Lysine Biosynthesis: Synthesis of Enzyme Inhibitors and Substrates

A Thesis submitted in part fulfilment
of the requirements of the degree
of Doctor of Philosophy

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Science is not proven

Dedicated to my wife Helen and children Alexander and Emma

Acknowledgements

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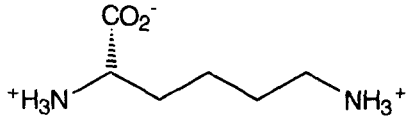
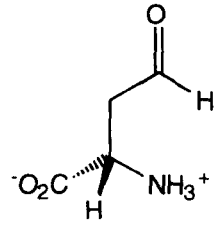
Summary

Two distinct biosynthetic pathways to the essential amino acid L-lysine **(A)** are found in nature. The α-aminoadipate pathway operates in fungi and yeasts. The diaminopimelate (DAP) pathway occurs in bacteria and higher plants. Our studies were concerned with the DAP pathway and particularly with the first two steps of this pathway. These steps involve condensation of L-aspartate-β-semialdehyde (ASA) **(B)** with pyruvate **(C)** to form L-2,3-dihydrodipicolinic acid (DHDPS) **(D)** and subsequent reduction to L-2,3,4,5-tetrahydrodipicolinic acid (THDPA) **(E)**. The first step is catalysed by the enzyme dihydrodipicolinate synthase (DHDPS). The second step is catalysed by the enzyme dihydrodipicolinate reductase (DHDPR) and utilises NADPH as a co-factor.



(A)



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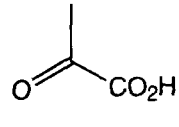

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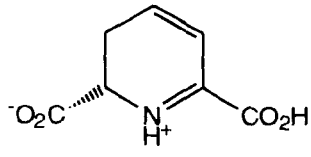

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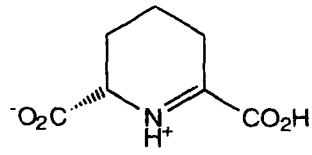
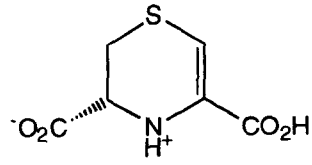


(E)

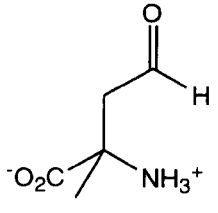
Our primary objective was the inhibition of this biosynthetic pathway. Inhibitors of this pathway have potential as antibacterial or herbicidal agents. A number of substrate analogues of the DHDPS and DHDPR enzymes were prepared and tested as inhibitors of these enzymes. In previous studies by our group, heterocyclic compound **(F)** showed promising activity. In our work, a number of analogues of compound **(F)** were prepared. Inhibition studies with these compounds constituted a valuable insight into the characteristics of these enzymes. The level of inhibition with these compounds and for a range of other substrate analogues indicate high substrate selectivity for the enzymes.



(F)

The condensation catalysed by DHDPS is mechanistically interesting. In chemical terms, C-C bond formation is commonly a high energy process involving highly reactive compounds such as organometallic agents or strong bases. In such cases the strategy must be directed towards protecting other functionality or introducing it at a later stage. Clearly, achieving this task under mild conditions with the high regio- and stereoselectivity associated with enzymic catalysis could be of tremendous advantage.

Once again, however, we were restricted by the high substrate specificity of the DHDPS enzyme. In earlier studies by our group, Dr J.E. McKendrick found evidence of substrate activity for 2- and 3-methyl substituted ASA, **(G)** and **(H)**, respectively. In our work, an improved preparation of these compounds was developed and the subsequent biotransformations with DHDPS were examined both qualitatively and quantitatively. Interestingly, compound **(G)** was shown to display a greater substrate activity than ASA. The preparation of ASA methyl ester **(I)** was achieved. This compound also displayed a moderate level of substrate activity and was the only compound found to show substrate activity for a DHDPS/DHDPR coupled substrate assay.



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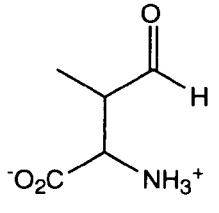

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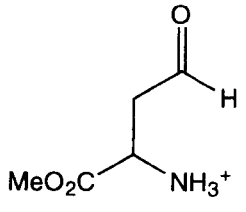
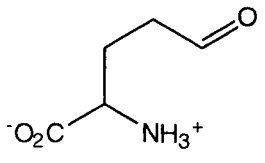


(I)

A significant proportion of our effort was concentrated on the investigation of glutamate-γ-semialdehyde (GSA) **(J)**, the higher homologue of ASA, as a substrate of DHDPS. Problems were encountered with earlier studies in this area because of cyclisation of GSA, even in protected forms. To counter this problem two novel strategies were considered. The first involved *in situ* enzymic deprotection of the *N*-acetyl derivative of GSA. Although this was successful for *N*-Acetyl-ASA, problems with cyclisation were once again experienced for the GSA equivalent. The second strategy utilises the reversible nature of enzymic catalysis. The synthesis of the proposed 7-membered heterocyclic product **(K)** of DHDPS catalysed condensation of GSA and pyruvate is not a trivial task. Some progress was made and further direction detailed. Further validation for this study was demonstrated on preparation of 5-hydroxyproline **(L)**. Compound **(L)** is a cyclic equivalent of GSA and was found to display clear evidence of substrate activity.



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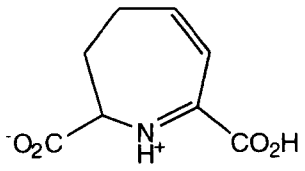

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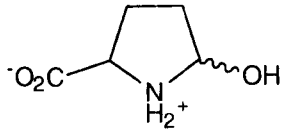


(L)

One other application of our 'reverse' strategy for enzymic activity was successfully implemented. This involved the development of an inhibition assay for detection of DHDPR activity. The natural substrate of DHDPR, **(D)**, has very low stability. Consequently, inhibition studies on this enzyme are most commonly performed by means of a DHDPS/DHDPR coupled assay. By synthesising the enzymic product of DHDPR, **(E)**, we have developed a 'reverse' assay for inhibition studies on DHDPR alone. The synthesis of compound **(E)** was previously achieved by our group. A selection of our synthetic inhibitors was evaluated by this new enzyme assay.

Contents

Abbreviations

AAA	α-Aminoadipate
Ac	Acetyl
Acetyl CoA	Acetyl co-enzyme A
Ar	Aromatic
aq.	Aqueous
ASA	Aspartic acid β-semialdehyde
ATP	Adenosine triphosphate
BOC	*tert*-Butyloxycarbonyl
Bn	Benzyl
br.	Broad
BTEAC	Benzyltrimethylammonium chloride
Bu	Butyl
c.	Concentrated
°C	Degrees centigrade
Cal	Calorie
CBZ	Carbobenzyloxy
CI	Chemical Ionisation
cm	Centimeters
d	Doublet
DAP	αα'–Diaminopimelic acid
DCC	Dicyclohexylcarbodiimide
DCU	*NN'*-Dicyclohexylurea
DDQ	2,3-Dichloro-4,5-dicyano-1,4-benzoquinone
DEAE-Sephacel	Diethylaminoethyl-sephacel
dec.	Decomposition
DHDPA	2,3-Dihydrodipicolinic acid
DHDPR	Dihydrodipicoliniate reductase
DHDPS	Dihydrdipicolinate synthase
DHT	3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid
DMAP	4-Dimethylaminopyridine
DMF	*NN*-Dimethyl formamide
DMS	Dimethylsulfide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenylhydrazine
DPA	Dipicolinic acid
ee	Enantiomeric excess

EI	Electron Impact
ESMS	Electrospray mass spectrometry
Et	Ethyl
FAB	Fast atom bombardment
FMOC	9-Fluorenylmethyl carbamate
FT	Fourier transform
GC	Gas chromatography
GSA	Glutamic acid γ-semialdehyde
h	Hours
HDPA	4-Hydroxy-2,3,4,5-tetrahydrodipicolinic acid
HPLC	High-performance liquid chromatography
Hz	Hertz
IC_{50}	Inhibtor concentration at 50% activity
IR	Infra-red
l	Litres
LDA	Lithium diisopropylamide
Lit.	Literature reference
m	Multiplet
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
mmol	Millimole
mol	Moles
mp	Melting point
μM	Micromolar
nm	Nanometer
nM	Nanomolar
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAG	*N*-Acetylmuramic acid
NAM	*N*-Acetylglucosamine
NBS	*N*-Bromosuccinimide
NMO	4-Methylmorpholine *N*-oxide
NMR	Nuclear magnetic resonance
p	$-\log_{10}$
P	Protecting group
PCC	Pyridinium chlorochromate
pet. ether	Petroleum ether

Ph	Phenyl
PKA	Porcine kidney acylase
pMB	*para*-Methoxybenzyl
ppm	Parts per million
Pr	Propyl
PTSA	*para*-Toluenesulfonic acid
py	Pyridine
q	Quartet
RNA	Ribonucleic acid
s	Singlet
SDS-Page	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Succinyl CoA	Succinyl co-enzyme-A
t	Triplet
tert	Tertiary
THDPA	2,3,4,5-Tetrahydrodipicolinic acid
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TPAP	Tetra-*n*-propylammonium perruthenate
UV	Ultraviolet

Chapter 1 α-Amino Acids, Proteins and Enzymes

1.01 Importance of α-Amino Acids[1]

There are a very large number of amino acids known to occur in nature. Of these, the most important are the twenty α-amino acids which almost exclusively comprise the structure of all known proteins. Proteins are found in all living cells. They are the principal material of skin, muscles, tendons and nerves as well as enzymes, antibodies and many hormones. The twenty amino acids most commonly found in proteins are listed in **Figure 1**. All of these α-amino acids (with the exception of glycine) have the L-stereochemistry and the highly specific stereoenvironment that results when a number of these amino acids are joined through peptide linkages is crucial to the high chemical selectivity of these biomolecules. Proteins are the end products of the genetic processes of every living organism **(Scheme 1)** and are the workhorses of all biological transformations.

Scheme 1 The Genetic Process

Amino acids are amphoteric in nature; that is, they contain both an acid function in the carboxyl group and a basic function in the amine group. In their commonly encountered neutral aqueous environment they exist mainly in the doubly ionic or zwitterionic form shown in **Figure 1**. As a result the free amino acids display similar chemical behaviour to ionic solids: high melting points; solubility in polar solvents; and very low acidity and basicity constants. For example, glycine has $K_a = 1.6 \times 10^{-10}$ and $K_b = 2.5 \times 10^{-12}$. At low pH the carboxylate group of the amino acid is protonated and it exists as an ammonium cation. At high pH the ammonium group of the amino acid is deprotonated and it exists as a carboxylate anion. The nature of the ionic species determines how the amino acid will migrate in an electric field. The hydrogen ion concentration of a solution in which a particular amino acid does not migrate towards either electrode in a magnetic field is an important physical constant known as the isoelectric point. An amino acid usually shows its lowest solubility in a solution at its isoelectric pH. The value of this constant

is dependent on the ratio of acidic and basic groups, including those in the side chain. Glycine, glutamic acid and lysine have isoelectric pH values of 5.97, 3.22 and 9.74, respectively.[2]

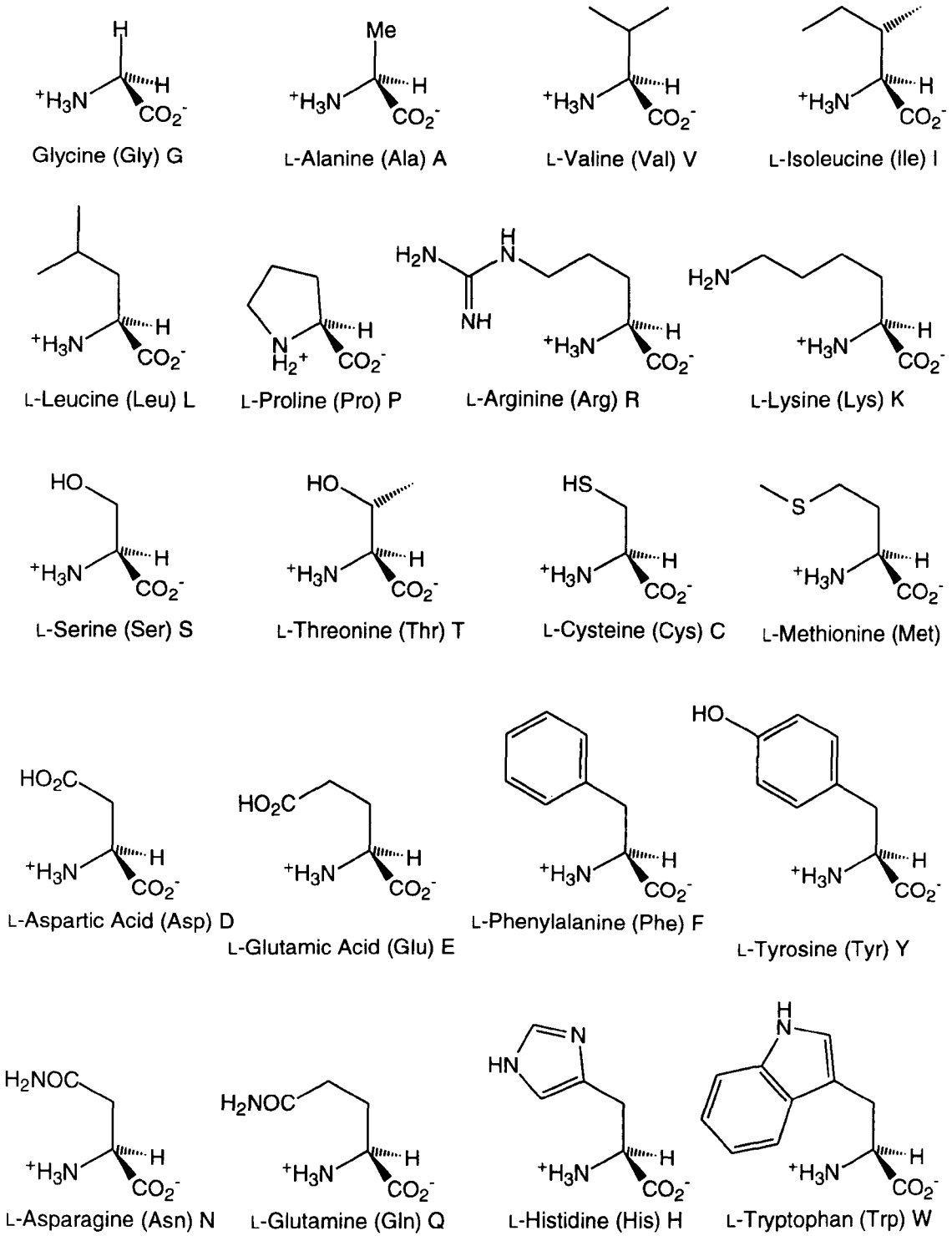
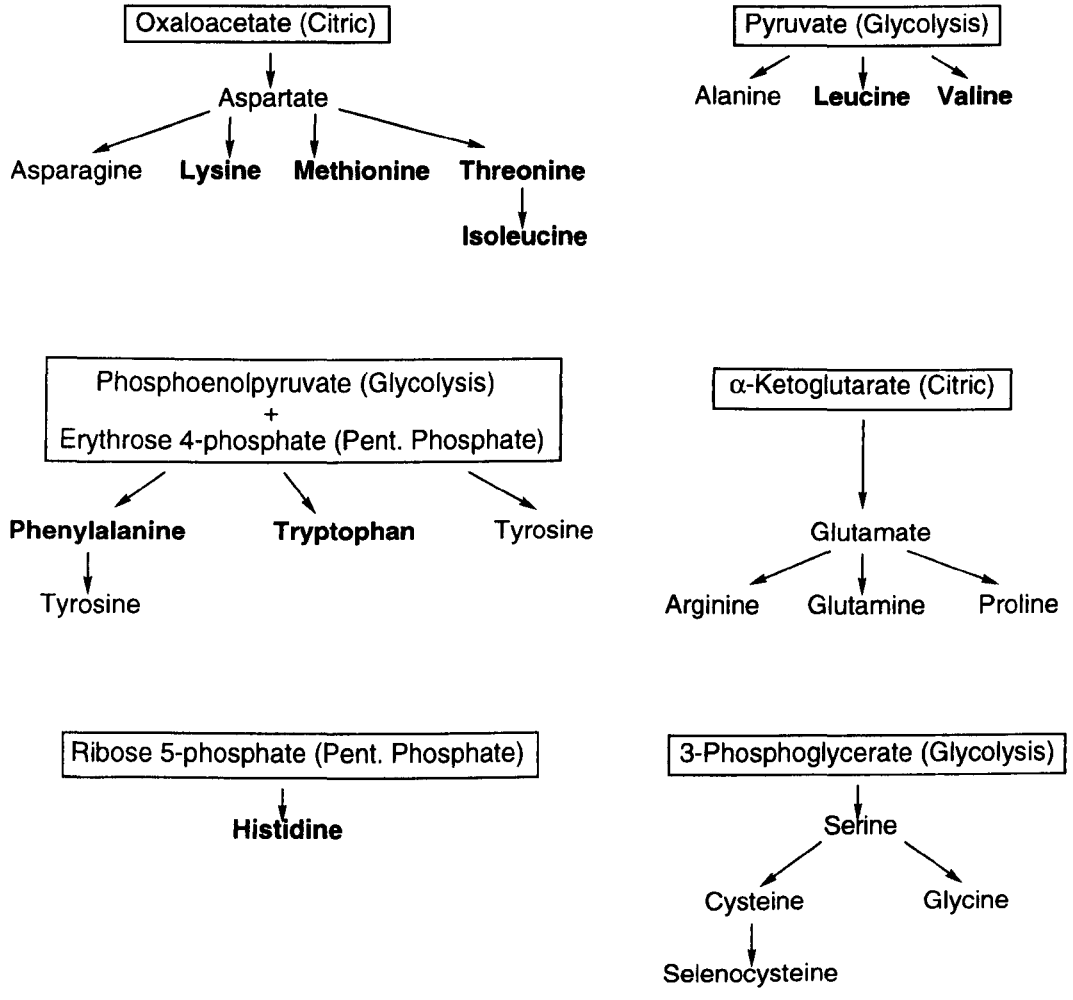


Figure 1 The 'Natural' α-Amino Acids

1.02 Biosynthesis of α-Amino Acids[3]

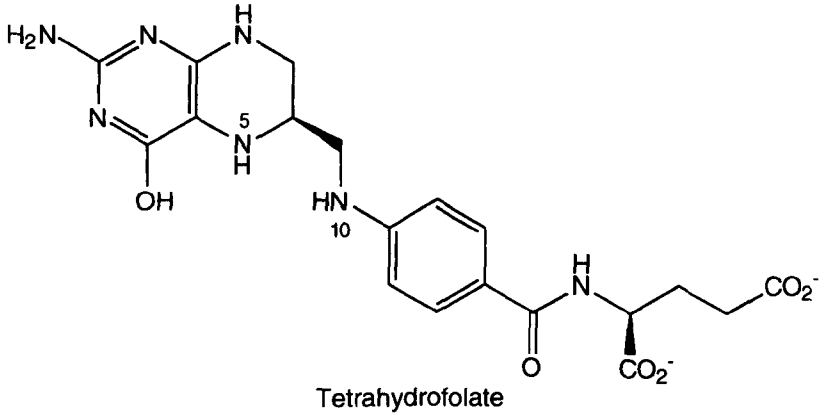
The entire basic set of twenty one genetically encoded α-amino acids, including the less commonly occurring selenocysteine, are derived from six intermediates produced from only three biosynthetic pathways: glycolysis; the pentose phosphate pathway and the citric acid cycle. Bacteria such as *Escherichia coli* and plants can synthesise the entire set required for protein synthesis. Humans can only synthesise twelve of the genetically encoded α-amino acids. These are known as the 'nonessential' amino acids: alanine; arginine; asparagine; aspartate; cysteine; glutamate; glutamine; glycine; proline; selenocysteine; serine and tyrosine. The remaining nine 'essential' amino acids must be supplied in the diet: histidine; isoleucine; leucine; lysine; methionine; phenylalanine; threonine; tryptophan and valine. The pathways to the amino acids are summarised in **Scheme 2**. The essential amino acids are in bold type and the biochemical pathway from which the precursor is derived is bracketed.



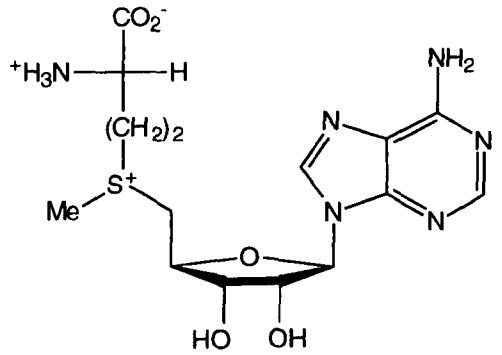
Scheme 2 Biosynthetic Routes to the α-Amino Acids

The synthesis of the carbon skeleton of the α-amino acids from the biochemical precursors is performed by simple enzyme controlled transformations. The transformations in the case of essential amino acids are generally more complex.

Two prosthetic groups, tetrahydrofolate and *S*-adenosylmethionine, play important roles in the synthesis of the carbon skeletons **(Figure 2)**. Tetrahydrofolate is a vitamin for mammals and must be supplied in the diet. In amino acid synthesis it acts as a supply of an activated methyl group. The key parts of the molecule are the N-5 and N-10 positions **(Figure 2)** which can carry methyl, methylene, formyl, formimino (-CHNH-) or methenyl (-CH=) groups. Tetrahydrofolate can also act as a one carbon acceptor in degradation reactions. *S*-Adenosylmethionine is a somewhat more reactive source of methyl groups and consequently is responsible for the majority of biosynthetic methylations. *S*-Adenosylmethionine is synthesised by transfer of an adenosyl group from adenosyltriphosphate (ATP) to the sulfur atom of methionine. The methionine can be regenerated from the *S*-adenosylhomocysteine byproduct by hydrolysis to homocysteine and subsequent methylation with N^5-methyltetrahydrofolate.



Tetrahydrofolate



S-Adenosylmethionine

Figure 2 Structure of Tetrahydrofolate and *S*-Adenosylmethionine

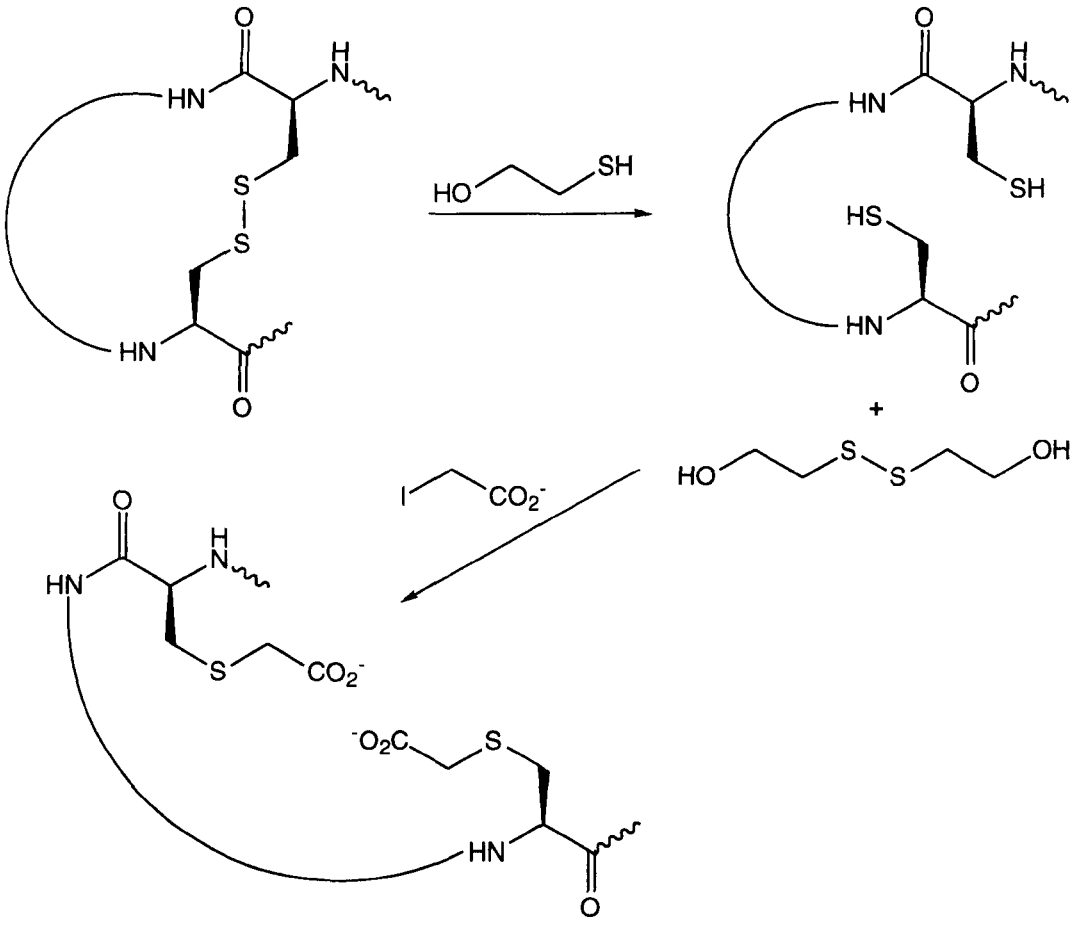
The incorporation of nitrogen into α-amino acids is another important biosynthetic feature. Nitrogen is the major constituent of atmospheric gases. N_2 gas from this source is reduced to water soluble ammonium ions (NH_4^+) by nitrogen-fixing microorganisms. The NH_4^+ reacts with α-ketoglutarate, NADPH and H^+ to produce glutamate, under catalysis by the enzyme glutamate dehydrogenase. Further biosynthetic reaction with NH_4^+ and ATP, catalysed by gluamine synthetase, produces glutamine. The α-amino group of most amino acids comes from a transamination reaction with glutamine.

1.03 Protein Structure[4,5]

Proteins are large macromolecules with molecular masses typically in the region 10 000 to 1 000 000 Daltons. They consist of a large number of α-amino acid units covalently linked through amide bonds to form long unbranched chains. With twenty amino acids arranged in varying number and order an almost infinite library of compounds is possible. At physiological pH, the amino acids can be classified into three forms by the polarity of their side chains: non-polar; neutral polar and charged polar. As a result proteins differ enormously in structure for both steric and electronic reasons. The sequence of these side chain groups is unique to each protein and gives each its distinct biochemical properties.

The shape of a protein can be described in terms of primary, secondary and tertiary structure. Primary structure is the basic covalent structure made up of peptide bonds and disulfide linkages between distant neighbouring cysteine residues. To determine the composition of a protein the peptide is hydrolysed in acid solution, since alkaline solution causes racemization. The resulting amino acid mix is then analysed by ion exchange chromatography or, after conversion into their methyl esters, by gas chromatography. Determination of protein sequence is a somewhat more difficult task and is accomplished by a combination of reductive cleavage, partial hydrolysis and terminal residue analysis. The first step of sequencing is the reductive cleavage of the covalent disulfide linkages and protection of the resulting cysteine groups by alkylation **(Scheme 3)**. In practice it is not feasible to extend the analysis of stepwise removal of terminal residues beyond about 20 residues because of the interference from the accumulation of residues. It is therefore necessary to subject the chain first to partial hydrolysis. The resulting fragments are then identified by terminal residue analysis. This technique

relies on the fact that there is a free amino group α to a peptide link at one end of the peptide chain and a free carboxylate group at the other end.

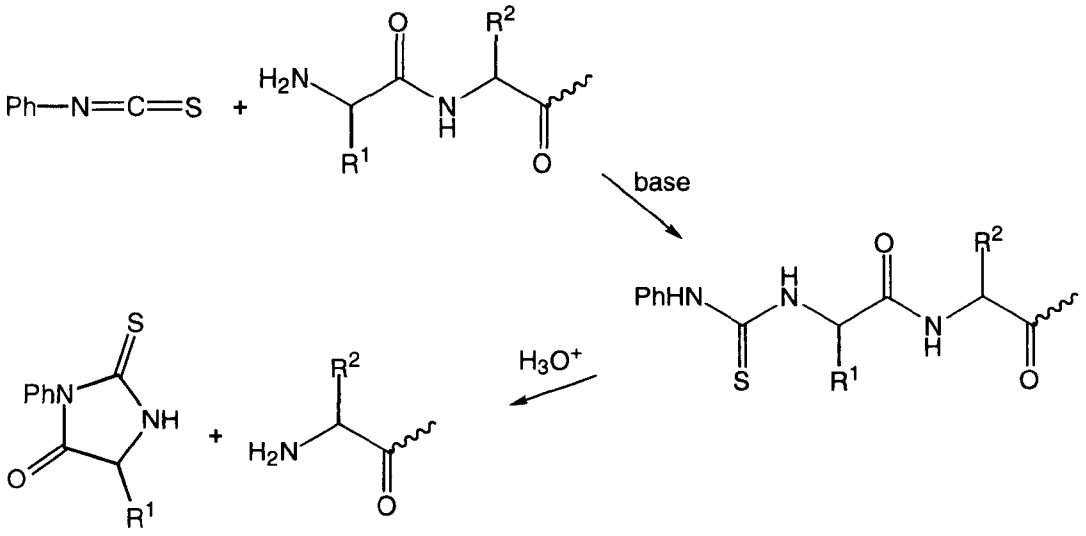


Scheme 3 Reductive Cleavage of S-S Crosslinks

The most commonly used method of terminal residue analysis involves selectively hydrolysing the *N*-terminal residue after treatment with phenyl isocyanate. The phenyl isothiocyanate reacts with the free amino group to form a thiourea. Mild hydrolysis with aqueous hydrochloric acid selectively removes the *N*-terminal residue, as a phenylthiohydantoin, and leaves the rest of the peptide intact **(Scheme 4)**. It is also possible to remove the *C*-terminal residue by selective hydrolysis with the enzyme carboxypeptidase, found in the pancreas. Standard notation is to list the protein sequence with the *N*-terminal end containing a free amino group on the left and the *C*-terminal end containing a free carboxyl group on the right.

X-Ray studies of dipeptides indicate that the entire amide group is flat. The oxygen, carbonyl carbon, nitrogen and three other atoms attached all lie in a plane. The carbon to nitrogen distance of 1.32 Å compared to the usual 1.47 Å for a carbon-nitrogen single bond indicates considerable double bond

character (~ 50%). As a result the angles around both the carbonyl carbon and the nitrogen are similar to those observed around a trigonal sp^2 carbon.



Scheme 4 Terminal Residue Analysis

Different proteins have different proportions of acidic and basic side chains and hence have different isoelectric points. The movement of the protein towards a particular electrode is not only dependent on the pH of the medium, which controls the magnitude of the net charge, but is also reliant on the molecular size and shape. This difference in behaviour in an electric field is the basis of electrophoresis. Electrophoresis is one method of separation and analysis of protein mixtures.

Secondary structure describes the way in which the protein chains are arranged in space to form coils, sheets or compact spheroids. The environment around the protein, aqueous or non-aqueous, plays a key role in the arrangement of the chains and side groups. As observed for nucleic acids and colloidal aggregates, proteins tend to hold their polar amide linkages and side chains to the outside where they are surrounded by a polar aqueous environment. The non-polar hydrophobic side chains are commonly held within this structure to the exclusion of water. The importance of the environment around the protein poses a problem for the protein crystallographer. The high vacuum environment required for X-ray analysis can cause the protein to distort by unfolding. Crystal structure of a normally folded protein is required to predict the sequence of events promoted within the active site. With this information and the skills of the molecular modeller, compounds can be designed to interact with the protein for chemotherapeutic or other useful purposes. Fortunately the skills of the protein crystallographer

are well advanced in crystallisation techniques and this problem is surmountable. The shape of the protein is principally determined by the intramolecular hydrogen bonds between distant N-H and C=O groups in amide bonds. This interaction has a stabilising effect of the order of 5 to 10 Kcal per mole per hydrogen bond. And since the attraction is electrostatic, and not governed by molecular orbital theory, then the N-H····O=C bond is close to linear. The simplest arrangement that fulfils this criterion is the linear β-sheet interaction **(Figure 3)**. For a linear peptide chain the repeat distance between similarly orientated alternate residues is 7.2 Å. It is however noteworthy from **Figure 3** that alternate side chains lie on the same side of the sheet. The result of this significant steric interaction is that the β-sheet structure is only observed when the neighbouring side chains concerned are small in size. Even when the side chains are small enough to permit this structure, the chain undergoes a puckering to accommodate the steric requirements. The protein silk fibron closely approaches the fully extended flat sheet structure and has a repeat distance of 7.0 Å. This protein is composed of 84% glycine, alanine and serine residues. When the side chains are larger the thermodynamically favoured conformation is the familiar α-helical shape that is observed with DNA **(Figure 4)**. Again, the predominant factor in adopting this structure is the thermodynamic gain of the hydrogen bonding interactions that hold it together. The stereochemistry of the individual amino acid residues imposes specific steric requirements that direct the formation of the highly stereospecific right handed helix. The α-helix has 3.6 amino acid residues per turn and has a repeat distance of 1.5 Å, measured along the helical axis.

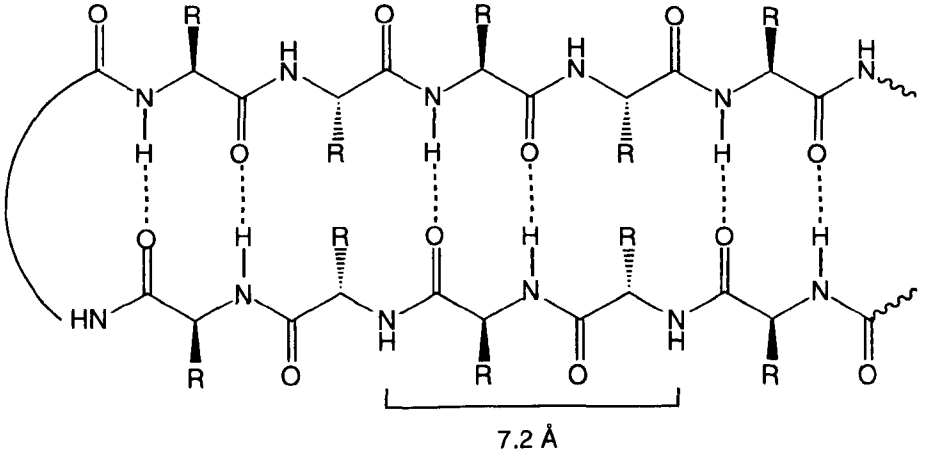


Figure 3 β-Sheet Structure

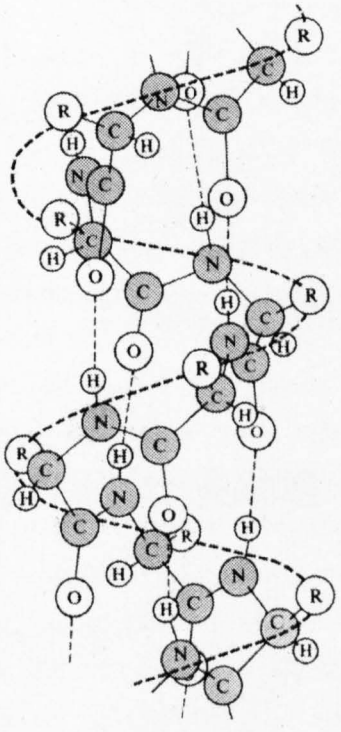


Figure 4 α-Helical Protein Structure

The influence of the amino acids proline and 4-hydroxyproline on secondary structure is worth consideration. When incorporated in the peptide chain both lack the N-H bond necessary for hydrogen bonding and impose significant steric requirements for the pseudo co-planar amide group and pyrrolidine ring. As a result, these residues can effect severe distortions or termination of both α-helical or β-sheet secondary structure.

Tertiary structure concerns the spatial arrangement of remote amino acid residues. The distinction between secondary and tertiary structure is not clear-cut. Both involve hydrogen bond interactions. Perhaps a simple interpretation would be to consider secondary structure as uniform or ordered and tertiary structure as random interaction between incidental neighbours. The disulfide bonds between cysteine residues, which have been discussed here as part of the covalent primary structure, are commonly considered as tertiary structure. Tertiary structure is clearly thermodynamically dictated and in some cases a degree of order is observed. It is suggested that each helix can itself be coiled into a superhelix with one turn for every 35 turns of the α-helix.

1.04 Enzymes, Active Sites and Co-enzymes

Proteins have a number of functions in living systems. The greatest diversity of function is observed in the field of protein-based biological catalysts, enzymes. Every physical function of a living system is in some way controlled or influenced by the action of these enzymes. The highly stereospecific structure of proteins ensures a selective function of each and every enzyme. Enzymes were originally considered to bind substrates into an 'active site' by a highly stereospecific 'lock and key' mechanism. Later structural evidence showed that most enzymes undergo a conformational change to accommodate substrates.[6] This 'induced fit' hypothesis accounts for the activity of enzymes commonly observed with 'unnatural' substrates of varing structural diversity. When in position, the substrate is located next to activated groups, protected from the exterior environment, and undergoes a chemical transformation. The catalytic mechanism is then completed by the release of the transformed substrate and regeneration of the catalyst by release of a used cofactor or reverse reaction with a co-substrate.

The specific function of enzymes is a product of evolution. Each enzyme has evolved through genetic processes to promote a specific biological function. Evolution is the process of altering the biological function of an organism for the purpose of increasing its chances of survival. It is estimated that it takes in the region of ten million years for one amino acid substitution to occur.[4] Such a change is due to a change in base sequence in a molecule of nucleic acid. A very small change in amino acid sequence can make a significant difference to the function of a protein. Replacement of one of the 300 residues, valine for glutamic acid, in haemoglobin is responsible for the fatal disease of sickle-cell anaemia.

Commonly the chemical transformation promoted at the active site of an enzyme is performed by a non-peptide portion held within the protein structure specifically for this purpose. This non-peptide portion is called a prosthetic group or co-enzyme and the protein is known as a conjugated protein.[4] An example of this is the tetrapyrrole structure of 'haem' **(Figure 5)** which chelates iron and is responsible for oxygen transport in mammals. Haem is held within a peptide portion (globin) of the protein haemoglobin. In our work we encountered the reducing enzyme dihydrodipicolinate reductase (DHDPR), responsible for the reduction of L-2,3-dihydrodipicolinic acid (DHDPA) to L-2,3,4,5-tetrahydrodipicolinic acid (THDPA) **(Chapter 2, Scheme 11)**. The reduction is performed by hydride transfer from the reduced form of the co-enzyme nicotinamide adenine dinucleotide phosphate

(NADPH) **(Figure 5)**. Metal ions such as zinc and magnesium are also frequently involved as co-factors in enzyme-catalysed reactions.

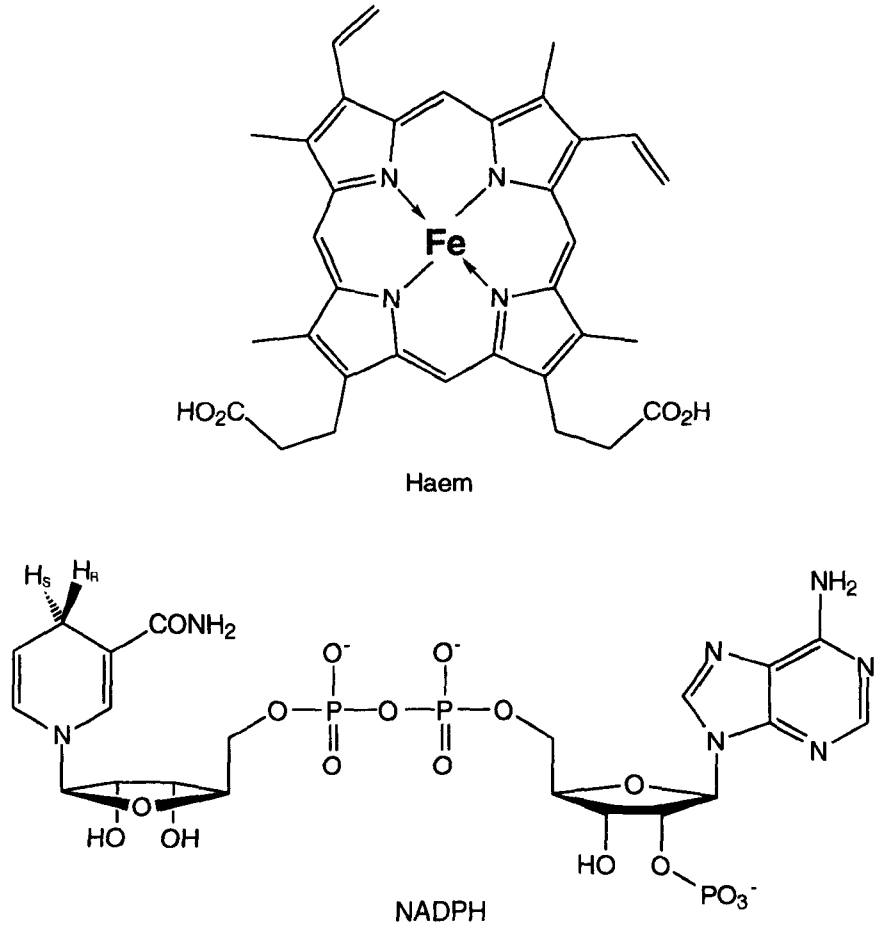


Figure 5 Structure of the Co-catalysts Haem and NADPH

1.05 Enzyme Catalysis[7]

Enzymes, like all catalysts, speed up the rate of attainment of equilibrium. They do not affect the position of equilibrium and are regenerated in their original form after reaction. The rate of any chemical transformation is determined by the difference in free energy between the starting materials and the high energy transition state. This energy difference is known as the free energy of activation, $\Delta G^{\ddagger}$. A catalyst is a species that can lower the energy of the transition state through a stabilising interaction or by offering an alternative lower energy route to the products. Commonly, enzyme catalysis proceeds through a number of 'low energy' intermediates and thus offers an 'optimum' route from starting materials to products **(Figure 6)**.

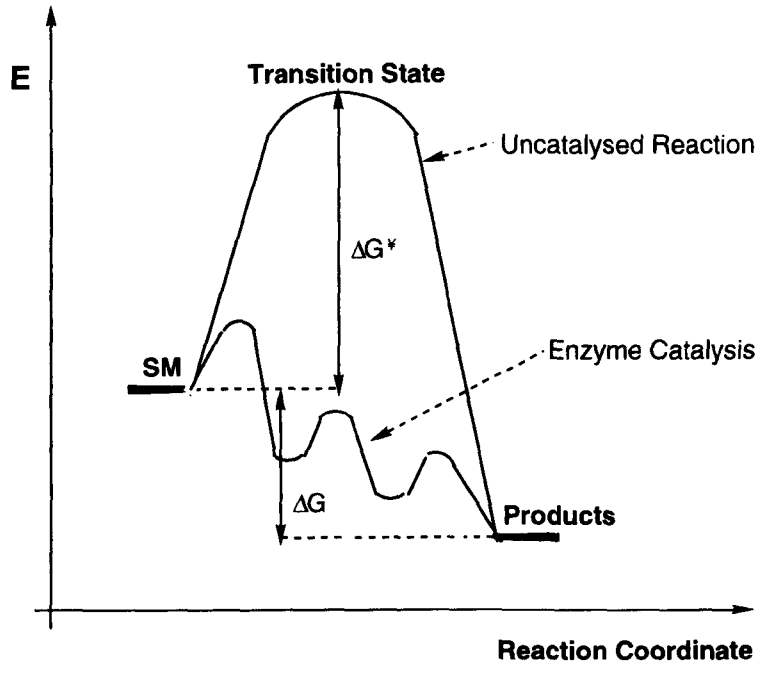


Figure 6 Energy Profile for an Uncatalysed and an Enzyme Catalysed Transformation

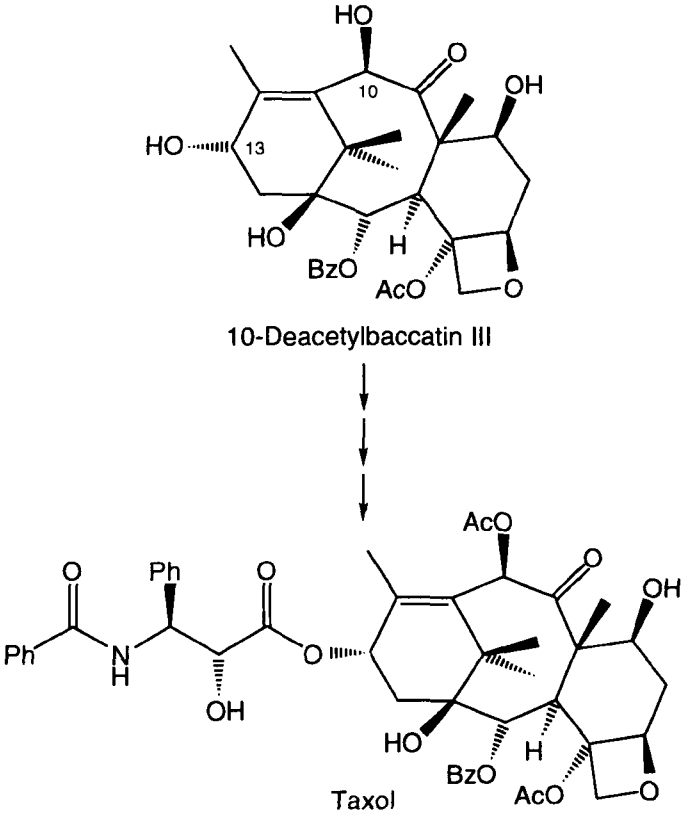
The nature of enzymes as catalysts dictates their effectiveness even at concentrations much lower than that of their substrates. However, in living systems many intercellular enzymes are present in relatively high localised concentrations, which may even exceed the concentration of the substrates. The substrates of these enzymes are likely to exist predominantly in their enzyme-bound form. Clearly this is a product of the evolutionary process, protecting unstable substrates from undesirable reactions. We might predict that the DHDPR enzyme is one such case. The substrate DHDPA is readily irreversibly oxidised by atmospheric oxygen to the aromatic derivative dipicolinic acid (DPA).

1.06 Enzymes as Chemotherapeutic Targets

Each biological entity is unique in its existence. Both modern and premodern medicine owe their successes to the inhibition of biological processes. If we target a biological transformation specific to an organism we can potentially selectively destroy the organism. The commonly targeted transformations are those of nucleic acids, enzymes and cell signalling processes. Many chemotherapeutic agents are derived from the massive library of compounds available from natural sources. In early days whole plants were used for medicinal purposes. Through time and scientific

advancement it has become possible to identify and isolate active constituents from the cocktail of chemicals of the natural source. We can draw an interesting analogy between early medicine and the current rapidly expanding field of combinatorial chemistry. Many modern day medicines are still based on the discovery of lead compounds from natural sources.

In modern drug development an understanding of biological processes at a molecular level allows us to design compounds for a specific purpose. The synthesis of drug compounds may be performed from scratch using readily available chemicals or by chemical derivatisation of a biological product which can be produced in sufficient bulk. The latter of the two methods is useful when the target compound requires many synthetic steps or involves formation of a complex stereochemical structure. An example of this is the recent much publicised anticancer drug Taxol™ **(Scheme 5)**. This compound occurs naturally at very low levels in the bark of *Taxus brevifolia*, the Pacific Yew Tree. Although the total synthesis of this compound has been achieved,[8,9,10] the method used for commercial purpose[11,12] involves a relatively short convergent synthesis utilising the readily available and more easily renewable biochemical intermediate 10-deacetylbaccatin III, from the leaves of *Taxus baccata L* **(Scheme 5)**.



Scheme 5 The Semisynthesis of Taxol

The importance of enzymes as biological catalysts has already been mentioned. Most biological transformations are kinetically disfavoured and rely on enzymes to promote the reaction. By inhibiting the action of enzymes we can effectively stop an undesirable process. The greatest problem in inhibiting enzymic activity is the selectivity of action. Many unrelated organisms have evolved different enzymes for important common purposes. Frequently the active sites of these enzymes have a great deal in common. As the acceptance of a substrate into an active site is made possible by an induced fit mechanism, then the inhibitor of one enzyme would very likely inhibit structurally related enzymes by a similar degree and would therefore display poor selectivity of action. This problem is equally troublesome in targeting cell signalling processes and nucleic acids. A clear illustration of this can be seen with the current problems in selective cancer chemotherapy.

Inhibition of enzymic activity can be either reversible or irreversible.[13] An irreversible inhibitor binds very strongly, either covalently or non-covalently, and essentially does not dissociate from the enzyme. Reversible inhibition is characterised by rapid dissociation and reassociation of the enzyme-inhibitor complex.

A competitive inhibitor is one which binds reversibly to the enzyme at the active site and therefore prevents the substrate from binding. Competitive inhibitors slow the rate of enzymic catalysis by reducing the number of substrate molecules binding to the active site. This effect can be minimised by increasing the ratio of substrate to inhibitor so that binding of the substrate is favoured. Molecules which are close analogues of the transition state of enzymic processes are commonly good competitive inhibitors.

A non-competitive inhibitor is one which binds away from the active site but disrupts the enzymic structure in such a way that the efficiency of the catalysis is diminished. A non-competitive inhibitor works by decreasing the turnover number rather than the number of substrate molecules bound to the active site. As a result the inhibition effect cannot be overcome by simply increasing the ratio of substrate to inhibitor. This subtle difference allows us to distinguish between the types of inhibition by studying the kinetics. If the extent of inhibition increases with concentration of inhibitor then competition for the active site is evident. This is a simplified view of inhibition study. Commonly inhibitors can bind both at and away from the active site and therefore display mixed inhibition.

1.07 Enzymes for Chemical Uses[14]

In designing inhibitors we look for analogues of similar shape and electronic structure to substrates or transition state intermediates. Usually the inhibitor is suitably functionalised so it will bind to the enzyme but not undergo enzyme catalysed reaction. Similarly, unnatural enzyme substrates are closely modelled on the natural substrates or transition state intermediates of the enzyme but differ in that they are suitably functionalised so as to undergo an enzyme catalysed chemical transformation. Individual enzymes vary enormously in their substrate specificity. Enzymes that display low substrate specificity are generally more useful as biotransformation catalysts as they will accommodate a variety of unnatural substrates. The fermentation of sugar into ethanol by yeast is a biotransfomation process which has been known for a very long time.

Proteins are an obvious source for the isolation of the genetically encoded amino acids. The oldest method of production of these important primary metabolites involves enzyme catalysed degradation of proteins. Indeed, the amino acids L-cysteine, L-glutamate, L-lysine, L-glutamine and L-arginine are still produced by microbiologically based processes.

Enzymes play an equally important commercial role in the production of secondary metabolites. Penicillins and cephalosporins are produced in fungi by *Penicillium* spp. and *Cephalosporium* spp. respectively. The annual world production of penicillin-G **(1)** and penicillin-V **(2)** **(Figure 7)** is approximately 12 000 tonnes. Many other commercially important penicillins are produced by chemical derivatisation of 6-aminopenicillanic acid **(3)** **(Figure 7)**. This important intermediate is produced from **(1)** or **(2)** by the bacterium *E. coli* or by the appropriate amidase isolated from the micro-organism.

Biotransformation catalysts play many other important roles in both small, laboratory scale and large, industrial scale chemical transformations. Tightly controlled enzyme structure ensures highly regioselective and stereoselective recognition processes and therefore highly selective catalytic activity. Following the recommendations of the International Union of Biochemistry enzymes are categorised into six groups: Oxidoreductases; Transferases; Hydrolases; Lyases; Isomerases; and Ligases. Oxidoreductases catalyse oxidation and reduction reactions involving the addition or removal of oxygen and hydrogen. An enzyme catalysing the reduction of a ketone to a secondary alcohol would fall into this class. Transferases catalyse the transfer of groups, such as amino groups, from one molecule to another. Hydrolases catalyse hydrolysis of molecules such as

esters and amides. Lyases catalyse the addition of HX (X ≠ OH) to alkene, imine and carbonyl groups. Isomerases catalyse isomerization process such as racemisation and epimerization. Ligases catalyse condensation reactions with the formation of C-O, C-N and C-C bonds.

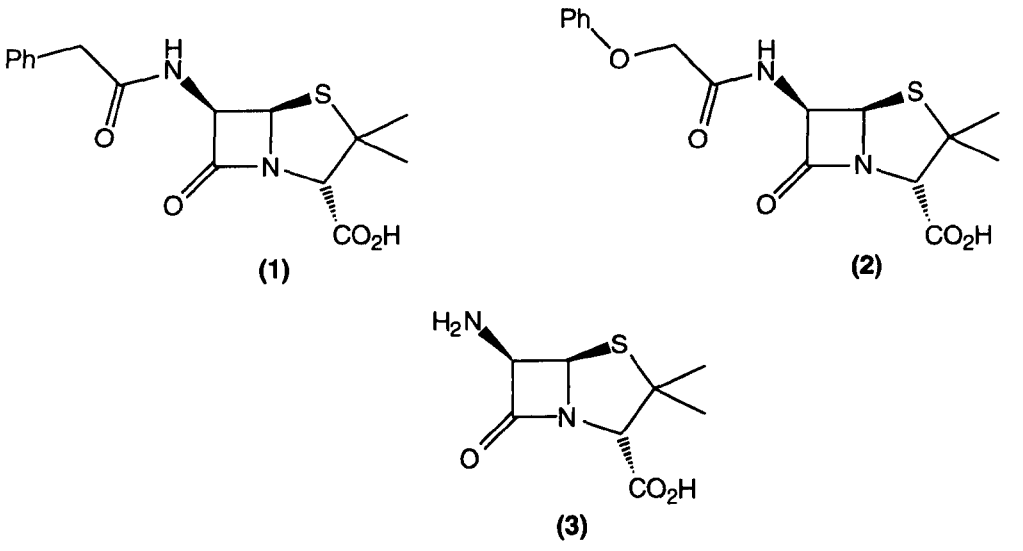


Figure 7 Commercially Important Penicillins

When using enzymes for biotransformations we can use either the whole cell or purified enzymes. There are advantages and disadvantages to each. When using whole cells we do not need to add any of the expensive co-factors required. Whole cells are cheap and readily available. However, in using whole cells we can encounter problems such as undesirable side reactions and difficult work up from large aqueous volumes. Transport of substrates into the cells and of products out of the cells, may also be troublesome. Using purified enzymes makes biotransformations easier to control and monitor. The transformations are highly selective, the work up is simplified and the use of organic co-solvents can be better tolerated. Purified enzymes can also be crystallised for X-Ray diffraction and modelling studies. The major disadvantage of using purified enzymes is the expense. Isolation of enzymes in reasonable yield often requires the organism to be genetically engineered to overexpress the required protein. Purification is also a difficult process, usually requiring several chromatographic steps to separate the required enzyme from the mixture of proteins and other biomolecules. Using purified enzymes may also require the addition of co-factors. Co-factor recycling may also be necessary.

1.08 Protein Purification

Electrophoresis, along with chromatographic techniques are the key tools in the purification of proteins.[15] Very high levels of purity can be achieved by crystallisation of pre-purified protein. Indeed the uniform structure of the ordered crystal lattice is essential for three dimensional structural elucidation by X-ray analysis.

The principle behind electrophoresis was mentioned briefly in section 1.03. The migration of each individual component in a mixture of macromolecules in an electric field depends on the field strength, the net charge on the macromolecule and the frictional coefficient. The frictional coefficient depends on the mass and shape of the migrating compound and the viscosity of the medium. Electrophoretic separations are most commonly performed on gels but can also be done on solid materials such as paper (cellulose). Polyacrylamide gels are often the ones of choice because they are chemically inert and easily manufactured with control over pore size. Separations by electrophoresis are roughly in the order of mass, with the smallest compounds migrating more quickly. pH gradients can also be used in electrophoretic separations because at the isoelectric pH of a protein the net charge is zero and the 'electrophoretic mobility' is also zero.

Electrophoretic techniques are limited in that they do not supply large quantities (milligrams) of the proteins in their native form. This is necessary for three dimensional structural and mechanistic studies. Other characteristics such as size, solubility, charge and specific binding affinity can be utilised to purify proteins. Proteins can be separated from small molecules by dialysis through a semipermable membrane. Centrifugation can be used in unison with dialysis to force the equilibrium towards removal of the small molecules, essentially the impurities are 'washed out.'

Another widely used technique is gel filtration which relies on size distribution **(Figure 8)**. The sample passes through a column containing a polymeric carbohydrate stationary phase with fixed pore sizes. Smaller molecules enter the pores and interact strongly. Larger molecules cannot enter the pores and so pass through the column more quickly. Gel filtration can be used to separate much larger quantities of protein than electrophoresis but the resolution is lower.

Ion exchange chromatography **(Figure 8)** separates molecules on the basis of their net charge. For example a macromolecule with a net positive charge will bind strongly to a stationary phase containing carboxylate groups (cation exchanger), whereas a macromolecule with a net negative charge will pass through this system very quickly. pH gradients are used in ion exchange

chromatography to vary net charges when required. Salt solutions can also be used to 'displace' the bound macromolecules in a competitive manner.

Affinity chromatography **(Figure 8)** is a powerful method of protein purification. A substrate of the enzyme is incorporated in the stationary phase, say glucose. The enzyme 'recognises' the glucose and binds strongly. Other proteins and impurities are washed through. The desired protein is then displaced by washing with a solution of 'free' glucose. All of these purification techniques have been used by different groups to purify DHDPS from a variety of sources.

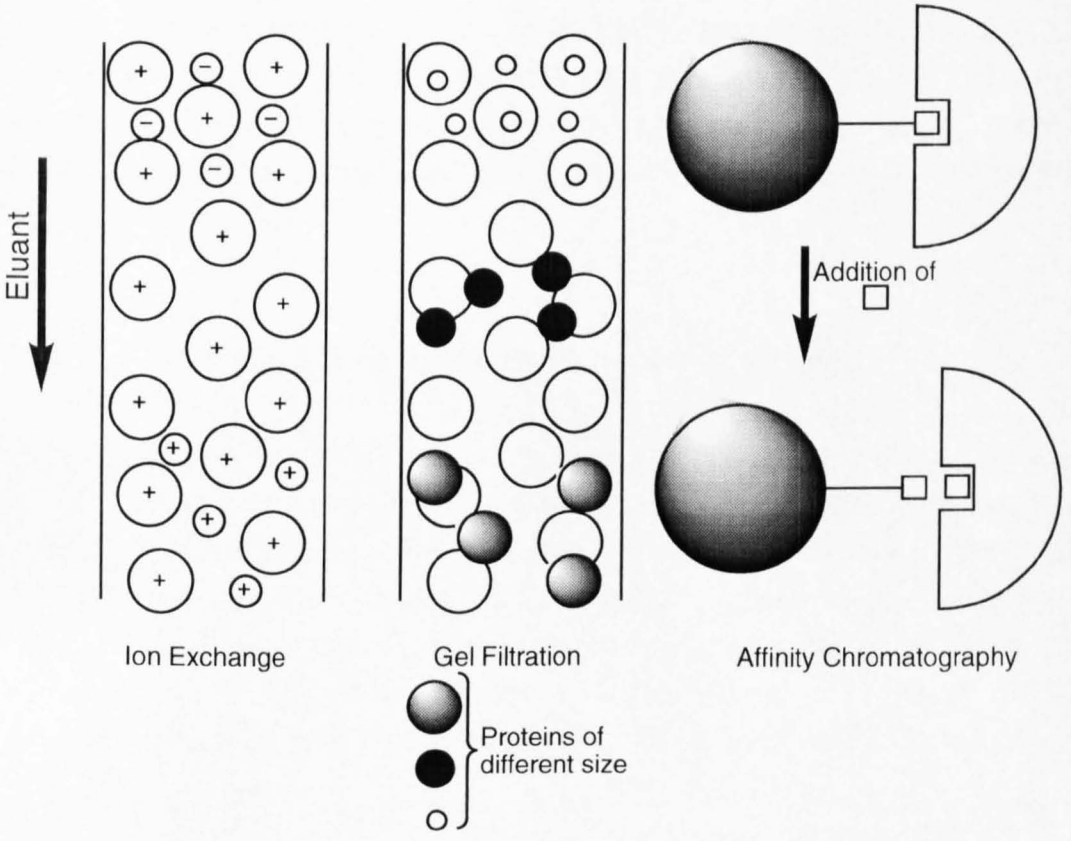


Figure 8 Chromatographic Techniques

Recently the new technique of displacement chromatography has proved to be of value.[16] Displacement chromatography involves loading of the impure material onto a stationary phase as normal. The column is then eluted with a low molecular weight polyelectrolyte displacer (M_w < 2000) which totally displaces the other components from the adsorbent material. This results in the formation of a highly concentrated zone of components and competition for the adsorbent sites. As the displacer travels down the column the poorer binding components are eluted in the solvent flow first. This high

concentration technique results in excellent resolution with potential for separating closely related materials and for large scale industrial purifications which suffer from loss of resolution on scale up. Separation of the desired product from the displacer can be troublesome but ultrafiltration is a useful procedure to achieve this end.

Chapter 2 The Biochemistry of L-Lysine

2.01 Introduction

In this chapter we will concentrate on the biochemical importance of the α-amino acid L-lysine. The need for lysine in mammalian nutrition and the chemical synthesis of this amino acid will be discussed. The biosynthetic pathways to this amino acid found in fungi, plants and bacteria and the value of targeting these pathways for chemotherapeutic benefit will be considered. Our studies are concentrated on the transformations promoted by the first two enzymes of the diaminopimelate (DAP) biosynthetic pathway to L-lysine: dihydrodipicolinate synthase (DHDPS) and dihydrodipicolinate reductase (DHDPR). These enzymes will be discussed in detail. In the following chapters we will look at the synthesis and testing of potential inhibitors and substrates of these enzymes.

2.02 The Importance of L-Lysine

The requirement for lysine in mammalian diets dictates the commercial importance of this amino acid. The estimated daily nutritional requirements of the nine 'essential' amino acids are detailed in **Table 1**. Proteins from meat, fruit, vegetables and dairy produce are the main sources of amino acids in the human diet. In the body proteins are metabolised by hydrolase enzymes into their amino acid units. The free amino acids are then available for the genetically controlled synthesis of proteins essential for sustaining life.

Amino acid	Required daily amount (mg/kg)
Isoleucine	10
Leucine	14
Lysine	12
Methionine	13
Phenylalanine	14
Threonine	7
Tryptophan	4
Valine	10

Table 1 Nutritional Requirements of the Essential Amino Acids

The nutritional value of proteins in the diet is dependent on the quantity and balance of their constituent amino acids. The balance of amino acids in the diet is very important because protein synthesis is restricted by deficient levels of any one of the amino acids. In plant protein, and especially cereal protein, L-lysine is the limiting amino acid. It is common practice for food manufacturers to supplement their produce with synthetic sources of important nutrients such as L-lysine.

As well as the life sustaining role of L-lysine in proteins, L-lysine also plays an important role as a metabolite in the biosynthesis of some alkaloids. Alkaloids are a very large and diverse family of nitrogen-containing natural products with a wide range of biological activities. Typical examples are: cocaine; nicotine; mescaline; morphine and strychnine **(Figure 9)**.

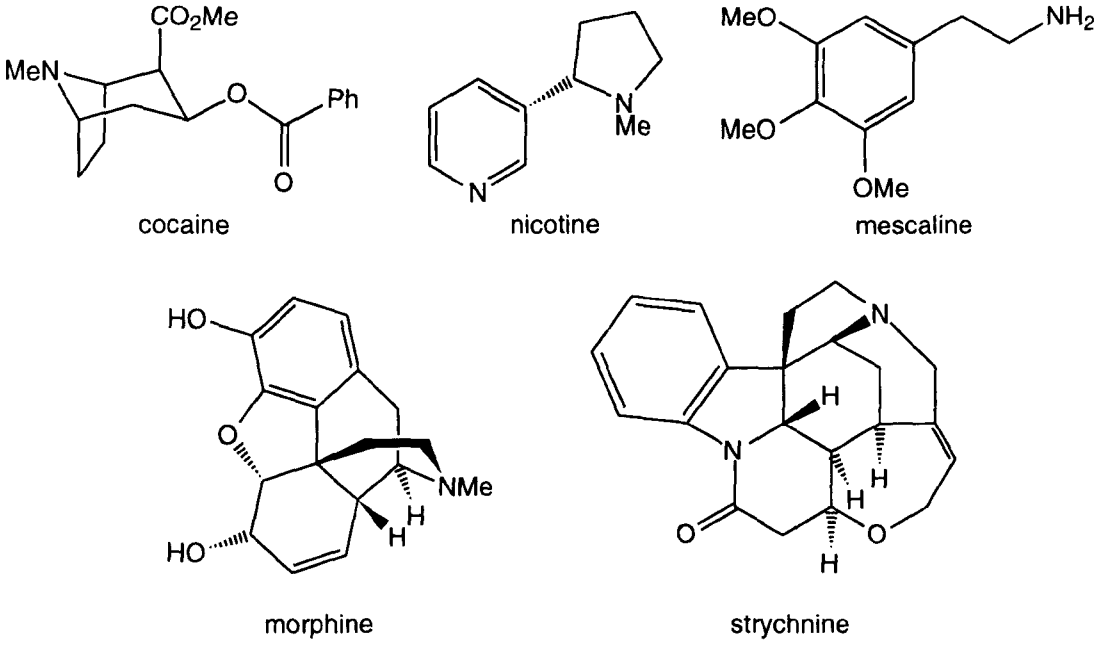
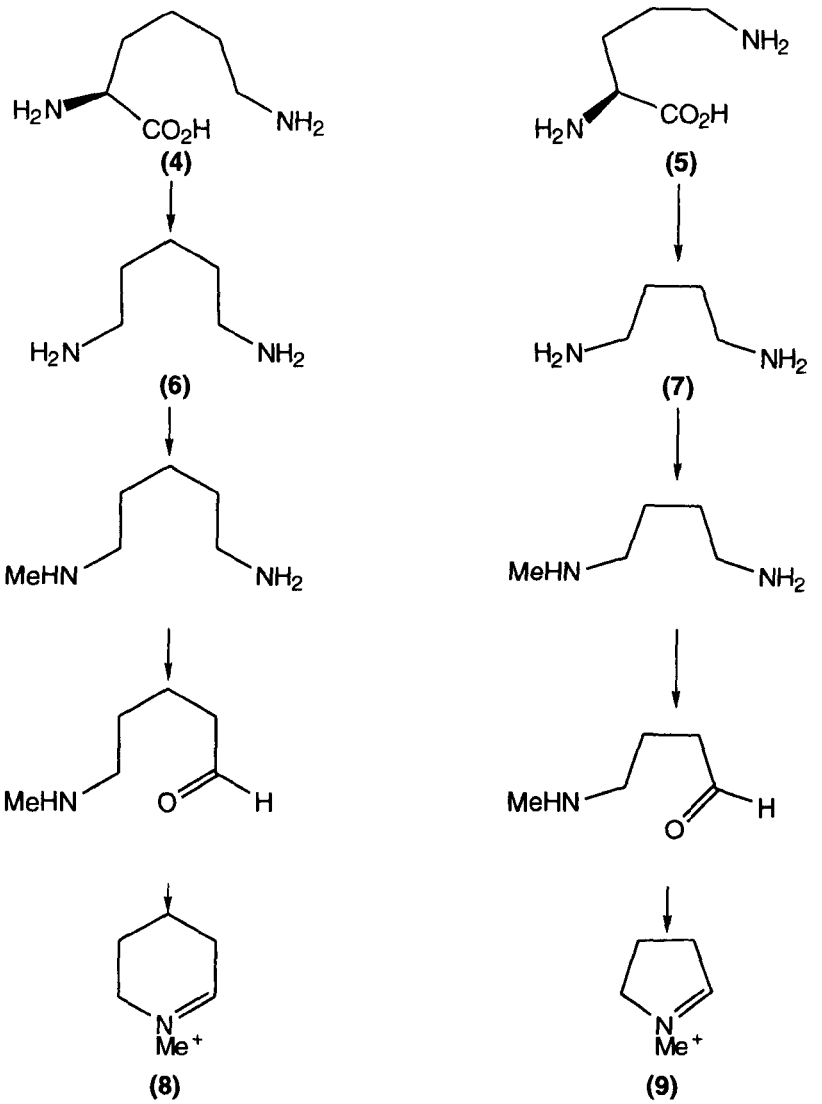


Figure 9 Biologically Active Alkaloids

The early stages of the biosynthetic pathways involving L-lysine **(4)** and its lower homologue L-ornithine **(5)** to form monocyclic systems are very similar **(Scheme 6)**.[17] The amino acids undergo decarboxylation to the diamines cadaverine **(6)** and putrescine **(7)**, respectively. *N*-Methylation by *S*-adenosylmethionine, transamination (primary amine to aldehyde) and subsequent cyclisation produces the iminium salts **(8)** and **(9)**. By this pathway and variations on it L-lysine and L-ornithine are incorporated into pyrrolidine, piperidine, pyrrolizidine and quinolizidine alkaloids such as hyoscyamine, pseudopelletierine, retronecine and lupinine **(Figure 10)**.[18] The biosynthesis of the pyrrolidine alkaloid hyoscyamine **(10)**, from the iminium salt **(9)** is illustrated in **Scheme 7**.[19]



Scheme 6 Lysine and Ornithine in the Biosynthesis of Alkaloids

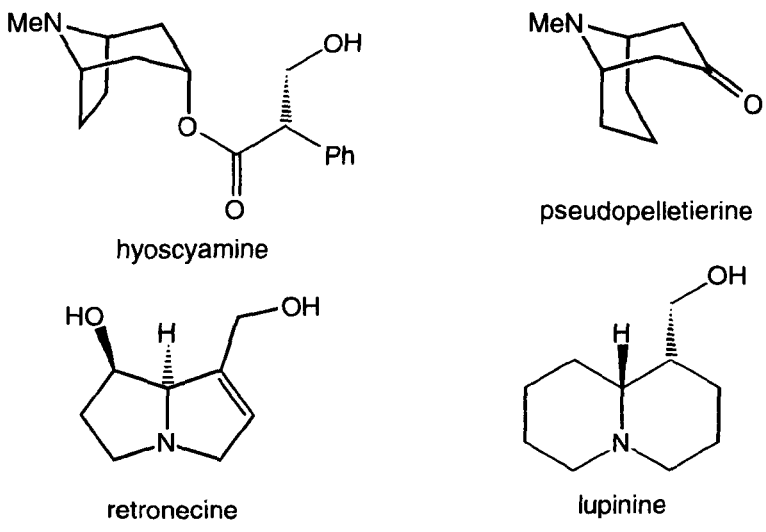
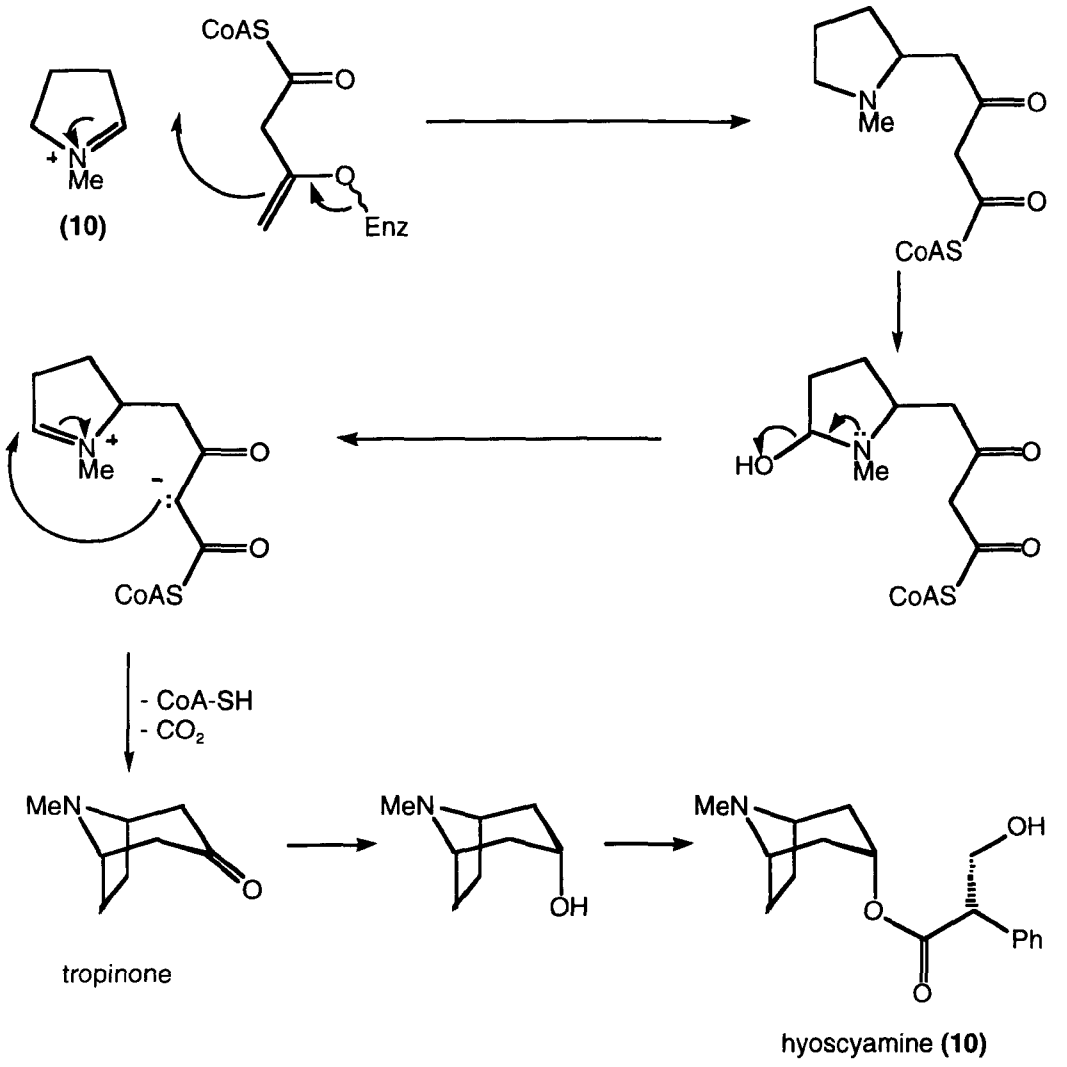


Figure 10 Alkaloids Derived from Lysine and Ornithine



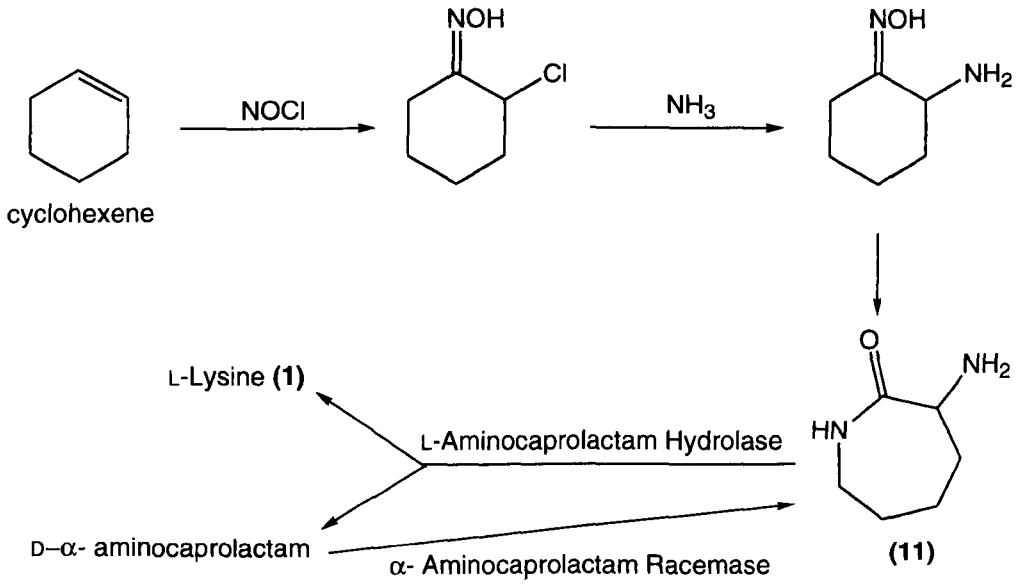
Scheme 7 Biosynthesis of Hyoscyamine

2.03 Commercial Production of L-Lysine

The important biochemical role of the generic α-amino acids means that there is an industrial demand for all of them for research and commercial purposes. All of the amino acids found in proteins are synthesised industrially. Monosodium glutamate and L-lysine are produced in the largest bulk with annual production of 370 000 and 40 000 tons, respectively.[20] Commercial production of amino acids is performed by extraction from proteins, enzymatic processes and by chemical synthesis. Extraction from proteins involves their formation by fermentation by microorganisms. Modern genetic engineering techniques allow the production of most proteinogenic amino acids by fermentation methods. Auxotrophic mutants are microorganisms which lack

one or more of the enzymes required for the biosynthesis of an amino acid. Mutants of *Corynebacterium glutamicum* and *Escherichia coli* which lack homoserine are found to accumulate L-lysine.[21] Lysine is a member of the aspartate family of amino acids. Blocking the route at the branching point to homoserine directs the biosynthesis towards lysine (see **Scheme 10**). Regulatory mutants are genetically modified to prevent the slowing or stoppage of the production of the required material. This usually involves the mutation of a protein in the biosynthetic pathway so that the organism cannot respond to the appropriate signalling mechanisms. L-Lysine can be produced in this manner by the mutated bacterium *Brevibacterium flavum*.[22]

Industrial production of L-lysine from the synthetic unit DL-α-aminocaprolactam (**11**) utilises enzymatic processes.[23] Biotransformation of L-α-aminocaprolactam with L-aminocaprolactam hydrolase produces L-lysine and the resulting D-aminocaprolactam is recycled by the enzyme α-aminocaprolactam racemase (**Scheme 8**).



Scheme 8 Commercial Production of L-Lysine

Chemical synthesis of the 'natural' amino acids is generally less useful because of the requirement for enantiomerically pure material. Although the field of asymmetric synthesis is well developed, the cost of chiral auxiliaries and catalysts counter the viability of this methodology for commercial purpose. Chemical synthesis is however very useful for the synthesis of 'unnatural' and novel amino acids. The synthesis of novel amino acids is very important in enzyme chemistry. Many enzyme metabolites are amino acids and the

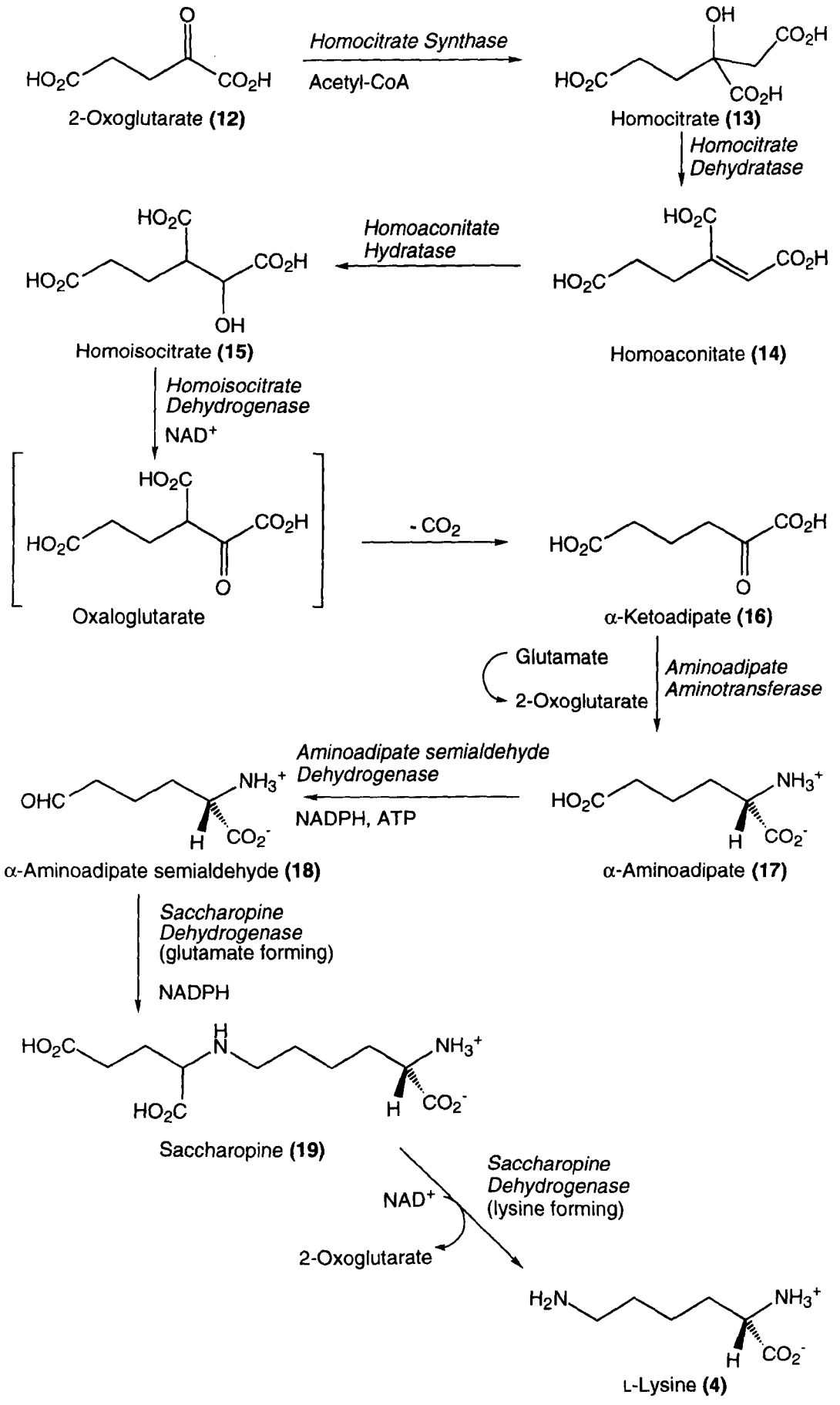
synthesis of novel analogues of enzyme substrates is key to the study of the behaviour of an enzyme. Indeed, the synthetic work involved in our studies of the enzymes DHDPS and DHDPR has been almost exclusively in the field of amino acid chemistry. Lysine can be synthesised by a number of classical chemical procedures for the synthesis of α-amino acids such as alkylation of glycine derivatives; the Strecker synthesis and Curtius, Hofmann and Schmidt rearrangements. The synthesis of α-amino acids will be reviewed in the next chapter. Resolution of 'natural', 'unnatural' and novel racemic α-amino acids can be achieved by enzymic and diastereomeric derivatisation techniques. In our work we have utilised the enzymic resolution of *N*-acetylated amino acids by the enzyme porcine kidney acylase (PKA) and we will discuss this in the following chapters.

2.04 Biosynthesis of L-Lysine

The biosynthesis of L-lysine in nature is observed *via* two mutually exclusive pathways.[24] The α-aminoadipate (AAA) pathway **(Scheme 9)** is adopted in yeasts and fungi and the diaminopimelate (DAP) pathway **(Scheme 11)** is found in bacteria and higher plants. In the next section we will briefly discuss the AAA pathway. The remainder of this chapter concentrates on a detailed review of the DAP pathway.[25]

2.05 The AAA Biosynthetic Pathway to L-Lysine

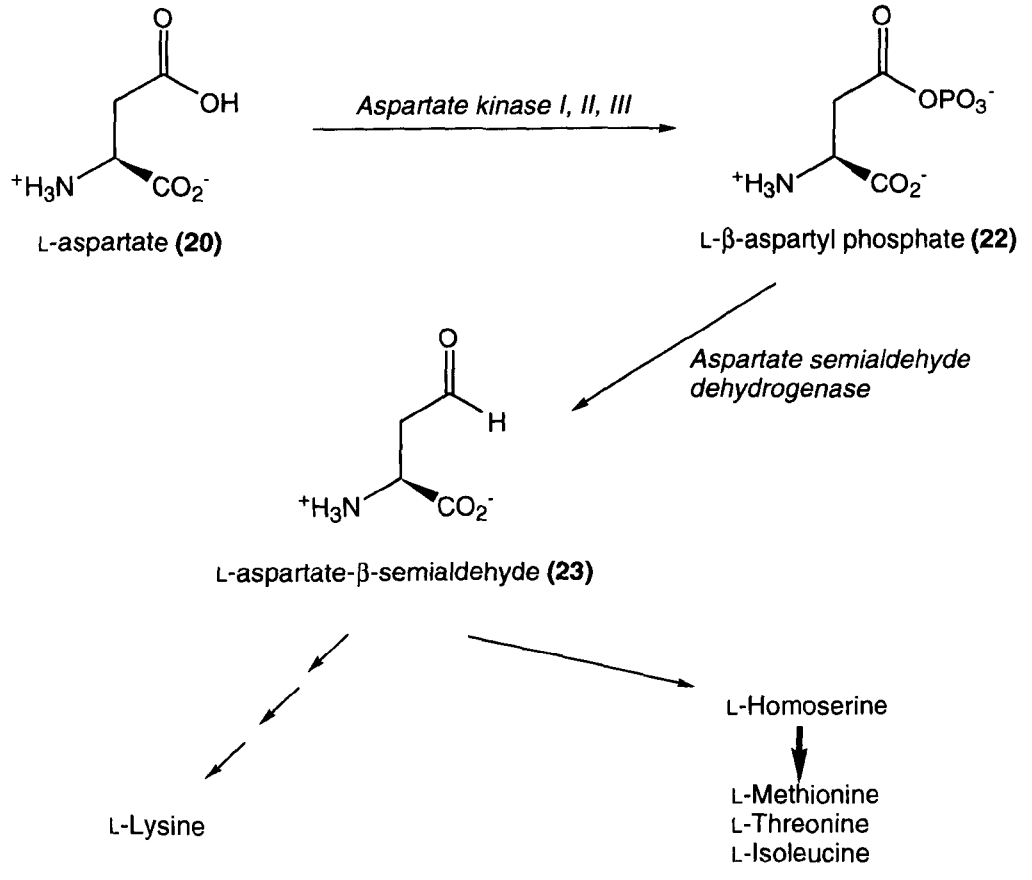
The starting point for the AAA pathway is 2-oxoglutarate **(12)**, a product of the Krebs cycle. Labelling studies with ^{14}C-acetate in *Saccharomyces cerevisiae*[26] and *Torulopis utilis*[27] have shown that the carbon skeleton of this important metabolite is derived from acetate units. The first step of the AAA pathway involves carboxylation of 2-oxoglutarate with acetyl-CoA to form homocitrate **(13)**. Homocitrate is isomerised to homoisocitrate **(15)** by dehydration to homoaconitate **(14)** and subsequent rehydration. Oxidation, by NAD^+, and decarboxylation gives α-ketoadipate **(16)**. The key amino acid intermediate α-aminoadipate **(17)** is produced by aminotransfer to α-ketoadipate from glutamate and reduction. Regiospecific reduction gives α-aminoadipate semialdehyde **(18)**, which undergoes condensation with glutamate and further reduction to give saccharopine **(19)**. Lysine **(4)** and 2-oxoglutarate **(12)** are then produced from saccharopine by reaction with NAD^+.



Scheme 9 The AAA Biosynthetic Pathway to L-Lysine

2.06 The DAP Biosynthetic Pathway to L-Lysine

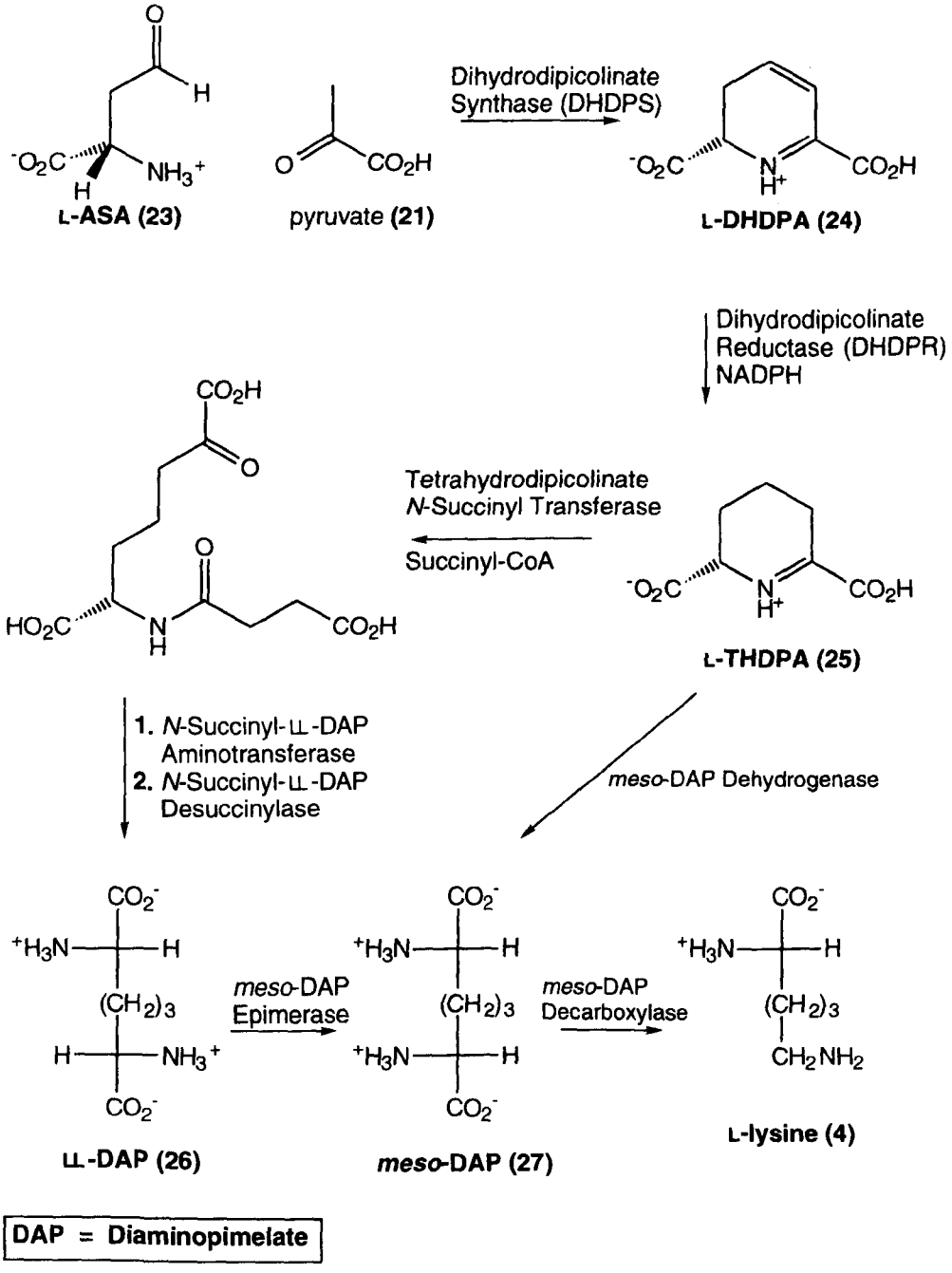
In Chapter 1 we mentioned that lysine, asparagine, methionine, threonine and isoleucine belong to the aspartate family of amino acids **(Scheme 10)**. Aspartate **(20)** is derived from oxaloacetate *via* the citric acid cycle. In plants and bacteria, lysine is synthesised from aspartate by the diaminopimelate pathway. The basic carbon skeleton of lysine is formed by the condensation of aspartate and pyruvate **(21)** units. Biosynthesis of the aspartate family of amino acids involves the formation of the common intermediate aspartate-β-semialdehyde **(23)**.[28] This important metabolite results from selective phosphorylation and reduction of aspartate, catalysed by aspartate kinase and aspartate semialdehyde dehydrogenase, respectively **(Scheme 10)**. At this point the biosynthetic pathway branches. Biosynthesis of lysine proceeds through a chain extending condensation with pyruvate. The other amino acids are biosynthesised *via* homoserine.



Scheme 10 The Aspartate Family of Amino Acids

The DAP biosynthetic pathway to L-lysine is illustrated in **Scheme 11**. L-Dihydrodipicolinate synthase (DHDPS) catalyses the condensation of L-aspartate-β-semialdehyde (L-ASA) **(23)** with pyruvate **(21)** to produce L-2,3-dihydrodipicolinic acid (L-DHDPA) **(24)**.[29] Recent X-Ray and NMR studies by

Blickling and co-workers[30] have shown that the product of DHDPS at pH values above 9 is *(2S,4S)*-4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (HDPA) **(33) (Scheme 14)**. Rapid decomposition of HDPA was noted at physiological pH. UV studies suggest that the decomposition proceeds by dehydration and subsequent oxidation to the stable aromatic derivative dipicolinic acid (DPA) **(28)**.



Scheme 11 The DAP Biosynthetic Pathway to L-Lysine

The second step of the DAP pathway involves reduction by nicotinamide adenine dinucleotide phosphate (NADPH) under catalysis by the enzyme L-dihydrodipicolinate reductase (DHDPR) to L-2,3,4,5-tetrahydrodipicolinic acid (L-THDPA) **(25)**. It is not certain if the substrate of the second enzyme is HDPA **(33)** or L-DHDPA **(24)**, although rapid dehydration at physiological pH is likely.

At this point the pathway splits; there are three known pathways to *meso*-DAP **(27)** and L-lysine **(4)**. The succinylase[31,32] and dehydrogenase[33,34,35] pathways are illustrated in **Scheme 11**. The third is the acetylase[36] pathway which differs from the succinylase pathway only in that an acetyl group is transferred on ring opening of THDPA **(25)**. Dual pathways are known to occur in some bacteria. The bacteria *Corynebacterium glutamicum*[37] and *Mycobacterium bovis*[38] are known to utilise both the succinylase and dehydrogenase pathways from THDPA **(25)** to *meso*-DAP **(27)** and lysine **(4)** and *Bacillus maceranas*[39] is known to use both the acetylase and dehydrogenase pathways.

In the dehydrogenase pathway L-THDPA is converted directly into *meso*-DAP **(27)** by the enzyme *meso*-diaminopimelate dehydrogenase.

The first step of the succinylase pathway is the L-tetrahydrodipicolinate *N*-succinyltransferase catalysed succinylation of L-THDPA by succinyl-coenzymeA. Subsequent amination by L-glutamate is catalysed by the enzyme *N*-succinyl-LL-diaminopimelate aminotransferase. Desuccinylation, catalysed by the enzyme *N*-succinyl-LL-diaminopimelate desuccinylase, produces LL-diaminopimelic acid **(27)**, which is selectively epimerised to *meso*-DAP **(28)** by the enzyme *meso*-diaminopimelate epimerase. *meso*-DAP **(28)** is common to all three pathways and is selectively decarboxylated to L-lysine **(4)** by the enzyme *meso*-diaminopimelate decarboxylase.

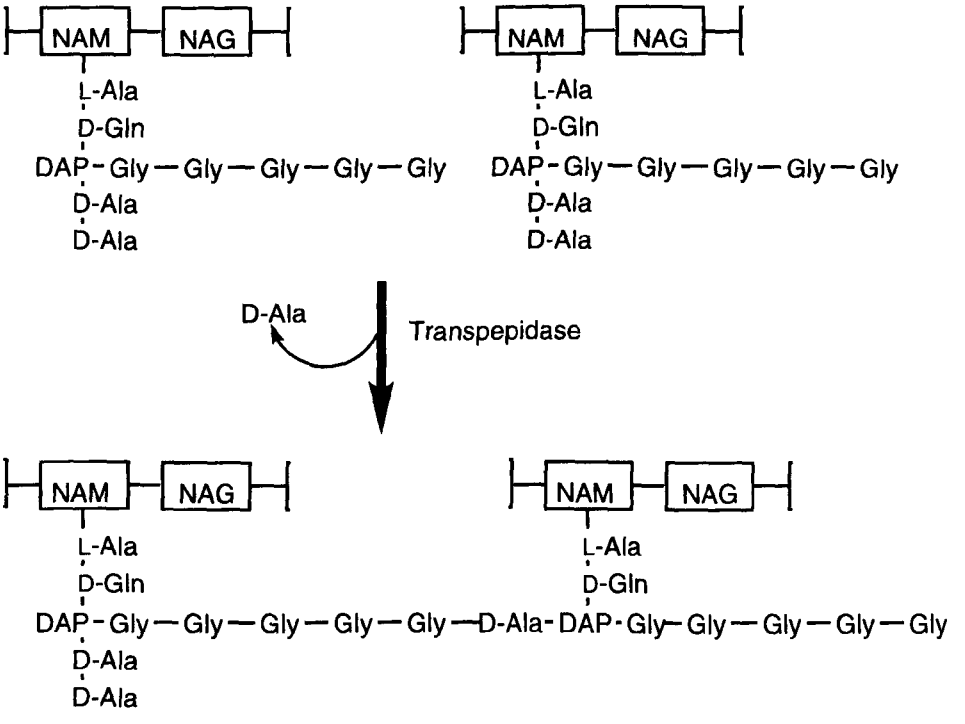
The later branching of pathways makes the search for potent inhibitors of the first two enzymes of the DAP biosynthetic pathway to L-lysine and *meso*-DAP particularly attractive. By inhibiting the pathway prior to the branching point we have potential for broad spectrum antibacterial activity.

2.07 Inhibition of Lysine Biosynthesis

A good understanding of a biological system can be of great advantage if we wish to utilise it. By far the most common example of this is the design of enzyme inhibitors for the purpose of blocking biological reactions. A very large proportion of modern day chemotherapeutic medicines rely on specifically

inhibiting undesirable biological transformations in the body. Similarly, enzyme inhibitors play an important role in agriculture, for the same reason. Understanding of a biological system can also be of great use to the biochemist and geneticist in manipulating the overexpression of a desired protein for isolation and purification. Indeed, we have relied heavily on our biochemical colleagues for the isolation and purification of the DHDPS and DHDPR enzymes for our studies.

The biosynthesis of lysine in plants and bacteria is not fully understood. The earlier enzymes of this pathway, namely the condensing enzyme dihydrodipicolinate synthase (DHDPS) and the reductive enzyme dihydrodipicolinate reductase (DHDPR), have received little attention. Our studies have concentrated on the first two steps of this biosynthetic pathway, catalysed by DHDPS and DHDPR. Labelling studies have confirmed the occurrence of *meso*-2,6-diaminopimelic acid (*meso*-DAP) **(38)** in the pathway to lysine in pea chloroplasts.[40] *meso*-DAP is utilised in the cell wall biosynthesis of many Gram positive and Gram negative bacteria. *meso*-DAP is attached to a *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) polymer *via* a short peptide unit and is employed in a cross-linking role between pentapeptide units **(Scheme 12)**. This key role of *meso*-DAP lends mechanical strength to the bacterial cell wall.[41]



Scheme 12 Bacterial Cell Wall Biosynthesis

Clearly in the DAP biosynthetic route to L-lysine we have all the attributes for targeting for antibacterial agents. Firstly, the amino acid is 'essential' for mammals and must therefore be supplied as part of the diet. Consequently enzyme inhibitors of the DAP pathway are likely to display low levels of mammalian toxicity. Secondly, by inhibiting the production of *meso*-DAP, we are denying the bacteria a key metabolite. Analogies can be drawn with the mode of action of the penicillin antibiotics where disruption of the bacterial cell wall biosynthesis results in osmotic stress and ultimate cell rupture.

2.08 DHDPS as a Biotransformation Catalyst

Another area of growing interest in enzyme chemistry is study of biotransformations with unnatural substrates. The DHDPS enzyme is of particular interest here because there are relatively few known enzymes which catalyse carbon-carbon bond formation with unnatural substrates.[42] It is understandable that achieving this goal with the high regioselectivity and stereoselectivity and mildness of conditions commonly associated with enzymes is of great value in organic chemistry.

One problem encountered in using isolated enzymes such as purified DHDPS is the regeneration of co-factors. In some cases this problem has been solved on a scale of several moles of substrate. The condensation catalysed by DHDPS involves imine formation between amino and ketone groups followed by aldol condensation and dehydration between an activated methyl group and an aldehyde. This process involves the loss of two molecules of H_2O and does not involve any co-factors. Another factor in our favour is that the product of the condensation, the thermodynamically disfavoured DHDPA, is rapidly oxidised in air to the aromatic derivative dipicolinic acid. This forces the conversion of substrates into product to completion but unfortunately removes the stereocentre in the process. Recent studies[30] suggest that keeping the pH above 9 will preserve the *S*-stereochemistry at both the 2 and 4 positions.

Because of the high substrate selectivity displayed by enzymes, inhibitors and unnatural substrates are very commonly closely related structural analogues of the natural substrates. In the following chapters we will discuss the synthesis of the natural substrates of the DHDPS and DHDPR enzymes and the synthesis of potential substrates and inhibitors modelled on each of the natural substrates.

2.09 Dihydrodipicolinate Synthase (EC 4.2.1.52, dapA)

Characterisation of DHDPS has been achieved from bacteria and a range of plants including maize seedlings (*Zea mays L.*),[43] spinach (*Spinacia oleracea*)[44] and wheat germ (*Triticium aestivum*). The enzyme has been purified from wheat cell suspension cultures, tobacco leaves (*Nicotiana sylvestris*) and bacteria (*E. coli*[29]). The protein has been sequenced in *E. coli*, *C. glutamicum*, wheat and maize[45,46] and has been shown to exist as homotetramers with four identical sub-units.

For our studies we have used DHDPS purified from *E. coli* and are grateful to our biochemical colleagues here at Glasgow University for the overexpression and purification of both the DHDPS and DHDPR enzymes from this source. Purification of DHDPS of over 5000 fold from crude cellular extracts of *E. coli W* was first performed by Shedlarski and Gilvarg.[29] They were able to study the enzyme and found that it was indeed a homotetramer comprising of four identical subunits. The molecular weight of the sub unit was determined to be 33 000 by SDS polyacrylamide gel electrophoresis.[47] The amino acid sequence[45] shows an absence of the amino acid methionine from the protein. The nucleotide sequence of the *dap*A gene locus predicts that the molecular weight of the protein produced from that gene sequence should be in the region of 31 372. The molecular mass of the native protein was estimated by the more gentle technique of gel permeation chromatography. The molecular weight obtained by this technique was 134 000; in agreement with the proposed existence of a tetrameric native form of DHDPS.[29,47,48]

Purification of DHDPS from pea (*Pisum sativum*) was achieved by Schar and co-workers.[49] The protein from this source proved to have some interesting characteristics. The protein is a homotrimer of molecular weight 127 000 and sub-unit molecular weight 43 000. It was found that the native protein contained 22 units of methionine in its composition, compared to zero in the composition of *E. coli* DHDPS. This suggests that these DHDPS enzymes are of a different evolutionary origin. Interestingly, the initial step of the enzymic mechanism was found to be common, namely, the formation of a Schiff base between pyruvic acid and the enzyme.

2.10 Purification of DHDPS

A typical purification procedure for DHDPS, summarising the relative activity after each step, is detailed in **Table 2**. This method for the purification of *E. coli* DHDPS was elucidated by Borthwick,[47,50] a former biochemical co-worker here at Glasgow University. The cells were grown by incubation of an overproducing strain of *E. coli* (MV1190/pDA2) in MM63 minimal growth medium supplemented with ampicillin at 30 °C for 48 h. The cells were broken in a French press at 95 MPa and centrifuged to remove particulate matter. Heating to 70 °C and further centrifugation removed further unwanted proteinogenic material. The sample was then chromatographed on an DEAE-Sephacel anion exchange resin, eluting with an increasing gradient of potassium chloride (0.1 to 1.0 M). The fractions showing DHDPS activity by UV assay were pooled and concentrated by dialysis. Ammonium sulfate was added to a final concentration of 0.5 M and the sample was further chromatographed on a phenyl-Sepharose column (hydrophobic interaction). The column was eluted with a decreasing linear gradient of potassium chloride (0.0 to 0.5 M) then fractions were pooled and dialysed as previously. Final chromatographic purification on a Mono-Q FPLC column (HR 10/10) was carried out by eluting with a two phase gradient of 0.1 to 0.5 M potassium chloride over 20 minutes and then a gradient of 0.1 to 0.5 M for a further 40 minutes. The active fractions were pooled and dialysed once more and then stored as a 50% buffered aqueous / glycerol solution at -20 °C. the overall purification using this method was 49 fold. The enzyme can be obtained free from glycerol by centrifugation in a Centricon, a membrane filtration device, washing with approximately 4 ml of water.

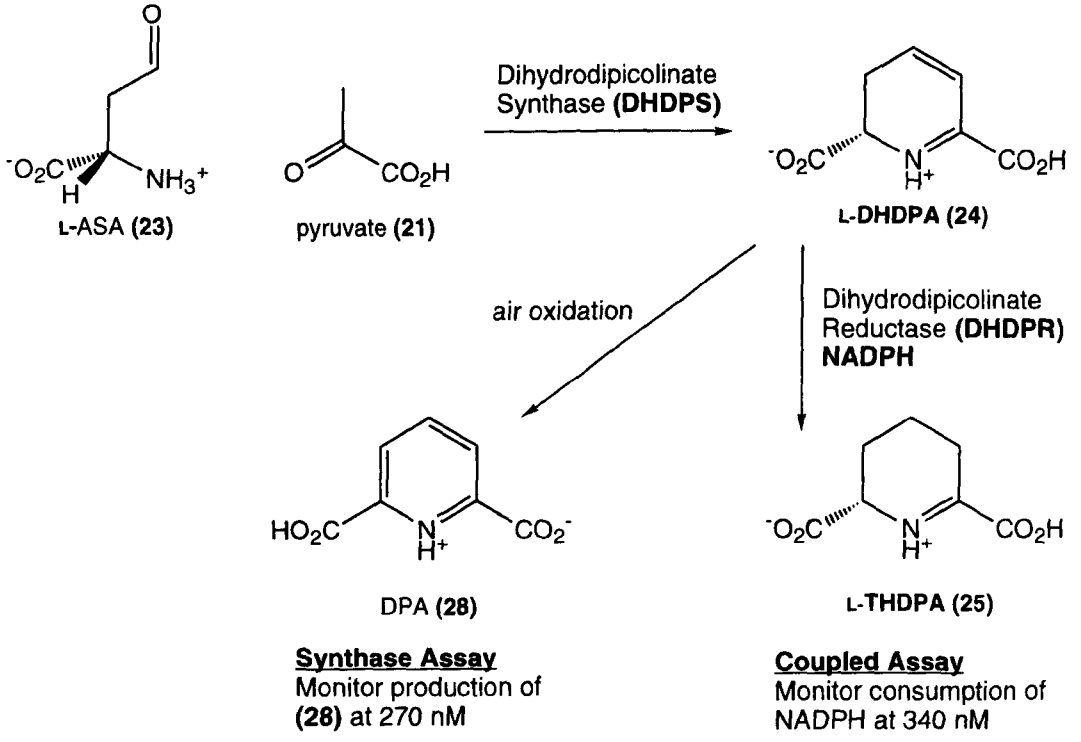
Step	Volume (ml)	Conc. (mg/ml)	Total (mg)	Activity (I.U.)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	38	31	1180	1240	1.1	100	1.0
Heat step	30	10	300	940	3.1	75	3.0
DEAE-Sephacel	170	0.43	72	2710	38	218	35
Phenyl-Sepharose	192	0.25	48	2110	44	170	42
Mono-Q	35	1.0	35	1820	52	146	49

Table 2 Purification of DHDPS from E. coli (MV1190/pDA2)

2.11 Characterisation of DHDPS Activity

Three assay systems have been developed to monitor DHDPS activity. The first involves monitoring the interaction between the enzymic product, DHDPA, and *o*-aminobenzaldehyde at 540 nm **(OAB assay)**.[51] The self condensing nature of *o*-aminobenzaldehyde, the chemical instability of DHDPA and the unidentified nature of the adduct make this a rather ambiguous assay. For this reason we have not used it in our studies.

The second and most commonly used assay involves monitoring the production of the aromatic compound dipicolinic acid (DPA) **(28)** at 270 nm **(synthase assay)**. DPA is produced by spontaneous air oxidation of DHDPA **(24)** **(Scheme 13)**. In Chapter 6 we will detail the limitations of this assay for inhibition studies. We will also briefly mention our development of a related new assay system for the qualitative detection of substrate activity with DHDPS. This assay involves *in situ* deprotection of *N*-acetylated amino acids with the enzyme porcine kidney acylase (PKA) and is useful for unstable substrates.



Scheme 13 Established Assay Systems for DHDPS and DHDPR

The third assay is a coupled assay system involving both the DHDPS and DHDPR enzymes **(coupled assay)**. In this assay the consumption of the

co-factor, NADPH, is monitored at 340 nm **(Scheme 13)**. This assay is not subject to the same limitations as the previously mentioned synthase assay and has proved to be of great value in our studies. Limitations arise in inhibition studies when we wish to determine the competitive nature with respect to the second enzyme, DHDPR. In Chapter 6 we will detail our work on a new assay system for the DHDPR enzyme alone.

Using these assay systems with DHDPS derived from a number of sources the K_m value for ASA **(23)** has been found to vary between 0.4 mM and 3.1 mM. The K_m value for pyruvate **(21)** varies between 0.5 mM and 11.8 mM. Kinetic studies in *E. coli*,[52] wheat[53] and maize[54] (*Z. mays L.*) suggest that the enzymic reaction involves binding of pyruvate **(21)**, with loss of a molecule of water, followed by the binding and condensation with ASA **(23)**. In *E. coli* DHDPS displays Ping Pong BiBi kinetics.[29,48,53]

2.12 Mechanistic Studies on DHDPS

Electrospray Mass Spectrometry (ESMS) is a mild method of studying compounds with large molecular masses. The technique involves formation of polycations by the polyprotonation of the compound in a 0.2% formic acid solution in water/acetonitrile (1:1). A fine spray of this solution is then introduced into the mass spectrometer under high vacuum. Under these conditions the solvents are rapidly evaporated, leaving the 'dry' polycationic species. The polycations are then deflected by an electrostatic/magnetic field for detection. This technique is also of use for large molecules other than peptides, such as nucleic acids and man-made polymers and macrocycles. An illustration of an ESM spectrometer is shown in **Figure 11**.

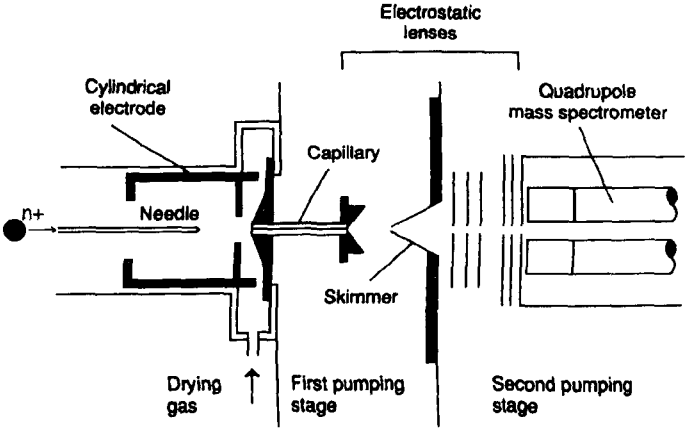
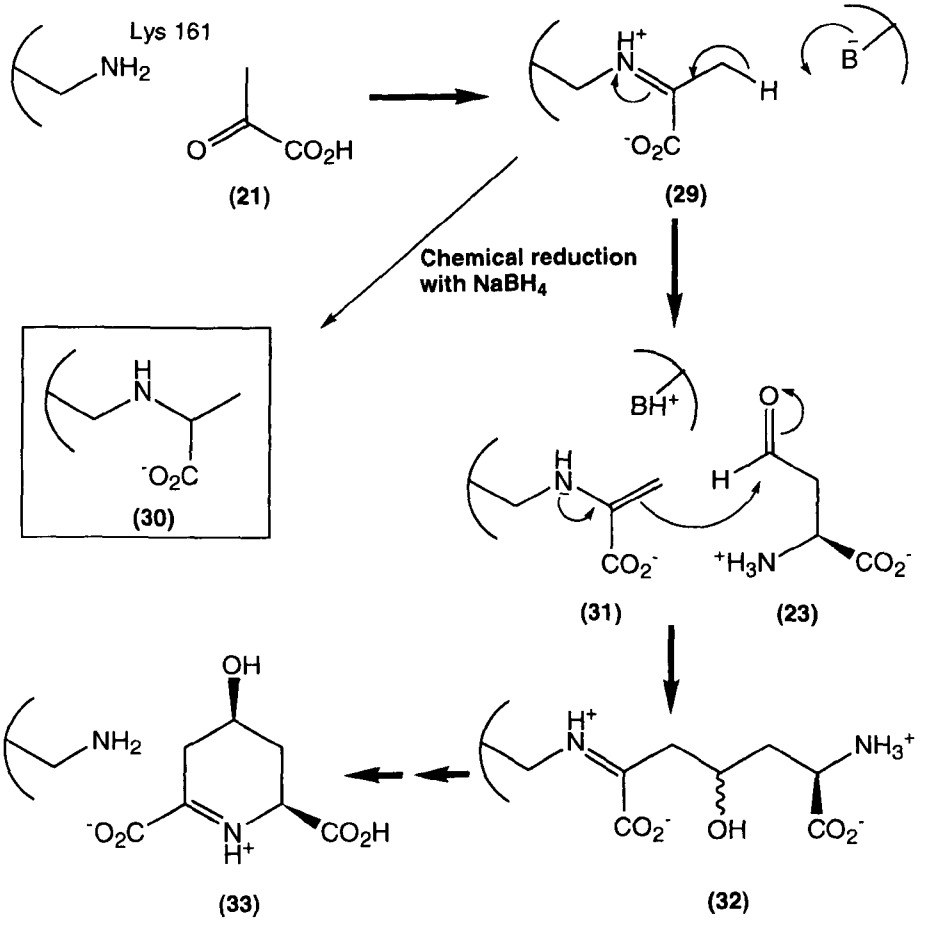


Figure 11 An Electrospray Mass Spectrometer

Using ESMS it has been established that the first step in the biological reaction catalysed by DHDPS is the binding of pyruvate to a lysine residue on the enzyme[50] **(Scheme 14)**. It is known that pyruvate **(21)** binds via the ε-amino group of lysine residue-161 in *E. coli* DHDPS.[29,48,53]

One problem associated with the detection of any reaction intermediate is the short lifetime and low concentration of these states. Although observation of the first formed DHDPS/pyruvate imine intermediate **(29)** can be observed by ESMS as a very weak signal, the imine **(29)** can be selectively reduced by $NaBH_4$, irreversibly binding the pyruvate to the enzyme **(Scheme 14)**. As a result, the chemically reduced enzyme intermediate **(30)** can be easily observed by ESMS **(Figure 12)**. The parent enzyme has a molecular weight of 31 272. The reduced intermediate **(30)** has a molecular weight of 31 344. The peak at 31 404 Daltons in the electrospray mass spectrum is presently unidentified.



Scheme 14 **Study of the Mechanism of the Condensation Catalysed by DHDPS**

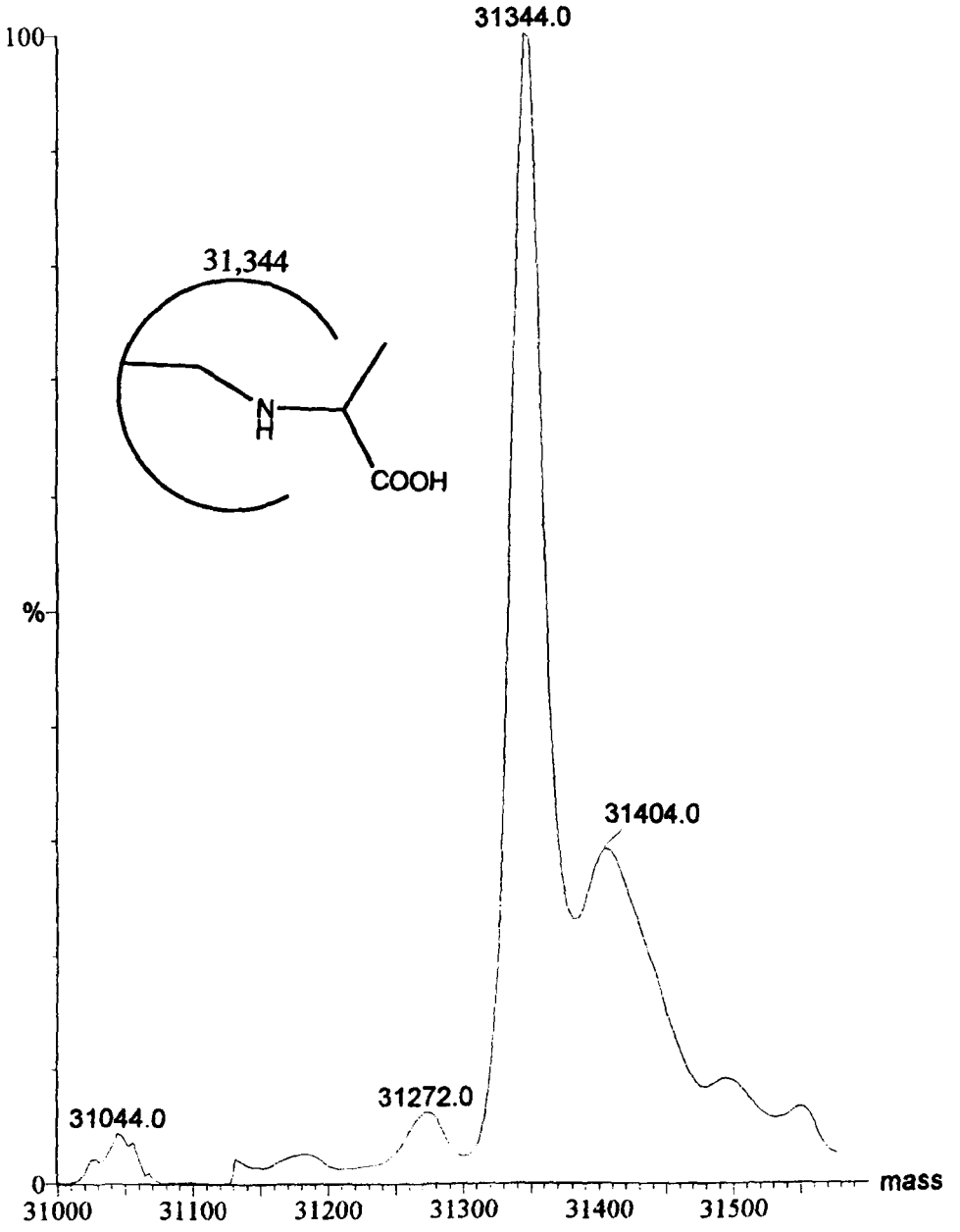


Figure 12 $NaBH_4$ Reduced Pyruvate/DHDPS Intermediate (30)

The observation of the binding of ASA **(23)** to DHDPS by ESMS has been rather inconclusive, either with ASA on its own, with pyruvate, or with the reduced intermediate **(30)**. As a result little is known about the binding of ASA to form the condensed intermediate **(32)**. Chemically reducing the intermediate **(29)**, or the enamine **(31)**, blocks condensation with the ASA. Early evidence suggests that the ASA may bind with this deactivated complex **(30)**. Further investigation of this is required. The cavity that accommodates ASA could interact through a covalent or an electrostatic binding mechanism. The early evidence indicates a covalent binding. However, if the aldehyde group is held as an imine, then trapping it with $NaBH_4$ should be possible. Clearly there are too many possibilities to draw any firm conclusions. It is likely that the ASA-bound intermediate is too short lived for detection or trapping and it is notable that the imine intermediate **(32)** has also not been observed. X-Ray studies of Blickling and co-workers[30] have however given some clues to this binding (see **Scheme 15**).

Blickling and co-workers[30] further identified the product of the condensing enzyme at pH 9 as *(2S,4S)*-4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (HDPA) **(33)**. This was achieved using 1D and 2D ^1H and ^{13}C NMR spectroscopy. Labelling studies with pyruvate, [3-^{13}C]-pyruvate and [2,3-^{13}C]-pyruvate allowed them to assign the signals. The assignment of ^1H NMR signals for unlabelled HDPA is illustrated in **Figure 13**.

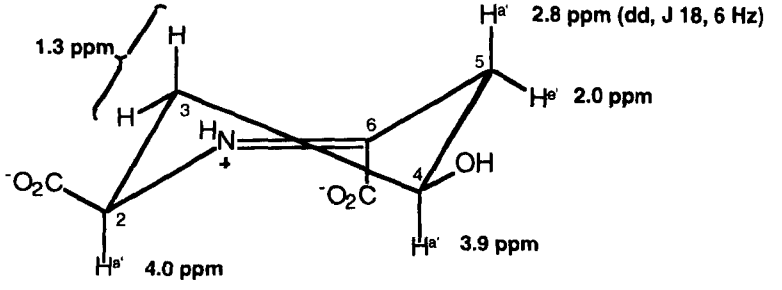


Figure 13 Assignment of ^1H NMR Signals for HDPA (33)

2.13 The Crystal Structure of DHDPS

The crystallisation and elucidation of the three dimensional structure of *E. coli* DHDPS has been achieved at 2.5 Å resolution by Huber and co-workers[55] **(Figure 14)**. The active site is a cavity formed by two monomers. Pyruvate binding residue Lys 161 lies at the bottom of a cavity 10 Å wide by 30 Å long. The entrance to this cavity is formed by two aspartate residues (Asp 187 and Asp 188). Interestingly, this adjacent aspartate relationship is common in all known DHDPS sequences. This implicates an important mechanistic role for this grouping.

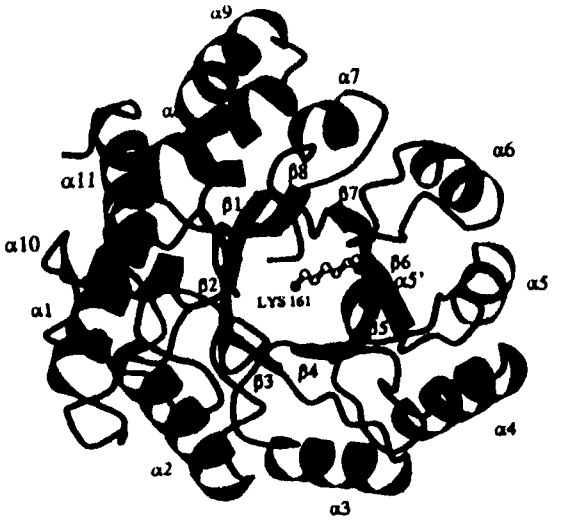
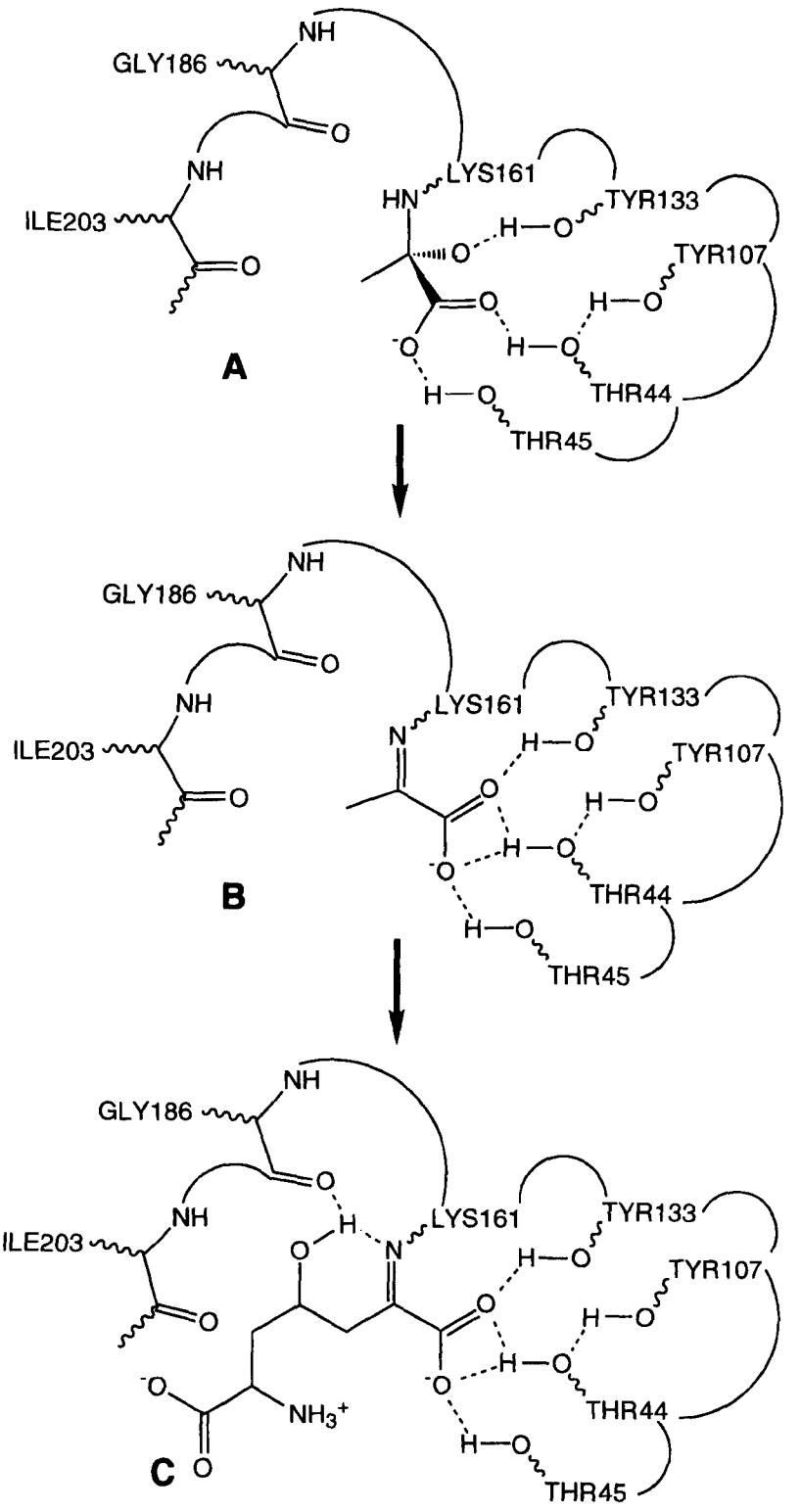


Figure 14 The Crystal Structure of *E. coli* DHDPS

X-Ray studies by Blickling and co-workers[30] involving DHDPS with pyruvate and pyruvate/succinate-β-semialdehyde identified the key interactions of the amino acid residues: Thr 44; Thr 45; Try 107; Try 133; Lys 161; Gly 186 and Ile 203. The binding role of these residues for the proposed biosynthetic mechanism are summarised in **Scheme 15**. Isoleucine residue 203 is considered to play a catalytic role in enamine formation from **B** prior to aldol condensation with ASA. The binding of the OH group of the condensed intermediate **C** was determined by experiments using pyruvate and succinate-β-semialdehyde.

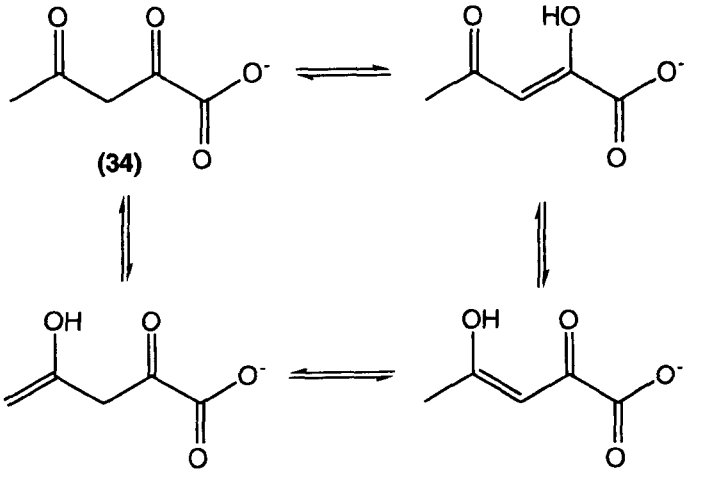


Scheme 15 Binding Interactions of DHDPS

2.14 Activity of DHDPS with Unnatural Substrates

A number of substrate analogues of DHDPS have been investigated as inhibitors. The enzyme has proved to be highly substrate specific. Pyruvate analogues such as phosphoenolpyruvate, phenylpyruvate, α-ketobutyrate, α-ketoglutarate, oxaloacetate and fluoropyruvate are not recognised by the enzyme. Analogues of L-ASA such as *N*-acetyl-ASA,[51] succinate semialdehyde,[51] and D-ASA[56] also show no activity.

The best inhibitor of DHDPS reported to date is acetopyruvic acid **(34)**, K_i 5 μM.[57] This compound has structural similarities with both ASA and pyruvate. Acetopyruvate exists in a number of tautomeric forms **(Scheme 16)** and is a less effective inhibitor at pH values above 8. This suggests that the most effective tautomer is in greater concentration at lower pH values.

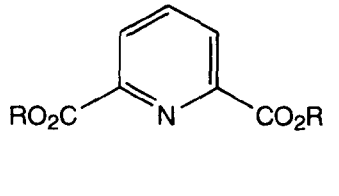


Scheme 16 Tautomerisation of Acetopyruvate

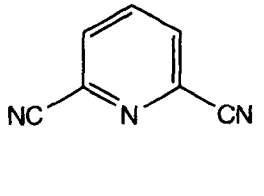
Bromopyruvic acid was found to inhibit DHDPS (K_i 1.6 mM for *E. coli* and 1.8 mM for wheat).[58] Coggins and co-workers showed by ESMS that it was possible to mono-alkylate DHDPS with four equivalents of this acid and that the enzyme retained 72 % activity under these conditions.[50] This suggests that alkylation occurs close to but not at the active site. Treatment with an excess of bromopyruvic acid resulted in further alkylation (ESMS) and significant inhibition of activity.

Dipicolinic acid inhibits the *E. coli* DHDPS with an IC_{50} of 1.2 mM.[48] This prompted a systematic investigation of numerous heterocyclic analogues of both dipicolinic acid **(28)** and DHDPA **(24)** by our group in Glasgow.[59] Substituted pyridine and piperidine derivatives were found to show moderate inhibition of *E. coli* DHDPS with IC_{50} values of less than 1 mM **(Figure 15)**. The best of the inhibitors were the dinitrile **(36)**, the *N*-oxide **(37)** of pyridine-2,6-dicarboxylic acid, the diimidate **(38)**, the ditetrazole **(39)** and chelidonic

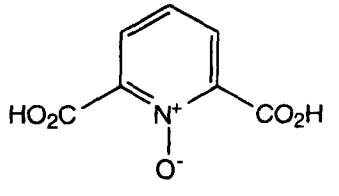
acid **(41)**. Detailed kinetic studies[60] showed that compounds **(36)** and **(37)** were non-competitive inhibitors with respect to either substrate. The X-Ray studies of Blickling and co-workers[30] showed that DPA **(28)** does not bind directly into the active site but does however coordinate with mechanistically important residues Thr 44 and Tyr 133. The studies also showed that esters were poorer inhibitors than their acid congeners; a planar distribution of the substituents was preferred around the nitrogen atom; and that mono-substituted ring systems also displayed poorer inhibition. *In vivo* studies of both DPA **(28)** and chelidonic acid **(41)** have shown inhibitory activity against the fungus *Phytophthora infestas*.[61]



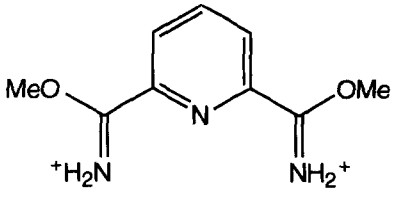
(28) R = H, IC_{50} 0.46 mM
(35) R = Me, IC_{50} 4.6 mM



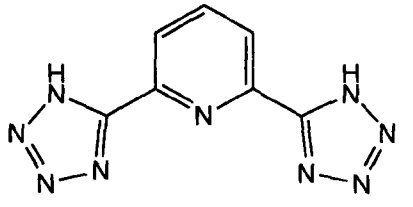
(36) IC_{50} 0.30 mM



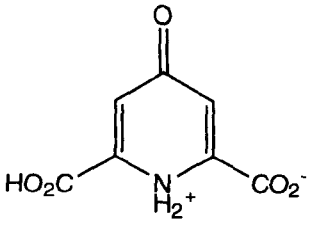
(37) IC_{50} 0.25 mM



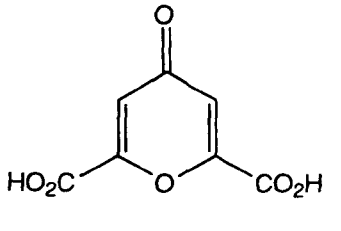
(38) IC_{50} 0.20 mM



(39) IC_{50} 0.25 mM



(40) IC_{50} 3.2 mM



(41) IC_{50} 0.30 mM

Figure 15 Heterocyclic Inhibitors of DHDPS

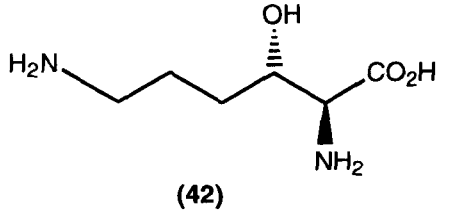
Lysine plays a regulatory role in plants and some bacteria. In wheat DHDPS L-lysine is a weak competitive inhibitor with respect to ASA. In the bacteria *E. coli* and *B. sphaericus*, IC_{50} values of 1 mM and 0.6 mM were observed, respectively.[62] L-lysine binds at the interface of two monomers of *E. coli*. DHDPS.[30] Two lysine molecules are involved, in contact with both monomers and with each other. This interaction causes an appreciable conformational strain on the protein structure. Inhibition by L-lysine has not

been observed in other bacterial stains.[63] L-Lysine is a potent allosteric inhibitor of a range of plant DHDPS enzymes **(Table 3)**.

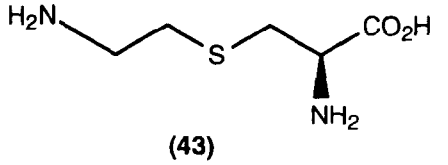
Plant	IC_{50} (μM)
wheat germ[64]	11
tobacco[65]	15
spinach[66]	20
maize[54]	23
wheat[53]	51

Table 3 Allosteric Inhibition of DHDPS by L-Lysine

L-Lysine analogues such as *threo*-β-hydroxy-L-lysine **(42)** and (2-aminoethyl)-L-cysteine (AEC) **(43)** show similar patterns of activity, but to a lesser extent. For wheat, compound **(42)** is inhibitory with an IC_{50} of 141 μM, as is AEC **(43)** with an IC_{50} of 288 μM.[53] Other plant DHDPS enzymes show similar patterns of inhibition: AEC **(43)** inhibits DHDPS from tobacco[67] with an IC_{50} of 120 μM, and the enzyme from spinach with an IC_{50} of 400 μM.[64]



(42)



(43)

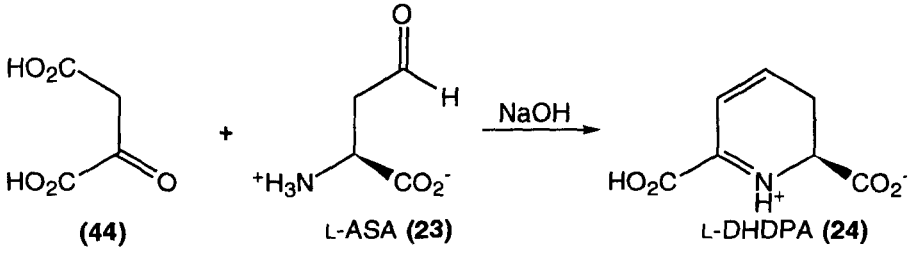
2.15 Dihydrodipicolinate Reductase (EC 1.3.1.26, *dap*B)

Dihydrodipicolinate reductase (DHDPR) catalyses the reduction of 2,3-dihydrodipicolinic acid (DHDPA) **(24)** to 2,3,4,5-tetrahydrodipicolinic acid (THDPA) **(25)** **(Scheme 11)** by hydride transfer from NADPH. This enzyme has been purified from *E. coli*,[68,69] maize,[70] and *Bacillus* spp.[71] DHDPR activity has also been detected in *Chlamydomonas*, corn, soybean and tobacco.[72] The *E. coli dap B* gene has been sequenced by two groups. The resulting peptide is predicted to have 273 amino acid units[73] and a molecular weight of 28 798. Overexpression, in *E. coli*, and subsequent ESMS studies on the protein have shown the molecular weight to be 28 758 ± 8.[74] The native protein is a homotetramer. The maize enzyme shows similar behaviour towards substrates and inhibitors but has a molecular weight of approximately 80 000 for the native protein.[70]

2.16 Characterisation of DHDPR Activity

DHDPA is stable only if stored in basic solution at pH values greater than 10. The instability of the substrate DHDPA **(24)** makes the task of monitoring DHDPR activity rather difficult. DHDPA rapidly undergoes air oxidation to DPA **(28)**. Indeed, it is this oxidative process that is utilised in the synthase assay for DHDPS activity.

Two assay systems have been developed for DHDPR involving the synthesis of DHDPA. L-DHDPA **(24)** has been synthesised chemically[70] by the condensation of L-ASA **(23)** with oxaloacetic acid **(44)** in alkaline solution **(Scheme 17)**. L-DHDPA was precipitated as its barium salt. The compound was unstable and was stored at -80 °C. Only freshly prepared samples could be used in assays. Kimura[75] synthesised DPA **(28)** by the direct condensation of ASA and pyruvate in alkaline solution. DPA was only produced when an aliquot of the reaction mix was incubated at 37 °C and pH 6.1 for 60 min. It was proposed that the formation of DPA under these conditions was by a disproportionation reaction of DHDPA. The final yield of DPA was enhanced by the presence of $HgCl_2$ in the reaction mixture. It was considered that the mercuric ions were acting as electron acceptors in the oxidation of DHDPA to DPA.

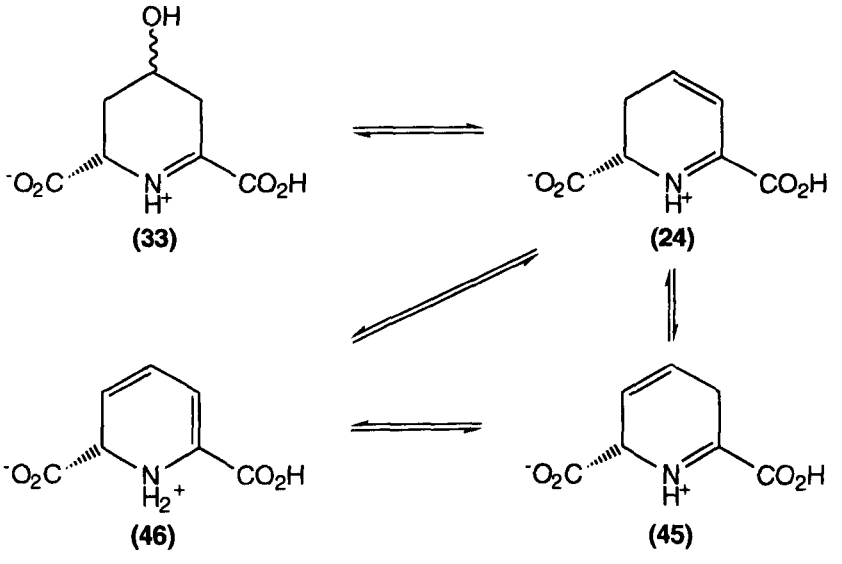


(44) L-ASA **(23)** L-DHDPA **(24)**

Scheme 17 Chemical Synthesis of L-DHDPA

Shedlarski and Gilvarg[29] synthesised L-DHDPA enzymically by the DHDPS-catalysed condensation of L-ASA **(23)** and pyruvate **(21)**. We have already mentioned this method for monitoring DHDPS activity in section 2.11 (coupled assay) and acknowledged limitations in quantifying results from combined enzyme systems. Shedlarski and Gilvarg suggested that the product of condensation of L-ASA **(23)** and pyruvate **(21)** is the heterocyclic compound 2,5-dihydrodipicolinic acid **(45)** in equilibrium with 2,3-dihydrodipicolinic acid **(24)** and 4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid **(33)** **(Scheme 18)**. The recent mechanistic studies[30] suggest that the latter, **(33)**, is the immediate enzymic product. Dehydration of compound **(33)** at

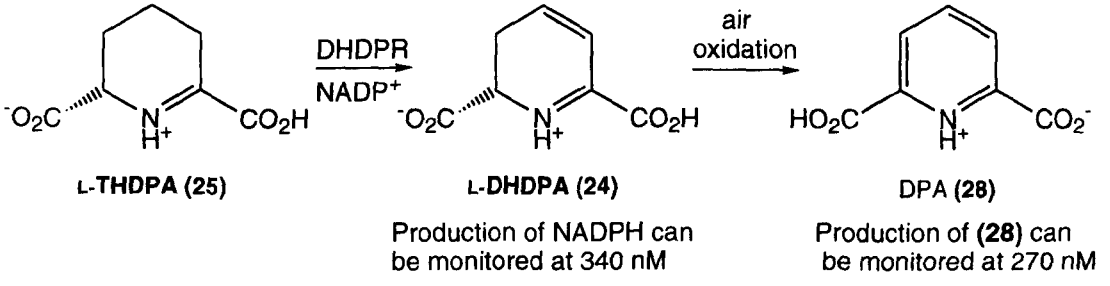
physiological pH is a rapid process and would likely lead to the thermodynamically favoured compound **(24)**. Furthermore we would acknowledge a likelihood of the presence of the conjugated enamine, 1,2-dihydrodipicolinic acid **(46)**.



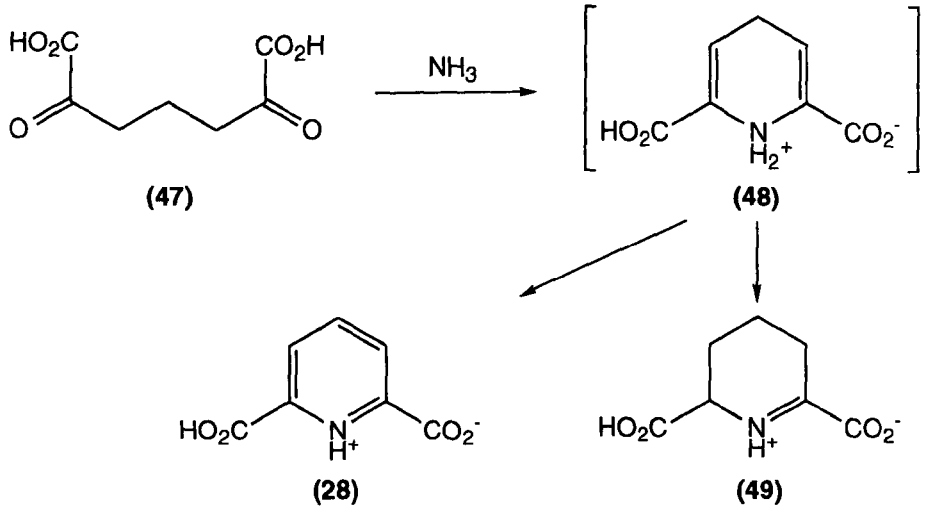
Scheme 18 Products of the Condensation Catalysed by DHDPS

In the two assay systems using DHDPR, activity is assessed spectroscopically by the consumption of the co-factor NADPH at 340 nm. The instability of DHDPA makes both of these assays of rather limited value. In our studies we noted that at the preferred substrate solution pH (> 10) the activity of DHDPR is significantly lowered. For these reasons we have developed a new assay system involving the reverse reaction of DHDPR with its natural product L-THDPA **(25)** and co-factor $NADP^+$ **(reverse reductase assay)**. The L-DHDPA **(24)** produced by this process is irreversibly oxidised to DPA **(28)**, thus driving the process to completion. The assay can be monitored spectroscopically by the production of NADPH at 340 nm or by the production of DPA **(28)** at 270 nm **(Scheme 19)**. The synthesis of THDPA is important for the study of the DHDPR enzyme and as a substrate of the tetrahydrodipicolinate-*N*-succinyl transferase and *meso*-DAP dehydrogenase enzymes. Shapshak[76] claimed to have made D-THDPA by the treatment of DL-DAP **(27)** with the L-amino acid oxidase from *Neurospora crassa*. Unfortunately no chemical or spectroscopic evidence of the formation of D-THDPA was provided. Kimura and Sasakawa[77] reported the formation of DL-THDPA **(49)** and DPA **(28)** by the disproportionation of 1,4-dihydrodipicolinic acid **(48)**, formed by treatment of αα'-dioxopimelic acid **(47)** with ammonia, either in the presence or absence of oxygen **(Scheme 20)**. NMR spectra of

the products of this reaction produced in the absence of oxygen showed a mixture.[78] Isolation of the required product proved impossible.

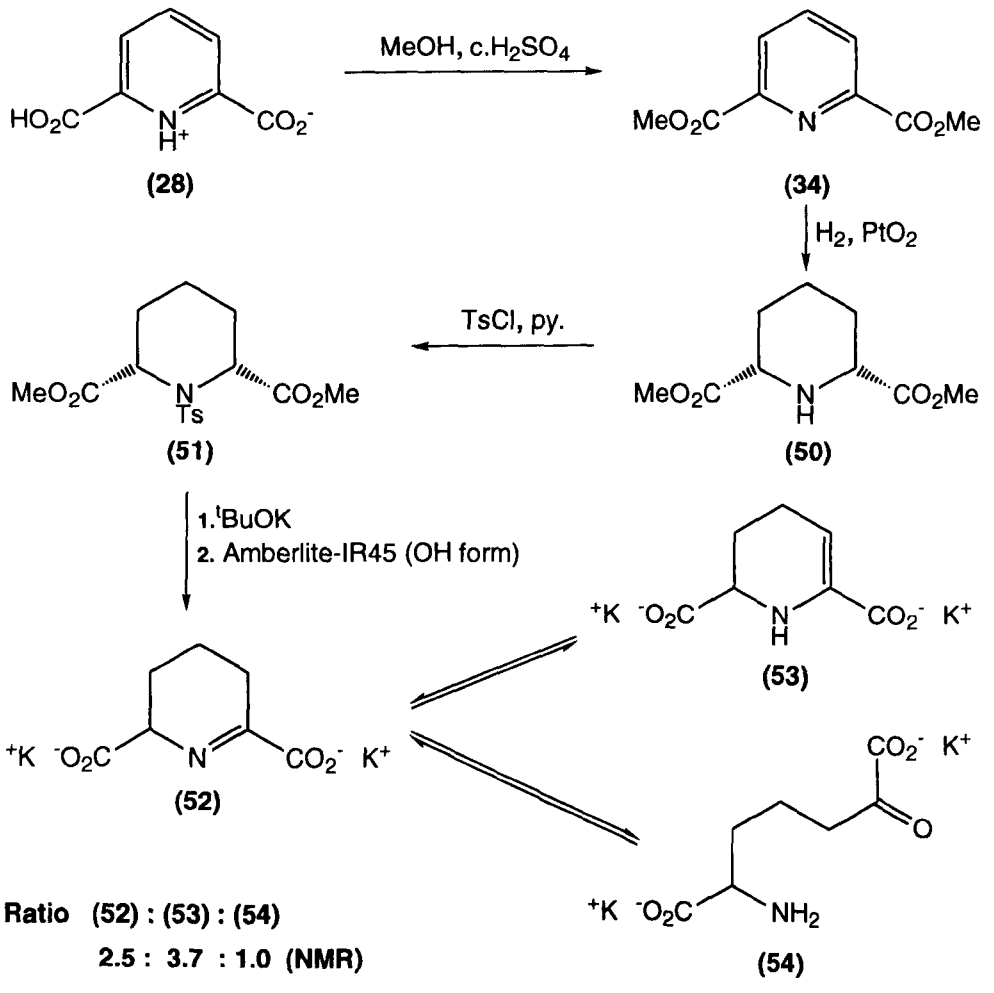


Scheme 19 Development of a New Assay for DHDPR Activity



Scheme 20 Kimura and Sasakawa Synthesis of DL-THDPA

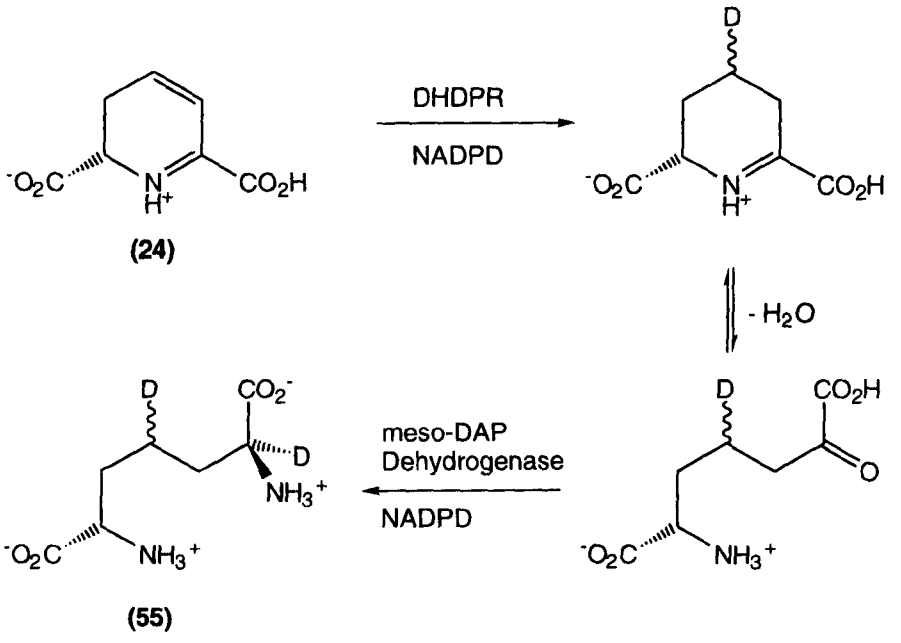
These problems were some of the first encountered in the project by our previous group members. After extensive research, a new chemical synthesis of racemic THDPA as its stable dipotassium salt **(52)**, involving the elimination of p-toluenesulfinic acid from dimethyl *cis-N*-tosyl-piperidine-2,6-dicarboxylate **(51)** by *tert*-BuOK was established[78,79] **(Scheme 21)**. Detailed study of the product by NMR spectroscopy showed it to exist in equilibrium with the enamine **(53)** and the open chain form **(54)** in the ratio 2.8:3.7:1, respectively. We have used this synthetic method for producing THDPA for the development of our reverse reductase assay.



Scheme 21 Synthesis of THDPA

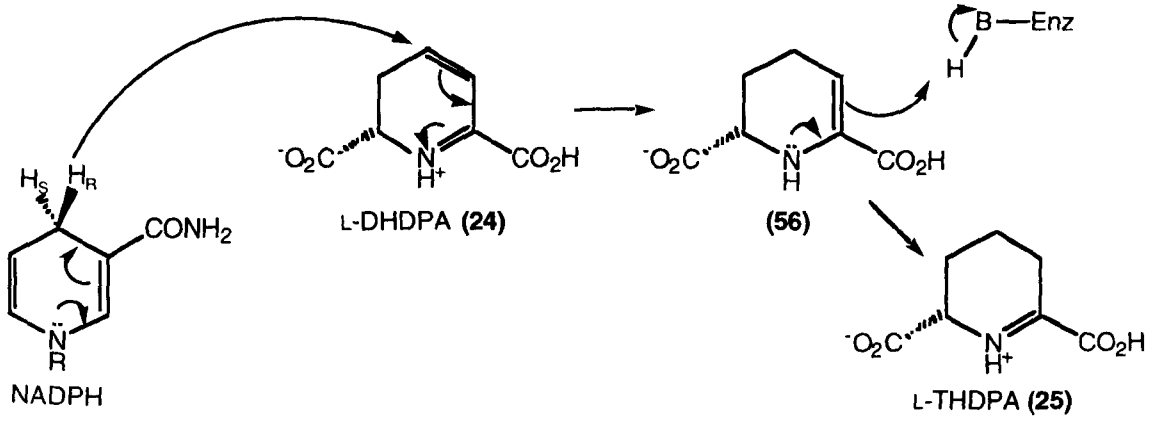
2.17 Mechanistic Studies on DHDPR

Production of significant quantities of DHDPR from an overexpressing strain of *E. coli* allowed Reddy and co-workers to investigate the enzymic mechanism.[60] It was found that the enzyme could utilise both NADH and NADPH as a co-factor. NADH was the better cofactor with a K_m (1.6 μM) approximately four times lower than that obtained for NADPH (5.6 μM). This is unusual because the rate of reaction with NADPH was approximately twice that observed when NADH was used (V_{rel} 100 for NADPH and 62 for NADH). Previously, tritium labelling studies had proved that NADPH donates its *pro*-R hydrogen to DHDPA **(24)**. Reddy and co-workers investigated the position of hydride addition to DHDPA by deuterium labelling. DHDPA was reduced by NADPD under DHDPR catalysis and the product was then carried through to produce doubly deuterated *meso*-DAP **(55)**, using *meso*-DAP dehydrogenase **(Scheme 22)**. It is known that *meso*-DAP dehydrogenase transfers its *pro*-S hydrogen to the substrate to form the D-stereocentre.[60]



Scheme 22 Investigation of the Mechanism of DHDPR

Comparison of the 500 MHz ^{1}H NMR spectra of unlabelled **(A) (27)** and labelled **(B) (55)** confirmed the donation of the *pro*-R hydrogen of NADPD to the 4-position of L-DHDPA **(24) (Figure 16)**. In the doubly labelled sample **(55)** the signal at δ 1.83 ppm was not observed. The signal at δ 4.35 ppm has a very similar coupling pattern in each case but is only half the intensity in the labelled sample. No attempt was made to distinguish the two C-4 proton signals to determine the face of the deuterium delivery onto L-DHDPA **(24)**. Thus the proposed mechanism **(Scheme 23)** involves transfer of the 4-*pro*-R hydride from the cofactor to the β-position of the αβ-unsaturated immonium ion, generating the enamine **(56)**. Enzyme assisted protonation and subsequent release completes the reduction of L-DHDPA **24)** to L-THDPA **(25)**.



Scheme 23 Proposed Mechanism for DHDPR

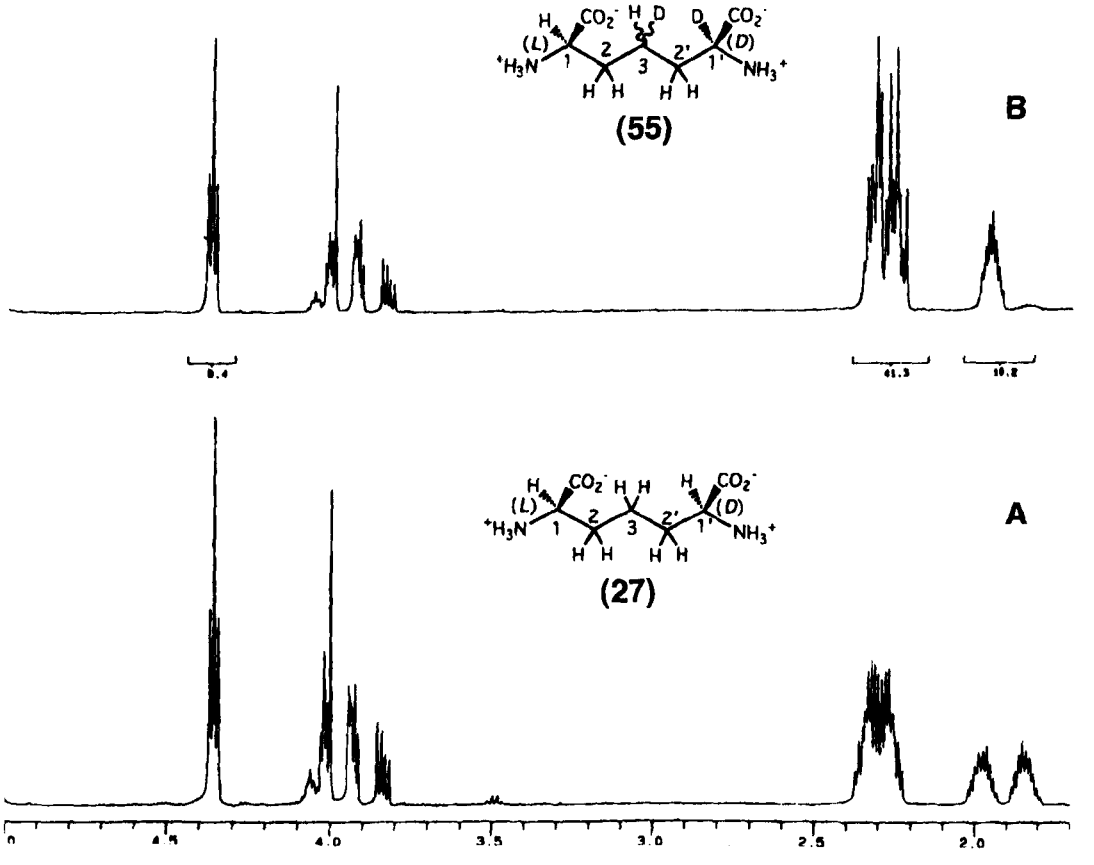


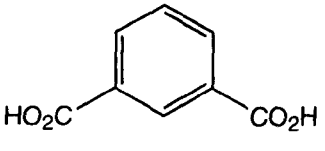
Figure 16 500 MHz ^{1}H NMR Spectra of Labelled (55) and Unlabelled (27) *meso*-DAP[74]

2.18 Crystal structure of DHDPR

The crystal structure of *E. coli* DHDPR and the bound NADPH co-factor at 2.2 Å resolution was reported by Scapin and co-workers.[80] The crystallisation was achieved for both the apoprotein and for the protein/NADPH complex. It is thought that a group of positively charged residues (His 159, His 160, Arg 161, His 162 and Lys 163) form the substrate binding site. Theoretical studies with DHDPA showed that these residues accommodate the substrate efficiently. A 30° rotation of the protein is required to bring the substrate and co-factor together for reaction. Further crystallographic investigations involving DHDPR with bound $NADP^+$ and DPA **(28)** are currently ongoing.

2.19 Inhibition of DHDPR

Dipicolinic acid **(28)** is a linear competitive inhibitor of *E. coli* DHDPR with respect to DHDPA **(24)**, with a K_i of 26 μM, and inhibits non-competitively with respect to NADPH, with a K_i of 330 μM. Inhibition by dipicolinic acid **(28)** is taken as evidence that it is the cyclic form, and not any ring opened form, of DHDPA **(24)** that is the substrate of the enzyme. Dipicolinic acid **(28)** was also found to be an inhibitor of DHDPR isolated from maize with a K_i value of 0.9 mM.[70] As with the *E. coli* enzyme the maize DHDPR binds the product in its cyclic form. A number of analogues of DHDPA **(24)** were also found to inhibit maize DHDPR **(Table 4)**.[70] *iso*-Phthalic acid **(57)** showed moderate inhibition with an IC_{50} value of 2 mM. Compounds with only one carboxylate group such as pipecolinic acid **(58)** and picolinic acid **(59)** were much less effective with IC_{50} values of greater than 20 mM. Pyridinedicarboxylic acids such as 2,5-pyridinedicarboxylic acid **(60)** were found not to be inhibitory.



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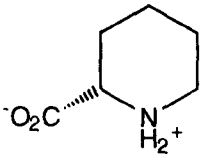

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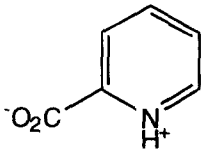

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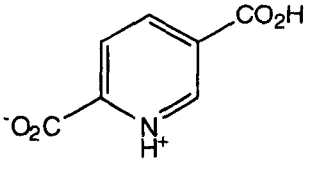


(60)

Inhibitor	% inhibition			
	20 mM	10 mM	5 mM	1 mM
Picolinic acid **(59)**	24	4	8	0
Pipecolinic acid **(58)**	23	30	0	0
Iso-phthalic acid **(57)**	44	20	0	0
Isocinchomeronic acid **(60)**	-	-	50	0
Dipicolinic acid **(28)**	-	-	-	100

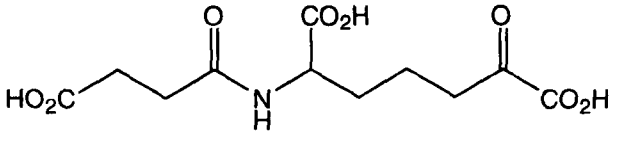
Table 4 Inhibition of Maize DHDPR

2.20 Alternative Targets for Inhibition of DHDPS/DHDPR

THDPA **(24)** is a substrate for the enzymes L-tetrahydrodipicolinate *N*-succinyltransferase, *meso*-diaminopimelate dehydrogenase and L-tetrahydrodipicolinate *N*-acetyltransferase. Competitive inhibitors and substrates of these enzymes, particularly cyclic ones, are likely to show similar binding characteristics with both the DHDPR and DHDPS enzymes. Efficient binding into the active site of the enzyme is key to our search for potential inhibitors and substrates.

2.21 L-Tetrahydrodipicolinate *N*-succinyltransferase (*dapD*)

L-THDP *N*-succinyltransferase catalyses the ring opening succinylation of L-THDPA **(25)** to the stable aliphatic derivative *N*-succinyl-α-amino-ε-ketopimelic acid **(61)** (see **Scheme 11**). The co-factor succinyl CoA supplies the succinyl group which serves to protect against recyclisation.



(61)

Berges *et al.*[32] investigated a number of cyclic and acyclic substrate analogues as inhibitors of L-THDP *N*-succinyltransferase. In general, the cyclic substrate analogues were shown to be poorer inhibitors **(Figure 17)**.

The trends noted here are very similar to those noted previously for heterocyclic inhibitors of DHDPS (see **Figure 14**). DPA **(28)**, chelidamic acid **(40)** and chelidonic acid **(41)** show lower levels of inhibition in this case. It was noted that the *trans*-isomers were better inhibitors than the corresponding *cis*-compounds. 2-Hydroxytetrahydropyran-2,6-dicarboxylate **(62)** was found to be a very potent inhibitor (K_i 58 μM). It was proposed that compound **(62)** may be a transition state analogue of the hydrated intermediate **(63)**. Related synthetic work in this area has been conducted by Roberts *et al.*[81]

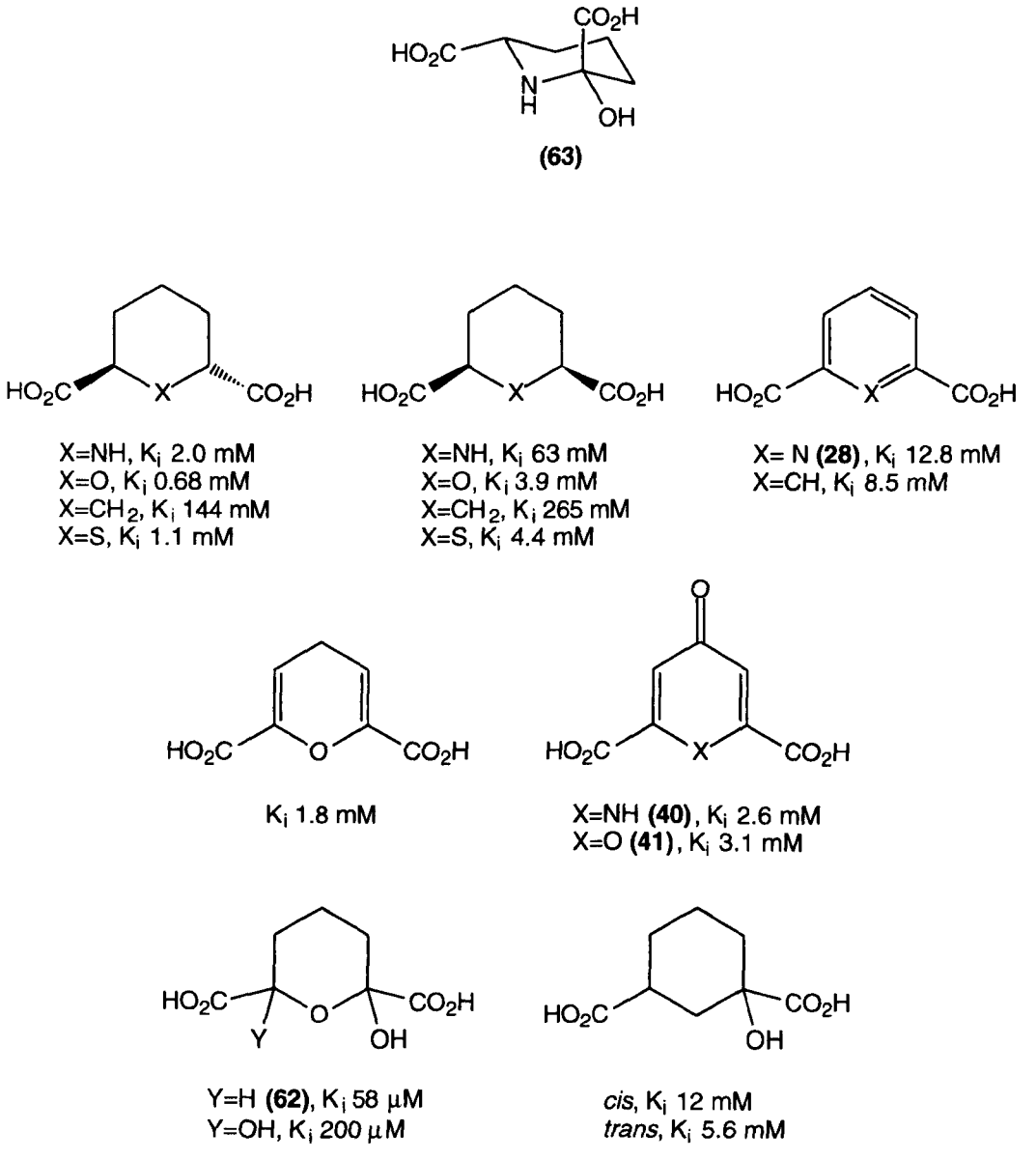
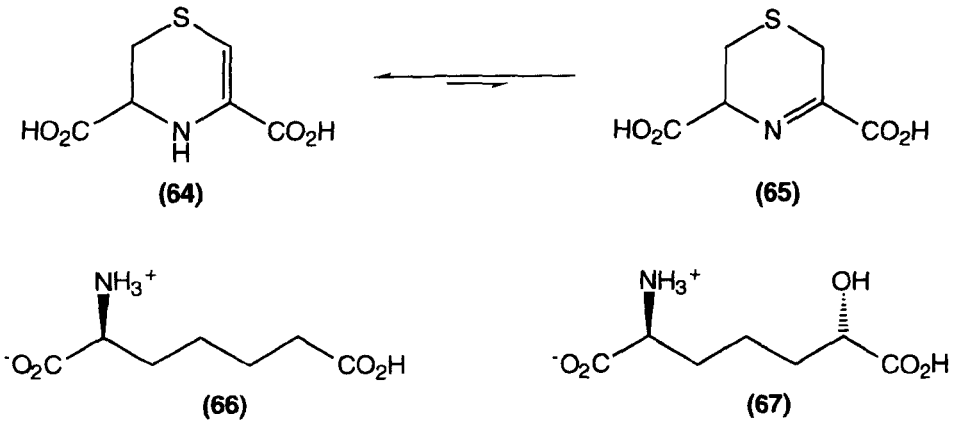


Figure 17 Cyclic inhibitors of L-THDP Succinyltransferase

3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid (DHT) **(64)** and L-2-aminopimelate **(66)** were found to be substrates of L-THDP succinyltransferase **(Table 5)**. The ^{1}H NMR spectrum of compound **(64)** shows it to exist mainly as an enamine. Compound **(64)** has a K_m value of 2 mM because only a very small proportion is present as the imine form **(65)** **(Scheme 24)**. The activity of compound **(64)** with the DHDPS and DHDPR enzymes will be discussed in chapter 7. LL-α-Amino-ε-hydroxypimelate **(67)** and L-α-amino-D-ε-hydroxypimelate were also shown to display good substrate activity. Compound **(67)** was succinylated at 43% of the rate of **(66)** and a stereoisomeric mixture was succinylated at 121% of the rate of **(66)**.



Scheme 24 Substrates of L-THDP Succinyltransferase

Substrate	$K_{m(app)}$	$V_{m(app)}$
THDPA **(25)**	20 μM	21 μmol/min
(64)	2 mM	9.8 μmol/min
(66)	1 mM	14 μmol/min

Table 5 Substrates of L-THDP Succinyltransferase

Berges *et al.*[32] found that acyclic compounds were generally better inhibitors of L-THDP *N*-succinyltransferase **(Figure 18)**. D-2-Aminopimelate **(68)** was a reasonable inhibitor with a K_i value of 0.76 mM compared to 4.5 mM for pimelic acid **(69)**. D- and L-Aminoadipic acid **(70)**, DL-aminosuberic acid **(71)** and D- and L-norleucine **(72)** were very poor inhibitors of the enzyme. This indicates that both carboxylate groups are required for efficient binding to the enzyme and the chain length is equally important. LL-DAP **(26)** was found to be a poor inhibitor (K_i 19 mM) and *meso*-DAP **(27)** showed no inhibition. Replacement of a methylene group with a sulfur atom had little effect as DL-2-amino-5-thiapimelic acid **(73)** was found to have a K_i value of 1.1 mM. D- and L-2-hydroxypimelic acid **(74)** and the conformationally restricted compound, (2*E*,5*E*)-γ-ketoheptadienedioic acid **(75)**, were shown to be good inhibitors with K_i values of 0.19, 0.33 and 0.53 mM, respectively.

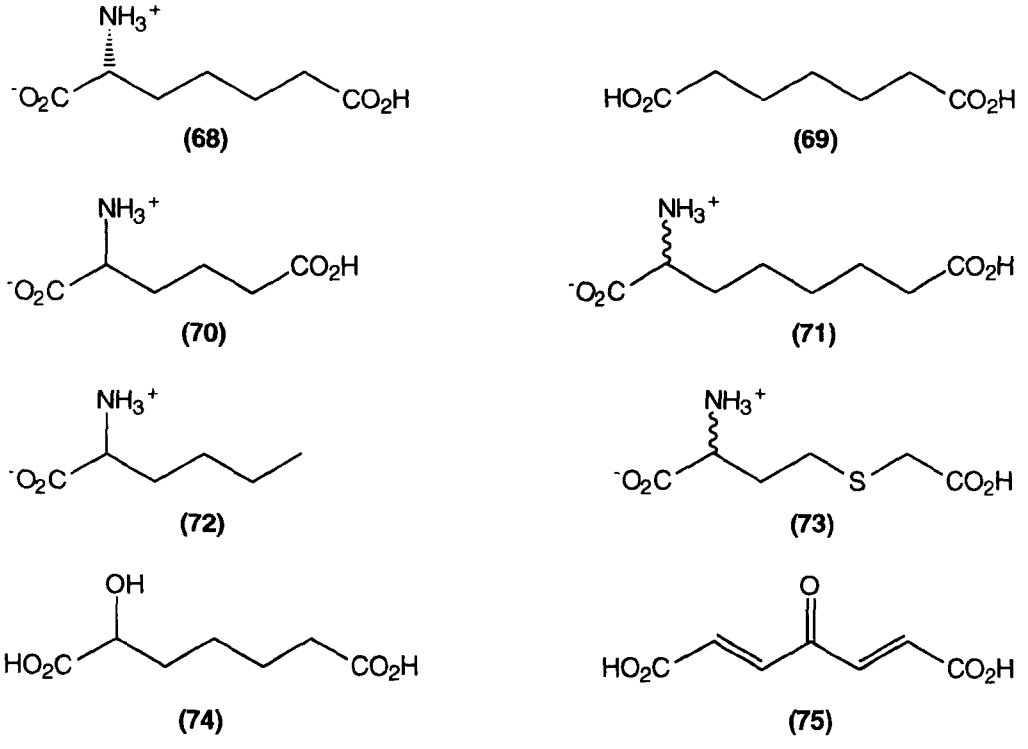


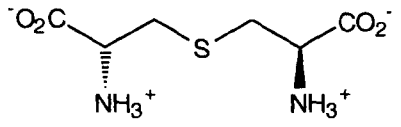
Figure 18 Acyclic Inhibitors of L-THDP *N*-succinyltransferase

2.22 L-Tetrahydrodipicolinate Dehydrogenase (EC 1.4.1.16, *ddh*)

L-THDP dehydrogenase catalyses the direct conversion of L-THDPA **(25)** into *meso*-DAP **(27)**, utilising NADPH and ammonia (see **Scheme 11**).

Lam *et al.*[82] investigated the activity of several DAP analogues against L-THDP dehydrogenase from *Bacillus sphaericus* and LL-DAP epimerase from *E. coli*. They found that the enzymes were both specific for the *meso*-geometry of their natural substrates. The dehydrogenase enzyme was not appreciably inhibited by a range of DAP analogues. LL-Lanthionine **(76)** was a weak noncompetitive inhibitor with respect to *meso*-DAP with a K_i value of 38 mM. *N*-Hydroxy-DAP **(77)**, *N*-amino-DAP **(78)** and 4-methylene DAP **(79)** showed 22%, 4% and 4% substrate activity, respectively, compared to *meso*-DAP.

meso-Lanthionine was a weak competitive inhibitor of the epimerase enzyme with a K_i of 0.18 mM, compared to 0.42 mM and 9.1 mM for the LL-**(76)** and DD-stereoisomers respectively. *N*-Hydroxy-DAP **(77)** was found to be a very potent inhibitor with a K_i of 5.6 μM. *N*-Amino-DAP **(78)** also inhibited with a K_i value of 2.9 mM.



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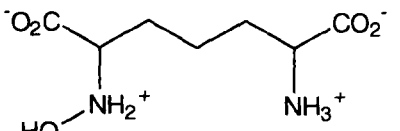

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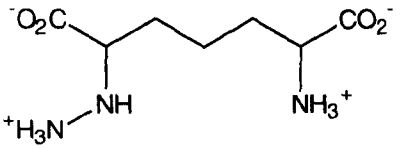

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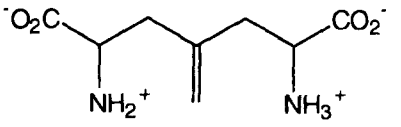
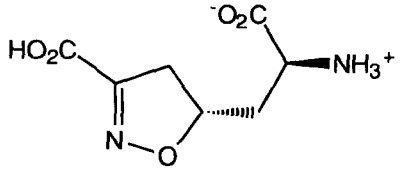


(79)

Abbott *et al.*[83] conducted a similar study involving heterocyclic analogues of THDPA **(25)** **(Figure 19)**. Isoxazole **(80)** was found to be a very potent competitive inhibitor of the dehydrogenase enzyme with respect to THDPA **(25)** with a K_i of 4.2 μM. The diastereoisomer **(81)** and imidazole **(82)** were much poorer inhibitors showing only 13% and 7% inhibition at 1 mM. Interestingly compounds **(83)** and **(84)**, lacking the side chains which make them close analogues of DAP are very poor inhibitors (7.5% and 2% at 7.75 mM respectively). Heterocycles **(80** to **84)** were very much poorer inhibitors of LL-DAP epimerase from *E. coli.*



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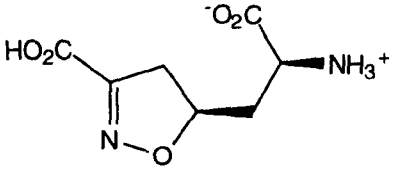

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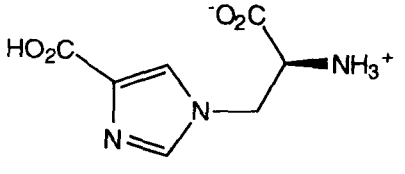

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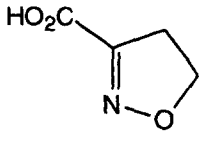

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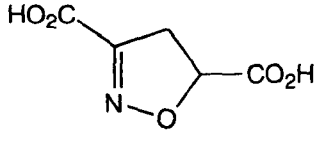
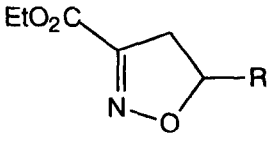


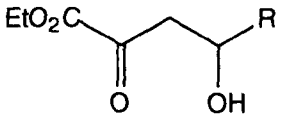
(84)

Figure 19 Heterocyclic Inhibitors of L-THDP Succinyltransferase

The inhibition of DHDPS by simple isoxazoles **(85** to **88)** and their open chain derivatives **(89** to **91)** was investigated by Dr J.E. McKendrick,[84] a former member of our group **(Figure 20)**. The ring opening was achieved by Pd catalysed hydrogenolysis of the N-O bond. These compounds also showed low levels of inhibition **(Table 6)**.



R = CN **(85)**
R = CO_2Me **(86)**
R = COMe **(87)**
R = CHO **(88)**



R = CO_2Me **(89)**
R = COMe **(90)**
R = CH_2OH **(91)**

Figure 20 Inhibitors of DHDPS

Compound	Inhibition of DHDPS at 1 mM
(85)	8%
(86)	12%
(87)	11%
(88)	5%
(89)	5%
(90)	7%
(91)	9%

Table 6 Inhibition of DHDPS by Isoxazoles and Open-chain Derivatives

2.23 Conclusion

In this chapter we have discussed the biochemical role played by of L-lysine. The value of investigating the first two steps of the DAP biosynthetic pathway to L-lysine has been in detailed. In the following chapters we will consider the synthesis and biological evaluation of metabolites and analogues of the DHDPS and DHDPR enzymes.

Chapter 3 Synthesis of Aspartate-β-semialdehyde and Analogues

3.01 Introduction

In this chapter we will briefly review the basic principles behind the chemical synthesis of α-amino acids. The role of proctecting groups in amino acid synthesis will be considered. The synthesis of aspartate-β-semialdehyde (ASA) and analogues from allylglycine precursors will be discussed in detail.

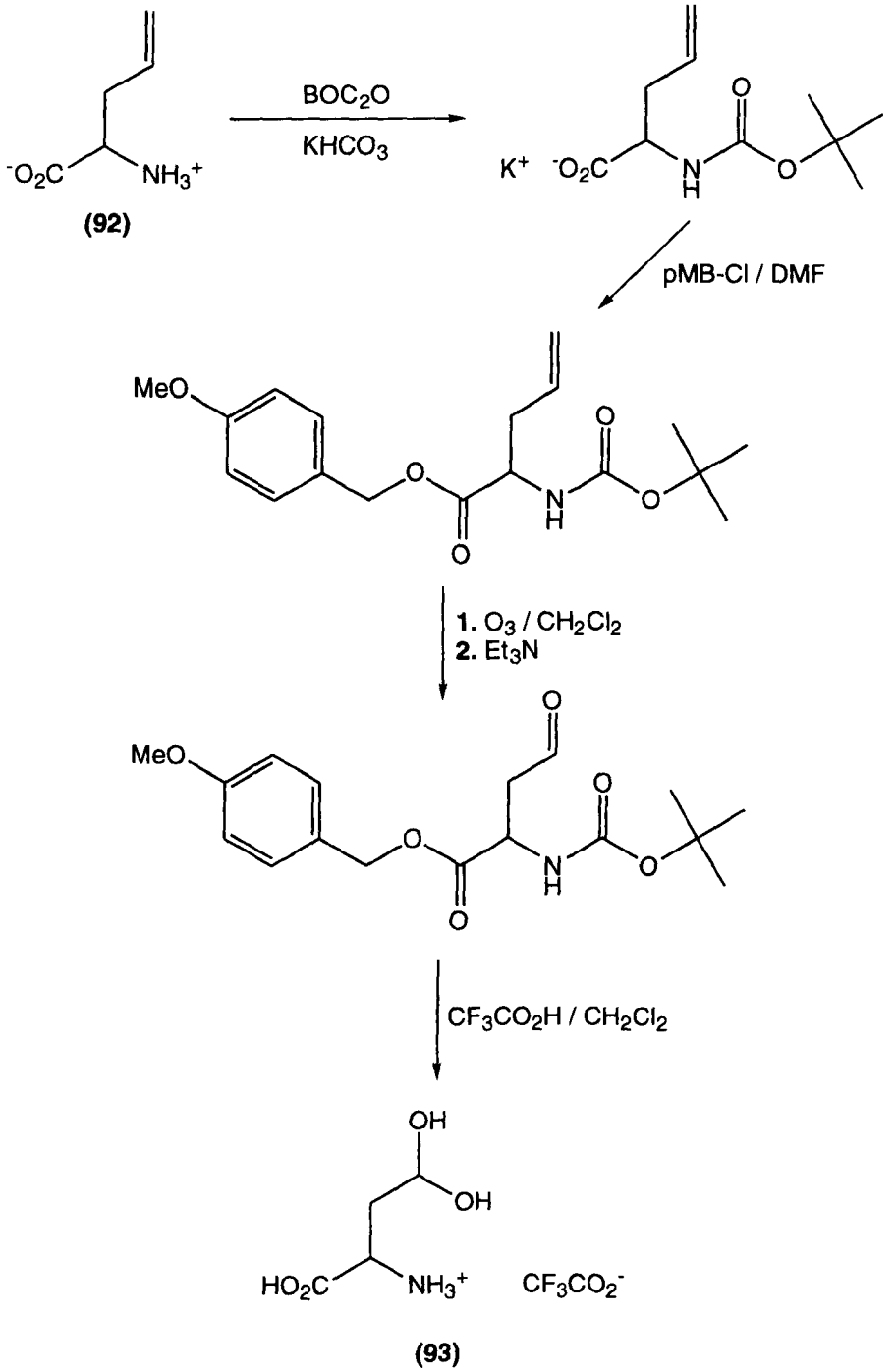
L-Aspartate-β-semialdehyde L-(ASA) **(23)** plays a key role in the DAP biosynthetic pathway to L-lysine (**Scheme 11**, Chapter 2). In Chapter 2 we discussed the importance of ASA, derived from oxaloacetate *via* the citric acid cycle. The amino acids lysine, methionine, threonine and isoleucine are biosynthesised from L-ASA **(23)**. Our studies in Glasgow have concentrated primarily on the DHDPS catalysed condensation of ASA and pyruvate **(21)** to produce the heterocyclic intermediate L-DHDPA **(24)**. Purification of the second enzyme of the DAP pathway, DHDPR, by our biochemical colleagues here at Glasgow has allowed us to extend our studies over the last two years.

It is of fundamental importance to the detailed study of any enzyme that a ready supply of the enzyme's natural substrates is available. These substrates are required for biological testing and mechanistic studies. The synthetic route may also allow us to make a number of analogues for testing as potential enzyme inhibitors or substrates. We are fortunate in that one substrate of the DHDPS enzyme, pyruvate, is a common metabolite and readily available as its pure, water soluble, sodium salt.

The first synthesis of DL-ASA was reported in 1954 by Black and Wright.[85] Ozonolysis of DL-allylglycine **(92)** in 1M HCl at 0 °C produced the required aldehyde. Black and Wright did not present any characterisation data for the ASA they produced but showed it to be reduced by homoserine dehydrogenase. In order to produce ASA in a form that could be isolated, characterised and stored, a former member of our group, Dr D.W. Tudor modified the Black and Wright synthesis. It was established that the purity and stability of ASA is greatly enhanced if the starting material **(92)** was first protected at both the acid and amine groups. After ozonolysis, the doubly protected aspartate semialdehyde was deprotected using trifluoroacetic acid, to yield the product as the stable trifluoroacetate salt of DL-aspartate-β-semialdehyde hydrate **(93)**, which was suitable for work with the DHDPS enzyme[56] **(Scheme 25)**. The overall yield for the four step process from **(92)** was 14%. Both stereoisomers of ASA have been prepared by this route from

enantiomerically pure starting material. D-ASA was shown not to be a substrate of *E. coli* DHDPS.[56]

In our work, further modifications of protecting groups and reaction conditions have been implemented to optimise the purity of product and reproducibility of the synthesis. This will be discussed in section 3.06.



Scheme 25 Synthesis of ASA

3.02 Synthesis of Amino Acids

The number of naturally occurring non-proteinogenic amino acids is of the order of 1000. Rapid expansion within the fields of chemical synthesis and semisynthesis of natural products and automated synthesis of polypeptides on solid supports ensure a constant demand for many amino acids. To satisfy this requirement there is a need for reliable synthetic methods for their preparation. Understandably, the research devoted to this task and detailed in the literature is truly immense. There is of course no definitive right or wrong way to set about the synthesis of a particular target and factors such as stereochemical requirements, economics, scale and environmental considerations must be taken into account. With so many options a good starting point is the many literature reviews in this area.[86] Notable in this regard are the extensive studies of O'Donnell[87] and Duthaler[88] and the textbooks of Williams[89] and Jones.[90]

The stereospecific nature of most enzymes dictates the demand for stereochemically pure material. As a consequence, for most applications in this field, there is a requirement for enantiomerically pure building blocks. To this end, the synthesis of amino acids is weighted towards asymmetric techniques, although enantiomeric resolution by chemical or enzymic methodology has appreciable value.

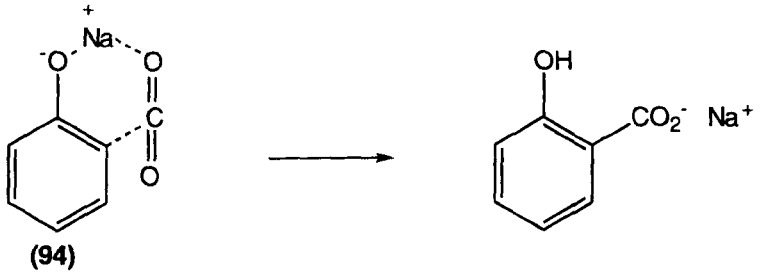
As the literature concerning the synthesis of amino acids is so extensive, we will concentrate exclusively on α-amino acids, outlining the principles of their synthesis. In section 3.04 we will briefly discuss some of the asymmetric routes to allylglycines.

Principally, there are five ways to synthesise α-amino acids: introduction of the acid group; introduction of the amino group; alkylation at the α-position; derivatisation of suitably functionalised amino acids and rearrangement of appropriate precursors.

Introduction of an Acid Group

The methods by which an acid group can be introduced during a synthesis are limited. The Kolbe-Schmitt reaction utilises carbon dioxide as a carboxylate synthon in the reaction with a phenoxide anion **(Scheme 26)**. In theory, this could be applied to the synthesis of amino acids, using a 1,3-dithiane as the nucleophile and generating an α-keto acid after hydrolysis. However, the role of the counter ion is very important in the Kolbe-Schmitt

reaction. Chelation to the carbon dioxide serves to activate the electrophile and generates a favourable six-membered transition state **(94)**.

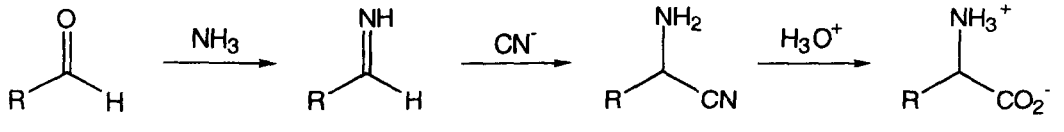


(94)

Scheme 26 The Kolbe-Schmitt Reaction

An alternative method, introducing the carboxylate group as the nucleophile, utilises the cyanide anion. This is the basis of the Strecker synthesis for amino acids, first reported in 1850.[91] Condensation of ammonia or a primary amine with an aldehyde yields an imine. Treatment with cyanide and hydrolysis of the resulting α-aminonitrile yields the α-amino acid **(Scheme 27)**. This method is highly versatile provided the alkyl side chain can withstand the reaction conditions, particularly the harsh conditions required for hydrolysis. Acid hydrolysis is commonly preferred since basic conditions would promote racemisation in asymmetric cases.

Carboxylate groups can also be intoduced by oxidation of primary alcohols. Oxidation of 2-amino-2-alkylethanolic compounds could produce α-amino acids. 2-Amino-2-alkylethanolic compounds could be produced by basic hydrolysis of terminal aziridines.



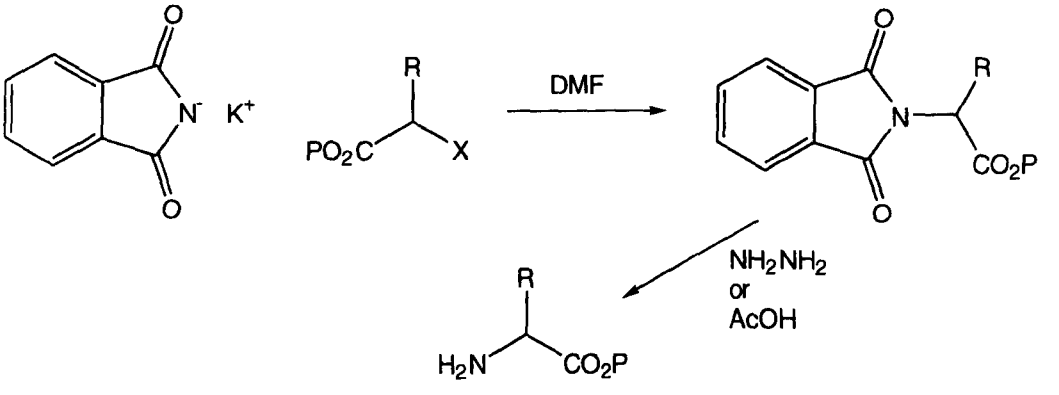
Scheme 27 The Strecker Synthesis of α-Amino Acids

Introduction of the Amino Group

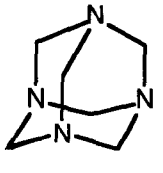
Amino groups can be introduced in the final step of α-amino acid synthesis by addition of ammonia equivalents to α-keto or α-halo acids. Halogenation α to an acid group can be easily achieved by a variety of methods.[92] Overalkylation is potentially a problem when using ammonia and a number of alternative strategies may be considered to introduce primary amino groups, such as: the Gabriel synthesis[93] **(Scheme 28)**; addition of the

azide anion followed by reduction; treatment with hexamethylenetetraamine[94] **(95)** and subsequent cleavage in ethanolic HCl; reaction with *bis*-(trimethylsilyl)amide[95] **(96)** and hydrolysis.

Amino groups can be introduced as electrophilic species using 1-chloro-1-nitrosocyclohexane **(97)**. Standard enolate chemistry can thus be employed to incorporate the amino group. Classical methodology by Oppolzer *et al*.[96] has been used to achieve this in an asymmetric manner.



Scheme 28 Amination of α-Halo Acids



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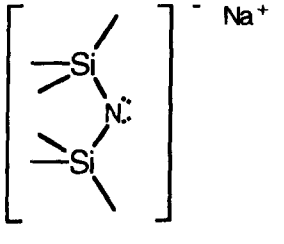

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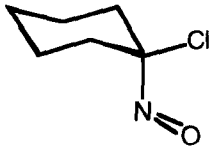
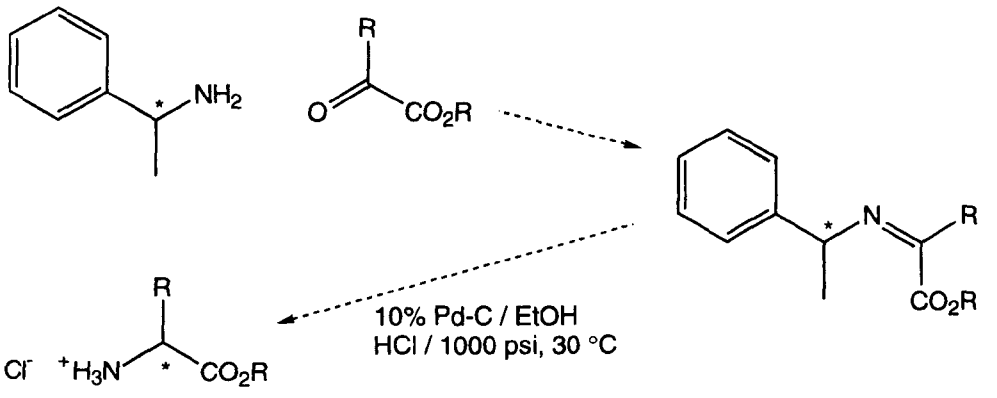


(97)

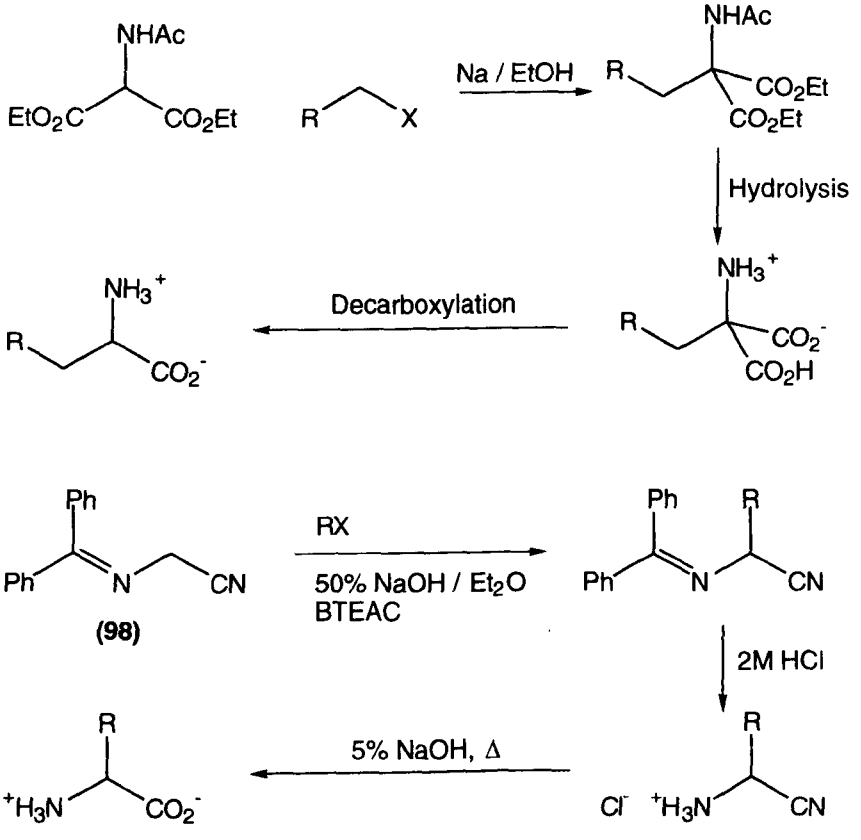
Reaction between ammonia and highly reactive α-keto acids to yield an α-imino acid is relatively straightforward. Mild reduction conditions, such as $NaBH_4$, must then be chosen to reduce the imine selectively in the presence of the carboxylic acid or equivalent group. A speculative method of achieving this in an asymmetric manner, linked to the asymmetric Strecker synthesis of Patel and Worsley,[97] is illustrated in **Scheme 29**. In this case it is also possible to introduce stereoselectivity at the final reduction step by catalytic asymmetric hydrogenation.[98]



Scheme 29 Amination of α-Keto Acids

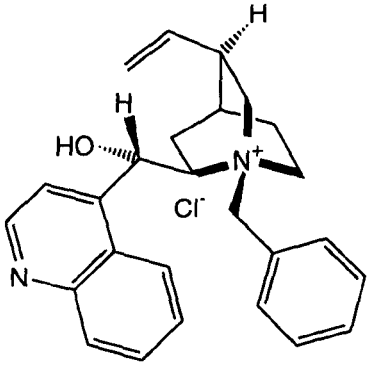
Alkylation of Glycine Equivalents

Modification of the carbon skeleton of glycine by alkylation can be achieved by nucleophilic or electrophilic reactions of appropriate glycine equivalents. *N*-Acylaminomalonates and *N*-benzylimino acid equivalents are important nucleophilic glycine equivalents in this regard **(Scheme 30)**.



Scheme 30 Alkylation of Nucleophilic Glycine Equivalents

Both are readily deprotonated at the α-position by virtue of their acidic hydrogens. Condensation with appropriate alkyl halides and hydrolysis yields the desired amino acids. *N*-Benzylimino acid equivalents such as *N*-(diphenylmethylene)aminoacetonitrile **(98)** are normally alkylated under phase transfer conditions to avoid over-alkylation.[99] O'Donnell and co-workers[100] have performed asymmetric alkylations by this method using chiral *Cinchona*-derived phase transfer catalysts **(99)** and **(100)**. Catalyst **(99)** favours formation of the *(R)*-enantiomer and catalyst **(100)** gives mainly the *(S)*-enantiomer. Reaction with allyl bromide under these conditions in the presence of catalyst **(99)** produced the protected allylglycine in 78% yield and 62% ee.



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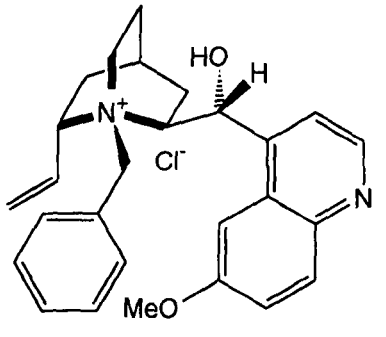
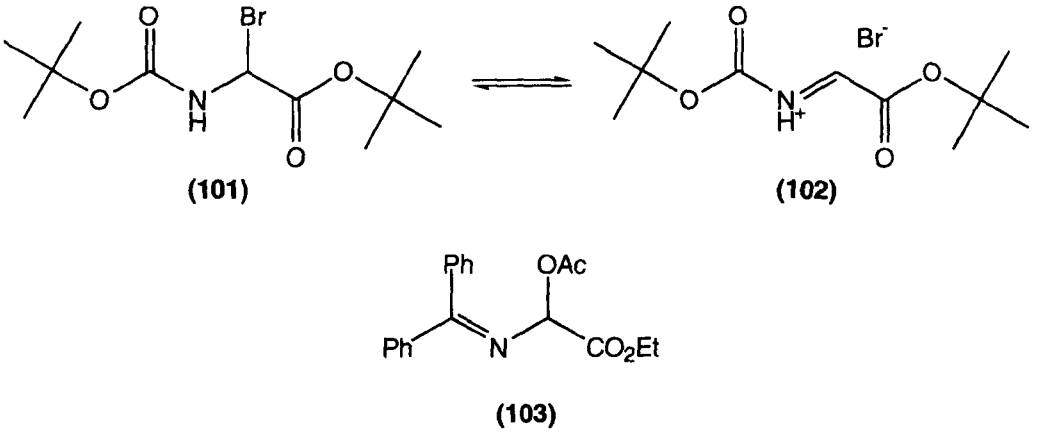


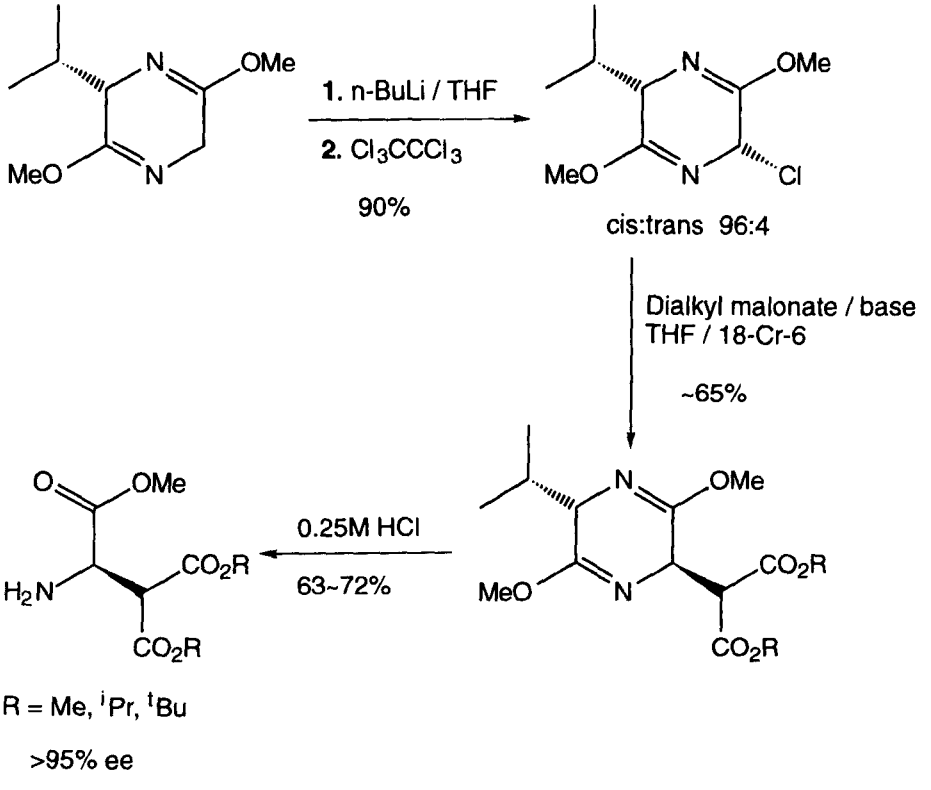
(100)

Electrophilic glycine equivalents are also of great value in amino acid synthesis. α-Bromo-*N*-BOC-glycine *tert*-butyl ester **(101)** can be prepared in high yield by irradiating the protected amino acid in the presence of *N*-bromosuccinimide in CCl_4.[101,102] Alkylation of the α-bromo species by Grignard reagents and deprotection yields the required amino acid.[103] Protected α-hetero α-amino acids are known to exist in equlibrium with their imine forms **(102)** **(Scheme 31)**. Loss of chirality at the α-position suggests that this would not be a useful procedure for asymmetric synthesis. However, as for many asymmetric syntheses, induction from further chiral functionallity can be a very useful technique. An example of this from Schollkopf and co-workers[104] is illustrated in **Scheme 32**.

Another important electrophilic glycine equivalent is the commercially available 2-acetoxy-*N*-(diphenylmethylene)glycine ethyl ester **(103)** which has been shown to be a very useful reagent in condensation with a wide range of carbon nucleophiles.[105]



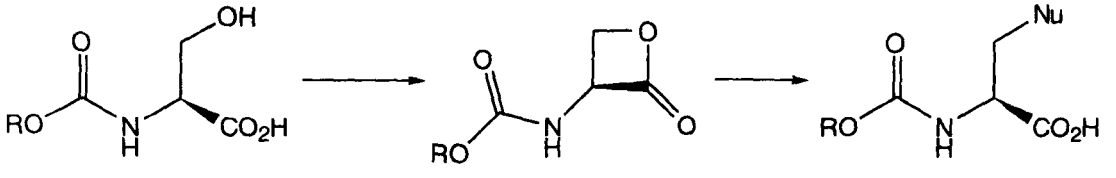
Scheme 31 Electrophilic Glycine Equivalents



Scheme 32 Asymmetric Alkylation of Electrophilic Glycine Equivalents

Derivatisation of Commercially Available α-Amino Acids

Partial synthesis of amino acids from suitably functionalised and readily available amino acid precursors can be a very useful procedure. This technique has been used for the synthesis of ASA from allylglycine **(92)** **(Scheme 25)**. An advantage of this method is the opportunity to utilise the chiral pool for stereochemical purity. An elegant use of this methodology by Vederas and co-workers[106] involves the displacement of the hydroxyl group of D- or L-serine by a wide range of nucleophiles. Intramolecular Mitsunobu esterification generates a reactive β-lactam **(104)** from *N*-protected serine **(Scheme 33)**. There are restrictions to this process in the case of C-C bond formations. Deprotonation of the acidic NH proton by reactive organometallic reagents can lead to oxazoline or oxazolinone formation by nucleophilic attack at the β-positon and the carbonyl of the β-lactone, respectively. Problems associated with regioselectivity of these hard nucleophiles could also be envisaged. Vederas and co-workers[107] performed selective alkylations in high yield using doubly protected amino-β-lactams and relatively soft dialkylcuprate nucleophiles.



N-Protected L-Serine
R = ^{t}Bu or Bn

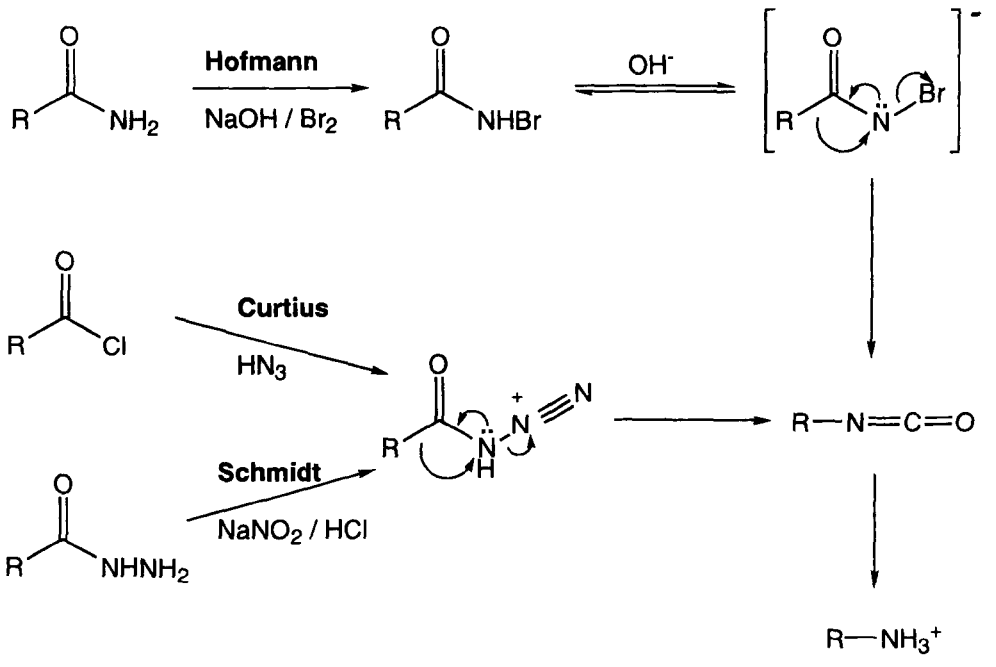
(104)

Scheme 33 **Derivatisation of L-Serine**

Rearrangements Leading to α-Amino Acids

A number of rearrangement reactions have also been employed in the synthesis of amino acids. The Hofmann, Curtius and Schmidt rearrangements all involve the replacement of a carboxyl function of a malonic equivalent by an amino group and proceed through a common isocyanate intermediate after migration of the alkyl group. Hydrolysis of an isocyanate yields a carbamic acid which rapidly decarboxylates under these conditions to yield the amino group **(Scheme 34)**.

In section 3.05 we will discuss the synthesis of allylglycine and analogues by a Claisen rearrangement of allylic esters.



Scheme 34 Hofmann, Curtius and Schmidt Rearrangements

3.03 Protecting Groups in α-Amino Acid Chemistry

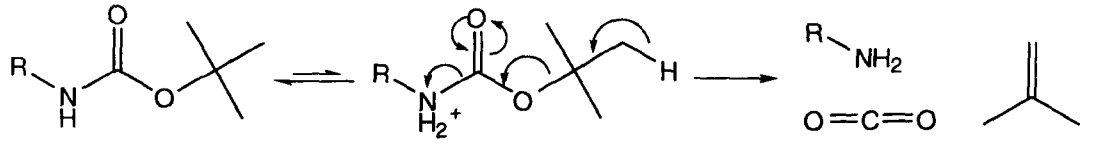
Protecting groups play a very important role in synthetic chemistry. Functional groups which are liable to take part in undesirable side-reactions for a particular process are first protected by transformation to a suitably unreactive derivative. Good synthetic strategy involves introducing the most reactive moiety at the latest possible stage, by further chemical transformation or simply by deprotection. In Sheehan's[108] total synthesis of penicillin V the highly reactive β-lactam ring was introduced at the final step by DCC coupling of the β-amino acid precursor.

To be useful for a particular task suitable protecting groups must fulfil certain criteria: the functional group to be protected must be selectively derivatised in good yield; the derivatised compound should ideally be easily crystallisable; the protected group must be inert to the conditions of any further reactions; after reaction the original functional group should be regenerated in high yield by deprotection under suitably mild conditions.

Clearly, as for synthesis of amino acids, protecting groups play an immensly important role in synthetic chemistry. Unsurprisingly, the field of protecting group chemistry is well developed. The many literature reviews[109] and texbooks, such as those by Greene and Wuts[110] and by Kocienski,[111] are useful starting points when deciding on synthetic strategies.

Amino acids are an interesting class of compound with regard to protecting groups in that the amino and carboxylic acid groups can readily react with each other. Fortunately the process involves a simple and reversible proton exchange to form the familiar zwitterion. At high temperatures α-amino acids are known to self-condense, forming 6-membered *bis*-lactam dimers. The simple proton exchange between acid and amino group has a fundamental effect on the properties of amino acids. The biological implications were discussed in chapter 1. Chemically, the uncharacteristically high polarity of amino acids means they are only soluble in highly polar protic solvents. Many synthetic applications require a less polar aprotic medium. For this reason the protection of amino acids for synthetic manipulation is almost routine. Carboxylic acids are most commonly protected as esters but can also be protected as amides and hydrazides. The latter two are somewhat less useful because harsh conditions are generally required for their cleavage. Amino groups are most commonly protected as carbamates but can also be protected as amides and as a range of other derivatives. In the synthesis of THDPA **(Scheme 21)** the amino group of the piperidine was effectively protected by tosylation, although in this case for the purpose of activation towards elimination.

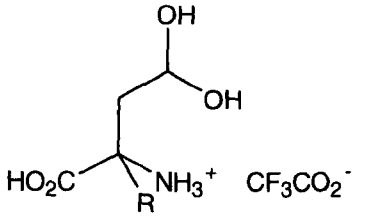
In our studies we have extensively used the protection of the amino and acid groups as *tert*-butoxycarbonyl (carbamate) and *tert*-butyl ester, respectively. These protecting groups have all the required attributes and can both be removed in the final step by treatment with anhydrous acid (see **Scheme 35**). Protecting both the acid and amino groups also allows us to use standard organic purification and analytical techniques such as chromatography in organic media. Chromatography in aqueous media is generally of lower resolution. On a preparative scale, removal of large quantities of water is considerably more difficult.



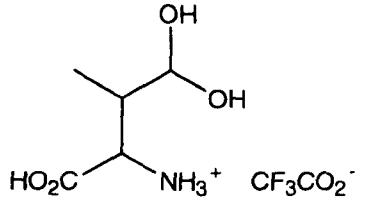
Scheme 35 Deprotection of *tert*-Butylcarbamates

3.04 Synthetic Objectives

Previous studies within our group have shown that analogues of L-ASA **(23)**, including D-ASA, are generally very poor inhibitors of the DHDPS enzyme. Importantly, D-ASA was also shown not to be a substrate of the enzyme.[56] These results indicate a high substrate specificity for the synthase enzyme. Dr Tudor reported poor inhibition characteristics for a number of ASA analogues functionalised at the aldehyde, amino and carboxylate groups.[112] Dr S.J. Connell[113] prepared oxime, methoxime, semicarbazone and thiosemicarbazone derivatives of ASA. These compounds showed low to moderate levels of inhibition with DHDPS. Dr J.E. McKendrick prepared a number of alkyl substituted ASA analogues[114] **(Figure 21)**. Once again these compounds were found to be very poor inhibitors. A key result however was that 2-methyl-ASA hydrate trifluoroacetate **(105)** and 3-methyl-ASA hydrate trifluoroacetate **(107)** were reported to show substrate activity with DHDPS. Compound **(107)** was reported to show 14% substrate activity. Interestingly the 2-ethyl **(106)**, 3,3-dimethyl **(108)** and 2,3-dimethyl **(109)** analogues did not show substrate activity. In Chapter 2 we briefly mentioned the potential value of enzyme mediated C-C bond formation with unnatural substrates. DHDPS catalysed biotransformations with 2-methyl-ASA and 3-methyl-ASA will be discussed in chapter 6.



R = Me **(105)**
R = Et **(106)**



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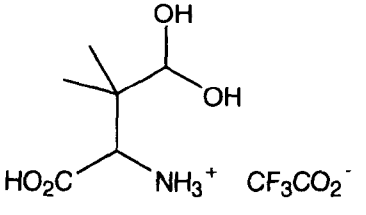

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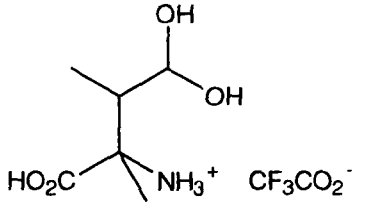
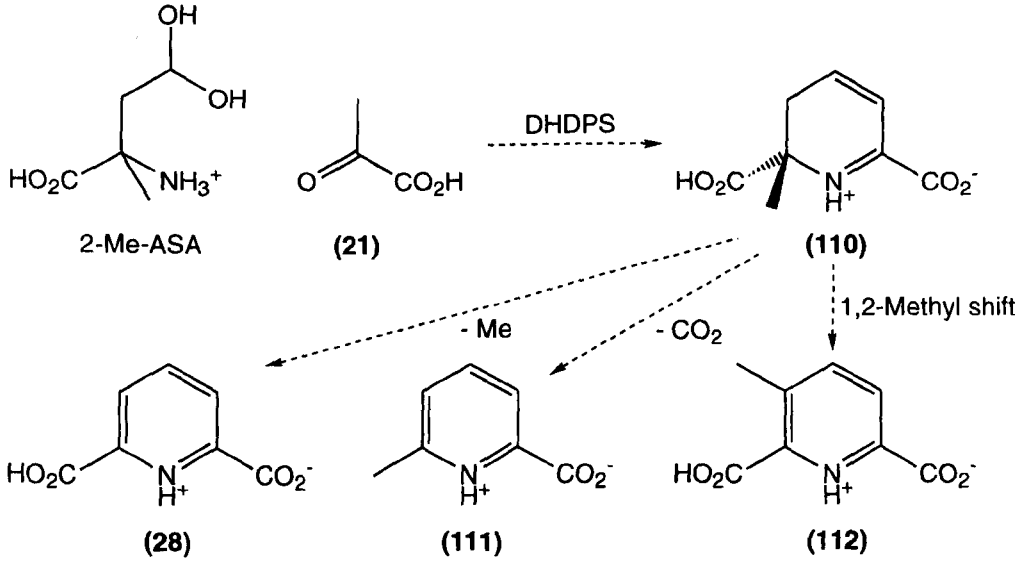


(109)

Figure 21 Alkylated ASA Analogues

Our key objectives were therefore directed towards further understanding the nature of the interaction between DHDPS and compounds **(105)** and **(107)**. Study of these compounds allows us a greater understanding of the mechanistic features of the enzyme and indicate any commercial potential. An important element of this study was the revision of the synthetic route to these compounds and further optimisation of existing methods. The syntheses were chosen with potential asymmetric application in mind, but, at this preliminary stage the stereoselection was left to the DHDPS enzyme. Although it is extremely likely that the enzyme will select only the L-stereoisomer in each case, this would have to be proved on further development.

2-Me-ASA is a very interesting case because it was shown that the product of DHDPS-catalysed condensation with pyruvate **(21)** had an absorbance maximum at 271 nm, similar to DPA **(28)**. This evidence strongly suggests that the product is an aromatic compound. Complete substitution at the 2-position in 2-Me-ASA should prevent aromatisation. The expected diene product **(110)** would be expected to have a UV absorbance maximum at approximately 240 nm. Although there is clearly a thermodynamic drive for aromatisation there appears no obvious mechanism for the event. Prior to further investigation it was considered that there were three possible routes to aromatisation: decarboxylation; demethylation; through a 1,2-methyl shift **(Scheme 36)**. This will be discussed further in chapter 6.

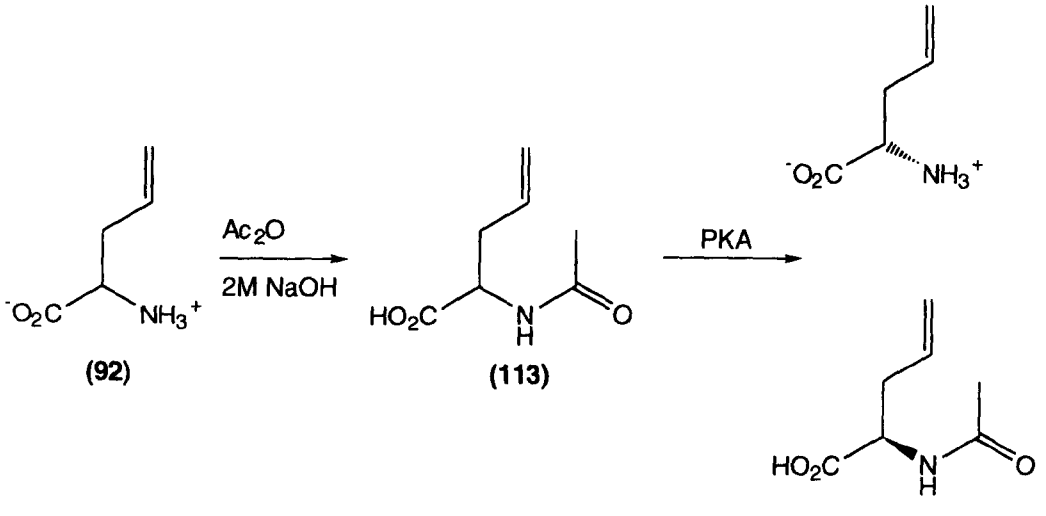


Scheme 36 2-Me-ASA as a Substrate of DHDPS

3.05 Literature Syntheses of Allylglycines

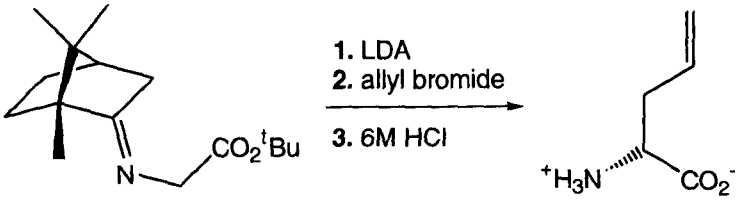
Allylglycine **(92)** is a key intermediate in Black and Wright s[85] synthesis of ASA. For our studies a readily available supply of allylglycine and analogues was required. Analogues of allylglycine are not commercially available and must therefore be synthesised. To achieve this objective we are interested in adaptable synthetic routes. In an ideal case the synthesis could be directed asymmetrically towards either enantiomer and would involve a minimum number of protection and deprotection steps. In this section we will briefly discuss the synthesis of allylglycine by classical asymmetric methodology. The methods used previously within our group will be considered. In section 3.08 we will detail two other synthetic routes used in our current work.

Allylglycine is commercially available as its D- and L-stereoisomers, as well as racemic material **(92)**. DL-Allylglycine can be resolved by acetylation and subsequent enantioselective cleavage catalysed by the enzyme porcine kidney acylase (PKA) **(Scheme 37)**.[115]



Scheme 37 Enzymic Resolution of Allylglycine

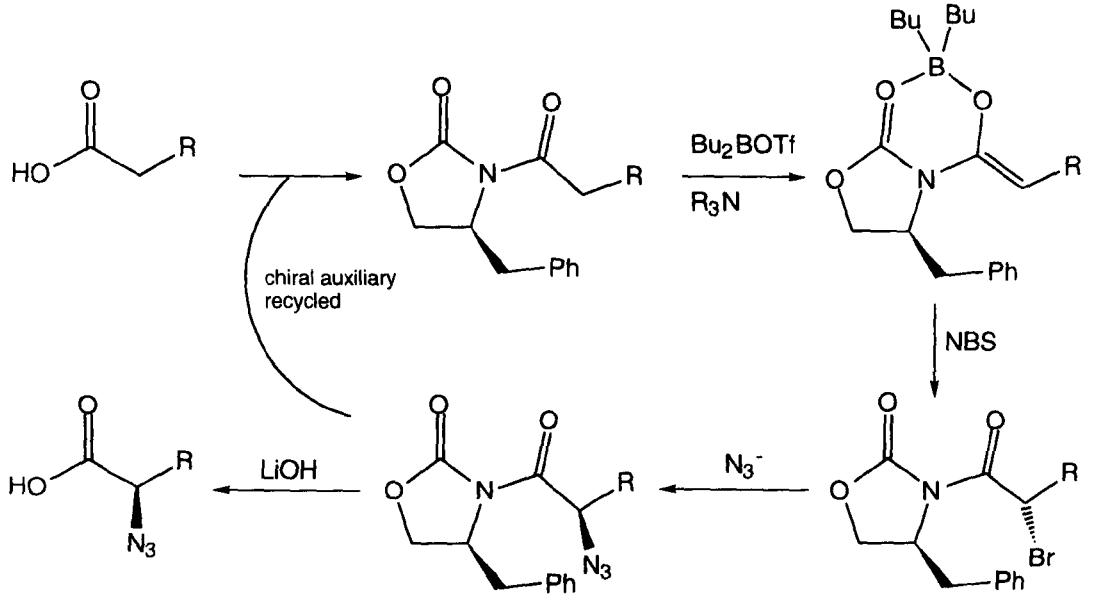
McKintosh *et al.*[116] produced D-allylglycine **(115)** by alkylation of camphor imine **(114)** **(Scheme 38)**. The desired product was isolated in 85% yield and 76% ee after hydrolysis. It is possible that the *tert*-butyl ester could be retained under milder deprotection conditions. This synthesis could however not be adapted to produce the opposite stereoisomer.



(114) (115)

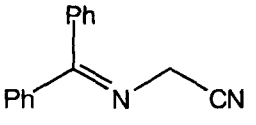
Scheme 38 Asymmetric Synthesis of D-Allylglycine

Evans and co-workers[117] have synthesised optically active allylglycines by the asymmetric introduction of the amino group **(Scheme 39)**. Williams and co-workers[118] have also achieved this by asymmetric alkylation of chiral glycine equivalents. In these cases the enantioselectivity is induced from chiral esters and oxazinones (2,3,5,6-tetrahydro-4*H*-oxazin-2-ones), respectively. Similar work by Oppolzer and co-workers,[119] involving introduction of the amino group to chiral esters, could also be adapted for this purpose.

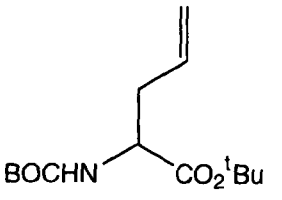


Scheme 39 Asymmetric Amino Acid Synthesis by Evans Methodology

Previous studies within our group by Dr McKendrick evaluated a number of synthetic routes to allylglycines: alkylation of nucleophilic glycine equivalent **(98)** under phase transfer conditions; direct alkylation of *N*-BOC-allylglycine *tert*-butyl ester **(116)**; and Claisen rearrangement of *N*-protected glycine and alanine allylic esters. In the remainder of this section we will consider each of these in detail.



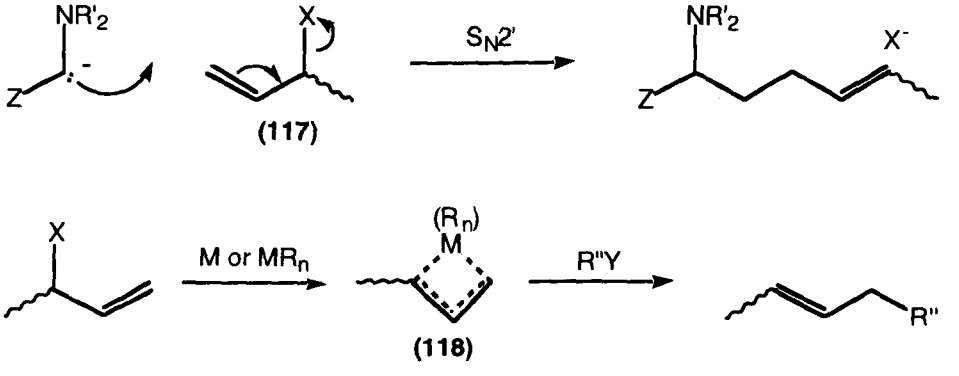
(98)



(116)

Alkylations of Amino Acids

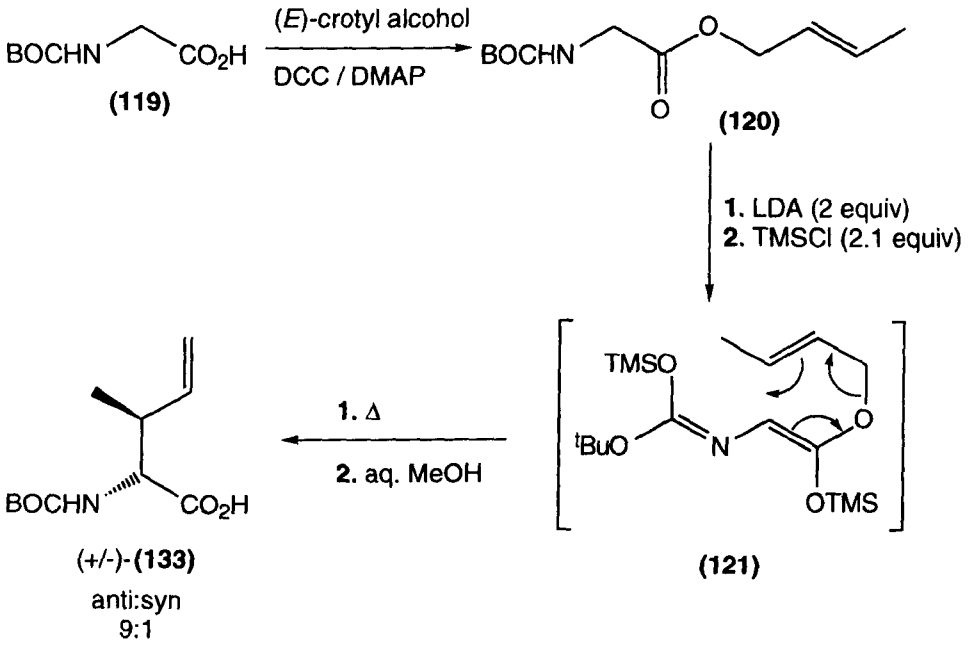
Synthesis of 3-substituted allylglycines by alkylation of glycine equivalents can be troublesome. Skinner *et al.*[120] synthesised 3-methylallylglycine in approximately 5% yield by alkylation of ethyl acetamidocyanoacetate. Electrophilic allylic equivalents **(117)** can undergo competing S_N2 conjugate additions and nucleophilic allylic equivalents **(118)** react more commonly through the less substituted position **(Scheme 40)**. These effects can be controlled in some cases by chelation (see later). Dr McKendrick used the two alkylation methods for the synthesis of 2-substituted allylglycines only. Both routes presented some difficulty. The phase transfer route to unsubstituted glutamate analogues is discussed in the next chapter.



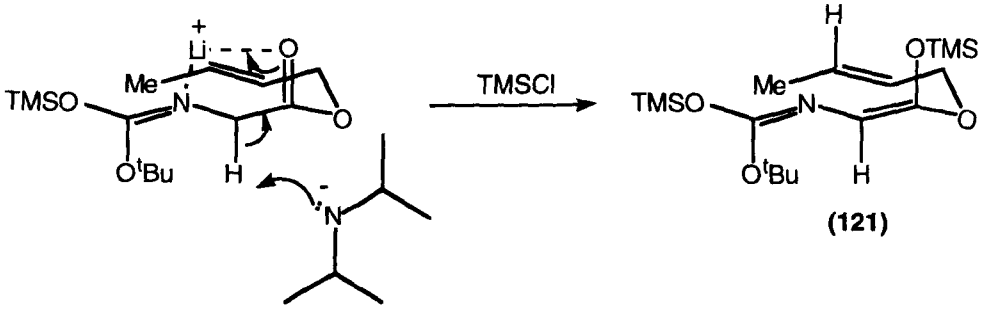
Scheme 40 Side Reactions with Allylic Equivalents

Clasien Rearrangement Route to Allylglycines

The Claisen rearrangement route to allylglycines from *N*-protected glycine and alanine allylic esters, originally reported by Bartlett and Barstow,[121] is illustrated in **Scheme 41**. A combination of chelation control in formation of the enol ether and steric factors favouring the positioning of the bulky group in a pseudo-equatorial position for the rearrangement, favour anti-diastereoselection in this case **(Scheme 42)**. Addition of chelating agents such as $ZnCl_2$ can improve diastereoselectivity.[122]



Scheme 41 **Claisen Rearrangement Route to Allylglycines**



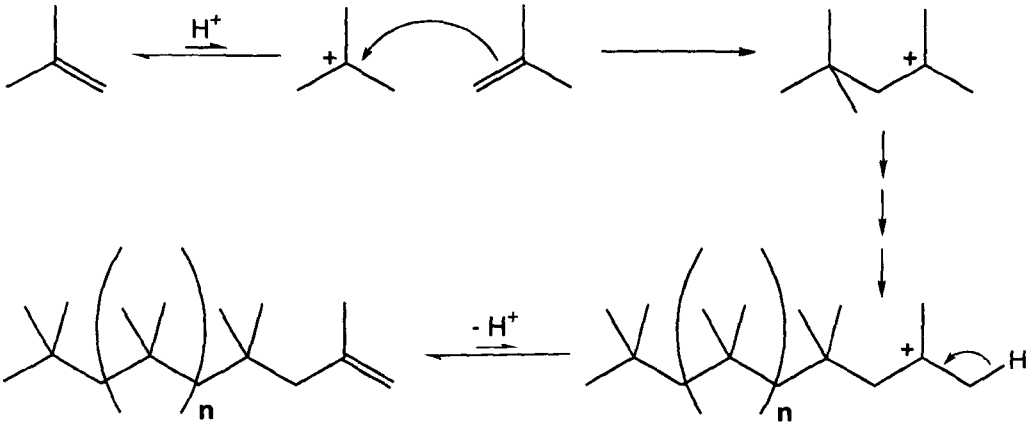
Scheme 42 **Stereochemical Control in the Claisen Rearrangement**

3.06 Synthesis of Aspartate-β-semialdehyde

Other than the original synthesis of ASA by Black and Wright and that of Robins and co-workers there has been one other synthesis reported by Gerrard and co-workers.[123] L-ASA was isolated by a multi-step process involving enzymic resolution and protection of the aldehyde function as an enol ether. A number of syntheses of protected ASA have been reported. The aldehyde functionality of ASA is useful for further structural manipulation. A modification of the method of Cooper *et al.*[124] by Baldwin and Flinn[125] yielded protected L-ASA by PCC oxidation of L-homoserine derived from L-methionine. Protected L-ASA was isolated in 40% yield for the one-pot

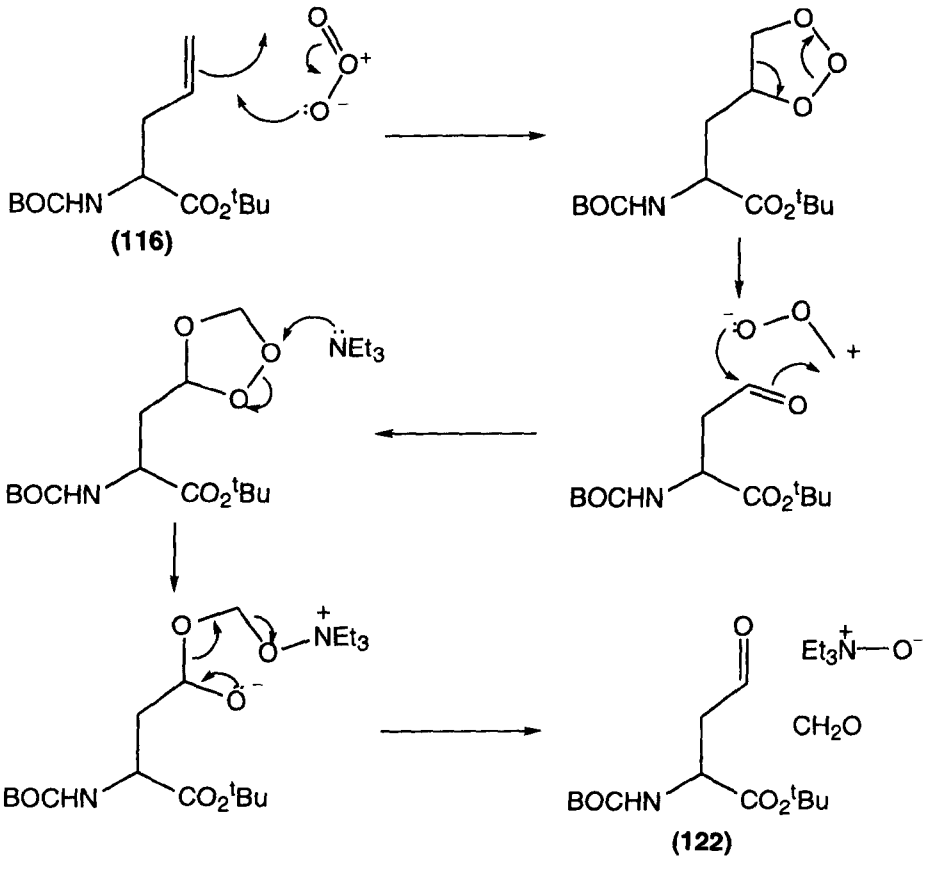
process. Similar studies by Rapoport and co-workers[126] resulted in the preparation of L-*N*-BOC-ASA *tert*-butyl ester L-**(122)** from L-aspartic acid. The 7 step process involved Et_3SiH/Pd/C reduction of a thioester to generate the aldehyde function.

For our studies the ozonolysis of protected allylglycine was the preferred method **(Scheme 25)**. Previous work within our group had determined that the purity of the ASA synthesised was enhanced when the acid function was protected as a *tert*-butyl ester rather than the *para*-methoxybenzyl ester. It is also noteworthy that the *para*-methoxytoluene byproduct must also be removed after deprotection. One disadvantage of protecting the acid as a *tert*-butyl ester was the poor recovery of material (43%). Formation of *tert*-butyl esters is most commonly achieved by treating a solution of the acid with isobutylene in the presence of a catalytic quantity of $c.H_2SO_4$. Reversible protonation of isobutylene generates the thermodynamically stable *tert*-butyl cation which condenses with the carboxylate nucleophile. A high concentration of isobutylene in the reaction mix results in significant self-addition **(Scheme 43)**. It is very important to realise that for this reaction with an amino acid a catalytic quantity of $c.H_2SO_4$ is actually greater than one equivalent. The first equivalent of $c.H_2SO_4$ protonates the amino acid, generating the acid salt.



Scheme 43 Cationic Polymerisation of Isobutylene

In our work it was found that side reactions were minimised and the yield of ester was improved to 83% if the reaction mixture was cooled to -78 °C, prior to addition of isobutylene. After addition of $c.H_2SO_4$ the suspension was stirred for 6 h at -78 °C and then at room temperature for a further 12 h. After BOC protection of the amino function with di-*tert*-butyldicarbonate (BOC_2O), ozonolysis generated the desired protected aldehyde **(122)**. Ozone added to the olefin by a [3 + 2] dipolar cycloaddition **(Scheme 44)**.



Scheme 44 Mechanism for the Ozonolysis of Alkenes

Deprotection of *N*-BOC-ASA *tert*-butyl ester **(122)** proved to be a key process. Assay of ASA hydrate trifluoroacetate **(93)** with DHDPS indicated a poor yield (~40%). In order to develop a reliable biotransformation system for further development it was considered very important to optimise the purity of our ASA. It appears likely that the conditions of deprotection (50% TFA in CH_2Cl_2, room temperature) promote decomposition. To address this problem a number of alternatives were considered, including *in situ* generation of ASA by enzymic cleavage of its *N*-acetyl analogue. This process is discussed in detail in the next chapter.

Rapoport and co-workers[127] reported selective cleavage of *N*-BOC protecting groups in the presence of *tert*-butyl esters by treatment with 500 mol% of 1M HCl in anhydrous EtOAc at room temperature. Consequently, it was considered that alternative protecting groups to the *tert*-butyl ester could be more appropriate for our studies. One protecting group which can be removed under milder conditions is the cumyl ester. Brunwin and Lowe[128] showed that cumyl esters could be selectively removed in the presence of a β-lactam and a *tert*-butyl ester by anhydrous HCl in CH_2Cl_2 at 0 °C for 3 min.

N-BOC-allylglycine cumyl ester **(123)** was prepared in 72% yield by DCC coupling of the acid with cumyl alcohol. However, no further

investigations of compound **(123)** proved necessary because consecutive investigation of the deprotection of *N*-BOC-ASA *tert*-butyl ester **(122)** with anhydrous HCl in EtOAc proved to be very successful. Treating the protected amino acid **(122)** with 1000 mol% of >2M HCl in anhydrous EtOAc at 0 C for 4 h resulted in quantitative recovery of ASA as a white hydrochloride salt. As for the previous deprotection with TFA, NMR spectroscopy in D_2O showed the compound to exist in its hydrated form **(124)** in solution. The advantage of this method over TFA deprotection is the ease of handling of the HCl solution and the simple removal of the volatile byproducts under reduced pressure. TFA is a difficult material to handle and colour changes in stock solutions suggest a tendency for decomposition. In reaction with protected amino acid **(122)** the trifluoroacetate salt **(93)** produced is a yellow coloured salt. One minor disadvantage of the deprotection, common to both TFA and HCl, is the tendency of the product to retain solvent. The ^1H NMR spectrum of compound **(124)** is illustrated in **Figure 22**. Signals are observed for both EtOAc and Et_2O (used for trituration). Extensive drying under high vacuum, with gentle heating, did not remove this solvent.

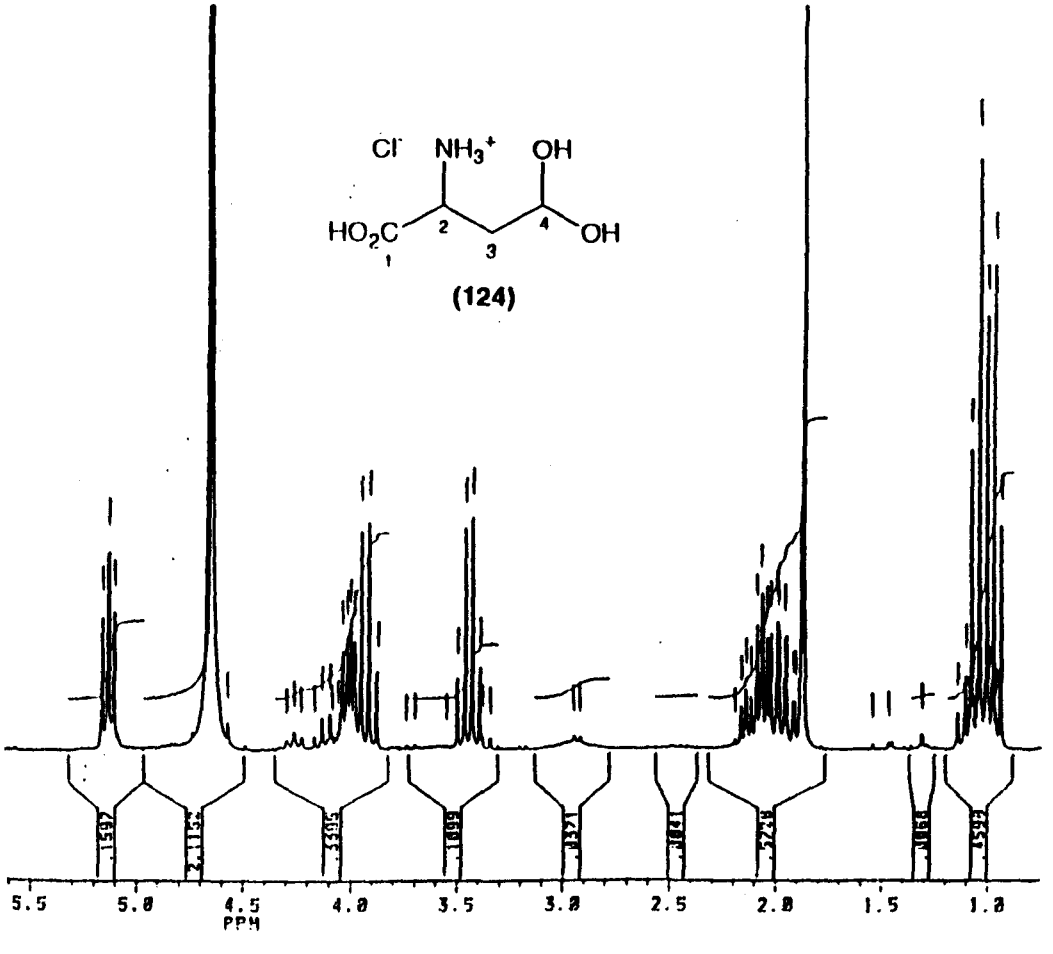
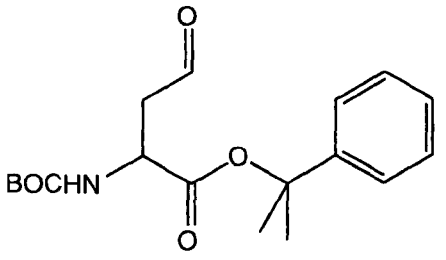


Figure 22 The ^1H NMR Spectrum of Compound (124)



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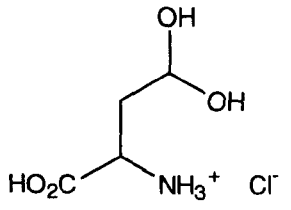
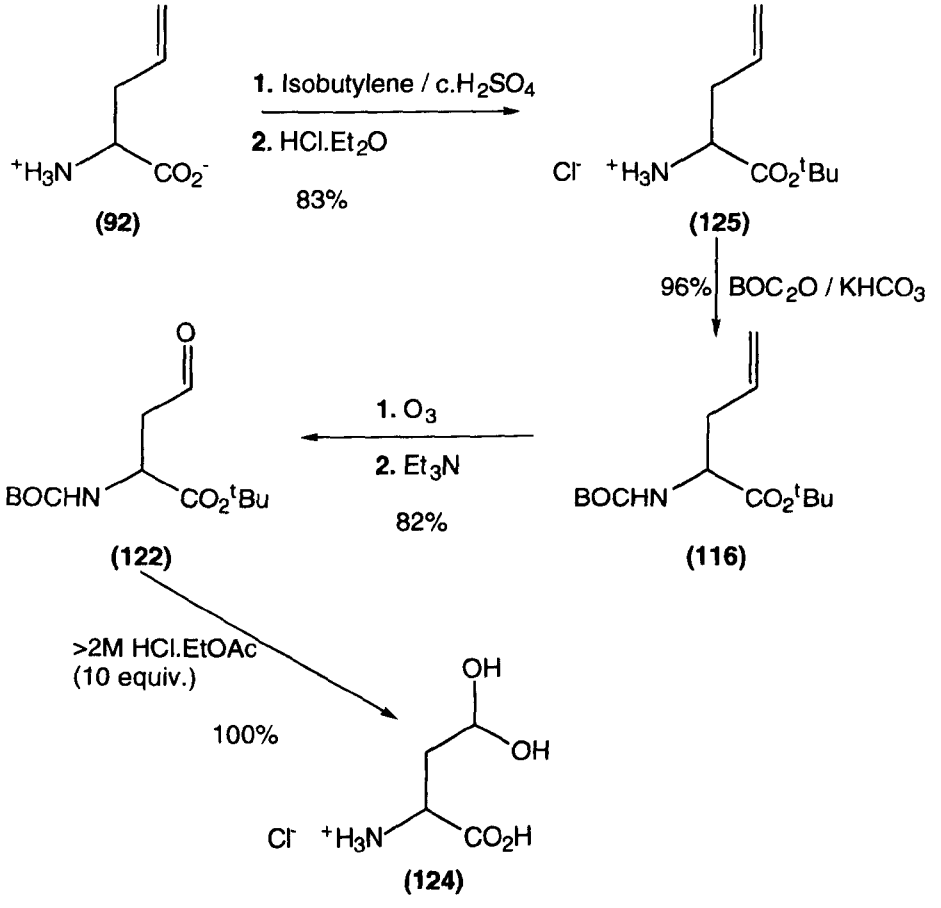


(124)

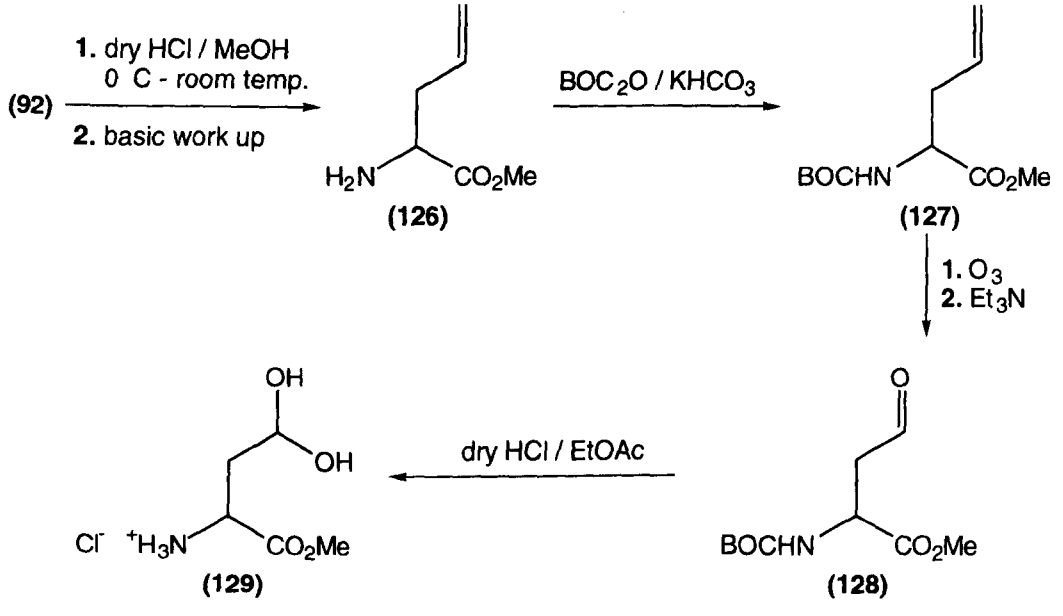
ASA hydrate hydrochloride **(124)**, produced by this method, was shown to display 218% relative activity when compared to its trifluoroacetate analogue **(93)** in a standard DHDPS assay. UV studies suggest a >80% conversion of L-ASA hydrate hydrochloride into DPA on DHDPS catalysed condensation with pyruvate **(21)**. The modified synthesis of ASA is detailed in **Scheme 45**. The overall yield of compound **(124)** from allylglycine was 66%.



Scheme 45 Modified synthesis of ASA

3.07 Synthesis of ASA Methyl Ester

This modified synthesis of ASA was further utilised for the synthesis of aspartate-β-semialdehyde methyl ester hydrochloride **(129)** **(Scheme 46)**. Previous attempts to make the trifluoroacetate salt of this compound had resulted in decomposition on deprotection with TFA. The milder HCl.EtOAc deprotection allowed us to negotiate this problem. Allylglycine methyl ester **(126)** was prepared in 33% yield from allylglycine **(92)** by stirring in methanolic HCl and basic work up. Protection with BOC_2O gave *N*-BOC-allylglycine methyl ester **(127)** in 90% yield. Subsequent ozonolysis gave *N*-BOC-aspartate-β-semialdehyde methyl ester **(128)** in 53% yield. Deprotection, as described, yielded the desired amino ester **(129)** quantitatively.



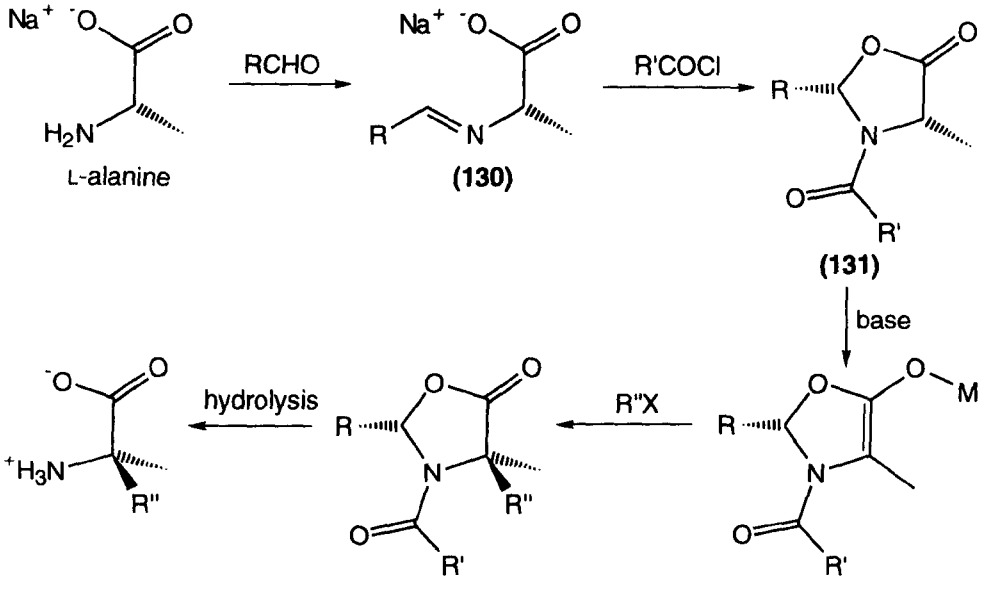
Scheme 46 **Synthesis of ASA Methyl Ester**

3.08 Synthesis of Methyl Substituted ASA Analogues

In our work we examined a number of strategies to substituted allylglycines for the synthesis of ASA analogues: alkylations of amino acids; the Claisen rearrangement route used by Dr McKendrick and zinc coupling of α-imino esters and allylic bromides. The merits of each are discussed in the remainder of this section.

Alkylations of Amino Acids

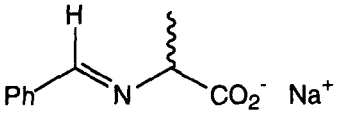
In this work the direct alkylation of amino acids by the ≅self-reproduction of chirality method of Seebach and Fadel[129] was investigated **(Scheme 47)**. In the cyclisation step the bulky acid chloride, commonly pivaloyl chloride, approaches away from the neighbouring methyl group of the *trans*-imine **(130)**. Resulting attack of the carboxylate group on the opposite face generates the *(2S,4S)-cis*-oxazolidinone **(131)** in high diastereoselective yield. After treatment with base the resulting enolate undergoes alkylation away from the bulky R-group in the 2-position. The overall result is retention of stereochemistry at the 2-position of the final substituted amino acid. Berner and co-workers[130] used this methodology for the asymmetric synthesis of 2-methylallylglycine. Further development by Alonso and Davis,[131] using ferrocene carboxaldehyde to form the imine **(130)**, resulted in recovery of 2-benzyl-L-alanine in 63% overall yield and >98% ee.



Scheme 47 Self-Reproduction of Chirality

In our work the usefulness of this alkylation process was investigated by attempts to alkylate DL-alanine. The oxazolidinone produced will favour the racemic *cis*-diastereoisomers and the amino acid isolated after hydrolysis will be racemic. Essentially we are using the oxazolidinone as a doubly *N*-protected amino acid equivalent. Treating sodium DL-alanate and 4Å molecular sieves in anhydrous ethanol with benzaldehyde gave sodium *trans*-*N*-benzylimino-DL-alanate **(132)** in 86% yield. NMR spectra of compound **(132)** were recorded in CD_3OD as it was found to be readily hydrolysed in D_2O. In the ^1H NMR spectrum there are signals at δ 1.37 and 3.90, ^3J 6.9 Hz,

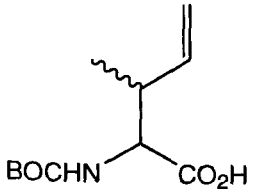
for the protons in the 3- and 2-positions, respectively. The imine proton is found at δ 8.22. There is no evidence in either the ^{1}H or ^{13}C NMR spectra for a *cis/trans* isomeric mixture. Attempts to produce the corresponding oxazolidinone by cyclisation with pivaloyl chloride were unsuccessful. On treating imine **(132)** in anhydrous dichloromethane with pivaloyl chloride at -20 °C a colour change was observed. However, after leaving the reaction at room temperature overnight, aqueous work up yielded mainly pivaloyl byproducts. Further attempts at cyclisation by the varied conditions described by Seebach and Fadel[129] were also unsuccessful.



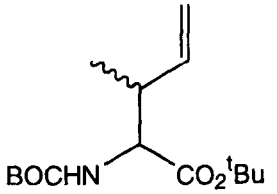
(132)

Claisen Rearrangement of Allylic Esters

To investigate the usefulness of the Claisen rearrangement for preparation of allylglycines, *N*-BOC-glycine **(119)** and *N*-BOC alanine **(133)** were prepared in quantitative yields by standard protection with BOC_2O. DCC coupling of compound **(119)** with *(E)*-crotyl alcohol produced ester **(120)** in 85% yield. Attempts to promote rearrangement of compound **(120)** by the method of McKendrick were successful but the yield (37%) was disappointing (see **Scheme 41**). The highly reactive dianion produced prior to quenching with TMSCl may be unstable, with side reactions such as isocyanate formation possible. It is likely that these problems could be avoided by doubly protecting the amino group. One method of achieving this is detailed in the next chapter. Another minor disadvantage of this route is that alternative protecting and deprotecting conditions, avoiding c.H_2SO_4, must be used for acid **(134)**. DCC coupling of the acid with *tert*-BuOH gave the desired ester **(135)** in 43% yield. Further investigations with *N*-acetylglycine allyl ester **(136)** produced a rearranged product that was not easily purified.



(134)



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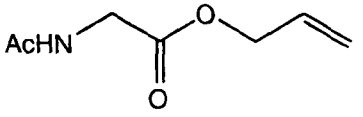
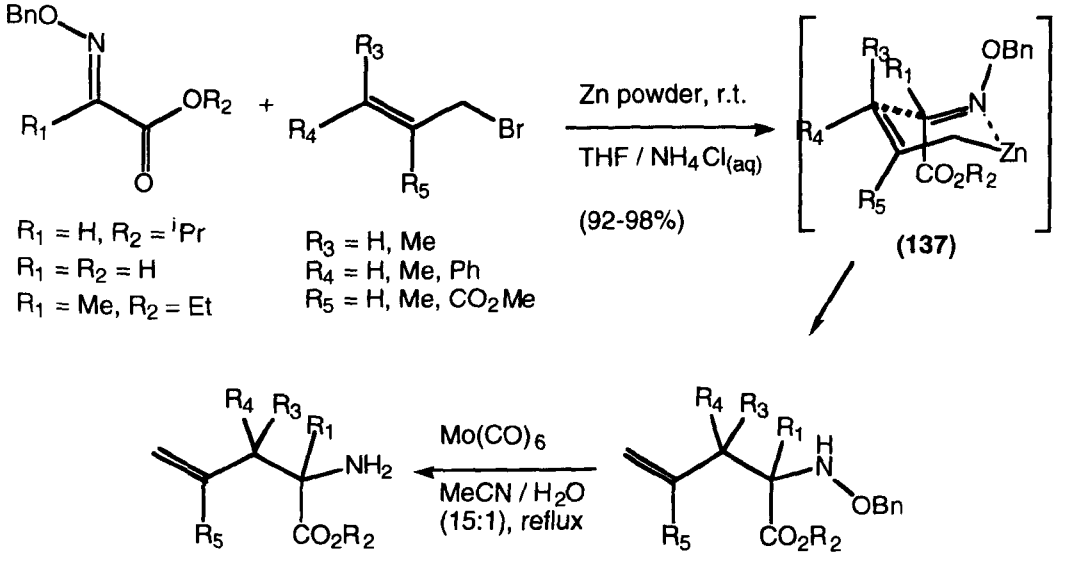


(136)

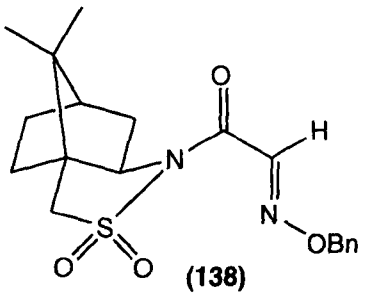
Zinc Coupling of α-Imino Acids and Allylic Bromides

The metal mediated coupling of allylic halides with aldehydes and ketones in aqueous media has been well documented. The reaction is similar to the Reformatski reaction of α-haloesters with aldehydes and ketones.[132] Luche *et al.*[133] reported highly selective reaction of aldehydes over ketones with allylic halides. More importantly, the tin or zinc induced reactions are completely regiospecific. After insertion the metal chelates to the oxygen of the carbonyl. Alkylation then occurs via a six-membered transition state away from the end that originally bore the halogen (see **Scheme 48**). Recent studies by Hanessian and Yang[134] have extended this methodology to the synthesis of allylic α-amino acids **(Scheme 48)**. Coupling the allylic halide to α-benzyloxoimino derivatives of glyoxalates and pyruvates produced the *N*-benzyloxoamino acids. Cleavage of the N-O bond by $Mo(CO)_6$ yielded the desired allylglycines.

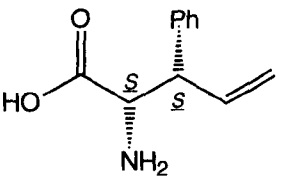


Scheme 48 Zinc Coupling Route to Allylglycines

Hanessian and Yang[134] further reported that the process could be performed with high stereoselective control using the amide of α-benzyloxoiminoglyoxylic acid and Oppolzer s *(1S)*-(-)-2,10-camphorsultam **(138)**. Treating compound **(138)** with allyl bromide yielded *(S)*-allylglycine in high yield and 93% ee after deprotection. When *trans*-cinnamyl bromide was used *(2S,3S)*-3-phenylallylglycine **(139)** was isolated in 88% yield and 99% ee. The stereochemistry was determined by X-ray analysis of the *N*-benzyloxoamino camphorsultam derivative.

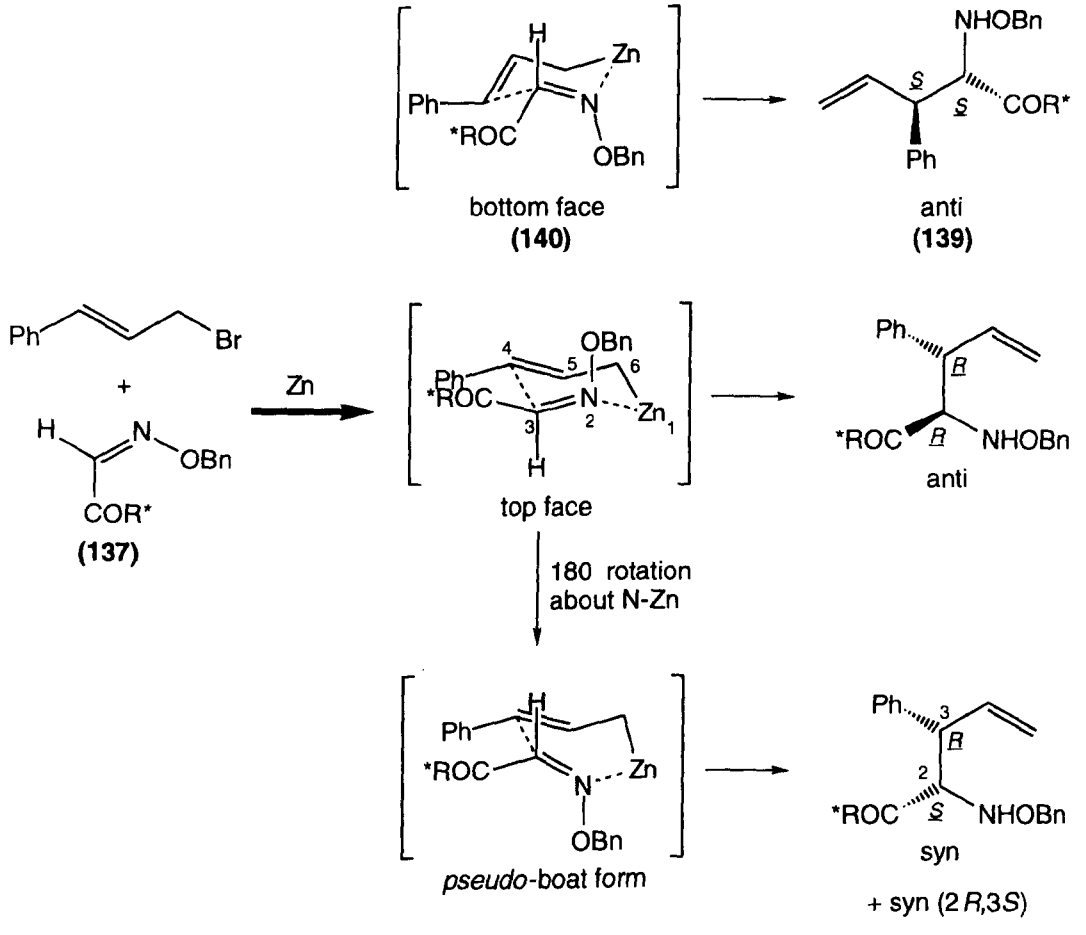


(138)



(139)

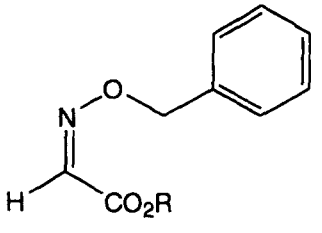
To achieve this stereochemistry the phenyl group and amide group must be *anti* in the *pseudo*-chair transition state **(140)**. It is possible to achieve a *syn* arrangement by rotating 180 about interaction 1-2 (N- -Zn) or bond 5-6 (C-C) but the resulting *pseudo*-boat form is sterically more hindered **(Scheme 49)**. The homochiral camphorsultam directs attack to the *Si*-face (bottom as drawn) of the imine. It is worthwhile noting that to achieve this *anti*-arrangement of phenyl group and amide group the imine must have the *Z*-stereochemistry, contrary to that drawn for the transition state by Hanessian and Yang[134] **(137) (Scheme 48)**.



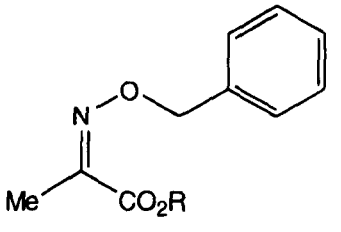
Scheme 49 Stereochemistry of Zinc Coupling

This route to 2- and 3-methyl substituted allylglycines proved to be very useful in our work. The synthesis of these compounds and subsequent conversion into the corresponding ASA analogues is detailed in the rest of this section.

Following the method of Ottenheijm and co-workers[135] α-benzyloxoiminoglyoxylic acid **(141)** and α-benzyloxoiminopyruvic acid **(142)** were both prepared in 99% yield by coupling the corresponding acids with *O*-benzylhydroxylamine hydrochloride in water. Attempts to couple compound **(141)** directly with allyl bromide by the method of Hanessian and Yang[134] produced a product that was difficult to purify. As a result it was decided to protect the acid function as the *tert*-butyl ester. Treating compounds **(141)** and **(142)** with isobutylene and $c.H_2SO_4$ gave α-benzyloxoiminoglyoxylate *tert*-butyl ester **(143)** and α-benzyloxoiminopyruvate *tert*-butyl ester **(144)** in 91% and 89% yield, respectively. Both compounds were isolated as colourless oils after chromatography. The imine proton of compound **(143)** is found at δ 7.47 in the ^1H NMR spectrum. The corresponding methyl signal of compound **(144)** is found at δ 1.96 in the ^1H NMR spectrum and δ 11.6 in the ^{13}C NMR spectrum. The NMR spectra show no evidence of a mixture of *cis*- and *trans*-isomers. It is proposed by the argument outlined earlier that the compounds have exclusively *cis*-geometry.

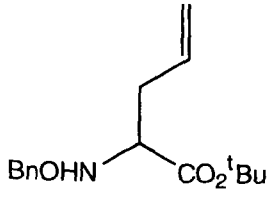


(141) R = H
(143) R=^{t}Bu

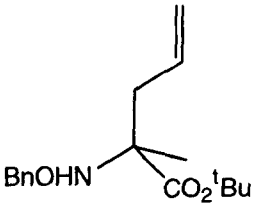


(142) R = H
(144) R=^{t}Bu

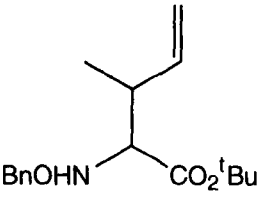
Zinc-induced coupling of compounds **(143)** and **(144)** with allyl bromide according to the method of Hanessian and Yang[134] produced protected allylglycine **(145)** and protected 2-methylallylglycine **(146)** in quantitative yield. In the ^1H NMR spectrum of compound **(146)** the signal for the 3-Me is found as a singlet at δ 1.28. Synthesis of the protected 2-methylallylglycine **(147)** required more planning.



(145)



(146)



(147)

The necessary bromide, crotyl bromide, was purchased as a mixture of 85% crotyl bromide and 15% 3-bromo-1-butene. To counter this, the quantity was adjusted and the activated zinc powder was added in two portions. As expected, the primary crotyl bromide reacted faster than the secondary isomer and the desired compound **(147)** was isolated in 99% yield without contamination. Inspection of the ^{1}H NMR **(Figure 23)** and ^{13}C NMR **(Figure 24)** spectra confirm the presence of diastereoisomers. The 3-Me is found as a doublet at δ 0.95, ^{3}J 6.9 Hz, in the ^{1}H NMR spectrum. GC analysis of the product showed the diastereomeric ratio to be 100:41. We would predict by our earlier argument that the *anti*-arrangement of ester and methyl group in a *pseudo*-chair transition state would be favoured. This would give a racemic mixture of the *(2S,3S)*- and *(2R,3R)*-stereoisomers as the major component. Assuming that our *(E)*-crotyl bromide and *(Z)*-**(143)** are stereochemically pure then the minor component of the mixture, racemic *(2S,3R)*- and *(2R,3S)*-**(147)**, must have arisen through a *syn*-arrangement of ester and methyl group in a *pseudo*-boat transition state.

The next step of the syntheses involves the cleavage of the N-O bond to generate the free amino function and benzyl alcohol as a byproduct. Literature procedures for this process involve catalytic hydrogenolysis or cleavage by $Mo(CO)_6$.[136,137] Since catalytic hydrogenation also reduces the olefinic function[134] then only the latter process was considered. Molybdenum promoted cleavage of N-O bonds proceeds through ligand exchange, reductive cleavage and release. The reaction is performed by heating a solution of the compound and 0.7 equivalents $Mo(CO)_6$ in 6% aqueous MeCN. The small volume of water assists in the release of the product by ligand exchange. In our work, early attempts to utilise this process were rather low yielding. After further investigations it was found that adding 1% c.NH_3 (aq) to organic solvents for chromatographic purification improved the yield. It was considered that the ammonia further assisted in the release of the product from the molybdenum. Treatment of compounds **(145)** and **(146)** by this method gave allylglycine *tert*-butyl ester **(148)** and 2-methylallylglycine *tert*-butyl ester **(149)** in 43% and 48% yield, respectively. Further developments revealed that extracting the product into 1M HCl (aq), basifying (NaOH) and reextracting into EtOAc gave the pure product in high yield without chromatography. 3-Methylallylglycine *tert*-butyl ester **(150)** was prepared by this method from compound **(147)** in 77% yield. The work up involving acidic extraction is a very messy process, involving large solvent volumes, but produces a lighter coloured purer product in high yield.

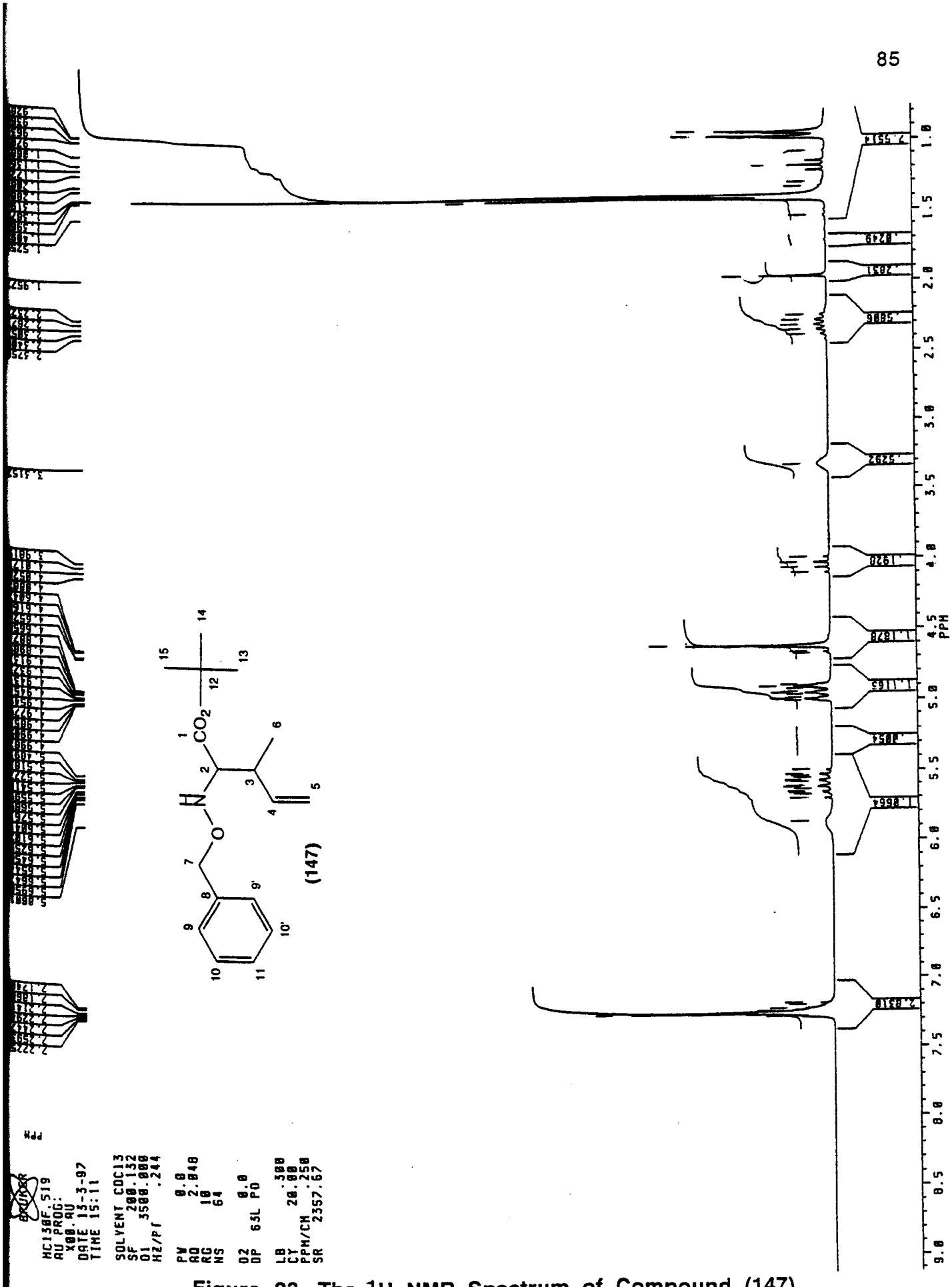


Figure 23 The ^{1}H NMR Spectrum of Compound (147)

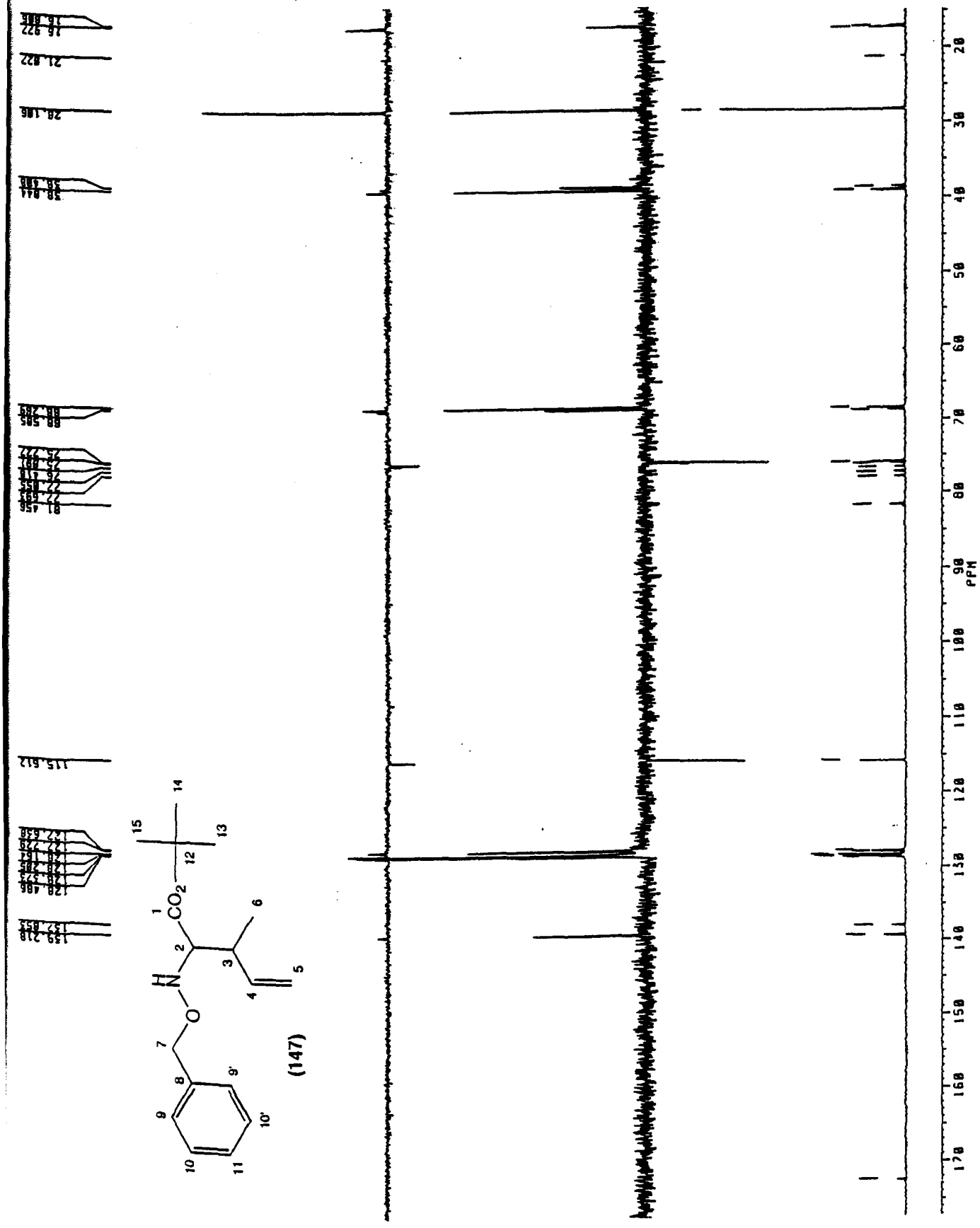
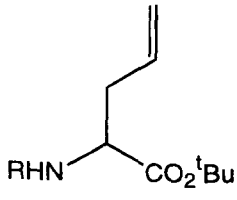


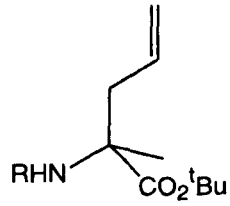
Figure 24 The ^{13}C NMR Spectrum of Compound (147)

After successfully preparing the substituted allylglycines the remaining steps to the aspartate-β-semialdehydes were performed as described previously for allylglycine *tert*-butyl ester hydrochloride **(125)** in section **3.06**. Reaction of compounds **(149)** and **(150)** with BOC_2O produced *N*-BOC-2-methylallylglycine*tert*-butyl ester **(151)** and *N*-BOC-3-methylallylglycine-*tert*-butyl ester **(135)** in 48% and 85% yield, respectively. Subsequent ozonolysis produced aldehydes **(152)** and **(153)** in 72% and 59% yield, respectively. The aldehyde signal of protected 2-Me-ASA **(152)** in the ^1H NMR spectrum was coupled to the diastereomeric protons at the 3-position: δ 9.38; ^3J 1.9 and 1.2 Hz. Corresponding aldehyde signals for protected 3-Me-ASA **(153)** were found as singlets in the^1H NMR spectrum at δ 9.63 and 9.68 for the major and minor diastereoisomers, respectively. Deprotection by HCl in anhydrous EtOAc gave quantitative yields of 2-methylaspartate-β-semialdehyde hydrate hydrochloride **(154)** and 3-methylaspartate-β-semialdehyde hydrate hydrochloride **(155)**. In the ^1H NMR spectrum of compound **(154)** the proton at the 4-position was coupled to the diastereomeric protons at the 3-position: δ 5.04; ^3J 6.7 and 5.1 Hz. In the ^{13}C NMR spectrum C-4 was found at δ 88.2. The corresponding ^{13}C NMR signals for compound **(155)** were found at δ 91.8 and δ 92.0 for the major and minor diastereomers, respectively.

The enzyme studies with 2-methyl-ASA, 3-methyl-ASA and ASA methyl ester will be discussed in chapter 6.



(148) R = H
(116) R = BOC



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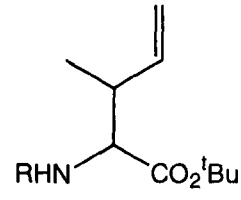
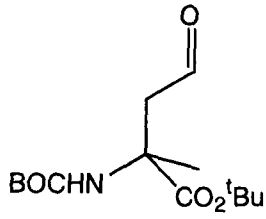
(151) R = BOC



(150) R = H
(135) R = BOC



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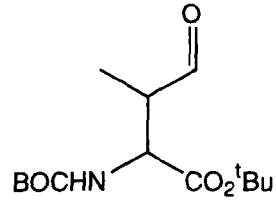

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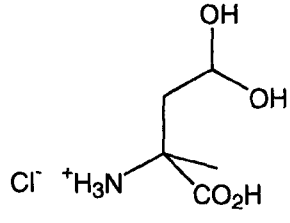

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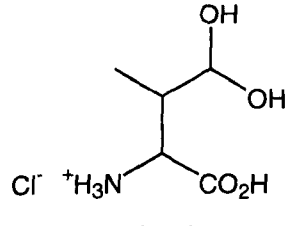
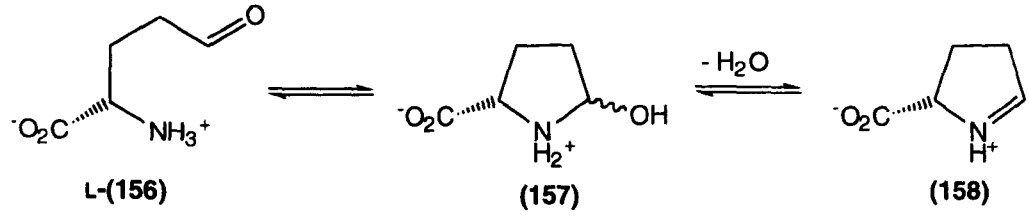


(155)

Chapter 4 Investigation Towards Glutamate-γ-semialdehyde as a Substrate of DHDPS

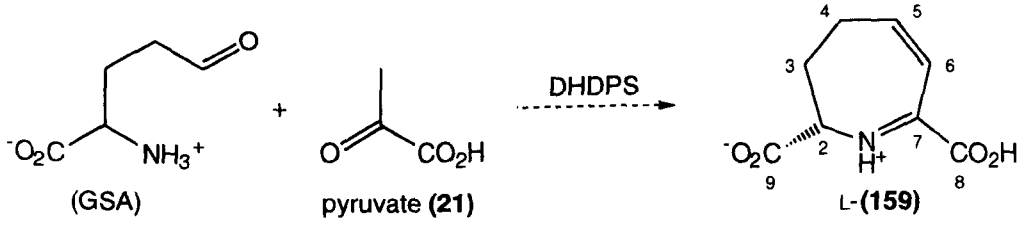
4.01 Introduction

L-Glutamate-γ-semialdehyde L-(GSA) L-**(156)** is the higher homologue of the natural substrate of the DHDPS enzyme, L-aspartate-β-semialdehyde L-(ASA) **(23)**. L-GSA **(156)** is known to exist in equilibrium with the aminol **(157)** and the cyclic imine, 3,4-dihydro-*2H*-pyrrole-2-carboxylic acid **(158)** **(Scheme 50)**. It is involved in the biosynthesis of L-proline from L-glutamic acid.[138]



Scheme 50 Cyclisation of GSA

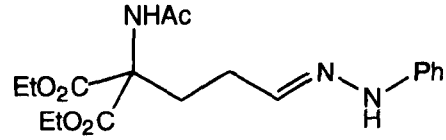
It was considered that this close analogue of ASA could exhibit substrate activity with DHDPS. The likely product of successful DHDPS catalysed condensation of pyruvate and GSA would be the seven-membered heterocycle L-3,4-dihydro-*2H*-azepine-2,6-dicarboxylic acid L-**(159)** **(Scheme 51)**. This heterocycle has an interesting ring oxidation state and biotransformation of GSA could open a number of new areas of study. Investigation of the inhibitory characteristics of analogues of heterocycle **(159)** with DHDPS and DHDPR as well as the latter succinylase, acylase and dehydrogenase enzymes would be of interest.



Scheme 51 GSA as a Substrate of DHDPS

4.02 Literature Preparations of GSA

3,4-Dihydro-*2H*-pyrrole-2-carboxylic acid **(158)** was prepared in racemic form by Strecker[139] on deprotection of alkylated glycine equivalent, γ,γ-dicarboethoxy-γ-acetamidobutyraldehyde phenylhydrazone **(160)**. Eto and co-workers[140] achieved the *in situ* preparation of DL-GSA by acidic hydrolysis of GSA dimethyl acetal **(161)**. No characterisation was presented for GSA but they successfully synthesised tryptophan by condensing the product with phenylhydrazine and subsequent Fischer indole formation. It is worthwhile noting that the condensation of phenylhydrazine with the cyclic imine **(158)** would be an equally viable route. GSA dimethyl acetal **(161)** was prepared by the method of Okuda[141] **(Scheme 52)**. Michael addition of diethyl nitromalonate **(162)** to acrolein and *in situ* acetal formation generated alkylated glycine equivalent **(163)**. Deprotection, reduction of the nitro group and purification yielded the desired acetal **(161)**.



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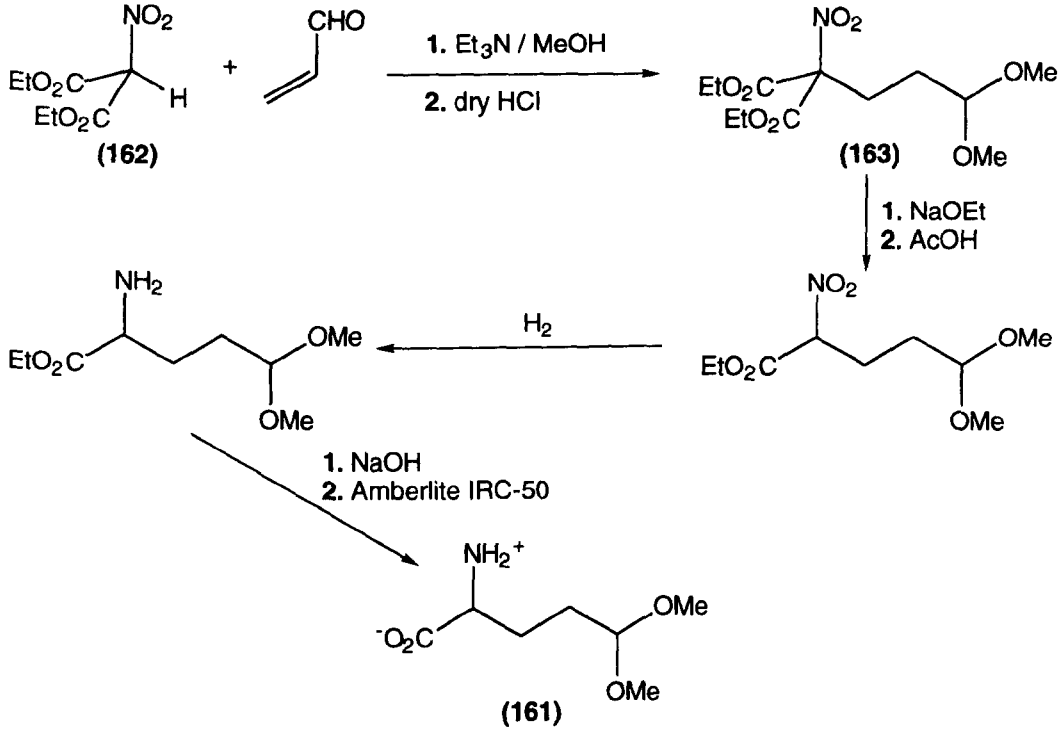


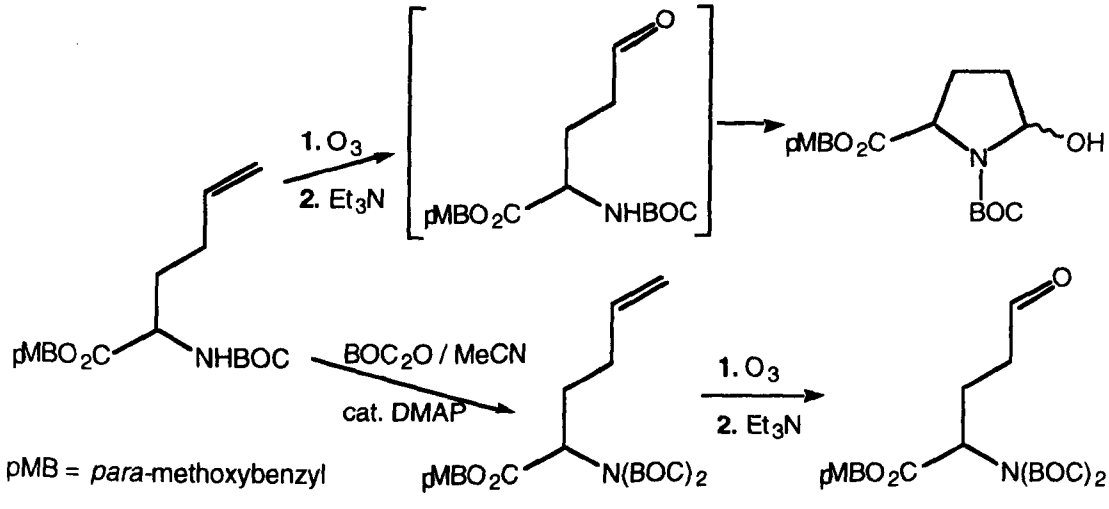
(162) **(163)** **(161)**

Scheme 52 Synthesis of GSA Dimethyl Acetal (161)

The stability of GSA in aqueous solution is concentration dependent. Williams and Frank[142] reported that GSA underwent 30% decomposition in 1 h at 1 M but decomposed at a lesser rate in less concentrated solutions. Eto and co-workers[140] noted that GSA could be preserved in aqueous solution by adding either phenylhydrazine, sodium hydrogensulfate, hydroxylamine or semicarbazide.

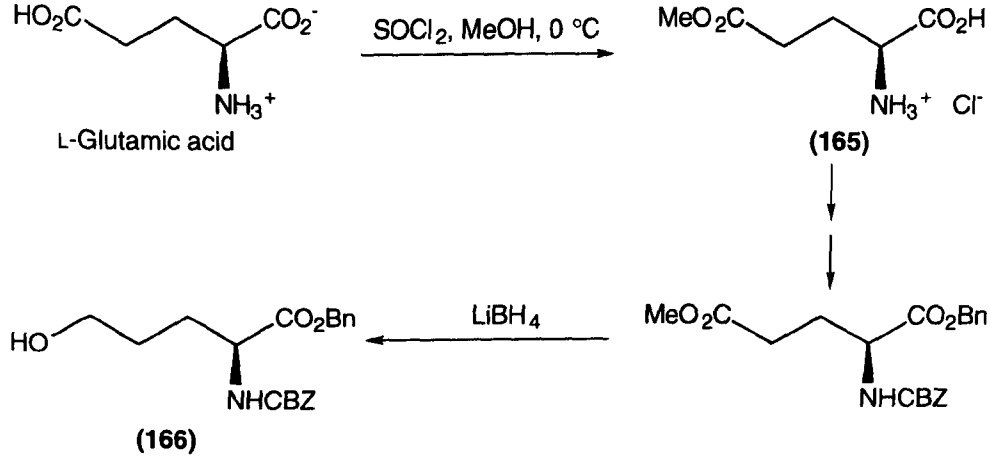
In recent work by Bearne and Wolfenden[143] compound **(158)** was prepared by periodic acid oxidation of allo-δ-hydroxylysine. Proton NMR studies showed that compound **(158)** was in equilibrium with GSA (0.05% at physiological pH). At low pD values the spectrum was complex in the region of δ 2 to 5, but signals were observed for the free aldehyde (GSA) **(156)** at δ 9.8. At pD 6.2 two well separated signals at δ 8.7 and 5.8 were observed for the imine **(158)** and hydrated aldehyde, respectively. The chemical shift of the imine proton varied with pD. Importantly, Bearne and Wolfenden made no mention of the intermediate 5-hydroxyproline **(157)** (see **Scheme 50**). The chemical shift of the proton in the 5-position of compound **(157)** would be very similar to the corresponding signal of hydrated GSA.

Our group previously investigated a number of synthetic routes to GSA. Dr S.J. Connell extensively studied the alkylation of a number of glycine equivalents with 4-bromo-1-butene.[144] Phase transfer alkylation with *N*-(diphenylmethylene)aminoacetonitrile **(98)** was found to be the most effective route to the higher homologue of allylglycine, 2-amino-5-hexenoic acid **(164)**. Ozonolysis of compound **(164)**, after protection, resulted in spontaneous cyclisation **(Scheme 53)**. This cyclisation was noted in a similar process by Olsen *et al.*[145] Doubly protecting the amino function, according to the method of Grehn and Ragnarsson,[146] prevented cyclisation on ozonolysis **(Scheme 53)**. Deprotection with TFA produced impure material.



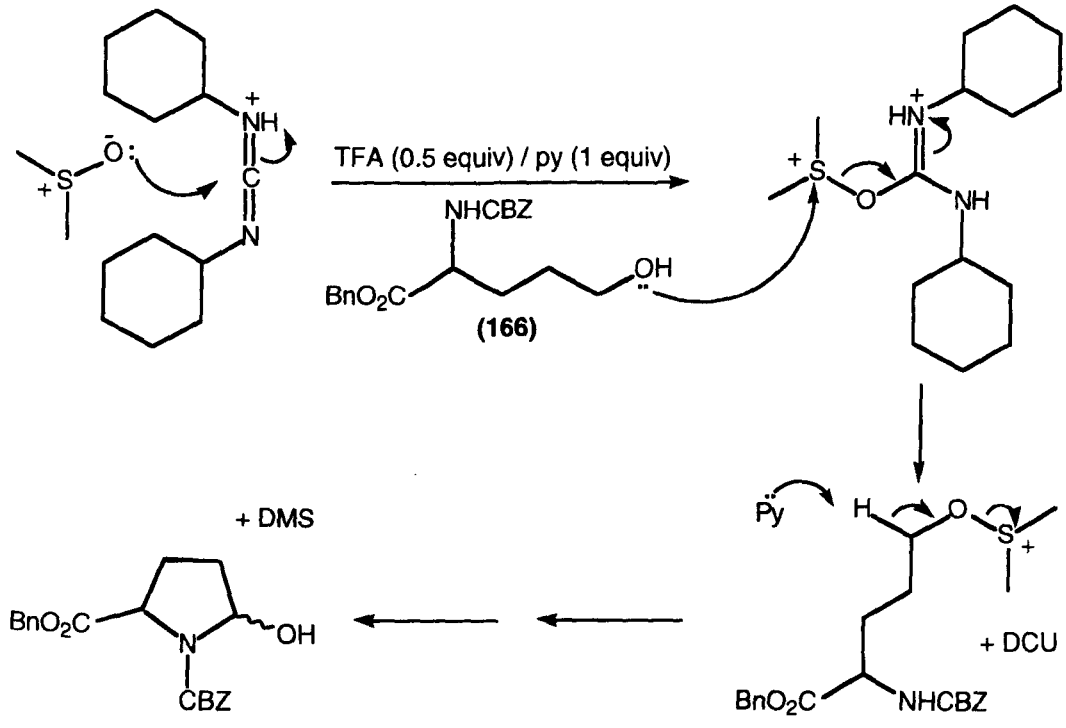
Scheme 53 Synthesis of Protected GSA

Derivatisation of glutamic acid is a useful method of synthesising GSA analogues. The synthesis requires non-symmetrical substitution of the two acid groups of glutamic acid. In earlier studies by our group Dr J.M. McKendrick attempted the synthesis of GSA by the derivatisation of glutamic acid **(Scheme 54)**.[147] The key step involves selective protection of the glutamic acid as the δ-methyl ester hydrochloride **(165)**. After further protection the methyl ester was selectively reduced with $LiBH_4$. Attempts to oxidise the resulting alcohol **(166)** to the aldehyde once again resulted in cyclisation. The oxidation was performed by a modified Swern type oxidation, according to the method of Moffat[148] **(Scheme 55)**. The advantage of derivatisation routes is that a number of glutamic acid derivatives are readily available in enantiomerically pure form.

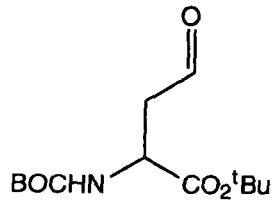


Scheme 54 **Non-Symmetrical Derivatisation of Glutamic Acid**

There are a number of alternative methods for the non-symmetrical derivatisation of glutamic and aspartic acid reported in the literature. Rapoport and co-workers[126] detailed one such way, generating the free β-acid function with aspartic acid in 5 steps. DCC coupling with EtSH and subsequent reduction with Et_3SiH and Pd/C yielded the protected ASA **(117)**. Bold *et al.*[149] incorporated the γ-aldehyde function into doubly amino protected glutamate derivative **(167)** by a Rosenmund reduction reaction. Lee and Miller[150] achieved the same transformation on acid chloride **(167)** by reduction with the sterically hindered hydride donors tributyltin hydride or lithium tri-*tert*-butoxyaluminium hydride.



Scheme 55 Modified Swern Oxidation



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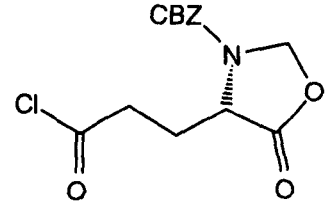
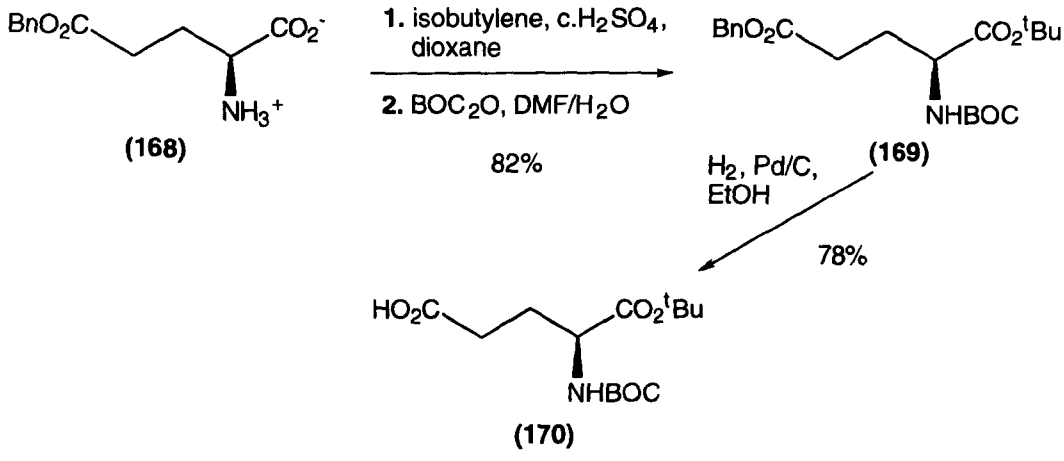
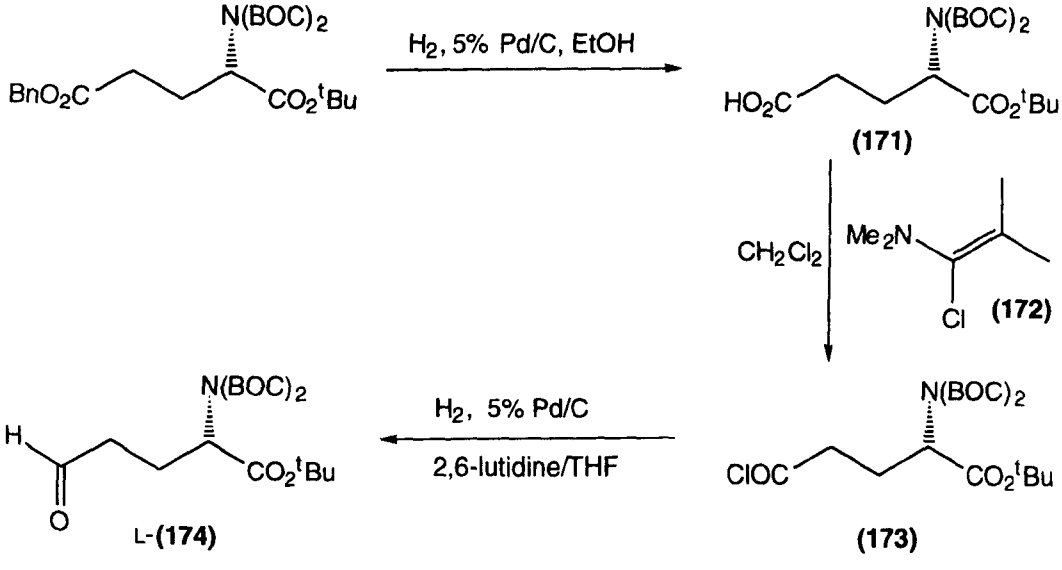


(167)

δ-Benzyl L-*N*-BOC-glutamate *tert*-butyl ester **(169)** can be prepared from the commercially available L-glutamate δ-benzyl ester **(168)** by standard methodology **(Scheme 56)**.[145] Hydrogenolysis then generates the free δ-acid **(170)** for further manipulation. Bold *et al.*[149] prepared *bis*-*N*-BOC-L-glutamate-γ-semialdehyde *tert*-butyl ester L-**(174)** from compound **(169)**. The key step involves generating the aldehyde from the acid chloride **(173)** by Rosenmund reduction **(Scheme 57)**. The acid chloride **(173)** is prepared from the corresponding acid by treatment with enamine **(172)**.[151] In the presence of acid labile protecting groups this reagent avoids potential problems associated with conventional acidic chlorinating agents. Compound **(174)** is of particular interest to us because, as discussed in Chapter 3, it can be deprotected in one step under anhydrous conditions and easily purified.



Scheme 56 Synthesis of Acid (170)



Scheme 57 Rosenmund Reduction to Generate Aldehyde

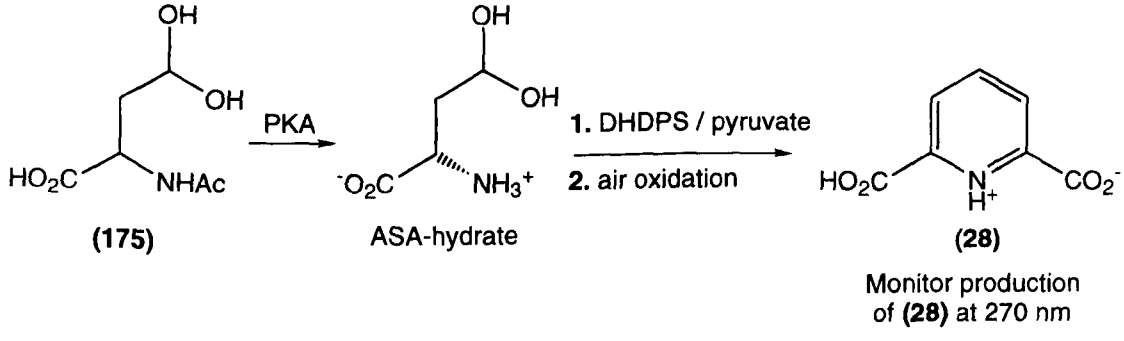
4.03 Objectives

With little mention of GSA in the literature and lack of any characterisation data the low stability of this compound is clearly indicated. It was notable that the cyclic analogues prepared previously by our group showed no evidence of the open chain aldehyde in the NMR spectra. If the equilibrium concentration of GSA is very low then it may be very difficult to

realise the substrate potential with DHDPS. For these reasons it was considered in our current work that alternative strategies were required.

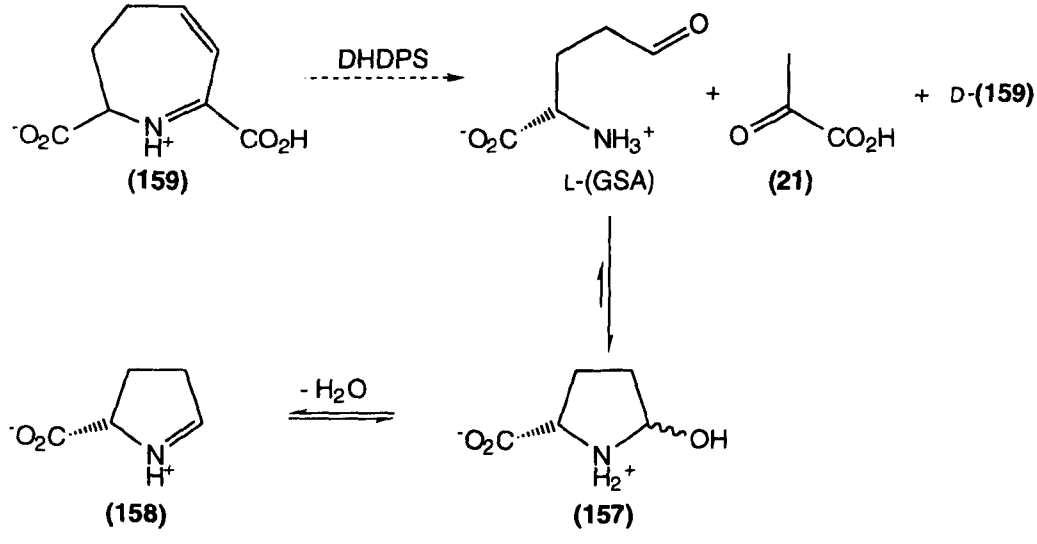
It was considered that *in situ* deprotection of an appropriate aliphatic precursor would be worthwhile. Ideally, to achieve this task the aldehyde would be protected to avoid cyclisation. Aldehydes can be protected as imines, acetals and other similar derivatives. It is likely that imine protected GSA would also cyclise. Although acetal-protected GSA would not cyclise the acidic conditions required for deprotection are not suitable for enzyme work. One other possibility is protection of the aldehyde as a silyl enol ether. This protecting group however would not be stable in an aqueous environment.

An alternative method of avoiding cyclisation of GSA, prior to *in situ* deprotection, is the protection of the amino function. In section **3.05** we mentioned the enzymic resolution of racemic *N*-acetylallylglycine **(113)** by the enzyme porcine kidney acylase (PKA) **(Scheme 37)**. It was considered that this process could also be applied to *N*-acetylated ASA and GSA. Enzymic cleavage of these compounds by PKA under physiological conditions would generate the required L-amino acids *in situ*. To validate this theory our first task was the synthesis of *N*-acetyl-ASA. The preparation and evaluation of *N*-acetyl-ASA as an inhibitor of DHDPS has been performed previously by our group.[152] *N*-Acetyl-ASA was found to exist in its hydrated form **(175)** in solution. In our work compound **(175)** was prepared by the same method. *In situ* deprotection by PKA and DHDPS catalysed condensation with pyruvate proved successful. The assay system used for this purpose **(Scheme 58)** will be discussed further in Chapter 6. Having successfully developed this new assay system for testing protected amino acids the next objective was the synthesis of *N*-acetyl-GSA. This will be discussed in section 4.05.



Scheme 58 **Development of a New Assay for Detecting Substrate Activity**

In our studies one other entirely different strategy for investigating the biological activity of GSA with DHDPS was considered. One advantage of working with enzymes is the reversible nature of catalysis. If the synthesis of a potential substrate is troublesome then investigation of the reverse biological process is a possible alternative. In this case the cyclisation of GSA would be a favourable event, displacing the equilibrium in our favour. The study of heterocycle **(159)** as a substrate of DHDPS is summarised in **Scheme 59**. The chemical synthesis of heterocycle **(159)** is however not a trivial matter. In section 4.06 the synthetic strategy towards this heterocycle will be discussed.



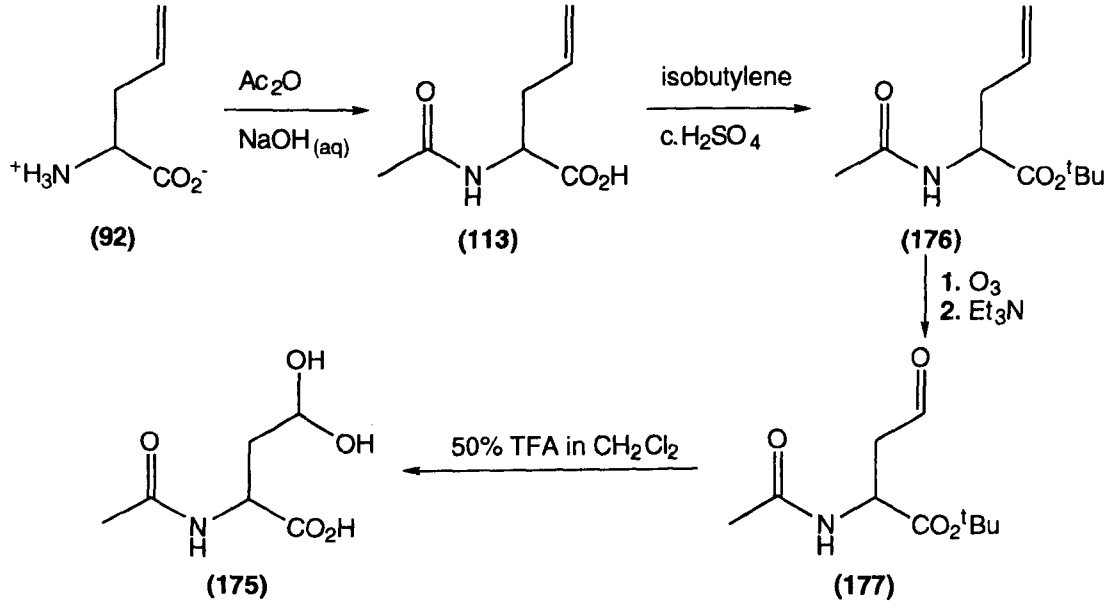
Scheme 59 **Alternative Strategy for Investigating GSA as a Substrate of DHDPS**

4.04 Synthesis of *N*-Acetylaspartate-β-semialdehyde

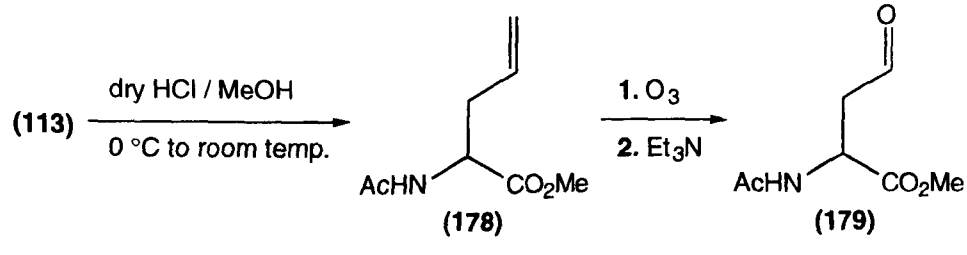
DL-*N*-Acetyl-ASA was required for developing the new assay system for *in situ* generation of amino acids. DL-*N*-Acetylallylglycine **(113)** was synthesised in 74% yield from DL-allylglycine **(92)** by treatment with acetic anhydride in NaOH (aq) according to the method of Black and Wright.[115] The methyl signal is found at δ 1.97 in the ^{1}H NMR spectrum and at δ 22.3 in the ^{13}C NMR spectrum. DL-*N*-Acetyl-ASA hydrate **(175)** was then synthesised from compound **(113)** by the method of Tudor[152] **(Scheme 60)**. Esterification of compound **(113)** with isobutylene produced *N*-acetylallylglycine *tert*-butyl ester **(176)** in 95% yield. Ozonolysis generated the desired aldehyde **(177)** in 82% yield. The aldehyde signal is found at δ 9.73 in the ^{1}H NMR spectrum and at δ = 199.4 in the ^{13}C NMR spectrum. Deprotecting the acid function of compound **(177)** with 50% TFA in

anhydrous CH_2Cl_2 gave the desired DL-*N*-acetyl-ASA hydrate **(175)** in 59% yield. In the ^1H NMR spectrum the proton in the 4-position was observed to couple to the diastereomeric methylene protons in the 3-position; δ 4.94, ^3J 6.6 and 4.9 Hz.

Development of the assay involving *in situ* deprotection of compound **(175)** allowed the original detection of substrate activity in ASA-methyl ester **(129)**. Prior to the revision of the TFA deprotection of ASA and analogues, compound **(129)** was found to decompose at this stage in the synthesis. *N*-Acetyl-ASA methyl ester **(179)** was synthesised by a modification of the method detailed above **(Scheme 61)**. Testing of compound **(179)** in the assay involving the PKA and DHDPS enzymes showed moderate levels of substrate activity.



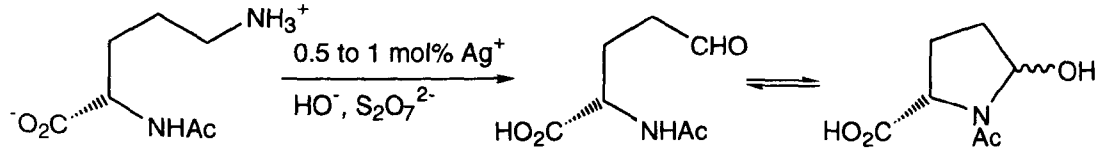
Scheme 60 Synthesis of *N*-Acetyl-ASA



Scheme 61 Synthesis of N-Acetyl-ASA Methyl Ester

4.05 Attempted Synthesis of *N*-Acetylglutamate-γ-semialdehyde

Having successfully synthesised *N*-acetyl-ASA by the method of Tudor[152] it was decided to use the same methodology toward the higher homologue *N*-acetyl-GSA **(181)**. It was considered possible that the amide **(181)** would undergo a similar cyclisation to that observed for the carbamate analogues prepared previously by our group. The greater electron withdrawing effect on the nitrogen lone pair in the amide compared to the carbamate was however in our favour. The synthesis of *N*-acetyl-L-GSA has been reported previously by Konodo.[153] *N*-Acetyl-L-GSA **(181)** was prepared by the persulfate oxidation of *N*-α-acetyl-L-ornithine **(180)** **(Scheme 62)**. The only characterisation presented was the microanalysis of the 2,4-DNP derivative. It is feasible that 2,4-DNP could condense with the cyclic form **(182)**.



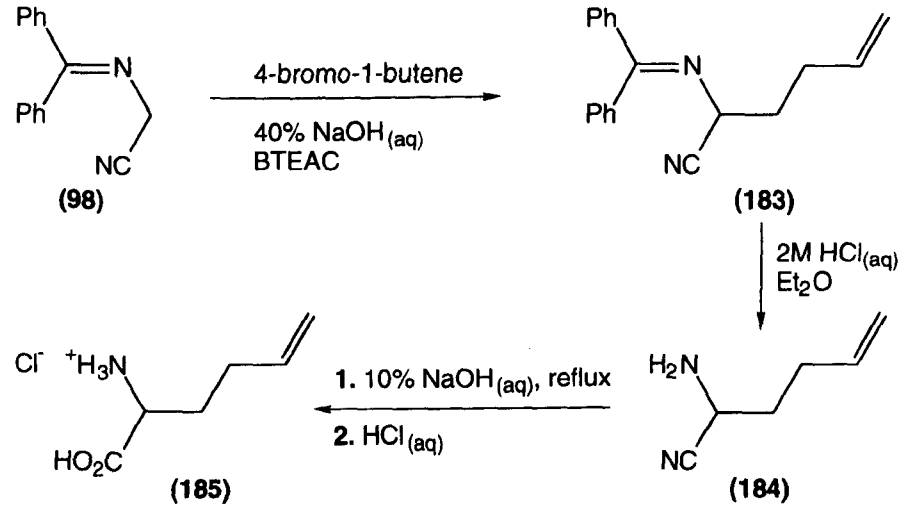
(180) **(181)** **(182)**

Scheme 62 **Literature Preparation of *N*-Acetyl-GSA (181)**

In order to use the methodology detailed in the previous section it was necessary to synthesise first the higher homologue of allylglycine, 2-amino-5-hexenoic acid **(164)**. Compound **(164)** has been synthesised previously by our group and isolated as its hydrochloride salt **(185)**[154] **(Scheme 63)**. This was achieved from glycine equivalent *N*-(diphenylmethylene)-aminoacetonitrile **(98)** by the phase transfer alkylation method of O'Donnell *et al.*[99]

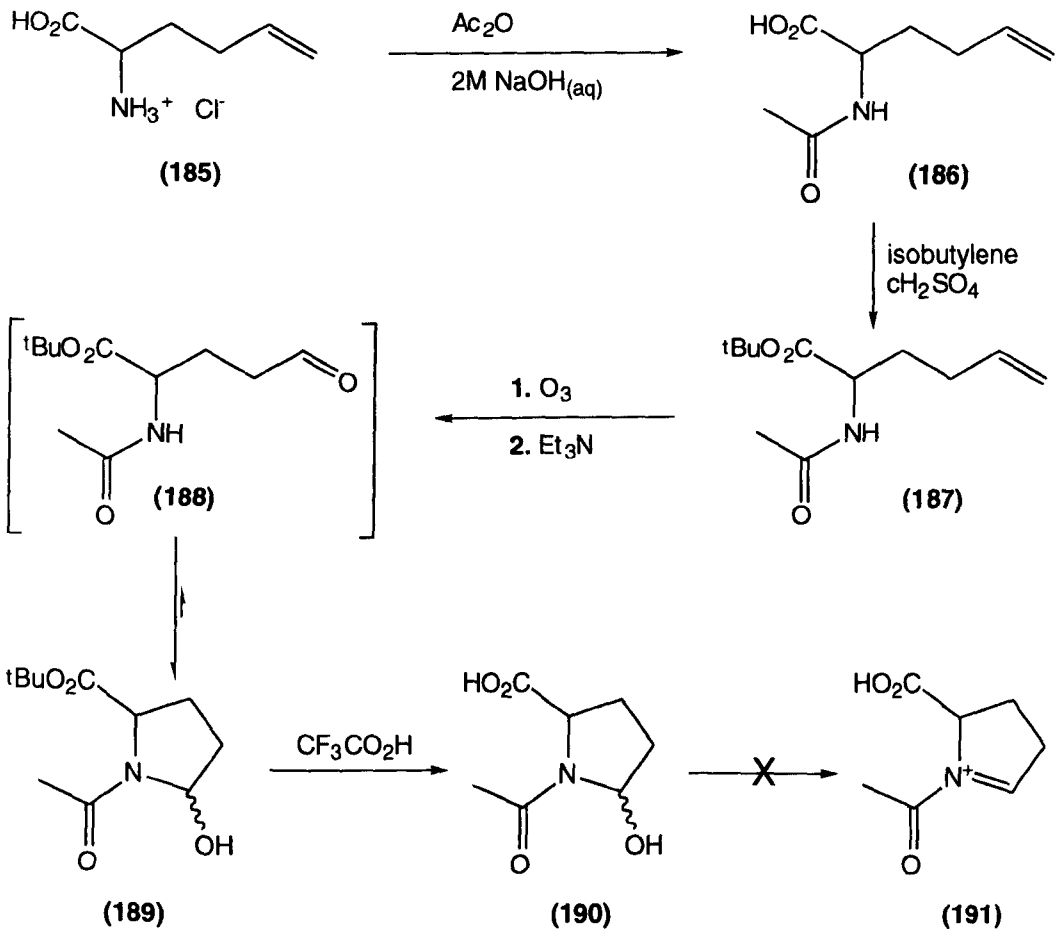
In our work, DL-2-(diphenylmethyleneamino)-5-hexenonitrile **(183)** was synthesised in 92% yield from *N*-(diphenylmethylene)aminoacetonitrile **(98)** and 4-bromo-1-butene in a two phase system (50% aqueous NaOH/dichloromethane). Deprotonation of **(98)** was performed in the aqueous medium. The resulting stabilised anion reacts with the alkyl halide in the organic medium. Benzyltriethylammonium chloride (BTEAC) ensures a significant equilibrium concentration of the anion of **(98)** in this medium. Mild

acidic hydrolysis of compound **(183)** gave the corresponding aminonitrile **(184)** in 61% yield after basic work up. The nitrile function is significantly more robust to the conditions of hydrolysis required for the imine group and more forcing conditions are necessary for this purpose. The hydrolysis was performed by heating at reflux in both NaOH and $Ba(OH)_2$ solution. Removal of excess barium ions in the $Ba(OH)_2$ (aq) hydrolysis by precipitation as $BaCO_3$ is an advantage but was lower yielding. Compound **(185)** was isolated in approximately 70% yield by hydrolysis in 5% NaOH and subsequent acidification. Removal of the NaCl byproduct was considered unnecessary although this could have been achieved by ion exchange chromatography. The olefinic signals are found at δ 5.25 and 5.98 in the ^1H NMR spectrum and δ 117.9 and 137.8 in the ^{13}C NMR spectrum.



Scheme 63 Synthesis of 2-Amino-5-hexenoic Acid Hydrochloride (185)

Compound **(185)** was protected as previously for allylglycine **(Scheme 64)**. Treatment with acetic anhydride in NaOH solution gave *N*-acetyl-2-amino-5-hexenoic acid **(186)** in approximately 84% yield. The ^1H NMR spectrum of compound **(186)** is illustrated in **Figure 25**. *N*-Acetyl-2-amino-5-hexenoate *tert*-butyl ester **(187)** was prepared in 86% yield from compound **(186)** by treatment with isobutylene and c.H_2SO_4. The *tert*-butyl group is found at δ 1.47 and δ 26.3 in the ^1H and ^{13}C NMR spectra, respectively.



Scheme 64 **Attempted Preparation of *N*-Acetyl-GSA (181)**

Unfortunately, ozonolysis of compound **(187)** resulted in a 77% recovery of the cyclic compound *N*-acetyl-5-hydroxyproline *tert*-butyl ester **(189)**. The NMR spectra are fairly complex showing the presence of both diastereoisomers and rotamers. Signals are found at δ 5.49 and 5.75 for the proton in the 4-position. A very small signal is observed at δ 9.7 indicating a very small equilibrium concentration of the aliphatic aldehyde **(188)**. The ^{13}C NMR spectrum shows that the product is also a mixture of rotamers. Signals for C-5 are found between δ 80.2 and 81.4. There are eight signals observed for the two carbonyl carbons between δ 168.9 and 169.9. There is no evidence of any aldehyde carbonyl signal. Deprotection of compound **(189)** with TFA yielded *N*-acetyl-5-hydroxyproline **(190)** in 53% yield. The NMR spectra of compound **(190)** were less complex. In the ^{1}H NMR spectrum a signal was found at δ 5.49 for the 5-position. The corresponding signals for C-5 were found at δ 83.2 and 83.6 in the ^{13}C NMR spectrum **(Figure 26)** and the diastereomeric ratio was determined as close to 1:1. There was no evidence in either of the spectra for any of the acyclic aldehyde or iminium ion **(191)**. The biological analysis of compound **(190)** with the DHDPS enzyme will be discussed in chapter 6.

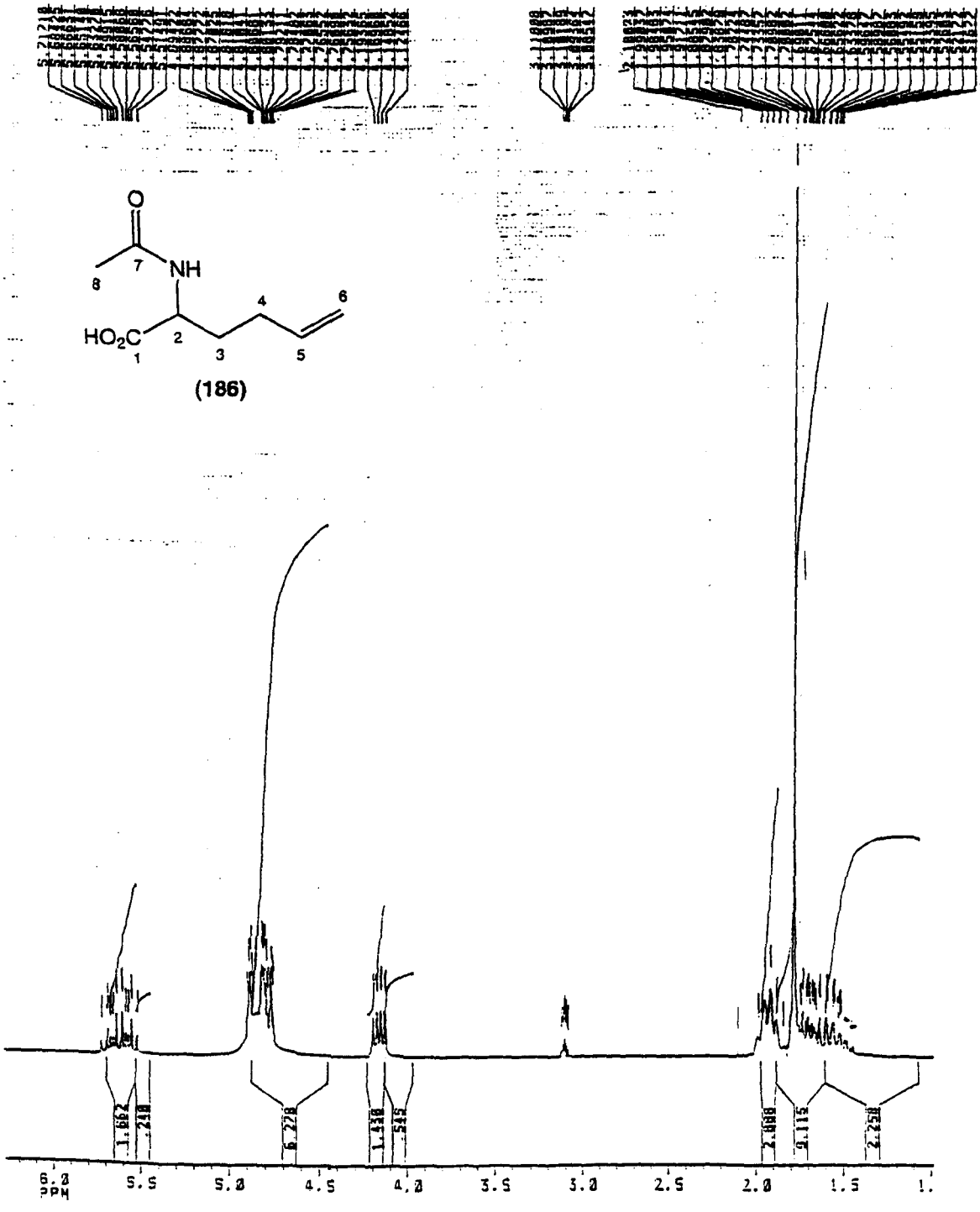


Figure 25 ^{1}H NMR Spectrum of *N*-Acetyl-2-amino-5-hexenoic acid (186)

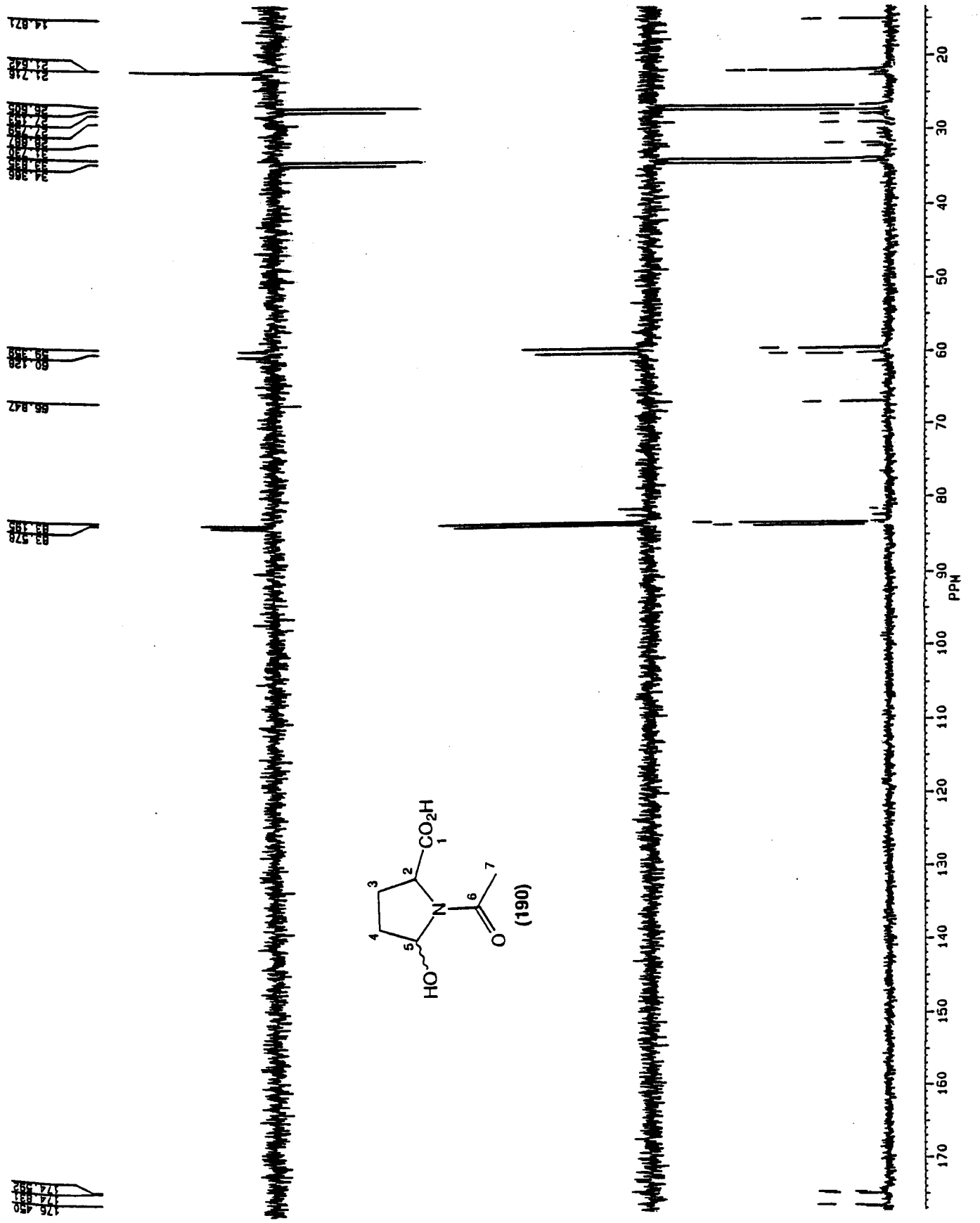
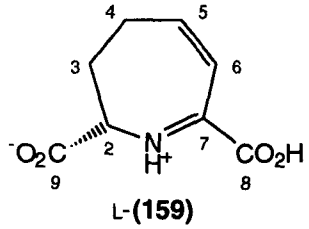


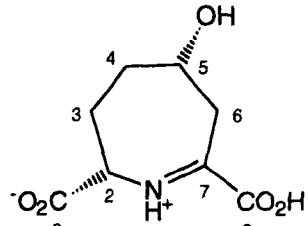
Figure 26 ^{13}C NMR Spectrum of *N*-Acetyl-5-hydroxyproline (190)

4.06 Strategy Towards the Enzymic Product of GSA with DHDPS

The evidence suggests that GSA will exist almost exclusively in a cyclic form in aqueous solution. This makes it very difficult to gauge its potential as a substrate. Clearly the most feasible method for investigating the biological activity of GSA with DHDPS is the study of the reverse process (see **Scheme 57**). The proposed product of the DHDPS catalysed condensation of GSA with pyruvate is the seven membered heterocycle L-**(159)**. However, the recent studies by Blickling and co-workers[30] indicate a likelihood that the hydrated precursor, *(2S,5S)*-5-hydroxy-3,4,5,6-tetrahydro-*4H*-azepine-2,6-dicarboxylic acid *(2S,5S)*-**(192)**, may be the product. Consequently, the synthetic strategy should be applicable to both heterocycles **(159)** and **(192)**.

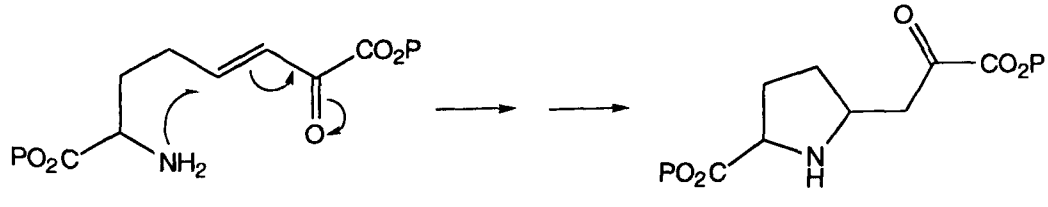


L-**(159)**



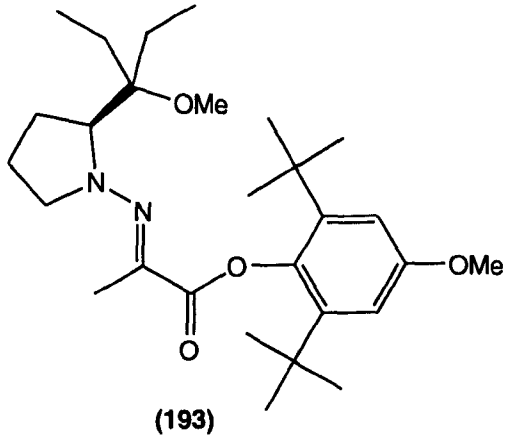
(192)

A number of synthetic strategies to heterocycle **(159)** have to be discounted. Where conjugate addition is possible on cyclisation five membered ring formation is both the thermodynamically and kinetically favoured outcome. An example of this is illustrated in **Scheme 65**. To overcome this problem it was considered that the unsaturation should be introduced after cyclisation. This strategy also assures the required *cis*-geometry.



Scheme 65 **Five Membered Ring Formation**

After consideration of a number of synthetic strategies towards heterocycles **(159)** and **(192)** it was decided that the direct condensation of protected GSA and pyruvate was the most viable route **(Scheme 66)**. Enders and co-workers[155] have reported the use of pyruvate derivative **(193)** in the LDA promoted condensation with aldehyde electrophiles. It was therefore proposed that condensation of pyruvate derivative **(144)** with *bis-N*-BOC-glutamate-γ-semialdehyde *tert*-butyl ester **(174)** followed by ozonolysis and deprotection of the amino function would yield the required heterocycle **(194)**. In section 4.02 **(Scheme 57)** the synthesis of *bis-N*-BOC-L-glutamate-γ-semialdehyde *tert*-butyl ester L-**(174)** by Bold and co-workers[149] was discussed.



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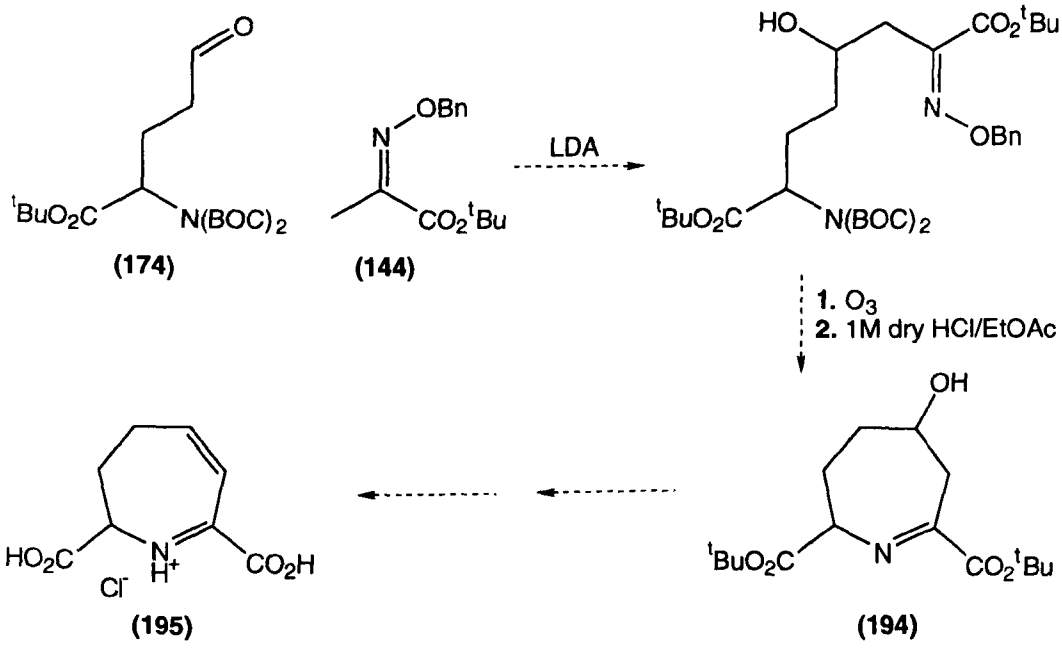


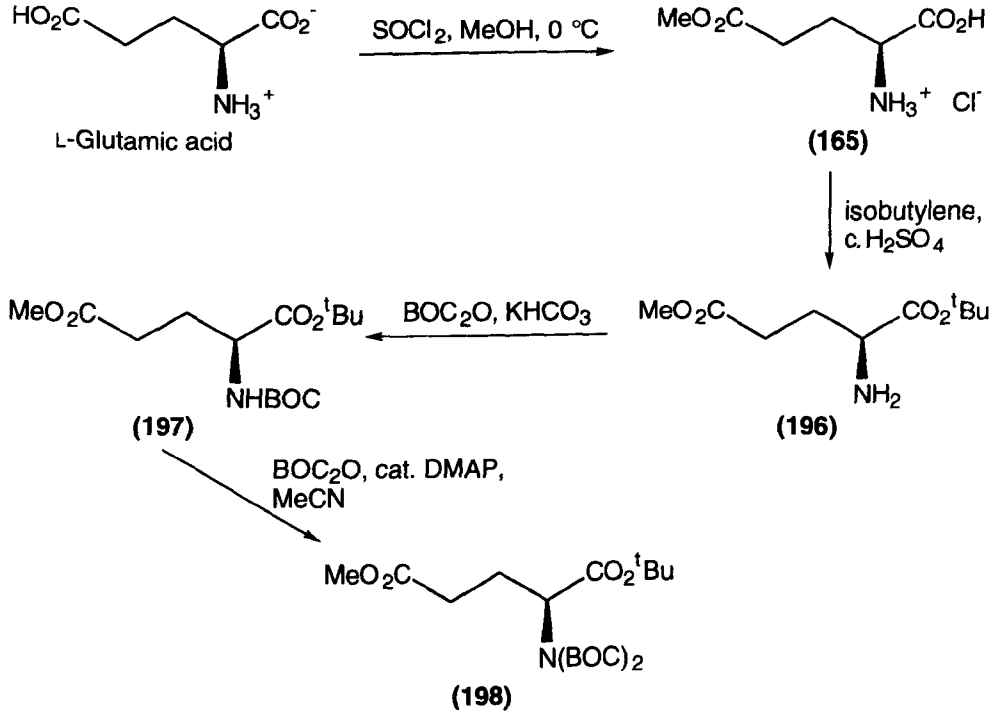
(174) **(144)** **(195)** **(194)**

Scheme 66 **Proposed Synthesis of the Azepine Ring System**

On preparation of heterocycle **(194)** the reaction conditions could then be tailored for or against elimination. Elimination to form the dihydroazepine **(195)** could be driven by tosylation of the alcohol function. It is likely however that the thermodynamically favoured extension of conjugation would occur spontaneously on deprotection of the *tert*-butyl ester groups in anhydrous acid. Indeed, it might be necessary to protect the alcohol to avoid this elimination until required.

4.07 Non-Symmetrical Derivatisation of Glutamic Acid

In our work, a variation on the derivatisation of glutamic acid used by Dr McKendrick **(Schemes 54** and **55)** was investigated for the synthesis of *bis*-*N*-BOC-GSA *tert*-butyl ester **(174)**. δ-Methyl L-glutamate hydrochloride **(165)** was prepared in quantitative yield by the method of McKendrick **(Scheme 67)**.[147] The methyl signal is found at δ 3.48 in the ^{1}H NMR spectrum. The α-acid function was protected as the *tert*-butyl ester **(196)** in 88% yield by treatment with isobutylene and c.H_2SO_4. *N*-BOC protection was achieved in 71% yield by treating compound **(196)** with BOC_2O and $KHCO_3$. In order to prevent cyclisation on generation of the γ-aldehyde a second *N*-BOC group was then introduced by the method of Ragnarsson and co-workers.[146]

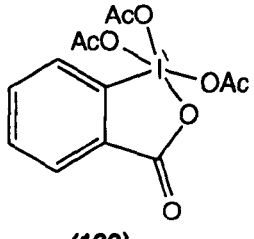


Scheme 67 Protection of Glutamic Acid

Treating δ-methyl L-*N*-BOC-glutamate *tert*-butyl ester **(197)** with BOC_2O and catalytic DMAP in MeCN produced the desired δ-methyl L-*NN-bis*-BOC-glutamate *tert*-butyl ester **(198)** in 67% yield. The signals for the *tert*-butyl ester and *bis*-*N*-BOC groups are found at δ 1.33 and 1.39, respectively, in the ^1H NMR spectrum. The corresponding signals are found at δ 27.7 and 27.8, respectively, in the ^{13}C NMR spectrum.

4.08 Synthesis of *bis*-*N*-BOC-GSA *tert*-Butyl Ester (174)

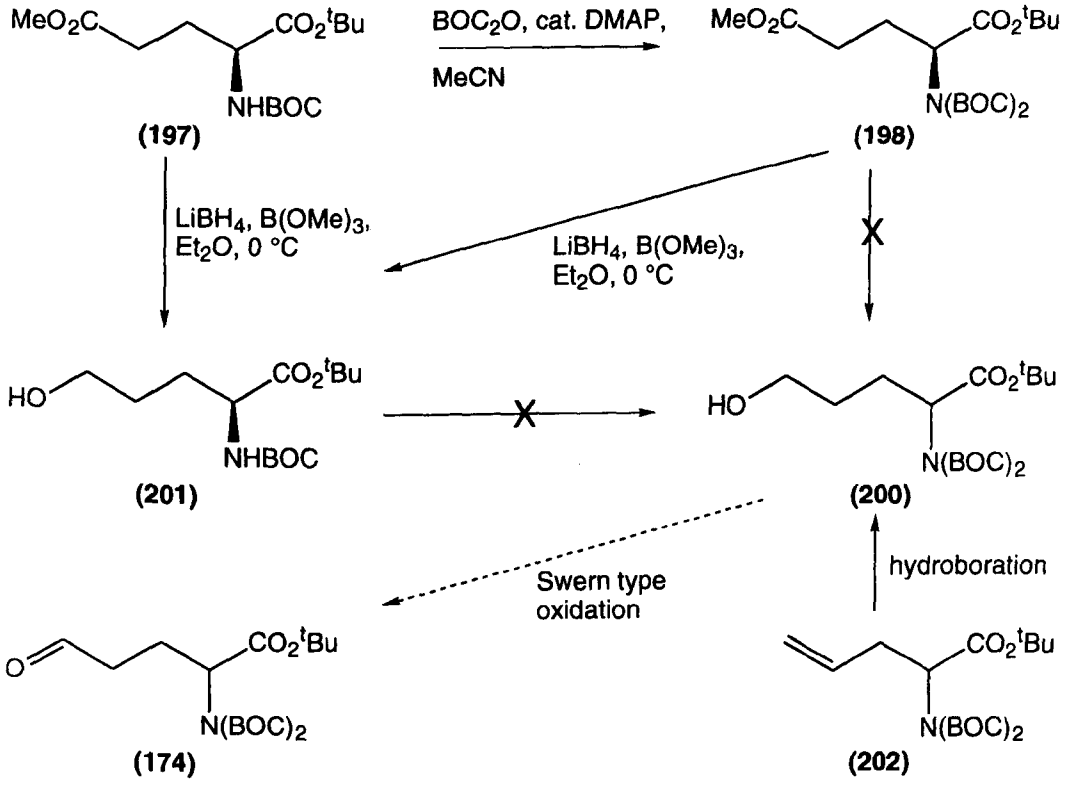
Having successfully protected L-glutamic acid our next objective was the selective transformation of the γ-methyl ester into an aldehyde. To achieve this, selective reduction of the methyl ester to the corresponding alcohol and controlled oxidation to the aldehyde was proposed. There are a number of classical techniques for this oxidation, such as the Moffat oxidation, detailed in **Scheme 55**, PCC oxidation[156] and oxidation catalysed by Dess-Martin periodinane **(199)**.[157]



(199)

Brown and Narasimhan[158] have reported the trimethylborate-catalysed selective reduction of esters over other reducible groups by lithium borohydride under a variety of conditions. In our studies it was found that the selectivity could be extended to the reduction of methyl esters over *tert*-butyl esters using trimethylborate in a catalytic role. Unfortunately, reduction of compound **(198)** with lithium borohydride in the presence of trimethylborate also resulted in cleavage of one of the BOC groups **(Scheme 68)**. The resulting product, *tert*-butyl L-2-(*tert*-butoxycarbonylamino)-5-hydroxypentanoate **(201)**, was isolated in high yield (~ 90%). It was therefore decided that the selective reduction was best performed on the mono-BOC precursor **(197)**. After optimisation of the conditions it was found that the reaction proceeded efficiently in anhydrous ether at -10 °C, yielding compound **(201)** in 78% yield from compound **(197)**. In the ^1H NMR spectrum **(Figure 29)** the protons in the 5-position are found as a triplet at δ 3.59, ^3J 6.1 Hz and the broad OH signal was found at δ 2.42. Problems were

however encountered in attempting to introduce a second BOC group with compound **(201)**. This problem arises from the nucleophilic nature of the alcohol function. Unfortunately, time restraints did not allow us to develop an alcohol protection/deprotection strategy to overcome this problem. On reflection, this is a highly efficient and economical route to non-symmetrically substituted glutamates which can easily be performed on a large scale and should be equally applicable to aspartates.

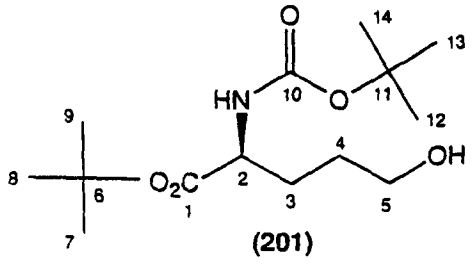


Scheme 68 Towards Aldehyde (174)

In order to further the strategy toward heterocycle **(159)**, in a tightly restricted time-frame, a two step preparation of the important intermediate **(174)** was performed **(Scheme 69)**. Starting from the previously prepared *N*-BOC-allylglycine *tert*-butyl ester **(116)** a further butoxycarbonyl protecting group was introduced to the amino function. This was performed as described previously, with BOC_2O and catalytic DMAP in MeCN, yielding the desired doubly protected amine **(202)** in 76% yield. The *bis*-*N*-BOC groups are observed at δ 1.43 in the ^1H NMR spectrum and at δ 27.9, 82.5 and 152.2 in the ^{13}C NMR spectrum for the primary, quaternary and carbonyl carbons, respectively. The second step towards compound **(174)** involved a 'one-pot' hydroboration/oxidation. This process for conversion of terminal olefins into aldehydes was recently described by Yates.[159] After treatment of compound **(202)** with borane.DMS *in situ* oxidation was achieved by treating with 4-

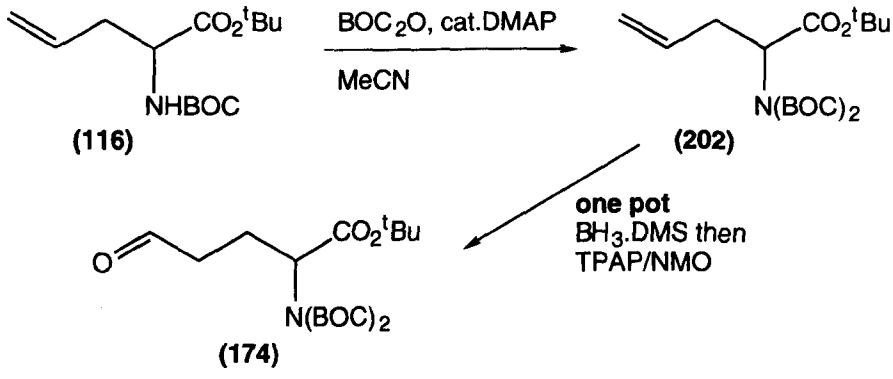
methylmorpholine-*N*-oxide (NMO) and catalytic tetra-*n*-propylammonium perruthenate (TPAP). The desired aldehyde **(174)** was isolated in 36% yield after work up and purification. It is likely that the low yield is due to the ligating affinity of nitrogen and oxygen lone pairs for ruthenium. Aldehyde **(174)** has a mp of 42-44 °C and carbonyl stretches at 1786, 1732 and 1696 cm^{-1} in the IR spectrum. The distinctive aldehyde signal is found at δ 9.70 and δ 201 in the ^{1}H and ^{13}C NMR spectra, respectively.

Although without the stereocontrol of the previous derivatisation route and not as economical, this simple route allowed the preparation of a small quantity of the racemic material for further development of the strategy towards heterocycle **(159)**.



(201)

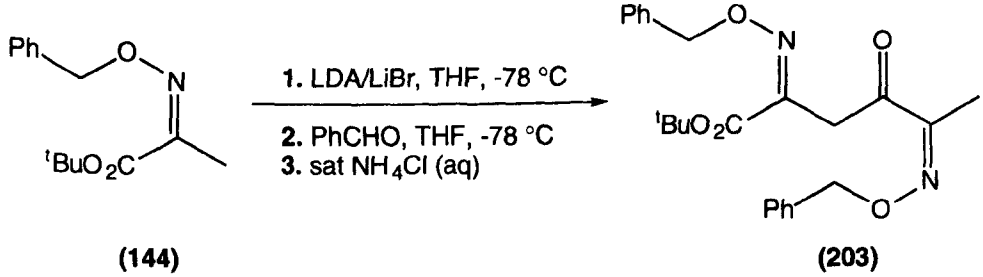
Figure 29 The ^{1}H NMR Spectrum of Alcohol (201)



Scheme 69 Alternative Synthesis of Compound (174)

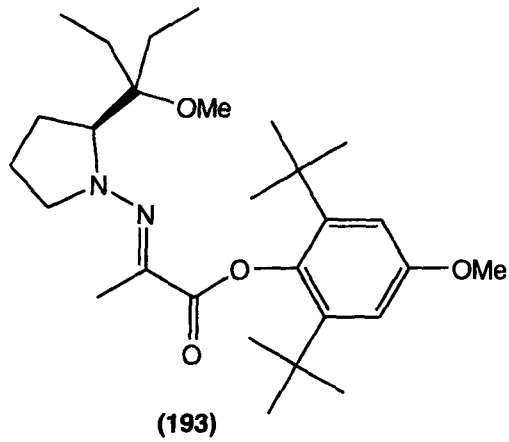
4.09 Condensation of Pyruvate with Aldehyde (174)

Following preparation of aldehyde **(174)** the next step towards heterocycle **(192)** was the condensation with a pyruvate equivalent (see **Scheme 66**). It was decided to attempt the condensation of the previously prepared pyruvate analogue **(144)** (see section 3.08) with benzaldehyde, thus developing the methodology without using our limited supply of aldehyde **(174)**. Unfortunately, when this condensation was attempted, following the method of Enders and co-workers,[155] a 66% recovery of **(203)** which is the self-condensed pyruvate derivative **(144)** was obtained **(Scheme 70)**. This indicates that the *tert*-butyl ester has insufficient steric bulk to prevent self condensation. To counter this problem we need to move more closely towards the bulkier 2,6-di-*tert*-butyl-4-methoxyphenyl ester **(193)** used by Enders and co-workers.[155] One disadvantage of using a phenyl ester such as **(193)** is that we would require the introduction of a further deprotection step for removal. Phenyl esters are readily cleaved under basic conditions (H_2O_2, H_2O, DMF, pH 10.5, 20 °C, 15 min).[160] Alternate bulky protecting groups such as cumyl esters ($-CO_2CPh(Me)_2$) and trityl esters ($-CO_2(Ph)_3$) would also be worthy of consideration. Clearly, further development of this method is required, which we did not have time to complete.



(144) **(203)**

Scheme 70 Self-Condensation of Pyruvate Derivative (144)



(193)

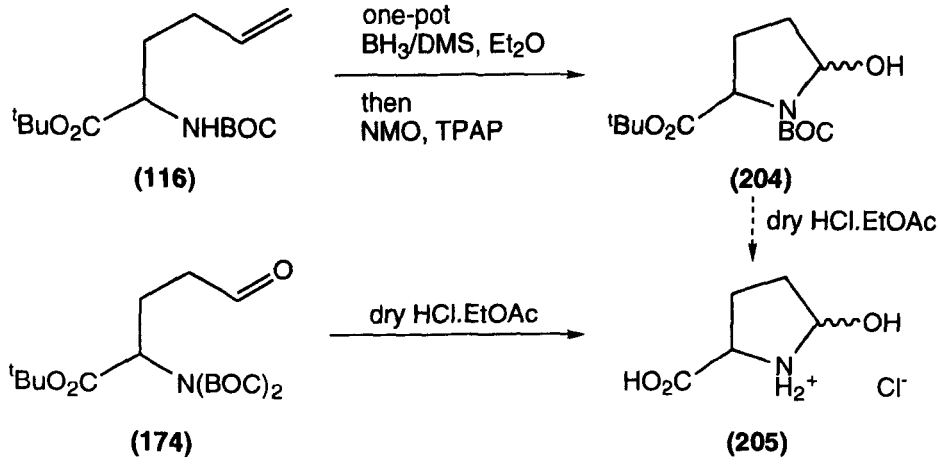
4.10 Synthesis of 5-Hydroxyproline (157)

5-Hydroxyproline **(157)** is the immediate product of cyclisation of GSA. Consequently, the study of the interaction of this compound with the DHDPS enzyme is of interest to us. The only record of compound **(157)** which could be found in the literature concerned its synthesis and dated back to 1926. In this article Abderhalden and Schwab detailed the synthesis of compound **(157)** by the hydrogenation of pyroglutamic acid in acetic acid.[161] Unsurprisingly, the characterisation presented for this compound was somewhat limited. Abderhalden and Schwab further noted the instability of this compound, characterised by a colour change on exposure to light.

In our work, it was considered that the preparation of *N*-BOC-5-hydroxyproline *tert*-butyl ester **(204)** would allow us to generate the desired amino acid under milder conditions. The 'one-pot' hydroboration/oxidation method of Yates,[159] discussed previously, was used for the synthesis of compound **(204)** directly from compound **(116)** **(Scheme 71)**. The yield for the transformation was only 16% after purification. The ^{1}H NMR spectrum shows evidence of a diastereoisomeric product mix, in a close to 1:1 ratio. The signal for the proton in the 5-position is found as a multiplet at δ 5.49.

Preparation of 5-hydroxyproline could be achieved by the deprotection of either compound **(204)** or aldehyde **(174)** **(Scheme 71)**. On examination of the characterisation data of these two compounds it was decided that aldehyde **(174)** was the purer of the two and thus presented greater chance of success. Consequently, the desired amino acid was prepared by treating aldehyde **(174)** with anhydrous HCl in EtOAc. The NMR spectra showed evidence for the preparation of 5-hydroxyproline hydrochloride **(205)**, such as disappearance of carbamate carbonyls in the ^{13}C NMR spectrum and the presence of the expected signals. However, not

unexpectedly, the low stability of this compound meant its purity was not completely satisfactory. It must therefore be stressed that this method can only be described as a crude preparation and the biological results detailed in Chapter 6 should be considered similarly.



Scheme 71 Synthesis of 5-Hydroxyproline Hydrochloride (205)

4.11 Conclusions

In this chapter we have detailed two novel indirect strategies towards the investigation of GSA as a substrate of the DHDPS enzyme. The argument presented supports the view that our target compound, glutamate-γ-semialdehyde (GSA), is of low stability and is only ever present in very small equilibrium concentrations. The lack of any conclusive evidence in the literature supports this argument. Consequently, it is considered that it would be very difficult to derive meaningful conclusion from attempts to study direct interaction between GSA and the DHDPS enzyme. Our alternative strategy, however, has scope for success. One could query the value of achieving our objective, proving that GSA is a substrate of DHDPS, when clearly the instability of GSA dictates that little could be gained. We would argue that there are potentially two significant gains from our 'reverse' strategy. Firstly we would gain knowledge that analogues of both GSA and the seven-membered heterocycle have potential as inhibitors or substrates of DHDPS and related enzymes. And secondly we would have discovered a mild stereospecific method for producing GSA and possibly other analogues.

The first strategy involved *in situ* generation of GSA from its *N*-acetylated precursor by enzymic cleavage. This theory was validated by the synthesis and successful performance of a two enzyme assay system of the *N*-

acetylated natural substrate. *N*-Acetyl-GSA was however observed to cyclise immediately upon preparation. This result discounts this strategy for this particular target but might be applied to other potential metabolites of DHDPS and to other biotransformations. Furthermore, the basic idea of *in situ* generation of GSA could be possible by alternate biotransformations.

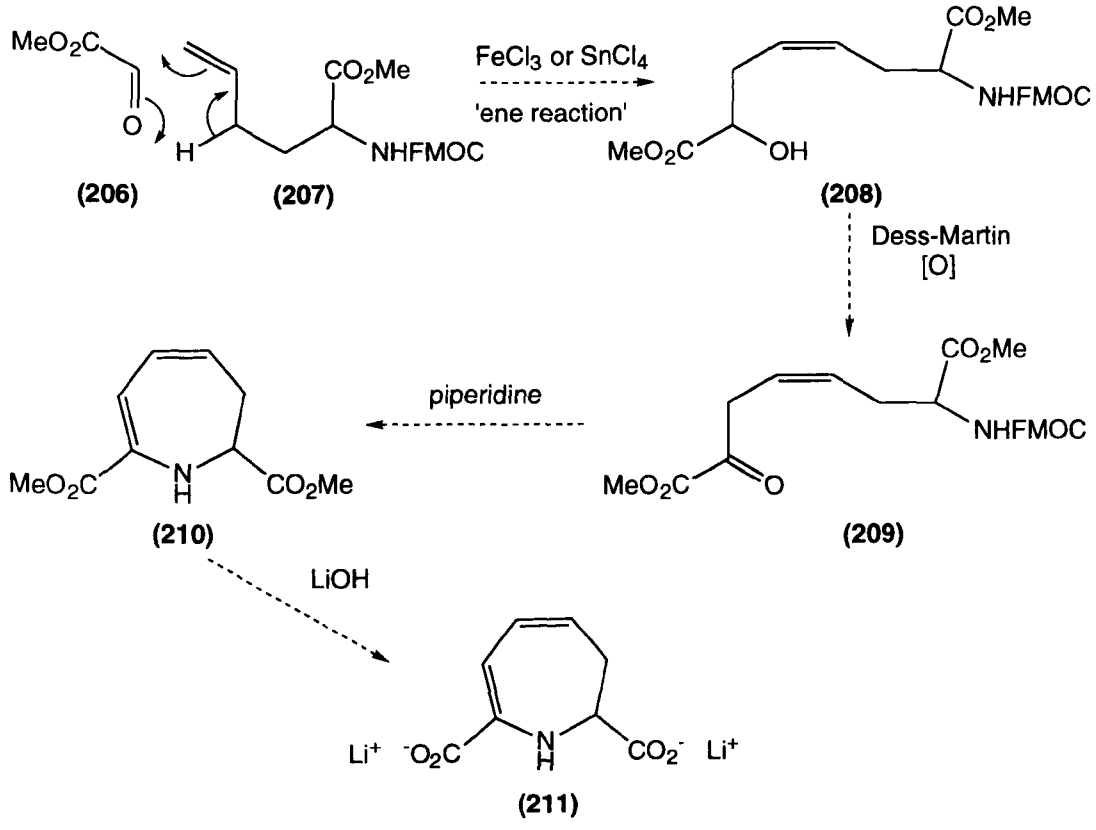
Our second strategy involved the investigation of the reverse process between the DHDPS enzyme and its likely product with GSA. Clearly the synthesis of the proposed seven membered heterocycle **(159)** is not a trivial task. However, we have established some sound methodology towards this end. In particular, the non-symmetrical derivatisation route of glutamic acid, detailed in Section 4.08, is recommended for future adaptation of this strategy and for other relevant studies.

4.12 Future Work

There is scope for further developing the 'reverse' strategy for the investigation of GSA as a substrate of DHDPS. To that end, the proposed synthesis of heterocycle **(159)** by condensation of pyruvate and GSA units, will require amendment. The problem arises from generation of an enolate from the α-keto moiety **(144)**. Intramolecular interaction can readily occur between the enolate and the ester unless there is significant steric restriction. In Section 4.09 we discussed the possibility of using bulkier pyruvate units. Alternatively, a pyruvate analogue with a non-electrophilic acid equivalent, such as one derived from pyruvonitrile, could be considered. Similarly, a derivatised free acid of pyruvate could be considered for generating the double anion for condensation with the GSA equivalent.

One alternative strategy for the synthesis of the dihydro-*(2H)*-azepine ring system is detailed in **Scheme 72**. Key to this process is the construction of the carbon skeleton prior to cyclisation. We have proposed an 'intermolecular ene' reaction between methyl glyoxylate **(206)** and protected amino acid **(207)** for this purpose. The synthesis of the parent amino acid of compound **(207)** was detailed in Section 4.05 **(Scheme 62)**. Use of a Lewis acid catalysed 'ene reaction' for similar purpose has previously been reported by Vederas and co-workers.[162] Earlier investigations of this process by Agouridas *et al.*[163] indicated that when the steric bulk of the olefin was remote from the reaction site the *cis*-geometry was favoured (2:1). The next step of the synthesis involves oxidation of the secondary alcohol **(208)** to the α-keto moiety **(209)**. Vederas and co-workers[162] reported that this transformation was achieved efficiently by Dess-Martin oxidation. One difference in our

proposed method from that of Vederas and co-workers[162] is the use of the 9-fluorenylmethyl carbamate (FMOC) group for protecting the amino function. This protecting group is stable to both Lewis acids and oxidation. After oxidation, the FMOC group can be selectively removed from α-keto ester **(209)** by treatment with piperidine. This should lead to spontaneous cyclisation and dehydration to yield the dihydro-*(2H)*-azepine ring system **(210)**. Hydrolysis of dimethyl ester **(210)** by the method of Vederas and co-workers[162] would give the desired heterocycle **(211)**.



Scheme 72 Alternative Synthesis of the Dihydro-(*2H*)-Azepine Ring

Chapter 5 Synthesis of Inhibitors of DHDPS and DHDPR

5.01 Introduction

Previously, in section 2.06 and 2.07 the potential of blocking the DAP biosynthetic route to lysine with compounds which may have antibacterial or herbicidal activity has been discussed. In particular, it was noted that blocking the pathway at one of the first two steps, prior to branching into succinylase, acylase or dehydrogenase pathways to *meso*-DAP **(27)**, had potential for a broader spectrum of activity. In section 2.14 and section 2.19 we reviewed work previously done on inhibition of DHDPS and DHDPR, respectively. The necessary binding nature of competitive inhibitors dictates, almost without exception, that each is a close substrate, transition state or product analogue of the enzyme or receptor in question. In sections 2.20 to 2.22 we mentioned an application of this property to further our objective of inhibiting the DHDPS and DHDPR enzymes. In this chapter we will consider two such cases: analogues of pyruvic acid **(21)** and sulfur-containing heterocyclic analogues of DHDPA **(24)** and THDPA **(25)**. Our interest in these areas is justified by examination of previous findings. The synthesis of appropriate analogues to further the investigation is discussed. The biological activity of these compounds and conclusions therefrom are detailed in the next chapter.

5.02 Pyruvic Acid Analogues as Substrates of DHDPS

Since substrates and competitive inhibitors can be considered to interact with the active site of an enzyme then one should consider previous work in both areas for further development of inhibitors. Earlier studies within our group by R.A. Campbell, J.E. McKendrick and principally by Dr P. Mallon indicated a positive UV substrate assay for the simple pyruvate analogues methyl pyruvate **(212)**, ethyl pyruvate **(213)** and pyruvamide **(214)** **(Figure 30)**. Further investigation showed that all of these compounds have almost identical electrospray mass spectra with DHDPS, after reduction with $NaBH_4$, to that of pyruvate and DHDPS **(Figure 12)**. From this we can conclude that it is likely that the esters **(212)** and **(213)** are hydrolysed to pyruvate before combining with the enzyme. The amide **(214)** is not so clear a case. It is very much less likely that this compound could be hydrolysed to pyruvate under the mild conditions of the assay. We also have the further problem that the

reduced DHDPS-pyruvamide adduct would have the same molecular weight as that for the reduced DHDPS-pyruvate adduct **(30)**. We could possibly navigate around this problem by investigating an analogue with a higher molecular weight such as *NN*-dimethylpyruvamide **(215)**. Ideally we would like to isolate, purify and identify products of our biotransformations. Some of the problems associated with this are discussed in the next chapter.

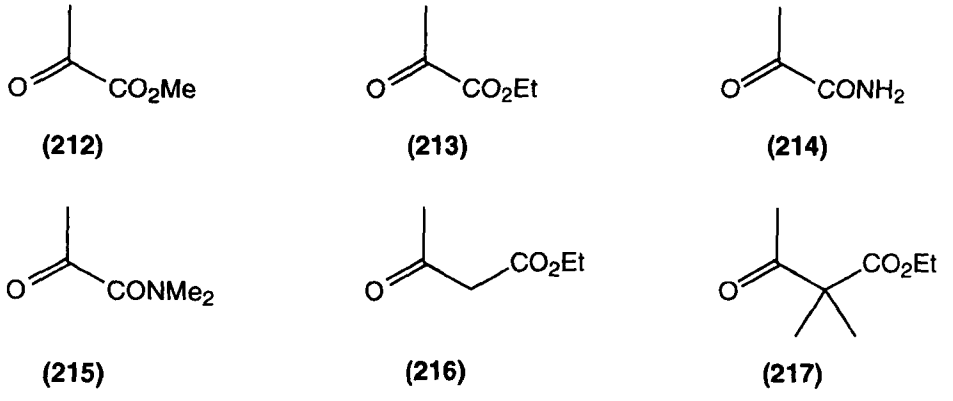
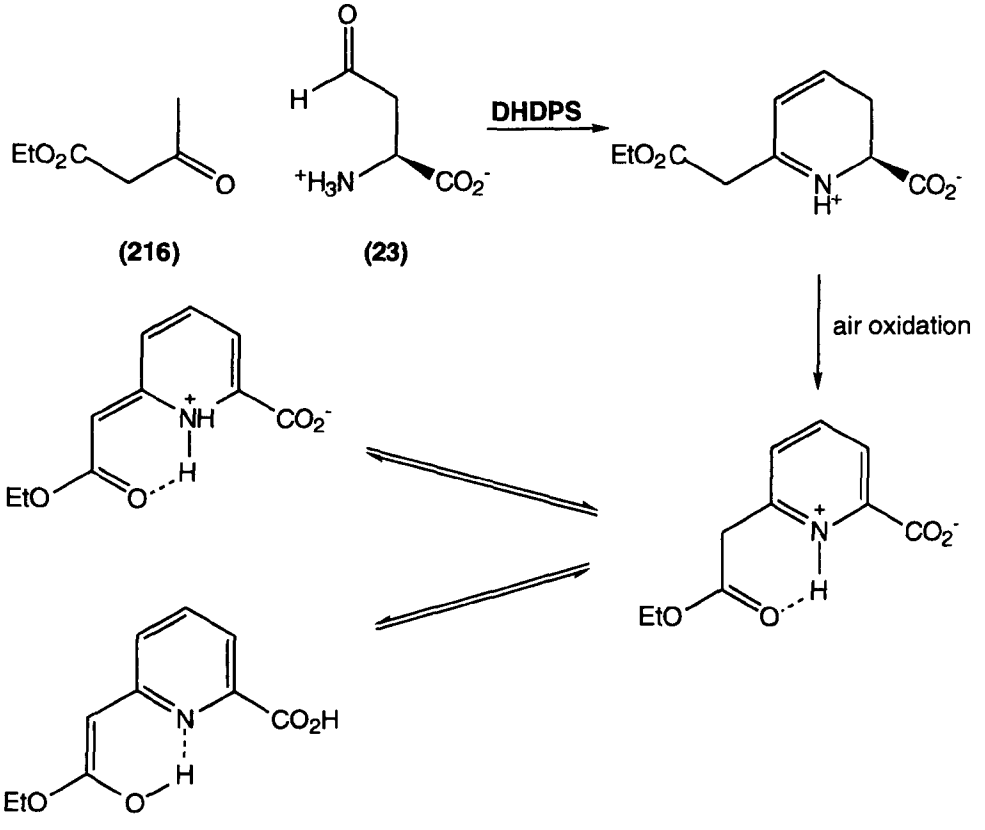


Figure 30 Pyruvate Analogues with Potential Substrate Activity.

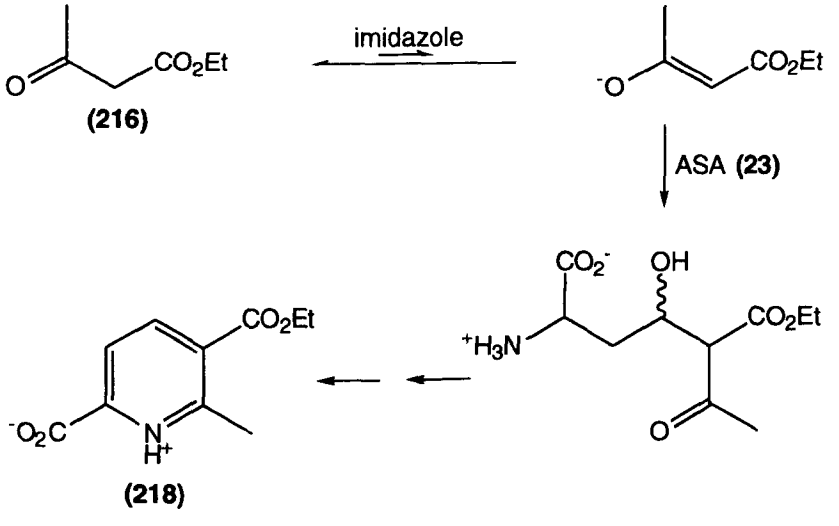
Further studies by Dr. Mallon have shown substrate activity for ethyl acetoacetate **(216)** with DHDPS. On scaling up the process in the presence of ASA a small quantity of an amorphous solid was isolated. Treatment of this solid with ethereal diazomethane and subsequent GCMS analysis showed two peaks. The *m/z* value of the major product coincided with that of the expected biotransformation product. Problems were encountered however, with the interpretation of the NMR and IR spectra. It was considered that the problem could be in some part be due to the likely tautomerisation of the product **(Scheme 73)**. This could possibly be avoided by the study of the derivative, ethyl αα-dimethylacetoacetate **(217)**. The synthesis of compound **(217)** should be straightforward and the removal of the two reactive α-hydrogens could be of further benefit. Dr Mallon also noted problems with attempted repetition of the biotransformation of **(216)**.

In our work, we found that the free acid analogue of compound **(216)**, rather unexpectedly showed no substrate activity by UV. This may be related to its high reactivity. It is supplied as its lithium salt and it is quite likely that at the assay pH of 7.4 it readily enolises and undergoes self condensation and possibly decarboxylation. Furthermore it was found by UV assay that the 'biotransformation' with ethyl acetoacetate **(216)** in place of pyruvate proceeded with equal efficiency without DHDPS enzyme. For these reasons we were led to conclude that the interaction of **(216)** with ASA was a non-

enzymic aldol interaction producing the pyridine analogue **(218)** **(Scheme 74)**.



Scheme 73 **Ethyl acetoacetate (216) as a Possible Substrate of DHDPS**



Scheme 74 **Non-Enzymic Reaction of Ethyl acetoacetate (216)**

5.03 Pyruvic Acid Analogues as Inhibitors of DHDPS

On the basis of the promising results for unnatural substrate activity of pyruvate analogues, demonstrating a non-specificity in substrate selectivity, it was recognised that there is scope for inhibition of the DAP pathway to L-lysine at this point. Interest in this area was first explored by our former group members L. Couper and D. Tudor, and some promising results were achieved. The most effective pyruvate-based inhibitors[164] of DHDPS were found to be: methyl pyruvate semicarbazone **(219)**; methyl pyruvate thiosemicarbazone **(220)**; the ethyl hydrazinoacetate derivative **(221)** of methyl pyruvate; the methyl hydrazinocarboxylate derivative **(222)** of methyl pyruvate; ethyl bromopyruvate semicarbazone **(223)**; ethyl bromopyruvate methyloxime **(224)** and αα-dioxopimelic acid **(225)** **(Figure 31)**. The results from inhibition studies with these compound are summarised in **Table 7**.

Compound	Inhibition of DHDPS
(219)	10% at 0.1 mM
(220)	10% at 0.1 mM
(221)	62% at 0.1 mM
(222)	100% at 0.5 mM
(223)	50% at 0.1 mM
(224)	14% at 0.1 mM
(225)[165]	75% at 0.1 mM

Table 7 Pyruvate Inhibitors of DHDPS

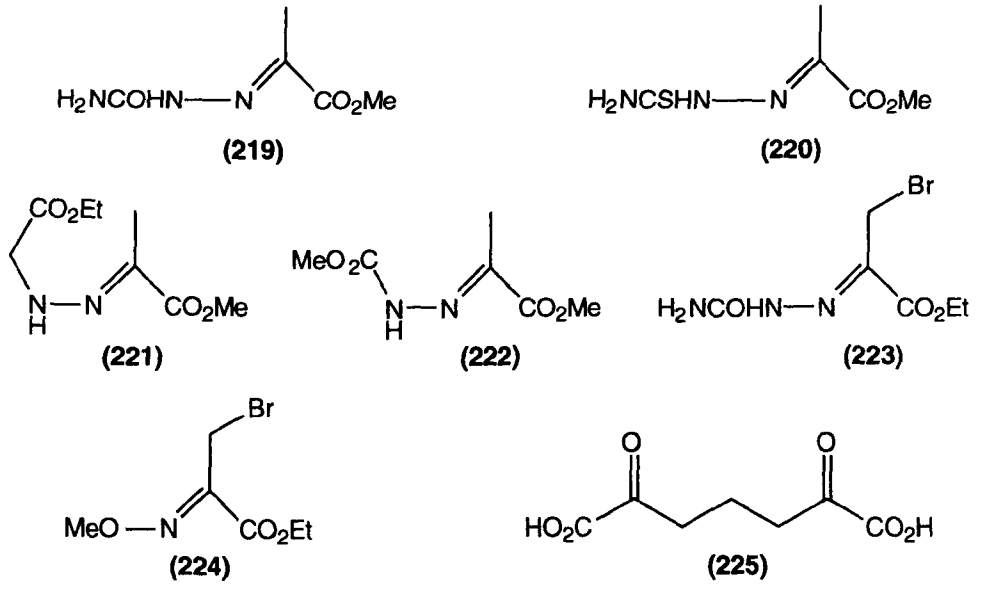


Figure 31 Pyruvate Inhibitors of DHDPS

5.04 Synthesis of Pyruvic Acid Analogues

As pyruvic acid is a fairly simple molecule, there are relatively few variations on its structure that we can explore. Experience has shown that despite their simple appearance, small compounds of this type are often highly reactive and therefore difficult to work with and purify. Pyruvic acid itself is a rather unstable compound exhibiting a tendency to decarboxylate and polymerise. Indeed, pyruvic acid has been noted to polymerise even in frozen aqueous solution.[166] There are a number of synthetic transformations leading to α-keto acids and these have been well documented in a review by Cooper and co-workers.[167] Our studies have followed on from the recent work of our group and concentrated on the derivatisation of pyruvate esters. Pyruvate esters are generally easier to handle than their free acid counterparts and in most cases can be purified by crystallisation or chromatography.

In our recent work to further this investigation derivatisation of methyl pyruvate with the amino acids homoserine, homocysteine and homocystenic acid was attempted. These compounds, **(226)**, **(227)** and **(228)**, respectively **(Figure 31)**, are structurally related to the more effective pyruvate derivatives found at that stage. They are similarly functionalised to the proposed enzyme intermediate and importantly, are very unlikely to participate in cyclisation.

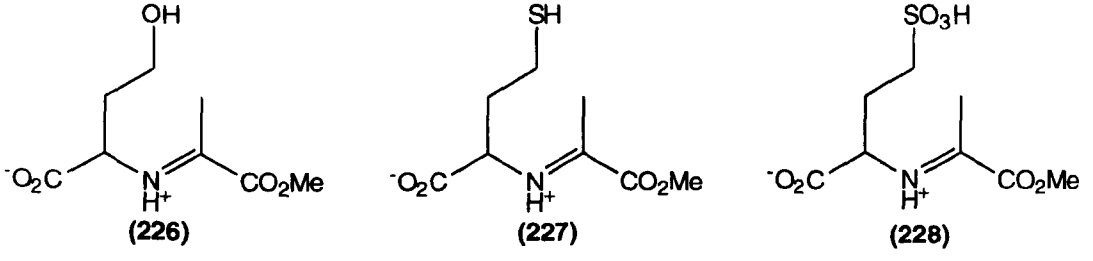


Figure 31 Original Targets for the Extension of the Study into Inhibition of DHDPS.

However, although the formation of Schiff bases between amino acids and ketones has been reported,[168] we found that the extensive functionality present here posed us a number of problems. Also, the zwitterion nature of the products made separation from any unreacted starting amino acids rather difficult. We found great difficulty in forming the required product with methyl pyruvate and cysteine under a range of conditions - acidic or basic catalysis. We also attempted removing the water produced with activated molecular sieves (4Å) under a N_2 atmosphere. To simplify the synthesis we also attempted to form a Schiff base using the less functionalised amino acid

alanine. With this amino acid an exothermic process accompanied by a colour change (clear to yellow) was observed almost immediately. The ^1H NMR spectrum however was not easily identifiable and the material proved to be difficult to purify.

The ethyl ester analogues of some of the more effective pyruvate inhibitors of the DHDPS enzyme, compounds **(229)** to **(232)**, along with known compound **(220)** for comparison, were synthesised by the method of Vogel[169] **(Figure 32)**. The derivatives were isolated in yields of 67 to 98% after crystallisation from ethanol/water.

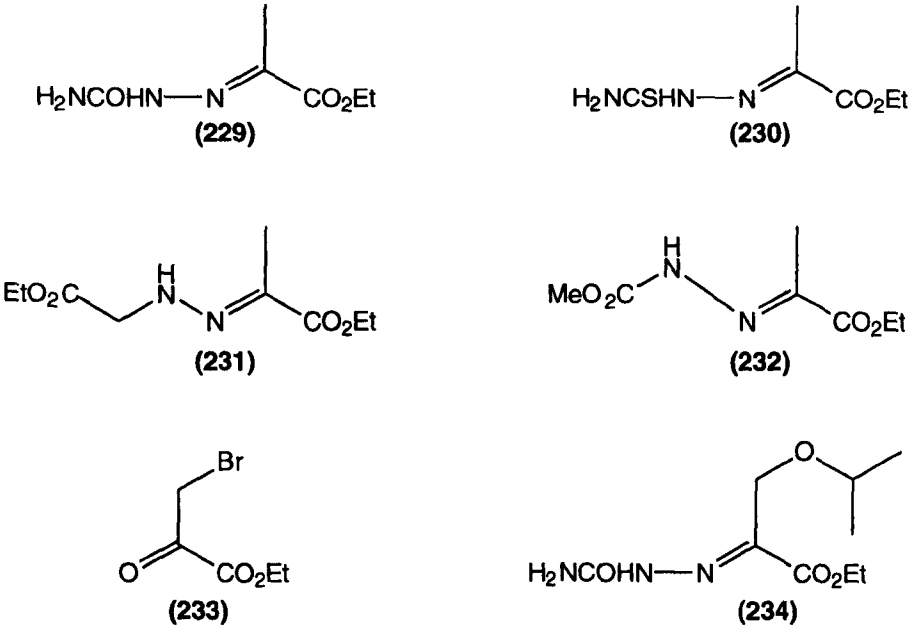


Figure 32 Other Pyruvate Analogues Synthesised.

The synthesis of derivatives of ethyl bromopyruvate **(233)** was much more troublesome. The extra electrophilic site not surprisingly reduces the yield of our desired product, in some cases introducing further possibilities such as cyclisation, and in all cases there is a requirement for extensive and often difficult purification steps. Known compound **(224)** was synthesised in low yield (50%) by direct condensation of ethyl bromopyruvate **(233)** and methoxylamine hydrochloride in ethanol. The synthesis of the semicarbazone derivative of ethyl bromopyruvate **(233)**, however, caused us a great deal of problems. The method used by D. Tudor[164] did not detail any purification steps and was therefore of little use to us. The polar nature of the compound limited us to using alcohol for chromatographic separation. The only workable purification system we found involved using an $iPrOH/CHCl_3$ solvent mix on a silica column. Unfortunately it appears that the alcohol displaces the bromine

under these conditions to yield the corresponding *iso*propyl ether **(234)**, in excellent purity but poor yield (5%).

The activity of these compounds with the DHDPS and DHDPR enzymes and the associated problems are discussed in detail in the next chapter. The results did not warrant any further investigation in this area.

5.05 Analogues of DHDPA and THDPA as Inhibitors of DHDPS and DHDPR

We will consider DHDPA **(24)** and THDPA **(25)** together because of their structural similarity. Due to their low stability, as experienced with the synthesis of these metabolites, we find that our search for analogues most commonly involves changing certain structural features markedly in order to introduce stabilisation. As a consequence our investigation has focused almost exclusively on the search for inhibitors. An exception could be made for those compounds that show substrate activity with DHDPS. By means of a DHDPS/DHDPR coupled assay, discussed in more detail in the next chapter, we can test for dual enzymic activity. DHDPR is of less interest as a biotransformation catalyst, since the reduction reaction is chemically less interesting, and secondly, because of the restriction on potential substrates imposed by the stability problems.

Prior to the recent purification of DHDPR by our biochemical colleagues, a number of analogues of DHDPA and THDPA were tested as inhibitors of DHDPS. In section 2.14 we detailed earlier studies by our group concerning the inhibition of DHDPS by pyridine and piperidine analogues **(Figure 15)**. Notable from this study[59] was that esters were poorer inhibitors than their carboxylic acid analogues. Likewise, monoacid systems were poorer inhibitors than their diacid counterparts. Most interestingly, the saturated piperidine analogues were very much poorer inhibitors. As these piperidines have similar polarity to their pyridine equivalents, this suggests a high substrate 'shape' selectivity tending towards the planar ring system.

5.06 Thiazines and Thiazoles as Inhibitors of DHDPS

Our interest in thiazines and thiazoles as inhibitors of the DHDPS enzyme was originally aroused by our former group members D. Tudor[170] and S.J. Connell.[171] Compound L-**(64)** and compounds **(235)** to **(239)** showed

some interesting activity **(Figure 33)**. The results from inhibition studies with these compounds with DHDPS are detailed in **Table 8**.

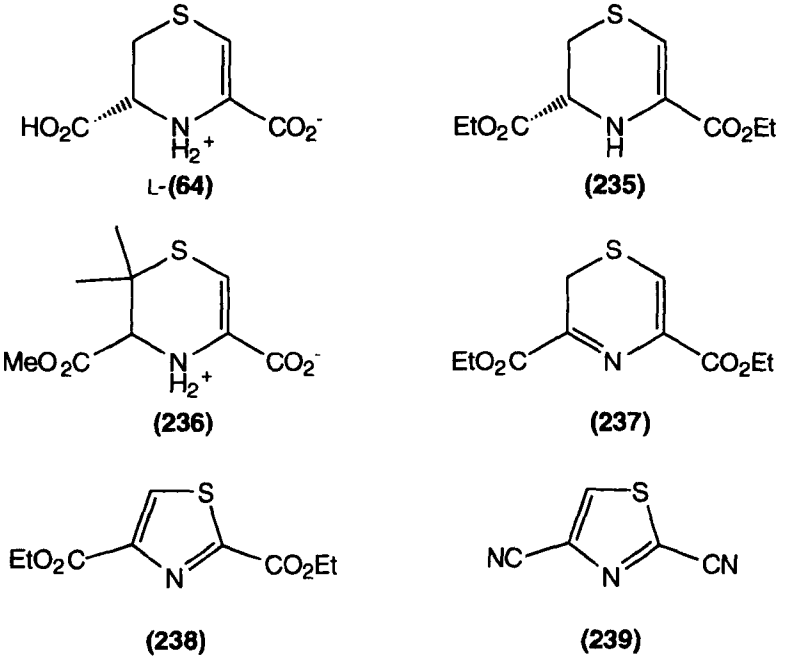


Figure 33 Thiazines and Thiazoles with Interesting Inhibitory Properties of DHDPS

Compound	Inhibition of DHDPS
L-(64)	88% at 0.5 mM, 0% at 0.1 mM
(235)	10% at 0.1 mM
(236)	20% at 0.1 mM
(237)	30% at 0.5 mM
(238)	15% at 0.1 mM
(239)	18% at 0.1 mM

Table 8 Inhibition of DHDPS by Thiazines and Thiazoles

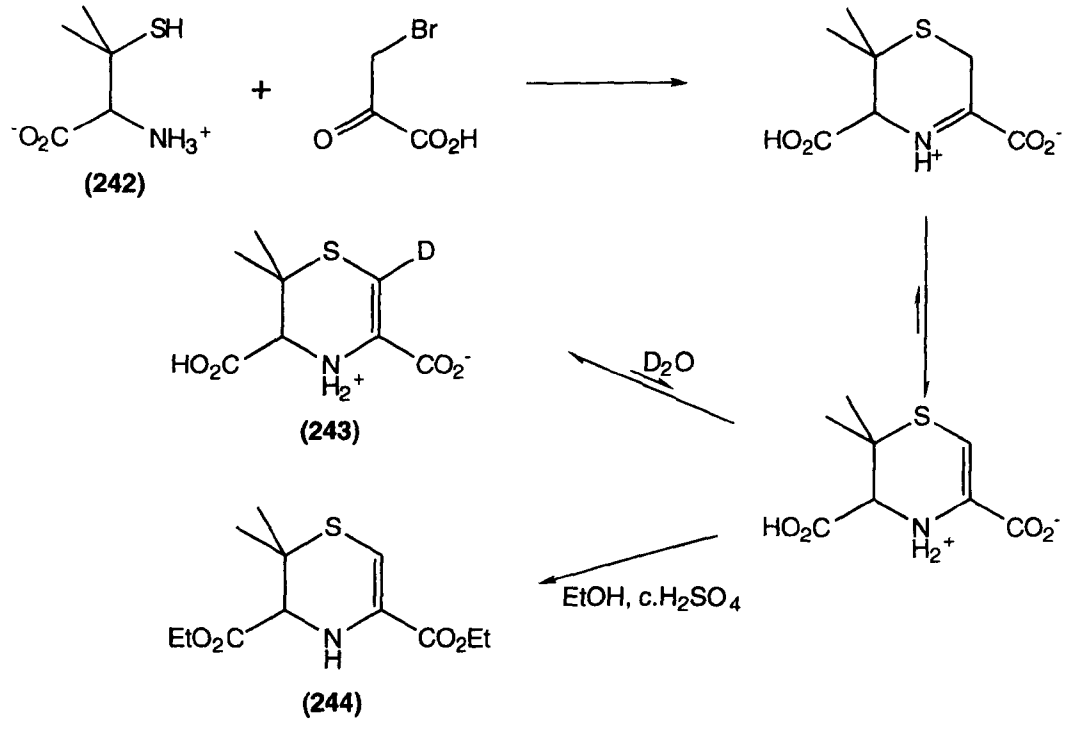
It is difficult to draw firm conclusion from some of these early results. In our recent work it was found that employing the DHDPS/DHDPR coupled assay for inhibition studies helped in obtaining reliably reproducible results. Extension of this library of compounds has allowed us to build up a valuable inhibition profile of the DHDPS/DHDPR enzymes incorporating steric, electrostatic and stereochemical factors.

5.07 Synthesis of Thiazines

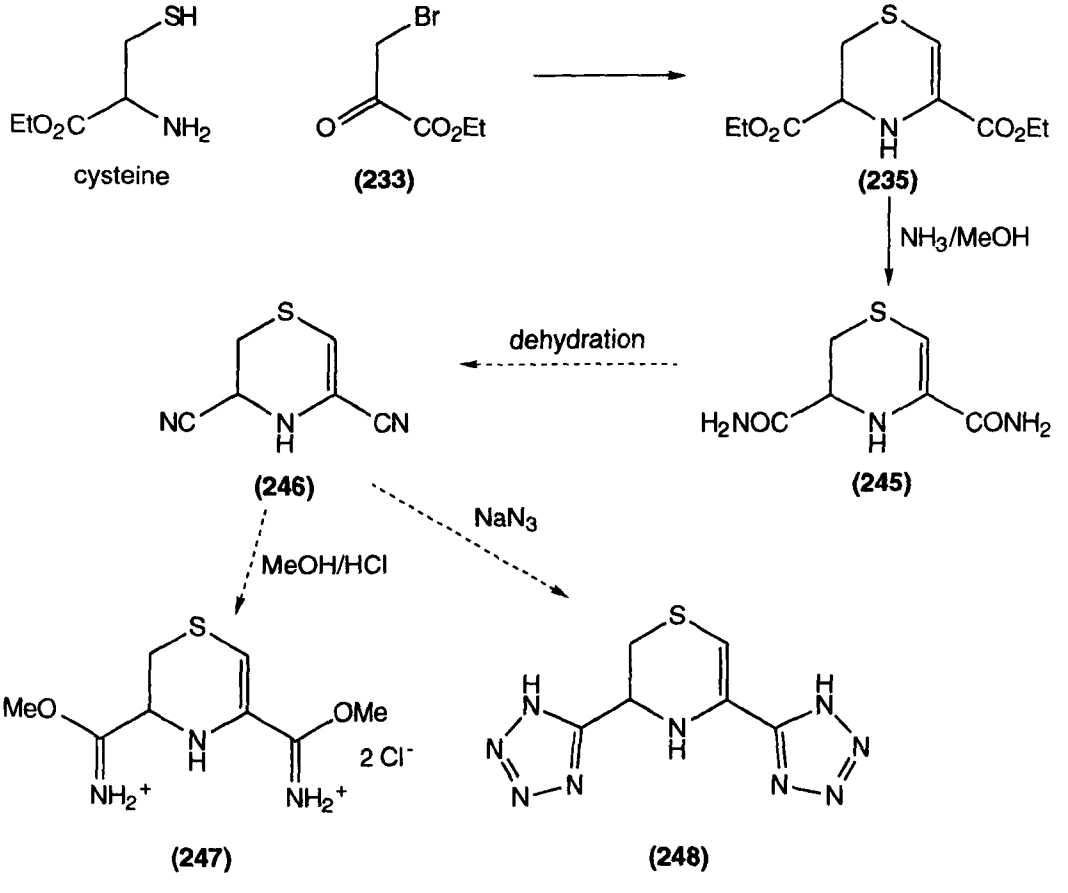
As with previous studies[59,172] compound L-**(64)** was synthesised by the direct condensation of L-cysteine and bromopyruvic acid in water. Similarly, the enantiomer D-**(64)** was synthesised from D-cysteine. The separate enantiomers were isolated in 88% and 83 % yield, respectively. They were shown to have optical rotations of -70.7° and +70.6°, respectively, in methanolic solution and the NMR spectra show no evidence of the imine or open chain forms. Diethyl *(R)*-3,4-dihydro-*2H*-1,4-thiazine-3,5-dicarboxylate **(235)** was synthesised, in 87% yield, by the direct condensation of L-cysteine ethyl ester and ethyl bromopyruvate, according to the method of Berges and Taggart.[173] The dimethyl ester **(240)** and di-*tert*-butyl **(241)** esters were also synthesised by direct esterification of compound L-**(64)** by classical methodology. The respective ester signals were found as singlets in the ^1H NMR spectra at δ 3.71 and 3.72 for compound **(240)** and δ 1.44 and 1.46 for compound **(241)**.

2,2-Dimethyl substituted thiazine analogues **(243)** and **(244)** were synthesised from DL-penicillamine **(242)**. Compound **(244)** was prepared in low yield (7%) by direct condensation with bromopyruvic acid in glacial acetic acid and subsequent esterification. The molecular ion for the parent compound was found at *m/z* 273 in the mass spectrum. The free acid analogue **(243)** was an interesting case. It was first isolated as a solid in an NMR tube while investigating the failure of the desired compound to precipitate from an aqueous solution. The subtle differences in solvation between D_2O and H_2O were enough to promote precipitation. It is likely that other small changes in the physical conditions, such as polarity of the medium and temperature, would have similar effect. The exchange of a proton for a deuterium in the 3-position of bromopyruvic acid suggests a rapid equilibration between imine and enamine forms **(Scheme 75)**. In the ^1H NMR spectrum a small signal at δ 6.09 is observed for unexchanged protons in the 6-position. Once again there is no evidence in either the ^1H or ^{13}C NMR spectra for the imine form.

Imidate and tetrazole groups are known to be good carboxylic acid mimics in biological systems. In earlier studies by J.E. McKendrick[59] pyridine-2,6-dimethylimidate **(38)** and pyridine-2,6-ditetrazole **(39)** **(Scheme 15)** were found to show interesting activity with the DHDPS enzyme. It was considered that the thiazine equivalents **(247)** and **(248)** may also be of interest. Diimidates and ditetrazoles can be synthesised via the corresponding dinitrile **(246)** **(Scheme 76)**.

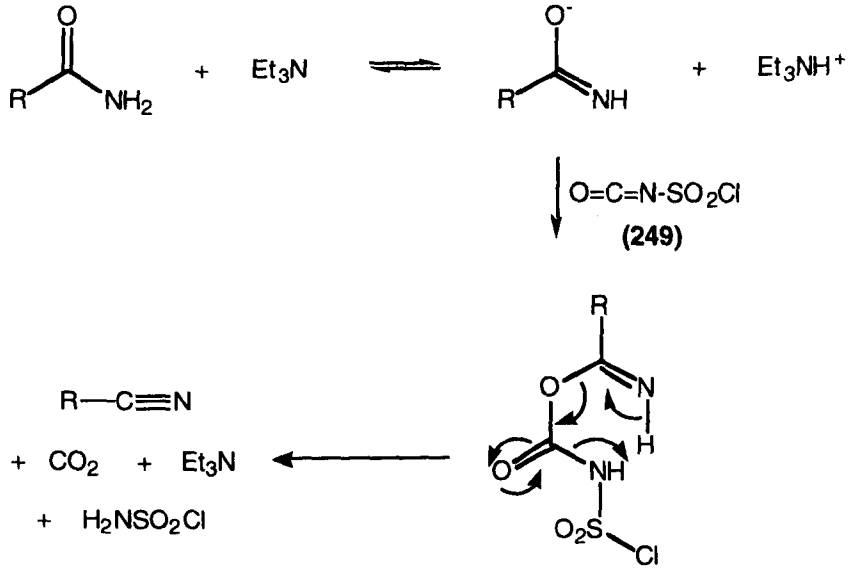


Scheme 75 Synthesis of 2,2-Disubstituted Thiazines



Scheme 76 Synthesis of Imidates and Tetrazoles

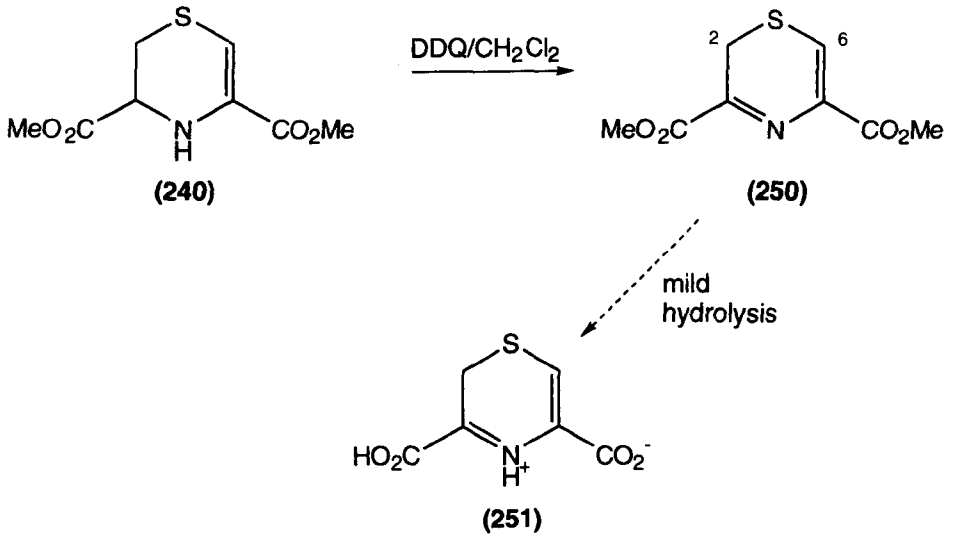
Treating compound **(235)** with anhydrous methanolic ammonia gave the corresponding dicarboxamide **(245)** in 59% yield. Unfortunately, the forcing conditions required for the dehydration of 3,4-dihydro-2*H*-1,4-thiazine-3,5-diamide **(245)** to the dinitrile **(246)** proved too severe. Even mild dehydrating agents such as chlorosulfonyl isocyanate[174] **(249)** **(Scheme 77)** effected decomposition. This transformation has previously been achieved for the corresponding thiazole **(238)** and pyridine **(35)**. We therefore conclude, that without aromatic stabilisation, decomposition is observed under the forcing conditions required.



Scheme 77 **Dehydration by Chlorosulfonyl Isocyanate (250)**

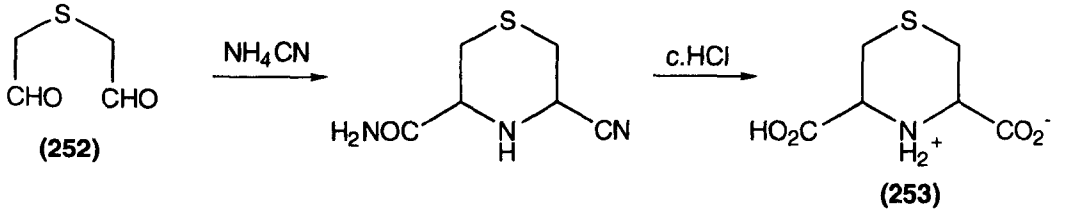
Another variation on the thiazine ring system that we are interested in are different ring oxidation states. Varying the extent of unsaturation of the ring results in a change of shape of the ring and orientation of the acid groups.

Treating the thiazine dimethyl ester **(240)** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) resulted in oxidation to dimethyl 1,4-thiazine-2,5-dicarboxylate **(250)** in 72% yield **(Scheme 78)**. Interestingly, in the ^{1}H NMR spectrum a coupling is observed (1.2 Hz) between the methylene protons in the 2-position (δ 3.39) and the olefinic proton in the 6-position (δ 7.66). Hydrolysis of compound **(250)** was attempted by a variety of methods: including acidic and basic hydrolysis; treatment with $NaBH_4/I_2$;[175] treatment with $AlCl_3$/DMS;[176] and treatment with KO^tBu/H_2O (4:1) (a source of anhydrous hydroxide).[177] Although there were signs of success for most of these methods, a combination of low stability and difficulty in purification meant that it was impossible to isolate the desired product **(251)**.



Scheme 78 Preparation of the Fully Oxidised Thiazine

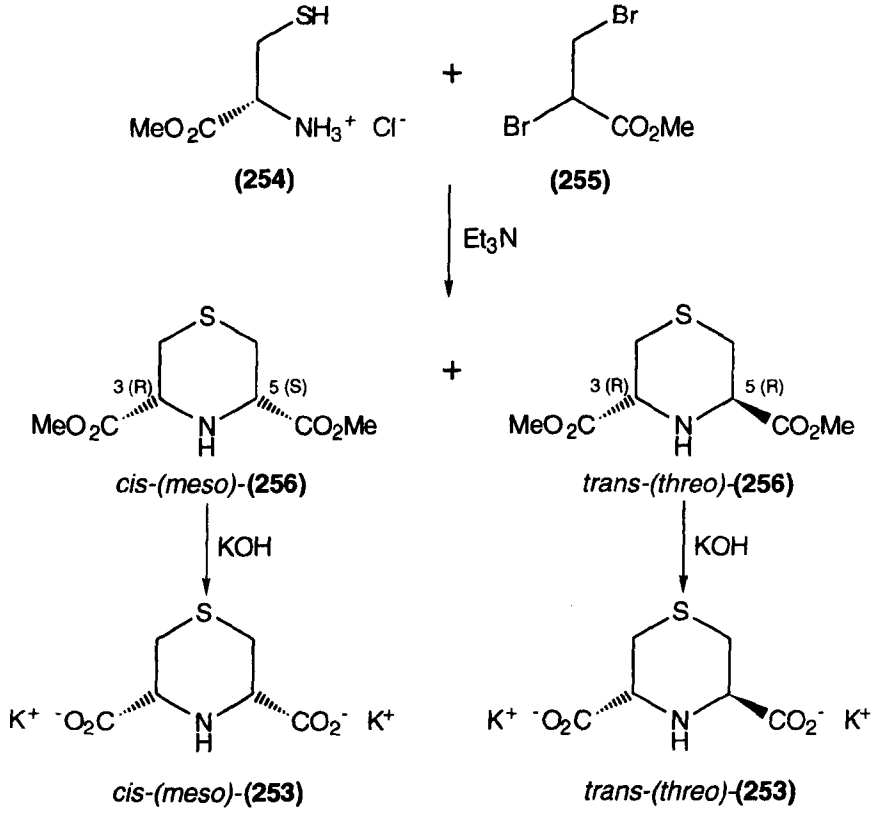
Fortunately, the preparation of the saturated ring system was a somewhat easier task. Thiomorpholine-3,5-dicarboxylic acid **(253)** has been prepared, as early as 1937, by Coghill.[178] Compound **(253)** was formed by treating thiodiacetaldehyde **(252)** with ammonium cyanide and subsequent hydrolysis **(Scheme 79)**.



Scheme 79 Synthesis of Thiomorpholine-3,5-dicarboxylic Acid (253)

In our work, we chose to follow the more recent method of Lucente and co-workers[179] for the preparation of dimethyl thiomorpholine-3,5-dicarboxylate **(256)**. Direct condensation of L-cysteine methyl ester hydrochloride **(254)** and racemic methyl 2,3-dibromopropionate **(255)** yielded the desired diester **(256)** as a mixture of *cis*- and *trans*-isomers in 75% overall yield **(Scheme 80)**. The two diastereoisomers were separated by chromatography on silica gel. The compounds were identified by GCMS analysis ($\underline{M}^+$ 219) and the diastereomeric ratio was found to be 60:40 in favour of the *cis*-isomer. The *pseudo*-axial/axial arrangement of ester groups in the *cis*-isomer was thermodynamically favoured. Interestingly, in accord with the findings of

Lucente and co-workers,[179] the *trans*-isomer was found to be the more polar of the two. The relative polarities were indicated by their respective R_f values on silica gel. Furthermore, in the ^{13}C NMR spectrum of the chiral *trans*-isomer we do not see separate signals for C-2 and C-6 or for C-3 and C-5. Conclusive distinction between the *meso*- and *threo*- diastereoisomers is therefore found in their optical rotations, which compare with the literature.[179] *cis*- and *trans*-Isomers of thiomorpholine-3,5-dicarboxylic acid **(253)** were prepared by basic hydrolysis of the individual diesters **(256)** to yield the desired compounds in high yield **(Scheme 80)**. This method of hydrolysis was suggested by Portalone and co-workers[180] and was shown not to result in racemisation.



Scheme 80 Alternative Synthesis of Compound (253)

5.08 Synthesis of Thiazoles and Thiazolidines

Our interest in five membered heterocycles followed from the earlier results within our group for the thiazoles **(238)** and **(239)** **(Table 8)**. As for the six-membered heterocycles, it was considered necessary to have carboxylic acid groups, or equivalents, adjacent to the nitrogen atom. A

number of possible targets were identified from an extensive literature search **(Figure 34)**.

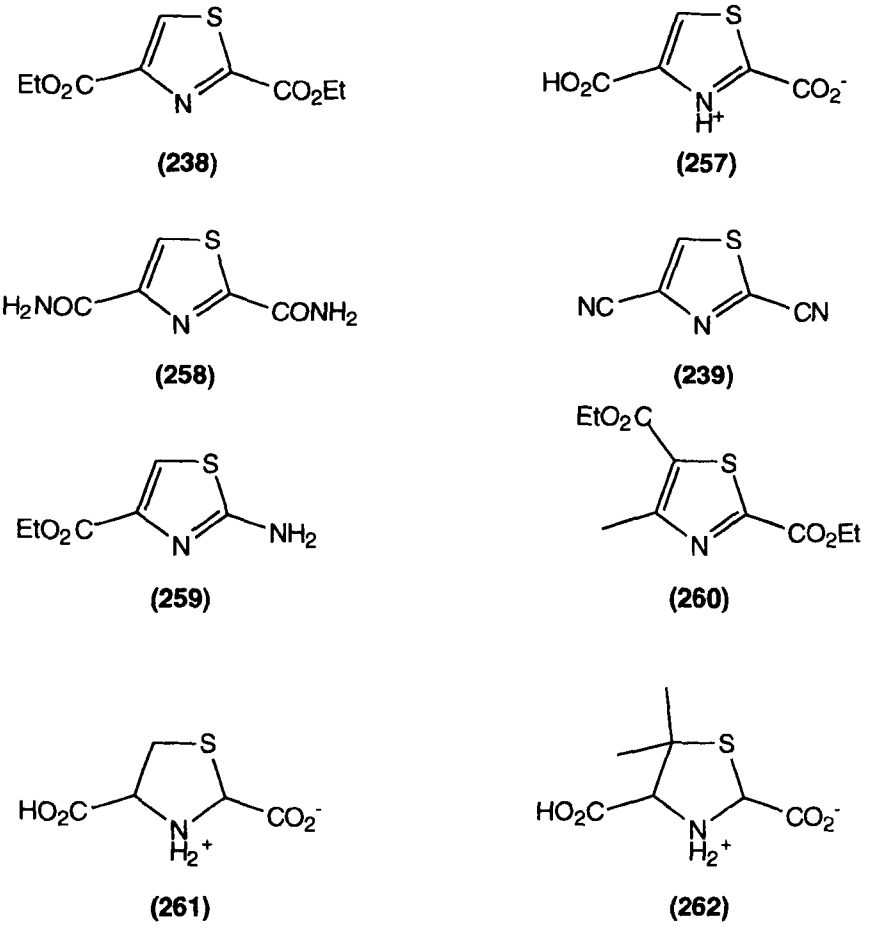
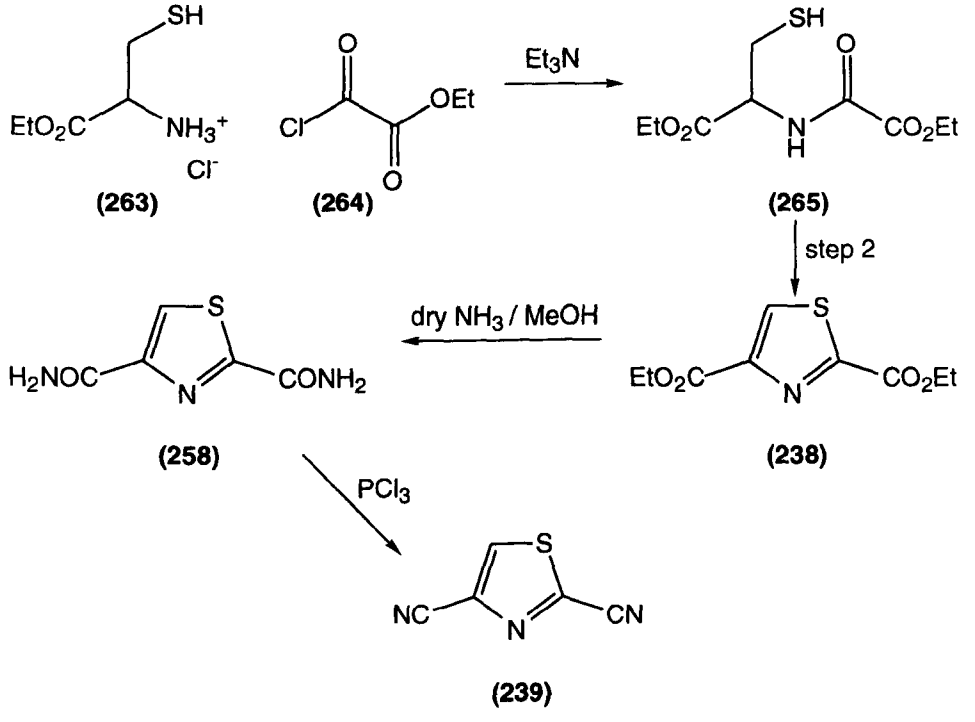


Figure 34 Target Thiazoles and Thiazolidines

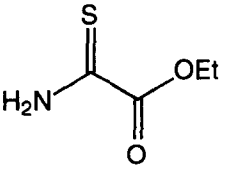
It was originally intended to synthesise thiazole-2,4-dimethylimidate and thiazole-2,4-ditetrazole from dinitrile **(239)**, as detailed for the 6-membered equivalent in **Scheme 76**. The preparation of dinitrile **(239)** is detailed in **Scheme 81**. The aliphatic precursor **(265)** to heterocycle **(238)** was prepared, in 94% yield, on treating cysteine ethyl ester hydrochloride **(263)** with ethyl oxalyl chloride **(264)**. In the ^1H NMR spectrum of compound **(265)** the thiol proton is observed as a triplet (δ 1.55, ^3J 9.0 Hz), by virtue of a coupling through sulfur with the adjacent methylene protons. When two equivalents of ethyl oxalyl chloride were used double substitution on cysteine, at both nitrogen and sulfur, was observed in 84% yield. The cyclisation/oxidation of precursor **(265)** (step 2, **Scheme 81)** proved troublesome. The transformation was achieved in 14% yield with P_4S_{10}, according to the method of Tudor,[181] but problems with disposal of the byproducts made this route impractical on a large scale. Attempted cyclisation

with PCl_3, $POCl_3$ (Bischler-Napieralski[182] type cyclisation) or with catalytic quantities of acid (PTSA) or base (DMAP) under dehydrating conditions, were all shown to favour elimination of H_2S. Any product that was formed in each of these cases was found to present further purification problems (TLC). A related cyclisaton was found in the literature[183] using anhydrous HCl in Et_2O. However, when this transformation was attempted, dimeric derivative **(267)** was recovered as the product after approximately ten weeks reaction time (see **Scheme 82**).

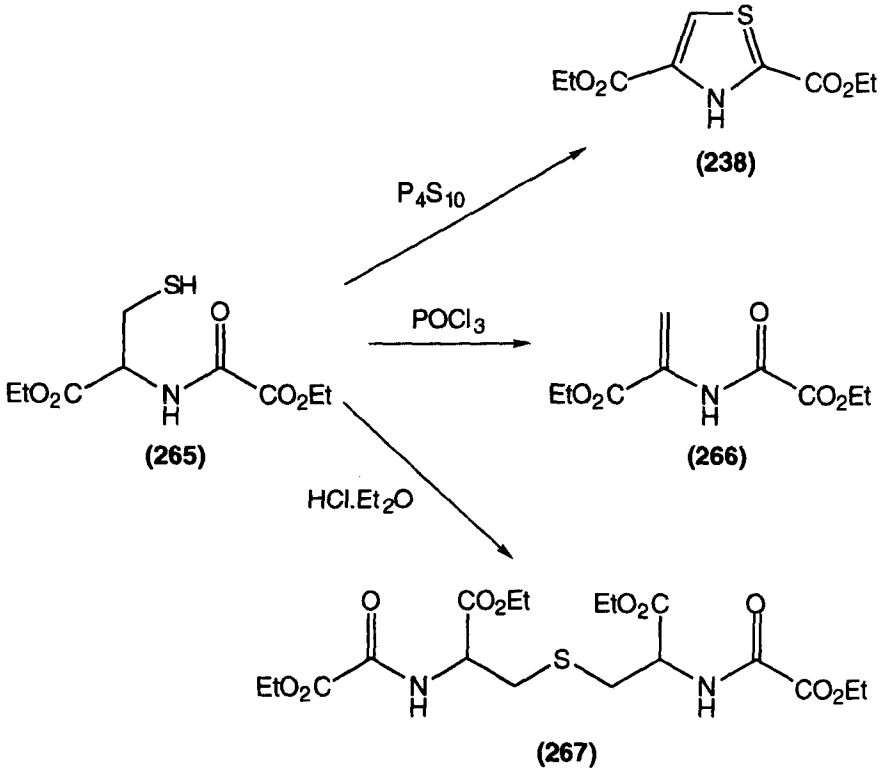


Scheme 81 Synthesis of the Dinitrile (239)

Because of the problems associated with the cyclisation of compound **(265)** an alternative synthesis of thiazole diester **(238)** was also used. Direct condensation of ethyl bromopyruvate and ethyl thiooxamate **(268)**, according to the method of Erlenmeyer *et al.*,[184] yielded a 60% recovery of the desired heterocycle. The single aromatic proton is found at δ = 8.42 in the ^1H NMR spectrum. Hydrolysis of compound **(238)** in $EtOH/KOH_{(aq)}$, adapted from the method of Erlenmeyer *et al.*,[184] gave the thiazole diacid **(257)** in 23% yield.



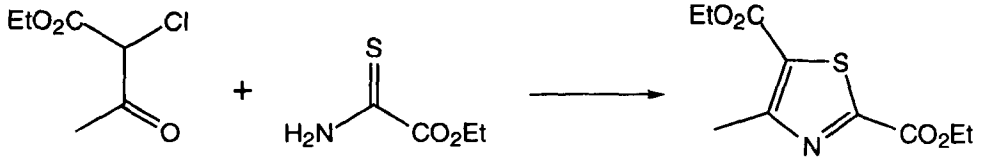
(268)



Scheme 82 Attempted Cyclisation of Compound (265)

Treating compound **(238)** with anhydrous methanolic ammonia resulted in precipitation of the dicarboxamide derivative **(258)**. Subsequent dehydration of the dicarboxamide by phosphorus oxychloride resulted in a 44% recovery of the target thiazine dinitrile **(239)**. Signals for the nitrile carbons are found in the ^{13}C NMR spectrum at δ 111 and 112. Inhibition results, detailed in the next chapter, dictated that no attempt was made to transform the dinitrile **(239)** into the dimethylimidate or ditetrazole.

Ethyl 2-amino-1,3-thiazole-4-carboxylate **(259)** was prepared by a similar process to that of compound **(238)**. Direct condensation of ethyl bromopyruvate with thiourea yielded a 61% recovery of the desired heterocycle. In the ^{1}H NMR spectrum, a signal is observed for the aromatic proton at δ 7.60. Diethyl 4-methyl-1,3-thiazole-2,5-dicarboxylate **(260)** was synthesised from ethyl 2-chloroacetoacetate **(269)** and ethyl thiooxamate **(268)** according to the method of Boon[185] **(Scheme 83)**. Heterocycle **(260)** was isolated in 35% yield. Signals for the aromatic carbons are found in the ^{13}C NMR spectrum at δ 127, 159 and 159.



(269) (268) (260)

Scheme 83 Synthesis of Heterocycle (260)

Thiazolidine-2,4-dicarboxylic acid **(261)** and 5,5-dimethylthiazolidine-2,4-dicarboxylic acid **(262)** were synthesised as diastereomeric mixtures by the method of Bentley *et al.*[186] Direct condensation of glyoxylic acid with L-cysteine and DL-penicillamine gave respective recoveries of 43% and 56%. The molecular ions are found at *m/z* =177.0096 and *m/z* = 205.0393 in the high resolution mass spectra for compounds **(261)** and **(262)**, respectively.

5.09 Conclusion

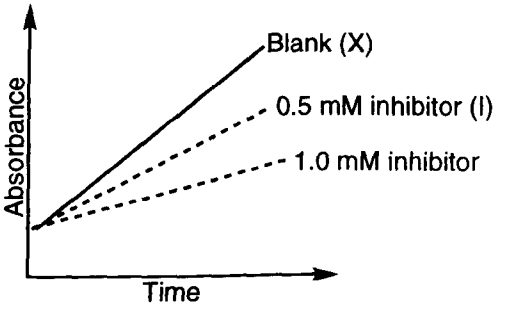
In this chapter we have described the synthesis of a number of analogues of pyruvate and heterocyclic analogues of DHDPA and THDPA. The results of inhibition studies on these compounds, while informative on function of the DHDPS and DHDPR enzymes, do not vindicate further investigation in this area. These results along with those of the our substrate studies are discussed in detail in the following chapter. In conclusion therefrom we elaborate a possible alternate direction for inhibition of these enzymes.

Chapter 6 Biological Results and Discussion

6.01 Introduction

In previous chapters we described the preparation of substrates and analogues of the DHDPS and DHDPR enzymes. In this chapter we discuss the biological evaluation of these potential inhibitors and substrates and limitations thereon. In conclusion, we summarise this research project and indicate areas of possible further study.

In sections 2.11 and 2.16 we discussed established assay systems for characterisation of DHDPS and DHDPR activity, respectively. Ideally, a suitable assay for monitoring enzymic activity involves observation of a 'real time' change in a physical property related to concentration. Suitable for this task is the observation of change in UV absorbance with respect to time. In accord with Beer's Law, UV absorbance is directly proportional to concentration. It is also possible, however, to monitor the extent of conversion post-reaction. A number of alternative quantitative analytical techniques are available for this evaluation, such as: UV, MS, NMR, fluorescence spectroscopy and radiolabelling. Commonly these analytical techniques are linked to LC systems to separate the analyte in question from other 'noise.' The advantages of a 'real time' assay are that we can see the results instantly, need not develop a purification procedure and can compare with a single standard. For the standard or 'blank' run the assay is performed with the enzyme and substrates at pre-set concentrations. Subsequent assays differ only in that varying levels of inhibitor are present. The extent of inhibition is determined by direct comparison of gradients of the absorption versus time curves **(Figure 35)**.



$$\% \text{ inhibition} = \frac{X-I}{X} \times 100$$

Figure 35 Typical Inhibition Profile

Substrate assays are performed in a similar manner where the potential substrate replaces a substrate in the assay at the same concentration. Due regard must be given to alteration of the chromophore when comparing activity with the blank.

6.02 Standard Assays for DHDPS and DHDPR

The DHDPS 'synthase' assay and DHDPS/DHDPR 'coupled' assay detailed in section 2.11 **(Scheme 13)** were used for the evaluation of our inhibitors.

The standard 1 ml synthase assay has ASA and pyruvate at 1 mM, imidazole buffer (pH 7.4) at 100 mM and 10 µl of purified DHDPS in 50% aqueous glycerol. Activity was determined by the change in absorbance at 270 nm with respect to time.

The standard 1 ml coupled assay has the substrates and buffer at the same concentration. NADPH co-factor was present at 0.250 mM. The DHDPS and DHDPR enzymes (10 µl) in 50% aqueous glycerol were added as 10 fold and 250 fold diluted solutions (H_2O), respectively. Activity was determined by the change in absorbance at 340 nm with respect to time.

Aqueous stock solutions of ASA, pyruvate and NADPH were prepared at 20 mM and stored, along with the enzymes, at less than -20 °C. Stocks of aqueous imidazole buffer were prepared at 1 M and stored at 4 °C.

6.03 Limitations of the DHDPS 'Synthase' Assay

Although the 'synthase' assay has previously been extensively used by our group, great difficulties were experienced with this assay. Many of our studies, including all those on previously tested 'inhibitors,' proved to be irreproducible. In some cases we observed an apparent negative inhibition, where the enzymic reaction appeared to be promoted by the potential inhibitor. In some cases the estimated 'inhibition' could vary by more than 100% for successive repetitions of an experiment. The difficulties created by these early observations were a massive hurdle, and took in excess of a year to overcome. It was acknowledged at an early stage that a major problem was the high levels of UV absorption by our potential inhibitors at the assay wavelength, 270 nm. It is notable that almost all saturated carbonyl-containing compounds absorb strongly at this wavelength. This problem was compounded by the fact that our inhibitors were not particularly efficient and

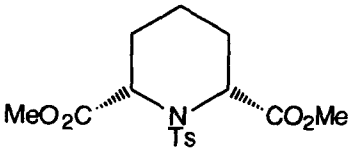
were studied at relatively high concentrations, in the region of 1 mM. As a result, very little of the incident UV beam passed through the sample to the detector. This makes the experimental error a significant factor. Extensive studies with ethyl pyruvate thiosemicarbazide **(230)**, which displayed consistent 'negative inhibition,' showed that a positive assay could be obtained in the absence of either of the natural substrates or in the absence of DHDPS. It is this non-enzymic interaction that is responsible for the apparent 'negative' result. It is not difficult to envisage a nucleophilic/electrophilic interaction between the carbonyl-containing substrates of DHDPS and the nucleophilic compound **(230)**. Furthermore, we could predict that this would be a common observation with all 'nucleophilic' inhibitors. Not surprisingly, therefore, we find this apparent 'negative inhibition' to be very common amongst many of our potential inhibitors which could be considered as nucleophilic.

We can therefore picture a series of events with our problem UV assays. With a number of competing processes, we can see that the kinetics of this system are greatly complicated. Coupled with the problem of significant experimental error, this leads to irreproducibility of results and makes it almost impossible to study the chemical transformation of interest. Consequently we have to question many of our previous investigations in this area. We should also consider that if the natural substrates are important metabolites in living systems, such as pyruvate is in mammals, then we have direct evidence that a number of our nucleophilic inhibitors are likely to display significant toxic effects.

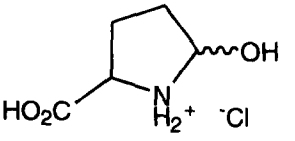
Successful reproducible results were obtained with the synthase assay for compounds **(51)** and **(205)** as well as for the commercially available DPA **(28)**, chelidonic acid **(270)** and glyoxylic acid **(271)**. These results are summarised in **Figure 36**. Interestingly, none of these compounds can be considered as nucleophilic.

The results for compounds **(28)** and **(270)** are similar to our previous studies. The structural similarity of these compounds with the enzymic product, DHDPA **(24)**, indicates towards their competitive nature. Similarly, compound **(271)** most likely binds directly into the active site of DHDPS enzyme in direct competition with pyruvate. There may be scope in studying other pyruvate analogues lacking enolisable protons in the 3-position, such as fluoro-substituted pyruvates. However, the highly toxic nature of such compounds must be borne in mind. The result for compound **(270)** also suggests possibilities for investigating alternate heterocyclic systems. Compound **(51)** was an intriguing case. Clearly this compound has little resemblence to the substrates and product. It is most likely that inhibition by compound **(51)** is of

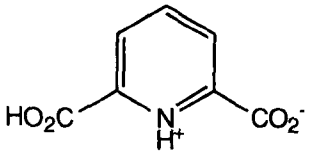
a non-competitive nature. Most interesting however was the result for compound **(205)**. This compound is the heterocyclic precursor to GSA **(156)**. Although the level of inhibition was low, there is suggestion that GSA may be accepted into the active site of DHDPS. In chapter 4 we have concentrated exclusively on this goal. This result in conjunction with complementary substrate studies is discussed further in section 6.07.



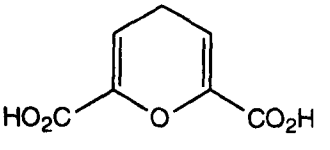
(51) IC_{50} 0.94 mM



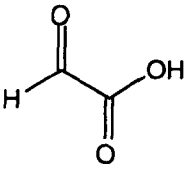
(205) IC_{50} 3.9 mM



(28) IC_{50} 0.4 mM



(270) IC_{50} 0.31 mM



(271) IC_{50} 1.0 mM

Figure 36 Results for Inhibition of DHDPS

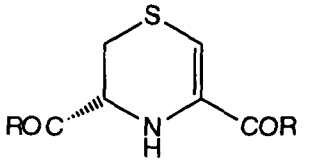
6.04 The DHDPS/DHDPR 'Coupled' Assay.

The availability of the DHDPR enzyme proved crucial to the solution of our testing problems. This enzyme allowed us to utilise the 'coupled' assay system for testing of our compounds. Examination of the consumption of NADPH at 340 nm almost totally excludes the observation of the non-enzymic reactions seen at 270 nm and greatly reduces the experimental error associated with the absorption of UV light at this wavelength. A further bonus is that we are testing our potential inhibitors against both DHDPS and DHDPR. This is significantly important for analogues of L-DHDPA **(24)** and THDPA **(25)**, the respective natural substrate and product of DHDPR.

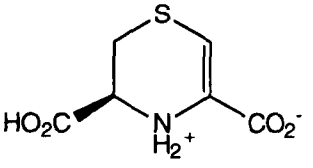
Although some minor problems were encountered, such as the non-enzymic consumption of NADPH by the thiosemicarbazone derivatives **(220)** and **(230)**, this assay system has proved invaluable.

In section 5.04 the synthesis of a number of analogues of pyruvate was detailed. Of these compounds, only ethyl pyruvate semicarbazone **(229)** was found to display modest inhibition of DHDPR/DHDPS. The calculated IC_{50} value for compound **(229)** was 3.8 mM.

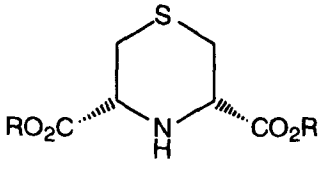
The syntheses of thiazine, thiazole and thiazolidine analogues of DHDPA and THDPA were detailed in section 5.07 and 5.08. The results from inhibition studies with DHDPS/DHDPR are summarised in **Figure 37**.



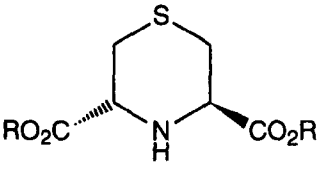
L-**(64)** R = OH, **IC_{50} 0.038 mM**
(235) R = OEt, **IC_{50} 3.9 mM**
(245) R = NH_2, **IC_{50} 1.0 mM**



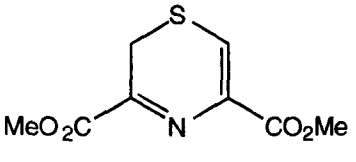
D-**(64)** **IC_{50} 0.42 mM**



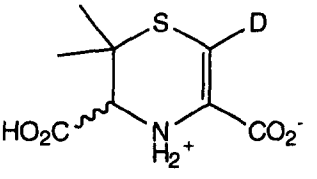
cis-**(253)** R = H, **IC_{50} 0.40 mM**
cis-**(256)** R = Me, **no inhibition**



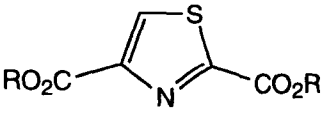
trans-**(253)** R = H, **IC_{50} 3.8 mM**
trans-**(256)** R = Me, **no inhibition**



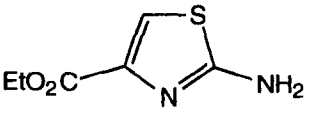
(250) no inhibition



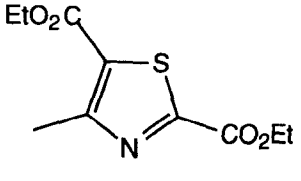
(243) no inhibition



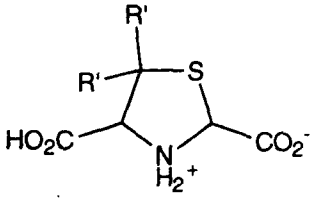
(238) R = H, **IC_{50} 2.7 mM**
(257) R = Et, **no inhibition**



(259) IC_{50} 6.6 mM



(260) IC_{50} 12.7 mM



(261) R' = H, **IC_{50} 1.0 mM**
(262) R' = Me, **IC_{50} 2.8 mM**

Figure 37 Results for Inhibition of DHDPS/DHDPR

A typical inhibition profile is illustrated graphically for compound L-**(64)** in **Figure 38**.

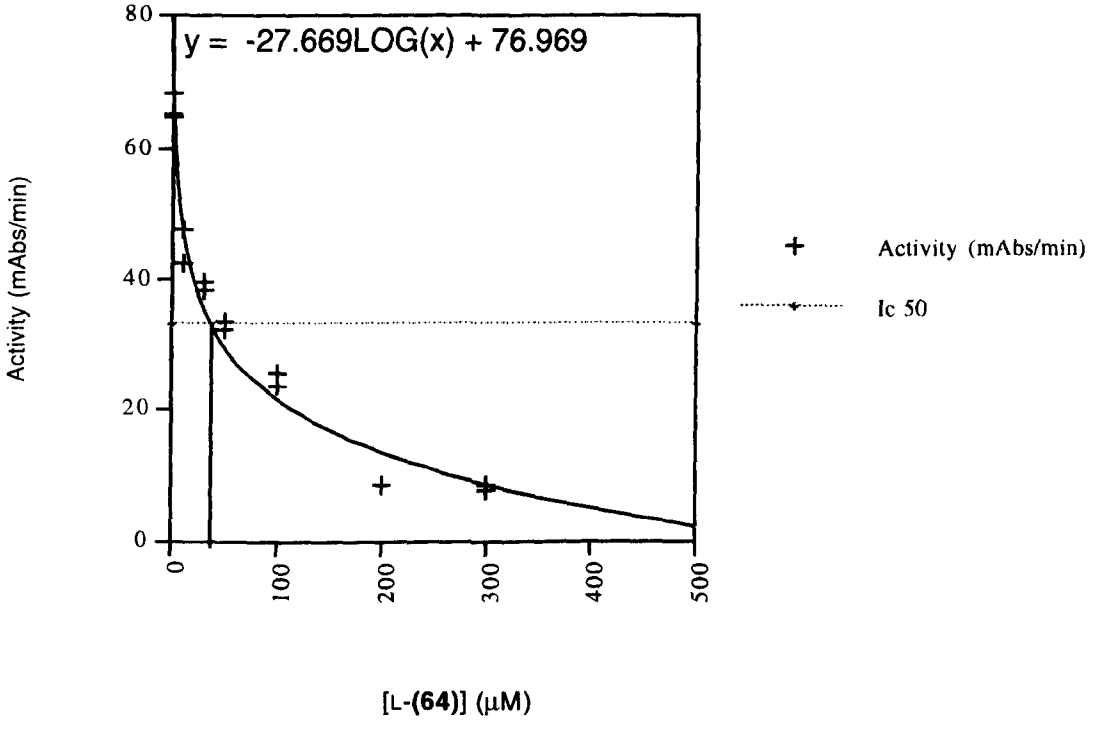


Figure 38 A Typical Inhibition Profile for Inhibition of DHDPS/DHDPR

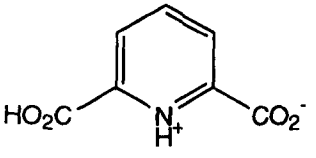
From these inhibition studies it is clear that a number of the earlier results in this area were somewhat misleading. We need only compare the results obtained for the diacid L-**(64)** and the corresponding diester **(235)** to see that esters are very much poorer inhibitors. Unsurprisingly, the hydrogen bonding ability of the carboxylate function is key to binding. This, along with the essential binding of the β-keto function of pyruvate **(Scheme 14)**, explains the poor results obtained for the pyruvate ester derivatives.

The results for inhibition of DHDPR/DHDPS by the sulfur-containing heterocycles, detailed in **Figure 37**, are a valuable insight into the binding characteristics of the enzymes. It is interesting to note the significant difference in inhibition between the different ring oxidation states. This indicates that very small changes in the orientation of the acid groups have a marked effect on the binding ability. Conversely, the ability of the enzymes to accommodate the bulky sulfur group in the 4-position readily, suggests a degree of variability in this region. It would be an interesting exercise to investigate if polar heteroatoms were equally tolerated in this position. It was not surprising that the enantiomer of compound **(64)** displayed different inhibitory properties.

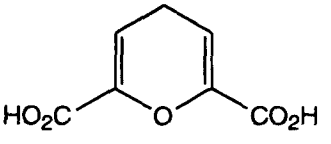
The stereocentre of this compound is in the corresponding position to that of ASA for the natural biotransformation. Somewhat more surprising were the results for *cis*- and *trans*-isomers of thiomorpholine-3,5-dicarboxylic acid **(253)**. The *cis*-isomer is a considerably better inhibitor of DHDPS/DHDPR. This result could be incorporated into an argument for the strict geometrical requirements of the two acid groups and could have further mechanistic/stereochemical implications.

The five-membered ring system appears a poorer model for inhibition. Interestingly, the saturated system has greater inhibitory activity in this case. Once again strict structural and geometrical requirements are indicated for the hydrophilic portion with suggested tolerance of structural variability in the hydrophobic portion.

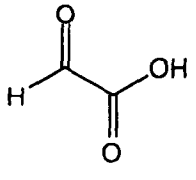
A number of commercially available compounds were shown to exhibit no inhibition with DHDPS/DHDPR: lithium acetoacetate; ethyl acetoacetate; ethyl 2-hydroxybutanoate; ethyl acetopyruvate; and acetylacetone. Other commercially available compounds, which had shown signs of inhibition for the 'synthase' assay, did show similar trends for the coupled assay. The results for DPA **(28)**, chelidonic acid **(270)** glyoxylic acid **(271)** and chelidamic acid **(272)** are detailed in **Figure 39**.



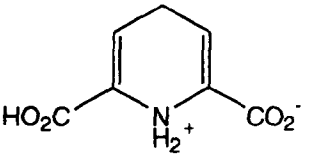
(28) IC_{50} **0.46 mM**



(270) IC_{50} **<0.3 mM**



(271) IC_{50} **0.31 mM**

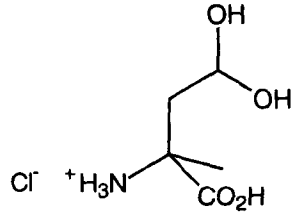


(272) IC_{50} **3.2 mM**

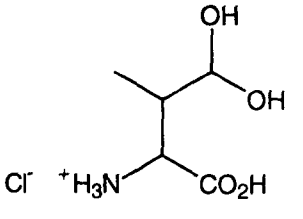
Figure 39 Results for Inhibition of DHDPS/DHDPR by Commercially Available Compounds

The competitive nature of substrates of DHDPS makes inhibition studies with these compounds a valuable exercise. For this purpose we are fortunate that, with the exception of ASA methyl ester **(129)**, these compounds are not substrates for the DHDPS/DHDPR coupled system.

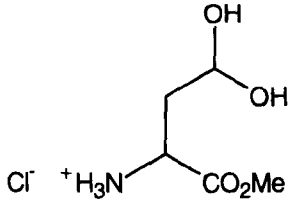
Consequently, the extent of inhibition when these substrates are at 1 mM, the same as ASA in the assay, is a direct indication of competition for the active site. These results are presented along with positive inhibition results for other synthetic compounds in **Figure 40**.



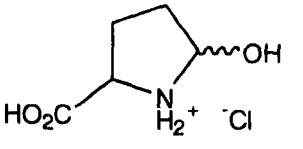
(154) 61% inhibition at 1 mM



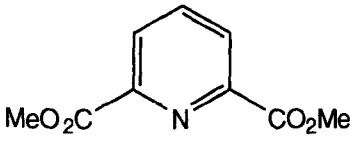
(155) 30% inhibition at 1 mM



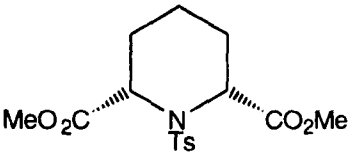
(129)
Evidence of Inhibition,difficult to guage because of substrate activity



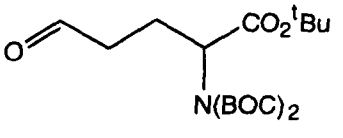
(205) 28% inhibition at 1 mM



(34) IC_{50} 4.6 mM



(51) IC_{50} 2.8 mM



(174) IC_{50} 3.0 mM

Figure 40 Inhibition of DHDPS/DHDPR by Substrates of DHDPS and Other Synthetic Compounds

In this case, we would consider a 50% inhibition value as an indication of equal competition for the active site between the 'natural' and 'unnatural' substrates. In that respect, the results for these compounds are promising. In particular, the result for compound **(154)** suggests a preference for this compound over the 'natural' substrate. The result for 5-hydroxyproline **(205)** was similarly encouraging and offers further indication of substrate potential

for GSA **(156)**. These results are considered further, from a substrate perspective, in section 6.07.

The result for compound **(51)** is interesting when compared with the result for the 'synthase' assay (IC_{50} value of 0.94 mM). The lower level of inhibition in the coupled system could suggest that the second enzymic transformation is rate-determining for the 'coupled' assay. Ideally, in an optimised two enzyme assay, both steps would proceed with equal velocity.

6.05 Development of a New DHDPR Assay

Previously in section 2.16 we mentioned the novel idea of utilising the reverse process to that promoted by the DHDPR enzyme as an assay system in a 'reverse reductase' assay **(Scheme 17)**. Once again the theory is based on the reversible nature of enzymic catalysis. If the substrate is too unstable to synthesise then the product could be made and the reverse process examined. The instability of the DHDPA product of the reverse process is in our favour, because, by undergoing a spontaneous irreversible oxidation to DPA **(28)** displacement of the equilibrium generates more DHDPA. In developing this assay we had the option of monitoring the production of DPA **(28)** at 270 nm, as for the 'synthase' assay, or monitoring the reduction of the $NADP^+$ co-factor to NADPH at 340 nm.

The first stage of development involved the synthesis of the product of DHDPR, namely THDPA **(25)**. Fortunately, this task has been performed previously by our group[78,79] and is detailed in Section **2.16 (Scheme 21)**. Starting from DPA **(28)**, esterification and catalytic hydrogenation of the aromatic ring proceeded in high yield. Tosylation of nitrogen on piperidine **(50)** by tosyl chloride in pyridine was achieved in 58% yield after crystallisation. The key step involves elimination of tosic acid, promoted by potassium *tert*-butoxide, followed by purification of **(25)** on ion-exchange resin and crystallisation from MeOH. The elimination was found to progress efficiently. Problems were however encountered with the crystallisation of **(25)**. A large volume of MeOH was required to dissolve the crude material, which did not crystallise on cooling. Further development of the purification method is required. For our purpose the crude amorphous material was recovered from the methanolic solution and evaluated by ^1H NMR spectroscopy. The material was estimated as 58 wt% pure which corresponded to 87% conversion from dimethyl *N*-tosyl-*cis*-piperidine-2,6-dicarboxylate **(51)**. This material was suitable for evaluating the merits of the assay.

The assay was performed by monitoring the change in absorption, at 340 nm, of a solution containing THDPA, $NADP^+$, imidazole buffer (pH 7.4) and DHDPR enzyme. High absorption at 270 nm by the assay cocktail meant that monitoring at this wavelength was not viable. The activity was found to be rather low with the components at similar concentrations to those described for the 'synthase' and 'coupled' assays. The activity was improved by increasing the concentration of enzyme, substrate or co-factor. It was considered that a higher pH would favour production of NADPH. Investigation of the assay at pH 8.0 and 9.0 showed this not to be the case. The optimum assay conditions for the 1 ml assay were therefore chosen as: 3 mM THDPA; 0.5 mM $NADP^+$; 100 mM imidazole buffer (pH 7.4) and 10 µl of a five fold diluted solution (H_2O) of purified DHDPR in 50% aqueous glycerol. A typical assay 'blank' is illustrated in **Figure 41**. Higher activity was possible at increased concentrations but a combination of economy of valuable material and optimised linear activity/time gradient made this the preferred system.

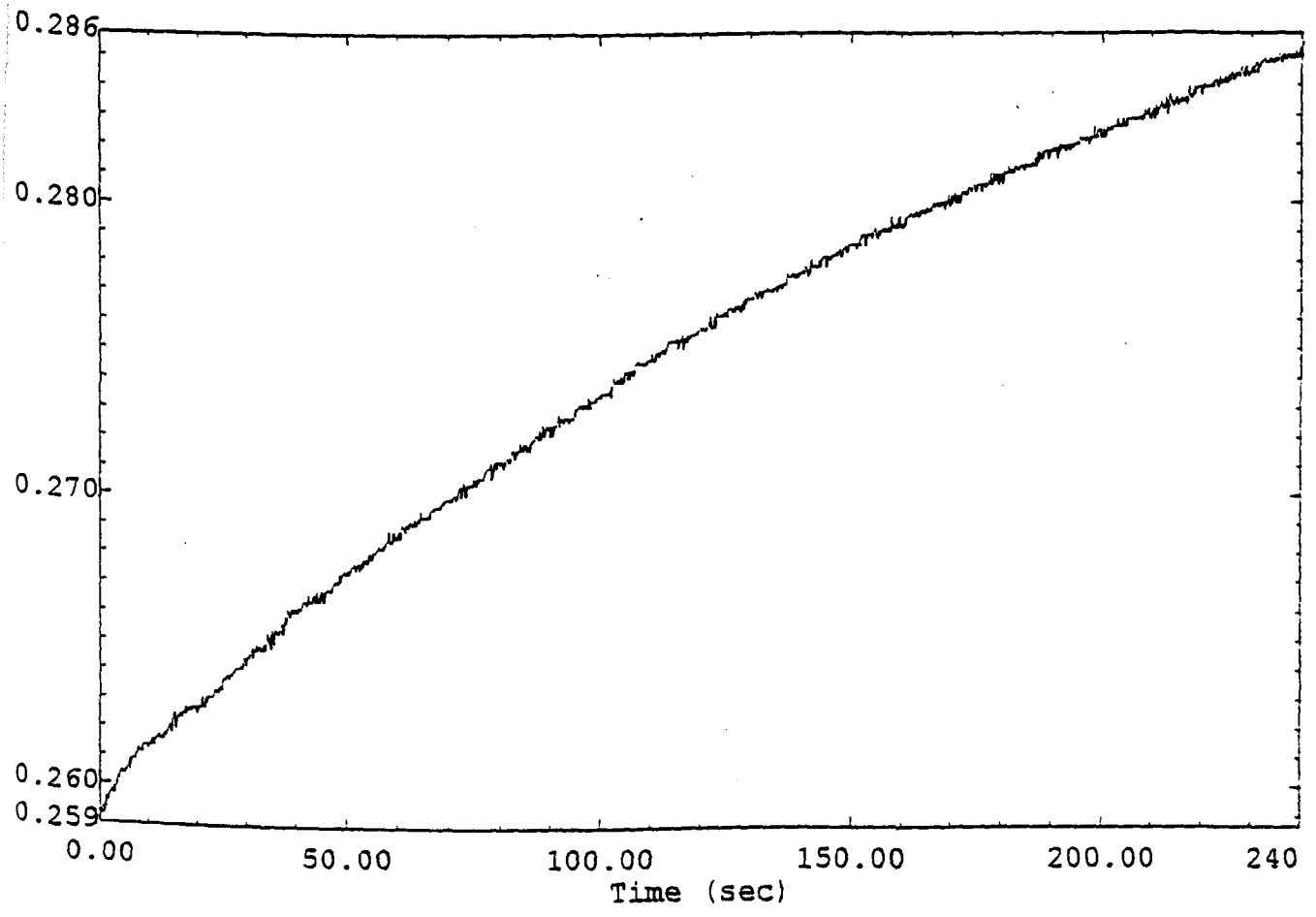
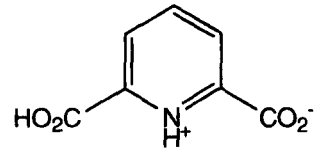


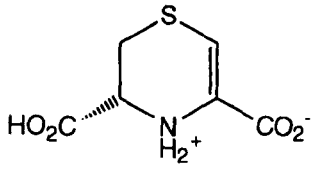
Figure 41 Graph of Absorbance/Time for a 'Reverse Reductase' Assay

6.06 Inhibition of DHDPR

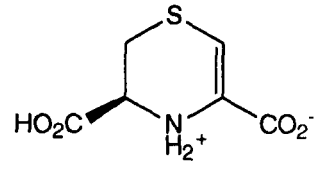
Using our newly developed 'reverse reductase' assay a number of our inhibitors were tested against DHDPR alone: compounds **(28)**; L-**(64)**; D-**(64)**; *cis*-**(253)** and *trans*-**(253)**.



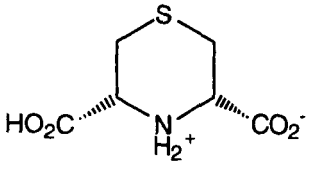
DPA **(28)**



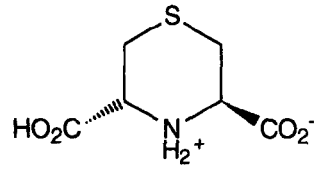
L-**(64)**



D-**(64)**



cis-**(253)**



trans-**(253)**

The thiomorpholine analogues *cis*- and *trans*-**(253)** showed very little inhibition of DHDPR. Only the *cis*-isomer had a measurable IC_{50} value, estimated at > 10 mM. A small increase in activity between respective inhibitor concentrations of 1 and 3 mM was attributed to non-enzymic interactions.

The IC_{50} value for D-**(64)** was estimated as 3.3 mM but significant variability was observed between replicate runs. The enantiomer L-**(64)**, which had proven the most potent inhibitor in our earlier studies, was similarly troublesome. Initial investigation suggested an IC_{50} value < 1 mM. However, a more detailed study again indicated problems of increasing activity with increasing inhibitor concentration. The resulting estimated IC_{50} value was 9.1 mM.

The assay system worked consistently well with DPA **(28)** to give an estimated IC_{50} value of 1.7 mM. The inhibition profile for this compound is illustrated graphically in **Figure 42**.

In conclusion to our study of this new assay we emphasise that these results constitute a preliminary investigation. A number of inconsistencies have been described. These problems may be resolved by removing

impurities from the assay mixture. This would be achieved by improved preparation of THDPA. Critically however it is stressed that the assay 'blanks' displayed consistency throughout this study.

These provisional results, when compared with those for the DHDPS/DHDPR 'coupled' assay, appear to suggest a greater specificity for the DHDPR enzyme over the DHDPS enzyme. This is indicated by significantly higher levels of inhibition for the 'coupled' system. The positive result for DPA **(28)** over L-**(64)** is opposite to the findings for the DHDPS/DHDPR coupled assay. This hints that the DHDPS enzyme is responsibile for structural/geometric tolerance around the hydrophobic portion of the metabolite, suggested in conclusion to our results detailed in **Figure 37** (section 6.04). Clearly, further investigation is required.

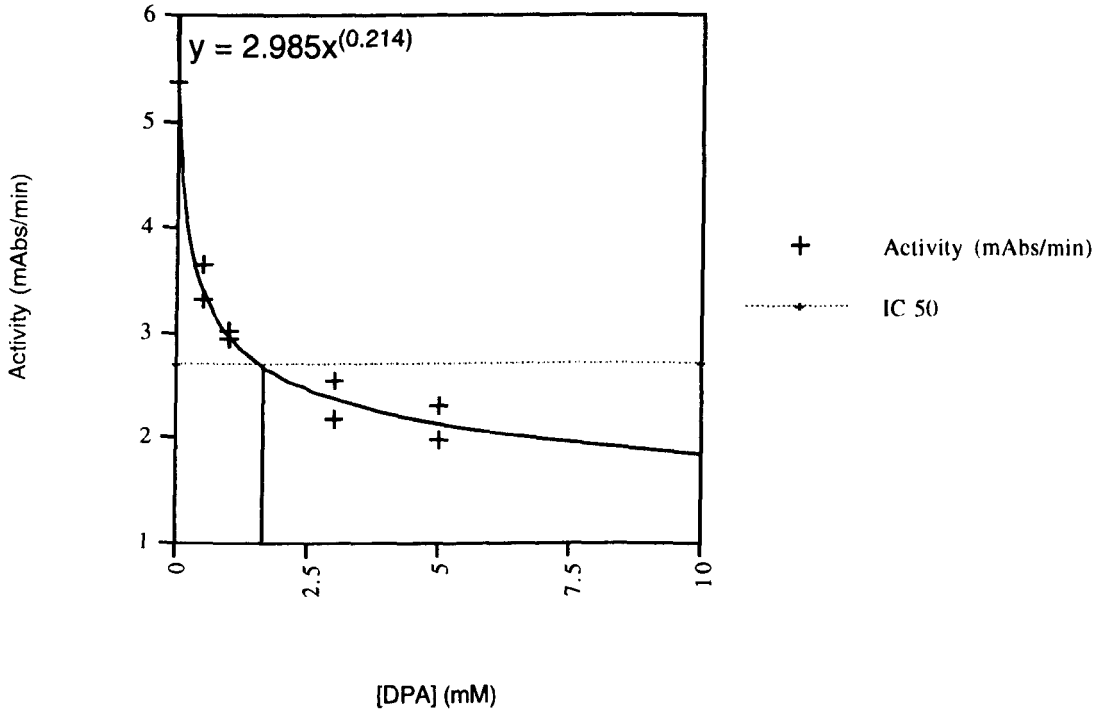


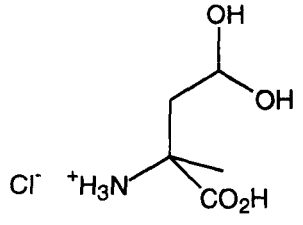
Figure 42 **'Reverse Reductase' Inhibition Profile for DPA (28)**

6.07 Biotransformations with DHDPS

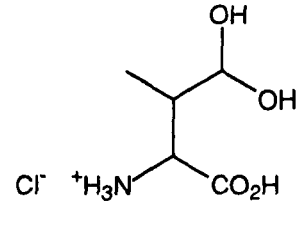
A standard DHDPS substrate assay was performed in a similar manner to the previously described DHDPS 'synthase' inhibition assay. The 'unnatural' metabolite analogue simply replaces the metabolite in the assay. The 'unnatural' metabolite was examined at different concentrations but the

relative activity was considered as that when the concentration is the same as the assay 'blank' (1 mM). Change in the UV chromophore must be given due regard.

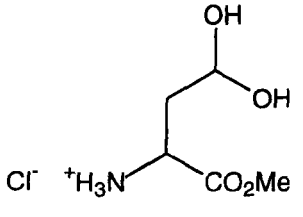
In section 5.02 we considered earlier work on pyruvate **(21)** analogues as substrates of DHDPS. In this section we concentrate on ASA **(23)** analogues. The compounds of interest are illustrated in **Figure 43**. The results quoted are relative to ASA by measure of change in absorbance with time at 270 nm. The results compare favourably with our inhibition studies on these compounds **(Figure 40)**. As with our inhibition studies the high specificity of DHDPS means that little structural variation is tolerated for metabolites.



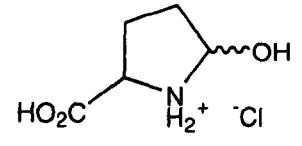
(154) 313% activity at 1 mM



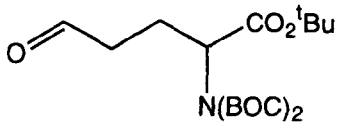
(155) 9% activity at 1 mM



(129) 14% activity at 1 mM



(205) 12% activity at 1 mM



(174) 3% activity at 1 mM

Figure 43 Relative Substrate Activity of ASA Analogues

Activity for compound **(129)** was first detected using our newly developed DHDPS/Porcine Kidney Acylase (PKA) coupled assay described in **Scheme 58**. This assay involves the replacement of the ASA analogue with an *N*-acylated equivalent and addition of 100 µl of a 1 mg/ml solution of PKA to the assay. When ASA (1 mM) is replaced by *N*-acetyl-ASA **(175)** (1 mM)

the assay has approximately 80% of the activity of a standard 'synthase' assay. This can be increased to 100% by adding extra PKA. By this method the relative activity of *N*-acetyl-ASA methyl ester **(179)** compared to *N*-acetyl-ASA **(175)** was found to be 7%.

Interestingly, of all of the substrates tested, only compound **(129)** displayed dual substrate activity with both DHDPS and DHDPR by means of a 'coupled' substrate assay. Surprisingly the relative dual activity was found to be 45%. It is not clear why greater substrate activity is displayed for the DHDPR/DHDPS system compared to DHDPS alone.

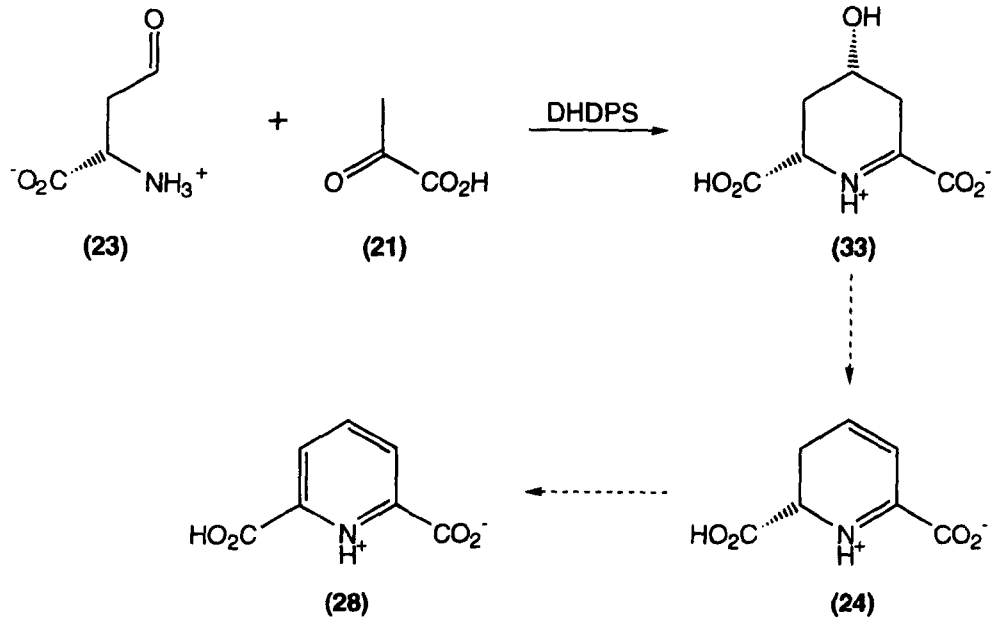
The positive result for 5-hydroxyproline hydrochloride **(205)** is encouraging. Once again it must be stressed that our preparation of this compound produced a crude product (section 4.10). There were slight variations in replicate assays and evidence (UV) of non-enzymic interactions. However, this compound displayed positive results for inhibition of DHDPS **(Figure 36)** and clear substrate activity indicated by enhancement of activity with DHDPS present. Although requiring further validation, these results indicate for the first time that the open chain equivalent, GSA **(156)**, has potential as a substrate of DHDPS. Strategies for this task were considered in detail in Chapter 4.

Most intriguing of all was the result for 2-methyl-ASA-hydrate hydrochloride **(154)**. The measured relative activity was 313% and the assay reaction proceeded to completion in just 10 minutes. This biotransformation was briefly discussed in Section 3.04. It has always been considered that in studying the 'natural' biotransformation **(Scheme 84)** at 270 nm we were monitoring the production of dipicolinic acid **(28)**. The UV absorbance maximum for the assay involving compound **(154)** was at 271 nm. Compound **(154)** cannot readily aromatise because of the methyl group in the 2-position. If the product was the expected diene **(110)** **(Scheme 36)** then the expected UV absorbance maximum would be around 240 nm. To achieve aromatisation a high energy process such as demethylation, decarboxylation or a 1,2-methyl shift would have to occur **(Scheme 36)**.

The biotransformation was briefly studied by HPLC. The system used was a 125 mm C-8 reversed-phase column, isocratically eluted with 0.1% TFA in water at 1 ml/min. The retention time (R_t) for DPA **(28)** on this system was 16.9 min. When a aliquot (50 µl) of the assay mixture for the 'natural' substrates was chromatographed a peak which may be due to the metabolic product was identified at R_t = 14.7 min. When the assay mixture containing compound **(154)** in place of ASA was chromatographed a new peak was identified at R_t = 7.5 min. This peak was not due to compound **(154)**. Attempts to isolate the unnatural product from a 100 mM scale assay by HPLC were

unsuccessful because of overlapping impurities. Further analysis under different HPLC conditions would be required for this task.

It is interesting to note that the product of the assay with compound **(154)** appears to have significantly shorter retention on the reverse phase system. This suggests that it is a more polar compound, possibly even structurally different. Perhaps the product is released from the enzyme prior to cyclisation. However, this would require an alternative release mechanism from the enzyme.



Scheme 84 The 'Natural' Biotransformation of DHDPS

Clearly there are a number of unanswered questions in this case and further research is required. In our opinion, conversion of diene **(110)** into an aromatic system **(Scheme 36)** requires too high an energy input to be feasible. The most plausible explanation is that we are simply observing the formation of the diene **(110)**. That being the case, then the same may be true for the assay with ASA. The product of the 'natural' assay would be DHDPA **(24)**. This of course does not explain why the UV absorbance maxima is at 271 nm or why the relative rate is so much higher than that observed for ASA.

DHDPA formation is necessary for the next step in the biosynthetic mechanism, hydride transfer to the 4-position. Blickling and co-workers[30] have shown that the product of DHDPS catalysed condensation of ASA and pyruvate at pH values above 9 is HDPA **(33)**. From the argument presented here and from a thermodynamic point of view we would propose that the

dehydration of HDPA to DHDPA is enzymically promoted by DHDPS and that the product of the DHDPS enzyme with its 'natural' substrates at physiological pH is indeed DHDPA.

One further investigation was performed in the field of biotransformations with DHDPS. The potential of altering enzyme specificity in an organic medium was considered. The secondary/tertiary structure of the DHDPS protein might be altered in such a medium. The study was conducted on a standard 'synthase' assay (1 ml) containing 80% volume of organic solvent. The solvents studied were: MeOH; EtOH; acetone; acetonitrile; dioxane; THF and ethylene glycol. The activities were all in the region of 5 to 10%, except for THF which had an activity of 15%. This was considered too low an activity for further investigation. With hindsight, however, we should perhaps have looked at lower proportions of organic solvent and compared activities with 'unnatural' substrates.

In conclusion to our substrate studies we can say that we are once again restricted by high substrate specificity. There are some interesting observations and further anomalies to investigate. Isolation of the enzymic product from biotransformation with compound **(154)** would be an interesting exercise and should reveal further clues to the mechanism of DHDPS. Undoubtedly, the most promising result was a suggestion of enzymic activity for 5-hydroxyproline hydrochloride **(205)**. This not only suggests possible substrate activity for GSA **(156)** but also gives general indication that the enzyme may be able to work with 7-membered rings. This along with the speculated structural/geometric tolerance away from the amino and acid groups, discussed in conclusion to section 6.05, could indicate potential for other 7-membered heterocycles as inhibitors of DHDPS.

6.08 Final Conclusions and Future Direction

In completion of this project we reflect on the challenges that we have faced, progress we have made and indicate areas of further study.

In our search for inhibitors of the DHDPS and DHDPR enzymes we were somewhat disappointed with the results. For this reason we would not recommend further study on these particular types of inhibitor. However, we have gained valuable insight into the specific nature of the enzymes. It is felt that there is some structural/geometric tolerance within the active site of DHDPS away from the electrostatic binding site.

The highly specific nature of the enzymes mean that there are few substrate analogues which display substrate activity. One could question the value of investigating DHDPS as a biotransformation catalyst where a narrow spectrum of activity is apparent. Once again we would point towards gains in mechanistic understanding for potential benefit in inhibition studies.

Most promising and pleasing were our studies on GSA as a potential substrate (Chapter 4). Despite fighting a seemingly lost cause for a appreciable period, we feel that with the positive result for 5-hydroxyproline hydrochloride **(205)**, we have presented an argument to warrant further investigation in this area. Furthermore, we would consider our novel strategy of investigating the reverse process as the best way to further this study. On a cautious note however, we would recommend further verification of the result for compound **(205)**. This would require an improved preparation of this compound.

Finally, for future inhibition studies of DHDPS we would suggest the study of six- and seven-membered heterocycles with alternative heteroatoms in appropriate positions. The two acid groups adjacent to a central polar heteroatom should be preserved for efficient electrostatic binding in the active site.

Chapter 7 Experimental

7.01 General Notes

The characterisation data are presented in accord with the *Journal of the Chemical Society, Perkin Transactions 1*, 'Instructions for authors (1996)'[187]

All melting points were measured with a Gallenkamp melting point apparatus and are uncorrected. Infra red spectra were recorded on a Philips analytical PU9800 FTIR spectrometer. Nuclear magnetic resonance spectra were recorded on a Bruker AM200 SY or WP200 SY operating at 200 MHz (δ_H) or 50 MHz (δ_C) or a Bruker AM360 SY operating at 360 MHz (δ_H) or 90 MHz (δ_C). Mass spectra (MS) were recorded on an updated AEI MS12 or MS902 spectrometers. The majority of MS were obtained using electron-impact ionisation (EI) mode. Chemical ionisation (CI) mode and fast atom bombardment (FAB) mode were used where stated.

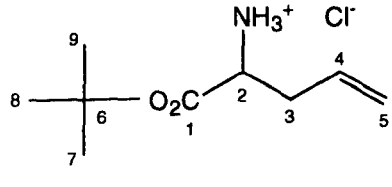
Analytical TLC was carried out using Merck silica gel 60 F_{254} aluminium backed plates of 0.25 mm thickness. Compounds were visualised using UV light or by staining with iodine or vanillin solution, with heat development. Column chromatography was carried out using Merck silica gel 60.

All solvents and reagents were of analytical grade unless otherwise stated. Aqueous solutions were freeze dried using a Christ Alpha 1-4 freeze drier. All organic solvents and reagents specified as purified or dried were treated according under the standard purification and drying techniques detailed by Perrin and Armarego.[188]

Numbering of compounds is used to aid the identification of signals in the ^{13}C and ^{1}H NMR spectra.

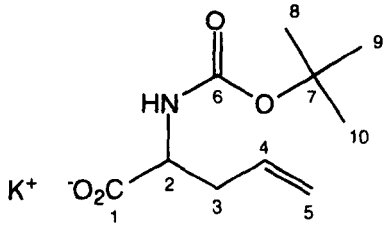
7.02 Experimental to Chapter 3

DL-Allylglycine *tert*-butyl ester hydrochloride (125)[189]



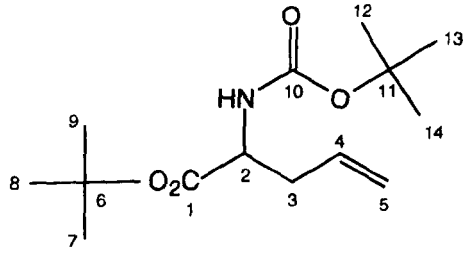
Compound **(125)** was synthesised from DL-allylglycine (3.45 g, 30.00 mmol) and isobutylene (~ 50 ml) in anhydrous dichloromethane (200 ml) and c.H_2SO_4 (4.5 ml, excess) according to the method of McKendrick.[189] The desired ester was isolated as a white solid 5.19 g (24.99 mmol, 83.3%); mp 134-136 °C (lit.[190] 134-135 °C) R_f 0.26 (free amine, in EtOAc); (Found: M^+, 172.1338; C, 52.08; H, 8.78; N, 6.73. $C_9H_{18}NO_2$ requires M, 172.1338; C, 52.05; H, 8.78; N, 6.75%).

Potassium salt of DL-*N-tert*-butoxycarbonylallylglycine (273)[56]



Compound **(273)** was synthesised from DL-allylglycine (2.30 g, 20.00 mmol), $KHCO_3$ (2.20 g, 22.00 mmol) and di-t-butyl dicarbonate (4.37 g, 20.00 mmol) in 1,4-dioxane (25 ml) and water (50 ml) according to the method of Robins and co-workers.[56] The desired carbamate was isolated as a white solid, 4.93 g (19.46 mmol, 97.3%); δ_H (200 MHz, D_2O) 1.27 (9H, s, 8, 9, 10-H_3), 2.25 (2H, m, 3-H_2), 2.77 (1H, m, 2-H), 4.98 (2H, m, 5-H_2) and 5,61 (1H, m, 4-H); δ_C (50 MHz) 28.6 (C-8, 9, 10), 37.3 (C-3), 56.6 (C-2), 81.9 (C-7), 118.9 (C-5), 134.8 (C-4), 166.5 (C-6) and 180.2 (C-1).

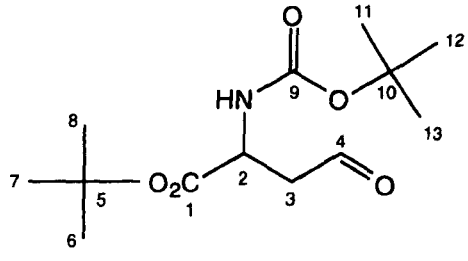
DL-*N-tert*-Butoxycarbonylallylglycine *tert*-butyl ester (116)[190]



Compound **(116)** was synthesised from compound **(125)** (4.15 g, 20.00 mmol), $KHCO_3$ (3.36 g, 40.00 mmol) and di-t-butyl dicarbonate (4.37 g, 20.00 mmol) in 1,4-dioxane (60 ml) and water (60 ml) according to the method of

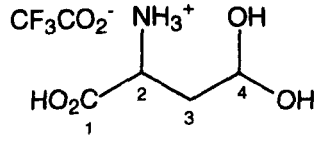
Tudor.[190] The desired carbamate was isolated as a colourless oil and did not require further purification, 5.23 g (19.27 mmol, 96.4%). New data: R_f 0.25 (10% EtOAc in hexane); (Found: M^+, 271.1802. $C_{14}H_{25}NO_4$ requires M, 271.1784).

DL-*N*-*tert*-Butoxycarbonylaspartate-β-semialdehyde *tert*-butyl ester (122)[190]



Compound **(122)** was synthesised from compound **(116)** (2.55 g, 9.40 mmol) in anhydrous dichloromethane (50 ml), treating with triethylamine (2.62 ml, 18.80 mmol), according to the method of Tudor.[190] Purification was achieved on a silica column, eluting with 33% ether in hexane, to yield the desired aldehyde as white solid, 2.10 g (7.67 mmol, 81.6%); mp 39-40 °C; R_f 0.41 (50% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 273.1556; C, 57.29; H, 8.56; N, 4.95. $C_{13}H_{23}NO_5$ requires M, 273.1576; C, 57.12; H, 8.48; N, 5.12%).

DL-Aspartate-β-semialdehyde hydrate trifluoroacetate (93)[189]

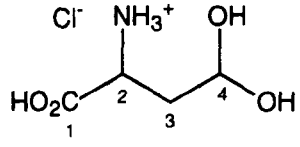


Compound **(93)** was synthesised from compound **(122)** (565 mg, 2.07 mmol) in anhydrous dichloromethane (10 ml) and analytical grade trifluoroacetic acid (10 ml) according to the method of McKendrick.[189] Removal of the solvent *in vacuo*, after 2h at 0 °C, under a nitrogen atmosphere, gave a residue which was triterated in ether at room temperature for 18 h. The desired amino acid was isolated as a cream coloured solid by filtration and dried in a desiccator (P_2O_5), 422 mg (1.69 mmol, 81.8%); mp 63-65 °C (Lit.[56] 63-65 °C); (Found: MH^+, 136.0617. $C_4H_{10}NO_4$ requires M, 136.0610).

General procedure 1:

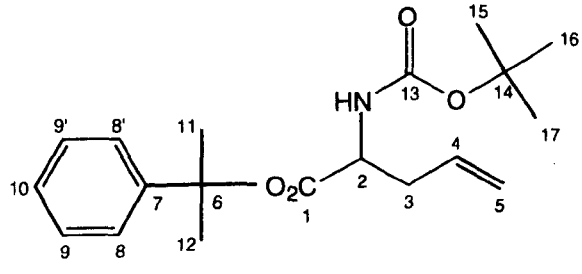
The doubly protected amino acid was stirred in anhydrous HCl in EtOAc (10 equiv.), under nitrogen, at 0 °C for 4 h. The HCl, solvent and byproducts were removed *in vacuo* and the resulting oily solid was washed with ether (3 x 20 ml). The etherial washings were decanted and the sample was dried in a desiccator (P_2O_5).

DL-Aspartate-β-semialdehyde hydrate hydrochloride (124)[85]



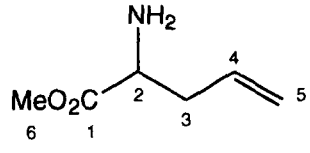
Compound **(124)** was synthesised from compound **(122)** (500 mg, 1.83 mmol) and 2.16M anhydrous HCl in EtOAc (8.47 ml, 18.30 mmol) according to **general procedure 1**. The product was isolated as a hygroscopic white solid which was dried in a desiccator (P_2O_5) and stored at -20 °C, 367 mg (2.14 mmol, 117% due to solvation); mp 79-80 °C (dec.); ν_{max}(KBr disc)/cm^{-1} 2924, 1790, 1740; δ_H (200 MHz, D_2O) 2.04 (2H, m, 3-H_2), 4.00 (1H, dd, J 7.7, 4.4 Hz, 2-H) and 5.13 (1H, t, J 5.8 Hz, 4-H); δ_C (50 MHz) 37.2 (C-3), 50.8 (C-2), 88.7 (C-4) and 172.4 (C-1). The ^{1}H NMR spectrum shows the presence of solvent in the solid lattice, which is not removed on extensive drying under high vacuum. This material was suitable for work with the DHDPS enzyme. This compound did not give a satisfactory mass spectrum.

DL-*N*-*tert*-Butoxycarbonylallylglycine cumyl ester (123)



To a solution of **(273)** (4.31 g, 20.00 mmol), DCC (4.56 g, 22.00 mmol) and DMAP (100 mg) in anhydrous dichloromethane (50 ml), under nitrogen, at room temperature, was added cumyl alcohol (2.72 g, 20.00 mmol). The resulting suspension was stirred at this temperature for 18 h. The mixture was filtered, cooled to -20 °C for 2 h and filtered again to remove the final traces of DCU byproduct. Removal of the solvent *in vacuo* yielded a yellow oil which was purified on a silica dry flash column, eluting with 5% EtOAc in hexane, to give the pure ester as a white solid, 4.83 g (14.49 mmol, 72.4%); mp 64-66 °C; R_f 0.45 (33% EtOAc in hexane); (Found: M^+-$CO_2C(Me)_2Ph$, 170.1185; C, 4.48; H, 7.83; N, 67.84. $C_{19}H_{27}NO_4$ requires M-$CO_2C(Me)_2Ph$, 170.1181; C, 68.44; H, 8.16; N, 4.20%); ν_{max}(KBr disc)/cm^{-1} 3348, 2979, 1738, 1706, 1526, 1448, 1363; δ_H (200 MHz, $CDCl_3$) 1.33 (9H, s, 15, 16, 17-H_3), 1.69 (6H, s, 11, 12-H_3), 2.47 (2H, m, 3-H_2), 4.25 (1H, m, 2-H), 4.90 (1H, br d, J 8.0 Hz, N-H), 5.05 (2H, m, 5-H_2), 5.60 (1H, m, 4-H) and 7.11 to 7.31 (5H, m, Ar-H's); δ_C (50 MHz) 28.3 (C-15, 16, 17), 28.5 (C-11, 12), 36.8 (C-3), 53.2 (C-2), 79.6 and 83.0 (C-6 and C-14), 118.9 (C-5), 124.3 and 128.2 (C-8, 8' and C-9, 9'), 127.2 (C-10), 132.4 (C-4), 145.1 (C-7) 155.5 (C-13) and 170.6 (C-1); *m/z* 170 (M^+-$CO_2C(Me)_2Ph$, 5.7%), 119, 91, 77, 70 and 57 (100%).

DL-Allylglycine methyl ester (126)[83]

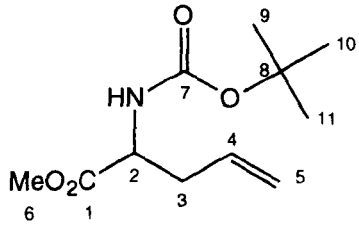


Compound **(126)** was prepared by treating a solution of DL-allylglycine (1.00g, 8.69 mmol) in methanol (30 ml) with anhydrous HCl at 0 °C until the solid dissolved. The solution was left to stand for 18 h. After this time, the solvent was removed *in vacuo*. The resulting residue was treated with 0.5 M aqueous NaOH (20 ml) and extracted into EtOAc (3 x 20 ml). The organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield the desired ester as a colourless oil, 371 mg (2.87 mmol, 33.1%); R_f 0.19 (33% EtOAc in hexane); (Found: MH^+, 130.0863. $C_6H_{12}NO_2$ requires M, 130.0868); ν_{max}($CHCl_3$)/cm^{-1} 3025, 2954, 1736, 1439, 1217; δ_H (200 MHz, $CDCl_3$) 1.57 (2H, s, N-H_2), 2.36 (2H, m, 3-H_2), 3.48 (1H, dd, J 7.1, 5.2 Hz, 2-H), 3.65 (3H, s, 6-H_3), 5.07 (2H, m, 5-H_2) and 5.68 (1H, m, 4-H); δ_C (50 MHz) 39.0 (C-3), 51.6 (C-2), 53.7 (C-6), 118.5 (C-5), 133.3 (C-4) and 175.5 (C-1); *m/z* (CI/NH_3) 130 (MH^+, 100%).

General procedure 2:

Di-*tert*-butyl dicarbonate ($(BOC)_2O$) was added to a solution of the amino acid and $KHCO_3$ in dioxane/water (1:1) and stirred for 18 h at room temperature. The solvents were removed *in vacuo* and the resulting residue was suspended in water and extracted with EtOAc. The combined organic extract was dried, filtered and concentrated.

DL-*N*-*tert*-Butoxycarbonylallylglycine methyl ester (127)[191]

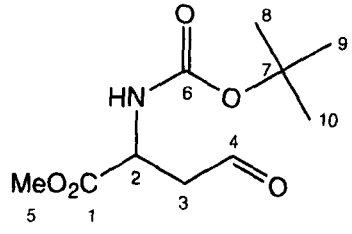


Compound **(127)** was prepared from compound **(126)** (360 mg, 2.79 mmol), $KHCO_3$ (586 mg, 5.85 mmol) and di-*tert*-butyl dicarbonate (662 mg, 3.07 mmol) in 1,4-dioxane (20 ml) and water (20 ml) according to **general procedure 2**. After reaction the solvents were removed in vacuo and the resulting residue was suspended in water (15 ml) and extracted with EtOAc (3 x 15 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated in vacuo to yield the desired carbamate as a colourless oil, 578 mg (2.52 mmol, 90.4%); R_f 0.42 (33% EtOAc in hexane); (Found: MH^+, 230.1369. $C_{11}H_{20}NO_4$ requires M, 230.1392); $\nu_{max}(CDCl_3)/cm^{-1}$ 3023, 3017, 2982, 1741, 1711, 1501; δ_H (200 MHz, $CDCl_3$) 1.38 (9H, s, 9, 10, 11-H_3), 2.44 (2H, m, 3-H_2), 3.65 (3H, s, 6-H_3), 4.30 (1H, m, 2-H), 5.06 (2H, m, 5-H_2), 5.15 (1H, br s, N-H) and 5.64 (1H, m, 4-H); δ_C (50 MHz) 28.1 (C-9, 10, 11), 36.5 (C-3), 52.0 (C-6), 52.8 (C-2), 79.6 (C-8), 118.8 (C-5), 132.3 (C-4), 155.1 (C-7) and 172.4 (C-1); *m/z* (CI/NH_3) 247 (M-NH_4^+, 100%), 230 (MH^+, 25%), 208, 191, 174.

General procedure 3:

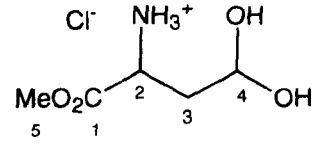
Ozone gas was bubbled through a solution of the protected amino acid in anhydrous dichloromethane at -78 °C. Triethylamine (2 equiv.) was added to the resulting blue coloured solution, a colour change to light yellow was observed and the solution was allowed to warm slowly to room temperature. The solvent was removed *in vacuo* and the resulting residue purified by column chromatography.

DL-*N-tert*-Butoxycarbonylaspartate-β-semialdehyde methyl ester (128)



Compound **(128)** was prepared by ozonolysis of compound **(127)** (540 mg, 2.36 mmol) in anhydrous dichloromethane (30 ml) according to **general procedure 3**. After 1 h, the reaction was quenched by the addition of Et_3N (2 equiv.). Purification was achieved on a silica dry flash column, eluting with 20% EtOAc in hexane, to yield the desired aldehyde as a coluorless oil, 290 mg (1.25 mmol, 53.1%); R_f 0.23 (50% EtOAc in hexane); (Found: MH^+, 232.1186. $C_{10}H_{18}NO_5$ requires M, 232.1185); ν_{max}(KBr disc)/cm^{-1} 3024,1711, 1501; δ_H (200 MHz, $CDCl_3$) 1.37 (9H, s, 8, 9, 10-H_3), 2.99 (2H, m, 3-H_2), 3.68 (3H, s, 5-H_3), 4.54 (1H, m, 2-H), 5.41 (1H, br m, N-H) and 9.66 (1H, s, 4-H); δ_C (50 MHz) 28.1 (C-8, 9, 10), 45.8 (C-3), 48.4 (C-5), 52.6 (C-2), 80.1 (C-7), 155.3 (C-6), 171.5 (C-1) and 199.4 (C-4); *m/z* (CI/NH_3) 149 ($M-NH_4^+$, 100%), 232 (MH^+, 12.6%), 219, 193, 175, 163, 149.

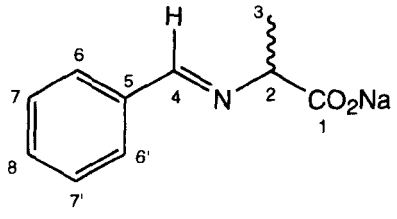
Methyl DL-aspartate-β-semialdehyde hydrate hydrochloride (129)



Compound **(128)** (151 mg, 0.653 mmol) was dissolved in anhydrous EtOAc (3 ml) and treated with 2.5M anhydrous HCl in EtOAc (2.61 ml, 6.53 mmol) according to **general procedure 1**. The product was isolated as a hygroscopic light yellow solid which was dried in a desiccator (P_2O_5) and stored at -20 °C, 112 mg (0.603 mmol, 92.4%); mp 62-64 °C (dec.); (Found: M^+, 132.0670. $C_5H_{10}NO_3$ requires M, 166.0661); ν_{max}(KBr disc)/cm^{-1} 3435, 1712, 1635, 1385; δ_H (200 MHz, D_2O) 2.07 (2H, m, 3-H_2), 3.67 (3H, s, 5-H_3), 4.13 (2H, dd, J 7.4, 4.4 Hz, 2-H) and 5.13 (1H, dd, J 6.0, 4.8 Hz, 4-H); δ_C (50 MHz) 37.2 (C-3), 50.7 (C-5), 54.5 (C-2), 88.5 (C-4) and 171.1 (C-1); *m/z* (FAB/glycerol) 132 (M^+, 22%), 115, 88. The ^1H NMR spectrum shows the

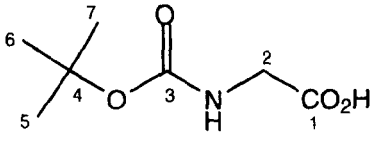
presence of solvent in the solid lattice, which is not removed on extensive drying under high vacuum. This material was suitable for work with the DHDPS enzyme.

N-Benzylimino-DL-alanine sodium salt (132)[192]



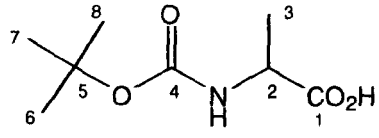
This was adapted from the method of Davis and co-workers.[131] DL-Alanine (4.45 g, 50.00 mmol) and freshly activated 4Å molecular sieves (20 g) were added to a solution of sodium ethoxide in anhydrous ethanol, prepared by adding sodium metal (1.15 g, 50.00 mmol) to anhydrous ethanol (50 ml) in a flame dried flask under a stream of nitrogen with cooling (ice bath). The resulting suspension was stirred at room temperature, under nitrogen for 18 h. The suspension was filtered to remove the molecular sieves, washing with $MgSO_4$ dried analytical grade ethanol (250 ml) to solublise the product. The filtrate was concentrated *in vacuo*, washed with $MgSO_4$ dried analytical grade acetone (2 x 50 ml) to remove unreacted benzaldehyde, filtered and dried in a desiccator (P_2O_5) to yield the desired imine as a white solid, 8.57 g (43.05 mmol, 86.1%); (Found: MH^+, 177.0781. $C_{10}H_{10}NO_2$ requires MH, 177.0790); ν_{max}(KBr disc)/cm^{-1} 3423 (br), 2978, 2867, 1642, 1595; δ_H (200 MHz, $CDCl_3$) 1.37 (3H, d, J 6.9 Hz, 3-H_3), 3.90 (1H, q, J 6.8 Hz, 2-H), 7.31 and 7.69 (5H, 2 x m, Ar-H) and 8.22 (1H, s, 4-H); δ_C (50 MHz) 21.0 (C-3), 72.7 (C-2), 129.5, 129.6 and 131.8 (Ar C-H), 137.6 (C-5), 163.2 (C-4) and 180.7 (C-1); *m/z* (FAB) 222 ($M+Na^+$, 100%), 176 (M^+, 7.8%).

N-tert-Butoxycarbonylglycine (119)[193]



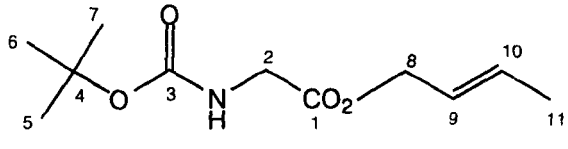
Compound **(119)** was synthesised from glycine (11.26 g, 150.0 mmol), $KHCO_3$ (24.88 g, 180.00 mmol) and di-*tert*-butyl dicarbonate (36.01 g, 165.0 mmol) in 1,4-dioxane (100 ml) and water (100 ml) according to **general procedure 2**. After reaction the mixture was washed with ether (100 ml) and the aqueous portion was acidified to pH 2 with 2M HCl (aq) before extraction with EtOAc (3 x 100 ml). The combined organic extract was dried (Na_2SO_4), filtered and concentrated in vacuo to yield the desired carbamate as a white solid, 26.02 g (148.5 mmol, 99.0%); mp 88-89 °C (Lit.[193] 87-88 °C).

L-*N-tert*-Butoxycarbonylalanine (133)[194]



Compound **(133)** was synthesised from alanine (13.36 g, 150.0 mmol), $KHCO_3$ (24.88 g, 180.0 mmol) and di-*tert*-butyl dicarbonate (36.01 g, 165.0 mmol) in 1,4-dioxane (100 ml) and water (100 ml) according to **general procedure 2**. After reaction the mixture was washed with ether (100 ml) and the aqueous portion was acidified to pH 2 with 2M HCl (aq) before extraction with EtOAc (3 x 100 ml). The combined organic extract was dried (Na_2SO_4), filtered and concentrated in vacuo to yield the desired carbamate as a white solid, 27.59 g (145.8 mmol, 97.2%); mp 79-81 °C (Lit.[194] 80-82 °C).

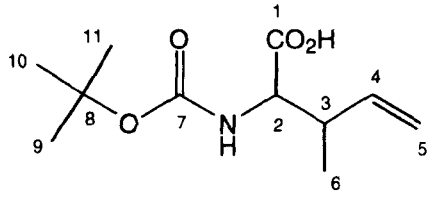
N-tert- Butoxycarbonylglycine crotyl ester (120)[189]



To a solution of compound **(119)** (7.01 g, 40.00 mmol), DCC (8.25 g, 40.00 mmol) and DMAP (100 mg) in anhydrous dichloromethane (100 ml), under nitrogen, at room temperature, was added crotyl alcohol (2.88 g, 40.00 mmol). The resulting suspension was stirred at this temperature for 18 h. The mixture was filtered, cooled to -20 °C for 2 h and filtered again to remove the final traces of DCU byproduct. The filtrate was washed with sat. $NaHCO_3$ (aq) (2 x 100 ml) and the organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a yellow oil. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to give the pure ester as a

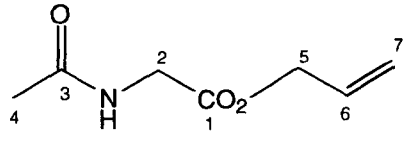
colourless oil, 7.83 g (34.16 mmol, 85.4%); R_f 0.40 (33% EtOAc in hexane); The IR and low resolution mass spectra were consistent with the literature;[189] (Found: M^+-$CH_2CH{=}CH(Me)$, 174.0772. $C_7H_{12}NO_4$ requires M, 174.0766); δ_H (200 MHz, $CDCl_3$) 1.39 (9H, s, 5, 6, 7-H_3), 1.67 (3H, dd, J 6.3, 1.3 Hz, 11-H_3), 3.85 (2H, d, J 5.6 Hz, 2-H_2), 4.52 (2H, dd, J 6.5, 0.9 Hz, 8-H_2), 5.10 (1H, br-s, N-H), 5.52 (1H, m, 9-H) and 5.75 (1H, m, 9-H); δ_C (50 MHz) 17.7 (C-11), 28.2 (C-5, 6, 7), 42.3 (C-2), 65.9 (C-8), 79.8 (C-4), 124.4 (C-10), 132.1 (C-9), 155.8 (C-3) and 170.1 (C-1).

DL-2-(*tert*-Butoxycarbonylamino)-3-methyl-4-pentenoic acid (134)[189]



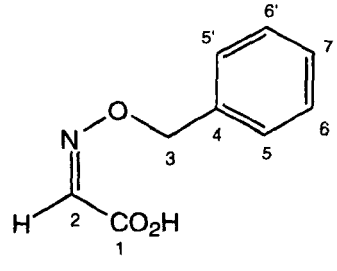
Compound **(134)** was synthesised according to the method of McKendrick.[189] Compound **(120)** (3.44 g, 15.00 mmol) in anhydrous THF (15 ml) was added to diisopropylamine (3.19 g, 31.50 mmol) and 1.6M BuLi in hexanes (19.69 ml, 31.5 mmol) in anhydrous THF (30 ml). Subsequent addition of TMS chloride (3.42 g, 31.50 mmol) and methanol (15 ml), followed by the recommended 'work up' procedure yielded a diastereomeric mixture the desired amino acids as a yellow solid, 1.26 g (5.49 mmol, 36.6%); R_f 0.23 (50% EtOAc, 1% AcOH in hexane); δ_H (200 MHz, $CDCl_3$) 1.02 (3H, d, J 7.0 Hz, 6-H_3), 1.37 (9H, s, 9, 10, 11-H_3), 2.65 (1H, m, 3-H), 4.29 (1H, dd, J 8.8, 4.6 Hz, 2-H), 5.04 (2H, m, 5-H_2), 5.69 (1H, m, 4-H), 8.65 (1H, br s, N-H) and 10.9 (1H, ,br s, CO_2-H); δ_C (50 MHz) 15.0 (C-6), 28.3 (C-9, 10, 11), 40.3 (C-3), 57.2 (C-2), 80.1 (C-8), 116.2 (C-5), 138.5 (C-4), 155.5 (C-7) and 176.2 (C-1), (signals visible for the minor diastereoisomer 14.7 (C-4), 39.9 (C-2) and 58.8 (C-3). Estimated diastereomeric ratio of 1:9 in favour of *anti*-diastereomer.

N-Acetylglycine allyl ester (136)



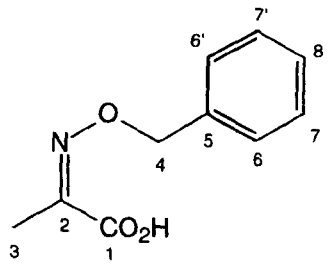
To a solution of *N*-acetylglycine (5.85 g, 50.00 mmol), DCC (10.32 g, 50.00 mmol) and DMAP (200 mg) in anhydrous dichloromethane (50 ml), under nitrogen, at room temperature, was added allyl alcohol (2.90 g, 50.00 mmol). The resulting suspension was stirred at this temperature for 18 h. The mixture was filtered, cooled to -20 °C for 2 h and filtered again to remove the final traces of DCU byproduct. Removal of the solvent *in vacuo* yielded a dark yellow oil which was purified on a silica dry flash column, eluting with 25% EtOAc in hexane, to give the pure ester as a lightly coloured solid, 6.31 g (40.15 mmol, 80.3%); mp 50-51 °C; R_f 0.32 (EtOAc); (Found: M^+, 157.0734; C, 54.62; H, 7.37; N, 9.15. $C_7H_{11}NO_3$ requires M, 157.0739; C, 53.49; H, 7.37; N, 8.91%); ν_{max}(KBr disc)/cm^{-1} 3266, 3086, 1738, 1644, 1565; δ_H (200 MHz, $CDCl_3$) 1.74 (3H, s, 4-H_3), 3.73 (2H, d, J 5.5 Hz, 5-H_2), 4.33 (2H, m, 2-H_2), 5.00 (2H, m, 7-H_2), 5.60 (1H, m, 6-H) and 6.71 (1H, br s, N-H); δ_C (50 MHz) 21.0 (C-4), 39.6 (C-2), 64.2 (C-5), 117.1 (C-7), 129.8 (C-6), 168.1 and 169.2 (C-1 and C-3); *m/z* 157 (M^+, 2.1%), 116, 100, 72 (100%) and 42.

α-Benzyloxoiminoglyoxylic acid (141)[135]



Compound **(141)** was synthesised from glyoxylic acid (3.00 g, excess) in water (50 ml) and *O*-benzylhydroxylamine hydrochloride (4.79 g, 30.00 mmol) in water (250 ml) according to the method of Ottenheijm and co-workers.[135] After extraction into dichloromethane (2 x 100 ml), the organic extract was dried (Na_2SO_4), filtered and concentrated *in vacuo* to yield the desired *O*-alkylimine as a white solid, 5.33 g (29.72 mmol, 99.0%); mp 74-75 °C (Lit.[135] 78-80 °C); (Found: M^+-N=C(H)CO_2H, 107.0496; C, 60.09; H, 4.85; N, 7.73. C_7H_7O requires M, 107.0497; C, 60.32; H, 5.06; N, 7.82%); ν_{max}(KBr disc)/cm^{-1} 3420 (br), 3033, 2947, 1712, 1679, 1590, 1438; δ_H (200 MHz, $CDCl_3$) 5.29 (2H, s, 3-H_2), 7.36 (5H, s, Ar-H), 7.55 (1H, s, 2-H) and 11.80 (1H, br s, CO_2-H); δ_C (50 MHz) 78.4 (C-3), 128.6 (C-2), 135.6 (C-4), 140.9 (br) (Ar C-H) and 166.5 (C-1); *m/z* 179 (M^+, 0.2%), 108, 91 (100%), 77, 65, 51.

α-Benzyloxoiminopyruvic acid (142)[135]

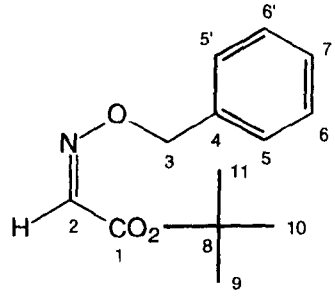


Compound **(142)** was synthesised from sodium pyruvate (3.50 g, excess) in water (50 ml) and *O*-benzylhydroxylamine hydrochloride (4.79 g, 30.00 mmol) in water (150 ml) according to the method of Ottenheijm.[135] After extraction into dichloromethane (2 x 100 ml), the organic extract was dried (Na_2SO_4), filtered and concentrated *in vacuo* to yield the desired *O*-alkylimine as a white solid, 5.72 g (29.61 mmol, 98.7%); mp 77-79 °C (Lit.[135] 83-85 °C); (Found: M^+, 193.0721; C, 62.29; H, 5.62; N, 7.43. $C_{10}H_{11}NO_3$ requires M, 173.0738; C, 62.16; H, 5.74; N, 7.25%); ν_{max}(KBr disc)/cm^{-1} 3027, 2973, 2937, 2589, 1697, 1609, 1450; δ_H (200 MHz, $CDCl_3$) 2.09 (3H, s, 3-H_3), 5.31 (2H, s, 4-H_2), 7.38 (5H, s, Ar-H) and 10.00 (1H, s, CO_2-H); δ_C (50 MHz) 10.7 (C-3), 78.0 (C-4), 128.3, 128.5, 128.6 (Ar C-H), 136.1 (C-5), 148.7 (C-2) and 165.5 (C-1); *m/z* 193 (M^+, 0.1%), 108, 91 (100%), 77, 65, 51.

General procedure 4:

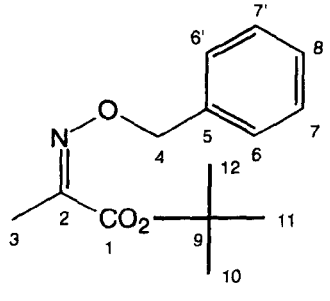
Isobutylene (20 to 50 ml, excess) was added, via a cold finger condenser, to a suspension of the amino acid in anhydrous dichloromethane at -78 °C. Concentrated H_2SO_4 was added and the suspension stirred at -78 °C for 8 h and then at room temperature for a further 10 h. After this time, the resulting solution was basified to pH 9 with sat. $NaHCO_3$ and the two phase mixture was stirred rapidly for 30 min. The organic portion was then washed with brine (3 x 100 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo*.

α-Benzyloxoiminoglyoxylate *tert*-butyl ester (143)[134]



Compound **(143)** was synthesised from compound **(141)** (2.00 g, 11.16 mmol) and isobutylene (~ 20 ml) in anhydrous dichloromethane (100 ml) and c.H_2SO_4 (2 ml) according to **general procedure 4**. Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then 5% EtOAc in hexane, to yield the desired ester as a colourless oil, 2.39 g (10.16 mmol, 91.0%); R_f 0.35 (10% EtOAc in hexane); (Found: M^+, 235.1207. $C_{13}H_{17}NO_3$ requires M, 235.1208); ν_{max}($CHCl_3$ solution)/cm^{-1} 3006, 2980, 1728, 1710, 1598; δ_H (200 MHz, $CDCl_3$) 1.53 (9H, s, 9, 10, 11-H_3), 5.27 (2H, s, 3-H_2), 7.25 to 7.39 (5H, m, Ar-H_3 and 7.47 (1H, s, 2-H); δ_C (50 MHz) 28.0 (C-9, 10, 11), 77,8 (C-3), 82.6 (C-8), 128.4, 128.5, 128.6 (Ar C-H), 136.1 (C-4), 142.4 (C-2) and 160.9 (C-1); *m/z* 235 (M^+, 0.2%), 179, 162, 91 (100%), 77, 57; GC (CP Sil 5CB; 30 m x 0.32 mm i.d. x 0.12 μm) 14.84 min.

α-Benzyloxoiminopyruvate *tert*-butyl ester (144)

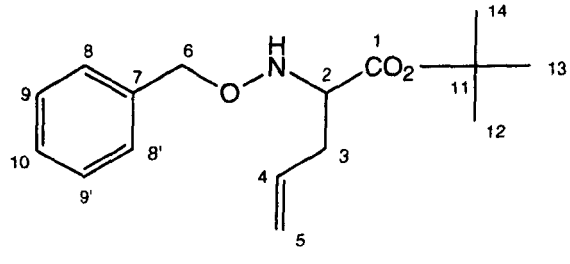


Compound **(144)** was synthesised from compound **(142)** (5.80 g, 30.00 mmol) and isobutylene (~ 30 ml) in anhydrous dichloromethane (200 ml) and c.H_2SO_4 (1.5 ml) according to **general procedure 4**. Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then 5% EtOAc in hexane, to yield the desired ester as a colourless oil, 6.67 g (26.75 mmol, 89.2%); R_f 0.37 (10% EtOAc in hexane); (Found: M^+, 249.1357. $C_{14}H_{19}NO_3$ requires M, 249.1364); ν_{max}(thin film)/cm^{-1} 2980, 2935, 1613, 1452, 1370, 1335; δ_H (200 MHz, $CDCl_3$) 1.47 (9H, s, 10, 11, 12-H_3), 1.96 (3H, s, 3-H_3), 5.21 (2H, s, 4-H_2) and 7.21 to 7.32 (5H, s, Ar-H); δ_C (50 MHz) 11.6 (C-3), 28.0 (C-10, 11, 12), 77.3 (C-4), 82.2 (C-9), 128.1, 128.3, 128.4 (Ar C-H), 136.8 (C-5), 150.3 (C-2) and 162.7 (C-1); *m/z* 249 (M^+, 0.1%), 193, 176, 91 (100%), 77, 57.

General procedure 5:

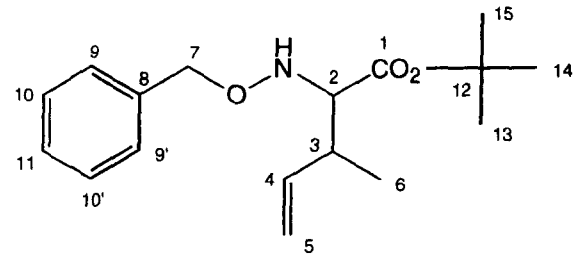
This was adapted from the communications of Hanessian and Yang[134] and Luche and co-workers,[133] regarding the zinc induced coupling of allyl bromides and aldehydes, ketones or imines. The allyllic bromide (1.4 equiv.) was added via a Gilson pipette to a solution of the *O*-alkyloximine t-butyl ester in sat. NH_4Cl (aq) and THF (5 : 1 respectively). Freshly activated zinc dust (1.8 equiv.) was then added in small portions to the stirred two phase solution at room temperature. The reaction was monitored by TLC and was generally complete within 5 min. The suspension was filtered to remove the zinc residues, extracted with EtOAc (3 x 100 ml) and the organic extract was dried (Na_2SO_4), filtered and concentrated *in vacuo* to a colourless oil.

DL-*N*-Benzyloxoallylglycine *tert*-butyl ester (145)



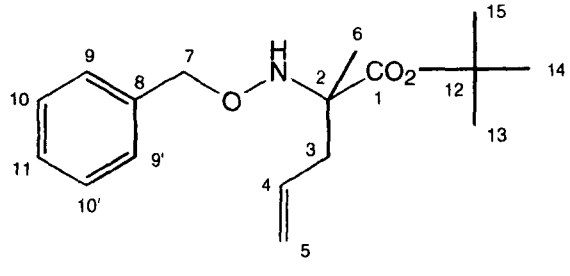
Compound **(145)** was synthesised from compound **(143)** (1.18 g, 5.01 mmol), freshly distilled allyl bromide (595 µl, 7.00 mmol) and freshly activated zinc powder (588 mg, 9.00 mmol) in sat. NH_4Cl (aq) (20 ml) and THF (4 ml) according to general **procedure 5**, to yield the desired amino acid as a colourless oil, 1.37 g (4.94 mmol, 98.6%); R_f 0.26 (10% EtOAc in hexane); (Found: M^+, 277.1676. $C_{16}H_{23}NO_3$ requires M, 277.1677); ν_{max}(thin film)/cm^{-1} 2974, 2927, 1729, 1450, 1367; δ_H (360 MHz, $CDCl_3$) 1.48 (9H, s, 12, 13, 14-H_3), 2.32 (2H, m, 3-H_2), 3.58 (1H, t, J 6.7 Hz, 2-H), 4.72 (2H, s, 6-H_2), 5.08 (2H, m, 5-H_2), 5.71 (1H, m, 4-H), 5.77 (1H, br s, N-H) and 7.31 (5H, m, Ar-H); δ_C (50 MHz) 28.1 (C-12, 13, 14), 33.9 (C-3), 63.6 (C-2), 76.0 (C-6), 81.6 (C-11), 117.9 (C-5), 127.7 (C-4), 128.2, 128.3, 133.2 (Ar C-H), 137.7 (C-7) and 172.4 (C-1); *m/z* 277 (M^+, 0.1%), 221, 176, 108, 91 (100%), 77, 57.

DL-2-(Benzyloxoamino)-3-methyl-4-pentenoate *tert*-butyl ester (147)



Compound **(147)** was synthesised from compound **(143)** (5.10 g, 21.67 mmol), crotyl bromide (85% pure) (3.67 ml, equivalent to 30.34 mmol) and freshly activated zinc powder (1.98 g, 30.34 mmol) in sat. NH_4Cl (aq) (20 ml) and THF (4 ml) according to general **procedure 3**. A further 1 g of powdered zinc was added after 30 min and the mixture was left for another 10 min before 'work up' to yield the desired amino acid as a colourless oil, 6.25 g (21.45 mmol, 99.0%); R_f 0.36 (10% EtOAc in hexane); (Found: MH^+, 292.1918 $C_{17}H_{25}NO_3$ requires MH, 292.1913); ν_{max}(thin film)/cm^{-1} 2977, 2932, 1730, 1454, 1368, 1156; δ_H (200 MHz, $CDCl_3$) 0.95 (2H, d, J 6.9 Hz, 6-H_3), 1.39 (9H, s, 13, 14, 15-H_3), 2.31 (1H, m, 3-H), 3.31 (1H, ,br m, 2-H), 4.60 (2H, s, 7-H_2), 4.94 (2H, m, 5-H_2), 5.59 (1H, m, 4-H), 5.86 (1H, br s, N-H) and 7.22 (5H, m, Ar-H). Complementary signals visible for minor diastereoisomer at δ = 0.96, 1.41 and 4.62 ppm; δ_C (50 MHz) 17.0 (C-6), 28.1 (C-13, 14, 15), 38.8 (C-3), 68.3 (C-2), 75.7 (C-7), 81.5 (C-12), 115.6 (C-5), 127.6, 128.2, 128.4 (Ar C-H), 137,9 (C-8), 139.2 (C-4) and 172.3 (C-1). Complementary signals for minor diastereoisomer at δ = 16.8, 38.4, 68.6, 75.9, 81.7, 127.7, 128.2, 128.5 and 172.4 ppm; *m/z* (CI) 292 (MH^+, 100%), 236, 197, 190, 146, 108, 91; GC (CP Sil 5CB; 30 m x 0.32 mm i.d. x 0.12 μm) major diastereoisomer 18.84 min, minor diastereoisomer, 19.06 min.

DL-2-(Benzyloxoamino)-2-methyl-4-pentenoate *tert*-butyl ester (146)



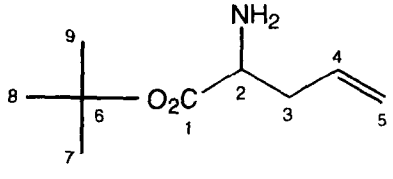
Compound **(146)** was synthesised from compound **(144)** (6.31 g, 25.31 mmol), freshly distilled allyl bromide (3.01, 35.43 mmol) and freshly activated zinc powder (2.98 g, 45.56 mmol) in sat. NH_4Cl (aq) (125 ml) and THF (25 ml) according to general **procedure 5**, to yield the desired amino acid as a colourless oil, 6.98 g (23.95 mmol, 94.6%); R_f 0.32 (10% EtOAc in hexane); (Found: M^+, 291.1819. $C_{17}H_{25}NO_3$ requires M, 291.1834); ν_{max}(thin film)/cm^{-1} 2979, 2932, 1731, 1453, 1369; δ_H (360 MHz, $CDCl_3$) 1.28 (3H, s, 6-H_3), 1.47 (9H, s, 13, 14, 15-H_3), 2.37 (2H, m, 3-H_2), 4.74 (2H, d, J 1.3 Hz, 7-H_2), 5.11 (2H, m, 5-H_2), 5.77 (1H, m, 4-H), 5.99 (1H, br s, N-H) and 7.30 (5H, m, Ar-H); δ_C (50 MHz) 19.7 (C-6), 28.0 (C-13, 14, 15), 39.9 (C-3), 65.4 (C-2), 76.6 (C-7), 81.2 (C-12), 118.6 (C-5), 127.6, 127.9, 128.2 (Ar C-H), 132.7 (C-4), 137.9 (C-8) and 173.7 (C-1); *m/z* 291 (M^+, 0.1%), 190, 91 (100%), 77, 57.

General procedure 6:

Adapted from the method of Cicchi *et al.*[137] and that of Nitta and Kobayashi,[136] the *N*-alkyloxide was dissolved in acetonitrile and water (15 : 1 respectively) and $Mo(CO)_6$ (0.7 equiv.) was added. The reaction vessel was degassed three times, filled with nitrogen and heated to reflux for 3 h. The $Mo(CO)_6$ dissolved on heating and a colour change from colourless to yellow to black was observed within 1 h. After cooling the solvents were removed *in vacuo* to leave a black coloured residue which was suspended in ether (50 ml) and extracted with 1M HCl (aq) (3 x 50 ml). The combined aqueous extract was basified to pH > 12 with 40% NaOH (aq) and extracted with EtOAc (3 x 200 ml). The combined EtOAc extract was dried ($MgSO_4$), filtered and concentrated to yield the desired amine as a lightly coloured oil.

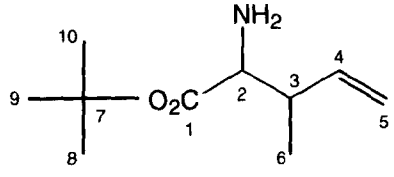
Alternatively, after reflux the cooled reaction mixture was filtered through a silica plug flushing with an excessive volume of 1% c.NH_3 (aq) in EtOAc. The filtrate was monitored in portions by TLC. The combined washings were concentrated *in vacuo* with ethanol and the product was further purified on a silica dry flash column, eluting with 1% c.NH_3 (aq) in EtOAc to yield the pure amine as a darkly coloured oil.

DL-Allylglycine *tert*-butyl ester (148)



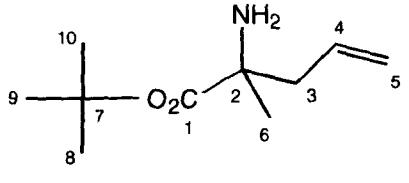
Compound **(148)** was synthesised from compound **(145)** (1.35 g, 4.87 mmol) and $Mo(CO)_6$ (899 mg, 3.41 mmol) in acetonitrile (60 ml) and water (4 ml) according to **general procedure 6**. The reaction mixture was 'worked up' using the chromatographic method described, to yield the desired amine as a dark coloured oil, 356 mg (2.08 mmol, 42.7%); R_f 0.26 (EtOAc); (Found: MH^+, 172.1338. $C_9H_{17}NO_2$ requires MH, 172.1338); δ_H (360 MHz, $CDCl_3$) 1.33 (9H, s, 7, 8, 9-H_3), 1.91 (2H, br s, N-H_2), 2.25 (2H, m, 3-H_2) 3.26 (1H, dd, J 6.8, 5.3 Hz, 2-H), 4.99 (2H, m, 5-H_2) and 5.59 (1H, m, 4-H). The IR, ^{13}C NMR and low resolution mass spectra were consistent with that of compound **(125)** (hydrochloride salt).

DL-3-Methylallylglycine *tert*-butyl ester (150)



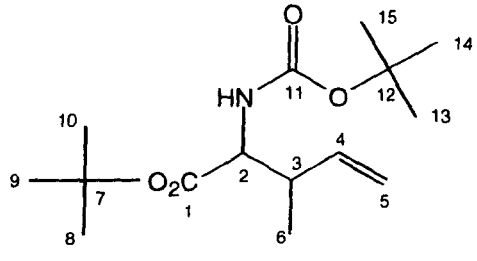
Compound **(150)** was synthesised from compound **(147)** (874 mg, 3.00 mmol) and $Mo(CO)_6$ (554 mg, 2.10 mmol) in acetonitrile (45 ml) and water (3 ml) according to **general procedure 6**. The reaction mixture was 'worked up' using the acid extraction method described, to yield the desired amine as a yellow oil 430 mg (2.32 mmol, 77.4%); R_f 0.14 (EtOAc); (Found: MH^+, 186.1496. $C_{10}H_{19}NO_2$ requires MH, 186.1494); $\nu_{max}(CHCl_3)/cm^{-1}$ 3020, 2980, 2934, 1723, 1369, 1216, 1155; δ_H (200 MHz, $CDCl_3$) 0.93 (3H, d, J 7.0 Hz, 6-H_3), 1.39 (9H, s, 8, 9, 10-H_3), 2.00 (2H, br s, N-H_2), 2.49 (1H, m, 3-H), 3.26 (1H, d, J 4.6 Hz, 2-H), 5.00 (2H, m, 5-H_2) and 5.67 (1H, m, 4-H). Complementary signals visible for minor diastereoisomer at δ 1.01 and 3.14 ppm; δ_C (50 MHz) 13.9 (C-6), 27.9 (C-8, 9, 10), 41.5 (C-3), 58.5 (C-2), 80.9 (C-7), 115.3 (C-5), 139.9 (C-4) and 173.6 (C-1). Complementary signals visible for minor diastereoisomer at δ 16.4, 27.8, 41.7, 59.1, 116.0, 138.6 and 173.7 ppm; *m/z* (CI) 186 (MH^+, 100%), 147, 130, 117, 96, 84.

DL-2-Methylallylglycine *tert*-butyl ester (149)



Compound **(149)** was synthesised from compound **(146)** (2.91 g, 10.00 mmol) and $Mo(CO)_6$ (1.85 g, 7.00 mmol) in acetonitrile (150 ml) and water (10 ml) according to **general procedure 6**. The reaction mixture was 'worked up' using the chromatographic method described, to yield the desired amine as a yellow oil, 890 mg (4.80 mmol, 48.0%); R_f 0.11 (EtOAc); (Found: M^+-O^{t}Bu, 112.0762.$C_{10}H_{19}NO_2$ requires M-O^{t}Bu, 172.0762); ν_{max}(thin film)/cm^{-1} 2978, 2933, 1726, 1457, 1369, 1143; δ_H (200 MHz, $CDCl_3$) 1.22 (3H, s, 6-H_3), 1.39 (9H, s, 8, 9, 10-H_3), 1.75 (2H, br s, N-H), 2.16 and 2.43 (2H, 2 x m, 3-H_2), 5.07 (2H, m, 5-H_2) and 5.66 (1H, m, 4-H); δ_C (50 MHz) 26.3 (C-6), 27.9 (C-8, 9, 10), 45.1 (C-3), 57.4 (C-2), 80.8 (C-7), 119.0 (C-5), 133.0 (C-4) and 176.3 (C-1); *m/z* (CI) 186 (M^+, 46.6%), 158, 147, 129, 112, 96, 84.

DL-*N*-*tert*-Butoxycarbonyl-3-methylallylglycine *tert*-butyl ester (135)



Method 1

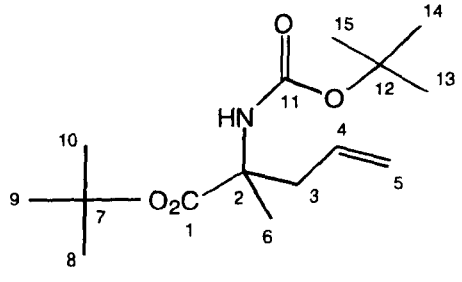
To a solution of compound **(134)** (1.26 g, 5.49 mmol), DCC (1.78 g, 8.64 mmol) and DMAP (10 mg) in anhydrous dichloromethane (30 ml), under nitrogen, at room temperature, was added *tert*-butyl alcohol (640 mg, 8.64 mmol). The resulting suspension was stirred at this temperature for 18 h. The mixture was filtered, cooled to -20 °C for 2 h and filtered again to remove the final traces of DCU byproduct. The filtrate was washed with sat. $NaHCO_3$ (aq) (2 x 100 ml) and the organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a brown oil. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to give the desired ester as a lightly coloured oil, 680 mg (2.38 mmol, 43.4%).

Method 2

Compound **(135)** was synthesised from compound **(150)** (1.75 g, 9.45 mmol), $KHCO_3$ (1.04 g, 10.40 mmol) and di-*tert*-butyldicarbonate (2.27 g, 10.40 mmol) in 1,4-dioxane (50 ml) and water (50 ml) according to **general**

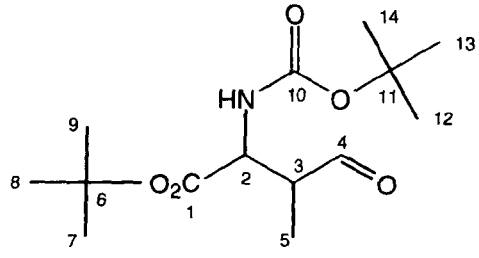
procedure 2. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired carbamate as a colourless oil, 2.28g (7.99 mmol, 84.5%); R_f 0.46 (20% EtOAc in hexane); (Found: M^+, 285.1935. $C_{15}H_{27}NO_4$ requires M, 285.1940); ν_{max}(thin film)/cm^{-1} 3372, 2975, 2934, 1711, 1513; δ_H (200 MHz, $CDCl_3$) 0.98 (3H, d, J 6.9 Hz, 6-H_3), 1.37 and 1.39 (18H, 2 x s, 8, 9, 10- and 13, 14, 15-H_3), 2.55 (1H, m, 3-H), 4.11 (1H, m, 2-H), 4.86 (1H, br m, N-H), 5.00 (2H, m, 5-H_2) and 5.65 (1H, m, 4-H). Complementary signal visible for minor diastereoisomer at δ 1.01 ppm; δ_C (50 MHz) 15.2 (C-6), 28.0 and 28.2 (C-8, 9, 10 and C-13, 14,15), 40.8 (C-3), 57.6 (C-2), 79.5 and 81.7 (C-7 and C-12), 115.6 (C-5), 138.9 (C-4), 155.4 (C-11) and 170.6 (C-1). Complementary signals visible for minor diastereoisomer at δ 15.9, 40.3, 57.9,81.8, 116.2, 137.9, 155.6 and 170.7 ppm; *m/z* 285 (M^+, 0.5%), 229, 186, 174, 158, 146, 130, 102, 83, 57 (100%).

DL-*N*-*tert*-Butoxycarbonyl-2-methylallylglycine *tert*-butyl ester (151)



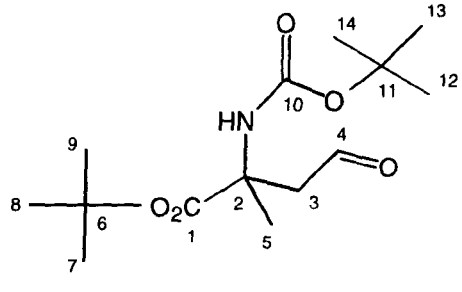
Compound **(151)** was synthesised from compound **(149)** (750 mg, 4.05 mmol), $KHCO_3$ (410 mg, 4.05 mmol) and di-*tert*-butyldicarbonate (1.09 g, 5.00 mmol) in 1,4-dioxane (20 ml) and water (20 ml) according to **general procedure 2**. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired carbamate as a colourless oil, 558 mg (1.96 mmol, 48.3%); R_f 0.29 (10% EtOAc in hexane); (Found: M^+, 285.1941. $C_{15}H_{27}NO_4$ requires M, 285.1940); ν_{max}(thin film)/cm^{-1} 3426, 2980, 2934, 1716, 1494; δ_H (200 MHz, $CDCl_3$) 1.36 and 1.39 (18H, 2 x s, 8, 9, 10- and 13, 14, 15-H_3), 1.43 (3H, s, 6-H_3), 2.44 (2H, dd, J 13.8, 7.2 Hz, 3-H_2), 5.00 and 5.07 (2H, 2 x m, 5-H_2), 5.20 (1H, br s, N-H) and 5.61 (1H, m, 4-H); δ_C (50 MHz) 23.2 (C-6), 27.8 and 28.2 (C-8, 9, 10 and C-13, 14, 15), 41.1 (C-3), 59.1 (C-2), 78.9 and 81.4 (C-7 and C-12), 118.8 (C-5), 132.6 (C-4), 154.1 (C-11) and 173.0 (C-1); *m/z* (CI) 286 (MH^+, 100%), 230, 186, 130, 84, 58.

DL-*N*-*tert*-Butoxycarbonyl-3-methylaspartate-β-semialdehyde *tert*-butyl ester (153)



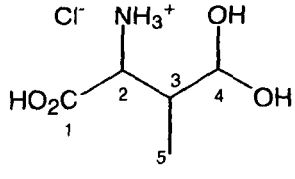
Compound **(135)** (296 mg, 1.04 mmol) was ozonised for 60 min in anhydrous dichloromethane (30 ml) and the resulting ozonide quenched with triethylamine (290 µl, 2.08 mmol), according to **general procedure 3**. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired aldehyde as a colourless oil, 175 mg (0.61 mmol, 58.6%); R_f 0.33 (33% EtOAc in hexane); (Found: MH^+, 288.1807; C, 58.55; H, 8.59; N, 4.82. $C_{14}H_{25}NO_5$ requires MH, 288.1811; C, 58.51; H, 8.77; N, 4.87%); ν_{max}(thin film)/cm^{-1} 2982, 2937, 1713, 1601, 1499, 1370; δ_H (200 MHz, $CDCl_3$) 1.04 (3H, d, J 7.2 Hz, 5-H_3), 1.38 and 1.41 (18H, 2 x s, 7, 8, 9- and 12, 13, 14-H_3), 2.97 (1H, m, 3-H), 4.58 (1H, dd, J 7.9, 3.7 Hz, 2-H), 5.32 (1H, br s, J 7.7 Hz, N-H) and 9.63 (1H, s, 4-H). Complementary signals for minor diastereoisomer at δ 0.91, 1.36, 2.79, 4.67 5.22 and 9.68 ppm; δ_C (50 MHz) 8.50 (C-5), 27.7 and 28.2 (C-7, 8, 9 and C-12, 13, 14), 49.0 (C-3), 53.8 (C-2), 80.0 and 83.1 (C-6 and C-11), 155.4 (C-10), 169.2 (C-1) and 201.0 (C-4). Complementary signals for minor diastereoisomer at δ 8.2, 27.8, 28.1, 49.4, 53.6, 80.1, 82.9, 155.2, 169.6 and 201.1 ppm; *m/z* (CI) 288 (MH^+, 36.9%), 249 (100%), 232, 188, 86.

DL-*N*-*tert*-Butoxycarbonyl-2-methylaspartate-β-semialdehyde *tert*-butyl ester (152)



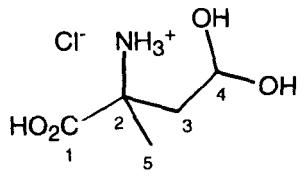
Compound **(151)** (843 mg, 2.95 mmol) was ozonised for 90 min in anhydrous dichloromethane (30 ml) and the resulting ozonide quenched with triethylamine (823 µl, 5.91 mmol), according to **general procedure 3**. Purification was achieved on a silica column, eluting with 10% EtOAc in hexane, to yield the desired aldehyde as a white solid, 611 mg (2.13 mmol, 72.1%); mp 47-48 °C; R_f 0.25 (20% EtOAc in hexane); (Found: M^+, 288.1821; C, 58.35; H, 8.96; N, 4.93. $C_{14}H_{25}NO_5$ requires MH, 288.1811; C, 58.51; H, 8.77; N, 4.87%); ν_{max}(KBr disc)/cm^{-1} 3372, 2975, 2934, 1716, 1512; δ_H (200 MHz, $CDCl_3$) 1.08 and 1.13 (18H, 2 x s, 7, 8, 9- and 12, 13, 14-H_3), 1.20 (3H, s, 5-H_3), 2.60 and 3.04 (2H, dd and br d respectively, J 16.9, 1.1 Hz and J 17.0 Hz, 3-H_2), 5.29 (1H, br s, N-H) and 9.38 (1H, dd, J 1.9, 1.2 Hz, 4-H); δ_C (50 MHz) 24.0 (C-5), 27.5 and 28.1 (C-7, 8, 9 and C-12, 13, 14), 49.2 (C-3), 58.6 (C-2), 79.4 and 82.2 (C-6 and C-11), 154.2 (C-10), 172.2 (C-1) and 199.6 (C-4); *m/z* (CI) 288 (MH^+, 73.7%), 249 (100%), 232, 193, 186, 176, 86.

DL-3-Methylaspartate-β-semialdehyde hydrate hydrochloride (155)



Compound **(155)** was synthesised from compound **(153)** (300 mg, 1.04 mmol) in anhydrous EtOAc (3 ml) and 2.5M anhydrous HCl in EtOAc (4.18 ml, 10.44 mmol) according to **general procedure 1**. The product was isolated as a hygroscopic white solid which was dried in a desiccator (P_2O_5) and stored at -20 °C, 189 mg (1.02 mmol, 98.1%); mp 87 °C (dec.); ν_{max}(KBr disc)/cm^{-1} 3419, 2978, 1790, 1739, 1598, 1509; δ_H (200 MHz, D_2O) 1.32 (2H, s, 5-H_3), 2.22 (1H, m, 3-H), 3.99 (1H, m, 2-H)-H_3) and 4.93 (1H, m, 4-H); δ_C (50 MHz) 9.7 (C-5), 40.2 (C-3), 54.8 (C-2), 92.0 (C-4) and 172.4 (C-1). The ^1H NMR spectrum shows the presence of solvent in the solid lattice, which is not removed on extensive drying under high vacuum. The spectra are further complicated by the presence of diastereomers. This compound did not give a satisfactory mass spectrum, but was however suitable for work with the DHDPS enzyme.

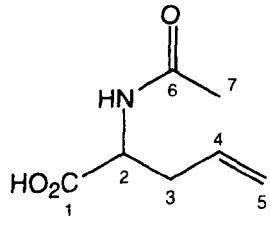
DL-2-Methylaspartate-β-semialdehyde hydrate hydrochloride (154)



Compound **(154)** was synthesised from compound **(161)** (190 mg, 0.66 mmol) in 2.16M anhydrous HCl in EtOAc (3.06 ml, 6.61 mmol) according to **general procedure 1**. The product was isolated as a hygroscopic white solid which was dried in a desiccator (P_2O_5) and stored at -20 °C, 151 mg (0.81 mmol, 123% due to solvation); mp 92 °C (dec.); ν_{max}(KBr disc)/cm^{-1} 3428, 2923, 1789, 1733, 1633; δ_H (200 MHz, D_2O) 1.38 (3H, s, 5-H_3), 1.84 and 2.07 (2H, 2 x dd, J 15.0, 6.7 Hz and 15.0, 5.1 Hz respectively, 3-H_2) and 5.04 (1H, dd, J 6.7, 5,1 Hz, 4-H). Signals observed for solvation at 0.95 (t), 3.34 (q) and 3.42 (q) ppm; δ_C (50 MHz) 23.3 (C-5), 43.2 (C-3), 58.9 (C-2), 88.2 (C-4) and 174.8 (C-1); *m/z* 150 (M^+, 3.2%), 136, 120 (100%), 97. This compound did not give a satisfactory mass spectrum.

7.03 Experimental to Chapter 4

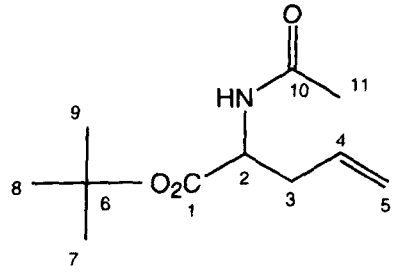
DL-*N*-Acetylallylglycine (113)[115]



This was prepared according to the method of Black and Wright.[115] Freshly distilled acetic anhydride (7.55 ml, 80.00 mmol) was added, via a syringe pump, over a period of 90 min to a solution of DL-allylglycine (4.60 g, 40.00 mmol) in 2M NaOH (aq) (20 ml) at 0 °C. The pH was carefully monitored and kept at pH > 9.5 by addition of 40 % NaOH (aq) as required. After addition of the anhydride the solution was acidified to pH 1 with c.HCl (aq) (~ 10 ml) and extracted with EtOAc (3 x 150 ml). The combined organic extract was dried (Na_2SO_4), filtered and concentrated *in vacuo* to a white solid. Purification was achieved by crystallisation from acetone / pet. ether (40-60 °C) to yield the desired amide as colourless plates, 4.68 g (29.78 mmol, 74.4%); mp 109-112

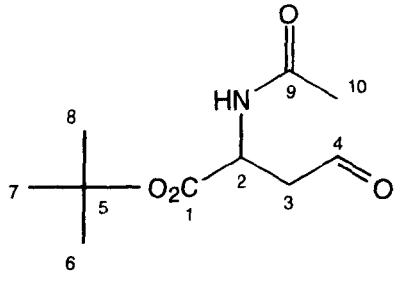
°C (acetone / pet. ether(40/60)) (Lit.[195] 114 °C); (Found: M^+, 157.0745; C, 53.27; H, 7.10; N, 8.93. $C_7H_{11}NO_3$ requires M, 157.0739; C, 53.49; H, 7.05; N, 8.91%); ν_{max}(KBr disc)/cm^{-1} 3342, 2934, 1722, 1596, 1544; δ_H (200 MHz, CD_3OD) 1.97 (3H, s, 7-H_3), 2.52 (2H, m, 3-H_2), 4.44 (1H, dd, J 8.2, 5.2 Hz, 2-H), 5.11 (2H, m, 5-H_2), and 5.78 (1H, m, 4-H); δ_C (50 MHz) 22.3 (C-7), 36.9 (C-3), 53.4 (C-2), 118.6 (C-5), 134.5 (C-4), 173.2 and 174.7 (C-1 and C-6); *m/z* 157 (M^+, 1.3%), 139, 116, 98, 71 and 42 (100%).

DL-*N*-Acetylallylglycine *tert*-butyl ester (176)[190]



Compound **(176)** was synthesised from compound **(113)** (1.57 g, 10.00 mmol) and isobutylene (~ 30 ml) in anhydrous dichloromethane (100 ml) and c.H_2SO_4 (1.5 ml) according to **general procedure 4**. Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then EtOAc to yield the desired ester as a white solid, 2.03 g (9.53 mmol, 95.3%); mp 53-55 °C; R_f 0.20 (50% EtOAc in hexane); (Found: C, 61.86; H, 8.70; N, 6.52. $C_{11}H_{19}NO_3$ requires C, 61.97; H, 8.92; N, 6.57%). The NMR spectra are consistent with the literature.[190]

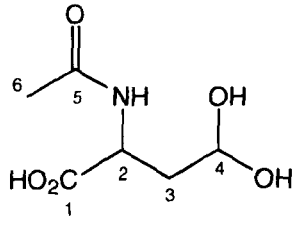
DL-*N*-Acetylaspartate-β-semialdehyde *tert*-butyl ester (177)[190]



Compound **(176)** (1.07 g, 5.00 mmol) was ozonised in anhydrous dichloromethane (30 ml) and the resulting ozonide quenched with triethylamine

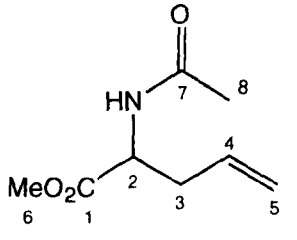
(1.39 ml, 10.00 mmol), according to **general procedure 3**. Purification was achieved on a silica dry flash column, eluting with 25% EtOAc in hexane, to yield the desired aldehyde as a colourless oil, 887 mg (4.12 mmol, 82.4%); R_f 0.29 (EtOAc); (Found: MH^+, 216.1239. $C_{10}H_{17}NO_4$ requires MH, 216.1236). The NMR spectra are consistent with the literature.[190]

DL-*N*-Acetylaspartate-β-semialdehyde hydrate (175)[190]



Compound **(175)** was synthesised from compound **(177)** (350 mg, 1.63 mmol) in anhydrous dichloromethane (2 ml) and analytical grade trifluoroacetic acid (2 ml) according to the method of Tudor.[190] Removal of the solvent *in vacuo*, after 1h at -9 °C under a nitrogen atmosphere, gave a residue which was triterated in ether at room temperature for 18 h. The desired acid was isolated as a white solid by filtration and dried in a desiccator (P_2O_5), 170 mg (0.96 mmol, 58.9%); mp 98-100 °C; δ_H (200 MHz, D_2O) 1.84 (3H, s, 6-H_3), 1.90 (2H, m, 3-H_2), 4.28 (1H, dd, J 8.5, 5.3 Hz, 2-H) and 4.94 (1H, dd, J 6.6, 4.9 Hz, 4-H); δ_C (50 MHz) 22.4 (C-6), 38.8 (C-3), 50.6 (C-2), 88.7 (C-4), 174.9 and 176.0 (C-1 and C-5).

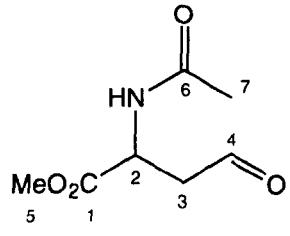
DL-*N*-Acetylallylglycine methyl ester (178)[162]



A solution of compound **(113)** (2.00 g, 12.73 mmol) in methanol (50 ml) at 0 °C was saturated with anhydrous HCl gas (~ 15 min). The solution was then stirred for 18 h at room temperature. After concentration *in vacuo* the resulting residue was purified on a silica dry flash column eluting with 50%

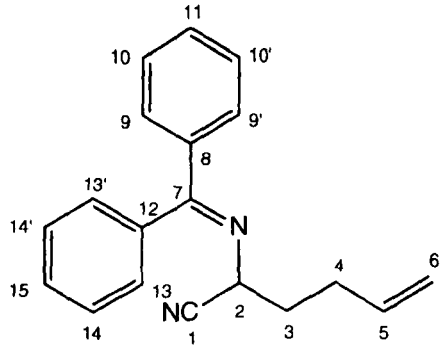
EtOAc in hexane to yield the desired ester as an oily white solid, 1.63 g (9.52 mmol, 74.8%); R_f 0.32 (EtOAc); (Found: M^+, 171.0911;. $C_8H_{13}NO_3$ requires M, 171.0895); ν_{max}(thin film)/cm^{-1} 3282 (br), 3078, 2954, 1748, 1656, 1547, 1437, 1375; δ_H (200 MHz, $CDCl_3$) 1.75 (3H, s, 8-H_3), 2.26 (2H, m, 3-H_2), 3.47 (3H, s, 6-H_3), 4.41 (1H, m, 2-H), 4.82 and 4.88 (2H, 2 x m, 5-H_2), 5.43 (1H, m, 4-H) and 6.16 (1H, br d, J 6.0 Hz, N-H); δ_C (50 MHz) 21.3 (C-8), 34.7 (C-3), 50.0 (C-2), 50.6 (C-6), 117.4 (C-5), 130.6 (C-4), 168.2 and 170.7 (C-1 and C-7); *m/z* 171 (M^+, 4.9%), 139, 130, 112, 88 (100%), 70.

DL-*N*-Acetylaspartate-β-semialdehyde methyl ester (179)[196]



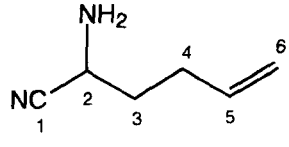
Compound **(178)** (1.00 g, 5.84 mmol) was ozonised in anhydrous dichloromethane (50 ml) and the resulting ozonide quenched with triethylamine (1.63 ml, 11.68 mmol), according to **general procedure 3**. Purification was achieved on a silica dry flash column, eluting with EtOAc, to yield the desired aldehyde as a colourless oil, 694 mg (4.05 mmol, 69.4%); R_f 0.16 (EtOAc); (Found: MH^+, 174.0791. $C_7H_{12}NO_4$ requires M, 174.0766; ν_{max}(KBr disc)/cm^{-1} 3024, 3009, 1748, 1678, 1508; δ_H (200 MHz, $CDCl_3$) 1.96 (3H, s, 7-H_3), 2.93 (2H, d, J 4.9 Hz, 3-H_2), 3.67 (3H, m, 5-H_3), 4.82 (1H, m, 2-H), 6.89 (1H, br d, J 7.5 Hz, N-H) and 9.65 (1H, s, 4-H); δ_C (50 MHz) 22.6 (C-7), 45.2 (C-3), 46.9 (C-2), 52.5 (C-5), 170.0 and 171.1 (C-1 and C-6) and 199.4 (C-4); *m/z* 173 (M^+, 3.0%), 141, 130, 114 (100%), 86.

DL-2-(Diphenylmethyleneamino)-5-hexenonitrile (183)[99]



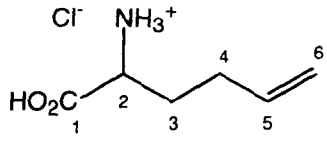
Compound **(183)** was synthesised from *N*-(diphenylmethylene)-aminoacetonitrile (11.01 g, 50.00 mmol), benzyl triethylammonium chloride (BTEAC) (1.01 g, 4.44 mmol) and 4-bromo-1-butene (8.10 g, 60.00 mmol) in 50% NaOH (aq) (12 g, 150.0 mmol) and toluene (11 ml) according to the method of O'Donnell and Eckrich,[99] for a related synthesis. After reaction the reaction mix was diluted with water (400 ml) and extracted with dichloromethane (4 x 200 ml). The combined organic extract was washed with water (3 x 100 ml), brine (100 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to a red oil. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired compound as a yellow oil, 12.65 g (46.11 mmol, 92.2%); R_f 0.35 (10% EtOAc in hexane); δ_H (200 MHz, $CDCl_3$) 2.00 (2H, m, 4-H_2), 2.20 (2H, m, 3-H_2), 4.24 (1H, t, J 6.6 Hz, 2-H), 4.97 (2H, m, 6-H_2), 5.67 (1H, m, 5-H) and 7.18 to 7.82 (10H, m, Ar-H's); δ_C (50 MHz) 29.5 (C-4), 33.9 (C-3), 52.3 (C-2), 116.0 (C-6), 119.5 (C-1), 127.4, 128.2, 129.3, 131.2 and 136.3 (Ar C-H), 130.0 (C-5), 135.2 and 138.4 (C-8 and C-12) and 173.1 (C-7). The NMR spectra are consistent with the literature.[197]

DL-2-Amino-5-hexenonitrile (184)[197]



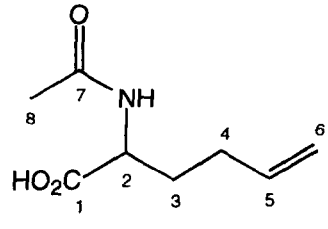
Compound **(184)** was synthesised from **(183)** (6.86 g, 25.00 mmol) in 3M HCl (aq) (25 ml) and ether (25 ml) according to the method of Connell.[197] After reaction the aqueous portion was washed with ether (25 ml) and basified with 2M NaOH (aq) (50 ml). The desired amine was then extracted into EtOAc (3 x 50 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to a colourless oil, 1.66 g (15.07 mmol, 60.8%); R_f 0.20 (50% EtOAc in hexane); (Found: M^+-H, 109.0785 $C_6H_{10}N_2$ requires M-H, 109.0766); δ_H (200 MHz, $CDCl_3$) 1.64 (2H, br s, N-H_2), 1.75 (2H, m, 3-H_2), 2.14 (2H, m, 4-H_2), 3.62 (1H, t, J 7.2 Hz, 2-H), 5.01 (2H, m, 6-H_2) and 5.72 (1H, m, 5-H); δ_C (50 MHz) 29.6 (C-3), 34.3 (C-4), 42.7 (C-2), 116.4 (C-6), 122.2 (C-1) and 136.3 (C-5). The NMR spectra are consistent with the literature.[197]

DL-2-Amino-5-Hexenoic acid hydrochloride (185)[197]



A suspension of **(184)** (2.47 g, 22.42 mmol) in 5% NaOH (aq) (100 ml) was heated to reflux for 24 h. After cooling the mixture was acidified to pH 1 with c.HCl (aq), washed with EtOAc (2 x 100 ml), concentrated *in vacuo*, and dried in a desiccator (P_2O_5) to yield the desired amino acid as a white solid, 10.66 g (including 125 mmol, 7.29 g NaCl) (estimated 3.37 g, 15.38 mmol **(185)**.NH_4Cl, 68.6%) The characterisation data was consistent with the literature:[197] δ_H (200 MHz, D_2O) 2.18 and 2.32 (4H, 2 x m, 3- and 4-H_2), 4.25 (1H, m, 2-H), 5.25 (2H, m, 6-H_2) and 5.98 (1H, m, 5-H); δ_C (50 MHz) 29.6 and 30.1 (C-3 and C-4), 53.7 (C-2), 117.9 (C-6), 137.8 (C-5) and 173.4 (C-1).

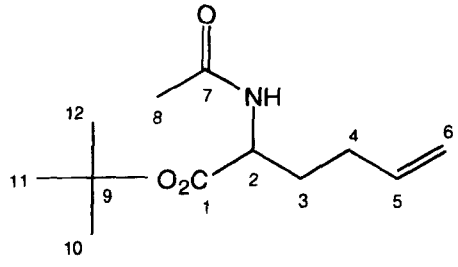
DL-*N*-Acetyl-2-amino-5-hexenoic acid (186)[198]



A suspension of **(184)** (220 mg, 2.00 mmol) in 5% NaOH (aq) (20 ml) was heated to reflux for 24 h. On cooling, the solution was washed, acidified and concentrated as described previously to give compound **(185)**. Freshly distilled acetic anhydride (1.89 ml, 20.00 mmol) was added, via a syringe pump, over a period of 120 min to a solution of the crude **(185)** (4.60 g, 40 mmol) in 2M NaOH (aq) (5 ml) at 0 °C. The pH was carefully monitored and kept at > 9.5 by addition of 40% NaOH (aq) as required. After addition of the anhydride the solution was acidified to pH 1 with c.HCl (aq) (~ 7 ml) and extracted with EtOAc (5 x 50 ml). The combined organic extracts were dried (Na_2SO_4), filtered and concentrated *in vacuo* to a yellow oil. Purification was achieved by crystallisation from acetone/pet. ether (40-60 °C) to yield the desired amide as colourless plates, 197 mg (1.15 mmol, 57.5% for two steps from compound **(184)**); mp 110-113 °C (Lit.[198] 114-116 °C); R_f 0.27 (30% MeOH in $CHCl_3$); (Found: M^+, 171.0896; C, 55.82; H, 7.32; N, 7.99. $C_8H_{13}NO_3$ requires M, 178.0895; C, 56.12; H, 7.65; N, 8.18%); ν_{max}(KBr disc)/cm^{-1} 3346, 1717, 1598,

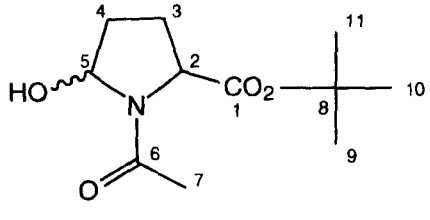
1544; δ_H (200 MHz, CD_3OD) 1.65 (2H, m, 4-H_2), 1.78 (3H, s, 8-H_3), 1.93 (2H, m, 3-H_2), 4.16 (1H, dd, J 9.0, 4.8 Hz, 2-H), 4.76 to 4.89 (3H, br m, N-H and 6-H_2) and 5.61 (1H, m, 5-H); δ_C (50 MHz) 24.7 (C-8), 33.4 and 34.3 (C-3 and C-4), 55.5 (C-2), 118.5 (C-6), 140.7 (C-5), 175.8 and 177.9 (C-1 and C-7); *m/z* 170 (M^+-H, 0.7%), 126, 117, 84 and 43 (100%).

DL-*N*-Acetyl-2-amino-5-hexenoic acid *tert*-butyl ester(187)



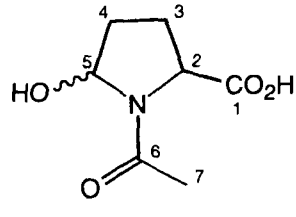
Compound **(187)** was synthesised from compound **(186)** (950 mg, 5.55 mmol) and isobutylene (~ 30 ml) in anhydrous dichloromethane (80 ml) and c.H_2SO_4 (1 ml) according to **general procedure 4**. Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then 20% EtOAc in hexane to yield the desired ester as a white solid, 1.08 g (4.77 mmol, 86.0%); mp 39-42 °C; R_f 0.48 (EtOAc); (Found: M^+-O^tBu, 154.0863; C, 63.48; H, 9.31; N, 6.22. $C_8H_{12}NO_2$ requires M, 154.0864; C, 63.40; H, 9.31; N, 6.22%); ν_{max}(KBr disc)/cm^{-1} 3300, 2978, 1736, 1654, 1552, 1150; δ_H (200 MHz, $CDCl_3$) 1.47 (9H, s, 10, 11, 12-H_3), 1.61 to 2.22 (4H, m, 3- and 4-H_2), 2.02 (3H, s, 8-H_3), 4.52 (1H, m, 2-H), 5.03 (2H, m, 6-H_2), 5.79 (1H, m, 5-H) and 6.31 (1H, br d, J 7.4 Hz, N-H); δ_C (50 MHz) 21.5 (C-8), 26.3 (C-10, 11, 12), 27.7 and 30.3 (C-3 and C-4), 50.6 (C-2), 80.4 (C-9), 113.8 (C-6), 135.6 (C-5), 168.1 and 170.2 (C-1 and C-7); *m/z* 154 (M^+-O^tBu, 3.9%), 126, 84 (100%), 57, 43.

DL-*N*-Acetyl-5-hydroxyproline *tert*-butyl ester (189)



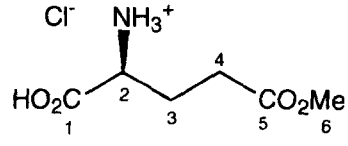
Compound **(187)** (984 mg, 4.33 mmol) was ozonised for 2 h in anhydrous dichloromethane (30 ml) and the resulting ozonide quenched with triethylamine (1.39 ml, 10 mmol), according to **general procedure 3**. Purification was achieved on a silica dry flash column, eluting with 50% EtOAc in hexane, yielded a mixture of diastereoisomers of compound **(189)** as a white solid, 767 mg (3.35 mmol, 77.3%); mp 83-85 °C; R_f 0.21 and 0.26 (EtOAc); (Found: M^+, 229.1329. $C_{11}H_{19}NO_4$ requires M, 229.1314); ν_{max}(KBr disc)/cm^{-1} 3240 (br), 2976, 1742, 1730, 1628, 1616; δ_H (200 MHz, $CDCl_3$) 1.45, 1.47, 1.48 and 1.48 (9H, 4 x s, 9, 10, 11-H_3), 1.80 to 2.57 (4H, m, 3- and 4-H_2), 2.30 (3H, s, 7-H_3), 4.22 to 4.43 (2H, m, 2- and O-H), 4.49 and 5.75 (1H, 2 x m, 5-H); δ_C (50 MHz) 19.3, 19.9, 20.1 and 20.6 (C-7), 24.4, 24.9, 26.7, 27.1, 28.8, 29.5, 32.1 and 33.3 (C-3 and C-4), 26.2 (C-9, 10, 11), 57.7, 57.9, 59.1 and 59.4 (C-2), 79.5, 80.6 and 80.8 (C-8), 80.2, 80.7 and 81.4 (C-5), 168.9, 169.1, 169.3, 169.5, 169.6, 169.8 and 169.9 (C-1 and C-6); *m/z* 229 (M^+, 0.1%), 128, 86 (100%), 69, 57, 43. The NMR spectra are complicated by the presence of both diastereomers and rotomers.

DL-*N*-Acetyl-5-hydroxyproline (190)



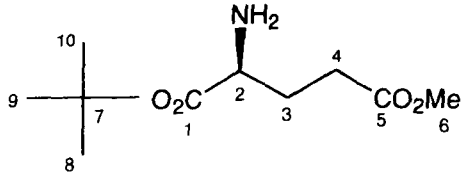
To a solution of **(189)** (370 mg, 1.61 mmol) in anhydrous dichloromethene (5 ml) at -9 °C, under a nitrogen atmosphere, was added analytical grade trifluoroacetic acid (3 ml) and the solution was stirred at this temperature for 2 h. Removal of the solvent *in vacuo* gave a residue, which was triterated in ether at room temperature for 18 h. The desired diastereomeric mixture of acids were isolated a white hygroscopic solid by filtration and dried in a desiccator (P_2O_5), 147 mg (0.85 mmol, 52.7%); mp 48-50 °C; (Found: M^+-H_2O, 155.0579. $C_7H_9NO_3$ requires M, 155.0582); ν_{max}(KBr disc)/cm^{-1} 3442 (br), 1736 (br), 1632 (br); δ_H (200 MHz, D_2O) 1.31, 1.91 and 2.29 (4H, 3 x m, 3- and 4-H_2), 2.05 and 2.08 (3H, 2 x s, 7-H_3), 4.16 and 4.35 (1H, 2 x m, 2-H) and 5.49 (1H, m, 5-H); δ_C (50 MHz) 21.6 and 21.7 (C-7), 26.6 and 27.2 (C-3), 33.8 and 34.4 (C-4), 59.4 and 60.1 (C-2), 83.2 and 83.6 (C-5), 174.6, 174.8, 176.4 and 176.5 (C-1 and C-6); *m/z* 155 (M^+-H_2O, 12.6%), 113, 69 (100%) and 42.

δ-Methyl L-glutamate hydrochloride (165)[147]



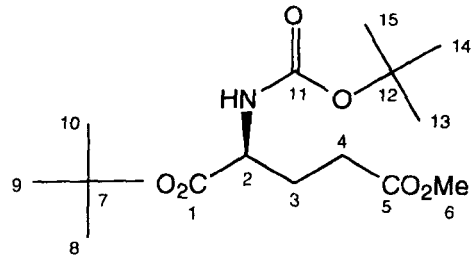
This was prepared according to the method of McKendrick.[147] Thionyl chloride (10.95 g, 92.04 mmol) was added slowly to a stirred suspension of L-glutamic acid (10.07 g, 68.44 mmol) in HPLC grade methanol (50 ml) at 0 °C. The resulting solution was stirred at this temperature for a further 15 min and then at room temperature for 45 min. Ether (200 ml) was added and a white solid precipitated. After a further 30 min at room temperature the solid was filtered, washed with ether (2 x 50 ml) and dried in a desiccator (P_2O_5) to yield the desired ester as a white solid, 13.35 g (67.55 mmol, 98.7%); mp 147-149 °C; (Found: M^+, 162.0763; C, 36.45; H, 6.05; N, 6.97. $C_6H_{12}NO_4$ requires M, 162.0766; C, 36.46; H, 6.12; N, 7.09%).

δ-Methyl L-glutamate *tert*-butyl ester (196)[199]



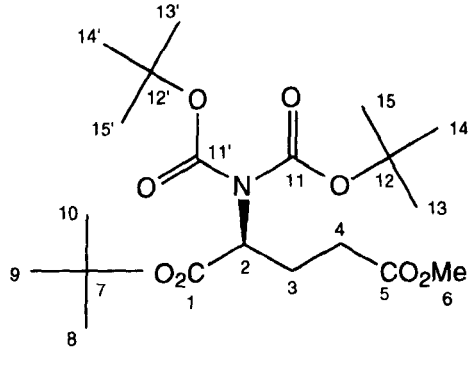
Compound **(196)** was synthesised from compound **(165)** (10.00 g, 50.60 mmol) and isobutylene (~ 30 ml) in anhydrous dichloromethane (250 ml) and c.H_2SO_4 (3 ml) according to the method of McKendrick.[199] Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then EtOAc to yield the desired ester as a colourless oil, 9.70 g (44.64 mmol, 88.2%); R_f 0.25 (EtOAc); (Found: MH^+, 218.1390. $C_{10}H_{20}NO_4$ requires M, 218.1387).

δ-Methyl L-*N*-*tert*-butoxycarbonylglutamate *tert*-butyl ester (197)[200]



Compound **(197)** was synthesised from compound **(196)** (9.55 g, 43.95 mmol), $KHCO_3$ (4.84 g, 48.34 mmol) and di-*tert*-butyldicarbonate (10.55 g, 48.34 mmol) in 1,4-dioxane (50 ml) and water (50 ml) according to **general procedure 2**. Purification was achieved on a silica dry flash column, eluting with 10% EtOAc in hexane, to yield the desired carbamate as a white solid, 9.95 g (31.35 mmol, 71.3%); mp 62-63 °C; R_f 0.11 (10% EtOAc in hexane); (Found: MH^+, 318.1918; C, 56.81; H, 8.57; N, 4.35. $C_{15}H_{28}NO_6$ requires M, 318.1916; C, 56.76; H, 8.57; N, 4.41%); ν_{max}(KBr disc)/cm^{-1} 3350, 2982, 1728, 1702, 1530, 1162; δ_H (200 MHz, $CDCl_3$) 1.41 and 1.44 (18H, 2 x s, 8, 9, 10- and 13, 14, 15-H_3), 1.88 and 2.14 (2H, 2 x m, 3-H_2), 2.37 (2H, m, 4-H_2), 3.65 (3H, s, 6-H_3), 4.15 (1H, m, 2-H) and 5.08 (1H, br d, J 8.0 Hz, N-H); δ_C (50 MHz) 27.8 and 28.2 (C-8, 9, 10 and C-13, 14, 15), 27.9 (C-3), 30.0 (C-4), 51.6 (C-6), 53.3 (C-2), 79.6 and 82.0 (C-7 and C-12), 155.3 (C-11), 171.2 and 173.2 (C-1 and C-5); *m/z* 317 (M^+, 0.2%), 216, 188, 174, 160, 116, 84, 57 (100%).

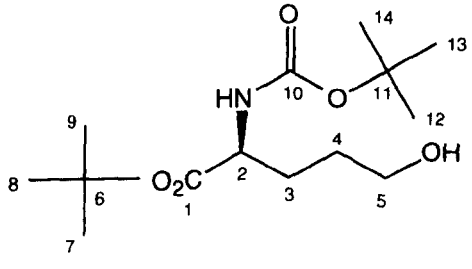
δ-Methyl L-*NN*-*bis*-*tert*-butoxycarbonylglutamate *tert*-butyl ester (198)



DMAP (98 mg, 0.80 mmol) was added to a solution of compound **(197)** (2.54 g, 8.00 mmol) in anhydrous acetonitrile (20 ml), under a nitrogen atmosphere. Di-*tert*-butyldicarbonate (1.92 g, 8.80 mmol) in anhydrous acetonitrile (5 ml) was then added and the resulting solution was stirred at room

temperature for 18 h. The orange solution was concentrated *in vacuo* at room temperature, diluted with ether (40 ml) and washed with 1M $KHSO_4$ (aq) (3 x 30 ml), sat. $NaHCO_3$ (aq) (30 ml) and sat. brine (30 ml). The organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a yellow oil. Purification was achieved on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired dicarbamate as a colourless oil, 2.29 g (5.49 mmol, 68.6%); R_f 0.29 (20% EtOAc in hexane); (Found: MH^+, 418.2433. $C_{20}H_{36}NO_8$ requires M, 418.2440); $\nu_{max}(CHCl_3$ solution)/cm^{-1} 3026, 2983, 1786, 1734, 1696, 1369; δ_H (200 MHz, $CDCl_3$) 1.33 (9H, s, 8, 9, 10-H_3), 1.39 (18H, s, 13, 14, 15- and 13', 14', 15'-H_3), 2.04 (2H, m, 3-H_2), 2.38 (2H, m, 4-H_2), 3.55 (3H, s, 6-H_3) and 4.68 (1H, m, 2-H); δ_C (50 MHz) 24.4 (C-3), 27.7 (C-8, 9, 10), 27.8 (C-13, 14, 15 and C-13', 14', 15'), 30.6 (C-4), 51.4 (C-6), 57.9 (C-2), 81.1 (C-7), 82.7 (C-12 and C-12'), 152.1 (C-11 and C-11'), 169.1 and 173.0 (C-1 and C-5); *m/z* 418 (MH^+, 0.1%), 316, 232, 216, 160, 116, 84, 57 (100%).

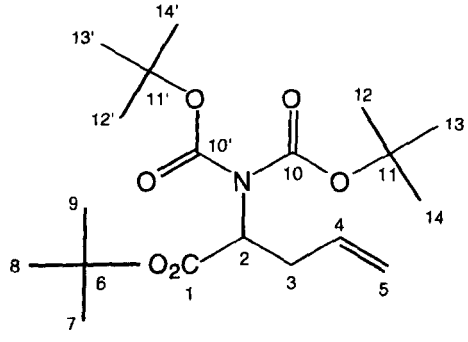
tert-Butyl L-2-(_tert_-butoxycarbonylamino)-5-hydroxypentanoate (201)[201]



Compound **(201)** was prepared by a method adapted from that of Brown and Narasimhan.[158] To a solution of compound **(197)** (2.12 g, 6.68 mmol) in anhydrous ether (20 ml), at -10 °C (NH_4Cl / ice bath) under nitrogen, was added lithium borohydride (145 mg, 6.68 mmol). On addition of trimethylborate (0.2 ml, 1.79 mmol) a rapid exothermic reaction with evolution of gas was observed. The solution was stirred at -10 °C for 90 min before addition of more trimethylborate (0.544 ml, 4.88 mmol). Stirring was continued at -10 °C for a further 2.5 h before quenching with sat. NH_4Cl (aq) (20 ml) and extraction with EtOAc (3 x 30 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a white solid. Purification was achieved on a silica dry flash column, eluting with 20% EtOAc in hexane, to yield the desired alcohol as an oily white solid, 1.50 g (5.18 mmol, 77.6%); R_f 0.23 (50% EtOAc in hexane); (Found: M^+, 290.1954. $C_{14}H_{28}NO_5$ requires M, 290.1967); $\nu_{max}(CHCl_3$ solution)/cm^{-1} 3436, 2982, 2254, 1709, 1501, 1369; δ_H (200 MHz, $CDCl_3$) 1.37 and 1.40 (18H, 2 x s, 7, 8, 9- and 12, 13, 14-H_3), 1.47 - 1.85 (4H,

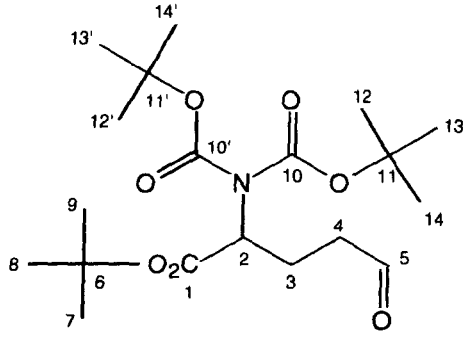
br m, 3- and 4-H_2), 2.42 (1H, br s, O-H), 3.59 (2H, t, J 6.1 Hz, 5-H_2), 4.13 (1H, br dd, J 13.7, 7.2 Hz, 2-H) and 5.18 (1H, br d, J 7.8 Hz, N-H); δ_C (50 MHz) 27.9 and 28.2 (C-7, 8, 9 and C-12, 13, 14), 28.2 (C-3), 29.6 (C-4), 53.5 (C-2), 62.0 (C-5), 79.7 and 81.8 (C-6 and C-11), 155.5 (C-10) and 171.9 (C-1); *m/z* 290 (M^+, 0.5%), 188, 160, 132, 88, 71, 57 (100%).

DL-*NN*-bis-(*tert*-Butoxycarbonyl)allylglycine *tert*-butyl ester (202)



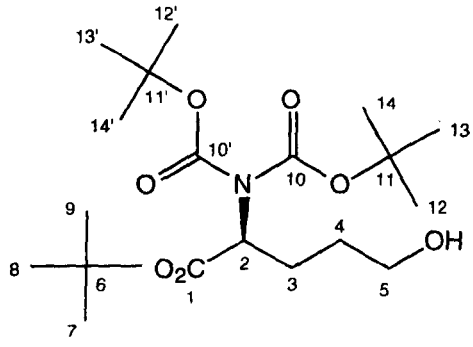
Compound **(202)** was prepared according to the method of Ragnarsson and co-workers[146] for a related synthesis. DMAP (191 mg, 1.56 mmol) was added to a solution of compound **(116)** (4.24 g, 15.60 mmol) in anhydrous acetonitrile (20 ml), under a nitrogen atmosphere. Di-t-butyldicarbonate (3.75 g, 17.16 mmol) in anhydrous acetonitrile (10 ml) was then added and the resulting solution was stirred at room temperature for 18 h. The yellow solution was concentrated *in vacuo* at room temperature, diluted with ether (50 ml) and washed with 1M $KHSO_4$ (aq) (3 x 30 ml), sat. $NaHCO_3$ (aq) (30 ml) and sat. brine (30 ml). The organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a yellow oil. Purification was achieved on a silica column, eluting with 3% EtOAc in hexane, to yield the desired dicarbamate as a white solid, 4.38 g (11.79 mmol, 75.6%); mp 70-71 °C; R_f 0.36 (10% EtOAc in hexane); (Found: MH^+, 372.2386; C, 61.52; H, 8.99; N, 3.64. $C_{19}H_{33}NO_6$ requires MH, 372.2386; C, 61.43; H, 8.95; N, 3.77%); ν_{max}(KBr disc)/cm^{-1} 2988, 2974, 1737, 1694, 1381, 1368; δ_H (200 MHz, $CDCl_3$) 1.38 (9H, s, 7, 8, 9-H_3), 1.43 (18H, s, 12, 13, 14- and 12', 13', 14'-H_3), 2.65 (2H, m, 3-H_2), 4.76 (1H, dd, J 9.9, 5.3 Hz, 2-H), 4.99 (2H, m, 5-H_2) and 5.66 (1H, m, 4-H); δ_C (50 MHz) 27.8 (C-7, 8, 9), 27.9 (C-12, 13, 14 and C-12', 13', 14'), 33.8 (C-3), 58.2 (C-2), 81.1 (C-6), 82.5 (C-11 and C-11'), 117.4 (C-5), 134.3 (C-4), 152.2 (C-10 and C-10') and 169.2 (C-1); *m/z* (CI) 372 (MH^+, 39.3%), 333 (100%), 316, 289, 272, 233, 172.

DL-*NN-bis*-(*tert*-Butoxycarbonyl)glutamate-γ-semialdehyde *tert*-butyl ester (174)



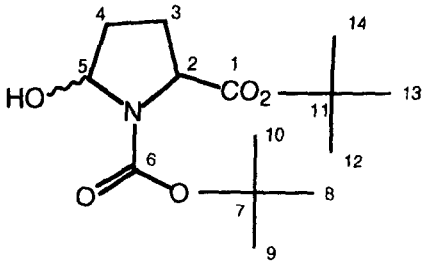
Compound **(174)** was prepared according to the method of Yates[159] for a related synthesis. To a solution of compound **(202)** (371 mg, 1.00 mmol) in anhydrous ether (5 ml) was added borane.DMS (2M complex in THF) (165 µl, 0.33 mmol) under a nitrogen atmosphere and the solution was stirred at room temperature for 3 h. Anhydrous dichloromethane (3 ml), freshly activated 4Å molecular sieves (6 g) and 4-methylmorpholine-*N*-oxide (NMO) (351 mg, 3.00 mmol) were added and the suspension was stirred for a further 1 h at room temperature. Tetra-n-propylammonium perreuthinate (TPAP) (20 mg, 57 µmol) was added and the suspension was stirred at room temperature for 10 min. A colour change from brown to jet black was observed. The reaction mix was filtered through a silica plug, washed with EtOAc (3 x 100 ml) and monitored by TLC. The crude product was concentrated to a yellow oil and then purified on a silica dry flash column, eluting with 5% EtOAc in hexane, to yield the desired aldehyde as a white solid, 140 mg (0.36 mmol, 36.1%); mp 42-44 °C; R_f 0.20 (20% EtOAc in hexane); (Found: M^+, 388.2332; C, 58.90; H, 8.55; N, 3.48. $C_{19}H_{34}NO_7$ requires M, 388.2335; C, 58.89; H, 8.58; N, 3.61%); ν_{max}($CHCl_3$ solution)/cm^{-1} 3690, 3022, 2983, 2360, 1786, 1732, 1696, 1369; δ_H (200 MHz, $CDCl_3$) 1.38 (9H, s, 7, 8, 9-H_3), 1.43 (18H, s, 12, 13, 14- and 12', 13', 14'-H_3), 2.07 (2H, m, 3-H_2), 2.45 (2H, m, 4-H_2), 4.67 (1H, dd, J 9.6, 5.1 Hz, 2-H) and 9.70 (1H, s, 5-H); δ_C (50 MHz) 21.9 (C-3), 27.8 (C-7, 8, 9), 27.9 (C-12, 13, 14 and C-12', 13', 14'), 40.6 (C-4), 58.0 (C-2), 81.4 (C-6), 82.9 (C-11 and C-11'), 152.3 (C-10 and C-10'), 169.1 (C-1) and 201.1 (C-5); *m/z* (CI/NH_3) 388 (MH^+, 58%), 349, 288, 270 (100%), 249, 193, 170.

tert-Butyl L-2-(bis-*tert*-butoxycarbonylamino)-5-hydroxypentanoate (200)



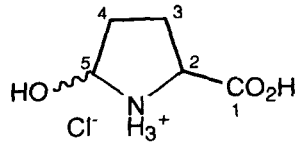
Compound **(200)** was prepared by a method adapted from that of Vogel.[202] To a solution of compound **(202)** (743 mg, 2 mmol) in anhydrous ether (10 ml) under a nitrogen atmosphere at room temperature was added BH_3.DMS (2M in THF) (0.5 ml, 1 mmol) and the solution was stirred for 3 h. After this time excess borane was quenched by the addition of water (0.5 ml). When evolution of H_2 gas had subsided 3% NaOH (aq) (0.5 ml, 3.75 mmol) was added and followed by careful dropwise addition of 30% w/v H_2O_2 (aq) (0.4 ml, 3.53 mmol). No significant rise in temperature was noted and the solution was stirred at room temperature for a further 1 h. Water (20 ml) was then added the product was extracted into EtOAc (3 x 20 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a colourless oil. Purification was achieved on a silica dry flash column eluting with 20% EtOAc in hexane to yield the desired alcohol as a colourless oil, 336 mg (0.86 mmol, 43.1%); R_f 0.35 (50% EtOAc in hexane); (Found: MH^+, 390.2488;. $C_{19}H_{36}NO_7$ requires M, 390.2491); $\nu_{max}(CHCl_3)/cm^{-1}$ 3022, 2983, 1733, 1694, 1369 ; δ_H (200 MHz, $CDCl_3$) 1.37 (9H, s, 7, 8, 9-H_3), 1.43 (18H, s, 12, 13, 14- and 12', 13', 14'-H_3), 1.62 (2H, m, 4-H_2), 1.83 (2H, m, 3-H_2), 3.65 (1H, m, 2-H) and 4.66 (1H, m, 5-H_2); δ_C (50 MHz) 25.6 (C-3), 27.8 (C-7, 8, 9), 27.9 (C-12, 13, 14 and C-12', 13', 14'), 29.4 (C-4), 58.6 (C-2), 62.1 (C-5), 81.2 (C-6), 82.8 (C-11 and C-11'), 152.4 (C-10 and C-10') and 169.8 (C-1); *m/z* (CI/NH_3) 390 (MH^+, 55%), 351, 307 (100%), 290, 251, 191.

DL-*N*-*tert*-Butoxycarbonyl-5-hydroxyproline *tert*-butyl ester (204)[149]



To a solution of compound **(116)** (543 mg, 2.00 mmol) in anhydrous ether (10 ml) was added borane.DMS (2M complex in THF) (500 µl, 0.50 mmol) under a nitrogen atmosphere and the solution was stirred at room temperature for 3 h. Anhydrous dichloromethane (6 ml), freshly activated 4Å molecular sieves (10 g) and 4-methylmorpholine-*N*-oxide (NMO) (703 mg, 6.00 mmol) were added and the suspension was stirred for a further 1 h at room temperature. Tetra-n-propylammonium perreuthinate (TPAP) (35 mg, 100 µmol) was added and the suspension was stirred at room temperature for 5 min. A colour change from brown to jet black was observed. The reaction mix was filtered through a silica plug, washed with EtOAc (3 x 100 ml) and monitored by TLC. The crude product was concentrated to a yellow oil and then purified on a silica column, eluting with 33% ether in hexane, to yield the desired compound as a colourless oil, 89 mg (0.31 mmol, 15.5%) The characterisation data was consistent with the literature:[149] R_f 0.43 (ether); δ_H (200 MHz, $CDCl_3$) Signals visible for both diastereoisomers: 1.37, 1.38, 1.39 and 1.41 (18H, 4 x s, 8, 9, 10 and 12, 13, 14-H_3), 1.72 -2,44 (4H, br m, 3- and 4-H_2), 3.60 and 3.80 (1H, 2 x s, O-H), 4.13 (1H, m, 2-H) and 5.49 (1H, m, 5-H).

DL-5-Hydroxyproline hydrochloride (205)[161]



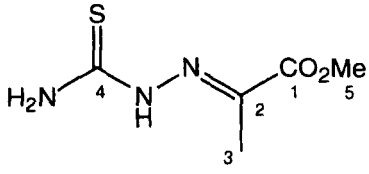
Compound **(174)** (200 mg, 0.52 mmol) was dissolved in anhydrous EtOAc (2.5 ml) and treated with 2.5M anhydrous HCl in EtOAc (4 ml, 10 mmol) according to **general procedure 1**. The product was isolated as a hygroscopic white solid, which was dried in a desiccator (P_2O_5) and stored at -20 °C, 79 mg (0.47 mmol, 90.4%); ν_{max}(KBr disc)/cm^{-1} 3427, 2980, 1738, 1396; δ_H (200 MHz, D_2O) 1.5 to 2.0 (4H, br m, 3- and 4-H_2), 4.08 (1H, dd, J 5.5, 4.4 Hz, 2-H) and 6.30 (1H, m, 5-H); δ_C (50 MHz) 28.3 (C-3), 42.9 (C-4), 49.7 (C-2), 86.6 (C-5) and 169.0 (C-1). There is evidence of doubling of signals in the ^{13}C NMR spectrum due to the presence of diastereoisomers. This compound did not give a satisfactory mass spectrum.

7.04 Experimental to Chapter 5

General Procedure 7:

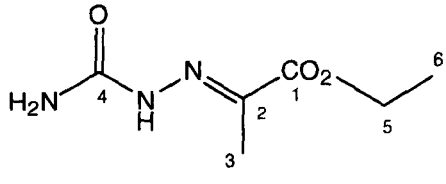
Imines were prepared according to the method of Vogel.[203] The semicarbazide or hydrazine and sodium acetate (2 equiv.) were dissolved in water and a solution of the pyruvic ester (1 equiv.) in ethanol was added. Sufficient ethanol was added to ensure homogeneity. After about 30 min at room temperature the resulting solid was filtered and recrystallised from water/ethanol.

Methyl pyruvate thiosemicarbazone (220)[204]



Compound **(220)** was synthesised from thiosemicarbazide (910 mg, 10.00 mmol) in 1M HCl (aq) (10 ml) and methyl pyruvate (1.02 g, 10.00 mmol) in water (20 ml) and ethanol (20 ml) according to **general procedure 7**. After crystallisation from water / ethanol the product was isolated as white prisms, 1.49 g (8.45 mmol, 84.5%); The characterisation data was consistent with the literature:[204] mp 134-136 °C; R_f 0.18 (33% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 175.0409. $C_5H_9N_3O_2S$ requires M, 175.0415); $\lambda_{max}(H_2O)$/nm 292 (ε/dm^{3}mol^{-1}cm^{-1} 27830).

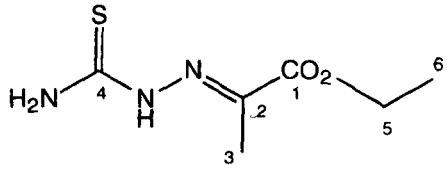
Ethyl pyruvate semicarbazone (229)



Compound **(229)** was synthesised from semicarbazide hydrochloride (558 mg, 5.00 mmol) and ethyl pyruvate (581 mg, 5.00 mmol) in water (20 ml) and ethanol (10 ml) according to **general procedure 7**. After crystallisation from ethanol the product was isolated as white prisms, 1.70 g (9.81 mmol, 98.1%); mp 200-201 °C (EtOH); R_f 0.27 (5% MeOH in $CHCl_3$); (Found: M^+, 173.0793. $C_6H_{11}N_3O_3$ requires M, 173.0800); $\lambda_{max}(H_2O)$/nm 258 (ε/dm^{3}mol-

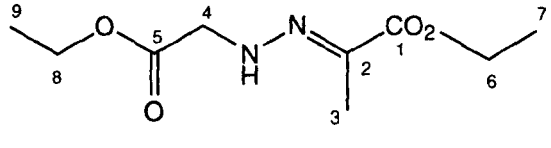
$^{1}cm^{-1}$ 11970); ν_{max}(KBr disc)/cm^{-1} 3507, 3322, 3174, 1698, 1599, 1296; δ_H (200 MHz, D_6-DMSO) 1.28 (3H, t, J 7.1 Hz, 6-H_3), 2.02 (3H, s, 3-H_3), 4.21 (2H, q, J 7.1 Hz, 5-H_2), 6.55 (2H, br s, N-H_2) and 9.94 (1H, s, N-H); δ_C (50 MHz) 12.3 (C-3), 14.1 (C-6), 60.8 (C-5), 136.3 (C-2), 156.1 (C-1) and 164.4 (C-4); *m/z* 173 (M^+, 0.1%), 130, 100, 83, 73, 57 (100%).

Ethyl pyruvate thiosemicarbazone (230)



Compound **(230)** was synthesised from thiosemicarbazide (910 mg, 10.00 mmol) in 1M HCl (aq) (10 ml) and ethyl pyruvate (1.16 g, 10.00 mmol) in water (90 ml) and ethanol (10 ml) according to **general procedure 7**. After filtration the product was isolated as a white solid, 581 mg (3.35 mmol, 67.0%); mp 137 °C; R_f 0.36 (33% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 189.0575; C, 37.91; H, 5.79; N, 22.19. $C_6H_{11}N_3O_2S$ requires M, 189.0572; C, 38.04; H, 5.85; N, 22.28%); λ_{max}(H_2O)/nm 291 (ε/$dm^3mol^{-1}cm^{-1}$ 34360); ν_{max}(KBr disc)/cm^{-1} 3424, 3270, 3206, 3164, 1712, 1605, 1507; δ_H (200 MHz, $CDCl_3$) 1.35 (3H, t, J 7.1 Hz, 6-H_3), 2.16 (3H, s, 3-H_3), 4.31 (2H, q, J 7.1 Hz, 5-H_2), 7.06 (1H, br s, N-H) and 7.60 (2H, br s, N-H_2); δ_C (50 MHz) 12.1 (C-3), 14.1 (C-6), 62.1 (C-5), 108.9 (C-4), 139.1 (C-2) and 180.0 (C-1); *m/z* 189 (M^+, 8.7%), 116 (100%), 75, 57, 43.

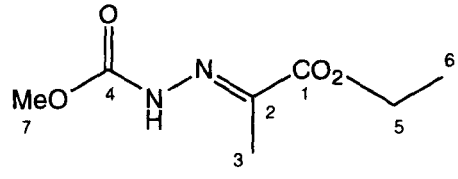
Ethyl hydrazinoacetate derivative of ethyl pyruvate (231)



To a suspension of ethyl hydrazinoacetate hydrochloride (464 mg, 3.00 mmol) and dry Et_3N (0.42 ml, 3.00 mmol) in anhydrous dichloromethane (10 ml) under a nitrogen atmosphere was added ethyl pyruvate (348 mg, 3.00 mmol). The reaction mixture was then stirred at room temperature for 40 h. After this time the solution was concentrated to a deep yellow coloured oil which was purified on a silica column, eluting with 50% EtOAc in pet. ether (40/60 °C), to

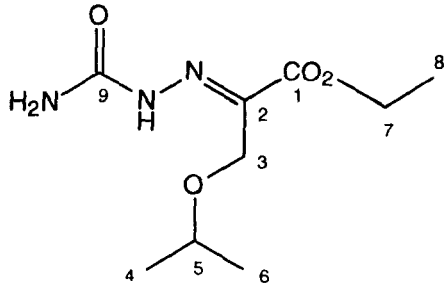
yield the desired imine as a white solid, 480 mg (2.22 mmol, 74.0%); mp 108-109 °C ; R_f 0.31 (50% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 216.1107; C, 49.83; H, 7.54; N, 12.76. $C_9H_{16}N_2O_4$ requires M, 216.1110; C, 49.99; H, 7.46; N, 12.96%); ν_{max}(KBr disc)/cm^{-1} 3352, 2982, 1730, 1697, 1581, 1373, 1324; δ_H (200 MHz, $CDCl_3$) 1.28 and 1.33 (6H, 2 x t, J 7.1 and 7.1 Hz, 7- and 9-H_3), 2.02 (3H, s, 3-H_3), 4.20 and 4.29 (4H, 2 x q, J 7.1 and 7.1 Hz, 6- and 8-H_2) and 6.04 (1H, br m, N-H); δ_C (50 MHz) 11.1 (C-3), 14.1 and 14.2 (C-7 and C-9), 52.4 (C-4), 61.4 and 61.8 (C-6 and C-8), 134.8 (C-2), 153.6 (C-1) and 170.2 (C-5); *m/z* 216 (M^+, 8.4%), 171, 143, 115, 97, 69 (100%), 55, 42.

Methyl hydrazinocarboxylate derivative of ethyl pyruvate (232)



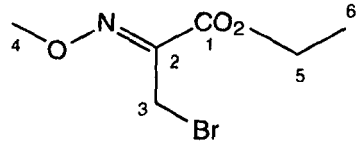
Compound **(232)** was synthesised from methyl hydrazinocarboxylate (1.80 g, 20.00 mmol) in 1M HCl (aq) (20 ml) and ethyl pyruvate (2.32 g, 10.00 mmol) in ethanol (10 ml) according to **general procedure 7**. After filtration the product was isolated as white prisms, 3.07 g (16.29 mmol, 81.4%); mp 134-136 °C (EtOH / acetone); (Found: M^+, 188.0792; C, 44.75; H, 14.92; N, 6.28. $C_7H_{12}N_2O_4$ requires M, ;188.0797; C, 44.68; H, 14.89; N, 6.43%); λ_{max}(H_2O)/nm 250 (ε/$dm^3mol^{-1}cm^{-1}$ 17260); ν_{max}(KBr disc)/cm^{-1} 3240, 3175, 1698, 1611, 1450, 1375; δ_H (200 MHz, $CDCl_3$) 1.25 (3H, t, J 7.1 Hz, 6-H_3), 2.05 (3H, s, 3-H_3), 3.75 (3H, s, 7-H_3), 4.21 (2H, d, J 7.1 Hz, 5-H_2) and 9.03 (1H, br s, N-H); δ_C (50 MHz) 11.6 (C-3), 14.0 (C-6), 53.4 (C-7), 61.9 (C-5), 141.3 (C-2), 154.4 (C-1) and 164.4 (C-5); *m/z* 188 (M^+, 1.3%), 143, 129, 115 (100%), 83, 73, 59.

Ethyl 3-*iso*-propoxypyruvate semicarbazone (234)



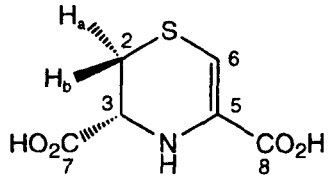
To a solution of semicarbazide hydrochloride (1.12 g, 10.00 mmol) in water (10 ml) at 0 °C was added ethyl bromopyruvate (1.95 g, 10.00 mmol). After 20 min the acidic suspension was neutralised with sat. $NaHCO_3$ (aq) and the crude mixture of products (TLC) was filtered off. Purification was achieved on a silica column, eluting with 10% ^{i}PrOH in CH_3Cl, to yield compound **(234)** as a white solid, 123 mg (0.53 mmol, 5.3%); mp 72-73 °C; R_f 0.49 (10% ^{i}PrOH in CH_3Cl); (Found: M^+-^{i}Pr, 188.0684; C, 46.76; H, 7.50; N, 18.08. $C_9H_{17}N_3O_4$ requires M-^{i}Pr, 188.0672; C, 46.74; H, 7.41; N, 18.17%); $\lambda_{max}(H_2O)$/nm 260 (ε/dm^{3}mol^{-1}cm^{-1} 25420); ν_{max}(KBr disc)/cm^{-1} ; δ_H (200 MHz, CD_3OD) 1.20 (6H, d, J 6.1 Hz, 4- and 6-H_3), 1.33 (3H, t, J 7.1 Hz, -H_3), 3.67 (1H, sept, J 6.1 Hz, 5-H), 4.30 (2H, q, J 7.1 Hz, 7-H_2) and 4.58 (2H, s, 3-H_2); δ_C (50 MHz) 14.4 (C-8), 22.1 (C-4 and C-6), 63.1 (C-7), 64.0 (C-3), 74.0 (C-5), 137.5 (C-2), 165.2 (C-1) and 168.3 (C-9); *m/z* 231 (M^+, 0.5%), 173, 158, 130, 100, 84, 73, 55 and 43 (100%).

Ethyl bromopyruvate methyl oxime (224)[204]



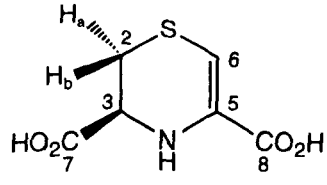
To a solution of methoxylamine hydrochloride (835 mg, 10.00 mmol) in water (10 ml) was added a solution of ethyl bromopyruvate (1.95 g, 10.00 mmol) in ethanol (10 ml) and the resulting solution was stirred at room temperature for 72 h. Removal of the solvent with ethanol *in vacuo* resulted in an oily solid, which was washed through an alumina plug with dichloromethane (50 ml) and reconcentrated to yield the pure oxime as a yellow coloured oil, 1.13 g (5.05 mmol, 50.5%); The characterisation data was consistent with the literature:[204] R_f 0.56 (50% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 224.9835. $C_6H_{10}Br^{81}NO_3$ requires M, 224.9824); $\lambda_{max}(H_2O)$/nm 234 (ε/dm^{3}mol^{-1}cm^{-1} 10720); *m/z* 225 (M^+ (Br^{81}), 11.2%), 223 (M^+ (Br^{79}), 11.0%), 181, 179, 152, 150, 120, 122, 106, 76, 41 (100%).

(*R*)-3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid L-(64)[173]



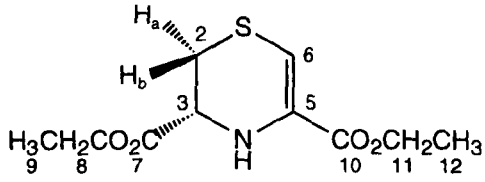
Bromopyruvic acid monohydrate (7.76 g, 46.48 mmol) was added to a solution of L-cysteine hydrochloride monohydrate (7.42 g, 42.25 mmol) in water (30 ml) and mixed in a sonic bath at room temperature for 15 min. The resulting precipitate was filtered, washed with water (3 x 10 ml) and dried in a desiccator (P_2O_5) to yield a white solid, 7.02 g (37.00 mmol, 87.6%) The characterisation data was consistent with the literature:[205] mp 254-260 °C (dec. at 160 °C); $[\alpha]_D^{20}$ -70.7° (*c* 2.439, MeOH); (Found: M^+, 189.0096; C, 38.04; H, 3.61; N, 7.41. $C_6H_7NO_4S$ requires M, 189.0094; C, 38.09; H, 3.73; N, 7.40%); $\lambda_{max}(H_2O)$/nm 307 (ε/dm^{3}mol^{-1}cm^{-1} 5200).

(*S*)-3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxylic acid D-(64)



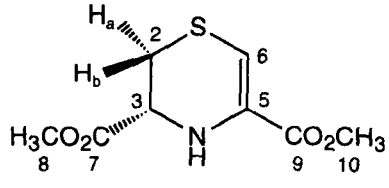
Bromopyruvic acid monohydrate (209 mg, 1.25 mmol) was added to a solution of D-cysteine hydrochloride monohydrate (200 mg, 1.14 mmol) in water (2 ml) and mixed in a sonic bath at room temperature for 15 min. The resulting precipitate was filtered, washed with water (3 x 5 ml) and dried in a desiccator (P_2O_5) to yield a white solid, 178 mg (0.94 mmol, 82.5%); mp 254-260 °C (dec. at 160 °C); $[\alpha]_D^{20}$ +70.6° (*c* 0.445, MeOH); (Found: M^+, 189.0096; C, 37.72; H, 3.45; N, 7.24. $C_6H_7NO_4S_5$ requires M, 189.0094; C, 38.09; H, 3.73; N, 7.40%). δ_H (200 MHz; D_2O/NaOD) 2.69 (1H, dd, J 12.5, 6.8 Hz, 2-H_a), 2.87 (1H, dd, J 12.5, 3.0 Hz, 2-H_b), 3.73 (1H, dd, J 6.8, 3.0 Hz, 3-H) and 5.72 (1H, s, 6-H); δ_C (50 MHz; D_6-DMSO) 25.7 (C-2), 52.2 (C-3), 97.7 (C-6), 128.6 (C-5), 168.4 (C-8) and 171.7 (C-7). All spectral data were the same as compound L-**(64)**.

Diethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (235)[205]



Compound **(235)** was synthesised from L-cysteine ethyl ester hydrochloride (9.28 g, 50.00 mmol), triethylamine (13.9 ml, 100.0 mmol) and ethyl bromopyruvate (6.27 ml, 50.00 mmol) in anhydrous dichloromethane (100 ml) according to the method of Tudor.[205] After 18h, at room temperature under a nitrogen atmosphere, the suspension was washed with water (2 x 100 ml) and the organic portion was dried (Na_2SO_4), filtered and concentrated *in vacuo* to a red oil. Purification was achieved on a silica gel dry flash column, eluting with 5% EtOAc in hexane, to yield the desired thiazine as a yellow oil, 10.67 g (43.50 mmol, 87.0%); R_f 0.40 (33% EtOAc in hexane); (Found: M^+ 245.0731. $C_{10}H_{15}NO_4S$ requires M, 245.0722); $\lambda_{max}(H_2O)$/nm 308 (ε/$dm^3mol^{-1}cm^{-1}$ 5350).

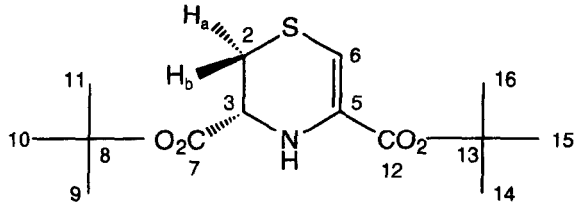
Dimethyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (240)



To a suspension of L-**(64)** (3.00 g, 15.86 mmol) in methanol (70 ml) was added concentrated sulfuric acid (1 ml) and the mixture was heated to reflux for 18 h. After this time the solvent was removed *in vacuo* and the resulting residue was carefully neutralised with sat. $NaHCO_3$ (aq) (50 ml) and extracted with ethyl acetate (2 x 50 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield a red oil. Purification was achieved on a silica column, eluting with 10 % EtOAc in hexane, to yield the desired diester as a lightly coloured solid, 2.10 g (9.67 mmol, 61.0%); mp 39-41 °C; R_f 0.44 (50% EtOAc in hexane); (Found: M^+, 217.0390; C, 44.33; H, 5.07; N, 6.22. $C_8H_{11}NO_4S$ requires M, 217.0409; C, 44.23; H, 5.10; N, 6.45 %); $\lambda_{max}(H_2O)$/nm 310 (ε/$dm^3mol^{-1}cm^{-1}$ 12150); ν_{max}(thin film)/cm^{-1} 3402 (br), 2954, 1744, 1710, 1606, 1438 and 1256; δ_H (200 MHz; $CDCl_3$) 2.95 (1H, ddd, J 12.4, 6.5, 0.66 Hz, 2-H_a), 3.14 (1H, ddd, J 12.4, 3.0, 0.78 Hz, 2-H_b), 3.71 and 3.72 (6H, 2 x s, 8 and 10-H_3), 4.21 (1H, dd, J 6.5, 3.0 Hz 3-H), 4.53 (1H, br s, N-H)

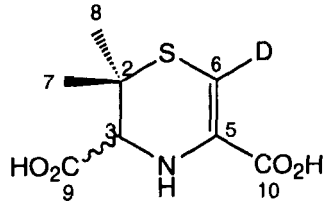
and 6.09 (1H, s, 6-H); δ_C (50 MHz) 26.7 (C-2), 52.1 and 52.7 (C-8 and C-10), 52.9 (C-3), 101.4 (C-6), 127.7 (C-5), 162.6 (C-9) and 170.6 (C-7); *m/z* 217 (M^+, 26.8%), 158, 126 (100%), 98, 87, 54 and 44.

Di-*tert*-butyl (*R*)-3,4-dihydro-2*H*-1,4-thiazine-3,5-dicarboxylate (241)



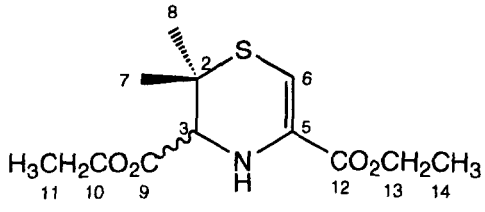
Compound **(241)** was synthesised from compound L-**(64)** (3.78 g, 20.00 mmol) and isobutylene (~ 20 ml) in anhydrous dichloromethane (250 ml) and c.H_2SO_4 (3 ml) according to **general procedure 4**. Purification was achieved on a silica dry flash column, eluting with hexane to remove the polyisobutylene byproduct and then 5% EtOAc in hexane, to yield the desired ester as a yellow coloured oil, 4.84 g (16.06 mmol, 80.3%); R_f 0.58 (50% EtOAc in hexane); (Found: M^+, 301.1350. $C_{14}H_{23}NO_4S$ requires M, 301.1348); ν_{max}(KBr)/cm^{-1} 3398, 2975, 1744, 1697, 1498; δ_H (200 MHz, $CDCl_3$) 1.44 and 1.46 (18H, 2 x s, 9, 10, 11- and 14, 15, 16-H_3), 2.89 (1H, dd, J 12.3, 7.0 Hz, 2-H_a), 3.13 (1H, dd, J 12.3, 2.2 Hz, 2-H_b), 4.08 (1H, m, 3-H), 4.66 (1H, br s, N-H) and 6.02 (1H, s 6-H); δ_C (50 MHz) 26.9 (C-2), 27.9 and 28.0 (C-9, 10, 11 and C-14, 15, 16), 53.5 (C-3), 81.4 and 82.3 (C-8 and C-13), 99.8 (C-6), 129.2 (C-5), 161.4 (C-12) and 169.2 (C-7); *m/z* 301 (M^+, 3.4%), 189, 169, 144, 126, 98, 72 and 57 (100%).

DL-6-Deutero-3,4-dihydro-2,2-dimethyl-2*H*-1,4-thiazine-3,5-dicarboxylic acid (243)



Bromopyruvic acid monohydrate (835 mg, 5.00 mmol) and DL-penicillamine (746 mg, 5.00 mmol) were dissolved in D_2O (5 ml), enclosed and left to stand at room temperature for 18 h. Precipitation of a white solid began after about 1 h. The suspension was filtered and the solid portion dried in a desiccator (P_2O_5) to yield the desired thiazine as a white solid, 795 mg (3.64 mmol, 72.9 %) mp 146-150 °C (dec.) (D_2O); (Found: M^+, 218.0479. $C_8H_{10}DNO_4S$ requires M, 218.0472); $\lambda_{max}(H_2O)$/nm 310 (ε/dm^{3}mol^{-1}cm^{-1} 6190); ν_{max}(KBr)/cm^{-1} 3390, 2974 (br), 1706, 1660, 1424 and 1295; δ_H (200 MHz; D_2O/CD_3OD) 1.20 and 1.38 (6H, 2 x s, 7- and 8-H_3), and 3.72 (1H, s, 3-H); δ_C (50 MHz) 25.4 and 28.4 (C-7 and C-8), 40.9 (C-2), 63.5 (C-3), 102.3 (C-6), 127.7 (C-5), 165.4 (C-10), and 173.2 (C-9); *m/z* 218 (M^+, 2.0%), 154, 128, 112, 82, 74, 59 and 44 (100%).

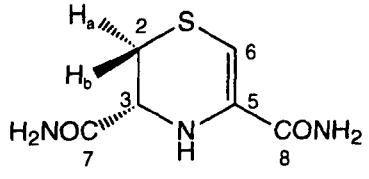
Diethyl DL-3,4-dihydro-2,2-dimethyl-2*H*-1,4-thiazine-3,5-dicarboxylate (244)



A solution of DL-penicillamine (2.00 g, 13.40 mmol) in glacial acetic acid (10 ml) was added to a stirred solution of bromopyruvic acid (2.24 g, 13.40 mmol) in glacial acetic acid (4 ml) and the resulting solution was stirred at room temperature overnight. After this time the solvent was removed under vacuum, freshly dried ethanol (50 ml) and c.H_2SO_4 (0.4 ml) were added and the solution was heated at reflux for 2 h. After cooling, the excess solvent was removed *in vacuo* and the resulting residue was suspended in sat. $NaHCO_3$ (aq) (50 ml) and extracted with EtOAc (3 x 50 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated. Purification was achieved on a silica column, eluting with 20% EtOAc in hexane, to yield the desired thiazine diester as a yellow oil, 240 mg (0.88 mmol, 6.6%); R_f 0.49 (50% EtOAc in hexane); (Found: M^+, 245.0726. $C_{10}H_{15}NO_4S$ requires M, 245.0722); $\lambda_{max}(H_2O)$/nm 310 (ε/dm^{3}mol^{-1}cm^{-1} 7020); ν_{max}(thin film)/cm^{-1} 3409 (br), 2982, 1738, 1704, 1607, 1462, 1369, 1250 and 1026; δ_H (200 MHz, $CDCl_3$) 1.22 and 1.39 (6H, 2 x s, 7- and 8-H_3), 1.23 and 1.24 (6H, 2 x t, J 7.1 Hz and 7.1 Hz, 11- and 14-H_3), 3.77 (1H, br d, J 2.8 Hz, 3-H), 4.17 (4H, 2 x q, J 7.1 Hz, 10- and 13-H_2), 4.74 (1H, br s, N-H) and 6.18 (1H, s, 6-H); δ_C (50 MHz) 12.6 (C-11 and C-14), 23.2 and 25.9 (C-7 and C-8), 38.9 (C-2), 59.5 and 59.8 (C-10 and C-13), 61.0 (C-3), 100.3 (C-

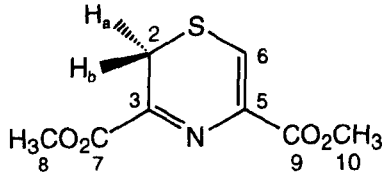
6), 124.6 (C-5), 160.7 (C-12) and 168.2 (C-9); *m/z* 273 (M^+, 17.5%), 260, 200,154 (100%), 126, 82, 71, 55 and 41.

(*R*)-3,4-Dihydro-2*H*-1,4-thiazine-3,5-dicarboxamide (245)[205]



Compound **(245)** was synthesised from compound **(235)** (10.67 g, 43.50 mmol) in methanol (200 ml), saturated with anhydrous ammonia gas, according to the method of Tudor.[205] After 48 h at room temperature, filtration yielded the desired diamide as a cream coloured solid, 4.84g (25.85 mmol, 59%); mp 204-206 °C (MeOH); R_f 0.38 (30% MeOH in $CHCl_3$); (Found: M^+, 187.0409. $C_6H_9N_3O_2S$ requires M, 187.0415); $\lambda_{max}(H_2O)$/nm 299 (ε/dm^{3}mol^{-1}cm^{-1} 2640); *m/z* 172, 158, 142, 126 (100%), 98, 72 and 54.

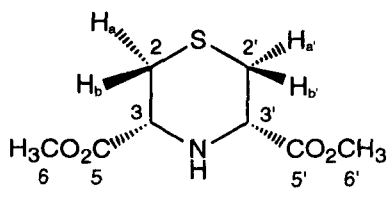
Dimethyl 1,4-thiazine-3,5-dicarboxylate (250)

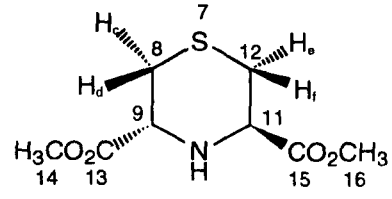


A solution of **(240)** (1.09 g, 5.00 mmol) in dichloromethane (20 ml) was added dropwise to a solution of DDQ (1.25 g, 5.50 mmol) in dichloromethane (70 ml) and stirred for 1 h at room temperature. The resulting suspension was filtered through celite and the filtrate was washed with sat. $NaHCO_3$ (3 x 100 ml). The organic portion was dried (Na_2SO_4), filtered and concentrated *in vacuo* to a orange coloured solid. Purification was achieved on a silica dry flash column eluting with 25% EtOAc in hexane, followed by crystallisation from ether/hexane to yield the desired thiazine as a yellow crystalline solid, 778 mg (3.61 mmol, 72.3%); mp 84-85 °C (Et_2O); R_f 0.16 (50% EtOAc in hexane); (Found: M^+, 215.0255; C, 44.75; H, 4.19; N, 6.47. $C_8H_9NO_4S$ requires M, 215.0252; C, 44.64; H, 4.22; N, 6.51%); $\lambda_{max}(H_2O)$/nm 371, 264 and 222 (ε/dm^{3}mol^{-1}cm^{-1} 4180, 7222 and 10320 respectively); ν_{max}(KBr)/cm^{-1} 3052,

2950, 1709, 1496, 1438, 1340, 1258, 1232 and 1089; δ_H (200 MHz, $CDCl_3$) 3.39 (2H, d, J 1.2 Hz, 2-H_2), 3.90 and 3.96 (6H, 2 x s, 8- and 10-H_3) and 7.66 (1H, t, J 1.2 Hz, 6-H); δ_C (50 MHz) 20.42 (C-2), 52.49 and 53.44 (C-8 and C-10), 128.86 (C-6), 136.69 and 137.44 (C-3 and C-5), 162.92 and 163.66 (C-7 and C-9); *m/z* 215 (M^+, 73.9%), 184, 170, 155 (100%), 128, 97, 84, 70 and 59.

cis- and *trans*-Dimethyl thiomorpholine-3,5-dicarboxylate (256)[179]



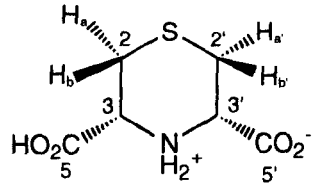


cis- and *trans*-**(256)** were synthesised from methyl DL-2,3-dibromopropionate (2.62 ml, 20.00 mmol) and dry triethylamine (2.79 ml, 20 mmol) in anhydrous ethanol (40 ml) and L-cysteine methyl ester hydrochloride (3.44 g, 20.00 mmol) and dry triethylamine (5.58 ml, 40 mmol) in anhydrous ethanol (50 ml) according to the method of Paradisi *et al.*[179] After cooling the solvent was removed *in vacuo* and the residue was suspended in water (50 ml) and extracted with EtOAc (3 x 50 ml). The combined organic extract was dried ($MgSO_4$), filtered and concentrated to an oil. The ratio of *cis* to *trans* diastereoisomers in the product was shown to be 40.3 : 59.7 respectively (GC). Careful purification on a silica column, eluting with 5% and then 10% EtOAc in hexane, resulted in the separation of the pure diastereoisomers.

<u>*cis*-Diastereoisomer *(meso)*:</u> lightly coloured solid, 1.49 g (6.81 mmol, 34.1%) mp 40-41 °C (Lit.[179] 43-44 °C); R_f 0.46 (EtOAc); $[\alpha]_D^{20}$ 0° (c 1.0, MeOH); (Found: M^+, 219.0556; C, 43.70; H, 5.98; N, 6.24. $C_8H_{13}NO_4S$ requires M, 219.0565; C, 43.82; H, 5.98; N, 6.39%); $\lambda_{max}(H_2O)$/nm 975 (ε/$dm^3mol^{-1}cm^{-1}$ 2400); δ_H (200 MHz, $CDCl_3$) 2.59 (2H, dd, J 13.5, 10.5 Hz, 2-H_a and 2'-$H_{a'}$), 2.68 (1H, s, N-H), 2.72 (2H, dd, J 13.5, 2.6 Hz, 2-H_b and 2'-$H_{b'}$), 3.64 (2H, dd, J 10.6, 2.6 Hz, 3- and 3'-H) and 3.70 (6H, s, 6 and 6'-H_3); δ_C (50 MHz) 28.9 (C-2 and C-2'), 52.4 (C-6 and C-6'), 59.4 (C-3 and C-3'), and 170.8 (C-5 and C-5'); *m/z* 219 (M^+, 8%), 160 (100%), 132, 114, 100, 87, 74, 54 and 42.

trans-Diastereoisomer *(threo)*: lightly coloured solid, 1.81 g (8.26 mmol, 41.3%); mp 30-34 °C (Lit.[179] 60 °C); R_f 0.37 (EtOAc); $[\alpha]_D^{20}$ -71.8° (c 1.0, MeOH) (Lit.[179] -73°); (Found: M^+, 219.0556; C, 43.64; H, 5.86; N, 6.20. $C_8H_{13}NO_4S$ requires M, 219.0565; C, 43.82; H, 5.98; N, 6.39%); $\lambda_{max}(H_2O)$/nm 976 (ε/dm^{3}mol^{-1}cm^{-1} 2410); δ_H (200 MHz, $CDCl_3$) 2.79 (5H, m, 8- and 12-H_2 and N-H), 3.70 (6H, s, 14- and 16-H_3), and 3.94 (2H, dd, J 6.2, 4.0 Hz, 9- and 11-H); δ_C (50 MHz) 29.6 (C-8 and C-12), 52.1 (C-14 and C-16), 55.1 (C-9 and C-11) and 171.8 (C-13 and C-15); *m/z* 219 (M^+, 10%), 160 (100%), 132, 114, 100, 87, 74, 54 and 42.

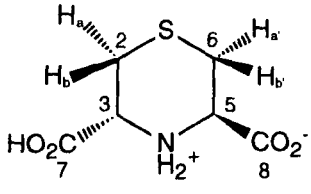
cis-(*meso*)-Thiomorpholine-3,5-dicarboxylic acid, *cis*-(253)[178]



A suspension of compound *cis*-**(256)** (210 mg, 0.96 mmol) in 0.200 M KOH (aq) (9.58 ml, 2 equiv.) was heated at reflux for 18 h. After cooling the solution was washed with ether (2 x 10 ml). The aqueous portion was adjusted to pH 1 by the careful addition of c. HCl (aq). Concentration under reduced pressure and drying in a desiccator (P_2O_5) yielded compound *cis*-**(253)**.HCl.2KCl as a light yellow coloured solid, 332 mg (0.88 mmol, 92.0%); (Found: M^+, 192.0320 $C_6H_9NO_4S$ requires MH, 192.0330). ν_{max}(KBr disc)/cm^{-1} 3426 (br), 2960 (br), 1761, 1741, 1378, 1192; δ_H (200 MHz, D_2O) 3.00 (4H, m, 2- and 2'-H_2) and 4.16 (2H, dd, J 11.7, 3.3 Hz, 3- and 3'-H); δ_C (50 MHz) 26.7 (C-2 and C-2'), 59.1 (C-3 and C-3') and 170.2 (C-5 and C-5'); *m/z* (FAB) 191 (M^+, 100%), 154, 136, 107, 90, 78, 40.

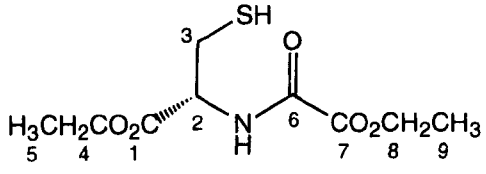
The literature[178] melting point for the free amino acid is 253-254 °C (dec.). The isomer isolated from the aqueous recrystallisation was not specified.

trans-(3*R*,5*R*)-Thiomorpholine-3,5-dicarboxylic acid, *trans*-(253)[178]



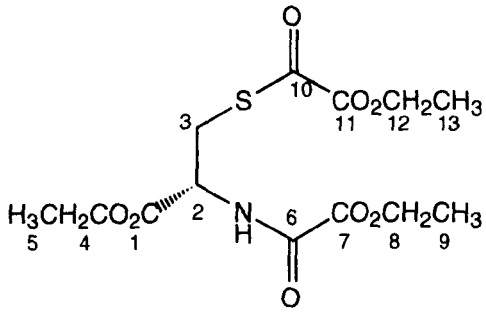
A suspension of compound *trans*-**(256)** (548 mg, 2.50 mmol) in 0.200 M KOH (aq) (25 ml, 2 equiv.) was heated at reflux for 18 h. After cooling the solution was washed with ether (2 x 20 ml). The aqueous portion was adjusted to pH 1 by the careful addition of c.HCl (aq). Concentration under reduced pressure and drying in a desiccator (P_2O_5) yielded compound *trans*-**(253)**.HCl.2KCl as a light yellow coloured solid, 930 mg (2.47 mmol, 98.7%); (Found: M^+, 191.0255. $C_6H_9NO_4S$ requires M, 191.0252); ν_{max}(KBr disc)/cm^{-1} 3460, 3410, 3005, 1664, 1579; δ_H (200 MHz, D_2O) 2.89 (4H, m, 2- and 6-H_2) and 4.17 (2H, m, 3- and 5-H); δ_C (50 MHz, D_6-DMSO) 27.4 (C-2 and C-6), 54.8 (C-3 and C-5) and 170.7 (C-7 and C-8); *m/z* 191 (M^+, 13.7%), 173, 146, 100 (100%), 73.

(*R*)-*N*-Ethoxyoxalylcysteine ethyl ester (265)[205]



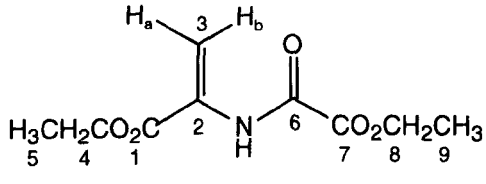
Ethyl oxalyl chloride (4.47 ml, 40.00 mmol) was added slowly over 10 min to a stirred suspension of L-cysteine ethyl ester hydrochloride (7.43 g, 40.00 mmol) and triethylamine (11.15 ml, 80.00 mmol) in anhydrous dichloromethane (100 ml), at room temperature under a nitrogen atmosphere. A rapid evolution of HCl gas and exotherm was observed. The reaction mix was allowed to cool to room temperature and stirred for a total of 3 h. The resulting suspension was washed with water (3 x 100 ml) and the organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a lightly coloured oil. Purification on a silica gel dry flash column, eluting with 25% EtOAc in hexane, yielded the desired amide as a colourless oil, 9.40 g (37.71 mmol, 94.3%); R_f 0.38 (50% EtOAc in hexane); (Found: M^+, 249.0666. $C_9H_{15}NO_5S$ requires M, 249.0671); ν_{max}(thin film)/cm^{-1} 3395, 3020, 1740, 1705, 1515; δ_H (200 MHz; $CDCl_3$) 1.32 and 1.40 (6H, 2 x t, J 7.2 and 7.2Hz, 5- and 9-H_3), 1.55 (1H, t, J 9.0 Hz, S-H), 3.08 (2H, dd, J 9.0, 4.5 Hz, 3-H_2), 4.28 (2H, dq, J 7.2, 1.2 Hz, 4- or 8-H_2), 4.38 (2H, q, J 7.2 Hz, 4- or 8-H_2), 4.84 (1H, dt, J 7.8, 4.5 Hz, 2-H) and 7.99 (1H, br d, J 7.6 Hz, N-H_2); δ_C (50 MHz) 12.2 and 12.4 (C-5 and C-9), 24.5 (C-3), 52.5 (C-2), 60.5 and 61.6 (C-4 and C-8), 154.6 (C-1), 158.1 (C-7) and 167.2 (C-6); *m/z* 249 (M^+, 0.5%), 204, 176, 158, 148, 132, 118 (100%) and 101.

(*R*)-*N,S*-Diethoxyoxalylcysteine ethyl ester (274)



To a suspension of L-cysteine ethyl ester hydrochloride (1.87 g, 10.00 mmol) in anhydrous dichloromethane (40 ml), under a nitrogen atmosphere, was added ethyl oxalyl chloride (2.23 ml, 20.00 mmol) and the solution was stirred at room temperature for 48 h. After this time the reaction mix was washed with water (3 x 20 ml) and the organic portion was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a yellow oil. Purification was achieved on dry silica flash column, eluting with 25% EtOAc in hexane, to yield compound **(274)** as a colourless oil, 2.92 g (8.36 mmol, 83.6%); R_f 0.30 (50% EtOAc in hexane); (Found: M^+, 349.0825. $C_{13}H_{19}NO_8S$ requires M, 349.0831) ν_{max}(KBr)/cm^{-1} 3353 (br), 2986, 1738, 1699, 1525, 1292, 1202 and 1025 ; δ_H (200 MHz, $CDCl_3$) 1.32 (3H, t, J 7.1 Hz, 5-H_3), 1.40 (6H, t, J 7.1 Hz, 9- and 13-H_3), 3.48 and 3.63 (2H, 2 x dd, J 14.2, 5.9 Hz and 14.2, 4.8 Hz, 3-H_a and 3-H_b), 4.26 (2H, t, J 7.1 Hz, 4-H_2), 4.37 and 4.38 (4H, 2 x q, J 7.1Hz and 7.1 Hz, 8- and 12-H_2), 4.87 (1H, m, 2-H_2) and 7.76 (1H, br d, J 7.7 Hz, N-H); δ_C (50 MHz) 13.9, 14.1 and 14.2 (C-5, C-9 and C-13), 30.5 (C-3), 51.9 (C-2), 62.7, 63.5 and 63.9 (C-4, C-8 and C-12), 158.45 (C-10), 159.57 (C-6), 168.66, 168.72 and 168.93 (C-1, C-7 and C-11); *m/z* 349 (M^+, 1.7%), 276, 248 (100%), 202, 176, 158, 118, 102, 86, 74, 59 and 42.

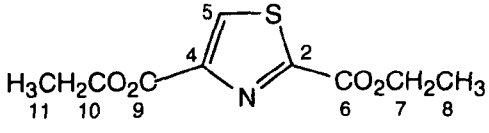
N-Ethoxyoxalyl-2-methyleneglycine ethyl ester (266)



Ethyl oxalyl chloride (1.12 ml, 10.00 mmol) was added slowly to a suspension of L-cysteine ethyl ester hydrochloride (1.86 g, 10.00 mmol) and Et_3N (10 ml, excess) in anhydrous dichloromethane under a nitrogen

atmosphere at 0 °C. The suspension was then stirred at room temperature for 2 h after which time the first step, substitution at nitrogen, was adjudged to be complete (TLC). The reaction mixture was recooled to 0 °C and phosphorus trichloride (0.87 ml, 10.00 mmol) was added slowly. After 10 min at 0 °C the reaction was quenched by adding water (50 ml) and stirred for a further 10 min. The organic portion was separated, washed with water (50 ml), dried ($MgSO_4$), filtered and concentrated to a red oil. Purification was achieved on a silica column, eluting with 25% EtOAc in hexane, to yield compound **(266)** as a white solid, 160 mg (0.74 mmol, 7.4%); mp ~200 °C (dec.); R_f 0.48 (50% EtOAc in hexane); (Found: M^+, 215.0796. $C_9H_{13}NO_5$ requires M, 215.0794); ν_{max}(KBr)/cm^{-1} 3411 (br), 2988, 1718, 1514, 1257 and 1018; δ_H (200 MHz, $CDCl_3$) 1.29 and 1.33 (6H, 2 x t, J 7.1 Hz and 7.1 Hz, 5- and 9-H_3), 4.25 and 4.31 (4H, 2 x q, J 7.1 Hz and 7.1 Hz, 4- and 8-H_2), 5.98 (1H, d, J 1.5 Hz, 3-H_b) (coupling lost by irradiating at 9.26 ppm), 6.63 (1H, s, 3-H_a), and 9.26 (1H, br s, N-H); δ_C (50 MHz) 13.8 and 13.9 (C-5 and C-9), 62.3 and 63.5 (C-4 and C-8), 110.7 (C-3), 130.4 (C-2), 154.5 (C-6), 159.7 and 162.9 (C-1 and C-7); *m/z* 215 (M^+, 25.8%), 142, 114, 96, 86, 72, 57, 49 and 42 (100%).

Diethyl 1,3-thiazole-2,4-dicarboxylate (238)[184]



Method 1

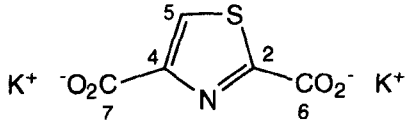
Compound **(238)** was synthesised from compound **(265)** (7.48 g, 30.00 mmol) and phosphorus pentasulfide (13.34 g, 30.00 mmol) in freshly distilled dried pyridine (50 ml) according to the method of Tudor.[205] An exotherm was noted prior to heating and it was observed that the solid quickly dissolved on heating. After reflux, the solvent was removed *in vacuo* and the resulting residue was suspended in water (200 ml) and extracted with EtOAc (3 x 100 ml). The organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a brown residue. Purification was achieved on a silica column, eluting with 10% EtOAc in hexane, to yield the desired thiazole as an orange solid, 992 mg (4.33 mmol, 14.4%).

Method 2

Compound **(238)** was synthesised from ethyl thiooxamate (2.04 g, 15.34 mmol) and ethyl bromopyruvate (3.09 g, 20.00 mmol) in ethanol (20 ml)

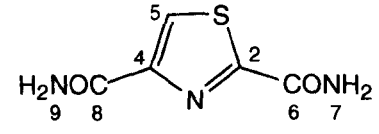
according to the method of Erlenmeyer and co-workers.[184] After heating at reflux for 3 h the solvent was removed *in vacuo*, the resulting residue dissolved in sat. Na_2CO_3 (aq) (25 ml) and extracted with ether (3 x 25 ml). The organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a red oil. Purification was achieved on a silica column, eluting with 20% EtOAc in hexane, followed by crystallisation from ether/pet. ether (40-60 °C) yielded the desired thiazole as a white crystalline solid, 2.12 g (9.26 mmol, 60%) The characterisation data was consistent with the literature:[206] mp 43-44 °C (Lit.[184] 43-44 °C); R_f 0.40 (50% EtOAc in hexane); (Found: C, 47.36; H, 4.73; N, 6.01. $C_9H_{11}NO_4S$ requires C, 47.16; H, 4.80; N, 6.11%); $\lambda_{max}(H_2O)$/nm 272, 209 (ε/$dm^3mol^{-1}cm^{-1}$ 7020, 19930 respectively).

1,3-Thiazole-2,4-dicarboxylic acid dipotassium salt (257)[184]



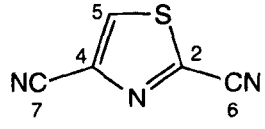
Compound **(257)** was prepared from a method adapted from that of Erlenmeyer and co-workers.[184] Compound **(238)** (713 mg, 3.11 mmol) was dissolved in ethanol (4 ml) and a solution of KOH (397 mg, 7.08 mmol) in water (0.5 ml) was added. The resulting suspension was heated to 60-70 °C in a sonic bath for 1 h. The resulting solid was dissolved in water, precipitated with ethanol, filtered and dried in a desiccator (P_2O_5). Purification was achieved by crystallisation from ~5% aqueous ethanol to yield the desired dicarboxylate salt as a cream coloured solid, 410 mg (1.64 mmol, 23.2%); mp 360-362 °C (dec. at ~320 °C); $\lambda_{max}(H_2O)$/nm 268 (ε/$dm^3mol^{-1}cm^{-1}$ 4820); ν_{max}(KBr)/cm^{-1} 3424 (br), 3076, 1609, 1485, 1462, 1365 and 1097; δ_H (200 MHz, D_2O) 8.04 (1H, s, 5-H) δ_C (50 MHz) 129.5 (C-5), 154.0 (C-4), 166.8 (C-2) and 169.8 (C-6 and C-7).

1,3-Thiazole-2,4-dicarboxamide (258)[206]



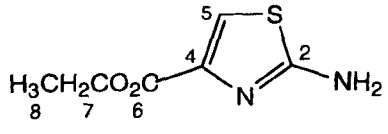
Compound **(258)** was synthesised from compound **(238)** (865 mg, 3.77 mmol) in methanol (20 ml), saturated with anhydrous ammonia gas, according to the method of Connell[206] to yield the desired diamide as a white solid, 550 mg (3.21 mmol, 85.2%); δ_H (200 MHz, $CDCl_3$) 7.76 and 8.21 (4H, 2 x br s, 7- and 9-H_2) and 8.46 (1H, s, 5-H); δ_C (50 MHz) 129.0 (C-5), 150.4 (C-4), 160.5 (C-2), 161.8 and 163.0 (C-6 and C-7).

1,3-Thiazole-2,4-dinitrile (239)[206]



Compound **(239)** was synthesised from compound **(258)** (470 mg, 2.75 mmol) and phosphorus oxychloride (2.56 ml, 10 equiv.) in 1,1,2,2-tetrachloroethane according to the method of Connell[206] to yield the desired dinitrile as white needles, 162 mg (1.20 mmol, 43.6%); mp 82-83 °C; R_f 0.46 (50% EtOAc in hexane); δ_H (200 MHz, $CDCl_3$) 8.35 (1H, s, 5-H); δ_C (50 MHz) 111.0 and 112.0 (C-6 and C-7), 129.3 (C-4), 134.6 (C-5) and 138.5 (C-2).

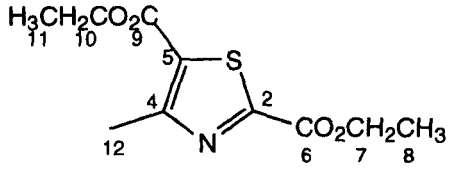
Ethyl 2-amino-1,3-thiazole-4-carboxylate (259)



Ethyl bromopyruvate (6.28 ml, 50.00 mmol) was added to a suspension of thiourea (3.81 g) and triethylamine (6.97 ml, 50.00 mmol) in ethanol (100 ml) and stirred at room temperature for 72 h. After this time, saturated aqueous Na_2CO_3 (25 ml) was added to the suspension and the residual solid was filtered. The filtrate was concentrated *in vacuo* and purified on a silica column, eluting with 30% EtOAc in hexane, followed by crystallisation from acetone/pet. ether (40-60 °C) to yield compound **(259)** as an orange solid, 5.25 g (30.48 mmol, 61.0%); mp 169-171 °C; R_f 0.35 (EtOAc); (Found: M^+, 172.0304; C, 41.80; H, 4.70; N, 16.20. $C_6H_8N_2O_2S$ requires M, 172.0306; C , 41.85; H, 4.68; N, 16.27%); $\lambda_{max}(H_2O)$/nm 281, 238 and 210 (ε/$dm^3mol^{-1}cm^{-1}$ 4750, 7430 and 20510 respectively); ν_{max}(KBr)/cm^{-1} 3440, 3264, 3126, 1690, 1618, 1538, 1340

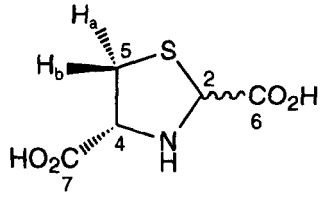
and 1242; δ_H (200 MHz, D_6-acetone) 1.17 (3H, t, J 7.1 Hz, 8-H_3), 4.11 (2H, q, J 7.1 Hz, 7-H_2), 6.91 (2H, br s, N-H_2), and 7.30 (1H, s, 5-H); δ_C (50 MHz) 14.6 (C-8), 60.9 (C-7), 117.2 (C-5), 143.9 (C-4), 161.9 (C-2), and 169.5 (C-6); *m/z* 172 (M^+, 56.0%), 144, 127, 100 (100%), 84, 71, 58 and 44.

Diethyl 4-methyl-1,3-thiazole-2,5-dicarboxylate (260)[185]



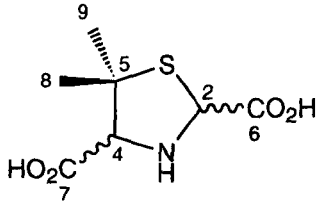
Compound **(260)** was synthesised from ethyl 2-chloroacetoacetate (0.484 ml, 3.50 mmol), ethyl thiooxamate (466 mg, 3.50 mmol) and ethanol (10 ml) according to the method of Boon.[185] After reflux the ethanol was removed *in vacuo*, the residue was neutralised with sat. $NaHCO_3$ (aq) (25 ml) and extracted with Et_2O (3 x 25 ml). The combined organic extract was dried ($MgSO_4$), filtered, and concentrated. Purification was achieved on a silica column, eluting with 10% EtOAc in hexane to yield the desired thiazole as a cream coloured solid, 298 mg (1.22 mmol, 35.0%); mp 53-54 °C (Lit. 59 °C); R_f 0.26 (25% EtOAc in hexane); (Found: M^+, 243.0565; C, 49.42; H, 5.29; N, 5.65. $C_{10}H_{13}NO_4S$ requires M, 243.0565; C, 49.37; H, 5.39; N, 5.76%); $\lambda_{max}(H_2O)$/nm 293, 246 and 206 (ε/$dm^3mol^{-1}cm^{-1}$ 12490, 6340 and 12370 respectively); ν_{max}(KBr)/cm^{-1} 2989, 1740, 1723, 1518, 1444, 1268, 1233 and 1104; δ_H (200 MHz, $CDCl_3$) 1.40 and 1.45 (6H, 2 x t, J 7.1 Hz, 8- and 11-H_3), 2.81 (3H, s, 12-H_3), 4.38 and 4.50 (4H, 2 x q, J 7.1 Hz, 7- and 10-H_2); δ_C (50 MHz) 14.0 (C-8 and C-11), 17.4 (C-12), 61.7 and 62.6 (C-7 and C-10), 127.4 (C-5), 158.9 (C-4), 159.4 (C-2), 161.0 and 161.3 (C-6 and C-9); *m/z* 243 (M^+, 15.0%), 198, 171 (100%), 143, 126, 113, 98, 85, 68 and 45.

(2*R*,4*R*)- and (2*S*,4*R*)-Thiazolidine-2,4-dicarboxylic acid (261)



L-Cysteine hydrochloride (4.00 g, 25.40 mmol) was suspended in ethanol (10 ml) and warmed to 70 °C. Glyoxylic acid (2.76 g, 30.00 mmol) was added in portions over 5 min to the resulting solution and a yellow colouration was observed. The solution was stirred at 70 °C for a further 5 min and the solvent was removed *in vacuo* to yield a yellow oil. Water (50 ml) was added to the mixture and the solution was left to crystallise at 0° C for 72 h. The white crystals were filtered off and dried in a desiccator (P_2O_5), 1.95 g (11.01 mmol, 43.3%); mp 179-183 °C (dec. at 170 °C) (H_2O); (Found: M^+, 177.0096; C, 34.07; H, 4.11; N, 7.49. $C_5H_7NO_4S$ requires M, 177.0096; C, 33.89; H, 3.98; N, 7.91%); ν_{max}(KBr)/cm^{-1} 3432 (br), 3049, 1719, 1628, 1542, 1413, 1384, 1334 and 1272; δ_H (200 MHz, CD_3OD) 1.20 (2H, m, 5-H_2), 4.13 (1H, m, 4-H) (decoupling at 1.20 ppm simplifies this multiplet), 4.50 and 4.53 (1H, 2 x d, J 6.4 Hz and 5.9 Hz, two diastereomeric forms of 2-H); δ_C (50 MHz, D_2O / NaOD) 38.4 (C-5), 68.0 and 69.4 (C-2 and C-4), 177.5 and 178.2 (C-6 and C-7); *m/z* 177 (M^+, 0.3%), 132, 118, 86 (100%), 60 and 44.

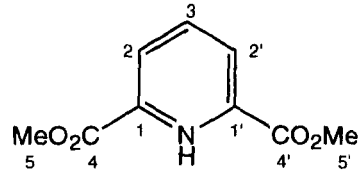
DL-5,5-Dimethylthiazolidine-2,4-dicarboxylic acid (262)[186]



Compound **(262)** was synthesised from DL-Penicillamine hydrochloride (1.24 g, 6.70 mmol) and glyoxylic acid (2 g, excess) in ethanol (10 ml) by the same method as that used for **(261)** and according to the method of Bentley *et al.*[186] After 20 min at 70 °C the solvent was removed *in vacuo*, water (20 ml) was added and the solution was left to crystallise at 0° C for 72 h. The desired thiazolidine was isolated as white needles after filtration and drying in a desiccator (P_2O_5), 773 mg (3.77 mmol, 56.2%); mp 198-199 °C (dec. at 175 °C) (H_2O); (Found: M^+, 205.0393; C, 40.12; H, 5.36; N, 6.50. $C_7H_{11}NO_4S$ requires M, 205.0409; C, 33.89; H, 3.98; N, 7.91%); ν_{max}(KBr)/cm^{-1} 3432 (br), 2987, 1727, 1640, 1587, 1461, 1375, 1282 and 1247; δ_H (200 MHz, D_6-DMSO) 1.20 and 1.59 (6H, 2 x s, 8- and 9-H_3), 3.65 (1H, s, 4-H), 4.95 (1H, s, 2-H) and 13.01 (weak, br s, 6- and 7-OH); δ_C (50 MHz) 26.6 and 27.5 (C-8 and C-9), 57.7 (C-5), 61.9 (C-4), 73.9 (C-2), 170.1 and 172.4 (C-6 and C-7); *m/z* 205 (M^+, 3.1%), 160 (100%), 114, 100, 86, 75, 55 and 43.

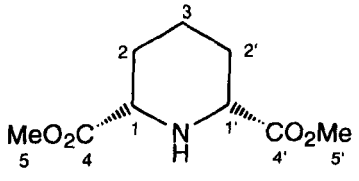
7.05 Experimental to Chapter 6

Dimethyl dipicolinate (34)[79]



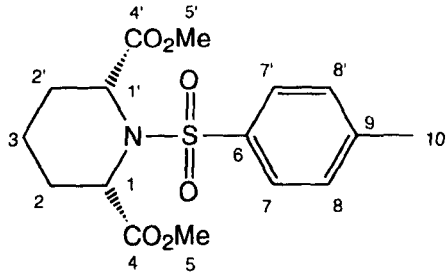
Compound **(34)** was synthesised from dipicolinic acid (16.71 g, 100.0 mmol) in methanol (200 ml) and c.H_2SO_4 (2 ml) according to the method of Robins and co-workers.[79] After reflux (30 h) the solvent was removed *in vacuo*, the residue was dissolved in sat. $NaHCO_3$ (aq) (100 ml) and extracted with $CHCl_3$ (2 x 100 ml). The combined organic extract was then washed with water (50 ml), dried ($MgSO_4$), filtered and concentrated to yield the desired diester as white prisms, 17.93 g (91.87 mmol, 91.9%); mp 118-120 °C (Lit.[79] 117-119 °C); R_f 0.18 (50% EtOAc in pet. ether (40/60 °C)); (Found: M^+, 196.0579. $C_9H_{10}NO_4$ requires M, 196.0610); $\lambda_{max}(H_2O)$/nm 222 (ε/$dm^3mol^{-1}cm^{-1}$ 9710).

Dimethyl *cis*-piperidine-2,6-dicarboxylate (50)[79]



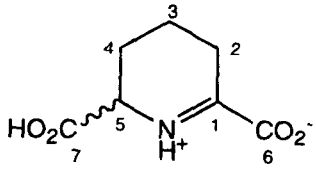
Compound **(50)** was synthesised from compound **(34)** (5.86 g, 30.00 mmol) in $CHCl_3$ (100 ml) by PtO_2 (3 x 100 mg) catalysed hydrogenation, according to the method of Robins and co-workers.[79] The reaction was monitored by TLC and the catalyst was added in three portions over an eight day period when deemed appropriate. A colour change from brown to black for the catalyst indicates reductive degradation. Filtration through celite with methanol (200 ml), followed by concentration and recrystallisation from methanol yielded the desired piperidine as a white solid, 5.65 g (28.07 mmol, 93.6%); mp 208-210 °C (Lit.[79] 210-212 °C); R_f 0.77 (50% EtOAc in Pet. Et. (40/60)); (Found: M^+, 201.1024. $C_9H_{15}NO_4$ requires M, 201.1001).

Dimethyl *N*-(4-toluenesulfonyl)-*cis*-piperidine-2,6-dicarboxylate (51)[79]



Compound **(51)** was synthesised from compound **(50)** (1.01 g, 5.00 mmol) and *p*-toluenesulfonyl chloride (1.91 g, 10.00 mmol) in freshly dried pyridine (10 ml) according to the method of Robins and co-workers[79] to yield the desired tosylate as white needles, 1.03 g (2.89 mmol, 57.9%); mp 59-61 °C (Lit.[79] 58-61 °C); R_f 0.42 (50% EtOAc in hexane); (Found: M^+, 355.1094. $C_{16}H_{21}NO_6S$ requires M, 355.1089); $\lambda_{max}(H_2O)$/nm 275 (ε/dm^{3}mol^{-1}cm^{-1} 1170).

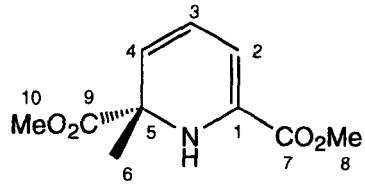
Dipotassium salt of DL-2,3,4,5-tetrahydrodipicolinate (52)[79]



Compound **(52)** was synthesised from compound **(51)** (400 mg, 1.13 mmol) and potassium *tert*-butoxide (450 mg, 4.01 mmol) in dry dichloromethane (10 ml) according to the method of Robins and co-workers.[79] Crystallisation from methanol proved to be very difficult to reproduce. The purity of the crude material (417 mg) was estimated as approximately 58% by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum indicates the presence of *tert*-butoxide and *para*-toluenesulfinate impurities. The crude material is a yellow solid and was found to be suitable for work with the DHDPR enzyme; 242 mg (0.98 mmol, 86.7%); The characterisation data was consistent with the literature:[79] δ_H (200 MHz, D_2O) 1.47 to 2.01 (6H, m, 2-, 3- and 4-H_2) and 3.46 (1H, dd, J 7.9, 3.5 Hz, 5-H). Signals visible for the enamine form at δ = 3.97 (1H, m, 5-H) and 5.47 (1H, t, J 4.1 Hz, 2-H).

7.06 Biotransformations with DHDPS

Dimethyl DL-1-hydro-2-methylpyridine-2,6-dicarboxylate (110)



In earlier chapters we have discussed typical enzyme inhibition and substrate assays. These assays were performed on an analytical scale. In order to attempt isolation of an enzymic product for full characterisation it was necessary to scale up the process to a preparative level. To achieve this goal solutions of sodium pyruvate (400 mM) and compound **(154)** (400 mM) were prepared in distilled water. Aliquots (250 µl) of these solutions were added in turn to a 1M aqueous imidazole buffer (pH 7.4; 250 µl) at room temperature in a 1 ml (0.5 cm) quartz cuvette. The enzymic reaction was initiated on addition of purified DHDPS in 50% aqueous glycerol (20 µl). The progress of the reaction was monitored spectrophotomerically at 370 nm. The absorbance maxima of the product is at 271 nm, monitoring the 'tail end' of this increasing absorbance ensures that we remain within the limits of detection for the incident UV beam. The enzymic reaction was complete after 90 min. The analysis of the enzymic reaction was discussed in chapter 6.

A number of variants of this assay were successfully performed with lesser concentrations of compound **(154)** and other substrates, including the natural substrate **(124)**. Results from these assays were similarly discussed in chapter 6.

References

1 R.T. Morrison and R.N. Boyd, *'Organic Chemistry'* (Fifth Edition), Allyn and Bacon Inc. (London), 1987, 1345-1352.

2 *'Handbook of Chemistry and Physics'* (72nd Ed.), CRC Press (Boston), 1991, **7**-1.

3 L. Stryer, *'Biochemistry'* (Fourth Edition), W.H. Freeman and Co. (New York), 1995, 713-723.

4 R.T. Morrison and R.N. Boyd, *'Organic Chemistry'* (Fifth Edition), Allyn and Bacon Inc. (London), 1987, 1355-1374.

5 P.F. Leadlay, *'An Introduction to Enzyme Chemistry'*, The Royal Society of Chemistry (Cambridge), 1978, 11-18.

6 T. Palmer, *'Understanding Enzymes'* (Second Edition), Ellis Horwood Ltd. (Chichester, UK), 80-84.

7 P.F. Leadlay, *'An Introduction to Enzyme Chemistry'*, The Royal Society of Chemistry (Cambridge), 1978, 24-32.

8 K.C. Nicolaou, Z. Yang, J.J. Liu, H. Ueno, P.G. Nantermet, R.K. Guy, C.F. Claiborne, J Renaud, E.A. Couladouros, K. Paulvannan and E.J. Sorensen, *Nature,* 1994, **367**, 630; K.C. Nicolaou and R.K. Guy, *Angew. Chem. Int. Ed. Engl.*, 1995, **34**, 2079.

9 R.A. Holton, C. Somoza, H.-B. Kim, F. Liang, R.J. Biediger, P.D. Boatman, M. Shindo, C.C. Smith, S. Kim, H. Nadizadeh, Y. Suzuki, C. Tao, P. Vu, S. Tang, P. Zhang, K.K. Murthi, L.N. Gentile and J.H. Liu, *J. Am. Chem. Soc.*, 1994, **116**, 1597.

10 J.J. Masters, J.T. Link, L.B. Snyder, W.B. Young and S.J. Danishefsky, *Angew. Chem. Int. Ed. Engl.*, 1995, **34**, 1723.

11 J-N Denis, A.E. Green, D. Guenard, F. Gueritte-Voegelein, L. Mangatal and P. Potier, *J. Am. Chem. Soc.*, 1988, **110**, 5917.

12 J-N Denis, A. Correa and A.E. Green, *J. Org. Chem.*, 1990, **55**, 1957.

13 L. Stryer, *'Biochemistry'* (Fourth Edition), W.H. Freeman and Co. (New York), 1995, 196-200.

14 H.G. Davies, R.H. Green, D.R. Kelly and S.M. Roberts, *'Biotransformations in Preperative Organic Chemistry'*, Academic Press (London), 1989, 1-12.

15 L. Stryer, *'Biochemistry'* (Fourth Edition), W.H. Freeman and Co. (New York), 1995, 45-52.

16 J. Gerstner, *Chemistry in Britain*, 1997, **33**, 40.

17 J. Mann, *'Chemical Aspects of Biosynthesis'*, Oxford University Press (Oxford), 1994, Chapter 6.

18 J. Mann, *'Secondary Metabolism'*, Oxford University Press (Oxford), 1992, 192.

19 R.R. Robins, T.W. Abraham, A.J. Parr, J. Eagles and N.J. Walton, *J. Am. Chem. Soc.*, 1997, **119**, 10929 and T.W. Abraham and E. Leete, *ibid.*, 1995, **117**, 8100.

20 O.P. Ward, *'Fermentation Biotechnology'*, Open University Press (Milton Keynes), 1989.

21 S. Kinoshitaa, K. Nakayama and S. Kitada, *J. Gen. Appl. Microbiol.*, 1954, **4**, 128.

22 K. Sano and I. Shiro, *J. Gen. Appl. Microbiol.*, 1970, **16**, 373.

23 T. Fufkumara, *Agric. Biol. Chem.*, 1976, **40**, 1687.

24 J.H. Vogel, *American Naturalist*, 1964, **98**, 435.

25 R.J. Cox, *Nat. Prod. Rep.*, 1996, **13**, 29-43.

26 C. Gilvarg and K. Bloch, *J. Biol. Chem.*, 1951, **193**, 339.

27 M. Strassman and S. Weinhouse, *J. Am. Chem. Soc.*, 1953, **75**, 1680.

28 N-Y. Chen, S-Q. Jiang, D.A. Klein and H. Paulus, *J. Biol. Chem.*, 1993, **268**, 9448.

29 J.G. Shedlarski and C. Gilvarg, *J. Biol. Chem.*, 1970, **245**, 1362.

30 S. Blickling, C. Renner, B. Laber, H-D. Pohlenz, T.A. Holak and R. Huber, *Biochem.*, 1997, **36**, 24.

31 S.H. Kindler and C. Gilvarg, *J. Biol. Chem.*, 1960, **235**, 3532.

32 D.A. Berges, W.E. DeWolf, G.L. Dunn, S.J. Newmann, J. Schmidt, J. Taggart and G. Gilvarg, *J. Biol. Chem.*, 1986, **261**, 6160.

33 H. Misono and K. Soda, *Agric. Biol. Chem.*, 1980, **44**, 227.

34 H. Misono and K. Soda, *J. Biol. Chem.*, 1980, **255**, 10599.

35 P.J. White, *J. Gen. Microbiol.*, 1983, **129**, 739.

36 G. Sundharadas and C. Gilvarg, *J. Biol. Chem.*, 1967, **242**, 3983.

37 B. Schrumpf, A. Schwarzer, J. Kalinowski, A. Puhler, L. Eggeling and H. Sahm, *J. Bacteriol.*, 1991, **173**, 4510.

38 J.D. Cirillo, T.R. Weisbrod, A. Banerjee, B.R. Bloom and W.R. Jacobs, *J. Bacteriol.*, 1994, **176**, 4424.

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

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

41 J.M. Ghuysen in *'Topics in Antibiotic Chemistry'*, (P.G. Sammes, Ed.), Ellis Horwood Ltd. (Chichester, UK),1980, Vol. 5.

42 H.G. Davies, R.H. Green, D.R. Kelly and S.M. Roberts, *' Biotransformtions in Preparative Organic Chemistry'*, Academic Press (London),1989, Ch. 5.

43 R.M. Cheshire and B.J. Miflin, *Phytochemistry*, 1975, **14**, 695.

44 R. M. Wallsgrove and M. Mazelis, *Phytochemistry*, 1981, **20**, 2651.

45 F. Richaud, C. Richaud, P. Ratet and J.C. Patte, *J. Bacteriol.*,1986, **166**, 297.

46 S. Bonnassie, J. Oreglia and A.M. Sicard, *Nucleic Acid Res.*, 1990, **18**, 6421.

47 E. Borthwick, *M.Sc. Thesis*, University of Glasgow, 1991, 27-43.

48 B.Laber, F-X. Gomis-Ruth, M.J. Romao and R. Huber, *Biochem. J.*,1992, **288**, 691.

49 C. Dereppe, G. Bold, O. Gishalba, E. Ebert and H.P. Schar, *Plant Physiol.*, 1992, **98**, 813.

50 E. Borthwick, S.J, Connell, D.W. Tudor, D.J. Robins, A. Shneier, C. Abell and J.R. Coggins, *Biochem. J.*, 1995, **305**, 521.

51 Y. Yugari and C. Gilvarg, *J. Biol. Chem.*, 1965, **240**, 4710.

52 E. Borthwick, *M.Sc. Thesis*, University of Glasgow, 1991, 55-71.

53 R. Kumpaisal, T. Hashimoto and Y. Yamada, *Plant Physiol.*, 1987, **85**, 145.

54 D.A. Frisch, B.G. Gengenbach, A.M. Tommey, J.M. Sellner, D.A. Somers and D.E. Myres, *Plant Physiol.*, 1991, **96**, 444.

55 C. Mirwaldt, I. Korndorfer and R. Huber, *J. Mol. Biol.*,1995, **246**, 227.

56 D.W. Tudor, T. Lewis and D.J. Robins, *Synthesis*, 1993, 1061.

57 W.E. Karsten, *FASEB J.*, 1995, **9**, A1298.

58 R. Kumpaisal, T. Hashimoto and Y. Yamanga, *Agric. Biol. Chem.*, 1989, **53**, 355.

59 L. Couper, J.E. McKendrick, D.J. Robins and E.J.T. Chrystal, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2267.

60 J.E. Mckendrick, *Ph.D. Thesis*, University of Glasgow, 1995, 154.

61 D.R. Walters, A. McPherson and D.J. Robins, *Mycol. Res.*, 1997, **101**, 329.

62 A.T.M. Bartlett and P.J. White, *J. Gen. Biochem.*, 1986, **132**, 3169.

63 A. Selli, F. Crociani, D. DiGinola, F. Fara, G. Grisetig and D. Matteuzzi, *Italian J. Biochem.*, 1994, **43**, 29.

64 M. Mazelis, F.R. Whatley, and J. Whatley, *FEBS Lett.*, 1977, **84**, 236.

65 M. Ghislain, V. Frankard and M. Jacobs, *Planta*, 1990, **180**, 480.

66 K. Sonntag, L. Eggeling, A.A. De Graaf and H. Sahm, *Eur. J. Biochem.*, 1993, **213**, 1325.

67 I. Negrutui, A. Cattoir-Reynearts, I. Verruggen and M. Jacobs, *Theor. Appl. Genet.*, 1984, **68**, 11.

68 W. Farkas and C. Gilvarg, *J. Biol. Chem.*, 1965, **240**, 4717.

69 H. Tamir and C. Gilvarg, *J. Biol. Chem.*, 1974, **249**, 3034.

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

71 K. Kimura and T. Goto, *J. Biochem.*, 1977, **81**, 1367.

72 S.P. Chatterjee, B.K. Singh and C. Gilvarg, *Plant Mol. Biol.*, 1994, **26**, 285.

73 J. Bouvier, C. Richaud, F. Richaud, J-C. Patte and P. Stragier, *J. Biol. Chem.*, 1984, **259**, 14829.

74 S.G. Reddy, J.C. Blanchard and J.C. Sacchettini, *Biochemistry*, 1995, **34**, 3492.

75 K. Kimura, *J. Biochem.*, 1974, **75**, 961.

76 P. Shapshak, *Diss. Abstr. Int. B*, 1970, **31**, 521.

77 K. Kimura and T. Sasakawa, *J. Biochem.*, 1975, **78**, 381.

78 E.J.T. Chrystal, L. Couper and D.J. Robins, *Tetrahedron Lett.*, 1992, **33**, 2717.

79 E.J.T. Chrystal, L. Couper and D.J. Robins, *Tetrahedron*, 1995, **51**, 10241.

80 G. Scapin, J.S. Blanchard and J.C. Sacchettini, *Biochemistry*, 1995, **34**, 3502.

81 J.L. Roberts, J. Borgese, C. Chan, D.D. Keith and C-C. Wei, *Heterocycles*, 1993, **35**, 115.

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

83 S.D. Abbott, P. Lane-Bell, K.P.S. Sidhu and J.C. Vederas, *J. Am. Chem. Soc.*, 1994, **116**, 6513.

84 J.E. Mckendrick, *Ph.D. Thesis*, University of Glasgow, 1995, Chapters 4 and 7.

85 S. Black and N.G. Wright, *J. Biol. Chem.*, 1954, **213**, 51.

86 R.M. Williams, *Aldrichimica Acta.*, 1992, **24**, 11. (Stereoselective synthesis of α-amino acids); P. Cintas, *Tetrahedron*, 1991, **47**, 6079 (Stereoselective synthesis of α-amino acids); *'Amino Acids, Peptides and Proteins'* (Vol. 25), Royal Society of Chemistry, 1994; U. Schollkopf, *Pure Appl. Chem.*, 1983, **55**, 1799. (Metallated Bis-lactim Ethers of 2,5-Diketopiperazines); U. Schollkopf, *Top. Curr. Chem.*, 1983, **109**, 65. (Stereoselective synthesis of α-amino acid); P.D. Bailey, A.N. Boa and J. Clayson, 1995, **2**, 173. (Synthesis of α-Amino Acids via α-Cation Equivalents); R. Breslow, J Chmielewski, D. Foley, B. Johnson, N. Kumabe, M. Varney and R. Mehra, *Tetrahedron*, 1988, **44**, 5515. (Amino Acid Synthesis using Artificial Transaminase Enzymes); M.A. Verkhovskaya and I.A. Yamskov, *Russ. Chem. Rev. (Engl. Transl.)*, 1991, **60**, 1163. (Enzymic Resolution of Amino Acids).

87 M.J. O'Donnell, *Tetrahedron*, 1988, **44**, 5253-5614.

88 R.O. Duthaler, *Tetrahedron*, 1994, **50**, 1539-1650.

89 R.M. Williams, *'Synthesesof Optically Active α-Amino Acids,'* Pergamon Press.(Oxford), 1985.

90 J. Jones, *'Amino Acid and Peptide Synthesis'*, Oxford University Press (UK), 1992.

91 A. Strecker, *Liebigs Ann. Chem.*, 1850, **75**, 27.

92 J. March, Advanced Organic Chemistry (4th Ed.), John Wiley and Sons, New York, 1992, p 590.

93 For a review see: M.S. Gibson and R.W Bradshaw, *Angew. Chem. Int. Ed. Engl.*, 1968, **7**, 919.

94 For a review see: N. Blazevic, D. Kolbah, B. Belin, V. Sunjic and F. Kajfez, *Synthesis*, 1979, 161-176.

95 W. Bestmann, *Chem. Ber.*, 1984, **117**, 1250.

96 W. Oppolzer, *Pure Appl. Chem.*, 1990, **62**, 1241; W. Oppolzer and O. Tamura, *Tetrahedron Lett.*, 1990, **31**, 991; W. Oppolzer, O. Tamura and J. Deerberg, *Helv. Chim. Acta*, 1992, **75**, 1965, W. Oppolzer and Ph. Lienard, *ibid.*, 2572; W. Oppolzer, P. Cintas-Moreno, O. Tamura and F. Cardinaux, *ibid.*, 1993, **76**, 187.

97 M.S. Patel, M. Worsley, *Can. J. Chem.*, 1970, **48**, 1881.

98 For a brief overview with references: K.C. Nicolaou and E.J. Sorensen, *'Classics in Total Synthesis'*, VCH Publishers (New York), 1996, p 358.

99 M.J. O'Donnell and T.M. Eckrich, *Tetrahedron Lett.*, 1978, **47**, 4625.

100 M.J. O'Donnell, W.D. Bennett and S. Wu, *J. Am. Chem. Soc.*, 1989, **111**, 2353.

101 R. Kober, W. Hammes and W. Steglich, *Agnew. Chem. Int. Ed. Engl.*, 1982, **21**, 203.

102 R. Kober and W. Steglich, *Liebigs Ann. Chem.*, 1983, 599.

103 P. Munster and W. Steglich, *Synthesis*, 1987, 223.

104 U.Schollkopf, H-J. Neubauer and M. Hauptreif, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 1066.

105 M.J. O'Donnell and W.D. Bennett, *Tetrahedron*, 1988, **44**, 5389.

106 L.D. Arnold, T.H. Kalantar and J.C. Vederas, *J. Am. Chem. Soc.*, 1985, **107**, 7105. Also see: N. Kucharczyk, B. Badet and F. LeGoffic, *Synth. Commun.*, 1989, **19**, 1603.

107 L.D. Arnold, T.H. Kalantar and J.C. Vederas, *J. Am. Chem. Soc.*, 1985, **107**, 7105; L.D. Arnold, J.C.G. Drover and J.C. Vederas, *J. Am. Chem. Soc.*, 1987, **109**, 4649.

108 J.C. Sheehan and K.R. Henery-Logan, *J. Am. Chem. Soc.*, 1957, **79**, 1262; J.C. Sheehan and K.R. Henery-Logan, *ibid.*, 1959, **81**, 3089.

109 For example: K. Jarowicki and P. Kocienski, *Contemporary Organic Synthesis*, 1995, **2**, 315.

110 T.W. Greene and P.G.M. Wuts, *'Protective Groups in Organic Synthesis'* (2nd Ed.), John Wiley and Sons (Chichester), 1991.

111 P.J. Kocienski, *'Protecting Groups,'* Georg Theme Verlag (Stuttgart), 1994. See also: H. Kunz and H. Waldmann, *'Comprehensive Organic Synthesis'* (Vol. 6), Pergamon Press (Oxford), 1991.

112 D.W. Tudor, *PhD Thesis*, University of Glasgow, 1992, 98.

113 S.J. Connell, *PhD Thesis*, University of Glasgow, 1995, 82.

114 J.E. McKendrick, *PhD Thesis*, University of Glasgow, 1995, pages 74, 95 and 152.

115 S. Black and N.G. Wright, *J. Biol. Chem.*, 1955, **213**, 39.

116 J.M. McKintosh, R.K. Leavitt, P. Mishra, K.C. Cassidy, J.E. Drake and R. Chadha, *J. Org. Chem.*, 1988, **53**, 1947.

117 D.A. Evans, J.A. Ellman and R.L. Dorow, *Tetrahedron Lett.*, 1987, **28**, 1123. See also: D.A. Evans, T.C. Britton and R.L. Dorow, *J. Am. Chem. Soc.*, 1986, **108**, 6395; D.A. Evans, T.C. Britton and R.L. Dorow, *Tetrahedron.*, 1988, **44**, 5525; D.A. Evans and T.C. Britton, *J. Am. Chem. Soc.*, 1987, **109**, 6881.

118 P.J. Sinclair, D. Zhai, J. Reibenspies and R.M. Williams, *J. Am. Chem. Soc.*, 1986, **108**, 1103; R.M. Williams, P.J. Sinclair, D. Zhai and D. Chen, *J. Am. Chem. Soc.*, 1988, **110**, 1547; R.M. Williams and M-N. Im, *Tertrahedron Lett.*, 1988, **29**, 6075.

119 W. Oppolzer and R. Moretti, *Helv. Chim. Acta*, 1986, **69**, 1923; W. Oppolzer and R. Moretti, *Tetrahedron*, 1988, **44**, 5541; W. Oppolzer, R. Pedrosa and R. Moretti, *Tetrahedron Lett.*, 1986, **27**, 831.

120 C.G. Skinner, J. Edelson and W. Shive, *J. Am. Chem. Soc.*, 1961, **83**, 2281.

121 P.A. Bartlett and J.F. Barstow, *J. Org. Chem.*, 1982, **47**, 3933.

122 U. Kazmaier, *Angew. Chem. Int. Ed. Engl.*, 1994, **33**, 998.

123 C.V. Coulter, J.A. Gerrard, J.A.E. Kraunsoe, D.J. Moore and A.J. Pratt, *Tetrahedron*, 1996, **52**, 7127.

124 R.D.G. Cooper, F. Jose, L. McShane and G.A. Koppel, *Tetrahedron Lett.*, 1978, 2243.

125 J. E. Baldwin and A. Flinn, *Tetrahedron Lett.*, 1987, **28**, 3605.

126 S.C. Bergmeier, A.A. Cobas and H. Rapoport, *J. Org. Chem.*, 1993, **58**, 2369.

127 F.S. Gibson, S.G. Bergmeier and H. Rapoport, *J. Org. Chem.*, 1994, **59**, 3217.

128 D.M. Brunwin and G. Lowe, *J. Chem. Soc., Perkin Trans.1*, 1973, 1321.

129 D. Seebach and A. Fadel, *Helv. Chim. Acta*, 1985, **68**, 1243.

130 A. Frauer, M. Mehlfuhrer, K. Thirring and H. Berner, *J. Org. Chem.*, 1994, **59**, 4215.

131 F. Alonso and S.D. Davis, *Tetrahedron Asymmetry*, 1995, **6**, 353.

132 For a review see: A. Furstner, *Synthesis*, 1989, 571-590.

133 C. Petrier, J. Einhorn and J-L. Luche, *Tetrahedron Lett.*, 1985, **26**, 1449; C. Petrier, and J-L. Luche, *J. Org. Chem.*, 1985, **50**, 912.

134 S. Hanessian and R-Y Yang, *Tetrahedron Lett.*, 1996, **37**, 5273 and 8997.

135 J.D.M. Herscheid, J.H. Colstee and H.C.J. Ottenheijm, *J. Org. Chem.*, 1981, **46**, 3346.

136 M. Nitta and T. Kobayashi, *J. Chem. Soc. Perkin Trans. 1*, 1985, 1401.

137 S. Cicchi, A. Goti, A. Brandi, A. Guarna and F. DeSarlo, *Tetrahedron Lett.*, 1990, **31**, 3351.

138 H.J. Vogel and B.D. Davis, *J. Am. Chem. Soc.*, 1952, **74**, 109.

139 H.J. Strecker, *J. Biol. Chem.*, 1960, **235**, 2045.

140 H. Eto, C. Eguchi and T. Kagawa, *Bull. Chem. Soc. Jpn.*, 1989, **62**, 961.

141 T. Okuda, *Bull. Chem. Soc. Jpn.*, 1957, **30**, 358.

142 I. Williams and L. Frank, *Anal. Biochemistry*, 1975, **64**, 85.

143 S.L Bearne and R. Wolfenden, *Biochemistry*, 1995, **34**, 11515.

144 S.J. Connell, *PhD Thesis*, University of Glasgow, 1995, Chapter 4.

145 R.K. Olsen, K. Ramasamy and T. Emery, *J. Org. Chem.*, 1984, **49**, 3527.

146 L. Grehn and U. Ragnarsson, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 510, also K. Gunnarsson, L. Grehn and U. Ragnarsson, *Agnew. Chem. Int. Ed. Eng.*, 1988, **27**, 400.

147 J.E. McKendrick, *PhD Thesis*, University of Glasgow, 1995, 121.

148 J.G. Moffat, *Organic Syntheses (Collective Vol. 5)*, 1973, 242.

149 G. Bold, H. Steiner, L. Moesch and B. Walliser, *Helv. Chim. Acta*, 1990, **73**, 405.

150 B.H. Lee and M.J. Miller, *Tetrahedron Lett.*, 1984, **25**, 927.

151 A. Devos, J. Remion, A-M. Frisque-Hesbain, A. Colens and L. Ghosez, *J. Chem. Soc. Chem. Commun.*, 1979, 1180.

152 D.W. Tudor, *PhD Thesis*, University of Glasgow, 1992, 94.

153 S. Kondo, *Laid-Open Patent Gazette*, 1980, Laid-Open No. 80-38306.

154 S.J. Connell, *PhD Thesis*, University of Glasgow, 1995, 100.

155 D. Enders, H. Dyker and G. Raabe, *Angew. Chem. Int. Ed. Engl.*, 1993, **32**, 421.

156 H.C. Brown, S.U. Kulkarni, C.G. Rao and V.D. Patil, *Tetrahedron*, 1986, **42**, 5515.
157 D.B. Dess and J.C. Martin, *J. Org. Chem.*, 1983, **48**, 4155.
158 H.C. Brown and S. Narasimhan, *J. Org. Chem.*, 1982, **47**, 1606.
159 M.H. Yates, *Tetrahedron Lett.*, 1997, **38**, 2813.
160 G.W. Kenner and J.H. Seely, *J. Am. Chem. Soc.*, 1972, **94**, 3259.
161 E. Abderhalden and E. Schwab, *Z. Phisiol. Chem.*, 1926, **153**, 88.
162 R.J. Cox, W.A. Sherwin, L.K.P. Lam and J.P. Vederas, *J. Am. Chem. Soc.*, 1996, **118**, 7449.
163 K. Agouridas, J.M. Girodeau and R. Pineau, *Tetrahedron Lett.*, 1985, **26**, 3115.
164 D. Tudor, *Ph. D. Thesis*, University of Glasgow, 1993, Chapter 4.
165 L. Couper, *Ph. D. Thesis*, University of Glasgow, 1991, 155.
166 H. Goldfine, *Biochim. Biophys. Acta.*, 1960, **40**, 557.
167 A.J.L. Cooper, J.Z. Ginos and A. Meister, *Chem. Rev.*, 1983, **83**, 321.
168 J.P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, J. Wiley, New York, 1961, Vol. 1, 593 and Vol. 3, 1906.
169 A.I. Vogel, *Practical Organic Chemistry*, Longman, London, 5th Ed., 1989, 1258.
170 D. Tudor, *Ph. D. Thesis*, University of Glasgow, 1993, 121.
171 S.J. Connell, *Ph. D. Thesis,* University of Glasgow, 1995, 113.
172 D. Tudor, *Ph. D. Thesis*, University of Glasgow, 1993, 199.
173 D.A. Berges and J.J. Taggart, *J. Org. Chem.*, 1985, **50**, 413.
174 G.H. Olah, Y.D. Vankar and A. Garcia-Luna, *Synthesis*, 1979, 227.
175 D.H.R. Barton, L. Bould, D.L.J. Clive, P.D. Magnus and T. Hase, *J. Chem. Soc. (C)*, 1971, 2204.
176 M. Node, K. Nishide, M. Sai and E. Fujita, *Tetrahedron Lett.*, 1978, **52**, 5211 and M. Node, K. Nishide, M. Sai, K. Fuji and E. Fujita, *J. Org. Chem.*, 1981, **46**, 1991.
177 P.G. Gassman and W.M. Schenk, *J. Org. Chem.*, 1977, **42**, 919.
178 R.D. Coghill, *J. Am. Chem. Soc.*, 1937, **59**, 801. See also related articles: H.I. Miner, E.O. Hook and R.D. Coghill, *ibid.*, 1940, **62**, 1613 and 1615.
179 M.P. Paradisi, G.P. Zecchini, I. Torrini and G. Lucente, *J. Heterocyclic Chem.*, 1990, **27**, 1661.
180 G. Portalone, A. Cassetta, G.P. Zecchini and I. Torrini, *Acta Cryst. (C)*, 1993, **49**, 976.
181 D. Tudor, *Ph. D. Thesis*, University of Glasgow, 1993, 212.
182 For a review see: G. Fodor and S. Nagubandi, *Tetrahedron*, 1980, **36**, 1279.

183 *The Chemistry of Penicillin*, Princeton University Press, 1949, 470.
184 H. Erlenmeyer, J. Junod, W. Guex and M. Erne, *Helv. Chim. Acta*, 1948, **31**, 1342.
185 W.R. Boon, *J. Chem. Soc.*, 1945, 601.
186 R. Bentley, A.H. Cook and J.A. Elvidige, *J. Chem. Soc.*, 1949, 3216.
187 Royal Society of Chemistry, *J. Chem. Soc., Perkin Trans. 1*, 1996, ix.
188 D.D. Perrin and L.F. Armarego, *'Purification of Laboratory Chemicals'*, 1992, Pergamon press, UK.
189 J.E. McKendrick, PhD Thesis, University of Glasgow, 1995, Section 8.2.
190 D.W. Tudor, PhD Thesis, University of Glasgow, 1993, Section 6.2.
191 O. Kitagawa, T. Hanano, N. Kikuchi and T. Taguchi, *Tetrahedron Lett.*, 1993, **34**, 2165.
192 E. Altmann, K. Nebel and M. Mutter, *Helv. Chim. Acta*, 1991, **74**, 800.
193 Aldrich Catalogue of Fine Chemicals, 1992 / 1993, 230.
194 Aldrich Catalogue of Fine Chemicals, 1994 / 1995, 261.
195 *Dictonary of Organic Compounds (6 th Ed.)*, Chapman and Hall, 1996, A-0-03350.
196 N.J. Turner and G.M. Whitesides, *J. Am. Chem. Soc.*, 1989, **111**, 624.
197 S.J. Connell, PhD Thesis, University of Glasgow, 1995, section 7.3.
198 J.E. Baldwin, C. Hulme and C.J. Schofield, *J. Chem. Res. Miniprint*, 1992, 1517.
199 J.E. McKendrick, PhD Thesis, University of Glasgow, 1995, Section 8.4.
200 D.W. Sullins, T.A. Bobik, R.S. Wolfe and K.L. Rinehart, *J. Am. Chem. Soc.*, 1993, **115**, 6646.
201 B.H. Lee, G.J. Gerfen and M.J. Miller, *J. Org. Chem.*, 1984, **49**, 2418.
202 A.I. Vogel, Textbook of Practical Organic Chemistry, 4th ed., Longman, London and New York, 1978, 376.
203 A.I. Vogel, Textbook of Practical Organic Chemistry, 4th ed., Longman, London and New York, 1978, 1112.
204 D.W. Tudor, PhD Thesis, University of Glasgow, 1993, Section 6.2.
205 D.W. Tudor, PhD Thesis, University of Glasgow, 1993, Section 6.3.
206 S.J. Connell, PhD Thesis, University of Glasgow, 1995, Section 7.4.