Synthesis of Enzyme Inhibitors of Lysine Biosynthesis

A thesis presented in part fulfilment
of the requirement for the
Degree of Doctor of Philosophy.

by
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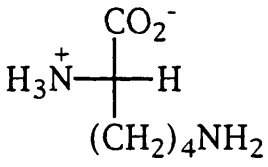
Finally, thankyou to my husband Graham.

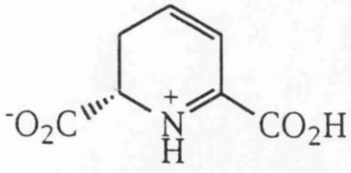
Summary.

There are two distinct biosynthetic pathways to the essential amino acid L-lysine (A). The diaminopimelate pathway to L-lysine occurs in higher plants and bacteria whereas the α-aminoadipate pathway operates in fungi and yeasts. This thesis is concerned with the initial step of the diaminopimelate pathway to L-lysine, catalysed by the enzyme dihydrodipicolinate synthase (DHDPS). The mechanism of formation of L-2,3-dihydrodipicolinate (L-2,3-DHDPA) (B), the product of the reaction catalysed by DHDPS, was investigated. The synthesis and testing of potential inhibitors of DHDPS was also studied.



(A)

L-Aspartic acid β-semialdehyde (L-ASA) (C) is a substrate of DHDPS. A former co-worker of ours, Dr. David Tudor, had previously prepared and isolated DL-ASA as the trifluoroacetate salt. The synthesis of DL-ASA was carried out in this work in order to have a supply of DL-ASA for testing of compounds with DHDPS. Manipulation of the synthetic route to DL-ASA (C) allowed us to prepare several imine derivatives (D). These were tested for inhibitory activity with DHDPS. The trifluoroacetate salt of the thiosemicarbazone derivative (E) was a good inhibitor, showing 46% inhibition at 0.1mM with DHDPS.



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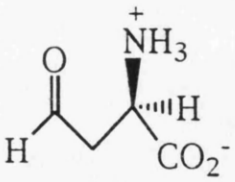

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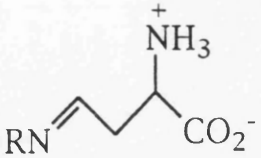

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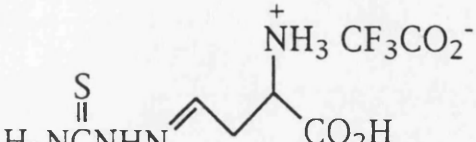
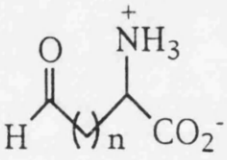


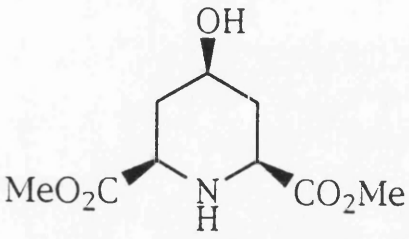
(E)

The synthesis of DL-glutamic acid γ-semialdehyde (DL-GSA) (F) and DL-aminoadipic acid δ-semialdehyde (DL-AASA) (G) was studied. The preparation of the trifluoroacetate salts of DL-GSA (F) and DL-AASA (G) was achieved and these compounds have been tested as inhibitors and substrates of DHDPS.



n = 2 (F)
n = 3 (G)

A number of heterocyclic analogues of L-2,3-DHDPA (B) were prepared and tested for inhibitory effects with DHDPS. Dimethyl 4-hydroxy-piperidine-*cis*-2,6-dicarboxylate (H) showed only 29% inhibition at 5mM with DHDPS. Commercially available 2,2,6,6-tetramethyl-4-piperidinol (I) and 2,2,6,6-tetramethyl-4-piperidone (J) were more effective inhibitors, showing 46% and 22% inhibition at 0.5 mM respectively. The oxime (K) and methyloxime (L) derivatives were also reasonable inhibitors at 0.5 mM. 1,3-Thiazole-2,4-dinitrile (M) was an effective inhibitor, showing 18% inhibition at 0.1 mM.



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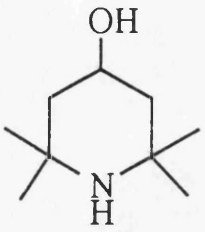
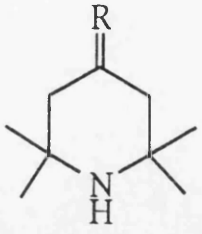
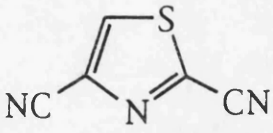


(I)



R = O (J)
R = NOH (K)
R = NOMe (L)



(M)

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Chapter 3 - Synthesis of L-Aspartic Acid β-Semialdehyde and Derivatives.

Chapter 7 - Experimental.

Abbreviations.

acetyl-CoA	:	acetyl co-enzyme A
ATP	:	adenosine triphosphate
α	:	alpha
AAA	:	alpha-aminoadipate
AASA	:	α-aminoadipic acid δ-semialdehyde
L-ASA	:	L-aspartic acid β-semialdehyde
β	:	beta
Boc	:	*t*-butoxycarbonyl
Cbz	:	carboxybenzyl
δ	:	delta
DAP	:	diaminopimelate
DDQ	:	2,3-dichloro-5,6-dicyanobenzoquinone
L-2,3-DHDPA	:	L-2,3-dihydrodipicolinic acid
DHDPR	:	dihydrodipicolinate reductase
DHDPS	:	dihydrodipicolinate synthase
DHT	:	3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid
DMF	:	dimethyl formamide
DPA	:	dipicolinic acid
d	:	doublet
ESMS	:	electrospray mass spectrometry
ε	:	epsilon
E. coli	:	*Escherichia coli*
Fig.	:	figure
γ	:	gamma
GC	:	gas chromatography
gem	:	geminal

GSA	:	glutamic acid γ-semialdehyde
IR	:	infra-red
λ	:	lambda
LDA	:	lithium diisopropylamide
MHz	:	megahertz
m p	:	melting point
μM	:	micromolar
m g	:	milligram
m l	:	millilitre
m M	:	millimolar
mmol	:	millimole
M	:	molar
m	:	multiplet
n m	:	nanometre
NADH	:	nicotinamide adenine dinucleotide
NADPH	:	nicotinamide adenine dinucleotide phosphate
NMR	:	nuclear magnetic resonance
p	:	para
PCC	:	pyridinium chlorochromate
PDC	:	pyridinium dichromate
q	:	quartet
s	:	singlet
SDS-PAGE	:	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
succinyl CoA	:	succinyl co-enzyme A
L-2,3,4,5-THDPA	:	L-2,3,4,5-tetrahydrodipicolinic acid
THF	:	tetrahydrofuran
TLC	:	thin layer chromatography

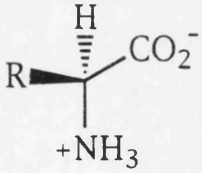
TFA	:	trifluoroacetic acid
t	:	triplet
UV	:	ultraviolet

Chapter 1 - L-Lysine : An Essential Amino Acid.

Introduction.

α-Amino acids are found in living organisms in both their free forms and joined by amide linkages in peptides and proteins. With the development of chromatographic techniques in the last 40 years, the number of known, naturally occurring amino acids has risen to around 1000. As amino acids are finding increasing use in the synthesis of pharmaceuticals, agricultural products, the food industry and materials science, the synthetic methods used to prepare both natural and structurally diversified α-amino acids are becoming increasingly important.

There are about 20 different protein α-amino acids, the structures of which were determined in the 100 years since Braconnet first characterised glycine and leucine in 1820. With the exception of glycine, all are optically active and usually have the L-configuration at the α-carbon atom. The general structure of the proteinogenic α-amino acids is shown in **Fig. 1**.



R = $(CH_2)_4NH_2$ (1)

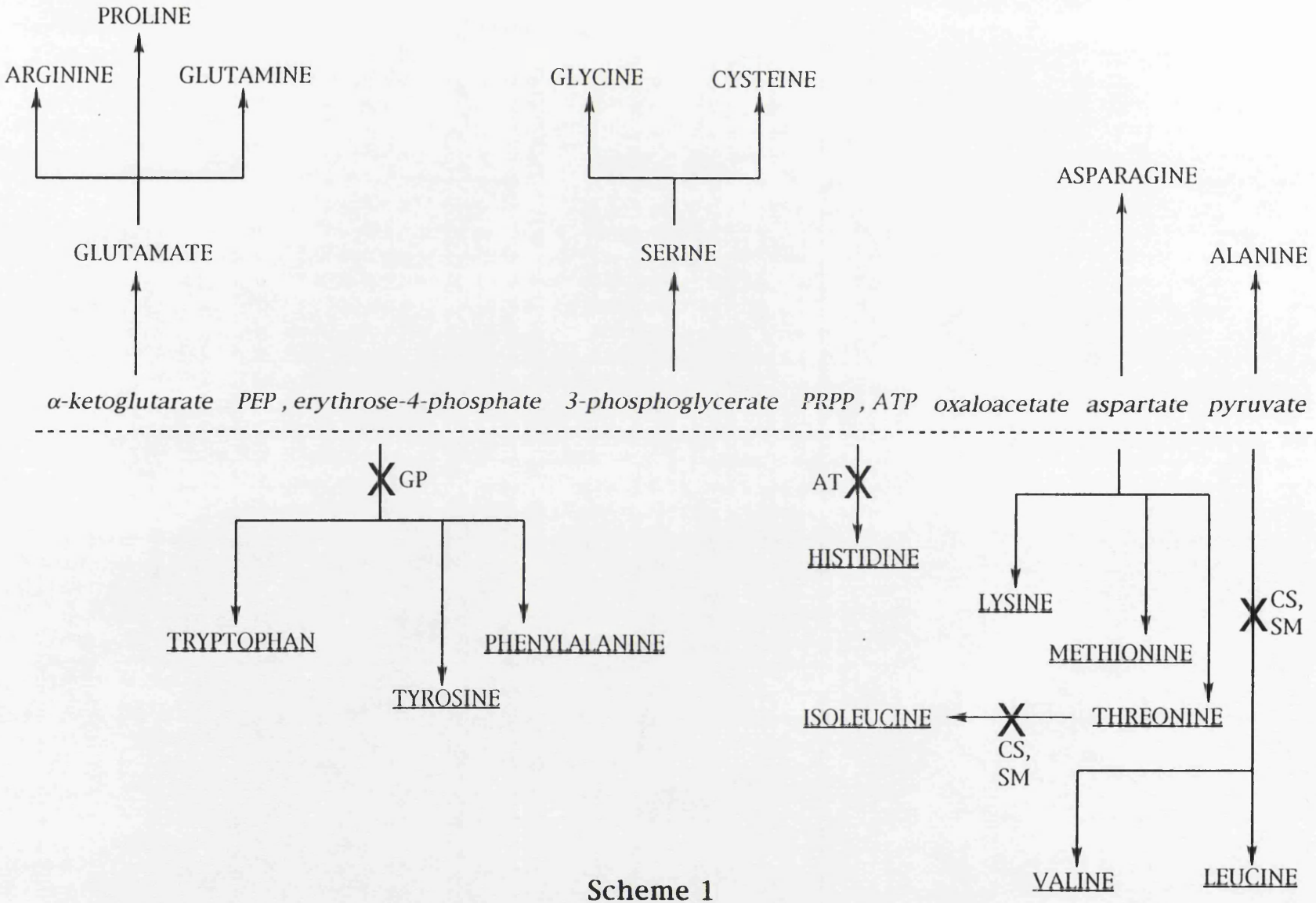
Fig. 1

An important proteinogenic α-amino acid is L-lysine (1) (R = $(CH_2)_4NH_2$ in **Fig. 1**). Its importance will be discussed in Section 1.2.

1.1 Amino Acid Biosynthetic Enzymes as Targets of Herbicide/Antibiotic Action.

Many metabolic differences between the plant and animal kingdoms exist. Plants, unlike animals, obtain energy from light via the process known as photosynthesis. They must also synthesise their own plant-specific hormones and cell walls. Plants and animals further differ in their ability to synthesise amino acids and vitamins. While plants are able to make all these compounds, animals must obtain vitamins and the essential amino acids from their diet. **Scheme 1** shows an overview of amino acid biosynthesis. The common metabolic intermediates are written in italics. Amino acids essential to the mammalian diet are underlined. The metabolic pathways shown below the broken line do not occur in mammals, but are found in plants and bacteria, and are therefore potential targets for selectively toxic herbicides or antibiotics.

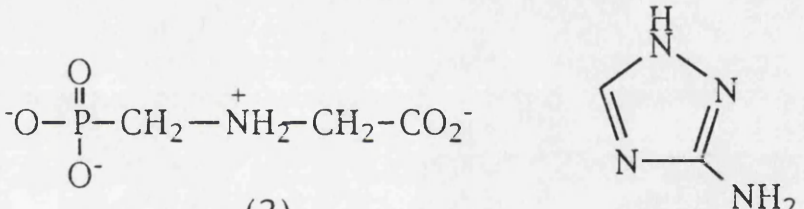
A number of herbicides are known to act by inhibiting essential amino acid biosynthesis. The biosynthetic pathways which each herbicide inhibits are marked by a cross in **Scheme 1**. Glyphosate (GP) (2) is a nonselective herbicide which interferes with steps of the common aromatic amino acids pathway.[1,2] Aminotriazole (AT) (3) was patented as a broad spectrum herbicide in 1954 by the American Chemical Paint Company. It was found to antagonize histidine biosynthesis in bacteria and yeast by blocking the activity of imidazole glycerol phosphate dehydratase.[3] The sulfonylurea herbicides, chlorosulfuron (CS) (4) and sulfometuron methyl (SM) (5) are notable for their high potency and low mammalian toxicity.[4] While sulfometuron methyl (5) is a broad-spectrum non-selective


PROLINE
ARGININE
GLUTAMINE
GLUTAMATE
GLYCINE
CYSTEINE
SERINE
ASPARAGINE
ALANINE
α-ketoglutarate
PEP , erythrose-4-phosphate
3-phosphoglycerate
PRPP , ATP
oxaloacetate
aspartate
pyruvate
GP
TRYPTOPHAN
PHENYLALANINE
TYROSINE
AT
HISTIDINE
LYSINE
METHIONINE
CS,
SM
ISOLEUCINE
THREONINE
CS,
SM
VALINE
LEUCINE

Scheme 1

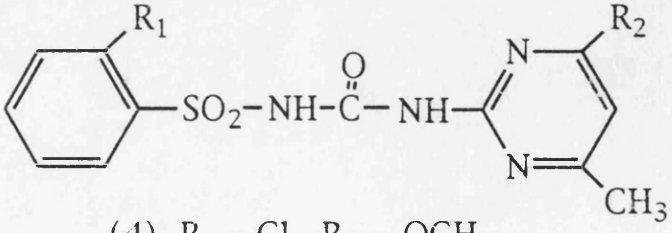
herbicide, chlorosulfuron (4) is selective and can be applied to prevent the growth of weeds without detriment to cereal crops. Both these compounds inhibit the biosynthetic pathways to branched chain amino acids.

One potential target for herbicide/antibiotic action which has received relatively little attention is the biosynthetic pathway to lysine (1). Our research group at Glasgow has been investigating the early stages of the biosynthetic pathway to lysine (1) as found in bacteria and plants. This thesis details the biosynthetic pathway to lysine (1) and describes the synthesis and testing of a range of compounds as potential inhibitors of lysine (1) biosynthesis.



(2)

(3)



(4) $R_1 = Cl$, $R_2 = OCH_3$

(5) $R_1 = CO_2CH_3$, $R_2 = CH_3$

1.2 The Importance of L-Lysine (1).

L-Lysine (1) is economically one of the most important amino acids. Mammals lack the ability to make L-lysine (1) and so must consume it within their diet. In other words, it is an essential amino acid for human and animal nutrition. The nutritive value of protein depends on the quantity and balance of its constituent essential amino acids. **Table 1** shows the estimated daily requirements of adults.

Amino Acid	Amount (mg/kg)
Isoleucine	10
Leucine	14
Lysine	12
Methionine and cysteine	13
Phenylalanine and tyrosine	14
Threonine	7
Tryptophan	4
Valine	10

Table 1

It is necessary to consume these essential amino acids daily and simultaneously. If one essential amino acid is supplied at an inadequate rate or at a different time from the other amino acids, the level of utilization of all the amino acids is lowered to that of the deficient, limiting amino acid. L-Lysine (1) is a limiting amino acid in plant protein, especially cereal protein. The nutritional value of plant

protein can be improved by adding small amounts of the limiting amino acid to food and animal feed. This amino acid fortification is important both nutritionally and economically as, along with DL-methionine, L-lysine (1) is indispensable for the production of animal feeds. Commercially, the largest amounts of L-lysine (1) are produced by the bacteria *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum*.[5] The production of L-lysine (1) will be discussed in more detail in Section 1.3.

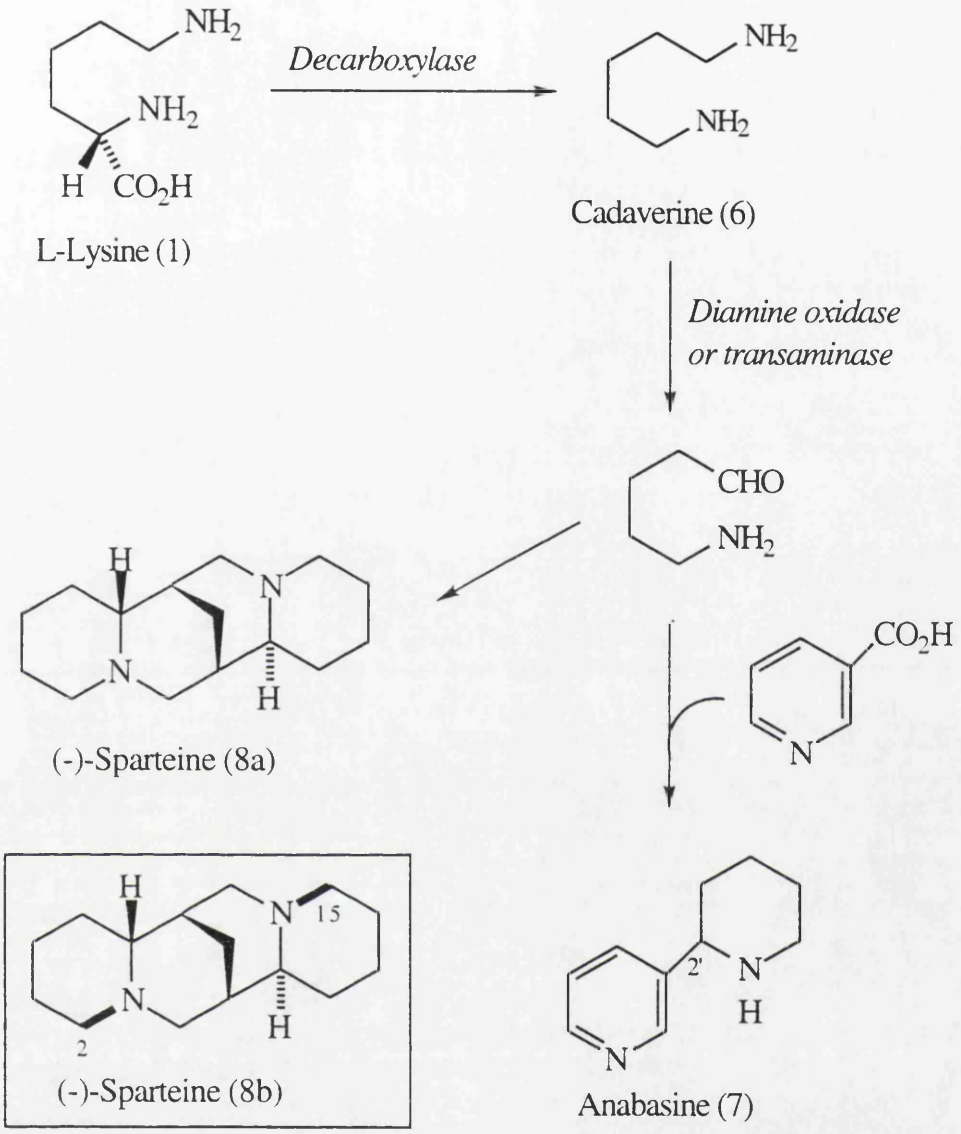
The biosynthesis of L-lysine (1) in bacteria and plants is incompletely understood. This will be discussed in Section 1.4. Labelling studies[6] have established that *meso*-2,6-diaminopimelic acid (29) is an intermediate in the biosynthesis of L-lysine (1). *meso*-2,6-Diaminopimelic acid (29) is a building block of the peptidoglycan of most Gram-negative bacteria as well as Gram-positive bacteria. It forms part of the cross-linking moiety between adjacent peptide chains of the cell wall peptidoglycans.[7] Inhibitors of peptidoglycan biosynthesis can have powerful antibiotic properties.[8,9] The enzymes of peptidoglycan processing are therefore viable targets for the rational design of novel antibacterial agents. The absence of the peptidoglycan and L-lysine (1) biosynthetic networks in mammals suggests that inhibitors may possess antibacterial and/or herbicidal activity, without mammalian toxicity. The design of inhibitors will be discussed in more detail in Section 1.5 and Chapters 3, 4, 5 and 6.

The conversion of L-lysine (1) into alkaloids is an important biological process which has been studied for many years. Alkaloids are a large and diverse group of natural products which contain a nitrogen heterocycle, are found mainly in plants, and exhibit a wide

range of biological activities. Large numbers of alkaloids have been isolated. This has resulted in an increase in the understanding of their biological properties and their pharmacological, toxicological and ecological significance in nature. The use of isotopically labelled precursors in feeding experiments has helped us to understand the complex manner in which alkaloids are biosynthesised. Alkaloids are formed mostly from a few α-amino acids (lysine, ornithine, phenylalanine, tryptophan and tyrosine) along with mevalonate and acetate. Alkaloids are important in chemistry, industry and medicine. In the last category, alkaloids find use in the following types of medicinal activity: anticholinergic (scopolamine); antihypertensive (rescinnamine); antimalarial (quinine); antitumour (vinblastine); cardiac depressant (quinidine); gout suppressant (colchicine); narcotic analgesic (morphine); tranquiliser (reserpine).

There are two main classes of alkaloids which utilise L-lysine (1) as a precursor, namely the piperidine alkaloids and the quinolizidine alkaloids. **Scheme 2** outlines the biosynthetic routes taken by L-lysine (1) in the production of a piperidine alkaloid, anabasine (7), and a quinolizidine alkaloid, sparteine (8a).

Many higher plants possess the necessary metabolic pathways for the synthesis of piperidine alkaloids. Robins *et al.* [10] studied the biosynthesis of anabasine (7) in transformed root cultures of *Nicotiana rustica* and *N. tabacum* using cadaverine (6) precursors labelled with deuterium. These labelling studies indicated that the *pro-R* hydrogen of cadaverine (6) is retained and that the *pro-S* hydrogen is lost at the position which becomes C-2' of anabasine (7).



Scheme 2

Quinolizidine alkaloids are important because of their toxicity to humans and livestock as constituents of poisonous plants and because some of them exhibit potentially useful pharmacological activities. These alkaloids are commonly found in species of the genus *Lupinus* from the plant family Fabaceae (formerly Leguminosae). The quinolizidine skeleton is built up from two or three C_5 units derived from L-lysine (1) via its decarboxylation product cadaverine (6). A common quinolizidine alkaloid is sparteine (8a). It has been isolated

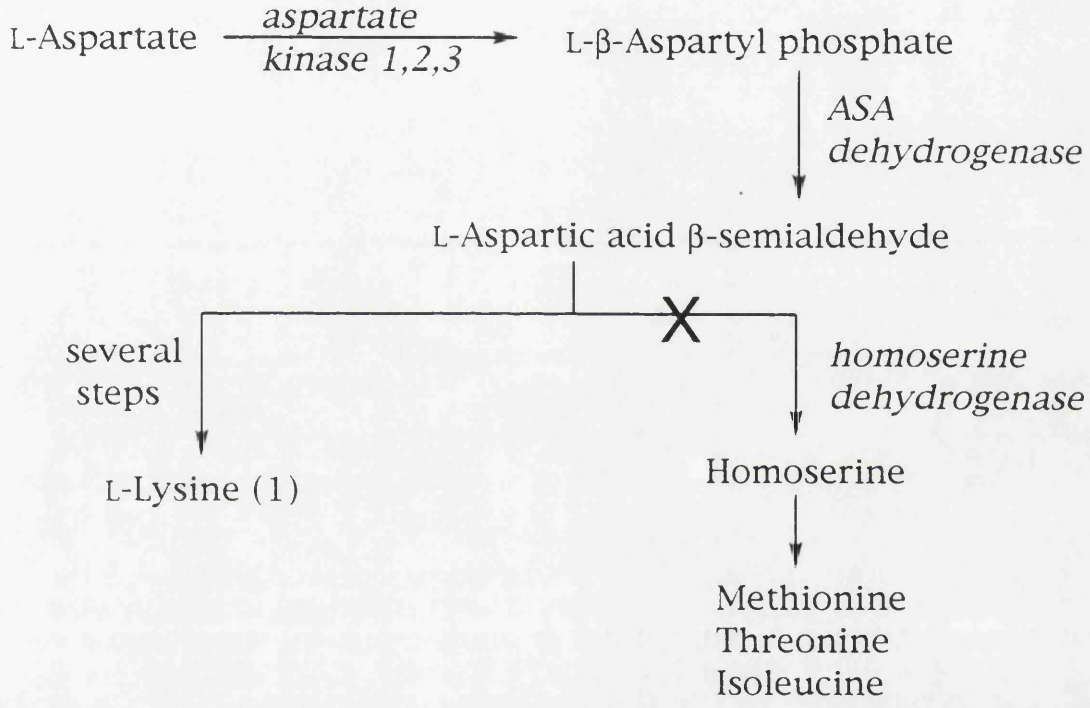
from many plant species including *Spartium scoparium,*[11] *Sarothamnus scoparius*[12] and *Lupinus lindeniancus.*[13] Careful labelling studies by the groups of Robins[14] and Spenser[15] showed that there were equal levels of ^{13}C-enrichment for 6 carbon atoms after feeding [^{13}C-^{15}N]-1,5-diaminopentane (cadaverine), and that there were two ^{13}C-^{15}N doublets around the signals for C-2 and C-15. This confirmed that two moles of cadaverine are incorporated into the two outer rings as shown in (8b) **(Scheme 2)**. The biosynthesis of quinolizidine alkaloids has recently been reviewed.[15a]

1.3 Production of L-Lysine (1).

The amino acid industry has been steadily expanding since monosodium glutamate was first marketed as a flavouring material in 1909. All the amino acids occurring in proteins are now produced industrially. Amino acids can be produced by extraction from protein hydrolysates, by fermentation with the aid of microorganisms, by enzymatic processes and by chemical synthesis. L-Lysine (1) can be produced industrially by direct fermentation methods (using auxotrophic and regulatory mutants) and by a biotransformation process involving both synthetic and enzymic methodology.

With the advancement of modern genetic biochemical techniques, it is possible to produce most proteinogenic amino acids by fermentation methods. Monosodium glutamate and L-lysine (1) are produced in the largest amounts, with annual world production levels of 370 000 and 40 000 tons respectively.[16] Auxotrophic mutants are microorganisms which lack one or more enzymes required for biosynthesis of an amino acid. The use of homoserine-less mutants of

C. glutamicum and *Escherichia coli* leads to the accumulation of L-lysine (1).[17] The absence of homoserine dehydrogenase in these mutants blocks the pathway from aspartic semialdehyde to homoserine. This results in the accumulation of L-lysine (1), the end product of a branched pathway **(Scheme 3).**

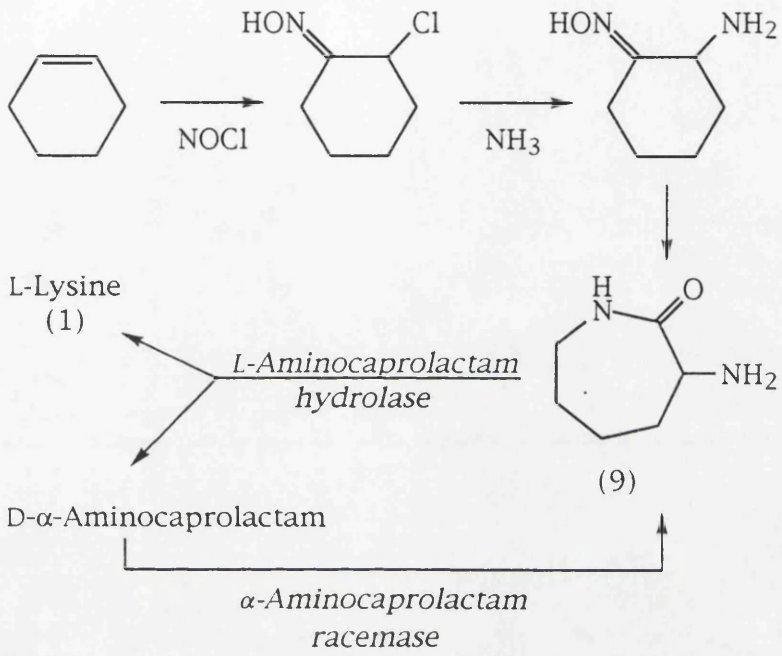


Scheme 3

In regulatory mutants of microorganisms the loss of feedback control leads to the accumulation of excess amino acids. L-Lysine (1) is produced by the regulatory mutant of *B. flavum.*[18]

An enzymatic process for the production of L-lysine (1) utilizes DL-α-aminocaprolactam (9), synthesised from cyclohexene.[19] Simultaneous addition of L-aminocaprolactam hydrolase (*Cryptococcus laurentii*) and aminocaprolactam racemase (*Achromobacter obae*)

results in the complete enzymic conversion into L-lysine (1) **(Scheme 4)**.



Scheme 4

1.4 Biosynthesis of L-Lysine (1).

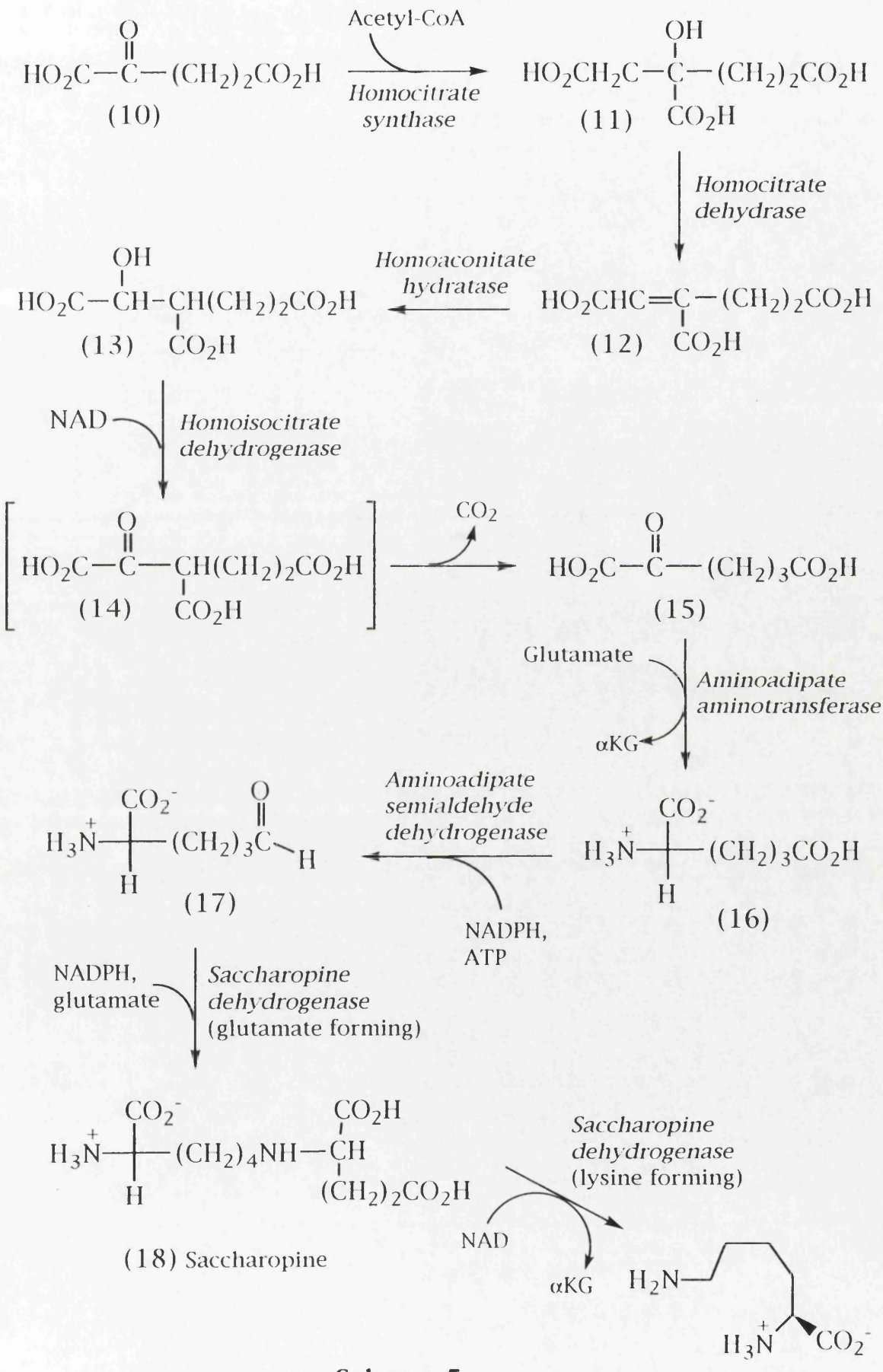
L-Lysine (1) is biosynthesised via two mutually exclusive pathways: the α-aminoadipate (AAA) pathway and the diaminopimelate (DAP) pathway. The AAA pathway is characteristic of the Euglenids, some Phycomycetes (Chytridiales, Blastocladiales and Mucorales), yeasts and higher fungi (Ascomycetes and Basidiomycetes). It starts with the reaction between α-ketoglutarate (10) and acetyl-CoA and involves the synthesis of α-aminoadipic acid (16) and saccharopine (18) as intermediates in the biosynthesis of L-

lysine (1). The DAP pathway occurs in bacteria and higher plants and involves the initial condensation of L-aspartic acid β-semialdehyde (20) with pyruvate (24). Several unique enzyme catalysed reactions result in the production of L-lysine (1). *meso*-2,6-Diaminopimelic acid (29) is an important intermediate of the DAP biosynthetic pathway to L-lysine (1).

The α-Aminoadipate Pathway to L-Lysine (1).

Metabolic labelling studies with ^{14}C-acetate have shown that, in *Saccharomyces cerevisiae*, the carbon skeleton of L-lysine (1) is derived from acetate.[20] The proposed pathway for the formation of the carbon chain of L-lysine (1) in yeast **(Scheme 5)** involves a condensation of acetate with α-ketoglutarate (10) to yield homocitrate (11) followed by a series of reactions which are analogous to the formation of α-ketoglutaric acid via reactions of the citric acid cycle.[21] The resultant α-aminoadipate (16) is converted into L-lysine (1) via α-aminoadipate semialdehyde (17) and saccharopine (18).

L-Lysine (1) biosynthesis has been investigated in detail in *S. cerevisiae*.[22] The enzymes of α-aminoadipate biosynthesis, namely homocitrate synthase, homoaconitate hydratase, homoisocitrate

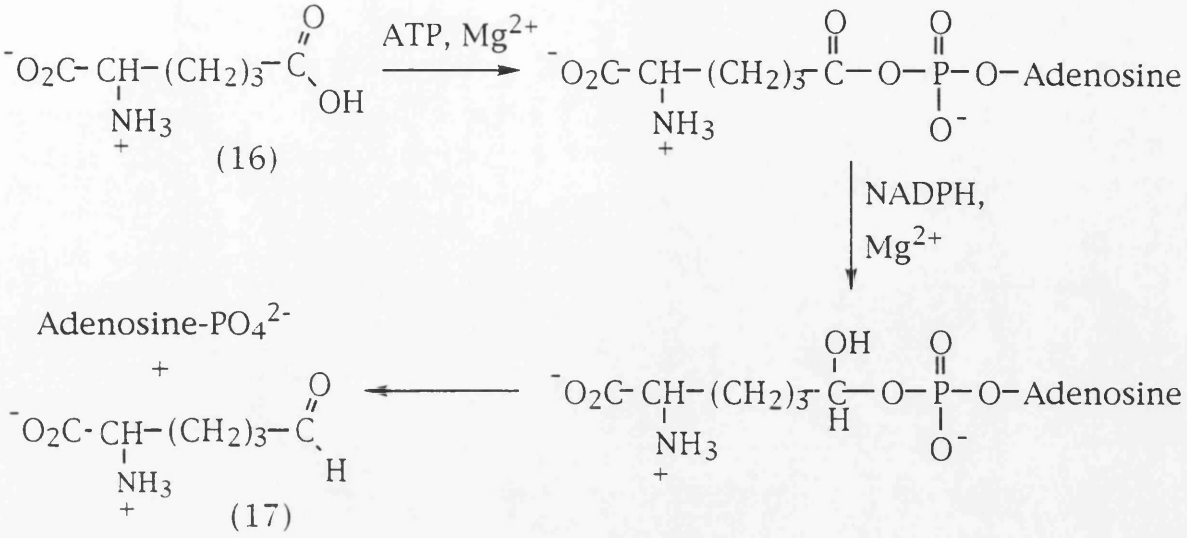

Acetyl-CoA
Homocitrate synthase
(10)
(11)
Homocitrate dehydrase
Homoaconitate hydratase
(12)
(13)
NAD
Homoisocitrate dehydrogenase
(14)
CO2
(15)
Glutamate
αKG
Aminoadipate aminotransferase
(16)
Aminoadipate semialdehyde dehydrogenase
NADPH, ATP
(17)
NADPH, glutamate
Saccharopine dehydrogenase (glutamate forming)
(18) Saccharopine
Saccharopine dehydrogenase (lysine forming)
NAD
αKG
(1) L-Lysine

Scheme 5

dehydrogenase and aminoadipate aminotransferase, are localised in the mitochondria. A second aminoadipate aminotransferase and the enzymes catalysing the last three steps of L-lysine (1) biosynthesis are nonmitochondrial.[23] Intracellular free L-lysine (1) is sequestered in vacuoles of *S. cerevisiae*.[24]

The first intermediate of the AAA pathway is formed by the condensation of acetyl-CoA with α-ketoglutarate (10). This reaction is catalysed by an ATP and CoA-dependent enzyme, homocitrate synthase. The second intermediate, homoaconitate (12), is formed from the dehydration of homocitrate (11). This is catalysed by homocitrate dehydrase. Homoaconitate hydratase then catalyses the formation of homoisocitrate (13). Action of homocitrate dehydrogenase results in the reversible oxidation of homoisocitrate (13) to oxaloglutarate (14). This process has been shown to be both NAD and Mg^{2+} dependent.[25]

Spontaneous decarboxylation results in α-ketoadipate (15). Aminoadipate aminotransferase catalyses the pyridoxal phosphate-dependent transamination of α-ketoadipate (15) to α-aminoadipate (16).[23] The conversion of α-aminoadipate (16) into α-aminoadipic semialdehyde (17) is a multistep reaction catalysed by a single ATP, Mg^{2+} and NADPH-dependent enzyme, aminoadipate semialdehyde dehydrogenase.[26] The reduction of α-aminoadipate (16) to α-aminoadipic semialdehyde (17) in *Saccharomyces* has been shown to be a three step process. The amino acid first reacts with ATP to form an adenylyl derivative. This is then reduced in the presence of NADPH. The reduced adenylyl derivative is then cleaved to form α-aminoadipic semialdehyde (17) **(Scheme 6)**.



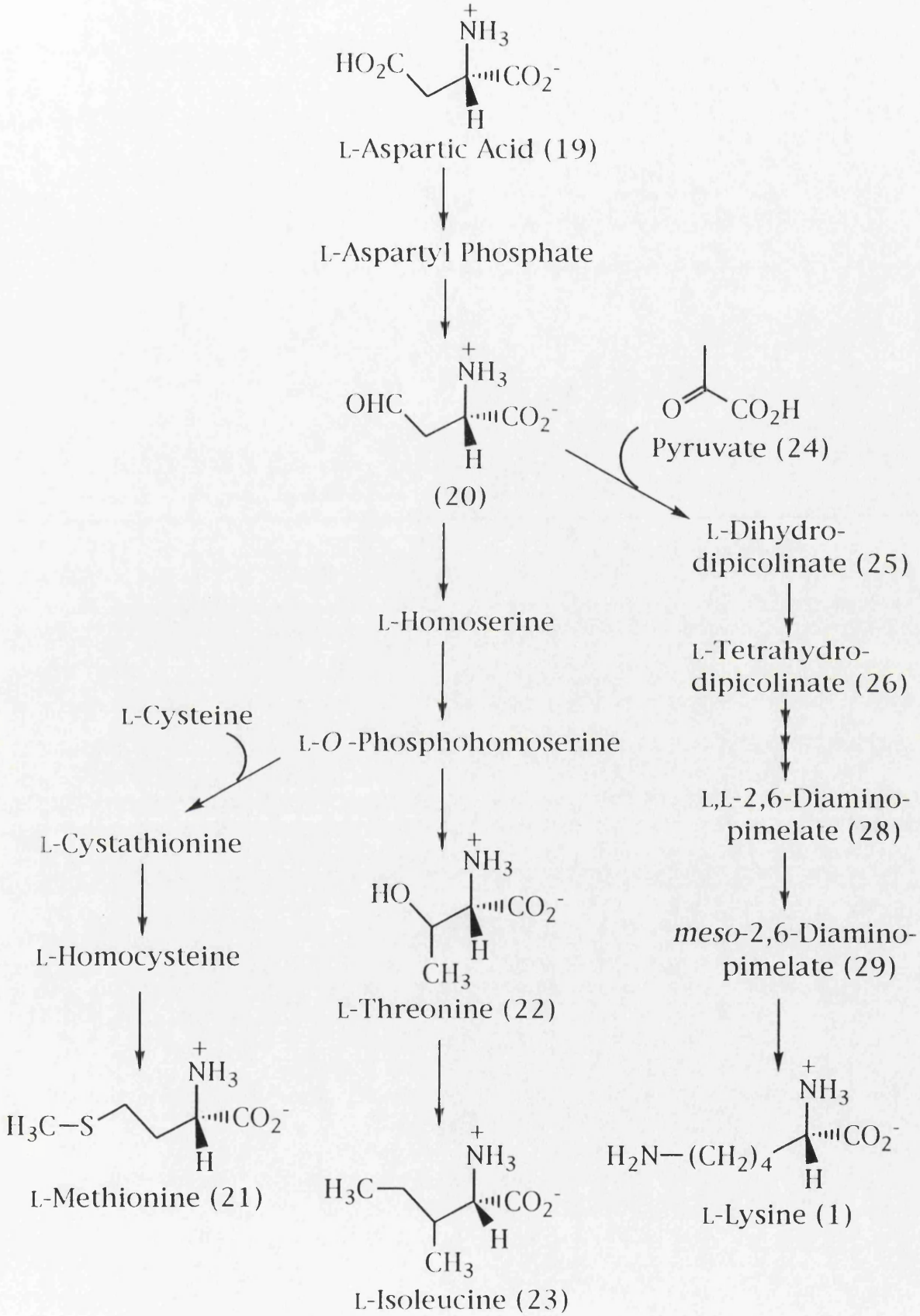
Scheme 6

Saccharopine dehydrogenase (glutamate forming) and saccharopine dehydrogenase (lysine forming) catalyse the formation of saccharopine (18) and L-lysine (1) respectively.[27]

The Diaminopimelate Pathway to L-Lysine (1).

L-Lysine (1), methionine (21), threonine (22) and isoleucine (23) belong to the aspartate family of amino acids. In *Escherichia coli*, they each derive part or all of their carbon atoms from aspartate (19) and share a common biosynthetic pathway **(Scheme 7)**.

In plants and bacteria, L-lysine (1) is produced via the diaminopimelate pathway[28] from L-aspartic acid β-semialdehyde (L-ASA) (20). Seven unique enzyme catalysed reactions are required for the synthesis of L-lysine (1) **(Scheme 8)**. Most of the enzymes have been isolated from *E. coli* and characterised. However, enzymes catalysing only the first and last of these reactions have been isolated from plants.

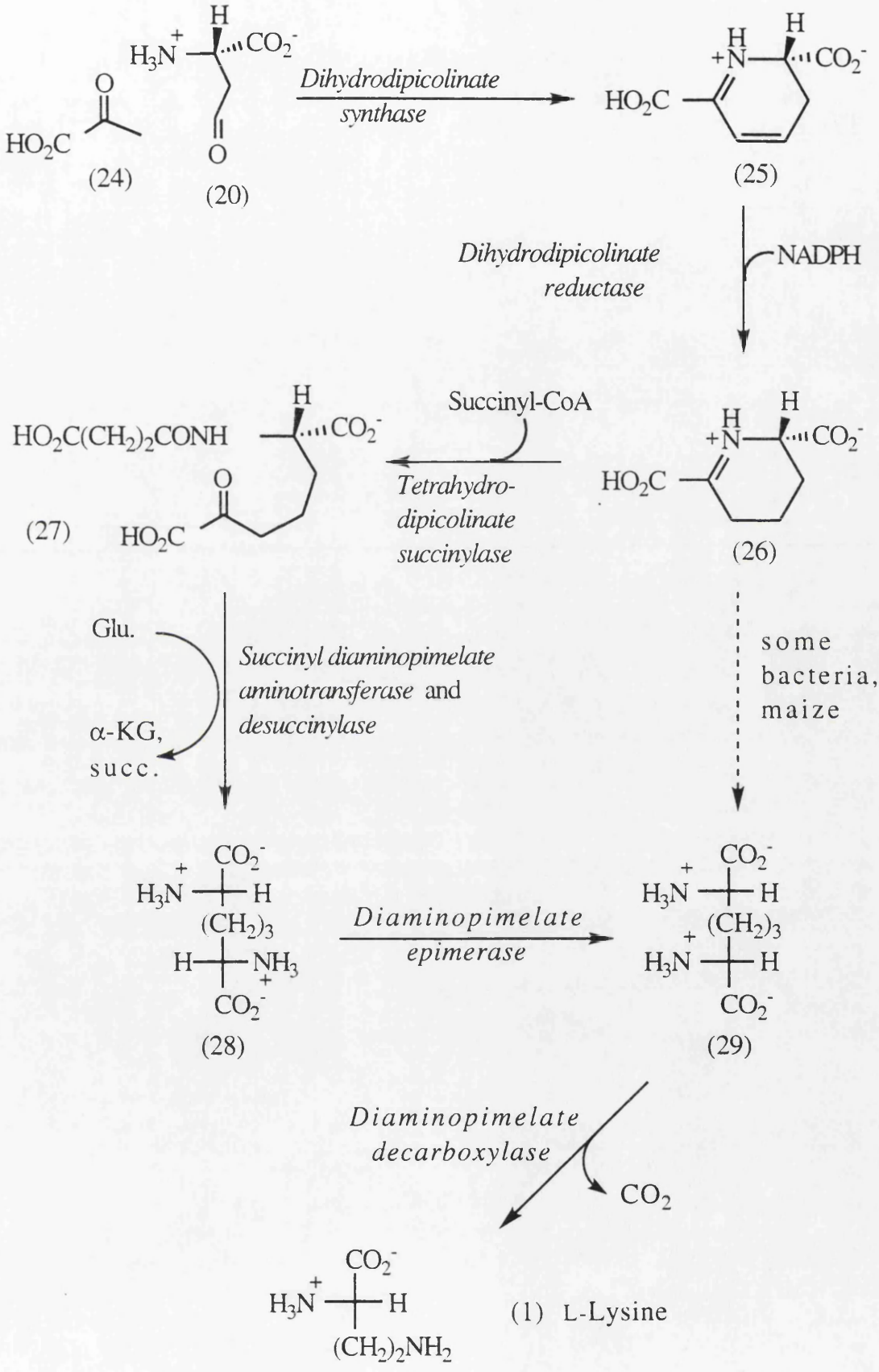

L-Aspartic Acid (19)
L-Aspartyl Phosphate
(20)
Pyruvate (24)
L-Dihydro-dipicolinate (25)
L-Tetrahydro-dipicolinate (26)
L,L-2,6-Diamino-pimelate (28)
meso-2,6-Diamino-pimelate (29)
L-Lysine (1)
L-Homoserine
L-Cysteine
L-O-Phosphohomoserine
L-Cystathionine
L-Homocysteine
L-Methionine (21)
L-Threonine (22)
L-Isoleucine (23)

Scheme 7

The first step unique to L-lysine (1) biosynthesis is the condensation of L-ASA (20) and pyruvate (24). This is catalysed by dihydrodipicolinate synthase and presumably proceeds in several stages. Nucleophilic attack by the methyl carbon of pyruvate (24) upon the aldehyde carbon of L-ASA (20) would result in carbon-carbon bond formation. Loss of water followed by spontaneous ring closure and the loss of a second molecule of water results in the product, dihydrodipicolinate (25). An NADPH-dependent reduction of dihydrodipicolinate (25) results in the formation of tetrahydrodipicolinate (26). Succinylation serves to protect the 2-amino group during the synthesis of the diamino intermediate. Desuccinylation results in L,L-diaminopimelate (28). This is followed by epimerisation at one of the L-centres to give *meso*-2,6-diaminopimelate (29). Decarboxylation at the D-centre of *meso*-2,6-diaminopimelate (29) gives L-lysine (1).

An alternative biosynthetic route, which bypasses several stages of the proposed DAP pathway has been found to operate in some bacteria and maize. The enzyme, *meso*-diaminopimelate dehydrogenase, directly converts L-2,3,4,5-tetrahydrodipicolinic acid (26) into *meso*-2,6-diaminopimelate (29).

The diaminopimelate pathway to L-lysine (1) will be discussed in more detail in Chapter 2.


Dihydrodipicolinate synthase
(24)
(20)
(25)
Dihydrodipicolinate reductase
NADPH
Succinyl-CoA
Tetrahydro-dipicolinate succinylase
(27)
(26)
Glu.
α-KG, succ.
Succinyl diaminopimelate aminotransferase and desuccinylase
some bacteria, maize
Diaminopimelate epimerase
(28)
(29)
Diaminopimelate decarboxylase
CO2
(1) L-Lysine

Scheme 8

1.5 Design of Inhibitors of the DAP Pathway to L-Lysine.

meso-2,6-Diaminopimelate (29) has been found to be an important building block of the peptidoglycan in bacterial cell walls.[7] As mentioned earlier, inhibitors of peptidoglycan biosynthesis should provide selective antibacterial action, without mammalian toxicity.

LL-2,6-DAP (28), *meso*-DAP (29) and L-lysine (1) are biosynthetically closely related in the DAP biosynthetic pathway **(Scheme 8)**. Epimerisation of LL-2,6-DAP (28) results in *meso*-DAP (29) and subsequent decarboxylation gives L-lysine (1). It follows that inhibitors of the DAP pathway to L-lysine (1) may possess antibacterial properties with no mammalian toxicity. It is believed that the DAP pathway to L-lysine (1) in plants is analogous to that found in bacteria. Therefore, inhibitors may have additional herbicidal activity.

The synthesis of L-aspartic acid β-semialdehyde (20) was achieved within our group by Dr. D. Tudor. This will be discussed in Chapter 3. A number of derivatives of L-aspartic acid β-semialdehyde (20) were prepared in this work. The synthesis and testing of these compounds will be discussed in Chapter 3. L-Glutamic acid γ-semialdehyde (122) and L-aminoadipic acid δ-semialdehyde (17) are homologues of L-aspartic acid β-semialdehyde (20). Chapter 4 of this thesis deals with their synthesis and testing.

The first synthesis of the proposed intermediate of the DAP pathway, L-2,3,4,5-tetrahydrodipicolinic acid (26), was achieved by Dr. L. Couper, a former co-worker. This will be discussed in Chapter 2. Analogues of L-2,3,4,5-tetrahydrodipicolinic acid (26) and L-2,3-dihydrodipicolinic acid (25) have been prepared. The synthesis and

test results of these heterocyclic analogues will be discussed in Chapters 5 and 6.

Chapter 2 - The Diaminopimelate Pathway to L-Lysine.

Introduction.

Bacteria and some plants have been shown to biosynthesise L-lysine (1) via the diaminopimelate (DAP) pathway **(Scheme 8)**. Whereas all seven of the enzymes of the DAP pathway have been identified in *Escherichia coli*, only four have been isolated and characterised from plants. The initial step on the pathway involves the condensation of L-aspartic acid β-semialdehyde (20) and pyruvate (24). The enzyme involved in this reaction is dihydrodipicolinate synthase (DHDPS). A further six, unique enzyme catalysed reactions result in L-lysine (1). In this Chapter, reported studies on each of the enzymes of the DAP pathway in bacteria and higher plants will be reviewed. This will include discussions of enzyme isolation and purification and mechanistic and inhibitor studies.

2.1 Dihydrodipicolinate Synthase.

L-Lysine (1) biosynthesis via the DAP pathway in bacteria and higher plants starts with aspartate (19) and shares the first two reactions with the synthesis of L-methionine (21), L-threonine (22) and L-isoleucine (23) **(Scheme 7)**.[29] The first step unique to L-lysine (1) biosynthesis is the condensation of pyruvate (24) and L-aspartic acid β-semialdehyde (L-ASA) (20). This is catalysed by the enzyme dihydrodipicolinate synthase (DHDPS).

DHDPS has been characterised from bacteria and a wide range of higher plants including spinach (*Spinacia oleracea*), wheat germ (*Triticum aestivum* L.), maize seedlings (*Zea mays* L.) and has been highly purified from wheat cell suspension cultures and tobacco leaves (*Nicotiana sylvestris*). The complete amino acid sequences of DHDPS from *E. coli*, *Cornynebacterium glutamicum*, wheat and maize have been reported.[30,31] These enzymes have been shown to exist as homotetramers, composed of four identical subunits.

Shedlarski and Gilvarg purified DHDPS 5000-fold from crude extracts of *E. coli* W.[32] The protein was shown to be homogeneous by polyacrylamide gel electrophoresis and to bear a net negative charge in the pH range 6.0 to 9.2. Amino acid analyses indicate the complete absence of methionine. The purified protein showed a single band upon a denaturing gel (SDS-PAGE) corresponding to a molecular mass of 33,000.[33] This is in good agreement with the molecular weight of 31,372 predicted from the nucleotide sequence of the *dapA* gene.[30] Since the molecular mass of the native protein has been estimated at 134,000 by gel permeation chromatography, DHDPS is most likely to be a tetramer composed of four subunits with identical molecular masses.[32,33,34] In bacteria, DHDPS is subject to two different types of control. In *E. coli* [35] and *Bacillus sphaericus* [36] the DAP pathway is characterised by a lysine feedback inhibition of DHDPS. In *C. glutamicum* [37] and some *Bacillus* [38,39] species there exists a lysine-insensitive form of DHDPS.

In 1975, Cheshire and Miflin first reported a partial purification of DHDPS from maize (*Z. mays* L.).[40] Mazelis and co-workers detected DHDPS activity in a range of both dicotyledonous and monocotyledonous species including corn, bean shoot, cabbage leaf, spinach leaf, potato tuber and squash fruit and succeeded in

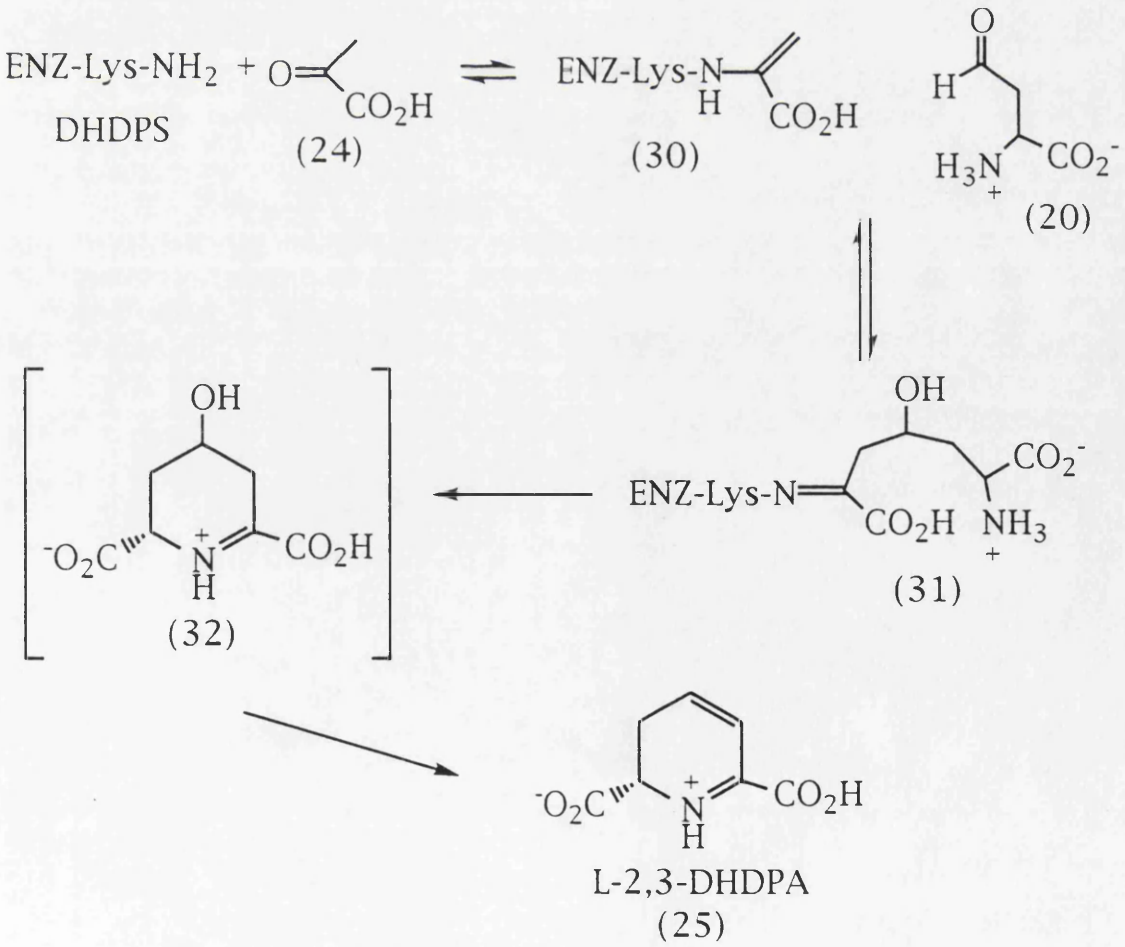
partially purifying DHDPS from wheat germ.[41] Wallsgrove and Mazelis obtained partially purified DHDPS from spinach leaves (*S. oleracea* L.) by chromatography and gel filtration.[42] They found the molecular weight of the enzyme to be 115,000. The DHDPS of spinach leaves was found to be localised in the chloroplast where the pH optimum is pH 8.2, close to the stromal pH of chloroplasts in the light.[43] DHDPS was purified 5100-fold from suspension cultured cells of wheat (*Triticum aestivum* var. Chinese Spring) by Kumpaisal and co-workers in 1987.[44] An average molecular weight of 123,000 was obtained from gel filtration with a subunit molecular weight of between 32,000 to 35,000. The enzyme exhibited maximum activity at pH 8.0.

Ghislain and co-workers[45] isolated and characterised DHDPS from *N. sylvestris*. The synthase was localised in the chloroplasts and identified as a soluble stroma enzyme by enzymatic and immunological methods. The molecular weight of the enzyme was determined to be 164,000. By carrying out isotopic labelling experiments with ^{14}C-pyruvate, the enzyme was shown to be composed of four identical subunits of 38,500.

A more recent isolation of the enzyme was from maize leaves (*Z. mays* L.) by Frisch and co-workers.[46] They obtained a 30,000-fold purification and a molecular weight of 130,000 for the native enzyme. The subunit molecular weight of maize DHDPS was estimated to be 38,000 on SDS-PAGE.

2.2 Mechanism of Dihydrodipicolinate Synthase.

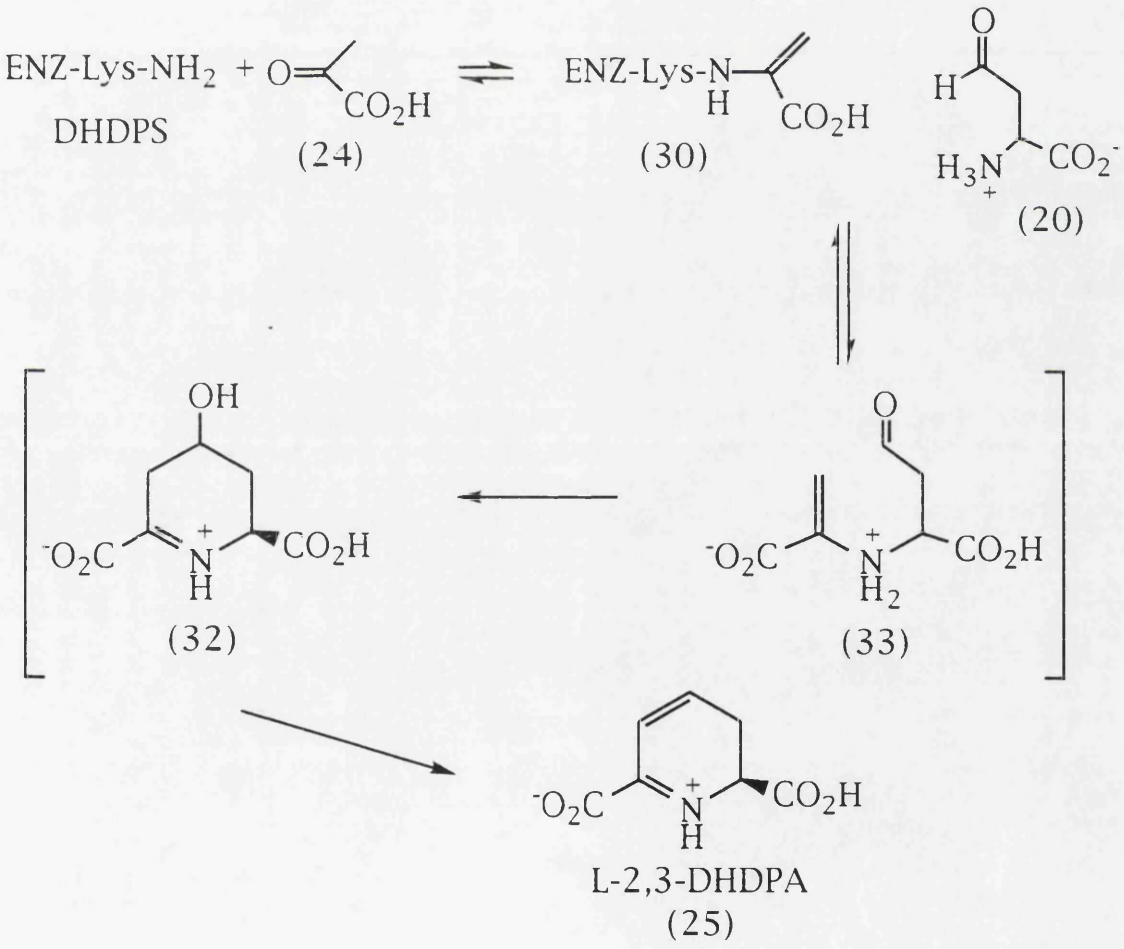
The sequence of steps in the mechanism of formation of L-2,3-dihydrodipicolinic acid (L-2,3-DHDPA) (25) from L-aspartic acid β-semialdehyde (L-ASA) (20) and pyruvate (24) has not yet been fully elucidated. It is believed that pyruvate (24) binds to the enzyme via an amino group of a lysine residue.[32,44] One possible mechanism is C-C bond formation between the aldehyde group of (20) and the methyl carbon of the activated pyruvate (30) to give the enzyme bound intermediate (31). Ring closure would result in 4-hydroxy-L-2,3,4,5-THDPA (32). Loss of a mole of the elements of water gives the enzymic product (25) **(Scheme 9)**.



Scheme 9

An alternative mechanism could involve the initial formation of the intermediate (33). This would be followed by a ring closing step to give 4-hydroxy-L-2,3,4,5-THDPA (32). Again, the loss of a mole of the elements of water would give the enzyme product (25) **(Scheme 10).**

Previous studies with various aldolases have shown that imine formation between a keto substrate and an ε-amino group of a lysine residue on the enzyme is an obligatory step in enzyme-catalysed condensation reactions.[47]



Scheme 10

Shedlarski and Gilvarg[32] have shown that there is irreversible loss of enzyme activity upon the addition of sodium

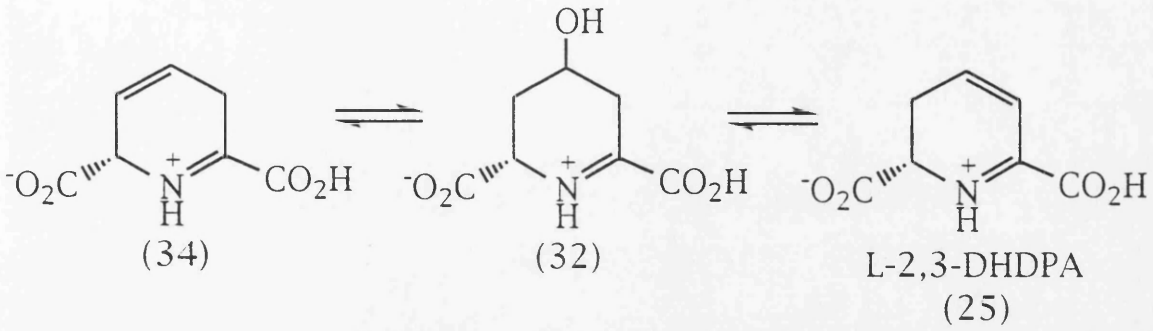
borohydride only in the presence of pyruvate (24) with DHDPS isolated from *E. coli*. This suggests that the formation of a Schiff's base intermediate between DHDPS and pyruvate (24) takes place in the enzymatic reaction. They also established, by chromatographic and electrophoretic methods, that the Schiff's base is between the carbonyl group of pyruvate (24) and an ε-amino group of a lysine residue attached to the protein. More recently, in an X-ray study of DHDPS, Laber *et al.* have identified Lys-161 as the active site lysine residue of DHDPS.[34] Sequence alignment and comparison revealed that Lys-161 is the only lysine residue conserved in the five characterised DHDPS isoenzymes.

From kinetic studies carried out on DHDPS isolated from wheat suspension cultures, Kumpaisal *et al.* [44] have proposed that the reaction proceeds via a 'ping-pong' mechanism. Pyruvate (24) first binds to the enzyme to form a Schiff's base (K_m 11.76 mM). K_m is the Michaelis constant, the dissociation constant of the enzyme-substrate complex. Water is then released, followed by the binding of L-ASA (20) (K_m 0.88 mM). Nucleophilic attack of the pyruvyl enamine on the L-ASA aldehyde group could then take place to give intermediate (31).

Kinetic studies carried out by Laber *et al.* [34] with *E. coli* DHDPS have also given results consistent with a ping-pong mechanism. K_m values of 0.57 mM and 0.55 mM for pyruvate (24) and DL-ASA (20) respectively were achieved.

The product of the enzymic reaction catalysed by DHDPS was found to be extremely labile, severely limiting efforts for its thorough characterisation. It has been suggested that the proposed product, L-2,3-DHDPA (25), would be expected to be in equilibrium with L-2,5-dihydrodipicolinic acid (34) and 4-hydroxy-L-2,3,4,5-THDPA (32)

(Scheme 11).[32] It is not clear which of these compounds is the immediate product of the enzymatic reaction.



Scheme 11

2.3 Inhibition of Dihydrodipicolinate Synthase.

In general, L-lysine (1) acts as an allosteric inhibitor, tightly controlling its own biosynthesis by shutting down the lysine-specific branch of the biosynthetic pathway when levels are high.

The DHDPS enzyme from spinach leaves (*Spinacia oleracea* L.), isolated by Wallsgrove and Mazelis[42] is strongly inhibited by L-lysine (1) with an IC_{50} of 20 μM. The IC_{50} value denotes the concentration of inhibitor required to produce 50% inhibition. *S*-(2-Aminoethyl)-L-cysteine (SAC), a lysine analogue, was less effective with an IC_{50} of 0.4 mM.

Frisch *et al.* [46] found, that in enzyme isolated from maize, several lysine analogues inhibited DHDPS activity (**Table 2**). However, none was as effective as L-lysine (1) itself. Analogues missing the α-amino group (ε-aminocaproic acid) or the ε-amino group (norleucine) were not inhibitory, indicating the importance of both amino groups.

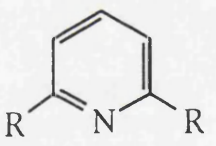
DHDPS isolated from *E. coli* by Laber *et al.*[34] was 50% inhibited by 1.0 mM L-lysine (1), by 1.2 mM sodium dipicolinate and by 4.6 mM *S*-(2-aminoethyl)-L-cysteine. No significant inhibition of DHDPS activity was observed in the presence of analogues of pyruvate (24) such as 3-fluoropyruvate, α-oxobutyrate, phenylpyruvate and oxaloacetate. However, 3-bromopyruvate competitively inhibited the condensation reaction with respect to pyruvate with a K_i value of 1.6 mM.

	Concentration (μM)		
	25	100	1000
	% Inhibition		
L-Lysine (1)	51	97	100
Lysine ethyl ester	14	89	95
threo hydroxylysine	4	83	100
SAC	0	18	93
Arginine	6	8	55
δ-Hydroxylysine	0	2	45
D-Lysine	0	0	27
DAP (28)	0	0	0
ε-Aminocaproic acid	0	0	0
Norleucine	0	0	0

Table 2 (Ref. 46)

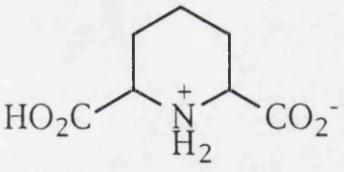
Within our group at Glasgow, a number of analogues of L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (26) have been tested on DHDPS isolated and purified from *E. coli*.[48] These compounds, which have a common structural feature of a 5- or 6-membered heterocyclic system containing nitrogen substituted with acid mimics at the α- or α, α'-positions, were prepared by Dr. L. Couper and Dr. D.W. Tudor.

Dipicolinic acid (35) showed significant inhibition of DHDPS at 0.5 mM, as did some of its derivatives. An IC_{50} value of 0.2 mM was observed for both the diimidate (36) and the *N*-oxide derivatives of dipicolinic acid (35). Compounds with only one carboxyl group such as pipecolinic acid, proline and picolinic acid and their derivatives showed less inhibition of DHDPS. Poor inhibition was observed with the fully saturated piperidine-2,6-dicarboxylic acid (37) analogues.[48]



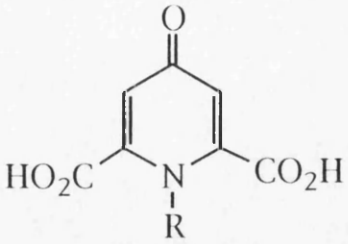
R = CO_2H (35)

R = $C(OMe)=NH_2^+ Cl^-$ (36)

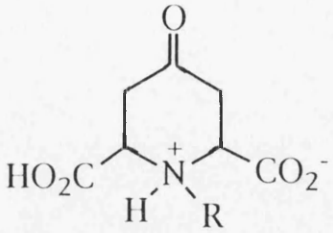


(37)

Chelidamic acid (38) and its *N*-substituted derivatives showed good inhibition of DHDPS at low concentrations. *N*-Methylchelidamic acid (39) gave the best inhibition of 63% at 0.1 mM. The diesters of chelidamic acid and their *N*-substituted derivatives also showed good inhibition. Interestingly, piperid-4-one-2,6-dicarboxylic acid (40) and its *N*-methyl derivative (41) also showed good inhibition, with values of 30% and 54% respectively at 0.1 mM.

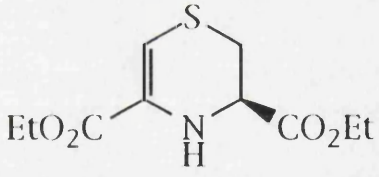


R = H (38)
R = Me (39)

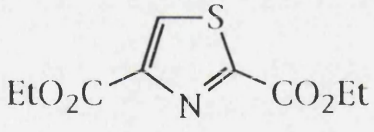


R = H (40)
R = Me (41)

Sulfur analogues of L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (26) showed good inhibition of DHDPS. Diethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (42) gave 13% inhibition at 0.1 mM. Diethyl 1,3-thiazole-2,4-dicarboxylate (43) showed 15% inhibition at 0.1 mM.



(42)



(43)

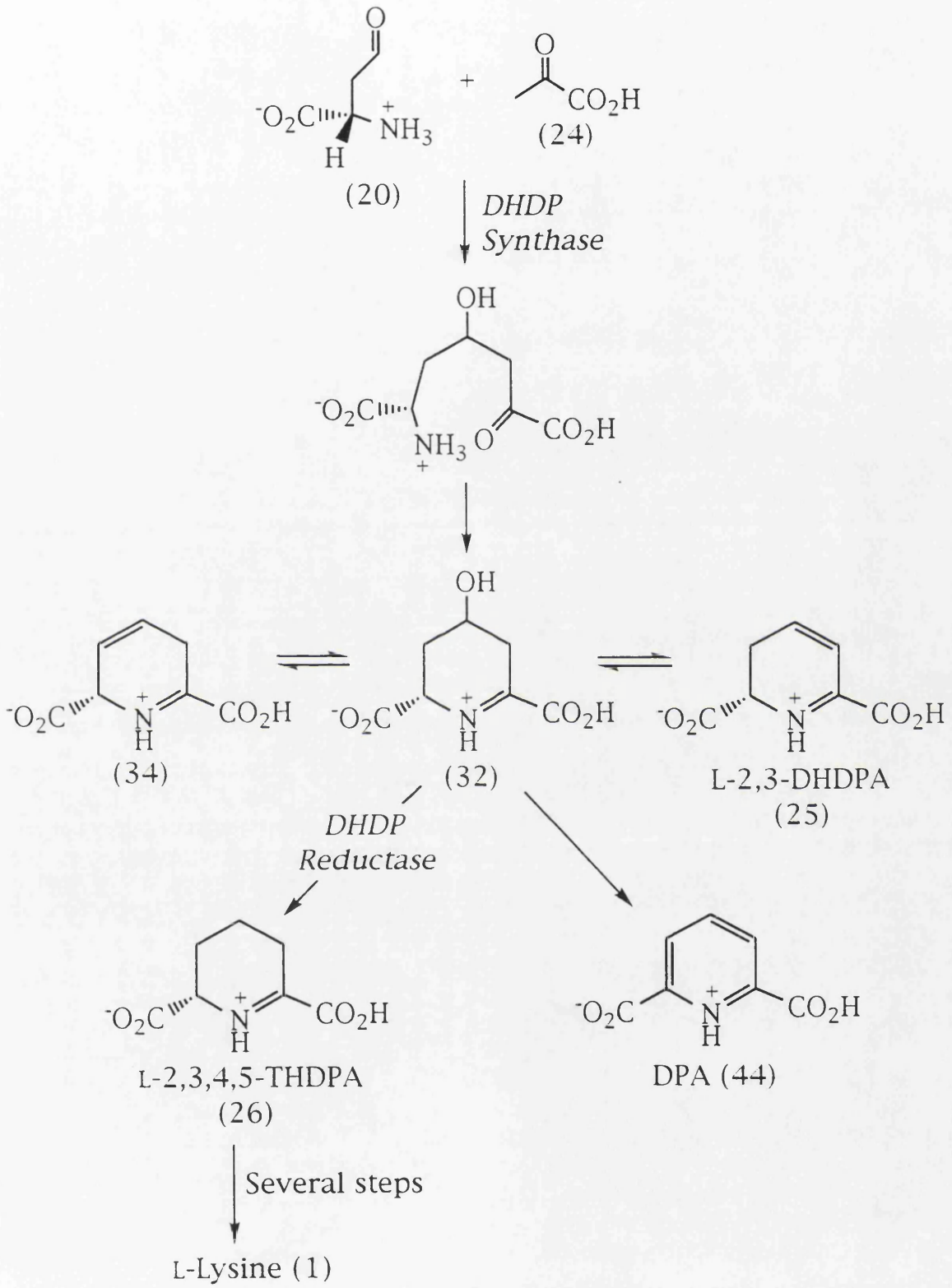
2.4 Dihydrodipicolinate Reductase.

The enzyme dihydrodipicolinate reductase (DHDPR) catalyses the pyridine nucleotide-linked reduction of L-2,3-DHDPA (25) to L-2,3,4,5-THDPA (26) **(Scheme 12)**.

The reductase was first described in *E. coli* by Farkas and Gilvarg.[49] Tamir and Gilvarg[50] succeeded in purifying the protein to homogeneity. A molecular weight of 110,000 was calculated and the K_m value obtained for L-2,3-DHDPA (25) was 9.0 μM. The pH optimum was 7.0 with either NADPH or NAPH as the cocatalyst.

DHDPR was purified 100-fold from crude extracts of both *Bacillus cereus* and *B. megaterium*.[51] The molecular weights of the proteins were 155,000 and 150,000 respectively. The reductases were inhibited noncompetitively by dipicolinic acid (44) with respect to L-2,3-DHDPA (25) and the K_i values were 85 μM and 140 μM respectively.

DHDPR has also been isolated and partially purified by Tyagi *et al.* from maize kernels.[52] The crude maize extract and the partially purified enzyme were assayed for DHDPR by their ability to restore the capability of crude extracts of a mutant *E. coli* (defective in DHDPR) to synthesise *meso*-2,6-diaminopimelate (29) from L-ASA (20) and pyruvate (24). The K_m value obtained for L-2,3-DHDPA (25) was 0.43 mM and for NADPH the K_m was 46 μM. The enzyme had a pH optimum close to 7.0 and was much more temperature labile than the bacterial enzyme. Its molecular weight was 80,000.


(20)
(24)
DHDP Synthase
(34)
(32)
L-2,3-DHDPA (25)
DHDP Reductase
L-2,3,4,5-THDPA (26)
DPA (44)
Several steps
L-Lysine (1)

Scheme 12

2.5 Mechanism of Dihydrodipicolinate Reductase.

2.5.1 L-2,3-Dihydrodipicolinic Acid (L-2,3-DHDPA).

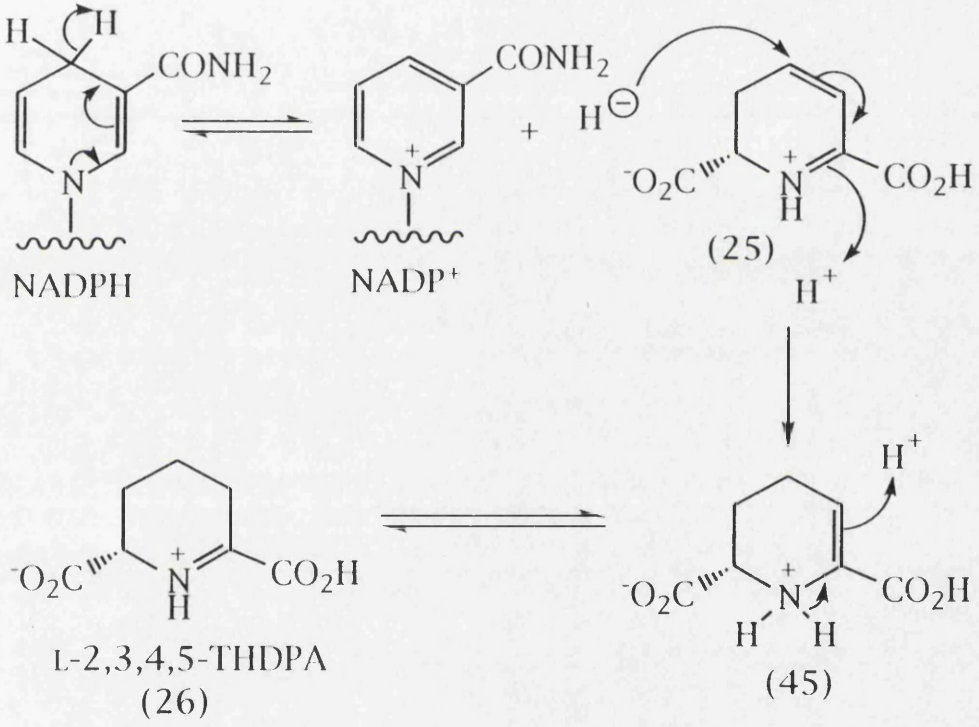
L-2,3-DHDPA (25) is extremely unstable and its isolation has not yet been achieved. Shedlarski and Gilvarg[32] suggested that L-2,5-dihydrodipicolinic acid (34) might be the reaction product of the pyruvate-ASA condensing enzyme and that this isomer may be in equilibrium with L-2,3-DHDPA (25) and 4-hydroxy-3,4,5,6-tetrahydrodipicolinic acid (32) **(Scheme 12)**. It is not clear which of these three compounds is the immediate product of the enzymatic condensation.

Tyagi *et al.*[52] described the synthesis of L-2,3-DHDPA (25) by the condensation of L-ASA (20) with oxaloacetic acid in alkali. L-2,3-DHDPA (25) was precipitated as its barium salt. The compound was stored in water at -80 °C and was found to be unstable at room temperature at neutral pH. Only freshly prepared material was used in enzyme assays.

2.5.2 Mechanism of Formation of L-2,3,4,5-Tetrahydrodipicolinic Acid (L-2,3,4,5-THDPA).

The reduction of L-2,3-DHDPA (25) to L-2,3,4,5-THDPA (26) by dihydrodipicolinate reductase requires the use of NADPH as a co-enzyme. NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate, is the major electron donor in reductive biosynthesis. When a substrate is reduced, the hydride anion is

transferred from the nicotinamide ring of NADPH to produce $NADP^+$. The reduced co-enzyme has a prochiral centre and the process is stereospecific. The reduction may involve a 1,4-addition of the hydride anion to the conjugated system to form L-1,2,3,4-tetrahydrodipicolinic acid (45) which could tautomerise to L-2,3,4,5-THDPA (26) **(Scheme 13)**. It has not yet been established whether the enzyme donates the *pro-S* or the *pro-R* hydrogen of its co-factor, NADPH.



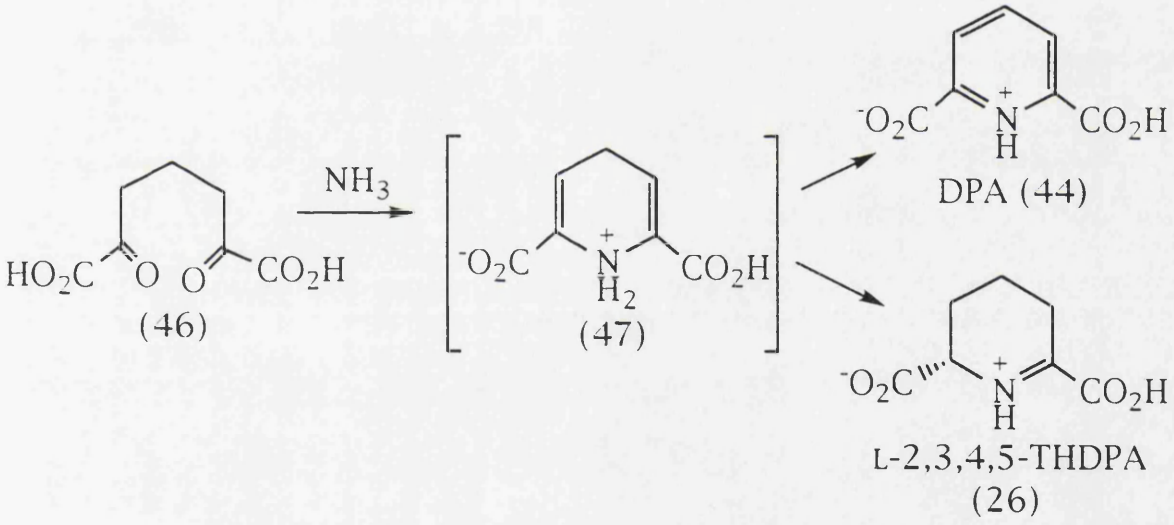
Scheme 13

2.5.3 L-2,3,4,5-Tetrahydrodipicolinic Acid.

Shapshak[53] claimed the formation of the D-isomer of THDPA by treatment of DL-2,6-diaminopimelic acid (28) with the L-amino

acid oxidase from *Neurospora crassa*, but no chemical or spectroscopic evidence for the product was provided.

Kimura and Sasakawa[54] reported the formation of DPA (44) and L-2,3,4,5-THDPA (26) on cyclisation of αα'-dioxopimelic acid (46) with ammonia. Since the reaction rate was not affected by the presence or absence of oxygen, they assumed that the initial product, 1,4-dihydrodipicolinic acid (47), disproportionated to DPA (44) and L-2,3,4,5-THDPA (26) **(Scheme 14)**. Evidence for the formation of the products was provided by UV absorption (for DPA) and by colour reactions with ninhydrin and *o*-aminobenzaldehyde (for L-2,3,4,5-THDPA).

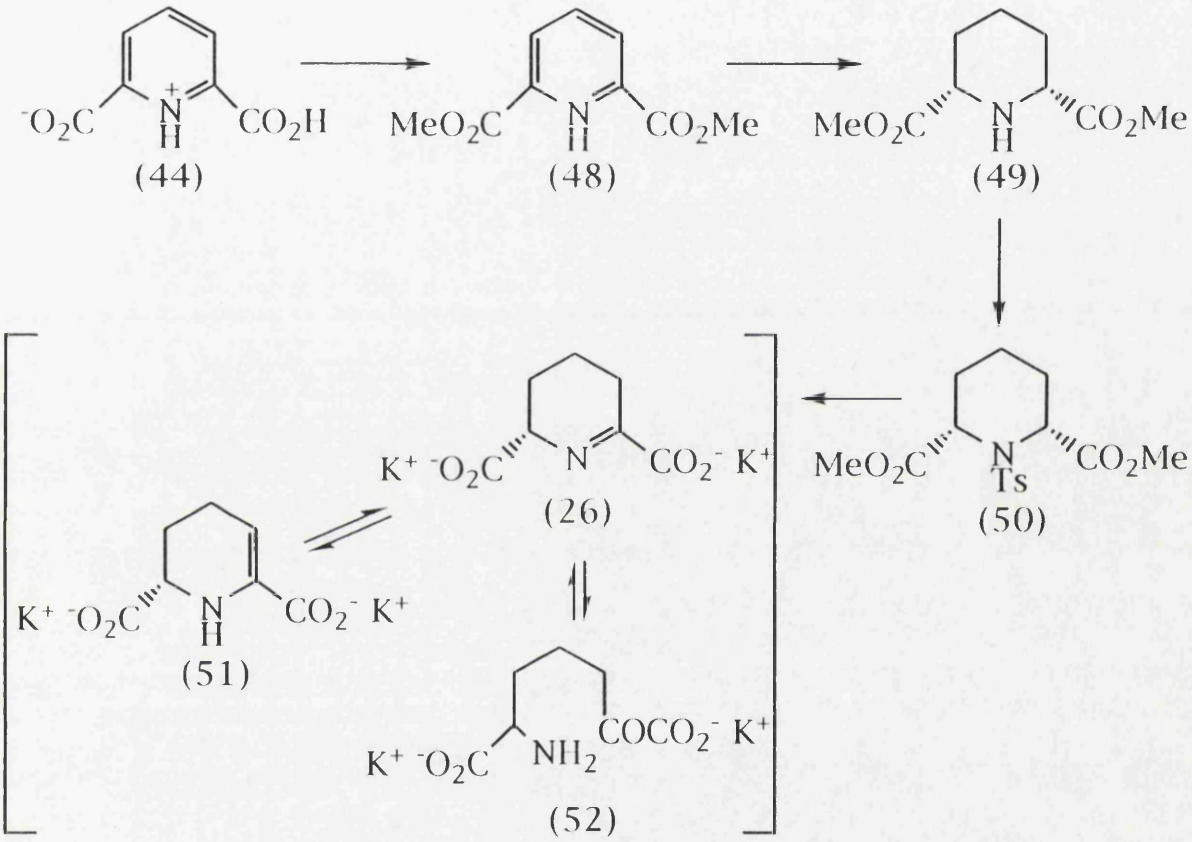


Scheme 14

The potassium salt of L-2,3,4,5-THDPA (26) has been prepared by the elimination of *p*-toluenesulfinic acid from the *N*-toluenesulfonyl (tosyl) derivative (50) of dimethyl *cis*-piperidine-2,6-dicarboxylate (49) at Glasgow by Couper *et al.* **(Scheme 15)**.[55]

Dimethyl dipicolinate (48) was made from DPA (44) by heating at reflux in methanol and conc. sulfuric acid. Hydrogenation of

(48) gave dimethyl *cis*-piperidine-2,6-dicarboxylate (49) in good yield. Dimethyl *N*-tosyl-*cis*-piperidine-2,6-dicarboxylate (50) was synthesised from (49) by stirring overnight in pyridine with toluenesulfonyl chloride.



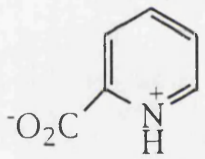
Scheme 15

The elimination was carried out using potassium *t*-butoxide in dichloromethane at room temperature. The ester groups were simultaneously cleaved during the reaction. Purification was achieved by stirring the reaction product in distilled water with the weak anion exchanger Amberlite IR-45 (hydroxide form) for 24 hours. ^{1}H and ^{13}C NMR data indicated that the potassium salt of L-2,3,4,5-THDPA (26)

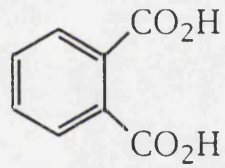
exists in solution in equilibrium with the corresponding enamine (51) and an open chain form (52). The potassium salt of L-2,3,4,5-THDPA (26) was shown to be a substrate for *meso*-DAP dehydrogenase by incubation with the enzyme and observation of the disappearance of NADPH. This dehydrogenase is used by some bacteria in the biosynthesis of L-lysine (1),[56] and L-2,3,4,5-THDPA (26) is converted directly into *meso*-DAP (29) using NADPH. *meso*-DAP Dehydrogenase will be discussed in more detail in Section 2.12.

2.6 Inhibition of Dihydrodipicolinate Reductase.

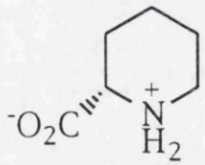
Theoretically, L-2,3-DHDPA (25) could exist in the open chain form with an amino group and a keto group at the α and ε positions or as a cyclic compound **(Scheme 12)**. The DHDPR enzyme isolated from *E. coli* by Tamir and Gilvarg[50] was inhibited by dipicolinic acid (DPA) (44). It was found to be a competitive inhibitor with a K_i value of 1.0 mM. α-Picolinic acid (53) and isophthalic acid (54) were also found to be inhibitors of the enzyme. The high affinity and competitive nature of the inhibition shown by DPA (44) and to a lesser extent, isophthalic acid (54), strongly suggests that a cyclic form of the substrate binds to the active site on the enzyme.



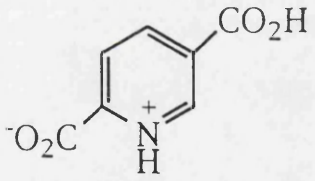
(53)



(54)



(55)



(56)

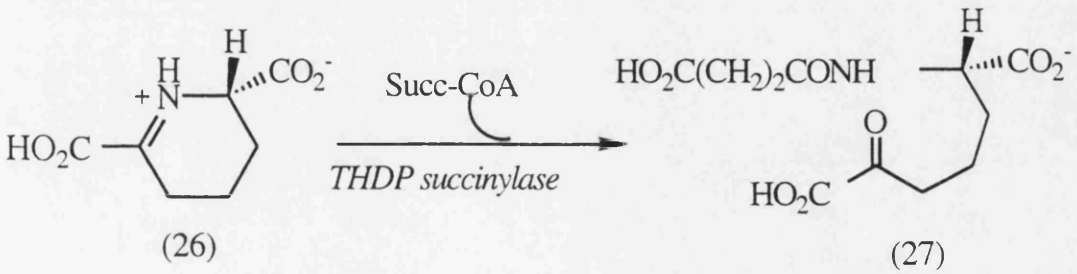
Tyagi *et al.* [52] found that the DHDPR isolated from maize was also inhibited by DPA (44), with a K_i value of 0.9 mM, indicating that, as in *E. coli*, the substrate of the enzyme is the cyclic form of L-2,3-DHDPA (25) rather than its open chain counterpart. A number of other compounds with similar structures to L-2,3-DHDPA (25) also inhibited the reductase **(Table 3)**.

Inhibitor	% Inhibition			
	20 mM	10 mM	5mM	1.5 mM
α-Picolinic acid (53)	24	4	8	0
L-Pipecolic acid (55)	23	30	0	0
Isophthalic acid (54)	44	20	0	0
Isocinchomeronic acid (56)	-	-	50	0
Dipicolinic acid (44)	-	-	-	100

Table 3

2.7 Tetrahydrodipicolinate Succinylase.

Tetrahydrodipicolinate (THDP) succinylase catalyses the *N*-succinylation of L-2,3,4,5-THDPA (26) by succinyl-coenzyme A to form succinyl-ε-keto-α -aminopimelate (27) **(Scheme 16)**.



(26) (27)

Scheme 16

The purification of THDP succinylase was not fully investigated until recently, largely due to the unavailability of its substrate, L-2,3,4,5-THDPA (26), required for the enzyme assay. Simms *et al.* [57] purified THDP succinylase 1900-fold from crude

extracts of *E. coli.* The L-2,3,4,5-THDPA (26) required for the enzyme assay was enzymatically prepared by the oxidative deamination of *meso*-DAP (29) in the presence of NADPH and *meso*-DAP dehydrogenase. The protein was found to be homogeneous by polyacrylamide gel electrophoresis and consists of two subunits each with a mass of 31,000. The pH optimum of the enzyme is 8.2. The equilibrium of the reaction lies predominantly in favour of product formation but the reverse reaction has been demonstrated *in vitro.*

THDP Succinylase is a sulfhydryl enzyme. In the absence of mercaptoethanol the enzyme lost 80% of its activity if kept at 4 °C for 4 days. No loss of enzymatic activity was observed when the same enzyme solution was stored in 0.001 M mercaptoethanol under the same conditions. Furthermore, known inhibitors of sulfhydryl enzymes were found to be effective in inhibiting THDP succinylase.

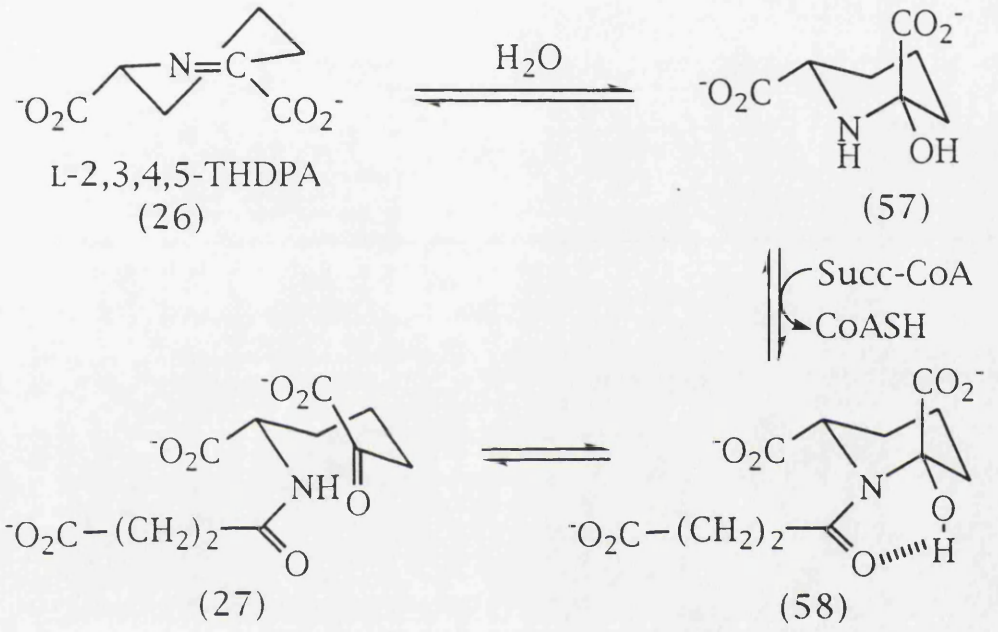
The acyl donor for the reaction is succinyl-CoA in *E. coli.* In *Brevibacterium lactofermentum,* both succinyl-CoA and acetyl-CoA can serve as the acyl-donor.[58]

2.8 Mechanism of THDP Succinylase.

Berges *et al.* [59] have proposed a stereochemical model for the succinylation of L-2,3,4,5-THDPA (26) from studies on the active site of THDP succinylase of *E. coli* **(Scheme 17).**

They tested cyclic and acyclic analogues of L-2,3,4,5-THDPA (26) for their ability to act as substrates or inhibitors of the succinylase. From these experiments, they were able to draw some conclusions regarding the catalytic mechanism of the enzyme and the structural requirements of the active site. The proposed model

involves the initial binding of L-2,3,4,5-THDPA (26) to the active site of the enzyme. Water adds to the double bond, *cis* to the 6-carboxyl group, producing a *trans*-piperidine-2,6-dicarboxylate (57). The hydrated product is then succinylated (58) and ring opening results in succinyl-ε-keto-α -aminopimelate (27).



L-2,3,4,5-THDPA (26)

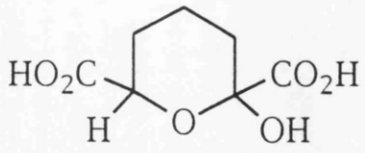
(57)

(27)

(58)

Scheme 17

An important piece of evidence for this model is the strong inhibition of the enzyme observed with 2-hydroxytetrahydropyran-2,6-dicarboxylic acid (59). With regard to the proposed mechanism, (59) would be expected to be a transition state analogue because of its similarity to the proposed hydrated intermediate (57). Its very low K_i value of 58 nM is consistent with this expectation.

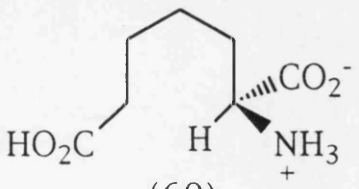


(59)

2.9 Inhibition of THDP Succinylase.

2.9.1 Acyclic Analogues of L-2,3,4,5-THDPA (26).

Gilvarg *et al.*[59] found D-2-aminopimelate (D-2-AP) (60) to be a reasonable acyclic inhibitor of the enzyme, with a K_i value of 0.41 mM. The presence of two carboxylate groups was shown to be important for binding to the enzyme. Furthermore, the spatial separation of the carboxyl groups was important for binding and substrate activity. D- and L-2-aminoadipic acid (one methylene group less than D-2-AP) and DL-2-aminosuberic acid (one methylene group more than D-2-AP) were very poor inhibitors of the enzyme. Substituting a sulfur for a methylene group had little effect as DL-2-amino-5-thiapimelic acid was a reasonable inhibitor, with a K_i value of 1.1 mM.



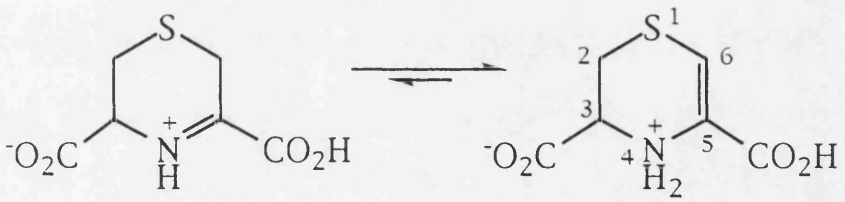
(60)

Introducing a hydroxyl group α to a carboxylate group was effective in increasing inhibition of the enzyme. Both enantiomers of 2-hydroxypimelic acid had high affinity for the enzyme with K_i

values of 0.33 mM and 0.19 mM respectively. The hydroxyl group may be involved in hydrogen-bonding with the enzyme. DL-Dihydroxypimelic acid was also a relatively good inhibitor with a K_i value of 0.14 mM.

2.9.2 Cyclic Analogues of L-2.3.4.5-THDPA.

The only cyclic compound other than L-2,3,4,5-THDPA (26) found to be a substrate for the succinylase was 3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid (DHT) (61).[59] Only the enamine tautomeric form of DHT (61) was observed in the ^1H NMR spectrum of the disodium salt in aqueous solution **(Scheme 18)**. The higher K_m value of DHT (61) may be explained by the fact that the imine form, which is used by the enzyme as a substrate, makes up only a small fraction of the total DHT (61).



(61)

Scheme 18

Other cyclic compounds were found to be inhibitors and are listed in **Table 4**. The best inhibitor was 2-hydroxytetrahydropyran-2,6-dicarboxylic acid (59). This was a competitive inhibitor with respect to L-2,3,4,5-THDPA (26) with a K_i value of 0.06 μM.

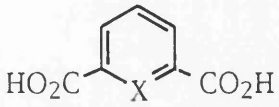
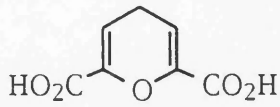
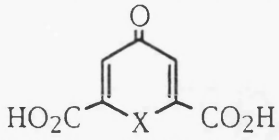
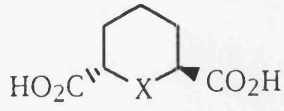
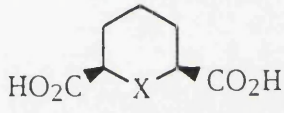
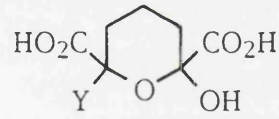
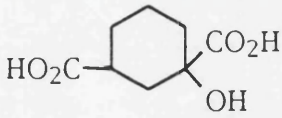
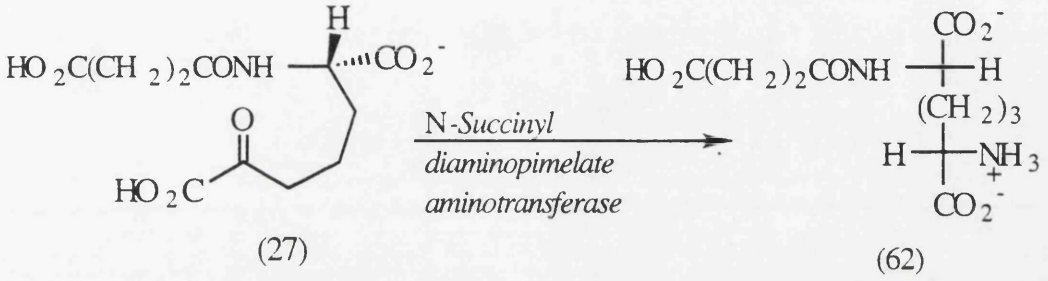
Compound	X	Name	K_i (μM)
	N CH	Dipicolinic acid Benzene-2,6-dicarboxylic acid	12,800 8,500
		Pyran-2,6-dicarboxylic acid	1,800
	NH O	Chelidamic acid Chelidonic acid	2,600 3,100
	NH O	*trans*-Piperidine-2,6-dicarboxylic acid *trans*-Tetrahydropyran-2,6-dicarboxylic acid	2,000 680
	NH O	*cis*-Piperidine-2,6-dicarboxylic acid *cis*-Tetrahydropyran-2,6-dicarboxylic acid	63,000 3,900
	Y=H Y=OH	2-Hydroxytetrahydropyran-2,6-dicarboxylic acid 2,6-Dihydroxytetrahydropyran-2,6-dicarboxylic acid	0.06 0.20
		*trans*-2-Hydroxycyclohexane-2,6-dicarboxylic acid *cis*-2-Hydroxycyclohexane-2,6-dicarboxylic acid	5,600 12,000

Table 4 (Ref. 59)

2.10 *N*-Succinyl Diaminopimelate Aminotransferase.

N-Succinyl diaminopimelate aminotransferase catalyses the interconversion of *N*-succinyl-α-amino-ε-ketopimelate (27) and *N*-succinyl-L-diaminopimelate (62) **(Scheme 19)**. The succinylated intermediate is aminated at the ε-position with glutamate as the amino group donor.

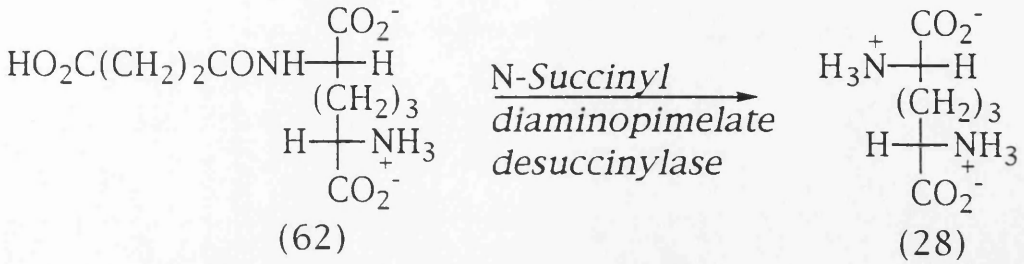


Scheme 19

The enzyme has been isolated and partially purified from *E. coli* by Peterofsky and Gilvarg.[60] The aminotransferase is pyridoxal phosphate dependent and is inhibited by hydroxylamine. Treatment of the enzyme at 0 °C with 0.05 M hydroxylamine causes 95% inhibition of enzyme activity.

2.11 *N*-Succinyl Diaminopimelate Desuccinylase.

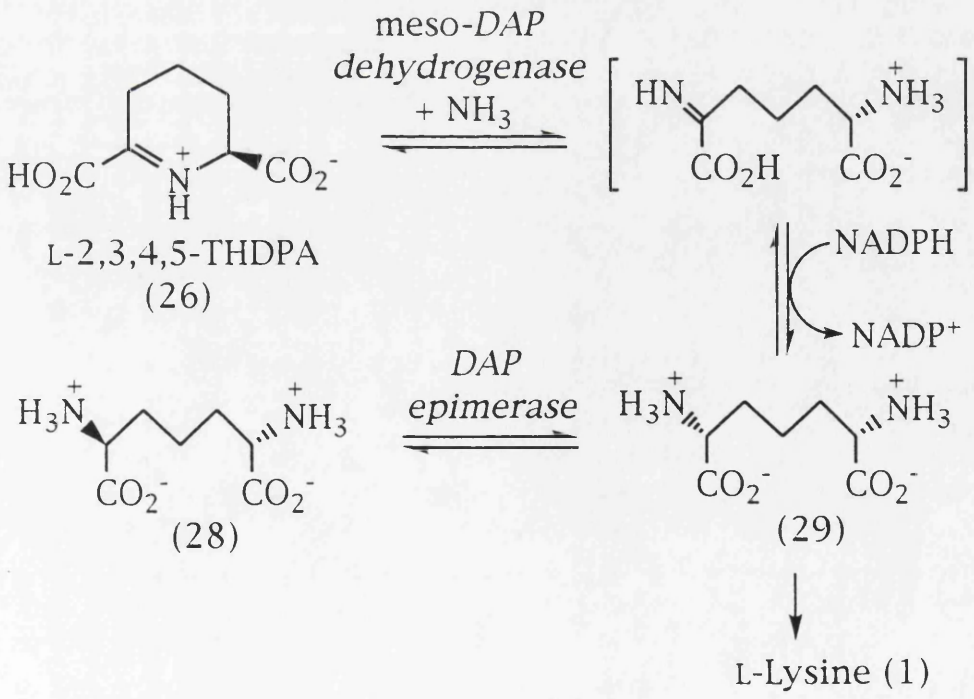
N-Succinyl diaminopimelate desuccinylase catalyses the hydrolysis of *N*-succinyl-L-diaminopimelate (62) to L,L-diaminopimelate (28) and succinate **(Scheme 20)**. The enzyme has been partially purified from *E. coli* by Kindler and Gilvarg.[61] It has a pH optimum of 8 and has been shown to require Co^{2+} for activity.



Scheme 20

2.12 *meso*-Diaminopimelate Dehydrogenase.

Certain bacteria possess an alternative pathway for *meso*-2,6-DAP (29) production and can transform the early precursor, L-2,3,4,5-THDPA (26) directly into *meso*-2,6-DAP (29) using *meso*-diaminopimelate (DAP) dehydrogenase **(Scheme 21)**.



Scheme 21

Like other amino acid dehydrogenases, this NADPH-dependent enzyme reduces a primary imine functionality, formed from the cleavage of the L-2,3,4,5-THDPA (26) ring by transamination with ammonia.

At neutral or slightly basic conditions, the dehydrogenase normally catalyses the reductive amination of L-2,3,4,5-THDPA (26) to *meso*-2,6-DAP (29) with concommitant conversion of NADPH into $NADP^+$, which allows continuous spectrophotometric assay at 340 nm. In contrast, pH 10.5 is the optimum for the reverse reaction, oxidative deamination of *meso*-2,6-DAP (29).

Misono and Soda[62] purified the protein to homogeneity from extracts of *Bacillus sphaericus*. The molecular weight of the enzyme was determined to be approximately 80,000. Optimum activity was observed at pH 10.5 when the enzyme was found to deaminate exclusively the *meso* form of 2,6-DAP (29), but did not act upon the D- and L-isomers. This process was shown to require $NADP^+$ as a cofactor. The K_m values were 2.5 mM for *meso*-2,6-DAP (29) and 0.83 μM for $NADP^+$.

Misono *et al.* [63] also purified *meso*-DAP dehydrogenase 400-fold from *C. glutamicum*. The enzyme had a molecular weight of 70,000 and consisted of two identical subunits. The pH optima for deamination and amination were about 9.8 and 7.9 respectively. The Michaelis constants were 3.1 mM for *meso*-2,6-DAP (29), 0.12 mM for $NADP^+$, 0.28 mM for L-2-amino-6-ketopimelate, 36 mM for ammonia and 0.13 mM for NADPH. The enzyme was detected in a wider range of bacterial species than previously reported, suggesting that the alternative lysine biosynthetic pathway (utilising *meso*-DAP dehydrogenase) is more widespread than realised. Both the DAP and

meso-DAP dehydrogenase pathways were found to operate in *C glutamicum.*

In plant systems, Wenko *et al.* [64] have isolated and purified *meso*-DAP dehydrogenase from *Glycine max* embryos. Maximum enzyme activity was observed at pH 8.0. From preliminary molecular weight determinations, the enzyme was found to have no subunit structure and had an apparent molecular weight of 67,000.

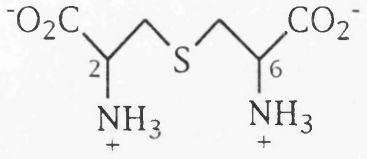
2.13 Mechanism of *meso*-DAP Dehydrogenase.

The mechanism of *meso*-DAP dehydrogenase has not yet been fully established. Studies by Misono and Soda[56] on the enzyme from *B. sphaericus* showed that it donates the 4-*pro-S* hydrogen of its co-factor NADPH to the substrate in the reductive reaction and is highly specific for the *meso*-isomer of DAP. In the active site of the dehydrogenase there is a non-essential thiol group[65] and a tryptophan residue.[56] Product inhibition studies indicated that the sequence of addition of substrates in the oxidative deamination is $NADP^+$ followed by *meso*-2,6-DAP (29) and that the order of release of products is ammonia, then L-2,3,4,5-THDPA (26) and finally NADPH.

2.14 Inhibition of *meso*-DAP Dehydrogenase.

Lam *et al.*[66] examined the interaction of several diaminopimelate analogues with *meso*-DAP dehydrogenase from *B. sphaericus*. Of the diaminopimelate analogues tested, only LL-

lanthionine (63) was found to be a weak competitive inhibitor with respect to *meso*-2,6-DAP (29) with a K_i of 38 mM.

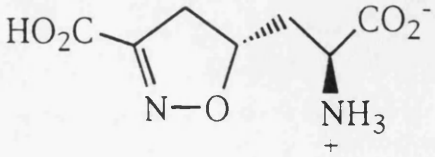


(63) LL-lanthionine (2*S*, 6*S*)

(64) *meso*-lanthionine (2*R*, 6*S*)

Some other analogues tested acted as substrates for the enzyme. The enzyme was shown to exhibit strict specificity for *meso*-isomers and barely accommodates substitutions in the main carbon chain. *meso*-Lanthionine (64) acted as a substrate for the enzyme, with a K_m of 5.8 mM compared with 1.1 mM for *meso*-2,6-DAP (29).

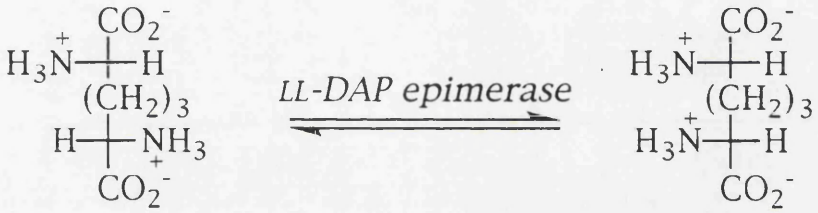
In a more recent report, Abbot *et al.*[67] described the synthesis and testing of heterocyclic analogues of *meso*-2,6-DAP (29) as inhibitors of *meso*-DAP dehydrogenase. They found (*2S, 5S'*)-2-amino-3-(3-carboxy-2-isoxazolin-5-yl)propanoic acid (65) to be a potent and specific inhibitor of the dehydrogenase. At pH 7.5 or 7.8 (65) shows competitive inhibition (K_i 4.2 μM) with L-2,3,4,5-THDPA (26) for the forward reaction and noncompetitive inhibition (K_i 23 μM) with *meso*- 2,6-DAP (29) for the reverse process.



(65)

2.15 LL-Diaminopimelate Epimerase.

Some bacteria can synthesise *meso*-2,6-DAP (29) directly from L-2,3,4,5-THDPA (26). This reaction is catalysed by *meso*-DAP dehydrogenase and is discussed in detail in Section 2.12. However, in most bacteria and higher plants, the enzyme LL-diaminopimelate (LL-DAP) epimerase catalyses the interconversion of LL- (28) and *meso*-2,6-DAP (29) **(Scheme 22).**



(28) (29)

Scheme 22

LL-DAP epimerase was first detected over 30 years ago in *E. coli*[68] but was only recently purified and characterised. Wiseman and Nicols[69] achieved the first purification of LL-DAP epimerase from *E. coli*. In order to facilitate the purification of LL-DAP epimerase, they took advantage of the fact that the alternative pathway for *meso*-2,6-DAP (29) synthesis is utilised by some bacteria[62] to develop a rapid, convenient assay for LL-DAP epimerase. In the alternative pathway, L-2,3,4,5-THDPA (26) is transformed directly into *meso*-2,6-DAP (29) by reductive amination using ammonia and NADPH, catalysed by *meso*-DAP dehydrogenase. This reaction is reversible and allowed the coupling of the epimerisation of LL-2,6-DAP (28) to *meso*-2,6-DAP (29) with the *meso*-DAP dehydrogenase catalysed oxidation of

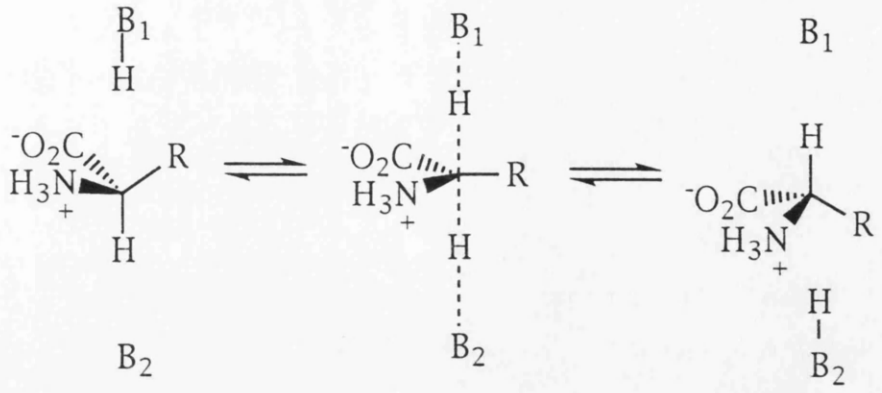
meso-2,6-DAP (29) by $NADP^+$. The production of NADPH can be continually monitored at 340 nm.

The epimerase was found to have a molecular weight of 45,000. It irreversibly loses activity at pH 6 and below. Activity is lost in a few hours at pH 8 in the absence of reducing thiols such as dithiothreitol. Enzyme inactivated in this manner is partially reactivated by thiols. The enzyme is also inactivated by iodoacetamide. This all suggests that the enzyme has a free thiol, necessary for activity.

Richaud *et al.*[70] recently determined the location of the *dapF* gene, encoding for LL-DAP epimerase. They were able to isolate the gene from a high-copy-number plasmid, thereby producing large amounts of the enzyme.

2.16 Mechanism of LL-DAP Epimerase.

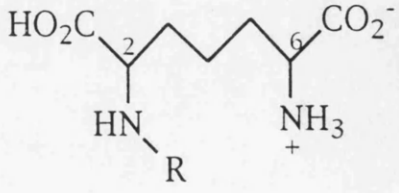
LL-DAP Epimerase is mechanistically interesting. It interconverts LL-2,6-DAP (28) and *meso*-2,6-DAP (29) without the aid of cofactors, metal ions or reducible imine or keto functionalities. This unusual transformation, which is analogous to that catalysed by proline and glutamate racemases, appears to proceed via an intermediate having anionic character at the α-carbon.[71] The two-base in-line mechanism proposed for the action of LL-DAP epimerase on LL-2,6-DAP (28) is shown in **Scheme 23**. B_1 and B_2 designate enzymic acid/bases of unspecified structure. At least one of these groups is thought to be a cysteine sulfhydryl moiety.[69]



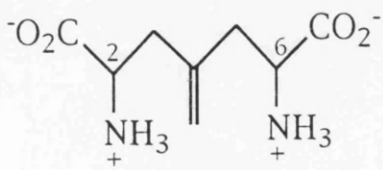
Scheme 23

2.17 Inhibition of LL-DAP Epimerase.

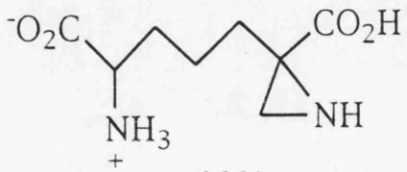
Lam *et al.*[66] synthesised and tested a number of analogues of *meso*-2,6-DAP (29) for inhibition of LL-DAP epimerase of *E. coli*. *meso*-Lanthionine (64) and the LL-isomer (63) were competitive inhibitors with K_i values of 0.18 mM and 0.42 mM respectively. Oxidation of the sulfur atom to a sulfoxide or sulfone drastically lowered the affinity for the enzyme's active site. *N*-Hydroxy DAP (66) was the most potent competitive inhibitor with a K_i of 0.0056 mM. *N*-Amino DAP (67) is weaker with a K_i of 2.9 mM. 4-Methylene-DAP (68) is a noncompetitive inhibitor with K_i 0.95 mM.



R = OH (66)
R = NH_2 (67)

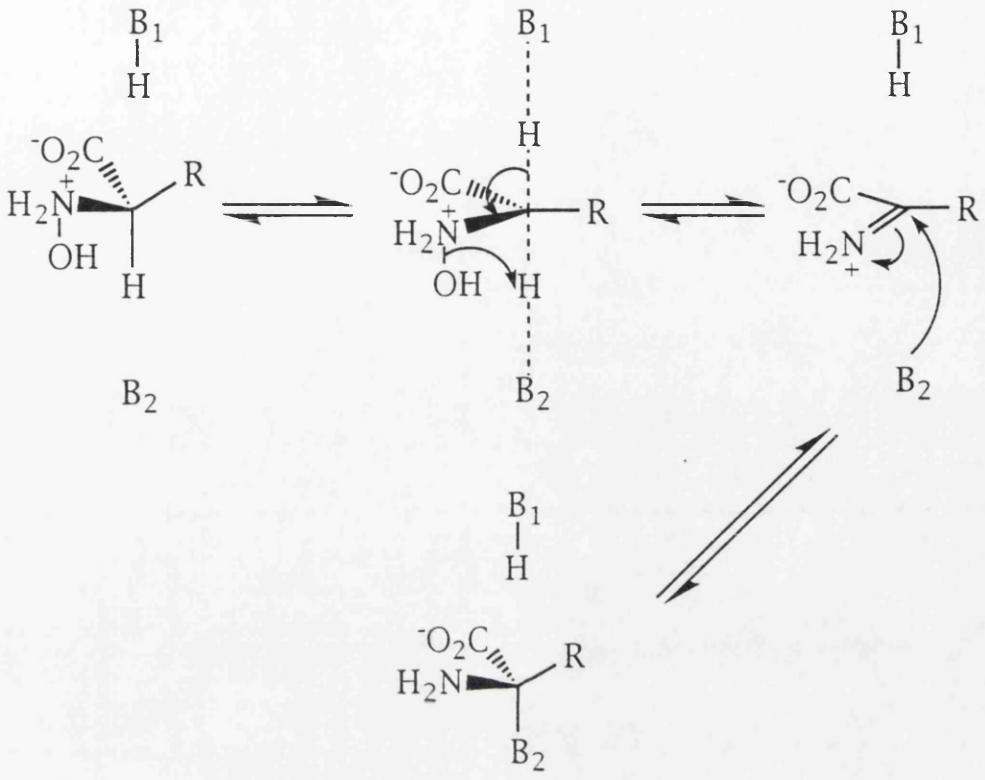


(68)



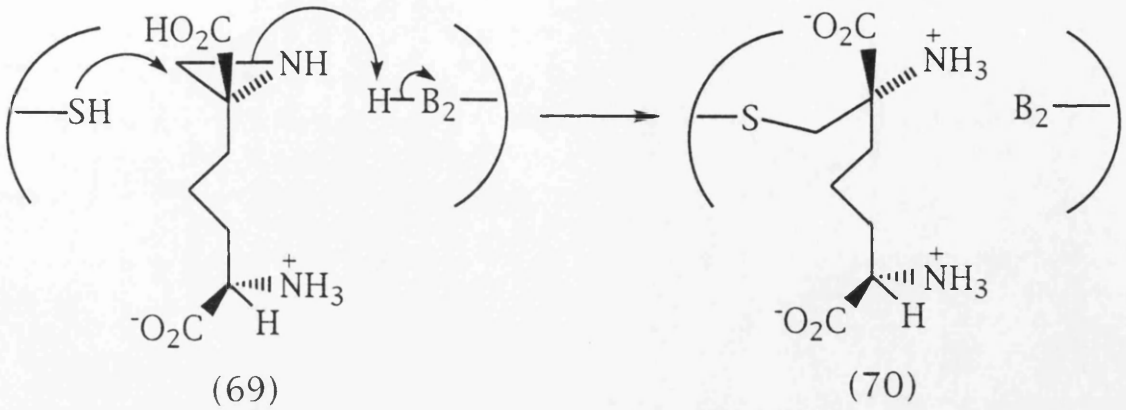
(69)

A proposed mechanism for the interaction of the epimerase with *N*-hydroxy DAP (66) is shown in **Scheme 24.**



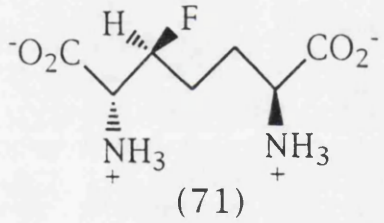
Scheme 24

Higgins *et al.*[72] found that LL-DAP epimerase is irreversibly inhibited by 2-(4-amino-4-carboxybutyl)-2-aziridine carboxylic acid (AZIDAP) (69). This irreversible inactivation results in the active site modification of a cysteine residue. Sequence determination of the protein shows this active site cysteine to be Cys 73 in the overall sequence. The proposed concerted mechanism with AZIDAP (69) as inhibitor involves the formation of an AZIDAP-cysteine adduct (70) **(Scheme 25).**

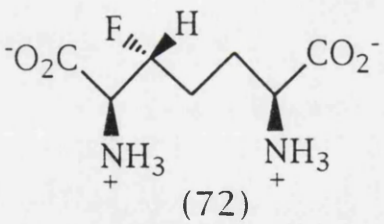


Scheme 25

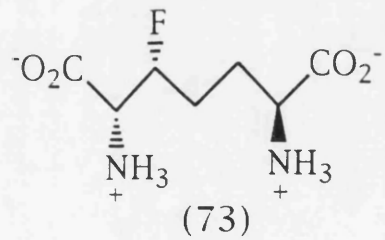
Gelb *et al.*[71] synthesised several fluorinated analogues of *meso*-2,6-DAP (29) in stereochemically pure form. The compounds were shown to be potent inhibitors of LL-DAP epimerase. Compounds (71), (72), (73) and (74) gave IC_{50} values of 4, 10, 25 and 8 μM respectively.



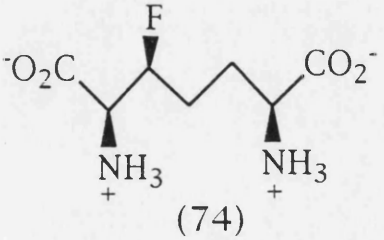
(71)



(72)



(73)

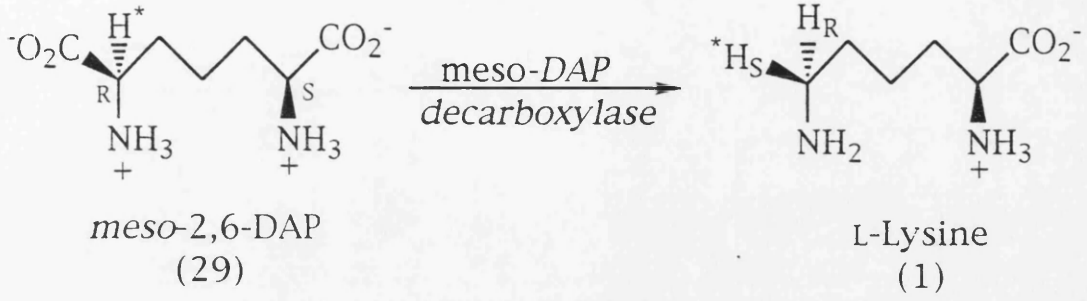


(74)

More recently, Abbot *et al.*[67] synthesised and tested a number of heterocyclic analogues of *meso*-2,6-DAP (29). However, none showed any inhibition of LL-DAP epimerase.

2.18 *meso*-Diaminopimelate Decarboxylase.

The enzyme *meso*-diaminopimelate decarboxylase catalyses the pyridoxal 5'-phosphate decarboxylation of the D-centre of *meso*-2,6-diaminopimelate (29) to give L-lysine (1) **(Scheme 26)**.[73,74]



meso-2,6-DAP
(29)

L-Lysine
(1)

Scheme 26

Dewey and Work[75] first detected the enzyme in *E. coli*. Asada *et al.*[76] purified the enzyme to homogeneity from *Bacillus sphaericus*. The molecular weight of the decarboxylase was determined to be approximately 80,000. The enzyme shows maximum activity in the pH range from 6.8 to 7.8 and decarboxylates exclusively the *meso*-form of 2,6-diaminopimelate (29). The DD- and LL-isomers of 2,6-diaminopimelate are neither substrates nor inhibitors of the enzyme. *meso*-Diaminopimelate decarboxylase has also been isolated from higher plants,[77,78,79] where it is localised solely in the chloroplasts. The molecular weight in higher plants varies between 75,000 for wheat germ[79] and 85,000 for maize endosperm. Estimates of the K_m range from 0.1 to 0.3 mM.

2.19 Mechanism of *meso*-Diaminopimelate Decarboxylase.

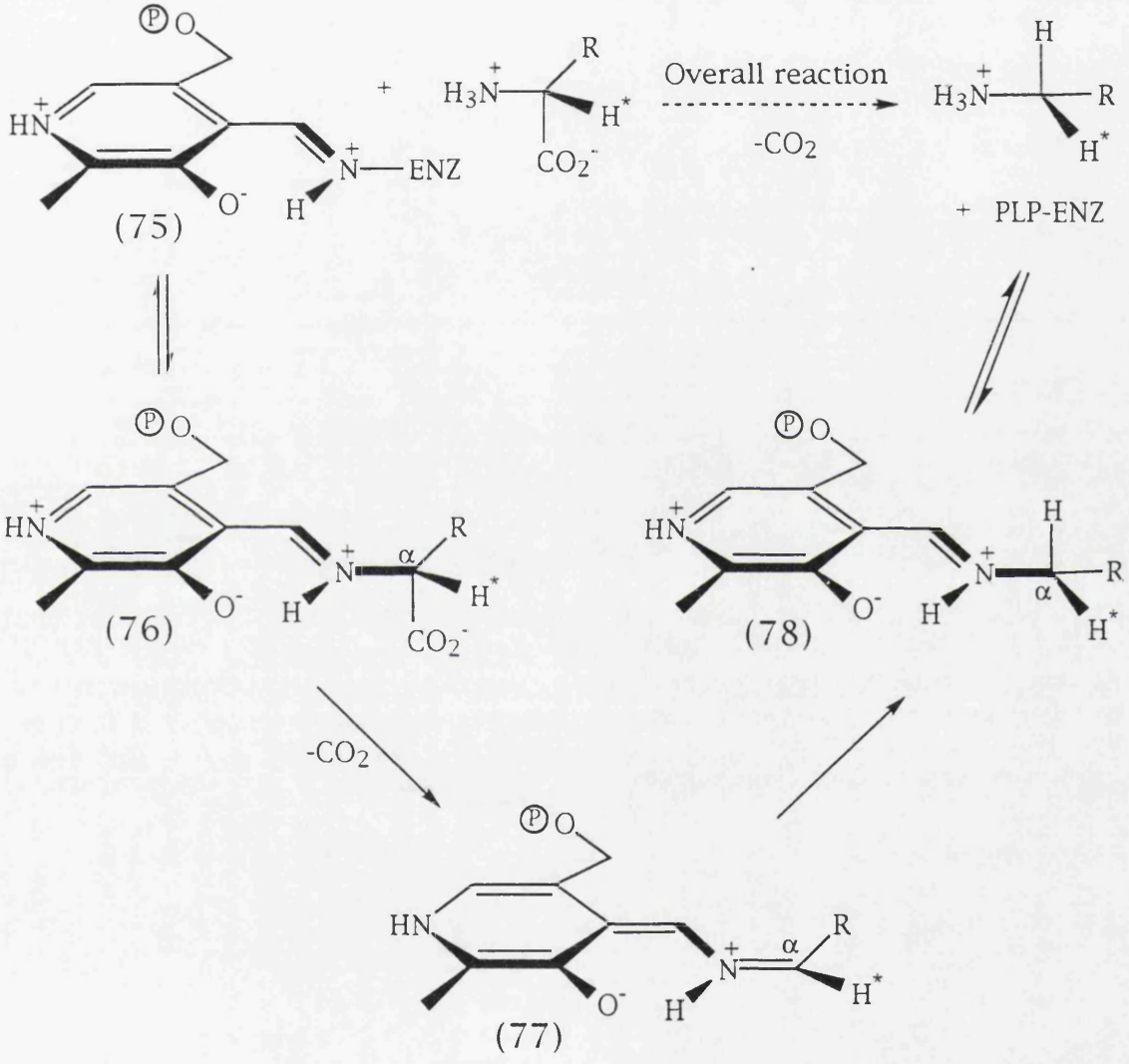
meso-2,6-Diaminopimelate decarboxylase is the only pyridoxal 5'-phosphate-dependent α-decarboxylase known to act on a D-amino acid. Soda *et al.*[80] found the enzyme from *B. sphaericus* to operate with inversion of configuration, in direct contrast to other pyridoxal 5'-phosphate-dependent α-decarboxylases for which the stereochemistry of the reaction has been determined. In all other cases examined, the reaction proceeds with retention of configuration - that is, the incoming proton occupies the same stereochemical position as did the departing carboxyl group.[81,82,83]

Kelland and co-workers[84] carried out a detailed mechanistic analysis of the decarboxylases from wheat germ and *B. sphaericus* using two-dimensional ^{1}H-^{13}C heteronuclear NMR shift correlation spectroscopy with ^{2}H decoupling. Both enzymes were found to operate with inversion of configuration.

In pyridoxal 5'-phosphate-dependent enzyme catalysed reactions, the bond to be broken in the substrate is expected to be perpendicular to the plane of the conjugated π system of the substrate-pyridoxal 5'-phosphate complex.[85] In decarboxylases, it is the bond between the α-carbon and the carboxyl carbon that must be aligned in this way. Where retention of configuration occurs, protonation must take place on the same face as of the planar carbanionic intermediate as did decarboxylation. However, in the case of either *B. sphaericus* or wheat germ *meso*-DAP decarboxylase, the proton must attach to the opposite face of the carbanionic intermediate to that from which the carboxyl group left.

The first step in the mechanism is the formation of the Schiff's base (75) between the cofactor and the enzyme (**Scheme**

27). Addition of *meso*-2,6-DAP (29) gives the imine (76). Carbon dioxide is then lost to give the intermediate (77). The cofactor essentially 'stores' the electrons of the cleaved bond until protonation can occur to give (78). The resulting imine (78) is then hydrolysed to give L-lysine (1).



Scheme 27

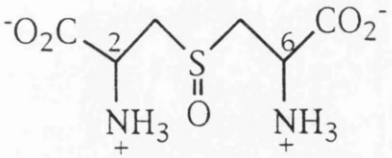
Asada *et al.*[80] have proposed two possible explanations for the inversion of configuration at the α-carbon. First, the catalytic group which donates a proton to the α-carbon atom of the anionic intermediate exists on the opposite side of the plane to that of

decarboxylation. Secondly, a drastic conformational change occurs leading to the rotation of the Schiff base plane and thereby to protonation from the same direction as the initial decarboxylation step. Conformational changes that occur upon substrate binding have been suggested for aspartate aminotransferase[86] and tyrosine decarboxylase.[87] However, a rotation of almost 180° after the formation of the anionic intermediate seems improbable because of the necessity of an extremely large structural rearrangement.

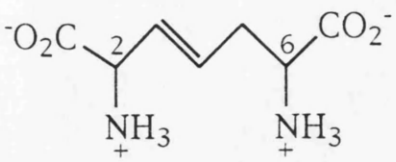
2.20 Inhibition of *meso*-DAP Decarboxylase.

Vederas *et al.*[88] synthesised a number of analogues of 2,6-diaminopimelic acid (29) and tested them for inhibition of *meso*-DAP decarboxylases from *B. sphaericus* and wheat germ. Lanthionine sulfoxides (79) (all stereochemistries) were found to be good inhibitors of both enzymes, giving 50% inhibition at 1mM. The *N*-modified analogues of 2,6-DAP, (66) and (67), were the most potent competitive inhibitors. The K_i values for *B. sphaericus* and wheat germ carboxylases with *N*-hydroxydiaminopimelate (66) (mixture of isomers) were 0.91 and 0.71 mM respectively. For *N*-aminodiaminopimelate (67) (mixture of isomers) the K_i values were 0.10 and 0.084 mM.

Girodeau *et al.*[89] synthesised unsaturated analogues of 2,6-DAP (29) and tested them with *meso*-DAP decarboxylase of *E. coli*. *trans*-3,4-Didehydro-DAP (80) was found to be the most potent inhibitor of *meso*-DAP decarboxylase.

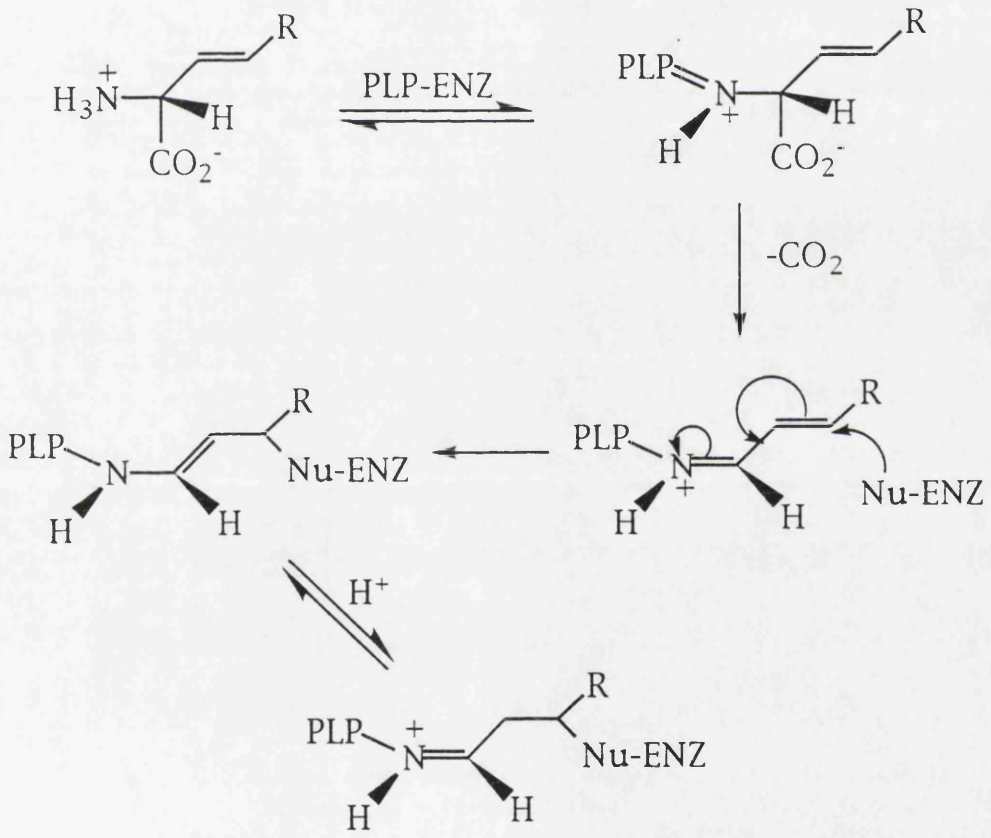


(79)



(80)

Scheme 28 shows the proposed mechanism for the irreversible inhibition of a pyridoxal 5'-phosphate-dependent decarboxylase by a vinylic amino acid.



Scheme 28

Chapter 3 - Synthesis of L-Aspartic Acid-β-Semialdehyde and Derivatives.

Introduction.

L-Aspartic acid β-semialdehyde (L-ASA) (20a) is an important intermediate in the biosynthesis of L-lysine (1), L-threonine (22) and L-methionine (21) **(Scheme 7)**. L-ASA (20a), along with pyruvate (24), is a substrate of the enzyme dihydrodipicolinate synthase. This enzyme catalyses the first unique step in the biosynthesis of L-lysine (1) in bacteria and higher plants.

L-ASA (20a) has also become an important synthetic intermediate. A range of polyfunctional non-protein and unnatural amino acids can be accessed by manipulation of the aldehyde functionality.[90,91,92]

In this Chapter there will be a brief description of the use of protecting groups in amino acid synthesis followed by a review of the Black and Wright synthesis of L-ASA (20a), first reported in 1955. Dr. D. Tudor, a former coworker of ours at Glasgow, carried out an improved synthesis of L-ASA (20a).[94] This procedure has been followed in order to prepare DL-ASA (20) for enzyme assays and testing of compounds and for use in the preparation of derivatives of DL-ASA (20). The preparation and test results of these derivatives will also be detailed in this Chapter.

3.1 Protecting Groups in αAmino Acid Chemistry.

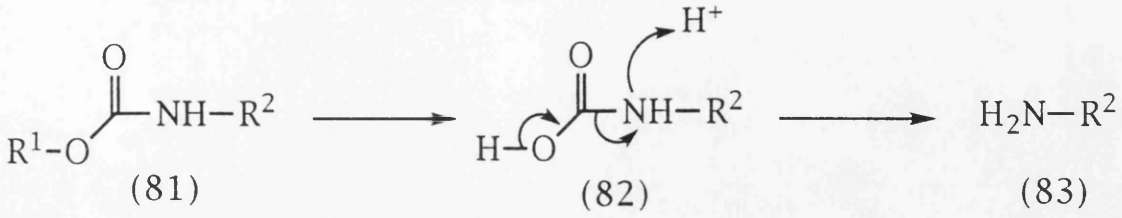
The selective use of protecting groups is of paramount importance in α-amino acid chemistry. The protection of both the carboxyl and amino functionalities of α-amino acids masks their zwitterionic character and allows for easier handling of the compounds in organic solvents. A protective group must fulfill a number of requirements. It must react selectively in good yield to give a protected substrate that is stable when subjected to further reaction conditions. The protective group should have a minimum of additional functionality to avoid further sites of reaction. Finally, the protective group must be selectively removed by reagents which do not destroy any new functionality within the molecule.

α-Amino Protection.

The nucleophilic reactivity of an α-amino group can be supressed by draining its electron density into an appropriate substituent or by concealing it with a sterically hindered substituent.

Amino groups are easily converted into alkoxycarbonylamino derivatives (81), also known as urethanes or carbamates. These can be considered to be a combination of both amide and ester functionalities. As amides, the nucleophilic reactivity of the nitrogen of (81) is greatly reduced. As esters, they can be degraded by alkyl-oxygen fission to the corresponding carbamic acids (82) which undergo spontaneous decarboxylation to regenerate the amine (83) **(Scheme 29)**. [In a number of the mechanisms drawn, it should be

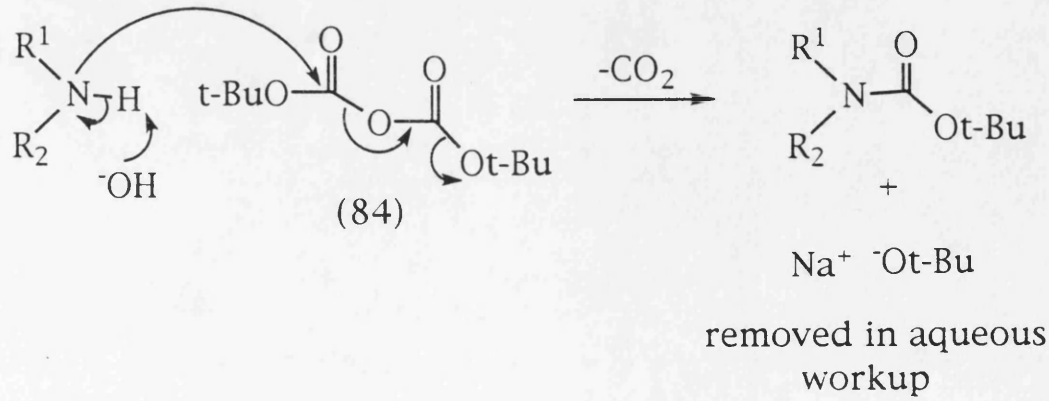
noted that the carbonyl group is involved in the mechanism, but arrows have been omitted for clarity.]



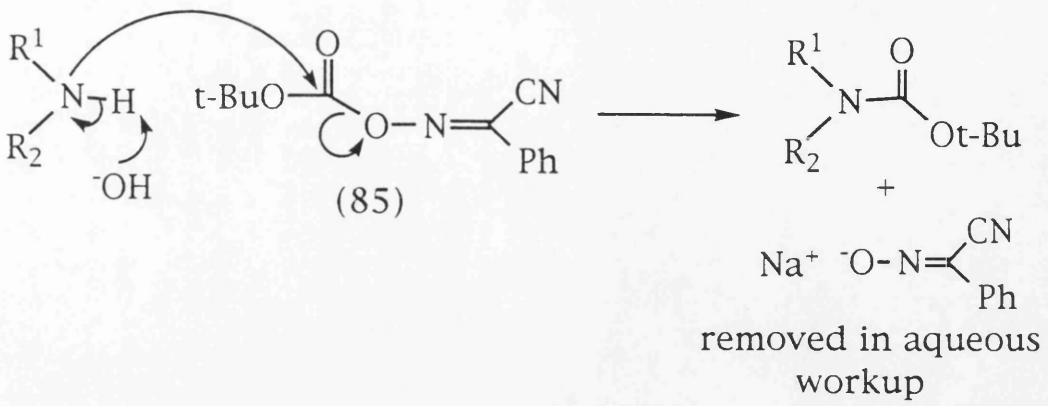
Scheme 29

Many carbamates have been used as protective groups. The carbamates which have been used in our amino acid work include the *t*-butoxycarbonyl (Boc) group and the benzyloxycarbonyl (Cbz) group.

The Boc group is not hydrolysed under basic conditions and is inert to hydrogenolysis. There are many reagents available for the formation and removal of the Boc group.[94] The two most common reagents for introducing the Boc group are di-*t*-butyl dicarbonate[95] (84) and 2-(*t*-butyloxycarbonyloxyimino)-2-phenylacetonitrile[96] (Boc-ON) (85). The mechanisms of formation of Boc-protected amines are shown in **Schemes 30** and **31**.

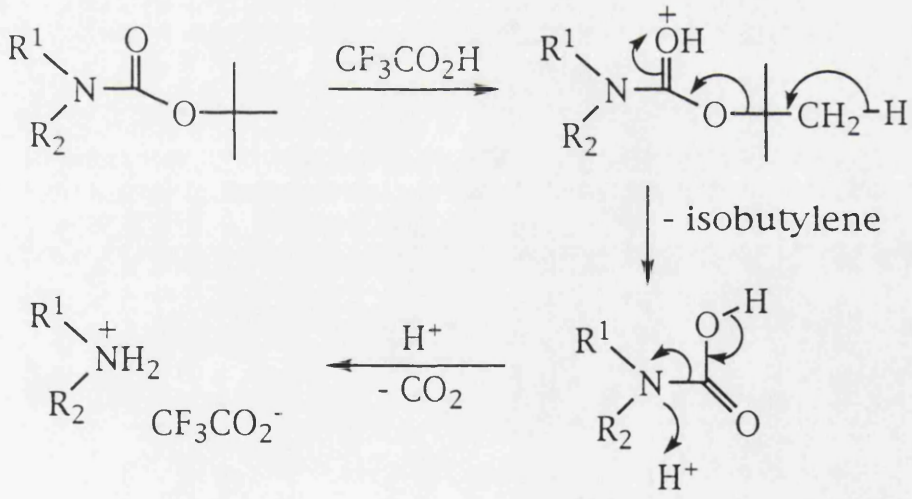


Scheme 30



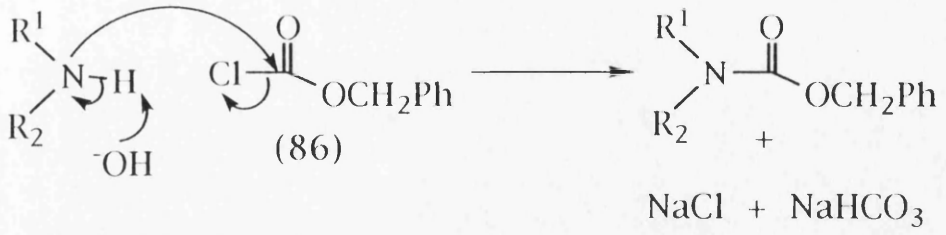
Scheme 31

Removal of the Boc group is conveniently carried out by dissolution in trifluoroacetic acid at room temperature for one hour (Scheme 32).[97]



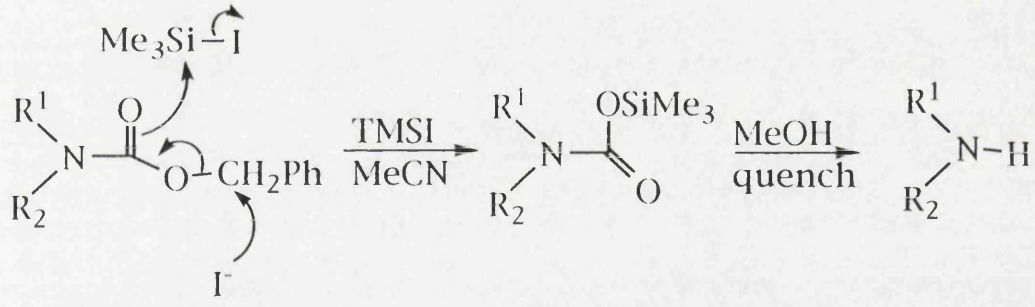
Scheme 32

The Cbz group was first described as an amino protecting group by Bergmann and Zervas in 1932.[98] It is normally introduced using benzyl chloroformate (86) under basic conditions (Scheme 33).



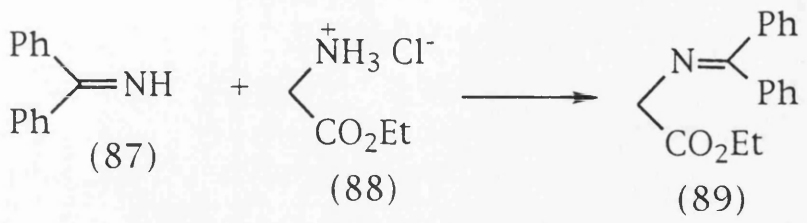
Scheme 33

The Cbz group is stable under mild acidic conditions. It can be removed by catalytic hydrogenolysis with a variety of catalysts and conditions, by hydrogen bromide in acetic acid or by use of a Lewis acid such as trimethylsilyl iodide **(Scheme 34)**.[99]



Scheme 34

A number of imine derivatives has been prepared as amine protective groups. The *N*-diphenylmethyleneamine derivative of glycine (89) can be prepared from an exchange reaction with benzophenoneimine (87) and glycine ethyl ester hydrochloride (88) **(Scheme 35)**.[100,101] The protective imine group can be cleaved under mild acidic conditions to yield the free amine.

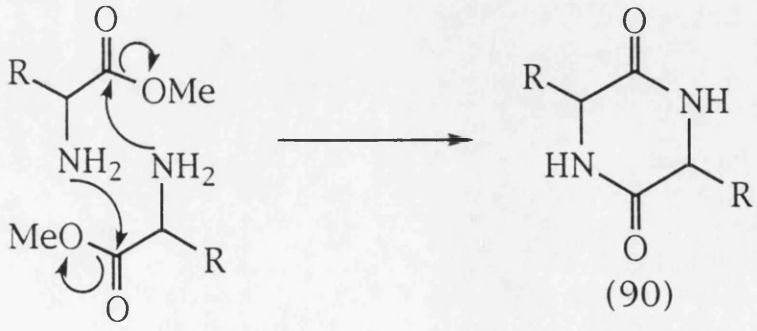


Scheme 35

α-Carboxyl Protection.

The usual means of carboxyl protection is esterification. Methyl esters provide good carboxyl protection and are not affected at ambient temperature by trifluoroacetic acid, catalytic hydrogenolysis or hydrogen bromide in acetic acid, so the selective removal of amino-protecting groups presents no difficulty.

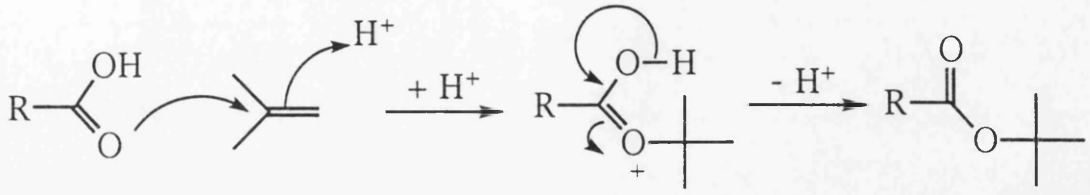
α-Amino acid methyl esters are often isolated as salts, or must be stored at low temperatures, as the methyl ester free bases can dimerise to form the corresponding diketopiperazines (90) **(Scheme 36)**.



Scheme 36

t-Butyl esters are relatively hindered esters and are stable under mild basic conditions. There are several reagents used to form

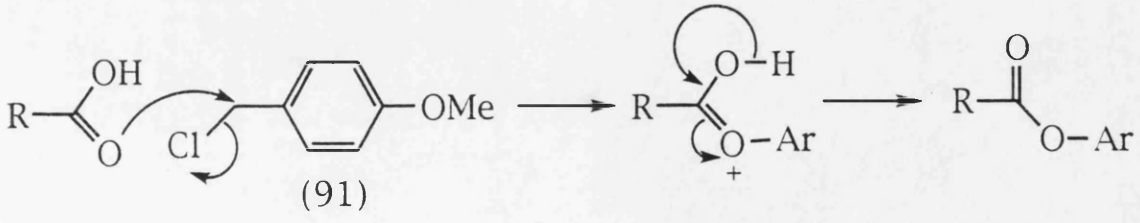
t-butyl esters. The most common procedure involves the use of isobutylene and concentrated sulfuric acid at room temperature for one day **(Scheme 37)**.[102]



Scheme 37

t-Butyl esters are cleaved by moderately acidic conditions, similar to those required for the removal of the Boc group **(Scheme 32)**. Thus the Boc and the *t*-butyl ester protecting groups are often used together in amino acid chemistry as they can both be removed in one step.

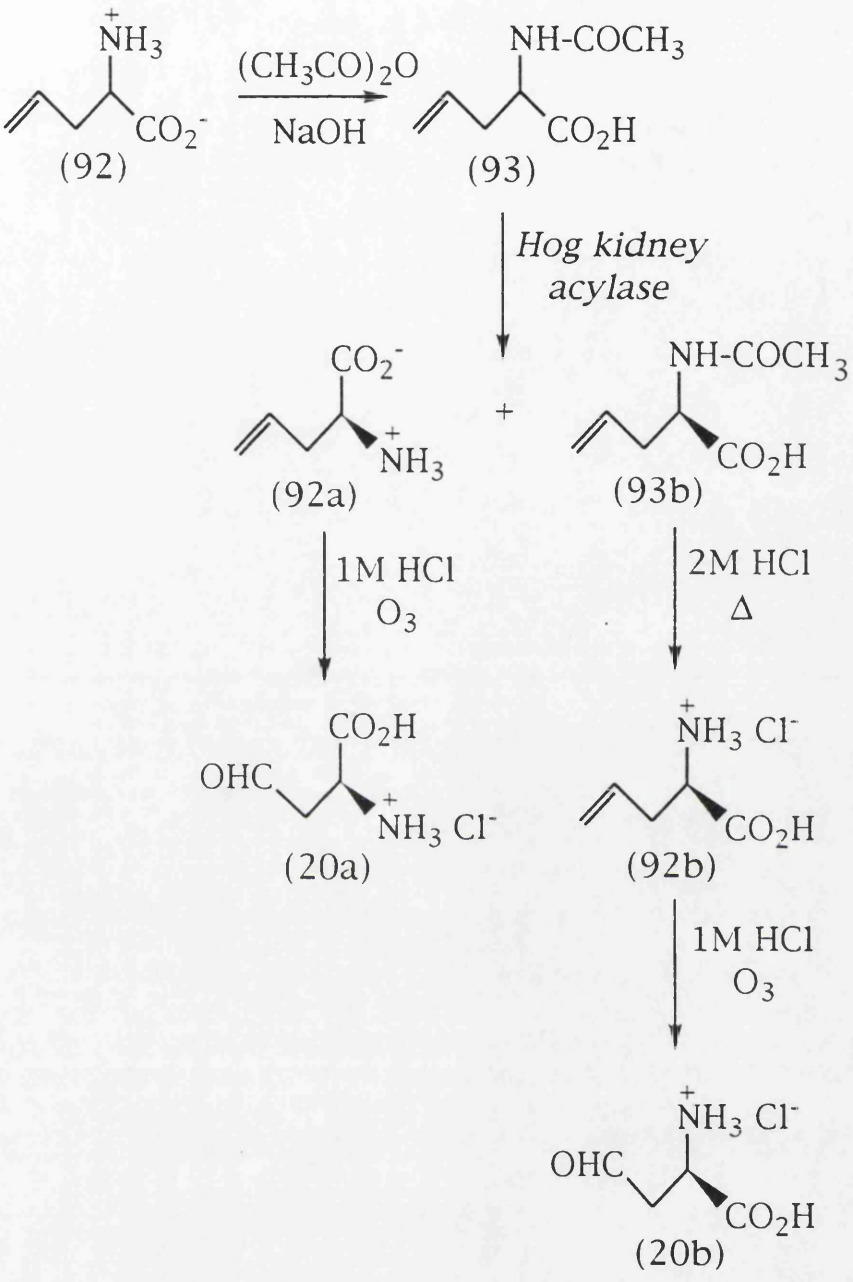
p-Methoxybenzyl esters can be prepared from the carboxylic acid and *p*-methoxybenzyl chloride (91) **(Scheme 38)**.[103] They can be removed under the same conditions as *t*-butyl esters and Boc groups.



Scheme 38

3.2 Black and Wright Synthesis of L-Aspartic Acid β-Semialdehyde.

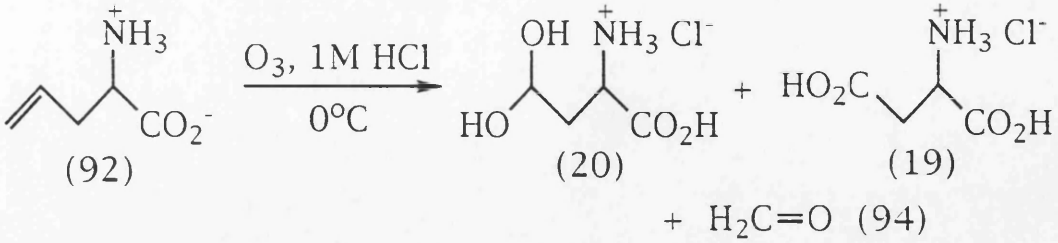
Black and Wright reported the first synthesis of the separate isomers of aspartic acid-β-semialdehyde (20) from DL-allylglycine (92) **(Scheme 39).**[104] DL-Allylglycine (92) was resolved into its D- (92b) and L-isomers (92a) by asymmetric enzymatic hydrolysis of the *N*-acetyl derivative (93). *N*-Acetyl-DL-allylglycine (93) was prepared from DL-allylglycine (92) using 2M sodium hydroxide and acetic anhydride. Hydrolysis of *N*-acetyl-DL-allylglycine (93) using hog kidney acylase gave L-allylglycine (92a) and unchanged *N*-acetyl-D-allylglycine (93b). L-Allylglycine (92a) crystallised on cooling. *N*-Acetyl-D-allylglycine (93b) was hydrolysed in 2M hydrochloric acid to give D-allylglycine (92b). Upon ozonolysis of D- (92b) and L-allylglycine (92a), D- (20b) and L-aspartic acid-β-semialdehyde (20a) were formed. Only the L-isomer was found to be a substrate of DHDP synthase. Black and Wright found that the aspartic acid β-semialdehyde was reasonably stable in acid solution at 0 °C. They were unable to isolate the product as the solid salt. Identification of the product of ozonolysis relied on the fact that it could be converted into homoserine by homoserine dehydrogenase.



Scheme 39

When our group repeated the Black and Wright procedure, the product obtained by ozonolysis was analysed by ^{1}H NMR spectroscopy. This indicated that DL-ASA (20) was present along with formaldehyde (94) and DL-aspartic acid (19) **(Scheme 40)**. No aldehyde signal was observed in the ^{1}H NMR spectrum, therefore the

aldehyde presumably exists as the hydrate. Attempts to purify the mixture using ion-exchange chromatography were unsuccessful.



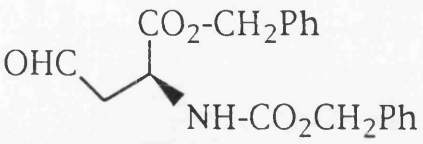
Scheme 40

3.3 Modified Synthesis of L-Aspartic Acid β-Semialdehyde.

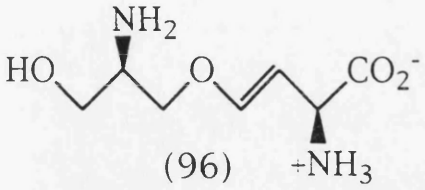
Our group at Glasgow required a readily available, pure form of DL- (20) or L-ASA (20a) for inhibitor and kinetic studies with the enzyme DHDP synthase. DL-ASA (20) prepared by the method of Black and Wright[104] could be used for the enzyme assay. However, this material was impure and available only as an acidic solution of variable concentration which meant that careful buffering of the assay was necessary. Other methods for the formation of DL- (20) or L-ASA (20a) were therefore investigated.

Several reports of the synthesis of L-ASA (20a) doubly protected on the amino and carboxyl groups had appeared in the literature. Keith *et al.*[91] used *N*-benzyloxycarbonyl-L-aspartic acid semialdehyde benzyl ester (95) in their total synthesis of the amino acid rhizobitoxine (96). Fushiya *et al.*[105] used protected L-ASA in the synthesis of avenic acid (97) and 2'-deoxymugineic acid (98), amino acids with an iron-chelating activity. Baldwin and Flinn[90] prepared L-ASA (20a) doubly protected on the amino and carboxyl groups from L-methionine (21) via homoserine in their route to more complex

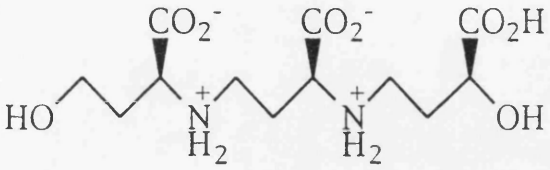
amino acids. However, no deprotection step to prepare free L-ASA (20a) was described by any of these authors.



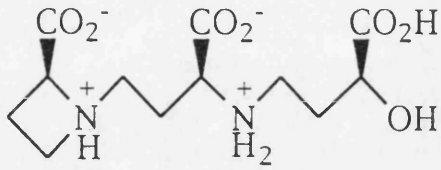
(95)



(96)



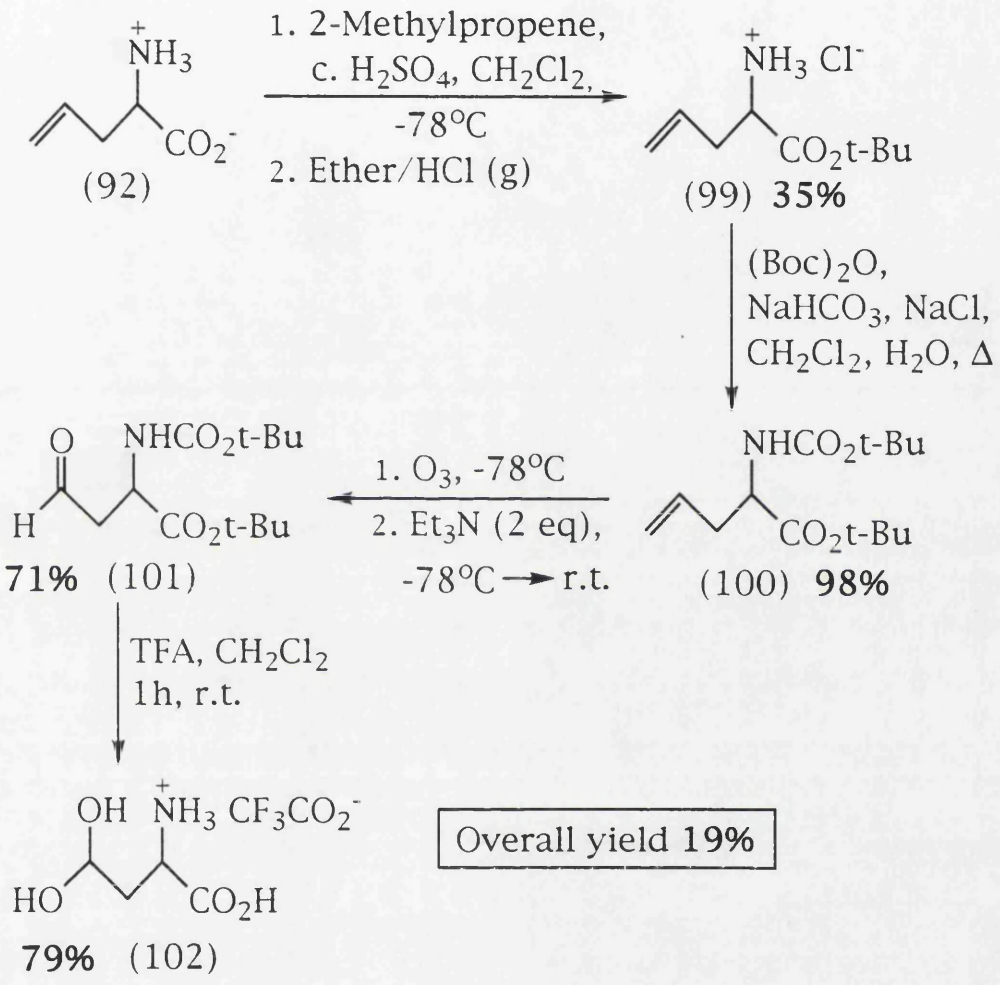
(97)



(98)

Our group reported the synthesis of the trifluoroacetate salts of D- (20b), L- (20a) and DL-ASA (20).[93] These were prepared from the ozonolysis of doubly protected allylglycine followed by careful deprotection with trifluoroacetic acid. The synthetic procedure was followed in this work in order to provide a supply of DL-ASA (20) for use in the preparation and testing of potential inhibitors of DHDP synthase. DL-Allylglycine (92) was converted into its *t*-butyl ester derivative using 2-methylpropene and a catalytic amount of conc. sulfuric acid **(Scheme 41)**.[95] The product was precipitated as the hydrochloride salt (99). The *N*-*t*-butoxycarbonyl (Boc) derivative (100) was prepared by treatment of (99) with di-*t*-butyl dicarbonate under basic conditions.[106] Ozonolysis of (100) at -78°C in dichloromethane and subsequent reductive decomposition of the ozonide gave the protected aspartic acid β-semialdehyde derivative (101). The mechanism of formation and decomposition of the ozonide

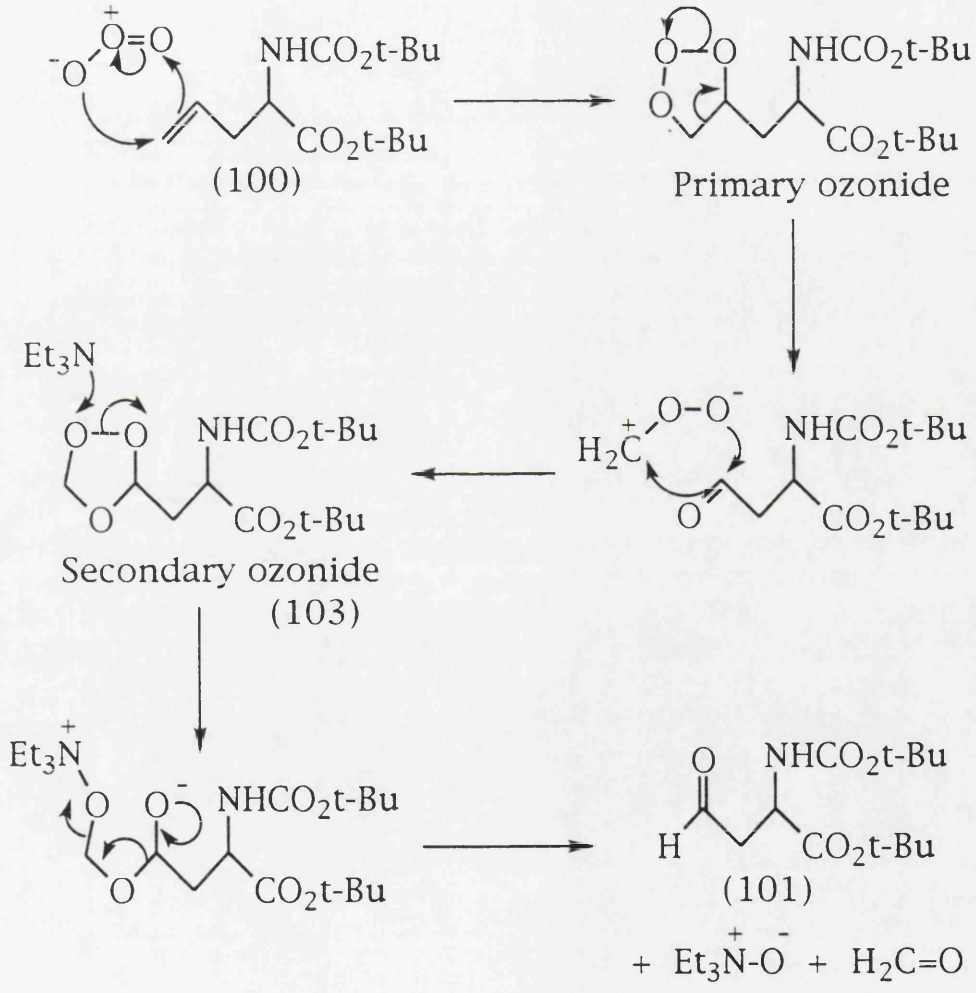
(103) is shown in **Scheme 42**. The ^{1}H and ^{13}C NMR spectra of (101) confirmed the presence of the aldehyde, i.e. there was a singlet at δ_H 9.74 and a signal at δ_C 199.4.



Scheme 41

Removal of the Boc group and the *t*-butyl ester was carried out in one step by stirring (101) in trifluoroacetic acid and dichloromethane for one hour at room temperature under an atmosphere of nitrogen. Removal of the solvent *in vacuo* gave an oily residue which solidified to a pale yellow solid upon addition of ether.

^1H and ^{13}C NMR data indicated that the product exists as a hydrate (102), i.e. no signal for an aldehyde proton was observed and there was a double doublet at δ_H 5.1 for the 4-H and a signal at δ_C 89.4 for C-4. The trifluoroacetate salts of L- (102a) and DL-ASA (102) were shown to act as substrates for DHDP synthase. The D-isomer (102b) was not a substrate of the enzyme.

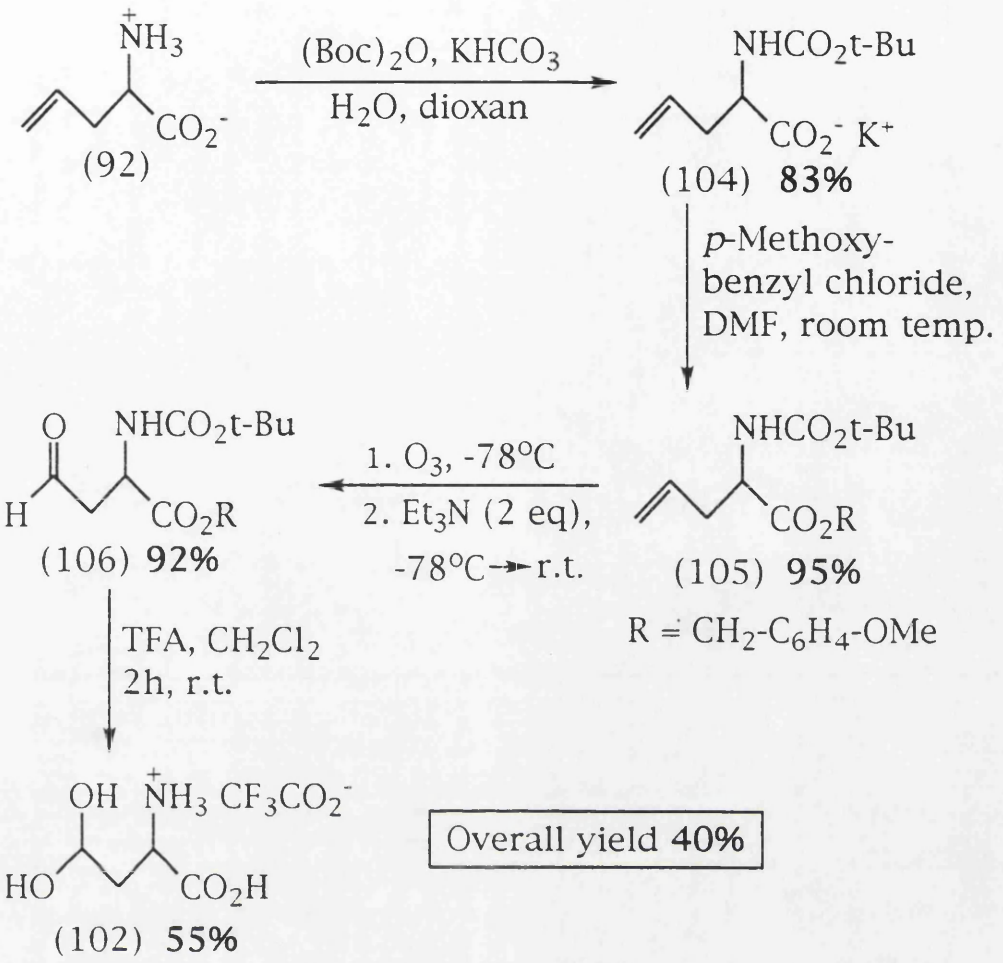


Scheme 42

This modified synthesis can provide our group at Glasgow with a stable, pure and easily handled form of DL-ASA (102) for use in the study of DHDP synthase.

One drawback of the synthesis of DL-ASA (102) as shown in **Scheme 41** is the low-yielding first step, namely the formation of the *t*-butyl ester of allylglycine (99) using 2-methylpropene and conc. sulfuric acid. The highest yield obtained for this reaction was only 35%.

Replacing the *t*-butyl ester with the *p*-methoxybenzyl ester **(Scheme 43)** improves the overall yield of the TFA salt of DL-ASA (102).[93] This synthesis was originally carried out by Dr. David Tudor in our group and was followed in this work. The potassium salt of *N*-*t*-butoxycarbonyl-DL-allylglycine (104) was prepared from DL-allylglycine (92) using di-*t*-butyl dicarbonate and potassium bicarbonate in water and dioxan.[107] The *p*-methoxybenzyl ester (105) was made from (104) using *p*-methoxybenzyl chloride in DMF. Ozonolysis of (105) gave the aldehyde (106). Removal of the *p*-methoxybenzyl ester and the Boc group was carried out in one step by stirring (106) in trifluoroacetic acid for two hours. The product was isolated as a pale yellow solid which had identical spectral data to the TFA salt of DL-ASA (102) prepared by the method shown in **Scheme 41**. This material was shown to act as a substrate of DHDP synthase.



Scheme 43

3.4 Studies with DHDP Synthase.

Isolation and Purification of DHDP Synthase.

The enzyme dihydrodipicolinate synthase catalyses the condensation of aspartic acid β-semialdehyde (20) and pyruvate (24) to form dihydrodipicolinate (25).[35] The gene encoding this enzyme in *E. coli* (*dap A*) has been cloned and sequenced[30] and the over-expressed enzyme has been purified and crystallised.[34]

The DHDP synthase required for use in our biochemical experiments can be routinely obtained from an over-expressing strain of *E. coli*. This work was initially carried out by Emma Borthwick (Biochemistry Department, University of Glasgow).[108] Plasmid pDA2 carrying the *dap A* gene of *E. coli* was obtained as a gift from Dr. P. Stragier, Institut Pasteur, Paris. It was transformed into *E. coli* MV1190 (*Rec* A^-) to produce the over-expressing strain MV1190/pDA2, the source of our enzyme. The specific activity of DHDP synthase from the over-expressed strain was 52 units/mg. [One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmole of product per minute.]

Standard Assay System for DHDP Synthase.

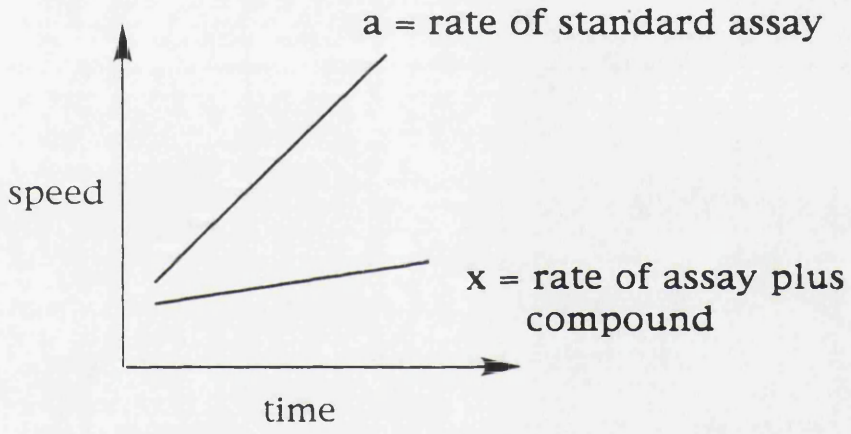
The enzyme was assayed at 25 °C by monitoring the formation of dipicolinic acid at 270 nm using a Gilford model 2600 spectrophotometer. The initial product of the reaction, L-2,3-DHDPA (25), is unstable and is spontaneously oxidised *in vitro* to DPA (44) which absorbs with a λ_{max} value at 270 nm **(Scheme 12)**. The assay product was isolated by HPLC and its UV and ^{1}H NMR spectra were shown to be indistinguishable from those obtained from an authentic sample of dipicolinic acid (44).[108]

This assay has enabled us to test analogues and derivatives of L-aspartic acid β-semialdehyde (20a), pyruvate (24), L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (26) for substrate or inhibitor activity of DHDP synthase.

The standard 1 ml assay consisted of 100 μM imidazole buffer, 1 mM aspartic acid β-semialdehyde (102), 1 mM pyruvate

(24) and 16 absorbance units of DHDP synthase. Various concentrations of compounds were studied, usually 5 mM, 1 mM, 0.5 mM and 0.1 mM. Each result is an average over three assay runs. The level of inhibition was measured as a percentage of the standard rate as shown by the equation and graph in **Fig. 2.**

Significant inhibition was taken to be about 10% inhibition at 0.5 mM of the compound being tested. All test data obtained are detailed in Chapters 3, 4, 5, 6 and in the experimental section of Chapter 7.



$$\frac{a - x}{a} \times 100 = \text{Inhibition (\%)}$$

Fig. 2

Electrospray Mass Spectrometry of DHDP Synthase.

Electrospray mass spectrometry (ESMS) is a relatively gentle technique used to examine samples with high molecular weights. Ionisation takes place at atmospheric pressure and moderate

temperatures, thus no fragmentation of the molecules occurs. ESMS can be used to determine or confirm the molecular weight of some proteins and to establish the presence of any post-translational modifications.

A homogeneous sample of our DHDP synthase was analysed by ESMS by Andrea Shneier and Dr. C. Abell (University of Cambridge) on a VG BioQ mass spectrometer. The observed molecular weight was 31 272 (average of eleven determinations). The ESMS spectrum of DHDP synthase is shown in **Fig. 3**.[109]

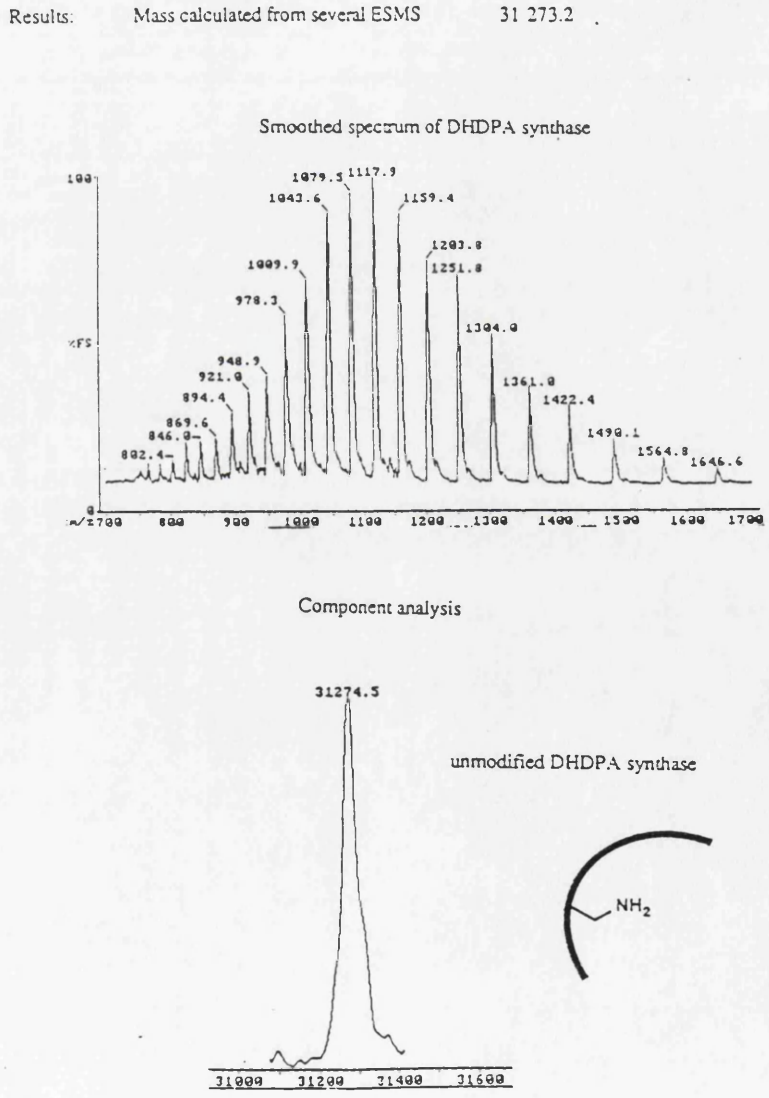
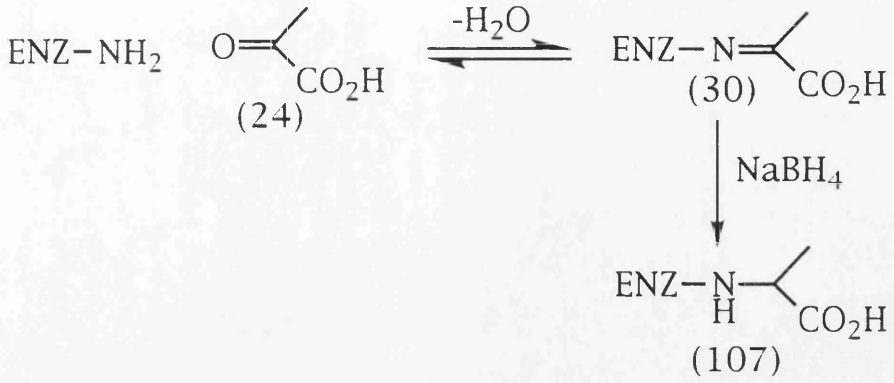


Fig. 3

Richaud *et al.*[30] had previously calculated the mass of DHDP synthase as 31 372 (100 greater than that observed by ESMS), based on the DNA-derived amino acid sequence. Unpublished amino acid sequence data by Laber indicate that one glutamic acid residue is in fact a glycine residue. This reduces the calculated mass to 31 300, still 28 larger than the observed mass. The difference of two methylene groups between the predicted and observed values is well within the resolution of the mass spectrometer. The spectrum shows no significant tailing, indicating that the mass observed is real and not a consequence of poor resolution. Furthermore, this result has been repeated by ourselves with the assistance of Dr. A. Pitt (University of Strathclyde). The discrepancy between the experimentally observed weight and the molecular weight calculated from the derived amino acid sequence indicates that the amino acid sequence must contain at least one more error.

Mechanistic Studies of DHDP Synthase.

The mechanism of DHDP synthase is believed to involve initial binding of pyruvate (24) as an imine to an active site lysine residue.[32,34] We have shown that there is irreversible loss of enzymatic activity of DHDP synthase upon addition of sodium borohydride only in the presence of pyruvate (24). This suggests that the formation of an imine intermediate (30) between DHDP synthase and pyruvate (24) occurs. Reduction of the imine (30) by borohydride results in the irreversible formation of (107) **(Scheme 44)**.



Scheme 44

Imine formation between pyruvate (24) and DHDP synthase has been detected directly using ESMS, without borohydride treatment (**Fig. 4**).[33] When DHDP synthase was mixed with pyruvate (24) immediately prior to injection into the mass spectrometer two peaks were observed, corresponding to native protein (A) and the imine adduct (B) (observed MW 31 346, calculated MW 31 343).

DHDP Synthase was incubated with pyruvate (24) and inactivated by the addition of sodium borohydride. The ESMS of this sample showed a single protein species, indicating that all the active sites had been modified. The observed MW was 31 347 (4 determinations) compared to a calculated MW of 31 345 for the reduced imine. In a control experiment with borohydride, in the absence of pyruvate (24), no loss of enzyme activity was observed and analysis by ESMS showed only unmodified enzyme.

As the proposed mechanism requires initial imine formation between the enzyme and pyruvate (24), it was not expected that any adduct between DHDP synthase and DL-aspartic acid β-semialdehyde (102) would be observed. There was no loss of enzyme activity upon addition of sodium borohydride in the presence of DL-ASA (102) and

the ESMS of the sample showed only unmodified enzyme. This confirmed that there is no imine formation between DHDP synthase and DL-ASA (102) in the enzyme reaction.

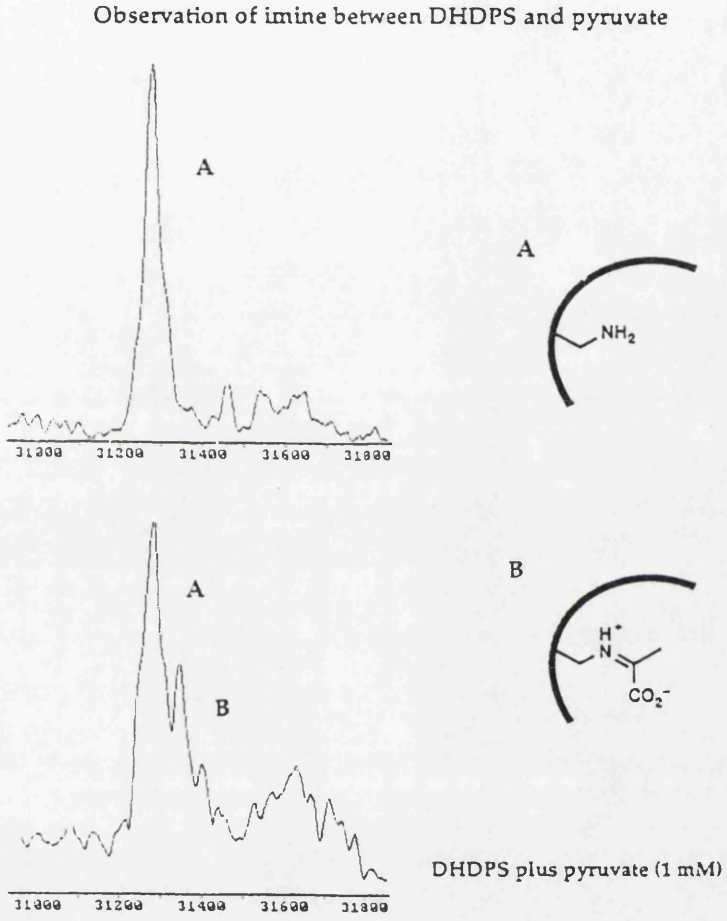


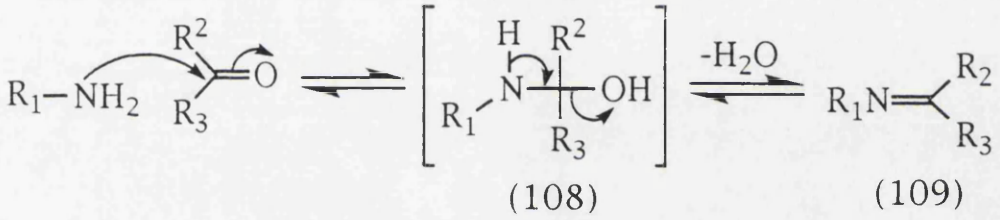
Fig. 4

Further mechanistic studies of DHDP synthase, using ESMS and NMR spectroscopy, are being undertaken by our co-workers John McKendrick and Dr. Philip Mallon.

3.5 Synthesis and Test Results of Derivatives of L-Aspartic Acid β-Semialdehyde.

Primary amines can add to aldehydes and ketones to give imines[110] which are stable enough for isolation. Imines are sometimes referred to as Schiff's bases.

The general mechanism of formation of imines formed from the reaction between a carbonyl compound and a primary amine is shown in **Scheme 45**. The initial *N*-substituted hemiaminal (108) is unstable and loses water to form the stable imine (109).

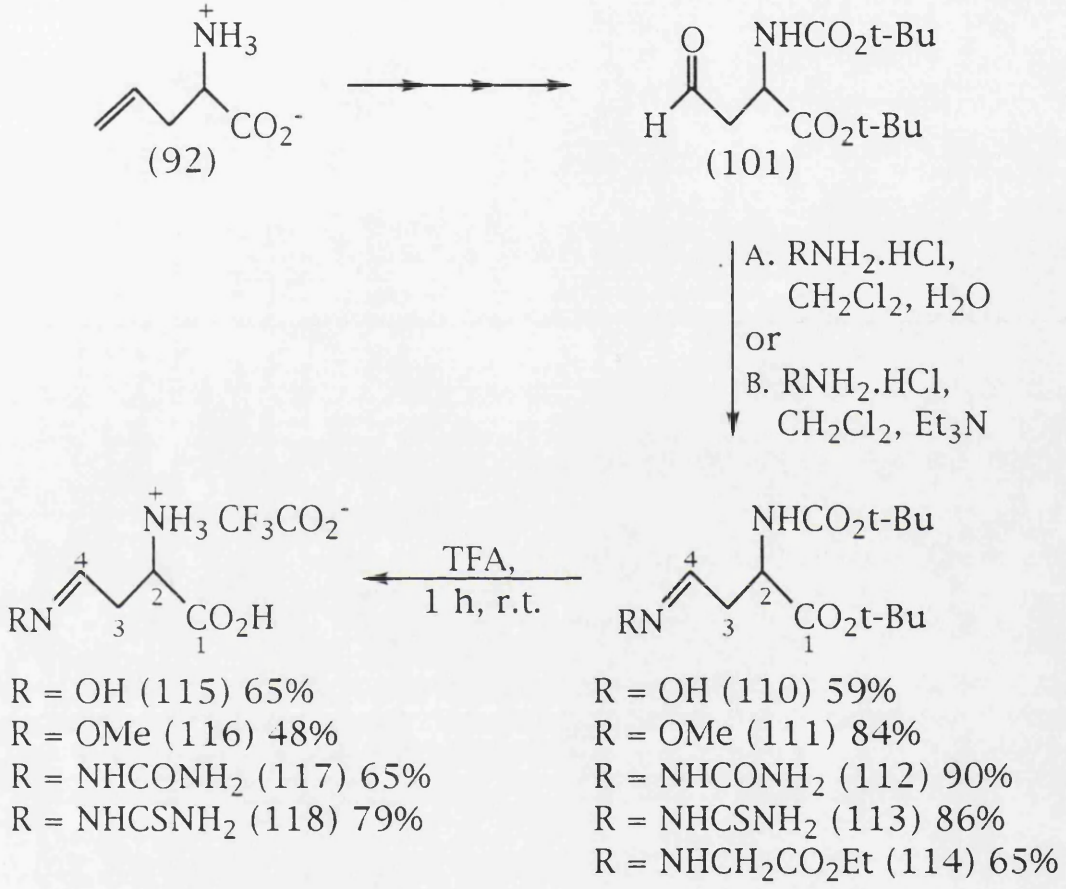


(108) (109)

Scheme 45

A number of imine derivatives of DL-ASA (20) were synthesised in this work and tested as inhibitors of DHDP synthase. The oxime (110), methyloxime (111), semicarbazone (112), thiosemicarbazone (113) and ethyl hydrazinoacetate (114) derivatives of protected DL-ASA (101) were all prepared under similar reaction conditions in 59%, 84%, 90%, 86% and 65% yields respectively **(Scheme 46)**. A solution of diprotected DL-ASA (101) in dichloromethane was added to a solution of the respective amine hydrochloride in water and the two-phase mixture was rapidly stirred for 24 hours. Extraction of the resultant mixture with dichloromethane, concentration and purification by chromatography, if necessary, gave the desired products. Alternatively, these imine

derivatives could be prepared by treatment of diprotected DL-ASA (101) with the appropriate amine hydrochloride and triethylamine in dichloromethane under an atmosphere of nitrogen. Concentration *in vacuo* and purification by column chromatography gave the desired products.



Scheme 46

Analysis by ^{1}H NMR spectroscopy showed the presence of a mixture of the *cis*-(*Z*-) and *trans*-(*E*-) isomers of the oxime (110), methyloxime (111) and the semicarbazone (112). For example, the ^{1}H NMR spectrum of the semicarbazone derivative (112) showed two triplets in 2:1 ratio, one at δ_H 7.11 (J 3.8 Hz) for the 4-H of the *E*-isomer and another at δ_H 6.53 (J 5.6 Hz) for the 4-H of the *Z*-isomer.

Deprotection of compounds (110), (111), (112) and (113) was achieved by stirring in dichloromethane and trifluoroacetic acid for one hour at room temperature to give the corresponding trifluoroacetate salts (115), (116), (117) and (118) in reasonable yields **(Scheme 46)**. Attempted deprotection of the ethyl hydrazinoacetate derivative (114) gave an oily residue. There were no signals in the ^{1}H NMR spectrum corresponding to the ethyl ester, indicating that compound (114) was degraded under the acidic conditions.

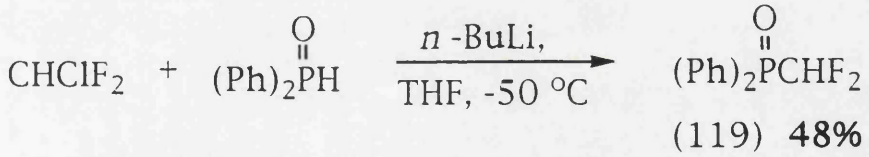
The protected methyloxime derivative (111) and the protected semicarbazone derivative (112) showed no significant inhibition of DHDP synthase at 1mM. However, the protected thiosemicarbazone derivative (113) gave some inhibition, showing 33% at 0.25 mM. The protected ethyl hydrazinoacetate derivative (114) gave 17% inhibition at 0.5 mM. The oxime derivative (110) has not yet been tested.

Of the deprotected derivatives, only the trifluoroacetate salt of the thiosemicarbazone, compound (118), gave any significant inhibition below 1.0 mM. This was a very good inhibitor showing 46% inhibition at 0.1 mM.

3.6 Attempted Synthesis of Difluoro Analogues of DL-ASA (20).

1,1-Difluoroalkenes are a class of fluoroorganic molecules with interesting chemical and biological properties.[111,112] They are potential mechanism-based enzyme inhibitors[113] and can be used as isosteric replacements for a carbonyl group.[114] Edwards *et al.*[115] reported a new reagent for the conversion of carbonyl compounds into 1,1-difluoroalkenes - difluoromethyldiphenylphosphine oxide (119). We decided to synthesise (119) in order to react it with diprotected DL-ASA (101) in an attempt to prepare the 1,1-difluoroalkene derivative (120).

Difluoromethyldiphenylphosphine oxide (119) was prepared by addition of chlorodifluoromethane to a solution of diphenylphosphine oxide and *n*-butyllithium in THF at -50°C **(Scheme 47)**. Concentration *in vacuo* and purification on a silica gel column gave compound (119) as a white solid in 48% yield.



Scheme 47

The ^1H NMR spectrum of (119) **(Fig. 5)** shows a double triplet at δ_H 6.35 ($^2J_{HF}$ 50.0 Hz and $^2J_{PH}$ 22.4 Hz) for the methine proton.

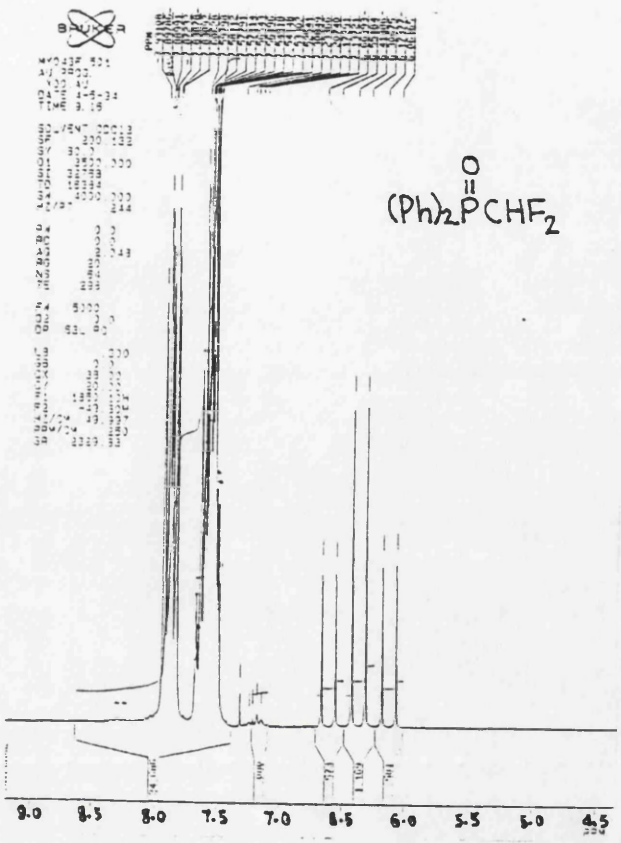
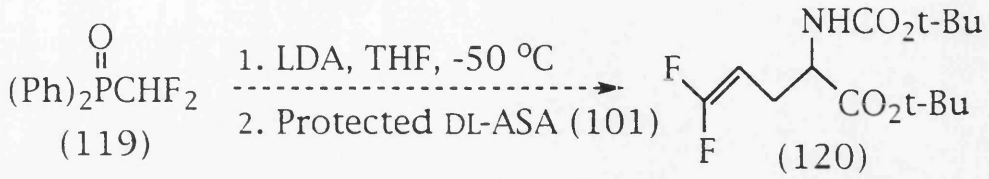


Fig. 5

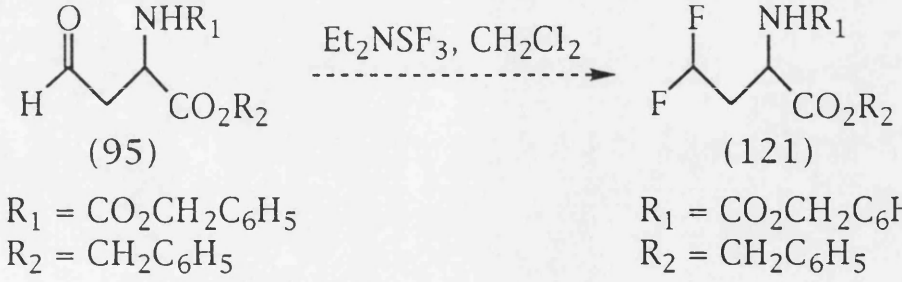
A solution of diprotected DL-ASA (101) in THF was added dropwise at -50°C to a solution of (119) and lithium diisopropylamide in THF **(Scheme 48)** according to the procedure of Edwards *et al.*[115] However, only starting materials were recovered from the reaction mixture - the 1,1-difluoroalkene (120) was not formed. Time constraints prevented further investigation of this reaction. It may be

that fresh reagents, longer reaction times or higher temperatures are required.



Scheme 48

The *gem*-difluoro analogue (121) would be an interesting compound to test as a potential inhibitor of DHDP synthase. However, attempts to synthesise (121) from diprotected DL-ASA (95) and diethylaminosulfur trifluoride (DAST)[116] have so far proved unsuccessful (Scheme 49).



$R_1 = CO_2CH_2C_6H_5$
$R_2 = CH_2C_6H_5$

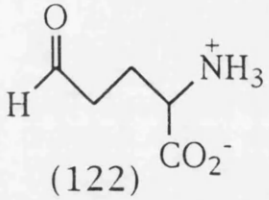
$R_1 = CO_2CH_2C_6H_5$
$R_2 = CH_2C_6H_5$

Scheme 49

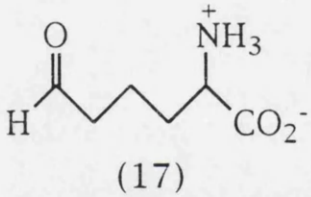
Chapter 4 - Synthesis of Homologues of L-Aspartic Acid β-Semialdehyde.

Introduction.

L-Aspartic acid β-semialdehyde (20a) is a substrate of dihydrodipicolinate synthase, the first enzyme unique to L-lysine (1) biosynthesis in plants and bacteria. The homologues of L-ASA (20a) are glutamic acid γ-semialdehyde (GSA) (122) and α-aminoadipic acid δ-semialdehyde (AASA) (17). While synthesising and testing potential inhibitors of DHDP synthase, we decided that GSA (122) and AASA (17) would be interesting compounds to test as inhibitors and/or substrates of the enzyme.



(122)

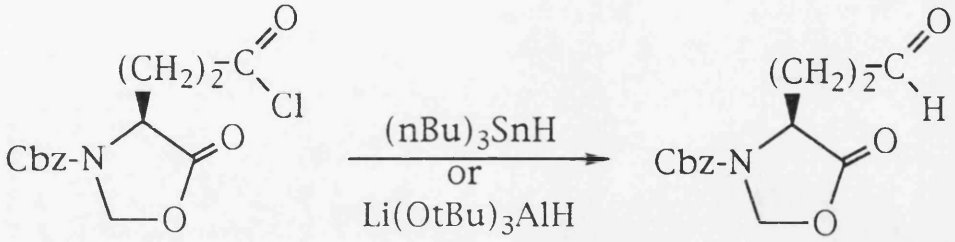


(17)

Both GSA (122) and AASA (17) are biologically important. GSA (122) is a common intermediate in the enzymatic interconversions of glutamic acid, proline and ornithine.[117] AASA (17) has been identified as a precursor of saccharopine (18), a key intermediate in lysine (1) biosynthesis by yeast.[118]

There are several reports in the literature detailing the synthesis of protected forms of GSA (122). Bold *et al.*[92] prepared GSA (122) doubly protected on the amine and the carboxyl functionalities via the Rosenmund reduction reaction. The doubly protected semialdehyde was used directly in further transformations. Lee and

Miller[119] made the protected semialdehyde by reduction of the corresponding acid chloride (123) with a hydride agent such as tributyltin hydride or lithium tri-*tert*-butoxyaluminohydride **(Scheme 50)**. The protected semialdehyde (124) was used for further transformations.

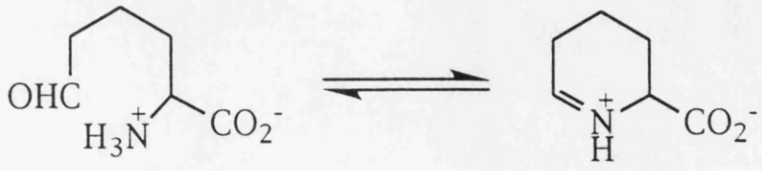


(123) (124)

Scheme 50

A Japanese patent in 1980 described a process for preparing optically active *N*-acetylglutamic acid γ-semialdehyde from oxidation of optically active *N*-acetylornithine with persulfate. The product was formed as an aqueous solution and was not isolated.

Rodwell[120] described the synthesis of an aqueous solution of an equilibrium mixture of α-aminoadipic acid δ-semialdehyde (AASA) (17) and Δ'-piperidine-6-carboxylate (125) **(Scheme 51)**.



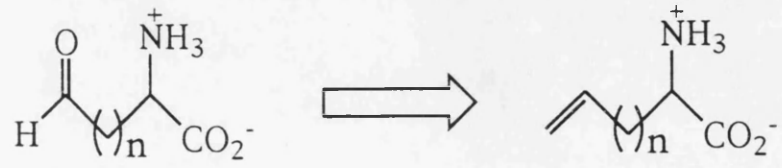
(17) (125)

Scheme 51

The synthetic procedure was analogous to that used by Black and Wright[104] in the preparation of L-aspartic acid β-semialdehyde (20a) in that the product existed as an acidic aqueous solution, formed by ozonolysis of the corresponding unsaturated amino acid.

In the same article Rodwell reported the biosynthesis of and isolation of L-α-aminoadipic acid δ-semialdehyde (L-AASA) (17) from DL-pipecolate using pipecolate dehydrogenase (*Pseudomonas putida*) as a catalyst. The equilibrium mixture of (17) and (125) was isolated as the hydrochloride salt - a pale yellow, hygroscopic amorphous solid, mp 75-79°C. No spectral data were provided although the material was optically active and was converted into L-α-amino-adipate by α-aminoadipate δ-semialdehyde NAD oxidoreductase.[121]

We wanted to synthesise and isolate GSA (122) and AASA (17) as their trifluoroacetate salts by using synthetic routes similar to the one we had developed for the production of DL-aspartic acid β-semialdehyde (102),[93] as detailed in Chapter 3. This would entail protection of the appropriate amino acid, followed by ozonolysis to afford the aldehyde and subsequent deprotection to give the trifluoroacetate salt of the semialdehyde. The starting amino acids that we required were 2-amino-5-hexenoic acid (126) and 2-amino-6-heptenoic acid (127) **(Scheme 52)**. These are not commercially available.



n = 2 GSA (122)
n = 3 AASA (17)

n = 2 (126)
n = 3 (127)

Scheme 52

This Chapter will contain a brief overview of general α-amino acid synthetic methodology followed by a detailed description of the routes we used to synthesise the amino acids (126) and (127) and the corresponding semialdehydes (122) and (17). The use of chiral *bis*-lactim ethers in the synthesis of optically active amino acids will also be discussed.

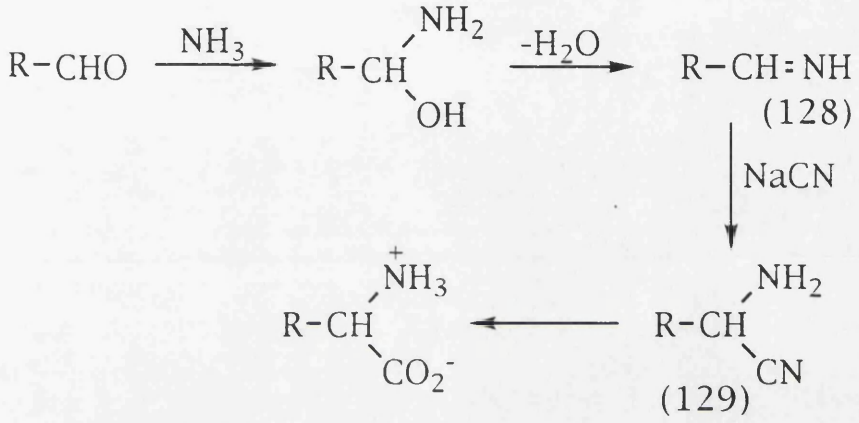
4.1 General α-Amino Acid Synthesis.

The increasing demand for α-amino acids for use in food products, drugs and as starting materials in organic synthesis has led to an enormous increase in methods available for their production. New and general synthetic routes to α-amino acids are of interest for both the large-scale production of known amino acids as well as the preparation of rare and/or unknown amino acids. A review by O'Donnell[122] details some recent synthetic methods developed for use in α-amino acid chemistry. More traditional methods include the Strecker synthesis, amination of α-halogen acids, rearrangement reactions and alkylation of malonate/glycine derivatives. Each of these synthetic strategies will be briefly discussed in this section.

The Strecker Synthesis.

α-Aminonitriles can be prepared in one step by the treatment of an aldehyde or ketone with sodium cyanide and ammonium chloride. This is known as the Strecker synthesis. A possible mechanism is shown in **Scheme 53**. Initial addition of

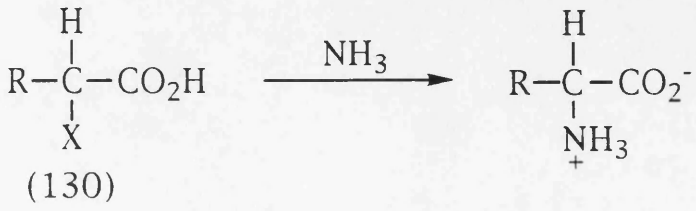
ammonia to the aldehyde followed by loss of water would give the imine (128). Sodium cyanide could then attack the imine to form the aminonitrile (129). As nitriles are readily hydrolysed to carboxylic acids, this is often a convenient method for the preparation of α-amino acids, although it can be limited by the accessibility of some aldehydes.



Scheme 53

Amination of α-Halogen Acids.

The ammoniolysis of α-halogen acids (130) is, in general, high yielding and convenient and remains a useful method for the synthesis of α-amino acids **(Scheme 54)**.

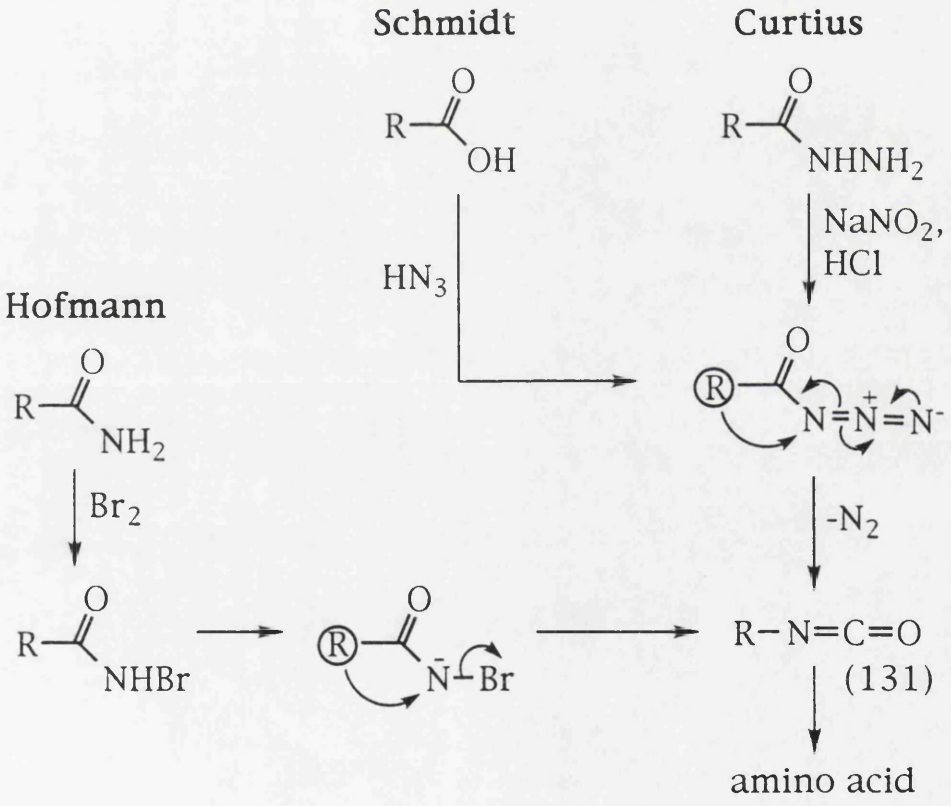


Scheme 54

If the requisite α-halo acid reactants are not available, these may be synthesised by either bromination of the appropriate malonic ester derivative or by the Hell-Volhard-Zelinsky procedure.

Rearrangement Reactions Leading to αAmino Acids.

The Curtius, Hofmann and Schmidt rearrangements[123] have all been used for the synthesis of α-amino acids *via* the corresponding isocyanate (131) (Scheme 55).



Scheme 55

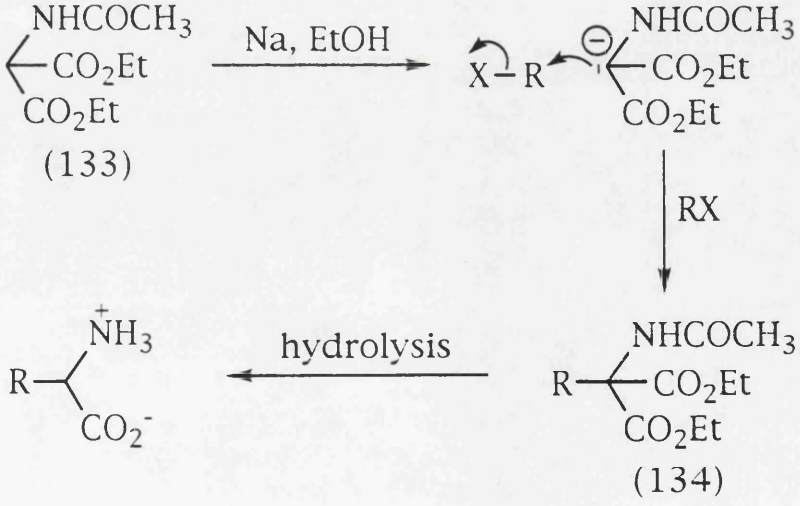
Alkylation of Malonate/Glycine Derivatives.

Compounds that contain strong electron-withdrawing groups (Z) on a carbon atom with an attached hydrogen, can easily be converted into the corresponding enolate anions (132) **(Scheme 56)**. These enolate anions can attack alkyl halides, resulting in alkylation.

(132)

Scheme 56

The Sorenson method of amino acid synthesis applies this reaction to diethyl acetamidomalonate (133). Hydrolysis and decarboxylation of the alkylated product (134) results in the amino acid **(Scheme 57)**.

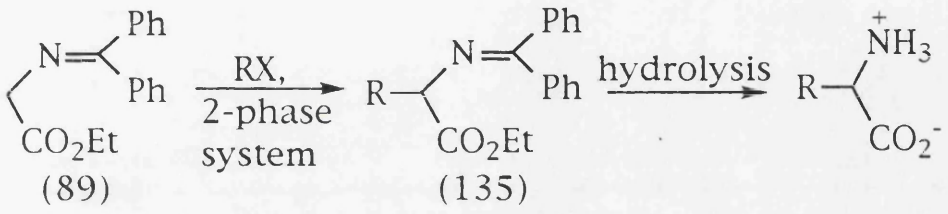


(133) (134)

Scheme 57

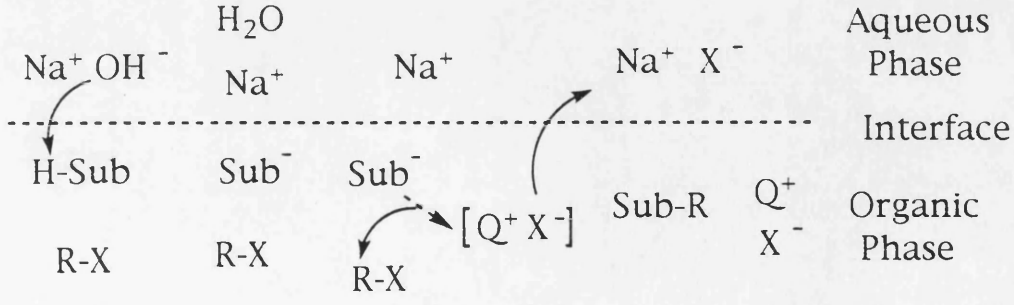
Phase-transfer reactions provide a potentially attractive synthetic route for the preparation of higher amino acids from

derivatives of the simplest, glycine. The Schiff base (89) is an active methylene compound in which both the amino acid and carboxylate groups of glycine have been protected towards proton abstraction by groups which will stabilise an adjacent carbanion. The use of phase-transfer reagents in a two-phase system can bring about alkylation of (89). The alkylated Schiff base (135) can then be hydrolysed to the corresponding amino acid **(Scheme 58)**.



Scheme 58

The classic phase-transfer experiment involves an aqueous-organic two-phase system and a phase-transfer catalyst, usually an ammonium salt (Q^+X^-). **Scheme 59** shows the stages in the phase-transfer catalysed alkylation of a weak C-H acid (H-Sub).



Scheme 59

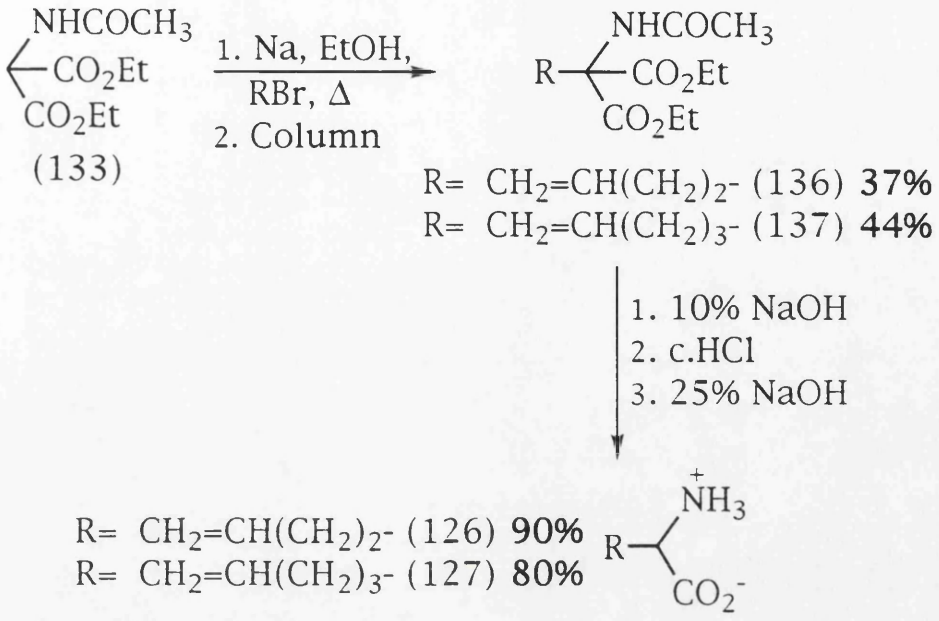
The catalyst ion pair (Q^+X^-) acts partly by facilitating deprotonation but mainly by detaching the substrate anions attached

at the phase boundary, with the release of the original anion of the catalyst into the water and fast subsequent reaction with the alkylating agent. This assumption is supported by the finding that a number of phase-transfer reactions proceed without a catalyst but are accelerated by one.[124]

4.2 Synthesis of Required Amino Acids.

Alkylation of Malonate Derivatives.

Our initial attempt at the synthesis of 2-amino-5-hexenoic acid (126) and 2-amino-6-heptenoic acid (127) was based on a method previously described by Rodwell.[120] The appropriate alkyl halide was added dropwise to a refluxing solution of sodium and diethyl acetamidomalonate (133) in ethanol to give the alkylated product **(Scheme 60)**. The alkyl halides used were 4-bromobut-1-ene and 5-bromopent-1-ene which gave the crystalline derivatives (136) and (137) respectively in no more than 44% yield after purification by column chromatography. Sequential ester hydrolysis, decarboxylation and amide hydrolysis of compounds (136) and (137) gave the amino acids (126) and (127) which were isolated as their hydrochloride salts.



Scheme 60

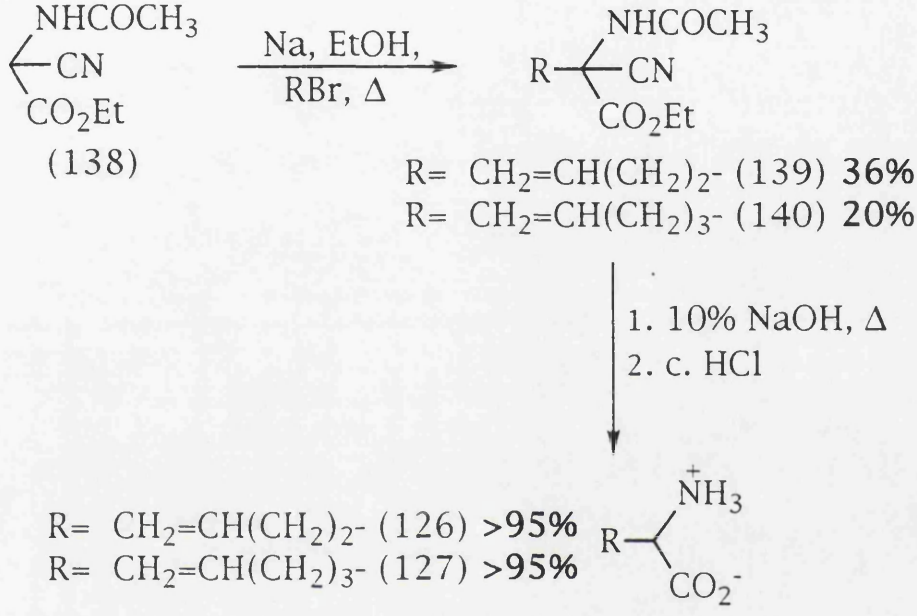
Sodium chloride, formed as a side product in the final stages of **Scheme 60**, was not removed but the amount was estimated and carried on through the next stage of the synthesis.

Both amino acids (126) and (127) were water soluble white solids. The ^{1}H NMR spectra of both (126) and (127) had distinctive multiplets at around δ_H 4.98 and 5.72 for the olefinic protons and a triplet at around δ_H 3.85 for the hydrogen of the α-carbon. The ^{13}C NMR spectra both showed signals at around δ_C 174 for the acid carbons.

Although this procedure was straightforward and used readily available, inexpensive starting materials the overall yields were low and purification by column chromatography was necessary after the first stage.

The reaction was repeated using a different malonate derivative, ethyl acetamidocyanoacetate (138) **(Scheme 61)**. The alkylated derivatives (139) and (140) were recrystallised from ethanol and water to give white crystalline solids, mp 98-101 °C and

43-45 °C respectively. The ^{13}C NMR spectra showed signals at around δ_C 116.4 for the carbons of the nitriles. As before, sequential hydrolysis and decarboxylation gave the amino acids (126) and (127), as the hydrochloride salts.



Scheme 61

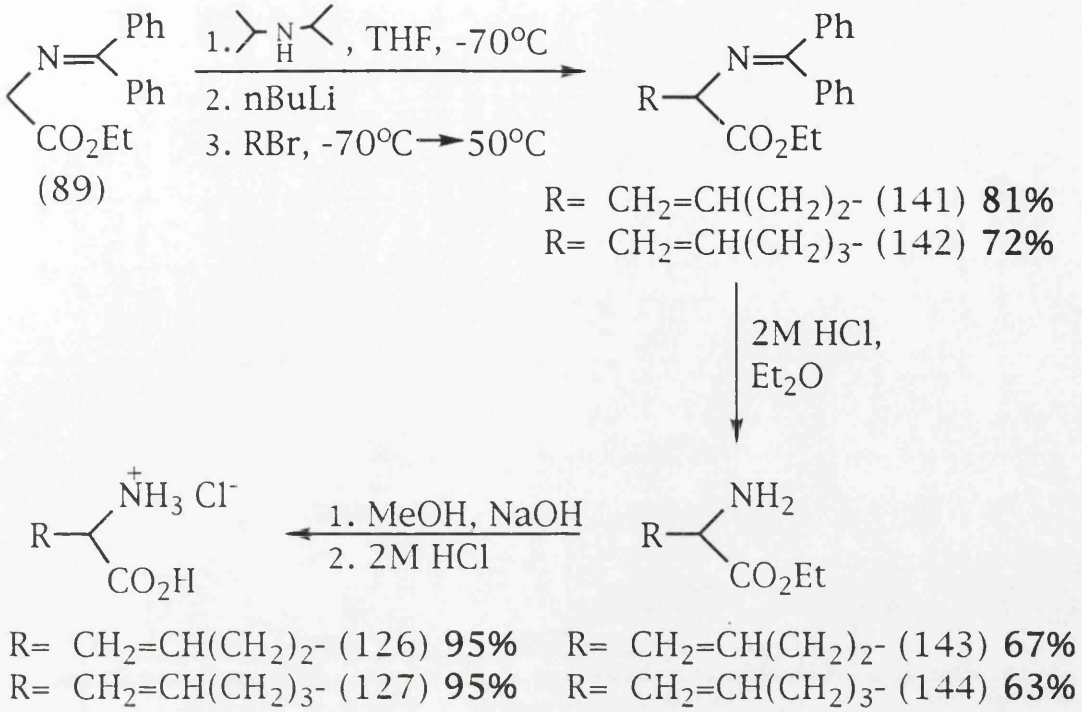
The hydrochloride salts of the amino acids, (126) and (127), showed no inhibition of DHDP synthase at 1 mM. The alkylated malonate derivatives, compounds (136), (137), (139) and (140), showed only slight inhibition of DHDP synthase at 5 mM

As both the synthetic routes detailed in **Schemes 60** and **61** are low-yielding and as we required several grams of the amino acids, other methods of α-amino acid synthesis were investigated.

Alkylation of Schiff's Base Derivatives of Glycine.

The protected glycine derivative, *N*-(diphenylmethylene)-glycine ethyl ester (89), was alkylated under anhydrous reaction conditions **(Scheme 62)**. The base used was lithium diisopropylamide (LDA), formed *in situ* from the dropwise addition of *n*-butyl lithium to diisopropylamine in dry THF at -70 °C. Slow addition of the appropriate bromoalkene with subsequent warming to 50 °C resulted in good yields of the monoalkylated products. Compounds (141) and (142) were isolated as dark red oils which each showed one spot by TLC analysis. The ^{1}H NMR, ^{13}C NMR and mass spectra all confirmed the formation of (141) and (142).

The benzylidene protective groups of (141) and (142) were removed under mild acidic two-phase conditions. Basification and extraction of the aqueous layers yielded the free amino acid esters, (143) and (144). Compounds (143) and (144) were subjected to mild basic hydrolysis followed by acidification and freeze-drying of the aqueous portions to yield the amino acids (126) and (127) as their hydrochloride salts.

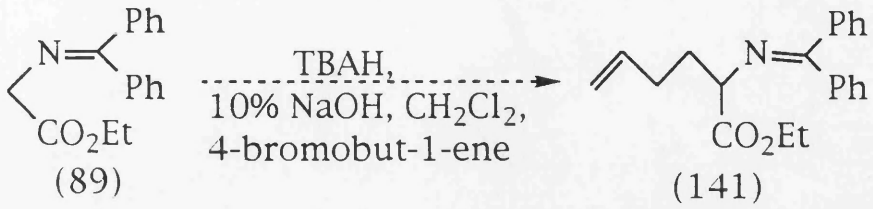


Scheme 62

Although the overall yields and purity of compounds were improved upon with this route, the need for strong, expensive bases, low temperatures and anhydrous reaction conditions dissuaded us from applying this route to larger scale preparations of the amino acids. This reasoning led us to investigate the use of phase-transfer methods in the synthesis of our amino acids. Phase-transfer techniques are noteworthy because of their simplicity and use of inexpensive reagents and solvents. This can favour their application in larger scale preparations.

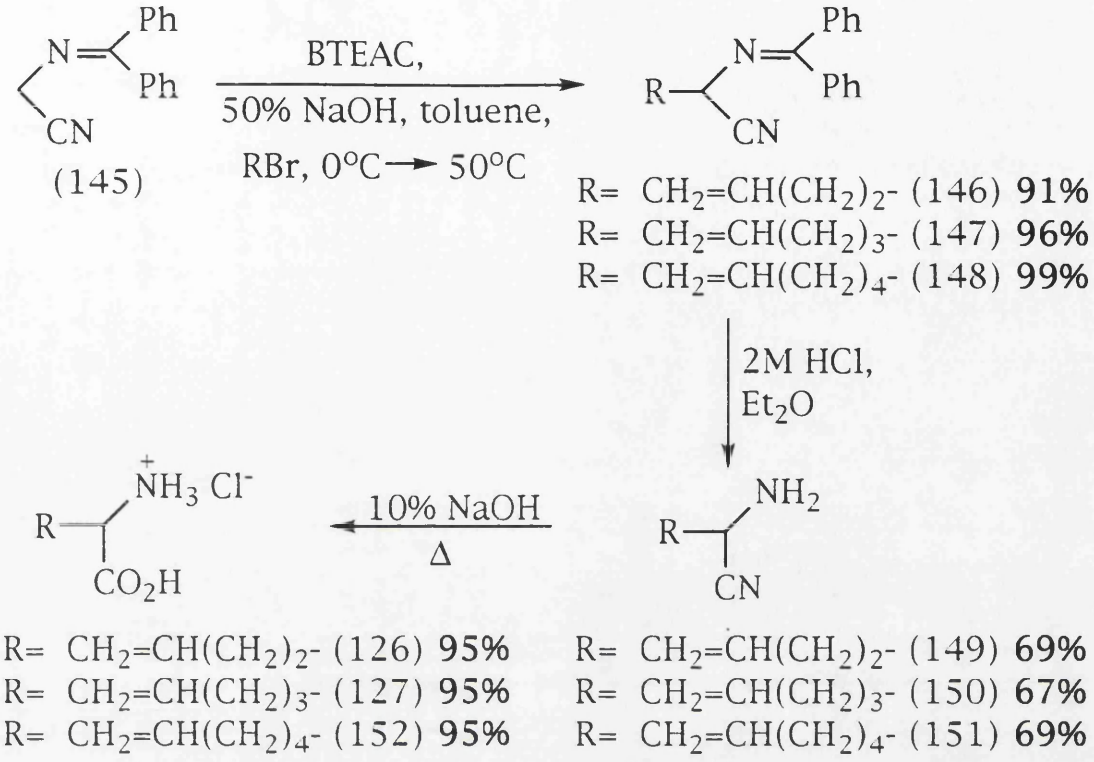
An attempt to alkylate *N*-(diphenylmethylene)glycine ethyl ester (89) under phase-transfer conditions was made. A two-phase mixture of (89), tetrabutylammonium hydrogen sulfate (TBAH), 4-bromobut-1-ene, 10% aqueous sodium hydroxide and dichloromethane was stirred at room temperature overnight (**Scheme 63**).[125] G.C. analysis of the organic portion of the reaction

mixture indicated that no alkylated material was present. The main peak of the GC spectrum may have been due to the free acid of (89). This would be formed from hydrolysis of the ester under the basic reaction conditions.



Scheme 63

The use of a different Schiff base, *N*-(diphenylmethylene)-aminoacetonitrile (145), allows stronger basic conditions to be employed without affecting the starting materials.[126] The catalytic phase-transfer alkylation of (145) was carried out with benzyltriethyl ammonium chloride (BTEAC) as the phase-transfer catalyst and 50% aqueous sodium hydroxide as the base **(Scheme 64)**.



Scheme 64

The alkylated products, (146), (147) and (148), were synthesised in high yield and without the need for further purification. Only monoalkylated compounds were observed. Removal of the benzylidene groups under mildly acidic conditions gave the aminonitriles (149), (150) and (151). Alkaline hydrolysis of the nitrile groups yielded the amino acids (126), (127) and (152) which were isolated as the hydrochloride salts. This route was suitable for the multi-gram preparation of the required amino acids.

None of the compounds detailed in **Schemes 62** and **64** showed significant inhibition of DHDP synthase. The alkylated Schiff's base derivatives, compounds (141), (142), (146), (147) and (148), interfered with the enzyme assay due to their insolubility. The amino acid esters, (143) and (144), were poor inhibitors. They both showed around 15% inhibition at 1 mM. The aminonitriles, (149) and (150), did not show any levels of inhibition below 1 mM.

An approximation of the overall yields of amino acids obtained by the different synthetic methods described in this Chapter is shown in **Table 5**.

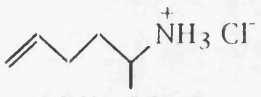
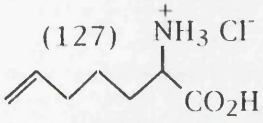
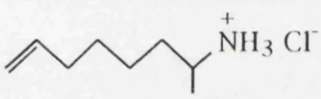
	Anhydrous reaction conditions		Phase-transfer
	Na/EtOH	LDA	50% NaOH
 (126)	33%	52%	60%
(127) 	35%	43%	61%
 (152)	-	-	61%

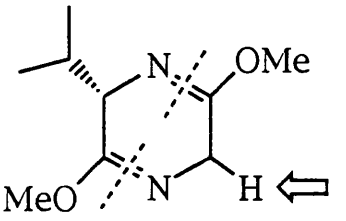
Table 5

4.3 Optically Active α-Amino Acids.

The possibility of synthesising optically active 2-amino-5-hexenoic acid (126) and 2-amino-6-heptenoic acid (127) was investigated.

There are several well documented methods used in the asymmetric synthesis of α-amino acids.[127] Schollkopf *et al.* developed the enantioselective synthesis of α-amino acids from the reaction of lithiated *bis*-lactim ethers of 2,5-diketopiperazines with electrophiles.[128] This asymmetric approach is based upon heterocyclic chemistry with the following requirements. The heterocycle is composed of a lower amino acid (e.g. glycine or racemic alanine) and a

a chiral auxiliary. The most studied and widespread *bis*-lactim ether is (153), that made from L-valine and glycine **(Fig. 6)**. The heterocycle has an acidic hydrogen next to the potential amino group and has two sites susceptible to hydrolysis (---).

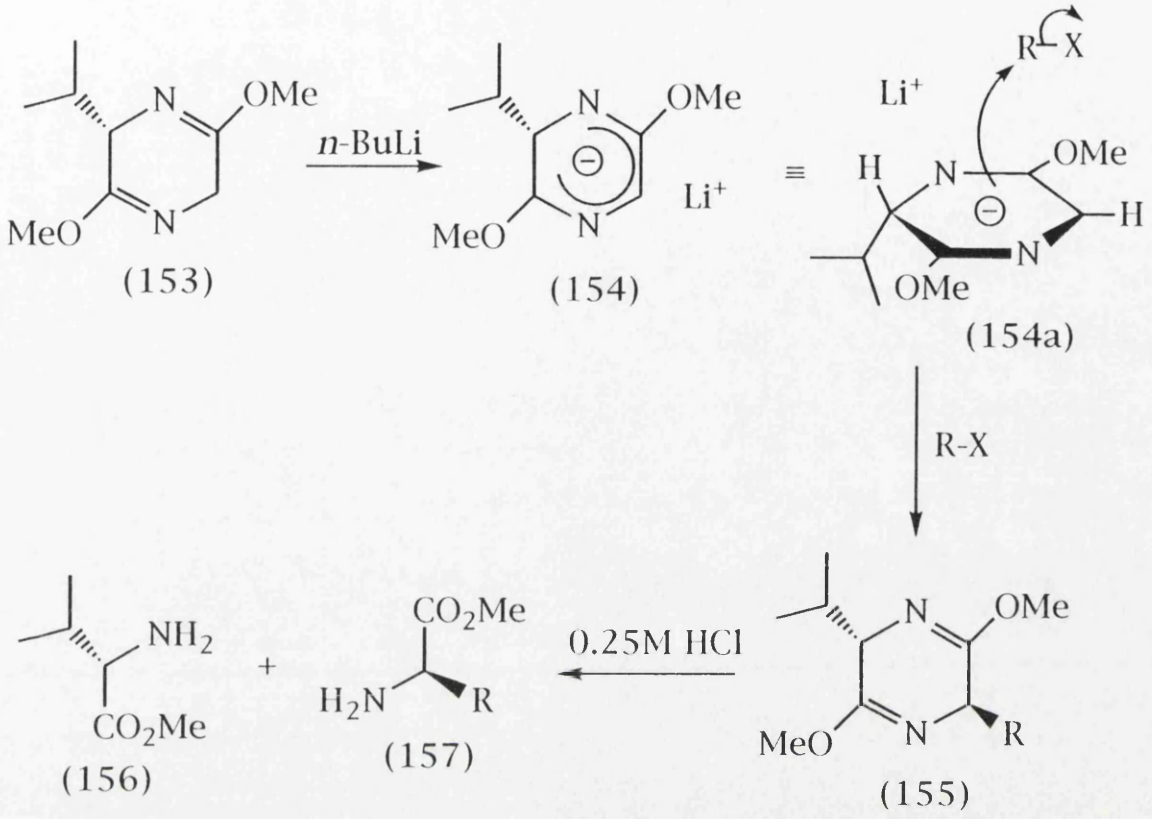


(153)

Fig. 6

Bis-lactim ethers react readily with *n*-butyl lithium to give the lithiated heterocycles (154) which possess a prochiral carbon atom **(Scheme 65)**. Electrophiles can be introduced both regio- and diastereo- selectively to the anion (154) to give the alkylated product (155). The high diastereoselectively of this reaction, often > 95%, can be explained if we consider the lithium compound as a planar dihydropyrazine anion (154a), one diastereotopic side of which is strongly shielded by the comparatively large isopropyl group of the chiral auxiliary.

Subsequent mild hydrolysis of the alkylated heterocycle (155) liberates the chiral auxiliary (156) and the new amino acid methyl ester (157). If (156) and (157) differ sufficiently in boiling points, the esters can be separated by bulb-to-bulb or fractional distillation.[129] Chromatographic techniques may also be used, after further hydrolysis to the amino acids.



Scheme 65

Several alkylated *bis*-lactim ethers were prepared (**Table 6**). The starting *bis*-lactim ethers were commercially available. Alkylation of the lithiated anions with 4-bromobut-1-ene resulted in good yields of the alkylated heterocycles, compounds (158), (160), (162) and (164) as clear oils. Diastereomeric excess (de) values were estimated to be around 84-95% from ^{1}H and ^{13}C NMR spectra or by gas chromatography. The ^{13}C NMR spectrum of (158) is shown in **Fig. 6.**

Hydrolysis of each of the alkylated *bis*-lactim ethers was readily achieved by stirring with 0.25-1.0 M aqueous HCl solution at room temperature for several hours. This yielded a 1:1 mixture of the chiral auxiliary (either L-valine methyl ester or D-valine methyl ester) and the desired amino acid methyl ester, compounds (159), (161),

(163) or (165), in quantitative yields. Under these conditions practically no 2,5-diketopiperazines (90) were formed. However, after several weeks storage under a sealed atmosphere of nitrogen at 4 °C, insoluble solid material was observed in some of the samples. This was evidence that formation of 2,5-diketopiperazines was eventually occurring.

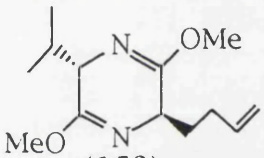
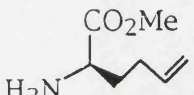
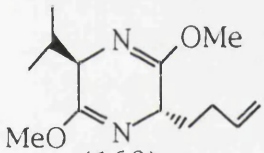
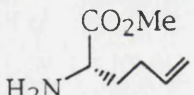
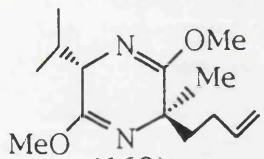
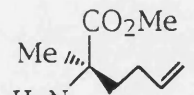
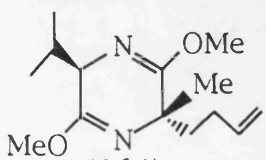
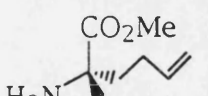
Alkylated *bis*-lactim ether	% Yield	% de	Products of hydrolysis
 (158)	78	84	L-Val-OMe +  (159)
 (160)	79	85	D-Val-OMe +  (161)
 (162)	95	>95	L-Val-OMe +  (163)
 (164)	99	>95	D-Val-OMe +  (165)

Table 6

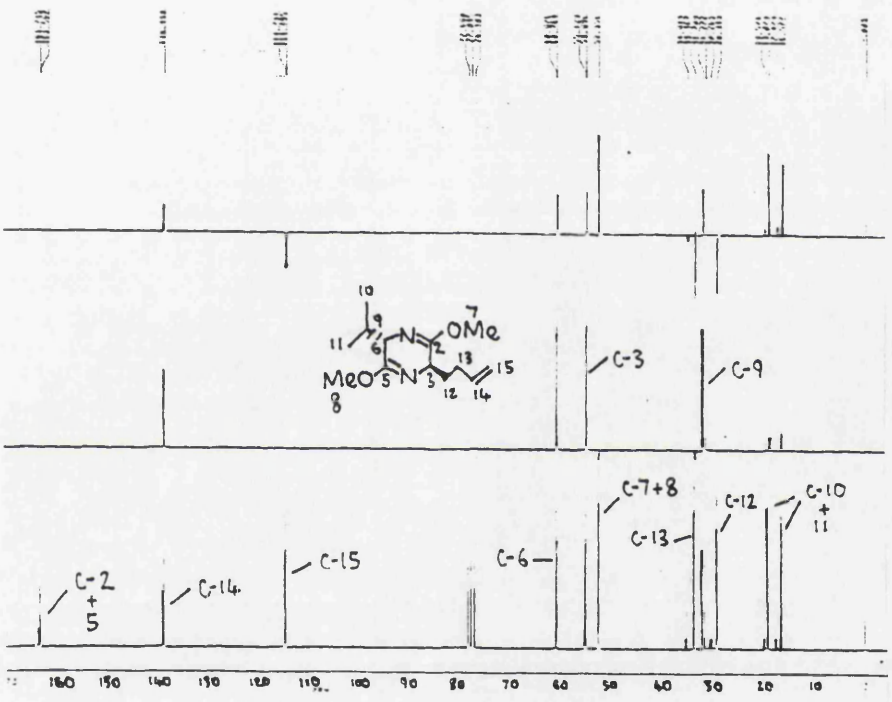


Fig. 6

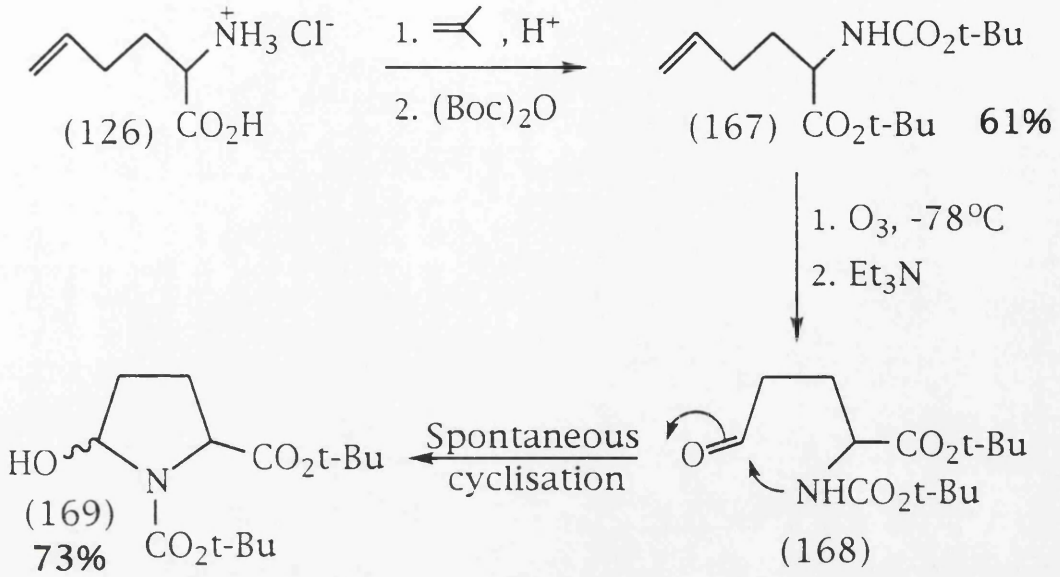
The separation of the valine auxiliaries from the required amino acid esters proved extremely difficult. This is not very suprising considering the similarities in molecular weight and functionality of the compounds being investigated. No suitable solvent system was found for separation using chromatography. The best

separation was attained by careful fractional distillation. For example, microdistillation of the mixture of D-valine methyl ester and compound (165) gave a sample of the amino acid methyl ester (165). Analysis of the ^1H NMR spectrum showed this to be approximately 75% pure.

Better separation of the products of hydrolysis is necessary before this synthetic procedure can be considered a viable route to optically active 2-amino-5-hexenoic acid (126) and 2-amino-6-heptenoic acid (127).

4.4 Attempted Synthesis of Glutamic Acid γ-Semialdehyde and α-Aminoadipic Acid δ-Semialdehyde.

Our first attempts at synthesising glutamic acid γ-semialdehyde (122) involved a synthetic route similar to that previously used in our synthesis of DL-aspartic acid β-semialdehyde (102). The amino acid, 2-amino-5-hexenoic acid (126) was doubly protected on the amino and carboxyl functionalities as the *t*-butoxycarbonyl derivative and the *t*-butyl ester respectively **(Scheme 66).**

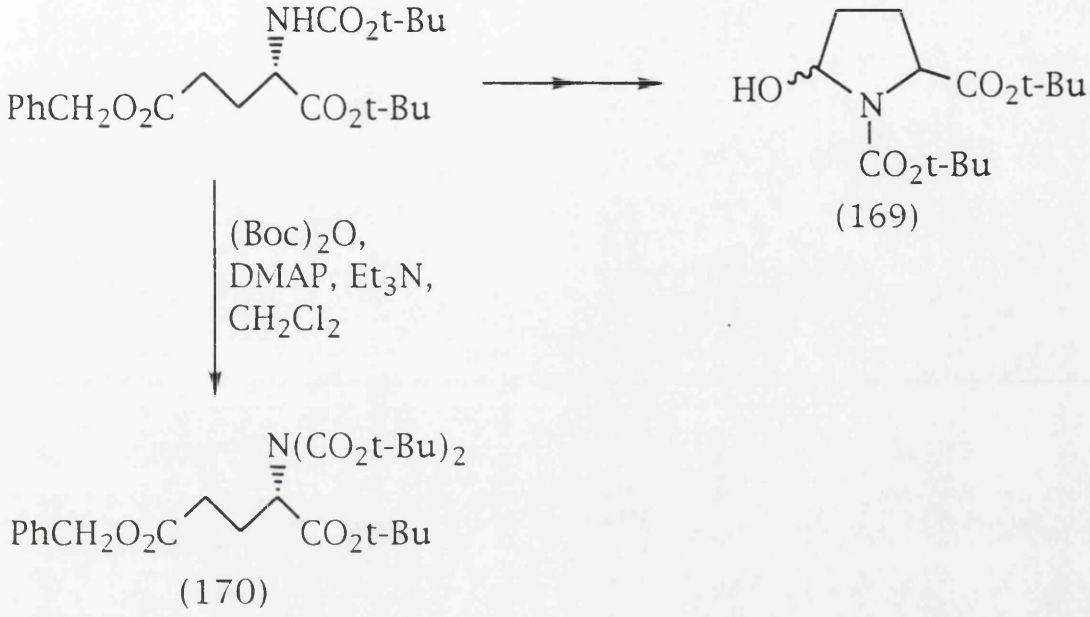


Scheme 66

This doubly protected material (167) was then subjected to ozonolysis at -78 °C followed by reductive work-up with triethylamine to yield around 73% of a pale yellow oil. However, analysis by ^{1}H and ^{13}C NMR showed that the aldehyde (168) had not been isolated. Instead, spontaneous intramolecular cyclisation took place between the carbamate nitrogen and the aldehyde group to give the 5-hydroxyproline derivative (169). The mass spectrum showed a peak at *m/z* 270, corresponding to the parent ion of (169), less a hydroxyl group, and the ^{1}H and ^{13}C NMR spectra were more complicated than expected due to the presence of diastereoisomers of (169).

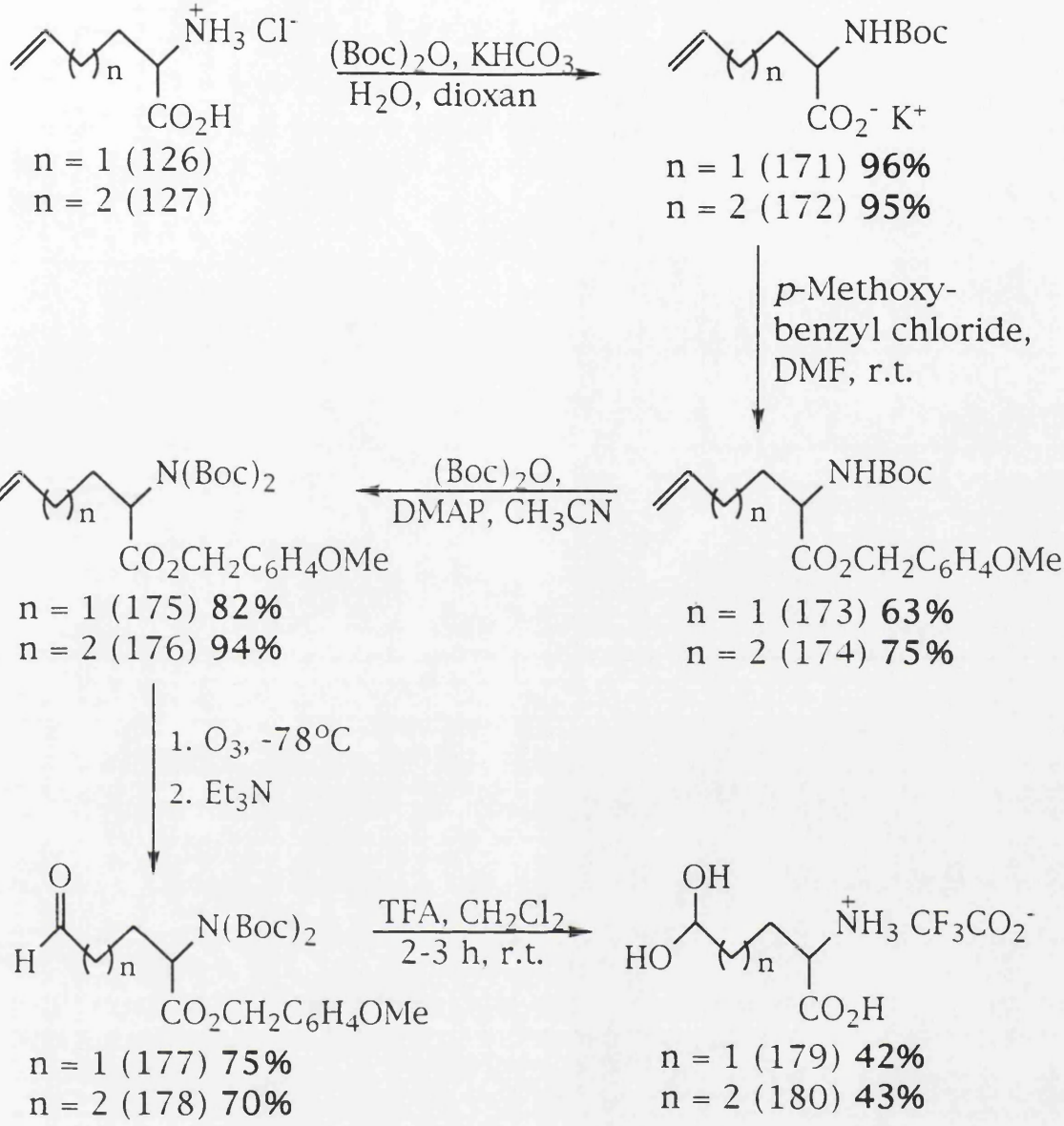
A literature search revealed that other workers had encountered this same cyclisation problem with similar compounds.[92,130] A solution to this problem lies within the work of Grehn and Ragnarsson.[131,132] They described the synthesis of *N*-$(Boc)_2$ compounds by the exhaustive *t*-butoxycarbonylation of amide nitrogens using di-*t*-butylcarbonate and a catalytic amount of 4-

dimethylaminopyridine. Bold *et al.*[92] used this method to synthesise an *N*-(Boc)$_2$ derivative of glutamic acid, (170), in order to prevent cyclisation to the 5-hydroxyproline compound (169) **(Scheme 67)**.



Scheme 67

We applied the method of Grehn and Ragnarsson to our synthetic routes of GSA (122) and AASA (17) in order to avoid intramolecular cyclisation at the oxidation stage. We also decided to use the *p*-methoxybenzyl ester as the carboxyl protecting group as opposed to the *t*-butyl ester. This resulted in higher overall yields and gave aromatic signals in the ^{1}H NMR spectra, making them easier to interpret.



Scheme 68

The doubly protected amino acids (173) and (174) were prepared in good overall yields (**Scheme 68**) as similarly described in the synthesis of DL-ASA (102) in Chapter 3. The *N*-$(Boc)_2$ derivatives (175) and (176) were prepared using $(Boc)_2O$ and a catalytic amount of 4-dimethylaminopyridine (DMAP). (175) and (176) were isolated as yellow oils in 82 and 94% yields respectively. Analysis of each of (175) and (176) by ^1H NMR showed a broad singlet at around δ_H 1.45 in each spectrum, integral 18H,

corresponding to the two Boc groups. The ^{13}C NMR spectra each had signals at around δ_C 155 and 152 for the amide carbonyl carbons.

Ozonolysis of compounds (175) and (176) followed by reductive work-up and purification by silica gel chromatography gave compounds (177) and (178) as pale orange oils in around 75% yield. The ^{1}H NMR spectra of (177) and (178) had singlets at δ_H 9.76 and 9.75 respectively for the aldehyde proton and the ^{13}C NMR spectra had signals at δ_C 201.0 and 201.8 for the aldehyde carbons. The mass spectra also supported the formation of the aldehydes.

A solution of (177) in dichloromethane was stirred with trifluoroacetic acid under an atmosphere of nitrogen at room temperature for 2-3 hours. The reaction mixture was concentrated *in vacuo*, taken up in distilled water and washed with ethyl acetate. The aqueous layer was freeze-dried to yield a cream-coloured, hygroscopic solid, mp 57-58 °C. Analysis of the solid by ^{1}H NMR and ^{13}C NMR spectroscopy indicated the presence of the trifluoroacetate salt of DL-glutamic acid γ-semialdehyde hydrate (179). The ^{1}H NMR spectrum showed broad multiplets at δ_H 1.84-2.30 (3- and 4-H_2), 4.16 (2-H) and 5.40 (5-H). The sample also contained impurities (possibly isobutylene polymer derivatives). No attempts have been made to purify the impure material.

Removal of the protecting groups of compound (178) was achieved under similar conditions as for compound (177). Freeze-drying of the aqueous layer yielded a yellow, hygroscopic solid, mp 59-61 °C. The ^{1}H NMR and ^{13}C NMR spectra indicated an impure sample of the trifluoroacetate salt of DL-α-aminoadipic acid δ-semialdehyde hydrate (180). The ^{1}H NMR spectrum had broad signals at 1.35-2.32 (3-, 4- and 5-H_2) and 3.80 (2-H). The expected signal corresponding to the methine proton of C-6 appears to be hidden by

the broad water peak. No attempts to purify this material were undertaken.

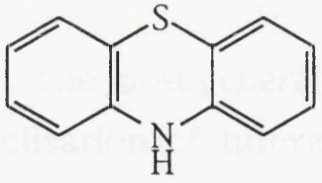
The potassium salts of the *N*-Boc amino acids, compounds (171) and (172), showed no inhibition of DHDP synthase at 1 mM. The remaining intermediates of the synthetic pathway shown in **Scheme 68**, compounds (173)-(178), were found to be insoluble in the enzyme assay mixture. The impure sample of DL-glutamic acid γ-semialdehyde hydrate (179) showed no inhibition of DHDP synthase. DL-α-aminoadipic acid δ-semialdehyde hydrate (180) was a poor inhibitor of DHDP synthase, showing only 27% inhibition at 1.0 mM.

Chapter 5 - Synthesis of Sulfur Analogues of L-2,3-DHDPA and L-2,3,4,5-THDPA.

Introduction.

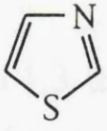
In recent years an ever-increasing number of organosulfur compounds have been introduced into medicine and agriculture. These compounds contain sulfur in a variety of forms and oxidation states such as thioethers, sulfoxides, sulfones, thiols and thiones. The sulfur atom may form part of a heterocyclic ring system, often in combination with other heteroatoms such as nitrogen. Around 30% of the chemicals currently used as insecticides, herbicides, plant growth regulators and fungicides contain sulfur.

Sulfur heterocycles commonly encountered in medicinal and agricultural applications include phenothiazine (181), thiophene (182), thiazole (183) and thiazolidine (184).

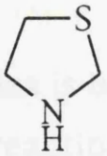


(181)

(182)

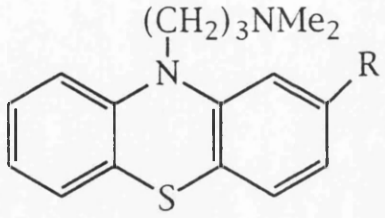


(183)



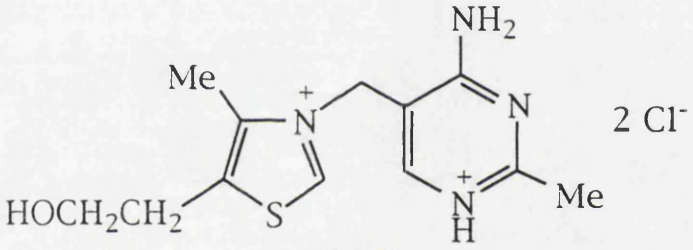
(184)

Phenothiazine drugs are used in the treatment of psychiatric illness and as sedatives, anti-histamines and tranquilisers.[133] Two important phenothiazine drugs are chlorpromazine (185) and trifluprom azine (186).



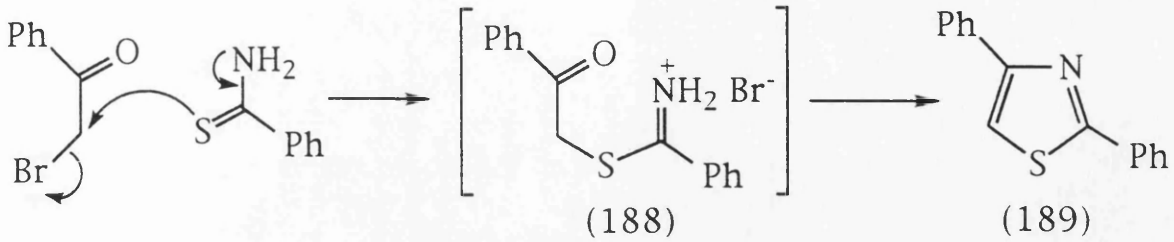
R = Cl (185)
R = CF_3 (186)

The 1,3-thiazole ring system is often found in natural products as it can be produced by the cyclisation of cysteine residues in peptides. An important natural product is vitamin B_1 (187) which contains both a pyrimidine and a 1,3-thiazole ring system.



(187)

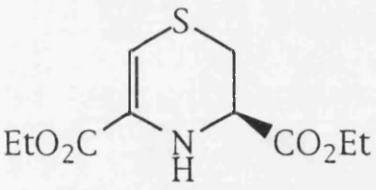
The most general synthetic route to 1,3-thiazoles is based on the cyclisation of intermediates formed from the reaction of α-halocarbonyl compounds and thioamides. This is known as the 'Hantzsch thiazole synthesis' after A. Hantzsch who first introduced the synthesis in 1888.[134] An example of the Hantzsch thiazole synthesis is shown in **Scheme 69**. Nucleophilic attack by sulfur on the carbon atom bearing the halogen atom results in the acyclic intermediate (188). Cyclisation and dehydration occur to give the product, 2,4-diphenyl-1,3-thiazole (189).



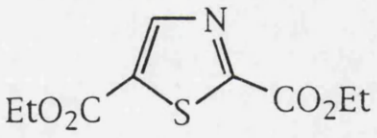
(188) (189)

Scheme 69

A number of sulfur analogues of L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (26) had been previously synthesised within our group and tested with DHDP synthase.[135] Among the best inhibitors tested were diethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (42) and diethyl 1,3-thiazole-2,4-dicarboxylate (43). Compound (42) gave 10% inhibition at 0.1 mM with DHDP synthase and (43) showed 15% inhibition at 0.1 mM.



(42)



(43)

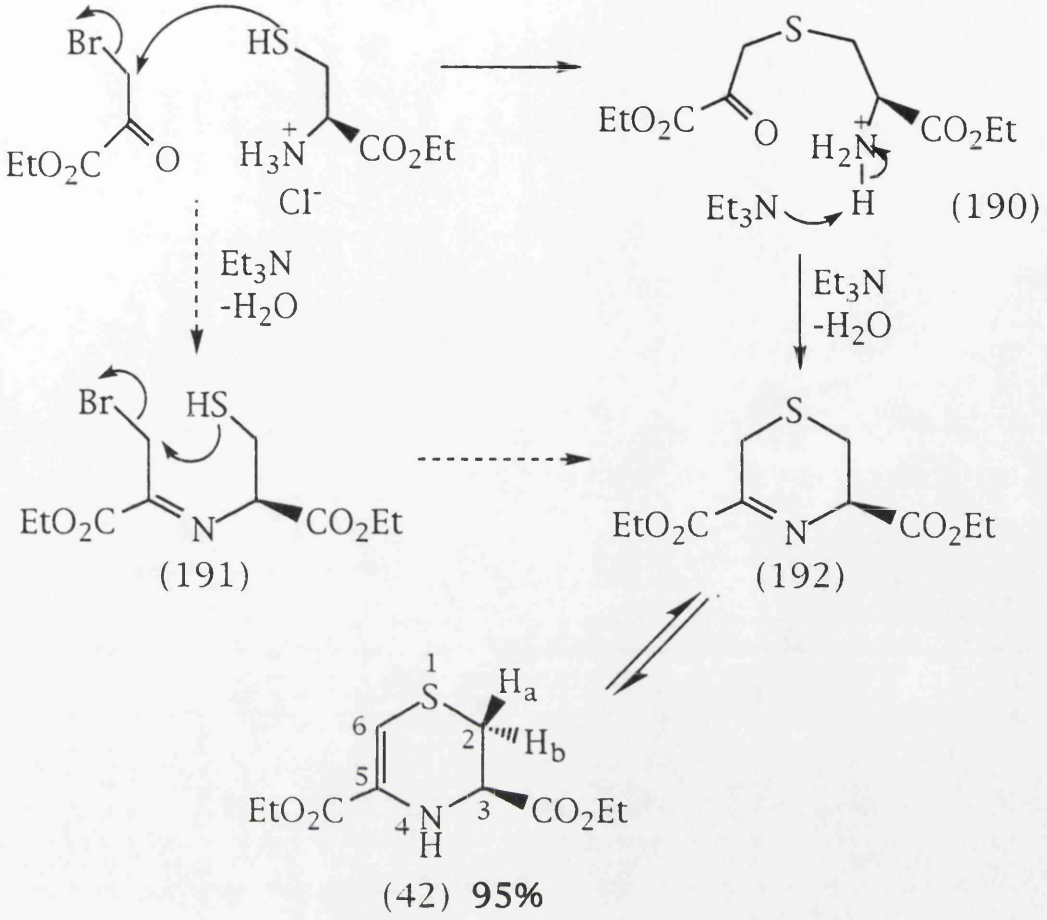
In this work compounds (42) and (43) were synthesised and kinetic studies were carried out to determine whether they showed competitive or non-competitive inhibition with DHDP synthase. A number of other sulfur heterocycles were also prepared and tested with DHDP synthase.

5.1 Synthesis and Test Results of 1,4-Thiazines.

Berges and Taggart[136] first reported the preparation and isolation of diethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (42) as an orange syrup. We followed their procedure by treating L-cysteine ethyl ester hydrochloride with ethyl bromopyruvate in dichloromethane in the presence of triethylamine **(Scheme 70)**. Purification by column chromatography yielded compound (42) as a golden orange syrup in 95% yield.

There are two possible mechanisms for the formation of (42) from L-cysteine ethyl ester hydrochloride and ethyl bromopyruvate.

Nucleophilic attack by sulfur on the carbon atom bearing the bromine would result in the acyclic intermediate (190). This could be followed by cyclodehydration and tautomerisation to give the more stable enamine form (42). Alternatively, attack of the amino group on the keto functionality of ethyl bromopyruvate could occur first to give the imine intermediate (191). Subsequent cyclisation via nucleophilic attack by sulfur on the carbon atom bearing the bromine would give the ketimine (192). Tautomerisation would give the more stable enamine (42).



Scheme 70

The ^{1}H NMR spectrum of (42) **(Fig. 7)** indicated that only the enamine form was present. There is a singlet at δ_H 6.17 for the olefinic proton at C-6. A well defined ABX splitting pattern is observed between the C-2 methylene protons and the adjacent C-3 proton. The 2a-H at C-2 gives a signal at δ_H 2.99 and a coupling constant of 6.7 Hz with the 3-H. The 2b-H at C-2 gives a signal at δ_H 3.25 and a coupling constant of 2.9 Hz with the 3-H. The geminal coupling constant between 2a-H and 2b-H is 12.1 Hz.

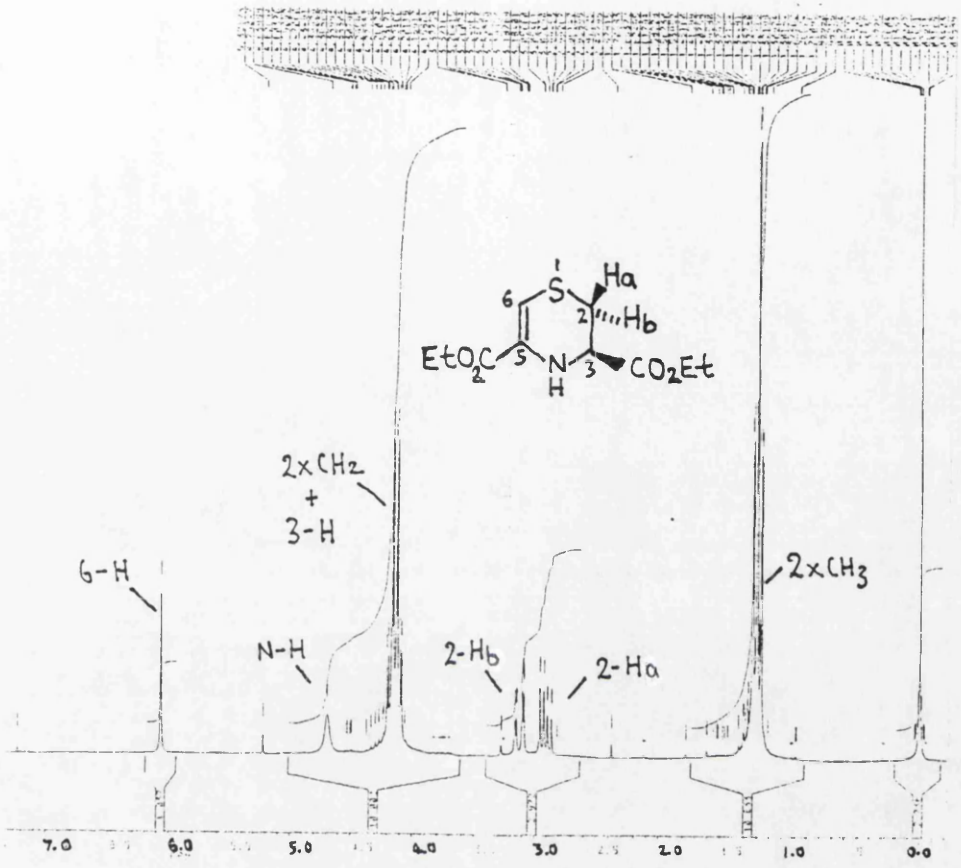
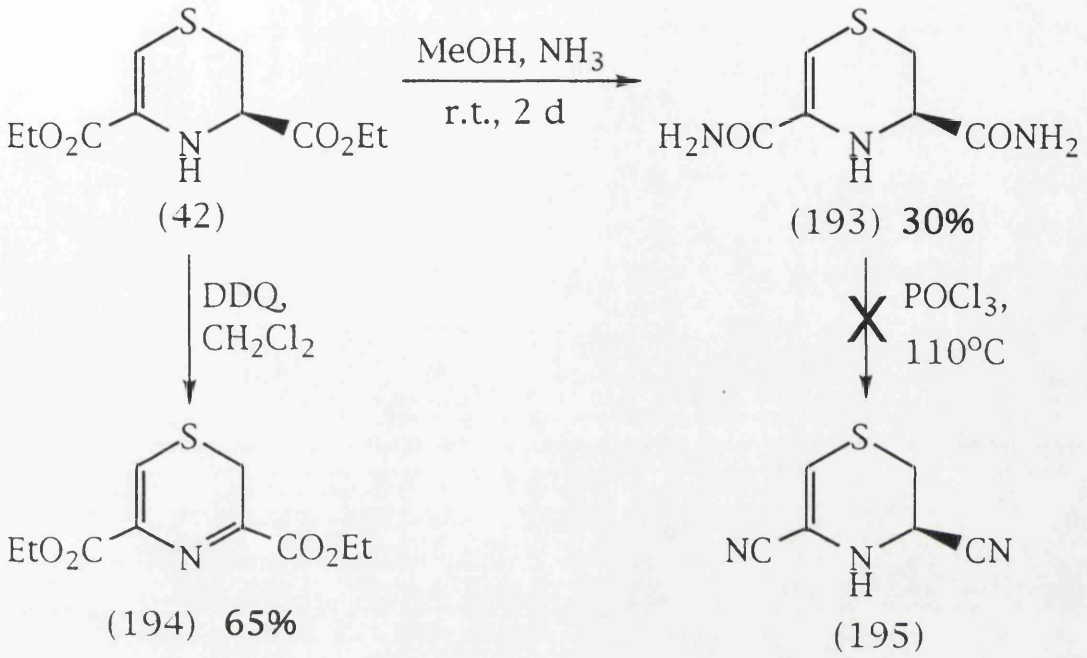


Fig. 7

3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxamide (193), the diamide derivative of (42), was prepared in 30% yield by stirring the diester (42) in methanolic ammonia solution **(Scheme 71)**. The diamide (193) precipitated out of solution as an orange solid.

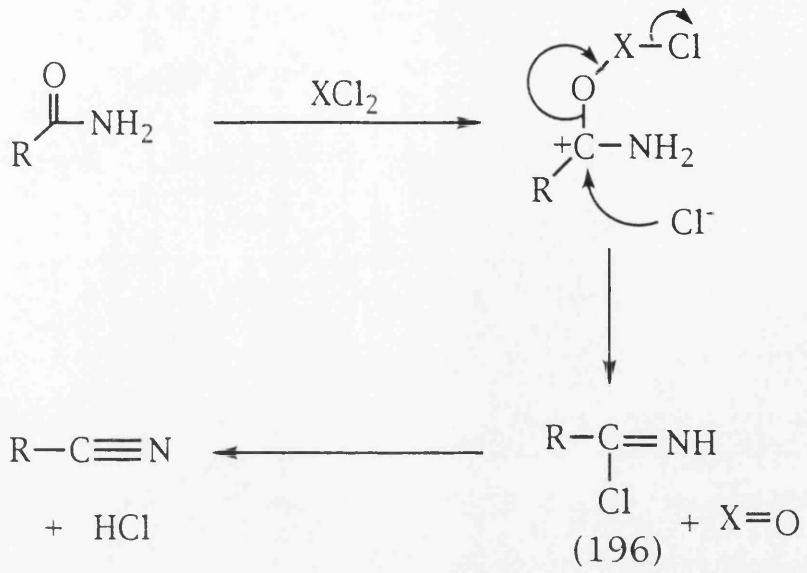
Diethyl 2*H*-1,4-thiazine-3,5-carboxylate (194) was prepared by treating a solution of the diester (42) in dichloromethane with the hydride abstractor, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) **(Scheme 71)**. DDQ is a reactive quinone which is reduced to the corresponding hydroquinone.[137] The 1,4-thiazine (194) was isolated as a yellow oil in 65% yield. The methylene protons of C-2 gave a

doublet at δ_H 3.32 (J 1.2 Hz) in the ^1H NMR spectrum. The proton of C-6 gave a triplet at δ_H 7.60 (J 1.2 Hz).



Scheme 71

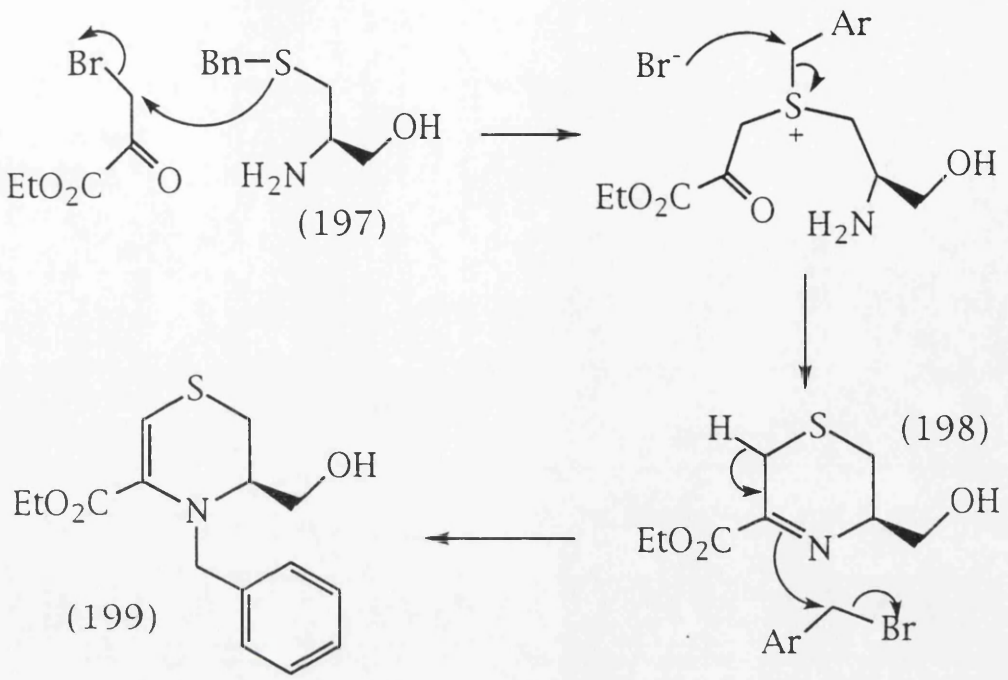
Nitriles can be prepared by the dehydration of amides. Dehydrating reagents commonly used include phosphorus pentoxide, phosphorus pentachloride and phosphorus oxychloride. The general reaction mechanism for the dehydration of amides by inorganic acid halides is shown in **Scheme 72**. Initial complexation of the acid halide with the amide results in polarisation of the C-O bond. Nucleophilic attack by a chloride anion results in elimination of XO (X= PCl_3, or POCl) to form the imidoyl chloride (196). Loss of hydrogen chloride gives the nitrile.



Scheme 72

Attempts to synthesise the dinitrile (195) by treating the diamide (193) with phosphorus oxychloride in 1,1,2,2-tetrachloroethane were unsuccessful **(Scheme 71)**. The diamide (193) decomposed under the harsh reaction conditions.

S-Benzyl-L-cysteinol (197) is commercially available. It was expected that treatment of (197) with an equimolar amount of ethyl bromopyruvate would give the half ethyl ester/alcohol (198) **(Scheme 73)**. However, nucleophilic attack by nitrogen upon benzyl bromide formed *in situ* resulted in formation of the N-benzyl derivative (199) as an orange oil in 73% yield. Analysis by TLC showed only one spot and the mass spectrum recorded the parent ion at *m/z* 293. In the ^{1}H NMR spectrum the methine proton of C-6 gave a singlet at δ_H 6.88 and the aromatic protons gave a multiplet at δ_H 7.38.



Scheme 73

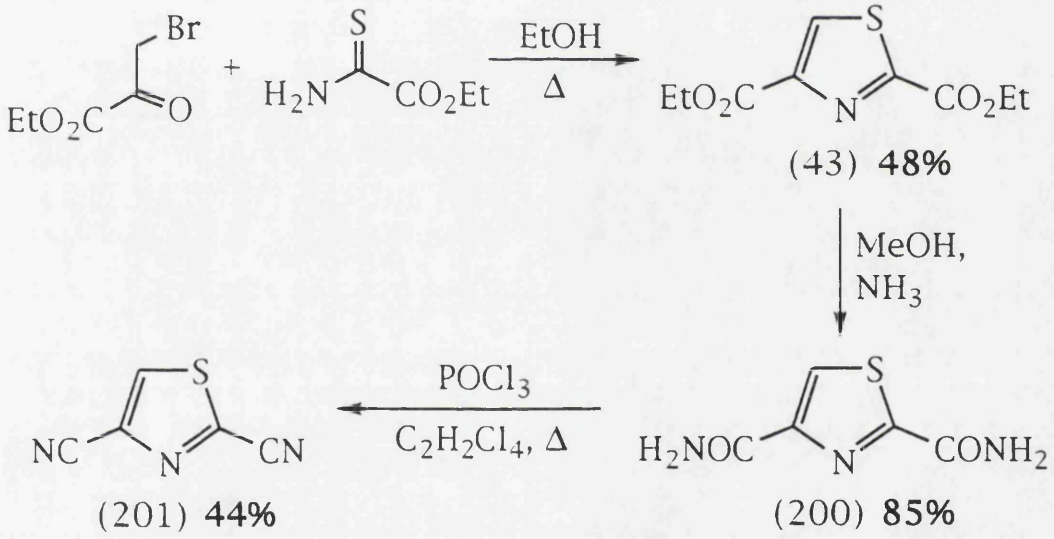
Test Results.

In this work, diethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (42) showed 15% inhibition at 0.1 mM with DHDP synthase and further kinetic studies (Lineweaver Burk plots) showed it to be a competitive inhibitor against DL-ASA (102), with K_i= 0.06 mM, and a noncompetitive inhibitor against pyruvate (24), with K_i= 0.05 mM.

The high insolubility of the diamide derivative (193) in both organic and aqueous solvent systems prevented us from obtaining accurate test results of (193) with DHDP synthase. Solubility problems were also encountered with the testing of compounds (199) and (194).

5.2 Synthesis and Test Results of 1,3-Thiazoles.

Diethyl 1,3-thiazole-2,4-dicarboxylate (43) was prepared by treating ethyl thioxamate with ethyl bromopyruvate in ethanol **(Scheme 74)**. Purification on a neutral alumina column eluting with 20% ethyl acetate in hexane gave a cream solid in 48% yield. The ^{1}H NMR spectrum showed a singlet at δ_H 8.45 for the proton at C-5 and the ^{13}C NMR spectrum showed a signal at δ_C 132.3 for C-5. The mechanism of formation of (43) is similar to that of 2,4-diphenyl-1,3-thiazole (189) **(Scheme 69)**.



Scheme 74

1,3-Thiazole-2,4-dicarboxamide (200) was prepared in 85% yield by treating diethyl 1,3-thiazole-2,4-dicarboxylate (43) with a solution of methanolic ammonia at room temperature **(Scheme 74)**. The ^{13}C NMR spectrum showed two signals at δ_C 161.8 and 163.0 for the two amide carbonyls.

1,3-Thiazole-2,4-dinitrile (201) was prepared from the diamide (200) by treatment with phosphorus oxychloride in 1,1,2,2-

tetrachloroethane (**Scheme 74**).[138] Recrystallisation from ethanol gave (201) as white needles in 44% yield. The ^{1}H NMR spectrum showed only one signal at δ_H 8.35 for the 5-H. The ^{13}C NMR spectrum had signals at δ_C 111.0 and 112.0 for the two nitrile carbons.

Test Results.

Diethyl 1,3-thiazole-2,4-dicarboxylate (43) showed 15% inhibition at 0.1 mM with DHDP synthase and further kinetic studies (Lineweaver Burk plots) showed it to be a competitive inhibitor against DL-ASA (102), with K_i= 0.16 mM, and a noncompetitive inhibitor against pyruvate (24), with K_i= 0.06 mM.

The diamide derivative (200) was highly insoluble under the assay conditions, thus no accurate test results were obtained for this compound. However, the dinitrile derivative (201) was a good inhibitor of DHDP synthase, showing 18% inhibition at 0.1 mM.

Chapter 6 - Synthesis of *cis*-2,6- and *cis*-2,4,6-Substituted Piperidines.

Introduction.

Alkaloids containing a *cis*-2,6-disubstituted piperidine ring (Fig. 8) occur frequently in nature and have been shown to exhibit significant biological activity.[139] Several *cis*-2,6-dialkylpiperidines found in venomous myrmicine ants have fungicidal, insecticidal and repellant properties.[140]

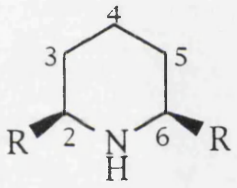
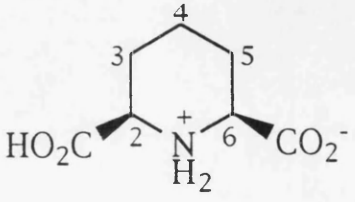


Fig. 8

cis-2,6-Piperidinedicarboxylic acid (37) has some structural similarity to the intermediates of the first two enzymic steps of the diaminopimelate pathway to L-lysine (1), namely L-2,3-dihydrodipicolinic acid (25) and L-2,3,4,5-tetrahydrodipicolinic acid (26).

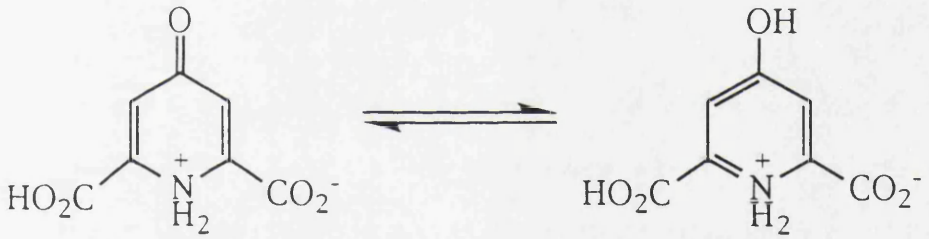


(37)

Preparation of *cis*-2,6-piperidinedicarboxylic acid (37) can be readily carried out by a number of methods.[141,142] This Chapter describes the synthesis and testing of derivatives of *cis*-2,6- and *cis*-2,4,6-piperidines as potential inhibitors of DHDP synthase.

6.1 Synthesis and Testing of Substituted Piperidine Derivatives.

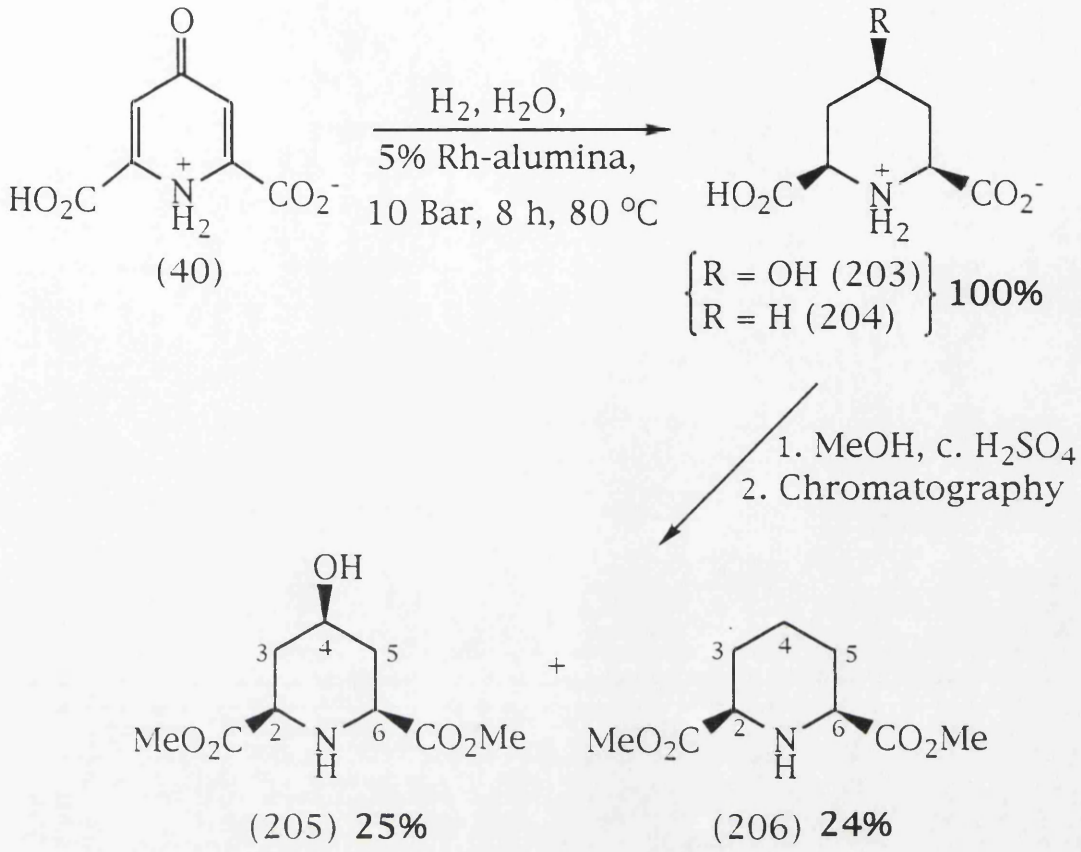
Chelidamic acid (40) is commercially available. The structure of chelidamic acid may be drawn as an equilibrium mixture of the γ-pyridone (40) and the hydroxypyridine (202) **(Scheme 75)**.



(40) (202)

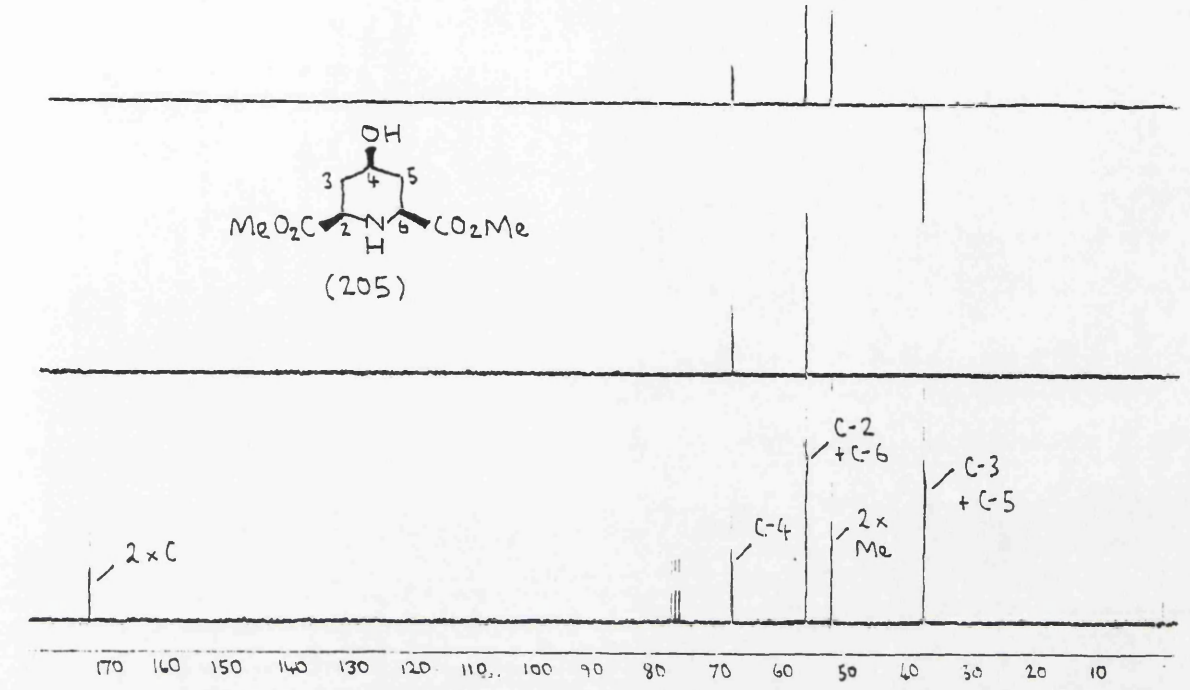
Scheme 75

We subjected chelidamic acid (40) to catalytic hydrogenation with 5% rhodium-alumina catalyst in water under 10 Bar hydrogen for 8 hours at 80 °C.[143] The resultant light brown solid was found to be a mixture of 4-hydroxy-piperidine-*cis*-2,6-dicarboxylic acid (203) and piperidine-*cis*-2,6-dicarboxylic acid (204) **(Scheme 76)**. Subsequent esterification with methanol gave an approximately 1:1 mixture of the dimethyl esters, (205) and (206), which were readily separated by column chromatography.



Scheme 76

Compound (205) was isolated as a white solid, mp 134-136 °C. The ^{1}H NMR spectrum of (205) showed a singlet at δ_H 3.76 for the six hydrogens of the two methyl esters and a multiplet at δ_H 3.74 for the hydrogen at C-4. The ^{13}C NMR spectrum had a signal at δ_C 68.0 for C-4. Compound (206) was also a white solid, mp 92-94 °C. Both the ^{1}H NMR and ^{13}C NMR spectra of (206) differed from that of compound (205). The ^{1}H NMR spectrum of (206) lacked a signal around δ_H 3.74 for the methine hydrogen at C-4 and the ^{13}C NMR spectrum had a signal at δ_C 24.1 for C-4. The ^{13}C NMR spectra of compounds (205) and (206) are shown in **Fig. 9**.


OH
3
4
5
MeO2C
2
N
H
6
CO2Me
(205)
C-2
+C-6
C-3
+C-5
C-4
2x
Me
2xC
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10

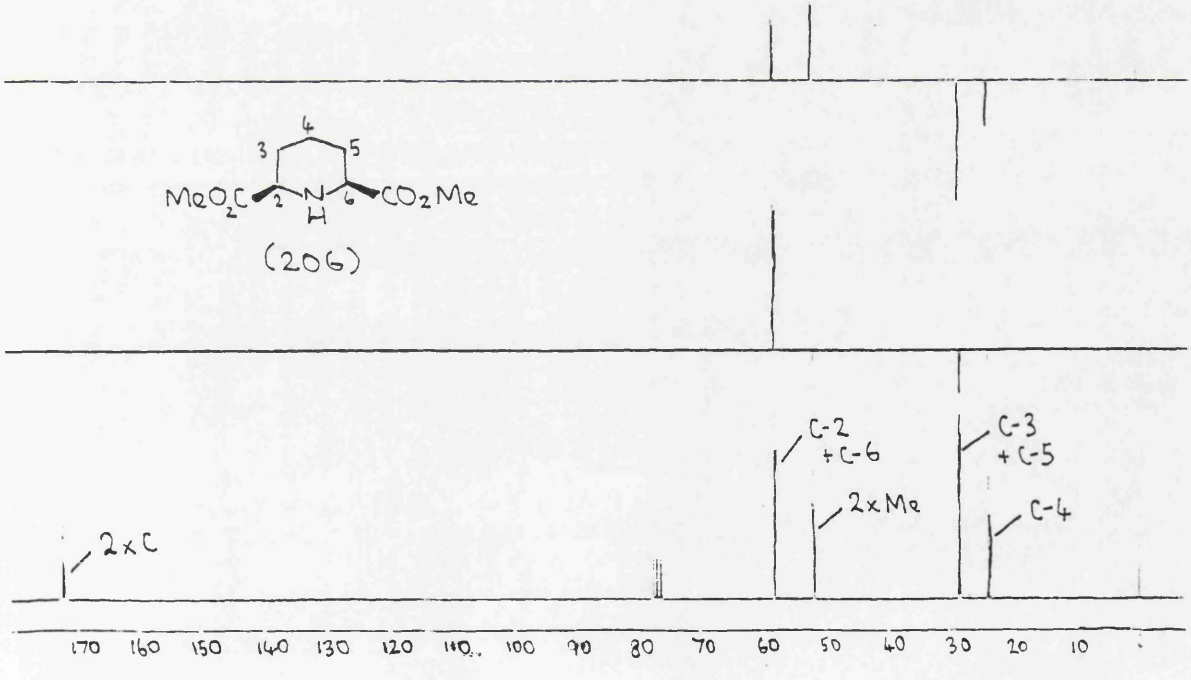
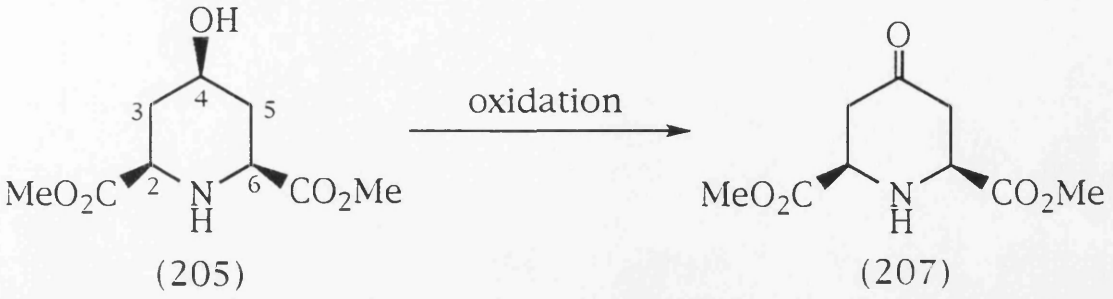

4
3
5
MeO2C
2
N
H
6
CO2Me
(206)
C-2
+C-6
C-3
+C-5
2xMe
C-4
2xC
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10

Fig. 9

Attempts were made to oxidise the 4-hydroxy group of (205) to the corresponding ketone, (207) **(Scheme 77).**



(205) (207)

Scheme 77

The various oxidation methods tried for the conversion of (205) into (207) are listed in **Table 7.** Attempted oxidation of (205) with dimethylsulfoxide and *N,N'*-diisopropylcarbodiimide[143] failed to afford the ketone (207). The use of chromate-based oxidative reagents allows for relatively mild and selective reaction conditions.[144] Chromic acid on a polymer support is commercially available. A solution of (205) in dichloromethane was stirred with polymer-supported chromic acid under an atmosphere of nitrogen for several hours. Analysis by TLC indicated that no reaction had occurred and only starting material was recovered.

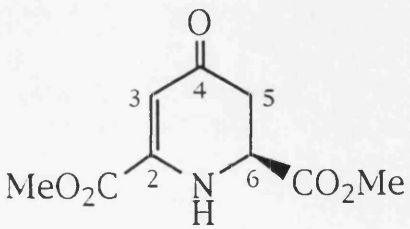
Pyridinium chlorochromate (PCC) adsorbed onto alumina was prepared by adding alumina to a solution of PCC and evaporating to dryness.[145] A solution of (205) in dichloromethane was stirred vigorously with PCC on alumina. Analysis of the reaction mixture by TLC indicated that no starting material remained. However, subsequent workup failed to afford a product.

The use of pyridinium dichromate (PDC) with freshly activated molecular sieve powder and a small amount of anhydrous acetic acid has been reported to effect the oxidation of alcohols.[146] A

solution of (205) in dichloromethane was treated with the above reagents at room temperature. Purification of the reaction mixture by column chromatography yielded a pale coloured solid. This solid showed only one spot on TLC but analysis by GCMS showed it to be a 1:1 mixture of the ketone (207) (*M* H^+, 216) and the over-oxidised enone (208) (*M* H^+, 214). The ^1H NMR spectrum showed three singlets at δ_H 3.80, 3.82 and 3.91 corresponding to the three different methyl esters present. There was a singlet at δ_H 5.78 for the methine proton at C-3 of (208).

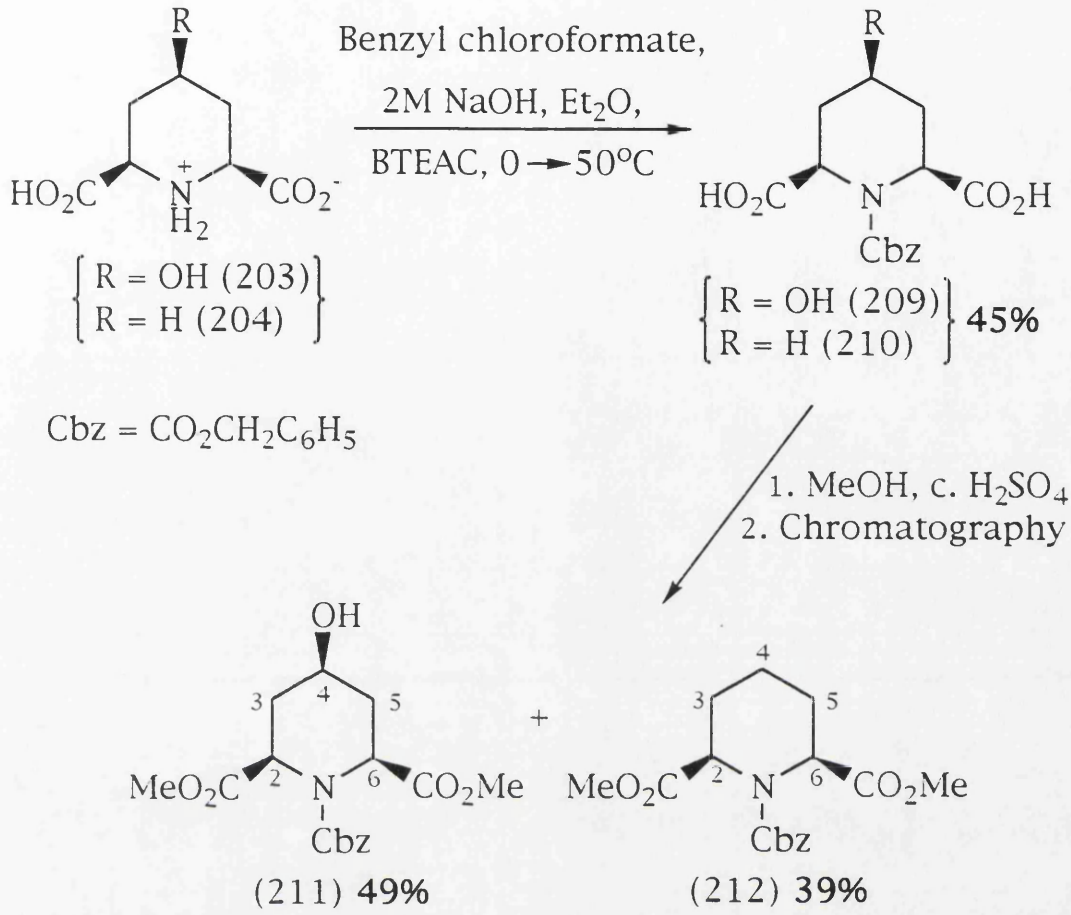
Reagents	Result
DMSO, *N,N'*-diisopropylcarbodiimide, TFA, pyridine, toluene	No product
Chromic acid-polymer, CH_2Cl_2	Starting material
PCC-alumina, CH_2Cl_2	No product
PDC, activated 3A sieves, glac. AcOH, CH_2Cl_2	1:1 Mixture of (207) and (208)

Table 7



(208)

The ring nitrogen can first be protected as the benzyloxycarbonyl (Cbz) derivative before derivatisation of the 4-hydroxy group is attempted. Benzyl chloroformate was added dropwise at 0 °C to a solution of a mixture of compounds (203) and (204) in 2M aqueous sodium hydroxide solution **(Scheme 78)**. This produced a mixture of the *N*-protected compounds (209) and (210). Subsequent esterification of the mixture of (209) and (210) with methanol in the presence of acid gave a clear oil which showed two distinct spots by TLC. Separation was achieved on a silica gel column eluting with ethyl acetate to give dimethyl 4-hydroxy-*N*-benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylate (211) as a clear oil in 49% yield and dimethyl *N*-benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylate (212) in 39% yield.

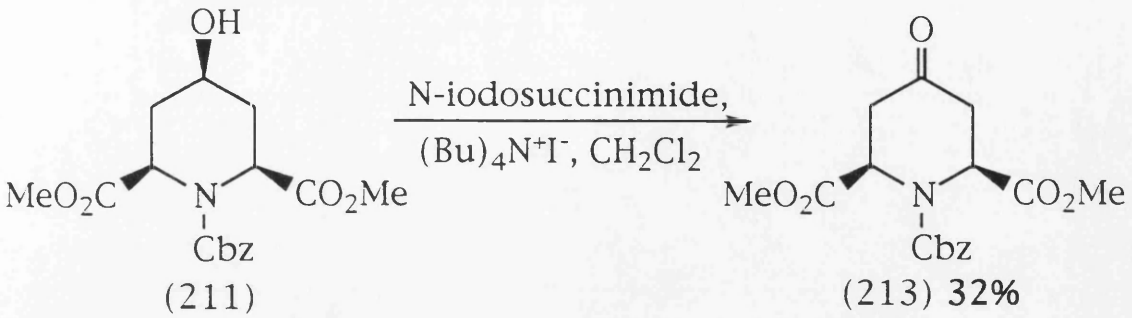


Scheme 78

The IR spectrum of compound (211) had a signal at around 3470 cm^{-1} for the free hydroxyl group. The ^1H NMR spectrum of (211) showed a broad doublet at δ_H 3.59 (J 3.6 Hz) which was absent after a D_2O exchange experiment. This signal was due to the free hydroxyl group. The ^{13}C NMR spectrum of compound (212) had a signal at δ_C 16.5 for C-4.

Oxidation of compound (211) to the ketone was best achieved using *N*-iodosuccinimide and tetra-*N*-butylammonium iodide in dichloromethane at room temperature **(Scheme 79)**.[147] Purification of the reaction mixture was achieved on a silica gel column eluting with 50% ethyl acetate in hexane to give (213) as a pale solid, mp 74-

76 °C. The ^{13}C NMR spectrum had a signal at δ_C 202.7 for the quaternary carbon at C-4.



Scheme 79

The Cbz-protecting group could possibly be removed *via* treatment with trimethylsilyl iodide. This reagent has been reported to convert benzyl carbamates into the free amines in the presence of methyl esters.[99] The deprotection was attempted in this work, however time limits did not allow for the satisfactory completion of this synthesis.

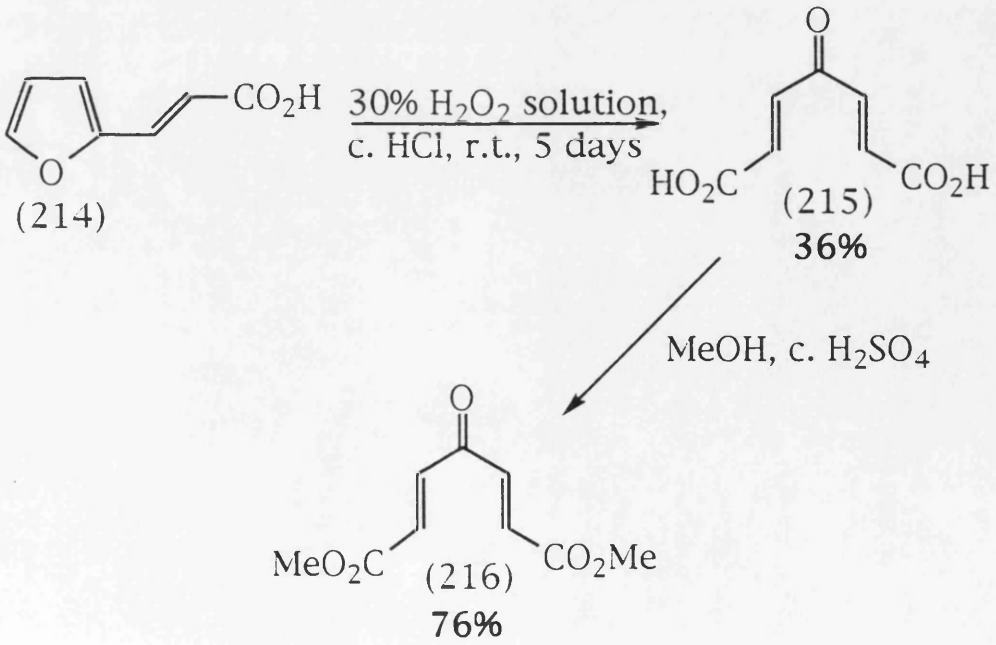
Test Results.

Dimethyl 4-hydroxy-piperidine-*cis*-2,6-dicarboxylate (205) and dimethyl piperidine-*cis*-2,6-dicarboxylate (206) were both poor inhibitors of DHDP synthase, showing only 29% and 6% inhibition respectively at 5mM. The mixture of dimethyl 4-piperidone-*cis*-2,6-dicarboxylate (207) and the enone (208) showed 22% inhibition at 2.5 mM. Neither of the Cbz-derivatives, compounds (211) and (212), showed any inhibition at 5 mM. Compound (213) was also a poor inhibitor, giving only 12% inhibition at 5 mM.

6.2 Diammonium Piperidin-4-one-2,6-dicarboxylate.

1,4-Pentadien-3-one-1,5-dicarboxylic acid (215) was prepared by stirring a suspension of 2-furanacrylic acid (214) in hydrogen peroxide and conc. hydrochloric acid **(Scheme 80)**.[148] The product precipitated out of solution as an orange solid. The IR spectrum of (215) had peaks at 1700 and 1665 cm^{-1} for the acid and the αβ-unsaturated ketone respectively. The mass spectrum showed a parent ion at *m/z* 170. Further signals due to the loss of the carboxylic acid groups were observed.

As (215) was insoluble in all deuteriated solvents available for NMR spectroscopy, thus making characterisation difficult, the dimethyl ester (216) was prepared by heating (215) with methanol and acid **(Scheme 80)**. Recrystallisation from ethyl acetate yielded an orange solid, mp 169-171 °C. The ^1H NMR spectrum of (216) had a singlet at δ_H 3.84 for the two methyl esters and an AB system at δ_H 6.82 and 7.34 (*J*15.9 Hz) due to the olefinic protons **(Fig. 10)**.



Scheme 80

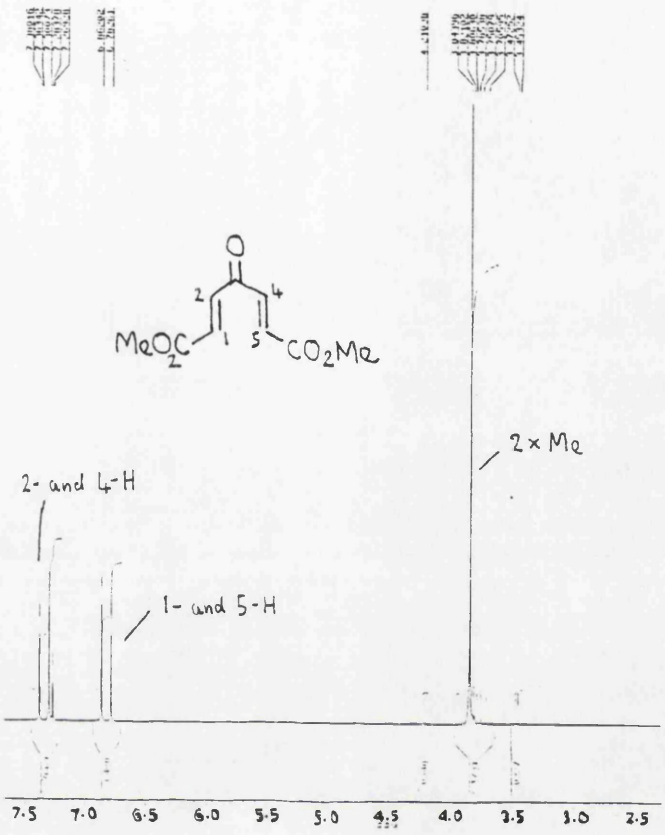
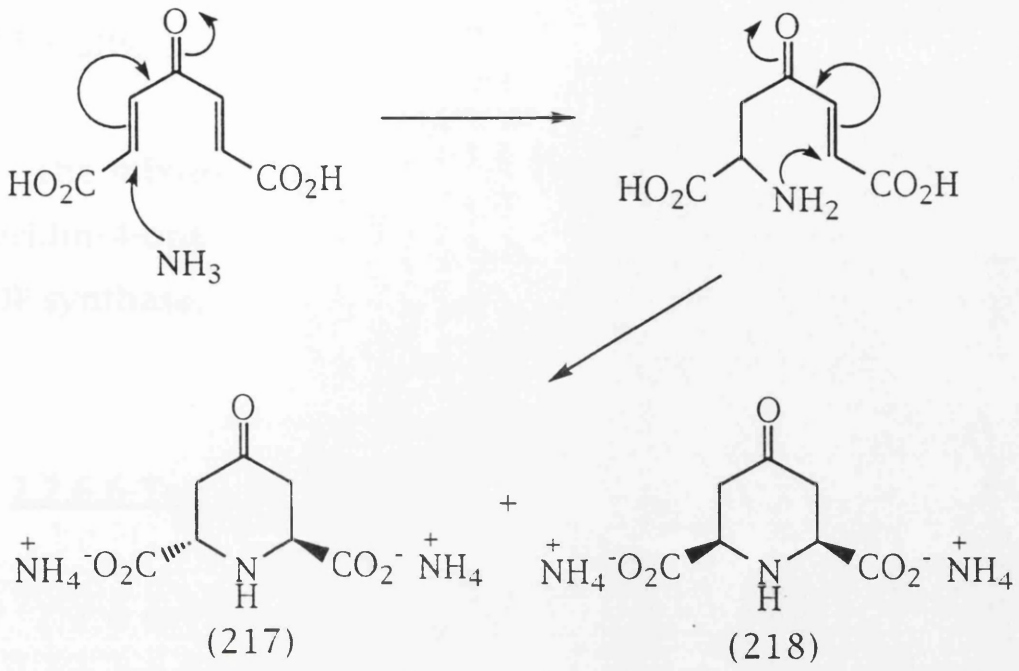


Fig. 10

The αβ-unsaturated carbonyl system of 1,4-pentadien-3-one-1,5-dicarboxylic acid (215) can undergo Michael additions with nucleophiles such as ammonia. We carried out this reaction according to the procedure of Hermann and Dreiding[143] by heating at reflux a mixture of (215) and conc. ammonia solution **(Scheme 81)**. A mixture of diammonium *cis*- (218) and *trans*-piperidin-4-one-2,6-dicarboxylate (217) was obtained as a brown solid.



Scheme 81

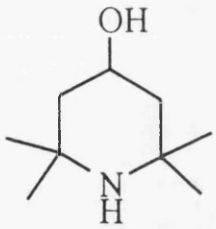
The ^{1}H NMR spectrum showed a broad multiplet at δ_H 2.28-2.33 for the methylene protons of C-3 and 5 of both the *cis*- (218) and *trans*-isomers (217). The multiplet at δ_H 4.05 was assigned to the C-2 and 6 protons of the major isomer. This could be the *cis*-isomer (218) as both carboxyls are likely to be equatorial, thus keeping destabilising 1,3-diaxial interactions to a minimum. The multiplet at δ_H 4.20 was assigned to the C-2 and 6 protons of the minor isomer, possibly the *trans*-isomer (217). Purification and separation of the mixture of (217) and (218) proved impossible as no suitable TLC solvent system was found and attempted recrystallisation from water or methanol failed to yield any solid material.

Test Results.

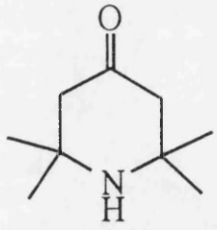
The mixture of the diammonium salts of *cis*- (218) and *trans*-piperidin-4-one-2,6-dicarboxylate (217) showed good inhibition of DHDP synthase, giving around 30% inhibition at 0.1 mM.

6.3 2,2,6,6-Tetramethylpiperidine Derivatives.

2,2,6,6-Tetramethyl-4-piperidinol (219) and 2,2,6,6-tetramethyl-4-piperidinone (220) are both commercially available. As they have some structural similarity to the first two intermediates of the DAP biosynthetic pathway, L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (26), we decided to test them for inhibitory effects on DHDP synthase.

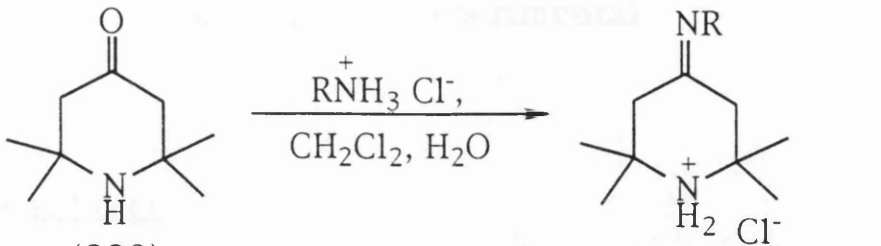


(219)



(220)

The oxime (221) and methyloxime (222) derivatives of 2,2,6,6-tetramethyl-4-piperidinone (220) were prepared by stirring (220) in a two-phase system of dichloromethane and water with the appropriate amine at room temperature **(Scheme 82)**. The products were isolated as the hydrochloride salts after trituration with ether/HCl.



(220)

R = OH (221) **67%**
R = OMe (222) **11%**

Scheme 82

The ^{1}H NMR spectrum of the oxime (221) had a singlet at δ_H 1.28 for the twelve hydrogens of the four methyl groups. There were a further two singlets at δ_H 2.30 and 2.58 for the methine hydrogens at C-3 and C-5. The IR spectrum of (221) had a strong peak at 3280 cm^{-1} for the hydroxyl group of the oxime.

The ^{1}H NMR spectrum of the methyloxime (222) had an additional singlet at δ_H 3.87 for the methyl hydrogens of the methoxy group.

Test Results.

2,2,6,6-Tetramethyl-4-piperidinol (219) and 2,2,6,6-tetramethyl-4-piperidinone (220) were reasonable inhibitors of DHDP synthase at 0.5 mM, showing 46% and 22% inhibition respectively. Derivatisation of the carbonyl functionality of (220) had no significant effect upon inhibitory activity, the oxime (221) and methyloxime (222) derivatives showing 8% and 23% inhibition at 0.5 mM.

Chapter 7 - Experimental

7.1 General Notes.

All melting points were measured with a Gallenkamp Melting Point Apparatus and are uncorrected. Infra-red spectra were obtained on a Philips analytical PU9800 FTIR spectrometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrometer operating at 90 MHz (δ_H) or with a Bruker WP-200 SY spectrometer operating at 200 MHz (δ_H) or 50 MHz (δ_C). Low resolution mass spectra were determined with a VG updated A.E.I. MS spectrometer and high resolution mass spectra were determined with a VG updated MS 902 spectrometer.

Analytical TLC was carried out on Kieselgel 60 F_{254} plastic sheets of 0.25 mm thickness. Spots were viewed under a UV lamp and were developed by iodine vapour. Column chromatography was carried out using 70-230 mesh silica gel.

All solvents and reagents were of analytical grade unless otherwise stated. Aqueous solutions were freeze-dried on a Christ Alpha 1-4 freeze drier. Organic solvents were dried with magnesium sulfate and evaporated on a Buchi rotary evaporator under water-pump vacuum with slight heating. Dichloromethane was distilled from calcium hydride; ethanol was distilled from magnesium turnings and iodine; triethylamine was distilled from potassium hydroxide; tetrahydrofuran was distilled from sodium pieces and benzophenone; dimethylformamide was distilled from silica gel; and acetonitrile was dried with calcium hydride.

7.2 Experimental to Chapter 3.

General Procedure [1] - Ozonolysis of Protected Allylglycine.

A solution of diprotected allylglycine (1.0 mmol) in dichloromethane (15 ml) was flushed with ozone at -78 °C until a pale blue colour persisted for 10 min. Excess ozone was removed by flushing the reaction flask with nitrogen. The ozonide was decomposed with triethylamine (2.0 mmol) at -78 °C with stirring and allowed to warm to room temperature overnight. Dichloromethane was removed *in vacuo* and ether was added to precipitate triethylamine *N*-oxide. The mixture was filtered and ether was removed *in vacuo* to give a gluey oil. Purification was achieved on a silica gel column eluting with ether to give the aldehyde.

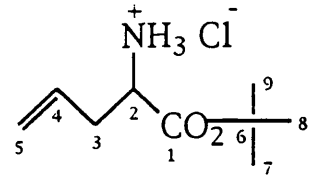
General Procedure [2] - Deprotection of Aspartic Acid βSemialdehyde Analogues using Trifluoroacetic Acid.

Trifluoroacetic acid (2 ml) was added to a solution of the aspartic acid β-semialdehyde analogue (1.0 mmol) in dichloromethane (2 ml) under nitrogen atmosphere. The mixture was stirred for 1 h and removal of the solvents *in vacuo* gave a yellow residue. Trituration with ether precipitated a light yellow solid. The solid was filtered and dried under vacuum to give the desired product.

General Procedure [3] - Imine Derivatisation of Protected Aspartic Acid β-Semialdehyde.

To a solution of the hydrochloride salt of the amine (1 equiv.) and triethylamine (1 equiv.) in dichloromethane (8 ml) under a nitrogen atmosphere was added dropwise a solution of **(101)** (1.0 mmol). The mixture was stirred at room temperature for 12 h. Removal of the solvent *in vacuo* left an oily residue. Ether (10 ml) was added with stirring to precipitate triethylamine hydrochloride. The mixture was filtered and the solvent was removed *in vacuo*. Purification was achieved where necessary on a neutral alumina column eluting with the appropriate solvent system.

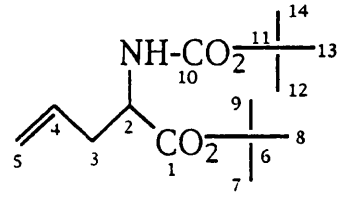
DL-Allylglycine *t*-Butyl Ester Hydrochloride (99).[102]



2-Methylpropene (100 ml) was added to a stirred suspension of DL-allylglycine (4.77 g, 41.5 mmol) in dichloromethane (100 ml) at -78 °C. Conc. sulfuric acid (5 ml) was added dropwise over 10 min. The mixture was stirred at -78 °C for 15 min then allowed to warm to room temperature and stirred for 18 h with a dry ice condenser fitted. The resulting clear solution was carefully basified to pH 8 with sodium bicarbonate solution. The organic layer was separated, washed with brine solution (2 x 50 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo* to give a yellow oil. A white precipitate

resulted on addition of a solution of dry HCl gas in ether (20 ml). The white solid was filtered and dried under vacuum, 2.72 g (32% yield), mp 115-118 °C; ν_{max} (KBr disc) 2980, 2880, 1735, 1570 and 1500 cm^{-1}; δ_H (200 MHz) (D_2O) 1.32 (9H, s, 7-, 8- and 9-H_3), 2.54 (2H, dd, 3-H_2), 3.94 (1H, dd, 2-H), 5.15 (2H, m, 5-H_2) and 5.60 (1H, m, 4-H); δ_C (50 MHz) 28.3 (C-7, 8 and 9), 35.4 (C-3), 53.8 (C-2), 86.9 (C-6), 122.7 (C-5), 131.2 (C-4) and 169.6 (C-1); *m/z* 130, 70 (100%), 57, 43 and 41 (Found: M^+, 172.1332; C, 52.08; H, 8.78; N, 6.73. $C_9H_{18}NO_2$ requires *M*, 172.1338; C, 52.05; H, 8.67; N, 6.75%).

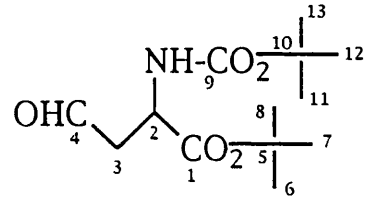
DL-*N*-*t*-Butoxycarbonylallylglycine *t*-Butyl Ester (100).[106]



To a stirred suspension of **(99)** (3.00 g, 14.4 mmol) in dichloromethane (40 ml) was added sodium bicarbonate (1.21 g, 1 equiv.) in water (15 ml), sodium chloride (2.5 g) and di-*t*-butyldicarbonate (3.32 ml, 1 equiv.). The mixture was heated at reflux for 1.5 h then cooled to room temperature and the organic layer was separated. The aqueous layer was extracted with dichloromethane (2 x 60 ml) and the combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil, 3.84 g (98% yield), R_F 0.53 (25% EtOAc/hexane); ν_{max} ($CHCl_3$) 3425, 2980, 1710 and 1495 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.41 (18H, br s, 7-, 8-, 9-, 12-, 13- and 14-H_3), 2.45 (2H, m, 3-H_2), 4.20 (1H, m, 2-H), 5.06 (3H, m, 5-H_2 and NH) and 5.67 (1H, m, 4-H); δ_C (50 MHz) 27.9 (C-7, 8

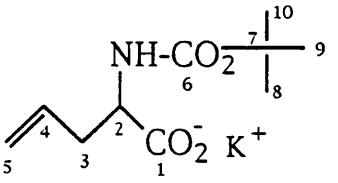
and 9), 28.3 (C-12, 13 and 14), 36.8 (C-3), 53.2 (C-2), 79.5 and 81.7 (C-6 and 11), 118.7 (C-5), 132.5 (C-4), 155.1 (C-10) and 171.1 (C-1); *m/z* 230, 170, 130, 114, 70, 57 (100%) and 41 (Found: C, 61.95; H, 9.01; N, 5.25. $C_{14}H_{25}NO_4$ requires C, 61.91; H, 9.21; N, 5.16%).

DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester (101).



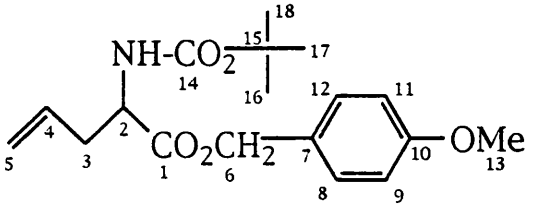
A solution of **(100)** (2.30g, 8.48 mmol) in dichloromethane (80 ml) was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to give a light yellow oil, 1.65 g (71% yield), R_F 0.63 (ether); ν_{max} ($CHCl_3$) 3425, 2980, 1710 and 1495 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.44 (18H, br s, 6-, 7-, 8-, 11-, 12- and 13-H_3), 2.98 (2H, m, 3-H_2), 4.48 (1H, m, 2-H), 5.40 (1H, br d, NH) and 9.74 (1H, s, 4-H); δ_C (50 MHz) 27.9 (C-6, 7 and 8), 28.3 (C-11, 12 and 13), 36.8 (C-3), 46.3 (C-2), 80.0 and 82.6 (C-5 and 10), 155.3 (C-9), 169.9 (C-1) and 199.4 (C-4); *m/z* 172, 118, 72, 57 (100%) and 41 (Found: C, 56.95; H, 8.32; N, 5.20. $C_{13}H_{23}NO_5$ requires C, 57.07; H, 8.41; N, 5.12%).

Potassium Salt of DL-*N*-*t*-Butoxycarbonylallylglycine (104).[107]



To a solution of DL-allylglycine (5.00 g, 43.4 mmol) in water (10 ml) was added dioxan (10 ml), potassium bicarbonate (4.78 g, 1.1 equiv.) and di-*t*-butyl dicarbonate (9.98 ml, 1.0 equiv.) with continuous stirring at room temperature for 18 h. The solvents were removed *in vacuo* to give a white solid, 9.11 g (83% yield); ν_{max} (KBr disc) 3360, 2985, 1675, 1590 and 1530 cm^{-1}; δ_H (200 MHz) (D_2O) 1.27 (9H, s, 8-, 9- and 10-H_3), 2.25 (2H, m, 3-H_2), 2.77 (1H, m, 2-H), 4.98 (2H, m, 5-H_2) and 5.61 (1H, m, 4-H); δ_C (50 MHz) 28.6 (C-8, 9- and 10), 37.3 (C-3), 56.6 (C-2), 81.9 (C-7), 118.9 (C-5), 134.8 (C-4), 166.5 (C-6) and 180.2 (C-1); *m/z* 214 (M^+, 0.3%), 112, 59 (100%) and 41.

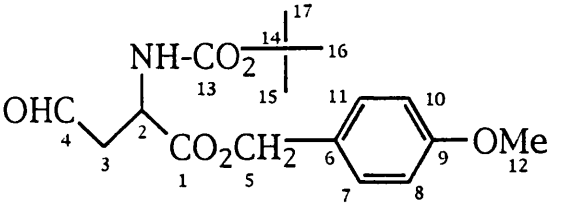
DL-*N*-*t*-Butoxycarbonylallylglycine *p*-Methoxybenzyl Ester (105).[107]



To a solution of **(104)** (8.10 g, 31.9 mmol) in DMF (20 ml) was added 4-methoxybenzyl chloride (4.64 ml, 1.0 equiv.) with continuous stirring for 48 h. DMF was removed with xylene *in vacuo*

and the resultant residue was partitioned between dichloromethane (50 ml) and aqueous sodium bicarbonate solution (60 ml). The organic portion was washed with water (4 x 80 ml), dried ($MgSO_4$) and the solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 60% ether in hexane to give a clear oil, 10.2 g (95% yield), R_F 0.57 (50% Et_2O/hexane); ν_{max} ($CHCl_3$) 3440, 3020, 2985, 1720, 1710, 1620, 1515 and 1500 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.43 (9H, s, 16-, 17- and 18-H_3), 2.50 (2H, dd, 3-H_2), 3.79 (3H, s, 13-H_3), 4.39 (1H, m, 2-H), 5.10 (5H, m, 5-H_2, 6-H_2 and NH), 5.67 (1H, m, 4-H), 6.89 (2H, d, *J* 8 Hz, 9- and 11-H) and 7.30 (2H, d, *J* 8 Hz, 8- and 12-H); δ_C (50 MHz) 28.3 (C-16, 17 and 18), 36.7 (C-3), 53.0 (C-2), 55.2 (C-13), 66.9 (C-6), 79.7 (C-15), 113.7 (C-7), 113.9 (C-8 and 12), 119.1 (C-5), 127.4 (C-11), 130.2 (C-9 and 11), 132.2 (C-4), 159.9 (C-14) and 171.9 (C-1); *m/z* 335 (M^+, 1.0%), 279, 170, 121 (100%), 70 and 57 (Found: M^+, 335.1720. $C_{18}H_{25}NO_5$ requires *M*, 335.1730).

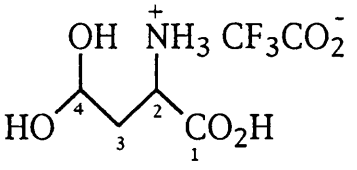
DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *p*-Methoxybenzyl Ester (106).



A solution of **(105)** (9.98 g, 29.75 mmol) in dichloromethane (60 ml) was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to

give a light orange oil, 9.24 g (92% yield), R_F 0.80 (Et_2O); ν_{max} ($CHCl_3$) 3430, 3030, 2980, 1735, 1690, 1620 and 1500 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.42 (9H, s, 15-, 16- and 17-H_3), 3.04 (2H, t, *J* 4.8 Hz, 3-H_2), 3.81 (3H, s, 12-H_3), 4.60 (1H, br m, 2-H), 5.11 (2H, s, 5-H_2), 5.42 (1H, br d, NH), 6.89 (2H, d, *J* 8 Hz, 8- and 10-H), 7.27 (2H, d, *J* 8 Hz, 7- and 11-H) and 9.70 (1H, s, 4-H); δ_C (50 MHz) 28.2 (C-15, 16 and 17), 46.0 (C-3), 48.8 (C-2), 55.2 (C-12), 67.4 (C-5), 80.2 (C-14), 113.9 (C-7 and 11), 115.1 (C-6), 128.7 (C-9), 130.2 (C-8 and 10), 160.0 (C-13), 171.2 (C-1) and 199.5 (C-4); *m/z* 337 (M^+, 0.5%), 281, 202, 137, 121 (100%), 72 and 57.

Trifluoroacetate Salt of DL-Aspartic Acid β-Semialdehyde Hydrate (102).[93]



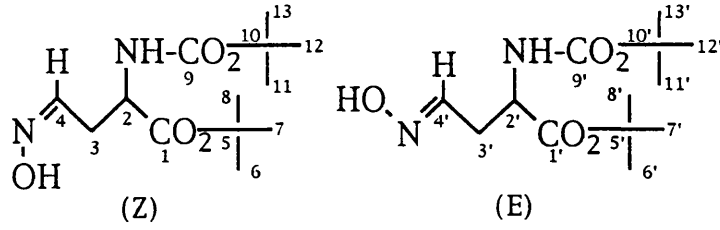
Method 1

Compound **(101)** (400 mg, 1.83 mmol) was deprotected with trifluoroacetic acid (3 ml) according to general procedure [2] to give a pale yellow solid, 336 mg (79% yield), mp 63-65 °C (lit.[93], 63-65 °C); ν_{max} (KBr disc) 3420 (broad), 2925, 1675 and 1645 cm^{-1}; δ_H (200 MHz) (D_2O) 1.98 (2H, m, 3-H_2), 3.82 (1H, dd, 2-H) and 5.10 (1H, t, 4-H); δ_C (50 MHz) 37.8 (C-3), 51.9 (C-2), 89.2 (C-4) and 173.9 (C-1); *m/z* 137 (MH^+, 12.1%), 120, 92, 69, 44 (100%) and 43 (Found: MH^+, 137.0445. $C_4H_{11}NO_4$ requires M, 137.0450).

Method 2

A solution of **(106)** (750 mg, 2.22 mmol) in trifluoroacetic acid (2 ml) and dichloromethane (2 ml) was stirred at room temperature under nitrogen atmosphere for 2 h. The solvent was removed *in vacuo* to give an oily residue. This was partitioned between water (5 ml) and ethyl acetate (5 ml). The aqueous layer was separated and washed with ethyl acetate (3 x 5 ml). Removal of the solvent *in vacuo* gave a yellow solid, 264 mg (48% yield) with identical spectral data to those obtained for compound **(102)** made by Method 1 above.

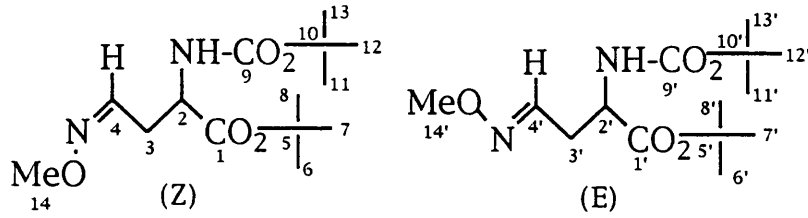
Z- (110a) and *E*-DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester Oxime (110b).



(Z) (E)

A solution of **(101)** (660 mg, 1.96 mmol) in dichloromethane (5ml) was added to a solution of hydroxylamine hydrochloride (136 mg, 1 equiv.) in dichloromethane (3 ml) according to general procedure [3] to give a clear oil. Purification was achieved on a silica gel column eluting with 60% ether in hexane to afford a clear oil, 389 mg (59% yield) R_F 0.65 (50% EtOAc/hexane); ν_{max} ($CHCl_3$) 3590, 3430, 3020, 2980, 1720, 1690 and 1500 cm^{-1}; ^1H NMR and ^{13}C NMR analysis showed that both the geometric isomers of the oxime were present in the ratio (*Z*) 2 : (*E*) 1; δ_H (200 MHz) ($CDCl_3$) 1.45, 1.46, 1.47 and 1.52 (4 x s, 6-, 6'-, 7-, 7'-, 8-, 8'-, 11-, 11'-12-, 12'-, 13- and 13'-H_3), 2.70 and 2.82 (2 x t, 3- and 3'-H_2), 4.36- 4.46 (m, 2- and 2'-H), 5.42 and 5.60 (2 x br d, 2 x NH), 6.81 and 7.40 (2 x t, 4- and 4'-H) and 9.05 (br s, 2 x N-OH); δ_C (50 MHz) 27.8, 28.2 and 28.5 (C-6, 6', 7, 7', 8, 8', 11, 11', 12, 12', 13 and 13'), 33.0 (C-3 and 3'), 51.5 and 51.8 (C-2 and 2'), 80.0 and 82.5 (C-5, 5', 10 and 10'), 147.2 (C-4 and 4'), 170.9 and 171.2 (C-1, 1', 9 and 9'); *m/z* 230, 187, 172, 159, 131, 87 and 57 (100%).

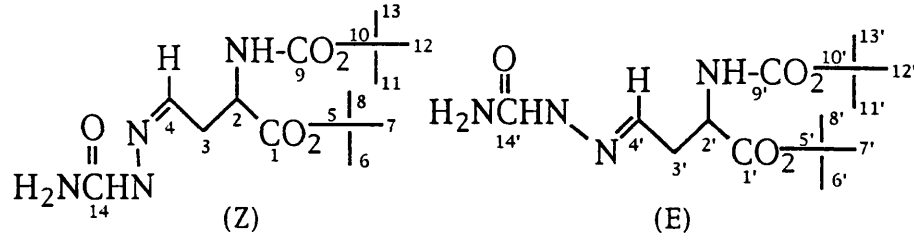
Z- (111a) and *E*-DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester Methyloxime (111b).



(Z) (E)

A solution of **(101)** (300 mg, 1.10 mmol) in dichloromethane (2 ml) was added to a solution of methoxylamine hydrochloride (101 mg, 1.1 equiv.) in water (2 ml). The two-phase system was stirred at room temperature overnight. The reaction mixture was extracted with dichloromethane (3 x 5 ml) and the combined organic extracts were dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a yellow oil, 279 mg (84% yield), R_F 0.85 and 0.67 (10% EtOAc/Et_2O); ^1H NMR and ^{13}C NMR analysis showed that both the geometric isomers of the methyloxime had been formed in a 1:1 ratio; δ_H (200 MHz) ($CDCl_3$) 1.45 (s, 6-, 6'-, 7-, 7'-, 8-, 8'-, 11-, 11'-, 12-, 12'-, 13- and 13'-H_3), 2.68 (2 x m, 3 and 3'-H_2), 3.81 and 3.88 (2 x s, 14- and 14'-H_3), 4.36 (m, 2 and 2'-H), 5.43 (br, 2 x $NHCO_2$), 6.71 and 7.33 (2 x t, 4- and 4'-H); δ_C (50 MHz) 27.9 and 28.3 (C-6, 6', 7, 7', 8, 8', 11, 11', 12, 12', 13 and 13'), 33.0 (C-3 and 3'), 51.5 (C-2 and 2'), 61.5 and 61.7 (C-14 and 14'), 77.2 and 82.4 (C-5, 5', 10 and 10'), 146.2 and 146.5 (C-4 and 4'), 155.2 (C-9 and 9') and 171.1 (C-1 and 1'); *m/z* 302 (M^+, 0.2%), 201, 100, 85 and 57 (100%).

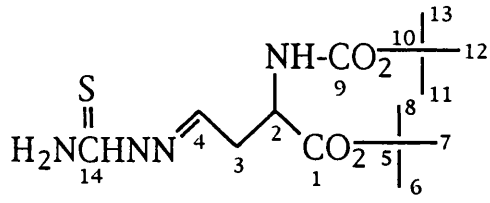
Z- (112a) and *E*-DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester Semicarbazone (112b).



(Z) (E)

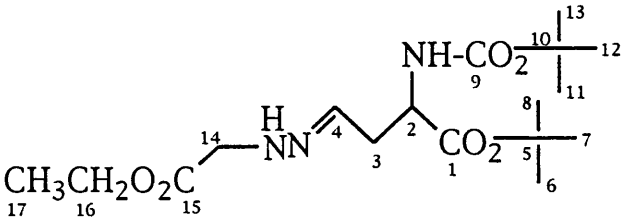
A solution of **(101)** (350 mg, 1.28 mmol) in dichloromethane (5 ml) was added to a solution of semicarbazide hydrochloride (143 mg, 1 equiv.) and triethylamine (0.18 ml, 1 equiv.) in dichloromethane (2 ml) according to general procedure [3]. Removal of the solvent *in vacuo* gave a white solid, 379 mg (90% yield), mp 57-60 °C, R_F 0.57 (10% MeOH/Et_2O); ν_{max} ($CHCl_3$) 3520, 3400, 1725, 1690 and 1150 cm^{-1}; ^1H NMR and ^{13}C NMR analysis showed that both the geometric isomers of the semicarbazone had been formed in a 1:2 ratio; δ_H (200 MHz) ($CDCl_3$) 1.44 (s, 6-, 6'-, 7-, 7'-, 8-, 8'-, 11-, 11'-, 12-, 12'-, 13- and 13'-H_3), 2.72 (m, 3- and 3'-H_2), 4.47 (m, 2- and 2'-H), 5.43 and 5.74 (2 x br d, 2 x NH), 5.74 (br, -$CONH_2$), 6.53 (t, *J* 5.6 Hz, 4-H), 7.11 (t, *J* 3.8 Hz, 4'-H), 9.42 and 10.00 (2 x br s, 2 x -CONHN); δ_C (50 MHz) 27.9 and 28.3 (C-6, 6', 7, 7', 8, 8', 11, 11', 12, 12', 13 and 13'), 35.3 (C-3 and 3'), 51.0 (C-2 and 2'), 79.9 and 82 3 (C-5, 5', 10 and 10'), 139.7 (C-4 and 4'), 155.3 (C-9 and 9'), 158.3 (C-14 and 14') and 171.3 (C-1 and 1'); *m/z* 330 (M^+, 0.3%), 229, 218 (100%), 201, 159, 112 and 57 (Found: M^+, 330.1876. $C_{14}H_{26}N_4O_5$ requires *M*, 330.1903).

DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester Thiosemicarbazone (113).



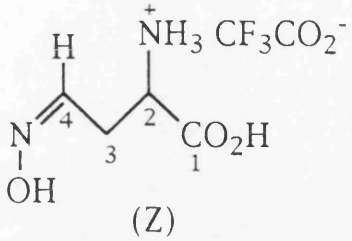
To a solution of thiosemicarbazide (78 mg, 0.86 mmol) in water (3 ml) was added a solution of the diprotected aldehyde (**101**) (236 mg, 0.86 mmol) in dichloromethane (5 ml). The two-phase system was stirred rapidly at room temperature for 48 h. The mixture was extracted with dichloromethane (2 x 8 ml) and the combined organic solvents were dried ($MgSO_4$). Removal of solvent *in vacuo* gave a white solid. Purification was achieved on a silica gel column eluting with diethyl ether to give a white solid, 256 mg (86% yield), mp 65-68 °C, R_F 0.55 (Et_2O); ν_{max} (KBr disc) 3435, 3170, 2980, 1710, 1520 and 1155 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.44 (18H, s, 6-, 7-, 8-, 11-, 12- and 13-H_3), 2.78 (2H, m, 3-H_2), 4.52 (1H, m, 2-H), 5.30 (1H, br d, -$NHCO_2$), 6.38 (br s, -$CSNH_2$), 7.30 (1H, t, *J* 3.6 Hz, 4-H) and 10.13 (br s, -CONHN); δ_C (50 MHz) 28.0 and 28.3 (C-6, 7, 8, 11, 12 and 13), 35.5 (C-3), 50.7 (C-2), 80.2 and 82.7 (C-5 and 10), 142.4 (C-4), 155.3 (C-9), 171.3 (C-1) and 178.5 (C-14); *m/z* 346 (M^+, 10.6%), 290, 216, 189, 144 and 57 (100%) (Found: M^+, 346.1681; C, 48.39; H, 7.56; N, 16.17. $C_{14}H_{26}N_4O_4S$ requires *M*, 346.175; C, 48.54; H, 7.56; N, 16.17%).

DL-*N*-*t*-Butoxycarbonylaspartic Acid β-Semialdehyde *t*-Butyl Ester Ethyl Hydrazinoacetate (114).

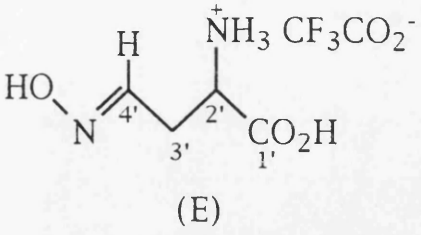


A solution of **(101)** (200 mg, 0.73 mmol) in dichloromethane (5 ml) was added to a solution of ethyl hydrazinoacetate hydrochloride (113 mg, 1 equiv.) and triethylamine (1 equiv.) in dichloromethane (2 ml) according to general procedure [3]. Removal of the solvent *in vacuo* gave a yellow oil. Purification was achieved on neutral alumina column eluting with 60% ethyl acetate in hexane to give a pale solid, 178 mg (65% yield), R_F 0.83 (60% EtOAc/hexane); ν_{max} ($CHCl_3$) 3410, 1730, 1710 and 1150 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.25 (3H, t, *J* 7.1 Hz 17-H_3), 1.42 (18H, s, 6-, 7-, 8-, 11-, 12- and 13-H_3), 2.63 (2H, m, 3-H_2), 3.82 (2H, s, 14-H_2), 4.19 (2H, q, *J* 7.1 Hz, 16-H_2), 4.40 (1H, m, 2-H), 5.25 (1H, br, -$NHCO_2$) and 6.97 (1H, t, 4-H); δ_C (50 MHz) 14.2 (C-17), 27.9 and 28.3 (C-6, 7, 8, 11, 12 and 13), 35.1 (C-3), 50.8 (C-14), 52.0 (C-2), 61.0 (C-16), 79.6 and 81.9 (C-5 and 10), 138.6 (C-4), 155.7 (C-9), 170.7 and 171.3 (C-1 and 15); *m/z* 373 (M^+, 2.7%), 316, 272, 200, 155 (100%) and 127 (Found: M^+, 373.2195. $C_{17}H_{31}N_3O_6$ requires *M*, 373.2203).

Trifluoroacetate Salt of *Z*- (115a) and *E*-DL-Aspartic Acid β-Semialdehyde Oxime (115b).



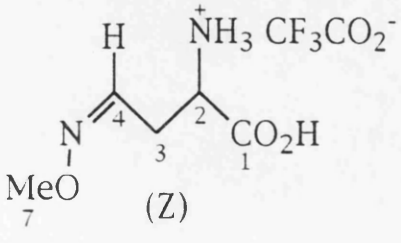
(Z)



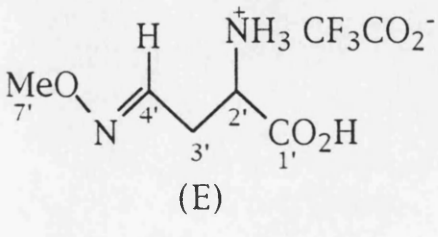
(E)

A mixture of **(110a)** and **(110b)** (208 mg, 0.59 mmol) was deprotected with trifluoroacetic acid according to general procedure [2] to give a pale syrup, 128 mg (65% yield); ν_{max} (thin film) 3350, 2980, 2520, 1730, 1670 and 1430 cm^{-1}; δ_H (200 MHz) (D_2O) 2.75 (br m, 3- and 3'-H_2), 4.05 (br m, 2- and 2'-H), 6.80 and 7.34 (2 x t, 4- and 4'-H); δ_C (50 MHz) 30.4 and 34.3 (C-3 and 3'), 51.3 (C-2 and 2'), 147.8 and 148.0 (C-4 and 4'), 172.3 and 174.1 (C-1 and 1'); *m/z* 132 (M^+, 0.9%), 116, 98, 87, 69 and 57 (100%) (Found: M^+, 132.0541. $C_4H_8N_2O_3$ requires *M*, 132.0534).

Trifluoroacetate Salt of *Z*- (116a) and *E*-DL-Aspartic Acid β-Semialdehyde Methyloxime (116b).



(Z)

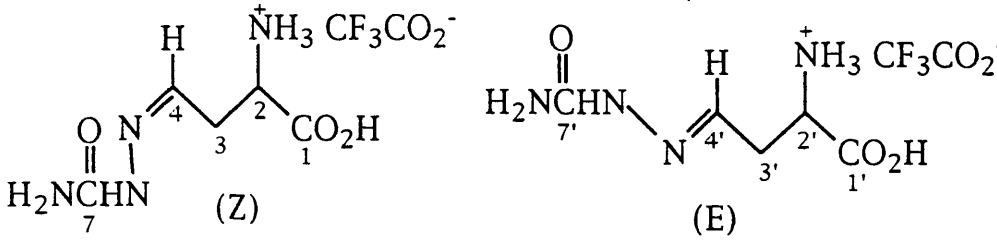


(E)

A mixture of **(116a)** and **(116b)** (150 mg, 0.50 mmol) was deprotected with trifluoroacetic acid according to general procedure

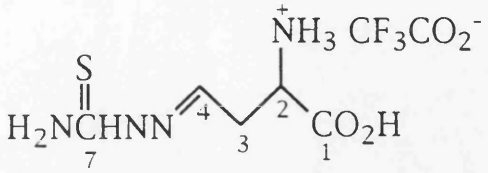
[2] to give a yellow solid, 62 mg (48% yield); ν_{max} (KBr disc) 2980, 2530, 1730, and 1430 cm^{-1}; δ_H (200 MHz) (D_2O) 2.68 (br m, 3- and 3'-H_2), 3.65 (br s, 7- and 7'-H_3), 3.81 (br m, 2- and 2'-H), 6.76 and 7.34 (2 x br m, 4- and 4'-H); δ_C (50 MHz) 37.6 (C-3 and 3'), 51.8 and 52.2 (C-2 and 2') and 62.2 (C-7 and 7').

Trifluoroacetate Salt of *Z*- (117a) and *E*-DL-Aspartic Acid β-Semialdehyde Semicarbazone (117b).



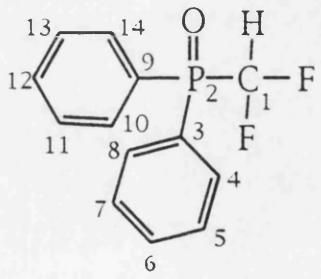
A mixture of **(112a)** and **(112b)** (159 mg, 0.48 mmol) was deprotected with trifluoroacetic acid according to general procedure [2] to give a pale green solid, 90 mg (65% yield), mp 57-59 °C; ν_{max} (KBr disc) 3455, 3000-2500 (broad), 2985, 1730, 1670, 1590, 1530 and 1205 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 2.76 (2H, br m, 3- and 3'-H_2), 4.14 (1H, br t, 2- and 2'-H) and 7.20 (br m, 4- and 4'-H); δ_C (50 MHz) 32.7 (C-3 and 3'), 51.1 (C-2 and 2'), 142.0 (C-4 and 4') and 169.5 (C-1 and 1'); *m/z* 128, 114, 111, 69, 57 and 44.

Trifluoroacetate Salt of DL-Aspartic Acid β-Semialdehyde Thiosemicarbazone (118).



Compound **(113)** (129mg, 037 mmol) was deprotected with trifluoroacetic acid according to general procedure [2] to give a pale green solid, 89 mg (79% yield), mp 65-70 °C; ν_{max} (KBr disc) 3425, 3175, 3000-2500 (broad), 1730, 1675, 1610, 1530 and 1200 cm^{-1}; δ_H (200 MHz) (D_2O) 2.87 (2H, br m, 3-H_2), 4.12 (1H, t, *J* 5.3 Hz, 2-H) and 7.27 (1H, br t, 4-H); δ_C (50 MHz) 32.8 (C-3), 51.0 (C-2), 145.3 (C-4) and 173.2 (C-1); *m/z* 144, 129, 114 and 57.

Difluoromethyldiphenylphosphine Oxide (119).[115]



To a solution of diphenylphosphine oxide (4.50 g, 22.3 mmol) in THF (100 ml) at -50 °C was added *n*-butyl lithium (15.3 ml, 1.1 equiv.). After 15 min, chlorodifluoromethane (approx. 100 ml) was added and the mixture was stirred at -50 °C for 2 h followed by 10 h at room temperature with a dry ice condenser in place. The reaction

mixture was concentrated *in vacuo* and the residue was taken up in dichloromethane (50 ml) and washed with water (2 x 50 ml). The organic portion was concentrated *in vacuo* to give a brown solid. Purification was achieved on a silica gel flash column eluting with 33% ethyl acetate in toluene to give white crystals, 2.70 g (48% yield), mp 92-94 °C (lit.[115], 93-94 °C), R_F 0.52 (33% EtOAc/toluene); ν_{max} (KBr disc) 3030, 2960, 1590, 1440, 1210 and 1040 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 6.35 (1H, dt, $^2J_{HF}$ 50.0 Hz and $^2J_{PH}$ 22.4 Hz, 1-H), 7.60 (6H, m, 5-, 6-, 7-, 11-, 12- and 13-H) and 7.89 (4H, dd, $^3J_{HH}$ 6.6 Hz and $^3J_{PCCH}$ 11.8 Hz, 4-, 8-, 10- and 14-H); δ_C (50 MHz) 116.0 (C-1), 127.0 (C-3 and 9), 129.8 (C-4,8,10 and 14), 132.8 (C-5, 7, 11 and 13) and 134.3 (C-6 and 12); δ_F (188 MHz) -132.6 (dd, $^2J_{HF}$ 48.9 Hz and $^2J_{PF}$ 69.8 Hz); *m/z* 252 (M^+, 0.6%), 201 (100%) and 77 (Found: M^+, 252.0506; C, 61.95; H, 4.38. $C_{13}H_{11}F_2OP$ requires *M*, 252.0516; C, 61.90; H, 4.36%)

7.3 Experimental to Chapter 4.

General Procedure [1] - Alkylation of Malonate Derivatives.[120]

A solution of the bromoalkene (49.6 mmol) in distilled ethanol (10 ml) was added dropwise to a solution of sodium (50.0 mmol) and the malonate derivative (44.6 mmol) heated to reflux in distilled ethanol (40 ml). The mixture was heated at reflux for 1 h, cooled to room temperature and the brown supernatant layer was decanted from the small amount of sediment. The solvent was removed *in vacuo* to give a brown residue. This was taken up in dichloromethane and washed with water and brine, dried ($MgSO_4$)

and the solvent was removed *in vacuo*. Purification was achieved by recrystallisation or column chromatography when necessary.

General Procedure [2] - Hydrolysis and Decarboxylation of Alkylated Malonate Derivatives.

A mixture of the alkylated malonate derivative (6.4 mmol) in ethanol (15 ml) and 10% aqueous sodium hydroxide (15 ml) was heated at reflux overnight. The reaction mixture was cooled to room temperature and solvent was removed *in vacuo*. The residue was taken up in water (15 ml), acidified with conc. hydrochloric acid (Congo red) and the mixture was heated at reflux for 3 h. The reaction was cooled and carefully neutralised with aqueous sodium bicarbonate solution (pH 7). The solvent was removed *in vacuo*, the residue was dissolved in 25% aqueous sodium hydroxide and the solution was heated at reflux overnight. After cooling to room temperature, the reaction mixture was diluted with water and acidified with conc. hydrochloric acid. Removal of the solvent *in vacuo* gave a brown solid. Purification was achieved by taking the brown solid up in hot ethanol and filtering through celite. Removal of the solvent *in vacuo* gave the hydrochloride salt of the amino acid.

General Procedure [3] - Alkylation of *N*-(Diphenylmethylene)aminoacetonitrile (145).[126]

To a solution of *N*-(diphenylmethylene)aminoacetonitrile (145) (9.1 mmol) in toluene (8 ml) was added 50% aqueous sodium hydroxide solution (5 ml). The resulting two-phase system was stirred vigorously. Upon addition of benzyltriethylammonium chloride (0.8 mmol) the reaction became deep red in colour. The bromoalkene (10.9 mmol) was added dropwise over 1 h at 0 °C with stirring. The reaction mixture was stirred at 0 °C for an additional 1 h then heated gently at 40 °C overnight. The reaction mixture was poured into a separatory funnel containing dichloromethane (15 ml) and water (20 ml). The aqueous portion was extracted with dichloromethane (3 x 15 ml) and the combined organic portions were washed with water (3 x 20 ml) and brine (1 x 20 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a dark red oil.

General Procedure [4] - Removal of Dibenzylidene Protecting Group.

To a solution of the protected amine (7 mmol) in diethyl ether (15 ml) was added 2M aqueous hydrochloric acid solution (15 ml). The two-phase system was stirred vigorously for 4 h. The aqueous layer was washed with diethyl ether (20 ml) and the organic fractions were discarded. The aqueous layer was carefully basified with saturated sodium bicarbonate solution and was extracted with ethyl acetate (3 x 25 ml). The combined organic fractions were washed with brine (1 x 25 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield an orange oil.

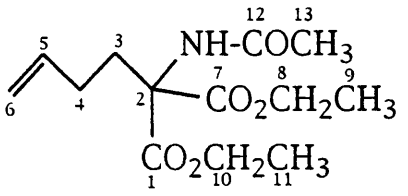
General Procedure [5] - Alkylation of Schollkopf-Hartwig Reagents.[128]

To a stirred solution of the Schollkopf-Hartwig reagent (1 equiv.) in dry THF (20 ml) at -70 °C under an atmosphere of nitrogen was added n-butyllithium (1.1 equiv.). The mixture was stirred for 10 min at this temperature and then treated dropwise with a solution of the bromoalkene (1 equiv.) in THF (8 ml). After 4 h at -70 °C the mixture was allowed to warm to room temperature and was left stirring overnight. The solvent was removed *in vacuo* and the residue was taken up in ether (20 ml) and extracted with water (3 x 20 ml). The ethereal portion was dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to yield an orange oil. Purification was achieved where necessary on a silica gel column eluting with 25% diethyl ether in hexane.

General Procedure [6] - Acid Hydrolysis of Alkylated Schollkopf-Hartwig Reagents.[128]

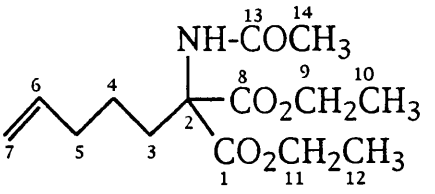
A suspension of the alkylated Schollkopf-Hartwig reagent (10 mmol) in 1.0 M HCl (50 ml) was stirred for 16 h at room temperature. The mixture was extracted once with diethyl ether, which was rejected. The aqueous layer was concentrated *in vacuo* and the residue was dissolved in a small amount of water, covered with ether and vigorously shaken with conc. ammonia solution until pH 8-10. The aqueous portion was extracted with ether (2 x 20 ml) and the combined organic portions were dried ($MgSO_4$). Ths solvent was removed *in vacuo* to yield a pale oil.

Diethyl 3-Butenylacetamidomalonate (136).



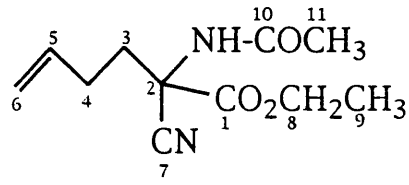
A solution of 4-bromobut-1-ene (6.00 g, 44.44 mmol) in distilled ethanol (10 ml) was added dropwise to a solution of sodium (1.23 g, 1.2 equiv.) and diethyl acetamidomalonate (9.65 g, 1 equiv.) heated to reflux in ethanol (50 ml) according to general procedure [1] to give an orange oil. Purification was achieved on a silica gel column eluting with 40% ethyl acetate in hexane to give a pale solid, 4.46 g (37% yield), mp 47-48 °C, R_F 0.30 (50% EtOAc/hexane); ν_{max} ($CHCl_3$) 3410, 2980, 1735, 1675 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.26 (6H, t, *J* 7.12 Hz, 9- and 11-H_3), 1.92 (2H, m, 3-H_2), 2.04 (3H, s, 13-H_3), 2.45 (2H, m, 4-H_2), 4.24 (4H, q, *J* 7.12 Hz, 8- and 10-H_2), 4.98 (2H, m, 6-H_2), 5.73 (1H, m, 5-H) and 6.78 (1H, br s, N-H); δ_C (50 MHz) 13.9 (C-9 and 11), 23.1 (C-13), 27.9 and 31.2 (C-3 and 4), 62.5 (C-8 and 10), 66.2 (C-2), 115.4 (C-6), 136.9 (C-5), 167.0 (C-12) and 168.1 (C-1 and 7); *m/z* 271 (M^+, 4.3%), 226, 198, 156 (100%) and 82 (Found: M^+, 271.1417; C, 57.51; H, 7.77; N, 5.11. $C_{13}H_{21}NO_5$ requires *M*, 271.1420; C, 57.55; H, 7.80; N, 5.16%).

Diethyl 4-Pentenylacetamidomalonate (137).



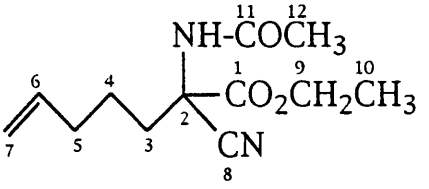
A solution of 5-bromopent-1-ene (6.00 g, 40.26 mmol) in distilled ethanol (10 ml) was added dropwise to a solution of sodium (1.04 g, 1.1 equiv.) and diethyl acetamidomalonate (8.75 g, 1 equiv.) heated to reflux in ethanol (50 ml) according to general procedure [1] to give a yellow oil. Purification was achieved on a silica gel column eluting with 50% ethyl acetate in hexane to give a white solid, 5.07 g (44% yield), mp 42-45 °C, R_F 0.40 (50% EtOAc/hexane); ν_{max} ($CHCl_3$) 3410, 2980, 1735, 1675 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.26 (8H, t and m, *J* 7.12 Hz, 10-, 12-H_2 and 4-H_2), 2.04 (5H, m and s, 3-H_2 and 14-H_3), 2.32 (2H, m, 5-H_2), 4.24 (4H, q, *J* 7.12 Hz, 9- and 11-H_2), 4.97 (2H, m, 7-H_2), 5.72 (1H, m, 6-H) and 6.83 (1H, br s, N-H); δ_C (50 MHz) 14.0 (C-10 and 12), 23.1 (C-4), 31.7 and 33.3 (C-3 and 5), 62.5 (C-9 and 11), 66.5 (C-2), 115.0 (C-7), 137.9 (C-6), 168.2 and 169.0 (C-1, 8 and 13); *m/z* 285 (M^+, 7.6%), 212 (100%), 170 and 96 (Found: *M* $^+$, 285.1575; C, 58.89; H, 7.98; N, 4.90. $C_{14}H_{23}NO_5$ requires *M*, 285.1577; C, 58.95; H, 8.07; N, 4.91%).

Ethyl 3-Butenylacetamidocyanoacetate (139).



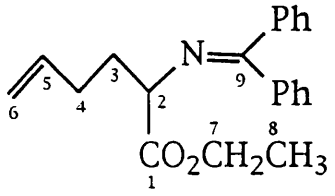
A solution of 4-bromobut-1-ene (5.00 g, 37.03 mmol) in distilled ethanol (10 ml) was added dropwise to a solution of sodium (0.85 g, 1.3 equiv.) and ethyl acetamidocyanoacetate (4.85 g, 1.0 equiv.) heated to reflux in ethanol (50 ml) according to general procedure [1] to give a brown residue. Recrystallisation from ethanol/water gave white needles, 2.31 g (36% yield), mp 98-101 °C, R_F 0.54 (EtOAc); ν_{max} ($CDCl_3$) 3440, 3400, 2980, 1750, 1690 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.36 (3H, t, *J* 7.12 Hz, 9-H_3), 2.07 (3H, s, 11-H_3), 2.24 (4H, m, 3- and 4-H_2), 4.32 (2H, q, *J* 7.12 Hz, 8-H_2), 5.11 (2H, m, 6-H_2), 5.74 (1H, m, 5-H) and 6.96 (1H, br s, N-H); δ_C (50 MHz) 13.9 (C-9), 22.3 (C-11), 28.0 (C-3), 35.0 (C-4), 56.9 (C-2), 63.7 (C-8), 116.4 (C-7), 116.9 (C-6), 135.1 (C-5), 166.2 and 170.2 (C-1 and 10); *m/z* 224 (M^+, 0.2%), 179, 109 (100%) and 82 (Found: M^+, 224.1164; C, 58.79; H, 7.23; N, 12.47. $C_{11}H_{16}N_2O_3$ requires *M*, 224.1161; C, 58.91; H, 7.19; N, 12.49%).

Ethyl 4-Pentenylacetamidocyanoacetate (140).



A solution of 5-bromopent-1-ene (5.73 g, 38.46 mmol) in distilled ethanol (10 ml) was added dropwise to a solution of sodium (0.96 g, 1.1 equiv.) and ethyl acetamidocyanoacetate (5.95 g, 34.96 mmol) heated to reflux in ethanol (50 ml) according to general procedure [1] to give a brown oil. Recrystallisation from ethanol/water gave white needles, 1.59 g (20% yield), mp 43-45 °C, R_F 0.22 (Et_2O); ν_{max} ($CHCl_3$) 3440, 3400, 1750, 1690, 1645 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.39 (3H, t, *J* 7.12 Hz, 10-H_2), 1.5-2.2 (6H, m, 3-, 4- and 5-H_2), 2.07 (3H, s, 12-H_3), 4.33 (2H, q, *J* 7.12 Hz, 9-H_2), 5.06 (2H, m, 7-H_2), 5.75 (1H, m, 6-H) and 6.72 (1H, br s, N-H); δ_C (50 MHz) 14.0 (C-10), 22.4 (C-12), 23.0 (C-4), 32.7 and 35.5 (C-3 and 5), 57.1 (C-2), 63.7 (C-9), 116.1 (C-7), 116.5 (C-8), 136.8 (C-6), 166.4 and 170.1 (C-1 and 11); *m/z* 238 (M^+, 0.7%), 195, 165 and 123 (100%) (Found: M^+, 238.1318; C, 60.63; H, 7.52; N, 11.68. $C_{12}H_{18}N_2O_3$ requires *M*, 238.1317; C, 60.50; H, 7.56; N, 11.76%).

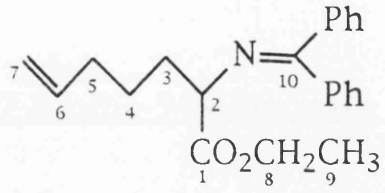
Ethyl *N*-(Diphenylmethylene)-2-amino-5-pentenoate (141).



n-Butyllithium (2.5 M solution in hexanes) (13.17 ml, 32.9 mmol) was added dropwise to a solution of diisopropylamine (5.0 ml, 1.1 equiv.) in dry tetrahydrofuran (150 ml) at -20 °C under a nitrogen atmosphere. The pale orange solution was then stirred at 0 °C for 20 min. After cooling to -70 °C, a solution of *N*-(diphenylmethylene)-glycine ethyl ester (8.0 g, 29.9 mmol) in tetrahydrofuran (20 ml) was added dropwise over 10 min. The reaction became deep red in colour and was stirred at -70 °C for a further 20 min. before dropwise addition of 4-bromobut-1-ene (3.65 ml, 35.9 mmol). The reaction was allowed to warm to room temperature and was then heated gently at 50 °C for 16 h. Upon cooling the reaction mixture was poured into water (100 ml) and extracted with ethyl acetate (3 x 100 ml). The combined organic fractions were washed with water (1 x 100 ml), saturated brine solution (1 x 100 ml), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to give a dark red oil, 7.82 g (81% yield), R_F 0.65 (50% EtOAc/hexane); ν_{max} (thin film) 3025, 2980, 1735, 1660, 1600, 1580 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.26 (3H, t, *J* 7.14 Hz, 8-H_3), 2.02 (4H, m, 3- and 4-H_2), 4.06 (2H, t, 2-H), 4.16 (2H, q, *J* 7.18 Hz, 7-H_2), 4.94 (2H, m, 6-H_2), 5.74 (1H, m, 5-H) and 7.15-7.83 (aromatic protons); δ_C (50 MHz) 14.2 (C-8), 30.2 and 33.0 (C-3 and 4), 60.9 (C-7), 64.9 (C-2), 115.0 (C-6), 128.1, 128.6, 130.2 and 136.4 (aromatic carbons), 137.8 (C-5), 170.6 (C-9) and 172.3 (C-1); *m/z* 320 (M^+-H,

99%), 292, 248 and 193 (Found: M^+-H, 320.1638. $C_{21}H_{23}NO_2$ requires M-H, 320.1651).

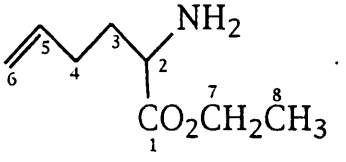
Ethyl *N*-(Diphenylmethylene)-2-amino-6-hexenoate (142).



n-Butyllithium (2.5 M solution in hexanes) (13.17 ml, 32.9 mmol) was added dropwise to a solution of diisopropylamine (5.0 ml, 1.1 equiv.) in dry tetrahydrofuran (150 ml) at -20 °C under nitrogen atmosphere. The pale orange solution was then stirred at 0 °C for 20 min. After cooling to -70 °C, a solution of *N*-(diphenylmethylene)-glycine ethyl ester (8.0 g, 29.9 mmol) in tetrahydrofuran (20 ml) was added dropwise over 10 min. The reaction became deep red in colour and was stirred at -70 °C for a further 20 min. before dropwise addition of 5-bromopent-1-ene (3.9 ml, 32.9 mmol). The reaction was allowed to warm to room temperature and was then heated gently at 50 °C for 16 h. Upon cooling the reaction mixture was poured into water (100 ml) and extracted with ethyl acetate (3 x 100 ml). The combined organic fractions were washed with water (1 x 100 ml), saturated brine solution (1 x 100 ml), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to give a dark red oil, 8.91 g (72% yield), R_F 0.64 (50% EtOAc/hexane); ν_{max} (thin film) 3025, 2980, 1735, 1660, 1600, 1580 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.27 (3H, t, *J* 7.11 Hz, 9-H_3), 1.38 (2H, m, 4-H_2), 1.91 (4H, m, 3- and 5-H_2), 4.01 (1H, t, 2-H), 4.12 (2H, q, *J* 7.12 Hz, 8-H_2), 4.90 (2H, m, 7-H_2), 5.76

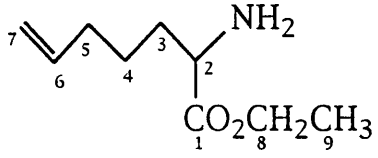
(1H, m, 6-H) and 7.15-7.83 (aromatic protons); δ_C (50 MHz) 14.2 (C-9), 25.3 (C-4), 33.1 and 33.4 (C-3 and 5), 60.8 (C-8), 65.4 (C-2), 114.6 (C-7), 128.0, 128.6, 130.1 and 136.5 (aromatic carbons), 138.5 (C-6), 170.3 (C-10) and 172.4 (C-1); *m/z* 334 (M^+, 41%), 262 (100%), 193 and 165 (Found: M^+-H, 334.1804. $C_{22}H_{25}NO_2$ requires *M*-H, 334.1807).

Ethyl 2-Amino-5-pentenoate (143).



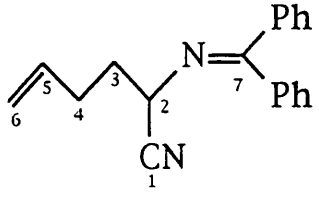
A solution of **(141)** (7.5 g, 22.0 mmol) in diethyl ether (70 ml) was stirred vigorously with 2M HCl (70 ml) according to general procedure [4] to give an orange oil, 2.3 g (67% yield); ν_{max} ($CHCl_3$) 3390, 3335, 3080, 2980, 1730 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.29 (3H, t, *J* 7.11 Hz, 8-H_3), 1.76 (4H, br m, 3-H_2 and NH_2), 2.15 (2H, m, 4-H_2), 3.44 (1H, m, 2-H), 4.14 (2H, q, *J* 7.11 Hz, 7-H_2), 5.05 (2H, m, 6-H_2) and 5.81 (1H, m, 5-H); δ_C (50 MHz) 14.3 (C-8), 29.9 and 34.1 (C-3 and 4), 53.9 (C-2), 60.9 (C-7), 115.4 (C-6), 137.6 (C-5) and 176.1 (C-1); *m/z* 157 (M^+, 1.2%), 112, 84 (100%) and 67.

Ethyl 2-Amino-6-hexenoate (144).



A solution of **(142)** (8.26 g, 24.62 mmol) in diethyl ether (100 ml) was stirred vigorously with 2M HCl (100 ml) according to general procedure [4] to give a dark orange oil, 2.64 g (63% yield); ν_{max} ($CHCl_3$) 3400, 3330, 3080, 2980, 1740 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.28 (3H, t, *J* 7.12 Hz, 9-H_3), 1.41-1.79 (6H, m, 3- and 4-H_2 and NH_2), 2.10 (2H, m, 5-H_2), 3.43 (1H, br, 2-H), 4.17 (2H, q, *J* 7.12 Hz, 8-H_2), 4.97 (2H, m, 7-H_2) and 5.78 (1H, m, 6-H); δ_C (50 MHz) 14.3 (C-9), 24.7 (C-4), 33.4 and 34.4 (C-3 and 5), 54.4 (C-2), 114.7 (C-6) and 138.3 (C-7); *m/z* 171 (M^+, 1.1%), 126, 98 (100%) and 81.

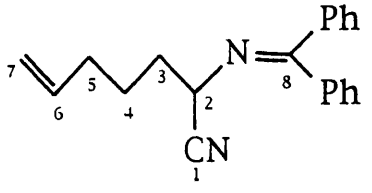
N-(Diphenylmethylene)-2-amino-5-pentenyl Cyanide (146).



To a vigorously stirred two-phase mixture of *N*-(diphenylmethylene)aminoacetonitrile (2.0 g, 9.1 mmol), benzyltriethylammonium chloride (0.18 g), 50% sodium hydroxide solution (10 ml) and toluene (10 ml) at 0 °C was added 4-bromobut-1-ene (1.11 ml, 10.9 mmol) according to general procedure [3] to give a dark red oil, 2.26 g (91% yield), R_F 0.60 (50% EtOAc/hexane); ν_{max} ($CHCl_3$)

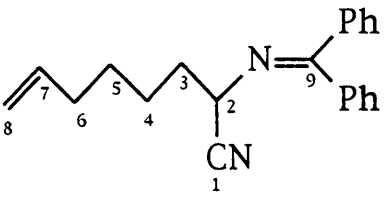
3080, 3010, 2980, 1660, 1610, 1580 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 2.01 (2H, m, 3-H_2), 2.16 (2H, m, 4-H_2), 4.23 (1H, t, 2-H), 4.97 (2H, m, 6-H_2), 5.69 (1H, m, 5-H) and 7.10-7.52 (aromatic protons); δ_C (50 MHz) 29.6 and 34.0 (C-3 and 4), 52.34 (C-2), 116.0 (C-6), 119.6 (C-1), 127.4, 128.2, 128.9 and 135.2 (aromatic carbons), 136.3 (C-5) and 173.1 (C-7); *m/z* 273 (M^+-H, 100%), 219, 180, 165, 116 and 77 (Found: M^+-H, 273.1365. $C_{19}H_{18}N_2$ requires *M*-H, 273.1392).

N-(Diphenylmethylene)-2-amino-6-heptenonitrile (147).



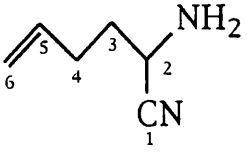
To a vigorously stirred two-phase mixture of *N*-(diphenylmethylene)aminoacetonitrile (6.0 g, 27.2 mmol), benzyltriethylammonium chloride (0.55 g), 50% sodium hydroxide solution (20 ml) and toluene (20 ml) at 0 °C was added 5-bromopent-1-ene (3.87 ml, 32.7 mmol) according to general procedure [3] to give a dark red oil, 7.56 g (96% yield), R_F 0.66 (50% EtOAc/hexane); ν_{max} ($CHCl_3$) 3080, 3010, 2980, 1660, 1620, 1600, 1580 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.53 (2H, m, 4-H_2), 2.01 (4H, m, 3- and 5-H_2), 4.23 (1H, t, 2-H), 5.03 (2H, m, 7-H_2), 5.71 (1H, m, 6-H), 7.10 and 7.79 (aromatic protons); δ_C (50 MHz) 24.7 (C-4), 32.9 and 34.2 (C-3 and 5), 52.9 (C-2), 115.3 (C-7), 119.7 (C-1), 128.2, 129.3 129.7 and 138.4 (aromatic carbons) and 137.6 (C-6); *m/z* 287 (M^+-H, 83%), 219, 208, 180, 165, 116 (100%), and 77 (Found: M^+-H, 287.1531. $C_{20}H_{23}N_2$ requires *M*-H, 287.1549).

N-(Diphenylmethylene)-2-amino-7-octenonitrile (148).



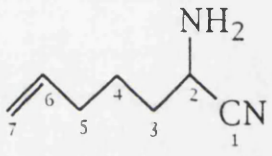
To a vigorously stirred two-phase mixture of *N*-(diphenylmethylene)aminoacetonitrile (6.0 g, 27.2 mmol), benzyltriethylammonium chloride (0.55 g), 50% sodium hydroxide solution (10 ml) and toluene (10 ml) at 0 °C was added 6-bromohex-1-ene (4.38 ml, 32.7 mmol) according to general procedure [3] to give a dark red oil, 8.15 g (99% yield), R_F 0.68 (50% EtAOc/hexane); ν_{max} ($CHCl_3$) 3080, 3010, 2980, 1660, 1620, 1600. 1580 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.39 (4H, m, 4- and 5-H_2), 1.92 (4H, m, 3- and 6-H_2), 4.21 (1H, t, 2-H), 4.99 (2H, m, 8-H_2), 5.77 (1H, m, 7-H) and 7.17-7.67 (aromatic protons) ; δ_C (50 MHz) 24.9 and 28.1 (C-4 and 5), 33.4 and 34.7 (C-3 and 6), 53.0 (C-2), 114.7 (C-8), 119.7 (C-1), 127.4, 128.2, 129.0 and 135.2 (aromatic carbons), 138.3 (C-7) and 172.7 (C-9) ; *m/z* 301 (M^+-H, 36%), 219, 208, 180, 165 116 (100%), and 77 (Found: M^+-H, 301.1710. $C_{21}H_{22}N_2$ requires *M*-H, 301.1704).

2-Amino-5-hexenonitrile (149).



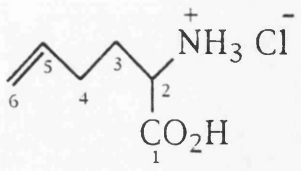
A solution of **(146)** (5.00 g, 18.22 mmol) in diethyl ether (25 ml) was stirred vigorously with 2M HCl (25 ml) according to general procedure [4] to give an orange oil, 1.38 g (69% yield); ν_{max} ($CHCl_3$) 3390, 3340, 3080, 2980, 2230 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.66 (2H, br, NH_2), 1.85 (2H, m, 3-H_2), 2.29 (2H, m, 4-H_2), 3.69 (1H, br m, 2-H), 5.10 (2H, m, 6-H_2) and 5.76 (1H, m, 5-H); δ_C (50 MHz) 29.6 and 34.3 (C-3 and 4), 42.7 (C-2), 116.5 (C-6), 122.1 (C-1) and 136.2 (C-5); *m/z* 110 (M^+, 11%), 84 and 68.

2-Amino-6-heptenonitrile (150).



A solution of **(147)** (11.5 g, 39.9 mmol) in diethyl ether (40 ml) was stirred vigorously with 2M HCl (40 ml) according to general procedure [4] to give an orange oil, 3.32 g (67% yield); ν_{max} ($CHCl_3$) 3390, 3340, 3080, 2980, 2230 and 1640 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.57 (2H, m, 4-H_2), 1.69 (2H, br s, NH_2), 1.85 (4H, m, 3- and 5-H_2), 3.69 (1H, br m, 2-H), 5.12 (2H, m, 7-H_2) and 5.78 (1H, m, 6-H); δ_C (50 MHz) 24.5 (C-4), 29.6 and 34.3 (C-3 and 5), 42.7 (C-2), 116.5 (C-7), 122.1 (C-1) and 136.2 (C-6); *m/z* 124 (M^+, 1.1%), 98, 82 (100%) and 68.

2-Amino-5-hexenoic Acid Hydrochloride (126).



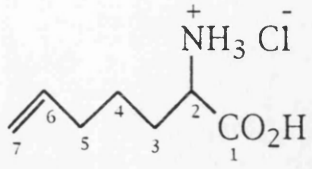
Method 1

A mixture of compound **(149)** (2.00 g, 18.16 mmol) and 10% sodium hydroxide solution (20 ml) was heated at reflux overnight. The reaction mixture was cooled, carefully acidified with conc. hydrochloric acid and washed with ethyl acetate (3 x 20 ml). The aqueous layer was freezed dried to give a pale solid, 4.06 g (135% yield-due to sodium chloride impurity); ν_{max} (KBr disc) 3000-2500 (broad), 1735, 1655, 1490 and 960 cm^{-1}; δ_H (200 MHz) (D_2O) 1.8-2.2 (4H, m, 3- and 4-H_2), 3.75 (1H, br t, 2-H), 5.08 (2H, m, 6-H_2), 5.82 (1H, m, 5-H); δ_C (50 MHz) 29.9 and 30.9 (C-3 and 4), 55.5 (C-2), 117.4 (C-6), 138.3 (C-5) and 175.9 (C-1); *m/z* 130 (M^+, 0.1%), 84 (100%) and 36/38 (3:1) (Found: M^+, 130.0844. $C_6H_{12}NO_2$ requires *M*, 130.0868).

Method 2

2-Amino-5-hexenoic acid hydrochloride (126) was prepared from compound **(136)** (1.73 g, 6.38 mmol) according to general procedure [2] as a pale solid, 1.01 g (95% yield). This compound had identical spectral data to those quoted for the material synthesised by Method 1 above.

2-Amino-6-heptenoic Acid Hydrochloride (127).



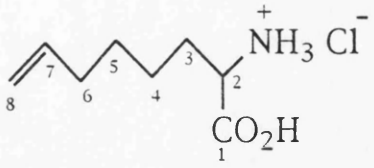
Method 1

A mixture of compound **(150)** (2.80 g, 22.55 mmol) and 10% sodium hydroxide solution (25 ml) was heated at reflux overnight. The reaction mixture was cooled, carefully acidified with conc. hydrochloric acid and washed with ethyl acetate (3 x 25 ml). The aqueous layer was freezed dried to give a pale solid, 5.59 g (138% yield-due to sodium chloride impurity); ν_{max} (KBr disc) 3420, 3200-2500 (broad), 1730, 1640, 1510 and 950 cm^{-1}; δ_H (200 MHz) (D_2O) 1.28 (2H, m, 4-H_2), 1.7-2.0 (4H, m, 3- and 5-H_2), 3.83 (1H, br t, 2-H), 4.82 (2H, m, 7-H_2) and 5.65 (1H, m, 6-H); δ_C (50 MHz) 24.1, 30.0 and 33.1 (C-3, 4 and 5), 53.8 (C-2), 116.1 (C-7), 139.1 (C-6) and 173.4 (C-1); *m/z* 144 (M^+, 0.3%), 98, 56 (100%) and 36/38 (3:1) (Found: M^+, 144.1027. $C_7H_{14}NO_2$ requires *M*, 144.1025).

Method 2

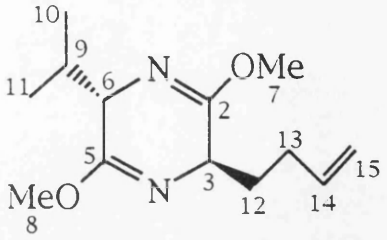
2-Amino-6-hexenoic acid hydrochloride (127) was prepared from compound **(137)** (2.03 g, 7.11 mmol) according to general procedure [2] as a pale solid, 1.10 g (86% yield). This compound had identical spectral data to those quoted for the material synthesised by Method 1 above.

2-Amino-7-octenoic Acid Hydrochloride (152).



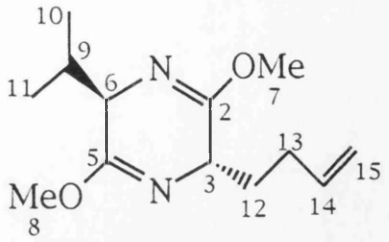
A mixture of compound **(151)** (2.10 g, 15.19 mmol) and 10% sodium hydroxide solution (20 ml) was heated at reflux overnight. The reaction mixture was cooled, carefully acidified with conc. hydrochloric acid and washed with ethyl acetate (3 x 20 ml). The aqueous layer was freezed dried to give a pale solid, 4.12 g (140% yield-due to sodium chloride impurity); ν_{max} (KBr disc) 3420, 3200-2500 (broad), 1735, 1640 and 1500 cm^{-1}; δ_H (200 MHz) (D_2O) 1.38 (4H, m, 4- and 5-H_2), 1.8-2.0 (4H, m, 3- and 6-H_2), 3.92 (1H, br t, 2-H), 4.97 (2H, m, 8-H_2) and 5.83 (1H, m, 7-H); δ_C (50 MHz) 24.6, 28.5, 30.7 and 33.5 (C-3, 4, 5 and 6), 54.6 (C-2), 115.6 (C-8), 140.6 (C-7) and 174.4 (C-1); *m/z* 158 (M^+, 0.1%), 112 (100%) and 36/38 (3:1).

(3*R*, 6*S*)-3-But-3-enyl-3,6-dihydro-6-isopropyl-2,5-dimethoxypyrazine (158).



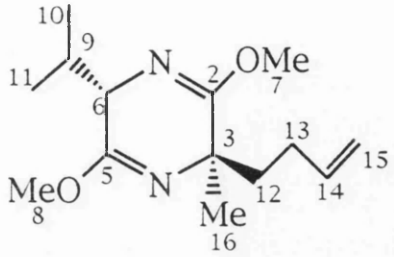
A solution of (2*S*)-(+)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (1.5 ml, 8.37 mmol) in THF (20 ml) was treated sequentially with n-butyllithium (3.68 ml of a 2.5 M solution in hexane, 9.21 mmol) and a solution of 4-bromobut-1-ene (0.94 ml, 9.21 mmol) in THF (8 ml) according to general procedure [5]. The resultant orange oil was purified on a silica gel column eluting with 25% ether in hexane to give a colourless oil, 1.55 g (78% yield), 84% de value by GC analysis. R_F 0.75 (EtOAc); ν_{max} (thin film) 3080, 2960, 2870, 1695, 1640, 1240 and 1015 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 0.68 (3H, d, *J* 6.8 Hz, 10-H_3), 1.06 (3H, d, *J* 6.9 Hz, 11-H_3), 1.76-2.07 (4H, m, 12- and 13-H_2), 2.26 (1H, m, 9-H), 3.67 and 3.70 (6H, 2 x s, 7- and 8-H_3), 3.94 (1H, t, *J* 3.4 Hz, 3-H), 4.04 (1H, m, 6-H), 4.97 (2H, m, 15-H_2) and 5.81 (1H, m, 14-H); δ_C (50 MHz) 16.6 and 19.1 (C-10 and 11), 28.9 (C-12), 31.7 (C-9), 33.4 (C-13), 52.4 (C-7 and 8), 54.9 (C-3), 60.8 (C-6),114.5 (C-15), 138.4 (C-14), 163.6 and 163.7 (C-2 and 5); *m/z* 238 (M^+, 7.8%), 223, 195, 183, 153 and 141 (100%). (Found: M^+, 238.1695. $C_{13}H_{22}N_2O_2$ requires *M*, 238.1709).

(3*S*, 6*R*)-3-But-3-enyl-3,6-dihydro-6-isopropyl-2,5-dimethoxypyrazine (160).



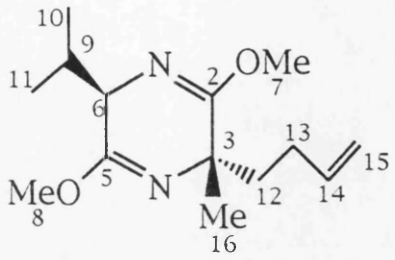
A solution of (2*R*)-(-)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (1.5 ml, 8.37 mmol) in THF (20 ml) was treated sequentially with n-butyllithium (3.68 ml of a 2.5 M solution in hexane, 9.21 mmol) and a solution of 4-bromobut-1-ene (0.85 ml, 8.37 mmol) in THF (8 ml) according to general procedure [5]. The resultant orange oil was purified on a silica gel column eluting with 25 % diethyl ether in hexane to give a clear oil, 1.58 g (79% yield), 85% de by GC analysis. R_F 0.77 (EtOAc); ν_{max} (thin film) 3075, 2960, 1695, 1640, 1235 and 1015 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 0.67 (3H, d, *J* 6.8 Hz, 10-H_3), 1.04 (3H, d, *J* 6.9 Hz, 11-H_3), 1.76-2.07 (4H, m, 12- and 13-H_2), 2.26 (1H, m, 9-H), 3.67 and 3.70 (6H, 2 x s, 7- and 8-H_3), 3.93 (1H, t, *J* 3.4 Hz, 3-H), 4.03 (1H, m, 6-H), 4.97 (2H, m, 15-H_2) and 5.80 (1H, m, 14-H); δ_C (50 MHz) 16.6 and 19.1 (C-10 and 11), 28.9 (C-12), 31.7 (C-9), 33.4 (C-13), 52.4 (C-7 and 8), 54.9 (C-3), 60.8 (C-6), 114.5 (C-15), 138.4 (C-14), 163.6 and 163.7 (C-2 and 5); *m/z* 238 (M^+, 6.5%), 223, 195 (100%), 183, 153 and 141. (Found: M^+, 238.1673. $C_{13}H_{22}N_2O_2$ requires *M*, 238.1681).

(3*R*, 6*S*)-3-But-3-enyl-3,6-dihydro-6-isopropyl-2,5-dimethoxy-3-methylpyrazine (162).



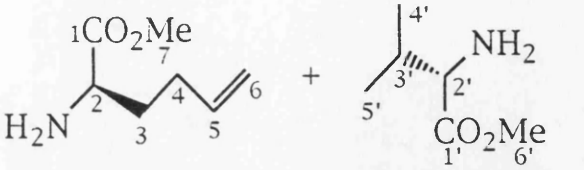
A solution of (2*S*, 5*SR*)-(+)-2,5-dihydro-3,6-dimethoxy-2-isopropyl-5-methylpyrazine (0.82 ml, 4.14 mmol) in THF (20 ml) was treated sequentially with n-butyllithium (2.84 ml of a 1.6 M solution in hexane, 4.55 mmol) and a solution of 4-bromobut-1-ene (0.42 ml, 4.14 mmol) in THF (5 ml) according to general procedure [5] to give a pale oil, 0.99 g (95% yield). R_F 0.76 (50% EtOAc/hexane); ν_{max} (thin film) 3080, 2970, 2840, 1690, 1640, 1240 and 1005 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 0.68 (3H, d, *J* 6.8 Hz, 10-H_3), 1.06 (3H, d, *J* 6.9 Hz, 11-H_3), 1.32 (3H, s, 16-H_3), 1.56-1.94 (4H, m, 12- and 13-H_2), 2.25 (1H, m, 9-H), 3.66 (6H, br s, 7- and 8-H_3), 3.92 (1H, d, *J* 3.4 Hz, 6-H), 4.91 (2H, m, 15-H_2) and 5.74 (1H, m, 14-H); δ_C (50 MHz) 16.9 and 19.4 (C-10 and 11), 28.7 (C-16), 29.1 (C-12), 31.0 (C-9), 40.5 (C-13), 52.1 (C-7 and 8), 58.2 (C-3), 61.2 (C-6), 114.1 (C-15), 138.6 (C-14), 162.0 and 165.2 (C-2 and 5); *m/z* 252 (M^+, 2.8%), 237, 209, 197, 195 and 155 (100%). (Found: M^+, 252.1829. $C_{14}H_{24}N_2O_2$ requires *M*, 252.1835).

(3*S*, 6*R*)-3-But-3-enyl-3,6-dihydro-6-isopropyl-2,5-dimethoxy-3-methylpyrazine (164).



A solution of (2*R*, 5*SR*)-(-)-2,5-dihydro-3,6-dimethoxy-2-isopropyl-5-methylpyrazine (2.5 ml, 12.61 mmol) in THF (40 ml) was treated sequentially with n-butyllithium (5.55 ml of a 2.5 M solution in hexane, 13.87 mmol) and a solution of 4-bromobut-1-ene (1.28 ml, 12.61 mmol) in THF (8 ml) according to general procedure [5] to give a pale oil, 3.15 g (99% yield). R_F 0.71 (50% Et_2O/hexane); ν_{max} (thin film) 3080, 2970, 2840, 1690, 1640, 1240 and 1005 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 0.68 (3H, d, *J* 6.8 Hz, 10-H_3), 1.06 (3H, d, *J* 6.9 Hz, 11-H_3), 1.32 (3H, s, 16-H_3), 1.56-1.94 (4H, m, 12- and 13-H_2), 2.25 (1H, m, 9-H), 3.66 (6H, br s, 7- and 8-H_3), 3.92 (1H, d, *J* 3.4 Hz, 6-H), 4.91 (2H, m, 15-H_2) and 5.74 (1H, m, 14-H); δ_C (50 MHz) 16.8 and 19.3 (C-10 and 11), 28.6 (C-16), 29.0 (C-12), 30.9 (C-9), 40.4 (C-13), 52.1 (C-7 and 8), 58.1 (C-3), 61.1 (C-6), 114.0 (C-15), 138.5 (C-14), 161.9 and 165.1 (C-2 and 5); *m/z* 252 (M^+, 2.3%), 237, 209, 197, 195 and 155 (100%). (Found: M^+, 252.1825. $C_{14}H_{24}N_2O_2$ requires *M*, 252.1837).

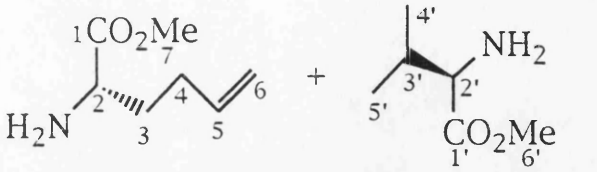
Mixture of Methyl (2*R*)-2-Amino-5-hexenoate (159) and Methyl L-Valinate.



(159) Methyl L-Valinate

A suspension of **(158)** (720 mg, 3.02 mmol) in 1.0 M HCl (20 ml) was stirred for 16 h at room temperature according to general procedure [6] to yield a light yellow oil, 325 mg. ^{1}H and ^{13}C NMR spectra indicated this to be a 1:1 mixture of the (*R*)-amino acid methyl ester (159) and methyl L-valinate. δ_H (200 MHz) ($CDCl_3$) 0.88 and 0.95 (6H, 2 x d, *J* 6.8 Hz and 6.8 Hz, 4'- and 5'-H_3), 1.48 (4H, br s, 2 x NH_2), 1.56-2.21 (5H, m, 3- and 4-H_2 and 3'-H), 3.27 (1H, d, *J* 5.0 Hz, 2'-H), 3.44 (1H, dd, *J* 7.8 Hz and 7.8 Hz, 2-H), 3.70 (6H, s, 7-H_3 and 6'-H_3), 5.00 (2H, m, 6-H_2) and 5.77 (1H, m, 5-H); δ_C (50 MHz) 17.2 and 19.3 (C-4' and 5'), 29.9 (C-3), 32.2 (C-3'), 34.1 (C-4), 51.7 and 51.9 (C-6' and C-7), 53.9 (C-2), 60.0 (C-2'), 115.4 (C-6), 137.6 (C-5), 176.1 and 176.5 (C-1 and C-1').

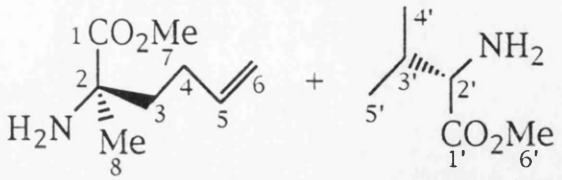
Mixture of Methyl (2*S*)-2-Amino-5-hexenoate (161) and Methyl D-Valinate.



(161) Methyl D-Valinate

A suspension of **(160)** (500 mg, 2.10 mmol) in 1.0 M HCl (20 ml) was stirred for 16 h at room temperature according to general procedure [6] to yield a light yellow oil, 205 mg. ^{1}H and ^{13}C NMR spectra indicated this to be a 1:1 mixture of the (*S*)-amino acid methyl ester (161) and methyl D-valinate. δ_H (200 MHz) ($CDCl_3$) 0.89 and 0.94 (6H, 2 x d, *J* 6.8 Hz and 6.8 Hz, 4'- and 5'-H_3), 1.49 (4H, br s, 2 x NH_2), 1.55-2.20 (5H, m, 3- and 4-H_2 and 3'-H), 3.27 (1H, d, *J* 5.0 Hz, 2'-H), 3.45 (1H, dd, *J* 7.8 Hz and 7.8 Hz, 2-H), 3.72 (6H, s, 7-H_3 and 6'-H_3), 5.00 (2H, m, 6-H_2) and 5.76 (1H, m, 5-H); δ_C (50 MHz) 17.2 and 19.3 (C-4' and 5'), 29.9 (C-3), 32.2 (C-3'), 34.1 (C-4), 51.7 and 51.9 (C-6' and C-7), 53.9 (C-2), 60.1 (C-2'), 115.4 (C-6), 137.6 (C-5), 176.2 and 176.5 (C-1 and C-1').

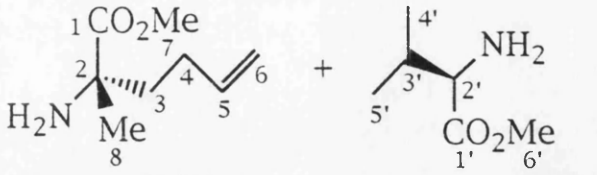
Mixture of Methyl (2*R*)-2-Amino-2-methyl-5-hexenoate (163) and Methyl L-Valinate.



(163) Methyl L-Valinate

A suspension of **(162)** (816 mg, 3.23 mmol) in 1.0 M HCl (30 ml) was stirred for 16 h at room temperature according to general procedure [6] to yield a light yellow oil, 646 mg. ^{1}H and ^{13}C NMR spectra indicated this to be a 1:1 mixture of the (*R*)-amino acid methyl ester (163) and methyl L-valinate. δ_H (200 MHz) ($CDCl_3$) 0.84 and 0.90 (6H, 2 x d, *J* 7.0 Hz and 6.9 Hz, 4'- and 5'-H_3), 1.34 (3H, s, 8-H_3), 1.58 (4H, br s, 2 x NH_2), 1.63-2.10 (5H, m, 3- and 4-H_2 and 3'-H), 3.30 (1H, d, *J* 5.0 Hz, 2'-H), 3.72 (6H, br s, 7-H_3 and 6'-H_3), 4.98 (2H, m, 6-H_2) and 5.77 (1H, m, 5-H); δ_C (50 MHz) 17.2 and 19.3 (C-4' and 5'), 26.5 (C-8), 28.5 (C-3), 32.2 (C-3'), 40.1 (C-4), 51.7 and 52.8 (C-7 and C-6'), 57.6 (C-2), 59.9 (C-2'), 114.9 (C-6), 137.9 (C-5), 176.1 and 178.0 (C-1 and C-1').

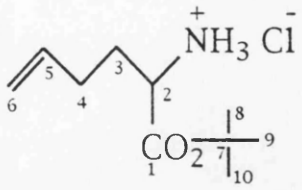
Mixture of Methyl (2*S*)-2-amino-2-methyl-5-hexenoate (165) and Methyl D-Valinate.



(165) Methyl D-Valinate

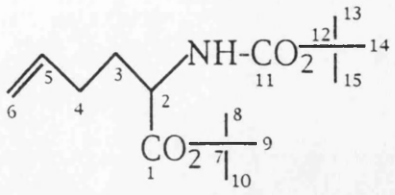
A suspension of **(164)** (2.50 g, 9.91 mmol) in 1.0 M HCl (60 ml) was stirred for 16 h at room temperature according to general procedure [6] to yield a colourless oil, 2.10 g. ^{1}H and ^{13}C NMR spectra indicated this to be a 1:1 mixture of the (*S*)-amino acid methyl ester (165) and methyl D-valinate. δ_H (200 MHz) ($CDCl_3$) 0.90 and 0.97 (6H, 2 x d, *J* 6.8 Hz and 6.9 Hz, 4'- and 5'-H_3), 1.33 (3H, s, 8-H_3), 1.64 (4H, br s, 2 x NH_2), 1.67-2.10 (5H, m, 3- and 4-H_2 and 3'-H), 3.29 (1H, d, *J* 5.0 Hz, 2'-H), 3.72 (6H, br s, 7-H_3 and 6'-H_3), 4.98 (2H, m, 6-H_2) and 5.77 (1H, m, 5-H); δ_C (50 MHz) 17.0 and 19.1 (C-4' and 5'), 26.2 (C-8), 28.5 (C-3), 32.0 (C-3'), 39.3 (C-4), 51.5 and 52.0 (C-7 and C-6'), 57.4 (C-2), 59.7 (C-2'), 114.7 (C-6), 137.7 (C-5), 175.8 and 177.8 (C-1 and C-1').

t-Butyl DL-2-Amino-5-hexenoate Hydrochloride (166).



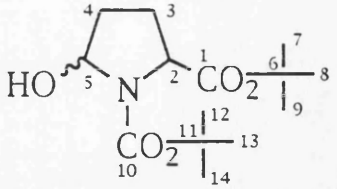
2-Methylpropene (excess) was added to a stirred suspension of **(126)** (0.60 g, 3.62 mmol) in dichloromethane (40 ml) at -78 °C. Conc. sulfuric acid (1.5 ml) was added dropwise over 10 min. The mixture was stirred at -78 °C for 15 min then allowed to warm to room temperature and stirred for 18 h with a dry ice condenser fitted. The resulting clear solution was carefully basified to pH 8 with sodium bicarbonate solution. The organic layer was separated, washed with brine solution (2 x 50 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo* to give a yellow oil. A white precipitate resulted on addition of a solution of dry HCl gas in ether (20 ml). The white solid was filtered and dried under vacuum, 0.27 g (33% yield), mp 132-133 °C; ν_{max} ($CHCl_3$) 2980, 2880, 1745, 1600 and 1500 cm^{-1}; δ_H (200 MHz) (D_2O) 1.34 (9H, s, 8-, 9- and 10-H_3), 1.85 (2H, m, 3-H_2), 2.02 (2H, m, 4-H_2), 3.84 (1H, t, 2-H), 4.95 (2H, m, 6-H_2) and 5.17 (1H, m, 5-H); δ_C (50 MHz) 28.2 (C-8, 9 and 10), 29.5 and 30.3 (C-3 and C-4), 54.0 (C-2), 86.8 (C-7), 117.5 (C-6), 137.6 (C-5) and 170.3 (C-1); *m/z* 186 (M^+, 0.6%), 129, 85 and 57 (100%) (Found: M^+, 186.1480; C, 54.21; H, 9.00; N, 6.20. $C_{10}H_{20}NO_2$ requires *M*, 186.1494; C, 54.16; H, 9.09; N, 6.32%).

t-Butyl DL-*N*-*t*-Butoxycarbonyl-2-amino-5-hexenoate (167).



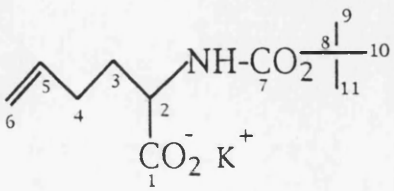
To a stirred suspension of **(166)** (0.64 g, 2.90 mmol) in dichloromethane (10 ml) was added sodium bicarbonate (0.24 g, 1 equiv.) in water (5 ml), sodium chloride (0.7 g) and di-*t*-butyldicarbonate (0.66 ml, 1 equiv.). The mixture was heated at reflux for 1.5 h then cooled to room temperature and the organic layer was separated. The aqueous layer was extracted with dichloromethane (2 x 40 ml) and the combined organic extracts weredried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 10% ethyl acetate/hexane to give a pale oil, 0.73 g (88% yield), R_F 0.37 (10% EtOAc/hexane); ν_{max} ($CHCl_3$) 3440, 2980, 1750, 1710 and 1500 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.47 (18H, br s, 8-, 9-, 10-, 13-, 14- and 15-H_3), 2.1-2.2 (4H, m, 3- and 4-H_2), 4.20 (1H, m, 2-H), 5.08 (3H, m, 6-H_2 and NH) and 5.70 (1H, m, 5-H); δ_C (50 MHz) 27.6 and 28.1 (C-8, 9, 10, 13, 14 and 15), 29.3 and 32.1 (C-3 and 4), 53.4 (C-2), 82.7 and 84.9 (C-7 and 12), 115.2 (C-6), 137.1 (C-5), 155.1 (C-11) and 171.7 (C-1); *m/z* 285 (M^+,0.1%), 212, 184, 128 and 57 (100%).

5-Hydroxy DL-*N*-*t*-Butoxycarbonylproline *t*-Butyl Ester (169).[92]



A solution of **(167)** (276 mg, 0.96 mmol) in dichloromethane (10 ml) was flushed with ozone at -78 °C according to general procedure [1], experimental to Chapter 3. Purification was achieved on a silica gel column eluting with diethyl ether to give a gluey white solid, 198 mg (72% yield), R_F 0.45 (Et_2O); ν_{max} ($CHCl_3$) 3590, 2980, 1735, 1695 and 1160 cm^{-1}; The ^1H NMR and ^{13}C NMR spectra indicate the presence of the diastereoisomers of compound **(169)**; δ_H (200 MHz) ($CDCl_3$) 1.48 (18H, 4 x s, 7-, 8-, 9-, 12-, 13- and 14-H_3), 1.84-2.42 (4H, br m, 3- and 4-H_2), 2.95-3.59 (1H, 4 x br d, OH), 4.17 (1H, br m, 2-H) and 5.61 (1H, br m, 5-H); δ_C (50 MHz) 28.0 and 28.3 (C-7, 8, 9, 12, 13 and 14), 30.9 and 32.2 (C-3 and 4), 59.9 and 60.0 (C-2), 80.7 (C-11), 82.3 and 82.5 (C-5), 85.2 (C-6), 146.8 (C-10) and 171.6 (C-1); *m/z* 270 (*M*-OH, 11.6%), 186, 170, 130, 68 and 57 (100%).

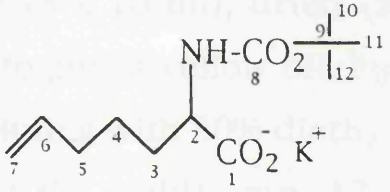
Potassium Salt of DL-*N*-*t*-Butoxycarbonyl-
2-amino-5-hexenoic Acid (171).



To a solution of **(126)** (1.00 g, 6.04 mmol) in water (10 ml) was added dioxan (7 ml), potassium bicarbonate (1.27 g, 2.1 equiv) and di-*t*-butyl dicarbonate (1.39 ml, 1 equiv) with continuous stirring

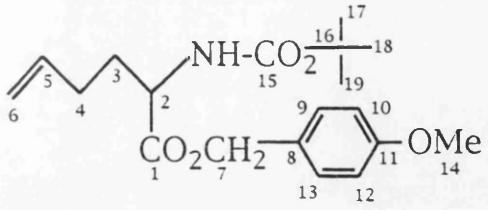
at room temperature for 18 h. The solvents were removed with ethanol *in vacuo* to give a white solid, 1.55 g (96 % yield); ν_{max} (KBr disc) 3420, 3080, 2980, 1690 and 1590 cm^{-1}; δ_H (200 MHz) (D_2O) 1.32 (9H, s, 9-, 10- and 11-H_3), 1.62 (2H, m, 3-H_2), 1.99 (2H, m, 4-H_2), 3.75 (1H, m, 2-H), 4.95 (2H, m, 6-H_2) and 5.79 (1H, m, 5-H); δ_C (50 MHz) 28.7 (C-9, 10 and 11), 30.5 and 31.9 (C-3 and 4), 56.4 (C-2), 81.8 (C-8), 116.3 (C-6), 139.1 (C-5), 163.5 (C-7) and 181.3 (C-1); *m/z* 228 (M H^+, 0.7%), 184, 171, 83 and 57.

Potassium Salt of DL-*N*-*t*-Butoxycarbonyl-2-amino-6-heptenoic Acid (172).



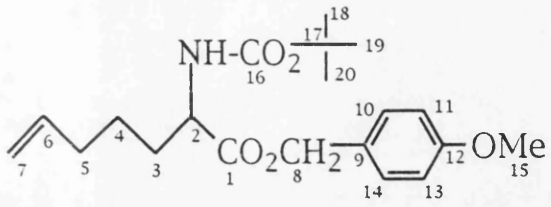
To a solution of **(127)** (1.00 g, 5.57 mmol) in water (10 ml) was added dioxan (8 ml), potassium bicarbonate (1.17 g, 2.1 equiv) and di-*t*-butyl dicarbonate (1.28 ml, 1 equiv) with continuous stirring at room temperature for 18 h. The solvents were removed with ethanol *in vacuo* to give a white solid, 1.49 g (95% yield); ν_{max} (KBr disc) 3430, 3080, 2980, 1680 and 1590 cm^{-1}; δ_H (200 MHz) (D_2O) 1.30 (9H, s, 10-, 11-, and 12-H_3), 1.49 (4H, br m, 3- and 4-H_2), 1.94 (2H, m, 5-H_2), 3.72 (1H, m, 2-H), 4.90 (2H, m, 7-H_2) and 5.76 (1H, m, 6-H); δ_C (50 MHz) 25.4 (C-4), 28.6 (C-10, 11 and 12), 32.2 and 33.5 (C-3 and 5), 56.9 (C-2), 81.8 (C-9), 115.5 (C-7), 140.3 (C-6), 162.1 (C-8) and 181.4 (C-1); *m/z* 198 (M^+-CO_2), 142, 124, 81 and 57 (100%) (Found M^+-CO_2, 198.1489. $C_{11}H_{20}NO_2$ requires M, 198.1489).

p-Methoxybenzyl DL-*N*-*t*-Butoxycarbonyl-2-amino-5-hexenoate (173).



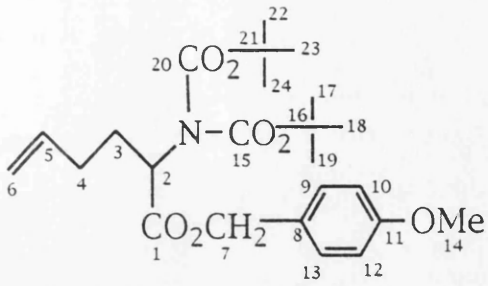
To a solution of **(171)** (0.50 g, 1.87 mmol) in DMF (3 ml) was added 4-methoxybenzyl chloride (0.27 ml, 1.0 equiv.) with continuous stirring for 48 h. DMF was removed with xylene *in vacuo* and the resultant residue was partitioned between dichloromethane (8 ml) and aqueous sodium bicarbonate solution (8 ml). The organic portion was washed with water (3 x 10 ml), dried ($MgSO_4$) and the solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 50% diethyl ether/hexane to give a white solid, 0.41 g (63% yield), mp 33-35 °C, R_F 0.38 (50% Et_2O/hexane); ν_{max} (KBr disc) 3360, 3080, 2980, 2835, 1750, 1690, 1610, 1580 and 1510 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.43 (9H, s, 17-, 18- and 19-H_3), 1.75 (2H, br m, 3-H_2), 2.04 (2H, br m, 4-H_2), 3.80 (3H, s, 14-H_3), 4.34 (1H, m, 2-H), 4.99 (2H, m, 6-H_2), 5.14 (2H, br d, 7-H_2), 5.73 (1H, m, 5-H), 6.88 (2H, d, 10- and 12-H) and 7.28 (2H, d, 9- and 13-H); δ_C (50 MHz) 28.3 (C-17, 18 and 19), 29.4 and 31.9 (C-3 and 4), 53.1 (C-2), 55.2 (C-14), 66.6 (C-7), 79.8 (C-16), 113.9 (C-10 and 12), 115.6 (C-6), 127.5 (C-8), 130.2 (C-9 and 13), 136.9 (C-5), 155.3 (C-15), 159.7 (C-11) and 172.7 (C-1); *m/z* 349 (M^+, 0.1%), 293, 184, 121 (100%) (Found: M^+, 349.1879; C, 65.48; H, 7.79; N, 4.01. $C_{19}H_{27}NO_5$ requires *M*, 349.1889; C, 65.32; H, 7.74; N, 4.01%).

p-Methoxybenzyl DL-*N*-*t*-Butoxycarbonyl-2-amino-6-heptenoate (174).



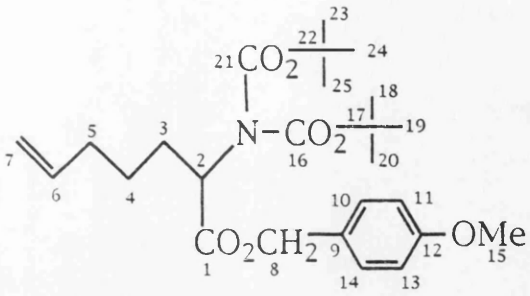
To a solution of **(172)** (0.90 g, 3.20 mmol) in DMF (8 ml) was added 4-methoxybenzyl chloride (0.46 ml, 1.0 equiv.) with continuous stirring for 48 h. DMF was removed with xylene *in vacuo* and the resultant residue was partitioned between dichloromethane (10 ml) and aqueous sodium bicarbonate solution (10 ml). The organic portion was washed with water (3 x 10 ml), dried ($MgSO_4$) and the solvent was removed *in vacuo* to give a yellow oil, 0.88 g (75% yield), R_F 0.30 (50% Et_2O/hexane); ν_{max} ($CHCl_3$) 3440, 3290, 2980, 1735, 1710, 1610 and 1515 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.43 (9H, s, 18-, 19- and 20-H_3), 1.75 (6H, br m, 3-, 4- and 5-H_2), 3.80 (3H, s, 15-H_3), 4.30 (1H, m, 2-H), 5.00 (4H, m, 7-H_2 and 8-H_2), 5.70 (1H, m, 6-H), 6.87 (2H, d, 11- and 13-H) and 7.27 (2H, d, 10- and 14-H); δ_C (50 MHz) 24.4 (C-4), 28.3 (C-18, 19 and 20), 32.1 and 33.2 (C-3 and 5), 53.4 (C-2), 55.3 (C-15), 66.8 (C-8), 79.8 (C-17), 113.9 (C-11 and 13), 115.0 (C-7), 127.6 (C-9), 130.2 (C-10 and 14), 137.6 (C-6), 155.5 (C-16), 159.7 (C-12) and 172.8 (C-1); *m/z* 363 (M^+, 0.9%), 307, 242, 198, 121 (100%) and 57 (Found M^+, 363.2039. $C_{20}H_{29}NO_5$ requires *M*, 363.2045).

p-Methoxybenzyl DL-*N*, *N*-Bis(*t*-Butoxycarbonyl)-2-amino-5-hexenoate (175).



To a solution of **(173)** (1.40 g, 4.01 mmol) in dry acetonitrile (10 ml) was added 4-dimethylaminopyridine (49 mg, 0.40 mmol) followed by di-*t*-butyldicarbonate (1.01 ml, 4.41 mmol). The mixture was stirred at room temperature for 18 h. The brownish reaction mixture was concentrated *in vacuo* at room temperature and the oily residue was partitioned between diethyl ether (20 ml) and 1M $KHSO_4$ solution (15 ml). The organic portion was washed with 1M $KHSO_4$ (2 x 15 ml), 5% sodium bicarbonate solution (15 ml), saturated brine solution (15 ml), dried ($MgSO_4$) and solvent was removed *in vacuo* to give an orange oil, 1.47 g (82% yield), R_F 0.60 (50% Et_2O/hexane); ν_{max} ($CHCl_3$) 3010, 2980, 2840, 1735, 1670, 1640 and 1500 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.45 (18H, s, 17-, 18-, 19-, 22-, 23- and 24-H_3), 2.10 (4H, br m, 3- and 4-H_2), 3.80 (3H, s, 14-H_3), 5.00 (5H, br m, 2-H, 6-H_2 and 7-H_2), 5.78 (1H, m, 5-H), 6.89 (2H, 10- and 12-H) and 7.26 (2H, 9- and 13-H); δ_C (50 MHz) 27.9 (C-17, 18, 19, 22, 23 and 24), 28.9 and 30.4 (C-3 and 4), 55.2 (C-2), 57.7 (C-14), 66.6 (C-7), 83.0 (C-16 and 21), 113.8 (C-10 and 12), 115.5 (C-6), 127.8 (C-8), 129.9 (C-9 and 13), 137.4 (C-5), 152.2 (C-15 and 20), 159.5 (C-11) and 170.8 (C-1); *m/z* 449 (M^+, 3.0%), 293, 121 (100%) and 57 (Found M^+, 449.2452. $C_{24}H_{35}NO_7$ requires *M*, 449.2413).

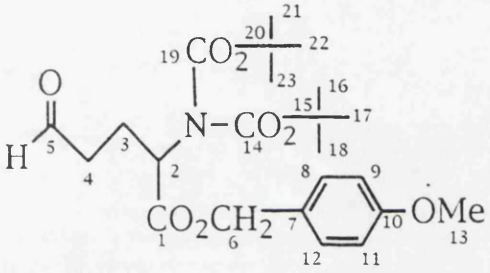
p-Methoxybenzyl DL-*N*, *N*-Bis(*t*-Butoxycarbonyl)-2-amino-6-heptenoate (176).



To a solution of **(174)** (2.10 g, 7.11 mmol) in dry acetonitrile (15 ml) was added 4-dimethylaminopyridine (87 mg, 0.71 mmol) followed by di-*t*-butyldicarbonate (1.80 ml, 7.82 mmol). The mixture was stirred at room temperature for 18 h. The brownish reaction mixture was concentrated *in vacuo* at room temperature and the oily residue was partitioned between diethyl ether (20 ml) and 1M $KHSO_4$ solution (20 ml). The organic portion was washed with 1M $KHSO_4$ solution (2 x 15 ml), 5% sodium bicarbonate solution (15 ml), saturated brine solution (15 ml), dried ($MgSO_4$) and solvent was removed *in vacuo* to give an orange oil, 3.09 g (94% yield), R_F 0.68 (50% EtOAc/hexane); ν_{max} ($CHCl_3$) 3010, 2980, 2840, 1735, 1670, 1640 and 1515 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.44 (18H, s, 17-, 18-, 19-, 23-, 24- and 25-H_3), 1.46 (2H, m, 4-H_2), 2.08 (4H, br m, 3- and 5-H_2), 3.80 (3H, s, 15-H_3), 5.00 (5H, br m, 2-H, 7-H_2 and 8-H_2), 5.76 (1H, m, 6-H), 6.87 (2H, 11- and 13-H) and 7.27 (2H, 10- and 14-H); δ_C (50 MHz) 25.5 (C-4), 27.9 (C-18, 19, 20, 23, 24 and 25), 28.9 and 33.2 (C-3 and 5), 55.3 (C-15), 58.1 (C-2), 66.6 (C-8), 82.9 (C-17 and 22), 113.8 (C-11 and 13), 114.9 (C-7), 127.9 (C-9), 129.9 (C-10 and 14), 138.3 (C-6), 152.2 (C-16 and 21), 159.5 (C-12) and 170.9 (C-1); *m/z* 463

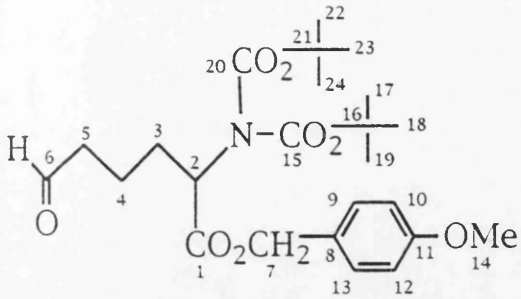
(M^+,10.7%), 363, 121 (100%) and 57 (Found M^+, 463.2581. $C_{25}H_{37}NO_7$ requires M, 463.2570).

DL-*N*, *N*-Bis(*t*-butoxycarbonyl)-glutamic Acid γ-Semialdehyde *p*-Methoxybenzyl Ester (177).



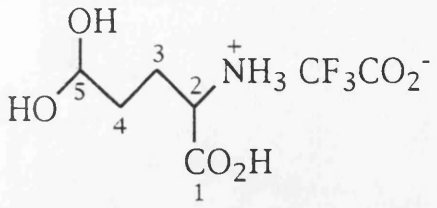
A solution of compound **(175)** (1.28 g, 2.85 mmol) in dichloromethane (35 ml) was saturated with ozone according to general procedure [1], experimental to Chapter 3. Purification was achieved on a silica gel column eluting with ether to give a pale oil, 970 mg (75% yield), R_F 0.55 (ether); ν_{max} ($CHCl_3$) 3020, 2840, 2730, 1735, 1710 and 1515 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.45 (18H, s, 16-, 17-, 18-, 21-, 22- and 23-H_3), 2.16 (2H, m, 3-H_2), 2.54 (2H, m, 4-H_2), 3.80 (3H, s, 13-H_3), 4.88 (1H, m, 2-H), 5.10 (2H, br s, 6-H_2), 6.87 (2H, 9- and 11-H), 7.27 (2H, 8- and 12-H) and 9.76 (1H, br s, 5-H); δ_C (50 MHz) 22.2 (C-3), 27.9 (C-16, 17, 18, 21, 22 and 23), 40.5 (C-4), 55.3 (C-13), 57.5 (C-2), 66.8 (C-6), 83.4 (C-15 and 20), 113.8 (C-9 and 11), 127.7 (C-7), 130.0 (C-8 and 12), 152.1 (C-14 and 19), 170.2 (C-1) and 201.0 (C-5); *m/z* 451 (M^+, 0.1%), 277, 186 and 121 (100%) (Found M^+, 451.2212. $C_{23}H_{33}NO_8$ requires M, 451.2206).

DL-*N*, *N*-Bis(*t*-butoxycarbonyl)-α-aminoadipic Acid δ-Semialdehyde *p*-Methoxybenzyl Ester (178).



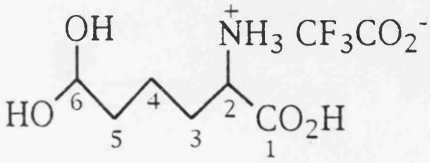
A solution of compound **(176)** (1.64 g, 3.54 mmol) in dichloromethane (40 ml) was saturated with ozone according to general procedure [1], experimental to Chapter 3. Purification was achieved on a silica gel column eluting with diethyl ether to give a yellow oil, 1.16 g (70% yield), R_F 0.71 (ether); ν_{max} ($CHCl_3$) 3010, 2980, 2840, 1740, 1710 and 1510 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.45 (18H, s, 17-, 18-, 19-, 22-, 23- and 24-H_3), 1.60-2.52 (6H, br m, 3-, 4- and 5-H_2), 3.80 (3H, s, 14-H_3), 4.86 (1H, m, 2-H), 5.09 (2H, 7-H_2), 6.86 (2H, 10- and 12-H), 7.27 (2H, 9- and 13-H) and 9.75 (1H, br s, 6-H); δ_C (50 MHz) 14.1 (C-4), 18.8 (C-3), 27.9 (C-17, 18, 19, 22, 23 and 24), 43.3 (C-5), 55.3 (C-14), 57.9 (C-2), 66.7 (C-7), 83.2 (C-16 and 21), 113.8 (C-10 and 12), 127.7 (C-8), 130.1 (C-9 and 13), 152.2 (C-15 and 20), 170.5 (C-1) and 201.8 (C-6); *m/z* 264, 137, 121, 82 and 57.

Trifluoroacetate Salt of DL-Glutamic Acid γ-Semialdehyde Hydrate (179).



A solution of compound **(177)** (490 mg, 1.1 mmol) in trifluoroacetic acid (3 ml) and dichloromethane (3 ml) was stirred at room temperature under an atmosphere of nitrogen for 2 h. The solvent was removed *in vacuo* to give an oily residue which was partitioned between distilled water (10 ml) and ethyl acetate (10 ml). The aqueous layer was separated, washed with ethyl acetate (3 x 10 ml) and freeze dried to give a cream coloured solid, 108 mg (40% yield), mp 57-58 °C; ν_{max} (KBr disc) 3435 (broad), 3000-2500 (broad), 1700, 1635 and 1200 cm^{-1}; δ_H (200 MHz) (D_2O) 1.5-2.5 (br m, 3- and 4-H_3), 4.16 (br m, 2-H) and 5.40 (br m, 5-H); δ_C (50 MHz) 31.1 and 38.7 (C-3 and 4), 55.3 (C-2), 60.2 (C-5) and 173.1 (C-1); *m/z* 113, 86, 69 and 41.

Trifluoroacetate Salt of DL-α-Aminoadipic Acid δ-Semialdehyde Hydrate (180).

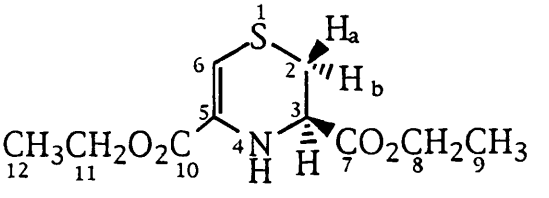


A solution of compound **(178)** (334 mg, 0.72 mmol) in trifluoroacetic acid (3 ml) and dichloromethane (3 ml) was stirred at

room temperature under an atmosphere of nitrogen for 2 h. The solvent was removed *in vacuo* to give an oily residue which was partitioned between distilled water (5 ml) and ethyl acetate (5 ml). The aqueous layer was separated, washed with ethyl acetate (3 x 8 ml) and freeze dried to give a yellow solid, 81 mg (43 % yield), mp °C; ν_{max} (KBr disc) 3435 (broad), 3000-2500 (broad), 1700, 1635 and 1200 cm^{-1}; δ_H (200 MHz) (D_2O) 1.35-2.32 (br m, 3-, 4- and 5-H_3) and 3.80 (br m, 2-H); δ_C (50 MHz) ; *m/z* 164, 114, 55 and 44.

7.4 Experimental to Chapter 5.

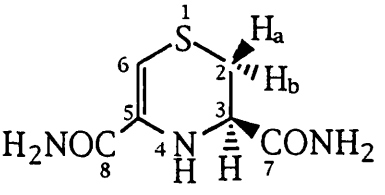
Diethyl (*R*)-3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (42).[136]



To a solution of L-cysteine ethyl ester (5.00 g, 26.9 mmol) and triethylamine (7.51 ml, 1.0 equiv) in dichloromethane (20 ml) under an atmosphere of nitrogen was added a solution of ethyl bromopyruvate (3.38 ml, 2.0 equiv) in dichloromethane (10 ml) with stirring. The mixture was stirred at room temperature for 12 h, washed with water (3 x 30 ml) and the organic portion was dried ($MgSO_4$). The solvent was removed *in vacuo* to give an orange oil. Purification was achieved on a silica gel column eluting with 20% ethyl acetate in hexane to give a yellow oil, 6.30 g (95% yield), R_F 0.28 (20% EtOAc/hexane); ν_{max} ($CHCl_3$) 3405, 3020, 2985, 1735, 1715 and 1605 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.30 (6H, 2 x t, *J* 7.1 Hz and *J* 7.2 Hz,

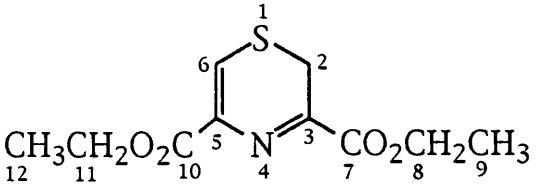
9- and 12-H_3), 2.99 (1H, dd, *J* 12.1 Hz and *J* 6.7 Hz, 2a-H), 3.25 (1H, dd, *J* 12.1 Hz and *J* 2.9 Hz, 2b-H), 4.26 (5H, 2 x q + m, *J* 7.1 Hz and *J* 7.2 Hz, 8- and 11-H_2 and 3-H), 4.79 (1H, br s, N-H) and 6.17 (1H, s, 6-H); δ_C (50 MHz) 14.1 and 14.2 (C-9 and 12), 26.9 (C-2), 53.1 (C-3), 61.2 and 61.8 (C-8 and 11), 101.1 (C-6), 128.2 (C-5), 162.3 (C-10) and 170.2 (C-7); *m/z* 245 (M^+, 37%), 172 (100%), 127 and 98 (Found: M^+, 245.0733. $C_{10}H_{15}NO_4S$ requires *M*, 245.0722).

3,4-Dihydro 2*H*-1,4-Thiazine-3,5-diamide (193).



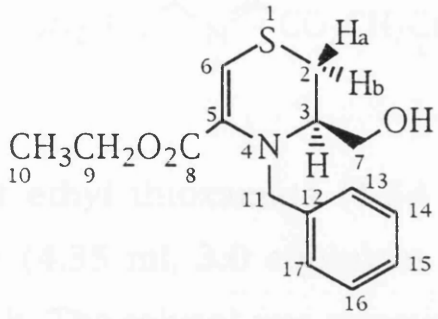
A solution of **(42)** (730 mg, 2.97 mmol) in methanolic ammonia (20 ml) was stirred at room temperature for 48 h. The resulting orange precipitate was filtered off and recrystallised from methanol to give a white solid, 167 mg (30% yield); ν_{max} (KBr disc) 3395, 3340, 1690, 1655, 1600 and 1490 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 2.54 (1H, dd, *J* 12.3 Hz and *J* 3.3 Hz, 2-H), 3.37 (1H, ddd, *J* 12.3 Hz, *J* 3.3 Hz and *J* 1.8 Hz, 2-H), 4.42 (1H, m, 3-H), 5.93 (2H, br s, NH_2), 6.33 (1H, d, *J* 1.8 Hz, 6-H) and 6.52 (2H, br s, NH_2); δ_C (50 MHz) 24.3 (C-2), 52.0 (C-3), 103.1 (C-6), 126.5 (C-5), 162.4 and 163.0 (C-7 and 8); *m/z* 172, 158, 142, 126 (100%), 98, 72 and 54.

Diethyl 2*H*-1,4-Thiazine-3,5-dicarboxylate (194).



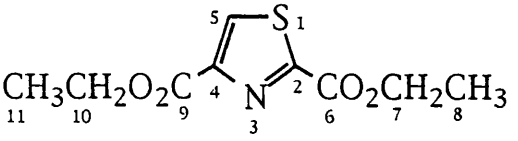
To a solution of 2,3-dichloro-5,6-dicyanobenzoquinone (611 mg, 2.69 mmol) in dichloromethane (6 ml) was added a solution of **(42)** (600 mg, 2.45 mmol) in dichloromethane (6 ml) at room temperature with continuous stirring for 1 h. The resultant reddish brown precipitate was filtered through Celite, washed with 5% aqueous sodium bicarbonate solution (3 x 10 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give an orange oil. Purification was achieved on a silica gel column eluting with 50% chloroform in ether to give a yellow oil, 385 mg (65% yield), R_F 0.41 (50% $CHCl_3$/ether); ν_{max} ($CHCl_3$) 3420, 2980, 1730, 1710 and 1465 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.34 (6H, 2 x t, 9- and 12-H_3), 3.32 (2H, d, *J* 1.2 Hz, 2-H_2), 4.30 (4H, 2 x q, 8- and 11-H_2) and 7.60 (1H, t, *J* 1.2 Hz, 6-H); δ_C (50 MHz) 14.1 and 14.2 (C-9 and 12), 20.6 (C-2), 63.0 and 63.3 (C-8 and 11), 128.3 (C-6), 137.2 and 137.9 (C-3 and 5), 167.5 and 168.9 (C-7 and 10); *m/z* 243 (M^+, 47.0%), 215, 169, 141,97, 45 and 29 (100%).

Ethyl *N*-Benzyl-3-hydroxymethyl-2*H*-3,4-dihydrothiazine-5-carboxylate (199).



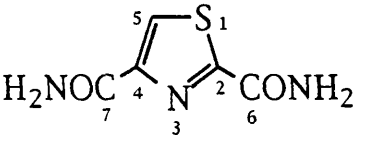
To a solution of S-benzyl-L-cysteinol (2.00 g, 10.14 mmol) in dichloromethane (15 ml) under an atmosphere of nitrogen was added dropwise a solution of ethyl bromopyruvate (1.27 ml, 1.0 equiv) in dichloromethane (5 ml). The mixture was stirred at room temperature for 18 h, washed with water (3 x 15 ml) and the organic portion was dried (Na_2SO_4). Removal of solvent *in vacuo* gave a dark orange oil which was purified on a silica gel column eluting with dichloromethane to give an orange oil, 1.51 g (73% yield), R_F 0.47 (CH_2Cl_2);.ν_{max} ($CHCl_3$) 3630, 3030, 2985, 1740, 1705, 1570, 1495, 1240 and 1075 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.32 (3H, t, *J* 7.1 Hz, 10-H_3), 2.67 (1H, m, 3-H), 3.40 (4H, m, 2- and 11-H_2), 4.27 (2H, q, *J* 7.2 Hz, 9-H_2), 4.30 (2H, m, 7-H_2), 6.88 (1H, s, 6-H) and 7.38 (5H, m, aromatic protons); δ_C (50 MHz) 14.2 (C-10), 21.7 (C-2), 53.3 (C-3), 58.5 (C-11), 60.9 (C-9), 61.0 (C-7), 116.8 (C-6), 127.6 (C-15), 128.5 (C-14 and 16), 128.9 (C-13 and 17), 130.2 (C-5), 137.9 (C-12) and 164.2 (C-8); *m/z* 293 (M^+, 9.5%), 262, 203, 172, 126 and 91.

Diethyl 1,3-Thiazole-2,4-dicarboxylate (43).



A solution of ethyl thioxamate (1.54 g, 11.56 mmol) and ethyl bromopyruvate (4.35 ml, 3.0 equiv) in ethanol (10 ml) was heated at reflux for 2 h. The solvent was removed *in vacuo* and ether (10 ml) was added to precipitate triethylamine hydrobromide. Filtration and removal of of the solvent *in vacuo* gave a yellow oil. Purification was achieved on a neutral alumina column eluting with 20% ethyl acetate in hexane to give a pale solid, 1.27 g (48% yield), mp 39-41 °C, R_F 0.50 (20% EtOAc/hexane); ν_{max} (KBr disc) 3110, 2985, 1720, 1485 and 1460 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.42 (6H, 2 x t, *J* 7.1 Hz, 8- and 11-H_3), 4.49 (4H, 2 x q, *J* 7.1 Hz, 7- and 10-H_2) and 8.45 (1H, s, 5-H); δ_C (50 MHz) 14.2 and 14.3 (C-8 and 11), 61.9 and 63.0 (C-7 and 10), 132.3 (C-5), 149.0 (C-4), 159.3 (C-2), 159.6 and 160.8 (C-6 and 9); *m/z* 229 (M^+, 6.3%), 184 (100%), 157 and 83 (Found: M^+, 229.0399; C, 47.36; H, 4.73; N, 6.01. $C_9H_{11}NO_4S$ requires *M*, 229.0409; C, 47.16; H, 4.80; N, 6.11%).

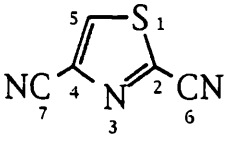
1,3-Thiazole-2,4-diamide (200).



A solution of **(43)** (865 mg, 3.77 mmol) in methanolic ammonia (20 ml) was stirred at room temperature for 48 h. The resultant

precipitate was filtered, washed with cold water and dried *in vacuo* to give a white solid, 550 mg (85% yield); ν_{max} (KBr disc) 3400 (broad), 3270, 3200, 1680, 1590 and 1485 cm^{-1}; δ_H (200 MHz) 7.76 (2H, br s, NH_2), 8.21 (2H, br s, NH_2), 8.46 (1H, s, 5-H); δ_C (50 MHz) 129.0 (C-5), 150.4 (C-4), 160.5 (C-2), 161.8 and 163.0 (C-6 and 7); *m/z* 171 (M^+,100%), 154, 128, 112, 84 and 70.

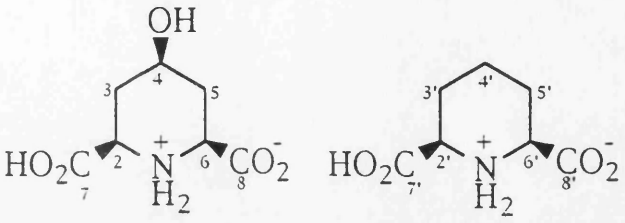
1,3-Thiazole-2,4-dinitrile (201).



A solution of compound **(200)** (470 mg, 2.75 mmol) in 1,1,2,2-tetrachloroethane was heated to 110 °C. Phosphorus oxychloride (2.56 ml, 10 equiv.) was added dropwise over 10 min and the mixture was heated at reflux for 1.5 h. The solution was decanted from remaining solid and the solvent was removed *in vacuo* to give a yellow residue. Chloroform (10 ml) was added and the solution was washed with 10% sodium carbonate solution (3 x 10 ml), water (2 x 10 ml), dried ($MgSO_4$) and the solvent was removed *in vacuo* to give a yellow oil. Recrystallisation from ethanol gave white needles, 162 mg (44% yield), mp 82-83 °C, R_F 0.46 (50% EtOAc/hexane); ν_{max} (KBr disc) 3100, 2240, 1610, 1465 and 1420 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 8.35 (1H, s, 5-H); δ_C (50 MHz) 111.0 and 112.0 (C-6 and 7), 129.3 (C-4), 134.6 (C-5) and 138.5 (C-2); *m/z* 135 (M^+, 55%), 83 (100%) and 58 (Found M^+, 134.9874. C_5HN_3S requires *M*, 134.9891).

7.5 Experimental to Chapter 6.

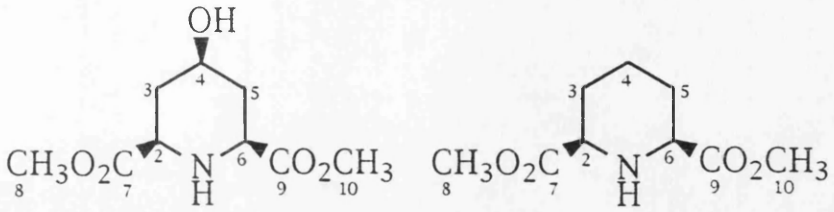
4-Hydroxypiperidine-*cis*-2,6-dicarboxylic Acid (203) and Piperidine-*cis*-2,6-dicarboxylic Acid (204).[143]



(203) (204)

An aqueous solution of chelidamic acid (5.0 g, 27.3 mmol) was hydrogenated in the presence of 5% rhodium-alumina catalyst (0.20 g, 4% by weight) for 8 h at 80 °C and 10 Bar. The reaction mixture was filtered and concentrated *in vacuo* to give a brown solid, 5.37 g (approximately quantitative yield); ν_{max} (KBr disc) 3465, 3170, 2980, 2510, 1710, 1635, 1355 and 1075 cm^{-1}; δ_H (200 MHz) (D_2O) 1.18-2.10 (10H, br m, 3-, 3'-, 4'-, 5- and 5'-H_2) and 3.39-3.58 (5H, br m, 2, 2'-, 4-, 6- and 6'-H); δ_C (50 MHz) 22.9 (C-4'), 26.0 (C-3' and 5'), 34.2 (C-3 and 5), 56.7 and 58.5 (C-2, 2', 6 and 6'), 66.3 (C-4), 172.6 and 173.4 (C-7, 7', 8 and 8').

Dimethyl 4-Hydroxypiperidine-*cis*-2,6-dicarboxylate (205) and Dimethyl Piperidine-*cis*-2,6-dicarboxylate (206).[143]



(205) (206)

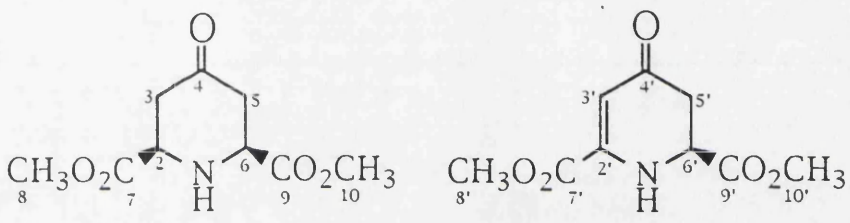
A mixture of **(203)** and **(204)** (1.00 g, approx. 5.5 mmol) in methanol (15 ml) and conc. sulfuric acid (1.0 ml) was heated at reflux for 18 h. The reaction mixture was cooled and water (15 ml) was added. The aqueous solution was basified with sodium carbonate (pH 8), extracted with chloroform (3 x 20 ml), dried ($MgSO_4$) and solvent was removed *in vacuo* to give a cream solid which showed two spots on TLC. Separation was achieved on a neutral alumina column eluting with ethyl acetate to give **(205)**, 285 mg (25% yield), mp 134-136 °C (lit.,[143] 134-135 °C), R_F 0.17 (EtOAc) and **(206)**, 253 mg, (24% yield), mp 92-94 °C (lit.,[143] 88-92 °C), R_F 0.67 (EtOAc).

Spectral data for compound **(205)**: ν_{max} (KBr disc) 3160, 2965, 1735, 1305 and 1070 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.36 and 2.33 (4H, 2 x m, 3- and 5-H_2), 1.93 (2H, br s, NH and OH), 3.40 (2H, m, 2- and 6-H), 3.74 (1H, m, 4-H) and 3.76 (6H, s, 8- and 10-H_3); δ_C (50 MHz) 37.6 (C-3 and 5), 52.3 (C-8 and 10), 56.3 (C-2 and 6), 68.0 (C-4) and 172.2 (C-7 and 9); *m/z* 217 (M^+, 0.4%), 158, 140 and 80 (100%) (Found: M^+, 217.0947; C, 49.81; H, 6.96; N, 6.33. $C_9H_{15}NO_5$ requires *M*, 217.0950; C, 49.77; H, 6.91; N, 6.45%).

Spectral data for compound **(206)**: ν_{max} (KBr disc) 3435, 3340, 2980, 2870, 1735, 1445 and 1220 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.43 and 2.00 (6H, 2 x br m, 3-, 4- and 5-H_2), 2.42 (1H, br s, NH), 3.40

(2H, m, 2- and 6-H) and 3.74 (6H, s, 8- and 10-H_3); δ_C (50 MHz) 24.1 (C-4), 28.6 (C-3 and 5), 52.1 (C-8 and 10), 58.4 (C-2 and 6) and 172.9 (C-7 and 9); *m/z* 201 (M^+, 0.8%), 142 (100%) and 82 (Found: M^+, 201.0997; C, 53.60; H, 7.49; N, 6.82. $C_9H_{15}NO_4$ requires *M*, 201.1001; C, 53.73; H, 7.46; N, 6.96%).

Dimethyl 4-Piperidone-*cis*-2,6-dicarboxylate (207) and 2,3-unsaturated analogue (208).

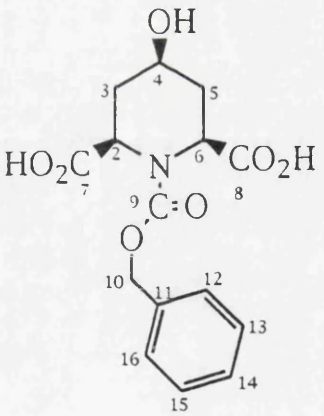


(207) (208)

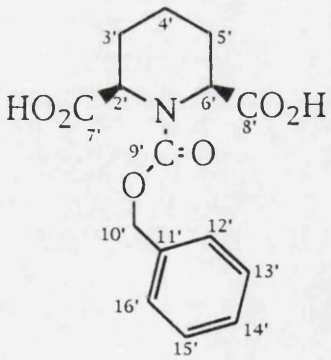
To a mixture of compound **(205)** (500 mg, 2.30 mmol) and pyridinium dichromate (1.30 g, 1.5 equiv.) in dry dichloromethane (10 ml) under a nitrogen atmosphere was added freshly activated, powdered 3A sieves (2 g) and glacial acetic acid (0.25 ml). The reaction mixture was stirred at room temperature for 2 h. Analysis by TLC indicated that no starting material remained. The reaction mixture was stirred with Celite (500 mg/mmol) for 20 min, filtered and solvent was removed *in vacuo* to give a dark brown solid. Puification was achieved on a silica gel column eluting with 2% methanol in dichloromethane to give 110 mg of a pale solid. Although this showed one spot on TLC (2% MeOH/CH_2Cl_2), GC analysis gave two distinct peaks, in approximately 1:1 ratio. Further analysis by GCMS indicated a mixture of the ketone (207) and the over-oxidised enone (208) to be present; ν_{max} (KBr disc) 3450, 3300, 3245, 3010, 2960,

1735, 1710, 1625 and 1590 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.68 (1H, br s, -NH), 2.64 (6H, m, 3-, 5- and 5'-H_2), 3.68 (2H, d, 2- and 6-H), 3.80 (6H, s, 8- and 10-H_3), 3.82 (3H, s, 10'-H_3), 3.91 (3H, s, 8'-H_3), 4.39 (1H, dd, 6'-H), 5.78 (1H, s, 3'-H) and 6.08 (1H, br s, -NH'); δ_C (50 MHz) 38.0 (C-5'), 44.2 (C-3 and 5), 52.7 (C-8 and 10), 53.1 and 53.5 (C-8' and 10'), 54.5 (C-6'), 56.9 (C-2 and 6), 102.3 (C-3'), 147.2 (C-2'), 163.3 (C-7'), 170.2 (C-9'), 171.0 (C-7 and 9), 193.5 (C-4') and 202.7 (C-4); *m/z* First peak: 215 (M^+), 156 (100%), 114 and 96. Second peak: 213 (M^+), 154 (100%) and 94.

4-Hydroxy-*N*-benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylic Acid (209) and *N*-Benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylic Acid (210).



(209)

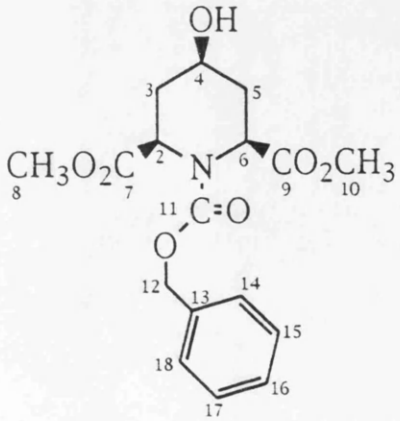


(210)

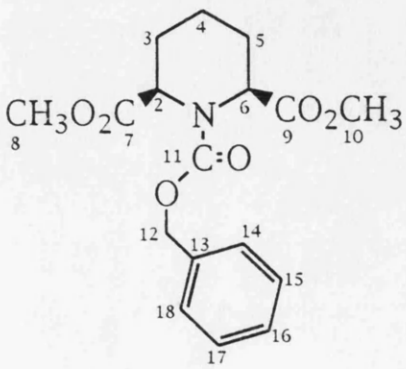
To a solution of the hydrogenation mixture, **(203)** and **(204)**, (2.0 g, approx. 11.0 mmol), and benzyl triethylammonium chloride (450 mg, 2.0 mmol) in 2M aqueous sodium hydroxide (10 ml) at 0 °C was added dropwise with stirring a solution of benzyl chloroformate (1.58 ml, 11.05 mmol) in diethyl ether (10 ml). The two-phase mixture was allowed to warm to room temperature and

was heated at 50 °C overnight with vigorous stirring. The aqueous layer was acidified with 1M hydrochloric acid and extracted with ethyl acetate (3 x 10 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a white, amorphous solid, 1.14 g, (approx. 35% yield); ν_{max} (KBr disc) 3485, 3035, 2955, 2540, 1695, 1630 and 1090 cm^{-1}. Analysis by ^1H NMR and ^{13}C NMR showed this to be a 1:1 mixture of compounds **(209)** and **(210)**. δ_H (200 MHz) ($CDCl_3/D_6$-DMSO) 1.39-2.40 (10H, m, 3-, 3'-, 4'-, 5- and 5'-H_2), 4.08 (1H, m, 4-H), 4.73 (4H, br d, 2-, 2'-, 6- and 6'-H), 5.16 (4H, br s, 10- and 10'-H), 7.32 (10H, s, 11-, 11'-, 12-, 12'-, 13-, 13'-, 14-, 14'-, 15-, 15'-, 16- and 16'-H) and 10.75 (4H, br s, 4 x -CO_2H); δ_C (50 MHz) 16.5 (C-4'), 25.9 (C-3' and 5'), 32.5 (C-3 and 5), 51.3 and 54.1 (C-2, 2', 6 and 6'), 59.9 (C-4), 67.5 (C-10 and 10'), 127.3, 127.8 and 128.2 (C-12, 12', 13, 13', 14, 14', 15, 15', 16 and 16'), 135.6 (C-11 and 11'), 155.5 (C-9 and 9'), 177.3 and 177.6 (C-7, 7', 8 and 8').

Dimethyl 4-Hydroxy-*N*-benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylate (211) and Dimethyl *N*-Benzyloxycarbonyl-piperidine-*cis*-2,6-dicarboxylate (212).



(211)



(212)

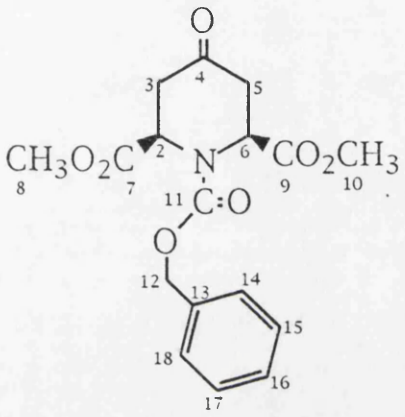
A mixture of **(209)** and **(210)**, (1.0 g, approx. 3.1 mmol), in methanol (20 ml) and conc. H_2SO_4 (0.5 ml) was heated at reflux overnight. The reaction mixture was cooled, water was added, and the solution was carefully neutralised with sodium carbonate (pH 8-9) and extracted with chloroform (3 x 15 ml). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a clear oil which showed two spots on TLC Separation was achieved on a silica gel column eluting with ethyl acetate to give **(211)**, 520 mg (49% yield), R_F 0.44 (EtOAc) and **(212)**, 410 mg (39% yield), R_F 0.66 (EtOAc).

Spectral data for compound **(211)**: ν_{max} ($CHCl_3$ solution) 3470, 3020, 2900, 2850, 1745, 1700 and 1090 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.84 and 2.42 (4H, 2 x m, 3- and 5-H_2), 3.59 (1H, d, *J* 3.6 Hz, -OH. Signal absent after D_2O exchange), 3.70 (6H, s, 8- and 10-H_3), 4.08 (1H, br m, 4-H), 4.95 (2H, dd, *J* 1.7 and 7.0 Hz, 2- and 6-H), 5.20 (2H, br s, 12-H_2) and 7.36 (5H, s, 14-, 15-, 16-, 17- and 18-H); δ_C (50 MHz) 31.9 (C-3 and 5), 50.5 (C-2 and 6), 52.3 (C-8 and 10), 61.7 (C-4), 68.0 (C-12), 127.9 (C-14 and 18), 128.1 (C-16), 128.4 (C-15 and 17), 136.4 (C-13), 155.8 (C11) and 173.4 (C-7 and 9); *m/z* 351 (M^+, 1.3%), 292, 260, 248, 230, 216 and 91 (100%) (Found: M^+, 351.1316. $C_{17}H_{21}NO_7$ requires *M*, 351.1318).

Spectral data for compound **(212)**: ν_{max} ($CHCl_3$ solution) 3015, 2955, 1740 and 1700 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.69 and 2.20 (6H, 2 x m, 3-, 4- and 5-H_2), 3.67 (6H, br s, 8- and 10-H_3), 4.89 (2H, br m, 2- and 6-H), 5.20 (2H, br d, 12-H_2) and 7.35 (5H, br s, 14-, 15-, 16-, 17- and 18-H); δ_C (50 MHz) 16.5 (C-4), 25.6 (C-3 and 5), 52.0 (C-8 and 10), 52.4 (C-2 and 6), 67.8 (C-12), 127.9 (C-14 and 18), 128.1 (C-16), 128.5 (C-15 and 17), 136.3 (C-13), 156.1 (C-11) and 171.6 (C-7

and 9); *m/z* 335 (M^+, 0.3%), 276, 232, 200, 140 and 91 (100%) (Found: M^+, 335.1387. $C_{17}H_{21}NO_6$ requires *M*, 335.1405).

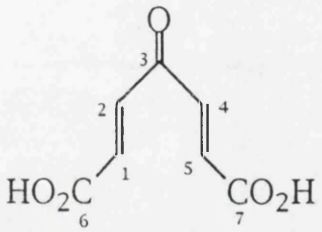
Dimethyl *N*-Benzyloxycarbonyl-4-piperidone-*cis*-2,6-dicarboxylate (213).



To a solution of N-iodosuccinimide (0.91 g, 4.06 mmol) and tetra-*N*-butylammonium iodide (300 mg, 0.81 mmol) in distilled dichloromethane (6 ml) was added compound **(211)** (285 mg, 0.81 mmol) in dichloromethane (4 ml). The dark solution was stirred at room temperature, under a nitrogen atmosphere for 12 h. The reaction mixture was diluted with saturated sodium thiosulfate solution (10 ml) and washed with water (3 x 8 ml). The organic portion was dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to give a dark yellow oil. Purification was achieved on a silica gel column eluting with 50% ethyl acetate in hexane to give a pale solid, 170 mg (32% yield), mp 74-76 °C, R_F 0.30 (50% EtOAc/hexane); ν_{max} (KBr disc) 3425, 3010, 2985, 1745, 1715 and 1700 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 2.84 (4H, m, 3- and 5-H_2), 3.65 and 3.76 (6H, 2 x s, 8- and 10-H_3), 5.01 (1H, br t, 2-H), 5.24 (3H, br d and t, 12-H_2 and 6-H)

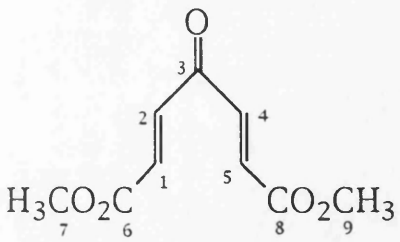
and 7.36 (5H, s, 14-, 15-, 16-, 17- and 18-H); δ_C (50 MHz) 39.7 and 40.0 (C-3 and 5), 52.7 and 52.8 (C-8 and 10), 53.3 and 53.6 (C-2 and 6), 68.6 (C-12), 128.1 (C-14 and 18), 128.4 (C-16), 128.6 (C-15 and 17), 135.5 (C-13), 155.1 (C-11), 171.0 (C-7 and 9) and 202.7 (C-4); *m/z* 349 (M^+, 6.4%), 290, 246, 214 and 91 (100%) (Found: M^+, 349.1178. $C_{17}H_{19}NO_7$ requires *M*, 349.1194).

1,4-Pentadien-3-one-1,5-dicarboxylic acid (215).[148]



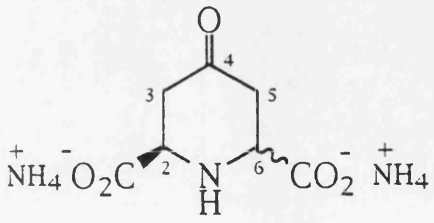
2-Furanacrylic acid (5.00 g, 36.0 mmol) in 30% aqueous hydrogen peroxide solution (50 ml) and conc. hydrochloric acid (5 ml) was stirred at room temperature for 5 d. The resultant orange precipitate was filtered, washed with methanol and dried to give a pale orange solid, 2.21 g (36% yield), mp 230-235 °C (lit.,[148] 243-245 °C); ν_{max} (KBr disc) 3450, 3035, 2650, 1700, 1665, 1640, 1410 and 1000 cm^{-1}; *m/z* 170 (M^+, 100%), 153 and 125 (Found: M^+, 170.0206; C, 49.24; H, 3.45. $C_7H_6O_5$ requires *M*, 170.0215; C, 49.42; H, 3.55).

Dimethyl 1,4-Pentadien-3-one-1,5-dicarboxylate (216).



A solution of **(215)** (250 mg, 1.47 mmol) in methanol (3 ml) and conc. sulfuric acid (0.2 ml) was heated at reflux for 30 min. The solution was concentrated *in vacuo* to give a brown residue. Crystallisation from ethyl acetate gave an orange solid, 220 mg (76% yield), mp 169-171 °C; ν_{max} (KBr disc) 3060, 2950, 1725, 1675, 1615, 1325 and 1000 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 3.84 (6H, s, 7- and 9-H_3), 6.82 and 7.34 (4H, AB system, J 15.9 Hz, 1-, 2-, 4- and 5-H); δ_C (50 MHz) 52.5 (C-7 and 9), 132.7 and 137.6 (C-1, 2, 4 and 5), 165.5 (C-6 and 8) and 195.1 (C-3); *m/z* 198 (M^+, 46%), 167 (100%) and 139 (Found: M^+, 198.0523. $C_9H_{10}O_5$ requires M, 198.0528).

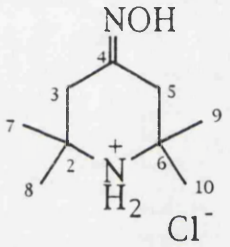
Mixture of the *cis*- (218) and *trans*- isomers of Diammonium Piperidin-4-one-2,6-dicarboxylate (217).



A solution of **(215)** (1.00 g, 5.88 mmol) in conc. ammonia solution (15 ml) was heated at reflux for 3 h. Concentration *in vacuo*

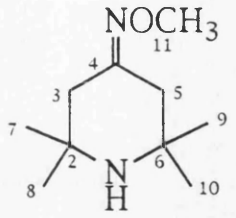
followed by drying over phosphorus pentoxide gave a dark brown solid, 940 mg (72% yield), mp >300 °C (lit.,[143] >300 °C); ν_{max} (KBr disc) 3450, 2970, 1620, 1590 and 1250 cm^{-1}; δ_H (200 MHz) (D_2O) 2.28-2.33 (4H, m, 3- and 5-H_2) and 4.20 (2H, m, 2- and 6-H); δ_C (50 MHz) 55.5 (C-3 and 5), 90.2 (C-2 and 6), 173.5 and 174.3 (C-7 and 8) and 201.3 (C-4); *m/z* 185 (-2 x H_2O, 11%), 143 and 79 (100%) (Found: 185.0735. $C_7H_{10}N_3O_3$ requires 185.0756).

2,2,6,6-Tetramethyl-4-piperidone Oxime Hydrochloride (221).



To a solution of 2,2,6,6-tetramethyl-4-piperidone (1.48 g, 6.18 mmol) in dichloromethane (5 ml) was added a solution of hydroxylamine hydrochloride (594 mg, 1.0 equiv.) in water (5 ml). The two-phase mixture was stirred rapidly at room temperature for 3 h. The resultant precipitate was filtered and triturated with ethereal HCl to give a pale solid, 854 mg (67% yield), mp 290-293 °C (decomp.); ν_{max} (KBr disc) 3280, 2955, 2820, 2600, 2480 and 1590 cm^{-1}; δ_H (200 MHz) (D_2O) 1.28 (12H, s, 7-, 8-, 9- and 10-H_3), 2.30 and 2.58 (4H, 2 x s, 3- and 5-H_2); δ_C (50 MHz) 27.3 and 27.6 (C-7, 8, 9 and 10), 34.7 and 41.1 (C-3 and 5), 59.4 and 59.6 (C-2 and 6) and 154.0 (C-4); *m/z* 170 (M^+, 41.7%), 155 (100%), 110 and 36/38 (3:1) (Found: M^+, 170.1423. $C_9H_{18}N_2O$ requires *M*, 170.1419).

2,2,6,6-Tetramethyl-4-piperidone Methoxime (222).



To a solution of 2,2,6,6-tetramethyl-4-piperidone (1.19 g, 6.85 mmol) in dichloromethane (5 ml) was added a solution of methoxylamine hydrochloride (572 mg, 1.0 equiv.) in water (5 ml). The two-phase mixture was stirred rapidly at room temperature for 48 h. The mixture was extracted with dichloromethane (2 x 10 ml), the combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to give a pale yellow solid, 112 mg (11% yield), mp 255-257 °C (decomp.); ν_{max} (KBr disc) 3440, 2960, 2815, 1580, 1400 and 1050 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.61 and 1.63 (12H, 2 x s, 7-, 8-, 9- and 10-H_3), 2.50 and 2.78 (4H, 2 x s, 3- and 5-H_2) and 3.87 (3H, s, 11-H_3); δ_C (50 MHz) 27.6 and 27.8 (C-7, 8, 9 and 10), 36.1 and 41.5 (C-3 and 5), 58.9 and 59.1 (C-2 and 6), 61.6 (C-11) and 151.1 (C-4); *m/z* 184 (M^+, 37%), 169, 153, 122 and 98 (100%) (Found: M^+, 184.1568. $C_{10}H_{20}N_2O$ requires *M*, 184.1576).

7.6 Test Data for Chapters 3, 4, 5 and 6.

The standard assay for DHDP synthase was set up by Borthwick,[108] details of which are given in Section 3.4, Chapter 3 of this thesis. The following tables show the percentage inhibition of DHDP synthase when tested with various concentrations of inhibitors.

Test Data for Chapter 3.

Compound no. or name	Experimental page no.	Inhibitor concentration 1 mM	0.5 mM	0.25 mM	0.1 mM
(111)	148	15%	0%	-	-
(112)	149	3%	-	-	-
(113)	150	100%	-	33%	0%
(114)	151	59%	17%	-	0%
(116)	152	14%	0%	-	-
(117)	153	22%	0%	-	-
(118)	154	100%	-	-	46%

Test Data for Chapter 4.

Compound no. or name	Experimental page no.	Inhibitor concentration 5 mM	4 mM	3 mM	1 mM
(126)	170	24%	-	-	0%
(127)	171	8%	-	-	0%
(152)	172	0%	-	-	-
(143)	165	-	-	53%	11%
(144)	166	-	40%	-	15%
(149)	168	-	-	50%	0%
(150)	169	-	51%	-	25%
(180)	191	-	-	-	27%

Test Data for Chapter 5.

Compound no. or name	Experimental page no.	Inhibitor concentration 1 mM	0.5 mM	0.25 mM	0.1 mM
(42)	192	85%	23%	-	13%
(43)	196	100%	100%	100%	15%
(201)	197	-	-	47%	18%

Test Data for Chapter 6.

Compound no. or name	Experimental page no.	Inhibitor concentration 5 mM	1 mM	0.5 mM	0.1 mM
(205)	199	29%	0%	-	-
(206)	199	6%	-	-	-
(207)/(208)	200	64%	11%		
(211)	202	0%	-	-	-
(212)	202	0%	-	-	-
(213)	204	12%	-	-	-
(217/218)	206	100%	65%	-	28%
(219)	-	-	89%	46%	-
(220)	-	-	90%	22%	-
(221)	207	-	-	8%	-
(222)	208	-	-	23%	-

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