STUDIES ON THE BIOSYNTHESIS OF PENICILLINS

Gordon A. Thomson

A Thesis Presented for
the Degree of
Doctor of Philosophy
University of Edinburgh
1984

This thesis is the original composition of the author's work, unless otherwise stated, and has not been submitted for any other degree. Certain results included in this thesis have already been published.

ACKNOWLEDGEMENTS

I would especially like to thank Dr. R.L. Baxter and Professor A.I. Scott for their help and guidance during the course of this work.

I would also like to thank Miss Caroline McEwan and Mr. S. Miller for technical assistance.

to Karen, to my Mum

and to my Dad

TABLE OF CONTENTS

EXPERIMENTAL

A B S T R A C T

A high yielding synthesis of the acyclic precursor of the penicillins, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) was developed. Using this synthesis the isotopically enriched ACV peptides, δ-(DL-α-aminoadipyl)-L-cysteinyl-D-(3R)-[4-^{13}C]-valine and δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine were prepared. In an extension to this work the tetrapeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine (LLD-ACVG) was prepared.

A putative intermediate in the biosynthesis of isopenicillin N, *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (LLD-AC(N-OH)V), was synthesised from *N*-hydroxy-D-valine benzyl ester.

A cell-free system capable of converting δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N was obtained from cultures of the cephalosporin producing fungus *Cephalosporium acremonium* CW19. The cell-free system was used to test the importance of the above synthesised compounds in penicillin biosynthesis. *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine was shown not to be a substrate for the enzyme "isopenicillin N synthetase" but to be a potent inhibitor of the LLD-ACV to isopenicillin N conversion. The tetrapeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine, was found to be a weak inhibitor of this conversion.

The isotopically enriched ACV peptides were used to confirm the stereochemistry of the valine C3 centre in biosynthesised δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and to allow preliminary ^{13}C n.m.r. studies on the biosynthesis of isopenicillin N.

LIST OF ABBREVIATIONS

7-ACA	7-aminocephalosporanic acid
AC(N-OH)V	*N*-[δ-(α-aminoadipyl)cysteinyl]-*N*-hydroxyvaline
ACV	δ-(α-aminoadipyl)cysteinylvaline
ACVG	δ-(α-aminoadipyl)cysteinylvalylglycine
ACyaV	δ-(α-aminoadipyl)cysteicylvaline
$[\alpha]_D$	specific rotation at the sodium D line wavelength of 589nm
a.m.u.	atomic mass units
6-APA	6-aminopenicillanic acid
Ar	aryl or aromatic
ATP	adenosine triphosphate
aq.	aqueous
br	broad
c	concentration (g/100ml)
cm	centimeter(s)
CoA	coenzyme A
cys	cysteine
d	doublet
DCC	dicyclohexylcarbodiimide
decomp.	decomposed
δ_C	^{13}C n.m.r. chemical shift(s)
δ_H	^{1}H n.m.r. chemical shift(s)
DMF	*N*,*N*-dimethylformamide
DTT	dithiothreitol
EI	electron impact
ether	diethyl ether

eV	electron volt(s)
FAB	fast atom bombardment
FID	free induction decay
g	gram(s) or acceleration due to gravity
GACV	glycyl–δ–(α–aminoadipyl)cysteinylvaline
GITC	2,3,4,6–tetra–*O*–acetyl–β–D–glucopyranosyl isothiocyanate
gly	glycine
h	hour(s)
1–HOBt	1–hydroxybenzotriazole
h.p.l.c.	high performance liquid chromatography
Hz	hertz
i.r.	infra–red
J	spin–spin coupling constant(s)
LLD–AC(N–OH)V	*N*–[δ–(L–α–aminoadipyl)–L–cysteinyl]–*N*–hydroxy–D–valine
LLD–ACV	δ–(L–α–aminoadipyl)–L–cysteinyl–D–valine
LLD–ACVG	δ–(L–α–aminoadipyl)–L–cysteinyl–D–valylglycine
LLD–ACyaV	δ–(L–α–aminoadipyl)–L–cysteicyl–D–valine
LLD–ACyaVG	δ–(L–α–aminoadipyl)–L–cysteicyl–D–valylglycine
lys	lysine
m	multiplet
M	molar
mg	milligram(s)
MHz	megahertz
MIC	minimum inhibitory concentration
µg	microgram(s)
µl	microlitre(s)
min	minute(s)

ml	millilitre(s)
mm	millimeter(s)
mmol	millimolar or millimole(s)
MOPS	morpholinopropanesulphonic acid
m.p.	melting point
m/s	meters per second
m/z	mass (m) to charge (z) ratio
N	normal
nm	nanometers
n.m.r.	nuclear magnetic resonance
ν	infra-red absorption frequencies
Ph	phenyl
p.p.m.	parts per million
p.s.i.	pounds per square inch
q	quartet
r.p.m.	revolutions per minute
s	singlet
sat.	saturated
sec	second(s)
sept.	septet
t	triplet
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
TsOH	4-toluenesulphonic acid
UV	ultraviolet
v	volume
V	volt(s)
val	valine
w	weight

I have attended the following lectures and seminars:-

Natural Products - Professor A.I. Scott (5 lectures).

Biosynthesis - Dr. T.J. Simpson (5 lectures).

Current Topics in Organic Chemistry - Organic Staff (15 lectures).

Bio-Organic Chemistry - Invited Speakers (5 lectures).

Modern Synthetic Methods in Organic Chemistry - Dr. G. Tennant (5 lectures).

Synthesis and Mechanism of Action of the β-Lactam Antibiotics - Glaxo Research (5 lectures).

Pulse Sequences and Applications in n.m.r. Spectroscopy - Dr. G. A. Morris (5 lectures).

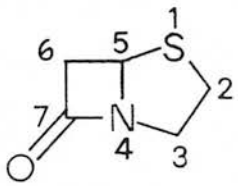
Medicinal Chemistry - Dr. R.M. Paton (5 lectures).

Professor Scott's research group seminars over a period of one year.

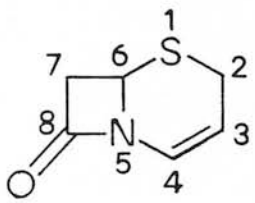
Various departmental colloquia and seminars, and Monday evening organic seminars over three years.

N O T E S

1. The penam[1] and cephem[2] ring systems are numbered non-systematically from the sulphur and not from the bridgehead nitrogen.



penam



3-cephem

The abbreviated nomenclature and numbering of β-lactam antibiotics is discussed in detail by Brown [3].

2. The synthesis and reactivity of β-lactams (azetidin-2-ones) and the general chemistry of the β-lactam antibiotics is introduced only where relevant and is not fully reviewed. Excellent reviews are available [4-23].

I N T R O D U C T I O N

1. BIOLOGICALLY ACTIVE β-LACTAMS: DISCOVERY AND DRUG DEVELOPMENT

1.1 THE PENICILLINS [17, 19-21, 24-28]

In 1929 Alexander Fleming published a report [29] in which he described the "inhibitory, bactericidal and bacteriolytic properties" of "penicillin". A contaminating mould, on a *Staphylococcus*-seeded plate, lysed the surrounding *Staphylococcus* colonies. Fleming tentatively identified this mould as *Penicillium rubrum* and noted that the filtrate, from seven day old nutrient broth cultures of *P. rubrum* had bactericidal properties. Fleming gave this active filtrate the name "penicillin" and made a number of important observations. "Penicillin" was found to be unstable at room temperature, to kill only growing staphylococci and to have negligible toxicity in animals and man (or, as Fleming noted, to be "no more toxic than the nutrient broth itself").

It was not until late 1937 that Florey and Chain, at Oxford, further investigated the lytic properties of "penicillin". Their initial assumption, based on Fleming's observations, that the active principle was a mould lysozyme was quickly dispelled. The active principle rapidly diffused through cellophane membranes indicating a low molecular weight compound. Early attempts to purify the compound were hampered by the small quantities of material produced, the elaborate purification procedure used, and the chemical instability of the material. Nevertheless by 1941 the value of penicillin in the treatment of infectious diseases had been successfully demonstrated [30, 31].

The economic problems faced by Britain during the war led to the establishment of a joint Anglo-American research and development programme aimed at improving the fermentation yield of penicillin and elucidating its molecular structure [25].

Coghill and co-workers at the Northern Regional Research Laboratories in Illinois were instrumental in securing the first objective [32-35]. All previous research had been carried out with surface cultures of *Penicillium notatum*. The isolation of high producing mutants of *Penicillium chrysogenum* (from X-ray irradiation) which could be grown in submerged, aerated cultures, greatly increased the yield of penicillin. The use of corn steep liquor in the culture medium, in place of the less readily available yeast extract, had an important impact on the structural studies taking place in both Britain and America.

Apart from the low purity of the initially available penicillin [36,37], it soon became apparent that British and American scientists were not studying the same compound. Inconsistent analytical and degradative results were obtained in different laboratories. Eventually, however, the degradative, synthetic and spectroscopic studies began to bear fruit [25, 26, 38]. The volume of work undertaken during this period is difficult to summarize and only the degradative pathways important to the assignment of the penicillin structure are shown (Figure 1).

The discrepancy between the British and American results lay solely in the carboxylic acids isolated upon hydrolysis of the penilloaldehydes ($RCONHCH_2CHO$ in Figure 1). In America phenylacetic acid was obtained, and at Oxford 2-hexenoic acid. Exhaustive as these studies were the available data allowed three possible structures; a fused β-lactam-thiazolidine structure (1), a thiazolidine-oxazolone (2) and, less likely, a tricyclic system (3).

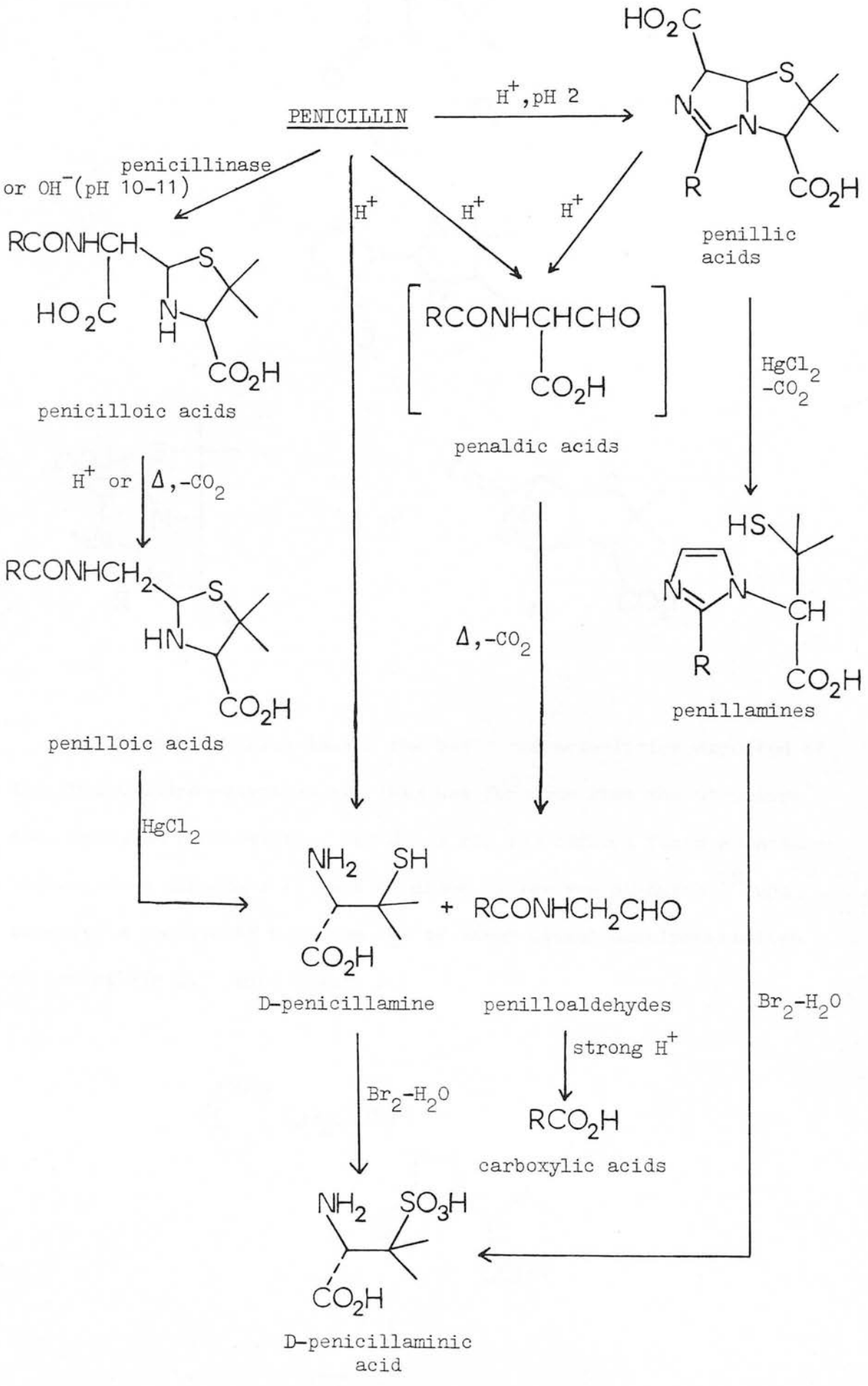
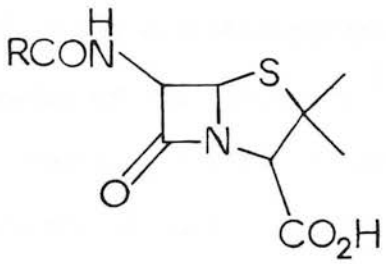

PENICILLIN
H+, pH 2
penicillinase
or OH− (pH 10-11)
H+
H+
H+
penillic acids
penicilloic acids
penaldic acids
HgCl2 -CO2
H+ or Δ, -CO2
penilloic acids
Δ, -CO2
penillamines
HgCl2
D-penicillamine
+ RCONHCH2CHO
penilloaldehydes
Br2-H2O
strong H+
Br2-H2O
carboxylic acids
D-penicillaminic acid

FIGURE 1



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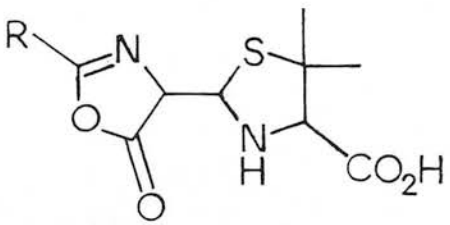

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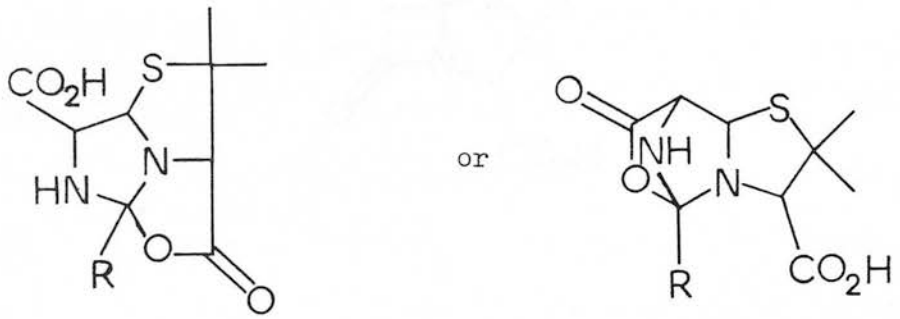
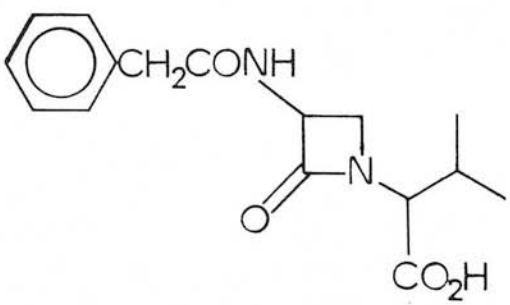


3

Although penicillin lacked the basic characteristics expected of the thiazolidine-oxazolone (2) this was for some time the structure most favoured by chemists. Evidence for the correct fused β-lactam-thiazolidine structure (1) was obtained by workers at Merck [25] who prepared a monocyclic β-lactam (4) by Raney nickel desulphurization of penicillin G. (See Figure 2)



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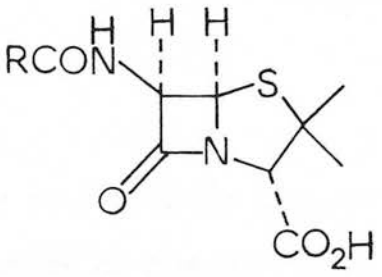
Finally, in 1945, X-ray chrystallographic analysis of penicillin provided conclusive proof of the structure [25] and confirmed the relative stereochemical configuration of the asymmetric centres in the molecule. The structures of various penicillins, isolated and identified during the period 1941-45, are shown in Figure 2.

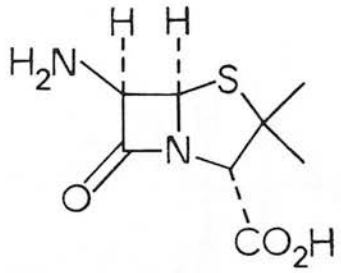


5

R	Penicillin
$CH_3CH_2CH{=}CHCH_2-$	F (2-pentenyl)
$CH_3CH{=}CHCH_2CH_2-$	(3-pentenyl)
$CH_3CH_2CH_2CH_2CH_2-$	Dihydro-F (*n*-amyl)
$PhCH_2-$	G (Benzyl)
$CH_3CH_2CH_2CH_2CH_2CH_2CH_2-$	K (*n*-heptyl)
$4\text{-}HOC_6H_4CH_2-$	X (*p*-hydroxybenzyl)

FIGURE 2

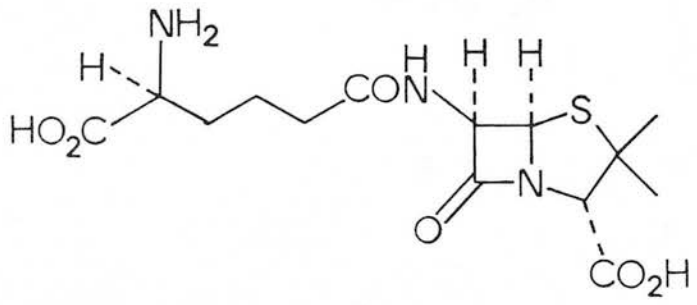
All the penicillins are side-chain acyl derivatives of a parent nucleus, 6-aminopenicillanic acid (6-APA) (6).



6

The production of penicillin G in corn steep liquor fermentations was due to the presence of 2-phenylethylamine [39]. Supplementation of the fermentation medium with phenylacetic acid further improved the yield of penicillin G [35]. Subsequently more than one hundred different penicillins were made by adding suitable side-chain precursors to the culture medium [25, 40-43]. Despite the large number of "biosynthetic" penicillins, this method is of limited utility. Only derivatives of monosubstituted acetic acids are produced [44] and, even then, not all monosubstituted acetic acids are incorporated as some prove toxic to the mould or are otherwise metabolized. Penicillin G and penicillin V (5, R=$PhOCH_2-$) are the only therapeutically useful penicillins obtained in this fashion [45].

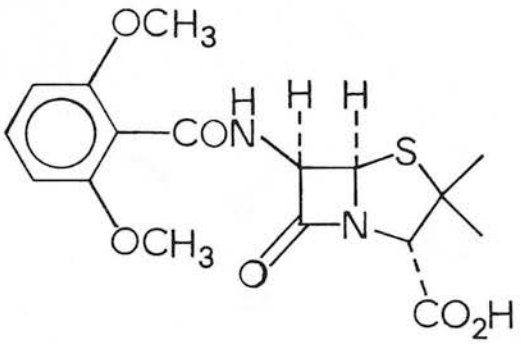
In 1957 workers at the Beecham Research Laboratories isolated small amounts of 6-aminopenicillanic acid (6-APA) [46, 47] (6) and isopenicillin N [48, 49] (7) from the culture fluid of *P. chrysogenum*, grown in the absence of side-chain precursors.



7

The action of bacterial, fungal or mammalian penicillin acylases on penicillin G or penicillin V allowed efficient production of 6-APA [50-55] and thus the potential of 6-APA as a precursor for the synthesis of penicillins with unnatural side-chains was quickly realised. Chemical methods for producing 6-APA were developed later [20, 55, 56].

The availability of 6-APA allowed the chemical introduction of an almost infinite variety of acyl side-chains [17, 20, 57] designed to improve the characteristics of penicillin. These "semi-synthetic" penicillins display increased acid stability [58], allowing oral administration of the drug, and improved resistance to pencillinase enzymes [58-79] (e.g. methicillin [59-61] (8) and oxacillin [65-67] (9)). Acids or penicillinases convert penicillins to biologically inactive compounds (see Figure 1).



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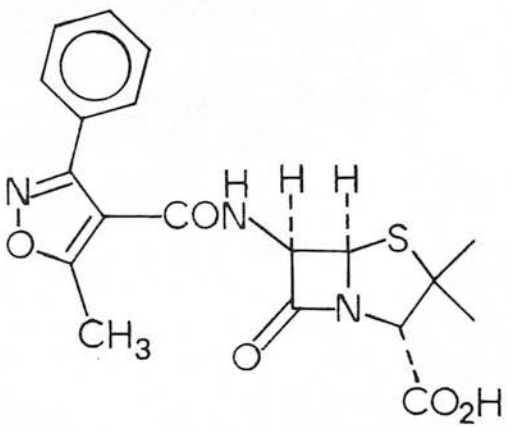
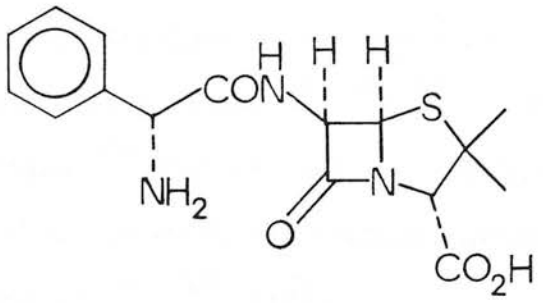


9

Unlike penicillin G the "semi-synthetic" penicillins show activity against many gram-negative bacteria [58, 80-92] including *Pseudomonas aeruginosa*, indole-positive *Proteus*, *Serratia*, *Providentia* and *Citrobacter* species [93-103] (e.g. ampicillin [80-85] (10), carbenicillin [93-96] (11) and piperacillin [102, 103] (12)).



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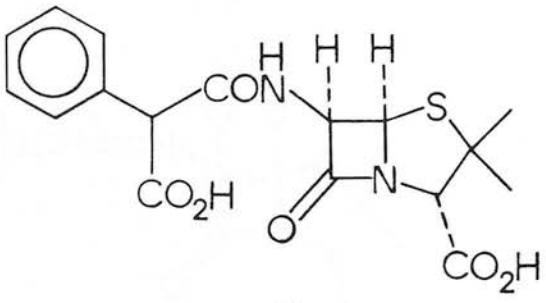

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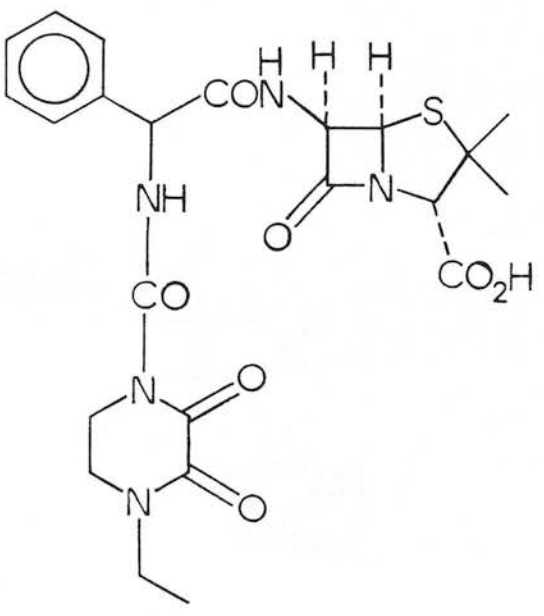
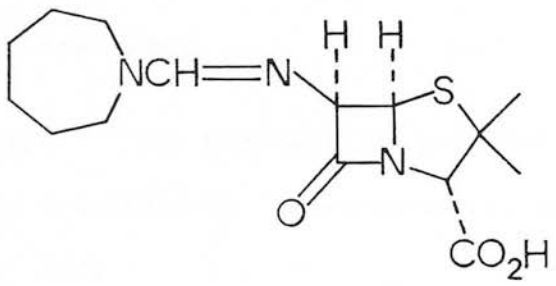


12

Only an extremely small number of chemical modifications to the basic acylated 6-aminopenicillanic acid structure (5) afford compounds with useful anti-bacterial activity [20, 58, 104]. The most important examples are mecillinam [105] (13) which is active against gram-negative bacteria and penicillins in which the carboxyl group has been replaced by a 5-tetrazolyl moiety [106, 107] (14).



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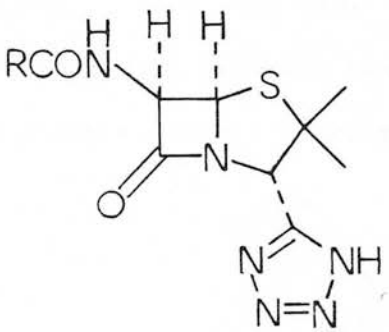

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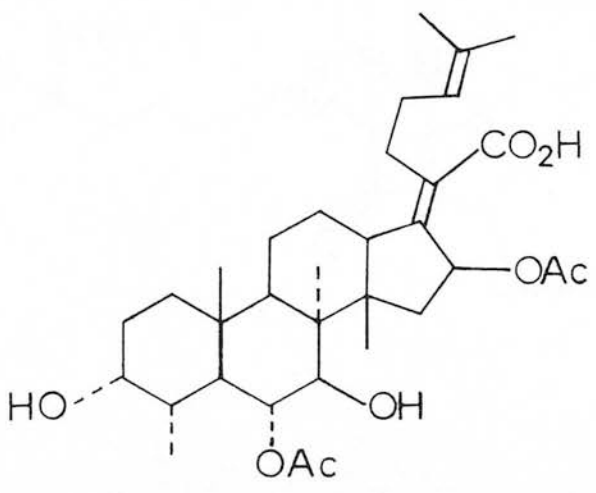
1.2 THE CEPHALOSPORINS [19, 28, 108-110]

Around the time that the structure of penicillin was being confirmed, Brotzu, in Sardinia, was studying the microbial flora of seawater near a sewage outlet. His search for antibiotic producing organisms led to the isolation of a fungus, similar to *Cephalosporium acremonium*, which inhibited the growth of both gram-positive and gram-negative bacteria. The filtrate from a particular strain of this fungus, grown in glucose-starch medium, was biologically active. A more active concentrate, obtained by ethanol precipitation of inactive materials, was used in the successful treatment of typhoid, paratyphoid A and B and brucellosis.

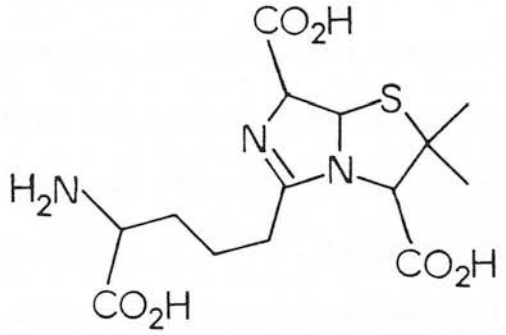
This work [111] was published in 1948 and in August or September of that year a culture of the organism was sent to Florey at Oxford for further study.

C. acremonium, grown in submerged aerated cultures, was filtered and the filtrate extracted with butyl acetate. Following counter-current distribution, one major and four minor antibiotics were isolated. The major component, cephalosporin P_1, inhibited only certain gram-positive bacteria [112-115] and was, therefore, not responsible for the wide spectrum antibiotic activity observed by Brotzu. Cephalosporin P_1 (15) was shown to have a tetracyclic triterpenoid structure [116-118].



15

The culture filtrate, after butyl acetate extraction, contained an antibiotic substance [113, 114] some 2-6 times more active than penicillin G against gram-negative organisms. A partially purified sample of this material, called cephalosporin N, was obtained after charcoal and alumina chromatography and countercurrent distribution [119]. The material was sufficiently pure to establish a molecular formula, and to allow degradative analyses. Acid hydrolysis produced D-α-aminoadipic acid, D-penicillamine and CO_2 [120]. The penillic acid (16) was obtained after treatment with dilute acid (pH 2.7) and this, along with other degradative results, confirmed the penicillin structure [121] (17). Cephalosporin N was identical to synnematin B [121, 122] and has since been renamed penicillin N.



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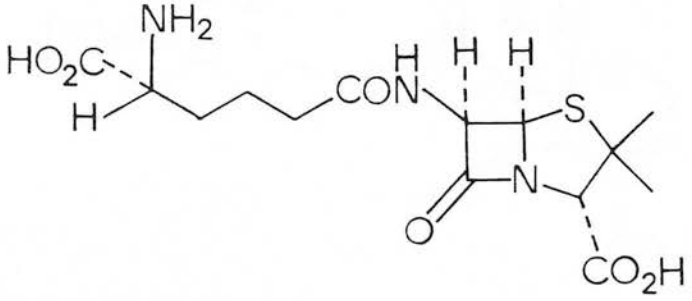
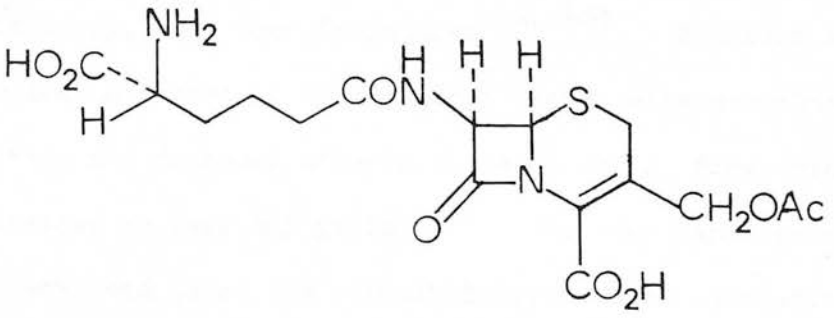


17

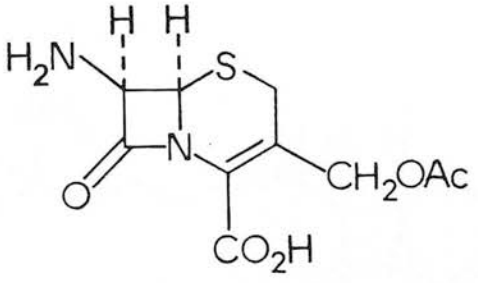
During purification of the penillic acid (16) by ion-exchange chromatography, a new material, cephalosporin C, was isolated in minute amounts [123, 124]. Although cephalosporin C showed only about 10% of the activity of penicillin N against a variety of organisms, its resistance to penicillinase hydrolysis aroused immediate interest. Mice infected with penicillin-resistant staphylococci were effectively treated using this new antibiotic and, in the hope that more powerful antibacterial compounds could be produced, semi-synthetically [125, 126] (as had happened with the penicillins), research was undertaken to establish the structure of cephalosporin C.

Initial degradative results [127] suggested a similarity to penicillin N. D-α- aminoadipic acid was obtained upon hydrolysis, the infra-red spectrum showed a strong β-lactam carbonyl absorption and valine was isolated after Raney nickel desulphurization and hydrolysis. However, penicillamine was not detected, the isolated valine was racemic and n.m.r. spectroscopy indicated the absence of the *gem* dimethyl groups characteristic of the penicillins. Based on these and other results [109, 128], Abraham and Newton proposed the fused β-lactam–dihydrothiazine structure (18) for cephalosporin C [129]. This structure accounted for the UV absorption maximum at 260nm [130, 131] and subsequently was confirmed by X-ray analysis [132].



18

To parallel the work undertaken on the penicillins, 7-amino-cephalosporanic acid (7-ACA, 19) was required.



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7-ACA had not been detected in the culture fluid of cephalosporin C producing organisms, and attempts to remove the D-α-aminoadipyl side-chain enzymically proved unsuccessful [133-138]. 7-ACA could be produced by hydrolysis [126] or acidolysis [139] of cephalosporin C but only in poor to moderate yields.

The first practical preparation of 7-ACA involved imine formation and hydrolysis [2] (Figure 3). The diazonium salt, generated in the presence of nitrosyl chloride and formic acid, is attacked intramolecularly by the amide carbonyl with elimination of nitrogen. The resultant imine is readily hydrolysed to afford 7-ACA in 40-50% overall yield.

A variety of methods for the preparation of 7-ACA, using this general strategy, have been introduced [55, 140]. Reaction of *N*, *N*-phthaloylcephalosporin C dibenzhydryl ester with phosphorus pentachloride and methanol affords an imino ether, from which 7-ACA can be obtained in over 80% yield [141]. Further improvements in yield are achieved using the trimethylsilyl ester derivatives of cephalosporin C or *N*, *N*-phthaloylcephalosporin C [142].

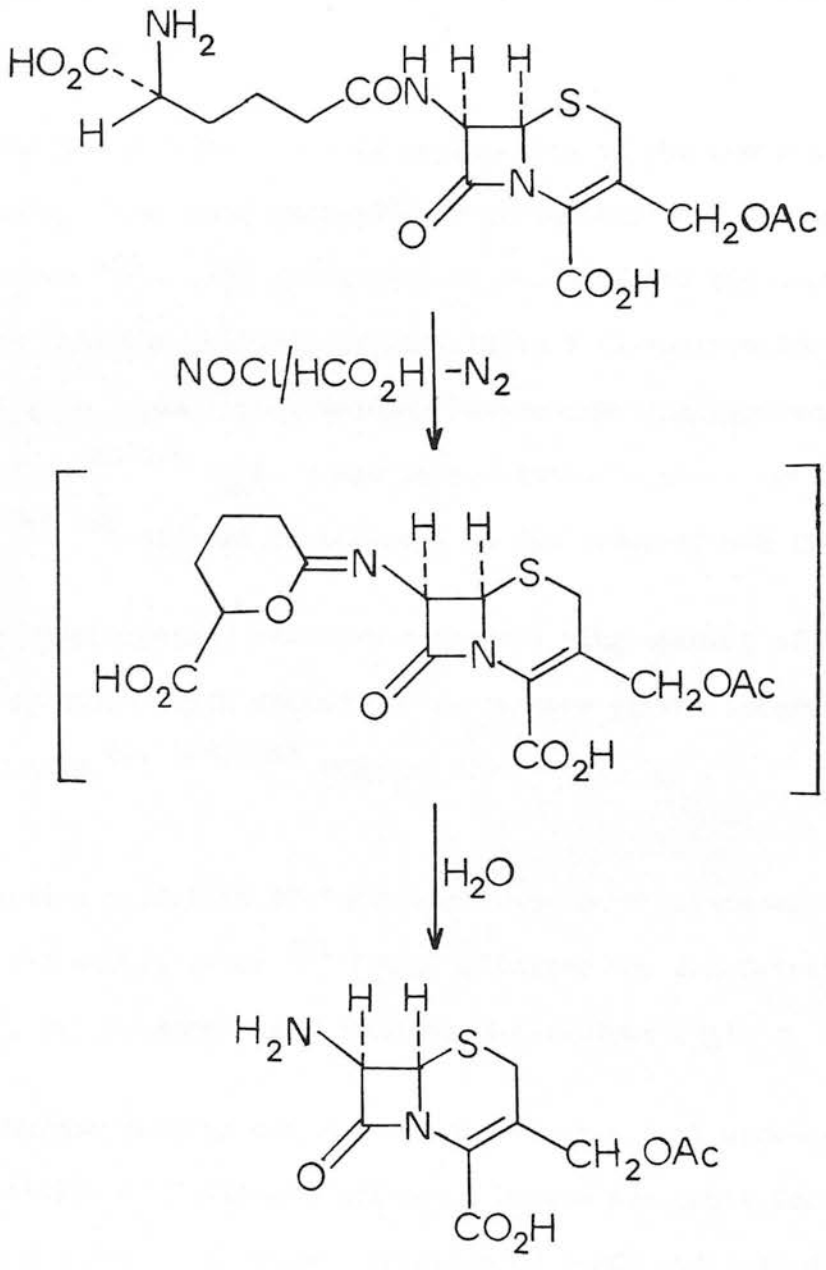

NH2
HO2C
H
CONH
H H H
S
N
O
CH2OAc
CO2H
NOCl/HCO2H
-N2
N
O
HO2C
H H
S
N
O
CH2OAc
CO2H
H2O
H2N
H H
S
N
O
CH2OAc
CO2H

FIGURE 3

More potent antibiotics could now be prepared by acylation of 7-ACA. However, a problem for the pharmaceutical industry was the supply of cephalosporin C. Even the most efficient industrial strains of *C. acremonium* produced cephalosporin C in only small amounts and, until recently [143], its extraction and purification had been time-consuming and wasteful.

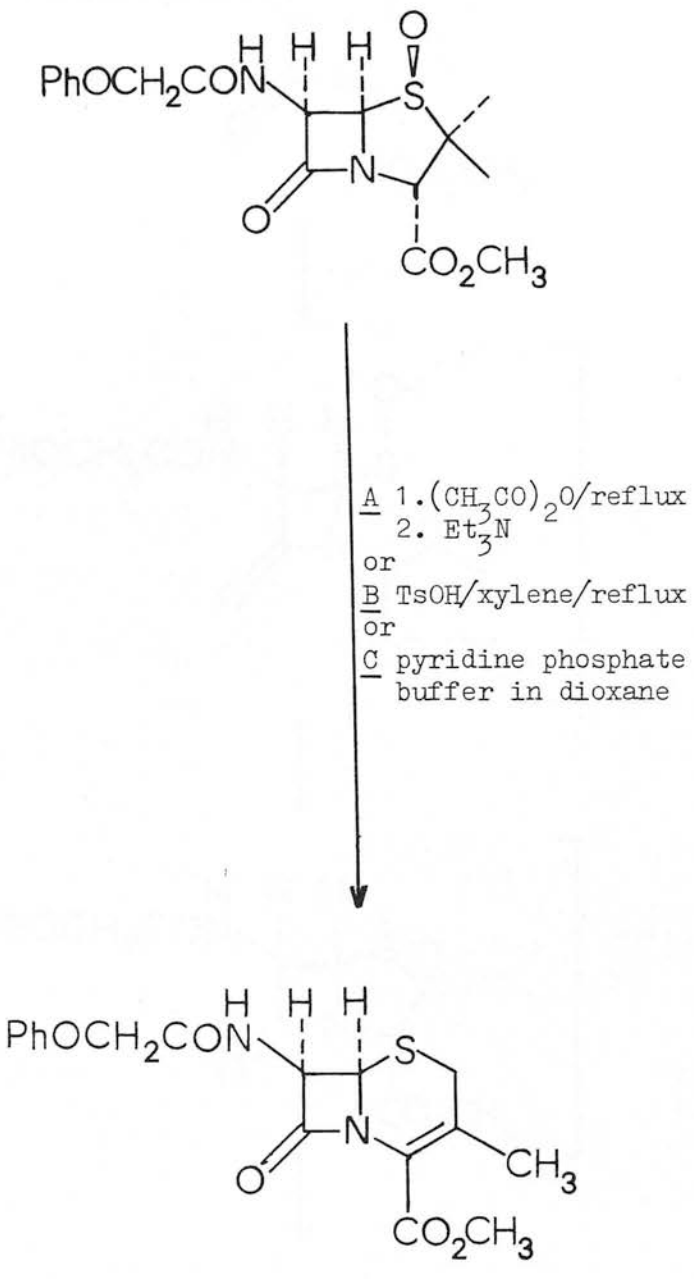
A possible solution to this problem lay in the chemical conversion of cheap, available penicillin V or penicillin G to cephalosporin C derivatives [144]. The conversion of the penam to the cephem nucleus involves a Pummerer reaction on penicillin V (S)-sulphoxide methyl ester, to give 7-phenoxyacetamido-3'-deacetoxycephalosporanic acid methyl ester [145-148] (20). Use of pyridine-phosphate buffers in dioxane [149, 150] allowed conversions in the order of 90% (Figure 4).

This rearrangement involves a thermal ring opening of the penicillin sulphoxide, with subsequent re-closure of the intermediate sulphenic acid [21, 144, 145] (Figure 5).

Selective oxidation of 7-phenoxyacetamido-3'-deacetoxycephalosporanic acid methyl ester [151] (20), or better the 2,2,2-trichloroethyl ester [152], can produce the 3'-acetoxy derivatives (21).

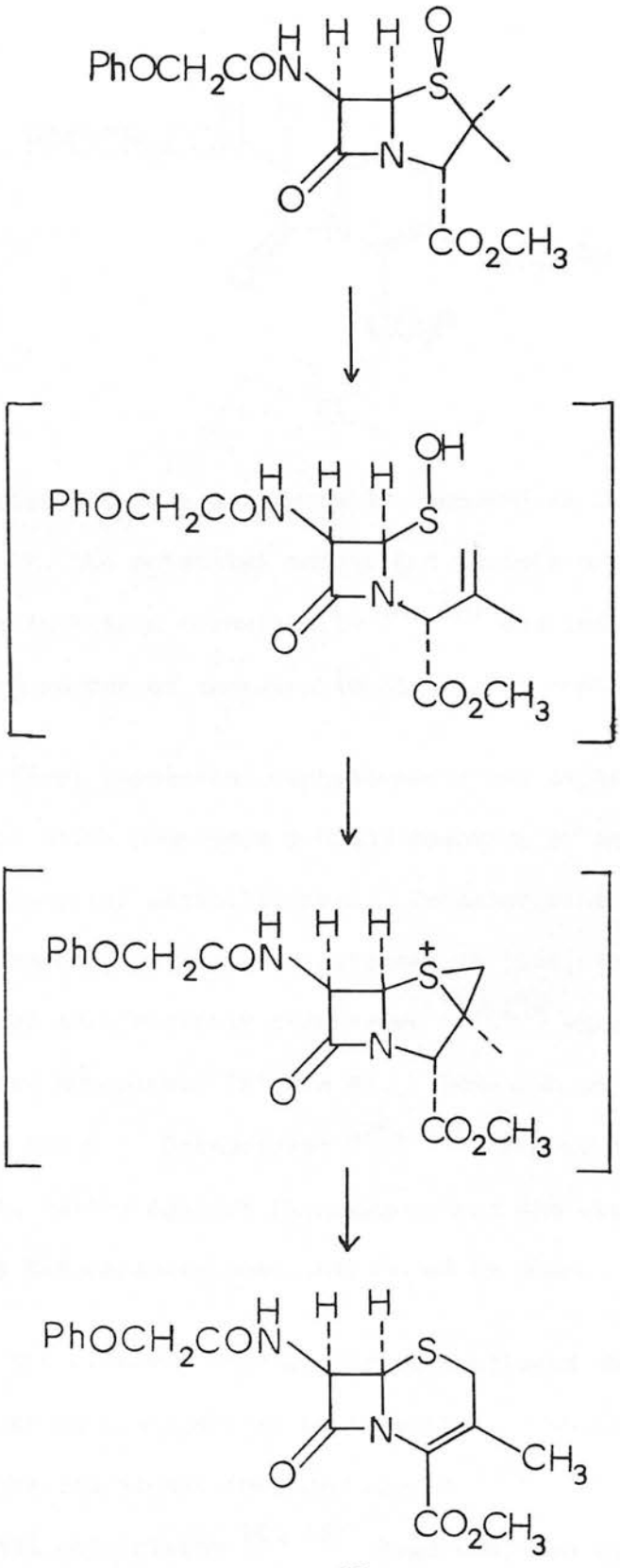
As acylase enzymes can remove the phenoxymethyl side-chain [134], a steady supply of 7-ACA and analogues became available for semi-synthetic studies. Chemical acylation of 7-ACA did indeed lead to the production of cephalosporins with improved antibacterial characteristics [58, 153].

The ready displacement of the 3' acetoxy group by N and S


PhOCH2CONH
H
H
H
O
S
N
O
CO2CH3
A 1.(CH3CO)2O/reflux
2. Et3N
or
B TsOH/xylene/reflux
or
C pyridine phosphate
buffer in dioxane
PhOCH2CONH
H
H
H
S
N
O
CH3
CO2CH3

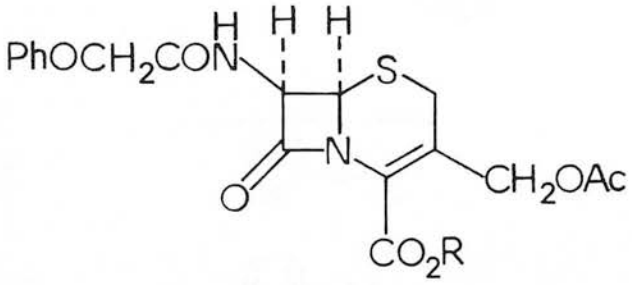
20

FIGURE 4


PhOCH2CONH
H
H
H
O
S
N
O
CO2CH3
OH
PhOCH2CONH
S
N
O
CO2CH3
PhOCH2CONH
S+
N
O
CO2CH3
PhOCH2CONH
S
N
O
CH3
CO2CH3

20

FIGURE 5

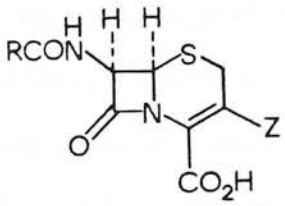


21

nucleophiles had been accidentally achieved in early experiments [154]. As a result, the potential number and variety of cephalosporin analogues increased dramatically [58, 153] and led to the development of a large number of therapeutically useful cephalosporins [155-157].

The first commercial cephalosporin was cephalothin [109, 158-162] (Figure 6) which possesses a broad spectrum of activity and stability to staphylococcal penicillinase. Cephaloridine [109, 162-168], with similar characteristics, was patented in 1965, followed by cefamandole [169-171] and more recently cefotaxime [172-174] which is resistant to β-lactamase hydrolysis (Figure 6). Research on the cephalosporins continues today. Ceftazidime [175, 176] (Figure 6), a broad spectrum antibiotic active against *Pseudomonas* spp and with good β-lactamase stability has recently been introduced by Glaxo.

All the clinical cephalosporins mentioned above lack oral activity and have to be administered by injection. While this is generally true of the cephalosporins, cephalexin [162, 168, 177-181], cefadroxil [182-184] and cefatrizine [185-187] (Figure 6) can be administered orally.



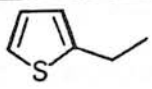
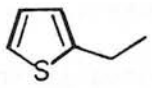
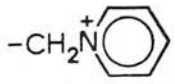
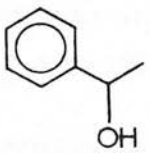
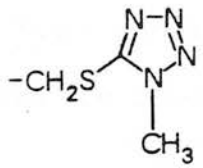
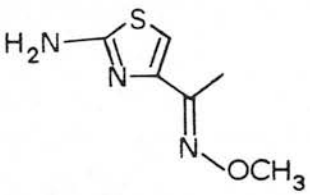
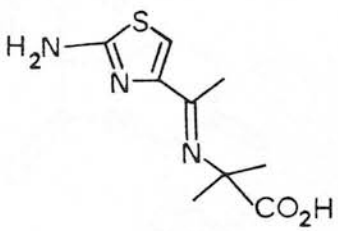
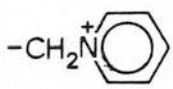
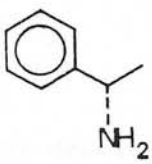
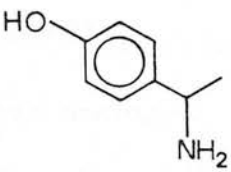
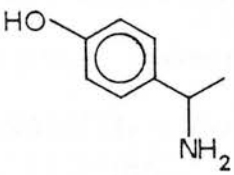
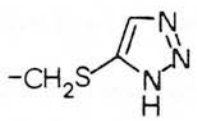
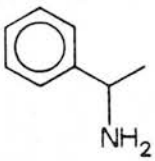
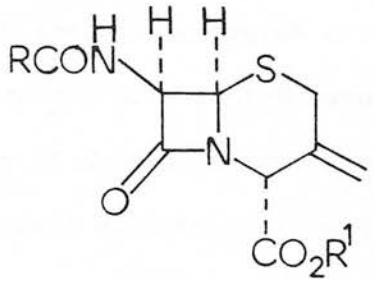
	R	Z
CEPHALOTHIN		$-CH_2OAc$
CEPHALORIDINE		
CEFAMANDOLE		
CEFOTAXIME		$-CH_2OAc$
CEFTAZIDIME		
CEPHALEXIN		$-CH_3$
CEFADROXIL		$-CH_3$
CEFATRIZINE		
CEFACLOR		$-Cl$

FIGURE 6

As with the penicillins, only acylation of 7β-aminocephalosporanic acid (19) produces biologically important compounds. Epimerization at the 7 position or introduction of other chemical groupings generally results in the loss of antibiotic activity.

Greater scope for variety exists at the 3 position where a substituted methylene is not essential for activity. Directly bonded methoxy, chloro or bromo derivatives can show enhanced antibacterial activity [188-190] and clinical potential, e.g. cefaclor [189, 191] (Figure 6). Key intermediates in the synthesis of these compounds are the 3-methylenecephams [188-190, 192-197] (22) which are conveniently prepared from penicillin sulphoxides.



22

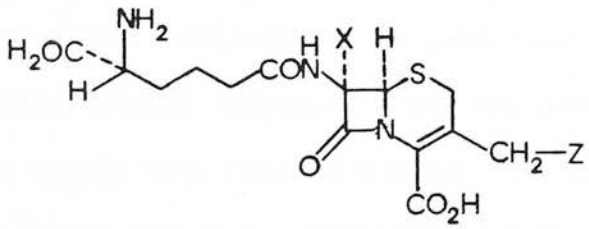
A most promising recent development has been the synthesis of 1-oxacephalosporins [198-200] which are some 4-10 times more active than their cephalosporin analogues. 1-oxacephalosporins, and others, have become known as "New Generation" β-lactam antibiotics. They display exceptional activity against many gram-negative organisms, including *E. coli*, *Klebsiella*, *Enterobacter*, *Serratia*, *Providencia*, *Citrobacter* and *Proteus* spp.[155].

1.3 THE CEPHAMYCINS [201, 202]

The discovery of the cephalosporin β-lactam antibiotics and scattered literature reports of various penicillin producing organisms [26, 203] prompted the major drug companies to undertake extensive screening programmes to identify novel β-lactam antibiotics and to isolate high producing strains of the known antibiotics.

From an initial total of 1852 cultures, workers at Lilly Research Laboratories selected two strains of *Streptomyces*, *S. lipmanii* and a new strain *S. clavuligerus*, for further study [204]. Three new antibiotics were obtained from the culture media following chromatographic separation and purification over charcoal, ion-exchange resin, Sephadex G-25, silica and microcrystalline cellulose. The physical-chemical properties of all three substances were similar to those of cephalosporin C. Acid hydrolysis produced D-α-aminoadipic acid, the UV spectra showed absorption maxima around 260nm and the i.r. spectra indicated the presence of β-lactam carbonyls. High resolution mass spectral analysis of *N*-acyl, dimethyl ester derivatives and n.m.r. studies on the parent systems confirmed the structures to be 7α-methoxycephalosporin C, *O*-carbamoyldeacetylcephalosporin C and *O*-carbamoyl-7α-methoxydeacetylcephalosporin C [205] (cephamycin C) (Figure 7). Both cultures also produced penicillin N.

At the same time, Merck scientists isolated and characterized the antibiotics cephamycin A, B and C from *Streptomyces* spp.[206-209] (Figure 7). Characterization of the cephamycins was followed by reports [210-218] of other naturally occurring cephalosporin derivatives, some of which are included in Figure 7.



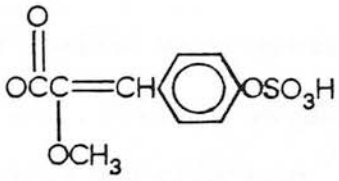
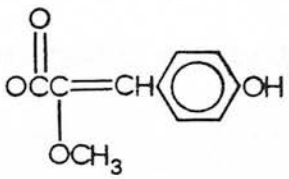
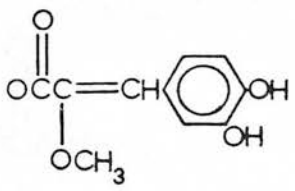
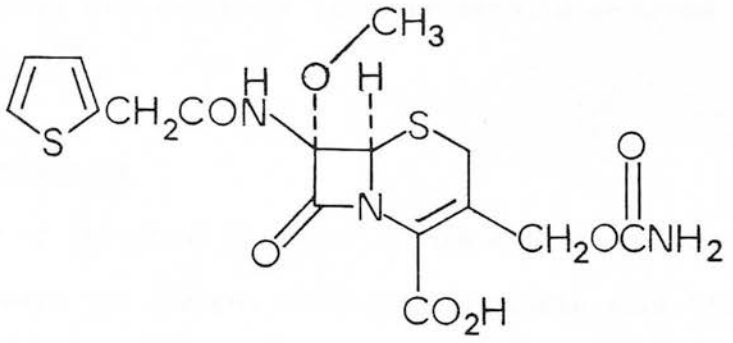
	X	Z
Deacetylcephalosporin C [210, 219]	H	OH
Deacetoxycephalosporin C	H	H
O-carbamoyldeacetylcephalosporin C	H	O_2CNH_2
3'-thiomethyldeacetoxycephalosporin C	H	SCH_3
7α-methoxycephalosporin C	OCH_3	OAc
Cephamycin A	OCH_3	
Cephamycin B	OCH_3	
Cephamycin C	OCH_3	O_2CNH_2
C 2801X	OCH_3	
7α-methoxydeacetoxycephalosporin C	OCH_3	H
7α-methoxydeacetylcephalosporin C	OCH_3	OH

FIGURE 7

The 7α-methoxy function of the cephamycins greatly increases the stability of these antibiotics to β-lactamase hydrolysis [220]. Although the natural cephamycins are not particularly potent antimicrobial agents they possess a slightly expanded spectrum of activity and significant effort has been expended in the synthesis of more potent analogues.

Side-chain cleavage [221, 222] and exchange [223] of the naturally occurring cephamycins has been carried out, but a more viable route to side-chain modified cephamycins has been direct 7α-methoxylation of cephalosporins [224-229]. A number of modified cephamycins have been prepared [155, 221, 223, 230] and are active against many cephalosporin resistant strains of *Enterobacter*, *Klebsiella*, *Proteus morganii* and *Serratia*. As yet cefoxitin [156, 231-233] (23) and cefotetan [234, 235] (24) are the only semi-synthetic cephamycins undergoing clinical evaluation.



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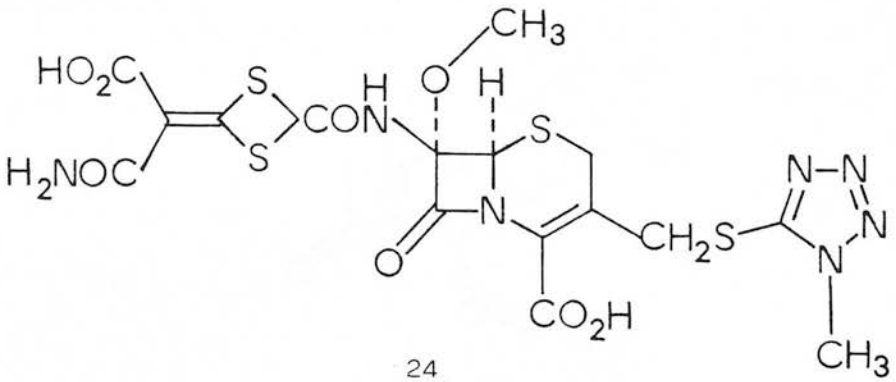
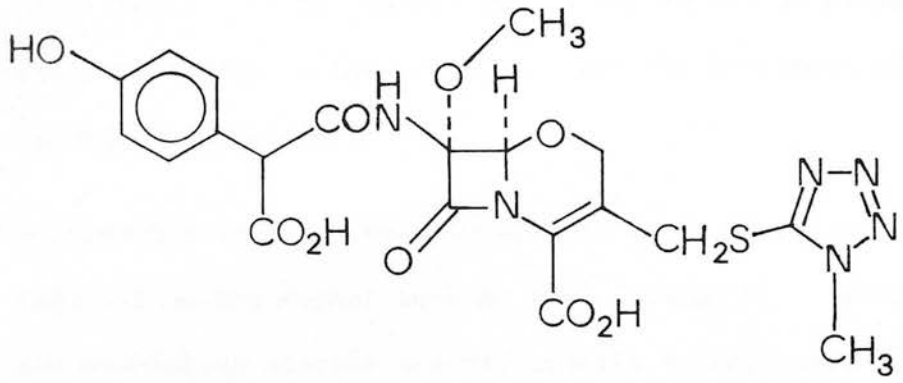


24

Shionogi scientists have combined the advantages of a 7α-methoxy group and a 1-oxacephem nucleus in the synthesis of a highly effective cephamycin analogue, moxalactam [155, 157, 200, 236-238] (25).



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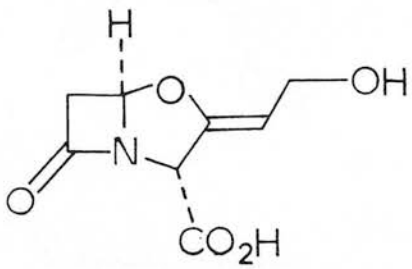
1.4 OTHER NATURALLY OCCURRING β-LACTAM ANTIBIOTICS

In recent years whole new families of β-lactam antibiotics have been isolated. Extensive screening programmes, using "super-sensitive" bioassay methods, have facilitated these discoveries and, though only a brief discussion is included here, a comprehensive review is available [199].

1.4.1 The Clavulanates

One of the first families of new β-lactam antibiotics to be discovered were the clavams of which clavulanic acid (26) is the most important member [239, 240].



26

Although only a weak antibiotic, clavulanic acid is a potent, irreversible inhibitor of many β-lactamases. Against penicillin resistant *Staphylococcus aureus*, the MIC value for ampicillin is reduced twenty-five thousand fold in the presence of 5 μg/ml of sodium clavulanate. These results have prompted the development of a clavulanic acid-amoxycillin "cocktail" for the treatment of penicillin resistant infections.

The biosynthesis of clavulanic acid is quite different from that of the penicillins and cephalosporins (see Section 3). Both glycerol and α-amino-δ-hydroxyvalerate are efficiently incorporated [241] (Figure 8).

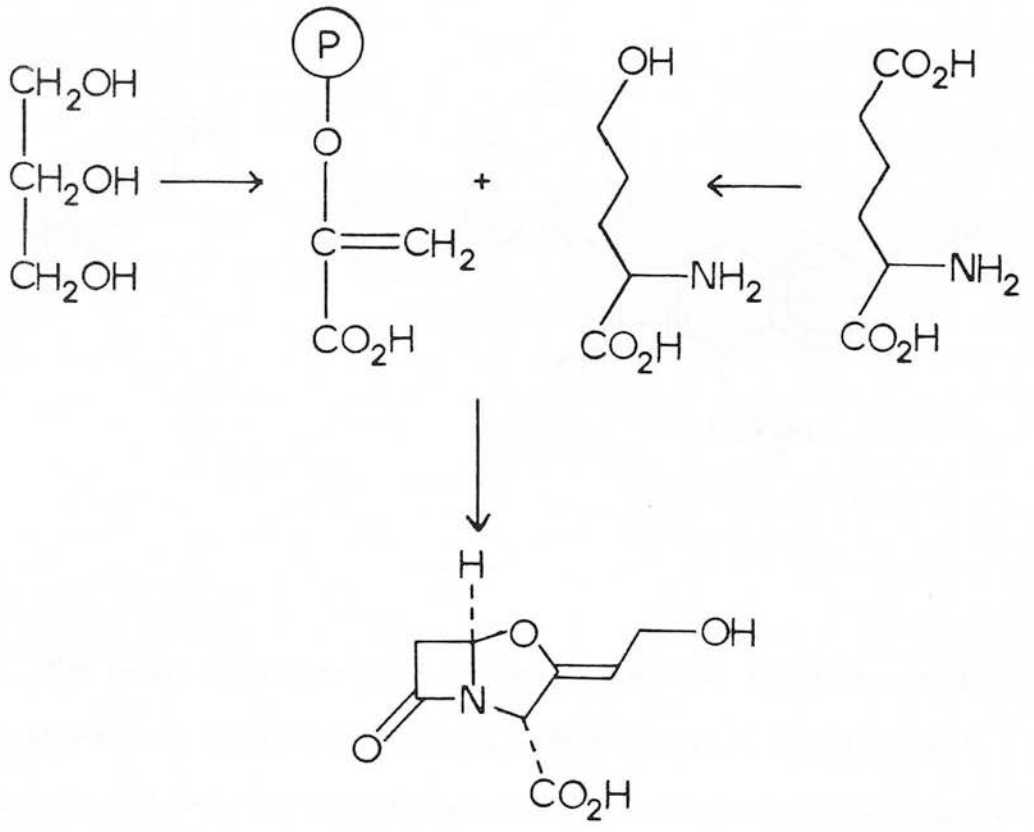
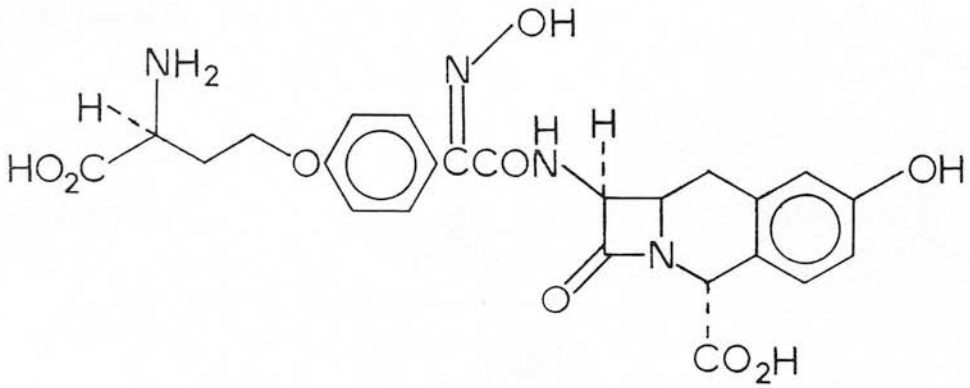


FIGURE 8

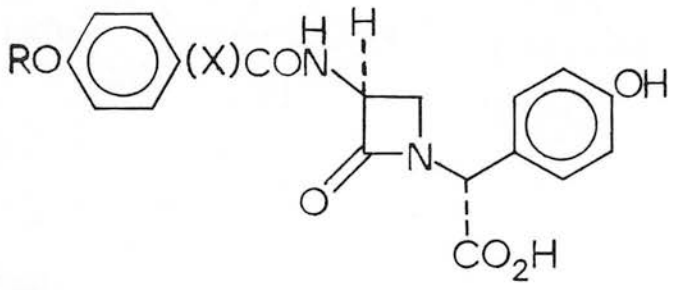
1.4.2 The Nocardacins

The nocardacins, isolated from *Nocardis uniformis* subsp. *tsuyamenensis*, were the first monocyclic ß-lactam antibiotics [242] (Figure 9). Nocardacins possess activity against only gram-negative bacteria, especially *P. aeruginosa*, *Proteus* and *Neisseria*, with nocardacin A being the most potent. Surprisingly the *in vivo* protection of mice from experimental infections was much greater than the *in vitro* results suggested. It is believed that the bacterial cell wall is modified by the nocardacin antibiotic in such a manner as to enhance the sensitivity of the bacterium to phagocytosis [199]. It is interesting to note that the fused analogue [243] (27) has negligible antibiotic activity.



27

The biosynthetic origin of the nocardacins has been studied [244, 245] and appears to involve a peptidal intermediate. L-methionine, L-serine and L-*p*-hydroxyphenylglycine are all precursors (Figure 10). Ring closure of serinyl peptides to ß-lactams has been achieved chemically [245, 246] and supports the proposed intermediacy of an open chain peptide.



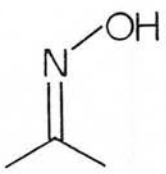
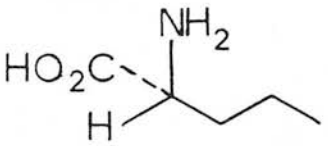
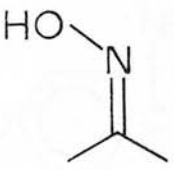
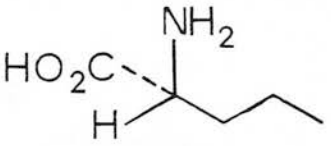
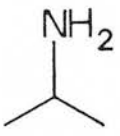
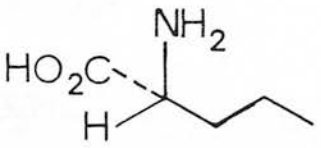
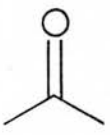
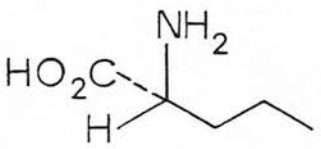
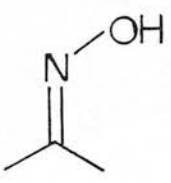
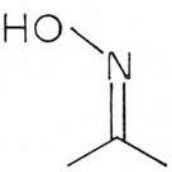
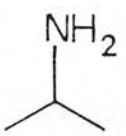
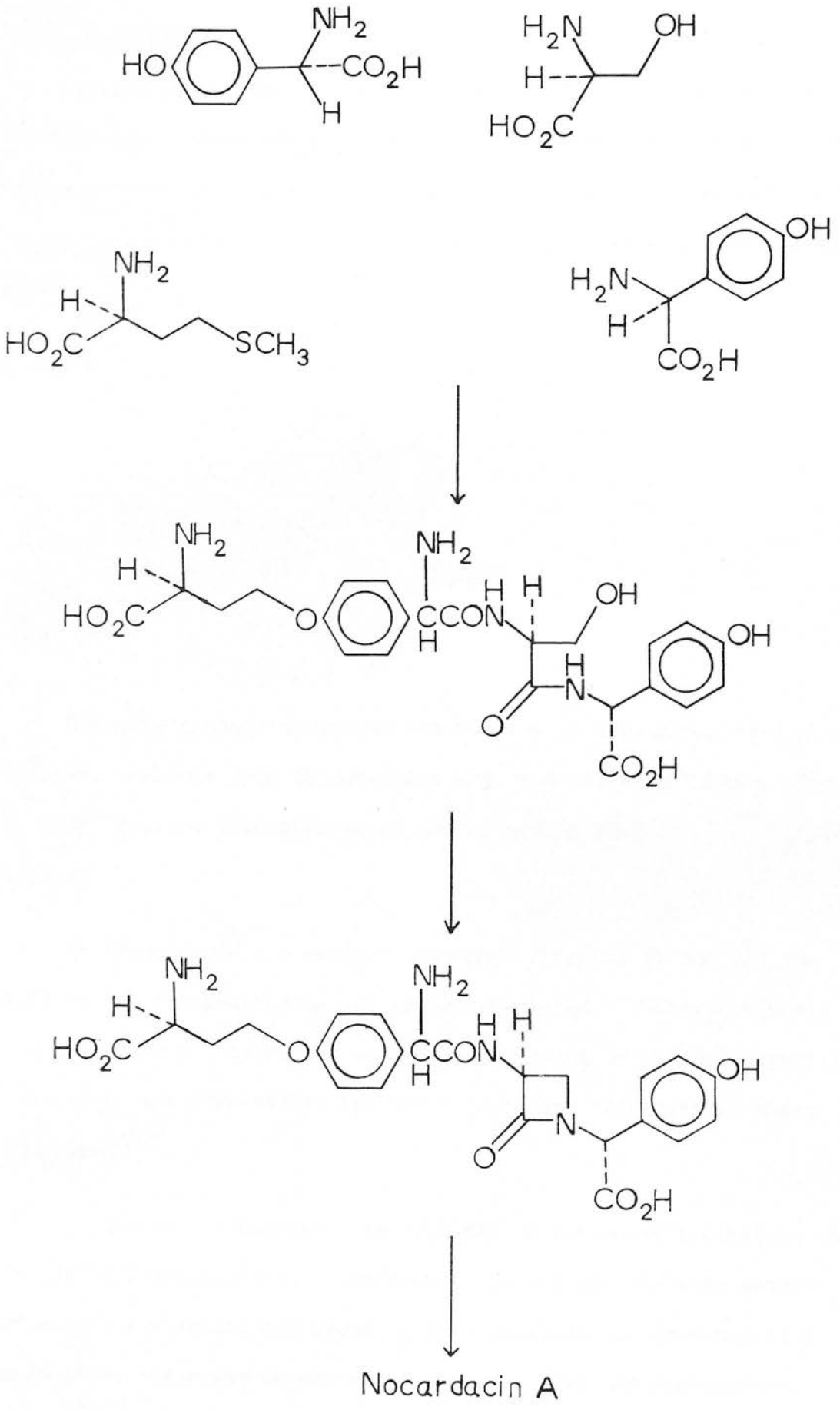
	X	R
Nocardacin A		
Nocardacin B		
Nocardacin C		
Nocardacin D		
Nocardacin E		H
Nocardacin F		H
Nocardacin G		H

FIGURE 9

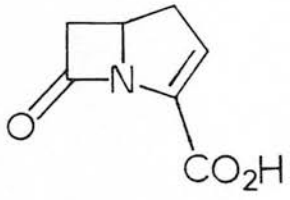


Nocardacin A

FIGURE 10

1.4.3 The Carbapenems

By far the largest group of new β-lactam antibiotics are the carbapenems. Over forty naturally occurring derivatives are known. Carbapen-2-em 3-carboxylic acid (28), the parent nucleus for all the carbapenem antibiotics, has been isolated from *Serratia* and *Erwinia* species [247].

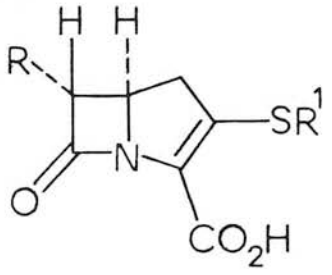


28

The more highly functionalised members of this group have almost all been isolated from *Streptomyces* spp. and are structurally very similar, bearing substituents at the C2 and C6 positions [199] (Figures 11-16).

In thienamycin and related compounds (Figures 11 and 12) the β-lactam ring substituents are *trans* orientated. This previously unknown stereochemistry and the *R* configuration of the hydroxyethyl side-chain are responsible for the β-lactamase stability of these compounds [267].

In general carbapenems are effective β-lactamase inhibitors and/or powerful antibiotics. Thienamycin is perhaps the most potent wide-spectrum antibiotic known. Gram-positive and gram-negative organisms, resistant or otherwise to ampicillin and cephalothin, *P. aeruginosa*, *Serratia marcescens* and the normally β-lactam resistant



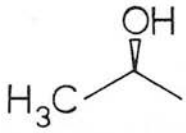
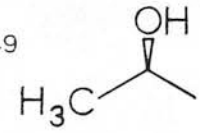
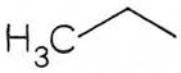
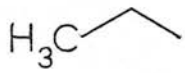
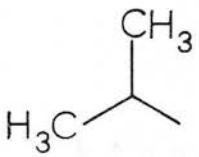
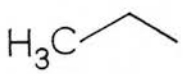
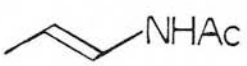
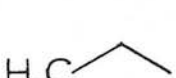
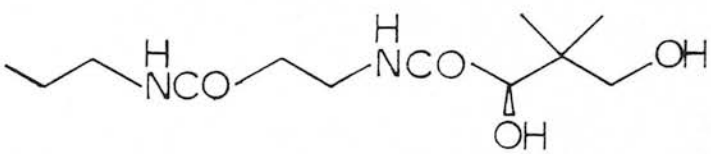
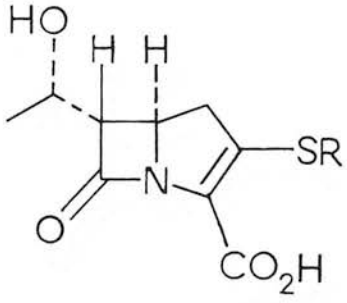
	R	R1
Thienamycin [248]		
Acetylthienamycin [249]		
NS-5 [249]		
PS-5 [250]		
PS-6 [251]		
PS-7 [251]		
OA-6129A [252]		

FIGURE 11



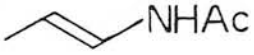
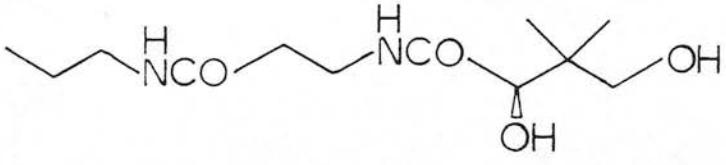
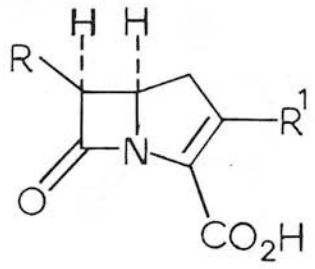
	R
Epithienamycin C[253], MM22381 [254]	
Epithienamycin D[253], MM22383 [254]	
OA-6129B_2 [252]	

FIGURE 12



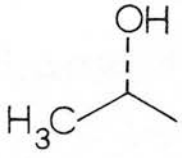
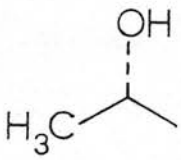
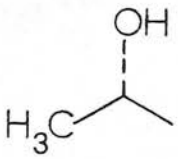
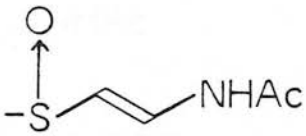
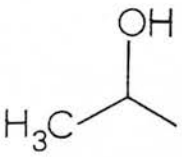
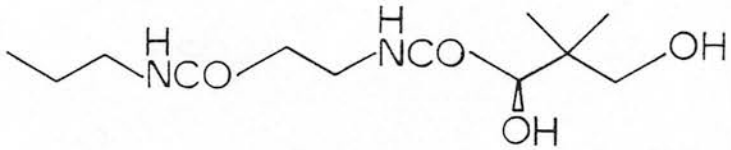
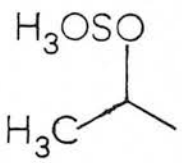
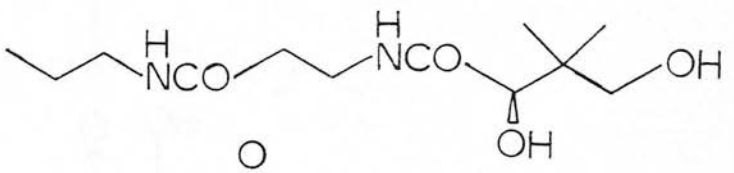
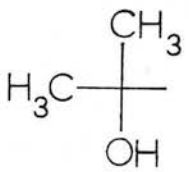
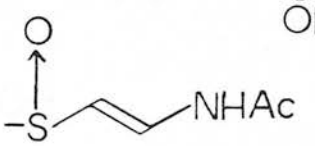
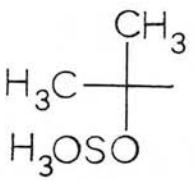
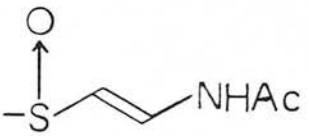
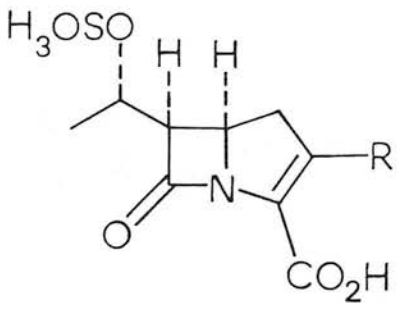
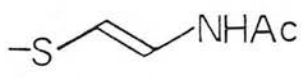
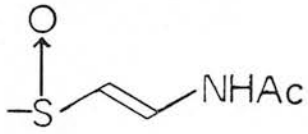
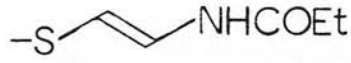
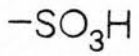
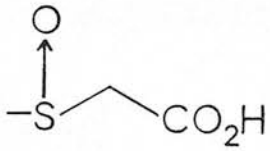
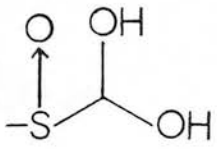
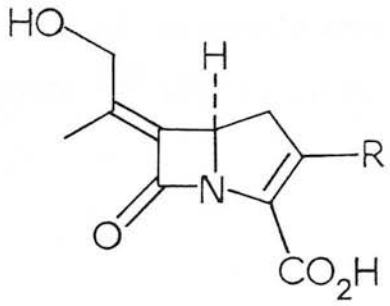
	R	R1
Epithienamycin A[253] MM22380[254]		
Epithienamycin B[253] MM22382[254]		
PA-31088-II[255]		
OA-6129B$_1$[252]		
OA-6129C[252]		
Carpetimycin A[256] C-19393H$_2$[257]		
Carpetimycin B[256] C-19393S$_2$[257]		

FIGURE 13



	R
Epithienamycin E[253], MM13902 [258]	
Epithienamycin F[253], MM17880 [259]	
MM4550[258], MC696-SY2-A [260]	
MM27690 [261]	
Pluramycin A [262]	
Pluramycin B [262]	
Pluramycin C [262]	

F I G U R E 14



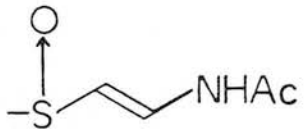
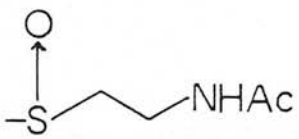
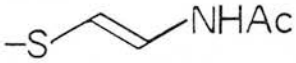
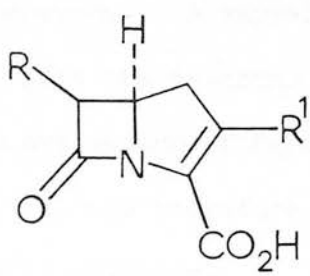
	R
Asparenomycin A [263]	
Asparenomycin B [263]	
Asparenomycin C [263]	
6643-X [264]	

FIGURE 15



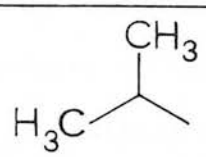
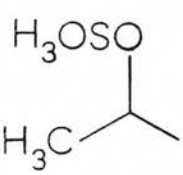
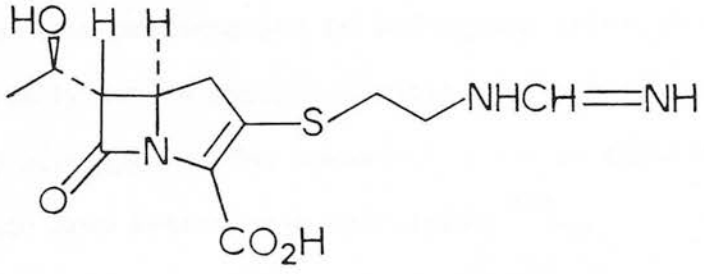
	R	R^1
PS-8 [265]		
SF-2103A [266]		$-SO_3H$

FIGURE 16

anaerobic *Bacteroides* are all extremely sensitive to thienamycin and *N*-formimidoyl thienamycin [268] (29) *in vitro*.



29

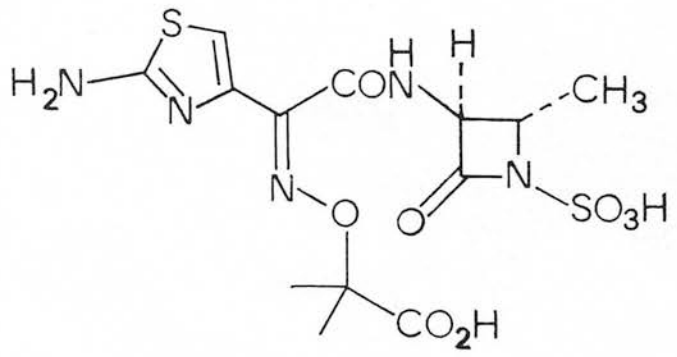
Results in man unfortunately are much less satisfactory due to renal metabolism of the compounds. A mammalian enzyme, dehydropeptidase I, rapidly hydrolyses the β-lactam ring of carbapenem antibiotics [269]. Therapeutic evaluation of Merck's semi-synthetic *N*-formimidoyl thienamycin (29) has therefore required co-administration of a dehydropeptidase I inhibitor.

Few biosynthetic results have been reported for the carbapenems though the apparent precursors of thienamycin are glutamic acid, cysteine and acetate. In contrast numerous total or partial syntheses of carbapenems and their precursors have been reported. The various synthetic strategies employed have been reviewed [270].

1.4.4 The Monobactams

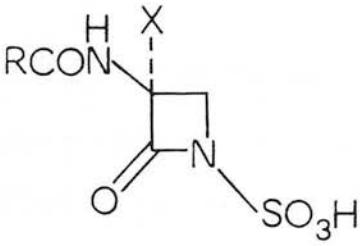
The most recently discovered family of β-lactam antibiotics, the monobactams [271-277], have been isolated from bacteria. The monobactams are monocyclic and possess a novel sulphamate grouping (Figure 17).

The antibacterial activity of the natural monobactams is restricted to gram-negative organisms and is much less than that of ampicillin. However, as with the cephamycins, the 3α-methoxy group confers β-lactamase stability. A highly potent, totally synthetic analogue, azthreonam [278, 279] (30), has been prepared and is undergoing clinical trials. Azthreonam is specifically active against aerobic gram-negative bacteria including *Pseudomonas aeruginosa*. The 4α-methyl group of azthreonam protects the antibiotic from β-lactamase hydrolysis [276].



30

No biosynthetic studies on the monobactams have been published though the amino-acids, alanine, glutamic acid, phenylalanine and/or tyrosine are presumably involved. The possibility also exists that serine may form the β-lactam ring, in a manner analogous to nocardacin biosynthesis, and that the immediate precursor may be a peptide as in the biosynthesis of penicillins, cephalosporins and nocardacins.



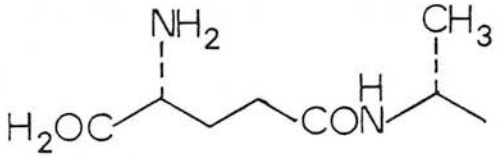
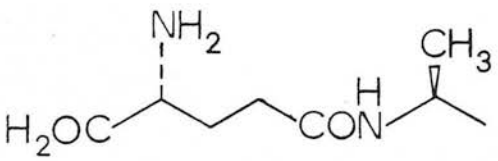
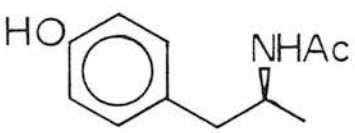
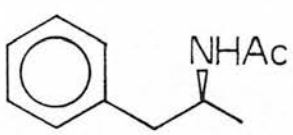
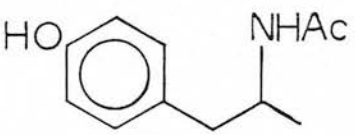
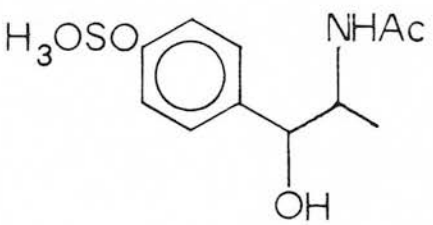
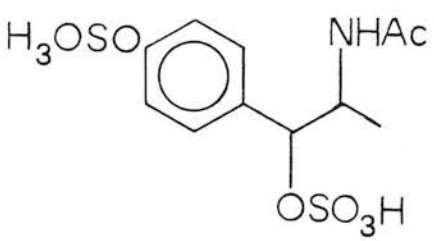
	X	R
Sulphazecin, SQ26445	$-OCH_3$	
Isosulphazecin	$-OCH_3$	
SQ26180	$-OCH_3$	$-CH_3$
SQ26700	$-H$	
SQ26823	$-OCH_3$	
SQ26875	$-OCH_3$	
SQ26970	$-OCH_3$	
SQ26812	$-OCH_3$	

FIGURE 17

As illustrated in this section the last decade has seen an explosive increase in the number of "non-classical" β-lactam antibiotics. Not only are their structures diverse but their natural sources have descended the evolutionary ladder from fungi to actinomycetes to bacteria. There seems little doubt that many other β-lactam compounds are present in nature and wait only to be discovered.

2. BACTERICIDAL MODE OF ACTION

Although most studies have employed penicillins, all β-lactam antibiotics are bactericidal because they interfere with bacterial cell wall biosynthesis [20, 58, 280-282].

The bacterial cell wall [283-286] contains many materials including teichoic acid, lipopolysaccharide and peptidoglycan [287, 288], a multiply cross-linked macromolecule which gives the cell rigidity and shape and protects the delicate semi-permeable cytoplasmic membrane from the potentially destructive high internal osmotic pressure.

The structure of peptidoglycan has been determined and, though differing in detail from species to species, can generally be represented by the *Staphylococcus aureus* structure proposed by Wise and Park [289, 290] (Figure 18). A polysaccharide backbone of alternating β-1,4-linked, D-*N*-acetylglucosamine and *N*-acetylmuramic acid moieties is cross-linked by peptide chains bound to the muramic acid carboxyls. This cross-linking is accomplished by attack of a $-(Gly)_5$ chain on the terminal D-ala-D-ala residue of a neighbouring pentapeptide, forming a new gly-D-ala bond and displacing D-alanine [291, 292] (Figure 19).

The enzymes responsible for the final cross-linking of the peptide chains are inhibited by β-lactam antibiotics [289, 293, 294]. Transpeptidases, D-alanine carboxypeptidases and endopeptidases are all inhibited, reversibly or irreversibly, by acylation at or near their active sites [295-298]. Ring strain, in bicyclic systems, or electron withdrawal in the monobactams, activates the β-lactam ring towards nucleophilic attack and acylation at the active site.

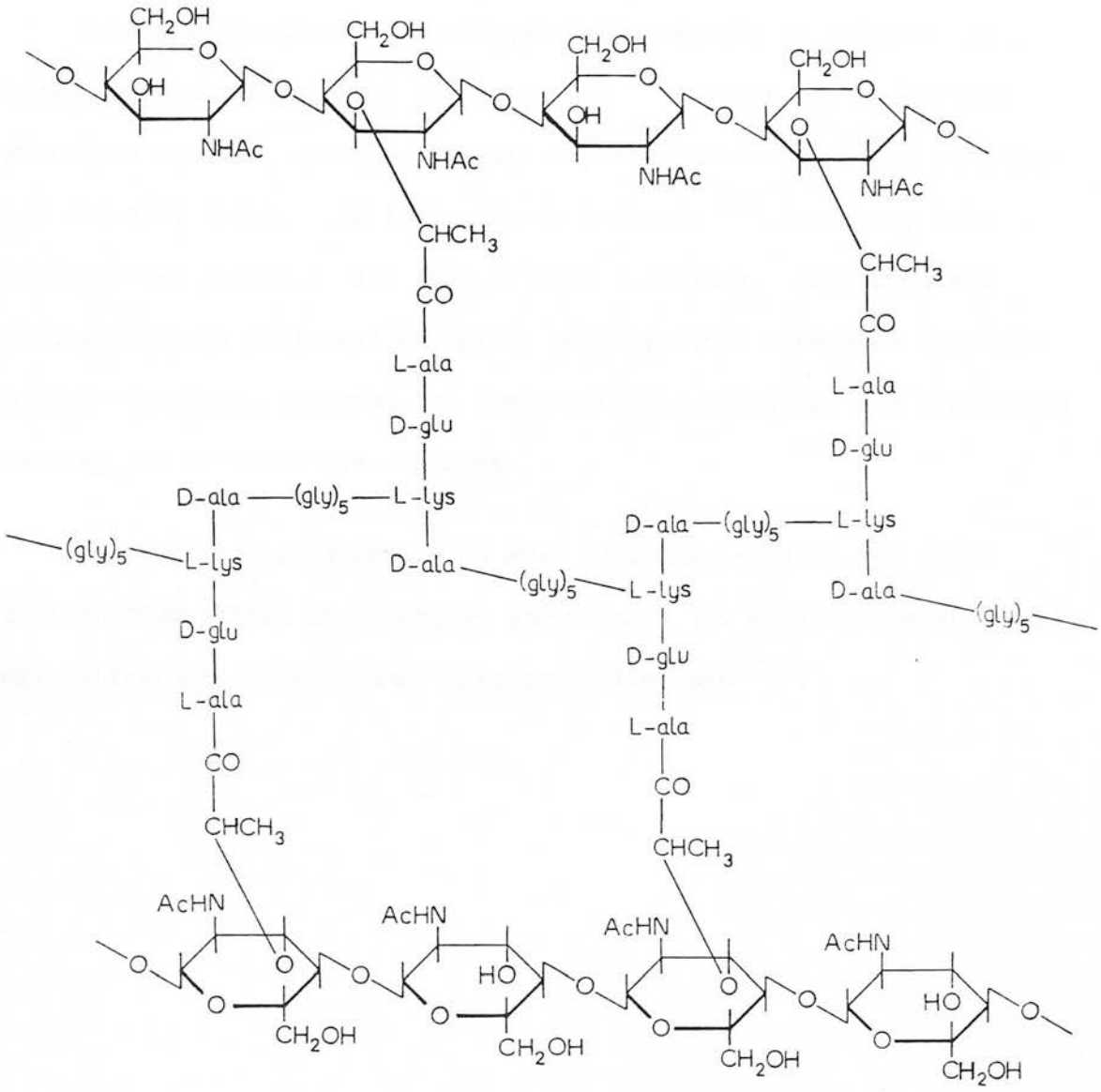


FIGURE 18

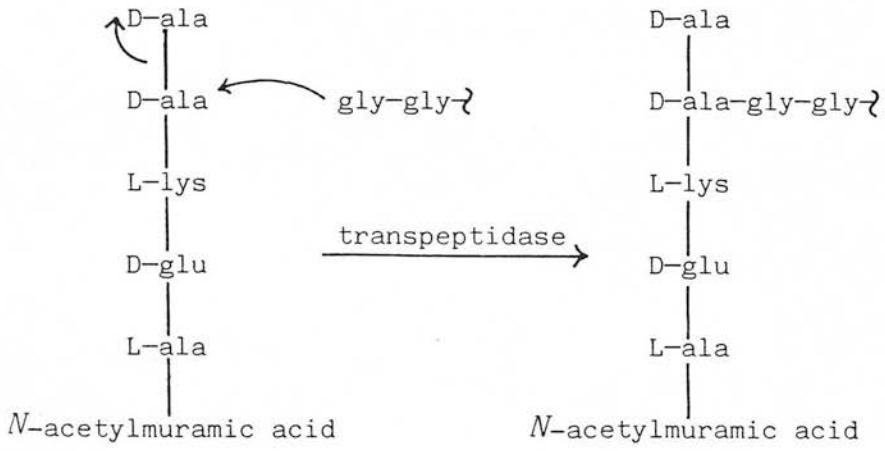


FIGURE 19

β-lactam treatment of bacterial cells results in reduced cross-linking and a weakening of the cell wall. A point may be reached where the internal osmotic pressure ruptures the cytoplasmic membrane and the cell lyses. As indicated by Rolinson [299], however, cell lysis is not essential for loss of cell viability. The impaired strength of the cell wall may place an additional strain on the cytoplasmic membrane, damaging its semi-permeable character and ultimately causing the death of the organism.

Why some bacteria should be more resistant to β-lactam antibiotics than others is a complex question. The various factors which may contribute to resistance have been discussed [300].

3. THE BIOSYNTHESIS OF PENICILLINS, CEPHALOSPORINS AND CEPHAMYCINS

It is convenient to discuss the biosynthesis of penicillins, cephalosporins and cephamycins in one section, as the biosynthetic pathways which lead to these natural products share a number of common intermediates.

Although studies on the biosynthesis of the β-lactam antibiotics have been continuous over the past forty years, most of the important observations have been made in the last ten years. Present knowledge of β-lactam biosynthesis is summarized in Figure 20. Various review articles are available [215, 301-306].

3.1 AMINO-ACID PRECURSORS

3.1.1 L-α-Aminoadipic Acid

A requirement for α-aminoadipic acid might be assumed from the structures of isopenicillin N, penicillin N, the cephalosporins and the cephamycins, but not necessarily for the solvent soluble penicillins. The fact that lysine inhibition of penicillin G synthesis could be reversed by addition of α-aminoadipic acid to the culture medium [307] suggests however that α-aminoadipic acid is an essential precursor of the solvent soluble penicillins*. Studies with lysine

* In fungi α-aminoadipic acid is a precursor of lysine [308]. In the presence of lysine, feedback inhibition of homocitrate synthetase prevents the production of α-aminoadipic acid [309]. In *Streptomyces* spp. α-aminoadipic acid is synthesised from lysine [310].

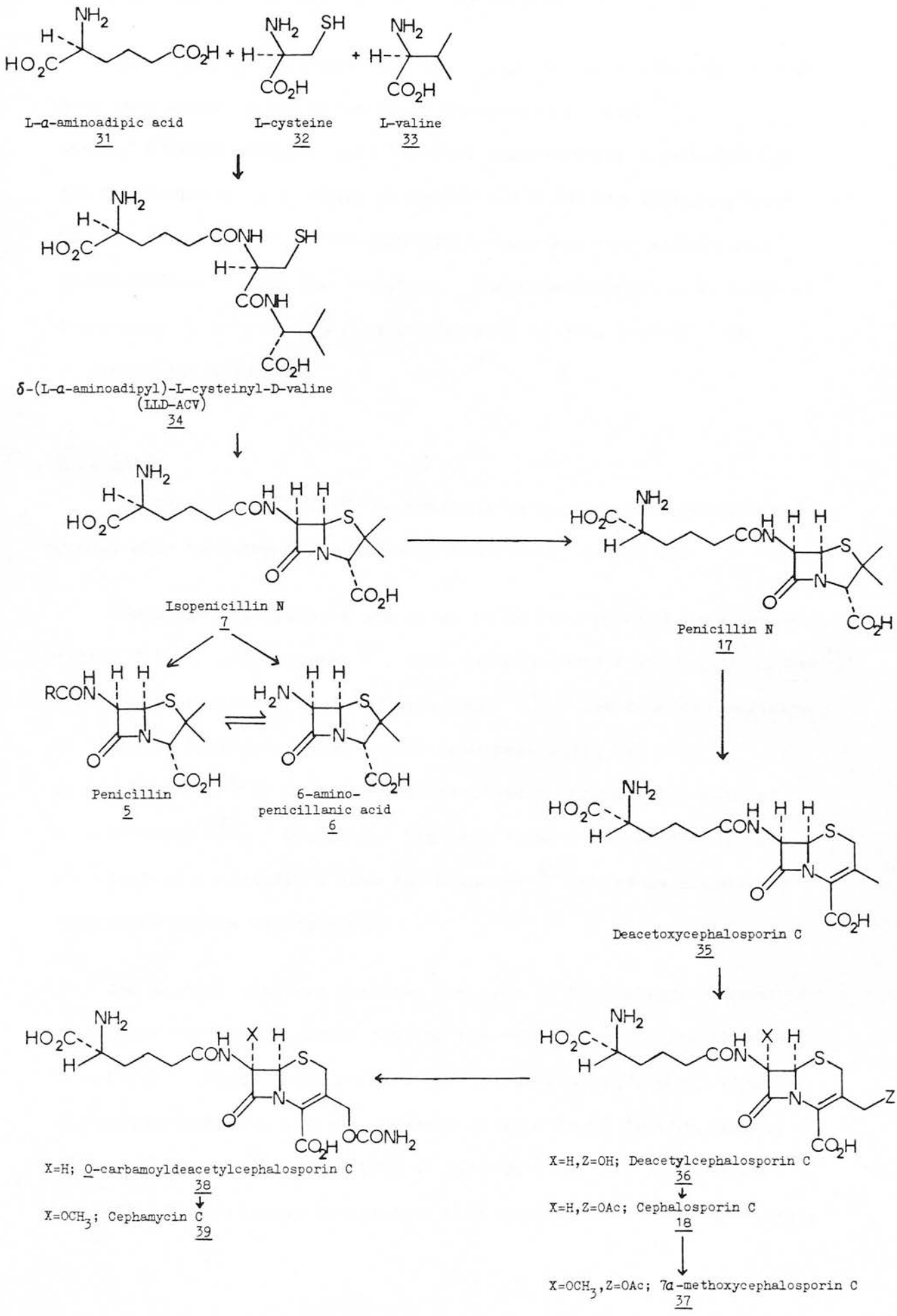

L-α-aminoadipic acid
31
L-cysteine
32
L-valine
33
δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
(LLD-ACV)
34
Isopenicillin N
7
Penicillin N
17
Penicillin
5
6-amino-
penicillanic acid
6
Deacetoxycephalosporin C
35
X=H,Z=OH; Deacetylcephalosporin C
36
X=H,Z=OAc; Cephalosporin C
18
X=OCH3,Z=OAc; 7α-methoxycephalosporin C
37
X=H; O-carbamoyldeacetylcephalosporin C
38
X=OCH3; Cephamycin C
39

FIGURE 20

auxotrophs of *P. chrysogenum* have confirmed this requirement [311].

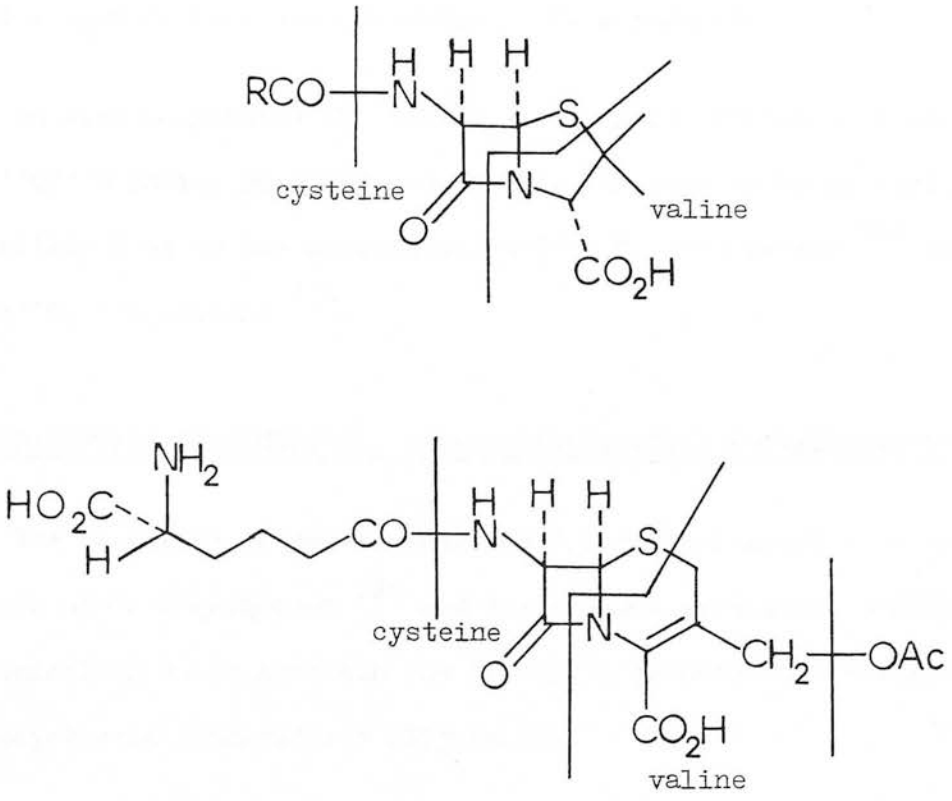
Likewise, lysine auxotrophs of *C. acremonium* produce antibiotics only when grown in the presence of α-aminoadipic acid [312]. DL-[1-^{14}C]-α-aminoadipic acid labelled cephalosporin C and although the α-aminoadipyl side-chain of penicillin N and the cephalosporins has the D-configuration L-α-aminoadipic acid was more efficiently incorporated [313] than the D-isomer. The α-aminoadipyl side-chain of cephamycin C, produced by *S. clavuligerus*, is also derived from α-aminoadipic acid [314].

3.1.2 L-Cysteine

The bicyclic nuclei of the penicillins and cephalosporins could conceivably be formed from cysteine and valine (Figure 21).

Radiolabelled cysteine was shown to be incorporated into penicillin G in *P. chrysogenum* [315], into cephalosporin C in *C. acremonium* [316] and into cephamycin C in *S. clavuligerus* [314]. The complete skeleton of cysteine is incorporated intact into penicillin G. DL-[3-^{14}C, ^{15}N, ^{35}S]-cysteine was effectively incorporated without loss of label [315]. L-cysteine has been shown to be a more efficient precursor of penicillin G than the D-isomer [317] which is presumably isomerized before incorporation.

The pivotal position cysteine occupies in the primary metabolism of sulphur in fungi explains many of the misleading results obtained in earlier studies. The ability of various *Penicillium* and *Cephalosporium* species to utilize sulphate or methionine depends largely on the primary metabolic control of each species [301, 318]. Thus the extremely efficient incorporation of sulphur from [^{35}S]-methionine



F I G U R E 21

into cephalosporins in *C. acremonium* is due to the rapid assimilation of methionine by the mycelia and its rapid conversion to cysteine within the cell.

3.1.3 L-Valine

The D-penicillamine fragment of penicillins [319-321] and the 2,3-dehydrovaline fragment of cephalosporin C [316, 321] and cephamycin C [314] are derived from L-valine. Radiolabelled L-valine was incorporated more efficiently into penicillin G [319, 320] than D-valine and although both isomers were equally effective precursors in a high producing strain of *P. chrysogenum* results from feeding D-[2-^{3}H]-valine, L-[U-^{14}C, ^{15}N]-valine and D-[U-^{14}C,^{15}N]-valine indicate that D-valine is not incorporated directly [322, 323]. Prior isomerization of D-valine to L-valine probably occurs [323, 324].

Similar results have been observed in *C. acremonium* [321].

As with L-cysteine the valine skeleton is incorporated intact. The $^{14}C/^{15}N$ ratios remained essentially the same in biosynthetised penicillin G as in the substrates, L-[1-^{14}C, ^{15}N]-valine [324] and L-[U-^{14}C, ^{15}N]-valine [323].

3.2 THE TRIPEPTIDE INTERMEDIATE, δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE

The isolation of δ-(α-aminoadipyl) cysteinylvaline from the mycelia of *P. chrysogenum* [325] and its obvious structural similarity to penicillin N led Arnstein and Morris to propose this tripeptide as a biosynthetic intermediate (Figure 22).

The stereochemistry of the *P. chrysogenum* tripeptide was later established [326-328] as δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) (34) and shown to be identical to material isolated from *C. acremonium* [329]. ACV has also been isolated from *S. clavuligerus* [330] and from the cephalosporin producing fungus *Paecilomyces persicinus* [331]. LLD-ACV is the only optical isomer of ACV to have been detected though related tri- and tetra-peptides of undefined stereochemistry are known [329, 331, 332].

The omnipresence of ACV in β-lactam producing fungi and actinomycetes strongly supports its role as an intermediate in penicillin and cephalosporin biosynthesis. This is discussed further in section 3.3 (page 51).

3.2.1 Biosynthesis of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine (LLD-ACV)

The biosynthesis of LLD-ACV has been studied in broken cell preparations from *C. acremonium* C91 and *P. chrysogenum*. The

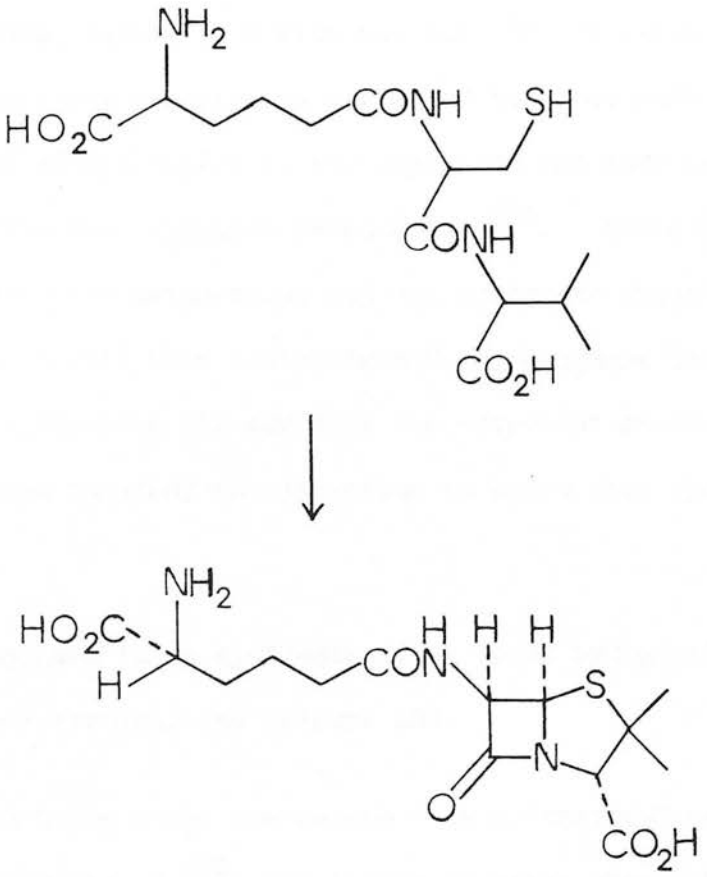


FIGURE 22

results [302, 333] from radiolabelled feeding experiments using the *C. acremonium* system suggest that LLD-ACV is synthesised from δ-(L-α-aminoadipyl)-L-cysteine and L-valine. No LLD-ACV was detected in this system when δ-(D-α-aminoadipyl)-L-cysteine, L-cysteinyl-L-valine or L-cysteinyl-D-valine were tested as precursors, nor was any production observed from the constituent amino-acids. Isolation of δ-(L-α-aminoadipyl)-L-cysteine from the mycelium of *S. clavuligerus* [306] supports the conclusion that LLD-ACV is biosynthesised from the *N*-terminal end, as is the case in glutathione biosynthesis [334].

Apparently contradictory results have been obtained with whole

P. chrysogenum cells [335, 336]. L-[2-^{3}H]-cysteinyl-L-[U-^{14}C]-valine was incorporated into penicillin G with the same ^{3}H:^{14}C ratio as the dipeptide. Subsequent experiments suggested that the most likely course of events involved hydrolysis of the dipeptide and construction of penicillin G from the liberated amino-acids [336]. Whole *C. acremonium* cells assimilate only amino-acids and not preformed dipeptides or tripeptides [337]. A cell free system from *P. chrysogenum* has been reported [338] to synthesise LLD-ACV from the component amino-acids, but has not been used to study the direction in which this synthesis occurs.

LLD-ACV appears to be synthesised, at least by *Cephalosporium* spp., from the *N*-terminal end (Figure 23).

Nothing is known about the enzyme "ACV synthetase", except that it may be membrane bound [333], and little is known about the activated forms of the amino-acids used by the enzyme. An α-aminoadipic acid - AMP ligase has been isolated [339] from *P. chrysogenum* and the AMP derivative of L-α-aminoadipic acid may well be the activated form of this amino-acid.

Elimination of one carboxyl oxygen atom from L-[$^{18}O_2$]-valine occurred during incorporation into penicillin V in *P. chrysogenum* [340]. This finding is consistent with the involvement of an enzyme bound form of valine, for example as a thioester, though not necessarily at the stage of LLD-ACV synthesis. However, recent results [341, 342] have shown that the loss of ^{18}O does not occur during cyclization of LLD-ACV to isopenicillin N (see Section 3.3).

Another interesting question, which remains as yet unanswered,

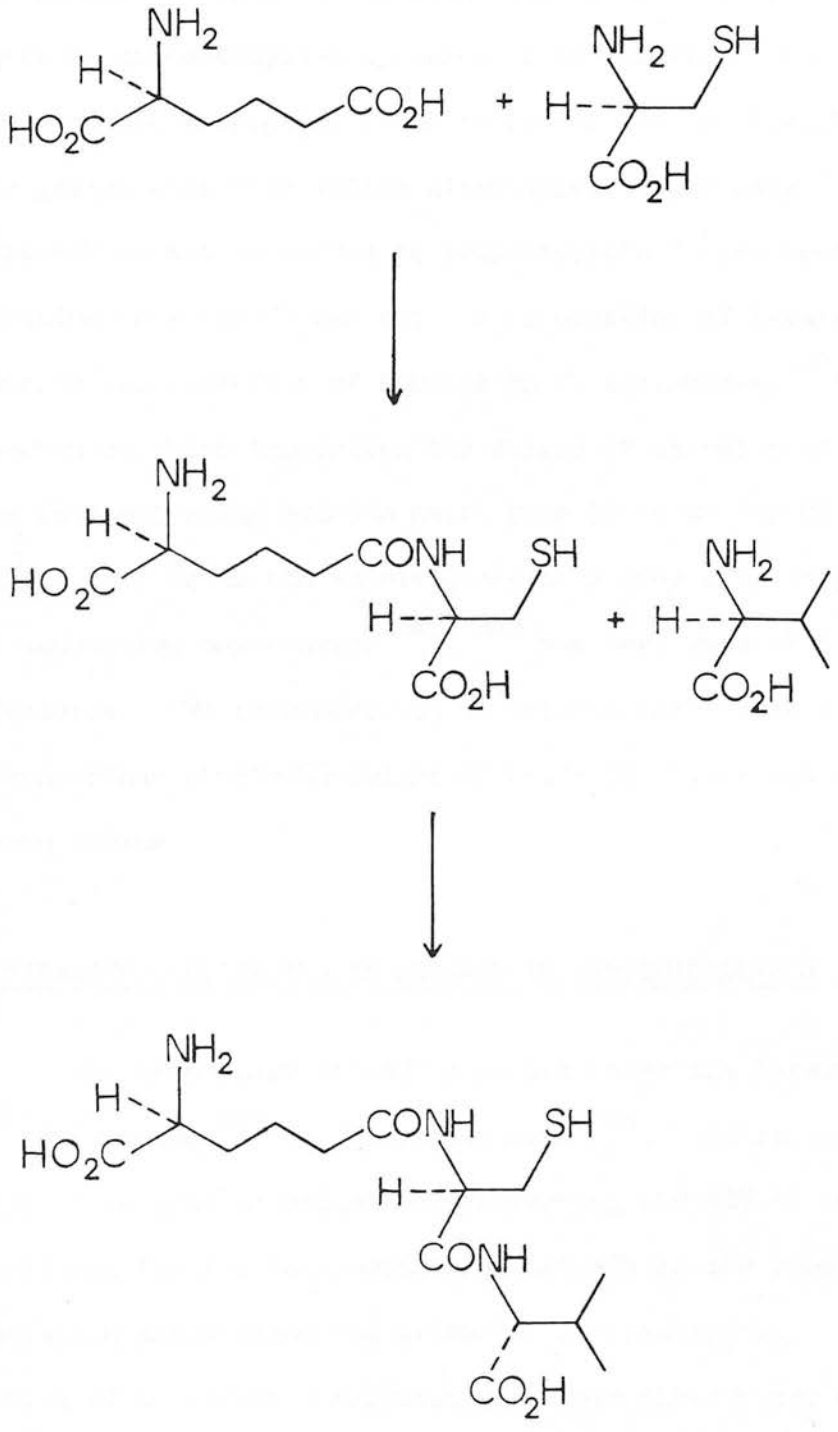

NH2
H
HO2C
CO2H
+
NH2
SH
H
CO2H
NH2
H
HO2C
CONH
SH
H
CO2H
+
NH2
H
CO2H
NH2
H
HO2C
CONH
SH
H
CONH
CO2H

FIGURE 23

is the point at which L-valine is isomerized to the D-valine residue of LLD-ACV. Is L-valine converted to D-valine then coupled with δ-(L-α- aminoadipyl)-L-cysteine or is L-valine first coupled to produce an LLL-tripeptide which is isomerized to LLD-ACV? Abraham has suggested that this latter alternative is unlikely [304] since synthetic LLL-ACV is not converted to isopenicillin N (see Section 3.3). Tritium from the C2 but not the C3 position of L-valine was lost during the synthesis of LLD-ACV in *C. acremonium* [343, 344]. Any mechanism which isomerizes the valine C2 chiral centre and involves an intramolecular hydride shift from C3 to C2 (or C2 to C3) is thus precluded, as is the intermediacy of a free 2, 3 dehydrovaline species. A supportive observation [345, 346] has been made with *P. chrysogenum* cultures. No incorporation of tritium into penicillin V was detected when either DL-[3-^{3}H]-valine or L-[3-^{3}H]-valine was added to the culture medium.

3.3 OXIDATIVE CYCLIZATION OF LLD-ACV TO ISOPENICILLIN N

The tripeptide LLD-ACV does not enter the intact mycelium of *C. acremonium* [302] or *S. clavuligerus* [215]. Until the development of cell-free systems capable of converting LLD-ACV to isopenicillin N, evidence for the intermediacy of LLD-ACV in the biosynthesis of the β-lactam antibiotics was primarily circumstantial. A lysine auxotroph of *C. acremonium* produced neither LLD-ACV nor antibiotics when grown in the presence of lysine, but produced both when grown in the presence of α-aminoadipic acid [312]. DL-[U-^{14}C]-valine, when fed to a *C. acremonium* culture, rapidly labelled this tripeptide immediately prior to antibiotic production [321].

Conversion of LLD-ACV to isopenicillin N has now been demonstrated in cell-free preparations derived from *P. chrysogenum* [347], *C. acremonium* [328, 348-354] and *S. clavuligerus* [330, 355, 356].

A lysed protoplast system from *C. acremonium* C91 [328, 357], in the presence of ATP, phosphoenalpyruvate and pyruvate kinase, converted δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4'-^{3}H]-valine into a substance which co-chromatographed with penicillin N and yielded tritium labelled penicillaminic acid following performic acid oxidation. Penicillin N was the only penicillin then known to be produced by *Cephalosporium* spp. and was assumed to be the product. However, the electrophoretic and chromatographic separations used could not have distinguished penicillin N from isopenicillin N. Subsequently [348] the antibiotic was identified as isopenicillin N on the basis of the behaviour of the α-aminoadipic acid, obtained by hydrolysis, towards L-amino-acid oxidase.

The same conclusion was reached by Demain and co-workers [349, 350] using protoplast lysates and active sonicates from an antibiotic-negative mutant, *C. acremonium* M-0198, blocked prior to LLD-ACV synthesis. Their cell-free preparations converted LLD-ACV to an antibiotic which was completely destroyed by penicillinase. The different bactericidal spectrum of the product from that of penicillin N (see Section 1.2) suggested that the product was isopenicillin N. Isolation of the biosynthesised material, chiral derivatization and h.p.l.c. comparison with authentic samples, has since confirmed this assignment [354]. Isopenicillin N has been detected in mycelial extracts from *C. acremonium* [352] and *S. tokunonensis* [358].

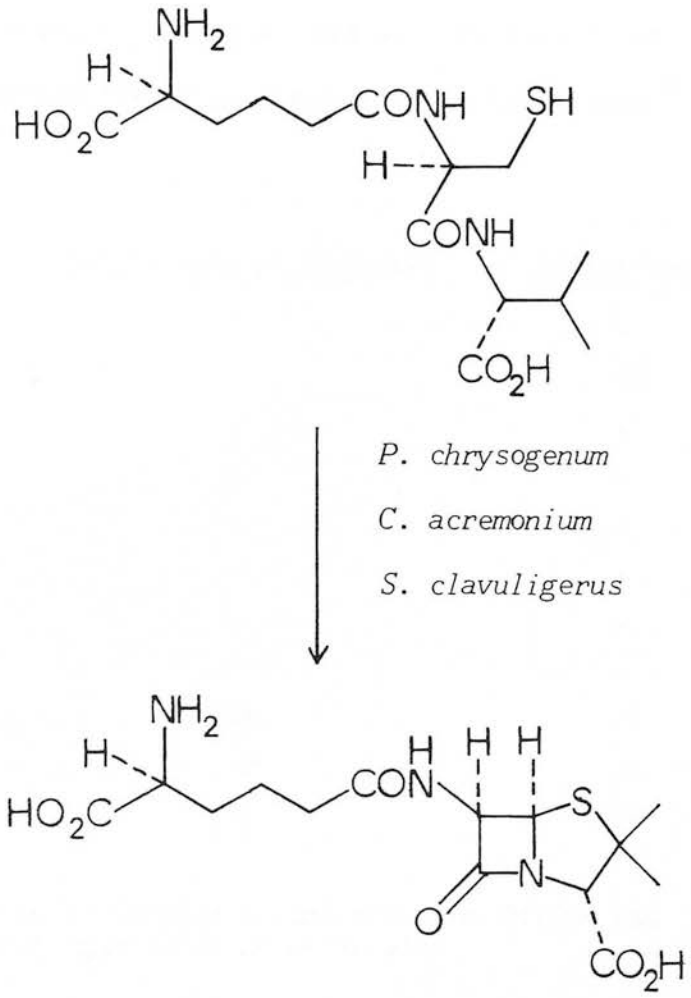
A cell-free system capable of cyclising LLD-ACV to isopenicillin

N has been obtained by grinding the mycelia of *C. acremonium* C91 in a Dyno-Mill [352]. A partially purified preparation allowed an 85% conversion of LLD-ACV to isopenicillin N and has been used to study this ring closure by direct ^{13}C and ^{1}H n.m.r. observation [351, 353].

Incubation of radiolabelled LLD-ACV with a protoplast lysate of *P. chrysogenum* produced isopenicillin N, identified as its penicilloic acid [347]. A number of methods to produce active cell-free extracts from *S. clavuligerus* have been reported [355]. Sonication proved not only to be the simplest method tested but also gave the most active preparations. Using this system LLD-ACV was converted to an antibiotic which appeared, on the basis of its activity against a variety of gram-positive and gram-negative organisms, to be a mixture of isopenicillin N and penicillin N [355]. Analysis by h.p.l.c. could not separate the two isomers but confirmed that reduction in tripeptide concentration, in the incubation mixture, was matched by an equal increase in penicillin concentration [356]. It has been shown [359] that *P. chrysogenum* mutants incapable of producing LLD-ACV are also incapable of producing penicillins. These results confirm the conclusion that the LLD-tripeptide is an obligatory intermediate in penicillin biosynthesis (Figure 24).

3.3.1 "Isopenicillin N Synthetase"

In the β-lactam producing fungi and *Streptomyces* the enzyme responsible for converting LLD-ACV to isopenicillin N would appear to be soluble and not membrane bound, despite the increased activity observed in the presence of Triton X-100. Molecular oxygen, ferrous ions and a reducing agent, preferably dithiothreitol (DTT) but also ascorbate, are essential for activity. Neither ATP nor 2-oxoglut-



F I G U R E 24

arate are required and other ions particularly Zn^{2+} are inhibitory. The enzyme has a molecular weight of approximately 32,000 a.m.u. and is stable at -20°C in tris-HCl buffer at pH7·0 for up to three months [350, 352, 355].

LLD-ACV is converted intact into isopenicillin N [328, 348, 351]. No label was lost from double-labelled material and no label was incorporated in the product when unlabelled LLD-ACV was incubated in the presence of DL-[4,4'-^{3}H]-valine.

To gain an insight on the active site of isopenicillin N synthetase various structural analogues and optical isomers of LLD-ACV have been synthesised and tested as substrates or inhibitors [328, 352, 360] (Figure 25).

Peptide	Active Product Yield(%)	Inhibition(%)*
LLD-ACV	100	-
LLL-ACV	-	30
DLD-ACV	-	-
LD-CV	-	-
LLD-GCV	-	-
LLD-AAbV	-	-
LLD-ASV	-	-
LLD-AAlV	-	-
LLD-ACI	36	40
LLD-ACIs	4	75
LLD-ACAb	10	78

* Inhibition is defined as the decrease (%) in the bioactivity generated after 30 min.

A=δ-(α-aminoadipic acid); Ab=α-aminobutyric acid; Al=alanine; C=cysteine; G=δ-glutamic acid; I=isoleucine; Is=alloisoleucine; S=serine; V=valine.

F I G U R E 25

The enzyme, though not totally substrate specific, tolerates only minor changes in the D-valine residue. The cysteinyl and δ-linked L-α-aminoadipyl residues are apparently essential for activity and may play a vital role in binding the tripeptide to the active site. It is possible that the L-α-aminoadipyl side-chain co-ordinates to, or bonds ionically with, some point on the enzyme surface and that the free thiol group of LLD-ACV forms a disulphide

bond with the enzyme. ^{17}O n.m.r. studies [341, 342] have shown that no thioesters, esters or amidines can be directly involved in binding LLD-ACV to the active site of the enzyme.

3.3.2 Mechanism of Formation of Isopenicillin N

As soon as ACV was muted as a possible precursor of the penicillin nucleus speculation arose on the mechanism by which this cyclization could occur. No intermediates have ever been isolated or detected by ^{13}C n.m.r. spectroscopy [351]. One major approach to elucidating the mechanism has involved the feeding of specifically labelled amino-acids and peptides. Results from such studies have proven valuable in examining the validity of various proposed mechanisms by comparing the predicted results with those obtained. Alternatively, putative intermediates have been synthesised and fed to cell-free systems or model reactions used to exemplify a potential mechanism. It has been generally assumed that cyclization occurs in two steps and that the β-lactam ring is formed first (Figure 26, path A). Although no experimental evidence exists to support these assumptions the alternative sequence of bond formation (Figure 26, path B) appears unlikely on the basis of recent feeding studies [361]. A concerted closure of both β-lactam and thiazolidine rings remains a possibility (Figure 26, path C).

Oxidation of cysteine and intramolecular attack by the valine nitrogen provides an attractive mechanism for the closure of the β-lactam ring (Figure 27).

The 2,3 dehydrocysteine species (42) is not an intermediate. Tritium from DL-[2-^{3}H]-cystine was retained in the penicillin G, isolated from *P. chrysogenum* [362]. In an extension to this work,

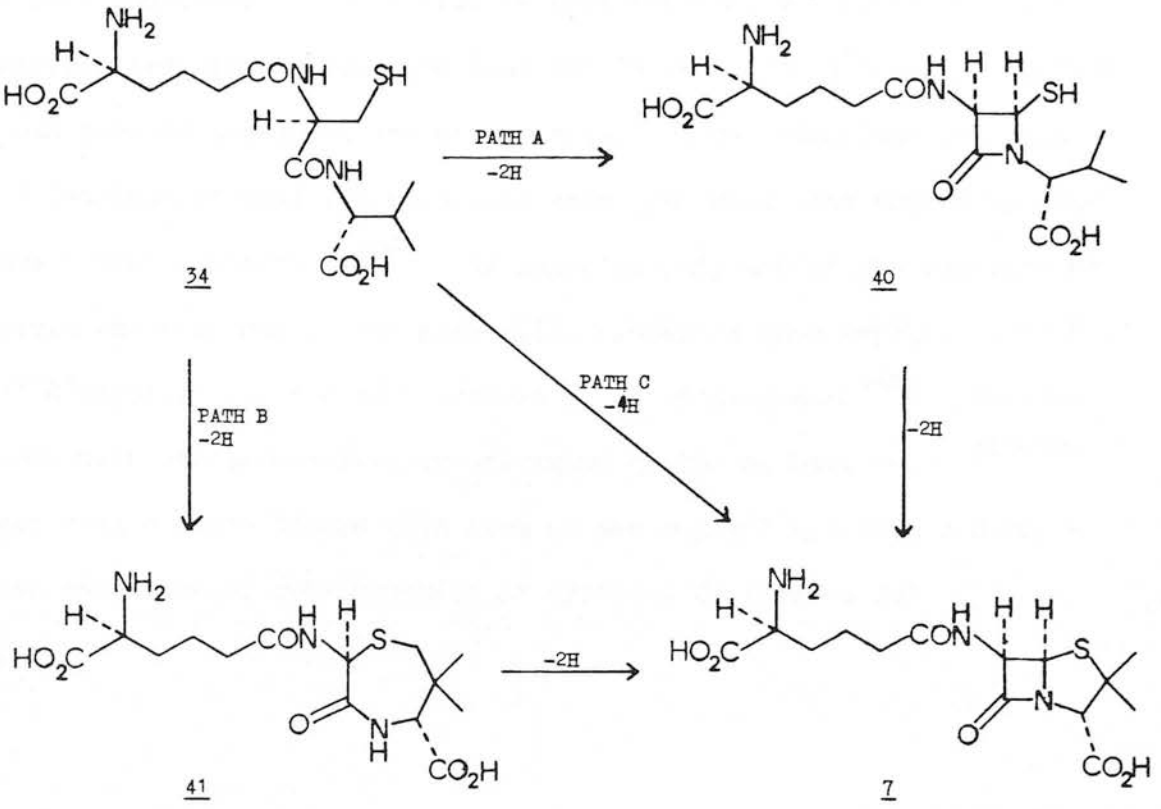


FIGURE 26

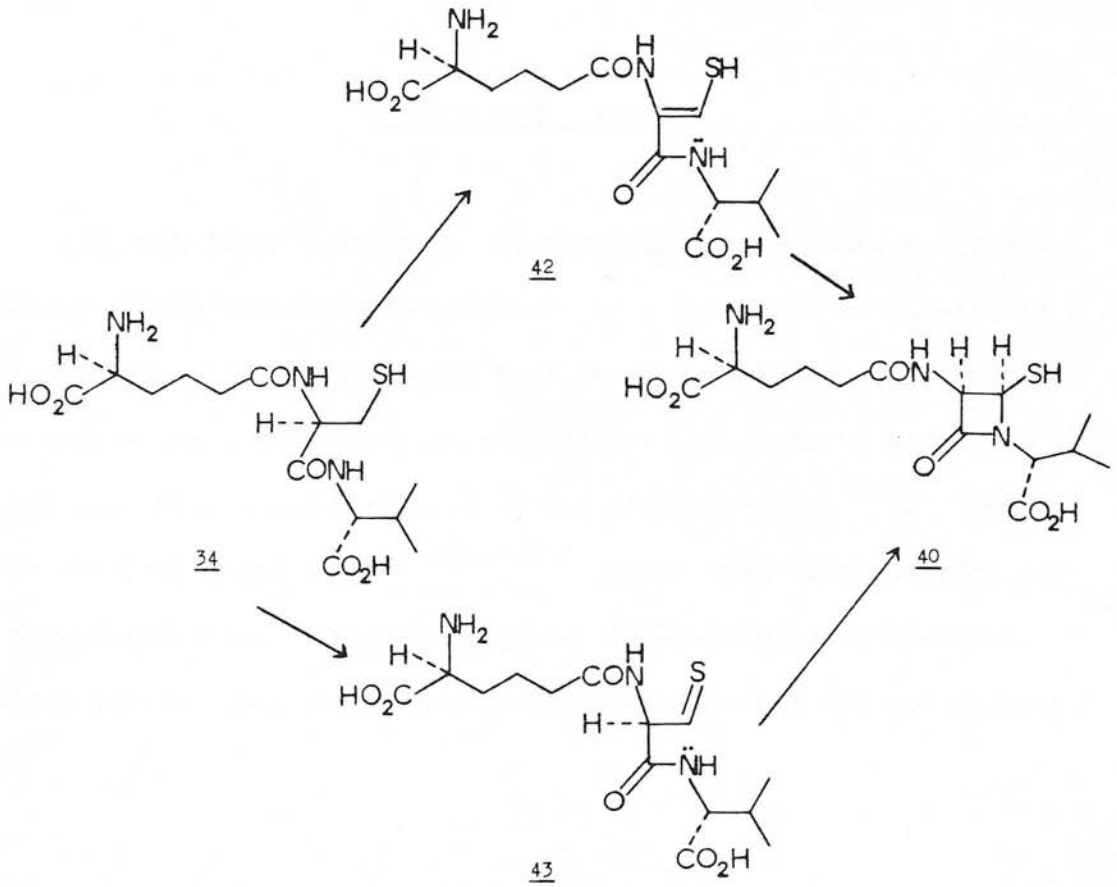
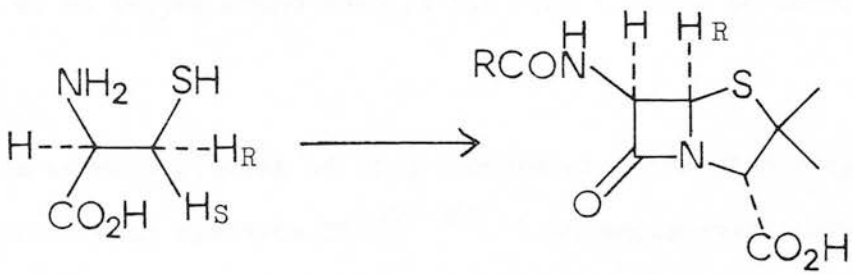


FIGURE 27

Bycroft confirmed [363] that tritium from L-[2-^{3}H, U-^{14}C]-cystine was incorporated without loss and that the ^{3}H from the isolated penicillin G was located solely at the C6 position. Model reactions in which 2,3 dehydrocysteinyl intermediates were generated also failed to produce ß-lactam products [364]. As expected only one of the cysteine C3 tritia was retained in the penicillin V isolated when L-[3,3,3',3'-$^3H_4$, U-^{14}C]-cystine was fed to a culture of *P. chrysogenum* [345]. Experiments with stereospecifically tritiated cysteines have shown [365-368] that ring closure occurs with loss of the 3-*pro*-S hydrogen and hence with retention of configuration at cysteine C3 (Figure 28).



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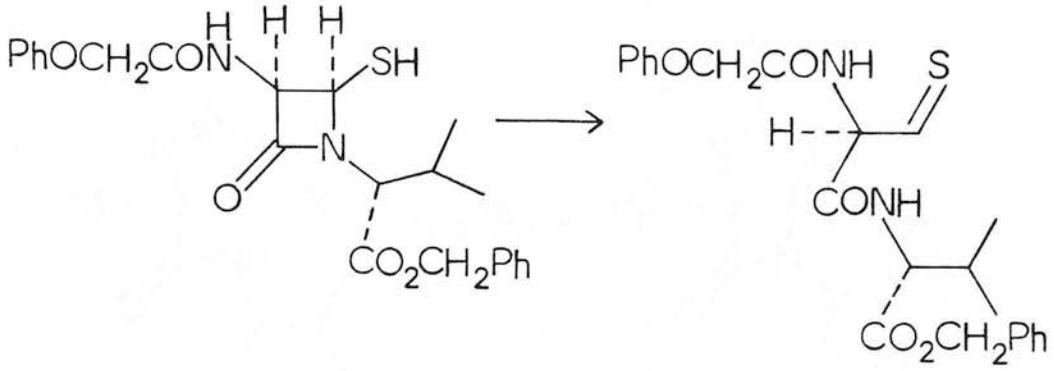
Although these results do not necessarily preclude the intermediacy of the thioaldehyde tautomer (43), oxidation of LLD-ACV to a thioaldehyde intermediate would have to proceed by a mechanism different from the known microbial oxidation of alcohols to aldehydes which generally occur with loss of the *pro*-R hydrogen [369]. Results from chemical model studies [370, 371] suggest that thioaldehydes are not intermediates. Chemically generated thioaldehydes enolized, polymerized or cyclized to a γ-lactam structure but did not produce

β-lactams. Unfortunately the model compounds tested all possessed a 2,3 dehydrovaline residue, then thought to be involved in thiazolidine ring closure. This is now known not to be the case and the reduced nucleophilicity of the valine nitrogen, due to amide and enamine resonance, may be the reason no β-lactam formation was observed. Consequently the thioaldehyde species (43) remains a possible, if unlikely, intermediate. In a formal reversion of the proposed mechanism the thioaldehyde (45) was produced [372] from the monocyclic β-lactam (44) (Figure 29).

Alternatively, hydroxylation at cysteine C3 could activate this position to nucleophilic attack. δ-(L-α-aminoadipyl)-L-3-hydroxy-cysteinyl-D-valine would be highly unstable but might exist long enough as an enzyme bound species for ring closure to occur (Figure 30).

The overall process of ring closure proceeds with retention of configuration at cysteine C3 [365-368]. As nucleophilic attack by the amide lone pair would involve inversion at the carbon centre, hydroxylations must also proceed with inversion. Most hydroxylations at aliphatic carbon, however, proceed with retention of configuration [373] and although a few exceptions have been reported [305] this unusual stereochemical requirement must cast doubt on the intermediacy of a 3-hydroxylated species. Other thioaldehyde equivalents, for example, -OR, -SR or S-Enzyme (for O-Enzyme in Figure 30) are valid alternatives. Model studies [374, 375], with related systems, have provided chemical analogy for ring closure (Figure 31).

Oxidation may occur at the valine nitrogen [376]. Hydroxamic acids are well known natural products produced by many micro-organ-



44 45

FIGURE 29

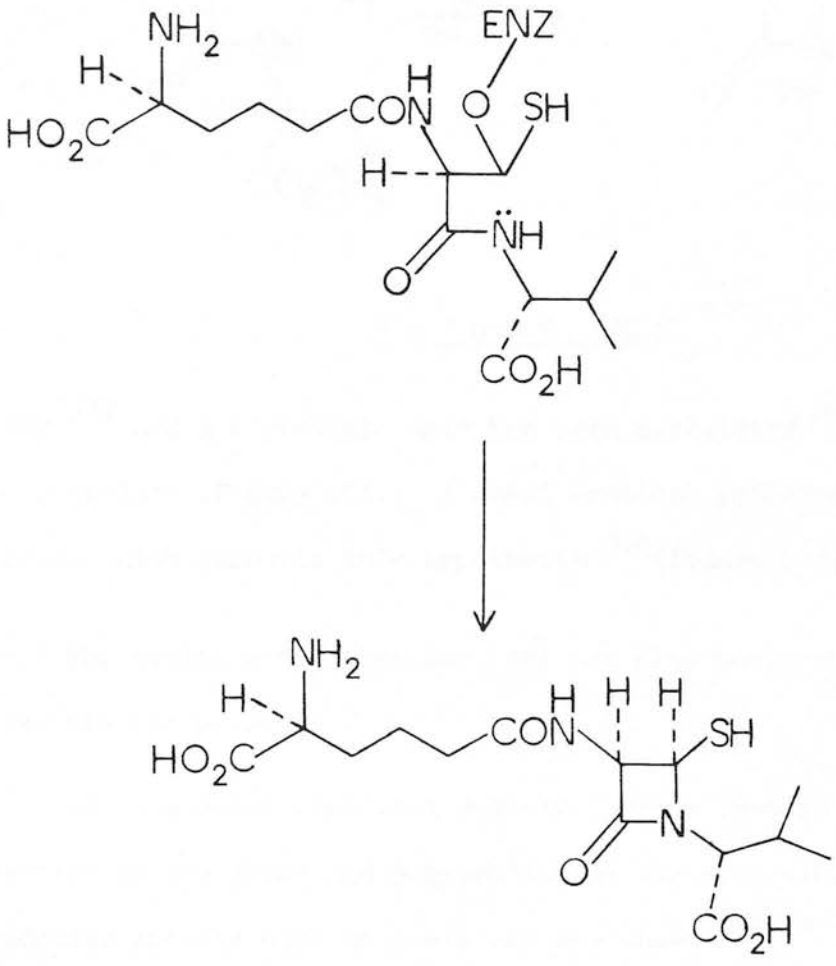
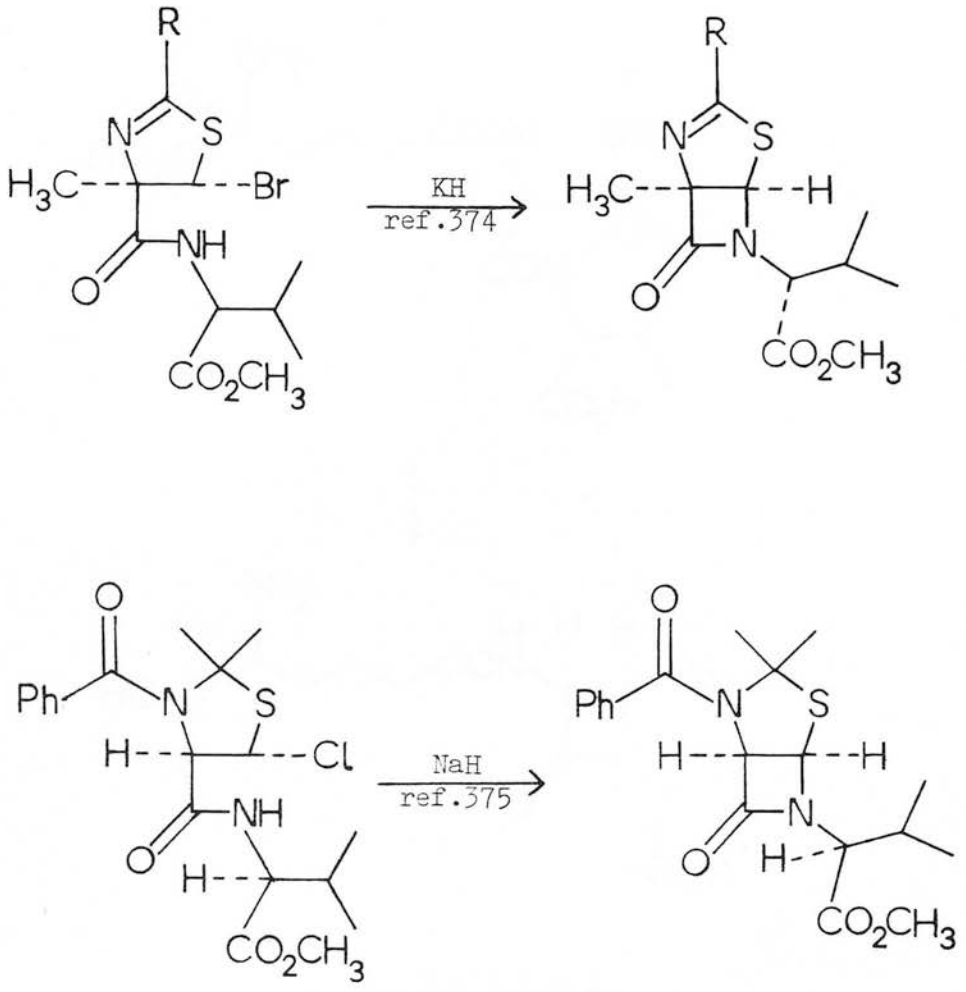


FIGURE 30



F I G U R E 31

isms [377] and a hydroxamic acid has been postulated [378] as a possible intermediate (Figure 32). A model chemical reaction has been described which supports this hypothesis [378] (Figure 33).

The cyclic orthothioamide (46) has also been proposed [379] as a possible intermediate .

All the model reactions described above involve internal protection of the thiol and dehydration of the acyl side-chain. Dehydrated species such as these are now known [341, 342] not to be intermediates in the cell-free conversion of LLD-ACV to isopenicillin N.

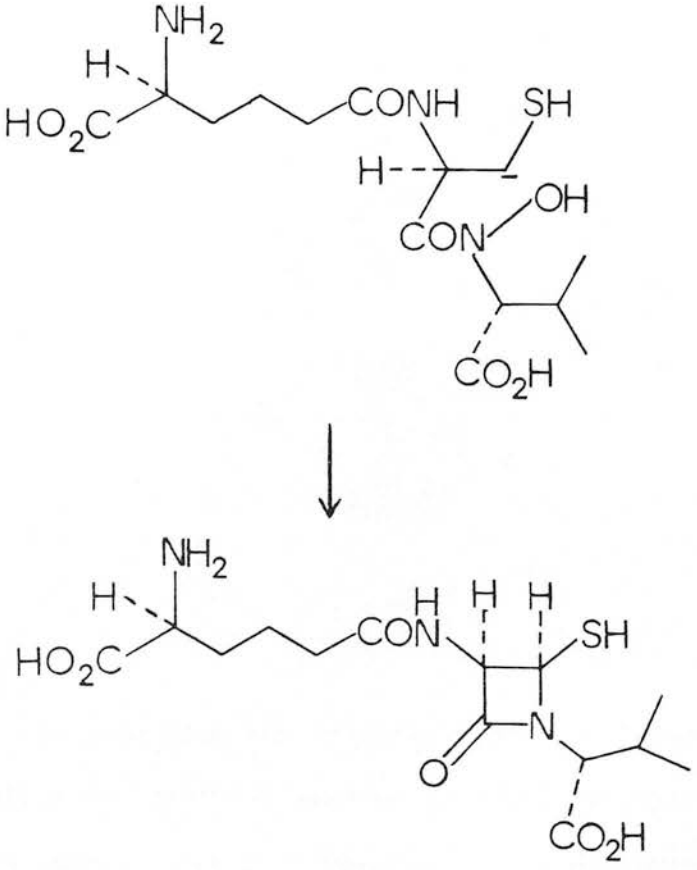


FIGURE 32

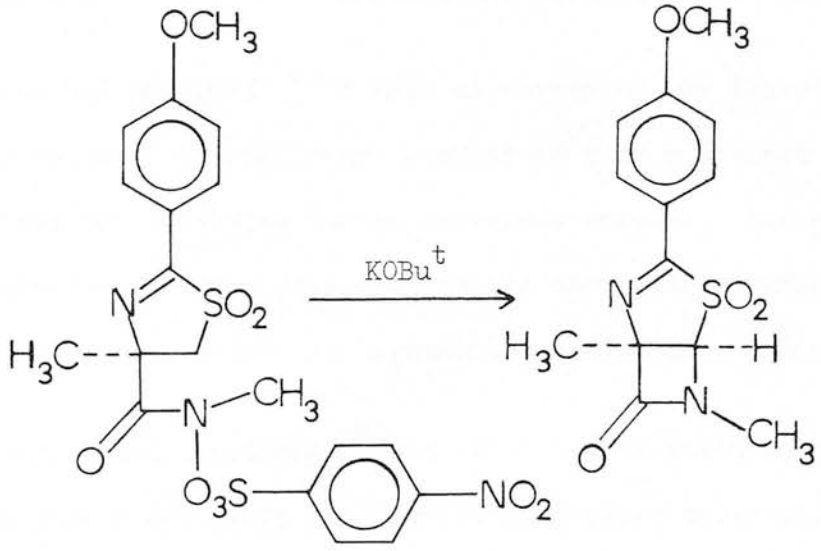
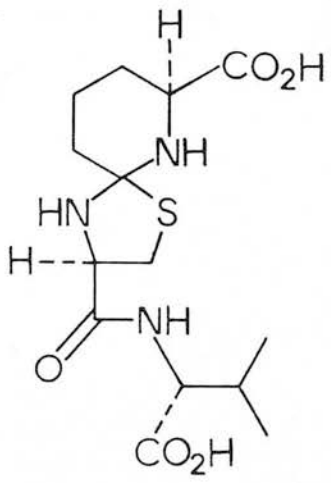


FIGURE 33

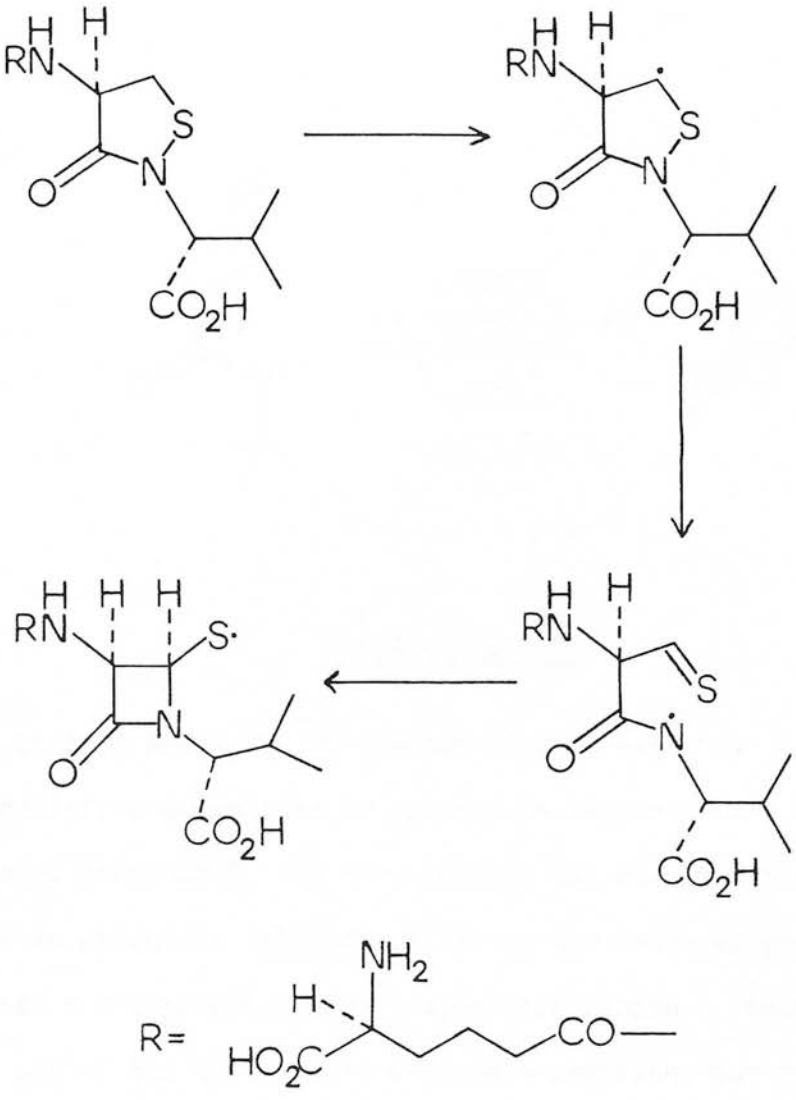


46

No label was lost from the isopenicillin N isolated after incubation of δ-(L-[1,1,6-$^{17}O/^{18}O$]-α-aminoadipyl)-L-cysteinyl-D-valine with a cell-free extract from *C. acremonium*. In a complementary experiment addition of $^{17}O/^{18}O$ water to the incubation mixture did not label isopenicillin N. These results eliminate the intermediacy of cyclic structures such as 46, but do not necessarily preclude an acyclic hydroxamic acid or a thioaldehyde equivalent as intermediates.

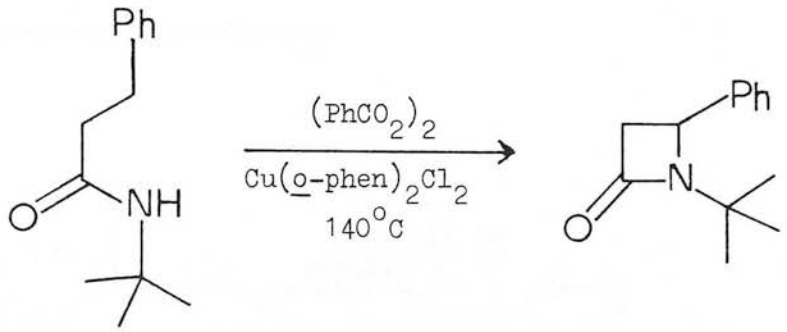
Baldwin has proposed [380] a radical mechanism for β-lactam formation. Isothiazolidinones, readily obtained from cysteinyl peptides [381, 382], may be substrates for an oxygenase enzyme. The generated carbon radical could then rearrange to the more stable sulphur radical, trapped as a symmetric or asymmetric disulphide (Figure 34).

However, model reactions [383] in which isothiazolidinonyl radicals were almost certainly produced did not yield detectable quantities of β-lactam materials. Baldwin has concluded that this



F I G U R E 34

mechanism is not involved in penicillin biosynthesis. β-lactams have been obtained, in small amounts, by treatment of a simple amide with di-*t*-butylperoxide [384] (Figure 35) but radical treatment of stereospecifically labelled *N*-*t*-butyldihydrocinnamamide resulted in complete loss of stereochemistry [385]. The previously mentioned stereochemical constraints on β-lactam formation appear therefore to eliminate any radical mechanisms involving free intermediates of this type.

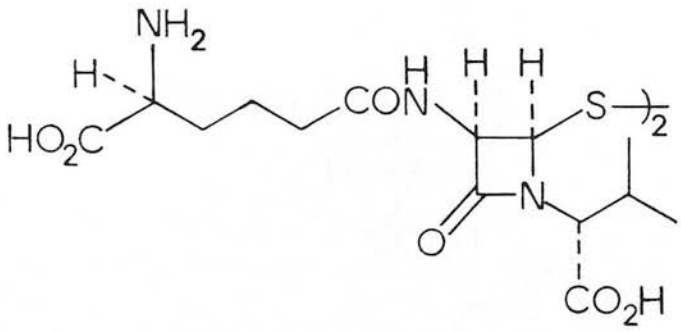


F I G U R E 35

In 1980 Adriaens [347] claimed to have detected an intermediate in the cell-free conversion of LLD-ACV to isopenicillin N. The material, known as compound Y, was not isolated but assigned the monocyclic β-lactam structure (40, Figure 26) on the basis of radiolabelling studies and comparison with a synthetic sample. Compound Y retained only one of the tritia from L-[3-$^3H_2$]-cysteine, but retained the valine C2, C3 and C4 hydrogens. The monocyclic β-lactam was reportedly synthesised (though details of this synthesis have not yet appeared in the literature) and was found to co-elute with compound Y from a cation-exchange column. These results are at variance with the reported syntheses [386, 387] of 1-(D-carboxy-2-methylpropyl)-3-L-(δ-L-α-aminoadipamido)-4-L-mercaptoazetidin-2-one (40). This material is now known to be extremely unstable particularly at alkaline pH. No mention was made of this instability in the original paper and indeed the monocyclic β-lactam was claimed to be stable to the alkaline isolation procedures used.

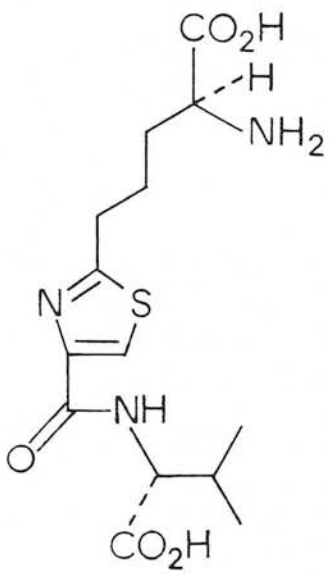
Compound Y could not be the more stable disulphide (47) as

extracted materials were reportedly stored overnight at pH8·2 in the presence of excess DTT; conditions known to destroy the disulphide [386]. Whatever the structure of compound Y, it is unlikely that it was the monocyclic β-lactam (40).

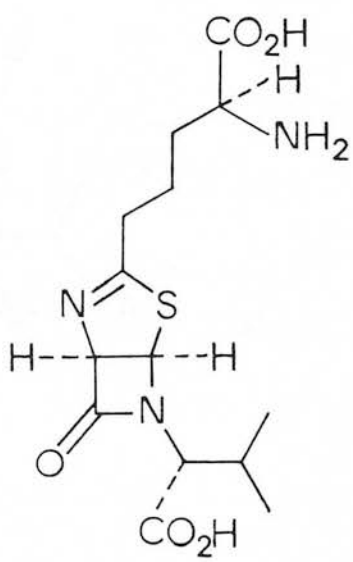


47

A speculative possibility is that compound Y might be the thiazole (48) or fused thiazoline-azetidinone(49).



48



49

Structure 48, while not an intermediate, fits the known radiolabelling results and analogous structures have been obtained from monocyclic β-lactams *via* thioaldehydes [372, 388] (Figure 36).

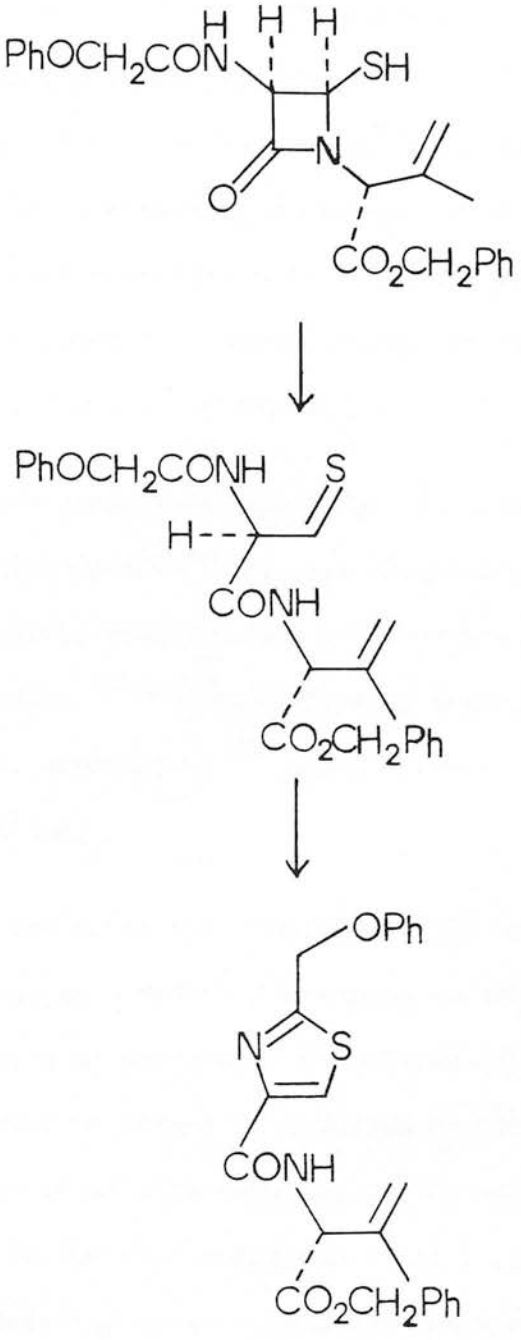

PhOCH2CONH
H
H
H
SH
N
O
CO2CH2Ph
PhOCH2CONH
S
H
CONH
CO2CH2Ph
OPh
N
S
CONH
CO2CH2Ph

FIGURE 36

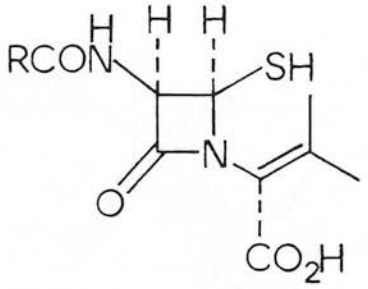
Perhaps the putative intermediate was a thioaldehyde, trapped as the thiazole (48) and subsequently shown to be the same as a rearranged product from the authentic monocyclic β-lactam (40). Baldwin has shown that compound 40 does open to an unstable thioaldehyde [386]. If such speculation is justified Adriaens' observations lend as much support to a thioaldehyde (or equivalent) intermediate as to the monocyclic structure.

The fused thiazoline-azetidinone (49), an internally protected form of the monocyclic species (40), was originally proposed by Cooper [389] as a possible biosynthetic intermediate. Nuclear magnetic resonance studies [341, 342] and feeding experiments with a cell-free extract from *C. acremonium* [390] have, however, eliminated 49 as a possible intermediate.

Incubation of the synthetic disulphide (47) with cell-free extracts of *P. chrysogenum* and *C. acremonium* in the presence and absence of DTT yielded no detectable quantities of isopenicillin N [386, 387]. These results cannot be inferred to eliminate the monocyclic β-lactam (40) as an intermediate, as the material undoubtedly decomposed rapidly in the test solutions. It is still possible that 40 exists transiently as an enzyme bound species. In conclusion, initial closure of the penicillin N4-C5 bond has still to be demonstrated.

Even if the monocyclic β-lactam is an intermediate the mechanism of its formation is unknown. A thioaldehyde, thioaldehyde equivalent, hydroxamic acid or organonometallic species [385] could be involved and each of these proposals remains viable.

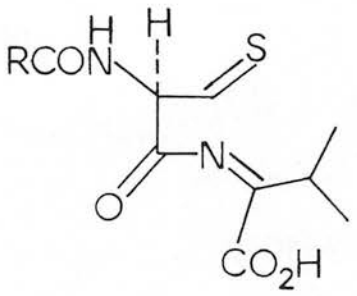
For a long time a2,3 dehydrovaline species (50) was assumed to be involved in thiazolidine ring formation [362].



50

Addition of the thiol across the double bond would effect ring closure and could readily explain the stereochemical inversion of L-valine to the D-penicillamine fragment of penicillins. This early assumption was challenged when ACV was found to already possess a D-valine residue and model reactions [371, 391] completely failed to yield penicillins.

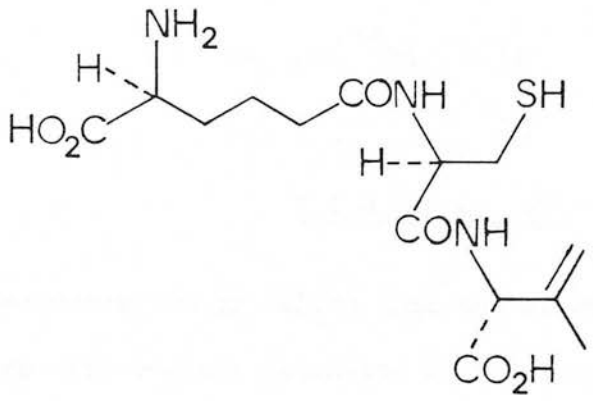
Tritium was retained in the penicillins isolated after incubation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-[2-^{3}H]-valine and δ-(L-α-aminoadipyl)-L-cysteinyl-D-[2-^{3}H,1-^{14}C]-valine with cell-free extracts from *C. acremonium* [328] and *P. chrysogenum* [347]. Such results are inconsistent with a free 2,3 dehydrovaline intermediate and also eliminate the intermediacy of an imine containing species [381] such as 51.



51

DL-[4,4'-$^2H_6$]-valine was fed to *C. acremonium* and *P. chrysogenum* and the isolated penicillins shown [392, 393] by mass spectral analysis to have retained all six deuterium atoms. The diastereoisomeric methyl groups of penicillin G were not labelled when D_2O was added to a *P. chrysogenum* culture [394]. Consequently no 3,4 dehydrovaline intermediates are involved in penicillin biosynthesis.

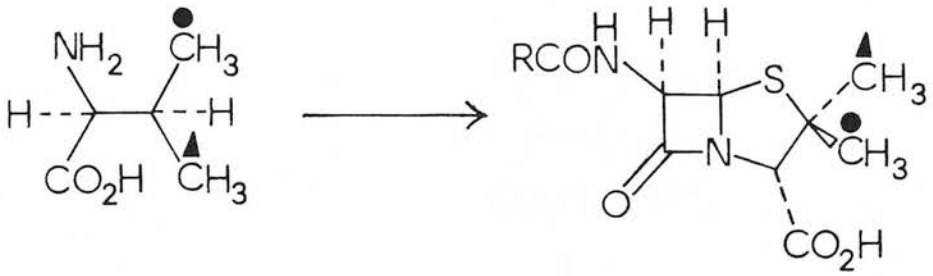
The conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-[2,4,4'-$^2H_7$]-valine to isopenicillin N in a cell-free system from *C. acremonium*, proceeded without loss of deuterium [395], as evinced from ^2H n.m.r. spectroscopy of the product, thus confirming earlier conclusions. The tripeptide (52) was recently synthesised [396] and found not to be a substrate for the cell-free production of isopenicillin N.



52

The stereochemical fate of the prochiral methyl groups of valine has been studied by several groups. Stereospecifically labelled precursors have been synthesised and fed and the biosynthesised penicillin V or penicillin N analysed by n.m.r. spectroscopy. Unfortunately the nomenclature used in this area is confusing and frequently incorrect. The correct nomenclature [397], and not necessarily the nomenclature used in the original literature, is used throughout the following discussion.

The β-methyl group of penicillin V was labelled when (2RS,3S)-[4-^{13}C]-valine was fed to *P. chrysogenum* [398] and in complementary experiments (2S,3R)-[4-^{13}C]-valine labelled the α-methyl of penicillin N [399] and penicillin V [400] (Figure 37).



▲ = $^{13}CH_3$, ● = $^{12}CH_3$ — 3R

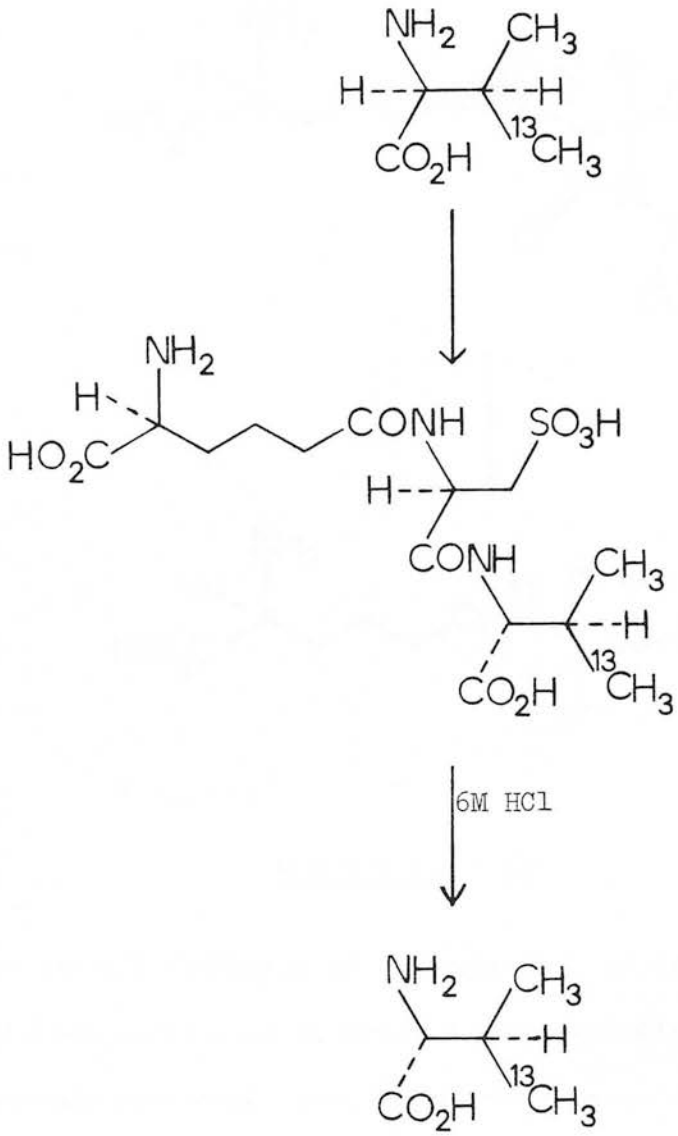
▲ = $^{12}CH_3$, ● = $^{13}CH_3$ — 3S

F I G U R E 37

Incorporation of valine into the bicyclic penicillin structure proceeds with overall retention of configuration at valine C3. Tritium studies [344] suggested that the first step, production of LLD-ACV, occurred with retention of configuration. Isolation of chirally labelled LLD-ACV as its sulphonic acid (ACyaV) later confirmed this [401]. When (2S,3R)-[4-^{13}C]-valine was fed to the β-lactam-negative mutant, *C. acremonium* N2, only the low field methyl group of LLD-ACyaV was labelled. Hydrolysis of the purified tripeptide gave (2R,3R)-[4-^{13}C]-valine [401] (Figure 38).

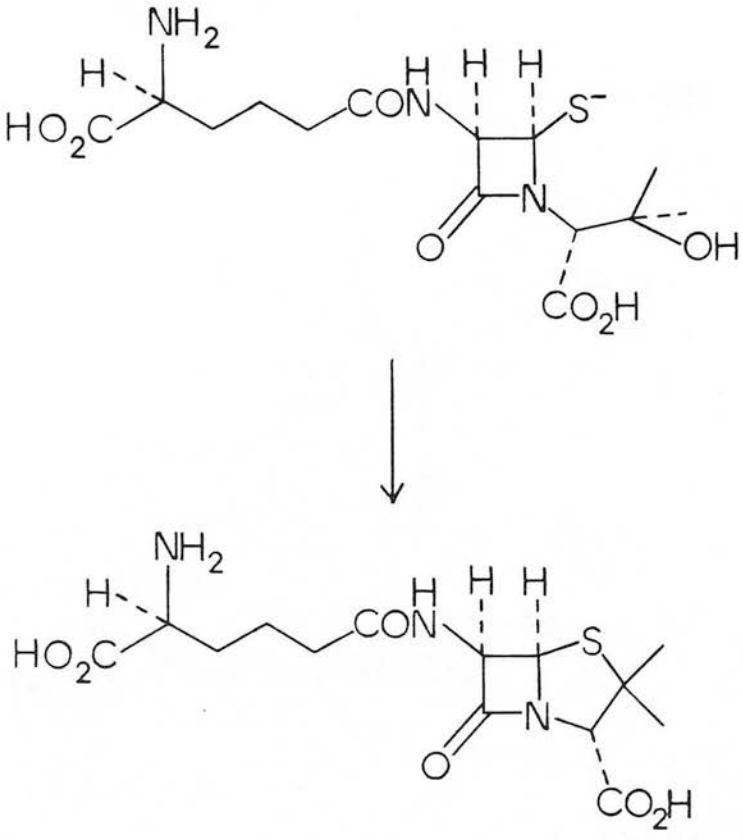
For all labelling results to be self-consistent the conversion of LLD-ACV to isopenicillin N must occur with retention of configuration at valine C3.

The incubation of chirally labelled LLD-ACV with a cell-free system and analysis of the synthesised isopenicillin N has not been reported. However, tripeptides related to LLD-ACV have been studied [353, 360] and produce the isopenicillin N analogues expected on the basis of the preceding stereochemical arguments.



F I G U R E 38

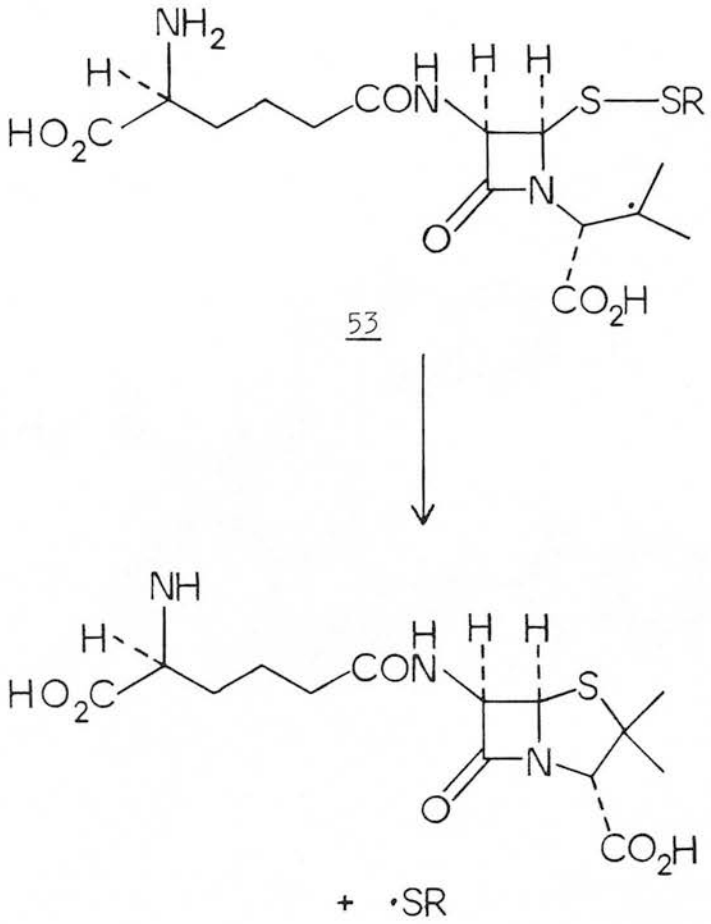
Tetrapeptides containing a 3-hydroxyvaline residue have been isolated from *C. acremonium* [329] and *Paecilomyces persicinus* [331]. It was proposed [305] that nucleophilic displacement of hydroxide by sulphur could effect ring closure (Figure 39).



F I G U R E 39

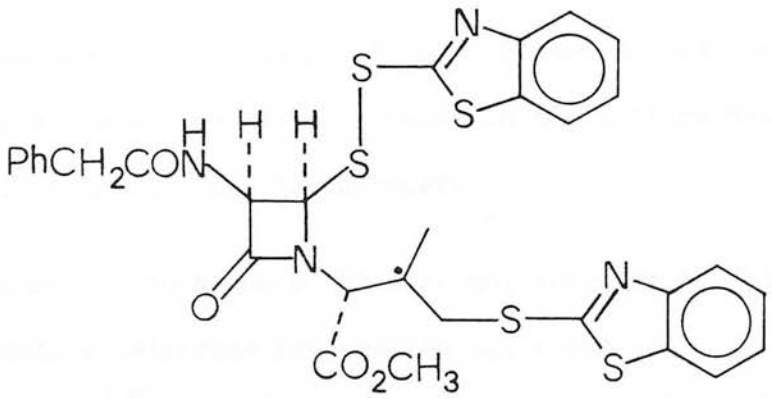
For overall retention of configuration at C2 (C3 of valine), hydroxylation would have to proceed with inversion of configuration. As previously mentioned, however, hydroxylation at aliphatic carbon generally proceeds with retention of configuration [373] and this proposal appears unlikely. An alternative mechanism [402] would satisfy the stereochemical requirements of the ring closure reaction, but recent feeding studies [361] have shown that δ-(L-α-aminoadipyl)-L-cysteinyl-3-hydroxy-D-valine is not incorporated into isopenicillin N.

Baldwin has suggested [403] that the 3-hydroxyvaline peptides are shunt products. The radical (53) generated by hydroxylase activity might be intramolecularly trapped to give isopenicillin N (Figure 40). Occasionally "normal" hydroxylation at the carbon centre would be observed.



F I G U R E 40

A model reaction has been reported [403] but this mechanism can only be acceptable and in agreement with the known stereochemical facts if bond formation is shown to be much faster than valine C2-C3 bond rotation. A radical intermediate (54) has been proposed [404] in the photochemical formation of a penam from an azetidin-2-one disulphide. Ring closure, in this instance, gave a mixture of epimers at C2.



54

At present is it not possible to say how LLD-ACV is converted to isopenicillin N. Many mechanisms have been eliminated and others appear unlikely, but no details are known as to the actual mechanism of ring closure. All intermediates may be enzyme bound, in which case a full understanding of the problem will require quantities of pure isopenicillin N synthetase. This enzyme appears fairly stable but its isolation has not yet been reported.

Isopenicillin N is the last common intermediate in the biosynthesis of the penicillins, cephalosporins and cephamycins. At this point the pathway branches to give the solvent soluble penicillins in *Penicillium* spp. and the cephalosporins in *Cephalosporium* and *Streptomyces* spp.

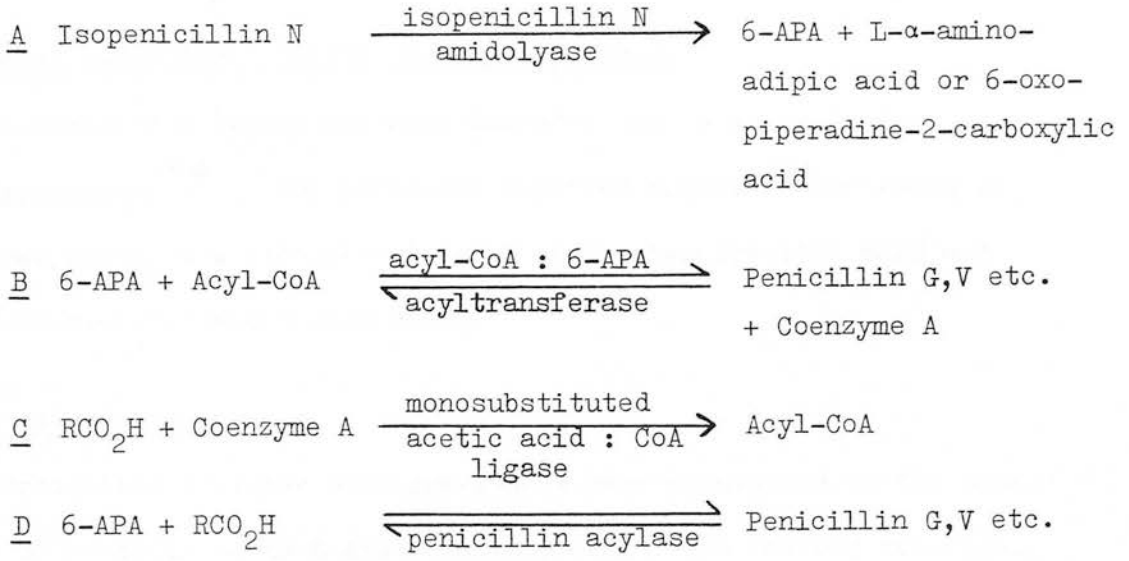
3.4 THE SOLVENT SOLUBLE PENICILLINS

The absolute requirement for L-α-aminoadipic acid in the biosynthesis of penicillins [307], and the presence of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine but not cysteinylvaline peptides in the mycelium of *P. chrysogenum* [325], suggests that isopenicillin N is a precursor of the solvent soluble penicillins. It was unclear whether 6-aminopenicillanic acid (6-APA) (6), found in the culture medium [46, 47], was an intermediate in this process.

Isopenicillin N and 6-APA were not taken up by intact mycelia and an active cell-free preparation was required. Such a system was reported [405] in 1975 and shown to catalyse the conversion of tritium labelled isopenicillin N and phenylacetyl-CoA to penicillin G. 6-APA but not penicillin N also gave penicillin G under identical conditions.

The inter-relationship of isopenicillin N, 6-APA and the solvent soluble penicillins is now more clearly understood [306]. Isopenicillin N is an obligate intermediate in the biosynthesis of the penicillins, but under the influence of various enzymes is hydrolysed to 6-APA. 6-APA is subsequently acylated to give, for example, penicillin G. Four classes of enzyme have been identified: isopenicillin N amidolyase, acyl-CoA:6-APA acyltransferase, monosubstituted acetic acid:CoA ligase and penicillin acylase (Figure 41).

As enzymes A and B are probably closely associated within the cell very little free 6-APA is observed.



F I G U R E 41

3.4.1 Isopenicillin N Amidolyase

Abraham and co-workers [306] have partially purified an enzyme from *P. chrysogenum* which hydrolyses isopenicillin N to 6-APA and L-α-aminoadipic acid. Cell-free extracts from *P. chrysogenum* were observed [347] to catalyse an acyltransfer reaction between labelled isopenicillin N and unlabelled 6-APA. Both results are consistent with the presence of an isopenicillin N amidolyase.

3.4.2 Acyl-CoA:6-APA Acyltransferase

The enzymic conversion of 6-APA and phenyacetyl-CoA to penicillin G has already been mentioned [405]. A purified acyltransferase [406] synthesised penicillins from the coenzyme A derivatives of various monosubstituted acetic acids. 6-APA acyltransferase activity has also been implied from radiolabelling studies [407].

3.4.3 Monosubstituted Acetic Acid: Coenzyme A Ligase

A coenzyme A ligase has been demonstrated in extracts from *P. chrysogenum* [408]. The partially purified enzyme [409] was found to condense coenzyme A with phenylacetic acid, phenoxyacetic acid and other monosubstituted acetic acids.

3.4.4 Penicillin Acylase

Penicillin acylases have previously been considered in the hydrolysis of penicillins to 6-APA (Section 1.1). The reverse reaction, formation of penicillin G from 6-APA and phenylacetic acid, has been observed [410] in extracts from *P. chrysogenum*.

Studies with bacterial penicillin acylases [411, 412] have suggested that this class of enzyme plays only a minor role in penicillin biosynthesis, and that enzymes A, B and C produce almost all the solvent soluble penicillins.

3.5 PENICILLIN N BIOSYNTHESIS

The conversion of isopenicillin N to penicillin N has been demonstrated [413] in a cell-free extract from *C. acremonium*. Incubation of isopenicillin N produced a penicillinase sensitive material which, when bioassayed against *Salmonella typhi*, proved to be penicillin N. The epimerase responsible is a soluble enzyme which appears to be extremely labile; only 15% of the activity remained after storage for 24 hours at −20°C.

Deacetoxycephalosporin C is produced from penicillin N (see Section 3.6). LLD-ACV [349, 414] or isopenicillin N [415] have been converted to cephalosporins by fresh cell-free extracts, presumably *via* penicillin N. This ability was lost following overnight storage

at −80°C. In repeated experiments using old cell-free extracts LLD-ACV was converted to only isopenicillin N, even though the systems remained capable of transforming penicillin N to deacetoxycephalosporin C [416]. Further evidence of epimerase activity has been provided using a *S. clavuligerus* cell-free preparation [355, 356]. Incubation of LLD-ACV and cofactors produced a mixture of isopenicillin N and penicillin N.

3.6 OXIDATIVE RING EXPANSION OF PENICILLIN N

As all cephalosporin producing species produce penicillin N, a possible precursor-substrate relationship has always been accepted. Early experiments [321] suggested this was not the case. However, the failure to isolate penicillin N negative − cephalosporin positive organisms when penicillin N positive − cephalosporin negative mutants were known [312], is readily explained if penicillin N is a precursor of the cephalosporins.

Incubation of penicillin N with cell-free extracts from *C. acremonium* produced a penicillinase resistant, β-lactamase sensitive antibiotic [216, 416-419] identified by h.p.l.c. [216, 419] and paper electrophoresis, paper chromatography and t.l.c. [416] as deacetoxycephalosporin C (35). Penicillin G and 6-APA were not substrates for the ring expansion enzyme.

The mechanism of ring expansion is unknown but published labelling experiments eliminate a number of possibilities. Thus C4, C4' deuterated valines, fed to *C. acremonium*, labelled cephalosporin C in the C2 and C3' positions with only the expected loss of two deuterium atoms [343, 393]. Neither position was labelled when the

incubation was repeated in 80% D_2O with unlabelled valine as substrate [393]. (2RS,3S)-[4-^{13}C]-valine [398] labelled cephalosporin C at C2 while (2S,3R)-[4-^{13}C]-valine [399] labelled the C3' position. Given the established labelling pattern of penicillins (Figure 37) the overall penicillin N-cephalosporin C conversion is believed to proceed as shown in Figure 42.

Thus the formation of deacetoxycephalosporin C does not involve the intermediacy of a free Δ^2 cephem or oxidation of a penicillin N methyl group to an aldehyde.

The penicillin N sulphoxide (55) was proposed [108] as an intermediate on the basis of analogy with the chemical conversion of penicillins to cephalosporins (Section 1.2). However, the β-sulphoxide of penicillin N (55) and β-acetoxymethylpenicillin N (56) have been tested in a cell-free system from *C. acremonium* and are not intermediates [380].

Optimal ring expansion activity in cell-free systems requires high aeration, an energy generating system, ferrous ions and ascorbic acid [417, 418, 420]. The enzyme was inhibited by potassium cyanide [417], zinc ions and copper ions [420], and appeared to be an oxygenase. When partially purified [421] the enzyme had properties similar to those of a 2-oxoglutarate-linked dioxygenase (Figure 43).

In this case penicillin N is the reduced substrate, SH, and the product, a hydroxylated species, may well be β-hydroxymethylpenicillin N (57). β-hydroxymethylpenicillin N has not yet been synthesised and tested [380] as a substrate for the ring expansion enzyme.

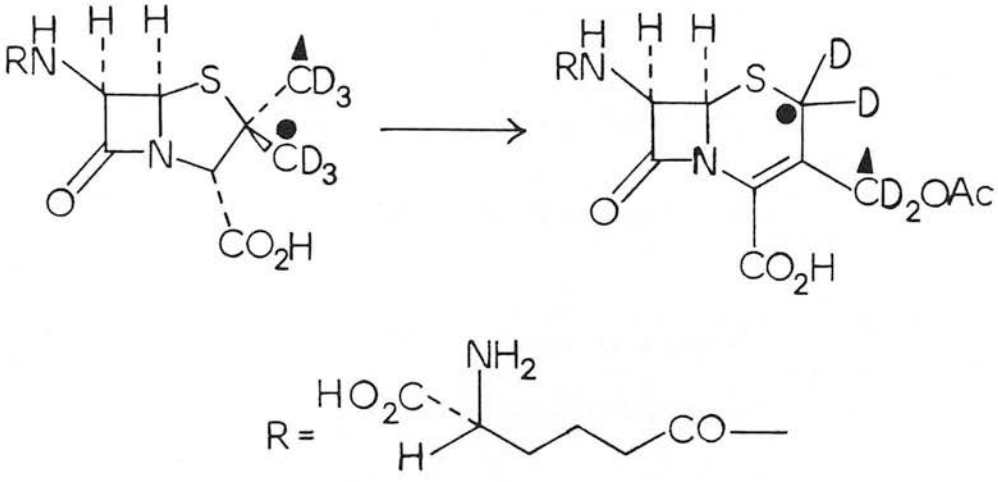
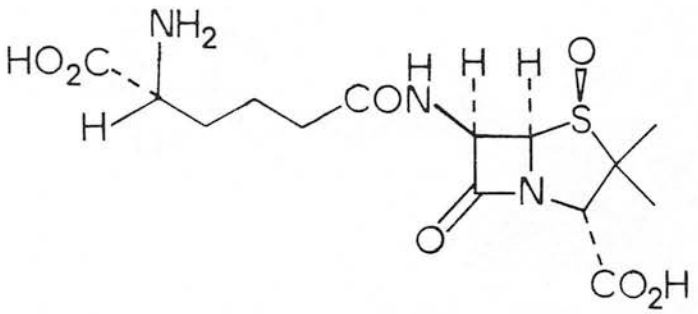
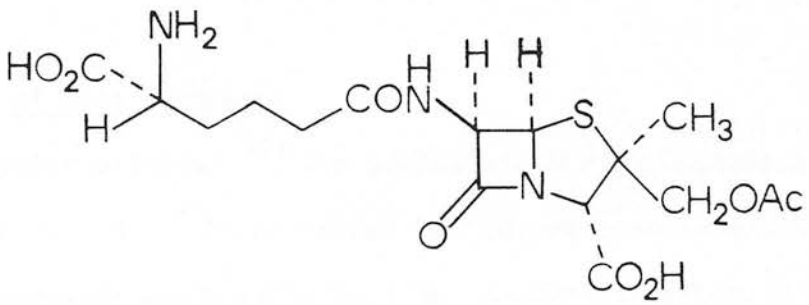


FIGURE 42



55



56

SH + O_2 + 2-oxoglutarate

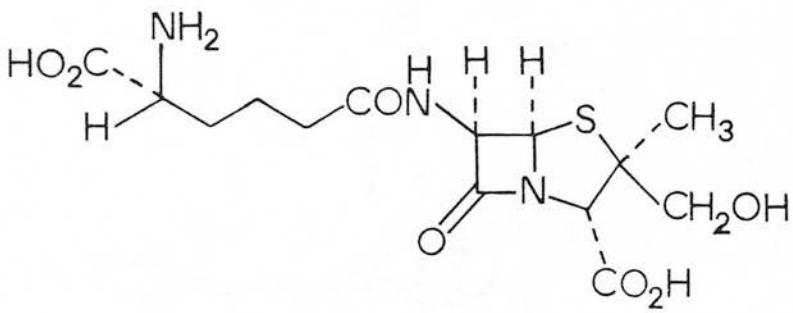
↓ dioxygenase

SOH + succinic acid + CO_2

SH = reduced substrate

SOH = oxidised substrate

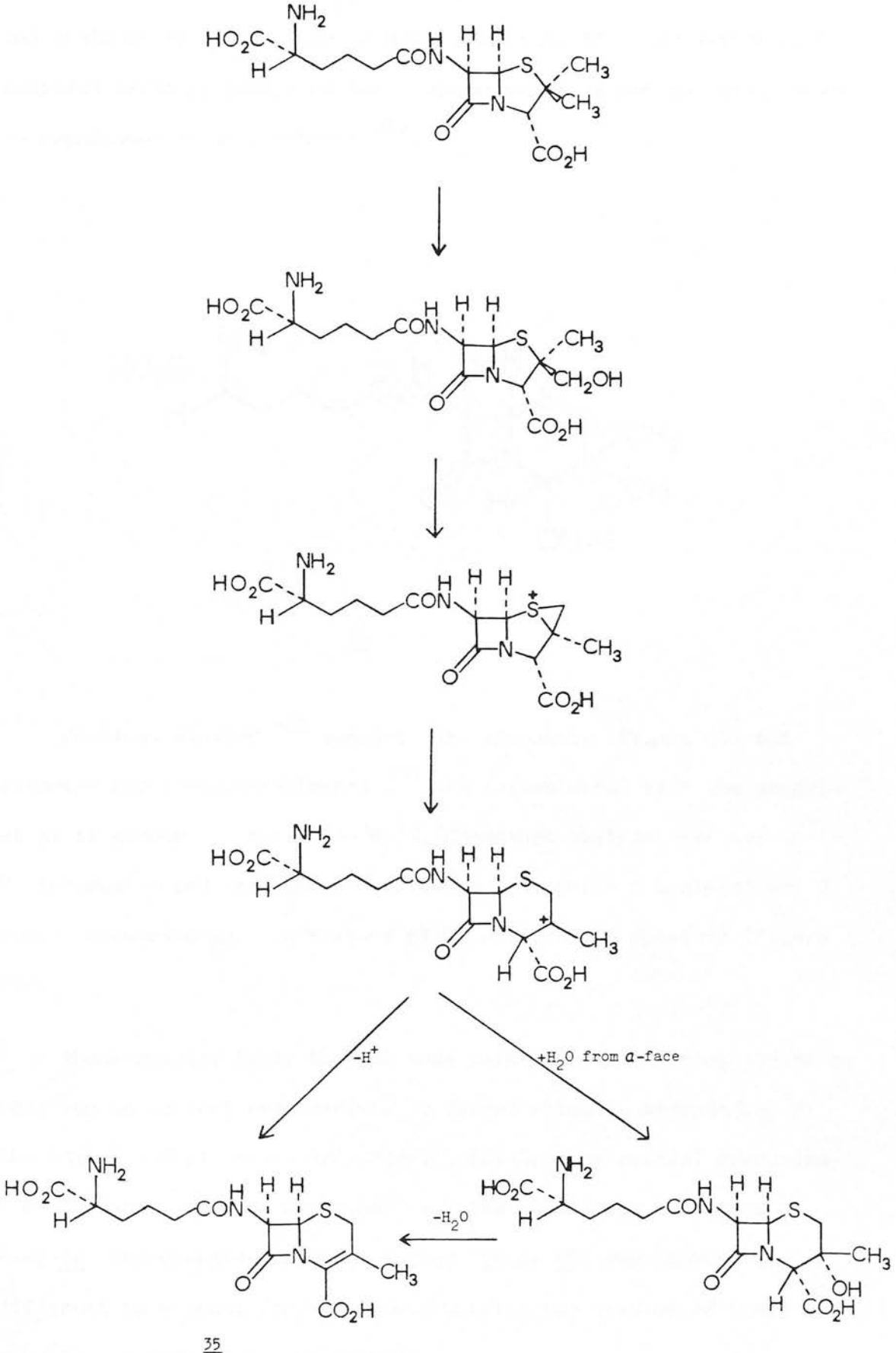
F I G U R E 43



57

3.6.1 Mechanism of Ring Expansion

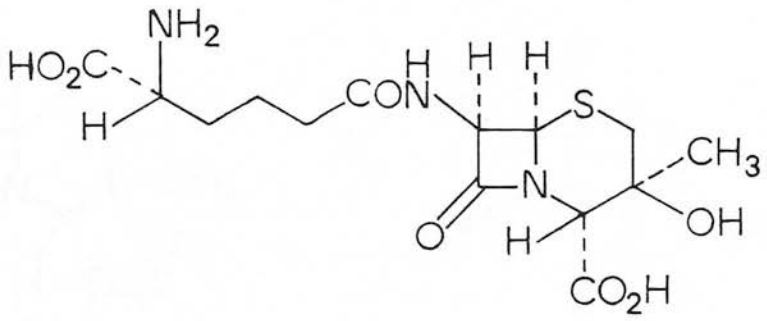
A popular proposal [306] for penicillin N ring expansion is outlined in Figure 44. Displacement of hydroxide or an activated form of hydroxide would give the β-episulphonium ion which could rearrange to a tertiary carbonium ion. Loss of H^+ or addition and trans-elimination of H_2O would yield deacetoxycephalosporin C (35).


NH2
HO2C
H
CON
H H H
S
CH3
CH3
N
O
CO2H
CH2OH
+
-H+
+H2O from α-face
-H2O
OH

35

FIGURE 44

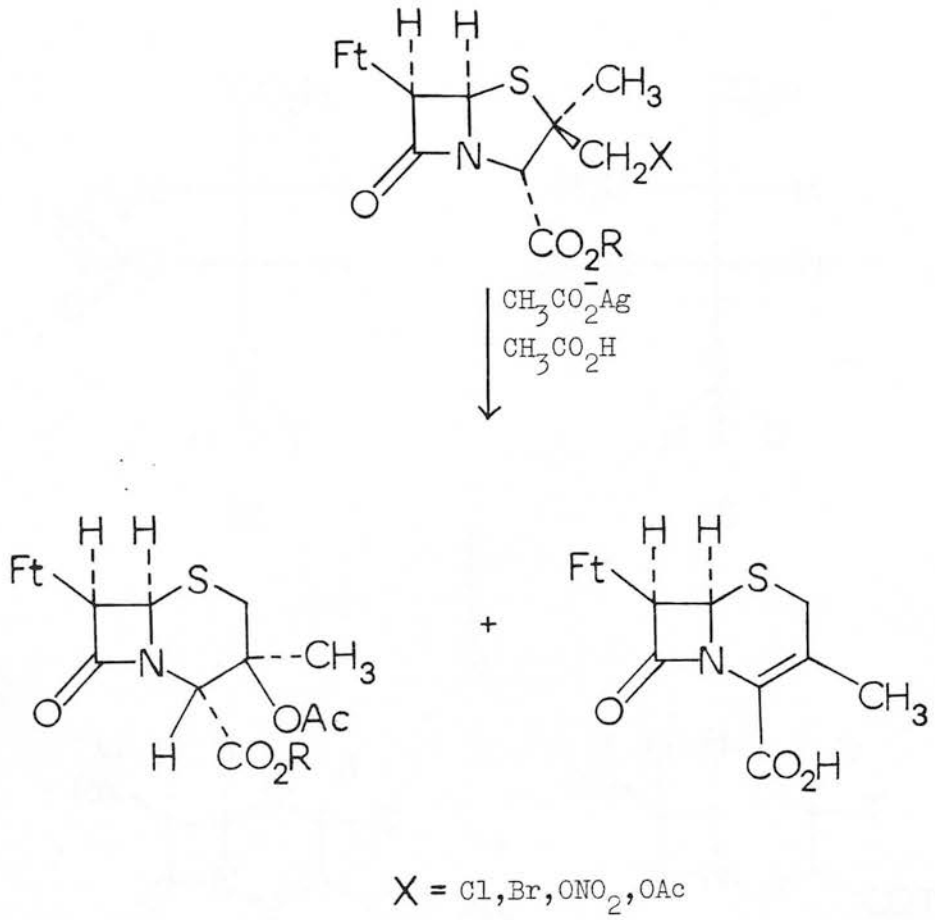
The isomeric alcohol (58) has been isolated [216] from *C. acremonium* and could arise by addition of H_2O to the more hindered ß-face of the proposed tertiary carbonium ion. Compound 58 is not an intermediate in cephalosporin biosynthesis [216].



58

Chemical studies [422] support this mechanism (Figure 45) but recently published experiments [423] are inconsistent with the proposal as it stands. Chiral [4-^{3}H, ^{2}H, ^{1}H]-methyl valines were fed to *C. acremonium* and the biosynthesised cephalosporin C analysed by ^{3}H n.m.r. spectroscopy. A mixture of C2 epimers was obtained (Figure 46).

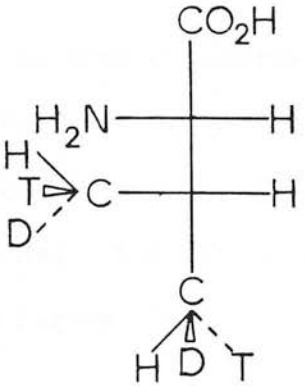
These results imply that at some point during the ring expansion reaction an achiral intermediate is formed which is independent of the chiral methyl valine precursor. Complete or partial randomization of label could be explained, but the consistent excess of 61 over 62, irrespective of which valine (59 or 60) was tested, is difficult to account for and no mechanistic explanation of these results was offered by the authors.



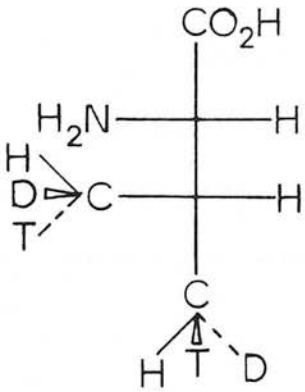
X = Cl, Br, ONO_2, OAc

F I G U R E 45

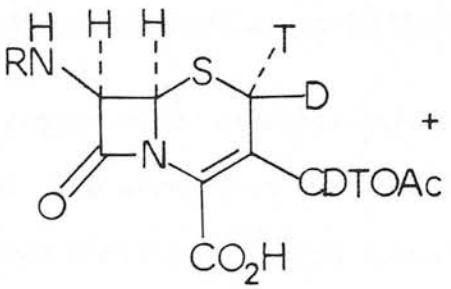
Further work is required to prove or disprove the proposed biosynthetic pathway (Figure 44) although some modification to this scheme appears essential. The intermediacy of the 3-exomethylene-cepham (63) in the conversion of penicillin N to deacetoxycephalosporin C has been discussed [216, 424, 425]. Results indicate that the exomethylene compound is not an intermediate, but under certain conditions can be converted [425] to deacetylcephalosporin C (36).



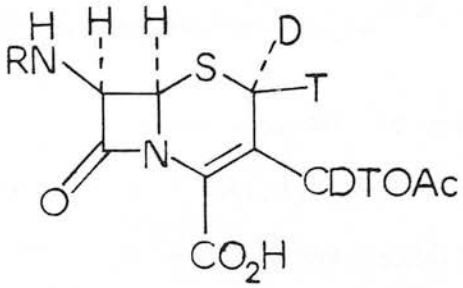
59



60



61



62

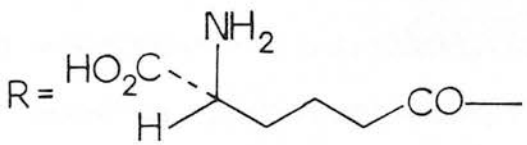
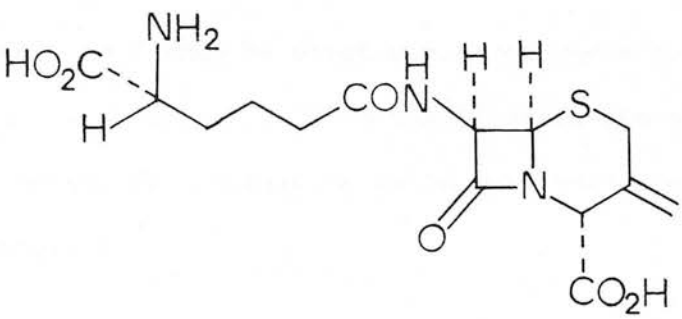


FIGURE 46



63

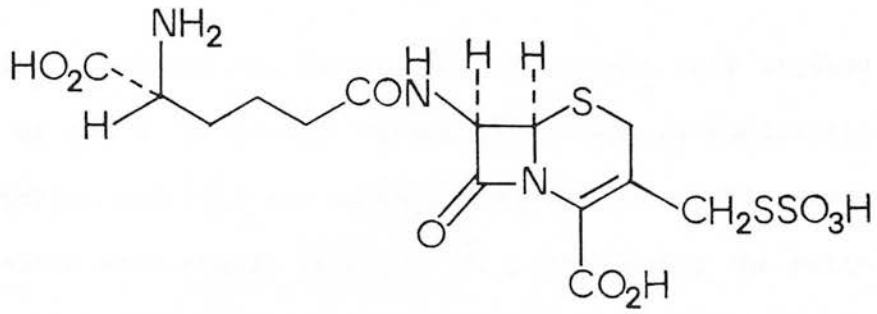
3.7 THE CEPHALOSPORINS AND CEPHAMYCINS

In some *C. acremonium* mutants [426, 427] deacetoxycephalosporin C is the final biosynthetic product, but in most β-lactam producing *Cephalosporium* and *Streptomyces* species additional oxidation, acylation and substitution reactions produce a wide variety of antibiotics (see Figure 7).

3.7.1 Functionalization at C3'

A substrate specific dioxygenase present in *C. acremonium* [428, 429] and *S. clavuligerus* [429] stereospecifically [423] hydroxylates deacetoxycephalosporin C to give deacetylcephalosporin C (36). The newly introduced hydroxyl group is derived from molecular oxygen [430, 431].

Acylation of deacetylcephalosporin C yields a number of derivatives. An acetyl-CoA:deacetylcephalosporin C *O*-acetyltransferase has been purified [427] from extracts of *C. acremonium* and found to catalyse the formation of cephalosporin C. A 3-hydroxmethylceph-3-em:*O*-carbamoyltransferase, from *S. clavuligerus* [432, 433], produced *O*-carbamoyldeacetylcephalosporin C from deacetylcephalosporin C and cephamycin C from 7α-methoxydeacetylcephalosporin C. Cephamycins A and B and compound C-2801X (Figure 7) may arise by acylation of a deacetylcephalosporin or by non-enzymic displacement of carbamate from cephamycin C. The C3' thiol substituted cephalosporins [212, 214, 426] may be formed by displacement of acetate from cephalosporin C. This may be enzymic, as in the biosynthesis of cysteine from *O*-acetylserine, or non-enzymic as in the synthesis [434] of 64 from cephalosporin C.



64

3.7.2 7α-Methoxylation

Studies [435] with a cell-free system from *S. clavuligerus* have shown that the methoxy substituent is introduced in two steps. A 2-oxoglutarate-linked dioxygenase incorporates molecular oxygen [431] into *O*-carbamoyldeacetylcephalosporin C. The resultant alcohol is rapidly methylated to give cephamycin C. The methyl donor is *S*-adenosylmethionine [314]. Cephalosporin C was likewise converted to 7α-methoxycephalosporin C but neither deacetoxycephalosporin C nor deacetylcephalosporin C were substrates for this enzyme [435] (Figure 20).

3.7.3 Miscellaneous Reactions

A number of modifications to the D-α-aminoadipyl side-chain of the cephalosporins have been observed. Some *Cephalosporium* strains oxidatively deaminate and decarboxylate the side-chain [436, 437] while others accumulate *N*-acetylated derivatives [438]. These compounds are not involved in cephalosporin biosynthesis.

3.8 ADDENDUM

Such is the current interest in β-lactam biosynthesis that since this manuscript was prepared several relevant publications have appeared in the literature.

Using model systems Easton and co-workers have studied the ring closure of the β-lactam and thiazolidine rings of penicillin. The isothiazolidinone (65) was converted to the monocyclic β-lactam (66) when treated with phenyl lithium [439]. Presumably the amide amion (67) was an intermediate in this reaction (Figure 47).

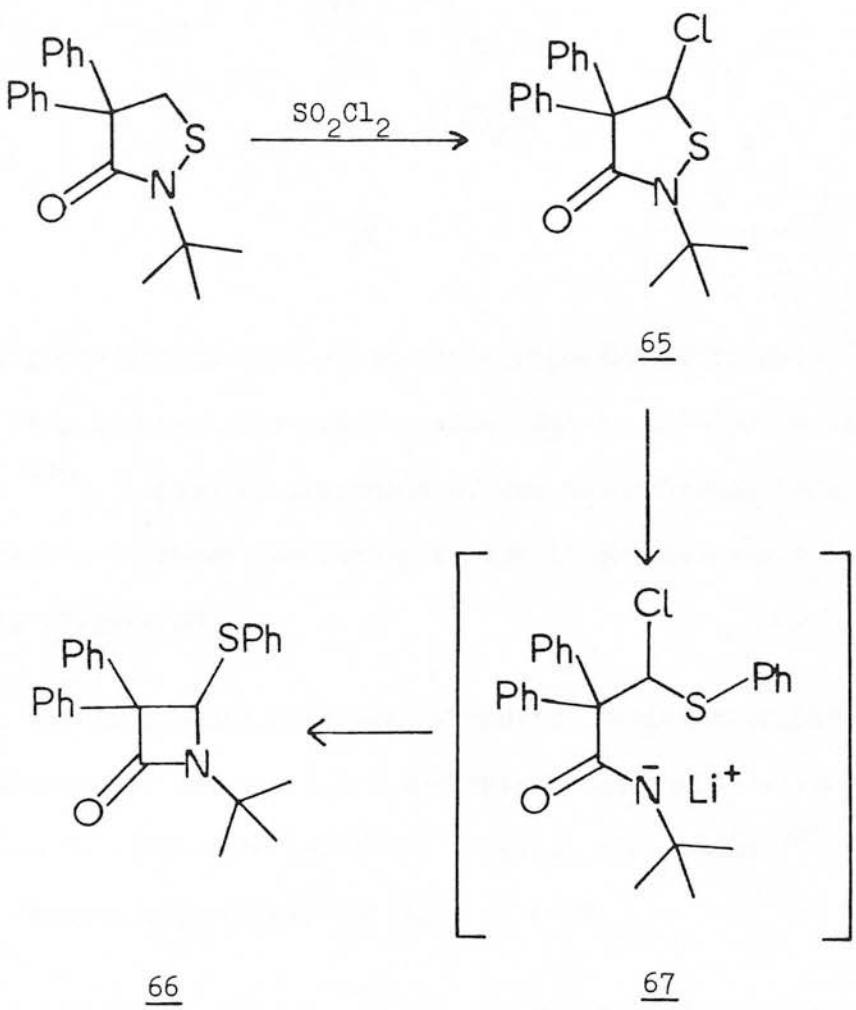
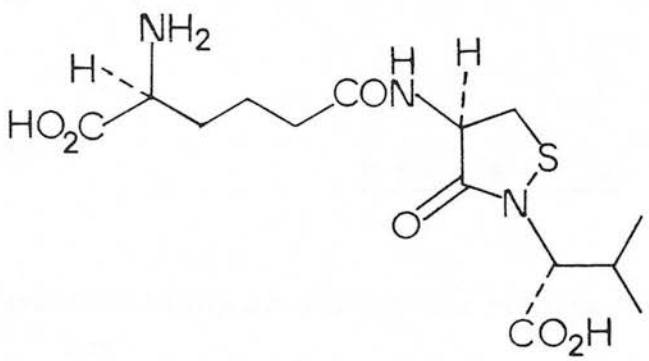


FIGURE 47

The mechanism of ring closure is similar to the model thioaldehyde equivalents previously discussed (see Figure 31). The analagous *in vivo* reaction could be tested by feeding the peptide derivative (68) to a cell-free system capable of converting LLD-ACV to isopenicillin N.

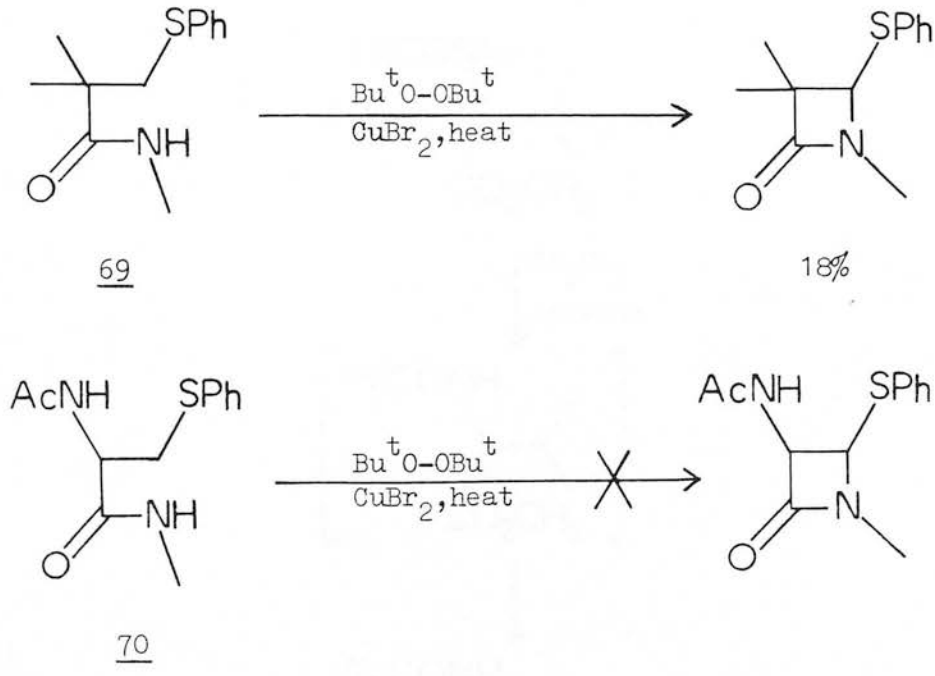


68

Using conditions similar to those reported by Baldwin [384, 385] radical ring closure of a simple amide (69) to a β-lactam was achieved [440]. However, treatment of the biosynthetic model (70) in an identical manner completely failed to produce any β-lactam materials (Figure 48).

The stereochemical problems of radical β-lactam formation have been discussed in Section 3.3.2 and this latest publication offers little new to contradict Baldwin's original conclusion [385] that a radical mechanism is highly unlikely *in vivo*.

In contrast a proposal [403] for the radical closure of the



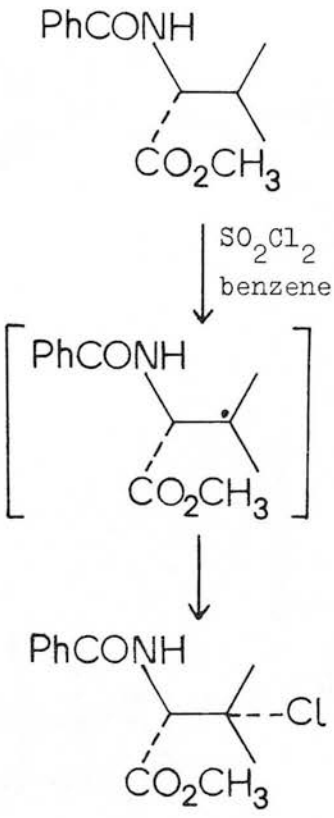
69 18%

70

F I G U R E 48

thiazolidine ring of penicillins remains a possibility (Figure 40). Easton [441] subjected *N*-benzoylvaline methyl ester to radical chlorination conditions. *N*-benzoyl-3-chlorovaline methyl ester was isolated as the major product (Figure 49), suggesting (perhaps not surprisingly) that hydrogen abstraction from the tertiary valine C3 position is most favoured. These results, together with the model studies discussed earlier [403], provide chemical evidence to support an *in vivo* closure of the thiazolidine ring by a radical mechanism.

In two communications from Baldwin and Abraham [442, 443] a highly purified sample of the single enzyme isopenicillin N synthetase [444] (molecular weight 37,000 a.m.u.) was used to investigate the mechanism of penicillin formation. Incubation of the modified substrate, α-(L-α-aminoadipyl)-L-cysteinyl-D-(α-aminobutyric) acid, with this enzyme, in the presence of $FeSO_4$, DTT, ascorbic acid, catalase and oxygen [445], afforded two β-lactam products in a 3:1 ratio (Figure 50).

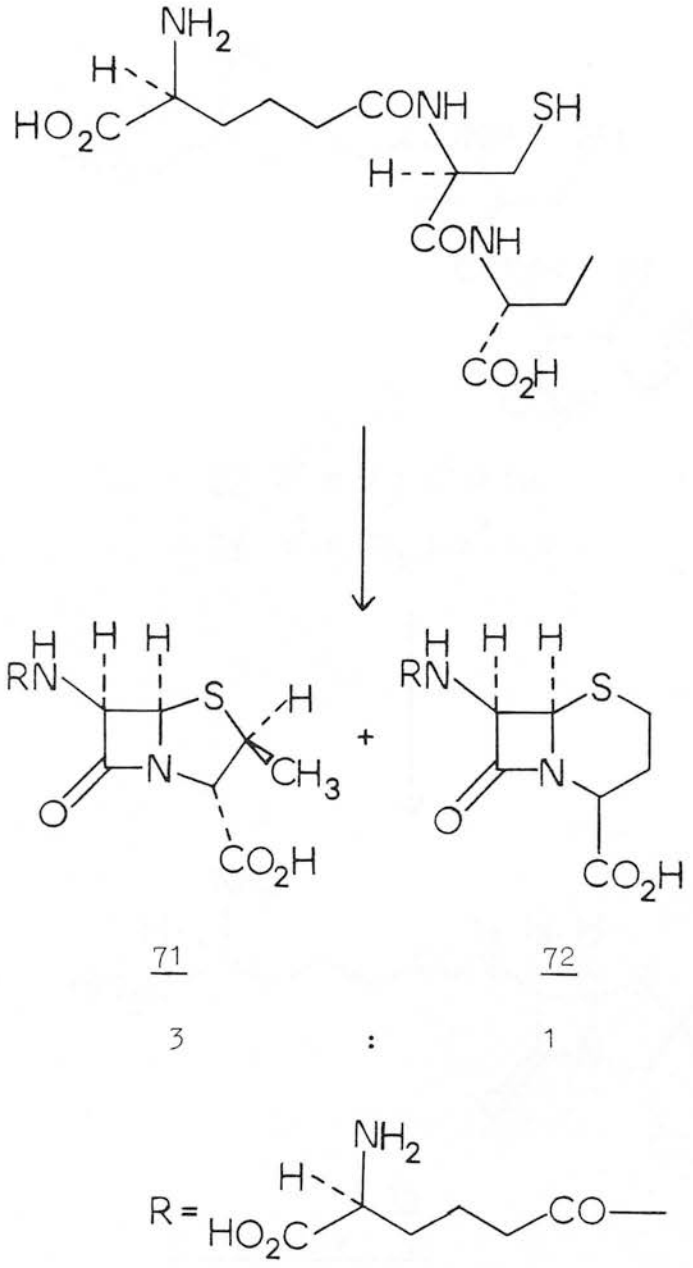


F I G U R E 49

One product was identified as the penicillin (71) reported previously[360] and the second material as the cepham (72) of undefined stereochemistry (Figure 50). The tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, gave only one product, isopenicillin N, when incubated with the pure enzyme.

To study the mechanism of thiazolidine ring closure the asymmetrically labelled substrates (73 and 74) were incubated with isopenicillin N synthetase. In both cases the penicillin (75) was the only penicillin isolated (Figure 51).

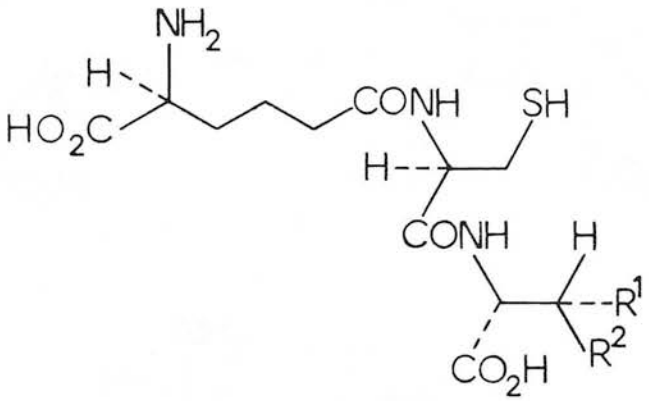
The authors conclude that these results strongly support a radical mechanism for the formation of the thiazolidine ring in



71 : 72

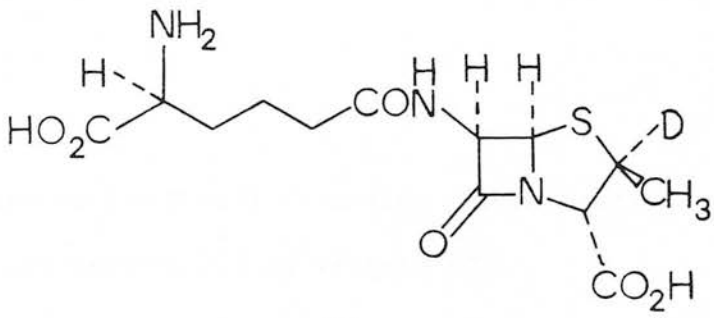
3 : 1

FIGURE 50



73 $R^1 = D$; $R^2 = CH_3$

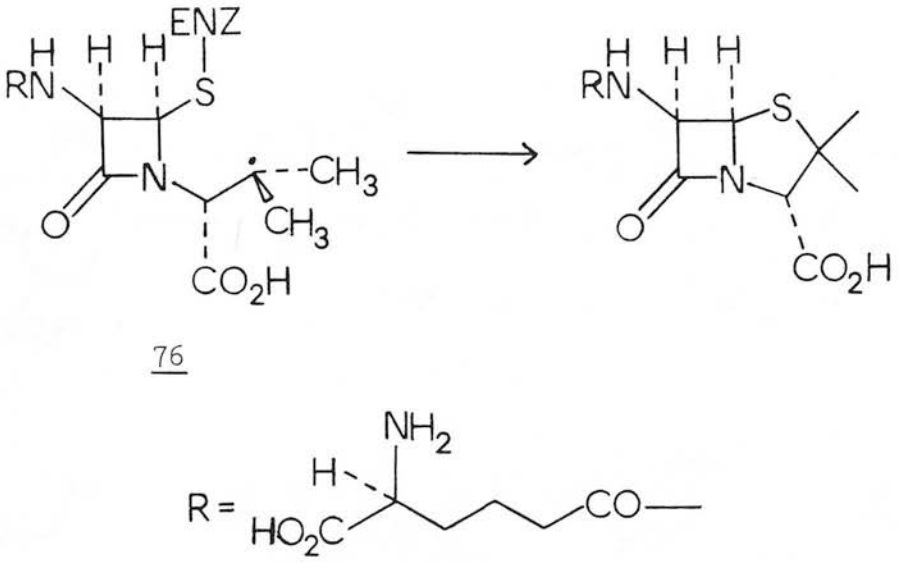
74 $R^1 = CH_3$; $R^2 = D$



75

F I G U R E 51

penicillin biosynthesis. Indeed if a simple assumption is made all the results can be readily explained. With the normal substrate LLD-ACV the valine isopropyl group is contained and sterically contained within a hydrophobic pocket at the active site of the synthetase enzyme. The time course of events is not known, but probably the β-lactam ring is first formed and the radical intermediate (76) then generated by hydrogen abstraction from the most reactive tertiary position. The isopropyl radical is sterically prevented from rotating



76

F I G U R E 52

and closes to isopenicillin N with retention of configuration at the valine C3 centre (see Section 3.3.2) (Figure 52).

With the modified substrate δ-(L-α-aminoadipyl)-L-cysteinyl-D-(α-aminobutyric) acid there is greater rotational freedom within the hydrophobic pocket. Any of the methyl or methylene hydrogens can, in a suitable conformation, present themselves to the radical initiator on the enzyme surface and be abstracted. With the stereospecifically deuterated tripeptides (73 and 74) a substantial kinetic isotope effect results in abstraction of hydrogen only from the α-aminobutyric acid C3 carbon (Figure 53). The radical (77) is free to rotate and adopts, presumably, the most stable conformation (78) (as only the penicillin (75) is isolated) before ring closure (Figure 53).

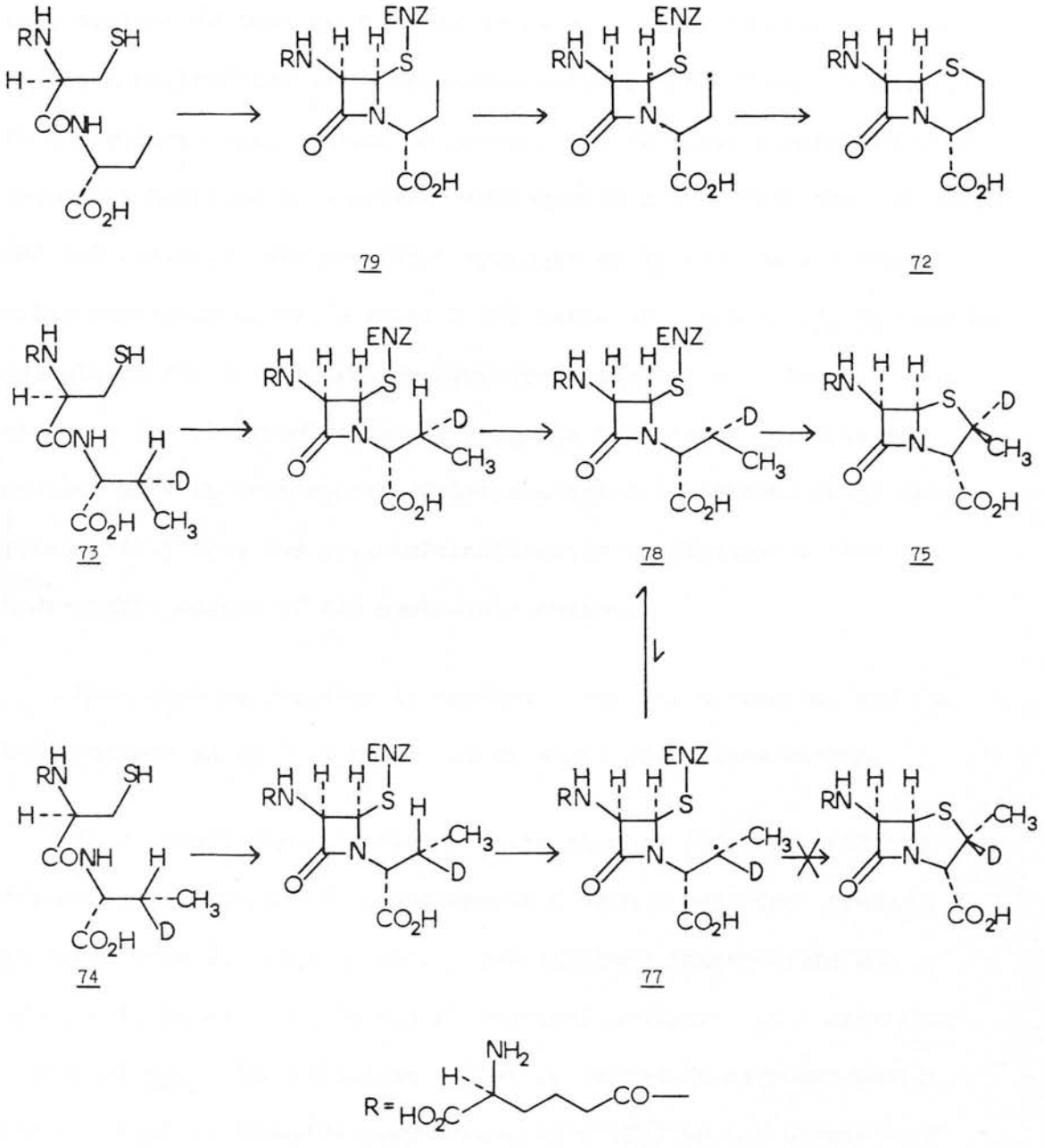
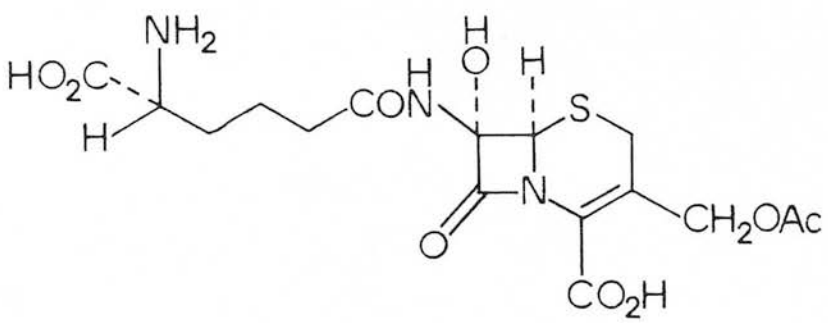


FIGURE 53

The observed 3:1 ratio of penam to cepham (Figure 50) is remarkably similar to that which would be calculated on the basis of random hydrogen abstraction from the α-aminobutyric acid C3 and C4 positions. In the radical chlorination of propane the relative reactivity of a secondary hydrogen to a primary hydrogens is 4:1. From the statistical 2:3 ratio of "abstractable" hydrogen on 79 and the 4:1 reactivity ratio mentioned above, a final 2·7:1 ratio of products (71:72) can be calculated for totally random hydrogen abstraction. This is very close to the observed ratio and suggests that the α-aminobutyric acid residue of δ-(L-α-aminoadipyl)-L-cysteinyl-D-(α-aminobutyric) acid is rotationally free and not conformationally constrained within the hydrophobic pocket of the synthetase enzyme.

More work is required to confirm a radical mechanism, and in this respect an *in vivo* e.s.r. study might prove interesting.

In a recent communication [446] the alcohol (80) was isolated following incubation of cephalosporin C with a cell-free system prepared from *S. clavuligerus*. The isolated intermediate was identical, by mass and ^{1}H n.m.r. spectral analysis, to a synthetic sample of 80. The cell-free system converted 7α-hydroxycephalosporin C (80) to 7α-methoxycephalosporin C (37) in the presence of *S*-adenosylmethionine (see Section 3.7.2).

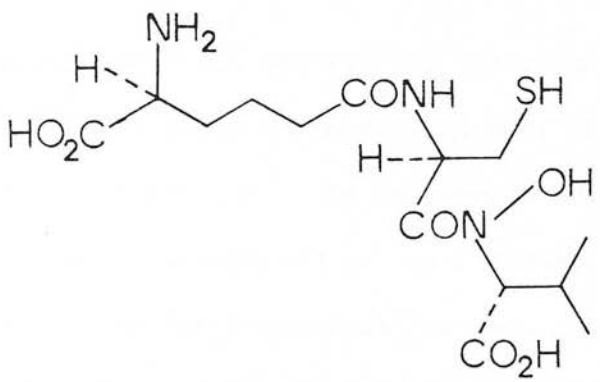


80

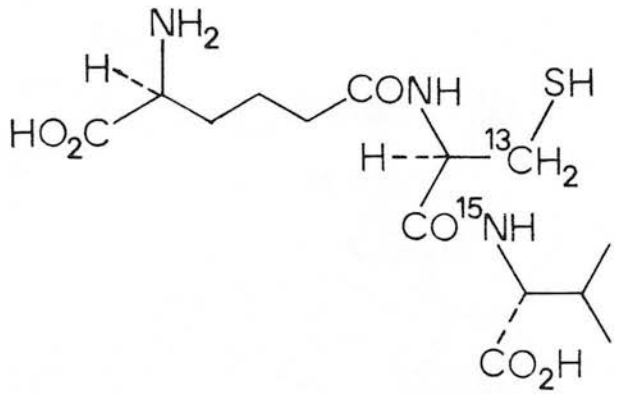
D I S C U S S I O N

4. SYNTHETIC TARGETS

In order to study the problem of isopenicillin N biosynthesis, two complementary approaches were proposed. The first was the synthesis of the putative intermediate [378] (81) (described in section 4.4), and to test this as a substrate, and the second was the synthesis of specifically ^{13}C, ^{15}N – labelled LLD-ACV (82). Direct *in vivo* ^{13}C n.m.r. observation of the cyclization of LLD-ACV to isopenicillin N (Section 3.3) could yield information on possible intermediates, particularly if these intermediates could be "trapped" by lowering the reaction temperature and reducing the rate of "intermediate" turnover. The problems associated with such cryoenzymological studies are discussed in detail elsewhere [447]. It was hoped that a $^{1}J_{CN}$ coupling would be observed in intermediates possessing the β-lactam ring and in the biosynthesised isopenicillin N. While the magnitude of one bond $^{13}C-^{15}N$ J values is dependent on geometry [448], a coupling of *ca* 7·0Hz between N4–C5 of penicillin G has been reported [336].



81

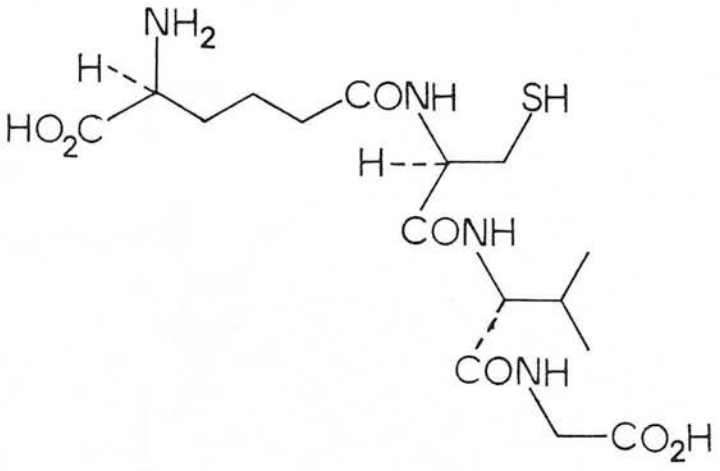


82

A pre-requisite for both approaches to this problem was the availability of a cell-free system capable of converting LLD-ACV to isopenicillin N (see Section 3.3).

In addition it was desired to synthesise δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine (LLD-ACVG) (83). A tetrapeptide with probably this sequence of amino-acids, but of undefined stereochemistry, has been isolated from the mycelia of *Cephalosporium* spp. [329]. The significance of ACVG in penicillin biosynthesis has never been investigated.

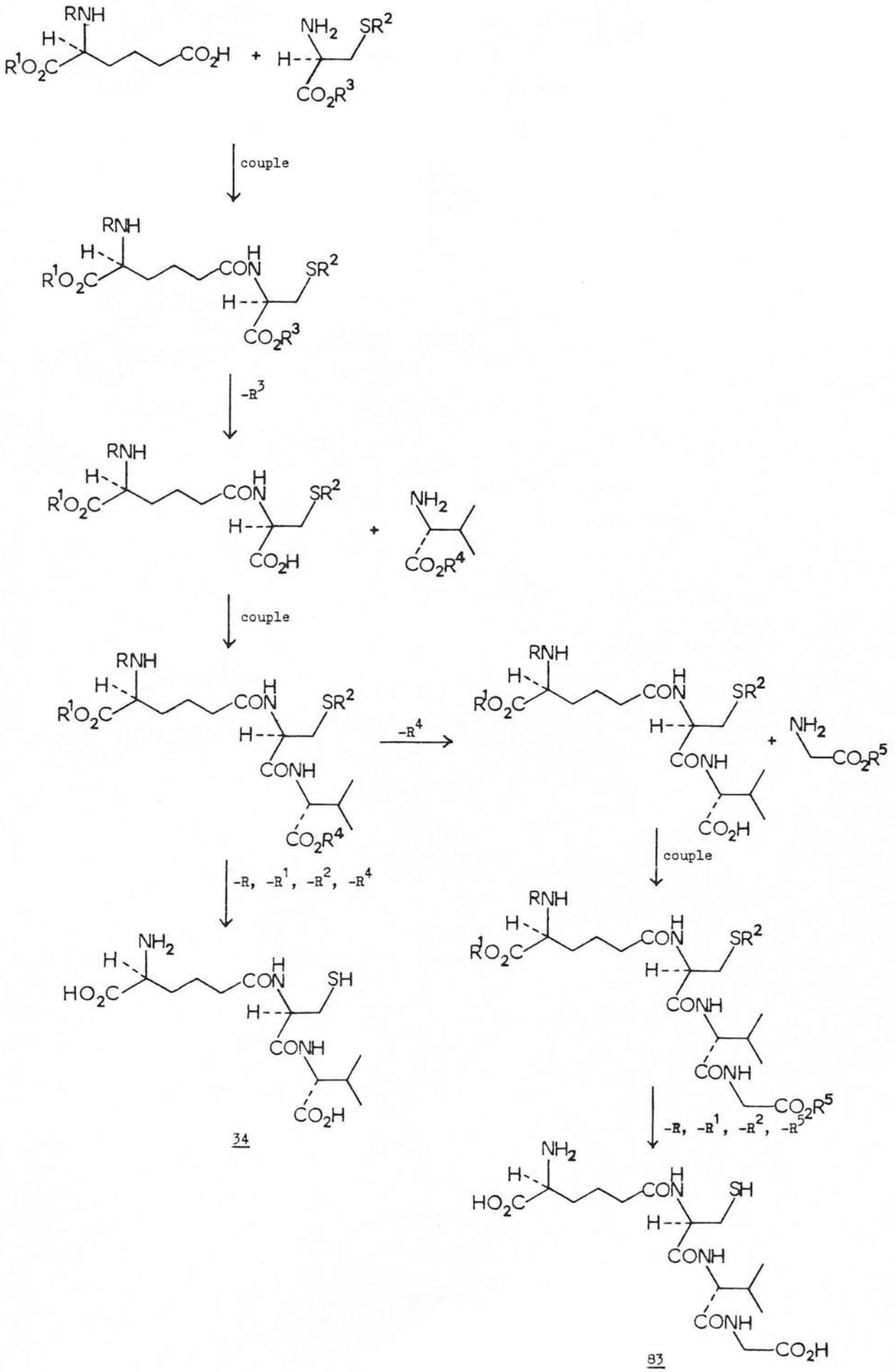
When this work was started the literature syntheses of LLD-ACV [328, 449, 450] all involved construction of the tripeptide from the *C*-terminal or valine end. As the target molecule (82) was to be labelled in the cysteine and valine residues, an alternative approach, construction from the *N*-terminal residue, appeared to be the most economical. A synthesis of LLD-ACV from the *N*-terminal residue has since been reported [451].



83

Peptide synthesis is in theory fairly straightforward. In practice, however, the problems associated with the protection, deprotection and coupling of amino-acids are complex, and these aspects have been discussed in detail in many publications [452-464]. The strategy for the synthesis of LLD-ACV (34) and LLD-ACVG (83) is outlined in Figure 54.

A successful synthesis of LLD-ACV by this route requires that the R^3 ester be selectively removed while the R, R^1 and R^2 protective groups are retained. In the synthesis of LLD-ACVG, R^3 and R^4 have to be selectively removed in the presence of R, R^1 and R^2. To optimize the final yield of LLD-ACV (34) and LLD-ACVG (83) it would be desirable if all the protective groups were removed in one deprotection step. The final synthesis [465] of LLD-ACV and LLD-ACVG which satisfied all these conditions is outlined in Figure 55.



34

83

FIGURE 54

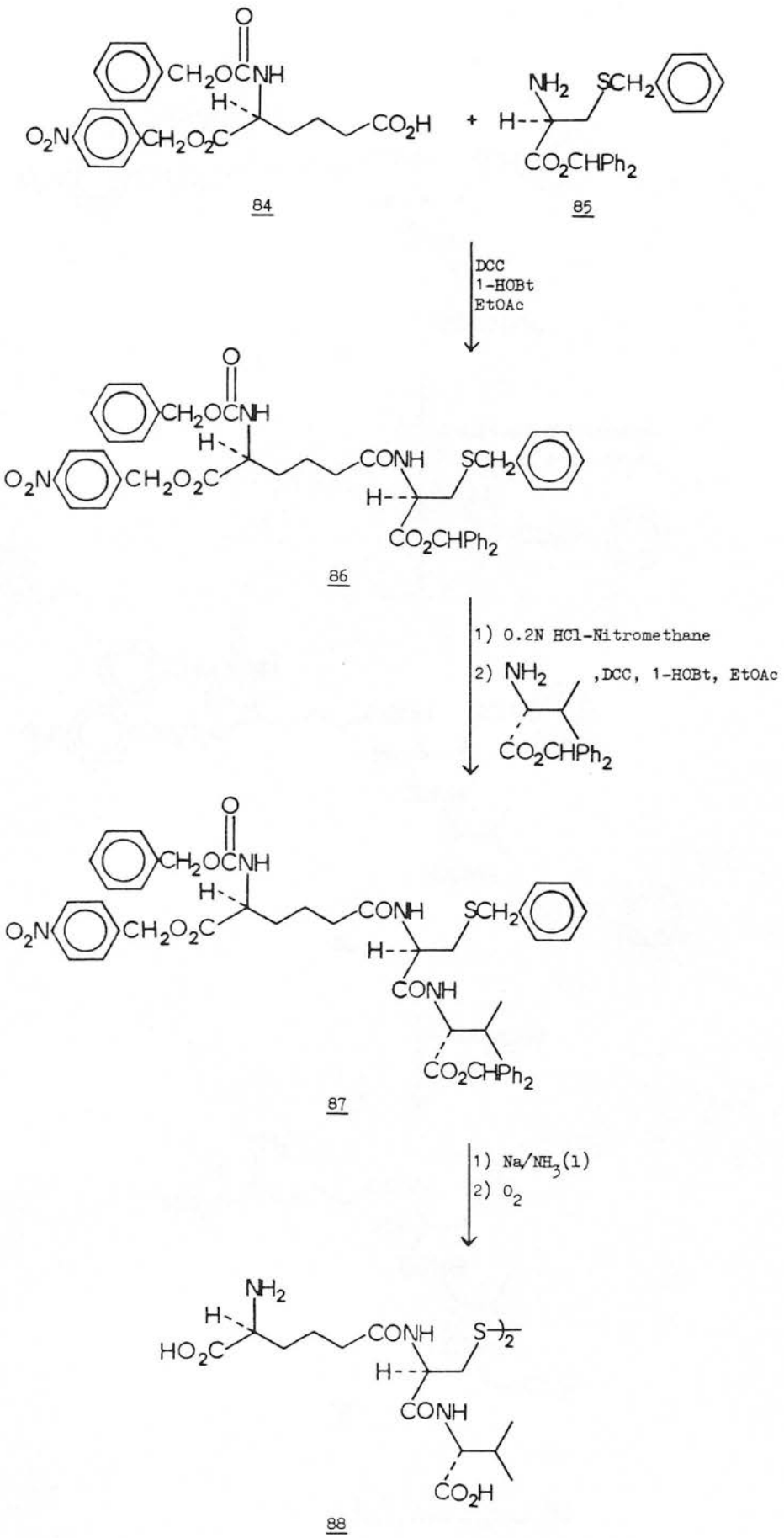
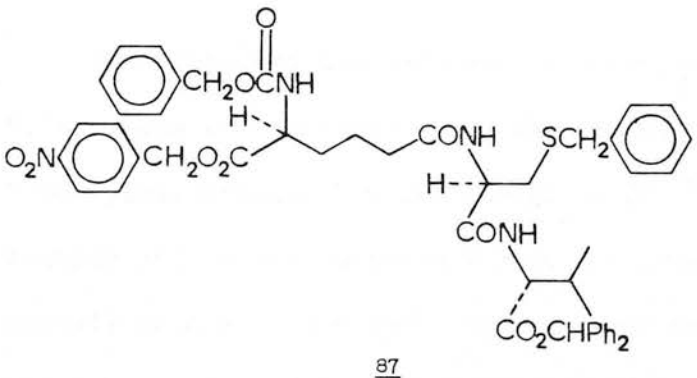

CH2OCNH
O2N
CH2O2C
CO2H
NH2
SCH2
CO2CHPh2
84
85
DCC
1-HOBt
EtOAc
CONH
86
1) 0.2N HCl-Nitromethane
2) ,DCC, 1-HOBt, EtOAc
87
1) Na/NH3(l)
2) O2
HO2C
S
88

FIGURE 55



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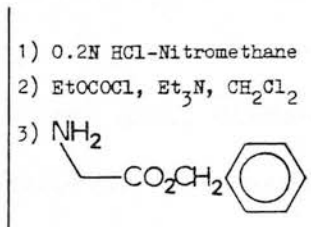

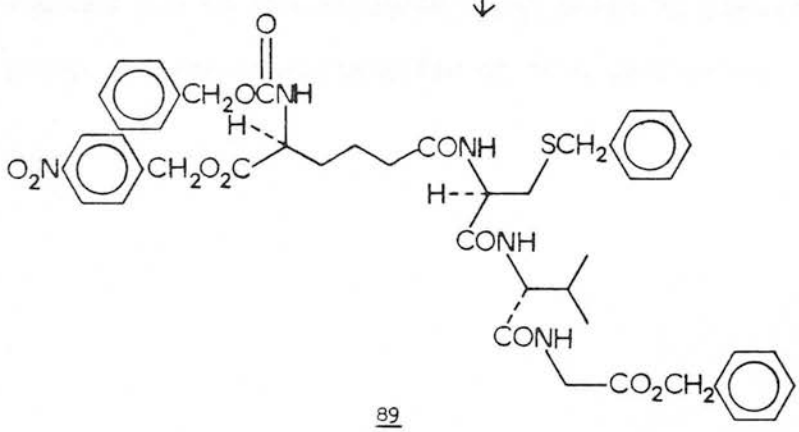

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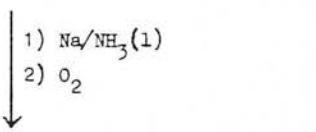
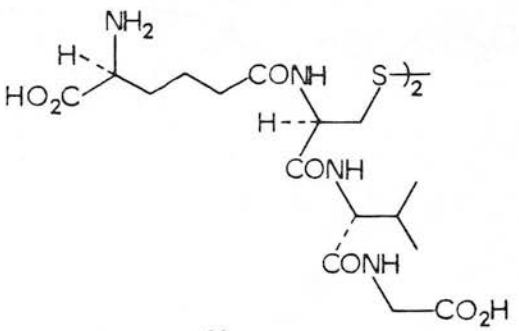


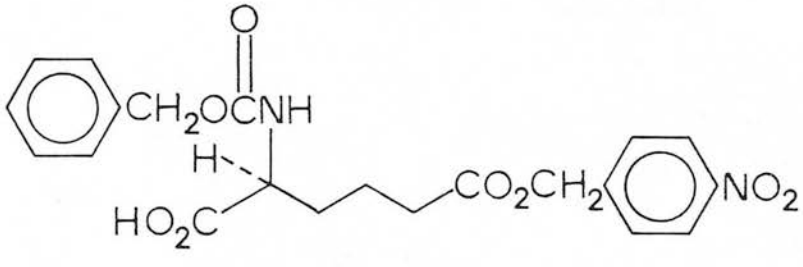


90

FIGURE 55

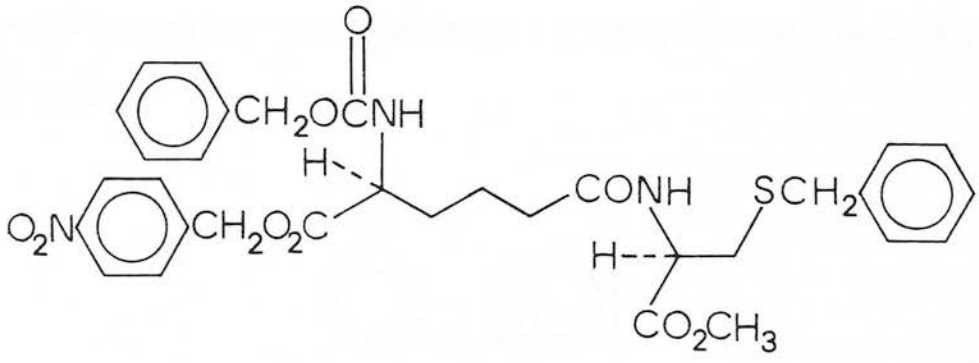
4.1 THE SYNTHESIS OF δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE

The protected L-α-aminoadipic acid (84) (R=$PhCH_2O_2C$-, R^1=4-$NO_2C_6H_4CH_2$-) was the derivative chosen for this work. Treatment of *N*-benzyloxycarbonyl-L-α-aminoadipic acid [466] with 4-nitrobenzyl-bromide and triethylamine afforded a mixture of the 1- and 6-(4-nitrobenzyl) esters, which could be separated by column chromatography. The desired 1-(4-nitrobenzyl) isomer (84) was isolated in 50% yield and the 6-(4-nitrobenzyl) isomer (91) in 24% yield. The mass spectra of the two derivatives were quite different and enabled the products to be readily distinguished. Fragmentation ions at m/z 250 and 206 in the spectrum of 84 arise by cleavage α to the amino-group and are characteristic of this derivative.



91

S-benzyl-L-cysteine (R^2=$PhCH_2$-) is commercially available. A number of ester protective groups were tested before the benzhydryl ester derivative (85) (R^3=Ph_2CH-) was finally selected. The methyl ester of *S*-benzyl-L-cysteine could be readily prepared in 90% yield by briefly refluxing the amino-acid in HCl saturated methanol. However attempts to selectively remove the methyl ester from synthesised *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine methyl ester (92) using an excess of LiI in DMF [467] or in refluxing pyridine [468] were unsuccessful.

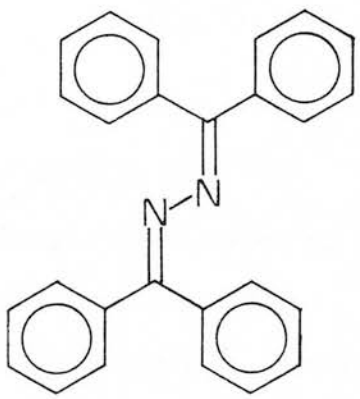


92

Since its introduction by Woodward [469, 470] the 2,2,2-trichloroethyl group has found extensive use in peptide synthesis as a protective group for carboxylate residues. Although the 2,2,2-trichloroethyl ester of *N*-benzyloxycarbonyl -*S*-benzyl-L-cysteine 2,2,2-trichloroethyl ester was rapidly and selectively removed at room

temperature using Zn/HOAc, the 2,2,2-trichloroethyl esters of *S*-benzyl-L-cysteine and D-valine could be prepared in only poor yield.

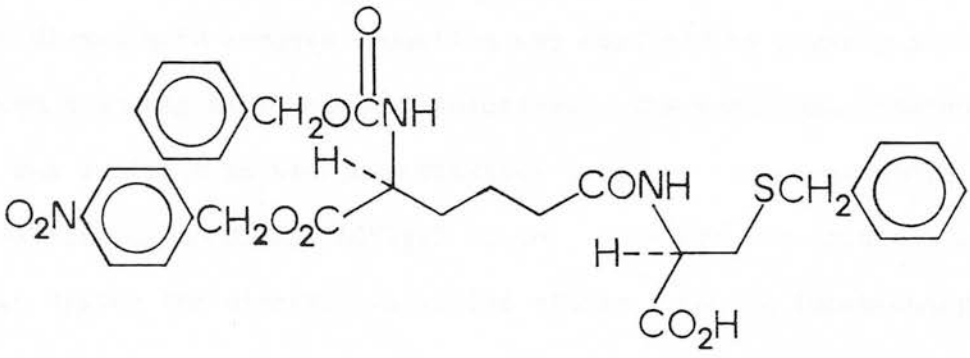
In contrast treatment of either amino-acid with 1•5 equivalents of diphenyldiazomethane in DMF afforded the corresponding benzhydryl esters in high yield [471]. Purification of the amino-acid benzhydryl esters as their 4-toluenesulphonic acid salts proved difficult due to the considerable quantities of crystalline acetophenone azine (93) produced as a by-product during the reaction [471]. However, it proved possible to use the crude benzhydryl esters in the coupling reactions (Figure 55) and to remove the contaminating acetophenone azine by column chromatography of the reaction mixture. Using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole [472], coupling of 84 and 85 proceeded in high yield to afford *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine benzhydryl ester (86).



93

To determine the optimum conditions for the selective removal of the benzhydryl ester small quantities of this dipeptide were subjected to various acidolytic conditions. Treatment with several

different concentrations of HBr—nitromethane [473] failed to selectively remove the benzhydryl ester, but treatment with 0·2N HCl—nitromethane [473] afforded only one product. Reaction of this product with diazomethane yielded material chromatographically identical with *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine methyl ester (92), prepared previously, indicating that the product was the free acid (94).



94

Condensation of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-L-cysteine (94) with crude D-valine benzhydryl ester using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole afforded fully protected LLD-ACV (87). The ^{1}H and ^{13}C n.m.r. spectra were fully consistent with the assigned structure. Only three α-carbon resonances were observed confirming that the material was one diastereoisomer and that no significant racemization had occurred during the coupling reactions.

Sodium-liquid ammonia reduction [474] of fully protected LLD-ACV (87) cleanly removed all the protective groups to yield the tripeptide

(34), which was isolated as its mercaptide and regenerated by passing a stream of hydrogen sulphide through an aqueous suspension of the mercaptide. However, the material obtained by freeze drying the resultant solution was shown by Ellman's procedure [475] (using 5,5'-dithiobis (2-nitrobenzoic acid) as test reagent) to be a 3:1 mixture of monomer (34) and dimer (88). Furthermore elemental analysis of the isolated peptide showed that it contained *ca* 4% inorganic contaminants.

To overcome the problem of inhomogeneity the crude product from the sodium-liquid ammonia reduction was oxidized by passing air through a weakly basic aqueous solution. The resultant disulphide [450] (88) was isolated in near quantitative yield by cation-exchange chromatography on Biorad AG50WX2 resin. The product obtained after freeze drying the ninhydrin-positive eluate from the ion-exchange column was shown to be homogeneous by paper electrophoresis, t.l.c. and h.p.l.c. (using a μ-Bondapak-NH_2 column with H_2O:AcOH (99·6:0·4) as eluant and UV detection at 214nm and 254nm). The ^{13}C n.m.r. spectrum of 88 showed ten distinct resonances and was similar to that described [451] for the monomer (34) except for the cysteinyl C3 which resonated at 38·5 p.p.m. The cysteinyl C3 resonance of the monomeric tripeptide is observed at 26·1 p.p.m. [451]. These results are in agreement with the reported [476] shifts of cystine C3 at 44·6 p.p.m. and cysteine C3 at 28·2 p.p.m.

Evidence from ^1H n.m.r. experiments confirmed that quantitative reduction of the disulphide to LLD-ACV (34) could be achieved using dithiothreitol (DTT) in neutral or slightly basic aqueous solution. The chemical shift of the cysteine 3H,2H and valine 3H protons are

quite distinct in the monomer and dimer. Thus a solution of 88 and a five fold excess of DTT in D_2O was stable and contained none of the monomeric species. When the solution was adjusted to neutral or weakly basic pH the ^1H n.m.r. spectrum indicated complete reduction to the monomer (34). Reduction occurred quantitatively and virtually immediately (at least in the 30 sec or so necessary to adjust the pH and return the sample to the probe).

The overall yield of LLD-ACV (88) from *S*-benzyl-L-cysteine was 40% and from D-valine, 62%.

4.2 THE SYNTHESIS OF ISOTOPICALLY ENRICHED ACV PEPTIDES

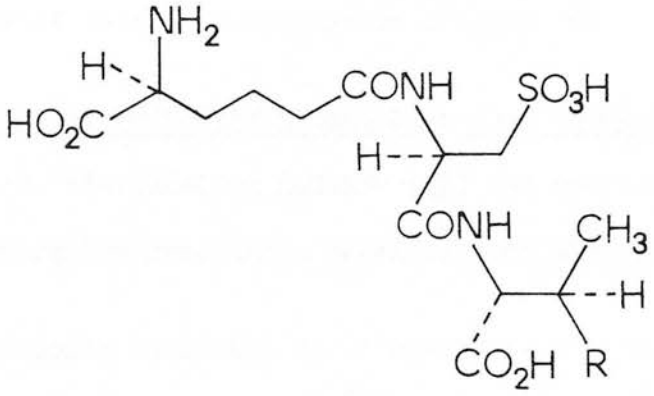
4.2.1 The Synthesis of δ-(DL-α-Aminoadipyl)-L-Cysteinyl-D-(3R)-[4-^{13}C]-Valine

With a satisfactory synthesis of LLD-ACV now available the synthesis of isotopically enriched species was undertaken.

As discussed in Section 3.3 the stereochemical fate of the valine C3 centre was of importance in elucidating the mechanism of thiazolidine ring formation. It has recently been shown [401] that (2S,3R)-[4-^{13}C]-valine* is incorporated into LLD-ACV (isolated as its sulphonic acid, LLD-ACyaV, 95) apparently without racemization of the valine C3 centre. Hydrolysis of the sulphonic acid (95) afforded D-valine enriched at the 3-*pro*-R methyl carbon. Assignment of the position of enrichment in the biosynthesised peptide was based on comparison of the ^{13}C n.m.r. spectra of the sulphonic acid and the D-valine from hydrolysis with the ^{13}C n.m.r. spectrum of authentic

*The correct stereochemical assignments and not those reported earlier [401] (on the basis of Aberhart's incorrect assignments [400]) are used throughout the following discussion.

(2R,3R)-[4-^{13}C]-valine. To confirm the stereospecificity of valine incorporation it was necessary to prepare the sulphonic acid (96) enriched specifically at the 3-*pro*-R methyl carbon.



95 R = CH_3

96 R = $^{13}CH_3$

Coupling of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-amino-adipyl)-*S*-benzyl-L-cysteine and D-valine benzhydryl ester enriched with synthetic (2R,3R)-[4-^{13}C]-valine benzhydryl ester afforded fully protected LLD-ACV. The (2R,3R)-[4-^{13}C]-valine required for this synthesis had previously been prepared in this laboratory [400] as a by-product in the preparation of (2S,3R)-[4-^{13}C]-valine. The D-valine isotopomer was diluted with D-valine to an enrichment of *ca* 2 atom%. The material was converted to its benzhydryl ester and coupled as previously described.

The fully protected LLD-ACV so obtained was deprotected and the

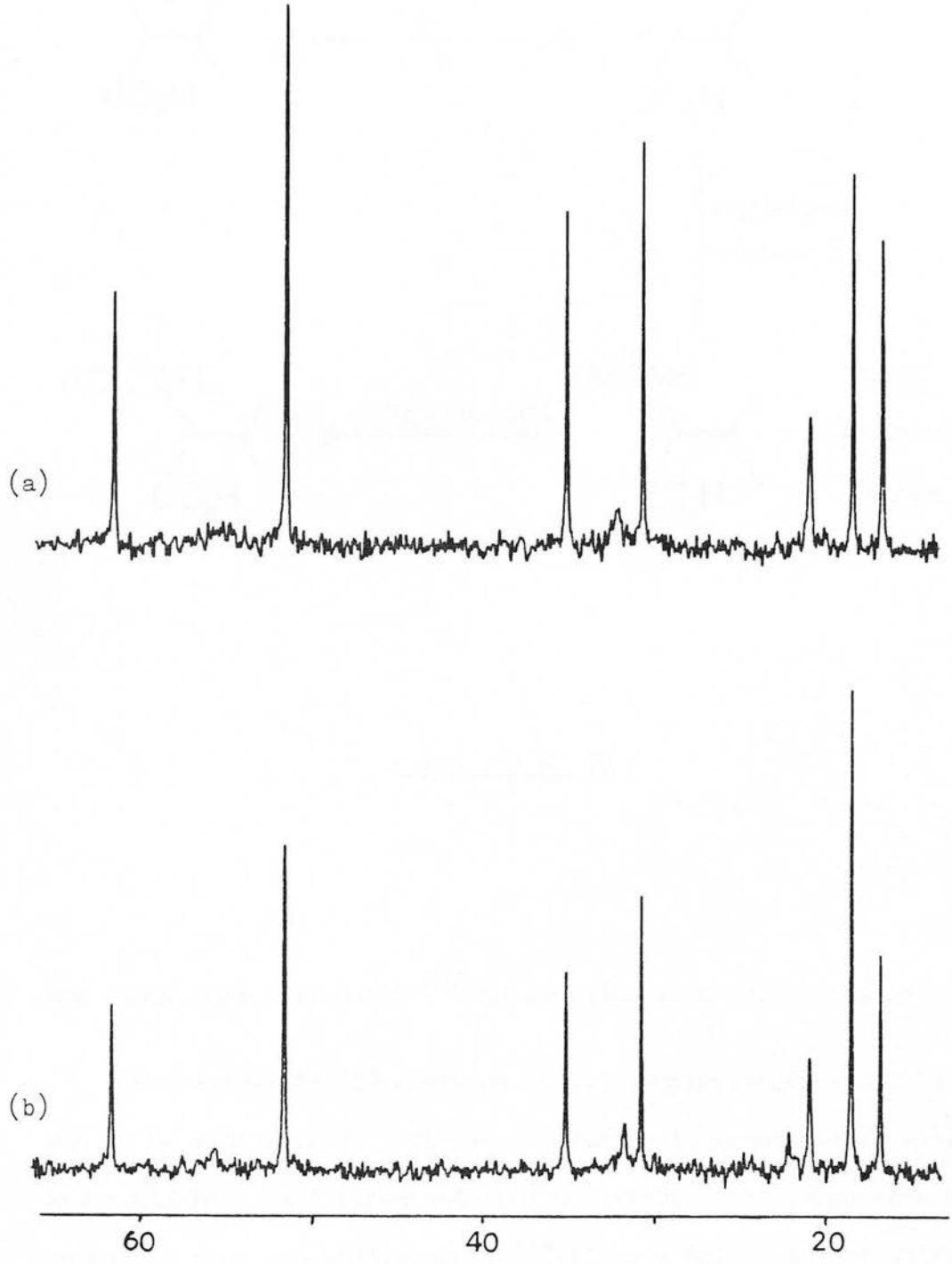
product oxidized with performic acid to afford δ-(DL-α-aminoadipyl)-L-cysteicyl-D-(3R)-[4-^{13}C]-valine (96). The ^{13}C n.m.r. spectrum was identical to that of unlabelled LLD-ACyaV (95) prepared in an analagous manner (Figure 56). The intensity of the signal at 18·7 p.p.m. was greater (2·1±0·1 x natural abundance) than that of the diastereoisomeric methyl group at 17·0 p.p.m. The results were in complete agreement with those previously published and confirmed the stereospecificity of valine incorporation (Figure 38).

4.2.2 The Synthesis of δ-(L-α-Aminoadipyl)-L-[3-^{13}C]-Cysteinyl-D-[^{15}N]-Valine

The ^{13}C, ^{15}N-labelled LLD-ACV (82) was synthesised as its disulphide using the procedures developed for the synthesis of 88.

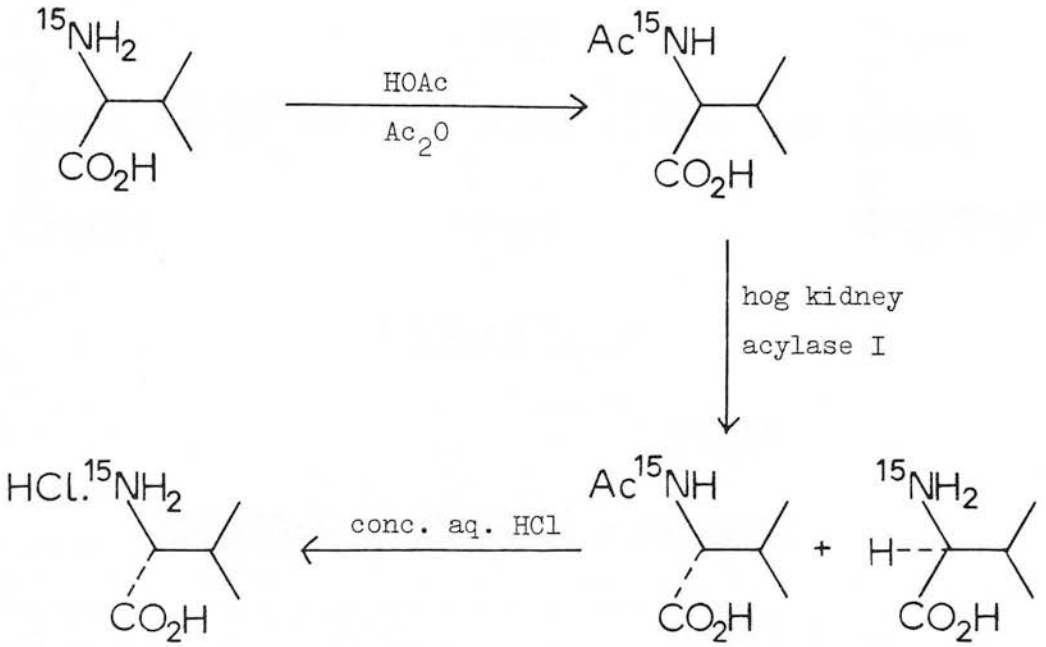
Commercially available DL-[^{15}N]-valine (95 atom% ^{15}N) was resolved using standard methods[477]. The labelled amino-acid was refluxed in a mixture of acetic acid and acetic anhydride to afford the *N*-acetyl derivative which was not isolated. Treatment of the crude product with hog kidney acylase I yielded L-[^{15}N]-valine and *N*-acetyl-D-[^{15}N]-valine which were separated by cation-exchange chromatography. The *N*-acetyl-D-[^{15}N]-valine was refluxed in concentrated aqueous HCl to obtain the required starting material, D-[^{15}N]-valine (Figure 57).

The ^{1}H n.m.r. spectrum of the D-[^{15}N]-valine hydrochloride salt displayed a three bond N-H coupling of 3·3Hz with the C3 proton but no two bond N-H coupling. A $^{1}J_{CN}$ of 6·0Hz was evident from the ^{13}C n.m.r. spectrum of the hydrochloride salt. A coupling of *ca* 3·0Hz has been reported [336] for L-[2-^{13}C, ^{15}N]-valine but the magnitude of the coupling constants may depend on the pH at which the spectra are recorded. The value of $^{2}J_{NH}$ in amino-acids(which is normally in



90MHz broad-band proton decoupled ^{13}C n.m.r. spectrum of (a) δ-(L-α-aminoadipyl)-L-cysteicyl-D-valine (sweep width 20kHz, number of scans 80808) and (b) δ-(DL-α-aminoadipyl)-L-cysteicyl-D-(3R)-[4-^{13}C]-valine (sweep width 20kHz, number of scans 49800).

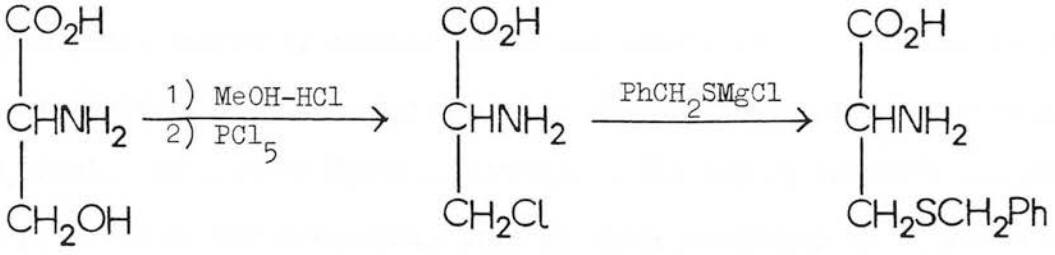
F I G U R E 56



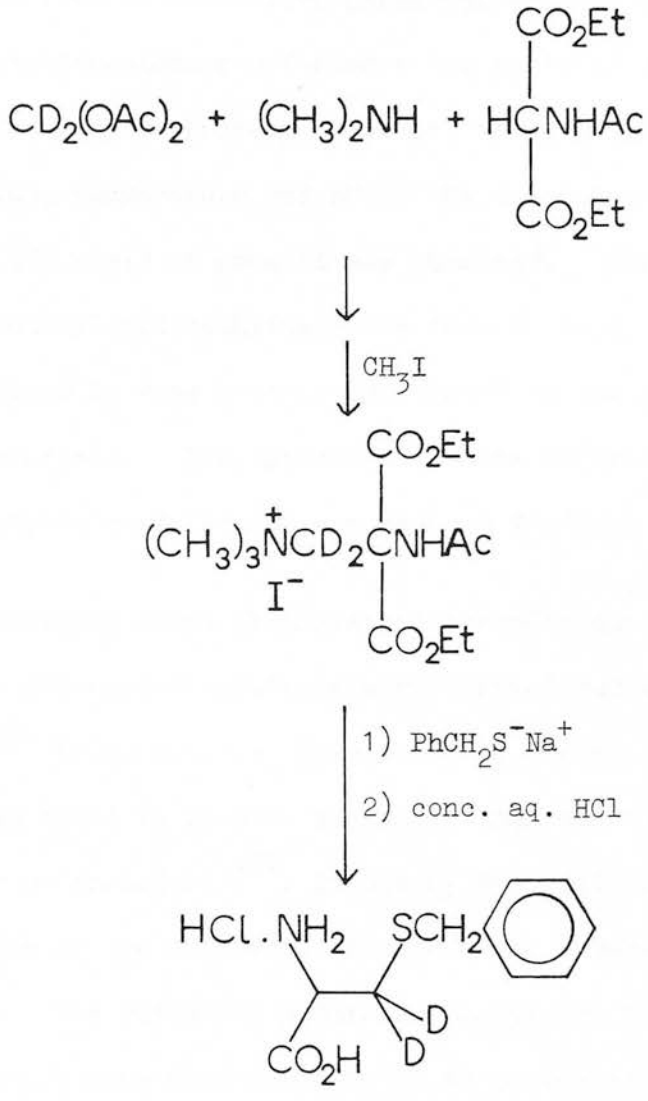
F I G U R E 57

the range 0-2Hz) is known [478] to decrease with decreasing pH.

S-benzyl-L-[3-^{13}C]-cysteine is not commercially available and had to be synthesised. DL-[3-^{13}C]-serine is commercially available and could have been converted to *S*-benzyl-DL-[3-^{13}C]-cysteine by the method of Wood and Middlesworth [479] (Figure 58). A more attractive route to the labelled amino-acid, however, was one based on Upson and Hruby's synthesis [480] of *S*-benzyl-DL-[3-$^{2}H_2$]-cysteine (Figure 59).



F I G U R E 58



F I G U R E 59

What was effectively a two step synthesis of *S*-benzyl-DL-cysteine could prove extremely economical if the relatively inexpensive [^{13}C]-paraformaldehyde could substitute for the paraformaldehyde equivalent $CD_2(OAc)_2$ employed by Upson and Hruby. The highly volatile diacetoxy -[$^2H_2$]-methane was presumably used in their synthesis as it could be easily prepared from the readily available dibromo-[$^2H_2$]-methane.

The reaction of unlabelled paraformaldehyde, dimethylamine and diethyl acetamidomalonate afforded a low yield of diethyl acetamido-dimethylaminomethylmalonate. However, by varying the reaction conditions, time, temperature and pH of the aqueous suspension, an acceptable 67% yield of product was obtained. The yield of diethyl acetamidodimethylaminomethylmalonate from a series of trial experiments was found to vary greatly and depend on the purity of the starting materials. The optimum pH of the reaction was the weakly acidic catalytic condition of the Mannich Reaction [481].

The remaining steps from diethyl acetamidodimethylaminomethyl-malonate to *S*-benzyl-DL-cysteine were carried out as previously described [480] (Figure 59). Resolution of the racemic *S*-benzyl-cysteine was found to be more difficult than had been anticipated. The literature procedure [477], involving *N*-acetylation and enzymic deacetylation of the L-isomer, afforded only moderate yields of L-cysteine. The refluxing $AcOH/Ac_2O$ conditions used to acylate *S*-benzyl-DL-cysteine were found to cause decomposition. Maintaining the temperature of the reaction below 60°C allowed *N*-acetylation without decomposition and, as observed with *S*-benzyl-L-cysteine, without racemization of the chiral centre of the amino-acid.

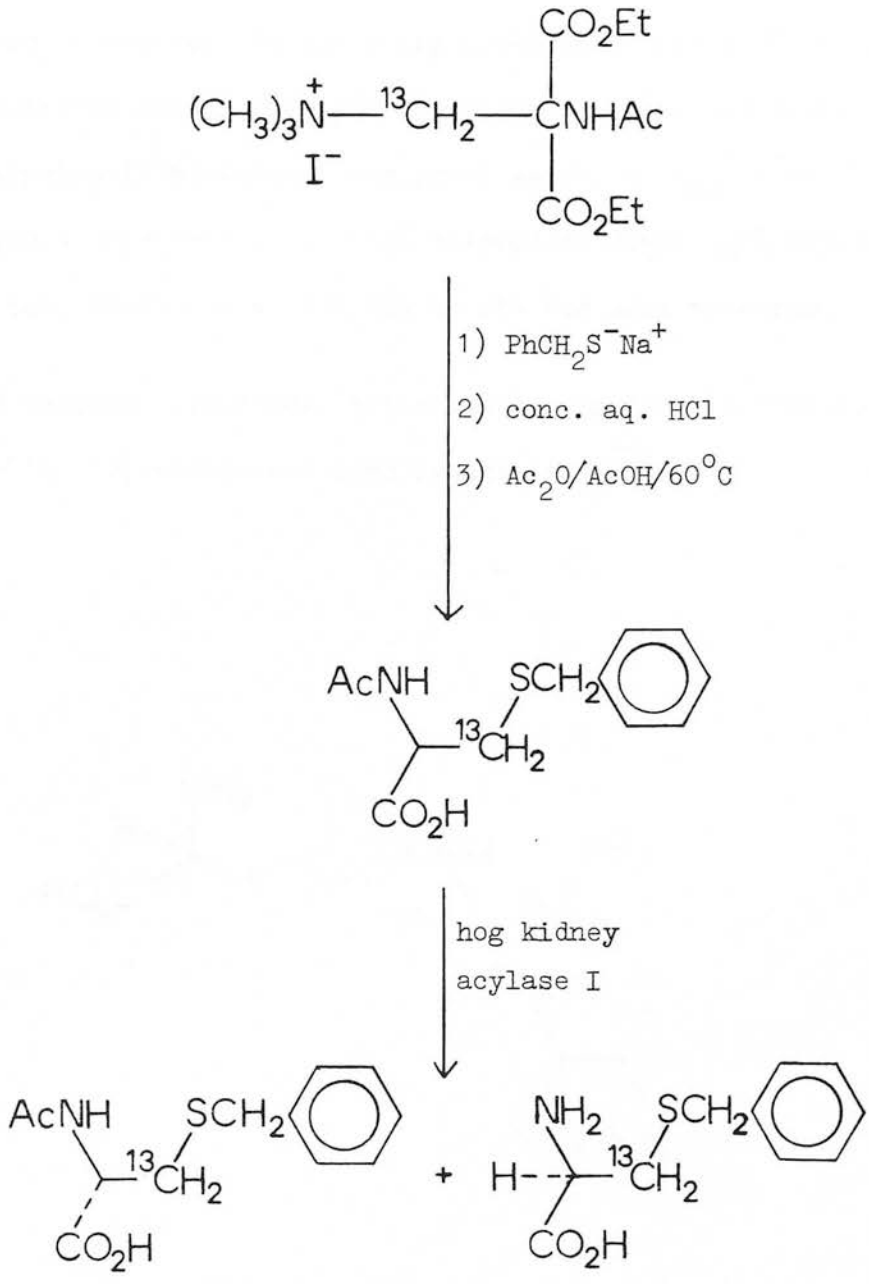
Despite careful attention to the optimum conditions derived

from trial reactions, when the synthesis was repeated using [^{13}C]-paraformaldehyde the final yield of product dropped considerably. This was presumably due to an impurity in the [^{13}C]-paraformaldehyde which was used as supplied by the manufacturer and was not resublimed. [^{13}C]-paraformaldehyde has also been used for the preparation of dimethoxy-[^{13}C]-methane in this laboratory. While yields of 90-95%, based on technical grade paraformaldehyde, are generally possible in this reaction, yields from [^{13}C]-paraformaldehyde are typically 60-70%. The source of the impurity is not known [482].

S-benzyl-L-[3-^{13}C]-cysteine was finally obtained by treating *S*-benzyl-DL-[3-^{13}C]-cysteine with AcOH/Ac_2O at 60°C and resolving the resultant racemic *N*-acetyl amino-acid with hog kidney acylase I. The mixture of *S*-benzyl-L-[3-^{13}C]-cysteine and *N*-acetyl-*S*-benzyl-D-[3-^{13}C]-cysteine was separated by cation-exchange chromatography (Figure 60).

Only one resonance at 36•73 p.p.m. was observed in the ^{13}C n.m.r. spectrum of *S*-benzyl-L-[3-^{13}C]-cysteine sodium salt. A number of ^{13}C-H couplings could be determined from the ^{1}H n.m.r. spectrum. The cysteine 3H protons formed the AB of an ABMX multiplet with $^{1}J_{CH}$ couplings of 140•7 and 140•3 Hz. A two bond ^{13}C-H coupling of 4•8Hz to the cysteine 2H proton and a three-bond ^{13}C-H coupling of 3•8Hz to the methylene protons of the *S*-benzyl group were also observed. The ^{1}H n.m.r. spectrum indicated a ^{13}C enrichment at cysteine 3C of $\geq$ 90%.

With D-[^{15}N]-valine and *S*-benzyl-L-[3-^{13}C]-cysteine now available the synthesis of δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine (82) was carried out in a manner analagous to that

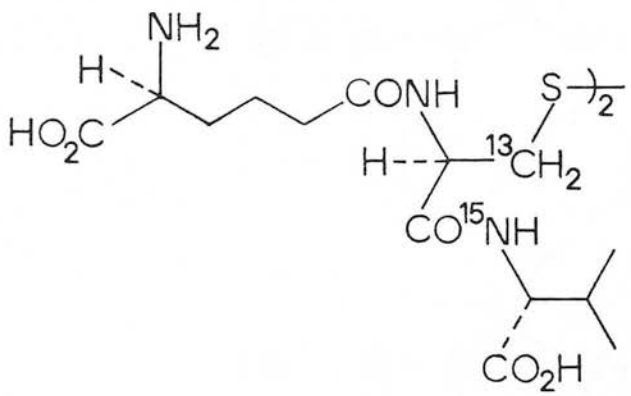


F I G U R E 60

described for the unenriched tripeptide (Section 4.1). The ^{13}C n.m.r. spectrum of the isolated disulphide (97) displayed one resonance at 39·17δ and in the ^{1}H n.m.r. spectrum a $^{1}J_{CH}$ coupling of 193·9Hz was observed for the cysteine 3H protons. All the amide protons exchanged in the D_2O solution and no ^{15}N-H doublet was

observed. However, in the fully protected species, *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine benzhydryl ester, a $^{1}J_{NH}$ of 91•3Hz was observed. The free [^{13}C, ^{15}N]-tripeptide dimer (97) displayed a base peak, (M+H) ion at m/z 729 in its FAB mass spectrum.

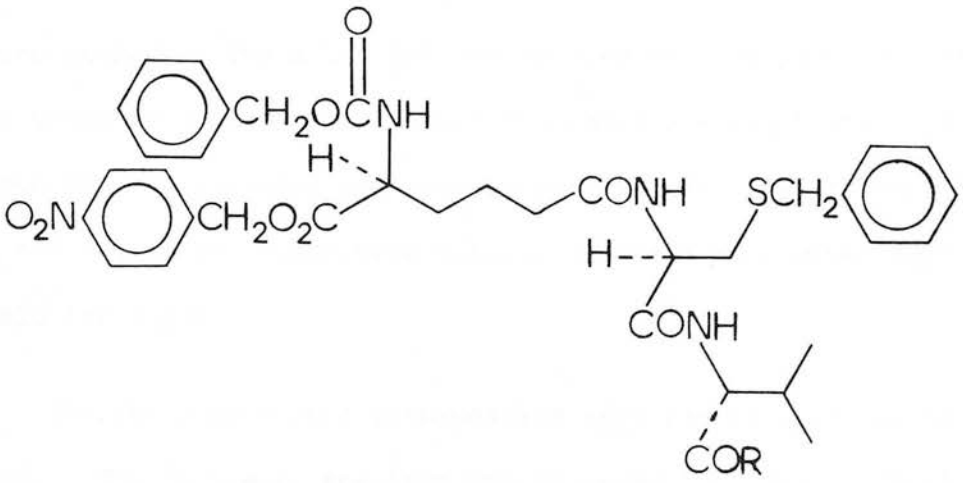
A related tripeptide, δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[3-^{13}C,^{15}N]-valine has been synthesised [451].

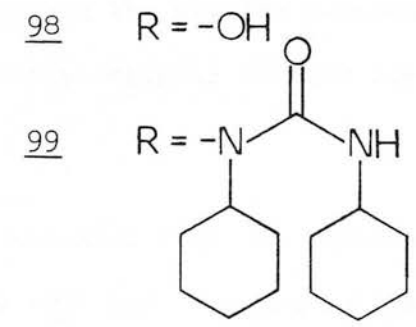


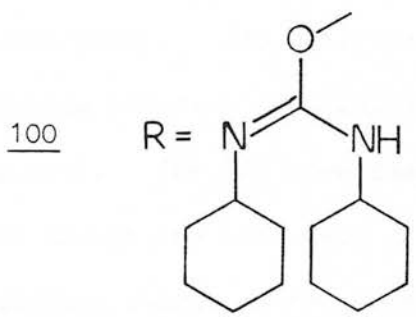
97

4.3 THE SYNTHESIS OF δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALYLGLYCINE

The nature of the protective groups used in the synthesis of LLD-ACV (Figure 55) was deliberately designed to allow easy access to the protected tetrapeptide (89). Thus treatment of 87 with 0•2N HCl-nitromethane selectively removed the benzhydryl ester yielding *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine (98). Coupling of 98 with glycine benzyl







ester could not be satisfactorily achieved with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The only material isolated from this reaction was the *N*-acyl urea (99). It is possible that the activated *O*-acyl derivative (100) is sterically hindered [456] to attack by 1-hydroxybenzotriazole or the added glycine benzyl ester and rearranges to the synthetically useless derivative (99).

Condensation was achieved in moderate yield using a mixed anhydride method. The acid (98) was treated with ethylchloroformate in the presence of pyridine to give the corresponding mixed anhydride which was not isolated but reacted *in situ* with glycine benzyl ester to afford, after preparative t.l.c., the desired product (89) in 46% yield (from 87).

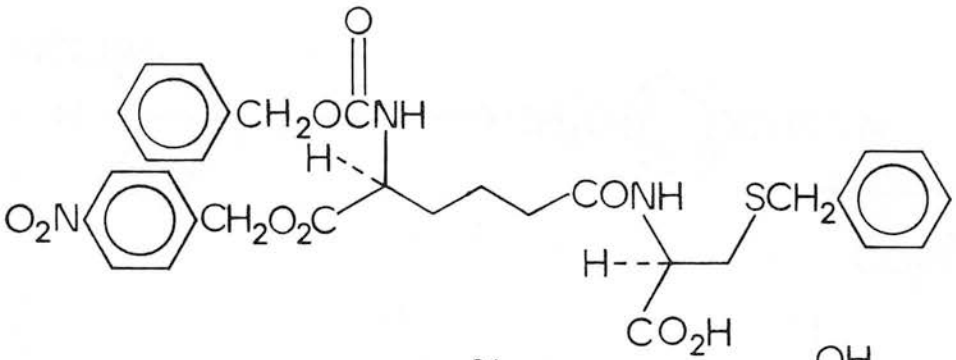
The fully protected tetrapeptide (89) was isolated analytically pure. The ^{1}H n.m.r. spectrum was recorded in $CDCl_3$:CD_3OD (1:1), as 89 was poorly soluble in $CDCl_3$. Although the presence of a CD_3OH resonance partially masked the glycine α-protons, the rest of the spectrum could be readily assigned and was consistent with the desired structure.

Fully protected LLD-ACVG (89) was deprotected as previously described for LLD-ACV (87) and isolated as its disulphide (90) after cation-exchange chromatography. The material was homogeneous by t.l.c. and h.p.l.c. (using a Waters μ-Bondapak-NH_2 column and H_2O:AcOH (99•6:0•4) as eluant). The FAB mass spectrum of 90 displayed a (M+H) ion at m/z 839 though the base peak at m/z 421 was of the monomer (83). The glycine α-protons resonated as a singlet at 3•20δ in the ^{1}H n.m.r. spectrum of 90.

The overall yield of 90 from *S*-benzyl-L-cysteine was 13% and from D-valine, 21%.

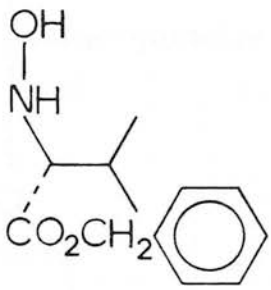
4.4 THE SYNTHESIS OF *N*-[δ-(L-α-AMINOADIPYL)-L-CYSTEINYL]-*N*-HYDROXY-D-VALINE

Based on the methodology used to synthesise LLD-ACV (88) it was hoped that the synthesis of *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-



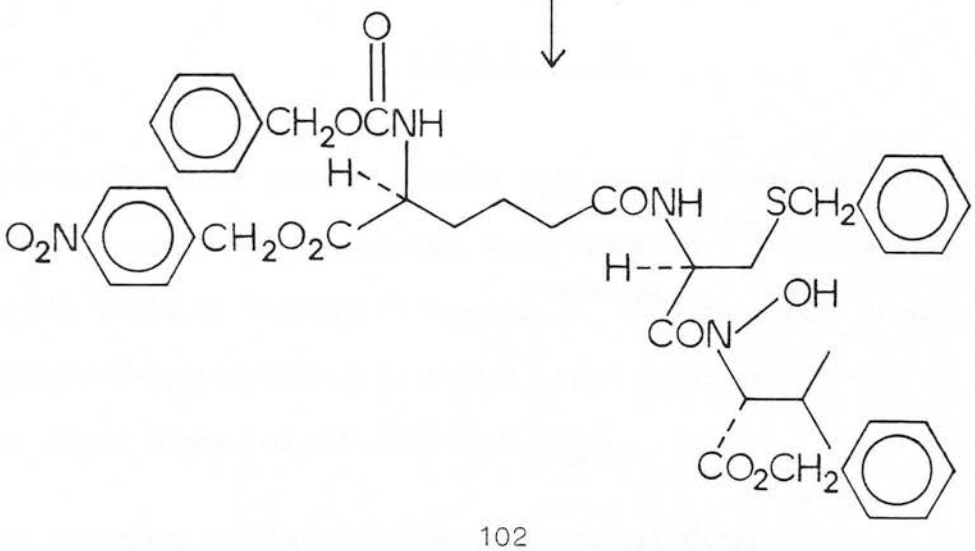
94

+



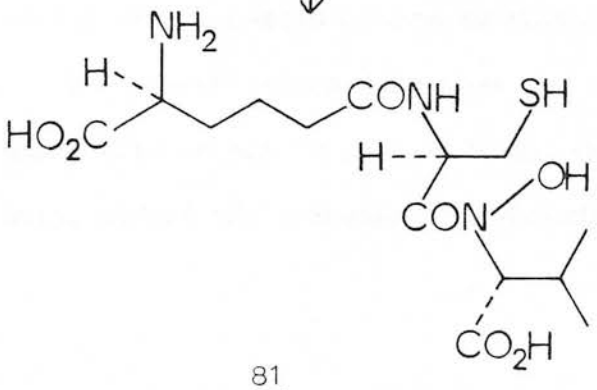
101

couple



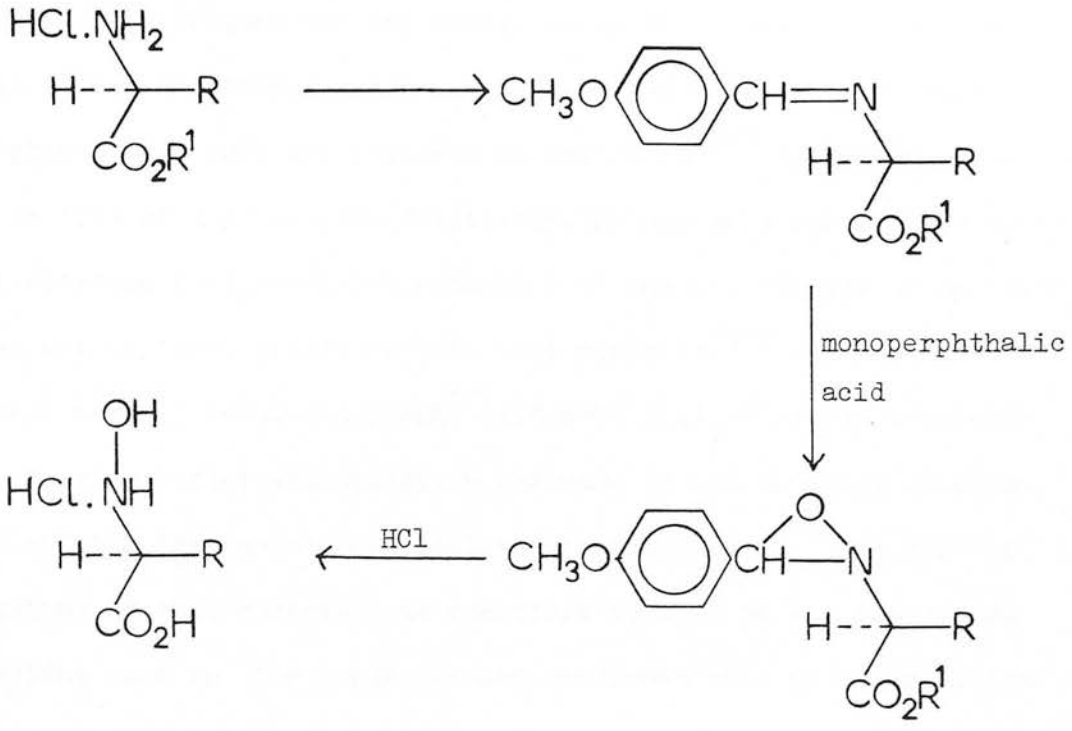
102

one step
deprotection



81

FIGURE 61



F I G U R E 62

hydroxy-D-valine (LLD-AC(N-OH)V) (81) would be straightforward (Figure 61). Compound 94 had already been prepared [465] and it seemed likely, on the basis of literature reports [483-485], that this compound would acylate *N*-hydroxy-D-valine benzyl ester (101) exclusively on nitrogen to afford protected LLD-AC(N-OH)V (102).

4.4.1 The Synthesis of *N*-Hydroxy-D-Valine Benzyl Ester

The synthesis of racemic *N*-hydroxy-amino-acids has been achieved [486-488] but there is only one brief report [489] in the literature on the indirect oxidation of amino-acid esters to chiral *N*-hydroxy-amino-acid derivatives. The communication describes the oxidation of the Schiff bases of amino-acid esters to oxaziridines, which, when heated in hydrochloric acid, afford the corresponding *N*-hydroxy-amino-acids (Figure 62).

In order to secure a one step deprotection of 102 to LLD-AC (N-OH)V (81) (Figure 61) the benzyl ester of D-valine was the derivative chosen as starting material. D-valine benzyl ester 4-toluenesulphonic acid salt was prepared as described [490] and shown by t.l.c. to be free of any D-valine 4-toluenesulphonic acid salt. The optical rotation ($[\alpha]_D$+3·0(c2·0,ethanol)) of the product was of opposite sign but of lower magnitude than that reported [491] for L-valine benzyl ester 4-toluenesulphonic acid salt ($[\alpha]_D$−3·5(c2·0,ethanol)). The D-valine ester was therefore refluxed in concentrated aqueous HCl and the free amino-acid isolated from solution. The optical rotation of this material was identical to that of the commercial D-valine used in the synthesis and confirmed that no racemization had occurred.

Treatment of D-valine benzyl ester with 4-methoxybenzaldehyde in benzene afforded the imine (103) in quantitative yield. Oxidation of the imine [492] with 3-chloroperbenzoic acid gave the oxaziridine (104) which was unstable and adjudged by ^{1}H n.m.r. spectroscopy to be a complex mixture of diastereoisomeric products. Attempted hydrolysis of the crude mixture with 1M or 2M aqueous-methanolic HCl afforded the highly crystalline D-valine benzyl ester 4-methoxybenzaldimine *N*-oxide (105) (Figure 63). The rearrangement of oxoziridines to *N*-oxides in the presence of an acid catalyst is known [489] and proceeds *via* a carbonium ion intermediate [492, 493]. At higher acid concentrations oxaziridines are successfully hydrolysed [492-494] (Figure 64).

The 4-methoxybenzaldimine derivative (R=4-$MeOC_6H_4$- in Figure 64) stabilises the carbonium ion allowing hydrolysis to proceed at a reasonable rate. Thus treatment of the 3-chloroperbenzoic acid

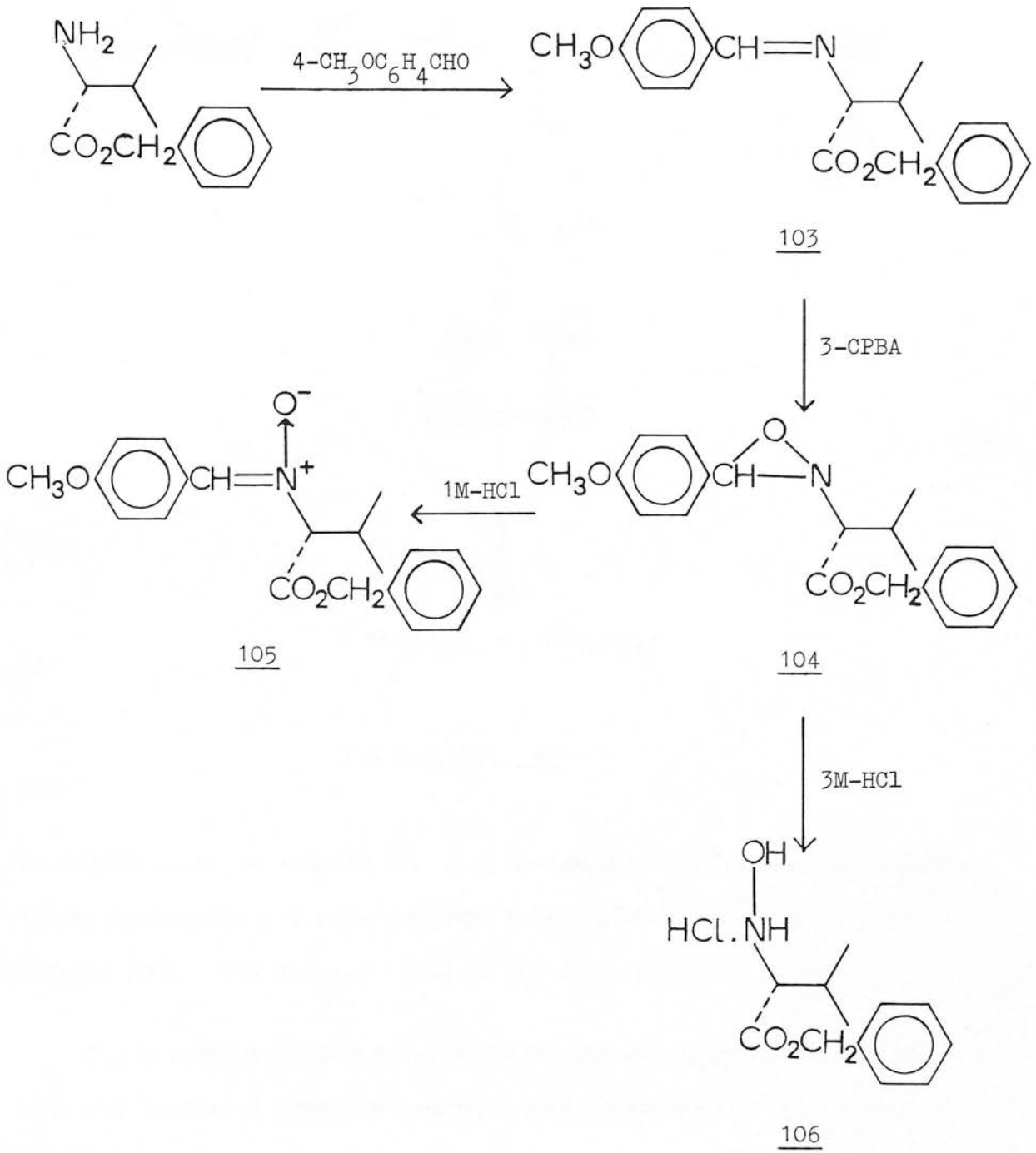
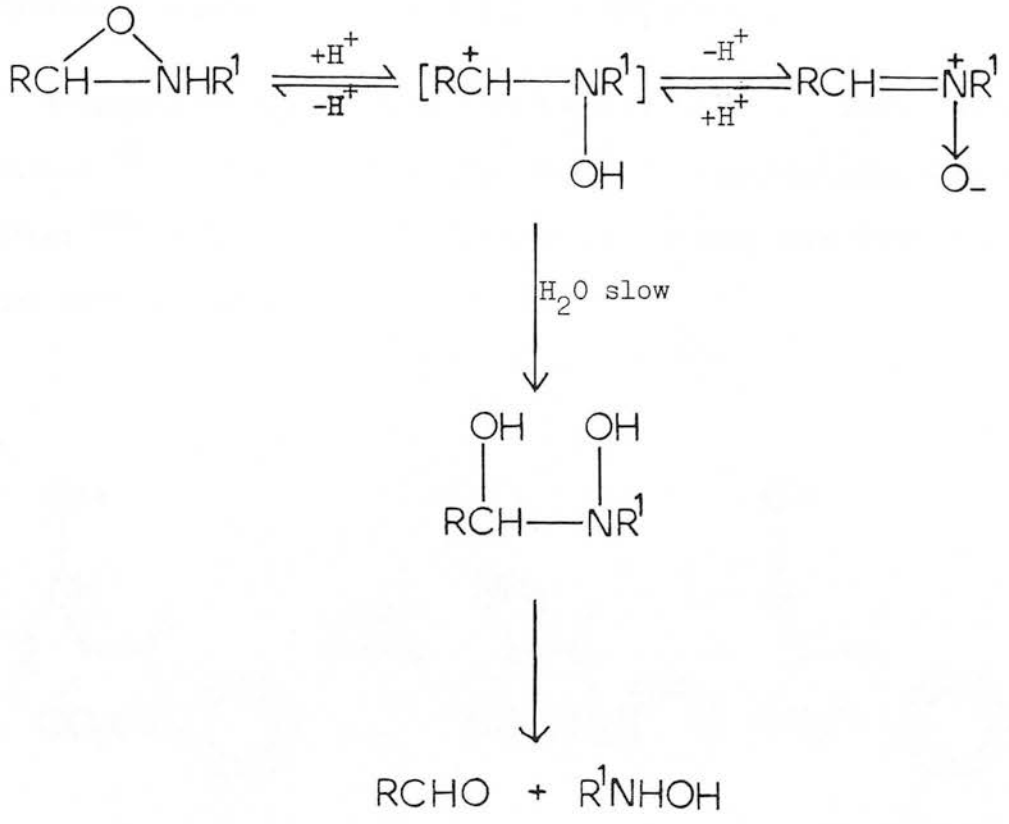

NH2
4-CH3OC6H4CHO
CH3O
CH=N
CO2CH2
103
3-CPBA
O
CH3O
CH
N
CO2CH2
104
1M-HCl
O−
CH3O
CH=N+
CO2CH2
105
3M-HCl
OH
HCl.NH
CO2CH2
106

FIGURE 63



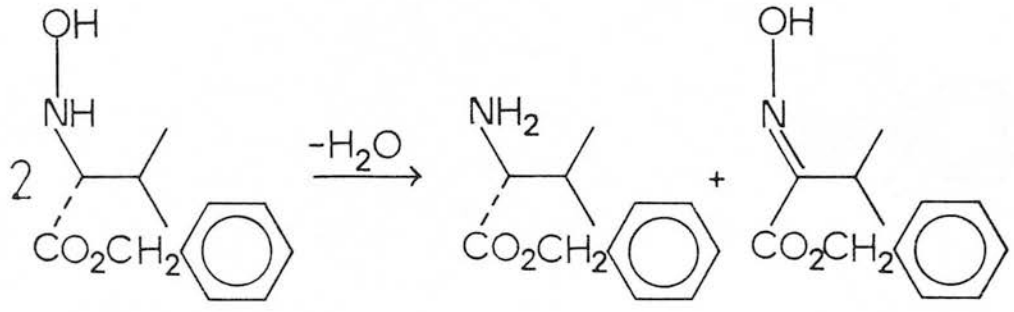
F I G U R E 64

oxidation products with 3M HCl in dioxane:H_2O(1:1) afforded the desired material, *N*-hydroxy-D-valine benzyl ester hydrochloride salt (106) (Figure 63). The overall yield of 106 from D-valine was 50%.

The structure of *N*-hydroxy-D-valine benzyl ester hydrochloride salt was confirmed from its spectroscopic properties. It is interesting to note that the valine C2 resonance, in the ^{13}C n.m.r. spectrum of 101 obtained from 106, was observed at 72·81 p.p.m. This compares with a chemical shift of 59·15δ for the valine C2 resonance of D-valine benzyl ester. Elemental analysis of the hydrochloride salt (106) was unsatisfactory and remained unsatisfactory even after three successive crystallizations. The free base (101) also yielded an unsatisfactory result but the 4-toluenesulphonic acid salt of

N-hydroxy-D-valine benzyl ester gave a satisfactory elemental analysis.

N-hydroxy-D-valine benzyl ester was found to be weakly ninhydrin-positive [495]. This may have been due to disproportionation of the product [496] (Figure 65), and reaction of the generated amino-acid ester with ninhydrin.

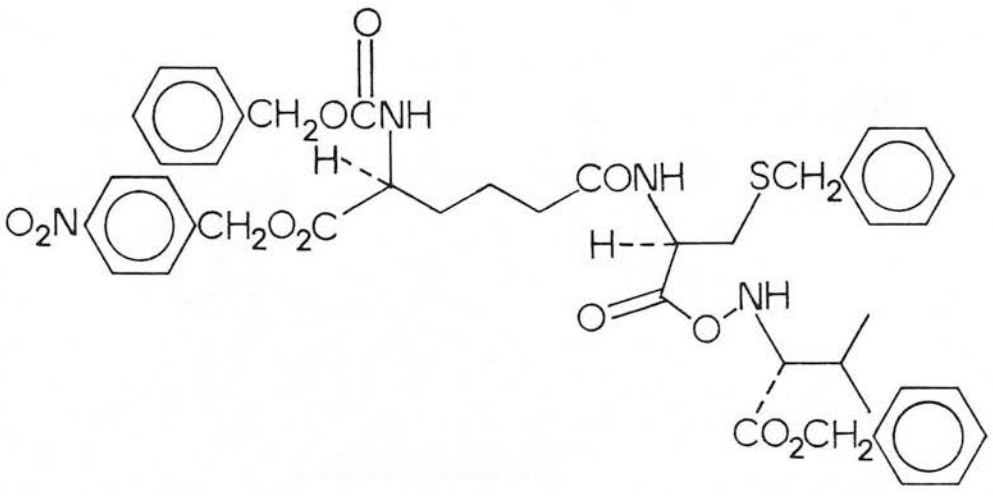


F I G U R E 65

The *N*-hydroxy-amino-acid (106) did not give a colour reaction with a 2% ethanolic solution of $FeCl_3$. All hydroxamic acids give a red or violet colour with ferric chloride in weak acid solution [497].

4.4.2 The Synthesis of *N*-[δ-(L-α-Aminoadipyl)-L-Cysteinyl]-*N*-Hydroxy-D-Valine

Reaction of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-amino-adipyl)-*S*-benzyl-L-cysteine (94) with *N*-hydroxy-D-valine benzyl ester (101), using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as coupling agents, afforded one major product isolated in 74% yield. This material was $FeCl_3$-negative and on the basis of mass spectroscopic evidence appeared to be the *O*-acyl derivative (107) rather than the desired hydroxamic acid (102).



107

This result was unexpected [483-485]. However, a search of the literature uncovered several examples [498-500] of *O*-acylation of *N*-alkyl hydroxylamines. In a mechanistic study, Jencks [500, 501] found that the reaction of 4-nitrophenyl benzoate with hydroxylamine gave rapid initial *O*-acylation. In the presence of excess hydroxylamine this product further reacted to yield benzohydroxamic acid. *O*-benzoylhydroxylamine was also converted to the thermodynamically more stable benzohydroxamic acid upon heating the pure liquid.

Attempts to rearrange the *O*-acyl derivative (107) by refluxing the product in EtOH or dioxane or heating the solid under an argon atmosphere failed to produce any $FeCl_3$-positive material. The only isolated material was *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (94) possibly obtained by α-proton abstraction (Figure 66).

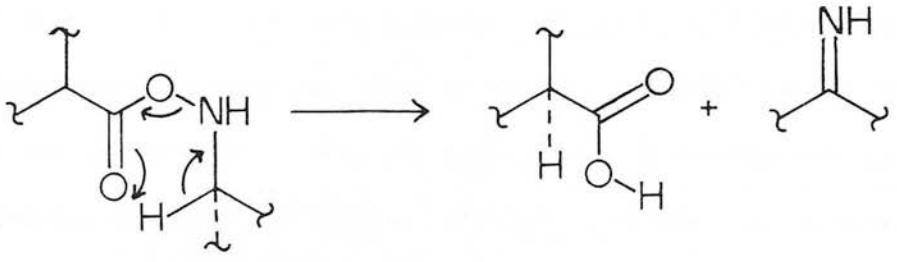
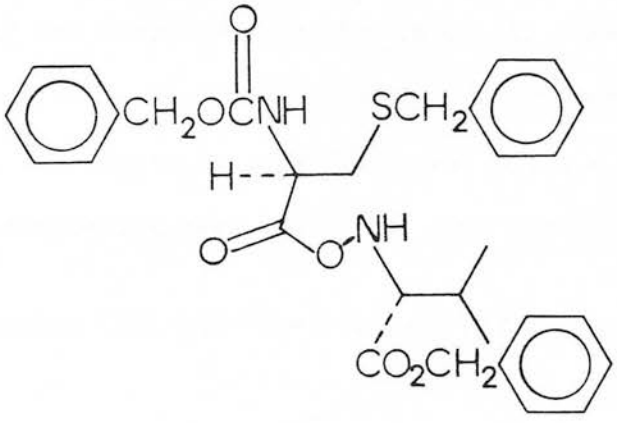


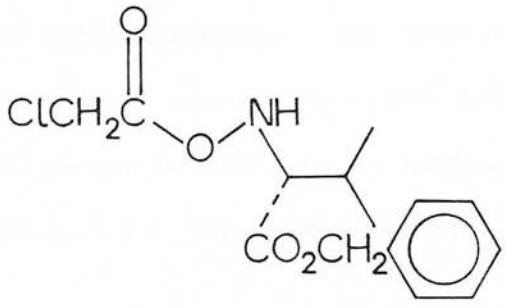
FIGURE 66

In an attempt to establish conditions which would isomerize *O*-acyl derivatives to hydroxamic acids, *O*-(*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-hydroxy-D-valine benzyl ester (108) was synthesised in 90% yield (by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole coupling of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine and *N*-hydroxy-D-valine benzyl ester). However, conditions could not be found which would transform 108 to the hydroxamic acid. Heating 108 in a variety of solvents, in the presence of acid, base, $FeCl_3$ and excess *N*-hydroxy-D-valine benzyl ester all failed to produce $FeCl_3$-positive material. In most cases decomposition to *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine was observed.



108

In Kolasa and Chimiak's successful synthesis [502] of *N*-hydroxy-peptidescoupling was achieved using *O*-protected derivatives of *N*-hydroxy-amino-acid esters. As *O*-acylation was evidently readily accomplished *N*-chloroacetoxy-D-valine benzyl ester (109) was synthesised but could not be successfully coupled with *N*-benzyloxy-carbonyl-*S*-benzyl-L-cysteine under a variety of conditions. The electron withdrawing chloroacetoxy substituent presumably reduces the nucleophilicity of the valine nitrogen, and may also sterically inhibit the reaction.

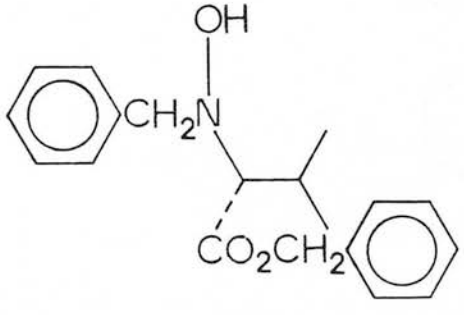


109

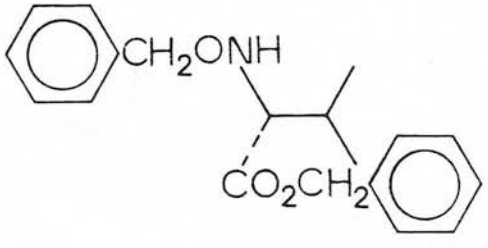
A trimethysilyl derivative of *N*-hydroxy-D-valine benzyl ester was prepared by reaction with *N*,*O*-bis(trimethysilyl)acetamide but was not characterized. Reaction of the crude material with chloro-acetic acid and dicyclohexylcarbodiimide in methylene chloride afforded a mixture of products including a minor $FeCl_3$-positive component. Attempts to synthesise and isolate the more stable *t*-butyldimethylsilyl derivative [503] were unsuccessful.

Alkylation of *N*-hydroxy-D-valine benzyl ester proved equally

unrewarding. Starting material was recovered when the salt (106) was treated with trityl chloride or benzyl bromide and triethylamine. Treatment with benzyl bromide and silver oxide [504] did yield a new product, but this was identified as the *N*-benzyl derivative (110), rather than the desired *O*-benzyl derivative (111), by comparing the shift of the benzyl methylene protons with reported [499] values and with those calculated from Shoolery's rules. The observed shift of the benzyl methylene was 3·87δ, while examples reported by Chimiak [499] for *O*-benzyl derivatives fall in the range 4·62-4·90δ. The calculated values for $ROCH_2Ph$ and R_2NCH_2Ph are 4.44_δ and 3.65_δ respectively. *N*-benzyloxy-D-valine benzyl ester (111) was prepared, though in very low yield, following reaction of *N*-hydroxy-D-valine benzyl ester with sodium hydride and benzyl bromide. The observed benzylation on nitrogen, with benzyl bromide and silver oxide, and on oxygen, with sodium hydride and benzyl bromide, is in complete agreement with theoretical predictions for the alkylation of hydroxylamine [505].



110

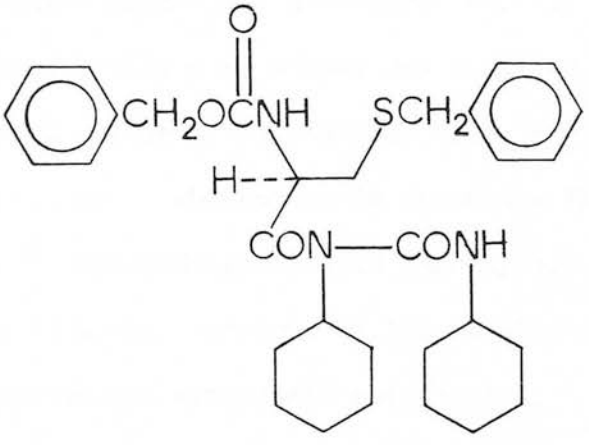


111

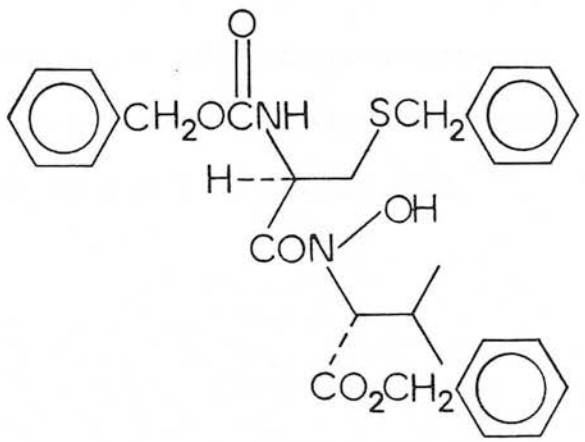
As direct *O*-protection of *N*-hydroxy-D-valine benzyl ester appeared impracticable, attention was focused on the coupling reaction in the hope that certain conditions would favour *N*- rather than *O*- acylation. The active 4-nitrophenyl esters, ethoxycarbonyl

mixed anhydrides or acid chloride derivatives of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (78) and *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine did not react with *N*-hydroxy-D-valine benzyl ester (101) to yield hydroxamic acids. No $FeCl_3$-positive materials were detected by t.l.c. of the reaction mixtures.

Treatment of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine and *N*-hydroxy-D-valine benzyl ester with dicyclohexylcarbodiimide in DMF gave a mixture of products, one of which was $FeCl_3$-positive. Chromatography on silica gel afforded *N*-(*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*,*N'*-dicyclohexylurea (112) isolated in 33% yield and the hydroxamic acid (113) isolated in impure form in 29% yield.



112



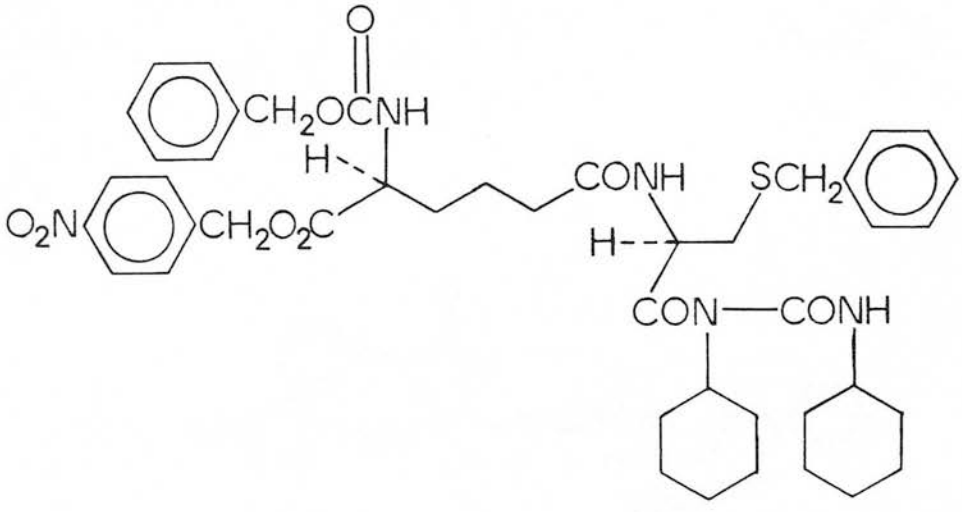
113

The hydroxamic acid (113) decomposed rapidly upon crystallization but an analytically pure sample was obtained. This material gave a strong red colour with ethanolic $FeCl_3$ and absorbed at 1670cm^{-1} in the i.r. spectrum. Absorption in the range 1670-1640 cm^{-1} has been reported [484] for hydroxamic acid carbonyls. A single resonance, at7.91δ in the ^{1}H n.m.r. spectrum of 113, readily exchanged with D_2O and was assigned to the hydroxamic acid proton.

In an attempt to reduce *N*-acyl urea (112) formation, the dicyclohexylcarbodiimide coupling was performed in methylene chloride [506], but none of the desired hydroxamic acid (113) was obtained. The only successful condition for the formation of 113 was the dicyclohexylcarbodiimide coupling in DMF.

When identical conditions were used to couple *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (94) and

N-hydroxy-D-valine benzyl ester, the only isolated product was *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*,*N'*-dicyclohexylurea (114). No $FeCl_3$-positive material was detected. The coupling condition is apparently very sensitive to the *N*-substituent of *S*-benzyl-L-cysteine. *N*-*t*-butoxycarbonyl-*S*-benzyl-L-cysteine likewise failed to condense with *N*-hydroxy-D-valine methyl ester to give a hydroxamic acid [507].



114

The successful formation of 113, however, suggested that LLD-AC(N-OH)V (81) could be produced by selective deprotection of 113, coupling of the product with *N*-benzyloxycarbonyl -L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (84) and deprotection of the resultant tripeptide (102) (Figure 67).

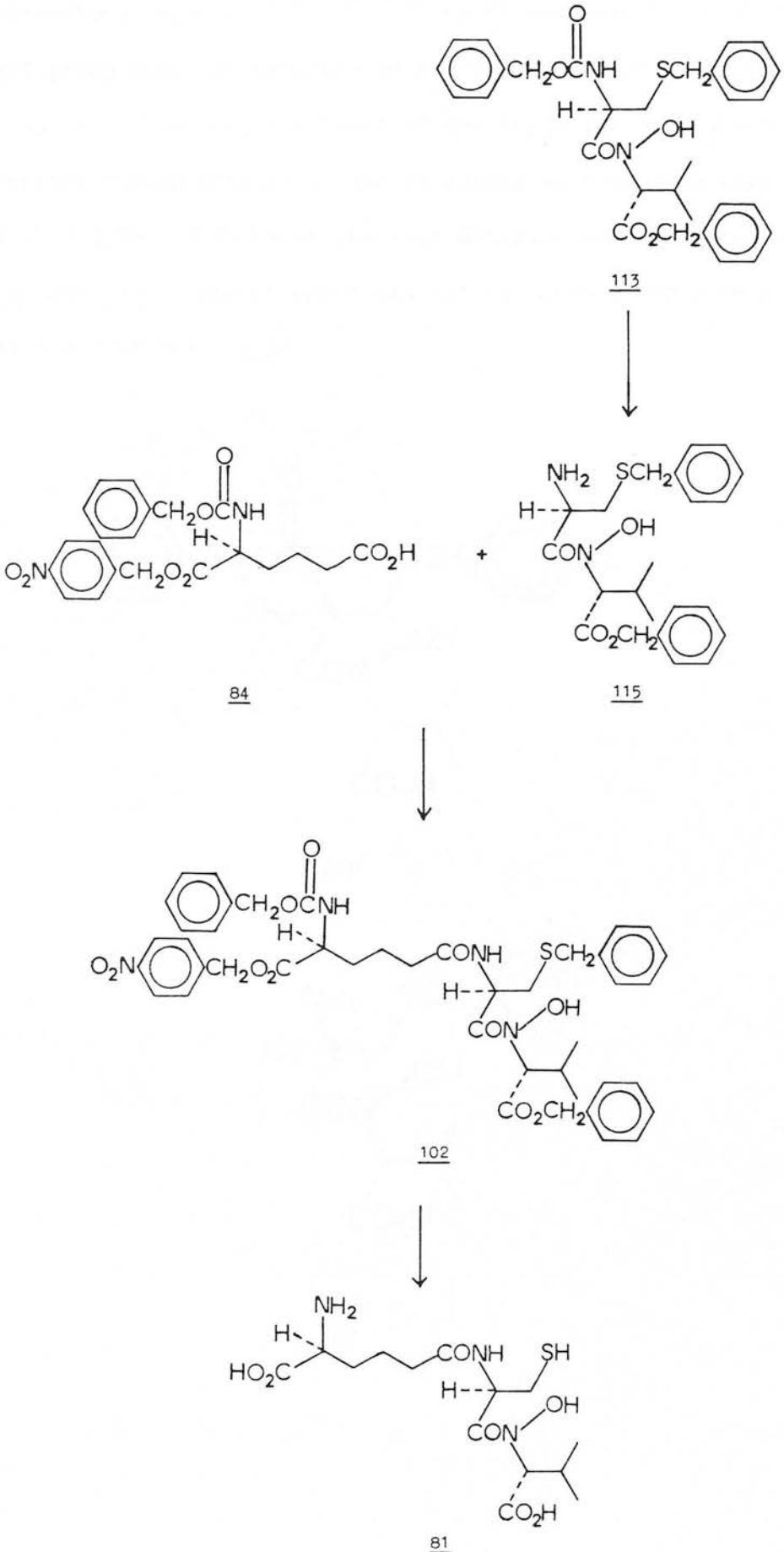
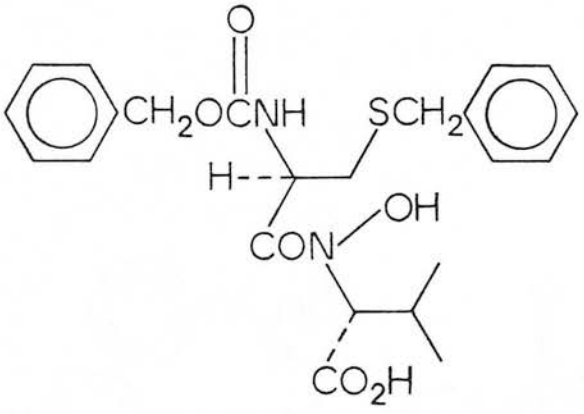
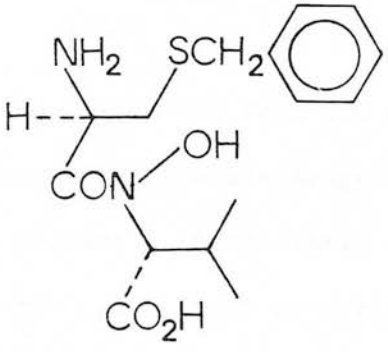

CH2OCNH
SCH2
H
OH
CON
CO2CH2
113
O2N
CH2O2C
CO2H
84
+
NH2
115
CONH
102
HO2C
SH
81

FIGURE 67

Literature reports [451, 508, 509] confirmed that a benzyloxycarbonyl group could be selectively removed in the presence of a benzyl ester. However, treatment of the dipeptide derivative (113) with various concentrations of HBr in acetic acid or with trifluoroacetic acid gave a mixture of starting material and the three products 115, 116 and 117. Conditions could not be found which gave predominantly the free base (115).



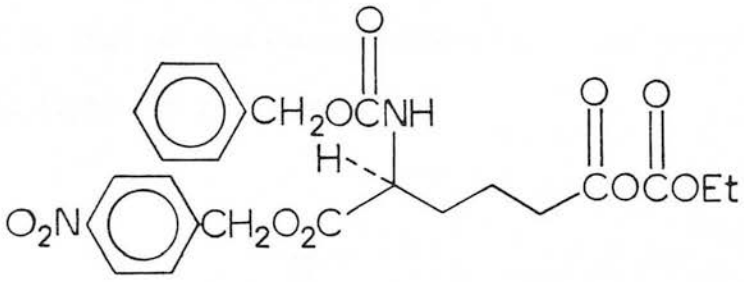
116



117

The desired product (115) was identified by t.l.c. as it gave a positive colour reaction with ninhydrin but no colour reaction with 2,6-dichlorophenolindophenol sodium salt [510]. Although 115 could be identified, it readily decomposed and could not be isolated by preparative t.l.c.

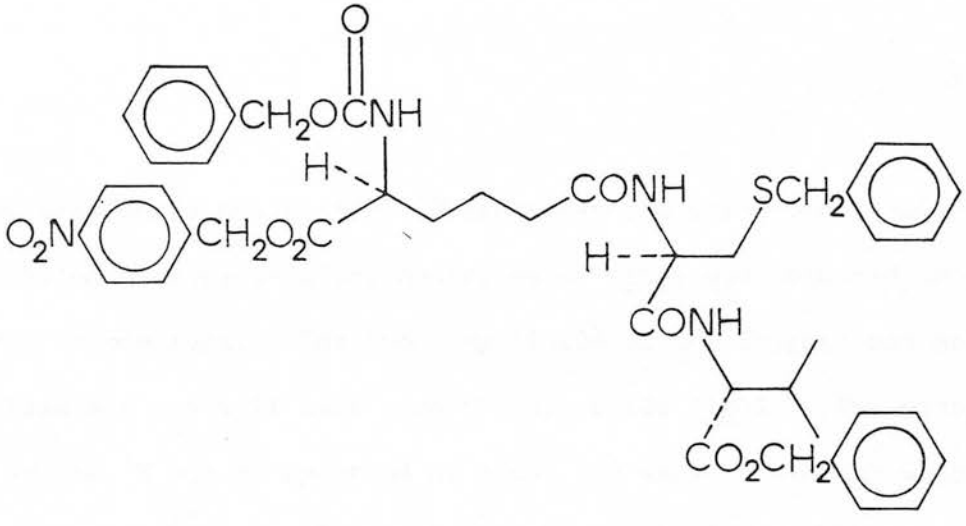
In a trial reaction the crude mixture of products (113, 115, 116 and 117) was treated with the ethoxycarbonyl mixed anhydride of *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (118).



118

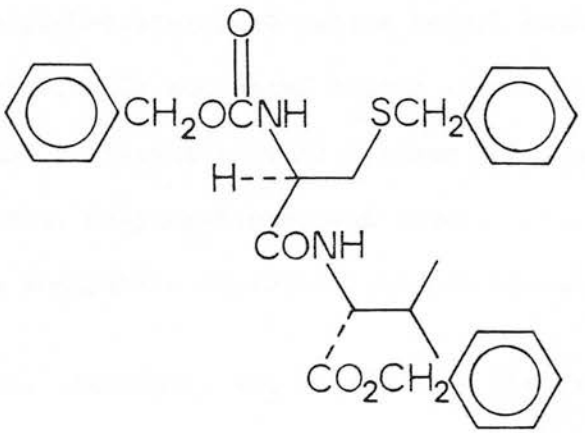
After 3½h at room temperature the reaction was stopped. An analytical t.l.c. of the reaction mixture suggested at least nine different materials were present. Fortunately there was only one major $FeCl_3$-positive material, and after extensive column chromatography this was isolated in 12% yield. The product was homogeneous by t.l.c. in a variety of solvent systems, but the ^1H and ^{13}C n.m.r. spectra of the material indicated that it was an almost 50:50 mixture of two compounds. A small quantity of the mixture was separated by reverse phase h.p.l.c. and the ^1H n.m.r. spectra of the individual

components examined. The least mobile component was $FeCl_3$-positive and appeared to be the desired hydroxamic acid tripeptide (102). The ^1H n.m.r. spectrum showed two amide NH resonances at 5·54 and 6·21 p.p.m. and one resonance at 8·34 p.p.m. assigned to the hydroxamic acid proton. The ^1H n.m.r. spectrum of the most mobile component contained three amide protons and no hydroxamic acid proton, but was otherwise similar to that of 102. It was initially assumed that this second component was *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (119) and indeed a subsequent ^1H n.m.r. spectrum of an authentic sample (prepared by coupling *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine and D-valine benzyl ester) was identical to that of the second component. The isolated LLD-ACV derivative (119) was $FeCl_3$-negative.



119

It is not entirely clear how the tripeptide (119) was formed in the above reaction. The most likely explanation, and one which can be inferred from later results, is that *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (120) was formed early in the synthesis.



120

As previously mentioned *N*-(*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-hydroxy-D-valine benzyl ester (113) was obtained in slightly impure form. The impurity (5-10% of the weight) was not identified but may well have been the dipeptide (120). The minor peaks in the ^{1}H n.m.r. spectrum of crude 113 were consistent with values reported [451] for 120, and a synthetic sample of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (120) (prepared by coupling *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine and D-valine benzyl ester) proved inseparable from 113 by analytical t.l.c.

No D-valine benzyl ester was detected in the synthetic sample

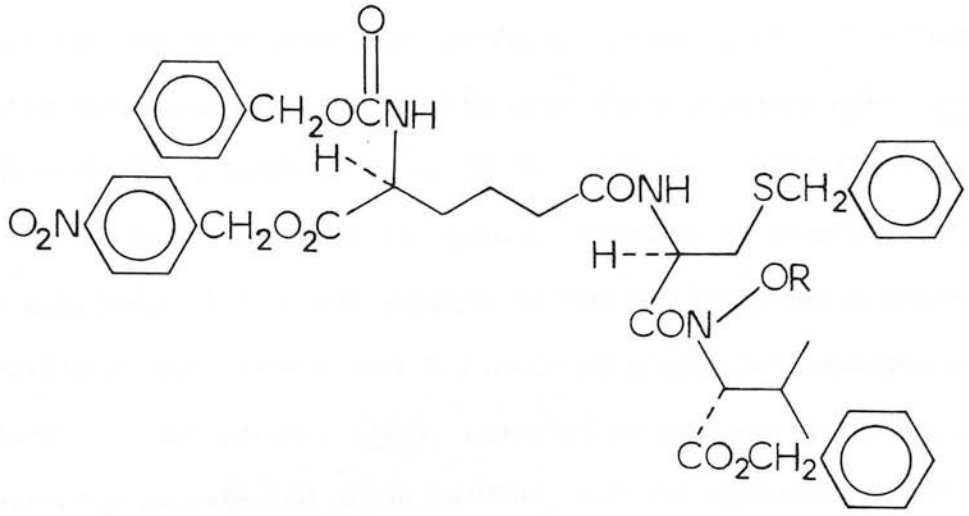
of *N*-hydroxy-D-valine benzyl ester used in the preparation of 113, but may have been formed during the coupling reaction by disproportionation of *N*-hydroxy-D-valine benzyl ester (Figure 65). In the presence of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine and dicyclohexylcarbodiimide the dipeptide (120) would be rapidly formed.

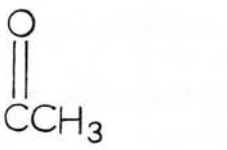
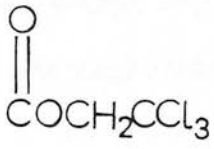
The mixture of *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (102) and *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (119) could not be chromatographically separated except in minute quantities by h.p.l.c. Many different solvent systems and stationery phases (silica, alumina, polyacrylamide and reverse phase C-18 silica) were tested but no separation of the components was observed.

Attention, therefore, was directed to the difference in functionality between the two components. It was hoped that a derivative of the hydroxamic acid (102) could be found which would allow chromatographic separation from the tripeptide (119).

The trimethylsilyl ester of the hydroxamic acid (121) could be prepared by treatment of the mixture with *N*,*O*-bis(trimethylsilyl) acetamide. This derivative, however, was too labile to effect separation of the mixture, and the *t*-butlydimethylsilyl ester, which would have been more stable [503], could not be prepared.

Reaction of 102 with acetic anhydride and pyridine afforded the *O*-acetyl derivative (122) which was stable and could be separated from the LLD-ACV tripeptide (119) by preparative t.l.c. Unfortunately the basic hydrolysis conditions required to remove the *O*-acetyl



	R
102	H
121	$Si(CH_3)_3$
122	
123	
124	CH_3
125	$CHPh_2$
126	CH_2Ph

group were incompatible with other functionalities in the molecule and a mixture of unidentified products was obtained. Trifluoroacetic anhydride reacted not only with the hydroxamic acid (102) but also with the tripeptide (119) in the mixture. Mild basic hydrolysis of the crude products did not lead to recovery of starting materials. The 2,2,2-trichloroethoxycarbonyl derivative (123) was prepared by reaction of the mixture with 2,2,2-trichloroethylchloroformate in pyridine. The product (123), isolated by preparative t.l.c., was apparently deprotected using Zn/HOAc, but the hydroxamic acid (102) was not recovered from the solution. The free hydroxamic acid may have complexed with the zinc.

In an attempt to alkylate the hydroxamic acid (102), the mixture of materials was treated with benzyl bromide and triethylamine, but after five days at room temperature the starting materials were recovered unchanged. Reaction with diazomethane afforded the methyl ester (124), but the more versatile diphenylmethyl derivative [473] (125) was not obtained from reaction with diphenyldiazomethane.

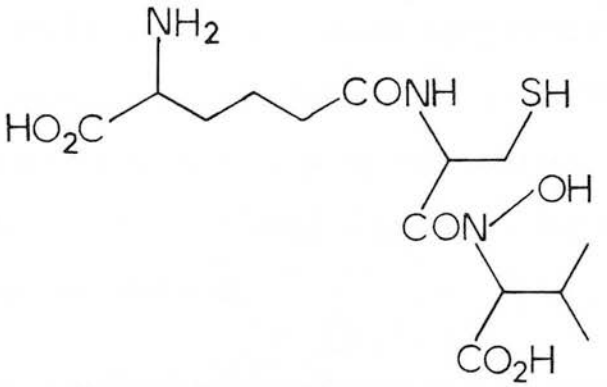
Finally treatment of the mixture (102 and 119) with benzyl bromide and silver oxide [504] in DMF yielded *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine benzyl ester (126) which was separated from unchanged 119 by column chromatography. The structure of the protected species (126) was confirmed from its spectroscopic characteristics. The material was $FeCl_3$-negative as anticipated, and in the ^1H n.m.r. spectrum a new benzyl methylene signal was observed as an AB multiplet centred at 5·06δ. In the ^{13}C n.m.r. spectrum of 126 the valine C2 resonance was observed at 66·23δ. Though this is not as

significant a downfield shift as observed for *N*-hydroxy-D-valine benzyl ester (101), the consistent shift of the α-carbon to high frequency appears to be indicative of the hydroxamic acid functionality. In *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (119) the valine α-carbon resonates at 57•47δ.

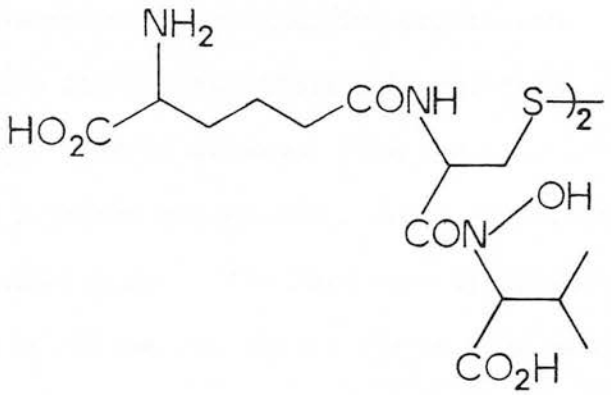
The derivative (126) was deprotected and isolated using the same procedures developed for the synthesis of LLD-ACV (88). Thus sodium-liquid ammonia reduction and air oxidation afforded a crude material which was purified by cation-exchange chromatography. Freeze-drying the ninhydrin-positive eluate yielded two products. These could be separated by preparative electrophoresis and, on the basis of FAB mass spectral analysis, were tentatively assigned the monomer and dimer structures, 127 and 128 respectively.

Both materials were $FeCl_3$-positive and yielded the same product (presumably *N*-[δ-(L-α-aminoadipyl)-L-cysteicyl]-*N*-hydroxy-D-valine) when oxidized with performic acid. Why the free thiol (127) was not completely oxidized to the disulphide (128) on oxygenation of a basic aqueous solution of 127 was unclear. The close proximity of the strongly acidic hydroxamic acid may decrease the pK_b of the sulphydryl group and reduce the rate of disulphide formation.

The ^1H n.m.r. and ^{13}C n.m.r. spectra of 127 and 128 were extremely complicated and adjusting the pH of the D_2O solutions had little simplifying effect on the ^1H n.m.r. spectra. As the mass spectra were consistent with the proposed structures, 127 ((M-H) ion at m/z 378•1327, $C_{14}H_{24}N_3O_7S$ requires m/z 378•1329) and 128 ((M-H) ion at m/z 755), the complexity of the n.m.r. spectra can be



127



128

attributed to each being a mixture of diastereoisomers. Racemization at one or more chiral centres may have occurred during the basic oxidation of the crude product or during the sodium-liquid ammonia deprotection of the fully protected species (126), particularly if a trace of moisture was present.

After eventually succeeding in producing AC(N-OH)V it was disheartening to find that the final product was not optically pure. It was resolved, therefore, to devise an improved synthesis of LLD-AC(N-OH)V (81). The knowledge gained during the above synthesis of 127 and 128 would help determine the new synthetic route. A more

acid labile *N*-protective group than the benzyloxycarbonyl derivative of 113 was required, yet too great a change in the *N*-protective group might result in failure to synthesise the hydroxamic acid (see page 134). The 4-methoxybenzyloxycarbonyl [511] derivative seemed ideal and the proposed synthesis of 81, using this protective group, is outlined in Figure 68. In order to test this route, LLD-ACV (119) was synthesised as shown in Figure 69.

N-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine (129) was prepared by reaction of *S*-benzyl-L-cysteine with 4-methoxybenzyloxycarbonyl azide under Shotten-Baumann conditions. The product was isolated as its dicyclohexylamine salt and fully characterized. The free acid (129) readily obtained from the dicyclohexylamine salt was coupled with D-valine benzyl ester using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The resultant dipeptide derivative (132) was isolated by column chromatography in near quantitative yield and subjected to various acidolytic conditions. It was found that removal of the 4-methoxybenzyloxycarbonyl group could be selectively achieved using a range of HCl-nitromethane concentrations. In practice HCl saturated nitromethane was employed. The deprotected species (133) could be isolated as its hydrochloride salt, but was most conveniently isolated as its 4-toluenesulphonic acid salt in 71% yield. The dicyclohexylcarbodiimide/1-hydroxybenzotriazole mediated coupling of the free base (133) and *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (84), afforded the fully protected LLD-ACV tripeptide (119) identical to previously prepared material. The overall yield of 119 from *S*-benzyl-L-cysteine was 36% and from D-valine, 50%.

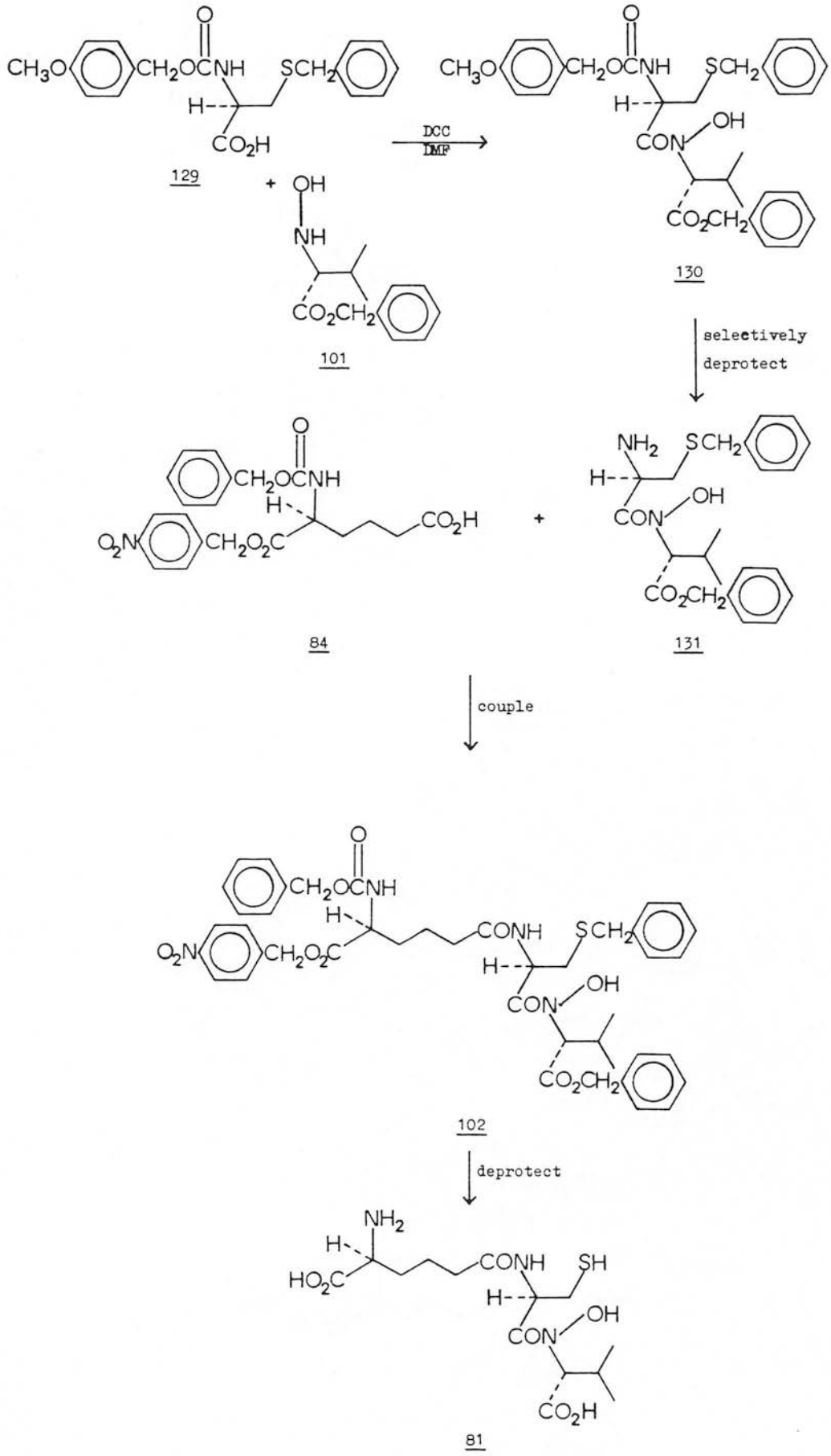
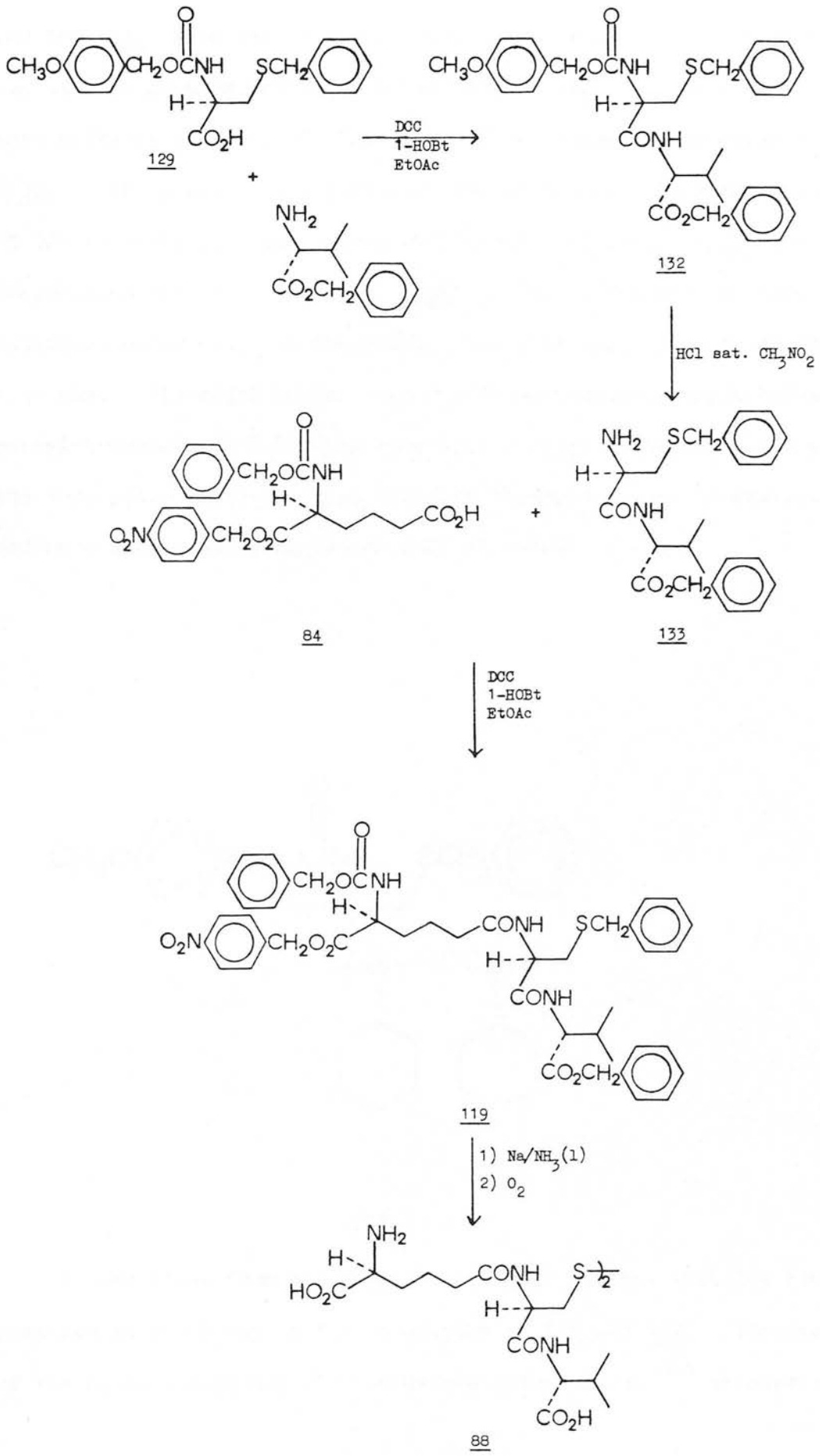

DCC
DMF
129
101
130
selectively
deprotect
84
131
couple
102
deprotect
81

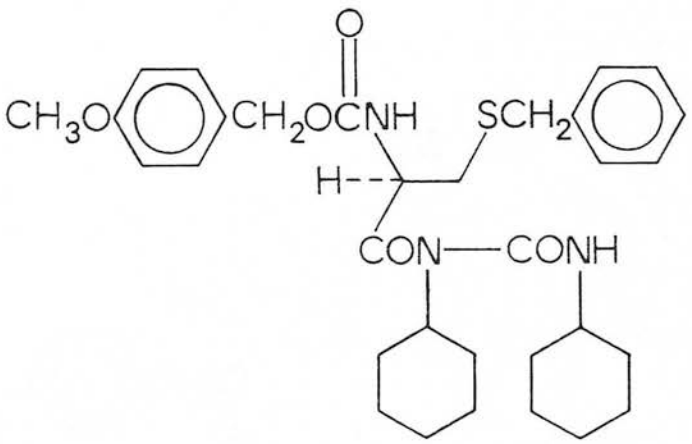
FIGURE 68



129 + 132 → 84 + 133 → 119 → 88

FIGURE 69

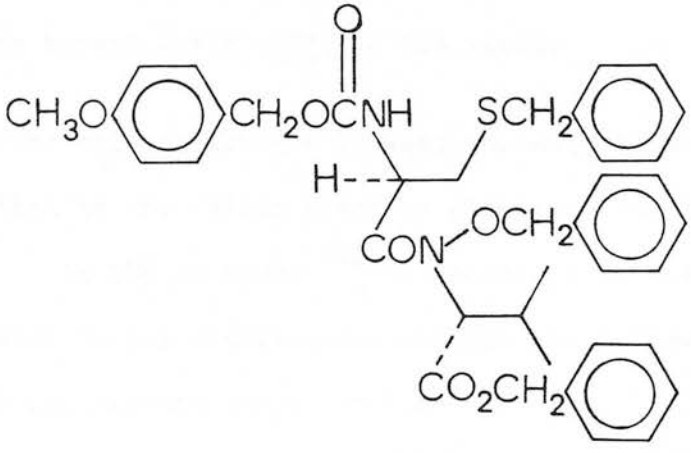
Encouraged by this short efficient synthesis of fully protected LLD-ACV (119) from the *C*-terminal end, an analagous route (Figure 68) was used to prepare protected LLD-AC(N-OH)V (102). The first and most critical step was the synthesis of the dipeptide hydroxamic acid (130). The previously established dicyclohexylcarbodiimide condensation of *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine (129) and *N*-hydroxy-D-valine benzyl ester (101) in DMF, afforded a mixture of materials which could be separated, with difficulty, by column chromatography. The major product was *N*-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*,*N*'-dicyclohexylurea (134) isolated in 22% yield. The more polar $FeCl_3$-positive material decomposed upon crystallization and as a result was obtained in only 8% overall yield.



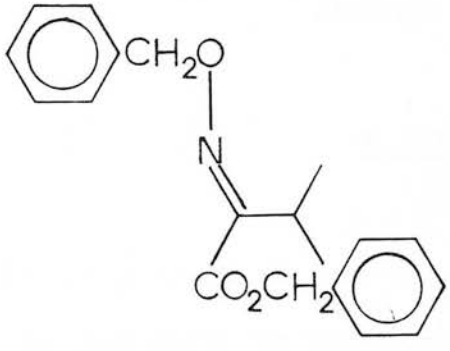
134

It was clear from the ^1H and ^{13}C n.m.r. spectra that the $FeCl_3$-positive material was in fact a mixture of 130 and 132. Treatment of the mixture with benzyl bromide and silver oxide [504] in distilled

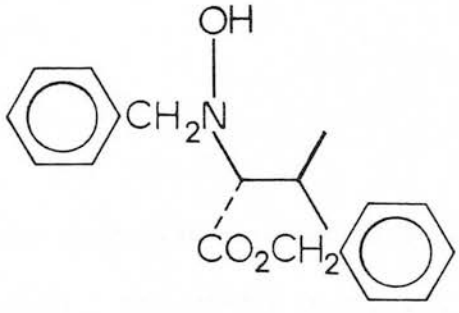
DMF did not give the *O*-benzyl derivative (135) as anticipated. The dipeptide (132) was recovered along with two new materials identified by ^{1}H n.m.r. and mass spectroscopy as *O*-benzyl-2-oxoisovaleric acid oxime benzyl ester (136) and *N*-benzyl-*N*-hydroxy-D-valine benzyl ester (110).



135



136



110

The isolated material (110) was identical to that produced during attempts to *O*-benzylate *N*-hydroxy-D-valine benzyl ester with benzyl bromide and silver oxide (see page 131). Presumably 110 arose in this case as in the previous case by reaction of free *N*-hydroxy-D-valine benzyl ester. How free *N*-hydroxy-D-valine benzyl ester came to be present in the reaction mixture, and how the minor component (136) was produced, is unknown. In contrast, the reaction proceeded smoothly and efficiently when repeated in non-distilled DMF to afford *N*-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-benzyloxy-D-valine benzyl ester (135) in 68% yield.

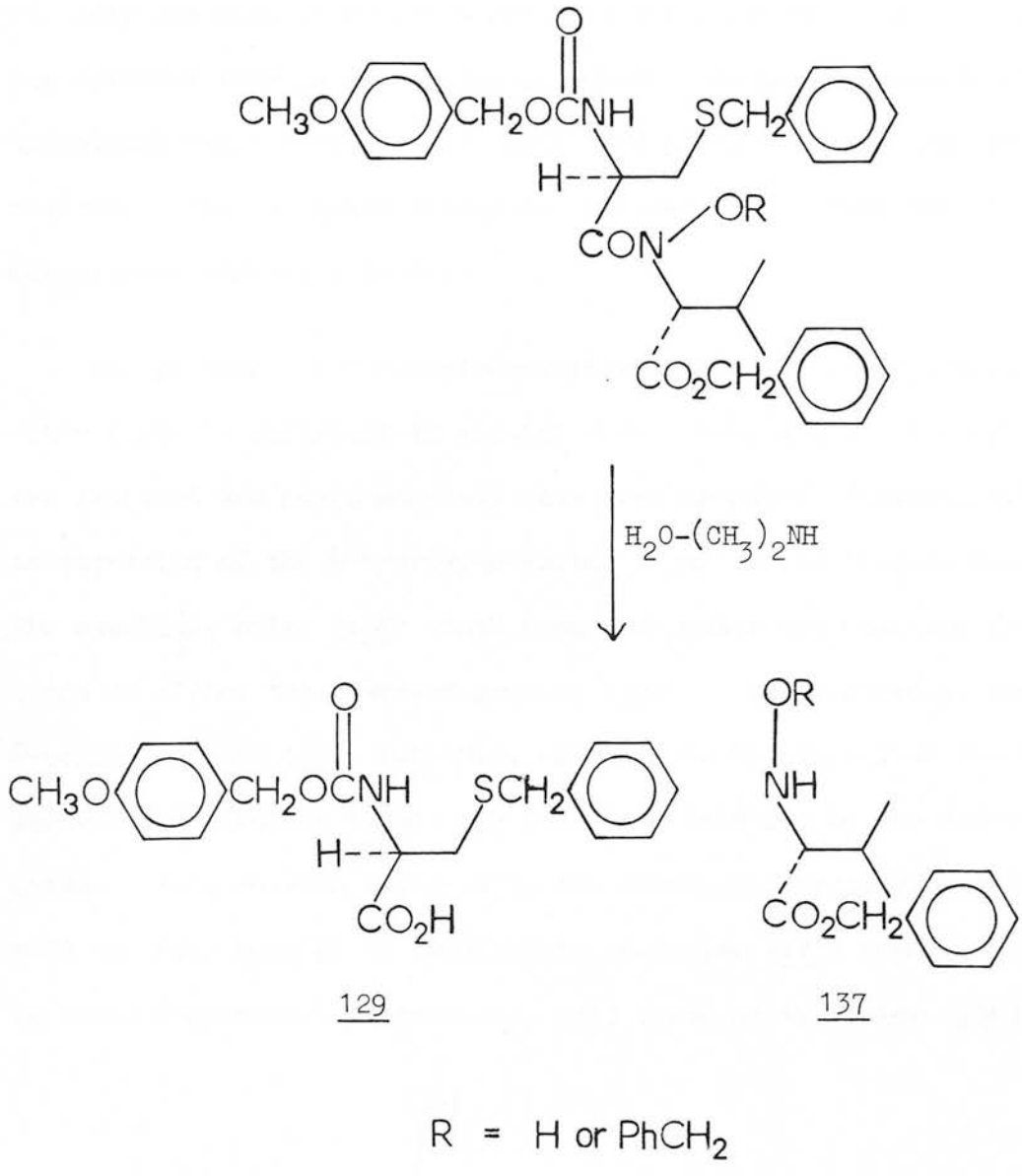
The only difference between the successful and failed reactions was that in the failed reaction distilled DMF had been used as solvent. As DMF is known [512] to decompose to dimethylamine and carbon-monoxide during distillation perhaps the following speculative proposal can explain these results.

Initially the benzylation reaction proceeds as expected but soon traces of moisture are produced (Figure 70).

$$Ag_2O + 2PhCH_2Br + 2ROH \longrightarrow 2ROCH_2Ph + 2AgBr + H_2O$$

F I G U R E 70

In the presence of dimethylamine, hydroxide ions would be formed and could hydrolyse the hydroxamic acid. Hydroxamic acids are hydrolysed to carboxylic acids and hydroxylamines far more readily than amides [484] and hydrolysis is almost certainly involved (Figure 71).



129 137

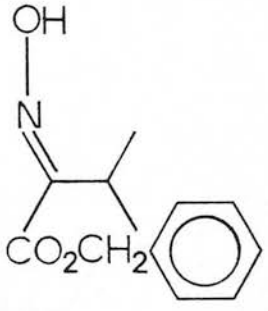
R = H or $PhCH_2$

FIGURE 71

The *N*-hydroxy-D-valine benzyl ester (137, R=H) so obtained would react to yield the observed *N*-benzyl-*N*-hydroxy-D-valine benzyl ester (110). The water produced from *N*-benzylation of the free *N*-hydroxy-D-valine benzyl ester (cf. Figure 70) would be available to hydrolyse more of the hydroxamic acid (130) yielding more *N*-hydroxy-D-valine benzyl ester.

This argument is tenuous, however. As can be seen from Figure 70, only one mole of water is produced for every two moles of starting material (ROH in Figure 70) consumed. As 130 was completely hydrolysed extra moisture must have been present in the reaction mixture. This is quite likely as it is known [512] that DMF is notoriously difficult to dry.

The presence of *O*-benzyl-2-oxoisovaleric acid oxime benzyl ester (136) is difficult to account for. Only 6mg of this material was isolated and could possibly have been produced following disproportionation of the *N*-hydroxy-D-valine benzyl ester (Figure 65). The resultant oxime (138) would benzylate under the reaction conditions to afford the observed product (136). Alternatively, the *O*-benzyl species (137, R=$PhCH_2$), obtained by hydrolysis of the fully protected derivative (135), may have been oxidized by the silver oxide. In a reverse argument to the above, the hydroxylamine (137, R=H) may have been first oxidized to the oxime (138) then benzylated to yield *O*-benzyl-2-oxoisovaleric acid oxime benzyl ester (136).

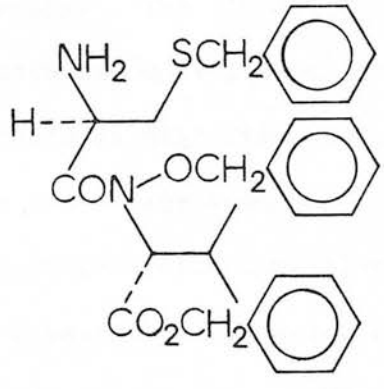


138

No oxime (138) nor D-valine benzyl ester (nor *N*-benzyl derivatives thereof) were observed, and studies to determine the mechanism of this reaction were not undertaken.

As has been mentioned *N*-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-benzyloxy-D-valine benzyl ester (135) could be prepared when non-distilled DMF was used as solvent. The resultant material (135) was $FeCl_3$-negative, and in the ^{13}C n.m.r. spectrum the valine C2 resonance was observed at 65·74δ and the benzyl methylene of the hydroxamate benzyl ester at 79·36δ.

Reaction of *N*-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-benzyloxy-D-valine benzyl ester (135) with HCl saturated nitromethane, followed by a basic aqueous work up, afforded *N*-(*S*-benzyl-L-cysteinyl)-*N*-benzyloxy-D-valine benzyl ester (139) as a crystalline solid. The material was ninhydrin-positive and homogeneous by t.l.c. A ^1H n.m.r. spectrum of 139 confirmed its purity, but when coupled with *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (84), using dicyclohexycarbodiimide and 1-hydroxybenzotriazole as coupling agents, an inseparable mixture of at least seven compounds was obtained.



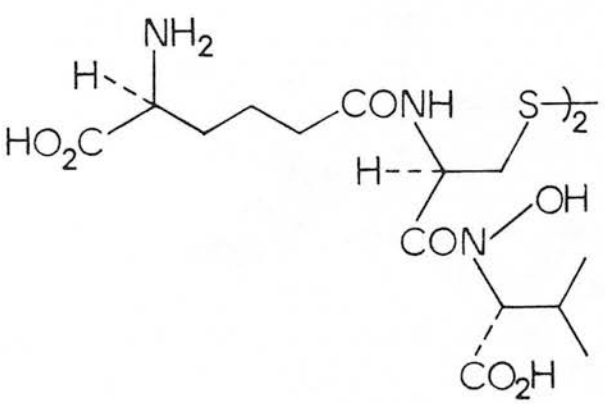
139

Fortunately, at the same time as this approach to the synthesis of the hydroxamic acid tripeptide (126) was proving unsuccessful, conditions were established which gave *N*-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-hydroxy-D-valine benzyl ester (130) in 20% yield and free of any contaminating *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (132). Thus a solution of *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine in non-distilled DMF was pre-treated with dicyclohexylcarbodiimide. Some five minutes later *N*-hydroxy-D-valine benzyl ester hydrochloride salt and pyridine were added and the solution stirred overnight. Following extensive column chromatography on silica the hydroxamic acid dipeptide (130) was isolated analytically pure. The valine C2 resonance was observed at 62·86δ in the ^{13}C n.m.r. spectrum of 130, and in the ^{1}H n.m.r. spectrum the hydroxamic acid proton resonated at 7·77δ.

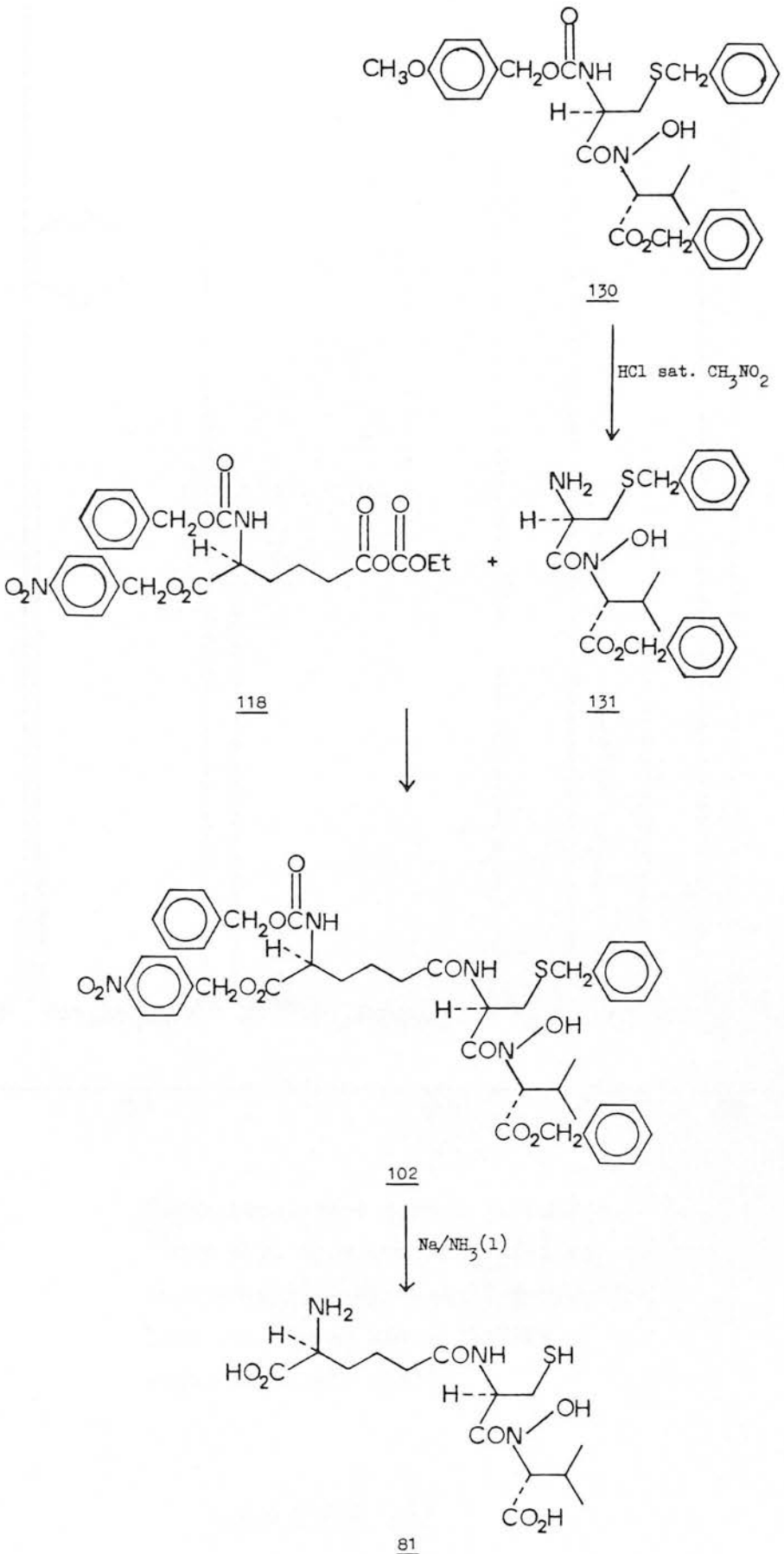
Deprotection of the hydroxamic acid (130) with HCl saturated nitromethane and coupling of the crude product with the ethoxycarbonyl mixed anhydride (118) afforded, after column chromatography, protected LLD-AC(N-OH)V (102) as a crystalline solid in 29% yield. The material was judged pure by elemental analysis, ^{1}H n.m.r. and ^{13}C n.m.r. spectroscopy. The ^{13}C n.m.r. spectrum was assigned on the basis of selective decoupling experiments. The valine C2 resonance was again found at high frequency, 63·10δ, compared with a shift of 57·47δ for the valine α-carbon in the ^{13}C n.m.r. spectrum of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (119). The material (102) was $FeCl_3$-positive and exhibited a (M+H) ion, as the base peak (m/z 829), in its FAB mass spectrum.

Attempts to deprotect *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (102) by catalytic reduction over charcoal supported palladium failed, even when one equivalent of catalyst was used. Sodium-liquid ammonia deprotection of 102 and purification of the crude product by cation-exchange chromatography afforded *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (81) in analytically pure form (Figure 72). The product was $FeCl_3$-positive and the ^1H n.m.r. spectrum could be unambiguously assigned by comparison with the ^1H n.m.r. spectra of other derivatives, described previously. The valine α-proton was observed as a sharp doublet at 4·66δ.

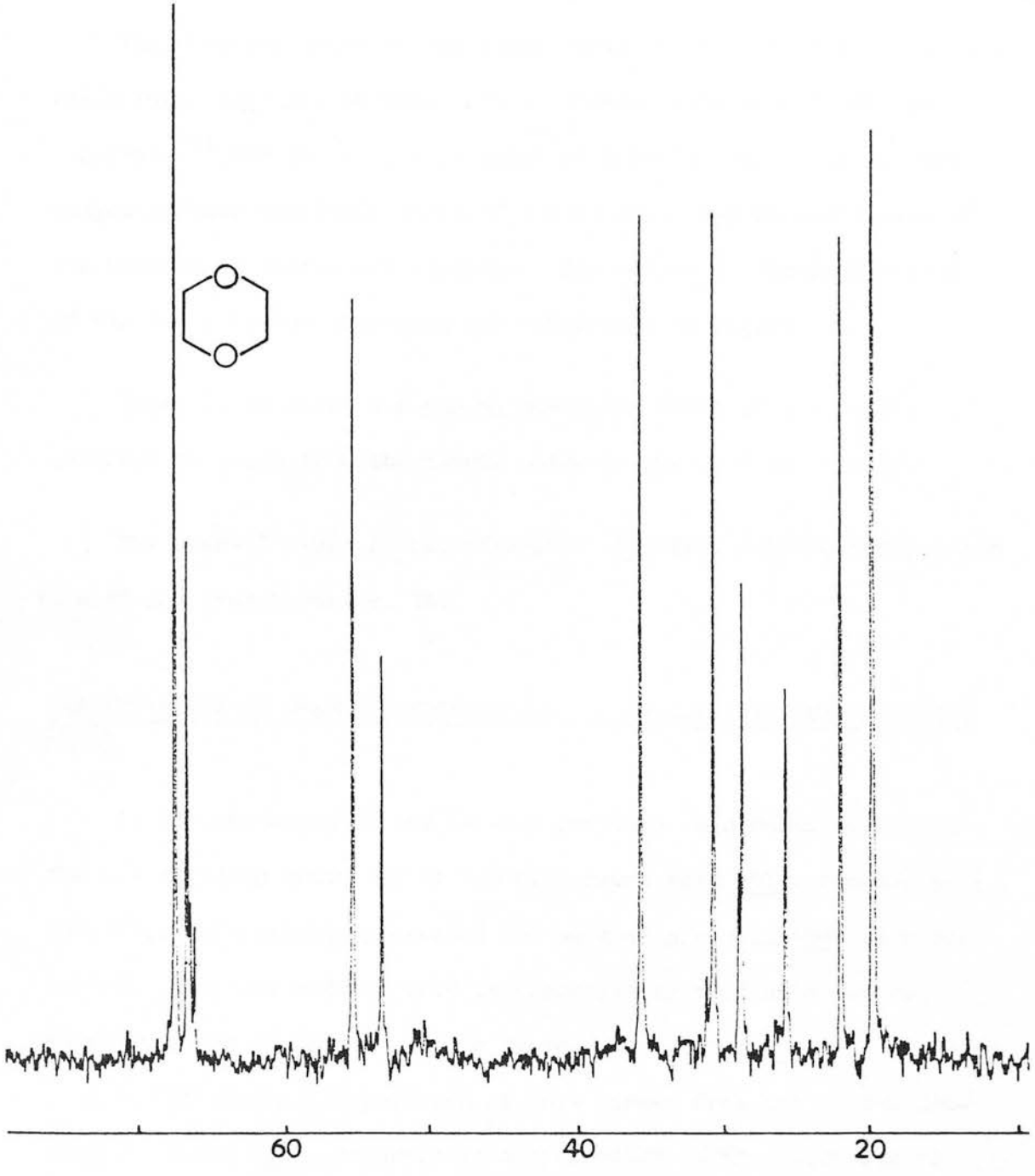
The material was identified as the monomer (81) and not the disulphide (140) from its ^{13}C n.m.r. spectrum (Figure 73). The cysteine C3 resonance at 25·55δ is characteristic of the free thiol. The chemical shift of cysteine C3 in the LLD-ACV monomer (34) is 26·10δ[451] and in the disulphide[465] (88), 38·49δ. After exposure to the atmosphere for several days the monomer (81) had oxidized to the disulphide (140). This was confirmed from subsequent ^{13}C n.m.r. and FAB mass spectral analysis; the base peak (M+H) ion at m/z 757 was of the dimer (140) and the cysteinyl C3 resonance was observed at 34.70 p.p.m.



140


CH3O
CH2OCNH
SCH2
H
OH
CON
CO2CH2
130
HCl sat. CH3NO2
O2N
CH2O2C
COCOEt
NH2
118
131
CONH
102
Na/NH3(l)
HO2C
SH
CO2H
81

F I G U R E 72



75MHz broad-band proton decoupled ^{13}C n.m.r. spectrum of N-[δ-(L-α-aminoadipyl)-L-cysteinyl]-N-hydroxy-D-valine (sweep width 15151Hz, number of scans 8687).

FIGURE 73

The chemical shift of the valine α-carbon in the originally isolated thiol (81) was 66·62δ. This compares with a shift of 59·30δ reported [451] for the valine α-carbon of LLD-ACV (34). As already suggested this downfield shift of the α-carbon may be indicative of the hydroxamic acid functionality. The valine C2 chemical shifts of various relevant compounds are summarized in Figure 74.

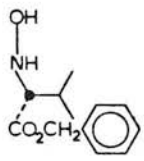
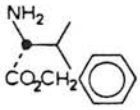
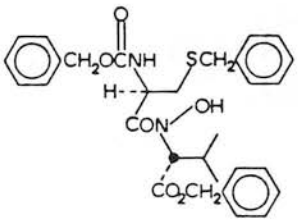
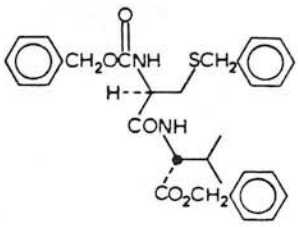
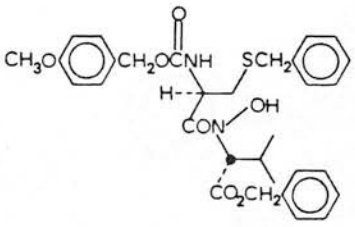
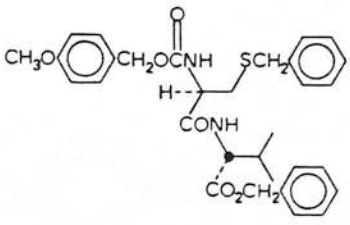
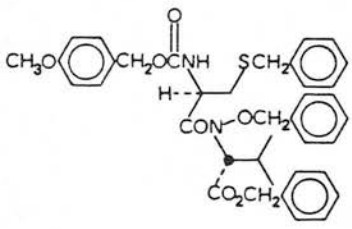
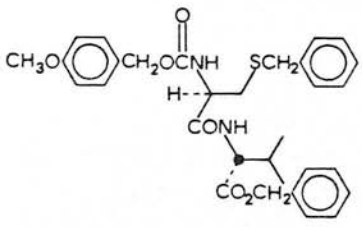
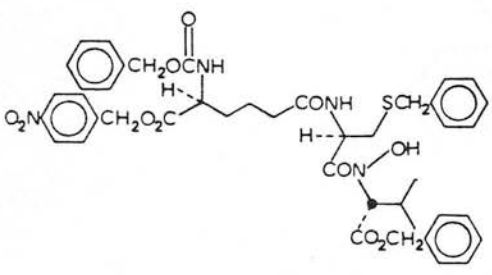
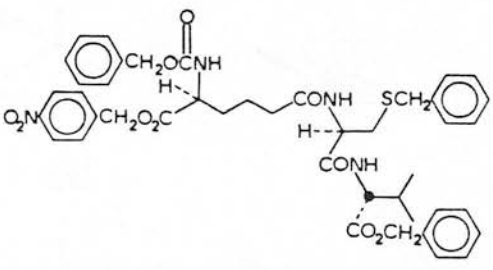
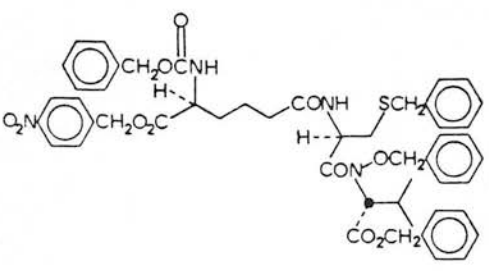
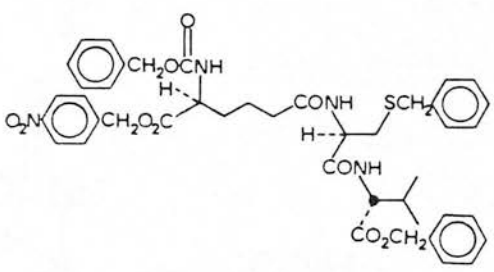
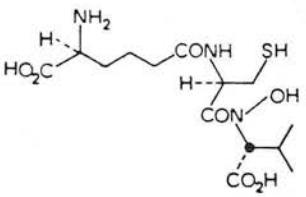
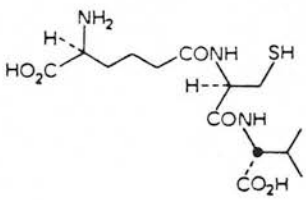
There is at least a 6 p.p.m. downfield shift of the valine α-carbon on going from the simple amide to the hydroxamic acid.

The overall yield of LLD-AC(N-OH)V (81) from *S*-benzyl-L-cysteine was 4% and from D-valine, 3%.

4.5 THE SYNTHESIS OF *N*-BENZYLOXYCARBONYL-L-α-AMINOADIPIC ACID 1-BENZYL ESTER

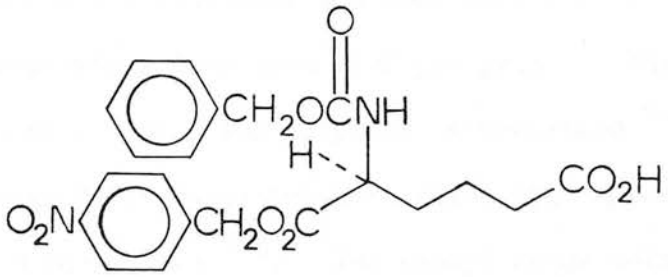
In the synthesis of the LLD-ACV peptides, and particularly in the low yielding synthesis of the hydroxamic acid (81), considerable quantities of suitably protected L-α-aminoadipic acid (84) were required. L-α-aminoadipic acid is commercially available but is expensive, and conversion to the desired derivative (84) was effected in only 39% yield. Separation of this isomer from the 6-(4-nitrobenzyl) isomer (91), produced as a by-product in the synthesis of (84), by column chromatography is difficult and restricts the preparation of 84 to a scale of about 7 millimolar.

To overcome the problem of expense and in order to produce large quantities of suitably protected L-α-aminoadipic acid, it was hoped to oxidize the L-lysine derivative (141) to *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-benzyl ester (142) (Figure 75). A similar strategy has been used for the conversion of L-lysine to L-α-aminoadipic acid [513].

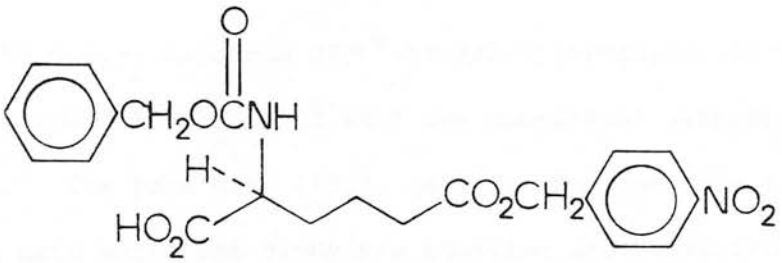
	Shift		Shift
	72.81		59.15
	-		57.3
	62.86		57.12
	65.74		57.12
	63.10		57.47
	66.23		57.47
	66.62		59.3

Comparison of the ^{13}C n.m.r. chemical shifts of the valine α-carbon of related compounds.

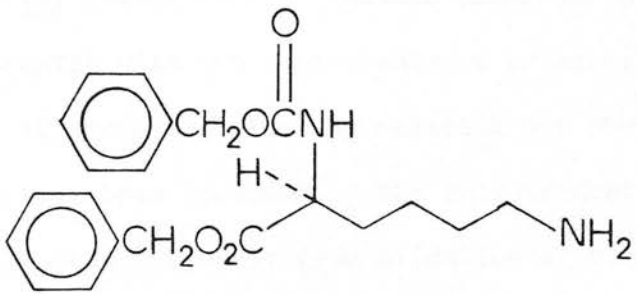
FIGURE 74



84



91



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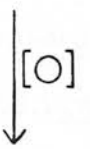
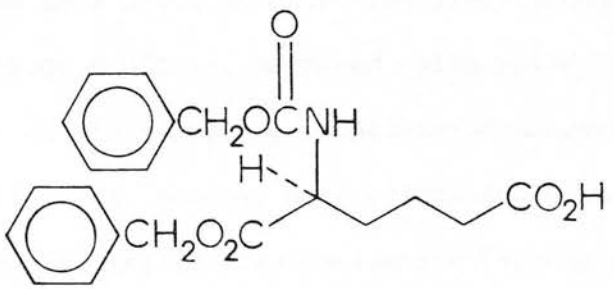




142

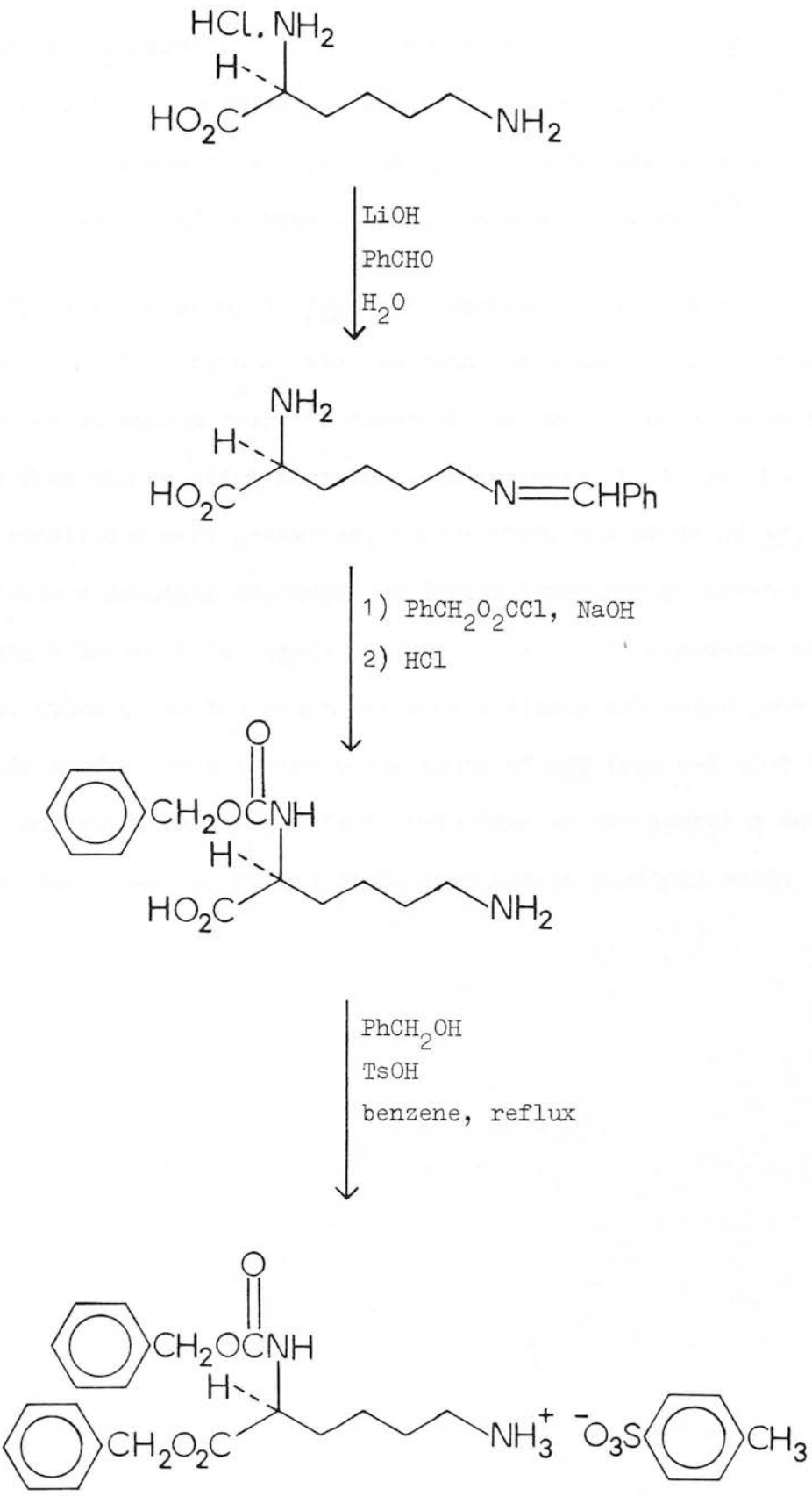
FIGURE 75

L-lysine is readily available and very much cheaper (£14 per kilogram) than L-α-aminoadipic acid (£20 per gram). The N^{ε}-benzylidene derivative of L-lysine was prepared as described [514] and converted to N^{α}-benzyloxycarbonyl-L-lysine using the improved procedures of Scott and co-workers [515]. The benzyl ester 4-toluenesulphonic acid salt of N^{α}-benzyloxycarbonyl-L-lysine was obtained by standard methods [490] in 92% yield (Figure 76).

The ^{1}H n.m.r. spectrum of N^{α}-benzyloxycarbonyl-L-lysine benzyl ester 4-toluenesulphonic acid salt was consistent with the proposed structure. The free base (141), readily obtained from the 4-toluenesulphonic acid salt, was ninhydrin positive and exhibited a parent ion at m/z 370 in its EI mass spectrum.

A solution of 141 in *t*-BuOH:0·1M aqueous phosphate buffer (pH6·0)(1:1) was treated with 2·5 equivalents of bromine. After gently heating for 12 hours the starting material was shown, by analytical t.l.c., to have been consumed. The only product isolated, however, was benzoic acid, probably from oxidation of benzyl alcohol produced during the reaction.

The use of potassium permanganate did allow direct oxidation of the amine (141) to the carboxylic acid (142). When 1·33 equivalents of $KMnO_4$ were added to a solution of N^{α}-benzyloxycarbonyl-L-lysine benzyl ester in *t*-BuOH:H_2O(2:1), buffered with solid $CaSO_4$, and the mixture heated for 40 min a brown precipitate of manganese dioxide formed. After filtration, several extractions and column chromatography of the extracts, the desired derivative N-benzyloxycarbonyl-L-α-aminoadipic acid 1-benzyl ester was isolated in 8% yield. The melting point, optical rotation and ^{1}H n.m.r. spectrum of the


HCl. NH2
H
HO2C
NH2
LiOH
PhCHO
H2O
NH2
H
HO2C
N=CHPh
1) PhCH2O2CCl, NaOH
2) HCl
O
CH2OCNH
H
HO2C
NH2
PhCH2OH
TsOH
benzene, reflux
O
CH2OCNH
H
CH2O2C
NH3+
-O3S
CH3

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L-α-aminoadipic acid derivative (142) were virtually identical to those reported by Baldwin [516], who had previously oxidized 141 to 142 by an indirect three-step process in an overall yield of 22%. A biomimetic oxidation of a protected lysine derivative to a protected L-α-aminoadipic acid derivative has also been reported [517].

The 8% yield of product (142) was disappointingly low but time did not allow optimization of the reaction conditions. Considerable quantities of unreacted starting material (as much as 50%) could be recovered from the reaction mixture which suggested that, if the reaction conditions were optimized, direct $KMnO_4$ oxidation of 141 could provide a feasible synthesis of *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-benzyl ester (142). Even an 8% yield represents an economical approach to the preparation of suitably protected L-α-aminoadipic acid. This indirect synthesis of 142 from L-lysine is some one hundred times cheaper (with reference to the starting amino-acid) than the direct synthesis of 84 from L-α-aminoadipic acid.

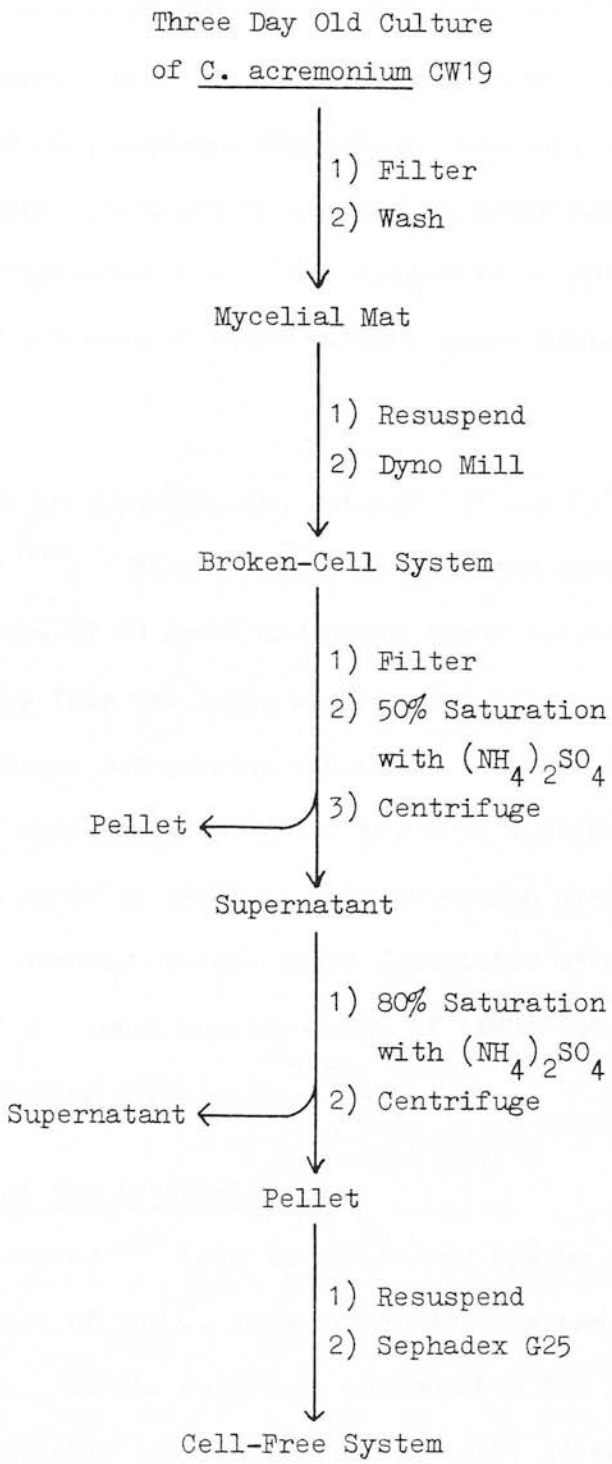
5. CELL-FREE PREPARATION AND FEEDING STUDIES

5.1 PREPARATION OF AN ACTIVE CELL-FREE SYSTEM

In 1981 Abraham reported [352] the preparation of a cell-free extract from the mechanically ruptured mycelium of *C. acremonium* C91. Using the procedures described in this paper it was possible to obtain an active cell-free system from *C. acremonium* CW19 (Figure 77).

A growth curve was established [518] for *C. acremonium* CW19. The results suggested that the mycelium should be harvested after 72h growth; prior to the onset of the stationary phase and cephalosporin C production. Centrifugation of the CW19 culture failed to pellet the cell mass, possibly due to a carbohydrate gradient, but the mycelium was readily collected by filtration of the growth medium. Mechanical grinding of the mycelium, resuspended in morpholinopropanesulphonic acid (MOPS) buffer (50mmol,pH7·2), in a Dyno Mill afforded a crude broken-cell system which was weakly active in converting synthetic LLD-ACV to an antibiotic substance (as determined from *B. subtilus* bioassay). However, control experiments indicated a large background antibiotic titre, presumably due to intracellular penicillins (and cephalosporins) released during cell rupture. This weak "synthetase" activity was completely lost after 24h at 0°C.

The crude broken-cell system was treated with ammonium sulphate and the 50-80% precipitate [352] dialysed overnight at 4°C. The cell-free system obtained in this manner was found to be only slightly


Three Day Old Culture
of C. acremonium CW19
1) Filter
2) Wash
Mycelial Mat
1) Resuspend
2) Dyno Mill
Broken-Cell System
1) Filter
2) 50% Saturation
with $(NH_4)_2SO_4$
3) Centrifuge
Pellet
Supernatant
1) 80% Saturation
with $(NH_4)_2SO_4$
2) Centrifuge
Supernatant
Pellet
1) Resuspend
2) Sephadex G25
Cell-Free System

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more active in converting LLD-ACV to an antibiotic than the crude broken-cell system. When the 50-80% precipitate fraction was immediately de-salted on a Sephadex G25 column, however, the eluate was found to be highly active and to contain no detectable quantities of penicillins or cephalosporins. The cell-free preparation could be stored at -20°C for several weeks without appreciable loss of "synthetase" activity.

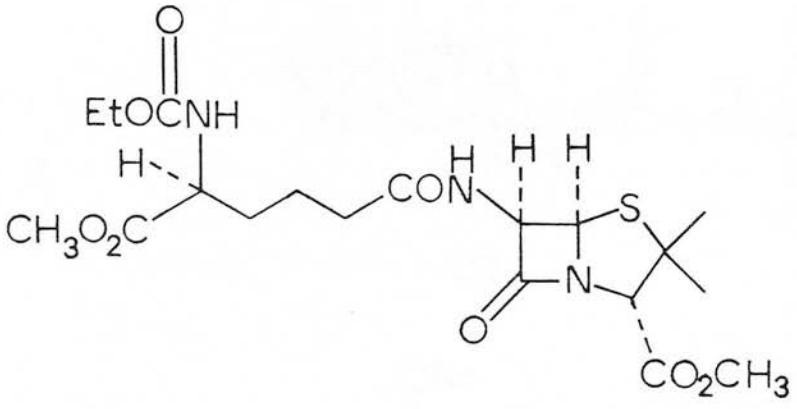
Experiments to determine the optimum DTT and Fe^{2+} concentrations were undertaken [352]. Maximum antibiotic titres were observed for DTT concentrations of *ca* 2mmol and above (some 3-4 x LLD-ACV concentration) but results from the $FeSO_4$ experiments were inconclusive. The antibiotic titre was not greatly dependent on the $FeSO_4$ concentration and the optimum concentration varied from one system to another. Why this should be so is unclear, but the enzyme system is fairly crude and might already contain small quantities of Fe^{2+}. Concentrations for DTT of 2mmol and for $FeSO_4$ of 1mmol were normally used in subsequent feeding experiments [352].

5.1.1 Identification of the Antibiotic

Abraham observed [352] that the cell-free system prepared by mechanical rupture of the *C. acremonium* C91 mycelium produced only isopenicillin N. As the epimerase responsible for penicillin N biosynthesis is highly labile (see Section 3·5) it was assumed that the cell-free system obtained from *C. acremonium* CW19 also produced only isopenicillin N.

The antibiotic activity produced from LLD-ACV by the CW19 cell-free system was completely destroyed when treated with 5 units of *B. cereus* penicillinase. Thus the material is a penicillin, either

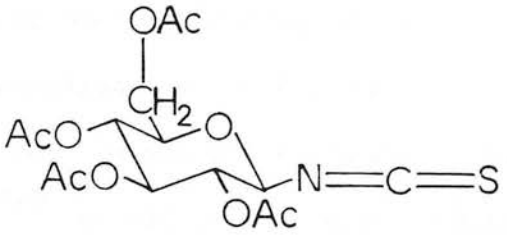
isopenicillin N or penicillin N, and this was further indicated by derivatization and mass spectral analysis of the antibiotic. LLD-ACV was incubated with the cell-free system and, using a procedure similar to that described by Baldwin [342], a small quantity of the *N*-ethoxycarbonyl dimethyl ester derivative (143) was isolated from solution.



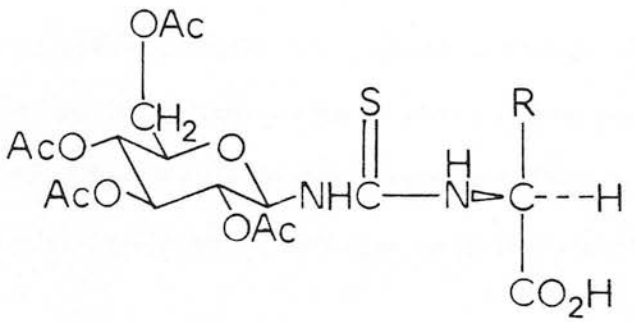
143

The EI mass spectrum exhibited a parent ion at m/z 459 and a base peak ion at m/z 230 (from α-cleavage of the aminoadipyl amide bond). The remainder of the mass spectrum was similar to that already reported [342] for 143.

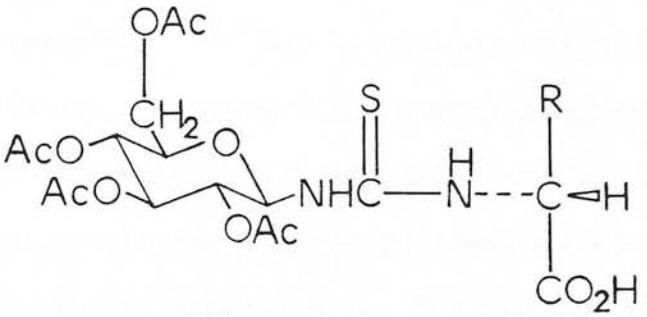
In a recent publication [354] the chiral derivatization of isopenicillin N and penicillin N and the h.p.l.c. separation of the diastereoisomers was described. Treatment of the penicillins with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [519] (GITC) (144) afforded the thiourea derivatives 145 and 146 (Figure 78) which were efficiently separated by reverse phase h.p.l.c.



144



145



146

R =

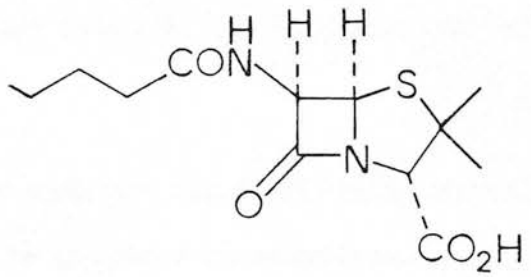


FIGURE 78

Authentic samples of penicillin N and isopenicillin N would be required in order to unambiguously identify the penicillin produced in the cell-free reaction. While the synthesis of both compounds has been reported [520-522], an alternative though related procedure appeared more attractive. If the penicillin derivative (143) was heated in concentrated acid, α-aminoadipic acid could be isolated from solution by preparative electrophonesis (cf. Figure 1). The chirality of the isolated α-aminoadipic acid would distinguish the penicillins (L-α-aminoadipic acid from isopenicillin N and D-α-aminoadipic acid from penicillin N) and could be determined from GITC derivatization and h.p.l.c. analysis of the amino-acid derivative.

The GITC derivatives of commercial L-α-aminoadipic acid (145, R= $-(CH_2)_3CO_2H$) and D-α-aminoadipic acid (146, R= $-(CH_2)_3CO_2H$), prepared as previously described [523], were separated by reverse phase h.p.l.c. using a Waters μ-Bondapak C18 analytical column and methanol: 10mmol aqueous phosphate buffer (pH 2•8)(37•5:62•5) as eluant. The thiourea group absorbs strongly at 254nm and facilitates UV detection of the chiral derivatives. The L-isomer was eluted before the D-isomer as has been reported [523] for the GITC derivatives of eleven other amino-acids. At a flow rate of 0•9ml/min the retention time for the L-isomer was 15•0 min and for the D-isomer 19•0 min.

The feasibility of this approach was thus demonstrated, but unfortunately 143 could not be prepared in quantities sufficient to establish the chirality of the α-aminoadipyl side-chain. On the basis of literature analogy [352], however, there seems little doubt that the penicillin produced is isopenicillin N and not penicillin N.

5.2 FEEDING STUDIES

Although the cell-free system was fairly crude, conversions of LLD-ACV to isopenicillin N in the order of 75% were routinely achieved. These results were determined from bioassay at optimum incubation conditions and with *Staph. aureus* as sensitive organism. An activity against *Staph. aureus* for cephalosporin C of 10 units/mg and for isopenicillin N of 80 units/mg was assumed [352].

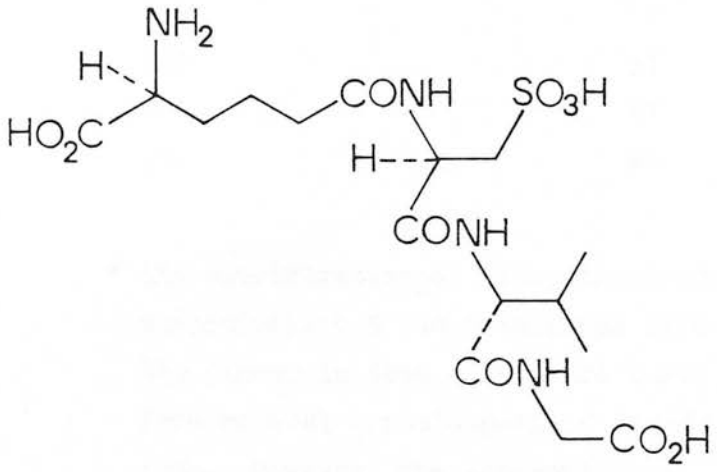
With an efficient cell-free system available the role of other peptides and peptide derivatives in penicillin biosynthesis could be investigated.

5.2.1 δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valylglycine

LLD-ACVG(83) is not a substrate for isopenicillin N synthetase. When the tetrapeptide was incubated with the cell-free system in the presence of DTT and $FeSO_4$ no antibiotic activity was detected by *Staph. aureus* bioassay. In a concurrent test LLD-ACV (34) was efficiently converted to isopenicillin N.

The LLD-ACVG tetrapeptide could be recovered from the incubation mixture as its sulphonic acid, δ-(L-α-aminoadipyl)-L-cysteicyl-D-valylglycine (LLD-ACyaVG) (147). An equivalent volume of methanol was added to the incubation mixture and the precipitated protein filtered. The filtrate was oxidized with performic acid and the sulphonic acid (147) isolated by cation-exchange chromatography with water as eluant.

When LLD-ACV and LLD-ACVG were co-incubated with the cell-free system antibiotic production was observed to decrease with increasing LLD-ACVG concentrations. The incubation mixtures contained LLD-ACV



147

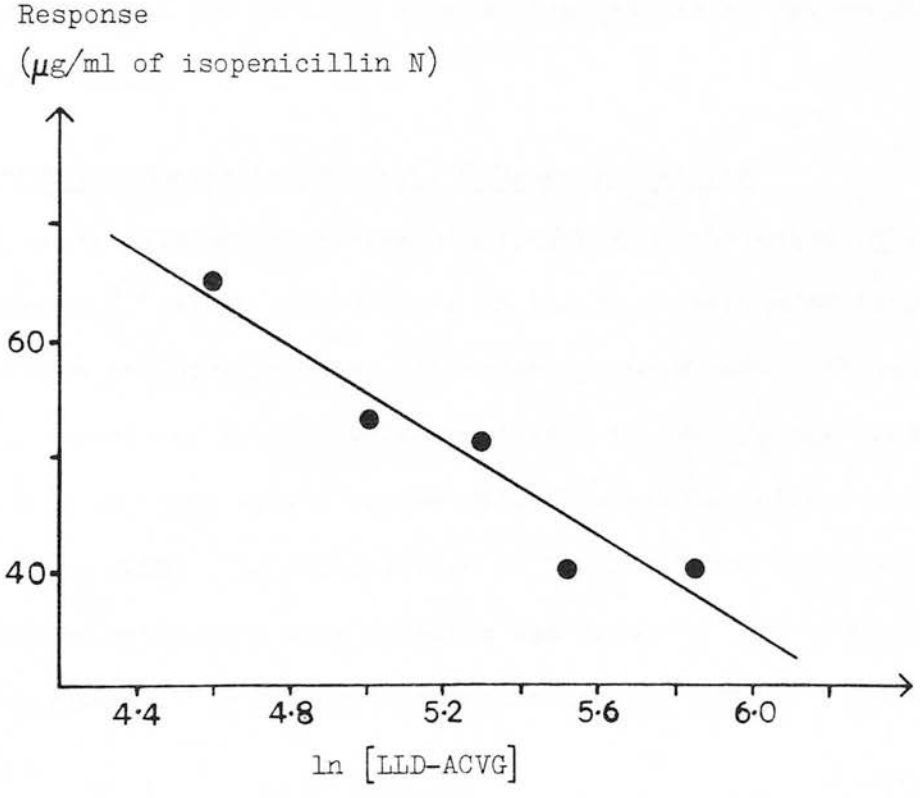
(125µg), cell-free preparation (500µl) and various quantities of LLD-ACVG (Figure 79). The volume of each flask was adjusted to 1·0ml with MOPS buffer (50mmol ,pH7·2) and dithiothreitol, to a final concentration of 2·5 mmol, and $FeSO_4$, to a final concentration of 1·0mmol, added. The mixtures were incubated at 26°C for 1½h then bioassayed against *Staph. aureus* (Figure 79).

Graphical interpretation of this data (Figure 80) supports the contention that LLD-ACVG is inhibiting the biosynthesis of isopenicillin N. It is not possible to deduce from this graph whether the inhibition is competitive or non-competitive, though the close structural similarity of LLD-ACVG and LLD-ACV favours competitive inhibition. Further kinetic studies were not undertaken as the crude nature of the cell-free system and the limitations of the bioassay method allow only gross measurements to be made. Nevertheless,

Conc. of LLD-ACVG (μg/ml)	Conc. of Isopenicillin N* (μg/ml)
0	89
100	65
150	53
200	51
250	40
350	40

* The concentration of biosynthesised isopenicillin N was determined from the linear ln dose - response curve from several cephalosporin C standards. However, the inherent assumptions[352] in the calculations mean that only the relative values and not the absolute values can be considered accurate.

F I G U R E 79



F I G U R E 80

the results suggest that LLD-ACVG is a poor inhibitor. Concentrations of LLD-ACVG double the original concentration of LLD-ACV were required to reduce isopenicillin N production by 50%.

The stereochemistry of the ACVG tetrapeptide isolated from *C. acremonium* is not known [329] but synthetic LLD-ACyaVG (147) has been reported [302] to behave slightly differently on paper electrophoresis to the sulphonic acid derivative of the isolated ACVG tetrapeptide. It is possible [302] that the isolated ACVG tetrapeptide is a diastereoisomer of LLD-ACVG. However, it has never been rigorously proven that the isolated tetrapeptide is δ-(α-aminoadipyl)-cysteinylvalylglycine[329]. A peptide with the same constituent amino acids but different primary structure, glycyl-δ-(α-aminoadipyl)-cysteinyl-valine (GACV), has been isolated from the mycelium of the β-lactam producing *Paecilomyces persicinus* P-10 [331]. The role of this tetrapeptide in penicillin biosynthesis has not been investigated.

It is quite possible that the ACVG and GACV peptides are simple shunt products and not relevant to the biosynthesis of the penicillins and cephalosporins.

5.2.2 *N*-[δ-(L-α-Aminoadipyl)-L-Cysteinyl]-*N*-Hydroxy-D-Valine

N-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (81) has been proposed [378] as an intermediate in the biosynthesis of isopenicillin N from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. It was of interest, therefore, to test this hypothesis by feeding the synthetic hydroxamic acid (140) to the active cell-free system obtained from *C. acremonium* CW19. In the presence of DTT and $FeSO_4$ no detectable quantities of antibiotic were produced (as observed from *Staph. aureus* bioassay). In the control incubation LLD-ACV was converted

to isopenicillin N though the apparent conversion yield in this experiment was only 20%.

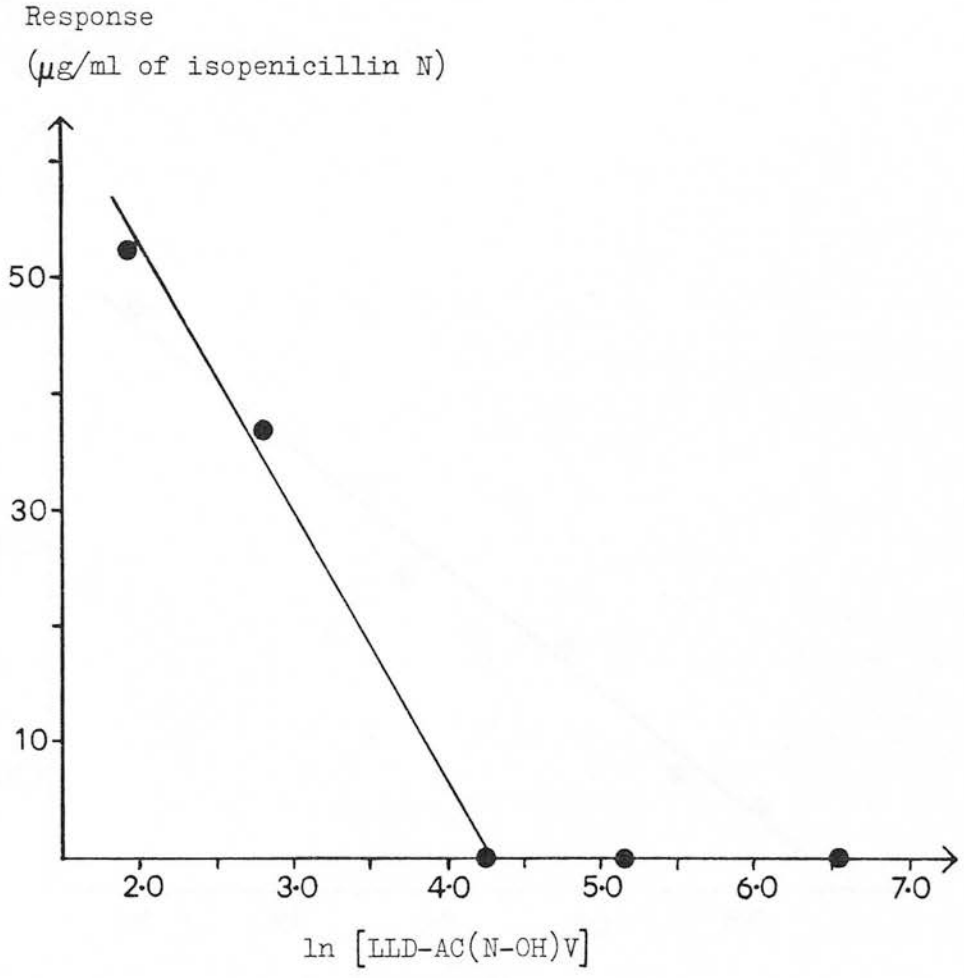
N-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine was however a powerful inhibitor of penicillin synthesis. Various quantities of LLD-AC(N-OH)V (140) were added to incubation mixtures containing LLD-ACV (at a final concentration of 350μg/ml), DTT and $FeSO_4$. The flasks were aerated on a rotary shaker at 26°C for 1h then bioassayed against *Staph. aureus* (Figure 81).

As the control conversion of LLD-ACV to isopenicillin N was disappointingly low the quantitative data may be suspect. However, it is apparent that *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (81) is a potent inhibitor of this conversion. Concentrations of the hydroxamic acid as low as 70μg/ml (0·18mmol) completely inhibited "synthetase" activity and, calculated from Figure 81, a hydroxamic acid concentration of 17μg/ml (0·04mmol) resulted in a 50% inhibition of penicillin synthesis. To check that the inhibition is a property of the tripeptide derivative (81) and not of any hydroxamic acid, an analogous set of experiments were performed in the presence of various concentrations of acethydroxamic acid (Figure 82). Incubation mixtures contained LLD-ACV (1mg/ml)(75μl), cell-free preparation (300μl), DTT (0·1M solution)(20μl), $FeSO_4$ (0·1M solution)(10μl), acethydroxamic acid and MOPS buffer (50mmol, pH7·2) to a final volume of 480μl. Each flask was shaken on a rotary shaker at 150 r.p.m. at 26°C for 1h and the incubation mixtures bioassayed against *Staph. aureus* (Figure 82).

Acethydroxamic acid proved inhibitory, but much higher concentrations of this simple hydroxamic acid were required to reduce

Conc. of LLD-AC(N-OH)V (μg/ml)	Conc. of Isopenicillin N* (μg/ml)
0	67
7	52
17	37
69	0
173	0
692	0

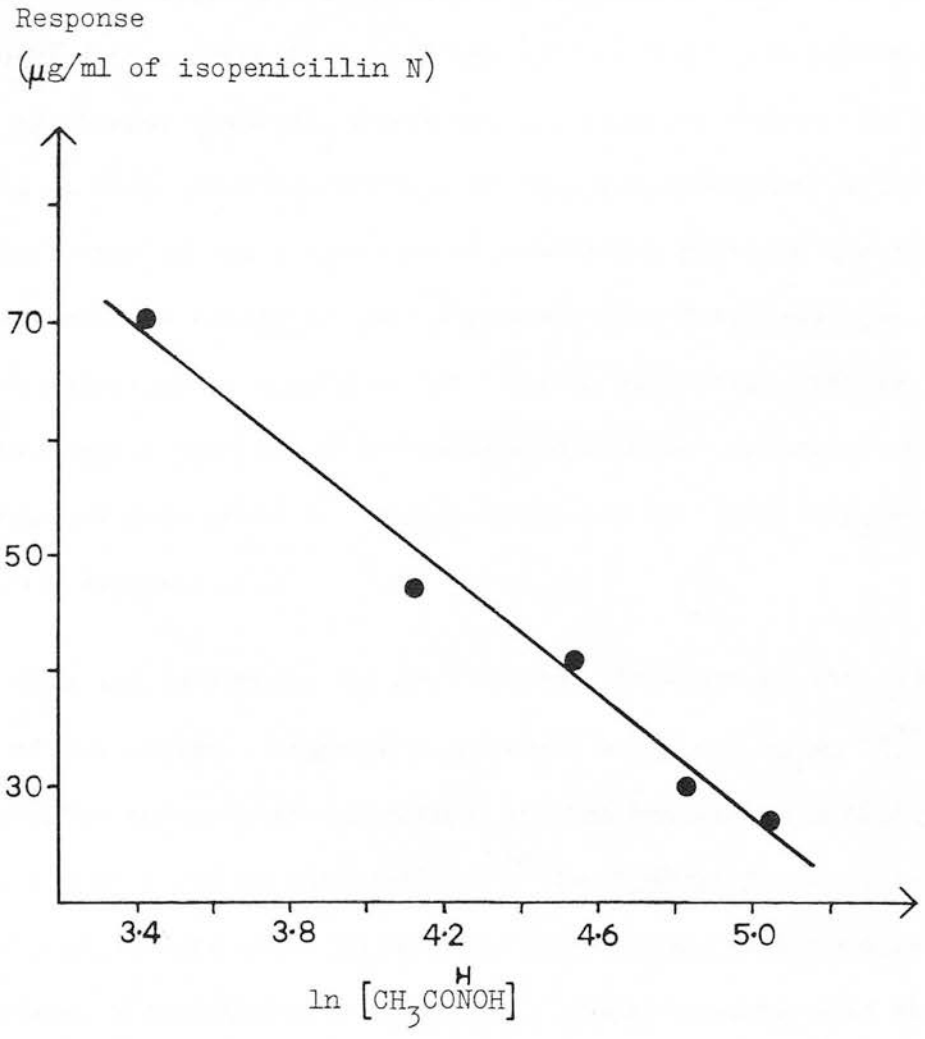
* See note at foot of Figure 79.



F I G U R E 81

Conc. of $CH_3\overset{H}{C}ONOH$ ($\mu g/ml$)	Conc. of Isopenicillin N* ($\mu g/ml$)
0	96
31	70
62	47
94	41
125	30
156	27

* See note at foot of Figure 79.



F I G U R E 82

isopenicillin N production. Production was halved at a concentration of 67μg/ml (0·91mmol); a concentration some twenty times greater than that required of *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (81) to elicit a similar reduction.

Acethydroxamic acid appears to inhibit isopenicillin N synthetase, and may do so by chelation to a metal at the active site of the enzyme. Influence at the active site, rather than elsewhere on the synthetase enzyme (i.e. competitive rather than non-competitive inhibition), can be inferred from the feeding studies with LLD-AC(N-OH)V (81). The hydroxamate derivative of LLD-ACV, due to its structural similarity with Arnstein's tripeptide (34), may be expected to bind more strongly to the active site than acethydroxamic acid and, as already observed, LLD-AC(N-OH)V inhibits isopenicillin N synthesis at much lower concentrations than acethydroxamic acid. If the influence on the enzyme was at some other point on the enzyme surface and due solely to the hydroxamic acid functionality, or due to the reduction of available Fe^{2+} in the incubation mixture, both acethydroxamic acid and *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine would be equally effective in inhibiting isopenicillin N synthesis.

This inhibition may be due to metal chelation at the active site of the enzyme. Peptide hydroxamic acids are known [524-527] to be powerful active site inhibitors of zinc containing metallopeptidases, and in a recent publication [528] the hydroxamic derivatives of L-amino-acids were shown to be potent inhibitors of *Aeromonas* aminopeptidase, a zinc-containing enzyme. Acethydroxamic acid also inhibited this enzyme but was a weaker inhibitor than the amino-acid

derivatives.

There is no evidence that Zn^{2+} is involved in penicillin biosynthesis, and indeed zinc ions inhibit isopenicillin N synthetase [350] (though zinc ions also inhibit *Pseudomonas aeruginosa* elastase, an enzyme which contains zinc at its active site [529]). Ferrous ions, however, are required by the cell-free system and may play a vital role at the active site of the synthetase enzyme. The assumption that the hydroxamic acid chelates enzyme bound ferrous ions is speculative and the mechanism by which *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine inhibits penicillin biosynthesis is not known.

5.2.3 δ-(L-α-Aminoadipyl)-L-[3-^{13}C]-Cysteinyl-D-[^{15}N]-Valine

The double labelled tripeptide, δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine (97), was prepared in order to study the synthesis of isopenicillin N by *in vivo* ^{13}C n.m.r. spectroscopy [530]. A number of experiments to test the feasibility of this approach concluded that no isopenicillin N was produced when LLD-ACV was incubated with the cell-free system in a 10mm n.m.r. tube. There is an absolute requirement for molecular oxygen [445] by the synthetase enzyme (Section 3.3.1), and poor oxygen diffusion in the n.m.r. tube was probably the reason no antibiotic production was observed. A stream of oxygen playing on the surface of the incubation mixture in a 10mm n.m.r. tube did not improve the situation, and ultimately the labelled tripeptide (82) had to be incubated with the cell-free system in the normal manner. The incubation mixture (labelled tripeptide (97) (5mg), DTT(1·6mg), $FeSO_4$(0·1M solution)(50μl) and cell-free extract (5ml)) was aerated on a rotary shaker at 26°C and aliquots removed at various times. The protein was precipitated by addition of

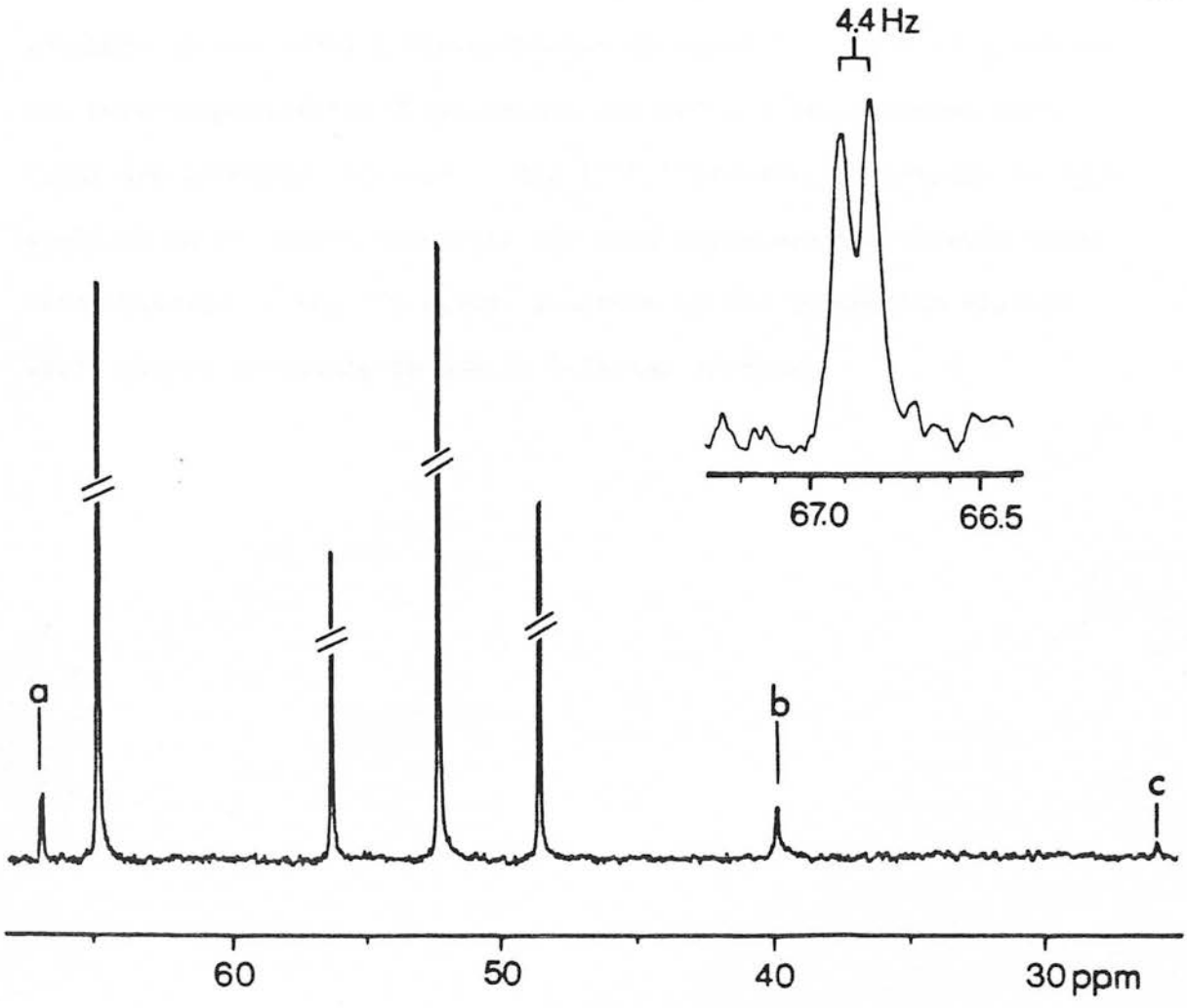
acetone and the ^{13}C n.m.r. spectrum of each de-proteinated aliquot recorded. Further purification of the δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine disulphide (97) improved the quality of the final spectrum [390] (Figure 83).

As expected the C5 of the biosynthesised isopenicillin N was observed as a doublet centred at 66•9 p.p.m. with a one bond ^{13}C-^{15}N coupling of 4•4 Hz. The chemical shift of the C5 carbon was very close to the value reported by Baldwin [351], but the observed $^{1}J_{CN}$ was smaller than the one bond N4-C5 coupling in the ^{13}C n.m.r. spectrum of ^{15}N enriched penicillin G [336].

Although aliquots of the incubation mixture were removed at short time intervals and the ^{13}C n.m.r. spectra of the deproteinated solutions recorded immediately, no new signals from potential monocyclic β-lactam intermediates were observed. It is possible that any intermediates are enzyme-bound and/or present in only low steady state concentration. In either case no ^{13}C n.m.r. signal would be observed [447].

The direct *in vivo* ^{13}C n.m.r. observation of the cell-free conversion of δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[3-^{13}C]-valine to [2,5-$^{13}C_2$]-isopenicillin N has been reported [351]. The incubation was performed in a 25mm n.m.r. tube using a cell-free extract of greater activity than available in this laboratory. There was no mention in the communication of any oxygen diffusion problems and no new ^{13}C resonances, assignable to biosynthetic intermediates, were observed.

The use of *in vivo* ^{13}C n.m.r. spectroscopy [530] may yet prove



50MHz broad-band proton decoupled spectrum of deproteinated incubation mixture (sweep width 6880Hz, number of scans 604). a: C5 of [5-^{13}C, 4-^{15}N]-isopenicillin N, b: cysteine C3 of [^{13}C, ^{15}N]-tripeptide dimer(97), c: cysteine C3 of [^{13}C, ^{15}N]-tripeptide monomer(82). No line broadening factor was applied during transformation of the FID.

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valuable in elucidating the mechanism of isopenicillin N biosynthesis but pure isopenicillin N synthetase and refined experimental conditions are probably required. The [^{13}C,^{15}N]-labelled tripeptide (82) would be an excellent substrate for such experiments as carbon doublets observed in the ^{13}C n.m.r. spectrum of the incubation mixture would almost certainly be due to β-lactam species.

E X P E R I M E N T A L

6. EXPERIMENTAL

6.1 MATERIALS AND METHODS

Electrophoresis was carried out at 80V/cm on Whatman 3MM paper using the apparatus described by Michl[531], pH 2.1, 3.5 and 6.5 buffers were prepared as described by Ambler[532]. Merck silica 60 (70-230 mesh) was used for column chromatography and thin layer chromatography (t.l.c.) was carried out on 200 x 200 x 1mm or 200 x 200 x 0.25mm layers of Merck $60F_{254}$ silica. Components were visualized by ultraviolet (UV) light, by exposure to iodine vapours or by colour reaction with ninhydrin, ferric chloride or 2,6-dichlorophenol indophenol sodium salt. High performance liquid chromatography (h.p.l.c.) was carried out using Waters or Gibson systems and prepacked Waters μ-Bondapak-C18 and Waters μ-Bondapak-NH_2 analytical columns. Samples were detected by their UV absorptions at 214, 254 or 313nm. Bio-Rad AG 50WX2 resin (200-400 mesh, H^+ form) was used for ion exchange chromatography.

Reagents were purchased from Fisons, Sigma, Aldrich, Fluka and Eastman Kodak and were routinely recrystallized or redistilled before use. [^{13}C]-paraformaldehyde (90 atom% ^{13}C) was obtained from Merck, Sharp and Dohme and DL-[^{15}N]-valine (95 atom% ^{15}N) from the Commissariat a L'Energie Atomique (CEA). Diphenyldiazomethane was made by an adaption to the literature procedure[533]. Typically, benzophenone hydrazone (1.96g, 10.0mmol), anhyd. Na_2SO_4 (2.84g, 20.0mmol), Yellow HgO (4.33g, 20.0mmol) and KOH sat. EtOH (1ml) were added to a 100ml pressure bottle containing dry *n*-hexane (30ml). The flask was sealed, shaken vigorously for 5h and the solution filtered.

Evaporation of the filtrate afforded a dark red crystalline solid, diphenyldiazomethane (1.94g, 100%). Solvents were dried and redistilled by standard procedures and organic extracts dried over $MgSO_4$, K_2CO_3 or Na_2SO_4.

Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Optical rotations were obtained using a Perkin-Elmer 141 Automatic Polarimeter and elemental analyses on a Perkin-Elmer 240 Elemental Analyser. UV/visible spectra were recorded on a Varian DMS90 spectrophotometer and infra-red (i.r.) spectra on Perkin-Elmer 298 and Perkin-Elmer 781 spectrophotometers. Unless otherwise stated, liquid samples were analysed as neat films and solid samples as solutions in CCl_4 or $CHCl_3$. The i.r. spectra were calibrated with the $1603cm^{-1}$ absorption of polystyrene. Nuclear magnetic resonance (n.m.r.) spectra were recorded on Varian EM360, Varian XL100, Varian CFT20, Bruker WP200SY, Bruker WM300WB and Bruker WH360 spectrometers. Spectra were measured on the δ scale with internal or external tetramethylsilane at 0.00δ, $CHCl_3$ at 7.25δ or $^{13}CDCl_3$ (centre peak) at 77.00δ. Mass spectra and exact mass measurements were recorded on an AEI MS902 spectrometer (electron impact (EI) at 70 eV) or a Kratos MS80RF-DS55 spectrometer using a fast atom bombardment (FAB) source.

Antibiotic titres were measured using a "hole" plate bioassay with *Bacillus subtilus* or *Staphylococcus aureus* as test organisms. The *B. subtilus* system was more sensitive to low concentrations of antibiotic but in practice proved less reliable for quantification of antibiotic levels. The *Staph. aureus* system was used to obtain quantitative data (limit of detection *ca* 10 units/ml of isopenicillin N).

Isopenicillin N was assumed [352] to have an activity of 80 units/mg and cephalosporin C an activity of 10 units/mg against *Staph. aureus*. Typically aliquots (100µl) of test solutions and cephalosporin C* or penicillin G standard solutions (100µl) were pipetted into the hole of the "hole" plate bioassay. The plates were allowed to stand at room temperature for 30min then incubated at 37°C for 16h. Clear inhibition zones were measured and test results determined from the linear ln dose - response graphs obtained from the standard solutions. Results were normally expressed as mg/ml cephalosporin C equivalents or as µg/ml of isopenicillin N. A 1 mg/ml solution of cephalosporin C* was assumed [352] to equal 90 µg/ml of isopenicillin N.

The Dyno-Mill used in the preparation of the cell-free systems was supplied by Willy A. Bachofen AG Maschinenfabrik, Basle.

6.2 AMINO-ACIDS AND AMINO-ACID PRECURSORS

Diethyl Acetamidodimethylaminomethylmalonate

Paraformaldehyde (1.00g, 33.3mmol), dimethylamine hydrochloride (2.84g, 35.0mmol) and diethyl acetamidomalonate (7.59g, 35.0mmol) were suspended in H_2O (10ml) and 3 drops of 1M-aq. HCl added. The suspension was heated to 100°C in a sealed flask until a clear solution was obtained and then for a further 30min. When cooled the solution was saturated with NaCl and adjusted to pH12 with 20%-aq. NaOH. The aqueous solution was extracted with ether (3x30ml), the extracts dried over Na_2SO_4 and evaporated to leave crude diethyl acetamidodimethylaminomethylmalonate which was crystallized from

*The cephalosporin C was 72% pure as judged by h.p.l.c. using the system of Miller and Neuss [534].

ether/*n*-hexane (6.18g,67%), m.p. 62-66°C; δ_H (60MHz,$CDCl_3$) 1.30 (6H,t,*J*7.0Hz,2xCH_2CH_3), 2.08 (3H,s,$COCH_3$), 2.28 (6H,s,$(CH_3)_2N$), 3.30 (2H,s,NCH_2C), 4.29 (4H,q,*J*7.0Hz,2xCH_2CH_3) and 7.12br (1H,s,NH).

Diethyl Acetamidodimethylamino-[^{13}C]-methylmalonate

The above procedure was repeated using [^{13}C]-paraformaldehyde (1.00g,33.3mmol) to give crude diethyl acetamidodimethylamino-[^{13}C]-methylmalonate (2.21g,25%), δ_H (60MHz,$CDCl_3$) 1.30 (6H,t,*J*7.0Hz, 2xCH_2CH_3), 2.08 (3H,s,$COCH_3$), 2.30 (6H,d,*J*5.0Hz,$(CH_3)_2N^{13}C$), 3.30 (2H,d,*J*131.0Hz,$N^{13}CH_2C$), 4.29 (4H,q,*J*7.0Hz,2xCH_2CH_3) and 7.03br (1H,s,NH). The material was used without further purification.

Diethyl Acetamidodimethylaminomethylmalonate Methyl Iodide

Diethyl acetamidodimethylaminomethylmalonate (6.18g,22.6mmol) was dissolved in dry ether (40ml) and iodomethane (14.00ml,150.9mmol) added. The solution was stirred at 40°C for 24h then chilled to 5°C for 12h to precipitate the crude product as a colourless solid (8.54g,91%), m.p. 160-170°C. Crystallization from MeOH/ether afforded diethyl acetamidodimethylaminomethylmalonate methyl iodide (8.18g,59% from paraformaldehyde), m.p. 171-173°C (lit.[480], m.p. 174-175°C; lit.[535], m.p. 171-173°C); δ_H (60MHz,D_2O) 1.38 (3H,t, *J*7.0Hz,CH_2CH_3), 1.40 (3H,t,*J*7.0Hz,CH_2CH_3), 2.27 (3H,s,$COCH_3$), 3.33 (9H,s,$(CH_3)_3N^+$), 4.46 (2H,q,*J*7.0Hz,CH_2CH_3), 4.48 (2H,q,*J*7.0Hz,CH_2CH_3) and 4.47 (2H,s,$^+NCH_2C$).

Diethyl Acetamidodimethylamino -[^{13}C]-methylmalonate Methyl Iodide

Repeating the above procedure using diethyl acetamidodimethylamino-[^{13}C]-methylmalonate (2.21g,8.0mmol) and iodomethane (5.00ml, 53.9mmol) afforded crude diethyl acetamidodimethylamino-[^{13}C]-methylmalonate methyl iodide (2.77g, 21% from [^{13}C]-paraformaldehyde), m.p.

160-170°C.

S-Benzyl-DL-Cysteine

Sodium ethoxide (1.22g,17.9mmol) was dissolved in dry ethanol (20ml) and benzyl mercaptan (1.48ml,12.6mmol) added. The solution was left for 5 min and diethyl acetamidodimethylaminomethylmalonate methyl iodide (4.99g,12.0mmol) added. The solution was refluxed under a N_2 atmosphere for 10 days, evaporated to dryness and the residue dissolved in $CHCl_3$ (20ml). The organic layer was washed with H_2O (10ml), sat. aq. NaCl (10ml) and dried over K_2CO_3. Evaporation left a yellow oil which was mixed with conc. aq. HCl (40ml) and refluxed for 5h. The solution was filtered through celite and evaporated to an off-white solid which was precipitated by dissolving in hot H_2O (20ml), adjusting to pH 5.4 with dil. aq. NH_3 and adding EtOH (40ml). The precipitate was washed with EtOH and dried to leave *S*-benzyl-DL-cysteine (1.72g,68%), m.p. 188-190°C (lit.[480], m.p. 209-211°C; lit.[536], m.p.215-216°C); δ_H (60MHz,TFA) 2.78br (2H,m, *cys*3H), 3.42 (2H,s,SC*H*$_2$Ph), 3.70br (1H,m,*cys*2H) and 6.90 (5H,s,Ar*H*). A small sample was crystallized from boiling H_2O, m.p. 207-209°C.

S-Benzyl-DL-[3-^{13}C]-Cysteine

A repeat experiment using NaOEt (0.66g,9.7mmol), benzyl mercaptan (0.80ml,6.8mmol) and diethyl acetamidodimethylamino-[^{13}C]-methylmalonate (2.71g,6.5mmol) in EtOH (10ml) gave *S*-benzyl-DL-[3-^{13}C]-cysteine which was recrystallized from boiling H_2O (0.91g,66%), m.p. 205°C.

Attempted Synthesis of *N*-Acetyl-*S*-Benzyl-DL-Cysteine

S-benzyl-L-cysteine (0.85g,4.0mmol) and Ac_2O (1.23ml,13.0mmol) were added to AcOH (10ml) and the mixture warmed at 60°C until a

clear solution was obtained. The solution was stirred a further 15 min at 60°C, cooled to room temperature and evaporated to a clear oil which was vigorously shaken with H_2O (20ml) for 30 min. Ethanol was added to give a clear solution from which a colourless solid was obtained by evaporation. The solid was dissolved in dry acetone, filtered through celite and crystallized from acetone/ether/*n*-hexane to afford *N*-acetyl-*S*-benzyl-L-cysteine (0.94g,92%). A small quantity of material was recrystallized from acetone/ether/*n*-hexane, m.p. 141-144°C (lit.[537], m.p. 143.5-145.5°C); $[\alpha]_D$-43.0° (c1.0,EtOH) [lit.[537],$[\alpha]_D$-46.1°(c1.0,95%EtOH) ; δ_H(60MHz,CD_3COCD_3) 1.58 (3H,s, CH_3CO), 2.42 (2H,AB of ABX,δ_A2.47,δ_B2.37,*J*3.5,1.5Hz,*cys*3H), 3.37 (2H,s,SCH_2Ph), 4.25 (1H,m,*cys*2H) and 6.92 (5H,s,Ar*H*).

N-Acetyl-*S*-Benzyl-DL-Cysteine

In an identical experiment to above, refluxing the AcOH/Ac_2O solution for 5 min and repeating the isolation procedure gave *N*-acetyl-*S*-benzyl-DL-cysteine (0.70g,69%), m.p. 155-157°C (lit.[477], m.p. 157°C); δ_H (60MHz,CD_3OD) 1.67 (3H,s,CH_3CO), 2.48 (2H,AB of ABX, δ_A2.53,δ_B2.42,*J*4.0,2.0Hz,*cys*3H), 3.43 (2H,s,SCH_2Ph), 4.27 (1H,m,*cys* 2H) and 6.98 (5H,s,Ar*H*).

N-Acetyl-*S*-Benzyl-DL-[3-^{13}C]-Cysteine

S-benzyl-DL-[3-^{13}C]-cysteine (0.85g,4.0mmol) and Ac_2O (1.23ml, 13.0mmol) in AcOH (10ml) were stirred at 60°C, as described above, and worked up by the same procedure to give *N*-acetyl-*S*-benzyl-DL-[3-^{13}C]-cysteine (0.92g,90%), m.p. 158-160°C; δ_H (60MHz,CD_3OD) 1.67 (3H,s,CH_3CO), 2.48 (2H, AB of ABMX, δ_A 2.53,δ_B2.42, *J*142.0, 4.0,2.0Hz,*cys*3H), 3.43 (2H,d,*J*4.0Hz,SCH_2Ph), 4.27 (1H,m,*cys*2H) and 6.98 (5H,s,Ar*H*).

S-Benzyl-L-Cysteine

N-acetyl-*S*-benzyl-DL-cysteine (1.01g,4.0mmol) was dissolved in H_2O (100ml), adjusted to pH 7.5 with 1M-aq. NH_3 and hog kidney acylase I (0.10g,1845 units/mg) added. The mixture was rocked gently at 37°C for 24h, the pH re-adjusted to 7.5 and hog kidney acylase I (0.08g,1845 units/mg) added. After a further 24h at 37°C the solution was heated to boiling for 5 min, cooled, and the denatured protein removed by filtration through celite. The filtrate was added to an ion-exchange column (3x20cm) and eluted with H_2O (600ml) followed by 1M-aq. NH_3 (400ml). The basic eluate was evaporated to give *S*-benzyl-L-cysteine (0.40g,95%) which was crystallized from H_2O/EtOH (0.15g,35%), $[\alpha]_D$-15.5° (*c*1.0,5N-aq.HCl) [lit.[477],$[\alpha]_D$-19.5° (5N-aq.HCl)]; $[\alpha]_D$+24.3 (*c*1.0,1N-aq.NaOH) [lit.[477],$[\alpha]_D$+25.5 (*c*1.0,1N-aq.NaOH)].

S-Benzyl-L-[3-^{13}C]-Cysteine

N-acetyl-*S*-benzyl-DL-[3-^{13}C]-cysteine (0.89g,3.5mmol) was treated in an analagous manner to yield *S*-benzyl-L-[3-^{13}C]-cysteine (0.31g,84%) which was crystallized from H_2O/EtOH (0.15g,40%), $[\alpha]_D$-16.7° (*c*1.0,5N-aq.HCl); δ_H (300MHz,D_2O/NaOD) 2.08 (2H,AB of ABMX, δ_A2.12,δ_B2.04,*J*140.7,140.3,13.5,5.3,6.7Hz,*cys*3H), 2.72 (1H,ddd,*J* 5.3,6.7,4.8Hz,*cys*2H), 3.12 (2H,d,*J*3.8Hz,SC*H*$_2$Ph) and 6.65-6.75 (5H, m,Ar*H*); δ_C (75MHz,D_2O/NaOD) 36.73 (t,*cys*C3).

N-Acetyl-D-Valine

DL-valine (1.00g,8.5mmol) was dissolved in AcOH(40ml), Ac_2O (20ml) added and the solution refluxed for 30 min. Water (300ml) was added and the solution evaporated to give a colourless solid which was suspended in H_2O (60ml). The pH was adjusted to 7.3

with 1M-aq. NH_3, hog kidney acylase I (30mg, 1845 units/mg) added, and the mixture gently rocked at 37°C for 2 days. The pH was re-adjusted to 7.3, a further aliquot of hog kidney acylase (20mg, 1845 units/mg) added, and rocking continued for 24h. The solution was boiled for 5 min, the denatured protein removed by filtration and the filtrate applied to an ion-exchange column (3x20cm). Elution with H_2O (700ml) gave a crude product which was crystallized from acetone/ether to afford *N*-acetyl-D-valine (0.41g,60%), m.p. 168-170°C (lit.[477], m.p. 168°C); $[\alpha]_D$+19.2° (c0.8,H_2O)[lit.[538],$[\alpha]_D^{25}$+20.0° (H_2O)]; δ_H (60MHz,D_2O) 0.82 (6H,d,*J*6.5Hz,*val*4,4'H), 1.83-2.30 (1H, m,*val*3H), 1.90 (3H,s,CH_3CO) and 4.07 (1H,d,*J*6.0Hz,*val*2H).

N-Acetyl-D-[^{15}N]-Valine

DL-[^{15}N]-valine (1.00g,8.5mmol) was treated in a similar manner to give *N*-acetyl-D-[^{15}N]-valine (0.46g,68%), m.p. 170-171°C; $[\alpha]_D$+ 18.8° (c0.3 H_2O); δ_H (60MHz,D_2O) 0.82 (6H,d,*J*6.5Hz,*val*4,4'H), 1.78-2.20 (1H,m,*val*3H), 1.90 (3H,d,*J*1.5Hz,$CH_3CO^{15}N$) and 4.03 (1H,dd, *J*6.0,1.5Hz,*val*2H).

D-Valine

N-acetyl-D-valine (0.32g,2.0mmol) was refluxed in conc.aq. HCl (10ml) for 2h and evaporated to dryness. The residue was dissolved in H_2O (0.8ml) and EtOH (3.2ml) and the pH adjusted to 4.5 with aniline. Chilling the solution to 5°C for 5h yielded a colourless solid, D-valine (0.20g,85%), δ_H (60MHz,D_2O) 1.02 (3H,d,*J*7.0Hz,*val*4H), 1.06 (3H,d,*J*7.0Hz,*val*4H), 2.00-2.53 (1H,m,*val*3H) and 3.59 (1H,d, *J*4.5Hz,*val*2H).

D-[^{15}N]-Valine

N-acetyl-D-[^{15}N]-valine (0.40g,2.5mmol) was refluxed in conc.aq. HCl (13ml) and worked up as above to yield D-[^{15}N]-valine (0.26g,88%), $[\alpha]_D$-30.3° ($_c$1.0,6N-aq.HCl) [lit.[538],$[\alpha]_D$-29.4° (6N-aq.HCl)]; δ_H (360MHz,D_2O/DCl) 0.34 (3H,d,*J*7.0Hz,*val*4H), 0.38 (3H,d,*J*7.0Hz,*val*4H). 1.65 (1H,ddsept.,*J*7.0,4.4,3.3Hz,*val*3H) and 3.14 (1H,d,*J*4.4Hz,*val*2H); δ_C (25MHz,D_2O/DCl) 18.18 (q,*val*4C), 18.94 (q,*val*4C), 30.35 (d,*val*3C), 60.44 (dd,*J*6.0Hz,*val*2C) and 173.85 (s,*val*1C).

S-Benzyl-L-Cysteine Methyl Ester Hydrochloride Salt

S-benzyl-L-cysteine (1.00g,4.7mmol) was dissolved in HCl saturated methanol (50ml) and refluxed for 1h. The volume of the solution was reduced to 25ml by evaporation and a colourless solid precipitated by addition of ether. Crystallization of the crude product from MeOH/ether gave *S*-benzyl-L-cysteine methyl ester hydrochloride salt (0.99g,80%), m.p. 150-152°C (lit.[539], m.p. 151-152°C); δ_H (60MHz,$CDCl_3$) 3.28br (2H,m,*cys*3H), 3.82 (2H,s,S*CH*$_2$Ph), 3.92 (3H, s,CO_2CH_3), 4.53br (1H,m,*cys*2H), 7.40 (5H,s,Ar*H*) and 9.07br (3H,s,NH_3^+).

S-Benzyl-L-Cysteine Benzhydryl Ester 4-Toluenesulphonic Acid Salt

S-benzyl-L-cysteine (0.21g,1.0mmol) and 4-toluenesulphonic acid monohydrate (0.21g,1.1mmol) were dissolved in DMF (10ml) and heated to 60°C. A solution of diphenyldiazomethane (0.29g,1.5mmol) in DMF (5ml) was added over 15 min to leave an orange solution which was evaporated to afford an off-white solid. Crystallization from acetonitrile/ether afforded *S*-benzyl-L-cysteine benzhydryl ester 4-toluenesulphonic acid salt (0.17g,31%), m.p. 156.5-158°C; $[\alpha]_D$-22.9° ($_c$7.9,MeOH) (Found:C,65.62; H,5.80; N,2.49. $C_{30}H_{31}NO_5S_2$ requires C,65.55; H,5.68; N,2.55%); δ_H (300MHz,$CDCl_3$) 2.21 (3H,s,

CH_3), 2.86 (2H,m,*cys*3H), 3.40 (2H,AB,δ_A3.47,δ_B3.34,*J*13.3Hz,SCH_2Ph), 4.36 (1H,t,*J*5.6Hz,*cys*2H), 6.77 (1H,s,$CHPh_2$), 6.88 (2H,d,*J*8.1Hz,Ar*H*), 7.04-7.23 (15H,m,Ar*H*), 7.62 (2H,d,*J*8.1Hz,Ar*H*) and 8.42br (3H,s,NH_3^+); $\nu(CDCl_3)$ 3010 (C-H), 2925 (C-H) and 1753 (C=O) cm^{-1}; m/z (EI) 376 (<1%), 210 (7%), 167 (100%) and 91 (100%).

N-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteine Dicyclohexylamine Salt

S-benzyl-L-cysteine (2.11g,10.0mmol) was suspended in H_2O (20ml), the pH adjusted to 9.0 with 2M-aq. NaOH and a solution of 4-methoxybenzyloxycarbonylazide in dioxan (20ml) added dropwise over 1h at room temperature. The solution was adjusted to pH 8.5 by addition of 2M-aq. NaOH and then stirred for 24h. The volume of the solution was reduced by evaporation and the solution washed with ether (50ml). The pH of the aqueous solution was adjusted to 1 by addition of 5M-aq. HCl and the solution extracted with EtOAc (3x50ml). The EtOAc extracts were dried over $MgSO_4$ and evaporated to give a yellow oil. Dicyclohexylamine (2.10ml,10.5mmol) was added and a colourless solid crystallized from EtOH/EtOAc, *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine dicyclohexylamine salt (3.95g,71%), m.p. 137-138°C; $[\alpha]_D$-13.3° (*c*1.0,MeOH) (Found: C,66.67; H,7.85; N,4.93. $C_{31}H_{44}N_2O_5S$ requires C,66.88; H,7.97; N,5.03%); δ_H (200MHz,$CDCl_3$) 1.07-2.03 (22H,m,*cyclohexyl*H), 2.83-3.00 (4H,m,*cys*3H and NH_2^+), 3.71 (2H,s, SCH_2Ph), 3.77 (3H,s,CH_3O), 4.24-4.27 (1H,m,*cys*2H), 5.03 (2H,s,OCH_2Ar), 5.90 (1H,d,*J*5.8Hz,N*H*), 6.85 (2H,d,*J*8.7Hz,Ar*H*) and 7.16-7.32 (7H,m,Ar*H*).

D-Valine Benzyl Ester 4-Toluenesulphonic Acid Salt

D-valine (1.17g,10.0mmol) and 4-toluenesulphonic acid monohydrate (2.09g,11.0mmol) were dissolved in a mixture of dry benzene (30ml) and benzyl alcohol (2.59ml). The solution was refluxed under an

argon atmosphere for 5 days in a Dean-Stark apparatus. Addition of ether (50ml) to the reaction mixture precipitated a colourless solid, D-valine benzyl ester 4-toluenesulphonic acid salt (3.70g,98%), m.p. 160-162°C (lit.[490],m.p. 158-160°C for L-isomer); $[\alpha]_D$+3.0° (c2.0, EtOH) [lit.[491], $[\alpha]_D$-3.5° (c2.0,EtOH) for L-isomer]; δ_H (60MHz, $CDCl_3$) 0.88 (3H,d,*J*7.0Hz,*val*4H), 0.91 (3H,d,*J*7.0Hz,*val*4H), 1.97-2.60 (1H,m,*val*3H), 2.32 (3H,s,CH_3), 3.85-4.08 (1H,m,*val*2H), 5.08 (2H,AB pattern,OCH_2Ph), 7.11 (2H,d,*J*8.0Hz,Ar*H*), 7.30 (5H,s,Ar*H*), 7.84 (2H, d,*J*8.0Hz,Ar*H*) and 8.27br (3H,s,NH_3^+).

A small sample was converted to the hydrochloride salt *via* the free amine, m.p. 141-143°C (lit.[540],m.p. 138°C for L-isomer); δ_H (200MHz,$CDCl_3$) 1.06 (3H,d,*J*5.4Hz,*val*4H), 1.09 (3H,d,*J*5.3Hz,*val*4H), 2.43 (1H,m,*val*3H), 3.94 (1H,d,*J*3.5Hz,*val*2H), 5.21 (2H,AB,δ_A5.27,δ_B 5.16,*J*12.1Hz,OCH_2Ph) and 7.30-7.40 (5H,m,Ar*H*); δ_C(20MHz,D_2O) 17.79 (q,*val*4C), 18.00 (q,*val*4C), 30.18 (d,*val*3C), 59.15 (d,*val*2C), 69.34 (t,OCH_2Ph), 129.68,129.84,135.51 (5xArC) and 170.47 (s,C=O).

N-Benzyloxycarbonyl-L-α-Aminoadipic Acid 1-(4-Nitrobenzyl) Ester

N-benzyloxycarbonyl-L-α-aminoadipic acid [466] (2.06g, 7.0mmol) was dissolved in DMF (5ml), triethylamine (1.02ml, 7.4mmol) added and the solution cooled to 0°C. 4-nitrobenzylbromide (1.59g, 7.4 mmol) was added in aliquots over 5h and the reaction mixture allowed to come to room temperature overnight. Saturated aqueous NaCl (25ml) was added, the solution adjusted to pH1 with conc.aq. HCl and extracted with EtOAc (4x30ml). The extracts were dried over $MgSO_4$, evaporated to a yellow oil and subjected to chromatography on silica (200g) with EtOAc:*n*-hexane (7:3) as eluant. The crude product was crystallized from EtOAc/*n*-hexane to yield *N*-benzyloxy-

carbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (1.22g,50%), m.p. 101-104°C; $[\alpha]_D$-8.0° (c2.0, acetone) (Found: C,58.38; H,5.08; N,6.46. $C_{21}H_{22}N_2O_8$ requires C,58.60; H,5.15; N,6.51%); δ_H(60MHz, $CDCl_3$) 1.45-2.05 (4H,m, *aminoadipyl* 3,4H), 2.37 (2H,t,*J* 5.5Hz, *aminoadipyl* 5H), 4.18-4.70 (1H,m, *aminoadipyl* 2H), 5.12 (2H,s,OCH_2Ar), 5.23 (2H,s,OCH_2Ar), 5.40 (1H,m,N*H*), 7.32 (5H,s,Ar*H*), 7.43 (2H,d, *J*9.0Hz,Ar*H*), 8.13 (2H,d,*J*9.0Hz,Ar*H*) and 8.75br (1H,s,CO_2H); m/z (EI) 430 (<1%) (M), 306 (2%), 250.1091 (19%,$C_{13}H_{16}NO_4$ requires 250.1079), 206.1168 (37%,$C_{12}H_{16}NO_2$ requires 206.1181) and 91 (100%).

N-benzyloxycarbonyl-DL-α-aminoadipic acid 1-(4-nitrobenzyl) ester was prepared in an analagous manner, m.p. 80-88°C.

6.3 δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE AND δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALYLGLYCINE

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(DL-α-Aminoadipyl)-*S*-Benzyl-L-Cysteine Methyl Ester

N-benzyloxycarbonyl-DL-α-aminoadipic acid 1-(4-nitrobenzyl) ester (100mg, 0.23mmol) was dissolved in dry benzene (10ml), $SOCl_2$ (36.3µl, 0.50mmol) added and the solution stirred for 4h. Evaporation left an oil which was dissolved in dry CH_2Cl_2 (5ml) and added dropwise to a solution of *S*-benzyl-L-cysteine methyl ester hydrochloride salt (69mg, 0.26mmol) and pyridine (41.3µl, 0.51mmol) in dry CH_2Cl_2 (5ml) at 0°C. The solution was stirred overnight at room temperature, washed with sat. aq. $NaHCO_3$ (10ml), H_2O (10ml), 1M-aq. HCl (10ml), H_2O (10ml), sat. aq. NaCl (10ml) and dried over Na_2SO_4. Evaporation to a colourless solid and crystallization from EtOAc/*n*-hexane yielded *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteine methyl ester (45mg, 30%),

m.p. 76-84°C remelting at 112-116°C; δ_H (200MHz, $CDCl_3$) 1.70-1.95 (4H,m,*aminoadipyl*3,4H), 2.16-2.27 (2H,m,*aminoadipyl*5H), 2.86 (2H, AB of ABMX pattern,*cys*3H), 3.67 (2H,s,SC*H*$_2$Ph), 3.71 (3H,s,CO_2CH_3), 4.41-4.44 (1H,m,*aminoadipyl*2H), 4.76 (1H,dt,*J*7.7,5.4Hz,*cys*2H), 5.10 (2H,s,OC*H*$_2$Ar), 5.23 (2H,s,OC*H*$_2$Ar), 5.43-5.47 (1H,m,N*H*), 6.09-6.15 (1H,m,N*H*), 7.22-7.33 (10H,m,Ar*H*), 7.48 (2H,d,*J*8.3Hz,Ar*H*) and 8.19 (2H,d,*J*8.3Hz,Ar*H*); m/z (EI) 637 (<1%) (M), 546 (7%) (M-91), 502 (7%), 208 (33%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(DL-α-Aminoadipyl)-*S*-Benzyl-L-Cysteine Benzhydryl Ester

S-benzyl-L-cysteine (0.42g, 2.0mmol) was converted to its benzhydryl ester 4-toluenesulphonic acid salt as previously described. The crude yellow oil obtained following evaporation of the DMF solvent was taken up in EtOAc (40ml), washed with sat. aq. $NaHCO_3$ (2x40ml), sat. aq. NaCl (40ml) and dried over Na_2SO_4. Evaporation left a yellow oil which was added to a solution of 1-hydroxybenzotriazole hydrate (0.28g, 2.1mmol) and *N*-benzyloxycarbonyl-DL-α-aminoadipic acid 1-(4-nitrobenzyl) ester (0.88g, 2.0mmol) in EtOAc (20ml). Dicyclohexylcarbodiimide (0.43g, 2.1mmol) was added and the mixture stirred overnight at room temperature. Oxalic acid dihydrate (13mg) was added and stirring continued a further 1h. The precipitated dicyclohexylurea was filtered and the filtrate washed with 1M-aq. HCl (30ml), H_2O (30ml), sat. aq. $NaHCO_3$ (30ml), H_2O (30ml), sat. aq. NaCl (30ml) and dried over $MgSO_4$. Evaporation afforded an oil which was chromatographed on silica (150g) with EtOAc:*n*-hexane (6:4) as eluant. Initial fractions yielded benzophenone azine, m.p. 167°C (lit.[541], m.p. 164-165°C); δ_H (60MHz,$CDCl_3$), 7.20-7.33 (20H,m,Ar*H*). Later fractions gave a semi-solid, crystallized from EtOAc/*n*-hexane

to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteine benzhydryl ester (0.99g, 63%), m.p. 73-77°C (Found: C,66.73; H,5.59; N,5.25. $C_{44}H_{43}N_3O_9S$ requires C,66.90; H,5.49; N,5.32%); δ_H (60MHz,$CDCl_3$) 1.65-2.03 (4H,m,*aminoadipyl* 3, 4H), 2.13-2.37 (2H,m,*aminoadipyl* 5H), 2.82-2.98 (2H,m,*cys* 3H), 3.63 (2H,s,SC*H*$_2$Ph), 4.30-4.56 (1H,m,*aminoadipyl* 2H), 4.86-5.03 (1H,m,*cys* 2H), 5.15 (2H,s,OC*H*$_2$Ar), 5.27 (2H,s,OC*H*$_2$Ar), 5.55 (1H,d,*J*7.5Hz,N*H*), 6.26 (1H,d,*J*8.0Hz,N*H*), 6.95 (1H,s,C*H*Ph$_2$), 7.32-7.58 (22H,m,Ar*H*) and 8.24 (2H,d,*J*8.5Hz,Ar*H*); ν(CH_2Cl_2) 3425 (N-H), 3035 (C-H), 2940 (C-H), 1730 (C=O) and 1675 (C=O)cm^{-1}; m/z (EI) 698 (<1%) (M-91), 622 (4%) (M-167), 578 (2%), 167 (100%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteine Benzhydryl Ester

S-benzyl-L-cysteine (0.42g, 2.0mmol) was converted to its benzhydral ester and coupled with *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (0.88g,2.0mmol), as described above for the DL-mixture, to give *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine benzhydryl ester (1.01g, 64%), m.p. 122-127°C; $[\alpha]_D$-5.6° (c1.0,CH_2Cl_2) (Found: C,66.90; H,5.58; N,5.10. $C_{44}H_{43}N_3O_9S$ requires C,66.90; H,5.49; N,5.32%); δ_H (360MHz,$CDCl_3$) 1.68-1.90 (4H,m,*aminoadipyl* 3,4H), 2.15-2.26 (2H, m,*aminoadipyl* 5H), 2.85 (2H,AB of ABX,δ_A2.91,δ_B2.79,*J*13.9,4.7,6.4 Hz,*cys* 3H), 3.58 (2H,AB,δ_A3.62,δ_B3.54,*J*13.4Hz,SC*H*$_2$Ph), 4.41 (1H,m, *aminoadipyl* 2H), 4.90 (1H,M of ABMX,*J*4.7,6.4,7.9Hz,*cys* 2H), 5.09 (2H,AB,δ_A5.12,δ_B5.06,*J*12.2Hz,OC*H*$_2$Ar), 5.21 (2H,AB,δ_A5.24,δ_B5.19,*J* 13.6Hz,OC*H*$_2$Ar), 5.47 (1H,d,*J*7.9Hz,N*H*), 6.19 (1H,d,*J*7.8Hz,N*H*), 6.86 (1H,s,C*H*Ph$_2$), 7.18-7.41 (20H,m,Ar*H*), 7.46 (2H,d,*J*8.5Hz,Ar*H*) and 8.17 (2H,d,*J*8.5Hz,Ar*H*); m/z (EI) 698 (<1%) (M-91), 622 (<1%)

(M-167), 578 (<1%), 167 (100%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-[3-^{13}C]-Cysteine Benzhydryl Ester

S-benzyl-L-[3-^{13}C]-cysteine (0.15g, 0.71mmol) was converted to its benzhydryl ester and coupled with *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (0.36g, 0.84mmol), as previously described, to give *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteine benzhydryl ester (0.36g, 64%), m.p. 120-122°C; δ_H (300MHz,$CDCl_3$) 1.69-1.92 (4H,m,*aminoadipyl* 3,4H), 2.16-2.26 (2H,m,*aminoadipyl* 5H), 2.85 (2H,AB of ABMX,δ_A 2.91, δ_B 2.79,*J*143.0,140.8,13.8,6.4,4.6Hz,*cys* 3H), 3.57 (2H,AB of ABX,δ_A 3.62,δ_B 3.54,*J*13.4,4.1,3.7Hz,SC*H*$_2$Ph), 4.41 (1H,m,*aminoadipyl* 2H), 4.85-4.89 (1H,m,*cys* 2H), 5.09 (2H,AB,δ_A 5.12,δ_B 5.06,*J*12.2Hz,OC*H*$_2$Ar), 5.21 (2H,AB,δ_A 5.24,δ_B 5.19,*J*13.4Hz,OC*H*$_2$Ar), 5.48 (1H,d,*J*8.1Hz,N*H*), 6.20 (1H,d,*J*7.6Hz,N*H*), 6.85 (1H,s,C*H*Ph$_2$), 7.17-7.36 (20H,m,Ar*H*), 7.46 (2H,d,*J*8.5Hz,Ar*H*) and 8.17 (2H,d,*J*8.5Hz,Ar*H*); m/z (EI) 699 (<1%) (M-91), 623 (6%) (M-167), 579 (4%), 167 (100%) and 91 (100%).

N-Benzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl-D-Valine Benzyl Ester

D-valine benzyl ester 4-toluenesulphonic acid salt (0.42g, 1.1mmol) was converted to the free base by suspension in EtOAc (20ml) and extraction with sat. aq. $NaHCO_3$ (20ml). The D-valine benzyl ester so obtained, *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine (0.35g, 1.0mmol) and 1-hydroxybenzotriazole (0.15g,1.1mmol) were dissolved in EtOAc (15ml). Dicyclohexylcarbodiimide (0.22g, 1.1mmol) was added in one portion and the solution stirred at room temperature overnight. Oxalic acid dihydrate (25mg) was added, the mixture stirred a further 1h and filtered. The filtrate was washed with 1M-aq. HCl (10ml), sat. aq. $NaHCO_3$ (10ml), sat. aq. NaCl (10ml) and dried over $MgSO_4$. Evaporation afforded a colourless

solid which was chromatographed on silica (30g) with an EtOAc:*n*-hexane (5:5) – EtOAc:*n*-hexane (7:3) gradient as eluant. The crude product was crystallized from EtOAc/*n*-hexane to yield *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (0.47g, 87%), m.p. 101-102°C (lit.[451], m.p. 99.5-101°C); $[\alpha]_D$-15.1° (*c*1.0, acetone) [lit.[451], $[\alpha]_D$-15.1° (*c*1.0, acetone)] (Found: C,67.14; H,6.24; N,5.14. $C_{30}H_{34}N_2O_5S$ requires C,67.39; H,6.41; N,5.24%); δ_H(200 MHz,$CDCl_3$) 0.82 (3H,d,*J*6.9Hz,*val*4H), 0.88 (3H,d,*J*6.9Hz,*val*4H), 2.15 (1H,dsept.,*J*6.9,4.8Hz,*val*3H), 2.81 (2H,AB of ABX,δ_A2.88,δ_B2.73,*J*14.1, 5.7,7.0Hz,*cys*3H), 3.73 (2H,s,SC*H*$_2$Ph), 4.29 (1H,m,*cys*2H), 4.56 (1H, dd,*J*4.7,8.8Hz,*val*2H), 5.12 (2H,s,OC*H*$_2$Ph), 5.15 (2H,AB,δ_A5.19,δ_B5.11, *J*12.2Hz,OC*H*$_2$Ph), 5.58 (1H,d,*J* 7.0 Hz,*cys*N*H*), 6.64 (1H,d,*J*8.9Hz,*val*N*H*) and 7.19-7.38 (15H,m,Ar*H*); m/z (EI) 534 (2%) (M), 443 (13%), 399 (22%), 335 (43%), 292 (53%) and 91 (100%).

N-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl-D-Valine Benzyl Ester

N-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine dicyclohexylamine salt (1.39g, 2.5mmol) was converted to the free acid by suspension in EtOAc (40ml) and extraction with 1M-aq. HCl (40ml). The *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine so obtained, D-valine benzyl ester (0.54g, 2.6mmol) and 1-hydroxybenzotriazole (0.35g, 2.6mmol) were dissolved in EtOAc (40ml) and dicyclohexylcarbodiimide (0.54g, 2.6mmol) added. The solution was stirred overnight at room temperature, oxalic acid dihydrate (15mg) added, and stirring continued for a further 1h. The dicyclohexylurea precipitate was filtered and the filtrate washed with 1M-aq. HCl (25ml), H_2O (25ml), sat. aq. $NaHCO_3$ (25ml), sat. aq. NaCl (25ml) and dried over $MgSO_4$. Evaporation left a crude solid which was filtered

through silica (5g) and crystallized from EtOAc/*n*-hexane to yield *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (1.31g, 93%), m.p. 117-118°C; $[\alpha]_D$-6.0° [c1.0,EtOH:CH_2Cl_2 (1:1)]; $[\alpha]_{365}$-23.0° [c1.0,EtOH:CH_2Cl_2(1:1)] (Found: C,66.17; H,6.28; N,5.15. $C_{31}H_{36}N_2O_6S$ requires C,65.94; H,6.43; N,4.96%); δ_H (200MHz,$CDCl_3$) 0.82 (3H,d,*J*6.9Hz,*val*4H), 0.89 (3H,d,*J*6.9Hz,*val*4H), 2.17 (1H,dsept.,*J*6.9,4.8Hz,*val*3H), 2.80 (2H,AB of ABX,δ_A2.87,δ_B2.74, *J*14.1,5.7,6.9Hz,*cys*3H), 3.72 (2H,s,SC*H*$_2$Ph), 3.79 (3H,s,OC*H*$_3$), 4.31 (1H,m,*cys*2H), 4.56 (1H,dd,*J*8.8,4.8Hz,*val*2H), 5.05 (2H,s,OC*H*$_2$Ar), 5.15 (2H,AB,δ_A5.19,δ_B5.10,*J*12.2Hz,OC*H*$_2$Ar), 5.59 (1H,d,*J*7.3Hz,*cys*NH), 6.69 (1H,d,*J*8.7Hz,*val*NH), 6.87 (2H,d,*J*8.7Hz,Ar*H*) and 7.22-7.33 (12H,m,Ar*H*); δ_C (20MHz,$CDCl_3$) 17.38 (q,*val*4C), 18.80 (q,*val*4C), 31.06 (d,*val*3C), 33.75, 36.33 (t,t,*cys*3C and SCH_2Ph), 54.06 (d,*cys*2C), 55.08 (q,OCH_3), 57.12 (d,*val*2C), 66.89 (t,2xOCH_2Ar), 113.78, 127.08, 127.65, 128.18, 128.31, 128.45, 128.81, 129.84, 135.11, 137.69, 155.78 (18xAr*C*), 159.51, 169.93 and 171.14 (3x*C*=O); m/z (EI) 473 (<1%) (M-91), 399 (23%), 292 (2%), 210 (4%), 121 (100%) and 91 (75%).

<u>*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl-*S*-Benzyl-L-Cysteine</u>

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine benzhydryl ester (0.40g, 0.51mmol) was dissolved in 0.2N-HCl $MeNO_2$ and stirred at room temperature for 1-2h or until the reaction was judged to be complete by analytical t.l.c. Evaporation afforded a crude solid which was chromatographed on silica (10g) with an EtOAc-EtOAc:HOAc (99:1) gradient as eluant. Initial fractions afforded chlorodiphenylmethane, δ_H (60MHz, $CDCl_3$) 6.08 (1H,s,C*H*Ph$_2$) and 7.18-7.40 (10H,m,Ar*H*). Later fractions gave crude *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-

0.31g, 98%) crystallized from EtOH/Et_2O/*n*-hexane (0.24g,
9-81°C; $[\alpha]_D$-16.6° [c1.0,CH_2Cl_2:MeOH(1:1)] (Found:
,5.44; N,6.49. $C_{31}H_{33}N_3O_9S$ requires C,59.70; H,5.33;
δ_H (300MHz,$CDCl_3$) 1.69-1.87 (4H,m,*aminoadipyl*3,4H),
H,m,*aminoadipyl*5H), 2.83-2.90 (2H,m,*cys*3H), 3.66 (2H,s,
1-4.42 (1H,m,2*H*), 4.69-4.71 (1H,m,2*H*), 5.07 (2H,AB,δ_A
,*J*12.0Hz,OCH_2Ar), 5.18 (2H,AB,δ_A5.20,δ_B5.16,*J*14.3Hz,
5 (1H,d,*J*7.9Hz,N*H*), 6.57 (1H,d,*J*7.2Hz,N*H*), 7.17-7.30
, 7.42 (2H,d,*J*8.5Hz,Ar*H*) and 8.15 (2H,d,*J*8.5Hz,Ar*H*);
(<1%), 429 (<1%), 322 (4%), 294 (6%), 249 (27%), 107
(100%).

ysteinyl-D-Valine Benzyl Ester Hydrochloride Salt and
phonic Acid Salt

hoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine
(1mg) was dissolved in HCl saturated $MeNO_2$ (50µl) at
ture. After 2 min the solvent was removed in a stream
gen and the crude product crystallized from CH_2Cl_2/*n*-
ford *S*-benzyl-L-cysteinyl-D-valine benzyl ester hydro-
t, m.p. 147-149°C; m/z (EI) 400 (3%) (M, free base),
2 (18%), 263 (12%), 166 (66%) and 91 (100%).

nalagous procedure addition of 4-toluenesulphonic acid
(1eq.) to the $MeNO_2$ solution, evaporation and crystall-
EtOH/ether gave *S*-benzyl-L-cysteinyl-D-valine benzyl
enesulphonic acid salt (71%), m.p. 200-205°C (Found:
.58; N,4.85. $C_{29}H_{36}N_2O_6S_2$ requires C,60.82; H,6.34;
δ_H (200MHz,$CDCl_3$) 0.81 (3H,d, 6.8Hz,*val*4H), 0.82 (3H,d,
), 2.11 (1H,dsept.,*J*6.8,5.6Hz,*val*3H), 2.24 (3H,s,CH_3),
pattern,*cys*3H), 3.64 (2H,AB,δ_A3.69,δ_B3.59,*J*12.9Hz,SCH_2Ph),

L-cysteine (0.31g, 98%) crystallized from EtOH/Et_2O/*n*-hexane (0.24g, 76%), m.p. 79-81°C; $[\alpha]_D$-16.6° [c1.0,CH_2Cl_2:MeOH(1:1)] (Found: C,59.59; H,5.44; N,6.49. $C_{31}H_{33}N_3O_9S$ requires C,59.70; H,5.33; N,6.74%); δ_H (300MHz,$CDCl_3$) 1.69-1.87 (4H,m,*aminoadipyl*3,4H), 2.16-2.23 (2H,m,*aminoadipyl*5H), 2.83-2.90 (2H,m,*cys*3H), 3.66 (2H,s, SC*H*$_2$Ph), 4.41-4.42 (1H,m,2*H*), 4.69-4.71 (1H,m,2*H*), 5.07 (2H,AB,δ_A 5.10,δ_B5.04,*J*12.0Hz,OC*H*$_2$Ar), 5.18 (2H,AB,δ_A5.20,δ_B5.16,*J*14.3Hz, OC*H*$_2$Ar), 5.65 (1H,d,*J*7.9Hz,N*H*), 6.57 (1H,d,*J*7.2Hz,N*H*), 7.17-7.30 (10H,m,Ar*H*), 7.42 (2H,d,*J*8.5Hz,Ar*H*) and 8.15 (2H,d,*J*8.5Hz,Ar*H*); m/z (EI) 481 (<1%), 429 (<1%), 322 (4%), 294 (6%), 249 (27%), 107 (96%) and 91 (100%).

S-Benzyl-L-Cysteinyl-D-Valine Benzyl Ester Hydrochloride Salt and 4-Toluenesulphonic Acid Salt

N-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (1mg) was dissolved in HCl saturated $MeNO_2$ (50µl) at room temperature. After 2 min the solvent was removed in a stream of dry nitrogen and the crude product crystallized from CH_2Cl_2/*n*-hexane to afford *S*-benzyl-L-cysteinyl-D-valine benzyl ester hydrochloride salt, m.p. 147-149°C; m/z (EI) 400 (3%) (M, free base), 383 (2%), 292 (18%), 263 (12%), 166 (66%) and 91 (100%).

In an analagous procedure addition of 4-toluenesulphonic acid monohydrate (1eq.) to the $MeNO_2$ solution, evaporation and crystallization from EtOH/ether gave *S*-benzyl-L-cysteinyl-D-valine benzyl ester 4-toluenesulphonic acid salt (71%), m.p. 200-205°C (Found: C,60.61; H,6.58; N,4.85. $C_{29}H_{36}N_2O_6S_2$ requires C,60.82; H,6.34; N,4.89%); δ_H (200MHz,$CDCl_3$) 0.81 (3H,d, 6.8Hz,*val*4H), 0.82 (3H,d, *J*6.8Hz,*val*4H), 2.11 (1H,dsept.,*J*6.8,5.6Hz,*val*3H), 2.24 (3H,s,C*H*$_3$), 3.01 (2H,AB pattern,*cys*3H), 3.64 (2H,AB,δ_A3.69,δ_B3.59,*J*12.9Hz,SC*H*$_2$Ph),

4.42 (1H,dd,*J*5.6,8.4Hz,*val*2H), 4.64br (1H,t,*J*6.3Hz,*cys*2H), 5.00 (2H, AB, δ_A5.08, δ_B4.91,*J*12.3Hz,OCH_2Ph), 7.02 (2H,d,*J*8.0Hz,Ar*H*), 7.11-7.28 (10H,m,Ar*H*), 7.71 (2H,d,*J*8.0Hz,Ar*H*) and 8.13 (1H,d,*J*8.4Hz,N*H*); m/z (EI) 400 (3%) (M, free base), 383 (4%), 309 (2%), 292 (14%), 263 (11%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(DL-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl-D-Valine Benzhydryl Ester

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteine (63mg, 0.10mmol) was prepared as described for the LL-species. D-valine (12mg, 0.10mmol) and 4-toluenesulphonic acid monohydrate (22mg, 0.10mmol) were dissolved in DMF (1ml) and heated to 50°C. Diphenydiazomethane (31mg, 0.16mmol) in DMF (1.5ml) was added dropwise over 1h to afford crude D-valine benzhydryl ester 4-toluenesulphonic acid salt. The DMF was removed *in vacuo*, the residue dissolved in EtOAc (4ml) and washed with sat. aq. $NaHCO_3$ (2x4ml), sat. aq. NaCl (4ml) and dried over Na_2SO_4. The crude D-valine benzhydryl ester, so obtained, the crude *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteine and 1-hydroxybenzotriazole (14mg, 0.10mmol) were dissolved in EtOAc (2ml) and dicyclohexylcarbodiimide (22mg, 0.11mmol) added. The mixture was stirred at room temperature overnight, oxalic acid dihydrate (1mg) added and after a further 1h the dicyclohexylurea filtered. The filtrate was washed with 1M-aq. HCl (2ml), H_2O (2ml), sat. aq. $NaHCO_3$ (2ml), H_2O (2ml), sat. aq. NaCl (2ml) and dried over Na_2SO_4. Evaporation to an oil which was chromatographed on silica (10g) with EtOAc:*n*-hexane (6:4) as eluant gave a crude solid crystallized from CH_2Cl_2/ether/*n*-hexane to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzhydryl

ester (65mg, 72%), m.p. 75-80°C (Found: C,66.36; H,6.15; N,6.03. $C_{49}H_{52}N_4O_{10}S$ requires C,66.20; H,5.90; N,6.30%); δ_H (300MHz,$CDCl_3$) 0.75 (3H,dd,*J*6.9,1.1Hz,*val*4H), 0.87 (3H,d,*J*7.0Hz,*val*4H), 1.68-1.85 (4H,m,*aminoadipyl*3,4H), 2.10-2.26 (3H,m,*aminoadipyl*5H and *val*3H), 2.74 (2H,AB of ABMX,δ_A2.80,δ_B2.68,*J*14.0,6.0,7.0,1.8,2.1Hz,*cys*3H), 3.72 (2H,s,S*CH*$_2$Ph), 4.36-4.41 (1H,m,2*H*), 4.55-4.66 (2H,m,2x2*H*), 5.09 (2H,AB,δ_A5.11,δ_B5.06,*J*11.9Hz,O*CH*$_2$Ar), 5.20 (2H,AB,δ_A5.23,δ_B5.16,*J* 13.7Hz,O*CH*$_2$Ar), 5.72-5.76 (1H,m,N*H*), 6.48-6.51 (1H,m,N*H*), 6.87 (1H, s,*CH*Ph$_2$), 6.91-6.98 (1H,m,N*H*), 7.17-7.38 (20H,m,Ar*H*), 7.41-7.45 (2H, m,Ar*H*) and 8.16 (2H,d,*J*8.9Hz,Ar*H*); m/z (EI) 797 (<1%) (M-91), 752 (<1%), 721 (<1%) (M-167), 309 (4%), 167 (84%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(DL-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl-D-(3R)-[4-^{13}C]-Valine Benzhydryl Ester

D-valine (11mg, 0.09mmol), mixed with (2R, 3R)-[4-^{13}C]-valine (1mg) was converted to its benzhydryl ester and coupled with *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteine as described above to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-(3R)-[4-^{13}C]-valine benzhydryl ester (56mg, 62%), m.p. 76-80°C; δ_C (20MHz, $CDCl_3$) 17.14 (*val*4C), 18.94 (*val*4C), 21.14 (*aminoadipyl*4C), 30.99, 31.30, 33.25, 35.04, 36.42 (*val*3C,*cys*3C,*aminoadipyl*3C and 5C and S*C*H$_2$Ph), 52.03, 53.70, 57.25 (*val*, *cys* and *aminoadipyl* 2C), 65.38, 66.98 (2xO*C*H$_2$Ar), 77.91 (*C*HPh$_2$), 123.70, 126.41, 126.79, 126.99, 127.28, 127.36, 127.64, 127.95, 128.05, 128.38, 128.84 (24xAr*C*), 136.03, 137.87, 139.25, 139.42, 142.33, 155.94 (6 x quaternary Ar*C*), 159.87, 170.06, 170.54, 171.77 and 172.20 (5x*C*=O).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl-D-Valine Benzhydryl Ester

D-valine (0.26g, 2.2mmol) was converted to its benzhydryl ester and coupled to *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (1.25g, 2.0mmol) as described above for the DL-α-aminoadipyl derivative to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzhydryl ester (1.16g, 65%), m.p. 77-80°C remelting at 133-135°C; $[\alpha]_{436}$ -11.9° (c 1.0, acetone) (Found: C,66.41; H,5.81; N,6.02. $C_{49}H_{52}N_4O_{10}S$ requires C,66.20; H,5.90; N,6.30%); δ_H (200MHz,$CDCl_3$) 0.75 (3H,d,*J*7.0Hz,*val*4H), 0.87 (3H,d,*J*6.8Hz,*val*4H), 1.67-1.90 (4H,m, *aminoadipyl*3,4H), 2.15-2.27 (3H,m,*aminoadipyl*5H and *val*3H), 2.74 (2H,AB of ABX,δ_A2.84,δ_B2.65,*J*14.0,5.6,7.3Hz,*cys*3H), 3.74 (2H,s, SC*H*$_2$Ph), 4.35-4.54 (2H,m, *aminoadipyl* and *cys* 2H), 4.61 (1H,dd,*J*4.5, 8.7Hz,*val*2H), 5.09 (2H,AB pattern,OC*H*$_2$Ar), 5.21 (2H,s,OC*H*$_2$Ar), 5.33 (1H,d,*J*7.9Hz,N*H*), 6.22 (1H,d,*J*7.1Hz,N*H*), 6.70 (1H,d,*J*8.7Hz,*val*N*H*), 6.88 (1H,s,C*H*Ph$_2$), 7.17-7.33 (20H,m,Ar*H*), 7.45 (2H,d,*J*8.5Hz,Ar*H*) and 8.17 (2H,d,*J*8.5Hz,Ar*H*); δ_C (25MHz,$CDCl_3$) 17.18 (*val*4C), 18.95 (*val* 4C), 21.17 (*aminoadipyl*4C), 30.99, 31.34, 33.36, 35.07, 36.48 (*val*3C, *cys*3C,*aminoadipyl*3C and 5C and SCH_2Ph), 52.07, 53.76, 57.29 (*val*, *cys* and *aminoadipyl*2C), 65.39, 66.96 (2xOCH_2Ar), 77.94 (CHPh$_2$), 123.69, 124.27, 125.37, 125.47, 125.62, 125.75, 126.02, 126.15, 126.42, 126.81, 127.26, 128.06, 128.37, 128.80 (24xArC), 136.05, 137.89, 139.28, 139.43, 142.34, 155.95 (6 x quaternary ArC), 159.85, 170.03, 170.49, 171.76 and 172.12 (5xC=O); m/z (EI) 797 (<1%) (M-91), 752 (<1%), 721 (<1%) (M-167), 167 (61%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-[3-^{13}C]-Cysteinyl-D-[^{15}N]-Valine Benzhydryl Ester

D-[^{15}N]-valine (57mg, 0.48mmol) was converted to its benzhydryl

ester and coupled with *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteine [prepared from *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteine benzhydryl ester (360mg, 0.46mmol)] as described above. The crude product was purified by column chromatography on silica (30g) with EtOAc:*n*-hexane (6:4) as eluant and crystallized from CH_2Cl_2/ether/*n*-hexane to afford *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine benzhydryl ester (222mg, 55%), m.p. 130-135°C; δ_H (300MHz,$CDCl_3$) 0.74 (3H,d,*J*6.9Hz,*val*4H), 0.86 (3H,d,*J*6.7Hz,*val*4H), 1.67-1.85 (4H,m, *aminoadipyl*3,4H), 2.15-2.24 (3H,m,*aminoadipyl*5H and *val*3H), 2.73 (2H,AB of ABMX,δ_A2.83,δ_B2.64,*J*142.5,141.0,14.0,5.4,7.2Hz,*cys*3H), 3.74 (2H,d,*J*3.4Hz,SC*H*$_2$Ph), 4.39-4.50 (2H,m,*aminoadipyl* and *cys*2H), 4.62 (1H,dd,*J*8.5,4.2Hz,*val*2H), 5.09 (2H,AB,δ_A5.11,δ_B5.06,*J*12.1Hz, OC*H*$_2$Ar), 5.21 (2H,AB,δ_A5.24,δ_B5.18,*J*13.4Hz,OC*H*$_2$Ar), 5.56 (1H,d,*J*7.8 Hz,N*H*), 6.24 (1H,d,*J*7.1Hz,N*H*), 6.72 (1H,dd,*J*91.3,8.5Hz,^{15}N*H*), 6.87 (1H,s,C*H*Ph$_2$), 7.18-7.31 (20H,m,Ar*H*), 7.46 (2H,d,*J*8.4Hz,Ar*H*) and 8.18 (2H,d,*J*8.4Hz,Ar*H*); δ_C (75MHz,$CDCl_3$) 33.38 (t,*cys*3C); m/z (EI) 799 (<1%) (M-91), 754 (<1%), 723 (<1%) (M-167), 167 (99%) and 91 (100%).

<u>*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl-D-Valine Benzyl Ester</u>

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (69mg, 0.11mmol), D-valine benzyl ester hydrochloride salt (30mg, 0.12mmol), triethylamine (17.1μl, 0.12mmol), 1-hydroxybenzotriazole (17mg, 0.13mmol) and dicyclohexylcarbodiimide (25mg, 0.12mmol) were dissolved in EtOAc (5ml) and stirred at room temperature overnight. Oxalic acid dihydrate (2mg) was added, stirring continued for a further 1h, and the dicyclohexylurea

precipitate filtered. The filtrate was washed with 1M-aq. HCl (5ml), H_2O (5ml), sat. aq. $NaHCO_3$ (5ml), H_2O (5ml), sat. aq. NaCl (5ml) and dried over $MgSO_4$. Evaporation afforded a colourless solid which was dissolved in the minimum of CH_2Cl_2, filtered and crystallized from CH_2Cl_2/ether to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (56mg, 62%).

Alternatively *S*-benzyl-L-cysteinyl-D-valine benzyl ester [prepared from *S*-benzyl-L-cysteinyl-D-valine benzyl ester 4-toluenesulphonic acid salt (172mg, 0.30mmol)], *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (136mg, 0.32mmol), 1-hydroxybenzotriazole (43mg, 0.32mmol) and dicyclohexylcarbodiimide (65mg, 0.32mmol) were dissolved in EtOAc (20ml) and stirred overnight at room temperature. Oxalic acid dihydrate (4mg) was added, stirring continued a further 1h and the solution filtered. The filtrate was washed with 1M-aq. HCl (20ml), sat. aq. $NaHCO_3$ (20ml), sat. aq. NaCl (20ml) and dried over $MgSO_4$. Evaporation left a colourless solid which was chromatographed on silica (25g) with EtOH:CH_2Cl_2:EtOAc:*n*-hexane (1:1:7:3) as eluant. The crude product was crystallized from CH_2Cl_2/ether/*n*-hexane to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester (188mg, 77%), m.p. 136-137°C; $[\alpha]_D$-13.6° (c1.0, acetone) (Found: C,63.27; H,5.78; N,6.90. $C_{43}H_{48}N_4O_{10}S$ requires C,63.53; H,5.95; N,6.89%); δ_H (200MHz,$CDCl_3$) 0.83 (3H,d,*J*6.9Hz,*val*4H), 0.89 (3H,d,*J*6.9Hz,*val*4H), 1.69-1.87 (4H,m,*aminoadipyl*3,4H), 2.12-2.21 (2H,m,*aminoadipyl*5H and *val*3H), 2.75 (2H,AB of ABX,δ_A2.85, δ_B2.66,*J*14.0,5.8,7.2Hz,*cys*3H), 3.76 (2H,s,SC*H*$_2$Ph), 4.45-4.54 (3H,m, *aminoadipyl*, *cys* and *val* 2H), 5.10 (2H,s,OC*H*$_2$Ar), 5.11 (2H,AB,

δ_A5.16, δ_B5.06, *J*12.2Hz, OC*H*$_2$Ar), 5.23 (2H,s,OC*H*$_2$Ar), 5.62 (1H,d,*J*8.2Hz, N*H*), 6.24 (1H,d,*J*7.3Hz,N*H*), 6.73 (1H,d,*J*8.7Hz,N*H*), 7.21-7.36 (15H,m, Ar*H*), 7.47 (2H,d,*J*8.5Hz,Ar*H*) and 8.18 (2H,d,*J*8.5Hz,Ar*H*); δ_C(50MHz, $CDCl_3$) 17.57 (*val*4C), 18.96 (*val*4C), 21.42 (*aminoadipyl*4C), 31.06, 31.42, 33.46, 35.23, 36.64 (*val*3C,*aminoadipyl*3C and 5C,*cys*3C and SCH$_2$Ph), 52.20, 53.93, 57.47 (*cys*,*val* and *aminoadipyl*2C), 65.48, 67.07 (3xOCH$_2$Ar), 123.78, 127.24, 128.02, 128.14, 128.29, 128.32, 128.47, 128.54, 128.64, 128.93 (19xAr*C*), 135.27, 136.21, 138.01, 142.48, 147.87 (5x quaternary Ar*C*), 156.05, 170.18, 171.33, 171.88 and 172.31 (5x*C*=O); m/z (EI) 335 (3%), 292 (5%), 249 (6%), 214 (14%), 166 (17%) and 91 (100%).

N-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl-D-Valylglycine Benzyl Ester

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzhydryl ester (200mg, 0.23mmol) was dissolved in 0.2N-HCl $MeNO_2$ for 1-2h. Evaporation left an oil which was chromatographed on silica (10g) with EtOAc:HOAc (99.5:0.5) as eluant to yield crude *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine. This crude product was dissolved in THF (3ml), pyridine (20.0µl, 0.25mmol) and ethyl chloroformate (23.8µl, 0.25mmol) added and the solution stirred at 0°C for 15 min. A solution of glycine benzyl ester 4-toluenesulphonic acid salt (84mg, 0.25mmol) and pyridine (20.0µl, 0.25mmol) in CH_2Cl_2(3ml) was added and the mixture allowed to come to room temperature overnight. The solvent was evaporated and the residue dissolved in EtOAc (15ml). The EtOAc solution was washed with 1M-aq. HCl (10ml), H_2O (10ml), sat. aq. NaCl (10ml) and dried over $MgSO_4$. Evaporation left a semi-solid which was purified by pre-

parative t.l.c. on silica with CH_2Cl_2:MeOH (19:1) as eluant and crystallized from CH_2Cl_2/EtOH/*n*-hexane to afford *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valyl-glycine benzyl ester (90mg, 46%), m.p. 155-158°C; $[\alpha]_D$-7.4° [c1.0, CH_2Cl_2:MeOH (1:1)] (Found: C,61.86; H,6.13; N,7.79. $C_{45}H_{51}N_5O_{11}S$ requires C,62.13; H,5.91; N,8.05%); δ_H [300MHz,$CDCl_3$:CD_3OD (1:1)] 0.88 (3H,d,*J*6.8Hz,*val*4H), 0.91 (3H,d,*J*6.8Hz,*val*4H), 1.66-1.85 (4H,m,*aminoadipyl*3,4H), 2.13-2.23 (3H,m,*aminoadipyl*5H and *val*3H), 2.68 (2H,AB of ABX,δ_A2.74,δ_B2.63,*J*13.8,7.4,6.5Hz,*cys*3H), 3.69 (2H,s, S*CH*$_2$Ph), 4.24 (1H,m,2H), 4.30 (1H,m,2H), 4.46 (1H,m,2H), 5.06 (2H, AB,δ_A5.09,δ_B5.03,*J*12.2Hz,O*CH*$_2$Ar), 5.11 (2H,s,O*CH*$_2$Ar), 5.20 (2H,AB, δ_A5.23,δ_B5.17,*J*13.5Hz,O*CH*$_2$Ar), 7.20-7.35 (15H,m,Ar*H*), 7.45 (2H,d, *J*8.6Hz,Ar*H*) and 8.15 (2H,d,*J*8.6Hz,Ar*H*); m/z (EI) 350 (<1%), 108 (53%) and 91 (100%).

δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzhydryl ester (100mg, 0.11mmol) was dissolved in refluxing liquid ammonia (10ml) under a nitrogen atmosphere and small pieces of sodium metal added until the blue colour persisted for 5 min. Ammonium acetate (*ca* 100mg) was added, the ammonia evaporated in a stream of dry nitrogen and the residue desiccated over conc. H_2SO_4. The product was isolated as follows:-

Procedure A: The residue was dissolved in 5% aq. HOAc (5ml), filtered and a 10% (w/v) solution of $Hg(OAc)_2$ in 5% aq. HOAc added dropwise to precipitate the tripeptide sulphide. The precipitate was separated by centrifugation and washed sequentially with degassed water (4x5ml), MeOH (2x5ml) and ether (5ml). The dried

tripeptide sulphide was resuspended in degassed water (2ml) and a stream of H_2S passed through the suspension for 15 min. The black HgS was separated by centrifugation, washed with degassed water (1ml) and the combined supernatant and washings filtered through celite. The filtrate was lyopholized to afford a mixture of δ-(L-α-amino-adipyl)-L-cysteinyl-D-valine and its disulphide as a colourless powder (32mg, 78%) (Found: C,44.75; H,6.85; N,11.10; ash,4%. $C_{14}H_{25}N_3O_6S$ requires C,46.30; H,6.87; N,11.57%); free thiol [475] 75%; m/z (FAB) 364 (100%) (M+H).

Procedure B: The residue was dissolved in 5% aq. HOAc (5ml), extracted with ether (2x2ml) and the aqueous layer lyopholized. The product was dissolved in water (5ml), adjusted to pH8 with dil. aq. NH_3 and the solution aerated for 2h. The solution was freeze dried, the residue dissolved in water and applied to an ion-exchange column (1.5x18cm) with a H_2O-1M-aq. pyridine gradient as eluant. The ninhydrin-positive fractions were lyopholized to give δ-(L-α-amino-adipyl)-L-cysteinyl-D-valine as its disulphide (39mg, 96%), m.p. 200-203°C (decomp.); $[\alpha]_D$-9.5° (c2.0,2M-aq.HCl) [lit.[449],$[\alpha]_D$-9.5° (c2.0,2M-aq.HCl); lit.[450],$[\alpha]_D$-11.0° (c2.0,2M-aq.HCl)] (Found: C,46.18; H,6.37; N,11.02. $C_{28}H_{48}N_6O_{12}S_2$ requires C,46.40; H, 6.67; N,11.59%); free thiol [475] 4%; δ_H (300MHz,D_2O) 0.31 (3H,d, *J*7.2Hz,*val*4H), 0.34 (3H,d,*J*7.9Hz,*val*4H), 1.08-1.30 (4H,m,*aminoadipyl* 3,4H), 1.53-1.60 (1H,m,*val*3H), 1.76-1.81 (2H,m,*aminoadipyl*5H), 2.48 (2H,AB of ABX,δ_A2.58,δ_B2.39,*J*14.1,5.7,8.4Hz,*cys*3H), 3.17br (1H,t, *J*5.8Hz,*aminoadipyl*2H) and 3.59 (1H,d,*J*5.8Hz,*val*2H); δ_C (75MHz,D_2O) 17.14 (*val*4C), 18.52 (*val*4C), 20.70 (*aminoadipyl*4C), 29.57, 30.07 (*val* and *aminoadipyl*3C), 34.56 (*aminoadipyl*5C), 38.49 (*cys*3C), 52.43, 59.51 and 59.77 (*aminoadipyl*, *cys* and *val* 2C); m/z (FAB)

725.2861 (100%) (M+H ,$C_{28}H_{49}N_6O_{12}S_2$ requires 725.2850).

δ-(L-α-Aminoadipyl)-L-[3-^{13}C]-Cysteinyl-D-[^{15}N]-Valine

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine benzhydryl ester (212mg, 0.24mmol) was deprotected as described above and purified by procedure B to afford δ-(L-α-aminoadipyl)-L-[3-^{13}C]-cysteinyl-D-[^{15}N]-valine as its disulphide (48mg, 55%), m.p. 195-200°C (decomp.); δ_H (200MHz, D_2O) 0.94 (3H,d,*J*7.8Hz,*val*4H), 0.98 (3H,d,*J*7.0Hz,*val*4H), 1.64-2.01 (4H,m,*aminoadipyl*3,4H), 2.10-2.22 (1H,m,*val*3H), 2.37-2.51 (2H,m,*aminoadipyl*5H), 2.80 (2H,AB of ABMX,δ_A2.90,δ_B2.70,*J*193.9,12.3, 3.6,6.9Hz,*cys*3H), 3.36-3.48 (1H,m,*cys*2H), 3.80 (1H,t,*J*6.0Hz,*aminoadipyl*2H) and 4.16 (1H,d,*J*5.6Hz,*val*2H); δ_C (50MHz,D_2O) 39.17 (t, *cys*3C); m/z (FAB) 729 (100%) (M+H) and 728 (45%).

δ-(L-α-Aminoadipyl)-L-Cysteicyl-D-Valine

δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and/or its disulphide (13mg, 0.04mmol) was dissolved in HCO_3H (1.5ml) and stirred at 0°C for 1h. Water was added and the solution freeze dried. The residue was purified by ion-exchange chromatography with water as eluant to yield δ-(L-α-aminoadipyl)-L-cysteicyl-D-valine (14mg, 95%), δ_C [90MHz,D_2O:H_2O (2:1),pH 10.5] 17.00 (*val*4C), 18.69 (*val*4C), 21.12 (*aminoadipyl*4C), 30.65 (*val*C3), 32.17 (*aminoadipyl*3C), 35.01 (*aminoadipyl*5C), 50.82 (*cys*2C and *cys*3C), 54.74 (*aminoadipyl*2C) and 60.66 (*val*2C); m/z (FAB) 412 (100%) (M+H).

δ-(DL-α-Aminoadipyl)-L-Cysteicyl-D-(3R)-[4-^{13}C]-Valine

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(DL-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-(3R)-[4-^{13}C]-valine benzhydryl ester (86mg, 0.10mmol) was deprotected as described above and purified by pro-

cedure A. The tripeptide was oxidized with HCO_3H and purified by ion-exchange chromatography with water as eluant to yield δ-(DL-α-aminoadipyl)-L-cysteicyl-D-(3R)-[4-^{13}C]-valine (14mg, 35%), δ_C [90MHz,D_2O:H_2O (2:1),pH10.5] 17.00 (*val*4C), 18.68 (*val*4C), 21.05 (*aminoadipyl*4C), 30.63 (*val*3C), 31.57 (*aminoadipyl*3C), 34.92 (*aminoadipyl*5C), 50.83 (*cys*2C and *cys*3C), 54.73 (*aminoadipyl*2C) and 60.67 (*val*2C); m/z (FAB) 412 (100%) (M+H).

δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valylglycine

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valylglycine benzyl ester (54mg, 0.06mmol) was dissolved in refluxing liquid ammonia (5ml) and treated with sodium metal as described for the synthesis δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. Purification using procedure B gave δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine as its disulphide (19mg, 73%), m.p. 230-235°C (decomp.) (Found: C,46.05; H,6.21; N,12.32. $C_{32}H_{54}N_8O_{14}S_2$ requires C,45.81; H,6.49; N,13.36%); δ_H (300MHz, D_2O) 0.25 (3H,d, *J*7.5Hz,*val*4H), 0.28 (3H,d,*J*7.5Hz,*val*4H), 1.00-1.22 (4H,m,*aminoadipyl* 3,4H), 1.44-1.52 (1H,m,*val*3H), 1.70br (2H,t,*J*6.7Hz,*aminoadipyl*5H), 2.40 (2H,AB of ABX,δ_A2.48,δ_B2.33,*J*13.9,7.4,5.8Hz,*cys*3H), 3.07br (1H, t,*J*5.6Hz,*aminoadipyl*2H), 3.20 (2H,s,*gly*2H) and 3.54 (1H,m,*val*2H); m/z (FAB) 839 (69%) (M+H) and 421 (100%).

6.4 *N*-[δ-(L-α-AMINOADIPYL)-L-CYSTEINYL]-*N*-HYDROXY-D-VALINE

D-Valine Benzyl Ester 4-Methoxybenzaldimine

D-valine benzyl ester 4-toluenesulphonic acid salt (1.14g, 3.0mmol) was converted to the free base, added to a solution of 4-methoxybenzaldehyde (0.38ml, 3.1mmol) in dry benzene (30ml) and

$MgSO_4$ (*ca*5g) added. The mixture was stirred for 12h at 30°C, filtered and evaporated to yield D-valine benzyl ester 4-methoxybenzaldimine (0.96g, 98%), δ_H (60MHz,$CDCl_3$) 0.95 (6H,d,*J*7.0Hz,*val*4,4'H), 2.13-2.68 (1H,m,*val*3H), 3.68 (1H,d,*J*7.0Hz,*val*2H), 3.77 (3H,s,CH_3O), 5.22 (2H,s,OCH_2Ph), 7.02 (2H,d,*J*8.5Hz,Ar*H*), 7.35 (5H,s,Ar*H*), 7.78 (2H,d,*J*8.5Hz,Ar*H*) and 8.20 (1H,s,C*H*=N); υ (film) 2960 (C-H), 1738 (C=O), 1635 (C=N), 1605 and 1510cm^{-1}.

Attempted Synthesis of *N*-Hydroxy-D-Valine Benzyl Ester Hydrochloride Salt

D-valine benzyl ester 4-methoxybenzaldimine (0.96g,3.0mmol) was dissolved in CH_2Cl_2 (15ml) and cooled to 0°C. A solution of 3-chloroperbenzoic acid (82% pure) (0.69g,4.0mmol) in CH_2Cl_2(15ml) was added dropwise over 15min and stirring continued at 0°C for a further 15min. The solution was filtered and the filtrate washed with sat. aq. $NaHCO_3$ (2x30ml), sat. aq. NaCl (30ml) and dried over Na_2SO_4. Evaporation left a crude orange oil. Treatment of the crude oil (222mg,0.68mmol) with 1M HCl-H_2O:MeOH (1:9) or 2M HCl-H_2O:MeOH (1:4) and crystallization of the product from EtOH/ether/*n*-hexane afforded D-valine benzyl ester 4-methoxybenzaldimine *N*-oxide (94mg, 40%) recrystallized from EtOH/ether/*n*-hexane (75mg, 32%), m.p. 100-102°C, $[\alpha]_D$+20.0 (*c*1.0,MeOH) (Found: C,70.25; H,6.86; N,4.12. $C_{20}H_{23}NO_4$ requires C,70.36; H,6.79; N,4.10%); δ_H (60MHz,$CDCl_3$) 1.04 (6H,d,*J*6.5Hz,*val*4,4'H), 2.40-2.90 (1H,m,*val*3H), 3.82 (3H,s, CH_3O), 4.30 (1H,d,*J*10.0Hz,*val*2H), 5.20 (2H,s,OCH_2Ph), 6.90 (2H,d, *J*9.0Hz,Ar*H*), 7.30-7.37 (6H,m,Ar*H* and C*H*=N) and 8.21 (2H,d,*J*9.0Hz, Ar*H*); υ (CCl_4) 2955 (C-H), 1740 (C=O) and 1608 cm^{-1}; m/z (EI) 341 (48%) (M), 206 (41%), 190 (86%), 151 (69%) and 91 (100%).

N-Hydroxy-D-Valine Benzyl Ester Hydrochloride Salt

In an identical experiment the crude orange oil was taken up in dioxane(50ml), 6M-aq. HCl (50ml) added and the solution stirred at room temperature for 1h. The solution was reduced in volume, extracted with ether (50ml) and evaporated to a semi-solid which was dessicated over P_2O_5 and crystallized from EtOH/ether/*n*-hexane to afford *N*-hydroxy-D-valine benzyl ester hydrochloride salt (0.40g, 52%), m.p. 118-126°C (Found: C,53.99; H,6.69; N,5.16. $C_{12}H_{18}ClNO_3$ requires C,55.49; H,6.99; N,5.39%); δ_H (200MHz,$CDCl_3$) 1.02 (3H,d,*J*6.9Hz,*val*4H), 1.11 (3H,d,*J*7.0Hz,*val*4H), 2.57 (1H,dsept., *J*5.2,6.9Hz,*val*3H), 4.01 (1H,d,*J*5.0Hz,*val*2H), 5.25 (2H,AB,δ_A5.30, δ_B5.21,*J*12.1Hz,OC*H*$_2$Ph), 6.09br (3H,s,$^+$N*H*$_2$-O*H*) and 7.31-7.40 (5H,m, Ar*H*); ν (KBr disc) 3440, 2970 (C-H), 2770, 1735 (C=O), 1205, 908, 759 and 700cm^{-1}; m/z (EI) 223 (11%) (M,free base), 178 (7%) and 91 (100%).

N-Hydroxy-D-Valine Benzyl Ester

N-hydroxy-D-valine benzyl ester hydrochloride salt (0.20g, 0.77 mmol) was suspended in EtOAc (10ml), washed with sat. aq. $NaHCO_3$ (2x5ml), sat. aq. NaCl (5ml) and dried over Na_2SO_4. Evaporation to a colourless solid and crystallization from *n*-hexane afforded *N*-hydroxy-D-valine benzyl ester (0.12g, 70%), m.p. 56-57°C; $[\alpha]_D^+$ 14.1°(c1.0,EtOH) (Found: C,62.95; H,7.60; N,6.38. $C_{12}H_{17}NO_3$ requires C,64.55; H,7.67; N,6.27%); δ_H (360MHz,$CDCl_3$) 0.93 (3H, d,*J*6.9Hz,*val*4H), 0.96 (3H,d,*J*6.9Hz,*val*4H), 2.02 (1H,dsept.,*J*6.3, 6.9Hz,*val*3H), 3.59 (1H,d,*J*6.3Hz,*val*2H), 5.22 (2H,AB,δ_A5.24,δ_B5.19, *J*12.3Hz,OC*H*$_2$Ph), 6.29br (2H,s,N*H*-O*H*) and 7.29-7.38 (5H,m,Ar*H*); δ_C [75MHz,CD_3SOCD_3:H_2O(1:1)] 19.92 (q,*val*4C), 20.58 (q,*val*4C), 29.80 (d,*val*3C), 67.64 (t,OCH$_2$Ph), 72.81 (d,*val*2C), 129.58, 129.82 and

130.04 (5xArC); m/z (EI) 223 (22%) (M), 178 (8%) and 91 (100%).

N-Hydroxy-D-Valine Benzyl Ester 4-Toluenesulphonic Acid Salt

N-hydroxy-D-valine benzyl ester (69mg,0.31mmol) and 4-toluenesulphonic acid monohydrate (64mg, 0.34mmol) were dissolved in methanol (2ml), evaporated and crystallized twice from CH_2Cl_2/*n*-hexane to yield *N*-hydroxy-D-valine benzyl ester 4-toluenesulphonic acid salt (106mg, 87%), m.p. 105-108°C; $[\alpha]_D$-3.4°(c1.0,MeOH) (Found: C,57.84; H,6.36; N,3.43. $C_{19}H_{25}NO_6S$ requires C,57.70; H,6.37; N,3.54%); δ_H (60MHz,$CDCl_3$) 0.93 (3H,d,*J*8.0Hz,*val*4H), 1.05 (3H,d, *J*6.0Hz,*val*4H), 2.15-2.60 (1H,m,*val*3H), 2.38 (3H,s,C*H*$_3$), 3.98 (1H,d, *J*5.0Hz,*val*2H), 5.22 (2H,s,OC*H*$_2$Ph), 7.17 (2H,d,*J*8.0Hz,Ar*H*), 7.35 (5H, s,Ar*H*) and 7.82 (2H,d,*J*8.0Hz,Ar*H*); m/z (EI) 223 (3%) (M, free base), 178 (3%), 172 (14%) and 91 (100%).

Attempted Syntheses of *N*-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (255mg, 0.41mmol) was dissolved in dry THF (5ml) at 0°C and pyridine (0.33ml, 4.1mmol) and ethylchloroformate (0.39ml, 4.1mmol) added. The solution was stirred for 15min, filtered and evaporated to yield an oil which was dissolved in CH_2Cl_2 (5ml) and added dropwise over 10min to a solution of *N*-hydroxy-D-valine benzyl ester (91mg, 0.41mmol) in CH_2Cl_2(5ml). The solution was stirred at room temperature for 2h, washed with 1M-aq. HCl (10ml), H_2O (10ml), sat. aq. NaCl (10ml) and dried over $MgSO_4$. Evaporation afforded a yellow oil which was chromatographed on silica (10g) with EtOAc: CH_2Cl_2:MeOH (500:500:1) as eluant to yield *O*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy

-D-valine benzyl ester (162mg, 48%).

Likewise, *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-amino-adipyl)-*S*-benzyl-L-cysteine (60mg, 0.10mmol), *N*-hydroxy-D-valine benzyl ester (23mg, 0.10mmol), 1-hydroxybenzotriazole (14mg, 0.10 mmol) and dicyclohexylcarbodiimide (20mg, 0.10mmol) were dissolved in MeCN (2ml) and stirred at room temperature for 5h. The solution was filtered, evaporated and the residue purified by column chromatography on silica (5g), with CH_2Cl_2:MeOH (19:1) as eluant. Crystallization of the product from CH_3OH/CH_2Cl_2/ether/*n*-hexane afforded *O*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (59mg, 74%), m.p. 81-84°C (Found: C,62.39; H,5.76; N,6.62. $C_{43}H_{48}N_4O_{11}S$ requires C,62.31; H,5.84; N,6.76%); $FeCl_3$-negative; δ_H (60MHz, $CDCl_3$) 0.94 (6H,d,*J*6.5Hz,*val*4,4'H), 1.57-1.85 (4H,m,*aminoadipyl*3,4H), 2.00-2.20 (2H,m,*aminoadipyl*5H), 2.62 (2H,d,*J*5.5Hz,*cys*3H), 3.57 (2H, s,SCH_2Ph), 3.60-3.65 (1H,m,*val*2H), 4.18-4.42 (1H,m,*aminoadipyl*2H), 4.65br (1H,t,*J*5.5Hz,*cys*2H), 5.00 (2H,s,OCH_2Ar), 5.08 (2H,s,OCH_2Ar), 5.52 (1H,d,*J*7.5Hz,N*H*), 5.98 (1H,d,*J*8.0Hz,N*H*), 7.08-7.17 (15H,m,Ar*H*), 7.38 (2H,d,*J*8.0Hz,Ar*H*) and 7.95 (2H,d,*J*8.0Hz,Ar*H*); m/z (EI) 721 (<1%), 622 (<1%), 546 (<1%), 372 (9%), 322 (<1%), 249 (7%), 124 (74%), 108 (38%), 107 (38%) and 91 (100%).

<u>*O*-[*N*-Benzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester</u>

N-benzyloxycarbonyl-*S*-benzyl-L-cysteine (138mg, 0.40mmol), *N*-hydroxy-D-valine benzyl ester (98mg, 0.44mmol), 1-hydroxybenzotriazole (59mg, 0.44mmol) and dicyclohexylcarbodiimide (91mg, 0.44 mmol) were dissolved in CH_3CN (4ml) and the solution stirred overnight at room temperature. The precipitated dicyclohexylurea was

filtered and the filtrate evaporated to a yellow oil which was dissolved in EtOAc (10ml). The EtOAc solution was washed with 1M-aq. HCl (5ml), H_2O (5ml), sat. aq. NaCl (5ml) and dried over $MgSO_4$. Evaporation and chromatography on silica (30g) with EtOAc:*n*-hexane (4:6) as eluant afforded *O*-[*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester as an oil (200mg, 91%), $FeCl_3$-negative; δ_H (300MHz,$CDCl_3$) 0.96 (3H,d,*J*6.5Hz,*val*4H), 0.98 (3H,d, *J*6.6Hz,*val*4H), 1.96-2.07 (1H,m,*val*3H), 2.69 (2H,AB of ABX,δ_A2.74, δ_B2.65,*J*14.2,5.1,6.4Hz,*cys*3H), 3.23-3.65 (1H,m,*val*N*H*), 3.63-3.73 (1H,m,*val*2H), 3.66 (2H,s,SCH_2Ph), 4.48-4.54 (1H,m,*cys*2H), 5.10 (2H, AB,δ_A5.12,δ_B5.07,*J*12.3Hz,OCH_2Ph), 5.13 (2H,AB,δ_A5.16,δ_B5.10,*J*12.2Hz, OCH_2Ph), 5.41 (1H,d,*J*8.5Hz,*cys*N*H*) and 7.21-7.38 (15H,m,Ar*H*); $\upsilon(CCl_4)$ 3430 (N-H), 2955 (C-H), 1735 (C=O), 1495 and 693cm^{-1}; m/z (EI) 393 (52%), 304 (61%), 260 (72%), 205 (85%), 108 (89%) and 91 (100%).

Attempted Synthesis of *N*-Benzyloxy-D-Valine Benzyl Ester

N-hydroxy-D-valine benzyl ester hydrochloride salt (100mg, 0.39mmol), benzyl bromide (50.5µl, 0.42mmol) and Ag_2O (98mg, 0.42 mmol) were added to DMF (0.6ml) and stirred at room temperature for 3 days. The mixture was filtered, evaporated and the residue dissolved in EtOAc (10ml). The EtOAc solution was washed with sat. aq. $NaHCO_3$ (2x5ml), sat. aq. NaCl (5ml) and dried over $MgSO_4$. Evaporation left a clear oil which was chromatographed on silica (10g), with a benzene-benzene:EtOAc (8:2) gradient as eluant, to yield unreacted *N*-hydroxy-D-valine benzyl ester (24mg, 28% recovery) and *N*-benzyl-*N*-hydroxy-D-valine benzyl ester (34mg, 39% based on recovered *N*-hydroxy-D-valine benzyl ester), δ_H(60MHz,$CDCl_3$) 0.91 (3H,d, *J*7.0Hz,*val*4H), 1.04 (3H,d,*J*7.0Hz,*val*4H), 1.95-2.52 (1H,m,*val*3H), 3.15 (1H,d,*J*9.0Hz,*val*2H), 3.87 (2H,s,NCH_2Ph), 5.15 (2H,s,OCH_2Ph),

5.30-5.52 (1H,s,O*H*) and 7.18-7.28 (10H,m,Ar*H*); $\upsilon(CCl_4)$ 3580 (O-H), 2965 (C-H), 1740 (C=O) and 695cm^{-1}; m/z (EI) 313 (41%) (M), 270 (28%), 222 (44%), 178 (99%), 162 (99%) and 91 (100%).

N-Chloroacetoxy-D-Valine Benzyl Ester

N-hydroxy-D-valine benzyl ester hydrochloride salt (50mg, 0.19 mmol) and pyridine (32.7µl, 0.40mmol) were dissolved in CH_2Cl_2(1.9ml) and 4-nitrophenychloroacetate [450] (44mg, 0.20mmol) added. The solution was stirred at room temperature for 24h, washed with 1M-aq. HCl (2ml), H_2O (2ml), sat. aq. NaCl (2ml) and dried over $MgSO_4$. Evaporation left an orange oil which was purified by preparative t.l.c. on silica with CH_2Cl_2:MeOH (19:1) as eluant to afford *N*-chloroacetoxy-D-valine benzyl ester (36mg, 62%).

Alternatively, *N*-hydroxy-D-valine benzyl ester (98mg, 0.44mmol), chloroacetic acid (38mg, 0.40mmol), 1-hydroxybenzotriazole (59mg, 0.44mmol) and dicyclohexylcarbodiimide (91mg, 0.44mmol) were dissolved in CH_3CN (4ml) and the solution stirred overnight and filtered. The filtrate was evaporated to an oil which was taken up in EtOAc (10ml), washed with 1M-aq. HCl (5ml), H_2O (5ml), sat. aq. NaCl (5ml) and dried over $MgSO_4$. Evaporation and column chromatography of the residue, on silica (10g) with EtOAc:*n*-hexane (4:6) as eluant afforded *N*-chloroacetoxy -D-valine benzyl ester as an oil (112mg, 93%), $FeCl_3$-negative; δ_H (60MHz,$CDCl_3$) 0.98 (6H,d,*J*7.0Hz, *val*4,4'H), 1.73-2.27 (1H,m,*val*3H), 3.58 (1H,d,*J*6.0Hz,*val*2H), 3.47 (2H,s,ClC*H*$_2$), 5.08 (2H,s,OC*H*$_2$Ph), 7.15-7.48 (1H,m,N*H*), 7.20 (5H,s, Ar*H*); $\upsilon(CCl_4)$ 3230 (N-H), 2960 (C-H), 1765 (C=O), 1740 (C=O) and 695cm^{-1}.

N-[*N*-Benzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-benzyloxycarbonyl-*S*-benzyl-L-cysteine (276mg, 0.80mmol) and *N*-hydroxy-D-valine benzyl ester (196mg, 0.88mmol) were dissolved in DMF (10ml). Dicyclohexylcarbodiimide (182mg, 0.88mmol) was added and the solution stirred at room temperature for 24h. The dicyclohexylurea precipitate was filtered, the filtrate added to 50% sat. aq. NaCl (10ml) and the mixture extracted with EtOAc (3x15ml). The EtOAc extracts were dried over $MgSO_4$ and evaporated to a yellow oil which was chromatographed on silica (30g) with a *n*-hexane–*n*-hexane: EtOAc (4:6) gradient as eluant. Initial fractions afforded, as a clear foam, *N*-[*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*,*N*'-dicyclohexylurea (147mg, 33%), $FeCl_3$-negative; δ_H (60MHz,$CDCl_3$) 1.03-1.95 (22H,m,*cyclohexyl*H), 2.68 (2H,AB of ABX pattern,*cys*3H), 3.60 (2H,s,SCH_2Ph), 3.93-4.17 (1H,m,*cys*2H), 4.49 (1H,d,*J*6.5Hz,N*H*), 4.95 (2H,AB,δ_A5.07,δ_B4.84,*J*12.0Hz,OCH_2Ph), 5.76 (1H,d,*J*6.5Hz,N*H*) and 7.12-7.16 (10H,m,Ar*H*); $\upsilon(CCl_4)$ 3430 (N-H), 3300 (N-H), 2930 (C-H), 2855 (C-H), 1710 (C=O), 1663 (C=O), 1495, 1217 and 695cm^{-1}; m/z (EI) 551 (<1%) (M), 426 (5%), 335 (12%), 291 (10%), 184 (35%), 125 (6%) and 91 (100%). Later fractions were $FeCl_3$-positive. Evaporation afforded a colourless solid (129mg, 29%) which was crystallized from EtOAc/*n*-hexane to yield *N*-[*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (72mg, 16%), m.p. 122-123°C; $[\alpha]_D$-39.0° (*c*1.0,$CHCl_3$) (Found: C,65.43; H,6.15; N,5.04. $C_{30}H_{34}N_2O_6S$ requires C,65.43; H,6.22; N,5.09%); $FeCl_3$-positive; δ_H (300MHz,$CDCl_3$) 0.96 (3H,d,*J*6.8Hz,*val*4H), 0.97 (3H,d,*J*6.8Hz,*val*4H), 2.34-2.46 (1H,m,*val*3H), 2.77 (2H,AB of ABX,δ_A2.86,δ_B2.68,*J*13.8,5.6, 7.3Hz,*cys*3H), 3.75 (2H,s,SCH_2Ph), 4.98 (1H,d,*J*7.9Hz,*val*2H), 5.08 (2H,AB,δ_A5.11,δ_B5.04,*J*12.2Hz,OCH_2Ph), 5.11-5.19 (1H,m,*cys*2H), 5.17

(2H,AB, δ_A5.21, δ_B5.13, *J*12.2Hz,OC*H*$_2$Ph), 5.59 (1H,d,*J*8.5Hz,N*H*), 7.18-7.42 (15H,m,Ar*H*) and 7.91 (1H,s,O*H*); υ($CHCl_3$) 3420 (N-H), 2960 (C-H), 1717 (C=O), 1670 (C=O), 1495 and 692cm^{-1}; m/z (EI) 550 (29%) (M), 489 (2%), 459 (2%), 276 (28%), 254 (38%), 137 (23%) and 91 (100%).

Attempted Synthesis of *N*-[*N*-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine dicyclohexylamine salt (1.33g, 2.4mmol) was converted to the free acid and dissolved in DMF (30ml). *N*-hydroxy-D-valine benzyl ester (0.54g, 2.4 mmol) and dicyclohexylcarbodiimide (0.51g, 2.5mmol) were added and the solution stirred at room temperature for 20h. The precipitated dicyclohexylurea was filtered and the filtrate evaporated to an oil which was filtered through silica (10g) and subjected to column chromatography on silica (200g) with an EtOAc:*n*-hexane (3:7)-EtOAc:*n*-hexane (7:3) gradient as eluant. Initial fractions afforded *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*,*N*'-dicyclohexylurea (0.31g, 22%) as an oil, δ_H (200MHz,$CDCl_3$) 1.12-1.40 (8H,m,*cyclohexyl*H), 1.59-1.95 (14H,m,*cyclohexyl*H), 2.72 (2H,AB of ABX, δ_A2.81, δ_B2.64, *J*13.8,5.7,8.1Hz,*cys*3H), 3.66 (2H,s,SC*H*$_2$Ph), 3.79 (3H,s,C*H*$_3$O), 4.08-4.21 (1H,m,N*H*), 4.54-4.59 (1H,m,*cys*2H), 5.00 (2H, AB, δ_A5.07, δ_B4.94, *J*11.8Hz,OC*H*$_2$Ar), 5.47 (1H,d,*J*6.3Hz,N*H*), 6.87 (2H, d,*J*8.7Hz,Ar*H*) and 7.14-7.34 (7H,m,Ar*H*); m/z (EI) 416 (<1%) (M-165), 365 (<1%), 291 (12%), 125 (11%), 121 (100%), 91 (48%) and 82 (47%). Later fractions were $FeCl_3$-positive and were evaporated to leave a colourless solid (0.29g, 21%) which was crystallized from EtOAc/*n*-hexane to yield a mixture of *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester and *N*-[*N*-4-methoxybenzyoxy-

carbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (0.11g, 8%).

Attempted Synthesis of *N*-[*N*-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Benzyloxy-D-Valine Benzyl Ester

The mixture of *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester and *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (166mg) was dissolved in distilled DMF (16.6ml). Benzyl bromide (272µl, 2.3mmol) and Ag_2O (265mg, 1.1mmol) were added and the mixture shaken, under an argon atmosphere, at room temperature and in the dark for 2h. The mixture was filtered, evaporated and filtered through silica (2g). Evaporation of the filtrate to a yellow oil and column chromatography on silica (10g) with EtOAc:*n*-hexane (2:8) as eluant afforded a mixture of products. The mixture was separated and purified by column chromatography on silica (10g) with toluene:*n*-hexane (1:9) as eluant to yield *O*-benzyl-2-oxoisovaleric acid oxime benzyl ester (6mg), $FeCl_3$-negative; δ_H (200MHz,$CDCl_3$) 1.17 (6H,d,*J*7.0Hz,*val*4,4'H), 3.42 (1H,sept.,*J*7.0Hz,*val*3H), 5.23 (2H,s,OC*H*$_2$Ph), 5.27 (2H,s,OC*H*$_2$Ph) and 7.29-7.40 (10H,m,Ar*H*); $\upsilon(CCl_4)$ 3035 (C-H), 2965 (C-H), 1732 (C=O), 1605 and 695cm^{-1}; m/z (EI) 311 (4%) (M), 294 (8%), 181 (48%) and 91 (100%), and *N*-benzyl-*N*-hydroxy-D-valine benzyl ester (39mg), $FeCl_3$-negative; δ_H (200MHz,$CDCl_3$) 0.95 (3H,d,*J*6.7Hz,*val*4H), 1.09 (3H,d,*J*6.7Hz,*val*4H), 2.28 (1H,dsept.,*J*6.7,8.9Hz,*val*3H), 3.21 (1H, d,*J*8.9Hz,*val*2H), 3.95 (2H,AB,δ_A4.00,δ_B3.89,*J*13.4Hz,NC*H*$_2$Ph), 5.25 (2H,AB,δ_A5.29,δ_B5.21,*J*12.2Hz,OC*H*$_2$Ph), 5.58 (1H,s,O*H*) and 7.27-7.45 (10H,m,Ar*H*); m/z (EI) 313 (4%) (M), 270 (30%), 222 (4%), 178 (15%) and 91 (100%).

N-[*N*-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Benzyloxy-D-Valine Benzyl Ester

The mixture of *N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valine benzyl ester and *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (150mg) was dissolved in non-distilled DMF (15ml) and benzyl bromide (153.6µl, 1.3mmol) and Ag_2O (150mg, 0.65mmol) added. The mixture was shaken at room temperature, in the dark and under an argon atmosphere for 5h. The mixture was filtered, the filtrate added to H_2O (15ml) and the aqueous layer extracted with EtOAc (4x30ml). The organic extracts were dried over $MgSO_4$ and evaporated to a clear oil which was chromatographed on silica (8g) with toluene:EtOAc (9:1) as eluant to afford *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine benzyl ester (118mg, 68%), $FeCl_3$-negative; δ_H (200MHz,$CDCl_3$) 0.92 (3H,d,*J*6.6Hz,*val*4H), 0.98 (3H,d,*J*6.7Hz,*val*4H), 2.51 (1H,m,*val* 3H), 2.61 (2H,AB of ABX,δ_A2.71,δ_B2.51,*J*13.9,4.9,8.8Hz,*cys*3H), 3.50 (2H,AB,δ_A3.57,δ_B3.43,*J*13.1Hz,SC*H*$_2$Ph), 3.79 (3H,s,C*H*$_3$O), 4.70 (1H,d, *J*9.9Hz,*val*2H), 4.98 (2H,AB,δ_A5.07,δ_B4.89,*J*9.4Hz,OC*H*$_2$Ar), 5.10 (2H, AB pattern,OC*H*$_2$Ar), 5.16-5.23 (1H,m,*cys*2H), 5.20 (2H,s,OC*H*$_2$ Ar), 5.56 (1H,d,*J*9.3Hz,N*H*), 6.88 (2H,d,*J*8.6Hz,Ar*H*) and 7.02-7.50 (17H,m,Ar*H*); δ_C (75MHz,$CDCl_3$) 19.49 (q,*val*4C), 19.72 (q,*val*4C), 27.93 (d,*val*3C), 33.63, 35.90 (t,t,*cys*3C and S*C*H$_2$Ph), 50.59 (d,*cys*2C), 55.04 (q,*C*H$_3$O), 65.74 (d,*val*2C), 66.66 (t,O*C*H$_2$Ar), 66.91 (t,O*C*H$_2$Ar), 79.36 (t, NO*C*H$_2$Ph), 113.79, 126.74, 128.18, 128.24, 128.39, 128.57, 128.76, 128.96, 129.24, 129.52, 129.65, 133.77, 135.10, 137.44 (24xAr*C*), 155.99, 170.09, 172.23 (3x*C*=O).

N-[*N*-4-Methoxybenzyloxycarbonyl-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteine dicyclohexylamine salt (1.39g, 2.5mmol) was converted to the free acid, and dissolved in DMF (25ml). Dicyclohexylcarbodiimide (0.52g, 2.5mmol) was added and the solution stirred at room temperature for 5min. *N*-hydroxy-D-valine benzyl ester hydrochloride salt (0.68g, 2.6mmol) and pyridine (0.20ml, 2.5mmol) were added and the solution stirred for 2h, reduced in volume to *ca* 15ml and stirred overnight. The precipitated dicyclohexylurea was filtered and the filtrate evaporated to a yellow oil which was taken up in EtOAc (30ml). The EtOAc solution was washed with 1M-aq. HCl (20ml), sat. aq. $NaHCO_3$ (20ml), sat. aq. NaCl (20ml) and dried over $MgSO_4$. Evaporation left an oil which was filtered through silica (8g) and partially purified by chromatography on silica (30g) with EtOAc:*n*-hexane (5:5) as eluant. Initial fractions afforded *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*,*N*'-dicyclohexylurea (0.32g, 22%) as an oil. The later $FeCl_3$-positive fractions were combined, evaporated and chromatographed on silica (25g) with EtOAc:*n*-hexane:HOAc (37.5:60:2.5) as eluant to yield *N*-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (0.29g, 20%), m.p. 124-126°C (Found: C,64.24; H,6.15; N,4.68. $C_{31}H_{36}N_2O_7S$ requires C,64.12; H,6.25; N,4.82%); $FeCl_3$-positive; δ_H(200MHz, $CDCl_3$) 0.96 (3H,d,*J*6.8Hz,*val*4H), 0.98 (3H,d,*J*6.7Hz,*val*4H), 2.31-2.48 (1H,m,*val*3H), 2.77 (2H,AB of ABX,δ_A2.85,δ_B2.68,*J*13.8,4.1,7.1Hz, *cys*3H), 3.75 (2H,s,SCH_2Ph), 3.78 (3H,s,CH_3O), 5.02 (1H,d,*J*10.1Hz, *val*2H), 5.11 (2H,AB,δ_A5.22,δ_B4.99,*J*12.4Hz,OCH_2Ar), 5.08-5.19 (1H, m,*cys*2H), 5.16 (2H,s,OCH_2Ar), 5.50 (1H,d,*J*8.4Hz,N*H*), 6.86 (2H,d, *J*8.6Hz,Ar*H*), 7.20-7.35 (12H,m,Ar*H*) and 7.77 (1H,s,O*H*); δ_C (75MHz,

$CDCl_3$) 19.18 (q,*val*4C), 19.31 (q,*val*4C), 28.82 (d,*val*3C), 33.29, 36.33 (t,t,*cys*3C and SCH_2Ph), 50.14 (d,*cys*2C), 55.04 (q,CH_3O), 62.86 (d,*val*2C), 66.77 (t,OCH_2Ar), 67.03 (t,OCH_2Ar), 113.75, 126.88, 127.01, 128.13, 128.30, 128.42, 128.85, 129.70, 134.91, 137.67 (18xArC), 156.31, 159.40 and 170.84 (3xC=O); m/z (EI) 580.2226 (<1%) (M, $C_{31}H_{36}N_2O_7S$ requires 580.2243), 441 (8%), 335 (16%), 292 (16%), 263 (15%), 121 (100%) and 91 (100%).

Attempted Synthesis of *N*-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteine (247mg, 0.40mmol) and *N*-hydroxy-D-valine (98mg, 0.44mmol) were dissolved in DMF (5ml), dicyclohexylcarbodiimide (90mg, 0.44mmol) added and the solution stirred at room temperature for 24h. The dicyclohexylurea was filtered, the filtrate added to 50% sat. aq. NaCl (5ml) and extracted with EtOAc (3x10ml). Evaporation left a yellow gum which was $FeCl_3$-negative. The yellow gum was subjected to chromatography on silica (30g) with EtOAc: *n*-hexane (5:5) as eluant to afford *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*,*N*'-dicyclohexylurea as the only isolable product (90mg, 27%), $FeCl_3$-negative; δ_H [60MHz,$CDCl_3$:CD_3OD (1:1)] 0.82-2.22 (28H,m,*aminoadipyl*3,4,5H and *cyclohexyl*H), 2.62-2.80 (2H,m,*cys*3H), 3.65 (2H,s,SCH_2Ph), 4.00-4.50 (3H,m,*cys* 2H ,*aminoadipyl*2H and N*H*), 5.03 (2H,s,OCH_2Ar), 5.17 (2H,s, OCH_2Ar), 6.43 (1H,d,*J*8.0Hz,N*H*), 7.17-7.43 (12H,m,Ar*H*) and 8.22 (2H, d,*J*8.0Hz,Ar*H*); m/z (EI) 722 (<1%), 550 (<1%), 390 (1%), 279 (69%), 225 (14%), 224 (20%) and 91 (100%).

Attempted Synthesis of *N*-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

Crude *N*-[*N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (1.52g, 2.8mmol) was dissolved in trifluoroacetic acid (20ml), refluxed for 40min and evaporated to a brown oil.

N-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (1.31g, 3.0mmol), triethylamine (0.46ml, 3.3mmol) and ethylchloroformate (2.64ml, 27.6mmol) were dissolved in dry benzene (30ml). The solution was stirred at room temperature for 1h, filtered and evaporated to a clear oil which was dissolved in CH_2Cl_2 (40ml). The crude material from the trifluoroacetic acid deprotection and triethylamine (1.92ml, 13.8mmol) were added to the CH_2Cl_2 solution, and the mixture stirred at room temperature for 3½h to leave a golden yellow solution. The CH_2Cl_2 was removed *in vacuo*, the residue dissolved in EtOAc (50ml), washed with 1M-aq. HCl (30ml), H_2O (30ml), sat. aq. $NaHCO_3$ (30ml), sat. aq. NaCl (30ml), and dried over $MgSO_4$. Evaporation left an orange oil which was chromatographed on silica (200g) with EtOAc:*n*-hexane (5:5) as eluant to afford a crude product. The crude material was further purified by column chromatography on silica (30g) with a benzene:EtOAc (9:1)-benzene:EtOAc (6:4) gradient as eluant to yield an almost 50:50 mixture of two compounds. A small quantity of the mixture was separated by h.p.l.c., using a Waters μ-Bondapak C18 analytical column and MeOH:H_2O (72:28) as eluant, to yield *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester, $FeCl_3$-negative; δ_H(200MHz, $CDCl_3$) 0.82 (3H, d, *J*6.9Hz, *val*4H), 0.88 (3H, d, *J*6.8Hz, *val*4H), 1.67-1.95

(4H,m,*aminoadipyl*3,4H), 2.12-2.25 (3H,m,*aminoadipyl*5H and *val*3H), 2.75 (2H,AB of ABX,δ_A2.84,δ_B2.66,*J*13.9,5.6,7.4Hz,*cys*3H), 3.76 (2H,s, SCH_2Ph), 4.39-4.55 (3H,m,*aminoadipyl*, *cys* and *val* 2H), 5.10 (2H,s, OCH_2Ph), 5.11 (2H,AB,δ_A5.16,δ_B5.06,*J*12.2Hz,OCH_2Ph), 5.23 (2H,s, OCH_2Ph), 5.61 (1H,d,*J*8.2Hz,N*H*), 6.23 (1H,d,*J*7.3Hz,N*H*), 6.72 (1H,d, *J*8.7Hz,N*H*), 7.27-7.37 (15H,m,Ar*H*), 7.47 (2H,d,*J*8.6Hz,Ar*H*) and 8.18 (2H,d,*J*8.6Hz,Ar*H*) and *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester, $FeCl_3$-positive; δ_H (200MHz,$CDCl_3$) 0.97 (6H,d,*J*6.5Hz,*val* 4,4'H), 1.67-1.87 (4H,m,*aminoadipyl*3,4H), 2.15-2.17 (2H,m,*aminoadipyl*5H), 2.41 (1H,dsept.,*J*7.8,6.6Hz,*val*3H), 2.76 (2H,AB of ABX, δ_A2.86,δ_B2.67,*J*13.8,5.8,7.7Hz,*cys*3H), 3.72 (2H,s,SCH_2Ph), 4.38 (1H, m,*aminoadipyl*2H), 4.92 (1H,d,*J*7.8Hz,*val*2H), 5.05-5.31 (7H,m,3xOCH_2Ar and *cys*2H), 5.54 (1H,d,*J*7.9Hz,N*H*), 6.21 (1H,d,*J*7.7Hz,N*H*), 7.18-7.39 (15H,m,Ar*H*), 7.46 (2H,d,*J*8.5Hz,Ar*H*), 8.20 (2H,d,*J*8.5Hz,Ar*H*) and 8.34 (1H,s,O*H*); m/z (EI) 369 (12%), 355 (11%), 263 (95%), 160 (40%), 114 (63%) and 91 (100%).

N-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Benzyloxy-D-Valine Benzyl Ester

The mixture of *N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine benzyl ester and *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (140mg) was dissolved in non-distilled DMF (14ml). Benzyl bromide (120.7µl, 1.0mmol) and Ag_2O (118mg, 0.51mmol) were added and the solution shaken at room temperature, in the dark, for 5h under an argon atmosphere. The solution was filtered through celite, evaporated and chromatographed on silica (25g) with an EtOAc:*n*-hexane (4:6)-EtOAc:*n*-hexane

(7:3) gradient as eluant to afford *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine benzyl ester (69mg) as an oil, $[\alpha]_D$-9.8° (c3.0, acetone); $FeCl_3$-negative; δ_H (200MHz,$CDCl_3$) 0.91 (3H,d,*J*6.6Hz,*val*4H), 0.97 (3H,d,*J*6.7Hz,*val*4H), 1.68-1.94 (4H,m,*aminoadipyl*3,4H), 2.21-2.27 (2H,m,*aminoadipyl*5H), 2.41-2.75 (3H,m,*cys*3H and *val*3H), 3.44 (2H, AB,δ_A3.51,δ_B3.37,*J*13.2Hz,SCH_2Ph), 4.41-4.43 (1H,m,*aminoadipyl*2H), 4.66 (1H,d,*J*9.8Hz,*val*2H), 5.06 (2H,AB,δ_A5.22,δ_B4.92,*J*9.3Hz,OCH_2Ar), 5.10 (2H,AB pattern,OCH_2Ar), 5.17 (2H,s,OCH_2Ar), 5.18 (2H,s,OCH_2Ar), 5.36-5.41 (1H,m,*cys*2H), 5.71 (1H,d,*J*8.0Hz,N*H*), 6.38 (1H,d,*J*8.9Hz, N*H*), 6.98-7.16 (5H,m,Ar*H*), 7.26-7.51 (17H,m,Ar*H*) and 8.15 (2H,d, *J*8.6Hz,Ar*H*); δ_C (75MHz,$CDCl_3$) 19.56 (*val*4C), 19.79 (*val*4C), 21.32 (*aminoadipyl*4C), 28.09, 31.37, 33.73, 35.23, 36.10 (*val*3C,*cys*3C, *aminoadipyl*3C and 5C and SCH_2Ph), 48.62 (*cys*2C), 53.95 (*aminoadipyl* 2C), 65.33 (OCH_2Ar), 66.23 (*val*2C), 66.95, 79.38 (3xOCH_2Ar), 123.63, 126.81, 127.85, 127.97, 128.23, 128.33, 128.41, 128.56, 128.73, 128.92, 129.41 (24xAr*C*), 133.90, 135.17, 136.23, 137.51, 142.42, 147.83 (6xAr*C*), 155.89, 169.86, 171.74, 171.87 and 172.39 (5x*C*=O).

Attempted Synthesis of *N*-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Benzyloxy-D-Valine Benzyl Ester

N-(*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl)-*N*-benzyloxy-D-valine benzyl ester (120mg, 0.18mmol) was dissolved in HCl sat.-$MeNO_2$ (6ml) and stirred at room temperature for 5min. Evaporation afforded an oil which was subjected to column chromatography on silica (8g) with EtOAc:*n*-hexane:HOAc (7:2:1) as eluant. The ninhydrin-positive fractions were combined, washed with sat. aq. $NaHCO_3$ (30ml), H_2O (30ml), sat. aq. NaCl (30ml) and dried over Na_2SO_4. Evaporation yielded *N*-[*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine

benzyl ester (75mg, 83%), m.p. 128-130°C; δ_H (200MHz,$CDCl_3$) 0.97 (3H,d,*J*7.0Hz,*val*4H), 0.99 (3H,d,*J*7.0Hz,*val*4H), 2.36-2.44 (3H,m,*val* 3H and -NH_2), 2.94 (2H,AB of ABX,δ_A3.21,δ_B2.69,*J*14.1,3.2,8.7Hz,*cys* 3H), 3.75 (2H,s,SCH_2Ph), 3.82 (1H,dd,*J*8.7,3.2Hz,*cys*2H), 3.85br (1H, d,*val*2H), 4.67 (2H,s,OCH_2Ph), 4.92 (2H,AB,δ_A4.97,δ_B4.86,*J*10.5Hz, OCH_2Ph) and 7.18-7.40 (15H,m,Ar*H*).

The *N*-[*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine benzyl ester (75mg, 0.15mmol) was dissolved in EtOAc (10ml). *N*-benzyloxycarbonyl -L-α-aminoadipic acid 1-(4-nitrobenzyl) ester (69mg, 0.16mmol), 1-hydroxybenzotriazole (21mg, 0.16mmol) and dicyclohexylcarbodiimide (32mg, 0.16mmol) were added and the solution stirred at room temperature overnight. Oxalic acid dihydrate (1.5mg) was added, the solution stirred a further 1h and filtered. The filtrate was washed with 1M-aq. HCl (10ml), sat. aq. $NaHCO_3$ (10ml), sat. aq. NaCl (10ml) and dried over $MgSO_4$. Evaporation afforded a complex mixture of at least seven products which could not be purified.

N-[*N*-Benzyloxycarbonyl-1-(4-Nitrobenzyl)-δ-(L-α-Aminoadipyl)-*S*-Benzyl-L-Cysteinyl]-*N*-Hydroxy-D-Valine Benzyl Ester

N-[*N*-4-methoxybenzyloxycarbonyl-*S*-benzyl-L-cysteinyl]-*N*-hydroxy -D-valine benzyl ester (295mg, 0.51mmol) was dissolved in HCl-sat. $MeNO_2$ (15ml) and stirred at room temperature for 3min. The solution was evaporated and the residue dissolved in EtOAc (20ml). The organic layer was washed with 5% aq. $NaHCO_3$ (20ml), sat. aq. NaCl (20ml) and dried over $MgSO_4$. The EtOAc was removed *in vacuo* to afford crude *N*-[*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester.

N-benzyloxycarbonyl-L-α-aminoadipic acid 1-(4-nitrobenzyl)

ester (240mg, 0.56mmol), triethylamine (81.4µl, 0.58mmol) and ethyl-chloroformate (487µl, 5.1mmol) were dissolved in dry toluene (20ml) and stirred at room temperature for 30min. The solution was filtered, evaporated and the residue dissolved in CH_2Cl_2(10ml). The crude *N*-[*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester and *N*-methylmorpholine (58.7µl, 0.53mmol) were added to the CH_2Cl_2solution and the mixture stirred at room temperature for 3½h. The solution was washed with 1M-aq. HCl (15ml), sat. aq. $NaHCO_3$ (15ml), sat. aq. NaCl (15ml) and dried over $MgSO_4$. Evaporation afforded a crude solid which was chromatographed on silica (25g) with EtOAc: *n*-hexane (5:5) as eluant. The $FeCl_3$-positive fractions were combined, evaporated and further purified by column chromatography on silica (25g) with EtOAc:*n*-hexane (6:4) as eluant to yield crude *N*-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (159mg, 38%) which was crystallized from $CHCl_3$/ether (120mg, 29%), m.p. 151-154°C; $[\alpha]_D$-15.3° (c1.0, CH_2Cl_2) (Found: C,62.28; H,5.94; N,6.72. $C_{43}H_{48}N_4O_{11}S$ requires C,62.31; H,5.84; N,6.76%); δ_H (200MHz, $CDCl_3$) 0.97 (6H,d,*J*6.8Hz,*val*4,4'H), 1.66-1.90 (4H,m,*aminoadipyl* 3,4H), 2.15-2.21 (2H,m,*aminoadipyl*5H), 2.41 (1H,dsept.,*J*7.8,6.8Hz, *val*3H), 2.76 (2H,AB of ABX,δ_A2.86,δ_B2.67,*J*13.8,5.7,7.7Hz,*cys*3H), 3.71 (2H,s,SC*H*$_2$Ph), 4.35-4.44 (1H,m,*aminoadipyl*2H), 4.92 (1H,d, *J*7.8Hz,*val*2H), 5.03-5.31 (7H,m,3xOC*H*$_2$Ar and *cys*2H), 5.55 (1H,d, *J*7.8Hz,N*H*), 6.20 (1H,d,*J*7.5Hz,N*H*), 7.20-7.36 (15H,m,Ar*H*), 7.46 (2H, d,*J*8.6Hz,Ar*H*), 8.18 (2H,d,*J*8.6Hz,Ar*H*) and 8.38 (1H,s,NO*H*); δ_C (75MHz,$CDCl_3$) 19.27 (q,*val*4C), 19.31 (q,*val*4C), 21.33 (t,*aminoadipyl* 4C), 28.68 (d,*val*3C), 31.13 (t,*aminoadipyl*4C), 32.74 (t,*cys*3C), 34.91, 36.23 (t,t,*aminoadipyl*5C and SCH_2Ph), 48.22 (d,*cys*2C), 53.75

(d, *aminoadipyl*2C), 63.10 (d,*val*2C), 65.35, 66.97 (t,t,3xO*C*H_2Ar), 123.64, 127.01, 127.90, 128.06, 128.15, 128.30, 128.39, 128.83 (19xAr*C*), 134.98, 136.09, 137.60, 142.43, 147.64 (5xAr*C*), 156.06, 170.35, 170.55, 171.84 and 172.93 (5x*C*=O); m/z (EI) 507 (<1%), 263 (4%), 214 (6%) and 91 (100%); m/z (FAB) 867 (54%) (M+K), 829 (100%) (M+H), 813 (55%) and 696 (62%).

Attempted Synthesis of *N*-[δ-(L-α-Aminoadipyl)-L-Cysteinyl]-*N*-Hydroxy-D-Valine

N-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl]-*N*-benzyloxy-D-valine benzyl ester (64mg, 0.07 mmol) was dissolved in refluxing liquid ammonia (5ml) under a N_2 atmosphere. Small pieces of sodium metal were added until the blue colour persisted for 5min. Ammonium acetate (*ca*50mg) was added and the ammonia blown off in a stream of dry N_2. The residue was dessicated over H_2SO_4 for 2h and dissolved in 5% aq. HOAc (5ml). The aqueous solution was filtered, washed with ether (5ml) and adjusted to pH8.5 with 1M-aq. NH_3. Oxygen gas was bubbled through the solution for 2h. The solution was freeze dried and the residue applied to an ion exchange column (15x1cm) with a H_2O-2M-aq. pyridine gradient as eluant. The ninhydrin-positive fractions were combined and lyopholized to afford a mixture of two products which were separated by preparative scale electrophoresis at pH6.5 to yield *N*-[δ-(α-aminoadipyl) cysteinyl]-*N*-hydroxyvaline (5mg, 19%), $FeCl_3$-positive; m/z (FAB) 378.1327 (M-H,$C_{14}H_{24}N_3O_7S$ requires 378.1329) and 360.1226 (M-H-H_2O,$C_{14}H_{22}N_3O_6S$ requires 360.1224) and its disulphide (6mg, 23%), $FeCl_3$-positive; m/z (FAB) 755 (M-H), 378.

N-[δ-(L-α-Aminoadipyl)-L-Cysteinyl]-*N*-Hydroxy-D-Valine

N-[*N*-benzyloxycarbonyl-1-(4-nitrobenzyl)-δ-(L-α-aminoadipyl)-

S-benzyl-L-cysteinyl]-*N*-hydroxy-D-valine benzyl ester (60mg, 0.07 mmol) was dissolved in refluxing liquid ammonia (20ml) under a N_2 atmosphere. Small pieces of sodium metal were added till the blue colour persisted for 3 min. Solid NH_4OAc (*ca*100mg) was added to leave a clear solution which was evaporated in a stream of dry N_2 gas. The residue obtained was dissolved in 10% aq. HOAc (5ml) washed with ether(5ml) and lyopholized. The residue was purified by ion exchange chromatography (18x1cm column) with a H_2O-1M-aq. pyridine gradient as eluant to afford *N*-[δ-(L-α-aminoadipyl)-L-cysteinyl]-*N*-hydroxy-D-valine (26mg, 95%), m.p. 157-160°C (decomp.); $[\alpha]_D$-30.8° (*c*0.5, H_2O); $[\alpha]_{546}$-33.6° (*c*0.5, H_2O) (Found: C,44.18; H,6.70; N,10.96. $C_{14}H_{25}N_3O_7S$ requires C,44.32; H,6.64; N,11.07%); $FeCl_3$-positive; δ_H (200MHz, D_2O) 1.03 (3H,d,*J*6.8Hz,*val*4H), 1.08 (3H, d,*J*6.8Hz,*val*4H), 1.68-2.02 (4H,m,*aminoadipyl*3,4H), 2.34-2.50 (3H,m, *aminoadipyl*5H and *val*3H), 3.01 (2H,AB of ABX,δ_A3.09,δ_B2.93,*J*14.0, 5.3,7.4Hz,*cys*3H), 3.82-3.87 (1H,m,*aminoadipyl*2H), 4.66 (1H,d,*J*8.8Hz, *val*2H) and 5.22 (1H,X of ABX,*J*5.2,7.3Hz,*cys*2H); δ_C (75MHz,D_2O,pH3) 19.57 (q,*val*4C), 19.65 (q,*val*4C), 21.73 (t,*aminoadipyl*4C), 25.55 (t,*cys*3C), 28.61 (d,*val*3C), 30.55 (t,*aminoadipyl*3C), 35.54 (t,*amino-adipyl*5C), 53.13 (d,*aminoadipyl*2C), 55.15 (d,*cys*2C), 66.62 (d,*val*2C), 174.94, 175.80 and 176.50 (3x*C*=O); m/z (FAB) 795 (35%) (M+K) and 757 (100%) (M+H).

6.5 *N*-BENZYLOXYCARBONYL-L-α-AMINOADIPIC ACID 1-BENZYL ESTER

N^{α}-Benzyloxycarbonyl-L-Lysine Benzyl Ester 4-Toluenesulphonic Acid Salt

N^{α}-benzyloxycarbonyl-L-lysine [515] (23.83g, 85.0mmol) and 4-toluenesulphonic acid monohydrate (16.98g, 89.3mmol) were dissolved

in a mixture of dry benzene (90ml) and benzyl alcohol (30ml). The solution was fitted with a Dean-Stark trap and refluxed under an Ar atmosphere for 18h. The solution was filtered, evaporated and the residue crystallized from EtOH/ether/*n*-hexane to afford N^{α}-benzyloxycarbonyl-L-lysine benzyl ester 4-toluenesulphonic acid salt (43.95g, 92%) m.p. 111-113°C (lit.[542], m.p. 113-115°C; lit.[543], m.p. 111-112°C); $[\alpha]_D$-17.9° (c2.0,MeOH) (Found: C,61.77; H,6.11; N,5.39. $C_{28}H_{34}N_2O_7S$ requires C,61.97; H,6.32; N,5.16%); δ_H (360MHz,$CDCl_3$) 1.16-1.25 (2H,m,*lys*4H), 1.41-1.63 (4H,m,*lys*3,5H), 2.26 (3H,s, CH_3), 2.69br (2H,m,*lys*6H), 4.24 (1H,m,*lys*2H), 5.02 (2H, AB,δ_A5.06,δ_B4.99,*J*12.1Hz,OCH_2Ph), 5.08 (2H,s,OCH_2Ph), 5.68 (1H,d, *J*8.0Hz,N*H*), 7.06 (2H,d,*J*8.0Hz,Ar*H*), 7.25-7.28 (10H,m,Ar*H*), 7.61 (3H, s,$-\overset{+}{N}H_3$) and 7.70 (2H,d,*J*8.0Hz,Ar*H*); m/z (EI) 370 (16%) (M,free base), 279 (<1%), 235 (1%), 206 (2%), 128 (18%) and 91 (100%).

N-Benzyloxycarbonyl-L-α-Aminoadipic Acid 1-Benzyl Ester

N^{α}-benzyloxycarbonyl-L-lysine benzyl ester 4-toluenesulphonic acid salt (1.68g, 3.0mmol) was converted to the free base and dissolved in *t*-BuOH:H_2O (2:1) (30ml). Potassium permanganate (0.63g, 4.0mmol) and $CaSO_4$ (2.45g, 18.0mmol) were added, the pH of the solution adjusted to 7 and the mixture heated to 60°C under an Ar atmosphere for 40min. A few drops of sat. aq. $Na_2S_2O_5$ were added, the mixture filtered through celite and the filtrate reduced in volume. Sat. aq. NaCl (20ml) was added, the pH adjusted to 1 with conc. aq. HCl and the solution extracted with EtOAc (3x30ml). The organic extracts were washed with 1M-aq. HCl (30ml), sat. aq. $NaHCO_3$ (2x30ml), sat. aq. NaCl (30ml) and dried over $MgSO_4$. The EtOAc solution was evaporated and the residue purified by filtration through silica (5g) and column chromatography on silica (25g) with EtOAc:*n*-hexane (5:5)

as eluant. The crude product was crystallized from EtOAc/CH_2Cl_2/ *n*-hexane to afford *N*-benzyloxycarbonyl-L-α-aminoadipic acid 1-benzyl ester (95mg, 8%) m.p. 87-89°C (lit.[516], m.p. 87-89°C); $[\alpha]_D$-13.1° (c2.0, acetone) [lit.[516], $[\alpha]_D$-13.3°(c2.0, acetone)] (Found: C,65.24; H,6.02; N,35.2. $C_{21}H_{23}NO_6$ requires C,65.44; H,6.02; N,3.63%); δ_H (200MHz, CD_3COCD_3) 1.72-2.02 (4H,m,*aminoadipyl*3,4H), 2.35-2.39 (2H,m,*aminoadipyl*5H), 4.30 (1H,m,*aminoadipyl*2H), 5.09 (2H,s,OC*H*$_2$Ph), 5.18 (2H,s,OC*H*$_2$Ph), 6.75 (1H,d,*J*8.0Hz,N*H*) and 7.30-7.41 (10H,m,Ar*H*).

6.6 CELL FREE SYSTEM FROM *CEPHALOSPORIUM ACREMONIUM* CW19

6.6.1 Culture and Growth Conditions

Vegatative slopes of *Cephalosporium acremonium* CW19 (ATCC 36225) were washed with sterile water (5ml) and 0.2ml aliquots aseptically transferred to each of twenty slopes of a defined sporulation agar[544]. The slopes were grown at 26°C for 5 days. One slope was used to inoculate 50ml of seed medium [544] in a 250ml flask. The cells were grown for 3 days on a rotary shaker at 26°C and 240 r.p.m. and *ca* 2ml of seed media used to inoculate 50ml of growth media [544] in each of forty 250ml flasks. The flasks were shaken on a rotary shaker at 240 r.p.m. and incubated at 26°C for up to 7 days. A growth curve for the organism was determined [518]. Four parameters were measured; the absorbance of the culture at 600nm, the dry weight of the mycelium, the pH of the culture medium and the concentration of cephalosporin C in the culture medium. In the range 72-84h the absorbance and dry weight reached a maximum and the pH dropped to a minimum. Cephalosporin C concentration increased from 96h onwards.

6.6.2 Preparation of Cell-Free System

Twelve 1000ml flasks containing 250ml of growth medium were inoculated with *ca* 10ml of a 3 day-old seed culture. The flasks were shaken at 250 r.p.m. and at 26°C for 3 days and the mycelial mass collected on Whatman 54 filter paper. The cell mass was thoroughly washed with morpholinopropanesulphonic acid (MOPS) buffer (50mmol, pH7.2), resuspended in the same buffer (400ml) and ground in a Dyno-Mill. The 300ml Dyno-Mill cell was cooled to 0°C and contained 240ml of glass beads. The tip of blade speed was 10m/s and the cell was fitted with a 0.03mm distance piece. The cell mass was pumped through the Dyno-Mill cell at 200ml/min. The homogenate was collected at 0°C, saturated to 50% with $(NH_4)_2SO_4$ and centrifuged at 20,000g for 30min. An oily film was removed from the surface of the supernatant and the supernatant 80% saturated with $(NH_4)_2SO_4$. The mixture was centrifuged at 20,000g for 30min and the supernatant discarded. The pellet was gently mixed with MOPS buffer (50mmol, pH7.2) (10ml), applied to a Sephadex G25 column (100ml) and eluted with MOPS buffer (50mmol, pH7.2). Fractions were collected and tested for the presence of SO_4^{2-} by addition of sat. aq. $BaCl_2$ to a small aliquot of each fraction. The UV absorbance at 280nm was measured and those fractions which were SO_4^{2-} -negative, and displayed the largest UV absorbance, were combined and stored at -20°C.

Occasionally the cell-free system was concentrated using an Amicon Ultrafilter fitted with a PM10 (molecular weight > 10,000 a.m.u.) filter under a nitrogen pressure of 56 p.s.i.

6.6.3 Co-Factor Requirements

The cell-free preparation (1.00ml) and LLD-ACV (1mg/ml solution in MOPS (50mmol, pH7.2) buffer) (0.25ml) were added to 10ml conical flasks. Various quantities of DTT and $FeSO_4$ were added to the flasks to achieve final concentrations of between zero and ten millimolar. The flasks were incubated at 26°C and shaken on a rotary shaker at 250 r.p.m. Aliquots (100µl) were removed from the incubation mixtures and bioassayed against *B. subtilus*. The results indicated a requirement for DTT with the optimum concentration in the range 2-5mmol. The requirement for ferrous ions by this system could not be accurately determined and appeared to vary from batch to batch. A final ferrous ion concentration of 1mmol was normally used [352] in subsequent feeding experiments. The optimum incubation period was 2h, although the reaction was shown to be 85% complete within 1h.

7. INDEX TO EXPERIMENTAL

For the preparation of compound X see page Y.

R E F E R E N C E S

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P U B L I C A T I O N S

J. CHEM. SOC. PERKIN TRANS. I 1983

Synthesis of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine and δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valylglycine

Gordon A. Thomson, A. Ian Scott * and Robert L. Baxter *
Chemistry Department, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ

An efficient synthesis of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV; 1) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine (ACVG; 2) *via* the protected tripeptide *N*-benzyloxycarbonyl-1-(*p*-nitrobenzyl)-δ-(L-α-aminoadipyl)-*S*-benzyl-L-cysteinyl-D-valine, benzhydryl ester (3) is described.

In the course of studies on the biosynthesis of the penicillins and their acyclic precursor,[1] δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV; 1),† we required an efficient synthesis of the tripeptide which could be utilised for the preparation of ^{13}C labelled isotopomers. In addition we wished to prepare δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine (ACVG; 2), for biosynthetic studies. A tetrapeptide with this sequence of amino-acids but of undefined stereochemistry has been isolated from a β-lactam producing *Cephalosporium sp.*[2] but its significance in penicillin biosynthesis is as yet unknown.

Five syntheses of the ACV tripeptide have been reported previously in the literature [3–6] each of which utilise different strategies and/or protecting groups. Two distinct routes have been employed involving elaboration from either the valine carboxy [3–6] or the amino-terminus.[6] Since it was our intention to prepare isotopomers labelled in the valine residue the overall yield from D-valine was of paramount importance and necessitated an approach using the second strategy. We report here a synthesis of ACV (1) by a route employing benzyl-based and benzhydryl-based protecting groups which offers the advantage of removal of the protecting groups by reduction with sodium in liquid ammonia [6] or selective deprotection of the valine carboxy-group to afford, in a single step, the acid (4) as the intermediate for the synthesis of ACVG (2).

Treatment of *N*-benzyloxycarbonyl-L-α-aminoadipic acid [7] (6a) with *p*-nitrobenzyl bromide and one equivalent of triethylamine in dimethylformamide afforded the ester (6b) in 50% yield. Coupling of this derivative with *S*-benzyl-L-cysteine, benzhydryl ester [8] (7) was carried out with 1-hydroxybenzotriazole and dicyclohexylcarbodi-imide [9] to give the fully protected dipeptide (8a). Removal of the benzhydryl protecting group was achieved in virtually quantitative yield by mild acid hydrolysis and the resulting dipeptide acid (8b) coupled with D-valine benzhydryl ester using 1-hydroxybenzotriazole and dicyclohexylcarbodi-imide to afford the fully protected tripeptide (3) in 22% overall yield from (6a).

Reduction of the protected ACV (3) with sodium in liquid ammonia gave the unprotected tripeptide (1). Isolation as the corresponding sulphide followed by regeneration with hydrogen sulphide afforded ACV in apparently 77% yield. However elemental analysis of the freeze-dried product showed it to contain *ca.* 4% inorganic contaminants.[6] The product was shown to be a 3 : 1 mixture of (1) and the corresponding disulphide (9) by Ellman's procedure [10] and by paper electrophoresis. To circumvent the inhomogeneity introduced by the above procedure the crude product from the reduction was oxidised by passing air through an aqueous solution and the resultant disulphide [5] (9) isolated by ion exchange chromatography. The product obtained in this manner, in 96% yield from (3), was homogeneous by paper electrophoresis, h.p.l.c., and t.l.c. and proved free of inorganic contaminants. Confirmation that the product was the desired disulphide (9) was obtained from the ^{13}C n.m.r. spectrum which showed a resonance at 38.5 p.p.m. corresponding to C-3 of the cystine residue. In contrast the spectrum of the monomeric tripeptide showed a peak at 26.1 p.p.m.[6] for C-3 of cysteine. Oxidation of (9) with performic acid [11] afforded the sulphonic acid (11) which proved to be chromatographically and spectroscopically identical with material prepared from ACV isolated from *Cephalosporium acremonium* N-2.[1]

With the protected tripeptide (3) in hand, synthesis of ACVG (2) could now be carried out in a straightforward manner. Deprotection of the valine carboxy-group by mild acid hydrolysis afforded the acid (4). Coupling of (4) with glycine benzyl ester using dicyclohexylcarbodi-imide and 1-hydroxylbenzotriazole as above gave only poor yields of the desired product, the major product being the corresponding *N*-acyl urea.[12] In contrast, treatment of (4) with ethyl chloroformate in the presence of pyridine to afford a mixed anhydride and subsequent reaction of the anhydride with glycine benzyl ester gave the desired product (5) in 46% yield from (3). Sodium–liquid ammonia reduction of (5) followed by oxidation and purification of the product by ion-exchange chromatography as described for (9) above gave the tetrapeptide disulphide (10).

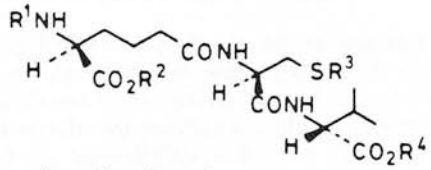
The disulphides (9) and (10) were quantitatively reduced to the respective monomers (1) and (2) with an excess of ethanethiol. The overall yields of ACV (ACVG) from D-valine and *S*-benzyl-L-cysteine by the route described were 68 (24%) and 43 (15%) respectively, which compare favourably with yields achieved in previous syntheses.[3–6]

Experimental

Electrophoresis was carried out at 50 V/cm on Whatman 3MM paper using the apparatus described by Michl,[13] pH 2.1 and 3.5 buffers were prepared as described by Ambler.[14] Merck silica G60 (70—230 mesh) was used for column chromatography and preparative t.l.c. was carried out on 200 × 200 × 1 mm layers of Merck 60GF254 silica. Thiol determinations were carried out using Ellman's procedure.[10] L-α-Aminoadipic acid $[\alpha]_D^{20}$ +74° (*c* 2.0, 5M-HCl) (lit.,[15] $[\alpha]_D$ +25°) was purchased from Sigma Chemical Co. Inc. Solvents were purified and dried by standard procedures and organic extracts typically dried over $MgSO_4$ or Na_2SO_4. N.m.r. spectra were recorded on Varian EM360, Bruker WM300, or Bruker WM360 spectrometers. Mass spectra (e.i.) were recorded on an AEI MS901 and a Kratos MS50 using a FAB source.

p-Nitrobenzyl N-Benzyloxycarbonyl-L-α-aminoadipate (6b).—*N*-Benzyloxycarbonyl-L-α-aminoadipic acid [7] (2.065 g, 7.00 mmol) was dissolved in dimethylformamide (5 cm^3),

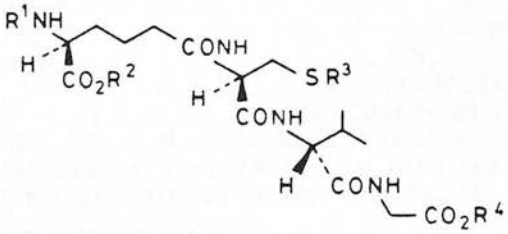
† α-Aminoadipyl = 5-amino-5-carboxypentanoyl



(1) $R^1 = R^2 = R^3 = R^4 = H$

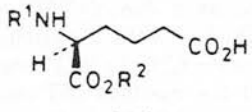
(3) $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$, $R^3 = PhCH_2$, $R^4 = Ph_2CH_2$

(4) $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$, $R^3 = PhCH_2$, $R^4 = H$

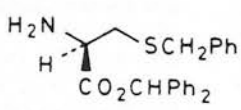


(2) $R^1 = R^2 = R^3 = R^4 = H$

(5) $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$, $R^3 = PhCH_2$, $R^4 = PhCH_2$



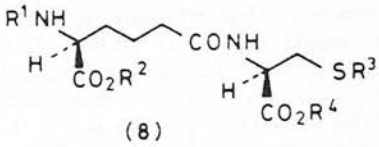
(6)



(7)

a, $R^1 = PhCH_2OCO$, $R^2 = H$

b, $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$

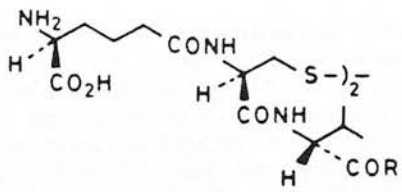


(8)

a, $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$, $R^3 = PhCH_2$, $R^4 = Ph_2CH$

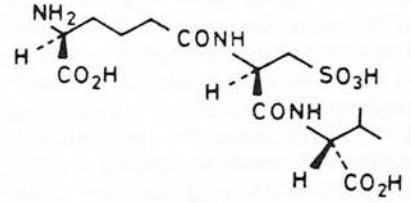
b, $R^1 = PhCH_2OCO$, $R^2 = p\text{-}NO_2C_6H_4CH_2$, $R^3 = PhCH_2$, $R^4 = H$

triethylamine (1.02 cm³, 7.35 mmol) added, and the olsution cooled to 0 °C. To the stirred solution, *p*-nitrobenzyl bromide (1.588 g, 7.35 mmol) was added in aliquots during 5 h. The reaction mixture was allowed to come to room temperature overnight, after which saturated aqueous NaCl (25 cm³) was added; the mixture was then adjusted to pH 1 with 5M-HCl and extracted with EtOAc (30 cm³ × 4). The extracts were evaporated to yield a yellow oil which was chromatographed on silica (200 g) with EtOAc–n-hexane (7 : 3) as eluant to afford (6b) which crystallised from EtOAc–n-hexane (1.22 g, 50%), m.p. 101—104 °C; $[\alpha]_D^{20}$ − 8.01° (*c* 2.0, acetone) (Found: C, 58.4; H, 5.1; N, 6.45. $C_{21}H_{22}N_2O_8$ requires C, 58.60; H, 5.15; N, 6.51%), δ(60 MHz, $CDCl_3$) 1.45—2.05 (4 H, m, 3, 4-H), 2.37 (2 H, t, *J* 5.5 Hz, 5-H), 4.18—4.70 (1 H, m, 2-H), 5.12 and 5.23 (4 H, 2s, benzyl-H), 5.4 (1 H, m, NH), 7.32 (5 H, s, ArH), 7.43 and 8.13 (4 H, 2 d, *J* 9 Hz, ArH),



(9) R = OH

(10) R = $NHCH_2CO_2H$



(11)

8.75 (1 H, s, CO_2H); *m/z* 430 (M^+), 306, and 250.1091 ($C_{13}H_{16}NO_4$ requires 250.1079), 206.1168 ($C_{12}H_{16}NO_2$ requires 206.1181).

N-*Benzyloxycarbonyl*-1-(p-*nitrobenzyl*)-δ-(L-α-*aminoadipyl*)-S-*benzyl*-L-*cysteine*, *Benzhydryl Ester* (8a).—*S*-Benzyl-L-cysteine (0.422 g, 2 mmol) was converted into the corresponding benzhydryl ester as previously described.[8] The crude product was added to a solution of (6b) (0.88 g, 2.05 mmol) and 1-hydroxybenzotriazole hydrate (0.284 g, 2.1 mmol) in EtOAc (10 cm³). To the stirred solution, dicyclohexycarbodi-imide (0.433 g, 2.1 mmol) in EtOAc (5 cm³) was added and the mixture stirred overnight. After addition of oxalic acid dihydrate (13 mg) the solution was stirred for 1 h, filtered, and the filtrate washed successively with aqueous HCl (1M; 30 cm³), water (30 cm³), and saturated aqueous $NaHCO_3$ (30 cm³), water (30 cm³) and saturated NaCl (30 cm³). Evaporation afforded an oil which was chromatographed on silica (150 g), with EtOAc–n-hexane (6 : 4) as eluant, to give (8a) which was crystallised from EtOAC–n-hexane (1.01 g, 64%), m.p. 122—127 °C; $[\alpha]_D^{20}$ −5.64° (*c* 1.0, CH_2Cl_2) (Found: C, 66.65; H, 5.6; N, 5.1. $C_{44}H_{43}N_3O_9S$ requires C, 66.90; H, 5.49; N, 5.32%), δ(360 MHz, $CDCl_3$) 1.68—1.90br (4 H, m, aminoadipyl 3,4-H), 2.15—2.26br (2 H, m, aminoadipyl 5-H), 2.85 (2 H, AB of ABX δ_A 2.91, δ_B 2.79, *J* 13.9, 4.7, 6.4 Hz, cys 3-H), 3.58 (2 H, AB; δ_A 3.62, δ_B 3.54, *J* 13.4 Hz, SCH_2Ph), 4.41 (1 H, m, aminoadipyl 2-H), 4.90 (1 H, M of ABMX, *J* 4.7, 6.4, 7.9 Hz, cys 2-H), 5.09 (2 H, AB; δ_A 5.12, δ_B 5.06, *J* 12.2 Hz; OCH_2Ar), 5.21 (2 H, AB, δ_A 5.24, δ_B 5.19, *J* 13.6 Hz, OCH_2Ar), 5.47 (1 H, d, *J* 7.9 Hz, NH), 6.19 (1 H, d, *J* 7.8 Hz, NH), 6.86 (1 H, s, $CHPh_2$), 7.18—7.41 (20 H, m, ArH), 7.46 and 8.17 (4 H, 2 d, *J* 8.5 Hz, ArH); *m/z* 698 (*M* − 91), 622 (*M* − 167), 167, and 91.

N-*Benzyloxycarbonyl*-1-(p-*nitrobenzyl*)-δ-(L-α-*aminoadipyl*)-S-*benzyl*-L-*cysteinyl*-D-*valine*, *Benzhydryl Ester* (3)—The protected dipeptide (8a) (0.59 g, 0.75 mmol) was dissolved in 0.2M HCl–nitromethane (19 cm³) and the solution stirred at room temperature for 5 h; it was then evaporated to give an oil which was subjected to column chromatography on silica (30 g), with EtOAc–n-hexane–HOAc (90 : 10 : 0.4) as eluant to afford the free acid (8b) (0.46 g, 98%) as a colourless foam.

The acid (8b) was added to a solution of 1-hydroxybenzotriazole hydrate (0.11 g, 0.83 mmol) and D-valine, benzhydryl ester [prepared from D-valine (97 mg, 0.83 mmol) as described [8]] in EtOAc (7.5 cm³). Dicyclohexylcarbodi-imide

(0.17 g) in EtOAc (5 cm^3) was added, the reaction mixture stirred overnight, treated with oxalic acid, and washed as described above. Evaporation of the organic fraction afforded crude (3) which was purified by column chromatography on silica (30 g), with EtOAc–n-hexane (6 : 4) as eluant, and crystallised from CH_2Cl_2–ether–n-hexane as colourless needles (0.47 g, 71%), m.p. 77—80 °C remelting at 133—135 °C; $[\alpha]_{436}^{20}$ −11.9° (*c* 1.0, acetone) (Found: C, 66.4; H, 5.8; N, 6.0. $C_{49}H_{52}N_4O_{10}S$ requires C, 66.20; H, 5.90; N, 6.30%); δ(300 MHz, $CDCl_3$) 0.75 (3 H, dd, *J* 6.9, 1.1 Hz, val 4-H), 0.87 (3 H, d, *J* 7.0 Hz, val 4-H), 1.68—1.85 (4 H, m, aminoadipyl 3, 4-H), 2.10—2.26 (3 H, m, aminoadipyl 5-H, val 3-H), 2.74 (2 H, AB of ABMX; δ_A 2.84, δ_B 2.68, *J* 14.0, 7.0, 6.1, 2.1, 1.8 Hz, cys 3-H), 3.72 (2 H, s, SCH_2Ph), 4.36—4.41 (1 H, m, aminoadipyl 2-H), 4.55—4.66 (2 H, m, cys 2-H, val 2-H), 5.09 (2 H, AB; δ_A 5.11, δ_B 5.06, *J* 11.9 Hz, OCH_2Ar), 5.20 (2 H, AB; δ_A 5.23, δ_B 5.17, *J* 13.7 Hz, OCH_2-Ar), 5.74 (1 H, dd, *J* 4.1, 7.8 Hz, NH), 6.50 (1 H, dd, *J* 7.3, 4.3 Hz, NH), 6.87 (1 H, s, $CHPh_2$), 6.95 (1 H, m, NH), 7.17—7.37br (20 H, m, ArH), 7.43 (2 H, m, ArH), and 8.16 (2 H, d, *J*, 8.9 Hz, ArH); *m/z* 797 (*M* − 91), 752, 721, 167, and 91.

N-*Benzyloxycarbonyl*-1-(p-*nitrobenzyl*)-δ-(L-α-*aminoadipyl*)-S-*benzyl*-L-*cysteinyl*-D-*valylglycine*, *Benzyl Ester* (5). The benzhydryl ester (3) (0.2 g, 0.23 mmol) was hydrolysed to the free acid (4) (0.16 g) as above and the product dissolved in dry THF (3 cm^3). Pyridine (20.0 μl, 0.25 mmol) was added, the solution cooled to 0 °C and a solution of ethyl chloroformate (23.8 μl, 0.25 mmol) in THF (2 cm^3) added in one portion. The solution was stirred for 15 min at 0 °C when a solution of glycine benzyl ester, toluene-*p*-sulphonic acid salt (83.6 mg) and pyridine (20.0 μl) in CH_2Cl_2 (3 cm^3) was added and the mixture allowed to warm to room temperature overnight. After evaporation of the solvent the residue was dissolved in EtOAc (15 cm^3), and the solution washed successively with aqueous HCl (1M; 10 cm^3), water (10 cm^3), and saturated aqueous NaCl (10 cm^3) and then dried, and evaporated. The residue was subjected to preparative t.l.c. on silica, with CH_2Cl_2–MeOH (19 : 1) as eluant, and the purified protected ACVG crystallised from CH_2Cl_2–EtOH–n-hexane as a microcrystalline solid (90 mg, 46%), m.p. 155—158 °C, $[\alpha]_D^{20}$ −7.4° [*c* 1.0, CH_2Cl_2–MeOH (1 : 1)] (Found: C, 61.85; H, 6.15; N, 7.8. $C_{45}H_{51}N_5O_{11}S$ requires C, 62.13; H, 5.91; N, 8.05%), δ[300 MHz, $CDCl_3$: CD_3OD (1 : 1)] 0.88 and 0.91 (6 H, 2 d, *J* 6.8 Hz, val 4-H), 1.66—1.85br (4 H, m, aminoadipyl 3, 4-H), 2.13—2.23br (3 H, m, aminoadipyl 5-H, val 3-H), 2.68 (2 H, AB of ABX; δ_A 2.74, δ_B 2.63, *J* 13.8, 7.4, 6.5 Hz, cys 3-H), 3.69 (2 H, s, SCH_2Ph), 4.24, 4.30, 4.46 (3 H, 3 m, 3 × 2-H), 5.06 (2 H, AB; δ_A 5.09, δ_B 5.03, *J* 12.2 Hz, OCH_2Ar), 5.11 (2 H, s, OCH_2Ar), 5.20 (2 H, AB; δ_A 5.23, δ_B 5.17, *J* 13.5 Hz, OCH_2Ar), 7.20—7.35 (15 H, m, ArH), and 7.45 and 8.15 (4 H, 2 d, *J* 8.6 Hz, ArH).

δ-(L-α-*Aminoadipyl*)-L-*cysteinyl*-D-*valine* (ACV; 1).—Typically the protected tripeptide (3) (100 mg, 0.12 mmol) was stirred in liquid NH_3 under N_2 and small pieces of freshly cut Na added until the blue colour persisted for 5 min. Solid NH_4OAc (*ca.* 100 mg) was added, the NH_3 evaporated under a stream of dry N_2, and the residue desiccated over H_2SO_4. The product was isolated as follows.

Procedure A. The residue was dissolved in 5% (v/v) aqueous HOAc (5 cm^3), the solution filtered and a 10% (w/v) solution of $Hg(OAc)_2$ in 5% (v/v) aqueous HOAc added slowly to the filtrate to afford a precipitate of the tripeptide sulphide. The precipitate was separated by centrifugation and washed sequentially with degassed water (5 cm^3 × 4), MeOH (5 cm^3 × 2), and ether (5 cm^3). The dried residue was resuspended in degassed water (2 cm^3) and a stream of H_2S passed through the suspension for 15 min. After separation by centrifugation the pellet of HgS was washed with water (1 cm^3) and the combined supernatant liquid and washings filtered through Celite. The filtrate was degassed *in vacuo* and lyophilized to afford a mixture of (1) and the corresponding disulphide (9) as a colourless powder (31.5 mg, 77%) (Found: C, 44.75; H, 6.85; N, 11.1; ash, 4%. $C_{14}H_{25}N_3O_6S$ requires C, 46.30; H, 6.87; N, 11.57%). Free thiol 75%, *m/z* (FABS) 364 [monomer (*M* + 1)].

Procedure B. The residue was dissolved in 5% (v/v) aqueous HOAc (5 cm^3), extracted with ether (2 cm^3 × 2), and the aqueous layer lyophilized. The residue was dissolved in water (5 cm^3), the pH adjusted to 8, and the solution aerated for 2 h. The solution was freeze dried, the residue dissolved in water (100 μl) and subjected to ion exchange chromatography on Biorad AG 50 × 2 resin (200—400 mesh, H^+, 18 × 1.5 cm) with a water–1M-pyridine gradient as eluant. Lyophilization of the ninhydrin positive fractions gave ACV disulphide as a colourless powder (39 mg, 96%), m.p. 200—203 °C (decomp.), $[\alpha]_D^{20}$ −9.5° (*c* 2.0, 2M-HCl) (lit.,[3] $[\alpha]_D$ −9.5°; lit.,[5] $[\alpha]_D$ −11.0°) (Found: C, 46.2; H, 6.35; N, 11.0. $C_{28}H_{48}N_6O_{12}S_2$ requires C, 46.40; H, 6.67; N, 11.59%), δ(300 MHz, D_2O) 0.27 (3 H, d, *J*, 6.9 Hz, val 4-H), 0.32 (3 H, d, *J*, 8.5 Hz, val 4-H), 1.06—1.27br (4 H, m, aminoadipyl 3,4-H), 1.54br (1 H, m, val 3-H), 1.76br (2 H, m, aminoadipyl 5-H), 2.40 (2 H, AB, cys 3-H), 3.18 (1 H, m, aminoadipyl 2-H), and 3.58 (1 H, m, val 2-H); δ_C (75 MHz, D_2O) 17.14 and 18.52 (val 4-C), 20.70 (aminoadipyl 4-C), 29.57 and 30.07 (val and aminoadipyl 3-C), 34.56 (aminoadipyl 5-C), 38.49 (cys 3-C), 52.43, 59.51, and 59.77 (3 × 2-C); *m/z* (FAB) 725 (*M* + 1) and 364.

δ-(L-α-*Aminoadipyl*)-L-*cysteinyl*-D-*valylglycine* (*ACVG*; 2).—Deprotection of the tetrapeptide derivative (5) (54 mg, 0.06 mmol) was carried out in a manner identical with that of (3) above. Isolation using procedure B afforded ACVG as its dimer (10), a colourless powder (19.4 mg, 74%), m.p. 230—235 °C (decomp.) (Found: C, 46.05; H, 6.2; N, 12.3. $C_{32}H_{54}N_8O_{14}S_2$ requires C, 45.8; H, 6.5; N, 13.35%), δ(300 MHz, D_2O) 0.25 and 0.28 (6 H, 2 d, *J* 7.5 Hz, val 4-H), 1.10br (4 H, m, aminoadipyl 3,4-H), 1.48br (1 H, m, val 3-H), 1.63—1.72 (2 H, m, aminoadipyl 5-H), 2.40 (2 H, AB of ABX; δ_A 2.48, δ_B 2.33, *J* 13.9, 7.4, 5.8 Hz, cys 3-H), 3.07 (1 H, t, *J* 5.6 Hz, aminoadipyl 2-H), 3.20 (2 H, s, gly 2-H), and 3.54 (1 H, m, val 2-H); *m/z* (FAB) 839 (*M* + 1) and 421.

Quantitative reduction of (10) to the monomer (2) was carried out by brief treatment with 5% (v/v) aqueous ethanethiol. The freeze-dried residue was homogeneous by electrophoresis and t.l.c. Reduction of (9) was carried out in a similar manner.

Acknowledgements

One of us (G. A. T.) acknowledges the support of the Science Faculty of Edinburgh University through the Sir David Baxter Scholarship. We thank Dr. R. P. Ambler of the Molecular Biology Department, Edinburgh University, for kind permission to use his electrophoresis apparatus, and Dr. D. Hazelby of Kratos Analytical Instruments Ltd., Manchester, for the FAB spectra.

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Received 29*th July* 1982; *Paper* 2/1309

J. CHEM. SOC., CHEM. COMMUN., 1984

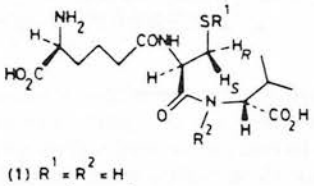
Synthesis and Biological Activity of δ-(L-α-Aminoadipoyl)-L-cysteinyl-*N*-hydroxy-D-valine:† a Proposed Intermediate in the Biosynthesis of the Penicillins

Robert L. Baxter,*[a] Gordon A. Thomson,[a] and A. Ian Scott*[b]

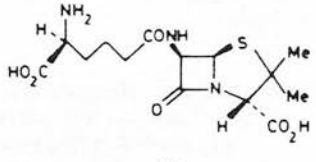
[a] *Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K.*
[b] *Center for Biological N.M.R., Texas A&M University, College Station, Texas 77843, U.S.A.*

δ-(L-α-Aminoadipoyl)-L-cysteinyl-*N*-hydroxy-D-valine (**3a**) has been prepared from the appropriately protected amino acids; (**3a**) was not converted into isopenicillin N (**2**) using a cell-free system from *Cephalosporium acremonium* but inhibited the formation of (**2**) from δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (**1**) by this system.

While it is now generally accepted that the cyclisation of the tripeptide, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (**1**) to isopenicillin N (**2**) is the final step in the series of reactions common to pencillin and cephalosporin biosynthesis,[1] the mechanisms by which the β-lactam and thiazolidine rings of the penam nucleus are elaborated *in vivo* remain unexplained. On the basis of *in vitro* analogy several mechanisms for the formation of the β-lactam ring have been proposed.[2,3] One such possibility involves enzymic hydroxylation at the nitrogen of the D-valine residue of (**1**) to generate a hydroxamic acid (**3a**) followed by abstraction of the cysteinyl 3-*pro-S* proton and ring closure with elimination of the *N*-hydroxy group or of the acyl group of an *N*-acyl derivative (**3b**) to afford an enzyme bound β-lactam derivative (**4**, R = enzyme).[2c,3] This route appears attractive since a number of microbial peptide hydroxamic acids have been isolated[4] and the feasibility of such a pathway has been demonstrated by a model chemical reaction[3] (Scheme 1). While recent results[5] have shown that the oxygen atoms of the α-aminoadipoyl residue of (**1**) are retained in the enzymic conversion of (**1**) into (**2**), precluding intermediacy of a thiazolinesulphone or a thiazoline species, the retention of the δ-carbonyl oxygen does



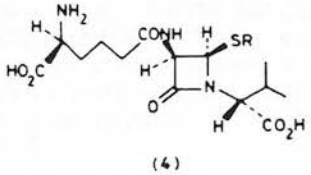
(**1**) $R^1 = R^2 = H$



(**2**)

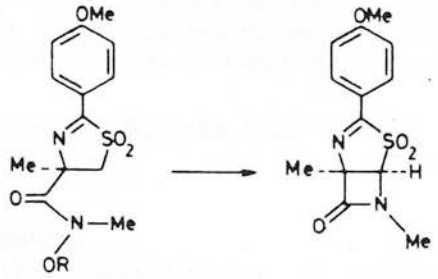
(**3**)a, $R^1 = H$, $R^2 = OH$

b, R^1 = enzyme, R^2 = Oacyl or OH



(**4**)

† δ-(L-α-aminoadipoyl) = 5-(5*S*)-amino-5-carboxypentanoyl.



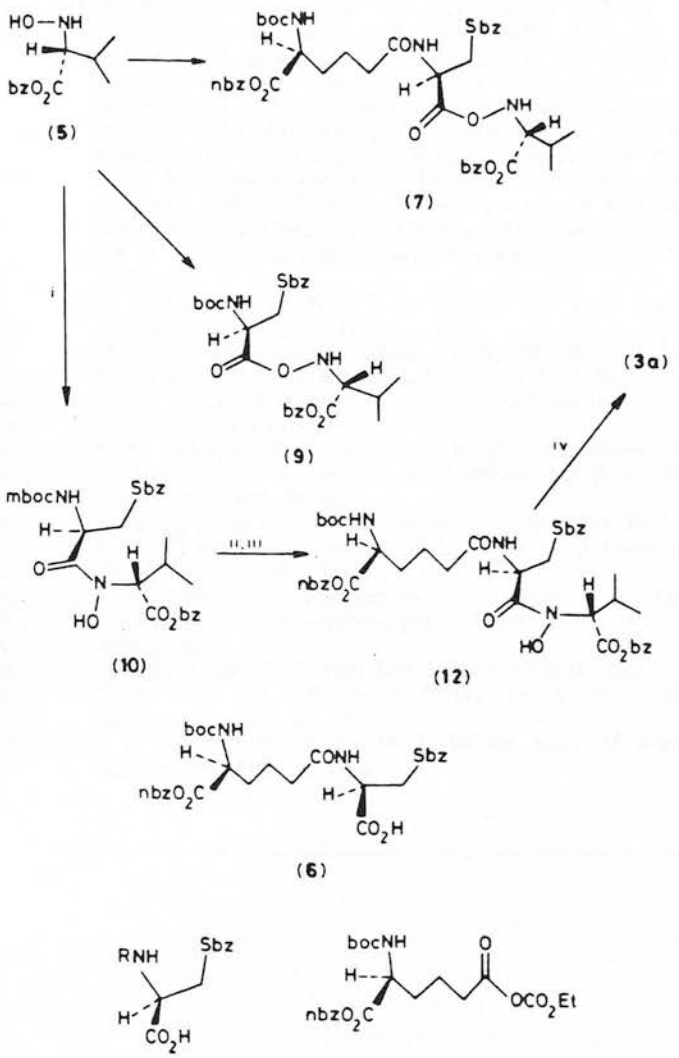
Scheme 1. R = 4-$NO_2C_6H_4SO_2$–.

not obviate the possible role of a linear *N*-hydroxy derivative such as (**3a**) as an intermediate. To test this hypothesis it was necessary to prepare the *N*-hydroxytripeptide (**3a**) and to evaluate it as a substrate.

Attempts to prepare a protected derivative of (**3a**) directly by acylation of (**5**)[6] with the protected dipeptide (**6**)[7] afforded only the *O*-acyl derivative (**7**). Similarly, acylation of (**5**) with *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine (**8a**) under a variety of peptide coupling conditions yielded the *O*-acyl derivative (**9**) as the major product. Selective *N*-acylation of (**5**) with *N*-(4-methoxybenzyloxycarbonyl)-*S*-benzyl-L-cysteine (**8b**) to give (**10**) and subsequent elaboration to the desired *N*-hydroxytripeptide (**3a**) were carried out as shown in Scheme 2.‡

The *N*-hydroxytripeptide (**3a**), gave a positive colour reaction with ferric chloride in solution and exhibited an ion at *m/z* 378 [378.1327, $(M - 1)^-$, $C_{14}H_{24}N_3O_7S$ requires 378.1329] in its negative ion mass spectrum (fast atom bombardment). The ^{13}C n.m.r. spectrum (75 MHz, D_2O) exhibited resonances at δ 19.57, 19.65 (val C-4, 4'), 21.73 (aaa C-4), 25.54 (cys C-3), 28.61 (val C-3), 30.55, 35.54 (aaa C-3, 5), 53.13, 55.15 (cys C-2, aaa C-2), and 66.61 p.p.m. (val C-2). The observation of the *N*-hydroxy-D-valine C-2 resonance at higher frequency than the chemical shift of the valine C-2 in the spectrum of (**1**) (δ 59.77 p.p.m.)[7] appears diagnostic of the hydroxamic acid structure. In the spectrum of *N*-hydroxy-D-valine benzyl ester (**5**) in D_2O the α carbon resonance appears at δ 72.81 p.p.m. while in the spectrum of (**12**) in $CDCl_3$ the corresponding carbon resonates at δ 63.10 p.p.m.

The *N*-hydroxytripeptide (**3a**) was administered to a partially purified enzyme system derived from homogenised cells of *C. acremonium* CW-19[1b] under conditions in which (**1**) was efficiently converted into (**2**).§ The resultant incubation mixture was assayed for isopenicillin N production using a hole-plate assay with *Staphococcus aureus*.[8] No significant antibiotic activity was detected and the *N*-hydroxytripeptide could be recovered unchanged from the incubation mixture suggesting that the compound is not directly involved as a free intermediate in the enzymatic conversion of (**1**) into (**2**).



Scheme 2. *Reagents*: i, (**8b**)–dicyclohexylcarbodi-imide (1 mol. equiv.)–dimethylformamide; ii, HCl–$MeNO_2$; iii, (**11**)–*N*-methylmorpholine–CH_2Cl_2; iv, Na–NH_3. Protecting groups, boc = benzyloxycarbonyl; mboc = 4-methoxybenzyloxycarbonyl; bz = benzyl; nbz = 4-nitrobenzyl.

However, addition of the *N*-hydroxytripeptide, at a concentration of 50 μM, to the crude enzyme system was found to completely inhibit formation of isopenicillin N from (**1**).

Peptide hydroxamic acids and *N*-acyl-*N*-hydroxypeptides are known to be powerful active site specific inhibitors of a number of zinc containing metallopeptidases.[9] While even relatively simple hydroxamic acids inhibit *Aeromonas* aminopeptidase, hydroxamic acids corresponding to L-amino acid amides which act as substrates for the metalloenzyme have been shown to be potent inhibitors.[10] It may be significant that acetohydroxamic acid also inhibits conversion of (**1**) into (**2**) by the *C. acremonium* enzyme system but only at concentrations higher than those required for inhibition by the *N*-hydroxytripeptide.¶

‡ Satisfactory elemental analyses and concordant spectroscopic data were obtained for compounds described in Scheme 2.

§ Incubations were carried out at 25 °C on a gyrorotatory shaker at 210 r.p.m. in 50 mM 3-(*N*-morpholino)propanesulphonic acid buffer, pH 7.2, containing 1.3 mM $FeSO_4$ and 2.5 mM dithiothreitol with a protein concentration of 5.5 mg/ml and substrate concentrations of 0.15—3.0 mM. For 0.3 mM (**1**) conversions were typically in the range 60—80% in 1 h. The limit of detection of the assay was 20 μg (**2**)/ml.

¶ Acetohydroxamic acid at 1 mM and (**3a**) at 40 μM were required for 50% inhibition of the conversion of (**1**) into (**2**).

We thank the S.E.R.C. for financial support. One of us (G. A. T.) acknowledges the award of a Sir David Baxter Scholarship from the University of Edinburgh.

Received, 26th September 1983; Com. 1279

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E R R A T A

Page 7	line 11	Read "penicillinase" for "pencillinase".
Page 52	line 5	Read "phosphoenolpyruvate" for "phosphoenal-pyruvate".
Page 78	Figure 41	Read "piperidine-2-carboxylic acid" for "piperadine-2-carboxylic acid".
Page 90	line 7	Read "amide anion" for "amide amion".
Page 91	lines 2-3	Read "analogous" for "analagous".
Page 95	lines 3-4	Read "sterically constrained" for "sterically contained".
Page 132	line 2	Read "(94)" for "(78)".
Page 140	line 12	Read "polyamide" for "polyacrylamide".
Page 161	line 22	Read "$CaCO_3$" for "$CaSO_4$".
Page 229	line 19	Read "$CaCO_3$" for "$CaSO_4$".
Page 230	line 5	Read "N,3.52" for "N,35.2".
Page 236	ref. 52	Read "G. N. Rolinson" for "G. N. Robinson".
Page 237	ref. 59	Read "G. N. Rolinson" for "G. N. Robinson".
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