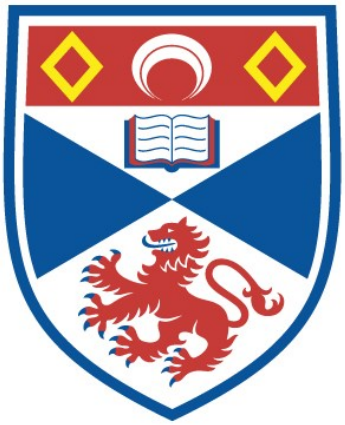
SYNTHESIS AND EVALUATION OF ENZYME INHIBITORS BASED ON AMINO- AND CYCLOPROPANE CARBOXYLIC ACIDS

Kamal Badiani

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1997

SYNTHESIS AND EVALUATION OF ENZYME INHIBITORS BASED ON AMINO- AND CYCLOPROPANE CARBOXYLIC ACIDS.

a thesis presented by

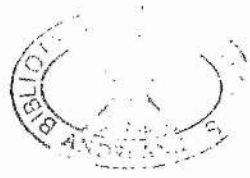
Kamal Badiani

to the

University of St. Andrews

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY



St. Andrews

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I was admitted as a research student in October 1992 and as a candidate for the degree of Ph.D. in October 1993; the higher study for which this is a record was carried out in the university of St. Andrews between 1992 and 1996.

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IN LOVING MEMORY OF MY GRANDFATHER,

SHREE HIRJI PARMANAND KOTECHA

&

TO ALL MY FAMILY

PAST, PRESENT AND FUTURE...

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Abstract

The coenzyme B_{12}- dependent enzyme, glutamate mutase (E. C. 5.4.99.1), catalyses the reversible carbon-skeleton rearrangement of (2*S*)-glutamic acid to (2*S*,3*S*)-3-methylaspartic acid. Glutamate mutase is the first enzyme on the mesaconate pathway. A variety of glutamate and 3-methylaspartate analogues (which also include isotopically labelled molecules), were synthesised as molecular probes of the enzyme.

Synthesis of stereospecifically labelled 3-ethylaspartic acid: (2*S*,3*S*)-[3'-C^2H_3], and (2*S*,3*S*)-[$C^2H_2C^2H_3$]-ethylaspartic acids were constructed using appropriately labelled iodoethane. (2*S*,3*S*)-2-Bromo-3-methylsuccinic acid was synthesised *via* the diazotization of (2*S*,3*S*)-3-methylaspartic acid, in the presence of bromide ion. (2*S*)-Methylsuccinic acid was synthesised by the catalytic hydrogenation of (2*S*,3*S*)-2-bromo-3-methylsuccinic acid. Biological studies of the synthesised compounds (including the labelled isotopomers) displayed no activity against glutamate mutase.

3-Methylaspartate ammonia-lyase, the second enzyme in the mesaconate pathway, catalyses the deamination of (2*S*,3*S*)-3-methylaspartic acid to mesaconic acid. A range of 1-substituted cyclopropane 1,2-dicarboxylic acids were synthesised using short efficient routes and were found to be good to potent inhibitors of 3-methylaspartase. X-ray crystallographic studies have determined the absolute stereochemistry. The mode of action of the most potent inhibitor, (1*S*,2*S*)-1-methylcyclopropane 1,2-dicarboxylic acid (20 μmol dm^{-3}), is consistent with it acting as a transition state analogue for the central substrate deamination reaction catalysed by the enzyme.

β-Amino acids are constituents of many biologically active peptides. A general procedure for the synthesis of α-substituted-β-amino acids has been developed. The synthesis involves a Baylis-Hillman amine catalysed conversion of methyl acrylate, with an appropriate aldehyde, to give the α-(hydroxyalkyl) acrylate. Bromination and subsequent azide displacement furnishes the azido alkene, which is catalytically hydrogenated, to furnish the β-amino ester.

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Abbreviations

PAL - phenylalanine ammonia-lyase

MAL - 3-methylaspartate ammonia-lyase

CBZ - carbobenzyloxy

BOC - ^{t}butoxycarbonyl

DMF - *N,N*-dimethylformamide

THF - tetrahydrofuran

DCM - dichloromethane

TFA - trifluoracetic acid

m-CPBA - *meta*-chloroperbenzoic acid

KHMDS - potassium *bis*(trimethylsilyl)amide

D - deuterium

NAD - nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide (reduced form)

ATP - adenosine tri-phosphate

t.l.c. - thin layer chromatography

h - hour

IR - infra-red spectroscopy

EPR - electron paramagnetic resonance

min - minutes

rt. - room temperature

V_{max} - maximum rate

K_m - michaelis constant, $\upsilon = V_{max}/2$

K_i - dissociation constant of inhibitor

IC_{50} - inhibitor concentration which reduces the rate by 50%.

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

CHAPTER 1

INTRODUCTION

1.1 Glutamic Acid Degradation By Anaerobic Bacteria

1.1.1 Introduction

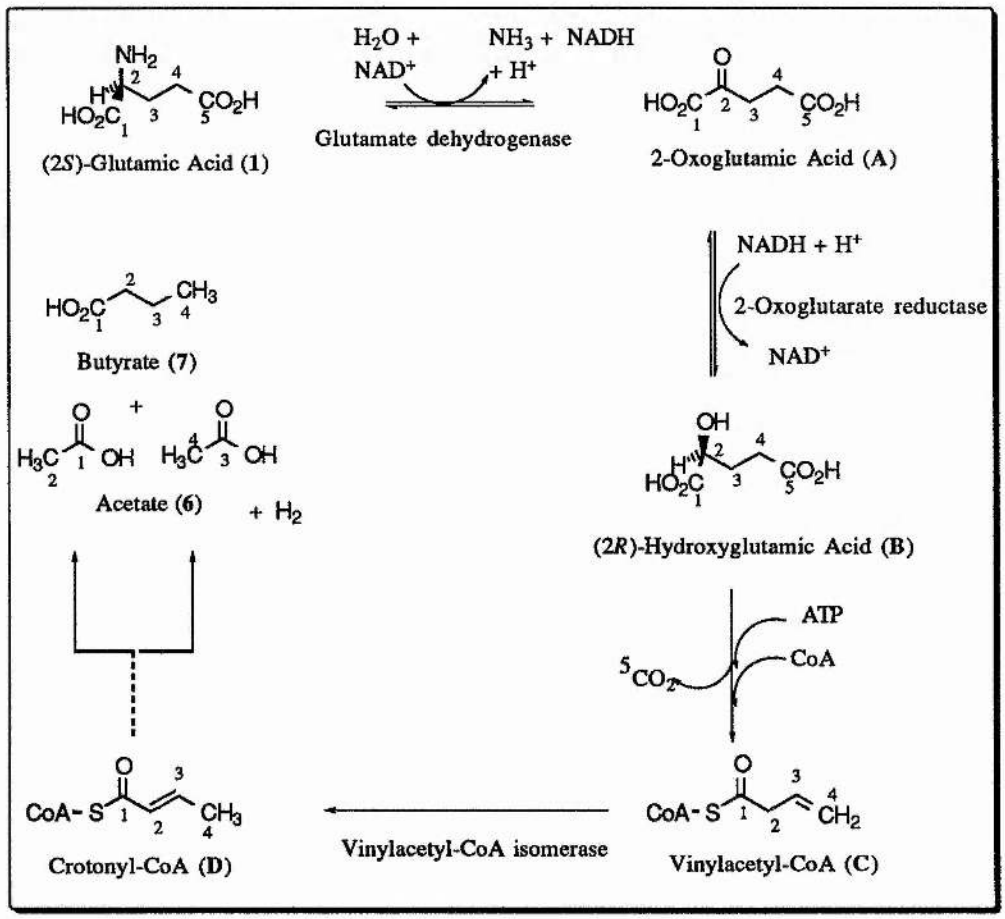
The study of amino acid degradation by anaerobic bacteria has in the past been complicated by the fact that such organisms require complex media for growth. However, developments within this area, have allowed for successful methods to be introduced for following the break down of all individual amino acids by such organisms. This difficulty was partially overcome by adding a high concentration of one amino acid to a medium containing low concentrations of other nutrients and determining the predominant products formed from the major substrate. In this way several investigators have demonstrated that single amino acids, including aspartic, glutamic, histidine, lysine, glycine, alanine, serine, amongst many others, can serve as major energy sources for selected species of anaerobic bacteria.

Amino acid degradations by anaerobic bacteria always involve oxidation and reduction reactions between one or more amino acids or non-nitrogenous compounds derived from amino acids.[1] The oxidation and reduction reactions are usually similar or identical to corresponding reactions catalysed by aerobic organisms, except for limitations imposed by the absence of molecular oxygen or other high potential oxidants.[2] Thus oxidations, deaminations, transaminations, and α-keto acid oxidations commonly occur in anaerobic amino acid degradation, but oxygenation reactions and fatty acid oxidations are excluded. The reduction reactions are more distinctive, since each organism must generate one or more electron acceptors of suitable potential from the amino acid(s) that it can metabolise.[2]

One of the major interests in this area of bioorganic chemistry is the determination of the metabolic pathways and enzymatic reactions which are involved in the degradation of selected amino acids by anaerobic bacteria. One such amino acid which has been extensively studied is the anaerobic degradation of glutamic acid.[3]

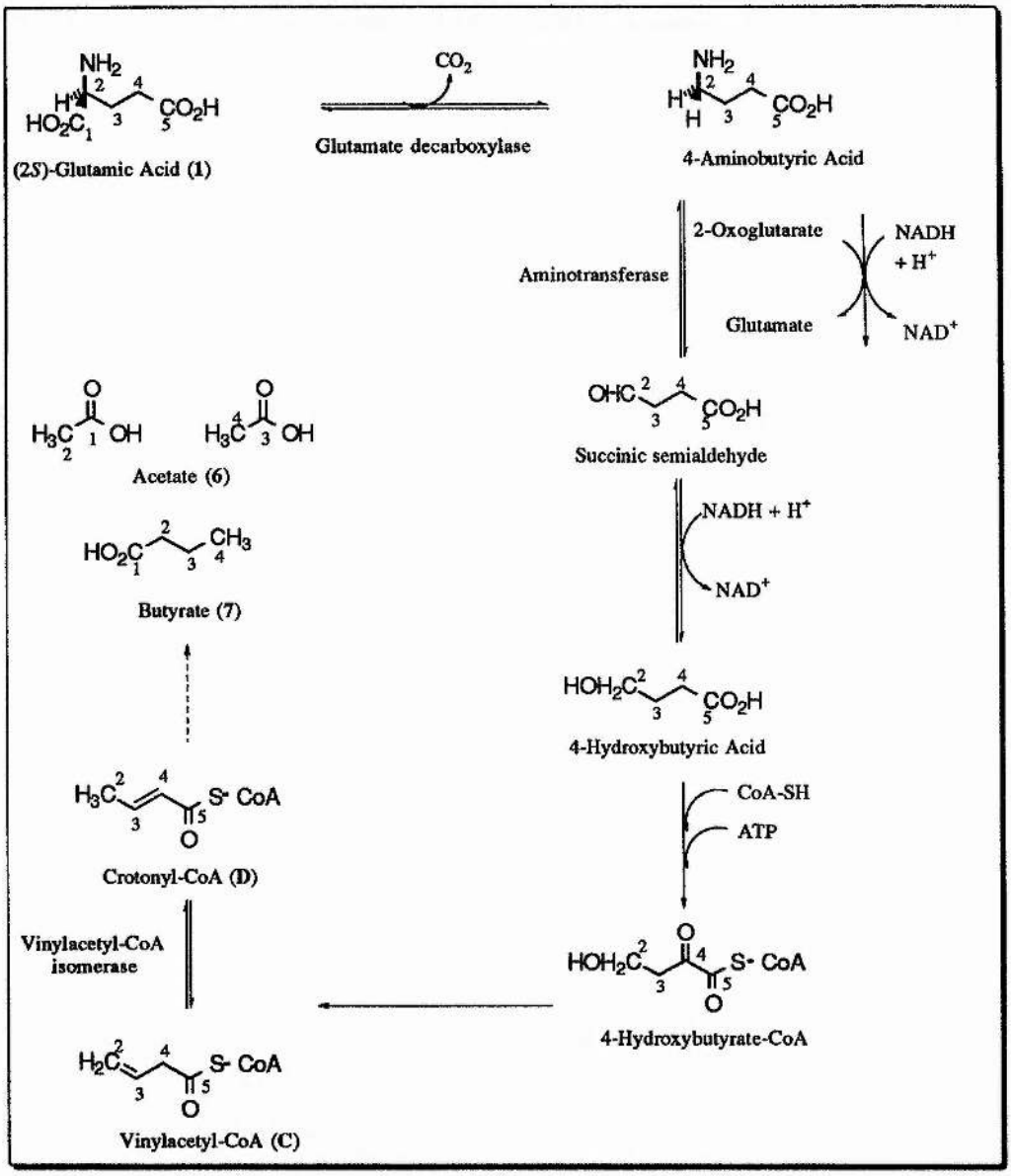
1.1.2 Anaerobic Pathways for Glutamate Assimilation

There are a variety of pathways which exist for the anaerobic and aerobic metabolism of glutamate. Several studies have been carried out to determine the possible pathways of glutamate catabolism in anaerobic bacteria.[3] Both *Clostridium tetanomorphum and Peptostreptococcus aerogenes* have been studied extensively and, although both of these bacteria decarboxylate glutamate at position C-5 (see relevant pathways below) and produce acetate and butyrate, different metabolic intermediates were recovered.[3-5] *C. tetanomorphum* utilises the methylaspartate pathway (Scheme 1.3, see p.4), and *P. aerogenes* was later shown to utilise the 2-oxoglutarate pathway (Scheme 1.1), in which successive intermediates are 2-oxoglutarate (**A**), 2-hydroxyglutarate (**B**), vinyl-acetyl-CoA (**C**), crotonyl-CoA (**D**); the latter is converted to acetate (**6**), butyrate (**7**) and hydrogen.



Scheme 1.1: Glutamate Catabolism via the 2-Oxoglutarate Pathway

A recent study[6] carried out on *Fusobacterium varium* ferments glutamic acid by yet another pathway; the 4-aminobutyrate pathway (Scheme 1.2). The 4-aminobutyrate pathway is linked with the 2-oxoglutarate pathway. Both pathways require glutamate dehydrogenase for the initial reaction and both use the same enzymes for the conversion of vinylacetyl-CoA (Scheme 1.2).

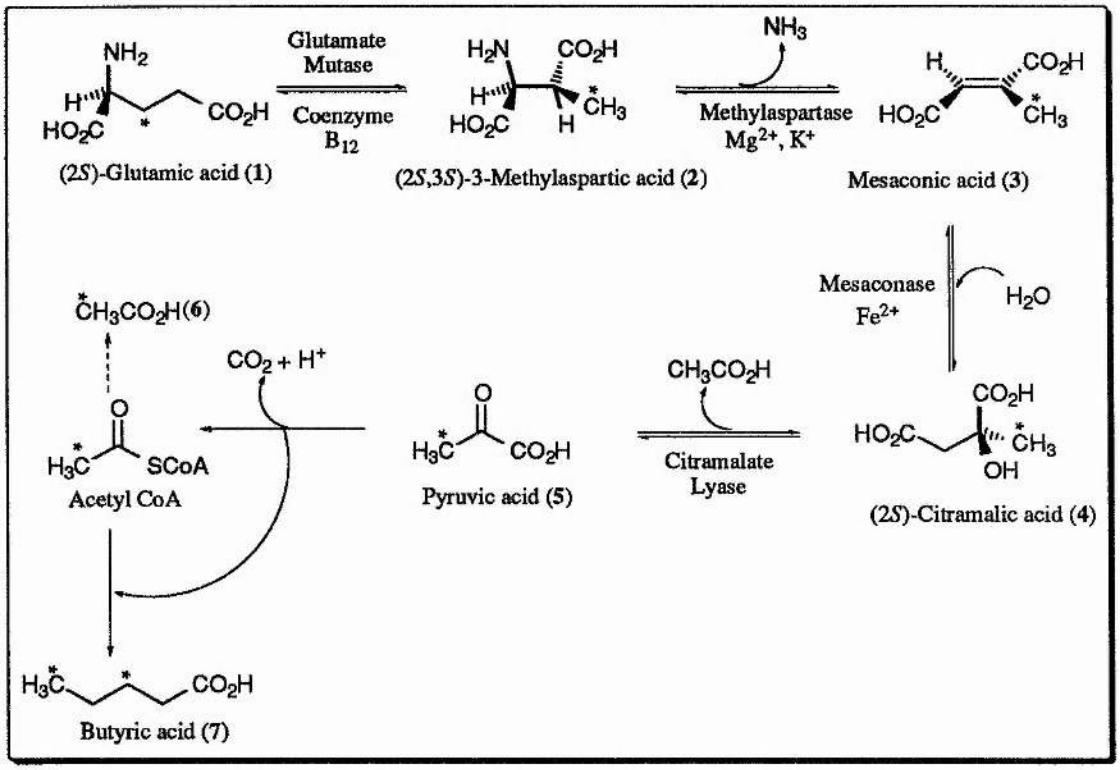


***Scheme 1.2:** Glutamate Catabolism via the 4-Aminobutyrate Pathway*

The 4-aminobutyrate pathway has only been detected in *F. varium* and *F. mortiferum*, both of which are normally isolated from the gastrointestinal tract.

1.1.3 The Methylaspartate (Mesaconate) Pathway

A significantly large number of bacteria assimilate the fermentation of (2*S*)-glutamic acid (**1**) *via* a very distinctive energy yielding pathway (Scheme 1.3). The final products from the pathway are acetate, butyrate, carbon dioxide and ammonia.



Scheme 1.3: *Glutamate catabolism via the methylaspartase pathway*

The methylaspartase pathway was first elucidated in 1958 by Barker and co-workers,[7] as a result of the fermentation studies carried out on the bacterium *Clostridium tetanomorphum*, strain H1 (American Type Culture Collection 15920). There is some evidence that this type of fermentation pathway also operates in the photosynthetic, purple non-sulphur bacteria, *Rhodospirillum rubrumi* and *Rhodopseudomanas spheroides*.[8] However, it is thought not to be the major catabolic pathway.[9] A similar pathway may also be used in an anabolic

sense, by the bacterium *Acetobacter suboxydans*[10,11] although it is not the primary route of glutamic acid assimilation.

The initial step of the pathway involves the enzyme glutamate mutase, which is required for the stereospecific, conversion of (2*S*)-glutamic acid (**1**) into (2*S*, 3*S*)-3-methylaspartic acid (**2**). The second step of the pathway, utilises the newly formed amino acid (**2**) for the production of mesaconic acid (**3**) ((E)-2-methylbutenedioic acid), utilising the second enzyme of the pathway, 3-methylaspartase.[12]

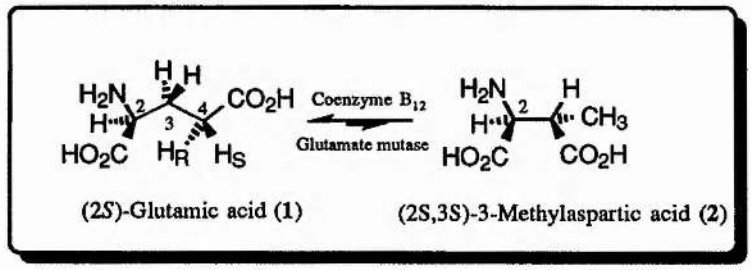
The next stage of the pathway involves the hydration of mesaconic acid, in an *anti-* fashion, *via* the action of mesaconase,[13] to give the tertiary alcohol, (2*S*)-citramalic acid (**4**). The final enzyme in the mesaconate pathway, is citramalate lyase[14], which catalyses a retro-aldol cleavage of (*S*)-citramalic acid to give pyruvic (**5**) and acetic acid (**6**).

Pyruvic acid is oxidatively decarboxylated to give acetyl-coenzyme A. Two acetyl-coenzyme A molecules form butryl coenzyme A and hence, butyric acid (**7**) is formed. Adenosine triphosphate (ATP) is also generated from the cleavage of the thioesters.

1.2 Glutamate Mutase

1.2.1 The Biological Role and the Discovery of Glutamate Mutase

The enzyme glutamate mutase (E.C.5.4.99.1) catalyses the reversible carbon-skeleton rearrangement of (2*S*)-glutamic acid (**1**) to (2*S*,3*S*)-3-methylaspartic acid (**2**) (Scheme 1.4) in the presence of coenzyme B_{12} (AdoCbl) (Fig.1.1).



Scheme 1.4: *Conversion of (2S)-Glutamic acid to (2S,3S)-3-Methylaspartic acid*

The enzyme was first discovered in the soil anaerobe *Clostridium tetanomorphum*[15] and is the first enzyme on the mesaconate pathway[16] (Scheme 1.3).

Coenzyme B_{12} (AdoCbl, Fig. 1.1) is believed to serve as a reversible carrier of free radicals. Homolysis of the cobalt-carbon bond, affords cob(II)alamin and a 5'-deoxyadenosyl radical, which gives rise to the formation of a new range of radicals derived from the appropriate substrate (Scheme 1.5).

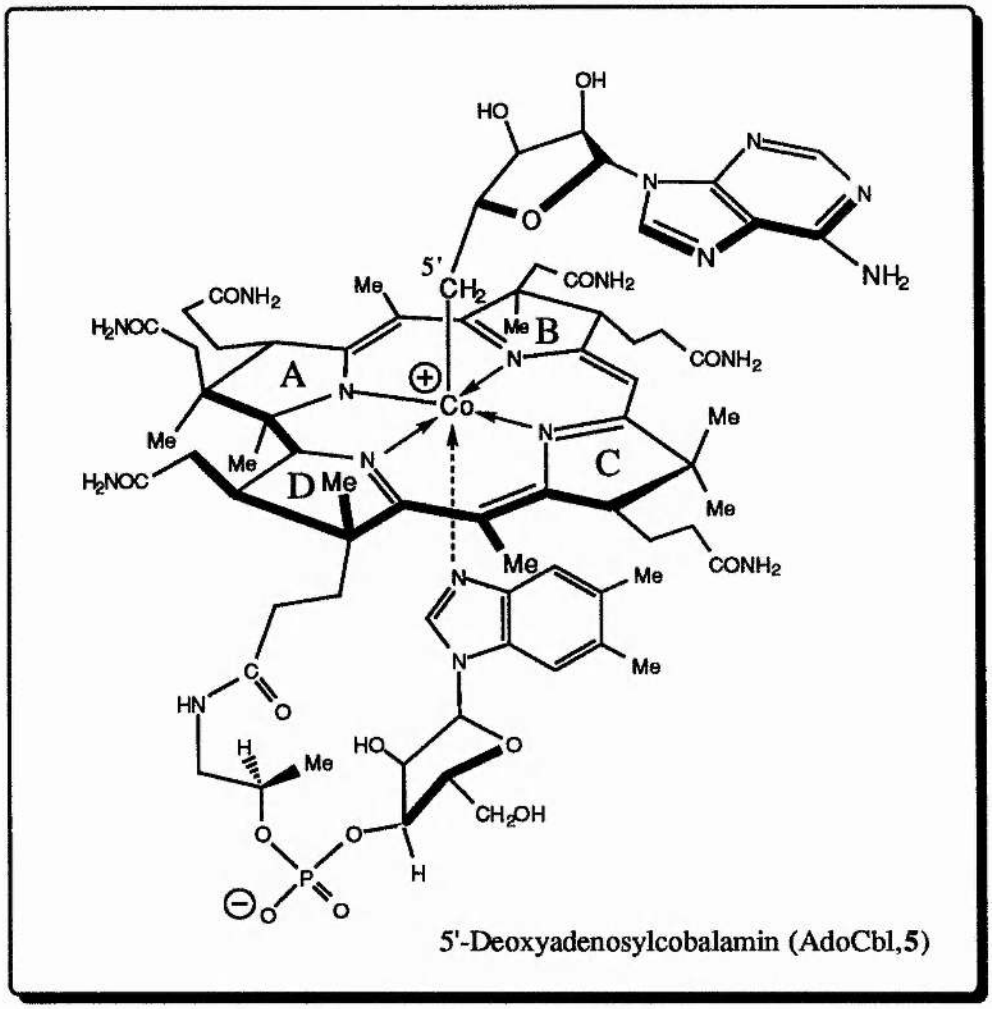
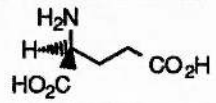
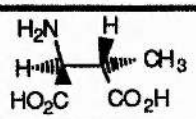
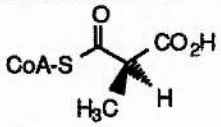
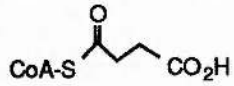
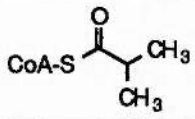
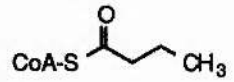
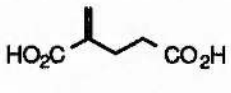
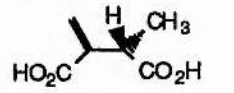
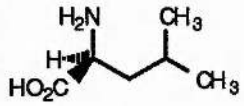
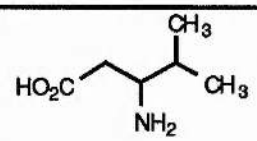
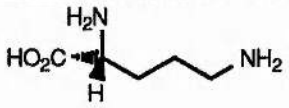
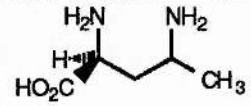
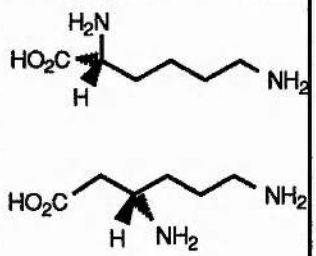
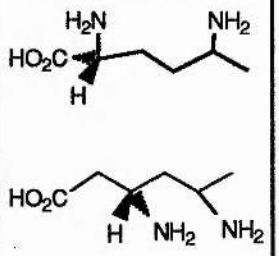
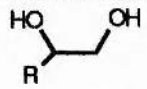
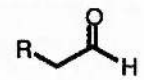
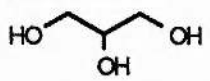
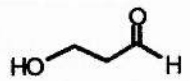
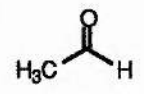


Figure 1.1: *Structure of 5'-Deoxyadenosylcobalamine, Coenzyme* B_{12}.

The glutamate mutase reaction is one of ten vicinal interchange reactions mediated by AdoCbl in which a functional group, (may vary widely in structure), swaps places with a hydrogen atom from an adjacent carbon atom (Table 1.1).

Enzyme	Substrate	Product	E.C. Number
1) (S)-Glutamate Mutase ⇌ *			5.4.99.1
2) (R)-Methylmalonyl-CoA mutase ⇌			5.4.99.2
3) Isobutyryl-CoA mutase ⇌			5.4.99.3
4) α-Methyleneglutarate mutase ⇌			5.4.99.4
5) (S)-Leucine 2,3-amino-mutase ⇌			5.4.3.7
6) (R)-Ornithine 4,5-amino-mutase ⇌			5.4.3.5
7) (R)- α-Lysine 5,6-amino -mutase ⇌ ((R)- β-Lysine mutase)			5.3.3.3
8) Diol dehydrase →			4.2.1.28
9) Glycerol dehydrase →			4.2.1.30
10) Ethanolamine ammonia lyase →			4.3.1.7

* Reaction reversibility under physiological conditions.

Table 1.1 *Coenzyme -B_{12}-dependent enzymes catalysing rearrangements*

atom. In four of these rearrangements (Table 1.1, entries 1-4) the migrating group is a substituted carbon atom.

The involvement of competent radical species has been shown for the carbon-skeleton rearrangement of methylmalonyl-CoA mutase[17] and glutamate mutase.[18] The EPR signal recorded for each haloenzyme complex, after the addition of the substrate, showed similar characteristics to those obtained for other B_{12}-dependent enzymes including diol dehydrases[17] and ethanolamine ammonia-lyase.[20]

1.2.2 Reaction Catalysed and Assays

The equilibrium for the glutamate mutase interconversion of (2*S*)-glutamic acid (**1**) to (2*S*,3*S*)-3-methylaspartic acid (**2**), favours the conversion of (2*S*,3*S*)-3-methylaspartic acid (**2**) to glutamic acid (**1**). Barker found that the K_{eq} at 30 °C and pH 8.2 was 10.78.[21] Although the optimum pH for the reaction is 8.5, the enzyme was still active between pH 6.0 and 9.88. The enzyme showed maximal activity at 38 °C and shows half maximal activity at 27 °C.[21] These findings were markedly different to those obtained for the activity for methylaspartase, which was maximal at 55 °C.[12]

The only known substrates for glutamate mutase to date, are the physiological substrates, (2*S*)-glutamic acid (**1**) and (2*S*, 3*S*)-3-methylaspartic acid (**2**).[22] The K_m for glutamic acid is 1.5 mM and for (2*S*, 3*S*)-3-methylaspartic acid is 0.5 mM.[23] Barker found that (2*R*)-glutamic acid, 3-ethylaspartic acid, α-,β- and γ-methyl-(2*S*/*R*)-glutamic acid, (2*S*)-glutamine, the γ-methyl ester of (2*S*)-glutamic acid and (*S*/*R*)-α-aminoadipic acid were not substrates for the enzyme.[23] However, more recently Buckel has shown that (2*S*,4*S*)-4-fluoroglutamic acid, (2*R*,3*R*/*S*)-3-fluoroglutamic acid, 2-methyleneglutaric acid, (*S*)-3-methylitaconic acid and itaconic acid are all competitive inhibitors for glutamate mutase[24] (Table 1.2).

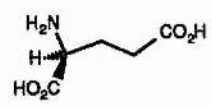
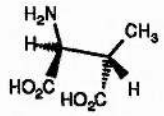
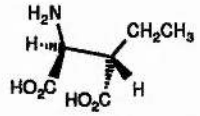
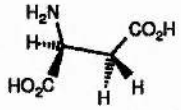
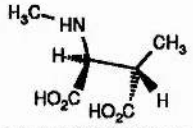
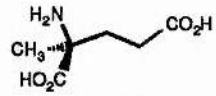
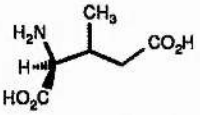
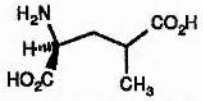
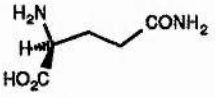
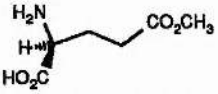
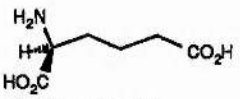
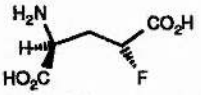
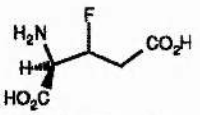
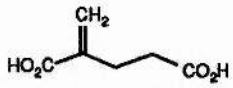
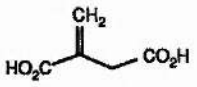
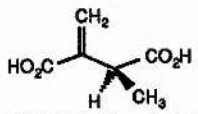
Compounds Tested	Substrate (s)	Inhibitor (s)
 (2*S*)-Glutamic Acid (1)  (2*S*,3*S*)-3-Methylaspartic Acid (2)	+	
 (2*R*)-Glutamic Acid  (2*S*,3*S*)-3-Ethylaspartic Acid (31, R=Et)	–	
 (2*S*)-Aspartic Acid (68)  *N*-Methyl (2*S*,3*S*)-3-Methylaspartic Acid (67)	–	
 α-Methylglutamic Acid  β-Methylglutamic Acid	–	
 γ-Methylglutamic Acid  (2*S*)-Glutamine	–	
 (2*S*)-Glutamic acid γ-methyl ester  α-Aminoadipic Acid	–	
 (2*S*,4*S*)-4-Fluoroglutamic Acid  (2*S*,3*S*/*R*)-3-Fluoroglutamic Acid	–	+
 2-Methyleneglutamic Acid  Itaconic Acid	–	+
 (*S*)-3-Methylitaconic Acid	–	+

***Table 1.2**: Compounds Tested With Glutamate Mutase*

Studies towards substrate and/or inhibitors for glutamate mutase, within our laboratories, have shown that (2*S*,3*R*) and (2*S*,3*S*)-3-methylglutamic acids were competitive inhibitors of glutamate mutase.[111]

Two assay systems have been developed to measure the biological activity of the mutase.[25] The first assay is based on the conversion of glutamate to mesaconate by the coupled action of glutamate mutase and β-methylaspartase. The chromophore of the double bond within the product mesaconic acid (3) provides convenient measure of the extent of the reaction. Although the method is reliable, some methylaspartate analogues which could give valuable information on the glutamate mutase reaction, are also substrates for β-methylaspartase and thus this would interfere with the coupled assay system. The anaerobic assay relies on the conversion of methyaspartic acid to glutamic acid under strictly oxygen-free conditions.

A series of coenzyme analogues have been examined by various groups,[26,27] to determine the flexibility of the coenzyme with the enzyme. The following changes were made to the heterocyclic base at the sixth ligand position of the coenzyme (Fig. 1.1), whilst conferring activity to the enzyme; 5'-hydroxy-benzimidazole, 2,6-diaminopurine, adenine, 5(6)-nitrobenzimidazole, 5(6),-aminobenzimidazole, 5(6)-trifluromethylbenzimidazole, benzimidazole, purine, 2-chloropurine, 2-thiopurine, 6-methylmercaptopurine and 5,6-dimethyl-benzimidazole. Those analogues found to be inactive included; corrinoid vitamins, methyl, carboxymethyl and 5-deoxyuridyl cobalamins and cobinamide coenzyme. The corrinoid vitamins have no deoxyadenosyl group and cobinamide coenzyme has no nucleotide. Thus, it can be inferred that a deoxyadenosyl moiety and a heterocyclic base are required for enzyme catalysis, but that fairly major alterations to the coenzyme can be made successfully.

1.2.3 Structure of the Holoenzyme

In 1966 Barker showed that the active form of glutamate mutase consisted of two polypeptide components. One, a large air-stable thiol containing subunit designated as component S (a calcium phosphate supernatant fraction) and a component E (a gel eluate fraction).[28,21] Barker purified each component and achieved a 20-25% yield of component S, and an 18% yield of component E.[21,28] Barker estimated that the molecular weight of component S was 17,000 Da and that of component E to be 128,000 Da.[21,28] The recent completion of the purification to homogeneity of both components by B. Hartzoulakis, in our own laboratories, has confirmed that the relative molecular masses of both components (E and S) to be the same as reported by Barker. This value is in accord with other recent reports from Holloway and Marsh (1993),[29] for the enzyme from *C. tetanomorphum*. Marsh has also cloned and sequenced the genes for components S[30] and E[29] from *C. tetanomorphum*. The *N*-terminal sequence for component S from *C. tetanomorphum* is almost identical to that from *C. cochlearium*.[24,30]

Over the past three years enormous advances have been achieved in establishing how the genes in the pathway for glutamic acid catabolism are organised within *C. tetanomorphum*. In 1992 the gene for 3-methylaspartase, the second enzyme in the pathway, was successfully sequenced and expressed in *E. coli*.[31] In accord with expectations derived from the determination of the molecular mass of the native homodimeric enzyme by size exclusion chromatography and by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE analysis), the gene was found to encode a polypeptide of 413 amino acid residues of molecular mass 44,539 Da. This work also identified two large but incomplete open reading frames up- and downstream of the *MeAsp* gene.[31] These were believed to code for the E-subunit of glutamate mutase and for mesaconase. The suggestion that the gene for the E-subunit existed immediately upstream of *MeAsp* was later confirmed when the complete gene

was sequenced.[29] The open reading frame for the E-subunit was shown to code for a polypeptide 485 amino acids of 53,708 Da.

Comparison of the E component with other proteins in the data bases failed to reveal any significant homologies, or any noteworthy similarities with other cobalamin-binding proteins. However, the comparison of E component with β-methylaspartase performed by Marsh identified a region of significant local homology (Fig. 1.2). Interestingly, the alignment includes the residue Ser-173 of β-methylaspartase, which has been implicated in the mechanism of deamination.[31,32] For β-methylaspartase a dehydroalanine moiety appears to be generated by the self catalysed dehydration of the serine residue (Ser-173) (see section 1.4). Furthermore, it is emerging that for enzymes which operate *via* the intermediacy of a dehydroalanine residue, there is a conserved nucleic acid deduced sequence motif, -Ser-Gly-Asp-,[31,33] where the serine residue becomes dehydrated.

```
                    170   *      180          190          200   210
β-Methylaspartase GAEINAVPVFAQSGDDRYDNVDKMIIKEADVLPHALINNVEE-KLGLKG-EKLLEYVK
                  ||:: : :: | : ::||:: : : |||::   ::|:|:  : : |:||  |:|| |:
Component E       GADLLPSTIDAYTRQNRYEECE-IGIKESEKAGRSLLNGFPGVNHGVKGCRKVLESVN
                    90       100            110          120          130          140
```

The aligned regions are G87 to N143 of the E component and G161 to K216 of β-methylaspartase. Identical residues are denoted by '|' and conserved residues by : ':'. The active-site serine of β-methylaspartase is identified by *.

Figure 1.2: *Alignment of the deduced amino acid sequence of Component E*[29] *and β-Methylaspartase*

Other work by Marsh, identified the gene for the S component from which it was deduced that the S component consisted of a 137-residue polypeptide of 14,748 Da.[30] Interestingly, these studies revealed that there is a single intervening gene, designated L, coding for a protein of 50,171 Da, between the genes for the two glutamate mutase components and immediately upstream of gene for the E-subunit, (Fig 1.3). No role has been ascribed for the product of the intervening gene.

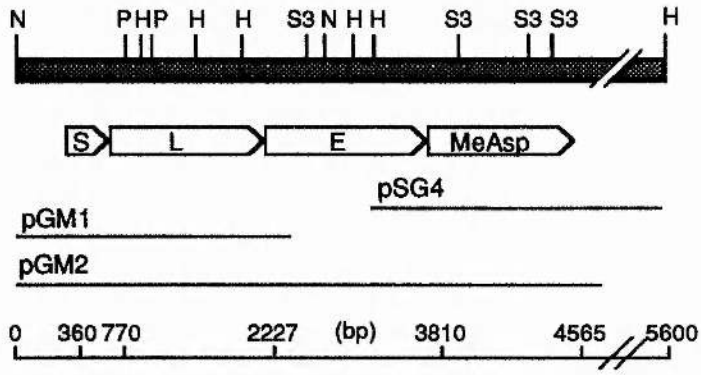


Figure 1.3: Organisation of the genes for glutamate mutase components S and E, protein L, and 3-methylaspartase in *Clostridium tetanomorphum*. Restriction sites for *Hind*III (H), *Nco*I (N), *Pst*I (P) and *Sau*3A(S3) are also indicated. Plasmids containing *C. tetanomorphum* genomic fragments are labelled pSG4 (Goda et al.[31]), pGM1 (Marsh and Holloway.[30]) and pGM2 (Holloway and Marsh.[29]).

MELKNKKWTDEEFFKQREEVLKQWPTGKEVDLQEAVDYLK	40	MEKKTIVLGVI	11
KVPTEKNFAKLVRAKEAGITLAQPRAGVALLDEHINLLRY	80	GSDCHAVGNKI	22
LQDEGGADLLPSTIDAYTRQNRYEECEIGIKESEKAGRSL	120	LDHSFTNAGFN	33
LNGFPGVNHGVKGCRKVLESVNLPLQARHGTPDSRLLAEI	160	VVNIGVLSSQE	44
IHAGGWTSNEGGGISYNIPYAKSVPIDKCLKDWQYCDRLV	200	DFINAAIETKA	55
GFYEEQGVHINREPFGPLTGTLVPPSMSNAVGITEALLAA	240	DLICVSSLYGQ	66
EQGVKNITVGYGECGNMLQDIAALRCLEEQTNEYLKAYGY	280	GEIDCKGLREK	77
NDVFVTTVFHQWMGGFPQDESKAFGVIVTATTIASLAGAT	320	CDEAGLKGIKL	88
KVIVKTPHEAIGIPTKEANASGIKATKMALNMLEGQRMPM	360	FVGGNIVVGKQ	99
SKELETEMAIIKAETKCILDKMFELGKGDLAVGTVKAFET	400	NWPDVEQRFKA	110
GVMDIPFGPSKYNAGKMMPVRDNLGCVRYLEFGNVPFTEE	440	MGFDRVYPPGT	121
LKNYNRERLAERAKFEGREVSFQMVIDDIFAVGKGRLIGR	480	SPETTIADMKE	132
PENK	485	VLGVE	137
Component E		**Component S**	

Figure 1.4: *The Amino Acid Sequences of Components E and S*

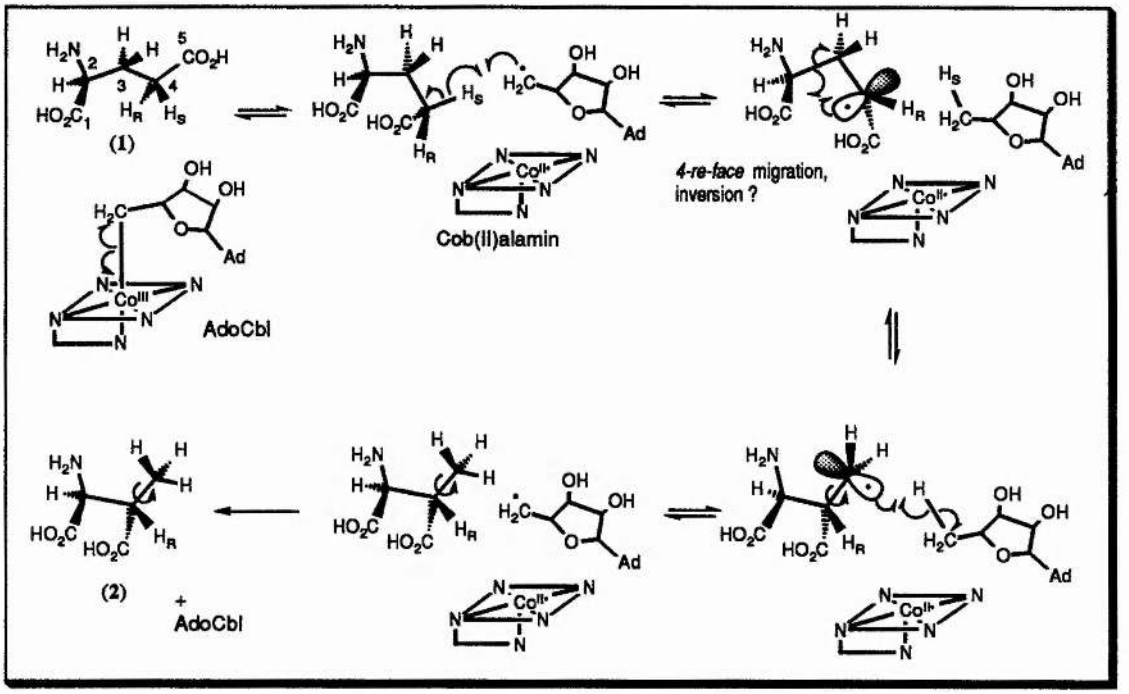
1.2.4 Substrates and the Rearrangement Step

The glutamate mutase rearrangement involves a reversible [1,2]-migration of C-2 of glutamic acid from C-3 to C-4, promoted by the enzyme. This type of rearrangement is the only known example, in which all three carbon atoms

group of the 5'-deoxyadenosine portion of the coenzyme during the rearrangement and that one of these hydrogen atoms is transferred back to the product radical when the rearrangement is complete. For the glutamate mutase system it has been demonstrated that tritium present in the 5'-deoxyadenosyl portion of the coenzyme at C-5' is transferred to the substrate.[35]

Given that Barker had determined that both the substrate and the product possess the same absolute configuration at C-2, the glycyl moiety must migrate to C-4 of glutamic acid with retention of configuration at C-2.[36] In order to define the stereochemical course of the rearrangement with respect to the other carbon centre, (C-3 of glutamic acid), Hartrampf and Buckel[37] prepared (2*S*)-glutamic acid labelled at C-3 and C-4 with all three isotopes of hydrogen in the expectation that the enzymic product would contain a chiral methyl group. However, analysis of the product indicated that the potentially chiral methyl group was, in fact, racemic. This is the expected result for a reversible reaction in which the product contains a torsiosymmetric methyl group, or where the putative methylene radical intermediate is able to rotate about the C-3-C-3' sigma bond prior to the hydrogen capture at C-3'. Thus, while it is tempting to suggest that the rearrangement reaction should proceed with inversion of configuration at C-3 of glutamic acid, because this outcome requires minimal motion (Scheme 1.5), it is evident that the stereochemical course of the reaction with respect to C-3 cannot be determined directly using the natural substrate.

involved are saturated.[27] It is believed that the homolysis of the cobalt-carbon bond in the coenzyme, is promoted by glutamate mutase, upon substrate binding. The 5'-deoxyadenosyl radical formed during the dissociation is believed to abstract the pro-S hydrogen from C-4 of glutamic acid. This action results in a [1,2]-shift involving the movement of the glycyl moiety from C-3 to C-4. The resulting product radical retrieves a hydrogen atom from C-5′ of the 5'-deoxyadenosine to give the product and regenerate the coenzyme.[34]

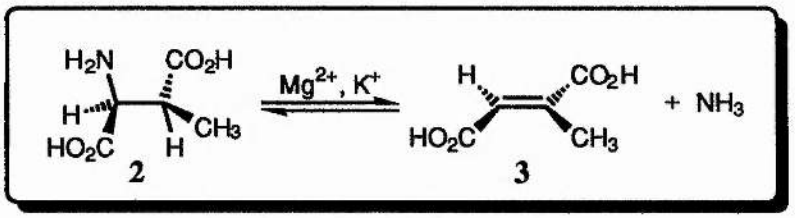


Scheme 1.5: Proposed Mechanism of the Glutamate Mutase Reaction.

Three carbon centres are involved in the glutamate mutase catalysed rearrangement reaction and it is evident that to fully understand the mechanism of the enzyme, it is necessary to determine the stereochemical course of the migrations with respect to each carbon centre. Early work by Sprecher *et al.*[34] demonstrated that the 4-*pro*-S hydrogen of glutamic acid migrates to become one of the three hydrogens of the methyl group of the product ((2*S*,3*S*)-3-methylaspartic acid) and is replaced by the migrating glycyl moiety with inversion of configuration at C-4 (Scheme 1.5). It is now generally believed that the migrating atom(s) becomes one of the three hydrogen atoms of the methyl

1.3 Methylaspartate Ammonia-Lyase (MAL)

Methylaspartase (E.C. 4.3.1.2) catalyses the deamination of (2*S*,3*S*)-3-methylaspartic acid (**2**) to give mesaconic acid (**3**) (Scheme 1.6). The enzyme is distributed among obligate anaerobic microorganisms[12] and was very recently found among facultative anaerobic bacteria.[38] The enzyme from the soil anaerobe, *Clostridium tetanomorphum*, has been the most extensively studied.[12]



Scheme 1.6: The 3-Methylaspartate Ammonia-Lyase Reaction

1.3.1 The Structure of 3-Methylaspartate Ammonia-Lyase

Methylaspartase from *Clostridium tetanomorphum* , has a molecular weight of 100,000 Da, as determined by sedimentation equilibrium measurements.[39] The enzyme has been cleaved into two equal molecular weight subunits, each weighing 50,000 Da, in the presence of 6 mol dm^{-3} guanidine hydrochloride.[39,40] However, work carried out by Hsiang and Bright reported that the enzyme, cleaves into four equal weight subunits (25,000 Da = 1 unit), when the enzyme is exposed to an 8-fold molar excess of *p*-chloromercuribenzoate. The molecular weight of each subunit was determined by density gradient sedimentation techniques.[41] Electrophoretic analysis of the enzyme, suggested that the enzyme possessed an $(ab)_2$ type structure,[42] however, more recent work by Cohen showed that the enzyme behaves as a homodimer having a molecular weight of 49,000 Da.[43] SDS-PAGE analysis, under a variety of conditions gives no indication of the homodimer splitting into smaller subunits. In addition, Wu and Williams reported that half the total number of arginine and lysine residues were present within the tryptic digests of the enzyme, as compared to the native enzyme.[32]

This work suggested that the enzyme was a dimer, consisting of two identical subunits.

The *Clostridium tetanomorphum* gene encoding methylaspartase, has been recently cloned, sequenced and expressed in *E. coli* by Goda *et al.*[31] The gene consists of 413 amino acid residues, comprising a total molecular weight of 45,539 Da (Fig. 1.5). This information about the structure of the enzyme unambiguously identifies methylaspartase to be a homodimer. The recombinant protein has been shown to be identical to the enzyme isolated directly from *C. tetanomorphum*.

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M K I V D V L C T P G L T G F Y F D D Q R A I K K  25
G A G H D G F T Y T G S T V T E G F T Q V R Q K G  50
E S I S V L L V L E D G Q V A H G D C A A V Q Y S  75
G A G G R D P L F L A K D F I P V I E K E I A P K  100
L I G R E I T N F K P M A E E F D K M T V N G N R  125
L H T A I R Y G I T Q A I L D A V A K T R K V T M  150
A E V I R D E Y N P G A E I N A V P V F A Q S G D  175
D R Y D N V D K M I I K E A D V L P H A L I N N V  200
E E K L G L K G E K L L E Y V K W L R D R I I K L  225
R V R E D Y A P I F H I D V Y G T I G A A F D V D  250
I K A M A D Y I Q T L A E A A K P F H L R I E G P  275
M D V E D R Q K Q M E A M R D L R A E L D G R G V  300
D A E L V A D E W C N T V E D V K F F T D N K A G  325
H M V Q I K T P D L G G V N N I A D A I M Y C K A  350
N G M G A Y C G G T C N E T N R S A E V T T N I G  375
M A C G A R Q V L A K P G M G V D E G M M I V K N  400
E M N R V L A L V G R R K                          413
```

***Figure 1.5:** The Primary Sequence of Methylaspartase*

Photo-oxidation studies, performed by Williams and Libano, have shown that the rate constant for the loss of enzyme activity, is almost identical to that for the cysteine sulfhydryl groups, suggesting that a thiol group was required for activity.[44] The enzyme was also known to be inactivated by the thiol alkylating agent, *N*-ethylmaleimide. Protection from inactivation studies, provided by various substrates and substrate analogues, had implied that the sulfhydryl group (cysteine residue) was proximate to the 3-H of (2*S*, 3*S*)-3-methylaspartate.[45]

EPR measurements by Fields and Bright, using manganese as the divalent cation, showed two metal ions were bound per enzyme molecule.[46] This lead to the suggestion that there are two active sites. The result correlates well with the number of mesaconic acid binding sites available, as determined by gel filtration of methylaspartase in buffer containing mesaconic acid.[32] Furthermore, Hsiang and Bright showed that the enzyme was completely inactivated by two equivalents of *p*-chloromercuribenzoate.[39] However, methylaspartase was insensitive to arsenite, indicating that the two essential thiol groups, were not located at the same active site.

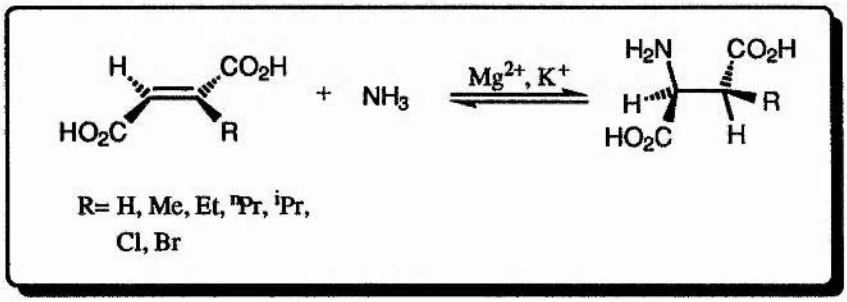
1.3.2 Substrate Specificity of 3-Methylaspartate Ammonia-Lyase

Due to the size of the methyl binding pocket, within the active site of 3-methylaspartase, the enzyme can tolerate a wide variety of 3-C substituted aspartic acids. The following compounds; (2*S*)-aspartate,[12] (2*S*,3*S*)-3-ethylaspartate,[47] (2*S*,3*S*)-3-^{n}propylaspartate, (2*S*,3*S*)-3-^{i}propylaspartate[47] and (2*S*,3*R*)-3-chloroaspartate[48] have all been shown to be substrates for 3-methylaspartase (Table 1.3). Interestingly, Asano and Kato have shown that (2*S*)-aspartic acid was not a substrate for MAL, from a facultative anaerobe, but catalysed the amination of fumaric acid to give (2*S*)-aspartic acid.[38]

Substrate	K_M (mM)	V_{max} ($\times 10^{-7}$ mol dm^{-3} S^{-1})
(2*S*,3*S*)-3-Methylaspartic acid	2.37±0.2	109.0
(2*S*)-Aspartic acid	10±0.82	0.80
(2*S*,3*S*)-3-Ethylaspartic acid	17.08±1.4	48.7
(2*R*,3*S*)-3-Chloroaspartic acid	>50	-
(2*S*,3*R*)-3-Methylaspartic acid	40	2.9

Table 1.3: *Substrates for Methylaspartase and Deamination Kinetic Parameters*

In early work within our laboratories, Akhtar *et al.* demonstrated that the enzyme could be used synthetically in the amination direction, to prepare 3-halogeno- and 3-alkylaspartic acids, of defined stereochemistry, from the corresponding substituted fumarates (Scheme 1.7 and Table 1.4).[49]



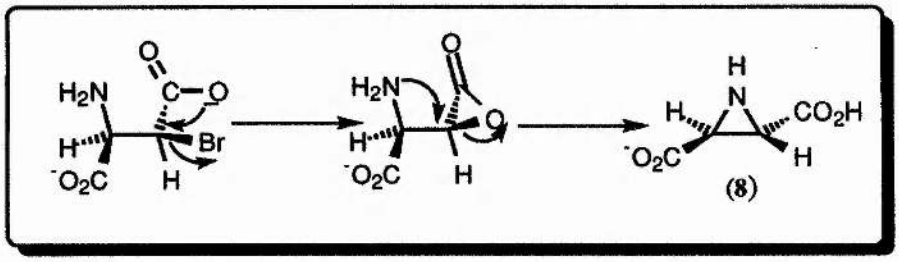
***Scheme 1.7:** Substrates for Methylaspartase*

Substrate	K_M	V_{max} ($x10^{-6}$ mol dm^{-3} min^{-1})
Fumaric acid	23±2.2	1702
Mesaconic acid	1.24±0.085	894
Ethylfumaric acid	1.05±0.2	583
Chlorofumaric acid	3.52±0.71	382
Bromofumaric acid	2.64±0.53	425
^{n}Propylfumaric acid	2.1±1.3	4.2
^{i}Propylfumaric acid	5.5±3.0	5.3
^{n}Butylfumaric acid	-	<0.05
Iodofumaric acid	-	<0.05

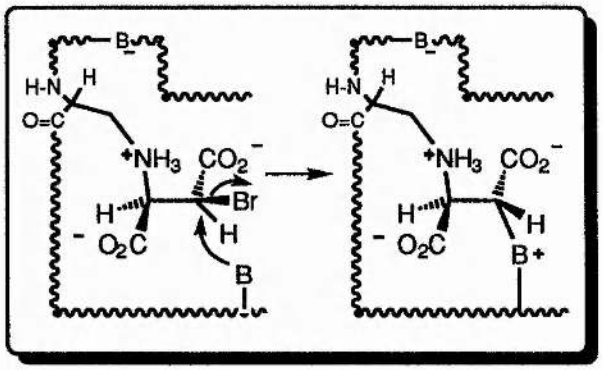
***Table 1.4:** Substrates for Methylaspartase and Amination Kinetic Parameters*

When fluorofumarate was incubated with 3-methylaspartase, (2*R*,3*S*)-3-fluoroaspartatic acid was formed. However, due to the competing side reactions, isolation of the fluoroaspartate was difficult. Bromofumarate was found to be a good substrate for 3-methylaspartase but the enzymic product, (2*R*,3*S*)-3-bromoaspartate, was unstable under the incubation conditions and was noted to

cyclise, giving rise to the formation of 2,3-aziridinedicarboxylate (Scheme 1.8; (8)). Bromofumarate was also found to cause inactivation of 3-methylaspartase as it was observed to behave as a suicide inhibitor. This observation may be explained due to the formation (2*R*,3*S*)-3-bromoaspartate.[50] This suggests that alkylation of an essential nucleophile at the active site of the enzyme might occur during the catalytic turnover (Scheme 1.9). It should also be noted that inactivation may also be caused by the formation of the aziridine (Scheme 1.8).



Scheme 1.8: The Formation of 2,3-Aziridinedicarboxylate

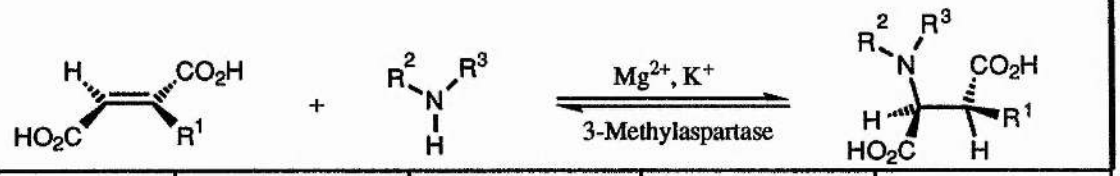


Scheme 1.9: Suicide Inhibition of Methylaspartase by (2R,3S)-3-Bromoaspartate

The C-3 deuteriated isotopomers of the 3-halogeno- and 3-alkylaspartic acids have also been prepared by performing the enzymatic incubations, in deuterium oxide.[49] It was shown by Barker that methylaspartase deaminated (2*S*,3*R*)-*erythro*-methylaspartic acid and that this diastereomer was generated by the enzyme, upon prolonged incubation with mesaconic acid.[12] In addition, Archer and Gani have demonstrated that the recombinant protein also catalysed the formation of (2*S*,3*R*)-*erythro*-methylaspartic acid. It was concluded that the

formation (or deamination) of (2*S*,3*R*)-*erythro*-methylaspartic acid, was inherent to the enzyme, and that the enzyme operates *via* a concerted *syn* elimination/addition reaction, during extended incubation times.[51] However, when the reaction was repeated for ethylfumaric acid, no activity was noted.[52]

Recent work within in our laboratories has shown that *N*-nucleophiles can serve as surrogates for ammonia in the direct amination of fumaric acids (Table 1.5).[53]



R^1	R^2	R^3	Conversion	Yield (%)
H	Me	H	55	34
Me	Me	H	54	35
Et	Me	H	60	35
Pr^n	Me	H	No reaction	—
Pr^i	Me	H	No reaction	—
H	Me	Me	70	38
Me	Me	Me	No reaction	—
H	NH_2	H	89	35
Me	NH_2	H	91	41
Et	NH_2	H	90	50
Pr^i	NH_2	H	90	—
H	OH	H	90	—
Me	OH	H	90	—
H	Et	H	5	—
H	OMe	H	80	30
Me	OMe	H	70	—
Et	OMe	H	No reaction	—

***Table 1.5:** Summary of the Enzyme Catalysed Addition Reaction*

Since hydrazine was shown to be an effective surrogate for ammonia, the feasibility of using hydroxylamine and methoxylamine as well alkylamines, as nucleophiles in the conjugate addition reactions was also determined (Table 1.4).[53] The rate of additions varied widely for different substrates. However, in general, hydrazine and hydroxylamine were found to be the fastest substrates and dimethylamine the slowest. The enzyme was also able to catalyse the addition of ethylamine to fumaric acid. However, the reaction occurs very slowly, on account of the steric constraints within the active site. The addition of hydrazine and hydroxylamine to chlorofumaric and bromofumaric acid was also catalysed by the enzyme. Remarkably, the enzyme catalyses the addition of dimethylamine to fumaric acid to give the expected product, (2*S*)-*N*,*N*-dimethylaspartic acid. Interestingly, only fumaric acid was able to support this type of reaction, (Fig. 1.6(**a**)). Insufficient space at the active site, and in particular, unfavourable steric interactions between the *N*-methyl groups and the alkyl group of the substituted fumaric acids probably account for the fact that the larger compounds were unable to act as Michael acceptors (Fig. 1.6 (**b**)).

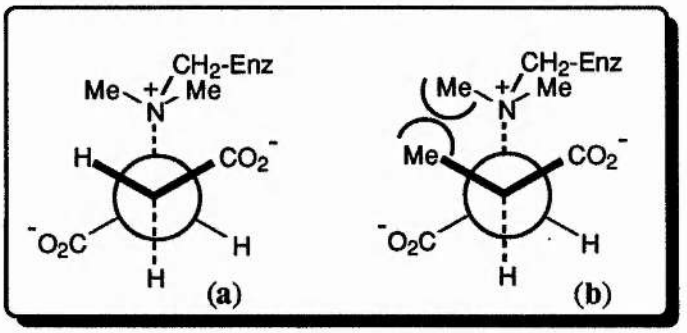


***Figure 1.6**: Plausible Geometry for the Enzymic Addition of Dimethylamine to Fumaric (**a**) and Mesaconic acid (**b**)*

The addition of hydrazine to mesaconic acid (**3**) follows the same stereochemical course as for the addition of ammonia and occurs at the 3-*si*-face at C-3 in an *anti*-fashion. Catalytic reduction of 2-hydrazino-3-methylsuccinic acid gave (2*S*,3*S*)-isomer of 3-methylaspartic acid (**2**), and was found to be identical, in all respects, with the authentic sample.[53]

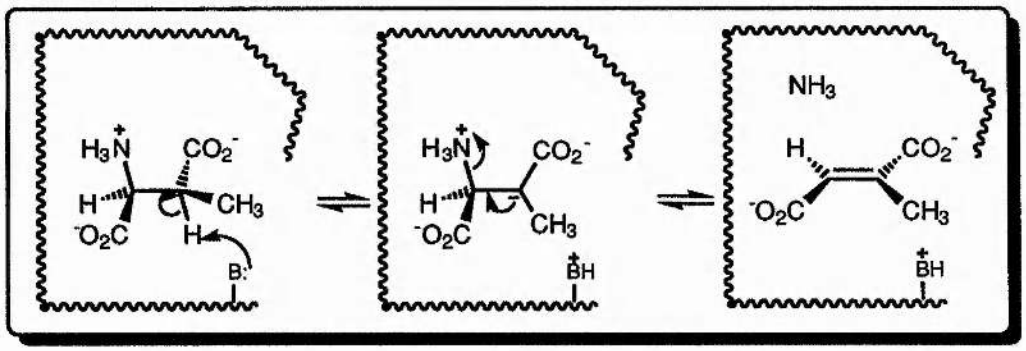
1.3.3 Kinetic Properties of 3-Methylaspartate Ammonia-Lyase

In 1959, Barker examined some of the fundamental characteristics of 3-methylaspartase. His work in this area revealed that the optimum pH for the enzyme is 9.7, and that the enzyme was most active at ~55 °C. However, the optimum pH for enzyme stability at 55 °C, was 6.7. The equilibrium constant, K_{eq}, for the deamination reaction at 25 °C and pH 9.7 was 0.3.[12] It was also shown that the enzyme required a divalent and a monovalent cation for enzyme activity.[12] Magnesium was found to be the best divalent metal ion for enzyme activity, although nickel, cobalt, zinc, iron, manganese and cadmium were also activators of the enzyme.[54] Work carried out by Bright and co-workers, showed that the order of activation to be: $Mg^{2+} > Mn^{2+} > Co^{2+} > Ni^{2+}$.[55] Calcium and strontium were found to be inhibitors of the enzyme. It was noted that the enzyme activity was directly related to the ionic radius of the cation and that those cations which possess an ionic radius of less than 1 Å were activators, whereas those larger than 1 Å were inhibitors of methylaspartase.[54] Of the monovalent cations, potassium was the most effective activator and the order of effectiveness for the monovalent cations was shown to be; $K^+ > NH_4^+ > Rb^+ > Li^+ > Na^+ > Cs^+$.[10] The K_m for potassium was 3 mM, under Barker's conditions. At high concentrations, however, potassium inhibited the reaction. The enzyme behaves autocatalytically in the absence of potassium, due to the formation of ammonium ion - a deamination product.

1.3.4 Mechanism of 3-Methylaspartate Ammonia-Lyase

Barker proposed that 3-methylaspartate ammonia-lyase, operated *via* a carbanion mechanism.[56,57] One of his main pieces of evidence was that (2*S*,3*R*)-*erythro*-3-methylaspartate was a substrate for the enzyme and he concluded that the carbanion intermediate generated at C-3 would be the same for both substrates. Using samples of (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid and unlabelled material, Bright showed that the rate of the deamination reaction was not

sensitive to substitution by deuterium at C-3 at pH 5.5, 7.5 and 9.5.[56] It should be noted that in these experiments the deuterated substrate contained a considerable amount of protium at C-3, and thus a small isotope effect may have been difficult to distinguish from the limits of experimental error. Furthermore, he found that ^{15}N-ammonia was incorporated into 3-methylaspartic acid at a negligible rate as compared to the C-3 hydrogen exchange with the solvent.[54] Thus hydrogen exchange could apparently occur without C-N bond cleavage. Bright concluded that the enzyme-substrate carbanion intermediate underwent hydrogen exchange before the elimination of ammonia and that after rate determining C-N bond cleavage ammonia and mesaconic acid were released from the active site rapidly. Hence, it was implied that 3-methylaspartase was the archetypal enzyme operating *via* an $E1_{cb}$ type mechanism (Scheme 1.10).



Scheme 1.10: *$E1_{cb}$ Mechanism of Methylaspartase*

However work carried out by Botting *et al.*, noted a primary deuterium isotope effect when the concentrations of potassium ion were lower than 50 mmol dm^{-3} (Table 1.6); thus indicating that the C-H bond cleavage was at least partially rate limiting and therefore, this questioned Bright's proposal.[58] Primary deuterium isotope effects were also measured for (2*S*)-aspartic acid and (2*S*,3*S*)-3-ethylaspartic acid, Table 1.6.

Substrate	K_M (mM)	V_{max} ($x10^{-7}$ mol dm^{-3} S^{-1})	V/K ($x10^{-4}$ S^{-1})
(2*S*)-Aspartic acid	10±0.82	0.80	0.076
(2*S*,3*R*)-[3-$^2H_1$]Aspartic acid	10±0.82	0.80	0.076
(2*S*,3*S*)-3-Methylaspartic acid	2.37±0.2	109.0	46.0
(2*S*,3*S*)-[3-^{2}H]-3-Methylaspartic acid	2.35±0.25	64.2	27.3
(2*S*,3*S*)-3-Ethylaspartic acid	17.08±1.4	48.7	2.85
(2*S*,3*S*)-[3-^{2}H]-3-Ethylaspartic acid	17.66±1.6	41.8	2.37

Table 1.6: *Kinetic Isotope Parameters*

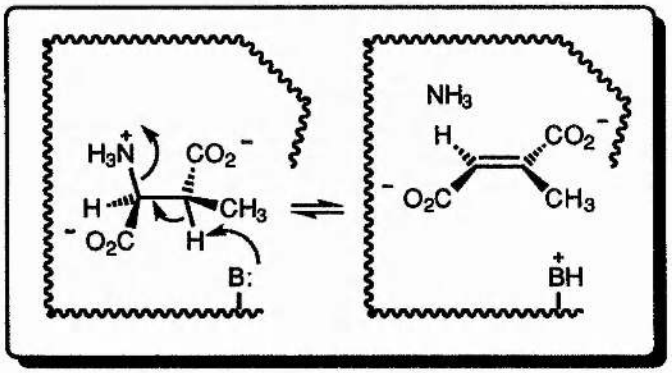
The absence of a deuterium isotope effect, for the deamination of aspartic acid, and the reduced isotope effect with 3-ethylaspartic acid, suggested that both (2*S*)-aspartic and (2*S*,3*S*)-3-ethylaspartic acids were potentially deaminated by a mechanism which was less concerted and one which contained a greater $E1_{cb}$ 'character'.[59] This can be rationalised as being due to the orbital alignment for a concerted elimination; which was considered optimal in the case of (2*S*,3*S*)-3-methylaspartic acid. Abstraction of the 3-C-H in the two slower reacting substrates generates a carbanion in which the torsion angle between the 2-C-N and 3-C-H bond is not optimal for the elimination of ammonia,[59] and hence there were no primary isotope effects were observed.

In (2*S*)-aspartate, a weak interaction of the 3-H with the hydrophobic methyl-binding pocket accounts for non-optimal orbital alignment of the carbanion and therefore no primary isotope effect. The substantially higher K_m for both substrate and product, than for the physiological compounds, suggests that substrate/product dissociation was not rate limiting. These results imply that 2-C-N bond cleavage was rate limiting and that 3-C-H bond cleavage was non-rate limiting, and thus it was concluded that (2*S*)-aspartic acid underwent deamination *via* an $E1_{cb}$ mechanism.[59]

In the case of (2*S*,3*S*)-3-ethylaspartate, strong interactions of the ethyl group with the hydrophobic methyl-binding pocket, resulted in non-optimal orbital

alignment of the carbanion, and therefore 3-C-H bond cleavage was non-rate limiting. However, the enzyme may still operate *via* an E2 mechanism since ethylfumarate binds more tightly in the active site than the physiological substrate, causing the product to debind and to become partially rate limiting.[48,59]

It was considered that the C-H bond cleavage was at least partially rate limiting for the natural substrate, and thus a need to distinguish between a balanced stepwise $E1_{cb}$ mechanism and an E2 type mechanism was necessary. Double isotope fractionation experiments, carried out by Botting *et al.* measured the kinetic isotope effect on C-N bond cleavage in the presence and absence of deuterium at C-3.[60] In a concerted (E2) mechanism, isotopic substitution at C-3 should not effect the kinetic isotope effect on C-N bond cleavage. However, a balanced stepwise $E1_{cb}$ mechanism would show a reduced kinetic isotope effect for C-N bond cleavage in the presence of deuterium at 3-C. Values of 1.0246 ± 0.0013 and 1.0241 ± 0.0009 were obtained for the ^{15}N isotope effect with protium and deuterium at 3-C respectively. These results indicated that the reaction followed a concerted pathway with a single transition state involving both 3-C-H and 2-C-N bond cleavages, *i.e.* by an E2 mechanism (Scheme 1.11).[60]

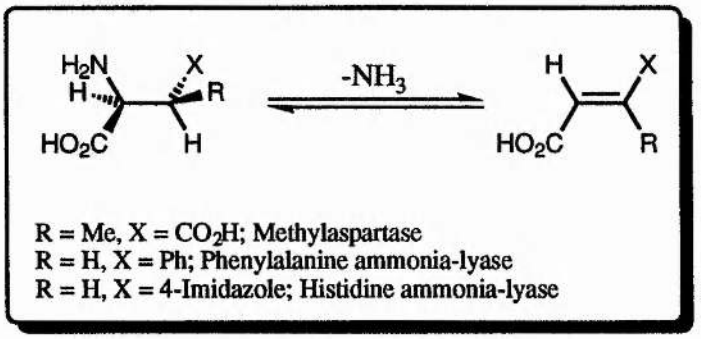


***Scheme 1.11:** E2 Mechanism of Methylaspartase*

Label exchange experiments were performed to explain the exchange reaction of the 3-C hydrogen of (2*S*,3*S*)-3-methylaspartic acid with the solvent. (2*S*,3*S*)-3-Methylaspartic and (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acids were incubated in tritiated water, thus allowing the rate of protium or deuterium wash-out from the substrates to be measured as tritium washed in. The primary deuterium

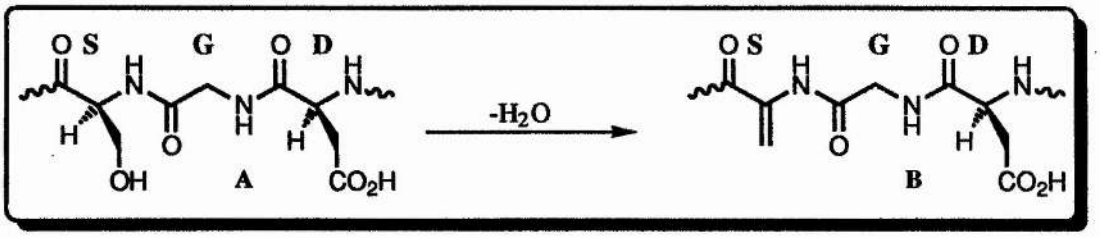
isotope effect on V_{max} for the exchange reaction at pH 9.0 was found to be 1.6.[61] The size of the isotope effect was not dependent on the ratio $V_{exchange}/V_{deamination}$, which showed large changes with pH. This suggested that the exchange reaction occurred after the concerted elimination step and that the step possesses a strong reversible commitment. It also indicates that the exchange did not occur at the free carbanion level, as $^{D}(V_{ex})$ was constant with pH.[61]

The ammonia-lyase enzymes, methylaspartase, phenylalanine ammonia-lyase[62] and histidine ammonia lyase[62], which catalyse the reversible elimination of ammonia from their respective amino acids to give the corresponding conjugated acids (Scheme 1.12), are believed to operate *via* mechanisms which require the formation of a dehydroalanine residue at the active site of the enzyme.



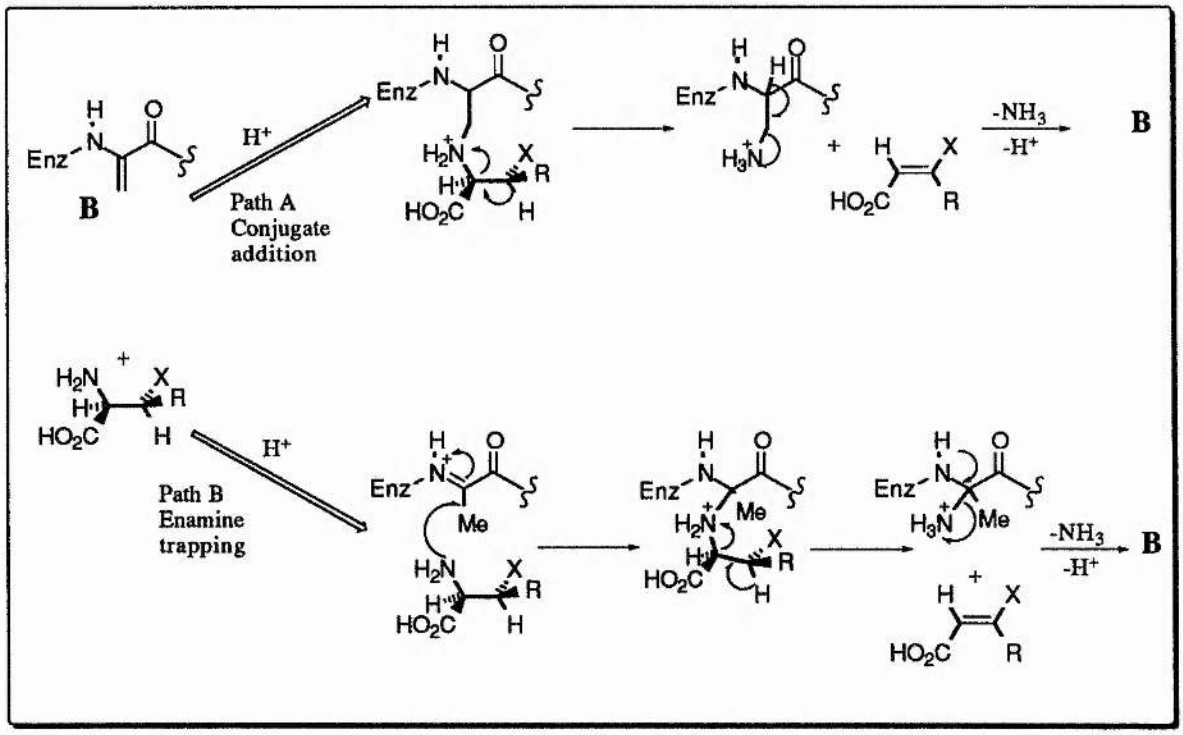
Scheme 1.12: *The General Reaction for the Ammonia-lyase Enzymes*

For methylaspartase the dehydroalanine residue moiety appears to be generated by the self-catalysed dehydration of a serine residue (Ser-173) within the polypeptide (**A**, Scheme 1.13).



Scheme 1.13: *Post-translational Formation of Dehydroalanine*

In the past it has been largely assumed that the dehydrolalanine residues within the ammonia lyases would undergo conjugate addition with the amino groups of the amino acid substrates to give substituted 2,3-diaminopropionic acid residues prior to the elimination of ammonia from the substrate (Scheme 1.14 path A)[62,63] Recent work within our laboratories reveal that such reactions are feasible. The authors also show that an alternative mechanism in which the dehydroalanine moiety behaves as an enamine and gives a conjugated imine which can then react with amines (Scheme 1.14 path B), should not be discounted as a mechanism of importance in enzymic deamination reactions.



Scheme 1.14: Plausible Routes for Amine Interaction with Dehydroalanine

Model studies were perfomed on the dehydroalanine residue (Fig. 1.7, A) by Gulzar, *et al.*[64]

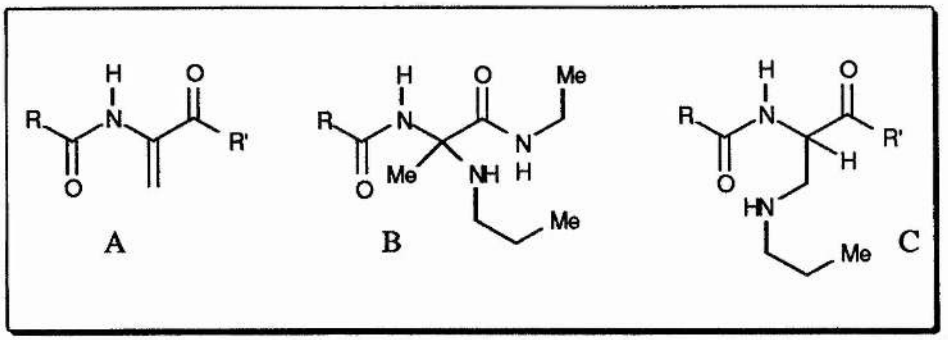


Figure 1.7: *The Reaction of Propylamine with Dehydroalanine Residues*

The studies show that when dehydroalanine (Fig. 1.7, A; where R = O^{t}Bu or OBn and R'=NHEt) is treated with propylamine, in methanol at room temperature, the sole product is the corresponding diaminal (Fig. 1.7, B). On the other hand, treatment of the dehydroalanine (Fig. 1.7 A; where R = O^{t}Bu or OBn, and R'=OMe) with propylamine, under the same reaction conditions, gave the corresponding conjugate addition product (Fig. 1.7, C). In addition, treatment of the dehydroalanine (Fig. 1.7, A; where R = Me and R'=OMe or NHEt) with propylamine, under the same reaction conditions, also gave the corresponding conjugate addition product (Fig. 1.7, C), thus indicating that the *N*-protecting group can profoundly influence the regioselectivity of the reaction. Taken together, the results of this study indicate that there is a fine balance between the α- and β-addition of *N*-nucleophiles to the dehydroalanine system. 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. Since the torsion about the amide bonds flanking the dehydroalanine residue in enzymes can be easily controlled to adjust the electronic properties of the putative Michael acceptor, either of the two mechanisms as shown in scheme 1.14 can operate.

1.3.5 Binding and Debinding Orders of 3-Methylaspartate Ammonia-Lyase

It was demonstrated by Bright and Silverman that (2*S*,3*S*)-3-methylaspartic acid is bound as the free amino acid rather than an amino acid-magnesium complex.[65] The experiment carried out to confirm this, was when 3-methylaspartic acid was incubated with methylaspartase, in the absence of magnesium ions, it was shown that mercuribenzoate had no inhibitory effect on the activity of the enzyme, thus suggesting that the substrate bound to the enzyme either first or in a random order with the divalent cation.[66] Kinetic analysis revealed that a random order, rapid equilibrium addition of methylaspartate and the divalent cation to the enzyme seemed most probable.

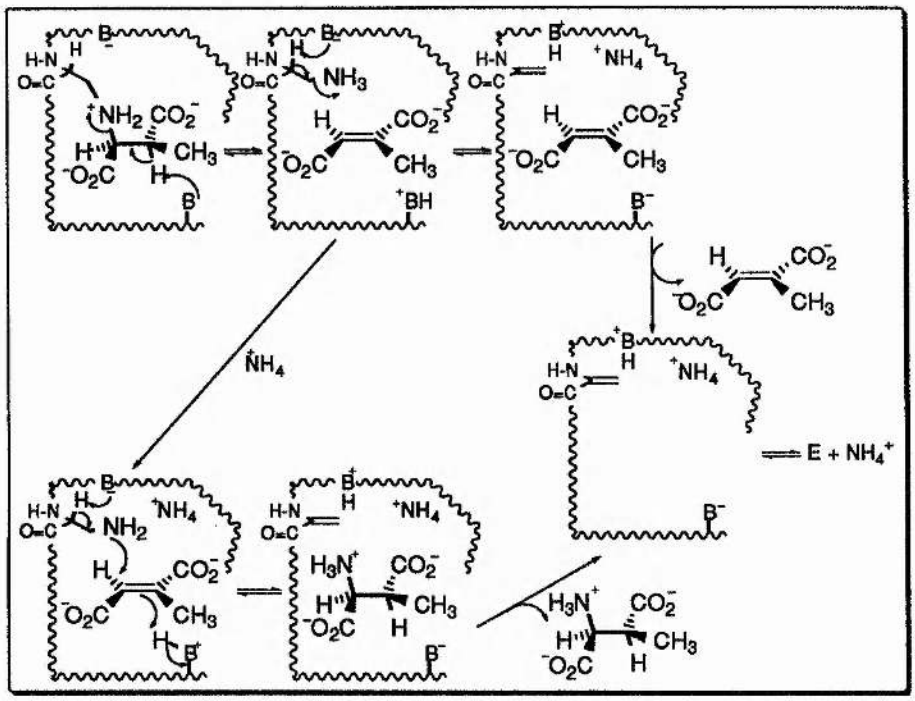
Botting and Gani showed that methylamine did not act as an activator of methylaspartase, but in fact behaved as a competitive inhibitor of 3-methylaspartic acid, in the deamination reaction and that of ammonia and mesaconic acid in the amination direction.[61] These experiments carried out, show that mesaconic acid and the ammonium ion are released at the same rate with random debinding. This hypothesis was also supported by the fact that mesaconic acid acts as a competitive inhibitor for (2*S*,3*S*)-3-methylaspartic acid.[61]

Bright observed that the ammonium ion can enhance the exchange rate, over that for normal deamination, and that ^{15}N-labelled ammonia was not incorporated into the substrate in an exchange reaction. From this observation, it appears that the increase in $V_{exchange}/ V_{deamination}$, is caused without cleavage of the C-N bond.[56] Botting and Gani showed that by incubating ^{15}N-labelled ammonia and [3-$^{2}H_3$]-mesaconic acid, with unlabelled (2*S*,3*S*)-3-methylaspartic acid, that the rapid formation of [^{15}N, *methyl* -$^{2}H_3$]-3-methylaspartic acid occurs. The singly labelled species were formed only slowly and could be accounted for by reaction with free unlabelled substrates. Thus the rate of mesaconic acid release by the enzyme was equal to the rate of ammonium ion release and that debinding was random.[61] This result confirms Bright's findings.[56]

Re-examination of Bright's work at low pH shows that the substrate and magnesium ion bound in a random steady state manner followed by potassium ion.[61] At pH 6.5 and high potassium ion concentrations, the binding order was compulsory, with that of potassium ion binding, after the substrate, and released before the first product.[61] At pH 9.0, magnesium ion bound before the substrate, in rapid equilibrium and the potassium ion bound after the substrate. At low magnesium ion concentrations, a binding order of: the substrate, then potassium and then magnesium was favoured. At low potassium ion concentrations, the substrate bound in the steady state co-operativity exists between the two active sites of the enzyme, which was in a new catalytic form, with a high affinity for potassium.[61]

Binding/ debinding experiments suggest that there was a slow step after C-N bond cleavage, but before the formation of the $E.NH_4^+$.Mes complex, which causes the products to partition between recombination (to give methylaspartic acid), and formation of the $E.NH_4^+$.Mes complex, from whence the product could escape.[61] Therefore, in labelled water, solvent-derived hydrogen could exchange with the conjugate acid on the enzyme (after the base removes the proton from the substrate at 3-C) to give labelled substrate. Since ammonium ion enhances the exchange reaction, the ion must prevent the formation of the $E.NH_4^+$.Mes complex. Ammonium ion could easily do this if the product site for ammonium ion is not occupied in the intermediate complex by the amino group of the substrate (Scheme 1.15).[61]

The evidence put forward for the second binding site for ammonia was firstly; that the rate of reaction was dependent upon $[NH_4^+]^2$, *i.e.* two molecules of ammonium ions were involved and secondly, ammonia behaves as a non-linear uncompetitive product inhibitor for the deamination process.



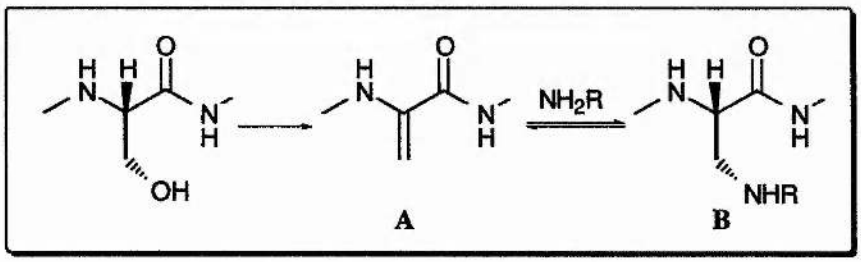
Scheme 1.15: Exchange Mechanism of 3-Methylaspartate Ammonia-lyase

1.3.6 Active Site Structure of 3-Methylaspartate Ammonia-Lyase

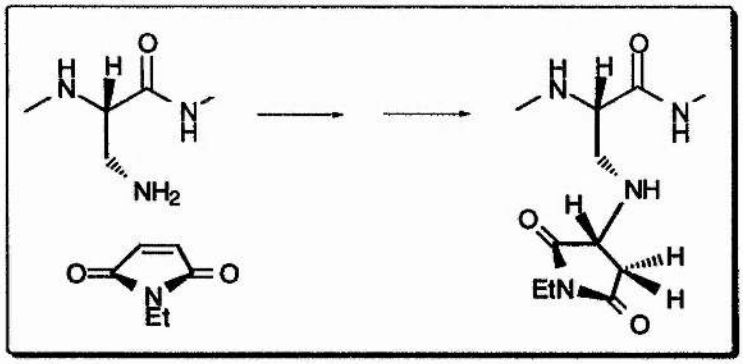
Enzymes containing a catalytic dehydroalanine residue such as histidine ammonia lyase and phenylalanine ammonia lyase, are inactivated by sodium borohydride; however, methylaspartase shows no inactivation in the presence of sodium borohydride.[31] In addition, phenylhydrazines cause partial-irreversible inactivation of MAL, with some inactivation protection afforded by the substrate; suggesting that an electrophilic prosthetic group is present at the active site.[31]

Wu and Williams showed that eight uniformly labelled peptides were formed upon treatment of methylaspartase with [1-^{14}C]-*N*-ethylmaleimide (thiol reacting compound), followed by tryptic digestion, and assumed that eight cysteine residues in the protein had been labelled.[32] There was also evidence, provided by Bright, which show the presence of sixteen cysteine residues per mole of enzyme.[39,40] Furthermore, an active site peptide was isolated and was believed to be labelled on a cysteine residue (with [1-^{14}C]-*N*-ethylmaleimide).[32]

This peptide corresponds to one of the eight uniformly labelled peptides obtained from the earlier experiment,[32] and as a consequence the amino acid composition was determined by total acid hydrolysis.[32] Upon attempting to match this amino acid composition to the primary structure of methylaspartase, there was no observable match if it was assumed to contain a cysteine. However, if the labelled residue was taken as a serine, a match was obtained. Moreover, from the primary structure, methylaspartase contains seven cysteine residues rather than eight. In order to explain the reaction of serine with *N*-ethylmaleimide, a post-translational modification of the serine to a dehydroalanine residue (Scheme 1.16, **A**) was invoked.[31] The dehydroalanine residue could conceivably be attacked by the amino group of the substrate to give a 2,3-diaminopropanoic acid residue (Scheme 1.16, **B**), which could then react with *N*-ethylmaleimide, alkylating the enzyme (Scheme 1.17).



Scheme 1.16: *Formation of the 2,3-Diaminopropanoate Residue*



Scheme 1.17: *The Reaction of N-Ethylmaleimide with 2,3-Diaminopropanoate*

1.4 β-Amino Acids

1.4.1 Introduction

Chemical synthesis of β-alanine, the simplest of the β-aminocarboxylic acids, was reported by Heinz in 1870[67] only 20 years after Strecker's classic, albeit inadvertent synthesis of α-alanine.[68] Within 30 years six additional syntheses of β-alanine were reported. Occurrence of β-alanine as a microbial catabolite of aspartate was reported as early as 1911[69] and carnosine (β-alanylhistidine) was identified in skeleton muscle in 1900.[70] β-amino acids were often included in early synthetic peptides and by the early 1900's, it was clear that proteins were composed exclusively of α-amino acids and β-amino acids were increasingly perceived as lying outside the mainstream of mammalian biochemistry.

This perception was strongly supported by the scientific developments that followed. Whereas many novel and complex β-amino acids, were isolated from plants and microorganisms as free molecules and components of peptides and antibiotics,[71] few β-amino acids were found in mammals, and their metabolism appeared, at least initially, to be simple.

Beyond their limited metabolic role, β-amino acids hold interest as analogues of α-amino acids. β-Amino acids have for example, been used to probe the structural specificity and topology of several α-amino acid-binding sites and have found use as enzyme specific alternative substrates. The limited normal metabolism of β-amino acids suggests that many β-amino analogues of α-amino acids will be metabolically stable *in vivo*; enzyme inhibitors may thus have extended half-lives when designed as β- rather than α-amino acids.[71] Similarly, incorporation of β-amino acids into peptides of pharmacological interest has in some cases been found advantageous in terms of biological activity, metabolic stability, or both.[71]

1.4.2 Nomenclature of β-Amino Acids

As illustrated in figure 1.8, there are three different β-amino acids which are plausible analogues of an α-amino acid. In type I analogues the α-amino group is 'moved' to the β-carbon whereas in type II or type III analogues an extra methylene group is inserted between the original carboxyl and amino groups. Type I and II analogues are named systematically as 3-amino derivatives of the parent acid but are frequently referred to by the nomenclature shown in figure 1.8. It is noted that there is some redundancy (β-leucine = β-homovaline, etc.); in such cases the type I name is preferred. α-Substituted-β-alanines (type III analogues) are named systematically as substituted propanoic acids or, less commonly, as 2-aminomethyl derivatives of the parent acid (*e.g.* 2-aminomethyl-4-methylpentanoic acid in the example shown). With the exception of β-proline (3-carboxypyrrolidine), α-substituted-β-alanines are not named by reference to the homologous α-amino acid. The prefix 'iso' is, however, used with some α-substituted-β-alanines. Isoserine and isocysteine are α-hydroxy- and α-mercapto-β-alanine, respectively; isothreonine is β-amino-α-hydroxybutanoic acid. Many di- and trisubstituted-β-alanines bear trivial names related to the plant, microorganism, or antibiotic in which they were discovered.[71]

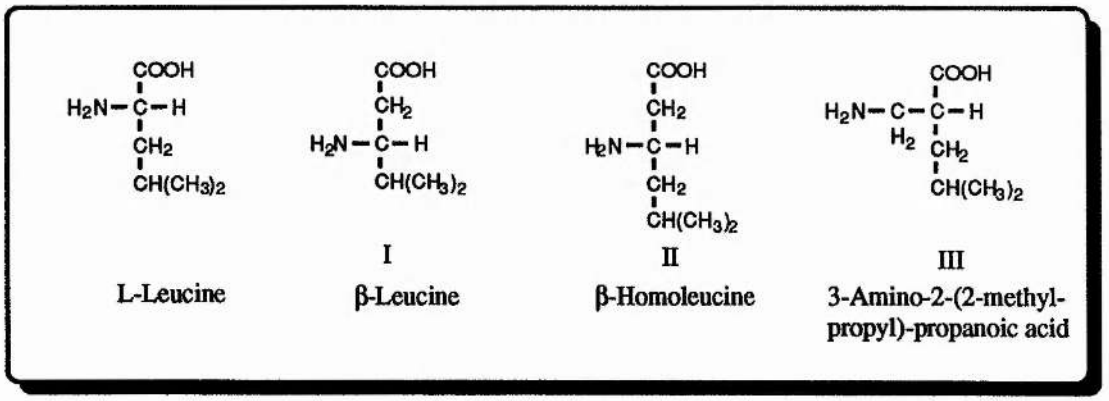


Figure 1.8: β-Amino Acid Analogues of L-Leucine

Both α- and β-substituted-β-alanines generally have at least one chiral carbon and thus occur in *R*- and *S*-configurations. Although all of the protein L-α-

amino acids except cysteine are *S*-enantiomers, the configurationally analogous β-amino acids may be *R* or *S*. The enantiomers shown in figure 1.8, all analogues of L-α-leucine, are *R*-β-leucine, *S*-β-homoleucine and *R*-3-amino-2-(2-methyl)propyl-propanoic acid. The D and L designations common to α-amino acids are frequently applied to type I and type II analogues by evaluating the configuration at the β-carbon as if it were the α-carbon. The D and L designations are less clearly assigned with type III analogues. *R*-β-Aminoisobutyrate, the type III analogue of L-alanine, is, for example, referred to as D-β-aminoisobutyrate. Correspondingly, in the sense illustrated in figure 1.8, *S*- or L-β-aminoisobutyrate is an analogue of D-alanine[72]

1.5 β-Amino Acids as Analogues of α-Amino Acids

1.5.1 β-Amino Acid-Containing Peptides

Early studies carried out by Abderhalden suggested that peptide bonds involving β-amino acids are resistant to enzymatic hydrolysis.[73,74] However, later studies tempered this view.[75] Interest in the synthesis, hydrolytic stability and conformational characteristics of β-amino acids-containing peptides continues.

As reviewed in depth by Drey,[71] several peptide antibiotics contain β-amino acid residues and analogues of these peptides and others (*e.g.* enkephalins) have been prepared in an effort to achieve greater stability or specificity of action. Since pure enantiomers of β-amino acids are not readily available, β-alanine is most frequently used to replace glycine, or less commonly, another α-amino acid. Several analogues have substantial or full biological activity, whereas in other cases the activity spectrum is modified. Replacement of the C-terminal Gly-NH_2 of vasopressin with β-AlaNH_2, for example yields an analogue with antidiuretic and uterotonic activity but without pressor activity.[76] On the other hand, 25 analogues of the sweetener Aspartame ((2*S*)-Asp-(2*S*)-Phe-OCH_3) were

synthesised with the Phe residue replaced by any several α- or β-substituted-β-alanines; none approached the sweetness of the parent molecule.[77]

Limited information on the topology of binding sites and on the conformational effects of inserting β-amino acids into peptides precludes reliable prediction of the biological activities of analogues.

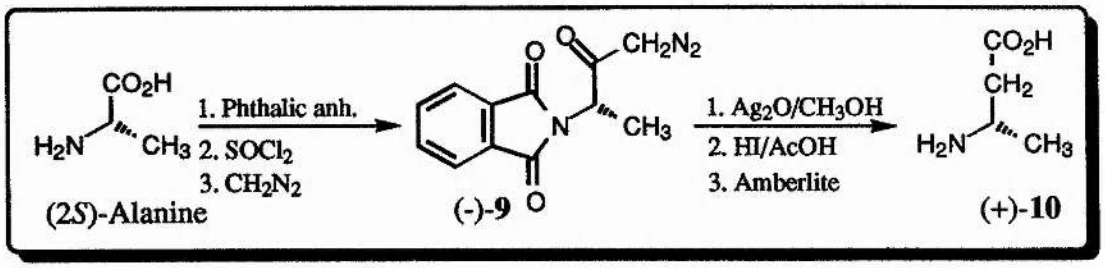
1.5.2 Chemical Synthesis of β-Amino acids.

β-Amino acids although less abundant than their α analogues, are also present in peptides,[78] and in free form they show interesting pharmacological effects.[79] Furthermore, β-amino acids are synthetic precursors of β-lactams,[80] which are potentially biologically active and of current interest.[81] In this respect, several methods for the synthesis of racemic β-amino acids have been developed,[82] but only recently has the preparation of enantiomerically pure compounds emerged as an important and challenging synthetic endeavour

The main procedures developed thus far for the enantioselective synthesis of β-amino acids can be tentatively separated into seven categories (*A-G*), as detailed below. (An example of each category will be mentioned briefly, within this section).

1.5.2.1 A. The "Chiral Pool"

The chiral pool refers to the utilisation of inexpensive, readily available natural products as substrates to be converted into enantiomerically pure β-amino acids *via* conventional organic synthesis. According to this strategy, Balenovic and co-workers[83] described the application of the Arndt-Eistert reaction to (-)-1-diazo-3-phthalimidobutan-2-one (**9**), prepared from (2*S*)-alanine, to give (+)-β-aminobutyric acid (**10**) (Scheme 1.17).

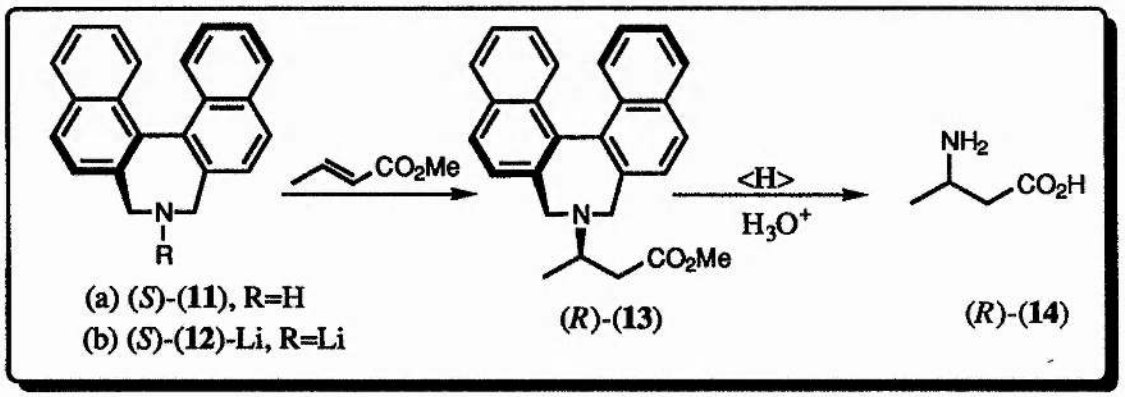


Scheme 1.17: Asymmetric Synthesis of β-Aminobutyric Acid

1.5.2.2 B. Asymmetric addition of amines to α,β-Unsaturated Esters

Terentev and co-workers reported the first example of an enantioselective addition of a chiral amine to crotonic acid,[84] nevertheless, the enantiomeric ratios obtained were quite low.

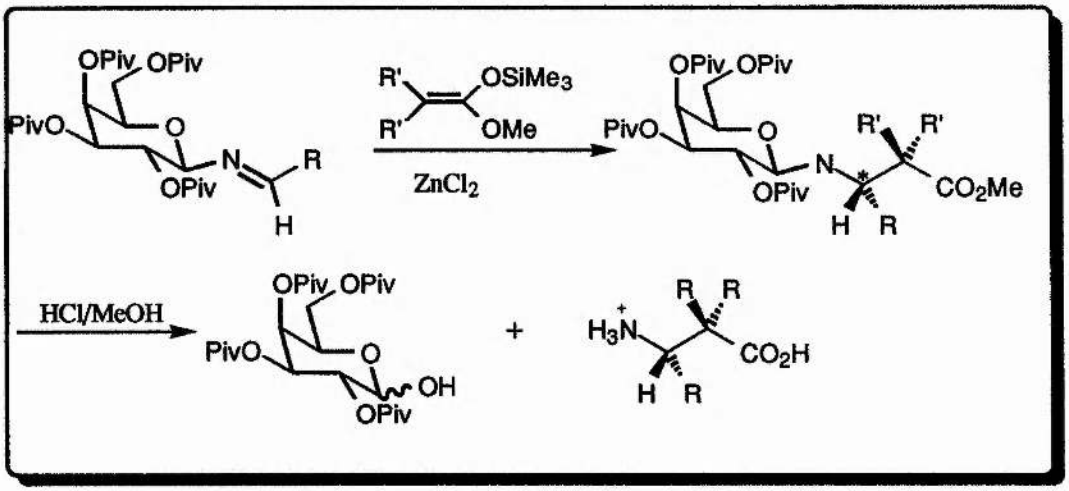
An improvement in this methodology, by Hawkins and co-workers,[85] gave better results, utilising the C_2 symmetric chiral secondary amine (**11**). Initially, treatment of (±)-(**11**) with methyl crotonate gave the amino ester (±)-**13** as a 78:22 mixture of diastereomers in 68% yield (Scheme 1.18). More useful Michael additions of the lithium amide (*S*)-(**12**)-Li occurred with very good diastereoselectivity to afford (*R*)-(**13**) as a 98:2 mixture of diastereomers in 81% yield (Scheme 1.18).



Scheme 1.18: Enantioselective Addition of Chiral Amines with α,β-Unsaturated Esters

1.5.2.3 C. Addition of C-Nucleophiles to Chiral Imines

Pioneering efforts in this direction were first reported by Furukawa and collaborators in 1978.[86] However low enantiomer ratios of the β-amino acids were obtained. More recently, Kunz and Schazenbach[87,88] described the asymmetric synthesis of *N*-unsubstituted β-amino acids *via* the diastereoselective Mannich reaction of Schiff bases of 2,3,4,6-tetra-*O*-pivaloyl-β-D-galactosylamine. One advantage of this method is the recovery of the chiral auxiliary group (Scheme 1.19).



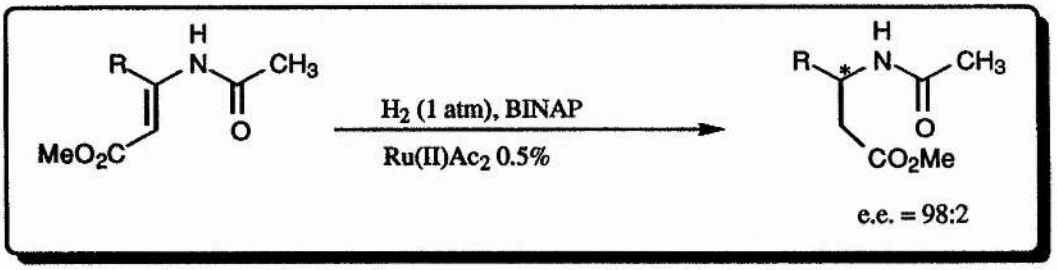
Scheme 1.19: Asymmetric Synthesis of N-Substituted β-Amino Acids

1.5.2.4 D. Enantioselective Hydrogenation of 3-amino-acrylic acid Derivatives

Early pioneering work in this area was carried out by Achiwa and Soga.[89] They performed asymmetric hydrogenation on prochiral, β-(acetylamino)acrylic acid derivatives, using biphosphines as the chiral ligands, to give the corresponding optically active product. Unfortunately, these reactions had only modest success, affording the desired products in low enantiomeric purities.

Improvement in the efficiency and understanding, in this area of asymmetric synthesis, was achieved by the Noyori group.[90] They demonstrated that BINAP-Ru(II) metal complexes [BINAP = 2,2'-bis(diarylphosphino)-1,1'-binaphthyl]

serve as excellent catalysts for enantioselective hydrogenation of β-substituted (*E*)-β-(acylamino)acrylic acids (Scheme 1.20).

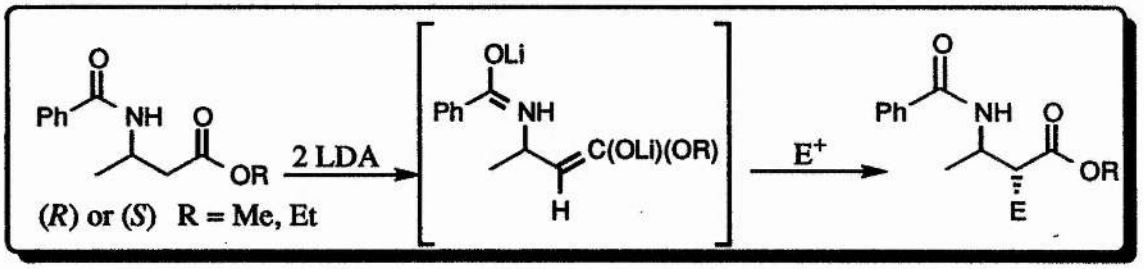


***Scheme 1.20:** Enantioselective Hydrogenation of 3-Amino Acid Derivatives*

Interestingly, the (Z) double bond isomers (which possess an intramolecular hydrogen bond between amide and ester groups) are more reactive but are hydrogenated with poor enantioselectivity with the opposite sense of enantioinduction.[90]

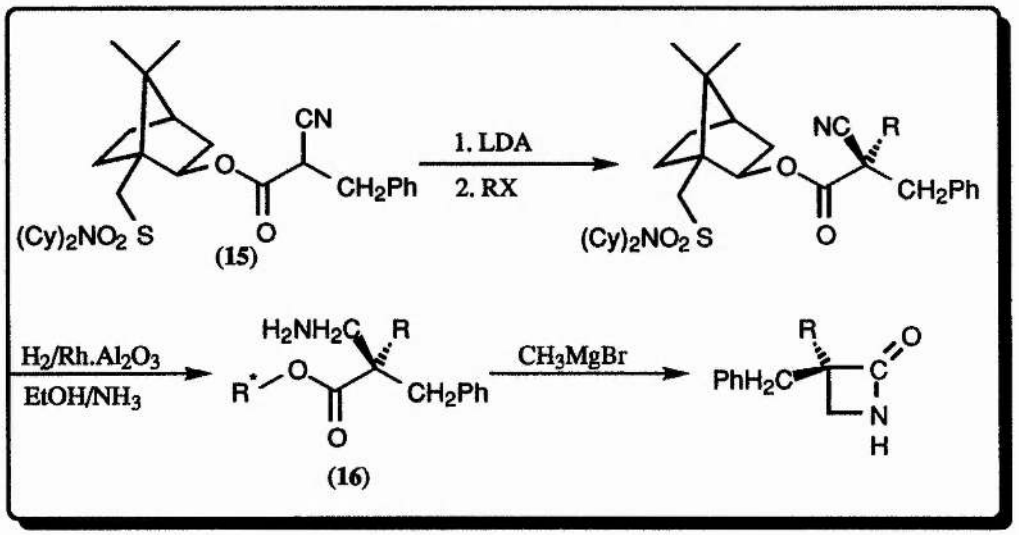
1.5.2.5 E. Stereoselective Alkylation of β–Amino Ester α-Enolates

N-Acyl-β-amino acid derivatives have been doubly deprotonated and then α-alkylated with moderate to excellent selectivity.[91] Enantiomerically pure 3-aminobutanoic acid derivatives were prepared by Seebach and Estermann[92] *via* dilithiated methyl- or ethyl-*N*-benzoyl-3-aminobutanoates (Scheme 1.21). The selectivity with which these reactions took place was usually very high, affording the enantiomerically pure product



***Scheme 1.21:** Synthesis of Enantiomerically Pure 3-Aminobutanoic Acid Derivatives*

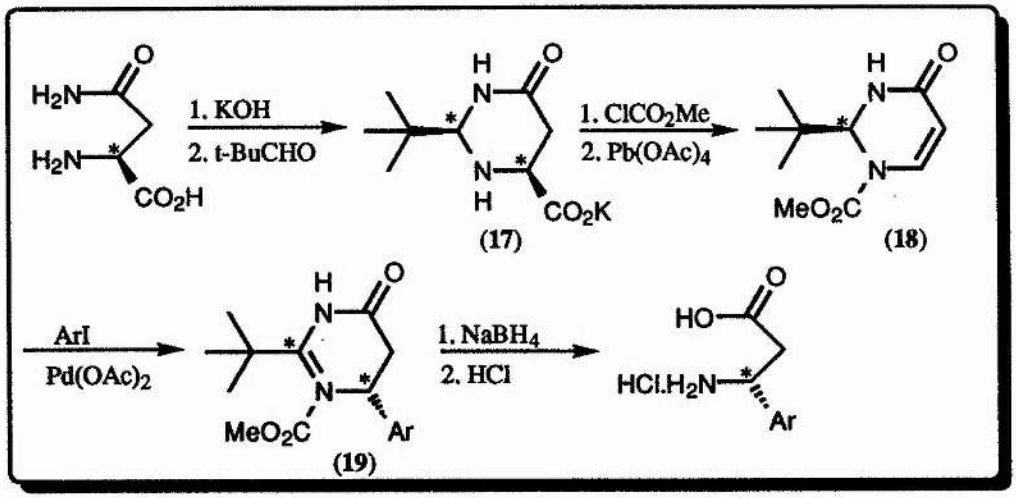
More recent work in this area by Cativela and coworkers[93] describe the diastereoselective alkylation of the enolate of isobornyl derivative (**15**). This alkylation takes place with very good yields and selectivity. The products were subsequently reduced to β-amino esters (**16**), which were then cyclised to β-lactams (Scheme 1.22).



Scheme 1.22: Stereoselective Alkylation of β-Amino Ester α-Enolates

1.5.2.6 F. Self-Regeneration of Stereogenic Centres

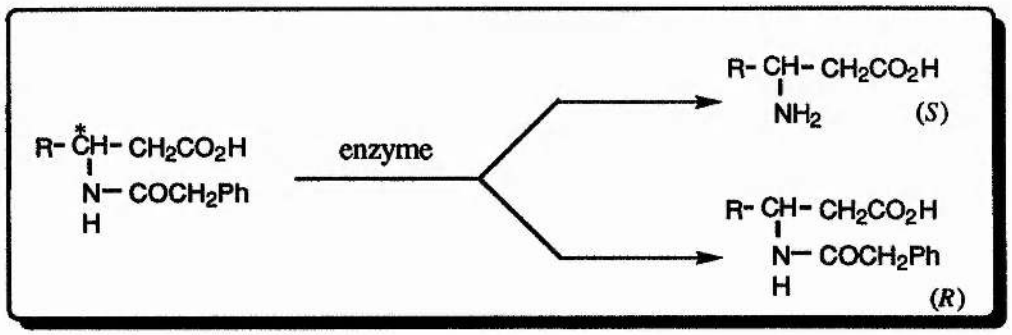
Konopelski *et al.*[94] developed a methodology in which the acetalisation of the potassium salt of (*S*)-asparagine formed pyrimidinone carboxylate (**17**) diastereoselectively. Carbomethoxy derivatisation of the secondary amine group followed by oxidative decarboxylation gave the unsaturated heterocycle (**18**). Although the original stereogenic centre is converted to a trigonal centre, the bulky *tert*-butyl group on the acetal carbon induces the stereoselective formal conjugate addition of aryl iodides in the presence of catalytic amounts of $Pd(OAc)_2$ to afford derivatives (**19**). Treatment of (**19**) with $NaBH_4$ followed by hydrolysis gave the desired enantiomerically pure β-amino acid hydrochlorides (Scheme 1.23).



Scheme 1.23: β-Amino Acids via Self-Regeneration of Stereogenic Centres

1.5.2.7 G. Enzymatic Methods

Recently, enantiomerically pure β-amino acids have been prepared by routes involving the enzymatic resolution of racemates.[95-97] For example, the fifty percent conversion of racemic *N*-phenylacetyl derivatives of β-amino acids with penicillin acylase allowed for the separation of the (*S*)-amino acid from the (*R*)-*N*-protected derivative (Scheme 1.24).[97]



Scheme 1.24: Enzymic Resolution of β-Amino Acid Racemates

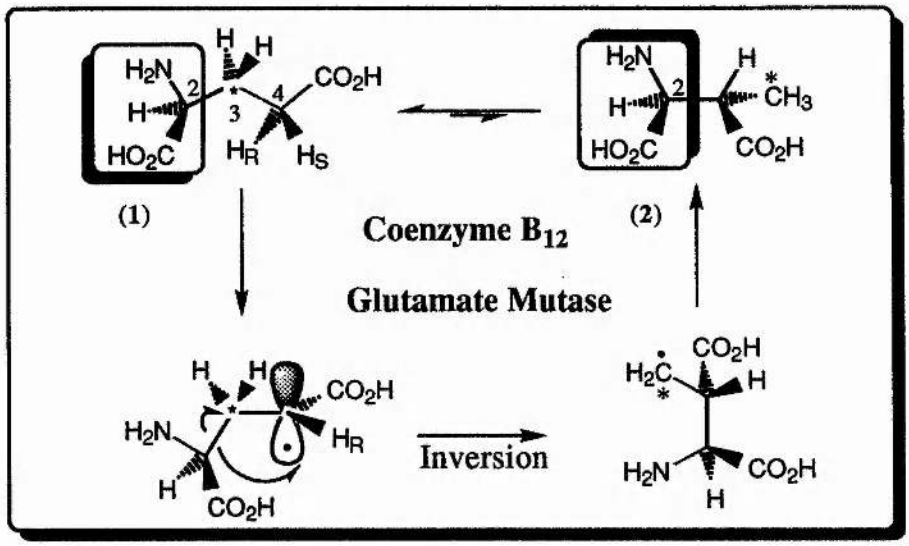
CHAPTER 2

RESULTS & DISCUSSION

2.0 Glutamate Mutase

2.1 Introduction

The transformation catalysed by glutamate mutase is a uni-uni reaction. (2*S*)-Glutamic acid (**1**) and (2*S*,3*S*)-3-methylaspartic acid (**2**) are the only known substrates to date.



Scheme 2.1: The Glutamate Mutase Reaction

The high selectivity of the enzyme for these two compounds,[22] severely restricts the physico-chemical methods that can be used in order to delineate details for the rearrangement. The discovery of appropriate molecular probes, substrates or inhibitors, which might interact with the enzyme and provide information about the mechanism and the radical chemistry involved, is therefore of highest priority.

No information is currently available on the three dimensional structure of glutamate mutase, and at present, there is no information known about the overall shape of the active site. Nevertheless, some data has recently been collected, and can be used to address three main issues. First, is the design of novel structures to probe the mechanism of the rearrangement in relation with the shape of the active site. A second issue, is highlighting the role of the

different functional groups on the natural substrates. Another important area which we have considered, is to look at a new class of transition-state analogues (Fig. 2.4), which can be used to gain valuable information about the active site of the enzyme. Finally, the various mechanistic aspects of the rearrangement step can be evaluated and a satisfactory working hypothesis can be proposed.

The structures initially identified as possible mechanistic probes were (±)-*cis/trans*-2,3-methanoaspartic acid (**21, 22**), (2*S*,3*S*)-[3'-$CH_2C^2H_3$]-3-ethylaspartic acid (**23**), (2*S*,3*S*)-[3'-$C^2H_2C^2H_3$]-3-ethylaspartic acid (**24**), 1-substituted cyclopropane 1,2-dioic acids (**25**) and (3*S*)-methylsuccinic acid (**26**). Our efforts towards these synthetic targets are discussed later in this chapter.

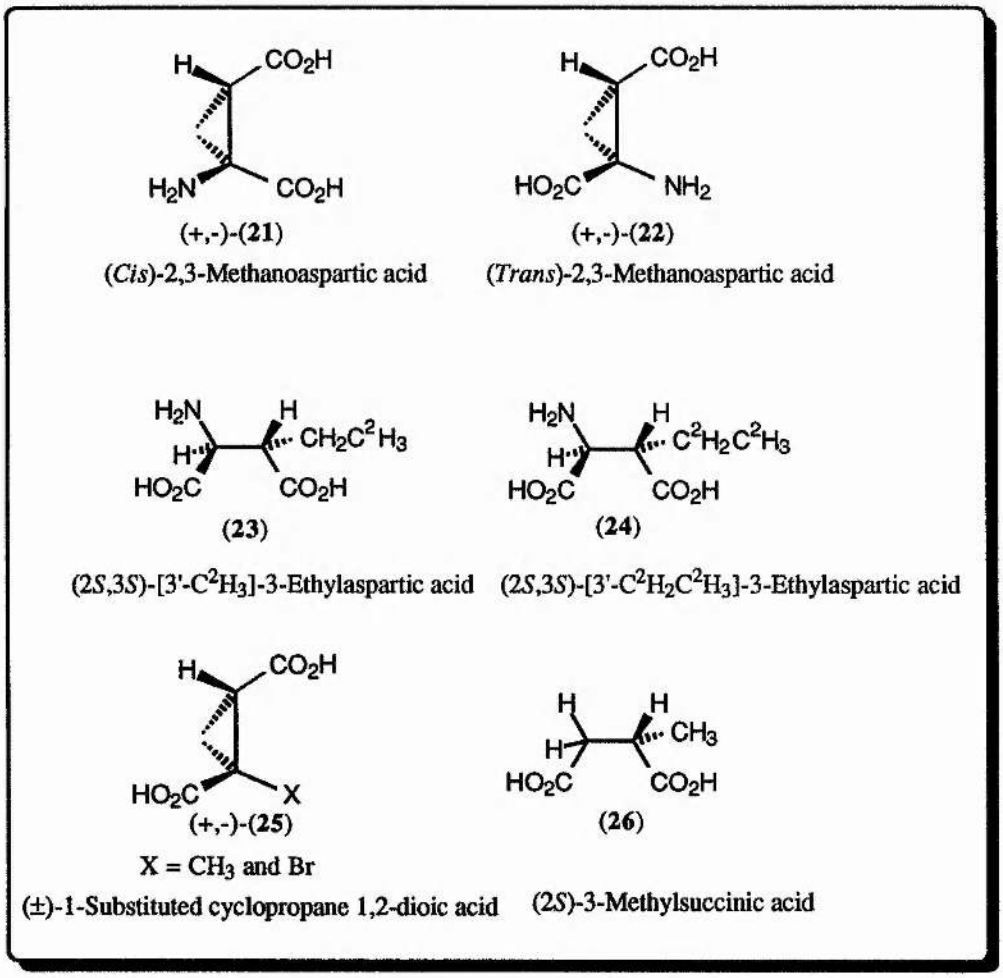
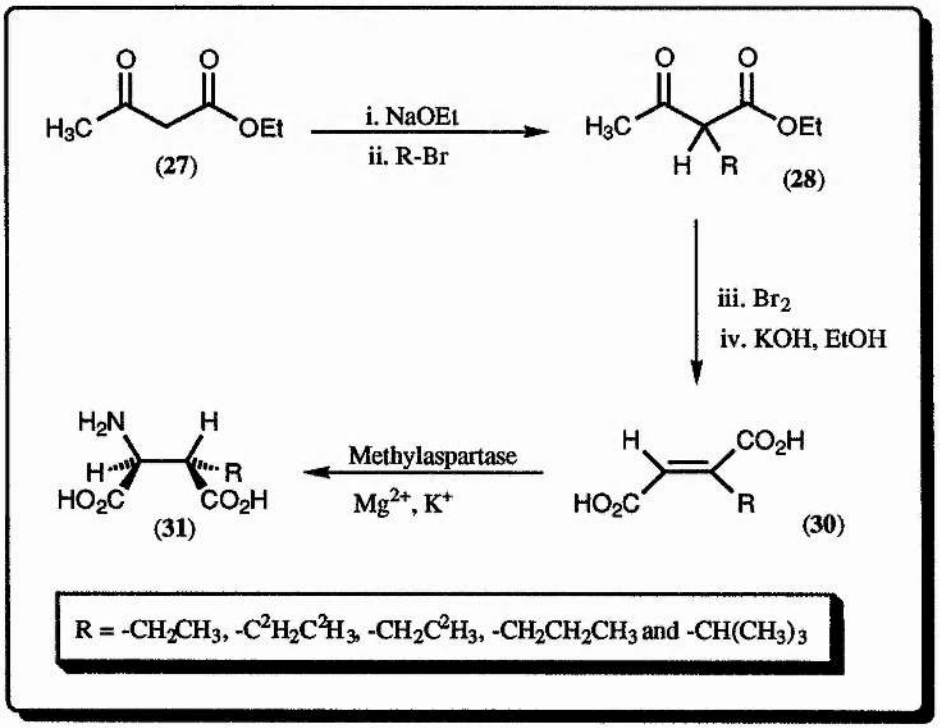


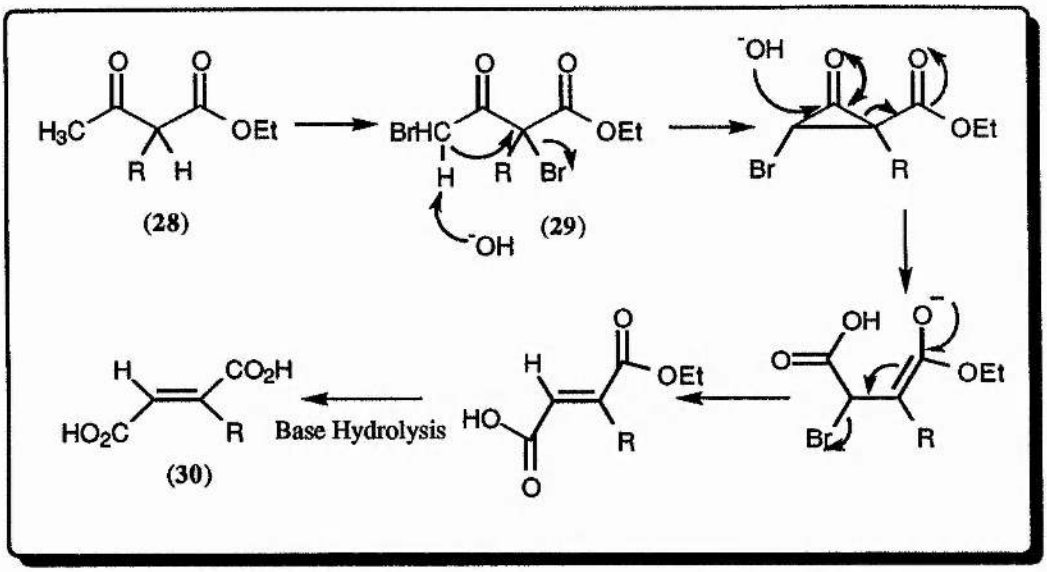
Figure 2.1: *Mechanistic Probes for the Enzyme Glutamate Mutase*

2.2 General Synthesis of 3-Alkyl Aspartic acids

It has been demonstrated in our laboratories,[49] that 3-alkylfumaric acids (**30**) undergo enzymic amination in the presence of β-methylaspartase, to give the corresponding 3-alkylaspartic acids (**31**, Scheme 2.2). The general preparation of 3-alkylfumaric acids (**30**) is shown in scheme 2.3, where ethyl acetoacetate is treated with anhydrous sodium ethoxide, generating a stabilised anion, which undergoes a nucleophilic attack on the appropriate alkyl halide, giving rise to the formation of the corresponding alkylated ester (**28**). The alkylated ester is treated with bromine (2 equivalents), forming the dibromoketoester (**29**). The dibromo-ketoester (**29**) then undergoes the Favorskii rearrangement (Scheme 2.3) to give the desired fumaric acid (**30**).



Scheme 2.2: General Synthesis of 3-Alkylaspartic Acids



Scheme 2.3: The Favorskii Rearrangement

Improved yields of the alkylated esters (**28**) were obtained by increasing the amount of alkyl halide (1.5 equivalents), used in the alkylation step. The percentage yields obtained of the alkylated esters ranged between 65 - 76%. The alkylated esters were then transformed into the desired fumaric acids as described in scheme 2.2, and the table below shows the isolated yields obtained.

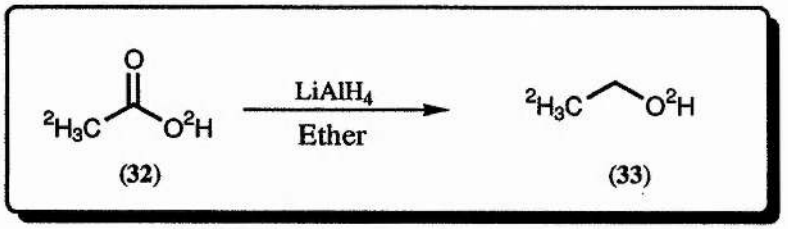
Compound 30 (R =)	% Yield	Mpt °C
$-CH_2CH_3$	45	195 - 197
$-C^2H_2C^2H_3$	50	195 - 197
$-CH_2C^2H_3$	50	190 - 192
$-CH_2CH_2CH_3$	45	172 - 173
$-CH(CH_3)_2$	48	180 - 183

Table 2.1: Synthesised Fumaric Acids

The next stage in the preparation of the 3-alkyl aspartic acids (**31**), involved the stereospecific enzymic conversion, of the appropriate fumarate, by β-methylaspartase, in the presence of Mg^{2+} and K^{+}.

2.2.1 Synthesis of Labelled 3-Ethylaspartic Acids.

[1,1,1-$^2H_3$]-Ethanol (**33**) was synthesised by the method of Friedman and Jurewicz, from the reduction of $C^2H_3CO_2{}^2H$ (**32**) by $LiAlH_4$ in dry ether, in 88% yield (Scheme 2.4).[98] Since the product of this type of reduction was low boiling and water soluble, isolation procedures usually recommended[99] was generally unsatisfactory. The efficient procedure employed by Friedman and Jurewicz, is based on the use of high-boiling ether alcohols as product liberating reagents and isolation of product by distillation directly from the reaction mixture. The reduction was conducted in 2-butoxyethanol, as this proved to be the most suitable alcohol.[98]



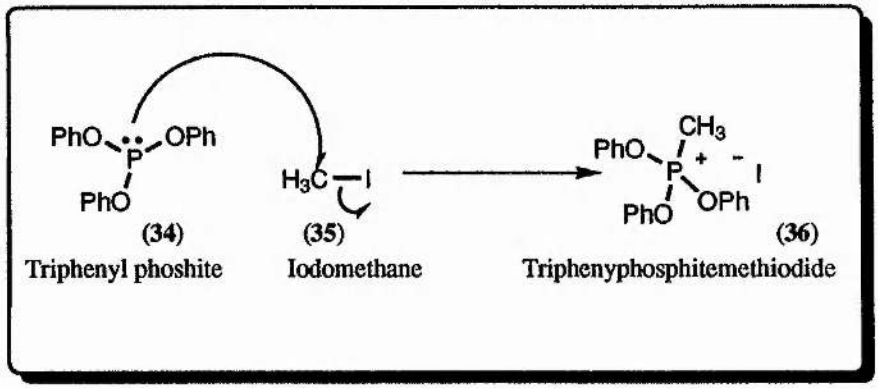
(**32**) (**33**)

Scheme 2.4: Synthesis of Labelled d_4-Ethanol

The preparative procedure, in the synthesis of the alkyl halides, was very simple; the alcohol was treated with triphenylphosphite methiodide (**36**, Scheme 2.5) (which need not be purified) and merely mixed in equivalent proportions; this is followed by reflux, and the product (alkyl halide) is worked up either by direct distillation or by washing out the phenol produced in the reaction, with alkali, followed by distillation.

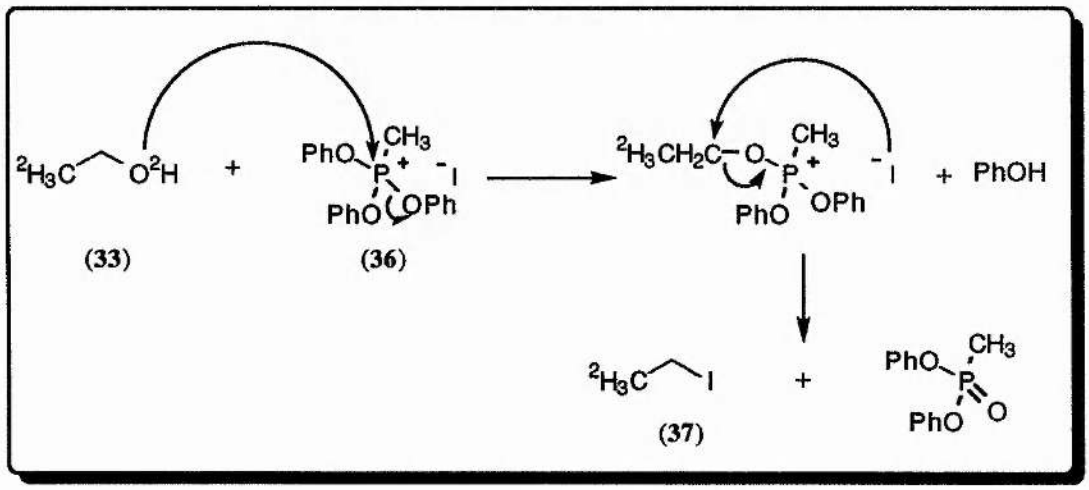
Triphenylphosphite methiodide (**36**) was easily prepared, by treating the triarylphosphite (**34**) with iodomethane, and the mixture then heated under reflux for 36 hours, with the exclusion of moisture. The temperature was

maintained between 110-115 °C, during the course of the reaction, as this effects the yield of the reaction. The quasi-phosphonium compound (**36**), was isolated as yellowish-brown needles, and the yield of the reaction was excellent, ranging between 90-95%; mpt 145-146 (lit.,[100] 146 °C).



Scheme 2.5: Synthesis of Triphenylphosphite Methiodide

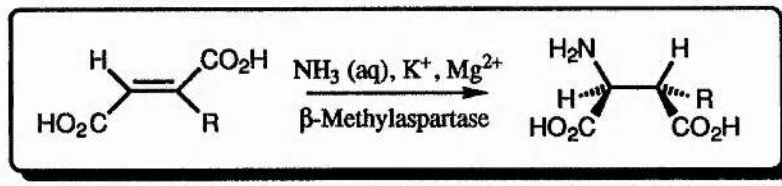
The synthesis of the alkyl halides employs the Arbuzov reaction, as demonstrated by Landauer *et al.*[100] The preparation of labelled iodoethane (**37**) was performed using the above procedure (Scheme 2.6), employing labelled ethanol (**33**), and treating it with the methiodide (**36**), after isolation, the yield of the labelled iodoethane (**37**), ranged between 65-70%.



Scheme 2.6: Synthesis of Labelled Ethyl [-$^2H_3$] iodide

2,2,2-$^2H_3$-Iodoethane (**37**) and commercially available perdeuteroiodoethane (**38**) were utilised in the synthesis of corresponding ethylfumaric acids (**30**,

R=CH_2C^2H3 and R=$C^2H_2C^2H3$) as shown in scheme 2.2. Following incubation of the labelled fumarates (**30**, R=CH_2C^2H3 and R=$C^2H_2C^2H3$) with β-methylaspartase, the correspondingly labelled ethylaspartic acids, were isolated in 55-59% yield, respectively.



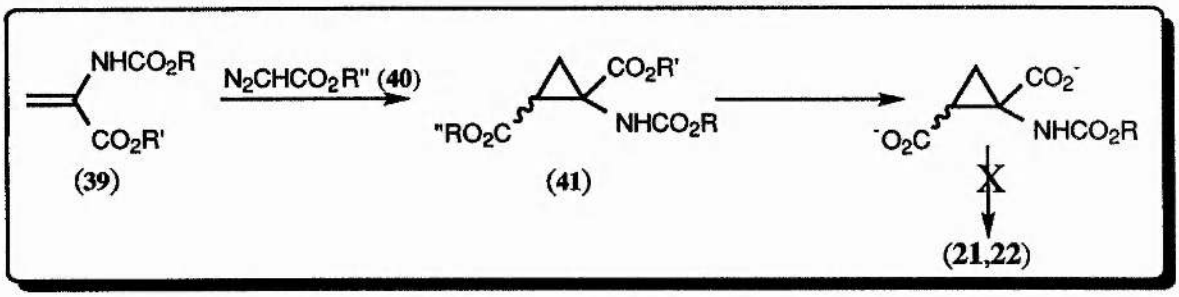
3-Alkyl Fumaric acid (30)	Yield (%)	Mpt °C
R= -CH3	67	272-276
R= $-CH_2CH_3$	59	244-245
R= $-C^2H_2C^2H_3$	59	256-258
R= $-CH_2C^2H_3$	55	255-256
R= $-CH_2CH_2CH_3$	55	235-236
R= $-CH(CH_3)_3$	45	240-242

2.3 Synthesis of (±)-*Cis/Trans*-2,3-Methanoaspartic Acid.

Cyclic analogues of glutamic acid[101] *e.g. cis/trans*-2,3-methanoaspartic acid (**21** and **22**), are molecules of considerable importance, due to their structural resemblance to a possible transition state for the rearrangement catalysed by glutamate mutase. A number of methods are available for the preparation of cyclopropanes bearing carboxyl substituents (See Chapter 2.7).[102,103]

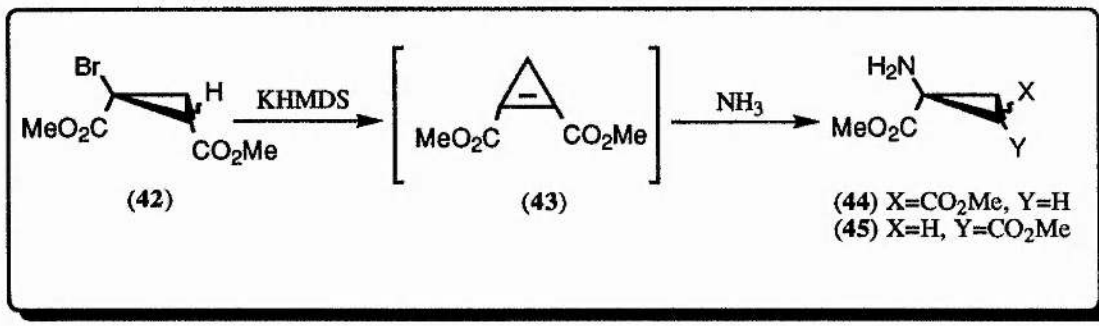
Recent approaches towards the synthesis of cyclopropane 1,2-dicaboxylates include the dipolar addition of diazo compounds (e.g. N_2CHCO_2Et (**40**)) to dehydroalanine derivatives (**39**, Scheme 2.7).[101] Both stereoisomers of compound (**41**) were prepared (the E isomer predominating). Although both carboxyl groups could be deprotected to give the free diacid uneventfully, no

cyclopropane was isolated when the amino group was liberated.[101] This is in accordance with earlier reports that 2-aminocyclopropanecarboxylic acids or esters undergo rearrangement with release of ring strain to form the corresponding imino compounds, which hydrolyse during work up.[102]



Scheme 2.7: Cyclopropanation via Dipolar Addition of Diazo Compounds

Contrary to the above report, one such synthesis of the desired amino acid (**41**) was published by Kraus *et al.*[104] in 1990 (Scheme 2.8). The authors of this work describe the preparation of the desired amino acids (**21** and **22**) *via* the bromo diester (**42**), which was readily synthesised in gramme quantities from glutaric acid.[104] The authors of this work report that dehydrobromination of (**42**), produced the amino diesters (**44**) and (**45**) in 35-55% yield, presumably *via* the reactive cyclopropene diester (**43**).



Scheme 2.8: Reported Synthesis of 2,3-Methanoaspartic Acid

The authors of this work suggest that the reaction proceeds *via* the regioselective addition of ammonia to the double bond of the cyclopropene diester (**43**), furnished during the course of the reaction. The structures identified as the desired amino diesters were assigned after comparison of their NMR spectra to that of dimethyl 1-^{t}butoxy-*cis*-cyclopropane-1,2-dicarboxylate.[105] The

amino diesters (**44** and **45**) were hydrolysed, to afford the amino acids (**21** and **22**), which were then separated from one another, by preparative HPLC (gradient elution using TFA in acetonitile). Despite the low yields obtained, the synthetic method to the desired compounds (**21** and **22**) seemed to be short and reliable.

Our efforts to reproduce the Kraus synthesis, gave, apparently, the required compounds in similar low yields to that Kraus had reported. The crude reaction mixture was purified using column chromatography on silica (3:1 hexane:ethyl acetate) and the desired compounds isolated (compound (**44**); t.l.c (3:1 hexane:ethyl acetate), R_f = 0.42, lit[104] R_f = 0.42) and (compound (**45**); t.l.c (3:1 hexane:ethyl acetate), R_f = 0.44, lit[104] R_f = 0.44).

On examination of the ^1H- and ^{13}C- NMR spectra, together with mass fragmentation and accurate mass spectrometric analysis, infra-red analysis and elemental analysis, it became apparent that compounds (**44**) and (**45**) were actually the *cis*- and *trans*- isomers of dimethyl-1-methoxycyclopropane-1,2-dicarboxylate (**46** and **47**). We were able to verify that Kraus had incorrectly identified his isolated compounds, by careful examination of his data, reproduced here in Table 2.3, and discussed below.

The reported NMR data obtained in deuterochloroform, is essentially identical to our own data and all of the signals assigned to the amino compound (**44**) match those obtained in our laboratories for the methoxy compound (**46**). The NMR spectra were performed in deuterated chloroform for both cases. In addition to the NMR data, the mass spectral data provided by Kraus was found to be incorrectly assigned. The authors obtained an accurate mass measurement for compound (**44**), which they identify as the parent ion peak. However, on re-examination we found this mass ion to be a fragmentation of the methoxy-compound (**46**), (*i.e.* $C_7H_9O_5$, which corresponds to the methoxy compound (**46**), in the absence of a CH_3^+ fragment).

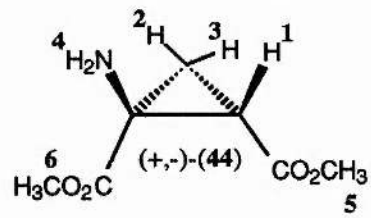
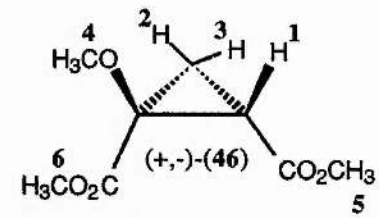
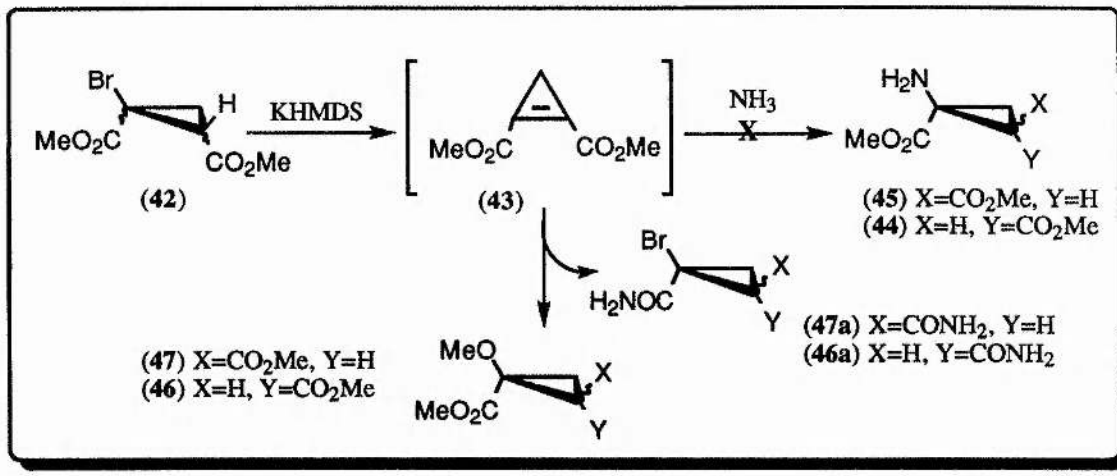
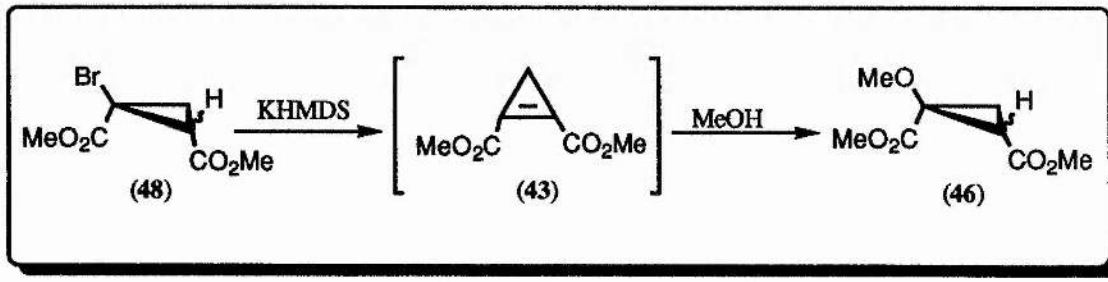
 (+,-)-(44) (*Cis*)-Dimethyl 1-aminocylopropane 1,2-dicarboxylate		 (+,-)-(46) (*Cis*)-Dimethyl 1-methoxycylopropane 1,2-dicarboxylate
NMR - data /(C^2HCl_3)	**Atom Label**	**NMR - data /(C^2HCl_3)**
1.53 (dd, *J* = 5.7, 10.2 Hz, 1H)	**1**	1.53 (dd, *J* = 5.8, 10.2 Hz, 1H)
1.88 (dd, *J* = 5.7, 10.2 Hz, 1H)	**2**	1.88 (dd, *J* = 5.8, 10.2 Hz, 1H)
2.28 (dd, *J* = 8.1, 10.2 Hz, 1H)	**3**	2.28 (dd, *J* = 8.1, 10.2 Hz, 1H)
3.46 (s, 2H)	**4**	3.46 (s, 3H)
3.69, 3.76 (2xs, 2x3H)	**5, 6**	3.69, 3.76 (2xs, 2x3H)
No ^{13}C Data Published		20.1 (C-3), 30.2 (C-2), 53.3 & 53.4 (2x CO_2CH_3), 58.2 (OCH_3), 66.4 (C-1) and 168.3 & 170.1 (2x CO_2CH_3)
MASS SPEC. - DATA		**MASS SPEC. - DATA**
MS - *m/z*		**MS - *m/z***
45, 55, 69, 75, **85, 101, 113, 129, 156, 173**		**85, 101, 113, 129, 156, 173**
Accurate Mass Measurement		**Accurate Mass Measurement**
***m/z* for $C_7H_{11}NO_4$**		***m/z* (CI) for $C_8H_{13}O_5$**
calculated 173.0688, **found** 173.0452		**calculated** 189.0759, **found** 189.0763
Mass ion found should be : *m/z* for $C_7H_9O_5$: calculated 173.0447 **found** 173.0452		

***Table 2.3**: A Comparison of the Spectral and Analytical Data for (44) and (45)*



***Scheme 2.9**: Unsuccessful Synthesis of Dimethyl 2,3-Methanoaspartate*

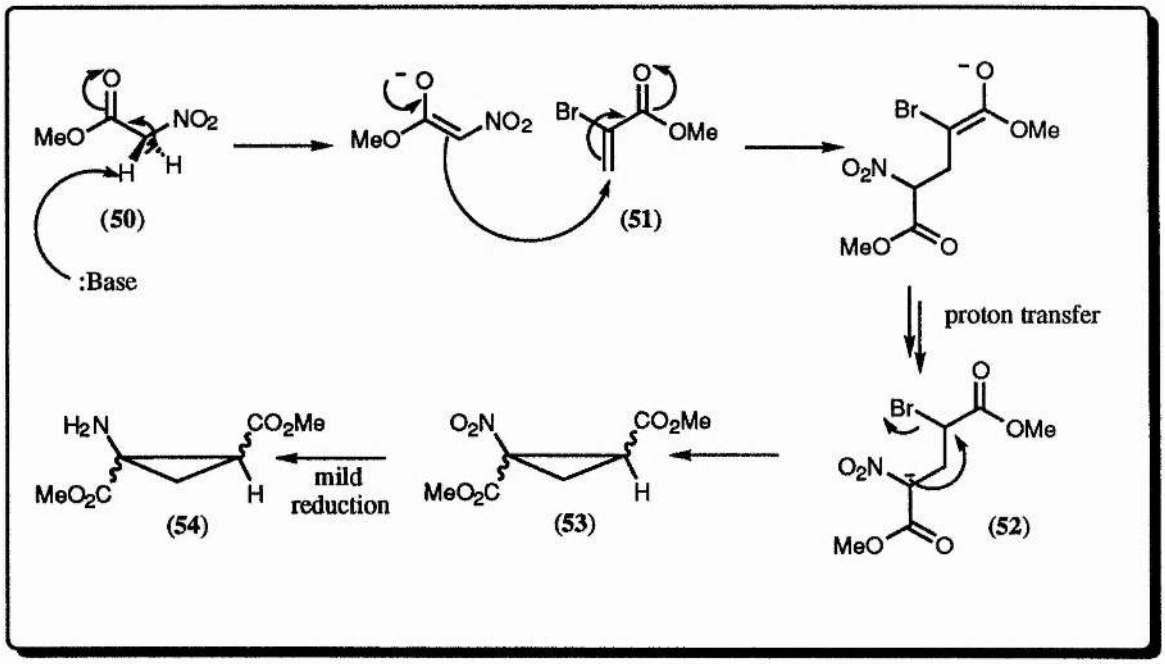
Since only moderate quantities of product (**46** and **47**) could be isolated (55-63%), we concluded that the formation of *cis/trans*-methoxycyclopropane (**46** and **47**, Scheme 2.9) was due to the loss of methanol from the ester groups contained within the bromocyclopropane (**42**). We suspect the side products to be the *bis*-amides (**46a, 47a**). The liberated methoxide anion, effects the elimination of HBr, to give the cyclopropene intermediate (**43**). Methoxide adds to the cyclopropene, giving the observed products (**46** and **47**, Scheme 2.9). In keeping with the results of Kraus, we found that by treating dimethyl *cis*-1-bromo-cyclopropane-1,2-dioate (**48**) with KHMDS, in the presence of anhydrous methanol, that the sole product isolated, was the *cis* isomer of dimethyl 1-methoxycyclopropane 1,2-dioate (**46**) (Scheme 2.10).



***Scheme 2.10**: Synthesis of Dimethyl 1-Methoxycyclopropane 1,2-Dicarboxylate*

In our attempt to synthesise the desired cyclopropane amino acids (**21** and **22**), we sought a different synthetic route. One such approach, was to synthesise dimethyl 1-nitrocyclopropane 1,2-dioate (**53**), which would then be reduced under mild conditions, to give the corresponding aminocyclopropane (**54**, Scheme 2.11).

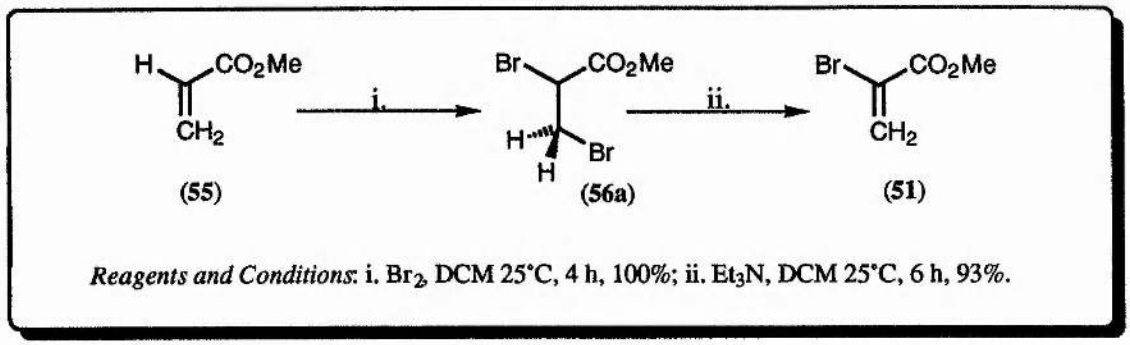
The synthesis towards the nitrocyclopropane (**53**), was expected to proceed *via* a 1,4-conjugate addition of commercially available, methyl nitroacetate (**50**) to methyl α-bromoacrylate (**51**), under mild basic conditions (Scheme 2.11).



***Scheme 2.11**: Proposed Synthetic Approach to Dimethyl 2,3-Methanoaspartate*

Potassium carbonate was employed as the base for initial abstraction of the acidic proton within methyl nitroacetate (**50**). The nitroacetate conjugate addition was expected to result in the rapid protonation of the intermediate enolate anion, (pK_B ~20) by a proton transfer, *via* the α-nitroacetate function (pK_B ~5.8),[106] giving rise to the more thermodynamically stable nitronate anion (**52**). The resulting anion would then undergo an electrocyclic ring closure, with concomitant loss of the bromide anion, to furnish the cyclopropane derivative (**53**).

The synthesis of methyl α-bromoacrylate (**51**) was achieved in quantitative yield, *via* bromination of the methyl acrylate (**55**), followed by dehydrobromination of the dibromo- compound, (**56a**, Scheme 2.12).



***Scheme 2.12**: Synthesis of 2-Halogenoacrylate Esters*

Attempts to follow the designed route (Scheme 2.11) for obtaining the nitrocyclopropane (**53**), led to the formation of a white crystalline solid (**57**). On examination of the ^{1}H- and ^{13}C- NMR, it was not fully conclusive as to whether or not, we had obtained our target compound (**53**). On comparison of ^{1}H- and ^{13}C- NMR with other 1-substituted cyclopropane 1,2-dioates, we noticed that the proton and carbon NMR shifts were too high {δH (200 MHz; C^2HCl_3) 3.70 (2H, m, CH_2), 3.88 (6H, s, 2x CO_2CH_3) and 5.15 (1H, dd, CH); δC (50.31 MHz; C^2HCl_3), 34.7 (CH_2), 53.3 & 53.7 (2x CO_2CH_3), 71.6 (CH), 106.1 (NO_2CCH_2-), 159.3 (CH_2-CO), 159.3 (CH_2-CO) and 169.4 (CH-CO)}. On the other hand, elemental, mass fragmentation and accurate mass measurement analysis, displayed the correct elemental composition to the target compound (**53**). Since the isolated compound (**57**) was a solid and readily crystallised, the structural identity was determined by X-ray crystallography. The result of such studies revealed that compound (**57**) was the isoxazoline-*N*-oxide (Fig. 2.2).

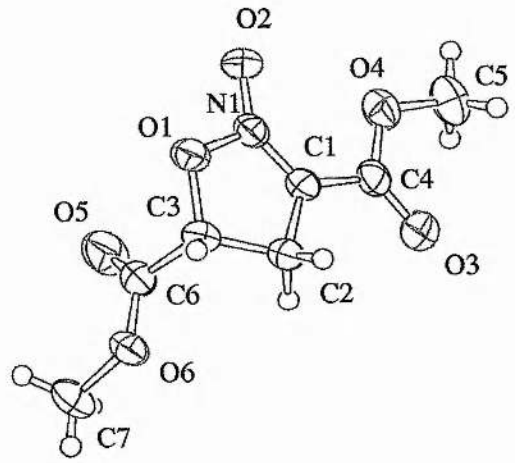
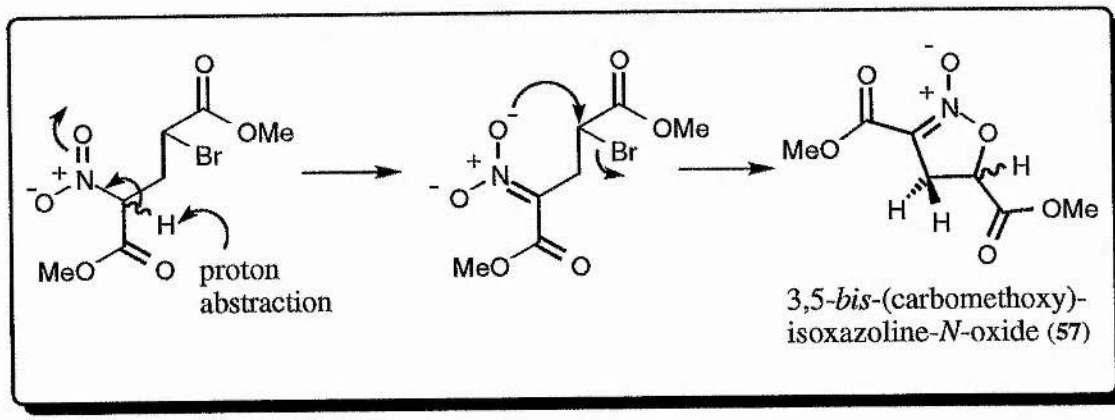


Figure 2.2: X-Ray Crystal Structure of the Synthesised Isoxazoline-N-Oxide

This result meant that an electrocyclic ring closure must operate *via* the stabilised nitro group, at C-4, with concomitant loss of the bromide anion and thus furnishing the isoxazoline-*N*-oxide (**57**, Scheme 2.13)

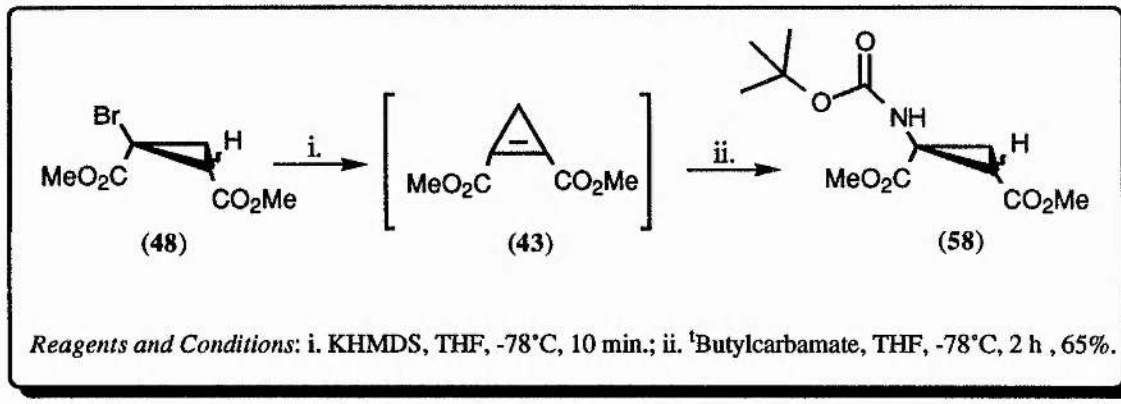


Scheme 2.13: Formation of the Isoxazoline-N-Oxide

2.3.1 Modification of Kraus Synthesis.

Having followed the work carried out by Kraus *et al.*[104] we were confident that the isolated products (**46** and **47**) were due to the addition of a methanol to the intermediate cyclopropene (Scheme 2.10) and not due to the proposed attack by ammonia (Scheme 2.9). In order to confirm our findings, we opted to expose the intermediate cyclopropene (**43**) to different nucleophiles. Since we had not

obtained the desired cyclopropane amino acids (**21** and **22**), when we treated the cyclopropene (**43**), with ammonia, it was our goal to try different nitrogen containing nucleophiles. We opted to attack the cyclopropene intermediate (**43**), with a nitrogen nucleophile, which had a similar pK_a to that of methanol (15.2). One such nitrogen containing compound would be a carbamate, which has a pK_a of ~17. ^{t}Butylcarbamate, was considered to be the ideal molecule to try with the cyclopropene intermediate (**43**), as the resultant cyclopropane would be the protected amino ester (**58**, Scheme 2.14).

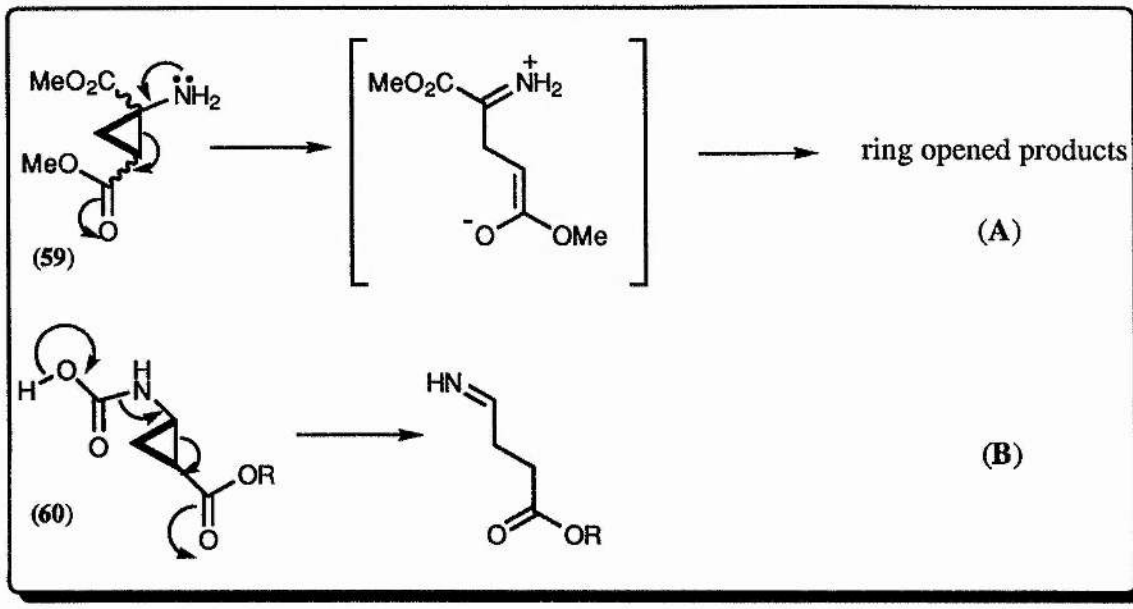


Reagents and Conditions: i. KHMDS, THF, -78°C, 10 min.; ii. ^{t}Butylcarbamate, THF, -78°C, 2 h , 65%.

***Scheme 2.14**: Synthesis of the Protected Cyclopropane Amino Acid*

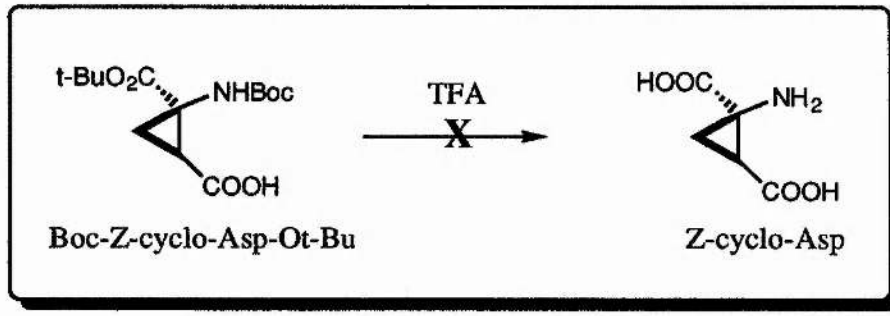
The protected cyclopropane amino acid (**58**), was obtained in 65% yield as a white crystalline solid after column chromatography (10% ethyl acetate: 90% hexane, R_f = 0.2), (*m/z* (Found: $[M + H]^+$, 274.1285. $C_{12}H_{19}NO_6$ Calc. for 274.1290). As expected on the basis of the previous experiment (Scheme 2.10) where the isolated product was the *cis*-regioisomer of the 1-methoxycyclopropane (**46**), we assigned compound (**58**) as the *cis*-regioisomer. However, in order to confirm our findings, NMR and X-ray crystallographic studies need to be performed.

Attempted removal of the ^{t}butoxycarbonyl group (^{t}BOC) (TFA, O °C to 25 °C) in compound (**58**), led to the destruction of the cyclopropane ring (**59**), presumably induced by amine assisted ring opening *via* the β-carboxylate (Scheme 2.15 A). A similar ring opening reaction was reported for compound (**60**, Scheme 2.15 B).[107]



Scheme 2.15: Ring Opening Reactions of 1-Aminocyclopropane Dicarboxylates

In addition, a similar report was published by Burgess *et al.*[108] (Scheme 2.16), where the isolated materials were the ring opened products as shown in scheme 2.15.



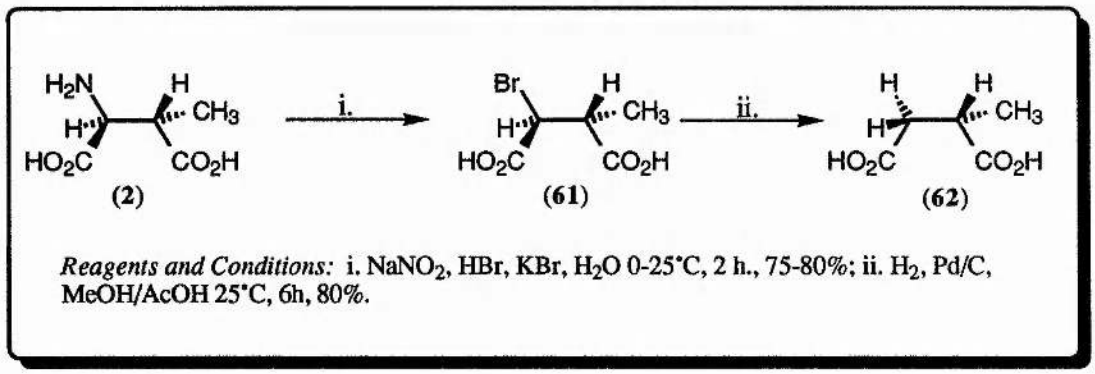
Scheme 2.16: Burgess's Attempted Synthesis of Z-Cyclo-Aspartic acid

Considering the importance of cyclopropanes with glutamate mutase, we have successfully synthesised a variety of 1-substituted cyclopropane dioic acids (**25**) and at present we are testing these compounds for biological activity against glutamate mutase. The synthesis of these molecules will be discussed in Chapter 2.7.

2.4 Synthesis of (2*S*)-Methylsuccinic Acid

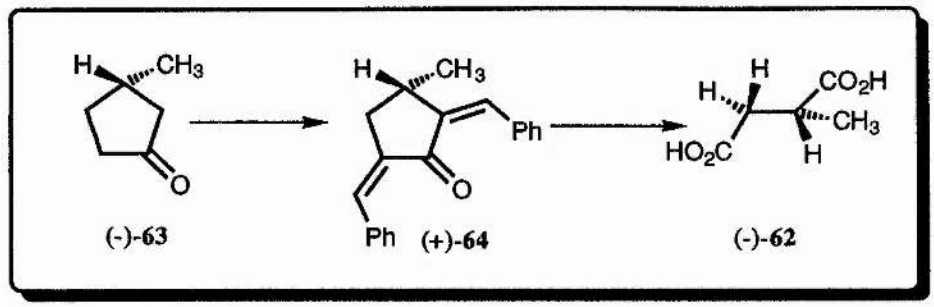
(2*S*)-Methylsuccinic acid (**62**) is an analogue of (2*S*,3*S*)-3-methylaspartic acid (**2**). This molecule is thought to be important in determining the active site geometry of glutamate mutase. Our aim was to determine whether one of the protons at C-3 of (2*S*)-methylsuccinic acid (**62**), would interact with the amino pocket within the active site of glutamate mutase, and if so, would there be any radicals generated after interaction with the cobalamin.

Our efforts towards obtaining the optically active compound (**62**, Scheme 2.17), involved the diazotization of (2*S*,3*S*)-3-methylaspartic acid (**2**) in the presence of bromide ion, thus yielding (2*S*,3*S*)-2-bromo-3-methylsuccinic acid (**61**). Catalytic hydrogenation of the bromo- compound (**61**) furnished the desired compound as a white solid, in excellent yield (81%), {$[\alpha]_D$ -13.7 (*c* 1.15, EtOH)} and m.p 113-114 °C (lit.,[109] {$[\alpha]_D$ -13.5 (*c* 1.15, EtOH)} and m.p 113-114.5 °C).



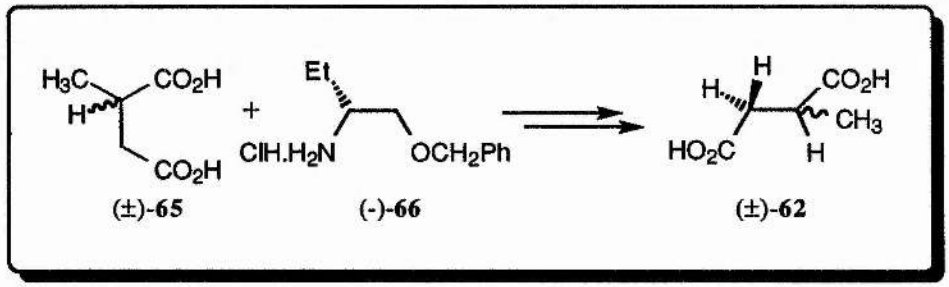
***Scheme 2.17**: Synthesis of (2S)-Methylsuccinic Acid*

We found that the method we employed, was very reliable and efficient towards obtaining enantiomerically pure (2*S*)-methylsuccinic acid (**62**). Other literature synthesis, involve the conversion of (-)-3-methylcyclopentanone (**63**) to (2*S*)-methylsuccinic acid (**62**) *via* the ozonolysis of the (-)-dibenzylidene derivative (**64**, Scheme).[110]



Scheme 2.18: Literature Synthesis of (2S)-Methylsuccinic Acid

More recent methods in obtaining enantiomerically pure (2*S*)-methylsuccinic acid (**62**), involve the resolution of the racemic acid (**65**), by means of solid salt formation with an optically active amine, such as (*R*)-(-)-2-amino-1-benzyl-oxybutane (**66**, Scheme 2.19).[109]



Scheme 2.19: Optical Resolution of (±)-α-Methylsuccinic Acid

2.5 Mechanistic Studies

2.5.1 (2*S*,3*S*)-3-Methylaspartate Analogues as Inhibitors or Substrates

(2*S*,3*S*)-Methylaspartic acid (**2**), *N*-methyl-(2*S*,3*S*)-methylaspartic acid (**67**)[53], (2*S*)-methylsuccinic acid (**62**), and aspartic acid (**68**) were tested with glutamate mutase by Dr. B. Hartzoulakis, as potential inhibitors of the enzyme.[111-112] The findings of this work reveal that there was no interaction between them, and the active holoenzyme. The above analogues of methylaspartic acid were also incubated with the enzyme, but no rearrangement products were detected.

(2*S*,3*S*)-2-Bromo-3-methylsuccinic acid (**61**) was another potentially promising molecule, but was found to react with the 2-mercaptoethanol present in the incubation and assay mixtures.[111]

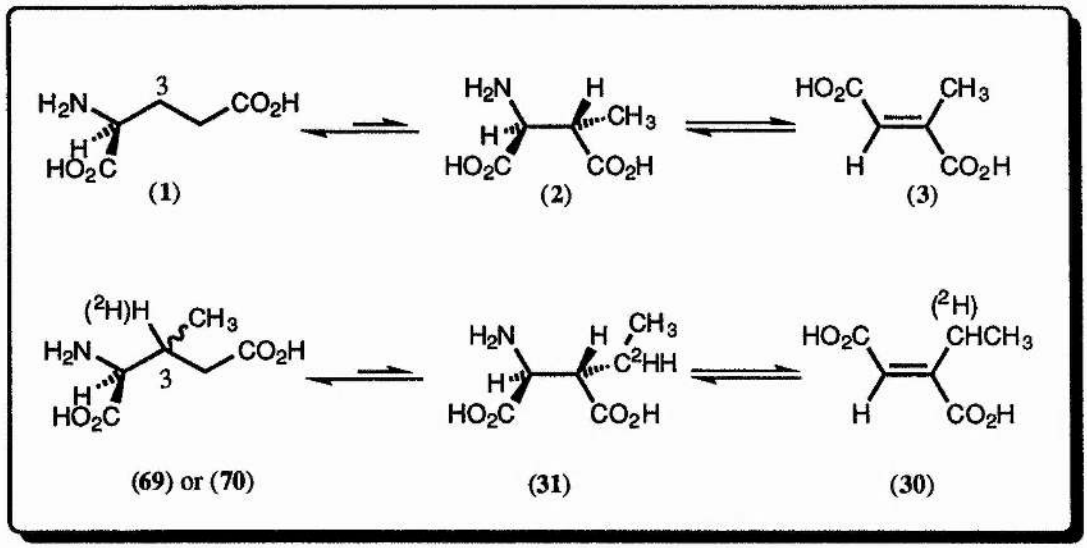
2.5.2 (2S,3S)-3-Ethylaspartic Acid as a Plausible Substrate and/or Inhibitor.

In order to gain further insights into defining the stereochemical course of the glutamate mutase reaction, with respect to the reaction at C-3 of glutamic acid, was a long standing objective of our research. As discussed earlier (Chapter 1.3.4), the stereochemical course of the rearrangement at C-3 cannot be easily defined due to the torsiosymmetry of the methyl group formed on 3-methylaspartic acid (**2**).

Barker had reported that 3-ethylaspartic acid (**31**, R= CH_2CH_3) was not a substrate for glutamate mutase. His conclusions were solely based on a chromatographic comparison (by t.l.c.) with the starting material. As a consequence, there was the possibility that if ethylaspartic acid (**31**, R= CH_2CH_3) was a slow binding substrate, as compared to 3-methylaspartic acid (**2**), the product may not have been detected. In addition, Barker used impure enzyme for his investigations, which would hinder any conclusions made about substrate specificity.

Our aim was to re-investigate the possibility of ethylaspartic acid (**31**, R= CH_2CH_3) serving as a useful substrate analogue, which would preserve the stereochemical information introduced by the enzyme, if rearrangement were to take place. This can be investigated by replacing one of the three protons of methylaspartic acid (**2**) with a methyl group and a second proton, with deuterium, thus forming the specifically labelled 3-ethylaspartic acid (**31**, R= $C^2H_2CH_3$, Scheme 2.20). We were initially confident that the presence of an additional methyl group on the substrate apart from reducing the rate of the reaction, would not interfere with the fundamental aspects of the rearrangement. Furthermore, the methyl group is in a suitable position which would allow it to

'lock' the methylene group of the 3-ethylaspartic acid (**31**, R= $C^2H_2CH_3$) in such an orientation that only one of the two prochiral protons present would be removed. The identity of the proton removed and the stereochemistry at the C-3 the (2*S*,3*R* or 2*S*,3*S*)-3-methylglutamic acid product ((**69** or **70**), Scheme 2.20), would be sufficient to establish if the rearrangement proceeds *via* an inversion or retention of configuration.



Scheme 2.20: Enzymic Route Defining the Stereochemical Course of Reaction.

2.5.3 Incubation Studies of 3-Ethylaspartic acid with Glutamate Mutase.

Incubation of 3-ethylaspartic acid (**31**, CH_2CH_3) with the mutase was carried out in our laboratories by Dr. B. Hartzoulakis. His findings revealed that there was no formation of 3-methylglutamic acid (**69** or **70**) as judged by ^{1}H-NMR spectroscopic analysis of the incubation solutions.

It was clear at this stage of our research, that 3-ethylaspartic acid (**31**, CH_2CH_3) was not a substrate for glutamate mutase and the satisfactory mechanistic explanation was simply that the ethyl- group was too big for the active site. The relevant information we sought, was whether or not the holoenzyme in the

presence of 3-ethylaspartic acid, activated the coenzyme and as a result, does this give rise to the generation of the 5'-adenosyl radical. If the 5'-adenosyl radical is formed, then a proton would almost certainly be abstracted from a molecule of 3-ethylaspartic acid, which would be occupying the active site. In short, the lack of rearrangement products was not conclusive as far as it concerned any hydrogen exchange between ethylaspartic acid and the coenzyme.

In order to investigate this possibility, our strategy was to synthesise a variety of deuterated ethylaspartates: (2*S*,3*S*)-[1'-C^2H_2]-3-ethylaspartic acid (Fig. 2.3, (**72**)), (this compound was prepared by S. Gulzar)[113], (2*S*,3*S*)-[2'-C^2H_3]-3-ethylaspartic acid (Fig. 2.3, (**73**)) and (2*S*,3*S*)-[3-$C^2H_2C^2H_3$]-3-ethylaspartic acid (Fig. 2.3, (**74**)). These molecules, where incubated with unlabelled 3-ethylaspartic acid or (2*S*)-glutamic acid in several different molar ratios. (2*S*,3*S*)-3-[3'-$^2H_3$]-3-Methylaspartic acid (**71**) (this compound was prepared by B. Hartzoulakis),[112] was also incubated with the enzyme in the presence of unlabelled 3-ethylaspartic acid. A combination of spectral techniques, such as ^1H- and ^2H-NMR spectroscopy, was employed so as to detect any exchange of deuterium.[112]

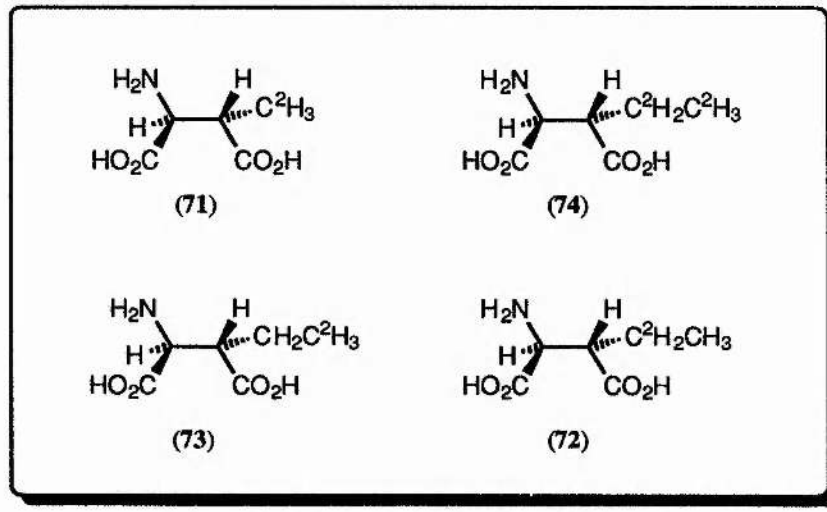
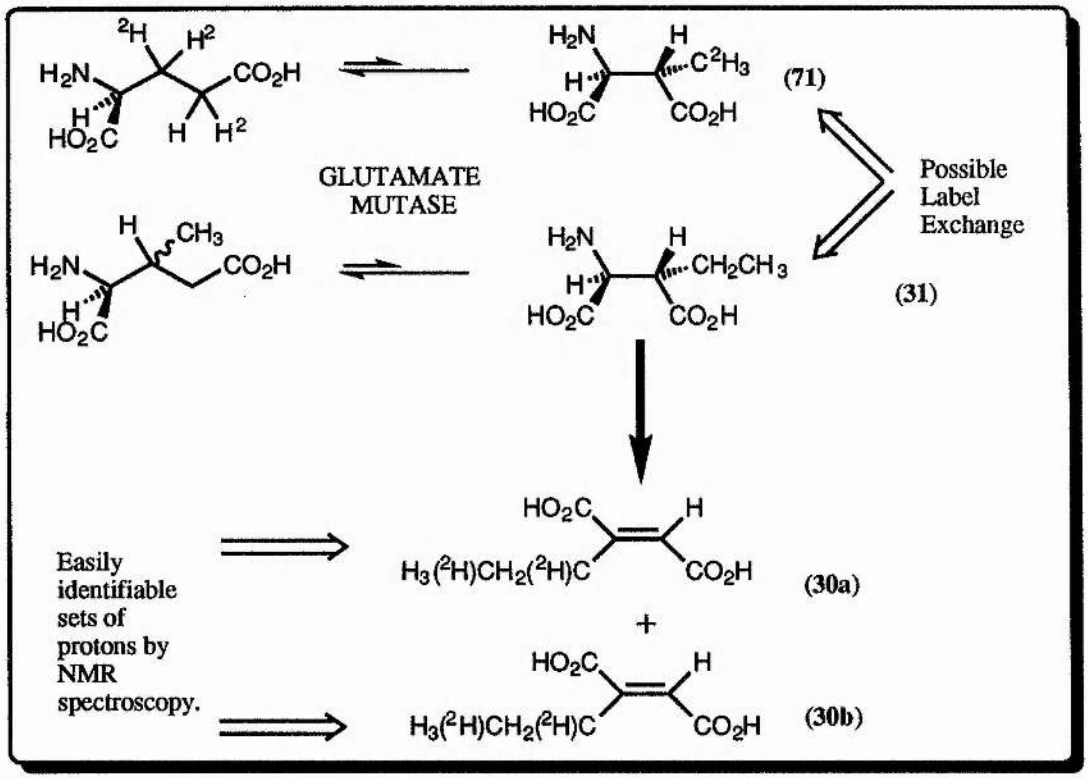


***Figure 2.3**: Deuterated 3-Ethyl Aspartic Acids Employed*

A mixture of [3'-$^2H_3$]-3-methylaspartic acid (**71**) and unlabelled ethylaspartic acid (**31**, R= CH_2CH_3), was incubated with pure glutamate mutase, under standard conditions. After an incubation period, β-methylaspartase was added,

the alkyl aspartic acids present were transformed into the alkyl fumarates (**30a**), (**30b**), followed by an ether extraction. If any deuterium label had been transformed from [3'-$^2H_3$]-methylaspartic acid into ethylaspartic acid (**31**, R= CH_2CH_3, Scheme 2.21) it would have been easily detected by means of ^2H-NMR spectroscopy, since now all three sets of protons present in the product mixture were different. Complete lack of deuterium exchange confirmed that the holoenzyme could not abstract hydrogens from either 3'- or the 4'-position of 3-ethylaspartic acid (**31**, R= CH_2CH_3).

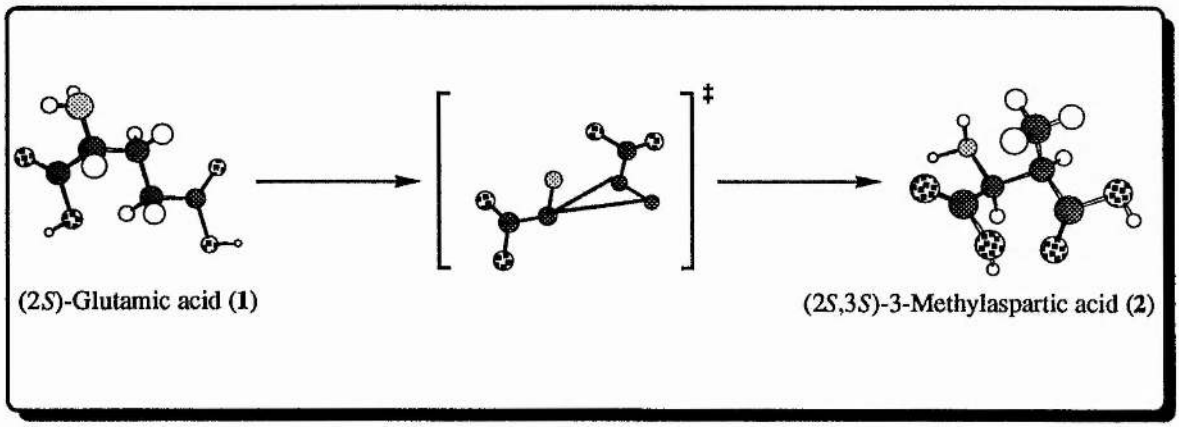


***Scheme 2.21**: Label Exchange experiments with glutamate mutase.*

Enzymic incubations of the labelled compounds mentioned have revealed no sign of protium/deuterium scrambling. This result concluded that (2*S*,3*S*)-3-ethylaspartic acid does not generate a radical within the active site of glutamate mutase and more likely, serves as an inhibitor.

2.5.4 Cyclopropanes as Molecular Probes for Glutamate Mutase

trans-2,3-Methanoaspartic acid (**22**), is a molecule of importance, for probing the mechanism of glutamate mutase. The cyclopropane amino acid (**22**) was envisaged to be a possible transition state analogue, for the rearrangement catalysed by glutamate mutase (Scheme 2.22).



***Scheme** 2.22: Glutamate Mutase Rearrangement via a Cyclopropane*

Our attempts to synthesise compound (**22**) were unsuccessful, however, we gained valuable information about the stability of this molecule. We have synthesised a variety of cyclopropane dioic acids (**25**, Chapter 2.7) where the substituent group in the C-1, position has been varied, according to the size of an NH_3^+ ion. It is conceivable that one of these molecules can be accommodated within the active site structure of glutamate mutase, and perhaps these molecules could behave as possible substrates and or inhibitors of the enzyme. This is the basis of future work within this research area.

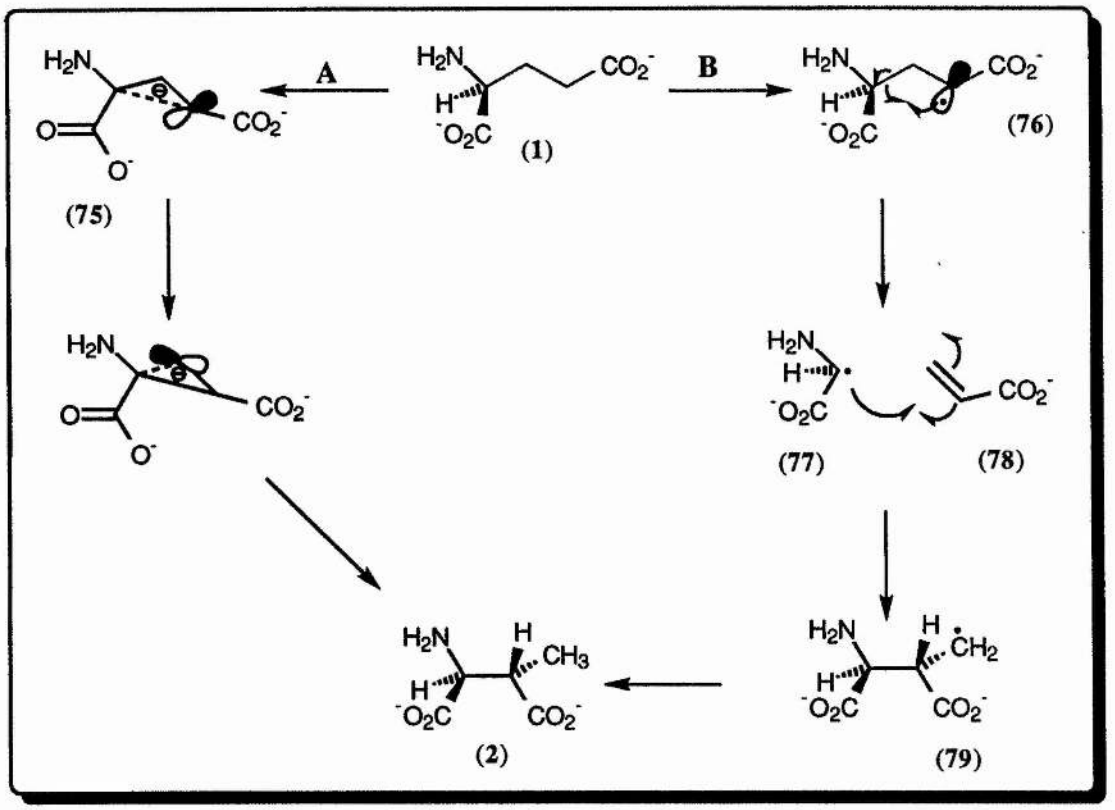
2.5.5 The Mechanism of the Rearrangement

Two main reaction mechanisms have been proposed for the glutamate mutase rearrangement (Scheme 2.23).

Merkekelbach and co-workers[114] suggested that the glutamate mutase rearrangement may proceed *via* an enolate cyclopropane anion (**75**) such as the control of stereochemistry of methylaspartate (C-3) by electronic rather than steric factors. Although it has been established that enzymes can generate enolates,[115] it is very difficult to anticipate a link between the generation of the 5'-adenosyl radical and the abstraction of a proton to afford the anion (**75**).[72,73]

An alternative involves proton abstraction by the 5'-adenosyl radical, to form the radical species (**76**) on C-4 of glutamic acid (Scheme 2.23, B). The radical species (**76**) can break down into two plausible intermediates, namely the glycyl radical (**77**) and the acrylic acid (**78**). These two intermediates are likely to be bound strongly to the enzyme and thus, are not likely to vacate the active site. It is reasonable to suggest that recombination of the two (**77** and **78**) is likely to occur, giving rise to the formation of 3-methylaspartic acid (**2**, Scheme 2.23, B).

Glycyl radicals such as (**77**), are considered to be very stable due to a combined action of an electron withdrawing (CO_2^-) and an electron-releasing group (NH_2).[118,119] This well documented phenomenon is called the *captodative effect* and the pair of the amine and the carboxyl group is one of the best known stabilising pairs known.



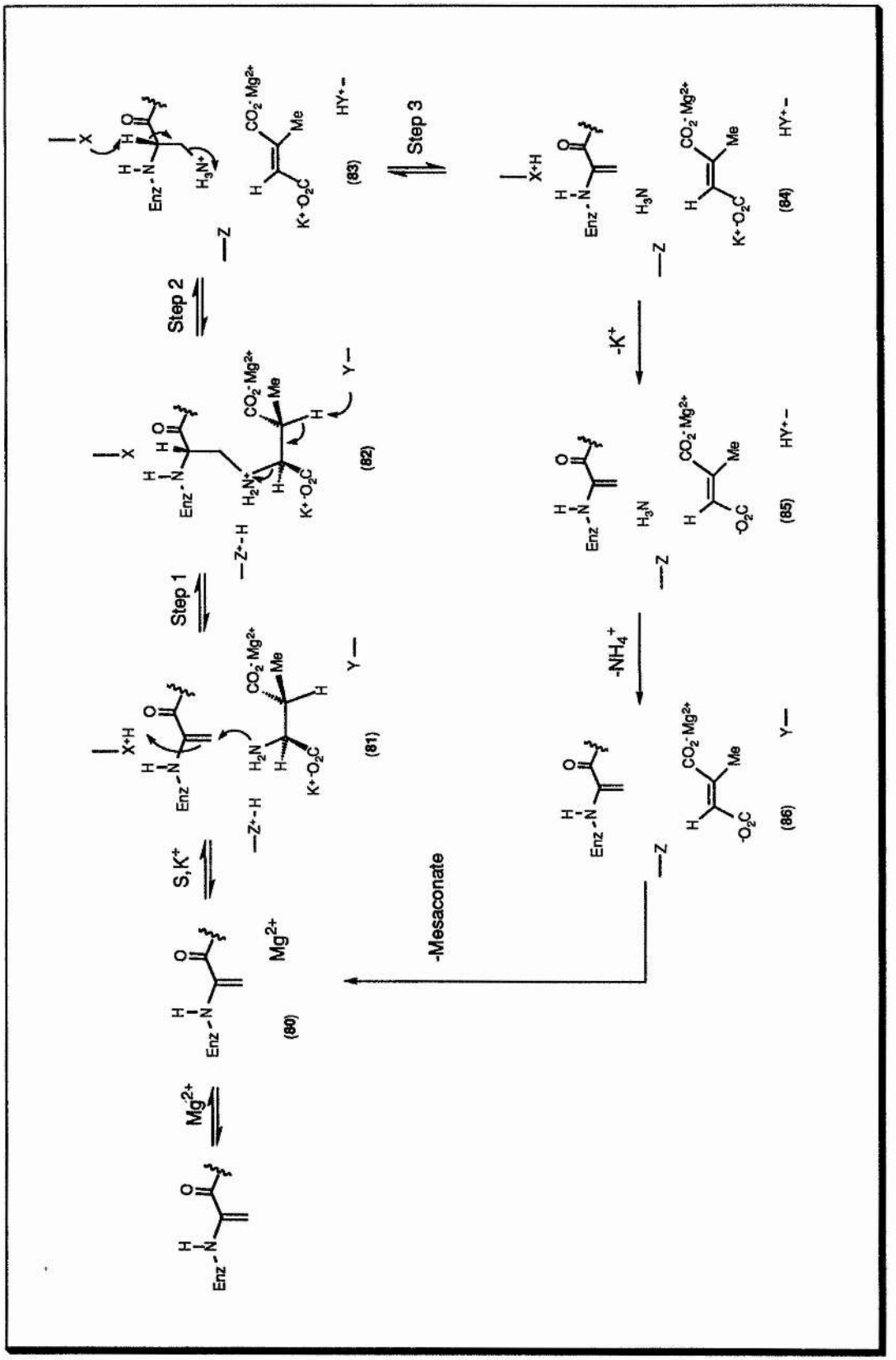
***Scheme 2.23**: Plausible Intermediates/Transition States for the Rearrangement*

2.6 Methylaspartase Ammonia-Lyase (MAL)

2.6.1 Introduction

The synthetic utility, together with the proposed elimination mechanism of 3-methylaspartase, have been established by the extensive work carried out within our research group since 1986.[49,58-60] Recent work has allowed us to tackle the problem and gain further details about the structural requirements necessary for enzyme catalysis. Results obtained from kinetic, inhibition and primary sequence studies for methylaspartase, suggest the presence of a dehydroalanine residue within the enzymes active site and that such a residue would be important for enzyme catalysis.[53,64]

As yet there is no X-ray crystallographic information available for any enzyme which operates *via* the intermediacy of a catalytic dehydroalanine residue. However, the enzyme in question, can be studied in the presence of potent reversible inhibitors. These molecules are likely to bind to the active site of an enzyme, in a manner similar to the substrate, but will remain unchanged. It is therefore envisaged, that such molecules can be used to co-crystallise the enzyme. By inspection of a three-dimensional model of the enzyme it is then possible to work out the active-site residues. Such compounds had not been described, and were therefore important to biological targets for MAL. Our aim was to design 1-substituted cyclopropane 1,2-dicarboxylic acids to serve as potential transition state (TS) analogues (Fig. 2.4), of the MAL reaction (Scheme 2.24).



Scheme 2.24: The Proposed Enzyme Mechanism

The transition states for the formation (Scheme 2.24, Step 1) and the break down (Step 3) of the covalent substrate (**82**) or covalent product (**83**) complexes are difficult to mimic because a large part of the TS structure involves protein-protein interactions. Therefore, attention was focused on a TS analogue for the central substrate deamination step (Step 2). In the flanking catalytically active complexes (**82**) and (**83**) in Scheme 2.24, the enzyme should exist in a closed conformation with both metal ions bound and in broad terms, the ground states should differ only in the C-3 dehydroalanine N-substrate bond length or distance and the hybridisation state at C-3 of the substrate. Substituted *trans*-cyclopropane 1,2-dicarboxylic acids (Figure 2.4) accurately mimic the expected TS structure for the substrate and it was hoped that such analogues might allow the simultaneous occupation of the cleft of the enzyme by ammonia.

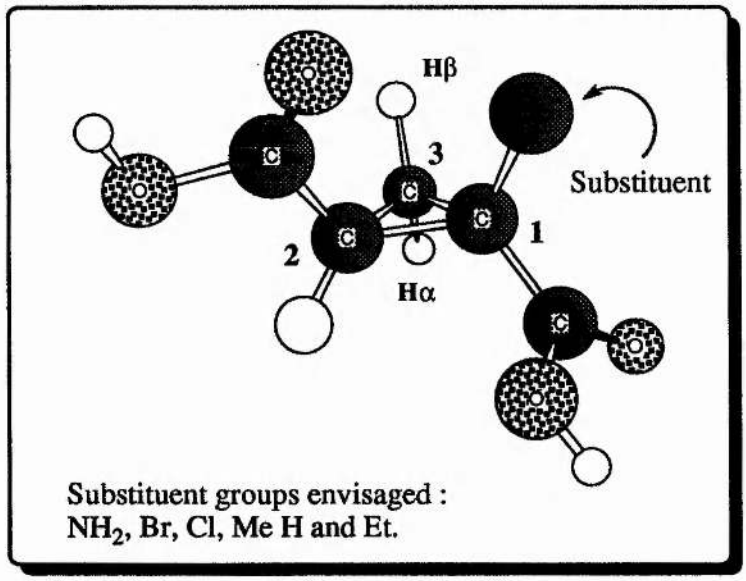


***Figure 2.4**: Substituted Cyclopropane Dicarboxylic Acids*

A variety of substituents were used as molecular probes for the enzyme active site. Our objective was to determine the size, electrostatic nature and hydrophobicity of the amino- binding pocket within the enzyme active site. This was done by introducing subtle variations in the substituent size. The partial

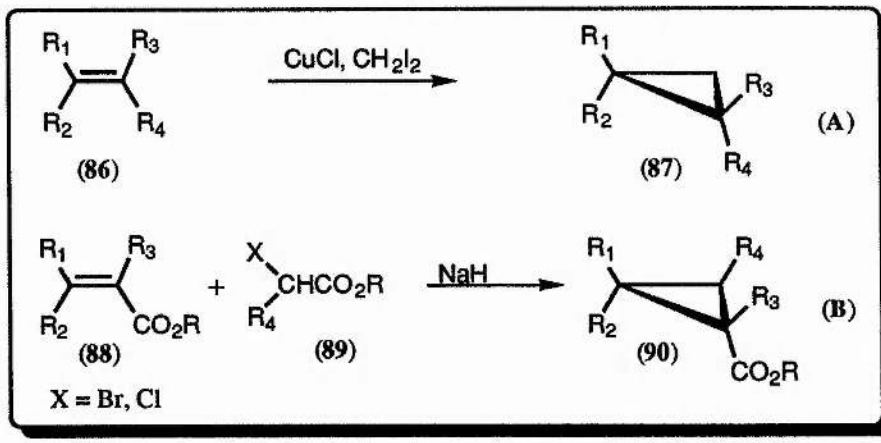
charge and hydrophobicity of the substituent were also varied as can be seen in Table 2.4.

Substituent Group	Vaan der Waals Radius / nM	Partial Charge	Hydrophobicity
–H	0.1	0	Hydrophobic
–Br	0.195	δ^-	Hydrophilic
–Cl	0.18	δ^-	Hydrophilic
–Me	0.21	0	Hydrophobic
$-NH_3^+$	0.2	+	Hydrophilic
–Et	0.36	0	Hydrophobic

***Table 2.4**: Van der Waals Radius of Cyclopropane Substituents*

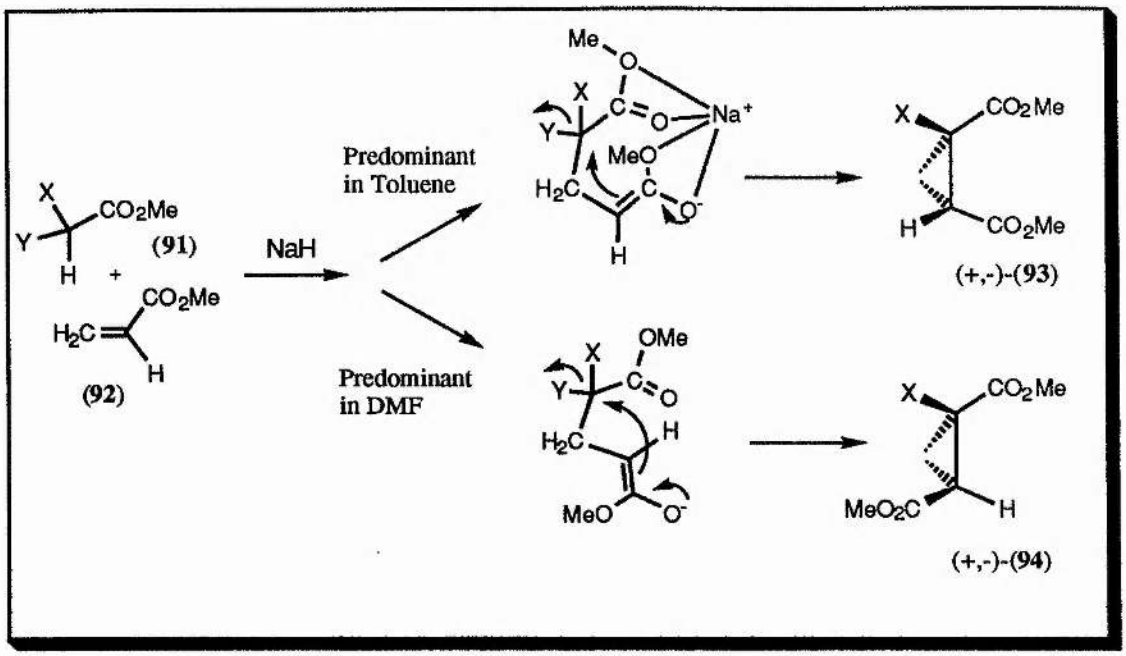
2.7 Synthesis of 1-Substituted Cyclopropane 1,2-Dicarboxylic Acids

1-Substituted cyclopropane-1,2-dicarboxylic acids (**25**) were thought to be molecules of considerable importance for MAL, as they closely resemble a possible transition state (TS), which may exist between the enzymic transformation of (2*S*,3*S*)-3-methylaspartic acid (**2**) to mesaconic acid (**3**). A variety of methods exist for the preparation of cyclopropane ring bearing carboxyl substituents (**87** and **90**). The Simmons-Smith[120] methylation of olefins (**86**, Scheme 2.25, A) and the use of halogenoacetates (**89**) together with the appropriate acrylate analogues (**88**), as developed by McCoy *et al.*[121] and McDonald and Reitz (Scheme 2.25, B),[122] are the best known approaches.



Scheme 2.25: *General Methods for the Preparation of Functionalised Cyclopropane Rings*

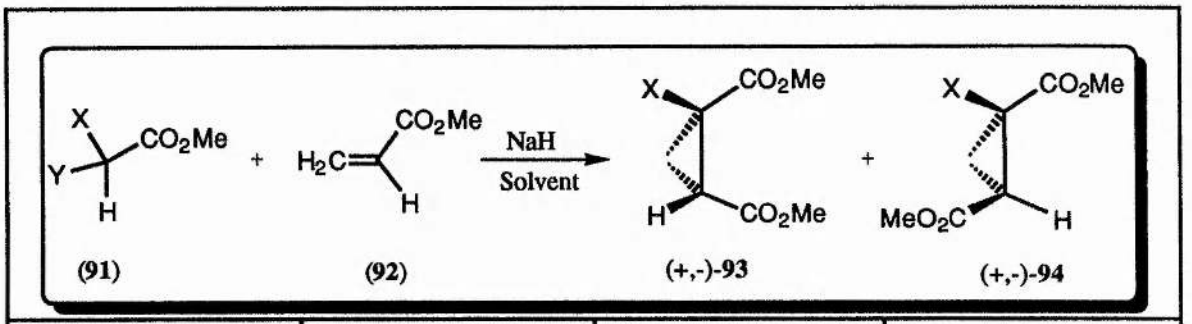
Our initial goal was to synthesise a variety of regioselectively pure (±) 1-sub-substituted cyclopropane 1,2-dicarboxylic acids (**25**). We considered attempting the preparation of these compounds *via* the Simmons-Smith reaction,[120] however, the low yields of similar cyclopropanes prepared by this method[122] discouraged us. On the other hand the alkali-metal hydride catalysed formation of 1-substituted cyclopropane 1,2-dicarboxylic esters (**93** and **94**) from 2-dihalogeno-ethanoate esters (**91**, Scheme 2.26) and acrylic ester (**92**) is well established and the *cis*-isomer (**93**, Scheme 2.26) is the predominant product, when the reaction is performed in a non-polar media[105,123]



Scheme 2.26: Regioselective Formation of 1-Substituted Cyclopropane 1,2-Dioates

The reaction is believed to proceed *via* the conjugate addition of the haloacetate (**91**) with the acrylate (**92**) and concomitant cyclisation giving rise to the formation of the cyclopropane compounds (**93** and **94**). The preference for the *cis*-isomer is believed to arise because the metal counter ion can chelate to both of the carboxylic ester groups of the initial conjugate addition product prior to the formation of the cyclopropane ring, Scheme 2.26. Since we required the *trans*-1,2-cyclopropanedioic acids (**96**), to test as potential inhibitors and expected that *trans*-1,2-cyclopropanedioate esters (**94**), would predominate in the absence of the metal ion chelating effect, the ratio of the *cis*- to *trans* - products formed in different solvents was examined (Table 2.5). In toluene, the ratios of products [(**93**) to (**94**)] formed respectively from the reaction of methyl 2-chloropropanoate (**91**, X= Me, Y= Cl) and methyl 2-dichloroethanoate (**91**, X,Y= Cl) and methyl 2-dibromoethanoate (**91**, X,Y= Br) with methyl acrylate (**92**) was *ca.* 10:1. However, in DMF, the ratio increased in favour of the *trans*- isomer to 0.73:1, for the 1-bromo derivatives (**93** and **94**, X = Br) to 1:3 for the 1-chloro derivatives (**93** and **94**, X = Cl) and to 1:5 for the 1-methyl derivatives (**93** and **94**, X = Me) in the best case Scheme 2.26. Isolated yields of the *trans*- isomer ranged from 42-65% (Table

2.5). The dihalogenoethanoate, monohalogenoethanoate esters (**91**, Scheme 2.26) were synthesised from the corresponding, commercially available acids, *via* the acid catalysed esterification in the presence of methanol.



(91) (92) (+,-)-93 (+,-)-94

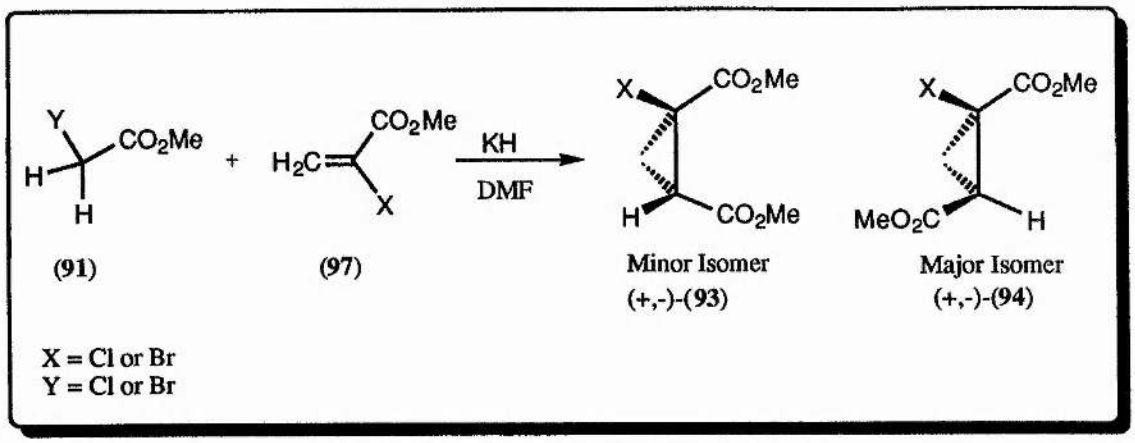
Substituent Group	Solvent employed	*Cis* : *Trans*	% Yield
X = CH_3	DMF	1 : 5	61
Y = Cl	Toluene	10 : 1	74
X = Br	DMF	2 : 3	42
Y = Br	Toluene	10 : 1	58
X = Cl	DMF	1 : 3	51
Y = Cl	Toluene	10 : 1	70
X = CH_2CH_3	DMF	1 : 5	65
Y = Br	Toluene	—	—

***Table 2.5**: Observed Regioselectivity in 1-Substituted Cyclopropane-1,2-Dioates*

(±)-*trans*-Diethyl cyclopropane-1,2-dioate (**94**, X= H), was not prepared under the above conditions, as this compound was commercially available.

2.7.1 Improved Synthesis of 1-Substituted Cyclopropane 1,2-Dioic Acids

In an attempt to enhance the regioselectivity, percentage yield and in a bid to lower the cost of the general reaction employed (Scheme 2.26), an alternative procedure was envisaged. The alternative method pursued, was based upon the general reaction, which had been developed by McCoy *et al.*[121,123] (Scheme 2.25, B).



***Scheme 2.27**: Improved Synthesis of Dimethyl-1-Substituted Cyclopropane-1,2-Dioates*

By placing the group which is retained in the cyclopropane product on the 2-position of the acrylate ester (**97**), such that the anion of a mono-halogenoethanoate ester (**91**), serves as the nucleophile in the conjugate addition reaction, was expected to reduce the steric repulsion in the transition state compared to the situation in which the anion is derived from a dihalo-genoethanoate ester (**91**, X,Y= halogen). Accordingly, the 2-halogenoacrylate esters (**97**, X= Cl or Br), were prepared and treated with 2-chloro- (**91**, Y= Cl) and 2-bromo- ethanoate methyl ester (**91**, Y= Cl), respectively in the presence of sodium or potassium hydride, Scheme 2.28. Under optimised conditions, reaction times were reduced to 6-12 h and the yields of the esters (**93, 94**, X = H, R = Cl; and X = H, R = Br) were increased to 80 and 83% respectively, after column chromatography on silica (Table 2.26).

Substituent Group	Solvent employed	Cis : Trans	% Yield
X = Br Y = Br	DMF	1 : 5	83
X = Cl Y = Cl	DMF	1 : 5	80

***Table 2.6**: Improved Synthesis of 1-Substituted Cyclopropane 1,2-Dioates*

The required dioic acids (**95, 96,** X = H, Me, Et, Cl and Br) were obtained in excellent yield (90-95%) through acid catalysed hydrolysis (Scheme 2.28), and each compound and synthetic intermediate, displayed the expected spectral and analytical properties (Table 2.7).

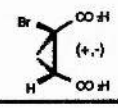
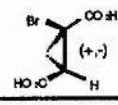
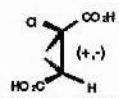
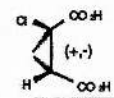
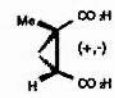
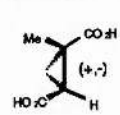
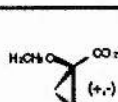
Cyclopropane	Mpt °C	Lit. Mpt °C	NMR Data (^1H, ^{13}C)
	140	140-141	^1H: 1.80 dd Hα, 2.02 dd Hβ, 2.60 dd 2-H. ^{13}C: 23.48 (C-3), 31.62 (C-2), 32.41 (C-1), 171.86, 173.67 (2xCO_2H).
	174-175	175	^1H: 1.73 dd Hα, 1.94 dd Hβ, 2.61 dd 2-H. ^{13}C: 25.68 (C-3), 32.21 (C-2), 37.4 (C-1), 171.8, 174.9 (2xCO_2H).
	178-180	—	^1H: 1.85 dd Hα, 2.05 dd Hβ, 2.80 dd 2-H. ^{13}C: 20.57 (C-3), 28.69 (C-2), 41.12 (C-1), 169.48, 170.2 (2xCO_2H).
	120-122	—	^1H: 1.81 dd Hα, 2.25 dd Hβ, 2.58 dd 2-H. ^{13}C: 25.1 (C-3), 35.4 (C-2), 46.08 (C-1), 173.48, 174.73 (2xCO_2H).
	139-140	139-141	^1H: 1.21 dd Hα, 1.34 s CH_3, 1.63 dd Hβ, 1.98 dd 2-H. ^{13}C: 17.8 (CH_3), 17.89 (C-3), 26.88 (C-2), 27.62 (C-1), 173.17, 174.68 (2xCO_2H).
	168-170	170	^1H: 1.25 dd Hα, 1.38 s CH_3, 1.70 dd Hβ, 2.28 dd 2-H. ^{13}C: 13.9 (CH_3), 21.8 (C-3), 27.8 (C-2), 29.1 (C-1), 174.18, 177.53 (2xCO_2H).
	158-160	—	^1H: 0.91 t (CH_3CH_2), 1.26 dd Hα, 1.46 dd Hβ, 1.75-1.9 m (CH_3CH_2), 2.31 dd 2-H. ^{13}C: 12.5 (CH_3CH_2), 21.3 (C-3), 22.1 CH_3CH_2), 28.3 (C-2), 34.2 (C-1), 174.5, 176.6. (2xCO_2H)
	176-177	177	^1H: 2.85 dd 3-H, 3.53 m 1-H + 2-H. ^{13}C: 18.7 (C-3), 25.9 (C-1 + C-2), 178.3 (2xCO_2H).

***Table 2.7**: Analytical Data for 1-Substituted Cyclopropane 1,2-Dioic Acids Synthesised*

(±)-*trans*-2,3-Methanoaspartic acids (**22**), is a molecule of considerable importance, due to its structural resemblance to a possible transition state, which may exist between step 1 and step 2 (Scheme 2.24). However, as discussed earlier within this chapter (Glutamate Mutase, p.51), this molecule was found to be unstable and as a consequence, we were unable to perform any kinetic studies on this compound.

2.8 Optical Resolution of 1-Substituted Cyclopropane 1,2-Dioic Acids.

The racemic cyclopropane compounds synthesised (**93**, (X= Me) and **94**, (X= H, Me, Et, Cl and Br)), were initially tested for biological activity against MAL (Table 2.9). We observed that the enzyme was inhibited by these compounds and that there was a significant degree of inhibition dependant on the substituent group (Table 2.9), placed on the cyclopropane dioic acid. In an attempt to probe the geometry of the active site and to identify the stereochemistry of the most active inhibitors, we sought to resolve the racemic cyclopropane dioic acids (**94**., (X= Me and Br)

1-Substituted cyclopropane 1,2-dicarboxylic acids, (Fig. 2.4), contain two stereogenic centres and it is possible to obtain up to four different enantiomers. The number of stereoisomers is of course dependant on the substituent group on the cyclopropane dioic acid. There are the (±)- forms of the regioselectively pure *cis* isomers and concomitantly, there are (±)- forms of the regioselectively pure *trans* isomers (Fig. 2.5).

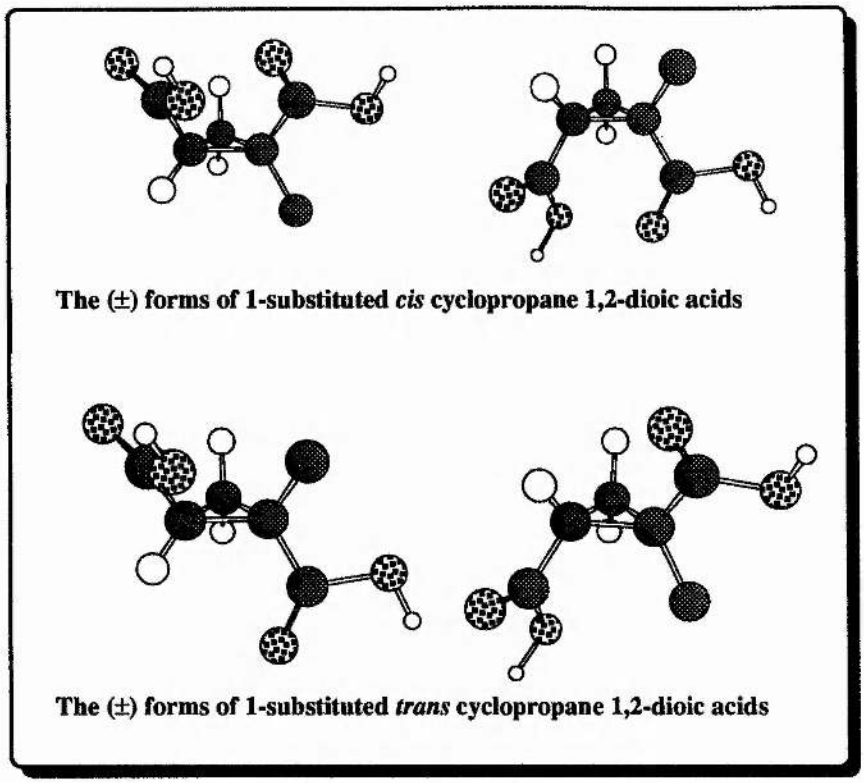


Figure 2.5: *Different Stereochemistries of 1-Substituted Cyclopropane 1,2-Dioic Acids*

There are reported procedures for the enzymic resolution of *meso*-dimethyl cyclopropane-, cyclobutane and cyclopentane dicarboxylates.[124,125] These procedures are reliable for 1-alkyl substituted cyclopropane dicarboxylates, however, they are not convenient for 1-halo-substituted cyclopropane dicarboxylates. Furthermore, these procedures are not designed for preparative scale synthesis.

Diastereomeric amides (A)/(B) (Fig. 2.6) with OH groups located in the γ or δ position to the carbonyl group [R_1 or R_2 = $(CH_2)_nOH$ and or X_1 or X_2 = $(CH_2)_{n-1}OH$, n= 2,3] (Fig. 2.6) show high separation factors in liquid absorption chromatography.[126]

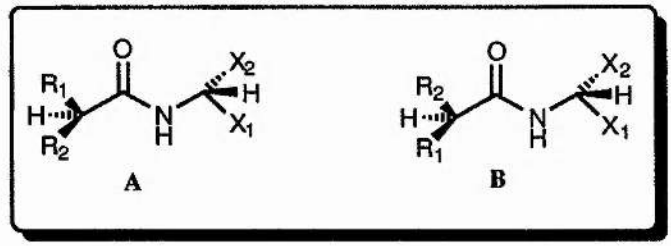
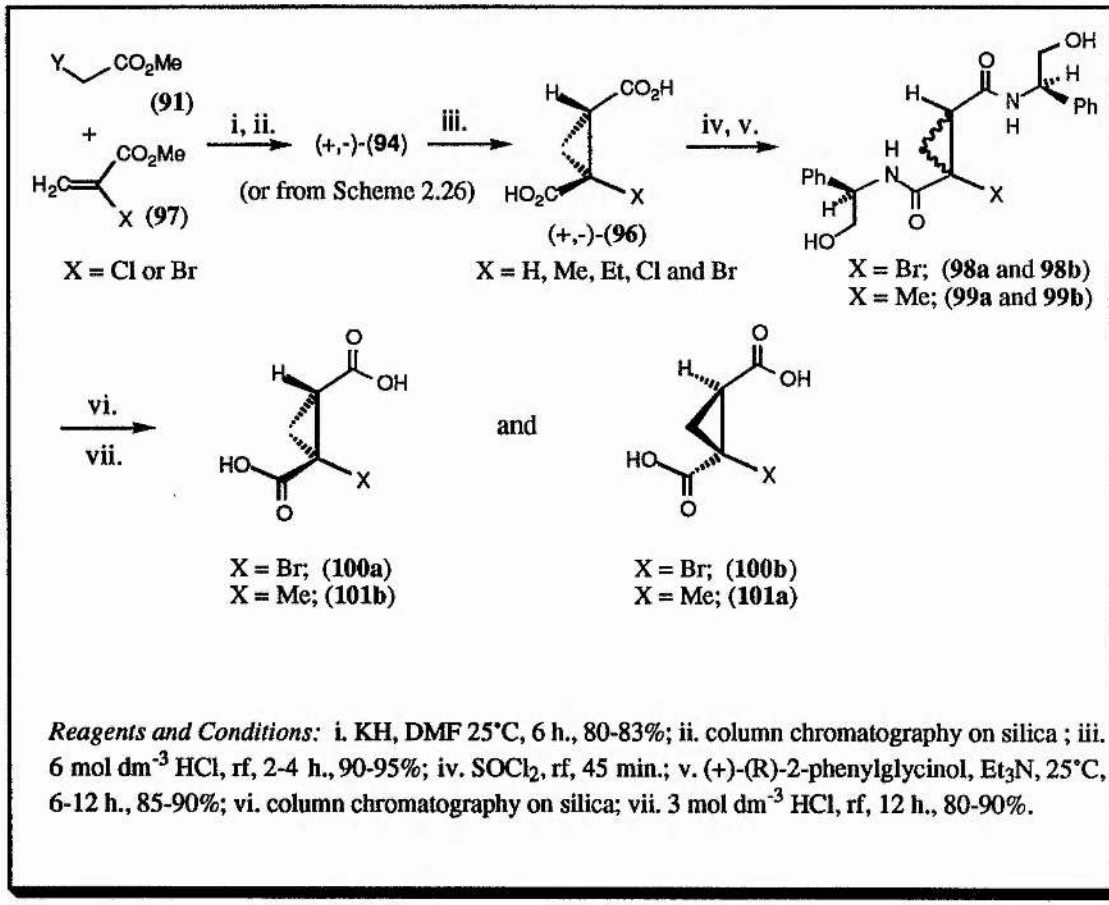


Figure 2.6: Diastereomeric amides containing polar substituents

To take advantage of this kind of chemistry, racemic 1-bromo- and 1-methyl-*trans*- dioic acids (**96**, X= Br and Me) were resolved by separating the diastereomeric *bis*-amide derivatives of (+)-(2*R*)-phenylglycinol[126] (**98a and 98b**) and (**99a and 99b**) by flash column chromatography on silica, Scheme 2.28.



Scheme 2.28: Diastereomeric Resolution of 1-Substituted Cyclopropane 1,2-Dioic Acids

Cyclopropane dioic acids (**96** X= Br, Me), were treated with neat thionyl chloride, to give the cyclopropane *bis*-acid chloride (quantitative yield). The *bis*-acid chloride was converted to the appropriate *bis*-amides (**98a,b** and **99a,b**), upon treatment with (+)-(*R*)-2-phenylglycinol. The diastereomeric amides (**98a,b** and **99a,b**), were resolved by column chromatography (Table 2.8). Finally, the separated diastereomers were each, easily hydrolysed under mild acid conditions, without causing the stereogenic centres to racemise. The required enantiomers of the 1-bromo and 1-methyl cyclopropane dicarboxylic acids (**100a,b** and **101a,b**), were isolated in excellent yield (85-90%).

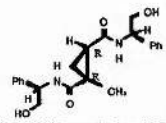
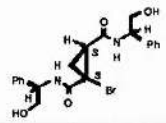
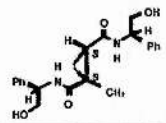
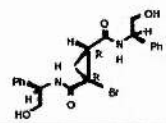
Diastereomeric *bis*-amide	Chromatography Solvent	R_f Value
	2 % Methanol in Ethyl acetate	0.21
	100 % Ethyl acetate	0.29
	2 % Methanol in Ethyl acetate	0.15
	100 % Ethyl acetate	0.15

***Table 2.8:** Chromatographic Polarities of the bis-Amides*

To determine the absolute configuration of each enantiomer, the most polar diastereomers of each *bis*-amides (**98b**) and (**99a**) was subjected to X-ray crystallographic analysis using the known (2*R*)-stereochemistry of the phenylglycinol moiety for reference in each case. The analysis revealed that the samples were derived from the (1*S*,2*S*)-1-bromo-1,2-cyclopropane-dicarboxylic

acid (**100b**) and *(1R,2R)*-1-methylcyclopropanedicarboxylic acid (**101a**) respectively, Figures 2.7 and 2.8.

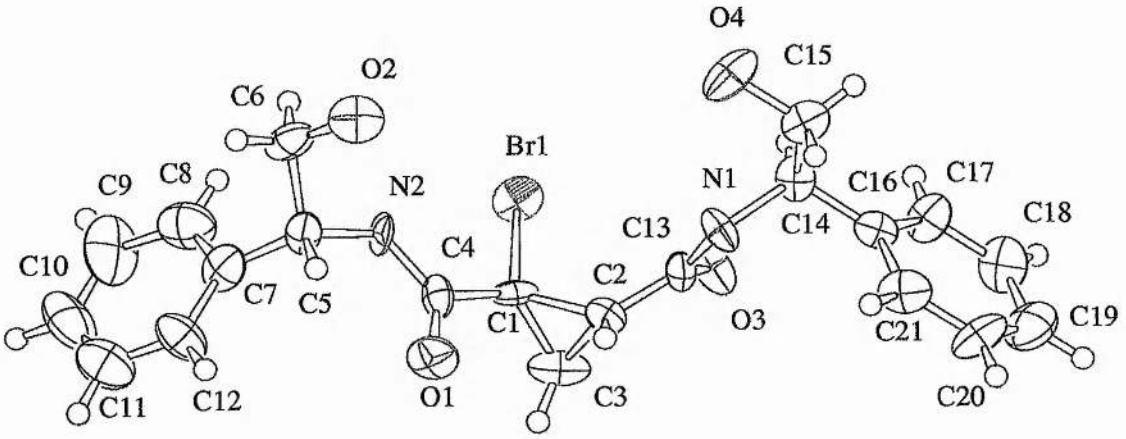


Figure 2.7 *X-Ray Crystal Structure of (1S,2S)-1-Bromo-trans-1,2-diamido[N-(2R)-phenyl ethanol] Cyclopropane*

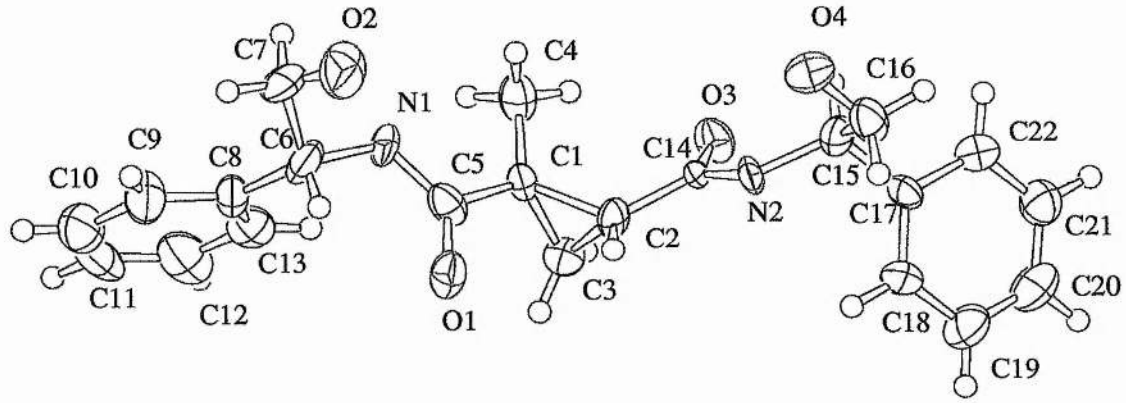


Figure 2.7: *X-Ray Crystal Structure of (1R,2R)-1-Methyl-trans-1,2-diamido[N-(2R)-phenyl ethanol] Cyclopropane*

2.9 Synthesis of 2-Methyl Oxirane and Aziridine 2,3-Dicarboxylic Acids.

Encouraging inhibition results (See Section 2.10), by (±)-1-methylcyclopropane 1,2-dioic acid (**96** X= Me), with MAL, prompted us to investigate whether the insertion of a heteroatom (such as oxygen and or nitrogen) in place of C-3 of the 1-substituted cyclopropane dioic acid (**96** X= Me), would have any effect on the mode and extent of inhibition against MAL. We envisaged, that perhaps due to the electrostatic nature of oxygen and nitrogen, a tighter binding may occur, between the heteroatom and the enzymes active site (Fig. 2.10 - where C-3 is a heteroatom). The two obvious synthetic targets to determine our hypothesis were; (±)-*trans*-2-methyl-oxirane-2,3-dicarboxylic acid (**102**) and (±)-*trans*-2-methylaziridine-2,3-dicarboxylic acid (**103**).

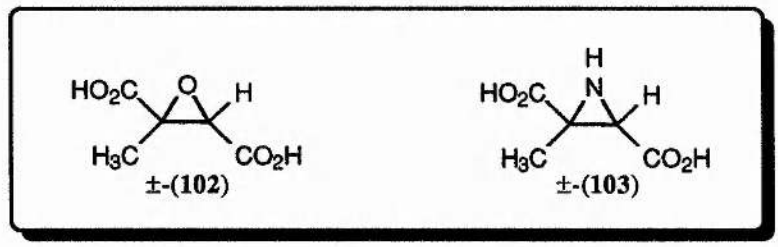
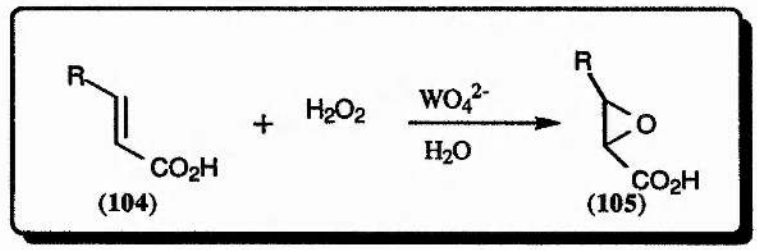


***Figure 2.9**: Desired Oxirane and Aziridine Dioic Acids*

Synthesis of the oxirane (**102**), was achieved *via* the method employed by Sharpless (Scheme 2.29).[127] This method involves the aqueous tungstate-catalysed epoxidation of an α,β-unsaturated acid (**104**) by hydrogen peroxide, to yield the corresponding epoxide (**105**).

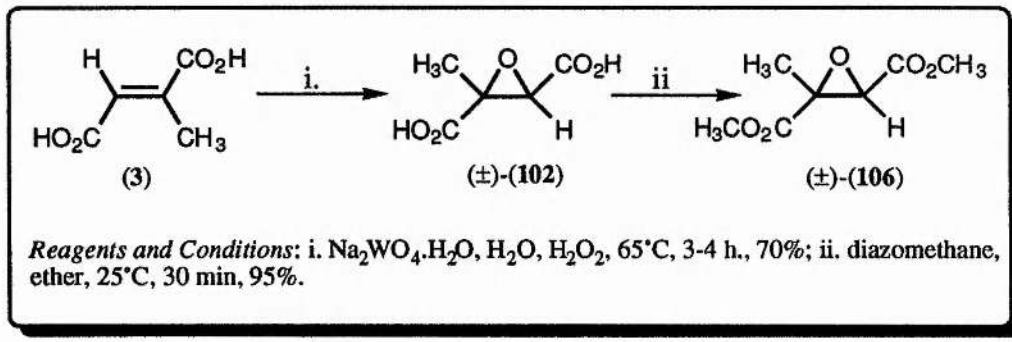


***Scheme 2.29**: Epoxidation of α,β-Unsaturated Acids*

This method of epoxidation was a modification of the original procedure described by Payne.[128] Sharpless utilised this modified procedure to epoxidise

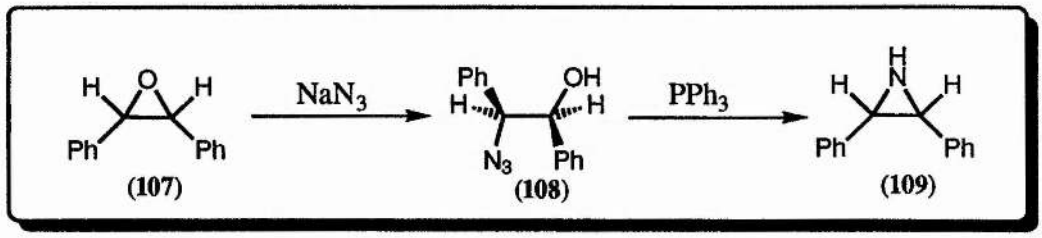
both di- and trisubstitued unsaturated acids. The epoxides isolated were synthesised in excellent yield.[127]

Mesaconic acid (**3**) was treated with hydrogen peroxide in the presence of sodium tungstate catalyst (Scheme 2.30) at pH 6.0. The reaction was carefully monitored by t.l.c and the pH kept between 5.8 and 6.8. The desired epoxide (**102**), was obtained in 70% yield, and was isolated as a white crystalline solid. The epoxide (**102**), was derivatised to the corresponding dimethyl ester (**106**), *via* diazomethane. The dimethyl ester (**106**), showed the required spectral and analytical data (R_f (ether/hexane (3:2)) = 0.47, literature, R_f (ether/hexane (3:2)) = 0.47;[129] *m/z* (Found: $[M+H]^+$, 175.0598. Calc. for $C_7H_{11}O_5$: 175.0606)).



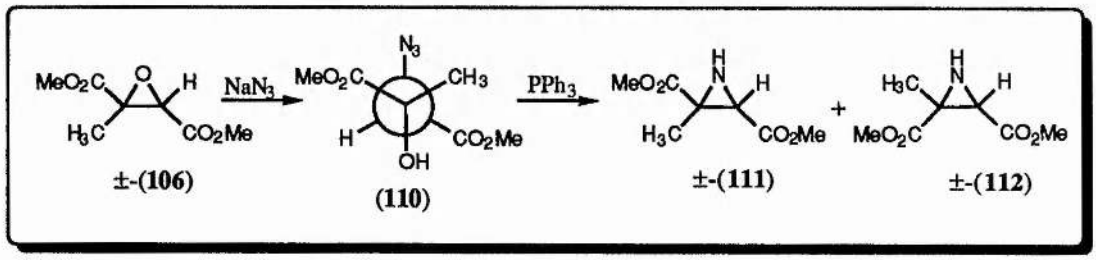
***Scheme 2.30**: Hydrogen Peroxide-Tungsten Catalysed Epoxidation of Mesaconic Acid*

Our approach towards obtaining the corresponding aziridine (**103**), was to employ the Blum reaction (Scheme 2.31). This reaction involves the synthesis of aziridines (**109**), from oxiranes (**107**), *via* azido alcohols (**108**), in the presence of phosphines.[130]



Scheme **2.31***: Blum, Aziridine Synthesis*

This work is currently being followed up within our laboratories, with the aim of obtaining the desired aziridine dicarboxylic acid (**103**, Scheme 2.32). Our approach involves the formation of the azido alcohol (**110**), from the ring opening reaction of the oxirane (**106**), by sodium azide, followed by the concomitant cyclisation of the azido alcohol (**110**), giving rise to the formation of the corresponding aziridines (**111** and **112**).



Scheme **2.32***: Proposed synthetic route towards Dimethyl-2-Methyl Aziridine 2,3-Dicarboxylate*

2.10 Kinetic Studies on 1-Substituted Cyclopropane 1,2-Dioic Acids

In order to test whether 1-substituted cyclopropane dioic acids behave as reversible inhibitors of MAL, kinetic (inhibition) studies were performed for each of the cyclopropane compounds. The results of our studies are shown in Table 2.9.

To determine the extent and the mode of inhibition, the kinetic turnover of (2*S*,3*S*)-3-methylaspartic acid (**2**) by the enzyme MAL (of fixed activity and of fixed

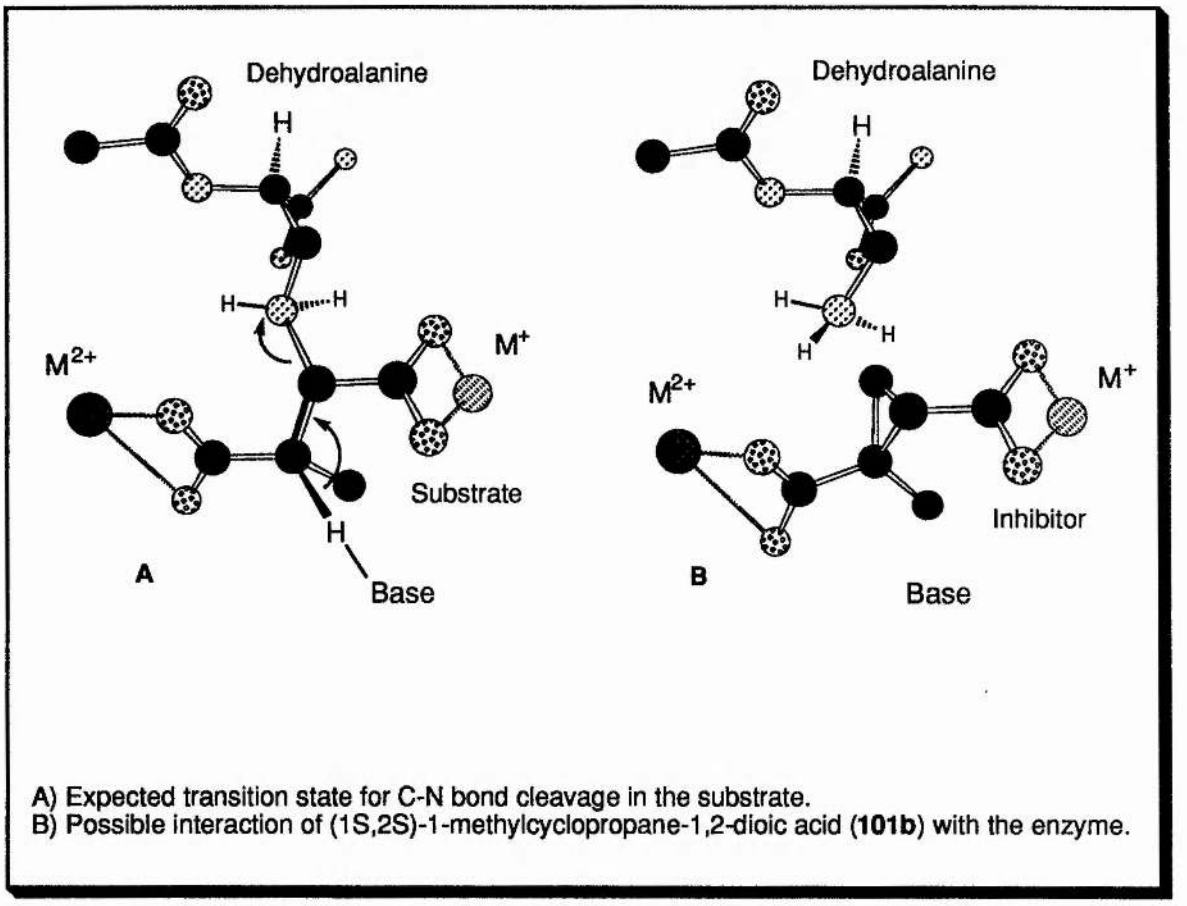
concentration), was examined in the presence of various concentrations of potential inhibitor.

The cyclopropane 1,2-dioic acids (Table 2.9), when tested for biological activity, the racemic *trans*-cyclopropane 1,2-dioic acids (**96**, X = H, Cl and Br), served as weak competitive inhibitors for the enzyme with K_i values of 7.69 mmol dm^{-3}, 4.15 mmol dm^{-3}, and 6.1 mmol dm^{-3}, respectively (Table 2.9). The racemic *cis*-1-methylcyclopropane 1,2-dioic acid (**95**, X= Me), was a very poor inhibitor (K_i 5.42 mmol dm^{-3}) but the corresponding *trans*-compound (**96**, X = Me) served as a good competitive inhibitor, K_i = 67 μmol dm^{-3}, Table 2.9. Note that the K_m for (2*S*,3*S*)-3-methylaspartic acid (**2**), is 2.3 mmol dm^{-3} and that the value of K_i for the competitive product inhibitor, mesaconic acid (**3**), is 3 mmol dm^{-3}.[131] Replacement of the methyl group in the best inhibitor by an ethyl group (**96**, X = Et), reduced the efficacy of the inhibitor by 10-fold (Table 2.9) in accord with the previous finding that K_m for (2*S*,3*S*)-3-ethylaspartic acid (**31**, R= Et), is large, 17.1 mmol dm^{-3}.[58] Thus it would appear that the series of inhibitors displayed the properties that might be expected if they were serving as TS analogues for the central substrate deamination step, Step 2, Scheme 2.24.

2.10.1 Inhibition Studies of the Resolved Cyclopropane Dioic Acids

When tested for activity the (1*S*,2*S*)-1-methylcyclopropane (**101b**) {$[\alpha]_D$ +191.4 (c 1.0, MeOH)} was a potent inhibitor (K_i = 20 μmol dm^{-3}) and was six times more effective than the (1*R*,2*R*)-antipode {$[\alpha]_D$ -191.4 (c 1.0, MeOH)}, whereas the (1*R*,2*R*)-1-bromocyclopropane (**100a**), {$[\alpha]_D$ +91.5 (c 1.0, MeOH)}, displayed a K_i of 920 μmol dm^{-3} and was at least six times more effective than the (1*S*,2*S*)-antipode (**100b**). Note that the spatial arrangement of similar groups is identical for the (1*S*,2*S*)-1-methylcyclopropane (**101b**), and the (1*R*,2*R*)-1-bromocyclopropane (**100a**), but that there are priority assignment changes. From these results, it is evident that each of the inhibitors prefer to bind with the 1-substituent in the pocket for the methyl group of the substrate. Thus each carboxylate group can

bind to the enzyme and metal ion cofactors as for the substrate with the cyclopropane methylene moiety disposed on the same face of the pseudo-plane described by C-1'-C-1,C-2-C-2' as the dehydroalanine residue (Fig. 2.7 and 2.8).



A) Expected transition state for C-N bond cleavage in the substrate.
B) Possible interaction of (1S,2S)-1-methylcyclopropane-1,2-dioic acid (**101b**) with the enzyme.

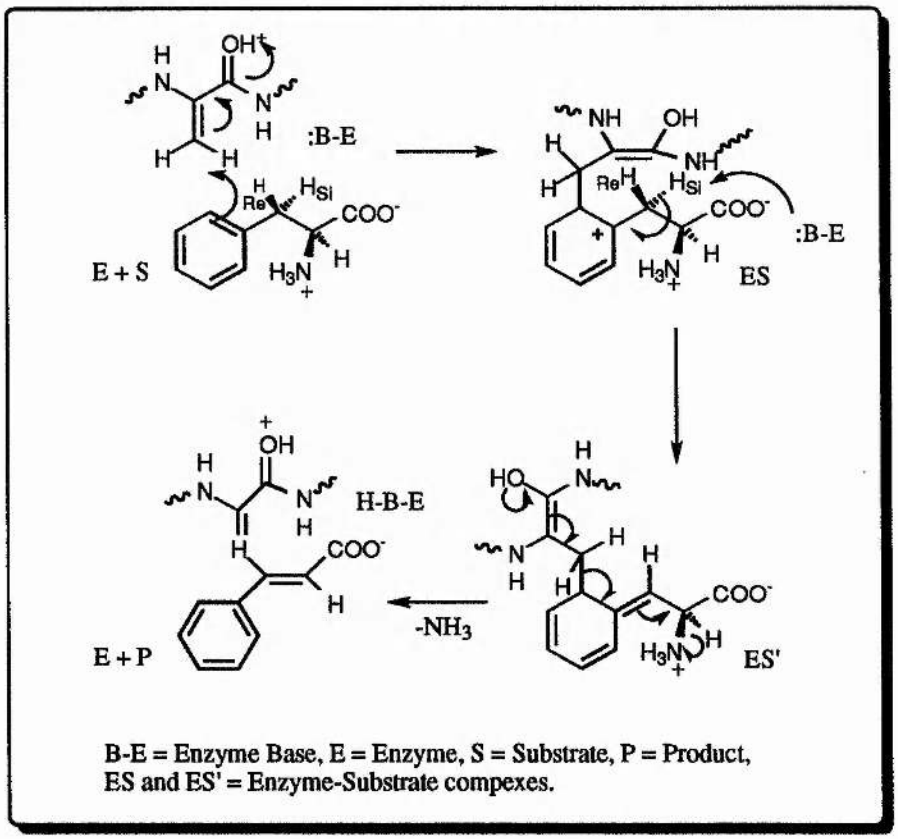
Figure 2.10: *Interaction of substrate and inhibitor with dehydroalanine*

This is in keeping with the observation that there is enough space to accommodate hydrazine or methylamine in place of ammonia and that such species support catalysis for the reverse reaction.[53]

2.10.2 Inhibition in the Presence of Ammonia.

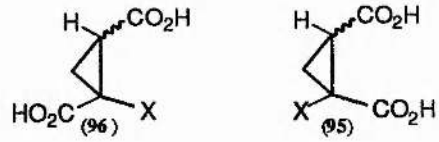
Given that compound (**101b**), can only bind to the enzyme as a partial substrate analogue in the absence of ammonia because the compound lacks an amino group and the ammonium ion is released before the dioic acid in the

forward reaction direction, Scheme 2.24, it was of interest to examine the inhibitory properties of the compound in the presence of ammonia. Accordingly a new set of kinetic experiments were performed in the presence of 10 mmol dm^{-3} ammonium chloride (~0.2 times K_m for the species behaving as a substrate in the reverse direction). Under these conditions compound (**101b**), behaved as a highly potent competitive inhibitor and displayed a reduced K_i value of 11 μmol dm^{-3} (Table 2.9, last entry). The result indicates that ammonia and the cyclopropane dioic acid can simultaneously bind to the enzyme and together mimic the TS structure for the central deamination process (Fig. 2.10, B). This result is at odds with the notion that the dehydroalanine residue of the related enzyme phenylalanine ammonia lyase, serves as a Friedel-Crafts donor rather than an amino group acceptor as has been recently suggested (Scheme 2.33).[132]



Scheme 2.33: Proposed Mechanism for PAL

2.10.3 Summary of Properties of Methylaspartase Inhibitors



Reaction Conditions	Stereo-chemistry	X	K_i (mmol dm^{-3})	Mode of Inhibition	Comp. No.
standard	*(+,-)-trans-*	H	7.69	competitive	96
standard	*(+,-)-cis-*	Me	5.42	competitive	95
standard	*(+,-)-trans-*	Me	0.067	competitive	96
standard	*(+,-)-trans-*	Et	0.69	competitive	96
standard	*(+,-)-trans-*	Cl	4.15	competitive	96
standard	*(+,-)-trans-*	Br	6.09	n.d., IC_{50}	96
standard	*(1R,2R)-trans-*	Br	0.92	mixed	100a
standard	*(1S,2S)-trans-*	Br	6.520	n.d., IC_{50}	100b
standard	*(1S,2S)-trans-*	Me	0.020	competitive	101b
standard	*(1R,2R)-trans-*	Me	0.120	competitive	101a
std + NH_4^+	*(1S,2S)-trans-*	Me	0.011	competitive	101b

***Table 2.9**: Kinetic Data for the Methylaspartase Inhibitors*

The enzyme was assayed according to the method of Barker,[12] where 1 unit of enzyme catalyses the formation of 1 μmol of mesaconic acid min^{-1} at pH 9.0 at 30 °C as determined by the increase in optical density (OD_{240}) at 240 nm, under the assay conditions.[12] The reaction rates were determined in triplicate under the standard assay conditions[58] for several different substrate and inhibitor concentrations and data was fitted using non-linear regression analysis. Errors in K_i values are within ±5% of the stated value.

2.11 Conclusions and Future Work

2.11.1 Cyclopropane Containing Inhibitors

The usefulness of rigid inhibitors for probing active conformations of enzyme substrates is well documented.[133] Their binding in the active site is entropically favoured and they can offer reliable information about the arrangements of the protein residues. Cyclopropane containing structures possess a number of advantages over unstrained inhibitors; for example they do not contain any additional atom(s) that will prevent their entrance into an active site. The cyclopropyl group is found as a basic structural element within a wide variety of naturally occurring compounds in plants and in microorganisms, both fungal and bacterial. It is also generated transiently in primary and secondary metabolism. Therefore, it is present in compounds of biological importance.[134] The cyclopropane chemical reactivity not only resembles that of an olefinic double bond but moreover, involves rearrangements: i.e., ring opening reactions, ring enlargements and ring contractions.[135,136]

Racemic 1-substituted cyclopropane-1,2-dioic acids which were kinetically assayed for inhibition against MAL, showed varied degrees of inhibition (Table 2.9). From the data obtained, we can speculate the kind of functionality which is required within the active site. Furthermore, we have shown for the first time that 1-substituted cyclopropane-1,2-dioic acids (Fig. 2.4) are biologically active against MAL. We envisage that these cyclopropane dioic acids, mimic a TS which exists during the catalytic turnover of (2*S*,3*S*)-3-methylaspartic acid (**2**) to mesaconic acid (**3**) (Scheme 2.24).

The impact of the stereochemistry on activity requires regioselective, as well as stereoselective synthesis of the three membered ring moiety. We observed that the regioselectively pure, *cis*-cyclopropane dioic acid tested (**95**, X= Me), was biologically active, but showed poor inhibition. On the other hand the regioselectively pure *trans*-cyclopropane dioic acids (**96**, Table 2.9), were much more potent inhibitors of MAL. Since, the best racemic *trans*-cyclopropane (**96**,

X=Me, (67 μmol dm^{-3})) possessed the methyl substituent in the 1-position of the cyclopropane, the only *cis*-cyclopropane dioic acid that was tested against MAL, was (**95**, X= Me, 5.42 mmol dm^{-3}). We concluded that no further kinetic inhibition experiments were necessary on the other, *cis*-cyclopropane dioic acids as we did not expect to find further information about the enzyme and we therefore focused our attention on the *trans*-cyclopropanes.

Enantiomerically pure *trans*-cyclopropane 1,2-dioic acids (**96**), showed good to potent inhibition (Table 2.9). From the inhibition data we could deduce the stereochemistry required by a potential substrate/inhibitor for the active site of MAL. We observed that compounds (**100a**) and (**101b**) were the more optically active compounds as compared to their counterparts (**100b**) and (**101a**).

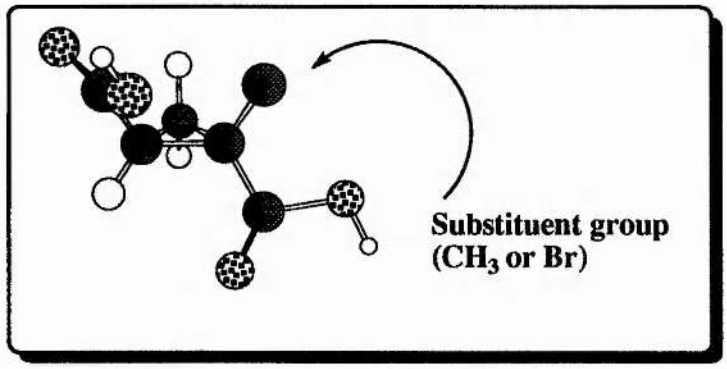


Fig. 2.11: The Conformational Requirements for MAL

The cyclopropane dioic acids studied in general bound reversibly to the active site of the enzyme and prevented the natural substrate (**2**), binding and *vice versa*. From the data collected we concluded that the cyclopropane dioic acids were competing for the active site against the natural substrate (**2**). The cyclopropane dioic acids, in general, behaved as competitive inhibitors of MAL. The only exception to this observation, was that of (1*R*,2*R*)-1-bromo-cyclopropane-1,2-dioic acid (**100a**), which displayed mixed inhibition. We consider this a significant result and we believe that the halogen within compound (**100a**), may be able to interact with one of the metal ions in the enzyme-bound complex. However, at this stage, this is a highly speculative suggestion. We envisage that supporting experimental work must be carried out,

to determine the cause of the mixed inhibition. This is one of the programmes which is currently under investigation in our research group.

Our determination of the inhibitory properties of the cyclopropane 1,2-dioic acids with ammonia was only conducted on cyclopropane (**101b**). The reason for this one study was because this compound (**101b**) was by far the best inhibitor. We do not expect that there would be a change in inhibition mode with the less good inhibitors and indeed, this work has a lower priority within our research programme.

X-ray crystallography has given a wealth of detail about the structures of enzymes. It is clear that we need to be able to locate the active site in MAL and examine carefully the mode of binding of the different inhibitors/substrates in order to pinpoint the functional amino acid side chains which are involved in the catalytic mechanism. Compound (**101b**), a potent competitive inhibitor of methylaspartase, which allows the simultaneous occupation of the active-site by ammonia, is ideally suited for gaining such information.

2.12 β-Amino Acids

2.12.1 Introduction

As mentioned in chapter 1 (Section 1.4), β-amino acids are used as constituents of many biologically active peptides. β-amino acids are used to replace their parent α-amino acid analogue and due to their limited normal metabolism, suggests that many β-amino acids are metabolically stable in *vivo;* enzyme inhibitors may thus have extended half-lives when designed as the β- rather than the α-amino acids. Similarly, incorporation of β-amino acids into peptides of pharmacological interest has in some cases been found advantageous in terms of biological activity, metabolic stability, or both (see section 1.4 for examples).

Our research in this area is focused on the synthesis and biological properties of peptidic phosphonamidate esters. These compounds are moderate to good inhibitors of the HIV-1 protienase, showing comparable potency to other inhibitor classes.[137-140]

Our target phosphonamidate inhibitor (Fig. 2.13) was modelled on the peptidic analogue of Ro-31-8959[158] (Roche's leading peptidic inhibitor of HIV-1 protienase, Fig. 2.12) and was designed to contain the biologically active β-amino acid (111).

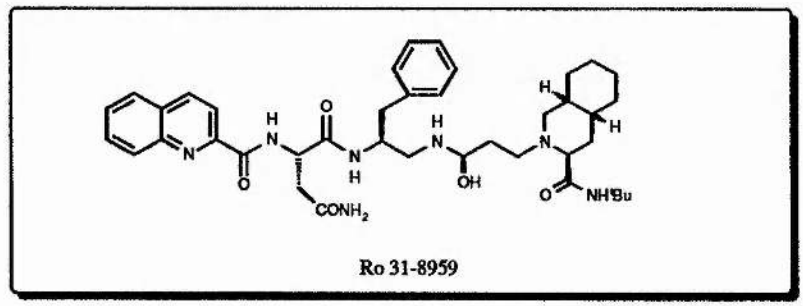


Figure 2.12: *Ro 31-8959, A Potent and Selective Inhibitor of HIV-1 Proteinase*

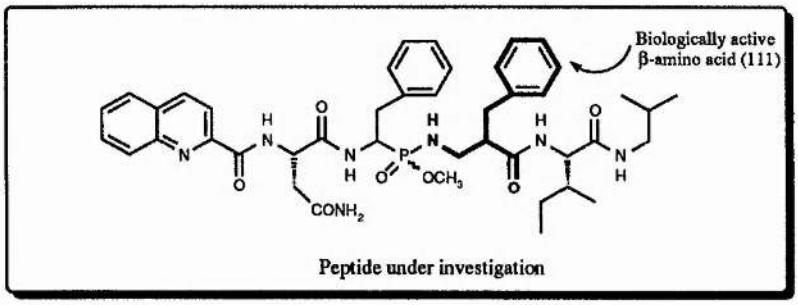
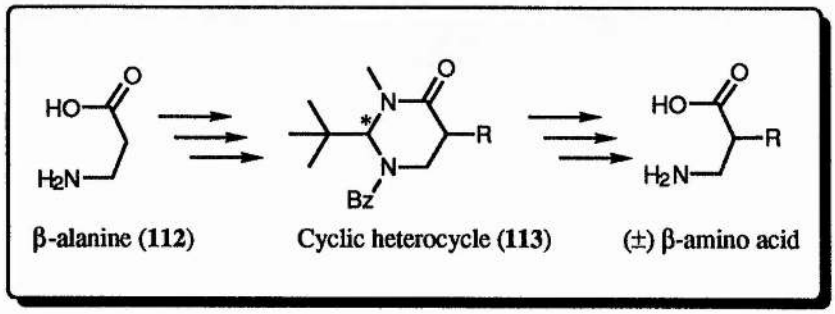


Figure 2.13: Target Peptide Containing β-Amino Acid

Our efforts were directed towards the synthesis of the β-amino acid (111) and initially it was our aim to obtain the amino acid (111) in a racemic form, so that its biological activity could be assessed by comparing the activity of peptide (Fig. 2.13), (which contains the β-amino acid (111)) against the same peptide which contains the in the parent α-amino acid.

2.13 Synthesis of (±)-α-Benzyl-β-Amino Propioinic acid

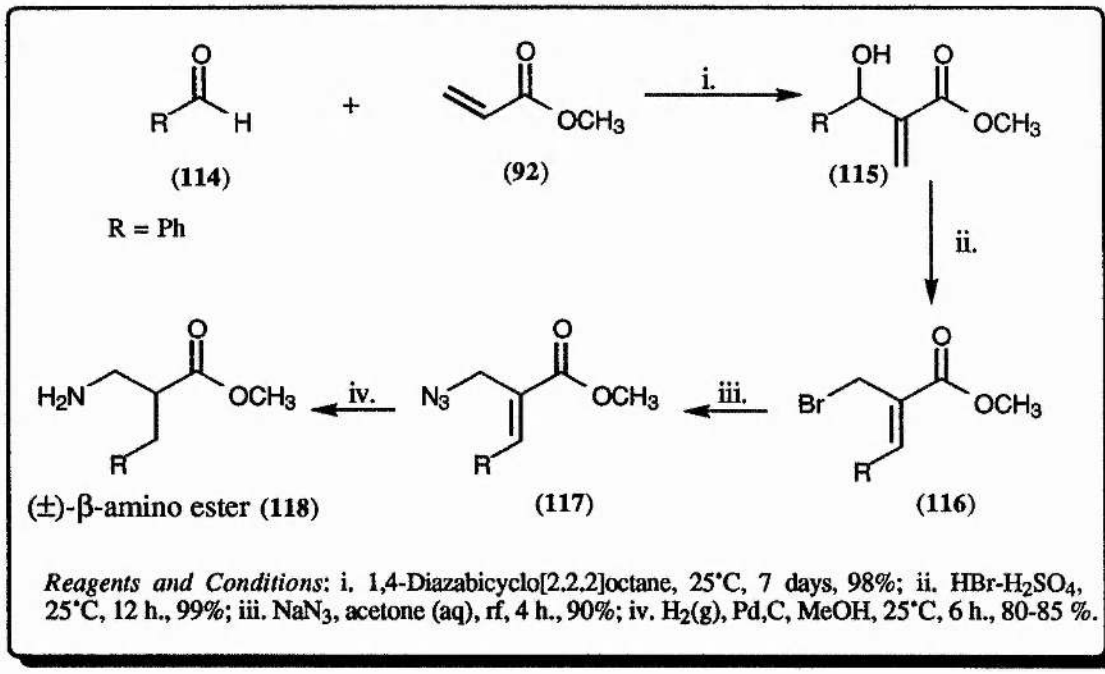
(±)-α-Benzyl-β-amino propionic acid (111), is an example of an α-substituted-β-amino acid and is a type III analogue of (2*S*)-alanine (see Fig. 1.7, Chapter 1). The β-amino acid (111), has received little attention in the literature and the only reported synthesis of this compound was achieved by Seebach *et al.* (Scheme 2.34),[141] in 1991. Seebach and co-workers synthesised a variety of α-substituted-β-amino acids, *via* β-alanine (112), as the starting material.



Scheme 2.34: Seebach's Synthesis of α-Substituted-β-Amino Acids

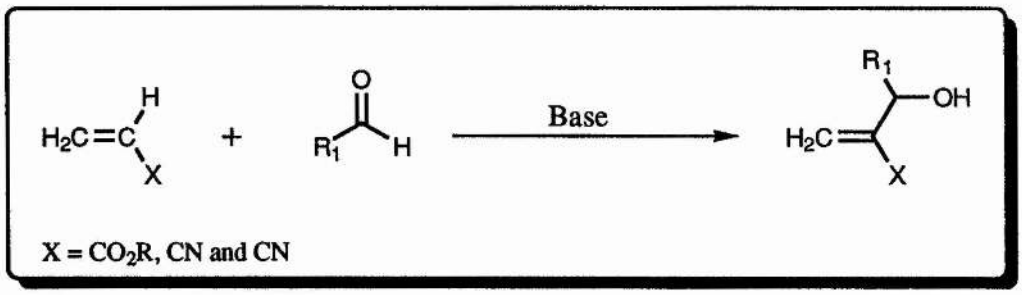
β-alanine (**112**), was converted into the heterocycle (2-*tert*-butyl perhydropyrimidin-4-one derivative, **113**), which was alkylated with high diastereoselectivity *via* the corresponding enolate. The high stereoselectivity observed in this reaction was due to steric hindrance generated by an axial disposition of the *tert*-butyl group at C(2) (**113**), which directs addition from the enolate face opposite to this group. The hydrolysis of the resulting adducts proceeds with 6 *N* hydrochloric acid to afford the (±)-α-substituted-β-amino acid.

Due to the severe reaction conditions which were employed in the synthesis of the cyclic heterocycle (**113**) and a generally tedious procedure overall, we sought to synthesise our target compound (**111**), *via* a different synthetic route (Scheme 2.35).



Scheme 2.35: Alternative Approach to α-Substituted-β-Amino Acids

The synthesis described in the above scheme (Scheme 2.35), represents a short and very efficient synthesis of (±)-α-substituted-β-amino acids. The first step of the reaction scheme, originates in a patent granted to Baylis and Hillman.[142] These authors describe the reaction between α,β-unsaturated esters, nitriles, amides or ketones together with a broad spectrum of aldehydes (Scheme 2.36).



Scheme 2.36: The Baylis Hillman Reaction

The reaction is carried out in the presence of a cyclic tertiary amine, such as 1,4-diazabicyclo[2.2.2]octane (DABCO, Fig. 2.13 A), which behaves as a catalyst for the reaction (Scheme 2.37).

The catalyst constitutes between 0.1% and 10% of the reactant weight. Reaction temperatures can be varied between 0 and 200 °C and the pressure may be atmospheric, however, the reaction can proceed at higher pressures or under vacuum. Solvents such as dioxane, THF, ethanol or chloroform are all suitable for the reaction, but in many cases the solvent is not required. Baylis and Hillman[142] refer to the use of tertiary cyclic amines such as DABCO (Fig. 2.13 A) and quinuclidine (Fig. 2.13 B), but non-cyclic tertiary cyclic amines such as triethylamine have also been employed by other researchers.[143]

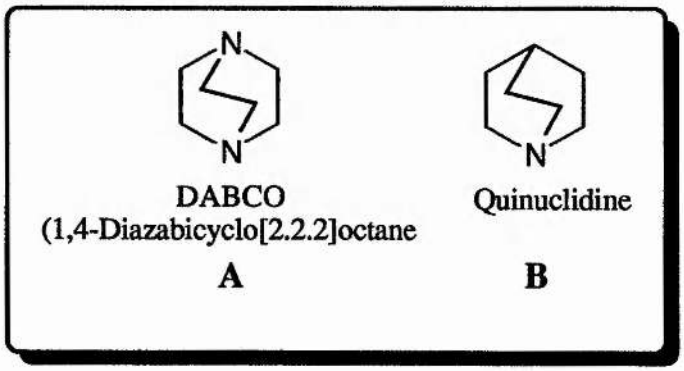
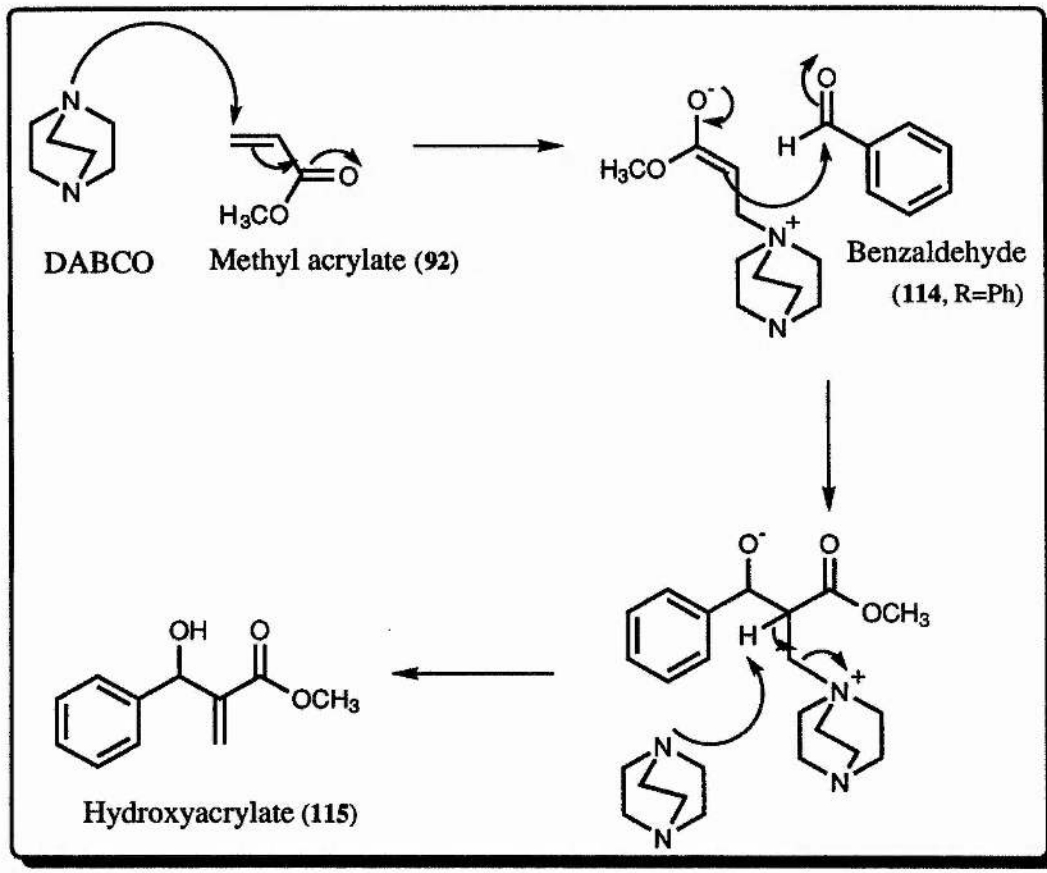


Figure 2.13: Tertiary Cyclic Amines as Catalysts for the Baylis-Hillman Reaction

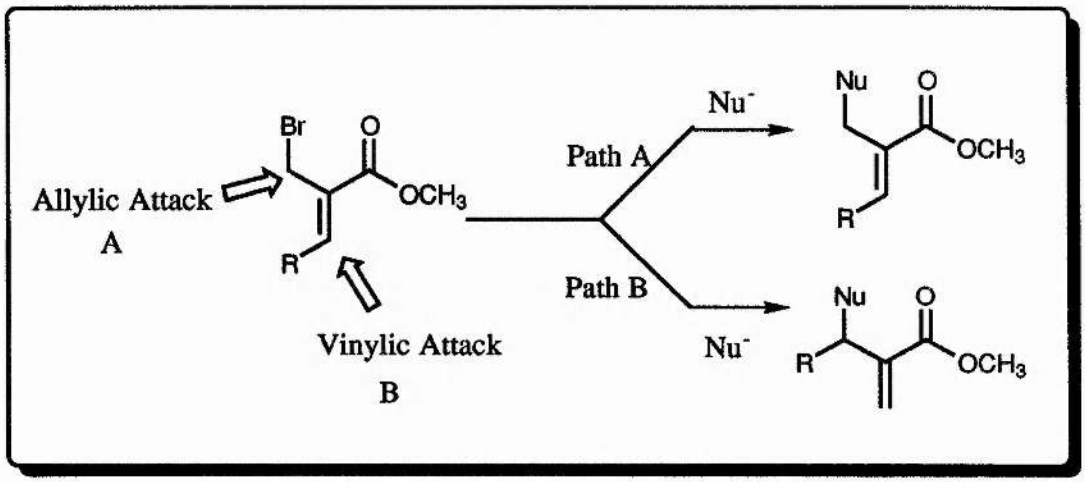


Scheme 2.37: Mechanism of the Baylis-Hillman Reaction

It seems reasonable to suggest that the first step in the synthesis, involves a Michael addition of the tertiary amine (Fig. 2.13 A), to the corresponding α,β-unsaturated carbonyl compound (**92**). This is concomitantly followed by a nucleophilic attack on the aldehyde (**114**, R= Ph), which is then followed by the subsequent elimination of the tertiary amine (Fig. 2.13 A). This type of mechanism was confirmed by Hoffman and Rabe,[144] who carried out conformation studies on this type of reaction.

Treatment of the hydroxyacrylate (**115**), with hydrobromic acid-sulphuric acid[145] led to the formation of the rearranged allylic bromo-ester (**116**), in quantitative yield (Scheme 2.35). The β-bromo ester (**116**), contains the labile carbonyl allyl system, and as such it is possible that it can undergo nucleophilic substitution at the allylic position or nucleophilic attack can occur at the vinylic

carbon, C-3 (Scheme 2.38), followed by concomitant rearrangement with the loss of the bromide anion.



Scheme 2.38: Different Pathways for Nucleophilic Attack/Substitution

It was our aim to follow the reaction so that a nitrogen nucleophile, such as sodium azide, would be able to substitute for the bromide anion, at the allylic position. We found that by carrying out the reaction in aqueous acetone and in the presence of sodium azide, under reflux conditions, that the sole product was allylic azide (**117**).

Having obtained the desired allylic azide (**117**), we attempted to reduce the azide and the double bond in one pot. We achieved this by hydrogenating compound (**117**), in the presence of (5%) palladium on carbon catalyst in neat methanol. The desired compound (**118**), was obtained in excellent yield (80-85%, Scheme 2.35). The spectral and analytical data of the β-amino ester (**118**), displayed the expected properties when compared to the literature compound.[141]

2.14 Asymmetric Synthesis of β-Amino Acids

Having obtained our target β-amino ester (**118**), in a short and efficient synthesis, we focused our attention towards obtaining enantiomerically pure β-amino acids, utilising our synthesis (Scheme 2.35).

Our first approach towards the synthesis of enantiomerically pure β-amino acids, was directed at inducing asymmetry on the prochiral carbon atom (C-2) in compound (**117**, Fig. 2.14).

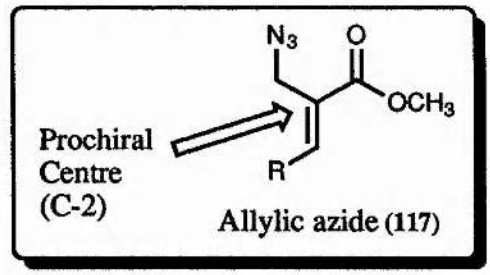
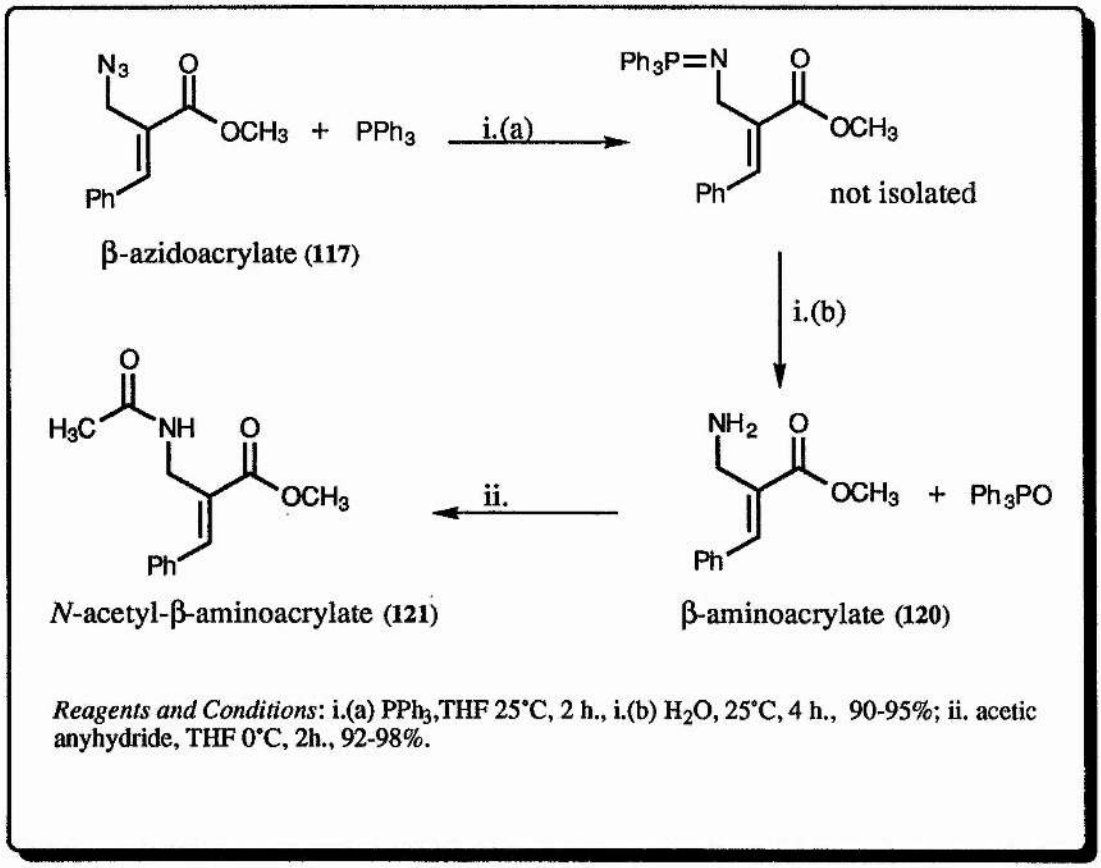


***Figure 2.14**: Introducing Chirality*

One method of inducing chirality at C-2 (**117**), was by the enantioselective hydrogenation of the olefin (**117**), in the presence of a chiral catalyst. As described in chapter 1 (Scheme 1.19), Nyori performed asymmetric hydrogenations on prochiral, α-(acylamino)acrylic acid derivatives, using BINAP-Ru(II) metal complexes to serve as excellent chiral catalysts (Scheme 1.19).[90]

The analogous β-(acylamino)acrylic ester (**120**, Scheme 2.39), was synthesised by the selective reduction of the azide functionality within compound (**117**), to the corresponding amino-acrylic ester (**120**). The selective reduction, was achieved in quantitative yield, using triphenylphosphine as the reducing agent.[146] The β-amino acrylate (**120**), was then treated with acetic anhydride to give the desired compound as a white crystalline solid (**121**, Scheme 2.39).



***Scheme 2.39**: Synthesis of Methyl-β-(acylamino)acrylate*

Commercially available ([Rhodium((*S*)-binap (norbornadiene)]perchlorate (**122**, Fig. 2.15), was hydrogenated in methanol at room temperature to produce norborane and two Rh complexes, (*S*)-**123** and **124**, (Fig. 2.15), in a ratio of 9 : 1 as described by Nyori.[90] The major, methanol-soluble complex was isolated as deep red prisms and was shown to have the same spectral properties as the literature compound. The minor complex (**124**), was insoluble in methanol and was isolated as a brown solid.

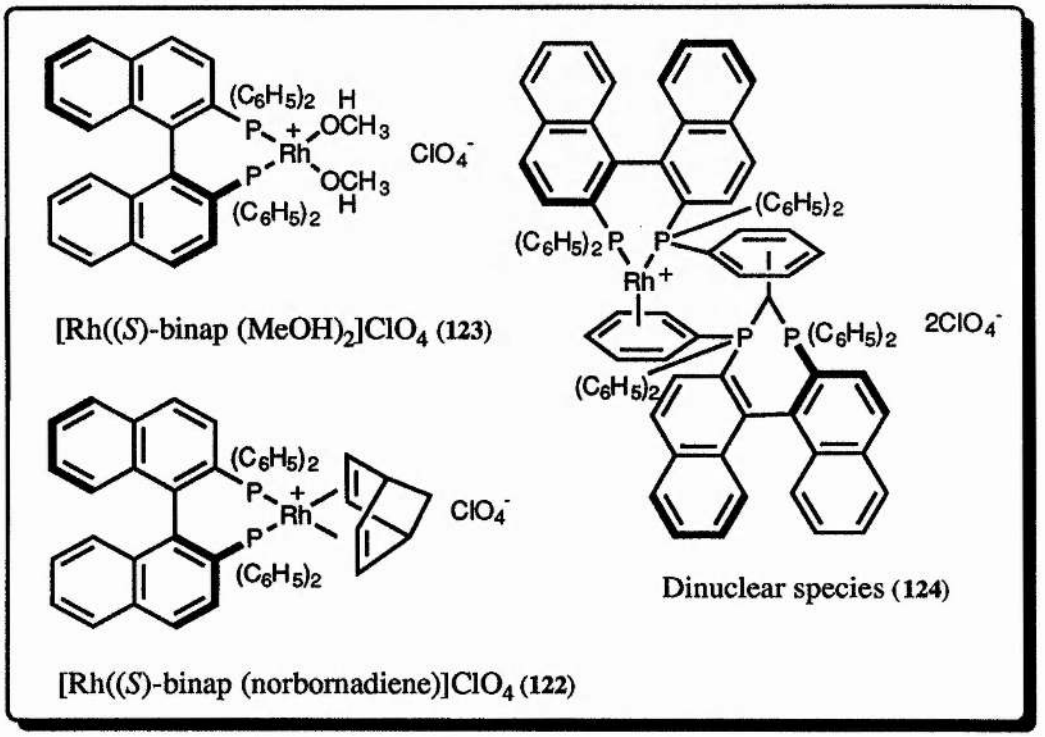
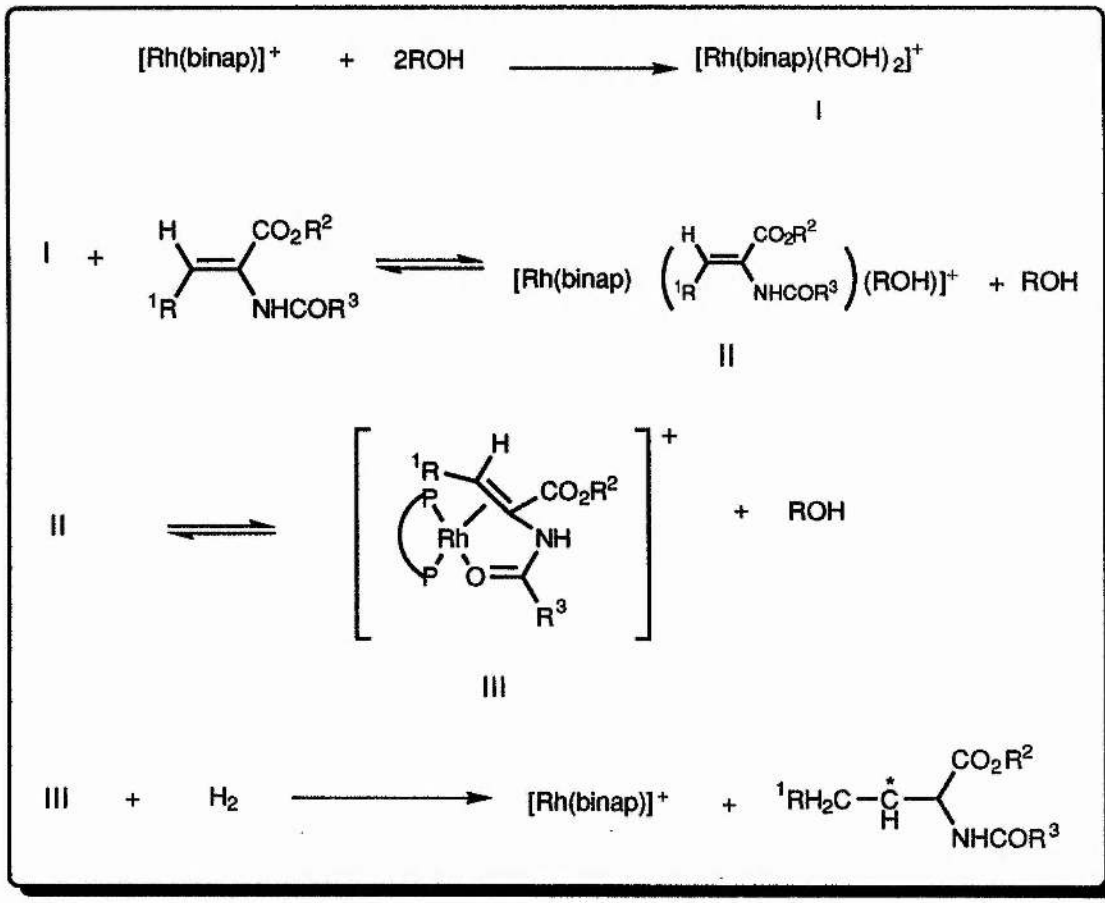


Figure 2.15: Rhodium-Binap Complexes

The β-(acylamino)acrylate (**121**), was dissolved in dry methanol and treated to the chiral catalyst (**123**), under an inert atmosphere of argon. This mixture was added to a pre-purged (sealed), pressure bottle, which was filled with 3 atmospheres of pure hydrogen. The reaction mixture was stirred and the reaction was followed by chromatography (t.l.c.). After several days, we observed no change in starting materials and it was concluded, that the chiral catalyst did not have any effect in co-ordination to the β-(acylamino)acrylate (**121**), as opposed to the case for the α-derivative according to literature (Scheme 1.19).[90]

We expected that the reaction between the β-(acylamino)acrylate (**121**), and the chiral catalyst (**123**), would have started in a similar manner, as is shown in scheme 2.40, where initial coordination of the olefinic substrate (**II**, Scheme 2.40), would chelate to the rhodium (Rh) atom (**III**, Scheme 2.40). Since we observed that the olefin (**121**), was not reduced, we concluded that the type of coordination as shown (**III**, Scheme 2.40), does not occur.



Scheme 2.40: Mechanism of Asymmetric Hydrogenations

Another approach towards the construction of enantiomerically pure β-amino acids, was to induce asymmetric induction, on the prochiral carbon atom, C-2 (Fig. 2.16). The induction was envisaged to be caused *via* a chiral auxiliary, which would be attached as the chiral ester (Fig. 2.16).

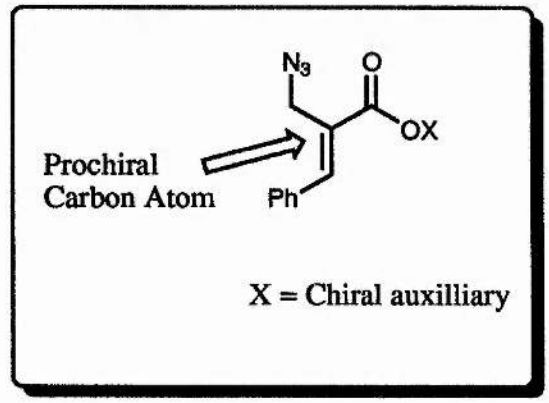
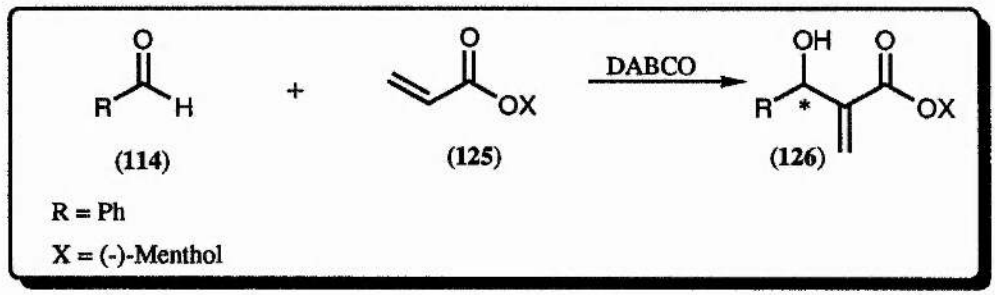


Figure 2.16: Chiral Induction on the Prochiral Centre

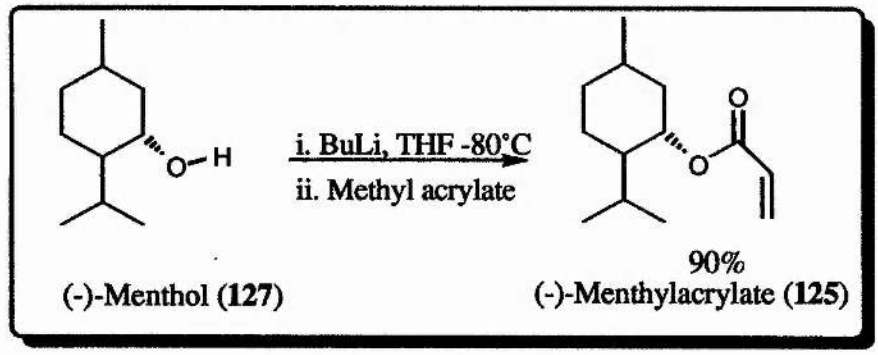
Once again, the synthetic route developed towards the synthesis of (±)-α-substituted-β-amino acids (Scheme 2.35), was utilised towards the synthesis of enantiomerically pure β-amino acids. We employed enantiomerically pure (-)-menthol (**127**), as the chiral auxiliary, which was expected to cause chiral induction on the azidoacrylate. (Fig. 2.16).

The synthesis of the optically active, 2-(1-hydroxyalkyl)acrylate (**126**), was achieved according to the method employed by Basavaiah,[147] where (-)-menthyl acrylate (**125**), was coupled to benzaldehyde (**114**, R= Ph), under the influence of DABCO (Scheme 2.41).



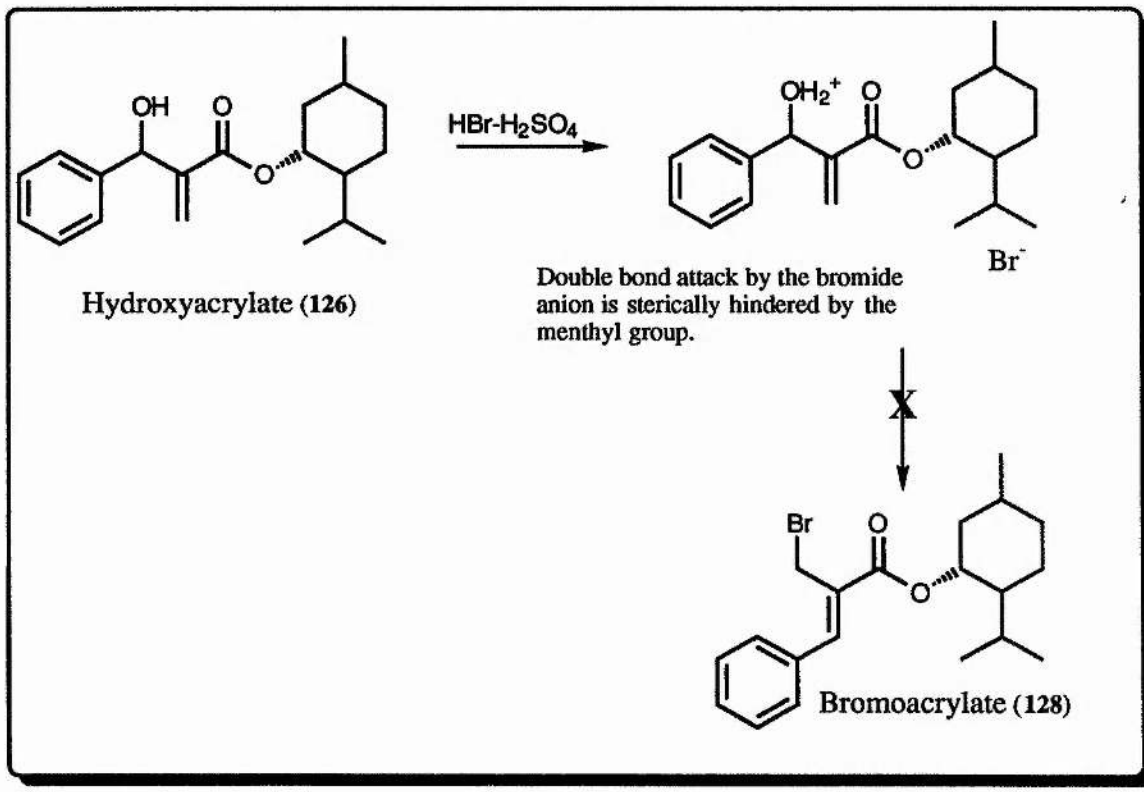
Scheme 2.41: Diastereoselective Synthesis of Hydroxyalkyl Acrylates

(-)-Menthyl acrylate (**125**), was easily prepared *via* the synthetic route developed by Meth-Cohn.[148] The reaction involves the efficient trans-esterification of methyl acrylate (**92**), with the chiral alcohol ((-)-menthol, **127**), in the presence of butyl-lithium in THF solution (Scheme 2.42).



Scheme 2.42: Transesterification of Methyl Acrylate

In an attempt to synthesise the bromoacrylate (**128**, Scheme 2.43), the hydroxy-acrylate (**126**), was treated with hydrobromic/sulphuric acid and the reaction was monitored by t.l.c. Since there was no observed change in starting materials and having changed the reaction conditions, such as reaction times and temperatures, we concluded that the bromide anion was sterically hindered from attacking the double bond in compound (**126**). We suggest that the steric hindrance is caused by the menthyl group (**126**, Scheme 2.43).

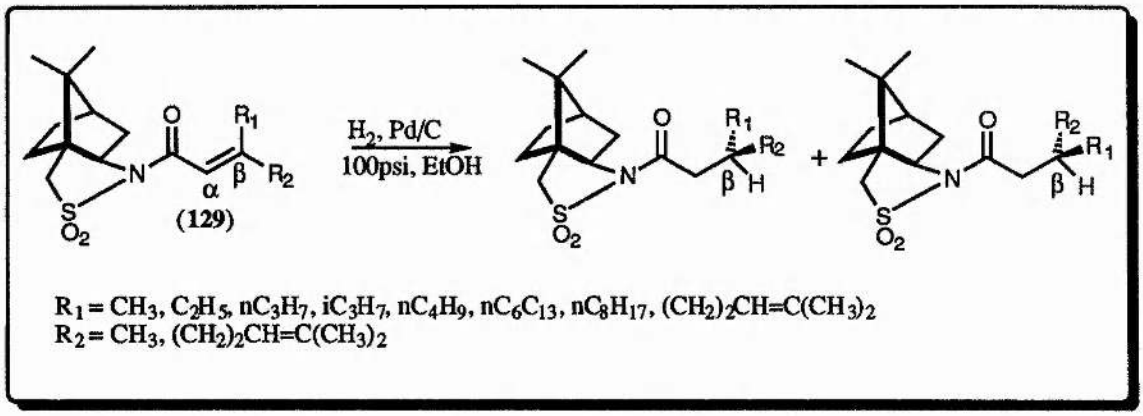


***Scheme 2.43**: Sterically Hindered Hydroxyalkylacrylate*

We have chosen to abandon the above approach (Scheme 2.43), as the optically active bromoacrylate (**128**), was not obtained *via* the synthetic approach as described in scheme 2.43.

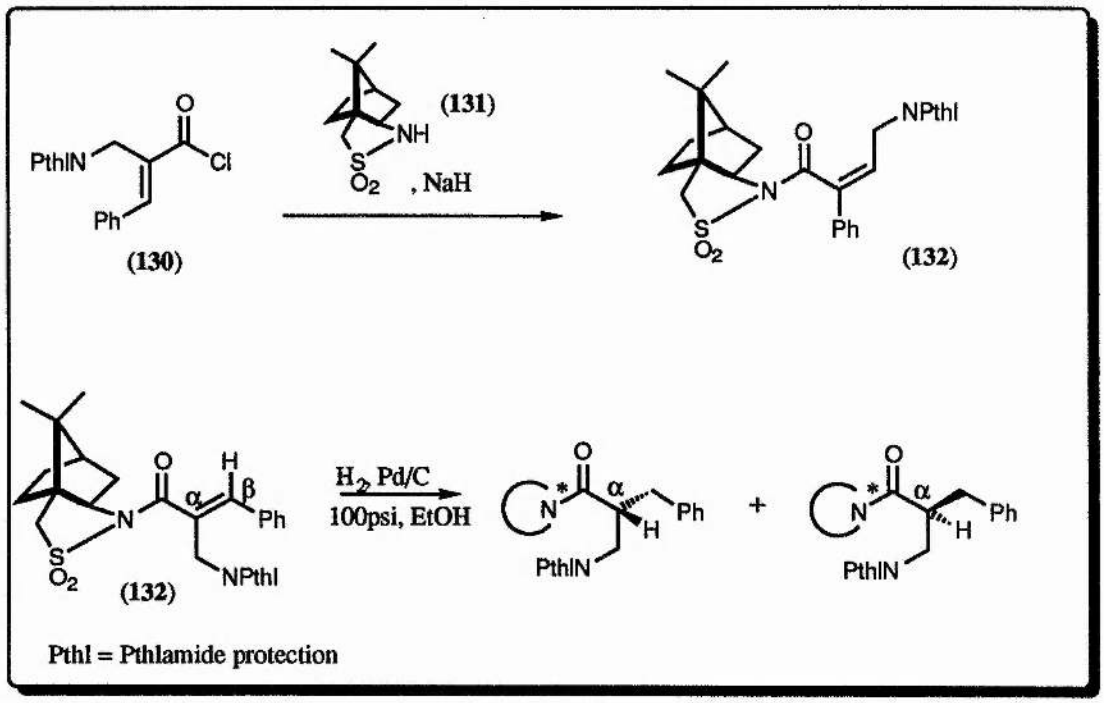
2.15 Conclusions and Future Work

The stereoface-selective addition of hydrogen to various olefinic bonds remains a practical and intellectual problem, despite the achievements of enantioselective hydrogenations of prochiral α-acylaminoacrylic acids.[90] Our approach to obtain stereoface-selective hydrogenation of the prochiral β-aminoacrylate (**120**), is to extend the work of Oppolzer.[149] Oppolzer and co-workers have obtained excellent stereoface differentiation on palladium catalysed hydrogenations of α,β-olefinic imides (**129**, Scheme 2.44), to provide, after saponification, β-substituted carboxylic acids in high enantiomeric purity (>95% enantiomeric excess).[149]



Scheme 2.45: Asymmetric Hydrogenations of Camphor Sultam-Imide-Conjugated Alkenes

We are currently following this approach, by synthesising the analogous chiral camphor sultam-imide conjugated alkene (**132**), followed by catalytic hydrogenation of the olefin (**132**, Scheme 2.45).



Scheme 2.45: Synthetic Approach Towards Enantiomerically Pure β-Amino Acids

The chiral imide **(132)**, can be synthesised by treating the camphor sultam **(131)**, with sodium hydride and the corresponding acid chloride **(130)**, to give the imide **(132)**.[149]

It is anticipated that asymmetry will be induced at the α-centre (Fig. 2.17, B) as opposed to the β-centre, as is the case for the trisubstituted olefinic compound (Fig. 2.17, A).

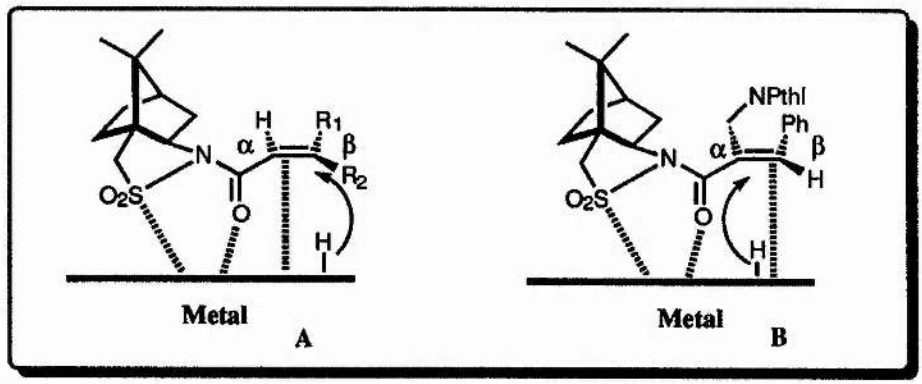


Figure 2.17: Diastereoface Differentiation on Pd/C Catalyst

CHAPTER 3

EXPERIMENTAL

3.0 Experimental

Synthesis of Substrates and Inhibitors

Elemental microanalyses were performed in the departmental micro-analytical laboratory. NMR spectra were recorded on a Bruker AM-300 (300 MHz; FT ^{1}H-NMR, and 75 MHz; ^{13}C-NMR), or a Varian gemini 200 (200 MHz; FT ^{1}H-NMR and 50.31 MHz; ^{13}C-NMR) spectrometers. ^{1}H-NMR spectra are described in parts per million downfield shift from TMS 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, sep - septet, m - multiplet, and br - broad), coupling constant (Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). ^{1}H-NMR were referenced internally on $^{2}H_2O$ (4.68 p.p.m.), $CHCl_3$ (7.27 p.p.m.) or DMSO (2.47 p.p.m.). ^{13}C-NMR were referenced on CH_3OH (49.9 p.p.m.), $C^{2}HCl_3$ (77.5 p.p.m.), or DMSO (39.70 p.p.m.).

I.R. spectra were recorded on a Perkin-Elmer 1710 FT IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies (υ) at 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, a Kratos MS-50 or by the SERC service at Swansea using a VG AZB-E. Fast atom bombardment spectra were recorded using glycerol as a matrix. Major fragments were given as percentages of the base peak intensity (100%). UV spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectrophotometers.

Flash chromatography was performed according to the method of Still *et al.*[102] using Sorbsil C 60 (40-60 μm mesh) silica gel. Analytical thin layer chromatography 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, or ninhydrin.

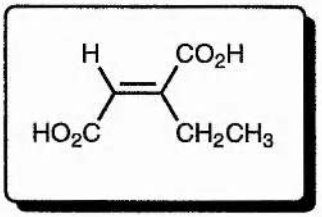
Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 23 °C on a Optical Activity AA-1000 polarimeter using 10 or 20 cm path length cells.

The solvents used were either distilled or of Analar quality. Light petroleum ether refers to that portion boiling between 40 and 60 °C. Solvents were dried according to literature procedures. Ethanol and methanol were dried using magnesium turnings. Isopropanol, isopentanol, DMF, toluene, CH_2Cl_2, acetonitrile, diisopropylamine, triethylamine, and pyridine were distilled over CaH_2. THF and diethyl ether were dried over sodium/benzophenone and distilled under nitrogen. Thionyl chloride was distilled from sulphur, and the initials fractions were always discarded. BuLi was titrated according to the method of Lipton.[150]

Diazomethane:

Potassium hydroxide (10 g) was dissolved in isopropanol (40 cm^3) with heating using the minimum amount of water (2-3 cm^3). Diethyl ether (40 cm^3) was added to the solution and the temperature was raised to 50 °C. Diazald (42 g), dissolved in ether (250 cm^3), was introduced into the solution over 45 min. The yellow distillate was collected in a flask immersed in an ice bath and the whole system was fitted with a drying tube. An additional amount of ether (100 cm^3) was added until the ether distilling over was colourless. The ethereal solution in the receiver (~400 cm^3) contained around 8 g of diazomethane.

3-Ethylfumaric Acid (30, R= CH_2CH_3)

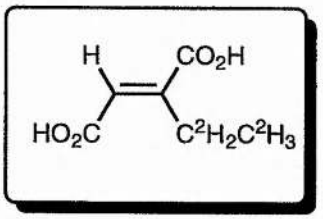


To a solution of sodium ethoxide, prepared by the addition of anhydrous ethanol (29 cm^3) to sodium (1.84 g, 80 mmol) was added ethyl acetoacetate (6 g, 53 mmol) dropwise over 10 min. The solution was stirred for an additional 5 min and ethyl iodide (10.8 g, 69 mmol) was slowly added dropwise. Following the addition, the reaction was heated at reflux for 2 h and was then allowed to cool. The solution was poured into water (40 cm^3) and then extracted with diethyl ether (3 x 40 cm^3). The pooled organic extracts were washed with water (3 x 20 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow oil, 2-ethyl acetoactetate ethyl ester (**28**, R= CH_2CH_3) (8.38 g, 65.5%). The product was used in the following reaction without further purification.

To a vigorously stirred solution of the ester (4 g, 25 mmol) in dry diethyl ether (30 cm^3) was slowly added bromine (8 g, 50 mmol) and the solution was then refluxed for 3 h. The solvent and hydrogen bromide, were removed under reduced pressure, to give the dibromide as a pale yellow oil (**29**, R= CH_2CH_3).

The dibromide (**29**, R= CH_2CH_3) was added slowly to a solution of ethanol (32 cm^3) containing powdered potassium hydroxide (9.6 g, 17.12 mmol) with rapid stirring. The mixture was heated at reflux for 30 min and was then steam distilled until 170 cm^3 of distillate had been collected. The acidified solution was extracted with diethyl ether (4 x 65 cm^3) and the pooled extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give an amorphous off-white solid. The product was decolourised with active charcoal and recrystallised from diethyl ether/hexane to give 3-ethylfumaric acid (**30**, R= CH_2CH_3) as white crystals (1.65 g, 45% overall yield), m.p. 195-197 °C (lit.,[49] 194-95 °C); υ_{max} (Nujol)/cm^{-1} 1688; δ_H (200 MHz; $^2H_2O/NaO^2H$) 0.9 (3H, t, *J* 7, CH_3), 2.35 (2H, q, *J* 7, CH_2) and 6.23 (1H, s, C=CH); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 13.81 (CH_3), 22.93 (CH_2), 128.05 (3-C), 147.52 (2-C) and 175.30 & 174.20 (2 x CO_2); *m/z* (EI) 126 (48%, $[M - H_2O]^+$) and 98 (100, $[M - CH_2O_2]^+$).

[$^2H_5$]-Ethylfumaric Acid (30, R= $C^2H_2C^2H_3$)



Ethyl acetoacetate (0.62 g, 4.78 mmol) was added dropwise to a stirred solution of sodium ethoxide in anhydrous ethanol (1.9 cm^3, 2.74 mol dm^{-3}, 5.2 mmol). After 15 min of further stirring, [$^2H_5$]-iodoethane (1.0 g, 6.2 mmol) was added dropwise. The reaction mixture was heated at reflux for 2 h and allowed to cool. The solution was poured into water (15 cm^3), extracted with diethyl ether (5 x 10 cm^3) and dried ($MgSO_4$). Solvent was removed under reduced pressure to

give [$^2H_5$]-2-ethyl acetoacetate ethyl ester (**28**, R= $C^2H_2C^2H_3$), as a yellow oil (0.63 g, 81%); δ_H (200 MHz; C^2HCl_3) 1.27 (3H, t, *J* 7, $CH_2C\underline{H}_3$), 2.22 (3H, s, CH_3), 3.31 (1H, s, CH) and 4.18 (2H, q, *J* 7, $C\underline{H}_2CH_3$); δ_C (75 MHz; C^2HCl_3) 13.96 ($CH_2\underline{C}H_3$), 21.1 (C^2H_3), 26.55 ($\underline{C}^2H_2C^2H_3$), 28.63 ($\underline{C}H_3CO$), 60.88 ($\underline{C}H_2CH_3$), 61.09 (CH), 169.71 ($\underline{C}O_2CH_2CH_3$) and 203.21 ($CH_3\underline{C}O$).

The crude ester was converted into [$^2H_5$]-ethylfumaric acid (**30**, R= $C^2H_2C^2H_3$), using the same procedure outlined for unlabelled ethylfumaric acid (**30**, R= CH_2CH_3). The crude product was recrystallised from diethyl ether/hexane to give [$^2H_5$]-ethylfumaric acid (**30**, R= $C^2H_2C^2H_3$) as an off white solid (0.21 g, 50%), m.p. 195-197 °C; (Found: $[M + H]^+$, 150.0819. $C_6H_4{}^2H_5O_4$ requires 150.0815); υ_{max} (Nujol)/cm^{-1} 2400-3500 and 1740; δ_H (200 MHz; $^2H_2O/NaO^2H$) 6.70 (1H, s, CH); δ_C (75 MHz; $^2H_2O/NaO^2H$) 12.36 (m, C^2H_3), 21.15 (m, C^2H_2), 128.50 (C-3), 151.0 (C-2) and 173.10 & 174.23 (2 x CO_2); *m/z* (CI) 150 (35%, $[M + H]^+$), 134 (20, $[M - OH]^+$), 106 (100, $[M + H - CO_2]^+$) and 57 (46, $[C_2HO_2]^+$).

[$^2H_3$]-Iodoethane (37)

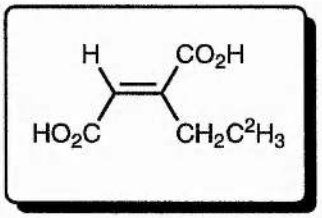
To a stirred suspension of $LiAlH_4$ (2.97 g, 78 mmol) and dry diethyl ether (30 cm^3) was added dropwise, dry labelled acetic acid ($C^2H_3CO_2H$) (5.0 g, 78 mmol) over 1 h and at 0 °C. The mixture was heated at reflux for 1 h and then hydrolysed with 2-butoxyethanol (31 cm^3, 234 mmol) whilst maintaining the temperature between 0 - 10 °C during the addition. The desired alcohol (**33**) was isolated by distillation (77-79 °C) (3.8 g, 100%) and was used in the next stage of the synthesis without further purification.

Triphenylphosphite (**34**) (31 g, 100 mmol) and methyl iodide (21 g, 148 mmol) were heated under reflux with exclusion of moisture for 36 h. The temperature of the refluxing mixture rose from 70 °C to 115 °C during the course of the reaction. Treatment of the product with anhydrous diethyl ether gave small yellowish-brown needles of the methiodide (**36**) which were repeatedly washed

with anhydrous ether and finally dried and weighed under reduced pressure (42 g, 94%), m.p. 145-146 °C (lit.,[100] 146 °C).

Triphenylphosphite methiodide (**36**) (22.14 g, 49 mmol) was dissolved in ethyl-2,2,2-$^2H_3$ alcohol (**33**) (2.0 g, 41 mmol) and left to stir at room temperature for 3 h. The iodide (**37**) was isolated by distilling the reaction mixture. The distillation was carried out under reduced pressure and collected in a trap at -80 °C. The condensate was collected and stored in the absence of light (4.65 g, 72%), b.p. 20 °C (0.5 mmHg). The labelled iodide was used in the preparation of the labelled fumarate (**30**, R= $CH_2C^2H_3$) without further purification.

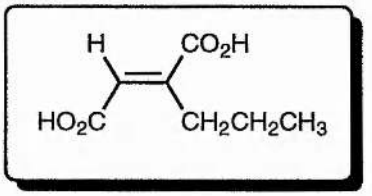
[$C^2H_3CH_2$]-Ethylfumaric Acid (30, R= $CH_2C^2H_3$)



Ethyl acetoacetate (2.56 g, 20 mmol), was added dropwise to a stirred solution of sodium ethoxide in anhydrous ethanol (8.0 cm^3, 2.75 mol dm^{-3}, 22 mmol). After 15 min of further stirring [$C^2H_3CH_2$]-iodoethane (**37**) (3.44 g, 22 mmol) was added dropwise. The reaction mixture was then refluxed for 2 h and allowed to cool. The solution was poured into water (50 cm^3) and extracted with diethyl ether (5 x 25 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give [$CH_2C^2H_3$]-2-ethyl acetoacetate ethyl ester (**28**, R= $CH_2C^2H_3$) as a yellow oil. The crude ester (**28**, R= $CH_2C^2H_3$) was purified by column chromatography (10% ethyl acetate/ 90% hexane) to yield the pure product as a colourless oil (2.32 g, 72%), δ_H (200 MHz; C^2HCl_3) 1.28 (3H, t, *J* 7.2, $CH_2C\underline{H}_3$), 1.85 (2H, d, *J* 7.4, $C\underline{H}_2C^2H_3$), 2.21 (1H, s, CH_3CO), 3.34 (1H, t, *J* 7.4, CH) and 4.22 (2H, q, *J* 7.1, $C\underline{H}_2CH_3$); δ_C (75 MHz; C^2HCl_3) 11.73 (C^2H_3), 13.96 ($CH_2\underline{C}H_3$), 21.22 ($\underline{C}H_2C^2H_3$), 28.59 ($\underline{C}H_3CO$), 61.08 ($\underline{C}H_2CH_3$), 61.2 (CH), 169.7 ($\underline{C}O_2CH_2CH_3$) and 203.13 ($CH_3\underline{C}O$).

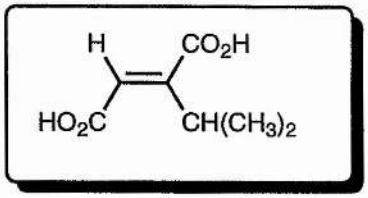
The ester (**28**, R= $CH_2C^2H_3$), was converted into [$C^2H_3CH_2$]-ethylfumaric acid using the same procedure outlined for unlabelled ethylfumaric acid (**30**, R= CH_2CH_3). The crude product was recrystallised from diethyl ether/hexane to give [$^2H_3$]-ethylfumaric acid as an off white solid (0.55 g, 50%), m.p. 190-192 °C; (Found: $[M + H]^+$, 148.0686. Calc. for $C_6H_6{}^2H_3O_4$: *m/z*, 148.0689); υ_{max} (Nujol)/cm^{-1} 1683; δ_H (300 MHz; $^2H_2O/NaO^2H$) 1.20 (3H, m, $CH_2C^2\underline{H}_3$), 2.35 (2H, s, $C\underline{H}_2C^2H_3$) and 6.28 (1H, s, CH); δ_C (75 MHz; $^2H_2O/NaO^2H$) 11.31 (C^2H_3), 19.98 ($CH_2C^2H_3$), 125.41 (CH), 143.36 ($\underline{C}$=CH) and 174.76 & 175.65 (2 x CO_2H); *m/z* (CI) 148 (48%, $[M + H]^+$), 130 (24, $[M + H - H_2O]^+$), 104 (100, $[M + H - CO_2]^+$) and 90 (26, $[C_2H_2O_2]^+$).

3-^{n}Propylfumaric Acid (30, R= $CH_2CH_2CH_3$)



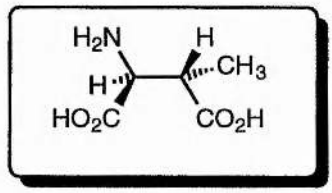
3-^{n}Propylfumaric acid (**30**, R= $CH_2CH_2CH_3$), was prepared from ethyl acetoacetate and ^{n}propyl bromide (1.5 eq), using the procedure described for 3-ethylfumaric acid (**30**, R= CH_2CH_3), to give an overall yield of 45% for the fumaric acid (**30**, R = $CH_2CH_2CH_3$), m.p. 172-173 °C (lit.,[49] 175 °C); υ_{max} (Nujol)/cm^{-1} 1700; δ_H (200 MHz; $^2H_2O/NaO^2H$) 0.75 (3H, t, *J* 7.5, CH_3), 1.28 (2H, m, *J* 7.5, $C\underline{H}_2CH_3$), 2.32 (2H, t, *J* 7.5 -C=C-$C\underline{H}_2$), and 6.28 (1H, s, C=CH); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 13.8 (CH_3), 22.0 (CH_2), 30.3 (CH_2), 128.4 (3-C), 143.4 (2-C) and 174.4 & 176.0 (2 x CO_2H).

3-^{i}Propylfumaric Acid (30, R= $CH(CH_3)_2$)



3-^{i}Propyl fumaric acid (**30**, R= $CH(CH_3)_2$) was prepared from ethyl acetoacetate and ^{i}propyl bromide, using the procedure described for 3-ethylfumaric acid (**30**, R= CH_2CH_3), in an overall 48% yield, m.p. 180-183 °C (lit.,[49] 183-184 °C); υ_{max} (Nujol)/cm^{-1} 1700; δ_H (200 MHz; $^2H_2O/NaO^2H$) 1.05 (6H, d, *J* 7, -$CH(C\underline{H}_3)_2$), 3.05 (1H, m, $C\underline{H}(CH_3)_2$) and 5.8 (1H, s, C=CH); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 13.8 (CH_3), 22.8 (CH_2), 128.0 (3-C) 148.0 (2-C) and 174.4 & 175.4 (2 x CO_2H).

(2*S*,3*S*)-3-Methylaspartic Acid (31, R= CH_3)

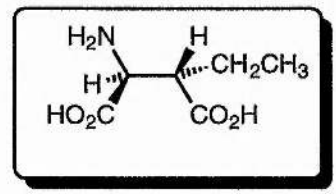


Diammonium mesaconate [mesaconic acid (13 g, 0.1 mol) and NH_3 to pH 7.0], potassium chloride (2.63 g, 4.5 mmol), magnesium chloride hexahydrate (2.13 g, 10.5 mmol), ammonium chloride (10.7 g, 200 mmol) and tris-buffer (6 g, 50 mmol) were dissolved in water (150 cm^3) one after the other. The pH of the solution was adjusted to 9.0 using KOH pellets. 3-Methylaspartate ammonia lyase (40 cm^3, 680 units/cm^3) was added to the reaction mixture and incubated at 30 °C until no further decrease in absorbance at 240 nm was observed (*ca.* 40 h).

The protein was denatured at 100 °C for 2 min and was removed by filtration on a Celite pad. The filtrate was evaporated to dryness and was crystallised at pH 3 from 2:1 water:ethanol (80 cm^3). The product was recrystallised from hot water/ethanol, to give pure product (10.1 g, 67%), m.p. 272-276 °C (lit.,[49] 276-278 °C); (Found: C, 40.70; H, 6.25; N, 9.50. Calc. for $C_5H_9NO_4$: C, 40.80; H, 6.15; N,

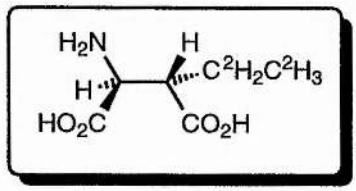
9.50%); *m/z* (Found: $[M + H]^+$, 148.0610. Calc. for $C_5H_{10}NO_4$: *m/z* 148.0607); $[\alpha]_D^{20}$ -10.1 (*c* 0.6 in H_2O), {lit.,[49] $[\alpha]_D^{20}$ -10 (*c* 0.42 in H_2O)}; υ_{max} (Nujol)/cm^{-1} 1680; δ_H (200 MHz; ^2H_2O, pH 9) 1.12 (3H, d, *J* 7.5, 3-CH_3), 3.10 (1H, m, (AB splitting), 3-H) and 4.10 (1H, d, *J* 3.0, 2-H); δ_C (75 MHz; ^2H_2O, pH 9) 12.15 (CH_3), 40.55 (CH), 55.86 (CH) and 175.70 & 171.00 (2 x CO_2); *m/z* (EI) 128 (12%, $[M - H_3O]^+$) and 102 (100, $[M - CO_2H]^+$).

(2*S*,3*S*)-3-Ethylaspartic Acid (31, R= CH_3CH_2)



(2*S*,3*S*)-3-Ethylaspartic acid (**31**, R= CH_3CH_2) was synthesised as described above for (2*S*,3*S*)-3-methylaspartic acid (**2**), using 3-ethylfumaric acid (**30**, R= CH_3CH_2) (0.5 g, 3.8 mmol) as the starting material. The product was obtained as white crystals (0.33 g, 59%), m.p. 244-245 °C (lit.,[49] 245-246 °C); $[\alpha]_D^{20}$ +14.8 (*c* 0.6 in 6 mol dm^{-3} HCl) {lit.,[49] $[\alpha]_D^{20}$ +15.0 (*c* 0.6 in 6 mol dm^{-3} HCl)}; δ_H (200 MHz; $^2H_2O/NaO^2H$) 1.00 (3H, t, *J* 8.3, $CH_2\underline{CH_3}$), 1.3 (2H, m, $\underline{CH_2}CH_3$), 2.55 (1H, m, 3-H) and 3.75 (1H, d, *J* 4.2, 2-H); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 11.5 (CH_3), 20.5 (CH_2), 46.1 (2-C), 55.3 (3-C) and 173.9 & 171.0 (2 x CO_2H).

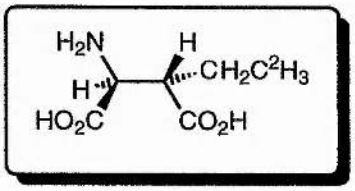
(2*S*,3*S*) -[$C^2H_2C^2H_3$]-3-Ethylaspartic Acid (31, R= $C^2H_2C^2H_3$)



[$C^2H_2C^2H_3$]-Ethylfumaric acid (**31**, R= $C^2H_2C^2H_3$) (0.23 g, 15.4 mmol) was suspended in water (10 cm^3) and then the pH adjusted to 9 (concentrated ammonia). This was then evaporated to dryness, to give the diammonium salt. The salt was re-dissolved in water (10 cm^3) and potassium chloride (7.5 mg, 0.1 mmol) and magnesium chloride hexahydrate (0.012 g, 0.2 mmol) was added. The pH was readjusted to 9, and after the addition of methylaspartase (25 cm^3/680 units per cm^3), the mixture was incubated at 30 °C until no further decrease in absorbance at 240 nm was observed.

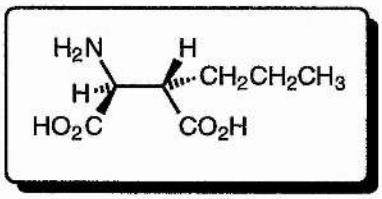
The protein was denatured at 100 °C for 2 min and was removed by filtration on a Celite pad. The filtrate was evaporated to dryness and was crystallised at pH 3 (the pH was adjusted to 3.0 by the addition of 12 mol dm^{-3} HCl). The product was recrystallised from hot water/ethanol to give pure product (0.15 g, 59%), m.p. 256-258 °C; *m/z* (Found: $[M + H]^+$, 167.1078. $C_6H_7{}^2H_5NO_4$ requires 167.1080); $[\alpha]_D^{22}$ +10.9 (*c* 1.0 in 0.5 mol dm^{-3} HCl); υ_{max} (Nujol)/cm^{-1} 2400-3500 and 1630; δ_H (200 MHz; $^2H_2O/NaO^2H$) 2.71 (1H, d, *J* 4, 3-CH) and 3.90 (1H, d, *J* 4, 2-CH); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 11.81 (3H, m, C^2H_3), 20.05 (2H, m, C^2H_2), 48.33 (C-3), 56.12 (C-2), and 173.40 & 179.97 (2 x CO_2); *m/z* (CI) 167 (59%, $[M + H]^+$), 149 (100, $[M + H - H_2O]^+$), 121 (96, $[M + H - CH_2O_2]^+$) and 77 (87, $[C_2H_7NO_2]^+$).

(2*S*,3*S*)-[$^2H_3$]-3-Ethylaspartic Acid (31, R= $CH_2C^2H_3$)



(2*S*,3*S*)-[$C^2H_3CH_2$]-3-Ethylaspartic acid (**31**, R= $CH_2C^2H_3$) was synthesised as described above for (2*S*,3*S*)-[$C^2H_3C^2H_2$]-3-ethylaspartic acid (**31**, R= $C^2H_2C^2H_3$) using 3-[$^2H_3$]-ethylfumaric acid (**30**, R= $CH_2C^2H_3$) (0.4 g, 27.2 mmol) as the starting material. The product was obtained as white crystals (0.25 g, 55%), m.p. 255-256 °C; (Found: $[M + H]^+$, 165.0948. $C_6H_9{}^2H_3NO_4$: requires 165.0955); $[\alpha]_D^{22}$ +10.6 (*c* 1.0 in 0.5 mol dm^{-3} HCl); υ_{max} (Nujol/cm^{-1}) 1733; δ_H (200 MHz; $^2H_2O/NaO^2H$) 1.65 (2H, m, $C\underline{H}_2C^2H_3$), 2.86 (1H, m, 3-CH) and 4.05 (1H, d, *J* 5, 2-CH); δ_C (75 MHz; $^2H_2O/NaO^2H$) 11.50 (m, C^2H_3), 21.80 (CH_2), 50.10 (C-3), 58.4 (C-2) and 175.30 & 181.10 (2 x CO_2H); *m/z* (CI) 165 (100%, $[M + H]^+$), 147 (20, $[M + H - H_2O]^+$) and 119 (27, $[M + H - CO_2]^+$).

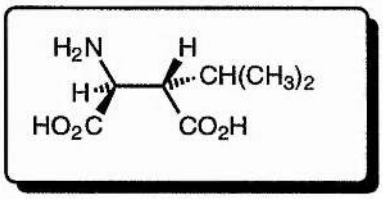
(2*S*,3*S*)-3-^{n}Propylaspartic Acid (31, R= $CH_3CH_2CH_2$)



(2*S*,3*S*)-3-n-Propylaspartic acid (**31**, R= $CH_3CH_2CH_2$) was prepared as described above for (2*S*,3*S*)-3-methylaspartic acid (**2**), using 3-^{n}propylfumaric acid (**30**, R= $CH_3CH_2CH_2$) (3.0 g, 19 mmol), as the starting material. Since this compound is a poor substrate an extended incubation time was required. Time-dependent enzyme activity loss therefore became significant, so fresh aliquots of enzyme were added at 48 h intervals. When no further decrease in absorbance was observed (*ca.* 2 weeks) the incubation mixture was heated to denature the enzyme. The solution was subjected to cation exchange chromatography on

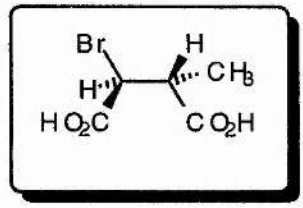
Amberlite IR-118(H), eluting with water and then 1.0 mol dm^{-3} aqueous ammonia. Fractions containing the amino acid were pooled and concentrated under reduced pressure. The residue was recrystallised from water/ethanol (1.8 g, 55%), m.p. 235-236 °C (lit.,[49] 235-237 °C); $[\alpha]_D^{22}$ +7.4 (*c* 0.5 in 6 mol dm^{-3} HCl), {lit.,[49] $[\alpha]_D^{22}$ +7.6 (*c* 0.5 in 6 mol dm^{-3} HCl)}; δ_H (200 MHz; $^2H_2O/NaO^2H$) 0.85 (3H, t, *J* 7.2, 3′ CH_3), 1.35 (2H, m, $C\underline{H}_2CH_3$), 1.6 (1H, m, 3′-$C\underline{H}CH_2CH_3$), 2.95 (1H, m, 3-CH) and 3.95 (1H, d, *J* 4.40, 2-CH); δ_C (50.31 MHz; ^2H_2O) 14.03 (CH_3), 21.29 (CH_2), 30.06 (CH_2), 46.26 (3-C), 56.201 (2-C), and 173.16 & 178.5 (2 x CO_2H).

(2*S*,3*S*)-3-^{i}Propylaspartic Acid (31, R= $CH(CH_3)_2$)



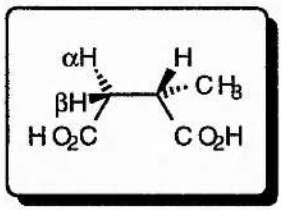
(2*S*,3*S*)-3-^{i}Propylaspartic acid (**31**, R= $CH(CH_3)_2$), was prepared as described above for (2*S*,3*S*)-3-^{n}propylaspartic acid (**31**, R= $CH_3CH_2CH_2$) using 3-^{i}propylfumaric acid (**30**, R= $CH(CH_3)_2$) (3.0 g, 19 mmol), as the starting material. The product was obtained as white crystals after recrystallisation from hot ethanol (1.5 g, 45%), m.p. 240-242 °C (lit.,[49] 239-242 °C); $[\alpha]_D^{20}$ +7.3 (*c* 0.4, in 6 mol dm^{-3} HCl), {lit.,[49] $[\alpha]_D^{20}$ +7.35 (*c* 0.4, 6 mol dm^{-3} HCl)}; δ_H (200 MHz; ^2H_2O) 0.9 (6H, dd, *J* 6.34 and 22.46, 3′-CH($C\underline{H}_3$)$_2$), 1.85 (1H, m, 3′-$C\underline{H}(CH_3)_2$), 2.6 (1H, dd, *J* 5.13 and 8.06, 3-CH), and 3.9 (1H, d, *J* 5.13, 2-CH); δ_C (50.31 MHz; ^2H_2O) 20.74 (CH_3), 21.43 (CH_3), 27.50 (CH_3), 53.56 (3′-CH), 55.50 (2-C) and 173.72 & 178.43 (2 x CO_2H).

(2*S*,3*S*)-2-Bromo-3-methyl Succinic Acid (61)



To a saturated solution of potassium bromide, was added 3-methylaspartic acid (**2**) (2.0 g, 13.6 mmol). The mixture was cooled to 0 °C, and a solution of hydrobromic acid (48%, 6.8 cm^3) was added dropwise, followed by sodium nitrite (1.9 g, 27.2 mmol) over 20 min. The reaction mixture was left to stir for 30 min at 0 °C and then left to stir for a further 2 h at room temperature. The reaction mixture was extracted with diethyl ether (3 x 20 cm^3) and the organic layers were combined, dried ($MgSO_4$) and concentrated under reduced pressure to yield a white solid, which was recrystallised using diethyl ether/light petroleum to yield (**61**) (2.35 g, 82%), m.p. 142-144 °C (lit.,[153] 142-144 °C); $[\alpha]_D^{23}$ -40.1 (*c* 0.6 in H_2O) {lit.,[153] $[\alpha]_D^{23}$ -40.42 (*c* 0.6 in H_2O)}; δ_H (200 MHz; ^2H_2O) 1.27 (3H, d, *J* 7, CH_3), 3.05 (1H, m, 3-H) and 4.51 (1H, d, *J* 8.5, 2-H); δ_C (50.31 MHz; ^2H_2O) 17.11 (CH_3), 46.18 (C-3), 50.59 (C-2) and 175.64 & 179.81 (2 x CO_2H).

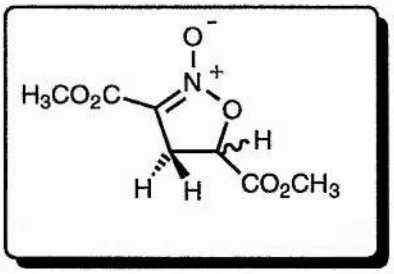
(2*S*)-Methyl Succinic Acid (65)



To a stirred solution of (2*S*,3*S*)-2-bromo-3-methyl succinic acid (**61**) (100 mg, 2.82 mmol) in aqueous methanol (1:1) (10 cm^3), was added palladium on activated carbon (10 mg). The mixture was then hydrogenated under an atmosphere of hydrogen, and the reaction mixture allowed to stir at room temperature for 6 h. The solvent was removed under reduced pressure to give a white solid which was recrystallised using ether/ light petroleum to yield (**65**) as

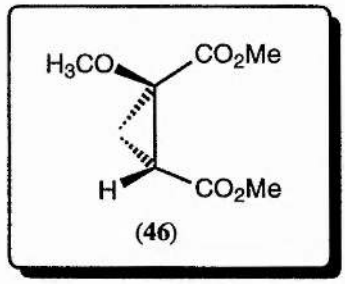
a white crystalline solid (51 mg, 81%), m.p. 113-114 °C (lit.,[109] 114-114.5 °C); $[\alpha]_D$ -13.7 (*c* 1.15 in Ethanol) {lit.,[109] $[\alpha]_D$ -13.5 (*c* 1.15 in Ethanol)}; υ_{max} (Nujol)/cm^{-1} 1699; δ_H (300 MHz; ^2H_2O, NaO^2H) 1.05 (3H, d, *J* 4, CH_3), 2.10 (1H, dd, *J* 4.2, H_α), 2.50 (1H, dd, *J* 4.2, H_β) and 2.65 (1H, m, 3-H); δ_C (75 MHz; ^2H_2O, NaO^2H) 15.30 (CH_3), 38.29 (C-3), 40.56 (C-2) and 180.03 & 183.78 (2 x CO_2H); *m/z* (CI) 114 (64%, $[C_5H_6O_3]^+$), 86 (88, $[C_2H_6O_2]^+$), 73 (100, $[C_3H_5O_2]^+$), 60 (24, $[C_2H_4O_2]^+$) and 45 (81, $[CO_2H]^+$).

(±)-3,5-*bis*-[Carbomethoxy] Isoxazoline-*N*-oxide (57)

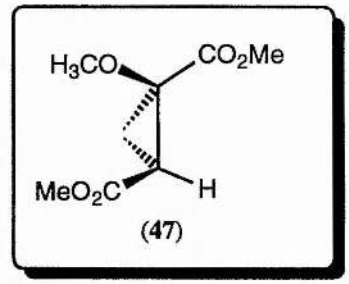


To a stirred solution of methyl nitroacetate (0.2 g, 1.7 mmol) in dichloromethane (15 cm^3) was added methyl α-bromoacrylate (**51**) (0.28 g, 1.7 mmol). Potassium carbonate (0.28 g, 2.0 mmol) was then added and the reaction mixture was left to stir for 48 h after which time the inorganic residue was filtered. The organic solution was evaporated under reduced pressure to give a yellow oil. The crude product was purified by flash chromatography on silica gel (50 % ethyl acetate in hexane) to yield a white crystalline solid (0.30 g, 90%), m.p. 64-66 °C; (Found C, 41.55; H, 4.45; N, 6.90. $C_7H_9NO_6$ requires C, 41.40; H, 4.35; N, 6.90%); *m/z* (Found: $[M - CO_2CH_3]^+$ 144.0291. $[M - CO_2CH_3]^+$ requires 144.0295); δ_H (200 MHz; C^2HCl_3), 3.70 (2H, m, CH_2), 3.88 (6H, s, 2 x OCH_3) and 5.15 (1H, dd, *J* 4, 8, CH); δ_C (50.31 MHz; C^2HCl_3) 34.70 (CH_2), 53.30 & 53.70 (2 x OCH_3), 71.60 (CH), 106.10 ($NO_2\underline{C}CH_2$), 159.30 (CH_2-$\underline{C}$=O) and 169.40 (CH-C=O); *m/z* (EI) 203 (3%, M^+), 172 (5, $[CH_3O]^+$), 144 (23, $[M - 2 \times CH_3OH]^+$), 59 (76, $[CO_2CH_3]^+$) and 43 (100, (CH_3OH)).

(±)-Dimethyl *cis/trans*-1-Methoxycyclopropane-1,2-dicarboxylate (46, 47)



(46)



(47)

Following the procedure reported by Kraus[104], to a solution of the bromo-diester (**42**) (1.0 g, 4.2 mmol) in dry liquid ammonia (30 cm^3) (liquid ammonia was dried by stirring with potassium amide) and dry THF (30 cm^3) at -78 °C was rapidly added potassium hexamethyl disilazide (KHMDS) (1.0 mol dm^{-3} in toluene, 4.2 mmol).

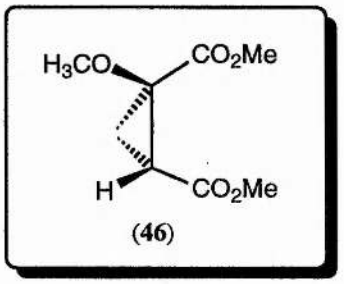
The resulting light yellow solution was stirred at -78 °C for 1 h. The reaction mixture was quenched with water (2.0 cm^3), extracted with dichloromethane (3 x 50 cm^3), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by flash chromatography (3:1 hexane:ethyl acetate) to afford pure dimethyl-*trans*-1-methoxycyclopropane-1,2-dioate (**47**) (0.1 g, 15%) and dimethyl-*cis*-1-methoxycyclopropane-1,2-dioate (**46**) (0.35 g, 48%). The overall yield of the reaction, varied from 55-63% over several runs.

Trans isomer (**47**); (Found: $[M+H]^+$, 189.0759. Calc. for $C_8H_{13}O_5$: *m/z*, 189.0763); υ_{max} (Neat)/cm^{-1} 3483, 3425, 1731 and 1680; δ_H (200 MHz; C^2HCl_3) 1.63 (1H, dd, *J* 5.4, 9.1, H_α), 1.91 (1H, dd, *J* 5.4, 7.5, H_β), 2.50 (1H, dd, *J* 7.5, 9.1 2-H), 3.41 (3H, s, OCH_3) and 3.73 & 3.80 (6H, 2 x s, 2 x CO_2CH_3); *m/z* (CI) 189 (100%, $[M + H]^+$), 157 (35, $[M + H - CH_3OH]^+$), 129 (30, $[M + H - C_2H_4O_2]^+$), 73 (85, $[C_2H_5O_2]^+$) and 59 (70, $[CO_2CH_3]^+$); R_f(hexane:ethyl acetate (3:1))= 0.44, (lit.,[104] R_f(hexane:ethyl acetate (3:1))= 0.44).

Cis isomer (**46**); (Found : $[M+H]^+$, 189.0754. Calc. for $C_8H_{13}O_5$: *m/z*, 189.0763); υ_{max} (Neat)/cm^{-1} 3481, 3423, 1730, 1683; δ_H (200 MHz; C^2HCl_3) 1.53 (1H, dd, *J* 5.8, 10.2, H_α), 1.88 (1H, dd, *J* 5.8, 8.1, H_β), 2.28 (1H, dd, *J* 8.1, 10.2, 2-H), 3.46 (3H, s, OCH_3) and 3.69 & 3.76 (6H, 2 x s, 2 x CO_2CH_3); δ_C (50.31 MHz; C^2HCl_3) 20.1 (C-3),

30.23 (C-2), 53.30, 53.40 (2 x $CO_2\underline{C}H_3$), 58.20 (OCH_3), 66.42 (C-1) and 168.33 & 170.10 (2 x $\underline{C}O_2CH_3$); *m/z* (CI) 189 (96%, $[M + H]^+$), 157 (100, $[M + H - CH_3OH]^+$) and 129 (29, $[M + H - C_2H_4O_2]^+$); R_f(hexane:ethyl acetate (3:1))= 0.42, (lit.,[104] R_f(hexane:ethyl acetate (3:1))= 0.42).

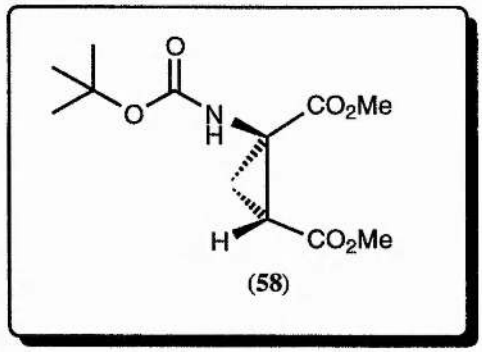
(±)-Dimethyl *cis*-1-Methoxycyclopropane-1,2-Dicarboxylate (46)



(46)

To a solution of the bromo-diester (**42**) (0.5 g, 2.1 mmol) in dry THF (30 cm^3) at -78 °C was added dry methanol (0.40 g, 12.6 mmol). To this mixture was rapidly added potassium hexamethyl disilazide (1.0 mol dm^{-3} in toluene, 2.52 mmol). The resulting pale yellow solution was stirred at -78 °C for 1 h and then allowed to warm up to room temperature. The reaction mixture was quenched with diethyl ether (20 cm^3). The ethereal solution was washed with water (3 x 10 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The desired compound was obtained as a pale yellow oil, (0.32 g, 80%), (Found: C, 50.75; H, 6.30. Calc. for $C_8H_{12}O_5$: C, 51.05; H, 6.45%); υ_{max} (Neat)/cm^{-1} 3480, 3423, 1731 and 1684; δ_H (200 MHz; C^2HCl_3) 1.53 (1H, dd, *J* 5.8, 10.2, H_α), 1.87 (1H, dd, *J* 5.8, 8.1, H_β), 2.27 (1H, dd, *J* 8.1, 10.2, 2-H), 3.46 (3H, s, OCH_3) and 3.70 & 3.76 (6H, 2 x s, 2 x CO_2CH_3); δ_C (50.31 MHz; C^2HCl_3) 19.61 (C-3), 29.66 (C-2), 52.2 and 52.57 (2 x $CO_2\underline{C}H_3$), 57.76 (OCH_3), 66.44 (C-1) and 168.27 & 171.21 (2 x $\underline{C}O_2CH_3$); R_f(hexane:ethyl acetate (3:1))= 0.42 (lit.,[104] R_f(hexane:ethyl acetate (3:1))= 0.42).

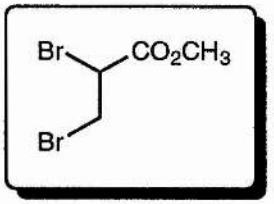
(±)-Dimethyl-*cis*-*N*-(tert-butoxycarbonyl)-2,3-methanoaspartate (58)



(58)

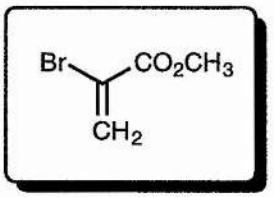
To a solution of the bromo-diester (**42**) (0.3 g, 1.27 mmol) in dry THF (15 cm^3) at -78 °C was added a dry THF solution containing ^{t}butylcarbamate (0.90 g, 7.62 mmol). To this mixture was rapidly added potassium hexamethyl disilazide (1.0 mol dm^{-3} in toluene, 1.65 mmol). The resulting pale yellow solution was stirred at -78°C for 1 h and then allowed to warm to room temperature. The reaction mixture was quenched with diethyl ether (10 cm^3). The ethereal solution was washed with water (3 x 5 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane:ethyl acetate (9:1)) to yield the desired compound as a white crystalline solid (0.23, 65%), m.p. 161-162 °C; *m/z* (Found: $[M + H]^+$, 274.1283. $C_{12}H_{20}NO_6$ requires 274.1291); υ_{max} (Nujol)/cm^{-1} 3284, 1722, 1653 and 1593; δ_H (200 MHz; C^2HCl_3) 1.46 (9H, s, -$C(CH_3)_3$), 1.7-1.9 (3H, m, 3-H_2, -NH), 2.74 (1H, t, *J* 6.5, 2-H) and 3.69 & 3.74 (6H, 2 x s, 2 x CO_2CH_3); δ_C (50.31 MHz; C^2HCl_3) 24.01 (C-3), 28.59 (-$C(\underline{C}H_3)_3$), 30.75 (C-2), 53.02, 53.26 (2 x $CO_2\underline{C}H_3$), 57.87 (C-1), 66. 47 (-$\underline{C}(CH_3)_3$), 156.91 ($\underline{C}$ONH) and 171.30 & 174.47 (2 x $\underline{C}O_2CH_3$); *m/z* 274 (23%, $[M + H]^+$), 218 (82, $[M + H - CO_2]^+$) and 174 (100, $C_7H_{14}NO_4]^+$).

Methyl 1,2-Dibromopropanoate (56a)



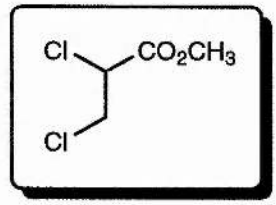
Bromine (5.57 g, 35 mmol) was added dropwise to a stirred solution of methyl acrylate (3.0 g, 35 mmol) in dichloromethane (50 cm^3). The reaction mixture was left to stir for 4 h and then the solution was concentrated under reduced pressure to yield the desired compound as a pale yellow oil. Compound (**56a**) (8.6 g, 100%), did not require purification and was used directly in the synthesis of (**51**), δ_H (200 MHz; C^2HCl_3) 3.68 (1H, m, BrC<u>H</u>-H), 3.88 (3H, s, CO_2CH_3), 3.98 (1H, m, BrCH-<u>H</u>) and 4.50 (1H, dd, BrC<u>H</u>).

Methyl α-bromoacrylate (51)



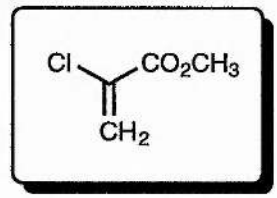
To a stirred solution of the dibromo- compound (**56a**) (4.0 g, 16.3 mmol) in dichloromethane (30 cm^3) was added a solution of triethylamine (2.50 g, 25.0 mmol) and the mixture stirred 6 h. The inorganic residue was filtered off and the filtrate was washed with acid (0.1 mol dm^{-3} HCl) (3 x 25 cm^3). The organic fraction was concentrated under reduced pressure to yield the desired compound as a colourless oil (2.50 g, 93%); *m/z* (Found: $[M + H]^+$, 164.9553. Calc. for $C_4H_6BrO_2$: *m/z*, 164.9551); δ_H (200 MHz; C^2HCl_3) 3.83 (3H, s, $CO_2C\underline{H}_3$), 6.28 & 6.90 (2H, 2 x s, C=C<u>H</u>$_2$); *m/z* (CI) 167 & 165 (100, 99%, $[M + H]^+$), 119 (16, $[CH_2O_2]^+$), 103 (26, $[C_2H_6O_2]^+$) and 87 (89, $[M + H - Br]^+$).

Methyl 1,2-Dichloropropanoate (56b)



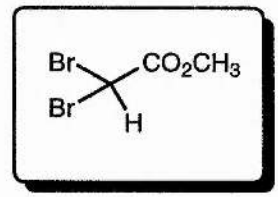
A stirred solution of methyl acrylate (5 g, 58.1 mmol) in dicloromethane (100 cm^3) was added a steady stream of chlorine gas at 0 °C for a period of 3-4 h (excess chlorine was passed into a sodium hydroxide scrubber). The reaction mixture was washed with a 10% solution of sodium carbonate, until the washings were no longer acidic. The organic phase was then washed with cold water (3 x 100 cm^3). The organic fraction was dried ($MgSO_4$), filtered and concentrated to give a yellow oil in quantitative yield, δ_H (200 MHz; C^2HCl_3) 3.7-4.0 (2H, m, CH_2Cl), 3.85 (3H, s, OCH_3) and 4.45 (1H, m, CHCl).

Methyl α-chloroacrylate (97, X= Cl)



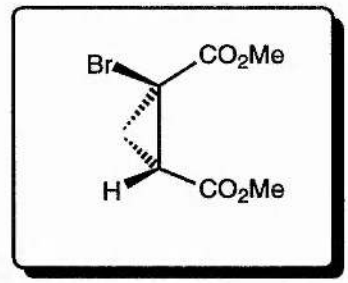
To a pre-cooled solution of (**56b**) (10 g, 64 mmol) in dichloromethane (100 cm^3) at 0 °C was added dropwise triethylamine (9.7 g, 96 mmol) and the reaction was stirred overnight at room temperature. The reaction mixture was filtered, and the filtrate was washed with 0.5 mol dm^{-3} HCl (2 x 50 cm^3), water (50 cm^3), saturated $NaHCO_3$ solution (2 x 50 cm^3) and finally water (150 cm^3) before being dried ($MgSO_4$). The solvent was removed under reduced pressure to give the desired compound as a pale yellow oil (7.7 g, 100%), δ_H (200 MHz; C^2HCl_3) 3.82 (3H, s, $CO_2C\underline{H}_3$), 6.0 & 6.51 (2H, 2 x s, $C{=}C\underline{H}_2$).

Methyl Dibromoacectate (91, X,Y= Br)



Dibromoacetic acid (10 g, 45 mmol) was dissolved in dry methanol (100 cm^3), and to this stirred solution at 0 °C was added concentrated H_2SO_4 (5 cm^3). The reaction mixture was left to stir overnight at room temperature. Saturated Na_2CO_3 solution (100 cm^3) was added and any excess methanol was removed under reduced pressure. The desired ester was extracted from the aqueous phase using diethyl ether (2 x 150 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and the solvent removed under reduced pressure to give methyl dibromoacetate (**91, X,Y= Br**) (9.5 g, 91%), b.p. 180-181 °C (lit.,[151] 182-183 °C); δ_H (200 MHz; C^2HCl_3) 4.85 (3H, s, OCH_3) and 5.82 (1H, s, $CHBr_2$); δ_C (75 MHz; C^2HCl_3) 31.70 (CH_3), 54.10 ($CHBr_2$) and 165.21 (CO_2).

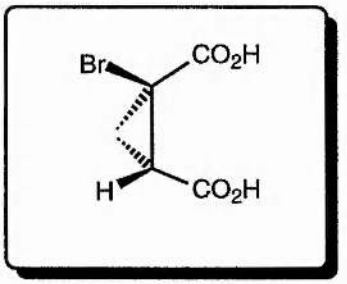
(±)-Dimethyl *cis*-1-Bromocyclopropane 1,2-dicarboxylate (93, X= Br)



A mixture of methyl dibromoacetate (**91**, X,Y= Br) (7 g, 30 mmol) and methyl acrylate (3.4 g, 40 mmol) was added to a stirred suspension of NaH (1.32 g, 33 mmol, 60% in mineral oil) in dry toluene (50 cm^3) at 0 °C. The reaction mixture was stirred at room temperature for 1 h, followed by refluxing for a period of 24 h. The excess metal hydride was destroyed by the addition of water (5 cm^3) and the volatile material was removed under reduced pressure to yield the residual oil, which was partitioned between water and ethyl acetate (1:1; v/v, 100

cm^3). After further extraction with ethyl acetate (3 x 50 cm^3), the organic fractions were pooled, dried ($MgSO_4$) and the solvent removed under reduced pressure, to give a yellow oil. The crude oil was a 10:1 mixture of the 1-bromo-*cis*-(**48**) and 1-bromo-*trans*-cyclopropane dicarboxylate dimethyl ester (**47**). The *cis* isomer was purified using flash chromatography on silica gel (5% ethyl acetate in hexane) (4.1g, 58%), υ_{max} (Neat)/cm^{-1} 3450, 3100, 1740, 1385, 1307, 1279, 1225, 1186 and 1106; δ_H (200 MHz; C^2HCl_3) 1.69 (1H, dd, *J* 7, 10, H_α), 2.12 (1H, t, *J* 7, H_β), 2.44 (1H, dd, *J* 7, 10, 2-H) and 3.60 & 3.73 (6H, 2s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3), 22.59 (C-3), 29.35 (C-2), 30.88 (C-1), 52.55 (C-2-CO_2CH_3), 53.51 (C-1-CO_2CH_3) and 167.80 & 169.08 (2 x CO_2); (R_f 0.33, 5% ethyl acetate in hexane).

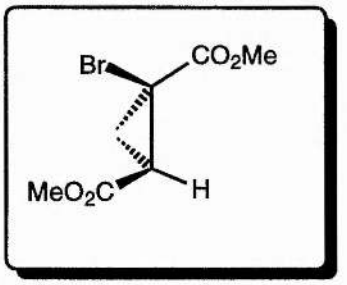
(±)-*cis*-1-Bromocyclopropane-1,2-dicarboxylic Acid (95, X= Br)



Dimethyl 1-bromocyclopropane-*cis*-1,2-dicarboxylate (**48**) (2 g, 8.4 mmol) was suspended in 6 mol dm^{-3} HCl (80 cm^3) and the solution was then refluxed for 2 h. To the solution was added KOH (2 mol dm^{-3}) until the pH was between 9-10. The aqueous phase was washed with dichloromethane (2 x 50 cm^3) acidified to pH 1 (6 mol dm^{-3} HCl) and extracted with ethyl acetate (3 x 50 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and removed under reduced pressure to give 1-bromo-*cis*-cyclopropane-1,2-dicarboxylic acid (**95**, X= Br) as a white solid which was recrystallised using ethyl acetate/light petroleum ether (1.75 g, 85%), m.p. 140 °C (lit.,[152] 140-141 °C); *m/z* (Found: $[M + NH_4]^+$, 225.9715. Calc. for $C_5H_5O_4Br$: 225.9715); υ_{max} (Nujol)/cm^{-1} 1700 (b), 1305, 1265 and 1212; δ_H (200 MHz; ^2H_2O) 1.80 (1H, dd, *J* 6, 10, H_α) 2.02 (1H, dd, *J* 7.5, 10, H_β) and 2.60 (1H, dd, *J* 7.5, 10, 2-H); δ_C (50.31 MHz; $^2H_2O/NaO^2H$) 23.48 (C-3), 31.62 (C-1), 32.41 (C-2) and

171.86 & 173.67 (2 x CO_2H); *m/z* (CI) 226 (38%, $[M + NH_4]^+$), 148 (100, [M + NH4 - $CH_4NO_3]^+$), 105 (17, $[C_2H_3NO_4]^+$) and 62 (15, $[CH_4NO_2]^+$).

(±)-Dimethyl *trans*-1-Bromocyclopropane-1,2-dicarboxylate (94, X= Br)



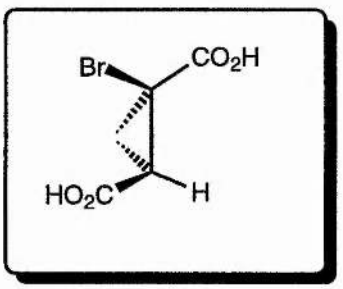
A mixture of methyl dibromoacetate (**91**, X,Y= Br) (10 g, 43 mmol) and methyl acrylate (4.8 g, 56 mmol), was added to a stirred suspension of NaH (1.9 g, 47 mmol, 60% in mineral oil) in dry DMF (50 cm^3) at 0 °C. The reaction mixture was stirred at 80 °C for 24 h. The excess metal hydride was destroyed by the addition of water (10 cm^3). Volatile material was removed under reduced pressure and the residual oil was partitioned between water and ethyl acetate (1:1; v/v, 100 cm^3). After further extraction with ethyl acetate (3 x 50 cm^3), the organic fractions were pooled, dried ($MgSO_4$) and the solvent was removed under reduced pressure to give a yellow oil. The crude oil was a 2:3 mixture of the 1-bromo-*cis*-(**48**) and 1-bromo-*trans*-cyclopropanedicarboxylate dimethyl ester (**47**) (both isomers 6.54 g, 60%, as determined by ^{1}H-NMR). The *trans*-isomer was purified using flash chromatography on silica gel (5% ethyl acetate in hexane) (3.9 g, 36%); υ_{max} (Neat)/ cm^{-1} 3460, 3100, 1750, 1385, 1375, 1320, 1280 and 1225; δ_H (200 MHz; C^2HCl_3) 1.85-2.05 (2H, m, H_α and H_β), 2.60 (1H, dd, *J* 9, 11, 2-H) and 3.77, 3.80 (6H, 2s, 2 x OCH_3); δ_C (50 MHz; C^2HCl_3) 22.58 (C-3), 29.93 (C-2), 30.43 (C-1), 52.69 (C-2-$CO_2\underline{C}H_3$), 34.09 (C-1-$CO_2\underline{C}H_3$) and 167.76 & 168.67 (2 x CO_2). (R_f 0.36, 5% ethyl acetate in hexane).

Method 2

(±)-Dimethyl *trans*-1-Bromocyclopropane-1,2-dicarboxylate (94, X= Br)

To a stirred suspension of potassium hydride (1.4 g, 12 mmol, 35% in mineral oil) in dry DMF (15 cm^3), was added methyl bromoacetate (**91**, X= Br, Y= H), (1.8 g, 12 mmol) and methyl α-bromoacrylate (**97**, X= Br) (2.0 g, 12 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 10 h. The excess metal hydride was destroyed by the addition of water (2 cm^3). Volatile material was removed under reduced pressure and the residual oil was partitioned between water and ethyl acetate (1:1; v/v, 50 cm^3). After further extraction with ethyl acetate (3 x 20 cm^3) the organic fractions were pooled, dried ($MgSO_4$) and the solvent was removed under reduced pressure to give a yellow oil. The crude oil was a 1:5 mixture of the 1-bromo-*cis*- (**48**) and 1-bromo-*trans*-cyclopropanedicarboxylate dimethyl ester (**47**) (both isomers 2.38 g 83%). The *trans*-isomer (**47**), was purified using flash chromatography on silica gel (5% ethyl acetate in hexane) (1.90 g, 66%), υ_{max} (Neat)/ cm^{-1} 3460, 3100, 1750, 1385, 1375, 1320, 1280 and 1225; δ_H (200 MHz; C^2HCl_3) 1.85-2.05 (2H, m, H_α & H_β), 2.60 (1H, dd, *J* 9, 11, 2-H), and 3.77 & 3.80 (6H, 2s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3) 22.58 (C-3), 29.93 (C-2), 30.43 (C-1), 52.69 (C-2-$CO_2\underline{C}H_3$), 34.09 (C-1-$CO_2\underline{C}H_3$) and 167.76 & 168.67 (2 x CO_2); (R_f 0.36, 5% ethyl acetate in hexane).

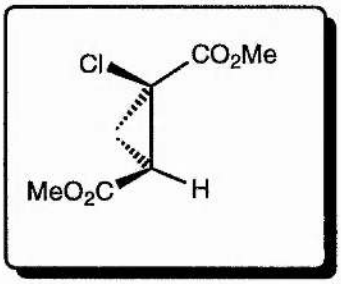
(±)-*trans*-1-Bromocyclopropane-1,2-dicarboxylic Acid (96, X= Br)



Dimethyl 1-bromocyclopropane-*trans*-dicarboxylate (**47**) (2 g, 8.4 mmol) was suspended in HCl (80 cm^3, 6 mol dm^{-3}) and the solution was refluxed for 2 h. To

the solution was added KOH (2 mol dm^{-3}) until the pH was between 9-10. The aqueous phase was washed with dichloromethane (2 x 50 cm^3), acidified to pH 1 (HCl, 6 mol dm^{-3}) and extracted with ethyl acetate (3 x 50 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and removed under reduced pressure to give 1-bromo-*trans*-cyclopropane-1,2-dicarboxylic acid (**96**, X= Br) as a white solid, which was recrystallised using ethyl acetate/ light petroleum (1.41 g, 80%), m.p. 174-5 °C (lit.,[152] 175 °C), (Found: C, 28.85; H, 2.30. Calc. for $C_5H_5BrO_4$: C, 28.70; H, 2.40);*m/z* (Found: $[M + NH_4]^+$, 225.9715 Calc. for $C_5H_9BrNO_4$: *m/z*, 225.9715); υ_{max} (Nujol)/cm^{-1} 1700 (b), 1290, 1242 and 1140; δ_H (200 MHz; ^2H_2O) 1.73, 1.94 (2H, m, H_α & H_β) and 2.61 (1H, dd, *J* 8, 9, 2-H); δ_C (50.31 MHz; ^2H_2O) 25.7 (C-3), 32.2 (C-2), 37.4 (C-1) and 171.8 & 174.9 (2 x CO_2); *m/z* (CI) 226 & 228 (40, 38%, $[M + NH_4]^+$), 148 (100, $[M + NH_4]^+ - [CH_4NO_3]^-$), 105 (19, $[C_2H_3NO_4]^+$) and 62 (16, $[CH_4NO_2]^+$).

(±)-Dimethyl *trans*-1-Chlorocyclopropane-1,2-dicarboxylate (94, X= Cl)



Compound (**94**, X= Cl) was prepared using methyl dichloroacetate (10 g, 70 mmol), and methyl acrylate (6.0 g, 70 mmol) in the presence of sodium hydride (3.08 g, 77 mmol, 60% in mineral oil) in dry DMF (100 cm^3) at 0 °C, using the procedure described for compound (**47**) (method 1). The crude-diester was a 3:1 mixture of the 1-chloro-*trans*- (**94**, X= Cl) and 1-chloro-*cis*-cyclopropane-dicarboxylate dimethyl ester (**93**, X= Cl) (both isomers 10.2g, 76%, as determined by ^1H-NMR). The *trans*-isomer was purified using flash chromatography on silica gel (20% ethyl acetate in hexane) to give the desired compound as a colourless oil (6.8g, 51%), (Found: $[M + H]^+$, 193.0259. Calc. for $C_7H_{10}ClO_4$: *m/z*,

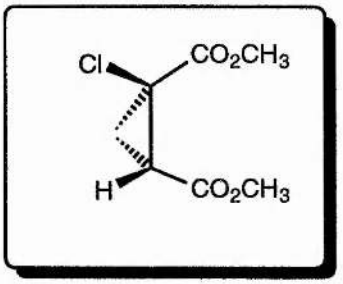
193.0268); υ_{max} (Neat)/cm^{-1} 2954, 1740, 1385, 1283, 1028 and 928; δ_H (200 MHz; C^2HCl_3) 1.92-1.96 (2H, d, *J* 8.5, H_α & H_β), 2.67 (1H, t, *J* 8.4, 2-H) and 3.75 & 3.77 (6H, 2 x s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3) 23.51 (C-3), 31.19 (C-2), 42.79 (C-1), 53.11 (C-2-$CO_2\underline{C}H_3$). 54.34 (C-1-$CO_2\underline{C}H_3$) and 167.90 & 169.41 (2 x CO_2); *m/z* (CI) 193 & 195 (30, 10%, $[M + H]^+$) and 161 & 163 (100, 31, $[M + H - CH_3OH]^+$); (R_f 0.32, 20% ethyl acetate in hexane).

Method 2

(±)-Dimethyl *trans*-1-Chlorocyclopropane-1,2-Dicarboxylate (94, X= Cl)

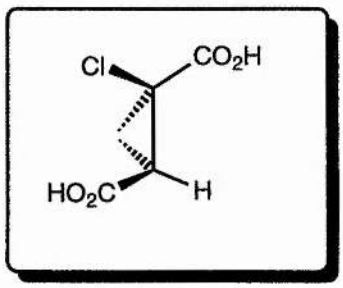
Compound (**94**, X= Cl), was prepared from methyl chloroacetate (1.8 g, 16.6 mmol) and methyl-α-chloroacrylate (**97**, X= Cl) (2.0 g, 16.6 mmol) in the presence of KH (1.9 g, 16.6 mmol, 35% in mineral oil) in dry DMF (15 cm^3) at 0 °C, using the modified procedure as described for method 2. The crude diester was a 5:1 mixture of the 1-chloro-*trans*-(**94**, X= Cl) and 1-chloro-*cis*-cyclopropane-dicarboxylate dimethyl ester (**93**, X= Cl) (both isomers 2.56 g, 80%). The *trans* isomer was purified using flash chromatography on silica gel (20% ethyl acetate in hexane) (2.0 g, 64%), υ_{max} (Neat)/ cm^{-1} 2957, 1735, 1384, 1284, 1027 and 924; δ_H (200 MHz; C^2HCl_3) 1.92-1.97 (2H, d, *J* 8.5, H_α & H_β), 2.70 (1H, dd, *J* 8.5, 2-H) and 3.76 & 3.79 (6H, 2 x s, 2 x OCH_3); δ_C(50.31 MHz; C^2HCl_3) 23.53 (C-3), 31.2 (C-2), 42.8 (C-1), 53.1 (C-2-$CO_2\underline{C}H_3$). 54.4 (C-1-$CO_2\underline{C}H_3$) and 167.9 & 169.4 (2 x CO_2); *m/z* (CI) 193 & 195 (100, 35%, $[M + H]^+$), and 161 & 163 (5, 4, $[M + H - CH_3OH]^+$); (R_f 0.32, 20% ethyl acetate in hexane).

(±)-Dimethyl *cis*-1-Chlorocyclopropane 1,2-dicarboxylate (93, X= Cl)



Compound (**93**, X=Cl), was prepared from methyl dichloroacetate (**91**, X, Y= Cl) (10 g, 70 mmol) and methyl acrylate (6.0 g, 70 mmol) using the procedure described for 1-bromo-*cis* -cyclopropanedicarboxylate dimethyl ester (**93**, X= Br). The crude diester was a 1:3 mixture of the 1-chloro-*trans*-cyclopropane-dicarboxylate dimethyl ester (**94**, X= Cl) and 1-chloro-*cis*- cyclopropane-dicarboxylate dimethyl ester (**93**, X= Cl). The *cis*-isomer was purified using flash chromatography on silica gel (20% ethyl acetate in hexane) (9.4 g, 70%), (Found: $[M + H]^+$, 193.0265. Calc. for $C_7H_{10}ClO_4$: m/z, 193.0268); υ_{max} (Neat)/cm^{-1} 2956, 1749, 1379, 1230 and 1122; δ_H (200 MHz; C^2HCl_3) 1.67 (1H, dd, J 3.5, 5, H_α), 2.13 (1H, dd, J 4, 5, H_β), 2.44 (1H, dd, J 4, 5, 2-H) and 3.75 & 3.82 (6H, 2 x s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3) 22.8 (C-3), 30.41 (C-2), 32.1 (C-1), 52.5 (C-2-$CO_2\underline{C}H_3$), 53.0 (C-1-$CO_2\underline{C}H_3$), and 166.6 & 169.0 (2 x CO_2); m/z (CI) 193 & 195 (100, 36%, $[M + H]^+$) and 161 & 163 (46, 15, $[M + H - CH_3OH]^+$); (R_f 0.26, 20% ethyl acetate in hexane).

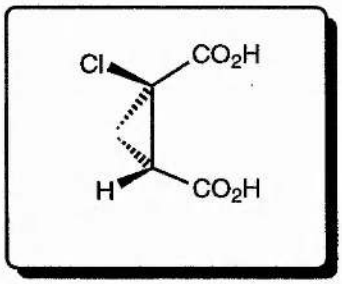
(±)-*trans*-1-Chlorocyclopropane-1,2-dicarboxylic Acid (96, X= Cl)



Compound (**94**, X= Cl) (3.0 g, 16 mmol) was used to prepare the cyclopropane-dioic acid (**96**, X= Cl), using the general procedure as described for compound (**96**,

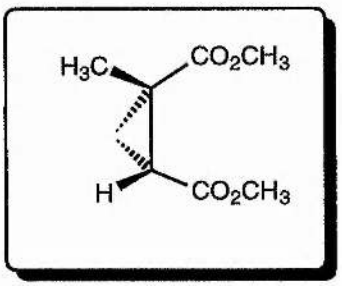
X= Br). The desired compound was isolated and recrystallised from ethyl acetate in hexane (1:9), to give a white crystalline solid (2.3 g, 90%), m.p. 178-180 °C; (Found: C, 36.55; H, 2.85. Calc. for $C_5H_5ClO_4$: C, 36.60; H, 3.05); *m/z* (Found: [M + H]$^+$, 164.9963. Calc. for $C_5H_6ClO_4$: *m/z*, 164.9955); υ_{max} (Nujol)/cm^{-1} 2925, 1697 and 1450; δ_H (200 MHz; ^2H_2O) 1.85 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 6, H_α), 2.05 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 9, H_β) and 2.80 (1H, dd, $J_{H\beta,2}$ 9,$J_{H\alpha,2}$ 6, 2-H); δ_C (50.31 MHz; ^2H_2O) 20.6 (C-3), 28.7 (C-2), 41.1 (C-1) and 169.5 & 170.2 (2 x CO_2); *m/z* (CI) 165 & 167 (100, 40%, [M + H]$^+$), 147 & 149 (59, 20, [M + H - H_2O]$^+$) and 129 (88, [M + H - 2 x H_2O]$^+$).

(±)-*cis*-1-Chlorocyclopropane-1,2-dicarboxylic Acid (95, X= Cl)



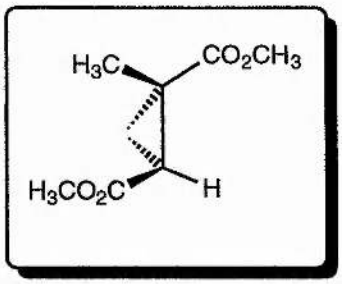
Compound (**93**, X= Cl) (3.0 g, 16 mmol) was used to prepare the cyclopropane-dioic acid (**95**, X= Cl), using the general procedure as described for compound (**95**, X= Br). The desired compound was isolated and recrystallised from ethyl acetate:hexane (1:9), to give a white crystalline solid (2.3 g, 90%), m.p. 120-122 °C; (Found: C, 36.90; H, 2.80. Calc. for $C_5H_5ClO_4$: C, 36.60; H, 3.05); *m/z* (Found: [M + H]$^+$, 164.9949. Calc. for $C_5H_6ClO_4$: *m/z*, 164.9955); υ_{max} (Nujol)/cm^{-1} 2923, 1699 and 1455; δ_H (200 MHz; ^2H_2O) 1.81 (1H, dd, *J* 7.5, 10, H_α), 2.25 (1H, t, *J* 10, H_β) and 2.58 (1H, t, *J* 10, 2-H); δ_C (50.31 MHz; ^2H_2O) 20.10 (C-3), 35.40 (C-2), 46.1 (C-1) and 173.5 & 174.7 (2 x CO_2H); *m/z* (CI) 165 (100%, [M + H]$^+$), 147 (59, [M + H - H_2O]$^+$) and 129 (88, [M + H - 2 x H_2O]$^+$)

(±)-Dimethyl *cis*-1-Methylcyclopropane-1,2-dicarboxylate (93, X= CH_3)



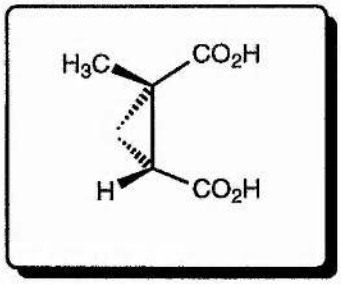
To a stirred suspension of sodium hydride (3.25 g, 80 mmol, 60% in mineral oil) in dry toluene (50 cm^3) was added a mixture of methyl α-chloropropionate (**91**, X= CH_3, Y= Cl) (10 g, 80 mmol) and methyl acrylate (7.0 g, 80 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then refluxed for 24 h. The excess metal hydride was destroyed by the addition of water (5 cm^3). Volatile material was removed under reduced pressure and the residual oil was partitioned between water and ethyl acetate (1:1; v/v, 100 cm^3). After further extraction with ethyl acetate (3 x 50 cm^3) the organic fractions were pooled, dried ($MgSO_4$) and solvent was removed under reduced pressure to give a yellow oil. The crude oil was a 10:1 mixture of the 1-methyl-*cis*-(**93**, X= CH_3) and 1-methyl-*trans*-cyclopropane dicarboxylate dimethyl ester (**94**, X= CH_3). The *cis*-isomer was purified using flash chromatography on silica gel (20% ethyl acetate in hexane) (10.2 g, 74%), *m/z* (Found: $[M + H]^+$, 173.0806. Calc. for $C_8H_{13}O_4$: *m/z*, 173.0814); δ_H (200 MHz; C^2HCl_3) 1.06 (1H, dd, *J* 8, 12, H_α), 1.39 (3H, s, CH_3), 1.8 (2H, m, H_β, 2-H), and 3.60 & 3.70 (6H, 2s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3) 20.20 (C-3), 21.55 (CH_3), 29.09 (C-2), 32.56 (C-1), 52.7 & 52.70 (2 x OCH_3) and 174.00 & 174.50 (2 x CO_2); *m/z* (CI) 173 (100%, $[M + H]^+$) and 73 (91, $[C_3H_5O_2]^+$); (R_f 0.18, 20% ethyl acetate in hexane);

(±)-Dimethyl *trans*-1-Methylcyclopropane-1,2-dicarboxylate (94, X= CH_3)



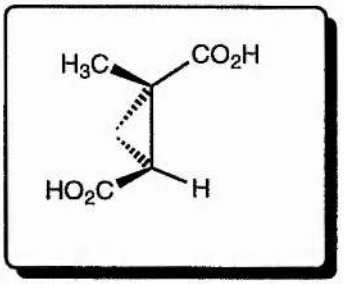
Compound (**94**, X= CH_3) was prepared using methyl α-chloropropionate (**91**, X= CH_3, Y= Cl) (10 g, 80 mmol) and methyl acrylate (7.0 g, 80 mmol) in the presence of sodium hydride (3.25 g, 80 mmol, 60% in mineral oil) in dry DMF (50 cm^3), at 0 °C, using the procedure described for (**94**, X= Cl). The crude oil was a 10:1 mixture of the 1-methyl-*trans* -(**94**, X= CH_3) and 1-methyl-*cis*-cyclopropane dicarboxylate dimethyl ester (**93**, X= CH_3). The *trans* isomer was purified using flash chromatography on silica gel (20 % ethyl acetate in hexane) (10.2 g, 74%), *m/z* (Found: $[M + H]^+$, 173.0821. Calc. for $C_8H_{13}O_4$: *m/z*, 173.0814); δ_H (200 MHz; C^2HCl_3), 1.29 (1H, dd, *J* 4, 7, H_α), 1.39 (3H, s, CH_3), 1.54 (1H, dd, *J* 4, 9, H_β), 2.30 (1H, dd, *J* 7, 9, 2-H) and 3.69 & 3.70 (6H, 2s, 2 x OCH_3); δ_C (50.31 MHz; C^2HCl_3) 13.52 (C-3), 21.57 (CH_3), 28.16 (C-2), 34.83 (C-1), 52.4 & 52.8 (2 x OCH_3) and 171.35 & 174.42 (2 x CO_2); *m/z* (CI) 173 (13, $[M + H]^+$), 97 (22, $[M + H - C_3H_8O_2]^+$) and 73 (100, $[C_3H_5O_2]^+$); (R_f 0.26, 20% ethyl acetate in hexane).

(±)-*cis*-1-Methylcyclopropane-1,2-dicarboxylic Acid (95, X= CH_3)



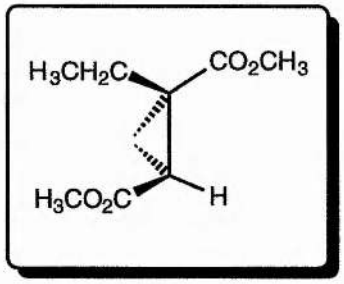
A suspension of the diester (**93**, X= CH_3) (3.0 g, 17 mmol) was in hydrochloric acid (3 mol dm^{-3}, 33 cm^3) was heated to reflux for 4 h. The solution was basified with potassium hydroxide (2 mol dm^{-3}) until the pH was 9-10. The aqueous phase was washed with dichloromethane (2 x 50 cm^3) and the aqueous phase was then acidified to pH 1 using hydrochloric acid (6 mol dm^{-3}). The acidified solution was then extracted with ethyl acetate (3 x 50 cm^3) and the organic phases were pooled, dried ($MgSO_4$) and concentrated to leave a white solid, which was recrystallised using ethyl acetate/hexane (1:9). The desired compound was isolated as a white crystalline solid (2.33 g, 95%), m.p. 139-140 °C (lit.,[123] 139-141 °C); (Found: C, 51.05; H, 5.85. Calc. for $C_6H_8O_4$: C, 50.00; H, 5.60); *m/z* (Found: $[M + H]^+$, 145.0509. Calc. for 145.0501); υ_{max} (Nujol)/cm^{-1} 1683 (b), 1321, 1264 and 1224; δ_H (300 MHz; ^2H_2O) 1.21 (1H, dd, $J_{3,3}$ 5, $J_{2,3}$ 8, H_α), 1.34 (3H, s, CH_3), 1.63 (1H, dd, $J_{3,3}$ 5, $J_{2,3}$ 6, H_β) and 1.98 (1H, dd, $J_{H\alpha,2}$ 8,$J_{H\beta,2}$ 6, 2-H); δ_C (75 MHz; ^2H_2O) 17.80 (CH_3), 17.89 (C-3), 26.88 (C-2), 27.62 (C-1) and 173.17 & 174.68 (2 x CO_2H); *m/z* (CI) 145 (84, $[M + H]^+$ and 127, (100, $[M + H - H_2O]^+$).

(±)-*trans*-1-Methylcyclopropane-1,2-dicarboxylic Acid (96, X= CH_3)



Compound (**96**, X= CH_3), was prepared in the same manner as compound (**95**, X= CH_3), using the diester (**94**, X= CH_3) (3.0 g, 17 mmol) as the starting material. The desired compound was recrystallised ethyl acetate:hexane (1:9) and isolated as a white crystalline solid (2.4 g, 98%), m.p. 168-170 °C (lit.,[123] 170 °C); (Found: C, 50.05; H, 5.65. Calc. for $C_6H_8O_4$: C, 50.0; H, 5.60); *m/z* (Found: $[M + H]^+$, 145.0509. Calc. for 145.0501); υ_{max} (Nujol)/cm^{-1} 1683 (b), 1307, 1246 and 1193; δ_H (200 MHz; $C^2H_3O^2H$) 1.25 (1H, dd, $J_{3,3}$ 6, $J_{2,3}$ 10, H_α), 1.38 (3H, s, CH_3), 1.70 (1H, dd, $J_{3,3}$ 6, $J_{2,3}$ 13, H_β) and 2.28 (1H, dd, $J_{H\alpha,2}$ 10,$J_{H\beta,2}$ 13, 2-H); δ_C (50.31 MHz; $C^2H_3O^2H$) 13.90 (C-3), 21.80 (CH_3), 27.80 (C-1), 29.10 (C-2) and 174.18 & 177.53 (2 x CO_2H); *m/z* (CI) 145 (78, $[M + H]^+$, 130 (22, $[M + H - CH_3]^+$), 127 (100, $[M + H - H_2O]^+$).

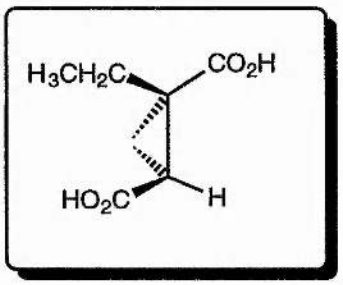
(±)-Dimethyl *trans*-1-Ethylcyclopropane-1,2-dicarboxylate (94, X= CH_2CH_3)



Compound (**94**, X= CH_2CH_3), was prepared using the general method as described for compound (**94**, X= CH_3). The preparation was carried out using methyl (±)-2-bromobutyrate (**91**, X= Br, Y= CH_2CH_3) (10 g, 55 mmol) and methyl acrylate (4.7 g, 55 mmol) in the presence of sodium hydride (60% disp. in mineral oil, 2.2 g, 55 mmol) and dry DMF (100 cm^3). The reaction temperature was

maintained between 40-60 °C. Following the general work up, the crude diester was found to be a mixture of the *trans*-1-ethyl- (**94**, X= CH_2CH_3) and *cis*-1-ethyl-cyclopropane dicarboxylate (**93**, X= CH_2CH_3) (5:1, as judged by ^{1}H-NMR). The *trans* isomer was purified using flash chromatography on silica gel (20% ethyl acetate in hexane) (6.2 g, 65%), *m/z* (Found: $[M + H]^+$, 187.0965 Calc. for $C_9H_{15}O_4$: *m/z*, 187.0970; υ_{max} (Nujol) /cm^{-1} 1735, 1725, 1395, 1248 and 1022; δ_H (200 MHz; C^2HCl_3) 0.70 (3H, t, *J* 9, $C\underline{H}_3CH_2$), 1.08 (1H, dd, *J* 5, 7, H_α), 1.24 (1H, dd, *J* 5, 10, H_β) 1.49 (1H, m, AB splitting, CH_3-C<u>H</u>-H) 1.65 (1H, m, AB splitting, *J* 5, 10, CH_3-CH-<u>H</u>) and 2.13 (1H, dd, *J* 7.5, 10, 2-H); δ_C (50.31 MHz; C^2HCl_3) 12.4 ($\underline{C}H_3CH_2$), 20.9 (C-3), 21.0 ($CH_3\underline{C}H_2$) 27.4 (C-2), 33.6 (C-1), 52.4 & 52.6 (2 x OCH_3) and 171.5 & 173.6 (2 x CO_2); *m/z* (CI) 187 (100%, $[M + H]^+$), 155 (28, $[M + H - CH_3OH]^+$) and 125 (8, $[M + H - 2 \times OCH_3]^+$); ($R_f$ 0.45, 20% ethyl acetate in hexane).

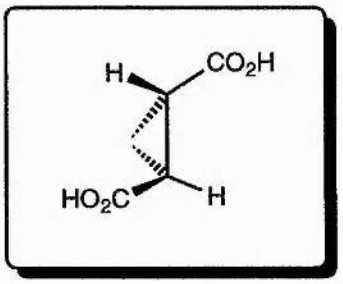
(±)-1-Ethyl-*trans*-cyclopropane-1,2-Dicarboxylic Acid (96, X= CH_2CH_3)



This compound (**96**, X= CH_2CH_3), was prepared in a manner identical to that for (**95**, X= CH_3), using the diester (**94**, X= CH_2CH_3) (4.0 g, 21.5 mmol) as the starting material. The desired compound was recrystallised ethyl acetate:hexane (1:9) and isolated as a white crystalline solid (3.23 g, 95%), m.p. 158-160 °C; (Found: C, 53.00; H, 6.45. Calc. for $C_7H_{10}O_4$: C, 53.15; H, 6.35); *m/z* (Found: $[M + H]^+$, 159.0663 Calc. for $C_7H_{11}O_4$: *m/z*, 159.0657); υ_{max} (Nujol) /cm^{-1} 2924, 1683, 1457 and 1377; δ_H (200 MHz; $C^2H_3O^2H$) 0.91 (3H, t, *J* 7, $C\underline{H}_3CH_2$), 1.26 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 6, H_α), 1.46 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 8, H_β) 1.75 (1H, m, AB splitting, *J* 7, CH_3-C<u>H</u>-H) 1.90 (1H, m, AB splitting, *J* 7, CH_3-CH-<u>H</u>) and 2.31 (1H, dd, $J_{H\alpha,2}$ 6, $J_{H\beta,2}$ 8, 2-H); δ_C (50.31 MHz; $C^2H_3O^2H$) 12.5 ($\underline{C}H_3CH_2$), 21.3 (C-3), 22.1 ($CH_3\underline{C}H_2$) 28.3 (C-2),

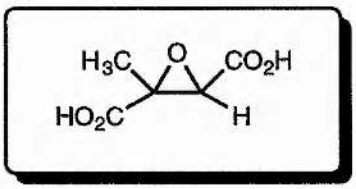
34.21 (C-1) and 174.50 & 176.60 (2 x CO_2H); *m/z* (CI) 159 (32%, $[M + H]^+$, 141 (100, $[M + H - H_2O]^+$) and 127 (19, $[C_7H_{11}O_3]^+$).

(±)-1,2-*trans*-Cyclopropanedicarboxylic Acid (96, X= H)



Compound (**96**, X= H), was prepared in a manner identical to that for compound (**95**, X= CH_3), using the commercially available (±)-diethyl trans-1,2-cylopropane dicarboxylate (5 g, 26.9 mmol) as the starting material. The desired compound was recrystallised ethyl acetate:hexane (1:9) and isolated as a white crystalline solid (3.3 g, 95%), m.p. 176-177 °C (lit.,[123] 177-177.5 °C); υ_{max} (Nujol)/cm^{-1} 1680, 1305, 1237 and 1190; δ_H (200 MHz; $C^2H_3O^2H$) 2.85 (2H, m, 3-CH_2) and 3.53 (2H, m, 1-H, 2-H); δ_C (50.31 MHz; $C^2H_3O^2H$) 18.73 (C-3), 25.94 (C-1, C-2) and 178.28 (2 x CO_2H); *m/z* (CI) 131 (8%, $[M + H]^+$, 108 (100, $[M - OH_6]^+$), 94 (45, $[M + H - 2 \times H_2O]^+$), 57 (47, $[CO_2H]^+$).

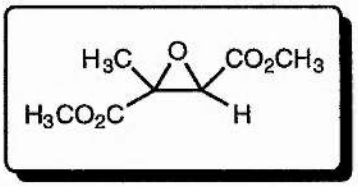
(±)-*trans*-2-Methyl-Epoxysuccinic Acid (102)



Mesaconic acid (**30** R= H) (20 g, 154 mmol), $Na_2WO_4.H_2O$ (5.1 g, 15.4 mmol) and water (450 cm^3) were stirred together at room temperature. The dissolution of the solid acid was aided by the periodic addition of potassium hydroxide (2 mol dm^{-3}). After the solid was completely dissolved the pH of the solution

was adjusted to 6.2 by the addition of potassium hydroxide (1 mol dm^{-3}) and / or H_2SO_4 (1 mol dm^{-3}). After 10 min of additional stirring at room temperature, an aqueous solution of hydrogen peroxide (6.3 g, 185 mmol) was added. The pH of the reaction mixture was kept between 5.8 and 6.8. The reaction mixture was heated to 65 °C for 3 h, followed by 75 °C for 1 h. The mixture was cooled down to room temperature and the mixture acidified to pH <2.0 with H_2SO_4 (6 mol dm^{-3}), saturated with ammonium sulphate, and extracted with diethyl ether (5 x 150 cm^3). The organic fractions were dried ($MgSO_4$), filtered and evaporated under reduced pressure, to yield a white solid, which was recrystallised from diethyl ether/ light petroleum (16.7 g, 74%), m.p. 165-167 °C; (Found: C, 41.10; H, 4.10. Calc. for $C_5H_6O_5$: C, 41.10; H, 4.15%); *m/z* (Found: $[M + H]^+$, 147.0295. Calc. for $C_5H_7O_5$: *m/z*, 147.0293); υ_{max} (Nujol)/cm^{-1} 1713; δ_H (200 MHz; ^2H_2O) 1.50 (3H, s, CH_3) and 3.89 (1H, s, 3-H); δ_H (50.31 MHz; ^2H_2O) 15.09 (CH_3), 60.51 (C-3), 61.35 (C-2) and 172.89 & 175.15 (2 x CO_2H); *m/z* (CI) 147 (13%, $[M + H]^+$), 129 (25, $[M + H - H_2O]^+$) and 103 (100, $[M + H - [CO_2]^+$).

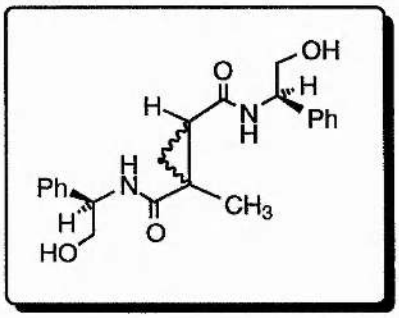
(±)-Dimethyl-*trans*-2-Methyl-Epoxysuccinate (106)



To a stirred solution of the epoxide (**102**) (0.5 g, 3.4 mmol) dissolved in a minimum volume of diethyl ether, was added dropwise, an ethereal solution of diazomethane at 0 °C. Diazomethane was added until the colour of the reaction mixture remained yellow, and the mixture was stirred for 30 min at room temperature. The reaction mixture was purged with nitrogen for 20 min, after which the solvent was removed under reduced pressure to give the desired ester (**106**) (0.58 g, 97%), *m/z* (Found: $[M + H]^+$, 175.0598. Calc. for $C_7H_{11}O_5$: *m/z*, 175.0606); υ_{max} (Neat)/cm^{-1} 2958, 1751, 1438 and 1387; δ_H (200 MHz; C^2HCl_3) 1.60 (3H, s, CH_3), 3.79 (3H, s, C-2 OCH_3) and 3.82 (4H, s, 3-H, C-4 OCH_3); *m/z* (CI) 175

(98%, $[M + H]^+$), 115 (100, $[M + H - C_2H_4O_2]^+$), 89 (10, $[C_3H_5O_3]^+$) and 73 (49, $[C_3H_5O_2]^+$); R_f(diethyl ether:hexane (3:2))= 0.47, (lit.,[129] R_f(diethyl ether:hexane (3:2))= 0.47).

(±)-1-Methyl *trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]cyclopropane (99)



To a dry solution of thionyl chloride (65.6 g, 486 mmol) was added in one portion the cyclopropanedicarboxylic acid (**96**, X= CH_3) (7.0 g, 48.6 mmol) under a stream of nitrogen. The reaction mixture was stirred under nitrogen for a period of 15 min, before being refluxed for a period of 45 min. The reaction mixture was allowed to cool down to room temperature, after which any excess thionyl chloride was removed under reduced pressure, leaving behind the diacid chloride as a solid crystalline product. The diacid chloride was dissolved in dry THF (20 cm^3) and was used immediately in the next stage of the synthesis, without purification.

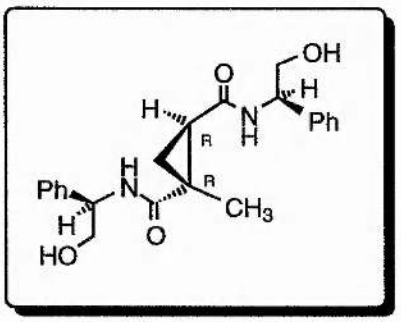
To a solution of (*R*)-2-phenylglycinol (13.3 g, 97.2 mmol) in dry THF (50 cm^3) was added triethylamine (11.7 g, 116.6 mmol) at 5 °C. To this stirred solution was added dropwise (over 30 mins), the dry THF solution of the diacid chloride (from above) under nitrogen. The reaction mixture was left to stir overnight at room temperature after which time the solvent was removed under reduced pressure leaving behind a solid residue. The residue was dissolved in ethyl acetate (100 cm^3) and was washed with HCl (1 mol dm^{-3}) solution (3 x 50 cm^3) followed by water (3 x 50 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and

concentrated under reduced pressure, to give the desired compound (**99**) as a white solid (16.53 g, 89%).

Chromatographic Resolution of (99)

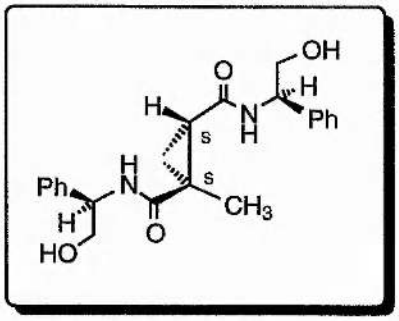
The mixture of diastereomers (**99**), were resolved on t.l.c. grade silica gel using gradient-elution column chromatography. The diastereomers were loaded onto a packed column and eluted first with neat ethyl acetate (1 bed volume), secondly, with 2% methanol in ethyl acetate (1 bed volume) and finally by eluting the column with 4% methanol in ethyl acetate (continuous flow). The (*R, R*)-diastereomer (**99a**) came off the column first (R_f 0.21 2% methanol in ethyl acetate), followed by the (*S,S*)-diastereomer (**99b**) (R_f 0.15 2% methanol in ethyl acetate).

(1*R*, 2*R*)-1-Methyl-*trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]cyclopropane (99a)



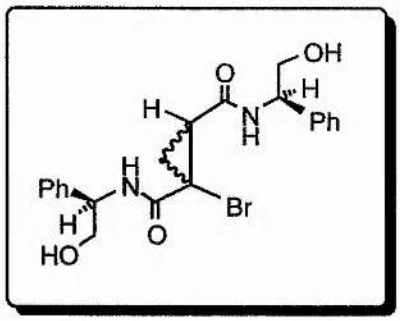
m.p. 60 °C; (Found C, 68.85; H, 7.0; N, 7.20. $C_{22}H_{26}N_2O_4$ requires C, 69.10; H, 6.85; N, 7.35%); *m/z* (Found: $[M + H]^+$, 383.1960. $C_{22}H_{27}N_2O_4$ requires 383.1971); $[\alpha]_D$ +30.6 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1}, 3438, 3355, 1634 and 1073; δ_H (300 MHz; $C^2H_3O^2H$) 1.33 (1H, dd, $J_{3,3}$ 7, $J_{2,3}$ 9, H_α), 1.55 (1H, dd, $J_{3,3}$ 7, $J_{2,3}$ 14, H_β), 1.67 (3H, s, CH_3), 2.45 (1H, dd, $J_{H\alpha,2}$ 9, $J_{H\beta,2}$ 14, 2-H), 3.80-4.10 (4H, m, 2 x $C\underline{H}_2OH$), 5.22 (2H, m, 2 x $C\underline{H}CH_2OH$) 7.30 (10H, m, 2 x C_6H_5) and 8.11 & 8.88 (2H, d, *J* 8, 2 x NH); δ_C (75 MHz; $C^2H_3O^2H$) 14.7 (CH_3), 20.1 (C-3), 29.3 (C-1), 30.52 (C-2), 58.3, 58.5 ($\underline{C}HCH_2OH$), 66.8, 67.1 ($\underline{C}H_2OH$), 128.9, 128.9, 129.3 and 130.4 (aromatic), 142.2 (ipso-C), 171.8 and 175.7 (2 x CO_2); *m/z* (CI) 383 (12%, $[M + H]^+$), 246 (10, $[M + H - C_8H_{11}NO]^+$), 138 (100, $[C_8H_{12}NO]^+$) and 127 (47, $[C_6H_9NO_2]^+$).

(1*S*, 2*S*)-1-Methyl-*trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]cyclopropane (99b)



m.p. 145-146 °C; *m/z* (Found: $[M + H]^+$, 383.1973. $C_{22}H_{27}N_2O_4$ requires 383.1971); $[\alpha]_D$ -125.8 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 3648, 3375, 1655 and 1075; δ_H (300 MHz; $C^2H_3O^2H$) 1.28 (1H, dd, $J_{3,3}$ 6, $J_{2,3}$ 10, H_α), 1.31 (3H, s, CH_3), 1.45 (1H, dd, $J_{3,3}$ 6, $J_{2,3}$ 13, H_β), 2.32 (1H, dd, $J_{H\beta,2}$ 13, $J_{H\alpha,2}$ 10, 2-H), 3.75 (4H, m, 2 x $C\underline{H}_2OH$), 5.05 (2H, m, 2 x $C\underline{H}CH_2OH$), 7.20-7.40 (10H, m, 2 x C_6H_5) and 7.95 & 8.75 (2H, d, *J* 8, 2 x NH); δ_C (75 MHz; $C^2H_3O^2H$) 13.93 (CH_3), 19.42 (C-3), 28.53 (C-1), 29.71 (C-2), 57.52 & 57.75 ($\underline{C}HCH_2OH$), 66.30, 66.0 ($\underline{C}H_2OH$), 127.91, 128.1, 128.53 and 129.66 (aromatic), 141.31 (ipso-C) and 171.83 & 175.74 (2 x CO_2); *m/z* (CI) 383 (100%, $[M + H]^+$), 264 (12, $[C_8H_9N]^+$) and 138 (2, $[C_8H_{12}NO]^+$).

(±)-1-Bromo-*trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]cyclopropane (98)



To a dry solution of thionyl chloride (3.49 g, 26 mmol) was added in one portion the cyclopropane dicarboxylic acid (**96**, X= Br) (0.54 g, 2.6 mmol), under a stream of nitrogen. The reaction mixture was stirred under nitrogen for a period of 15 min, before being refluxed for a period of 45 min. The reaction mixture was then allowed to cool down to room temperature after which time, any excess

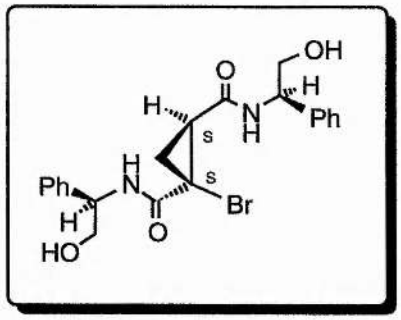
thionyl chloride was removed under reduced pressure leaving the diacid chloride as a solid crystalline product. The diacid chloride was dissolved in dry THF (5 cm^3) and was used immediately in the next stage of the synthesis, without purification.

To a solution of (*R*)-2-phenylglycinol (0.70 g, 5.2 mmol), in dry THF (10 cm^3) was added triethylamine (0.63 g, 6.2 mmol) at 5 °C. To this stirred solution, was added dropwise (over 30 min) the dry THF solution of the diacid chloride (from above), under nitrogen. The reaction mixture was left to stir overnight at room temperature, after which time the solvent was removed under reduced pressure, leaving behind a solid residue. The residue was dissolved in ethyl acetate (100 cm^3) and washed with HCl (1 mol dm^{-3}) solution (3 x 15 cm^3), followed by water (3 x 15 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and concentrated under reduced pressure to give the desired compound (**98**) as a white solid (0.98g, 85%).

Chromatographic Resolution of (98)

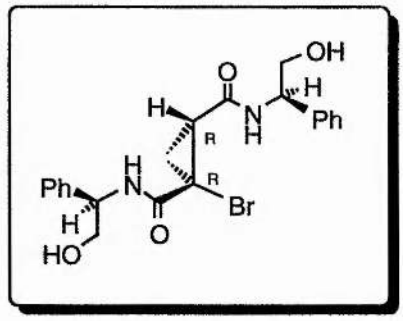
The mixture of diastereomers (**98**) were resolved on t.l.c. grade silica gel using column chromatography. The diastereomers were loaded onto a packed column and eluted with ethyl acetate. Diastereomer-(*S,S*) (**98b**) came off the column first (R_f of 0.39 in ethyl acetate), followed by diastereomer-(*R,R*) (**98a**) (R_f of 0.15 in ethyl acetate).

(1*S*, 2*S*)-1-Bromo-*trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]Cyclopropane (98b)



m.p. 148-150 °C; (Found C, 56.35; H, 5.15; N, 6.05. $C_{21}H_{23}N_2BrO_4$ requires: C, 56.40; H, 5.20; N, 6.25%); *m/z* (Found: $[M + H]^+$, 447.0927. $C_{21}H_{23}N_2BrO_4$ requires 447.0919); $[\alpha]_D$ -104 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 3651, 3384, 1658 and 1074 ; δ_H (200 MHz; $C^2H_3O^2H$) 1.83 (2H, m, H_α, H_β), 2.62 (1H, m, 2-H), 3.85 (4H, m, 2 x $C\underline{H}_2OH$), 5.10 (2H, m, 2 x $C\underline{H}CH_2OH$), 7.20-7.50 (10H, m, 2 x C_6H_5) and 8.41 & 8.91 (2H, d, *J* 8, 2 x NH); δ_C (75 MHz; $C^2H_3O^2H$) 21.6 (C-3), 31.3 (C-2), 34.7 (C-1), 57.7, 58.3 (2 x $\underline{C}HCH_2OH$), 65.8, 66.1 (2 x $\underline{C}H_2OH$), 127.8-129.6 (aromatic) and , 140.8 & 141.1 (2 x ipso-C) and 168.6 & 169.6 (2 x CO_2); *m/z* (CI) 449 & 447 (30, 29%, $[M + H]^+$), 369 (15, $[M + H - Br]^+$), 351 (18, $[M + H - BrH_2O]^+$), 138 (39, $[C_8H_{12}NO]^+$) and 58 (100, $[2 \times COH]^+$).

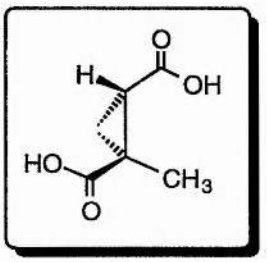
(1*R*, 2*R*)-1-Bromo-*trans*-1,2-diamido[*N*-(2*R*)-phenyl ethanol]cyclopropane (98a)



m.p. 110-111 °C; (Found C, 56.85; H, 5.15; N, 6.05. $C_{21}H_{23}N_2BrO_4$ requires: C, 56.40; H, 5.20; N, 6.25%); $[\alpha]_D$ +34.4 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 3650, 3378, 1661 and 1079 ; δ_H (200 MHz; $C^2H_3O^2H$) 1.84 (2H, d, H_α, H_β), 2.63 (1H, m, 2-H), 3.7-3.9 (4H, m, 2 x $C\underline{H}_2OH$), 5.05 (2H, m, 2 x $C\underline{H}CH_2OH$), 7.20-7.50 (10H, m, 2 x C_6H_5) and

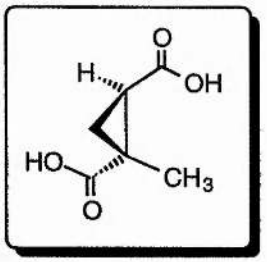
8.32 & 8.88 (2H, d, *J* 8, 2 x NH); δ_C (50.31 MHz; $C^2H_3O^2H$) 22.4 (C-3), 31.4 (C-2), 34.7 (C-1), 58.6 & 58.7 (2 x $\underline{C}HCH_2OH$), 66.1 & 66.5 (2 x $\underline{C}H_2OH$), 128.3-129.9 (aromatic), 141.1 and 141.3 (2x ipso-C) and 169.0 & 169.8 (2 x CO_2); *m/z* (CI) 447 (28%, $[M + H]^+$), 369 (10, $[M + H - Br]^+$), 351 (15, $[M + H - [H_2BrO]^+$) and 138 (35, $[C_8H_{12}NO]^+$).

(+)-(1*S*,2*S*)-1-Methyl-*trans*-cyclopropane-1,2-dioic Acid (101b)



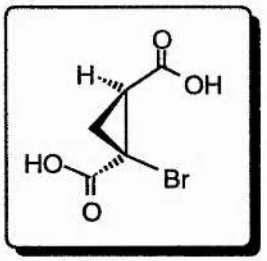
(1*S*,2*S*)-1-Methyl-*trans*-1,2-diamido[*N*-(2*R*)-phenylethanol]cyclopropane (**99b**) (1.49 g, 3.9 mmol) was suspended in a 3 mol dm^{-3} solution of H_2SO_4 (8 cm^3, 24 mmol), at 0 °C. This mixture was stirred for 10 min, before being heated to 70 °C. The reaction mixture was maintained at 70 °C for a period of 24 h after which time the reaction mixture was allowed to cool down to room temperature. The aqueous phase was extracted with diethyl ether (3 x 20 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and concentrated under reduced pressure to give the desired product as an off white solid. The crude product was recrystallised using ethyl acetate/ hexane (1:9), to leave the pure compound as a white crystalline solid (0.51 g, 91%), m.p. 168 °C; (Found: C, 49.80; H, 5.60. $C_6H_8O_4$ requires C, 50.0; H, 5.60%); *m/z* (Found: $[M + H]^+$, 145.0499. $C_6H_9O_4$ requires: 145.0500); $[\alpha]_D$ +191.4 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 2925, 1683, 1456 and 1377; δ_H (200 MHz; $C^2H_3O^2H$) 1.25 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 6, H_α), 1.40 (3H, s, CH_3), 1.55 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 8, H_β) and 2.29 (1H, dd, $J_{H\alpha,2}$ 6,$J_{H\beta,2}$ 8, 2-H); δ_C (50.31 MHz; $C^2H_3O^2H$) 13.9 (CH_3), 21.8 (C-3), 27.8 (C-1), 29.1 (C-2), 174.2 and 177.6 (2 x CO_2H); *m/z* (CI) 145 (94%, $[M + H]^+$), 130 (5, $[M + H - CH_3]^+$) and 127 (100, $[M + H - H_2O]^+$).

(-)-(1*R*,2*R*)-1-Methyl-*trans*-cyclopropane-1,2-dioic Acid (101a)



This compound (**101a**) was prepared in a manner identical to that for compound (**101b**), using the diastereomer (**99a**) (2.0 g, 5.24 mmol), and a 3 mol dm^{-3} solution of H_2SO_4 (10.5 cm^3, 31.4 mmol). The crude product was recrystallised using ethyl acetate/hexane (1:9) to leave the pure compound as a white crystalline solid (0.70 g, 93%), m.p. 168 °C; $[\alpha]_D$ -191.4 (*c* 1.0 in MeOH); (Found: C, 50.00; H, 5.55. $C_6H_8O_4$ requires: C, 50.00; H, 5.60%); *m/z* (Found: $[M + H]^+$ 145.0490. $C_6H_9O_4$ requires 145.0500); υ_{max}(Nujol)/cm^{-1} 2925, 1683, 1456 and 1377; δ_H (200 MHz; $C^2H_3O^2H$) 1.24 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 6, H_α), 1.38 (3H, s, CH_3), 1.54 (1H, dd, $J_{3,3}$ 4, $J_{2,3}$ 8, H_β) and 2.28 (1H, dd, $J_{H\alpha,2}$ 6, $J_{H\beta,2}$ 8, 2-H); δ_C (50.31 MHz; $C^2H_3O^2H$) 13.9 (CH_3), 21.8 (C-3), 27.8 (C-1), 29.1 (C-2) and 174.2 & 177.5 (2 x CO_2H); *m/z* (CI) 145 (100, $[M + H]^+$), 130 (13, $[M + H - CH_3]^+$) and 127 (83, $[M + H - H_2O]^+$).

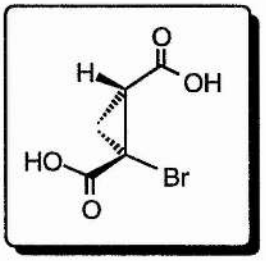
(-)-(1*S*,2*S*)-1-Bromo *trans*-cyclopropane-1,2-dioic Acid (100b)



This compound (**100b**) was prepared in manner identical to that for compound (**101b**), using the diastereomer (**98b**) (0.43 g, 0.96 mmol), and a 3 mol dm^{-3} solution of H_2SO_4 (1.9 cm^3, 5.8 mmol). The crude product was recrystallised using ethyl acetate/hexane (1:9) to leave the pure compound as a white crystalline solid (0.19 g, 95%), m.p. 174-175 °C; (Found: C, 28.85; H, 2.35.

$C_5H_5BrO_4$ requires C, 28.75; H, 2.40%); $[\alpha]_D$ -91.43 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 1699, 1290, 1242 and 1139; δ_H (200 MHz; ^2H_2O) 1.72, 1.94 (2H, m, H_α & H_β) and 2.60 (1H, m, 2-H); δ_C (50.31 MHz; ^2H_2O) 25.7 (C-3), 32.2 (C-2), 37.5 (C-1) and 171.8 & 174.9 (2 x CO_2H); *m/z* (CI) 209 & 211 (42, 38%, $[M + H]^+$), 191 & 193 (35, 33, $[M + H - H_2O]^+$) and 129 & 131 (100, 38, $[M + H - Br]^+$).

(+)-(1*R*,2*R*)-1-Bromo-*trans*-cyclopropane-1,2-dioic Acid (100a)



This compound (**100a**) was prepared in a manner identical to that for compound (**100b**), using the diastereomer (**98a**) (0.65 g, 1.45 mmol), and a 3 mol dm^{-3} solution of H_2SO_4 (2.9 cm^3, 8.73 mmol). The crude product was recrystallised using ethyl acetate/hexane (1:9), to leave the pure compound as a white crystalline solid (0.28 g, 92%), m.p. 174-175 °C; (Found: C, 28.95; H, 2.25. $C_5H_5BrO_4$ requires C, 28.75; H, 2.40%); *m/z* (Found: $[M + H]^+$, 208.9453. $C_5H_6BrO_4$ requires 208.9449); $[\alpha]_D$ +91.5 (*c* 1.0 in MeOH); υ_{max} (Nujol)/cm^{-1} 1699, 1291, 1245 and 1143; δ_H (200 MHz; ^2H_2O) 1.72 & 1.94 (2H, m, H_α & H_β) and 2.61 (1H, m, 2-H); δ_C (50.31 MHz; ^2H_2O) 25.7 (C-3), 32.3 (C-2), 37.4 (C-1) and 171.8 & 174.9 (2 x CO_2H); *m/z* (CI) 209 & 211 (33, 31%, $[M + H]^+$), 191 & 193 (31, 30, $[M + H - H_2O]^+$) and 129 &131 (100, 40, $[M + H - Br]^+$).

Enzyme Kinetics

3-Methylaspartate ammonia-lyase was purified by Dr. K. Morris from an *E. coli* cell growth containing the pSG4 plasmid.[31] The specific activity of the enzyme used in the kinetic studies, was 46-92 units (mg of protein)$^{-1}$.

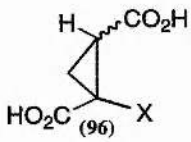
General Procedure for the Kinetic Inhibition Studies.

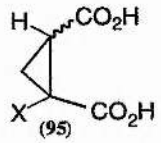
Experiments were performed at an incubation temperature of 30 °C, pH = 9, with[Mg^{2+}] ($MgCl_2.6H_2O$) = 20 mmol dm^{-3}, [K^+] (KCl) = 1 mmol dm^{-3}, tris buffer (tris[hydroxymethyl]aminomethane) = 500 mmol dm^{-3}, (2*S*,3*S*)-3-methylaspartic acid = 1-5 mmol dm^{-3} [inhibitor] (which was varied in concentration) and enzyme. The total volume of the enzyme assay ranged from 1-3 cm^3. The specific activity of the enzyme used in these studies, ranged from 46-92 units mg^{-1}, from which 25 μdm^3 was diluted with phosphate buffer (750 μdm^3, 10 mmol dm^{-3}). The kinetic experiments performed were allowed to equilibrate to 30 °C before being initiated by the addition of 10 μdm^3 of the diluted enzyme solution. Initial rates were measured in 1 or 3 cm^3 quartz cuvettes (10 mm path length) and each rate measurement was carried out in triplicate. The kinetic reaction was followed directly spectrophotometrically at 240 nm (Shimadzu-UV-2101 PC), under the stated conditions. Kinetic data was analysed with Lineweaver-Burk plots.

Determination of Inhibition via IC_{50} Assays

The assays were based on the general procedure as described above. The assays were compared by varying different inhibitor concentrations (0-10 mmol dm^{-3}) against a fixed concentration of substrate (2 mmol dm^{-3}).

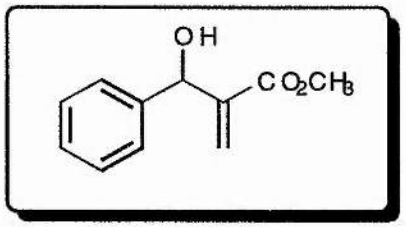
Summary of Assay Conditions for Methylaspartase Inhibitors





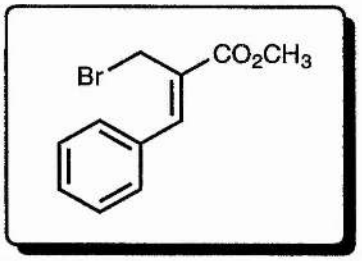
Reaction Conditions	Stereo-chemistry	X	[Inhibitor] (mmol dm^{-3})	[Substrate] (mmol dm^{-3})
standard	*(+,-)-trans-*	H	0, 20, 30, 40	1.0, 1.25, 1.67, 2.5, 5.0
standard	*(+,-)-cis-*	Me	0, 3, 7	1.0, 1.25, 1.67, 2.5, 5.0
standard	*(+,-)-trans-*	Me	0, 0.1, 0.25, 0.5	0.2,0.25,0.4,0.6,2.0
standard	*(+,-)-trans-*	Et	0, 1.0, 2.5, 5.0	1.0,1.25,1.67,2.5,5.0
standard	*(+,-)-trans-*	Cl	0, 1.0, 2.5, 5.0	1.0,1.25,1.67,2.5,5.0
standard	*(+,-)-trans-*	Br	0, 1, 2, 4, 8	2.0
standard	*(1R,2R)-trans-*	Br	0, 1.0, 2.5, 5.0	1.0,1.25,1.67,2.5,5.0
standard	*(1S,2S)-trans-*	Br	0, 3, 6, 10	2.0
standard	*(1S,2S)-trans-*	Me	0, 0.4, 0.8, 0.12	1.0,1.25,1.67,2.5,5.0
standard	*(1R,2R)-trans-*	Me	0, 0.5, 0.67, 1.0	1.0,1.25,1.67,2.5,5.0
std + NH_4^+	*(1S,2S)-trans-*	Me	0, 0.4, 0.8, 0.12	1.0,1.25,1.67,2.5,5.0

Methyl 3-Hydroxy 2-methylene-3-phenylpropenoate (115)



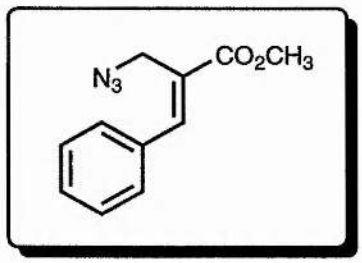
Benzaldehyde (24.6 g, 23.6 cm^3, 0.232 mol), methyl acrylate (13 g, 13.6 cm^3, 0.151 mol) and 1,4-diazabicyclo[2.2.2]octane (0.847 g, 75 mmol) were stirred together in a sealed flask for seven days at room temperature. The resultant mixture was washed successively with hydrochloric acid (6 mol dm^{-3}, 50 cm^3), sodium hydroxide (2 mol dm^{-3}, 50 cm^3) and saturated brine (50 cm^3). The organic portion was diluted with diethyl ether (50 cm^3), and then dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow oil, which was purified by distillation under reduced pressure to yield the desired compound (**115**) as a colourless oil, (26 g, 90%), b.p. 160 °C/18 mmHg; (Found: C 68.70; H, 6.00. Calc. for $C_{11}H_{12}O_3$ C, 68.50; H, 6.10%); υ_{max}(Neat)/cm^{-1} 1723, 1631 and 3467; δ_H (200 MHz; C^2HCl_3) 3.35 (1H, s, CH), 3.75 (3H, s, OCH_3), 5.57 (1H, s, CHO<u>H</u>), 5.89 & 6.38 (2H, s, C=CH_2) and 7.40 (5H, m, Ph); δ_C (50.13 MHz; C^2HCl_3) 52.40 (OCH_3), 73.52 (COH), 126.54, 127.23 and 128.30 (aromatic), 128.00 (C=<u>C</u>H_2), 141.81 (ipso-C), 142.56 (<u>C</u>=CH2) and 167.24 (<u>C</u>O_2CH_3).

Methyl (*E*)-2-Bromomethylcinnamate (116)



The hydroxy ester (**115**), (10 g, 50 mmol) was suspended in hydrobromic acid (48%, 290 mmol) and concentrated sulphuric acid (1.5 cm^3) and was stirred at room temperature for 12 h. The resulting solution was extracted with diethyl ether (3 x 50 cm^3) and the pooled organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow oil. This was purified by distillation to yield the bromo ester (**116**) as a clear pale yellow oil (11.2 g, 97%), b.p. 160 °C/5 mmHg; (Found: C 52.0; H, 4.5. Calc. for $C_{11}H_{11}O_2Br$: C, 52.0; H, 4.5%); υ_{max} (Neat)/cm^{-1} 1718 and 1626; δ_H (200 MHz; C^2HCl_3) 3.89 (3H, s, OCH_3), 4.40 (2H, s, CH_2Br), 7.85 (1H, s, C=CH) and 7.25 & 7.85 (5H, m, Ph); δ_C (50.13 MHz; C^2HCl_3), 27.4 (CH_2Br), 53.0 (OCH_3), 129.1-129.4 (aromatic), 130.2 (C=<u>C</u>H), 134.7 (ipso-C), 143.5 (<u>C</u>=CH) and 167.1 (<u>C</u>O_2CH_3).

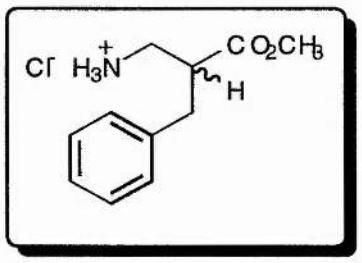
Methyl (*E*)-2-Azidomethylcinnamate (117)



The bromo ester, (**116**), (2.0 g, 10 mmol) was heated to reflux in 50% aqueous acetone (50 cm^3) for 4 h with sodium azide (0.616 g, 9.5 mmol). The resulting solution was concentrated to 25 cm^3 under reduced pressure, and the aqueous residue was then extracted with diethyl ether (3 x 50 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and the solvent was removed under reduced

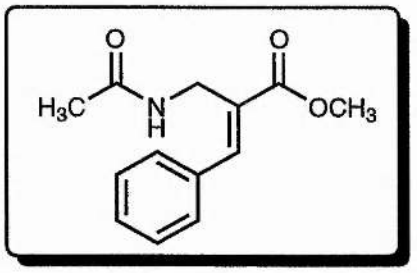
pressure to yield the desired product as a pale yellow opaque oil, (1.95 g, 90%). The isolated product (**117**) was used directly in the following step without any purification; υ_{max}(Neat)/cm^{-1} 2109, 1724 and 1632; δ_H (200 MHz; C^2HCl_3) 3.90 (3H, s, OCH_3), 4.19 (2H, s, CH_2N_3), 7.43 (5H, m, Ph) and 7.98 (1H, s, C=CH); δ_C (50.31 MHz; C^2HCl_3) 47.5 (CH_2N_3), 53.0 (OCH_3), 127.1, 129.3 &130.1, (aromatic), 130.2 (C=CH), 134.7 (ipso-C), 145.1 (C=CH) and 168.0 (CO_2CH_3); *m/z* (EI) 59 (45%, [$C_2H_3O_2^+$]), 77.(18, [$C_6H_6^+$]), 143 (100, [$C_{11}H_{11}^+$]) and 175 (68, [$C_{11}H_{11}O_2^+$]).

(±) Methyl 2-Benzyl-3-aminopropanoate. HCl (118)



A solution of the azido ester (**117**) (0.2 g, 0.92 mmol) in methanol (10 cm^3) was stirred overnight with 2% palladium on charcoal (0.04 g) under hydrogen. After the reaction was complete (as monitored by t.l.c.), the reaction mixture was filtered, and concentrated under reduced pressure. To the crude oil was added an aqueous solution of HCl (5 cm^3, 1 mol dm^{-3}) and the mixture was left to stir at 0 °C for 30 min. The mixture was extracted with diethyl ether (3 x 10 cm^3) and the aqueous phase was concentrated under reduced pressure. The solid residue, was recrystallised from water/ethanol to yield a white crystalline solid (0.125 g, 70%), m.p. 140 °C; (Found: [M + H]$^+$ 193.1094. $C_{11}H_{15}NO_2$ requires 193.1099); υ_{max} (Nujol)/cm^{-1} 1732; δ_H (200 MHz; ^2H_2O) 2.95 (2H, m, CH_2), 3.12 (3H, m, CH_2 & CH), and 3.71 (3H, s, OCH_3); δ_C (50.31 MHz; ^2H_2O) 38.28 (CH_3), 42.5 (CH), 47.5 (CH_2), 55.62 (CH_2-Ph), 130.10-140.03 (aromatic) and 177.53 (C=O); *m/z* (CI), 193 (58%, [M + H]$^+$), 176 (100, [$C_{11}H_{12}O_2$]$^+$) and 91 (34, [C_7H_7]$^+$).

Methyl [*N*-acetyl]-3-Aminomethylcinnamate (121)



To a stirred solution of methyl (Z)-2-azidomethyl-3-phenylprop-2-enoate (**117**) (5 g, 23 mmol) in a dry solution of THF (50 cm^3) was added triphenyl phosphine (6 g, 23 mmol) under anhydrous conditions. The resulting mixture was stirred for 1-2 h at room temperature until no further starting material remained (as judged by t.l.c). To the reaction mixture was added water (0.63 cm^3, 35 mmol) and stirring was continued for a further 4 h, after which time the THF was removed under reduced pressure. The remaining oil was triturated in cold diethyl ether which resulted in the precipitation of triphenylphosphine oxide. The triphenylphosphine oxide was filtered off and the trituration procedure repeated until no traces of the oxide could be detected by t.l.c. To the filtrate was added water (20 cm^3) and the mixture was extracted with diethyl ether (3 x 30 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and concentrated to yield the desired ester (**120**) as a pale yellow oil (4.1 g, 93%).

The ester (**120**) (1.0 g, 5.2 mmol) was dissolved in anhydrous THF (15 cm^3) and the solution was allowed to stir at 0 °C. To the cold solution was then added dropwise, acetic anhydride (2.67 g, 26.7 mmol) and the resulting mixture was stirred at 0 °C for 2 h. The reaction mixture was concentrated under reduced pressure. The solid residue was recrystallised from ethyl acetate/light petroleum, to yield the desired compound as a white crystalline solid (0.95 g, 78%), m.p. 133-134 °C; *m/z* (Found: $[M]^+$, 233.1048. $C_{22}H_{27}N_2O_4$ requires 233.1052); υ_{max} (Nujol)/cm^{-1}, 2253, 1701, 1438 and 1274; δ_H (200 MHz; C^2HCl_3) 2.0 (3H, s, CH_3), 3.86 (3H, s, -CO_2CH_3), 4.35 (2H, d, CH_2), 6.19 (1H, b, -NH), 7.31-7.64 (5H, m, Ph) and 7.8 (1H, s, -C=C<u>H</u>-); δ_C (50.31 MHz; C^2HCl_3) 23.83 (CH_2), 37.31 (<u>C</u>H_3CO), 52.77 (CO_2<u>C</u>H_3), 128.9-130.15 (aromatic), 132.48 (C=<u>C</u>H), 132.66 (ipso-C), 134.60 (<u>C</u>=CH)

168.85 ($CH_3\underline{C}O$) and 170.21 (CO_2CH_3); *m/z* (EI) 233 (30%, $[M]^+$), 201 (18, $[M - CH_3OH]^+$), 190 (100, $[M - C_2H_3O]^+$) and 173 (15, $[M - C_2H_6NO]^+$).

CHAPTER 4

APPENDIX

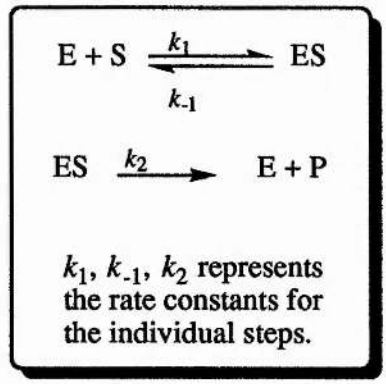
Appendix 1

Enzyme Kinetics

3-Methylaspartase catalyses a reaction in which a single substrate, (2*S*,3*S*)-methylaspartic acid (**2**), is converted into a single product, mesaconic acid (**3**) (Scheme 1.6). To understand some of the kinetic results presented here, simple Michaelis-Menten kinetics and inhibition of one substrate reactions have been described.

Michaelis-Menten Kinetics

Enzyme catalysis generally occurs *via* rapid and reversible formation of a complex, ES, between enzyme, E, and its substrate, S. The region of the enzyme to which the substrate binds, is known as the *active site* . This complex, breaks down in a slow step giving the product, P, and regenerating the enzyme (Scheme 4.1).



Scheme 4.1: *The Conversion of Substrate to Product in an Enzyme Catalysed Reaction*

In scheme 4.1, the breakdown of the ES complex to E + S is faster than the breakdown of ES complex to E + P. The equilibrium constant, *K*, is defined by:

$$K = \frac{[E][S]}{[ES]} \qquad \text{equation 1}$$

(where [E] and [S] are the concentrations of *free* enzyme and *free* substrate respectively). As the total concentration of substrate is much greater than the

total concentration of enzyme, and thus we can set $[S]_{free}$ little from $[S]_{total}$, and can be approximated to $[S]_{total}$.

The fractional saturation, F, of the enzyme is given by:

$$F = \frac{[ES]}{[E] + [ES]} \qquad \text{equation 2}$$

Thus, from equation 1,

$$F = \frac{\frac{[E][S]}{K}}{[E] + \frac{[E][S]}{K}} \qquad \text{equation 3}$$

The total concentration of enzyme is written $[E]_0$, then $[ES] = F[E]_0$; thus from equation 3,

$$[ES] = \frac{[E]_0 [S]}{K + [S]} \qquad \text{equation 4}$$

The rate of product formation, v, is determined by the rate of [ES] breakdown, such that $v = k_2$ [ES],

$$\therefore v = k_2 \frac{[E]_0 [S]}{K + S} \qquad \text{equation 5}$$

The maximum rate, V_{max}, will be observed when all the enzyme is in the form of the ES complex. (*i.e.* $V_{max} = k_2 [E]_0$)

$$v = \frac{V_{max} [S]}{K + [S]} \qquad \text{equation 6}$$

From this it can be seen that when [S] = K, the rate of product formation is half-maximal ($v = V_{max}/2$). K is termed the Michaelis constant, and is normally written as K_m. K_m is a crude measure of the affinity of an enzyme, for its substrate.

When interpreting kinetic data, it is often difficult to determine the limiting value of v against [S], and therefore, by implication K_m. This can be overcome by rearranging equation 6 so as to give a linear graphical representation. Some of the best known rearranged forms are given below:

(*i*) The *Lineweaver-Burk* plot[154]: This equation is obtained by taking reciprocals of the two sides of equation 6 yields:

$$\frac{1}{v} = \frac{K_m}{[S]} \frac{1}{V_{max}} + \frac{1}{V_{max}} \qquad \text{equation 7}$$

Thus a plot of $1/v$ against $1/[S]$ gives a straight line of slope K_m/V_{max}, with x and y intercepts of $-1/K_m$ and $1/V_{max}$ respectively (Fig. 4.1, A).

(*ii*) The *Eadie-Hofstee* plot[155,156]: Equation 6 can be rearranged to give:

$$\frac{v}{[S]} = \frac{V_{max}}{K_m} - \frac{v}{K_m} \qquad \text{equation 8}$$

Thus a plot of $v/[S]$ against v gives a straight line of slope $-1/K_m$ and with an *x*-axis intercept of V_{max} (Fig. 4.1, B).

(*iii*) The *Hanes* plot[157]: Equation 6 is rearranged to give:

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \qquad \text{equation 9}$$

Thus a plot of $[S]/v$ against [S] is linear with a slope of $1/V_{max}$ and an x-axis intercept of $-K_m$ (Fig. 4.1, C).

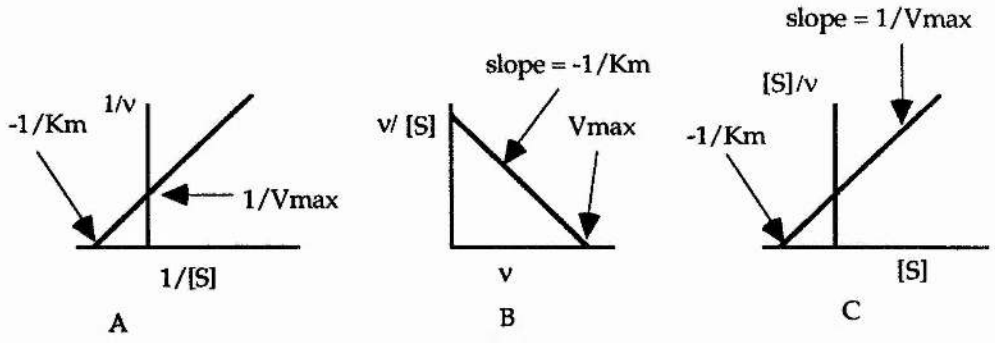


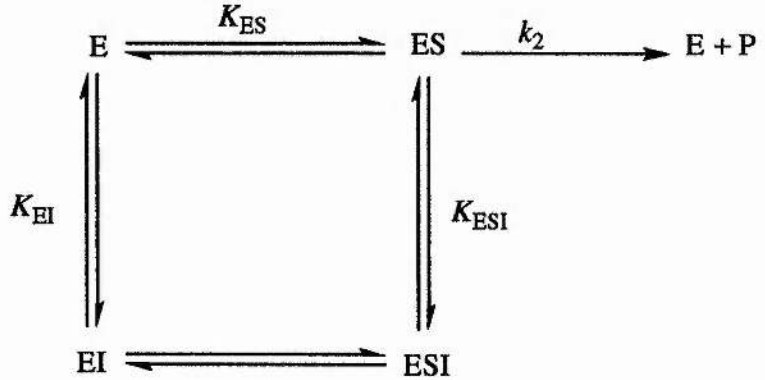
Figure 4.1: *Graphical Representation of Enzymic Data*

Inhibitors of enzyme-catalysed reactions are important as they can give an insight at the molecular level, into the active site structure and catalytic mechanism of the enzyme. Since the cyclopropane inhibitors designed for MAL, were found to behave as reversible inhibitors. I shall confine my attention to reversible inhibitors.

General Overview of Inhibition

Enzymes are known to be irreversibly inactivated by heat or chemical reagents. They can also be *reversibly* inhibited by the non covalent binding of ligands. There are four main types of reversible inhibition, each of which will be discussed here.

Enzyme-catalysed reactions usually follow the general scheme (Scheme 4.2), as below.



Scheme 4.2: *A General Overview of Inhibition in Enzyme-Catalysed Reactions*

We can follow this general scheme when dealing with different types of inhibition.

(*i.*) Competitive Inhibition:

In this case, the inhibitor, I, binds reversibly to the active site of the enzyme and prevents substrate, S, binding, and *vice versa*. I and S therefore compete for the active site and I is said to be a *competitive* inhibitor. It follows that $K_{ESI} = \infty$, and the general kinetic equation for the Lineweaver-Burk plot becomes:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{ES}}{V_{max}}\left[1 + \frac{[I]}{K_{EI}}\right]\frac{1}{[S]} \qquad \text{equation 10}$$

The effect of the inhibitor on the Lineweaver-Burk plot is shown in Fig. 4.2 V_{max} is unaffected but the apparent K_m is increased by a factor $(1 + [I]/K_{EI})$. Effectively, the inhibitor pulls some of the enzyme over into the form of the EI complex. When the concentration of S is increased sufficiently, the effect on the rate can be overcome.

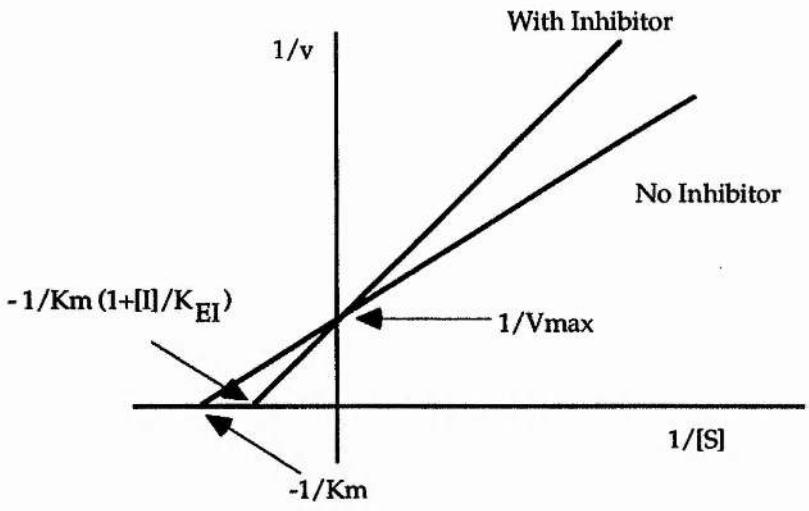


Figure 4.2: *Effect of a Competitive Inhibitor on a Lineweaver-Burk Plot*

The Lineweaver-Burk plot has the disadvantage of compressing the data points at high substrate concentrations into a small region and emphasising points at lower concentrations. It can therefore be inaccurate when determining

K_m values. K_i values can be determined by the application of a secondary plot. The secondary graph, is a plot of the gradient, m, from the Lineweaver-Burk plot, *versus* inhibitor concentration, [I], and is of the form:

$$m = \frac{K_m}{V_{max}} + \frac{K_m[I]}{V_{max}K_i} \qquad \text{equation 11}$$

It can be seen that the gradient of this secondary plot is $\frac{K_m}{V_{max}K_i}$ and the y-intercept is at $\frac{K_m}{V_{max}}$.

As $\frac{apparent\ K_m}{apparent\ V_{max}} = \frac{K_m}{V_{max}}\left(1+\frac{[I]}{K_i}\right)$, a plot of the $\frac{apparent\ K_m}{apparent\ V_{max}}$ *versus* inhibitor concentration, [I], also gives the same gradient and y-intercept as described above. The apparent K_m and V_{max} values are obtained *via* non-linear regression on the substrate, [S], *versus* the rate, (υ), plot.

(*ii*) Uncompetitive Inhibition:

When the inhibitor, I, cannot combine with E, but only combines with the ES complex, this is known as *uncompetitive* inhibition (*i.e.* when $K_{EI} = \infty$). The general kinetic equation used for uncompetitive inhibition is:

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1+\frac{[I]}{K_{ESI}}\right] + \frac{K_{ES}}{V_{max}}\frac{1}{[S]} \qquad \text{equation 12}$$

The effect of the inhibitor on the Lineweaver-Burk plot is shown in Fig. 4.4.

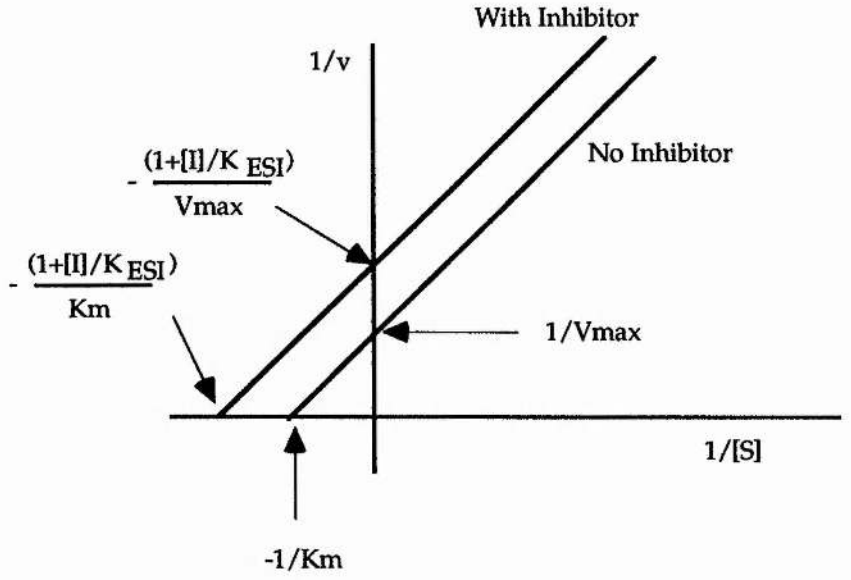


Figure 4.4: *Effect of an UnCompetitive Inhibitor on a Lineweaver-Burk Plot*

For uncompetitive inhibition, both K_m and V_{max} are affected to the same degree, by the inhibitor giving rise to parallel lines.

(iii) Non-Competitive Inhibition:

When the binding of substrate, S, to the enzyme does not affect the binding of inhibitor, I, (*i.e.* $K_{ESI} = K_{EI}$) then this is known as *non-competitive* inhibition. The general kinetic equation used for non-competitive inhibition is:

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1+\frac{[I]}{K_{EI}}\right] + \frac{K_{ES}}{V_{max}}\left[1+\frac{[I]}{K_{EI}}\right]\frac{1}{[S]} \quad \text{equation 13}$$

The effect of the inhibitor on the Lineweaver-Burk plot is shown in Fig. 4.3.

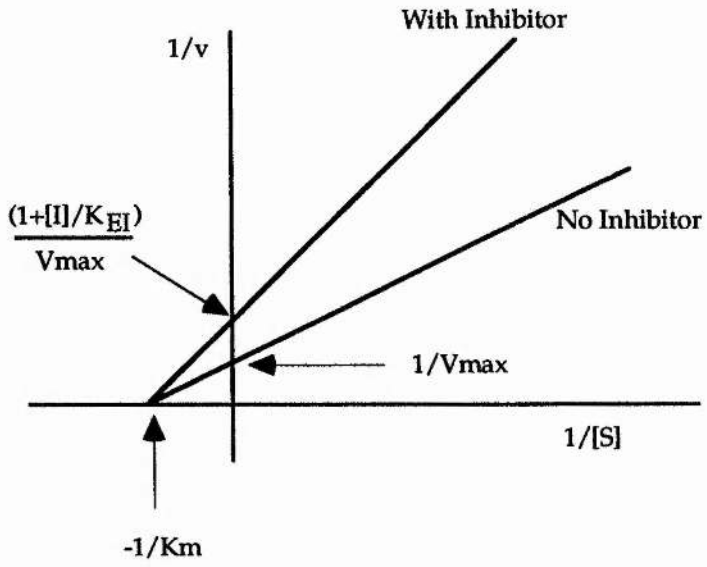


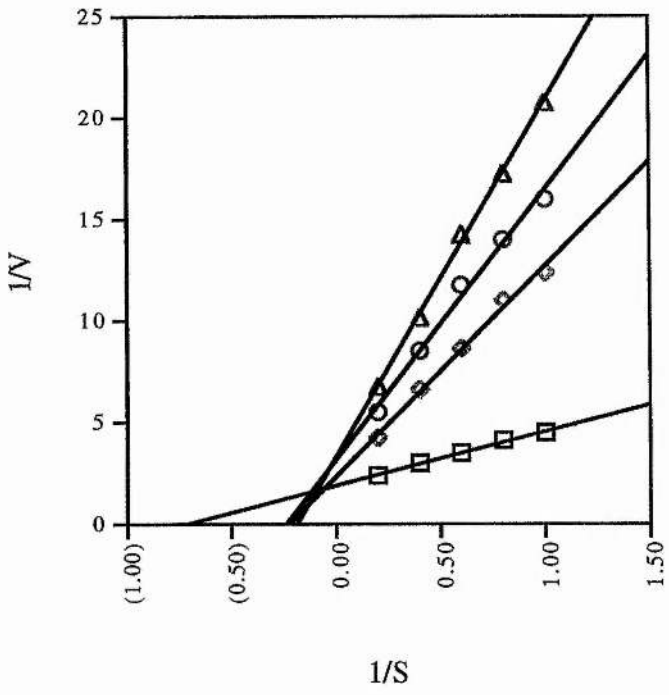
Figure 4.3: *Effect of a Non-Competitive Inhibitor on a Lineweaver-Burk Plot*

The K_m remains unaffected, but V_{max} is decreased by a factor of $1/(1+([I]/K_{EI}))$. Addition of inhibitor pulls both E and ES over into inactive forms (EI and ESI respectively), but does not effect the distribution between them. Hence, increasing the concentration of S cannot overcome the effect of the inhibitor on the rate.

(*iv*) Mixed Inhibition

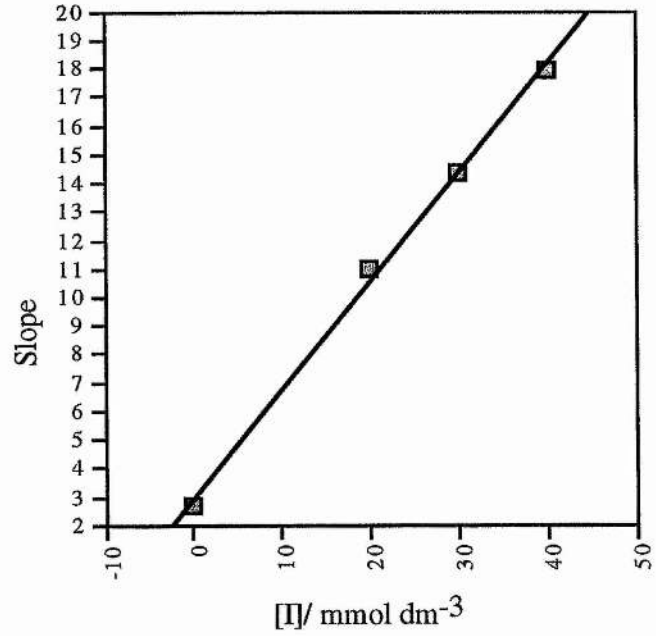
When the binding of substrate, S, to the enzyme affects the binding of inhibitor, I, such that K_{ESI} is different from K_{EI}, the inhibition is termed *mixed*. In this case, both K_m and V_{max} are altered. The cross-over point is found in either of the negative x quadrants.

Lineweaver-Burk Plot for (±)-*trans*-Cyclopropane 1,2-Dioic Acid



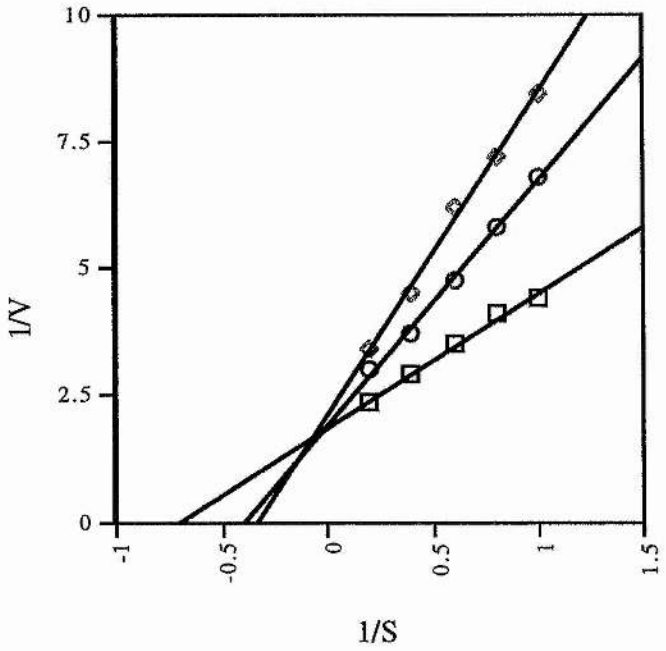
	App Km	App Vmax
0 mM [I]	1.48	0.55
20 mM [I]	5.62	0.51
30 mM [I]	5.91	0.33
40 mM [I]	5.46	0.4

Secondary Plot



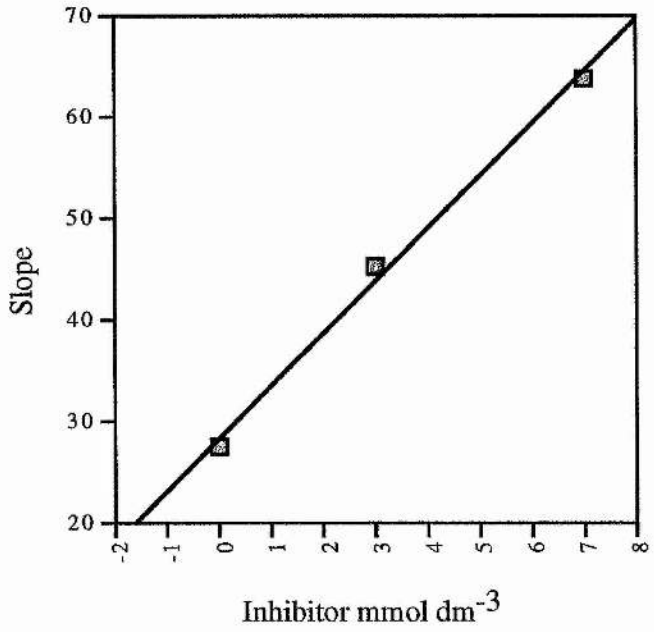
Slope $y = 0.381x + 2.931$ $r^2 = 0.997$

Lineweaver-Burk Plot for (±)-*cis*-1-Methylcyclopropane 1,2-Dioic Acid



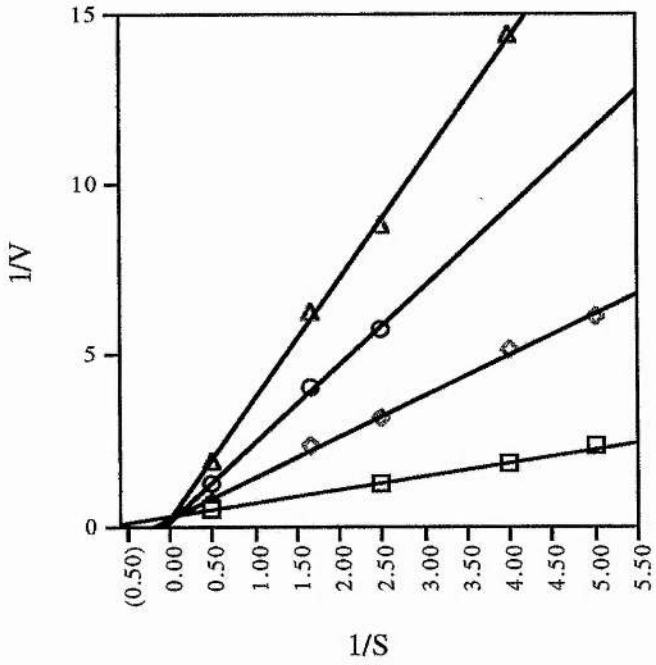
		App Km	App Vmax
□	0mM	1.51	0.55
◇	7.0mM	2.19	0.48
○	3mM	3.04	0.48

Secondary Plot



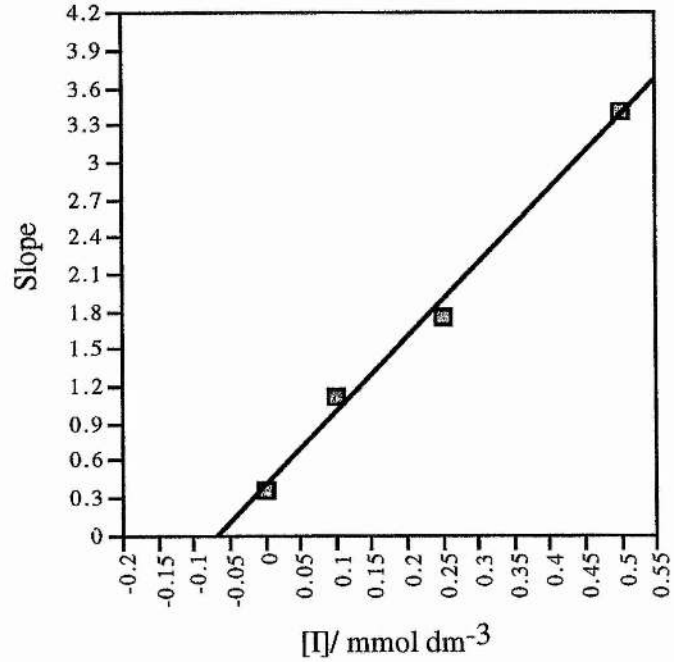
■ slope $y = 5.172x + 28.295$ $r^2 = 0.996$

Lineweaver-Burk Plot for (±)-*trans*-1-Methylcyclopropane 1,2-Dioic Acid



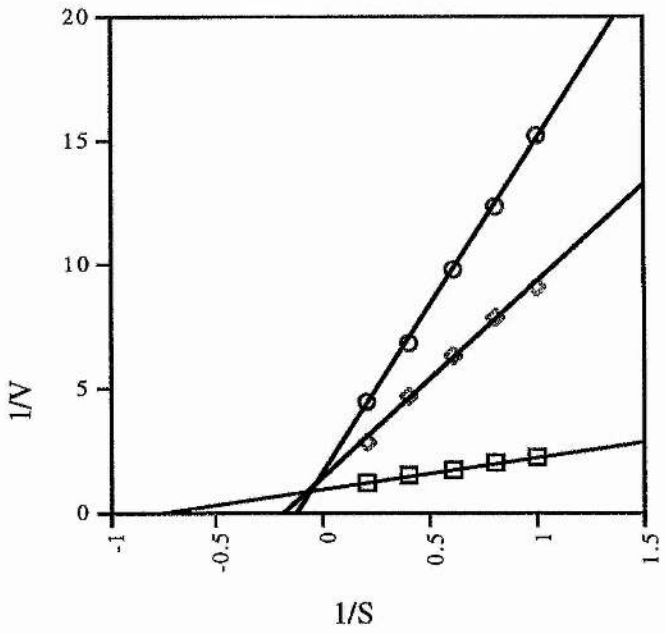
		App Km	App Vmax
□	0 mM [I]	0.91	2.52
◇	0.1 mM [I]	2.26	2.02
○	0.25 mM [I]	1.50	0.85
△	0.5 mM [I]	6.21	1.82

Secondary Plot



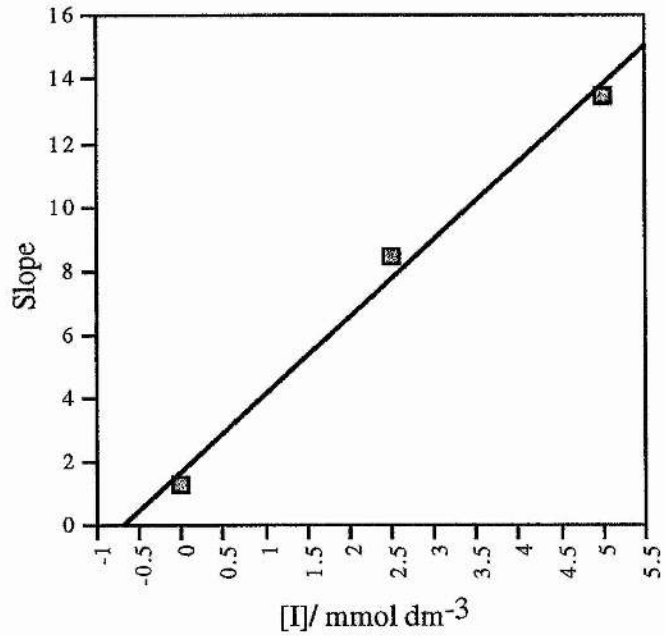
■ Slope $y = 5.947x + 0.399$ $r^2 = 0.993$

Lineweaver-Burk Plot for (±)-*trans*-1-Ethylcyclopropane 1,2-Dioic Acid



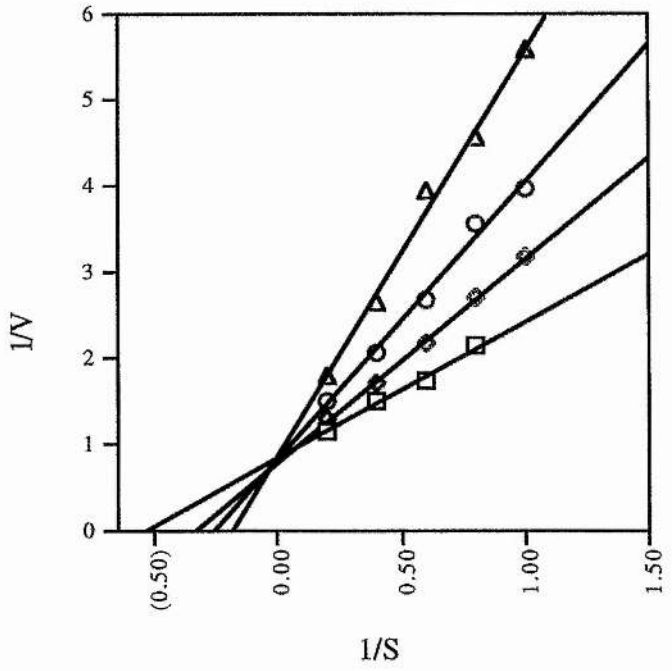
		App Km	App Vmax
□	0mM	1.36	1.04
◇	2.5mM	7.22	0.85
○	5.0mM	7.03	0.54

Secondary Plot



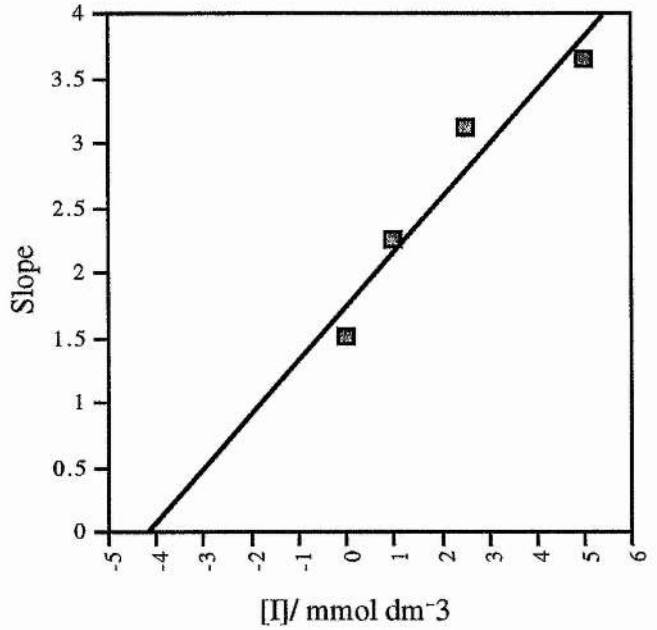
■ Slope $y = 2.433x + 1.676$ $r^2 = 0.989$

Lineweaver-Burk Plot for (±)-1-Chlorocyclopropane 1,2-Dioic Acid



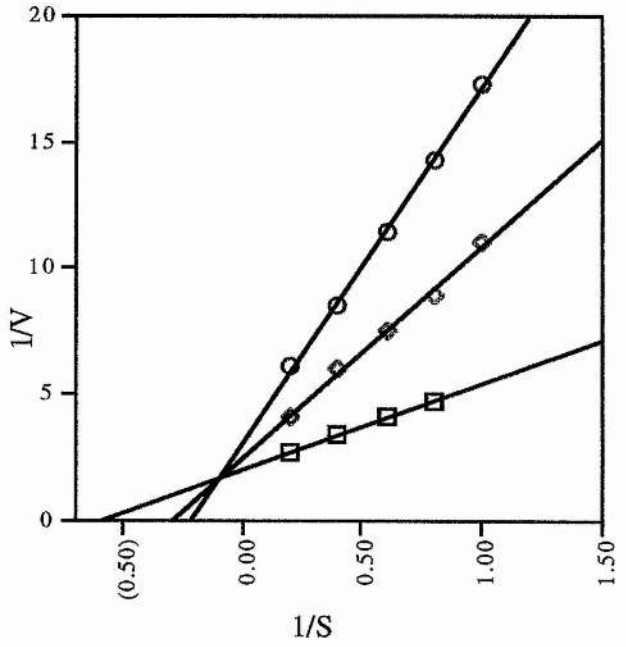
		App Km	App Vmax
□	0 mM [I]	1.75	1.16
◇	1.0 mM [I]	2.65	1.18
○	2.5 mM [I]	3.59	1.15
△	5.0 mM [I]	3.29	0.9

Secondary Plot



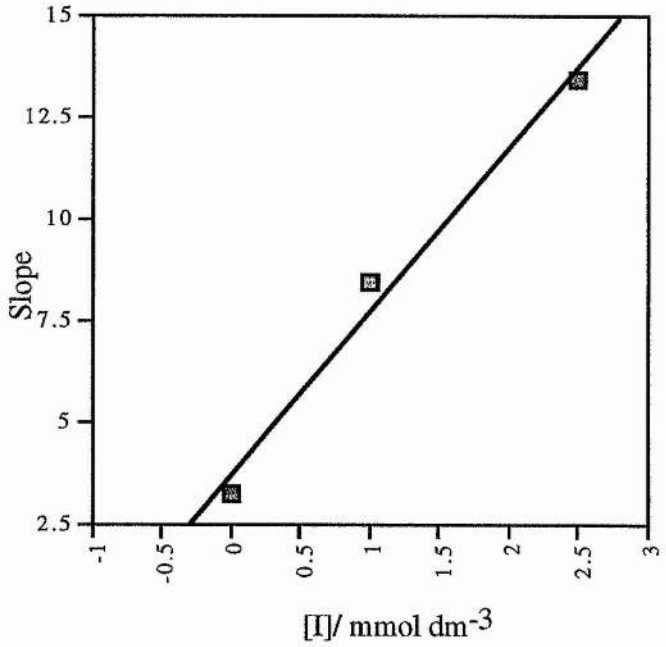
■ Slope $y = 0.420x + 1.743$ $r^2 = 0.925$

Lineweaver-Burk Plot for (1*R*,2*R*)-1-Bromocyclopropane 1,2-Dioic Acid



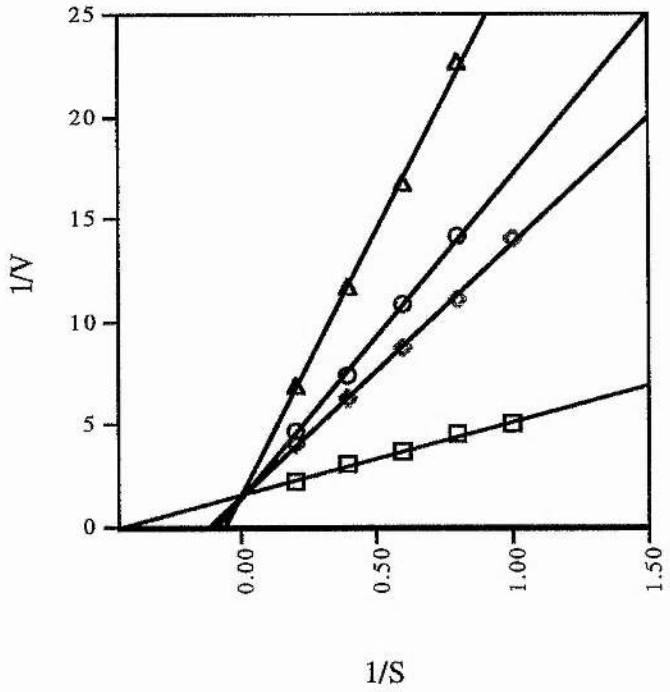
		App Km	App Vmax
□	0 mM [I]	1.63	0.5
◇	1.0 mM [I]	3.56	0.42
○	2.5 mM [I]	4.04	0.30

Secondary Plot



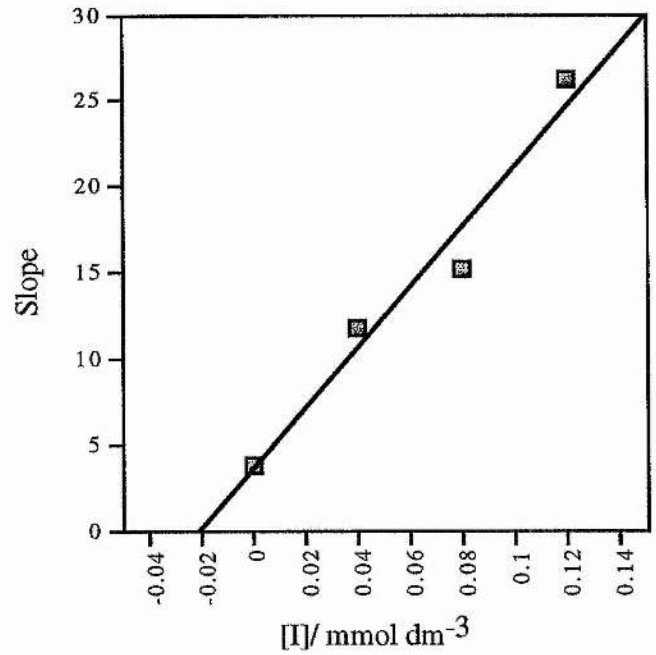
■ Slope $y = 4.024x + 3.708$ $r^2 = 0.984$

Lineweaver-Burk Plot for (1*S*,2*S*)-1-Methylcyclopropane 1,2-Dioic Acid



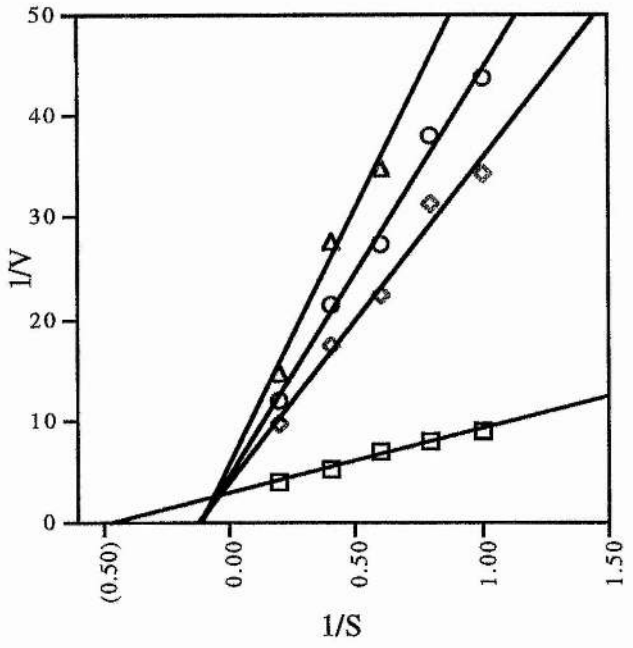
		App Km	App Vmax
□	0 mM [I]	2.60	0.69
◇	40uM [I]	6.93	0.59
○	80uM [I]	9.42	0.62
△	120uM [I]	16.5	0.63

Secondary Plot



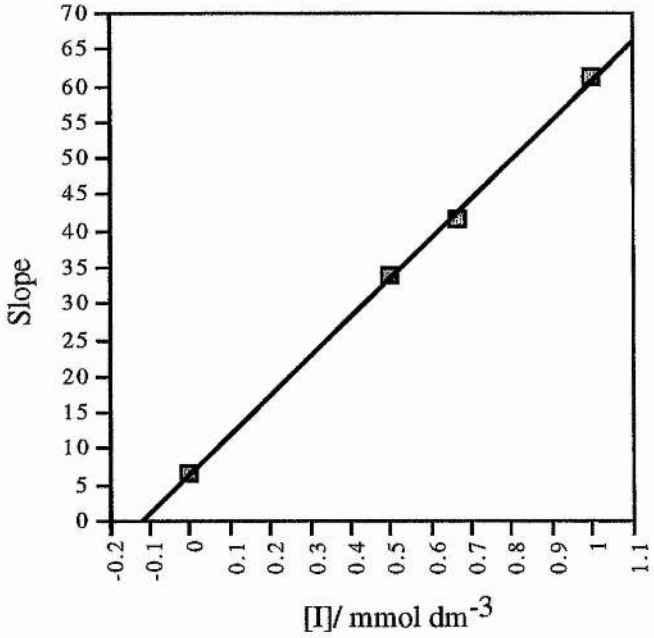
■ Slope $y = 176.750x + 3.620$ $r^2 = 0.963$

Lineweaver-Burk Plot for (1*R*,2*R*)-1-Methylcyclopropane 1,2-Dioic Acid



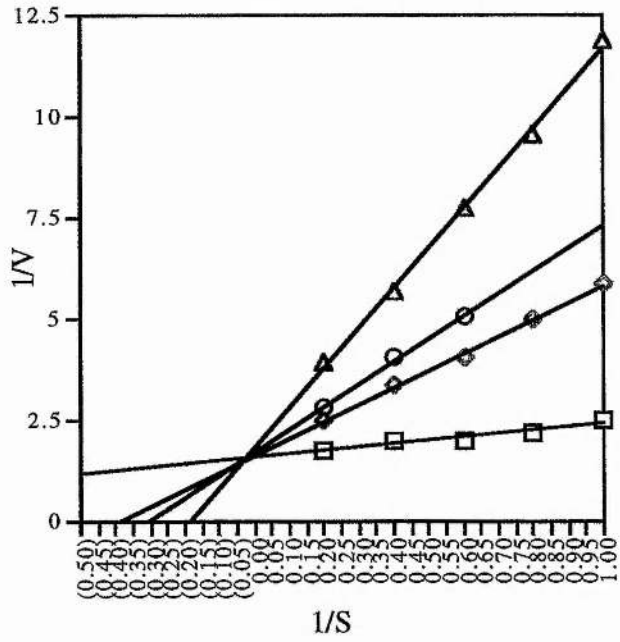
		App Km	App Vmax
□	0 mM [I]	2.51	0.37
◇	0.5 mM [I]	11.83	0.35
○	0.67 mM [I]	10.81	0.26
△	1.0 mM [I]	24.57	0.40

Secondary Plot



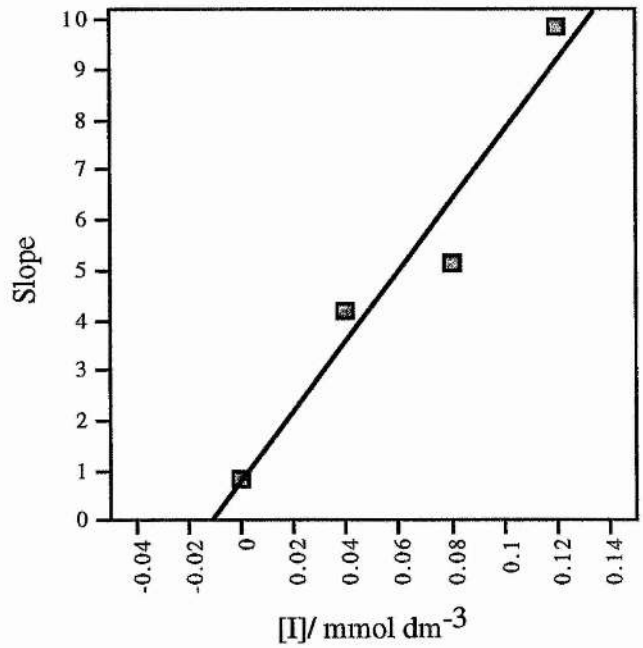
■ Slope $y = 54.231x + 6.477$ $r^2 = 0.999$

Lineweaver Burk Plot for (1*S*,2*S*)-1-Methylcyclopropane 1,2-Dioic Acid in the Presence of Ammonia (10 mmol dm^{-3})



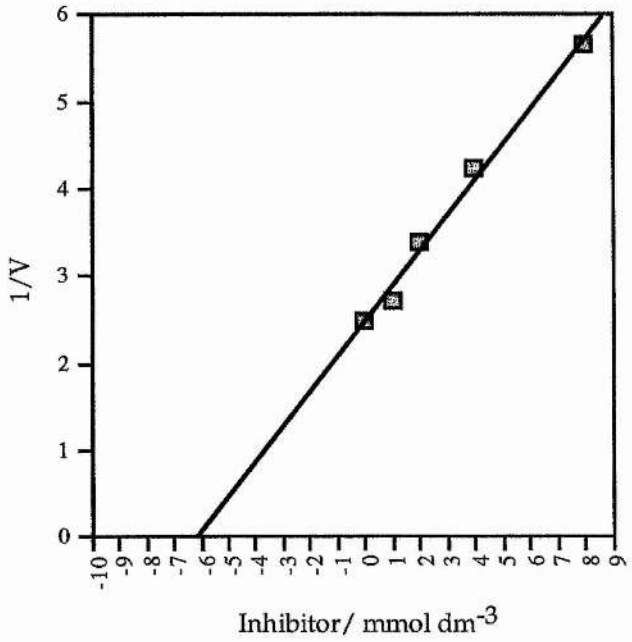
		App Km	App Vmax
□	0 mM [I]	0.5	0.62
◇	40uM [I]	2.47	0.60
○	80uM [I]	3.15	0.58
△	120uM [I]	4.57	0.49

Secondary Plot



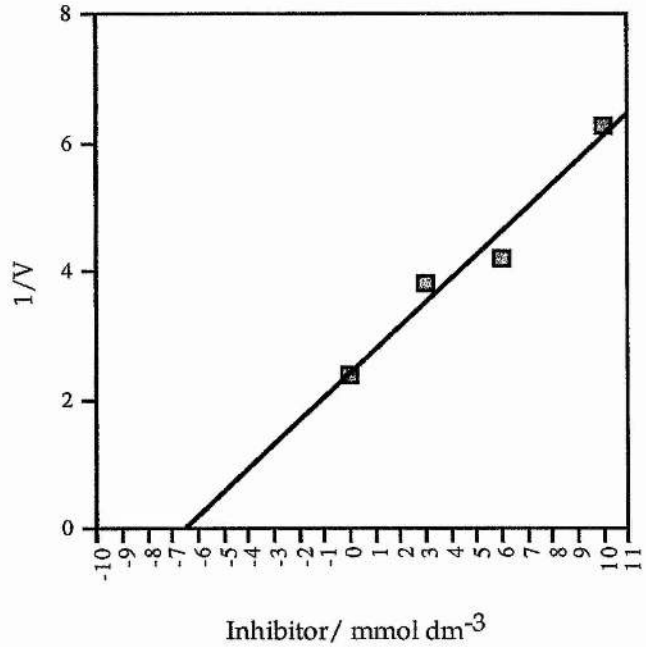
Slope $y = 70.056x + 0.805$ $r^2 = 0.943$

IC_{50} Plot of (±)-1-Bromocyclopropane 1,2-Dioic Acid



1/V $y = 0.406x + 2.476$ $r^2 = 0.991$

IC_{50} Plot of (1*S*,2*S*)--1-Bromocyclopropane 1,2-Dioic Acid



1/V $y = 0.369x + 2.406$ $r^2 = 0.961$

Crystallography Data

(1*R*,2*R*)-1-Methylcyclopropane 1,2-dicaboxylic acid (**101a**)
Intramolecular Bond Angles (°) Involving the Nonhydrogen Atoms

atom	atom	atom	angle	atom	atom	atom	angle
C(5)	N(1)	C(6)	120.7(9)	C(9)	C(8)	C(13)	118(1)
C(14)	N(2)	C(15)	120.7(9)	C(8)	C(9)	C(10)	120(1)
C(2)	C(1)	C(3)	57.9(6)	C(9)	C(10)	C(11)	122(1)
C(2)	C(1)	C(4)	119.5(8)	C(10)	C(11)	C(12)	118(1)
C(2)	C(1)	C(5)	114.5(9)	C(11)	C(12)	C(13)	120(2)
C(3)	C(1)	C(4)	119.3(9)	C(8)	C(13)	C(12)	121(1)
C(3)	C(1)	C(5)	114.1(9)	O(3)	C(14)	N(2)	123.6(9)
C(4)	C(1)	C(5)	117.8(9)	O(3)	C(14)	C(2)	124(1)
C(1)	C(2)	C(3)	59.0(6)	N(2)	C(14)	C(2)	112.8(9)
C(1)	C(2)	C(14)	123.2(9)	N(2)	C(15)	C(16)	107.3(9)
C(3)	C(2)	C(14)	119.8(9)	N(2)	C(15)	C(17)	113.2(8)
C(1)	C(3)	C(2)	63.1(7)	C(16)	C(15)	C(17)	108.2(9)
O(1)	C(5)	N(1)	125(1)	O(4)	C(16)	C(15)	113.8(9)
O(1)	C(5)	C(1)	119(1)	C(15)	C(17)	C(18)	124(1)
N(1)	C(5)	C(1)	115(1)	C(15)	C(17)	C(22)	118.4(9)
N(1)	C(6)	C(7)	108.9(9)	C(18)	C(17)	C(22)	117(1)
N(1)	C(6)	C(8)	110(1)	C(17)	C(18)	C(19)	123(1)
C(7)	C(6)	C(8)	117.3(9)	C(18)	C(19)	C(20)	119(1)
O(2)	C(7)	C(6)	112(1)	C(19)	C(20)	C(21)	121(1)
C(6)	C(8)	C(9)	120(1)	C(20)	C(21)	C(22)	121(1)
C(6)	C(8)	C(13)	121(1)	C(17)	C(22)	C(21)	119(1)

Intramolecular Distances (Å) Involving the Nonhydrogen Atoms

atom	atom	distance	atom	atom	distance
O(1)	C(5)	1.25(1)	C(6)	C(8)	1.55(1)
O(2)	C(7)	1.35(2)	C(8)	C(9)	1.40(1)
O(3)	C(14)	1.23(1)	C(8)	C(13)	1.35(1)
O(4)	C(16)	1.43(1)	C(9)	C(10)	1.36(2)
N(1)	C(5)	1.34(1)	C(10)	C(11)	1.36(2)
N(1)	C(6)	1.46(1)	C(11)	C(12)	1.39(2)
N(2)	C(14)	1.34(1)	C(12)	C(13)	1.39(2)
N(2)	C(15)	1.47(1)	C(15)	C(16)	1.55(2)
C(1)	C(2)	1.56(1)	C(15)	C(17)	1.53(1)
C(1)	C(3)	1.49(1)	C(17)	C(18)	1.36(1)
C(1)	C(4)	1.52(1)	C(17)	C(22)	1.40(1)
C(1)	C(5)	1.51(1)	C(18)	C(19)	1.35(1)
C(2)	C(3)	1.48(1)	C(19)	C(20)	1.36(2)
C(2)	C(14)	1.47(1)	C(20)	C(21)	1.33(1)
C(6)	C(7)	1.55(2)	C(21)	C(22)	1.37(1)

(1*S*,2*S*)-1-Bromocyclopropane 1,2-dicarboxylic acid (**100b**)

Intramolecular Bond Angles (°) Involving the Nonhydrogen Atoms

atom	atom	atom	angle	atom	atom	atom	angle
C(13)	N(1)	C(14)	125(2)	C(8)	C(7)	C(12)	114(2)
C(4)	N(2)	C(5)	122(2)	C(7)	C(8)	C(9)	122(2)
Br(1)	C(1)	C(2)	113(1)	C(8)	C(9)	C(10)	120(3)
Br(1)	C(1)	C(3)	115(1)	C(9)	C(10)	C(11)	119(3)
Br(1)	C(1)	C(4)	116(1)	C(10)	C(11)	C(12)	120(3)
C(2)	C(1)	C(3)	59(1)	C(7)	C(12	C(11)	125(2)
C(2)	C(1)	C(4)	120(1)	O(3)	C(13)	N(1)	120(2)
C(3)	C(1)	C(4)	122(2)	O(3)	C(13)	C(2)	123(2)
C(1)	C(2)	C(3)	56(1)	N(1)	C(13)	C(2)	117(2)
C(1)	C(2)	C(13)	125(2)	N(1)	C(14)	C(15)	110(2)
C(3)	C(2)	C(13)	124(2)	N(1)	C(14)	C(16)	112(1)
C(1)	C(3)	C(2)	65(1)	C(15)	C(14)	C(16)	111(2)
O(1)	C(4)	N(2)	125(2)	O(4)	C(15)	C(14)	108(2)
O(1)	C(4)	C(1)	117(2)	C(14)	C(16)	C(17)	123(2)
N(2)	C(4)	C(1)	118(2)	C(14)	C(16)	C(21)	123(2)
N(2)	C(5)	C(6)	109(1)	C(17)	C(16)	C(21)	114(2)
N(2)	C(5)	C(7)	116(2)	C(16)	C(17)	C(18)	123(2)
C(6)	C(5)	C(7)	113(2)	C(17)	C(18)	C(19)	121(2)
O(2)	C(6)	C(5)	112(2)	C(18)	C(19)	C(20)	116(2)
C(5)	C(7)	C(8)	119(2)	C(19)	C(20)	C(21)	122(2)
C(5)	C(7)	C(12)	127(2)	C(16)	C(21)	C(20)	125(2)

Intramolecular Distances (Å) Involving the Nonhydrogen Atoms

atom	atom	distance	atom	atom	distance
Br(1)	C(1)	1.97(2	C(5)	C(7)	1.49(3)
O(1)	C(4)	1.23(2)	C(7)	C(8)	1.46(3)
O(2)	C(6)	1.39(2)	C(7)	C(12)	1.34(3)
O(3)	C(13)	1.24(2)	C(8)	C(9)	1.36(3)
O(4)	C(15)	1.39(2)	C(9)	C(10)	1.38(3)
N(1)	C(13)	1.37(2)	C(10)	C(11)	1.37(3)
N(1)	C(14)	1.45(2)	C(11)	C(12)	1.37(3)
N(2)	C(4)	1.32(2)	C(14)	C(15)	1.53(3)
N(2)	C(5)	1.44(2)	C(14)	C(16)	1.50(2)
C(1)	C(2)	1.57(2)	C(16)	C(17)	1.40(3)
C(1)	C(3)	1.45(2)	C(16)	C(21)	1.36(2)
C(1)	C(4)	1.47(2)	C(17)	C(18)	1.38(2)
C(2)	C(3)	1.49(2)	C(18)	C(19)	1.41(3)
C(2)	C(13)	1.39(2)	C(19)	C(20)	1.35(3)
C(5)	C(6)	1.58(2)	C(20)	C(21)	1.37(2)

(±)-3,5-*bis*[Carbomethoxy]isoxazoline-*N*-oxide

Intramolecular Bond Angles (°) Involving the Nonhydrogen Atoms

atom	atom	atom	angle	atom	atom	atom	angle
N(1)	O(1)	C(3)	105.7(2)	O(1)	C(3)	C(2)	106.0(2)
C(4)	O(4)	C(5)	115.4(2)	O(1)	C(3)	C(6)	108.8(2)
C(6)	O(6)	C(7)	116.7(2)	C(2)	C(3)	C(6)	112.4(2)
O(1)	N(1)	O(2)	112.9(2)	O(3)	C(4)	O(4)	125.2(3)
O(1)	N(1)	C(1)	111.2(2)	O(3)	C(4)	C(1)	120.9(3)
O(2)	N(1)	C(1)	135.9(2)	O(4)	C(4)	C(1)	113.9(2)
N(1)	C(1)	C(2)	111.9(2)	O(5)	C(6)	O(6)	126.0(3)
N(1)	C(1)	C(4)	125.2(3)	O(5)	C(6)	C(3)	125.2(3)
C(2)	C(1)	C(4)	122.8(2)	O(6)	C(6)	C(3)	108.8(2)
C(1)	C(2)	C(3)	101.2(2)				

Intramolecular Distances (Å) Involving the Nonhydrogen Atoms

atom	atom	distance	atom	atom	distance
O(1)	N(1)	1.450(3)	O(6)	C(6)	1.323(3)
O(1)	C(3)	1.444(3)	O(6)	C(7)	1.453(3)
O(2)	N(1)	1.233(3)	N(1)	C(1)	1.295(3)
O(3)	C(4)	1.200(3)	C(1)	C(2)	1.489(4)
O(4)	C(4)	1.338(3)	C(1)	C(4)	1.468(4)
O(4)	C(5)	1.449(3)	C(2)	C(3)	1.529(4)
O(5)	C(6)	1.184(3)	C(3)	C(6)	1.518(4)

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