ASYMMETRIC SYNTHESIS OF AMINO ACID DERIVATIVES AS MOLECULAR PROBES FOR THE MECHANISM OF 3-METHYLASPARTATE AMMONIA-LYASE

Philippe Lasry

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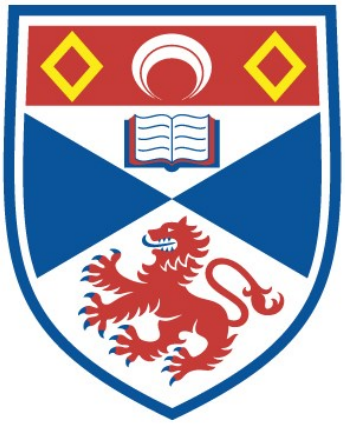
at the
University of St Andrews



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ASYMMETRIC SYNTHESIS OF AMINO ACID DERIVATIVES AS MOLECULAR PROBES FOR THE MECHANISM OF 3-METHYLASPARTATE AMMONIA-LYASE

a thesis presented by

Philippe Lasry

to the

University of St. Andrews

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

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I hereby certify that the candidate has fulfilled the conditions of Resolution appropriate to the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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à mes parents

Abstract

3-Methylaspartate ammonia-lyase is a bacterial enzyme that catalyses the reversible elimination of ammonia, in the presence of monovalent and divalent metal ions, from (2*S*,3*S*)-3-methylaspartic acid to yield mesaconic acid. The catalytic mechanism of the wild type enzyme obtained from *Clostridium tetanomorphum* involves a concerted elimination of ammonia and is thought to operate *via* the intermediacy of a dehydroalanine residue at the active site.

No direct evidence has been established so far concerning the existence of this dehydroalanine residue, except that 3-methylaspartate ammonia-lyase was found to be irreversibly inactivated by phenylhydrazine. Inactivation of the enzyme with labelled $^{15}N_{\beta}$-phenylhydrazine and subsequent ^{15}N-edited NMR spectra of the digested enzyme resulted in a proposal for the inhibition mechanism by phenylhydrazine. This mechanism involved the conjugate addition of phenylhydrazine onto the dehydroalanine moiety and the subsequent cyclisation to give a cyclic adduct. A synthetic model containing a ^{15}N atom of this adduct was synthesised and the NMR data obtained were compared with the spectra obtained earlier from the enzyme experiment.

In order to carry out mapping studies of the active site it was highly desirable to prepare molecules capable of reacting specifically and irreversibly at the active site.

To this end, the synthesis of aminofumaric acid and (2*S*,3*S*)-3-methyl *N*-amino aziridine dicarboxylic acid were attempted. (2*S*,3*R*) and (2*R*,3*R*)-3-Methylsuccinic acids were prepared in 5 steps from (2*S*,3*S*)-3-methyl aspartic acid. These compounds were found to behave as non-competitive inhibitors of β-methylaspartase. (2*S*)-3,3-Dimethylaspartic acid was also synthesised and found to be a competitive inhibitor having the same binding affinity as the natural substrate.

In order to prepare a β-electrophilic amino acid analogue of the physiological substrate, an asymmetric synthesis was designed that involved the stereoselective alkylation and acylation of suitably protected (2*S*,3*S*)-3-methylaspartic acid. The stereochemistry of the reaction was established *via* the X-ray structure of the camphanic derivative resulting from the alkylation of protected (2*S*,3*S*)-3-methylaspartic acid with chloroiodomethane. The X-ray structure of an acylated adduct was also determined. This method allowed diastereoselective synthesis of a highly functionalised amino acid, (2*S*,3*S*)-3-formyl-3-methylaspartic acid, in 7 steps which was found to be a powerful competitive inhibitor ($K_i = 0.57\ \mu M$). The inhibitory properties could be explained *via* the interaction of the cyclic furanyl form of the aldehyde. In addition, this synthesis also proved to be useful in the synthesis of a stable protected *trans*-(2*S*,3*S*)-3-methylazetidine 1,2-dicarboxylate diester for which a X-ray structure was determined.

Abbreviations

HAL-Histidine Ammonia-Lyase

AL- Aspartase Ammonia-Lyase

PAL - Phenylalanine Ammonia-Lyase

MAL - 3-Methylaspartate Ammonia-Lyase

Cbz - Carbobenzyloxy

Boc - *t*-Butoxycarbonyl

DMF - *N*, *N*-Dimethylformamide

THF - Tetrahydrofuran

DCM - Dichloromethane

TFA - Trifluoroacetic acid

m-CPBA - *meta*-Chloroperbenzoic acid

PMB - *p*-Methoxy Benzyl

D - Deuterium

t. l. c - Thin Layer Chromatography

h - hour

IR - Infra-red Spectroscopy

min - Minutes

rt - Room Temperature

V_{max} - Maximun rate

K_m - Michaelis constant

K_i - Dissociation constant of Inhibitor

Amino acid	Three letter code	One Letter code
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	ILe	I
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Serine	Ser	S
Threonine	Thr	T
Cysteine	Cys	C
Methionine	Met	M
Asparagine	Asn	N
Glutamine	Gln	Q
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Proline	Pro	P

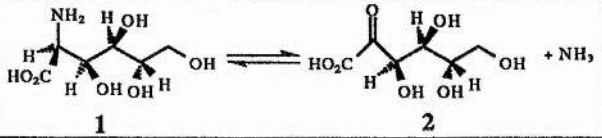
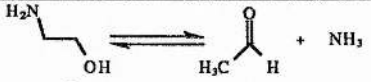
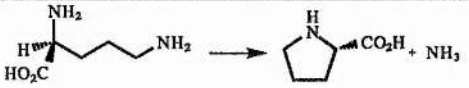
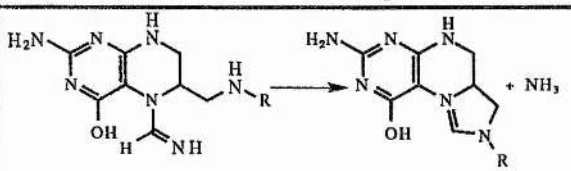
Contents

Chapter 1 : Introduction

1.1 The Ammonia-lyases

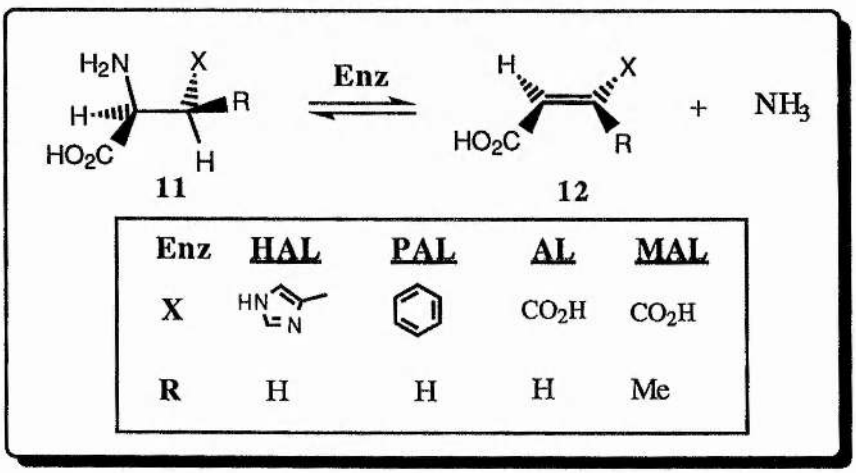
The elimination of ammonia from molecules is catalysed in natural systems by the group of enzymes known as the ammonia-lyases. This family can be broken down into a number of smaller sub-groups, each of which has a specific requirement for a different co-factor. These include those that require cobalamine, pyridoxal and nicotinamide (Table 1).

Table 1: *The ammonia-lyases*

ENZYME	COENZYME	REACTION CATALYSED
Glucosaminate ammonia-lyase (EC 4.3.1.9)[1]	PLP	 1 2
Ethanolamine ammonia-lyase (EC 4.3.1.1)[2]	B_{12}	 3 4
Ornithine *cyclo* -deaminase (EC 4.3.1.12)[3]	NAD^+	 5 6
Formimino-tetrahydrofolate *cyclo* -deaminase (EC 4.3.1.4)[4]	–	 7 8
3-Alanyl-CoA ammonia-lyase (EC 4.3.1.6)[5]	–	 9 10
"true ammonia-lyase"	–	See Scheme 1

One such sub-group of four enzymes is known as the "True" ammonia-lyases represented by histidine, phenylalanine, methylaspartate ammonia-lyases and aspartase. They have no formal requirements for any specific co-factors, other then the case of 3-methylaspartate ammonia-lyase which is known to require a Mg^{2+} and a K^{+} for activity.

These enzymes are all located on the catabolic pathway for amino acids (**11**) that includes 2*S*-histidine, 2*S*-phenylalanine, 2*S*-aspartic acid and (2*S*,3*S*)-3-methylaspartic acid, Scheme 1. Despite differences in the molecular structures of the substrates and the reaction mechanisms, all appeared to share a similar step, the removal of the β-proton and elimination of NH_3^{+}. The result is the formation of olefinic products (**12**) each containing a *trans* double bond, Scheme 1.[6,7,8]

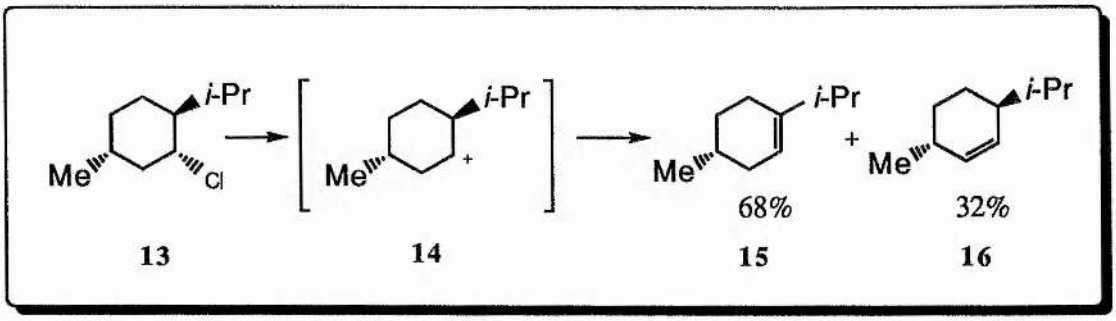


Scheme 1: *The 'true' ammonia-lyases*

The mechanism for elimination reactions in both synthetic and natural systems can be classified into three main types *viz.* E1, $E1_{cb}$ and E2:

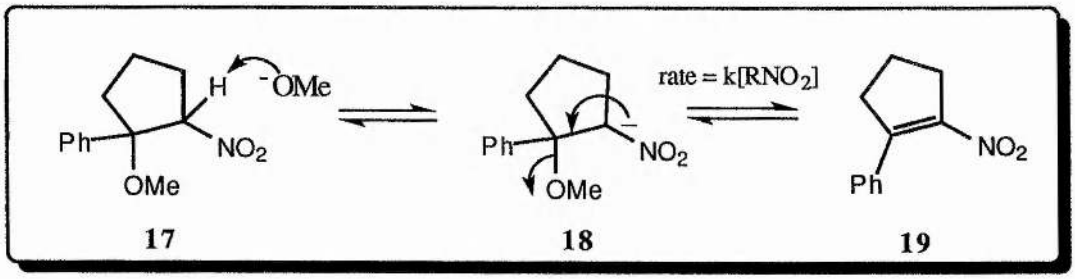
• E1: 1st order unimolecular elimination. The first and rate determining step involves the elimination of a leaving group to afford a carbocation. Subsequent removal of the β-proton by a weak base (commonly the solvent) affords the olefin product. An example is shown in Scheme 2, *viz.* the elimination of HCl from menthyl chloride (**13**). The elimination proceeds *via* the cation (**14**) to give regio-isomers (**15**) and (**16**). As the

elimination is stepwise, the stereochemical course of the reaction is not controlled by the orbital overlap in the substrate and can therefore proceed with *syn* or *anti* stereochemistry.



Scheme 2: *E1 elimination of HCl from menthyl chloride*

• $E1_{cb}$: 1st order unimolecular elimination from the conjugate base. The first step is the removal of an acidic proton to afford a carbanion. The resulting intermediate collapses, eliminating the leaving group to give an unsaturated product. Generally, this mechanism involves a poor leaving group such as methoxide ion, as illustrated for the nitro compound (**17**) in Scheme 3.[9,10] The rate-limiting step is the elimination of the leaving group from the anion intermediate (**18**) to give alkene (**19**).

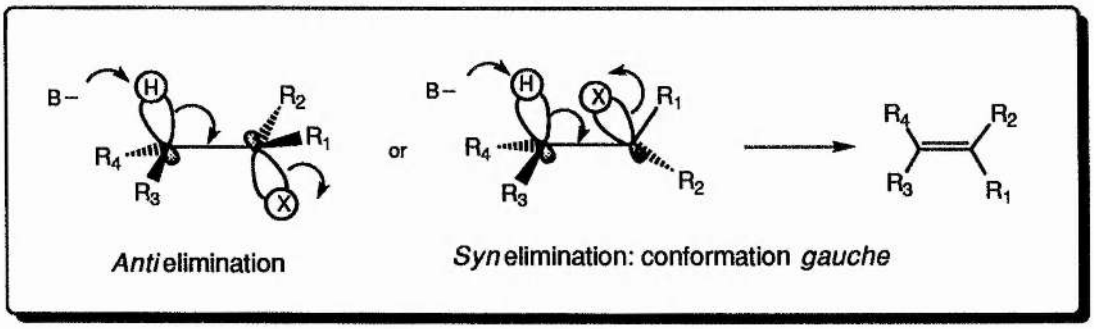


Scheme 3: *Example of $E1_{cb}$ elimination*

• E2: 2nd order bimolecular elimination. This is characterised by the simultaneous rate-determining fission of both C-X and C-H bonds. It is initiated by attack of a base at the β-proton and proceeds *via* a planar transition state. In synthetic reactions, this mechanism generally involves a strong base such as potassium *tert*-butoxide. Theoretically, the elimination can be *syn* and *anti*-periplanar; however in most cases, the *anti*-periplanar

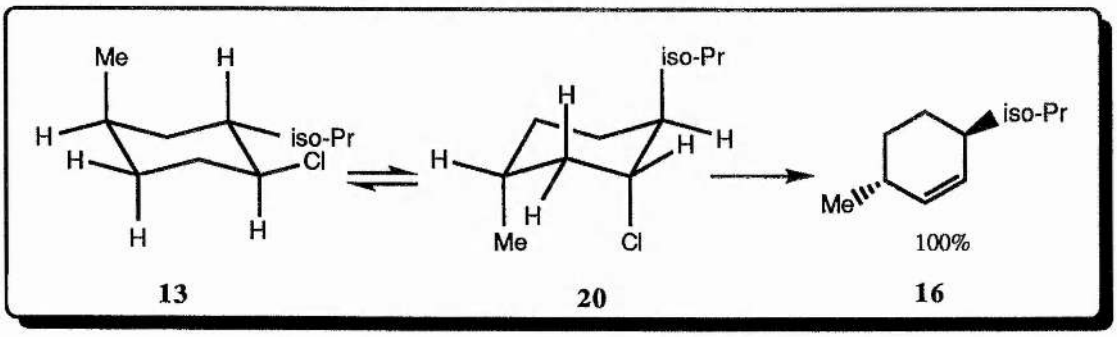
mechanism dominates. The coplanar relationship is preferred so that the orbitals on carbon can overlap to form a π bond as the elimination proceeds as shown in Scheme 4.

These stereochemical considerations do not apply for an $E1_{cb}$ mechanism since two distinct steps are involved, independent of each other.



Scheme 4: *Orbital overlaps for anti and syn elimination*

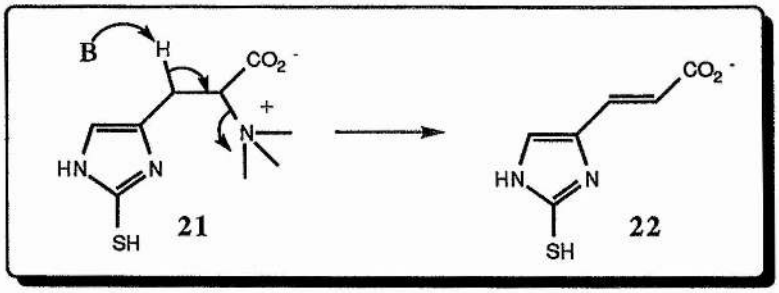
An example of where E2 elimination is favoured is the elimination of HCl from menthyl chloride (**13**). The elimination proceeds only from the chair conformation (**20**) that brings the proton to be abstracted and the leaving group to be trans to each other. As a result the reaction gives exclusively isomer (**16**, Scheme 5).



Scheme 5: *Example of anti elimination*

Havir and Hanson have reported that the ammonium groups ($-NH_3^+$) of α-amino acids are poor leaving groups, and as a result suggested that the mechanism for their elimination would be of the $E1_{cb}$ type.[11] However, they pointed out that in the case of the enzymatic elimination of the permethylated amino group ($-NMe_3^+$) of ergothionine (**21**) to

yield mercapto-uroconate (**22**), the elimination was stereospecific indicative of an E2 type mechanism (Scheme 6).[12]

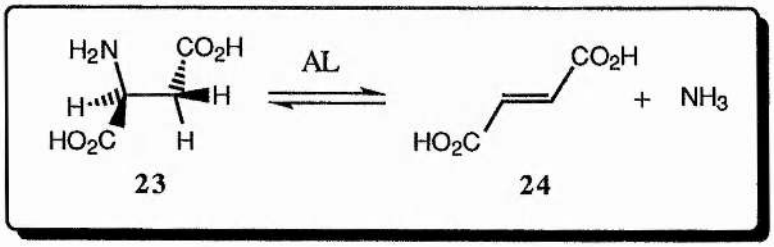


Scheme 6: *Elimination of permethylated ammonia from ergothionine (**21**)*

1.2 Aspartase

1.2.1 Kinetic properties

The enzyme aspartase catalyses the reversible deamination of (2*S*)-aspartic acid (**23**) to yield fumaric acid (**24**) and ammonia, Scheme 7.[13]



Scheme 7: *The aspartase catalysed reaction*

The enzyme has been identified in a wide range of organisms including bacteria, fungi and plants.[14,15] Halpern and Umbarger have reported that the physiological role for the enzyme is associated with the catabolism of aspartic acid.[16] Lartigues and Shimura have shown that the enzyme was active as a tetramer of 477 amino acid residue protomers possessing a subunit mass of 52 kDa.[17,18]

Aspartase from *Escherichia coli* has been extensively studied and showed a strong pH-dependence, with pK_a at 6.2 and 9.2. Above pH 8 (the optimal pH),[19] the enzyme

showed a strong dependence for divalent metal ions such as Mn^{2+}, Co^{2+}, Zn^{2+}. The role of these metal ions was shown to be simple activators of the enzyme between pH 8 and 9. However, above pH 9 the enzyme displayed an absolute requirement for divalent ions. The value of K_m for aspartic acid (in the presence of Mg^{2+}) was found to be 2.5 mmol dm^{-3}. However, when Mg^{2+} was substituted with Mn^{2+}, the K_m for the substrate was found to be 0.7 mmol dm^{-3}.[18] This 3.5 fold decrease of the K_m is attributed to the ability of Mn^{2+} to stabilise the enzyme-substrate complex. The effect is believed to result from the higher electronegativity of Mn^{2+} and smaller Van der Waals radius, which make it capable of binding to the β-carboxyl group of aspartatic acid more efficiently.[18] The role of the metal ion above pH 8 appears to be then to facilitate a better binding of the substrate at the active site and contribute in delocalising the developing negative charge on the β-carboxyl group. Hence the pK_a of the C-3 proton is decreased, making it more susceptible to removal by an active site base.

The enzyme *Escherichia coli* was also shown to be highly substrate specific, accepting only (2*S*)-aspartic acid (K_m = 2.5 mmol dm^{-3} at pH 7.9) and fumaric acid (K_m = 0.2 mmol dm^{-3} at pH 9.0). The amine nucleophile which in the *retro*-physiological reaction is ammonia (K_m = 112 mmol dm^{-3} at pH 8.0) could be replaced by hydroxylamine (K_m = 30 mmol dm^{-3} at pH 6.8).[20] The *N*-hydroxyaspartic acid resulting from fumariconic acid and hydroxylamine has been isolated and characterised. Many substrate analogues have been studied and all were shown to act as competitive inhibitors with the K_i-values of 0.2 to 30 mmol dm^{-3}, Table 2.[21] These results showed that in many cases the inhibitor binds more tightly to the active site than the physiological substrate.

Williams and Lartigue carried out detailed kinetic studies on the *Escherichia. coli.* enzyme, that revealed the presence of non-linear kinetics at above pH 8.0. They showed, however, that the addition of 2-5 mmol dm^{-3} (2*S*)-aspartic acid gave rise to linear kinetics. This effect was also observed with other compounds such as (2*S*)-glutamic acid, (2*R*)-alanine, (2*S*)-2-methylaspartic acid. None of these compounds were shown to act as either substrates or inhibitors, which suggested that they were interacting with an alternative site on the enzyme, remote from the active site.[21]

Table 2 : *Substrate analogues as inhibitors of aspartase*[21]

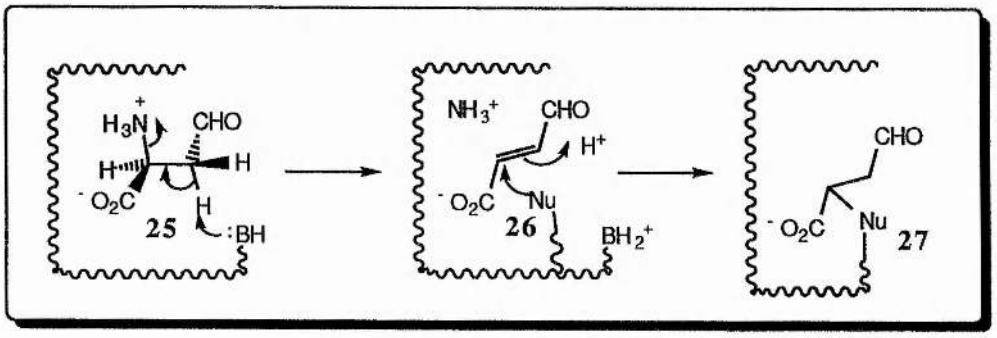
Analogues	R_1	R_2	K_i (mmol dm^{-3})
O-Phospho-(2*S*)-serine	NH_3^+	OPO_3^{2-}	0.2
(2*R*)-Malate	OH	COO-	0.66
(2*S*)-Malate	OH	COO-	31
Succinate	H	COO-	24
(2*S*,3*S*)-2-Amino-3-phosphono-propionate	NH_3^+	PO_3^{2-}	0.66
3-Nitropropionate	H	NO_2	0.83
Mercaptosuccinate	SH	COO-	1.6
(2*S**,3*S**)-2,3-Diphosphoglycerate	OPO_3^{2-}	OPO_3^{2-}	1.1
(2*S*)-2-Chlorosuccinate	Cl	COO-	1.7
(2*S**)-2-Bromosuccinate	Br	COO-	2.3
(2*S**)-2-Amino-4-phosphono-butyrate	NH_3^+	$CH_2PO_3^{2-}$	2.4

1.2.2 Active site structure of Aspartase

Important amino acid residues at the active site of aspartase were probed by a number of active site directed chemical modification reactions.[22,23,24] The enzyme was incubated with *N*-ethylmaleimide,[24] a chemical modifying agent known to selectively alkylate all cysteine residues, in the presence of an excess of substrate in order to protect the active site against modification. Any unreacted inhibitor was then removed by extensive dialysis along with the substrate. The active modified enzyme was then incubated with the fluorescent analogue, *N*-[-7-(dimethylamino)-4-methyl-5-coumarinyl] maleimide, in the absence of substrate. The enzyme was subsequently digested with the protease enzyme trypsin, which is known to cleave the peptide chain at the carboxyl group of Lys and Arg residues. Of the eleven cysteines found in the deduced amino acid

sequence of the enzyme, two were shown to have been modified by the fluorescent reagent. The two Cys residues were mapped and identified as Cys140 and Cys430.[25] Site directed mutagenesis experiments were performed and three mutants, C430T, C14OS and C140A, were examined.[26] In all three cases Murase *et al.* showed that the mutants were capable of catalysing the physiological reaction and that there were no substantial changes in the kinetic parameters for the mutants compared to those for the native enzyme.

Viola and Schindler showed that the incubation of (2*S*)-aspartic acid β-semi-aldehyde (L-ASA) (**25**, Scheme 8) with aspartase at 30 °C and pH 8.5, resulted in rapid time-dependent inactivation of the enzyme (K_i = 0.71 mmol dm^{-3} in absence of Mg^{2+}).[27] Following incubation with L-ASA, the inactive protein was treated with 2,4-nitrophenylhydrazine. A change in absorbance at 370 nm was observed which suggested that the inactivation had not resulted from reaction of a lysine residue with the aldehyde. Viola proposed that L-ASA may be accepted as a substrate for the enzyme and might consequently undergo elimination of ammonia to give fumaric acid semi-aldehyde (**26**) (FAA). This reactive alkene would be susceptible to irreversible nucleophile attack by a number of active site residues to give adduct (**27**) resulting in inactivation of the enzyme. In order to test this hypothesis, the proposed intermediate FAA (**26**) was synthesised and incubated with the enzyme. Rapid irreversible inhibition was observed which supported the role of L-ASA (**25**) as a mechanism based inhibitor.[28]

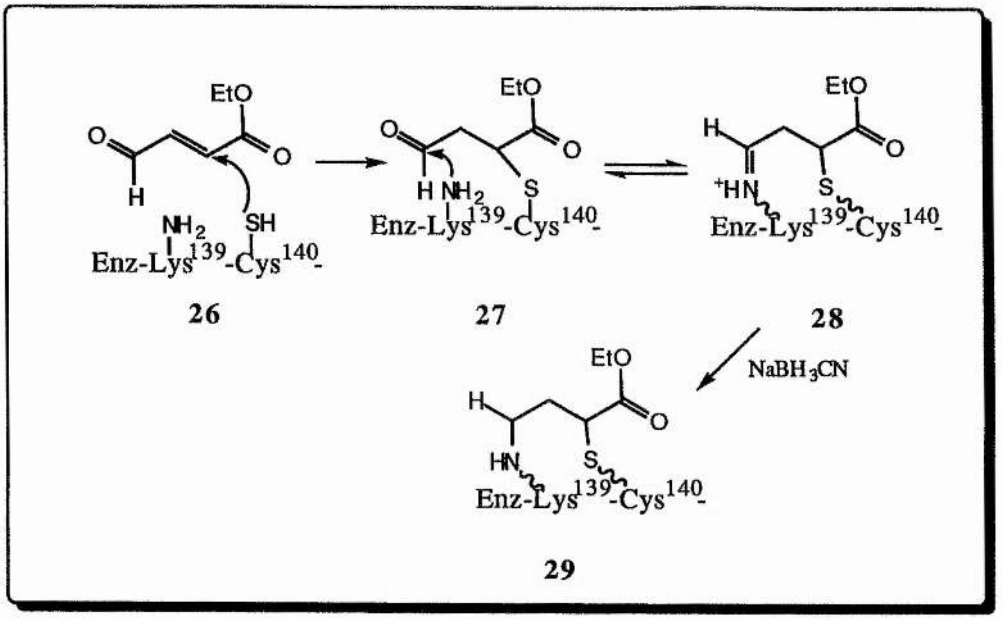


Scheme 8: *Proposed inhibition mechanism of aspartate β-semialdehyde*

Tryptic digestion of the inactivated enzyme allowed the isolation of a single peptide containing the modified amino acid which was characterised by mass spectrometry.

Edman degradation of the peptide identified Cys273 to be the site of modification by FAA. The role of this residue was examined by site directed mutagenesis. Mutation of the cysteine to both alanine and serine (C273A, C273S) did not have any effect on the catalytic properties of the enzyme. Hence, Giorgianni et *al.* proposed that Cys273 did not play a significant part in the catalytic reaction of the enzyme. The two mutants C273A and C273S were still inactivated by L-ASA, indicating the existence of further reactive centers for FAA in the active site of the enzyme.

To test this hypothesis the enzyme was directly inactivated with the more stable FAA (**26**), the inactivated enzyme was heated with sodium cyanoborohydride.[28] Under these conditions three modified amino acid residues were identified, Lys139, Cys140 and Cys273. The mutant K139I showed a 10-fold higher K_m for (2*S*)-aspartic acid, but no effect on the rate of inactivation by ASA or FAA was observed. This effect on the value K_m suggested that K139 was involved in substrate binding. The double mutants C140S/C273S were found not to be sensitive to either ASA (**25**) or FAA (**26**), and possessed similar kinetic parameters to those for the wild type enzyme. Presumably, the additional covalent modifications were introduced as a result of the borohydride reduction of a reactive species such as an imine. Giorgianni proposed that C140 and K139 were cross-linked within the active site possibly *via* the formation of an imine, thus giving intermediate (**28**), Scheme 9.[28] Reduction of the intermediate (**28**) presumably afforded adduct (**29**).



Scheme 9: *The proposed inactivation mechanism by FAA ethyl ester*

Farber and coworkers determined the X-ray crystal structure for aspartase in 1998 to a resolution of 2.8 Å.[29] They showed the role of Ser143 was probably to serve as the general acid, protonating the amino group prior to deamination (Figure 1). Further supporting evidence was obtained with the mutants S143G and S143T, prepared by site directed mutagenesis. In both cases the enzyme activity was found to be 1% of the activity of the wild type enzyme.[29,30]

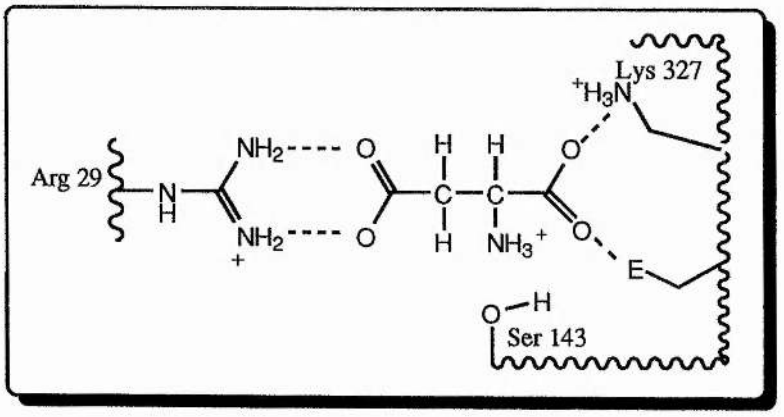


Figure 1: *Active site stucture of aspartase; E = unidentified residue to account for substrate specificity*

Furthermore, sequence similarity studies between acid sequences of aspartase and fumarase, isolated from different sources, showed that Ser143 was fully conserved. This conservation of Ser143 might indicate that this residue plays an important catalytic role in the enzyme.[30]

In order to identify the base responsible for abstraction of the β-proton Shi and coworkers prepared several mutants of both His26 and Asp10.[29] From the crystal structure of the enzyme both residues appeared to be in the correct position within the active site.[29] The mutant H26Q retained activity whereas a 4-5 fold decrease in activity was observed for D10A and D10N mutants. They also observed identical pK_a values for the D10N mutant and the wild type enzyme. [29] This similar values of pK_a indicated that the protonation of Asp10 was not responsible for the loss of activity below pH 7. Thus Shi and coworkers suggested that the most likely role for Asp10 was to participate in the formation of a hydrogen bond either, directly with the substrate, or, to assist in the orientation of a catalytic group within the active site. [29]

Examination of the active site pocket revealed that Arg29 was involved in binding to one of the carboxyl groups of the substrate.[29,30] Indeed, the mutation of Arg29 to alanine gave a mutant (R29A) which displayed a K_m value for aspartic acid of 40-fold lower than that of the native enzyme. Substrate analogues were prepared in which the β-carboxyl group was replaced by either a phosphate or a sulphate group. These molecules showed competitive inhibition (*versus* aspartate) with K_i-values ranging from 0.12 to 2.3 mmol dm^{-3}.[30] The mutant R29A was also tested with the compounds and again competitive inhibition was observed, however, the K_i values were higher by a factor of 2.5-10. Modification of the α-carboxyl group of the substrate to an amide gave an analogue that was a weak inhibitor of the native enzyme, and which did not inhibit the mutant R29A. Both experiments therefore suggested that Arg29 was involved in binding the β-carboxyl group of the substrate. [30]

1.2.3 Mechanism of Aspartase

Nuiry *et al.* carried out extensive kinetic studies on aspartase isolated from *Hafnia alvei*.[31] They showed that no primary isotope effect on V or V/K was observed for the

substrate (2*S*,3*S*)-[3-^{2}H] aspartic acid. However an ^{15}N primary isotope effect of 1.0239 ± 0.0014 was observed on V/K.[31] Porter and Bright found that 3-nitro-2-aminopropionate (**30**, Figure 2) was a powerful competitive inhibitor with $K_m(asp)/K_i$ = 220 at pH 9.0.[32] Thus they suggested that 3-nitro-2-aminopropionate (**30**) was acting as a transition state mimic. A comparison of the structure of 3-nitro-2-aminopropionate and the proposed transition state (**31**) supports this hypothesis.

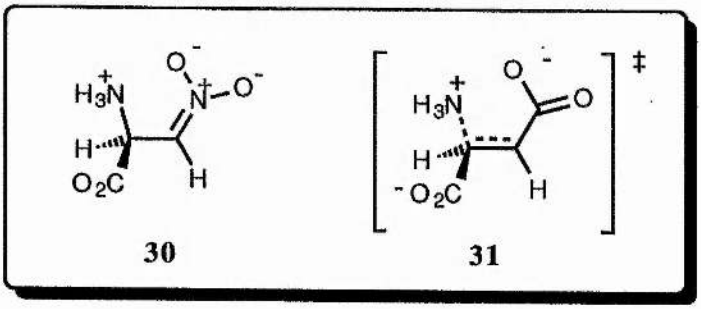
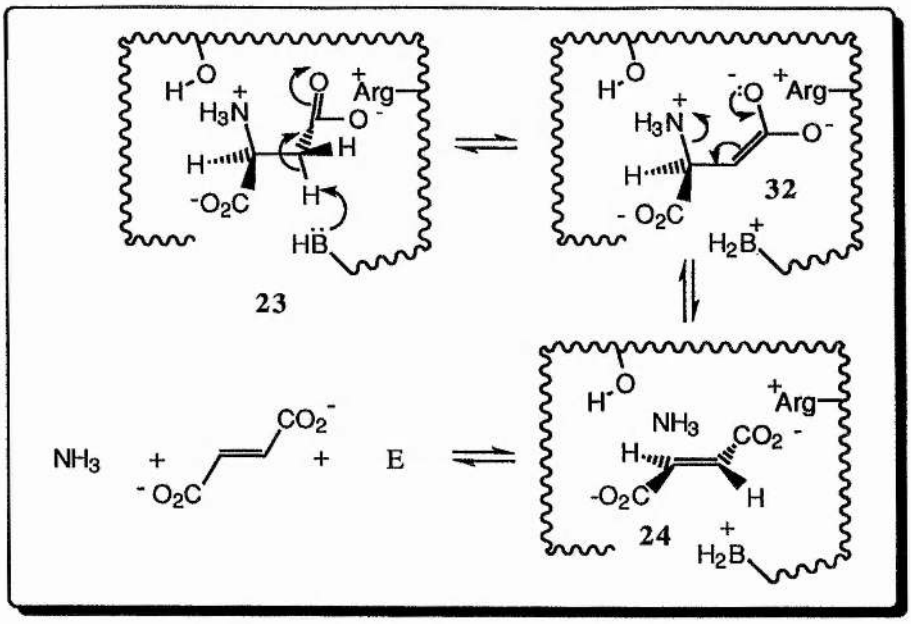


Figure 2: *3-Nitro-2-aminopropionate as transition state mimic*

The observation of ^{15}N primary isotope effect, the lack of a primary deuterium isotope effect and the strong competitive inhibition by 3-nitro-2-aminopropionate collectively provided strong evidence in support of $E1_{cb}$ mechanism for the overall elimination of ammonia from the substrate. The first step in this mechanism is the removal of the 3-*proR* hydrogen from enzyme bound aspartate (**23**) to give the corresponding carbanion (**32**). The resulting intermediate then eliminates ammonia to give the unsaturated product, fumarate (**24**), Scheme 10.

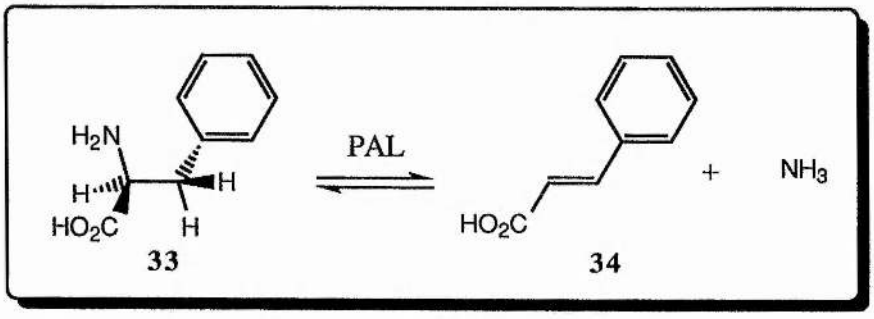


Scheme 10: *Mechanism of aspartase*

1.3 Phenylalanine ammonia-lyase

1.3.1 Kinetic proprieties

The enzyme phenylalanine ammonia-lyase catalyses the elimination of ammonia from (2*S*)-phenylalanine (**33**) to give cinnamic acid (**34**) (Scheme 11).[33]



Scheme 11: *Phenylalanine ammonia-lyase catalysed reaction*

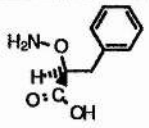
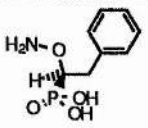
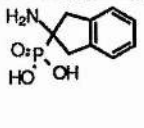
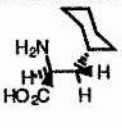
The enzyme is widely distributed in plants such as maize and potato and fungi for example, *Rhodoturela glutinis*. In these three sources the enzyme is in the catabolic pathway for phenylalanine.[34] The physiological role for the enzyme has been reported to

be involved in the production of secondary plant products such as lignin, coumarins and flavanoids for which cinnamic acid is the precursor.[35,36] In addition the phenolic compounds produced from cinnamic acid have been implicated in the process of disease resistance of plants. The enzyme from all three sources has been extensively studied. In all cases, the enzyme has been shown to be active as a homotetramer of subunit mass 320 kDa.[37,38]

Hanson and Havir showed that under saturating substrate conditions the pH optimum was 8.7.[39] Conway and Kosland identified two pK_a values at pH 7.25 and 10.25 from the bell-shaped pH-activity curve for the potato enzyme.[40] They noted that enzymes from potato and maize exhibited non-linear kinetics. For example, in the case of the enzyme extracted from potato, the apparent values of K_m for phenylalanine at pH 8.7 increased from 0.038 to 0.26 mmol dm^{-3} and the apparent V_{max} doubled as the substrate concentration increased. This increase in K_m values implies that after the addition of substrate, the affinity of the enzyme for (2*S*)-phenylalanine decreases. Addition of (2*R*)-phenylalanine caused a return to linear kinetics for the enzyme isolated from potato.[41,42] Hanson and Havir showed that (2*R*)-phenylalanine acted as a poor competitive inhibitor (K_i = 100 mmol dm^{-3}). Other molecules including (2*S*)-glutamic acid, (2*R*)-alanine and (2*S*)-2-methylaspartic acid were found to be non-inhibitory but also suppressed the non-linear kinetic behaviour. These observations provided strong evidence in support of the presence of an allosteric site. (2*R*)-Phenylalanine and other molecules are then able to bind to an alternative site different from the active site. This binding results in the suppression of the non-linear kinetics.

PAL enzymes from plants were shown to be capable of accepting molecules other than the physiological substrate (2*S*)-phenylalanine (K_m = 0.17 mmol dm^{-3}, V_{max} = 0.63 U/mg). These include *p*-chlorophenylalanine, *p*-fluorophenylalanine, *p*-nitrophenylalanine (K_m = 0.32 mmol dm^{-3}, V_{max} = 0.17 U/mg) and dihydrophenylalanine (K_m = 0.122 mmol dm^{-3}, V_{max} = 0.06 U/mg). In addition, Hanson and Havir observed that (2*S*)-tyrosine was a substrate but only for the enzyme from *Rhodoturela glutinis* and maize (K_m = 0.03-0.1 mmol dm^{-3}, pH 8.7).[43] Finally, Zon and Amrhein described a number of substrates analogues which showed competitive inhibition, Table 3.[44]

Table 3: *Inhibitors of phenylalanine ammonia-lyase*

Inhibitor	 35	 36	 37	 38
K_i	1.4 mmol dm^{-3}	1.5 mmol dm^{-3}	0.08 mmol dm^{-3}	1.8 mmol dm^{-3}

1.3.2 Active site structure

Important amino acid residues in the active site were probed by a number of chemical modification reactions. PAL from *R. Glutinis* was inactivated by the following thiol reagents: *p*-mercuribenzoate, *N*-ethylmaleimide and iodoacetic acid. In contrast, enzymes isolated from potato and maize were unaffected.[45] As an explanation, Hanson and Havir suggested that thiol groups might play a critical part in maintaining the conformation of these enzymes, presumably *via* the formation of disulfide bridges. These thiol groups therefore might not be directly involved in the catalytic mechanism.[45] In contrast, PAL from all three sources was completely inactivated by carbonyl directed chemical modifying reagents, such as potassium cyanide, semicarbazide, nitromethane, phenylhydrazine and sodium borohydride.[41] The presence of phenylalanine or cinnamic acid afforded protection against the inactivation.[41] Protection against inactivation by sodium borohydride was also afforded by phenylhydrazine. In addition, inhibiton by all the directed chemically modifying reagents was reversible, excepted for the case of sodium borohydride.[41] These results indicate that all the inhibitors were reacting specifically at the active site. In order to identify the site of modification, these experiments were repeated with corresponding radiolabelled inhibitors. Inactive protein was subjected to hydrolysis with 6 mol dm^{-3} HCl and the total amino acid content was determined using an automatic analyzer.

Treatment with [^{3}H]-$NaBH_4$ resulted in tritiated (2*RS*)-alanine which had been modified at the C-3 position.[38] The analogous experiment performed with [^{14}C]-KCN

treated enzyme was carried out and a radiolabelled [^{14}C]-aspartic acid residue was isolated. The label was localised in the β-carboxyl group.[43] Both observations imply that the original amino acid residue was a dehydroalanine. Examination of the deduced amino acid sequence of PAL from a variety of sources (Table 4), showed that Ser202 and Ser209 were located in a region of very high sequence homology. It was therefore suggested that the serine residues which are involved in these sequences might have an important catalytic role.

Table 4 : *The amino acid sequence comparison of PAL isolated from plants*

Source	Residues	AA sequence
PAL P. crispum	197-208	GTITASGDLVPLSYIA
PAL R. toruloides	205-216	GTISASGDLSPLSYIA
PAL R. rubra	211-222	GTISASGDLSPLSYIA
PAL O. sativa	184-195	GTITASGDLVPLSYIA
PAL L. esculentum	204-215	GTITASGDLVPLSYIA
PAL I. batatas	187-198	GTITASGDLVPLSYIA

Schuster and Retey prepared the mutants S202A and S209A by site directed mutagenesis, and they showed that the mutant S202A was inactive where as the S209A remained active.[46] As a result of these finding, they proposed that the dehydroalanine residue was derived through a post-translational modification of Ser202.

1.3.3 Mechanism of PAL

Cleland *et al.* carried out extensive kinetic studies on the PAL from *R. glutinus*. No primary isotope effect was observed over a wide pH range with [3-$^{3}H_2$]-phenylalanine. However, [3-$^{3}H_2$]-dihydrophenylalanine showed a primary isotope effect on V and V/K of 2.0.[47] These results established that for [3-$^{3}H_2$]-dihydrophenylalanine the abstraction of the β-proton was at least partially rate limiting.

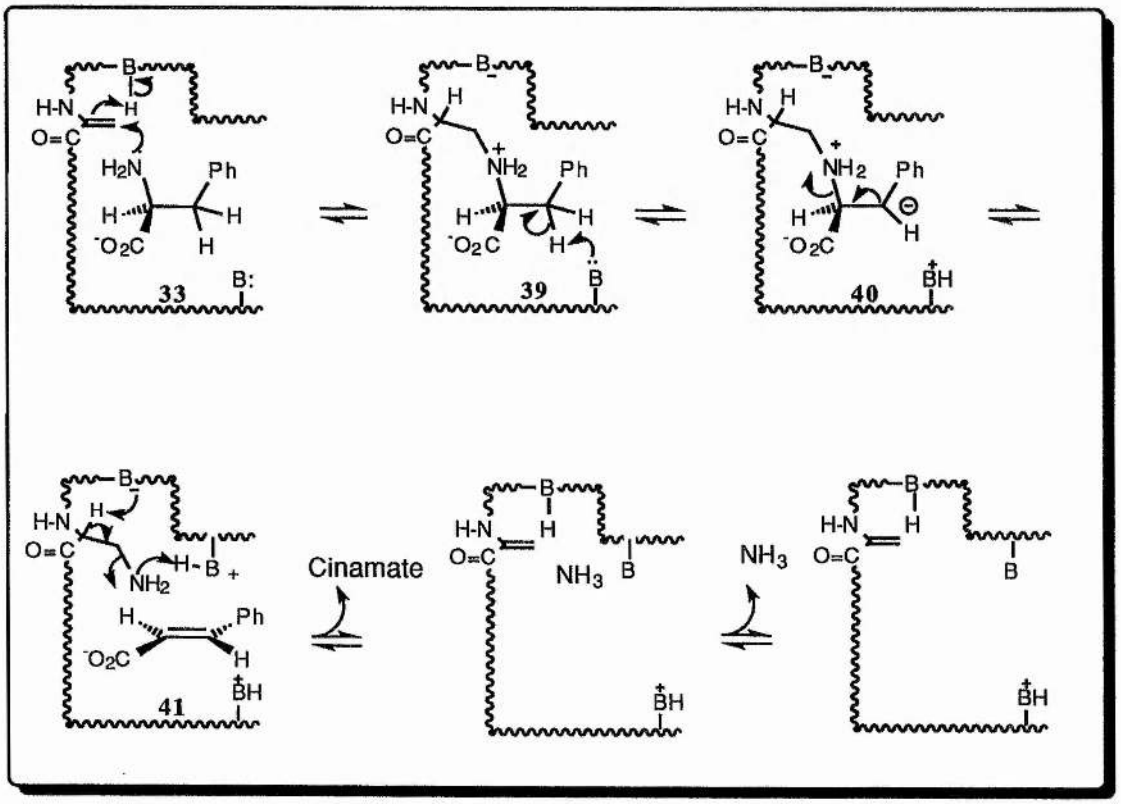
The primary nitrogen isotope effect was also determined at low and high pH (7.5 and 10), for dihydrophenylalanine, phenylalanine and their C-3 deuterated analogues. The results are shown in Table 5.

Table 5 : *^{15}N- isotope effects forPAL*

Substrate	$^{15}(V/K)_{low\ pH}$	$^{15}(V/K)_{high\ pH}$
phenylalanine	1.0170	1.0021
phenylalanine-d_2	1.0167	1.0010
dihydrophenylalanine	1.0228	1.0047
dihydrophenylalanine-d_2	1.0065	0.9921

Cleland *et al.* observed a primary nitrogen isotope effect for all four compounds. They showed that the isotope effect was pH-dependent, with the lowest value measured above pH 9. This pH value corresponds to the pK_a for the α-amino group of the substrate which had been determined to be 9.3 for dihydrophenylalanine and 9.1 for phenylalanine. They subsequently suggested that the unprotonated free amino form of the substrate was the specie bound by the enzyme. The ratio of the primary nitrogen isotope effect on V/K for dihydrophenylalanine and [3-$^2H_2$]-dihydrophenylalanine was found to be 1.0127. This difference between the two isotope effects is consistent with a stepwise mechanism which proceeds *via* an intermediate carbanion. The slight decrease in the $^{15}(V/K)$ observed with [3-$^2H_2$]-phenylalanine compared to phenylalanine is also consistent with an $E1_{cb}$ mechanism but was not sufficient to allow mechanistic distinction. This result therefore established that the mechanism of elimination for the substrate dihydrophenylalanine was balanced stepwise involving an intermediate carbanion. As the carbanion intermediate formed by deprotonation of phenylalanine is more stable than the analogous intermediate from dihydrophenylalanine, Cleland proposed that the carbanion mechanism $E1_{cb}$ applied with both substrates.

From the results of the experiments discussed above, a mechanism for the reaction catalysed by PAL was proposed. The first step is a nucleophilic attack of the substrate amine (**33**) on the dehydroalanine residue to generate the corresponding Michael adduct intermediate (**39**), Scheme 12. Deprotonation at the C-3 proton in the substrate then gives the corresponding carbanion (**40**) which eliminates an activated amino group to afford the cinnamate product, and an amino-enzyme (**41**). The amino-enzyme then undergoes elimination to regenerate the dehydroalanine residue, ammonia is then released.

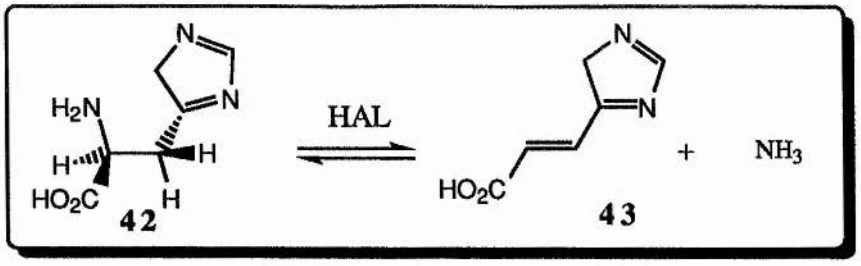


Scheme 12: *Mechanism of action of PAL*

1.4 Histidine ammonia-lyase

1.4.1 Kinetic proprieties

Histidine ammonia-lyase (EC 4.3.1.3) catalyses the deamination of histidine (**42**) to give ammonia and urocanic acid (**43**) (Scheme 13).



Scheme 13: *Histidase catalysed-reaction*

The enzyme is found in a wide range of bacteria,[48] mammals,[49] and in a limited number of plants.[50] In mammalian HAL has been isolated from the liver and the epidermis.[49] Deficiency of HAL in mammals leads to speech defects and mental retardation, a desease known as histidinemia.[51]

The HAL reaction was first believed to be irreversible,[52] but was subsequently shown that with prolonged incubation the reaction is reversible with an equilibrium constant of 3 to 5.[53]

The enzyme from *Pseudomonas fluorescens* has been the most extensively studied, and was shown to be a tetramer of subunit mass 222 kDa.[54] The enzyme has been reported to be most efficient between pHs 8.6 and 9.2 in Tris buffer,[55] and 8.5 and 9.3 in carbonate buffer.[56] The K_m value for histidine was found to be 10.0 mmol dm^{-3} and constant below pH 8 and to increase by up to 8-fold above pH 8.[56]

The activity of HAL from *fluorescems* has been found to be enhanced by incubation with divalent metal ions. The metals Mn^{2+}, Fe^{2+}, Cd^{2+} and Zn^{2+} were shown to afford a 2 to 10 fold increase in the enzyme activity.[57] As the enzyme is not dependent on metal ions for activity, this suggests that the metal is not required for catalysis.

Brand and Harper showed that the enzyme isolated from rat liver was competitively inhibited by substrates analogues which proved to be competitive inhibitors at pH 9.0, Table 6.[58] (2*S*)-Hydrazino-imidazolyl-propionic acid (**44**, Table 6) was an efficient competitive inhibitor with a K_i-value 7-fold lower than K_m value 10 mmol dm^{-3} for histidine.

Table 6: *Inhibitors of Histidase*

Inhibitor	K_i (mmol dm^{-3})
(2*R*)-Hydrazinoimidazolyl propionic acid (**44**)	0.075
(2*R*)-Histidine (**45**)	1.98
(2*S*)-Histidine hydroxymate (**46**)	0.42
(2*S**)-Hydrazinoimadazolyl propionic acid (**47**)	0.22
(2*S*)-Histidinol (**48**)	2.71

1.4.2 Active site structure of HAL

Amino acid residues at the active site of HAL were probed by a number of active site directed chemical modifying reagents. The enzyme was irreversibly inactivated by dicarbonyl reagents such as phenylglyoxylate which is known to react specifically with arginine residues.[59] Protection against this inactivation was achieved in the presence of the substrate,[59] indicating that an arginine residue is present at the active site.

Histidine ammonia-lyase was inhibited by nucleophilic reagents including phenylhydrazine, sodium bisulfite, nitromethane, potassium cyanide and sodium borohydride.[60] The enzyme was inactivated with [^{14}C]-KCN, [^{14}C]-KCH_2NO_2,[55] and ^{3}H-$NaBH_4$[61] and, following total amino acid analysis of the inactivated protein, [4-^{14}C]-aspartatic acid, 2,4-diamino-[^{14}C]-butyrate and 3-[^{3}H]-alanine, respectively, were isolated. The isolation of these radiolabelled molecules strongly suggests that a dehydroalanine residue exists at the active site.

Hernandez found that histidase from *P. putida* was irreversibly inactivated by (2*S*)-cysteine at pH 10.5 in the presence of oxygen, and that inactivation caused the formation of a new UV-absorbing species displaying λ_{max} at 340 nm.[62] Labelling experiments performed with (2*S*)-[^{35}S]-cysteine revealed that 4 moles of cysteine were bound per mole of enzyme tetramer, upon complete modification. The radiolabel, however, dissociated from the protein under denaturating conditions without loss of the 340 nm absorbance.

Inactivation of histidase by cyanide prevented the formation of the 340 nm species in subsequent (2*S*)-cysteine modification experiments, indicating that there was a common target site for modification of histidase by all these reagents. Trypsin and protease digestion showed that the strong absorbing species at 340 nm could be attributed to an octapeptide (Gly138-Ser-Val-Gly-Ala-Ser-Gly-Asp145), which contained an unidentified modification at Ser143 as shown by electrospray mass spectrometry. The results indicated that Ser143 was the binding site for an electrophilic co-factor required for histidase activity. In addition, Retey and coworkers prepared the alanine mutant, S143A, and showed that it was inactive.[63] Furthermore, the mutant could not incorporate [^{14}C]-KCN nor could generate the UV-absorbing species normally observed when (2*S*)-cysteine modifies wild-type histidase. Thus, it was suggested that Ser143 is a most likely precursor of the active site dehydroalanine.

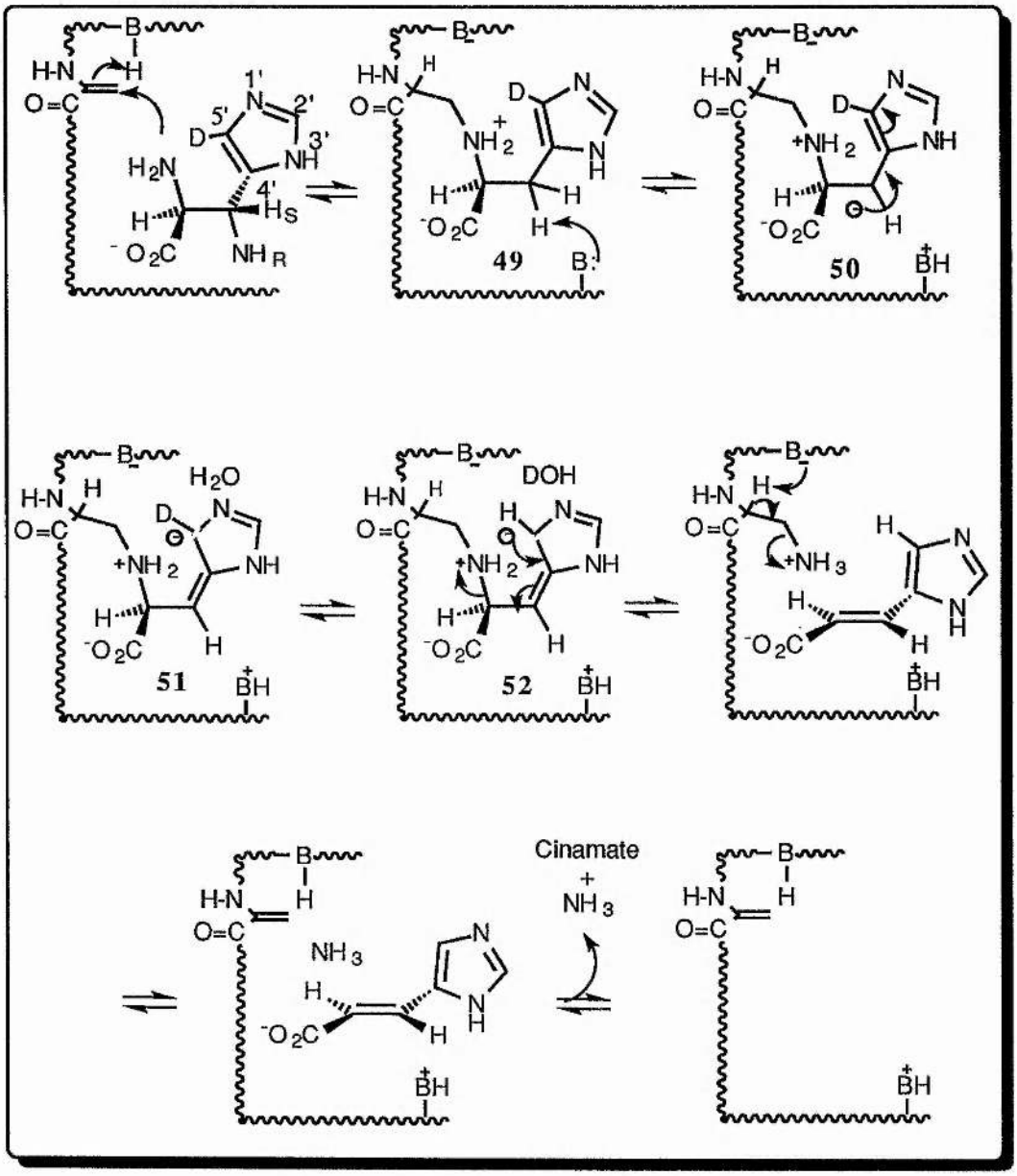
Langer showed that when Ser143 was replaced by cysteine, the mutant S134C retained full catalytic activity and possessed similar kinetic parameters to the native enzyme.[64] However when Ser143 was replaced by threonine, all enzymic activity was lost. Therefore, it is possible that a post-translational modification occurs in the active site with both Ser143 and Cys143, by elimination of H_2O and H_2S respectively affording the dehydroalanine residue. In the case of threonine, similar modification is not possible, presumably due to the extra methyl group.

1.4.3 Mechanism of HAL

Klee *et al.* undertook initial kinetic studies on HAL isolated from *Pseudomonas putida.* The results showed of a primary isotope effect on V with the substrates (2*S*)-histidine (^{D}(V) = 1.4-1.5) and 4-fluoro-histidine (^{D}(V)=1.7-2.0)[65] , indicating that the abstraction of the C-3 proton is at least rate limiting.

Furuta *et al.* carried out further studies with the enzyme isolated from *P. fluorescens*, investigating hydrogen exchange at the C-5' position in the substrate [3-$^{2}H_2$]-[5'-^{2}H]-histidine, Scheme 14.[66,67] Incubation of the enzyme with the trideuterated substrate at pH 9.0 resulted in a 44% loss of deuterium at the C-5'position after 24 h. In the absence of the enzyme, however, the deuterium atom at C-5' was completely retained.

Klee therefore suggested the observation of an enzyme catalysed deuterium hydrogen exchange at the C-5' position excluded the possibility of a concerted mechanism. Having established the presence of dehydroalanine at the active site and excluded a concerted mechanism for the elimination of NH_3, a stepwise $E1_{cb}$ mechanism for histidine ammonia-lyase was proposed (Scheme 14). Protonation at C-5' and subsequent re-aromatisation of intermediate (**52**) leads to the elimination of ammonia and the release of urocanate. Deamination of the dehydroalanine occurs *via* deprotonation at the peptide backbone to afford the regenerated protein in preparation for further catalytic cycles.

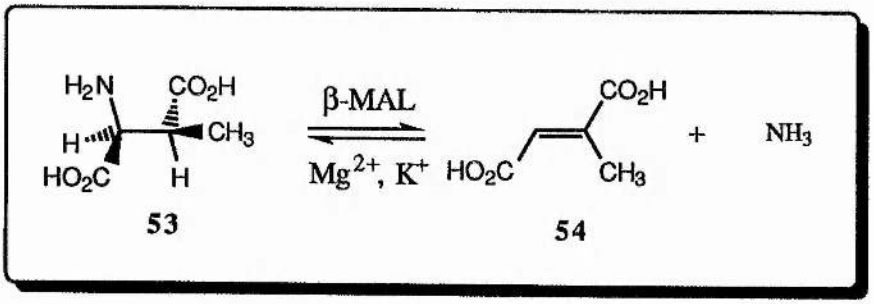


Scheme 14: *Mechanism of HAL*

1.5 (2*S*)–3–Methylaspartate ammonia-lyase

1.5.1 Origin and biological role

The enzyme 3-methylaspartase (MAL) catalyses the reversible elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid (**53**) to afford mesaconic acid (**54**).



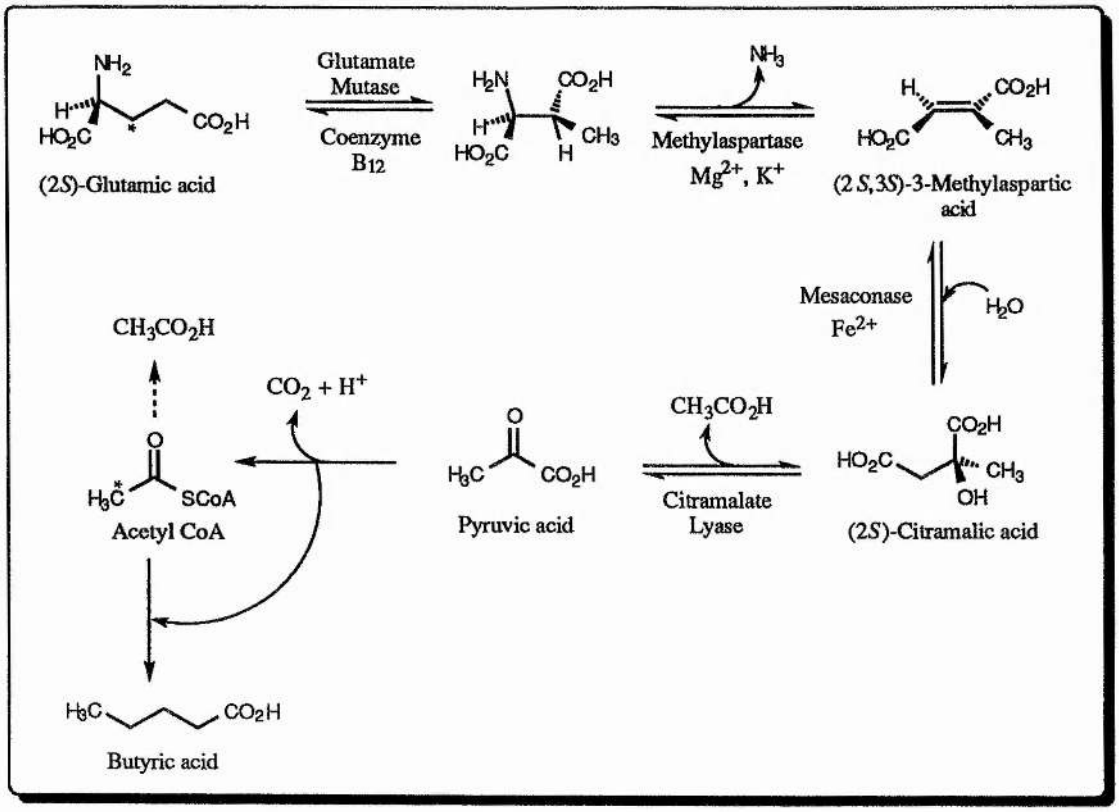
53 **54**

Scheme 15 : *Methylaspartase reaction*

The enzyme lies on the main catabolic pathway for glutamate in a limited number of anaerobic microorganisms (Scheme 8). (2*S*)-Glutamic acid undergoes carbon chain rearrangement which is catalysed by the coenzyme B_{12}-dependent enzyme glutamate mutase. The product 3-methylaspartic acid is then deaminated by 3-methylaspartase prior to stereospecific hydration catalysed by mesaconase. The resulting (2*S*)-citramalic acid is then cleaved to afford pyruvic acid and acetic acid which is catalysed by the hydratase enzyme citramalate lyase. Finally oxidative decarboxylation of pyruvic acid affords acetyl coenzyme A. The first metabolite produced by the pathway, acetyl coenzyme and butyric acid can then be employed as substrates in the citric acid cycle. This cycle is a series of reactions in the cytosol that bring about the catabolism of acetyl residues, liberating hydrogen equivalents, which upon oxidation, lead to the release of most of the free energy of tissue fuels.

The enzyme was first detected and isolated from cell-free extracts of the anaerobe *Clostridium tetanomorphum*.[68,69] Activity was determined by the use of UV spectroscopic assay by monitoring the formation of mesaconic acid (**54**, λ_{max} = 240 nm, ε = 3.85 x106 $M^{-1}cm^{-1}$ at pH 9.0 in 50 mmol dm^{-3} Tris-HCl buffer).[69] The molecular

weight of the native protein was found to be 100 kDa by sedimentation equilibrium experiments and was reported to possess an $(AB)_2$ structure.[70] Subsequent analysis of the gene sequence has indicated, however, that it exists as a homodimer of subunit mass 45.539 kDa.[71]



Scheme 16: *Catabolic pathway of glutamic acid in Clostridium tetanomorphum*

1.5.2 Substrate specificity

3-Methylaspartase was shown to catalyse the elimination of ammonia not only from the *threo* diastereoisomer of (**53**) but also for the *erythro*-(2*S*,3*R*)-3-methylaspartic acid (**55**), however, at a rate 100-fold less than the former. The enzyme was found to be non-substrate specific since several fumaric acid analogues were found to be accepted as substrate by the enzyme. The results are displayed in Table 7.[72] (2*S*)-Aspartic acid and a number of 3-alkyl homologues were also shown to be substrates of the enzyme (Table 8).

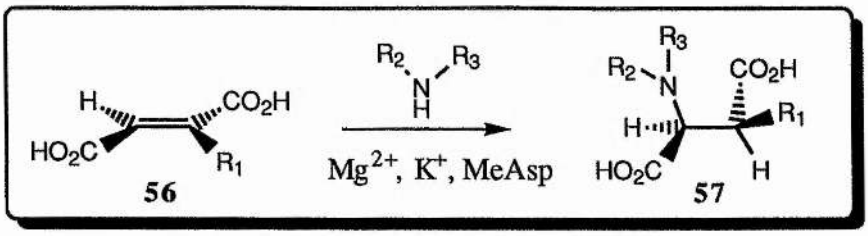
Table 7: *Kinetic parameters for the amination of substitued fumaric acids*[72]

Substrate	K_m (mmol dm^{-3})	V_{max} (%)
Mesaconic acid	1.24	100
Fumaric acid	23	190
Ethylfumaric acid	1.05	65
Bromofumaric acid	2.64	48
Isopropylfumaric acid	5.5	0.3

Table 8: *Kinetic parameters of aspartate analogues*[72]

Substrate	K_m (mmol dm^{-3})	V_{max} (%)
(2*S*,3*S*)-Methylaspartic acid	2.37	100
(2*S*,3*R*)-Methylaspartic acid	40	3
(2*S*,3*S*)-Ethylaspartic acid	17.08	45
(2*R*,3*R*)-Chloroaspartic acid	>50	
(2*S*)-Aspartic acid	10.5	0.7
(2*S*,3*S*)-N-Methyl-3-methylaspartic acid	1.4	0.04

Gulzar showed that in the reverse reaction direction, amine nucleophile, which is ammonia in the physiological reaction could be replaced by a variety of amine derivatives such as alkyl amines, hydroxylamines, or hydrazine (Scheme 17, Table 9).[73] Thus it was possible to obtain from alkylfumaric acids (**56**) various aspartate derivatives (**57**).



Scheme 17: *Amination of fumaric acids with different amines*[73]

Table 9: *Amine nucleophiles as substrate for MAL*[73]

R_1	R_2	R_3	Conv. (%)	R_1	R_2	R_3	Conv. (%)
H	Me	H	55	Me	NH_2	H	91
Me	Me	H	54	Et	NH_2	H	90
Et	Me	H	60	iPr	NH_2	H	90
Pr^n	Me	H	No reaction	H	OH	H	90
Pr^i	Me	H	No reaction	Me	OH	H	90
H	Me	Me	70	H	Et	H	5
Me	Me	Me	No reaction	H	NH	H	89

A variety of substrate analogues were tested as inhibitors of methylaspartate ammonia-lyase (Figure 3) and all showed competitive inhibition.[74,75] The inhibition constants are shown in Table 10.

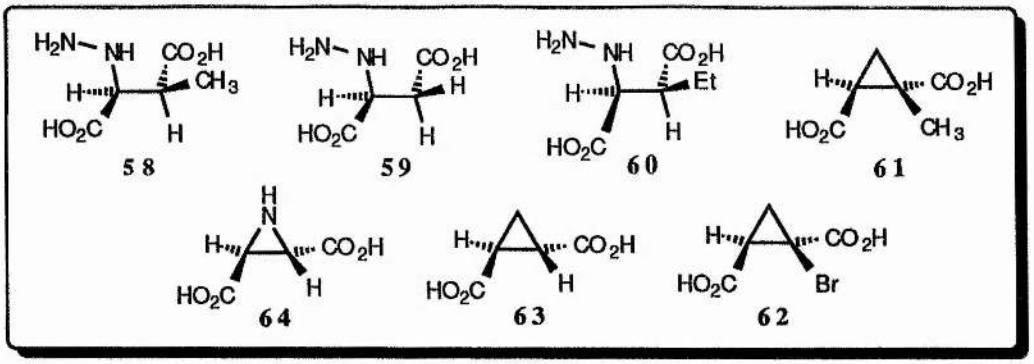


Figure 3: *K_i of some inhibitors of MAL*

Table 10*: Inhibition constants*

compounds N°	K_i mmol dm^{-3}	compounds N°	K_i mmol dm^{-3}
58	2.32	**62**	0.92
59	10.4	**63**	7.69
60	13.8	**64**	11
61	0.020		

The result showed that the hydrazinoaspartic analogues (**58-60**) were only weak inhibitors of the enzyme.[75] However the *trans*-(2*S*,3*S*)-methylcyclopropane dicarboxylic analogue (**61**) showed potent inhibition with a K_i = 20 μmol, 100-fold lower than the value K_m for (2*S*,3*S*)-3-methylaspartic acid.[74] This is consistent with the compound behaving as an analogue of the transition state, as shown in Figure 4. The (2*R*,3*R*)-bromocyclopropane analogue (**62**) showed mixed inhibition.[74] This indicates that this molecule can bind to the free enzyme and also to an enzyme-substrate or product complex. The poor inhibition by *trans*-cyclopropane dicarboxylic (**63**) acid showed the importance of the methyl group for binding. The analogous aziridine (**64**) was found to be a very weak inhibitor, K_i-value 11 mmol dm^{-3}.[76]

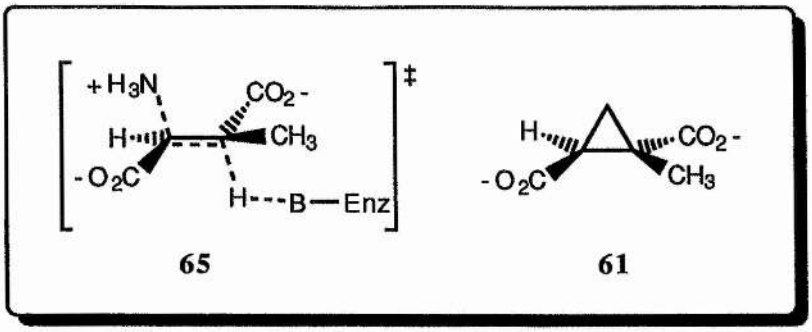


Figure 4: *Transition state analogue*

1.5.3 Kinetic properties of MAL and metal ion dependence

The enzyme displays Michaelis-Menten kinetics over the pH range studied (pH 6-10).[77,78] The pH rate profile (V_{max}) for the forward reaction was shown to exhibit a bell-shaped curve with a maximum at 9.7.[69] The enzyme is known to utilise a variety of monovalent ions. The order of effectiveness in activation is $K^+ > NH_4^+ > Rb^+ > Li^+ > Na^+ > Cs^+$.[78] The physiological monovalent ion is K^+, K_m = 3 mmol dm^{-3}, pH 9.7.[78] The enzyme has been found to behave autocatalytically at low monovalent concentration where the product ammonium ion cause activation.[78] A study of the role of the divalent ion in the activation of 3-methylaspartase was carried out with K^+ ion as the monovalent ion (in 50 mmol dm^{-3} potassium-acetate buffer at pH 5.1 with 125 mmol dm^{-3} K^+ ion). Although Mg^{2+} was found to afford maximal enzyme activity, six others divalent metal ions were also shown to activate the enzyme. The order of activation being Mg^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+}.[79] Moreover, it was shown that divalent metal ions possessing ionic radii of less than 1 Å were competitive inhibitors of 3-methylaspartase. These included Ca^{2+} ion (K_i = 27 mmol dm^{-3}) and Sr^{2+} ion (K_i = 2.11 mmol dm^{-3}).[79]

Bright and Silverman showed using kinetic and paramagnetic studies that the K_m-value of different metal ions was independent on the substrate concentration and that K_m was the a true dissociation constant for the enzyme-substrate complex.[80] They also showed that the true substrate was the free amino acid and not the metal-amino acid complex.[81]

At pH 9.0 Botting and Gani found that the K_m value for (2*S*,3*S*)-3-methyl aspartic acid varied with the concentration of Mg^{2+} ion and K^+ ion.[82] The influence of the metal ions on the value of K_m is shown in Figure 5.

Bright showed that the value of K_m for mesaconic acid was reduced by 4-fold by replacing Mg^{2+} ion by the more electronegative Co^{2+} ion.[83] This finding suggested that the metal could interact with the substrate in the active site, and that the more electronegative Co^{2+} ion was capable of binding more tightly to the enzyme-substrate complex than Mg^{2+} ion. Bright proposed that the role of the divalent ion was to decrease the pK_a of the C-3 hydrogen atom in order to ease its removal by an active site base. Finally, EPR experiments carried out by Fields and Bright using manganese as the divalent ion, showed that two metal ions were bound by enzyme molecule.[77]

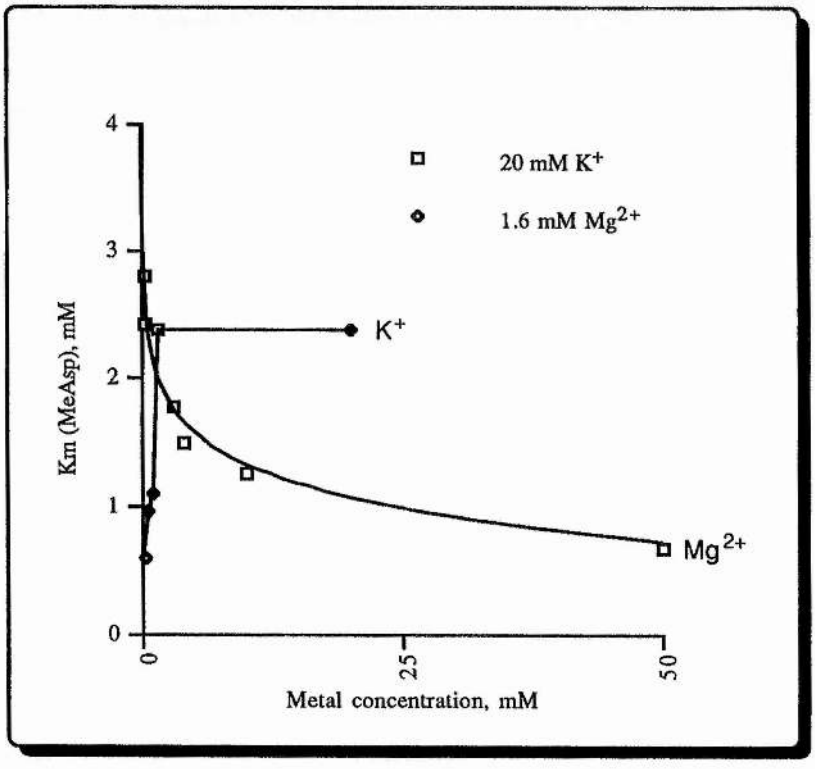


Figure 5*: Influence of metal ions concentration on* K_m

1.5.4 Early investigations of the mechanism

Bright's early investigation into the mechanism of deamination involved the reaction of mesaconate and ammonia with MAL in D_2O and L-*threo*-(2*S*,3*S*)-3-[^{2}H]-methylaspartic acid in H_2O.[84] In both cases exchange, at C-3, with the solvent was observed by ^{1}H-NMR spectroscopy solely.[80,84] In addition the rate of exchange at C-3 in *erythro*-(2*S*,3*R*)-3-methylaspartic acid was shown to occur at a rate greater than that for the overall reaction.[84,85] This finding along with the apparent lack of a primary isotope effect (for the deamination reaction over the pH range 5.5 - 10.5), all together with the fact that the enzyme is capable of catalysing the elimination of ammonia from L-*threo*-(2*S*, 3*R*)-3-methyl aspartic acid led to the proposal of a stepwise mechanism involving a carbanion intermediate (Scheme 19).[80,84]

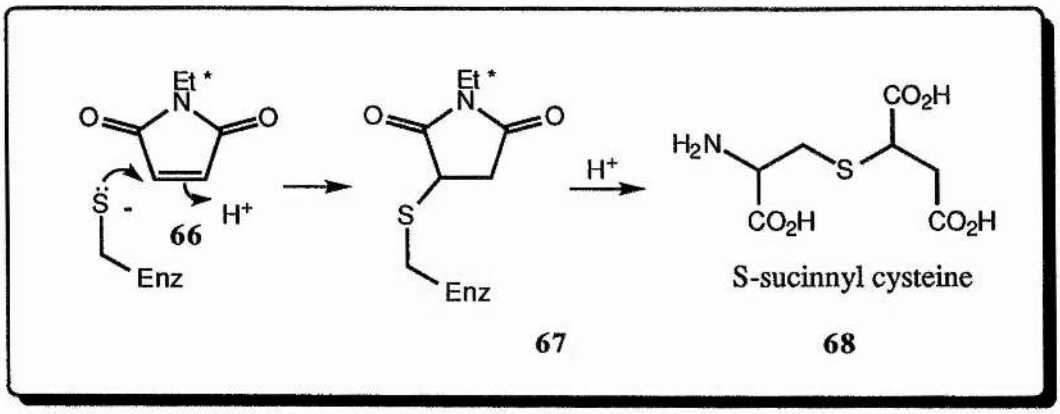
William and Libano showed that the rate of enzyme inactivation was proportional to the destruction rate of thiol groups by photo-oxidation.[86] Protection against inhibition was achieved in the presence of the substrate and a number of analogues. In the other hand divalent metal ions did not afford any protection. A titration experiment with *p*-chlorohydroxy-mercurybenzoate showed that one equivalent of inhibitor was consumed per sub-unit of enzyme.[87] This findings provided strong evidence to suggest that one cysteine was present in the active site, but that the Cys residue was not a binding site for the divalent metal ion.[87] Bright proposed that the active cysteine residue might act as the catalytic base and remove the proton at C-3 of the substrate.[80] This catalytic role was suggested on the basis of the analysis of the pH rate profile which showed a maximum rate at pH 9.7 that corresponds to the pK_a of a cysteine group (9.0 to 9.5). At pH 9.7 the thiol group can fulfill efficiently its role of base as it is fully deprotonated.

Attempts to identify the active site base involved labelling the enzyme with the chemical modifying reagent [1-^{14}C]-*N*-ethylmaleimide (**66**, Scheme 18) ([1-^{14}C]-NEM) and subsequent digestion with the protease enzyme trypsin, known to cleave the peptide chain at the carboxyl groups of Lys and Arg residues. This resulted in eight radiolabelled peptides (out of a theoretical total number or cleavage sites of 55). These were presumed to have resulted from S-alkylation of all of the available cysteine residues. The experiment

was repeated in the presence of excess substrate (acting to protect the active site) and unlabelled NEM. Unreacted inhibitor was denatured with 2-mercaptoethanol and excess of 2-mercaptoethanol was removed by dialysis along with the substrate. The enzyme was then treated with [^{14}C]NEM and the enzyme was then again subjected to tryptic digestion. In this case just one radiolabelled peptide was isolated and the amino acid composition was determined by hydrolysis with 6 mol dm^{-3} HCl. Analysis of the result showed the presence of 42 amino acids (Table 11). An unusual amino acid was observed which was attributed to a half-cysteine (**67**) (hydrolysis product of NEM-cysteine **68**), resulting from covalent addition by NEM (Scheme 18).[88]

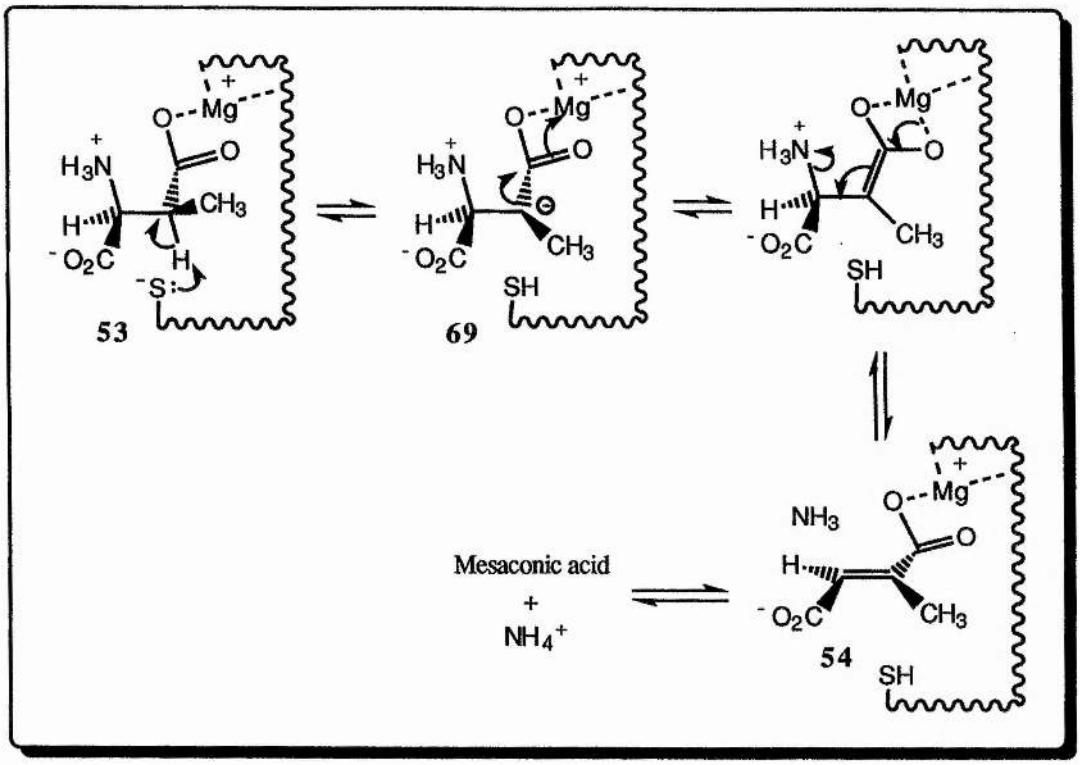
Table 11 : *Comparison of amino acid content of the labelled tryptic peptide isolated by Wu and Williams (NEM determined) and inferred amino acid sequence for 3-methylaspartase (NEM deduced).*

amino acid	3-methyl aspartase	NEM peptide determined	NEM peptide deduced
alanine	41	5	4
arginine	23	2	2
asparagine	18		3
aspartic acid	33		5
(Asp + Asn)	51	8	8
cysteine	7	1(half-cysteine)	0
glutamine	12		1
glutamic acid	29		3
(Glu + Gln)	41	4	4
glycine	38	8	2
histidine	7	0	0
isoleucine	29	2	2
leucine	27	1	0
lysine	28	2	2
methionine	17	1	1
phenylalnine	14	1	1
proline	13	2	2
serine	6	1	1
threonine	22	1	1
tryptophan	2		0
tyrosine	12	1	2
valine	35	3	5



Scheme 18: *Mechanism of inhibition of MAL by N-methylmaleimide and hydrolysis of resulting product*

Following these findings a mechanism for MAL was proposed that involves an active site cysteine residue as the catalytic base.[77] With the assistance of a magnesium ion bound at the β-carboxylate group of (2*S*,3*S*)-3-methylaspartic acid (**53**), the proton at C-3 of the substrate is removed to generate carbanion (**69**). Finally the carbanion eliminates ammonia to give mesaconic acid (**54**).



Scheme 19: *E1*$_{CB}$ *type mechanism for 3-methylaspartate ammonia-lyase*

1.5.5 Expression of 3-methylaspartase in *Escherchia Coli*

Goda *et al.* in this laboratory identified the *Clostridial* 3-methylaspartase gene by hybridisation studies using a ^{32}P-labelled synthetic oligonucleotide.[72] The N-terminal sequence of the sub-unit was determined using Edman degradation and a 77-mer oligonucleotide probe capable of encoding a 26-mer peptide from the N-terminus was synthesised. In position of codon degeneracy those nucleotides bases most frequently utilised in *Clostridial* genes were employed. The gene was mapped to a 2.2 kb sequence of *Clostridial* DNA contained within a Hind*III* restriction fragment. This enabled the encoding, sequencing and expression of 3-methylaspartase into *Escherichia coli* (plasmid pSG4), to yield the enzyme at a level of ~ 6% of the total soluble protein. Neal *et al.* in our group have performed a polymerase chain reaction to amplify the 3-methylaspartase gene which includes an extra region of DNA at the N-terminus to encode for a deca-His tag.[89] The amplified gene was finally expressed into the pET-16b *Escherichia coli* vector.[89]

1.5.6 Investigation on the nature of the active site base responsible for the abstraction of the H-3 hydrogen atom of (2S,3S)-3-methylaspartic acid

Babbitt and coworkers have examined the homology in the amino acid sequence of enzymes catalysing the removal of the α-protons of carboxylic acid (Table 12).[90] For two of these enzymes, mandelate racemase and muconate-lactonising enzyme, the 3-dimensional structures of the enzyme bound substrate and substrate analogues were known. These afforded the identification of the active site base for both enzymes as Lys166 and Lys169 respectively. The role of these Lys residues were also confirmed by site directed mutagenesis of these two. Hence, the sequence KXK was identified as the general motif for this class of enzymes. Alignment of the amino sequences of mandelate racemase and muconate-lactonising enzyme with the other members of this class of enzymes afforded the identification of the active site base of these later, see Table 12.

Table 12: *Sequence superposition in the region of the active site base for enzymes catalysing the abstraction of α-protons of carboxylic acids. Residues whose roles have been confirmed experimentally are designated by asterisks (*). All of the other active site residues are predicted.*

Enzyme	sequence number	AA-sequence	general base
Mandelate racemase	161-167	G F R A V **K T K** I	K166*
Galactonase dehydratase	141-147	G F D T F **K L N** G	(N146)
Glutarate dehydratase	208-214	G F K D F **K L K** G	K213
Muconate-lactonising enzyme	164-170	R H R V F **K L K** I	K169*
N-Acylamino acid racemase	158-164	G Y V R I **K L K** I	K163
3-methylaspartase	221-227	R D R I I **K L R** V	[R221/R226]
o-Succinylbenzoate synthase	152-159	G E K V A **K M K** V	K158
SynORF	138-144	G A S T F **K W K** I	K143

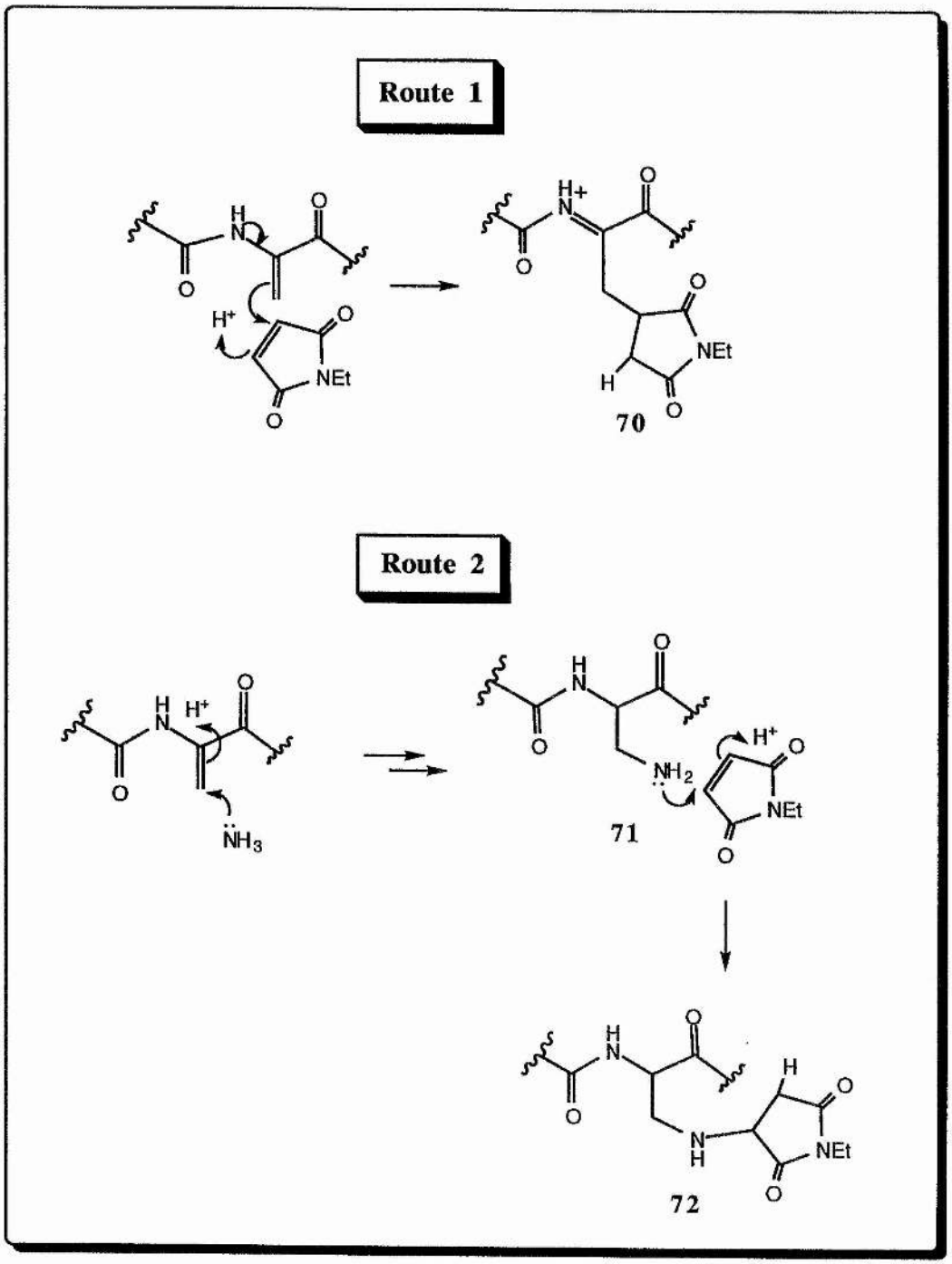
For most enzymes the conservation of the motif KXK was observed except for galactonase dehydratase (KLN) and 3-methylaspartase (KLR). As far as 3-

methylaspartase is concerned Arg226 was proposed to be the active site base responsible for the abstraction of the *pro-S* proton at C-3. This proposal was further supported by mutagenesis experiments with mandelate racemase in which the k_{cat} of the mutant K166R (ICR) was reduced 10^3-fold relatively to the wild type enzyme.[91] This suggested that the mutant enzyme was still able to catalyse the removal of the α-proton and therefore that an ARG residue was capable of participating as a general base catalyst. Thus it was suggested that the guanido functional group of an Arg residue could participate as the active site base in 3-methylaspartase as well.

1.5.7 A dehydroalanine residue in (2*S*)-3-methylaspartate ammonia-lyase

When the DNA sequence of the gene was established, a comparison of the inferred amino acid sequence and the amino acid content of the tryptic digest (isolated by Wu and Williams)[88] was carried out, with the intention of mapping the active site.

There appeared to be no correlation with peptides containing cysteines, however a very good match was found if the active site residue was serine (peptide deduced, Table 11). A 37-mer peptide, which spanned Lys147 to Lys183 was shown to contain a unique serine residue Ser173 (Figure 6). In order to explain the reaction of this residue with NEM, initial modification of Ser273 to form a dehydroalanine residue is required. This presumably occurs *via* a post-transitional dehydration. In order to account for the reaction of NEM with a dehydroalanine at the active site, two possibilities were considered. The first is an electrophilic reaction of dehydroalanine moiety (acylated enamine) as a nucleophile to give adduct (**70**, Scheme 20). The second involves the initial conjugate addition of ammonia resulting in a reactive nucleophile (**71**), which could then directly react with NEM to give the secondary amine (**72**) (route 2 in Scheme 20).



Scheme 20: *Proposed mechanisms of inhibition by NEM*

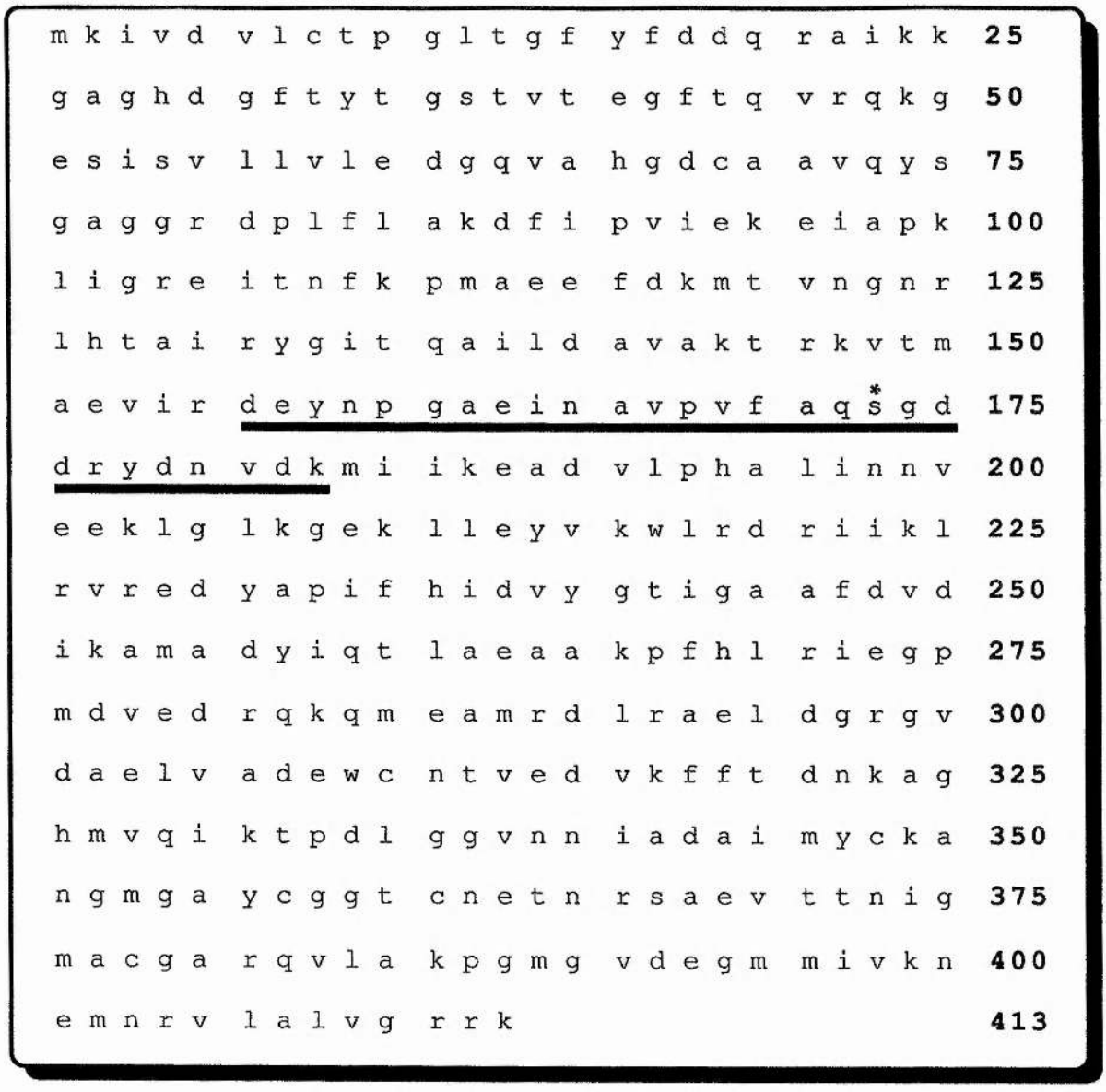


Figure 6: *Primary sequence of methylaspartase (the active site sequence is underlined)*

The presence of a dehydroalanine residue at the active site is consistent with the observed inhibition by hydrazine and a number of analogues. The proposed mode of inhibition being nucleophilic modification of the terminal methylene group. In contrast, Akhtar in this laboratory observed that anionic reagents such as potassium cyanide, nitromethane and sodium borohydride did not inhibit 3-methylaspartase.[92] The reasons for this may be due to the electronic environment surrounding the dehydroalanine residue. The presence of carboxylate chains for instance while favouring the binding of the amino group through hydrogen bondings, will repel any negatively charged reagent.

A series of chemical reactions were undertaken in our laboratory to model the reaction of dehydroalanine with amines.[93] The study showed that when a synthetic dehydroalanine model (**73**, Figure 7) was treated with propylamine, the sole product was the corresponding diaminal (**74**) resulting from nucleophilic addition at the α-position. Substituting the ethylamide group of the dehydroalanine with the more electron-withdrawing methyl ester gave model compound (**75**). Upon reaction with propylamine the ester afforded the product of conjugate addition, compound (**76**), as a result of nucleophilic addition at the β-position. Furthermore, the N-acylated model (**77**) also gave the corresponding conjugate addition product (**78**).

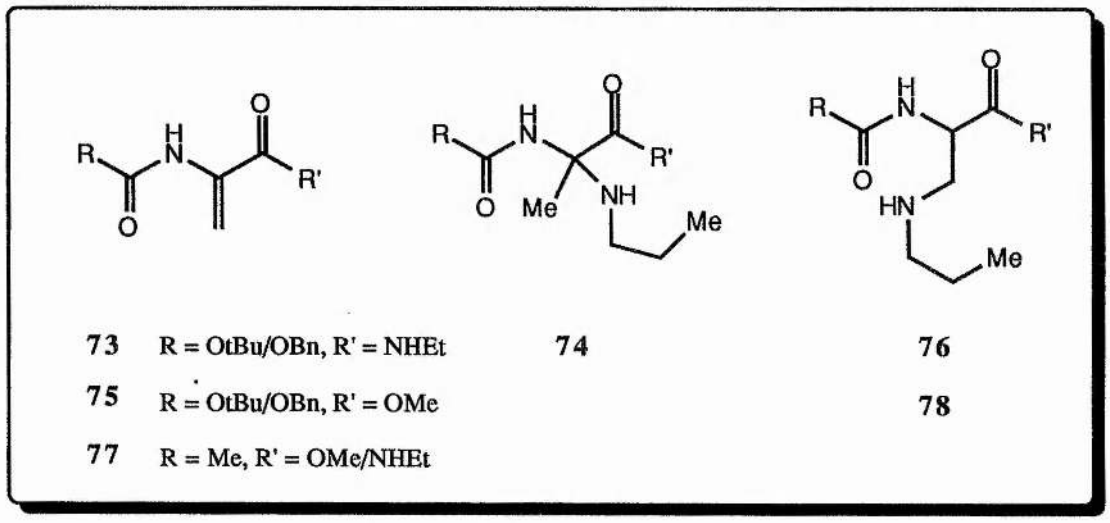
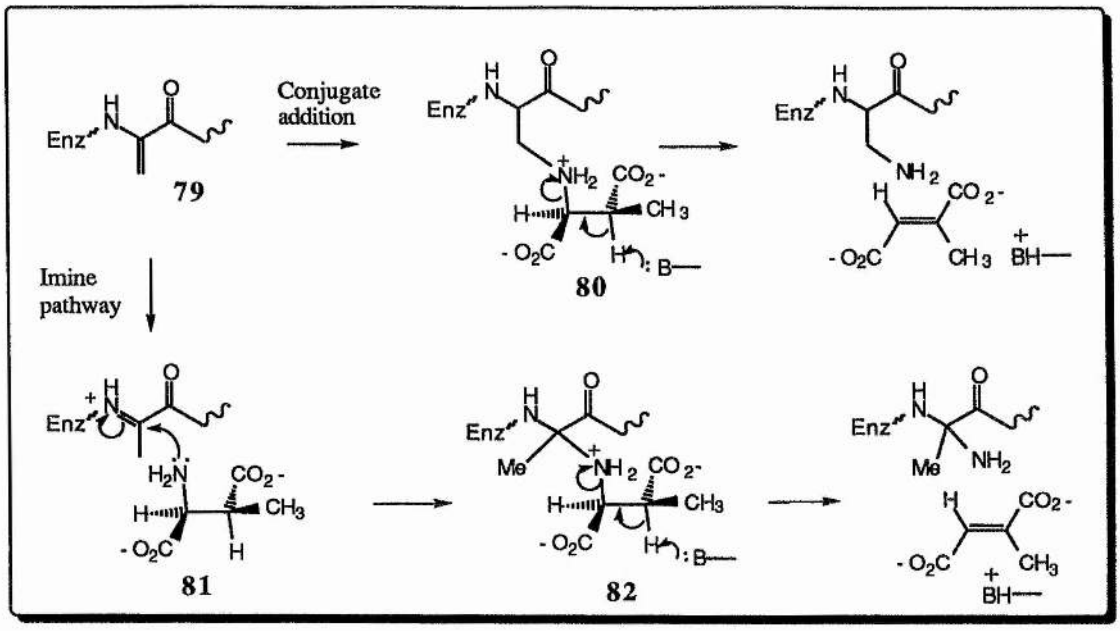


Figure 7: *The reaction of propylamine with dehydroalanine residues*

It appears that the loss of amide resonance in the C-1 carboxamide moiety on changing an N atom for an O atom promotes β-addition whilst a reduction in the delocalisation of the lone pair of the α-amino group on changing from acyl to urethane protection promotes α-addition. This has significant implications to the natural enzyme system, since torsion about the amide bond flanking the dehydroalanine residue can easily be affected by the surrounding of the protein. This would result in a change to the electronic properties of the dehydroalanine residue which would favour nucleophilic addition at either the α or the β-position, see Scheme 21.



Scheme 21: *Plausible routes for the interaction of the substrate with the dehydroalanine residue*

1.5.8 Update of the mechanism of (2*S*)-3-methylaspartate ammonia-lyase

Recently research in our laboratory has focused on the kinetics of the deamination reaction and has involved repeating some of the earlier studies.[82] In contrast to Bright findings,[80] a significant deuterium isotope effect was observed for L-*threo*-(2*S*, 3*S*)-3-[^{2}H]-methylaspartic acid of 1.7 on V_{max} and V/K for C-H bond cleavage at pH 9,[94] indicating that this step is at least partially rate limiting. The lack of an observation of this effect in Bright studies might have reflected the fact that only 87% of their substrate pool was deuterated. However, it was shown that at the high levels of K^+ ion used in Bright's work, the isotope effect is completely suppressed.[82]

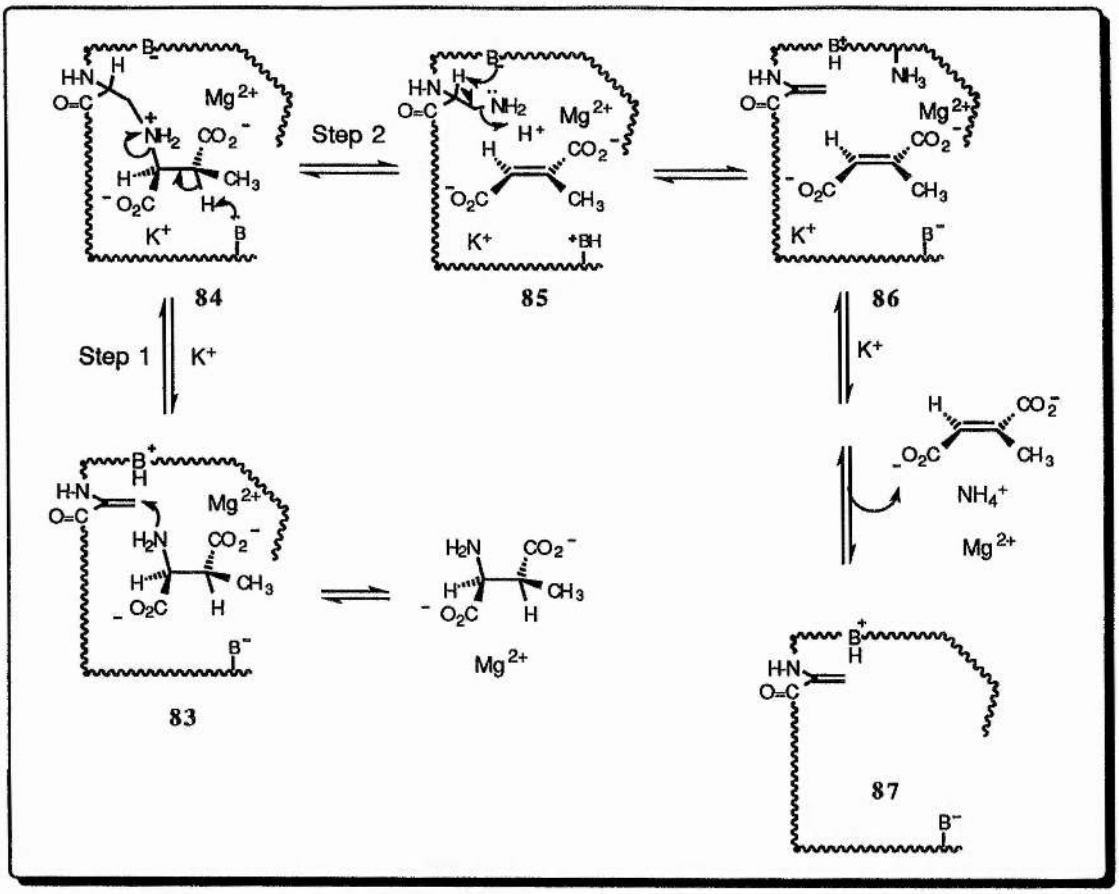
The influence of K^+ was further studied in detail by varying the concentration of both the mono- and divalent metal ion.[82] Botting and Gani, indeed, found that the concentration of K^+ had a profound effect on the reaction commitment of the C-H bond cleavage step and consequently on the deuterium isotope effect, in the range 1.6-50 mmol dm^{-3} (^{D}V(1.6 mmol dm^{-3} K^+) = 1.7, ^{D}V (50 mmol dm^{-3} K^+) = 1.00).[82] The isotope effect on V/K for *threo*-(3*S*,3*S*)-3-methyl aspartic acid, was found to increase (1.00 to 1.68) as the concentration of potassium was decreased (50 to 1.6 mmol dm^{-3}).

This gave strong evidence that K^+ had an effect on the forward commitment, suggesting that the binding order of K^+ was compulsory and must occur after the substrate.[82]

Botting and Gani noted with some interest that (2*S*)-aspartic acid, despite having a higher K_m than the physiological substrate did not show an isotope effect.[94,95] This indicated that the poor enzyme affinity for aspartic acid did not effect the isotopically sensitive step, suggesting that the initial reversible binding of substrates and products was not rate limiting.[94] Moreover, *erythro*-(2*S*,3*R*)-3-[^{2}H]-methyl aspartic acid, showed a $^D(V)/^H(V)$ of 6.79.[95] This large isotope effect showed clearly that C-H bond cleavage is the rate limiting step in the reaction. The corresponding effect on V/K was found to be lower ($^D(V/K)/^H(V/K) = 3.39$), indicating that any reverse steps preceding the isotopically sensitive step, are slow and must exert a forward commitment.[95]

As substrate debinding is fast the cause for the depressed value of $^D(V/K)$ was proposed to be the slow break-down of the covalent L-*erythro*-(2*S*,3*R*)-substrate enzyme complex to afford the Michaelis complex which is the reverse reaction of step 1, see Scheme 22.[94]

Further studies were carried out using double isotope ($^2H/^1H$, $^{15}N/^{14}N$) fractionation techniques to investigate the ^{15}N isotope effect for C-N bond cleavage in the absence and presence of a deuterium at C-3 of the substrate.[96] It was found that at pH 9.0 an isotope effect of 1.0246 on V/K was observed. This value of ~ 2.5% is quite large and therefore significant, leading to the conclusion that in addition to C-H bond cleavage, C-N bond cleavage is also partially rate limiting.[96] These observations suggested that the mechanism actually operates *via* a concerted pathway and not through a discrete carbanion intermediate as originally proposed.[82,96] This proposal was further supported by (2*S*,3*S*)-3-methyl cyclopropane 2,3-dicarboxylic acid (**61**) acting as transition state mimic ($K_i = 20\ \mu M$).[74]



Scheme 22: *Proposed mechanism for 3-methylaspartate ammonia-lyase*

In summary (2*S*,3*S*)-3-methyl aspartic acid is believed to form a covalent enzyme substrate from a dehydroalanine residue. After the formation of a Mg^{2+} enzyme-substrate complex (**83**) it is believed that the K^+ ion adds and the N-atom of the substrate attacks the dehydroalanine residue at C-3 to give the conjugate addition product (**84**). This covalent enzyme-substrate adduct then undergoes elimination to give a covalent ammonia-enzyme adduct (**85**) *via* a concerted process which is at least partially rate determining for the overall reaction. In subsequent steps it is believed that the amino-enzyme (**85**) is deaminated to give the product complex (**86**). The products are subsequently released generating the free enzyme (**87**).

1.6 Role of dehydroalanine in the mechanism of phenylammonia-lyase and histidase

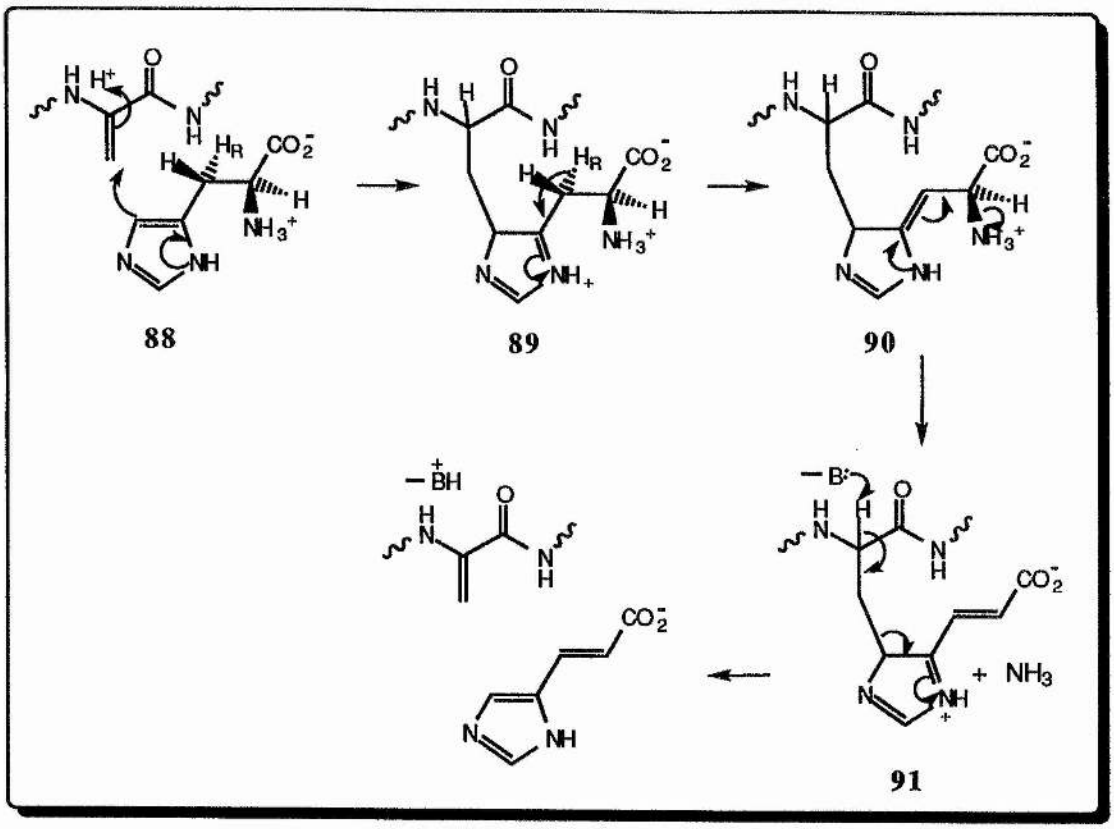
As was discussed earlier the presence of dehydroalanine at the active site of HAL was established, involving the preparation of a series of mutant enzymes (S143A, S143T and S143G), see p. 21. These mutants were assayed for activity using (2*S*)-histidine and the alternative substrate 5'-nitro histidine, see Table 13.[97,98]

Table 13: *Comparison of the kinetic parameters for wild-type enzyme and mutants with L-histidine (left) and 5'-nitro-L-histidine (right) as substrates* [98]

enzyme	K_m (mmol dm^{-3})	V_{max} (IU/mg)		K_m (mmol dm^{-3})	V_{max} (IU/mg)
wt	5.2	25		7.7	0.85
S143A	7.5	0.0214		9.8	0.87
S143T	3.1	0.00047		12.8	0.87
S143G	5.3	0.0047		4.0	0.52

The results showed that mutant S143G was capable of catalysing the deamination of 5'-nitro-histidine at a rate 100 times greater than for the natural substrate L-histidine. Retey *et al.* proposed that the nitro group present in the alternate substrate was mimicking the effect of the dehydroalanine residue, by lowering the pKa of the C-3 proton of 5'-nitro-histidine.

Hence, the role of the dehydroalanine in the native enzyme was proposed to be associated with assisting deprotonation of the β-hydrogen by lowering its pK_a. This effect on pK_a is achieved through a Friedel-Crafts type acylation of the substrate, see in Scheme 23.



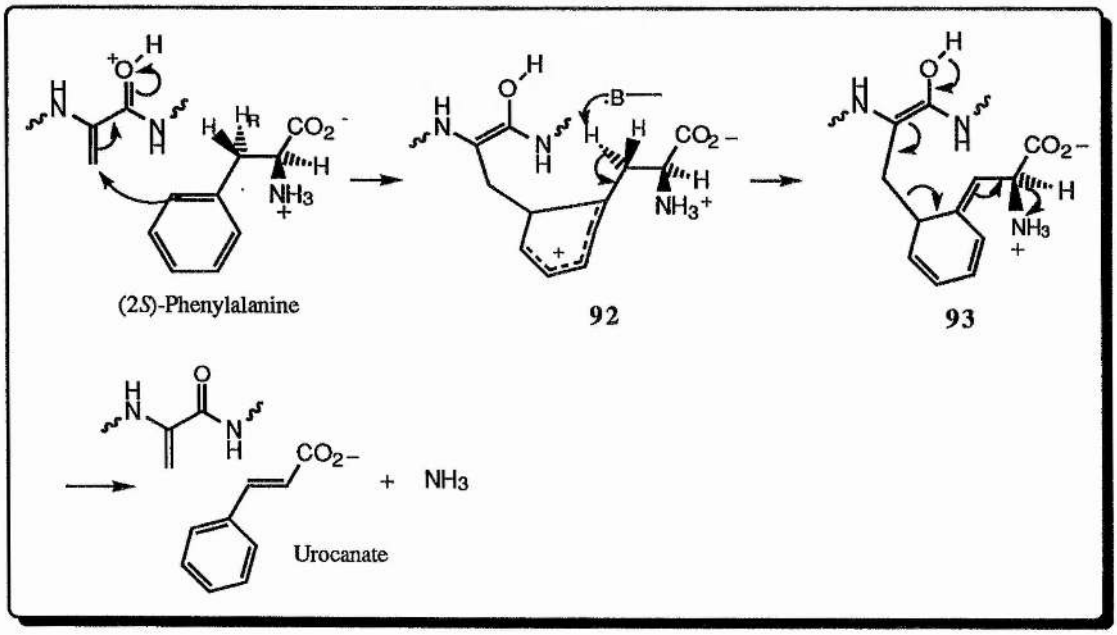
Scheme 23: *Friedel-Crafts type mechanism for histidase*

In an analogous series of experiments the phenyl ammonia-lyase mutants S202A, S202T were assayed with the alternate substrate *p*-nitrophenylalanine.[99,100] The results which are displayed in Table 14 showed that the mutants enzyme are 80 to 300 times more active with *p*-nitrophenylalanine than with phenylalanine. Wild type enzyme which has been treated with $NaBH_4$ was 15 times more active with the *p*-nitrated substrate analogue than with natural substrate.

These results suggest a similar role for the dehydroalanine as described previously for PAL. It was proposed that the electrons of the phenyl ring attack the dehydroalanine residue to generate an acyl cation (**92**) (Scheme 24). The acyl cation promotes the abstraction of the *proR* β-proton to generate structure (**93**). Finally, a cascade reaction involving regeneration of the dehydroalanine residue, re-aromatisation of the phenyl ring and elimination of ammonia, leads to the urocanate product.

Table 14: *Comparison of the kinetic parameters from wild-type PAL, the mutants S202A, S202T, and the $NaBH_4$ treated wild-type PAL (wt PAL red.) with p-NO_2 Phe as substrate*

Enzyme	K_m (mmol dm^{-3}) for *p*-NO_2Phe	V_{max} (U/mg) for Phe	V_{max} (U/mg) for *p*-NO_2Phe
wt PAL	0.32	0.56	0.17
S202A	0.84	1.1 E-4	0.008
S202T	1.14	2.7 E-5	0.01
wt PAL red	1.08	4.5 E-4	0.007



Scheme 24: *Friedel-Crafts acylation of the phenyl ring for phenylalanine ammonia-lyase*

Chapter 2 : A study of the inhibition of β-methylaspartase by phenylhydrazine

2.1 Introduction

Phenylalanine ammonia-lyase and histidine ammonia-lyase were both shown to be totally inactivated by anionic nucleophiles including the anion of nitromethane, potassium cyanide and potassium borohydride. Subsequent studies with the corresponding radiolabelled reagents highlighted the site of covalent modification to be the active site dehydroalanine residue. In addition phenylhydrazine was also shown to be an efficient irreversible inhibitor of the enzyme. Furthermore the enzymes which had been inactivated by phenylhydrazine, when treated with radiolabelled potassium cyanide did not incorporate any radioactivity. This strongly suggested that both cyanide and phenylhydrazine were reacting with the same catalytic group believed to be dehydroalanine residue.

Parallel studies with methylaspartase showed that the enzyme was irreversibly inactivated by phenylhydrazine derivatives, Table 15.[101]

Table 15: *Activity of 3-methylaspartase after incubation with a range of inhibitors. All incubations were carried out on ice in Tris-HCl buffer at pH 9.0 (50 mmol dm^{-3}) unless otherwise stated*

entry	Inhibitor	Conc. (mmol dm^{-3})	Activity/Time
1	4-nitrophenylhydrazine	2.5	2% 135 min
2	phenylhydrazine	10	100% 180 min
3	phenylhydrazine (30°C)	10	<10% 150 min

Anionic nucleophiles derived from cyanide, nitromethane and borohydride, however, had no effect on the enzyme activity. It was suggested that the lack of inhibition could be attributed to unfavorable electrophilic interactions between the anionic inhibitors and an active site which is likely to contain a number of negatively charged amino acid side chains required, possibly, for binding to the metal. This very polar environment is prone to produce a high energy barrier, between the free enzyme and inhibitor and the active site bound inhibitor.

In order to determine the site of covalent modification caused by phenylhydrazine, ^{14}C-phenylhydrazine was synthesised and incubated with 3-methylaspartase.[102] The resulting inactive enzyme was dialysed extensively to remove unreacted inhibitor whereupon it was found that despite remaining inactive the protein did not contain any radioactivity. Analysis of the protein by SDS-PAGE showed distinct bands consistent with cleavage of the subunit.

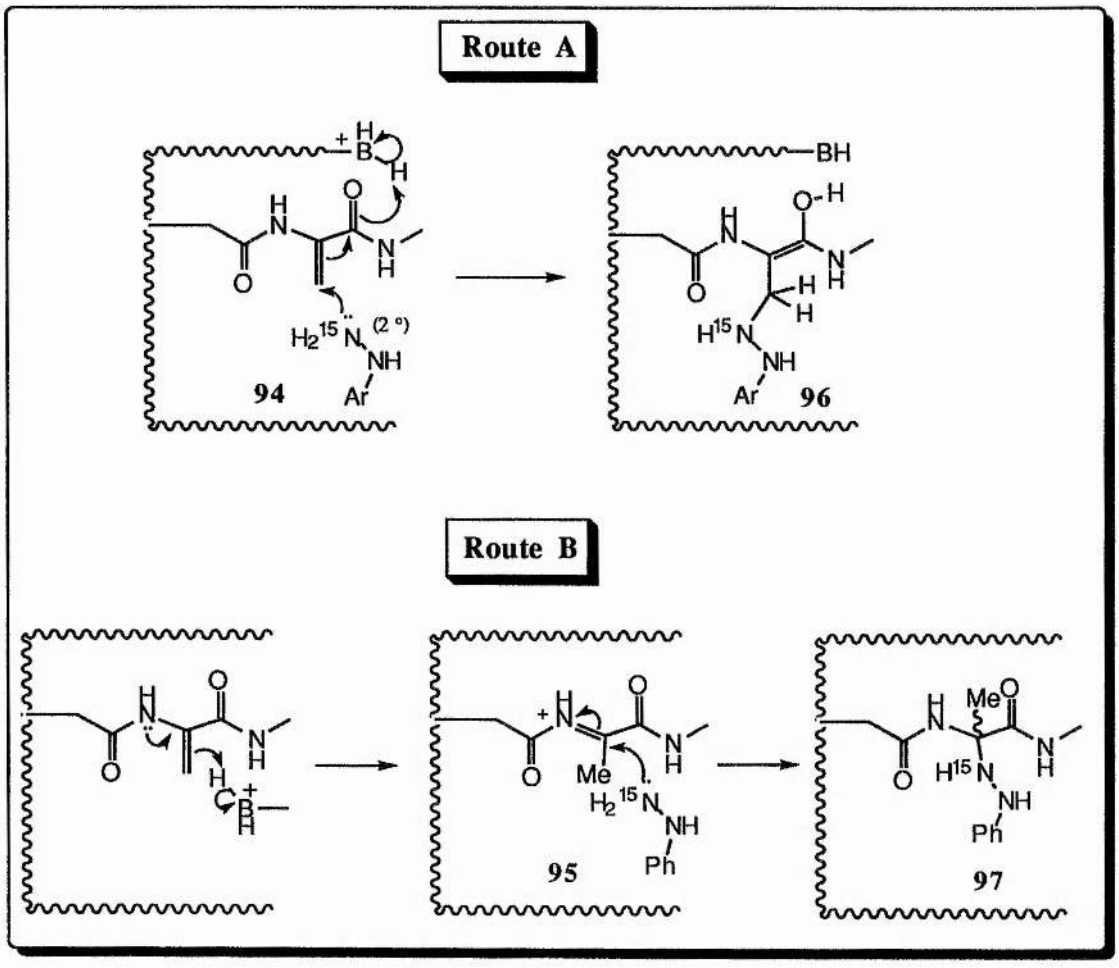
In order to gain further information on the mode of inhibition by hydrazine analogues the experiment was repeated in our laboratory by Dr. Morris, with 2-^{15}N-phenylhydrazine. The inactive dialysed protein was treated with trypsin and analysed by {^{15}N edited}-^{1}H-NMR spectroscopy.[103] In this NMR experiment, magnetisation of the nitrogen ^{15}N nucleus is transferred to the protons attached to it such that other ^{1}H signals are not observed. As a result the filtered spectrum obtained displays only the signals for the proton attached to the labelled nitrogen.

Analysis of the {^{15}N}-^{1}H-decoupled NMR spectra displayed the presence of a single sharp peak at 7.64 ppm corresponding to the incorporation of a ^{15}N atom into the protein. Removal of the ^{15}N-decoupling resulted in the transformation of the singlet into a clearly resolved doublet with a coupling constant of 80 Hz, see Figure 8, p. 50.

Four plausible mechanisms were proposed for the reaction of phenylhydrazine with dehydroalanine, see Scheme 25, p. 48.

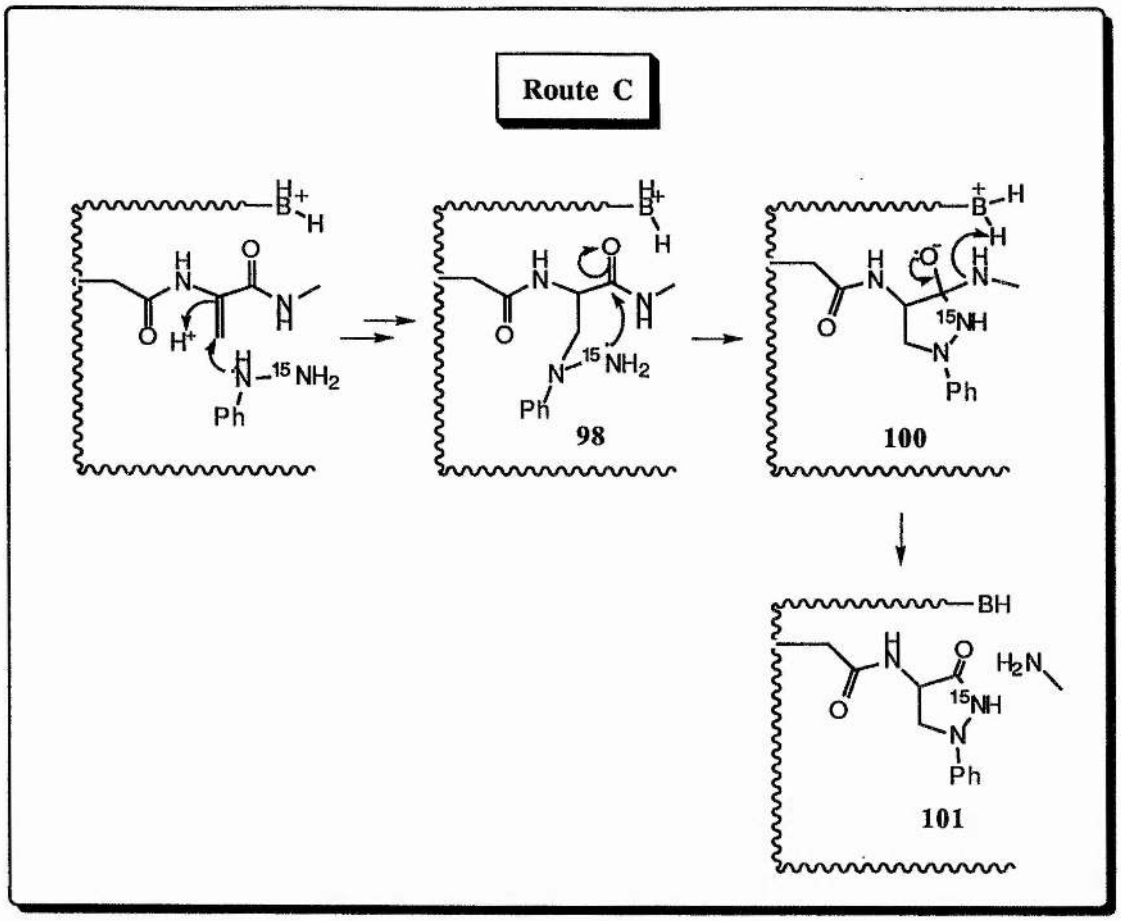
Routes A and B both involve nucleophilic attack of the N-1 nitrogen at the β– or α-position of the active site dehydroalanine residue, Scheme 25a. These were both abandoned due to inconsistency with the observed NMR spectra. Note that the product

(**96**) of route A would be expected to give a doublet of triplets in the decoupled {^{15}N}-NMR spectrum and product (**97**) of route B would give rise to a doublet of doublets.



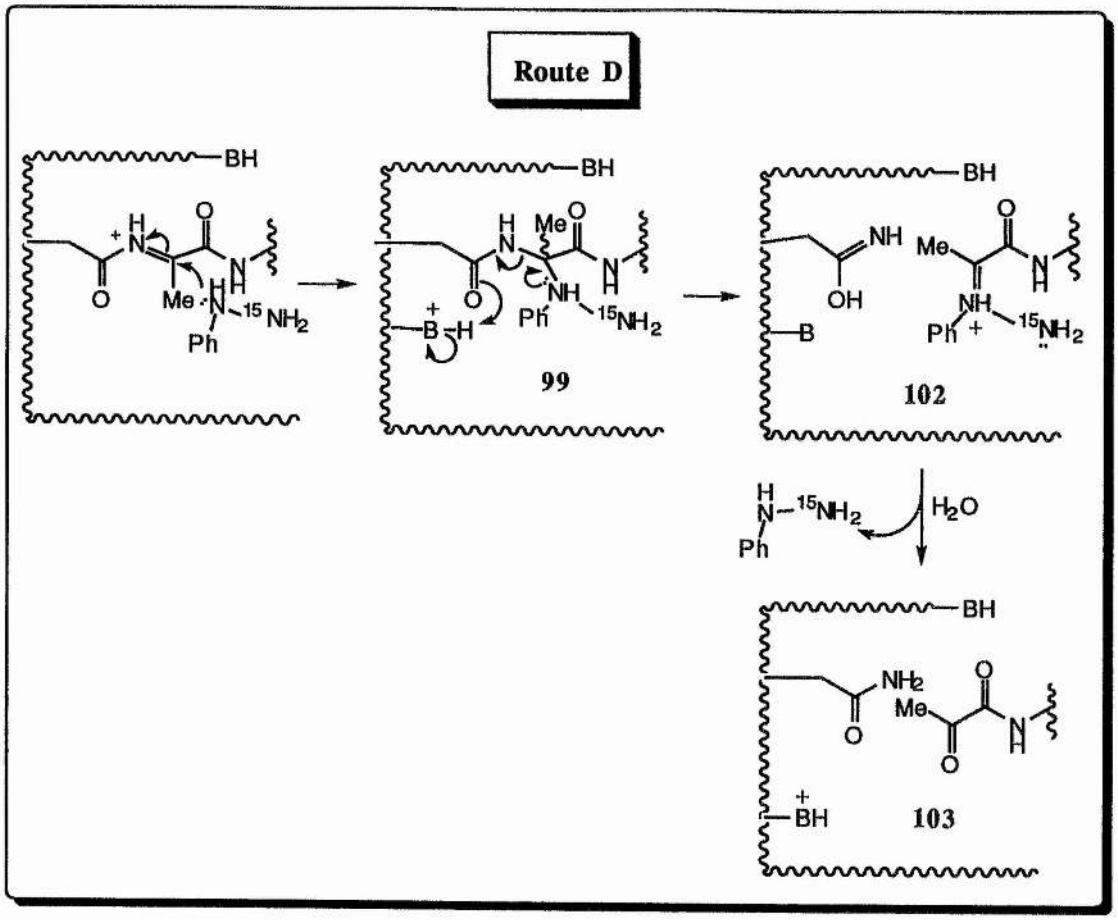
Scheme 25a: *Routes A and B as plausible mechanism of inhibition by phenylhydrazine*

Routes C and D involve attack of backbone dehydroalanine either at the β- or α-position *via* the more nucleophilic N-2 nitrogen to afford adducts (**98**) and (**99**), see Scheme 25b and Scheme 25c. Compound (**98**) can cyclise *via* attack of the primary amine onto the backbone carbonyl to form the cyclic product (**101**), see Scheme 25b. The expected {^{15}N}-NMR spectrum of this product would be a singlet, and is consistent with the observed NMR data.



Scheme 25b: *Routes C as a plausible mechanism of inhibition by phenylhydrazine*

The product of attack at the α-position (**99**, route D) can result in cleavage of the protein backbone to give Schiff's base (**102**) which upon hydrolysis releases phenylhydrazine and enzyme bound diketone (**103**), Scheme 25c. Hence, route D gives an explanation for the observed loss of radiolabel upon dialysis and appearance of bands in the SDS-PAGE attributed to cleaved subunits.



Scheme 25c: *Route D as a possible mechanism for the inhibition of 3-methylaspartase by phenylhydrazine*

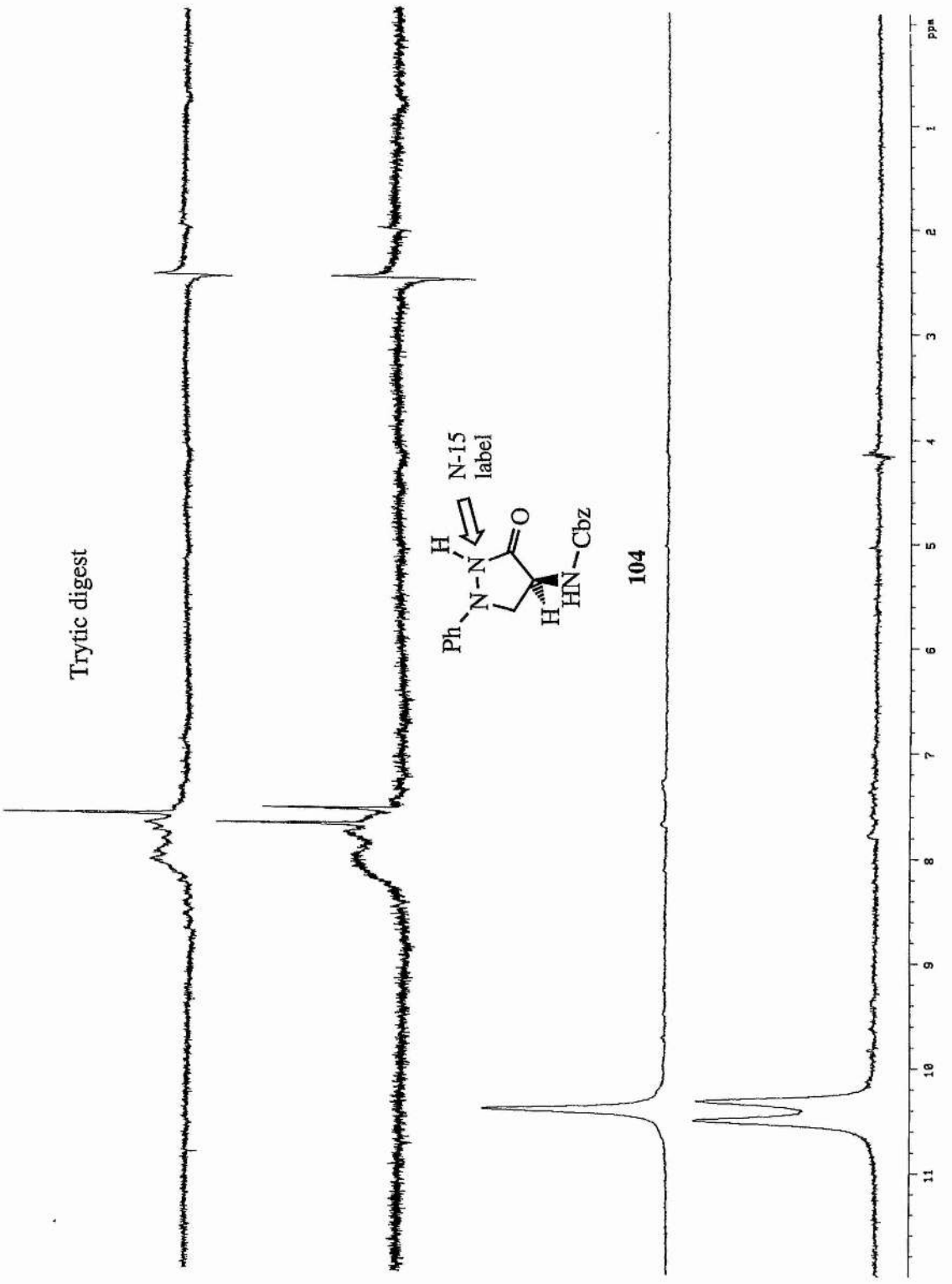
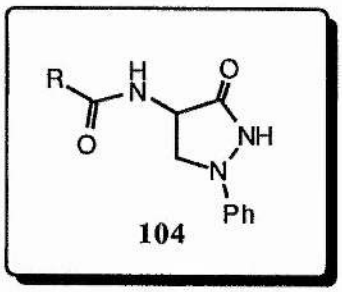


Figure 8: *{^{15}N}-edited-^{1}H-NMR of the triptic digest and of the model compound*

In order to gain further insight, it was decided to synthesise 4-amino-pyrazolidin-3-ones (**104**, R = CH_3 or CO_2Bn) as analogue of the cyclic product (**101**, Scheme 25b). The NMR characteristics could then be determined and compared with the experimental data obtained from the inactivation of the enzyme.



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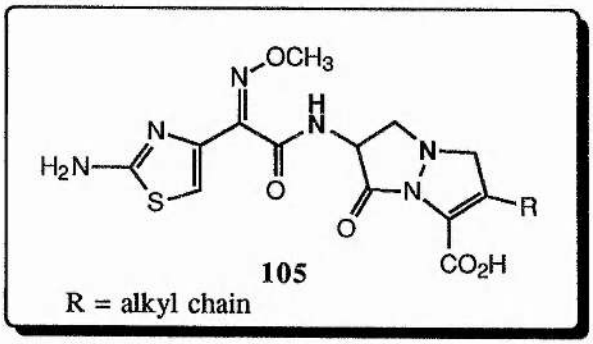
2.2 Synthesis of (4*S*)-1-phenyl-4-amino-pyrazolidin-3-one

Substituted 4-aminopyrazolidin-3-ones are common intermediates in the synthesis of bicyclic pyrazolidinones (aza-γ-lactams **105**) a potent new class of antibacterial agents.[104]

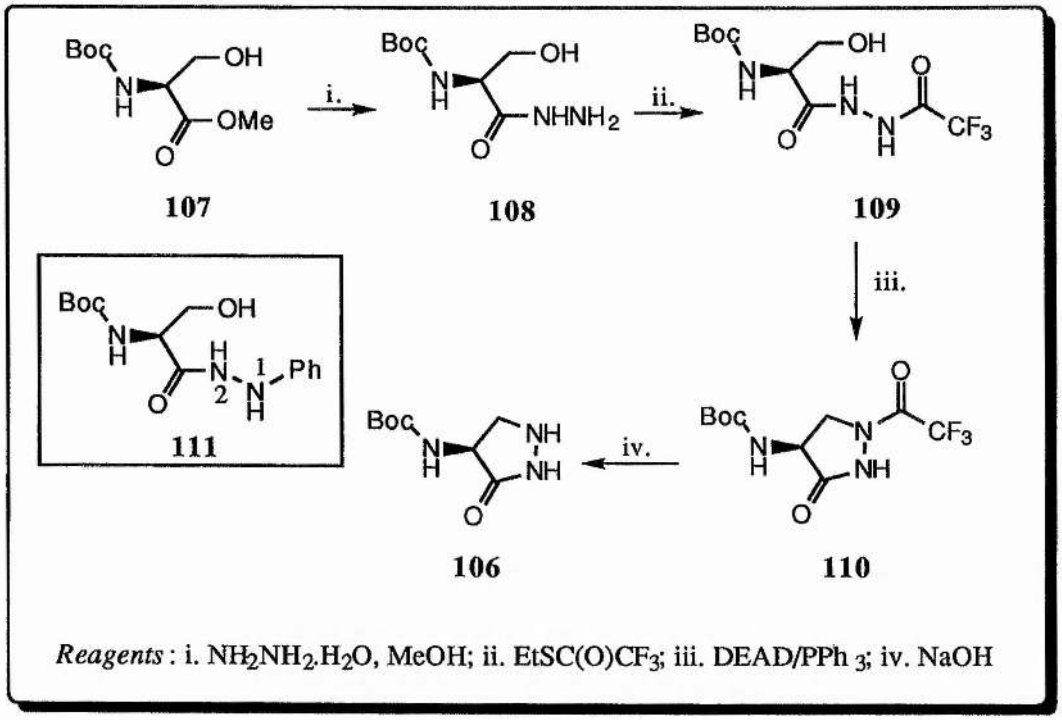


105

R = alkyl chain

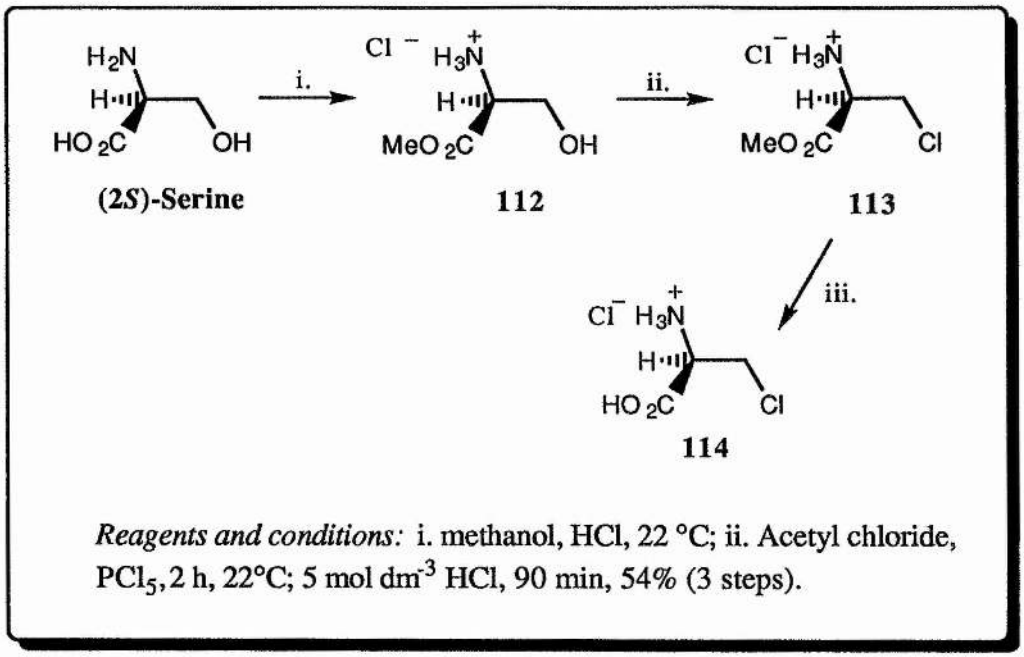
Holmes *et al.* described an asymmetric synthesis of *N*-Boc protected pyrazolidin-3-one (**106**),[105] see Scheme 26, starting from *N*-Boc methyl serine (**107**). Preparation of the hydrazide (**108**) was achieved from the methyl ester (**107**). The free amino group was then acylated with *S*-ethyl trifluoro-thioacetate to give the product (**109**). A

Mitsunobu reaction then afforded the pyrazolidino-3-one (**110**). This method takes advantage of the acidity of the N-1H bond, *i.e.* poor nucleophilicity making the hydroxy group the more reactive specie towards the Mitsunobu reagents.



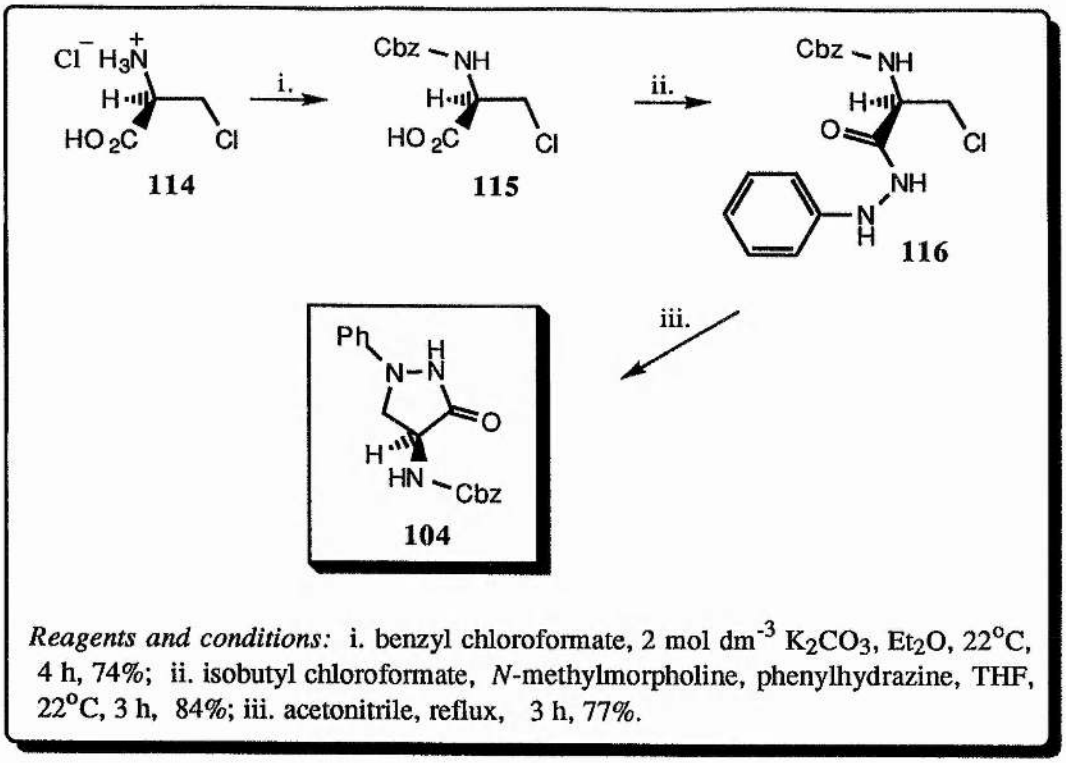
Scheme 26: *Holmes's synthesis of amino pyrazolidine-3-one*

The precursor to reach our target molecule (**104**) was thought to be hydrazide (**111**), see Scheme 26. However due to the strong nucleophilicity of its N-1 nitrogen, we choose a slightly different approach to our target pyrazolidin-3-one starting from chloroalanine. This was readily accessed in three steps from (2*S*)-serine (Scheme 27). Esterification of (2*S*)-serine was achieved by bubbling HCl gas through a solution of serine in methanol to give methyl ester hydrochloride (**112**) [mp 165 °C [lit.,[106] 167 °C]; $[\alpha]_D$ -2.3 (*c* 1 in H_2O) [lit.,[106] $[\alpha]_D$ -3.0 (*c* 1 in H_2O)]. The ester (**112**) was then reacted with phosphorus pentachloride in acetyl chloride according to the method of Fischer and Suzuki to give chloromethylester hydrochloride (**113**) which showed the require analytical data.[107] Finally hydrolysis of the methyl ester in refluxing 5 mol dm^{-3} aqueous hydrogen chloride (loss of the absorption at δ = 3.58 ppm), afforded the hydrochloride salt of chloroalanine (**114**) in 54% overall yield.



Scheme 27: *Synthesis of (2S)- chloroalanine*

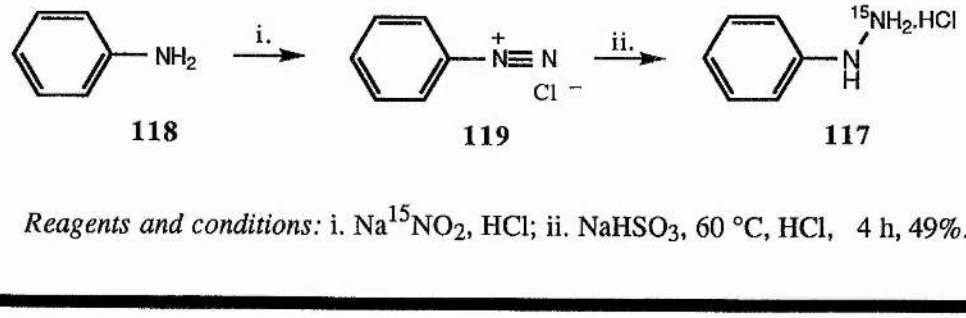
Reaction of chloroalanine (**114**) with benzylchloroformate in a biphasic mixture of diethyl ether and 2 mol dm^{-3} solution of potassium carbonate, gave benzyloxycarbonyl protected chloroalanine (**115**) in 74% yield (mp 122-3 °C [lit., 120 °C]), Scheme 28.[108] The *N*-benzyloxycarbonyl chloroalanine (**115**) was subsequently coupled to phenylhydrazine using anhydride peptide coupling methodology. The mixed anhydride was formed with isobutylchloroformate at low temperature in the presence of *N*-methylmorpholine. Phenylhydrazine was then added to form the chlorohydrazide (**116**) as a white solid in 84% yield [mp, 67-68 °C], which gave the required analytical data. Finally, the chlorohydrazide (**116**) was cyclised in refluxing acetonitrile to afford the 4-benzyloxycarboxylamino pyrazolidin-3-one product (**104**) in 77% yield [(mp, 108-110 °C; Found C, 65.50; H, 5.65; N, 13.35. requires for $C_{17}H_{17}N_3O_3$ C, 65.60; H, 5.5; 13.5]. ^{1}H-NMR spectra were recorded in DMSO due to high insolubility of the product in chloroform, and where signals associated with the acidic carbamate and amide protons were observed at δ 7.70 and 10.45 ppm.



Reagents and conditions: i. benzyl chloroformate, 2 mol dm^{-3} K_2CO_3, Et_2O, 22°C, 4 h, 74%; ii. isobutyl chloroformate, *N*-methylmorpholine, phenylhydrazine, THF, 22°C, 3 h, 84%; iii. acetonitrile, reflux, 3 h, 77%.

Scheme 28: *Synthesis of (4S)-1-phenyl-4- amino pyrazolidin-3-one (**104**)*

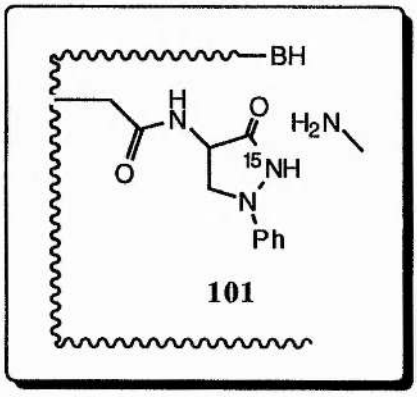
In order to compare the ^{15}N-NMR spectra of both the model compound and the enzyme system, [β-^{15}N]-pyrazolidin-3-one (**104**) was prepared. For this purpose [β-^{15}N]-phenylhydrazine (**117**) was synthesised according to a literature procedure, Scheme 29.[109] Diazotisation of aniline (**118**) using sodium [^{15}N]-nitrite in aqueous hydrochloride acid gave the required diazonium hydrochloride salt (**119**) which was found to be stable below 5 °C.



Reagents and conditions: i. $Na^{15}NO_2$, HCl; ii. $NaHSO_3$, 60 °C, HCl, 4 h, 49%.

Scheme 29: *Synthesis of β- [^{15}N]-phenylhydrazine*

This was reduced *in situ* with a freshly prepared solution of sodium sulfite in aqueous sodium hydroxide to afford the hydrochloride salt of [β-^{15}N]-phenylhydrazine (**117**) in 48% yield following recrystallisation from ethanol (mp 252 °C [lit., 250-254 °C]; (HRMS: found [M + H]$^+$, 110.0740. calc. for $C_6H_9N^{15}N$: 110.0736). Coupling with chloroalanine (**114**) gave the corresponding hydrazide which was subsequently cyclised *in situ* to afford labeled [^{15}N]-pyrazolidin-3-one (**104**). The labeled compound showed the same NMR data as for the non-labeled compound except that the N-2 amide proton was in this instance coupled with the N-15 atom to give a doublet with a coupling constant $J_{N\text{-}H}$ = 98 Hz.



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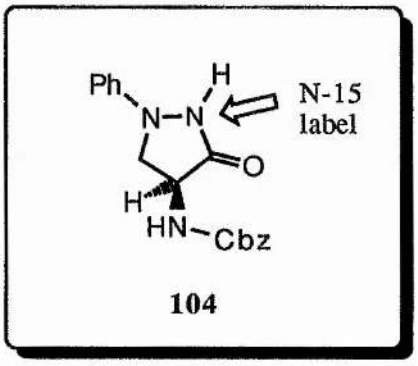


104

2.2.3 Conclusions

The {^{15}N}-edited-^{1}H-NMR spectra of the model compound showed the presence of a doublet at 10.45 ppm, with a coupling constant of 98 Hz. This was compared with the spectra from the tryptic digest of the protein which showed a doublet at 7.56 ppm with a coupling constant of 80 Hz (Figure 8). The difference in the chemical shifts of the amide proton between the model and the tryptic digest product, is not consistent with the proposed structure (**101**, see above).

It is interesting to note that the pyrazolidin-3-ones (**120**) and (**121**), described in the literature, showed a chemical shift for the amide proton respectively at 7.45 ppm and 7.39 ppm, see Figure 9.[110] This shows that the presence of a Cbz group on the N-1 nitrogen of the heterocycle (**121**) does not affect the chemical shift of the amide proton. On the other hand the large effect observed on the chemical shift of the amide proton for our system (**104**) is probably due to the presence of the phenyl substituent. The anisotropic region generated by the aromatic system could result in the deshielding of the amide proton of the pyrazolidin-3-one (**104**).

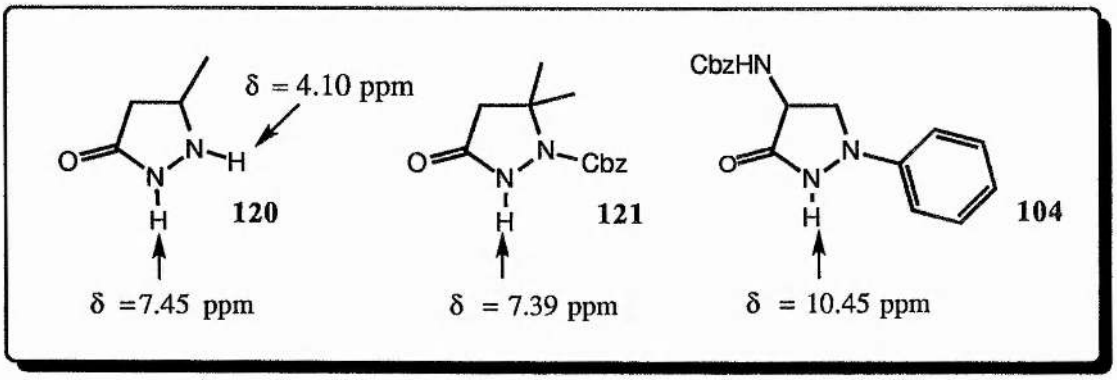
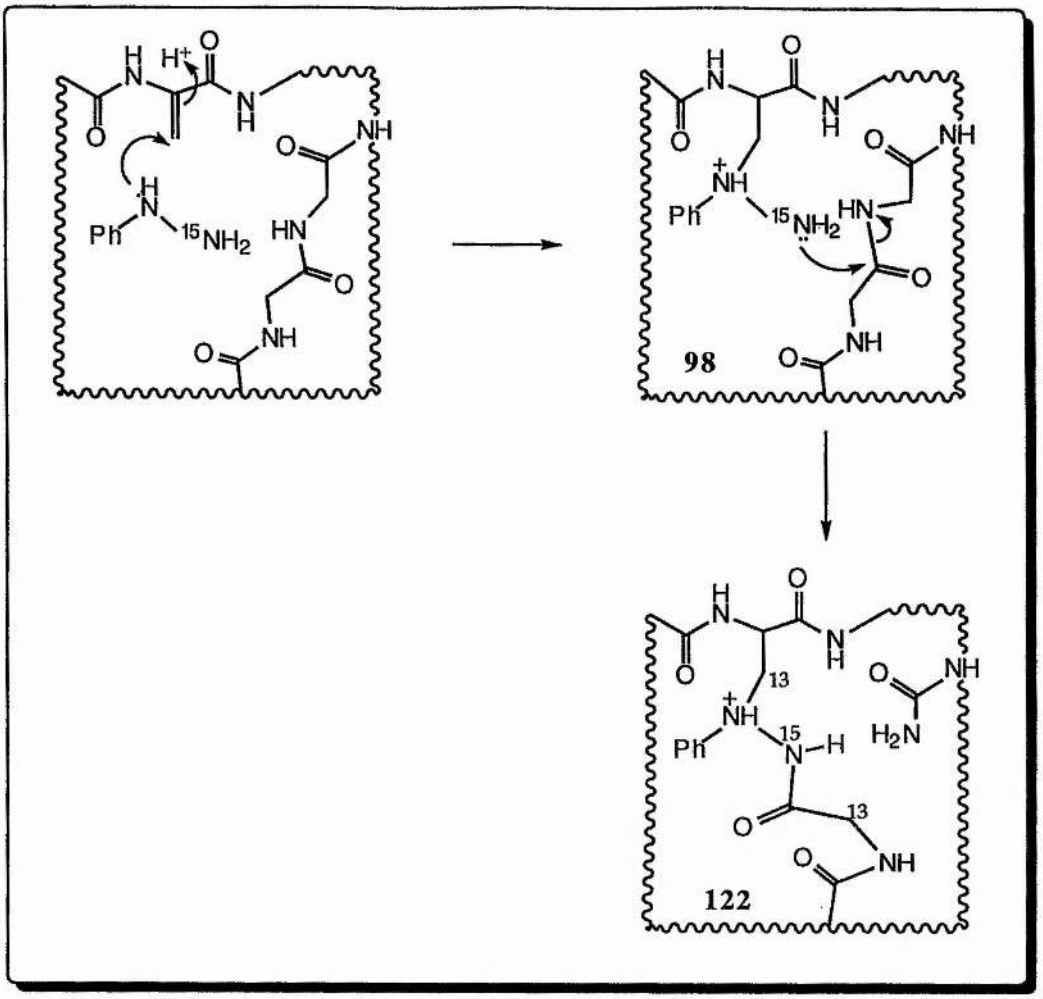


Figure 9: *Comparison of NMR data with literature compounds*

The proposed structure (**101**) therefore can not correspond to the $\{^{15}N\}$-^{1}H chemical shift observed at 7.56 ppm with the protein sample. An alternative explanation would be that the β-N atom of phenylhydrazine adduct (**98**) could have reacted with a near peptide chain in the active site to afford the structure (**122**), see Scheme 30.



Scheme 30: *Alternative mechanism for the inactivation of 3-methylaspartase by phenylhydrazine*

This structure would be expected to show a $\{^{15}N\}$-^{1}H chemical shift between 7.0 and 7.5 ppm since the phenyl substituent will have a very limited influence on the amide proton as the system is free to rotate. In order to test this proposal, work is currently underway to characterise all the spin system of the diazo bridge between the two peptide chains of the structure (**122**). This work consists in incorporating a $[^{13}C]$-label into all the amino acid residues of the protein and inactivating the resulting enzyme with $[\alpha$-$^{15}N]$-$[\beta$-$^{15}N]$-phenylhydrazine. It is hoped that the resulting adduct (**122**) can be completely assigned in this manner by transferring the magnetisation of the nitrogen ^{15}N-atom to the adjacent carbon ^{13}C-atom of the protein backbone.

Chapter 3 : Design and synthesis of potential inhibitors

3.1 Design of potential inhibitors

3.1.1 Transition state analogues

It was shown that (1*S*,2*S*)-1-methylcyclopropane 1,2-dicarboxylic acid (**61**) acted as a transition state mimic inhibitor for 3-methylaspartase with a K_i of 20 μM in the absence of ammonia. In the presence of ammonia, the value of K_i was reduced 2-fold to 11 μM, (**61**, Figure 10). Thus, the simultaneous binding of ammonia and the cyclopropane inhibitor at the active site of 3-methylaspartase is synergistic. It was hoped that the X-ray structure for the enzyme-inhibitor complex would provide a wealth of information on the different amino acid residues which are involved in binding the different species during the catalytic process. However, the structure would give no direct information on the participation of a dehydroalanine residue in the catalytic mechanism in the absence of ammonia. Of course it is not clear that ammonia would bind covalently since, clearly there is a site for ammonia ion binding as an activator also.[82]

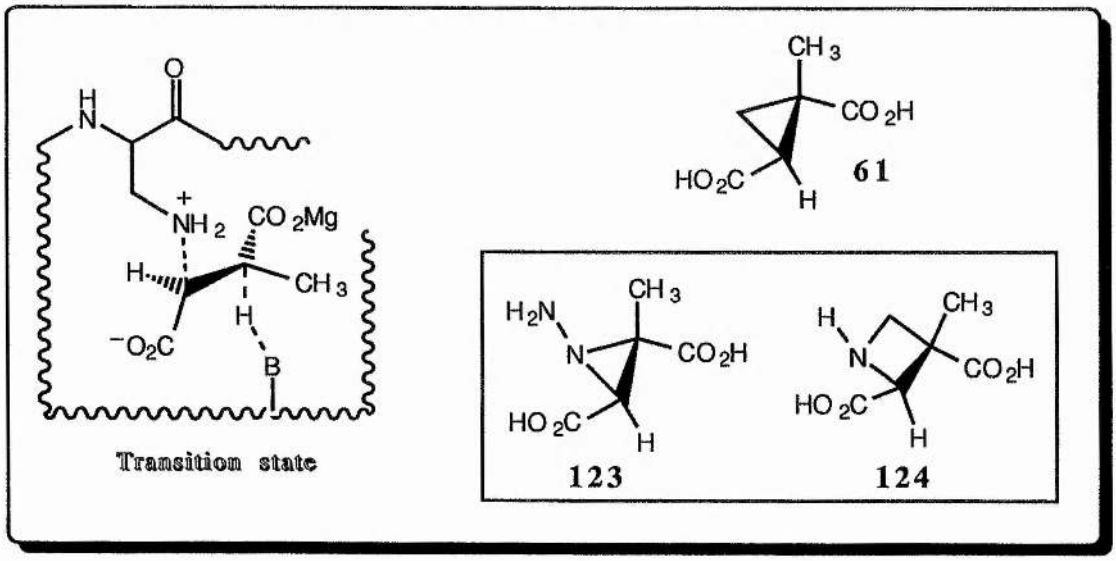


Figure 10: *Transition state analogues containing nitrogen*

In order to bridge this gap in our understanding we thought of designing molecules capable of mimicking the transition state for the elimination reaction (see Figure 10) and which contained a N-atom in a suitable position so that it could react with the active site dehydroalanine. To this end, we designed the *N*-amino aziridine dicarboxylic acid (**123**) and the azetidine dicarboxylic acid (**124**), see Figure. 10.

3.1.2 Substrate analogues

Previous studies in our laboratory had shown that 3-methylaspartase was irreversibly inactivated with hydrazine and various analogues including hydroxylamine and phenylhydrazines.[111] In contrast, (2*S*,3*S*)-3-methyl-2-hydrazinosuccinic acid (**58**) was not found to be an irreversible inhibitor of 3-methylaspartase but instead served as a competitive inhibitor with a K_i value of 2.32 mmol dm^{-3}.[75] This finding suggested that either the compound did not react with the dehydroalanine residue, or, the equilibrium lies in favour of the unbound species (**125**), see Figure 11.

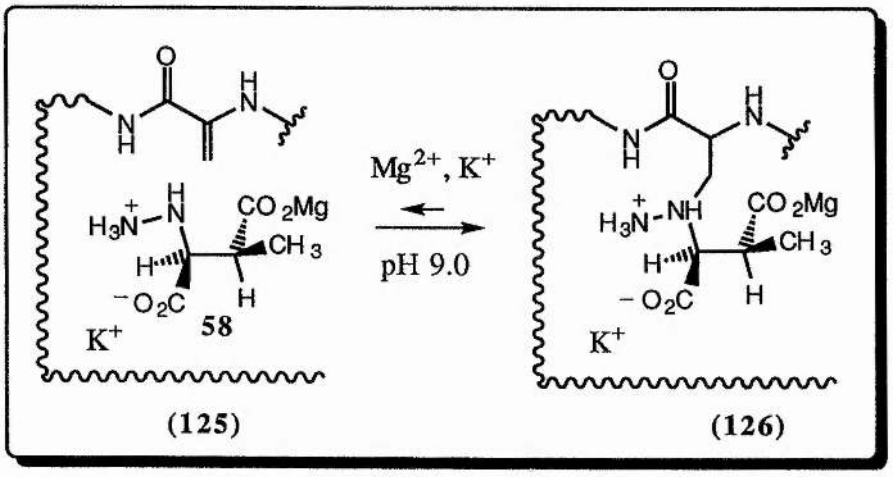


Figure 11: *Inhibition by (2S,3S)-3-methyl-2-hydrazinosuccinic acid (58)*

In order to investigate the possibility of irreversibly inactivating 3-methylaspartase *via* nucleophilic addition on the putative dehydroalanine residue, we thought of synthesising (2*S*)-3,3-dimethylaspartic acid (**127**), aminomethylfumaric acid (**128**), (2*S*,3*R*)-2-sulfanylsuccinic acid (**129a**) and (2*R*,3*R*)-2-sulfanylsuccinic acid (**129b**), Figure 12.

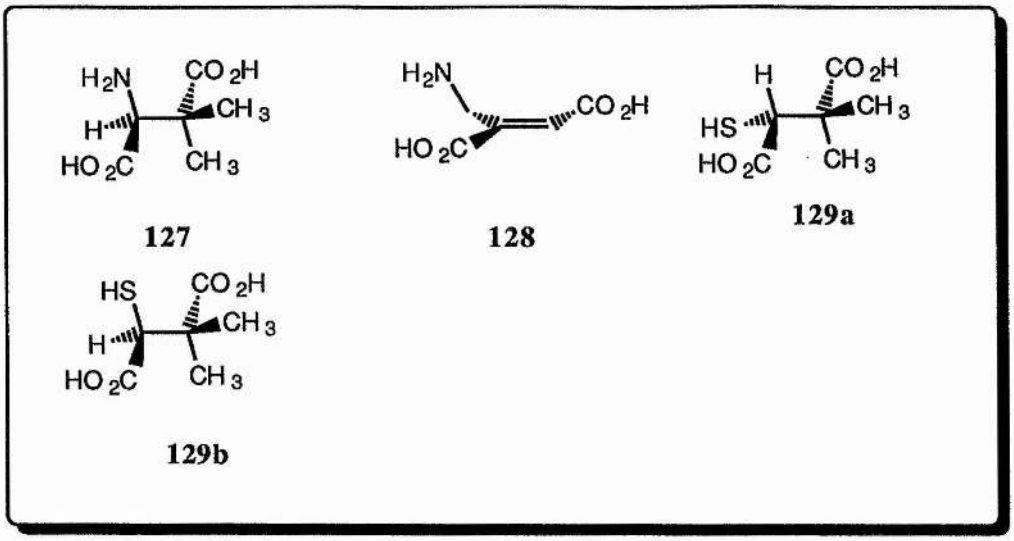
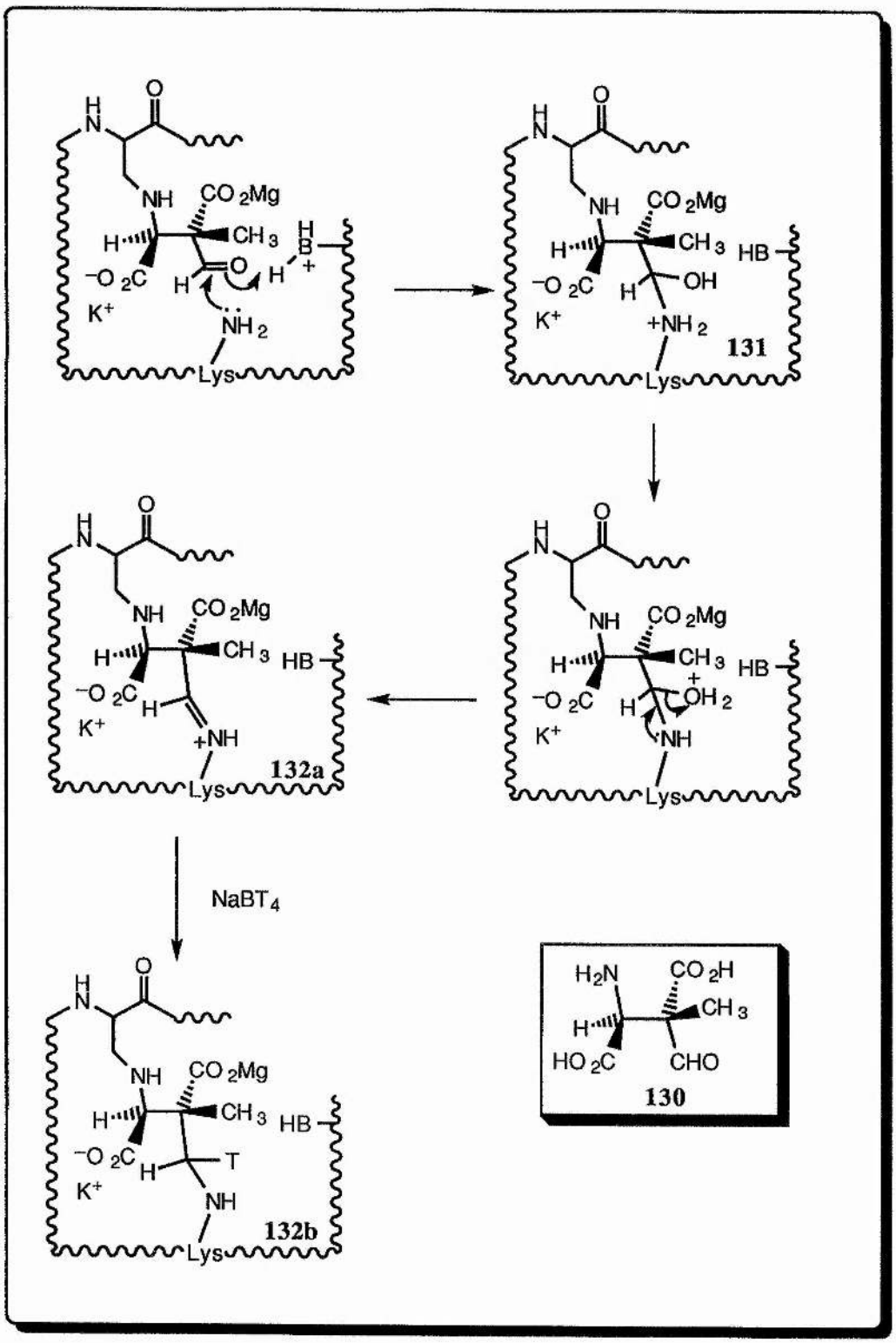


Figure 12: *Substrate analogues as potential inhibitors of ß-methylaspartase*

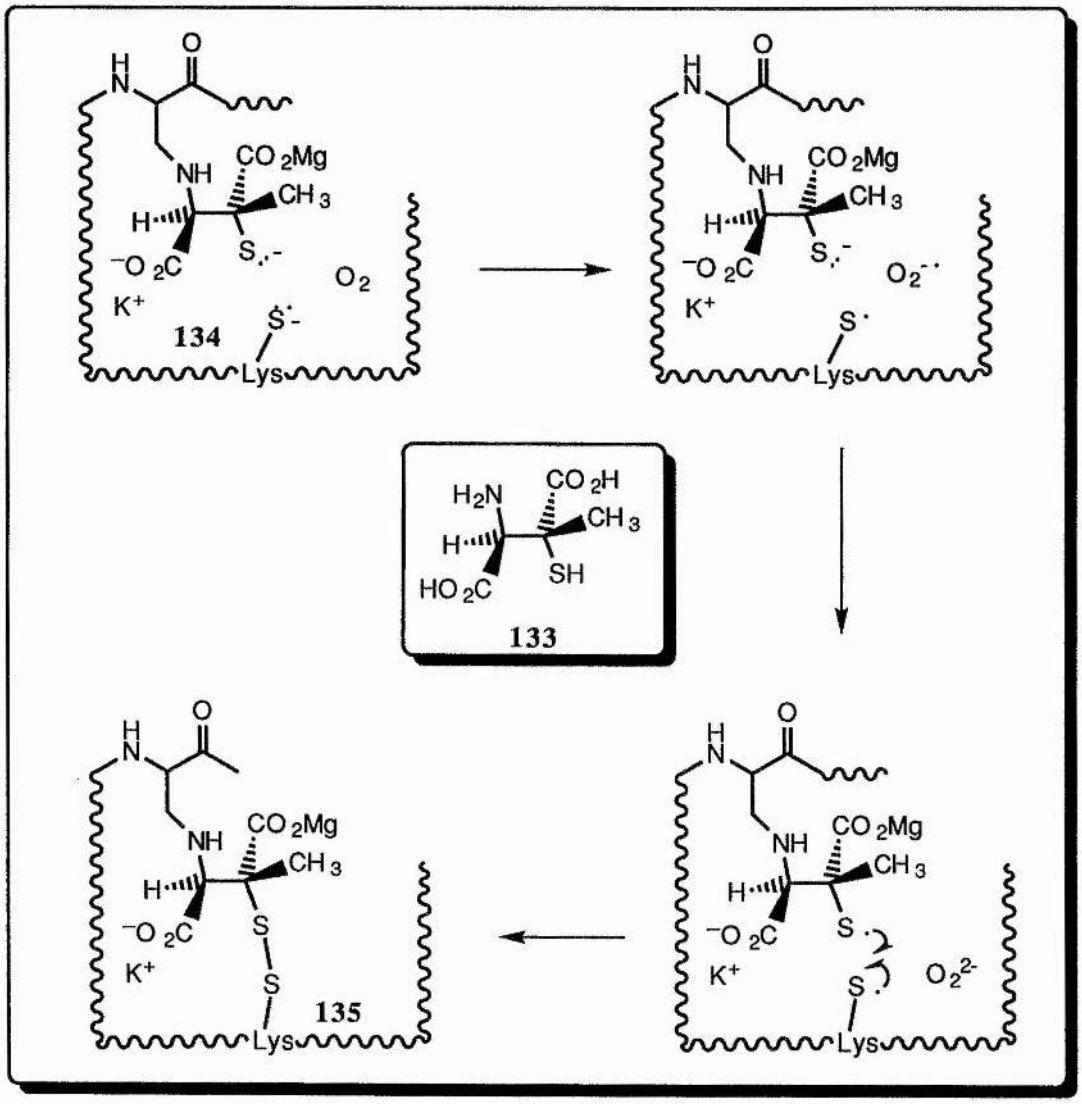
We reported ealier in the introduction (see p. 35) that Babbitt *et al.* suggested an Arg residue as the active site base for 3-methylaspartase. This proposal seems quite difficult to accept since the pK_a of the guanido group is 12. This pK_a-value implies that the guanido group is fully protonated at pH 9.7, pH where 3-methylaspartase was found to possess a maximum activity on V. A pK_a-value in the range 9.0 to 11.0 for the active site base would be more acceptable. A Lys residue (pK_a = 11.1) or a Cys residue (pK_a 9.0-9.5) seem to be in this respect better candidates for such a role.

In order to investigate the possibility that a Lys residue is the active site base for the removal of the 3-*pro-S* proton, we thought of synthesising (2*S*,3*S*)-3-formyl-3-methylaspartic acid (**130**). This highly functionalised amino acid should be able to bind at the active site and as such its aldehyde function might react with a Lys residue to give a covalent carbinolamine intermediate (**131**), see Scheme 31. Subsequent elimination of water should generate the imine (**132a**). Formation of such an intermediate would be of great interest for mapping the active site base since the imine can be reduced using labelled borohydride to afford a labelled dead end adduct (**132b**) by cross-linking between the dehydroalanine residue and the active site base.



Scheme 31: *Hypothetical cross-linking of the active site by 3-methyl-3-formyl aspartic acid*

Finally, investigation of the possibility that the active site base may be a Cys residue can be achieved with a compound containing a thiol group in the place of the C-3 proton. As such the sulfanyl α-amino acid (**133**) in the presence of oxygen would be capable to form a disulfide bridge with a near active site Cys residue. The occurrence of this reaction requires the concomitant occupation of the active site by oxygen and the inhibitor, Scheme 32.

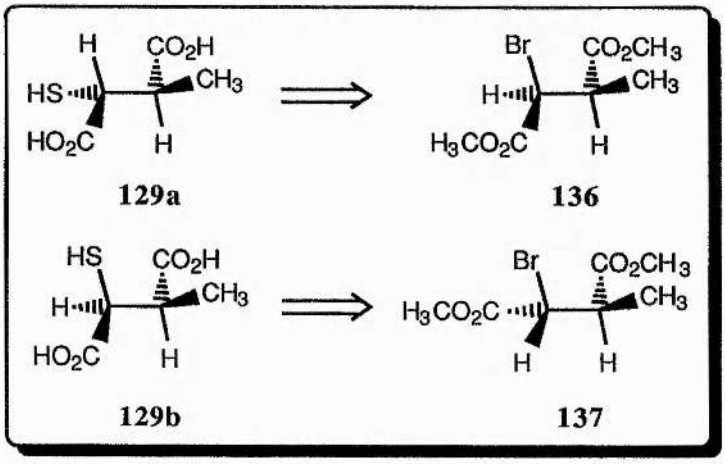


Scheme 32: *Hypothetical interaction of the sulfanyl α-amino acid in the active site of β-methylaspartase*

3.2 Synthesis of the potential inhibitors

3.2.1 Synthesis of (2*R*,3*R*)- and (2*S*,3*R*)-3-methyl-3-sulfanylsuccinic acids (129a) and (129b)

As no precedent in the literature was described for the succinic derivatives (**129a**) and (**129b**), we devised an asymmetric synthesis of both, respectively, starting from the bromides (**136**) and (**137**), see Scheme 33. This strategy was adopted since potassium thioacetate is a good reagent for the introduction of sulphur *via* alkyl bromides.[112]

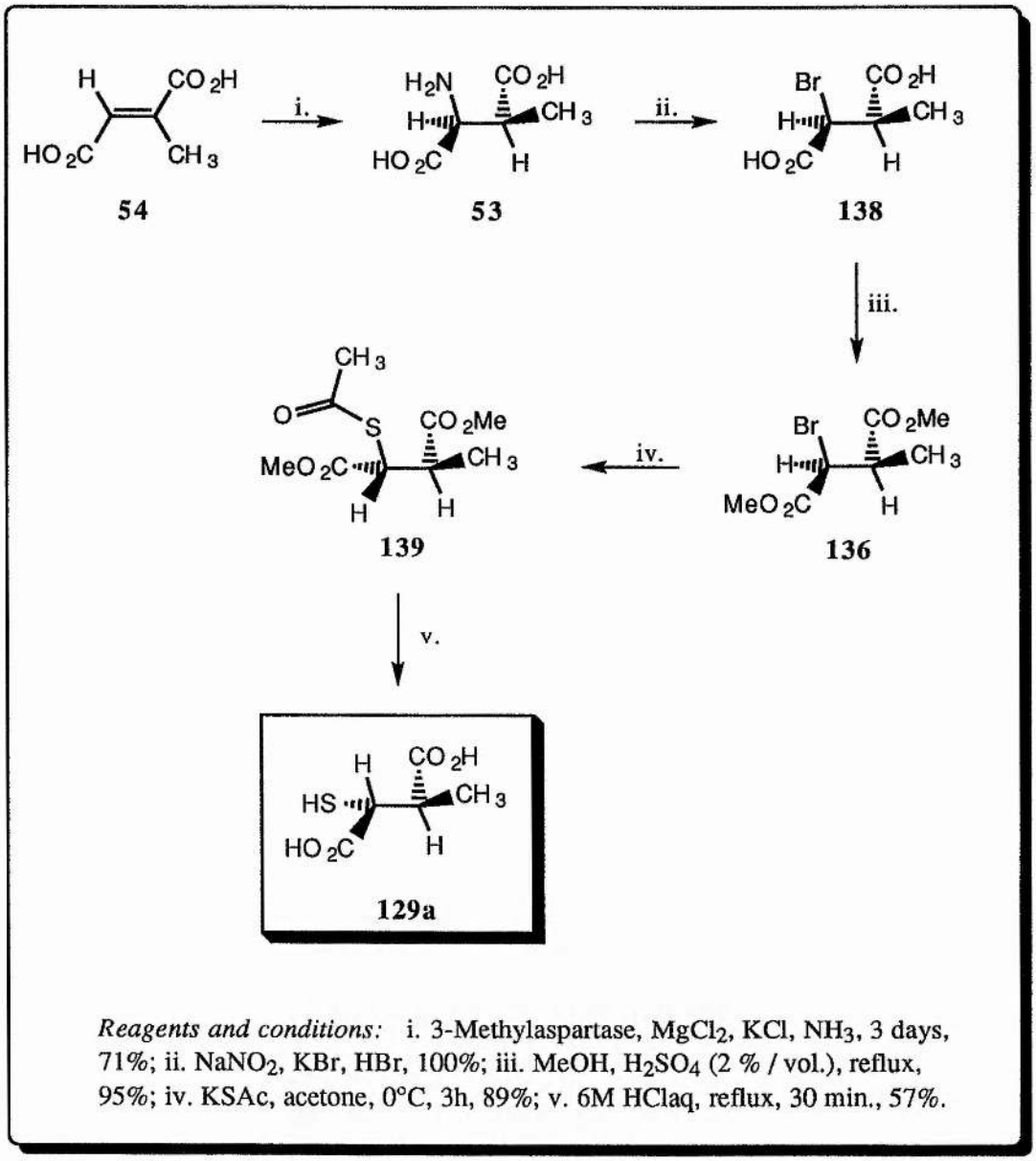


Scheme 33: *Precursors for the synthesis of the succinic acids (129a) and (129b)*

3.2.1.1 Synthesis of (2*R*,3*R*)-3-methyl-2-mercaptosuccinic acid (129a)

The bromide (**136**) was readily prepared in 3 steps from mesaconic acid (**54**) (Scheme 34).[114] Mesaconic acid was converted into (2*S*,3*S*)-3-methylaspartic acid (**53**) using ammonia in the presence 3-methylaspartase in 64-71% yield. The product was then diazotised with sodium nitrite in a saturated solution of potassium bromide. A 48% hydrogen bromide solution was used as the acid to enrich the solution with bromide ions.[113] The bromide (**138**) was isolated in quantitative yield as a pale yellow solid [mp 142-144°C; {lit.[114] 142-144 °C}. $[\alpha]_D$ -39.8 (*c* 0.7 in H_2O) {lit.[114] $[\alpha]_D$ -40.4 (*c* 0.6 in H_2O)]. The diacid was esterified directly in refluxing methanol with a catalytic amount of concentrated sulfuric acid (2% in vol.). The bromoester (**136**) was obtained this way in

95% yield. Substitution of bromide with potassium thioacetate, with inversion of configuration, in acetone gave the thio ester (**139**) which showed a new signal at 2.35 ppm due to the acetyl group in the ^{1}H-NMR spectrum. The reaction was found to be more facile at 0 °C, since the formation of some elimination by-product (dimethylmesaconate) which formed at higher temperature was avoided.



Reagents and conditions: i. 3-Methylaspartase, $MgCl_2$, KCl, NH_3, 3 days, 71%; ii. $NaNO_2$, KBr, HBr, 100%; iii. MeOH, H_2SO_4 (2 % / vol.), reflux, 95%; iv. KSAc, acetone, 0°C, 3h, 89%; v. 6M HClaq, reflux, 30 min., 57%.

Scheme 34: *Synthesis of (2R,3R)-3-methyl-2-sulphanylsuccinic acid*

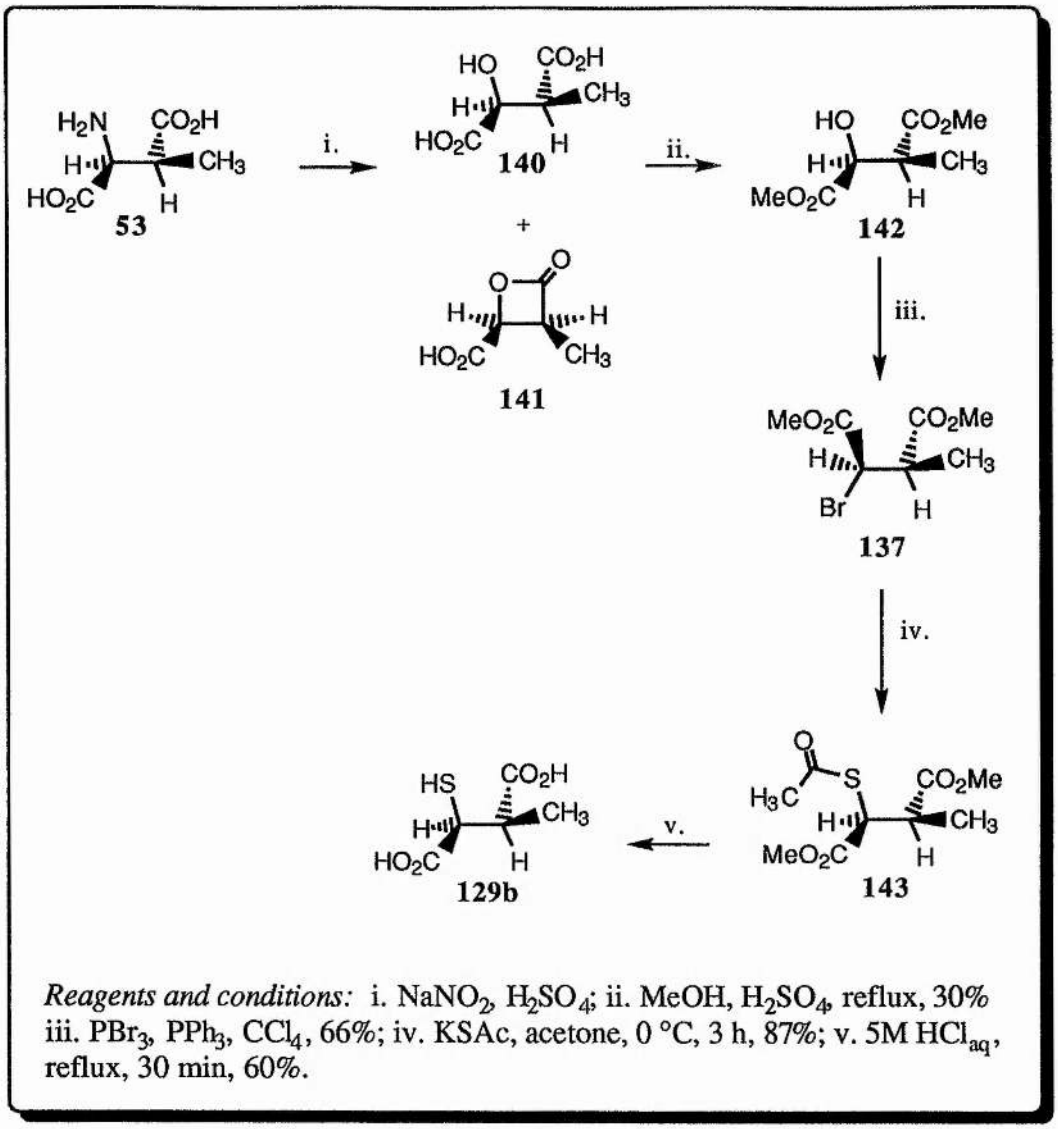
The full deprotection of the thioester (**129a**) was performed in refluxing 6 mol dm^{-3} HCl under an inert atmosphere to avoid the oxidative formation of disulphide bond.

The mercaptodiacid (**129a**) crystallised nicely upon cooling to room temperature and was collected by filtration in 57% yield as a white solid [mp 196-200 °C; $[\alpha]_D$ +40.9 (*c* 0.17 in MeOH); *m/z* (CI) 165 (100%, $[M + H]^+$)].

3.2.1.2 Synthesis of (2*S*,3*R*)-3-methyl-2-sulfanylsuccinic acid (129b)

A similar strategy to that described above was adopted for the synthesis of the (2*S*,3*R*) epimer, the sulfanyl (**129b**). However, we had to invert the stereochemistry twice to obtain an overall retention of configuration at C-2, since the diazotisation reaction occurs with retention of configuration.[115] For this purpose we chose to make the alcohol (**140**) by diazotisation of (2*S*,3*S*)- 3-methylaspartic acid (**53**, Scheme 35).

3-Methylaspartic acid was diazotised with sodium nitrite in dilute sulphuric acid. The reaction was cumbersome because dehydration of the alcohol to give mesaconic acid was very facile under such conditions. Moreover, the extraction of the product from the aqueous reaction mixture was difficult due to its high polarity. Nevertheless, a crude mixture of the alcohol (**140**) and possibly the propiolactone (**141**) was isolated as a thick yellow-orange oil which solidified upon standing. Without purification the residue was refluxed in methanol (2% H_2SO_4) to give the pure hydroxydiester (**142**) in 30% overall yield [(HRMS: found $[M + H]^+$ 177.0760. $C_7H_{13}O_5$ requires 177.0762), $[\alpha]_D$ +9.3 (*c* 17.7 in MeOH)] which displayed the expected ^{1}H- and ^{13}C-NMR spectral data.



Reagents and conditions: i. $NaNO_2$, H_2SO_4; ii. MeOH, H_2SO_4, reflux, 30% iii. PBr_3, PPh_3, CCl_4, 66%; iv. KSAc, acetone, 0 °C, 3 h, 87%; v. 5M HCl_{aq}, reflux, 30 min, 60%.

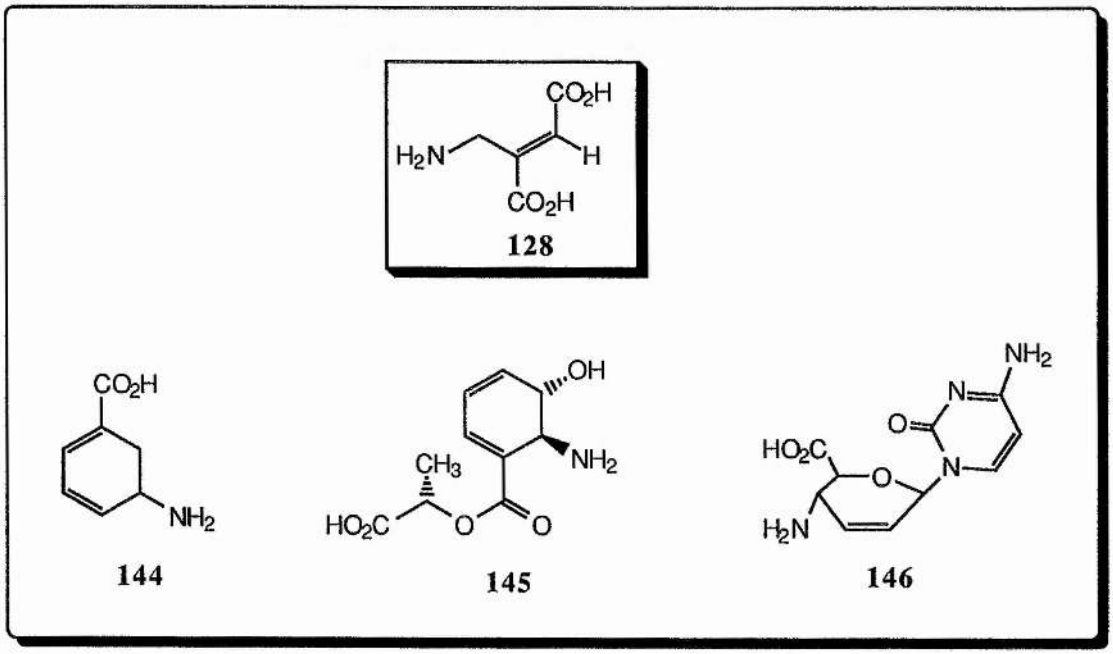
Scheme 35: *Synthesis of (2S,3R)-2-sulfanyl-3-methylsuccinic acid*

The alcohol (**142**) was brominated using triphenylphosphine and phosphorus tribromide.[116] This reaction occurred with inversion of configuration at C-2 and gave a 9:1 mixture (as determined by ^1H NMR spectroscopy) of the bromodiesters (**137**) and (**136**) respectively, in 66% total yield. By careful column chromatography most of the undesired diastereoisomer (**136**) was removed. As before, substitution of bromide in compound (**137**) by sodium thioacetate proceeded smoothly to give the (2*S*,3*R*)-thio ester (**143**) in 87% yield which showed a new signal at 2.39 ppm in the ^1H-NMR spectrum due to the acetyl group. Hydrolysis of this latter afforded the (2*R*,3*R*)-mercapto derivative

(**129b**) as a white solid [(HRMS found $[M + H]^+$ 165.0215. $C_5H_8O_4S$ requires 165.0221); mp 195-198 °C; $[\alpha]_D$ +37.0 (*c* 0.88 in MeOH)].

3.2.3 Synthesis of aminomethylfumaric acid (128)

Allylamines represent an important class of compounds, because of their occurrence in natural products such as gabaculine (**144**),[117] oryzoxymicin (**145**),[118] and cytosinine (**146**),[119] and in vinylogous unnatural polypeptide (**147**, Figure 13), which can adopt antiparallel sheet secondary structure.[120]



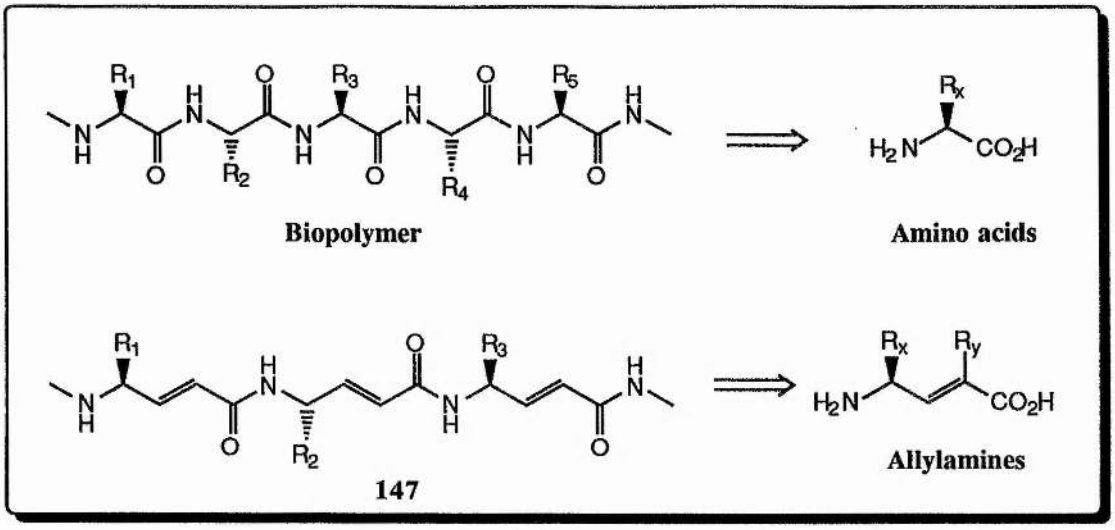
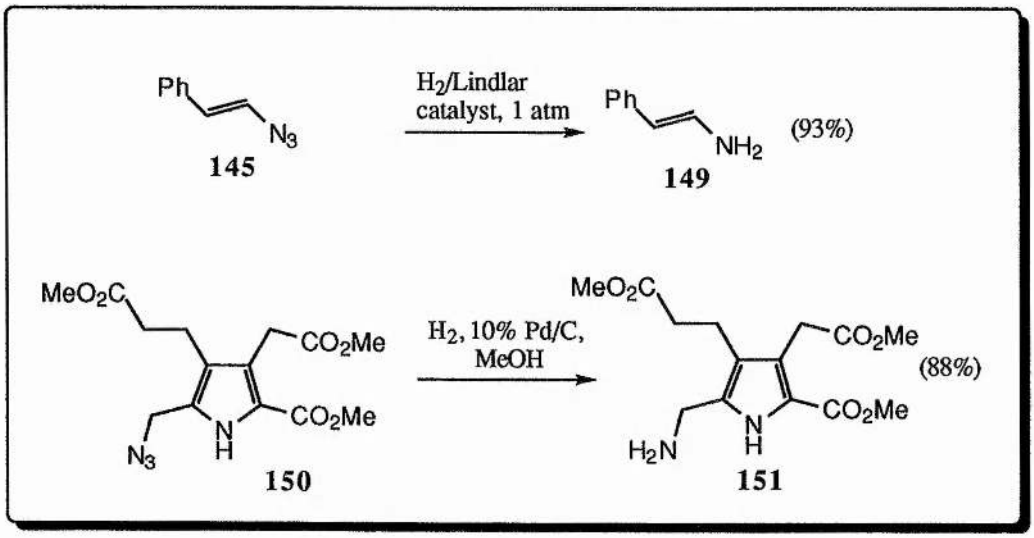


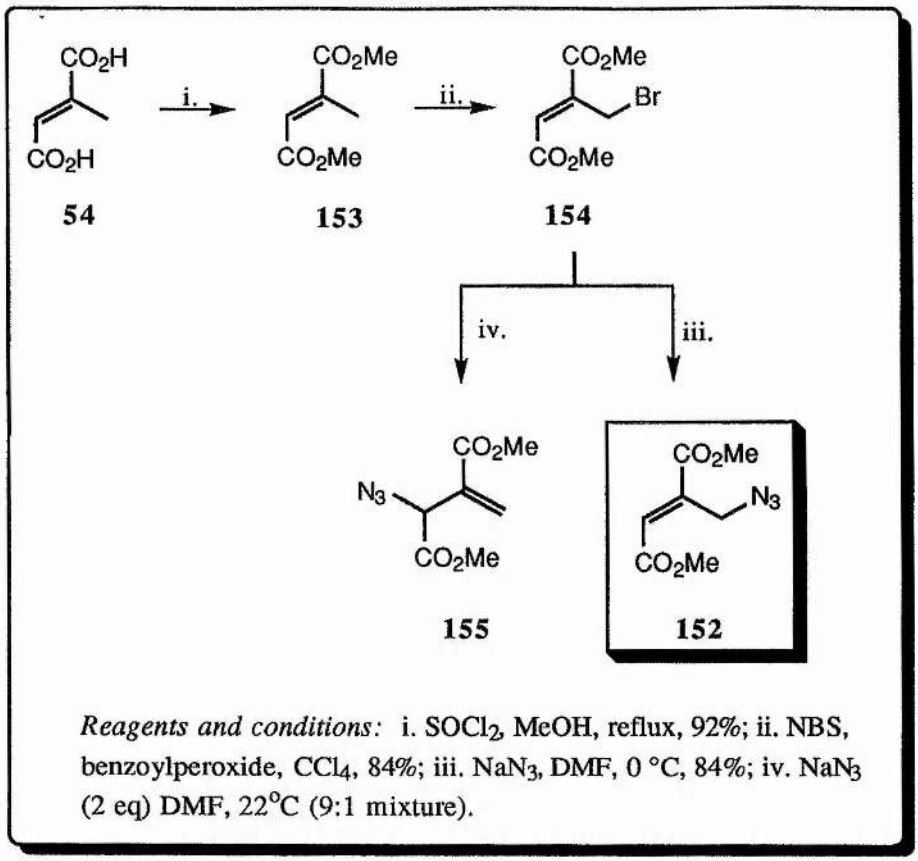
Figure 13: *Natural products containing the allyl amine function and vinologous polypeptides*

Allylamines can be prepared from their corresponding allyl azide.[121] Some cases of selective reduction into the corresponding allylic amines have been achieved using selective catalytic hydrogenation, see Scheme 36.



Scheme 36: *Synthesis of allylamines by catalytic hydrogenation of allylazide*

We planned to investigate a similar strategy for the vinyl azide (**152**) which can be synthesised from bromomethylfumarate diester (**154**, Scheme 37).



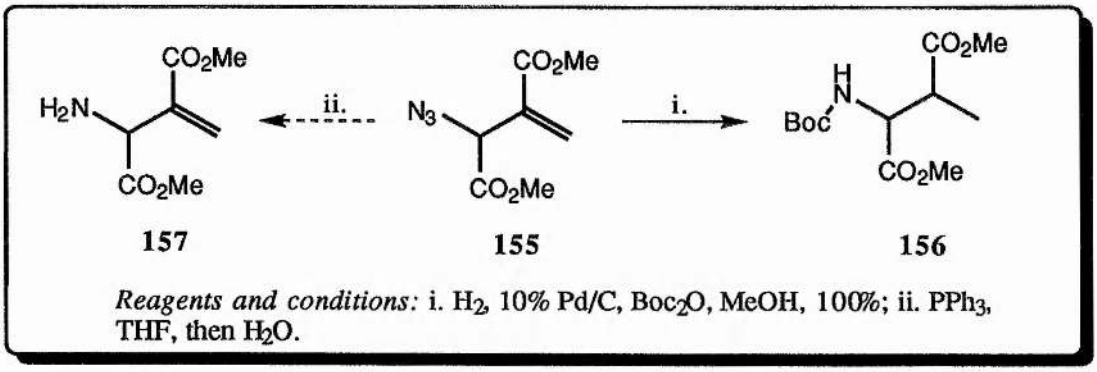
Reagents and conditions: i. $SOCl_2$, MeOH, reflux, 92%; ii. NBS, benzoylperoxide, CCl_4, 84%; iii. NaN_3, DMF, 0 °C, 84%; iv. NaN_3 (2 eq) DMF, 22°C (9:1 mixture).

Scheme 37: *Synthesis of target vinyl azide (152)*

Bromomethylfumarate diester (**154**) was prepared according to a literature procedure by radical induced bromination of dimethyl mesaconate.[122] Mesaconic acid (**54**) was esterified to its dimethyl ester (**153**) using thionyl chloride in methanol. Subsequent reaction of diester (**153**) with *N*-bromosuccinimide in the presence of a catalytic amount of benzoyl peroxide, afforded bromomethyl mesaconate (**154**) in 84% yield which showed a new signal at 4.71 pm in the ^{1}H-NMR spectrum corresponding to CH_2Br. Our aim was then to substitute the bromide with azide ion to give the azide (**152**). DMF was chosen as the solvent as this has been shown to be ideal for such substitution reactions.[123] The first attempt performed at room temperature gave a mixture of both regioisomers (**152**) [δ = 4.50 ppm for CH_2N_3] and (**155**) [δ = 4.83 ppm for CHN_3 and a two doublets at δ = 5.92 and 6.98 ppm for CH_2=]. However, when the reaction was carried out at 0 °C at high dilution only the desired vinyl azide (**152**) was formed and was isolated in 84% yield. The compound showed, in the IR spectrum, a

strong absorbtion at 2098 cm^{-1} corresponding to the azide stretch. It was also possible to reverse the selectivity towards the less thermodynamicaly favoured isomer (**155**) by using a minimum volume of DMF and two equivalents of sodium azide at room temperature. Hydrogenolysis of vinyl azide (**155**) in the presence of Boc anhydride in methanol was investigated. This gave a 2:1 mixture of benzyloxycarbonyl (2*S**,3*S**)- and (2*R**,3*S**)-3-methylaspartate diester (**156**) in quantitative yield, Scheme 38.

Also of interest is the possibility of synthesising a new unatural amino acid, using triphenylphosphine as the reducing agent to selectively reduced azide (**155**) into 3-methyleneaspartate diester (**157**).[124]

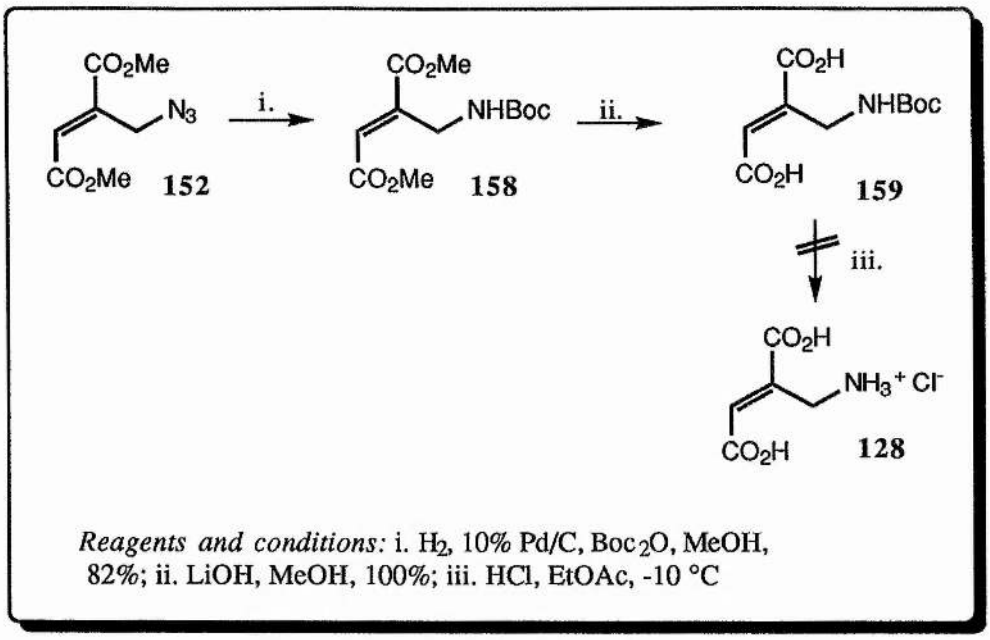


157 **155** **156**

Reagents and conditions: i. H_2, 10% Pd/C, Boc_2O, MeOH, 100%; ii. PPh_3, THF, then H_2O.

Scheme 38: *Synthesis of N-Boc 3-methylaspatate and 3-methylene aspartate (156) and 3-methyleneaspartate diester (157)*

Returning to the synthesis of the amino alkene (**128**), we first tried the hydrogenolysis of the azide (**152**) hoping that it would be possible to stop the reaction before the reduction of the double bond due to its poor nucleophilicity. The strategy proved to be successful and the N-Boc protected aminomethylfumarate diester (**158**) was isolated in 84% yield [IR, 1649 cm-1 (C=C stretch); [M+H]+ 274.1300. Requires for $C_{12}H_{20}NO_6$ 274.1291], Scheme 39.



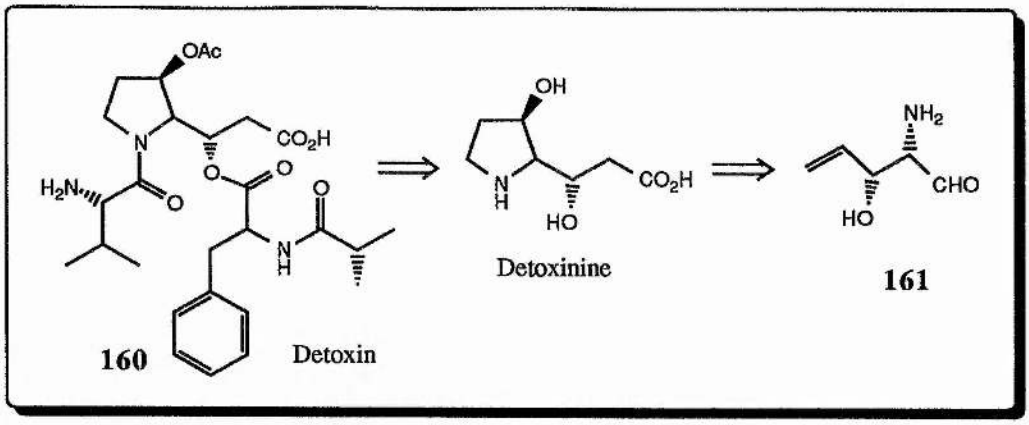
Scheme 39: *Synthesis of methylaminofumaric acid*

Deprotection of the carboxyl groups using lithium hydroxide followed by an acidic work-up quantitatively gave the diacid (**159**). Note that it was also possible to stop the reaction before the more hindered α-ester was hydrolysed. This offered the advantage of the selective incorporation of the amino alkene into peptides. Removal of the N-Boc group was carried out by passing HCl gas through an ethyl acetate solution of the alkene at low temperature. The ^{1}H NMR spectra of the resulting oily residue, however, revealed that there were a mixture of products, possibly due to addition of hydrogen chlorine across the double bond. An alternative protocol which is currently under investigation in our laboratory is the use of a milder reagent for the removal of Boc groups, *i.e.* trifluoroacetic acid.

3.2.4 Synthesis of (2*S*,3*S*)-3-methyl-3-formylaspartic acid (130)

3.2.4.1 Introduction on α- and β-amino aldehydes

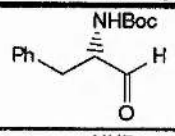
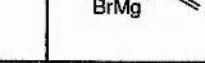
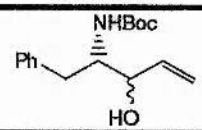
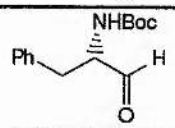
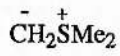
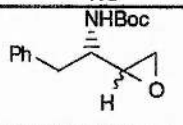
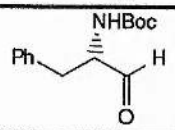
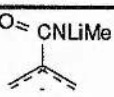
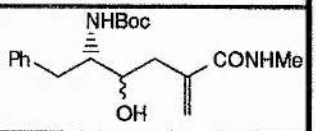
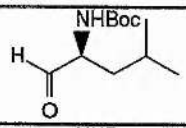
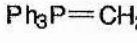
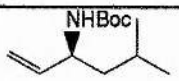
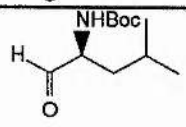
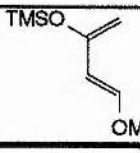
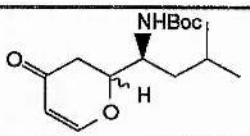
The synthesis of optically active *N*-protected α-amino aldehydes was shown to be important for the total synthesis of natural products of biological significance. As an example the antibacterial molecule detoxin (**160**) was synthesised from the α-amino aldehyde (**161**) derived from allyl glycine (Scheme 40).[125]



Scheme 40: *An α-amino aldehyde as a precursor to molecules of biological interest*

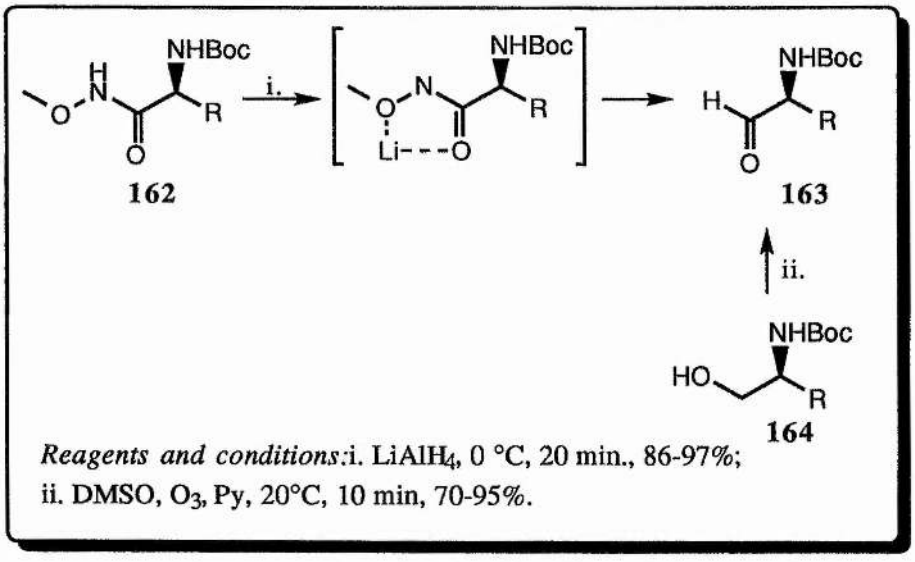
α-Amino aldehydes can be transformed into a wealth of very useful synthetic intermediates as is shown in Table 16.

Table 16: *Transformation of α-amino aldehydes*

E	α–amino aldehyde	Reagent	Product
1[126]			
2[127]			
3[128]			
4[129]			
5[130]			

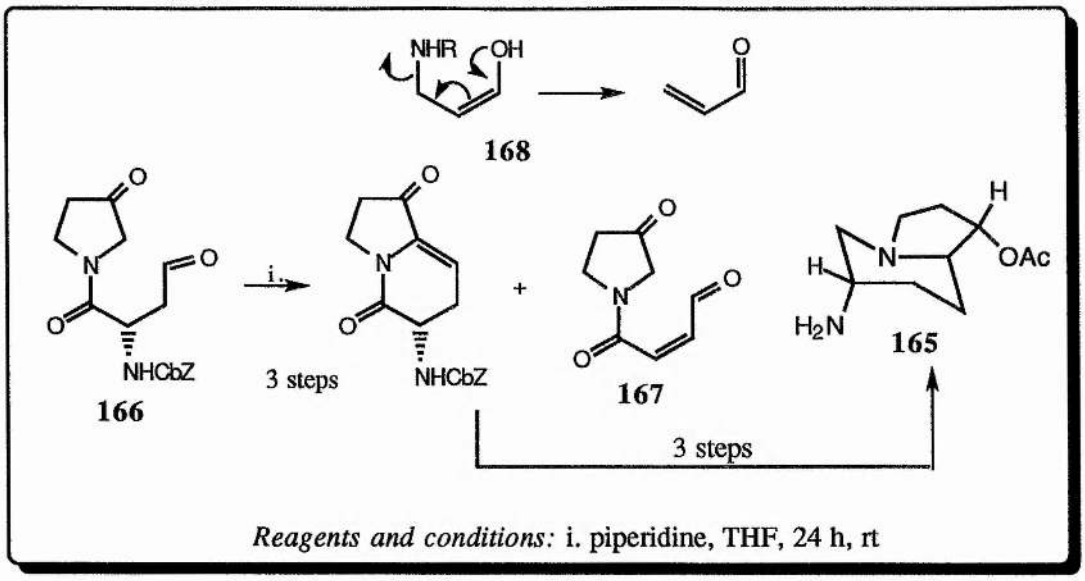
α-Amino aldehydes have been most generally obtained from α-amino acids *via* the reduction of their corresponding esters or active amides. For example the reduction of *N*-methoxy-*N*-methyl carboxamides (**162**) with lithium aluminium hydride gives, after

hydrolysis the aldehyde (**163**) (Scheme 41). This reaction proceeds *via* a stable lithium-chelated intermediate;[131] which prevents further reduction to the alcohol. A second approach is the oxidation of α-amino alcohols, which are obtained from the reduction of the corresponding α-amino acids (**164**).[132]



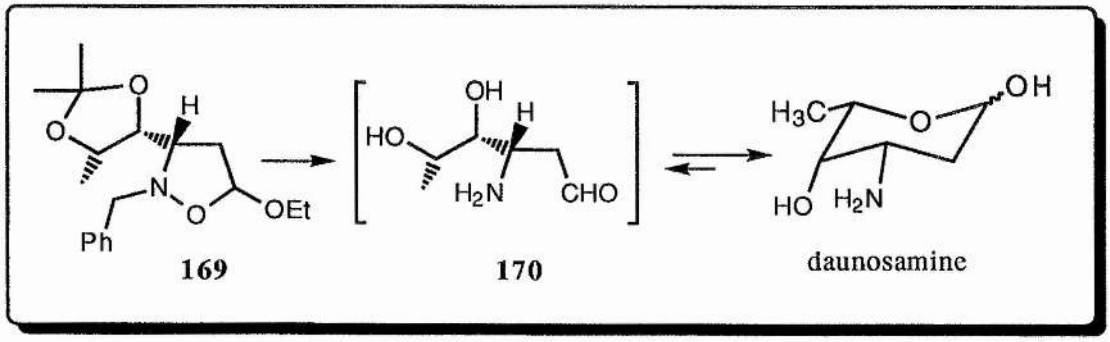
Scheme 41: *Synthesis of α-amino acids*

The situation was found to be less trivial with the homologous β-amino aldehydes which have found little use in organic synthesis due to the lack of general methods for their preparation and their inherent instability. Indeed, serious problems have been encountered due to the ability of free β-amino aldehydes to react in condensation reactions with the amino group,[133] and the facile β-elimination of the amino group.[134] For instance, the synthesis of (-)-slaframine (**165**), a fungal metabolite produced by *Rhizoctonia legumicola*, contains an example of elimination of a N-Cbz protected amino group from the β-amino aldehyde (**166**) to give the conjugated aldehyde (**167**) as a side product. This elimination proceeds via the enolic form of the aldehyde (**168**), see Scheme 42.



Scheme 42: *β-elimination of the amino group from β-amino aldehydes*

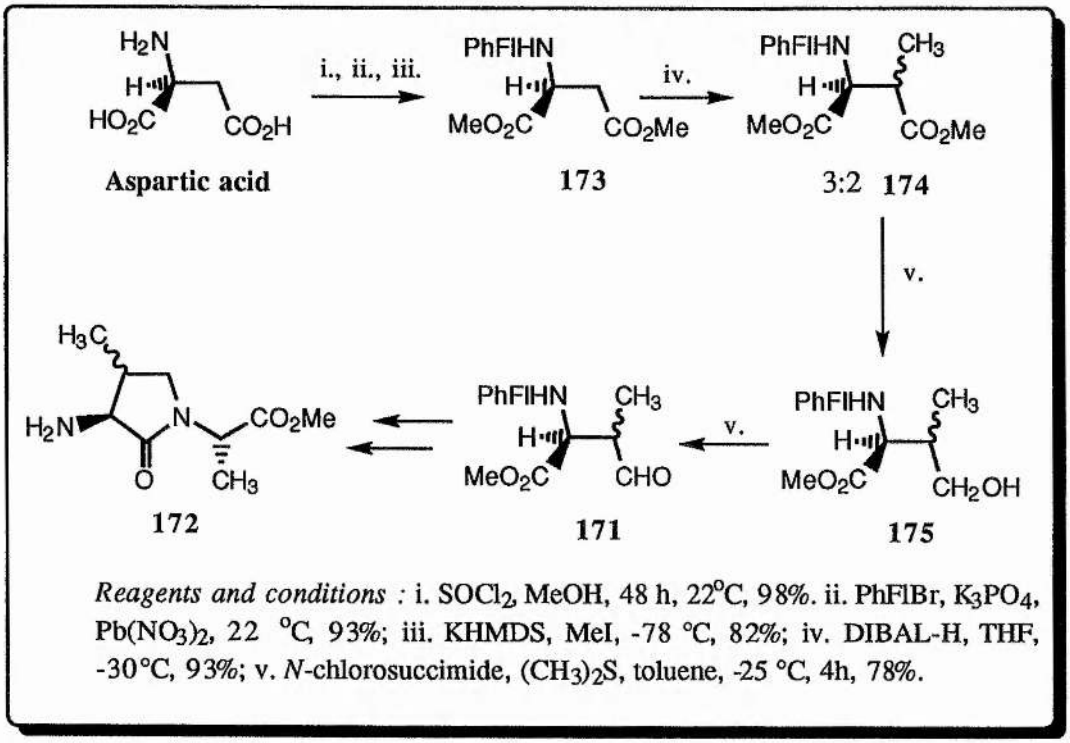
However, β-amino aldehydes showed excellent stability when masked as acetals, oxalates or hydrazones. Few efficient methods were reported in the literature for the preparation of protected non chiral or racemic β-amino aldehydes. Examples included ozonolysis of homoallylic amines,[135] nucleophilic substitution on β-chloro and bromo acetals with primary or secondary amines,[136] and conjugate addition of amine or imides to α,β-unsaturated aldehydes.[137,138] Chiral protected β-amino aldehydes were encountered in the literature as key intermediates for the synthesis of a number of compounds of biological interest. For instance, the synthesis of the amino sugar daunosamine, has involved the reductive ring opening of 5-alkoxyisoxazolidines (**169**) to afford the amino aldehyde (**170**), see Example 1 in Scheme 43.



Scheme 43: *Examples of use of amino aldehyde in synthesis*

3.2.4.2 Design of the synthesis

Scheme 44 and Scheme 45 show two examples of interest for the design of our target β-amino aldehyde (**130**). The first example by Rapoport is the synthesis of 3-methylaspartate diester β-semialdehyde (**171**) from aspartic acid, which provided a route for the synthesis of a conformationally constrained peptide (**172**).[139] This synthesis involved the use of 9-(9-phenylfluorenyl) as the protecting group for the nitrogen. This group allowed selective alkylation of aspartate diester (**173**) with methyl iodide using potassium hexamethylsilylamide as the base, to give an epimeric mixture of methylaspartate diester (**174**). The β-ester was then selectively reduced to the alcohol (**175**) using DIBAL-H. Finally, the alcohol was oxidised to the aldehyde (**171**) using *N*-chlorosuccinimide.

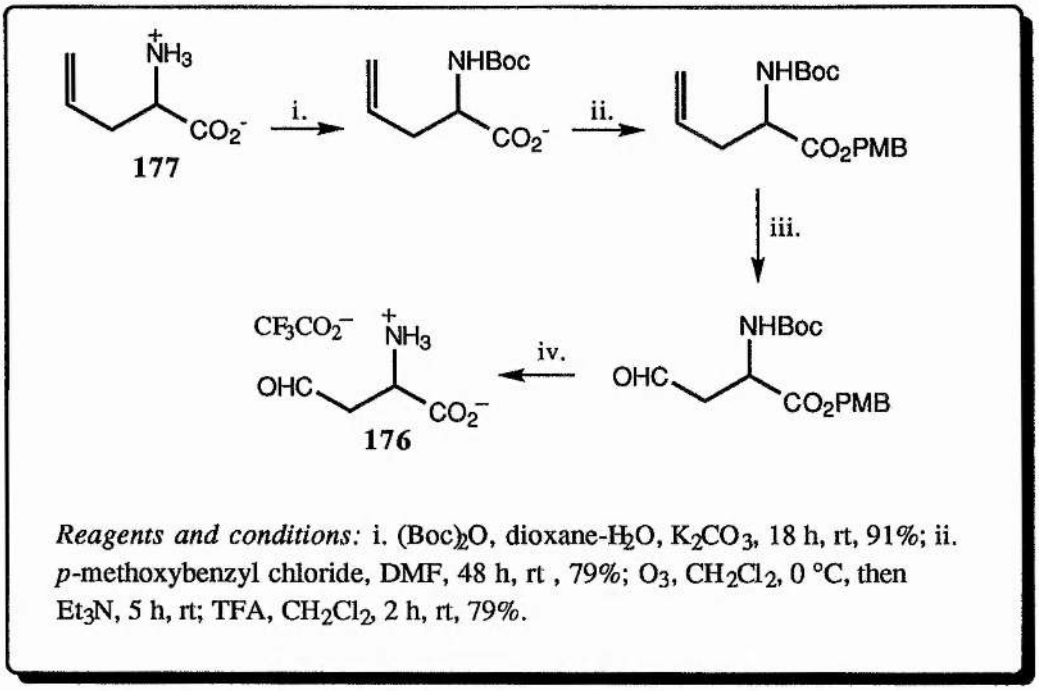


Reagents and conditions : i. $SOCl_2$, MeOH, 48 h, 22°C, 98%. ii. PhFlBr, K_3PO_4, $Pb(NO_3)_2$, 22 °C, 93%; iii. KHMDS, MeI, -78 °C, 82%; iv. DIBAL-H, THF, -30°C, 93%; v. *N*-chlorosuccimide, $(CH_3)_2S$, toluene, -25 °C, 4h, 78%.

Scheme 44: *Rapoport's synthesis of methylaspartate β-semialdehyde*

The second example is the synthesis by Robins *et al.* of aspartatic acid β-semialdehyde as a stable trifluoroacetate salt (**176**) from allyl glycine (**177**). This result was of interest since the production of aspartic acid β-semialdehyde was usually carried

out by ozonolysis of (2*S**)-allylglycine in 1mol dm^{-3} HCl at 0 °C, and used as such with often poor purity. Under these conditions aspartic acid β-semialdehyde was found to be stable only if kept at 0 °C in acidic solution.

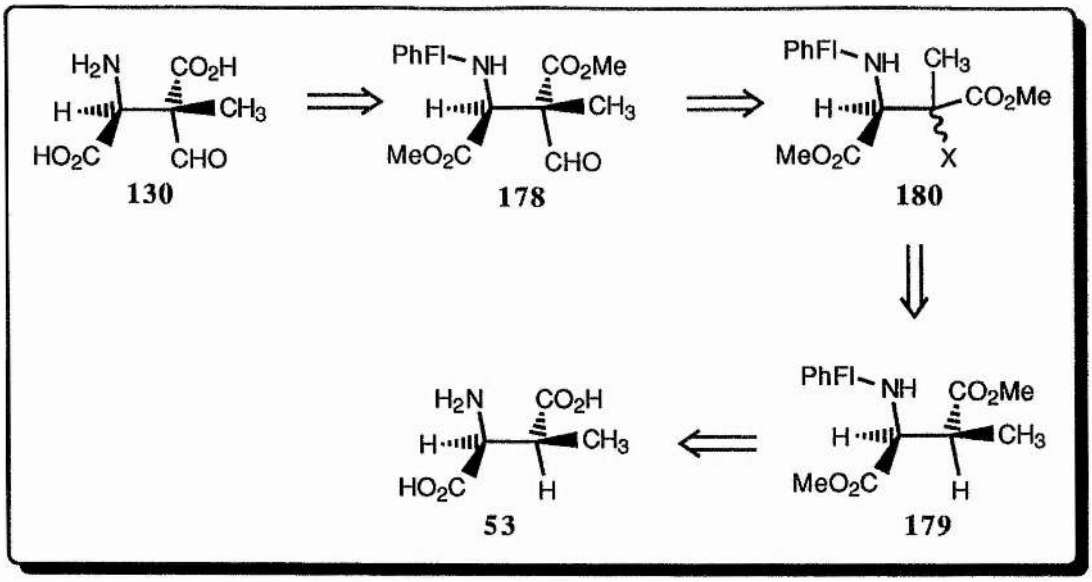


Reagents and conditions: i. $(Boc)_2O$, dioxane-H_2O, K_2CO_3, 18 h, rt, 91%; ii. *p*-methoxybenzyl chloride, DMF, 48 h, rt , 79%; O_3, CH_2Cl_2, 0 °C, then Et_3N, 5 h, rt; TFA, CH_2Cl_2, 2 h, rt, 79%.

Scheme 45: *Robins synthesis of aspartic acid β-semialdehyde*

To embark on the synthesis of the chiral highly functionalised amino acid (**130**) is a chemical challenge due to the inherent instability of β-amino aldehydes and the ease of decarboxylation of β-keto acids. We planned to synthesise the protected amino aldehyde (**178**) from suitably protected dimethylaspartate (**179**) available from (2*S*,3*S*)-3-methyl aspartic acid (**53**, Scheme 44). Introduction of a functional group such a cyano group, a nitro group or an ester may afford the product (**180**), which can be reduced to the corresponding aldehyde (**178**). Finally, suitable conditions would need to be investigated in order to deprotect this sensitive molecule to the free amino aldehyde (**130**).

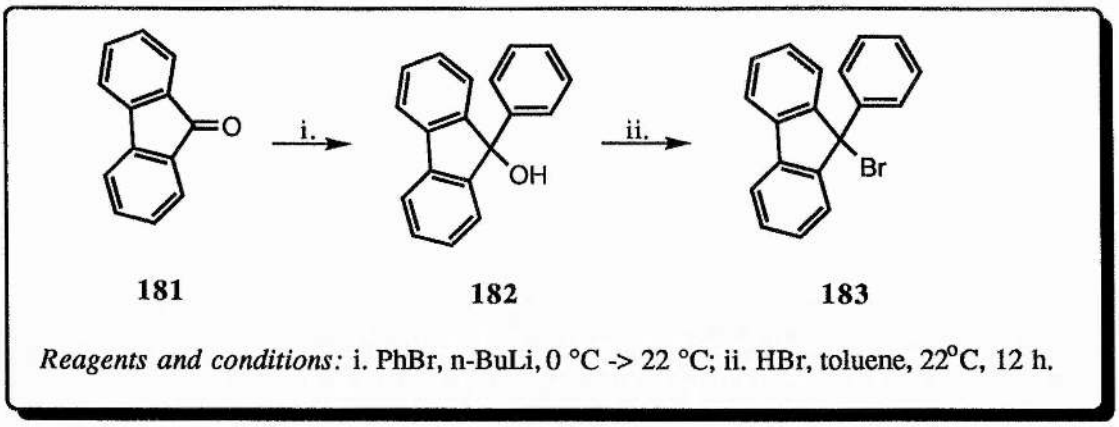
Our first objective was to investigate the regioselectivity of the alkylation of (2*S*,3*S*)-3- methylaspartate (**179**).



Scheme 46: *Retrosynthesis for (2S,3S)-3-formyl-3-methylaspartic acid (**130**)*

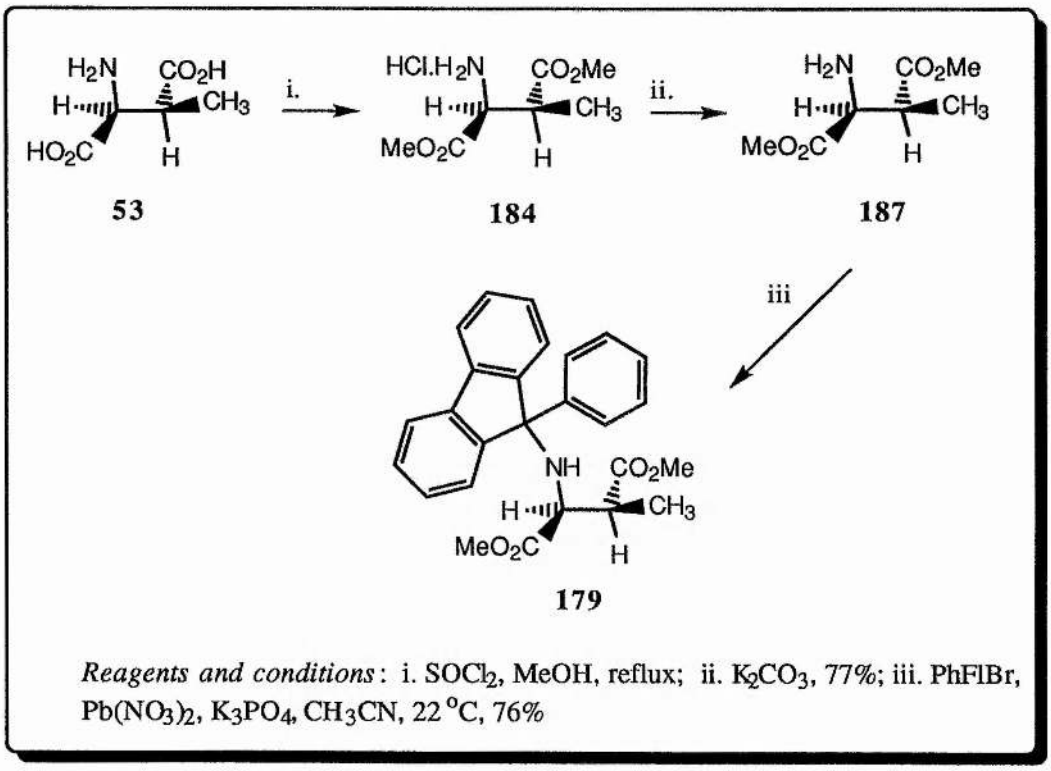
3.2.4.3 Synthesis of the precursor dimethyl (2*S*,3*S*)-*N*-9-(9-Phenylfluorenyl)-3-methylaspartate (179)

The first challenge was to find a way of selectively alkylating the readily available (2*S*,3*S*)-3-methylaspartic acid. For this purpose we chose to use (9-phenylfluorenyl) as the protecting group for the nitrogen. Its potential for sterically insulating the α-center by preventing the removal of the α-proton, hence leading to exclusive formation of the β-enolate ester, is well described by Rapoport.[140] This 9-(phenylfluorenyl) group was introduced *via* the corresponding bromide. Reacting fluorenone (**181**) with n-butyllithium yielded the alcohol (**182**) as a solid.[140] Treatment with an aqueous solution of hydrogen bromide then gave the 9-(9-phenylfluorenyl) bromide (**183**) in 86% yield over two steps.



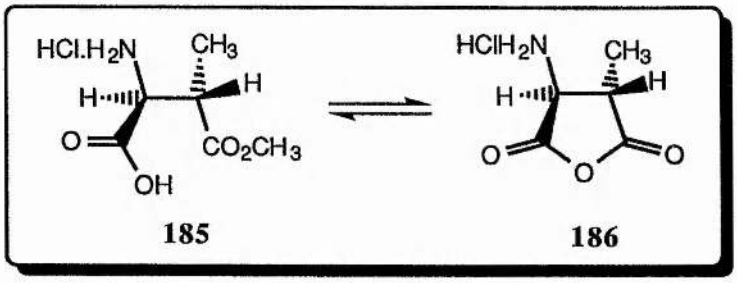
Scheme 47: *Synthesis of 9-(9-phenylfluorenyl) bromide*

Esterification of (2*S*,3*S*)-3-methylaspartic acid (**53**) to its dimethyl ester (**184**) was less straight forward. For comparison, the dimethyl ester of aspartic acid was obtained, in quantitative yield using one equivalent of thionyl chloride in methanol at room temperature.[141]



Scheme 48: *Synthesis of N-(9'-(9'-Phenylfluorenyl) (2S,3S)-3-methylaspartate diester (179)*

Surprisingly when the same conditions were applied for (2*S*,3*S*)-3-methylaspartic acid, only partial esterification occurred. This result can be explained from the favourable cyclisation of the β-methylester (**185**) to give the anhydride (**186**, Scheme 49).



185 **186**

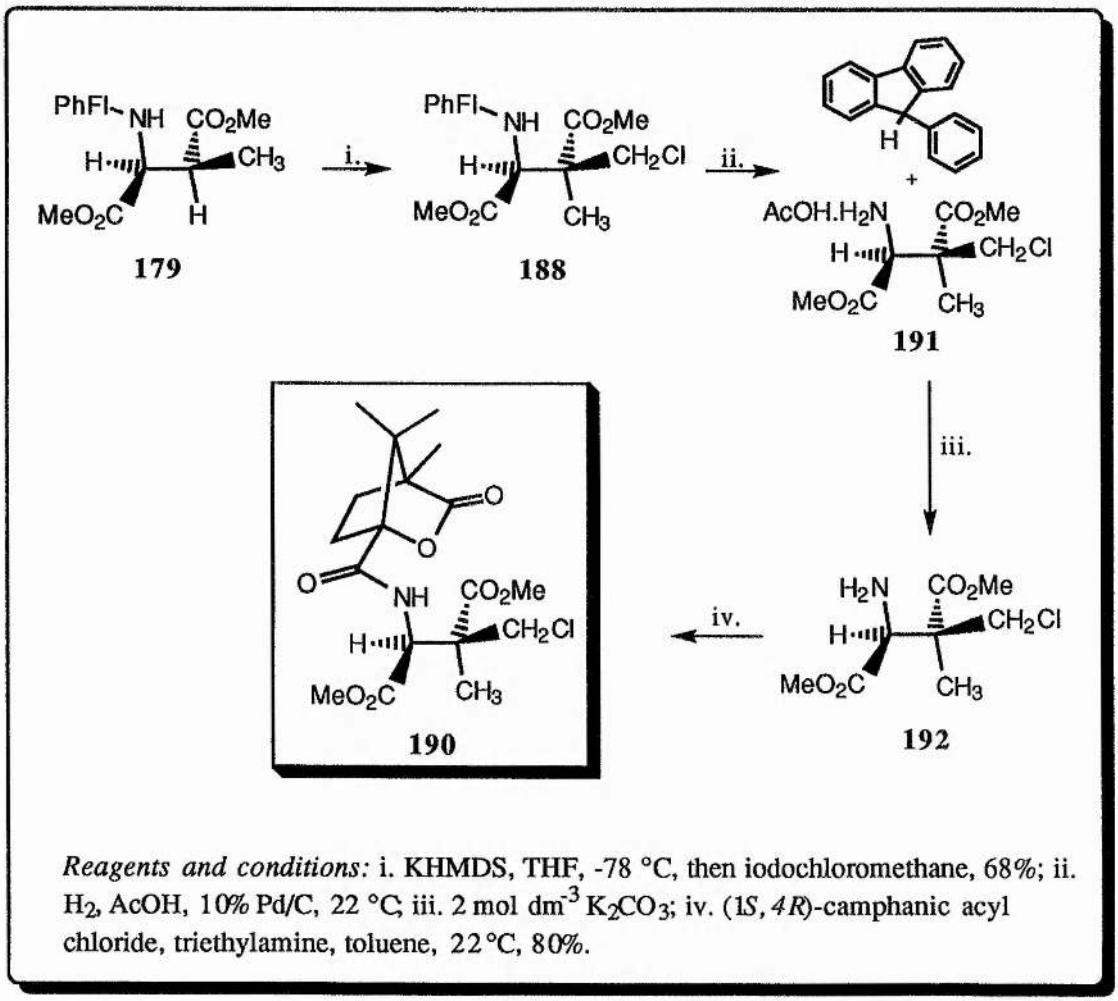
Scheme 49: *lactone formation during esterification*

In order to avoid this difficulty the methanolic mixture was refluxed in methanol using a large excess of thionyl chloride. The esterification which was monitored by ^{1}H NMR spectroscopy, was 95% completed after 8 h. The hydrochloride ester (**184**) was then converted to its free amine (**187**) in 77% overall yield using a 2 mol dm^{-3} solution of potassium hydrogencarbonate (Scheme 48). The free amine (**187**) was then alkylated with 9-(9-phenylfluorenyl) bromide (**183**) using the conditions published by Rapoport *et al.* The reaction occurred smoothly in acetonitrile in presence of the ion scavenger lead nitrate. The reaction time was shown to be crucial in order to obtain a moderate yield of the protected aspartate (**179**), as a solid, after purification by gel chromatography [mp, 75-80 °C, found C, 74.70; H, 5.75; N, 3.30. $C_{26}H_{25}NO_4$ requires C, 75.15; H, 6.05; N, 3.35; $[\alpha]_D$ -276 (*c* 1.23 in DCM)]. It is interesting to note that the amine proton displays a very sharp absorption at 3302 cm^{-1} in the IR spectrum revealing its inability to H-bond, due to the insulation afforded by the large 9-Phenylfluorenyl group.

3.2.4.4 Investigation of the regioselectivity of the alkylation

We wished first to investigate the regioselectivity of the alkylation of *N*-PhFl aspartate (**179**) (Scheme 50). We expected that the reaction would occur mainly with retention of configuration at the C-3 center, to give us the required isomer. In order to achieve this, we planned to alkylate the protected amino acid with iodochloromethane.

The bulky base potassium bis(trimethylsilyl)amide was chosen to generate the β-enolate ester at -78 °C. Addition of an equivalent of iodochloromethane led to the isolation of chloromethylaspartate (**188**) in 68% yield as a white solid (mp, 48-50 °C; found C, 70.20; H, 5.95; N, 3.00. $C_{26}ClH_{26}NO_4$ requires C, 69.90; H, 5.65; N, 3.00). The ^1H NMR spectrum of the crude mixture showed the presence of only one methyl group at 1.32 ppm. Note that for the corresponding (2*S*)-3,3-dimethylaspartate diester (**189**) methyl groups resonate at 1.08 and 1.24 ppm. The fact that the reaction could give a unique diastereoisomer was very exciting since this avoids any difficult separation. However, it was still to be determined if the diastereoisomer obtained was the one required.



Scheme 50: *Investigation of the stereochemistry of the reaction of the alkylation of aspartate diester (**179**) with chloroiodomethane*

We planned to synthesise the camphanic derivative (**190**) and to obtain its crystal structure. The phenylfluorenyl group of the chloromethylaspartate diester (**188**) was removed by hydrogenolysis in glacial acetic acid to give quantitatively the acetate salt of the amino ester (**191**). This latter was carefully converted to its free amine (**192**) with a potassium carbonate solution. The free amine was readily acylated with (1*S*,4*S*)-camphanoyl chloride in toluene, in the presence of triethyl amine, and the camphanic derivative (**190**) was obtained in 80% overall yield. Recrystallisation gave colourless crystals suitable for analysis by X-ray crystallography performed by Dr. P. Lightfoot [mp, 175-177 °C, $[\alpha]_D$ -4.7 (*c* 1.5 in DCM)], (Found C, 70.20; H, 5.95; N, 3.00. $C_{26}ClH_{26}NO_4$ requires C, 69.9; H, 5.65; N, 3.00).

Contrary to our original expectations, the crystal structure revealed that we had obtained the (2*S*,3*S*) isomer, see Figure 14. Thus, the alkylation had occurred with inversion of configuration at the C-3 center.

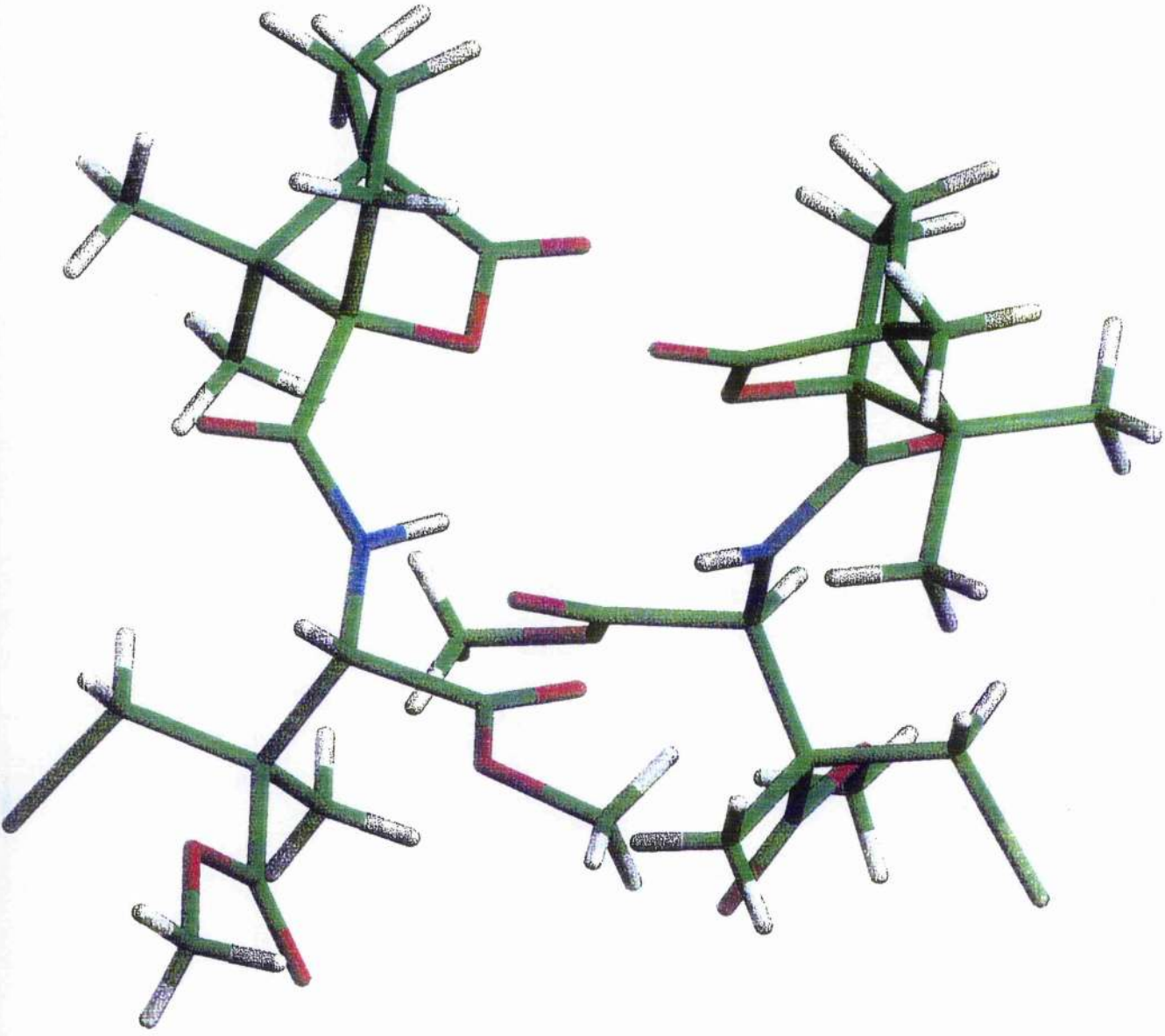


Figure 14*: Crystal structure of the camphanic derivative (**190**)*

The rationalization behind this result stands in the formation of the stable chelate (**193a**), (see, Figure 15) where the upper face (*Re*) is obstructed by the crowded phenylfluorenyl group. As illustrated in Figure 15, *lk* attack [face (*S*,*Si*)] of the electrophile induces an inversion of configuration at the C-3 center. In a similar maneer alkylation of the starting material (**179**) having the opposite stereochemistry at C-3 should afford chelate (**193b**) to give the mirror image of product (**188**), when the electophile (E^+) is chloroiodomethane.

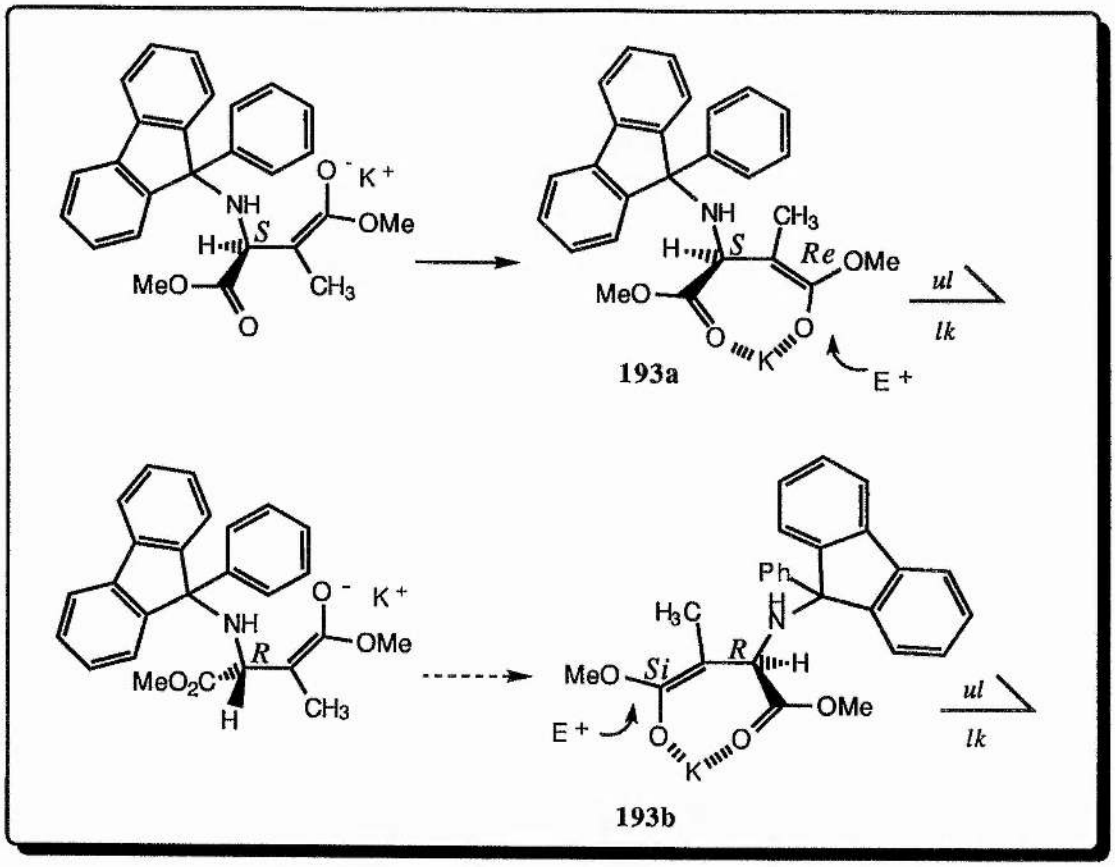


Figure 15: *Mechanistic considerations*

Note that the treatment of an epimeric mixture of *N*-(9-(9-phenylfluorenyl) 3-methylaspartate diester (**179a**, see below) with KHMDS and iodochloromethane at -78 °C, afforded a mixture of (2*S*,3*R*)-methylaspartate diester (**179b**) and (2*S*,3*S*)-chloromethylaspartate diester (**188**). In order to understand this result, the X-ray structure of (2*S*,3*S*)-3-methylaspartate diester (**179**) was determined by Dr. P. Lightfoot, see Figure 16, p. 86. The structure shows that the two esters groups are *anti* to each other. As a result, the proton at C-3 points down and thus is accessible to the bulky base. On the other hand, for the other isomer to keep the energetically favoured *anti* conformation, the proton at C-3 must face the phenylfluorenyl group. In this orientation the removal of this proton by a bulky base becomes highly unfavourable.

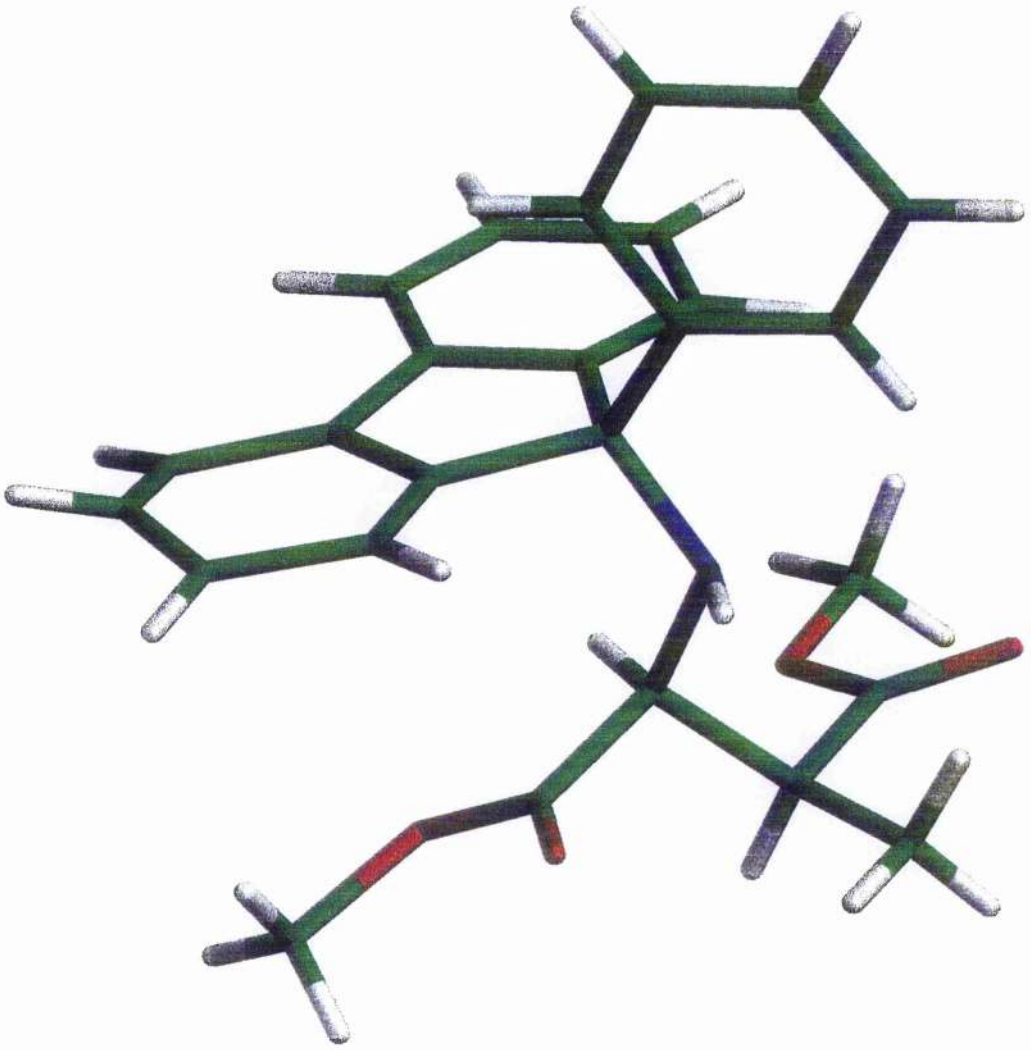
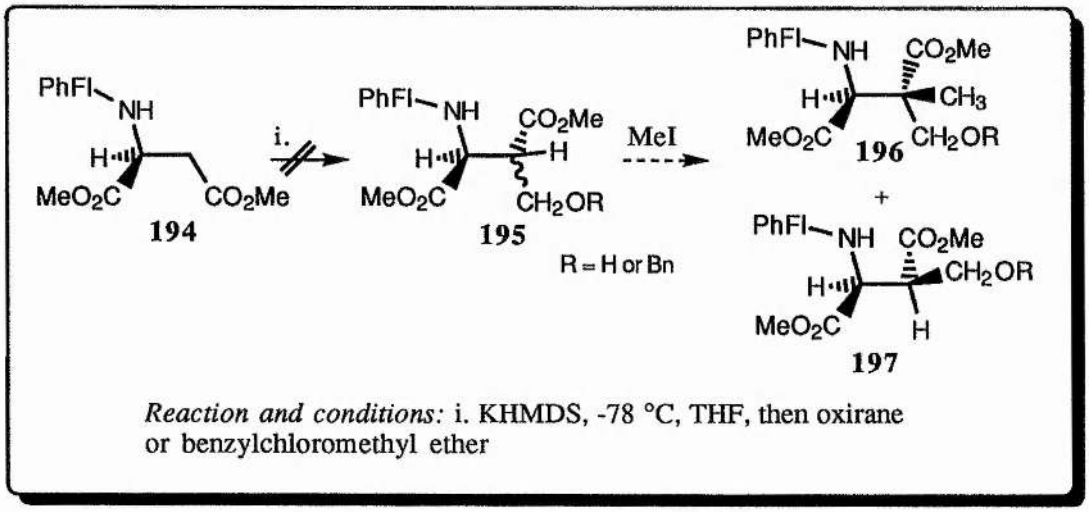


Figure 16: *Crystal structure of N-(9'-(9'-Phenylfluorenyl) (2S,3S)-3-methylaspartate* dimethyl *ester* (**179**)

We first thought of taking advantage of this result in order to obtain the require stereochemistry at C-3. The idea was to alkylate aspartate diester (**194**) with oxirane or benzylchloromethyl ether to obtain a diastereoisomeric mixture of compound (**195**, Scheme 51). Treatment of this mixture with methyliodide should give an easily separable mixture of alcohol (R = OH) or benzylether (R = Bn) (**196**) and (2*S*,3*S*)-isomer (**197**, Scheme 51). Unfortunately all attempts to alkylate aspartate diester (**194**) with either of these reagents were unsuccessful. In all cases the complete recovery of the starting material was possible.



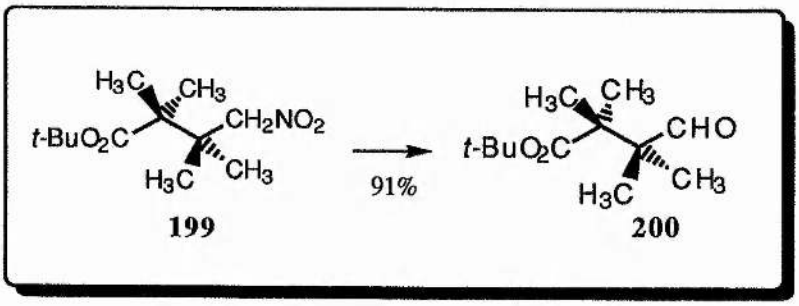
Reaction and conditions: i. KHMDS, -78 °C, THF, then oxirane or benzylchloromethyl ether

Scheme 51: *Attempt of retaining the stereochemistry at C-3 starting from aspartate diester* (**194**)

3.2.4.5 Route to target aldehyde (130) *via* (2*S*,3*S*)-3-iodo-3-methylaspartate diester

We thought of synthesising the primary nitromethylaspartate diester (**198**, see next page) as this compound can be hydrolysed to the corresponding aldehyde (**178**) *via* the Nef reaction.[142] This reaction involves hydrolysis of the *aci*-form of a primary nitro compound using a solution of sulphuric acid. A milder method, however, has since been developed that consists of the oxidation of the conjugate base of a primary nitro compound. This oxidation is carried out with potassium permanganate in neutral conditions. An example of this methodology that involves a nitro compound structurally

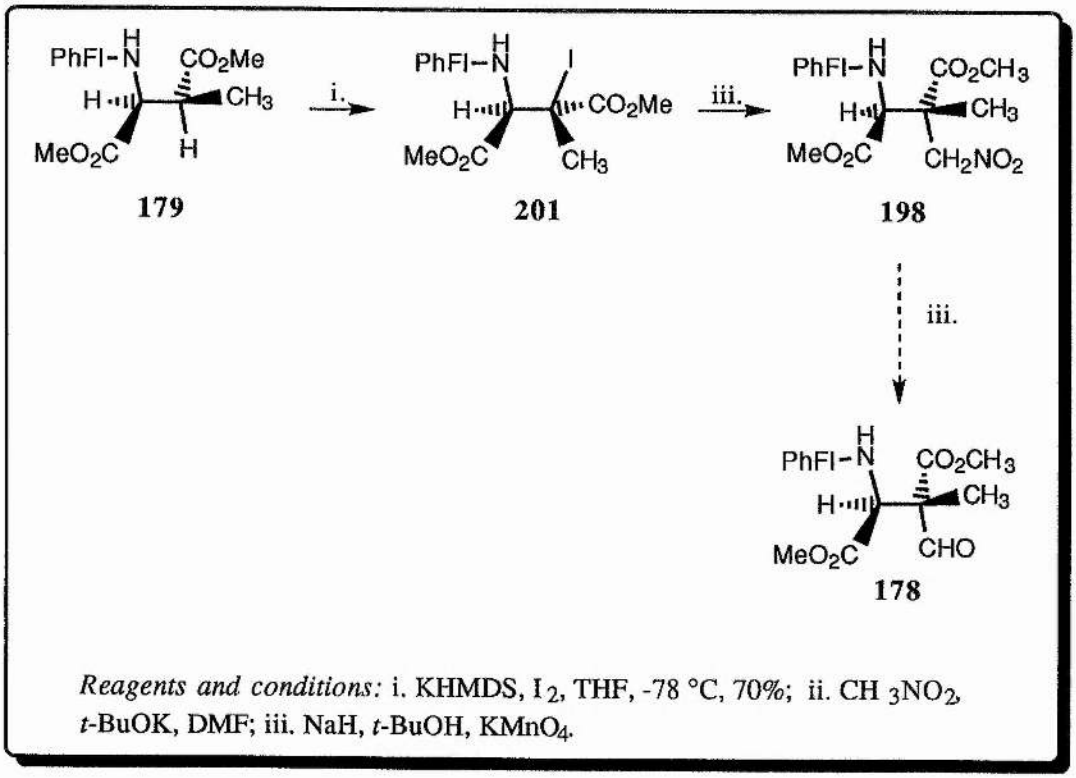
related to our desired nitromethylaspartate diester (**198**), has been described by Kornblum.[143] This consists of the conversion of the primary nitro *tert*-Butyl ester (**199**) into aldehyde (**200**) in 91% yield (Scheme 52).



199 **200**

Scheme 52: *Kornblum's modification of Nef reaction* via oxydation with permanganate *of the aci-nitro form a primary nitro compound*

We planned to synthesise nitromethylaspartate diester (**198**) from (2*S*,3*S*)-iodomethylaspartate diester (**201**, Scheme 53).



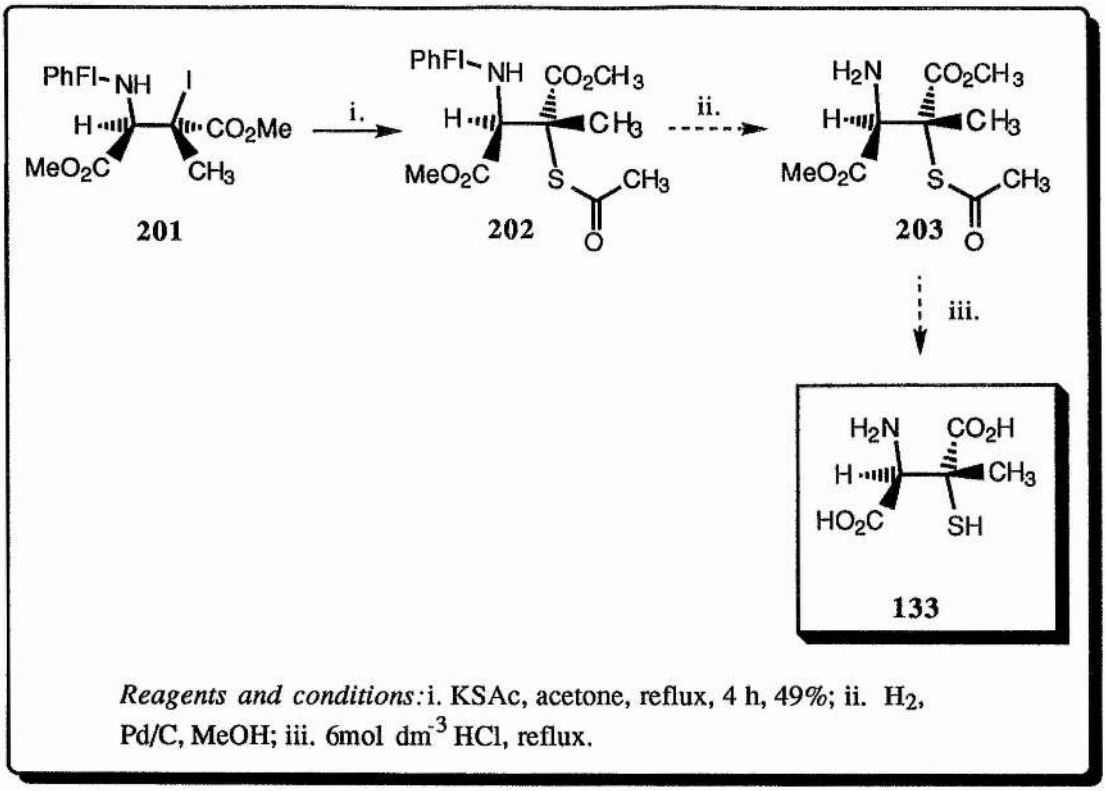
179 **201** **198** **178**

Reagents and conditions: i. KHMDS, I_2, THF, -78 °C, 70%; ii. CH_3NO_2, *t*-BuOK, DMF; iii. NaH, *t*-BuOH, $KMnO_4$.

Scheme 53: *Attempt synthesis of aldehyde* (**179**) *from iodo diester* (**199**)

This was prepared from methylaspartate diester (**179**) which was regiospecifically iodinated at the C-3 position by conversion to its enolate ion with potassium hexamethyldisyllyl amide in THF and treatment of this solution at -78 °C with iodine. The light sensitive iodomethylaspartate diester (**201**) was isolated in 70% yield as a white solid [mp, 72 °C]. It interesting to note that the methyl group is unusually deshielded and occurs at 2.24 ppm in ^{1}H-NMR spectrum, in comparison with the expected value of 1.65 ppm for a methyl group in the β-position of an iodo substituent. Iodomethylaspartate diester (**201**) was added to a solution of NaH (1eq) in DMSO under inert atmosphere at room temperature. Under these conditions, a 20% conversion of the starting material into a new product occurred. The product was thought to be the nitro compound (**198**) based upon the analysis of the ^{1}H NMR spectrum of the crude. The spectrum showed two new methyl ester signals (δ = 3.23 and 3.55 ppm) whom integration correlates with two doublets (δ = 4.80 and 4.88 ppm). This is consistent with the expected chemical shift for protons adjacent to a nitro group (δ = 4.4 ppm). Attempts to optimise the reaction conditions were not successful. Prolonged reactions times or an increase of the temperature led to unidentified side reactions. Due to the low yield of the desired product, at this stage of the synthesis, this route to the synthesis of the target aldehyde (**130**) was not pursued further.

In contrast iodomethylaspartate (**201**) could prove to be a viable route for the synthesis of (2*R*,3*R*)-3-methyl-3-sulfanylmethylaspartic acid (**202**, Scheme 54), a potential mechanism-based inhibitor of β-methylaspartase. Substitution of iodine by potassium thioacetate in refluxing acetone afforded the highly functionalised protected sulfanyl α-amino acid (**202**) in 49% yield as a colourless oil, [HRMS found: $[M + H]^+$, 490.1701. $C_{28}H_{28}NO_6S$ requires 490.1688], υ_{max} 1742 cm^{-1} (esters) and 1688 cm^{-1} (thioester).



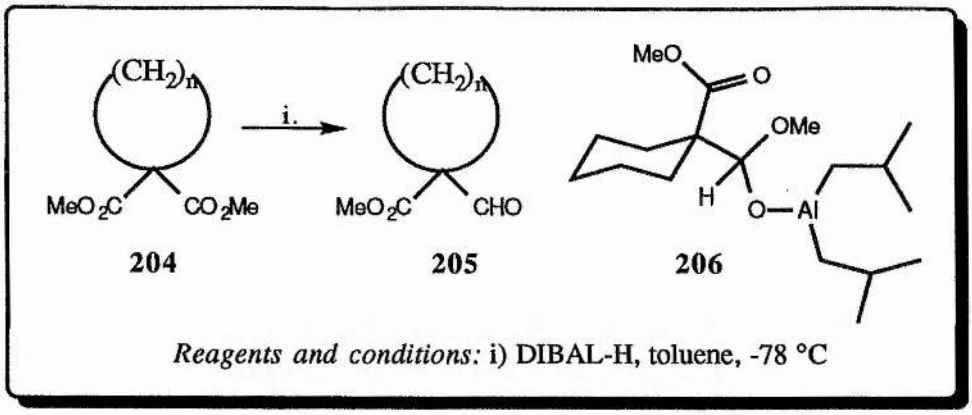
Reagents and conditions: i. KSAc, acetone, reflux, 4 h, 49%; ii. H_2, Pd/C, MeOH; iii. 6mol dm^{-3} HCl, reflux.

Scheme 54: *Synthesis of (2R,3R)-3-methyl-3-sulfanylaspartic acid*

Methods for the removal of the protecting groups are currently under investigation in our laboratory. Removal of the phenylfluorenyl group can be accomplished by catalytic hydrogenation as described previously. Finally cleavage of the esters and thio ester groups will be carried out in similar conditions to that described for the deprotection of the dimethyl thiosuccinates (*cf.* p. 65).

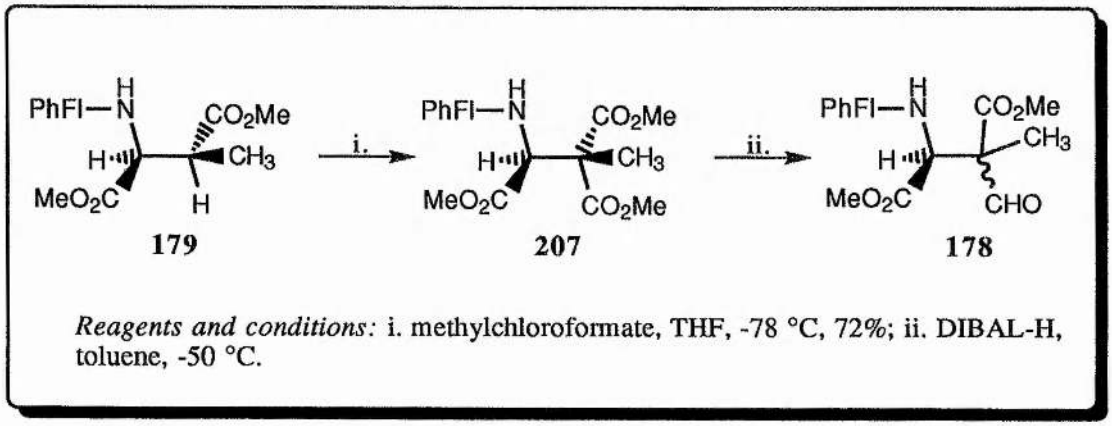
3.2.4.6 Route to aldehyde (130) *via* acylation of methylaspartate diester (179)

Burton *et al.* reported on a general method for the monoreduction of cycloalkyl geminal diesters (**204**), using excess DIBAL-H, to afford the aldehydic product (**205**) (Scheme 55).[144]



Scheme 55: *Burton's reduction of geminal diester into aldehyde*

The substituents on the aluminium complex act presumably as a blocking group that prevents reduction of the remaining ester group as shown for the reduction of 1,1-dimethyl ester cyclohexane (**206**). Intramolecular coordination of the aluminium metal with the one of the *gem*-diester carbonyl might also enhance this steric effect. This work persuaded us to synthesise trimethyl ester (**207**) by acylation of methylaspartate diester (**179**) with methylchloroformate (Scheme 56). We expected that the reduction of the *gem*-diester would be in favour of one diastereoisomer as we observed previously for the alkylation to give selectively one diastereoisomer of the aldehyde (**178**).

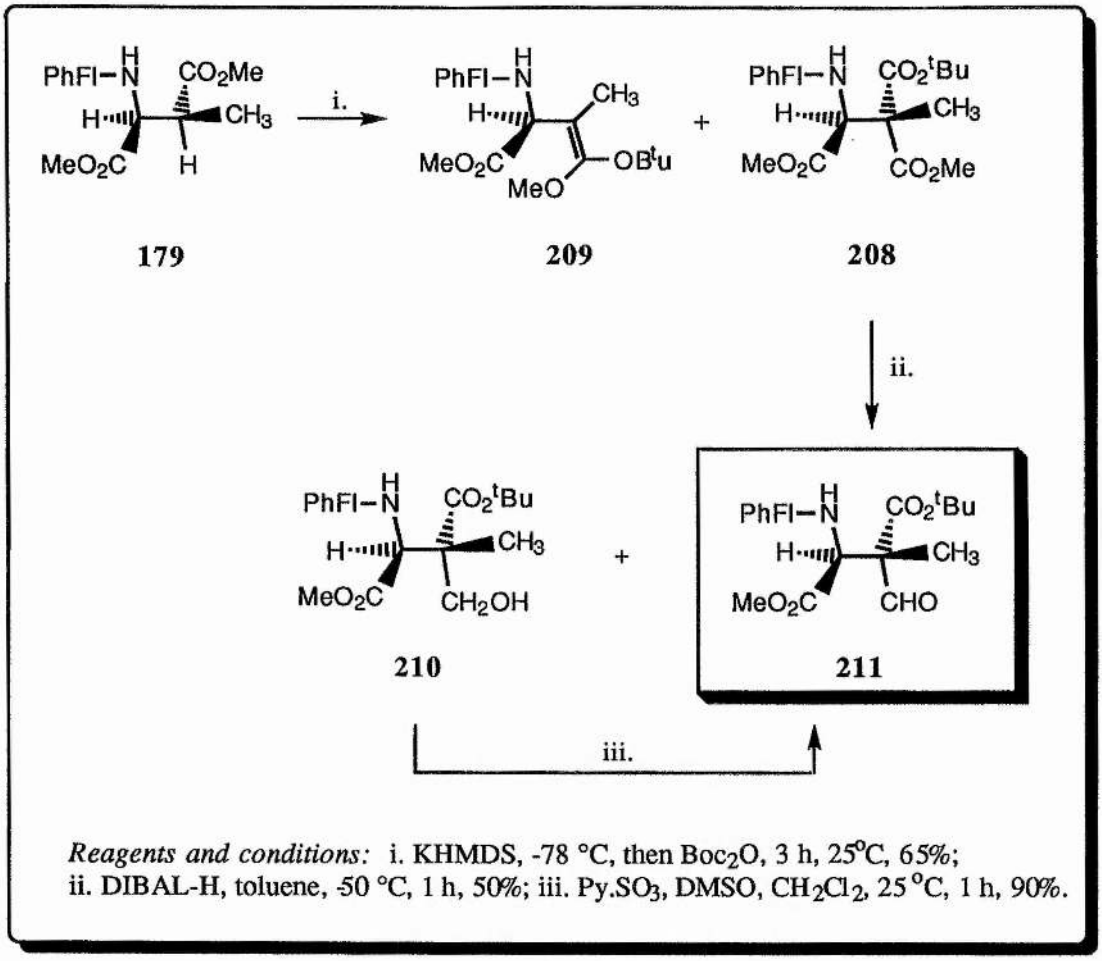


Scheme 56: *Synthesis of the β-amino aldehyde (178)*

Methylaspartate diester (**179**) was successfully acylated with methylchloroformate in a rapid reaction at -78 °C, to afford trimethyl ester (**207**) in 72% yield. The reaction

appeared to occur very quickly at -78 °C. Reduction was attempted with DIBAL-H in toluene, at -50 °C to afford a 1:1 diastereoisomeric mixture of aldehyde (**178**). The α-ester was not affected by the reduction conditions due the crowded environment imposed by the phenylfluorenyl group. The lack of selectivity in the reduction between the *proR* and the *proS* ester was disappointing. Nevertheless, we established that the required aldehyde can be obtained directly from the *gem*-diester. However, our aim was to obtain only the (2*S*,3*S*)-diastereoisomer of aldehyde (**178**) for synthetic purposes first, as we did not wish to complicate spectral interpretation during the deprotection steps. The deprotection of the α-ester of compound aldehyde (**178**) is expected to generate a five-membered ring lactone, and as a result producing a mixture of two diastereoisomers (see p. 96).

As a result we turned our attention towards bis(*tert*-butyloxycarbonyl) as the acylating agent. A higher temperature was needed in this case, and the reaction only worked at or above -20 °C. The enolate ester was acylated smoothly to yield a 7:3 mixture of the C-acylated product (**208**) and the O-acylated product (**209**). This latter was also isolated and characterised. It was noticed that the O-acylated and C-acylated products ratio was very dependent upon the dilution factor and the temperature. An optimised yield of 65% for the compound (**208**) was obtained but it was often difficult to obtain consistent results concerning the C- to O-acylation ratio. Attempts to use lithium ion instead of potassium ion gave no improvements. Crystals of the *tert*-butyl ester (**208**) were obtained for X-ray crystallography (Figure 17). The structure confirmed the (2*S*,3*S*) stereochemistry as was expected on the basis of our previous study , see p. 81.



Reagents and conditions: i. KHMDS, -78 °C, then Boc_2O, 3 h, 25°C, 65%; ii. DIBAL-H, toluene, -50 °C, 1 h, 50%; iii. $Py.SO_3$, DMSO, CH_2Cl_2, 25 °C, 1 h, 90%.

Scheme 57 : *Synthesis of the fully protected target aldehyde (211)*

In view of the hindered environment of the methyl ester, the conditions of the reduction were difficult to tune. Above -55 °C, no reduction was observed and below -45 °C the main product was the alcohol (**210**). The aldehyde (**211**) was obtained using three equivalents of DIBAL-H in toluene at -50°C for 1 h. Under these conditions, the aldehyde (**211**) was isolated in 50% yield as a white solid [mp, 65-68°C; (HRMS: found $[M + H]^+$, 486.2268. $C_{30}H_{32}NO_5$ requires 486.2280)]. Some alcohol (**210**) was also isolated, which was oxidised to the aldehyde using Parikh-Doering procedure in 95% yield.[145]

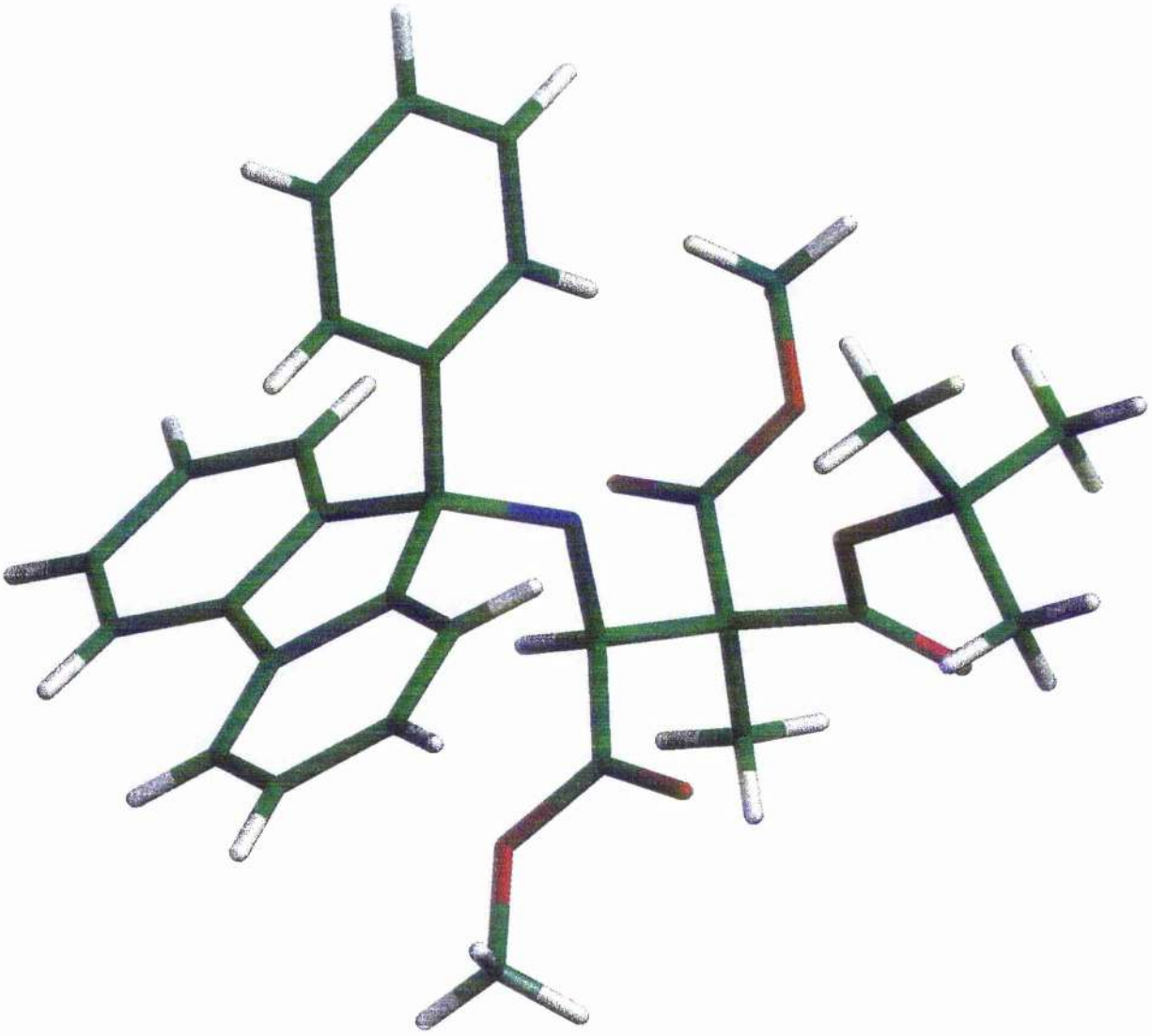
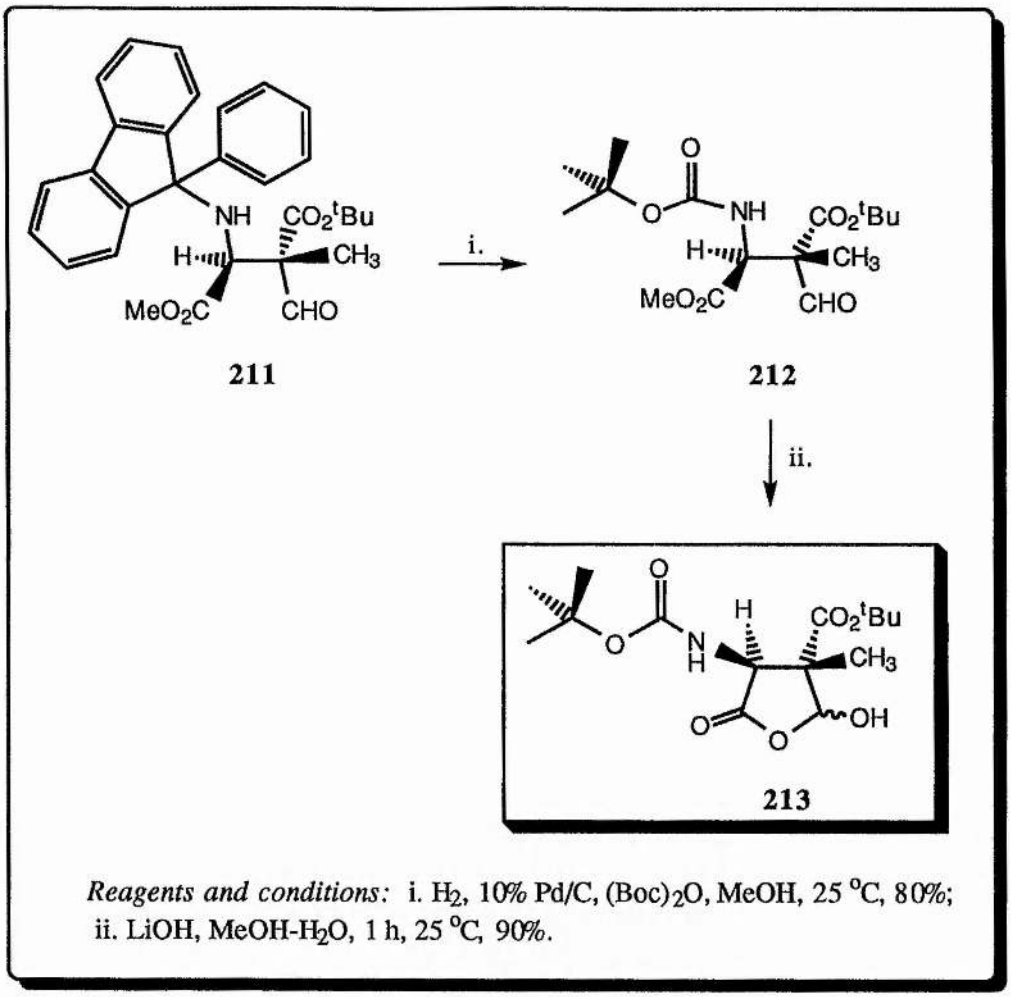


Figure 17 : *X-ray structure of the gem-diester* ***(208)***

At this stage we had to tackle the problem concerning the removal of the protecting groups. Removal of the methyl ester by standard saponification in methanol proved to be difficult presumably because of the hydrophobic shielding effect of the phenylfluorenyl group. We therefore, opted to change the *N*-PhFl group for the less bulky Boc group. This operation was performed in one pot using ~ 5 fold excess of $(Boc)_2O$ and the minimum of solvent under the hydrogenolysis conditions (Scheme 58). This method has the advantage of limiting any intermolecular condensation. Another concern was the possible reduction the aldehyde under palladium catalysed hydrogenation. However, some examples exist in the literature describing selective reduction of double bond in the presence of a ketone or aldehyde moiety.[146]

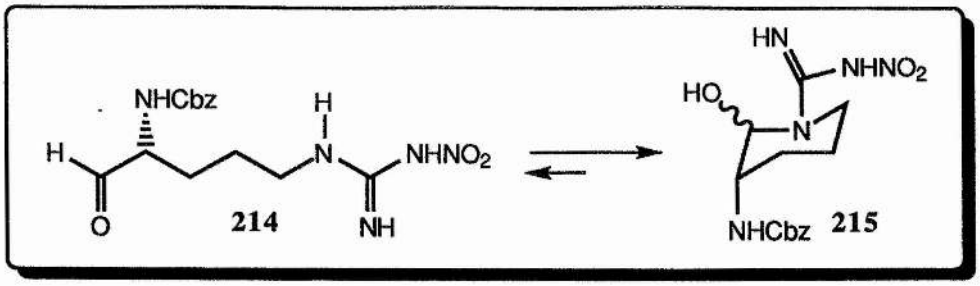
A tight monitoring of the progress of the hydrogenolytic cleavage of the phenylfluorenyl group by tlc, allowed the isolation of the *N*-Boc aldehyde (**212**) in excellent 80% yield. It was noticed that if the reaction was left for too long (over 6 hours), the yield dropped dramatically due to the reduction of the aldehyde.

The methyl ester was then removed using two equivalents of lithium hydroxide in methanol at room temperature. Note that a larger excess of base led to a complicated mixture of products. After careful acidification with 10% citric acid the hemiketal lactone (**213**) was isolated as a white solid [mp, 146-147 °C; $[\alpha]_D$ +41.1 (c 2 in THF); (found C, 54.25; H, 7.70; N, 3.95. $C_{15}H_{25}NO_7$ requires C, 54.35; H, 7.60; N, 4.20)]. The IR spectrum displayed two stretches at 1730 and 1711cm^{-1}, for the two carbonyls of the ester groups, and a medium stretch at 1772 cm^{-1}, for the carbonyl absorption of the lactone. The ^{1}H NMR spectrum in chloroform showed a ~ 1:3 ratio of the α- and β-epimers at 25 °C. Although, it was observed that this ratio changed with the temperature and/or the solvent used, for instance, a ~ 1:1 ratio was observed in methanol at 25 °C.



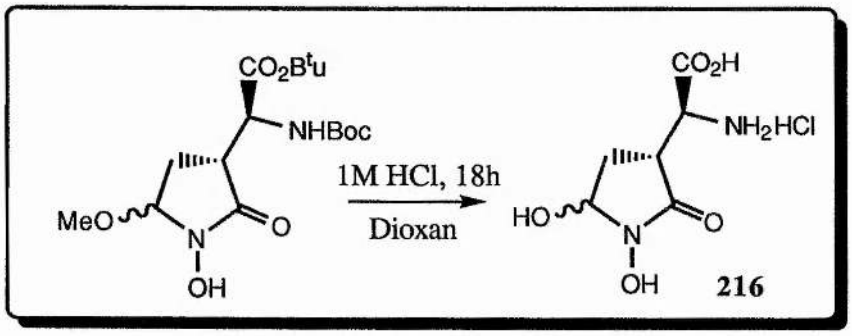
Scheme 58: *Synthesis of (1S*,2S,3S)-1-hydroxy-2-methyl-2-(tert-butyl)oxycarbonyl-3-(tert-butyloxycarbonyl)amino-butano-4-lactone (**213**)*

A related type of cyclisation was illustrated in the literature with Cbz-*N*-nitro-*S*-arginal (**214**). This α-amino aldehyde exists essentially as its cyclic carbinolamine (**215**, Scheme 59).[147] This result was somewhat remarkable as most α-amino aldehydes tend to racemise on silica gel, due to keto-enol tautomerism.[148] In this particular case racemisation was not observed.



Scheme 59: *Cyclic carbinolamine from Cbz-N-nitro-S-arginal*

Finally, we hoped to remove the Boc group and the *tert*-butyl ester with trifluoroacetic acid in dichloromethane. We initially tried in the same conditions used by Robins et al. These workers used a 1:1 ratio TFA/CH_2Cl_2 (0.6 ml/mmol of substrate) for 2 h at room temperature and they had to remove both a *p*-methoxybenzyl group and a Boc group. In our work, after 2 h only the Boc group seemed to have been removed as judged by ^1H NMR spectroscopy. As a model, the final deprotection step of Baldwin's synthesis of dealanylalahopcin (**216**), involving a Boc group, a *tert*-butyl ester and also an hemiketal required 18 h in 1 mol dm^{-3} aqueous hydrochloride and dioxan (Scheme 60).[149]

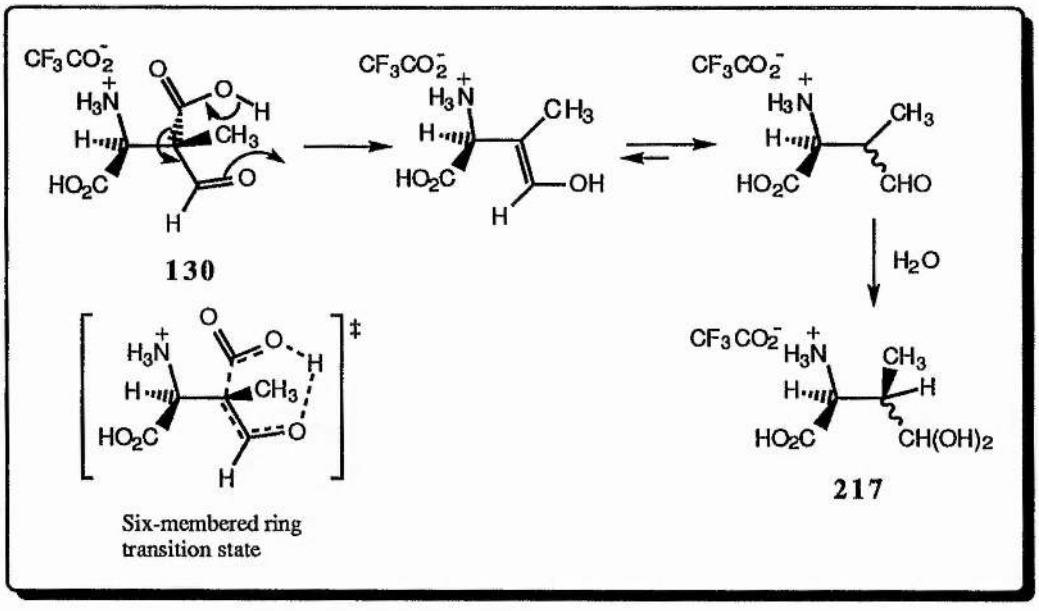


Scheme 60: *Model from Baldwin's synthesis of dealanylalahopcin*

The explanation is that *tert*-Butyl esters require harsher conditions for their removal than their carbamate homologues. The difference is ascribed to the easier protonation of the nitrogen of the carbamate.[150]

Hence, we decided to leave the reaction for a longer period of time at room temperature. After 24 h the mixture was poured into water and was extracted with dichloromethane. The aqueous layer was lyophilised and the ^1H NMR spectrum of the

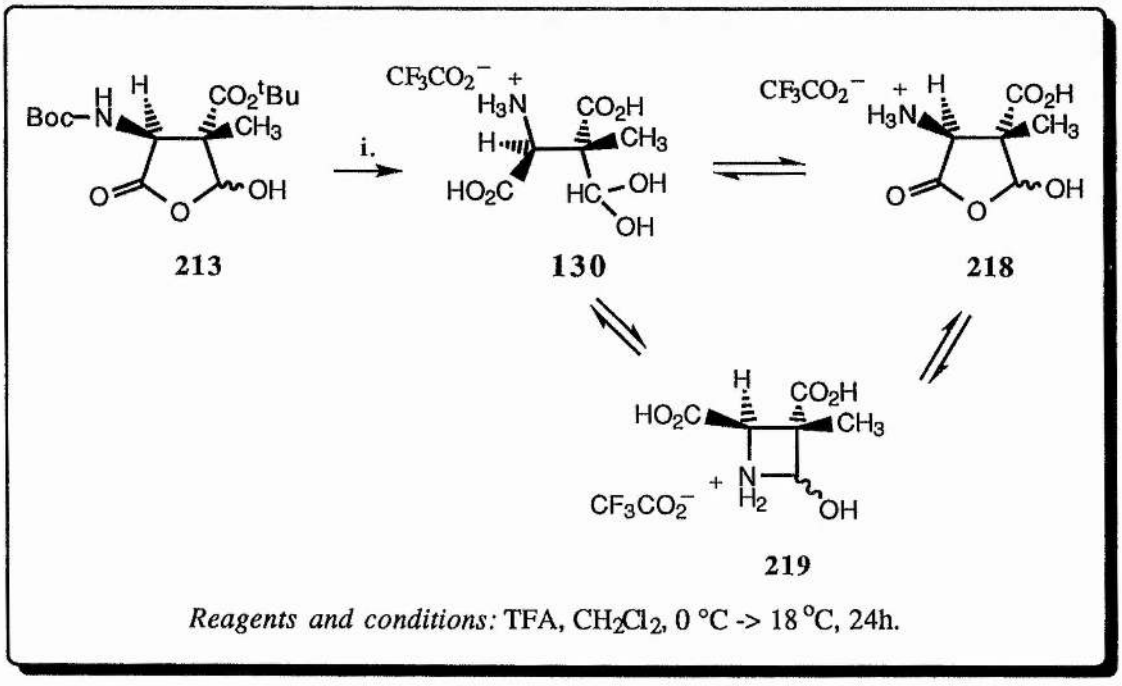
residue was recorded in ^2H_2O. Analysis revealed the presence of a mixture of diastereoisomers of the decarboxylated material as the *gem*-diol (**217**, Scheme 61), [δ_H (D_2O) 1.07 (3 H, d, CH3), 1.1 (3 H, d, CH3), 2.38 (1 H, m,C*H*CH3), 2.4 (1 H, m, C*H*CH3), 3.74 (1 H, d, CHN), 4.02 (1 H, d, CHN), 5.08 (1 H,d, C*H*OH), 5.13 (1 H, d, C*H*OH)].



Scheme 61: *Decarboxylation mechanism of the target aldehyde(**130**)*

This result was disappointing since we had hoped that the decarboxylation would have not occurred so readily. We tried to overcome this difficulty by using only 4 molar equivalents of TFA instead of a large excess. Under these conditions, after 30 h an aliquot was taken off the reaction mixture and was analysed by chemical ionisation mass spectroscopy. The spectrum displayed three major peaks at 130 (100%), 179 (30%) and 195 (30%). The peak at 195 corresponds to the protonated molecular ion of aldehyde (**130**) $[M + H]^+$. The reaction was then worked-up as before and lyophilisation gave a brownish residue. Purification by ion exchange chromatography (Amberlite IRC-50) afforded a white solid in 26% yield. The ^1H NMR spectrum was complicated and the broadness of the signals suggests a dynamic equilibrium. Two broad signals at 0.92 ppm

and 1.2 ppm were attributed to the methyl groups. Broad signals at 4.0 ppm and 4.3 ppm corresponding to the resonance of C*H*NH_3 and 5.0 ppm for C*H*(OH) were also observed. In addition, no decarboxylation product was observed under these conditions. The ^{13}C NMR spectrum showed five methyl groups, of which two were dominant. Signals located at 90 ppm suggested the presence of the masked aldehyde (gem diol or hemiketal). These NMR data and the broadness of the ^1H NMR spectrum, suggested the presence of an equilibrium mixture of mainly of the cyclic hemiketal (**218**), the hydrated aldehyde (**130**) and possibly traces of the four-membered ring carbinolamine (**219**), Scheme 62. This proposal was further supported by the chemical ionisation mass spectrum which displayed a *m/z* peak at 179 ($[M + H - H_2O]^+$) corresponding to the cyclic hemiketal (**218**), and 195 ($[M + H]^+$), for the hydrated aldehyde (**130**), (HRMS: found $[M + H]^+$, 195.0737. $C_6H_{13}NO_6$ requires 195.0743).

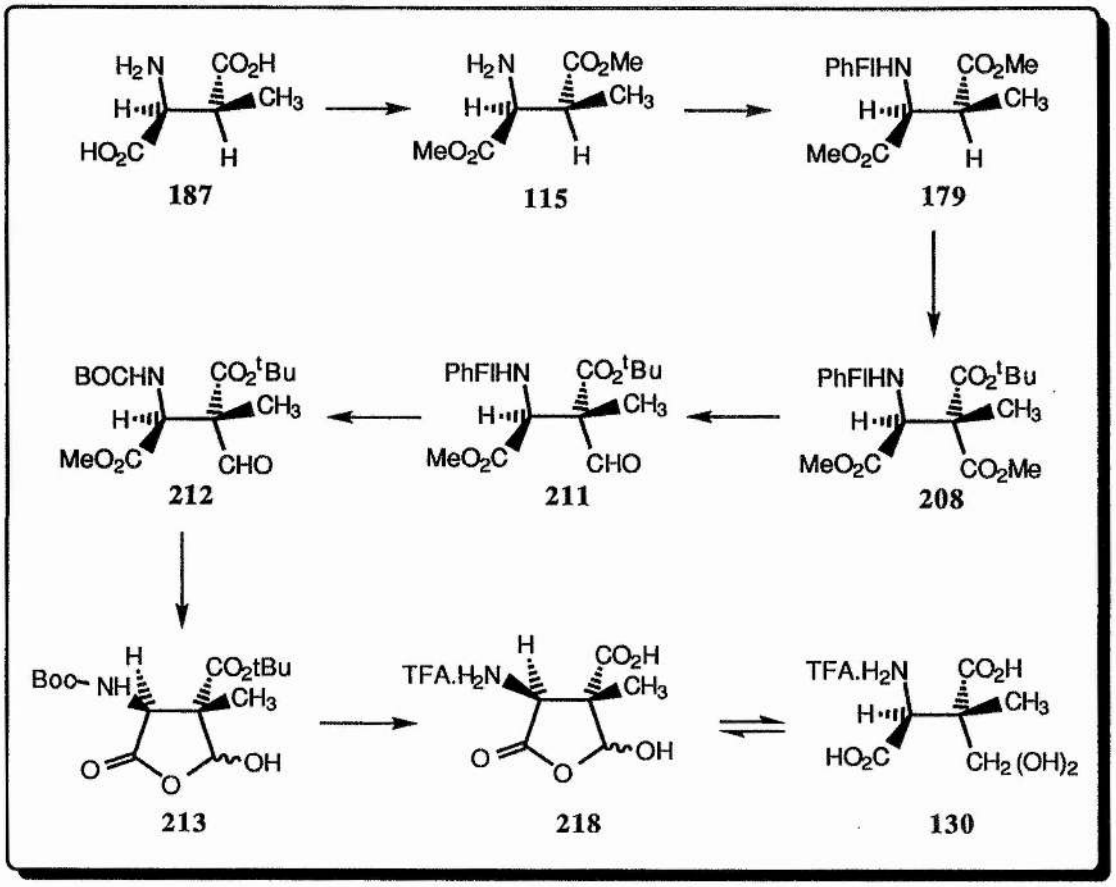


213 **130** **218** **219**

Reagents and conditions: TFA, CH_2Cl_2, 0 °C -> 18 °C, 24h.

Scheme 62: *Deprotection of the cyclic hemiketal (***213***)*

In summary, we were confident that we had successfully synthesised the required (2*S*,3*S*)-3-formyl-3-methylaspartic acid in 7 steps and in an overall yield of 3.6% (Scheme 63).

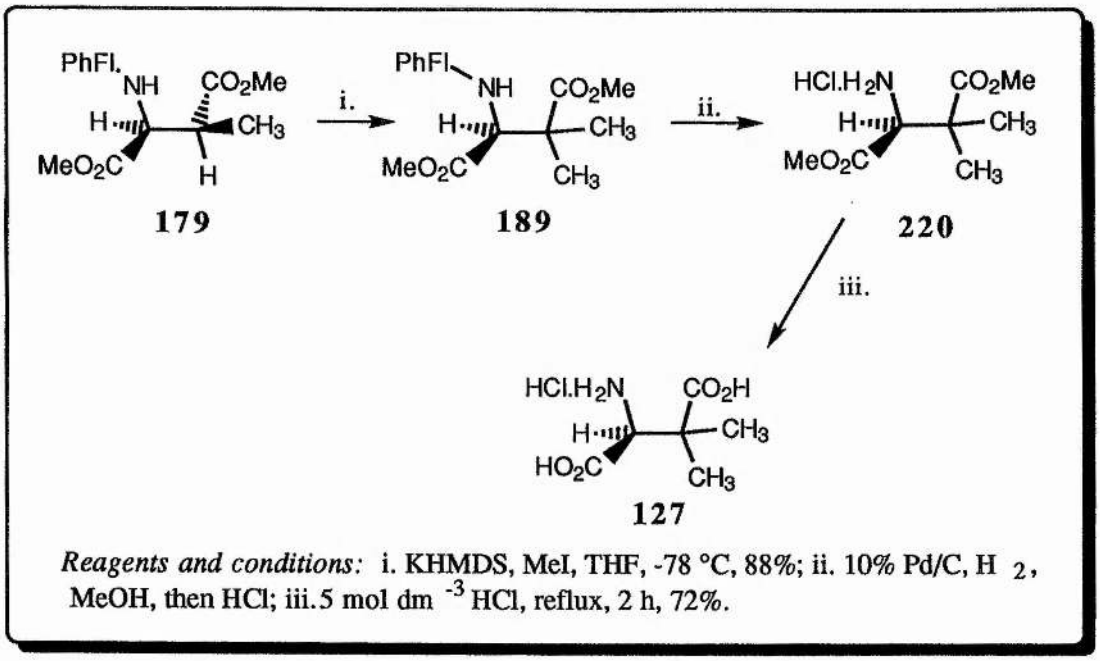


Scheme 63: *Synthesis of the target amino aldehyde* (**130**)

3.2.5 Synthesis of (2*S*)-3,3-dimethyl aspartic acid (127)

(2*S*)-3,3-Dimethylaspartic acid (**127**) was synthesised from *N*-9'-(9'-phenyl-fluorenyl) 3-methylaspartate diester (**179**), the synthesis of which was already described p. 80. Alkylation with methyl iodide after removal of the β-proton with KHMDS afforded dimethylaspartate diester (**189**) in 88% yield. The phenylfluorenyl group was removed by catalytic hydrogenolysis over 10% Pd/C in methanol and the resulting amino ester hydrochloride (**220**) was refluxed in 5 mol dm^{-3} HCl for 2 h to give the hydrochloride salt

of the amino acid (**127**) as a pale yellow solid in 72% yield, mp 97-100 °C, $[\alpha]_D$ -4.6 (c 1 in H_2O), (HRMS found: $[M + H]^+$, 162.0769. $C_6H_{12}NO_4$ requires 162.0766).



Reagents and conditions: i. KHMDS, MeI, THF, -78 °C, 88%; ii. 10% Pd/C, H_2, MeOH, then HCl; iii. 5 mol dm^{-3} HCl, reflux, 2 h, 72%.

Scheme 64: *Synthesis of* (2*S*)-3,3-dimethylaspartic acid (***127***)

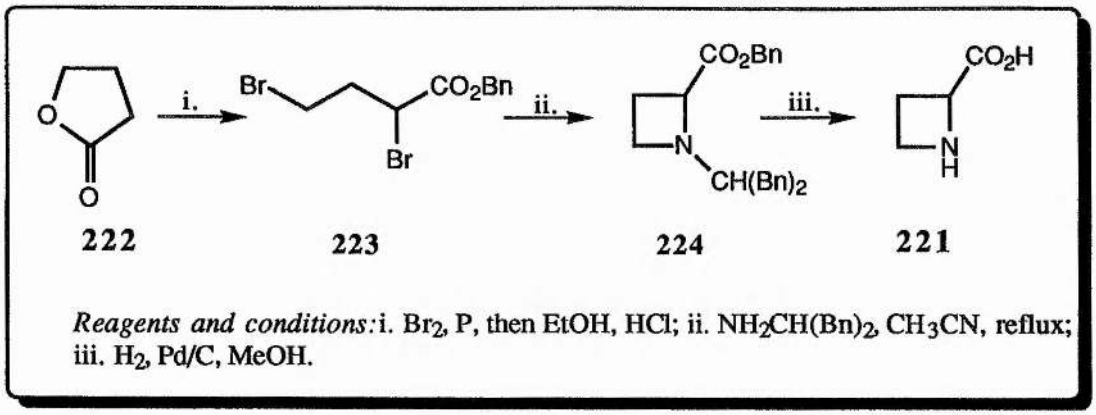
3.2.6 Synthesis of the (2*S*,3*S*)-3-methyl azetidine dicarboxylic acid (124)

3.2.6.1 Brief overview of azetidine carboxylic acid

Although, azetidines are valuable compounds in pharmaceutical and agrochemical research,[151] they are one of the most difficult amines to synthesize because of the unfavorable enthalpy of activation in four membered ring formation[152] and the high susceptibility to cleavage of the strain in the heterocyclic ring.[153] The ease of formation of the saturated nitrogen heterocycles was reported to decrease in the order 5 > 3 > 6 > 7 ~ 4.[154] The slower rate of four-membered ring formation was attributed to the less favorable enthalpy of activation since the entropy of activation was calculated to be the same for three- and four-membered rings.

(2*S*)-Azetidine-2-carboxylic acid (**221**), a lower homologue of proline, occurs in nature. It is a powerful proline agonist in plant tissue cultures and was first isolated by

Fowden in 1956.[151] Cromwell described one of the first syntheses of this important heterocycle from butyrolactone (**222**), Scheme 65.[155,156]

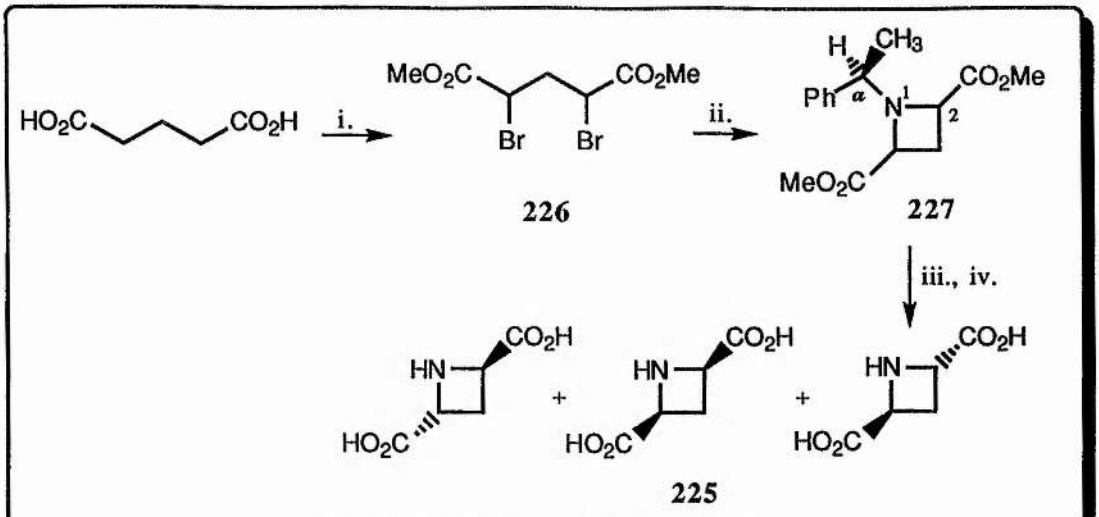


222 **223** **224** **221**

Reagents and conditions: i. Br_2, P, then EtOH, HCl; ii. $NH_2CH(Bn)_2$, CH_3CN, reflux; iii. H_2, Pd/C, MeOH.

Scheme 65: *Cromwell's Synthesis of azetidine-2-carboxylic acid (221)*

Another non-protein heterocyclic amino acid, that is not known in nature, azetidine 2,4-dicarboxylic acid (**225**) has been synthesised by Yamamoto *et al.*.[157] This group used a similar strategy to the one previously described, *viz.* reaction of 2,4-dibromo pentanedioate (**226**) and (*S*)-α-methylbenzylamine to give a mixture of three diastereoisomers [(α*S*,2*R*,4*R*), (α*S*,2*S*,4*S*), (α*S*,2*R*,4*S*)] of compound (**227**) which could be separated by silica gel.

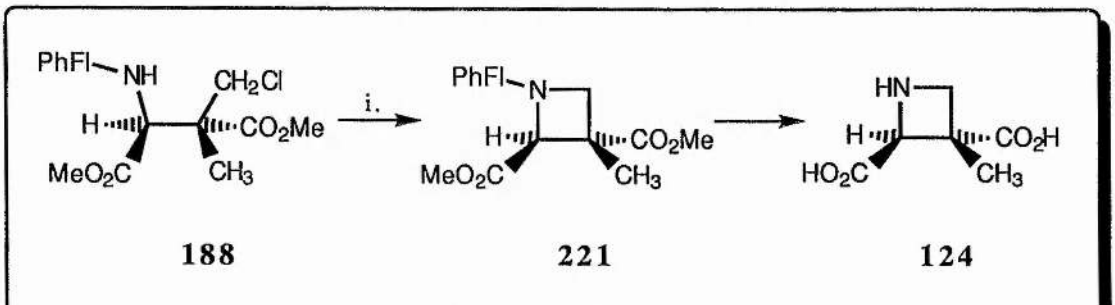


Reagents and conditions: i. $SOCl_2$, Br_2, MeOH; ii. (*S*)-methylbenzylamine, toluene-H_2O (K_2CO_3), reflux then separation by chromatography by silica gel; iii. H_2, $Pd(OH)_2$; iv. H_2O, relux, 15 h

Scheme 66: *Yamato's synthesis of 2,4-dicarboxylic azetidines (225)*

3.2.6.2 Synthesis of *trans*-(2*S*,3*S*)-azetidine 2,3-dicarboxylic acid (124)

The 3-chloromethyl 3-methylaspartate diester (**188**) is an ideal precursor to synthesise the azetidine (**124**), see Scheme 67. Preliminary cyclisation reactions, performed in refluxing acetonitrile or toluene were largely unsuccessful, and gave unreacted starting material, although in toluene traces of the azetidine were observed after prolonged reaction times. We decided then to use DMF and one equivalent of sodium iodide at 100-110 °C.



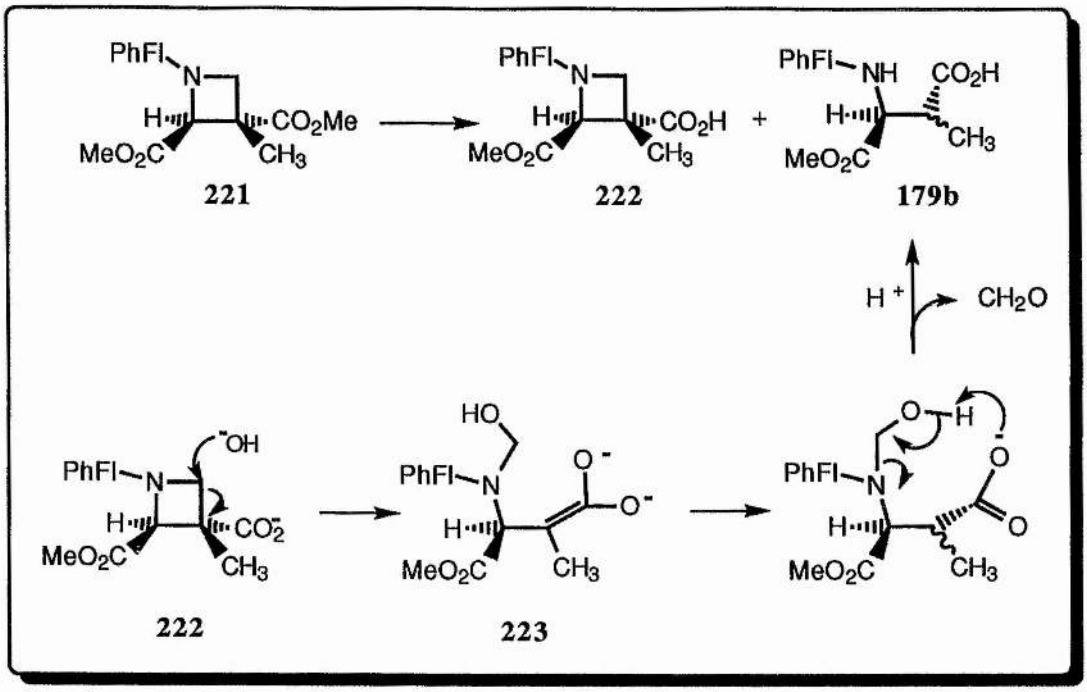
Reagents and conditions: i. DMF, NaI, 110 °C, 3h, 80%

Scheme 67: *Synthesis of the trans-(2S,3S)-azetidine dicarboxylic acid (124)*

Under these conditions the starting material completely disappeared after 3 h and a new, less polar product appeared on the tlc plate. The azetidine (**221**) was isolated in 80% yield as a white solid [mp, 102-106 °C, (found C, 76.10; H, 5.95; N, 3.25. $C_{27}H_{25}NO_4$ requires C, 75.85; H, 5.90; N, 3.25); $[\alpha]_D$ +144.3 (*c* 2 in DCM)].

It was noted however, that some product degradation occurred if the reaction was left too long. This was probably a consequence of the acid sensitivity of the phenylfluorenyl group,[158] and the presence of hydrogen iodide formed during the reaction. The problem could be avoided by introducing solid potassium bicarbonate into the reaction mixture. It was interesting to note that the infrared spectrum of the azetidine (**221**) displays two very distinct sharp absorption bands at 1756 and 1737 cm^{-1} for the carbonyl groups. What is remarkable also is the large separation in the ^1H NMR signals of the two methylene protons which occur as an AB-quartet with one doublet at 3.3 and one doublet at 3.74 ppm, J = 9.1 Hz. A colourless crystal was obtained by recrystallisation from petroleum ether-ethyl acetate (97:3) and was subjected for X-ray analysis. The three dimensional structure corroborated the previous stereochemical analysis, and confirmed the formation of the *trans* azetidine, see Figure 18, see p. 106.

The next problems to tackle were the deprotection steps. Initially, the saponification of the methyl esters using sodium hydroxide was attempted. Although it was possible to obtain the β-acid (**222,** Scheme 68), the α-ester was too hindered to react under these conditions. However, when a large excess of base was used to force the hydrolysis of the second methyl ester, a mixture of the azetidine (**222**) and some ring opened products were obtained. The acyclic products were identified as a diastereoisomeric mixture of (2*S*,3*RS*)-*N*-PhFl 3-methylaspartate dimethylester (**179b**).



Scheme 68: *Proposed mechanism of the ring opening of the azetidine (**221**) in basic conditions*

It seems that the instability under these conditions is largely due to the electrophilic properties of C-4. It is suggested that hydroxide ion attacks the C-4 carbon atom of the ring to generate an unstable hemiaminal (**223**, Scheme 68.).[159] Subsequent loss of formaldehyde gives the C-3 epimeric mixture of compound (**179b**).

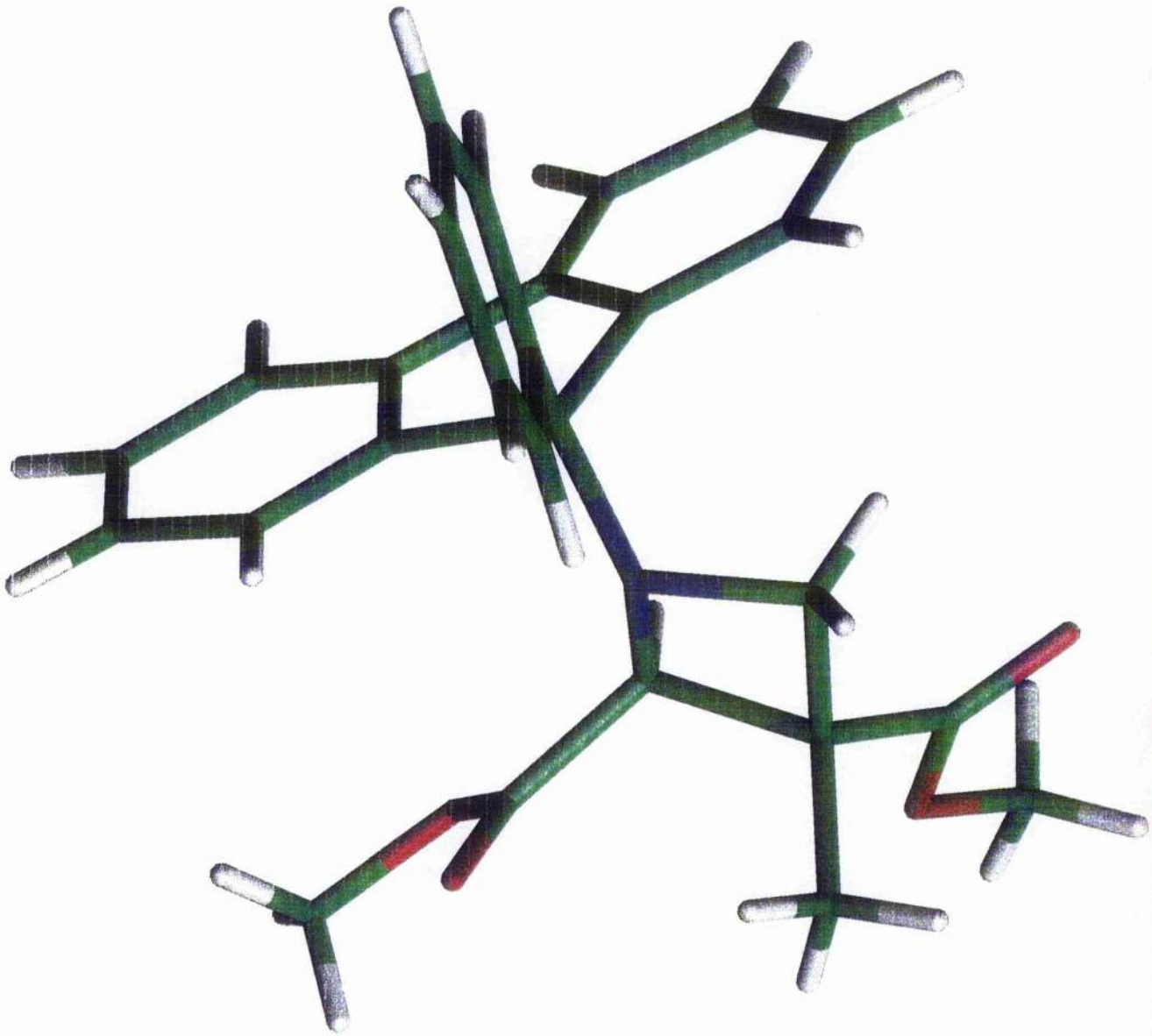
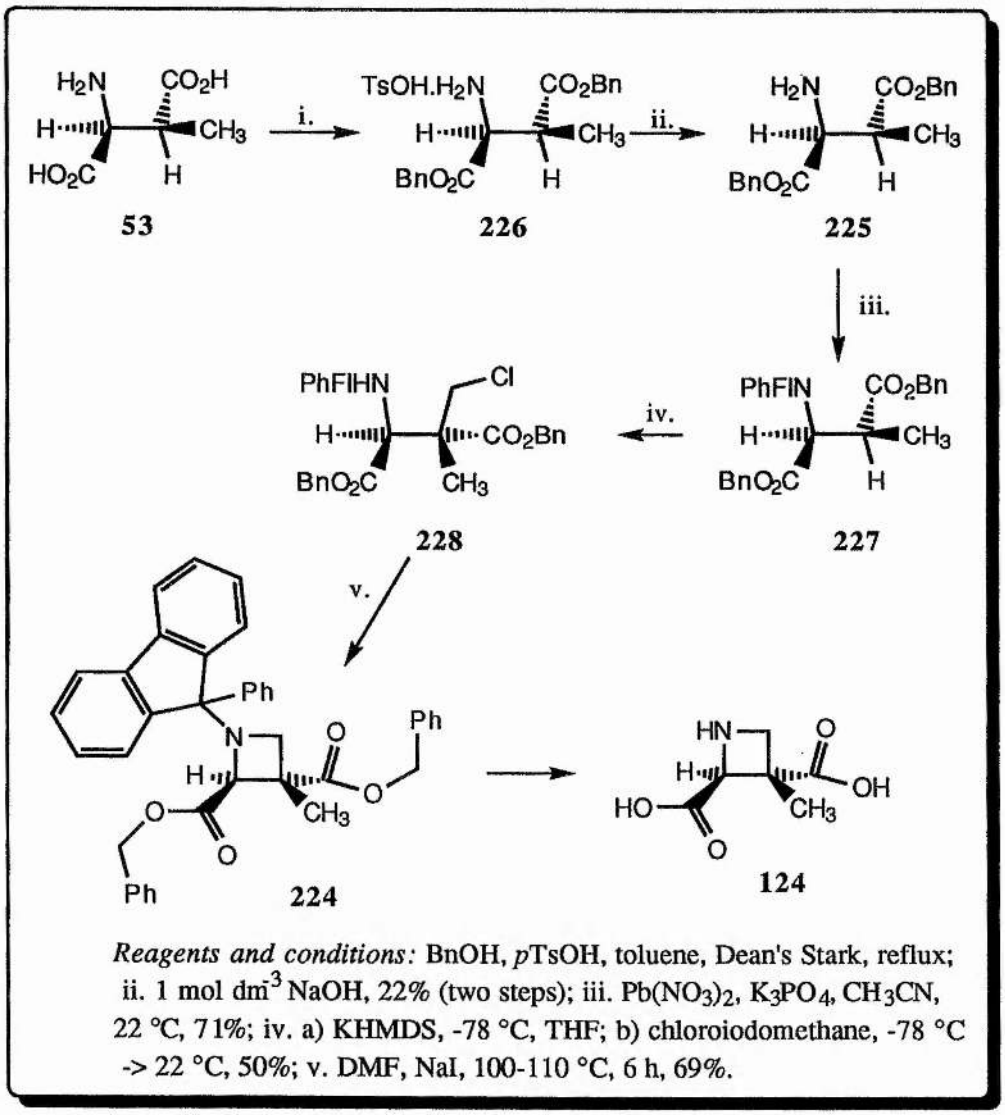


Figure 18: *Crystal structure of dimethyl trans-(2S,3S) azetidine 2,3-dicarboxylate (**221**)*

An alternative strategy to avoid this difficulty was to synthesise the corresponding dibenzylazetidine (**224**) from dibenzyl dibenzyl 3-methylaspartate (**225**), see Scheme 69. Diester (**225**) was prepared through esterification of (2*S*,3*S*)-3-methylaspartic acid (**53**) at reflux in a solution of benzyl alcohol and *p*-toluene sulfonic acid in toluene. The *p*-toluenesulfonic acid salt of the dibenzyl ester (**226**) was then converted to the free amine diester (**225**) with 1 mol dm^{-3} sodium hydroxide in 22% overall yield.



Reagents and conditions: BnOH, *p*TsOH, toluene, Dean's Stark, reflux; ii. 1 mol dm^{-3} NaOH, 22% (two steps); iii. $Pb(NO_3)_2$, K_3PO_4, CH_3CN, 22 °C, 71%; iv. a) KHMDS, -78 °C, THF; b) chloroiodomethane, -78 °C -> 22 °C, 50%; v. DMF, NaI, 100-110 °C, 6 h, 69%.

Scheme 69: *Synthesis of trans-(2S,3S0-3-methylazetidine-2,3-dicarboxylic acid (**124**)*

Subsequent protection of the free amine with 9-(9-phenylfluorenyl) bromide afforded dibenzyl *N*-9'-(-9'-phenylfluorenyl) 3-methylaspartate diester (**227**) in 71%

yield. Alkylation of protected dibenzyl ester (**227**) with chloroiodomethane afforded (2*S*,3*S*)-3-chloromethyl-3-aspartate (**228**) in 50% yield and subsequent cyclisation of the product, under the conditions previously described for the synthesis of dimethyl azetidine (**221**), gave the corresponding dibenzyl ester azetidine (**224**) in 69% yield.

Final deprotection of the amino and carboxyl groups was carried out in one step *via* hydrogenolysis over 10% Pd/C, in a methanolic solution of sodium hydrogencarbonate (2 eq). After purification by ion exchange chromatography (Amberlite IR-50) a gummy solid was obtained in 20% yield. The ^{1}H NMR spectrum in D_2O was consistent with the structure of azetidine (**124**, Figure 19), [δ_H (200 MHz; ^2H_2O) 1.22 (3 H, s, CH_3), 2.75 (1 H, s, NH), 3.56 and 4.15 (2 H, AB, *J* 10.7, CH_2N) and 4.78 (1 H, s, CH); δ_C (50.31 MHz; ^2H_2O) 22.2 (CH_3), 45.1 (quaternary), 60.1 (CH_2), 72.4 (CH)]. Unfortunately, further characterisation of the azetidine and enzyme studies were precluded due to apparent instability of this compound at room temperature. The instability of this compound seems to be related to the instability of other strained ring systems containing an amino group, see p. 115.

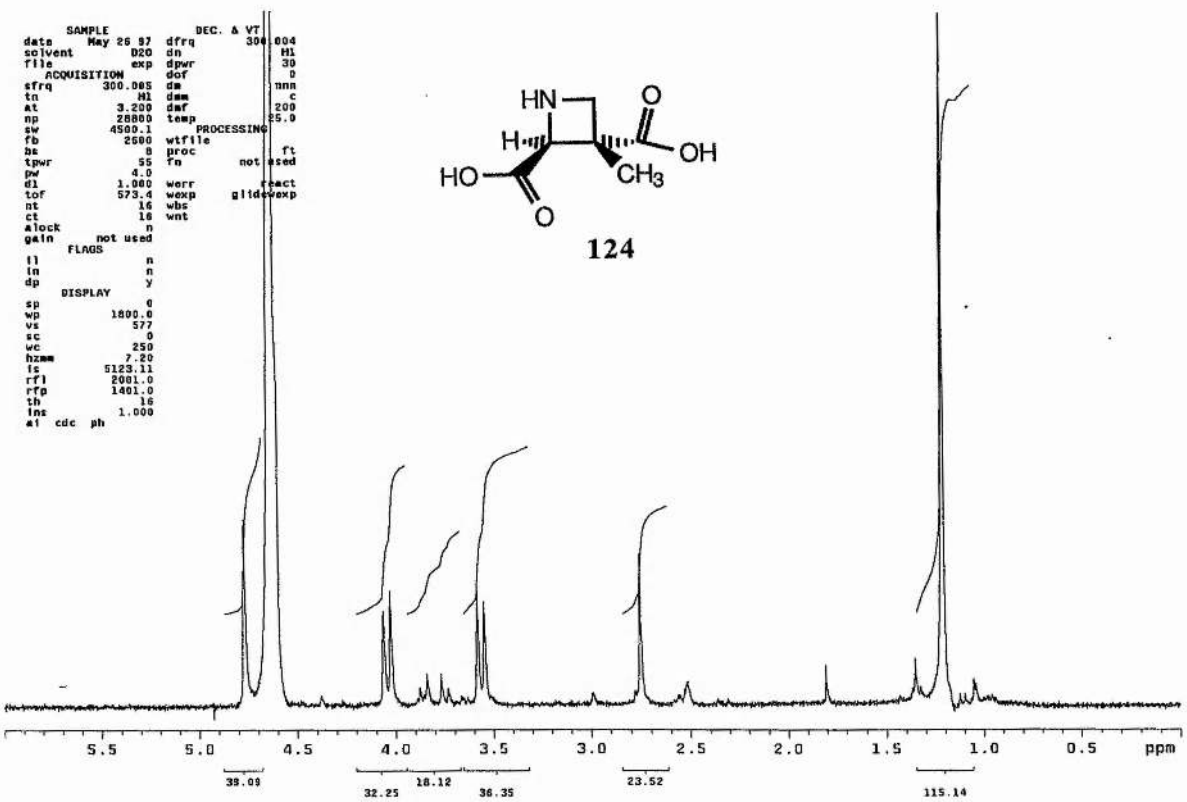
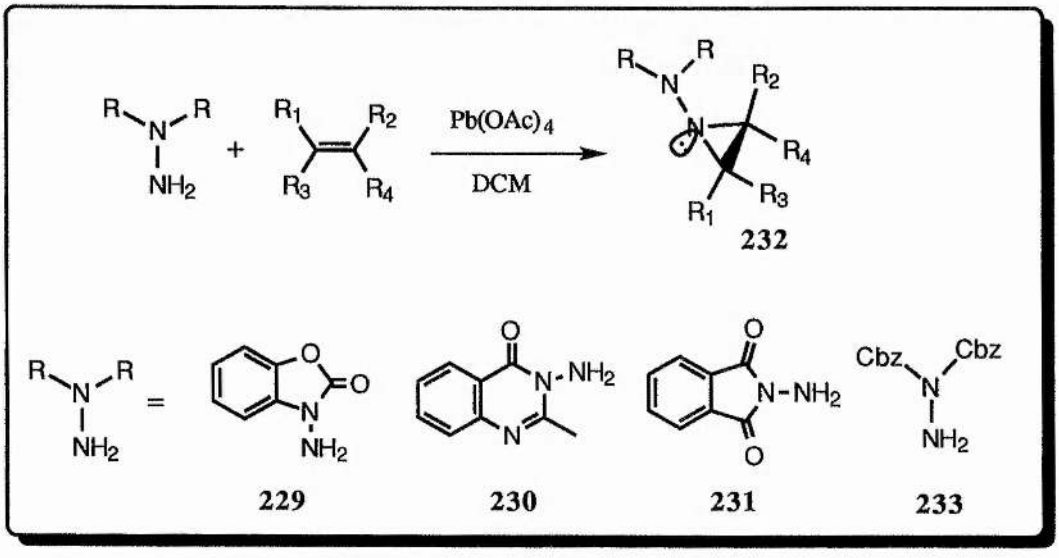


Figure 19: *^{1}H-NMR in D_2O of (2S,3S)-3-methyl-2,3- azetidine dicarboxylic acid (**124**)*

3.2.7 Synthesis of the *N*-aminoaziridinedicarboxylic acid (123)

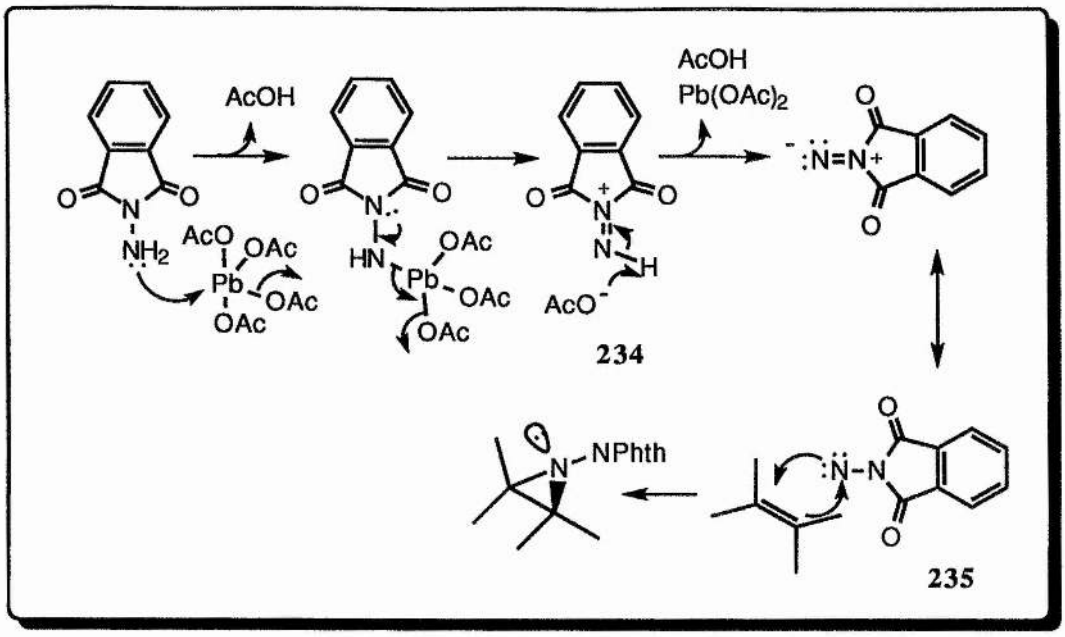
3.2.7.1 Introduction

The cycloaddition of carbenes to olefins has provided a interesting path to cyclopropanes, similarly the corresponding cycloaddition of nitrenes to alkenes is a versatile route to aziridines. This aziridination involves the oxidation of certain heterocyclic, *N*-amino compounds (1,1-disubstituted hydrazines) such as 3-aminobenzoxazol-2-one (**229**), 3-amino-2-methyl-quinazolone (**230**) or *N*-amino phthalamide (**231**), with lead tetraacetate in the presence of an olefin to afford the corresponding *N*-aminoaziridine (**232**, Scheme 69). It was also reported that *N*,*N*-dibenzyloxycarbonyl hydrazine (**233**, R = Cbz) can be used to give aziridines, although lower yields were obtained, especially for electrodeficient alkenes.[160]



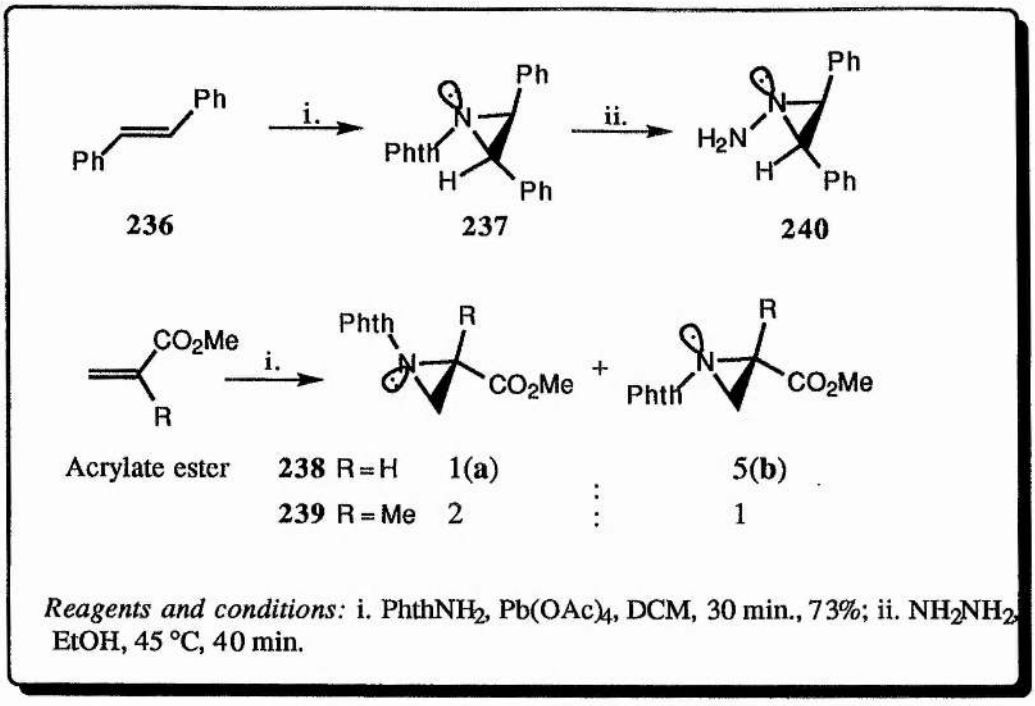
Scheme 69: *Oxydation of alkenes to form* N-*amino aziridine*

The reactive intermediate in these aziridinations was thought to be the *N*-nitrene (azaimide) which is formed *via* the oxidation of *N*-aminophthalamide by lead tetraacetate, Scheme 70. It seemed reasonable to suggest a mechanism in which *N*-aminophthalamide was oxidised to the intermediate diazenium acetate (**234**), lead(IV) tetraacetate being reduced to lead(II) diacetate. Finally, the diazenium (**234**) collapses to the reactive intermediate nitrene (**235**) which is stabilised by resonance.



Scheme 70: *Proposed mechanism for the aziridination of alkenes via phthalamidonitrene*

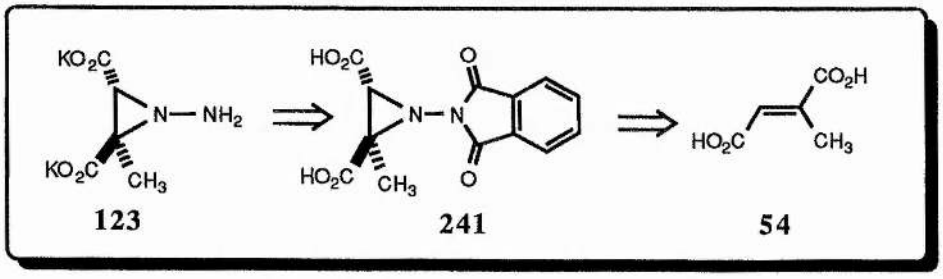
A wide variety of alkenes which contained alkyl chains were found to give aziridines, but acrylic esters and similar olefins in which the double bond was conjugated with an electron withdrawing group were shown to afford the best yield of aziridines.[161] This finding illustrates that amino-nitrenes have significant nucleophilic character. The reaction was shown to be completely *syn*-selective, for the oxidation of *N*-aminophthalamide in the presence of *trans*-stilbene (**236**) (Scheme 71), giving the *trans*-aziridine (**237**).[162] This result strongly suggests that the nitrenes react in the singlet state. Interestingly, it was reported that the ^{1}H NMR spectrum of methyl 1-phthalamidoaziridine-2-carboxylate (**238**), derived from methyl acrylate, displayed two singlets for the methyl group resonance which integrated in the ratio 1:5.[163] This observation corresponded to the presence of two rotamers, for which it was reasoned that the smaller signal represented the more sterically crowded rotamer(**238a**, Scheme 71). For the corresponding methacrylate ester adduct (**239**, R = Me), the introduction of a vicinal methyl group caused the rotamer (**239a**) to become preferred in the ratio 2:1.[163]



Reagents and conditions: i. $PhthNH_2$, $Pb(OAc)_4$, DCM, 30 min., 73%; ii. NH_2NH_2, EtOH, 45 °C, 40 min.

Scheme 71: *Synthesis of* N-*amino aziridines*

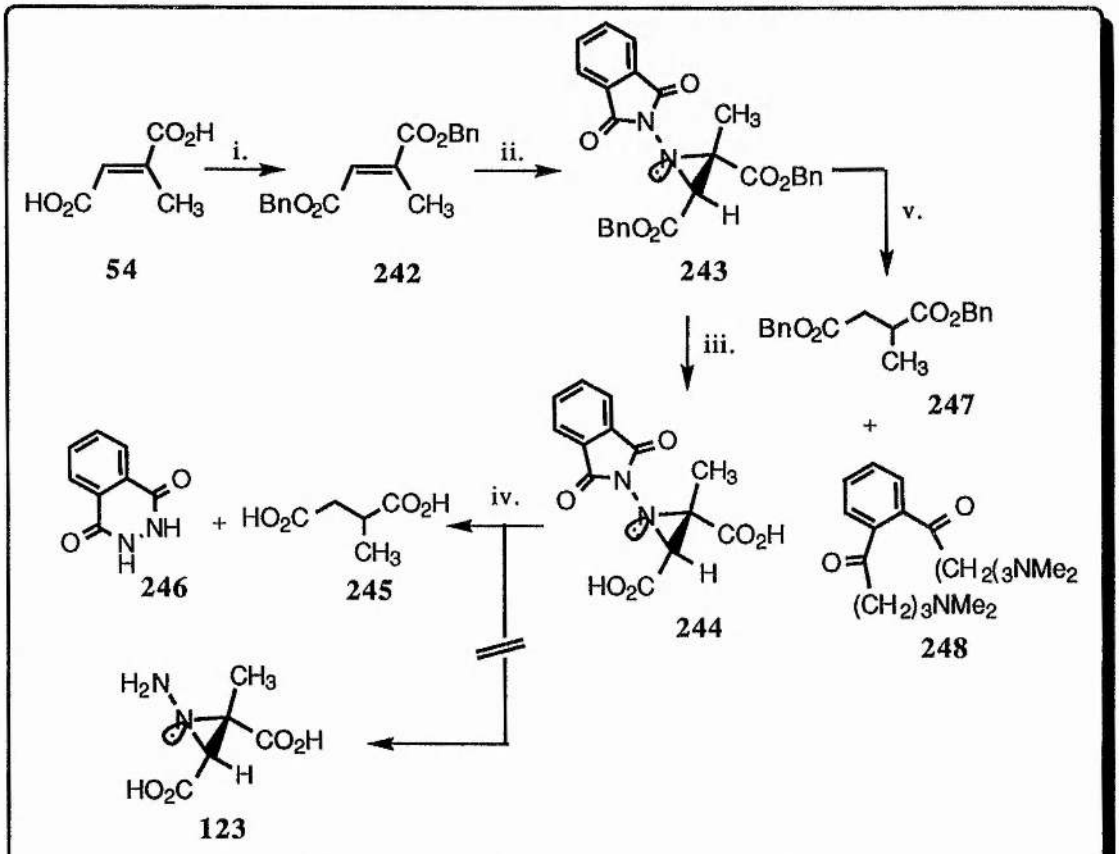
The removal of the phthalamido group of the 1-phthalamido-2,3-diphenyl aziridine (**237**) by hydrazinolysis has been reported, see Scheme 71, where the aziridine in ethanolic solution was warmed to 45 °C in presence of a large excess (>10 eq.) of hydrazine monohydrate, to afford the 1-aminoaziridine (**240**) in 94% yield.[164] It was also mentioned that the deprotection of the 1-phthalamidoaziridines containing electron withdrawing substituents like the aziridines (**238**) and (**239**), did not deprotect under above conditions, but no experimental details were given. Nevertheless, we planned to synthesise the target aminoaziridine (**123**) *via* the aziridine dicarboxylic acid (**241**) which could be prepared from mesaconic acid, see Scheme 72.



Scheme 72: *Retrosynthesis of the target amino aziridine (123)*

3.2.7.2 Results of the synthesis of compound (123)

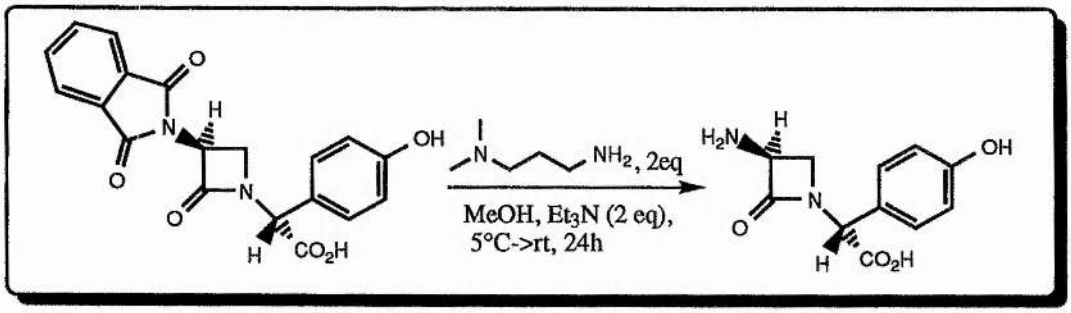
Mesaconic acid (**54**) was readily esterified to its dibenzyl ester derivative (**242**) using benzyl alcohol in the presence of tosic acid (Scheme 73). Reaction of this alkene with lead tetraacetate and *N*-aminophthalamide at 0 °C gave the *N*-phthalamidoaziridine (**243**) which was isolated as a colourless oil in 60% yield (HRMS found: $[M + H]^+$ 472.1642. $C_{27}H_{23}N_2O_6$ requires 472.1634). The ^1H NMR spectrum recorded in chloroform displayed one singlet at 1.74 ppm for the methyl group and one singlet at 4.31 ppm for the CH moiety. This suggested that only one invertomer existed for this compound at room temperature; presumably a sterically locked conformation as represented in Scheme 73.



Reagents and conditions: i. BnOH, pTsOH, CCl_4, 67%; ii. $Pb(OAc)_4$, DCM, $PhthNH_2$, 0 °C->22°C, 1 h, 60%; iii. H_2 , 10% Pd/C, EtOAc, 80%; iv. $NH_2NH_2.H_2O$ (1eq), H_2O, 22°C; v. *N*-dimethylpropylamine (2eq), Et_3N (2eq), MeOH, 71%, 22°C.

Scheme 73: *Synthesis of trans-N-amino-2-methyl-2,3-dicarboxyl aziridine (123)*

Both benzyl esters of compound (**243**) were removed by 1 atm of hydrogen in the presence of 10% Pd/C in ethyl acetate solution. The *N*-phthalimidoaziridine dicarboxylic acid (**244**) was isolated in 80% yield as a white solid after recrystallisation from diethyl ether (HRMS: found: $[M + H]^+$ 291.0629. $C_{13}H_{11}N_2O_6$ requires 291.0617). The diacid was dissolved in a solution of potassium carbonate (4 eq.) in the presence of one equivalent of hydrazine monohydrate. The resulting mixture was stirred at room temperature for a 24 h and was then lyophilised. The ^1H NMR spectrum recorded in ^2H_2O of the crude residue revealed the presence of a mixture containing 17% of starting material 41.5% of the diamide (**245**) and 41.5% of a new product. The new product {δ_H (200 MHz; $CDCl_3$)) 1.09 (3 H, s, C*H*$_3$), 2.10 (1 H, dd, C*H*), 2.50 (1 H, dd, C*H*), 2.60 (1 H, m, C*H*CH_3)} was identified as 2-methylsuccinic acid (**246**). Other phthalimidogroup cleaving reagents were also tried, such as aqueous ammonia and aqueous methylamine, but without success,[165] and in all cases 2-methylsuccinic acid (**246**) was formed. Deprotection of the aziridine (**243**) with *N*-dimethylpropylamine in methanol was also investigated.[166] This mild reagent, compared to hydrazine, had been used to remove the phthalimido group in presence of the β-lactam ring (Scheme 74).

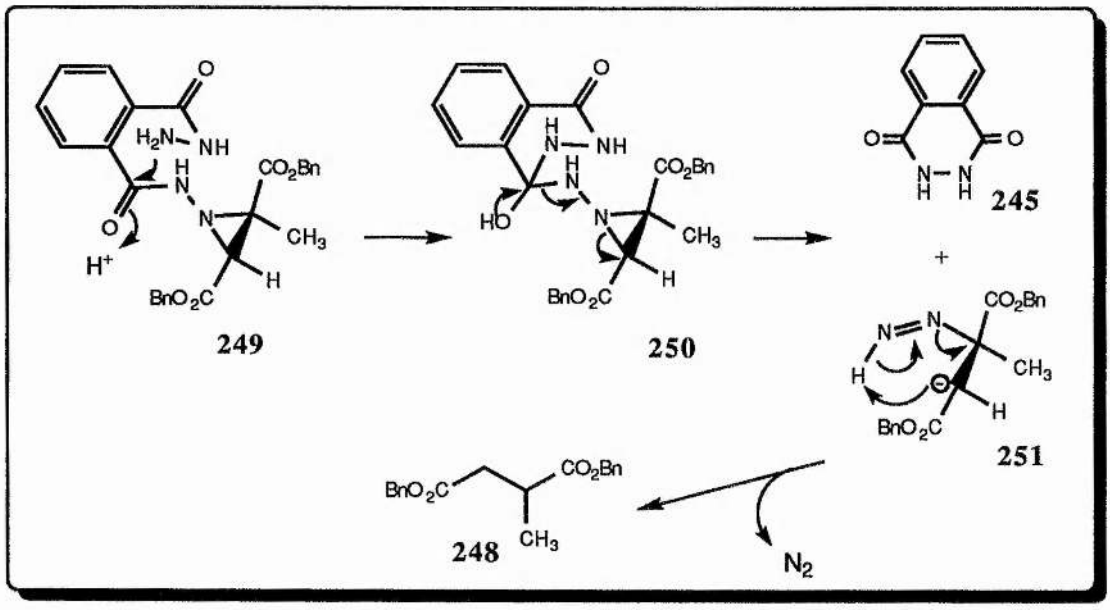


Scheme 74: *Deprotection of the* N*-phthalamido group*

Treatment of the aziridine (**243**) in methanol with *N*-dimethylpropylamine (2eq) and triethylamine (1eq.) for 20 h at room temperature gave two products as judged by ^1H NMR spectroscopy. These were the diamide (**247**) [δ_H(200MHz; $CDCl_3$) 1.85 (2 H, q, CH_2), 2.2 (6 H, s, CH_3), 2.34 (2 H, t, CH_2), 7.80 (5 H, m, CHar + NH)] and the dibenzyl 2-methylsuccinate (**248**) [δ_H(200 MHz, $CDCl_3$) 1.29 (3 H, d, CH_3), 2.52 (1 H, dd, CH), 2.85 (1 H, m, CH), 3.40 (1 H, m, CH), 5.15 (4 H, s, CH_2) and 7.38 (10 H, s,

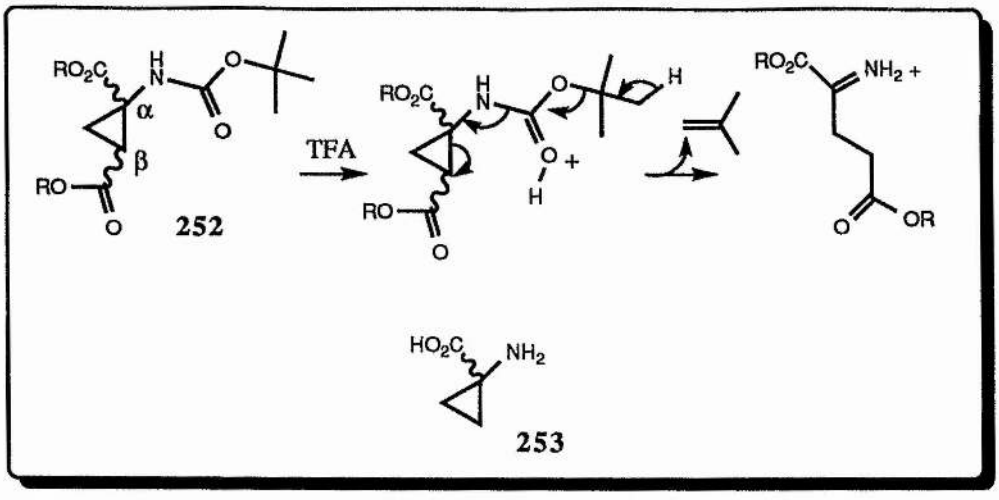
CHar)]. Dibenzyl 2-methylsuccinate was also formed when the removal of the phthalimido group was attempted using methylhydrazine in acetonitrile.[167]

It seems reasonable to propose a decomposition mechanism that first involves the formation of the hydrazide (**249**, Scheme 75). The nucleophilic primary amino group of this hydrazide could then attack the other amide carbonyl group intramolecularly, to afford the intermediate carbinolamine (**250**). This intermediate could then evolve to the phthalazine (**245**) in eliminating the diazene (**251**) which could decompose rapidly to give nitrogen and dibenzyl-3-methylsuccinate (**248**).



Scheme 75: *Proposed mechanism for the decomposition of aziridine (**249**)*

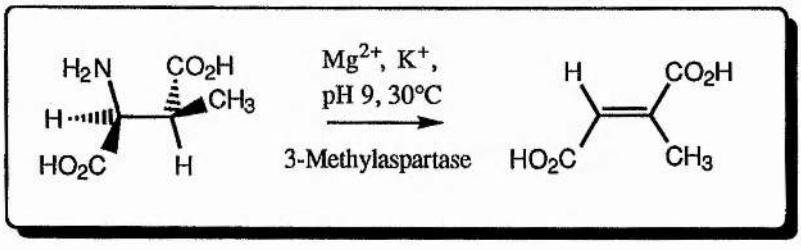
An analogy can be made with the instability of the aminocyclopropanedicarboxylate (**252**) which was shown to be unstable, yielding ring opening products, see Scheme 76.[168,169] The reason invoked for this instability was the presence of the carboxyl group in β-position of the amino group, the carboxyl group playing the role of an electron sink. On the other hand, 1-aminocyclopropane-1-carboxylic acid (**253**) is a stable compound.[170]



Scheme 76: *Instability of 1-amino cyclopropane 2-carboxylic acid (252)*

3.3 Kinetic studies

The inhibitory properties of the four synthesised potential inhibitors (see syntheses discussed in previously in chapter 4, p. 64) were determined using the continuous assay described in the experimental section. The enzyme activity was measured by monitoring the increase in absorbance at 240 nm due to the production of mesaconic acid (ε 3850 mol^{-1} cm^{-1}, pH 9, Scheme 77).

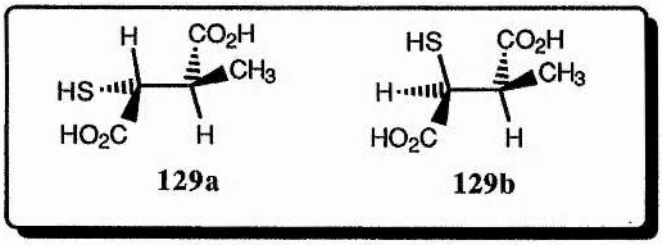


Scheme 77: *Assay of 3-methylaspartase*

3.3.1 Results and discussion

3.3.1.1 (2*S*,3*R*) and (2*R*,3*R*) 3-Methyl-2-sulfanyl succinic acid (129a,b)

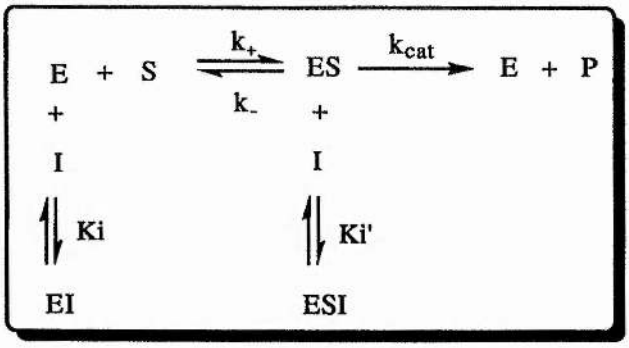
Irreversible inhibition by the sulfanyl compounds (**129a**) and (**129b**) was investigated by comparing the enzyme activity of incubations containing either the (2*S*,3*R*) or the (2*R*,3*R*) analogues at 50 mmol dm^{-3} concentration and at pH 9, with incubations containing only the enzyme. No loss of activity was observed up to 5 h after incubation. This suggests that the enzyme was not irreversibly inactivated in presence of the inhibitor.



Competitive inhibition was studied by measuring the change in absorbance at 240 nm, due to the production of mesaconic acid from (2*S*,3*S*)-3-methylaspartic acid. All rate measurements were conducted at pH 9 and 30 °C, in incubations containing 500 mmol dm^{-3} Tris-HCl buffer, 20 mmol dm^{-3} magnesium chloride, 1 mmol dm^{-3} potassium chloride and the substrate in the range 1-5 mmol dm^{-3}. Sulfanyl analogues in the range of 1-3 mmol dm^{-3} were used and each activity assay was carried out in triplicate. The results were analyzed using double reciprocal Lineweaver-Burk plots, see Graphs 1 and 2.

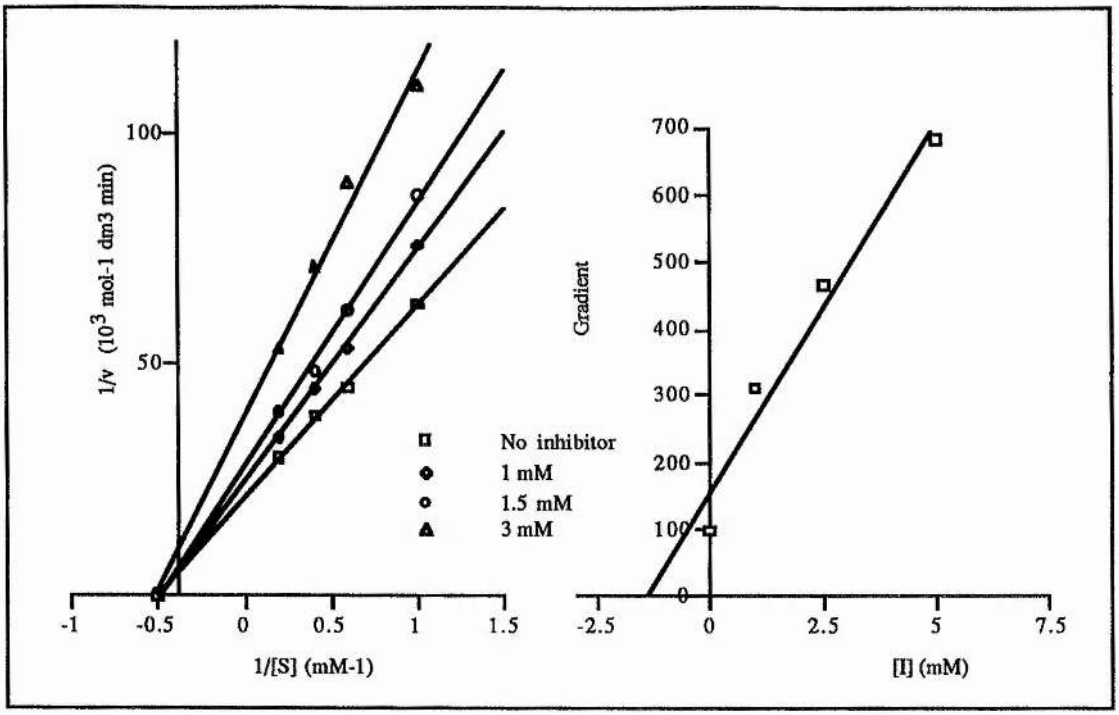
The results showed that in the presence of varying concentration of the inhibitor a change in V_{max} was observed, but with no change in the value of K_m. This is consistent with noncompetitive inhibition as described in Scheme 78. A plot of the gradient from the double reciprocal plots *vs.* inhibitor concentration allowed the K_i value to be determined at the x-axis intercept. This was calculated to be 1.4 mmol dm^{-3} for the (2*S*,3*R*) diastereoisomer and 3.4 mmol dm^{-3} for the other diastereoisomer (2*R*,3*R*). However, it should be noted that the plots show non-linear inhibition indicating that more than one molecule can bind to a form of the enzyme, or that the enzyme catalyses the slow elimination of H_2S from the inhibitor and that moieties from more than one molecule of

inhibitor can co-exist in a bound form. The most likely combination is H_2S plus the intact inhibitor.

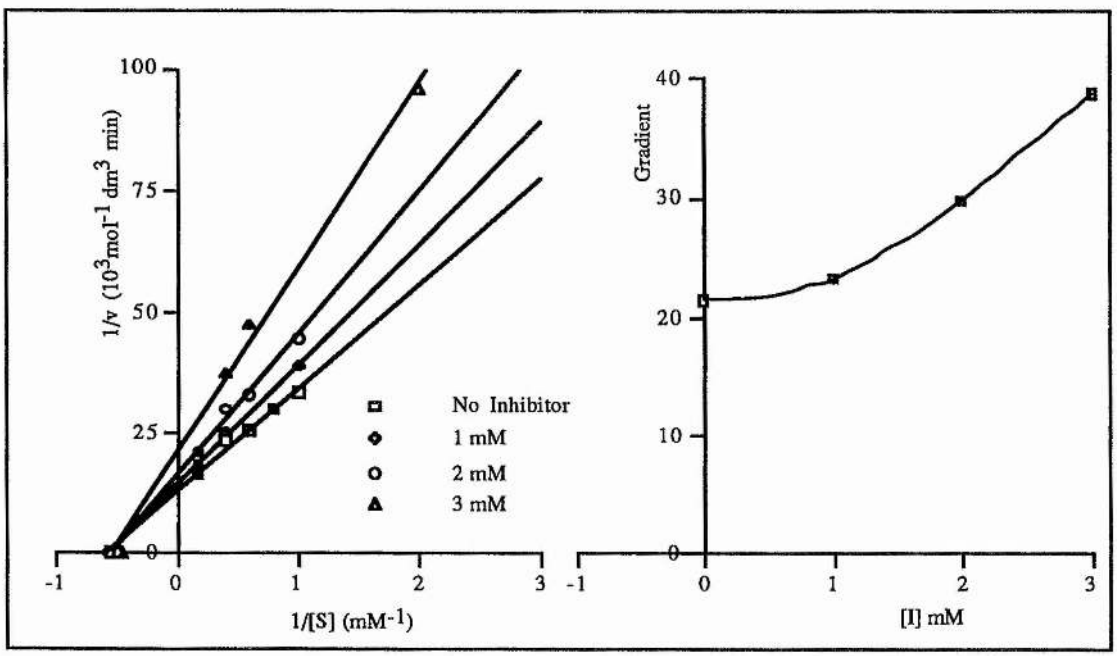


Scheme 78: *Noncompetitive inhibition*

Noncompetitive inhibition is observed when the inhibitor **I** interacts with the free enzyme **E** and also equally well with the enzyme substrate complex **ES**. This type of inhibition is commonly observed for multi-substrate systems where the inhibitor can interact with a co-substrate binding site. As far as 3-methylaspartase is concerned, there is reasonable experimental evidence to suggest that the enzyme active site contains a substantial pocket capable of binding the co-substrate ammonia and a number of analogous *N*-nucleophiles including hydrazine, hydroxylamine, methylamine, dimethylamine and ethylamine.[73] A second pocket can accommodate mesaconate acid and a number of analogous alkylfumarates including ethylfumarate and isopropylfumarate.

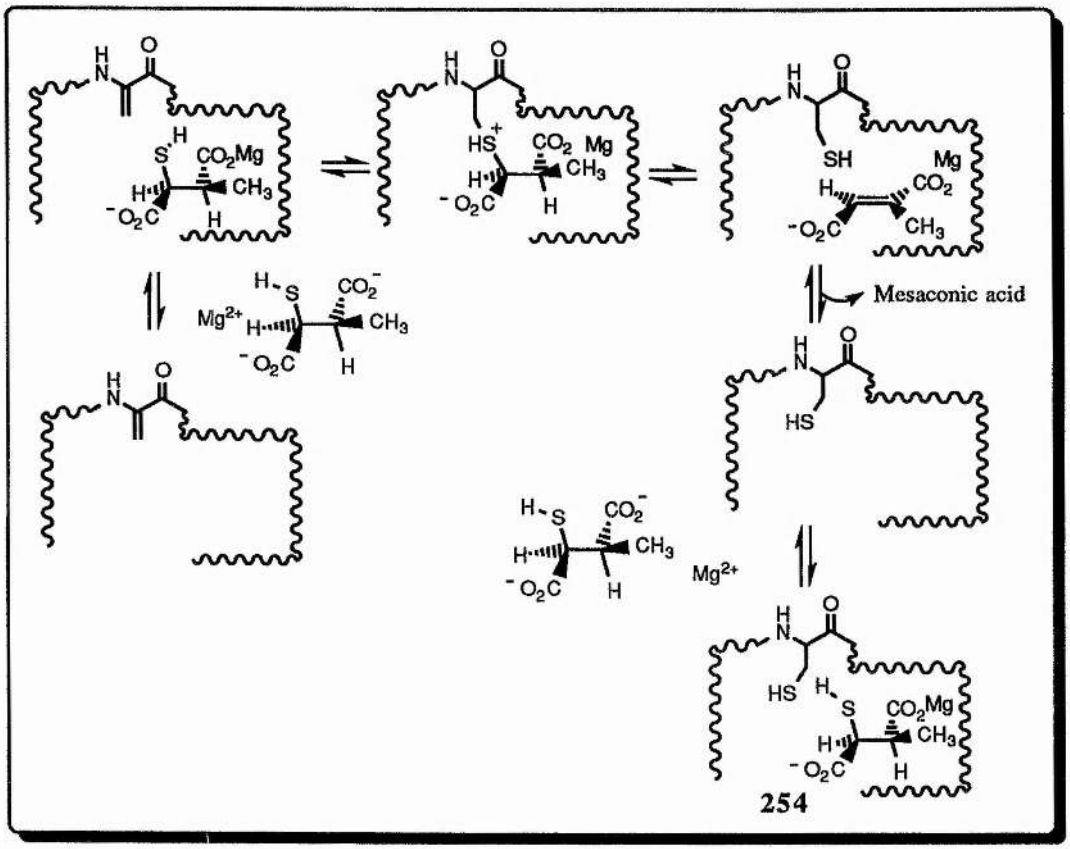


Graph 1 : *plots for (2S,3R)-3-methyl-2-sulpfanyl succinic acid (29b)*



Graph 2 : *plots for (2R,3R)-3-methyl-2-sulpfanyl succinic acid (29a)*

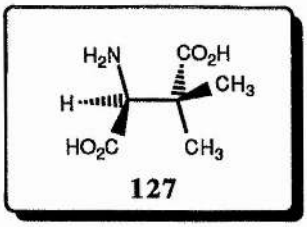
The observed inhibition could then come from the simultaneous binding of the inhibitor and/or a derivative (**254**) as illustrated in Scheme 79.



Scheme 79: *Proposed mechanism for the noncompetitive inhibition*

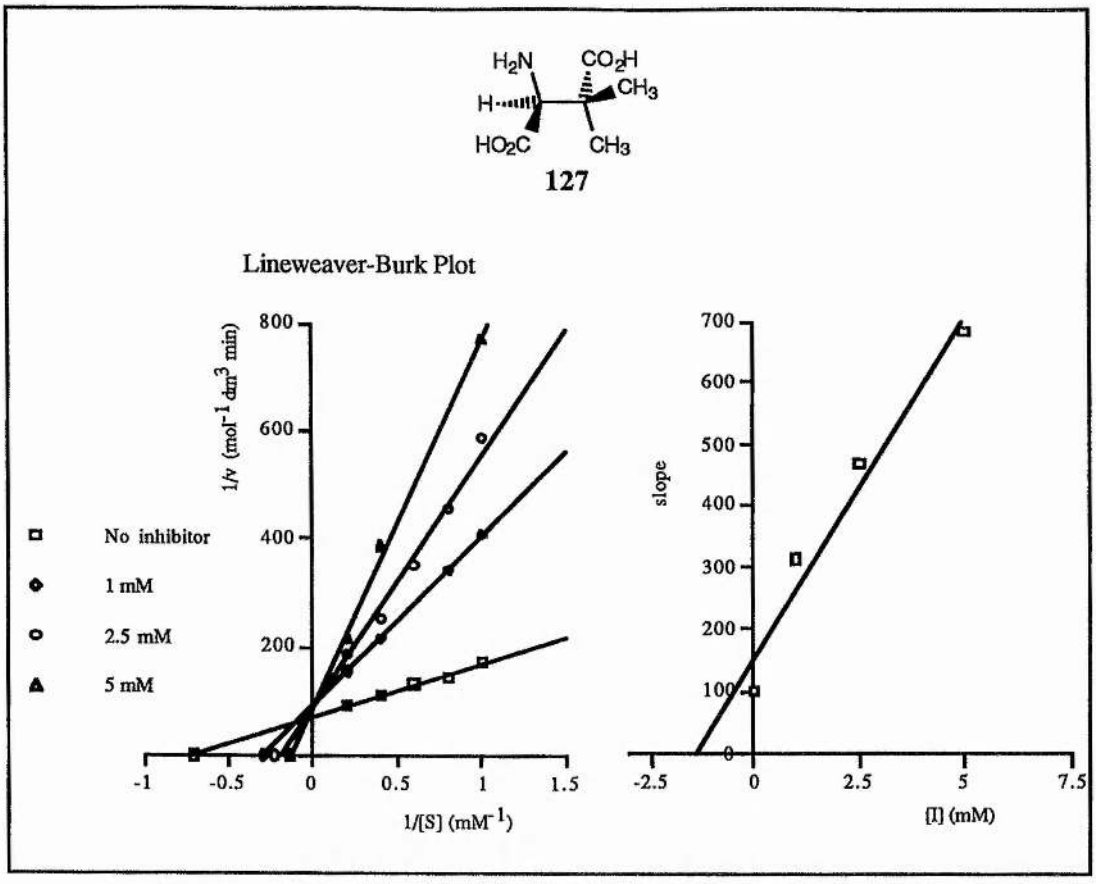
3.3.2.2 (2*S*,3*S*)-3,3-dimethylaspartic acid (123)

Irreversible inhibition by (2*S*,3*S*)-3-methylaspartic (**127**) was investigated by comparing the enzyme activity of incubation containing the compound at 50 mmol dm^{-3} concentration and at pH 9, to incubation containing only the enzyme at pH 9. No loss of activity was observed for up to 5 h after incubation. This suggested that there was no irreversible reaction between the enzyme and the inhibitor.



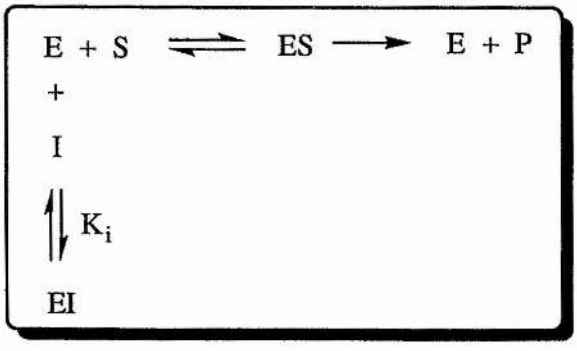
127

Competitive inhibition was studied by measuring the change in absorbance at 240 nm, due to the production of mesaconic acid from (2*S*,3*S*)-3-methyl aspartic acid. All rate measurements were conducted at pH 9 and 30 °C, with incubations containing 500 mmol dm^{-3} Tris-HCl buffer, 20 mmol dm^{-3} magnesium chloride, 1 mmol dm^{-3} potassium chloride and the substrate in the range 1-5 mmol dm^{-3}. Concentration of the inhibitor in the range of 1-5 mmol dm^{-3} were used and each activity assay was carried out in triplicate. The results were analysed using double reciprocal Lineweaver-Burk plots, see Graph 3.



Graph 3: *Plots for (2S)-3,3-dimethyl aspartic acid*

The results show that increasing the concentration of the inhibitor lead to a decrease in the observed rate of the reaction. In this case however K_m was reduced by a factor $(1+ [I]/K_i)$. This is consistent with reversible competitive inhibition as described in Scheme 80. A K_i-value of 1.3 mM was found. This value is comparable with the K_m of the natural substrate of 2.3 mmol dm^{-3}.



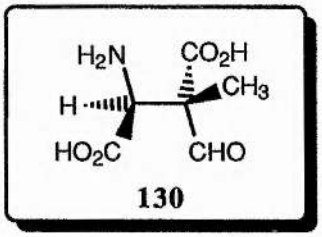
Scheme 80: *Competitive inhibition*

This suggests that exchanging the C-3 proton with a methyl group has no serious effect on the ability of the compound to bind. Therefore, it is evident that the active site has a sufficiently large pocket to accommodate the extra methyl group.

The observation that this molecule does not irreversibly inhibit the enzyme suggests either that the substrate analogue does not add on the postulated dehydroalanine or that this addition is reversible with the equilibrium favouring the unbound species. This result appears to contradict the observation that hydrazine and hydrazine analogues irreversibly inhibit 3-methylaspartase. However it may be that the conjugate addition of hydrazine to the dehydroalanine could result in a concomitant cleavage of the protein backbone as was discussed earlier, see p.48, or some other event that requires cleavage of the N-N bond to cause inactivation.

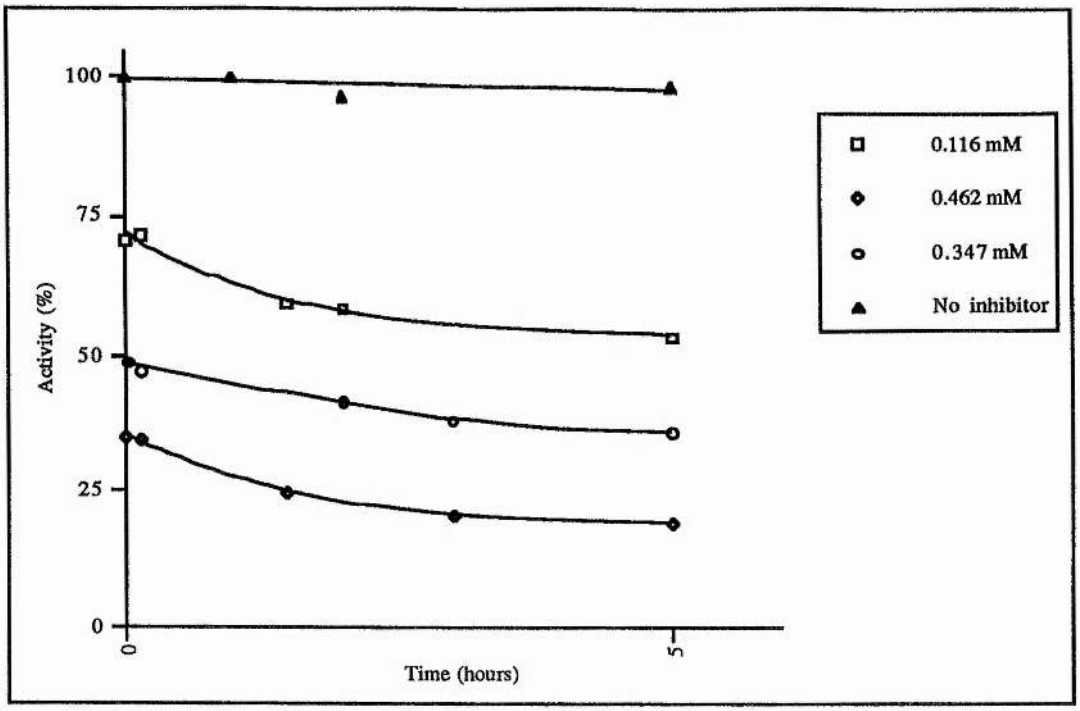
3.3.2.3 (2*S*,3*S*)-3-Formyl-3-methylaspartic acid (130)

Irreversible inhibition was studied by incubating the enzyme with the amino aldehyde (**130**) in the concentration range, 0.110-0.462 mmol dm^{-3} in the presence of 20 mmol dm^{-3} magnesium chloride and 1 mmol dm^{-3} potassium chloride in Tris-HCl buffer at pH 9.



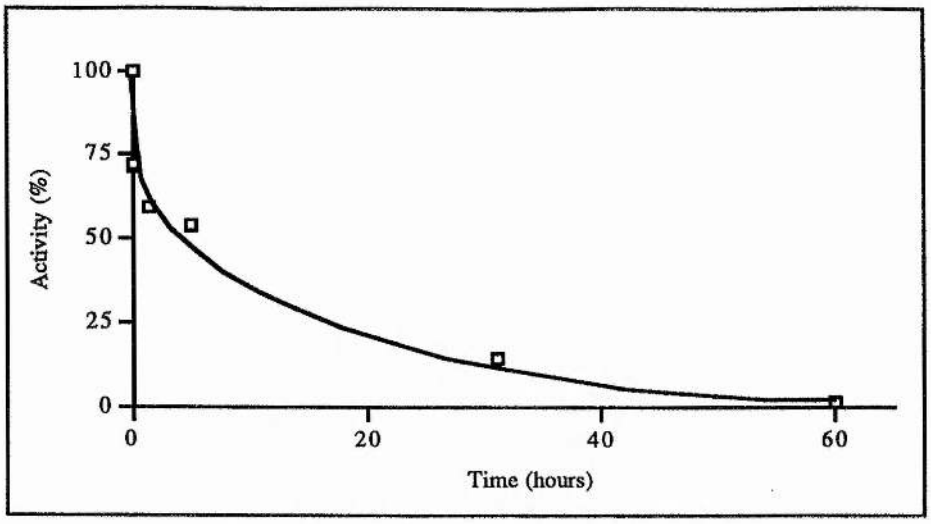
130

Aliquots were removed at regular time intervals and diluted with 1 cm^3 500 mmol dm^{-3} Tris-HCl pH 9 buffer containing 20 mmol dm^{-3} magnesium chloride and 1 mmol dm^{-3} potassium chloride. The activity was determined by measuring the change in absorbance at 240 nm. The percentage of remaining activity was represented as a function of time for the four inhibitor concentrations, see Graph 4.



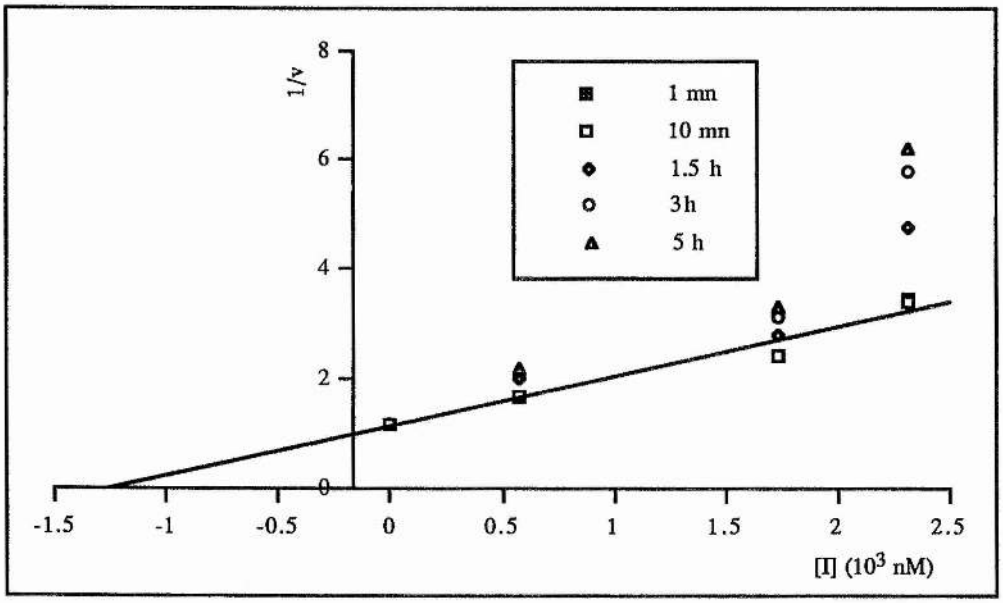
Graph 4: *percentage of remaining activity as a function of time and for different concentrations of inhibitor*

The results showed that the activity of the enzyme was reduced in the presence of the inhibitor. This effect was increased as the concentration of the inhibitor was raised. However, no time dependent inhibition was observed for the first 10 minutes. This was followed by a gradual decrease of activity over 5 h. The biphasic nature of the inhibition suggests that two different processes may be occurring. When the incubation was followed for a period of 60 h almost all of the enzyme activity was lost, compared with the retention of 80% activity in the control experiment (where inhibitor had been omitted), see Graph 5. This result suggests that two distinct events occur. A possible explanation is illustrated in Scheme 81. Initially a reversible inhibition step occurs, followed by a very slow irreversible inactivation.

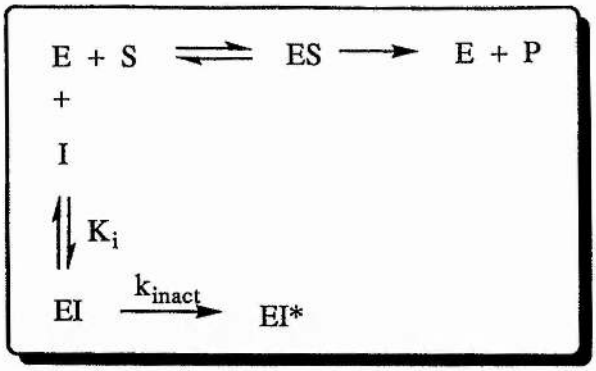


Graph 5: *Enzyme activity over a period of 60 h in the presence of 0.210 mM of aldehyde (**130**)*

The reciprocal of the observed rate, at the various intervals of time, was reported as a function of the concentration of inhibitor, see Graph 6.



Graph 6: *Reciprocal of the observed rate for various time intervals as a function of time*



Scheme 81: ***Proposed Inhibition scheme for(2S,3S)-3- formyl-3-methylaspartic acid (130)***

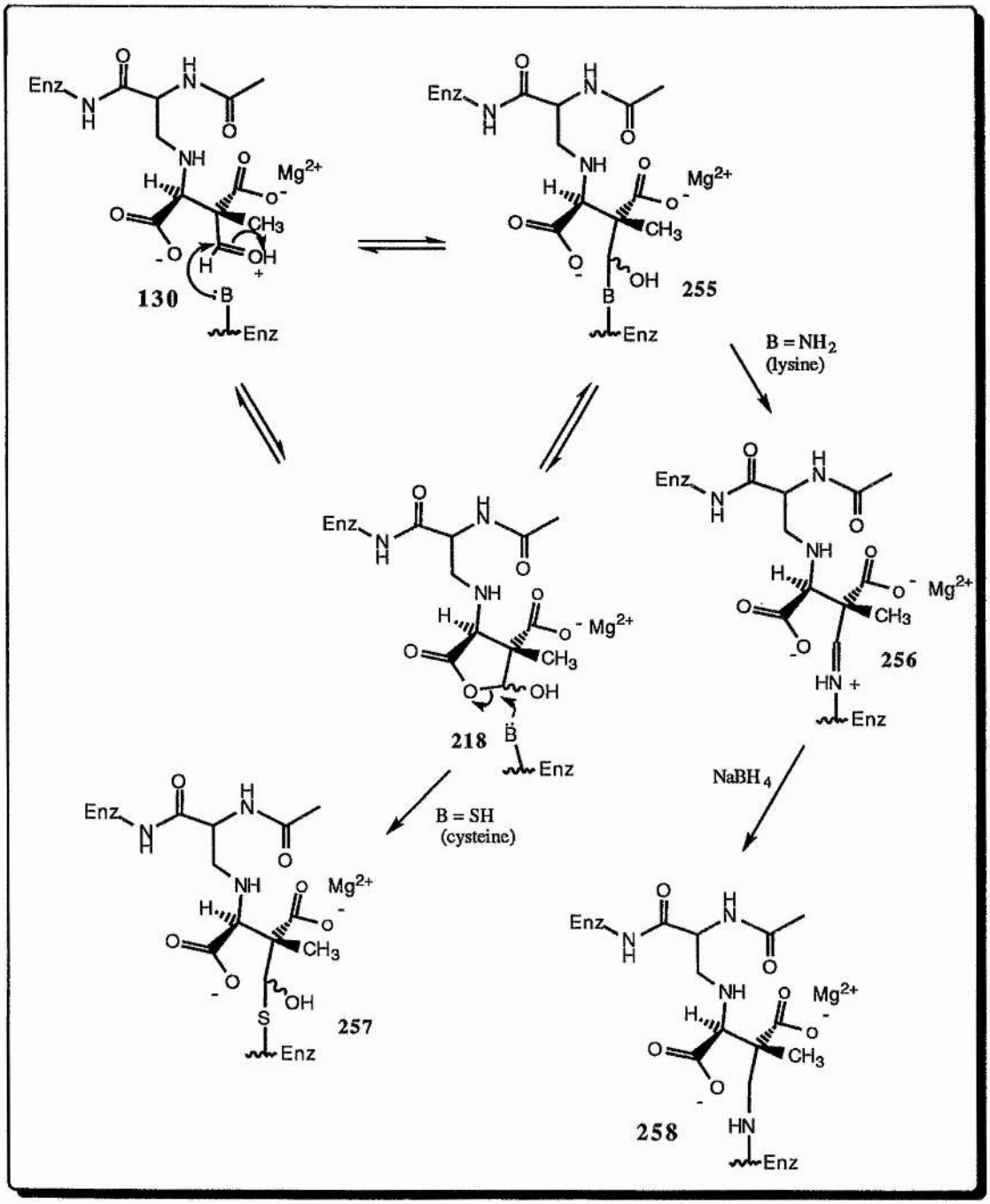
Graph 6 shows that during the first 10 min the linear relationships between the reciprocal of the rate and the concentration of inhibitor is maintained and the inhibition can be considered to be reversible. Hence the inhibition constant K_i can be estimated from a Lineweaver-Burk plot. A K_i-value of 0.6 μmol mol^{-3} was found which is 3.8 x 10^3 times lower than the K_m for the natural substrate.

An interaction of the cyclic hemiketal (**218**) with the enzyme active site can be envisaged to explain the excellent affinity of the enzyme for the molecule. The hemiketal can possibly mimic the transition state of the enzymic reaction due to its rigid structure. This could involve hydrogen bonding interactions between the base responsible for the abstraction of the proton at C-3 of the physiological substrate, see Scheme 82.

As far as the irreversible process is concerned, the catalytic base may react with the hemiketal to form the covalent reversible adduct (**255**). However the kinetic study gives no direct information on the nature of this active site base.

Work is currently underway in order to trap with sodium borohydride the possible intermediate imine (**256**) which would be expected to form if the base was a lysine residue. It is not excluded that a covalent irreversible adduct (**257**) would formed as well in the case that the active site base is a cysteine residue. The reaction of a thiolate group with hemiketal (**218**) would be expected to be occurring at a slower rate than the corresponding reaction with the amino group of a lysine residue. This could explain the

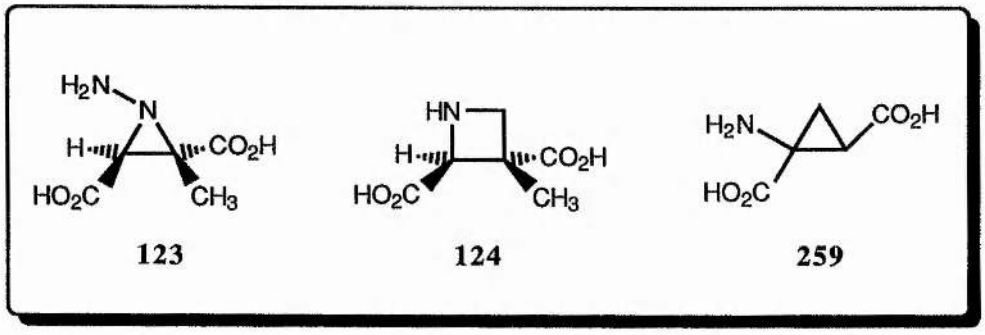
biphasic nature of the inhibition and the observed slow irreversible inactivation of 3-methylaspartase.



Scheme 82: *Proposed inhibtion mechanism for compound (130)*

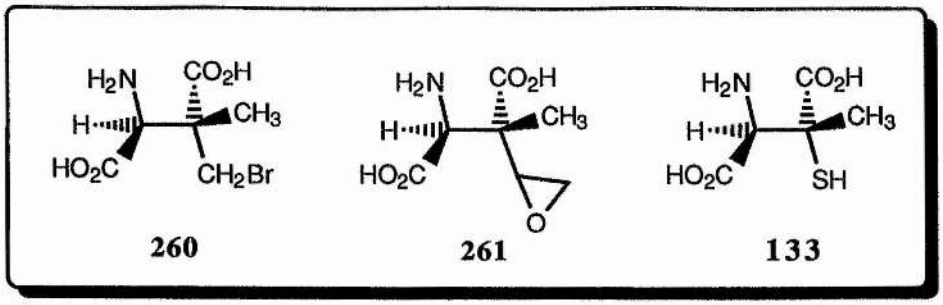
3.4 Conclusions and Future work

The synthesis of the transition state analogues azetidine (**124**) and *N*-aminoaziridine (**123**) showed that these compound were unstable in their unprotected forms. The azetidine is probably the closest structure analogue of the transition state of the β-methylaspartase reaction one can design. As such the instability of this molecule is not much of a surprise. This instability seems related to a general structure which is also present in *N*-amino aziridine (**123**) and aminocyclopropane (**259**) which were shown to be equally unstable. All these molecules contain a β-amino acid motif which is part of a highly strained structure, prone to ring opening.



123 **124** **259**

The work on the synthesis of (2*S*,3*S*)-3-methyl-3-formyl aspartic acid has allowed the development of an asymmetric synthesis for the preparation of β-electrophilic amino acids analogue of the natural substrate for 3-methylaspartate ammonia-lyase. This route gives an access to other molecules of interest as suicide inhibitors, including bromomethylaspartate diester (**260**) and the epoxide (**261**).



(3*S*,2*S*)-3-Formyl-3-methylaspartic acid was found to have a very good affinity for the active site of β-methylaspartase with a K_i-value of 0.56 μM which represents the best inhibitor known for this enzyme. This kind of affinity is expected for a transition state analogue such as 3-methylcyclopropane-1,2-dicarboxylic since the 3-membered ring can mimic the hybridisation state of the C-1 and C-2 in the transition state, expected to be between sp^2 and sp^3. As far the aldehyde (**130**) is concerned another type of interaction must be involved to explain its high affinity for the active site of 3-methylaspartase, may be a combination of the rigid structure of the hemiketal (**218**) and of the interactions between the carbonyl of the aldehyde (or hemiketal) and an active site base. The subsequent irreversible inhibition may possibly be due to the attack of a lysine residue or a cysteine residue which would formed an imine intermediate in the former case. If the active base is a cysteine residue, a relatively stable adduct should be formed causing irreversible inactivation of the enzyme. In order to investigate this possibility, the sulfanyl aminoacid (**133**), where the fully protected form (**202**) has been prepared successfully (see p. 90), and may be a powerful mechanism-based inhibitor. For this to happen, conditions that favour the formation of disulfide bridges must be discovered.

Chapter 4 : Experimental

5.1 Synthesis

5.1.1 Experimental procedure:

Elemental microanalyses were performed in the departmental micro-analytical laboratory. NMR spectra were recorded on a Bruker AM-300 (^{1}H, 300 MHz; ^{13}C 74.76 MHz), or a Varian Gemini 200 (^{1}H, 200 MHz; ^{13}C, 50.31 MHz) spectrometers. Chemical shifts are described in parts per million downfield shift from $SiMe_4$ and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double of doublets, m = multiplet, and br = broad), coupling constant (*J*/Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). ^{1}H-NMR spectra were referenced internally on ^2H_2O ($\delta \supseteq 4.68$), $CHCl_3$ (δ 7.27 ppm) or $(C^2H_3)_2SO$ (δ 2.47). ^{13}C-NMR spectra were referenced on CH_3OH (δ 49.9), C^2HCl_3 (δ 77.5), or $(CH_3)_2SO$ (δ 39.7).

IR spectra were recorded on a Perkin-Elmer 1710 FT IR spectrometer. The samples were prepared as Nujols mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies (υ) as absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE. Major fragments were given as percentages of the base peak intensity (100%). UV spectra were recorded on a Shimadzu UV-210PC spectrophotometer.

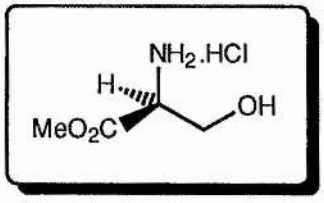
Melting points were taken on an electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at room temperature on a Optical Activity AA-1000 polarimeter using 10 or 20 cm path length cells and are given in 10^{-1} deg cm^2 g^{-1}.

Flash chromatography was performed using Fluka C 60 (40-60 μm mesh) according to the method of Still.[171] Analytical thin layer chromatography (TLC) was carried out on 0.25 mm precoated silica gel plates (Macherey-Nagel SIL g/UV254) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, permanganate, 2,4-dinitrophenylhydrazine or ninhydrin.

The solvents used were either distilled or of Analar quality and petroleum ether refers to that portion boiling between 40 and 60 °C. Solvents were dried according to literature procedures.[172] Ethanol and methanol were dried using magnesium turnings. Propan-2-ol, DMF, toluene, CH_2Cl_2, acetonitrile, triethylamine, *N*-methylmorpholine were dried over CaH_2. THF and diethyl ether were dried over sodium-benzophenone and distilled under nitrogen. Thionyl chloride was distilled over sulphur and the initial fractions were always discarded. BuLi was titrated according to the method of Lipton.[173]

All reactions unless otherwise stated were carried out at room temperature and under inert atmosphere.

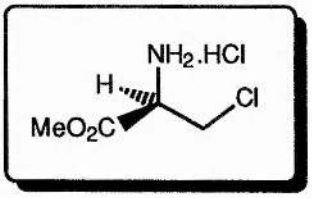
(2*S*)-Serine methyl ester hydrochloride (112)



To a stirred suspension of (2*S*)-serine (6 g, 57 mmol) in dry methanol (100 cm^3) was passed through HCl (generated with H_2SO_4/NaCl) at 0 °C. The resulting solution was stirred overnight and then concentrated to dryness under reduced pressure to give the hydrochloride ester as a white solid which was recrystallised from ethanol (8.6 g, 98%); mp 165 °C [lit.,[106] 167 °C]; $[\alpha]_D$ -2.3 (*c* 1 in H_2O) [lit., $[\alpha]_D$ -3.0 (*c* 1 in H_2O)]; υ_{max} (Nujol)/cm^{-1} 3380 (OH) and 1721 (C=O); δ_H (200 MHz; ^2H_2O) 3.82 (3 H, s, CH_3O),

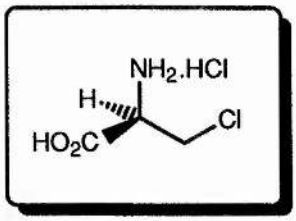
4.10 (2 H, qd, CH_2OH) and 4.25 (1 H, t, CH); δ_C (50.31 MHz; ^2H_2O) 56.5 (CH_3), 57.5 (CH_2), 62.0 (CH) and 172.0 (CO).

(2*S*)-Chloroalanine methyl ester hydrochloride (113)



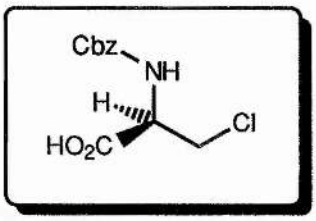
To a stirred solution of (2*S*)-serine methyl ester hydrochloride (**112**) (5 g, 32.3 mmol) in acetyl chloride (100 cm^3) was added dropwise phosphorus pentachloride (7.5 g, 35.84 mmol). The resulting mixture was stirred for 2 h and the precipitated product was then isolated by filtration to give the chloroalanine ester hydrochloride as a white solid. The product was washed with diethyl ether and dried (4.23 g, 76%); a small amount was recrystallised from 2-propanol/methanol (9:1), mp 130 °C (decomp.) [lit.,[107] 134 °C (decomp.)]; $[\alpha]_D$ -9.3 (*c* 1 in H_2O); (Found C, 27.85; H, 4.95; N, 7.9. Calc. for $C_4H_9Cl_2NO_2$ C, 27.6; H, 5.2; N, 8.05); υ_{max} (Nujol)/cm^{-1} 2960 and 2260 (NH_3^+) and 1760 (C=O); δ_H (200 MHz; ^2H_2O) 3.58 (3 H, s, CH_3), 3.85 (2 H, dd, CH_2) and 4.90 (1 H, t, CH); δ_C (50.31 MHz; $C^2H_3O^2H$) 40.7 (CH_3), 51.8 (CH), 52.7 (CH_2) and 165.6 (CO); *m*/z (CI) 138 and 140 (40%, $[(M + H]^+$) and 103 (100, $[(M + H - Cl]^+$).

(2*S*)-Chloroalanine hydrochloride (114)



A solution of (2*S*)-chloroalanine methyl ester (**113**) (2.5 g, 14.4 mmol) in 6 mol dm^{-3} HCl was refluxed for 90 min. The solvent was removed under reduced pressure to give chloroalanine hydrochloride (**113**) as a yellowish solid. This solid was recrystallised from methanol-diethyl ether (1:3) to yield a white crystalline solid (2.7 g, 73%), mp 168 °C [lit.,[107] 172 °C)]; $[\alpha]_D$ -9.4 (*c* 3.5 in H_2O); υ_{max} (Nujol)/ cm^{-1} 2820 (OH and NH) and 1750 (C=O); δ_H (200 MHz; ^2H_2O) 4.05 (2 H, dd, CH_2) and 4.46 (1 H, t, CH); δ_C (50 MHz; ^2H_2O) 44.8 (CH_2), 56.7 (CH) and 171.80 (CO); *m/z* (CI) 124 (100%, $[M + H - Cl]^+$), 88 and 86 (28, $[M + H - Cl]^+$).

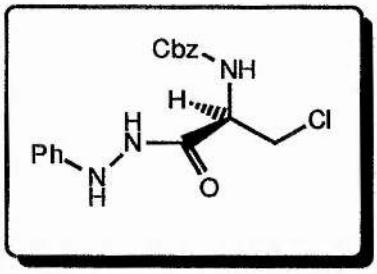
(2*S*)-*N*-Benzyloxycarbonyl-3-chloroalanine (115)



To a stirred solution of $NaHCO_3$ (80 cm^3, 2 mol dm^{-3}) and (2*S*)-3-chloroalanine (**114**) (5 g, 31.3 mmol) was added benzylchloroformate (4.9 cm^3, 34.4 mmol) in diethyl ether (25 cm^3). The resulting mixture was stirred vigorously for 4 h. The solution was then washed with diethyl ether (100 cm^3) and acidified to pH 1. The aqueous solution was then extracted with diethyl ether (3 x 50 cm^3), dried ($MgSO_4$) and the solvent was removed under reduced pressure to give *N*-benzyloxycarbonyl-3-chloroalanine as a white solid (6

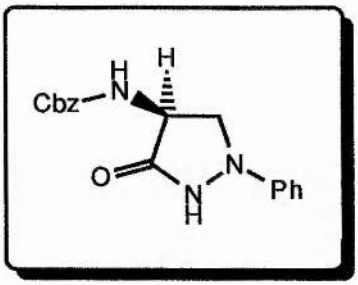
g, 74%), mp 122-3 °C [lit.,[106] 120 °C]; $[\alpha]_D$ +7.3 (*c* 1 in MeOH); υ_{max} (Nujol)/cm^{-1} 3400 (OH), 3000 (NH_3^+), 1740 (C=O ester) and 1650 (C=O amide); δ_H (200 MHz; C^2HCl_3) 3.85 (2 H, dd, C*H*$_2$Cl), 4.84 (1 H, m, C*H*NH), 5.15 (2 H, s, CH_2), 5.83 (1 H, d, NH) and 7.40 (5 H, s, Ar-H); δ_C (C^2HCl_3; 50.31 MHz) 55.1 (CH_2Cl), 66.5 (CH_2N), 68.1 (CH_2Ph), 128.7, 128.9 and 129.1 (Ar-CH), 136.2 (Ar-C quaternary), 156.5 (CO carbamate) and 172.9 (CO acid); *m/z* (EI) 258 (42%, [M - Cl]$^+$) and 91 (100, $C_7H_7^+$).

(2*S*)-*N*-Benzyloxycarbonyl-3-chloroalanine phenylhydrazide (116)



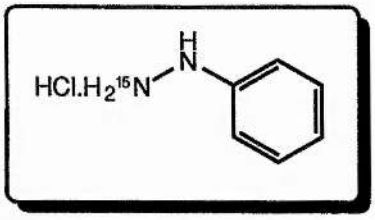
To a stirred solution of (2*S*)-*N*-benzyloxycarbonyl-2-chloroalanine (**115**) (4 g, 17 mmol) in dry THF was added 4-*N*-methylmorphiline (1.87 cm^3, 17 mmol) and isobutylchloroformate (2.18 cm^3, 17 mmol) at -10 °C. Phenylhydrazine (1.78 cm^3, 17 mmol) was added slowly after 5 min and the reaction mixture was stirred for 3 h. The solution was then filtered and the solvent was removed under reduced pressure to give a pale yellow solid which was recrystallised from acetone/ether to yield an off-white solid (0.11 g, 85%), mp 67-68 °C, (Found C, 58.95; H, 5.3; N, 12.1. $C_{17}H_{18}ClN_3O_3$ requires C, 58.7; H, 5.2; N, 12.1); $[\alpha]_D$ -36.7 (*c* 2.5 in MeOH); υ_{max} (Nujol)/cm^{-1} 3270 (NH), 2780 (CH), 2360, 1692 and 1654 (C=O amide); δ_H (300 MHz; d_6-DMSO) 3.80 (2 H, d, *J* 6.5, CH_2), 4.45 (1 H, t, *J* 6.5, C*H*CH$_2$Cl), 5.12 (2 H, s, C*H*$_2$Ph), 6.93 (5 H, m, Ar-H), 7.28 (5 H, m, Ar-H), 7.75 (1 H, d, *J* 12.3, N*H*CH) and 10.05 (1 H, s, N*H*NPh); δ_C (50.31 MHz; d_6-DMSO) 44.5 (*C*H$_2$Ph), 55.3 (CH_2Cl), 66.3 (CH), 112.9, 116.2, 119.5, 128.3, 128.5, 129.0, 129.3 and 129.6 (Ar-CH), 137.3 (Ar-C quaternary), 149.2 (CO amide), 156.5 (C-*ipso*) and 168.1 (CO ester); *m/z* (EI), 311 (30%, [M - Cl]$^+$), 220 (30) and 91 (100, $C_7H_7^+$).

(4*S*)-4-[(Benzyloxycarbonyl)amino]-1-phenylpyrazolidin-3-one (104)



A solution of the hydrazide (**116**) (0.4 g, 1.2 mmol) in acetonitrile was refluxed for 3 h. The solvent was then removed under reduced pressure to yield a pale yellow solid. This solid was purified by silica gel chromatography using ethyl acetate-petroleum ether (6:4) as the eluant to give a white solid (0.24 g, 67%), mp 108-110 °C; (HRMS: found $[M + H]^+$ 311.1277. Requires for $C_{17}H_{17}N_3O_3$ 311.1270), (Found C, 65.5; H, 5.65; N, 13.35. $C_{17}H_{17}N_3O_3$ requires C, 65.5; H, 5.5; N, 13.5); $[\alpha]_D$ -155.8 (*c* 1.4 in DMSO); υ_{max} (Nujol)/cm^{-1} 3340 (NH), 2923 and 2854 (Ar-CH), 1694 (large, C=O), 1597, 1518, 1458, 1241, 1187, 1140, 1086, 1032, 1006, 980 and 919; δ_H (300 MHz; d_6-DMSO) 3.58 (1 H, t, *J* 12.3, CH), 4.18 (2 H, dd, *J* 9.5, 12.3, $CH_\alpha H_\beta$), 4.40 (1 H, dd, *J* 9.5, 12.3, $CH_\alpha H_\beta$), 5.05 (2 H, s, CH_2Ph), 6.98 (3 H, m, Ar-H), 7.38 (7 H, m, Ar-H), 7.70 (1 H, d, *J* 10, NH carbamate) and 10.45 (1 H, s, NH amide); δ_C (50.31 MHz; C^2HCl_3) 50.6 (CH_2Ar), 58.3 (CH_2), 66.2 (CHNH), 116.1, 122.1, 129.0 and 129.5 (Ar-CH), 136.0 (Ar-C quaternary), 150.6 (CO carbamate), 156.3 (Ar-C quaternary) and 172.8 (CO); *m/z* (CI), 312 (100%, $[M + H]^+$) and 176 (30, $[M + H - PhN_2CO]^+$).

[^{15}N]-Phenylhydrazine hydrochloride (117)

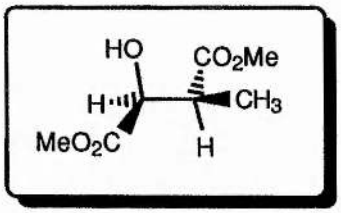


Freshly distilled aniline (0.33 cm^3) was added to an ice cooled solution of HCl (1 mol dm^{-3}). [^{15}N]-Sodium nitrite (0.25 g, 5.3 mmol) was then added in portions. A freshly prepared sodium sulfite solution (by bubbling sulphur dioxide through a sodium hydroxide solution [3.3 mol dm^{-3}, 6 cm^3] in the presence of phenylphthalein indicator until the solution became colourless [pH 8-10]) was added dropwise while the mixture was maintained at 0 °C by adding crushed ice (the solution turned orange). The resulting solution was heated up to 60-70 °C for 1 h and was then acidified with a concentrated HCl solution (1 mol dm^{-3}, 10 cm^3). The solution was then refluxed for a further 4 h and then reduced in volume under reduced pressure until precipitation of phenylhydrazine hydrochloride occurred. The precipitation of the product was completed by cooling the mixture in an ice bath. The product was filtered-off the solution and was recrystallised from ethanol to give a pale pink solid (0.3 g, 48.5%), mp 252 °C [lit.,[109] 250-254 °C]; (HRMS: found $[M + H]^+$, 110.0740. Calc. for $C_6H_9N^{15}N$: 110.0736); *m/z* (CI) 110 (100%, $[M + H]^+$).

(4*S*)-*N*-4-(Benzyloxycarbonyl)amino-1-phenyl-2-[^{15}N]-pyrazolidin-3-one (104, labelled)

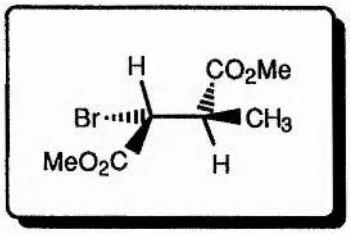
To a stirred solution of (2*S*)-*N*-benzyloxycarbonyl-2-chloroalanine (**115**) (0.43 g, 1.78 mmol) in dry THF was added 4-*N*-methylmorphiline (0.231 cm^3, 17 mmol) and isobutylchloroformate (0.270 cm^3, 17 mmol) at -10° C. ^{15}*N*-Phenylhydrazine hydrochloride (1.78 cm^3, 17 mmol) in DMF (2 cm^3) was slowly added after 5 min. The reaction mixture was then stirred at room temperature (22 °C) for 3 h. The solvent was removed under reduced pressure and the residue obtained was dissolved in ethyl acetate. The organic solution was washed with water (10 x 20 cm^3), brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a yellow solid which was dissolved in acetonitrile. The resulting solution was refluxed for 3 h and the solvent was removed under reduced pressure to yield the [^{15}N]-pyrazolidinone (**104**, labelled) as a pale yellow solid which was purified by silica gel chromatography as for (**104**) (0.28 g, 51%), mp 108-110 °C. All other spectroscopic data were identical to that for the non-labelled compound, excepted δ_H (300 MHz; d_6-DMSO) 10.45 (1 H, d, $J_{N\text{-}H}$ 98 Hz, ^{15}NH); *m/z* (CI) 313 (100%, $[M + H]^+$), 176 (36, $[M + H - PhN_2CO]^+$.

Dimethyl (2*S*,3*S*)-2-hydroxy-3-methylsuccinate (140)



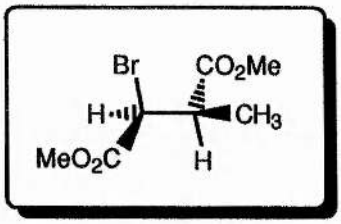
To an ice-cooled stirred solution of (2*S*,3*S*)-3-methylaspartic acid (**53**) (9.8 g, 66.7 mmol) and sulphuric acid (2 mol dm^{-3}, 100 cm^3) was added slowly a solution of sodium nitrite (13.8 g, 0.2 mol, 10 cm^3). The mixture was stirred overnight and the solvent removed under reduced pressure (30 °C) to obtain a yellow oil which was dissolved in methanol (100 cm^3). The resulting solution was refluxed for 2 h in the presence of concentrated sulphuric acid (2 cm^3). The methanol was removed under reduced pressure to give a yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (3:7) as the eluant to yield the hydroxy dimethyl ester as a colourless oil (3.5 g, 30%), (HRMS: found $[M + H]^+$ 177.0760. Requires for $C_7H_{13}O_5$: 177.0763); $[\alpha]_D$ +9.3 (*c* 17.7 in MeOH); υ_{max} (thin film)/cm^{-1} 3400 (OH) and 1745 (CO); δ_H (200 MHz; C^2HCl_3) 1.12 (3 H, d, *J* 9, CH_3), 2.90 (1 H, qd, *J* 4, 9, C*H*CH_3), 3.18 (1 H, d, *J* 5, CHO*H*), 3.69 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3) and 4.60 (1 H, dd, *J* 4, 5, C*H*OH); δ_C (50.31 MHz; C^2HCl_3) 11.1 (CH_3), 43.4 (CH*C*H_3), 52.0 and 53.3 (OCH_3), 71.0 (CHOH) and 174.0 and 174.1 (CO esters); *m/z* (CI) 177 (100%, $[M + H]^+$).

Dimethyl (2*R*,3*R*)-2-bromo-3-methylsuccinate (137)



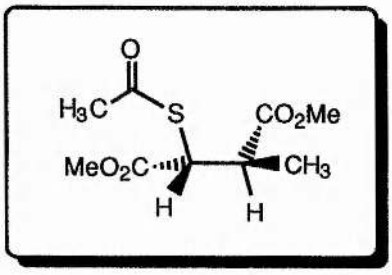
To an ice-cooled stirred solution of the hydroxy diester (**140**) (2.5 g, 14 mmol) in DCM (100 cm^3) was added triphenylphosphine (5.1 g, 19.6 mmol) and carbon tetrabromide (6.5 g, 19.6 mmol). The mixture was stirred for 2 h at 0-5 °C and 1 h at room temperature. The solvent was then removed under reduced pressure to yield a yellow oil which was dissolved in dichloromethane (30 cm^3). Addition of diethyl ether (80 cm^3) to this solution resulted in the precipitation of triphenyl oxide which was filtered-off. The filtrate was concentrated under reduced pressure to yield a 9:1 mixture of the (2*R*,3*R*) and (2*S*,3*R*) bromoesters. Pure (2*R*,3*R*) (**137**) was obtained as a colourless oil after silica gel chromatography using ethyl acetate-petroleum ether (2:8) as the eluant (2.2 g, 66%), $[\alpha]_D$ +35.3 (*c* 23.8 in MeOH), (HRMS: found $[M + H]^+$ 238.9910. Requires $C_7H_{12}O_4{}^{79}Br$: 238.9920); υ_{max} (neat)/cm^{-1} 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.22 (3 H, d, *J* 7, CH_3), 3.15 (1 H, qd, *J* 7, 10, C*H*CH_3), 3.70 (3 H, s, OCH_3), 3.75 (3 H, s, OCH_3) and 4.42 (1 H, d, *J* 10, CHBr); δ_C (50.31 MHz; C^2HCl_3) 15.8 (CH_3), 45.0 (*C*HCH_3), 45.9 (CHBr), 52.9 and 53.5 (OCH_3) and 169.0 and 173.2 (CO esters) ; *m/z* (CI) 239 and 241 (95, 85%, bromine isotopes, $[M + H]^+$) and 207 and 209 (100, bromine isotopes, $[M + H - OCH_3]^+$).

Dimethyl (2*S*,3*R*)-2-bromo-3-methylsuccinate (138)



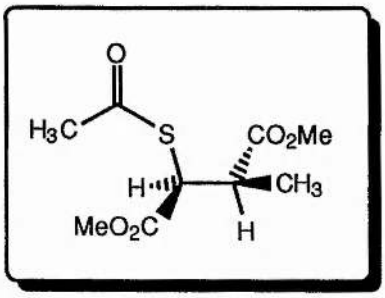
To an ice-cooled stirred saturated solution of potassium bromide (50 cm^3) and (2*S*,3*S*)-3-methylaspartic acid (**53**) (1.73 g, 11.8 mmol) was added sodium nitrite (1.62 g, 23.6 mmol) over a period of 45 min. The mixture was stirred at room temperature (22 °C) overnight and the resulting solution extracted with diethyl ether (2 x 40 cm^3). The combined organic extracts were dried ($MgSO_4$) and the solvent removed under reduced pressure to give a white solid (2.16 g, 82%) which was dissolved in methanol (50 cm^3) containing concentrated sulphuric acid (2 cm^3). This solution was refluxed for 1 h and then concentrated under reduced pressure. The resulting residue was dissolved in ethyl acetate and washed with NaOH (1 mol dm^{-3}) and brine. The combined organic layers were dried ($MgSO_4$) and the solvent removed under reduced pressure to give a pale yellow oil (2.7 g, 95%); $[\alpha]_D$ -46.9 (*c* 1 in MeOH); (HRMS: found $[M + H]^+$ 238.9910. Requires $C_7H_{12}O_4{}^{79}Br$: 238.9920); δ_H (200 MHz; C^2HCl_3) 1.38 (3 H, d, *J* 6, CH_3), 3.12 (1 H, qd, *J* 6, 9, $CHCH_3$), 3.68 (3 H, s, OCH_3), 3.78 (3 H, s, OH_3) and 4.45 (1 H, d, *J* 9, CHBr); δ_C (50.31 MHz; C^2HCl_3) 15.8 (CH_3), 43.7 ($CHCH_3$), 47.3 (CHBr), 52.8 and 53.6 (methyl esters) and 170.2 and 173.8 (CO esters). All other data were found to be identical to that for compound (**137**).

Dimethyl (2*R*,3*R*)-2-thioacetoxy-3-methylsuccinate (138)



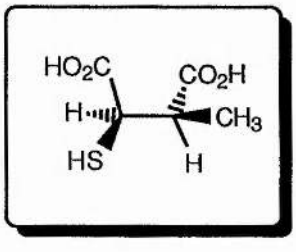
To an ice-cooled stirred solution of the bromodimethyl ester (**136**) (2 g, 8.4 mmol) in acetone (25 cm^3) was added potassium thioacetate (1 g, 8.4 mmol). The mixture was stirred for 3 h at 0 °C and then poured into water (50 cm^3). The aqueous mixture was extracted with ethyl acetate (3 x 25 cm^3) and the combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow oil. The compound was purified by silica gel chromatography using ethyl acetate-petroleum ether (2:8) as the eluant to give a colourless oil (1.7 g, 87%), (HRMS: found $[M + H]^+$ 235.0645. Requires for $C_9H_{14}O_5S$: 235.0640); $[\alpha]_D$ -129.2 (*c* 23.4 in DCM); υ_{max} (thin film)/cm^{-1} 1739 and 1701 (CO); δ_H (200 MHz; C^2HCl_3), 1.20 (3 H, d, *J* 7.5, CH_3), 2.35 (3 H, s, CH_3COS), 2.98 (1 H, qd, *J* 7.5, 9, $CHCH_3$), 3.64 (3 H, s, OCH_3), 3.66 (3 H, s, OCH_3) and 4.52 (1 H, d, *J* 9, CHSAc); δ_C (50.31 MHz; C^2HCl_3) 15.0 (CH_3), 30.8 (CH_3COS), 41.4 (CH), 47.0 (CHS), 52.8 and 53.3 (methyl esters), 171.5 and 174.4 (CO ester) and 183.0 (COS); *m/z* (CI), 235 (100%, $[M + H]^+$) and 203 (75, $[M + H - OCH_3]^+$).

Dimethyl (2*S*,3*R*)-2-thioacetoxy-3-methylsuccinate (143)



This compound was prepared from bromide (**137**) (1.55 g, 6.5 mmol) in a manner identical to that for (2*R*,3*R*)-2-thioacetoxy-3-methyl-succinate (**138**) (1.75g, 87%); (Found C, 46.15; H, 6.25. $C_9H_{14}O_5S$ requires C, 46.15 ; H, 6.0); $[\alpha]_D$ + 75.2 (*c* 23.3 in MeOH); υ_{max} (thin film)/cm^{-1} 1730 and 1705 (CO); δ_H (200 MHz; C^2HCl_3), 1.22 (3 H, d, *J* 9, CH_3), 2.39 (3 H, s, CH_3COS), 3.24 (1 H, qd, *J* 4.5, 9, C*H*CH_3), 3.68 (3 H, s, OCH_3), 3.72 (3 H, s, OCH_3) and 4.54 (1 H, d, *J* 4.5, CHSAc); δ_C (50.31 MHz; C^2HCl_3) 14.9 (CH_3), 30.4 (*C*H_3COS), 41.2 (CH), 47.9 (CHS), 52.4 and 53.2 (CO_2CH_3), 170.8 and 173.9 (CO) and 193.9 (COS); *m/z* (CI) 235 (100%, $[M + H]^+$), and 203 (75, $[M + H - OCH_3]^+$).

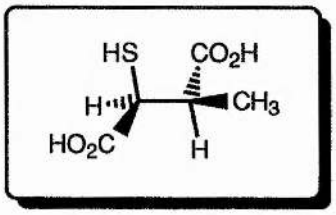
(2*S*,3*R*)-3-Methyl-2-sulfanylsuccinic acid (129a)



The thioacetoxy ester (**139**) (1 g, 4.3 mmol) was refluxed for 30 min in 6 mol dm^{-3} HCl (10 cm^3). The sulfanyldioic acid crystallised upon cooling at room temperature as white

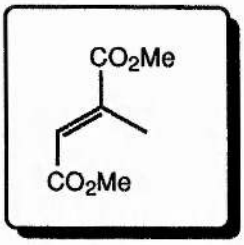
crystals which were collected by filtration (0.4 g, 57%), mp 190-195 °C; $[\alpha]_D$ +40.9 (*c* 0.17 in MeOH); (HRMS: found $[M + H]^+$ 165.0220. Requires for $C_5H_8O_4S$: 165.0220; υ_{max} (Nujol)/cm^{-1} 2900 (SH) and 1700 (CO); δ_H (200 MHz; $C^2H_3O^2H$) 1.25 (1 H, d, *J* 7.5, CH_3), 2.85 (1 H, qd, *J* 7, 10, C*H*CH_3), 3.48 (1 H, dd, *J* 7.5, 10, C*H*SH) and 3.72 (1 H, d, *J* 7.5, SH); δ_C (50 MHz; C^2HCl_3) 18.2 (CH_3), 47.4 (*C*HCH_3), 47.5 (CHSH) and 152.5 and 153.0 (CO acids); *m/z* (CI), 165 (100%, $[M + H]^+$), 147 (55, $[M + H - OH]^+$), 133 (13, $[M + H- SH]^+$) and 113 (85, $[M - OH - SH]^+$).

(2*R*,3*R*)-2-Sulfanyl-3-methylsuccinic acid (129b)



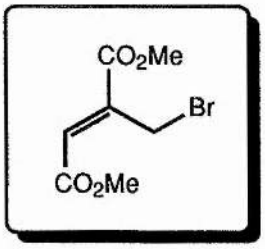
This compound was prepared from compound (**143**) (0.3 g, 1.3 mmol) in a manner identical to that for (2*S*,3*R*)-2-sulfanyl-3-methylsuccinic acid (**129a**). The desired compound (**129b**) was obtained as a white powder (0.126 g, 60%), mp 195-200 °C, (HRMS: found $[M + H]^+$ 165.0215. Requires for $C_5H_8O_4S$: 165.0220); $[\alpha]_D$ +37.0 (*c* 0.88 in MeOH); υ_{max} (Nujol)/cm^{-1} 3500-2500 (very large, OH acid + SH), 2922, 2652, 1700 (large, CO), 1461, 1422 (small), 1378, 1292, 1192, 1060 and 938; δ_H (200 MHz; $C^2H_3O^2H$) 1.35 (1 H, d, *J* 7, CH_3), 2.82 (1 H, qd, *J* 7, 10, C*H*CH_3), 3.50 (1 H, d, *J* 10, C*H*SH) and 3.70 (1 H, d, *J* 7.5, SH); δ_C (50.31 MHz; $C^2H_3O^2H$) 15.4 (CH_3), 44.4 (*C*HCH_3), 45.3 (CHSH) and 175.5 and 177.6 (CO_2H); *m/z* (CI) 165 (100%, $[M + H]^+$), 147 (55, $[M + H - OH]^+$), 133 (13, $[M + H - SH]^+$) and 113 (85, $[M - OH - SH]^+$).

Dimethyl mesaconate (153)



To a stirred solution of mesaconic acid (10 g, 76.9 mmol) in methanol (100 cm^3) was added dropwise thionyl chloride (11.2 cm^3, 154 mmol) at 0 °C. The resulting solution was refluxed for 2 h and the methanol was removed under reduced pressure. The residue obtained was dissolved in ethyl acetate (50 cm^3) and the resulting solution washed with sodium hydroxide (20 cm^3, 1 mol dm^{-3}) and brine. The organic phase was then dried ($MgSO_4$) and ethyl acetate was removed under reduced pressure to give a colourless oil (11.2 g, 92%); δ_H (200 MHz; C^2HCl_3) 2.25 (3 H, s, CH_3), 3.70 (3 H, s, CO_2CH_3), 3.8 (3 H, s, CO_2CH_3) and 6.7 (1 H, s, CH); δ_C (50.31 MHz; C^2HCl_3) 14.5 (CH_3), 51.8 and 52.7 (CO_2CH_3), 126.6 (quaternary), 114.1 (CH), 164.4 and 167.7 (CO); *m/z* (CI) 161 (100%, $[M + H]^+$).

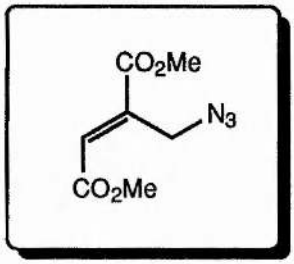
Dimethyl *cis*-2-bromomethyl-2-ene-1,4-butanedioate (154)



To a stirred solution of dimethyl mesaconate (**153**) (10 g, 63 mmol) in carbon tetrachloride (25 cm^3) was added *N*-bromosuccinimide (11.25 g, 63.2 mmol) and benzoyl

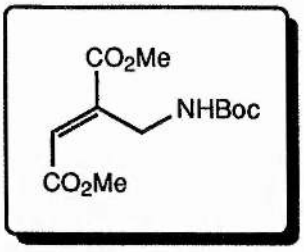
peroxide (0.550 g). The mixture was refluxed gently for 2 h while exposed to an incandescent light (1000 watts). The resulting brown-orange solution was washed with water (3 x 40 cm^3) and the organic layer dried ($MgSO_4$). Carbon tetrachloride was removed under reduced pressure to give a yellow oil (12.6 g, 84%); υ_{max} (thin film)/cm^{-1} 2955, 1731 (large, CO), 1643 (small, C=C), 1435, 1371, 1286 (CH_2Br); δ_H (200 MHz; C^2HCl_3) 2.17 (3 H, s, CH_3), 3.82 (3 H, s, CO_2CH_3), 3.87 (3 H, s, CO_2CH_3), 4.71 (2 H, s, CH_2) 6.81 (1 H, s, CH); δ_C (50.31 MHz; C^2HCl_3) 22.8 (CH_3), 62.2 (CO_2CH_3), 63.6 (CO_2CH_3), 128.8 (CH_2) and 165.5 and 165.6 (CO).

Dimethyl *trans*-2-azidomethyl-2,3-butanedioate (152)



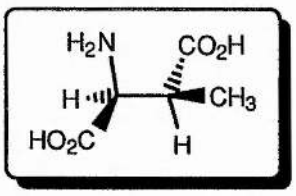
To an ice-cooled stirred solution of the vinyl bromide (**154**) (2 g, 8.4 mmol) in DMF (50 cm^3) was added sodium azide (0.6 g, 9.24 mmol). The yellow mixture was stirred for 30 min at 0 °C and water (100 cm^3) was added. The resulting solution was extracted with ethyl acetate (2 x 50 cm^3) and the combined organic layers were washed successively with water (10 x 15 cm^3), brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a yellowish oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (25:75) as the eluant to afford the azido alkene (**152**) as a colourless oil (1.4 g, 84%); υ_{max} (thin film)/cm^{-1} 2955, 2097 (N_3), 1727 (C=O), 1649 (C=C), 1434, 1249, 1078, 903 and 791; δ_H (200 MHz; C^2HCl_3) 3.80 (3 H, s, CO_2CH_3), 3.85 (3 H, s, CO_2CH_3), 4.50 (2 H, s, CH_2N_3) and 6.98 (1 H, s, CH); δ_C (50.31 MHz; C^2HCl_3) 45.9 (CH_2N_3), 52.8 and 53.4 (OCH_3), 130.7 (=CH), 140.4 (quaternary) and 165.6 and 166.4 (CO).

Dimethyl 2-*N*-(*tert*-butyloxocarbonyl)aminomethylbutan-2-ene-dioate (158)



To a stirred solution of the azide (**152**) (1 g, 5 mmol) in methanol was added Boc_2O (2.18 g, 10 mmol) and 10% Pd/C (0.1 g). The mixture was stirred 4 h under 1 atm of H_2 and was filtered through Celite. Methanol was removed under reduced pressure to yield a colourless oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (3:7) as the eluant to yield pure (**158**) (1.16 g, 85%); (HRMS: found $[M + H]^+$ 274.1300. Requires for $C_{12}H_{20}NO_6$: 274.1290); υ_{max} (thin film) 3403 (NH), 2994, 1731 (CO), 1649 (C=C), 1512, 1103, 806; δ_H (200 MHz; C^2HCl_3) 1.34 (9 H, s, *t*-Bu), 3.72 (3 H, s, CO_2CH_3), 3.75 (3 H, s, CO_2CH_3), 4.32 (2 H, d, *J* 10, CH_2), 5.2 (1 H, broad, NH) and 6.74 (1 H, s, CH=C); δ_C (50.31 MHz; C^2HCl_3) 28.7 (*t*-Bu), 37.8 (CH_2), 52.5 (CO_2CH_3), 53.1 (CO_2CH_3), 79.8 (quaternary, *t*-Bu), 128.9 (*C*H=C), 144.0 (CH=*C*), 155.8 (NCO) and 166.1 and 166.9 (CO); *m/z* (CI) 274 (80%, $[M + H]^+$), 218 (100, $[M + H - t\text{-Bu}]^+$) and 174 (70, $[M - CO_2\ t\text{-Bu}]^+$).

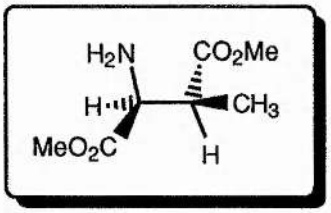
(2*S*,3*S*)-3-Methylaspartic acid (53)



To a stirred suspension of mesaconic acid (13 g, 0.1 mol) in water (20 cm^3) was added a concentrated ammonia solution until the pH of the solution was 9. The solvent was

removed under reduced pressure and the residue dissolved in water (150 cm^3) containing $MgCl_2$ $6H_2O$ (0.2 g), KCl (0.05 g) and β-methylaspartase (200 units). The resulting solution was left to stand for 4 days at 30°C and then heated up to 100 °C for 5 min and filtered. Adjustment of the pH of the filtrate to 3 resulted in the precipitation of 3-methylaspartic acid (**53**) which was collected by filtration and recrystallised from H_2O to give white crystals (9-10 g, 64-71%), mp 272-275 °C [lit.,[174] 276-278 °C]; $[\alpha]_D$ - 8.4 (*c* 1 in H_2O), [lit.,[175] $[\alpha]_D$ -10.0 (*c* 0.42 in H_2O)]; (Found C, 40.6; H, 6.2; N, 9.45. $C_5H_9NO_4$ requires C, 40.8; H, 6.15; N, 9.5); υ_{max} (Nujol)/cm^{-1} 1680 (CO); δ_H (200 MHz; $^2H_2O/NaO^2H$) 1.12 (3 H, *J* 7.5, CH_3), 3.10 (1 H, m, C*H*CH_3) and 4.10 (1 H, d, *J* 3.0, C*H*NH_2); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 12.2 (CH_3), 40.6 (CH), 55.9 (CH) and 171.0 and 175.7 (CO); *m/z* (CI) 148 (100%, $[M + H]^+$).

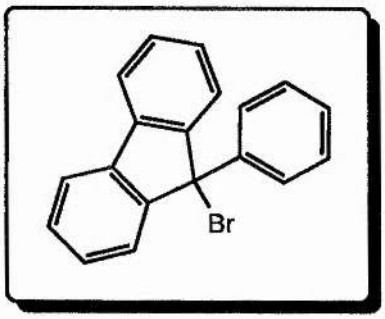
Dimethyl (2*S*,3*S*)-3-methylaspartate (187)



To a suspension of (2*S*,3*S*)-3-methylaspartic acid (**53**) (6 g, 40.8 mmol) in dry methanol (200 cm^3) was added dropwise thionyl chloride (11.9 cm^3, 163.2 mmol) at 0 °C. The solution was stirred until complete dissolution of the amino acid and refluxed for 5 h. The solvent was removed under reduced pressure to afford a colourless oil which was dissolved in cold water (100 cm^3). The pH of the solution was adjusted to 9 with a cold K_2CO_3 solution (2 mol dm^{-3}) and extracted with ethyl acetate (8 x 40 cm^3). The combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to yield a pale yellow oil (5.5 g, 77%), $[\alpha]_D$ +24.9 (*c* 17.5 in DCM); υ_{max} (thin film)/cm^{-1} 3392 (NH_2), 2955 (CH), 1736 (C=O), 1438, 1217, 996 and 856; δ_H (200 MHz; C^2HCl_3) 1.03 (3 H, s, CH_3), 1.06 (3 H, s, CH_3), 1.17 (2 H, s (broad), NH_2), 2.84 (1 H, qd, C*H*CH_3), 3.61 (3 H, s, CO_2CH_3), 3.64 (3 H, s,

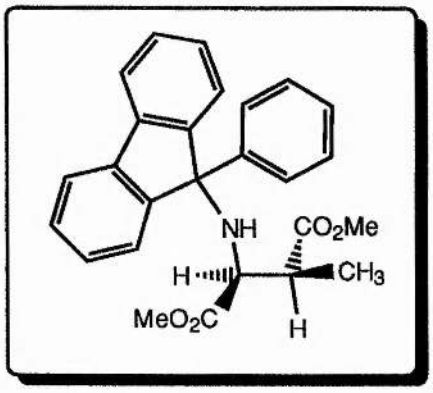
CO_2CH_3) and 3.83 (1 H, d, CH); δ_C (50 MHz; C^2HCl_3) 43.1 (CH_3), 52.4 and 52.6 (CO_2CH_3), 56.4 (CH) and 174.6 and 174.7 (CO); *m/z* (CI) 177 (100%, $[M + H]^+$).

9-Bromo-9-phenylfluorene (183)



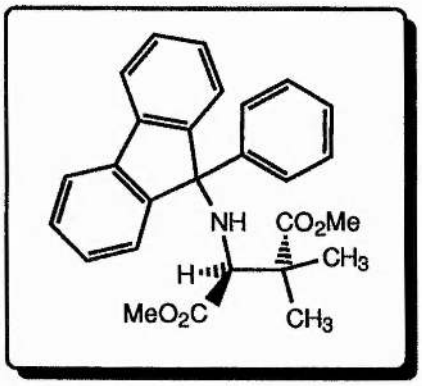
To a stirred solution of bromobenzene (15.8 cm^3, 0.149 mol) in diethyl ether (200 cm^3) was added n-butyl lithium (1.6 mol dm^{-3}, 75.6 cm^3, 0.1788 mol). The mixture was stirred at 0 °C for 30 min and a solution of fluorenone (27 g, 0.15 mol) in THF was added dropwise over 1 h, while keeping the temperature below 5 °C. The reaction mixture was finally let to warm-up to room temperature for 4 h and the reaction was quenched by adding cold water (300 cm^3) and diethyl ether (200 cm^3). The organic phase was washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give the alcohol (**182**) as a yellow solid which was dissolved in toluene (100 cm^3). The resulting solution was mixed with aqueous hydrogen bromide (48%, 50 cm^3) and stirred vigorously for 8 h. The organic phase was washed with water (100 cm^3), brine and the solvent was removed under reduced pressure to give a pale yellow solid (38 g, 80%) which was recrystallised from petroleum ether (60/80), mp 98-99 °C [lit.[158] mp 98-99 °C]; δ_H (200 MHz; C^2HCl_3) 7.40 (11 H, m, Ar-H) and 7.8 (2 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 80.6 (C-9), 117.1, 121.9, 122.5, 124.2, 125.3, 125.5 and 126.1 (Ar-CH) and 136.7, 140.4 and 147.6 (Ar-C); *m/z* (CI) 322 (40%, $[M + H]^+$ and 241 (100, $PhFl^+$).

Dimethyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-methylaspartate (179)



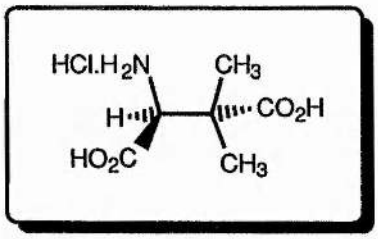
To a stirred solution of 3-methylaspartate (**187**) (2.5 g, 14.3 mmol) in dry acetonitrile (120 cm^3) was added $Pb(NO_3)_2$ (7.5 g, 22.6 mmol), K_3PO_4 (7.5 g, 40.5 mmol) and 9-phenylfluorenylbromide (5 g, 15.6 mmol). The resulting mixture was stirred 3 h and then filtered through Celite which was subsequently washed thoroughly with DCM. The solvent was removed under reduced pressure to give a thick yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether as the eluant (15/85) to yield a colourless solid (4.5 g, 76%), mp 75-80 °C; (Found C, 74.7 ; H, 5.8 ; N, 3.3. $C_{26}H_{25}NO_4$ requires C, 75.15 ; H, 6.05; N, 3.40); $[\alpha]_D$ -276 (*c* 1.23 in DCM); υ_{max} (Nujol)/cm^{-1} 3302 (sharp band, NH), 2926 and 2855 (large, Ar-CH), 1739 (CO ester), 1459, 1377 and 1204; δ_H (200 MHz; C^2HCl_3) 1.22 (3 H, d, *J* 7.5, CH_3), 2.55 (1 H, m, C*H*CH_3), 2.95 (1 H, dd, *J* 10, 7.5, C*H*NH), 3.08 (1 H, d, *J* 10, NH), 3.28 (3 H, s, CO_2CH_3), 3.50 (3 H, s, CO_2CH_3) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 11.9 (CH_3), 43.1 (*C*HCH_3), 51.5 and 51.9 (OMe), 57.3 (CH), 72.6 (*N*-C-9), 119 .8, 125.5, 125.9, 126.4, 127.1, 127.2 and 127.7 (Ar-CH), 139.8, 141.1, 144.4 and 148.4 (Ar-C quaternary) and 173.5 and 174.7 (CO); *m/z* (CI), 416 (15, $[M + H]^+$), 242 (100, $PhFl^+$) and 176 (40%, $[M - PhFl]^+$).

Dimethyl (2*S*)-*N*-9'-(9'-phenylfluorenyl)-3,3-dimethylaspartate (189)



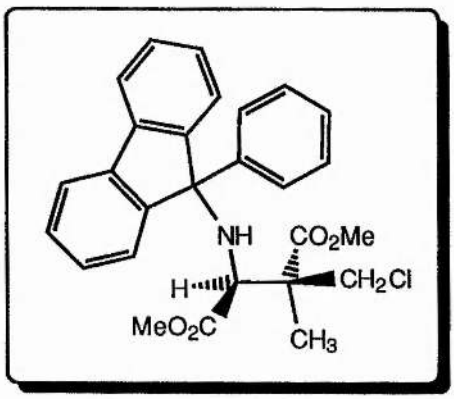
To a stirred solution of *N*-PhFl aspartate (**179**) (1.6 g, 3.9 mmol) in THF (25 cm^3) was added KHMDS (0.5 mol dm^{-3} in toluene, 7.8 cm^3) at -78 °C. After 30 min methyliodide (0.369 g, 2.4 mmol) was added to the yellow solution of the enolate and the resulting mixture was stirred 2 h at -78 °C. The reaction was then quenched with a saturated ammonium chloride solution (1 cm^3) and poured into water (100 cm^3). The resulting solution was extracted with ethyl acetate (3 x 30 cm^3) and the combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give a colourless oil (1.42 g, 85%); $[\alpha]_D$ -312 (*c* 1 in DCM); δ_H (200 MHz; C^2HCl_3) 1.08 (3 H, CH_3), 1.24 (3 H, s, CH_3), 2.78 (1 H, d, *J* 12.5, CH), 3.13 (1 H, d, *J* 12.5, NH), 3.18 (3 H, s, OCH_3), 3.58 (3 H, s, OCH_3) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 21.3 (CH_3), 22.8 (CH_3), 46.9 (C-3 quaternary), 51.8 and 52.5 (OCH_3), 61.4 (CH), 73.1 (C-9' quaternary), 120.4, 126.5, 127.4, 127.7, 127.7, 127.9, 128.1 and 128.9 (Ar-CH), 140.4, 141.9, 145.3, 148.7 and 148.9 (Ar-C quaternary) and 174.7 and 176.9 (CO); *m/z* (CI) 430 (10%, $[M + H]^+$), 415 (30, $[M + H - CH_3]^+$) and 241 (100, $PhFl^+$]).

(2*S*)-3,3-Dimethylaspartic acid hydrochloride (127)



A stirred solution of *N*-PhFl dimethylaspartate (**189**) (0.55 g, 1.28 mmol) 10% Pd/C (0.2 g) in methanol (20 cm^3) was stirred 10 h under 1 atm of H_2. The resulting solution was filtered through Celite which was subsequently washed thoroughly with water and methanol. The resulting aqueous solution was extracted with ethyl acetate and the aqueous layer was concentrated under reduced pressure. The resulting residue was dissolved in aqueous HCl (5 mol dm^{-3}, 20 cm^3) and refluxed for 3 h. The solvent was removed under reduced pressure to give a pale yellow solid which was purified by ion exchange chromatography (Amberlite IR-50H^+) eluted with water to give a white powder (0.18 g, 72%), mp 97-100 °C, $[\alpha]_D$ -4.6 (*c* 1 in H_2O), (HRMS: found $[M + H]^+$, 162.0770. Requires for $C_6H_{12}NO_4$ 162.0765); υ_{max}(Nujol)/cm^{-1} 3450 (NH and COOH), 2867, 2146, 1731 and 1678 (CO), 1459, 1225 and 855; δ_H (200 MHz; ^2H_2O) 0.98 (3 H, s, CH_3), 1.12 (3 H, s, CH_3) and 4.14 (1 H, s, CH); δ_C (50.31 MHz; ^2H_2O) 22.8 (CH_3), 24.3 (CH_3), 45.7 (CH), 60.7 (quaternary) and 172.0 and 180.1 (CO); *m/z* (CI) 162 (100%, $[M + H]^+$).

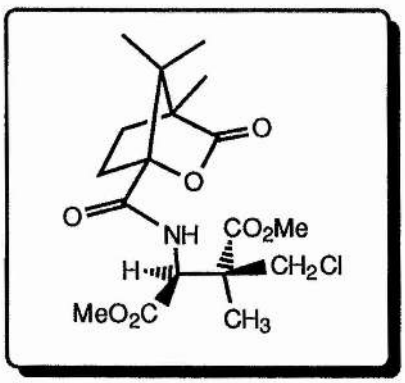
Dimethyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-chloromethyl-3-methyaspartate (188)



To a stirred solution of *N*-9-phenylfluorenyl methylaspartate (**179**) (4 g, 9.6 mmol) in THF (50 cm^3) was added KHMDS (0.5 mol dm^{-3} in toluene, 19.3 cm^3, 9.6 mmol) at -78 °C. After 30 min iodochloromethane was added (0.7 cm^3, 9.6 mmol) to the yellow solution which was then warmed up slowly to room temperature overnight. A saturated solution of ammonium chloride (1 cm^3) was added and the resulting solution was poured into water (100 cm^3). The aqueous mixture was extracted with ethyl acetate (3 x 50 cm^3) and the combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give a white solid (3 g, 68%), mp 48-40 °C; (Found C, 70.2; H, 5.95 ; N, 3.0. $C_{27}H_{26}{}^{35}ClNO_4$ requires C, 69.9 ; H, 5.65 ; N, 3.0); $[\alpha]_D$ -272 (*c* 14.6 in DCM); υ_{max} (Nujol)/cm^{-1} 3745 (NH), 2224 (CH), 2361, 1738 (C=O), 1459, 1377 (CH_2Cl) and 736; δ_H (300 MHz; C^2HCl_3) 1.32 (3 H, s, CH_3), 2.75 (1 H, d, *J* 10.5, C*H*NH), 3.14 (1 H, d, *J* 10.5, NH), 3.22 (3 H, s, CO_2CH_3), 3.54 (3 H, s, CO_2CH_3), 3.61 (2 H, d, AB quartet, *J* 11.0, CH_2Cl) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 11.9 (CH_3), 43.1 (CH_2Cl), 51.5, 51.9 (CO_2CH_3), 57.3 (C-3 quaternary), 72.6 (CHNH), 120.0, 125.5, 126.4, 127.1, 127.2, 127.9 and 130.8 (Ar-CH), 141.1, 144.4, 147.9 and

148.4 (Ar-C quaternary) and 173.5 and 174.7 (CO); *m/z* (CI) 465 and 467 (15%, chlorine isotopes $[M + H]^+$), 429 (5, $[M + H - Cl]^+$), 356 (40) and 241 (100, $PhFl^+$).

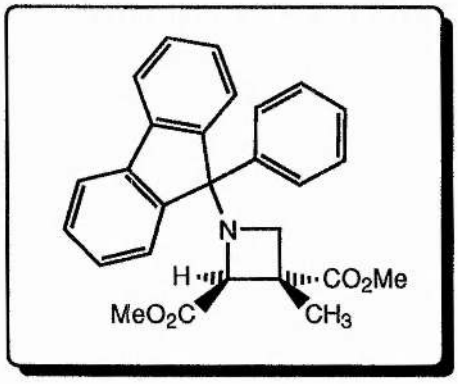
Dimethyl (2*S*,3*S*)-*N*-[(1'S,4'S)-camphanoyl]-3-chloromethyl-3-methylaspartate (190)



A solution of *N*-PhFl chloromethylaspartate (**179**) (0.83 g, 1.79 mmol) in methanol-acetic acid (10:1, 11 cm^3) in the presence of 10% Pd/C (0.4 g) was stirred 10 h under 1 atm of H_2. The reaction mixture was filtered through celite and the filtrate concentrated under reduced pressure. The residue was dissolved into water (75 cm^3) and the pH adjusted to 4 with diluted HCl (1 mol dm^{-3}). The resulting solution was washed with ethyl acetate (2 x 30 cm^3), its pH adjusted to 9 and then was extracted with ethyl acetate (3 x 40 cm^3). The combined organic extracts were washed with brine, dried ($MgSO_4$) and were concentrated under reduced pressure to give (**192**) as a yellowish oil. To a stirred solution of this oil in DCM (10 cm^3) was added triethyl amine (1.79 mmol) and (2*S*,4*S*)-(-)-camphanyl chloride (0.095 g, 0.44 mmol) in toluene (2 cm^3). This mixture was stirred for 3 h and the reaction was quenched with water (30 cm^3). The aqueous solution was then extracted with ethyl acetate (3 x 15 cm^3) and the combined organic extracts were washed with a HCl solution (1 mol dm^{-3}), with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow solid which was recrystallised from ethyl acetate-petroleum ether to give colourless crystals (0.11 g, 64%), mp 175-177 °C; (Found C, 53.5 ; H, 6.45; N, 3.4. $C_{18}H_{26}ClNO_7$ requires C, 53.55; H, 6.5; N, 3.5); $[\alpha]_D$ -4.7 (*c* 1.5 in

DCM); υ_{max}(thin film)/cm-1 3377 (NH), 2930 and 2860 (CH), 1797 (CO lactone), 1728 (CO ester), 1687 (CO amide), 1517, 1459, 1381, 1273, 1124, 1073 and 743 (C-Cl); δ_H (500 MHz; C^2HCl_2) 0.92 (3 H, s, CH_3), 1.08 (6 H, s, 2 x CH_3), 1.27 (3 H, s, CH_3), 1.65 (1 H, m, CH), 1.91 (2 H, m, 2 x CH), 2.45 (1 H, m, CH), 3.71 (3 H, s, CH_3), 3.73 (3 H, s, CH_3), 3.83 (2 H, AB , *J* 11, CH_2Cl), 5.13 (1 H, d, *J* 9.5, C*H*NH) and 7.18 (1 H, d, *J* 9.5, NH); δ_C (125.8 MHz; C^2HCl_2) 10.1, 17.1, 17.2 and 18.1 (CH_3), 29.6 and 31.2 (CH_2), 49.6 (CH_2Cl), 51.5 ($C(CH_3)$), 51.3 and 53.4 (CO_2CH_3), 55.4 (CHNH), 55.4, 55.8 and 92.8 (quaternary) and 168.1, 170.0, 173.1 and 178.0 (CO); *m/z* (CI) 404 (80%, $[M + H]^+$), 368 (30, $[M - Cl]^+$) and 199 (100, [M - Cl - camphanyl]$^+$).

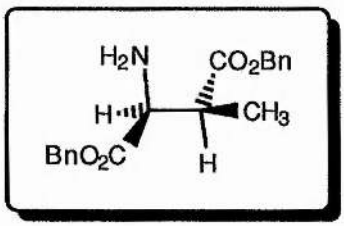
Dimethyl *trans*-(2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-methylazetidine 2,3-dicarboxylate (221)



A stirred solution of (2*S*,3*S*)-3-chloromethyl-3-methylaspartate diester (**188**) (0.7 g, 1.6 mmol) and sodium iodide (0.255 g, 1.7 mmol) in DMF (30 cm^3) was heated to 100-110 °C for 3 h. Water (50 cm^3) was added to the reaction mixture which was then extracted with ethyl acetate (2 x 25 cm^3). The combined organic layers were washed with water (10 x 30 cm^3), brine and the solvent was removed under reduced pressure to give a yellow oil. This oil was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85, R_f 0.31) as the eluant to give a white solid (0.53 g, 80%). A sample was dissolved in hot petroleum-ether/ethyl acetate (97:3, 0.1 cm^3) and let to stand at 16 °C for 7 days, in a closed sample vial, to afford few crystals suitable for X-ray, mp 102-106 °C

(HRMS: found C, 76.10 ; H, 5.95; N, 3.25; $[M + H]^+$, 427.1790. $C_{27}H_{25}NO_4$ requires C, 75.85; H, 5.90; N, 3.30; $[M + H]^+$ 427.1785); $[\alpha]_D$ +144.3 (*c* 2 in DCM); υ_{max} (Nujol)/cm^{-1} 2936, 2858 (large, Ar-CH), 1756 and 1737 (sharp bands, C=O), 1464, 1381, 1201, 1176 and 1157; δ_H (500 MHz; C^2HCl_3) 1.45 (3 H, s, CH_3), 3.30 (1 H, d, *J* 9.1, CH_2), 3.75 (3 H, s, CO_2CH_3), 3.35 (3 H, s, CO_2CH_3), 3.68 (1 H, s, CH), 3.74 (1 H, d, *J* 9.1, CH_2) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 19.4 (CH_3), 41.7 (CH_2Cl), 51.7 and 52.7 (CO_2CH_3), 56.3 (C-3 quaternary), 64.3 (CH-2), 76.3 (C-9' quaternary), 120.0, 127.4, 127.7, 127.8, 128.0, 128.9, 129.1 and 129.4 (Ar-CH), 140.2, 141.4, 142.6, 145.3 and 146.8 (Ar-C quaternary) and 170.7 and 174.7 (CO); *m/z* (CI) 427 (20%, $[M + H]^+$), 368 (25, $[M - CO_2Me]^+$) and 241 (100, $PhFl^+$).

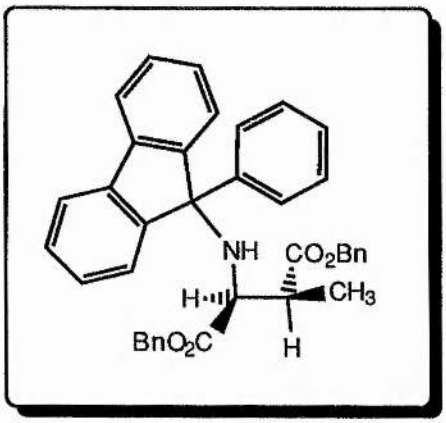
Dibenzyl (2*S*,3*S*)-3-methylaspartate diester (225)



A stirred solution of (2*S*, 3*S*)-3-methylaspartic acid (**53**) (4 g, 27.1 mmol), benzyl alcohol (18 cm^3, 166 mmol) and *p*-toluene sulfonic acid (6.4 g, 33.9 mmol) in toluene was refluxed for 4 h using a Dean's Starck apparatus. The resulting solution was warmed up to room temperature and washed with sodium hydroxide (100 cm^3, 1 mol dm^{-3}). The aqueous phase was extracted with ethyl acetate (3 x 30 cm^3) and the combined organic extracts were washed with HCl (2 mol dm^{-3}, 2 x 50 cm^3). The pH of the combined aqueous washes was adjusted to 10 with sodium hydroxide (1 mol dm^{-3}) and the resulting solution extracted with ethyl acetate (3 x 30 cm^3), washed with brine and dried (K_2CO_3). The solvent was removed under reduced pressure to give the dibenzyl ester as a colourless oil (2 g, 22%); $[\alpha]_D$ - 0.63 (*c* 16.3 in DCM); δ_H (200 MHz; C^2HCl_3) 1.16 (3 H, d *J* 7,

CH_3), 1.72 (2 H, s, NH_2), 3.02 (1 H, qd *J* 4.5, 7, CH), 5.12 (2 H, s; CH_2Ph), 5.15 (2 H, s, CH_2Ph) and 7.4 (10 H, s, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 11.5 (CH_3), 43.3 (*C*HCH_3), 56.4 ($CHNH_2$), 67.0, 67.5 (CH_2Ph), 128.6, 128.7, 128.9 and 129.0 (Ar-CH), 135.8 and 136.2 (Ar-C-*ipso*) and 174.0 (2 x C=O); *m/z* (CI) 328 (100%, [M + H]$^+$), 192 (25) and 91 (55, $C_7H_7^+$).

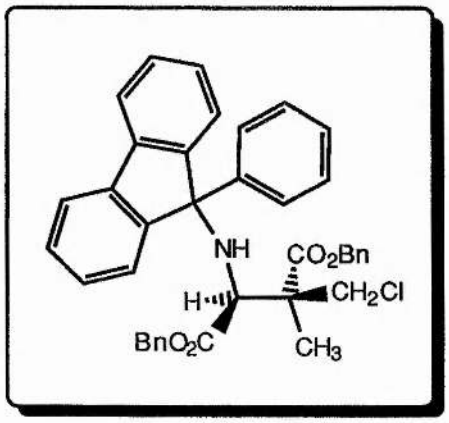
Dibenzyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-methylaspartate (227)



To a stirred solution of dibenzyl methylaspartate (**225**) (1.36 g, 4.2 mmol) in acetonitrile (20 cm^3) was added potassium phosphate (1.7 g, 12.6 mmol), lead nitrate (1.7 g, 6.3 mmol) and 9-phenylfluorenylbromide (1.45 g, 4.5 mmol). The mixture was stirred 8 h and then filtered through Celite. The solvent was removed under reduced pressure to give a thick yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to yield a white solid (1.7 g, 71%), mp 61-63 °C; (HRMS: found [M + H]$^+$ 568.2475. Requires for $C_{38}H_{34}NO_4$ 568.2490); $[\alpha]_D$ -290 (*c* 1 in DCM; υ_{max} (Nujol)/cm^{-1} 2926, 1737 (CO), 1463 (C-O) and 1371; δ_H (200 MHz; C^2HCl_3) 1.18 (3 H, d, *J* 7, CH_3), 2.61 (1 H, qd, *J* 5, 7, CH), 3.11 (2 H, m, C*H*N*H*), 4.75 (2 H, AB, *J* 17.5, CH_2Ph), 4.90 (2 H, AB, *J* 17.5, CH_2Ph) and 7.38 (23H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 13.0 (CH_3), 44.7 (*C*HCH_3), 58.2 (CHNH), 66.8 (CH_2Ph), 67.1 (C-9'), 120.4, 120.5, 120.6, 125.3, 125.8, 126.2, 126.6, 127.0, 127.7, 127.8, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9 and 129.6 (Ar-CH), 135.7, 136.4,

140.1, 140.4, 141.7, 145.1, 148.7, 149.1 and 151.0 (Ar-C quaternary) and 173.5 and 174.6 (C=O); *m/z* (CI) 568 (20%, $[M + H]^+$), 241 (100, $PhFl^+$) and 91 (60, $C_7H_7^+$).

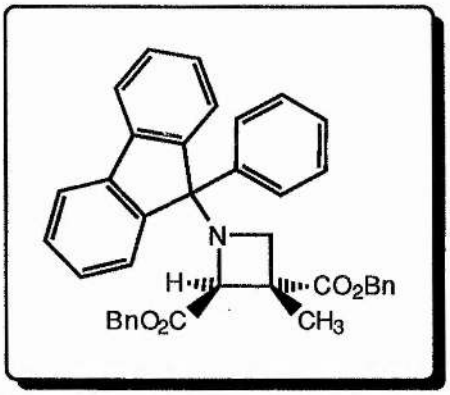
Dibenzyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-chloromethyl-3-methylaspartate (228)



To a stirred solution of *N*-PhFl dibenzyl methylaspartate (**227**) (0.410 g, 0.65 mmol) in THF (10 cm^3) was added KHMDS (0.5 mol dm^{-3} in toluene, 13 cm^3) at -78 °C. After 30 min, iodochloromethane (47.4 mm^3, 0.65 mmol) was added and the solution was warmed-up slowly to room temperature overnight. A saturated ammonium chloride solution (1 cm^3) was added and the resulting solution was poured into water (50 cm^3). The aqueous solution was extracted with ethyl acetate (3 x 20 cm^3) and the combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a thick yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give chloromethylaspartate (**162**) as a white solid (0.220 g, 50%), mp 90-93 °C (HRMS: found 616.2230. Requires for $C_{39}H_{35}O_4{}^{35}Cl$ 616.2255); $[\alpha]_D$ -270 (*c* 1 in DCM); υ_{max} (Nujol)/cm^{-1} 1730 (CO); δ_H (200 MHz; C^2HCl_3) 1.34 (3 H, s, CH_3), 3.00 (1 H, d, CH_2Cl), 3.24 (1 H, d, CH), 3.45 (1 H, d, NH), 3.88 (1 H, d, CH_2Cl), 4.50 (4 H, m, CH_2Ph) and 7.30 (23H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 14.6 (CH_3), 50.5 (CH_2Cl), 52.4 (CCH_3), 59.9 and 66.4 (CH_2Ph), 72.7 (C-9'), 119.7, 119.8, 125.7, 125.9, 126.9,

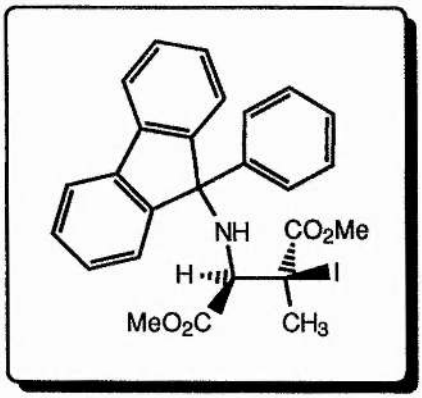
127.1, 127.2, 127.4, 127.6, 127.9, 128.1, 128.2 and 128.3 (Ar-CH), 134.4, 135.3, 139.0 and 143.7 (Ar-C quaternary) and 171.7 and 172.4 (CO); *m/z* (CI) 616 and 618 (10%, $[M + H]^+$), 580 (50, $[M + H - Cl]^+$) and 241 (100, $PhFl^+$).

Dibenzyl *trans*-(2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-methylazetidine 2,3-dicarboxylate (224)



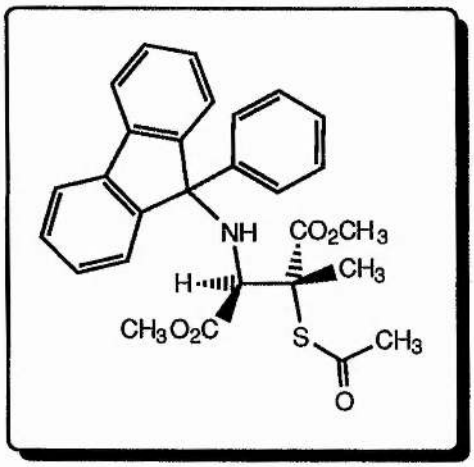
A stirred solution of dibenzyl chloromethylaspartate (**228**) (0.31 g, 0.67 mmol) and sodium iodide (0.1 g, 0.66 mmol) in DMF (15 cm^3) was heated to 100-110 °C for 6 h. The reaction mixture was then poured into water (30 cm^3) and the resulting solution was extracted with ethyl acetate (2 x 5 cm^3). The combined organic extracts were washed with water (8 x 15 cm^3), brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a brown residue which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give the pure azetidine as a colourless oil (0.2 g, 69%); $[\alpha]_D$ +110 (*c* 2 in DCM); υ_{max} (thin film)/cm^{-1} 1720 (CO); δ_H (200 MHz; C^2HCl_3) 1.56 (CH_3), 3.38 (1 H, d, CH_2), 3.84 (CHN), 3.85 (CH_2), 4.88 (2 H, s, CH_2Ph), 5.02 (2 H, s, CH_2Ph) and 7.45 (23H, m, Ar-CH); δ_C (50.31 MHz; C^2HCl_3) 19.5 (CH_3), 42.1 (CCH_3), 56.2 (CH), 64.4 (CH_2), 66.3 and 67.1 (CH_2Ph), 119.9, 120.6, 125.4, 126.0, 127.4, 127.8, 127.9, 128.0, 128.1, 128.3, 128.5, 128.6, 128.7, 128.8, 128.9, 129.0, 129.1, 129.4 and 129.6 (Ar-CH), 136.3, 136.4, 140.2, 140.3, 141.5, 142.6, 145.4 and 146.8 (Ar-C quaternary) and 170.0 and 174.0 (CO); *m/z* (CI) 579 (5%, $[M + H]^+$), 258 (90, $[C_{19}H_{15}N + H]^+$) and 241 (100, $PhFl^+$).

Dimethyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-iodo-3-methylaspartate (201)



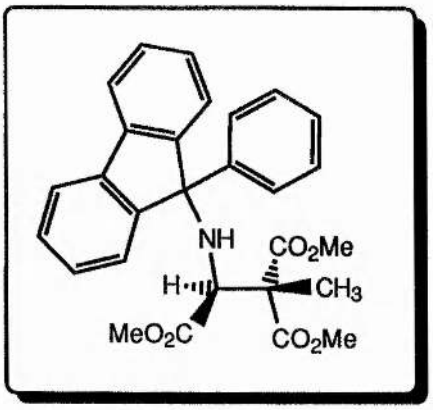
To a stirred solution of *N*-PhFl aspartate diester (**179**) (2 g, 4.8 mmol) in THF (30 cm^3) cooled to -78 °C was added KHMDS (0.5 mol dm^{-3} in toluene, 9.6 cm^3). The yellow solution was stirred 30 min at -78 °C and iodine (1.4 g, 5.5 mmol) in THF (5 cm^3) was added. The resulting mixture was stirred 3 h at -78 °C and then warmed-up slowly to room temperature overnight. A saturated ammonium chloride solution was added (2 cm^3), water (40 cm^3) and the resulting aqueous solution was extracted with ethyl acetate (3 x 20 cm^3). The combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was then removed under reduced pressure to give a yellow oil which was purified, in darkness,by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give the light sensitive iodo compound (**201**) as a white solid (1.83 g, 70%); mp 72 °C; $[\alpha]_D$ -265 (*c* 1 in DCM); υ_{max} (Nujol)/cm^{-1} 1725 (CO, large); δ_H (200 MHz; C^2HCl_3) 2.24 (3 H, s, CH_3), 3.18 (3 H, s, CO_2CH_3), 3.16 (1 H, d, CH), 3.38 (1 H, d, NH), 3.67 (3 H, s, CO_2CH_3) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 26.3 (CH_3), 40.0 (C-3), 52.0 (CO_2CH_3), 53.8 (CO_2CH_3), 72.8 (C-9' quaternary), 120.4, 125.3, 126.4, 126.9, 127.0, 127.9, 128.9 and 129.1 (Ar-CH), 140.4, 141.9, 144.9, 147.0 and 147.8 (Ar-C) and 172.3 and 173.2 (CO); *m/z* (CI), 542 (10%, $[M + H]^+$), 482 (30, $[M + H - CO_2Me]^+$), 414 (75, $[M + H - I]^+$) and 242 (100, $PhFl^+$).

Dimethyl (2*R*,3*R*)-*N*-9'-(9'-phenylfluorenyl)-3-methyl-3-thioacetoxyaspartate (202)



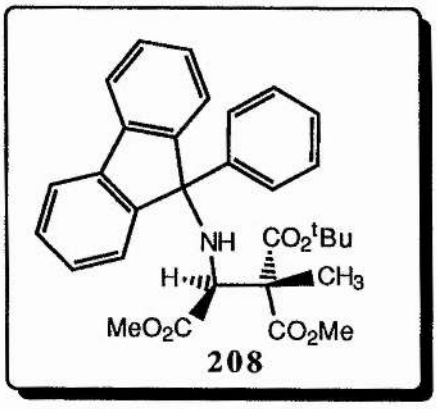
To a stirred solution of *N*-PhFl iodoaspartate (**201**) (70 mg, 0.130 mmol) in acetone (10 cm^3) was added potassium thioacetate (42 mg, 0.36 mmol). The solution was refluxed for 4 h and was poured into water (30 cm^3). The aqueous solution was then extracted with ethyl acetate (3 x 15 cm^3) and the combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to obtain a yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (gradient 15:85 and 20:80) as the eluant to give a colourless oil (30 mg, 49%), (HRMS: found $[M + H]^+$, 490.1700. Requires for $C_{28}H_{28}NO_6S$ 490.1690); υ_{max} (thin film)/cm^{-1} 3400 (NH), 2950 (CH), 1742 (C=O), 1688 (C=O, thio acetate), 1450, 1249, 1117 and 735; δ_H (200 MHz; C^2HCl_3) 1.68 (3 H, s, CH_3), 2.28 (3 H, s, CH_3COS), 3.0 (1 H, d, *J* 10.5, CH), 3.15 (3 H, s, CO_2CH_3), 3.17 (1 H, d, NH), 3.64 (3 H, s, CO_2CH_3), and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 17.3 (CH_3), 20.2 (CH_3COS), 49.5 (CO_2CH_3), 50.4 (*C*SCO), 50.6 (CO_2CH_3), 57.0 (CH), 70.5 (C-9' quaternary), 117.7, 123.1, 124.5, 125.2, 125.5, 126.2, 126.3, 126.3 and 126.5 (Ar-CH), 139.1, 142.1, 144.8 and 144.9 (Ar-C quaternary), 168.6 and 169.7 (CO ester) and 192.9 (COS); *m/z* (CI), 490 (15%, $[M + H]^+$) and 241 (100, $PhFl^+$).

Dimethyl (2*S*)-*N*-9'-(9-phenylfluorenyl)-3,3-dimethylaspartate (207)

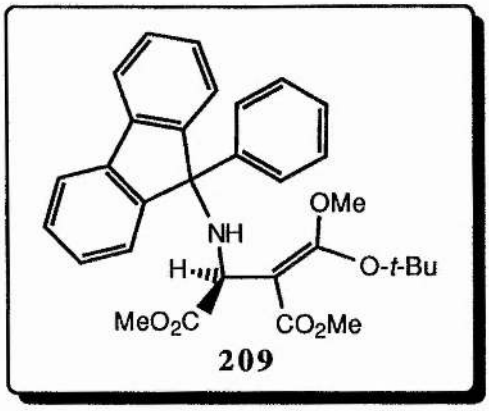


To a stirred solution of dimethyl aspartate (**179**) (0.26 g, 0.63 mmol) in THF (15 cm^3) was added KHMDS (0.5 mol dm^{-3} in toluene, 1.25 cm^3) at -78 °C. After 20 min methylchloroformate (49 mm^3, 0.63 mmol) was added and the resulting mixture left to stirr for 1 h at -78 °C and was warmed up slowly to room temperature overnight. The reaction was quenched with a cold solution of saturated ammonium chloride (0.5 cm^3) and was poured into water (50 cm^3). The aqueous solution was extracted with ethyl acetate (3 x 15 cm^3) and the combined organic extracts were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a thick pale red oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to yield a colourless oil (0.214 g, 72%), (HRMS: found $[M + H]^+$, 474.1923. Requires for $C_{28}H_{28}NO_6$ 474.1916); δ_H (200 MHz; C^2HCl_3) 1.48 (CH_3), 3.17 (OCH_3), 3.4 (2 H, m, CH and NH), 3.74 (OCH_3), 3.77 (OCH_3) and 7.45 (Ar-H); δ_C (50.31 MHz; C^2HCl_3) 18.5 (CH_3), 52.0 and 53.1 (OCH_3), 59.8 (quaternary), 59.8 (CH), 72.0 (C-9' quaternary), 120.3, 120.4, 126.4, 126.6, 126.7, 127.7, 127.9 and 128.8 (Ar-CH) 140.5, 141.9, 145.5, 147.9 and 148.9 (Ar-C quaternary) and 170.9, 171.5 and 173.6 (CO); *m/z* (CI), 474 (65%, $[M + H]^+$), 416 (20, $[M + H - CO_2CH_3]^+$) and 241 (100, $PhFl^+$).

Dimethyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-(*tert*-Butyl)-oxycarbonyl-3-methylaspartate (208) and Methyl (2*S*)-2-[9'-(9'-phenylfluorenyl)amino]-3-methyl-4-*tert*-butoxy-4-methoxy but-3-ene-1-oate (209)



208



209

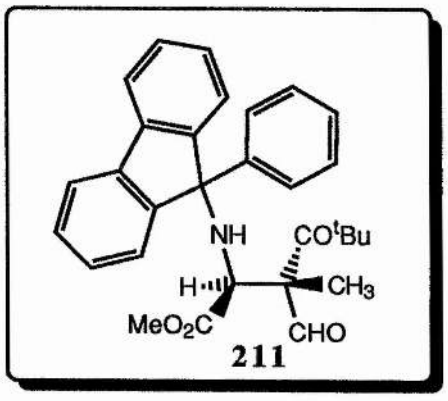
To a stirred solution of methylaspartate (**87**) (5 g, 12 mmol) in THF (30 cm^3) was added KHMDS (0.5 mol dm^{-3} in toluene, 28.9 cm^3) at -78 °C. The yellow solution was stirred at -78 °C for 20 min before adding di-*tert*-butyldicarbonate (1 mol dm^{-3} in THF, 12 cm^3) and then warmed-up to -30 °C for 1 h. The resulting solution was warmed-up slowly to room temperature overnight. The reaction mixture was quenched with a saturated solution of ammonium chloride (5 cm^3) and poured into water (100 cm^3). The aqueous solution was extracted with ethyl acetate (3 x 50 cm^3) and the combined organic extracts were dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85) as the eluant to give (**208**) ($R_f = 0.3$) as a white solid (4 g, 65%) and (**209**) ($R_f = 0.35$) as a colourless oil (1.2 g, 19%).

Compound (**208**); mp 133-135 °C (HRMS: found $[M + H]^+$, 516.2385. Calc. for $C_{31}H_{34}NO_6$ 516.2400); $[\alpha]_D$ -261.3 (*c* 15.0 in DCM); υ_{max} (Nujol)/cm^{-1} 3365 (sharp band, NH), 2946 (large, CH aromatic), 1742, 1727 (shoulder, C=O), 1469, 1381, 1332, 1274, 1240 and 1157; δ_H (200 MHz; C^2HCl_3) 1.28 (3 H, s, CH_3), 1.52 (9 H, s, $C(CH_3)_3$), 3.04 (3 H, s, OCH_3), 3.27 (1 H, d, *J* 16.0, CH), 3.78 (3 H, s, OCH_3), 3.80 (1 H, d, *J* 16.0, NH) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 19.1 (CH_3),

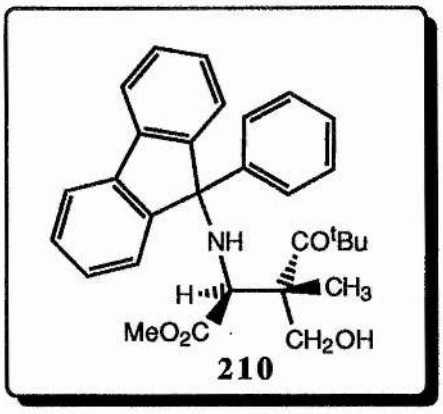
27.5 ($C(CH_3)_3$), 50.9 and 52.1 (CO_2CH_3), 57.9 (C-3), 58.9 (CH), 72.0 (C-9' quaternary), 81.8 ($C(CH_3)_3$), 119.2, 119.4, 125.5, 126.7, 126.9, 127.0, 127.9 and 128.1 (Ar-CH), 141.0, 144.8, 147.1 and 148.1 (Ar-C quaternary), and 164.0, 166.7 and 168.0 (CO); *m/z* (CI) 516 (25%, $[M + H]^+$), 456 (85, $[M + H - CO_2CH_3]^+$), 283 (70) and 242 (100, $[PhFl + H]^+$).

Compound (**209**): (HRMS: found $[M + H]^+$, 516.2385. Requires for $C_{31}H_{34}NO_6$ 516.2400); υ_{max} (thin film)/cm^{-1} 3500 (NH), 3100 (CH), 1766 (CO); δ_H (200 MHz; C^2HCl_3) 1.54 (9 H, s, $C(CH_3)_3$), 1.56 (3 H, s, CH_3), 3.01 (3 H, s, OCH_3), 3.3 (1 H, s, CH), 3.45 (3 H, s, CO_2CH_3), 3.75 (1 H, s, NH) and 7.45 (13 H, m, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 10.8 (CH_3), 28.0 ($C(CH_3)_3$), 52.7 (OCH_3), 55.2 (CH), 57.1 (CO_2CH_3), 73.8 (C-9' quaternary), 83.9 (C-3), 103.8 (C-4), 120.2, 120.3, 126.1, 126.7, 127.8, 128.1, 128.2, 128.7 and 128.8 (Ar-CH), 140.4, 141.8, 145.3, 148.6 and 149.0 (Ar-C quaternary) and 174.9 (C=O); *m/z* (CI), 516 (30%, $[M + H]^+$) and 241 (100, $PhFl^+$).

3-*tert*-Butyl-1-methyl (2*S*,3*S*)-*N*-9'-(9'-phenylfluorenyl)-3-formyl-3-methylaspartate (211) and 3-*tert*-Butyl-2-methyl (2*S*,3*S*)-*N*-9'(9'-phenylfluorenyl)-3-hydroxymethyl-3-methylaspartate (210)



211



210

Reduction of triester (**208**) to aldehyde (**211**) [method 1]:

To a stirred solution of the *tert*-Butyl ester (**208**) (0.1 g, 0.19 mmol) in toluene (3 cm^3) was added dropwise DIBAL-H (1 mol dm^{-3} in toluene, 0.871 cm^3) while maintaining the temperature at -50 °C. The reaction mixture was left 1 h at -50 °C and was quenched with a cold saturated ammonium chloride solution (1 cm^3) at -50 °C. The resulting mixture was stirred vigorously at room temperature for 30 min and filtered. The solid residue was washed several times with ethyl acetate (20 cm^3). The filtrate was poured into water (20 cm^3) and extracted with ethyl acetate (2 x 10 cm^3). The combined organic extracts were washed with brine, dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (15:85, R_f 0.3) as the eluant. The 2,4-dinitrophenylhydrazine positive fractions were concentrated to give pure aldehyde (**211**) as a white solid, (46 mg, 50%). Some alcohol (**210**) was also isolated as a white solid, (R_f 0.15), (9.2 mg, 10%).

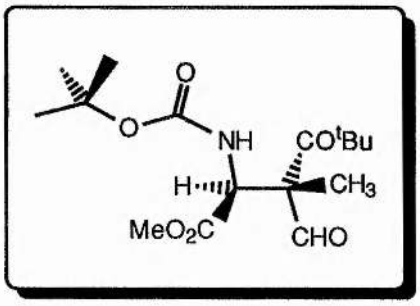
Compound (**211**): mp 38-40 °C; (HRMS: found $[M + H]^+$, 486.2270. Requires for $C_{30}H_{32}NO_5$ 486.2280); $[\alpha]_D$ -265 (*c* 9.3 in DCM); υ_{max} (Nujol)/cm^{-1} 3400 (NH), 2926, 1786 (C=O aldehyde), 1736 (C=O), 1461, 1377, 1154 and 846; δ_H (200 MHz; C^2HCl_3) 1.06 (3 H, s, CH_3), 1.48 (9 H, *t*-Bu), 3.02 (1 H, d, NH), 3.17 (3 H, s, CO_2CH_3), 3.17 (3 H, s, CO_2CH_3), 3.64 (1 H, d, CH), 7.43 (13 H, m, Ar-H) and 9.68 (1 H, s, CHO); δ_C (50.31 MHz; C^2HCl_3) 16.5 (CH_3), 28.5 ($C(CH_3)_3$), 52.1 (CH), 60.7 (CO_2CH_3), 73.0 (C-9' quaternary), 83.2 ($C(CH_3)_3$), 120.4, 120.6, 126.2, 126.4, 126.9, 127.9, 128.3, 128.5, 128.9, 129.0 and 129.2 (Ar-CH), 141.9, 144.7, 148.1 and 148.3 (Ar-C quaternary) 169.5 and 172.9 (CO esters) and 199.2 (CO aldehyde); *m/z* (CI) 486 (10%, $[M + H]^+$), 457 (45, $[M + H - CHO]^+$), 396 (50, $[M + H - CO_2t\text{-Bu}]^+$) and 241 (100, $PhFl^+$).

Compound (**210**): mp 78-80 °C; υ_{max} (Nujol)/cm^{-1} 3491 (broad, OH), 3345 (NH), 2945, 1746 (CO), 1449, 1376 and 1152; δ_H (200 MHz; C^2HCl_3) 0.94 (3 H, s CH_3), 1.49 (9 H, s, in DCM (0.25 cm^3) *t*-Bu), 2.78 (1 H, d, *J* 12.5, CH), 3.18 (3 H, s, CO_2CH_3), 3.64 (4 H, m, NH and OH and CH_2) and 7.45 (13 H, m, Ar-Hl); δ_C (50.31 MHz; C^2HCl_3) 19.3 (CH_3), 28.5 ($C(CH_3)_3$), 51.5 (CH_2OH), 51.9 (CO_2CH_3), 60.9 (CH), 68.5 (C-3), 76.9 (C-9' quaternary), 81.9 ($C(CH_3)$), 120.2, 120.5, 126.1, 126.3, 127.2, 127.7, 127.8, 128.5, 128.9 and 129.1 (Ar-CH), 140.7, 141.7, 144.5, 148.1 and 148.4 (Ar-C quaternary), 173.4 and 173.9 (CO); *m/z* (CI) 488 (40%, $[M + H]^+$), 460 (15, $[M + H - CO]^+$), 428 (35, $[M + H - CO_2Me]^+$) and 241 (100, $PhFl^+$).

Oxydation of alcohol (**210**) to aldehyde (**211**) [method2]:

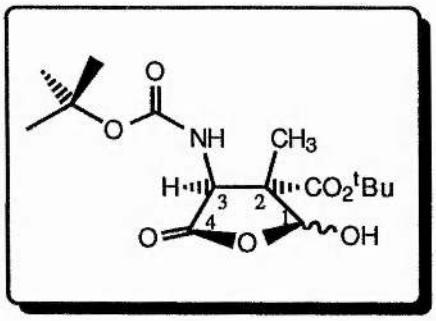
To a stirred solution of alcohol (**210**) (50 mg, 0.1 mmol) in DCM (0.25 cm^3) was added triethylamine (0.1 cm^3, 0.5 mmol), DMSO (0.3 cm^3, 0.5 mmol) and pyridine-SO_3 complex (0.110 g, 0.5 mmol). The reaction was stirred 1 h, quenched with water (20 cm^3). The resulting solution was extracted with ethyl acetate (3 x 10 cm^3) and the combined organic layers were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to yield a yellow oil which was purified as in method 1 to give aldehyde (**211**) (43 mg, 90%).

3-*tert*-Butyl-2-methyl (2*S*,3*S*)-*N*-benzyloxycarbonyl-3-formyl-3-methylaspartate (212)



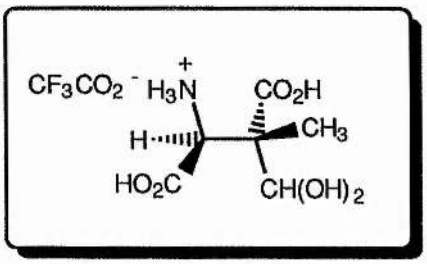
To a stirred solution of *N*-PhFl aldehyde (**211**) (0.26 g, 0.53 mmol) was added $(Boc)_2O$ (1 g, 4.5 mmol) and 10% Pd/C (0.07 g) in methanol (5 cm^3).The mixture was stirred under 1 atm of H_2 for 2 h (monitoring by tlc) and filtered through Celite which was subsequently washed thoroughly with methanol (20 cm^3). The filtrate was concentrated under reduced pressure to give a pale yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (2:8, R_f 0.38) as the eluant to yield *N*-Boc aldehyde (**212**) as a colourless oil (0.2 g, 80%), (HRMS: found $[M + H]^+$, 346.1805. Requires for $C_{16}H_{28}NO_7$ 346.1865); $[\alpha]_D$ -16.8 (*c* 7.6 in DCM); υ_{max} (thin film)/cm^{-1} 3375 (NH), 3000 (CH), 1800 (small, C=O aldehyde), 1730 (large, C=O ester + carbamate), 1500, 1366 and 1156; δ_H (200 MHz; C^2HCl_3) 1.37 (3 H, s, CH_3), 1.42 (9 H, s, *t*-Bu), 1.46 (9 H, s, *t*-Bu), 3.69 (3 H, s, CO_2CH_3), 5.00 (1 H, d, *J* 16, CH), 5.25 (1 H, d, *J* 16, NH) and 9.71 (1 H, s, CHO); δ_C (50.31 MHz; C^2HCl_3) 16.1 (CH_3), 27.5, 27.9 (*t*-Bu), 52.2 (CH), 55.5 and 59.5 ($C(CH_3)$), 80.0 (C-3 quaternary), 155.4 (CO, carbamate), 169.0 (CO, *t*-Bu ester), 170.4 (CO, methyl ester), and 199.2 (CO aldehyde); *m/z* (CI) 376 (25%, $[M + H]^+$), 320 (30, $[M + H-$ *t*-Bu$]^+$) and 264 (100).

(1*S*,2*S*,3*S*)-1-hydroxy-2-methyl-2-(*tert*-Butyl)oxycarbonyl-3-(*tert*-Butyloxycarbonyl)amino-butano-4-lactone (213)



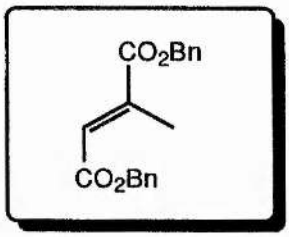
To a stirred solution of aldehyde (**212**) (0.15 g, 0.44 mmol) in methanol (5 cm^3) was added LiOH (0.9 mol dm^{-3}, 1 cm^3). The mixture was stirred 1 h and was acidified with 10% citric acid (5 cm^3) and diluted with water (20 cm^3). The resulting solution was extracted with ethyl acetate (3 x 10 cm^3) and the combined organic layers were washed with water (3 x 10 cm^3), brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give butan-4-lactone (**213**) as a white powder (0.116 g, 80%) which was further purified for microanalysis by silica gel chromatography using ethyl acetate-petroleum ether (3:7) as the eluant (R_f = 0.22), mp 146-147 °C, (Found C, 54.30; H, 7.70 ; N, 3.95. $C_{15}H_{25}NO_7$ requires C, 54.40 ; H, 7.60 ; N, 4.20); $[\alpha]_D$ +41.1 (*c* 2.0 in THF); υ_{max} (Nujol)/cm^{-1} 3384 (large, OH), 2853 and 2919 (large, CH_3), 1772 (medium, C=O lactone), 1730 (C=O, ester), 1711 (C=O carbamate), 1526, 1460, 1370, 1322, 1251 and 1161; δ_H (200 MHz; C^2HCl_3) 1.24 (3 H, s, CH_3), 1.28 (3 H, s, CH_3), 1.50 (18H, s, C(C*H*$_3$)), 1.52 (18H, s, C(C*H*$_3$)), 4.76 and 4.99 (1 H, d, *J* 8.7, 2 x C*H*NH), 5.33 and 5.45 (1 H, d, *J* 9, 2 x NH), 5.63 (2 H, m (broad), 2 x C*H*OH), 6.04 (2 H, s (broad), 2 x OH); δ_C (75.4 MHz; C^2HCl_3) 11.9 and 15.7 (CH_3), 27.7 and 28.1 (C(*C*H$_3$)), 54.6 and 55.4 (*C*(CH$_3$)$_3$), 56.2 and 56.3 (C-2 quaternary), 81.8 and 83.3 (CH-3), 100.7 (CH-1), 156.2 (NH*C*O$_2$) and 170.7, 172.8 and 173.8 (CO); *m/z* (CI) 332 (2%, $[M + H]^+$), 316 (1, $[M + H - OH]^+$) and 220 (100%, [M + H -2 x *t*-Bu]).

(2*S*,3*S*)-3-Formyl-3-methylaspartate trifluoroacetate salt (130)



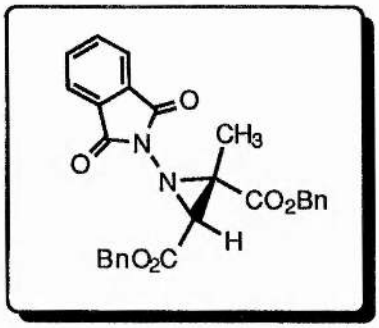
To a stirred solution of butano-4-lactone (**213**) (10 mg g, 30 μmol) in DCM (1 cm^3) was added TFA (10 mm^3, 130 μmol). The mixture was stirred for 30 h under argon and then poured into cold water (5 cm^3). The resulting solution was extracted with DCM (3 x 3 cm^3), the organic layers were discarded and the aqueous layer was left 30 min under high vaccuum to give a pale yellow oil which was passed through a weakly acidic Amberlite IRC-50 ion exchange column. The column was eluted with water and the clear and ninhidrin positive fractions were collected and lyophilised to yield a white powder (0.00239 g, 26%), (HRMS: found $[M + H]^+$ 195.0740. Requires for $C_6H_{13}NO_6$ 195.0745); δ_H (200 MHz; ^2H_2O) 0.92 (3 H, s(br), CH_3),.1.20 (6 H, s(br), 2 x CH_3), 4.0 (1 H, s (br), CHN), 4.15 (3 H, m (br), 2 x C*H*N and C*H*OH), 5.00 (2 H, m(br), 2 x C*H*OH [lactone]); δ_C (75.4 MHz; ^2H_2O) 11.7 and 12.1 (CH_3), 39.3 and 39.95 (CHN), 55.5 (br, quaternary), 91.0 and 91.3 (C*H*OH) 115.5 and 119.1 (CF_3), 162.9 and 163.3 (CF_3CO_2), 170 9 and 176.4 (br, CO); *m/z* (CI) 195 (30%, $[M + H]^+$), 179 (30, $[M + H - OH\}^+$) and 130 (100, $[M - NH_3 - CH_2O]$.

Dibenzyl mesaconate (242)



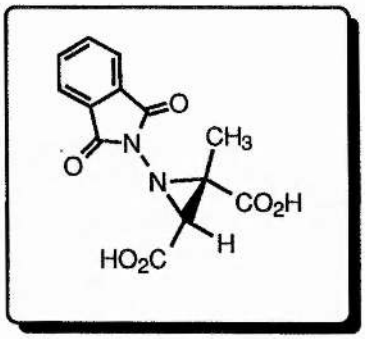
A stirred solution of mesaconic acid (5 g, 38.4 mmol), benzyl alcohol (8 cm^3, 76.8 mmol) and *p*-toluene sulfonic acid (0.73 g) in CCl_4 (20 cm^3) was refluxed using a Dean and Stark apparatus until no more water was collected. The solvent was then removed under reduced pressure to give a residue which was dissolved in ethyl acetate and washed with sodium hydroxide (100 cm^3, 2 mol dm^{-3}). The resulting organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to yield a yellow oil which was purified by silica gel chromatography using ethylacetate-petroleum ether (1:9) as the eluant to yield a colourless oil (8 g, 67%), υ_{max} (thin film)/cm^{-1} 3043, 1727 (C=O), 1658 (C=C), 1463, 1249 and 1108; δ_H (200 MHz; C^2HCl_3) 2.38 (3 H, s, CH_3), 5.12 (2 H, s, CH_2Ph), 5.15 (2 H, s, CH_2Ph), 6.90 (1 H, s, CH), 7.40 (10 H, s, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 15.0 (CH_3), 67.0 and 67.9 (CH_2), 127.2, 127.3 and 128.9 (Ar-CH), 129.1 (CH=), 135.9 and 136.0 (Ar-CH), 144.7 (quaternary C=), 166.1 and 167.8 (CO); *m/z* (CI) 311 (72%, $[M + H]^+$)

Dibenzyl *trans*-(2*S,3*S**)-2-methyl-*N*-phthalyl aziridine 2,3-dicarboxylate (243)**



To a stirred ice-cooled solution of dibenzyl mesaconate (**242**) (0.646 g, 2.1 mmol) and *N*-aminophthalamide (0.371 g, 2.28 mmol) in dry DCM (20 cm^3) was added by portion $Pb(OAc)_4$ (1 g, 2.25 mmol). The mixture was stirred at 0 °C for 30 min and left to stir overnight at room temperature. The resulting yellow solution was filtered through Celite and the solvent was removed under reduced pressure to give a residue which was dissolved in ethyl acetate. The resulting solution was washed with water (50 cm^3) and the aqueous phase was extracted with ethyl acetate (2 x 25 cm^3). The combined organic extracts were washed with brine, dried ($MgSO_4$) and the solvent was removed under reduced pressure to yield a thick yellow oil which was purified by silica gel chromatography using ethyl acetate-petroleum ether (3:7) as the eluant to afford *N*-phthalamido aziridine (**243**) as a colourless oil (0.6 g, 60%), (Found $[M + H]^+$ 472.1645. Requires for $C_{27}H_{23}N_2O_6$ 472.1635; υ_{max} (thin film)/cm^{-1} 2955, 1737 (large, CO), 1625 (very small), 1449, 1390, 1303, 1206, 1147 and 713; δ_H (200 MHz; C^2HCl_3) 1.74 (3 H, s, CH_3), 4.31 (1 H, s, CH), 5.08 (2 H, AB, CH_2), 5.44 (2 H, s, CH_2), 7.35 (11 H, m, Ar-H) and 7.70 (3 H, m, Ar-CH); δ_C (50.31 MHz; C^2HCl_3) 14.6 (CH_3), 50.1 (C-3), 50.9 (CH), 68.1 (CH_2), 68.9 (CH_2), 123.0, 129.0, 129.1, 130.4, 134.6 and 135.5 (Ar-CH), 164.5 (CO amide) and 166.1 and 166.4 (CO ester); *m/z* (CI) 472 (10%, $[M + H]^+$), 295 (20) and 148 (100, $[PhtNH_2]^+$).

trans-(2_S_*,3_S_*)-2-Methyl-_N_-phtalamido aziridine 2,3-dicarboxylic acid (244)



A solution of the dibenzyl ester (**242**) (0.6 g, 1.35 mmol) in ethyl acetate (15 cm^3) and 10% Pd/C (5%/weight) was stirred under 1 atm of H_2 for 48 h. The catalyst was then filtered-off through Celite and the filtrate was concentrated under reduced pressure to yield a pale yellow solid which was recrystallised from diethyl ether (0.3 g, 80%), mp 140 ℃ (decomp.); (Found $[M + H]^+$ 291.0629. Requires for $C_{13}H_{11}N_2O_6$ 291.0617); υ_{max} (Nujol)/cm^{-1} 3500-2600 (OH acid), 2926, 2361, 1736 (multiple bands, CO), 1459, 1381, 1264, and 1157; δ_H (200 MHz; C^2HCl_3) 1.4 (3 H, s, CH_3), 3.85 (1 H, s, CH) and 7.45 (4 H, s, Ar-H); δ_C (50.31 MHz; C^2HCl_3) 18.9 (CH_3), 53.7 (C-3), 55.0 (CH), 81.7, 82.3 and 82.9 (Ar-C quaternary), 168.6 (CON) and 172.4 and 172.7 (CO_2H) ; *m/z* (CI), 291 (5%, $[M + H]^+$), 203 (65) and 148 (100, $[PhtNH_2]^+$).

5.2 Enzyme Kinetics

3-Methylaspartase was purified by S. Richardson from an *E. Coli.* cell growth containing the pSG4 plasmid. The specific activity of the enzyme used in the kinetic studies, was 46-92 units (mg of protein)$^{-1}$.

5.2.1 β-Methylaspartase activity

An aliquot (5 mm^3) of suitably diluted enzyme solution was added to assay buffer (total vol. 1 cm^3, pH 9) containing 3-methyl aspartase (4 mmol dm^{-3}), Tris.HCl (500 mmol dm^{-3}, pH 9), magnesium chloride hexahydrate (20 mmol dm^{-3}), potassium chloride (1 mmol dm^{-3}) in a quartz cuvette (10 mm pathlength) at 30°C. The increase in absorbance at 240 nm due to the production of mesaconic acid (ε 3850 mol^{-1}cm^{-1}, pH 9) was monitored. One unit of enzyme corresponds to the formation of 1 μmol of mesaconic acid in 1 minute.

5.2.2 General Procedure for the kinetic Inhibition studies

Incubations of the volume range 1-3 cm^3 containing Tris.HCl (500 mmol. dm^{-3}), magnesium chloride (20 mmol dm^{-3}), potassium chloride (1 mmol dm^{-3}), 3-methylaspartic acid (1.00, 1.25, 1.67, or 5 mmol dm^{-3}) and the inhibitor (0-5 mmol dm^{-3}) were pre-equilibrated at 30°C. The reactions were initiated by addition of 3-methylaspartase and were followed spectrophotometrically at 240 nm in thermostated quartz cells of 10 mm path. Each rate determination was performed in triplicate and the kinetic data was analysed graphically using double reciprocal plots. The K^{app}_m.values were calculated using non-regression analysis with the software Enzfitter.[176] The fitted parameters were then used to draw a line through the data points on the double reciprocal plots.

Appendix

Atomic coordinates of:

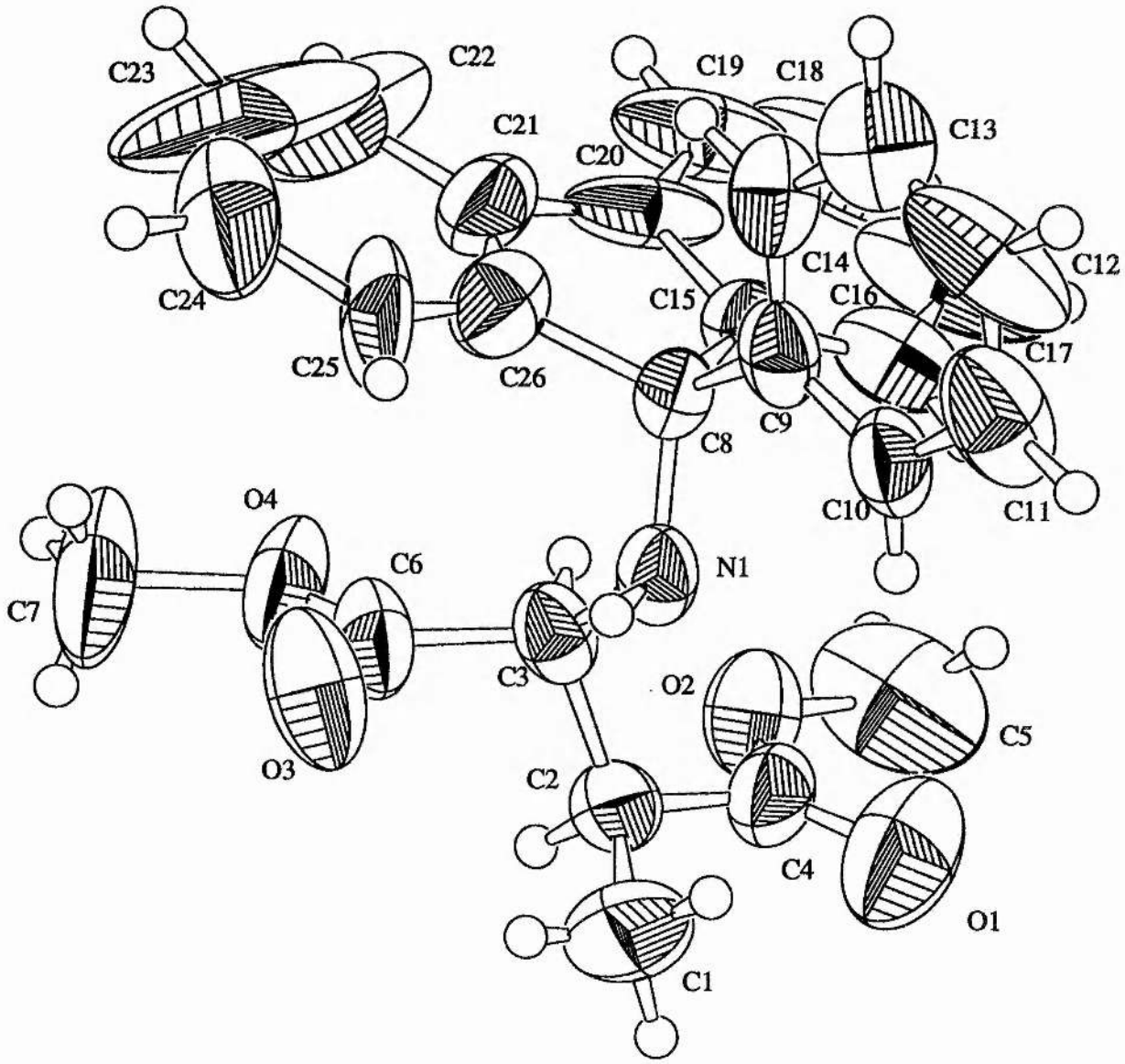
1. Dimethyl (2*S*,3*S*)-*N* -(9'-(9'-phenylfluorenyl)-3-methylaspartate (**179**)

2. Dimethyl (2*S*,3*S*)-*N*-[(1S',4S')-camphanoyl]-3-chloromethyl-3-methylaspartate (**190**)

3. Dimethyl (2*S*,3*S*)- *N* -(9'-(9'-phenylfluorenyl)-3-(*tert*-butyl)-oxycarbonyl-3-methylaspartate (**208**)

4. Dimethyl *transi*-(2*S*,3*R*)-*N*-((9'-(9'-phenylfluorenyl)-3-methylazetidine 2,3-dicarboxylate (**221**)

1 - Dimethyl (2*S*,3*S*)-*N* -(9-(9-phenylfluorenyl)-3-methylaspartate (179)



Data Collection

A colorless block crystal of $C_{26}H_{25}O_4N$ having approximate dimensions of 0.50 x 0.40 x 0.30 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7S diffractometer with graphite monochromated Mo-Kα radiation.

Cell constants and an orientation matrix for data collection , obtained from a least-squares refinement using the setting angles of 20 carefully centered reflections in the range $6.51 < 2\theta < 11.82°$ corresponded to a primitive monoclinic cell with dimensions:

$a = 9.461(3)\ \AA$

$b = 10.716(4)\ \AA \qquad \beta = 97.21(2)°$

$c = 11.350(2)\ \AA$

$V = 1141.5(6)\ \AA^3$

For Z = 2 and F.W. = 415.49, the calculated density is 1.21 g/cm^3. Based on the systematic absences of:

0k0: $k \neq 2n$

packing considerations, a statistical analysis of intensity distribution, and the successful solution and refinement of the structure, the space group was determined to be:

$P2_1$ (#4)

The data were collected at a temperature of 20 $\pm$ 1°C using the ω-2θ scan technique to a maximum 2θ value of 50.0°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.28° with a take-off angle of 6.0°. Scans of $(1.68 + 0.35 \tan \theta)°$ were made at a speed of 16.0°/min (in omega). The weak reflections ($I < 15.0\sigma(I)$) were rescanned (maximum of 4 scans) and the counts were accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm and the crystal to detector distance was 235 mm, The computer-controlled slits were set to 9.0 mm (horizontal) and 13.0 mm (vertical).

Data Reduction

Of the 2271 reflections which were collected, 2137 were unique ($R_{int} = 0.035$). The intensities of three representative reflection were measured after every 150 reflections. No decay correction was applied.

The linear absorption coefficient, μ, for Mo-Kα radiation is 0.8 cm^{-1}. Azimuthal scans of several reflections indicated no need for an absorption correction. The data were corrected for Lorentz and polarization effects. A correction for secondary extinction was applied (coefficient = 4.58136e-06).

A. Crystal Data

Empirical Formula	$C_{26}H_{25}O_4N$
Formula Weight	415.49
Crystal Color, Habit	colorless, block
Crystal Dimensions	0.50 X 0.40 X 0.30 mm
Crystal System	monoclinic
Lattice Type	Primitive
No. of Reflections Used for Unit Cell Determination (2θ range)	20 (6.5 - 11.8°)
Omega Scan Peak Width at Half-height	0.28°
Lattice Parameters	a = 9.461(3)Å b = 10.716(4) Å c = 11.350(2) Å β = 97.21(2)° V = 1141.5(6) Å^3
Space Group	$P2_1$ (#4)
Z value	2
D_{calc}	1.209 g/cm^3
F_{000}	440.00
μ(MoKα)	0.81 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku AFC7S

Table 1. Atomic coordinates and B_{iso}/B_{eq}

atom	x	y	z	B_{eq}
O(1)	0.3466(7)	-0.0236	0.5470(6)	10.3(3)
O(2)	0.1919(6)	0.120(1)	0.4705(6)	7.3(2)
O(3)	-0.1085(7)	-0.026(1)	0.7869(7)	9.6(3)
O(4)	-0.1723(5)	0.122(1)	0.6526(5)	7.4(2)
N(1)	0.1781(6)	0.082(1)	0.7916(5)	4.7(2)
C(1)	0.143(1)	-0.150(1)	0.654(1)	8.7(3)
C(2)	0.1112(8)	-0.022(1)	0.5970(7)	5.1(2)
C(3)	0.0698(7)	0.077(1)	0.6884(7)	4.8(2)
C(4)	0.2291(9)	0.023(1)	0.5363(9)	6.2(3)
C(5)	0.299(2)	0.170(2)	0.4037(9)	12.4(5)
C(6)	-0.0790(9)	0.050(1)	0.7176(9)	6.3(3)
C(7)	-0.3279(8)	0.107(2)	0.6768(9)	12.1(4)
C(8)	0.1945(7)	0.204(1)	0.8540(6)	4.0(2)
C(9)	0.3070(8)	0.193(1)	0.9596(8)	4.9(2)
C(10)	0.4105(8)	0.104(1)	0.9676(8)	5.9(2)
C(11)	0.520(1)	0.103(1)	1.064(1)	8.9(4)
C(12)	0.520(1)	0.193(2)	1.1489(9)	10.0(4)
C(13)	0.419(1)	0.283(1)	1.1448(9)	7.1(3)
C(14)	0.3136(8)	0.281(1)	1.0502(7)	5.4(2)
C(15)	0.2333(8)	0.303(1)	0.7705(7)	4.5(2)
C(16)	0.347(1)	0.309(1)	0.7108(8)	6.8(3)
C(17)	0.373(2)	0.409(2)	0.640(1)	11.9(6)
C(18)	0.271(3)	0.503(2)	0.629(2)	13.4(8)
C(19)	0.160(2)	0.498(2)	0.681(1)	10.3(5)

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

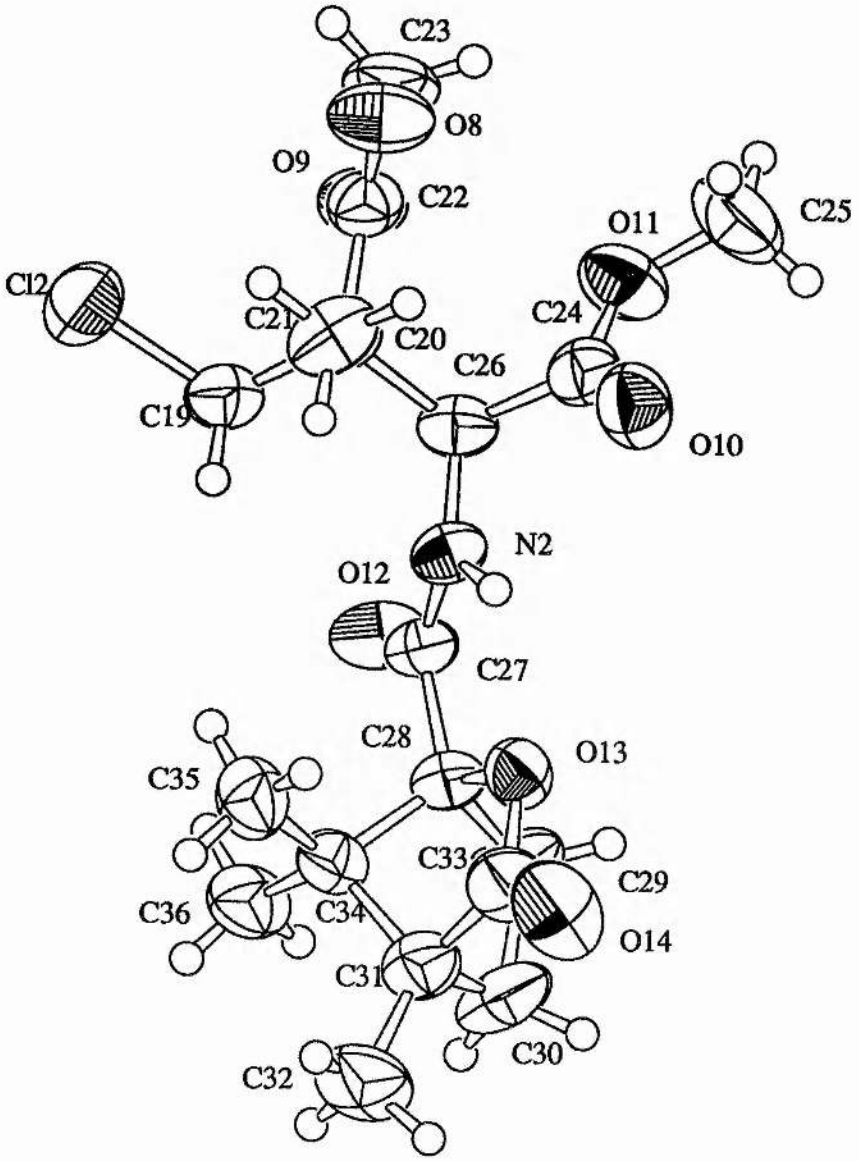
atom	x	y	z	B_{eq}
C(20)	0.133(1)	0.398(1)	0.7562(9)	7.5(3)
C(21)	0.015(1)	0.365(2)	0.825(1)	7.5(3)
C(22)	-0.113(2)	0.430(3)	0.827(2)	17.1(9)
C(23)	-0.177(4)	0.388(4)	0.909(3)	24(1)
C(24)	-0.171(1)	0.277(2)	0.965(2)	14.6(7)
C(25)	-0.0395(9)	0.207(2)	0.9587(9)	9.7(4)
C(26)	0.0471(9)	0.255(1)	0.8855(8)	6.1(3)
H(1)	0.0647	-0.1770	0.6915	10.3952
H(2)	0.1598	-0.2088	0.5938	10.3952
H(3)	0.2259	-0.1450	0.7106	10.3952
H(4)	0.0310	-0.0315	0.5383	6.1790
H(5)	0.0677	0.1559	0.6509	5.7616
H(6)	0.2622	0.2423	0.3614	14.8331
H(7)	0.3232	0.1092	0.3491	14.8331
H(8)	0.3809	0.1922	0.4564	14.8331
H(9)	-0.3351	0.1283	0.7571	14.5417
H(10)	-0.3871	0.1611	0.6257	14.5417
H(11)	-0.3573	0.0233	0.6626	14.5417
H(12)	0.4086	0.0415	0.9077	7.0661
H(13)	0.5918	0.0402	1.0700	10.6261
H(14)	0.5944	0.1923	1.2135	11.9545
H(15)	0.4211	0.3448	1.2050	8.5235
H(16)	0.2414	0.3429	1.0464	6.4998
H(17)	0.4131	0.2412	0.7174	8.1962

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
H(18)	0.4554	0.4141	0.6015	14.3425
H(19)	0.2847	0.5734	0.5802	16.1038
H(20)	0.0933	0.5648	0.6702	12.3326
H(21)	-0.1465	0.4959	0.7748	20.5262
H(22)	-0.2427	0.4446	0.9374	30.1452
H(23)	-0.2460	0.2465	1.0045	17.4644
H(24)	-0.0172	0.1332	1.0033	11.7180
H(25)	0.1546	0.0210	0.8470	5.6024

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(cc^*)^2 + 2U_{12}aa^*bb^*\cos\gamma + 2U_{13}aa^*cc^*\cos\beta + 2U_{23}bb^*cc^*\cos\alpha)$$

2 - Dimethyl (2*S*,3*S*)-*N*-[(1S',4S')-camphanoyl]-3-chloromethyl-3-methylaspartate (190)



Experimental

Data Collection

A colorless block crystal of $C_{18}H_{26}ClNO_7$ having approximate dimensions of 0.50 x 0.40 x 0.35 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7S diffractometer with graphite monochromated Mo-Kα radiation.

Cell constants and an orientation matrix for data collection , obtained from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $23.17 < 2\theta < 24.96°$ corresponded to a primitive monoclinic cell with dimensions:

a = 12.052(5) $\AA$

b = 13.664(4) $\AA$ $\quad \beta = 98.11(3)°$

c = 12.703(4) $\AA$

V = 2071(1) $\AA^3$

For Z = 4 and F.W. = 403.86, the calculated density is 1.29 g/cm^3. Based on the systematic absences of:

0k0: k $\neq$ 2n

packing considerations, a statistical analysis of intensity distribution, and the successful solution and refinement of the structure, the space group was determined to be:

$P2_1$ (#4)

The data were collected at a temperature of 20 $\pm$ 1°C using the ω-2θ scan technique to a maximum 2θ value of 50.0°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.09° with a take-off angle of 6.0°. Scans of (0.94 + 0.35 tan θ)° were made at a speed of 16.0°/min (in omega). The weak reflections ($I < 15.0\sigma(I)$) were rescanned (maximum of 4 scans) and the counts were accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm and the crystal to detector distance was 235 mm, The computer-controlled slits were set to 9.0 mm (horizontal) and 13.0 mm (vertical).

Data Reduction

Of the 4014 reflections which were collected, 3826 were unique ($R_{int} = 0.025$). The intensities of three representative reflection were measured after every 150 reflections. No decay correction was applied.

The linear absorption coefficient, μ, for Mo-Kα radiation is 2.2 cm^{-1}. Azimuthal scans of several reflections indicated no need for an absorption correction. The data were corrected for Lorentz and polarization effects.

A. Crystal Data

Empirical Formula	$C_{18}H_{26}ClNO_7$
Formula Weight	403.86
Crystal Color, Habit	colorless, block
Crystal Dimensions	0.50 X 0.40 X 0.35 mm
Crystal System	monoclinic
Lattice Type	Primitive
No. of Reflections Used for Unit	
Cell Determination (2θ range)	25 (23.2 - 25.0°)
Omega Scan Peak Width	
at Half-height	0.09°
Lattice Parameters	a = 12.052(5)Å b = 13.664(4) Å c = 12.703(4) Å β = 98.11(3)°
	V = 2071(1) $Å^3$
Space Group	$P2_1$ (#4)
Z value	4
D_{calc}	1.295 g/cm^3
F_{000}	856.00
μ(MoKα)	2.21 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku AFC7S

Table 1. Atomic coordinates and B_{iso}/B_{eq}

atom	x	y	z	B_{eq}
Cl(1)	1.5429(1)	0.6003	1.1114(1)	8.00(4)
Cl(2)	1.2703(1)	0.6910(2)	0.2830(1)	6.55(4)
O(1)	1.4551(3)	0.8056(3)	0.9298(3)	6.8(1)
O(2)	1.3506(3)	0.7853(3)	1.0599(2)	5.68(9)
O(3)	1.1755(3)	0.6683(3)	0.7347(2)	5.42(8)
O(4)	1.1976(3)	0.7934(3)	0.8488(2)	6.19(9)
O(5)	1.1446(3)	0.5205(3)	1.0612(2)	5.89(9)
O(6)	1.0895(3)	0.3821(3)	0.8244(2)	6.38(9)
O(7)	1.0604(5)	0.2523(4)	0.7149(3)	12.8(2)
O(8)	1.4843(3)	0.5373(3)	0.4478(3)	7.1(1)
O(9)	1.3667(2)	0.4521(3)	0.3351(2)	5.57(9)
O(10)	1.3029(3)	0.4466(3)	0.6834(3)	6.18(9)
O(11)	1.3663(3)	0.3593(3)	0.5551(3)	6.38(10)
O(12)	1.0034(2)	0.4537(3)	0.4036(2)	5.66(9)
O(13)	0.9730(2)	0.5239(3)	0.6697(2)	4.58(7)
O(14)	0.8976(3)	0.5861(4)	0.8038(2)	8.2(1)
N(1)	1.2037(3)	0.5388(3)	0.9004(3)	3.70(8)
N(2)	1.1264(3)	0.5086(3)	0.5442(3)	4.08(8)
C(1)	1.4020(4)	0.5845(4)	1.0535(3)	5.2(1)
C(2)	1.3713(3)	0.6469(3)	0.9527(3)	4.19(9)
C(3)	1.4328(3)	0.6124(4)	0.8616(4)	5.4(1)
C(4)	1.4000(4)	0.7558(4)	0.9784(4)	4.7(1)
C(5)	1.3617(6)	0.8873(5)	1.0877(5)	8.9(2)
C(6)	1.1999(4)	0.6984(4)	0.8199(4)	4.18(8)

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
C(7)	1.1641(7)	0.8608(5)	0.7615(4)	10.2(2)
C(8)	1.2433(3)	0.6381(3)	0.9201(3)	3.54(8)
C(9)	1.1501(3)	0.4918(3)	0.9717(3)	3.92(9)
C(10)	1.1020(4)	0.3913(4)	0.9372(3)	4.12(8)
C(11)	0.9880(5)	0.3673(5)	0.9674(6)	8.8(2)
C(12)	0.9820(5)	0.2557(5)	0.9452(5)	7.9(2)
C(13)	1.0908(4)	0.2333(4)	0.9065(4)	5.3(1)
C(14)	1.1216(5)	0.1241(4)	0.9045(5)	8.8(2)
C(15)	1.0807(6)	0.2828(4)	0.8019(5)	7.4(1)
C(16)	1.1770(4)	0.3022(4)	0.9776(4)	6.0(1)
C(17)	1.2901(4)	0.3076(4)	0.9372(7)	12.3(2)
C(18)	1.1907(8)	0.2877(5)	1.0952(5)	14.6(3)
C(19)	1.2066(3)	0.5966(4)	0.3537(4)	5.3(1)
C(20)	1.2891(4)	0.5602(4)	0.4506(3)	4.6(1)
C(21)	1.3226(4)	0.6413(4)	0.5314(4)	5.8(1)
C(22)	1.3947(4)	0.5161(4)	0.4125(3)	4.8(1)
C(23)	1.4578(4)	0.4008(5)	0.2993(4)	7.0(2)
C(24)	1.3021(4)	0.4263(4)	0.5935(4)	4.6(1)
C(25)	1.4458(4)	0.3116(5)	0.6322(5)	8.3(2)
C(26)	1.2250(3)	0.4753(3)	0.5027(3)	4.3(1)
C(27)	1.0241(3)	0.4854(3)	0.4939(4)	4.09(9)
C(28)	0.9290(3)	0.5030(4)	0.5603(3)	4.11(9)
C(29)	0.8482(4)	0.4161(4)	0.5598(4)	5.6(1)
C(30)	0.7485(4)	0.4653(4)	0.6077(4)	6.8(1)

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
C(31)	0.7882(4)	0.5719(4)	0.6272(4)	5.4(1)
C(32)	0.6943(4)	0.6419(5)	0.6494(4)	7.7(2)
C(33)	0.8897(4)	0.5643(4)	0.7148(4)	5.3(1)
C(34)	0.8488(3)	0.5907(4)	0.5281(3)	4.45(9)
C(35)	0.9128(4)	0.6879(4)	0.5354(4)	5.9(1)
C(36)	0.7766(4)	0.5835(5)	0.4213(3)	5.9(1)
H(1)	1.3901	0.5175	1.0354	6.2168
H(2)	1.3546	0.6026	1.1042	6.2168
H(3)	1.4146	0.5460	0.8456	6.5564
H(4)	1.4107	0.6516	0.8003	6.5564
H(5)	1.5114	0.6185	0.8823	6.5564
H(6)	1.4390	0.9030	1.1050	10.6870
H(7)	1.3296	0.9259	1.0291	10.6870
H(8)	1.3244	0.8998	1.1472	10.6870
H(9)	1.1650	0.9258	0.7881	12.1368
H(10)	1.2149	0.8555	0.7111	12.1368
H(11)	1.0907	0.8447	0.7287	12.1368
H(12)	1.2084	0.6632	0.9771	4.2329
H(13)	0.9843	0.3814	1.0400	10.6343
H(14)	0.9298	0.4015	0.9239	10.6343
H(15)	0.9755	0.2203	1.0084	9.4432
H(16)	0.9206	0.2403	0.8925	9.4432
H(17)	1.1262	0.0978	0.9742	10.6204
H(18)	1.0656	0.0900	0.8584	10.6204

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

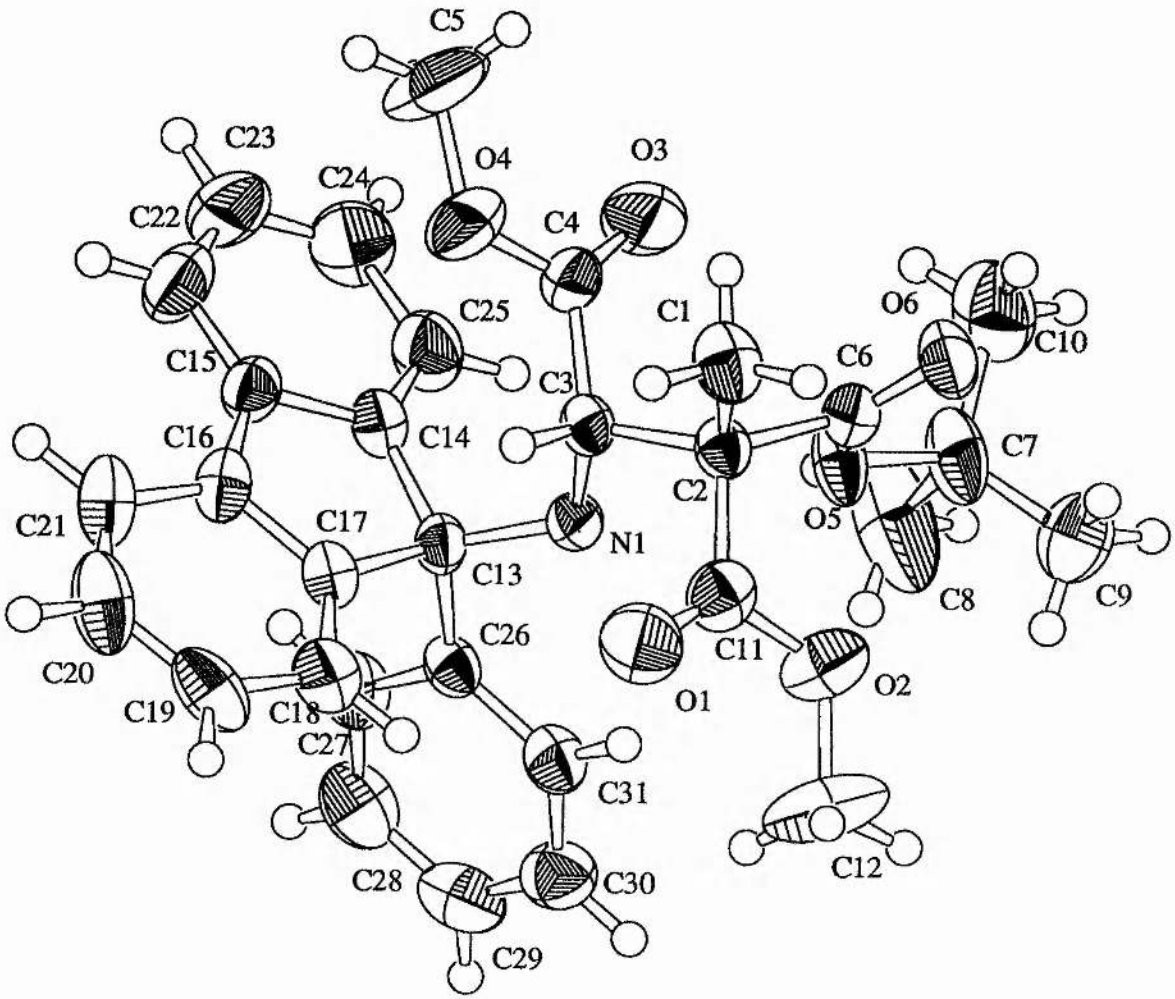
atom	x	y	z	B_{eq}
H(19)	1.1918	0.1168	0.8795	10.6204
H(20)	1.3296	0.2481	0.9534	14.7835
H(21)	1.2787	0.3173	0.8624	14.7835
H(22)	1.3323	0.3605	0.9707	14.7835
H(23)	1.1191	0.2847	1.1179	17.4880
H(24)	1.2302	0.2286	1.1130	17.4880
H(25)	1.2319	0.3411	1.1294	17.4880
H(26)	1.1414	0.6221	0.3782	6.3794
H(27)	1.1863	0.5432	0.3073	6.3794
H(28)	1.3701	0.6151	0.5906	6.9480
H(29)	1.3611	0.6913	0.4992	6.9480
H(30)	1.2572	0.6678	0.5543	6.9480
H(31)	1.4975	0.3650	0.3568	8.4108
H(32)	1.4297	0.3570	0.2439	8.4108
H(33)	1.5071	0.4464	0.2734	8.4108
H(34)	1.4075	0.2794	0.6827	9.9262
H(35)	1.4868	0.2651	0.5978	9.9262
H(36)	1.4958	0.3588	0.6674	9.9262
H(37)	1.2029	0.4277	0.4498	5.2001
H(38)	0.8250	0.3927	0.4897	6.7781
H(39)	0.8805	0.3642	0.6032	6.7781
H(40)	0.6812	0.4628	0.5586	8.2546
H(41)	0.7369	0.4346	0.6722	8.2546
H(42)	0.7240	0.7059	0.6618	9.2050

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
H(43)	0.6370	0.6428	0.5899	9.2050
H(44)	0.6640	0.6201	0.7104	9.2050
H(45)	0.9578	0.6925	0.6029	7.0580
H(46)	0.9591	0.6905	0.4810	7.0580
H(47)	0.8609	0.7406	0.5270	7.0580
H(48)	0.7252	0.6366	0.4130	7.0736
H(49)	0.8228	0.5859	0.3666	7.0736
H(50)	0.7366	0.5235	0.4170	7.0736
H(51)	1.206(3)	0.512(3)	0.834(3)	3.5(9)
H(52)	1.120(3)	0.522(3)	0.619(3)	4.4(9)

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(cc^*)^2 + 2U_{12}aa^*bb^*\cos\gamma + 2U_{13}aa^*cc^*\cos\beta + 2U_{23}bb^*cc^*\cos\alpha)$$

3 - Dimethyl (2*S*,3*S*)- *N* -(9-(9-phenylfluorenyl)-3-(*tert*-butyl)-oxycarbonyl-3-methylaspartate (208)



Experimental

Data Collection

A colorless block crystal of $C_{31}H_{32}NO_6$ having approximate dimensions of 0.50 x 0.40 x 0.35 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7S diffractometer with graphite monochromated Mo-Kα radiation.

Cell constants and an orientation matrix for data collection , obtained from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $22.39 < 2\theta < 24.89°$ corresponded to a primitive monoclinic cell with dimensions:

a = 9.222(3) $\AA$

b = 15.897(3) $\AA$ $\quad \beta = 109.78(2)°$

c = 10.357(2) $\AA$

V = 1428.7(6) $\AA^3$

For Z = 2 and F.W. = 514.60, the calculated density is 1.20 g/cm^3. Based on the systematic absences of:

0k0: k $\neq$ 2n

packing considerations, a statistical analysis of intensity distribution, and the successful solution and refinement of the structure, the space group was determined to be:

$P2_1$ (#4)

The data were collected at a temperature of 20 $\pm$ 1°C using the ω-2θ scan technique to a maximum 2θ value of 50.0°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.31° with a take-off angle of 6.0°. Scans of (1.63 + 0.35 tan θ)° were made at a speed of 16.0°/min (in omega). The weak reflections ($I < 15.0\sigma(I)$) were rescanned (maximum of 4 scans) and the counts were accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm and the crystal to detector distance was 235 mm, The computer-controlled slits were set to 9.0 mm (horizontal) and 13.0 mm (vertical).

Data Reduction

Of the 2778 reflections which were collected, 2610 were unique ($R_{int} = 0.036$). The intensities of three representative reflection were measured after every 150 reflections. No decay correction was applied.

The linear absorption coefficient, μ, for Mo-Kα radiation is 0.8 cm^{-1}. Azimuthal scans of several reflections indicated no need for an absorption correction. The data were corrected for Lorentz and polarization effects. A correction for secondary extinction was applied (coefficient = 2.47835e-06).

Table 1. Atomic coordinates and B_{iso}/B_{eq}

atom	x	y	z	B_{eq}
O(1)	0.7998(5)	0.2699	0.3752(4)	6.0(1)
O(2)	0.5437(5)	0.2656(4)	0.3193(4)	5.4(1)
O(3)	0.6499(5)	0.5564(4)	0.5346(5)	6.3(1)
O(4)	0.9042(4)	0.5435(4)	0.5834(4)	5.3(1)
O(5)	0.4294(3)	0.4073(4)	0.4188(3)	4.14(10)
O(6)	0.4273(4)	0.4564(4)	0.2142(4)	4.33(10)
N(1)	0.7011(4)	0.3688(4)	0.6132(4)	2.91(10)
C(1)	0.7384(6)	0.4428(5)	0.2750(5)	4.4(2)
C(2)	0.6646(5)	0.3994(4)	0.3706(5)	2.9(1)
C(3)	0.7575(5)	0.4198(4)	0.5223(5)	2.7(1)
C(4)	0.7599(7)	0.5133(4)	0.5475(5)	3.7(1)
C(5)	0.9210(9)	0.6334(5)	0.6169(8)	8.3(2)
C(6)	0.4913(6)	0.4256(4)	0.3245(6)	3.4(1)
C(7)	0.2599(6)	0.4166(5)	0.3927(6)	5.0(2)
C(8)	0.2482(7)	0.3862(7)	0.5242(8)	9.5(3)
C(9)	0.1730(7)	0.3617(6)	0.2716(8)	6.4(2)
C(10)	0.2183(7)	0.5085(6)	0.3685(8)	6.7(2)
C(11)	0.6802(7)	0.3051(4)	0.3582(5)	3.9(2)
C(12)	0.550(1)	0.1742(5)	0.3142(9)	8.2(3)
C(13)	0.7991(5)	0.3598(4)	0.7585(5)	2.8(1)
C(14)	0.8258(6)	0.4400(4)	0.8424(5)	3.3(1)
C(15)	0.9858(6)	0.4571(4)	0.9000(5)	3.5(1)
C(16)	1.0691(6)	0.3901(5)	0.8603(5)	3.4(1)
C(17)	0.9658(6)	0.3329(4)	0.7771(5)	3.0(1)

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

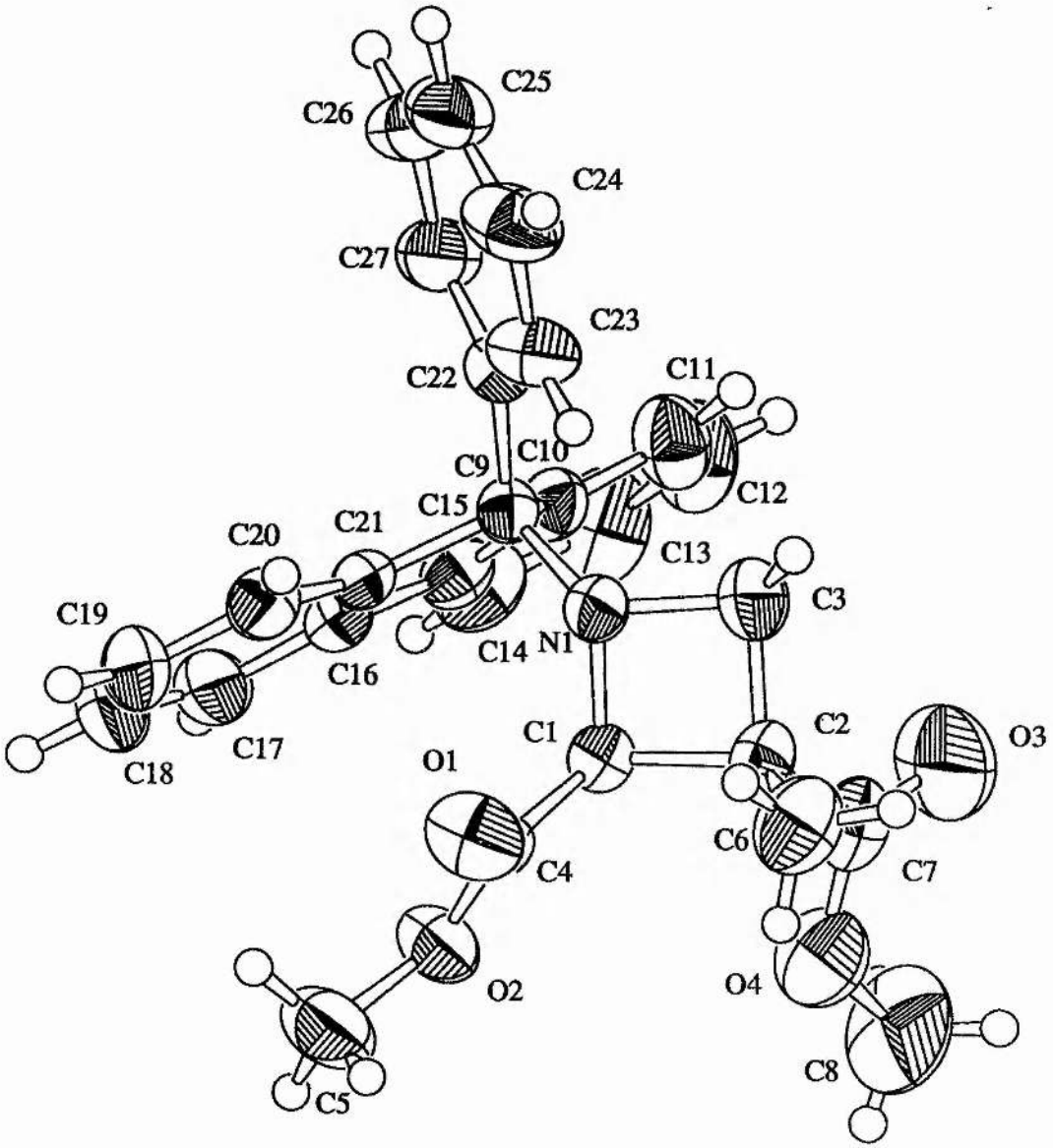
atom	x	y	z	B_{eq}
C(18)	1.0160(6)	0.2631(5)	0.7280(5)	4.2(1)
C(19)	1.1751(7)	0.2490(5)	0.7662(6)	4.8(2)
C(20)	1.2777(6)	0.3050(6)	0.8506(6)	5.5(2)
C(21)	1.2285(6)	0.3748(5)	0.8983(5)	4.8(2)
C(22)	1.0361(7)	0.5278(5)	0.9794(6)	5.1(2)
C(23)	0.9298(9)	0.5811(5)	1.0044(7)	6.1(2)
C(24)	0.7733(8)	0.5635(5)	0.9507(7)	5.9(2)
C(25)	0.7224(7)	0.4927(5)	0.8684(6)	4.9(2)
C(26)	0.7279(5)	0.2904(4)	0.8210(5)	3.0(1)
C(27)	0.7667(6)	0.2830(5)	0.9614(5)	4.2(2)
C(28)	0.7127(7)	0.2171(5)	1.0187(6)	5.0(2)
C(29)	0.6152(7)	0.1583(5)	0.9368(7)	4.9(2)
C(30)	0.5742(6)	0.1648(5)	0.7979(7)	4.5(2)
C(31)	0.6291(6)	0.2306(5)	0.7392(5)	3.7(1)
H(1)	0.7330	0.5020	0.2838	5.3161
H(2)	0.6844	0.4269	0.1827	5.3161
H(3)	0.8431	0.4260	0.2995	5.3161
H(4)	0.8609	0.4031	0.5378	3.2702
H(5)	0.8876	0.6441	0.6926	9.9541
H(6)	0.8603	0.6651	0.5399	9.9541
H(7)	1.0262	0.6491	0.6401	9.9541
H(8)	0.3093	0.4208	0.5971	11.4092
H(9)	0.2840	0.3298	0.5396	11.4092
H(10)	0.1437	0.3885	0.5201	11.4092

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
H(11)	0.2021	0.3046	0.2924	7.6598
H(12)	0.1972	0.3785	0.1932	7.6598
H(13)	0.0654	0.3674	0.2532	7.6598
H(14)	0.1111	0.5155	0.3518	7.9981
H(15)	0.2424	0.5278	0.2914	7.9981
H(16)	0.2751	0.5400	0.4473	7.9981
H(17)	0.6040	0.1529	0.4032	9.8431
H(18)	0.6006	0.1577	0.2525	9.8431
H(19)	0.4476	0.1523	0.2835	9.8431
H(20)	0.9446	0.2249	0.6692	4.9784
H(21)	1.2122	0.2006	0.7335	5.7674
H(22)	1.3851	0.2946	0.8759	6.5633
H(23)	1.3005	0.4129	0.9568	5.7067
H(24)	1.1430	0.5399	1.0170	6.1697
H(25)	0.9641	0.6301	1.0586	7.2701
H(26)	0.7011	0.5996	0.9701	7.1274
H(27)	0.6153	0.4811	0.8304	5.8805
H(28)	0.8315	0.3241	1.0195	5.0596
H(29)	0.7434	0.2122	1.1157	6.0287
H(30)	0.5766	0.1135	0.9766	5.8324
H(31)	0.5075	0.1240	0.7407	5.3686
H(32)	0.5990	0.2347	0.6422	4.4094

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(cc^*)^2 + 2U_{12}aa^*bb^*\cos\gamma + 2U_{13}aa^*cc^*\cos\beta + 2U_{23}bb^*cc^*\cos\alpha)$$

4 - Dimethyl *trans*-(2*S*,3*R*)-*N*-((9-(9-phenylfluorenyl)-3-methylazetidine 2,3-dicarboxylate (221)



EXPERIMENTAL DETAILS

A. Crystal Data

Empirical Formula	$C_{27}H_{25}NO_4$
Formula Weight	427.50
Crystal Color, Habit	colorless, block
Crystal Dimensions	0.40 X 0.35 X 0.35 mm
Crystal System	orthorhombic
Lattice Type	Primitive
No. of Reflections Used for Unit	
Cell Determination (2θ range)	20 (6.7 - 12.7°)
Omega Scan Peak Width	
at Half-height	0.26°
Lattice Parameters	a = 10.801(7)Å b = 21.728(6) Å c = 9.916(7) Å
	V = 2327(1) Å^3
Space Group	$P2_12_12_1$ (#19)
Z value	4
D_{calc}	1.220 g/cm^3
F_{000}	904.00
μ(MoKα)	0.82 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku AFC7S
Radiation	MoKα (λ = 0.71069 Å)

Table 1. Atomic coordinates and B_{iso}/B_{eq}

atom	x	y	z	B_{eq}
O(1)	0.6978(3)	0.1466(1)	0.3697(3)	5.52(9)
O(2)	0.5597(3)	0.0966(1)	0.4962(3)	4.80(8)
O(3)	0.5584(5)	-0.0686(2)	0.1112(4)	9.5(1)
O(4)	0.6077(4)	-0.0548(1)	0.3256(4)	7.3(1)
N(1)	0.5421(3)	0.1127(1)	0.1410(3)	3.27(8)
C(1)	0.5558(4)	0.0754(2)	0.2648(4)	3.22(9)
C(2)	0.6371(4)	0.0290(2)	0.1820(5)	4.1(1)
C(3)	0.5861(4)	0.0611(2)	0.0554(5)	4.8(1)
C(4)	0.6143(4)	0.1108(2)	0.3800(5)	3.8(1)
C(5)	0.6006(6)	0.1320(2)	0.6122(5)	6.8(2)
C(6)	0.7766(5)	0.0349(2)	0.2016(6)	6.0(1)
C(7)	0.5963(5)	-0.0367(2)	0.1996(6)	5.1(1)
C(8)	0.5664(8)	-0.1170(2)	0.3579(7)	11.0(2)
C(9)	0.4195(4)	0.1420(2)	0.1151(4)	3.15(10)
C(10)	0.3142(4)	0.0958(2)	0.1033(5)	3.7(1)
C(11)	0.2880(5)	0.0554(2)	-0.0002(5)	5.5(1)
C(12)	0.1879(5)	0.0150(2)	0.0151(6)	6.8(2)
C(13)	0.1152(5)	0.0162(3)	0.1282(7)	7.2(2)
C(14)	0.1377(5)	0.0578(2)	0.2301(6)	5.7(1)
C(15)	0.2371(4)	0.0979(2)	0.2163(5)	3.7(1)
C(16)	0.2809(4)	0.1477(2)	0.3044(4)	3.7(1)
C(17)	0.2328(5)	0.1691(2)	0.4256(5)	5.2(1)
C(18)	0.2889(6)	0.2202(3)	0.4822(5)	6.1(2)
C(19)	0.3891(6)	0.2485(2)	0.4230(5)	6.2(2)

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
C(20)	0.4388(4)	0.2259(2)	0.3031(4)	4.4(1)
C(21)	0.3830(4)	0.1751(2)	0.2453(4)	3.28(10)
C(22)	0.4318(4)	0.1866(2)	-0.0025(4)	3.54(10)
C(23)	0.5442(5)	0.2066(2)	-0.0490(5)	4.8(1)
C(24)	0.5518(6)	0.2513(2)	-0.1495(5)	6.1(1)
C(25)	0.4457(6)	0.2775(2)	-0.2001(5)	5.8(1)
C(26)	0.3308(5)	0.2579(3)	-0.1556(5)	6.0(2)
C(27)	0.3237(5)	0.2121(2)	-0.0578(5)	5.0(1)
H(1)	0.4793	0.0574	0.2909	3.8426
H(2)	0.5218	0.0390	0.0113	5.6902
H(3)	0.6476	0.0723	-0.0084	5.6902
H(4)	0.6869	0.1261	0.6249	8.1606
H(5)	0.5842	0.1744	0.5970	8.1606
H(6)	0.5572	0.1186	0.6901	8.1606
H(7)	0.7976	0.0223	0.2903	7.1686
H(8)	0.8178	0.0094	0.1380	7.1686
H(9)	0.8005	0.0765	0.1885	7.1686
H(10)	0.4806	-0.1206	0.3392	13.1694
H(11)	0.6113	-0.1457	0.3048	13.1694
H(12)	0.5809	-0.1249	0.4508	13.1694
H(13)	0.3367	0.0550	-0.0800	6.5610
H(14)	0.1703	-0.0138	-0.0543	8.1246
H(15)	0.0484	-0.0119	0.1361	8.6562
H(16)	0.0866	0.0591	0.3079	6.8530

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
H(17)	0.1645	0.1494	0.4678	6.2228
H(18)	0.2571	0.2362	0.5643	7.2986
H(19)	0.4246	0.2838	0.4642	7.4882
H(20)	0.5087	0.2447	0.2626	5.2176
H(21)	0.6180	0.1899	-0.0121	5.7694
H(22)	0.6304	0.2635	-0.1832	7.2528
H(23)	0.4512	0.3093	-0.2657	6.9953
H(24)	0.2575	0.2756	-0.1915	7.1993
H(25)	0.2450	0.1981	-0.0281	6.0005

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(cc^*)^2 + 2U_{12}aa^*bb^*\cos\gamma + 2U_{13}aa^*cc^*\cos\beta + 2U_{23}bb^*cc^*\cos\alpha)$$

References

1. J. M. Merrick, S. Roseman, *J. Biol. Chem.*, 1960, **235**, 1274-1280.
2. T. J. Carty, B.M. Babior, R. H. Abeles, *J. Biol. Chem.*, 1971, **246**, 6313-6317.
3 R. N. Costilow, L. Laycock, *J. Biol. Chem.*,1971, **246**, 6655-6660.
4. J. C. Rabinowitz, W. E. Pricer, Jr., *J. Am. Chem. Soc.*, 1956, **78**, 5702-5704.
5. P.R. Vagelos, J.M Earl, E. R. Stadtman, *J. Biol. Chem.*, 1959, **234**, 490-497.
6. S. Sawa, A. Tanaka, Yuzoinouye, T. Hirasawa and K. Soda, *Biochem. Biophys. Acta*, 1974, **350**, 354-357.
7. H. J. Bright, R. E. Ludin and L. L. Ingraham, *Biochemistry*, 1964, **3**, 1224-1230.
8. Zon and B. Laber, *Phytochemistry*, 1988, **27**, 711-714.
9. M. Albortz and K. T. Douglas, *J. Chem. Soc., Perkin Trans II*, 1982, 331.
10. F. Bordwell, A. Knipe and K. Yee, *J. Am. Chem. Soc.*, 1970, **92**, 5945.
11. E. Havir and K. Hanson, *The Enzymes*, 1973, 3d ed.P. Boyer. vol. 7, p. 75.
12. J. B. Wolff, *J. Biol. Chem.*, 1962, **237**, 874.
13. J. H. Quastel and B. Woolf, *Biochem. J.*, 1926, **20**, 545.
14. A. I. Virtanen and J. Tarnanen, *Biochemistry*, 1932, **250**, 193.
15. Y. Kurata, *Exptl. Cell. Res.*, 1962, **28**, 424.
16. Y. Halper and H. Umbarger, *J. Bacteriol.*, 1960, **80**, 285.
17. J. S. Takagi, N. Ida, M. Tokushige, H. Sakamoto and Y. Shimura, *Nucleic Acids Res.*, 1985, **13**, 2063-2074.
18. W. V. R. Williams and D. J. Lartigues, *J. Biol. Chem.*, 1967, **242**, 2973-2978.
19. V. R. Williams and J. M. Lartigue, *J. Biol. Chem.*, 1959, **234**, 490.
20. T. F. Emery, *Biochemistry*, 1963, **2**, 1041.
21. C. J. Falzone, W. E. Karsten, J. D. Conley and R. E. Viola, *Biochemistry*, 1988, **27**, 9089-9093.
22. N. Ida and M. Tokushige, *J. Biochem.*, 1984, **96**, 1315-1321.
23. W. E. Karsten, J. R. Hunsley and R. E. Viola, *Anal. Biochim.*, 1985, **287**, 60-67.
24. K. Mizuta and M. Tokushige, *Biochem. Biophys. Acta*, 1975, **403**, 221-231.
25. N. Ida and M. Tokushige, *J. Biochem.*, 1985, **98**, 793-797.
26. S. Murase, J. S. Takagi, Y. Higashi, H. Imaishi, N. Yumoto and M. Tokushige, *Biochem. Biophys. Res. Commun.I*, 1991, **177**, 414-419.
27. J. F. Schindler and R. E. Viola, *Biochemistry*, 1994, **33**, 9365-9370.

28. F. Giorgianni, S. Beranová, C. Wesdemiotis and R. Viola, *Biochemistry*, 1995, **34**, 3529-3535.
29. W. Shi, J. Dunbar, M. K. Jayasekera, R. E. Viola and G. K. Farber, *Biochemistry*, 1997, **36**, 9136-9144.
30. M. K. Jayasekera, W. Shi, G. K. Farber and R. E. Viola, *Biochemistry*, 1997, **36**, 9145-9150.
31. I. Nuiry, J. D. Hermes, P. D. Weiss, C-Y. Chan and P. J. Cook, *Biochemïstry*, 1984, **23**, 5168-5175.
32. D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, 1980, **255**, 4772.
33. J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, **236**, 2692-2698.
34. K. R. Hanson and E. A. Havir, in 'The biochemistry of plants', ed. P. D. Stumpf and E. E. Conn., Academic Press New-York, 1981, **7**, 577-625.
35. K. Hahlbrock and D. Scheel, *Annu. Rev. Plant. Physiol. Mol. Biol.*, 1989, **40**, 237- 369.
36. H. J. Gilbert and M. Tully, *J. Bacteriol.*, 1982, **150**, 498-505.
37. K. R. Hanson and E. A. Havir, *Biochemistry*, 1973, **12**, 1583-1591.
38. K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1-17.
39. P. V. Subba Rao, K. Moore, and G. H. N. Towers, *Can. J. Biochem.*, 1967, **45**, 1863.
40. A. Conway and D. E. Koshland, *Biochemistry*, 1968, **7**, 4011.
41. K. R. Hanson and E. A. Havir, *Biochemistry*, 1968, **7**, 1904-1914.
42. K. R. Hanson and E. A. Havir, *Methods in Enzymology*, 1970, **17A**, 575-581.
43. K. R. Hanson and E. A. Havir, *Biochemistry*, 1975, **14**, 1620-1626.
44. J. Zon and N. Amrhein, *Liebigs. Ann. Chem.*, 1992, 625-628.
45. K. R. Hanson and E. A. Havir, *The Enzymes*, 1972, vol. VII, p.145, ed. P. B. Boyer, Academic Press.
46. B. Schuster and J. Retey, *FEBS Lett.*, 1994, **349**, 252-254.
47. J. D. Hermes, P. W. Weiss and W. W. Cleland, *Biochemistry*, 1985, **24**, 2959-2967.
48. T. G. Lessie and F. C. Neidhardt, *J. Bacteriol.*, 1967, **93**, 1800.
49. V. G. Zannoni and B. N. La Du, *J. Biol.*, 1963, **88**, 160.
50. H. Ruis and H. Kindl, *Phytochemistry*, 1971, **10**, 2627.
51. B. N. La Du, *Methods in Enzymology*, 1971, **17b**, 895.
52. H. Weissbach, B. G. Redfield and S. J. Undenfriend, *J. Biol. Chem.*, 1957, **229**, 953.

53. W. S. Collins, J. F. Metzger and A. D. Johnson, *J. Immunol.*, 1972, **108**, 852-856.
54. M. M. Rechler, *J. Biol. Chem.*, 1969, **244**, 551-559.
55. I. L. Givot, T. A. Smith and R. H. Abeles, *J. Biol. Chem.*, 1969, **244**, 6341-6353.
56. A. Peterkofsky, *J. Biol. Chem.*, 1962, **237**, 787-795.
57. C. B. Klee, *J. Biol. Chem.*, 1972, **247**, 1938.
58. L. M. Brand and A. E. Harper, *Biochemistry*, 1976, **15**, 1814-1821.
59. P. J. White and K. E. Kendrick, *Biochem. Biophys. Acta*, 1993, **1163**, 273-279.
60. T. A. Smith, F. H. Cordelle and R. H. Abeles, *Arch. Biochem. Biophys.*, 1967, **120**, 724.
61. R. B. Wickner, *J. Biol. Chem.*, 1969, **244**, 6550-6552.
62. D. Hernandez, J. G. Stroh and A. T. Philips, *Arch. Biochem. Biophys.*, 1993, **307**, 126-132.
63. M. Langer, G. Reck, J. Reed and J. Retey, *Biochemistry*, 1994, **33**, 6462-6467.
64. M. Langer, A. Lieber and J. Retey, *Biochemistry*, 1994, **33**, 14034-14038.
65. C. B. Klee, K. L. Kirk, L. A. Cohen and P. McPhie, *J. Biol. Chem.*, 1975, **250**, 5033-5040.
66. T. Furuta, H. Takahashi and Y. Kasuya, *J. Am. Chem. Soc.*, 1990, **112**, 3633-3636.
67. T. Furuta, H . Takahashi, H. Shibasaki and Y. Kasuya, *J. Biol. Chem.*, 1992, **267**, 12600-12605.
68. D. D. Woods and C. E. Clifton, *Biological Journal*, 1938, **32**, 345.
69. H. A. Barker, R. D. Smyth, R. M. Wilson and H. Weissbach, *J. Biol. Chem.*, 1959, **234**, 320.
70. V. R. Williams and W. Y. Libano, *Biochem. Biophys. Acta*, 1962, **118**, 144.
71. S. K. Goda, N. P. Minton, N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 10747-10756.
72. M. Akhtar, M. A. Cohen and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1986, 1290-1291.
73. M. S. Gulzar, M. Akhtar and D. Gani, *J. Chem. Soc. Perkin Trans 1*, 1997, 649-655.
74. K. Badiani, P. Lightfoot and D. Gani, *J. Chem. Soc. Chem. Commun.*, 1996, 675-677.
75. M. S. Gulzar, Mechanistic and structural studies on 3-methylaspartase ammonia-lyase, Ph. D. Thesis, University of St. Andrews, 1994
76. N. R. Thomas, Ph. D Thesis, University of Southampton

77. G. A. Fields and H. J. Bright, *Biochemistry*, 1970, **9**, 3801.
78. H. J. Bright and L. L. Ingraham, *Biochim. Biophys. Acta*, 1960, **44**, 586.
79. H. J. Bright, *Biochemistry*, 1967, **6**, 1191-1203.
80. H. J. Bright, *J. Biol. Chem.*, 1964, **239**, 2307-2315.
81. H. J. Bright and R. Silverman, *Biochem. Biophyd. Acta*, 1964, **81**, 177-180.
82. N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 1509-1520.
83. H. J. Bright, *J. Biol. Chem.*, 1965, **240**, 1198-1210.
84. H. J. Bright, L. L. Ingraham and R. E. Lundin, *Biochem. Biophys. Acta*, 1964, **81**, 576.
85. C. H. Archer and D. Gani, *J. Chem. Soc. Chem. Commun.*, 1993, 140-142.
86. V. R. Williams and W. Y. Libano, *Biochem. Biophys. Acta*, 1966, **118**, 144-156.
87. M. W. Hsiang and H. J. Bright, *J. Biol. Chem.*, 1967, **242**, 3079-3088.
88. W. T. Wu and V. R. Williams, *J. Biol. Chem.*, 1968, **243**, 5644-5650.
89. T. Neal, M. Ryan and D. Gani, 1996, unpublished rsults
90. P. C. Babbitt, M. S. Hasson, J. E. Wedekind, D. R. J. Palmer, W. C. Barrett, G. H. Reed, I. Rayment, D. Ringe, G. L. Kenyon and J. A. Gerlt, *Biochemistry*, 1996, **35**, 16489-16501.
91. A. T. Kallarakal, B. Mitra, J. W. Kozarich, J. A. Gerlt, J. R. Clifton, G. A. Petsko and G. L. Kenyon, *Biochemistry*, 1995, **34**, 2788.
92. M. Akthar, J. Pollard, S. Richardson, P. Lasry, unpublished results.
93. M. S. Gulzar, K. B. Morris and D. Gani, *J. Chem. Soc. Chem. Commun.*, 1995, 1061.
94. N. P. Botting, M. Akhtar, M. A. Cohen and D. Gani, *J. Chem. Soc.Chem. Commun.*, 1987, p. 1371-1373.
95. N. P. Botting, M. A. Cohen, M. Akhtar and D. Gani, *Biochemistry*, 1988, **27**, 2953.
96. N. P. Botting, A. Jackson and D. Gani, *J. Chem. Soc.Chem. Commun.*, 1989, 1583-1585.
97. M. Langer, A. Pauling and J. Rétey, *Angew. Chem.*, 1995, **107**, 1585-1587.
98. M. Langer, A. Pauling, and J. Rétey, *Angew. Chem. Int. Ed. Engl.*, 1995, **34**, 1464-1465.
99. J. Rétey, *Naturwissenschaften*, 1996, **83**, 439-447.
100. B. Schuster and J. Rétey, *Proc. Natl. Acad. Sci.*, USA, 1995, **92**, 8433-8437.
101. J. Pollard and D. Gani, unpublished results
102. M. Akthar and D. Gani, unpublished results
103. K. Morris and T. Rutherford, unpublished results

104. L. N. Jungheim and S. K. Sigmund, *J. Org. Chem.*, 1987, **52**, 4007-13.
105. R. E. Holmes and D.A. Neel, *Tet. Lett.*, 1990, **39**, 5567-5570.
106. Dictionary of Organic Chemistry, 1982,Chapman & Hall, 5th Edition, vol. 1, pp. 624.
107. Fischer and Suzuki, *Chem. Ber.*, 1907, **40**, 4193.
108. M. Szekerke, *Acta. Chim. Acad. Sci. Hung.*, 1964, **41**, 337-40.
109. *Organic Syntheses*, Collective Volume I, 1932, J. Wiley and Sons, London
110. S. T. Perri, S. C. Slater, S. G. Toske and J. D. White, *J. Org. Chem.*, 1990, **55**, 6037-6047.
111. M. Akhtar and D. Gani, unpublished results
112. D. Horton and M. L. Wolfrom, *J. Am. Chem. Soc.*, 1962, **27**, 1794-1800.
113. Y. Murakami, K. Koga and S-I Yamada, *Chem. Pharm. Bull.*, 1978, **26**, 307-308.
114. A. P. Mehrota, Ph. D. Thesis, University of St. Andrews, 1995.
115. P. Brewster, F. Hiron, E. D. Hugues, C. K. Ingold and P. A. D. Rao, *Nature*, 1950, **166**, 178.
116. a) M. F. Beatty, C. Jennings-White and M. A. Avery, *J. Chem. Soc. Chem. Commun.*, 1991, 351-52. b) A. Wagner, M-P. Heitz and C. Mioskowski, *Tetrahedron Lett.*, 1989, **30**, 557-558.
117. R. Rando and F. Bangerter, *J. Am. Chem. Soc.*, 1977, **99**, 5141.
118. C. Teng and B. Ganem, *Tetrahedron Lett.*, 1982, **23**, 313.
119. T. Kondo, H. Nakai and T. Goto, *Tetrahedron Lett.*, 1973, **29**, 1801.
120. M. Hagihara, N. J. Antony, T. Stout, J. Clardy and S. Schreiber, *J. Am. Chem. Soc.*, 1992, **114**, 6568-6569.
121. For a review see, R. B. Cheikh, R. Chaabouni, A. Laurent, P. Misson and A. Nafti, *Synthesis*, 1983, 685-700.
122. R. P. Nelson, J. M. McEuen and R. G. Lawton, *J. Org. Chem.*, 1969, **34**, 1225-29.
123. R. G. Micetich, S. N. Maitti and P. Spevak, *Synthesis*, 1986, 292-296.
124. M. Vaultier, N. Knouzi and R. Carriét, *Tetrahedron Lett.*, 1983, **24**, 763-764.
125. Y. Ohfune and H. Nishio, *Tetrahedron Lett.*, 1984, **35**, 4133.
126. G. J. Hanson, T. J. Lindberg, *J. Org. Chem.*, 1985, **50**, 5399.
127. B. E. Evans, K. E. Rittle, C. F. Homnick, J. P. Springer, J. Hirshfield and D. F. Veber, *J. Org. Chem.*, 1985, **50**, 4615.
128. D. J. Kempf, *J. Org. Chem.*, 1986, **51**, 3921.
129. J. R. Luly, J. F. Dellaria, J. J. Plattner, J. L. Soderquist and N. J. Yi, *J. Org. Chem.*, 1987, **52**, 1487.

130. S. Danishefsky, S. Kobayashi and J. F. Kerwin, *J. Org. Chem.*, 1982, **47**, 1981.
131. S. Nahm and S. Weireb, *Tetrahedron. Lett.*, 1981, **22**, 3815.
132. Y. Hamada and T. Shioiri, *Chem. Pharm. Bull.*, 1982, **30**, 1921.
133. A. Whol and M. Losantisch, *Ber*, 1905, **38**, 4170.
134 C. Mannich, B. Lesser and F. Silten, *Ber*, 1932, **65**, 378.
135. K. Brands and U. Pandit, *Heterocycles*, 1990, **30**, 257.
136. A. Whol and M. Losantisch, *Ber.*, 1905, **38**, 4170.
137. A. Chesney and I. Mako, *Synth. Commun.*, 1990, **20**, 3167.
138. O. Moe and D. Warner, *J. Am. Chem. Soc.*, 1949, **71**, 1251.
139. J-P. Wolf and H. Rapoport, *J. Org. Chem.*, 1989, **54**, 3164-3173.
140. W. D. Lubell and H. Rapoport, *J. Org. Chem.*, 1985, **50**, 1239
141. P. Gmeiner, P. L. Feldman, M. Y. Chu-Moyer and H. Rapoport, *J. Org. Chem.*, 1990, **55**, 3068-3074.
142. J. March, Advanced Organic Chemistry, 4ed, 1992, Wiley-Interscience, p. 886-887.
143. N. Kornblum and A. S. Erickson, *J. Org. Chem.*, 1981, **46**, 1037-1039.
144. a) C. R. Davis, D. C. Swenson and J. Burton, *J. Org. Chem.*, 1993, **58**, 6843-6850. b) L. I. Zakharkin and I. M. Khorlina, *Tetrahedron Lett.*, 1962, **14**, 619-629. c) For a review on DIBAL-H see E. Winterfeldt, *Synthesis*, 1975, 617-630.
145. R. J. Parikh and W. E. Doering, *J. Am. Chem. Soc.*, 1967, **89**, 5505-5507.
146. Jerry March, 4th ed., p. 771
147. Jurczak and Glebiowski, *Chem. Rev.*, 1989, **89**, 149-164.
148. A. Ito, R. Takahashi and Y. Baba, *Chem. Pharm. Bull.*, 1975, **23**, 3081.
149. J. E Baldwin, R. M. Adlington, D. W. Gollins and C. J. Schofield, *Tetrahedron*, 1990, **46**, 4733-4748.
150. J. Jones, *Amino Acid and Peptide synthesis*, Oxford Chemistry Primers, Oxford University Press, pp 23-24.
151. P. J. Peterson and L. Fowden, *Nature*, 1963, **200**, 148.
152. E. Juaristi and D. Madrigal, *Tetrahedron*, 1989, **45**, 629.
153. J. A. Moore, *Heterocycle Compounds with Three- and Four- Membered Ring*, Part 2, Chap. VII, J. Wiley & Sons, 1964, pp887,900 and 902.
154. A. Martino, C. Galli, P. Gargano and L. Mandolini, *J. Chem. Soc. Perkin Trans. II*, 1985, 1345.
155. E. Doomes and N. Cromwell, *J. Org. Chem.*, 1968, 310.
156. N. H. Cromwell and B. Philips, *Chem. Rev.*, 1979, **79**, 331-358.
157. J. Hoshiro, J. Hiraoka, Y. Hata, S. Sawada and Y. Yamamoto, *J. Chem. Soc. Perkin Trans*, 1995, 693.

158. B. D. Christie and H. Rapoport, *J. Org. Chem.*, 1985, **50**, 1239-1246.
159. a) Forlani, Marianucci, Todesco, *J. Chem. Res.*, 1984, 126; b) Chudeck, Foster and Young, *J. Chem. Soc. Perkin Trans. 2*, 1985, 1285.
160. R. Milcent, M. Guevrekian-Soghomoniantz and G. Barbier, *J. Heterocyclic Chem.*, 1986, **23**, 1845-1848.
161. D. J. Anderson, T. L. Gilchrist, D. C. Horwell and C. W. Rees, *J. Chem. Soc. Chem. Comm*, 1969, 146.
162. L. A. Carpino and R.K. Kirkley, *J. Am. Chem. Soc.*, 1969, 1784-1785.
163. D. J. Anderson, T. L. Gilchrist. D. C. Horwell and C. W. Ress, *J. Chem. Soc.*, 1970, 576-582.
164. C. W. Rees, *Helvetica Chimica Acta*, 1972, **55**.
165. S. Wolfe and S. K. Hasan, *Canadian J. Chem.*, 1970, **48**, 3572-3579.
166. T. Kamiya, M. Hashimoto, O. Nakaguchi and T. Oku, *Tetrahedron*, 1979, **35**, 323-328.
167. A. L. Smith, C.-K. Hwang, E. Pitsinos, G. R. Scarlato and K. C. Nicolaou, *J. Am. Chem. Soc.*, 1992, **114**, 3134-3116.
168. K. Burges, K.Ho and D. Moye-Sherman, *Synlett.*, 1994, 575.
169. K. Badiani, Ph. D. thesis, St. Andrews University, 1996.
170. C. H. Stammer, *Tetrahedron*, 1990, **7**, 2231-2254.
171. W. C. Still, M. Khan and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923-2925.
172. DD. Perrin and W. L. F. Armarego, *Purification of Laboratory Chemicals*, 3rd Edition, Pergamon Press
173. M. F. Lipton , C. M. Sorenson and A. C. Sadler, *J. Organomet. Chem.*, 1980, **186**, 155.
174. M. Akhtar, N. P. Botting, M. A. Cohen and D. Gani, *Tetrahedron*, 1987, **43**, 5899-5908.
175. M. Akthar, PhD thesis, 1988, University of Southampton.
176. Enzfitter, a non-linear regression analysis program for the IBM PC, R. J. Leatherbarrow, BIOSOFT, 1987, Cambridge