A Study of Two Key Enzymes in the Diaminopimelate Pathway to L-Lysine

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

by

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"The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact."

Thomas Henry Huxley (1825-1895),
Ib.viii. *Biogenesis and Abiogenesis.*

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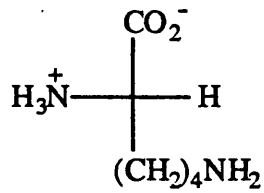
Finally, I dedicate this thesis to my beautiful wife Margaret for her patience and understanding during my University career and for typing this thesis.....*my inspiration.*

Summary

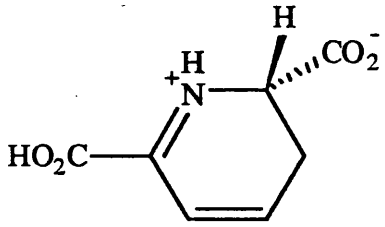
There are two distinct biosynthetic pathways to the essential amino acid L-lysine (A). The diaminopimelate pathway to L-lysine (A) occurs in higher plants and bacteria whereas the α-aminoadipate pathway to L-lysine (A) operates in fungi and yeasts. This thesis is concerned with the first two steps in the diaminopimelate pathway to L-lysine (A), catalysed by dihydrodipicolinate synthase (DHDPS) and dihydrodipicolinate reductase (DHDPR). In particular, the synthesis of L-aspartic acid-β-semialdehyde (L-ASA) (C), a substrate of the first enzyme and the mechanism of formation of L-2,3-dihydrodipicolinate (L-2,3-DHDPA) (B), the product of the reaction catalysed by DHDPS, was studied. In addition the synthesis of compounds which might be inhibitors of DHDPS was studied.



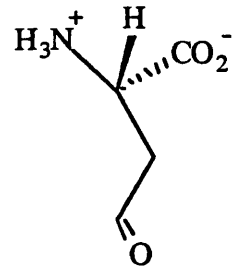
(A)

L-Aspartic acid-β-semialdehyde (C) is an important intermediate in the biosynthesis of L-lysine (A), L-threonine and L-methionine. The trifluoroacetate salts of the L- and D-isomers of aspartic acid-β-semialdehyde were prepared from L- and D-allylglycine respectively. Our Biochemistry co-workers have isolated and purified DHDPS and have set up an assay system for this

enzyme. Using this assay system we confirmed that L-ASA (C) is a substrate for DHDPS whereas the D-isomer is not.

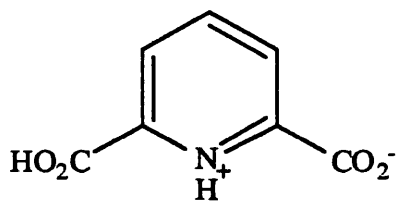


(B)



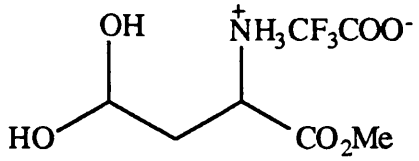
(C)

Having pure L-ASA (C) allowed us to carry out precise NMR spectroscopic experiments and biochemical experiments to investigate the mechanism of DHDPS. We confirmed that the product from the enzymic reaction is dipicolinic acid (D).



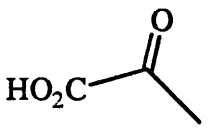
(D)

Manipulation of the synthetic route to L-ASA (C) allowed us to prepare a range of aspartic acid-β-semialdehyde analogues and derivatives. Most of these compounds were tested for potential inhibitor or substrate activity with DHDPS. In particular the trifluoroacetate salt of DL-aspartic acid-β-semialdehyde methyl ester hydrate (E) showed no substrate activity however, 14% inhibition was observed with this compound at 0.5 mM.



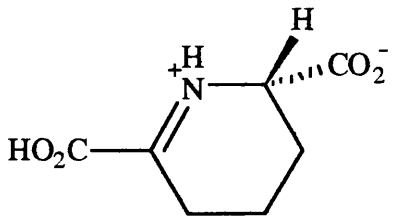
(E)

The other substrate of DHDPS in the diaminopimelate pathway to L-lysine (A) is pyruvate (F). A range of pyruvate (F) and bromopyruvate derivatives were synthesised and tested as substrates or inhibitors of DHDPS. Methyl pyruvate has shown substrate activity with DHDPS. In general, the bromopyruvate derivatives were better inhibitors than the pyruvate derivatives.

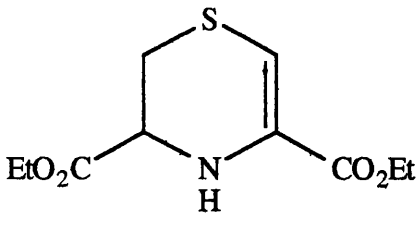


(F)

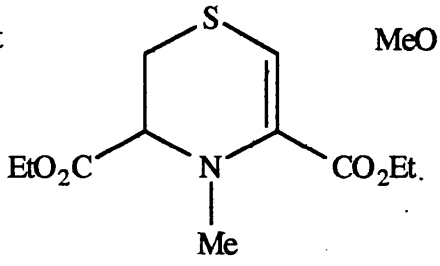
A range of sulphur analogues of L-2,3-DHDPA (B) and L-2,3,4,5-tetrahydrodipicolinic acid (L-2,3,4,5-THDPA) (G) were synthesised and tested for inhibitor activity with DHDPS. The 3,4-dihydro-1,4-thiazines, in particular the diethyl ester (H) and its *N*-methyl analogue (I), showed good inhibition at 0.1 mM with DHDPS. The 3,4-dihydro-2,2-dimethyl-1,4-thiazine diester (J) showed the best inhibition with 20% at 0.1 mM. The 3,4-dihydro-1,4-thiazines were prepared by reacting L-cysteine derivatives with ethyl bromopyruvate in dichloromethane in the presence of triethylamine.



(G)



(H)



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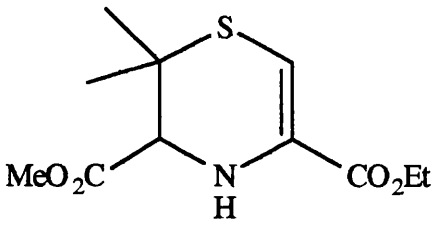
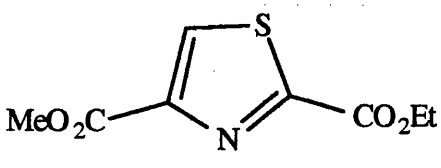
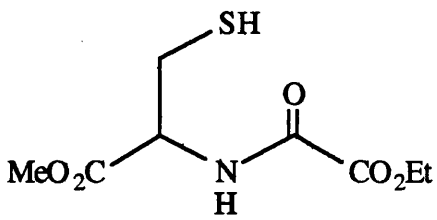


(J)

The 1,3-thiazoles that were prepared showed very good inhibition with DHDPS. The diester (K) showed 21% inhibition at 0.1mM with DHDPS. This compound was prepared by treatment of the mercapto-amide (L) with phosphorus pentasulphide in pyridine. The mercapto-amide (L) was prepared by treating L-cysteine methyl ester hydrochloride with ethyl oxalyl chloride in dichloromethane in the presence of triethylamine.



(K)



(L)

Table of Contents

Abbreviations

acetyl CoA	:	acetyl co-enzyme A
ATP	:	adenosine triphosphate
α	:	alpha
AAA	:	alpha-aminoadipate
L-AAO	:	L-amino acid oxidase
L-ASA	:	L-aspartic acid-β-semialdehyde
β	:	beta
BOC	:	*t*-butoxycarbonyl
CBZ	:	carboxybenzyl
m-CPBA	:	*meta*-chloroperbenzoic acid
CD	:	circular dichromism
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-2H-1,4-thiazine-3,5-dicarboxylic acid
DMF	:	dimethyl formamide
2,4-DNP	:	2,4-dinitrophenyl hydrazone
DPA	:	dipicolinic acid
d	:	doublet
ε	:	epsilon
E. coli	:	Escherichia coli

γ	:	gamma
gem	:	geminal
IR	:	infra-red
λ	:	lambda
MHz	:	megahertz
μM	:	micromolar
mg	:	milligram
ml	:	millilitre
mmol	:	millimolar
M	:	molar
m	:	multiplet
nm	:	nanometre
NADH	:	nicotinamide adenine dinucleotide
NADPH	:	nicotinamide adenine dinucleotide phosphate
NMR	:	nuclear magnetic resonance
o	:	ortho
p	:	para
q	:	quartet
s	:	singlet
succinyl CoA	:	succinyl co-enzyme A
L-2,3,4,5-THDPA	:	L-2,3,4,5-tetrahydrodipicolinic acid
TLC	:	thin layer chromatography
t	:	triplet
UV	:	ultraviolet

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

Introduction

Amino acids are found in living organisms in both their free forms and bound by amide linkages in peptides and proteins. There are approximately 20 different protein α-amino acids. The composition of proteins varies widely. In some cases only a few amino acids constitute the bulk of the protein. However, more typically, most of the amino acids are present. The structures of the amino acids have all been determined following the characterisation of glycine and leucine by Braconnet in 1820, with threonine being the last to be isolated in pure form in 1925.[1-3] With the exception of glycine, they are all optically active and exist mainly in the L-forms. An important member of this group of protein amino acids is L-lysine (1). Its importance will be discussed in Section 1.1.

1.1. The Importance of L-Lysine.

L-Lysine (1) is economically one of the most important amino acids. It is an essential amino acid for human nutrition and is frequently the limiting amino acid in plant protein in terms of nutritional quality. Mammals lack the ability to make L-lysine (1) and so must consume it within their diet. Commercially, the largest amounts are produced by the bacteria *Corynebacterium glutamicum, Brevibacterium flavum and Brevibacterium lactofermentum.*[4] The production of L-lysine (1) will be discussed in more detail in Section

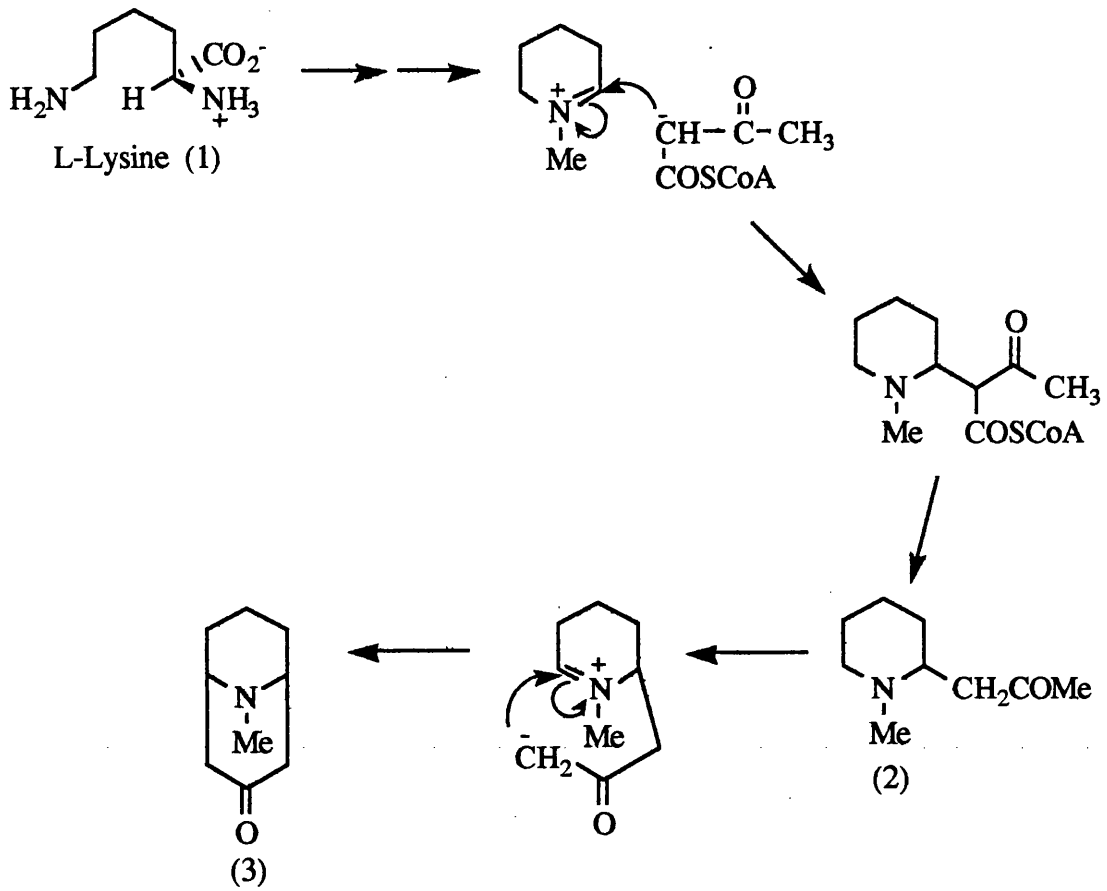
1.2. The biosynthesis of L-lysine (1) in bacteria and plants is incompletely understood. This will be discussed in Section 1.3. *meso* -2,6-Diaminopimelic acid (28), an intermediate in the biosynthesis of L-lysine (1), is a building block for the cell wall cross-linking material of most bacteria. Enzyme inhibitors of lysine biosynthesis should therefore possess anti-bacterial and/or herbicidal activity without mammalian toxicity. The design of inhibitors will be discussed in more detail in Section 1.4 and Chapters 4, 5, 6 and 7.

The conversion of L-lysine (1) into alkaloids is an important biological process which has been studied for many years. Alkaloids are a diverse group of natural products containing nitrogen as part of a ring system and are found mainly in plants. They exhibit a wide range of biological activities. The isolation of large numbers of alkaloids has been accompanied by an increase in understanding of their biological properties and their pharmacological, toxicological and ecological significance in nature. Experiments with isotopically labelled precursors have helped us to understand the complex manner in which alkaloids are biologically synthesised. More importantly, understanding the biosynthesis of alkaloids, starting from simple amino acid precursors, has assisted the design of biomimetic synthetic routes. In addition synthesis of analogues of biosynthetic intermediates may lead to inhibition of particular enzymes and hence these analogues may have useful biological activity.

There are two main classes of alkaloids which utilise L-lysine (1) as the precursor, namely the piperidine alkaloids and the

quinolizidine alkaloids. Both these types of alkaloids occur in a limited number of plant families.

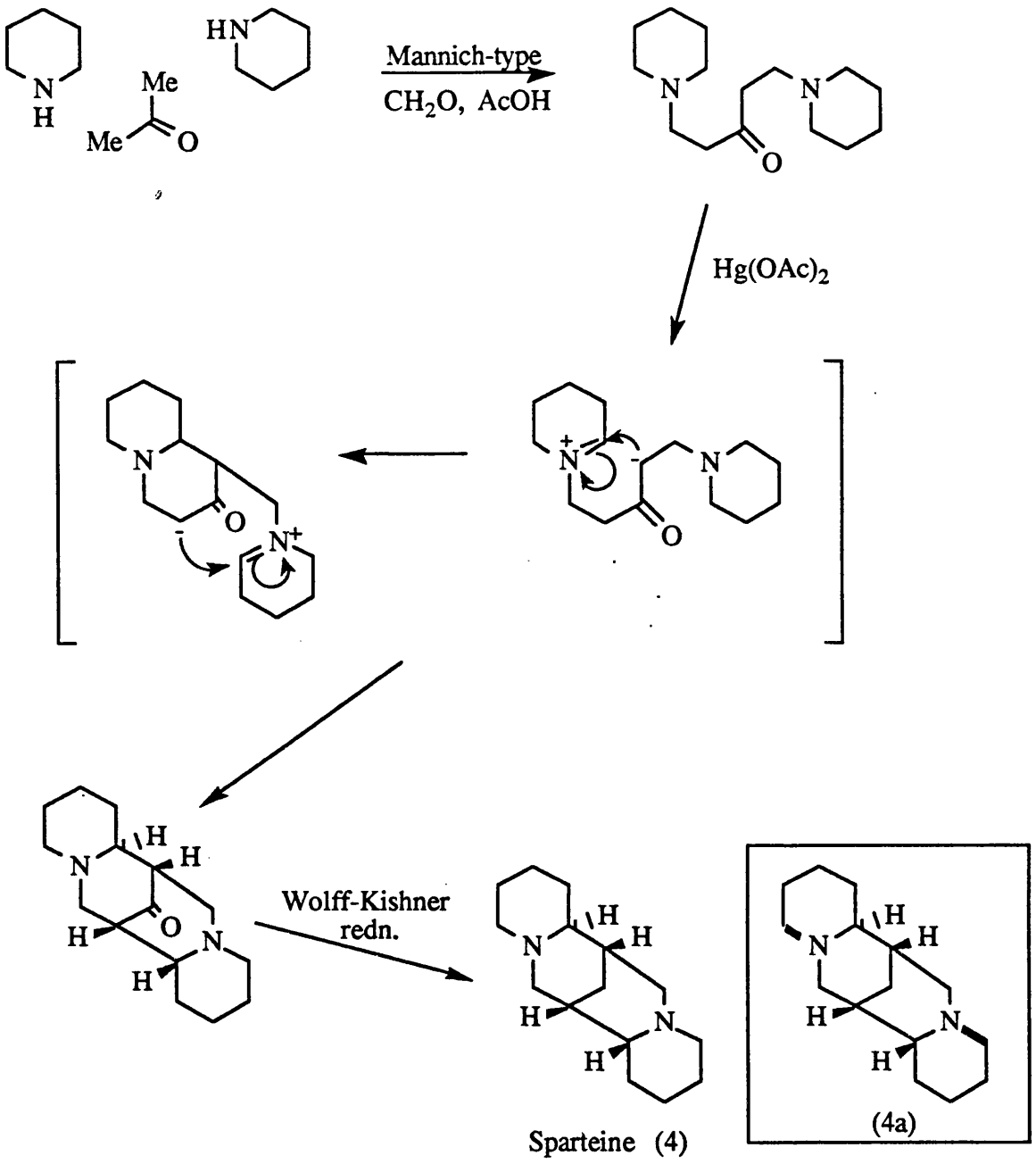
Many higher plants possess the necessary metabolic pathways for the synthesis of piperidine alkaloids. In each case these alkaloids incorporate one or more piperidine rings. A typical piperidine alkaloid is pseudopelletierine (3), isolated from the bark of *Punica granatum L.* (pomegranate).[5] Its probable metabolic pathway from L-lysine (1) via *N*-methylisopelletierine (2) is shown below (**Scheme 1**).



Scheme 1 : Pseudopelletierine biosynthesis.

Quinolizidine alkaloids are sometimes known as the lupin alkaloids since they occur widely, but not exclusively, in species of the genus *Lupinus* from the plant family Fabaceae (formerly Leguminosae). Their biosynthesis from L-lysine (1) starts with a decarboxylation in an analogous fashion to that of the pyrrolizidine alkaloids from ornithine.[6] Thereafter the two biosynthetic pathways differ. The route to the pyrrolizidine alkaloids is via a later symmetrical intermediate (C_4-N-C_4), whereas the formation of quinolizidine alkaloids does not involve a corresponding C_5-N-C_5 intermediate. The most common quinolizidine alkaloid is sparteine (4). It has been isolated from many plant species including *Spartium scoparium*,[7] *Sarothamnus scoparius*,[8] *Lupinus lindeniancus*[9] *and Lupinus laxus Rydb.*[10] A particularly good 'biomimetic' total synthesis of this compound has been carried out by Van Tamelen **(Scheme 2)**. Careful labelling studies by the groups of Robins[11] and Spenser[12] 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. This confirmed that two moles of cadaverine are incorporated into the two outer rings of sparteine as shown in (4a) **(Scheme 2)**. The steps by which 3 molecules of cadaverine form sparteine are not understood.

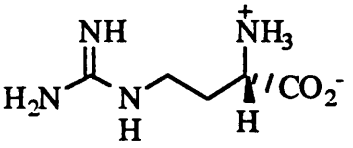
L-Lysine (1) and L-arginine (5) play an important role in molecular interactions in biological systems because of their basic groups. It is well established that L-lysine (1) and L-arginine (5) are indispensable requirements for normal growth of microbes,[13-16] plants[17-19] and animals.[20-22] Furthermore, an antagonistic role



Scheme 2

between these two amino acids has also been observed in living organisms.[23,24] Antagonism existing between L-lysine (1) and L-arginine (5) has been clearly illustrated by their effects on animal

systems. Either oral or peritoneal administration of a large dosage of L-lysine (1) or L-arginine (5) has been found to produce a highly significant change in the growth of transplantable tumours in mice.[25,26] Thus L-lysine (1) strongly inhibited the tumour growth while L-arginine (5) promoted the growth of the tumour. On the other hand, the D-enantiomers produced an opposite effect on tumor growth: D-lysine promoted and D-arginine inhibited this growth.



L-Arginine (5)

From the above considerations it is obvious that L-lysine (1) plays a crucial role in nature. Hence, lysine biosynthesis in both micro-organisms and plant systems, and particularly its regulation, is of considerable practical importance. There are two major pathways to L-lysine (1). Both the α-aminoadipate (AAA) and the 2,6-diaminopimelate (DAP) pathways will be discussed in Section 1.3.

1.2. Production of L-Lysine.

The remarkable progress in the technology of amino acid production, based on biochemistry and genetics, has made the amino acid industry of great importance for human life today. In particular, amino acids have many diverse uses in foods. They are

largely responsible for the taste of many types of foods, including soy sauce, seafood, and cheese. Alanine and glycine are used as sweeteners, for example, and synthetic mixtures of protein amino acids are used in special diets, *eg.* a low phenylalanine amino acid mixture is used to prevent the mental retardation effects of phenylketonuria, a metabolic inability to degrade phenylalanine in the normal way.

L-Lysine (1) is now produced by direct fermentation with auxotrophic and regulatory mutants and by enzymatic methods. The production of L-lysine and L-ornithine represents the first industrial utilisation of auxotrophs for amino acid production. The first report describes lysine production by homoserine auxotrophs of *Micrococcus glutamicus* grown at suboptimal concentrations of the required amino acids methionine and threonine.[27]

An enzymatic method for lysine production uses the yeast *Cryptococcus laurentii,* which hydrolyses α-aminocaprolactam, a synthetic intermediate in the manufacture of nylon, to lysine.[28] A strain of *Achromobacter obae* can racemise D- and L-α-amino-caprolactam, and cells of both organisms were combined in an efficient enzymatic process for lysine production.[29]

1.3. Biosynthesis of L-Lysine.

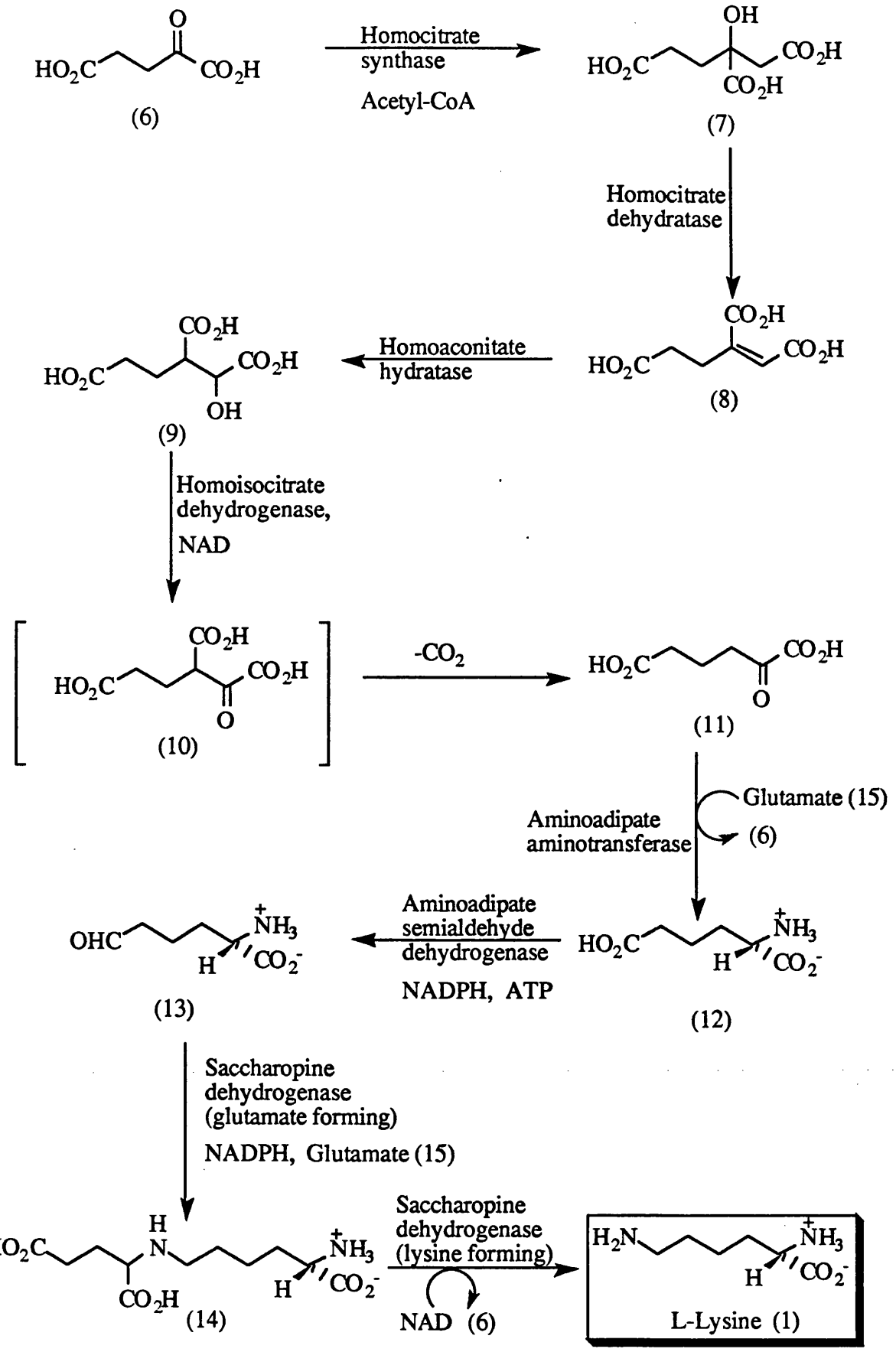
There are two distinct biosynthetic pathways for the synthesis of the essential amino acid L-lysine (1). In organisms such as Euglenoids, some Phycomycetes (*eg.* Chytridiales, Blastocladiales and Mucorales), as well as yeasts and higher fungi such as Ascomycetes

and Basidiomyces, L-lysine (1) is synthesised via the intermediate α-aminoadipic acid (12) derived from 2-oxoglutarate (6) and acetyl coenzymeA (acetyl CoA) **(Scheme 3).** In bacteria, some Phycomycetes (*eg.* Hypochytriales, Suprolegniales and Leptomytales) and higher plants the pathway is characterised by the condensation of L-aspartic acid-β-semialdehyde (21) and pyruvate (22) and the formation of the intermediate *meso*-2,6-diaminopimelate (28)[30] **(Scheme 4).**

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

α-Aminoadipate (12), α-ketoadipate (11) and α-ketoglutarate (6) have long been known as products of lysine catabolism in animals.[31,32] Proof that these compounds are part of a biosynthetic cycle to produce L-lysine (1) came from studies of lysine mutants, which cannot make lysine for themselves but can produce lysine when their growth media are supplemented with the above compounds. These studies led to the establishment of the following biosynthetic pathway **(Scheme 3).**

There are nine enzymes involved in the pathway and most of them have been isolated and reasonably well characterised. The starting substrate is 2-oxoglutarate (6) which is a product of the Krebs cycle.[33] This is condensed with acetyl CoA to form homocitrate (7). The reaction is catalysed by homocitrate synthase.[34] The next reaction in the pathway is the conversion of homocitrate (7) into homoaconitate (8) catalysed by the enzyme



Scheme 3 : α-Aminoadipate Pathway

homocitrate dehydratase. This enzyme has not yet been isolated. Homoaconitate (8) is then hydrolysed to homoisocitrate (9) catalysed by homoaconitate hydratase.[35]

The next step in the process involves the reduction of homoisocitrate (9) to α-ketoadipate (11) via the proposed intermediate oxaloglutarate (10). This step is catalysed by homoisocitrate dehydrogenase[36] and needs the coenzyme Nicotinamide Adenine Dinucleotide (NAD) for the reaction to take place. The reaction is believed to proceed via oxaloglutarate (10), which is an enzyme bound intermediate. Whilst bound to the enzyme decarboxylation occurs to give the product, α-ketoadipate (11). This is then converted into α-aminoadipate (12) via the enzyme aminoadipate aminotransferase[37] with glutamate as the amino group donor. Pyridoxal phosphate is used as a cofactor during the transamination.

The processes detailed so far take place within the mitochondria of the organism and all the enzymes used are found in the mitochondria. However the last three steps of the process take place in the cytoplasm. This means that α-aminoadipate (12) must be transported from the mitrochondria to the cytoplasm to be finally converted into L-lysine (1).

The pathway to L-lysine (1) continues with the conversion of α-aminoadipate (12) into α-aminoadipate semialdehyde (13) catalysed by aminoadipate semialdehyde dehydrogenase.[38,39] The enzyme is cocatalysed by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and Adenosine Triphosphate (ATP) and the reaction is believed to happen in two stages. The first step involves

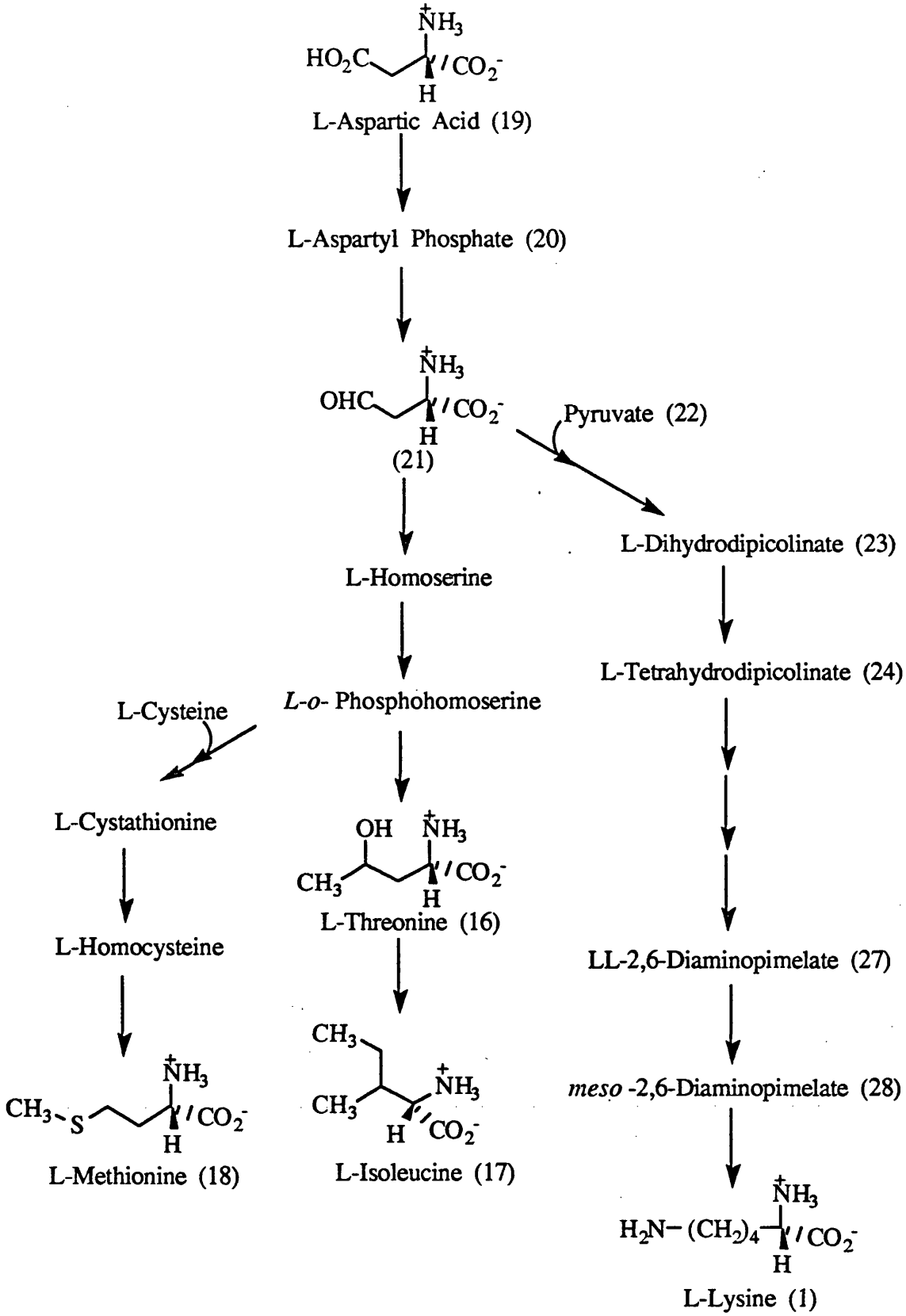
the conversion of α-aminoadipate (12) into δ-adenyl-α-aminoadipate catalysed by ATP. In the second step the above enzyme bound intermediate is converted into α-aminoadipate semialdehyde (13) co-catalysed by NADPH.

The synthesis of L-lysine (1) from α-aminoadipate semialdehyde (13) is carried out in two steps which invove first of all the condensation of α-aminoadipate semialdehyde (13) with glutamate (15) followed by reduction to form saccharopine (14) in the presence of NADPH. This step is catalysed by saccharopine dehydrogenase (glutamate forming).[40] The final step in the pathway involves the conversion of saccharopine (14) into L-lysine (1) and α-ketoglutarate (6) using saccharopine dehydrogenase (lysine forming)[40] in the presence of NAD. It is known that this enzyme has a very ordered mechanism in which NAD and saccharopine (14) both bind to the enzyme and L-lysine (1), α-ketoglutarate (6) and NADH are released in that order. The α-aminoadipate pathway to L-lysine (1) will not be discussed any further in this thesis.

The Diaminopimelate (DAP) Pathway to L-Lysine.

The use of radioactive precursors of lysine and intermediates of the proposed pathway have confirmed that the synthesis of L-lysine (1) in bacteria is via *meso*-2,6-diaminopimelate (28).[41-43]

The first two enzymes of the pathway, aspartate kinase and aspartate semialdehyde dehydrogenase, are common to the synthesis of threonine (16), isoleucine (17) and methionine (18) (Scheme 4).

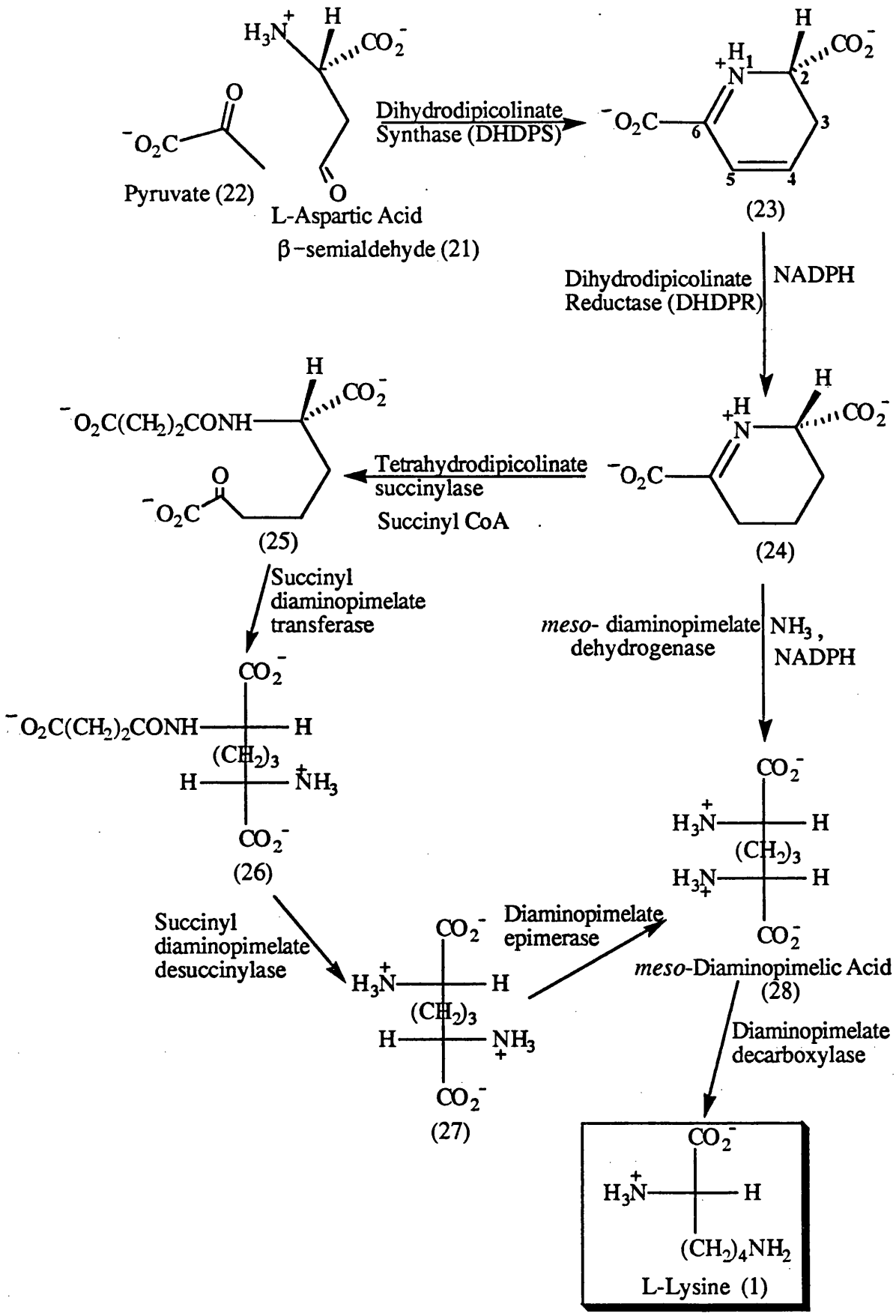


Scheme 4 : Aspartate family of amino acids

These three are known as the aspartate family of amino acids. Aspartate kinase catalyses the formation of aspartyl phosphate (20) from aspartic acid (19). It has been detected in a variety of plant tissues including maize,[44,45] pea,[46] barley,[47,48] wheat,[49,50] spinach[51] and carrot.[52,53] Aspartate semialdehyde dehydrogenase catalyses the conversion of aspartyl phosphate (20) into aspartic acid-β-semialdehyde (21). This enzyme has been shown to be present in pea[54] and a range of maize tissues.[55]

A further seven unique enzyme catalysed reactions are required for the synthesis of L-lysine (1) from pyruvate (22) and L-aspartic acid-β-semialdehyde (21) **(Scheme 5).** These enzymes have been characterised in *Escherichia coli.* However, only enzymes catalysing the first two and last two steps have been isolated from plants and characterised.

Dihydrodipicolinate Synthase (DHDPS) catalyses the condensation of L-aspartic acid-β-semialdehyde (21) with pyruvate (22) to give L-2,3-dihydrodipicolinic acid (23). The reaction presumably proceeds in several steps. Carbon-carbon bond formation must occur between the aldehyde group of L-aspartic acid-β-semialdehyde (21) and the methyl carbon of pyruvate (22) with loss of the elements of water. Imine formation also takes place with the loss of a second mole of the elements of water. DHDPS has been isolated from bacteria, such as *E. coli*,[56] and from plant systems, such as maize[45] and wheat.[57] The second stage in the biosynthesis is the NADPH dependent reduction of L-2,3-dihydropicolinic acid (23) to L-2,3,4,5-tetrahydrodipicolinic acid (24) catalysed by



Scheme 5 : Diaminopimelate Pathway to L-Lysine

dihydrodipicolinate reductase (DHDPR). DHDPR has been isolated from maize.[58]

Enzymes which catalyse the next three steps in the pathway have not been isolated from plants, but have been isolated from micro-organisms. The NADPH dependent reduction of L-2,3-dihydrodipicolinic acid (2) to L-2,3,4,5-tetrahydrodipicolinic acid (24) is followed by succinylation of the amino group with succinyl CoA. This reaction is catalysed by tetrahydrodipicolinate succinylase (THDPS). Succinylation serves to protect the amino group of (24) during the synthesis of LL-2,6-diaminopimelate (27). The *N*-succinyl intermediate (25) is aminated at the ε-position in an amino transferase reaction with glutamate as the amino group donor. The enzyme which catalyses this step is succinyl diaminopimelate transferase. Desuccinylation of *N*-succinyl diaminopimelic acid (26) with succinyl diaminopimelate desuccinylase results in the formation of LL-2,6-diaminopimelic acid (27). This is epimerised to *meso*-2,6-diaminopimelic acid (28) catalysed by diaminopimelate epimerase. Finally decarboxylation at the D-centre of *meso*-2,6-diaminopimelic acid (28) gives L-lysine (1). Diaminopimelate decarboxylase has been detected in *E. coli*,[59] and in maize[60] and wheat germ.[61,62]

An unusual enzyme, *meso*-diaminopimelate dehydrogenase, has been detected in maize.[63] It seems in maize that there could be an alternative biosynthetic route which bypasses several stages of the proposed pathway and directly converts L-2,3,4,5-tetrahydrodipicolinic acid (24) into *meso*-2,6-diaminopimelate (28). The diaminopimelate pathway to L-lysine (1) is discussed in more detail in Chapter 2.

The enzymic reactions that we have investigated are the dihydrodipicolinate synthase (DHDPS) and dihydrodipicolinate reductase (DHDPR) steps of the diaminopimelate pathway to L-lysine (1) found in bacteria.

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

meso-2,6-Diaminopimelic acid (28) is a building block of the peptidoglycan of most Gram-negative as well as Gram-positive bacteria. It is introduced into this network as part of the cross-linking moiety between polysaccharide fibres.[64,65]

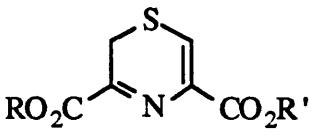
Inhibitors of peptidoglycan biosynthesis such as β-lactams,[45] fosfomycin,[66] D-cycloserine,[67] L-alanyl-L-aminophosphonic acid[68] and vancomycin, for instance, have powerful antibiotic properties. Hence, the enzymes of peptidoglycan processing appear to be good targets for the rational design of new antibacterial agents.[66] The absence of the peptidoglycan network in mammals also implies that such an approach to inhibitors of the DAP pathway to L-lysine (1) should provide selective toxicity against bacteria.

It is noteworthy that LL-2,6-DAP (27), *meso*-2,6-DAP (28) and L-lysine (1) are biosynthetically closely related (**Scheme 5**) as lysine is formed from LL-2,6-DAP by epimerisation followed by decarboxylation of *meso*-2,6-DAP (28).[64,69] Hence, potential inhibitors of the DAP pathway to L-lysine (1) should have antibacterial properties with no mammalian toxicity. It is assumed that the DAP pathway to lysine in plants is analogous to the enzymic

production of lysine in bacteria. Therefore, inhibitors may be produced which have antibacterial and/or herbicidal activity.

The synthesis of L-aspartic acid-β-semialdehyde (21) was first reported by Black and Wright in 1955.[70] In Chapter 3 we report an improved synthesis of this starting substrate. Mechanistic studies carried out using this compound and biological results obtained doing this work will also be discussed in Chapter 3. Analogues of L-aspartic acid-β-semialdehyde (21) have been synthesised and tested as inhibitors of DHDP Synthase. This will be discussed in Chapter 4. Derivatives of pyruvate (22) have been prepared. The interesting test results that they showed will be discussed in Chapter 5.

The first synthesis of the proposed intermediate L-2,3,4,5-tetrahydrodipicolinic acid (24) was achieved within our group by Dr. L. Couper. This will be discussed in Chapter 2. Analogues of L-2,3,4,5-tetrahydrodipicolinic acid (24) and L-2,3-dihydrodipicolinic acid (23) with an additional heteroatom in the ring, namely 1,4-thiazines (29) and 1,3-thiazoles (30), have been synthesised. Their preparation and test results will be discussed in Chapters 6 and 7 respectively.



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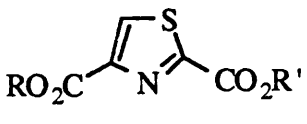

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Chapter [2] - The Diaminopimelate (DAP) Pathway to L-Lysine.

Introduction

The diaminopimelate (DAP) pathway of lysine biosynthesis has been mainly studied in bacteria and is also considered to be the major pathway operating in higher plants. Among the seven enzymes of *Escherichia coli* involved in the DAP pathway from L-aspartic acid-β-semialdehyde (21) to L-lysine (1), only four of them have been identified in plants. In this Chapter reported studies on each of the enzymes of the DAP pathway in bacteria and in higher plants will be reviewed. This will include a discussion on enzyme isolation and purification, mechanistic studies carried out on each particular enzymic step, and compounds which have been synthesised and tested as substrates or inhibitors for individual enzymes.

2.1 Dihydrodipicolinate Synthase.

Dihydrodipicolinate synthase (DHDPS) catalyses the condensation of pyruvic acid (22) and L-aspartic acid-β-semialdehyde (21) to form L-2,3-dihydrodipicolinic acid (23). It is a branch point enzyme in the aspartate family of amino acids (**Scheme 4**), which leads to L-lysine (1) via 7 enzymic steps and the

intermediate *meso*-2,6-DAP (28). DHDPS has been detected in a broad range of species and tissues.[45,71]

In bacteria DHDPS is subjected to two different types of control: the *E. coli* DAP pathway is characterised by a lysine feedback inhibition of DHDPS, while *Bacillus* species have a lysine insensitive form of DHDPS.[72,73] DHDPS has been purified 5000-fold from crude extracts of *E. coli W*.[74] The enzyme has been shown to be homogenous by polyacrylamide gel electrophoresis and bears a negative charge in the pH range 6.0 to 9.2. Its molecular weight was determined as 134,000 daltons.

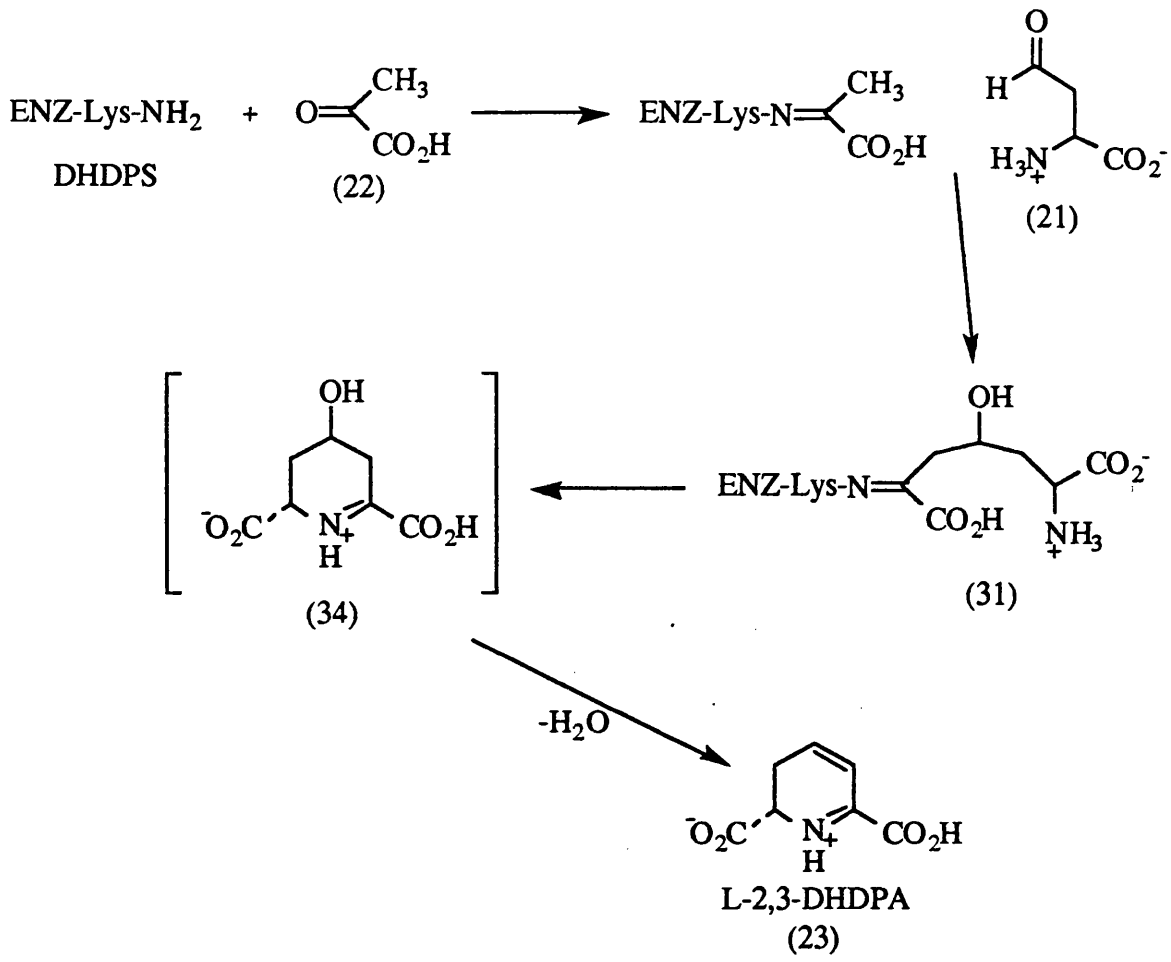
All plant DHDPS enzymes characterised at the regulatory level belong to the lysine sensitive type. The plant enzyme has been detected in chloroplasts of spinach leaves and is presumed, as are the other enzymes of the plant DAP pathway, to be active only in such organelles.[75,76] There are four reports which focus on higher plant DHDPS. The enzyme was first demonstrated by Cheshire and Miflin[45] in 1975 using maize seedlings as the source of the enzyme. The spinach leaf DHDPS was partially purified and characterised in 1981 by Wallsgrove and Mazelis.[77] They succeeded in producing a fraction of the enzyme from spinach leaves that was purified 87-fold and had a specific activity of 836 units mg^{-1} protein.

Wheat DHDPS, in a semi-pure form, was more recently used to investigate the kinetics and the inhibitory features of DHDPS.[52] The enzyme was purified about 5100-fold from suspension cultured cells of wheat (*Triticum aestivum* var. Chinese Spring). The enzyme has an average molecular weight of 123,000 daltons as determined by gel filtration and exhibited maximum activity at pH 8.0.

The most recent isolation of the enzyme was from *Nicotiana sylvestris* by Ghislain *et al.*[7,8] The synthase was localised in the chloroplasts and identified as a soluble stromal enzyme by enzymatic and immunological methods. It has been purified and characterised and the molecular weight of the enzyme was shown to be 164,000 daltons by an electrophoretic method. By carrying out isotopic labelling experiments with ^{14}C-pyruvate, the enzyme was shown to be composed of four identical sub-units of 38,500 daltons.

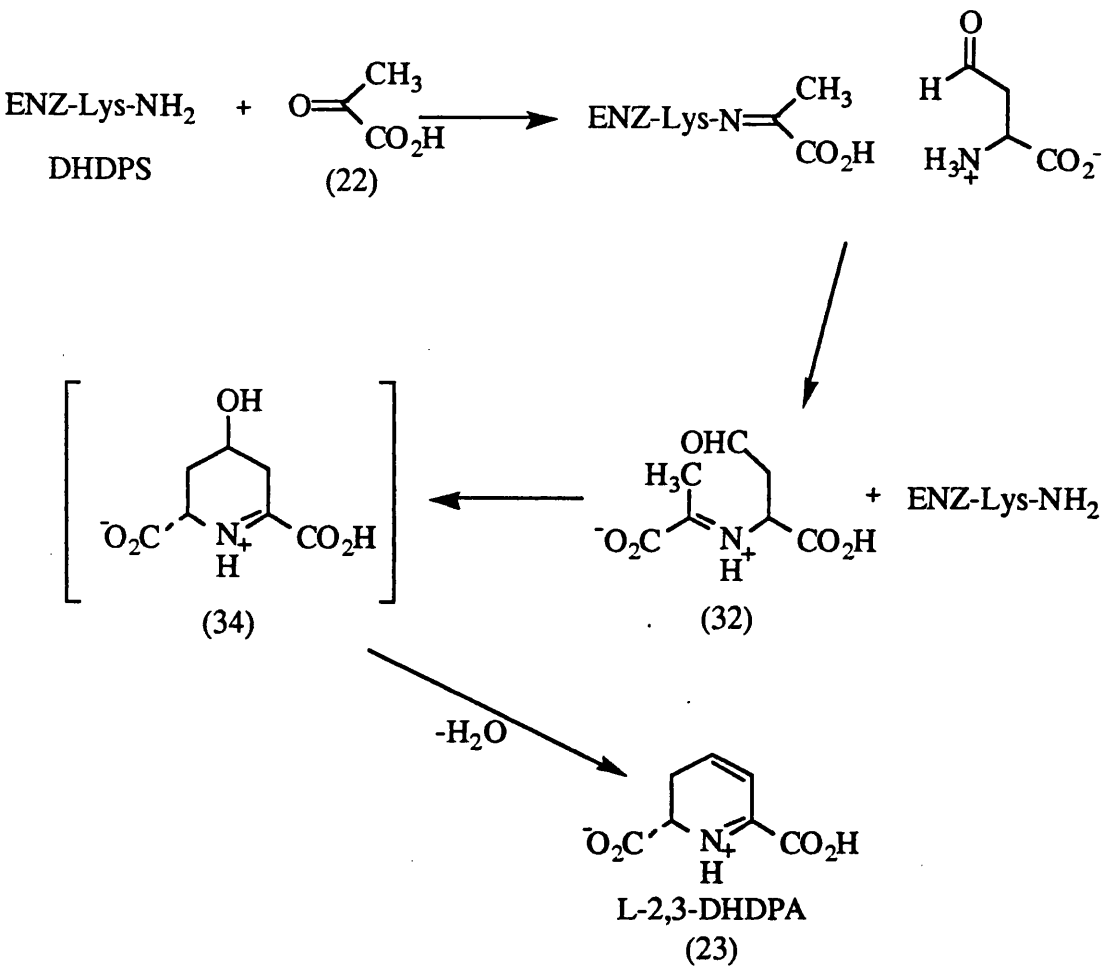
2.2 Mechanism of Dihydrodipicolinate Synthase.

The sequence of steps in the mechanism of formation of L-2,3-dihydrodipicolinic acid (L-2,3-DHDPA) (23) from L-aspartic acid-β-semialdehyde (21) and pyruvate (22) has not yet been fully established. Pyruvate binds to the enzyme via an amino group on the lysine.[57,74] One possible mechanism is a C-C bond formation between the aldehyde group of (21) and the methyl carbon of (22) in an addition reaction to give the enzyme bound intermediate (31). This would be followed by ring closure to give 4-hydroxy-L-2,3,4,5-THDPA (34). Loss of a mole of the elements of water gives the enzymic product (23) **(Scheme 6).** An alternative mechanism could be first of all the formation of the imine intermediate (32). This would be followed by a ring closing addition step to give 4-hydroxy-L-2,3,4,5-THDPA (34). As before, the loss of a mole of the elements of water would give the enzymic product (23) **(Scheme 7).**



Scheme 6

Kinetic studies carried out by Schedlarski and Gilvarg[74] on DHDPS isolated from *E. coli* suggest that the enzyme functions in C-C bond formation by first of all forming an imine linkage with pyruvate (22). They have shown that there is irreversible loss of enzymatic activity upon the addition of sodium borohydride only in the presence of pyruvate (22). This suggests that the formation of a Schiff's base intermediate between DHDPS and pyruvate (22) takes place in the enzymic reaction. Moreover they have established by chromatographic and electrophoretic methods that the Schiff's base is



Scheme 7

between the carbonyl group of pyruvate (22) and an ε-amino group of a lysine group attached to the protein.

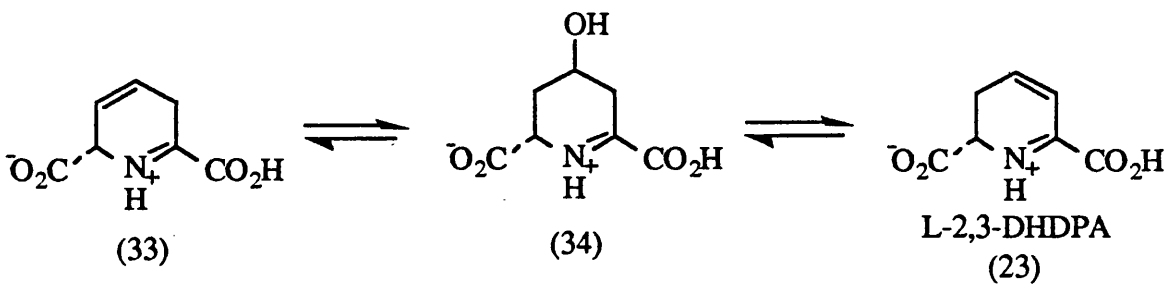
From kinetic studies caried out on DHDPS isolated from wheat suspension cultures, Kumpaisal *et al*[5 7] have proposed that the reaction proceeds in a 'ping-pong' mechanism. First of all pyruvate (22) binds to the enzyme to form a Schiff's base (K_M 11.76 mM). K_M is the Michaelis constant. This is the ≃ dissociation constant of the enzyme-substrate complex. Water is then released, followed by binding of L-aspartic acid-β-semialdehyde (21) (K_M 0.88 mM).

Footnote :

B. Laber, F. X. Gomis-Rüth, M.J. Romao and R. Hober, Biochem. J., 1992, 288, 691 report crystals of DHDP Synthase and K_M of pyruvate of 0.57mM.

Nucleophilic attack of the pyruvyl enamine on the L-aspartic acid-β-semialdehyde aldehyde group could then take place to give intermediate (31). The mechanism of DHDPS will be discussed further in Chapters 3 and 4.

The product of the enzymatic reaction catalysed by DHDPS was found to be very labile, severely limiting efforts for its thorough characterisation. It has been suggested that the proposed product, L-2,3-DHDPA (23), would be expected to be in equilibrium with L-2,5-dihydrodipicolinic acid (33) and 4-hydroxy-L-2,3,4,5-THDPA (34) **(Scheme 8).**[74] It is not clear which of these compounds is the immediate product of the enzymatic reaction.



Scheme 8

2.3. Inhibition of Dihydrodipicolinate Synthase.

The DHDPS enzyme from *N. sylvestris*, isolated by Ghislain *et al*,[78] is strongly inhibited by lysine with an ID_{50} of 15 μM. The ID_{50} value denotes the dose of inhibitor required to produce 50% inhibition.* *S*-(2-Aminoethyl)-L-cysteine and γ-hydroxylysine, two

* As long as $[S] = K_M$.

lysine analogues, were found to be only weak inhibitors. An analogue of pyruvate (22), 2-oxobutyrate, competitively inhibited the enzyme and was found to act at the level of the pyruvate-binding site.

With DHDPS isolated from wheat cultures[51] allosteric inhibition was observed with increasing concentrations of L-lysine (1) and its structural analogues, including *threo*-4-hydroxy-L-lysine and *S*-(2-aminoethyl)-L-cysteine, with respective ID_{50} values of 51, 141 and 288 mM. These amino acids were competitive inhibitors with respect to L-aspartic acid-β-semialdehyde (21) and non-competitve inhibitors with respect to pyruvate (22). The wheat enzyme was also inhibited by Zn^{2+}, Cd^{2+} and Hg^{2+} and also by sulphydryl inhibitors such as *p*-(hydroxymercuri)benzoic acid and *p*-chloromercuribenzenesulphonic acid.

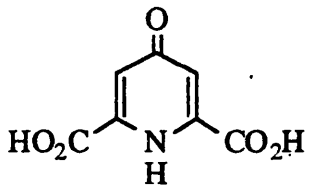
Within our group at Glasgow, Borthwick[121] has tested analogues of L-2,3-DHDPA (23) and L-2,3,4,5-THDPA (24) on DHDPS isolated from *E. coli.* These compounds, which include dipicolinic acid (36) analogues, chelidamic acid (35) analogues and piperidine-2,6-dicarboxylic acid (37) analogues, were synthesised by Dr. L. Couper.

Chelidamic acid (35) and its *N*-substituted analogues showed good inhibition of DHDPS at low concentrations. *N*-Methylchelidamic acid showed the best inhibition, 63% was observed at 0.1 mM. The diesters of chelidamic acid and their *N*-substituted analogues also showed good inhibition.

Dipicolinic acid (36) showed significant inhibition of DHDPS at 0.5 mM, as did some its analogues. Changing the diacid to a diester, a dicyano, a diimidate or a ditetrazole derivative had little effect on

the amount of inhibition. Poor inhibition was observed with the fully saturated piperidine-2,6-dicarboxylic acid (37) analogues. However piperid-4-one-2,6-dicarboxylic acid and its *N*-methyl derivative showed good inhibition.

Compounds with only one carboxyl group such as pipecolinic acid, proline and picolinic acid and their analogues showed little or no inhibition of DHDPS, showing the importance of two carboxylic acid groups in binding to the active site of the enzyme.



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Inactivation of Wheat DHDPS by 3-Bromopyruvate.[80]

3-Bromopyruvate was first introduced by Meloche[79] as an active site directed alkylating reagent and has proved to be quite versatile in labelling pyruvate binding sites of many enzymes.[80] Kumpaisal *et al.*[81] have studied the inhibitory effects of 3-bromopyruvate on DHDPS isolated from wheat cultures. The assay system they used was developed by Yugari and Gilvarg[56] and involved *o*-aminobenzaldehyde. The chromophore of the product formed from L-2,3-DHDPA (23) and *o*-aminobenzaldehyde was observed by its spectrophotomeric absorbance at 540 nm.

They have shown that 3-bromopyruvate inhibits wheat DHDPS considerably at 1 mM. Kinetic studies have shown that this compound inhibits in a competitive manner with respect to pyruvic acid (22) and in a non-competitive manner with respect to L-aspartic acid-β-semialdehyde (21). The calculated K_I for 3-bromopyruvate was 1.88 mM. K_I is the dissociation constant of the non-productive enzyme-inhibitor complex.

From the results of Kumpaisal *et al.* it appears that 3-bromopyruvate inactivates DHDPS by alkylating nucleophilic amino acid residues at or near the active site. The competitive type of inhibition with respect to pyruvate and the protection by pyruvate against inactivation indicate that reacting groups at or near the pyruvate-binding site of DHDPS are modified by 3-bromopyruvate.

The pseudo first order kinetics observed for the initial inactivation with 3-bromopyruvate is consistent with a two step binding process. The first process is probably a rapid, reversible Schiff's base formation between the enzyme and the inhibitor, followed by a slow, irreversible alkylation step. The K_M of pyruvate (11.8 mM) is considerably larger than the K_I of 3-bromopyruvate (1.8 mM). The electron withdrawing bromine in 3-bromopyruvate promotes Schiff's base formation between the amino group of an amino acid residue of the enzyme and the carbonyl carbon of 3-bromopyruvate, resulting in a higher affinity of 3-bromopyruvate than of pyruvate.

2.4. Biosynthesis of Dipicolinic Acid (36) in *Bacillus subtilis.*

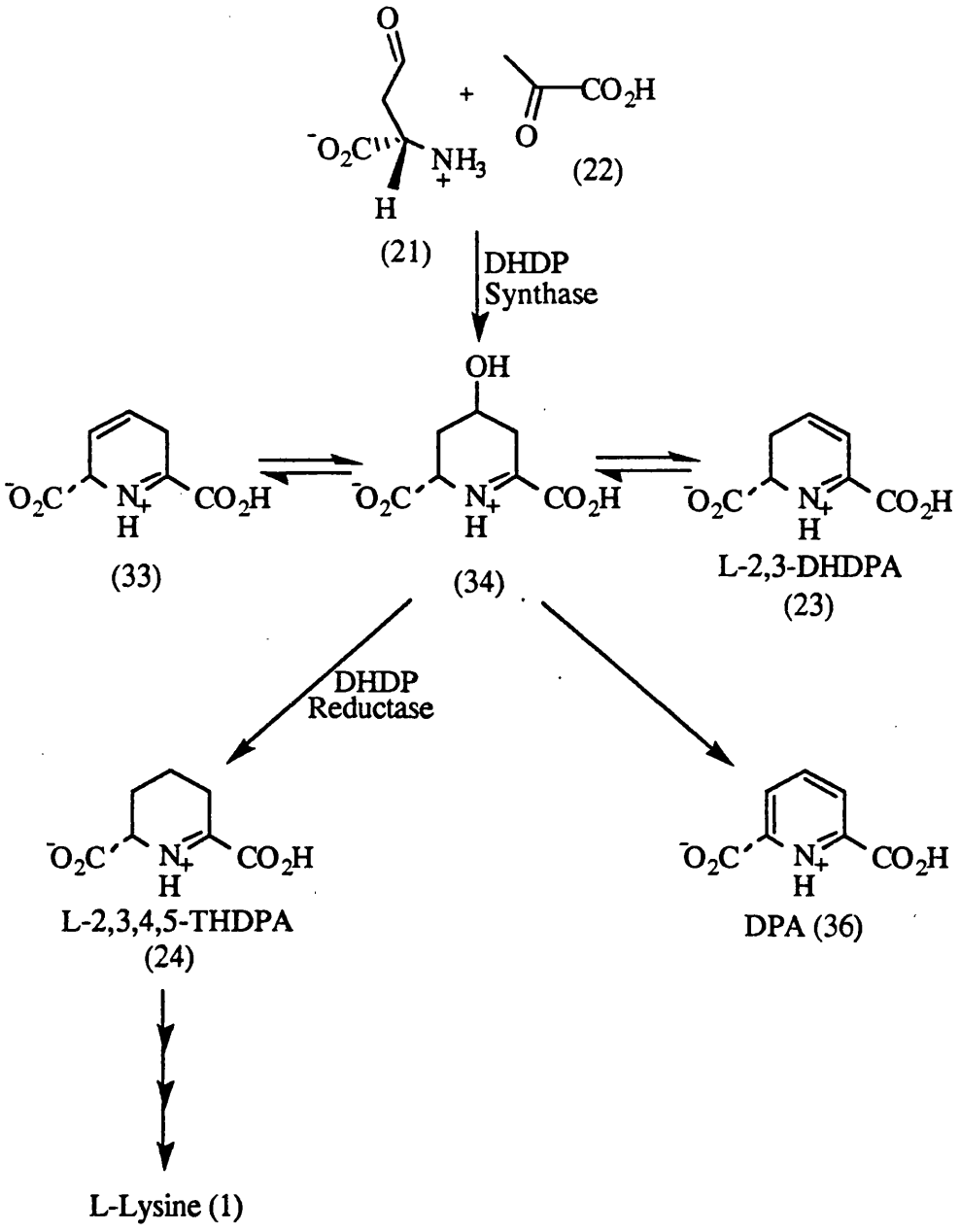
In 1966 Bach and Gilvarg[82] demonstrated that cell free extracts prepared from sporulating cultures of *Bacillus megaterium* can carry out the net synthesis of dipicolinic acid (DPA) (36) from pyruvate (22) and L-aspartic acid-β-semialdehyde (21). Subsequent results on DPA (36) formation in spore forming bacteria have suggested that the reaction leading to the synthesis of DPA (36) from L-2,3-DHDPA (23), which is the reaction product of DHDP Synthase in *E. coli*,[56] occurs as a branch of the pathway for lysine biosynthesis in bacteria[83-85] **(Scheme 9).**

It is difficult to demonstrate the conversion of L-2,3-DHDPA (23) into DPA (36) as an isolated reaction, since the substrate is extremely unstable. It has not been confirmed whether the conversion is an enzymic process or just spontaneous air oxidation of L-2,3-DHDPA (23).

The chemical synthesis of DPA (36) from oxaloacetic acid and L-aspartic acid-β-semialdehyde (21) was investigated by Kimura in 1973.[86] This work will be discussed in more detail in Chapter 3.

2.5. Dihydrodipicolinate Reductase.

The enzyme dihydrodipicolinate reductase (DHDPR) catalyses a pyridine nucleotide linked reduction of L-2,3-dihydrodipicolinic acid (23) to L-2,3,4,5-tetrahydrodipicolinic acid (24) in the DAP pathway to L-lysine (1) **(Scheme 9).**



Scheme 9

The enzyme was first described by Farkas and Gilvarg in 1965 from *E. coli*.[89] More recently, Tamir and Gilvarg[87] modified their procedure by carrying out additional purification steps which led to a preparation of this enzyme from *E. coli* that was homogeneous as judged by sedimentation pattern and gel filtration and was at least

95% pure as judged by gel electrophoresis. The molecular weight was found to be 110,000 daltons; the K_M value obtained for L-2,3-DHDPA (23) was 9.0 μM, and the pH optimum for the reaction was 7.0 with either NADH or NADPH as the co-catalyst.

DHDPR has also been isolated and partially purified by Tyagi *et al.*[58] from three week old maize kernels. 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* to synthesise *meso*-2,6-diaminopimelate (28) from L-aspartic acid-β-semialdehyde (21) and pyruvate (22). In a study of its properties, the K_M value obtained for L-2,3-DHDPA (23) 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 daltons.

2.6. Mechanism of Dihydrodipicolinic Reductase.

L-2,3-Dihydrodipicolinic Acid (23)

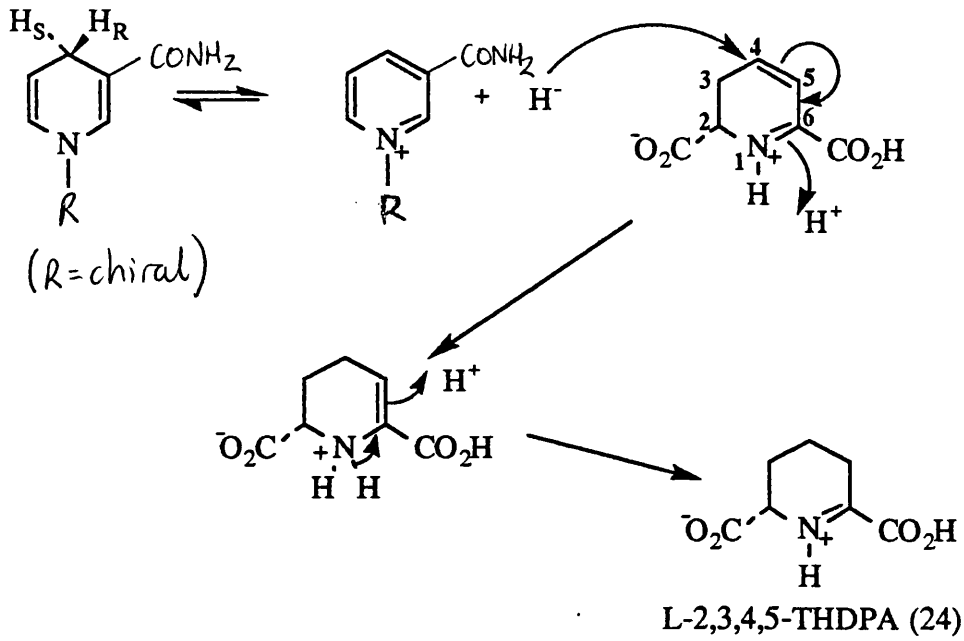
The substrate for this enzymic step is the proposed product of DHDP Synthase, L-2,3-DHDPA (23). As previously mentioned, this product is very labile and is immediately oxidised by either an enzymic or a chemical process to dipicolinic acid (DPA) (36) and, as such, the purification and characterisation of L-2,3-DHDPA (23) has proved very difficult. Kimura[86] suggested that L-2,3-DHDPA (23) might exist in equilibrium with L-2,5-DHDPA (33) and 4-hydroxy-L-

2,3,4,5-THDPA (34) (**Scheme 8**). Theoretically it is also possible for L-2,3-DHDPA (23) to exist as an open chain compound. It has not been established which is the true physiological substrate of the reductase enzyme although it has been assumed to be L-2,3-DHDPA (23).

For the studies carried out on maize DHDPR,[53] L-2,3-DHDPA (23) was synthesised by the condensation of L-aspartic acid-β-semialdehyde (21) with oxaloacetic acid in alkali. The reaction was accompanied by evolution of a stoichiometric amount of CO_2. L-2,3-DHDPA (23) was partially purified and isolated as the barium salt. The compound was stored in water at -80 °C due to its instability at room temperature at neutral pH. As such, only freshly synthesised compound was used in the enzyme assays. This reaction will be discussed further in Chapter 3.

Mechanism of Formation of L-2,3,4,5-Tetrahydrodipicolinic Acid (24).

The enzymes that catalyse hydride transfer reactions have been shown to fold into two distinct domains with cofactor in one domain and substrate in the other. 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 hydride anion attacks at the 4-position of L-2,3-DHDPA (23) (**Scheme 10**). It has not yet been established whether the enzyme donates the *pro-S* or the *pro-R* hydrogen of its co-factor NADPH.



Scheme 10

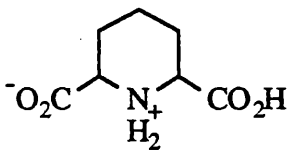
L-2,3,4,5-Tetrahydrodipicolinic Acid (24).

L-2,3,4,5-THDPA (24) is the second intermediate on the DAP pathway to L-lysine (1). It is formed from the NADPH dependent reduction of L-2,3-DHDPA (23) catalysed by DHDPR. There have been several syntheses claimed of this intermediate.

Shapshak[88] reported the synthesis of D-2,3,4,5-THDPA by treatment of *meso*-2,6-diaminopimelic acid (28) with L-amino acid oxidase isolated from *Neurospora crassa*. There was no chemical or spectroscopic evidence given for the product he made.

Farkas and Gilvarg[89] investigated the enzymic reduction of L-2,3-DHDPA (23) to L-2,3,4,5-THDPA (24). DHDPR was isolated from *E. coli* and partially purified. Reduction of the enzymic reaction product (24) with sodium borohydride gave piperidine-2,6-

dicarboxylic acid (37). This was characterised by comparison of its R_F value with authentic piperidine-2,6-dicarboxylic acid. The solvent system used was methanol-water-pyridine (8:1:1). The R_F values were identical.

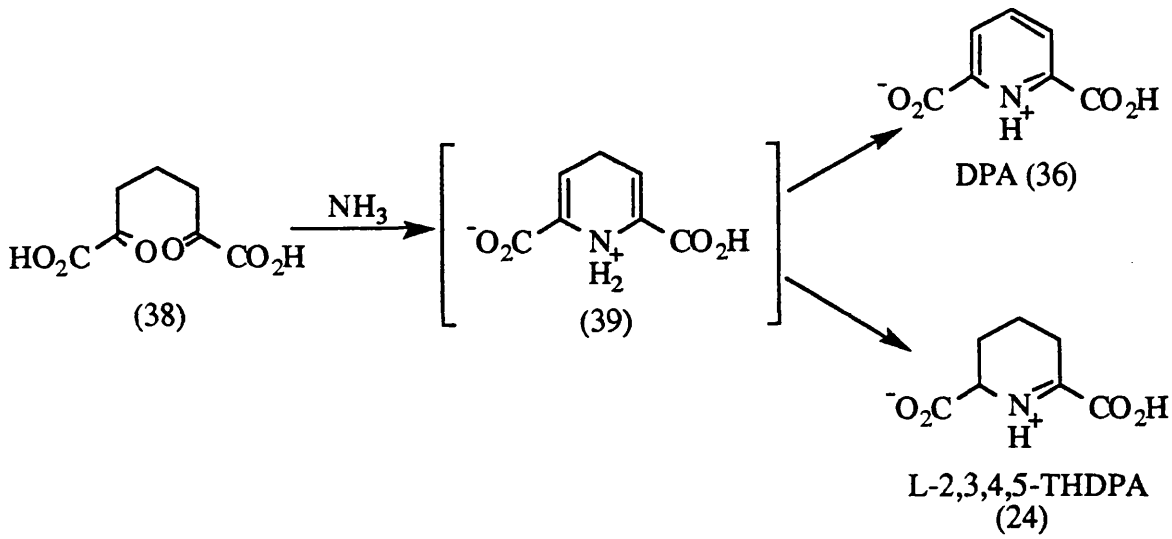


(37)

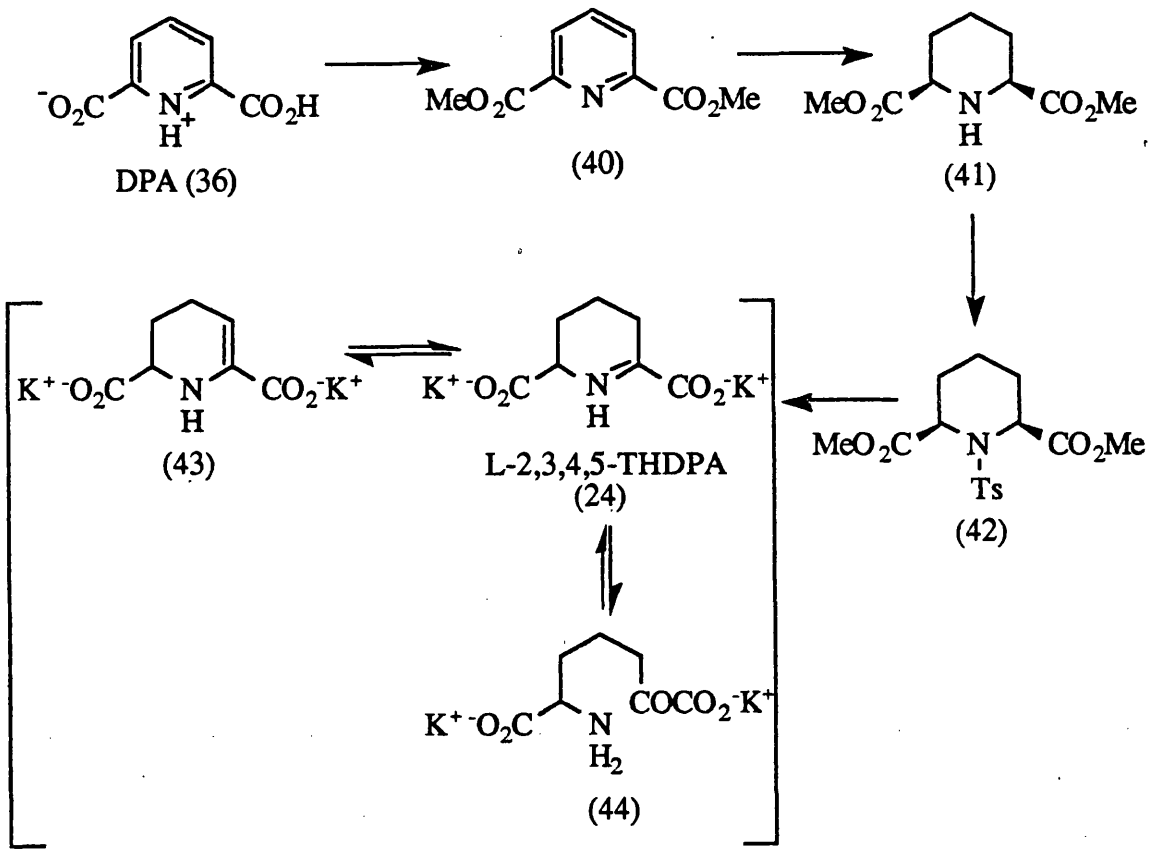
Kimura and Sasakawa[90] reported the formation of DPA (36) and L-2,3,4,5-THDPA (24) on cyclisation of αα'-dioxopimelic acid (38) with ammonia. Since the reaction rate was not affected by the presence or absence of oxygen, they assumed that the presumed initial product, 1,4-dihydrodipicolinic acid (39), disproportionated to DPA (36) and L-2,3,4,5-THDPA (24) **(Scheme 11)**. The products were identified by UV absorption (DPA at 270 nm) and by colour reactions with ninhydrin and *o*-aminobenzaldehyde (L-2,3,4,5-THDPA).

The potassium salt of L-2,3,4,5-THDPA (24) has been prepared by the elimination of *p*-toluenesulphinic acid from the *N*-toluenesulphonyl derivative of dimethyl *N*-tosyl-*cis*-piperidine-2,6-dicarboxylate (42) by one of my co-workers Dr. L. Couper **(Scheme 12)**.[91]

Dimethyl *N*-tosyl-*cis*-piperidine-2,6-dicarboxylate (42) was synthesised from dimethyl *cis*-piperidine-2,6-dicarboxylate (41) by stirring it overnight in pyridine with toluenesulphonyl



Scheme 11



Scheme 12

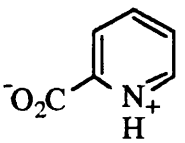
chloride. Hydrogenation of dimethyl dipicolinate (40) gave (41) in good yield. Dimethyl dipicolinate (40) was made from dipicolinic acid (36) by heating at reflux in methanol and conc. sulphuric acid.

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 1R-45 (hydroxide form) for 24 hours to remove *p*-toluenesulphinic acid. ^{1}H and ^{13}C-NMR data indicated that L-2,3,4,5-THDPA (24) exists in solution in equilibrium with the corresponding enamine (43) and an open chain form (44). THDPA (24) was shown to be unstable at neutral or acidic pH.[91]

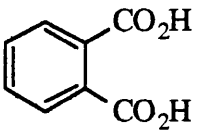
2.7. Inhibition of Dihydrodipicolinate Reductase.

The DHDPR enzyme isolated from *E. coli* by Tamir and Gilvarg[87] was inhibited by DPA (36). It was found to be a competitive inhibitor with the substrate with a K_I value of 1 mM. α-Picolinic acid (45) and isophthalic acid (46) were also found to be inhibitors of the reaction. The latter was also shown to be competitive with the substrate with a K_I of 15 mM. The high affinity and competitive nature of the inhibition shown by DPA (36) and to a lesser extent, isophthalic acid (46) strongly suggests that a cyclic form of the substrate binds to the active site on the enzyme.

With the plant DHDPR isolated from maize by Tyagi *et al.*,[58] α-picolinic acid (45), L-pipecolic acid (47), isophthalic acid (46) and



(45)



(46)

isocinchomeronic acid (48) all inhibited the enzyme activity. These compounds all have similar structures to L-2,3-DHDPA (23) (**Table 1**).

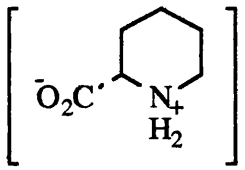
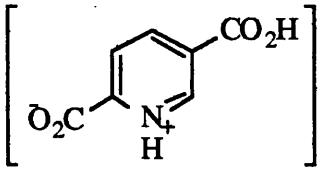
INHIBITOR	% INHIBITION			
	20 mM	10 mM	5 mM	1.5 mM
α-Picolinic Acid (45)	24	4	8	0
L-Pipecolic Acid (47) 	23	30	0	0
Isophthalic Acid (46)	44	20	0	0
Isocinchomeronic Acid (48) 	–	–	50	0
Dipicolinic Acid (36)	–	–	–	100

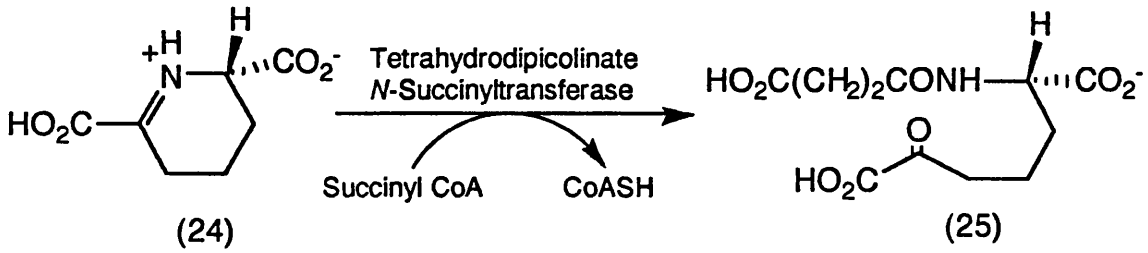
Table 1

Dipicolinic acid (36) was by far the most potent inhibitor that they tested. It was a competitive inhibitor with a K_I value of 0.9

mM. This is analogous to the inhibition shown by DPA (36) on bacterial DHDPR. Similar to the conclusion reached with bacterial DHDPR, competitive inhibition of the reductase reaction by DPA (36) suggests that the substrate for this enzyme is in the ring form rather than the open chain form.

2.8. Succinyl CoA:Tetrahydrodipicolinate *N*-Succinyltransferase.

Succinyl CoA:Tetrahydrodipicolinate *N*-Succinyltransferase (Succinyl CoA:THDPS) catalyses the *N*-succinylation of L-2,3,4,5-THDPA (24) by succinyl coenzyme A to form L-2-(succinylamino)-6-oxopimelic acid (25) **(Scheme 13).** Succinylation serves to protect the amino group during the synthesis of LL-2,6-diaminopimelate (27). Either acetyl CoA or succinyl CoA can be used for the acylation step used in protection in micro-organisms.



(24) (25)

Scheme 13

Succinyl CoA:THDPS was first isolated from *E. coli* by Gilvarg in 1961[92] and more recently, enzyme activity has been demonstrated in *Brevibacterium* by Tosaka and Takinami.[93] However, the

isolation, physical characterisation and initial kinetic studies of this enzyme have only relatively recently been described by Simms *et al.* in 1984.[94]

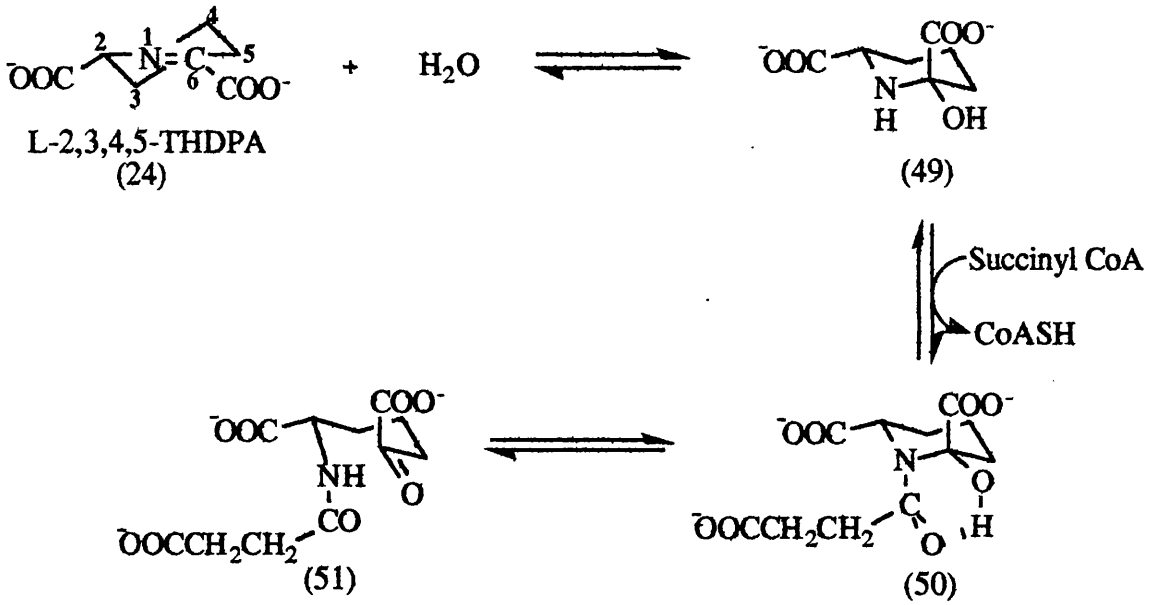
2.9. Mechanism of Succinyl CoA:Tetrahydrodipicolinate *N*-Succinyltransferase.

In 1986 Gilvarg *et al.*[95] studied analogues of L-2,3,4,5-THDPA (24) for their ability to act as substrates or inhibitors of the succinylase. From the results obtained and studies on the interactions of these compounds at the active site of the enzyme they were able to draw certain conclusions concerning the catalytic mechanism of the enzyme and the structural requirements of the catalytic site. From these conclusions they proposed a stereochemical model for the succinylation of L-2,3,4,5-THDPA (24) **(Scheme 14)**. This involves the succinylase enzyme first binding L-2,3,4,5-THDPA (24). This is followed by the hydration of the imine group where water adds to the double bond *cis* to the C-2 carboxyl group to give 2-hydroxypiperidine-2,6-dicarboxylic acid (49) in which the two carboxyl groups are *trans* to each other. The hydrated product (49) is then succinylated to give (50). This is followed by ring opening to give the acyclic product L-2-(succinylamino)-6-oxopimelic acid (51).

The carboxyl groups are the primary ligands involved in the binding of L-2,3,4,5-THDPA (24) to the succinylase enzyme. The hydration of the imine occurs enzymically, but the enzyme does not necessarily catalyse ring opening. Opening of the ring may occur in a

concerted manner during or after succinylation. It is also possible that ring opening occurs after dissociation of the succinylated THDPA (50) from the enzyme.

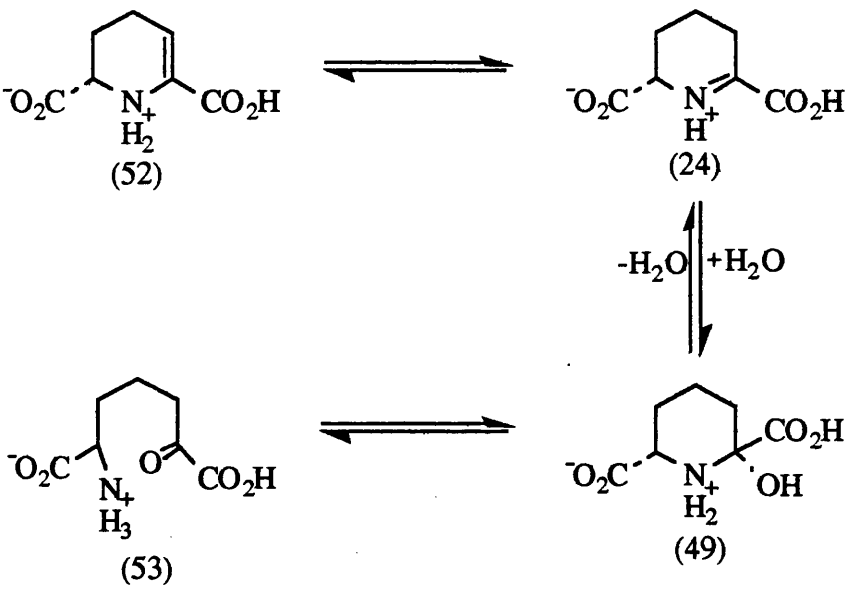
In aqueous solution, L-2,3,4,5-THDPA (24) can exist in a number of forms which are shown in **Scheme 15.** In Section 2.10



Scheme 14

certain acyclic analogues of L-2,3,4,5-THDPA (24) will be shown to be substrates for the succinylase enzyme and, as such, it is reasonable to postulate that the open chain form (53) of L-2,3,4,5-THDPA could be the true substrate of the enzyme. Gilvarg *et al.*[95] however, have proved that this is not the case. They carried out experiments to measure the spontaneous rate of ring opening of L-2,3,4,5-THDPA (24) using 2,4,6-trinitrobenzenesulphonic acid as a trapping agent for primary amines. Their results showed that the non-enzymatic rate of ring opening is insignificant compared to the enzyme-catalysed

rate of succinylation. Hence, the acyclic form of L-2,3,4,5-THDPA (53) is unlikely to be the true enzyme substrate although it undoubtedly exists in aqueous solution, but only as a small fraction of the total THDPA.



Scheme 15

2.10. Inhibition of Succinyl CoA:Tetrahydrodipicolinate *N*-Succinyltransferase.

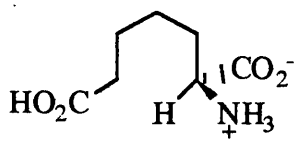
As previously mentioned, inhibitor and substrate studies have been carried out on succinyl CoA:THDPS by Gilvarg *et al.*[9 5] In the succinylase enzyme assay, transfer of the succinyl group from succinyl CoA was followed by measuring the appearance of the free sulphydryl group of CoASH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent). The enzyme assay contained 100 m M

potassium phosphate, 0.5 mM DTNB, L-2,3,4,5-THDPA (24) and the succinylase enzyme. Compounds were tested initially at 5 mM. The reaction was started with the addition of succinyl CoA and was followed by looking at the spectrophotomeric absorbance at 412 nm. The compounds which were tested could be split into 2 categories: acyclic and cyclic analogues of L-2,3,4,5-THDPA (24).

Acyclic Analogues of L-2,3,4,5-THDPA.

DL-2-Aminopimelic acid was previously found by Simms[96] to be both a substrate and an inhibitor of succinyl CoA:THDPS. Gilvarg *et al.*[95] investigated this further by resolving the racemic mixture of DL-2-aminopimelic acid and testing the enantiomers separately. L-2-Aminopimelic acid (54) was found to be a good substrate and showed a V_{max} similar to the natural substrate, L-2,3,4,5-THDPA (24), but had a K_M approximately 50-fold higher. D-2-Aminopimelic acid was not a substrate of the succinylase, showing that stereochemistry at the α-carbon is important in determining substrate activity. However, D-2-aminopimelic acid was a competitive inhibitor of the enzyme with respect to L-2,3,4,5-THDPA (24) with a K_I value of 0.76 mM.

Related acyclic compounds were also tested for inhibitor activity in the succinylase assay. Increasing the chain length of (54) by one methylene group as in DL-2-aminosuberic acid or by



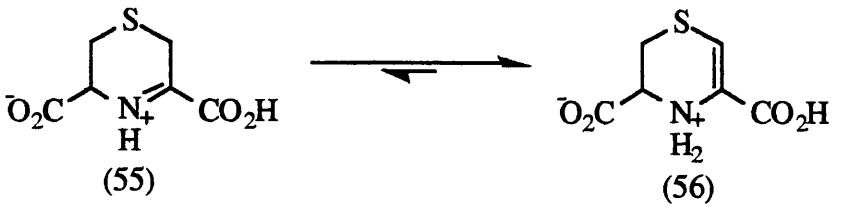
(54)

decreasing by one methylene group as in DL-2-aminoadipic acid resulted in neither inhibition nor substrate activity. Substituting a sulphur for a methylene as in L-2-amino-4-thiaadipic acid gave a high K_I value of 125 mM and little, if any, substrate activity. DL-2-Aminothiapimelic acid gave a K_I of 1.1 mM, similar to the K_I for (54). Close investigation of this compound also revealed that it had a low level of substrate activity. 2-Oxopimelic acid was a weak inhibitor. However, both enantiomers of 2-hydroxypimelic acid had better affinity for the enzyme than the corresponding enantiomers of (54), although L-2-hydroxypimelic acid did not have any substrate activity. L-2-Amino-D-6-hydroxypimelic acid was found to be a better substrate than (54). DL-2,6-Dihydroxypimelic acid was a relatively good inhibitor with a lower K_I value than (54).

Cyclic Analogues of L-2,3,4,5-THDPA.

A number of cyclic compounds were also tested on succinyl CoA:THDPS. Only one was found to be a substrate, namely 3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid (DHT) (55). This compound had a V_{max} about one-half of that of the natural substrate L-2,3,4,5-THDPA (24). Its K_M was slightly higher than L-2-aminopimelic acid (54). This can be explained by the fact that only the enamine tautomeric form of DHT (56) **(Scheme 16)** was

observed in the ^1H NMR spectrum of the disodium salt in aqueous solution. Hence, the high K_M of DHT (55) may be explained because the imine form, which is used as a substrate of the succinylase, makes up only a small fraction of the total DHT.



(55) (56)

Scheme 16

Other cyclic compounds were found to be inhibitors and are listed in **Table 2.** When compared to L-2,3,4,5-THDPA (24), compounds containing fully unsaturated rings (*eg.* pyridine, benzene and pyran analogues) were all weak inhibitors with K_I values at least 50-fold higher than the K_M for L-2,3,4,5-THDPA (24).

Fully saturated rings (*e g.* piperidine, tetrahydropyran, cyclohexane and tetrahydrothiopyran analogues) were also weak inhibitors. A notable feature of these compounds is that *trans*-isomers are better inhibitors than the corresponding *cis*-compounds. As was found with the acyclic compounds, placing a hydroxy group alpha to the carboxyl group greatly enhanced the inhibition. The best inhibition was shown by 2-hydroxytetrahydropyran-2,6-dicarboxylic acid (57). This was a competitive inhibitor with respect to L-2,3,4,5-THDPA (24) with a K_I value of 0.06 μM.

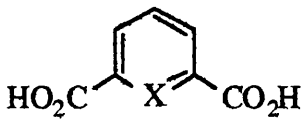
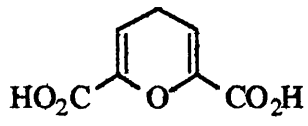
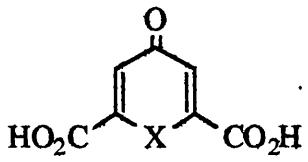
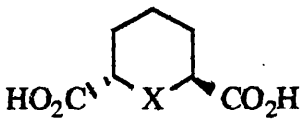
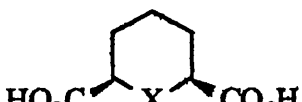
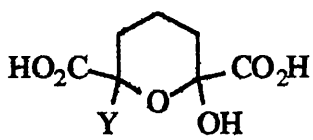
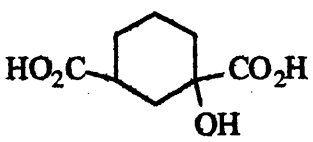
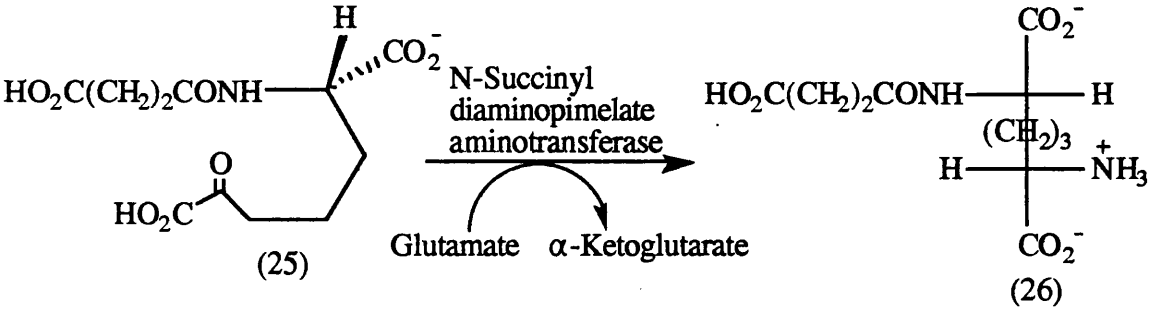
STRUCTURE	X	NAME	K_i (μM)
	N	Dipicolinic Acid	12,800
	CH	Benzene-2,6-dicarboxylic Acid	8,500
		Pyran-2,6-dicarboxylic Acid	1,800
	NH	Chelidamic Acid	2,600
	O	Chelidonic Acid	3,100
	NH	*trans*- Piperidine-2,6-dicarboxylic Acid	2,000
	O	*trans*-Tetrahydropyran-2,6-dicarboxylic Acid	680
	NH	*cis*- Piperidine-2,6-dicarboxylic Acid	63,000
	O	*cis*-Tetrahydropyran-2,6-dicarboxylic Acid	3,900
	Y = H (57)	2-Hydroxytetrahydropyran-2,6-dicarboxylic Acid	0.06
	Y = OH	2,6-Dihydroxytetrahydropyran-2,6-dicarboxylic Acid	0.20
		*trans*-2-Hydroxycyclohexane-2,6-dicarboxylic Acid	5,600
		cis-2-Hydroxycyclohexane-2,6-dicarboxylic Acid	12,000

Table 2

2.11. *N*-Succinyl Diaminopimelate Aminotransferase.

N-Succinyl diaminopimelate aminotransferase catalyses the conversion of L-2-(succinylamino)-6-oxopimelic acid (25) into L-2-(succinylamino)-6-aminopimelic acid (26). The succinylated intermediate is aminated at the ε-position with glutamate as the amino group donor **(Scheme 17).**



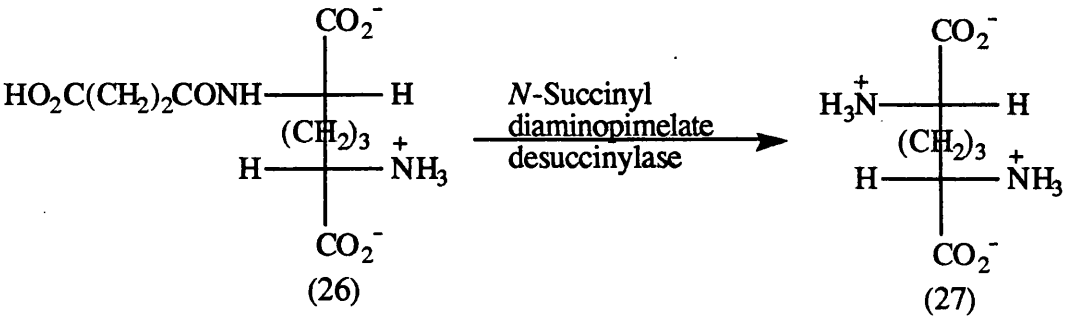
Scheme 17

N-Succinyl DAP aminotransferase has been isolated from *E. coli* and has been partially purified and characterised by Peterofsky and Gilvarg.[97] The aminotransferase is pyridoxal phosphate dependent and like most pyridoxal dependent enzymes the aminotransferase is inhibited by hydroxylamine. Treatment of the enzyme at 0 °C with 0.05 M hydroxylamine causes an inhibition of 95%.

2.12. *N*-Succinyl Diaminopimelate Desuccinylase.

N-Succinyl diaminopimelate desuccinylase catalyses the formation of LL-2,6-diaminopimelic acid (27) by the desuccinylation of L-2-(succinylamino)-6-aminopimelic acid (26) **(Scheme 18).**

The enzyme was detected in *E. coli* by Gilvarg[98] and has been partially purified by Kindler and Gilvarg.[99] The enzyme requires Co^{2+} for activity.

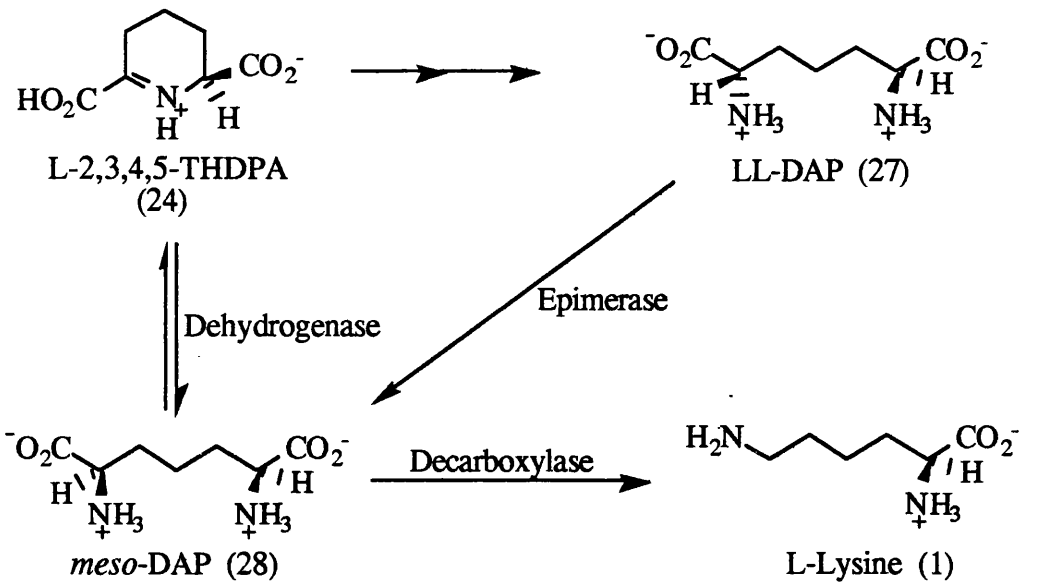


Scheme 18

2.13. LL-Diaminopimelate Epimerase.

Several variations of *meso*-2,6-DAP (28) biosynthesis exist in different bacterial strains.[100] Most bacteria (and higher plants) convert LL-2,6-DAP (27) into the *meso*-form (28) catalysed by LL-diaminopimelate epimerase,[101-103] but some bacteria, such as *Bacillus sphaericus*, bypass the LL-form (27) by directly converting L-2,3,4,5-THDPA (24) into *meso*-2,6-DAP (28) using *meso*-

diaminopimelate dehydrogenase (Scheme 19).[100,104-106] *meso*-Diaminopimelate dehydrogenase will be discussed in Section 2.15.

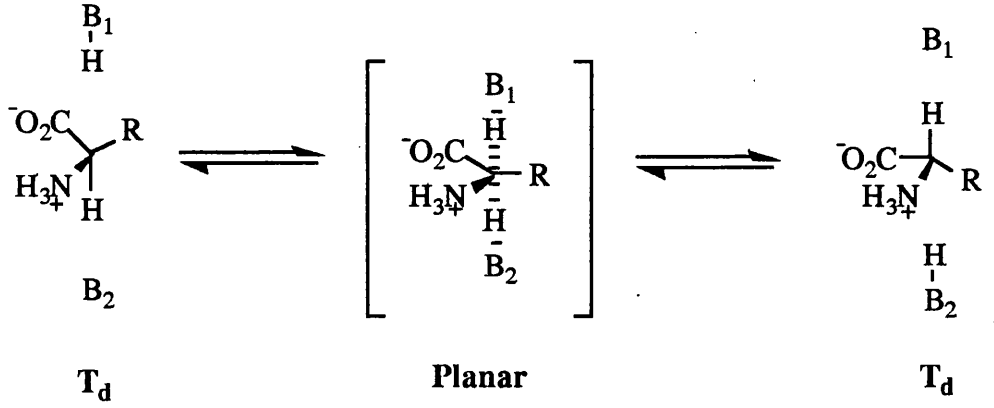


Scheme 19

LL-Diaminopimelate epimerase was detected over 30 years ago in *E. coli,* but was only recently purified and fully characterised by modification of the method of Wiseman and Nichols.[101] A purification of 2500-fold was achieved in overall 31% yield. The purified LL-DAP epimerase had a specific activity of 35 units mg^{-1}. It exists as an active monomer of molecular weight 34,000 daltons and requires no co-factor and no metal. Tyagi and co-workers[102] have isolated LL-diaminopimelate epimerase from two to three week old maize leaves.

The mechanism of LL-DAP epimerase resembles that of proline racemase.[107] It uses an active site thiol group in the deprotonation-

protonation process of diaminopimelate epimerisation. Therefore, some anionic character might be expected to develop at the α-carbon centre and hence, the carbon framework at that site should be planar in the transition state (**Scheme 20**).

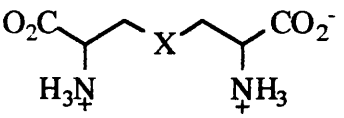


Scheme 20

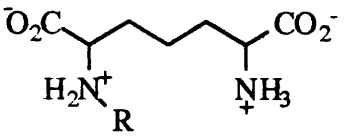
2.14. Inhibition of LL-Diaminopimelate Epimerase.

Lam *et al.*[108] investigated the interaction of analogues of *meso*-2,6-DAP (28) with diaminopimelate epimerase from *E. coli.* The analogues which were tested for enzyme activity are shown in **Scheme 21.** A coupled enzyme assay was used to measure the amount of inhibition. The conversion of LL-2,6-DAP (27) into *meso*-2,6-DAP (28) by the epimerase was measured by further transformation of *meso*-2,6-DAP (28) into L-2,3,4,5-THDPA (24) by

excess *meso*-DAP dehydrogenase with formation of NADPH. NADPH formation was followed by observing the absorbance at 340 nm.



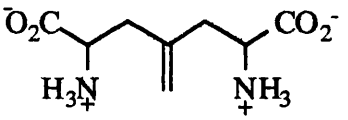
X = S (58)
X = SO (59)
X = SO_2 (60)



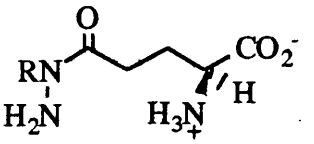
R = OH (61)
R = NH_2 (62)

a. 2R, 6S c. 2S, 6S

b. 2S, 6R d. 2R, 6R



(63)



R = H (64)
R = Me (65)

Scheme 21

None of the DAP analogues tested irreversibly inactivated the epimerase. *meso*-Lanthionine (58ab) and LL-lanthionine (58c) were both good competitive inhibitors with K_I values of 0.67 mM and 0.42 mM respectively. The corresponding DD-isomer (58d) was at least 20 times less effective as an inhibitor. This is in accord with the stereochemical requirements of the enzyme for its natural substrates.

The LL- and DD-isomers of both lanthionine sulphoxides (59c and 59d) and lanthionine sulphones (60c and 60d) showed no detectable effects on the epimerase, but their corresponding *meso*-

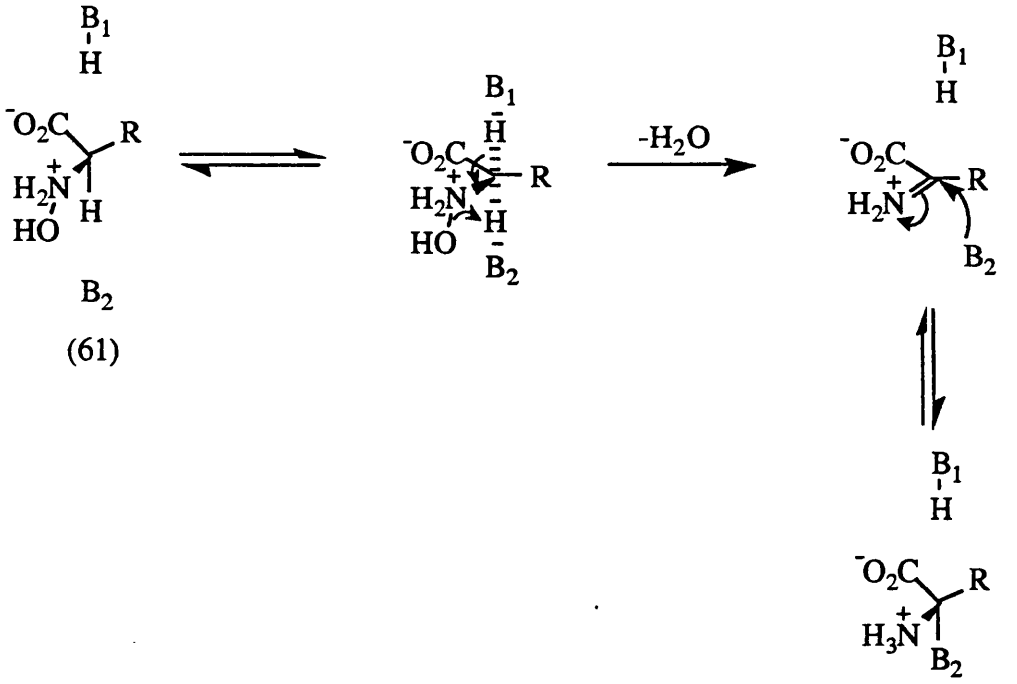
isomers (59ab and 60ab) were very weak competitive inhibitors with respective K_I values of 11 mM and 21 mM. The reduced activity of the lanthionine sulphoxides and sulphones could be due to electronic effects or to changes in the overall geometry.

N-Hydroxydiaminopimelate (61) proved to be an extremely potent competitive inhibitor with a K_I value of 5.6 μM for the mixture of isomers. The reason for this extremely good inhibition is unknown, but it may be that elimination of water from (61) occurs to generate an α-imine bound to the enzyme as a planar transition state analogue (**Scheme 22**). This could then be attacked by the active site thiol at the α-carbon in a reversible fashion.

N-Aminodiaminopimelic acid (62) was a less effective competitive inhibitor with a K_I value of 2.9 mM. This may be due to the inability of the terminal hydrazine amino group to leave through elimination. 4-Methylenediaminopimelic acid (63) was found to be a non-competitive inhibitor with a K_I value of 0.95 mM. Neither (64) or (65) showed any significant inhibition of the epimerase.

2.15. *meso*-Diaminopimelate Dehydrogenase.

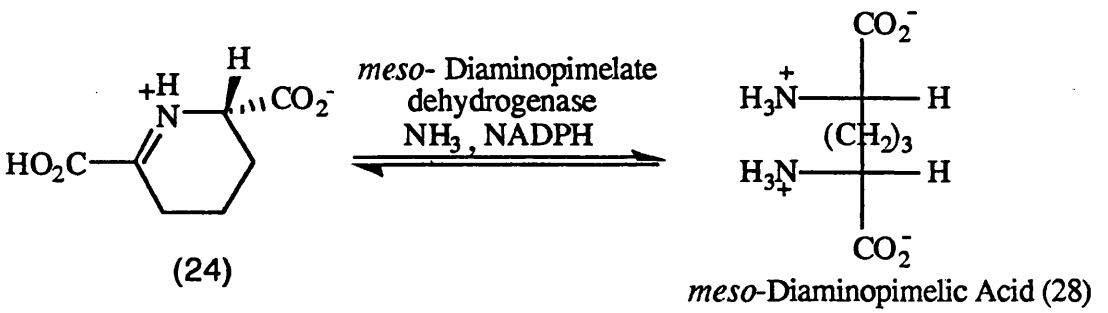
meso-Diaminopimelate (DAP) dehydrogenase catalyses the direct conversion of L-2,3,4,5-THDPA (24) into *meso*-2,6-DAP (28) in one enzymatic step. The reaction is reversible and is co-catalysed by



(61)

Scheme 22

NADPH and ammonia. It has been described as a reductive transamination reaction **(Scheme 23).**



(24)

meso-Diaminopimelic Acid (28)

Scheme 23

In 1976, Misono and co-workers[109] demonstrated *meso*-DAP dehydrogenase activity in *Bacillus sphaericus.* This work was further extended in 1983 by White[110] and was extensively studied

by Misono and co-workers.[105,109,111] They purified the enzyme to homogeneity. SDS-polyacrylamide gel electrophoresis showed the enzyme to consist of two identical subunits, each with a molecular weight of 41,000 daltons. The enzyme showed maximum activity at pH 10.5. The occurrence of *meso*-DAP dehydrogenase has also been described for a few other bacterial species.[105] It is worth noting that the enzyme has not been detected in *E. coli.*

In plant systems, Wenko *et al.*[112] 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 daltons.

The mechanism of *meso*-DAP dehydrogenase has not yet been fully established. Careful studies by Misono and Soda[105] on the dehydrogenase 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[113] and a tryptophan residue.[105] Product inhibition studies indicated that the sequence of addition of substrates in the oxidative deamination is $NADP^+$ followed by *meso*-2,6-DAP (28) and that the order of release of products is ammonia, then L-2,3,4,5-THDPA (24) and finally NADPH.

2.16. Inhibition of *meso*-Diaminopimelate Dehydrogenase.

Lam *et al.*[108] tested analogues of *meso*-2,6-diaminopimelic acid (28) for substrate and inhibitor activity on the dehydrogenase enzyme from *B. sphaericus*.[105] The dehydrogenase assay system monitored NADPH formation spectrophotomerically at 340 nm.

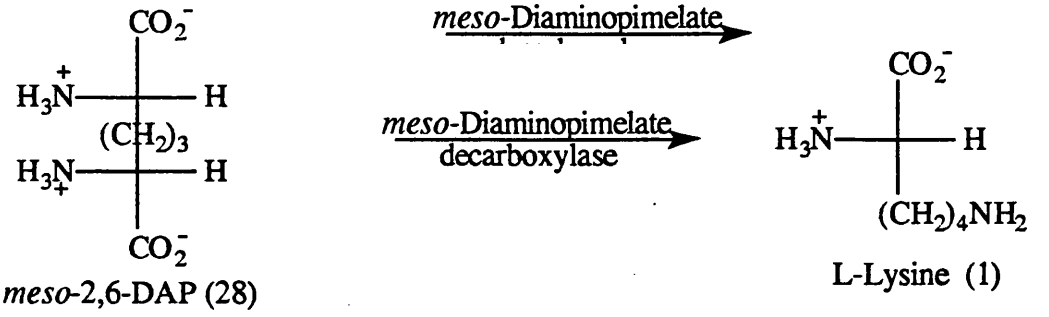
From substrate studies they found that the enzyme is stereospecific for *meso*-isomers and barely accommodates substituents on the carbon chain. *meso*-Lanthionine (58ab) was a poor substrate (K_M 5.8 mM) compared to *meso*-2,6-DAP (28) (K_M 1.1 mM), with a relative V_{max} 1% of that observed for the natural substrate. The sulphoxide (59) and the sulphone (60) of lanthionine were even poorer substrates. However, the *meso*-isomers of *N*-hydroxy- (61ab), *N*-amino- (62ab) and 4-methylenediaminopimelic acid (63ab) were found to be good substrates of the dehydrogenase enzyme.

The sulphoxides (59c and d), sulphones (60c and d) and DD-lanthionine (58d) showed very slight competitive inhibition. LL-Lanthionine (58c) is a weak non-competitive inhibitor with respect to *meso*-2,6-DAP (28) with a K_I of 38 mM.

2.17. *meso*-Diaminopimelate Decarboxylase

meso-Diaminopimelate (DAP) decarboxylase catalyses the final step in the synthesis of L-lysine (1) by the pyridoxal phosphate

dependent decarboxylation of *meso*-2,6-DAP (28) exclusively at the D-centre to give L-lysine (1) (Scheme 24).[62,114] The enzyme is highly specific for the *meso*-isomer, and the DD- and LL-isomers of 2,6-DAP are neither substrates nor effective inhibitors. *meso*-DAP decarboxylase is the only pyridoxal dependent α-decarboxylase known to act on a D-amino acid.



Scheme 24

meso-DAP decarboxlase has been studied in bacteria[69,115] and in higher plants,[59-61,116] where it is localised solely in the chloroplasts.[75] White and Kelly[117] have purified the enzyme from *E. coli* and have shown that it has a native molecular weight of 200,000 daltons as calculated from its sedimentation coefficient.

The molecular weight of the enzyme in higher plants varies between 75,000 daltons for wheat germ[61] and 85,000 daltons for maize endosperm.[60] Estimates of the K_M for *meso*-2,6-DAP (28) vary between 0.1 and 0.3 mM. The wheat enzyme was extracted from a wheat germ acetone powder in 50 mM potassium phosphate buffer (pH 7.0). The enzyme had a specific activity of 283 pkat mg^{-1}

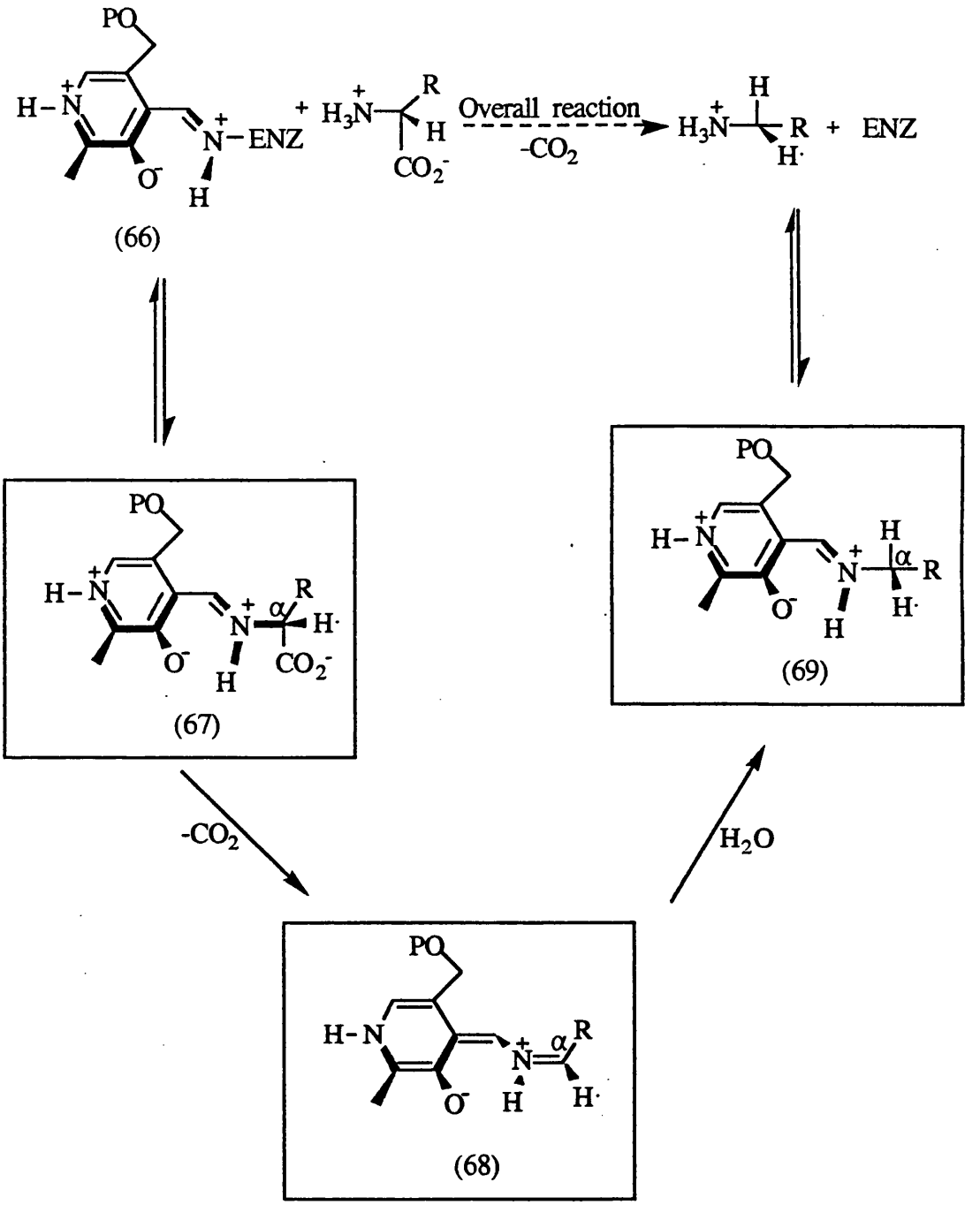
protein. A particularly rich source of the maize enzyme came from maize endosperm harvested 28 days after pollination. After purification this enzyme had a specific activity of 783 nkat mg^{-1} protein. (Katal is the SI Unit of enzyme activity and is the amount of enzyme that converts 1 micromole of substrate into product per second. It is an inconveniently large unit and is used much less than the international 'unit' of enzyme activity.)

2.18. Mechanism of *meso*-Diaminopimelate Decarboxylase

Kelland *et al.*[62] carried out a detailed analysis of this plant enzyme mechanism by two-dimensional ^{1}H-^{13}C heteronuclear NMR shift correlation spectroscopy with ^{2}H decoupling. From their results they proposed that the mechanism of *meso*-DAP decarboxylase in plants was comparable to the known mechanism operating in *Bacillus sphaericus*[69] **(Scheme 25).**

During decarboxylation of α-amino acids by pyridoxal phosphate-dependent decarboxylases the bond between the α-carbon and the carboxyl carbon of the substrate is expected to be nearly perpendicular to the plane of the cofactor's conjugated π-system.[118-120]

The first step in the mechanism is the formation of the Schiff's base (66) between the co-factor and *meso*-2,6-DAP (28) **(Scheme 25)**. Addition of *meso*-2,6-DAP (28) gives the imine (67). The carboxyl group is *trans*-antiparallel with the imine. Carbon dioxide is then lost to give the intermediate (68). The co-factor essentially



Scheme 25

stores the electrons of the cleaved bond until protonation from the solvent can occur to give (69). The resulting imine (69) is then

hydrolysed to give L-lysine (1). All pyridoxal phosphate dependent α-decarboxylases proceed with retention of configuration except for *meso*-DAP decarboxylase which shows inversion.[62,69]

2.19. Inhibition of *meso*-Diaminopimelate Decarboxylase.

With higher plant *meso*-DAP decarboxylase Mazelis and Crevelling[61] and Sodek[60] have shown that at concentrations up to 1 mM lysine has little effect on enzyme activity. At 20 mM however, lysine inhibited the wheat germ *meso*-DAP decarboxylase by 60%. It is unlikely that inhibition at this high concentration would indicate any physiological feedback regulation.

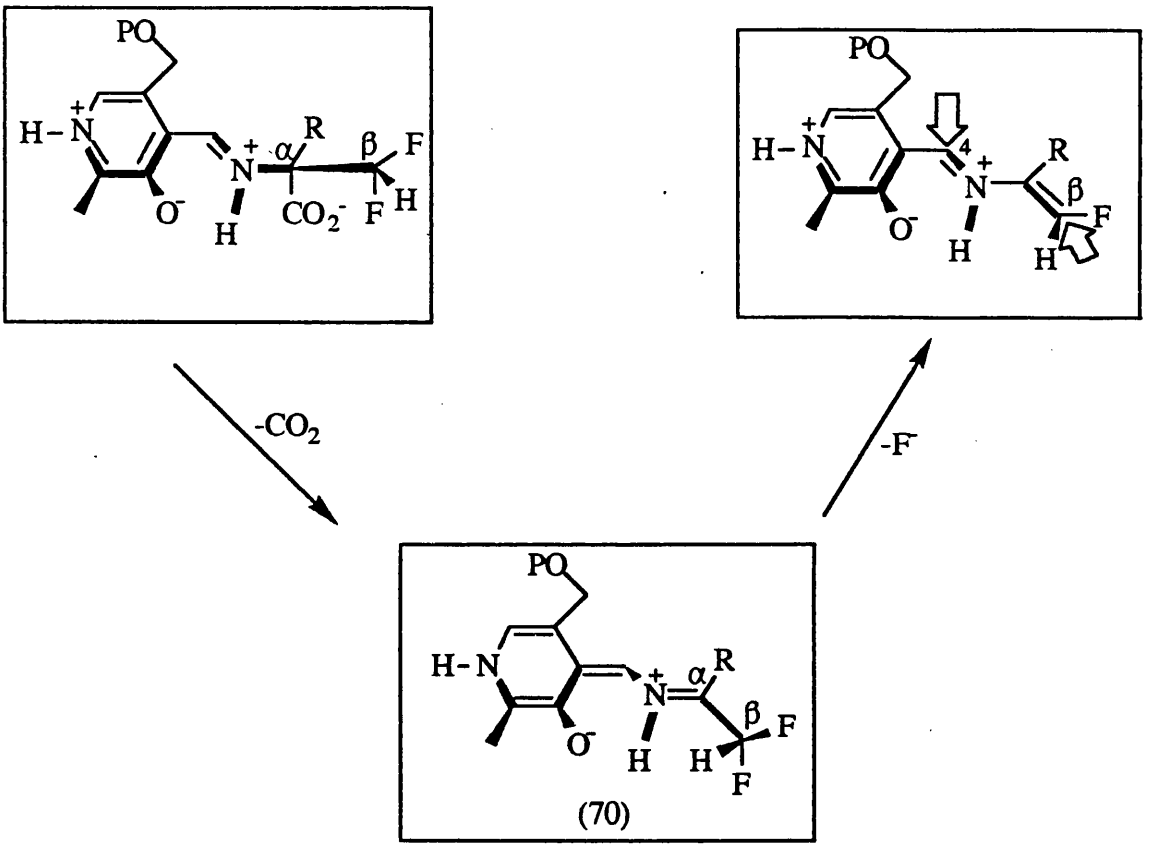
Vederas *et al.*[122] have synthesized and tested analogues of *meso*-2,6-DAP (28) for enzyme activity with *meso*-DAP decarboxylase from *Bacillus sphaericus*[123] and from wheat germ (*Triticum vulgaris*).[61] Enzyme activity was assayed by measuring the release of $^{14}CO_2$ from [1,7-^{14}C]-DAP.[62] Rates of $^{14}CO_2$ production were analysed by the statistical method of Wilkinson.[124]

There are two potential modes of inhibition of pyridoxal phosphate-dependent α-decarboxylases, β-elimination and *N*-modification. Both will be discussed below.

β-Elimination

Substrate analogues which have a leaving group attached to a β-carbon of the α-amino acid can undergo elimination to generate an

electron-deficient conjugated system (70) (Scheme 26). Stereoelectronic considerations for elimination reactions[125] require that the bond between the β-carbon and its attached leaving group must be aligned nearly perpendicular to the plane of the conjugated co-factor-inhibitor complex. The leaving group may be above or below this plane, depending on the side chain R.[126]



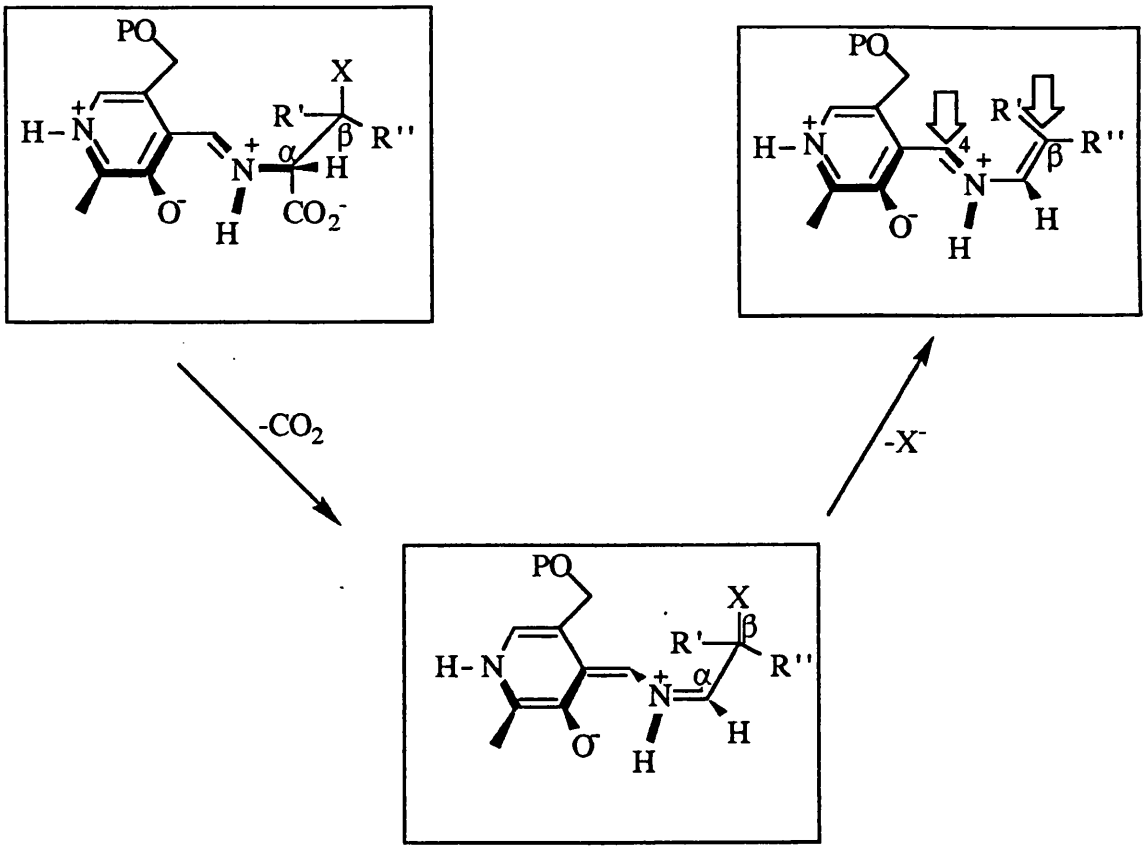
Scheme 26

The electron-deficient conjugated system is highly reactive and is prone to attack by nucleophilic species at the β-carbon or at C-4 of the co-factor.[127,128] This would result in direct covalent attachment

of the co-factor-inhibitor complex to a group in the enzyme active site, and as such, would result in irreversible loss of enzyme activity.

α-Difluoromethyldiaminopimelate was expected to be a potent inhibitor due to extensive precedent with other pyridoxal phosphate-dependent α-decarboxylases. However, Vederas *et al.*[122] found no irreversible or even strong competitive inhibition. This suggested that α-difluoromethyldiaminopimelate cannot bind to the enzyme active site. Apparently both plant and bacterial *meso*-DAP decarboxylases enforce the stringent stereochemical requirement for the DL-isomer of the substrate by a 'tight-fit' in the region surrounding the α-carbon and do not permit replacement of the α-H by a larger group.

To solve this problem, Vederas *et al.*[122] investigated the elimination of a group 'X' at the β-carbon of the side chain (**Scheme 27**). The sulphoxides (59) and sulphones (60) were investigated because a number of pyridoxal phosphate-catalysed β-eliminations of sulphur-containing groups are known[129,130] and the parent *meso*-lanthionine (58) is decarboxylated by *meso*-DAP decarboxylase. The LL- (59 and 60c) and *meso*-isomers (59 and 60ab) of lanthionine sulphoxide and sulphone are competitive inhibitors but did not show inactivation of the decarboxylase. The sulphoxides were found to be better inhibitors than sulphones, possibly due to secondary binding of the sulphoxide functionality.



Scheme 27

N-Modification

An alternative approach to inhibition of the decarboxylase is modification of the substrate nitrogen. Cooper and Griffiths[131] have observed that *N*-hydroxyglutamate irreversibly inhibits pyridoxal phosphate-dependent glutamate decarboxylase.

N-hydroxydiaminopimelate was tested for inhibition of *meso*-DAP decarboxylase by Vederas *et al.*[122] They found it to be a good competitive inhibitor of both the wheat germ and the bacterial

enzyme. However the compound showed no irreversible inactivation.

N-Aminodiaminopimelate has been found to have inhibition constants in the range 1-2 μM with a pyridoxal phosphate-dependent histidine decarboxylase[132] and with aspartate aminotransferase.[133] With *meso*-DAP decarboxylase this compound was found to be a good competitive inhibitor with a K_I of 100 μM for the bacterial enzyme and a K_I of 84 μM for the wheat germ enzyme.

Conclusion

The diaminopimelate pathway to L-lysine (1) has been reasonably well studied in bacteria. However, in higher plants the pathway is not completely understood. In bacteria all seven enzymes of the pathway have been isolated and characterised. In higher plants only four of the enzymes have been isolated. Although the main intermediates in the pathway, in both bacteria and plants, have been established, the mechanisms and stereochemistry of some of the steps are not fully understood.

The synthesis and testing of potential inhibitors has been carried out in some detail for the latter enzymes in the pathway. However, very few inhibitor studies have been undertaken for the earlier steps in the pathway, particularly the first and second steps, catalysed by DHDPS and DHDPR.

The syntheses of two important intermediates in the pathway, L-2,3-DHDPA and L-2,3,4,5-THDPA, have been attempted by many

groups. However, the purification and characterisation of these intermediates has proved very difficult due to their instabiltiy.

The synthesis of the starting substrate in the pathway, L-aspartic acid-β-semialdehyde, has been carried out by Black and Wright.

Chapter [3] - Synthesis of L-Aspartic Acid-β-semialdehyde and Analogues.

Introduction

L-Aspartic acid-β-semialdehyde (21a) is an important intermediate in the biosynthesis of L-lysine (1), L-threonine (16) and L-methionine (18) (**Scheme 4**). The biosynthesis of L-threonine (16) and L-methionine (18) proceeds *via* L-homoserine after reduction of L-aspartic acid-β-semialdehyde (21a) catalysed by homoserine dehydrogenase.[134-136] Furthermore, in the DAP pathway to L-lysine (1) (**Scheme 5**), which occurs in bacteria and higher plants, the most intriguing step is the condensation of L-aspartic acid-β-semialdehyde (21a) with pyruvate (22) catalysed by DHDP Synthase to give L-2,3-DHDPA (23). Further enzyme catalysed reactions of L-2,3-DHDPA (23) lead to L-lysine (1).

L-Aspartic acid-β-semialdehyde (21a) is derived biosynthetically from L-aspartic acid (19) (**Scheme 4**) *via* L-aspartic acid-β-phosphate (20). Formation of L-aspartic acid-β-phosphate (20) from L-aspartic acid (19) is catalysed by aspartate kinase.[44-53] Aspartate semialdehyde dehydrogenase catalyses the pyridine nucleotide reduction of L-aspartic acid-β-phosphate to give L-aspartic acid-β-semialdehyde (21a).[54,55]

L-Aspartic acid-β-semialdehyde (21a) has also become an increasingly important synthetic intermediate.[137] The aldehyde function provides a useful handle for manipulation, and this allows access into a range of polyfunctional non-protein and unnatural amino acids. The use of L-aspartic acid-β-semialdehyde (21a) as a

synthetic intermediate will be discussed in more detail in Sections 3.3 and 3.6.

The first reported chemical synthesis of L-aspartic acid-β-semialdehyde (21a) was by Black and Wright in 1955.[70] However, very little chemical evidence was provided for the compound. In this chapter there will be described a brief selection of synthetic methods and the use of protecting groups in α-amino acid synthesis. The Black and Wright synthesis of L-aspartic acid-β-semialdehyde (21a) will be reviewed followed by a discussion on our improved synthesis of this compound and biochemical results obtained with L-aspartic acid-β-semialdehyde (21a). Finally, the synthesis of analogues of L-aspartic acid-β-semialdehyde (21a) will be discussed along with the test data obtained on DHDP Synthase with these compounds.

3.1. α-Amino Acid Synthesis.

The synthesis, resolution and physiochemical characterisation of α-amino acids and their derivatives continue to be areas of highly active research. There are many well established and, more recently, newer methods for the synthesis of α-amino acids.[138-140] These can be grouped into three main categories: alkylation of glycine derivatives; condensation and substitution reactions in which the amino- or carboxyl-function is introduced to complete the synthesis; and lastly rearrangement reactions. Each of these synthetic strategies will be briefly discussed in this section.

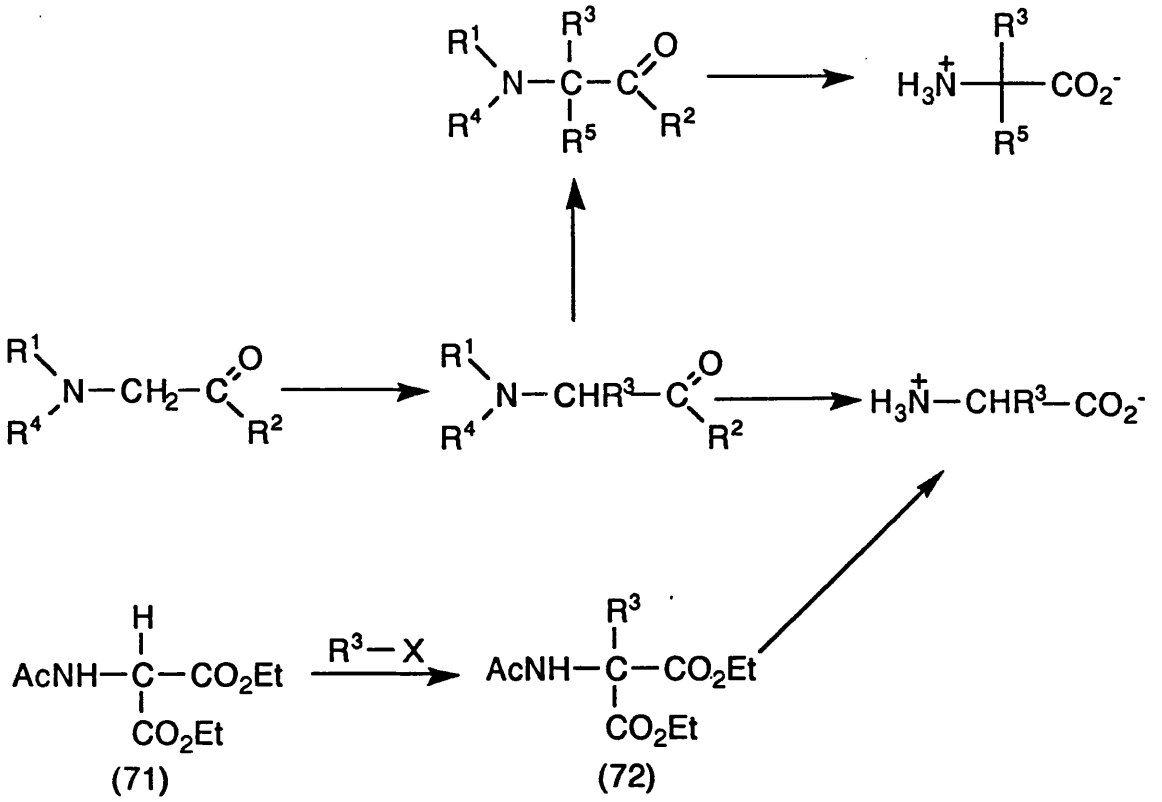
Asymmetric synthesis of α-amino acids is undergoing considerable development and several successful methods have been established.[141] However, asymmetric synthesis of α-amino acids will not be discussed in this section. The use of α-amino acids as starting materials for the synthesis of other α-amino acids is an increasingly important technique. Examples of this strategy will be shown in the Black and Wright[70] synthesis, and in our modified synthesis of L-aspartic acid-β-semialdehyde in Sections 3.2 and 3.3 respectively.

Alkylation of Glycine Derivatives to give α-Amino Acids.

Alkylation of acetamidomalonate esters and other *N*-acylaminomalonates remains the most frequently chosen route to α-amino acids with side chains which can withstand the hydrolysis step involved at the end of the synthesis **(Scheme 28)**.

A typical procedure from diethyl acetamidomalonate (71) involves reaction of the alkyl halide with it to give the crystalline alkylated malonate (72). This is hydrolysed with refluxing dilute aqueous sodium hydroxide. Excess alkali is removed with a strong cation exchange resin. This brings about hydrolysis to the malonic acid, which is decarboxylated by boiling in water.

Another well documented alkylating method for the synthesis of α-amino acids is alkylation of benzylidene glycine ester (73), a Schiff's base. However, overalkylation problems arise with this method giving mixtures of mono- and di-alkylated products. The yields of these reactions are not very good.



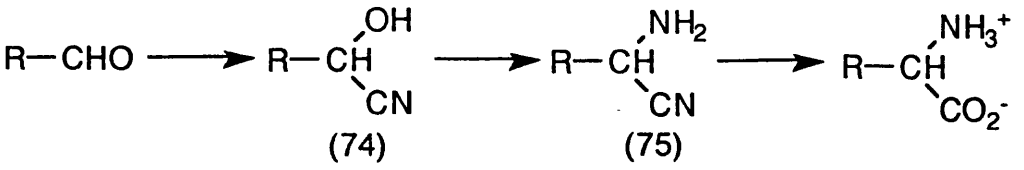
Scheme 28

$$PhCH{=}NCH_2{-}CO_2R$$

(73)

Condensation and Substitution Reactions to give α-Amino Acids.

The Strecker synthesis (**Scheme 29**) is a well established condensation reaction yielding α-aminoalkanenitriles (75) which, on hydrolysis, give the corresponding α-amino acid. There are many variations of the Strecker synthesis and the yields are variable.[142]



Scheme 29

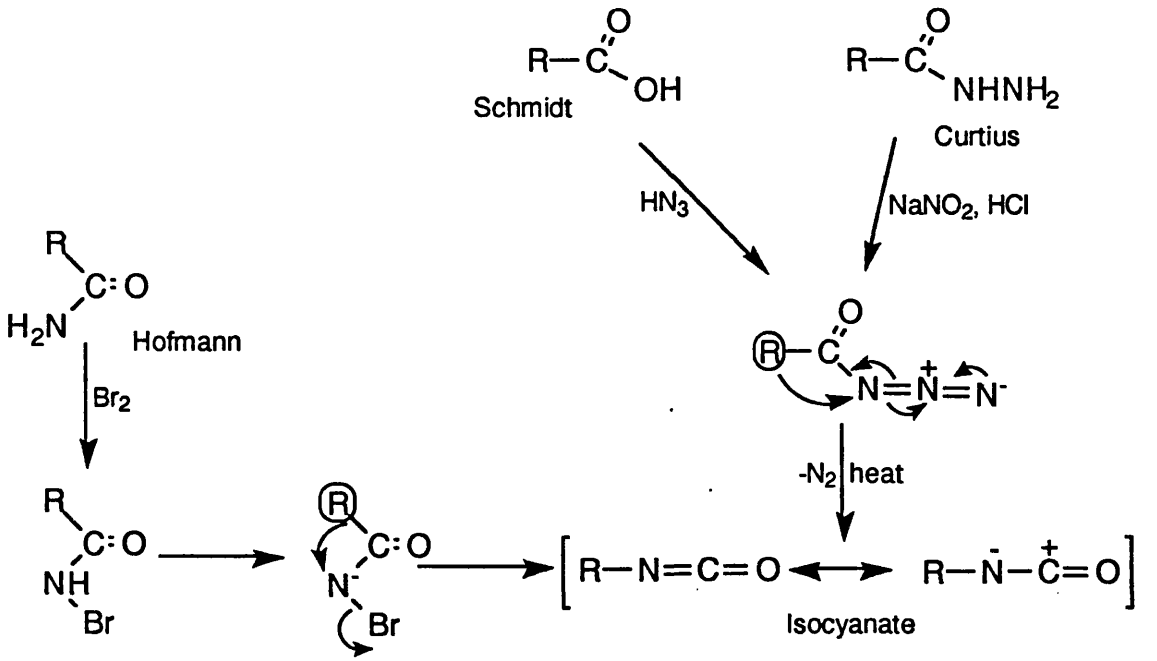
The first step in the Strecker synthesis involves the formation of the cyanohydrin (74) from the aldehyde using sodium cyanide, acetic acid and methanol. The aminonitrile (75) is formed from the cyanohydrin (74) by treatment with ammonium hydroxide and ammonium chloride. Hydrolysis of the aminonitrile (75) with 10M HCl gives the α-amino acid.

Substitution reactions leading to α-amino acids, which have been used over many years, are based on the easy availability of α-halogeno-alkanoic acids and α-hydroxy- or epoxy-alkanoic acids. The introduction of the nitrogen function can either be as the free amino group using ammonia to give the α-amino acid, or if an amine is used, *N*-alkylated α-amino acids can be prepared.

Rearrangement Reactions leading to α-Amino Acids.

The Curtius, Hofmann and Schmidt rearrangements[142a] have all been used for the synthesis of α-amino acids *via* the corresponding isocyanate (Scheme 30). However, these rearrangement reactions are now only rarely used for this purpose in

view of the availability of efficient, more convenient, alternative general methods for amino acid synthesis.



Scheme 30

Protecting Groups in α-Amino Acid Synthesis.

Protecting groups are of paramount importance in α-amino acid synthesis. Temporary protection of the α-amino group and of the α-carboxyl group eliminates the dipolar character of the starting zwitterionic amino acids. This allows the synthesis of α-amino acids to be carried out in organic solvents, and not under more difficult aqueous conditions. The protecting group must fulfil a number of requirements. It must react selectively in good yield to give a

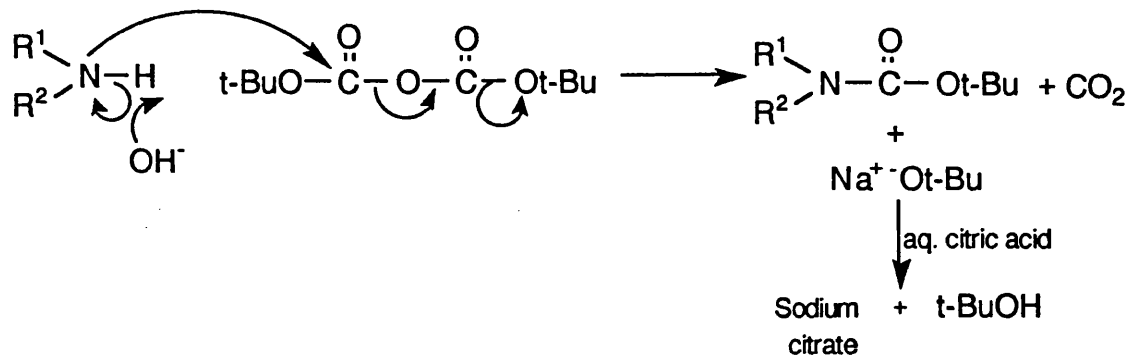
protected substrate that is stable to the conditions used in the rest of the planned synthesis. The protecting group must also be selectively removed in good yield by readily available reagents which do not attack the rest of the molecule. Finally, the protecting group should have a minimum of additional functionality to avoid further sites of reaction.

The protecting groups which have been mainly used in our amino acid synthesis are the *t*-butoxycarbonyl (BOC) group and the benzyloxycarbonyl or carboxybenzyl (CBZ) group for *N*-protection and the *t*-butyl and benzyl esters for protecting the carboxyl group. These protecting groups meet all of the above requirements.

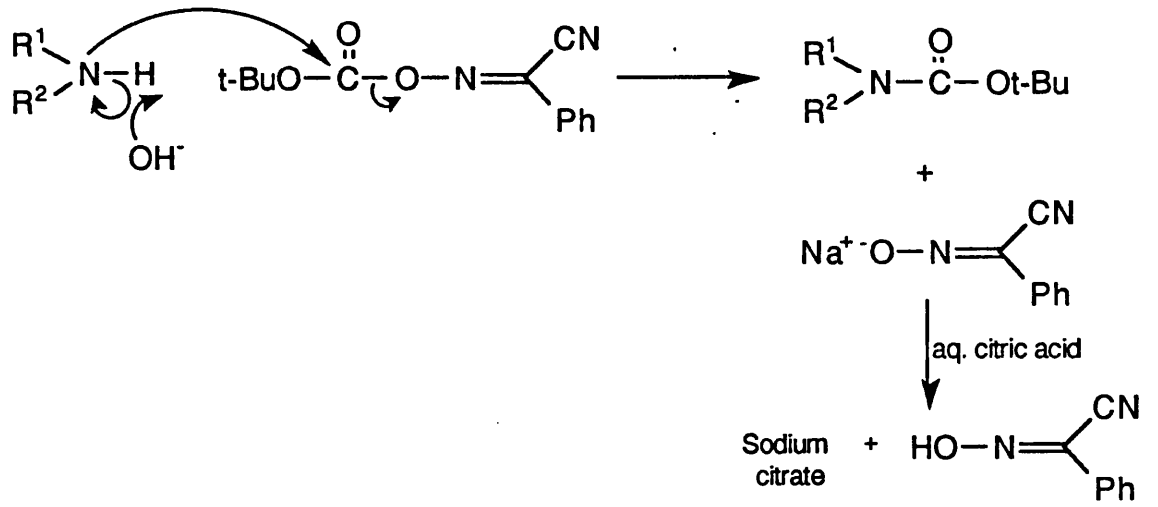
There are many reagents available for the formation and removal of the widely used BOC group. The two most common reagents for putting on the BOC group are di-*t*-butyl dicarbonate[143] and 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile[144] under basic conditions. The yields vary between 70 and 95%. The mechanisms for putting on the BOC group using both these reagents are shown in **Scheme 31.**

Removal of the BOC group is carried out under mild acidic conditions. The most common method of removal is stirring the protected compound in trifluoroacetic acid at room temperature for one hour **(Scheme 32).**[145] Thiophenol is sometimes used with trifluoroacetic acid to act as a scavenger for *t*-butyl cations. This prevents possible alkylation of the deprotected product.

Di-*t*-butyl dicarbonate



2-(*t*-Butoxycarbonyloxyimino)-2-phenylacetonitrile

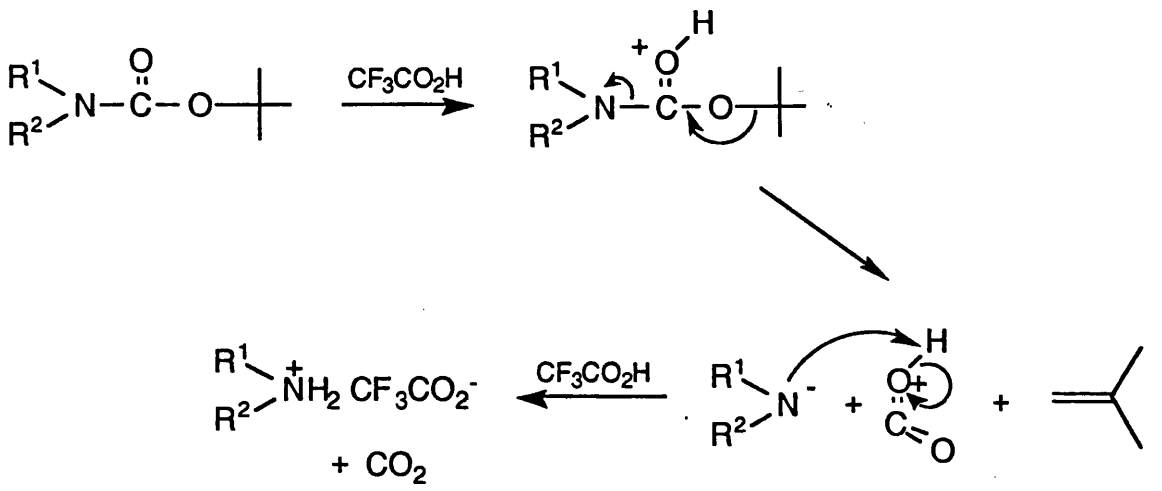


Scheme 31

t-Butyl esters are stable under mild basic conditions and are very popular for the protection of the carboxyl group. There are several reagents used to form *t*-butyl esters. The most common procedure used is isobutylene and conc. sulphuric acid at room temperature for one day.[146]

t-Butyl esters are cleaved under similar conditions to the BOC group and so the BOC/*t*-butyl ester protecting groups for α-amino

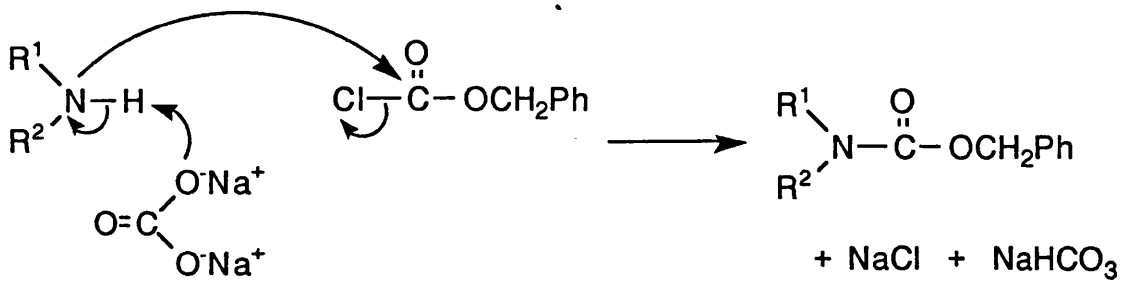
acid synthesis are commonly used together since they can be both removed in one synthetic step.



Scheme 32

The CBZ group is widely used in peptide synthesis since it can easily be removed by catalytic hydrogenolysis.[147] It is usually put on using benzyl chloroformate under basic conditions (Scheme 33).

Benzyl esters are easily prepared using benzyl bromide in dimethylformamide.[148] They are cleaved under the same catalytic hydrogenolysis conditions as the CBZ group and so the CBZ/benzyl ester protecting groups for α-amino acid synthesis are commonly used together since, like the BOC/*t*-butyl ester protecting groups, they can both be removed in one synthetic step.

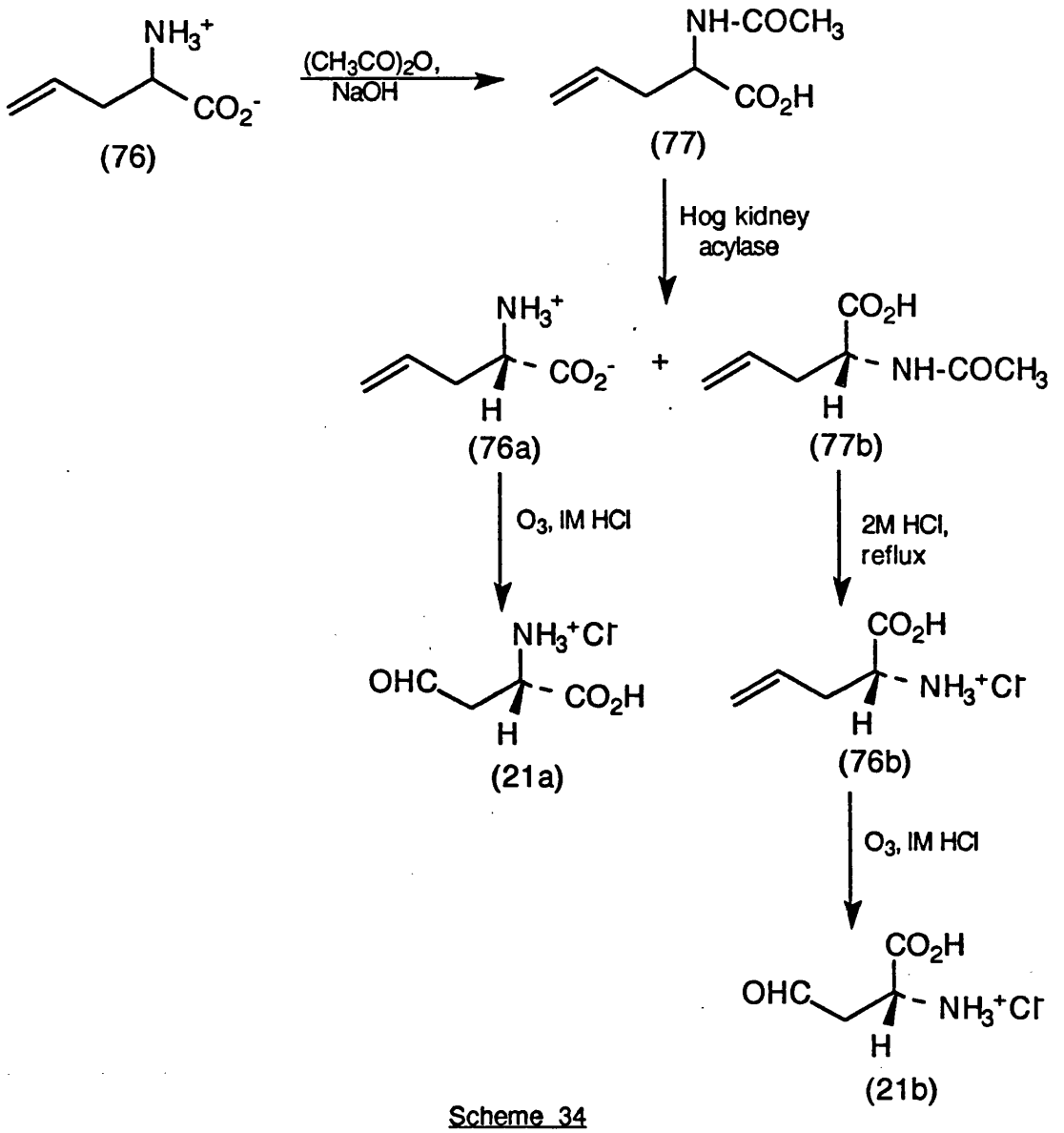


Scheme 33

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

Black and Wright reported the first synthesis of the separate enantiomers of aspartic acid-β-semialdehyde (21) from DL-allylglycine (76) (Scheme 34).[70] Resolution was achieved by enantioselective enzymic hydrolysis of the *N*-acetyl derivatives of DL-allylglycine. *N*-Acetyl-DL-allylglycine (77) was prepared from DL-allylglycine (76) by Sorrenson's method using 2M sodium hydroxide and acetic anhydride. Hydrolysis of *N*-acetyl-DL-allylglycine (77) using hog kidney acylase gave L-allylglycine (76a) and unchanged *N*-acetyl-D-allylglycine (77b). L-Allylglycine (76a) crystallised on cooling. *N*-Acetyl-D-allylglycine (77b) was hydrolysed by heating at reflux in 2M hydrochloric acid to give D-allylglycine (76b). Upon ozonolysis of D- (76b) and L-allylglycine (76a), D- (21b) and L-aspartic acid-β-semialdehyde (21a) were formed. Only the L-isomer (21a) was found to be a substrate of DHDP Synthase. Black and Wright found that L-aspartic acid-β-semialdehyde (21a) was reasonably stable in acid solution at 0 °C. However it deteriorated rapidly in neutral solution or in the dry state. Attempts by them to

dry the acid solution of L-aspartic acid-β-semialdehyde (21a) by evaporation at low temperatures also resulted in substantial losses of the enzymatically reactive compound.

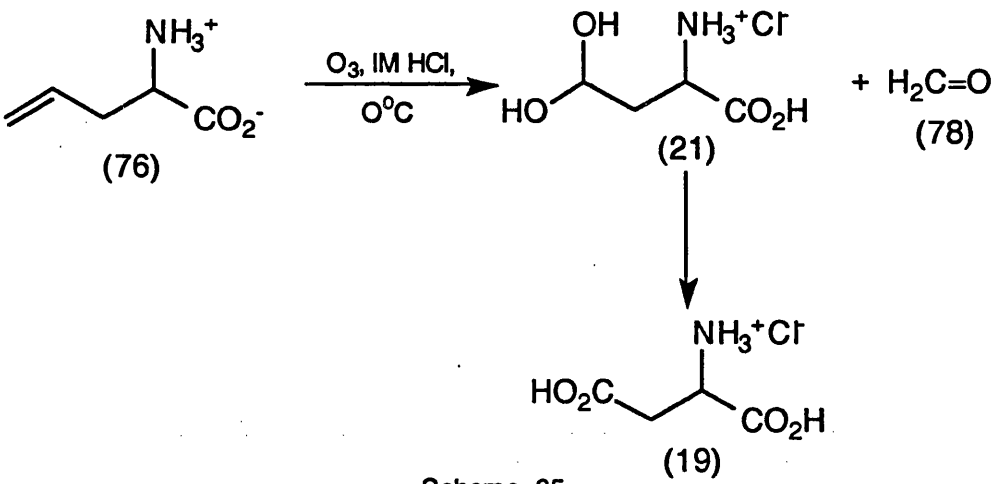


Scheme 34

Black and Wright found that the usual aldehyde derivatives did not form readily with this substance and hence, the identity of L-

aspartic acid-β-semialdehyde (21a) rested largely on the fact that it could be converted into L-homoserine. This enzymic process was catalysed by homoserine dehydrogenase.[134-136]

When the Black and Wright procedure was repeated by us, i.e. ozonolysis of DL-allylglycine (76) in 1M HCl at 0 °C, we found that DL-aspartic acid-β-semialdehyde (21) was formed, along with a mixture of other products. To identify these products the reaction was repeated in D_2O and DCl and followed by ^1H NMR spectroscopy. There were signals present which were consistent with the presence of DL-aspartic acid-β-semialdehyde (21), formaldehyde (78) and DL-aspartic acid (19) **(Scheme 35)**. DL-Aspartic acid (19) is presumably formed by oxidation of DL-aspartic acid-β-semialdehyde (21). No aldehyde proton was observed in the ^1H NMR spectrum therefore the aldehyde probably exists as the hydrate.

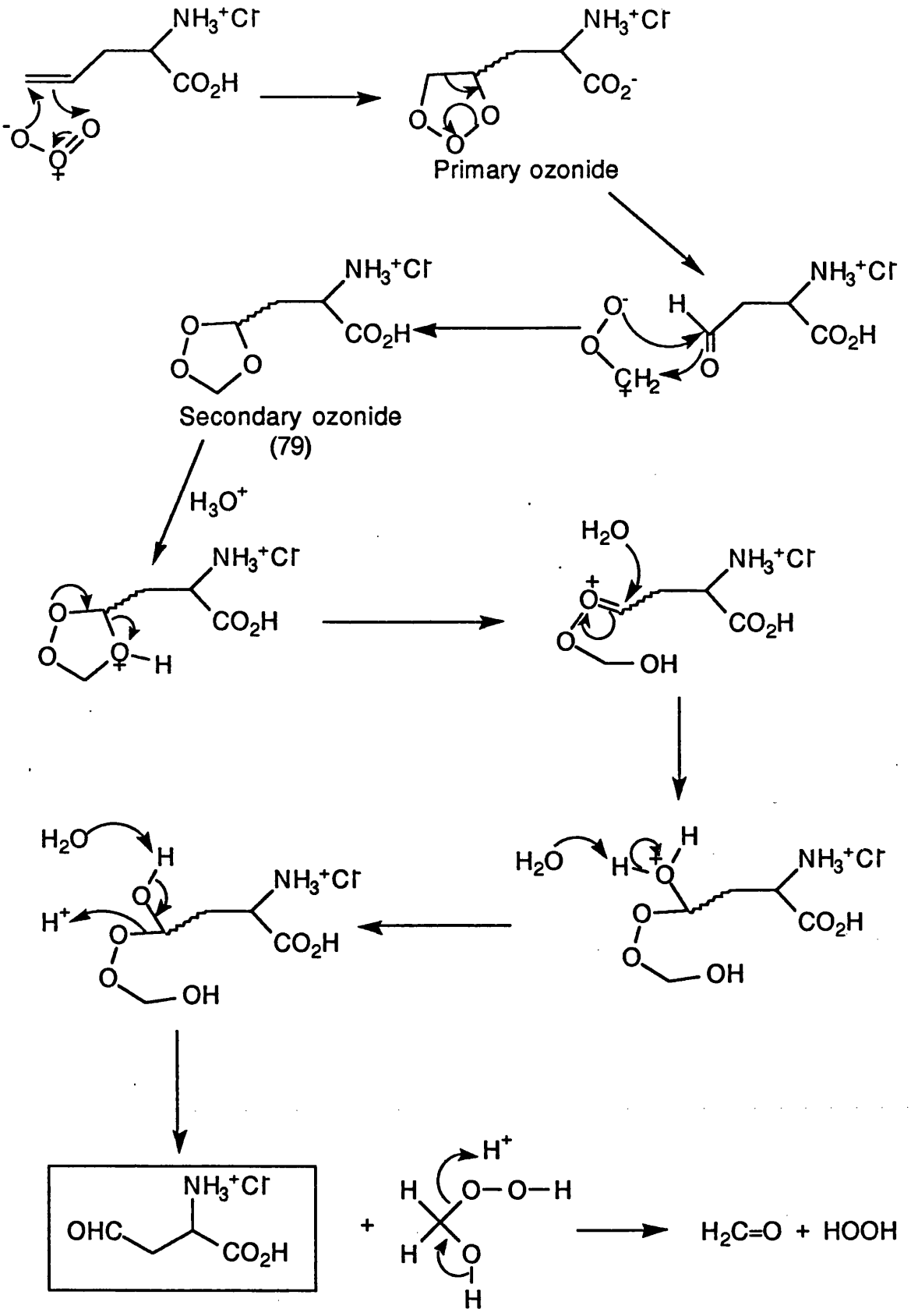


Scheme 35

Ion-exchange column chromatography was attempted to purify the mixture. However the concentration of DL-aspartic acid-β-semialdehyde (21) was found to be less after ion-exchange chromatography. This is probably due to the instability of DL-aspartic acid-β-semialdehyde (21) at room temperature.

The concentration of DL-aspartic acid-β-semialdehyde (21) in the acid solution mixture was calculated by converting DL-aspartic acid-β-semialdehyde (21) into DL-homoserine catalysed by homoserine dehydrogenase.[134-136] This work was carried out by Emma Borthwick in the Biochemistry Dept. at Glasgow University. The enzyme activity was monitored by the decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$. The change in concentration of NADPH during the reaction could be calculated. Since 1 mole of NADPH reacts with 1 mole of L-aspartic acid-β-semialdehyde (21a), the concentration of L-aspartic acid-β-semialdehyde (21a) is equivalent to the change in concentration of NADPH. The concentration of the solution prepared under similar conditions with different batches of material was between 0.3 M and 0.4 M.

A possible mechanism of formation and decomposition of the ozonide is shown in **Scheme 36.** The secondary ozonide (79) decomposes as it is formed in the acidic solution. The mechanism of decomposition involves hydrolytic cleavage of the ether linkage of (79) followed by decomposition to the aldehyde and formaldehyde. Aspartic acid-β-semialdehyde (21) and formaldehyde (78) can then be further oxidised to aspartic acid and formic acid by hydrogen peroxide.


NH3+Cl-
CO2H
CO2-
Primary ozonide
Secondary ozonide
(79)
H3O+
H2O
OH
OHC
H2C=O + HOOH

Scheme 36

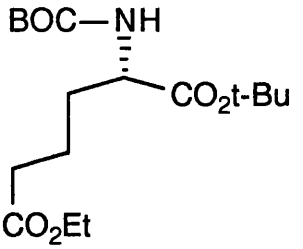
3.3. Modified Synthesis of L-Aspartic Acid-β-semialdehyde.

In order to study the mechanism of DHDP Synthase and to carry out precise kinetic and inhibitor studies with this enzyme we required a synthesis of pure L-aspartic acid-β-semialdehyde (21a). We also required to make L-aspartic acid-β-semialdehyde (21a) in a protected form which was easy to handle, could be stored for long periods of time, and the protecting groups could be easily removed under mild reaction conditions in one synthetic step.

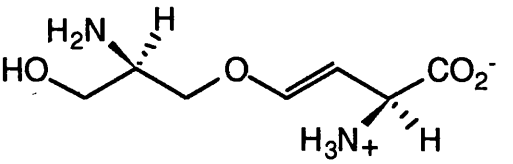
Our initial strategy was to start with the naturally occurring amino acid DL-homoserine. Double protection at the α-amino and α-carboxyl groups followed by oxidation of the primary hydroxyl group to the aldehyde would give protected DL-aspartic acid-β-semialdehyde derivatives. Purification could be carried out at this stage. Finally careful deprotection would give the desired DL-aspartic acid-β-semialdehyde (21).

The oxidation of the primary hydroxyl group of DL-homoserine analogues has been reported in the literature by several groups. Ramsamy *et al.*[149] carried out the oxidation of *N*-*t*-butoxycarbonyl-L-homoserine *t*-butyl ester to give *N*-*t*-butoxycarbonyl-L-aspartic acid-β-semialdehyde *t*-butyl ester using a chromium (VI) trioxide-pyridine complex in methylene chloride.[150] These compounds were intermediates in the synthesis of *N*-*t*-butoxycarbonyl-L-α-aminoadipic acid 1-*t*-butyl 6-ethyl diester (80), a suitably protected amino acid for use in peptide synthesis. Keith and co-workers used the same oxidising conditions to prepare *N*-carboxybenzyl-L-aspartic

acid-β-semialdehyde benzyl ester from its homoserine analogue in the total synthesis of the naturally occurring amino acid rhizobitoxine (81).[151]

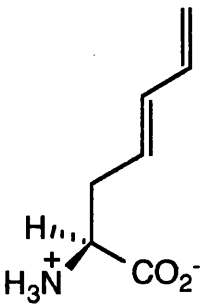


(80)

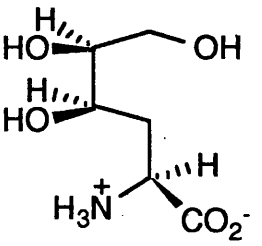


(81)

Baldwin and Flinn[137] prepared *N*-*t*-butoxycarbonyl-L-aspartic acid-β-semialdehyde *p*-methoxybenzyl ester from its homoserine analogue using pyridinium chlorochromate and sodium acetate in methylene chloride.[152] This protected aspartic acid-β-semialdehyde derivative was further converted into the more complex amino acids, L-2-aminohept-4,6-dienoic acid (82) and 2*S*,4*R*,5*S*-2-amino-4,5,6-trihydroxyhexanoic acid (83).



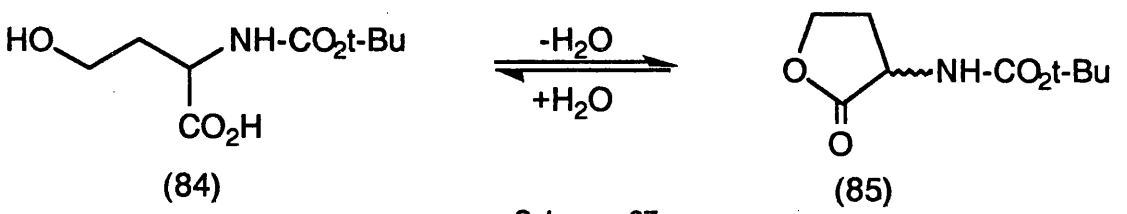
(82)



(83)

Unfortunately when we tried to prepare *N*-*t*-butoxycarbonyl-DL-homoserine (84), the first intermediate in our proposed synthetic pathway to *N*-*t*-butoxycarbonyl-DL-aspartic acid-β-semialdehyde *t*-

butyl ester, the reaction yields were low and TLC analysis showed two strong spots. ^{1}H and ^{13}C NMR and IR spectroscopic analysis showed that we had a 50/50 mixture of the desired product (84) and the lactone (85) (**Scheme 37**). The ^{1}H NMR spectrum had two *t*-butoxycarbonyl signals, there were 3 carbonyl absorption bands in the IR spectrum: 1777 (lactone), 1725 (acid) and 1705 (broad) (carbamate) and the ^{13}C NMR spectrum had double the expected amount of signals. Ozinskas and Rosenthal[153] have previously prepared the lactone (85) in their synthesis of L-calanine and γ-functional 2-aminobutyric acid derivatives. A variety of conditions using di-*t*-butyl dicarbonate and 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile were tried with no better results. At this stage it was decided to abandon homoserine as the starting material and go back to allylglycine and modify the Black and Wright method.

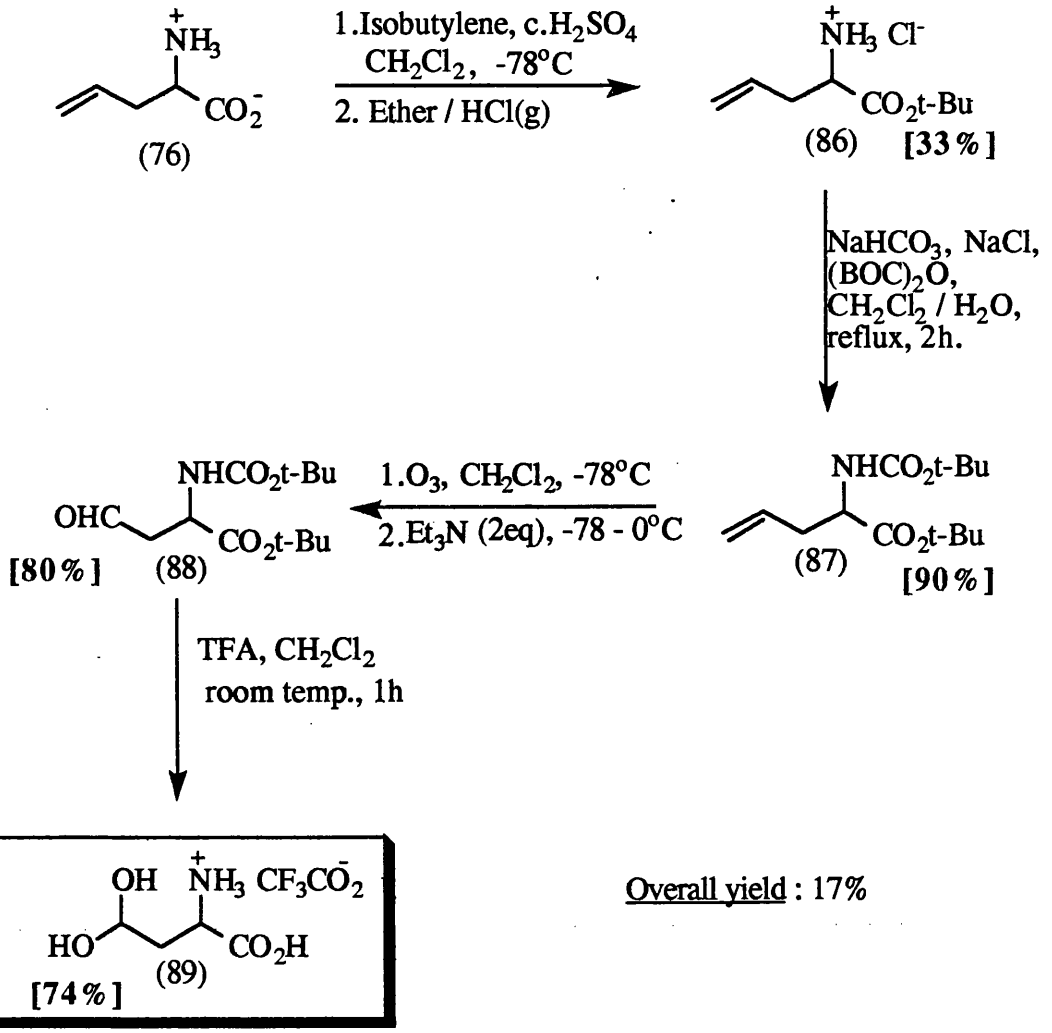


(84) (85)

Scheme 37

DL-Allylglycine (76) was converted into its *t*-butyl ester using 2-methylpropene and sulphuric acid (**Scheme 38**).[143] The product was precipitated as the hydrochloride salt (86). The *N*-*t*-butoxycarbonyl (BOC) derivative (87) was prepared in 90% yield by treatment of (86) with di-*t*-butyl dicarbonate under basic conditions.[163] The doubly protected material (87) was subjected to

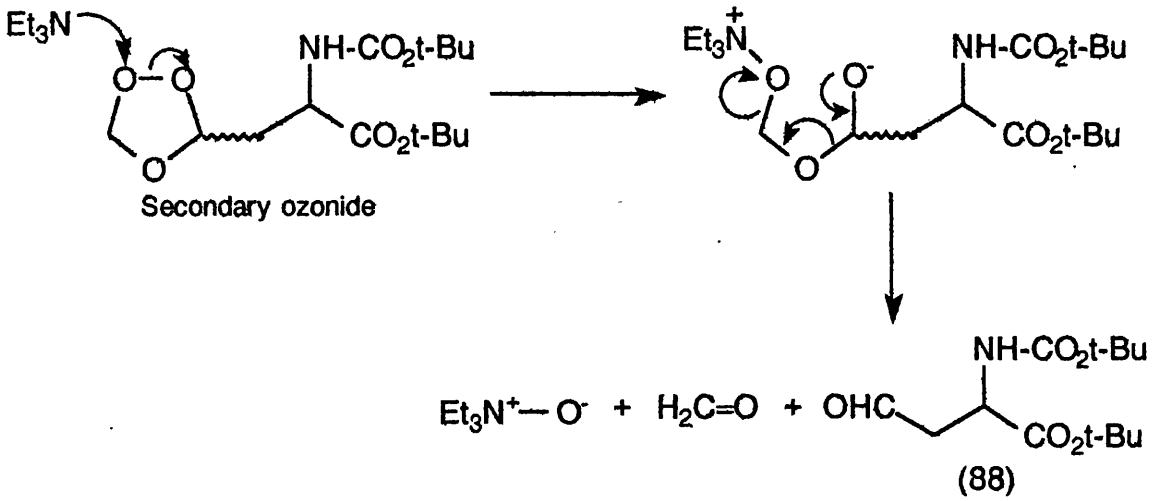
ozonolysis at -78 °C in methylene chloride and the ozonide was decomposed with triethylamine to give the protected aspartic acid-β-semialdehyde derivative (88). The mechanism of ozonide decomposition is shown in **Scheme 39.** Purification of (88) was carried out using flash column chromatography and elution with ether to give a clear oil in 80% yield.



Overall yield : 17%

Scheme 38

The L-isomer of *N*-*t*-butoxycarbonylaspartic acid-β-semialdehyde t-butyl ester had identical spectroscopic data to the aldehyde prepared by Ramsamy and co-workers[149] from oxidation of *N*-*t*-butoxycarbonyl-L-homoserine *t*-butyl ester using a chromium trioxide-pyridine complex.



Scheme 39

Deprotection of the BOC group and the *t*-butyl ester was carried out in one step by stirring (88) in trifluoroacetic acid in methylene chloride for one hour at room temperature under a nitrogen atmosphere. Removal of the solvent *in vacuo* gave a yellow oily residue which solidified giving a light yellow solid when ether was added. The yield was 74% and the solid had mp 64-66 °C. ^{1}H and ^{13}C NMR data indicated that the product exists as a hydrate (91), i.e. no aldehyde proton was observed and there was a double doublet at δ_H 5.1 for the 4-H and a signal δ_C 89.4 for C-4. The ^{1}H NMR spectrum of (89) is shown in **Figure 1.** The product has correct

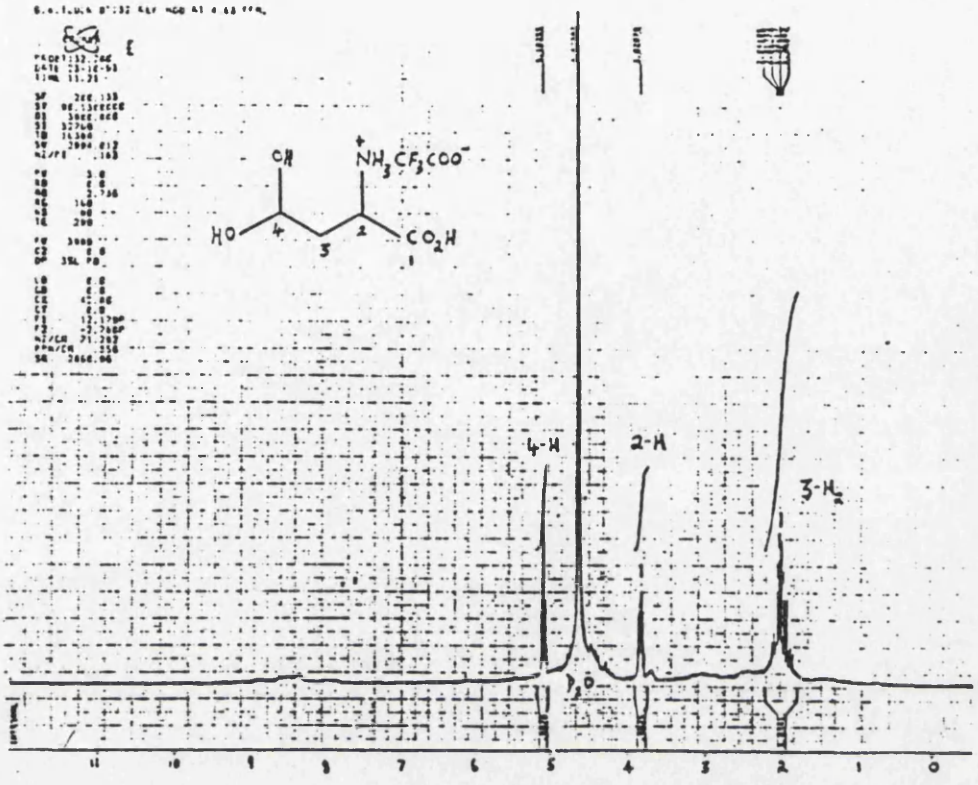


FIGURE 1 : ^{1}H NMR SPECTRUM OF ASPARTIC ACID-β-SEMIALDEHYDE HYDRATE, TRIFLUOROACETATE SALT.

microanalysis and accurate mass data. It exists as the trifluoroacetate salt. This material is stable for several months if kept dry under a nitrogen atmosphere at 0 °C.

This synthesis was repeated for D- (76b) and L-allylglycine (76a). The L-isomer of aspartic acid-β-semialdehyde hydrate (89a) had $[\alpha]_D^{16}$ +3.33° and mp 63-64 °C, whereas the D-isomer (89b) had $[\alpha]_D^{16}$ -3.15° and mp 63-65 °C. The CD spectrum of D- (89b) and L-aspartic acid-β-semialdehyde hydrate (89a) were mirror images (**Figure 2**). The D-isomer had Δ_E -1.0 at 202 nm. This is in

agreement with the general rule that aliphatic D-amino acids give negative CD curves around 203 nm, and vice-versa for L-amino acids.

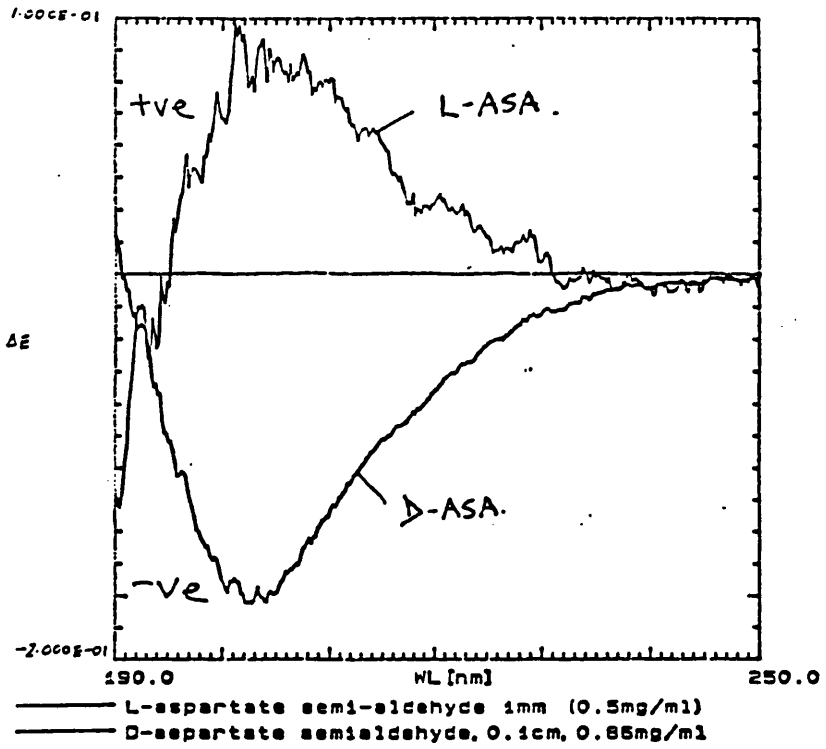
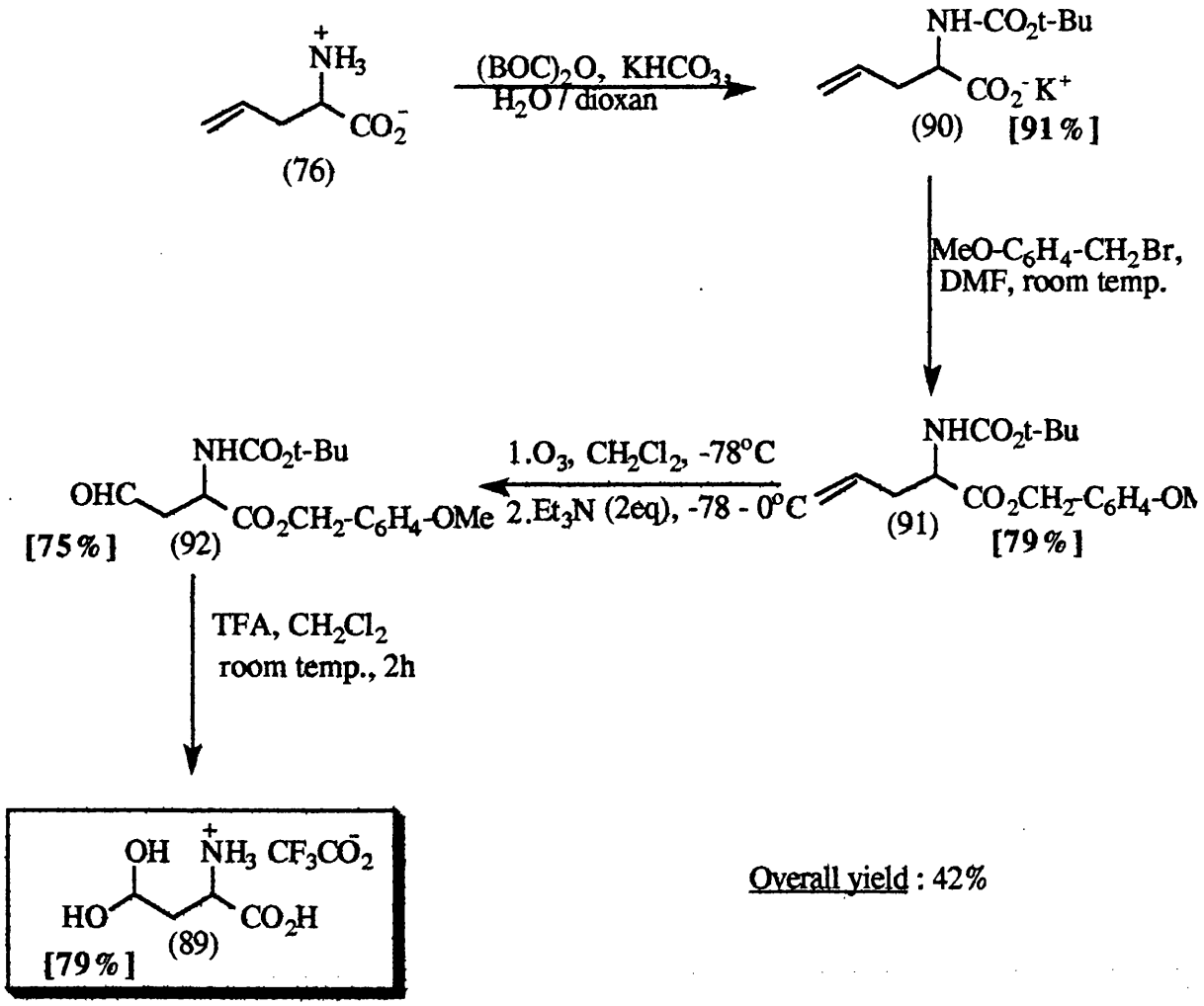


FIGURE 2 : CD CURVES OF D- AND L-ASPARTIC ACID-β-SEMIALDEHYDE.

The overall yield of formation of DL-aspartic acid-β-semialdehyde hydrate (89) from DL-allylglycine (76) was 17%. The reason for this low overall yield is the poor yield of the first step in the synthesis, namely formation of the *t*-butyl ester of allylglycine (86) using isobutylene and conc. sulphuric acid. This step was repeated several times under various different reaction conditions, but no yield higher than 33% was obtained. The low yield is presumably due to formation of isobutylene polymers. These remain

in the organic solvent when the *t*-butyl ester is precipitated as the hydrochloride salt.

To overcome this problem it was decided to change slightly the synthesis by replacing the *t*-butyl ester with the anisyl (*p*-methoxybenzyl) ester **(Scheme 40).**[154]



Overall yield : 42%

Scheme 40

This ester was put on in 79% yield giving an overall yield of formation of DL-aspartic acid-β-semialdehyde hydrate (89) of 42%.

The *p*-methoxybenzyl ester can also be easily removed by treatment with trifluoroacetic acid.[156]

The potassium salt of *N*-*t*-butoxycarbonyl-DL-allylglycine (90) was prepared in 91% yield from DL-allylglycine (76) using di-*t*-butyl dicarbonate and potassium bicarbonate in a water/dioxan mixture. This procedure was used by Baldwin *et al.* to prepare the potassium salt of *N*-*t*-butoxycarbonyl-L-homoserine.[157] Compound (90) was converted into the *p*-methoxybenzyl ester (91) in 79% yield using *p*-methoxybenzyl bromide in DMF. The same ozonolysis procedure was used as before to give the aldehyde (92). The L-isomer of this aldehyde has already been prepared by Baldwin and Flinn[137] as an intermediate in their total syntheses of the more complex amino acids (82) and (83).

Deprotection was carried out in one step by stirring (92) in trifluoroacetic acid for two hours to give the hydrate of the trifluoroacetate salt of DL-aspartic acid-β-semialdehyde (89). This product had identical physical properties and spectroscopic data to those formed from the synthesis using the *t*-butyl ester.

3.4. Biochemical Results with L-Aspartic Acid-β-semialdehyde and Pyruvate.

DHDP Synthase.

DHDP Synthase used in our biochemical experiments was purified and over expressed from *E. coli*.[121] This work was

undertaken by Emma Borthwick (Biochemistry Dept., University of Glasgow). The *E. coli* strain MV1190 (*Rec* A^-) was transformed by pDA2 to produce an overexpressing strain for DHDP Synthase. pDA2, a pUC9 derived plasmid containing the gene encoding for DHDP Synthase, was obtained from Dr. P. Stragier in Paris. The specific activity of DHDP Synthase from the over-expressed strain is 52 units/mg.

A homogeneous sample of DHDP Synthase was analysed by electrospray mass spectrometry (ESMS) by Andrea Schneier and Dr. C. Abell (University of Cambridge).[157a] A molecular mass of 31 272.3 Da (eleven determinations) was obtained for the enzyme. The electrospray mass spectrum of DHDP Synthase is shown in **Figure 3.**

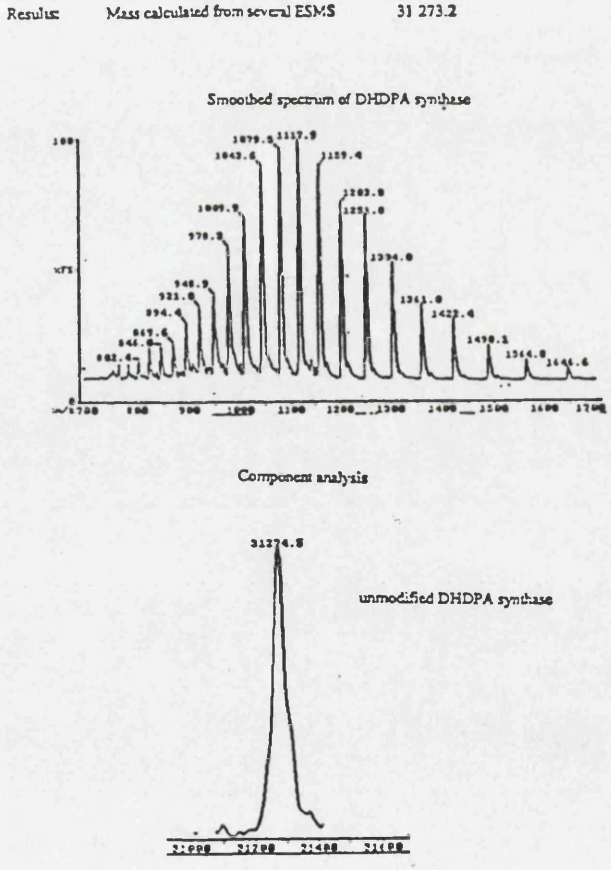


FIGURE 3 : ELECTROSPRAY MASS SPECTROMETRY OF DHDP SYNTHASE.

The *E. coli* DHDP Synthase has previously been sequenced and the mass calculated as 31 372.3. The difference between the mass calculated and that observed is within the resolution of the VG BioQ mass spectrometer (0.1%).

The molecular mass of 31 272.3 is taken as correct for all subsequent studies of DHDP Synthase. The masses of all enzyme-substrate complexes analysed by ESMS are based on this mass.

L-Aspartic Acid-β-semialdehyde

The trifluoroacetate salts of DL- (89) and L-aspartic acid-β-semialdehyde hydrate (89a) were shown to act as substrates for the DHDP Synthase reaction. The D-isomer (89b) did not act as a substrate. This is in agreement with the results obtained by Black and Wright.[70]

The DL- (89) and L-isomers (89a) were also shown to be substrates for homoserine dehydrogenase[134-136] by incubation with the enzyme and observation of the disappearance of NADPH. The D-isomer (89b) did not act as a substrate. Homoserine dehydrogenase was partially purified using the method of Bachi and Cohen.[155]

Schiff's Base Formation Between DHDP Synthase and Pyruvate.

One of my co-workers, Susanne Connell, has shown that there is irreversible loss of enzymatic activity of DHDP Synthase upon the addition of sodium borohydride only in the presence of pyruvate (22). This suggests that the formation of a Schiff's base intermediate

between DHDPS and pyruvate (22) takes place in the enzymatic reaction. This is in agreement with the results reported by Schedlarski and Gilvarg[74] and by Kumpaisal *et al.*[57]

Andrea Schneier and Dr. C. Abell (University of Cambridge)[157a] have obtained direct evidence for Schiff's Base Formation Between DHDP Synthase and Pyruvate (22). A sample of catalytically active DHDP Synthase was incubated with pyruvate (22) for five minutes at 0 °C. Sodium borohydride was then added, resulting in 85 % inactivation of the enzyme.

ESMS of this sample (Figure 4) showed a single protein species, indicating that all the active sites were modified and confirming that the tetrameric enzyme does not exhibit half-sites reactivity. The molecular mass of the protein, 31 347.1 (4 determinations) corresponds to that of the reduced Schiff's base with pyruvate and DHDP Synthase. The calculated molecular mass of this species is 31 345.4. The ESMS of DHDP Synthase, pyruvate and borohydride is shown in **Figure 4.**

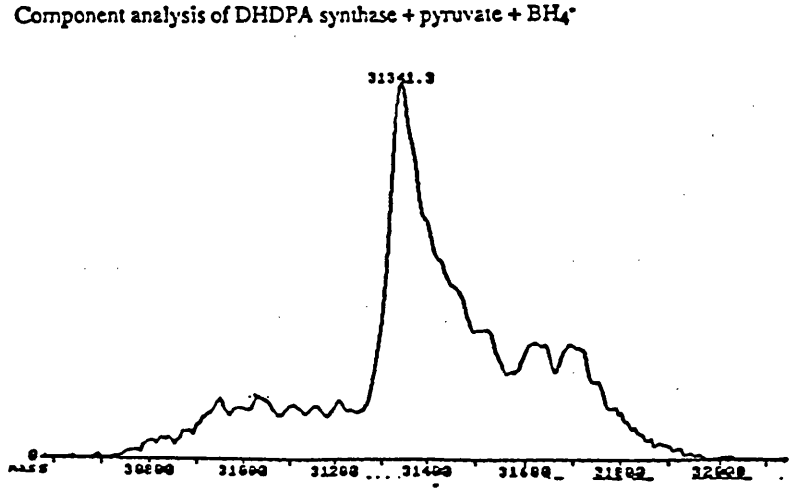


FIGURE 4 : ELECTROSPRAY MASS SPECTROMETRY OF DHDP SYNTHASE + PYRUVATE + BOROHYDRIDE.

An identical sample of DHDP Synthase was treated with sodium borohydride in the absence of pyruvate (22). No loss of enzyme activity was observed and the ESMS analysis showed only unmodified enzyme.

Similar studies were carried out in which DHDP Synthase was incubated with L-aspartic acid-β-semialdehyde hydrate (89a). However, no loss of enzymatic activity was observed upon the addition of sodium borohydride and the ESMS of the sample showed only unmodified DHDP Synthase. This suggests that there is no Schiff's formation base between DHDPS and L-aspartic acid-β-semialdehyde hydrate (89a) in the enzymic reaction.

Further work must be carried out using ESMS to investigate the mechanism of formation of L-2,3-DHDPA (23).

Standard Assay System for DHDP Synthase.

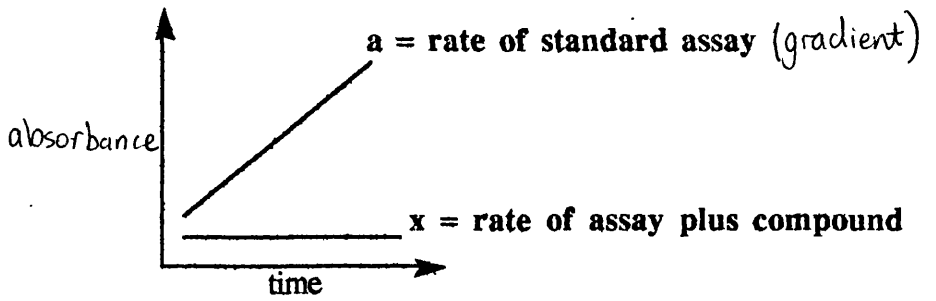
Borthwick[121] set up a standard assay screen for DHDP Synthase by monitoring the rate of formation of dipicolinic acid (36) at 270 nm. The product of the reaction, *in vivo*, is L-2,3-DHDPA (23) which is unstable and could not be the product absorbing at 270 nm. L-2,3-DHDPA (23) is oxidised in air to DPA (36) which absorbs at 270 nm (**Scheme 9**). This assay has enabled us to test analogues of both substrates, L-aspartic acid-β-semialdehyde hydrate (89a) and pyruvate (22), and the pathway intermediates L-2,3-DHDPA (23) and L-2,3,4,5-THDPA (24) for substrate or inhibitor activity.

The standard 1 ml assay consisted of 100 mM imidazole buffer, 1 mM L-aspartic acid-β-semialdehyde (89a), 1 mM pyruvate (22)

and 16 units of DHDP Synthase. Three concentrations of compound were studied, 1 mM, 0.5 mM and 0.1 mM, unless other dilutions were required. Each result shown is an average over three assay runs.

The level of inhibition was measured by a percentage of the standard rate as shown in the following equation and graph :

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



Significant inhibition was taken to be about 10% inhibition at 0.5 mM of the compound being tested. All test data obtained is shown in Section 6.4 - Experimental Chapter. Due to the lack of time no K_I or K_M values for any of the compounds tested have been obtained.

3.5. NMR Spectroscopic Studies.

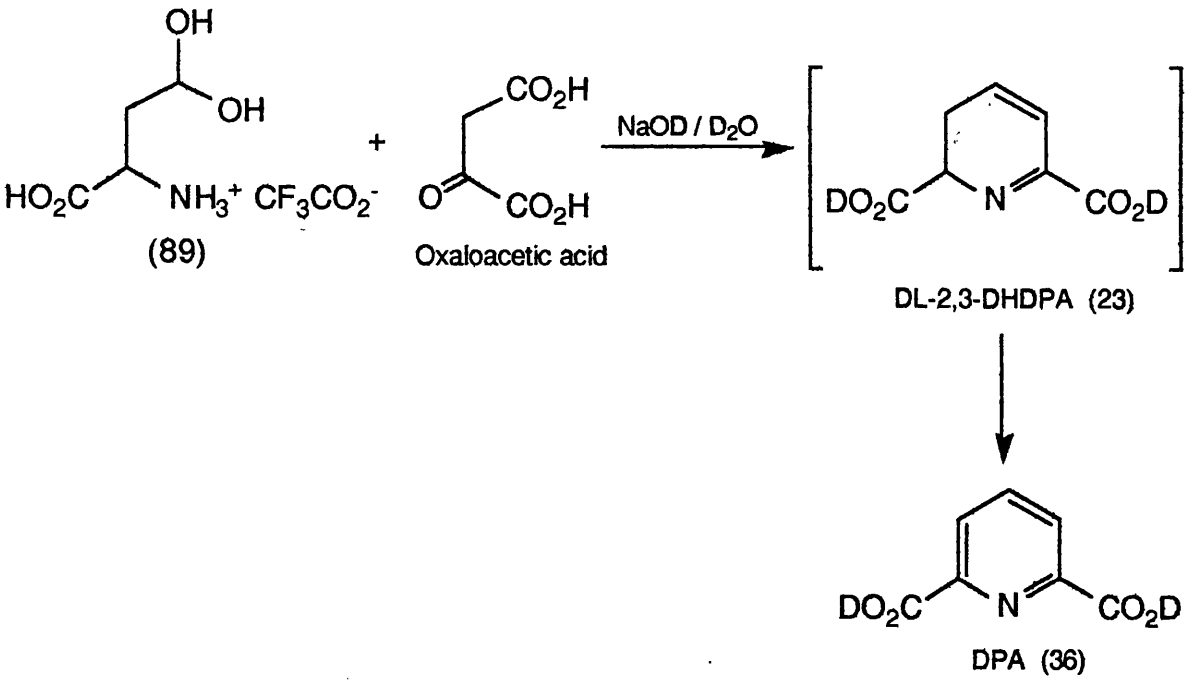
Our Biochemistry co-workers have shown that by incubating L-aspartic acid-β-semialdehyde hydrate (89a) and pyruvate (22) with

DHDP Synthase and following the reaction by UV spectroscopy the product obtained has a UV spectrum with λ_{max} at 270 nm.

To identify the absorbing species a portion of the assay solution was freeze dried, dissolved in D_2O and a ^1H NMR spectrum at 200 MHz was run at room temperature. The spectrum was very noisy and several impurities were present. A strong singlet at δ 7.95 ppm corresponded to DPA (36). The standard ^1H NMR spectrum of DPA (36) is a singlet at δ 8.35 ppm. The difference in δ may be due to pH differences. Further study of this product from the enzymic reaction in an NMR spectrometer with higher field strength would be desirable to see if splitting of the signal can be observed.

A similar non-enzymic reaction between DL-aspartic acid-β-semialdehyde hydrate (89a) and oxaloacetic acid under basic conditions in D_2O was followed by ^1H NMR spectroscopy (**Scheme 41**).

The product from this reaction was DPA (36) (singlet δ 8.35 ppm) after a period of two hours *via* some intermediate which has a vinyl signal at δ 5.47 ppm. This intermediate signal was formed instantaneously then slowly disappeared as the aromatic (DPA) signal increased over the two hour period. A ^{13}C NMR spectrum of the reaction product was consistent with the authentic ^{13}C NMR spectrum of DPA (36), i.e. there were 4 signals at δ_C 127.7 (C-3 and -5), 139.4 (C-4), 148.2 (C-2 and -6) and 165.6 (2 x acid groups). Unfortunately due to lack of time this interesting result was not studied further.



Scheme 41

3.6. Synthesis and Test Results of Analogues of L-Aspartic Acid-β-semialdehyde.

Analogues of DL-aspartic acid-β-semialdehyde hydrate (89) have been synthesised and preliminary testing for substrate or inhibitor activity on DHDP Synthase has been carried out.

DL-Allylglycine (76) and the intermediates in the synthetic pathway to DL-aspartic acid-β-semialdehyde hydrate (89) (**Scheme 38**), DL-allylglycine *t*-butyl ester hydrochloride (86), DL-*N*-*t*-butoxycarbonylallylglycine *t*-butyl ester (87) and DL-*N*-*t*-butoxycarbonylaspartic acid-β-semialdehyde *t*-butyl ester (88) have

been tested for inhibitor activity. DL-Allylglycine (76) showed poor inhibition (14% at 1 mM) and compounds (86), (87) and (88) showed no inhibition.

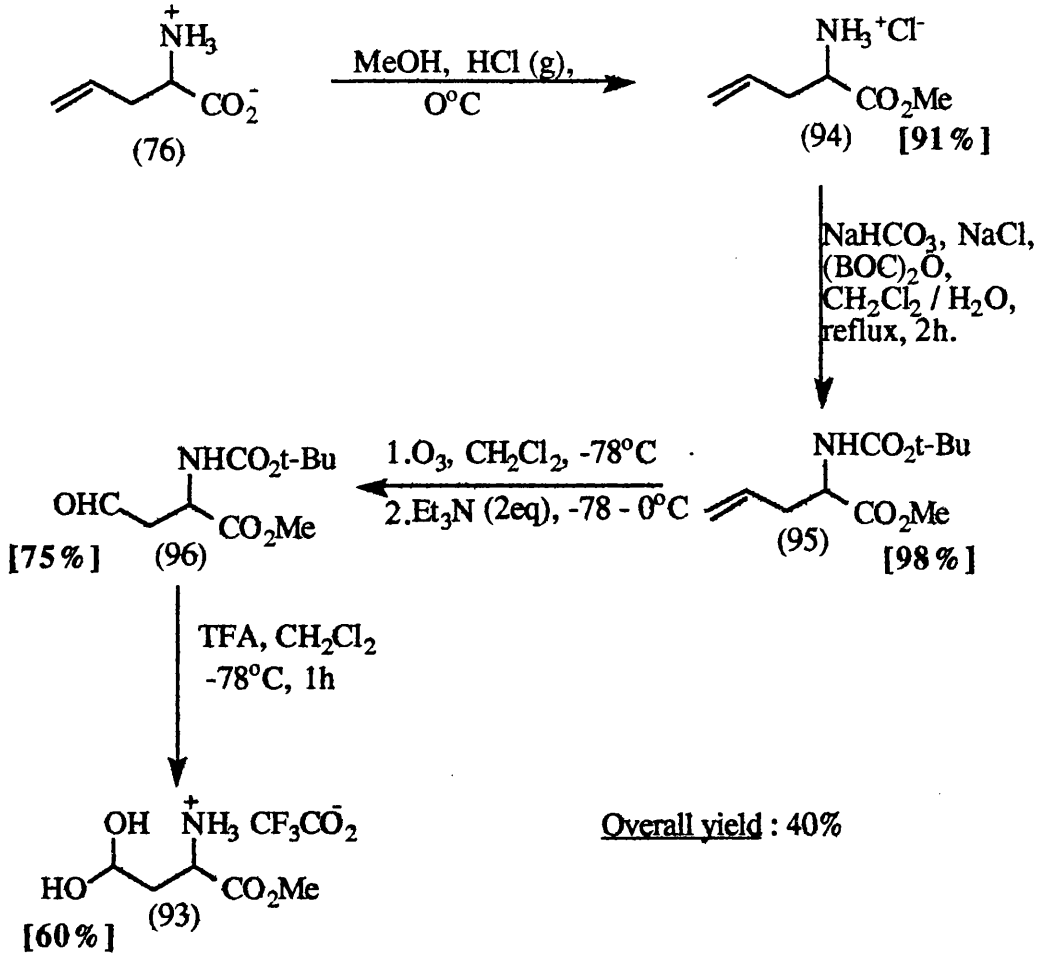
Trifluoroacetate Salt of DL-Aspartic Acid-β-semialdehyde Methyl Ester Hydrate.

The trifluoroacetate salt of the methyl ester of DL-aspartic acid-β-semialdehyde hydrate (93) was prepared in a similar manner to DL-aspartic acid-β-semialdehyde (89) (**Scheme 42**), starting from DL-allylglycine (76). The overall yield of formation was 40%.

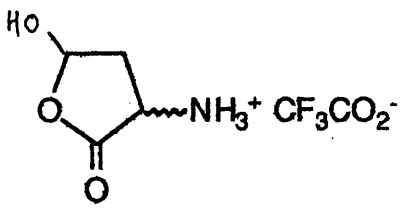
DL-Allylglycine methyl ester hydrochloride (94) was prepared in 91% yield by stirring DL-allylglycine (76) in methanol and flushing the solution with HCl gas at 0 °C. Protection of the nitrogen using di-*t*-butyldicarbonate gave (95). Ozonolysis was carried out as before to give the aldehyde (96).

However problems arose with the deprotection of (96). Under the same conditions as before, using trifluoroacetic acid at room temperature a yellow syrup was obtained which proved to be the lactone (97) by ^1H NMR and IR analysis, i.e. no methyl ester signal was observed in the ^1H NMR spectrum and the IR spectrum had a υ_{max} at 1770 cm^{-1}. To solve this problem the deprotection was carried out with the same reagents at -78 °C. Removal of the solvent *in vacuo* gave a yellow solid in 60% yield which was shown by spectroscopic analysis to be the methyl ester (93), i.e. there was a

singlet at δ_H 3.63 and a signal at δ_C 171.3 for the methyl ester and the IR spectrum had a υ_{max} at 1750 cm^{-1}.



Scheme 42



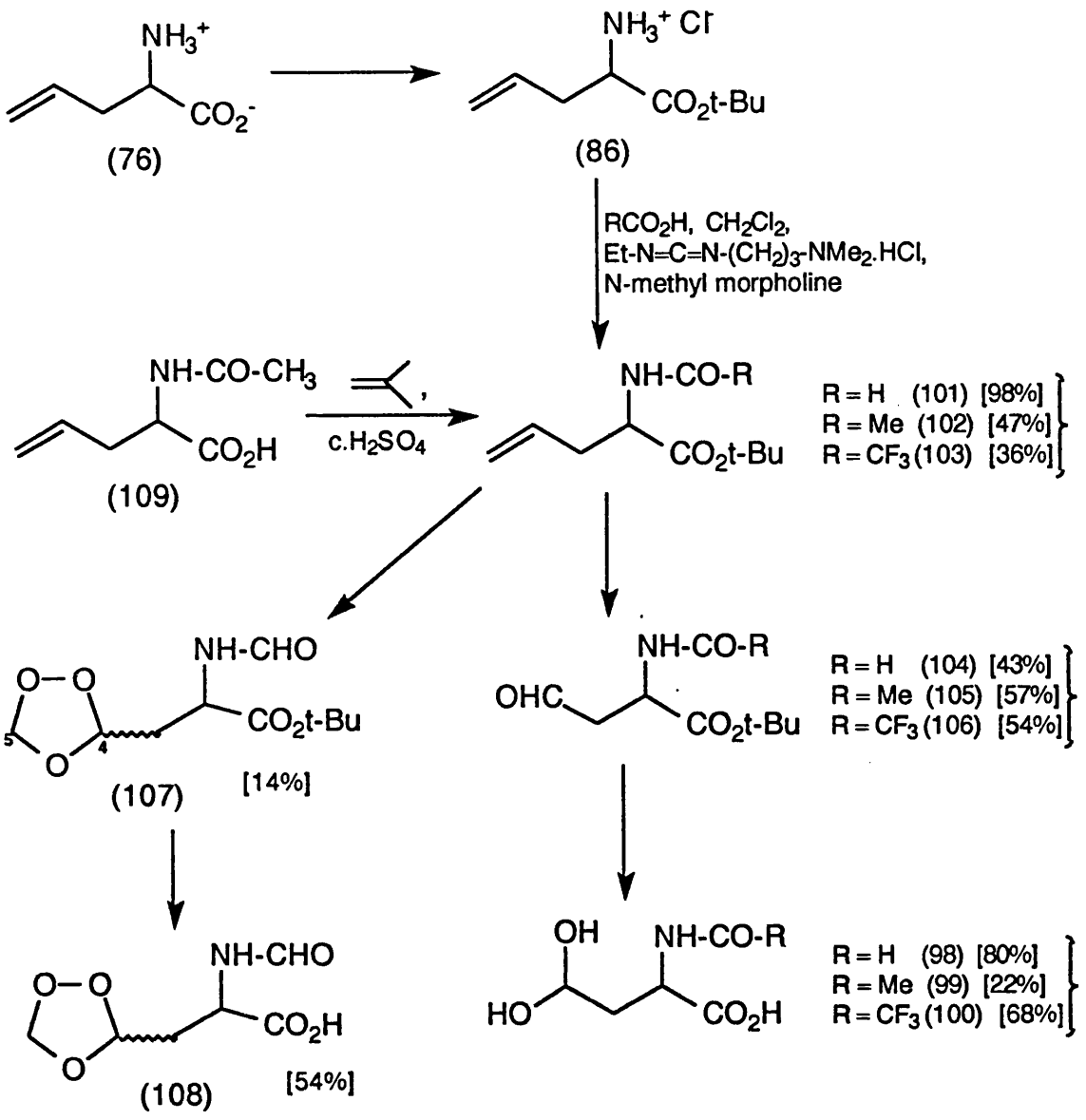
(97)

When tested as a substrate, no rate was observed for the methyl ester (93), but, 14% inhibition was found at 0.5 mM. Similarly DL-*N*-*t*-butoxycarbonylaspartic acid-β-semialdehyde methyl ester (96) showed 5% inhibition at 0.5 mM.

DL-*N*-Formyl-, DL-*N*-Acetyl- and DL-*N*-Trifluoroacetyl-aspartic acid-β-semialdehyde Hydrates.

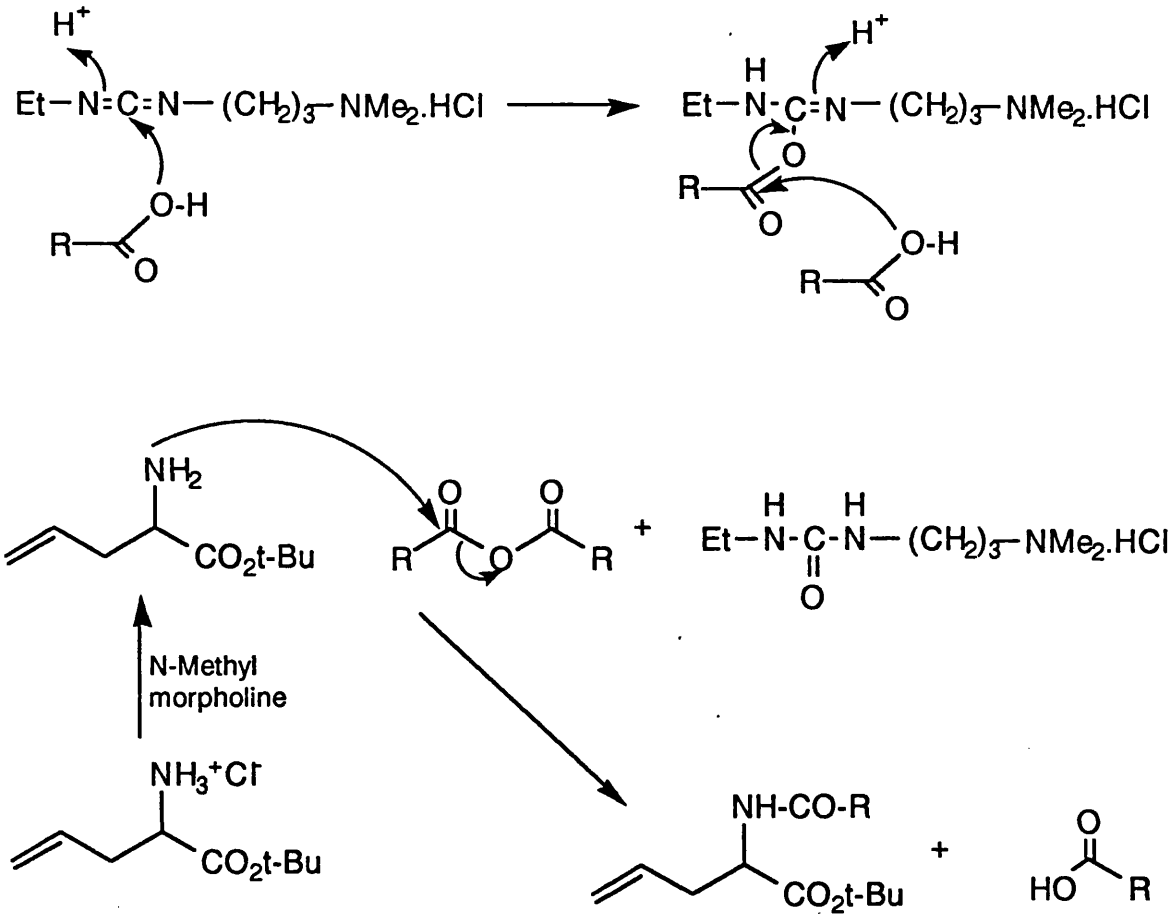
The same synthetic route was used to form the *N*-amido analogues DL-*N*-formyl- (98) and DL-*N*-trifluoroacetylaspartic acid-β-semialdehyde hydrates (100) **(Scheme 43)**. The *t*-butyl ester (86) has been previously described. The *N*-formyl- (101) and *N*-trifluoroacetyl amides (103) were prepared in 98% and 36% yields using formic acid and trifluoroacetic acid respectively with the coupling agent, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide.[158] This is a particularly good coupling agent to use as the resultant urea compound can be easily removed as the acid salt of the amine by washing the reaction mixture with 5% aqueous citric acid in the work-up. The mechanism for the coupling reaction is shown in **Scheme 44.**

Ozonolysis of the trifluoroacetyl amide (103) gave the aldehyde (106) in 54% yield. However, with the ozonolysis of the *N*-formyl amide (101) under the same conditions and the same reductive work-up using two equivalents of triethylamine at -78 °C no aldehyde was formed. Instead, spectroscopic analysis showed that the ozonide (107) had been isolated in 14% yield, i.e. the ^{1}H and ^{13}C



Scheme 43

NMR spectra showed no aldehyde signals. The ^{13}C NMR spectrum had signals at δ_C 67.3 ppm for C-5 and δ_C 91.2 ppm for C-4 (**Figure 5**). There was no possibility of this product being starting material (101) because the expected signals for C-5 and C-4, at δ_C 118.9 and 131.9 respectively, were not observed in the ^{13}C NMR spectrum.



Scheme 44

To solve this problem, the reductive work-up was changed to four equivalents of triethylamine at room temperature. This gave the desired aldehyde (104) in 57% yield. Compounds (104), (106) and (107) were all deprotected by stirring in trifluoroacetic acid and dichloromethane at room temperature for one hour to give the products (98), (100) and (108) in 80%, 68% and 54% yields respectively. The IR spectra of (98) and (100) showed υ_{max} at 1665 and 1655 cm^{-1} respectively for the amide carbonyl bands.

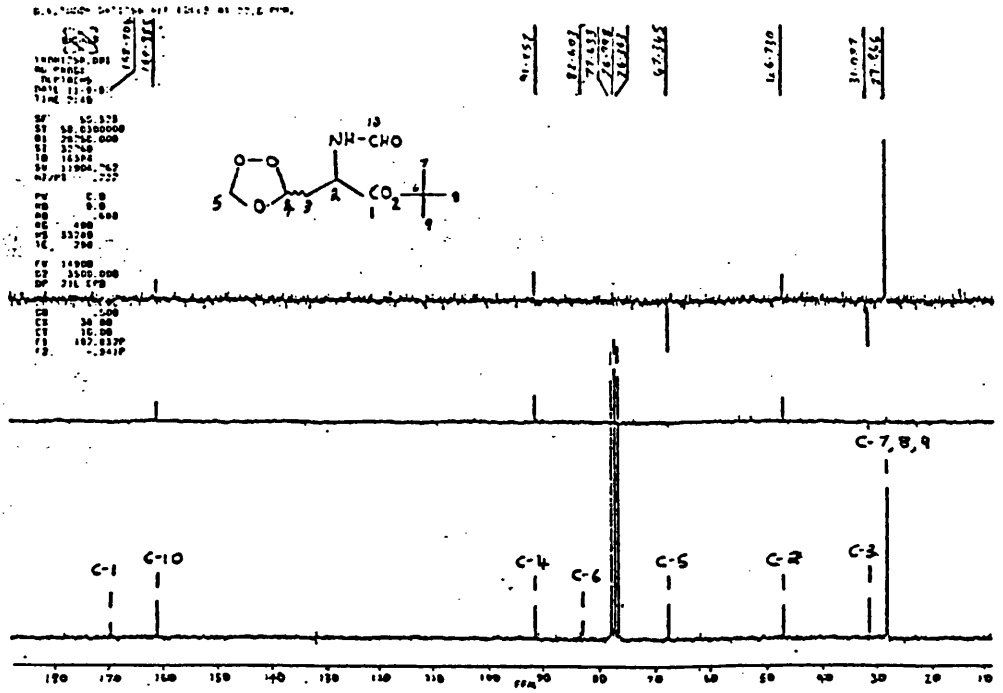


FIGURE 5.

N-Acetyl-DL-allylglycine *t*-butyl ester (102) was prepared from the commercially available *N*-acetyl-DL-allylglycine (109) using isobutylene as previously described. Purification was achieved on a silica gel column eluting with 15% ethyl acetate in hexane to give a clear oil in 47% yield. Ozonolysis followed by deprotection gave first of all the aldehyde (105) in 57% yield, i.e. there was a signal at δ_H 9.68 for the aldehyde proton and a signal at δ_C 199.4 for C-4, and then the desired product (99) in 72% yield.

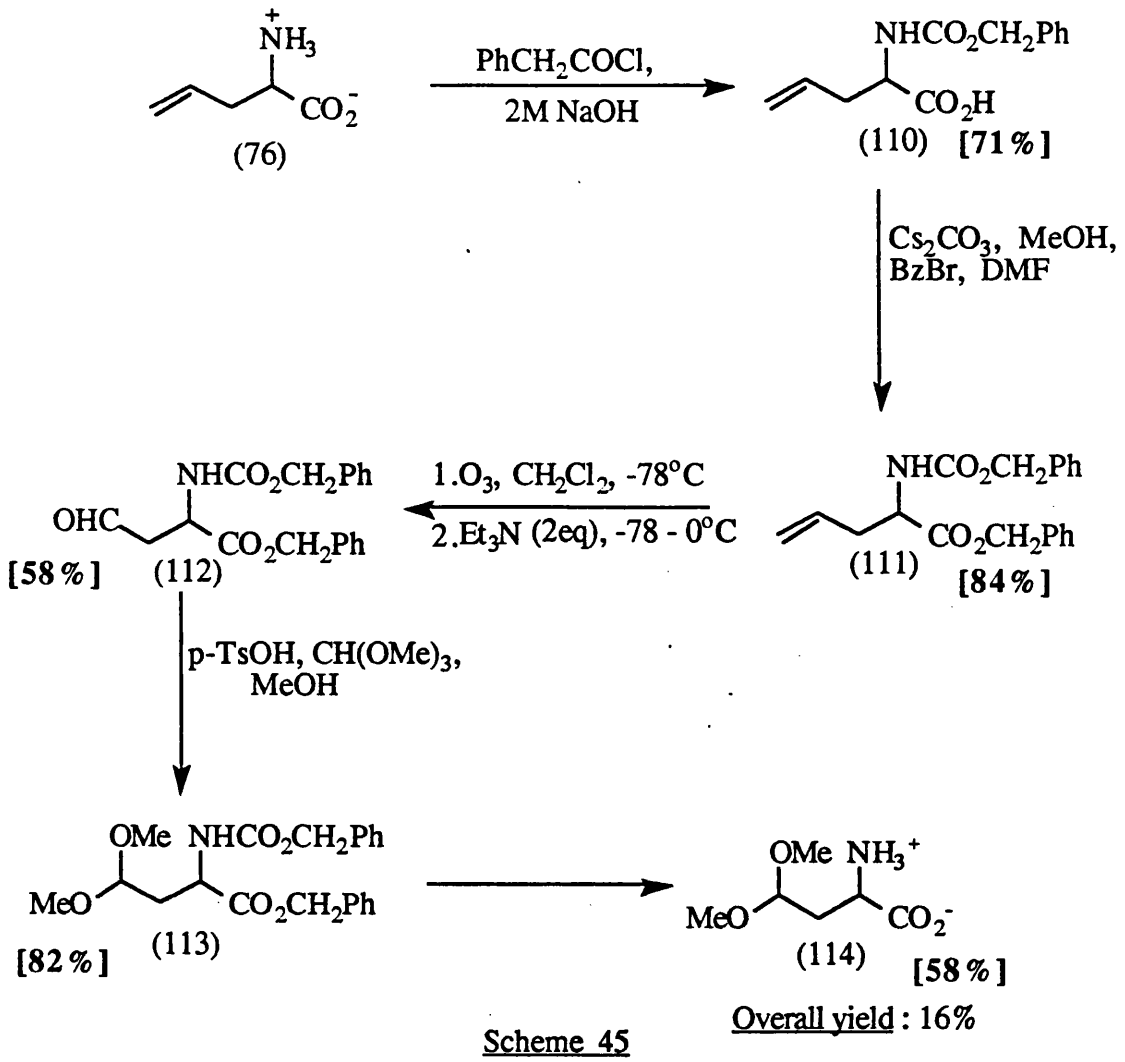
Unfortunately due to lack of time only DL-*N*-formylaspartic acid-β-semialdehyde hydrate (98), DL-*N*-formylaspartic acid-β-semialdehyde *t*-butyl ester (104) and DL-*N*-formylaspartic acid-β-semialdehyde ozonide *t*-butyl ester (107) have been tested for inhibitor activity. Compounds (98) and (104) both showed poor

activity with approximately 10% inhibition at 1 mM. The ozonide (107) showed no inhibition.

DL-Aspartic Acid-β-semialdehyde Dimethyl Acetal.

Acylation of DL-allylglycine (76) with benzyl chloroformate yielded the corresponding *N*-protected amino acid (110) in 71% yield (**Scheme 45**). Treatment of (110) with benzyl bromide in dimethylformamide at room temperature gave an 84% yield of *N*-carboxybenzyl-DL-allylglycine benzyl ester (111).[148] Ozonolysis of (111) in dichloromethane at -78 °C led to the aspartic acid-β-semialdehyde derivative (112) in 58% yield. The corresponding dimethyl acetal (113) was formed in 82% yield upon heating the aldehyde (112) in methanol/trimethyl orthoformate solution at reflux temperature for two hours.[159] Catalytic reduction of (113) gave DL-aspartic acid-β-semialdehyde dimethyl acetal in 58% yield.[160] The overall yield from DL-allylglycine was 16%.

The L-isomers of the protected dimethyl acetal (113) and the aspartic acid-β-semialdehyde derivative (112) have been previously prepared by Keith *et al.*[151] in their total synthesis of the naturally occurring amino acid rhizobitoxine (81). They started with L-homoserine and used the same protecting groups to give *N*-carboxybenzyl-L-homoserine benzyl ester. Oxidation of this protected homoserine derivative with chromium trioxide-pyridine complex[150] in dichloromethane gave the aldehyde (112). The corresponding dimethyl acetal (113) was formed in essentially



Scheme 45

quantitative yield upon heating the aldehyde (112) in methanol/trimethyl orthoformate solution at reflux temperature in the presence of a catalytic amount of ammonium chloride.

The compounds prepared by Keith *et al.*[151] had identical spectroscopic and physical data to the aldehyde (112) and the dimethyl acetal (113) prepared in our synthesis of DL-aspartic acid-β-semialdehyde dimethyl acetal (114). Unfortunately, due to lack of time (114) has not been tested for substrate or inhibitor activity.

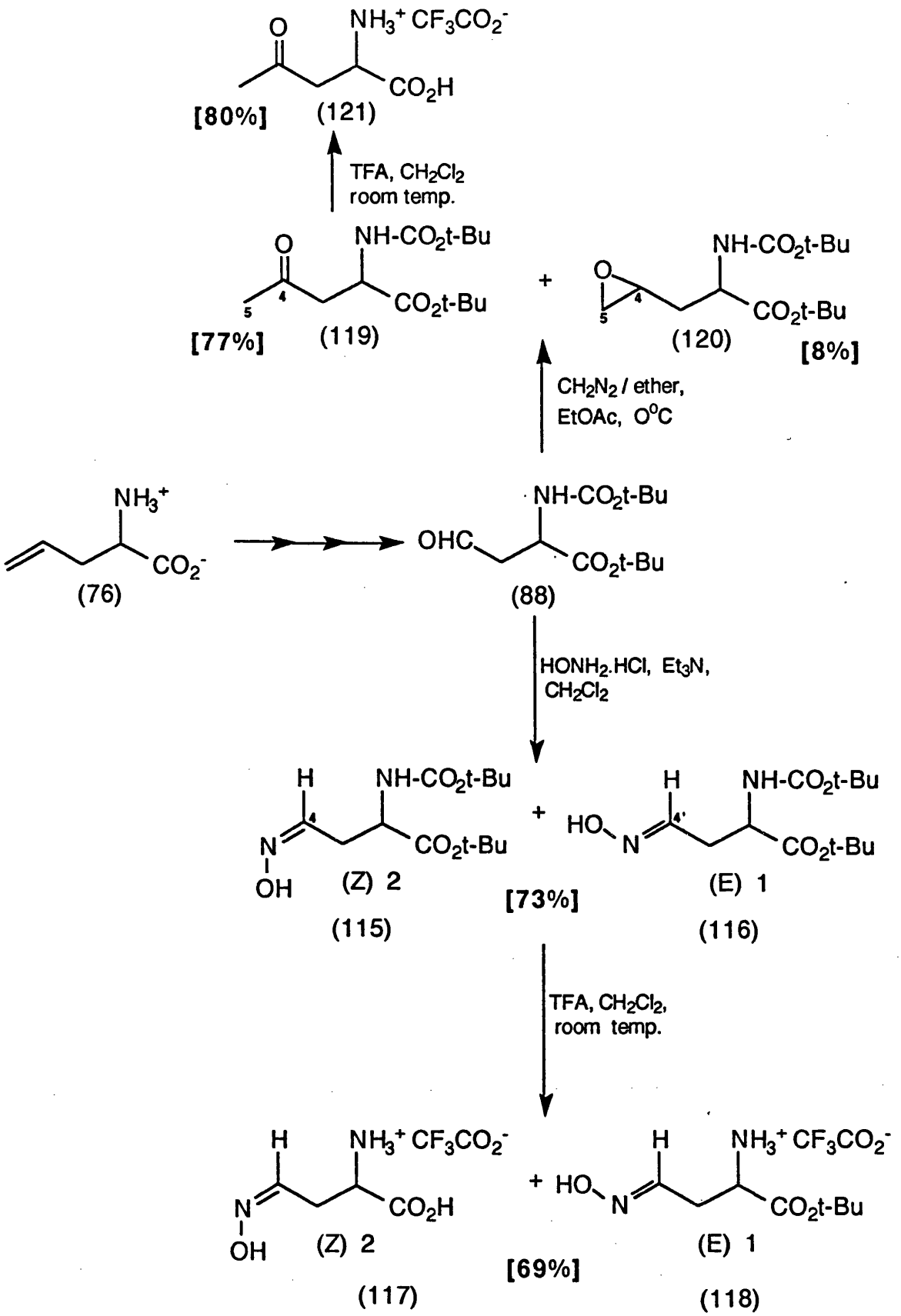
Trifluoroacetate Salt of DL-Aspartic acid-β-semialdehyde Oxime.

Reaction of *N*-*t*-butoxycarbonylaspartic acid-β-semialdehyde (88) with hydroxylamine hydrochloride and triethylamine in dichloromethane gave a 2 : 1 mixture of the *Z*- (115) and *E*- oxime (116) in 73% yield (**Scheme 46**). The ^{1}H NMR spectrum showed a doublet of doublets at δ_H 6.80 for 4-H (*Z*-isomer) and at δ_H 7.39 for 4'-H (*E*-isomer). The *Z*-isomer (115) 4-H is shielded by the lone pair on the adjacent nitrogen and, as expected, comes at a lower chemical shift than that of the *E*-isomer (116) 4'-H.

Deprotection of (115) and (116) using trifluoroacetic acid as previously described gave the *Z*- (117) and the *E*-isomer (118) of the desired product in 69% yield. The oximes have not been yet tested for inhibitor activity.

Trifluoroacetate Salt of DL-2-Amino-4-oxopentanoic Acid and DL-2-Amino-4-epoxypentanoic Acid.

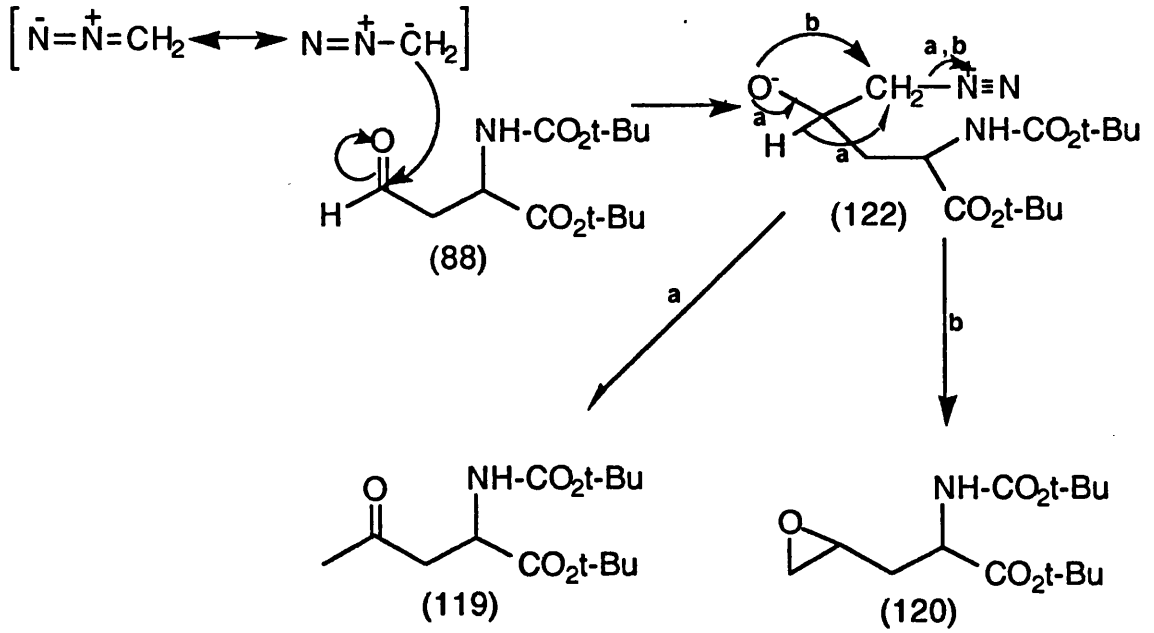
Treatment of a solution of (88) in ethyl acetate with a solution of diazomethane in ether gave a mixture of the methyl ketone (119) and the epoxide (120) in 77% and 8% yields respectively (**Scheme 46**).[161] Separation was achieved on a silica gel column eluting with 50% ether in hexane. The ^{1}H NMR spectrum of (119) had a singlet at δ_H 2.17 for the methyl ketone, and a signal at δ_C 206.6 C-4 in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum of (120) had a multiplet at δ_H


NH3+ CF3CO2-
O
CO2H
[80%]
(121)
TFA, CH2Cl2
room temp.
NH-CO2t-Bu
CO2t-Bu
[77%]
(119)
+
(120)
[8%]
CH2N2 / ether,
EtOAc, 0°C
NH3+
CO2-
(76)
OHC
(88)
HONH2.HCl, Et3N,
CH2Cl2
H
HO
N
OH
(Z) 2
(115)
[73%]
(E) 1
(116)
TFA, CH2Cl2,
room temp.
[69%]
(117)
(118)

Scheme 46

2.45-2.63 for the protons at C-5 and at δ_H 2.91-3.00 for the protons at C-4. The ^{13}C NMR spectrum had signals at δ_C 48.1 for C-5 and δ_C 49.8 for C-4.

The mechanism of the reaction of the aldehyde (88) with diazomethane involves attack at the aldehyde carbon to give the intermediate (122) (**Scheme 47**). This compound can either lose nitrogen to form the epoxide (120) or hydrogen can migrate with the loss of nitrogen to give the methyl ketone (119).

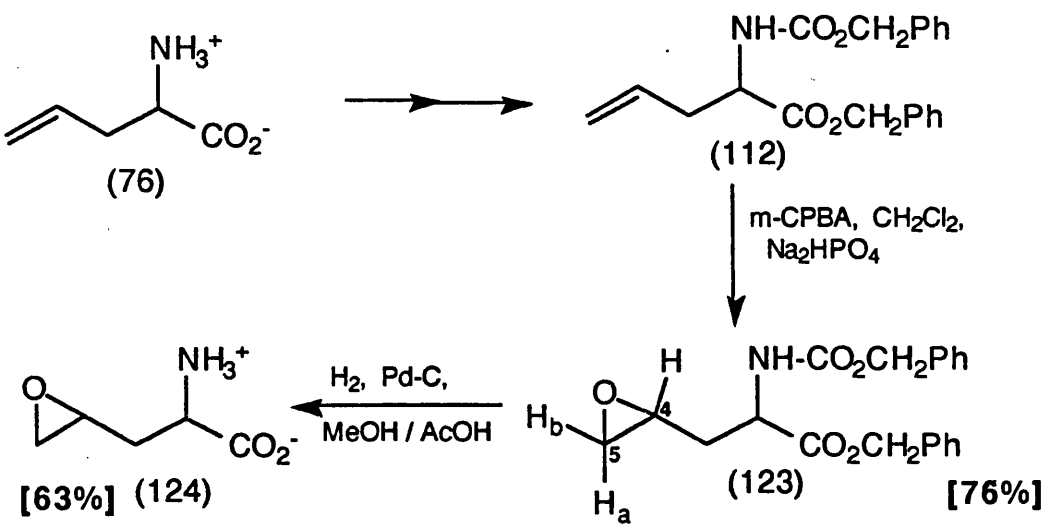


Scheme 47

Deprotection of the methyl ketone (119) was easily carried out using trifluoroacetic acid to give the trifluoroacetate salt of DL-2-amino-4-oxopentanoic acid (121) in 80% yield. However, there was not enough material to carry out the deprotection of the epoxide

(120). It was decided to prepare 2-amino-4,5-epoxypentanoic acid (124) by an alternative route.

Treatment of DL-*N*-carboxybenzylallylglycine benzyl ester (111), previously described in the synthesis of DL-aspartic acid-β-semialdehyde dimethyl acetal (114), with a solution of *m*-chloroperbenzoic acid in dichloromethane at 0 °C followed by bringing the pH of the reaction to 8 with Na_2HPO_4 buffer and stirring at room temperature for three hours gave the epoxide (123) in 76% yield (**Scheme 48**).[162] There was a doublet of doublets at δ_H 2.69



Scheme 48

for the 5b-H in the ^1H NMR spectrum. The coupling constants were *J* 4.8 Hz and *J* 2.6 Hz. There was a doublet of doublets at δ_H 2.69 for the 5a-H with coupling constants *J* 8.7 Hz and *J* 4.8 Hz and a multiplet at δ_H 2.88-3.02 for the 4-H. The ^{13}C NMR spectrum had signals at δ_C 46.7 for C-5 and δ_C 48.9 for C-4. Catalytic hydrogenolysis of (123)

gave the desired epoxide (124) in 63% yield. The methyl ketone (121) showed no inhibition at 1 mM. The epoxide (124) has not yet been tested for inhibitor activity.

Conclusion.

L-Aspartic acid-β-semialdehyde (21a) is an important intermediate in the biosynthesis of L-lysine (1), L-threonine (16) and L-methionine (18). It has also become an important intermediate in the synthesis of more complex non-protein and unnatural amino acids.

The first synthesis of L-aspartic acid-β-semialdehyde (21a) was reported in 1955 by Black and Wright.[70] This is the only reported chemical synthesis of this compound. We now report an improved synthetic route to both the pure L- (89a) and D-isomers (89b) of the trifluoroacetate salt of aspartic acid-β-semialdehyde hydrate starting from the commercially available amino acid allylglycine (76). It appears as a light yellow solid and can be stored at 0 °C for several months.

Having L-aspartic acid-β-semialdehyde hydrate (89a) in pure form has enabled us to set up a more accurate and reliable assay system to carry out kinetic studies on potential substrates or inhibitors of DHDP Synthase. Using this assay system we have confirmed that L-aspartic acid-β-semialdehyde hydrate (89a) is a substrate for the enzyme and that the D-isomer (89b) is not. We have also shown that L-aspartic acid-β-semialdehyde hydrate (89a)

does not form a Schiff's base with DHDP Synthase in the enzyme reaction whereas, as previously reported in the literature, pyruvate (22) does. Having pure L-aspartic acid-β-semialdehyde hydrate (89a) has also allowed us to carry out precise NMR spectroscopic experiments to investigate the mechanism of DHDP Synthase. We have confirmed that the product which can be isolated after carrying out the enzymic reaction is DPA (36).

Manipulation of the synthetic route to DL-aspartic acid-β-semialdehyde hydrate (89) has allowed us to prepare a range of aspartic acid-β-semialdehyde analogues and derivatives. Most of these compounds have been tested for potential inhibitor or substrate activity.

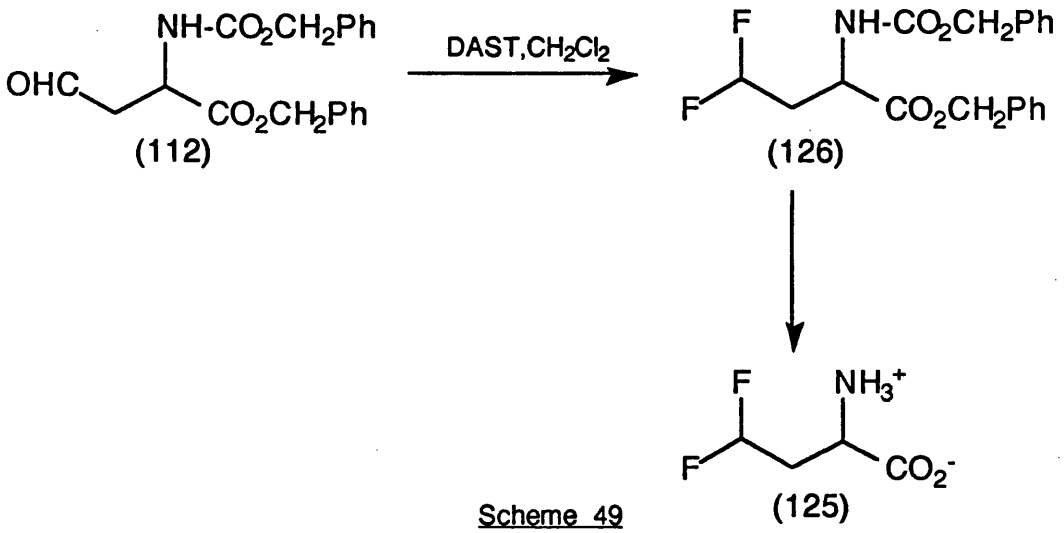
3.7. Future Work.

Accurate kinetic studies to get reliable K_I values should be carried out with compounds which are showing at least 10% inhibition at 0.5 mM. It must also be determined if the inhibition is competitive or non-competitive.

Using ^1H NMR spectroscopy to look at the mechanism of DHDP Synthase is important to establish the structures of intermediates which are formed during the enzymatic reaction. Identification of enzyme intermediates will help in proposing synthetic targets which may have useful enzymic activity. More NMR spectroscopic experiments are required at low temperatures under oxygen free conditions. This may slow down the enzymic reaction, thus giving more time to identify the enzyme intermediates.

From the test data which we already have it appears that substitution at the nitrogen group, as in the *N*-amido aspartic acid-β-semialdehyde analogues, does not lead to any inhibition. However, the methyl ester (92) showed slight inhibition and as such, larger ester groups should be investigated. Aldehyde derivatives should be prepared.

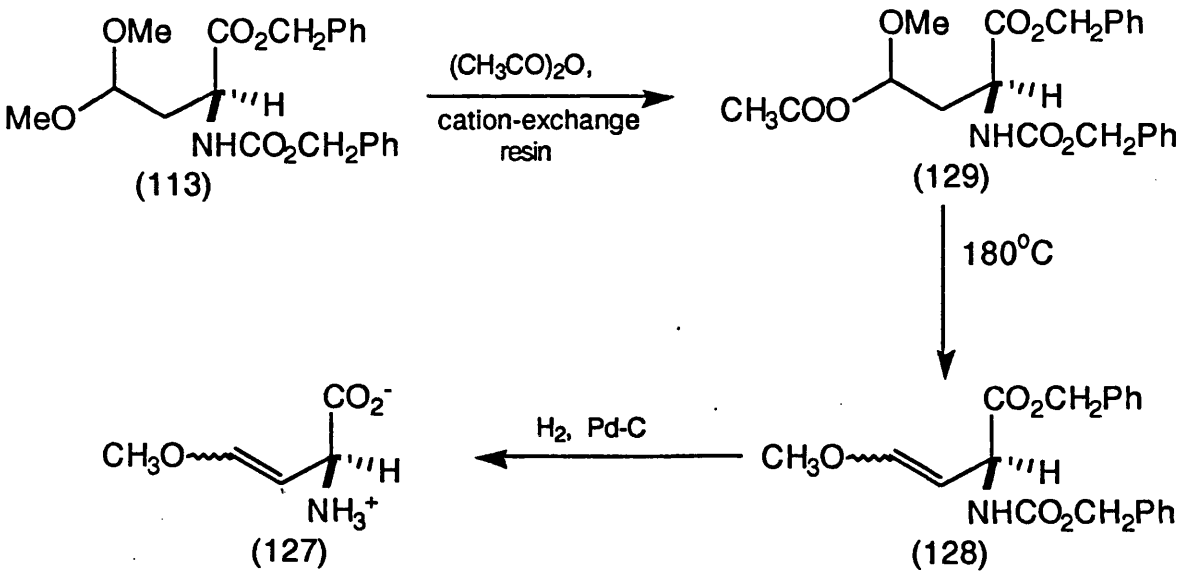
The *gem*-difluoro analogue (125) would be an interesting compound to test for activity. This could be prepared from (112) using diethylaminosulphur trifluoride (DAST)[162] to give the protected *gem*-difluoro compound (126). Catalytic hydrogenolysis should give the desired product (125) **(Scheme 49).**



Scheme 49

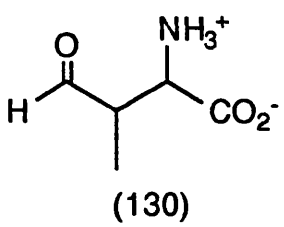
The enol ether (127) would also be an interesting compound to test for substrate or inhibitor activity on DHDP Synthase. The protected enol ether (128) has already been prepared by Keith *et al.*[151] from the dimethyl acetal (113) **(Scheme 50).**

Treatment of acetal (113) with acetic anhydride and dry cationic exchange resin at 50 °C afforded the more labile hemiacetal ester (129). Pyrolysis of this compound under reduced pressure at 180 °C gave a mixture of the *trans*- and *cis*-enol ether (128).



Scheme 50

Finally substitution at C-3 of DL-aspartic acid-β-semialdehyde hydrate (89) has not yet been considered. This might be possible starting from the enol ether (128). DL-3-Methylaspartic acid-β-semialdehyde (130) and DL-3-fluoroaspartic acid-β-semialdehyde (131) would be interesting targets.



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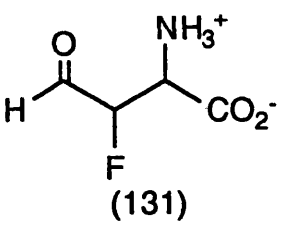

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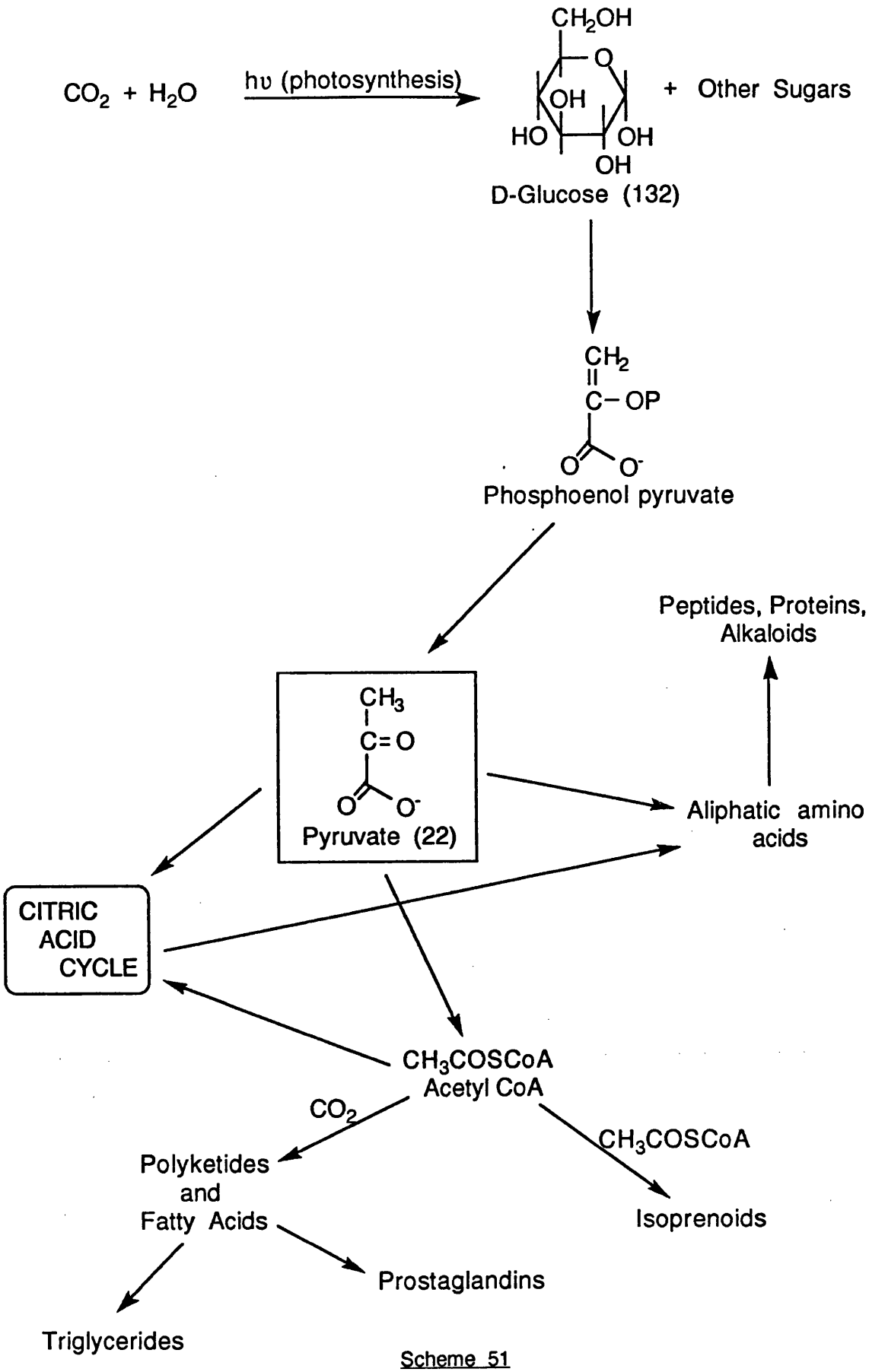
Chapter [4] - Synthesis of Derivatives of Pyruvate and Bromopyruvate.

Introduction

All cells have the capacity to metabolise glucose (132) from the photosynthesis of carbon dioxide and water. Glucose (132) is by far the most important sugar. It can be converted into adenosine triphosphate (ATP), as well as small organic molecules which are the building blocks of other biosynthetic pathways. In the absence of oxygen, glucose (132) is converted into pyruvate (22) **(Scheme 51)**, via a process known as glycolysis; but in the presence of oxygen, glucose (132) may be completely oxidised to carbon dioxide *via* the citric acid cycle,[164] with concomitant production of ATP. The products of the citric acid cycle are simple keto-acids, dicarboxylic acids, carbon dioxide and most important of all ATP. The simple keto-acids react with ammonia or its equivalent to produce aliphatic amino acids.

A product of glycolysis, pyruvate (22), can be further converted into acetylcoenzyme A, which is of pivotal importance for both primary and secondary metabolism. Acetylcoenzyme A may be used as a building block for the biosynthesis of fatty acids and polyketides which can be further coverted into triglycerides, polyphenols, polyacetylenes, prostaglandins and macrocyclic antibiotics. It can also be used as a building block for the biosynthesis of isoprenoids. Pyruvate (22) may also enter the


CO2 + H2O
hυ (photosynthesis)
CH2OH
O
OH
HO
OH
OH
+ Other Sugars
D-Glucose (132)
CH2
C–OP
O
O-
Phosphoenol pyruvate
Peptides, Proteins, Alkaloids
CH3
C=O
O
O-
Pyruvate (22)
Aliphatic amino acids
CITRIC ACID CYCLE
CH3COSCoA
Acetyl CoA
CO2
CH3COSCoA
Polyketides and Fatty Acids
Isoprenoids
Prostaglandins
Triglycerides

Scheme 51

citric acid cycle to combine with oxaloacetate to produce citrate and other keto-acids such as α-ketoglutarate, which can be converted into glutamate and other amino acids.

As previously mentioned, pyruvate (22) and the products of the citric acid cycle can be converted into aliphatic amino acids, which are the building blocks for peptides, proteins, alkaloids and penicillins. One such example of pyruvate (22) being converted into an aliphatic amino acid is the DAP pathway to L-lysine (1). Pyruvate (22) and L-aspartic acid-β-semialdehyde (21a) are the two substrates involved in this pathway. DHDP Synthase catalyses the condensation of pyruvate (22) with L-aspartic acid-β-semialdehyde (21a) to give L-2,3-DHDPA (23). A further six enzyme catalysed reactions of L-2,3-DHDPA (23) lead to L-lysine (1) **(Scheme 5)**.

The sequence of steps in the mechanism of formation of L-2,3-DHDPA (23) from pyruvate (22) and L-aspartic acid-β-semialdehyde (21a) has not yet been fully established. However, Schedlarski and Gilvarg[74] and Kumpaisal et al.[57] have shown that the enzyme functions in carbon-carbon bond formation by first of all forming an imine linkage with pyruvate (22), i.e. DHDP Synthase forms a Schiff's base intermediate with pyruvate (22) in the enzymic reaction.

In this Chapter a range of pyruvate and bromopyruvate analogues have been synthesised and tested for either substrate or inhibitor activity with DHDP Synthase. The results obtained with these compounds will be discussed at the end of each section.

4.1. Synthesis of Pyruvate Derivatives.

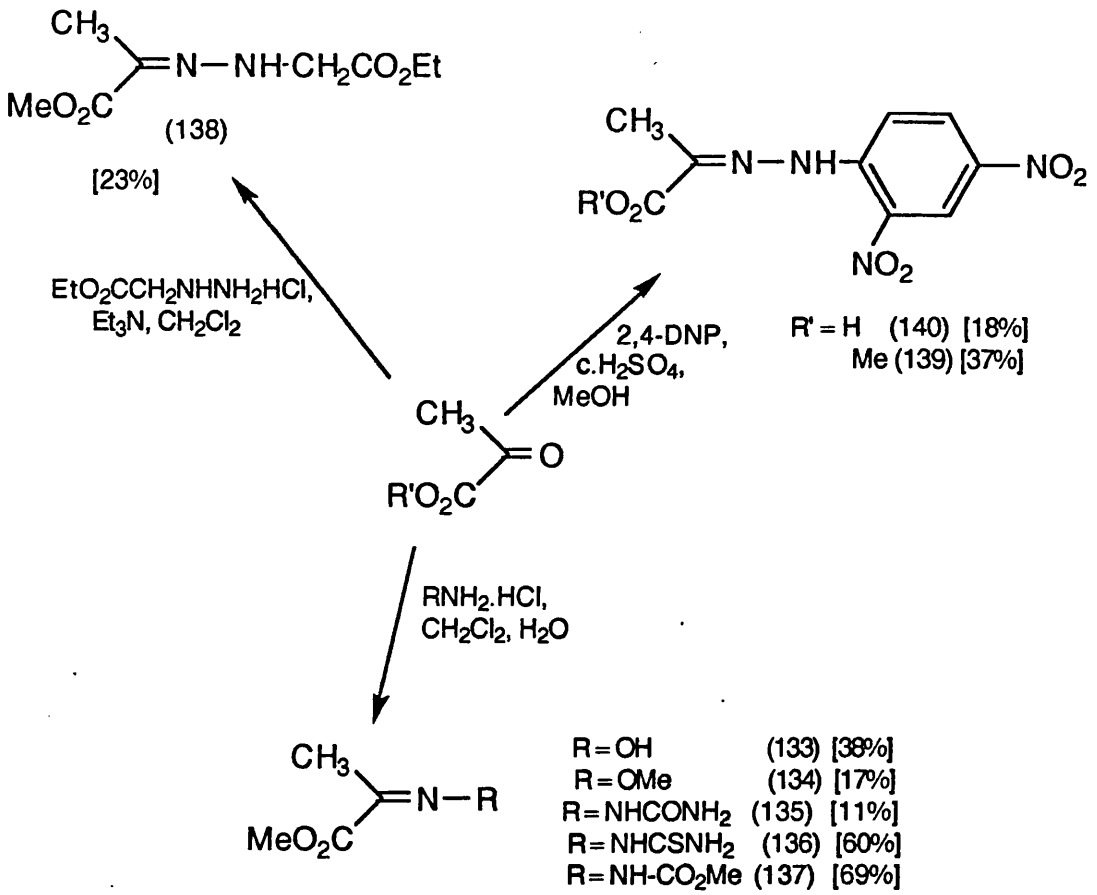
A number of imine and hydrazine derivatives of pyruvate (22) and methyl pyruvate have been synthesised and tested with DHDP Synthase.

The oxime (133), methyloxime (134), semicarbazone (135), thiosemicarbazone (136) and methyl hydrazinocarboxylate (137) derivatives of methyl pyruvate were all prepared under the same reaction conditions in 38%, 17%, 11%, 60% and 69% yields respectively **(Scheme 52)**, i.e. a solution of methyl pyruvate 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, followed by drying and concentration gave the desired products.

Thiosemicarbazide was prepared in 47% yield by heating ammonium thiocyanate and hydrazine hydrate at reflux in water for three hours.

The ethyl hydrazinoacetate derivative of methyl pyruvate (138) was prepared by treatment of methyl pyruvate with ethyl hydrazinoacetate hydrochloride and triethylamine in dichloromethane under a nitrogen atmosphere. The yield of the reaction was 23% after purification using column chromatography.

The 2,4-dinitrophenylhydrazones (2,4-DNPs) of methyl pyruvate (139) and pyruvic acid (140) were prepared according to the method of Vogel[165] in 37% and 18% yields respectively.



Scheme 52

Test Results.

The oxime (133) and methyloxime (134) showed no inhibition at 0.5 mM with DHDP Synthase. The thiosemicarbazone (136) and semicarbazone (135) were both very good inhibitors showing approximately 10% inhibition at 0.1 mM. However, by far the best inhibition was shown by the ethyl hydrazinoacetate derivative (138). This compound showed 62% inhibition at 0.1 mM. Unfortunately due to lack of time K_I values have not yet been obtained. It has also not yet been established if (138) is a competitive or a non-competitive inhibitor.

The methyl hydrazinocarboxylate derivative (137) showed 100% inhibition at 0.5 mM, but at 0.1 mM no inhibition was observed. This sudden loss of activity is unusual and further biochemical experiments must be carried out to establish why this happens.

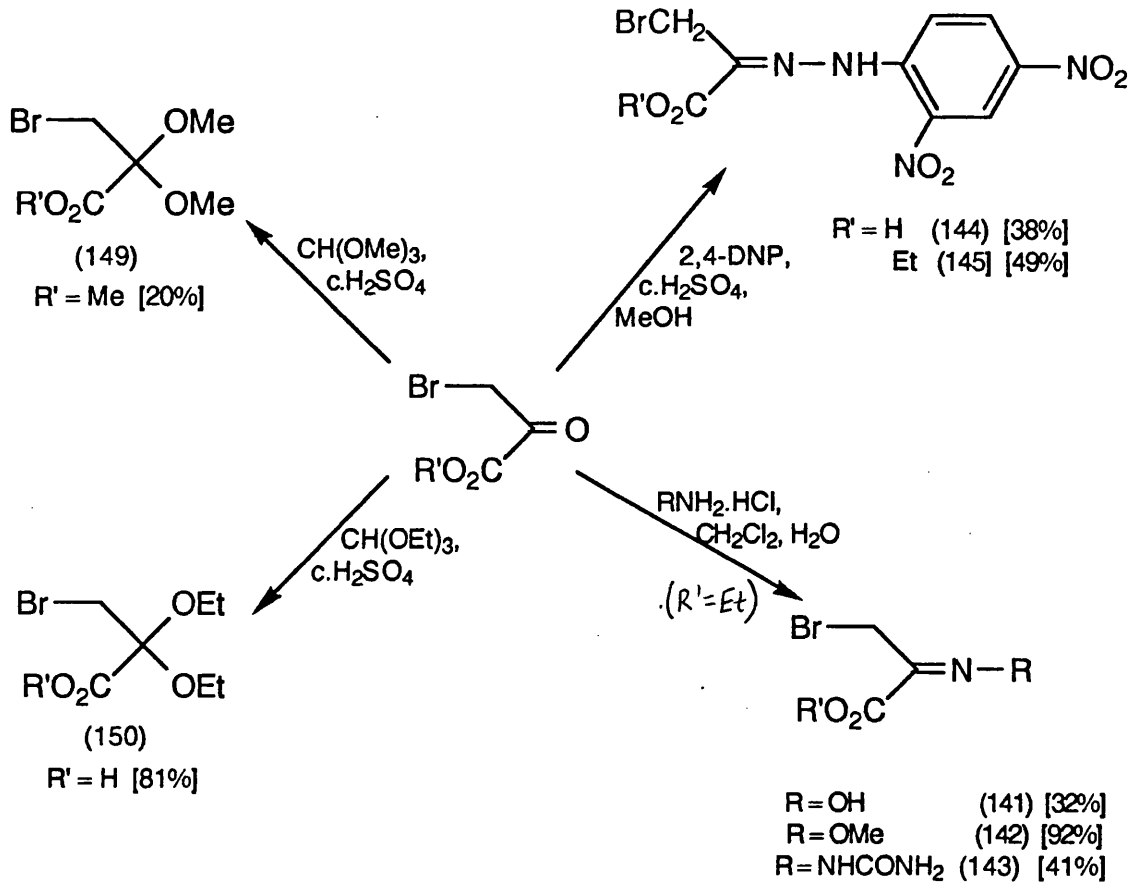
The 2,4-DNP derivatives (148) and (149) did not dissolve particularly well in water/ethanol mixtures. These compounds did not show very good inhibition. This may be due to the fact that they were only poorly soluble and that they might interfere with the assay system at 270 nm, due to their extensive conjugation.

Methyl pyruvate has been shown to act as a substrate with DHDP Synthase. This compound shows half as good a rate in the enyme assay as that of the natural substrate pyruvate (22).

4.2. Synthesis of Bromopyruvate Derivatives.

A range of imine and hydrazine derivatives of ethyl bromopyruvate and bromopyruvic acid hydrate have also been synthesised and tested for inhibitor activity with DHDP Synthase.

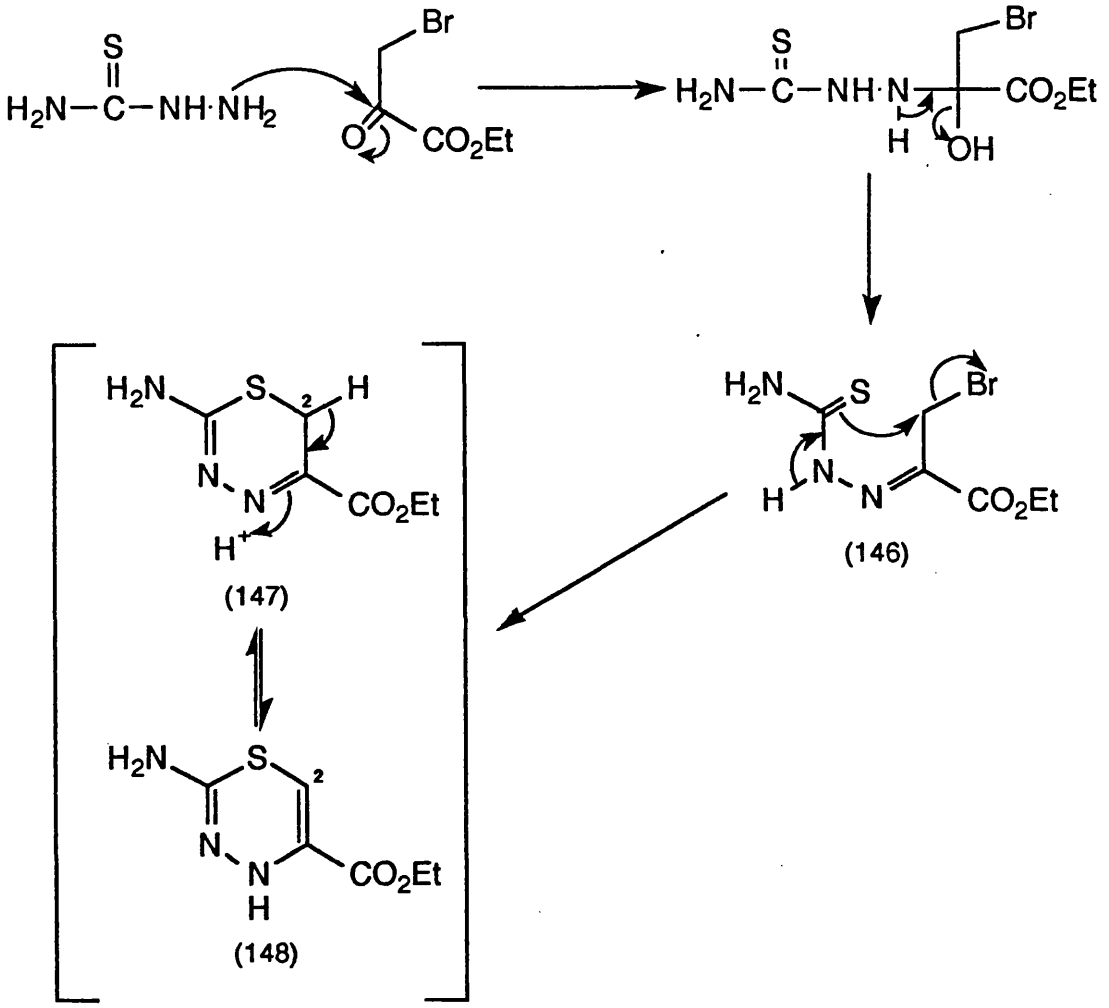
The oxime (141), methyloxime (142) and semicarbazone (143) derivatives of ethyl bromopyruvate **(Scheme 53)** were all prepared in a dichloromethane/water two-phase reaction with the respective amine hydrochloride, in the same manner as the methyl pyruvate derivatives. The respective yields for each of the products were 32%, 92% and 41%. The 2,4-DNP derivatives (144) and (145) were prepared according to the procedure of Vogel.[165]



Scheme 53

Attempts to prepare the thiosemicarbazone of ethyl bromopyruvate (146) using the dichloromethane/water two-phase system proved unsuccessful. Instead ^{1}H NMR analysis of the reaction product showed a 1 : 1 mixture of the cyclic products (147) and (148) **(Scheme 54)**. The ^{1}H NMR spectrum of the mixture showed an AB system for the methylene group at the 2-position in (147) at δ_H 5.33 and 6.28 and the vinyl proton in (148) appeared at δ_H 6.97 **(Figure 6)**. The ^{13}C NMR spectrum showed all the expected signals for the two cyclic products. TLC analysis of the mixture showed one

spot. Column chromatography proved unsuccessful in separating the two products. The mechanism of formation of these cyclic compounds presumably proceeds *via* the thiosemicarbazone (146) as shown in **Scheme 54.**



Scheme 54

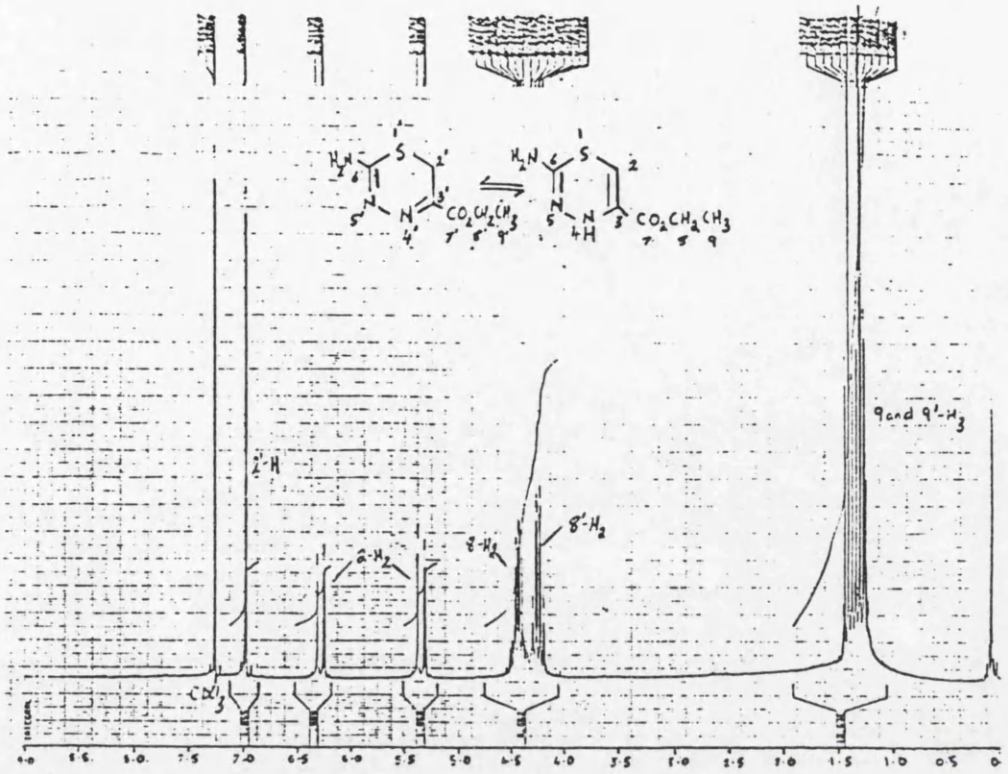


FIGURE 4.

The dimethyl acetal of methyl bromopyruvate (149) was prepared in 20% yield from bromopyruvic acid hydrate using trimethyl orthoformate and conc. sulphuric acid. The diethyl acetal of bromopyruvic acid (150) was prepared in 81% yield by similar treatment of bromopyruvic acid with triethyl orthoformate and conc. sulphuric acid.

Test Results.

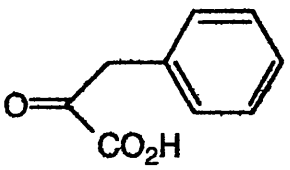
Bromopyruvic acid hydrate showed very little inhibition at 1 mM with DHDP Synthase. This result is in contrast to the results of Kumpaisal *et al.*[81] They found that bromopyruvic acid inhibits

wheat DHDPS considerably at 1 mM using an assay system devised by Yugari and Gilvarg[5 6] involving *o*-aminobenzaldehyde. Further kinetic studies by Kumpaisal *et al.*[8 1] showed that bromopyruvic acid hydrate inhibits wheat DHDPS in a competitive manner with respect to pyruvate (22) and in a non-competitive manner with respect to L-aspartic acid-β-semialdehyde (21a). They found the K_I value for bromopyruvic acid to be 1.82 mM.

The oxime (141) showed very little inhibition at 1 mM. However, the methyloxime (142) showed very good inhibition with 14% at 0.1 mM. Further kinetic studies are required with the methyloxime (142). The semicarbazone (143) was a very impressive inhibitor showing 50% inhibition at 0.1 mM. Further investigations are required with this compound to obtain an accurate K_I value and to determine whether it is a competitive or non-competitive inhibitor.

Like the pyruvate 2,4-DNP derivatives, the bromopyruvate 2,4-DNP derivatives (153) and (154), showed very little inhibition. Unfortunately due to lack of time the dimethyl acetal (149) and diethyl acetal (150) have not yet been tested for enzyme activity with DHDP Synthase.

Commercially available phenylpyruvic acid (151) and sodium mercaptopyruvate (152) showed no inhibition with DHDP Synthase.



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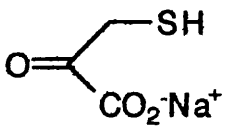


(152)

Conclusions.

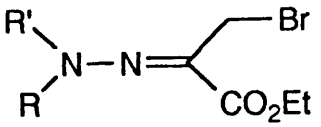
A range of pyruvate and bromopyruvate derivatives have been synthesised and tested, along with commercially available pyruvates, for enzyme activity with DHDP Synthase.

Methyl pyruvate has shown substrate activity with DHDP Synthase, whereas bromopyruvic acid hydrate, phenylpyruvic acid and sodium mercaptopyruvate showed no substrate activity. Further investigations are required to obtain an accurate K_M value for methyl pyruvate. Other pyruvate esters should be tested for substrate activity.

The bromopyruvates were better inhibitors than the pyruvates i.e. the bromopyruvate oxime (141), methyloxime (142) and semicarbazone (143) all showed better inhibition than their corresponding pyruvate derivatives, (133), (134) and (135).

In particular, the ethyl hydrazinoacetate derivative of methyl pyruvate (138) and the semicarbazone of ethyl bromopyruvate (143) were very good inhibitors. Accurate K_I values should be calculated for these compounds and the nature, competitive or non-competitive, of the inhibition should be determined.

Further imine and hydrazone derivatives of pyruvate and bromopyruvate should be synthesised and tested for inhibitor activity with DHDP Synthase. The simple hydrazine (153), methylhydrazine (154) and dimethylhydrazine (155) derivatives of ethyl bromopyruvate would be interesting targets which should be tested for enzyme activity with DHDP Synthase.

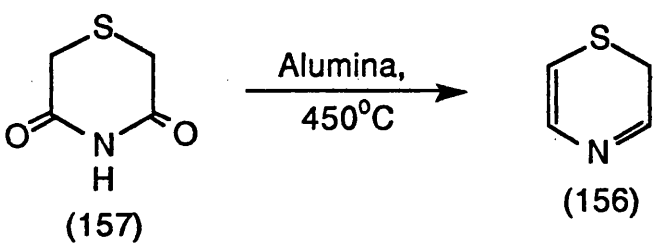


R = R' = H (153)
R = H, R' = Me (154)
R = R' = Me (155)

Chapter [5] - Synthesis of Sulphur Analogues of L-2,3-DHDPA (23) and L-2,3,4,5-THDPA (24).

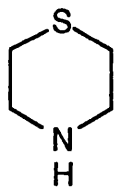
Introduction.

1,4-Thiazine (156) was first reported in 1948 by Barkenbus and Landis.[167] They obtained a 13% yield of 1,4-thiazine (156) by passing thiodiglycolic acid imide (157) over alumina on pumice at 450 °C (**Scheme 55**). Since that early discovery considerable attention has been given to the synthesis of compounds containing a tetrahydro-1,4-thiazine (158) or, as it is more commonly known, a thiamorpholine ring system.[168,169] In particular, there are many drugs which are widely used in medicine based on the dibenzo derivative of 1,4-thiazine (159). These drugs have the trivial name of phenothiazines. They are important in the management of psychiatric illness and have other useful properties which enable them to be used as sedatives, anti-histamines and in the treatment of nausea. Phenothiazines have also shown good specificity and high potency as tranquillisers for animals. Burke *et al.*[169a] have reported that 10-(3-dimethylaminopropyl)-2-(trifluoromethyl)-phenothiazine hydrochloride shows very good tranquillising activity.



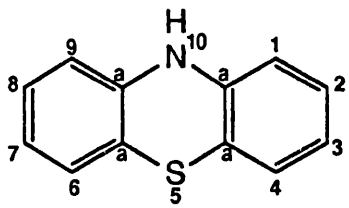
(157) (156)

Scheme 55

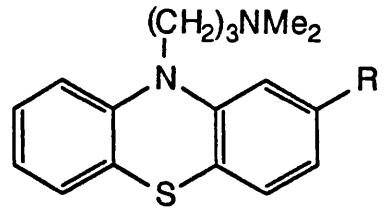


(158)

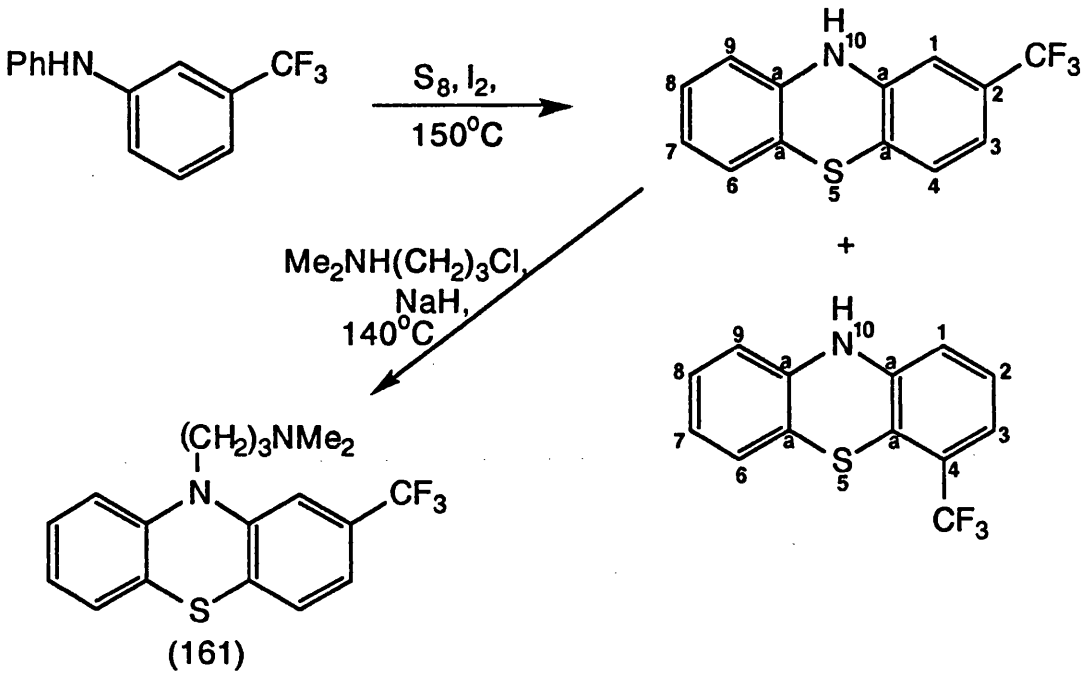
Two important phenothiazine drugs are chlorpromazine (160) and triflupromazine (161). The synthesis of triflupromazine (161) is shown in **Scheme 56.**



(159)



(160) R = Cl
(161) R = CF_3



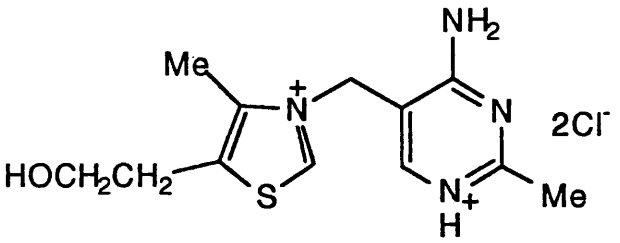
Scheme 56

The reaction of diarylamines with sulphur, with iodine being used as a catalyst,[170] is one of the oldest and most widely used methods of synthesis of phenothiazine drugs. A major disadvantage of this method is the 1:1 formation of both the 2-(trifluoromethyl)-phenothiazine and the 4-(trifluoromethyl)-phenothiazine isomers. The 2-isomer has a melting point of 187-188 °C, whereas the 4-isomer has a melting point of 72-73 °C. The 2-isomer is also less soluble than the 4-isomer and so separation can be quite easily achieved.

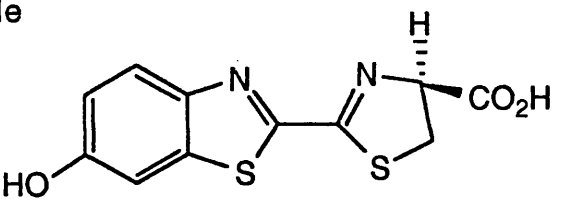
The 1,3-thiazole ring system is quite commonly found in natural products, since it can be produced by the cyclisation of cysteine residues in peptides. The most important of these is vitamin B_1 (thiamine) (162), which contains both a pyrimidine and a 1,3-thiazole ring system. The bleomycin antibiotics, which have antitumour properties, are complex aminoglycosidic structures containing 1,3-thiazole units. Firefly luciferin (163) is a benzothiazolyl derivative: the bioluminescence is produced by photo-oxygenation at the asymmetric centre. Several modern semisynthetic β-lactams, such as cefotaxime (164), contain 2-amino-1,3-thiazole units in the side chain.

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 strategy was first introduced by Hantzsch[171] and is commonly known as the 'Hantzsch thiazole synthesis'. The reaction has been carried out not only with thioamides, but also with thioureas, thiosemicarbazides and other compounds containing the -N-C=S structural unit. An example of the

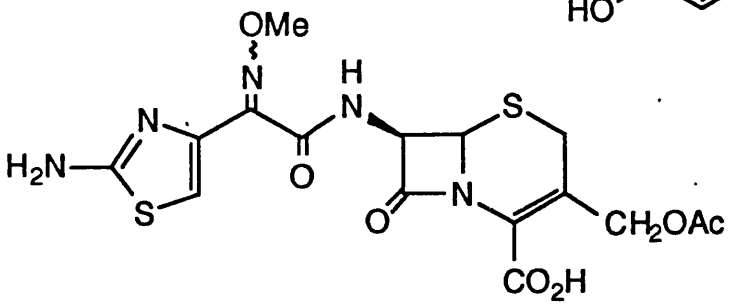
Hantzsch thiazole synthesis to produce 2,4-diphenyl-1,3-thiazole (165) is shown in Scheme 57.



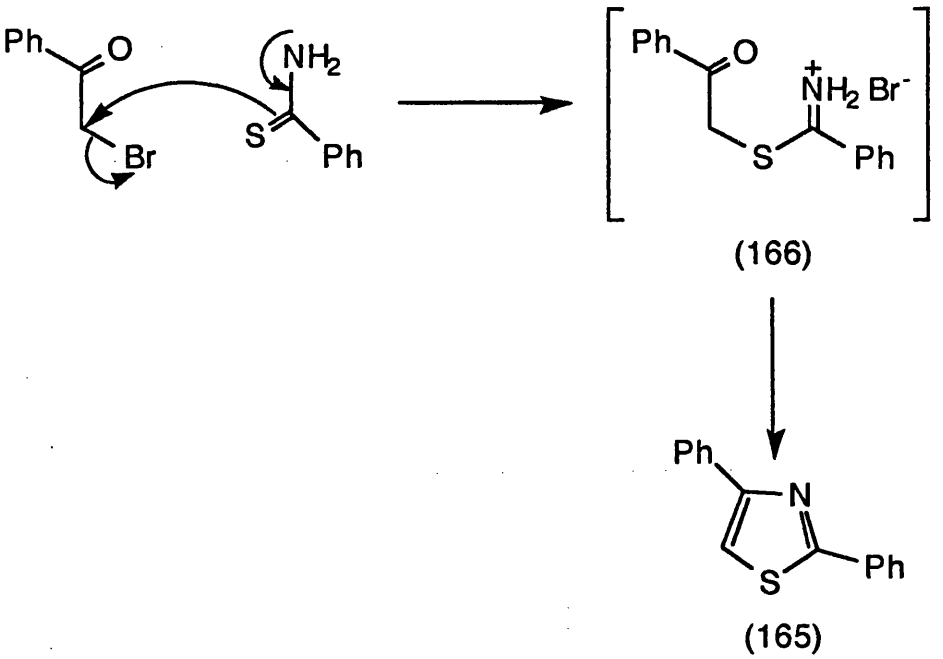
(162)



(163)



(164)



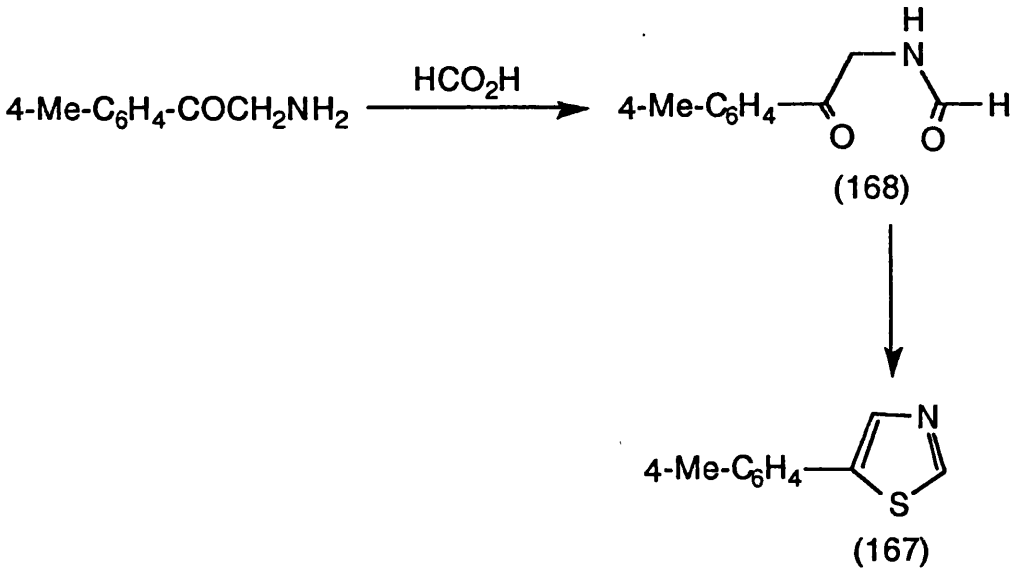
(166)

(165)

Scheme 57

The mechanism of the reaction goes by way of nucleophilic attack by sulphur on the carbon atom bearing the halogen to form an acyclic intermediate (166), which has been isolated in a few cases. Cyclisation, followed by dehydration, takes place to give the desired 1,3-thiazole (165). The reaction is normally carried out in ethanol.

Another important method of 1,3-thiazole synthesis is the cyclodehydration of α-acylaminoketones (168) with phosphorus pentasulphide.[172] An example of this method is the synthesis of 4-(4-methylphenyl)-1,3-thiazole as shown in **Scheme 58.**



Scheme 58

5.1. Synthesis and Test Results of 1,4-Thiazine Derivatives.

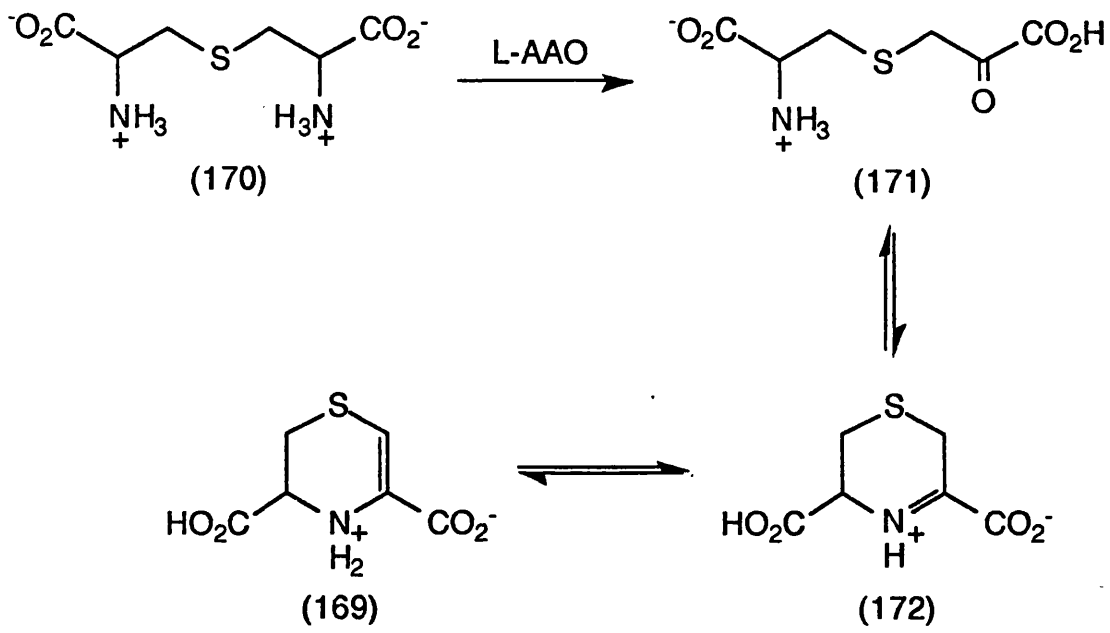
3,4-Dihydro-2*H*-1,4-Thiazines.

3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid (3,4-DHT) (169) hydrobromide was first reported by Ricci *et al.*[173] in their studies on the products of L-lanthionine (170) oxidation with snake venom L-amino acid oxidase (L-AAO). They showed that L-lanthionine (170) was oxidised by snake venom L-AAO with the release of one mole of ammonia gas per mole of L-lanthionine (170). From spectrophotomeric, chromatographic and analytical data they identified the mono-keto derivative of lanthionine (171) as the first enzymatic product of the reaction **(Scheme 59)**. This then cyclised into 2,3-dihydro-6*H*-1,4-thiazine-3,5-dicarboxylic acid (ketimine form) (172) which tautomerised to the more stable 3,4-DHT (enamine form) (169).

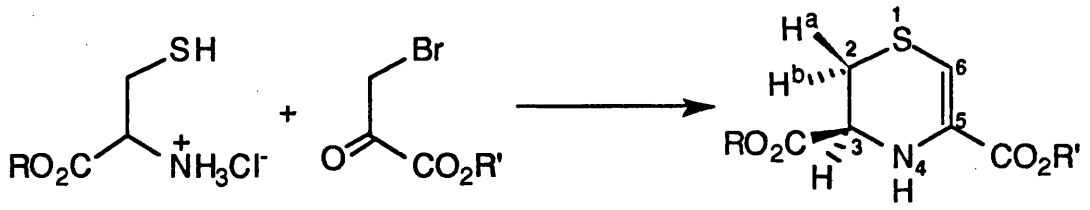
Ricci *et al.*[173] prepared authentic 3,4-DHT (enamine form) (169) by stirring equimolar concentrations of bromopyruvic acid hydrate and L-cysteine hydrochloride in water **(Scheme 60)**. The product crystallised in the monomeric form from the aqueous solution. The properties of the synthetic material were found to be similar to those exhibited by the enzymatic product.

We prepared 3,4-DHT (169) in 55% yield according to the procedure of Ricci *et al.*[173] **(Scheme 60)**. The ^{13}C NMR spectrum showed only the enamine form (169) i.e. there was a signal at δ_C 97.7 for C-6 and at δ_C 128.6 for C-5. No signal was observed for the methylene group at C-6 of the ketimine form (172). The ^{1}H NMR

spectrum showed a singlet at δ_H 5.94 for the proton at C-6 of the enamine form (169). The ^{13}C NMR spectrum of 3,4-DHT (enamine form) (169) is shown in **Figure 7.**



Scheme 59



		Conditions	Yield
(169)	: R=R'=H	H_2O	55%
(175)	: R=R'=Ethyl	CH_2Cl_2 / Et_3N	96%
(176)	: R=Ethyl, R'=H	$CHCl_3$ / Et_3N	20%
(177)	: R=H, R'=Ethyl	$CHCl_3$ / Et_3N	-

Scheme 60

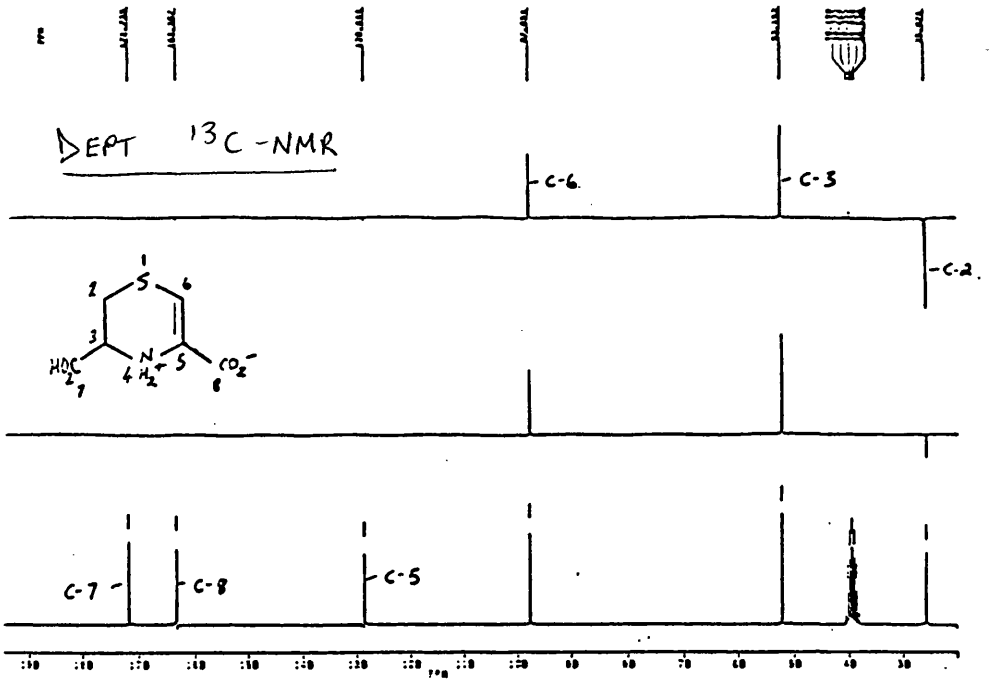
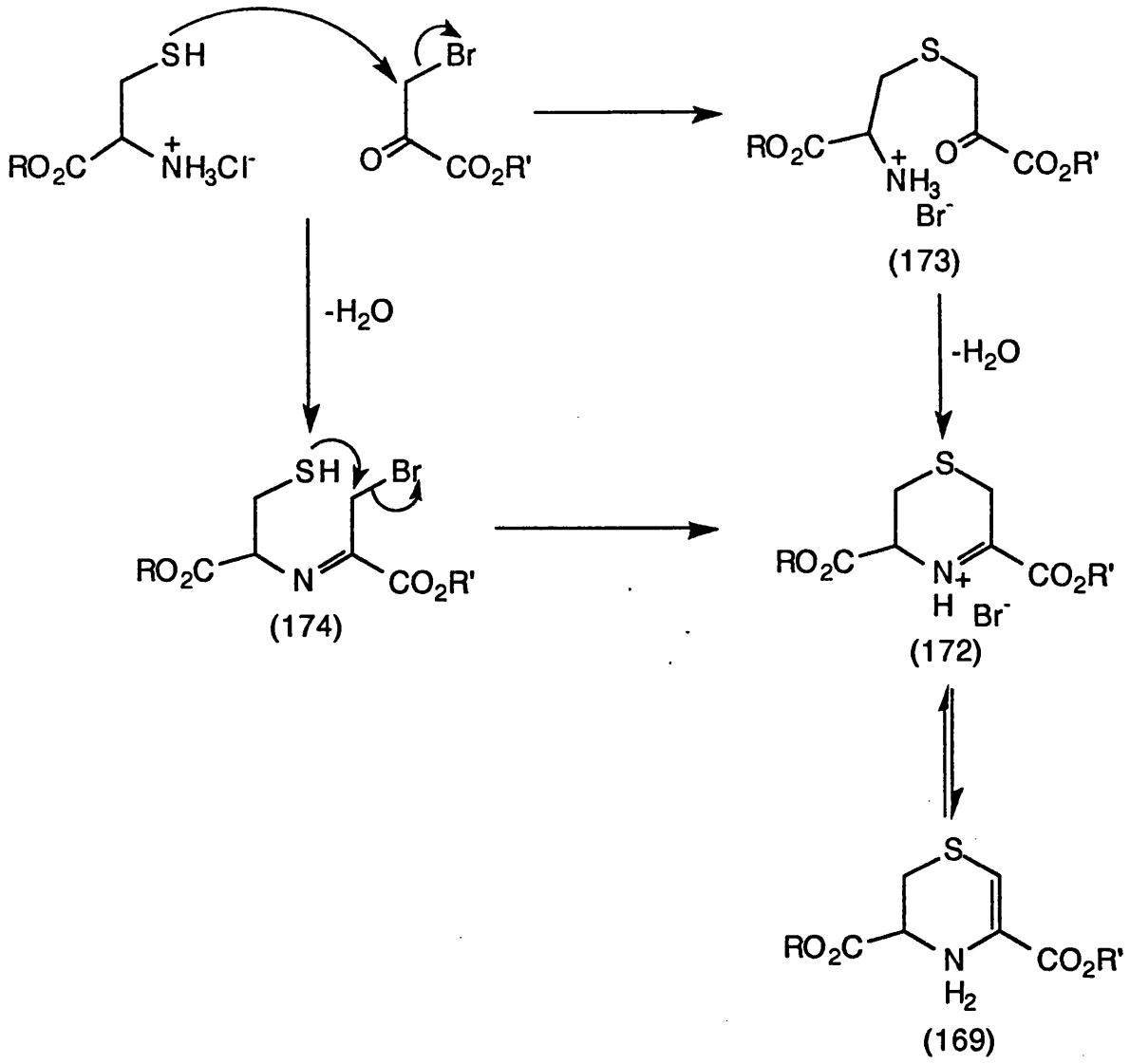


FIGURE 7 : ^{13}C NMR SPECTRUM OF 3,4-DIHYDRO-1,4-THIAZINE DIACID.

There are two possible mechanisms for the formation of 3,4-DHT (169) from L-cysteine and bromopyruvic acid. One possibility is nucleophilic attack by sulphur on the carbon atom bearing the bromine to form an acyclic intermediate (173) **(Scheme 61)**. This could then undergo cyclodehydration to give 3,4-DHT (169). An alternative mechanism is to reverse these two steps i.e. first of all formation of the imine intermediate (174) by attack of the amino group on the keto group of bromopyruvic acid with the loss of the elements of water. This could be followed by cyclisation via nucleophilic attack by sulphur on the carbon atom bearing the bromine **(Scheme 61)**. It has not yet been established which of these mechanisms actually occurs.



Scheme 61

Diethyl (R)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid (175), the diethyl ester derivative of 3,4-DHT, was prepared in 90% by treating L-cysteine ethyl ester hydrochloride with ethyl bromopyruvate in dichloromethane in the presence of triethylamine (Scheme 60). The compound exists as a golden brown syrup and was first reported by Berges and Taggart[174] from their studies on

3,4-DHT (169). The mechanism of formation of the diester (175) is presumably similar to that proposed for the diacid (169).

The ^1H NMR spectrum of the diester (175) is shown in **Figure 8**. The spectrum shows an interesting long range coupling between the methylene protons at C-2 and the vinyl proton at C-6 with a coupling constant of 0.7 Hz. There is a well defined ABX splitting pattern between the methylene protons at C-2 and the adjacent proton at C-3. The diastereotopic protons at C-2 are easily distinguishable from their coupling with the proton at C-3. The 2a-H at C-2 has a signal at δ_H 2.97 and a coupling constant of 6.7 Hz with the proton at C-3 whereas the 2b-H at C-2 has a signal at δ_H 3.18 and a coupling constant of 2.9 Hz with the proton at C-3. The coupling constant between the geminal 2a-H and 2b-H is 12.1 Hz.

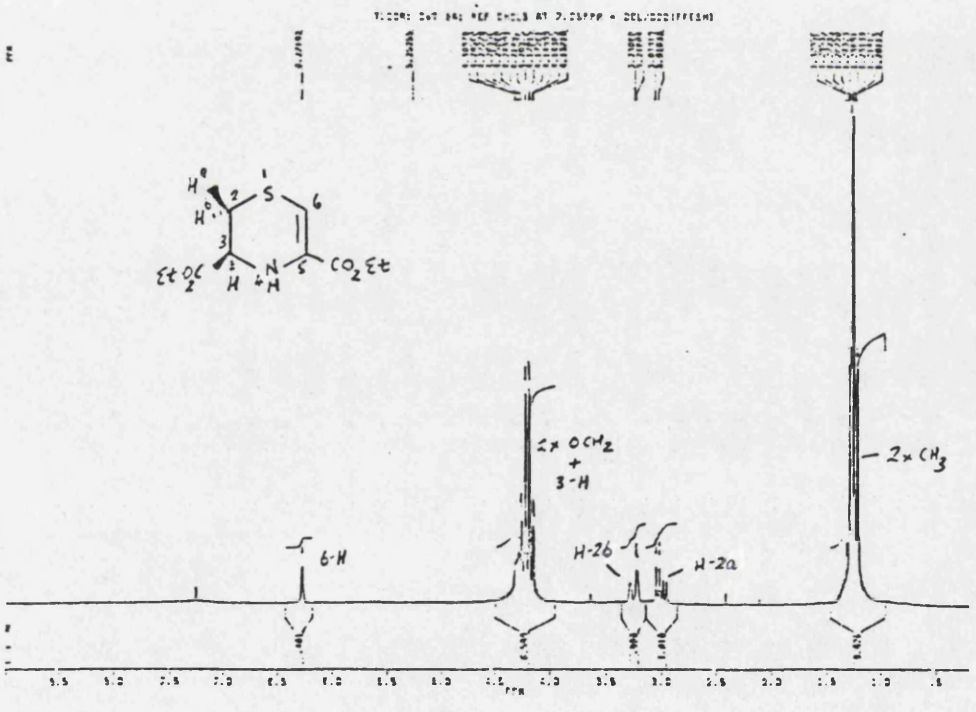


FIGURE 8 : ^1H NMR SPECTRUM OF 3,4-DIHYDRO-1,4-THIAZINE DIESTER.

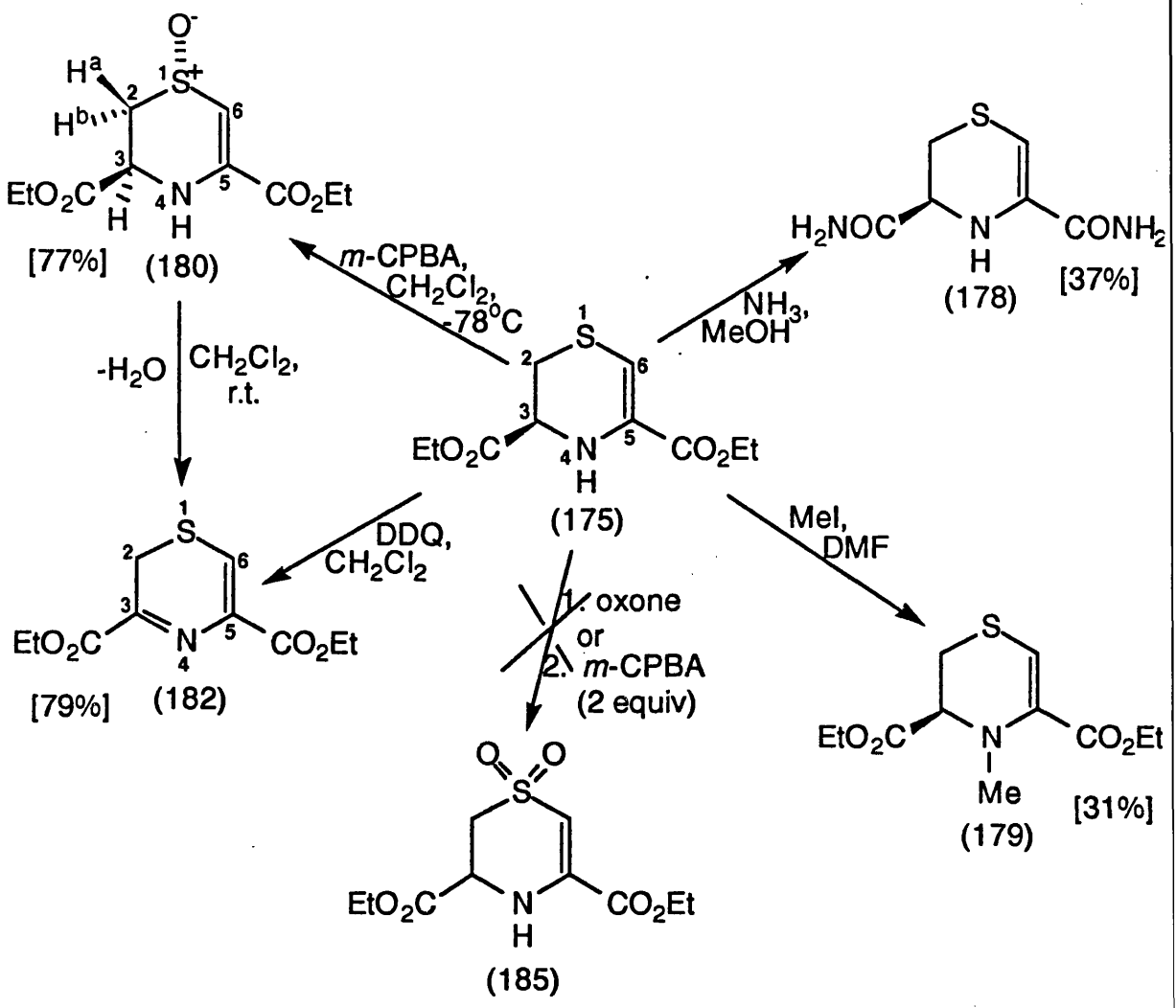
3-Carboethoxy-3,4-dihydro-2*H*-1,4-thiazine-5-carboxylic acid (176) (**Scheme 60**), the 3,4-DHT derivative containing one free and one esterified carboxyl group, was prepared in 20% yield by treating L-cysteine ethyl ester hydrochloride and bromopyruvic acid hydrate in chloroform in the presence of triethylamine. The work-up for the reaction involved extracting the product from chloroform as the sodium salt using 5% aqueous sodium bicarbonate solution. The aqueous layer was then acidified and the product was extracted into organic solvent. Evaporation *in vacuo* and drying gave the desired product as a yellow oil. This extensive work-up may contribute to the reaction being low yielding. The spectrophotomeric and analytical data of this compound are compatible with those of the diester (175).

3-Carbomethoxy-3,4-dihydro-2*H*-1,4-thiazine-5-carboxylic acid, the methyl ester derivative of (176), was previously reported by Strokov[175] from his studies on the condensation of methyl esters of α-amino-β-mercaptocarboxylic acids with bromopyruvic acid.

Attempts to prepare 5-carboethoxy-3,4-dihydro-2*H*-1,4-thiazine-3-carboxylic acid (177) (**Scheme 60**), the other derivative of 3,4-DHT (169) containing one free and one esterified carboxyl group, proved unsuccessful. Treating L-cysteine hydrochloride with ethyl bromopyruvate in chloroform in the presence of triethylamine under various reaction conditions gave a dark brown residue which showed several spots on TLC analysis and had no vinyl signal for the proton at C-6 in the ^{1}H NMR spectrum.

3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxamide (178) (**Scheme 62**), the diamide derivative of 3,4-DHT (169), was

prepared in 37% yield by stirring the diester (175) in a solution of methanolic ammonia. The diamide (178) is insoluble in organic solvents and precipitated as an orange solid from the methanolic solution.



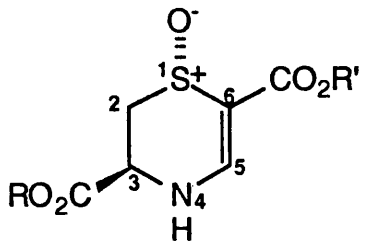
Scheme 62

Diethyl 3,4-dihydro-4-methyl-2*H*-1,4-thiazine-3,5-dicarboxylate (179) (**Scheme 62**), the *N*-methyl derivative of the diester (175), was prepared in 31% yield by treating the diester (175) with methyl iodide in dimethyl formamide (DMF). Excess DMF was removed by azeotroping the reaction mixture with two volumes of water and concentrating the solution *in vacuo.* Excess methyl iodide and hydroiodic acid, formed *in situ* during the reaction, were removed by dissolving the reaction mass in ethyl acetate and then washing the organic phase with aqueous ammonia solution. The ^{1}H NMR spectrum of the product showed a signal at δ_H 2.70 and the ^{13}C NMR spectrum had a signal at δ_C 42.3 for the *N*-methyl group.

Diethyl *trans*-3,4-dihydro-1-oxo-2*H*-1,4-thiazine-3,5-dicarboxylate (180) (**Scheme 62**), the sulphoxide derivative of the diester (175), was prepared in 77% yield according to the procedure of Berges and Taggart.[174] A solution of the diester (175) in dichloromethane at -78 °C was treated with one equivalent of *meta*-chloroperbenzoic acid (*m*-CPBA). The product was purified on a silica gel column eluting with 50% chloroform in methanol to give an amber syrup. The spectrophotomeric and physical data of the product obtained were identical to those obtained by Berges and Taggart.[174]

Berges and Taggart[174] assigned the *trans*-stereochemistry to the sulphoxide (180) by comparing the ^{1}H NMR spectrum of the sulphoxide (180) with the spectra obtained by Kitchen and Stoodley[176] for *trans*-3,4-dihydro-1-oxo-2*H*-1,4-thiazine-3,6-dicarboxylates (181). To confirm this assignment they carried out NMR aromatic solvent induced studies on the sulphoxide (180).

It was previously reported by Strom *et al.*[177] and Fraser *et al.*[178] that benzene solvates the positive end of the sulphoxide



(181)

dipole and is therefore closer to the vicinal proton *trans* (2a-H) to the sulphoxide (**Figure 9**) and shields it more than the proton *cis* (2b-H) to it. Hence 2a-H (δ_H 2.25) has a lower chemical shift than 2b-H (δ_H 3.41). The large coupling of 3-H to 2a-H and its small coupling to 2b-H indicates that 3-H is *trans* to 2a-H and is therefore *cis* to the sulphoxide. Hence the ester group at C-3 must be *trans* to the sulphoxide.

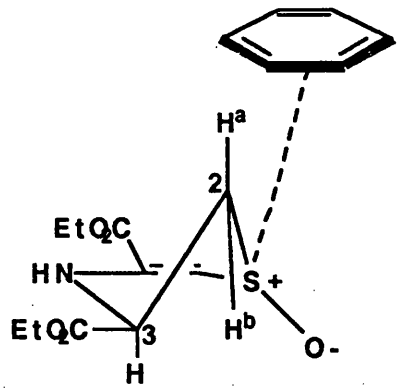
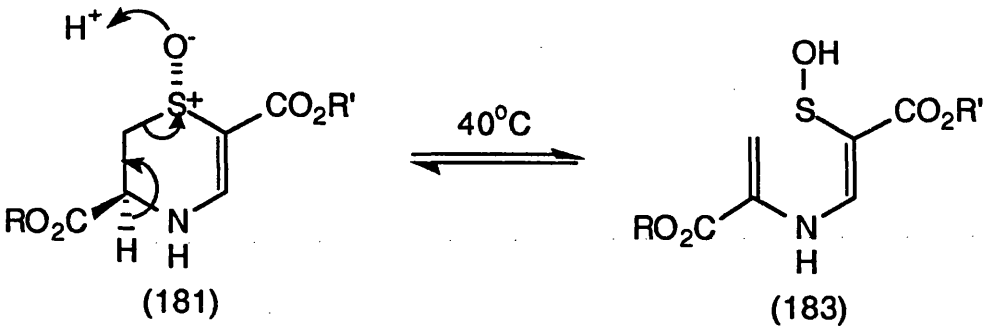


FIGURE 9

Berges and Taggart[174] found that the sulphoxide (180) is remarkably labile. On standing overnight in dichloromethane at room temperature it was transformed into the 1,4-thiazine (182) (**Scheme 62**). The ^{1}H and ^{13}C NMR spectra of the 1,4-thiazine (182) were quite different from those of the sulphoxide (180). The ^{1}H NMR spectrum of the 1,4-thiazine (182) had a doublet at δ_H 3.33 for the protons at C-2 [*J* 1.2 Hz] and a triplet at δ_H 7.59 for the proton at C-6 [*J* 1.2 Hz]. The ^{13}C NMR spectrum had a signal at δ_C 20.17 for C-2 and a signal at δ_C 128.22 for C-6. There were also two signals at δ_C 136.92 and 137.57 for C-3 and C-5.

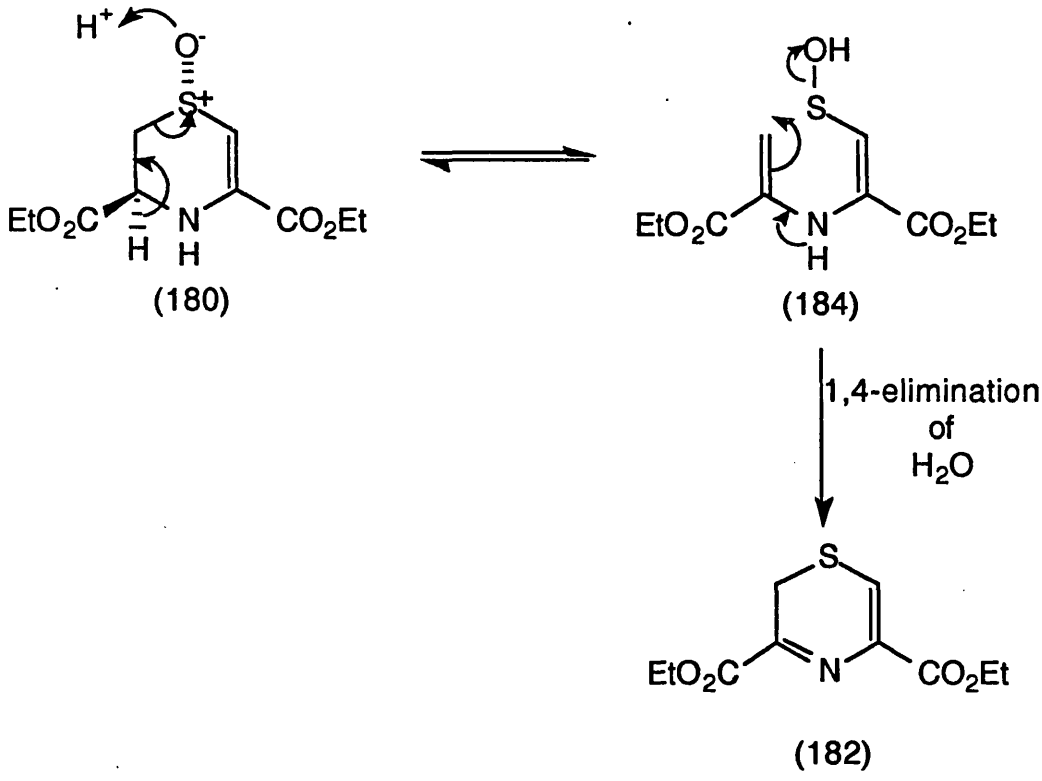
No such precedent for such a mild sulphoxide dehydration was found in the literature. However, Stoodley and Wilkins[179] reported the thermal (ca. 40 °C) racemisation of *trans*-3,4-dihydro-1-oxo-2*H*-1,4-thiazine-3,6-dicarboxylates (181) and provided evidence that this reaction involves a cycloelimination to produce an achiral sulphenic acid (183) which recloses to give back the *trans*-sulphoxide as a racemate (**Scheme 63**).



(181) (183)

Scheme 63

Since the sulphoxide (180) obtained by Berges and Taggart[174] was racemic it would be reasonable to conclude that the mechanism for dehydration of the sulphoxide (180) must go via the sulphenic acid (184) (Scheme 64). In addition to being able to reclose to give racemised sulphoxide, the ring-opened sulphenic acid (184) can also undergo 1,4-elimination of water to give the 1,4-thiazine (182). Yoshida *et al.*[180] reported the 1,2-elimination of water from a sulphenic acid intermediate. However Stoodley did not report any dehydration reactions for his sulphoxides.



Scheme 64

To confirm that the 1,4-thiazine (182) is the sulphoxide dehydration product Berges and Taggart[174] prepared authentic 1,4-thiazine (182) by treating a solution of the diester (175) in

dichloromethane with the hydride abstractor, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (**Scheme 62**). They obtained a yellow oil which gave ^{1}H NMR, IR and UV spectra identical with those obtained for the sulphoxide dehydration product.

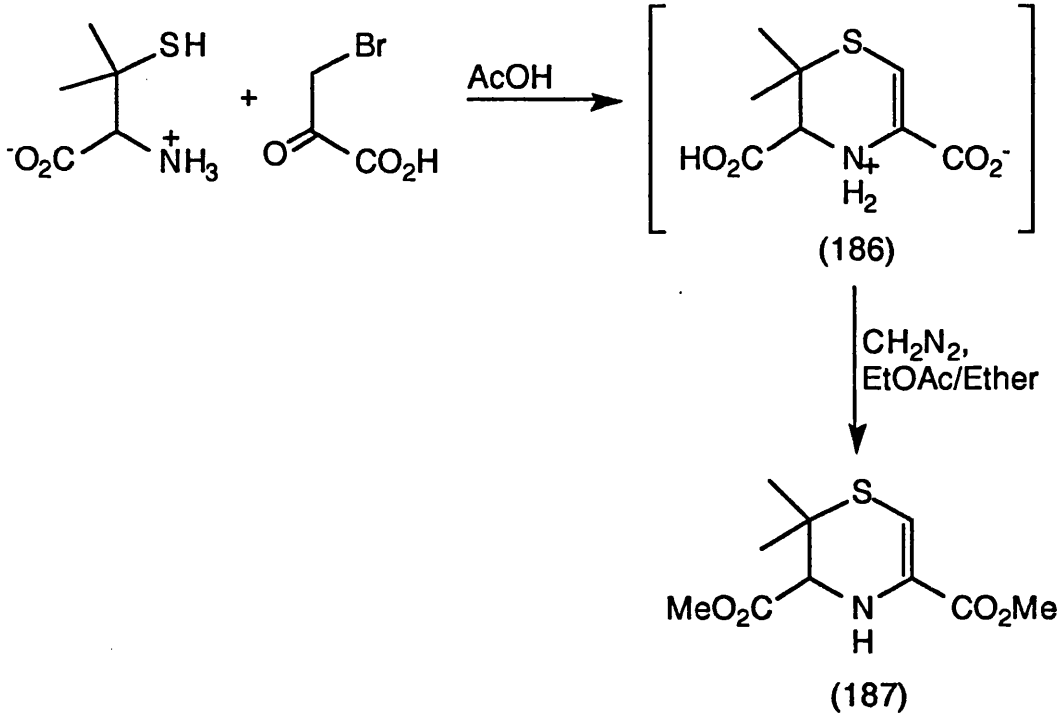
We repeated the synthesis of the 1,4-thiazine (182) in 79% yield according to the procedure of Berges and Taggart.[174] Both the sulphoxide (180) and 1,4-thiazine (182) will be tested for inhibitory activity on DHDP Synthase.

Attempts to prepare the sulphone (185) (**Scheme 62**) from the diester (175) using oxone (potassium peroxymonosulphate),[181] and two equivalents of *m*-CPBA proved unsuccessful in both cases. The reason for this may be due to stereochemical bulk at the sulphur centre of the 3,4-dihydro-1,4-thiazines.

3,4-Dihydro-2,2-dimethyl-2*H*-1,4-thiazines.

DL-3,4-Dihydro-2,2-dimethyl-2*H*-1,4-thiazine-3,5-dicarboxylic acid (186) hydrobromide, the 2,2-dimethyl derivative of 3,4-DHT, was prepared by treating DL-penicillamine with bromopyruvic acid in glacial acetic acid (**Scheme 65**). Removal of the solvent *in vacuo* gave a brown solid. The ^{1}H NMR spectrum of this material showed two signals at δ_H 1.20 and δ_H 1.37 for the geminal methyl groups at C-2 and a signal at δ_H 5.98 for the proton at C-6. Purification of this compound proved very difficult. To confirm that this brown solid was the correct product, the compound was converted into the dimethyl ester (187) using a solution of diazomethane in ether. The yield of the dimethyl ester (187) from DL-penicillamine was 61%.

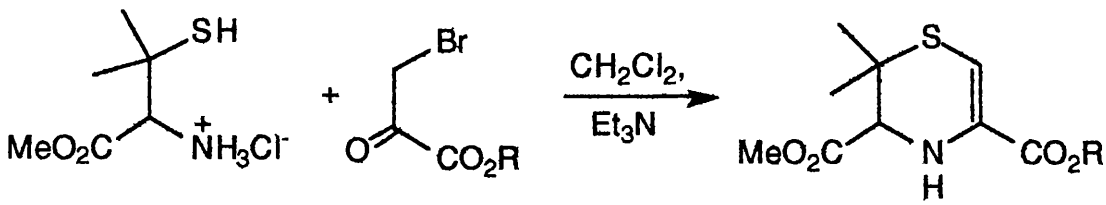
yield of the dimethyl ester (187) from DL-penicillamine was 61%. The ^{13}C NMR spectrum of this compound showed two signals at δ_C 24.8 and 27.5 for the geminal methyl groups at C-2, two signals at δ_C 52.0 and 52.1 for the saturated and unsaturated methyl esters, and a signal at δ_C 102.3 for C-6.



Scheme 65

Ethyl 3-Carbomethoxy-3,4-dihydro-2,2-dimethyl-2*H*-1,4-thiazine-5-carboxylate (188) was prepared in 90% yield by treating DL-penicillamine methyl ester hydrochloride with ethyl bromopyruvate in dichloromethane in the presence of triethylamine (**Scheme 66**). This compound had similar ^1H and ^{13}C NMR spectra to those of the dimethyl ester (187) apart from the ester signals. The

geminal methyl groups at C-2 and a signal at δ_H 6.19 for the proton at C-6.



R = Et [90%] (188)
R = H [24%] (189)

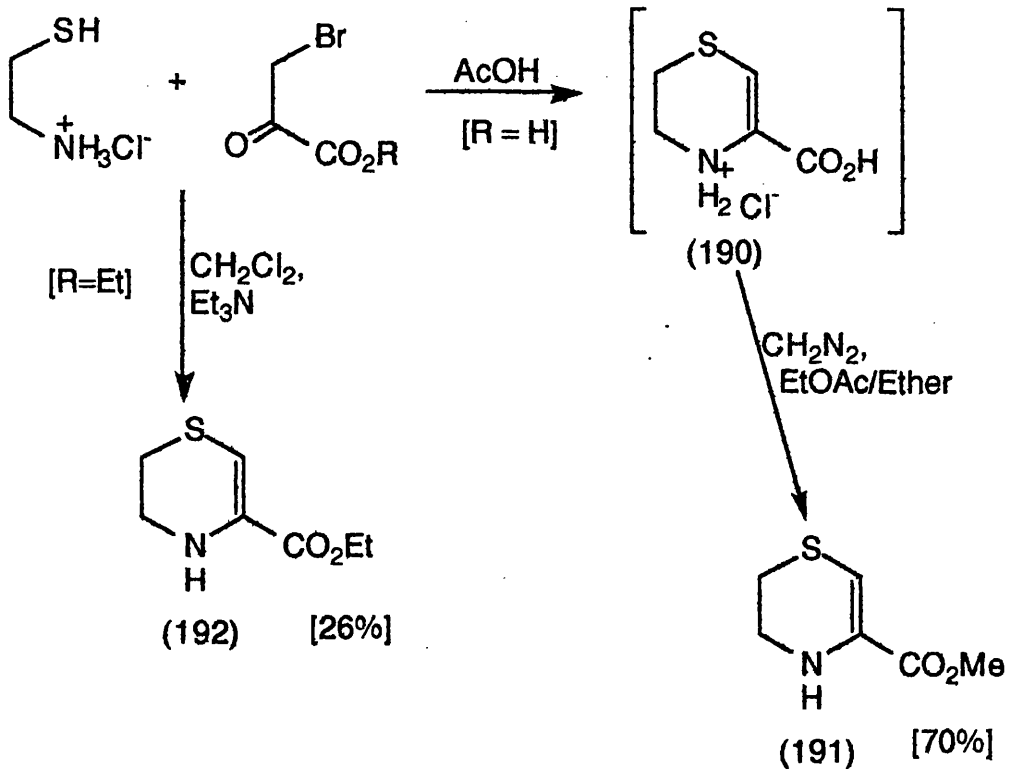
Scheme 66

Methyl 3,4-dihydro-2,2-dimethyl-2H-1,4-thiazine-5-carboxylic acid-3-carboxylate (189) **(Scheme 66)**, a derivative of the di-acid (186) containing one free and one esterified carboxyl group, was prepared in 24% yield by treating DL-penicillamine methyl ester hydrochloride with bromopyruvic acid in chloroform in the presence of triethylamine. Like the half-acid/half-ester derivative of 3,4-DHT (176) the yield of this reaction was low presumably due to the extensive work-up involved. The ^1H and ^{13}C NMR spectra of this compound were similar to those obtained for the diesters (187) and (188).

The mechanism of formation of the 2,2-dimethyl 3,4-DHT derivative is presumably the same as that proposed for the 3,4-DHT derivatives **(Scheme 61)**.

3,4-Dihydro-2H-1,4-thiazine-5-carboxylic acids.

3,4-Dihydro-2*H*-1,4-thiazine-5-carboxylic acid hydrochloride (190) (Scheme 67), a derivative of 3,4-DHT (169) with no carboxyl



Scheme 67

group at C-3, was prepared by stirring a solution of 2-aminoethanethiol hydrochloride and bromopyruvic acid in glacial acetic acid at room temperature for 1 hour. Removal of the solvent *in vacuo* gave an off-white solid which showed a signal at δ_H 6.25 for the proton at C-6 in the ^1H NMR spectrum. Purification of this material proved difficult and, as in the case of the diacid (186), the compound was converted into the methyl ester (191) by adding a solution of diazomethane in ether to a solution of the acid (190) in

ethyl acetate. The yield of the methyl ester (191) from 2-aminoethanethiol hydrochloride was 70%. The ^{1}H NMR spectrum showed multiplicities at δ_H 2.86 for the protons at C-2 and at δ_H 3.62 for the protons at C-3 and a singlet at δ_H 6.05 for the proton at C-6. The ^{13}C NMR spectrum showed a signal at δ_C 40.4 for C-2, a signal at δ_C 62.5 for C-3 and a signal at δ_C 102.3 for C-6.

Ethyl 3,4-dihydro-2*H*-1,4-thiazine-5-carboxylate (192) (**Scheme 67**) was prepared in 26% yield by treating 2-aminoethanethiol hydrochloride with ethyl bromopyruvate in dichloromethane in the presence of triethylamine. The ^{1}H and ^{13}C NMR spectra of (192) were similar to those obtained for the methyl ester (191).

Test Results.

The 3,4-dihydro-1,4-thiazines showed very strong inhibition with DHDP Synthase. The diethyl ester (175) and the dihydrothiazine derivative with one free and one esterified carboxyl group (176) both showed approximately 10% inhibition at 0.1 mM with DHDP Synthase. Further kinetic studies are required to determine whether the inhibition is competitive or non-competitive. The diacid (169) showed 88% inhibition at 0.5 mM; however, at 0.1 mM no inhibition was observed. The reasons for this sudden loss of inhibition going from 0.5 mM to 0.1 mM are not fully understood and further investigation is required. The diamide (178) has not yet been tested for inhibitory activity with DHDP Synthase.

The *N*-methyl derivative (179) showed just as good inhibition as the diester (175) with 11% inhibition at 0.1 mM. However, the sulphoxide (180) and the 1,4-thiazine (182) were poorer inhibitors. The sulphoxide (180) showed no inhibition with DHDP Synthase and the 1,4-thiazine (182) only showed 30% inhibition at 0.5 mM.

The 3,4-dihydro-2,2-dimethyl-1,4-thiazines showed slightly more inhibition than the 3,4-dihydro-1,4-thiazines. The dimethyl ester (187) and the half acid/half ester derivative (189) both showed approximately 20% inhibition at 0.1 mM. Further kinetic studies are required to determine the type of inhibition involved.

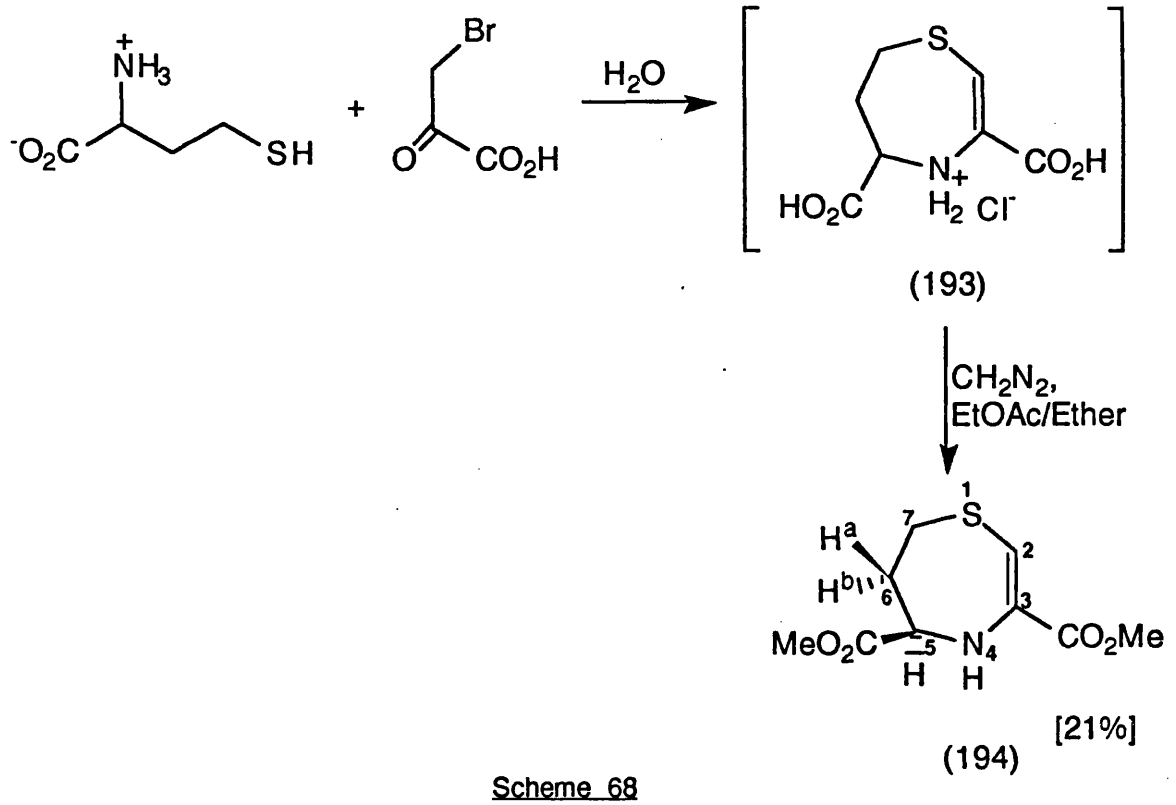
The mono-carboxylate 3,4-dihydro-1,4-thiazine analogues were poor inhibitors with respect to the dicarboxylate 3,4-dihydro-1,4-thiazine derivatives. The ethyl ester (192) showed 5% inhibition at 0.1 mM with DHDP Synthase.

5.2. Synthesis of 1,4-Thiazepine Derivatives.

4,5,6,7-Tetrahydro-4*H*-1,4-thiazepine-3,5-dicarboxylic acid hydrochloride (193) **(Scheme 68)**, a seven-membered analogue of 3,4-DHT (169), was prepared by treating DL-homocysteine with bromopyruvic acid in water. The compound was isolated as a yellow solid. The ^1H NMR showed a signal at δ_H 6.27 for the proton at C-2. Purification of this diacid proved difficult and so the compound was converted into the dimethyl ester (194) by treating a solution of the diacid (193) in ethyl acetate with a solution of diazomethane in ether. The diester (194) was easily purified on a neutral alumina

column eluting with 25% ethyl acetate in hexane to give a yellow oil. The yield of the diester (194) from DL-homocysteine was 21%.

The ^1H NMR spectrum of the dimethyl ester (194) showed a multiplet at δ_H 2.07 for the 6b proton and a multiplet at δ_H 2.40 for the protons at C-7. There is an interesting signal at δ_H 2.72 for the



Scheme 68

6a proton. It appears as a doublet of double double doublets (dddd) showing a long range coupling with the vinyl proton at C-2 (J 1.2 Hz), a coupling with the proton at C-5 (J 3.0 Hz), a coupling with the adjacent protons at C-7 (J 5.5 Hz) and a geminal coupling with the 6b proton (J 14.2 Hz). The ^{13}C NMR spectrum of this compound shows a signal at δ_C 29.9 for C-6, a signal at δ_C 33.1 for C-7 and a signal at δ_C

57.5 for C-5. The vinyl signal at δ_C 107.2 corresponds to C-2. The ^{13}C NMR spectrum of the diester (194) is shown in **Figure 10.**

The mechanism of formation of the 1,4-thiazepine diacid (193) from DL-homocysteine and bromopyruvic acid is presumably similar to that proposed for the 1,4-thiazines **(Scheme 61).** The 4,5,6,7-tetrahydro-1,4-thiazepine diacid (193) and dimethyl ester (194) have not yet been tested for inhibitory activity with DHDP Synthase.

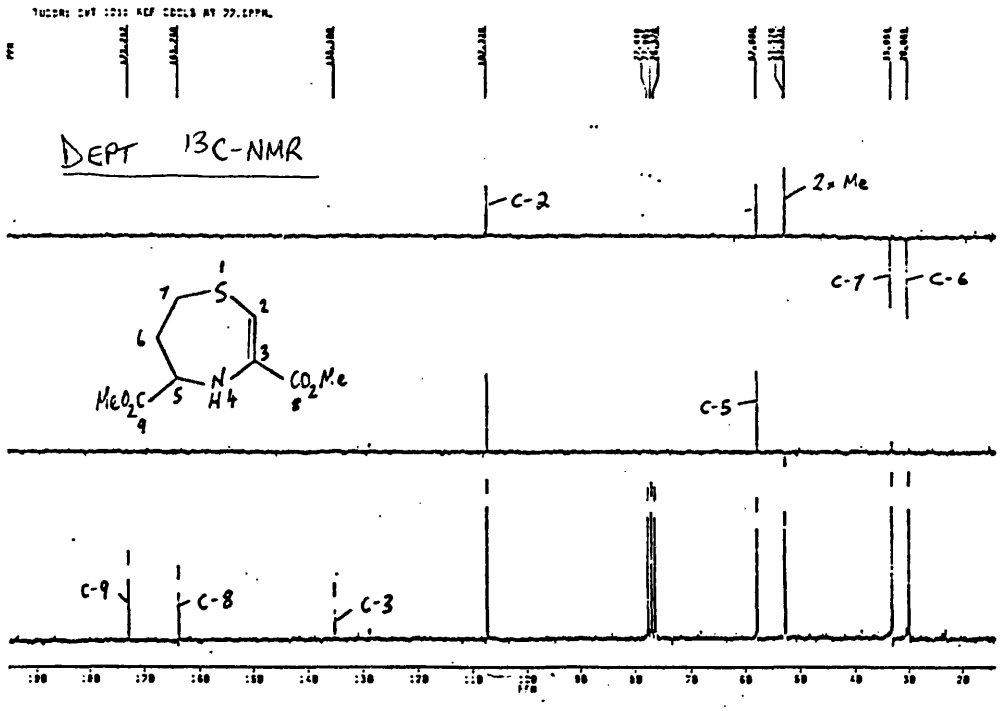
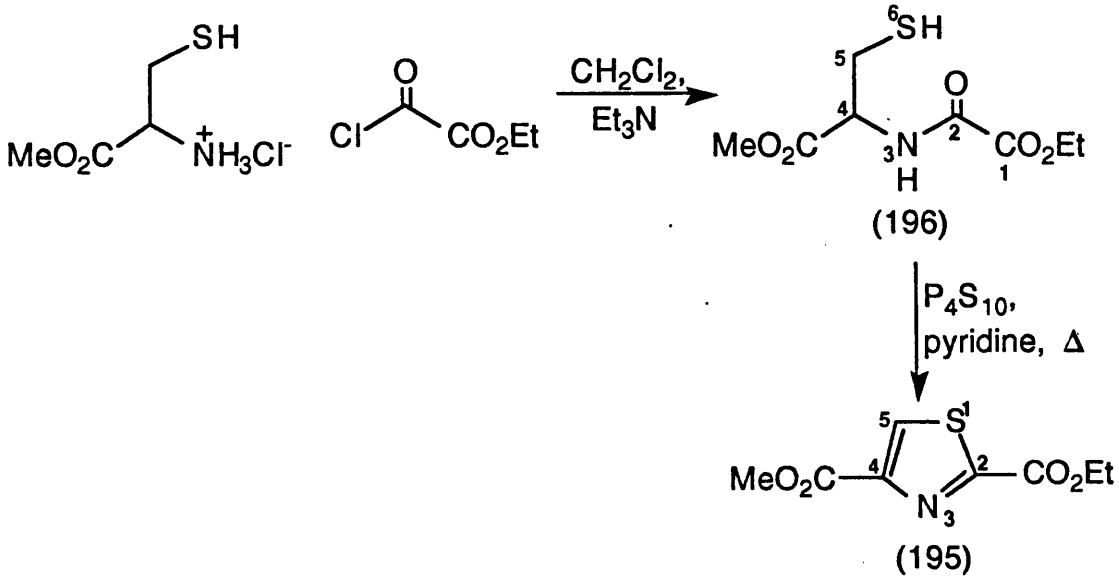


FIGURE 10 : ^{13}C NMR SPECTRUM OF THIAZEPINE DIESTER.

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

Ethyl 4-carbomethoxy-1,3-thiazole-2-carboxylate (195) was prepared in 40% yield by cyclisation of the mercapto-amide (196) **(Scheme 69).** The cyclisation was induced by treatment of the

mercapto-amide (196) with phosphorus pentasulphide in pyridine.[172] The product was purified on a silica gel column eluting with 5% ethyl acetate in hexane to give the desired 1,3-thiazole (195) as a yellow oil. The ^1H NMR spectrum of the 1,3-thiazole (195) showed a singlet in the aromatic region at δ_H 8.40 for the proton at C-5 and the ^{13}C NMR spectrum showed a signal at δ_C 132.4 for C-5.

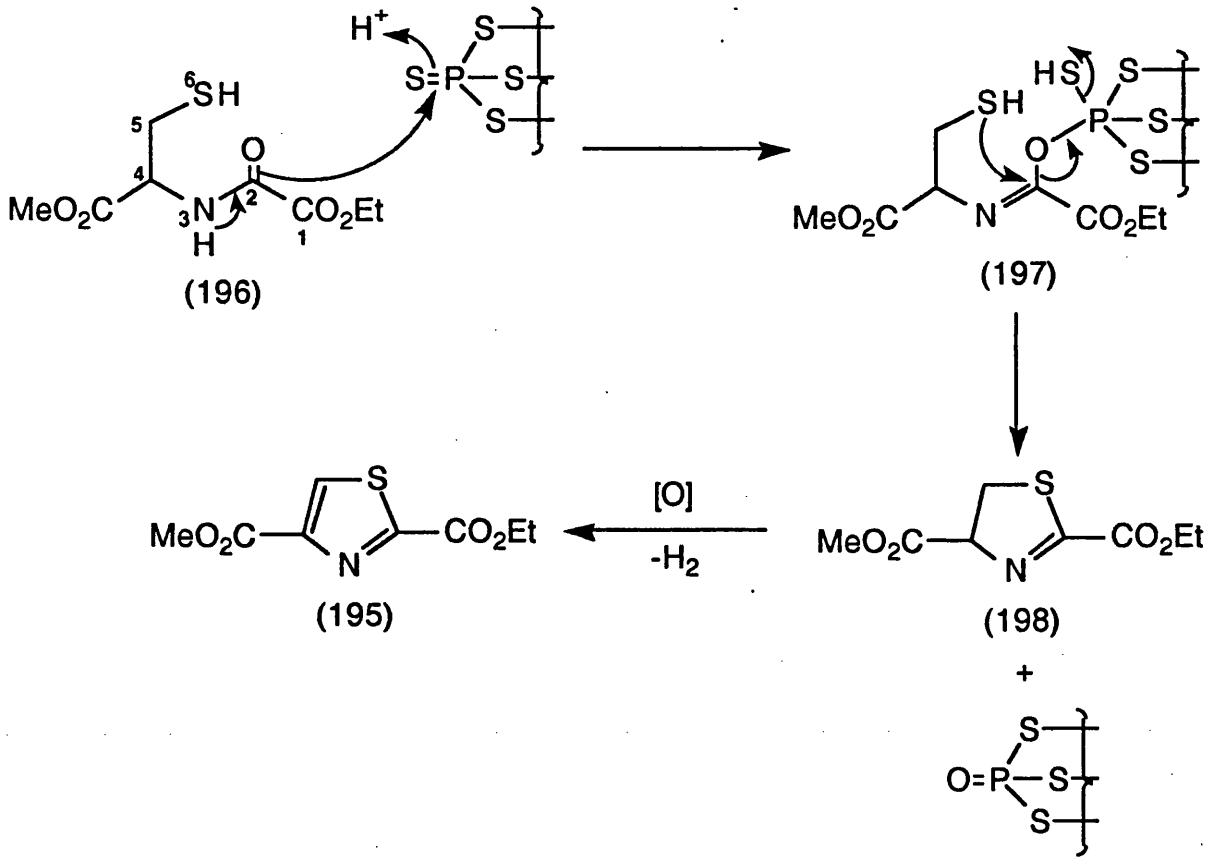


Scheme 69

The mechanism of the cyclisation presumably involves attack of the amide carbonyl group on phosphorus pentasulphide to give the intermediate phosphorus complex (197) (**Scheme 70**). Cyclisation then takes place via nucleophilic attack by the mercapto group at position 6 on the carbonyl at C-2 to give the 4,5-dihydro-1,3-thiazole intermediate (198). The driving force for the cyclisation is the formation of the strong phosphorus-oxygen double bond in the phosphorus complex which is eliminated. The mechanism is

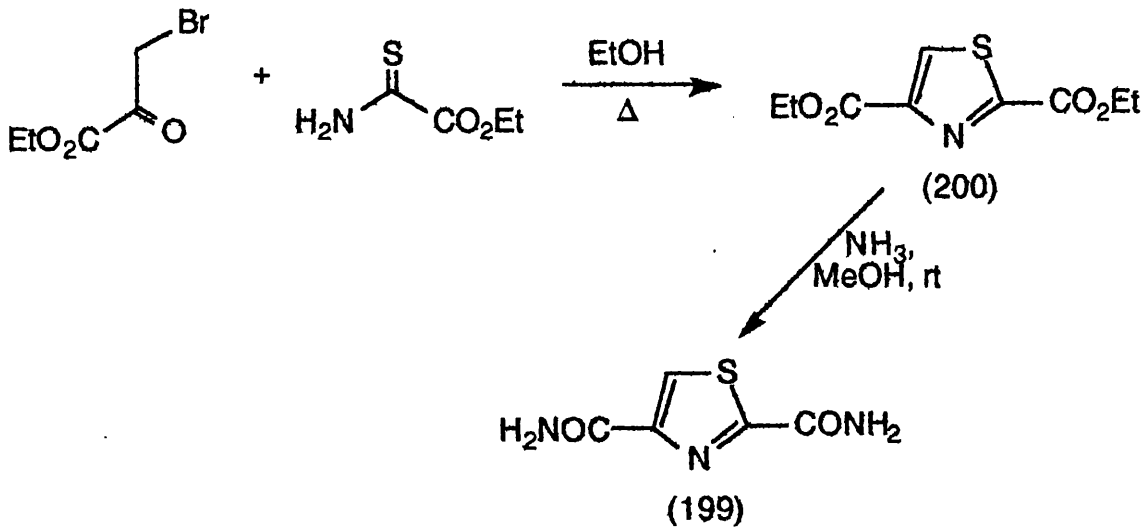
completed with spontaneous aromatisation of the 4,5-dihydro-1,3-thiazole intermediate (198) to the 1,3-thiazole (195) in air.

The mercapto-amide (196) was prepared in 94% yield by treating L-cysteine methyl ester hydrochloride with ethyl oxalyl chloride in dichloromethane in the presence of triethylamine. The compound was purified on a silica gel column eluting with 25% ethyl acetate in hexane to give a clear oil. The ^1H NMR spectrum of (196) showed a multiplet at δ_H 3.07 for the protons at C-5 and a multiplet at δ_H for the proton at C-4.



Scheme 70

1,3-Thiazole-2,4-dicarboxamide (199) was prepared in 90% yield by treating diethyl 1,3-thiazole-2,4-dicarboxylate (200) with a saturated solution of methanolic ammonia at room temperature (**Scheme 71**). The ^{13}C NMR spectrum of (199) showed two signals at δ_C 161.9 and 163.1 for the two amide carbonyls. The IR spectrum showed two signals in the carbonyl region at 1680 and 1590 cm^{-1}.



Scheme 71

The diethyl ester (200) was prepared in 92% yield by treating ethyl thioxamate with ethyl bromopyruvate in ethanol. The compound was purified on a neutral alumina column eluting with 25% ethyl acetate in hexane to give a yellow oil. The ^1H NMR and ^{13}C NMR spectra of the diethyl ester (200) were similar to those of the ester (195). The mechanism of formation of (200) is similar to that of 2,4-diphenyl-1,3-thiazole (165) (**Scheme 57**). This is an example of the Hantzsch thiazole synthesis.[171]

Test Results.

The 1,3-thiazole derivatives showed strong inhibition with DHDP Synthase. The methyl/ethyl ester 1,3-thiazole (195) produced 21% inhibition at 0.1 mM with DHDP Synthase and the diethyl ester 1,3-thiazole (200) showed 15% inhibition at 0.1 mM. Kinetic studies have still to be carried out to determine whether the 1,3-thiazole derivatives are competitive or non-competitive inhibitors. The diamide (199) has not yet been tested for inhibitory activity with DHDP Synthase.

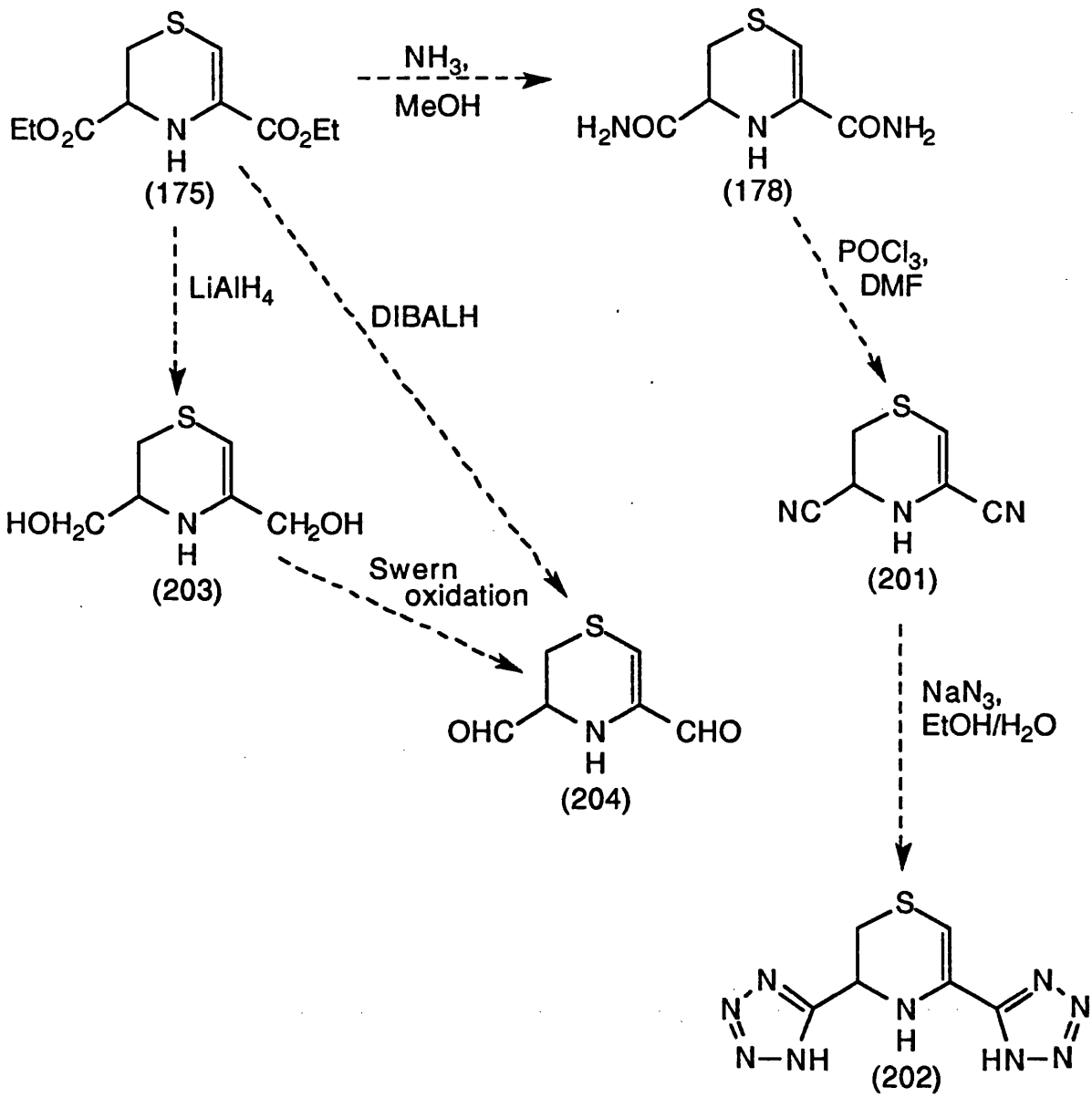
5.4. Conclusions and Future Work.

A range of sulphur analogues of L-2,3-DHDPA (25) and L-2,3,4,5-THDPA (24) have been synthesised and tested for inhibitor activity with DHDP Synthase.

The 3,4-dihydro-1,4-thiazines displayed good inhibition at 0.1 mM with DHDP Synthase. In particular the diethyl diester (175) and the *N*-methyl derivative (179) showed 10% inhibition at 0.1 mM. Further biochemical experiments must be carried out to determine whether the inhibition is competitive or non-competitive.

The diamide (178) has not yet been tested for inhibitor activity with DHDP Synthase. However, the diacid (169) was not as good an inhibitor as the diester (175). This suggests that changes in the carbonyl group functionality may have an effect on the level of inhibition with DHDP Synthase. Larger diesters should be

investigated. The dinitrile (201) and ditetrazole (202) could be synthesised from the diamide (178) and the diol (203) and dialdehyde (204) could be prepared from the diester (175) (**Scheme 72**).

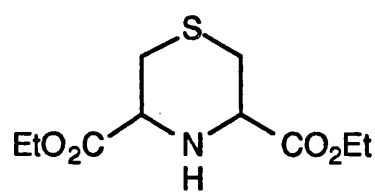


Scheme 72

The *N*-methyl derivative (179), as previously mentioned, showed good inhibition at 0.1 mM with DHDP Synthase. Further *N*-alkyl 1,4-thiazines should be synthesised where the alkyl group is sequentially increased in size to see what effect this has on the level of inhibition with DHDP Synthase.

Substitution at the 2-position of the 1,4-thiazine ring caused an increase in the level of inhibition observed with DHDP Synthase. The 2,2-dimethyl-3,4-dihydro-1,4-thiazines showed 20% inhibition at 0.1 mM whereas the 3,4-dihydro-1,4-thiazines showed 10% inhibition at 0.1 mM. Further substitution at the 2-position and at the 6-position should be investigated.

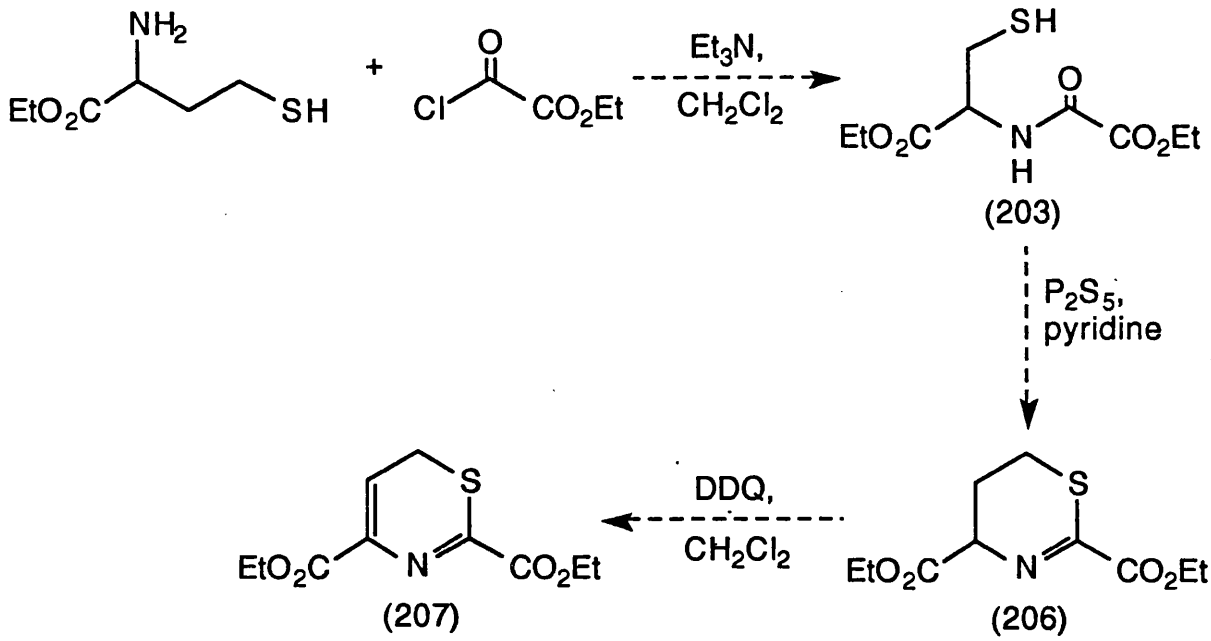
The 1,4-thiazine diester (182) was not as good an inhibitor with DHDP Synthase as the 3,4-dihydro-1,4-thiazine diester (175). The fully saturated 2,3,4,5-tetrahydro-1,4-thiazine diester (205) should be synthesised and tested for inhibitory activity with DHDP Synthase.



(205)

So far only 1,4-thiazines have been investigated for inhibitory activity with DHDP Synthase. Further thiazine derivatives should be synthesised and tested for inhibition of DHDP Synthase on the basis of the good inhibition results obtained with the 1,4-thiazines. These include the 4,5-dihydro-1,3-thiazines (206) and the 1,3-thiazine

(207) starting from DL-homocysteine ethyl ester and ethyl oxalyl chloride (**Scheme 73**).

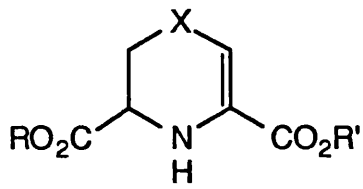


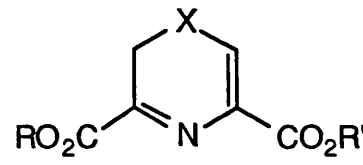
Scheme 73

Replacing the sulphur atom with an oxygen or a nitrogen would give the respective oxazines and pyrazines. A range of oxazine and pyrazine analogues should be synthesised and tested for inhibitory activity with DHDP Synthase. Possible targets are shown in **Scheme 74.**

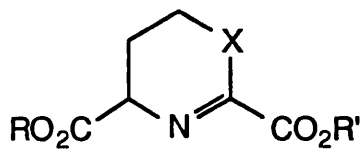
The 1,3-thiazoles showed very good inhibition with DHDP Synthase. The level of inhibition observed was 20% at 0.1 mM. As with the 3,4-dihydro-1,4-thiazines, changes in the carboxyl functionality produced slight changes in the level of inhibition, i.e. the methyl/ethyl ester (195) showed 21% inhibition at 0.1 mM whereas the diethyl ester (200) showed 15% inhibition at 0.1 mM.

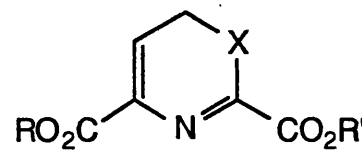
Hence, the diacid (208), dinitrile (209), ditetrazole (210), diol (211) and dialdehyde (212) should be synthesised and tested for inhibitory activity with DHDP Synthase (**Scheme 75**).





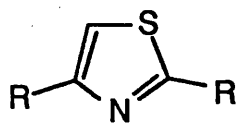
X = O 4,5-dihydro-1,4-oxazine 1,4-oxazine
X = N 4,5-dihydro-1,4-pyrazine 1,4-pyrazine

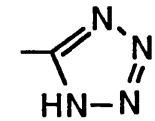




X = O 4,5-dihydro-1,3-oxazine 1,3-oxazine
X = N 4,5-dihydro-1,3-pyrazine 1,3-pyrazine

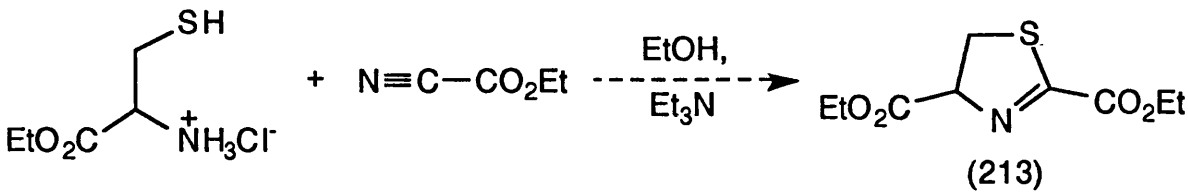
Scheme 74



R = -CO_2H (208)
R = -CN (209)
R =  (210)
R = -CH_2OH (211)
R = -CHO (212)

Scheme 75

The 4,5-dihydro-1,3-thiazole (213) could be prepared by treating a solution of L-cysteine ethyl ester with ethyl cyanoformate in ethanol in the presence of triethylamine **(Scheme 76).**



(213)

Scheme 76

Chapter [6] - Experimental

6.1. General Notes.

All melting points were measured with a Kofler hot-stage apparatus and are uncorrected. The optical rotations were measured with an optical Activity Ltd. AA-10 polarimeter. Infra-red spectra were obtained on a Perkin Elmer 580 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 12 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 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. Organic solvents were dried using magnesium sulphate and evaporated on a Buchi rotary evaporator under water-pump vacuum with slight heating. Dichloromethane was distilled from calcium hydride; methanol was distilled from magnesium turnings and iodine; triethylamine was distilled from potassium hydroxide; and dimethylformamide was distilled from silica gel.

Sodium deuteroxide was prepared by adding sodium metal (115 mg) to a cooled solution of D_2O (5 ml). It was stored under nitrogen at 0 °C.

6.2. Experimental to Chapter [3].

Synthesis of L-Aspartic Acid-ß-semialdehyde and Analogues.

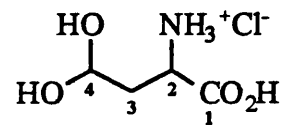
General Procedure [1] - Ozonolysis of Protected Allylglycine Analogues.

A solution of the protected allylglycine analogue (1.0 mmol) in dichloromethane (20 ml) was flushed with ozone 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 continued stirring for 5h at room temperature. Dichloromethane was removed *in vacuo* and ether (25 ml) was added to precipitate triethylamine *N*-oxide. The mixture was filtered and ether removed *in vacuo* to give a gluey oil. Purification was achieved using a silica gel column eluting with ether to give the desired aldehyde.

General Procedure [2] - Deprotection of Aspartic Acid-ß-semialdehyde Analogues using Trifluoroacetic Acid.[146]

Trifluoroacetic acid (1 ml) was added to a solution of the aspartic acid-ß-semialdehyde analogue (1.0 mmol) in dichloromethane (1 ml) under a nitrogen atmosphere. The mixture was stirred for 1 h and removal of the solvent *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.

The Hydrochloride Salt of DL-Aspartic Acid-ß-semialdehyde hydrate (21).[70]



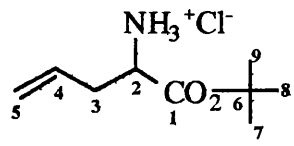
Ozone was bubbled through a solution of DL-allylglycine (0.50 g, 4.30 mmol) in 1M HCl (4.3 ml) at 0°C for 3 h to give DL-aspartic acid-ß-semialdehyde. The product was not isolated or purified as it is very unstable. The acidic solution was stored at O °C.

The ozonolysis reaction was repeated in D_2O and DCl and a ^1H NMR spectrum of the product was taken; δ_H (200 MHz) (D_2O + DCl) 1.38 (2H, m, 3-H_2) and 3.37 (1H, m, 2-H) and impurities at δ_H 2.24 (m) and 3.51 (m).

Attempted Preparation of DL-*N*-*t*-Butoxycarbonylhomoserine (84).

To a cooled solution of DL-homoserine (1.19 g, 10 mmol) in dioxan (20 ml) and water (10 ml) was added 1N sodium hydroxide (10 ml) with stirring. Di-*t*-butyl dicarbonate (1.1 equiv) was added to the cooled solution with continuous stirring for 30 min. The solution was concentrated *in vacuo* to a volume of 10 ml, cooled in an ice bath, covered with a layer of ethyl acetate (20 ml) and acidified to pH 2-3 with a dilute solution of $KHSO_4$. The mixture was extracted with ethyl acetate (2 x 10 ml) and the combined ethyl acetate extracts were washed with water. The organic layer was dried, filtered and the solvent was removed *in vacuo* to give a clear oil, 1.97 g (90%). The ^1H NMR spectrum of the product showed two *t*-butoxycarbonyl groups and the spectrum contained many peaks. The ^{13}C NMR spectrum had double the expected number of signals. The IR spectrum had three carbonyl absorptions at 1777 cm^{-1} (lactone), 1725 cm^{-1} (acid) and 1705 cm^{-1} (broad) (carbamate).

DL-Allylglycine *t*-Butyl Ester Hydrochloride (86).[146]

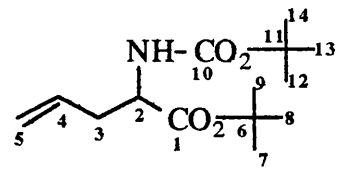


2-Methylpropene (50 ml) was added to a stirred suspension of DL-allylglycine (824 mg, 7.16 mmol) in dichloromethane (50 ml) at -78 °C. Conc. sulphuric acid (1 ml) was added dropwise over 10 min. The mixture was stirred for 15 min at -78 °C then gradually warmed to room temperature and continually stirred for 24 h. The resultant clear solution was carefully basified to pH 8 with sodium bicarbonate solution. The organic layer was separated, washed with brine solution (2 x 20 ml), dried (Na_2SO_4), filtered and concentrated to give a yellow oil. A white precipitate resulted on addition of a solution of dry HCl gas in ether (10 ml). The white solid was filtered and dried under vacuum, 490 mg (33% yield), mp 134-135°C; υ_{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), 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^+, 172.1342; C, 52.05; H, 8.80; N, 6.74. $C_9H_{18}NO_2$ requires *M*, 172.1338; C, 52.05; H, 8.67; N, 6.75%).

The L-isomer had $[\alpha]_D^{16}$ -16.50° (c = 1.4 in H_2O), mp 133-136 °C.

The D-isomer had $[\alpha]_D^{14}$ +16.07° (c = 1.4 in H_2O), mp 135-138 °C.

DL-*N*-*t*-Butoxycarbonylallylglycine *t*-Butyl Ester (87).[163]

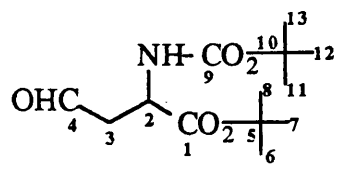


To a solution of sodium bicarbonate (121 mg) in water (7 ml), sodium chloride (253 mg) and di-*t*-butyldicarbonate (0.331 ml, 1 equiv.) was added a solution of **(86)** (300 mg, 1.44 mmol) in dichloromethane (20 ml) with stirring. The mixture was heated at reflux for 1.5 h then cooled to room temperature and the organic layer was separated. The aqueous layer was extracted with dichloromethane (2 x 20 ml) and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give an oil, 355 mg (90% yield), R_F 0.42 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3425, 2980, 1710 and 1495 cm^{-1}; δ_H (200 MHz) 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 (IH, m, 4-H); δ_C (50 MHz) 27.9 (C-7, -8 and -9), 28.3 (C-12, -13 and -14), 37.0 (C-3), 53.2 (C-2), 79.5 and 81.8 (C-6 and -11), 118.7 (C-5), 132.5 (C-4), 155.1 (C-10), 171.0 (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%).

The L-isomer had $[\alpha]_D^{16}$ +20.58° (c = 2.2 in CH_2Cl_2).

The D-isomer had $[\alpha]_D^{14}$ -20.32° (c = 2.2 in CH_2Cl_2).

DL-*N*-*t*-Butoxycarbonylaspartic Acid-ß-semialdehyde *t*-Butyl Ester (88).

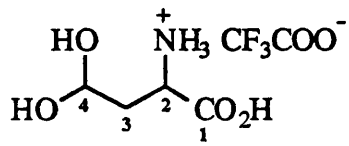


A solution of (87) (300 mg, 1.106 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to give a clear oil, 243mg (80% yield), R_F 0.5 (ether); υ_{max} ($CHCl_3$) 3425, 2980, 1710 and 1495 cm^{-1}; δ_H (200 MHz) 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 and 28.2 (C-6, -7, -8, -11, -12 and 13), 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, 56.92; H, 8.21; N, 5.31. $C_{13}H_{23}NO_5$ requires C, 57.07; H, 8.41; N, 5.12%).

The L-isomer had $[\alpha]_D^{16}$ +7.97° (c = 2.0 in CH_2Cl_2).

The D-isomer had $[\alpha]_D^{14}$ -7.80° (c = 2.0 in CH_2Cl_2).

Trifluoroacetate Salt of DL-Aspartic Acid-ß-semialdehyde Hydrate (89).

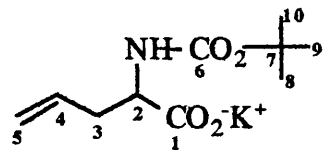


Compound (**88**) (100 mg, 0.73 mmol) was deprotected with trifluoroacetic acid (1 ml) according to general procedure [2] to give a pale yellow solid, 67 mg (74% yield), mp 64-66 °C; υ_{max} (KBr disc) 3420 (broad), 2925, 1675 and 1645 cm^{-1}; δ_H (200 MHz) (D_2O) 1.98 (2H, m, 3-H_2), 3.84 (1H, dd, 2-H) and 5.11 (1H, t, 4-H); δ_C (50 MHz) 37.9 (C-3), 52.0 (C-2), 89.4 (C-4) and 173.9 (C-1); *m / z* 137 (MH^+, 13.2%), 120, 92, 69, 44 (100%) and 43 (Found: MH^+, 137.0435. $C_4H_{11}NO_4$ requires *M*, 137.0450).

The L-isomer had $[\alpha]_D^{16}$ +3.33° (c = 1.5 in H_2O), mp 63-64 °C.

The D-isomer had $[\alpha]_D^{14}$ -3.15° (c = 1.5 in H_2O), mp 63-65 °C.

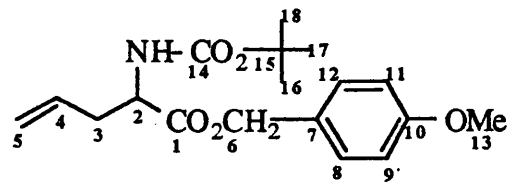
Potassium Salt of DL-*N*-*t*-Butoxycarbonylallylglycine (90).[157]



To a solution of DL-allylglycine (1 g, 8.69 mmol) in water (25 ml) was added dioxan (12 ml), potassium bicarbonate (957 mg, 1.1 equiv) and di-*t*-butyl dicarbonate (2 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, 2.0 g (91% yield); υ_{max} (KBr disc) 3360, 2980, 1675, 1595 and 1530 cm^{-1}; δ_H (200 MHz) (D_2O) 1.26 (9H, s, 8-, 9- and 10-H_3), 2.25 (2H, m, 3-H_2), 2.78 (1H, m,

2-H), 4.97 (2H, m, 5-H_2) and 5.60 (1H, m, 4-H); δ_C (50 MHz) 28.6 (C-8, -9 and -10), 37.3 (C-3), 56.5 (C-2), 81.9 (C-7), 118.9 (C-5), 134.9 (C-4), 166.5 (C-6) and 180.2 (C-1); *m/z* 214 (M^+, 0.3%), 112, 59 (100%) and 41.

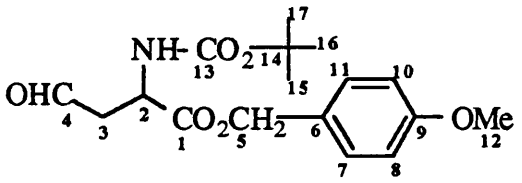
DL-*N*-*t*-Butoxycarbonylallylglycine *p*-Methoxybenzyl Ester (91).[157]



To a solution of **(90)** (1.50 g, 5.92 mmol) in DMF (10 ml) was added 4-methoxybenzyl chloride (0.9 ml, 1.04 equiv) with continuous stirring for 48 h. DMF was removed with xylene *in vacuo* and the resultant residue was partitioned between dichloromethane (20 ml) and aq. sodium carbonate solution (20 ml). The organic portion was washed with water (2 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 60% ether in hexane to give a clear oil, 1.56 g (79% yield), R_F 0.37 (50% ether / hexane); υ_{max} ($CHCl_3$) 3440, 3020, 2980, 1715, 1620, 1515 and 1500 cm^{-1}; δ_H (200 MHz) 1.43 (9H, s, 16-, 17- and 18-H_3), 2.51 (2H, dd, 3-H_2), 3.80 (3H, s, 13-H_3), 4.39 (1H, m, 2-H), 5.10 (5H, m, 5-H_2, 6-H_2 and NH), 5.66 (1H, m, 4-H), 6.89 (2H, d, *J* 8 Hz, 9- and 11-H) and 7.29 (2H, d, *J* 8 Hz, 8- and 12-H); δ_C (50 MHz) 28.3 (C-16, 17

and 18), 36.8 (C-3), 53.0 (C-2), 55.3 (C-13), 66.9 (C-6), 79.8 (C-15), 113.8 (C-7), 113.9 (C-8 and 12), 119.1 (C-5), 127.5 (C-11), 130.2 (C-9 and 11), 132.2 (C-4), 159.8 (C-14) and 171.9 (C-1); *m/z* 335 (M^+, 1.0%), 279, 170, 121 (100%), 70 and 57. (Found: M^+, 335.1718. $C_{18}H_{25}NO_5$ requires *M*, 335.1732).

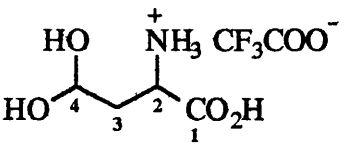
DL-*N*-*t*-Butoxycarbonylaspartic Acid-ß-semialdehyde *p*-Methoxybenzyl Ester (92).



A solution of compound (**91**) (1.20 g, 3.58 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to give a clear oil, 900 mg (75% yield), R_F 0.36 (ether); υ_{max} ($CHCl_3$) 3440, 3030, 2980, 1715, 1620 and 1515 cm^{-1}; δ_H (200 MHz) 1.42 (9H, s, 15-, 16- and 17-H_3), 3.03 (2H, t, 3-H_2), 3.80 (3H, s, 12-H_3), 4.58 (1H, m, 2-H), 5.10 (2H, s, 5-H_2), 5.42 (1H, br d, NH), 6.89 (2H, d, *J* 8 Hz, 8- and 10-H), 7.26 (2H, d, *J* 8 Hz, 7- and 11-H) and 9.69 (1H, s, 4-H); δ_C (50 MHz) 28.2 (C-15, -16 and -17), 46.0 (C-3), 48.8 (C-2), 55.3 (C-12), 67.5 (C-5), 80.2 (C-14), 114.0 (C-7 and -11), 115.1 (C-6), 128.6 (C-9), 130.2 (C-8 and 10), 159.8 (C-13), 171.1 (C-1) and 199.4 (C-4); *m/z* 337 (M^+, 0.7%), 281, 202, 137, 121

(100%), 72 and 57. (Found: M^+, 337.1538. $C_{17}H_{23}NO_6$ requires M, 337.1525).

Trifluoroacetate Salt of DL-Aspartic Acid ß-Semialdehyde Hydrate (89).

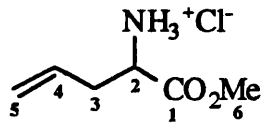


A solution of (92) (103 mg, 0.30 mmol) in trifluoroacetic acid (2 ml) was stirred at room temperature for 2 h. The solvent was removed *in vacuo* to give an oily residue. This was partitioned between water (10 ml) and ethyl acetate (10 ml). The aqueous layer was separated and washed with ethyl acetate (2 x 10 ml). Removal of the solvent *in vacuo* gave a yellow solid, 60 mg (79% yield); υ_{max} (KBr disc) 3420, 300, 1675, 1630 and 1400 cm^{-1}; δ_H (200 MHz) (D_2O) 2.01 (2H, m, 3-H_2), 3.80 (1H, dd, 2-H) and 5.18 (1H, m, 4-H); δ_C (50 MHz) 37.8 (C-3), 51.8 (C-2), 89.7 (C-4) and 174.2 (C-1); *m/z* 137 (MH^+, 17.5%), 119, 107, 93, 86, 69 and 44 (100%). (Found: MH^+, 137.0442. $C_4H_{11}NO_4$ requires M, 137.0431).

NMR Experiments with L-Aspartic Acid-β-semialdehyde (89) and Oxaloacetic Acid.

A solution of the trifluoroacetate salt of L-aspartic acid-β-semialdehyde (25 mg, 0.1 mol) in D_2O (1 ml) in a NMR tube was brought to pH 8 with sodium deuteroxide. Oxaloacetic acid (13 mg, 1 equiv.) was added to the tube with shaking and the reaction was followed by ^1H NMR spectroscopy (200 MHz). After 10 min two new signals appeared; a singlet in the vinyl-H region at δ 5.48 ppm and an aromatic singlet at δ 8.35 ppm in the integral ratio 3 : 1. Over a period of 2 h the strong vinyl signal gradually disappeared and the aromatic signal got stronger. The aromatic signal was shown to be dipicolinic acid (36) by ^{13}C NMR spectroscopy.

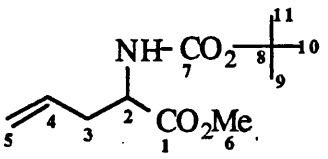
DL-Allylglycine Methyl Ester Hydrochloride (94).



A solution of DL-allylglycine (2.46 g, 0.021 mol) in methanol (60 ml) at 0 °C was saturated with dry HCl gas. The solution was brought to room temperature with continuous stirring for 1 h. Removal of the solvent *in vacuo* gave a clear oil, 3.2 g (91% yield); υ_{max} ($CHCl_3$) 3350, 2980 (broad), 1745 and 1500 c m^{-1}; δ_H (200 MHz) 2.72 (2H, dd, 3-H_2), 3.72 (3H, s, 6-H_3), 4.17 (1H, dd, 2-H), 5.17 (2H, m,

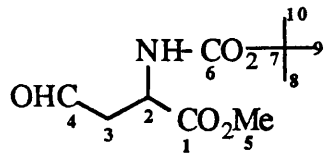
5-H_2), 5.73 (1H, m, 4-H) and 8.37 (2H, br s, NH_2); δ_C (50 MHz) 34.2 (C-3), 49.5 (C-6), 53.1 (C-2), 121.0 (C-5), 130.0 (C-4) and 169.2 (C-1); *m/z* 130 (MH^+), 129 (M^+, 0.1%), 88, 70, 43, 33 and 28 (100%). (Found: M^+, 129.0779. $C_6H_{11}NO_2$ requires M, 129.0789).

DL-*N*-*t*-Butoxycarbonylallylglycine Methyl Ester (95).[163]



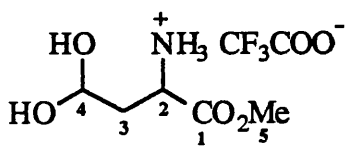
To a solution of sodium bicarbonate (1.52 g) in water (20 ml), sodium chloride (3.17 g) and di-*t*-butyl dicarbonate (4.16 ml, 1 equiv.) was added a solution of **(94)** (3.0 g, 0.018 mol) in dichloromethane (40 ml) with stirring. The mixture was heated at reflux for 1.5 h, cooled to room temperature and the organic layer was separated. The aqueous layer was extracted with dichloromethane (2 x 20 ml). The combined organic portions were dried (Na_2SO_4), filtered, and the solvent removed *in vacuo* to give a clear oil, 4.66 g (98% yield), R_F 0.46 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3430, 3020, 2980, 1805, 1740, 1710 and 1495 cm^{-1}; δ_H (200 MHz) 1.50 (9H, s, 9-, 10- and 11-H_3), 2.53 (2H, dd, 3-H_2), 3.72 (3H, s, 6-H_3), 4.30 (1H, dd, 2-H), 5.15 (3H, m, 5-H_2 and NH) and 5.60 (1H, m, 4-H); δ_C (50 MHz) 27.2 (C-9, -10 and -11), 28.1 (C-6), 36.6 (C-3), 52.8 (C-2), 85.0 (C-8), 118.9 (C-5), 132.2 (C-4), 146.6 (C-7) and 172.4 (C-1); *m/z* 188, 173, 170, 128, 113, 88, 70, 57 (100%) and 41.

DL-*N*-*t*-Butoxycarbonylaspartic Acid-ß-semialdehyde Methyl Ester (96).



A solution of compound **(95)** (8.17 g, 0.016 mol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to give a clear oil, 934 mg (26% yield), R_F 0.15 (ether); υ_{max} ($CHCl_3$) 3440, 3010, 2980, 1710 and 1500 cm^{-1}; δ_H (200 MHz) 1.39 (9H, s, 8-, 9- and 10-H_3), 3.05 (2H, m, 3-H_2), 3.70 (3H, s, 5-H_3), 4.05 (1H, m, 2-H), 5.40 (1H, br d, NH) and 9.68 (1H, s, 4-H); δ_C (50 MHz) 28.2 (C-8, -9 and -10), 45.9 (C-3), 48.5 (C-2), 52.7 (C-5), 80.2 (C-7), 171.4 (C-1) and 199.4 (C-4); *m/z* 172, 130, 115, 72, 59, 57 (100%) and 41.

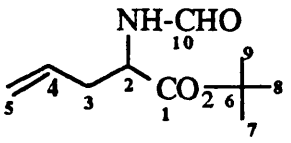
Trifluoroacetate Salt of DL-Aspartic Acid-ß-semialdehyde Hydrate Methyl Ester (93).



Trifluoroacetic acid (0.5 ml) was added to a solution of **(96)** (90 mg, 0.39 mmol) in dichloromethane at -78 °C with stirring under

a nitrogen atmosphere. The mixture was brought to 0 °C with continued stirring for 1h. Removal of the solvent *in vacuo* gave a yellow oil, 61 mg (60% yield); υ_{max} (KBr disc) 3425 (broad), 2960, 1750, 1675, 1520 and 1440 cm^{-1}; δ_H (200 MHz) (D_2O) 2.05 (2H, m, 3-H_2), 3.63 (3H, s, 5-H_3), 4.09 (1H, dd, 2-H) and 5.11 (1H, dd, 4-H); δ_C (50 MHz) 37.5 (C-3), 51.1 (C-2), 54.8 (C-5), 88.8 (C-4) and 171.3 (C-1); *m / z* 151, 150 (MH^+, 1.4%), 91, 89, 69 and 45 (100%). (Found : MH^+, 150.0550. $C_5H_{12}NO_4$ requires *M*, 150.0555).

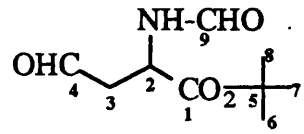
DL-*N*-Formylallylglycine *t*-Butyl Ester (101).[158]



1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide (911 mg, 2 equiv.) was added to a solution of formic acid (0.359 ml, 4 equiv.) in dichloromethane (15 ml) at 0 °C with stirring for 15 min. A cooled solution of **(86)** (570 mg, 2.38 mmol.) and N-methylmorpholine (0.523 ml, 2 equiv.) in dichloromethane (15 ml) was added and the resultant mixture was stirred in a water bath for 20 h. The reaction mixture was washed with 5% aq. citric acid (2 x 20 ml), aq. sodium bicarbonate soln. (2 x 20 ml) and brine soln. (2 x 20 ml). The organic portion was dried ($MgSO_4$), filtered and removal of the solvent *in vacuo* gave a clear oil, 468 mg (98% yield), R_F 0.55 (25% EtOAc /

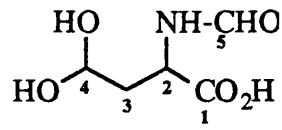
hexane); υ_{max} ($CHCl_3$) 3405, 3010, 1725, 1685 and 1490 cm^{-1}; δ_H (200 MHz) 1.47 (9H, s, 7-, 8- and 9-H_3), 2.55 (2H, m, 3-H_2), 4.67 (1H, dd, 2-H), 5,14 (2H, m, 5-H_2), 5.69 (1H, m, 4-H), 6.63 (1H, br d, NH) and 8.21 (1H, s, 10-H); δ_C (50 MHz) 27.8 (C-7, -8 and -9), 36.5 (C-3), 50.6 (C-2), 82.3 (C-6), 118.9 (C-5), 131.9 (C-4), 160.5 (C-10) and 170.3 (C-1); *m/z* 199 (M^+, 0.1%), 158, 143, 126, 98, 70 and 57 (100%). (Found: M^+, 199.1202. $C_{10}H_{17}NO_3$ requires 199.1208).

DL-*N*-Formylaspartic Acid-ß-semialdehyde *t*-Butyl Ester (104).



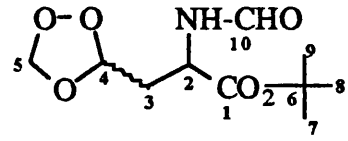
A solution of compound (**101**) (828 mg, 4.16 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. The ozonide was decomposed with triethylamine (232 mg, 4 equiv.) at room temperature with stirring for 12 h. Purification was achieved on a silica gel column eluting with 50% ethyl acetate in hexane to give a clear oil, 362 mg (43% yield), R_F 0.50 (ether); υ_{max} ($CHCl_3$) 3415, 3025, 2985, 1735, 1685 and 1500 cm^{-1}; δ_H (200 MHz) 1.44 (9H, s, 6-, 7- and 8-H_3), 3.08 (2H, dd, 3-H_2), 4.75 (1H, dd, 2-H), 6.58 (1H, br s, NH), 8.17 (1H, s, 9-H) and 9.70 (1H, s, 4-H); δ_C (50 MHz) 27.8 (C-6, -7 and -8), 45.7 (C-3), 46.7 (C-2), 83.3 (C-5), 160.6 (C-9), 169.1 (C-1) and 199.1 (C-4); *m/z* 172, 158, 128, 100, 72, 57 (100%) and 41.

DL-*N*-Formylaspartic Acid-ß-semialdehyde Hydrate (98).



Compound (**104**) (50 mg, 0.25 mmol) was deprotected with trifluoroacetic acid (0.5 ml) according to general procedure [2] to give a yellow syrup, 29mg (80% yield); υ_{max} (KBr disc) 3420 (broad), 2920, 1770, 1725, 1665 and 1520 cm^{-1}; δ_H (200 MHz) (D_2O) 1.70 (2H, m, 3-H_2), 4.20 (1H, m, 2-H), 4.70 (1H, m, 4-H) and 7.68 (1H, s, 5-H); δ_C (50 MHz) 38.9 (C-3), 49.3 (C-2), 88.8 (C-4), 164.9 (C-5) and 175.5 (C-1); *m/z* 163 (M^+, 0.8%), 144, 135, 119, 118, 44 and 28 (100%). (Found: M^+, 163.0488. $C_5H_9NO_5$ requires *M*, 163.0481).

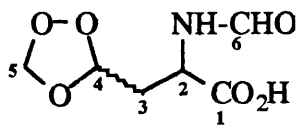
Ozonide (107) from DL-*N*-Formylallylglycne *t*-Butyl Ester.



A solution of compound (**101**) (400 mg, 2.01 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Reduction of the ozonide using triethylamine (2 equiv.) at -78 °C was unsuccessful. The ozonide was isolated using silica gel chromatography eluting with ethyl acetate to give a white solid, 70 mg (14% yield), R_F 0.47 (EtOAc); υ_{max} ($CHCl_3$) 3590, 3400,

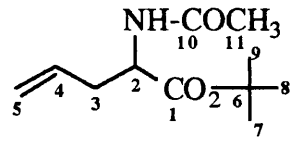
3010, 2980, 1735, 1680 and 1430 cm^{-1}; δ_H (200 MHz) 1.49 (9H, s, 7-, 8- and 9-H_3), 2.04-2.17 (2H, m, 3-H_2), 3.23 (1H, br s, NH), 4.68 (1H, dd, 2-H), 4.93-5.04 (2H, m, 5-H_2), 5.36-5.51 (1H, m, 4-H) and 8.23 (1H, s, 10-H); δ_C (50 MHz) 27.9 (C-7, -8 and -9), 31.1 (C-3), 46.7 (C-2), 67.3 (C-5), 82.6 (C-6), 91.2 (C-4), 161.0 (C-10) and 169.3 (C-1); *m / z* 146, 130, 100, 84, 72 and 57 (100%).

Ozonide (108) from deprotection of (107).



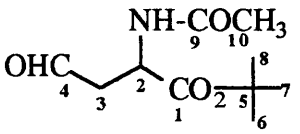
Trifluoroacetic acid (1 ml) was added to a solution of **(107)** (15 mg, 0.06 mmol) in dichloromethane (0.5 ml) at -78 °C with stirring under a nitrogen atmosphere. The mixture was brought to 0 °C with continued stirring for 1 h. Removal of the solvent *in vacuo* gave a yellow syrup, 6 mg (54% yield); υ_{max} (thin film) 3400 (broad), 2940, 2500 (broad), 1725, 1660, 1435 and 1410 cm^{-1}; δ_H (200 MHz) 2.10-2.41 (2H, m, 3-H_2), 4.41-4.62 (1H, m, 2-H), 4.78-5.10 (2H, m, 5-H_2), 5.23-5.34 (1H, m, 4-H) and 8.08 (1H, s, 6-H); *m/z* 129, 100, 84 and 55.

DL-*N*-Acetylallylglycine *t*-Butyl Ester (102).[146]



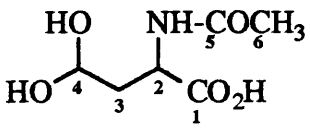
2-Methylpropene (100 ml) was added to a stirred suspension of DL-*N*-acetylallylglycine (3.1 g, 19.7 mmol) in dichloromethane (100 ml) at -78 °C. Conc. sulphuric acid (2.2 ml) was added dropwise to the mixture over 10 min. The mixture was stirred for 15 min at -78 °C then gradually warmed to room temperature and stirred for 24 h. The resultant clear solution was carefully basified to pH 8 with aq. sodium bicarbonate solution. The organic layer was separated, washed with brine (2 x 20 ml), dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 15% ethyl acetate in hexane to give an oil, 2.0 g (47% yield), R_F 0.2 (25% EtOAc / hexane); υ_{max} (KBr disc) 3440, 3280, 3080, 2975, 1735, 1650 and 1550 cm^{-1}; δ_H (200 MHz) 1.41 (9H, s, 7-, 8- and 9-H_3), 1.96 (3H, s, 11-H_3), 2.47 (2H, m, 3-H_2), 4.52 (1H, m, 2-H), 5.06 (2H, m, 5-H_2), 5.66 (1H, m, 4-H) and 6.15 (1H, br s, NH); δ_C (50 MHz) 23.1 (C-11), 27.9 (C-7, -8 and -9), 36.7 (C-3), 51.9 (C-2), 82.3 (C-6), 118.8 (C-5), 132.3 (C-4), 169.5 (C-10) and 170.9 (C-1); *m/z* 213 (M^+, 0.7%), 172, 157, 140, 116, 114, 112 (100%), 57 and 43. (Found: M^+, 213.1354; C, 61.86; H, 8.70; N, 6.52. $C_{11}H_{19}NO_3$ requires *M*, 213.1365; C, 61.97; H, 8.92; N, 6.57%).

DL-*N*-Acetylaspartic Acid-ß-semialdehyde *t*-Butyl Ester (105).



A solution of compound (**102**) (1.00 g, 4.69 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with 10% ethyl acetate in ether to give a clear oil, 582 mg (57% yield), R_F 0.17 (ether); υ_{max} ($CHCl_3$) 3430, 3000, 2980, 1730, 1670 and 1505 cm^{-1}; δ_H (200 MHz) 1.41 (9H, s, 6-, 7- and 8-H_3), 1.97 (3H, s, 10-H_3), 3.01 (2H, m, 3-H_2), 4.68 (1H, m, 2-H), 6.48 (1H, br s, NH) and 9.68 (1H, s, 4-H); δ_C (50 MHz) 22.9 (C-10), 27.7 (C-6, -7 and -8), 45.8 (C-3), 47.9 (C-2), 82.9 (C-5), 169.6 (C-9), 170.0 (C-1) and !99.4 (C-4); *m/z* 200, 172, 158, 142, 130, 114, 88, 72, 57 and 43 (100%).

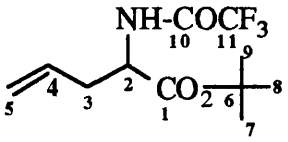
DL-*N*-Acetylaspartic Acid-ß-semialdehyde Hydrate (99).



Compound (**105**) (185 mg, 0.86 mmol) was deprotected with trfiluoroacetic acid according to general procedure [2] to give a yellow oil, 110mg (72% yield); υ_{max} (KBr disc) 3400 (broad), 3080, 2980, 1775, 1730, 1650, 1540 and 1435 cm^{-1}; δ_H (200 MHz) (D_2O) 1.83 (3H, s, 6-H_3), 1.91 (2H, m, 3-H_2), 4.28 (1H, dd, 2-H) and 4.93 (1H,

dd, 4-H); δ_C (50 MHz) 22.4 (C-6), 38.8 (C-3), 50.6 (C-2), 88.7 (C-4), 174.9 (C-5) and 176.0 (C-1); *m/z* 134, 132, 119, 116, 100, 87, 73 and 43 (100%).

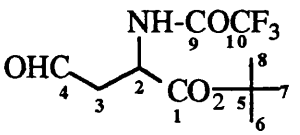
DL-*N*-Trifluoroacetylallylglycine *t*-Butyl Ester (103).[158]



1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide (1.43 g, 2 equiv.) was added to a solution of trifluoroacetic acid (0.74 ml, 4 equiv.) in dichloromethane (15 ml) at 0 °C with stirring for 15 min. A cooled solution of **(86)** (500 mg, 2.41 mmol) and *N*-methylmorpholine (0.531 ml, 2 equiv.) in dichloromethane (15 ml) was added and the resultant mixture was stirred in a water bath for 20 h. The reaction mixture was washed with 5% citric acid (2 x 20 ml), aq. sodium bicarbonate solution (2 x 20 ml) and brine solution (2 x 20 ml). The organic portion was dried ($MgSO_4$), filtered, and the solvent was removed *in vacuo* gave a yellow residue. Purification was achieved on a silica gel column eluting with 20% ethyl acetate in hexane to give a light yellow solid, 234 mg (36% yield), R_F 0.57 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3400, 3030, 2980, 1725 and 1530 cm^{-1}; δ_H (200 MHz) 1.49 (9H, s, 7,8 and 9-H_3), 2.63 (2H, m, 3-H_2), 4.55 (1H, m, 2-H), 5.18 (2H, m, 5-H_2), 5.67 (1H, m, 4-H) and 6.90 (1H, br s, NH); δ_C (50 MHz) 28.0 (C-7, -8 and -9), 36.0 (C-3), 52.3 (C-2), 83.5

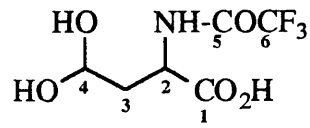
(C-6), 118.5 (C-11), 120.1 (C-5), 130.9 (C-4), 156.9 (C-10) and 169.2 (C-1); *m/z* 202, 173, 166, 159, 121 (100%), 70 and 57.

DL-*N*-Trifluoroacetylaspartic Acid-ß-semialdehyde *t*-Butyl Ester (106).



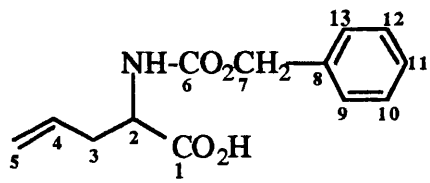
A solution of compound **(103)** (200 mg, 0.75 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with ether to give a clear oil, 108 mg (54% yield), R_F 0.39 (ether); υ_{max} ($CHCl_3$) 3395, 3030, 2980, 1725 and 1535 cm^{-1}; δ_H (200 MHz) 1.47 (9H, s, 6-, 7- and 8-H_3), 3.17 (2H, dd, 3-H_2), 4.67 (1H, m, 2-H), 5.40 (1H, br s, NH) and 9.73 (1H, s, 4-H); δ_C (50 MHz) 27.7 (C-6, -7 and -8), 44.6 (C-3), 48.2 (C-2), 84.0 (C-5), 114.9 (C-10), 152.0 (C-9), 167.8 (C-1) and 198.6 (C-4); *m/z* 251, 204, 176, 169, 140 and 57 (100%).

DL-*N*-Trifluoroacetylaspartic Acid-ß-semialdehyde Hydrate (100).



Compound **(106)** (124 mg, 0.461 mmol) was deprotected with trifluoroacetic acid according to general procedure [2] to give 72 mg of a yellow syrup, (68% yield); υ_{max} (KBr disc) 3415 (broad), 3050, 2910, 1770, 1730, 1655 and 1550 cm^{-1}; δ_H (200 MHz) (D_2O) 1.75 (2H, m, 3-H_2), 4.33 (1H, m, 2-H) and 4.85 (1H, m, 4-H); δ_C (50 MHz) 37.9 (C-3), 49.8 (C-2), 88.1 (C-4), 116.1 (C-6), 155.7 (C-5) and 175.4 (C-1); *m/z* 186, 122, 114, 97, 69 (100%), 51 and 44.

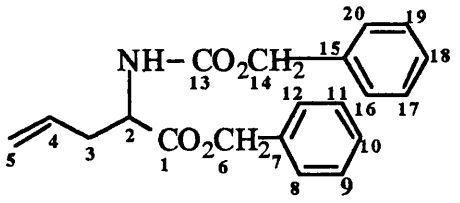
DL-*N*-Benzyloxycarbonylallylglycine (110).



A solution of DL-allylglycine (685 mg, 5.95 mmol) in 2M sodium hydroxide solution (8 ml) was cooled to 0 °C. Benzyl chloroformate (0.927 ml, 1.1 equiv.) and 4M sodium hydroxide solution (2 ml) were simultaneously added dropwise over 45 min with continued stirring and cooling to 0 °C. After 30 min the reaction mixture was washed with ether (3 x 15 ml). The aqueous portion was acidified to Congo Red with conc. hydrochloric acid and

extracted with ethyl acetate (2 x 20 ml). The combined organic extracts were dried ($MgSO_4$), filtered, and the solvent removed *in vacuo* to give a light yellow oil, 1.0 g (71% yield), R_F 0.28 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3440, 3020, 1720 and 1510 cm^{-1}; δ_H (200 MHz) 2.56 (2H, m, 3-H_2), 4.48 (1H, m, 2-H), 5.11 (2H, s, 7-H_2), 5.17 (2H, m, 5-H), 5.43 (1H, br d, NH), 5.60 (1H, m, 4-H) and 7.33 (5H, br s, 9-, 10-, 11-, 12- and 13-H); δ_C (50 MHz) 36.3 (C-3), 53.0 (C-2), 67.1 (C-7), 119.6 (C-5), 128.0, 128.2 and 128.5 (C-9, -10, -11, -12 and -13), 131.4 (C-4), 135.9 (C-8), 156.0 (C-6) and 176.1 (C-1); *m / z* 249 (M^+, 2.0%), 208, 204, 108, 91, 79, 51 and 41 (100%). (Found: M^+, 249.0998. $C_{13}H_{15}NO_4$ requires *M*, 249.0202).

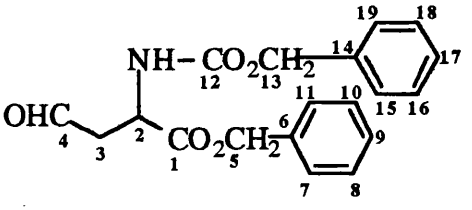
DL-*N*-Benzyloxycarbonylallylglycine Benzyl Ester (111).[148]



A solution of **(110)** (750 mg, 3.19 mmol) in methanol (15 ml) and water (2 ml) was titrated to pH 7 with 20% aq. caesium carbonate solution. The solvent was removed *in vacuo* and the residue was re-evaporated from DMF (2 x 10 ml). The resultant white solid was stirred with benzyl bromide (0.42 ml, 1.1 equiv.) in DMF (10 ml) at room temperature for 6 h. The solvent was removed *in vacuo* and on addition of water (50 ml) a white solid started to

precipitate. The reaction mixture was taken into ethyl acetate (50 ml), washed with water (2 x 20 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give a yellow oil, 900 mg (84% yield), R_F 0.27 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3440, 3020, 1720 and 1510 $c\,m^{-1}$; δ_H (200 MHz) 2.55 (2H, m, 3-H_2), 4.50 (1H, m, 2-H), 5.11 (4H, br s, 6- and 14-H_2), 5.12 (1H, br d, NH), 5.64 (1H, m, 4-H) and 7.35 (10H, br s, 8-, 9-, 10-, 11-, 12-, 16-, 17-, 18-, 19- and 20-H); δ_C (50 MHz) 36.6 (C-3), 53.3 (C-2), 67.1 and 67.0 (C-6 and -14), 119.4 (C-5), 128.0, 128.1, 128.3, 128.4 ,128.5 and 128.6 (C-8, -9, -10, -11, -12, -16, -17, -18, -19 and -20), 131.8 (C-4), 135.2 and 136.0 (C-7 and -15), 155.7 (C-13) and 171.5 (C-1); *m/z* 399 (M^+, 0.3%), 298, 204, 160, 107 and 91 (100%). (Found: M^+, 339.1477. $C_{20}H_{21}NO_4$ requires M, 339.1471).

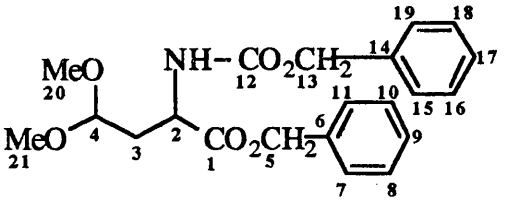
DL-*N*-Benzyloxycarbonylaspartic Acid-ß-semialdehyde Benzyl Ester (112).



A solution of compound (**111**) (850 mg, 2.50 mmol) in dichloromethane was saturated with ozone according to general procedure [1]. Purification was achieved on a silica gel column eluting with 35% ethyl acetate in hexane to give a clear oil, 494 mg

(58% yield), R_F 0.31 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3435, 3020, 1725 and 1510 cm^{-1}; δ_H (200 MHz) 3.07 (2H, m, 3-H_2), 4.66 (1H, m, 2-H), 5.10 and 5.16 (4H, 2 x s, 5- and 13-H_2), 5.73 (1H, br d, NH), 7.33 (10H, br s, 7-, 8-, 9-, 10-, 11-, 15-, 16-, 17-, 18- and 19-H) and 9.67 (1H, s, 4-H); δ_C (50 MHz) 45.7 (C-3), 49.1 (C-2), 67.1 and 67.6 (C-5 and 13), 128.0, 128.1, 128.2 ,128.5 and 128.6 (C-7, -8, -9, -10, -11, -15, -16, -17, -18 and -19), 135.0 and 136.0 (C-6 and -14), 155.8 (C-12), 170.5 (C-1) and 199.1 (C-4); *m/z* 341 (M^+, 0.1%), 312, 250, 206, 180, 162, 108 and 91 (100%). (Found: M^+, 341.1278. $C_{19}H_{19}NO_5$ requires *M*, 341.1263).

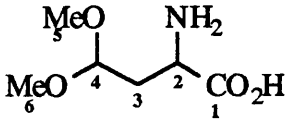
DL-*N*-Benzyloxycarbonylaspartic Acid-ß-semialdehyde Benzyl Ester Dimethyl Acetal (113).[159]



A solution of **(112)** (300 mg, 0.88 mmol), *p*-toluenesulphonic acid (42 mg, 0.25 equiv.) and trimethyl orthoformate (0.77 ml, 8 equiv.) in anhydrous methanol (2 ml) was heated to reflux for 2 h under a nitrogen atmosphere. The mixture was cooled to room temperature and diluted with ether (20 ml). The organic portion was washed with a 1:1 mixture of 5% sodium hydroxide solution and brine solution (2 x 10 ml), water (2 x 10 ml) then brine solution (2 x

10 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give an oil. Purification was achieved on a silica gel column eluting with ether to give a clear oil, 280 mg (82% yield), R_F 0.36 (ether); υ_{max} ($CHCl_3$) 3420, 3010, 2950, 1720 and 1505 cm^{-1}; δ_H (200 MHz) 2.11 (2H, br t, 3-H_2), 3.24 and 3.26 (6H, 2 x s, 20- and 21-H_3), 4.34 (1H, t, 4-H), 4.46 (1H, m, 2-H), 5.11 and 5.16 (4H, 2 x s, 5- and 13-H_2) and 7.35 (10H, br s, 7-, 8-, 9-, 10-, 11-, 15-, 16-, 17-, 18- and 19-H); δ_C (50 MHz) 34.6 (C-3), 51.0 (C-2), 53.5 and 53.7 (C-20 and -21), 66.9 and 67.1 (C-5 and -13), 102.2 (C-4), 127.7, 128.1, 128.4, 128.5, 128.6 and 128.8 (C-7, -8, -9, -10, -11, -15, -16, -17, -18 and -19), 135.3 and 136.2 (C-6 and -14), 155.9 (C-12) and 171.6 (C-1); *m/z* 356, 325, 252, 220, 143, 107, 91 (100%) and 75.

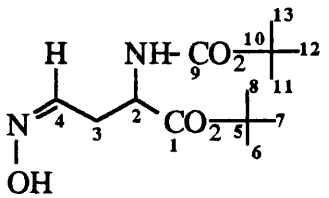
DL-Aspartic Acid-ß-semialdehyde Dimethyl Acetal (114).[160]



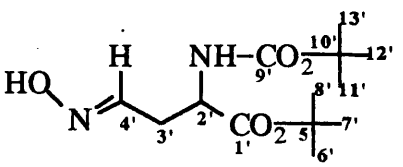
A solution of **(113)** (206 mg, 0.53 mmol) and 10% Pd-charcoal (40 mg) in anhydrous methanol (10 ml) and glacial acetic acid (1 ml) was left in a sonic bath under a hydrogen atmosphere for 20 h. The resultant black mixture was filtered through celite and the solvent was removed *in vacuo* to give an oil. Azeotroping with toluene removed traces of acetic acid. Trituration of the resultant oil with

ether gave a white solid, 50 mg (58% yield); υ_{max} (KBr disc) 3440, 3080, 2950, 2830, 2560, 1595 and 1505 cm^{-1}; δ_H (200 MHz) (D_2O) 2.00 (2H, m, 3-H_2), 3.23 and 3.24 (4H, 2 x s, 5- and 6-H_3), 3.65 (1H, dd, 4-H) and 4.34 (1H, t, 2-H); δ_C (50 MHz) 34.0 (C-3), 52.1 (C-2), 55.5 (C-5 and 6), 104.3 (C-4) and 174.5 (C-1); *m/z* 148, 132, 118, 102, 86, 75 (100%), 59 and 47.

<u>*Z*- (115) and *E*-DL-*N*-*t*-Butoxycarbonylaspartic Acid-ß-semialdehyde *t*-Butyl Ester Oxime (116).</u>



(Z)

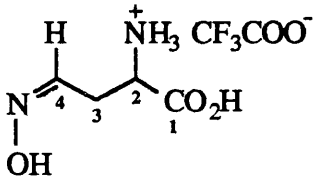


(E)

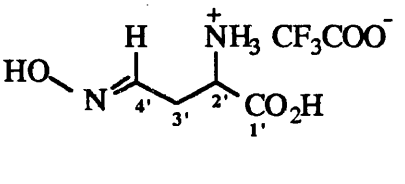
To a solution of hydroxylamine hydrochloride (74 mg, 1 equiv.) and triethylamine (0.15 ml, 1 equiv.) in dichloromethane (8 ml) under a nitrogen atmosphere was added dropwise a solution of **(88)** (290 mg, 1.06 mmol). The mixture was stirred at room temperature for 12 h. Removal of the solvent *in vacuo* 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* to give a clear oil. Purification was achieved on a silica gel column eluting with 5% ethyl acetate in ether to give a clear oil, 214 mg (73% yield), R_F 0.39 (20% EtOAc / hexane); υ_{max} ($CHCl_3$) 3590, 3430, 3020, 2985, 1715 and 1500 cm^{-1}; ^1H NMR and ^{13}C NMR analysis showed that both the geometric isomers of the oxime had

been formed in the ratio (117) (*Z*) 2 : (118) (*E*) 1; δ_H (200 MHz) 1.45, 1.46, 1.47 and 1.52 (4 x s, 6- and 6'-, 7- and 7'-, 8- and 8'-, 11- and 11'-, 12- and 12'-, 13- and 13'-H_3), 2.69 and 2.81 (2 x t, 3- and 3'-H_2), 4.35-4.45 (m, 2- and 2'-H), 5.40 and 5.60 (2 x br d, 2 x NH), 6.80 and 7.39 (2 x t, 4- and 4'-H) and 9.03 (h, 2 x N-0H); δ_C (50-MHz) 27.8, 28.2 and 28.6 (C-6 and -6', -7 and -7', -8 and -8', -11 and -11', -12 and -12', -13 and -13'), 32.9 (C-3 and -3'), 51.5 and 51.7 (C-2 and -2'), 80.0 and 82.5 (C-5 and -5', -10 and -10'), 147.2 (C-4 and -4'), 170.9 and 171.3 (C-1 and -1', -9 and -9'); *m/z* 230, 187, 172, 159, 131, 87 and 57 (100%).

Trifluoroacetate Salt of *Z*- (117) and *E*-DL-Aspartic Acid-ß-semialdehyde Oxime (118).



(Z)

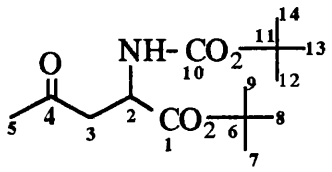


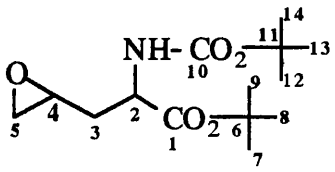
(E)

The mixture of **(115)** and **(116)** (125 mg, 0.456 mmol) was deprotected with trifluoroacetic acid (2 ml) according to general procedure [2] to give an amber syrup, 77 mg (69% yield); υ_{max} (thin film) 3350, 2980, 2520, 1730, 1670 and 1430 cm^{-1}; δ_H (200 MHz) 2.68-2.82 (m, 3- and 3'-H_2), 4.00-4.08 (m, 2- and 2'-H), 6.78 and 7.35 (2 x t, 4- and 4'-H); δ_C (50 MHz) 30.6 and 34.5 (C-3 and -3'), 51.4 and 51.5 (C-2 and -2'), 148.0 and 148.6 (C-4 and -4'), 172.5 and

174.4 (C-1 and -1'); *m/z* 132(M^+, 1.0%), 116, 98, 87, 69 and 57(100%). (Found: M^+, 132.0540. $C_4H_8N_2O_3$ requires M, 132.0535).

t-Butyl DL-2-*t*-Butoxycarbonylamino-4-oxopentanoate (119) and *t*-Butyl DL-2-*t*-Butoxycarbonylamino-4-epoxypentanoate (120).[161]





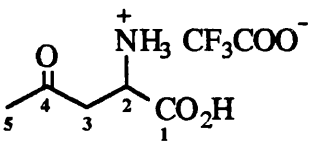
A solution of diazomethane in ether was added dropwise to a solution of **(88)** (230 mg, 0.84 mmol) in ethyl acetate (5 ml) with stirring and cooling to 0 °C until a yellow colour persisted for 15 min. The resultant mixture was stirred at room temperature for 12 h. Removal of the solvent *in vacuo* gave a clear oil. TLC analysis showed 2 main spots. Separation was achieved on a silica gel column eluting with 50% ether in hexane to give two compounds **119** and **120.**

(119) : 188 mg of a clear oil, (77% yield), R_F 0.18 (50% ether / hexane); υ_{max} ($CHCl_3$) 3440, 3030, 2980, 1715 and 1500 cm^{-1}; δ_H (200 MHz) 1.44 (18H, s, 7-, 8-, 9-, 12-, 13- and 14-H_3), 2.17 (3H, s, 5-H_3), 3.01 (2H, ddd, *J* 46 Hz, *J* 18 Hz, *J* 4.4 Hz, 3-H_2), 4.36 (1H, dd, *J* 18 Hz, *J* 4.4 Hz, 2-H) and 5.46 (1H, br d, NH); δ_C (50 MHz) 27.8 and 28.3

(C-7, -8, -9, -12, -13 and -14), 29.9 (C-5), 45.6 (C-3), 50.1 (C-2), 79.8 and 82.1 (C-6 and 11), 155.6 (C-10), 170.3 (C-1) and 206.6 (C-4); *m / z* 287 (M^+, 0.2%), 231, 186, 130, 86, 57 (100%) and 41. (Found: M^+, 287.1722. $C_{14}H_{25}NO_5$ requires *M*, 287.1733) and

(120) : 21 mg of a clear oil, (8% yield), R_F 0.08 (50% ether / hexane); υ_{max} ($CHCl_3$) 3500, 3430, 3020, 2985, 2940, 1715, 1500 and 910 cm^{-1}; δ_H (200 MHz) 1.42 (18H, s, 7-, 8-, 9-, 12-, 13- and 14-H_3), 2.11-2.19 (2H, m, 3-H_2), 2.45-2.63 (2H, m, 5-H_2), 2.91-3.00 (1H, m, 4-H), 4.42 (1H, m, 2-H) and 5.52 (1H, br d, NH); δ_C (50 MHz) 27.2 and 28.1 (C-7, -8, -9, -12, -13 and -14), 38.3 (C-3), 48.1 (C-5), 49.8 (C-4), 52.2 (C-2), 80.1 and 82.0 (C-6 and -11), 154.9 (C-10) and 171.7 (C-1); *m / z* 216, 186, 160, 130, 116, 86, 57 (100%) and 41.

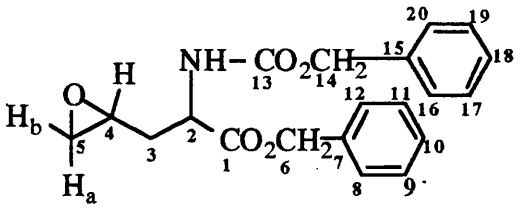
Trifluoroacetate Salt of DL-2-Amino-4-oxopentanoic Acid (121).



Compound **(119)** (60 mg, 0.21 mmol) was deprotected with trifluoroacetic acid (1 ml) according to general procedure [2] to give a yellow syrup, 40 mg (80% yield); υ_{max} (thin film) 3430 (broad), 2995, 2550, 1680, 1635 and 1400 cm^{-1}; δ_H (200 MHz) (D_20) 2.06 (3H, s, 5-H_3), 3.05-3.07 (2H, m, 3-H_2) and 3.93 (1H, dd, *J* 6.1 Hz and *J* 4.9

Hz, 2-H); δ_C (50 MHz) 30.3 (C-5), 43.6 (C-3), 50.2 (C-2), 173.9 (C-1) and 211.4 (C-4); *m/z* 131 (M^+), 114, 87, 69, 56 and 45. (Found: M^+, 131.0719. $C_5H_9NO_3$ requires M, 131.0727).

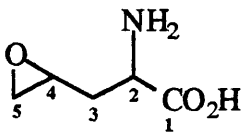
Benzyl DL-2-(Benzyloxycarbonyl)amino-4-epoxypentanoate (123).[162]



A solution of **(111)** (50 mg, 0.147 mmol) in dichloromethane (1 ml) was added dropwise to a solution of *m*-CPBA (31 mg, 1.2 equiv.) in dichloromethane (2 ml) at 0 °C with stirring under a nitrogen atmosphere. Na_2HPO_4 buffer was added to bring the pH of the mixture to 8 and stirring was continued at room temperature for 3 h. The resulting mixture was diluted with dichloromethane (10 ml), washed with 5% sodium bicarbonate solution (2 x 10 ml), dried ($MgSO_4$), and the solvent was removed *in vacuo* to give an oily residue. Purification was achieved on a silica gel column eluting with 30% ethyl acetate in hexane to give a clear oil, 39 mg (76% yield), R_F 0.12 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3430, 3010, 2955, 1620 and 1505 and 1245 cm^{-1}; δ_H (200 MHz) 1.81-2.17 (2H, m, 3-H_2), 2.40 (1H, dd, *J* 2.6 Hz and *J* 4.8 Hz, 5b-H), 2.60 (1H, dd, *J* 4.8 Hz and *J* 8.7 Hz, 5a-H), 2.88-3.02 (1H, m, 4-H), 4.46-4.64 (1H, m, 2-H), 5.11 and 5.19 (4H, 2 x s, 6- and 14-H_2), 5.62 (1H, br d, NH) and 7.35 (10H, br s, 8-,

9-, 10-, 11-, 12-, 16-, 17-, 18-, 19- and 20-H); δ_C (50 MHz) 35.4 (C-3), 46.7 (C-5), 48.9 (C-4), 52.4 (C-2), 67.1 and 67.5 (C-6 and -14), 128.1, 128.2, 128.4, 128.5 and 128.6 (C-8, --9, -10, -11, -12, -16, -17, -18, -19 and -20), 135.1 and 140.8 (C-7 and -15), 156.0 (C-13) and 171,5 (C-1); *m / z* 355(M^+, 3.2%), 220, 176, 107 and 91(100%). (Found: M^+, 355.1404. $C_{20}H_{21}NO_5$ requires *M*, 355.1418).

DL-2-Amino-4-epoxypentanoic Acid (124).



A solution of (**123**) (30 mg, 0.08 mmol) and 10% Pd-charcoal (6 mg) in anhydrous methanol (2 ml) and glacial acetic acid (0.5 ml) was left in a sonic bath under a hydrogen atmosphere for 20 h. The resultant black mixture was filtered through celite and the solvent was removed *in vacuo* to give a clear oil. Azeotroping with toluene removed traces of acetic acid. Trituration of the resultant oil with ether gave an off-white solid, 7 mg (63% yield); υ_{max} (KBr disc) 3025 (broad), 300, 2980, 1595, 1500 and 1240 cm^{-1}; δ_H (200 MHz) (D_2O) 2.10 (2H, m, 3-H_2), 2.41-2.44 (1H, m, 5b-H), 2.59-2.64 (1H, m, 5a-H), 3.01-3.03 (IH, m, 4-H) and 4.34-4.41 (1H, m, 2-H); δ_C (50 MHz) 35.1 (C-3), 46.1 (C-5), 50.1 (C-4), 52.1 (C-2) and 173.9 (C-1); *m / z* 114, 86, 74, 45 (100%).

6.3. Experimental to Chapter [4].

Synthesis of Derivatives of Pyruvate and Bromopyruvate.

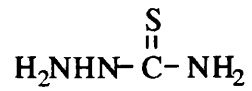
General Procedure [1] - Preparation of Imine Derivatives of Pyruvate and Bromopyruvate.

A solution of the amine hydrochloride (1 equiv.) in water (5 ml) was added to a solution of the pyruvate analogue (6.00 mmol) in dichloromethane (3 ml) and the 2-phase mixture was rapidly stirred at room temperature for 48 h. The resultant mixture was then extracted with dichloromethane (2 x 10 ml) and the combined organic extracts were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give the desired products (20-70% yield).

General Procedure [2] - Preparation of 2,4-Dinitrophenylhydrazone (DNP) Derivatives of Pyruvates and Bromopyruvates.[165]

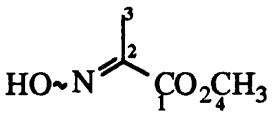
Conc. sulphuric acid (0.5 ml) was added to a solution of 2,4-dinitrophenylhydrazine (1.5 mmol) in methanol (5 ml) with stirring. The resultant orange precipitate was filtered and to the filtrate a solution of the pyruvate analogue (1 equiv) in methanol (2 ml) was slowly added with stirring. The mixture was cooled and a solid precipitated immediately. Filtration and drying under vacuum gave the desired 2,4-DNP derivative (20-50% yield).

Preparation of Thiosemicarbazide.



A solution of ammonium thiocyanate (2.20 g, 0.03 mol) and 98% hydrazine hydrate (0.54 ml, 0.01 mol) in water (10 ml) was heated to reflux for 3 h. After cooling the mixture was filtered to remove small traces of sulphur. The filtrate was then allowed to cool to room temperature over 10 h and thiosemicarbazide precipitated. The solid was filtered and washed with cold water to remove any unreacted ammonium thiocyanate. Drying under vacuum gave 500 mg of a white solid (47% yield), mp 178-181 °C (EtOH/H_2O); υ_{max} (KBr disc) 3485, 3370, 3265, 1645, 1620, 1533, 1286 and 100 cm^{-1}; *m/z* 91 (M^+, 69.8%), 60, 42 and 32 (100%). (Found: C, 13.09; H, 5.42; N, 46.06; S, 35.21. CH_5N_3S requires C,13.19; H, 5.49; N, 46.15; S, 35.16%).

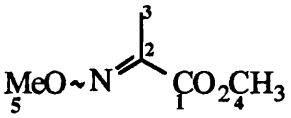
Methyl Pyruvate Oxime (133).



Methyl pyruvate (867 mg, 8.50 mmol) was reacted with hydroxylamine hydrochloride (607 mg, 1 equiv) according to general procedure [1] to give a white solid, 380 mg (38% yield), R_F 0.62 (50% $CHCl_3$/ether); υ_{max} (Nujol mull) 1730, 1460 and 1370 cm^{-1}; δ_H (200 MHz) 2.10 (3H, s, 3-H_3), 3.84 (3H, s, 4-H_3) and 9.90 (1H, br s, N-

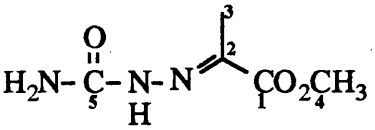
OH); δ_C (50 MHz) 10.5 (C-3), 52.7 (C-4), 149.2 (C-2) and 164.0 (C-1); *m / z* 117 (M^+, 29.6%), 85 (100%), 58 and 42. (Found: C, 40.92; H, 6.06; N, 11.82. $C_4H_7NO_3$ requires C, 41.05; H, 6.03; N, 11.96%).

Methyl Pyruvate Methyloxime (134).



Methyl pyruvate (0.90 ml, 9.98 mmol) was reacted with methoxylamine hydrochloride (4 g, 1 equiv.) according to general procedure [1] to give a yellow oil, 220 mg (17% yield) R_F 0.45 (EtOAc); υ_{max} (thin film) 2950, 1730, 1440 and 1330 cm^{-1}; δ_H (200 MHz) 1.99 (3H, s, 3-H_3), 3.80 and 4.00 (6H, 2 x s, 4- and 5-H_3); δ_C (50 MHz) 11.1 (C-3), 52.6 (C-4), 63.0 (C-5), 148.6 (C-2) and 164.0 (C-1); *m/z* 131 (M^+, 21%), 101, 84, 72, 59 and 42 (100%). (Found: M^+, 131.0585. $C_5H_9NO_3$ requires *M* ,131.0582).

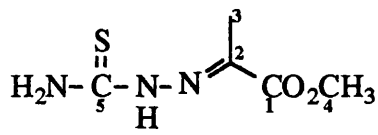
Methyl Pyruvate Semicarbazone (135).



Methyl pyruvate (0.56 ml, 6.17 mmol) was reacted with semicarbazide hydrochloride (850 mg, 1 equiv.) according to general procedure [1] to give a white solid, 110 mg (11% yield), mp 197-201

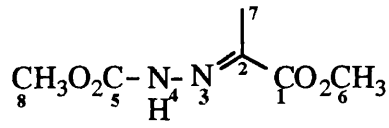
°C, R_F 0.19 (EtOAc); υ_{max} (Nujol mull) 1710, 1590, 1400 and 1380 c m^{-1}; δ_H (200 MHz) (D_6-DMSO) 1.74 (3H, s, 3-H_3), 2.67 (2H, br s, NH_2), 3.49 (3H, s, 4-H_3) and 5.83 (1H, br s, C-N<u>H</u>-N); δ_C (50 MHz) 11.3 (C-3), 51.5 (C-4), 135.9 (C-2), 155.9 (C-5) and 164.2 (C-1); *m/z* 159 (M^+, 3.6%), 128, 116, 100, 83, 57 and 44 (100%). (Found M^+, 159.0635. $C_5H_9N_3O_3$ requires *M*, 159.0644).

<u>Methyl Pyruvate Thiosemicarbazone (136).</u>



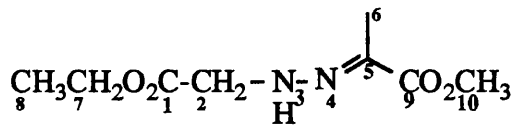
Methyl pyruvate (0.12 ml, 1.35 mmol) was reacted with thiosemicarbazide (150 mg, 1 equiv.) according to general procedure [1] to give a white solid, 141 mg (60% yield), R_F 0.48 (EtOAc); υ_{max} (KBr disc) 3520, 3440, 3240, 3160, 1725, 1630, 1610 and 1500 cm^{-1}; δ_H (200 MHz) (D_6-DMSO) 2.08 (3H, s, 3-H_3), 3.71 (3H, s, 4-H_3), 7.68 (1H, br s, NH) and 8.66 (2H, br s, NH_2); δ_C (50 MHz) 13.2 (C-3), 52.4 (C-4), 138.8 (C-2), 164.8 (C-1) and 179.9 (C-5); *m/z* 175 (M^+, 38.4%), 116 (100%), 75, 57 and 43.

Methyl Hydrazonocarboxylate (137) of Methyl Pyruvate.



Methyl hydrazinoacetate (200 mg, 2.22 mmol) was reacted with methyl pyruvate (0.2 ml, 1 equiv.) according to general procedure [1] to give a white solid, 270 mg (69% yield), R_F 0.30 (EtOAc); υ_{max} (KBr disc) 3240, 1750, 1730, 1710, 1615 and 1500 cm^{-1}; δ_H (200 MHz) 2.15 (3H, s, 7-H_3), 3.86 and 3.89 (6H, 2 x s, 6- and 8-H_3) and 8.96 (1H, br s, NH); δ_C (50 MHz) 11.5 (C-7), 52.9 and 53.7 (C-6 and 8), 140.9 (C-2), 154.3 (C-1) and 164.8 (C-5); *m / z* 174 (M^+1.1%), 83, 55 and 42. (Found: C, 41.19; H, 6.04; N, 15.97%. $C_6H_{10}N_2O_4$ requires, C, 41.38; H, 5.79; N, 16.08%).

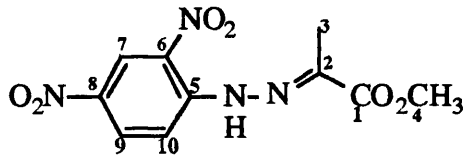
Ethyl Hydrazonoacetate (138) of Methyl Pyruvate.



To a solution of ethyl hydrazinoacetate hydrochloride (200 mg, 1.29 mmol) and triethylamine (131 mg, 1 equiv.) in dichloromethane (5 ml) under a nitrogen atmosphere was added methyl pyruvate (132 mg, 1 equiv.) at room temperature with stirring. After 12 h stirring the resultant yellow solution was washed with water (3 x 10 ml), dried (Na_2SO_4) and the solvent removed *in vacuo* to give an off-white solid. Purification was achieved on a silica gel column eluting

with ethyl acetate to give a white solid, 61 mg (23% yield), R_F 0.69 (EtoAc); υ_{max} (KBr disc) 3350, 2950, 1730, 1705, 1585 and 1450 cm^{-1}; δ_H (200 MHz) 1.28 (3H, t, *J* 7.2 Hz, 8-H_3), 2.03 (3H, s, 6-H_3), 3.82 (3H, s, 10-H_3), 4.22 (2H, q, *J* 7.2 Hz, 7-H_2), 4.23 (2H, m, 2-H_2) and 5.97 (1H, br s, NH); δ_C (50 MHz) 10.5 (C-6), 14.1 (C-8), 51.8 (C-10), 52.4 (C-2), 61.4 (C-7), 134.8 (C-5), 153.6 (C-9) and 170.8 (C-1); *m/z* 202 (M^+, 11.6%), 143, 129, 115, 97, 69 (100%) and 59. (Found: C, 47.79; H, 6.83; N, 13.93%. $C_8H_{14}N_2O_4$ requires C, 47.52; H, 6.93; N,13.86%).

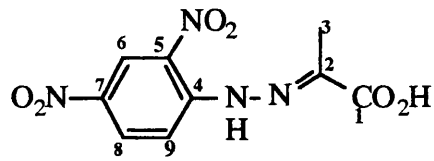
2,4-DNP Derivative (139) of Methyl Pyruvate.



Methyl pyruvate (0.46 ml, 5.05 mmol) was reacted with 2,4-dinitrophenylhydrazine (1.00 g, 1 equiv.) according to general procedure [2] to give a yellow solid, 520 mg (37% yield), mp 144-146 °C (EtOH), R_F 0.85 (50% $CHCl_3$ / ether); υ_{max} (KBr disc) 3306, 3080, 2360, 1730, 1620, 1590, 1550, 1510 and 1450 cm^{-1}; δ_H (200 MHz) 2.30 (3H, s, 3-H_3), 3.91 (3H, s, 4-H_3), 8.15 (1H, d, *J* 9.5 Hz, 10-H), 8.41 (1H, dd, *J* 9.5 Hz and *J* 2.5 Hz, 9-H), 9.15 (1H, d, *J* 2.5 Hz, 7-H) and 11.23 (1H, br s, NH); δ_C (50 MHz) 12.1 (C-3), 53.0 (C-4), 117.6 (C-7), 123.0 (C-9), 130.3 (C-10), 131.2 (C-5), 139.9 and 143.2 (C-6 and 8), 144.1 (C-2) and 164.4 (C-1); *m/z* 282 (M^+, 31.2%), 222, 181, 152,

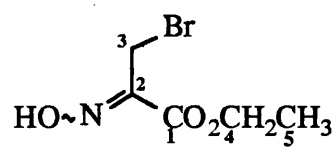
131, 72 and 43 (100%). (Found: M^+, 282.0599. $C_{10}H_{10}N_4O_6$ requires M, 282.0601).

2,4-DNP Derivative (140) of Pyruvic Acid.



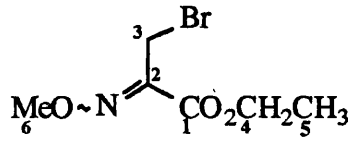
Pyruvic acid (0.35 ml, 5.05 mmol) was reacted with 2,4-dinitrophenylhydrazine (1.00 g, 1 equiv.) according to general procedure [2] to give an orange solid, 240 mg (18% yield), mp 203-206 °C; υ_{max} (KBr disc) 3350, 3290, 3100, 1725, 1615, 1590, 1515 and 1425 cm^{-1}; δ_H (200 MHz) (D_6-DMSO) 2.19 (3H, s, 3-H_3), 3.40 (1H, br s, NH), 8.09 (1H, d, J 9.5 Hz, 9-H), 8.48 (1H, dd, J 9.5 Hz and J 2.5 Hz, 8-H) and 8.87 (1H, d, J 2.5 Hz, 6-H); δ_C (50 MHz) 11.8 (C-3), 117.2 (C-6), 122.7 (C-8), 130.4 (C-9), 131.4 (C-4), 138.9 and 143.8 (C-5 and 7), 144.7 (C-2) and 165.1 (C-1); *m/z* 268 (M^+, 20.3%), 222, 152, 122, 91, 78 and 43 (100%). (Found: M^+, 268.0433. $C_9H_8N_4O_6$ requires M, 268.0443).

Ethyl Bromopyruvate Oxime (141).



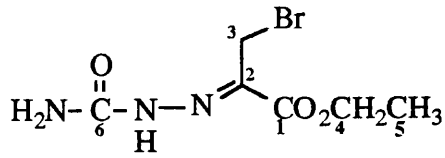
Ethyl bromopyruvate (0.64 ml, 5.13 mmol) was reacted with hydroxylamine hydrochloride (320 mg, 1 equiv.) according to general procedure [1] to give a white solid, 900 mg (83% yield), mp 77-79 °C, R_F 0.62 (50% $CHCl_3$ / ether); υ_{max} (KBr disc) 3290, 3060, 2980, 1725, 1470 and 1445 cm^{-1}; δ_H (200 MHz) 1.35 (3H, t, *J* 7.2 Hz, 5-H_3), 4.23 (2H, s, 3-H_2) and 4.35 (2H, q, *J* 7.2 Hz, 4-H_2); δ_C (50 MHz) 13.9 (C-5), 15.1 (C-3), 62.5 (C-4), 147.6 (C-2) and 161.6 (C-1); *m/z* 210 (M^+, 37.5%), 183, 165, 138, 129 (100%), 121, 93, 81 and 73. (Found: M^+, 210.9665; C, 28.61; H, 3.79; N, 6.42%. $C_5H_8BrNO_3$ requires *M*, 210.9668; C, 28.59; H, 3.84; N, 6.67%).

Ethyl Bromopyruvate Methyloxime (142).



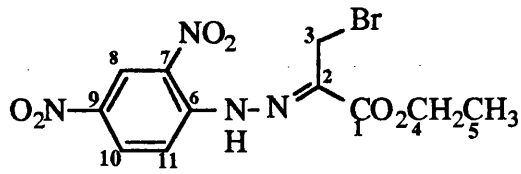
Ethyl bromopyruvate (0.64 ml, 5.13 mmol) was reacted with methoxylamine hydrochloride (520 mg, 1 equiv.) according to general procedure [1] to give a yellow oil, 1.06 g (92% yield), R_F 0.30 (EtOAc); υ_{max} (thin film) 3440, 2980, 2940, 1720, 1590 and 1460 cm^{-1}; δ_H (200 MHz) 1.29 (3H, t, *J* 7.3 Hz, 5-H_3), 4.09 (3H, s, 6-H_3), 4.11 (2H, s, 3-H_2) and 4.29 (2H, q, *J* 7.3 Hz, 4-H_2); δ_C (50 MHz) 13.9 (C-5), 15.8 (C-3), 62.1 (C-4), 64.0 (C-6), 147.1 (C-2) and 161.5 (C-1); *m/z* 224 (M^+, 50.4%), 179 (100%), 150, 144, 122, 101 and 72. (Found: M^+, 224.9835. $C_6H_{10}BrNO_3$ requires *M*, 224.9825).

Ethyl Bromopyruvate Semicarbazone (143).



Ethyl bromopyruvate (0.64 ml, 5.13 mmol) was reacted with semicarbazide hydrochloride (470 mg, 1 equiv.) according to general procedure [1] to give a white solid, 530 mg (41% yield), mp 142-143 °C, R_F 0.13 (30% MeOH / $CHCl_3$); υ_{max} (Nujol mull) 3500, 3340, 3160, 1700, 1610, 1585 and 1455 cm^{-1}; δ_H (200 MHz) 1.24 (3H, t, *J* 7.1 Hz, 5-H_3), 4.19 (2H, q, *J* 7.1 Hz, 4-H_2), 4.35 (2H, s, 3-H_2), 6.32 (2H, br s, NH_2) and 10.36 (1H, br s, NH); δ_C (50 MHz) 13.7 (C-5), 18.7 (C-3), 61.1 (C-4), 132.6 (C-2), 155.8 (C-6) and 162.4 (C-1); *m/z* 252 (M^+, 0.6%), 209, 178, 172, 129 (100%), 101 and 83. (Found: M^+, 252.9870; C, 28.22; H, 4.20; N, 16.25%. $C_6H_{10}BrN_3O_3$ requires *M*, 252.9887; C, 28.59; H, 4.00; N, 16.67%).

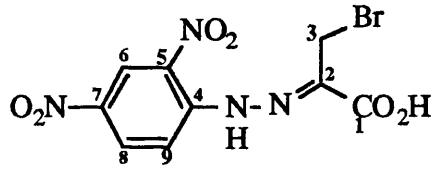
2,4-DNP Derivative (145) of Ethyl Bromopyruvate.



Ethyl bromopyruvate (0.16 ml, 1.28 mmol) was reacted with 2,4-dinitrophenylhydrazine (240 mg, 1 equiv.) according to general

procedure [2] to give a yellow solid, 235 mg (49% yield), mp 87-89 °C, R_F 0.66 (50% $CHCl_3$ / ether); υ_{max} ($CHCl_3$) 3260, 3100, 3015, 2990, 1710, 1615, 1585, 1525, 1505 and 1430 cm^{-1}; δ_H (200 MHz) 1.42 (3H, t, *J* 7.1 Hz, 5-H_3), 4.38 (2H, s, 3-H_2), 4.41 (2H, q, *J* 7.1 Hz, 4-H_2), 8.18 (1H, d, *J* 9.4 Hz, 11-H), 8.46 (1H, dd, *J* 9.4 Hz and *J* 2.5 Hz, 10-H), 9.16 (1H, d, *J* 2.5 Hz, 8-H) and 11.56 (1H, br s, NH); δ_C (50 MHz) 14.2 (C-5), 16.8 (C-3), 62.6 (C-4), 117.9 (C-8), 122.8 (C-10), 130.3 (C-11), 132.0 (C-6), 139.3 and 140.7 (C-7 and 9), 143.4 (C-2) and 162.1 (C-1); *m/z* 375 (M^+, 0.8%), 295, 195, 99 and 79.

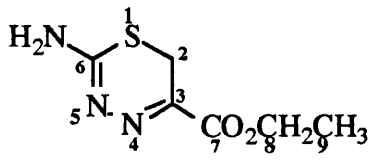
2,4-DNP Derivative (144) of Bromopyruvic Acid.



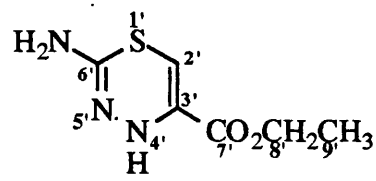
Bromopyruvic acid (210 mg, 1.26 mmol) was reacted with 2,4-dinitrophenylhydrazine (240 mg, 1 equiv.) according to general procedure [2] to give a yellow solid, 132 mg (38% yield), mp 115-117 °C, R_F 0.57 (50% $CHCl_3$ / ether); υ_{max} (KBr disc) 3440, 3190, 3110, 2940, 1720, 1690, 1620, 1590, 1500 and 1420 cm^{-1}; δ_H (200 MHz) (D_6-DMSO) 4.33 (2H, s, 3-H_2), 8.12 (1H, m, 9-H), 8.35 (1H, m, 8-H),and 9.08 (1H, m, 6-H); δ_C (50 MHz) 29.7 (C-3), 117.6 (C-6), 122.6 (C-8), 130.7 (C-9), 134.4 (C-4), 139.4 and 143.2 (C-5 and 7), 144.1 (C-2) and 165.1 (C-1); *m/z* 266, 180, 98, 78 and 64.

Attempted Preparation of Ethyl Bromopyruvate Thiosemicarbazone

Ethyl bromopyruvate (500 mg, 2.56 mmol) was reacted with thiosemicarbazide (233 mg, 1 equiv.) according to general procedure [1] to give a yellow oil, 310 mg (64% yield). TLC analysis (25% EtOAc / hexane) with iodine visualisation showed one main spot. Purification was achieved on a silica gel column eluting with 25% EtOAc in hexane. ^{1}H and ^{13}C NMR spectroscopic analysis showed that a 1:1 mixture of the cyclic products (147) and (148) had been formed.



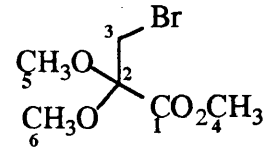
(147)



(148)

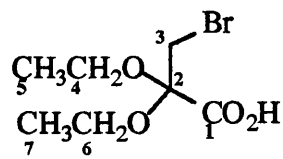
The spectroscopic data for these cyclic products is as follows; υ_{max} ($CHCl_3$) 3010, 2400, 1725, 1710, 1585 and 1510 cm^{-1}; δ_H (200 MHz) 1.30 and 1.41 (6H, 2 x t, *J* 7.14 Hz, 9- and 9'-H_3), 4.23 (2H, dq, *J* 7.14 Hz and *J* 1.00 Hz, 8'-H_2), 4.40 (2H, ddq, *J* 7.14 Hz and *J* 5.18 Hz, 8-H_2), 5.33 (1H, d, *J* 13.38 Hz, 2-H), 6.28 (1H, d, *J* 13.38 Hz, 2-H) and 6.97 (1H, s, 2'-H); δ_C (50 MHz) 14.1 (C-9 and -9'), 42.0 (C-2), 61.5 and 61.7 (C-8 and -8'), 113.3 (C-3'), 115.1 (C-2'), 132.2 (C-3), 148.2 (C-6), 159.1 (C-6'), 165.2 (C-7) and 170.7 (C-7').

Methyl 3-Bromo-2,2-dimethoxypropanoate (149).[166]



A solution of bromopyruvic acid hydrate (500 mg, 3.0 mmol), trimethylorthoformate (2 ml) and c. H_2SO_4 (0.03 ml) was stirred at room temperature for 18 h. The resultant mixture was diluted with dichloromethane (6 ml) and washed with water (2 x 10 ml) and brine solution (2 x 10 ml). The organic portion was dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give a clear oil, 132 mg (20% yield), R_F 0.78 (50% EtoAc / hexane); υ_{max} ($CHCl_3$) 3500, 3020, 2950, 1730 and 1430 cm^{-1}; δ_H (270 MHz) 3.38 (6H, s, 5- and 6-H_3), 3.63 (2H, s, 3-H_2) and 3.97 (3H, s, 4-H_3); *m/z* 168, 163, 120, 104 and 80 (100%).

3-Bromo-2,2-diethoxypropanoic Acid (150).[166]



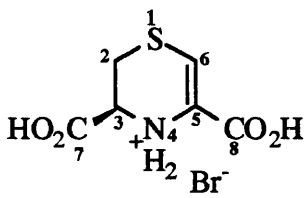
A solution of bromopyruvic acid hydrate (500 mg, 3.0 mmol), triethylorthoformate (2 ml) and c. H_2SO_4 (0.03 ml) was stirred at room temperature for 18 h. The resultant mixture was diluted with dichloromethane (6 ml) and washed with water (2 x 10 ml) and brine solution (2 x 10 ml). The organic solution was dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give a white solid, 499

mg (81% yield), R_F 0.33 (50% EtoAc / hexane); υ_{max} ($CHCl_3$) 3495, 3400, 3020, 2980, 1780, 1730 and 1440 cm^{-1}; δ_H (270 MHz) 1.28 (6H, 2 x t, *J* 6.7 Hz, 5 and 7-H_3), 3.60 (4H, dq, *J* 11.2 Hz and *J* 6.7 Hz, 4- and 6-H_2) and 3.63 (2H, s, 3-H_2); δ_C (50 MHz) 13.8 and 14.9 (C-5 and -7), 30.8 (C-3), 58.5 and 63.1 (C-4 and 6), 99.7 (C-2), 159.2 (C-1).

6.3. Experimental to Chapter [5].

Synthesis of Sulphur Analogues of L-2,3-DHDPA (23) and L-2,3,4,5-THDPA (24).

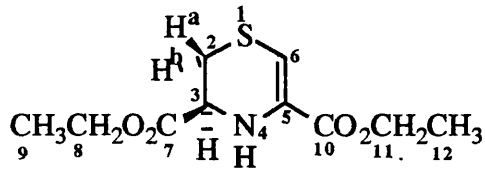
3,4-Dihydro-2H-1,4-thiazine-3,5-dicarboxylic Acid Hydrobromide (169).



To a solution of bromopyruvic acid (190 mg, 1.139 mmol) in water (1 ml) was added a solution of L-cysteine hydrochloride (200 mg, 1 equiv.) with stirring for 1 h. The resultant precipitate was filtered, washed with cold water and dried to give a white solid, 119 mg (55% yield); υ_{max} (KBr disc) 3343 (broad), 3090, 2361, 1691, 1626, 1601, 1468 and 1417 cm^{-1}; δ_H (200 MHz) (D_6-DMSO) 3.01 (2H, m, 2-H_2), 4.25 (1H, dd, 3-H), 5.17 (2H, br s, NH_2) and 5.94 (1H, s,

6-H); δ_C (50 MHz) 25.7 (C-2), 52.2 (C-3), 97.7 (C-6), 128.6 (C-5), 168.4 (C-7) and 171.7 (C-8); *m/z* 189 (M^+, 35.6%), 145, 126, 100, 54 and 44 (100%). (Found: M^+, 189.0094; C, 37.93; H, 3.70; N, 7.33; $C_6H_7NO_4S$ requires *M*, 189.0095; C, 38.09; H, 3.70; N, 7.41%).

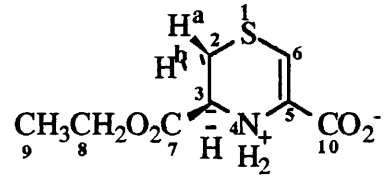
Diethyl (R)-3,4-Dihydro-2H-1,4-thiazine-3,5-dicarboxylate (175).



To a solution of L-cysteine ethyl ester hydrochloride (200 mg, 1.077 mmol) and triethylamine (0.16 ml, 2 equiv.) in dichloromethane (5 ml) under a nitrogen atmosphere was added dropwise a solution of ethyl bromopyruvate (0.135 ml, 1 equiv.) in dichloromethane (5 ml) with stirring. The mixture was stirred at room temperature for 12 h, washed with water (2 x 10 ml) and the organic portion was dried (Na_2SO_4). The solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 20% ethyl acetate in hexane to give a yellow oil, 145 mg (90% yield), R_F 0.36 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3405, 2980, 1740, 1700, 1605, 1475 and 1465 cm^{-1}; δ_H (200 MHz) 1.26 and 1.27 (6H, 2 x t, *J* 7.1 Hz and *J* 7.2 Hz, 9- and 12-H_3), 2.97 (1H, ddd, *J* 12.1 Hz, *J* 6.7 Hz and *J* 0.7 Hz, 2a-H), 3.18 (1H, ddd, *J* 12.1 Hz, *J* 2.9 Hz and *J* 2.9 Hz, 2b-H), 4.30 (5H, 2 x q + m, *J* 7.1 Hz and *J* 7.2 Hz, 8- and 11-H_2) and 6.14 (1H, t, *J* 0.7 Hz, 6-H); δ_C (50 MHz) 14.0

and 14.1 (C-9 and -12), 26.8 (C-2), 53.0 (C-3), 61.2 and 61.8 (C-8 and -11), 101.2 (C-6), 128.0 (C-5), 162.3 (C-10) and 170.2 (C-7); *m / z* 245 (M^+, 43.6%), 201, 172 (100%), 154, 139, 101, 98 and 73. (Found: M^+, 245.0731. $C_{10}H_{15}NO_4S$ requires *M*, 245.0722).

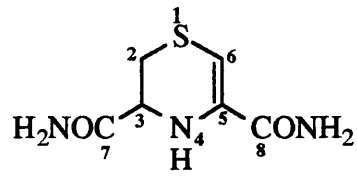
3-Carboethoxy-3,4-dihydro-2H-1,4-thiazine-5-carboxylic Acid (176).



To a solution of L-cysteine ethyl ester hydrochloride (100 mg, 0.54 mmol) and bromopyruvic acid (90 mg, 1 equiv.) in anhydrous chloroform (6 ml) under a nitrogen atmosphere was added triethylamine (0.21 ml, 2 equiv.) with stirring and cooling to 0 °C. The mixture was continuously stirred for 16 h then washed with water (2 x 5 ml) and 2M hydrochloric acid (2 x 5 ml). The organic portion was extracted with 5% sodium bicarbonate solution (10 ml). The aqueous portion was carefully acidified to pH 2 with c. HCl then extracted with chloroform (3 x 10 ml). The combined organic extracts were dried ($MgSO_4$) and removal of the solvent *in vacuo* gave a yellow oil, 20 mg (20% yield), R_F 0.32 (ether); υ_{max} ($CHCl_3$) 3400, 3020, 2410, 1740, 1690 and 1420 cm^{-1}; δ_H (200 MHz) 1.29 (3H, t, *J* 7.2 Hz, 8-H_3), 3.01 (1H, dd, *J* 12.4 Hz and *J* 6.7 Hz, 2a-H), 3.24 (1H, dd, *J* 12.4 Hz and *J* 2.3 Hz, 2b-H), 4.25 (3H, q + m, *J* 7.2 Hz, 7-H_2 and 3-H) and 6.36 (1H, s, 6-H); δ_C (50 MHz) 14.1 (C-9), 26.9 (C-2),

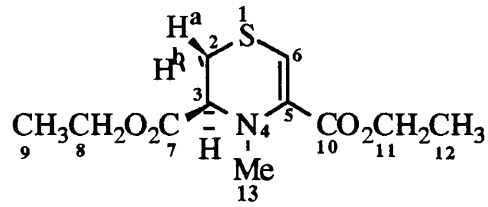
52.9 (C-3), 62.0 (C-8), 104.7 (C-6), 127.2 (C-5), 166.6 (C-10) and 170.1 (C-7); *m/z* 217 (M^+, 37.9%), 189, 172, 144, 126 (100%), 98 and 45.

3,4-Dihydro-2H-1,4-thiazine-3,5-diamide (178).



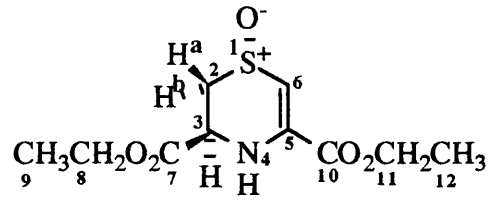
A solution of (175) (250 mg, 1.02 mmol) in methanolic ammonia (10 ml) was stirred at room temperature for 16 h. The resulting orange precipitate was filtered to give an orange solid. Recrystallisation from methanol gave a white solid, 71 mg (37% yield); υ_{max} (KBr disc) 3400, 3340, 2950, 1690, 1650, 1595, 1485 and 1435 cm^{-1}; δ_H (200 MHz) 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) 24.8(C-2), 52.4 (C-3), 103.2 (C-6), 126.8 (C-5), 162.6 and 163.1 (C-7 and -8); *m/z* 172, 158, 142, 126 (100%), 98, 72 and 54.

Diethyl 3,4-Dihydro-4-methyl-2H-1,4-thiazine-3,5-dicarboxylate (179).



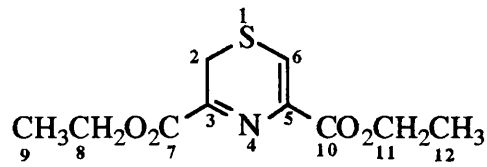
Methyl iodide (0.76 ml, 10 equiv.) was added to a solution of (175) (300 mg, 1.22 mmol) in DMF (3 ml) with stirring. The mixture was heated at reflux for 1 h then cooled to room temperature, diluted with water (3 ml) and the solvents were removed *in vacuo* to give an oily residue. Traces of DMF were removed by azeotroping the mixture with further volumes of water. The resulting oil was dissolved in ethyl acetate (5 ml) and washed with aq. ammonia solution. The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 50% ethyl acetate in hexane to give a yellow oil, 100 mg (31% yield), R_F 0.45 (EtOAc); υ_{max} ($CHCl_3$) 3000, 1750, 1710, 1580 and 1465 cm^{-1}; δ_H (200 MHz) 1.23 and 1.31 (6H, 2 x s, *J* 7.1 Hz, 9- and 12-H_3), 2.70 (3H, s, 13-H_3), 2.93 (1H, dd, *J* 12.7 Hz and *J* 3.3 Hz, 2a-H), 3.19 (1H, ddd, *J* 12.7 Hz, *J* 3.7 Hz and *J* 1.7 Hz, 2b-H), 4.19 (5H, m, 8- and 11-H_2 and 3-H) and 6.86 (1H, d, *J* 1.7 Hz, 6-H); δ_C (50 MHz) 14.1 and 14.3 (C-9 and -12), 21.8 (C-2), 42.3 (C-13), 60.1 (C-3), 60.9 and 61.5 (C-8 and -11), 116.6 (C-6), 132.4 (C-5), 163.3 (C-10) and 168.6 (C-7); *m/z* 259 (M^+, 21.6%), 186 (100%), 158, 113, 86 and 42. (Found: M^+, 259.0865. $C_{11}H_{17}NO_4S$ requires *M*, 259.0878).

Diethyl *trans*-3,4-Dihydro-1-oxo-2H-1,4-thiazine-3,5-dicarboxylate(180).



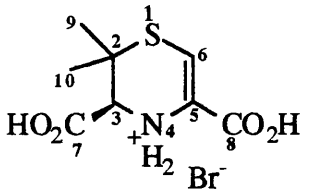
To a solution of (175) (490 mg, 2 mmol) in dichloromethane (10 ml) at -78 °C under a nitrogen atmosphere was added dropwise a solution of *m*-chloroperbenzoic acid (414 mg, 2.4 mmol) in dichloromethane (2 ml). The mixture was stirred at -78 °C for 20 min then warmed to room temperature and stirred for a further 30 min. The resultant yellow solution was washed with 5% sodium bicarbonate solution (2 x 5 ml), water (2 x 5 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give an amber syrup. Purification was achieved on a silica gel column eluting with 50% chloroform in methanol to give an amber syrup, 400 mg (77% yield), R_F 0.22 (50% $CHCl_3$ / MeOH), $[\alpha]_D = 0$; υ_{max} ($CHCl_3$) 3405, 3000, 1740, 1595, 1200 and 1025 cm^{-1}; δ_H (200 MHz) 1.32 (6H, 2 x t, 9- and 12-H_3), 2.25 (1H, dd, *J* 13.3 Hz, 2b-H), 3.41 (1H, br d, *J* 13.5 Hz, 2a-H), 4.25 (5H, 2 x q + m, 8- and 11-H_2 and 3-H), 6.24 (1H, br s, 6-H) and 6.38 (1H, br s, NH); δ_C (50 MHz) 14.0 and 14.2 (C-9 and -12), 44.2 (C-3), 44.3 (C-2), 62.8 and 62.9 (C-8 and -11), 97.4 (C-6), 137.1 (C-5), 161.9 (C-10) and 169.8 (C-7); *m/z* 261 (M^+, 1.0%), 245, 143, 70, 45 and 29 (100%).

Diethyl 2H-1,4-Thiazine-3,5-dicarboxylate (182).



To a solution of 2,3-dichloro-5,6-dicyanobenzoquinone (120 mg, 0.53 mmol) in dichloromethane (5 ml) was added a solution of (175) (115 mg, 0.47 mmol) in dichloromethane (2 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 15 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a silica gel column eluting with 50% chloroform in ether to give 102 mg (79% yield), R_F 0.46 (50% $CHCl_3$ / ether); υ_{max} (KBr disc) 3420, 2960, 1735, 1710 and 1465 cm^{-1}; δ_H (200 MHz) 1.35 (6H, 2 x t, 9- and 12-H_3), 3.33 (2H, d, *J* 1.2 Hz, 2-H_2), 4.30 (4H, 2 x q, 8- and 11-H_2) and 7.59 (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 63.4 (C-8 and -11), 128.2 (C-6), 137.2 and 137.9 (C-3 and -5), 167.5 and 168.8 (C-7 and 10); *m / z* 243 (M^+, 47.0%), 215, 169, 141, 97, 45 and 29 (100%).

3,4-Dihydro-2,2-dimethyl-2H-1,4-thiazine-3,5-dicarboxylic Acid Hydrobromide (186) and Dimethyl 3,4-Dihydro-2,2-dimethyl-2H-1,4-thiazine-3,5-dicarboxylate (187).



(186)

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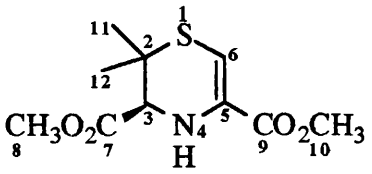
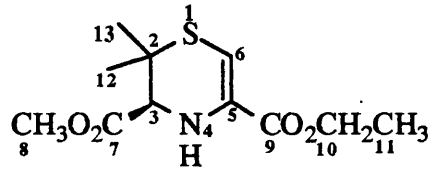


(187)

A solution of DL-penicillamine (500 mg, 3.35 mmol) in glacial acetic acid (2 ml) was added to a solution of bromopyruvic acid (559 mg, 1 equiv.) in glacial acetic acid (1 ml) with stirring. The mixture was stirred for 16 h at room temperature. The solvent was removed *in vacuo* to give a brown solid (186); δ_H (90 MHz) (D_6-DMSO) 1.20 and 1.37 (6H, 2 x s , 9 and 10-H_3), 3.70 (1H, s, 3-H), 5.98 (1H, s, 6-H), and 7.60 (2H, br s, NH_2); A solution of the brown solid in ethyl acetate (20 ml) was cooled to 0 °C then saturated with ethereal diazomethane and stirred at room temperature for 18 h. The resultant mixture was washed with water (2 x 20 ml), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a brown oil. Purification was achieved on a neutral alumina column eluting with 25% ethyl acetate in hexane to give an amber oil (187) 505 mg (61% yield), R_F 0.56 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3400, 3010, 2950, 1735, 1700, 1605 and 1435 cm^{-1}; δ_H (200 MHz) 1.23 and 1.40 (6H, 2 x s, 11- and 12-H_3), 3.73 (6H, s, 8- and 10-H_3), 3.81 (1H, d, *J* 3.8 Hz, 3-H), 4.75 (1H, br s, NH) and 6.20 (1H, s, 6-H); δ_C (50 MHz) 24.8 and 27.5 (C-11 and -12), 40.4 (C-2), 52.0 and 52.1 (C-8 and -10), 62.5 (C-

3), 102.3 (C-6), 125.8 (C-5), 162.6 (C-9) and 170.2 (C-7); *m/z* 245 (M^+, 45.0%), 186, 154 (100%), 126, 112 and 82. (Found: M^+, 245.0723; C, 49.02; H, 6.39; N, 5.50. $C_{10}H_{15}NO_4S$ requires *M*, 245.0721; C, 48.79; H, 6.12; N, 5.71%).

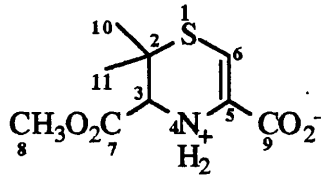
Ethyl 3-Carbomethoxy-3,4-dihydro-2,2-dimethyl-2H-1,4-thiazine-5-carboxylate (188).



To a solution of DL-penicillamine methyl ester hydrochloride (200 mg, 1.0 mmol) and triethylamine (0.28 ml, 2 equiv.) in dichloromethane (5 ml) under a nitrogen atmosphere was added dropwise a solution of ethyl bromopyruvate (0.13 ml, 1 equiv.) in dichloromethane (5 ml) with stirring. The mixture was stirred for 12 h at room temperature then washed with water (2 x 10 ml). The organic portion was dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a neutral alumina column eluting with 30% ethyl acetate in pet. ether (40-60 °C) to give a yellow oil, 234 mg (90% yield), R_F 0.61 (EtOAc); υ_{max} ($CHCl_3$) 3390, 3005, 2965, 1730, 1690, 1500, 1450 and 1425 cm^{-1}; δ_H (200 MHz) 1.22 (3H, s, 12- or 13-H_3), 1.25 (3H, s, *J* 7.1 Hz, 11-H_3), 1.39 (3H, s, 12- or 13-H_3), 3.71 (3H, s, 8-H_3), 3.80 (1H, d, *J* 3.7 Hz, 3-H), 4.18 (2H, q, *J* 7.1 Hz, 10-H_2), 4.75 (1H, br s, NH) and 6.19 (1H, s, 6-

H); δ_C (50 MHz) 14.1 (C-11), 24.7 and 27.5 (C-12 and -13), 40.3 (C-2), 52.1 (C-3), 61.0 (C-10), 62.5 (C-8), 101.8 (C-6), 126.0 (C-5), 162.1 (C-9) and 170.2 (C-7); *m/z* 259 (M^+, 44.0%), 200, 186, 154 (100%), 126, 112, 82 and 45. (Found: M^+, 259.0871. $C_{11}H_{17}NO_4S$ requires M, 259.0878).

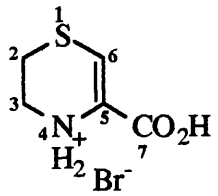
Methyl 3,4-Dihydro-2,2-dimethyl-1,4-thiazine-5-carboxylic acid-3-carboxylate (189).



To a solution of DL-penicillamine methyl ester hydrochloride (200 mg, 1.00 mmol) and bromopyruvic acid (167 mg, 1 equiv.) in anhydrous chloroform (10 ml) under a nitrogen atmosphere was added triethylamine (0.418 ml, 2 equiv.) with stirring and cooling to 0 °C. The mixture was continuously stirred for 16 h then washed with water (2 x 5 ml) and 2M HCl (2 x 10 ml). The organic portion was extracted with 5% sodium bicarbonate solution (2 x 10 ml) then carefully acidified to pH 2 with conc. HCl. The aqueous portion was extracted with chloroform (3 x 15 ml), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a yellow oil, 55 mg (24% yield); υ_{max} ($CHCl_3$) 3405, 3020, 2960, 1740, 1680, 1600, 1440 and 1425 cm^{-1}; δ_H (200 MHz) 1.29 and 1.46 (6H, 2s, 10 and 11-H_3), 3.78 (3H, s, 8-H_3), 3.87 (1H, d, *J* 7.4 Hz, 3-H) and 6.45 (1H, s, 6-H); δ_C (50 MHz) 25.0 and 27.7 (C-10 and 11), 40.9 (C-2), 52.9 (C-3), 62.5 (C-8), 105.7 (C-6),

129.3 (C-5), 148.5 (C-9) and 170.2 (C-7); *m/z* 231 (M^+, 30.6%), 186, 172, 154 (100%), 126 and 82.

3,4-Dihydro-2H-1,4-thiazine-5-carboxylic Acid Hydrobromide (190) and Methyl 3,4-Dihydro-2H-1,4-thiazine-5-carboxylate (191).



(190)

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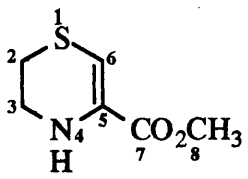
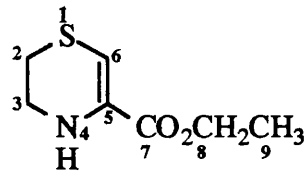


(191)

A solution of 2-aminoethanethiol hydrochloride (1.23 g, 0.011 mol) in glacial acetic acid (4 ml) was added to a solution of bromopyruvic acid hydrate (1.80 g, 1 equiv.) in glacial acetic acid (4 ml) at room temperature with stirring for 1 h. The resulting white precipitate was filtered, washed with ether and dried to give an off-white solid (190); δ_H (90 MHz) (D_6-DMSO) 2.45 (2H, m, 2-H_2), 2.93 (2H, m, 3-H_2), 6.25 (1H, s, 6-H) and 6.40 (2H, br s, NH_2); A solution of the white solid in ethyl acetate (30 ml) was cooled to 0 °C then saturated with ethereal diazomethane and stirred at room temperature for 16 h. The resultant mixture was washed with water (2 x 20 ml), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a yellow oil. Purification was achieved on a neutral alumina column eluting with 20% ethyl acetate in hexane to give 1.22 g of a yellow oil (191), (70% yield), R_F 0.64 (20% EtOAc / hexane); υ_{max} ($CHCl_3$) 3415, 3005, 2995, 2910, 1720, 1700 and 1625 cm^{-1}; δ_H (200 MHz) 2.86 (2H, m, 2-H_2), 3.62 (2H, m, 3-H_2), 3.80 (3H, s, 8-H_3), 4.98

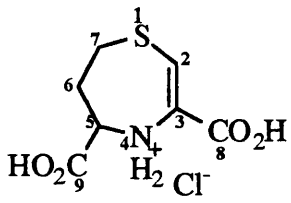
(1H, br s, NH) and 6.05 (1H, s, 6-H); δ_C (50 MHz) 27.1 (C-2), 41.4 (C-3), 51-3 (C-8), 102.9 (C-6), 128.5 (C-5) and 159.8 (C-7); *m/z* 159 (M^+, 23.5%), 144, 100, 72, 61 and 44 (100%).

Ethyl 3,4-Dihydro-2H-1,4-thiazine-5-carboxylate (192).



Ethyl bromopyruvate (0.552 ml, 1 equiv.) was added to a solution of 2-aminoethanethiol hydrochloride (500 mg, 4.40 mmol) and triethylamine (1.22 ml, 2 equiv.) in dichloromethane (15 ml) under a nitrogen atmosphere at room temperature with continuous stirring for 1 h. The resultant brown mixture was washed with water (3 x 15 ml), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a brown residue. Purification was achieved on a neutral alumina column eluting with 15% ethyl acetate in hexane to give a yellow oil, 200 mg (26% yield), R_F 0.73 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3420, 3020, 2980, 2920, 1715, 1695 and 1600 cm^{-1}; δ_H (200 MHz) 1.28 (3H, t, *J* 7.1 Hz, 9-H_3), 2.97 (2H, m, 2-H_2), 3.53 (2H, m, 3-H_2), 4.20 (2H, q, *J* 7.1 Hz, 8-H_2) and 6.17 (1H, s, 6-H); δ_C (50 MHz) 14.2 (C-9), 25.8 (C-2), 41.2 (C-3), 61.0 (C-8), 101.9 (C-6), 129.1 (C-5) and 162.7 (C-7); *m/z* 173 (M^+, 44.7%), 145, 132, 99, 72, 61, 45 and 29 (100%).

4,5,6,7-Tetrahydro-1,4-thiazepine-3,5-dicarboxylic Acid Hydrochloride (193) and Dimethyl 4,5,6,7-Tetrahydro-1,4-thiazepine-3,5-dicarboxylate (194).



(193)

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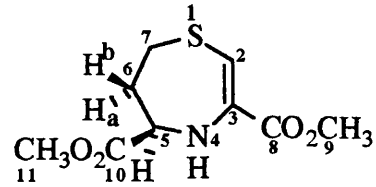
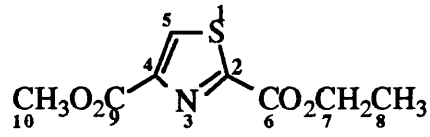


(194)

A solution of DL-homocysteine (1.19 g, 8.80 mmol) in water (2 ml) and 2M HCl (1 ml) was added to a solution of bromopyruvic acid (1.63 g, 1 equiv.) in water (3 ml) with continued stirring at room temperature for 1 h. The solvent was removed *in vacuo* to give a yellow solid (193); δ_H (90 MHz) (D_6-DMSO) 2.15 (2H, m, 6-H_2), 2.75 (2H, m, 7-H_2), 3.95 (1H, m, 5-H), 6.27 (1H, s, 2-H) and 8.10 (2H, br s, NH_2); A solution of the yellow solid in ethyl acetate (20 ml) was cooled to 0° C then saturated with ethereal diazomethane and stirred at room temperature for 18 h. The resultant mixture was washed with water (2 x 20 ml), dried (Na_2SO_4) and the solvent was removed *in vacuo* to give a yellow residue. Purification was achieved on a neutral alumina column eluting with 25% ethyl acetate in hexane to give a yellow oil (194), 425 mg (21% yield), R_F 0.54 (25% EtOAc / hexane); υ_{max} ($CHCl_3$) 3360, 3020, 2950, 1735, 1705, 1595 and 1435 c m^{-1}; δ_H (200 MHz) 2.07 (1H, m, 6b-H), 2.40 (2H, m, 7-H_2), 2.72 (1H, dddd, *J* 14.2 Hz, *J* 5.5 Hz, *J* 3.0 Hz and *J* 1.2 Hz, 6a-H), 3.75 and 3.76 (6H, 2 x s, 9- and 11-H_3), 4.77 (1H, ddd, *J* 14.2 Hz, *J* 5.5 Hz and *J* 1.2 Hz, 5-H), 4.97 (1H, br s, NH) and 6.09 (1H, s, 2-H); δ_C (50 MHz) 29.9 (C-6), 33.1 (C-7), 52.5 and 52.6 (C-9 and -11), 57.7 (C-5), 107.2 (C-

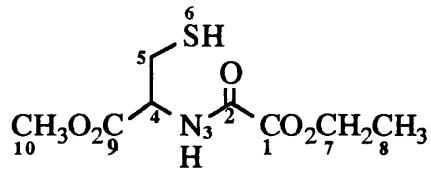
2), 135.1 (C-3), 163.7 (C-8) and 172.7 (C-10); *m/z* 231 (M^+, 23.4%), 172, 140, 112 (100%), 85 and 59. (Found: M^+, 231.0566. $C_9H_{13}NO_4S$ requires *M*, 231.0567).

Ethyl 4-Carbomethoxy-1,3-thiazole-2-carboxylate (195).



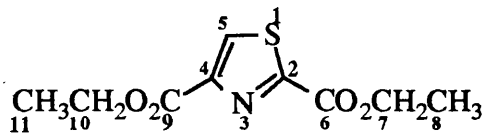
A solution of (196) (7.26 mg, 3.09 mmol) and phosphorus pentasulphide (1.37 g, 1 equiv.) in pyridine (20 ml) was heated to reflux with stirring for 4 h. Pyridine was removed *in vacuo* and the resultant residue was partitioned between water (100 ml) and ethyl acetate (40 ml). The organic portion was separated and the aqueous layer was extracted with ethyl acetate (2 x 20 ml). The combined organic layers were washed with water (3 x 20 ml), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give a dark yellow residue. Purification was achieved on a silica gel column eluting with 5% ethyl acetate in hexane to give a yellow oil, 265 mg (40% yield), R_F 0.60 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3120, 3000, 2960, 1720 and 1465 cm^{-1}; δ_H (200 MHz) 1.42 (3H, t, *J* 7.1 Hz, 8-H_3), 3.95 (3H, s, 10-H_3), 4.48 (2H, q, *J* 7.1 Hz, 7-H_2) and 8.40 (1H, s, 5-H); δ_C (50 MHz) 14.2 (C-8), 52.6 (C-10), 63.0 (C-7), 132.4 (C-5), 148.5 (C-4), 159.3 (C-2), 159.5 and 161.2 (C-6 and 9); *m/z* 215 (M^+, 9.2%), 184, 170, 156, 143 (100%), 111, 84 and 57.

Ethyl 3-Aza-4-carbomethoxy-5-mercapto-2-oxopentanoate (196).



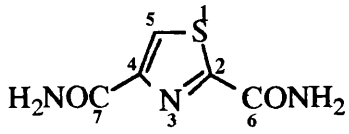
Ethyl oxalyl chloride (0.651 ml, 1 equiv.) was added to a solution of L-cysteine methyl ester hydrochloride (1g, 5.83 mmol) and triethylamine (1.62 ml, 2 equiv) in dichloromethane (20 ml) at room temperature with stirring. The resultant white suspension was stirred for 3 h, washed with water (3 x 20 ml), dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* to give an oily residue. Purification was achieved on a silica gel column eluting with 25% ethyl acetate in hexane to give a clear oil, 1.29 g (94% yield), R_F 0.48 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3395, 3020, 1740, 1705 and 1515 cm^{-1}; δ_H (200 MHz) 1.40 (3H, t, *J* 7.1 Hz, 8-H_3), 3.07 (2H, ddd, *J* 9.2 Hz, *J* 4.3 Hz and *J* 1.6 Hz, 5-H_2), 3.83 (3H, s, 10-H_3), 4.39 (2H, q, *J* 7.1 Hz, 7-H_2), 4.89 (1H, m, 4-H) and 7.94 (1H, br s, NH); δ_C (50 MHz) 13.8 (C-8), 26.2 (C-5), 52.9 (C-10), 54.0 (C-4), 63.3 (C-7), 159.7 (C-1), 169.3 (C-9) and 184.6 (C-2); *m/z* 235 (M^+, 2.5%), 234, 176, 162, 134, 102, 86 and 59.

Diethyl 1,3-Thiazole-2,4-dicarboxylate (200).



A solution of ethyl thioxamate (1.12 g, 8.41 mmol) and ethyl bromopyruvate (3.17 ml, 3 equiv.) in ethanol (20 ml) was heated at reflux for 2 h. The solvent was removed *in vacuo* and ether (20 ml) was added to precipitate triethylamine hydrobromide. Filtration and removal of the solvent *in vacuo* gave a yellow oily residue. Purification was achieved on a neutral alumina column eluting with 25% ethyl acetate in hexane to give a yellow oil, 1.80 g, (92% yield), R_F 0.39 (50% EtOAc / hexane); υ_{max} ($CHCl_3$) 3120, 3010, 1715, 1485 and 1460 cm^{-1}; δ_H (200 MHz) 1.35 and 1.38 (6H, 2 x t, *J* 7.1 Hz, 8- and 11-H_3), 4.38 and 4.43 (4H, 2 x q, *J* 7.1 Hz, 7- and 10-H_2) and 8.36 (1H, s, 5-H); δ_C (50 MHz) 14.0 and 14.1 (C-8 and -11), 61.7 and 62.9 (C-7 and -10), 132.1 (C-5), 148.8 (C-4), 159.1 (C-2), 159.4 and 160.6 (C-6 and 9); *m/z* 229 (M^+, 5.0%), 200, 184, 157, 156, 129,111 and 83.

1,3-Thiazole-2,4-diamide (199).



A solution of (200) (220 mg, 0.96 mmol) in methanol (15 ml) saturated with ammonia was stirred at room temperature for 18 h. The resultant precipitate was filtered, washed with cold water and dried *in vacuo* to give a white solid, 124 mg (90% yield); υ_{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) and 8.46 (1H, s, 5-H); δ_C (50 MHz) 128.9 (C-5), 150.4 (C-4), 160.5 (C-2), 161.9 and 163.1 (C-6 and 7); *m/z* 171(M^+, 100%), 154, 128, 112, 84 and 70.

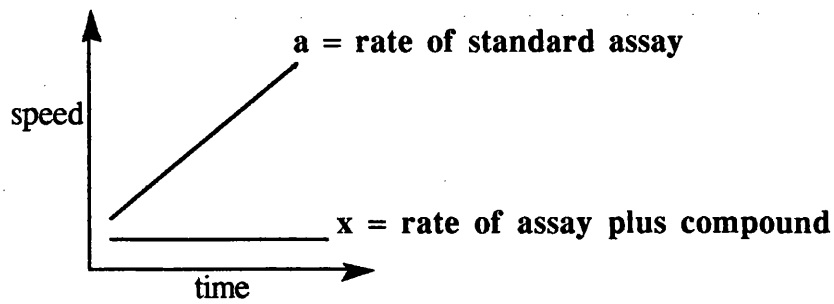
6.4. Test Data for Chapters [3], [4] & [5].

Borthwick[121] has set up a standard assay screen for DHDP Synthase by monitoring the rate of formation of dipicolinic acid (36) at 270 nm. The product of the reaction, *in vivo*, is L-2,3-DHDPA (23) which is unstable and could not be the product absorbing at 270 nm. L-2,3-DHDPA (23) is oxidised in air to DPA (36) which absorbs at 270 nm **(Scheme 9)**.

The standard assay consisted of 100 mM imidazole buffer, 1 mM L-aspartic acid-β-semialdehyde (89a), 1 mM pyruvate (22) and 16 units of DHDP Synthase. Three concentrations of compound were studied, 1 mM, 0.5 mM and 0.1 mM, unless other dilutions were required. Each result shown is an average over three assay runs.

The level of inhibition was measured by a percentage of the standard rate as shown in the following equation and graph :

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



Significant inhibition was taken to be about 10% inhibition at 0.5 mM of the compound being tested.

Test Data for Chapter [3].

Compound No. or Name	Experimental Page No.	Concentration of Inhibitor			
		1 mM	0.5 mM	0.25 mM	0.1 mM
DL-Allylglycine		14%	0	-	-
(86)	158	0	-	-	-
(87)	159	0	-	-	-
(96)	167	11%	5%	-	-
(93)	168		14%	0	-
(104)	169	11%	0	-	-
(98)	170	7%	0	-	-
(107)	170	0	-	-	-
(121)	184	0	-	-	-

Test Data for Chapter [4].

Compound No. or Name	Experimental Page No.	Concentration of Inhibitor			
		1 mM	0.5 mM	0.25 mM	0.1 mM
(133)	188	0	-	-	-
(134)	189	8%	0	-	-
(135)	189	100%	-	-	8%
(136)	190	100%	96%	-	8%
(137)	191	100%	100%	-	0
(138)	191	100%	100%	-	62%
(140)	193	100%	92%	-	0
(141)	194	3%	-	-	-
(142)	194	100%	-	-	14%
(143)	195	100%	100%	-	51%
(145)	196	65%	50%	-	38%
(144)	196	100%	78%	-	0
Phenylpyruvate		0	-	-	-
Mercaptopyruvate		0	-	-	-
Bromopyruvate		2	0	-	-

Test Data for Chapter [5].

Compound No. or Name	Experimental Page No.	Concentration of Inhibitor			
		1 mM	0.5 mM	0.25 mM	0.1 mM
(169)	199	100%	88%	-	0
(175)	200	87%	26%	-	13%
(176)	201	63%	37%	-	8%
(180)	204	78%	0	-	-
(182)	205	94%	31%	-	0
(188)	207	100%	79%	-	13%
(189)	208	100%	97%	-	19%
(192)	210	67%	25%	-	8%
(195)	212	100%	86%	-	21%
(200)	215	100%	100%	100%	15%

References

(1). J.P. Greenstein and M. Winitz, *'Chemistry of the amino acids'*, 1961, (Vols 1-3), John Wiley, New York.

(2). H.B. Vickery, *Adv. Protein, Chem.*, 1972, **2 6**, 82.

(3). A. Meister, *'Biochemistry of the Amino Acids'*, 1965, (Vol.1) 2nd edn, Academic Press, New York.

(4). A. Kleeman, W. Leuchtenberger, B. Hoppe and H. Tanner, Amino acids. In *'Ulmann's Encyclopedia of Industrial Chemistry'*, 1985, Vol. A2, 57-97.

(5). T. Tunaka, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, 1986, **3 4**, 650.

(6). D.J. Robins, *Chem. Soc. Rev.*, 1989, **1 8**, 375.

(7). G. Bellavita, *Farm. Sci. Tec.*, 1948, **3**, 424.

(8). D'aragona, *Bull. Chim. Farm.*, 1948, **8 7**, 240.

(9). T. Nakano, A.C. Spinelli, and A.M. Mendez, *J. Org. Chem.*, 1974, **3 9**, 3584.

(10). D.A. Marion, *Can. J. Chem.*, 1953, **3 1**, 181.

(11). J. Rana and D.J. Robins, *J. Chem. Soc. Perkin Trans. 1*, 1986, 1133.

(12). W.M. Golebiewski and I.D. Spenser, *J. Am. Chem. Soc.*, 1984, **1 0 6**, 7925.

(13). H.C. Rouse and W.R. Schlesinger,*Virology*, 1967, **3 3**, 513.

(14). G.R. Dubes, K.B. Moyer, B.L. Helliburton and R.K. Van de Bogart, *Acta. Virol.*, Prague, 1969, **1 3**, 8.

(15). S.M. Chang and S.C. Sung, *Bull. Inst. Chem. Acad. Sinica*, 1968, **1 5**, 1019.

(16). G. Pontecorvo, J.A. Roper, L.M. Hemmona, R.A. McDonald and A.W.J. Bufton, *Advanc. Genet.*, 1953, **5**, 141.

(17). B. Lerch and H. Stegeman, *Landw. Forsch.*, 1970, **2 3**, 181.

(18). L.G. Nickell and A. Maretzky, *Physiol. Plant* , 1969, **2 2**, 117.

(19). K. Furuhoshi and M. Yarazawa, *Plant Cell Physiol.*, 1970, **1 1**, 559.

(20). J.D. Jones, *J. Nutr.*, 1964, **8 4**, 313.

(21). B.L. O'Dell and F.J. Savage, *J. Nutr.*, 1966, **9 0**, 364.

(22). K.N. Boorman and H. Fisher, *Brit. Poultry Sci.*, 1966, **7**, 39.

(23). W.B. Roess and A.G. DeBusk, *J. Gen. Microbiol.*, 1968, **5 2**, 421.

(24). J.D. Jones, S.J. Petersburg and P.C. Burnett, *J. Nutr.*, 1967, **9 3**, 103.

(25). E. Tyihak and B. Szende, *Hung. Pat.*, 1967-1970, **1 5 8**, 979.

(26). B. Szende, 'The effect of amino acids on tumor growth', *Dissertation*, Budapest, 1973.

(27). S. Kinoshita, K. Nakayama and S. Kitada, *J. Gen. Appl. Microbiol.*, 1958, **4**, 128.

(28). T. Fukumura, *Agr. Biol. Chem.*, 1976, **4 0**, 1687.

(29). T. Fukumura, *Agr. Biol. Chem.*, 1977, **4 1**, 1327.

(30). H.J. Vogel, J.S. Thompson and G.D. Shockman, In '*Organisation and Control in Prokaryotic and Eukaryotic Cells*'. (H.P. Charles and B.C.J.G. Knight, Eds), 1970, 107-119, Cambridge, University Press, London and New York.

(31). H. Borsook, C.L. Deasy, A.J. Haagen-Smit, G. Keighley and P.H. Lowry, *J. Biol. Chem.*, 1948, **1 7 6**, 1395.

(32). D. Cavallini and B. Mondowi, *Arch. Sci. Biol.*, 1952, **3 6**, 468.

(33). J. March, '*Advanced Organic Chemistry: Reactions, Mechanisms and Structure,*' 1985, 116, 3rd Edn., Wiley & Sons, New York.

(34). A.F. Tucci and L.N. Ceci, *Arch. Biochem. Biophys.*, 1972, **153**, 742.

(35). M. Strassman and L.N. Ceci, *J. Biol. Chem.*, 1966, **241**, 5401.

(36). B. Rowley and A.F. Tucci, *Arch. Biochem. Biophys.*, 1970, **141**, 499.

(37). M. Matsuch and M. Ogur, *J. Biol. Chem.*, 1969, **244**, 5153.

(38). J.K. Bhattarjee, *Adv. Appl. Microbiol.*, 1970, **13**, 139.

(39). S. Sagisaka and K. Shimura, *J. Biochem. (Tokyo)*, 1962, **52**,155.

(40). H.P. Broquist, '*Methods In Enzymology*', (Colowick, S.P. and Kaplan, N.O., Eds), 1971, (Vol.178), 112-129, Academic Press, New York.

(41). B.L. Moller, *Plant Physiol.*, !974, **54**, 638.

(42). B.J. Miflin, and P.J. Lea, *Ann. Rev. Plant Physiol.*, 1977, **28**, 299.

(43). W.R. Mills, P.J. Lea and B.J. Miflin, *Plant Physiol.*, 1980, **65**, 1166.

(44). P.A. Bryan, R.D. Crawley, C.E. Bruner and J.K. Bryan, *Biochem. Biophys. Res. Commun.*, 1970, **41**, 1211.

(45). R.M. Cheshire and B.J. Miflin, *Phytochemistry*, 1975,**14**, 695.

(46). H. Aarnes and S.E. Rognes, *Phytochemistry*, 1974, **13**, 2717.

(47). P.R. Shewry and B.J. Miflin, *Plant Physiol.*, 1977, **59**, 69.

(48). S.W.J. Bright, B.J. Miflin and S.E. Rognes, *Biochem. Genet.*, 1982, **20**, 229.

(49). S.W.J. Bright, P.R. Shewry and B.J. Miflin, *Plant*, 1978, **139**, 119.

(50). Y. Yamada, R. Kumpaisal, T. Hashimoto, Y. Sugimoto and A. Susuki, *Plant Cell Physiol.*, 1986, **27**, 607.

(51). S. Kochhav, V.K. Kochhav and P.V. Sane, *Biochem. Biophys. Acta.*, 1986, **880**, 220.

(52). H.M. Davies and B.J. Miflin, *Plant Sci. Lett.*, 1977, **9**, 323.

(53). J.M. Relton, P.L.R. Bonner, R.M. Wallsgrove and P.J. Lea, *Biochem. Biophys. Acta.*, 1988, **953**, 48.

(54). K. Sasaoka, *Plant Cell Physiol.*, 1961, **2**, 231.

(55). B.G. Gegenbach, T.J. Walter, C.E. Green and K.A. Hibberd, *Crop. Sci.*, 1978, **18**, 472.

(56). C. Gilvarg and Y. Yugari, *J. Biol. Chem.*, 1965, **240**, 4710.

(57). R. Kumpaisal, T. Hashimoto and Y. Yamada, *Plant Physiol.*, 1987, **85**, 145.

(58). V.V.S. Tyagi, W.R. Farkas and R.R. Henke, *Plant Physiol.*, 1983, **73**, 687.

(59). H.J. Vogel and A.P. Hirvonen, '*Methods Of Enzymology*', (H. Tabor and C.W. Tabor Eds), 1971, Vol. XVII, 146-150, Academic Press, New York and London.

(60). L. Sodek, *Rev. Brazil. Bot.*, 1978, **1**, 65.

(61). M. Mazelis and R.K. Crevelling, *J. Food. Biochem.*, 1978, **2**, 29.

(62). J.G. Kelland, M.M. Palcic, M.A. Pickard and J.C. Vederas, *Biochemistry*, 1985, **24**, 3263.

(63). L.K. Wenko, R.W. Treick and K.G. Wilson, *Plant Mol. Biol.*, 1985, **4**, 197.

(64). G. Gottschalk, '*Bacterial Metabolism*', 1979, Springer-Verlag, New York.

(65). J.M. Ghuysen, *'Topics In Antibiotic Chemistry'*, P.G. Sammes, 1980, Vol. 5, Ellis Horwood Ltd., Chichester, U.K.

(66). E.F. Gale, E. Cundliffe, P.E. Reynolds, M.H. Richmond, M.J. Waring, *'The Molecular Basis Of Antibiotic Action'*, 1981, 2nd Ed., Wiley, London.

(67). M.P. Lambert and F.C. Newhaus, *J. Bacteriol.*, 1972, **1 1 0**, 978.

(68). J.G. Allen, F.R. Atherton, M.J. Hall, C.H. Hassal, S.W. Holmes, R.W. Lambert, L.J. Nisbet and P.S. Ringrose, *Antimicrob. Agent Chemother.*, 1979, **1 5**, 684.

(69). Y. Asada, K. Tunizawa, S. Sawada, T. Susuki, H. Misonu and K. Soda, *Biochem.*, 1981, **2 0**, 6881.

(70). S. Black and N. Wright, *J. Biol. Chem.*, 1955, **2 1 3**, 39.

(71). M. Mazelis, F.R. Whatley and J. Whatley, *FEBS Lett.*, 1977, **8 4**, 236.

(72). F. Yamakura, Y. Ikeda, K. Kimura and T. Sasakawa, *J. Biochem.*, 1974, **7 6**, 611.

(73). S.M. Hallings and D.P. Stahly, *Biochim. Biophys. Acta.*, 1976, **4 5 2**, 580.

(74). J.G. Schedlarski and C. Gilvarg, *J. Biol. Chem.*, 1970, **2 4 5**, 1362.

(75). M. Mazelis, B.J. Miflin and H.M. Pratt, *FEBS Lett.*, 1976, **6 4**, 197.

(76). R.M. Wallsgrove and M. Mazelis, *FEBS Lett.*, 1980, **1 1 6**, 189.

(77). R.M. Wallsgrove and M. Mazelis, *Phytochemistry*, 1981, **2 0**, 2651.

(78). M. Ghislain, V. Frankard and M. Jacobs, *Planta*, 1990, **1 8 0**, 480.

(79). H.P. Meloche, *Biochem. Biophys. Res. Commun.*, 1965, **1 8**, 277.

(80). F.C. Hartmann, '*Methods In Enzymology*', (W.B. Jakoby and M. Wilchek, Eds). 1977, Vol.46, 130-153, Academic Press, New York.

(81). R. Kumpaisal, T. Hashimoto and Y. Yamada, *Agric. Biol. Chem.*, 1989, **5 3**, 355.

(82). M.L. Bach and C. Gilvarg, *J. Biol. Chem.*, 1966, **2 4 1**, 4563.

(83). A.I. Aronson, E. Henderson and A. Tincher, *Biochem. Biophys. Res. Commun.*, 1967, **2 6**, 454.

(84). L.A. Chasin and J. Szulmajester, *Biochem. Biophys. Res. Commun.*, 1967, **2 9**, 648.

(85). A. Fukuda and C. Gilvarg, *J. Biol. Chem.*, 1968, **2 4 3**, 3871.

(86). K. Kimura, *J. Biochem.*, 1974, **7 5**, 961.

(87). H. Tamir and C. Gilvarg, *J. Biol. Chem.*, 1974, **2 4 9**, 3034.

(88). P. Shapshak, *Diss. Abstr. Int. B.*, 1970, **3 1**, 252.

(89). W. Farkas and C. Gilvarg, *J. Biol. Chem.*, 1965, **2 4 0**, 4717.

(90). K. Kimura and T. Sasakawa, *J. Biochem.*, 1975, **7 8**, 381.

(91). L. Couper, D.J. Robins and E.J.T. Chrystal, *Tetrahedron Lett.*, 1992, 2717.

(92). C. Gilvarg, *J. Biol. Chem.*, 1961, **2 3 6**, 1429.

(93). O. Tosaka and K. Takinami, *Agr. Biol. Chem.*, 1978, **4 2**, 95.

(94). S.A. Simms, W.H. Voige and C. Gilvarg, *J. Biol. Chem.*, 1984, **2 5 9**, 2734.

(95). C. Gilvarg, D.A. Berges, W.E. DeWolf, G.L. Dunn, D.J. Newman, S.J. Schmidt and J.J. Taggart, *J. Biol. Chem.*, 1986, **2 6 1**, 6160.

(96). S.A. Simms, *Ph.D. dissertation*, Princeton University, 1983.

(97). B. Peterofsky and C. Gilvarg, *J. Biol. Chem.*, 1961, **2 3 6**, 1432.

(98). C. Gilvarg, *J. Biol. Chem.*, 1959, **2 3 4**, 2955.

(99). S.H. Kindler and C. Gilvarg, *J. Biol. Chem.*, 1960, **235**, 3532.

(100). A.T.M. Bartlett and P.J. White, *J. Gen. Microbiol.*, 1985, **131**, 2145.

(101). J.S. Wiseman and J.S. Nichols, *J. Biol. Chem.*, 1984, **259**, 8907.

(102). V.V.S. Tyagi, R.R. Henke and W.R. Farkas, *Biochim. Biophys. Acta*, 1982, **719**, 363.

(103). P.J. White, B. Lejeune and E. Work, *Biochem. J.*, 1969, **113**, 589.

(104). S. Ishino, K. Yamaguchi, K. Shirahata and K. Araki, *Agric. Biol. Chem.*, 1984, **48**, 2557.

(105). H. Misono and K. Soda, *J. Biol. Chem.*, 1980, **255**, 10599.

(106). H. Misono, M. Ogasawara and S. Nagasaki, *Agric. Biol. Chem.*, 1986, **50**, 2729.

(107). W.J. Albery and J.R. Knowles, *Biochemistry*, 1986, **25**, 2572.

(108). L.K.P. Lam, L.D. Arnold, T.H. Kalantar, J.G. Kelland, P.M. Lane-Bell, M.M. Palcic, M.A. Pickard and J.C. Vederas, *J. Biol. Chem.*, 1988, **263**, 11814.

(109). H. Misono, H. Togawa, T. Yamamoto and K. Soda, *Biochem. Biophys. Res. Commun.*, 1976, **72**, 89.

(110). P.J. White, *J. Gen. Microbiol.*, 1983, **129**, 739.

(111). H. Misono and K. Soda, *Agric. Biol. Chem.*, 1980, **44**, 227.

(112). L.K. Wenko, R.W. Treick and K.G. Wilson, *Plant Molecular Biol.*, 1985, **4**, 197.

(113). H. Misono, S. Nagasaki and K. Soda, *Agric. Biol. Chem.*, 1981, **45**, 1455.

(114). M. Antia, D.S. Hoare and E. Work, *Biochem. J.*, 1957, **65**, 448.

(115). M. Lakshman, B.C. Stenoy and M.R.R. Rao, *J. Biosci.*, 1981, **3**, 89.

(116). Y. Shimura and H.J. Vogel, *Biochim. Biophys. Acta*, 1966, **118**, 396.

(117). P.J. White and B. Kelly, *Biochemistry*, 1965, **96**, 75.

(118). H.G. Floss and J.C. Vederas, '*Stereochemistry*' (C. Tamm, eds), 1982, 161-169, Elsevier/North-Holland Biomedical Press, Amsterdam.

(119). M. Akhtar, V.C. Emery and J.A. Robinson, '*The Chemistry of Enzyme Action*' (M.L. Page, editor), 1984, 303-372, Elsevier/North-Holland Biomedical Press, Amsterdam.

(120). M.M. Palcic and H.G. Floss, '*Pyridoxal Phosphate and Derivatives, Part A*' (D. Dolphin, R. Poulson and D. Avramovic, editors), 1986, 26-68, John Wiley and Sons, New York.

(121). E. Borthwick, M.Sc Thesis, University of Glasgow, 1991.

(122). J.G. Kelland, J.C. Vederas and M.A. Pickard, *J. Biol. Chem.*, 1988, **261**, 11814.

(123). Y. Asada, K. Tanizawa, Y. Kawabata, H. Misono and K. Soda, *Agric. Biol. Chem.*,1981, **45**, 1513.

(124). G.N. Wilkinson, *Biochem. J.*, 1961, **80**, 324.

(125). P. Deslongchamps, '*Stereoelectronic Effects in Organic Chemistry*', 1983, 252-290, Pergamon Press Ltd., Oxford.

(126). M.H. O'Leary and F.J. Piazza, *J. Am. Chem. Soc.*, 1978, **100**, 632.

(127). C. Walsh, *Annu. Rev. Biochem.*, 1984, **53**, 493.

(128). C. Walsh, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1983, **55**, 197.

(129). M.D. Tsai, J. Weaver, H.G. Floss, E.E. Conn, K.R. Crevelling and M. Mazelis, *Arch. Biochem. Biophys.*, 1978, **190**, 553.

(130). K. Soda, N. Esaki and H. Tanaka, *Agric. Biol. Chem.*, 1984, **48**, 383.

(131). A.J.L. Cooper and O.W. Griffiths, *J. Biol. Chem.*, 1979, **254**, 2748.

(132). S. Tanase, B.M. Guirard and E.E. Snell, *J. Biol. Chem.*, 1985, **260**, 6738.

(133). R.H. Yamada, Y. Wakabayashi, A. Iwashima and T. Hasegawa, *Biochim. Biophys. Acta*, 1984, **801**, 151.

(134). J.K. Bryan, *Biochim. Biophys. Acta*, 1969, **171**, 205.

(135). H. Aarnes, *Plant Sci. Letters*, 1977, **10**, 381.

(136). C.A. DiCamelli and J.K. Bryan, *Plant Physiol.*, 1980, **65**, 176.

(137). J.E. Baldwin and A. Flinn, *Tetrahedron Lett.*, 1987, **28**, 3605.

(138). G.C. Barrett, '*Chemistry and Biochemistry of the Amino Acids*', 1985, 246-296, Chapman and Hall.

(139). J.P. Greenstein and M. Winitz, '*Chemistry of the amino acids*', 1961, (Vols 1-3), 1-52, John Wiley and Sons, Inc., New York.

(140). Theodora W. Greene, '*Protective Groups in Organic Synthesis*', 1981, 152-187 & 218-223, John Wiley and Sons.

(141). J.W.P. Asimon and R.P. Seguin, *Tetrahedron*, 1979, **35**, 2797.

(142). D.K. Black and S.R. Landor, *J. Chem. Soc. (C)*, 1968, 281.

(142a).J. March, '*Advanced Organic Chemistry: Reactions, Mechanisms and Structure*,' 1985, 983, 3rd Edn., Wiley & Sons, New York.

(143). D.S. Tarbell, Y. Yamamoto and B.M. Pope, *Proc. Nat. Acad. Sci., U.S.A.*, 1972, **69**, 730.

(144). M. Itoh, D. Hagiwara and T. Kamiya, *Bull. Chem. Soc. Jpn.*, 1977, **5 0**, 718.

(145). B.F. Lundt, N.L. Johansen, A. Volund and J. Markussenl, *Int. J. Pept. Protein Res.*, 1978, **1 2**, 258.

(146). G.W. Anderson and F.M. Callahan, *J. Am. Chem. Soc.*, 1960, **8 2**, 3359.

(147). M. Bergmann and L. Zervas, *Ber.*, 1932, **6 5**, 1192.

(148). S.S. Wang, B.F. Gisin, D.P. Winter, R. Makofske, I.D. Kelusha, C. Tzougraki and J. Meienhofer, *J. Org. Chem.*, 1977, **4 2**, 1286.

(149). K. Ramsamy, R.K. Olsen and F.J. Frank, *Synth. Commun.*, 1982, 42.

(150). J.C. Collins, W.W. Hess and F.J. Frank, *Tetrahedron Lett.*, 1968, 3363.

(151). D.D. Keith, J.A. Tortora, K. Ineichen and W. Leimgruber, *Tetrahedron*, 1975, **3 1**, 2633.

(152). G. Piancatelli, A. Scettri and M. D'Auria, *Synthesis*, 1982, 245.

(153). A.J. Ozinskas and G.A. Rosenthal, *J. Org. Chem.*, 1986, **5 1**, 5047.

(154). G.C. Stelakatos and N. Argyropoulos, *J. Chem. Soc. (C).*, 1970, 964.

(155). P.T. Bachen and C.N. Cohen, *Methods Enzymol.*, 1969, **1 7 A**, 694.

(156). F.H.C. Stewart, *Aust. J. Chem.*, 1968, **2 1**, 2543.

(157). J.E. Baldwin, M. North and A. Flinn, *Tetrahedron*, 1988, **4 4**, 637.

(157a). A. Schneier, Ph. D. Thesis, University of Cambridge, 1992.

(158). F.M.F. Chen and N. Leo Benoiton, *Synth. Commun.*, 1979, 709.

(159). E. Wenkert and T.E. Goodwin, *Synth. Commun.*, 1977, 7, 409.

(160). W.H. Hartung and R. Simonoff, *Org. Reactions,* 1953, **V I I**, 263.

(161). C.D. Gutsche, *Org. Reactions,* **V I I I**, 372.

(162). L.N. Markovsku, V.E. Pashinnik and A.V. Kirsanov, *Synthesis,* 1973, 787.

(163). P. Munster and W. Steglich, *Synthesis,* 1987, 223.

(164). J. Mann, '*Secondary Metabolism*', 2nd Edn., 1987, p18-20, Oxford Science Publications.

(165). '*Vogel : Practical Organic Chemistry*', 3rd Edn., 344, Longman.

(166). J.L. Mattina and C.J. Mularski, *Tett. Let.,* 1983, **2 4**, 2059.

(167). Barkenbus and Landis, *J. Am. Chem. Soc.*, 1948, **7 0**, 684.

(168). Mousseron and Combes, *Bull. Soc. Chim. France,* 1947, 82.

(169). Culvenor, Davis and Heath, *J. Chem. Soc.*, 1949, 279.

(169a). J.C. Burke, H.L. Yale, G.L. Hassert and J.P. High, '1956 Meeting of The American Society for Pharmacology and Experimental Therapeutics', at French Lick Springs, Indiana, Nov. 8-10, 1956.

(170). H.L. Yale, F. Sowinski and J. Bernstein, *J. Am. Chem. Soc.*, 1957, **7 9**, 4375.

(171). T.S. Griffin, T.S. Woods and D.L. Klayman, *Adv. Heterocycl. Chem.*, 1975, **1 8**, 100.

(172). H. Erlenmeyer, J. Eckenstein, E. Sorkin and H. Meyer, *Helv. Chim. Acta,* 1950, **3 3**, 1271.

(173). G. Ricci, G. Federici, G. Lucente, M. Achilli and D. Cavallini, *Physiol. Chem. Phys.*, 1982, **1 4**, 193.

(174). D.A. Berges and J.J. Taggart, *J. Org. Chem.*, 1985, **5 0**, 413.

(175). I.T. Strukov, *Zhur. Obshchei Khim.*, 1958, **2 8**, 69.

(176). J. Kitchin and R.J. Stoodley, *Tetrahedron,* 1973, **2 9**, 3023.

(177). T.E. Strom, B.S. Snowden and P.A. Toldan, *J. Chem. Soc., Chem. Commun.*, 1969, 50.

(178). R.R. Fraser, T. Hurst, M.R. McLory, R. Viau and M.Y. Wigfield, *Int. J. Sulfur Chem. Part A.*, 1971, **1**, 133.

(179). R.J. Stoodley and R.B. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1974, 1572.

(180). A. Yoshida, S. Oida and E. Ohki, *Chem. Pharm. Bull.*, 1975, **2 3**, 2518.

(181). R.J. Kennedy, *J. Org. Chem.*, 1960, **2 5**, 1901.