>C</u>H$_3$), 20.88 (t, CHCH$_2$<u>C</u>H$_2$), 31.01 (t, CH<u>C</u>H$_2$CH$_2$), 34.48 (t, <u>C</u>H$_2$CO), 53.65 (d, <u>C</u>HCH$_2$CH$_2$), 60.05 and 62.60 (2 × d, NH<u>C</u>H<u>C</u>HS), 65.08 and 65.24 (2 × t, 2 × <u>C</u>H$_2$ArNO$_2$), 67.51 (t, <u>C</u>H$_2$Ph), 68.44 (s, <u>C</u>CH$_3$), 68.62 (d, <u>C</u>HCO$_2$Bn), 116.13 (dt, J_{C-F} 249 Hz, <u>C</u>HF$_2$), 123.29, 123.40, 127.64, 128.00, 128.33, 128.40 and 128.51 (7 × d, aromatic <u>C</u>H), 134.15 (s, aromatic C1 of CH_2Ph), 142.38 and 143.59 (2 × s, aromatic C1 of CH_2ArNO_2), 147.25 and 147.44 (2 × s, aromatic C4 of CH_2ArNO_2), 155.46 (s, NH<u>C</u>O$_2$PNB), 165.94, 167.19, 170.34 and 171.51 (4 × s, <u>C</u>=O)

m/z (FAB) : 822 ([MNa^+], 100%), 800 ([MH^+], 58 %)

(2<u>S</u>,3<u>R</u>,5<u>R</u>,6<u>R</u>)-1-Aza-3-difluoromethyl-3-methyl-6-[(5<u>R</u>)-5-amino-5-carboxypenta-namido]-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (155)

$C_{14}F_2H_{19}N_3O_6S$; MW 395



To a solution of (182) (240 mg, 0.3 mmol) in THF/H_2O [15 ml, 1:1(v/v)] was added $NaHCO_3$ (25 mg, 0.3 mmol, 1 eq) and 10% Pd/C (340 mg). The suspension was purged with hydrogen (5×) and stirred vigorously under 1 atmosphere of H_2 for 3h. The reaction mixture was filtered through a pad of celite, washing with H_2O, and the THF removed under vacuum. The aqueous solution was washed with EtOAc (3 × 20 ml) and freeze-dried to give the crude product. This was purified by chromatography [HPLC, Gilson system, solvent 10 mM NH_4HCO_3, flow rate 4 ml/min] to give the product (155) (retention time 6 min, *ca.* 50 mg by NMR calibration, 1.2 mmol, 40% yield).

δ_H (500 MHz, D_2O, HOD suppressed) : 1.54 (3H, s, $C\underline{H}_3$), 1.63-1.97 (4H, m, $CHC\underline{H}_2C\underline{H}_2$), 2.44 (2H, t, J 7 Hz, $CH_2C\underline{H}_2CO$), 3.72 (1H, t, J 6 Hz, $H3N^+C\underline{H}CO_2^-$), 4.67 (1H, s, $C\underline{H}CO_2H$), 5.40 (1H, d, J 3.7 Hz, $NHC\underline{H}CHS$), 5.72 (1H, br. s, $NHCHC\underline{H}S$), 5.98 (1H, t, J_{H-F} 56 Hz, $C\underline{H}F_2$)

m/z (ESMS) : 396 ([MH^+], 100%), 397 (32%), 398 (12%)

Incubation of (155) with DAOC/DACS

The 2β-difluoromethyl penicillin N (155) (1 mg) was incubated with DAOC/DACS (2 ml, 0.8 IU) according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR showed the presence of some new, very weak peaks in the β-lactam region. Attempted purification of the crude mixture by HPLC (Waters system, solvent 10 mM NH_4HCO_3, flow rate 1 ml/min) resulted in the isolation of no β-lactam products apart from the starting material (155).

Bioassay tests

A solution of 2β-difluoromethyl penicillin N (155) (0.4 mg/ml) was bioassayed according to the general procedure, to give the following inhibition zones (in mm) :

	E.coli (-)	*E. coli* (+)	*S. aureus*
(155)	29	0	18
(1) (25μg/ml)	24	0	22

The penicillin (155) (1 mg) was incubated with DAOC/DACS (6 ml, 0.03 IU) according to the general procedure. The crude incubation mixture was lyophilised and redissolved in H_2O (3 ml). This solution was tested for bioassay with the following results :

	E.coli (-)	*E. coli* (+)	*S. aureus*
incubation mixture	32	16	20
(1) (25μg/ml)	25	0	21

6.5 Experimental for Chapter 5

6.5.1 Experimental for Section 5.2

Incubations with recombinant DAOC/DACS

The incubations with soluble recombinant DAOC/DACS were performed with partially purified and purified enzyme according to the general procedure. The product composition was determined by 500 MHz ^{1}H-NMR analysis of the lyophilised crude incubation mixtures.

Incubations of different substrates were sometimes performed using different batches of enzyme, and so an overall comparison of conversion between the substrates was not possible. It was found, however, that a complete conversion of all the substrates could be achieved if the right amounts of enzyme and/or incubation times were used. The incubations with each substrate were repeated several times. It was also found that the activity was retained if the enzyme was exchanged from the TRIS buffer (50 mM TRIS.HCl, pH 7.5, 2 mM DTT) to an ammonium hydrogen carbonate buffer (10 mM NH_4HCO_3, 2 mM DTT) using a Sephadex PD-10 column, prior to the incubations. The ammonium hydrogen carbonate buffer is more suitable for ^{1}H-NMR observation of the crude incubation mixture as it is almost completly removed by lyophilisation. The results of the incubations are described in the table :

Substrate	Product of incubation
Penicillin N (1)	DAOC (2) + DAC (3) + trace of 3β-Hydroxycepham (40)
[3-^{2}H]Penicillin N (1d)	DAOC (2) + DAC (3) + 3β-Hydroxycepham (40)
DAOC (2)	DAC (3)
DAC (3)	3-Formyl cephalosporoates
3-Exomethylene (63)	DAC (3)
[4-^{2}H]-3-Exomethylene (63a)	DAC (3) + Spiroepoxide (122) + 3-Formyl cephal.
3β-Spiroepoxide (122)	3-Formyl cephalosporoates

6.5.2 Experimental for Section 5.3

Incubations with recombinant DAOCS

Incubations with soluble recombinant DAOCS were performed with partially purified enzyme (*ca.* 0.7 IU/ml) according to the general procedure for DAOC/DACS incubations. Incubation mixtures typically contained 0.3 ml of enzyme (*ca.* 0.21 IU), 200 µl cofactor solution and 0.5 mg of substrate in 0.5 ml H_2O. The enzyme was tested only in TRIS buffer (50 mM TRIS.HCl, pH 7.5, 2 mM DTT). All the test incubations were carried out with same batch of enzyme, and so a qualitative comparison of the extent of conversion between the different substrates was possible as described in the text. The incubations with each substrate were performed at least twice, and the results observed are described in the table :

Substrate	Product of incubation
Penicillin N (1)	DAOC (2) + trace of DAC (3)
[3-^{2}H]Penicillin N (1d)	DAOC (2) + trace of 3β-Hydroxycepham (40) (< 2%)
DAOC (2)	trace of DAC (3) (< 5%)
DAC (3)	No conversion
3-Exomethylene (63)	DAC (3)
[4-^{2}H]-3-Exomethylene (63a)	DAC (3) + Spiroepoxide (122) + 3-Formyl cephal.

APPENDIX A

Competitive kinetic Isotope Experiments

Appendix A

Competitive Kinetic Isotope Experiments

The use of isotope effects is one of the most important techniques for unravelling the mechanism of a chemical reaction. However, its application to biochemical systems is not straightforward, as the interpretation of an observed isotope effect is invariably complicated by the multistep nature of such reactions. In most enzymatic systems the maximal velocity is not dominated by a single step but depends on several "rate-contributing" or "partially rate-limiting" steps, which may lead to the reduction or suppression of an isotope effect on one of the steps.[278]

The steady-state kinetic behaviour of most enzymes generally obeys the Michaelis-Menten equation (1) :

$$v = \frac{V_{max}\,[S]}{K_m + [S]} \qquad (1)$$

where v = velocity
V_{max} = maximal velocity
$[S]$ = substrate concentration
K_m = substrate concentration at which $v = 1/2\ V_{max}$

Isotope effects are usually measured as changes in the kinetic parameters V_{max} and V_{max}/K_m, which are complex functions of several rate constants. Consequently, a reduction in the magnitude of one of these rate constants as a result of isotopic substitution will be expressed to a greater or lesser extent on either V_{max} or V_{max}/K_m, depending upon the relationships between the rate constants within these complex functions. As such, it is useful to define these relationships by the use of a model for the enzymatic reaction. A simple model to use is :

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} EP \xrightarrow{K_3} E + P$$

In this model, the enzyme-substrate complex (ES) is in thermodynamic equilibrium with the free enzyme (E) and substrate (S), and is transformed into the enzyme-product complex (EP) in the first irreversible step. Assuming the system is in steady-state equilibrium ([ES] and [EP] are constant), the following expressions can be derived :

as $v = K_3 [EP]$ and $[EP] = \frac{K_2}{K_3} [ES]$

$$v = K_2 [ES] \tag{2}$$

as $$\frac{\partial[ES]}{\partial t} = K_1[E][S] - K_{-1}[ES] - K_2[ES] = 0 \tag{3}$$

and $[E] = [E_o] - [ES] - [EP]$ where E_o = total amount of enzyme

it can be shown that :

$$[ES] = \frac{[E_o]\,[S]}{\frac{K_{-1} + K_2}{K_1} + \frac{K_2}{K_3}[S] + [S]} \tag{4}$$

Substituting (4) in equation (2) and rearranging, gives :

$$v = \frac{\frac{K_2K_3}{K_2 + K_3}[E_o]\,[S]}{\frac{K_3(K_{-1} + K_2)}{K_1\,(K_2 + K_3)} + [S]} \tag{5}$$

Expression (5) is the Michaelis-Menten equation for this system, showing that the expressions for V_{max} and V_{max}/K_m are :

$$V_{max} = \frac{K_2K_3}{K_2 + K_3}[E_o] \tag{6}$$

$$K_m = \frac{K_3\,(K_{-1} + K_2)}{K_1\,(K_2 + K_3)} \tag{7}$$

and

$$V_{max}/K_m = \frac{K_1\,K_2}{K_{-1} + K_2}[E_o] \tag{8}$$

From equations (6) and (8) it is clear that the expression for V_{max} contains only rate constants for events occurring after the formation of ES, and the expression for V_{max}/K_m contains only rate constants for events up to and including the formation of EP, that is, the first irreversible step (the same can be demonstrated for enzymatic models envolving more steps). As a consequence, isotope effects on V_{max} or on V_{max}/K_m give additional and complementary information about an enzyme catalysed process.

In general, the experimental design used for isotope effect experiments can be divided into three categories : noncompetitive, competitive intermolecular and competitive intramolecular experiments. In the noncompetitive approach, the kinetic parameters V_{max} and K_m for the natural and isotopically labelled substrate are measured independently, and differences in these values are interpreted as isotope effects on V_{max} or on V_{max}/K_m. In the intermolecular competitive approach, both labelled and unlabelled substrate are present in the incubation mixture at the same time, and the isotope effects are measured through the changes in isotopic distribution of the substrate and product, after incubation. Intramolecular competitive isotope effects were introduced to eliminate the possible masking effect of other steps with regard to the isotopically sensitive one. In these experiments, the substrate must have a minimum of two symmetrical sites that can be metabolised, and only one of them is labelled. Again, the isotopic distribution of the substrate and product can reveal the relative rate of reaction of each position. If the rate of interchange between the enzyme-bound orientations from which the labelled and unlabelled positions are metabolised is fast relative to the isotopically sensitive step, the intrinsic isotope effect for that step will be observed.

The intermolecular competitive approach is usually the prefered method for isotope effect studies, because only the isotopic distribution of substrate and product need to be measured, and so the experimental errors are usually very small, especially compared to noncompetitive methods where two sets of measurements taken in different conditions are compared. However, competitive experiments only give the relative rate of conversion of the labelled and unlabelled substrates and so the individual values of V_{max} and K_m cannot be obtained.

The overall rate of conversion v_T of an isotopic substrate mixture is given by[279] :

$$v_T = v + v' = \frac{V_{max}[S]}{[S] + K_m\,(1 + \frac{[S']}{K_m'})} + \frac{V_{max}'[S']}{[S'] + K_m'\,(1 + \frac{[S]}{K_m})} \qquad (9)$$

where v and v' are the partial reaction rates of the unlabelled and labelled substrates, S and S'. Equation (9) corresponds formally to the expression used for describing competitive inhibition, but it cannot be concluded that the labelled substrate acts necessarily as a competitive inhibitor. From equation (9) it can be shown that the ratio of the partial rates is always proportional to the ratio of the substrate concentrations :

$$\frac{v}{v'} = \frac{V_{max}/K_m}{V_{max}'/K_m'}\,\frac{[S]}{[S']} \qquad (10)$$

Equation (10) reveals that in competitive intermolecular experiments the ratio of the parameter V_{max}/K_m for labelled and unlabelled substrate is obtained. As this parameter contains only rate constants for events up to and including the first irreversible step, this type of experiments will provide information only about these events. As such, an isotope effect will be observed in competitive experiments only if the isotopically sensitive step is not preceded by an irreversible one. Isotope effects for steps occurring after an irreversible event can only affect the parameter V_{max}, and so will only be observed in noncompetitive experiments.

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