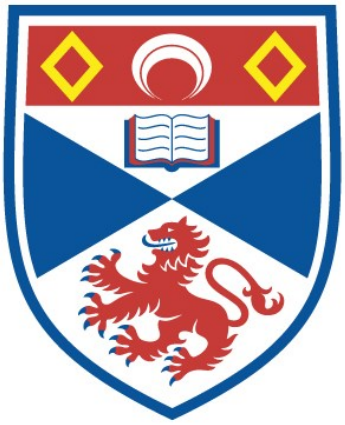
MECHANISTIC AND STEREOCHEMICAL STUDIES ON METHYLASPARTASE AND GLUTAMATE MUTASE

Catherine Helen Archer

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



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MECHANISTIC AND STEREOCHEMICAL STUDIES ON METHYLASPARTASE AND GLUTAMATE MUTASE

a thesis presented by

Catherine Helen Archer

to the

UNIVERSITY OF ST. ANDREWS

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St. Andrews

February 1993

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Declaration

I, Catherine Helen Archer, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed .. .. Date ..21st April 1993..

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on ..1st October 1990.... and as a candidate for the degree of Ph. D. on ..1st October 1990.....

Signed .... Date ..21st April 1993.

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph. D.

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To

My Parents

and

Nigel

It was the best of times,
it was the worst of times,
it was the age of wisdom,
it was the age of foolishness,

A Tale of Two Cities
Charles Dickens

Acknowledgements

Firstly may I thank my supervisor, Professor David Gani, without whom this thesis would not have been possible. He has been a great source of help, enthusiasm and inspiration. Also thanks to his bench allocation scheme in Southampton, I owe him my husband, Nigel, without whom this tome would also not have been completed. He has been steadfast in his support, encouragement, understanding and practical advice. He has also been a wonderful house husband during the weekends and evenings spent scribbling this thesis.

Dave should also be complemented on becoming professor in such a beautifully situated chemistry department. I will never forget my view over the West Sands from my bench. Thank you Dave for bringing us to St Andrews.

I also want to thank Mahmoud, our most senior of post-docs, who is a great source of wisdom both in the laboratory and the rest of life. He has been a great friend. Other members of the Gani group who deserve special thanks are Neil, with whom I seemed to share a flat with in Southampton, Paul, my big brother, and Tanmaya, the philosopher. The glutamate mutase group of Basil, Saeed and Kamal are also acknowledged - keep up the good work. Thanks also to the rest of the Gani group, past and present for making it 'the Gani group', and to Stacey for her friendship (and typing).

At Southampton I want to thank John Langley for mass spectra, David Turner for 500 MHz NMR spectra, and Prof. Alan Jackson for the ^{15}N isotope measurements.

In St Andrews I owe my thanks to Ray Mackie and Melanja in particular, for help with NMR time course experiments. I would also like to thank the whole department for making us feel so at home up here

Finally I want to thank my parents who have supported and encouraged me throughout my education.

Abstract

Preliminary studies have been undertaken on the enzyme, glutamate mutase. Stereochemically pure (2S,3S)-3-ethylaspartic acid has been synthesized. The turnover of this substrate analogue by glutamate mutase has been investigated. Possible reaction products have been synthesized. (2S,3S)-[1',1',2',2',2'-^{2}H]-3-Ethylaspartic acid has been prepared and a novel synthesis of [1-^{2}H]-ethanol has been investigated with the aim of preparing (2S,3S)-[1'-^{2}H]-3-ethylaspartic acid.

In order to investigate the mechanism of elimination of ammonia from (2S,3R)-3-methylaspartic acid, by the enzyme 3-methylaspartase, stereospecific routes to (2S,3R)-3-methylaspartic acid and [3-^{2}H]-(2S,3R)-3-methylaspartic acid have been explored. The compounds were obtained in high enantiomeric excess and with >97 % incorporation of deuterium into the latter. It has been demonstrated that 3-methylaspartase catalyses the direct elimination of ammonia from these substrates, presumably by a *syn*-elimination mechanism. The kinetic parameters, V_{max} and K_m, have been determined for both compounds at 1 and 50 mM potassium ion concentrations. A deuterium isotope effect on V_{max} ($^{D}(V)$) of 7.15 ± 2.74 was measured for the reaction at 1 mM potassium ion concentration. A large $^{D}(V)$ of 6.79 ± 0.92 was also observed at 50 mM potassium ion concentration, in contrast to results with the natural isomer which show the effect is completely suppressed at this potassium ion concentration. Values for $^{D}(V/K)$ were also obtained at 1 and 50 mM potassium ion concentrations. They were 3.39 ± 1.6 and 4.10 ± 1.3, respectively. The ^{15}N isotope effect on V/K was measured at 1 mM potassium ion concentration. A value of 1.0028 ± 0.0040 was observed for (2S,3R)-3-methylaspartic acid, and, a value of 1.0033 ± 0.0043 observed for [3-^{2}H]-(2S,3R)-3-methylaspartic acid.

Contents

Chapter 3 - Methylaspartase - Results and Discussion

List of Schemes

List of Figures

List of Tables

Abbreviations

AAT	aspartate aminotransferase
Ado	adenosine
ATCC	American *Type Culture* Collection
ATP	adenosine triphosphate
B	base
B_{12r}	cob(II)alamin
B_{12s}	cob(III)alamin
b.p.	boiling point
Cbz	carbobenzoxy
CoA	coenzyme A
Da	Daltons
DACM	N-(7-dimethylamino-4-methylcoumarynyl) maleimide
DEAE	diethylaminoethyl
decomp.	decomposed
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
ε	extinction coefficient
$E1_{cb}$	conjugate base unimolecular elimination
$(E1_{cb})_I$	conjugate base unimolecular elimination where hydrogen elimination is essentially irreversible
E2	bimolecular elimination
EC	Enzyme Catalogue
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Enz	enzyme
EPR	electron paramagnetic resonance
FPLC	fast protein liquid chromatography
GC	gas liquid chromatography
h	hour
I	ionic strength
K_{cat}	enzyme catalytic constant / turnover number
K_{eq}	equilibrium constant

K_i	enzymic inhibition constant
K_m	Michaelis constant
LDA	lithium diisopropylamide
m.p.	melting point
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance
Nu	nucleophile
OD	optical density
p.	page
PAGE	polyacrylamide gel electrophoresis
pD	$-\log[^2H^+]$, equivalent to (pH - 0.4)
PLP	pyridoxal phosphate
PMSF	phenylmethanesulphonyl fluoride
m.p.	melting point
R_p	distance travelled in an electric field / distance between electrodes
SDS	sodium dodecylsulphate
S_N2	nucleophilic bimolecular substitution
THF	tetrahydrofuran
tlc	thin layer chromatography
Tris	tris(hydroxymethyl)amino methane
UV	ultra-violet
$^D(V)$	V_H/V_D
$^D(\upsilon_{ex})$	deuterium isotope effect on υ_{ex}
$^D(V/K)$	$(V_H/V_D)/(K_H/K_D)$
$^{15}(V/K)$	nitrogen isotope effect on V/K
υ_{ex}	rate of exchange
V_{max}	maximum rate of substrate turnover at saturation

List of Amino Acids

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

CHAPTER ONE

INTRODUCTION

GLUTAMIC ACID

FERMENTATION

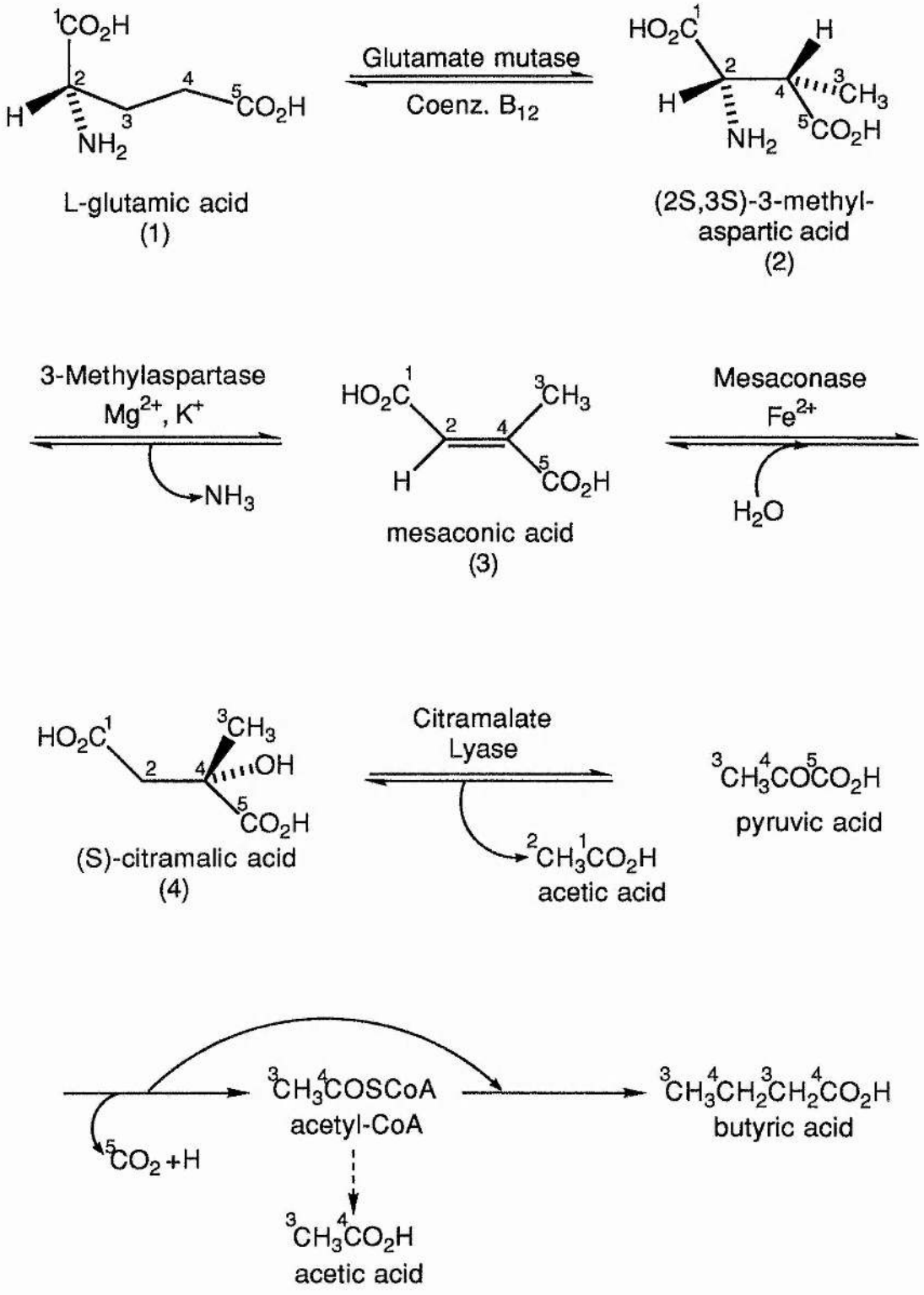
1.1.1 The Methylaspartic Acid Pathway

Glutamic acid (1) is used as the main energy source in certain obligate anaerobes[1]. A number of catabolic routes for glutamic acid exist. In the soil anaerobe *Clostridium tetanomorphum*, and several other Clostridial species, the route utilized is the 'methylaspartic acid pathway' (Scheme 1.1). This glutamic acid degradation pathway was first elucidated by H. A. Barker and co-workers[2] studying *Clostridium tetanomorphum.* This species of bacteria (*Clostridium tetanomorphum* strain H1 (ATCC 15920)) has been the enzyme source for most of the studies performed on the pathway.

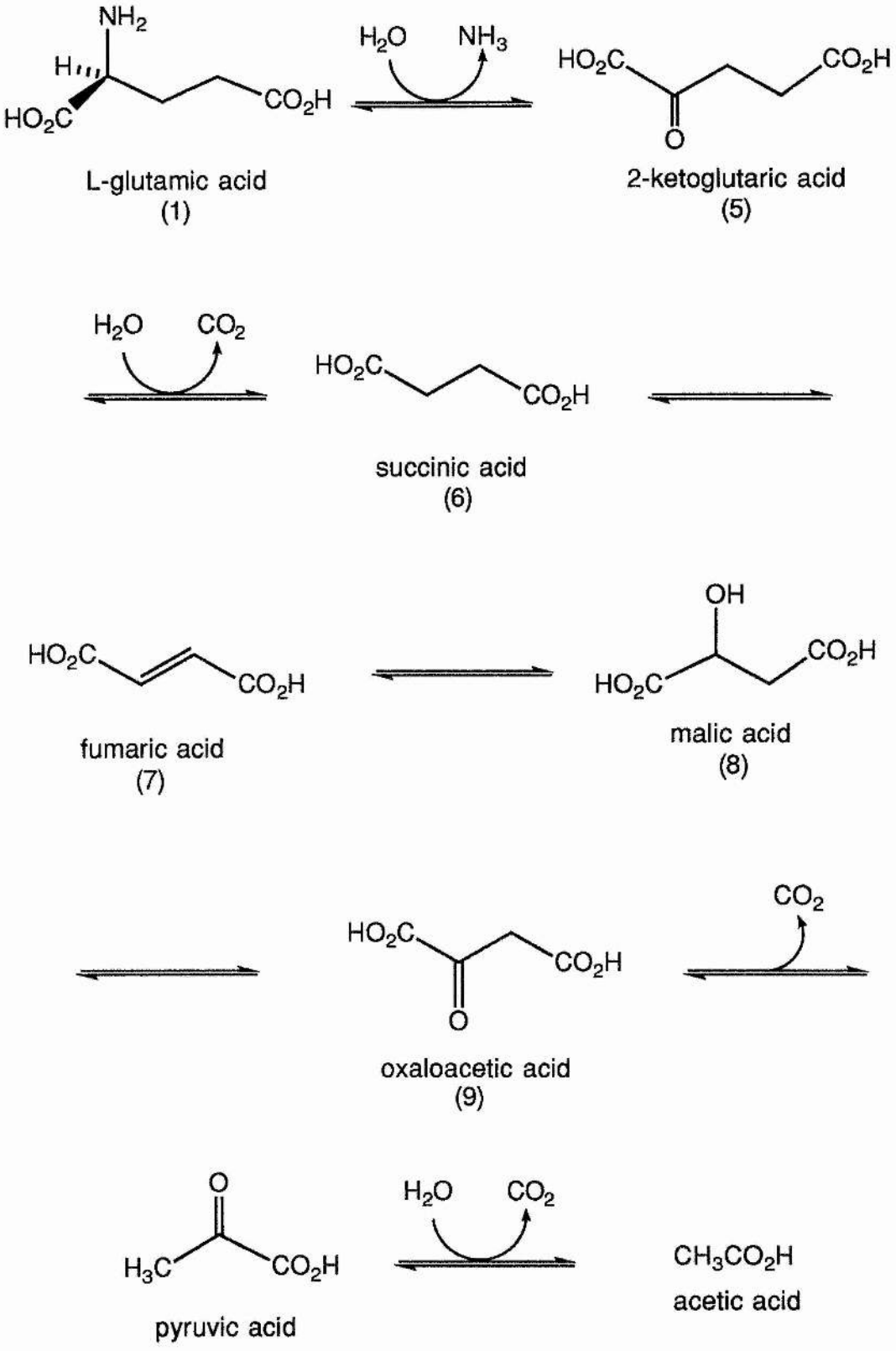
There is also some evidence that this fermentation route operates in the photosynthetic, purple nonsulphur bacteria, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* [3] (see section 1.1.5, p. 7.). However it is thought not to be the major catabolic pathway[4]. A similar pathway may also be used in an anabolic sense by the bacterium *Acetobacter suboxydans* [5,6] (see section 1.1.6, p. 10.), although it is not the primary route of glutamic acid assimulation[7].

1.1.2 Elucidation of the Methylaspartic Acid Pathway

The first step in the methylaspartic acid pathway (Scheme 1.1) is the carbon skeleton rearrangement of (2S)-glutamic acid (1) to (2S,3S)-3-methylaspartic acid (2). The reaction is catalysed by glutamate mutase, a coenzyme B_{12} -dependent enzyme[8]. The next step involves deamination of (2S,3S)-3-methylaspartic acid, by the enzyme, 3-methylaspartase[9], to give mesaconic acid (3) ((E)-2-methylbutenedoic acid). Ammonia is lost by an *anti-* elimination. Hydration of the double bond of mesaconic acid, in an *anti-* Markovnikov fashion, by mesaconase[10], gives (S)-citramalic acid (4). Citramalate lyase[11] then cleaves the molecule into pyruvic acid and acetic acid. The pyruvic acid is oxidatively decarboxylated to give acetyl-CoA. Two acetyl-CoA units form butyryl-CoA and, hence, butyric acid. Adenosine triphosphate (ATP) is generated from cleavage of the thioesters.



Scheme 1.1 The Methylaspartic Acid Pathway



Scheme 1.2 The Tricarboxylic Acid Cycle in an Oxidative Direction

A number of routes for glutamic acid degradation were postulated in the years following the initial observation, in 1937, that glutamic acid was converted to acetic acid, butyric acid, carbon dioxide, ammonia and hydrogen[1]. Quantitative analysis showed that only one mole of carbon dioxide was produced per mole of glutamic acid. This excluded any possible degradation pathway via α-ketoglutaric acid (5), succinic acid (6), fumaric acid (7), malic acid (8), oxaloacetic acid (9) and pyruvic acid (essentially the tricarboxylic acid cycle in an oxidative direction) (Scheme 1.2), which would yield at least three moles of carbon dioxide per mole of glutamic acid. However other pathways, *e.g.* the 2-hydroxyglutaric acid pathway (see section 1.1.3, Scheme 1.3), would produce one mole of carbon dioxide per mole of glutamic acid.

With the advent of specifically labelled ^{14}C-glutamic acids, it became possible to ascertain the fate of the individual carbon atoms of glutamic acid during fermentation. Wachsman showed, in 1955, that the labels derived from C-1 and C-2 of glutamic acid were found in acetic acid. The label at C-4 of glutamic acid enriched C-1 and C-3 of butyric acid and C-5 of glutamic acid was oxidized to carbon dioxide[12].

Paper chromatographic analysis of the products from the reaction of glutamic acid with a cell-free extract of *C. tetanomorphum* revealed the accumulation of a UV-absorbing acidic constituent. This compound was identified as mesaconic acid[13]. The structure was confirmed by showing that authentic mesaconic acid was a substrate for the extract. Thus, it was evident that the linear carbon chain of glutamic acid had been converted into a branched chain compound, mesaconic acid.

In further work, by Munch-Petersen and Barker, using [^{14}C]-glutamic acids, the fate of the carbon atoms in mesaconic acid was determined[14]. They discovered that C-1 and C-2 of glutamic acid were transferred together from C-3 of glutamic acid to C-4, using the numbering system of the glutamic acid carbon skeleton. Thus the bond between C-2 and C-3 of glutamic acid had cleaved and a new bond had formed between the original C-2 and C-4 of

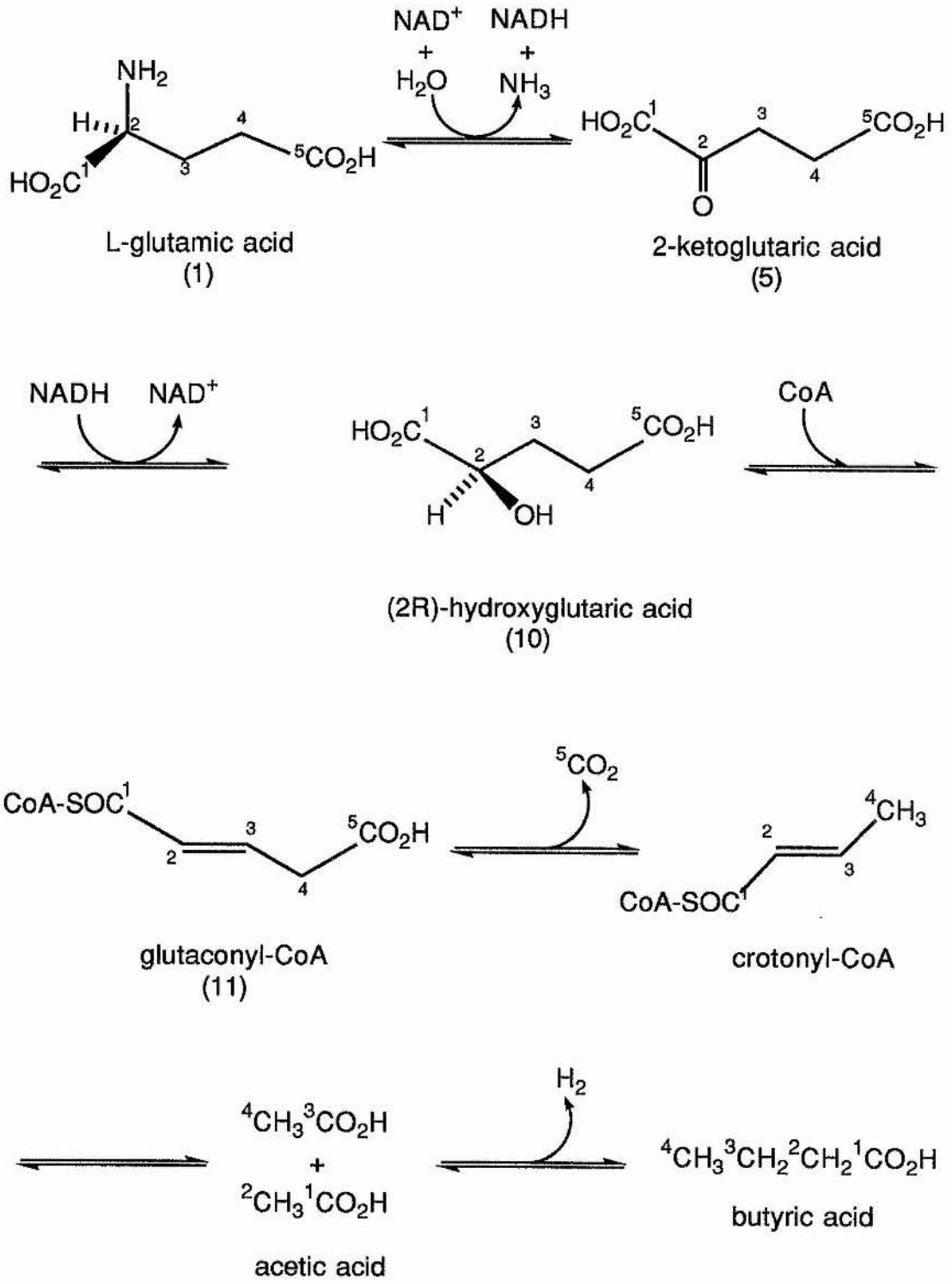
glutamic acid. The C-3 methylene group of glutamic acid had become the methyl group of mesaconic acid (see Schemes 1.1 and 1.14).

It was not obvious how glutamic acid was converted to mesaconic acid. In their investigations into the number and nature of the enzymes involved in the conversion, Munch-Petersen and Barker treated the crude cell-free extract from *C. tetanomorphum* with charcoal[15]. This extract was found to be completely inactive when incubated with glutamic acid. However, in the presence of ammonium ions, the extract utilized mesaconic acid[16]. A new amino acid accumulated. It was shown to be (2S,3S)-L-*threo* -3-methylaspartic acid[17]. This intermediate was the direct amination product of mesaconic acid. Hence it appeared that (2S)-L-glutamic acid was converted to the branched chain amino acid, (2S,3S)-3-methylaspartic acid, in one step. This reaction was dependent upon a novel cofactor which was inactivated by charcoal.

1.1.3 The 2-Hydroxyglutaric Acid Pathway

Whilst the methylaspartic acid pathway is important in certain anaerobes, other routes of glutamic acid degradation are also available[18]. One such route is the 2-hydroxyglutaric acid pathway (Scheme 1.3) elucidated by Westlake in 1966[19]. In this pathway, (S)-glutamic acid (1) is converted, via 2-ketoglutaric acid (5), to (2R)-hydroxyglutaric acid (10),[20] and eventually to acetic acid and butyric acid. Thus the linear carbon chain of glutamic acid remains intact in the butyric acid product. This was confirmed by the labelling pattern of butyric acid produced by the fermentation of ^{14}C-labelled glutamic acid in cell suspensions of *Peptococcus aerogenes* [19].

The decarboxylation of glutaconyl-CoA (11) liberates 30 kJ mol^{-1} free energy[21]. The organism uses this energy source to drive a sodium pump and thus establish an electrochemical sodium gradient which allows the transport of metabolites in and out of the cell.



Scheme 1.3 The 2-Hydroxyglutaric Acid Pathway

1.1.4 The Occurrence of the Two Pathways

Buckel and Barker[22] surveyed several species of bacteria, examining the butyric acid labelling patterns obtained when [4-^{14}C]-glutamic acid was fermented. This gave an indication of how extensively each pathway occurred.

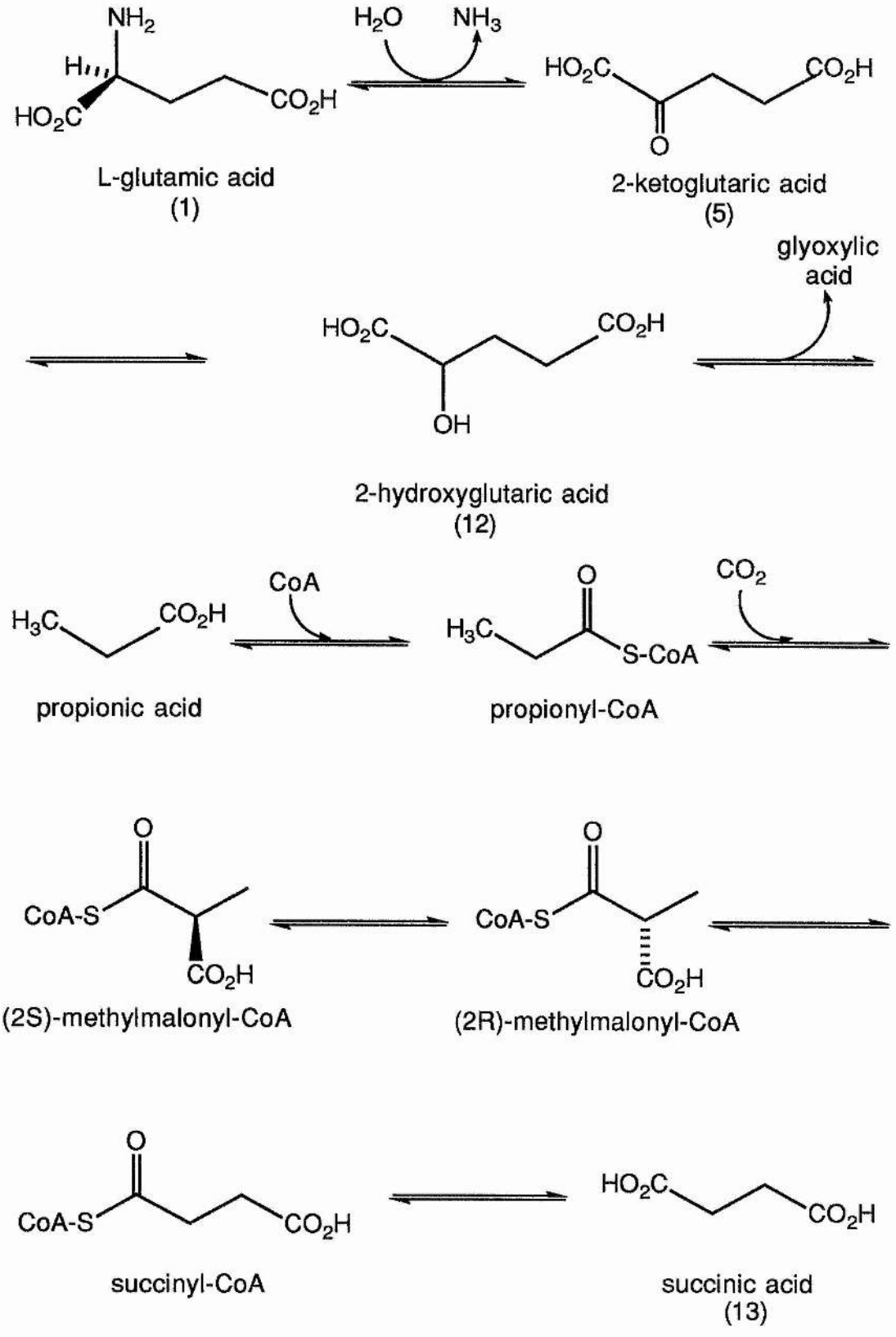
Butyric acid from the fermentation of [4-^{14}C]-glutamic acid *via* the methylaspartic acid pathway had labels at C-1 and C-3, as it was derived from the condensation of two identical acetyl groups, mainly derived from C-3 and C-4 of glutamic acid. In contrast the butyric acid, from the 2-hydroxyglutaric acid pathway, was labelled at only C-4, as no rearrangement of the carbon chain had occurred. Material from the methylaspartic acid pathway also contained twice the radioactivity of the butyric acid formed *via* the 2-hydroxyglutaric acid route. Assays for 3-methylaspartase and 2-ketoglutarate reductase, key enzymes from each pathway, were also performed[22].

The results showed that the 2-hydroxyglutaric acid pathway was used by a wide variety of bacteria *e.g. Peptococcus aerogenes, Acidaminococcus fermentans, Fusobacterium nucleatum, Fusobacterium fusiformis* and *Clostridium microsporum.* This latter microorganism may be wrongly classified as a *Clostridium* and may in fact be a *Fusobacterium* [22]. Thus, the 2-hydroxyglutaric acid pathway is used by representatives of several genera, whereas, the methylaspartic acid pathway seemed to be restricted mainly to Clostridia.

1.1.5 Glutamic Acid Metabolism in Photosynthetic Bacteria

In the photosynthetic bacteria, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*, 3-methylaspartic acid, mesaconic acid and citramalic acid were detected as intermediates[3,4]. However the methylaspartic acid pathway accounted for only 10 to 20 % of glutamic acid

metabolism[4]. The major proportion of glutamic acid (80 %) was converted to 2-ketoglutaric acid (5) and hence to 2-hydroxyglutaric acid (12) and succinic acid (13) (Scheme 1.4).



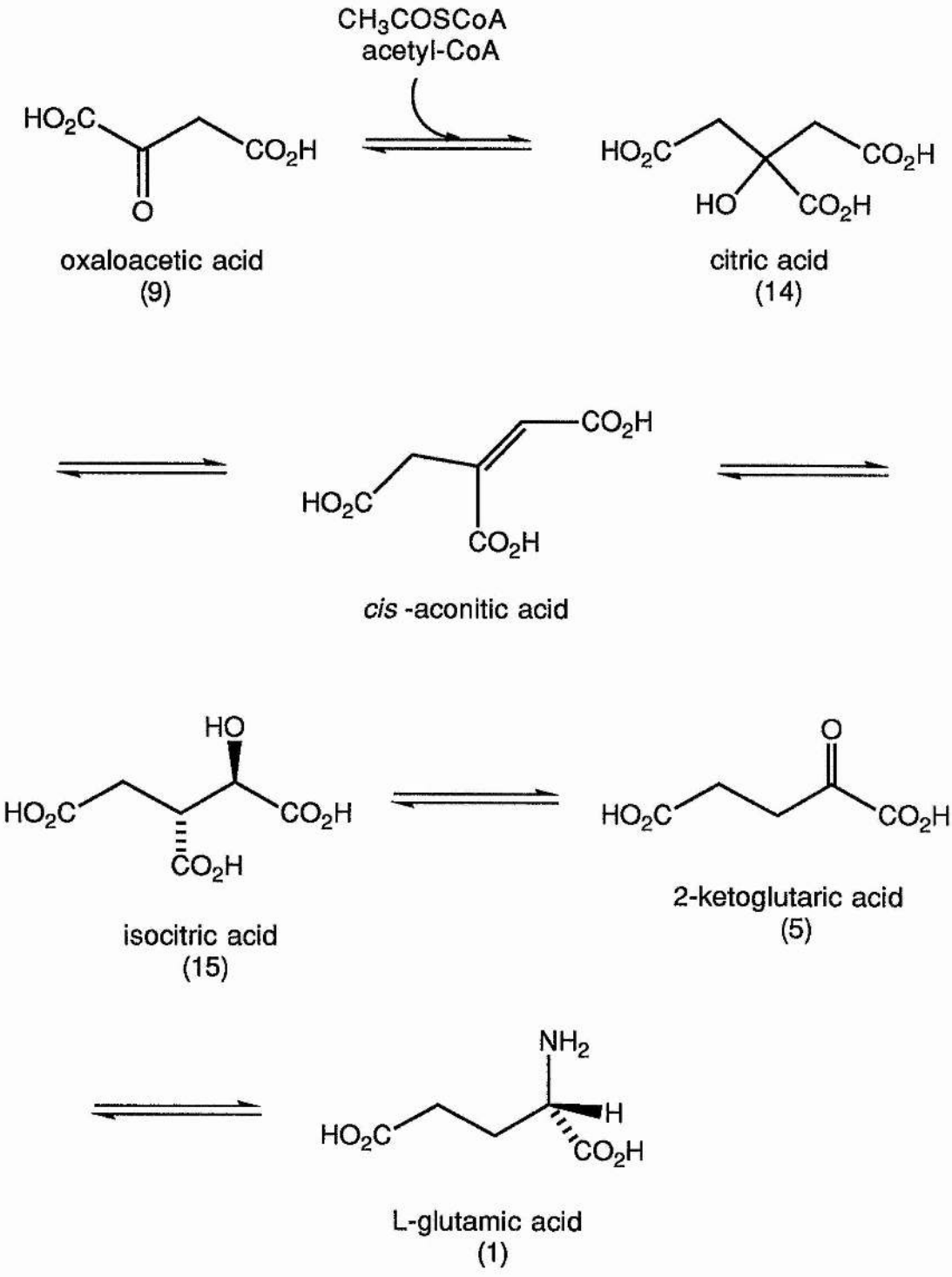
Scheme 1.4 The Conversion of Glutamic Acid to Succinic Acid

1.1.6 Glutamic Acid Biosynthesis in *Acetobacter suboxydans*

The obligate aerobe *Acetobacter suboxydans* appears to lack a functional tricarboxylic acid cycle[23]. However it does biosynthesize glutamic acid[24], presumably by another route. Cheldelin *et al.* reported that two pathways operated. One involved the condensation of glyoxylic acid and oxaloacetic acid (9), and the other was essentially the reverse of the methylaspartic acid pathway[25]. Administration of ^{14}C-labelled pyruvic acid and acetic acid to either growing cells or a cell free extract gave labelled glutamic acid and 3-methylaspartic acid. Citramalic acid and mesaconic acid were also demonstrated to be intermediates on the pathway. All intermediates were isolated and characterised.

However, the 3-methylaspartic acid isolated did not co-crystallize with synthetic (2R/S,3S)-3-methylaspartic acid, which was mainly in the *threo*-form. Also, the slow conversion rate of the isolated 3-methylaspartic acid to mesaconic acid on incubation with 3-methylaspartase from *Clostridium tetanomorphum* indicated that the L-*erythro*-isomer of 3-methylaspartic acid was utilized in this pathway[25]. Kato isolated Coenzyme B_{12} from *Acetobacter suboxydans* [26]. It may be present as the cofactor for glutamate mutase.

However, more recent workers failed to find any evidence for the methylaspartic acid pathway in *Acetobacter suboxydans* and showed that a third pathway was entirely responsible for glutamic acid biosynthesis[7]. They showed the bacteria lacked only the succinate dehydrogenase enzyme of the tricarboxylic acid cycle. Thus, cells grown on an acetate-1-^{14}C containing media showed 100 % incorporation of radioactivity at C-5 of glutamic acid. This labelling pattern would be expected from a partial tricarboxylic acid cycle, namely the aconitase-isocitrate dehydrogenase pathway (Scheme 1.5). In this route acetyl-CoA and oxaloacetic acid (9) combine to give citric acid (14) which is converted to isocitric acid (15), then to 2-ketoglutaric acid (5) and finally glutamic acid (1).



Scheme 1.5 The Aconitase - Isocitrate Dehydrogenase Pathway

COENZYME B_{12}-DEPENDENT

ENZYMES

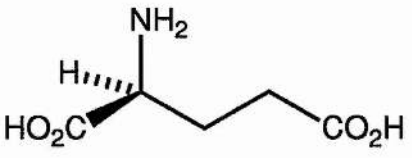
1.2.1 Coenzyme B_{12} Catalysed Reactions

The cofactor, coenzyme B_{12} (Figure 1.2), is required for the catalysis of a wide range of rearrangement reactions. These reactions all involve a vicinal interchange between a hydrogen atom and a group X (Scheme 1.6).

Scheme 1.6 Generalized Coenzyme B_{12} - Dependent Vicinal Interchange Rearrangement

The X group can vary from acyl or alkyl groups, which give carbon skeleton rearrangements, to alcohol or amine groups, as in the dehydratase and aminomutase catalysed rearrangements. The enzymes which catalyse such rearrangements are methylmalonyl-CoA mutase (EC 5.4.99.2)[27], methyleneglutarate mutase (EC 5.4.99.4)[28], glutamate mutase (EC 5.4.99.1)[29], isobutyryl-CoA mutase[30], diol dehydrase (EC 4.2.1.28)[31], glycerol dehydrase (EC 4.3.1.30)[32], ethanolamine ammonia lyase (EC 4.3.1.7)[33], β-lysine mutase (EC 5.4.3.3)[34,35], D-α-lysine mutase (EC 5.4.3.4)[36], D-ornithine mutase (EC 5.4.3.5)[37,38], L-leucine-2,3-aminomutase (EC 5.4.3.7)[39] and ribonucleotide reductase (EC 1.17.4.2)[40] (Scheme 1.7). Many of these enzymes contain two subunits; one which binds the coenzyme and another which has sulphydryl groups.

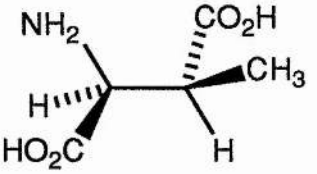
The four enzymes which catalyse carbon skeleton rearrangement reactions are particularly intriguing. Amongst these glutamate mutase is unique in that it catalyses a rearrangement in which the migrating group (X) is centred on an sp^3 carbon. The other coenzyme B_{12} catalysed carbon skeleton rearrangements all occur with migration from an sp^2 carbon.



L-glutamic acid

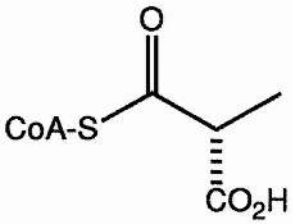
(1)

glutamate mutase



(2S,3S)-3-methyl-aspartic acid

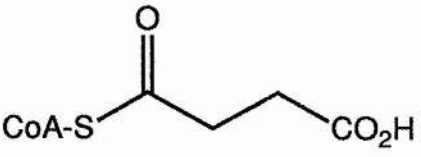
(2)



(2R)-methylmalonyl-CoA

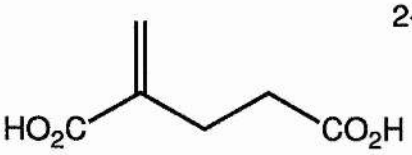
(21)

methylmalonyl-CoA mutase



succinyl-CoA

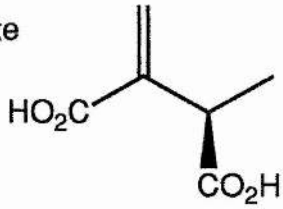
(22)



2-methyleneglutaric acid

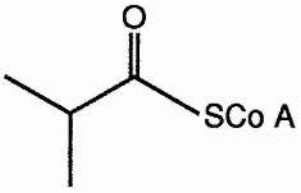
(28)

2-methyleneglutarate mutase



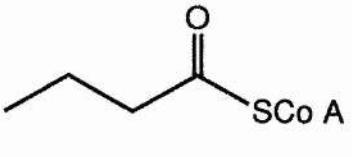
(3R)-3-methylitaconic acid

(29)

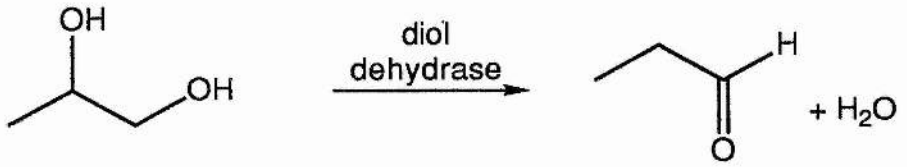


isobutyryl-CoA

isobutyryl-CoA mutase

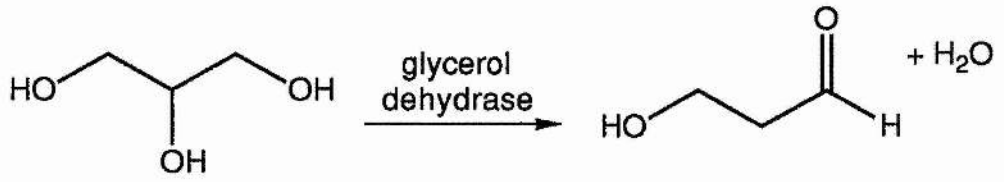


butyryl-CoA



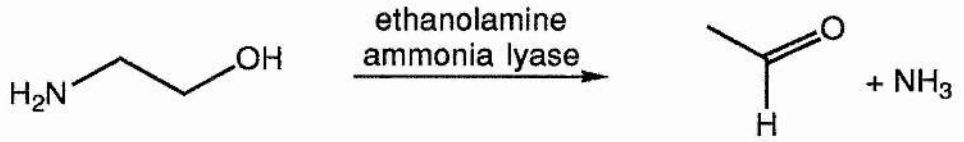
(R)-1,2-propanediol (30)
and
(S)-1,2-propanediol (31)

propionaldehyde
(32)



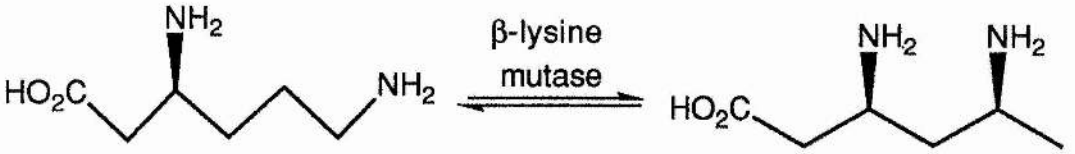
glycerol

β-hydroxypropionate



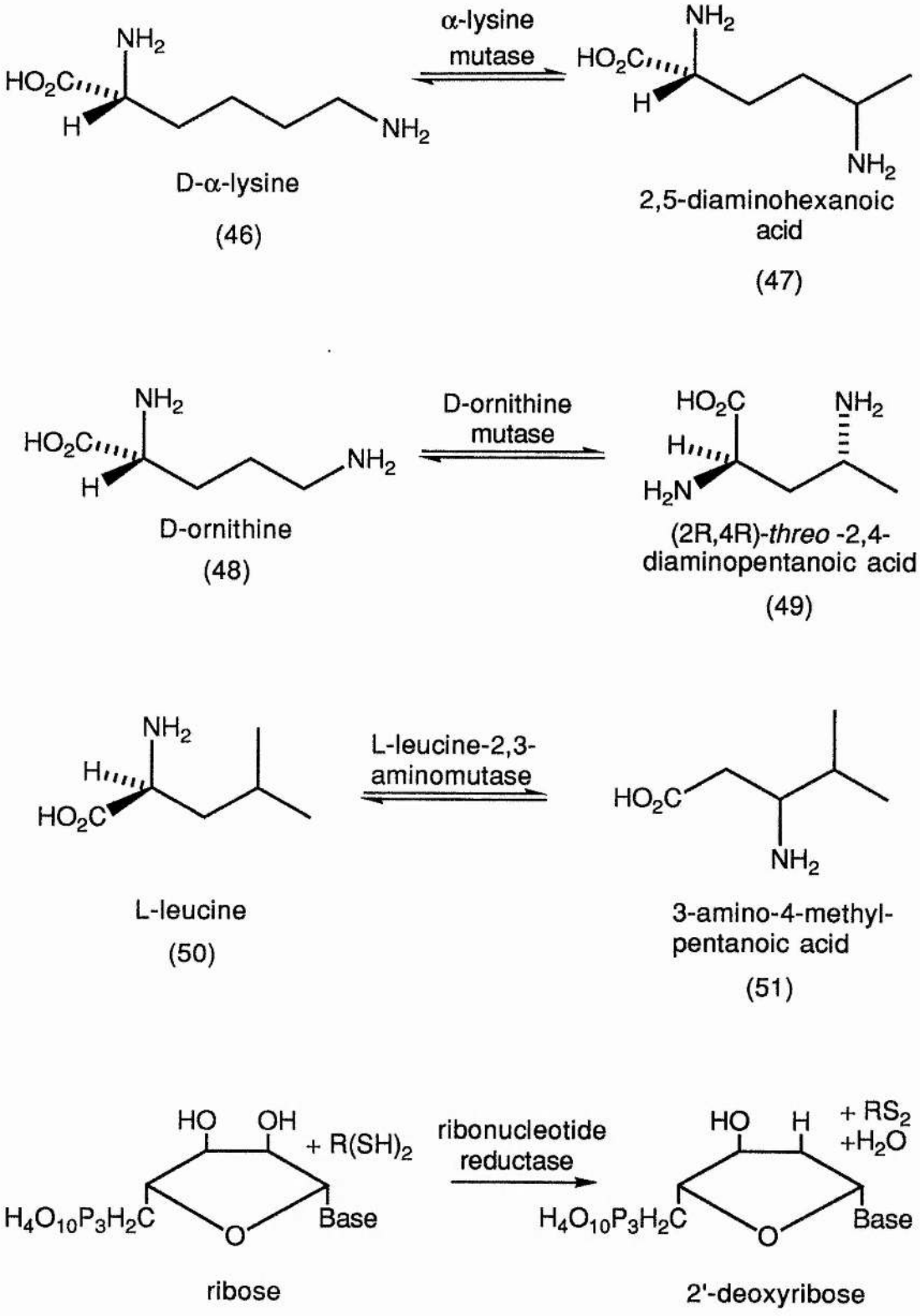
ethanolamine
(33)

acetaldehyde



(3S)-L-β-lysine
(44)

(3S,5S)-diaminohexanoic
acid
(45)



Scheme 1.7 The Rearrangements Catalysed by Coenzyme B_{12} - Dependent Enzymes

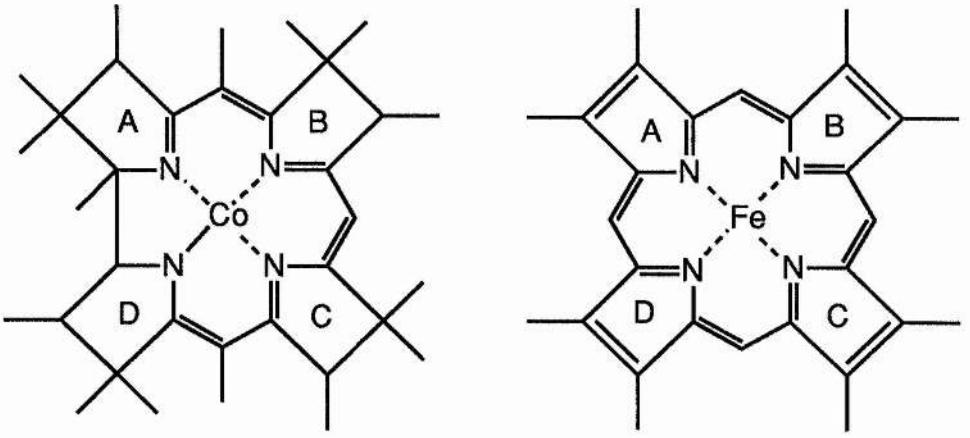
1.2.2 Coenzyme B_{12}

The B_{12} molecule is the most complex non-polymeric compound found in nature[41]. Its history began in 1926 when Minot and Murphy reported the effectiveness of whole liver treatment on pernicious anaemia[42], a disease which is now controlled by vitamin B_{12} injections. In 1934 they were awarded the Nobel Prize for Medicine and Physiology 'for their discoveries concerning liver therapy against anaemias'. The active factor in liver was isolated in a crystalline form simultaneously by Folkes[43] and Smith[44] in 1947. Its structure was determined in 1956 by Dorothy Hodgkin[45]. At the time it was the largest structure yet elucidated by X-ray crystallography. Two years later Barker isolated the biologically active form of the vitamin, known as coenzyme B_{12}[46,47]. Its structure was also confirmed by Hodgkin[48].

The molecule consists of a corrin ring with a cobalt ion at its centre. The corrin ring is a tetrapyrrole macrocycle and as such it is related to the porphyrin ring. However the corrin ring differs in several ways from the porphyrin system (Figure 1.1). In the corrin ring there is less unsaturation and the A and D pyrrole rings are linked directly. The substitution of ring D is reversed relative to rings A to C[49].

The four pyrrole nitrogen atoms are ligated to the cobalt at the centre. The two remaining positions above and below the plane of the ring are filled by further ligands whose nature determines the biological role of the corrin system. In mammalian systems the fifth ligand of coenzyme B_{12} is always a 5,6-dimethylbenzimidazole group, although some bacteria contain other benzimidazoles or adenine analogues as the heterocyclic base[50]. The base is bonded through its N-3 lone pair. The sixth position in the octahedral arrangement is occupied by an adenosyl moiety, *via* C-5', in the case of the coenzyme (Figure 1.2), a cyano group in the vitamin and a methyl group in the case of methylcobalamin. These sixth ligands are all attached by carbon-cobalt (C-Co) bonds. The B_{12} molecules are the only organometallic compounds known in nature[51]. The coenzyme form is easily inactivated by light, which catalyses cleavage of the C-Co bond[52].

Hydroxycobalamin then forms in solution.



Corrin Porphyrin

Figure 1.1 The Corrin and Porphyrin Rings

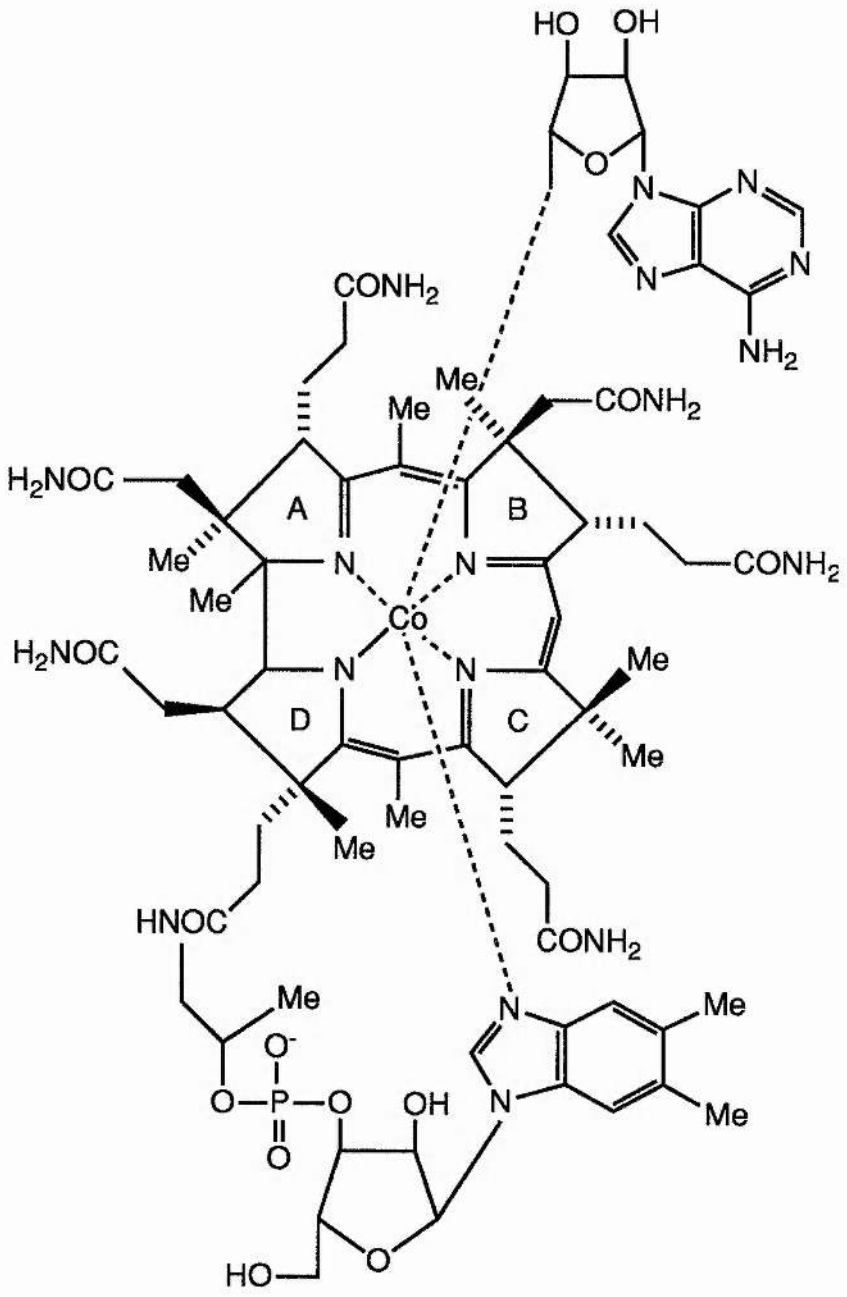


Figure 1.2 Coenzyme B_{12}

1.2.3 General Mechanism of Rearrangement

All the coenzyme B_{12}-dependent enzymes are thought to employ similar mechanisms for catalysis[51], although, due to the diversity of rearrangements catalysed (Scheme 1.7), finer details must obviously vary. A general mechanism[53] is shown in Scheme 1.8 and summarized below.

The reaction is initiated by an enzyme-induced homolytic dissociation of the C-Co bond of the coenzyme to generate a cob(II)alamin species and a 5'-deoxyadenosyl radical (Ado-$CH_2^{\bullet}$) (16). Next a hydrogen atom is abstracted from the substrate, by the 5'-deoxyadenosyl radical, to generate a substrate radical and 5'-deoxyadenosine (Ado-CH_3) (17). Rearrangement of the substrate radical then occurs, either directly or through further intermediate steps, to give a radical product (18). Finally the product radical abstracts a hydrogen atom, from 5'-deoxyadenosine, to give the product and regenerate the 5'-deoxyadenosyl radical (19) which recombines with the cob(II)alamin species to regenerate the coenzyme (20).

It is envisaged that the corrin ring becomes distorted when bound to the enzyme. The ring is pushed up, thus, increasing the steric repulsion between the ring and the 5'-deoxyadenosyl group and hence weakening the C-Co bond[54]. The initial homolysis of the C-Co bond may be brought about by additional strain caused by the substrate binding[53].

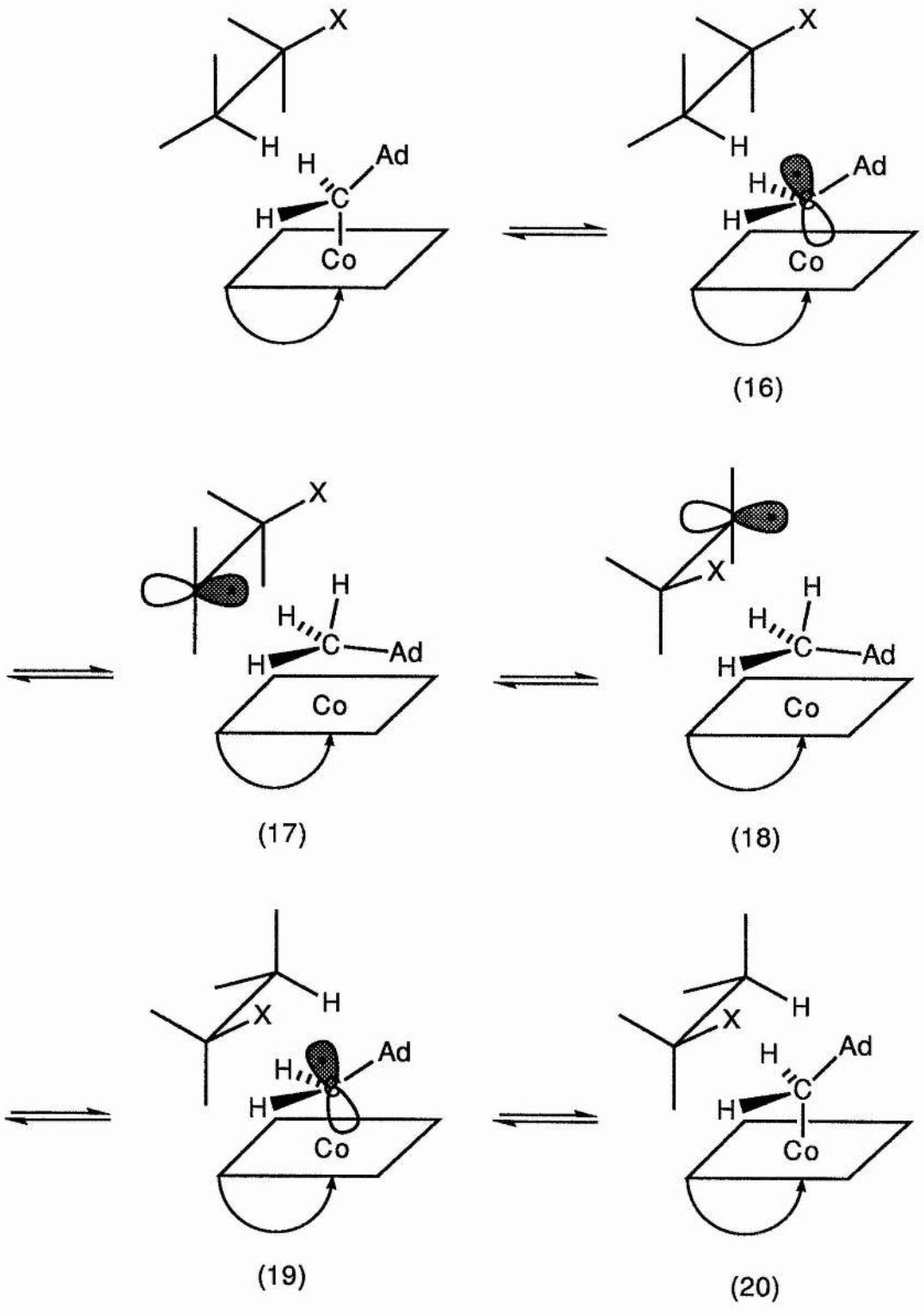
Cobalt (II) is paramagnetic, thus the cob(II)alamin (B_{12r}) species generated by homolytic cleavage of the C-Co bond of the coenzyme should give an electron paramagnetic resonance (EPR) signal. Such signals have been detected, by EPR techniques, in the diol dehydrase[55,56], ethanolamine ammonia lyase[57,58] and ribonucleotide reductase[59] systems, and recently in the methylmalonyl-CoA mutase[60] and glutamate mutase[61] systems. These results lend credence to the proposed mechanism.

There is also spectroscopic evidence, from Babior[62] and Abeles[63], for a carbon-centred free radical intermediate in the ethanolamine ammonia

lyase[62] and diol dehydrase[63] catalysed rearrangements. The use of substrate analogues, which reacted more slowly than the natural substrates, allowed such species to be identified.

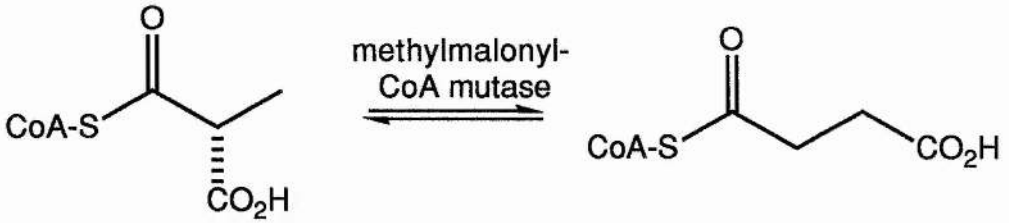
Rearrangement of the substrate radical, S$^{\bullet}$ could occur directly to give the product radical, or carbonium ion, carbanion or organocobalt intermediates could be invoked[54].

Frey and Abeles[64] demonstrated that if in the diol dehydrase catalysed reaction the migrating hydrogen was labelled, its label became scrambled with the two 5' methylene hydrogens. Thus, all three hydrogens had become equivalent, as would occur in the methyl group of 5'-deoxyadenosine. Babior obtained a similar result in experiments with the ethanolamine ammonia lyase system[65]. Abeles and Babior have also demonstrated the reversible formation of 5'-deoxyadenosine in these two enzyme systems[66,67].



Scheme 1.8 General Mechanism for Coenzyme B_{12} Rearrangements

1.2.4 Methylmalonyl-CoA Mutase



(2R)-methylmalonyl-CoA (21)

succinyl-CoA (22)

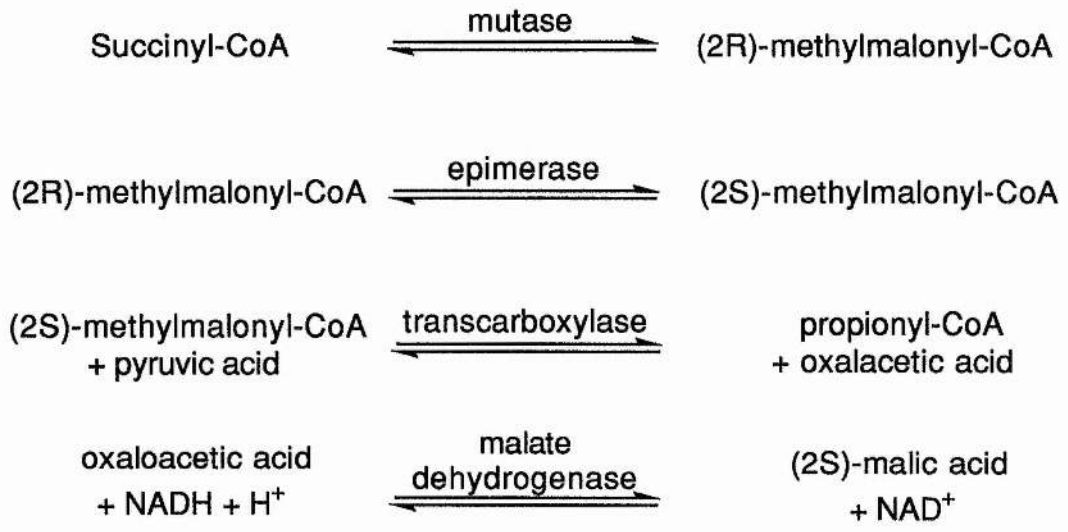
Scheme 1.9 Methylmalonyl-CoA Mutase Catalysed Rearrangement

The enzyme methylmalonyl-CoA mutase (EC 5.4.99.2) catalyses the interconversion of (2R)-methylmalonyl-CoA (21) and succinyl-CoA (22)[68] (Scheme 1.9). It is the only B_{12}-dependent mutase found in mammalian tissue[51] and is involved in propionic acid metabolism[69]. Here propionic acid, the degradation product of isoleucine and fatty acids, is activated as propionyl-CoA, carboxylated to (2S)-methylmalonyl-CoA and then epimerized to (2R)-methylmalonyl-CoA. The coenzyme B_{12}-dependent reaction, then, gives succinyl-CoA[70].

In bacteria, *e.g. Propionibacterium shermanii,* the metabolic flow is in the opposite direction, *i.e.* succinic acid is degraded to propanoic acid[71]. Again the pathway also requires an epimerase.

Wood's spectrophotometric assay for methylmalonyl-CoA mutase activity coupled together several enzyme reactions (Scheme 1.10)[72]. The other enzymes were present in excess, with respect to the mutase. The assay involved epimerization of the (2R)-methylmalonyl-CoA, produced by the reaction of the methylmalonyl-CoA mutase with succinic acid, to the (2S)-enantiomer, by methylmalonyl-CoA epimerase. This was then converted, by oxaloacetate transcarboxylase, in the presence of pyruvic acid, to propionyl-CoA and oxaloacetic acid. The oxaloacetic acid was then converted to (2S)-malic acid by malate dehydrogenase, a NAD^+-dependent enzyme. The

consumption of NADH, determined at 340 nm, gave a measure of methylmalonyl-CoA mutase activity.



Scheme 1.10 Methylmalonyl-CoA Mutase Assay

The molecular weight of methylmalonyl-CoA from sheep liver was determined, by Ochoa, to be 165 000 Da[73]. Retey showed the bacterial enzyme, isolated from *Propionibacterium shermanii,* had a molecular weight of 124 000 Da, with two subunits of approximately 65 000 Da[74]. The enzyme from both sources contained two moles of coenzyme per mole of enzyme[73,75]. In both sources of enzyme, the coenzyme was protected from inactivation by light, when bound to the enzyme[70]. The coenzyme also gave some protection to the mammalian enzyme from inactivation by thiol specific inhibitors such as N-ethylmaleimide and iodoacetate[73]. The bacterial enzyme did not appear to contain essential sulphydryl groups[75].

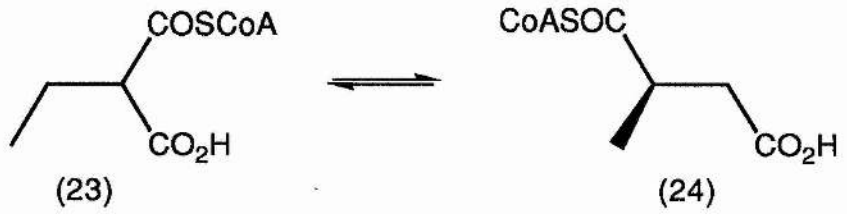
The pH optimum for the mammalian enzyme[76] was 7.0 and for the bacterial enzyme[75] 7.4. The equilibrium constant for the conversion of succinyl-CoA to methylmalonyl-CoA was measured by Wood[75], at 23.1. The K_m for succinyl-CoA was 34.5 μM and for methylmalonyl-CoA, 80 μM.

Eggerer and Swick used ^{14}C labelling experiments to establish that the CoA

thio ester group (COS-CoA) was the migrating group[77,78]. Wood[79] and Phares *et al.* [80] confirmed that migration was intramolecular. The steric course of the migration at C-2 was elucidated using deuterium labelling[81], and that at C-3, using the substrate analogue, ethylmalonyl-CoA (23)[82] (see also below). These experiments showed migration occurred with retention of configuration at both centres.

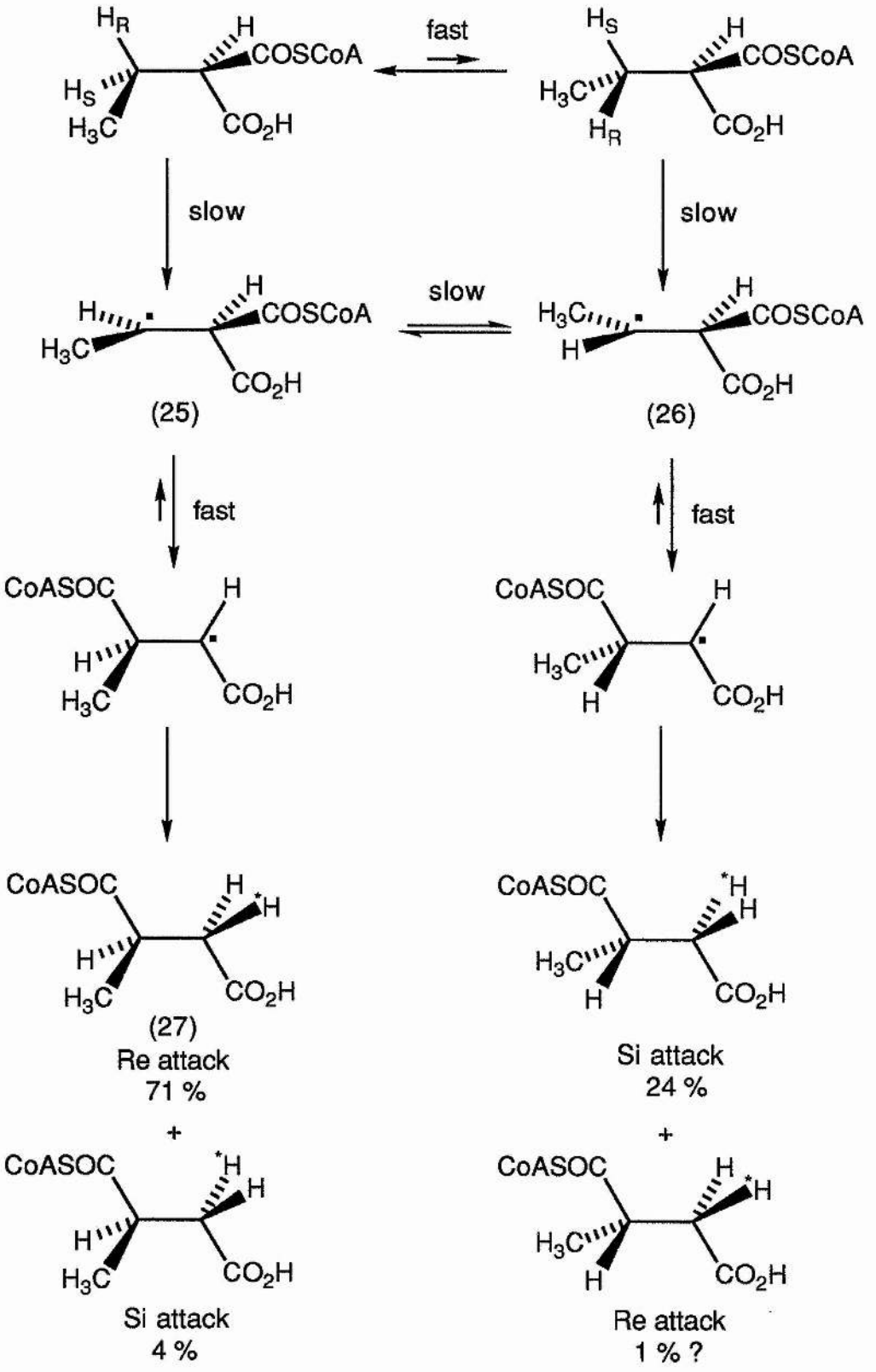
When monodeuteriated (2R)-[2-^{2}H]-methylmalonyl-CoA was used as the substrate, the succinyl-CoA produced contained up to 40 % unlabelled and dideuteriated material[81]. Retey[53] suggested that a proportion of the product radical underwent a 1-2 hydrogen shift, before reabstracting a hydrogen from 5'-deoxyadenosine. This would result in the product molecule being orientated in such a way that the reformed 5'-deoxyadenosyl radical could abstract a deuterium, to give the product radical again. The product radical could then reabstract a hydrogen, from the 5'-deoxyadenosine, to give unlabelled product. A monodeuteriated product radical could abstract the deuterium atom from the 5'-deoxyadenosine and so become dideuteriated.

The use of ethylmalonyl-CoA (23) to probe the methylmalonyl-CoA mutase system has helped to further elucidate the mechanism. Retey first showed that ethylmalonyl-CoA was a substrate for the enzyme in 1973[83]. The major product was (2R)-methylsuccinyl-CoA (24) (Scheme 1.11). Using ethylmalonyl-CoA stereospecifically labelled with deuterium at the methylene of the ethyl group, further details of the rearrangement were elucidated[82] (Scheme 1.12). Retey found the *pro*-R hydrogen was removed preferentially from the methylene of the ethyl group (25). However, there was also significant removal of the *pro*-S hydrogen (26) when the *pro*-R hydrogen was replaced by deuterium. This showed that hydrogen abstraction was a slower step than interconversion between the two conformations of the ethyl group. Rearrangement then gave the product radical to which a hydrogen was returned predominantly by *Re*-attack (27). Retey showed the reaction was not stereochemically clean, with some interconversion between the two possible conformations of the substrate radical, resulting in a mixture of final products.



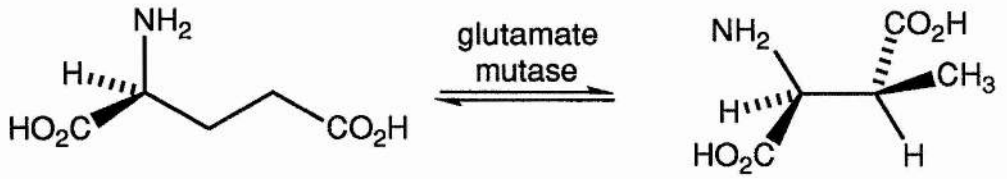
(23) (24)

Scheme 1.11 The Rearrangement of the Substrate Analogue, Ethylmalonyl-CoA



Scheme 1.12 Steric Course of Rearrangement of Ethylmalonyl-CoA

1.2.5 Glutamate Mutase



L-glutamic acid
(1)

(2S,3S)-3-methyl-
aspartic acid
(2)

Scheme 1.13 Glutamate Mutase Catalysed Rearrangement

The enzyme glutamate mutase catalyses the carbon skeleton rearrangement of L-glutamic acid (1) to (2S,3S)-3-methylaspartic acid (2) (Scheme 1.13)[8]. It is the first enzyme on the glutamic acid degradation pathway used by clostridia and some other species of bacteria (see section 1.1)[84]. The enzyme from *Clostridium tetanomorphum* has been most extensively studied[85].

Barker used two assays for glutamate mutase activity. A spectrophotometric assay detected the build up of mesaconic acid, when L-glutamic acid was incubated with glutamate mutase and 3-methylaspartase (the latter being in excess)[8]. The alternative anaerobic assay was based on the conversion of 3-methylaspartic acid to glutamic acid[8]. Aliquots were removed and assayed with 3-methylaspartase to measure how much 3-methylaspartic acid remained unconverted.

The enzyme consists of two components, designated Component S (a calcium phosphate supernatant fraction) and Component E (a gel eluate fraction)[86]. The purification of each component was accomplished by Barker, who achieved 20 - 25 % yields of Component S after 350-fold enrichment[87] and an 18 % yield of Component E after 180-fold enrichment[88]. Barker estimated the molecular weight of Component S to be 17 000 Da and that of Component E to be 128 000 Da.

The enzyme from *Clostridium cochlearium* was recently purified by Buckel[89]. He showed the molecular weight of Component S from this enzyme was 16 000 Da and that Component E was a homodimer of molecular weight 100 000 Da. Marsh[90], also confirmed, in 1992, that glutamate mutase from *Clostridium tetanomorphum* consisted of a monomeric Component S of molecular weight 15 000 Da and a homodimeric Component E with subunits of 50 000 Da. Marsh cloned and sequenced the genes for Components S[90] and E[91] from the *Clostridium tetanomorphum* source. The N-terminal sequences for Component S from *Clostridium cochlearium* are almost identical to those from the *C. tetanomorphum* source[89,90,92].

Barker reported that the purified Component E appeared faint pink in colour[88]. This was probably due to small amounts of bound corrinoid vitamins, rather than coenzyme, as the addition of coenzyme (and Component S) was required for activity. Barker found Component E was relatively stable if cationic buffers, *eg.* Tris were avoided. He noted that inactivation by such buffers was prevented by addition of substrate. This was evidence for the substrate binding to the E component.

Barker showed that Component S required incubation with mercaptoethanol, which reduces disulphide bonds, to produce the component in its monomeric, active form. He also found it easier to purify the protein in the reduced form, as it was chromatographically homogeneous. If allowed to oxidize, about 20 % of the S component took on the dimeric form. However, the component was more stable to storage in an oxidized state[87].

The amino acid composition, determined by Barker, for Component S[87], correlates well with the sequence obtained by Marsh[90]. The S Component contains 4 to 5 cysteine residues per molecule. Barker suggested, from the results of experiments with mercuribenzoate, iodoacetate and arsenite, that one cysteine was deeply buried, whereas two were always available to react with thiol specific reagents and two could form disulphide bonds[87].

Iodoacetate and mercuribenzoate both inhibited the activity of reduced Component S. However dimeric Component S was not inactivated by iodoacetate and upon reduction with mercaptoethanol exhibited activity. This demonstrated that a sulphydryl group which formed a disulphide bond in the oxidised dimer was partially responsible for enzyme activity. Arsenite also inhibited enzyme activity. Arsenite inactivation is evidence for vicinal dithiol groups.

Barker could not detect a stable complex between the mutase components by gel filtration techniques[87]. However he reasoned that the two components had appreciable affinity for each other as activity could be detected at very low protein concentrations.

The binding of the coenzyme to Component E was facilitated by Component S. Experiments showed that Component E alone bound one coenzyme molecule but with excess Component S it bound two molecules of coenzyme[87]. Component S alone did not bind coenzyme. Increasing the Component S : Component E ratio increased activity, until a maximum rate was reached. Further increases in the ratio of components had no effect on activity. However the K_m for the coenzyme continued to decrease. This indicated that the catalytic function of Component S was separate from its function of enhancing coenzyme binding. The ratio of Component E to Component S at half maximal enzyme activity was 1.6. The rate determined for various ratios of components gave a saturation curve with respect to either component[87].

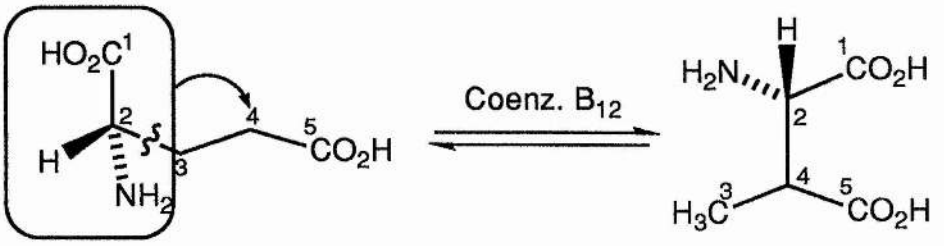
The equilibrium for the reaction favours the conversion of 3-methylaspartic acid to glutamic acid; the opposite direction to that of the physiological reaction. Barker measured K_{eq} at 30 °C and pH 8.2 as 10.7[8]. The enzyme showed most activity at pH 8.5 but was active between pH 6.0 and 9.8[8]. The pH of 8.2 was chosen for most experiments to avoid interference from mercaptoethanol in UV spectra. The enzyme showed maximal activity at 38 °C, and half maximal activity at 27 °C[8]. This was quite different to the temperature for maximal activity of methylaspartase, which was 55 °C[9].

The only substrates found for glutamate mutase were the physiological substrates, L-glutamic acid and 3-methylaspartic acid[8]. The K_m for glutamic acid was 1.5 mM and for 3-methylaspartic acid was 0.5 mM[85]. Barker tested D-glutamic acid, 3-ethylaspartic acid, α-, β- and γ-methyl-DL-glutamic acid, L-glutamine, the γ-methyl ester of L-glutamic acid and DL-α-aminoadipic acid for activity but no turnover was detected[8]. Buckel has shown that (2S,4S)-4-fluoroglutamic acid, (2R,3RS)-3-fluoroglutamic acid, 2-methyleneglutaric acid, (S)-3-methylitaconic acid and itaconic acid are competitive inhibitors for glutamate mutase[89]. He also recently obtained evidence for a paramagnetic species as an intermediate in the reaction with substrate, and also in the presence of the inhibitors, (2S,4S)-4-fluoroglutamic acid and 2-methyleneglutaric acid[61]. This may be the low-spin Co^{II} species, B_{12r}, which would be generated after homolytic cleavage of the coenzyme C-Co bond.

A series of coenzyme analogues have been examined by various groups. The following changes were made to the heterocyclic base at the sixth ligand position of the coenzyme whilst still conferring activity to the enzyme: 5-hydroxybenzimidazole[93], 2,6-diaminopurine, adenine, 5(6)-nitro-benzimidazole, 5(6)-aminobenzimidazole, 5(6)-trifluoromethyl-benzimidazole, benzimidazole[94], purine, 2-chloropurine, 2-thiopurine, 6-methylmercaptopurine and 5,6 dimethylbenzimidazole[95]. Those analogues found to be inactive included corrinoid vitamins, methyl, carboxymethyl and 5-deoxyuridyl cobalamins and cobinamide coenzyme[96]. As the corrinoid vitamins, without a deoxyadenosyl group, and the cobinamide coenzyme, without a nucleotide, were inactive, these experiments suggested that a deoxyadenosyl moiety and a heterocyclic base were required for activity, but that the enzyme was tolerant of fairly major alterations in the heterocyclic base.

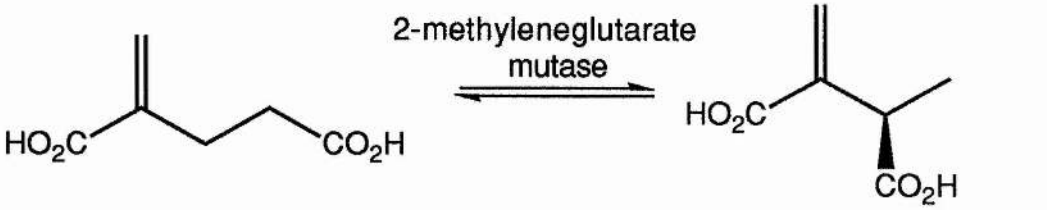
Stereochemical studies revealed the glutamate mutase catalysed rearrangement occurred *via* cleavage of the bond between C-2 and C-3 in the glutamic acid molecule, followed by transfer of the carboxyaminomethyl fragment thus formed, from C-3 to C-4, with inversion of configuration at C-

4[96,97]. This was accompanied by removal of a hydrogen from C-4 and addition of a hydrogen to C-3, thus forming a methyl group at this carbon (Scheme 1.14). No exchange of hydrogens with the solvent occurred[98]. More recently Hartrampf and Buckel fermented (2S,3R)- and (2S,3S)-[3-^{2}H, 3-^{3}H] glutamic acids with growing cells of *Clostridium tetanomorphum* and determined the chirality of the butyric acid produced[99]. They showed that racemization occurred at C-3, the methyl group of 3-methylaspartic acid.



Scheme 1.14 Schematic Diagram of Rearrangement Catalysed by Glutamate Mutase

1.2.6 Methyleneglutarate Mutase



2-methyleneglutaric acid (28)

(3R)-3-methylitaconic acid (29)

Scheme 1.15 2-Methyleneglutarate Mutase Catalysed Rearrangement

2-Methyleneglutarate mutase catalyses the rearrangement of 2-methyleneglutaric acid (28) to (3R)-3-methylitaconic acid (29) (Scheme 1.15)[100]. This reaction is a step in the fermentation of nicotinic acid to

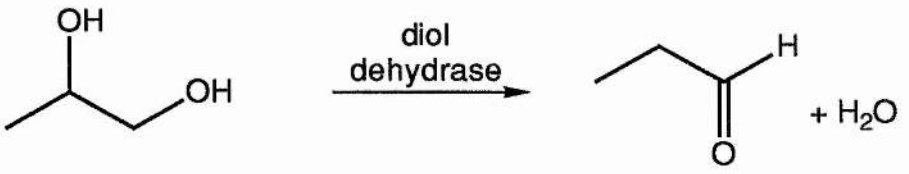
ammonia, propionic acid, acetic acid and carbon dioxide[101].

Buckel purified the enzyme from *Clostridium barkeri* [102]. He discovered it was a homotetramer, with a subunit molecular weight of 70 000 Da. The enzyme seemed to form a complex, stabilized by hydrophobic interactions, with 3-methylitaconate isomerase, the next enzyme on the pathway. The coenzyme remained active if the enzyme was purified in the dark[103], but was inactivated by exposure to light. Protection from inactivation of the coenzyme by light was given by the natural substrates. No evidence for any conformational strain was observed in the coenzyme whilst bound to the enzyme.

In order to assay for activity, Buckel coupled the mutase reaction to that of 3-methylitaconate isomerase, which converts the (R)-3-methylitaconic acid, produced by the mutase, to 2,3-dimethylmalic acid[102]. The UV absorbance due to the latter compound was measurable at 240 nm. The equilibrium constant for the conversion of (R)-3-methylitaconic acid to 2-methyleneglutaric acid was 0.26 ± 0.04, at I = 0.1 M and 25 °C[102]. The apparent K_m value for the coenzyme was 50 pM and for 2-methyleneglutaric acid 4 mM[102].

Buckel showed the rearrangement occurred with migration of the acrylic acid moiety from C-3 to C-4[104]. The stereochemistry of the reaction was investigated at C-4 (C-3 in (R)-3-methylitaconic acid), using (3R)-methyl-[3-^{2}H]-itaconic acid. Inversion of configuration was observed at this position[104]. Inversion of configuration also occurred in the glutamate mutase reaction but retention of configuration was observed in the methylmalonyl-CoA mutase reaction.

1.2.7 Diol Dehydrase



(2R)-1,2-propanediol (30)
and
(2S)-1,2-propanediol (31)

propionaldehyde
(32)

Scheme 1.16 Diol Dehydrase Catalysed Rearrangement

Diol dehydrase catalyses the irreversible conversion of both enantiomers of 1,2-propanediol (30,31) to propionaldehyde (32) (Scheme 1.16)[105]. Abeles showed the enzyme also converted glycerol to β-hydroxypropionaldehyde[31] and 1,2-ethanediol to acetaldehyde[105]. Toraya demonstrated that glycerol also caused irreversible inactivation of the enzyme[106]. The enzyme plays a role in glycerol fermentation in microorganisms[105]. Several methods of measuring diol dehydrase activity have been devised. Abeles coupled the rearrangement to a NAD^+-dependent alcohol dehydrogenase reaction and measured the decrease in absorbance due to NADH with time[107]. Other methods converted the aldehyde product of the diol dehydrase reaction to the 2,4-dinitrophenylhydrazone derivative[31] or azine derivative[108], which could be quantified

The molecular weight of diol dehydrase was determined, by Toraya, to be 230 000 ± 10 000 Da[109]. The enzyme had two components; Component F had a molecular weight of 26 000 Da and Component S, 200 000 Da[109]. Component S consisted of at least four different polypeptide chains. The enzyme appeared to bind one mole of coenzyme per mole of enzyme[110]. Both components were required for activity and coenzyme binding[111,112]. Reassociation of the components was promoted by the substrate[109]. The enzyme was irreversibly inactivated by exposure to oxygen[66], although protection was afforded by the substrate[113], suggesting the substrate bound close to the coenzyme.

The use of thiol-specific alkylating agents showed that Component S contained thiol group(s)[111,112]. Modification of the thiol group(s) prevented coenzyme binding[114].

Lee and Abeles showed diol dehydrase required a monovalent cation for activity[31]. Potassium, rubidium, ammonium and methylammonium cations were most active. Activity was thus related to ionic radius[115]. The monovalent cation was involved in binding the coenzyme to the enzyme.

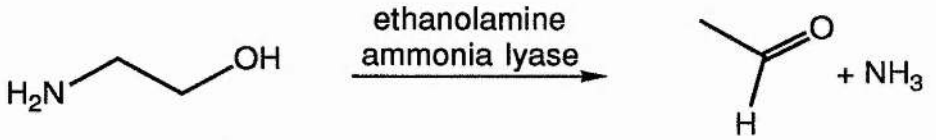
Diol dehydrase has a wide substrate specificity, accepting both enantiomers of 1,2-propanediol and an number of other glycols, including 3-fluoro-[116] and 3-chloro-1,2-propanediols[117]. Abeles showed that the *pro* -R hydrogen migrated stereospecifically in the reaction with (2R)-1,2-propanediol (30), and the *pro* -S hydrogen with (2S)-1,2-propanediol (31)[118,119]. No incorporation of solvent protons occurred[120]. Retey showed the hydroxyl group migrated stereospecifically, to form a gem-diol[121], from which water was then eliminated, again stereospecifically[122]. The deuterium isotope effect for the overall reaction was found to be 10 - 12[118,119], indicating that cleavage of the substrate C-H bond was rate-limiting. The tritium isotope effect for transfer of hydrogen from the substrate to the coenzyme was 20. Abeles measured isotope effects of 125 for tritium and 28 for deuterium, for the transfer of hydrogen from the coenzyme to the product[110].

Abeles and Fukui investigated the effect on enzyme activity of modifying the coenzyme[107,123]. Structure / activity correlations led to the following conclusions: firstly, very few changes could be made to the adenosyl group without loss of activity[108], secondly, the benzimidazoyl ligand was not involved in catalysis[124] and also that the coenzyme propionamide side chains interact with the enzyme to facilitate C-Co bond cleavage and stabilize radical intermediates[125]

In 1970, Schrauzer proposed that the diol dehydrase catalysed rearrangement occurred by a 1,2-hydride shift[126]. He accounted for the transfer of label between substrate and coenzyme by invoking an exchange

between labelled propionaldehyde and the coenzyme after the hydride shift. However, Abeles' measurements of the specific activities of the coenzyme, substrate and product, during the course of the reaction with 1,2-[1-^{3}H]-propanediol as substrate gave specific activity values for the coenzyme which were much larger than those for the substrate or product[127]. This was inconsistent with an equilibration process. Schrauzer also proposed that the enzyme abstracted a hydrogen from C-4' of the coenzyme[126]. Once again Abeles and co-workers showed that no labelling occurred at this centre during the course of the reaction[127]. Schrauzer's proposals show, perhaps, the folly of studying model systems in isolation from the enzyme reaction.

1.2.8 Ethanolamine Ammonia-Lyase



ethanolamine (33) — acetaldehyde

Scheme 1.17 Ethanolamine Ammonia-Lyase Catalysed Rearrangement

Ethanolamine ammonia-lyase catalyses the deamination of vicinal amino alcohols to carbonyl compounds and ammonia. The natural substrate for the enzyme is ethanolamine (33), which is converted to acetaldehyde and ammonia[128] (Scheme 1.17). The enzyme was first obtained by Kaplan and Stadtman from an uncharacterized *Clostridium* species in large quantities[129]. It was a large protein of molecular weight, 520 000 Da[130]. The enzyme had subunits of 36 000 Da and 51 000 Da arranged in two stacked rings with five or six subunits in each ring[131]. Between one and three moles of coenzyme were tightly bound to each mole of enzyme[132]. Experiments by Babior showed the enzyme had two active sites. Enzyme activity was destroyed by thiol-specific reagents[129].

Kaplan and Stadtman showed a monovalent cation was required for activity[129]. Potassium was most suitable but ammonium and rubidium also supported activity. Sodium and lithium were competitive inhibitors.

Babior showed, using [1-^{3}H]-ethanolamine that the hydrogen was removed from C-1 stereospecifically[65]. However racemization occurred when it was replaced at C-2[133].

The enzyme accepts a number of other substrates as well as ethanolamine. These include (S)-2-aminopropanol (34)[134], (R)-2-aminopropanol (35)[135] and 1-amino-2-propanol[136]. Babior measured the K_m values for the above substrates. The K_m for ethanolamine was 22 μM[137], for (S)-2-aminopropanol, 15.5 μM[138] and for (R)-2-aminopropanol, 63.6 μM[135].

(S)-2-Aminopropanol was deaminated at 1 - 2 % of the rate of ethanolamine[139]. The coenzyme was irreversibly destroyed in an abortive side reaction that occurred once every 300 - 400 catalytic cycles[62,139]. Retey and Babior showed the *pro* -S hydrogen was abstracted from C-1[135]. This hydrogen was transferred to C-2, where inversion occurred as the amino group left. Ammonia was eliminated from the *gem* -amino alcohol thus formed, to give propionaldehyde. Unlike the rearrangement of ethanolamine the reaction was, to a small extent, reversible[134].

(R)-2-Aminopropanol reacts at 12 % of the rate of the (S)-enantiomer[134]. Retey and Babior found that, with this substrate, the *pro* -S hydrogen was again removed from C-1[135]. Hence the hydrogen *threo* - with respect to the amino group was abstracted in the (R)-enantiomer but that *erythro* - to the amino group in the (S)-substrate. The hydrogen was replaced with retention of configuration at C-2, in contrast to the inversion seen with the (S)-enantiomer. The enzyme also catalysed the conversion of the (R)-enantiomer to the (S)-enantiomer[135].

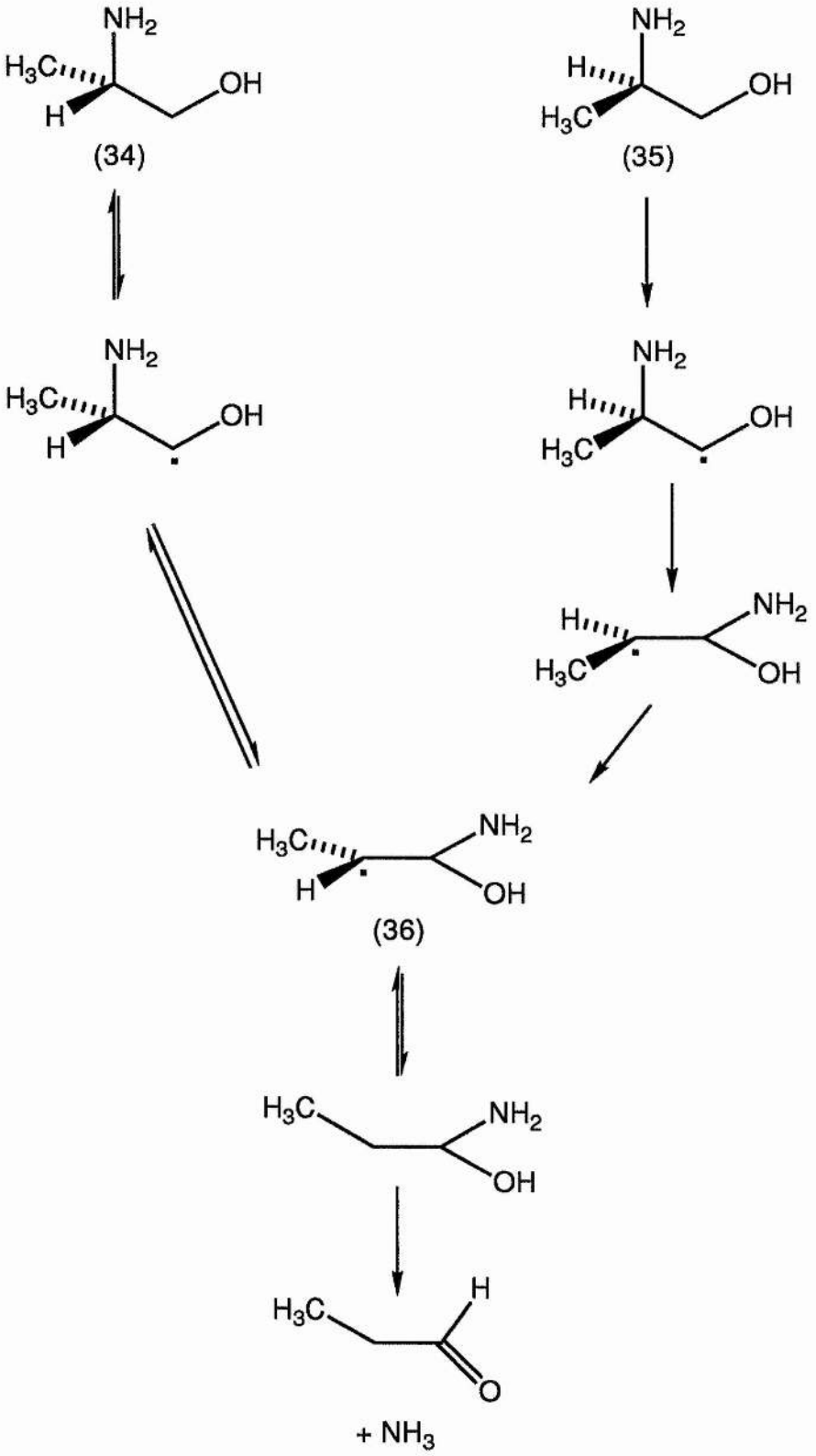
Babior found the turnover of 1-amino-2-propanol was very slow and that catalytic activity of the enzyme was lost after only a few turnovers[136]. The

reaction products were acetone and ammonia. Again a hydrogen was abstracted from the carbon bonded to the alcohol (C-2). In this case abstraction occurred from a secondary centre.

Babior demonstrated, using [1-^{3}H]-ethanolamine, that the hydrogen migrated *via* the 5' position of 5'-deoxyadenosine[140]. He also demonstrated the reversible formation of 5'-deoxyadenosine as an intermediate in the reaction[67,141]. EPR studies showed signals attributable to cob(II)alamin, the product of homolytic cleavage of the Co-C bond, and a substrate radical[57,58]. With 2-aminopropanol as substrate the radical was identified, using labelled substrates, as the 2-aminopropanol-1-yl radical[62]. The cob(II)alamin and substrate radicals signals appeared at the same rate, approximately twice that of the overall catalytic reaction.

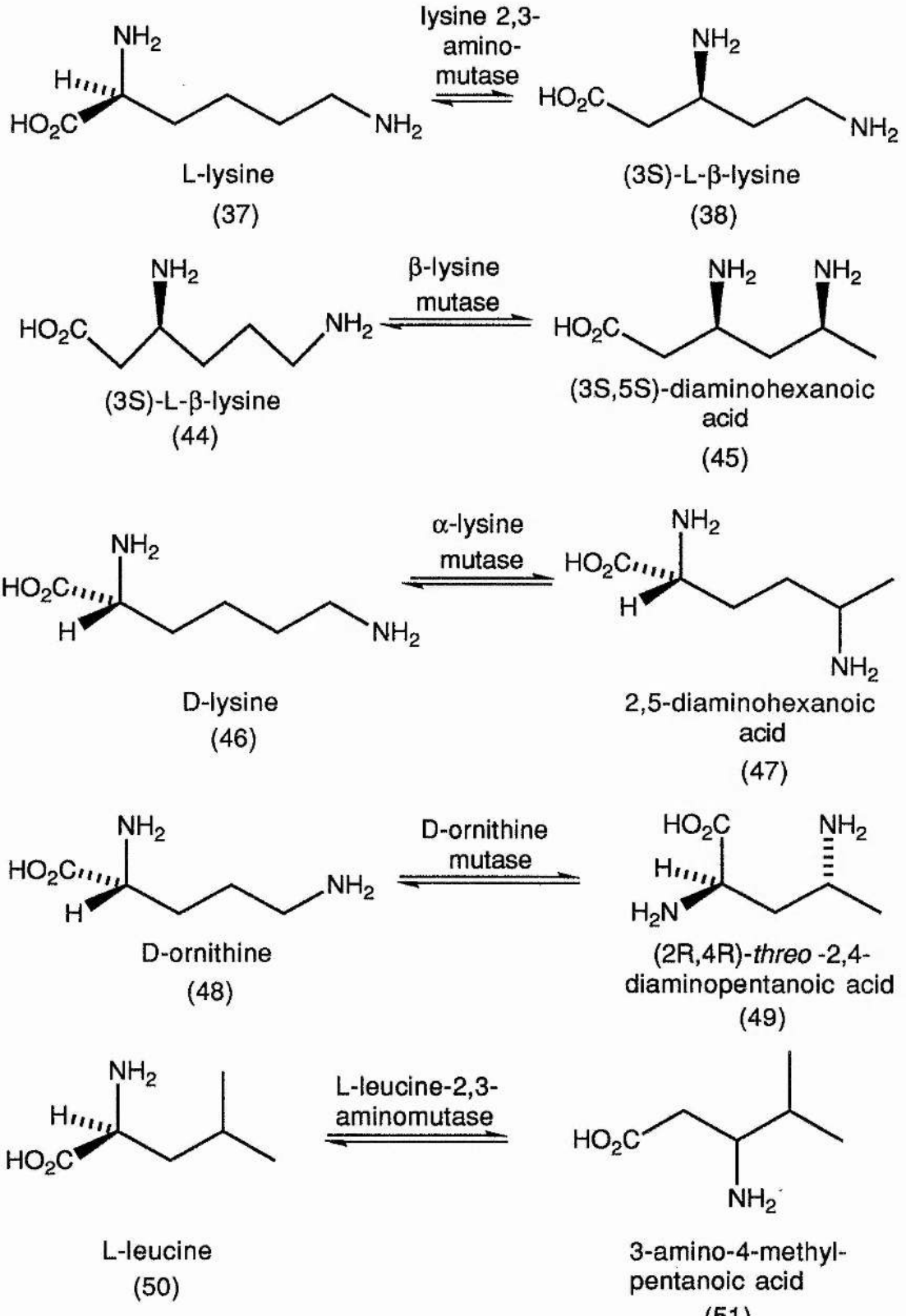
Using both deuterium and tritium labelled ethanolamine and [5'-^{3}H]-coenzyme B_{12}, Babior measured the tritium isotope effect for each of the two hydrogen transfer steps, the overall deuterium isotope effect and the deuterium isotope effect for the second hydrogen transfer step[137,142]. The overall deuterium isotope effect on K_m was 7.4. This was in close agreement with the value for the second hydrogen transfer step. Thus indicating the second hydrogen transfer step was rate limiting. There was an exceedingly large tritium isotope effect on the second hydrogen transfer step, of 160, which was not predicted from the deuterium isotope effect and has not been explained.

The isotope effects observed for (S)-2-aminopropanol (34) were similar to those for ethanolamine[138,62]. However the values for the (R)-enantiomer (35) were very different. They showed the first hydrogen transfer step was rate limiting with (R)-2-aminopropanol as substrate. Based on these isotope effects and the stereochemical results discussed above, Babior suggested a common intermediate for the two reactions, namely the product radical corresponding to the (S)-configuration (36) (Scheme 1.18)[143]. This required inversion of configuration at C-2 of the (R)-enantiomer after migration of the amino group.



Scheme 1.18 Mechanism for the Rearrangement of (S)- and (R)-2-Aminopropanol

1.2.9 The Aminomutases



L-lysine (37)

(3S)-L-β-lysine (38)

(3S)-L-β-lysine (44)

(3S,5S)-diaminohexanoic acid (45)

D-lysine (46)

2,5-diaminohexanoic acid (47)

D-ornithine (48)

(2R,4R)-*threo*-2,4-diaminopentanoic acid (49)

L-leucine (50)

3-amino-4-methyl-pentanoic acid (51)

Scheme 1.19 Aminomutase Catalysed Rearrangements

This group of enzymes includes four coenzyme B_{12}-dependent enzymes and the related lysine 2,3-aminomutase (Scheme 1.19). A survey of the aminomutases highlights how different systems have evolved to solve the same catalytic problem.

1.2.9.1 Lysine 2,3-Aminomutase

Lysine 2,3-aminomutase catalyses the rearrangement of L-α-lysine (37) to L-β-lysine (38)[144]. The enzyme is not coenzyme B_{12}-dependent but requires S-adenosyl-methionine (39) (Figure 1.3)[145], termed by Barker the 'poor man's adenosylcobalamin'[146], as cofactor for a reaction very similar to that catalysed by coenzyme B_{12}-dependent enzymes.

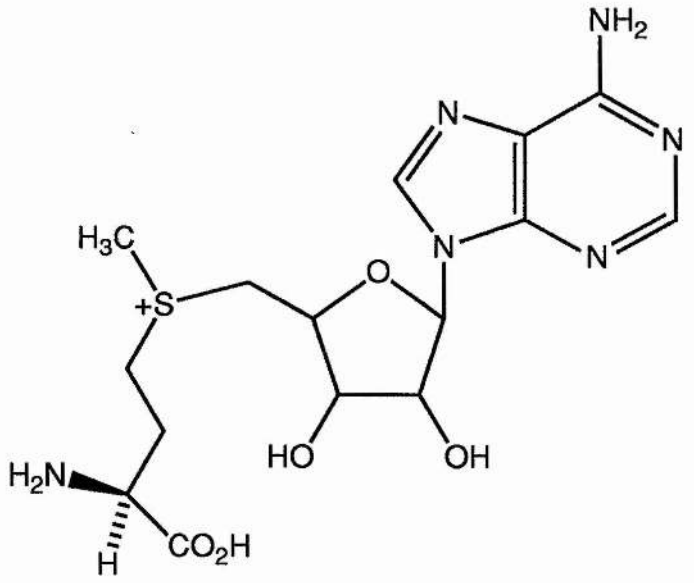


Figure 1.3 S-Adenosylmethionine

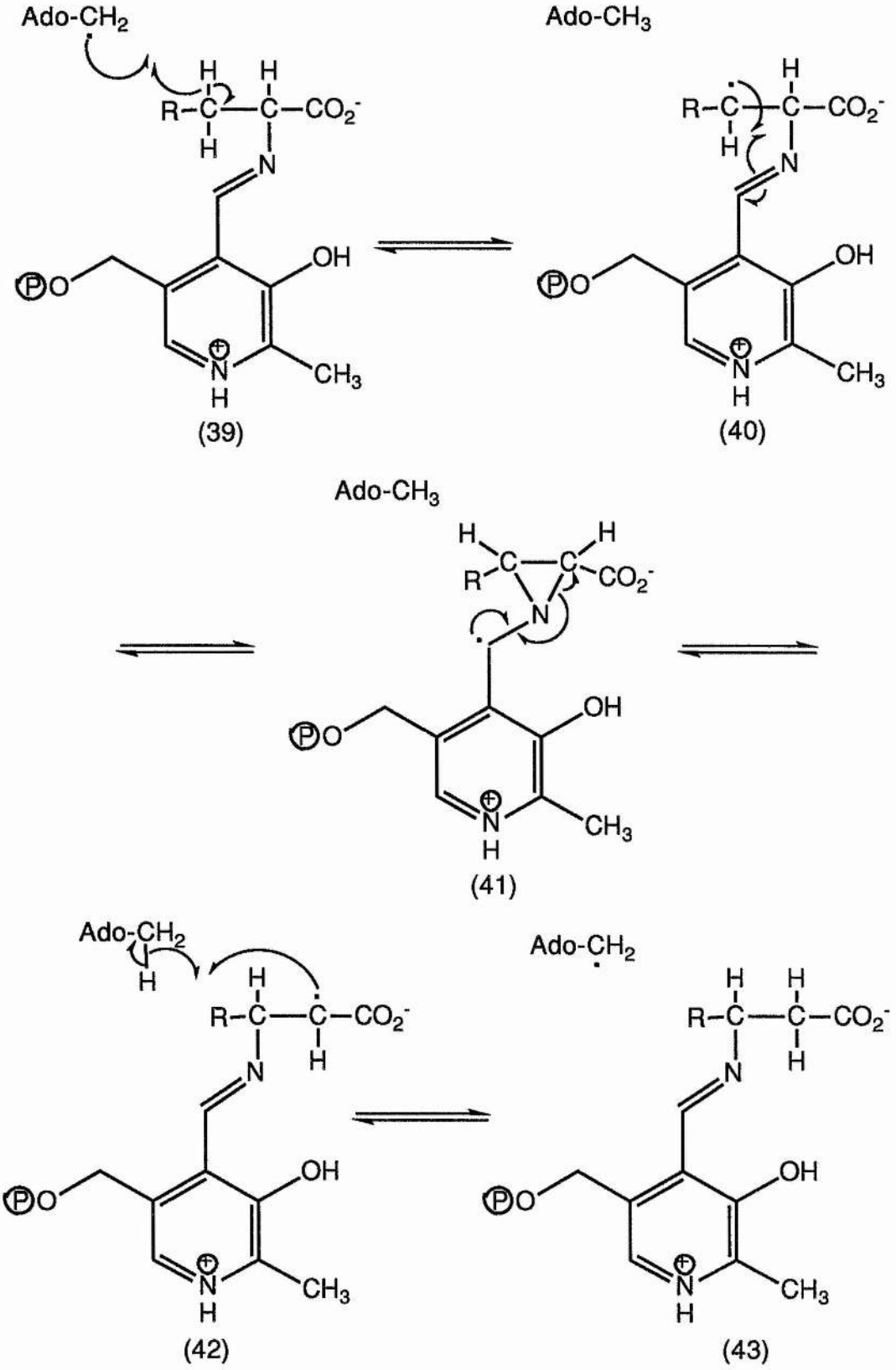
Lysine 2,3-aminomutase also requires pyridoxal phophate (PLP) (see Scheme 1.20), cobalt and iron sulphur centres for activity[147]. The related coenzyme B_{12}-dependent enzymes, β-lysine mutase[148], α-lysine aminomutase[149], D-ornithine aminomutase[150] and L-leucine 2,3-

aminomutase[39] (Scheme 1.19) are also PLP-dependent.

In Clostridia, lysine 2,3-aminomutase catalyses the first step in the degradation of lysine to ammonia and acetyl-CoA[151]. In other microorganisms the enzyme is responsible for the β-lysine derived acyl subunit found in some antibiotics[152].

Recent work on this system, by Frey, using lysine 2,3-aminomutase isolated from *Clostridium* SB4[153] showed the enzyme was a hexamer of molecular weight 285 000 Da[154]. The enzyme contained 12 to 13 iron and sulphur atoms, greater than three cobalt atoms and 5.5 moles of PLP, per hexamer.

Frey found the enzyme was activated by the reaction of S-adenosyl-methionine with the reduced metal (Fe-S) centre, leading to the formation of a reactive adenosyl-metal cofactor[154]. The role of the cobalt is not known. The adenosyl-metal cofactor then underwent reversible homolytic cleavage to give the 5'-deoxyadenosyl radical. The rearrangement mechanism was then very similar to that of a coenzyme B_{12}-dependent rearrangement (Scheme 1.8). However, during the reaction, the lysine molecule was bound to PLP (Scheme 1.20)[155]. PLP facilitated the 1,2-imino shift, by stabilizing the radical intermediates. Thus the rearrangement occurred as follows; a hydrogen was abstracted from the substrate (39), PLP-mediated rearrangement of the substrate radical occurred (40), to give an aziridine intermediate (41), a 1,2-imino shift produced the product radical (42) which reabstracted a hydrogen (43), to give the PLP-bound product.



Scheme 1.20 Mechanism for the Reaction Catalysed by Lysine 2,3-Aminomutase

Labelling experiments showed the 3-*pro*- R hydrogen of lysine was transferred to the 2-*pro*- R position in β-lysine. This was accompanied by concomitant migration of the amino group from C-2 to C-3[156]. There was no exchange of hydrogens with the solvent[144]. PLP probably plays a similar role in the coenzyme B_{12}-dependent aminomutase reactions, facilitating transfer of the amino group.

Frey performed EPR studies on lysine 2,3-aminomutase which indicated the presence of an organic radical intermediate[155]. Deuteriation of the substrate led to changes in the signal. This indicated the lysine carbon skeleton was the host for the radical centre. Changes in the hyperfine splitting patterns in the EPR spectra of [2-^{2}H]-lysine and [2-^{13}C]-lysine allowed further identification of the radical species. It appeared to be a Π-radical with the unpaired spin localized primarily in a p orbital on C-2 of β-lysine.

1.2.9.2 L-β-Lysine Mutase / D-α-Lysine Mutase

The clostridial enzyme L-β-lysine mutase catalyses the second step in the fermentation pathway of lysine to ammonia, acetic acid and butyric acid, the conversion of L-β-lysine (44) to (2S,5S)-*erythro* -3,5-diaminohexanoic acid (45) (Scheme 1.19)[157]. The protein complex responsible for L-β-lysine mutase activity also catalyses a D-α-lysine mutase reaction[150], which converts D-α-lysine (46) to 2,5-diaminohexanoic acid (47) (Scheme 1.19)[36]. The complex has a molecular weight of 250 000 Da[149,150]. Stadtman showed the complex from *Clostridium sticklandii* consisted of an E_1 and E_2 protein. E_1 bound the cobalamin coenzyme and E_2 contained sulphydryl groups. Both catalytic activities required coenzyme B_{12}, PLP, ATP, divalent and monovalent cations and a mercaptan[34,148].

1.2.9.3 D-Ornithine Mutase

D-Ornithine mutase, also found in Clostridia, catalyses the first step in the fermentation of ornithine to acetic acid, carbon dioxide, alanine and ammonia[37,38]. It catalyses the conversion of D-ornithine (48) to (2R,4R)-*threo* -2,4-diaminopentanoic acid (49) (Scheme 1.19). Baker showed the enzyme had a molecular weight of 200 000 Da with subunits of 90 000 Da and 12 800 Da[150]. D-ornithine mutase is also dependent on PLP and coenzyme B_{12}, but has no requirement for ATP or cations.

1.2.9.4 L-Leucine 2,3-Aminomutase

L-Leucine 2,3-aminomutase is widely distributed in bacteria, mammals, plants and yeast[39,158,159]. It was first discovered in *Clostridium sporogenes* where it catalyses the first step in the fermentation of L-leucine to isobutyric acid, the conversion of L-leucine (50) to β-leucine (51) (Scheme 1.19)[39]. The enzyme is also coenzyme B_{12} and PLP-dependent.

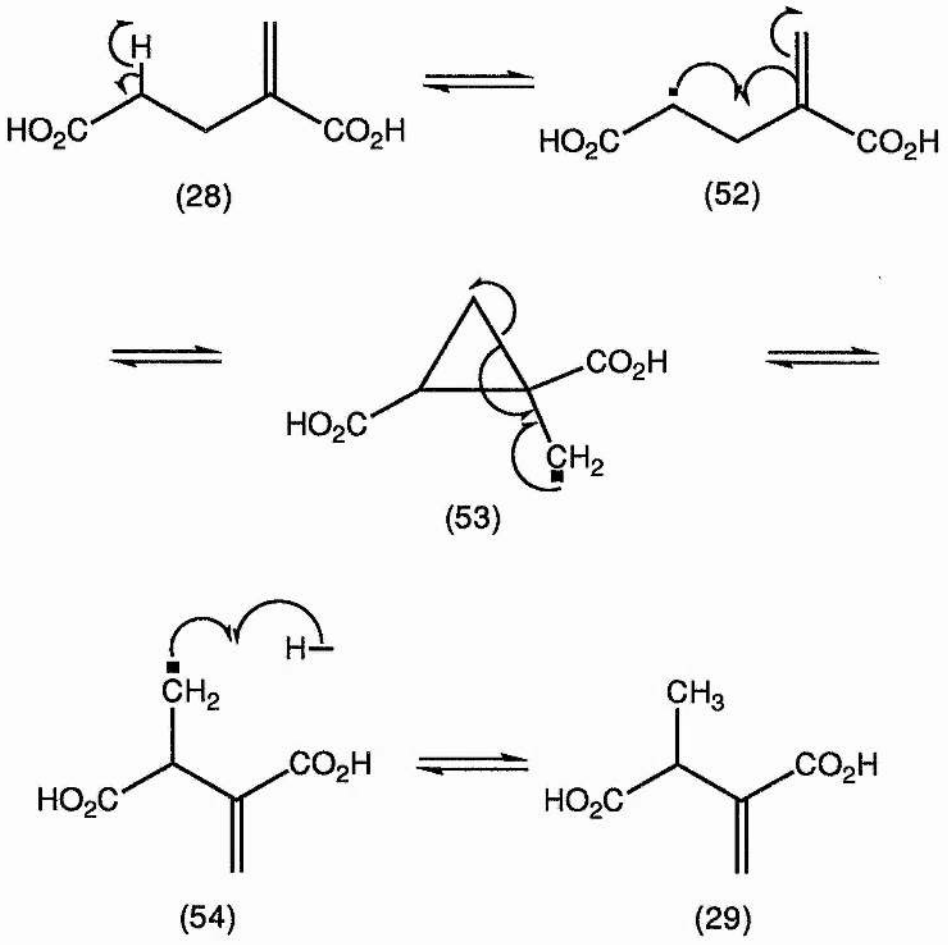
1.2.10 Model Reactions

Modelling coenzyme B_{12}-dependent reactions may provide an insight into the mechanisms of the enzyme catalysed reactions. Manipulation of the reaction conditions, to favour either free radical or cobalt-mediated mechanisms, gives a body of information which can be used to lend credence to the general reaction scheme postulated above (pp. 19 - 20, Scheme 1.8).

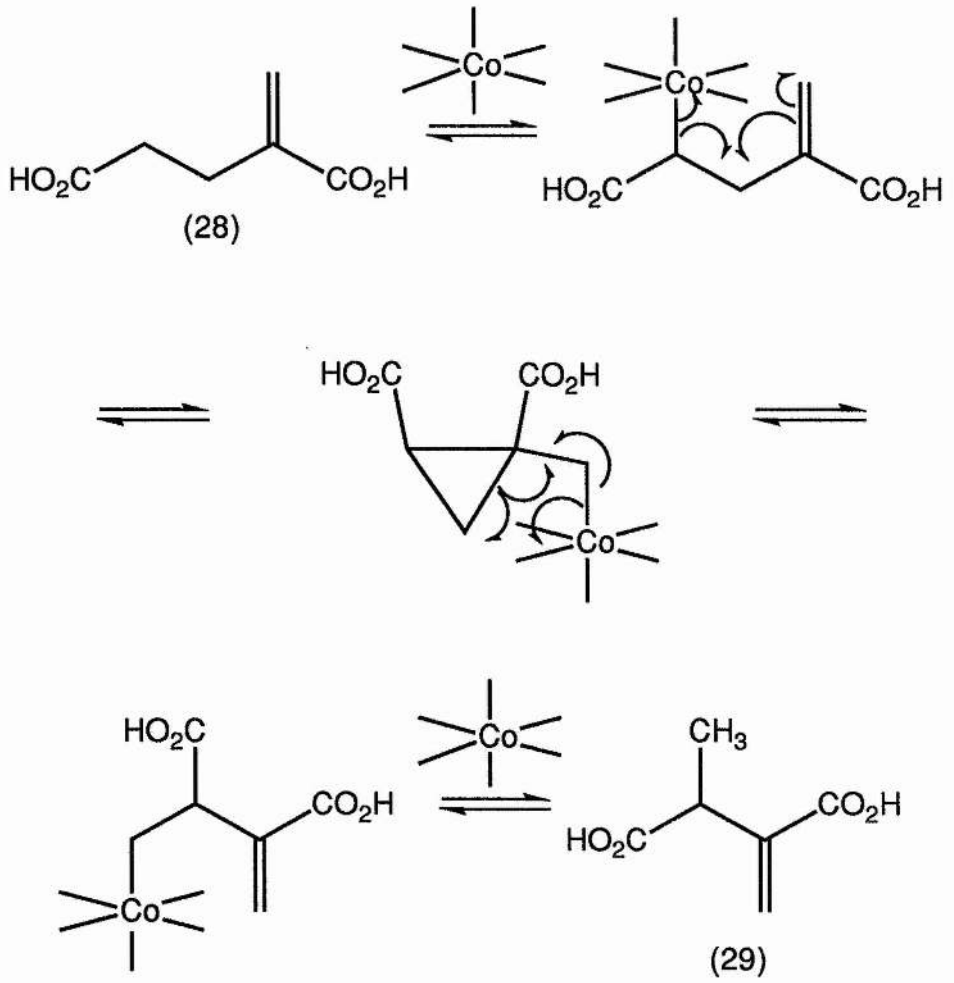
1.2.10.1 Models for 2-Methyleneglutarate mutase

The enzyme catalysed rearrangement of 2-methyleneglutaric acid (28) to methylitaconic acid (29) could occur by an entirely free radical mechanism (Scheme 1.21) in which a hydrogen is abstracted from 2-methyleneglutaric acid (28) to give the substrate radical (52), which rearranges to the cyclic intermediate (53), and then to the product radical (54). Reabstraction of the hydrogen gives the product (29). Scheme 1.22 shows an alternative cobalt-mediated mechanism.

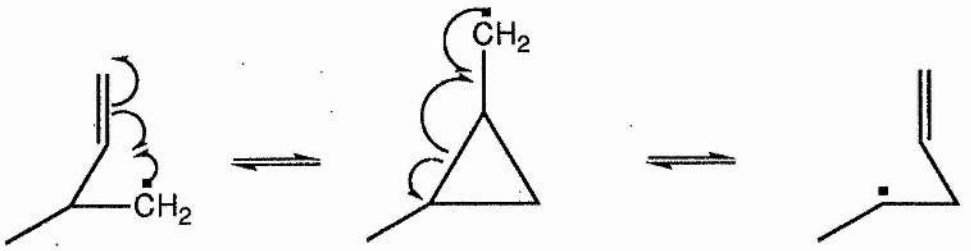
The free radical pathway (Scheme 1.21) has some chemical precedent, namely, rearrangement of the but-3-enyl radical (55), *via* a cyclopropylcarbinyl radical (56), to give the pent-1-enyl radical (57) (Scheme 1.23)[160]. Golding similarly showed that the 3-ethoxycarbonyl but-1-enyl radical (58) rearranged to the 4-ethoxycarbonyl but-1-enyl radical (59) by a free radical mechanism. Again a cyclic intermediate (60) can be envisaged (Scheme 1.24). A tin radical was used to initiate the reaction. The corresponding cobaloxime compounds did not rearrange[161]. However, Dowd showed, in a similar system, that the cobalt adduct of *bis*-(tetrahydropyranyl) methylitaconate (61) did rearrange, at room temperature, to give a mixture of 2-methyleneglutaric acid (28), the rearrangement product, methylitaconic acid (29) and butadiene 2,3-dicarboxylic acid (62) (Scheme 1.25)[162].



Scheme 1.21 Radical Mechanism for the Conversion of 2-Methyleneglutaric Acid to Methylitaconic Acid

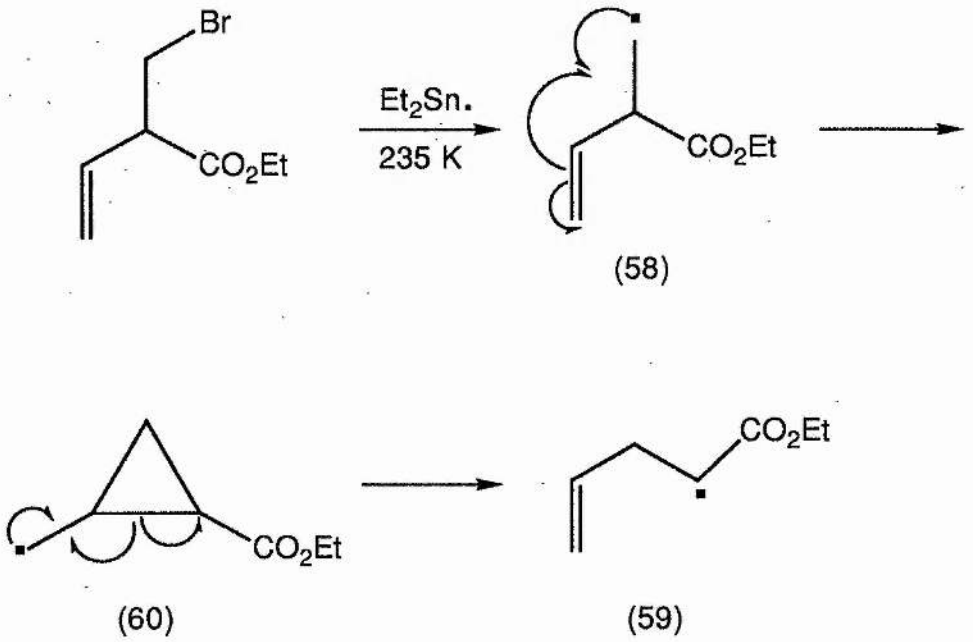


Scheme 1.22 Cobalt Mediated Mechanism for Conversion of 2-Methyleneglutaric Acid to Methylitaconic Acid



(55) (56) (57)

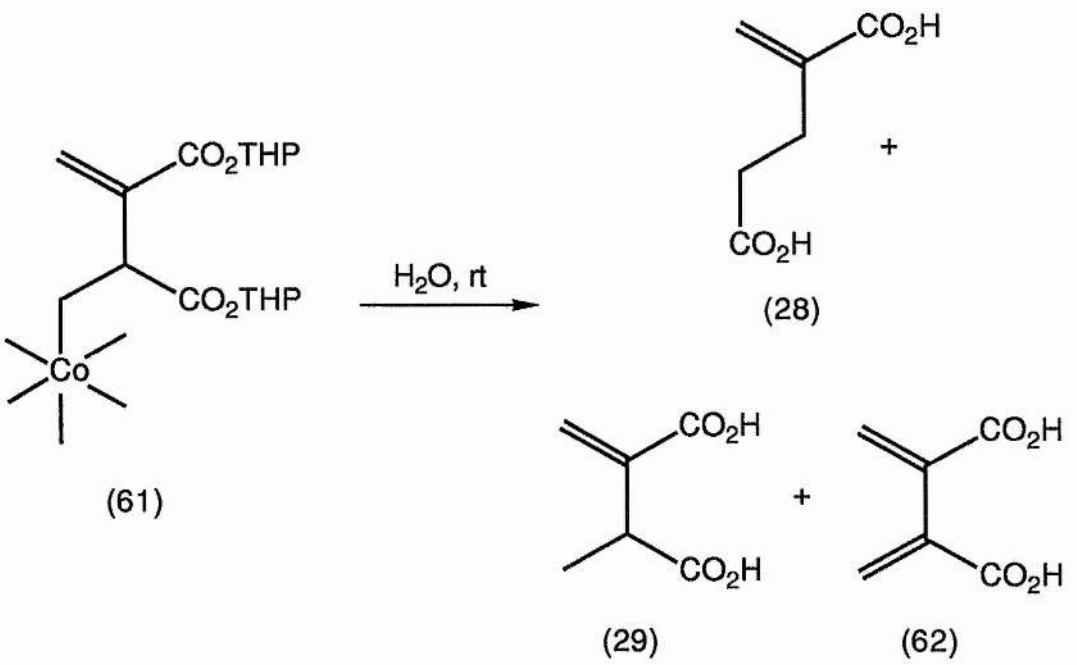
Scheme 1.23 Chemical Precedent for a Free Radical Mechanism for the 2-Methyleneglutarate Mutase Catalysed Rearrangent



(58)

(60) (59)

Scheme 1.24 Free Radical Model for the 2-Methyleneglutarate Mutase Catalysed Rearrangement



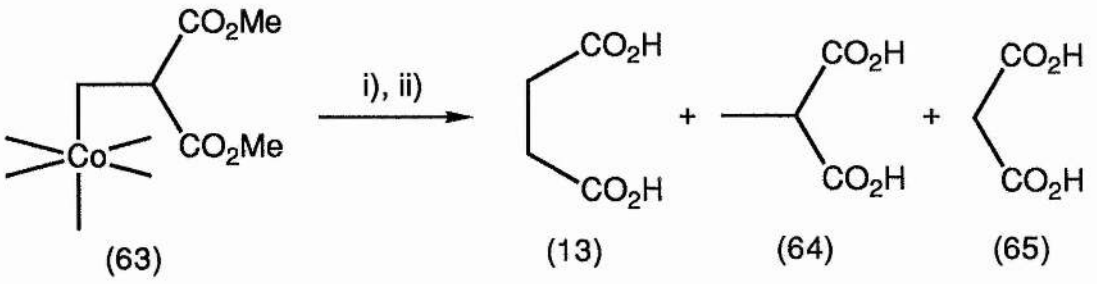
Scheme 1.25 Cobalt Mediated Model of the 2-Methyleneglutarate Mutase Catalysed Rearrangement

1.2.10.2 Models for Methylmalonyl-CoA Mutase

Similarly various model reactions have been designed to mimic the methylmalonyl-CoA mutase catalysed rearrangement. A variety of mechanisms have been shown to operate in these models. Dowd showed that the cobalt adduct of dimethyl methylmalonate (63) underwent rearrangement, in water, in the dark, at room temperature and physiological pH, to give succinic acid (13) in 3 % yield. Methylmalonic acid (64) and malonic acid (65) were also formed[163] (Scheme 1.26). Halpern also showed that cobalt adduct of diethyl 2,2-dimethylmalonyl thio ester (66) rearranged to give some diethyl methylsuccinyl thio ester (67) and some unrearranged material (68). However the bromo-analogue (69) also gave the rearrangement product (67)[164] (Scheme 1.27). Hence both cobalt-mediated and free radical mechanisms were shown to operate with this substrate. Lowe and Ingraham showed that yet another mechanism was

possible with their rearrangement of 1-carbethoxy-2-oxocyclopentyl-methyl(pyridinato)bis(dimethylglyoximato)cobalt(III) (70) to ethyl 3-oxocyclohexanecarboxylate (71) (Scheme 1.28)[165]. Such a ring expansion must occur *via* the carbanionic intermediates (72) and (73). Some ethyl 2-oxo-1-methylcyclopentanecarboxylate (74) was also formed.

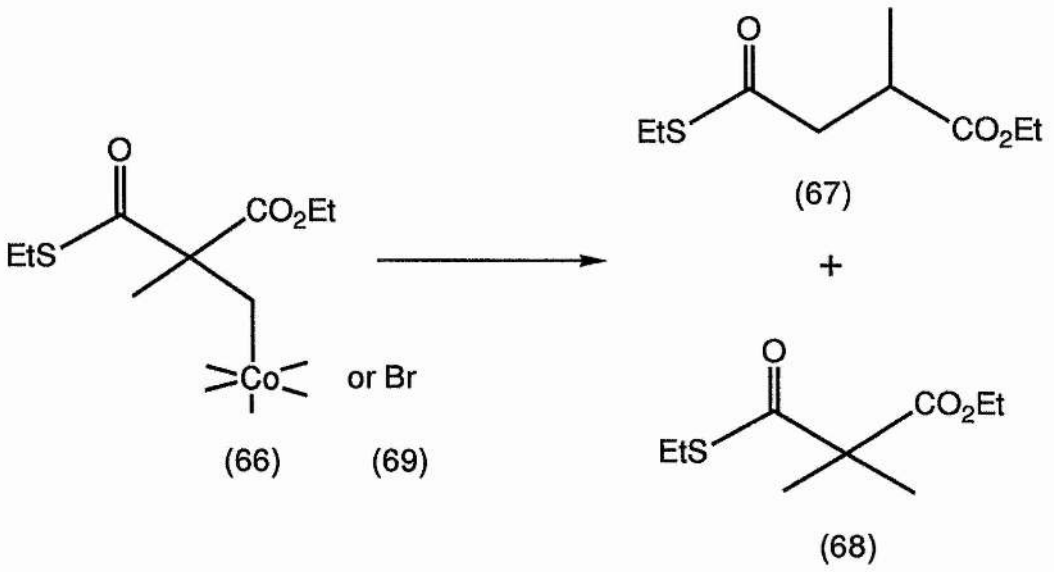
Retey devised a cobaloxime in which the substrate was covalently attached to side chains on the corrin ring[166]. The substrate radical formed by homolysis of the C-Co bond was therefore trapped close to the cobalt. Thus, the chemistry of the reaction was influenced by the cobalt. A transition state was postulated in which the cobalt interacted with the migrating CoA thio ester and with the carbon centres between which the CoA thio ester migrated. Retey's bridged cobaloxime system gave up to 95 % yield of the rearrangement product.



(63) (13) (64) (65)

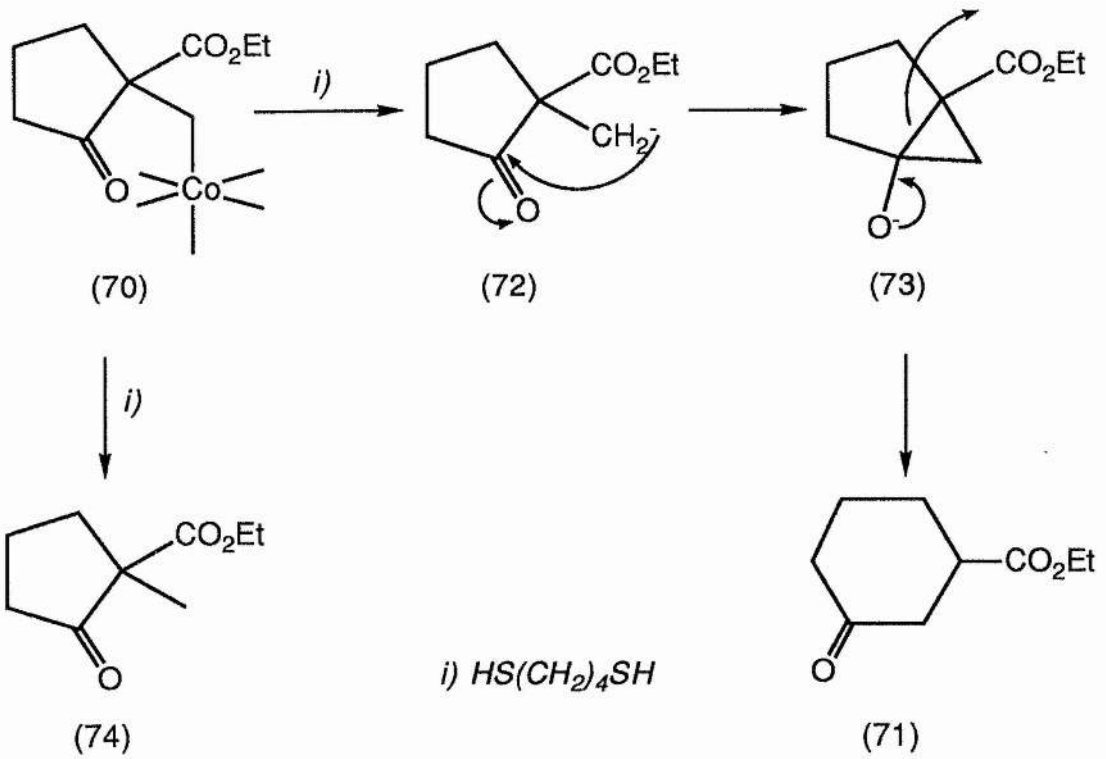
i) H_2O, 48 h, dark
ii) H^+

Scheme 1.26 Cobalt Mediated Rearrangement of Dimethyl Methylmalonic Acid



(66) (69) (67) (68)

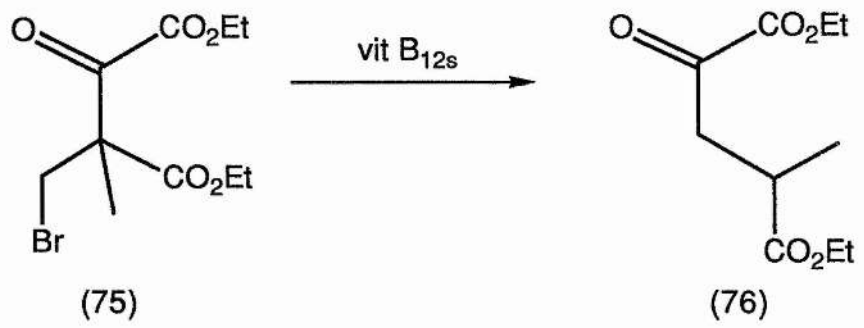
Scheme 1.27 Free Radical and Cobalt Mediated Rearrangements of Diethyl 2,2-Dimethylmalonyl Thio Ester



Scheme 1.28 Ionic Rearrangement Mechanism Mimicking the Methylmalonyl-CoA Mutase Catalysed Rearrangement

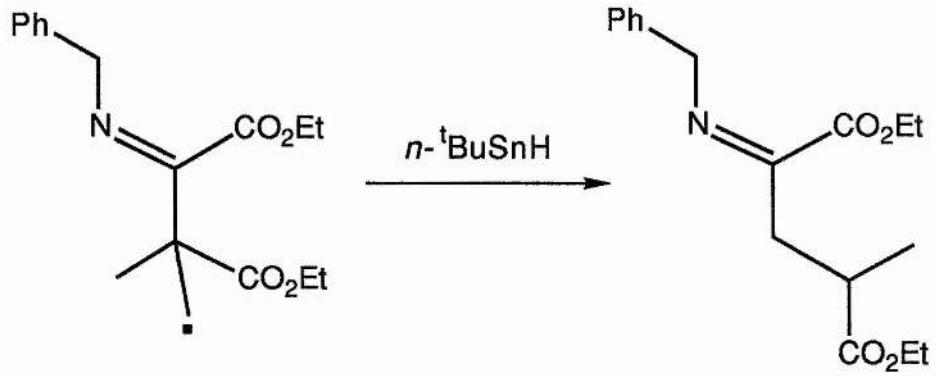
1.2.10.3 Models for Glutamate Mutase

Dowd showed diethyl 3-bromomethyl-3-methyl-2-oxosuccinate (75) rearranged in the presence of a vitamin B_{12} nucleophile to give diethyl 4-methyl-2-ketoglutaric acid (76) in 71 % yield (Scheme 1.29)[167]. This reaction serves as a partial model for the rearrangement of 3-methylaspartic acid to glutamic acid. However in contrast, the Schiff's base of diethyl 3-bromomethyl-3-methyl-2-oxosuccinate (75), namely, diethyl 3,3-dimethyl-2-benzylimino succinate (77), would not rearrange in the presence of vitamin B_{12s}, but did rearrange with tri-*n*-butyltin hydride, to give diethyl 4-methyl-2-benzylimino glutarate (78) (Scheme 1.30).



(75) (76)

Scheme 1.29 Cobalt Mediated Rearrangement of Methylaspartic Acid Mimic

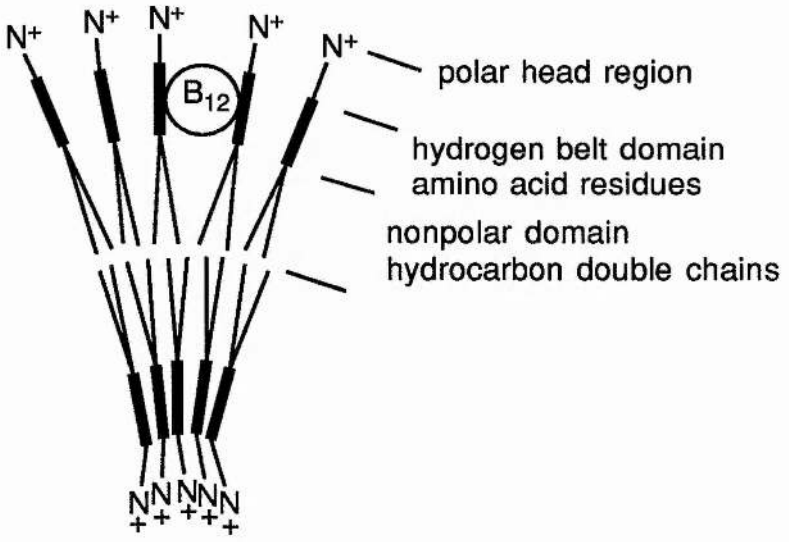


(77) (78)

Scheme 1.30 Free radical Rearrangement of Methylaspartic Acid Mimic

In an alternative approach, Murakami developed a hydrophobic microenvironment to use as a mimic for the apoenzyme[168,169,170]. This consisted of single-walled bilayer vesicles formed by aggregates of synthetic peptide lipids. Chemical modification of the cobalamin molecule made it hydrophobic and hence the coenzyme inserted into the apolar domain of the membrane (Figure 1.4). Diethyl 3-methylaspartic acid was successfully induced to rearrange to give diethyl glutamic acid, in this environment. Vanadium trichloride and atmospheric oxygen were used to generate the substrate radical, which then reacted with the hydrophobic cobalamin to give a C-Co bond. The C-Co bond was cleaved by photolysis. The membrane

served to keep the substrate radical and coenzyme in close proximity, allowing the cobalt ion to promote rearrangement.



$(CH_3)_3N^+(CH_2)_5CONHCH(CH_3)CON$ with $(CH_2)_nCH_3$ and $(CH_2)_nCH_3$

Peptide lipid - $N^+C_5Ala2C_{n+1}$

Figure 1.4 Peptide Lipid Single-Walled Bilayer Vesicle with Hydrophobic Vitamin B_{12} - an Artifical B_{12} Holoenzyme

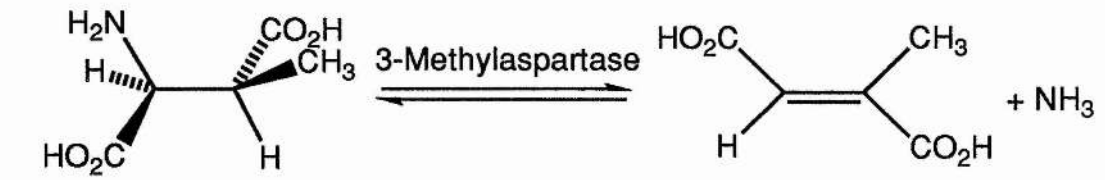
1.2.11 Conclusion

The experimental evidence available form both enzyme and model studies supports the hypothesis that catalysis involves homolytic cleavage of the coenzyme C-Co bond, to initiate the reaction. The high energy radical formed must be bound, by the enzyme, in such a way that it abstracts a hydrogen only from the substrate and does not undergo any radical quenching processes. The enzyme then induces the rearrangement which is possibly mediated by the coenzyme cobalt ion.

THE AMMONIA-LYASES

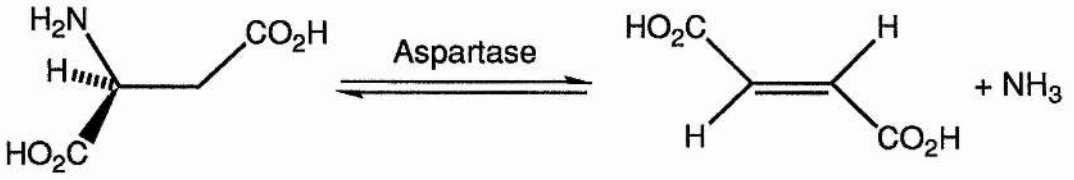
1.3.1 Ammonia - lyases

Ammonia-lyases are defined as enzymes which catalyse carbon to nitrogen (C-N) bond cleavage, to give ammonia and a double bond[171]. This constitutes a large group of enzymes which use a variety of coenzymes to bring about catalysis. For example, glucosaminate ammonia-lyase (EC 4.3.1.9)[172], serine sulphate ammonia-lyase (EC 4.3.1.10)[173] and carbamoyl-serine ammonia-lyase (EC 4.3.1.1)[174] require pyridoxal phosphate (PLP). Ethanolamine ammonia-lyase (EC 4.3.1.7)[175] requires coenzyme B_{12}, while ornithine *cyclo*-deaminase (EC 4.3.1.12)[176] requires nicotinamide (NAD^+). The remaining ammonia-lyases: formimino-tetrahydrofolate *cyclo*- deaminase (EC 4.3.1.4)[177], 3-alanyl-CoA ammonia-lyase (EC 4.3.1.6)[178], porphobilinogen deaminase (EC 4.3.1.8)[179], dihydroxyphenylalanine ammonia-lyase (EC 4.3.1.11)[180], methylaspartase (EC 4.3.1.2)[9], aspartase (EC 4.3.1.1)[181], histidase (histidine ammonia-lyase) (EC 4.3.1.3)[182] and phenylalanine ammonia-lyase (EC 4.3.1.5)[183] have no specific organic cofactors. The latter four enzymes have been studied in some detail and can be considered together. They all catalyse the elimination of ammonia from amino acids to give *trans*- carbon to carbon double bonds. 3-Methylaspartase catalyses the elimination of ammonia from (2S,3S)-3-methylaspartic acid (2) to give mesaconic acid (3). Aspartase catalyses the elimination of ammonia from aspartic acid (79) to give fumaric acid (7). Histidase catalyses the elimination of ammonia from (2S)-histidine (80) to give urocanic acid (81) and phenylalanine ammonia-lyase catalyses the elimination of ammonia from phenylalanine (82) to give *trans*-cinnamic acid (83) (Scheme 1.31)



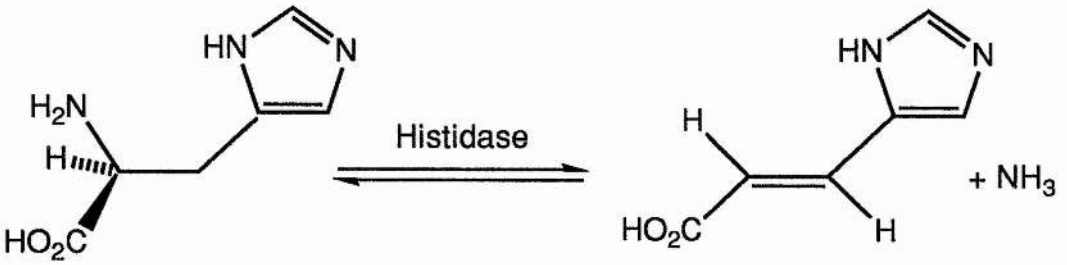
(2S,3S)-3-methylaspartic acid
(2)

mesaconic acid
(3)



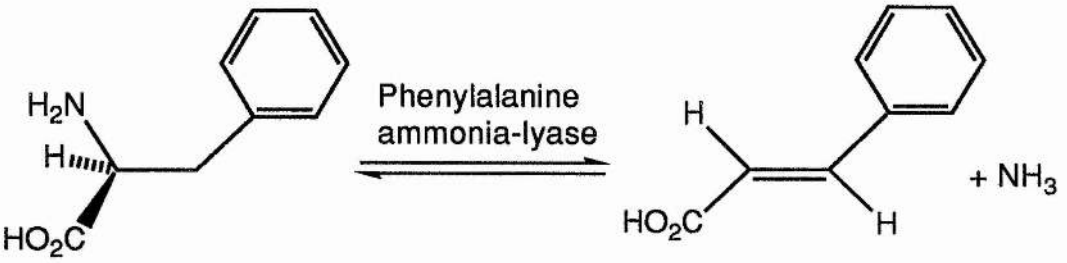
(2S)-aspartic acid
(79)

fumaric acid
(7)



(2S)-histidine
(80)

urocanic acid
(81)



(2S)-phenylalanine
(82)

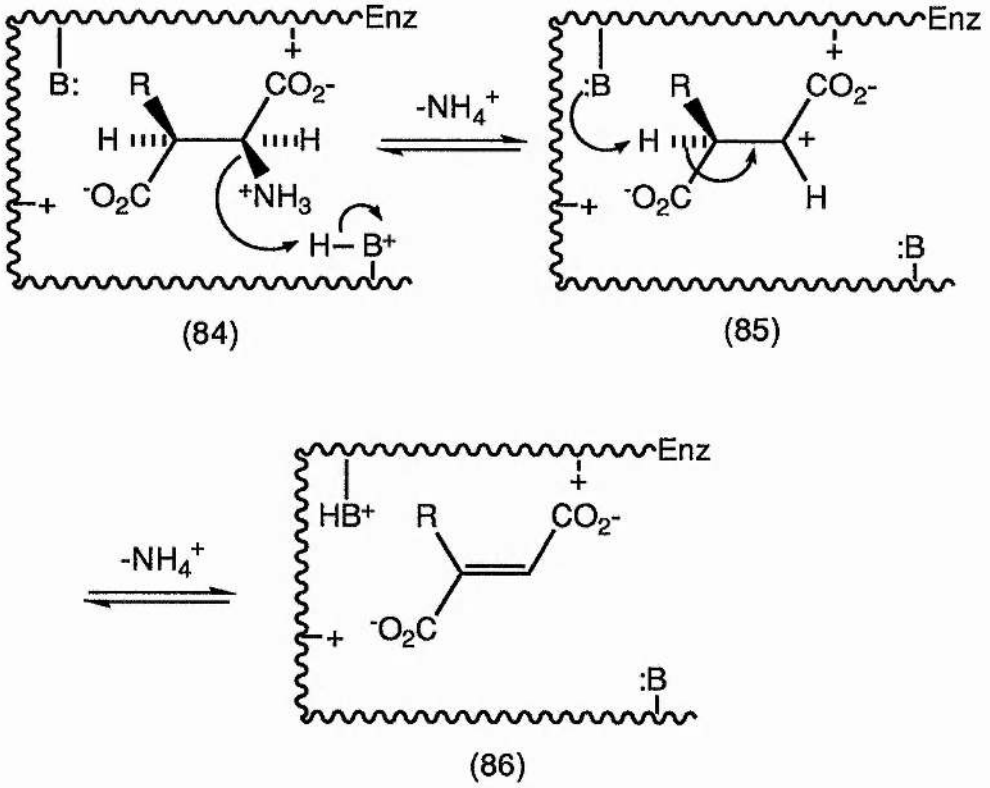
trans -cinnamic acid
(83)

Scheme 1.31 Ammonia-lyase Catalysed Reactions

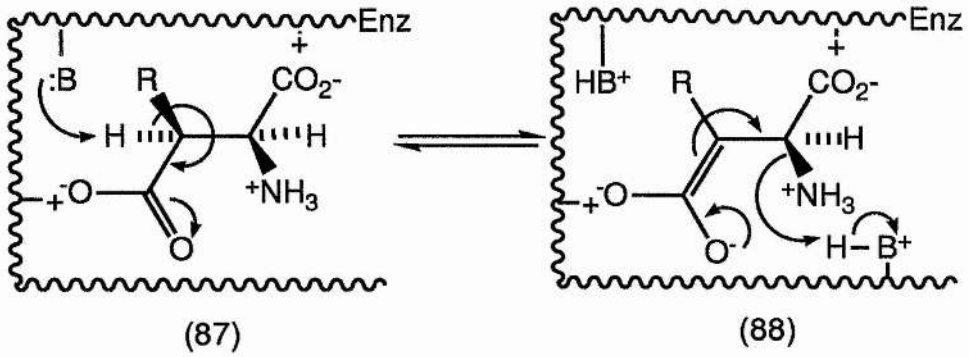
1.3.2 Elimination Mechanisms

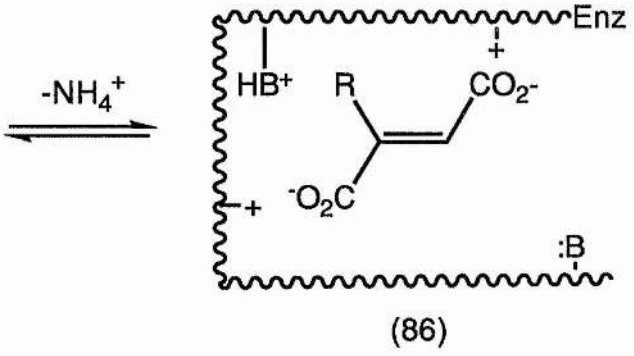
Considerable effort has been spent on elucidation of the mechanism of elimination of ammonia in these enzyme systems. Stereochemical studies have shown that ammonia is eliminated in an *anti*- fashion. However, these observations in isolation do not give insight into the mechanism of the elimination process. Carbonium ion (Scheme 1.32), carbanion (Scheme 1.33) and concerted (Scheme 1.34) mechanisms are all feasible, in principle. Kinetic studies have thus been used to differentiate between these mechanistic possibilities (see pp. 61, 67, 75 and 78).

In the carbonium ion mechanism (Scheme 1.32), ammonia is eliminated first (84). This leaves a carbonium ion at C-2 which is quenched by elimination of a hydrogen from C-3 (85). The double bond is thus formed (86) (Scheme 1.32). This mechanism would be disfavoured as it places a positive charge α- to a carboxyl group. This simplifies the problem to differentiation between carbanionic and concerted mechanisms. In the carbanion mechanism a hydrogen is removed from C-3 first (87). This carbanion is stabilized by the adjacent carboxyl group. Ammonia is then eliminated (88), to give the product (86) (Scheme 1.33). In the concerted mechanism a hydrogen is eliminated from C-3 and ammonia is eliminated from C-2 simultaneously (89), to give the product molecules (86) (Scheme 1.34).

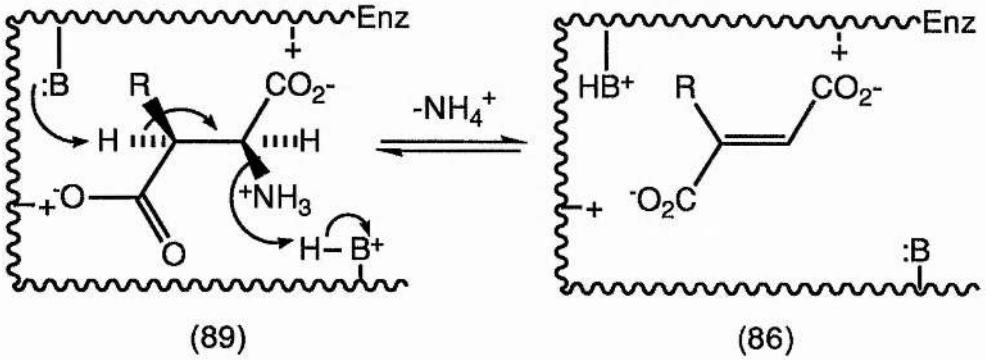


Scheme 1.32 Carbonium Ion Mechanism for the Elimination of Ammonia from an Aspartic Acid





Scheme 1.33 Carbanion Mechanism for the Elimination of Ammonia from an Aspartic Acid



Scheme 1.34 Concerted Mechanism for the Elimination of Ammonia from an Aspartic Acid

1.3.3 Aspartase

Aspartase occurs widely in bacteria[181,184], plants[185] and mammals[186], where it catalyses the reversible conversion of (2S)-aspartic acid (79) to fumaric acid (7) and ammonia (Scheme 1.31). The enzyme from *E. coli* W has been most thoroughly investigated[187]. Tokushige showed that aspartase from this source had a molecular weight of 193 000 Da and consisted of four identical subunits, each of 48 500 ± 500 Da, arranged in D_2 symmetry. He also detemined the primary sequence[188].

No alternative substrates to aspartic acid and fumaric acid have been found for aspartase, but the ammonia can be replaced by hydroxylamine to give N-hydroxyaspartic acid[189]. Viola *et al.* showed aspartase was an allosteric enzyme, *i.e.* it was activated by aspartic acid binding at a site remote from the active site[190]. Some substrate analogues were found for the allosteric site.

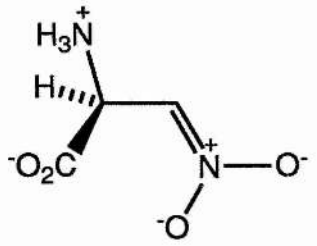
The enzyme displayed cooperative kinetics at alkaline pH, indicated by a sigmoidal relationship between the substrate concentration and the rate. At pH 6.0, however, it exhibited Michaelis-Menten kinetics[191]. Depue and Moat showed aspartase was activated by divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}) above pH 8.0, and that above pH 9.0 there was an absolute requirement for a divalent cation. However below pH 8.0 the enzyme was completely active in the absence of divalent cations[192]. Potassium also caused activation at high substrate concentrations (>20 mM), but inhibited at lower concentrations[187]. In the absence of added metal ions, the pH optimum, for the deamination reaction, dropped from 8.7 to 7.7. The K_m for aspartic acid was 1 mM[187].

The use of reagents to specifically modify certain amino acids showed there were two cysteine residues, and one or possibly two histidine residues, that were essential for enzyme activity[193,194]. The presence of aspartic acid and magnesium ions afforded protection to cysteine residues 140 and 430 from modification by N-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide

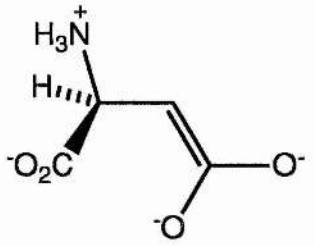
(DACM), a fluorescence reagent, specific for thiols[195]. Similarly the presence of aspartic acid, fumaric acid or chloride ions (an inhibitor) prevented inactivation of the histidine residue(s) by diethyl pyrocarbonate, a histidine specific modifying agent. Cysteine modification increased the K_m for aspartic acid, whereas modification of the histidine residue(s) caused inactivation. Tokushige therefore concluded that the histidine residue(s) was situated at the active site, where it acted as a general base, while the cysteines were required for binding, either at the active site or the remote aspartic acid binding site.

Nuiry's kinetic studies, using the enzyme isolated from *Hafnia alvei*, showed a negligible primary isotope effect of 1.04 on V_{max} and 1.02 on V/K for the deamination of (2S,3R)-[3-^{2}H]-aspartic acid. A significant ^{15}N primary isotope effect of 1.0239 ± 0.0014 was also observed on V/K[196]. These results indicated a two-step mechanism was occurring, in which C-N bond cleavage was, at least, partially rate limiting.

Porter and Bright showed 3-nitroalanine (90) bound very strongly to aspartase, especially in its ionized form (K_m/K_i = 220 above pH 9.0)[197]. They suggested that 3-nitroalanine was mimicking the stable *aci* -carboxylate intermediate (91) (Figure 1.5) of a carbanion mechanism (Scheme 1.33). Young showed that the 3-*pro* -R hydrogen of aspartic acid was abstracted as a proton from C-3, by one of the cysteine residues, prior to the rate limiting C-N bond cleavage[198]. The evidence therefore pointed to an $E1_{cb}$ mechanism for aspartase. However, the complicated cation effects and allosterism have still to be fully explained.



(90)



(91)

Figure 1.5 Ionized 3-Nitroalanine Mimicking the Intermediate of a Carbanionic Mechanism

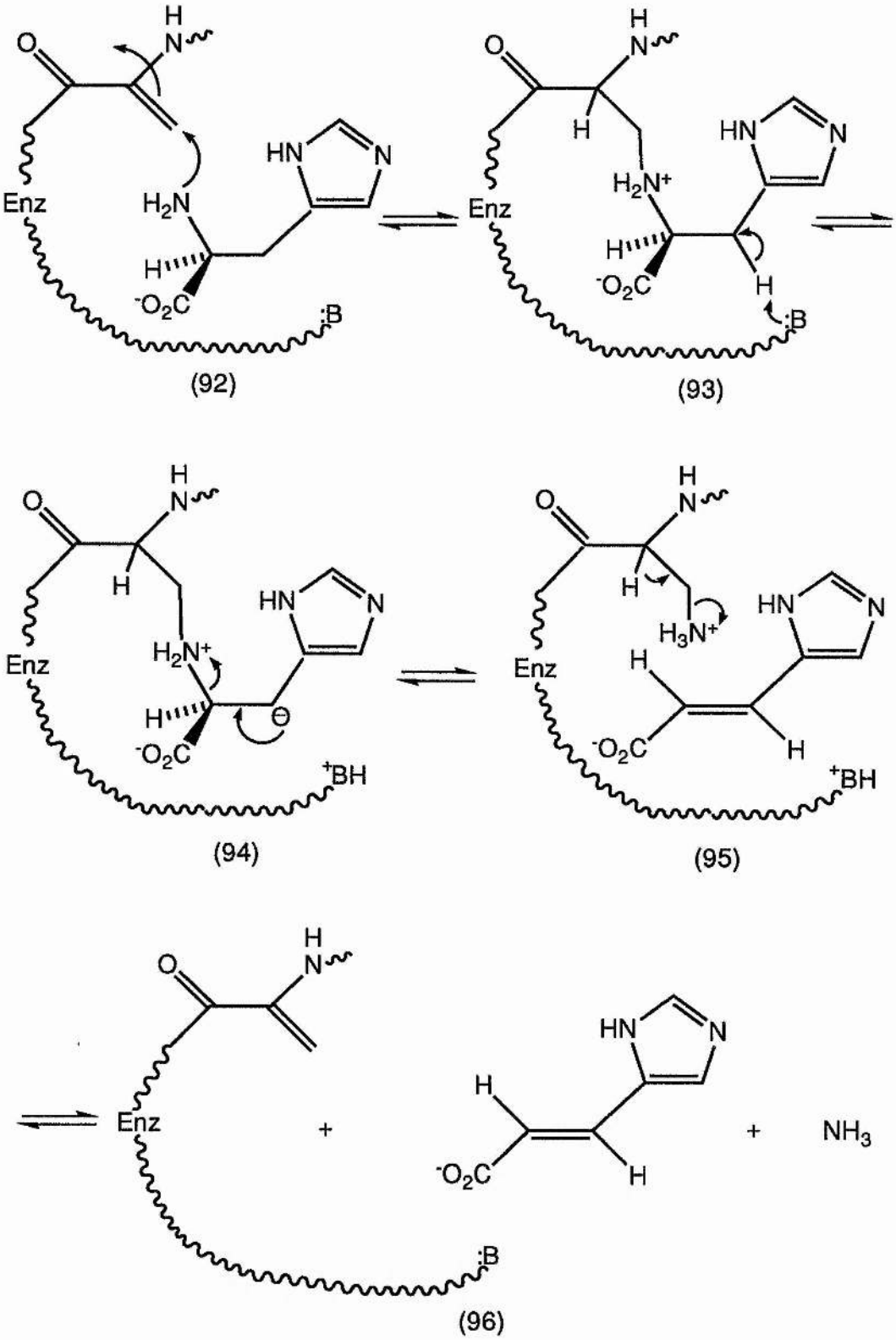
1.3.4 Histidase

Histidase catalyses the elimination of ammonia from (2S)-histidine (80) to give urocanic acid (81) (Scheme 1.31). Histidase is found in both mammals[199] and microbes[200]. In bacteria, it is the first enzyme in histidine catabolism[201]. Most studies have used the enzyme isolated from *Pseudomonas fluorescens*, which Rechler showed was a tetramer of molecular weight 222 000 Da[202]. Peterkofsky measured the K_m for histidine as 9 mM below pH 8.5 and 24 mM above pH 9.0. For urocanic acid the K_m was 7 mM at pH 9.0[203].

Abeles showed histidase was inhibited by sodium borohydride, but that protection was afforded by the presence of substrate[204]. Reduction with sodium borotritide, followed by acid hydrolysis of the inactive enzyme, gave tritiated alanine. This was the only labelled residue[205]. Similarly, ^{14}C-labelled nitromethane inactivated the enzyme. It was demonstrated that, after acid hydrolysis, the labelled molecule had reacted either by β-displacement of an unknown group, or β-addition at a dehydroalanine residue[206]. Taken together these results provided compelling evidence for the existence of a dehydroalanine residue at the active site. It was thought that the dehydroalanine residue was probably attacked, in a Michael fashion, by the amino group of the substrate (92) to give an enzyme bound intermediate (Scheme 1.35).

Peterkofsky showed, by labelling experiments in tritiated water, that the abstraction of a proton from C-3 (93) was stereospecific (Scheme 1.35)[203]. This gave a carbanionic intermediate (94)[207]. C-N bond cleavage then produced the urocanic acid product (95). Elimination was facilitated by delocalization of charge through the dehydroalanine system. The dehydroalanine residue was finally regenerated by loss of ammonia, to give the active enzyme and product (96)

In the original work on the enzyme, in 1962, Peterkofsky showed, through the use of ^{15}N ammonia, that exchange of ammonia into the substrate did not occur[203]. Peterkofsky also showed that incubation of histidine and ^{14}C-urocanic acid gave labelled histidine. However, in the absence of histidine the back reaction did not occur. Free ammonia and urocanic acid did not give the amination product. Furata and co-workers incubated L-[5'-^{2}H]-histidine with histidase to investigate the rate of hydrogen exchange at C-5'[208]. They showed the rate of increase in concentration of unlabelled urocanic acid did not depend on the rate of formation of urocanic acid. Unlabelled urocanic acid was still formed even after urocanic acid formation was complete. These results suggested the formation of an amino-enzyme complex, derived from histidine, as an intermediate in the reaction. The back reaction (amination of urocanic acid to give histidine) did not occur unless the enzyme had been incubated with histidine, to allow the amino-enzyme intermediate to form.

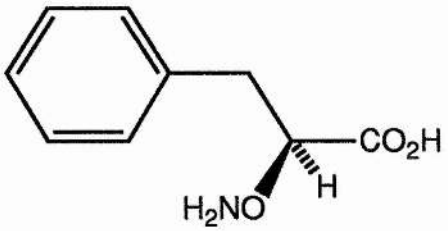


Scheme 1.35 Mechanism for Histidase Involving the Dehydroalanine Residue

1.3.5 Phenylalanine Ammonia-Lyase

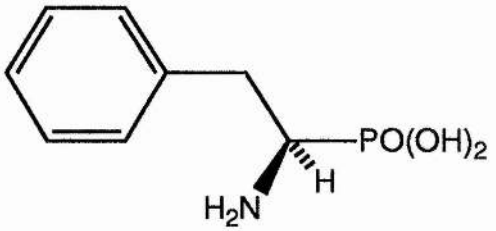
Phenylalanine ammonia-lyase catalyses the elimination of ammonia from (2S)-phenylalanine (82) to give *trans* -cinnamic acid (83) (Scheme 1.31). Phenylalanine ammonia-lyase is found in many plants and some lower organisms[209,210]. In higher plants the enzyme diverts phenylalanine into secondary metabolism[201]. The elimination product, *trans* -cinnamic acid, is further metabolized into a variety of phenylpropanoid compounds such as lignins, coumarins, stilbenes and anthocyanins[211]. In contrast, in microorganisms the enzyme has a catabolic role. The phenylalanine ammonia-lyases from maize, potatoes and the yeast-like fungus *Rhodotorula glutinis* have been most extensively studied.

Havir and Hanson showed phenylalanine ammonia-lyase, from all three sources, was a tetramer of molecular weight 330 000 Da[212,213]. The enzyme from potatoes was specific for (2S)-phenylalanine, but that from maize and *Rhodotorula glutinis* turned over both (2S)-phenylalanine and (2S)-tyrosine[214]. The potato enzyme turned over (2R)-phenylalanine but at 1/5000 of the rate for (2S)-phenylalanine. (2R)-Phenylalanine was also a competitive inhibitor[214]. The enzyme also accepted *p* -fluoro- and *p* -chloro-phenylalanine as substrates[215]. Hanson and Havir also showed that it acted on the cyclohexadienyl analogue, 3-(1,4-cyclohexadienyl)-L-alanine, to give *trans* -3-(1,4-cyclohexadienyl)acrylic acid[216]. Amrhein synthesized a variety of inhibitors for phenylalanine ammonia-lyase, including (S)-2-aminooxy-3-phenylpropanoic acid (97) (K_i = 1.4 nM)[217], (R)-(1-amino-2-phenylethyl)phosphonic acid (98) (K_i = 1.5 μM)[218] and 2-aminoindan-2-phosphonic acid (99) (K_i = 0.08 μM)[219].



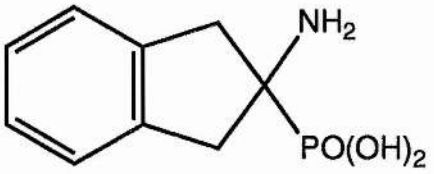
(S)-2-aminooxy-
3-phenylpropanoic acid

(97)



(R)-(1-amino-2-phenylethyl)
phosphonic acid

(98)



2-aminoindan-2-phosphonic acid

(99)

Figure 1.6 Inhibitors of Phenylalanine Ammonia-lyase

Hanson and Havir showed the deamination reaction did not follow Michaelis-Menten kinetics[214,215]. At the optimum pH (pH 8.7), the apparent K_m increased, from 0.038 mM to 0.26 mM, and the apparent V_{max} doubled, as the concentration of (2S)-phenylalanine was increased from 0.01 mM to 6.7 mM. Michaelis-Menten kinetics were restored by the presence of the competitive inhibitor (2R)-phenylalanine. The presence of the product, *trans* -cinnamic acid, also restored Michaelis-Menten kinetics and caused inhibition of the reaction. These observations suggested that phenylalanine ammonia-lyase was an allosteric enzyme. The enzyme was efficient at low substrate concentration but with increasing substrate concentration efficiency decreased. This system therefore controls the flow of phenylalanine and tyrosine metabolites into secondary metabolism.

Unlike the histidase catalysed reaction, the phenylalanine ammonia-lyase catalysed reaction was fully reversible; incubation of the enzyme with

labelled *trans*- cinnamic acid and ammonia gave labelled (2S)-phenylalanine. If unlabelled (2S)-phenylalanine and labelled *trans*-cinnamic acid were incubated together, labelled (2S)-phenylalanine was produced faster than expected for the back reaction. Havir and Hanson rationalized this result by invoking an amino-enzyme intermediate which allowed a partial back reaction (between labelled *trans*- cinnamic acid and the amino-enzyme intermediate to give (2S)-phenylalanine) to occur at such a rate.

Hanson and Havir found, as with histidase, that sodium borotritide, inactivated phenylalanine ammonia-lyase, giving, after acid hydrolysis of the inactive enzyme, [3-^{3}H]-alanine[220,221]. Similarly ^{14}C-nitromethane[213] caused inactivation of the enzyme, giving [4-^{14}C]-aspartic acid (the usual product of acid hydrolysis of nitroalkyl *aci*- salts) after acid hydrolysis.

Zon and Laber showed that the mechanism of elimination was stereospecific[222]. The *pro*- 3S hydrogen was eliminated. Cleland studied the mechanism of phenylalanine ammonia-lyase using deuterium and ^{15}N isotope effects[223]. (2S)-Phenylalanine gave a ^{D}(V/K) value of 1.15. The pH dependence of the ^{15}N isotope effects showed the active substrate was the monoanion. Extrapolated ^{15}(V/K) values for the unlabelled monoanion of (2S)-phenylalanine were 1.0021 and, for the deuteriated compound, 1.0010. This finding is in accord with a stepwise carbanion mechanism. Experiments of this type will be discussed in more detail in Chapter 3 (pp. 158 - 159).

The enzyme from yeast has been crystallized[224], but as yet no crystal structure has been published.

1.3.6 Methylaspartase

Methylaspartase catalyses the reversible elimination of ammonia from (2S,3S)-3-methylaspartic acid (2) to give mesaconic acid (3) (Scheme 1.31). It also catalyses a rapid exchange between the 3-H of the substrate and the solvent. Much of the early work carried out in the 1960's has been recently reinvestigated by Gani *et al.* However, it is presented here to provide a context for the more recent work.

1.3.6.1 Enzyme Structure

The enzyme from *Clostridium tetanomorphum* has a molecular weight of 100 000 Da, as determined by sedimentation equilibrium measurements[225]. Treatment with 6 M guanidine hydrochloride cleaved the enzyme into two subunits of 50 000 Da[226]. Hsiang and Bright reported that *p* -chloro-mercuribenzoate in a molar excess of 8-fold cleaved the enzyme into four subunits of 25 000 Da, as measured by density gradient sedimentation techniques[227]. The enzyme was assigned an $(\alpha\beta)_2$ subunit structure based on Wu's electrophoretic evidence, suggesting that the four subunits were not homogeneous[228]. However, in later work, Cohen showed the enzyme behaved as a homodimer of molecular weight 49 000 Da[229]. SDS-PAGE under a variety of conditions failed to show any evidence of splitting into smaller subunits. A tryptic digest of the enzyme performed in 1968 by Wu and Williams resulted in 55 peptides, corresponding to half the total number of arginine and lysine residues present in the enzyme. This was further evidence for the enzyme being a dimer of identical subunits[230]. Also Gani's N-terminal sequence analysis published in 1992 unambiguously identified the first 26 amino acids, indicating the presence of only one N-terminal sequence[231].

Photooxidation studies, performed by Williams and Libano, in 1966, showed that the rate constant for enzyme activity loss was nearly identical to that for

sulphydryl destruction, suggesting a sulphydryl group was required for activity[232]. The enzyme was also inactivated by the thiol alkylating agent, N-ethylmaleimide. Analysis of the protection from inactivation given by various substrates and substrate analogues implied that the sulphydryl group (cysteine residue) was in close proximity to C-3. Williams and Libano suggested that this cysteine was the base responsible for the removal the 3-H of (2S,3S)-3-methylaspartic acid.

1.3.6.2 Early Kinetic Work

A number of properties of 3-methylaspartase were described in the first major paper on the enzyme by Barker in 1959[9]. The pH optimum for 3-methylaspartase was 9.7. The enzyme was most active at 55 °C, although it lacked stability at this temperature. The equilibrium constant, K_{eq}, for the deamination reaction was determined as 0.3.

Barker showed a divalent and a monovalent cation were required for activity[9]. Magnesium was the most active divalent metal ion, although nickel, cobalt, zinc, iron, manganese and cadmium also served as cofactors[233]. Bright obtained the following order of activation: Mg^{2+} > Mn^{2+}> Co^{2+}> Ni^{2+} [234]. Calcium and strontium were inhibitors. It was noted that activity was related to ionic radii. Those ions with radii of less than 1 Å were activators, whereas those with larger radii were inhibitors[233]. A variety of monovalent cations activated the enzyme. Potassium was most effective and others less so, activity decreased in the following order; K^+ > NH_4^+ > Rb^+> Li^+ > Na^+ > Cs^+ [9]. The K_m for potassium was 3 mM, under Barker's conditions. At high concentrations potassium inhibited the reaction. The ammonium ion, being a deamination product as well as serving as a monovalent cationic activator, caused the enzyme to behave auto-catalytically in the absence of potassium.

1.3.6.3 Early Work on Binding Order

Bright and Silverman showed 3-methylaspartic acid bound as the free amino acid and not as an amino acid - magnesium complex[235]. Incubation of the enzyme, with 3-methylaspartic acid in the absence of magnesium, afforded the enzyme protection against inhibition by mercuribenzoate. Thus the substrate must bind to the enzyme either first or in a random order with the divalent cation[236]. Kinetic analysis showed that a random order, rapid equilibrium addition of the divalent cation and 3-methylaspartic acid to the enzyme was most likely.

Fields and Bright performed EPR measurements, using manganese as the divalent cation, which showed two metal ions were bound per enzyme molecule[237]. It was therefore suggested that the enzyme had two active sites. This correlated with the number of mesaconic acid binding sites found by gel filtration of 3-methylaspartase in buffer containing mesaconic acid[230]. Hsiang and Bright also showed the enzyme was inactivated by two equivalents of *p* -chloromercuribenzoate[225]. As the enzyme was insensitive to arsenite, the two essential thiol groups were not located at the same active site.

1.3.6.4 Evidence for a Carbanion Mechanism

In Bright's original studies, a primary deuterium isotope effect for the deamination of (2S,3S)-L-*threo* -3-methylaspartic acid was not observed[238]. It was concluded, therefore, that C-H bond cleavage was not rate limiting. Furthermore, exchange of the 3-H of the substrate with solvent was observed to occur at a rate faster than that for the deamination reaction. This finding supported the notion that a carbanion was formed at C-3 and that C-N bond cleavage was rate limiting. Bright also showed that ^{15}N-ammonia was incorporated into 3-methylaspartic acid at a neglible rate compared with 3-H exchange with the solvent[238]. This indicated that C-H and C-N bond cleavage were separate events and tended to exclude the possibility of a

concerted mechanism. The lack of ammonium ion exchange also ruled out the operation of a carbonium ion mechanism.

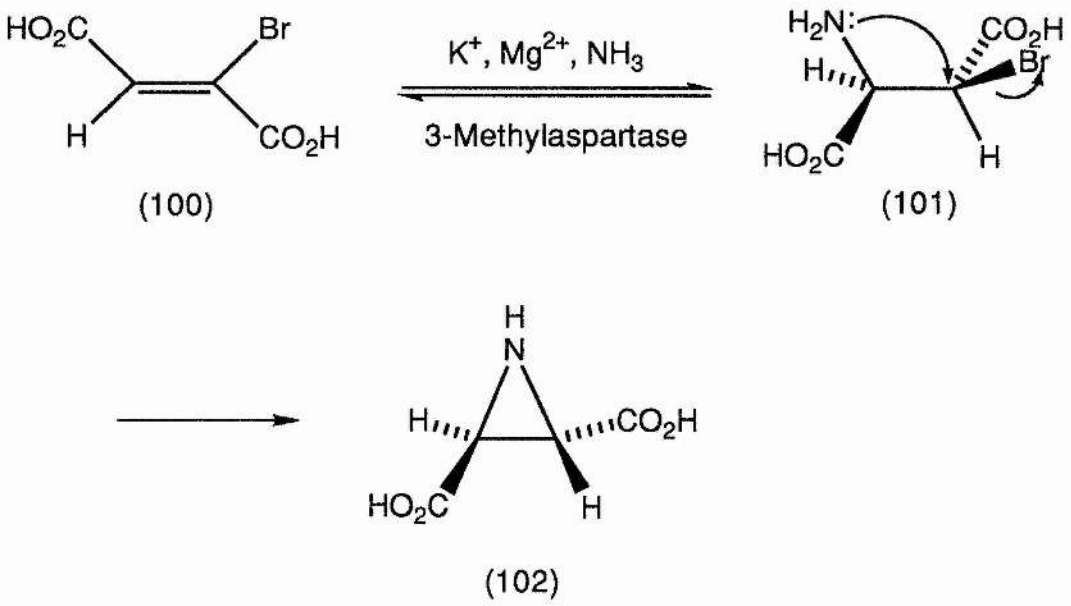
Preparations of 3-methylaspartase have also been reported to turnover (2S, 3R)-L-*erythro* -methylaspartic acid[9]. Barker suggested that this activity was an inherent property of 3-methylaspartase as the ratio of L-*erythro* - to L-*threo* - activity remained constant throughout the purification of the enzyme. He also noted that the rates of deamination of both compounds were dependent upon the potassium and magnesium ion concentrations and were inhibited by calcium ions. The fact that (2S,3R)-3-methylaspartic acid was a substrate for the enzyme also lent credence to a carbanion mechanism for 3-methylaspartase. The carbanion intermediate generated at C-3 would be identical for both substrates.

Thus, 3-methylaspartase was originally held as the archetypal example of an enzyme which operated by a carbanionic mechanism[239].

1.3.6.5 Substrate Specificity

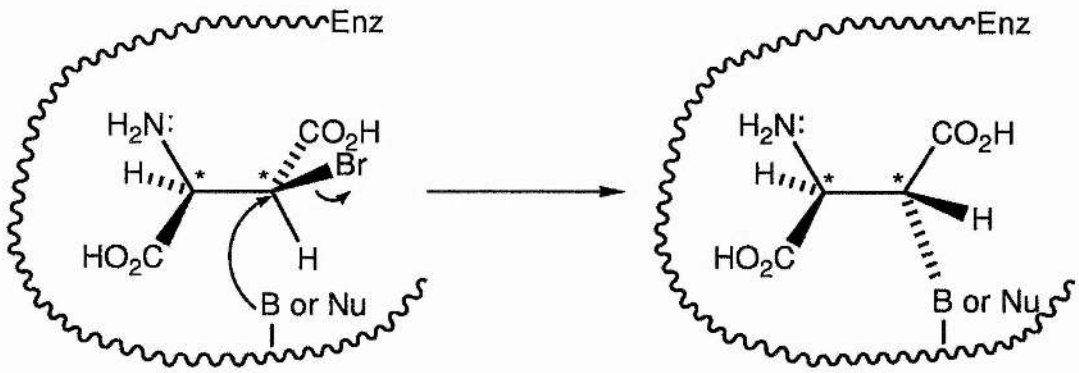
3-Methylaspartase tolerates a wide variety of substituents in place of the methyl group at C-3. L-Aspartic acid[9], 3-ethylaspartic acid[8], 3-*n*-propylaspartic acid, 3-*iso*- propylaspartic acid[240] and, more recently, 3-chloroaspartic acid[241] have been shown to be substrates. 3-Fluorofumaric acid and 3-bromofumaric acid (100) were also substrates[242]. Although (2R,3S)-3-fluoroaspartic acid was produced in low yields, a number of side products were also formed, resulting in a mixture of products. 3-Bromofumaric acid (100) was a good substrate for 3-methylaspartase. However the (2R,3S)-3-bromoaspartic acid (101) produced, being an α-bromo acid, was unstable under the incubation conditions and cyclized to form 2,3-aziridinedicarboxylic acid (102) (Scheme 1.36). Attack by an active site base or nucleophile (103) also occurred, causing alkylation (105), and hence inactivation, of the enzyme (Scheme 1.37). This would provide a means of identifying the active site base / nucleophile, as this residue would

be covalently linked to a labelled 3-bromoaspartic acid substrate.



(100) (101) (102)

Scheme 1.36 The Formation of 2,3-Aziridinedicarboxylic Acid from Bromofumaric Acid



(103) (104)

Scheme 1.37 Labelling of the Active Site Base / Nucleophile of 3-Methylaspartase

Gani used the enzyme to synthesize stereochemically pure 3-halogeno- and 3-alkyl aspartic acids from the correspondingly substituted fumaric acids. The C-3 deuteriated isotopomers were also prepared, by incubation of the appropriate fumaric acid with the enzyme in deuterium oxide[241]. Deuterium was thus incorporated stereospecifically at C-3. The kinetic parameters for amination of the substituted fumaric acids (Table 1.1) and deamination of the 3-substituted aspartic acids (Table 1.2) were determined[243].

Table 1.1 Kinetic Parameters for the Amination of Substituted Fumaric Acids

Substrate	K_m (mM)	V_{max} ($x10^{-6}$ mol dm^{-3} min^{-1})
Fumaric acid	23 ± 2.2	1702
Mesaconic acid	1.24 ± 0.08	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
iso-Propylfumaric acid	5.5 ± 3.0	5.3
n-Butylfumaric acid		<0.05
Iodofumaric acid		<0.05

Table 3.2 Kinetic Parameters for the Deamination of 3-Substituted Aspartic Acids

Substrate	K_m (mM)	V_{max} ($x10^{-6}$ mol dm^{-3} min^{-1})
3-Methylaspartic acid	2.37 ± 0.2	654
Aspartic acid	10.5 ± 0.82	4.8
3-Ethylaspartic acid	17.08 ± 1.4	292
3-Chloroaspartic acid	>50	382

The K_m values for the fumaric acids studied were very similar, with the exception of fumaric acid (see Table 1.1). The high K_m for fumaric acid indicated that it bound poorly. The magnitudes of V_{max} for amination of the smaller fumaric acids were all similar. The rate then dropped off dramatically with the *iso* -propyl- and *n* -propyl fumaric acids, while *n* -butyl- and iodofumaric acid did not react at all. It appeared that a point was reached when the alkyl / halogeno substituent was too large to fit in the methyl binding pocket of the enzyme active site.

There was more variation in V_{max} for the deamination reaction (see Table 1.2). 3-Ethylaspartic acid and 3-chloroaspartic acid were deaminated at about half the rate of the natural substrate, whereas aspartic acid was a much slower substrate. However in the amination direction the rates of reaction for fumaric acid and mesaconic acid are almost identical. This implied a step in the deamination reaction was more sensitive to the size of the substituent than any of the reverse reaction steps. This interesting result led to further investigations of the enzyme mechanism.

1.3.6.6 Primary Deuterium Isotope Effects

Primary deuterium isotope effects were measured for 3-methylaspartic acid, aspartic acid and 3-ethylaspartic acid (Table 1.3)[244].

Table 1.3 Kinetic Parameters for the Deamination of 3-Substituted Aspartic Acids and 3-Substituted-[3-^{2}H]-Aspartic Acids

Substrate	K_m (mM)	V_{max} (x10^{-6} mol dm^{-3} min^{-1})	$^D(V)$ & $^D(V/K)$
3-Methylaspartic acid	2.37 ± 0.2	654	
3-[3-^{2}H]-Methylaspartic acid	2.35 ±0.25	385.2	1.7 ± 0.3
Aspartic acid	10.5 ± 0.82	4.8	
[3-^{2}H]-Aspartic acid	10.5 ± 0.82	4.8	1.0
3-Ethylaspartic acid	17.08 ± 1.4	292	
3-[3-^{2}H]-Ethylaspartic acid	17.66 ± 1.6	250.6	1.16 ± 0.2

A significant primary deuterium isotope effect was seen on the deamination of 3-methylaspartic acid (see Table 1.3). This was contrary to the work of Bright who did not observe an isotope effect for this reaction. The presence of a primary deuterium isotope effect implied that C-H bond cleavage was at least partially rate limiting and threw into question the carbanion mechanism proposed by Bright[238]. Cleavage of the C-H was, necessarily, kinetically more important than previously assumed.

The lack of a deuterium isotope effect, for the deamination of aspartic acid, and the reduced isotope effect seen with the 3-ethyl substrate, compared to the natural substrate, suggested that both (2S)-aspartic and (2S,3S)-3-

ethylaspartic acid were deaminated by a mechanism greater in carbanion character than that of the natural substrate. This was rationalized by invoking a torsional distortion of the dihedral angle between the 2-C-N and 3-C-H bond, in these two substrates, away from the 180° required for a concerted *anti* -elimination and presumably attained for (2S,3S)-3-methylaspartic acid (105) (Figure 1.7). With aspartic acid the interaction between the 3-H and the methyl binding pocket (106) would be smaller than with the natural substrate. Conversely, there would be a strained interaction between this pocket and the ethyl group of 3-ethylaspartic acid (107). The unnatural substrates would be subject to poor orbital alignment in a carbanion intermediate. Hence C-H bond cleavage would become less rate limiting, as C-N bond cleavage became more difficult and, therefore, more rate limiting itself.

Ethylfumaric acid was noted to bind more tightly at the active site than the physiological substrate. Therefore product debinding may have become partially rate limiting with (2S,3S)-3-ethylaspartic acid as substrate, masking the chemical steps of the reaction. Hence the elimination of ammonia from 3-ethylaspartic acid may still occur concertedly.

With (2S)-aspartic acid the K_m values for both substrate and product were substantially higher than for the physiological compounds so substrate / product dissociation should not be rate limiting. It seems likely that (2S)-aspartic acid is deaminated by a carbanion mechanism. Exchange of the C-3 hydrogen with solvent occurred, for L-aspartic acid, at approximately one-tenth of the rate for 3-methylaspartic acid.

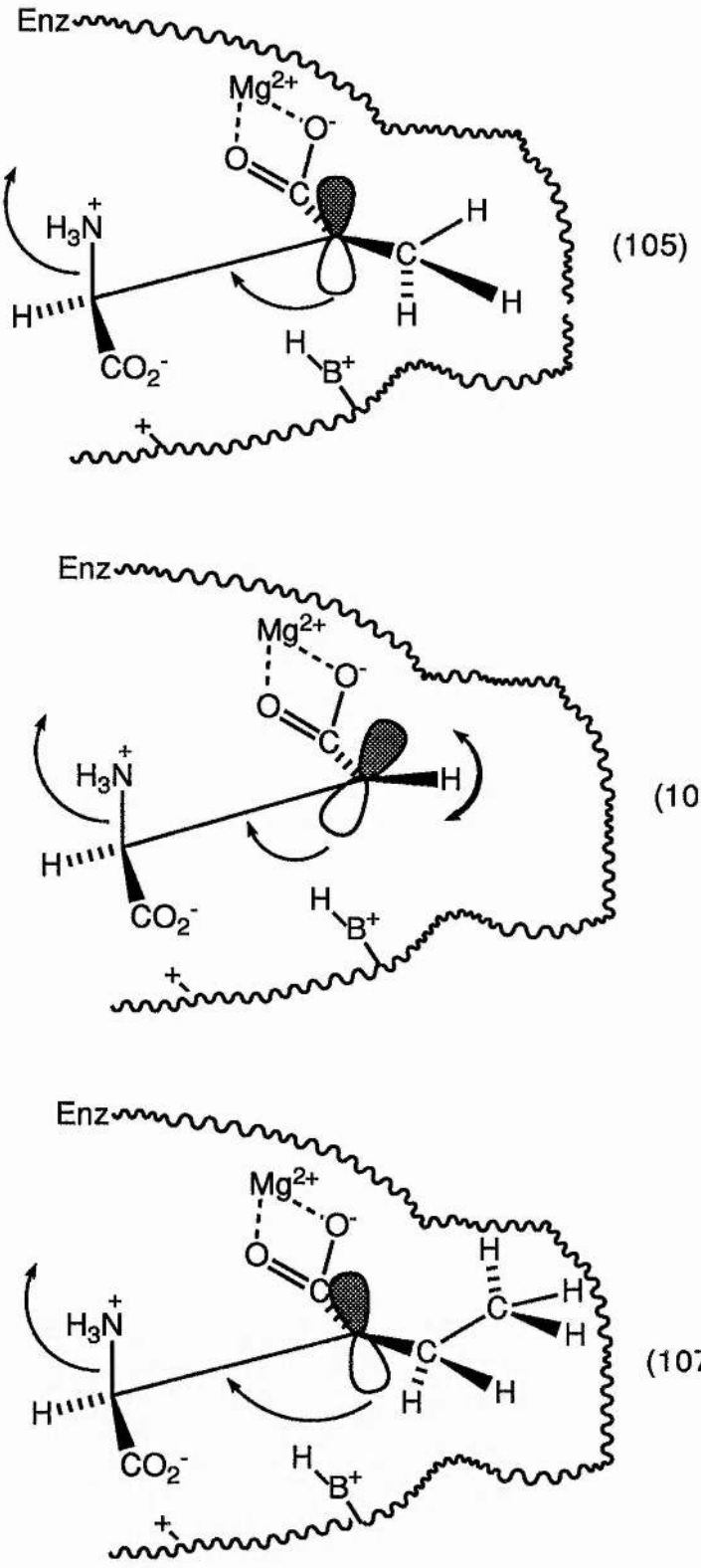


Figure 1.7 Active Site Binding of (2S,3S)-3-Methylaspartic Acid (105), (2S)-Aspartic Acid (106) & (2S,3S)-3-Ethylaspartic Acid (107) in 3-Methylaspartase

Bright's original experiments attempted to measure a primary deuterium isotope effect for the deamination of 3-methylaspartic acid at 50 mM potassium ion concentration[238]. However, at this potassium ion concentration the effect was masked[245]. It was only at lower potassium ion concentrations that the isotope effect was observed with the effect being maximal at 1 mM potassium ion concentration. The lower level of deuterium incorporation in Bright's substrate (87 % label compared with the >95 % deuterium incorporation Botting and Gani obtained) may also have helped mask the effect.

1.3.6.7 ^{15}N Isotope Effects and Double Isotope Fractionation

As a deuterium isotope effect had been observed, C-H bond cleavage was considered at least partially rate limiting. However this result provided no information as to whether a balanced stepwise carbanion mechanism or a concerted mechanism was occurring.

To distinguish between these two mechanisms, a double isotope fractionation experiment was performed[246]. In this experiment the kinetic isotope effect on C-N bond cleavage was measured in the presence and absence of deuterium at C-3 of 3-methylaspartic acid. In a concerted mechanism isotopic substitution at C-3 should have no effect on, or increase, the isotope effect on C-N bond cleavage. A balanced stepwise carbanion mechanism would show a reduced kinetic isotope effect for C-N bond cleavage in the presence of deuterium at C-3. (See p. 158 - 159 for a more detailed explanation.) Values of 1.0246 ± 0.0013 and 1.0241 ± 0.0009 were obtained for the ^{15}N isotope effect in the presence of protium and deuterium at C-3 respectively. As these values were identical, within experimental error, they implied that the reaction followed a concerted pathway with a single transition state involving both 3-C-H and 2-C-N bond cleavage.

For (2S)-aspartic acid a double isotope fractionation experiment showed a

smaller ^{15}N isotope effect in the presence of deuterium[247], as one would expect for a balanced stepwise mechanism. However, as no deuterium isotope effect was observed with aspartic acid[243], this result has yet to be fully explained.

1.3.6.8 Exchange Reactions

Further experiments showed that the exchange of the 3-H of (2S,3S)-3-methylaspartic acid with the solvent was consistent with a concerted mechanism[245]. (2S,3S)-3-Methylaspartic acid and (2S,3S)-[3-^{2}H]-3-methylaspartic acid were incubated in tritiated water. This allowed the rate of protium or deuterium wash-out from the substrates to be measured as tritium wash-in. The primary deuterium isotope effect on V_{max} for the exchange reaction ($^{D}(\upsilon_{ex})$) at pH 9.0 was measured as 1.6. The size of the isotope effect was not dependent on the ratio of the rates of exchange and deamination, which showed gross changes with pH. This suggested that exchange occurred after a slow step which followed C-H bond cleavage. It also indicated that exchange did not occur at the free carbanion level, as $^{D}(\upsilon_{ex})$ was constant with pH.

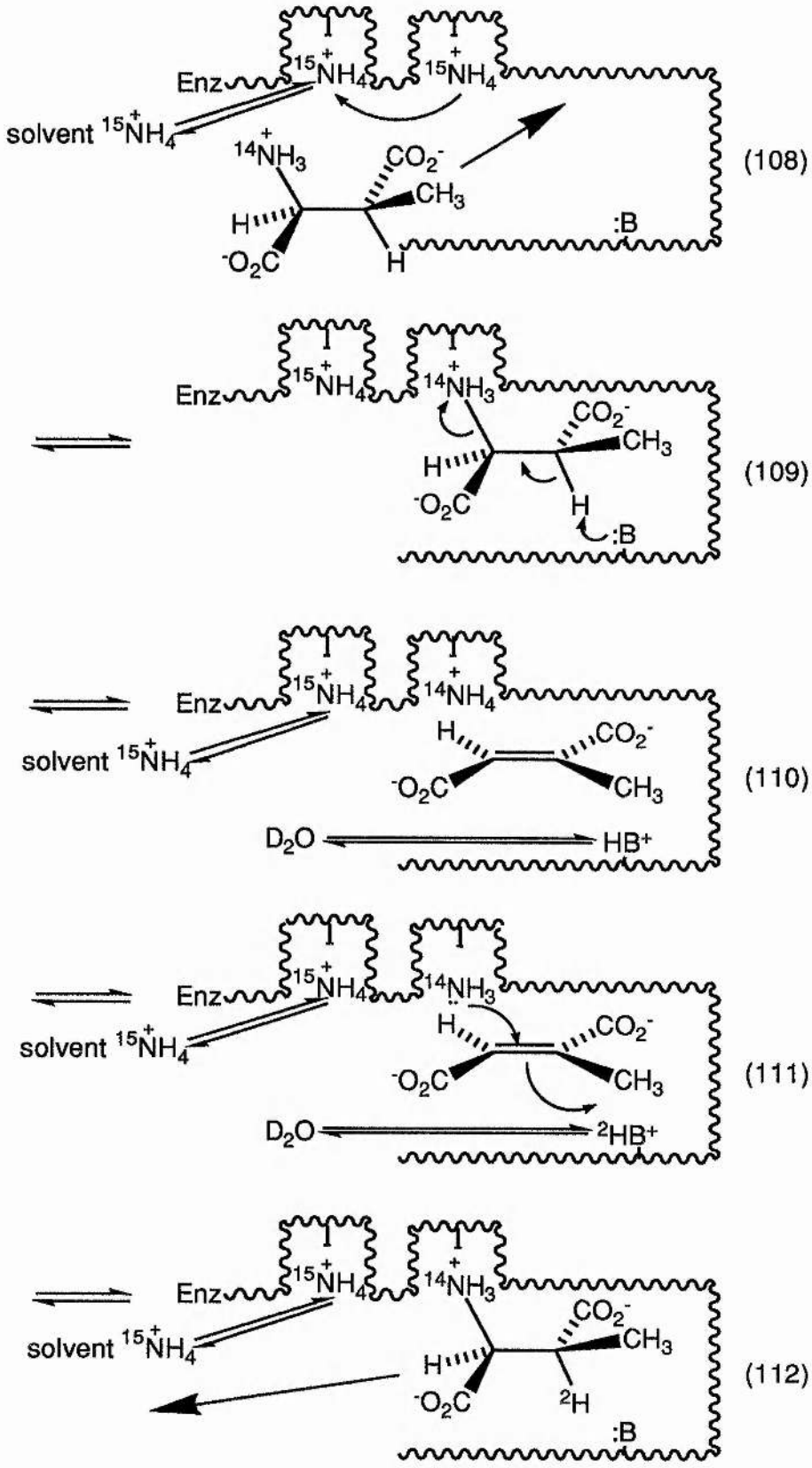
A product trapping experiment was devised to ascertain why ^{15}N-labelled ammonia was not incorporated into the substrate in an exchange reaction. Incubation of ^{15}N-labelled ammonia and [3-$^{2}H_3$]-mesaconic acid with unlabelled (2S,3S)-3-methylaspartic acid showed rapid formation of [^{15}N, *methyl* -$^{2}H_3$]-3-methylaspartic acid. The singly labelled species were formed only slowly and could be accounted for by reaction with free unlabelled substrates.

1.3.6.9 Binding and Debinding Orders

Methylamine was found not to be an activator of 3-methylaspartase and was a competitive inhibitor of 3-methylaspartic acid in the deamination reaction and of ammonia and mesaconic acid in the amination direction[245]. These two experiments showed that mesaconic acid and the ammonium ion were released at the same rate with random debinding. This hypothesis was also supported by the fact that mesaconic acid was a competitive inhibitor of (2S,3S)-3-methylaspartic acid. The increase in rate of hydrogen exchange observed with increasing ammonium ion concentration[238] was rationalized as being caused by retardation of the release of the products from the enzyme.

Gani suggested there was a slow step after C-N bond cleavage but before the formation of the $E.NH_4^+$.Mes complex, during which the ammonium ion was relocated within the active site. During this step, 3-H exchange also occurred. The substrates then either recombined to give 3-methylaspartic acid or formed the enzyme-product complex and dissociated.

The evidence presented for the second binding site for ammonia was firstly that the rate of reaction was observed to depend upon $[NH_4^+]^2$ *i.e.* two molecules of ammonia were involved. Secondly, ammonia acted as a non-linear uncompetitive product inhibitor for the deamination process. It was proposed that 3-methylaspartase reacted by the mechanism depicted in Scheme 1.38. In this mechanism unlabelled 3-methylaspartate enters an active site fully saturated with ^{15}N-ammonium ions (108), the ammonium ion from the first ammonium binding site is displaced. The 3-H and C-N bonds then break concertedly (109), leaving unlabelled ammonium bound at the first ammonium binding site (110). At this stage deuterium exchange can take place with the hydrogen removed from C-3 by the enzyme base, but exchange of ammonium at the first ammonium binding site is blocked by the product molecule. Reamination of mesaconate (111) gives [3-^{2}H]-3-methylaspartate, retaining an unlabelled amino group (112).



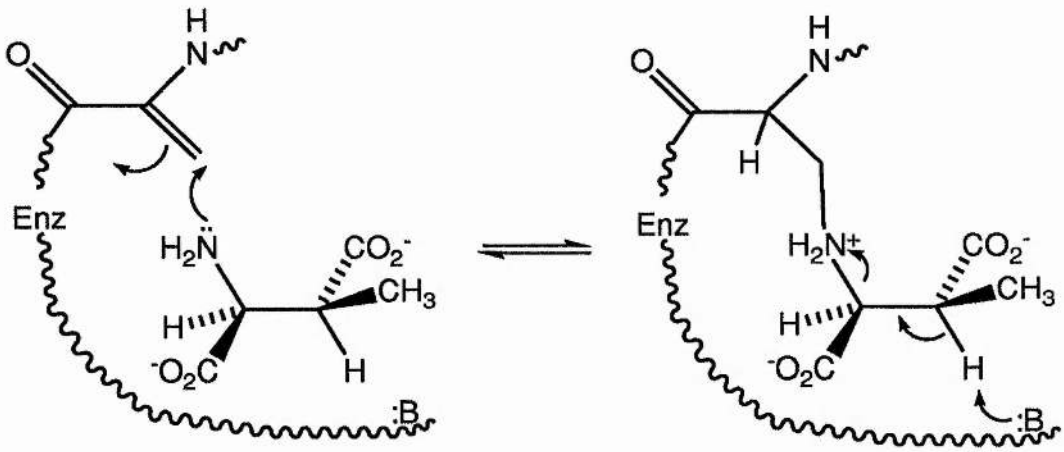
Scheme 1.38 Two Ammonia Binding Site Mechanism for 3-Methylaspartase

Reanalysis of Bright's work at low pH showed that the substrate and magnesium ion bound in a random steady state manner and then the potassium ion bound[245]. At pH 6.5 and high potassium ion concentrations the binding order was compulsory, with the potassium ion binding after the substrate, potassium was also released before the first product. At pH 9.0 the magnesium ion could bind before the substrate, which bound in rapid equilibrium; potassium 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. Cooperativity existed between the two active sites of the enzyme, which was in a new catalytic form with a high affinity for potassium.

1.3.6.10 Active Site Structure

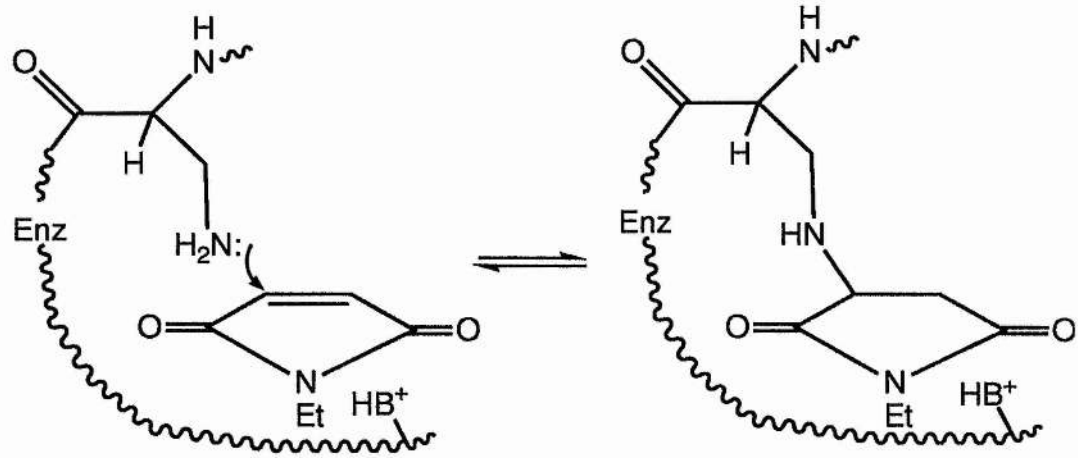
Sodium borohydride did not cause inactivation of 3-methylaspartase[248] as it did with the dehydroalanine residue containing enzymes, histidase[204] and phenylalanine ammonia-lyase[220]. However, phenylhydrazine caused irreversible inactivation of the enzyme, with some protection against inactivation being conferred by the substrate.

The tryptic peptide containing an active site residue selectively labelled with [1-^{14}C]-N-ethylmaleimide by Wu and Williams[230], for which the amino acid composition was known, could not be identified in the nucleotide base sequence of the enzyme obtained from the recent genetic work[231], if it was assumed that the labelled residue was a cysteine. However, if the labelled residue was taken as a serine a match was obtained. In order to explain the reaction of serine with N-ethylmaleimide, a post-translational modification of the serine to a dehydroalanine residue (113) was invoked (Scheme 1.39)[248]. The dehydroalanine residue could conceivably be attacked by the amino group of the substrate (114) to give a 2,3-diaminopropanoic acid residue (115) which could then react with N-ethylmaleimide, alkylating the enzyme (116) (Scheme 1.40).



(113) (114)

Scheme 1.39 Proposed Reaction of Putative Dehydroalanine Residue in the 3-Methylaspartase Active Site with 3-Methylaspartic Acid



(115) (116)

Scheme 1.40 Proposed Mechanism of Inactivation of 3-Methylaspartase by N-Ethylmaleimide

CHAPTER TWO

GLUTAMATE MUTASE

RESULTS AND DISCUSSSION

2.1 Introduction

The enzyme glutamate mutase catalyses the carbon skeleton rearrangement of L-glutamic acid to (2S,3S)-3-methylaspartic acid. Although a number of coenzyme B_{12}-dependent enzymes have recently been studied in some detail (see pp. 12 - 44), Barker's original studies remain the major literature source for glutamate mutase. The rearrangement has however been studied in terms of model reactions (see pp. 52 - 54).

Our approach to studying glutamate mutase involved, initially, purification of the enzyme to allow construction of a gene probe, followed by eventual cloning and over-expression of the enzyme in *E. coli*. It was envisaged that (2S,3S)-3-ethylaspartic acid, which could be accessed in a stereochemically pure form using 3-methylaspartase, may act as a substrate analogue for glutamate mutase. This molecule and its labelled variants could be used as mechanistic probes for the enzyme.

2.2 Detection of Glutamate Mutase Activity

Clostridium tetanomorphum cell paste, obtained on a service basis from the Department of Biology at the University of East Anglia, was the intended source for glutamate mutase. The bacteria were grown up on Barker's media[9] (Table 2.1) using growing conditions which were a modification of Barker's original method[249,229]. Barker obtained both glutamate mutase and 3-methylaspartase from bacteria grown under these conditions. The cell paste provided, after very little purification (see p. 167 -168), an extract rich in 3-methylaspartase. There was no significant removal of material from the substrate or product pools by contaminating activities. The activity of such enzymes on the methylaspartic acid pathway was suppressed under 3-methylaspartase assay conditions, as their cofactors were not present. Glutamate mutase requires coenzyme B_{12} for activity[250] and, similarly, mesaconase requires Fe^{2+} for activity[251].

Table 2.1 Media for Growing Up *Clostridium tetanomorphum*

Component	Concentration (g/l)
Sodium glutamate	10.62
Yeast extract	3.1
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	0.11
$MnCl_2.\ 2H_2O$	0.002
Na_2MoO_4	0.0025
$CaCl_2.\ 2H_2O$	0.015
$CoCl_2$	0.005
K_2HPO_4	5.4
KH_2PO_4	1.5
Glucose	7.2

All the enzymes on the glutamic acid fermentation pathway should be strongly expressed in *C. tetanomorphum* grown with glutamic acid as the major carbon source. Any differences in activity levels are attributable to the enzyme's physiological role in the cell. The first enzyme in the pathway is often the controlling enzyme, determining flux through the pathway. Any accumulation of intermediates is determined by the activity levels of the other enzymes in the pathway.

A crude extract was prepared, from *C. tetanomorphum* cell paste, based on the partial purification procedure for 3-methylaspartase (see p 167 - 168). Barker's purification procedures for the individual components of glutamate mutase indicated that both components should co-extract with the 3-methylaspartase under these conditions[87,88].

Barker used a spectrophotometric assay, to determine glutamate mutase activity, measuring the conversion of L-glutamic acid to mesaconic acid[8].

However, this method was not suitable for determining glutamate mutase activity in the crude enzyme extract as its specific activity was so low that the absorbance from contaminating proteins masked any absorbance changes due to mesaconic acid production.

Other methods for detecting activity were investigated. Attempts to observe the formation of 3-methylaspartic acid by tlc were not successful. Although a variety of solvent systems were evaluated, the two structural isomers, glutamic acid and 3-methylaspartic acid were not separable by tlc.

An alternative approach was to detect ammonia, the other product of the conversion of L-glutamic acid to mesaconic acid. Nessler's reagent[252] produces a yellow-brown colour on reaction with ammonia. Ammonia was detected qualitatively, but attempts to quantify the measurement by colour intensity comparison with standard solutions were not successful.

The conversion of L-glutamic acid to 3-methylaspartic acid and to mesaconic acid was followed successfully by ^{1}H NMR spectroscopy. Sodium glutamate was incubated with partially purified protein in Tris buffer containing the cofactors required for glutamate mutase and 3-methylaspartase activity. Aliquots were removed at various time intervals. After several hours signals due to 3-methylaspartic acid and to mesaconic acid were observable. A similar incubation of (2S,3S)-3-methylaspartic acid using calcium chloride to inhibit 3-methylaspartase activity showed some conversion to L-glutamic acid.

2.3 Purification of Glutamate Mutase

In contrast to 3-methylaspartase, where the specific activity of the partially purified extract was so high that no further purification was required for either the synthetic[241] or kinetic studies[245] performed, glutamate mutase is much less active. Therefore the enzyme required further purification before any further investigations could be made. The aim was to purify both

components to allow our molecular biology collaborators to obtain N-terminal amino acid sequences. Hence they could construct gene probes and scan the *C. tetanomorphum* DNA library available from work on the 3-methylaspartase gene (*MeAsp*)[231]. There is some evidence that the gene for mesaconase resides immediately 3' (downstream) of *MeAsp* [248]. If sequential expression of the genes encoding for the enzymes of the methylaspartic acid pathway occurred, the glutamate mutase gene would lie 5' (upstream) to *MeAsp.* An incomplete open reading frame (ORF), of 1.2 kb, beyond the 5' end of *MeAsp* has been identified and sequenced. This ORF was therefore too large to be the gene for Component S of glutamate mutase, but it was thought it could be the gene for Component E. This was confirmed recently by Marsh[91], who has sequenced both components. The gene for Component S was situated upstream of the gene for Component E, with a third gene of unknown function between the two.

Component S may not be expressed in an active form from the aerobe *E. coli*, as it contains air sensitive thiol groups which are active only when reduced[87]. However genetic engineering could replace these thiol groups with serine residues thus conferring air stability whilst, hopefully, retaining activity.

Over-expression of the glutamate mutase genes in *E. coli* is essential to give easy access to large amounts of protein. Repetitive full purification of the enzyme from *C. tetanomorphum* would be required to give access to sufficient enzyme for mechanistic experiments.

The purification of Component S with a reported molecular weight of 17 000 Da was attempted first, as entire amino acid sequences can be obtained for proteins of this size. It was intended to obtain Component S *via* a modification of Barker's protocol[87].

Component S containing fractions could not be identified by assaying for glutamate mutase activity, as the component was not active without Component E. Thus, molecular weight determination by SDS-PAGE

(polyacrylamide gel electrophoresis)[253], using Barker's molecular weight value (17 000 Da)[87], was the only guide to purification.

After lysis of the glutamate mutase containing cells, by sonication, isoelectric precipitation, at pH 4.6, removed many contaminating proteins[87]. SDS-PAGE of the supernatant showed two strong bands of low molecular weight, one slightly below 17 200 Da (myoglobin molecular weight marker) and one just above 12 300 Da (cytochrome c molecular weight marker). The former was considered most likely to be Component S. However, later work, by Mr. Hartzoulakis, showed the molecular weight of Component S was 15 000[254]. This value was confirmed by Marsh[90,91]. Component S from *C. cochlearium* had a molecular weight of 16 000 Da[89].

The next step involved ion exchange chromatography on DEAE-cellulose. Component S was reported to elute at 50 mM potassium phosphate concentration[87]. Fractions that eluted at this concentration were pooled. The next purification step, gel exclusion chromatography gave two, well separated, protein peaks. Calibration of the column elution times with molecular weight standards and SDS-PAGE showed that neither of these proteins were Component S. Component S was not relocated after the DEAE-cellulose chromatography step.

The alternative approach of eluting the supernatant from the isoelectric precipitation step from a gel exclusion column in the next step, removed the higher molecular weight proteins (>50 000 Da). However the remaining fractions did not contain enough protein for detection by SDS-PAGE, staining with Coomassie Brilliant Blue R.

Only the initial steps of the purification of Component E were attempted.

2.4 Mechanistic Probes

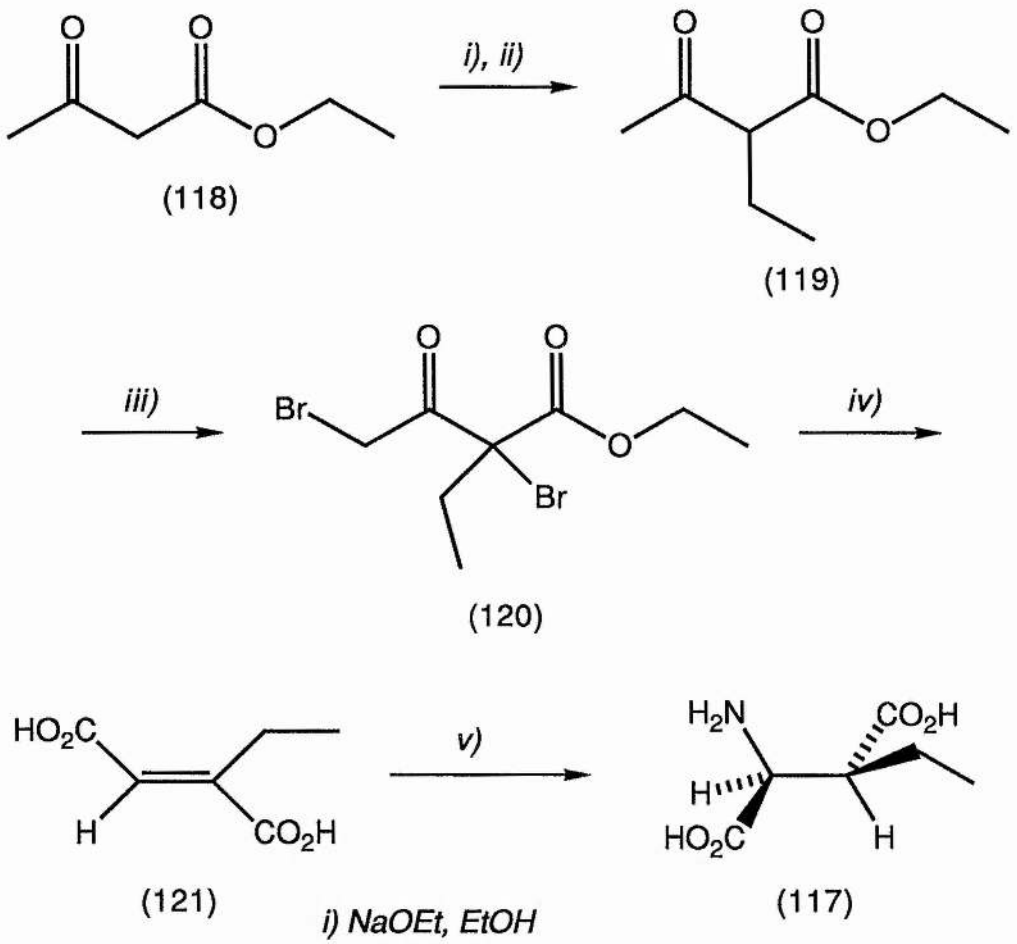
One approach to probing enzyme mechanism is the use of substrate analogues. However Barker reported that glutamate mutase was substrate specific, turning over only its natural substrates (see p. 30)[8]. Buckel showed isotopic labelling, introduced into the methyl group of 3-methylaspartic acid, was scrambled during the glutamate mutase catalysed rearrangement, probably by rapid rotation of the methylene radical intermediate[99]. Therefore it was not possible to elucidate from which side of the molecule the adenosyl radical attacked, in the natural substrate. Thus it was imperative to find another substrate for the enzyme. (2S,3S)-3-Ethylaspartic acid (117) or 3-fluoromethylaspartic acid were structurally closest to the natural substrate. The latter compound is electronically different from the natural substrate and so may react in a different manner, possibly inactivating the enzyme. 3-Ethylaspartic acid had the advantage that it may actually be a slow substrate for the enzyme. Also replacing one of the hydrogens with a methyl group, as in 3-ethylaspartic acid, would present the enzyme with a larger group to bind to, thus locking the conformation at this centre. The two remaining hydrogens could then be distinguished by isotopic labelling. (2S,3S)-3-Ethylaspartic acid was therefore the preferred synthetic target.

2.5 Synthesis of (2S,3S)-3-Ethylaspartic Acid (117)

The putative substrate analogue, (2S,3S)-3-ethylaspartic acid was synthesized in four steps from ethyl acetoacetate (118) (Scheme 2.1)[241]. Treatment with freshly prepared sodium ethoxide, followed by alkylation with ethyl bromide or ethyl iodide gave 2-ethylacetoacetate ethyl ester (119), which was isolated and purified by distillation. 2-Bromo-2-ethyl-4-bromo-acetoacetate ethyl ester (120) was prepared by reaction with bromine. A Favourskii rearrangement[255] of the dibromide, effected with potassium hydroxide as base, gave ethylfumaric acid (121).

In the literature synthesis of isocrotonic acid[256], Rappe showed potassium hydrogen carbonate gave significantly higher yields than potassium hydroxide, when used as the base in the Favorskii rearrangement (77 % *cf.* 21 %). However the reaction time was noticeably slower (2.5 h *cf.* 30 min). In our hands, there was no substantial improvement in the yield of ethylfumaric acid when potassium hydrogen carbonate was used.

Purification by steam distillation to remove the volatile impurities, diethyl ether extraction of the acidified aqueous solution and successive recrystallizations gave ethylfumaric acid as a white solid. This was incubated, for several days, with 3-methylaspartase and ammonia, to give (2S,3S)-3-ethylaspartic acid (117).



(118) (119) (120) (121) (117)

i) NaOEt, EtOH
ii) EtBr, reflux , 2 h
iii) Br_2, Et_2O, reflux, 3 h
iv) KOH / EtOH, reflux, 30 min
v) Mg^{2+}, K^+, H_2O, NH_3, methylaspartase

Scheme 2.1 Synthesis of (2S,3S)-3-Ethylaspartic acid

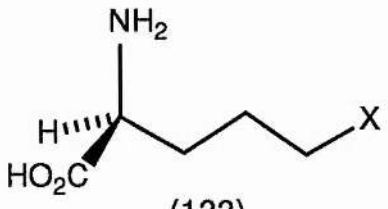
2.6 Experiments Using (2S,3S)-3-Ethylaspartic Acid

(2S,3S)-3-Ethylaspartic acid was incubated with adenosylcobalamin and a crude preparation of glutamate mutase. ^1H NMR spectra of aliquots removed at various time intervals during the incubation were recorded at 500 MHz. Signals attributable to an amino acid with a linear three carbon side chain and electron-withdrawing group on C-5, accumulated with time. Total correlation spectroscopy confirmed that the signals were linked.

Incubations in the absence of added coenzyme showed a two-fold decrease in production of this compound. The remaining turnover observed was attributed to active coenzyme still bound to the enzyme. A 1,3 carbon skeleton rearrangement of 3-ethylaspartic acid, initiated by removal of a hydrogen from C-2' of the ethyl group, would give such a six carbon amino acid.

Comparisons were made of the ^1H NMR spectra of the incubation product (122) with aminoadipic acid (123) and ornithine (124), in an attempt to identify the C-5 substituent (see Table 2.2).

Table 2.2 ^1H NMR Data For 5-Substituted Amino Acids



(122)

Substituent X	Chemical Shift - C-5 Hs (ppm)
-X (122)	3.04
-CO_2H (123)	2.25
-NH_2 (124)	3.40

Based on these chemical shifts 5-hydroxynorvaline (125) was proposed as the incubation product (122). This compound was synthesized to allow comparison of its ^1H NMR spectrum with that of the incubation product.

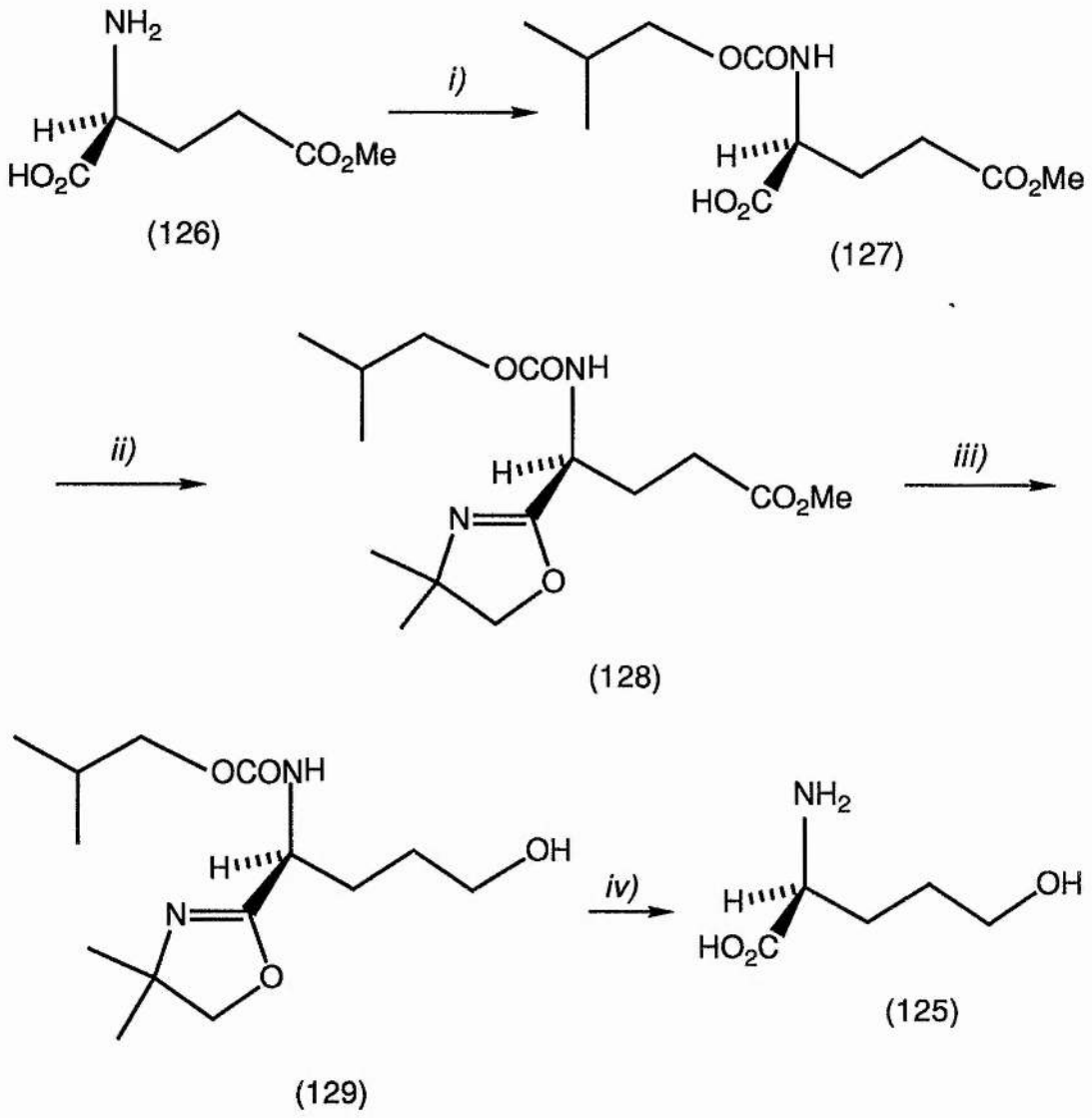
2.7 Synthetic Routes to 5-Hydroxynorvaline

Four synthetic routes were examined.

Nitrosation of N-acetyl ornithine with perchloric acid and sodium nitrite was unsuccessful[257]. Hydroboration of DL-allylglycine methyl ester with disiamylborane[258] was also unsuccessful, even when 3 equivalents of disiamylborane were used to compensate for any possible complexation between the amine and the borane.

Reduction of the γ-methyl ester of L-glutamic acid (126) with lithium aluminium hydride proved more successful. Protection of the α-carboxylic acid and amine groups was required, before the reduction step. Two protection methodologies were followed (Schemes 2.2 and 2.3).

In the first method, isobutyl chloroformate was used to protect the nitrogen (127) (Scheme 2.2). Protection of the α-carboxylic acid as the 4,4,-dimethyl-2-oxazoline (128)[259] proceeded smoothly, although care was required to ensure the oxazoline ring closed. However, the ester reduction, giving the protected alcohol (129) and subsequent deprotection reactions gave a mixture of products. Preparative tlc however gave a small amount of the required 5-hydroxynorvaline (125).

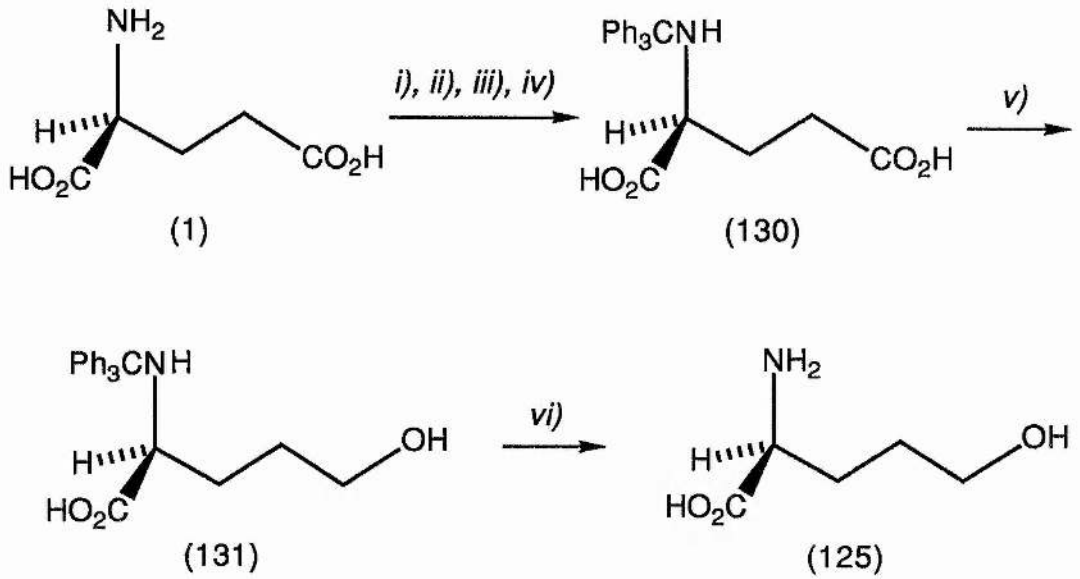


i) isobutylchloroformate, H_2O/toluene
ii) 2-amino 2-methyl 1-propanol, p TSA
iii) $LiAlH_4$, Et_2O
iv) 6 M HCl, reflux, 4 h

Scheme 2.2 Synthesis of 5-Hydroxynorvaline *via* the Oxazoline

The second route to 5-hydroxynorvaline used trityl chloride to protect the amine of glutamic acid in a one pot reaction (Scheme 2.3) [260,261]. Trimethylsilyl chloride was added first to protect the carboxylic acid groups.

Trityl chloride afforded protection of the amine and the trimethylsilyl groups were removed under mild acid conditions, to give N-trityl glutamic acid (130). In our hands, lithium aluminium hydride reduction to give N-trityl 5-hydroxynorvaline (131) did not go to completion and deprotection with 95 % acetic acid gave a mixture of 5-hydroxynorvaline (125) and glutamic acid (1).



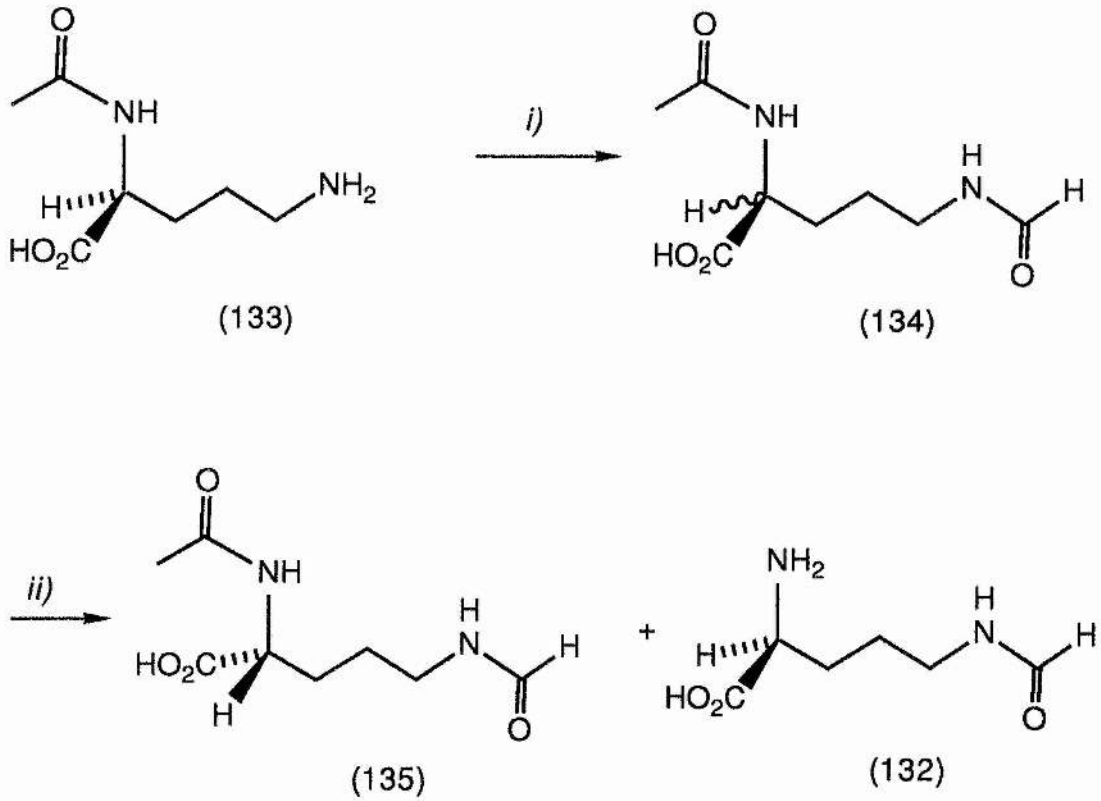
i) TMSCl, DCM, Et_3N
ii) MeOH
iii) Et_3N, Ph_3CCl
iv) MeOH
v) $LiAlH_4$, THF
vi) $AcOH/H_2O$ (95/5)

Scheme 2.3 Synthesis of 5-Hydroxynorvaline Using Trityl Protection

The chemical shift of the 5-Hs of 5-hydroxynorvaline was 3.02 ppm. Addition of a sample to the incubation mixture confirmed the signal was not coincident with that from the incubation product (Table 2.3).

2.8 Synthesis of δ-N-Formyl Ornithine

It seemed likely that the incubation product (122) possessed a nitrogen containing electron withdrawing group at C-5. One such compound was δ-N-Formyl ornithine (132). This compound was therefore synthesized, in order to allow the chemical shift of its 5-Hs to be compared with that of the incubation product. δ-N-Formyl ornithine (132) was synthesized from α-N-acetyl ornithine (133) (Scheme 2.4). Formic acetic anhydride, made from acetyl chloride and sodium formate[262], was added to α-N-acetyl ornithine in glacial acetic acid. The product, α-N-acetyl-δ-N-formyl ornithine (134), was obtained as a racemate at the α-centre. The enzyme acylase was used to remove the α-acetyl protection. Although the resultant α-N-acetyl-δ-N-formyl-D-ornithine (135) and δ-N-formyl-L-ornithine (132) mixture could not be separated, subtraction of the α-N-acetyl-δ-N-formyl-D-ornithine signals from the ^{1}H NMR spectrum of the mixture gave the ^{1}H NMR spectrum of δ-N-formyl-L-ornithine.



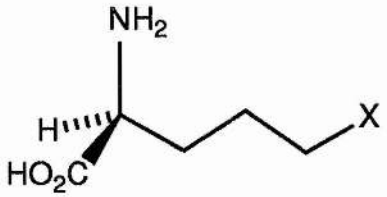
i) formic acetic anhydride, AcOH
ii) acylase

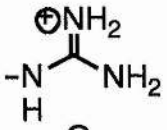
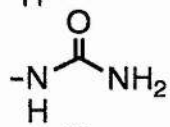
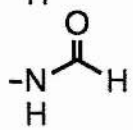
Scheme 2.4 Synthesis of δ-N-Formyl-L-Ornithine

2.9 Further NMR Data

The chemical shifts obtained from ^{1}H and ^{13}C NMR spectroscopy of arginine (136), citrulline (137) and δ-N-formyl-L-ornithine (132) were compared to those of the incubation product (Table 4.3).

Table 2.3 ^{1}H and ^{13}C NMR Data for 5-Substituted Amino Acids



Substituent X		^{1}H-Chemical Shift 5-Hs (ppm)	^{13}C-Chemical Shift (ppm) C-5	X
-X	(122)	3.04	42.0	158.0
$-CO_2H$	(123)	2.25		
$-NH_2$	(124)	3.40		
-OH	(125)	3.02		
	(136)	3.04	41.7	157.9
	(137)	3.05	39.8	162.3
	(132)	2.90	38.0	176.6

2.10 Attempted Isolation of Incubation Product

Isolation of the incubation product proved unsuccessful by both preparative tlc and ion exchange chromatography. However, acidification of the

incubation mixture and extraction with diethyl ether removed both ethylfumaric acid and mercaptoethanol from the mixture. This essentially left a mixture of ethylaspartic acid, the compound in question and another unknown compound typified by a doublet at 1.37 ppm. Subtraction of the 3-ethylaspartic acid signals from a ^{13}C NMR spectrum of this mixture left signals identical to those of arginine (136).

2.11 Conclusions

However, further incubations showed arginine was also produced in the absence of substrate. Thus, it would seem likely that it is produced by some protease action in the crude enzyme extract.

Mr Hartzoulakis has since shown, in similar incubations with purified glutamate mutase that (2S,3S)-3-ethylaspartic acid is indeed a substrate for glutamate mutase[254]. ^{1}H NMR spectra suggested that L-*erythro* -methyl glutamic acid was the initial rearrangement product, but that further rearrangement gave 3,3-dimethyl aspartic acid, typified by two singlets at 1.37 ppm, as observed in incubations with crude enzyme and interpreted as a doublet.

2.12 Synthesis of (2S,3S)-[$^{2}H_5$]-3-Ethylaspartic Acid

In order to follow in more detail any rearrangement of 3-ethylaspartic acid, access to appropriately labelled compounds was required. (2S,3S)-3-Ethylaspartic acid completely deuteriated in the ethyl group was synthesized *via* the route for unlabelled 3-ethylaspartic acid discussed previously (Scheme 2.1). The deuterium labelling was introduced by using [$^{2}H_5$]-ethyl iodide in the alkylation of ethyl acetoacetate. The 2-[$^{2}H_5$]-ethylacetoacetate ethyl ester (138) produced in a small scale reaction, was brominated. A Favourskii rearrangement of the dibromide gave [$^{2}H_5$]-3-ethylfumaric acid (139), typified by only one signal in ^{1}H NMR spectroscopy, a singlet at 6.59

ppm. This was enzymically aminated to (2S,3S)-[$^2H_5$]-3-ethylaspartic acid (140), which displayed ^1H NMR signals due to 2-H and 3-H at 2.71 and 3.90 ppm, respectively.

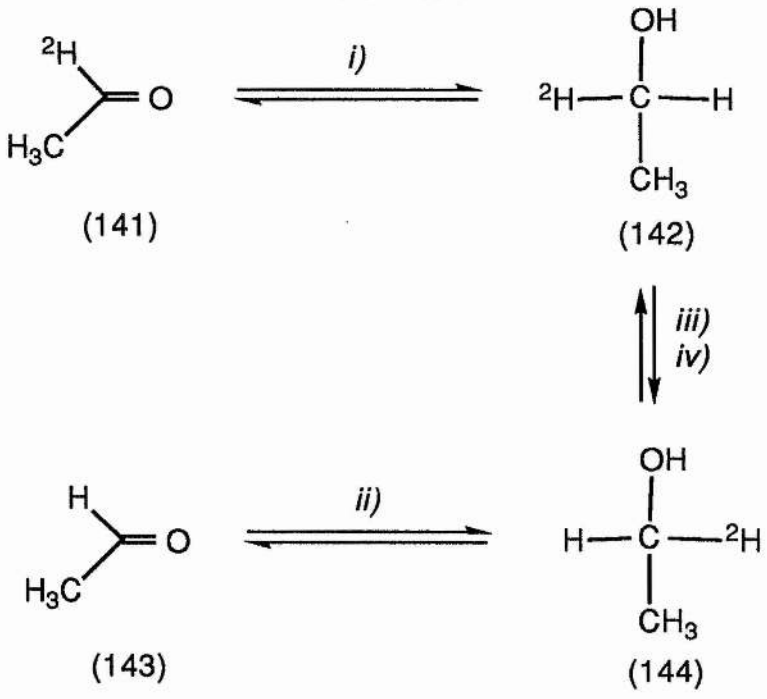
2.13 Synthesis of (2S,3S)-[1'-^{2}H]-3-Ethylaspartic Acid

3-Ethylaspartic acid chirally labelled at the 1' C of the ethyl group was required to attempt to establish from which side the 5'-deoxyadenosyl radical abstracted a hydrogen. In order to utilize the synthetic route previously employed (Scheme 2.1) a chirally labelled ethyl halide was required. Simple alkyl halides are extremely volatile compounds and therefore difficult to purify. The low melting point solid, ethyl tosylate (m. p. 33 -34 °C[263]) was, therefore, substituted for ethyl bromide / iodide in the reaction with the ethyl acetoacetate anion. The harder, bulkier oxygen-based leaving group proved not to be as efficient an electrophile as the alkyl halides. A mixture of compounds was obtained, from which 2-ethylacetoacetate ethyl ester (119 b) was isolated in 40 % yield by column chromatography on silica gel, eluting with ethyl acetate / petroleum ether (15 / 85). Access to chirally labelled ethyl tosylate was to be *via* the appropriately labelled ethanol.

2.13.1 Methods Available for the Synthesis of Chirally Labelled Ethanol

Loewus, Westheimer and Vennesland's classic demonstration of the stereospecificity of enzyme catalysed reactions[264] was also the first synthesis of chirally labelled ethanol. They showed that nicotinamide-(NAD^+) dependent yeast alcohol dehydrogenase stereospecifically reduced [1-^{2}H]-acetaldehyde (141) to [1-^{2}H]-ethanol (142) (Scheme 2.5). Reoxidation of the alcohol regenerated [1-^{2}H]-acetaldehyde. Similarly unlabelled acetaldehyde (143) was converted to [1-^{2}H]-ethanol (144) by reduction with deuteriated nicotinamide coenzyme, again with stereospecificity. Walden inversion of the tosylate using sodium hydroxide

cleanly converted one enantiomer to the other.



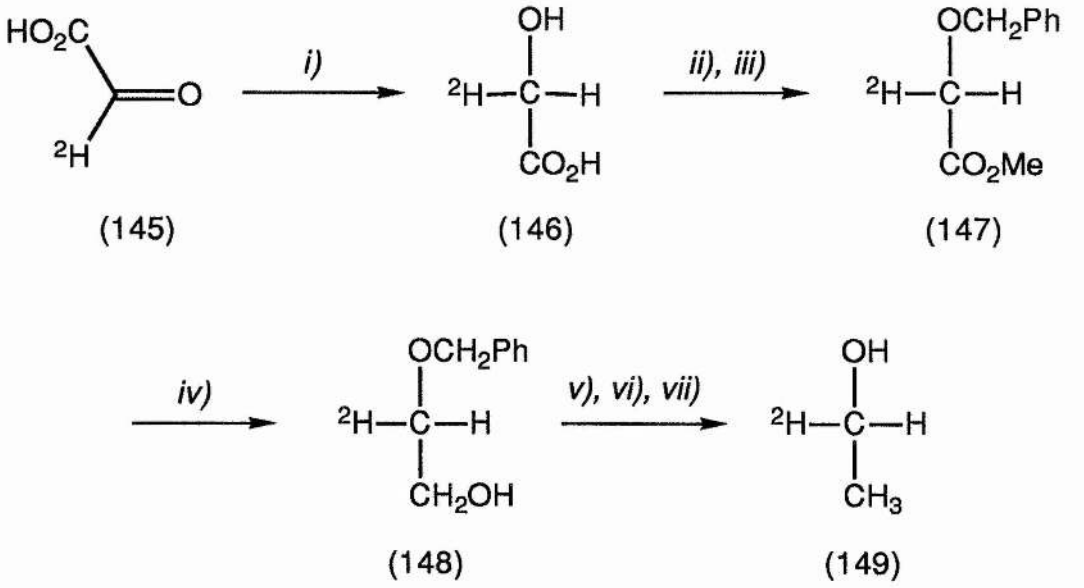
i) NADH, H^+, alcohol dehydrogenase
ii) NAD^2H, H^+, alcohol dehydrogenase
iii) TsCl, pyr
iv) NaOH

Scheme 2.5 The Stereospecific Nature of the Alcohol Dehydrogenase Reaction

A number of syntheses of [1-^{2}H]-ethanol of known stereochemistry followed, thus allowing the configuration of the ethanols produced enzymically to be established[265]. Several of these routes merit investigation.

Weber's synthesis of (S)-[1-^{2}H]-ethanol from [1-^{2}H]-glyoxylic acid[266] employed NAD^+-dependent muscle lactate dehydrogenase to convert [1-^{2}H]-glyoxylic acid (145) to (S)-[1-^{2}H]-glycolic acid (146). The methyl ester was then formed and the alcohol protected as the benzyl derivative (147). Reduction of the ester with $LiAlH_4$ gave the alcohol (148) which was activated with *p* -bromobenzenesulphonyl chloride. Reduction with $LiAlH_4$ introduced the methyl group and hydrogenation deprotected the alcohol to

give (S)-[1-^{2}H]-ethanol (149) (Scheme 2.6).

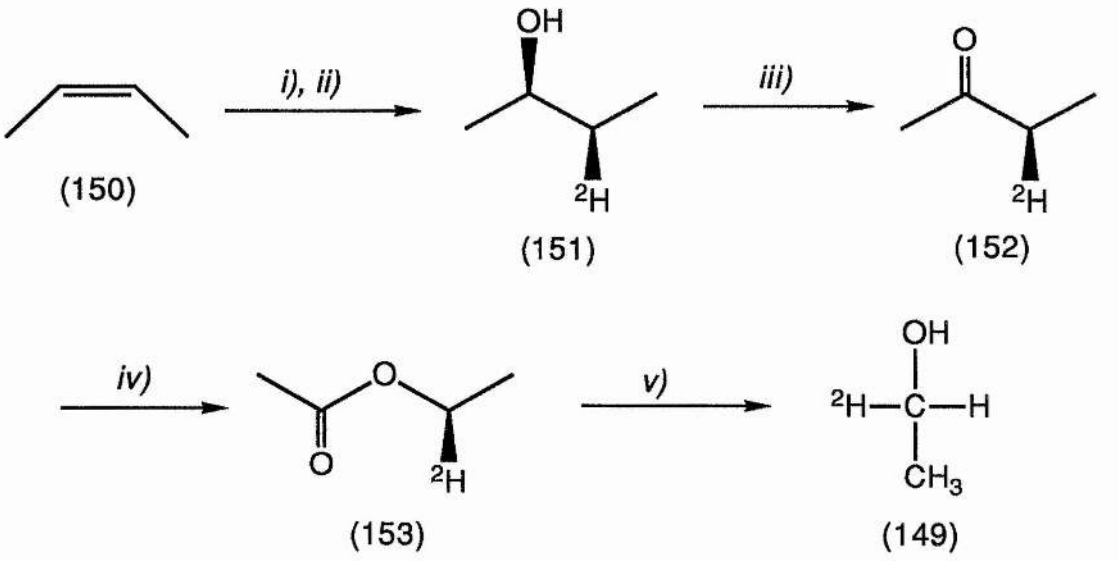


(145) (146) (147) (148) (149)

i) NADH, lactate dehydrogenase
ii) $Me_2C(OMe)_2$, H^+
iii) $PhCH_2Br$, Ag_2O
iv) $LiAlH_4$
v) p -$BrPhSO_3Cl$, pyr
vi) $LiAlH_4$
vii) H_2, Pd/C

Scheme 2.6 Synthesis of (S)-[1-^{2}H]-Ethanol from [2-^{2}H]-Glyoxylic acid

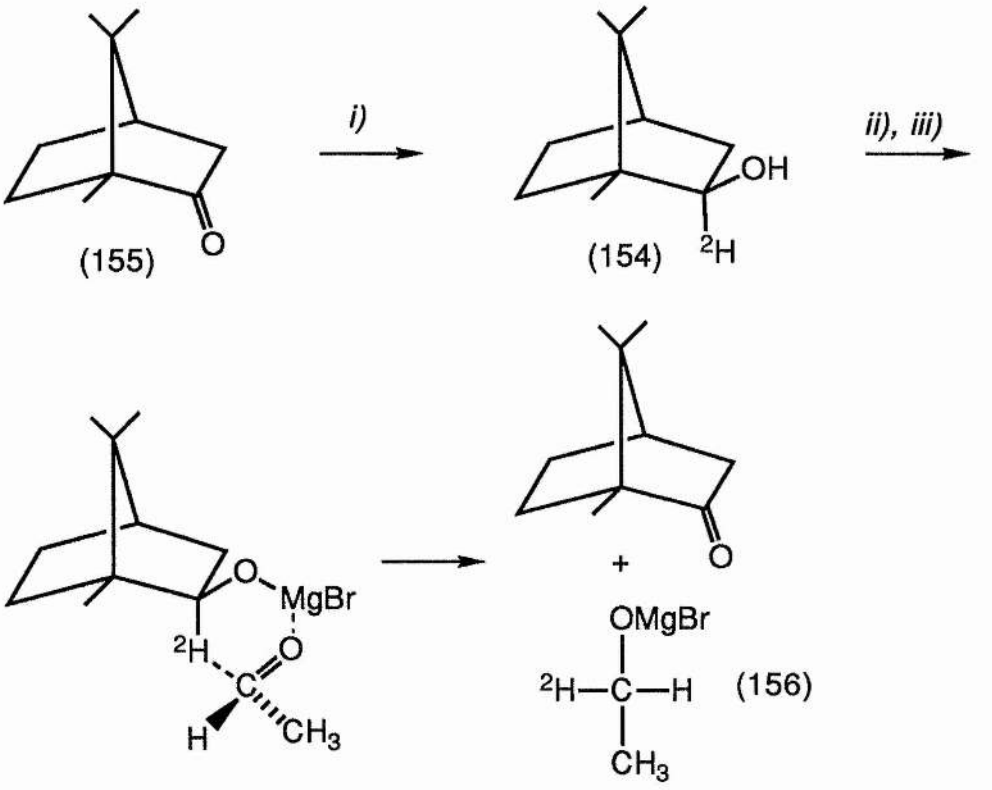
An attempt to introduce stereospecificity by the asymmetric hydroboration of 2-*cis* -butene (150) with deuteriated diisopinocampheylborane, giving (2R,3S)-[3-^{2}H]-butan-2-ol (151), was not entirely successful as the reaction gave some of the alternative, *erythro* isomer[267]. However, oxidation to the aldehyde (152), followed by a Baeyer-Villiger rearrangement gave [2-^{2}H]-ethylacetate (153), and hydrolysis to (S)-[1-^{2}H]-ethanol (149) proceeded smoothly (Scheme 2.7).



i) ^{2}*H- diisopinocampheylborane*
ii) H_2O_2
iii) Pt, O_2
iv) CF_3CO_3H, Na_2HPO_4
v) KOH

Scheme 2.7 Synthesis of (S)-[1-^{2}H]-Ethanol from *cis* -But-2-ene

Prochirality was also induced chemically, by a Grignard type reaction between acetaldehyde and α-deuteriated isoborneol (154), produced by reduction of (+)-camphor (155) with lithium aluminium deuteride. The magnesium bromide salt of (S)-[1-^{2}H]-ethanol (156) resulted (Scheme 2.8)[268].



i) $LiAl^2H_4$
ii) Mg, Br_2
iii) MeCOH

Scheme 2.8 Synthesis of (S)-[1-^{2}H]-Ethanol using (+)-Camphor

Enzymic syntheses of [1-^{2}H]-ethanol offer higher stereochemical purity, but are limited by the cost of the enzymes and NAD^+ cofactor. The isolation of the ethanol produced from the aqueous medium also proved difficult. Catalytic amounts of cofactor can be used, if it is regenerated *in situ* by a coupled reaction. Levi *et al* used an alcohol dehydrogenase / glucose dehydrogenase system[269]. Glucose dehydrogenase made an ideal recycling enzyme as the reaction equilibrium favoured reduction of NAD^+, and non-enzymic hydrolysis of the gluconic acid product drove the reaction to completion.

The alcohol dehydrogenase reaction was also coupled to the flavin enzyme,

diaphorase (Scheme 2.9)[270]. This coupling allowed the deuterium label to be introduced from the solvent. Solvent deuterium exchanged into the flavin (157) and was, thus, transferred to the nicotinamide (158). The opposite enantiomer was produced by stereospecific loss of deuterium from dideuteroethanol ([1,1-^{2}H]-ethanol).

$$\text{Flavin-H}_2 + {}^2\text{H}_2\text{O} \rightleftharpoons \underset{(157)}{\text{Flavin-}{}^2\text{H}_2} + \text{H}_2\text{O}$$

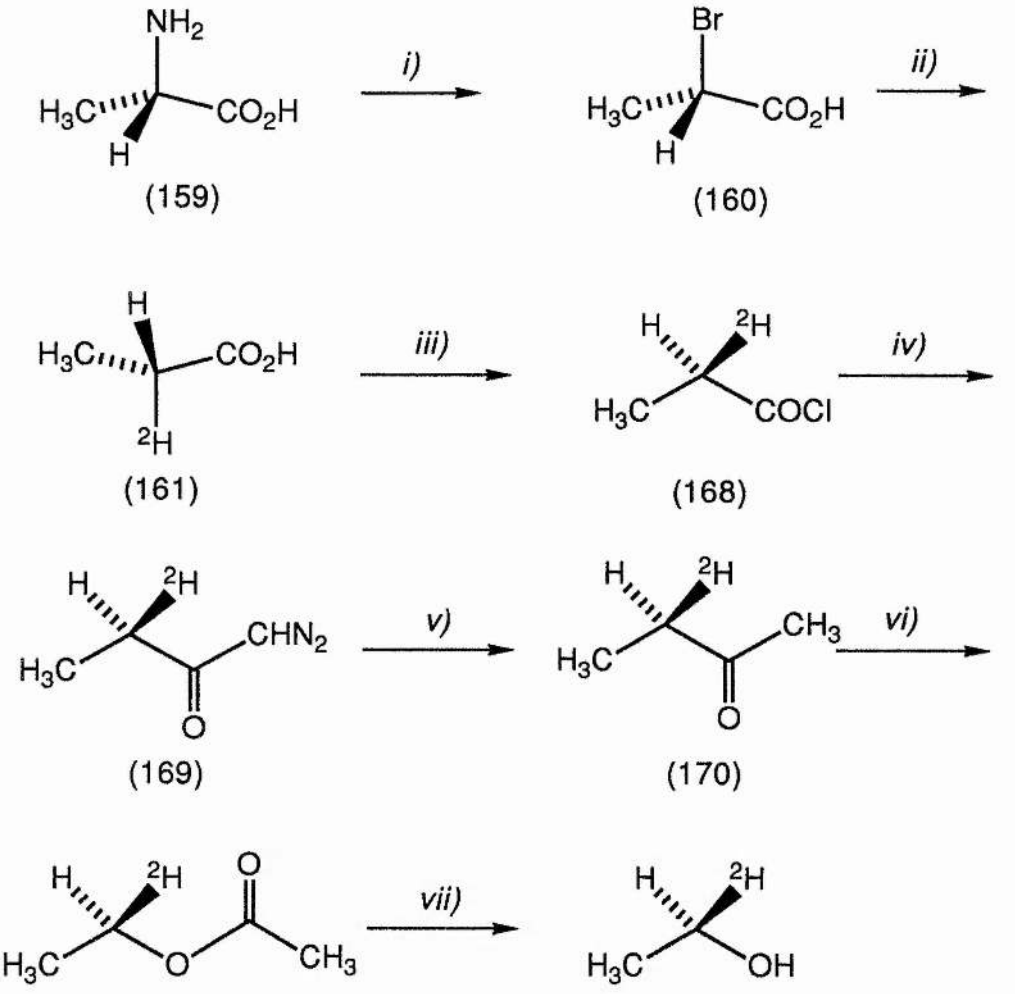
$$\text{Flavin-}{}^2\text{H}_2 + \text{NAD}^+ \rightleftharpoons \underset{(158)}{\text{NAD}^2\text{H}} + {}^2\text{H}^+ + \text{Flavin}$$

$$\text{CH}_3\text{CHO} + \text{NAD}^2\text{H} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{CH}^2\text{HOH} + \text{NAD}^+$$

Scheme 2.9 Diaphorase Coupled Synthesis of (R)-[1-^{2}H]-Ethanol

2.13.2 Attempted Synthesis of Chirally Labelled Ethanol

It was decided to employ a new chemical synthesis of [1-^{2}H]-ethanol. The route devised is shown in Scheme 2.10. The appropriate enantiomer of alanine (159) was the starting material. Chirality was introduced into the molecule using the reducing agent, lithium triethylborodeuteride (Super Deuteride). Subsequent steps involved activation of the acid group, formation of the methyl ketone and a Baeyer-Villiger rearrangement. Cleavage of the acetate then gave ethanol.



i) KBr, $NaNO_2$, HBr
ii) $LiEt_3BD$, THF
iii) PCl_5, $CHCl_3$
iv) CH_2N_2
v) HI, DCM
vi) m -CPBA, 1,1,1-TCE
vii) N_2H_4

Scheme 2.10 Synthetic Route to Chiral Ethanol

(2S)-Alanine (159) was first converted to (2S)-bromopropanoic acid (160), *via* a nitrosation / bromination reaction[271], which gave retention of configuration. A double inversion of stereochemistry actually occurred. The amine was nitrosated and then displaced by an intramolecular S_N2 reaction

to give the internal α-lactone. The lactone was then attacked by the bromide, again by a S_N2 mechanism, opening up the lactone, to give (2S)-bromopropanoic acid with overall retention of configuration. Lithium triethylborodeuteride[272] was then used to reduce the (2S)-bromopropanoic acid to (2R)-[2-^{2}H]-propanoic acid (161), with a further inversion of configuration[273]. Triethyl borate was produced as a side product of this reaction and proved difficult to remove. Attempted removal *in vacuo* resulted in loss of the low boiling propanoic acid as well. Separation by short path distillation was also unsuccessful, as was chelation with 1,1,1-tris(hydroxymethyl)ethane. The problem was noted in a similar literature synthesis[274] where spinning band column distillation was used, giving pure ethyl [2-^{2}H]-propanoate in only 10 % yield, the rest being contaminated with triethylborate.

The (2R)-[2-^{2}H]-propanoic acid produced by reduction with lithium triethyl-borodeuteride was not purified any further at this stage and the triethylborate carried through to the next step. It was expected not to interfere with subsequent reactions and might be removed more easily in a later step.

An alternative two step route from (2S)-bromopropanoic acid to (2R)-[2-^{2}H]-propanoic acid used by Prescott and Rabinowitz[275] in their synthesis of [2-^{2}H]-propionyl-CoA was considered. They used $LiAlD_4$ to introduce deuterium at C-2 with inversion of configuration and also to reduce the acid to an alcohol with the introduction of deuterium at C-1, giving [1,1,2-^{2}H]-*n*-propanol. This was oxidised to [2-^{2}H]-propanoic acid with chromic acid. However a number of purification steps were required to obtain the pure deuteriated acids, using preparative GC techniques that were not available in the group.

As unlabelled propanoic acid and other intermediates were available, the reaction conditions for the following steps were investigated without using labelled material.

Several attempts were made to synthesize propionyl chloride (162).

Treatment of propanoic acid with thionyl chloride yielded the acid chloride, as was confirmed by the distinctive C=O stretch in the IR spectrum at 1790 cm^{-1} and by comparison of spectral data with that for the authentic compound. However, the excess thionyl chloride could not be removed *in vacuo* as the boiling points of thionyl chloride and propionyl chloride were very similar (thionyl chloride: b.p. 75 - 76 °C[276] and propionyl chloride: b.p. 80 °C[277]). Vogel described the decomposition of excess thionyl chloride by addition of formic acid[278]. Accordingly, 96 % formic acid was added but the propionyl chloride was not isolable and seemed to have decomposed. The alternative oxalyl chloride had a lower boiling point (oxalyl chloride: b.p. 62 °C[276]). However, despite the greater difference in boiling points between oxalyl chloride and propionyl chloride, the excess oxalyl chloride still co-distilled with the propionyl chloride. Formation of the acid chloride using triphenylphosphine and carbon tetrachloride[279] was also pursued. However removal of the triphenylphosphine oxide by-product and unreacted triphenylphosphine proved difficult.

An alternative was to activate the acid as an anhydride instead of the acid chloride. Ethyl chloroformate was used to form the mixed anhydride, which was converted directly to the diazoketone without isolation. This reaction was partially successful in yielding the required diazoketone but there were a number of side products which could not be fully identified, so introducing a purification problem.

Synthesis of the acid chloride was readdressed. This was accomplished using phosphorus pentachloride[280] with chloroform as solvent. Propionyl chloride was co-distilled off with the chloroform, leaving the by-product, phosphorus oxychloride (b.p. 107.2 °C[276]) and any unreacted phosphorus pentachloride behind.

Diazomethane added to a solution of propionyl chloride in chloroform gave the diazoketone cleanly. Two equivalents of diazomethane were required; one equivalent was used to form the diazonium intermediate and the other to remove HCl from the intermediate, giving the diazoketone, chloromethane

and nitrogen. The diazoketone was fairly non-volatile (57 °C at 12 mm Hg[281]) and hence removal of the solvent *in vacuo* allowed the product to be isolated. It displayed a N=N stretching peak at 2105 cm^{-1} in the IR spectrum and, by ^1H NMR spectroscopy, a distinctive singlet at 5.23 ppm.

The diazoketone was dissolved in dichloromethane and reduced using aqueous hydrogen iodide to give butanone (164). The excess HI was removed using aqueous sodium thiosulphate (50 %). Such a concentrated sodium thiosulphate solution was used to prevent the butanone from partitioning into the aqueous layer. The material was purified by co-distillation of the butanone and dichloromethane. The C=O stretching peak observed by IR spectroscopy had shifted 30 cm^{-1} lower to 1710 cm^{-1}.

Baeyer-Villiger oxidation, using *m*- chloroperbenzoic acid then gave ethyl acetate (165). 1,1,2-Trichloroethane was used as solvent (1,1,2-trichloroethane: b.p. 113.7 °C[277]). The reaction was followed by ^1H NMR spectroscopy in which the shift of the methylene group from 2.45 ppm in butanone, to 4.13 ppm in ethyl acetate was particularly indicative. The reaction was cooled to cause precipitation of the contaminating chlorobenzoic acid and the solid filtered off. The ethyl acetate and 1,1,2-trichloroethane were co-distilled away from any further chlorobenzoic acid. The higher boiling solvent ensured that the ethyl acetate co-distilled over in the early part of the distillation. An alternative purification procedure, namely removal of chlorobenzoic acid by washing with aqueous bicarbonate solution, resulted in significant losses of ethyl acetate into the aqueous layer. The ethyl acetate was also purified by silica gel column chromatography, eluting with low boiling petroleum ether (b.p. 30 - 40 °C), which could be at least partially removed by distillation, to allow concentration of the ethyl acetate.

The acetate group was removed by reaction with hydrazine hydrate to give the required ethanol (166). Dioxane (b.p. 101 °C at 750 mmHg[282]) was initially used as solvent as ethanol (b.p. 78.5 °C[282]) co-distilled with it, leaving the non-volatile acetic hydrazide behind. However, the 1,1,2-

trichloroethane used in the previous step worked equally well.

The solvent / ethanol mixture was dried thoroughly and the ethanol converted to the tosylate (167), by reaction with tosyl chloride in pyridine[283].

However, when attempting the synthesis with labelled material, the diazoketone reaction failed to proceed smoothly. It was thought hydrogen chloride, present in the solution of (2R)-[2-^{2}H]-propionyl chloride (168) in chloroform, was disrupting the reaction. Using a larger excess of diazomethane did not improve the yield. Therefore a number of techniques were employed to remove hydrogen chloride. Firstly the freshly distilled chloroform was passed down an alumina column immediately prior to use. The reactions were also shielded from light and the solution of propionyl chloride in chloroform was distilled off N,N-dimethylaniline. This step successfully removed the hydrogen chloride, but some propionyl chloride was thought to have reacted with the N,N-dimethylaniline, lowering the yield. Potassium carbonate was added to the reaction of propionyl chloride with diazomethane immediately prior to concentration *in vacuo*, to quench any acid, preventing attack of the diazoketone as the solution was concentrated. The diazoketone was then redissolved in diethyl ether and the solid potassium carbonate removed by filtration. These precautions together ensured the smooth production of (3R)-[3-^{2}H]-1-diazobutan-2-one (169).

Reduction of (3R)-[3-^{2}H]-1-diazobutan-2-one with hydrogen iodide gave (3R)-[3-^{2}H]-butan-2-one (170). However, spectral analysis revealed that, in some reactions, up to 50 % of the deuterium label was lost. In the ^{1}H NMR spectrum extra splitting of the signals and inconsistent integrations were observed and two molecular ion peaks of equal intensity were revealed by mass spectroscopy. Loss of label presumably occurred by enol formation in the presence of a trace of acid. However, no procedural error could be linked to the loss of label. This step, therefore, proved unreliable.

Due to the large number of technical difficulties experienced with the synthesis and the build up of impurities caused by the non-isolable nature of

the volatile intermediates, it was decided not to pursue this route to chiral ethanol. If preparative GC were available the route might prove viable.

CHAPTER THREE

3-METHYLASPARTASE

RESULTS AND DISCUSSION

3.1 Introduction

3-Methylaspartase catalyses the conversion of (2S,3S)-L-*threo* -3-methylaspartic acid to mesaconic acid[9]. It has also been shown to turnover a number of 3-alkyl- and 3-halogeno- substituted fumaric acids[240,241], to give the L-*threo* -3-substituted aspartic acids in each case. The enzyme was considered to be the archetypal enzyme to operate *via* a carbanion mechanism[239]. The evidence for this mechanism included the lack of a primary deuterium isotope effect and that the rate of 3-H exchange exceeded the rate of mesaconic acid formation[238]. Also no ^{15}N- labelled ammonia was incorporated into the product, from the solution. It was also noted that both the *erythro* - and *threo* - isomers of 3-methylaspartic acid were substrates for the enzyme[9]. In a carbanion mechanism the two substrates would have a common intermediate upon loss of 3-H[239].

However, a re-examination of the enzyme by Botting and Gani showed there was a primary deuterium isotope effect of 1.7 on V_{max} at 1 mM potassium ion concentration, for the natural substrate[244]. This implied that C-H bond cleavage was at least partially rate limiting. A ^{15}N isotope effect on (V/K) of 2.5 % was also measured[246]. Hence C-N bond cleavage was also partially rate limiting. The reaction showed identical ^{15}N / ^{14}N isotope fractionation, when deuterium was substituted for hydrogen at C-3. This result indicated a concerted mechanism was operating and hence a carbanion mechanism was ruled out.

Given that the enzyme operated *via* a concerted mechanism, the ability of the enzyme to process the (2S,3R)-L-*erythro*- isomer of 3-methylaspartic acid required investigation. The assumption that both isomers gave rise to the same carbanionic intermediate was clearly no longer valid.

3.2 Confirmation that 3-Methylaspartase Catalyses the Elimination of Ammonia from (2S,3R)-3-Methylaspartic Acid

Confirmation was required as to whether 3-methylaspartase was responsible for the conversion of (2S,3R)-L-*erythro* -3-methylaspartic acid to mesaconic acid, or whether the reaction was due to a contaminating enzyme. Such a contaminating enzyme could either be an L-*erythro / threo* - epimerase or L-*erythro* -3-methylaspartase. Support for the latter notion came from the early studies on *Acetobacter suboxydans* [25], which showed the micro-organism was able to synthesis L-glutamic acid *via* a pathway which was essentially the reverse of the catabolic pathway used in *Clostridium tetanomorphum* (see p. 10). It seemed that *Acetobacter suboxydans* produced L-*erythro* -3-methylaspartic acid and not the L-*threo* - isomer used in *Clostridium tetanomorphum* . This implied the presence of L-*erythro* -3-methylaspartase and an L-*erythro* - specific glutamate mutase.

Barker sought to address the existence of a contaminating enzyme, in *Clostridium tetanomorphum*, by measuring the ratio of L-*erythro* - to L-*threo*- activity at various stages of the purification of 3-methylaspartase[9]. The ratio remained constant throughout. He also noted that the rates of deamination of both L-*threo* - and L-*erythro* -3-methylaspartic acid were dependent on potassium and magnesium ion concentration and inhibited by calcium ions. Botting recently confirmed that incubations of diammonium mesaconate, performed in the presence of potassium and magnesium ions at pH 9.0, with both partially purified clostridial 3-methylaspartase and clostridial 3-methylaspartase purified to homogeneity, gave, first, L-*threo* -3-methylaspartic acid and, after prolonged incubation, L-*erythro* -3-methylaspartic acid, in similar ratios independent of enzyme purity[284].

Cloning and over-expression of the 3-methylaspartase gene in *E. coli* [232] produced pure recombinant enzyme, free of any clostridial contaminants. Botting and Gani showed that incubations of diammonium mesaconate with enzyme from this source also produced L-*erythro* -3-methylaspartic acid[248]. Thus, L-*threo* -3-methylaspartase was capable of synthesizing the L-*erythro*

- isomer from mesaconic acid.

Given that one enzyme was responsible for both activities, it was of interest to investigate the L-*erythro* -3-methylaspartic acid reaction in more detail. The reaction could occur *via* epimerization of the L-*erythro* -isomer to the L-*threo* -isomer, from which ammonia could be eliminated, *via* a concerted *anti* -elimination mechanism, or direct *syn* -elimination of ammonia could occur. As the kinetics and mechanism of the reaction with the natural substrate were now so well understood[245], a study of the L-*erythro* -substrate would prove enlightening.

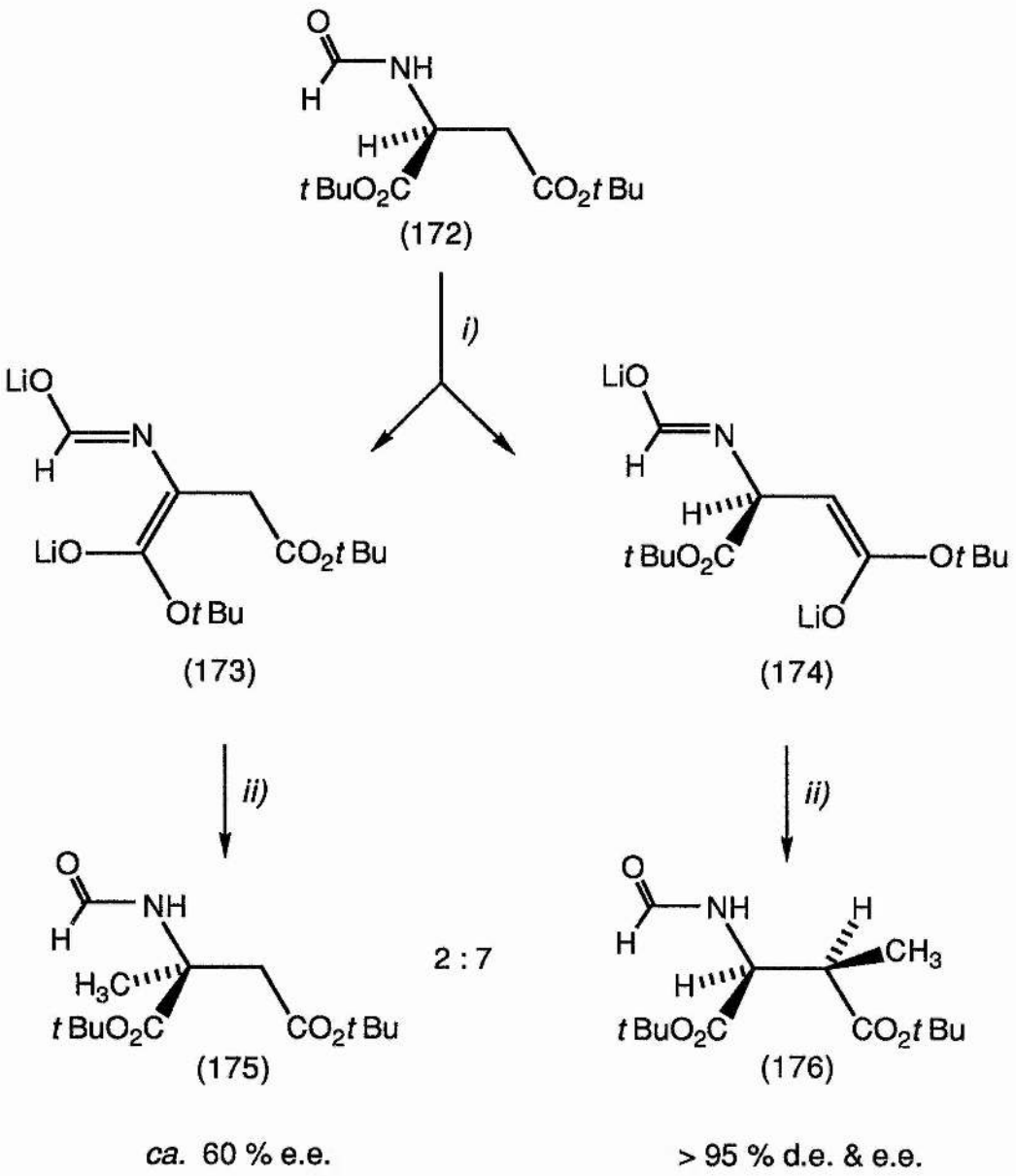
3.3 Possible Routes to (2S,3R)-3-Methylaspartic Acid

To investigate the reaction further, (2S,3R)-L-*erythro* -3-methylaspartic acid (171) was required. Barker incubated disodium mesaconate with *Clostridium tetanomorphum* cell-free extract, to obtain a mixture of L-glutamic acid, L-*threo* -3-methylaspartic acid and L-*erythro* -3-methylaspartic acid[9]. The L-glutamic acid was removed by elution through a Dowex 1-acetate column with 0.05 M acetic acid which gave a mixture of the two 3-methylaspartic acid isomers. This mixture was incubated with pure 3-methylaspartase until the rate of mesaconic acid production approached that expected for conversion of the L-*erythro* -isomer. The 3-methylaspartic acid remaining was isolated, to give L-*erythro* -3-methylaspartic acid, with less than 5 % contamination by L-*threo* -3-methylaspartic acid and L-glutamic acid. He also separated the two 3-methylaspartic acid isomers by paper electrophoresis in 0.1 M sodium formate buffer at pH 3.85, applying 1700 volts for 1 hour. The L-*threo* - isomer travelled faster than the L-*erythro* -isomer, having a R_P of 0.86 compared with 0.73 for the L-*erythro* -isomer[9].

It was decided however to utilize a synthetic route to (2S,3R)-3-methylaspartic acid. The route needed to be diastereoselective and flexible enough to allow the synthesis of (2S,3R)-[3-^{2}H]-3-methylaspartic acid as well.

3.3.1 Seebach's Synthesis of (2S,3R)-3-Methylaspartic Acid

Seebach's alkylation of di-*tert* -butyl-N-formyl-aspartic acid (172)[285] was considered (Scheme 3.1). In this synthesis, lithium diethylamide effected a clean double deprotonation of di-*tert* -butyl-(S)-N-formyl-aspartic acid in THF (-78 °C, 2 hours). The dilithio derivative (173 and 174) was then alkylated with iodomethane (-78 °C, 12 hours). This gave a mixture of α- and β-substituted aspartic acid derivatives (175 and 176) in a 2 : 7 ratio (60 - 70 % yield). The isomers could be separated chromatographically to give the diastereomerically and enantiomerically pure (2S,3R)-3-methylaspartic acid derivative (175), which was hydrolysed to give the free amino acid. However, the yields associated with this synthesis were intrinsically low.



i) $LiNEt_2$, THF, - 78 °C, 2 hr
ii) MeI, - 78 °C, 2 hr

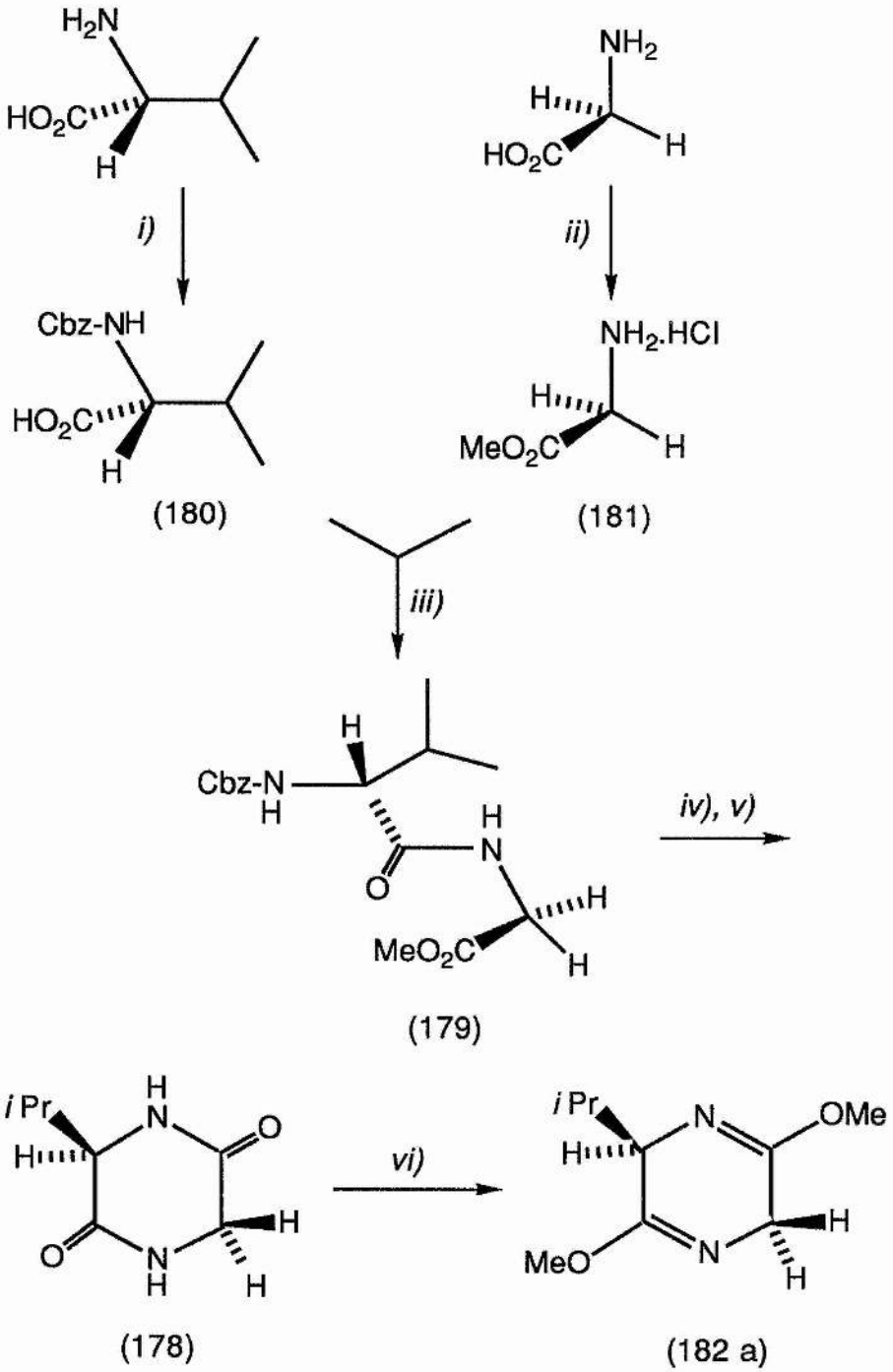
Scheme 3.1 Synthesis of (2S,3R)-3-Methylaspartic Acid from Di-*tert*-butyl-N-formylaspartic Acid

3.3.2 Schollkopf's Synthesis of (2S,3R)-3-Methylaspartic Acid

Schollkopf's *bis* -lactim ether methodology (Scheme 3.2)[286] offered a potentially higher yielding synthesis. This methodology involved alkylation of the lithiated *bis* -lactim ether (177) of *cyclo* -(D-Val-Gly) (178) (or variants) at the glycine α-centre. *Cyclo* -(D-Val-Gly) (178) was produced by cyclization of the dipeptide (179), formed from the appropriately protected amino acids (180 and 181). Methylation gave the *bis* -lactim ether (182 a), which could be deprotonated with *n*- butyl lithium. The *bis* -lactim ether anion (177) (Scheme 3.3) produced was relatively flat and hindered on one face by the valine side chain *iso*- propyl group. Thus the stereochemistry of alkylation at the glycine α-centre was controlled. The alkylating agent approached from the sterically less hindered face opposite to the *iso*- propyl group. Mild acid hydrolysis gave cleavage of the *bis* -lactim ether to yield alkylated amino acid derivatives of high enantiomeric excess.

This methodology was used, with aldehydes or ketones as the electrophile, to give compounds with two adjacent chiral centres. Thus 3-substituted serines were obtained with good selectivity at C-3, when the lithium ion was substituted with *tris* -dimethylaminotitanium *in situ*. However, formation of the *threo* - product was favoured[287].

This approach to generating two adjacent chiral centres was, therefore, not appropriate. An alkylating agent, with one of the chiral centres already in place, was required. The stereochemistry at the second chiral centre could then be introduced by the stereospecific nature of this reaction (see above). Methyl (2R)-bromopropanoate (183) was used as the alkylating agent (Scheme 3.3). This material was synthesized from D-alanine (184), by a nitrosation / bromination reaction (see p. 107) to give (2R)-bromopropanoic acid (185)[272], followed by esterification using diazomethane. Thus the *bis* -lactim ether ring (177) was alkylated to give (3S,6R)-2,5-dimethyoxy-3-((2'R)-2'-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine (186 a). Cleavage of the alkylated *bis* -lactim ether ring gave the required (2S,3R)-3-methylaspartic acid dimethyl ester (187) and D-valine methyl ester.

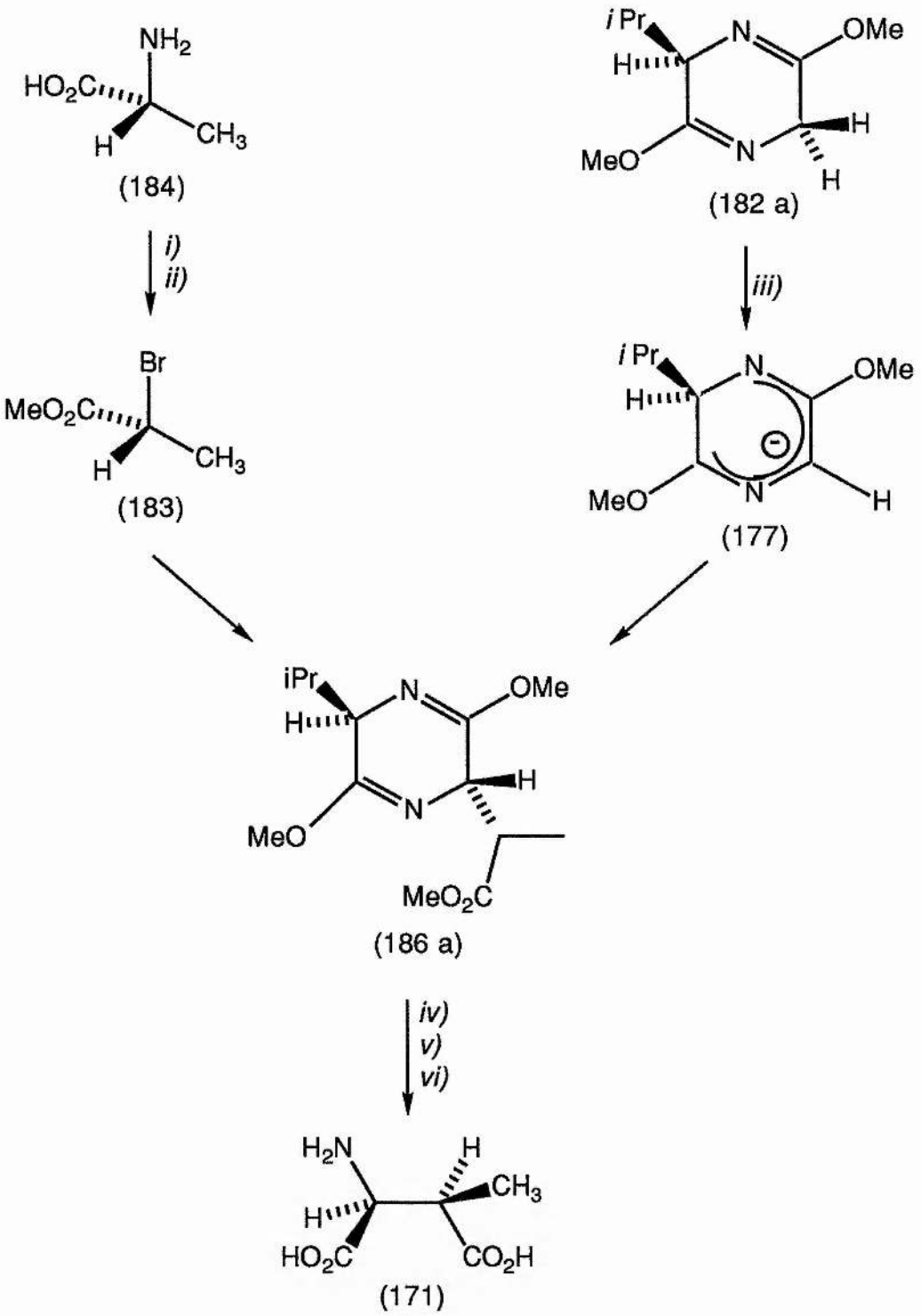


i) $PhCH_2OCOCl$, $NaHCO_3$, H_2O *iv) H_2/Pd/C, DCM/MeOH*

ii) $SOCl_2$, MeOH *v) $PhCH_3$, reflux, 12 h*

iii) NMM, iBuOCOCl, THF, DMF *vi) $[MeO]^+BF_4^-$, DCM*

Scheme 3.2 Synthesis of Schollkopf's *Bis* -Lactim Ether

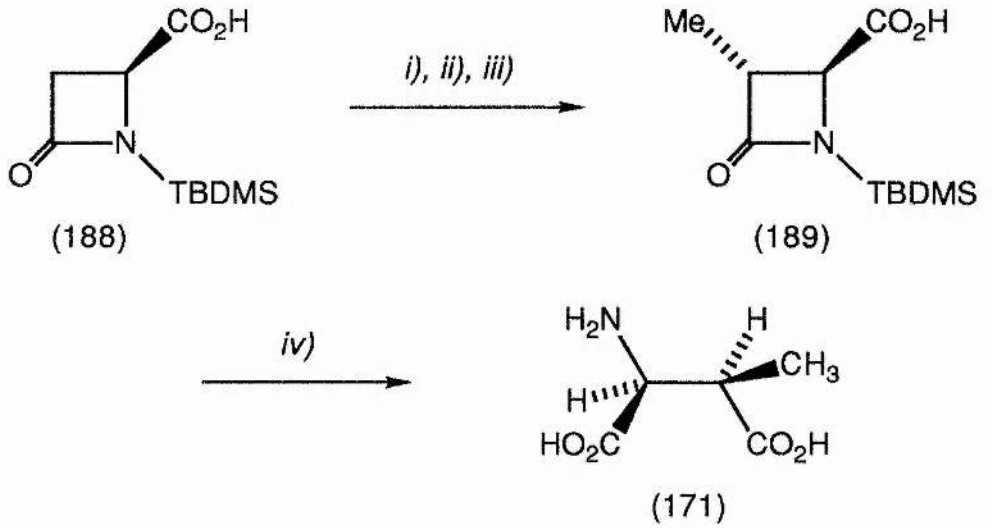


i) $NaNO_2$, HBr *iv) 0.1 M HCl, AcCN*
ii) CH_2N_2, Et_2O *v) 2 M NaOH*
iii) n BuLi, -80 °C, THF *vi) Prep tlc*

Scheme 3.3 Synthesis of (2S,3R)-3-Methylaspartic Acid Using Schollkopf's *Bis* -Lactim Ether Methodology

3.3.3 Hanessian's Synthesis of (2S,3R)-3-Methylaspartic Acid

A further methodology was published, by Hanessian[288], during the course of this work (Scheme 3.4). This route used (S)-1-*tert* -butyldimethylsilyl-4-carboxy-2-azetidinone (188) as a chiral template. Treatment with 2.1 equivalents of LDA in THF at 0 °C produced the dianion. Reaction with methyl iodide gave the 3-methylated product (189) with the methyl group *anti-* to the carboxyl group. Acid hydrolysis gave (2S,3R)-3-methylaspartic acid hydrochloride (171). However, only a 37 % yield of (S)-1-*tert* -butyldimethylsilyl-4-carboxy-3-methyl-2-azetidinone (189) was reported.

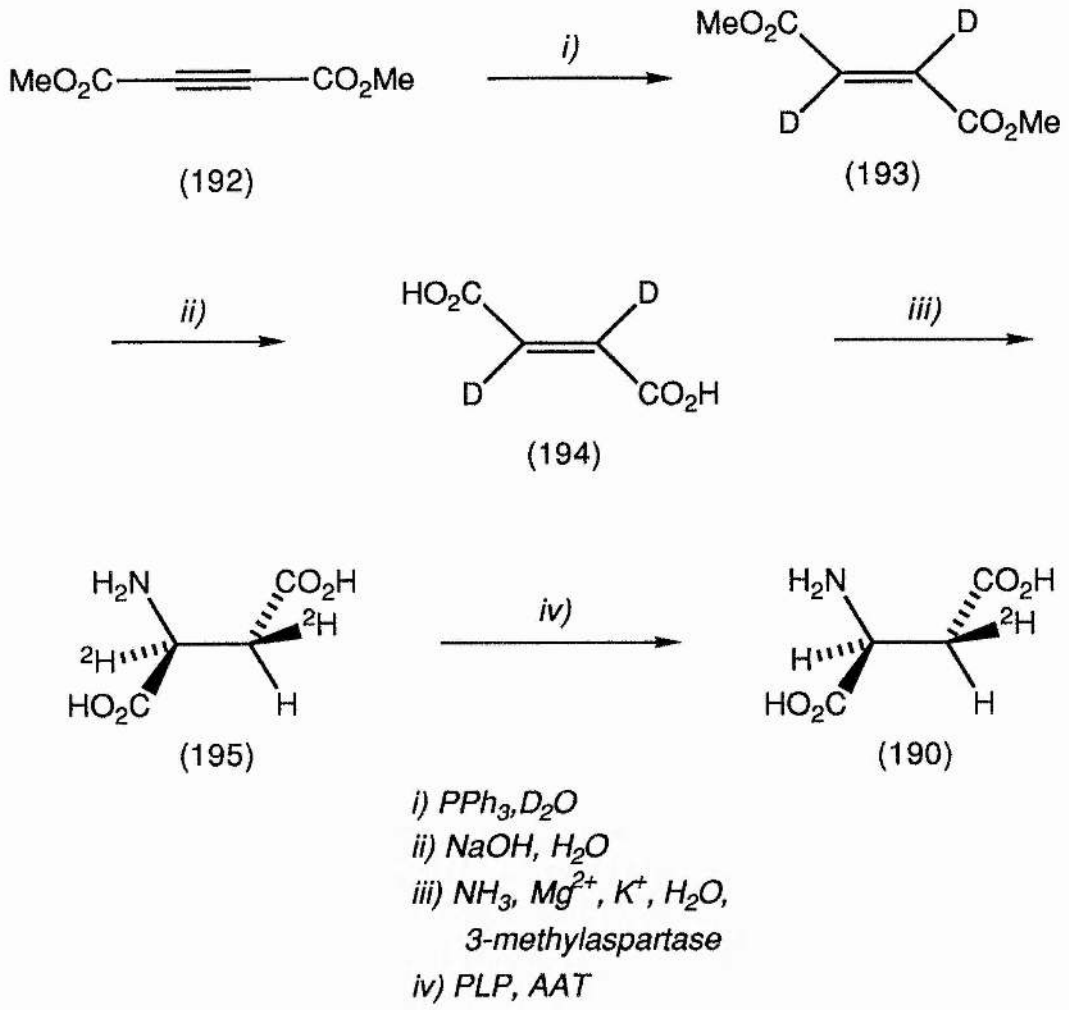


i) LDA, 0 °C
ii) MeI (2.2 eq), 0 °C, 2h
iii) aq. $KHSO_4$
iv) 6 N HCl, reflux 10 h

Scheme 3.4 Synthesis of (2S,3R)-3-Methylaspartic Acid from N-*t* -Butyldimethylsilyl (S)-4-carboxy-2-azetidone

3.3.4 Introduction of Deuterium into (2S,3R)-3-Methylaspartic Acid *via* Seebach's and Hanessian's Routes

Both Seebach's and Hanessian's methodologies used L-aspartic acid as starting material. Hence (2S,3S)-[3-^{2}H]-aspartic acid (190) was required for the synthesis of (2S,3R)-[3-^{2}H]-3-methylaspartic acid (191). The stereochemical control was introduced enzymically (Scheme 3.5). Firstly, reduction of dimethyl acetylenedicarboxylate (192) with triphenylphosphine, under deuteriating conditions, gave dimethyl dideuteriofumarate (193)[289], as a red/brown tar. Sublimation gave pure white dimethyl dideuteriofumarate, with one signal by ^{1}H NMR, at 3.81 ppm, due to the methyl ester. This was hydrolysed under basic conditions to dideuteriofumarate (194). Incubation of diammonium dideuteriofumarate with 3-methylaspartase, ammonia and potassium and magnesium ions, in water, for two days gave (2S,3S)-[2,3-^{2}H]-aspartic acid (195) in high enantiomeric excess, displaying a singlet by ^{1}H NMR, at 2.85 ppm, due to the 3-H. (2S,3S)-[3-^{2}H]-Aspartic acid (190) was obtained by incubation with aspartate aminotransferase, a PLP-dependent enzyme. The 2-H proton was observed at 4.12 ppm in the ^{1}H NMR spectrum.



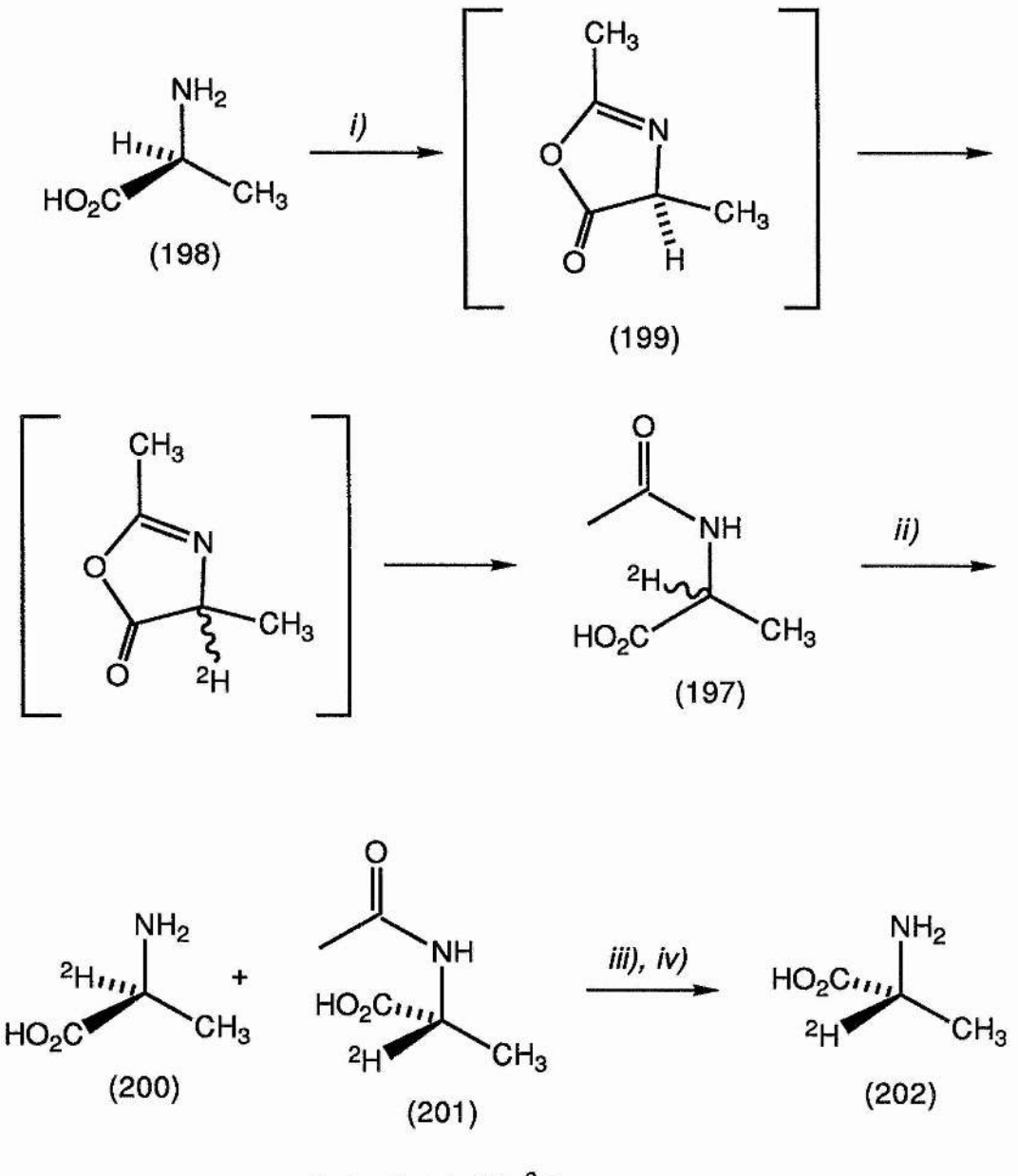
Scheme 3.5 Synthesis of (2S,3S)-[3-^{2}H]-Aspartic acid

3.3.5 Introduction of Deuterium Label into (2S,3R)-3-Methylaspartic Acid *via* Schollkopf's Synthesis

For the synthesis of (2S,3R)-[3-^{2}H]-3-methylaspartic acid (191) *via* the Schollkopf route, methyl (2R)-[2-^{2}H]-bromopropanoate (196) was required. A method for the racemic introduction of deuterium into α-amino acids was available[290]. Accordingly, Mr. Hartzoulakis produced the α-deuteriated racemate of N-acetyl-alanine (197), by the reaction of acetic anhydride and deuteriated acetic acid on L-alanine (198) (Scheme 3.6). The reaction

proceeded *via* an oxazolidine intermediate (199), from which hydrogen was abstracted, by acetate. Quenching with deuterium gave racemic [2-^{2}H]-N-acetyl-alanine. The enzyme acylase was then used to selectively deacetylate the L-enantiomer[291] giving a mixture of [2-^{2}H]-L-alanine (200) and [2-^{2}H]-N-acetyl-D-alanine (201). The two enantiomers were separated by preferential crystallization of the [2-^{2}H]-L-alanine. The D-enantiomer was then chemically deacetylated to [2-^{2}H]-D-alanine (202).

[2-^{2}H]-D-alanine was then converted to [2-^{2}H]-(2R)-bromopropanoic acid (203) and hence methyl (2R)-[2-^{2}H]-bromopropanoic acid (196), as outlined for the non-labelled reagent (p. 117).



i) Ac_2O, $MeCO_2{}^2H$
ii) acylase
iii) separate by crystallization
iv) 6 M HCl, reflux, 2h

Scheme 3.6 Synthesis of [2-^{2}H]-D-Alanine

3.4 Synthesis of (2S,3R)-3-Methylaspartic Acid Using Schollkopf's Methodology

The Schollkopf route to (2S,3R)-3-methylaspartic acid and (2S,3R)-[3-^{2}H]-3-methylaspartic acid was pursued as it seemed the potentially highest yielding synthesis. Previous members of the group had also already had experience of this chemistry.

3.4.1 Synthesis of the *Bis* -Lactim Ether

Two methods were available for the synthesis of *cyclo* -(D-Val-Gly) (178). The first involved coupling of D-valine N-carboxyanhydride with glycine methyl ester and then refluxing in toluene to complete cyclization. However, the formation of D-valine N-carboxyanhydride required the use of phosgene[292], so a mixed anhydride route[293] was favoured. In this route D-valine was protected as the N-carbobenzoxy derivative (180) using benzyl chloroformate. ^{1}H NMR spectroscopy showed benzyl signals at 5.13 and 7.35 ppm. Glycine was protected as the methyl ester hydrochloride (181) using thionyl chloride in methanol. This compound was typified by a methyl signal at 3.85 ppm in its ^{1}H NMR spectrum. The protected amino acids were then coupled using isobutyl chloroformate and N-methyl morpholine to give the dipeptide, N-Cbz-D-valine-glycine methyl ester (179). This was deprotected by catalytic hydrogenation and cyclization completed by refluxing in toluene, to give *cyclo* -(D-Val-Gly) (178). The two 6-Hs contributed from the glycine moiety exhibited considerably different chemical shifts, by ^{1}H NMR spectroscopy, (3.76 and 3.95 ppm) as a result of the two different environments above and below the ring. Care had to be taken to ensure the cyclic dipeptide was completely dry (*in vacuo*, 60 °C, 3 days) to achieve good yields in the next step.

The methylating agent, trimethyloxonium tetrafluoroborate, in the solid form, was initially used to convert *cyclo* -(D-Val-Gly) to the dihydropyrazine product (182 a). However, the moisture and air sensitivity of trimethyl-

oxonium tetrafluoroborate precluded that bottle's use in further reactions, once the contents had been subjected to the air contact required for weighing out. Therefore triethyloxonium tetrafluoroborate, which could be bought as a 1 M solution in dichloromethane, and hence transferred to the reaction vessel under anhydrous conditions, was used instead. This reagent was more soluble and so reacted quicker than trimethyloxonium tetrafluoroborate[294]. (3R)-2,5-Diethoxy-3-isopropyl-3,6-dihydropyrazine (182 b) was successfully synthesized and reacted with (R)-methyl-2-bromopropanoate in an alkylation reaction to give (3S,6R)-2,5-diethoxy-3-((2R')-methoxycarbonylethyl)-6-isopropyl-3,6-dihydropyrazine (186 b). However it proved cheaper to purchase the (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (182 a).

3.4.2 Optimization of the Alkylation Reaction

The literature method, for the formation of the *bis* -lactim ether anion and its subsequent alkylation, involved addition of *n* -butyl lithium to a stirred solution of the *bis* -lactim ether in THF at - 70 °C[294]. Stirring was continued at this temperature for 10 minutes and then a solution of the alkylating agent in THF added dropwise. After stirring for 5 hours at -70 °C the reaction was worked up. However, Thomas found that unless the reaction was allowed to warm up to - 65 °C, before the alkylating agent was added, the *bis* -lactim ether anion did not form[295]. Nonetheless the reaction still proved to be messy and low yielding. A detailed investigation was pursued in an attempt to improve the yield.

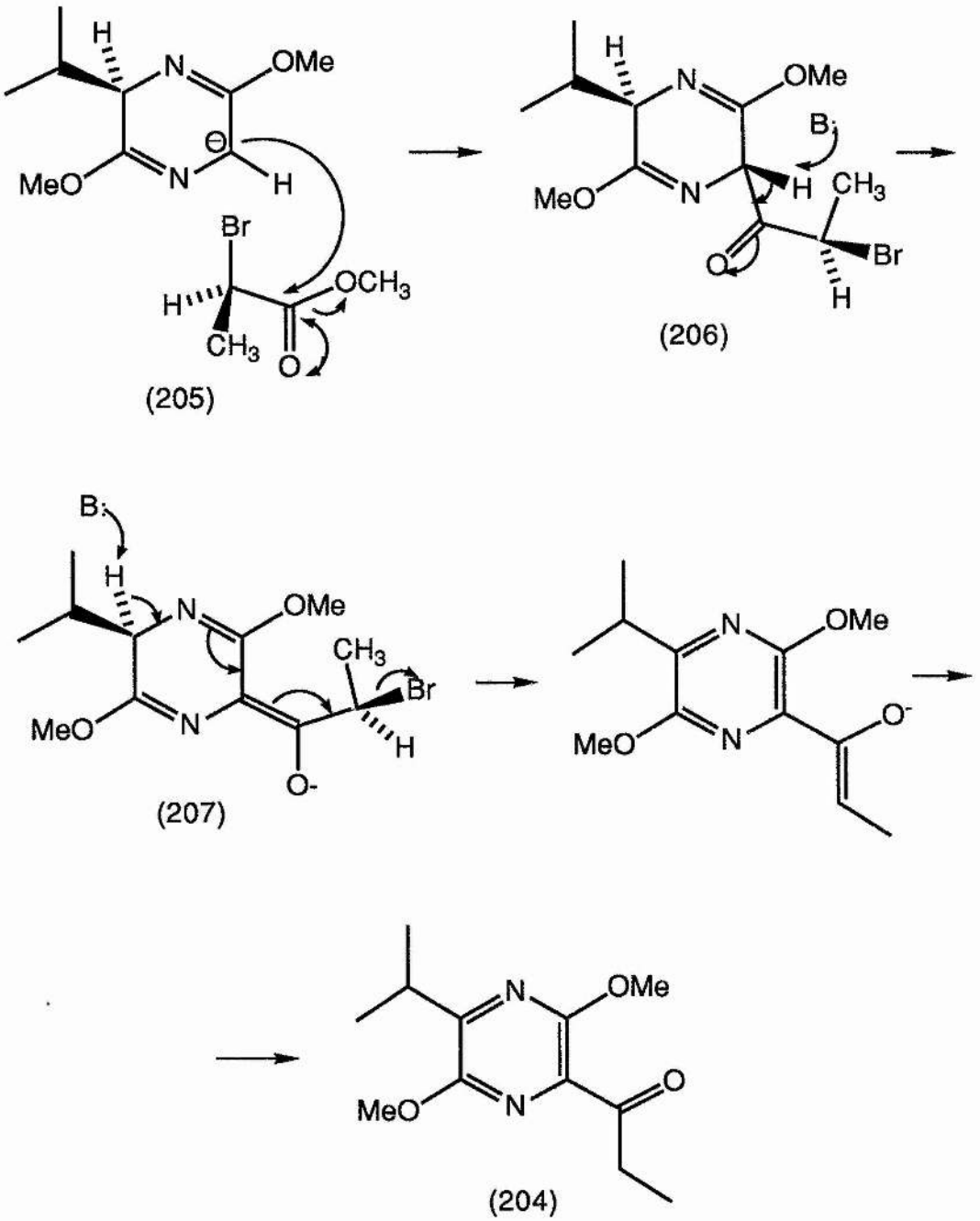
Unfortunately the reaction progress could not be followed by tlc, as the lithium salts prevented the formation of clear spots, even after quenching aliquots of the reaction mixture with phosphate buffer. ^{1}H NMR spectroscopy of an aliquot of reaction mixture was also unsuccessful without complete workup first.

Initial experiments utilized the 48 hour reaction times of Thomas[295], but

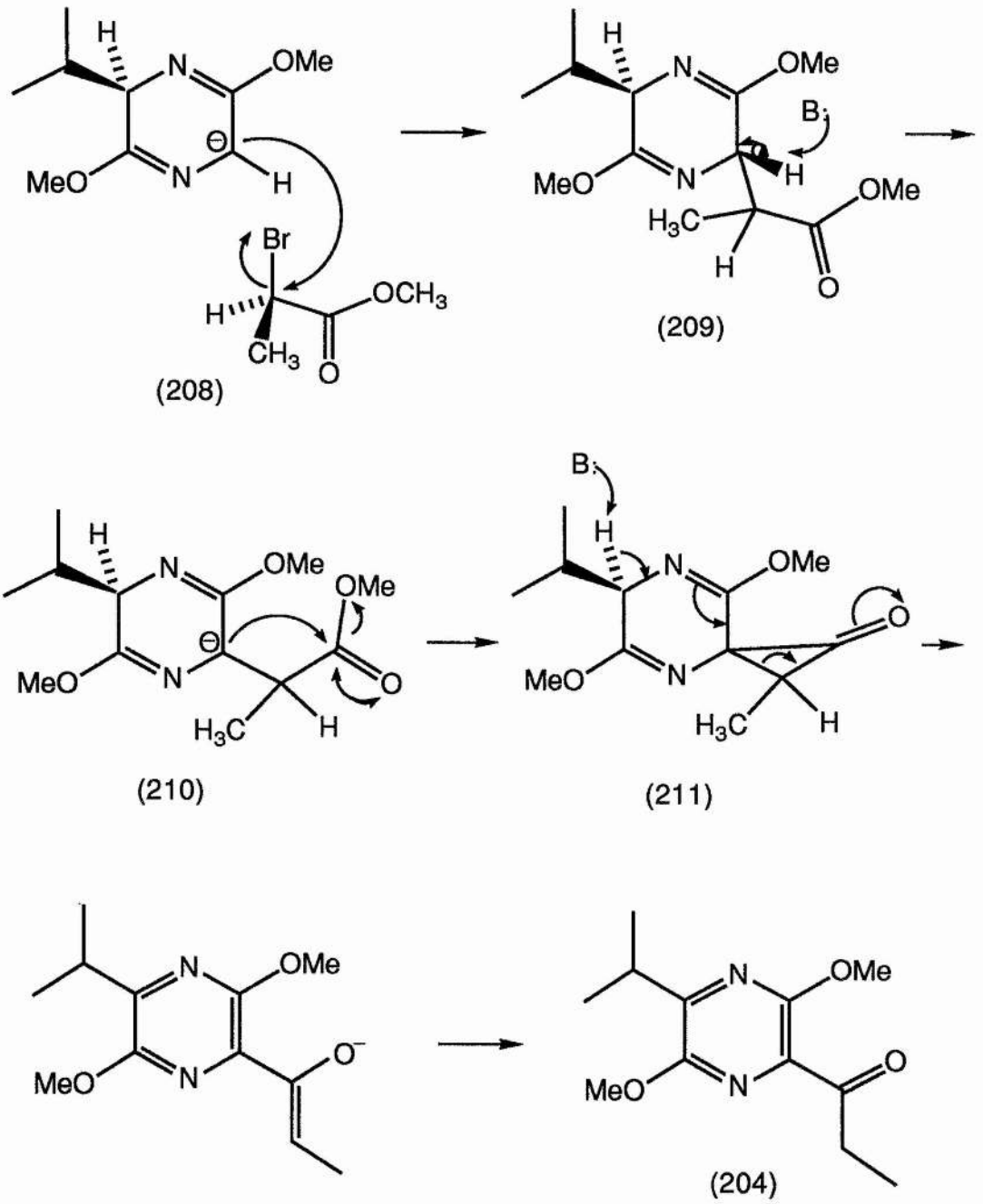
reinvestigation of the time scale of the reaction showed that the long reaction times reported were not necessary and may even have led to greater formation of an unwanted pyrazine side product (204) (Schemes 3.7 and 3.8). Interestingly, even when the reaction was performed at - 105 °C compared with the - 55 °C of Thomas (using an ethanol / liquid nitrogen bath with cryocool assistance), the reaction was complete after only 7 hours.

The required dihydropyrazine (186) and unwanted pyrazine (204) were easily distinguishable by ^{1}H NMR spectroscopy. The doublet signals from the isopropyl methyl groups of the valine side chain of the dihydropyrazine came upfield of the equivalent signals from the pyrazine (0.70 and 1.03 ppm *c.f.* 1.17 ppm). The signal from the hydrogen at the tertiary isopropyl centre of the dihydropyrazine was well separated from any other signals (at 2.25 ppm) and the triplet from the 6-H was downfield of any other signals (at 4.40 ppm). The pyrazine was most easily distinguished by the signal from the hydrogen at the tertiary isopropyl centre at 3.34 ppm.

Two mechanisms were proposed for the formation of 2,5-dimethoxy-3-propionyl-6-isopropylpyrazine (204) (Schemes 3.7 and 3.8). The *bis* -lactim ether anion could attack at the ester centre of methyl bromopropanoate (205), as shown in Scheme 3.7. Removal of the second proton from C-6 of the *bis* -lactim ether, would give the enolate (206). Loss of the proton from C-3 would lead to elimination of the bromide (207), thus forming the pyrazine (204). In the second mechanism (Scheme 3.8), bromine is eliminated first (208). However removal of the second proton from C-3 of the *bis* -lactim ether (209) gives an anion at C-3. Hence intramolecular attack of the ester can occur (210). Loss of the proton from C-6 of the *bis* -lactim ether would cause opening of the ring (211) to give the pyrazine (204).



Scheme 3.7 Pyrazine Formation by Attack at the Ester



Scheme 3.8 Pyrazine Formation by Attack at the Bromide

Excess base is required for both these mechanisms. However as the reaction produces base (OMe^-/Br^-) little control can be exercised here. Indeed, in reactions performed with less than 1 equivalent of *n* -butyl lithium, pyrazine formation was still high. However the *n* -butyl lithium in hexanes solution was titrated to determine its accurate molarity, thus ensuring only 1.1 equivalents of *n*- butyl lithium were added. Varying the quantity of alkylating agent (methyl (2R)-bromopropanoate) added from 5 equivalents through to 1.5 equivalents had no effect on the amount of pyrazine formed.

Both mechanisms indicate that enhancing the susceptibility of the C-2 centre of the alkylating agent to attack would be advantageous.

An attempt was made to make iodopropanoate, as iodide would be a superior leaving group to bromide, competing more favourably with the ester. However the nitrosation / iodination of D-alanine was not successful, due to the reaction of nitrous acid on hydroiodic acid.

Attack at C-2 of the alkylating agent would be favoured by increasing the steric bulk of its ester, due partially to simple steric reasons and also by reducing the rate of ester elimination. For example, isopropoxide is a poorer leaving group than methoxide, due to the larger inductive effect of the secondary alkyl group over the primary. Accordingly, the synthesis of isopropyl (2R)-bromopropanoate (212) was undertaken.

(2R)-Bromopropanoic acid was prepared *via* the nitrosation / bromination of D-alanine (see p. 107). The reaction occurs without racemization below 10 °C. Various reaction conditions were then employed in an attempt to prepare isopropyl (2R)-bromopropanoate (212) in good yield. However the reaction of isopropanol and thionyl chloride with (2R)-bromopropanoic acid always gave side products, which could not be removed. Decolourization of the brown oil produced and removal of excess isopropanol was achieved by column chromatography on silica gel, eluting with 10% diethyl ether in petroleum ether. However the contaminants were not separable.

Activation of the acid functionality by mixed anhydride methods, using N-methyl morpholine and isobutyl or ethyl chloroformate gave none of the required product.

No attempt was made to optimize the reaction further, but the isopropyl 2-bromopropanoate prepared was used to investigate how effective the isopropyl group was in reducing formation of the pyrazine. The ratio of alkylated *bis* -lactim ether to pyrazine formation was not improved in alkylation reactions with isopropyl 2-bromopropanoate over that for methyl 2-bromopropanoate.

There was also some doubt as to the chiral integrity of the isopropyl 2-bromopropanoate. Unfortunately the material was never pure enough to measure its optical activity. However synthesis of methyl 2-bromopropanoate by the thionyl chloride route proceeded cleanly and gave material with an optical rotation consistent with that from the diazomethane route ($[\alpha]_D$ + 55.6 ° (neat)). However the 3-methylaspartic acid produced from (3S,6R)-2,5-dimethoxy-3-((2'R)-isopropoxycarbonylethyl)-6-isopropyl-3,6-dihydropyrazine (213) was racemic. Alkylation of the *bis* -lactim ether anion with isopropyl 2-bromopropanoate gave two isolable products. The ^{1}H NMR spectra of both products displayed the correct splitting for the product molecule but slightly different chemical shifts. The triplet assigned to 3-H was most different, occurring at 4.58 ppm in one compound and 4.34 ppm in the other. Other differences of 0.08 ppm were seen in the septet signals from the hydrogen on the tertiary carbon of the isopropyl ester, and of 0.02 ppm in the methyl groups of the valine side chain. Other chemical shift differences were difficult to identify due to the complexity of the spectra. The mixture of compounds may be due to a mixture of stereochemistry at C-2'.

In order to ensure that the alkylating agent was added to the *bis* -lactim ether anion whilst still at the low temperature of the ethanol bath, it was contained in a side arm to the flask containing the anion. Turning the side arm allowed a quick addition of the alkylating agent, without warming up. Transfer by

syringe or catheter would allow the solution to warm up during addition. These conditions gave better ratios of product to pyrazine, although the yields were still only modest.

3.4.3 Purification of the Alkylated *Bis* -Lactim Ether

It was reported[295] that the alkylated *bis* -lactim ether (186) could be separated from the pyrazine (204) and unreacted *bis* -lactim ether (182) by column chromatography on silica gel, eluting with 15 % ethyl acetate in petroleum ether. However this solvent system was unsatisfactory. A system of 25 % diethyl ether in petroleum ether performed better. However the silica gel required pretreatment with triethylamine to prevent decomposition of the alkylated *bis* -lactim ether. Decomposition did not occur on neutral alumina, eluting with 10 % diethyl ether in petroleum ether. However although both systems separated the unreacted *bis* -lactim ether from the other two components, neither separated the alkylated *bis* -lactim ether and pyrazine well. As pure alkylated *bis* -lactim ether could not be obtained from one elution, only a short column of silica gel was used to remove baseline material.

3.4.4 Isolation of the Free Amino Acids

Schollkopf used acid hydrolysis to cleave the alkylated *bis* -lactim ether[294], giving the two amino acid esters, which Thomas reported were separable by column chromatography on silica gel, eluting with 50 % acetone in ethyl acetate[295]. However, this proved unsuccessful and no alternative solvent systems were found.

Base hydrolysis of the external ester left the *bis* -lactim ether ring intact. The pyrazine was removed by extraction with diethyl ether. Acidification of the aqueous layer to pH 5 allowed the (3S,6R)-2,5 dimethoxy-3-((2'R)-carbonylethyl)-6-isopropyl 3,6-dihydropyrazine to be extracted back into the

diethyl ether layer. However the product was not very clean and only achieved in 20 % yield from the *bis* -lactim ether, therefore this method was not pursued.

Acid hydrolysis of the alkylated *bis* -lactim ether / pyrazine mixture cleaved the alkylated *bis* -lactim ether, to give the D-valine methyl ester and (2S,3R)-3-methylaspartic acid dimethyl ester (187), whilst leaving the aromatic pyrazine intact. Extraction with toluene removed the pyrazine. The amino acid esters were then hydrolysed in 1 M NaOH, to give the free amino acids, which were separated by preparative tlc on cellulose plates, using 80 % methanol in ammonia as solvent. This solvent system was chosen over the standard 26 : 6 : 5 isopropanol : ammonia: water system as it gave a better separation of the two amino acids, taking them further off the baseline, in a shorter time.

The 3-methylaspartic acid obtained was a mixture of isomers. The degree of L -*threo* -contamination varied with preparations from 5 to 20 %. All samples were of high salt concentration and the (2S,3R)-3-methylaspartic acid would not crystallize out of solution.

It was not clear whether the contaminating isomer, (2S,3S)-3-methylaspartic acid, was formed due to poor stereoselective control of the alkylation reaction or during base hydrolysis of the esters. Therefore an acid hydrolysis (6 M HCl, 2 hour reflux) of the amino acid esters was also performed. However this also gave a mixture of the two isomers. This mixture did however contain less salt than the base hydrolysis product and therefore ran better on preparative tlc plates.

Nitrogen protection of the amino acid esters, obtained from cleavage of the *bis* -lactim ether ring, as carbobenzoxy derivatives, gave N-Cbz-3-methylaspartic acid dimethyl ester and N-Cbz valine methyl ester which could be separated by silica gel column chromatography, eluting with 50 % diethyl ether in petroleum ether. Acid hydrolysis of the N-Cbz methylaspartic acid dimethyl ester yielded (2S,3R)-3-methylaspartic acid as the

hydrochloride salt.

Some separation was also achieved between the L-*erythro* - and L-*threo* - isomers of N-Cbz-3-methylaspartic acid dimethyl ester, with the L-*erythro* - isomer eluting just before the L-*threo* - isomer.

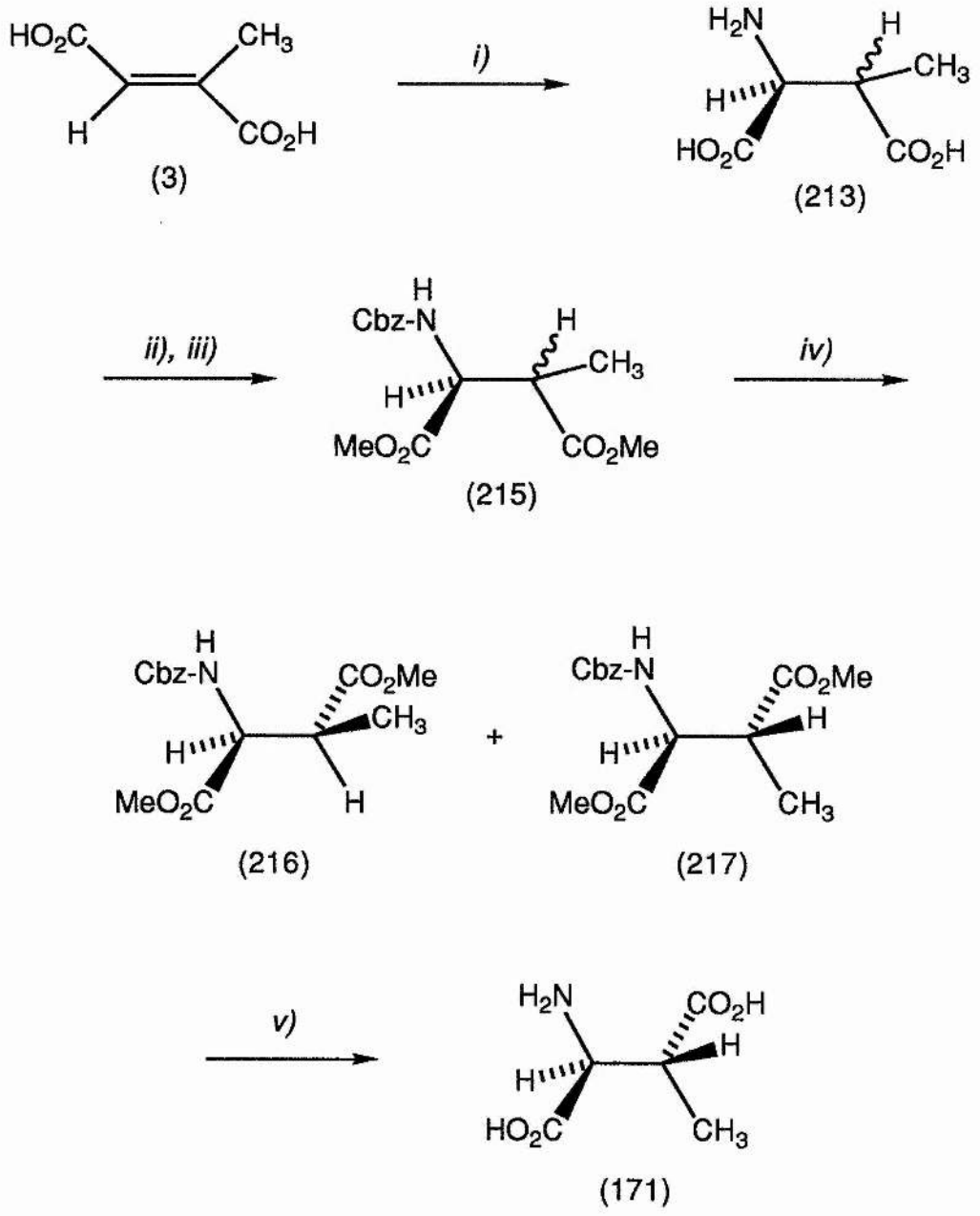
3.5 A New Route to (2S,3R)-3-Methylaspartic Acid and (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid

It has been shown that the two L-isomers of 3-methylaspartic acid were separable as their N-Cbz dimethyl esters. It was also known that prolonged incubation of diammonium mesaconate with 3-methylaspartase, magnesium and potassium ions and ammonia gave a mixture of both isomers. Thus (2S,3R)-3-methylaspartic acid could be produced by incubation with the enzyme and then separated from the (2S,3S)-isomer as a N-Cbz dimethyl ester. Furthermore incubation of diammonium mesaconate, with the enzyme, cofactors and ammonia, in deuterium oxide, would give [3-^{2}H]-3-methylaspartic acid.

Prolonged incubation (several weeks) of mesaconic acid (3) with 3-methylaspartase gave 80 - 85 % conversion to the 3-methylaspartic acid diastereomeric mixture (214) (Scheme 3.9). A ratio of up to 1 to 1 was achieved between the two diastereomers, distinguishable by their chemical shifts in ^{1}H NMR spectra. The L-*erythro*- isomer displayed signals at 1.31 and 3.81 ppm from the methyl and 2-H groups respectively, whereas the L-*threo*- isomer was characterized by signals at 1.20 and 4.06 ppm. The 3-H multiplet signals from the each isomer were coincident. The mixture of isomers of 3-methylaspartic acid was isolated from the incubation and the N-Cbz derivatives (215) formed using N-(benzyloxycarbonyloxy)-succinimide[296]. This reagent performs well in aqueous salt containing solutions. Conversion of the mixture of N-Cbz-3-methylaspartic acid diastereomers, to the diester (216) occurred cleanly with diazomethane and the two diastereomers (217 and 218) separated by column chromatography

on silica gel, eluting with 50 % diethyl ether in petroleum ether. Fractions were analysed by ^{1}H NMR spectroscopy as no separation was observable between the two isomers by tlc. Signals from the 3-Hs were separated by 0.3 ppm and the 2-Hs by 0.12 ppm. Fractions containing a mixture of isomers were recycled. Acid hydrolysis gave the hydrochloride salt, from which the free amino acid (171) was obtained, by addition of one equivalent of ammonia.

(2S,3R)-[3-^{2}H]-3-methylaspartic acid (191) was obtained by a similar route. Prolonged incubation of diammonium mesaconate with 3-methylaspartase, magnesium and potassium ions and ammonia, in deuterium oxide, gave a mixture of the L-diastereomers of [3-^{2}H]-3-methylaspartic acid (219), with greater than 95 % incorporation of deuterium. The mixture of diastereomers were protected as their N-Cbz dimethyl esters (216 b) and separated by column chromatography, as with the non-deuteriated analogue. Acid hydrolysis gave (2S,3R)-[3-^{2}H]-3-methylaspartic acid hydrochloride, from which the free amino acid was obtained.



i) 3-methylaspartase, NH_3, $MgCl_2$, KCl, H_2O
ii) Z-OSu, K_2CO_3 (aq)
iii) CH_2N_2, Et_2O
iv) chromatography
v) AcOH/HCl

Scheme 3.9 Synthesis of (2S,3R)-3-Methylaspartic Acid

3.6 ^{1}H-NMR Experiments Using (2S,3R)-3-Methylaspartic Acid and (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid

The deamination of (2S,3R)-L-*erythro*-3-methylaspartic acid was followed by ^{1}H NMR spectroscopy. The experiment was designed to investigate whether direct deamination, of the substrate, to mesaconic acid occurred, or whether the substrate epimerized first, to the (2S,3S)-L-*threo*- isomer and then deaminated to mesaconic acid.

It was required that the reamination of mesaconic acid to L-*threo* -3-methylaspartic acid was slow, in order to observe any epimerization of the L-*erythro* -substrate to the L-*threo*- isomer. Thus, ammonium-containing buffers were avoided and sodium hydroxide used to adjust the pD of the buffer. Imidazole was used as a buffer, as its ^{1}H NMR spectrum gave proton signals well downfield of any of the signals of interest (7.07 and 7.72 ppm). As this buffer had not previously been used in 3-methylaspartase incubations, it was confirmed that imidazole had no effect on the reaction by performing an incubation in Tris buffer for comparison. A substrate concentration of 5 mg / ml (34 mM) and cation concentrations of 1 mM potassium and 20 mM magnesium were used. Hence, for the deuterio-substrate, incubations were run above V_{max} / 2 and for the protio- substrate at approximately V_{max} / 2 (see p. 147 and p. 151). The incubations were performed in deuterium oxide. Spectra were recorded at T = 0 and at one hourly time intervals thereafter, for 15 hours. Further spectra were obtained at longer time intervals (Figures 3.1 and 3.2).

Some incubations of protio-substrate seemed to be contaminated by a small amount of L-*threo* -3-methylaspartic acid (*ca.* 2 %) at T = 0, typified by signals at 1.05 and 3.95 ppm, which remained in the incubation and was not deaminated immediately. This appeared to be in direct contradiction with UV spectroscopy results (see p. 142 - 143). However a ^{1}H NMR experiment, in which the signal at 3.95 ppm, coincident with the 2-H of L-*threo* -3-methylaspartic acid, was irradiated, showed no change in the signal at 1.05 ppm, coincident with the methyl group of L-*threo* -3-methylaspartic acid.

This confirmed the signals were not linked and, therefore, suggested they were not due to L-*threo*-3-methylaspartic acid contamination.

The ^{1}H NMR time course experiments showed that L-*erythro*-3-methylaspartic acid was deaminated directly to mesaconic acid (see Figure 3.1). Hence 3-methylaspartase would seem to have a true L-*erythro*-3-methylaspartase activity. The L-*threo* - isomer was detected only after several hours, presumably on conversion of enough material to ammonia and mesaconic acid to allow the back reaction to take place. The L-*threo*-3-methylaspartic acid formed was completely deuteriated at C-3, as expected from amination of mesaconic acid in deuterium oxide.

It can be concluded that, if epimerization of the L-*erythro*-isomer to the L-*threo* - isomer had occurred, the intermediate L-*threo* - isomer produced was not released before it was deaminated.

It should be noted that, there was no detectable exchange of solvent deuterium into the L-*erythro* - substrate. As proton signals on carbons adjacent to those bonded to deuterium are shifted slightly upfield from their unlabelled positions, the methyl signal from L-*erythro*-[3-^{2}H]-3-methylaspartic acid would be expected to be coincident with the upfield portion of the methyl doublet, thus reducing or even inverting the signpost effect on this signal. Even at long incubation times (17 hrs), the signal displayed a strong signpost effect, indicating that less than 2 % deuterium had been incorporated. Epimerization would be expected to be faster than the loss of ammonia. Indeed C-3 hydrogen exchange in the reaction of the L-*threo*-substrate with 3-methylaspartase occurs at a rate 5.1 times faster than that for the overall reaction, at pH 7.6[245]. Therefore, some incorporation of deuterium into the substrate would be expected if an epimerization / deamination reaction was occurring without release of the L-*threo* - intermediate. Thus the evidence suggested direct *syn*- elimination was taking place.

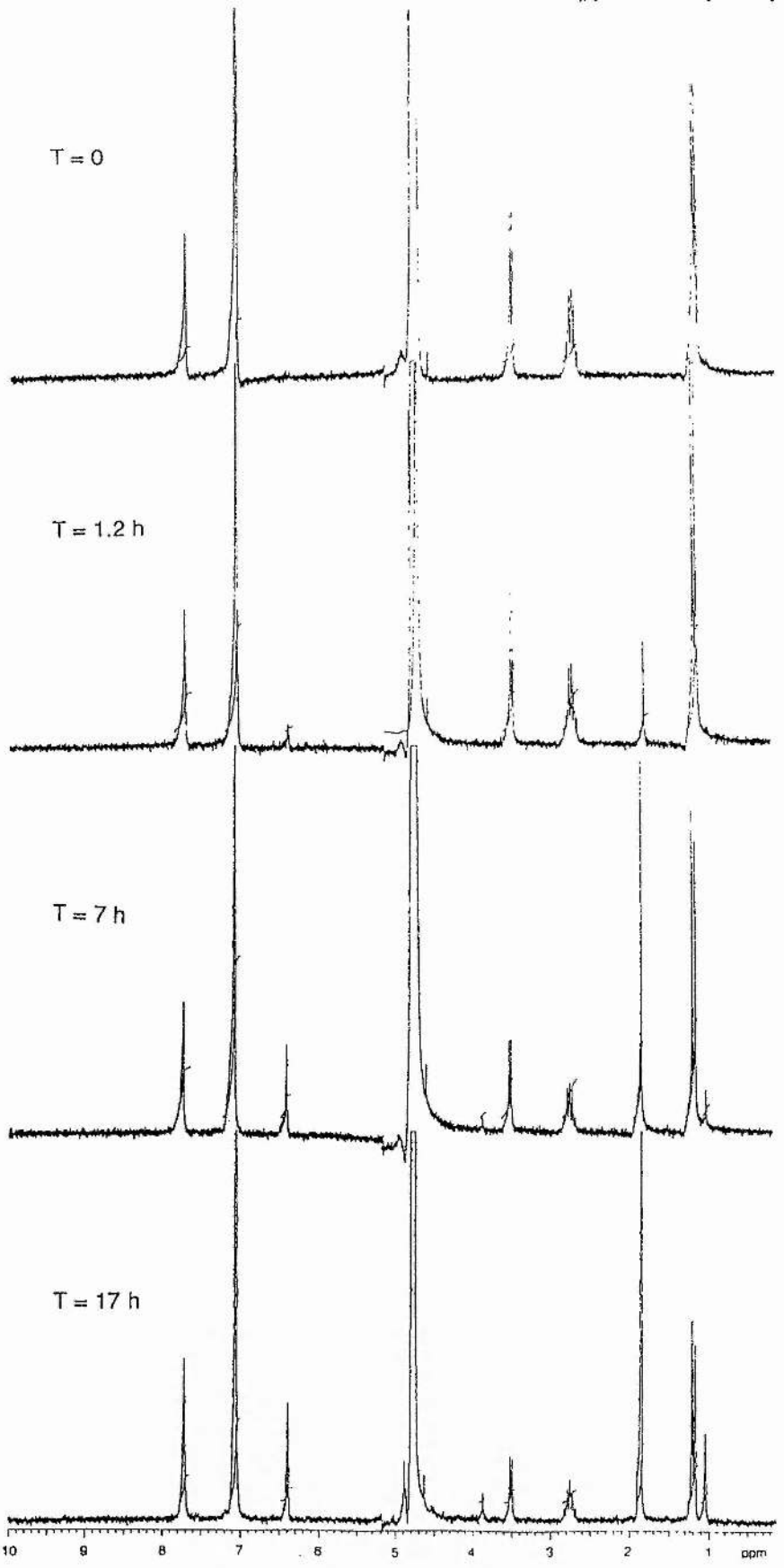


Figure 3.1 ^{1}H NMR Spectra Showing Time Course of the Deamination of (2S,3R)-3-Methylaspartic Acid

Similar incubations of the deuteriated substrate with 3-methylaspartase were also followed by ^{1}H NMR spectroscopy. They showed a similar pattern of product formation, but at a much slower rate (Figure 3.2). It therefore seemed likely that a deuterium isotope effect was operating.

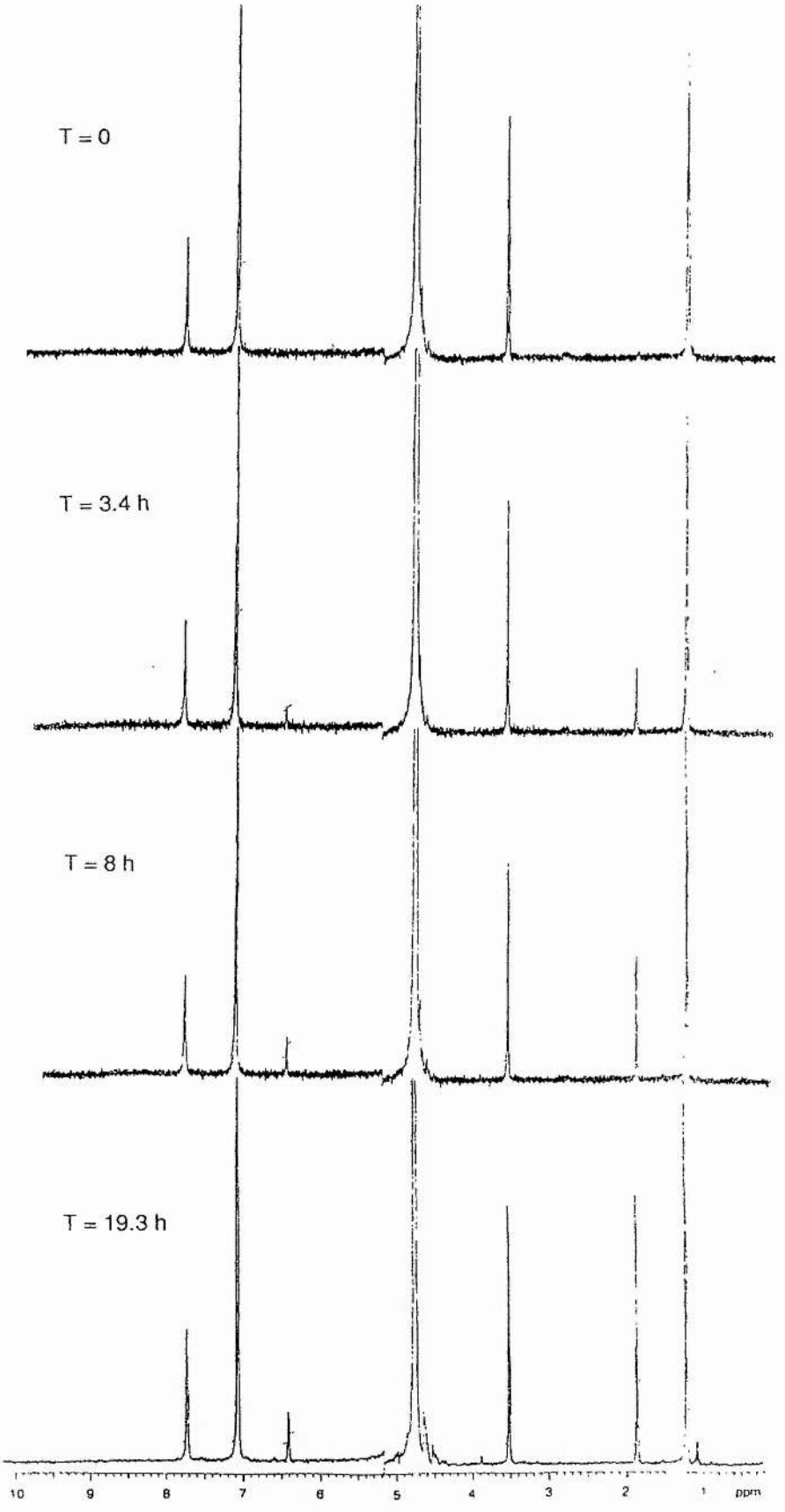


Figure 3.2 ^{1}H NMR Spectra Showing Time Course of the Deamination of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid

An incubation of equal quantities of deuteriated and non-deuteriated substrate with 3-methylaspartase was followed by ^1H NMR spectroscopy. This competition experiment showed 2.3 times more protiated substrate was deaminated in a given time than deuteriated substrate. This was further evidence of a substantial deuterium isotope effect.

3.7 Determination of Kinetic Parameters

The values of K_m and V_{max} were measured for the deamination of (2S,3R)-3-methylaspartic acid with 3-methylaspartase. A UV spectroscopic method for measuring the rate of production of mesaconic acid was available[9]. Mesaconic acid has a UV maximum, due to conjugation between its acids and the double bond, which occurs at less than 190 nm. However, the absorbance at 240 nm was chosen to give optical density (OD) changes of up to 2 OD units in a few minutes for the substrate concentrations used. The rate of production of mesaconic acid (deamination of (2S,3R)-3-methylaspartic acid) was determined for a range of substrate concentrations. The conditions chosen were those used to determine the kinetic parameters of 3-methylaspartase with the natural substrate, (2S,3S)-3-methylaspartic acid, by Botting and Gani[244,245]. (2S,3R)-3-Methylaspartic acid was dissolved in Tris buffer (0.5 M, pH 9.0) containing magnesium chloride (20 mM) and potassium chloride (1 mM) to give the required substrate concentration. Rates were measured at 30 ± 0.1 °C.

An initial burst of activity occurred and then a slower but steady rate was observed. One explanation for this behaviour was the formation of an enzyme-bound intermediate, which would react further, only slowly[297]. Thus the first equivalent of substrate would react quickly with the enzyme to produce stoichiometric amounts of the enzyme-bound intermediate and product (mesaconic acid). Subsequent turnover would be slower, as it would depend on the slow breakdown of the enzyme-bound intermediate to generate free enzyme, before further catalysis produced more product. However the size of the initial burst was shown to be independent of enzyme

concentration. Thus burst phase kinetics were not occurring.

The size of burst was, in fact, shown to be dependent on substrate concentration. Thus product inhibition could be responsible for this burst phenomenon. This seemed unlikely as a linear rate was achieved, rather than a sustained deceleration of rate as product concentration increased. Ammonia was shown not to deactivate the enzyme. Addition of 0.5 nmol ammonia gave a rate increase by a factor of 4.2 on the second steady rate. Confirmation that the cloned enzyme from *E. coli* had no contaminating enzymes which turned over mesaconic acid, thus giving an apparently suppressed rate of mesaconic acid formation, was obtained from incubation of a solution of mesaconic acid with enzyme. The absorbance at 240 nm remained constant over several hours.

The initial burst was due to turnover of the small amount (1 -2 %) of L-*threo* -3-methylaspartic acid present. This was confirmed by doping samples with a further 1 % of the L-*threo* - isomer prior to addition of the enzyme. The size of the burst doubled. Also addition of L-*threo* -3-methylaspartic acid to an incubation which had already reached the second steady rate caused a second burst. The size of the burst was consistent with complete conversion of the L-*threo* - isomer to mesaconic acid. The rate of the initial burst was also consistent with the rate expected from the L-*threo* -3-methylaspartic acid concentration calculated to be present.

Using 3 ml cuvettes (path length of 1), it was difficult to measure the second steady rate, *i.e.* the rate of reaction of L-*erythro* -3-methylaspartic acid. At high substrate concentrations the second steady rate was not achieved within the absorbance range of the spectrophotometer, as absorbance due to the production of mesaconic acid from the contaminating L-*threo* -isomer was too high (1.93 OD units for 50 mM solution).

This problem was overcome by using 0.3 ml cuvettes with a path length of 0.1. Change in absorbance is related to the path length by the following equation:

$$\Delta OD = C\varepsilon l$$

where ΔOD is change in absorbance (optical density), C is concentration, ε is the extinction coefficient and l, the path length. Therefore at a shorter path length, larger concentrations are measurable for the same OD change. By measuring the absorbance every 10 minutes, the distortion due to the initial high rate was minimised, as the L-*threo* -isomer was essentially consumed within the first 10 minutes. However, due to mixing problems 0.3 ml cuvettes did not give very consistent results.

In order to use 1 ml cuvettes (path length of 1), the rates at higher concentrations were measured at a wavelength of 270 nm, where the extinction coefficient was much lower (ε_{270} = 482.5, *c.f.* ε_{240} = 3850). For the lower substrate concentrations absorbtion measurements at 240 nm using 1ml cuvettes (with a path length of 1) were satisfactory.

Rates were obtained for the deamination of L-*erythro* -3-methylaspartic acid over a range of substrate concentrations at both 1 and 50 mM potassium ion concentrations. The data are presented graphically.

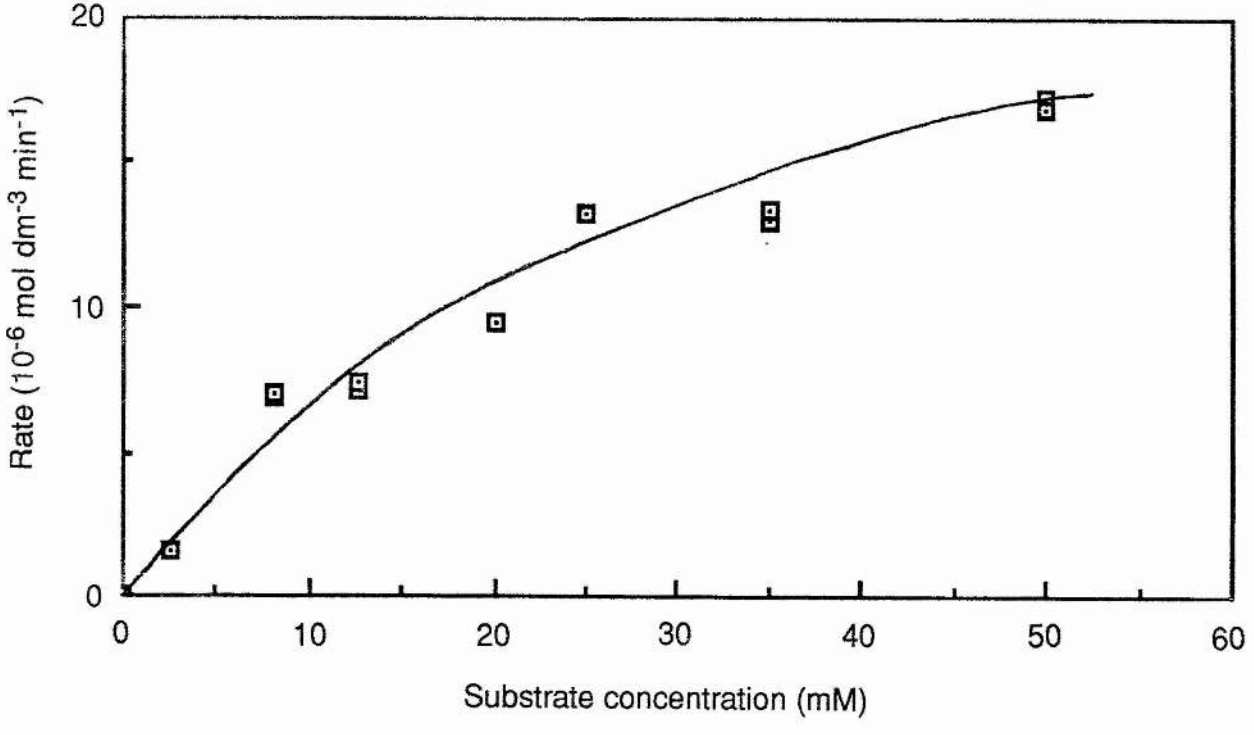


Figure 3.3 Graph Showing the Rate of Deamination for Various Concentrations of (2S,3R)-3-Methylaspartic Acid at 1 mM Potassium Ion Concentration

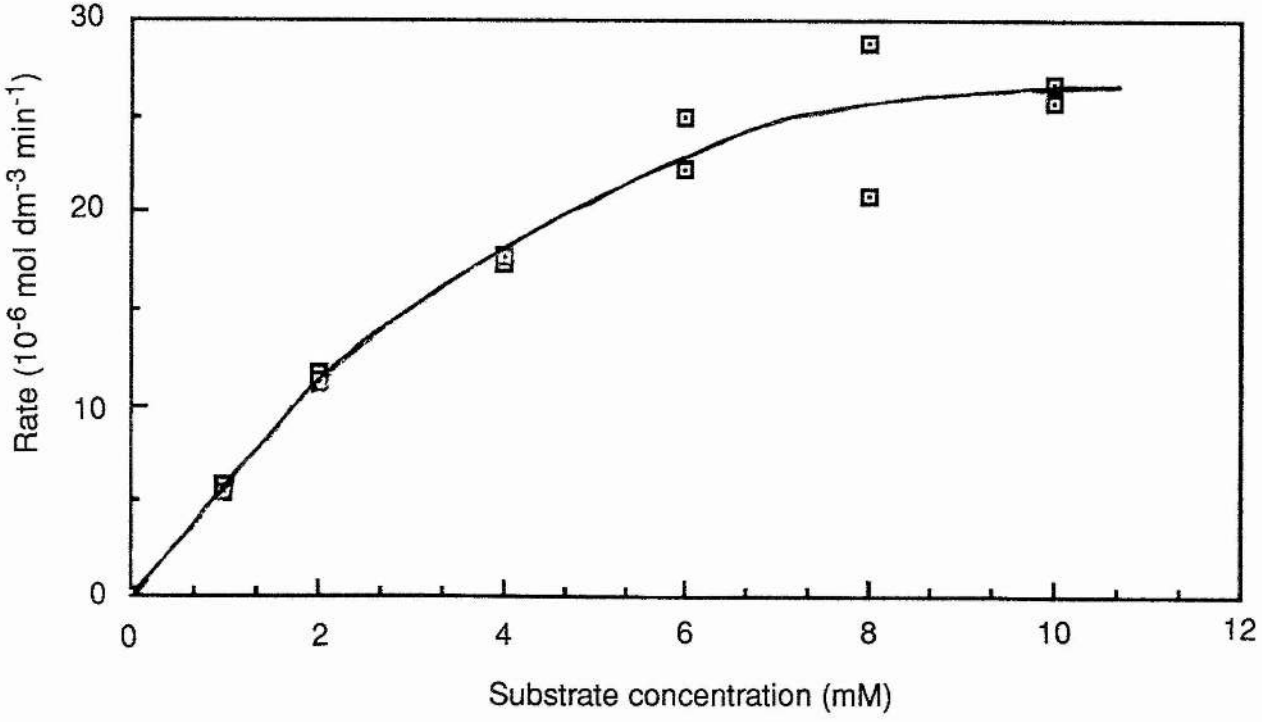


Figure 3.4 Graph Showing the Rate of Deamination for Various Concentrations of (2S,3R)-3-Methylaspartic Acid at 50 mM Potassium Ion Concentration

Thus, values of V_{max} and K_m were determined for the deamination of (2S,3R)-3-methylaspartic acid by 3-methylaspartase, at pH 9.0 in the presence of 20 mM magnesium chloride and at both 1 and 50 mM potassium chloride concentrations. These values are tabulated below (Table 3.1) with those obtained for the L-*threo* - isomer, by Botting and Gani[244], for comparison (Table 3.2).

Table 3.1 Kinetic Parameters for (2S,3R)-3-Methylaspartic Acid

[KCl] (mM)	K_m (mM)	V_{max} (10^{-6} mol dm^{-3} min^{-1}unit $enzyme^{-1}$)
1	40 ± 0.8	17.2 ± 0.3
50	5.2 ± 1.1	41.1 ± 4.0

Table 3.2 Kinetic Parameters for (2S,3S)-3-Methylaspartic Acid

[KCl] (mM)	K_m (mM)	V_{max} (10^{-6} mol dm^{-3} min^{-1}unit $enzyme^{-1}$)
1	2.37 ± 0.2	654 ± 65
50	0.67 ± 0.07	2089 ± 209

Thus, at 1 mM potassium ion concentration V_{max} for the L-*erythro* - substrate was 38 times less than for the natural substrate and at 50 mM potassium ion concentration, 51 times lower. Barker's values of 2.5 µmoles min^{-1} mg $protein^{-1}$ for V_{max} and 0.65 mM for the K_m of (2S,3R)-3-methylaspartic acid[9] were obtained at pH 9.76 in ethanolamine chloride buffer with 10 mM potassium chloride and 1 mM magnesium chloride and are, therefore, not

directly comparable with the values obtained from this work. However Barker's data showed a rate difference of 106 between deamination of the L-*erythro* - substrate and the L-*threo* - substrate, a much greater variation than that obtained here. He gave the same K_m value for both the L-*erythro* - and L-*threo* - substrates, whereas the K_m values obtained from this work were 17 times higher at 1 mM potassium ion concentration and 7.8 times higher at 50 mM potassium ion concentration, for the L-*erythro* - substrate . Barker made no mention of an initial burst due to contaminating L-*threo* - material but claimed no more than 95 % purity for his L-*erythro* -3-methylaspartic acid samples. Without repeating Barker's experimental conditions exactly, it is difficult to rationalize his results in the light of the findings of this work. However, the purpose of this work was to allow comparisons to be made with the kinetics data obtained, by Botting and Gani[244], for L-*threo* - isomer.

The kinetic parameters were also determined for (2S,3R)-[3-^{2}H]-3-methylaspartic acid at pH 9.0, 20 mM magnesium chloride and both 1 and 50 mM potassium chloride concentrations. At 50 mM potassium chloride concentration, an initial burst of activity, from the contaminating L-*threo*-isomer, was not discernible at low substrate concentrations. The rate of mesaconic acid formation due to deamination of the L-*erythro* - substrate tended to fall off with time, unlike with the undeuteriated substrate where the rate was linear. Thus, at higher substrate concentrations it was difficult to ascertain the point at which all the L-*threo* - substrate had been consumed. However, by taking tangents from the same part of the curve each time consistent data points were achieved.

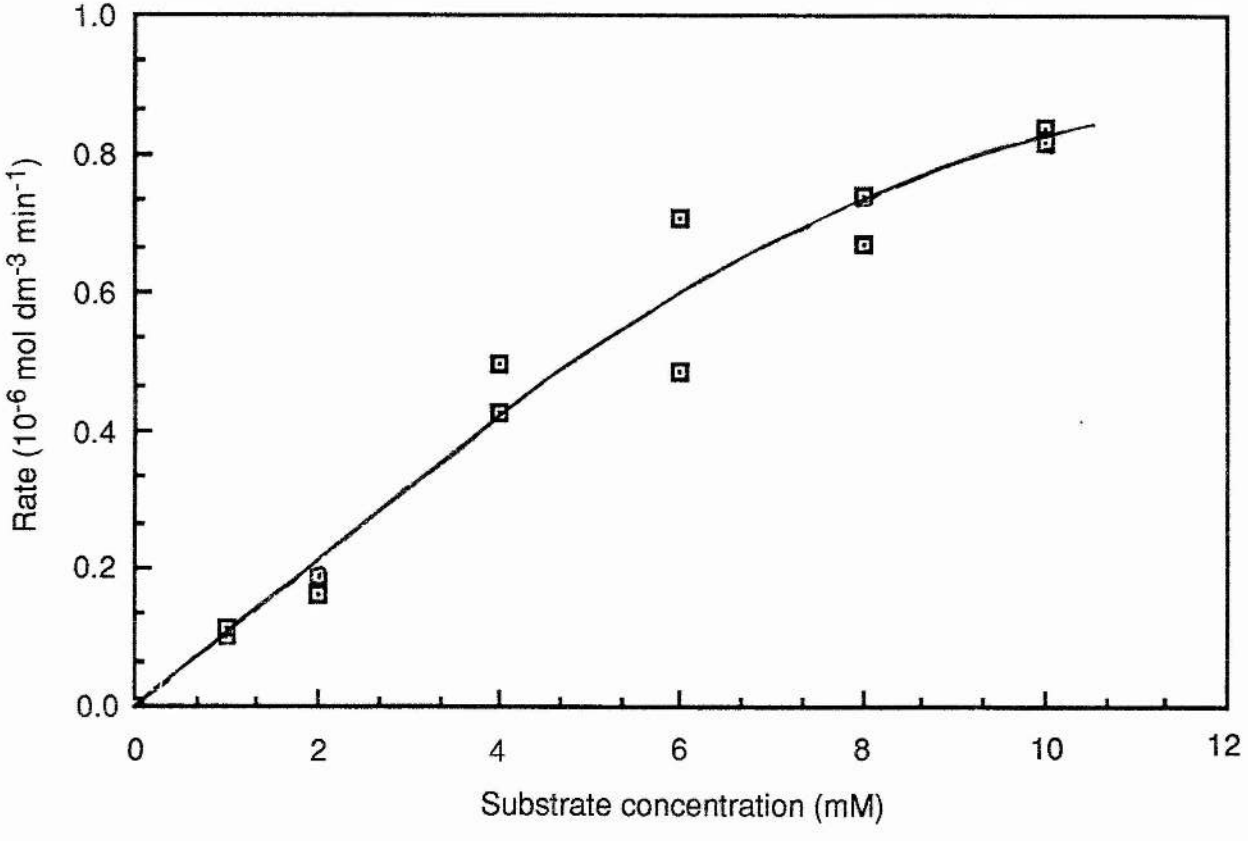


Figure 3.5 Graph Showing the Rate of Deamination for Various Concentrations of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid at 1 mM Potassium Ion Concentration

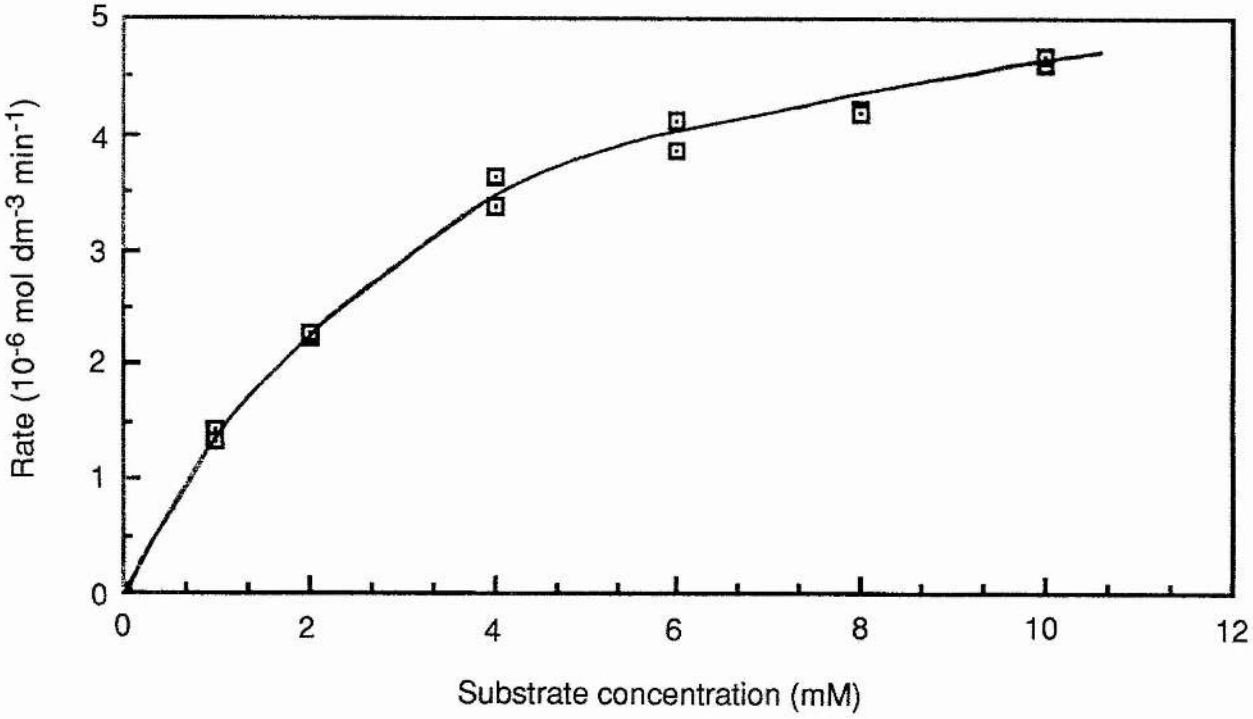


Figure 3.6 Graph Showing the Rate of Deamination for Various Concentrations of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid at 50 mM Potassium Ion Concentration

Thus the V_{max} and K_m values for the deuteriated substrate were calculated for the reaction carried out at pH 9.0 with 20 mM magnesium chloride and both 1 and 50 mM potassium chloride concentrations (Table 3.3). The values obtained, by Botting and Gani[244], for the L-*threo*- isomer are also presented (Table 3.4).

Table 3.3 Kinetic Parameters for (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid

[KCl] (mM)	K_m (mM)	V_{max} (10^{-6} mol dm^{-3} min^{-1}unit $enzyme^{-1}$)
1	18.8 ± 8.4	2.4 ± 0.78
50	3.14 ± 0.29	6.05 ± 0.23

Table 3.4 Kinetic Parameters for (2S,3S)-[3-^{2}H]-3-Methylaspartic Acid

[KCl] (mM)	K_m (mM)	V_{max} (10^{-6} mol dm^{-3} min^{-1}unit $enzyme^{-1}$)
1	2.35 ± 0.25	385 ± 39
50	0.67 ± 0.07	2089 ± 209

From these data the isotope effects ^DV (V_H / V_D) and ^{D}(V/K) ((V_H / V_D)/(K_H / K_D)) were calculated at each potassium ion concentration (Table 3.5). Again the values for the L-*threo* -isomer under the same conditions are shown for comparison (Table 3.6).

Table 3.5 Primary Deuterium Isotope Effects for (2S,3R)-3-Methylaspartic Acid

[KCl](mM)	^{D}V	$^{D}(V/K)$
1	7.15 ± 2.74	3.39 ± 1.6
50	6.79 ± 0.92	4.10 ± 1.3

Table 3.6 Primary Deuterium Isotope Effects for (2S,3S)-3-Methylaspartic Acid

[KCl](mM)	^{D}V	$^{D}(V/K)$
1	1.7	1.68
50	1.0	1.00

3.8 The Implications for the Mechanism of Elimination of Ammonia from (2S,3R)-3-Methylaspartic Acid

A very large isotope effect was observed on V_{max} at 1 mM potassium ion concentration which was diminished only very slightly at 50 mM potassium ion concentration. Hence C-H bond cleavage was almost completely rate limiting. The effect was not masked by any binding or debinding steps. Therefore, the V_{max} values obtained, apply directly to the rate of C-H bond cleavage. These results showed that the chemical steps involved in the deamination of the L-*erythro* - substrate were of much higher energy than for the L-*threo* - substrate.

Two mechanisms are consistent with these data; a concerted mechanism or a carbanion mechanism in which C-H bond cleavage is rate limiting. These

mechanisms are equivalent to the chemical E2 and $(E1_{cb})_l$ mechanisms.

In the E2 mechanism, both bonds cleave in the same step. The reaction is second order; first order with respect to substrate and first order with respect to base. In the $(E1_{cb})_l$ mechanism, the first step (the proton leaving) is the slow step of the reaction, and the formation of the product is faster than the return of the carbanion to the starting material. Hence the first step is essentially irreversible. This mechanism has an identical rate equation to the E2 mechanism: Rate = k[substrate][B]. Hence the two mechanisms are difficult to distinguish.

An E2 mechanism normally requires an *anti-* periplanar conformation, where the two groups involved in the reaction are *trans-* to one another with a dihedral angle of 180 °. This is the lowest energy conformation. However, a *syn-* elimination from a *syn-* periplanar conformation is also possible. Here the dihedral angle between the two groups is 0 °.

In purely chemical reactions *syn-* elimination can be favoured for steric reasons. Two examples are given below (Figure 3.7 and 3.8). In the first example (Figure 3.7) four conformations are shown[298]. In conformation (220) the asterixed proton is shielded on both sides by groups R and R', thus making it difficult for the base to approach. Conformation (221) is shielded only on one side. Therefore, an *anti-* elimination in this system would give mainly *cis-* product. Of the *syn-* periplanar conformations, conformation (222) is less eclipsed than (223) and hence the *trans-* product is favoured.

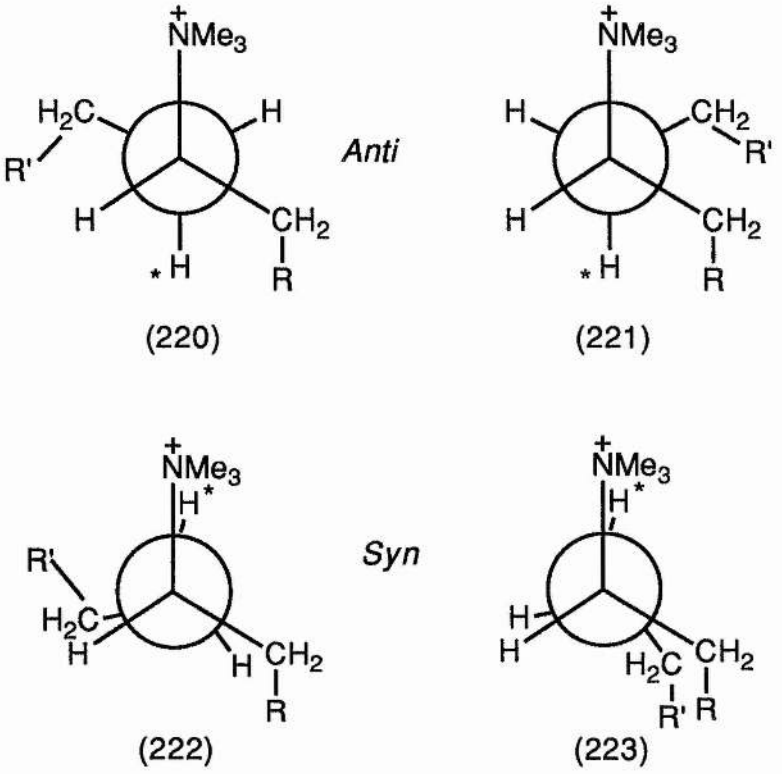


Figure 3.7 Conformations for Elimination of the Trimethylammonium Ion (I)

In the second example (Figure 3.8)[299] the *anti-* periplanar conformation (224) is more strained than the *syn-* conformation (225) due to the gauche interactions between the bulky groups R^1, R^2, and NMe_3^+.

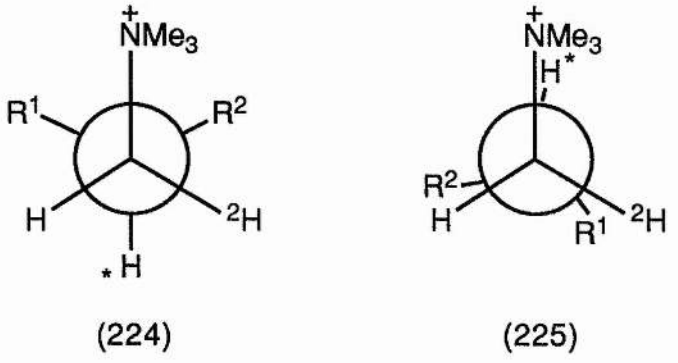
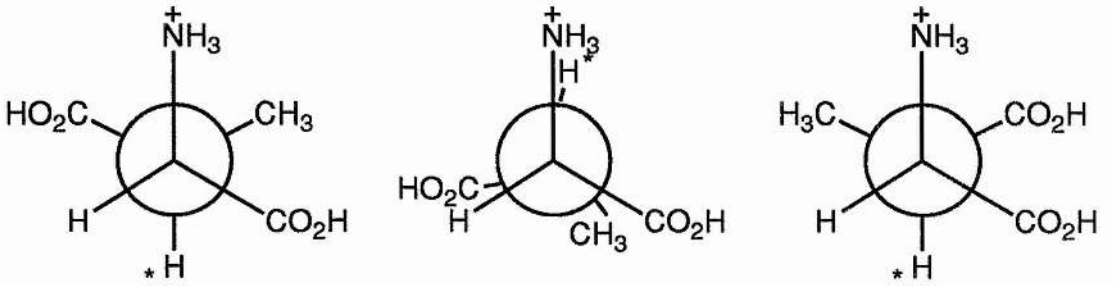


Figure 3.8 Conformations for Elimination of the Trimethylammonium Ion (II)

In the enzymic case, the natural L-*threo* - substrate adopts an *anti*-conformation (226) (Figure 3.9). As L-*erythro* -3-methylaspartic acid also gives the *trans*- elimination product, mesaconic acid, it must adopt a *syn*-conformation (227), if concerted elimination is to occur. The *cis*- product, itaconic acid, would be formed from an *anti*- elimination (228).



L-*threo* - (226)

L-*erythro* - (227)

L-*erythro* - (228)

Figure 3.9 Conformations for the 3-Methylaspartic Acid Isomers

3.9 Measurement of the ^{15}N Isotope Effect

In order to ascertain whether C-N bond cleavage was also rate limiting, the ^{15}N isotope effect on (V/K) was measured, using natural abundance L-*erythro* -3-methylaspartic acid. The ratios of ^{15}N to ^{14}N were measured at the start of the reaction, and after 20 % conversion of L-*erythro* -3-methylaspartic acid to mesaconic acid and ammonia[300]. The methods used ae discussed below. As C-^{14}N bond cleavage is faster than C-^{15}N, the ammonia produced initially contains a higher proportion of the lighter isotope. At 20 % conversion a sufficient proportion of each isotope has been turned over to allow the ratio to be determined satisfactorily.

The ratio of ^{15}N to ^{14}N in the starting material was obtained by degradation of L-*erythro* -3-methylaspartic acid, by Kjeldahl digestion[301], to release

ammonia. This digestion procedure involves boiling the amino acid in concentrated sulphuric acid and mercury sulphate, with potassium sulphate to raise the boiling temperature. The ammonia produced stayed in solution as the ammonium ion, in the acidic mixture. The ammonia was then distilled out into dilute sulphuric acid and oxidized to dinitrogen with hypobromite, by Professor Alan Jackson at the University of Southampton. The ratio of ^{15}N to ^{14}N was measured by mass spectrometry, using a dual-inlet isotope ratio mass spectrometer. This ratio represented the initial ratio of isotopes.

To determine the isotope effect, L-*erythro* -3-methylaspartic acid, from the same batch, was incubated in Tris buffer (0.5 M, pH 9.0) containing 1 mM potassium chloride and 20 mM magnesium chloride with sufficient enzyme to convert 20 % of the starting material to mesaconic acid and ammonia in approximately 4 hours. The reaction was followed by measuring the OD, at 240 nm, of 20 µl aliquots removed from the reaction at various time intervals and added to 3 ml of buffer. Once approximately 20 % of the material had been converted, the reaction was quenched by the addition of concentrated hydrochloric acid. The experiment was repeated a number of times. The ammonia was again extracted from the samples by Professor Alan Jackson and after oxidation to dinitrogen, the ^{15}N to ^{14}N ratio measured by mass spectrometry. The isotope effect, $^{15}(V/K)$, was calculated using the following equation:

$$^{15}(V/K) = \log (1 - f)/\log [1 - (fR/R_0)]$$

where f is the fraction of the reaction and R and R_0 are the isotope ratios in the substrate and the ammonia product respectively. This gave an isotope effect of 1.0028 ± 0.0040 for the reaction of L-*erythro* -3-methylaspartic acid with 3-methylaspartase. This value for $^{15}(V/K)$ is small and indicates that the ^{15}N isotope effect is very close to unity.

3.10 Double Isotope Fractionation Experiment

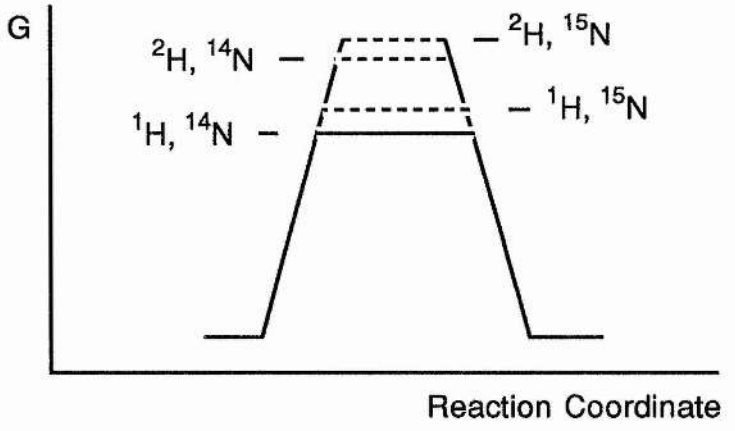
A second isotope fractionation experiment, which measured the $^{15}(V/K)$ isotope effect with [3-^{2}H]-(2S,3R)-3-methylaspartic acid as substrate, was also performed at the same time to investigate the effect of the presence of deuterium at C-3 on the ^{15}N isotope effect. The two experiments together constituted a double isotope fractionation experiment.

Such experiments are usually used to distinguish between a concerted and non-concerted mechanism, for example, a balanced stepwise carbanion mechanism, where C-N bond cleavage is rate limiting. In a concerted mechanism, isotopic substitution at C-3 should have no effect on the kinetic isotope effect for C-N bond cleavage. However, for a balanced stepwise carbanion mechanism the $^{15}(V/K)$ isotope effect would be reduced, in the presence of deuterium at C-3, if the C-N bond cleavage step is cleanly rate limiting and the forward commitment is low or zero. Figure 3.10 is a diagrammatic representation of the transition state energies for the chemical steps of the two reaction mechanisms. No binding or debinding steps are shown. Although the isotope effect is not just due to changes in transition state energy, but also effects the ground state energies, consideration of only the transition state energy changes simplifies the discussion (Figure 3.10).

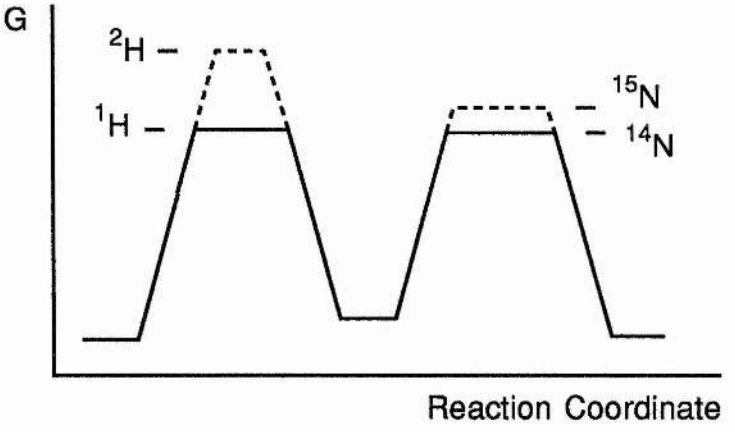
In a concerted mechanism, where C-N bond cleavage is rate determining, isotopic substitution at C-3 does not alter the $^{15}(V/K)$ effect. The energy difference in the transition state between the completely unlabelled substrate (^{1}H,^{14}N) and the deuteriated substrate (^{2}H,^{14}N) is the same as the energy difference between the nitrogen labelled substrate (^{1}H, ^{15}N) and the doubly labelled substrate (^{2}H, ^{15}N).

However a balanced stepwise carbanion mechanism shows a reduced kinetic isotope effect for C-N bond cleavage in the presence of deuterium at C-3. Here the energy difference between C-H and C-^{2}H bond cleavage is greater than the difference between C-^{14}N and C-^{15}N bond cleavage. Therefore the C-N bond cleavage step becomes less important to the overall

reaction, in the presence of deuteriated substrate and so the isotope effect decreases.



Concerted Mechanism



Balance Stepwise Mechanism

Figure 3.10 Transition State Energy Diagrams for ^{1}H, ^{2}H ,^{14}N and^{15}N Labelled Compounds

The double isotope fractionation experiment was performed as for the unlabelled substrate, by incubating (2S,3R)-[3-^{2}H]-3-methylaspartic acid with 3-methylaspartase until 20 % conversion to mesaconic acid and

ammonia had occurred. The ammonia was oxidised to nitrogen with hypobromite. The ratio of ^{15}N to ^{14}N in the ammonia produced during the reaction was compared with the ratio of nitrogen isotopes in a portion of unreacted substrate, which had undergone Kjeldahl digestion. A ^{15}N isotope effect of 1.0033 ± 0.0043 was obtained. This result is essentially identical, within experimental error, to the $^{15}(V/K)$ value obtained for the protio substrate.

The fact that the $^{15}(V/K)$ values are the same for the protio and deuterio substrates supports the notion that elimination occurs *via* a concerted mechanism. However the lack of a substantial observed $^{15}(V/K)$ effect , in the presence of a very large deuterium isotope effect, does not support this hypothesis. C-N bond cleavage seems not to be kinetically significant, whilst C-H bond cleavage is. It is therefore unlikely that both bond cleavages occur in the same step. This result can be rationalized by considering the differences between this substrate and the natural one. With the natural substrate C-H and C-N bond cleavage occur concertedly and fast. The chemical reaction occurs faster than the binding / debinding steps, as evidenced by the relatively small deuterium isotope effects obtained for both V and V/K, compared to those obtained with the L-*erythro*- substrate. With the latter substrate C-H bond cleavage is completely rate limiting. This would be expected as the C-H bond in the L-*erythro*- substrate is not positioned favourably for removal of the proton. However the amino acid portion remains the same in both substrates and so presumably both substrates experience the same binding interactions at this end of the molecule. In the reaction with the L-*erythro*- substrate, the hydrogen must be removed with difficulty from C-3, in a slow step, thus giving a large deuterium isotope effect. However once C-H bond cleavage has occurred, C-N bond cleavage could occur quickly. The enzyme is optimized to catalysis the latter cleavage, but is struggling with the first. Thus any isotope effect on C-N bond cleavage would be masked by the large isotope effect on C-H bond cleavage.

Another possible explanation for the lack of a $^{15}(V/K)$ effect can be found by

invoking a dehydroalanine residue at the active site of the enzyme, as proposed by Gani (see p. 82). If the substrate amine does indeed bind to this residue at the active site, then a C-N bond formation step and a further C-N bond cleavage step would be involved, as well as the substrate C-N bond cleavage step. The $^{15}(V/K)$ effects on these steps could mask any isotope effect due to substrate C-N bond cleavage. However, although there is some indirect support for the dehydroalanine mechanism, unequivocal evidence remains elusive.

3.11 Prolonged Incubation of Fumaric Acid and Ethylfumaric Acid with 3-Methylaspartase

Fumaric acid was incubated, in deuterium oxide, with 3-methylaspartase, ammonia and potassium and magnesium ions, for a number of weeks. ^{1}H-NMR spectra were recorded directly using aliquots of the incubation solution at various time intervals. The spectra initially showed conversion of fumaric acid to (2S,3R)-[3-^{2}H]-aspartic acid, as indicated by doublets of equal intensity, at 2.50 and 3.70 ppm. As the incubation progressed the intensity of the 3-H signal at 2.50 ppm decreased, relative to the 2-H signal. It seemed likely this was due to incorporation of deuterium into the second hydrogen position at C-3. A second signal at 3.60 ppm, which was attributable to (2S,3S)-[3-^{2}H]-aspartic acid, also appeared and showed a time dependent increase in intensity. All signals became broad singlets at long incubation times. It can be concluded that 3-methylaspartase also shows 'L-*erythro*-' activity with the substrate analogue, fumaric acid.

An incubation of 3-ethylfumaric acid, with 3-methylaspartase in buffered water containing the necessary cofactors, did not give any evidence for the formation of a second isomer of 3-ethylaspartic acid. The ^{1}H-NMR spectra were complicated by signals from both the substrate and product, however there was no indication of a second shifted 2-H proton in the 2-H region, which remains free of other signals. Therefore 3-methylaspartase seems not

to show L-*erythro* - activity with the ethyl analogue.

These results can be rationalized by considering the active site of 3-methylaspartase. The enzyme should have a binding pocket which specifically binds a methyl group and only be able to accomodate a hydrogen on the other side at C-3. However Gani showed that the enzyme accepted a number of other groups in place of the methyl functionality, indicating a degree of flexibility of binding at this centre. Also we have shown the enzyme can turn over the other C-3 isomer. The second (hydrogen) binding pocket must therefore just be able to accomodate a methyl group. It will therefore have no difficulty accomodating aspartic acid / fumaric acid. The reaction is slow as the substrate is not held at the optimum orientation for reaction, due to poor interactions with the methyl binding pocket (see p. 77). It would be unlikely that the enzyme could accomodate an ethyl group in its hydrogen binding pocket.

CHAPTER FOUR

CONCLUSIONS

AND

FURTHER WORK

Some evidence has been obtained which implies that glutamate mutase turns over the substrate analogue (2S,3S)-3-ethylaspartic acid. Improved access to the enzyme, through cloning of the genes for the two components of the enzyme and overexpression in *E. coli,* would allow more detailed studies on this enzyme. Further investigation of the nature of the reaction with (2S,3S)-3-ethylaspartic acid should yield important information about the mechanism of the reaction and the structure of the enzyme active site. One of the priorities would be the synthesis of methylglutamic acid and dimethylaspartic acid, in order to confirm whether these products are produced by the reaction of glutamate mutase with (2S,3S)-3-ethylaspartic acid. The reversiblity of the reaction can also be probed with these putative substrates. Labelling of the methylene of the ethyl group with deuterium will allow the stereochemical course of the reaction to be defined and will determine from which side the coenzyme abstracts a hydrogen. Initial studies on a route to chirally labelled ethanol were performed

Stereochemically pure (2S,3R)-3-methylaspartic acid and the [3-^{2}H]-analogue have been synthesized. Schollkopf's *bis*- lactim ether chemistry was explored as a possible route to these compounds but did not prove satisfactory. An alternative route based on enzymic synthesis of the (2S,3S) and (2S,3R)-isomers of 3-methylaspartic acid and their subsequent separation, was used.

It has been shown that 3-methylaspartase catalyses the deamination of (2S,3R)-3-methylaspartic acid at 1/38 the rate of the (2S,3S)-isomer. The K_m for the substrate was also determined and was much higher than that of the natural substrate indicating poorer binding. The deamination reaction was demonstrated to proceed by *syn*- elimination and not *via* epimerization of the (2S,3S)-isomer. A large deuterium isotope effect was measured for the deamination of (2S,3R)-3-methylaspartic acid, indicating that C-H bond cleavage was rate limiting. It was shown that the deamination of (2S,3R)-3-methylaspartic acid does not display a ^{15}N isotope effect on V/K. Hence C-N bond cleavage was not rate limiting.

Experiments suggest that 3-methylaspartase also shows *'erythro* activity' with fumaric acid as substrate. This phemomenon should be investigated further, in both the amination and deamination directions. [3-^{2}H]-Labelled aspartic acids would be useful for probing the latter.

Identification of the active site base, by labelling experiments with 3-bromoaspartic acid, together with the crystal structure of the enzyme, should provide vital information about the enzyme active site. In particular, with reference to the *'erythro* activity', it is necessary to determine whether a second active site base is responsible for removing the 3-H proton from (2S,3R)-3-methylaspartic acid.

CHAPTER FIVE

EXPERIMENTAL

Melting points were determined using an Electrothermal melting point apparatus and are uncorrected.

Elemental analyses were carried out in the departmental microanalytical laboratory and by the University College, London laboratory.

Optical rotations were measured at room temperature using Optical Activity Ltd. AA 100 and AA 1000 polarimeters. 10 cm Path-length cells were used. All optical rotations were recorded at ambient temperature.

Infrared spectra were recorded on a Perkin-Elmer 1500 series FT IR spectrometer or Perkin-Elmer 1310 and 1330 IR spectrometers. The samples were prepared as thin films between sodium chloride discs or as solutions in sodium chloride cells (thickness 0.1 nm). Absorption maxima are given in wavenumbers (cm^{-1}) relative to polystyrene standard.

^{1}H Nuclear magnetic resonance spectra were recorded at 90 MHz on a Jeol FX90Q, at 200 MHz on a Varian Gemini-200, at 270 MHz on a Jeol JNM-GX270, at 300 MHz on a Bruker AM-300, at 400 MHz on a Bruker ACP400 (by the SERC service at Warwick) and at 500 MHz on a Varian VXR500 instrument. NMR spectra are described in parts per million downfield shift from TMS or are referenced using the $CHCl_3$ signal (at 7.27 ppm), HOD signal (at 4.61 ppm) or the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-$^{2}H_4$ (at 0.00 ppm) and are reported consecutively as position (δ_H), relative integral multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, qn-quintet, m-multiplet, dd-double doublet, sp-septet and br-broad), coupling constant (J Hz), and assignment (numbering according to IUPAC nomenclature for the compound).[302]

^{13}C Nuclear magnetic resonance spectra were recorded at 22.6 MHz on a Jeol FX90Q, at 50.3 MHz on a Varian Gemini-200, at 67.8 MHz on a Jeol JNM-GX270 and at 75.47 MHz on a Bruker AM-300 and at 100.61 MHz on a Bruker ACP400 instrument by the SERC service at Warwick. Chloroform (at 77.20 ppm) or methanol (at 47.00 ppm) were used as reference signals.

^{2}H Nuclear magnetic resonance spectra were recorded at 61.44 MHz on a Bruker ACP400 instrument, by the SERC service at Warwick, and are quoted in ppm relative to $CDCl_3$ at 7.27 ppm.

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 ZAB E. Major fragments are given as percentages of the base peak intensity (100 %). Fast atom bombardment (FAB) spectra were recorded using glycerol as a matrix.

^{15}N / ^{14}N isotope ratios were measured, by Professor Alan Jackson at the University of Southampton, using a VG SIRA 10 dual-inlet isotope ratio mass spectrometer.

UV / vis spectra were obtained using a Pye-Unicam SP8-500 or SP8-100 UV / vis spectrometer.

Flash chromatography was performed according to the procedure of Still[303] using Sorbsil C 60 (40 - 60 μm) silica gel or Macherey-Nagel silica gel N.

Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Macherey-Nagel SIL G/UV_{254}), on 0.1 mm precoated cellulose plates (Macherey-Nagel CEL 400-10 UV_{254}) or precoated aluminum oxide plates (Macherey-Nagel F-284 (type E)). Compounds were visualised by UV fluorescence, iodine vapour, phosphomolybdic acid in ethanol, aqueous potassium permanganate or ninhydrin.

Protein concentrations were measured by UV absorption at 280 nm or by the method of Bradford[304].

Sonication was carried out with a Heat Systems-Ultrasonics Inc. Europa W-220F sonicator.

Centrifugation was carried out in a Beckman J2-21 centrifuge.

All equipment used in protein purification (peristaltic pumps, UV detectors, fraction collectors, FPLC pumps etc.) were purchased from LKB.

Electrophoresis was carried out with a discontinuous tris-glycine system according to the method of Laemmeli[253]. Gels were visualized with a Coomassie Brilliant Blue R stain.

Standard proteins for calibration of FPLC columns and gel electrophoresis were obtained from Sigma and BDH.

Solvents were dried and purified according to the methods of Perrin and Armarego.[305]

Enzymes other than 3-methylaspartase and glutamate mutase were obtained from Sigma Chemical Co., Poole, UK. and were used without further purification.

Partial Purification of 3-Methylaspartase from *Clostridium tetanomorphum*

Clostridium tetanomorphum strain H1 (ATCC 15920) was grown according to the method of Barker[249], using a modification of literature procedures, by Prof. C. Greenwood and Mr. A. Thompson, University of East Anglia.

1. *Clostridium tetanomorphum* cell paste (112 g) was added to potassium phosphate buffer (110 ml, 50 mM, pH 7.6). The mixture was stirred until thawed. Charcoal (10 g) was added and the mixture sonicated (power setting: 40) in bursts for 10 minutes, with care being taken not to allow the temperature to exceed 10 °C. The cell debris was spun down (30 000 g, 50 minutes) and discarded.

2. To the supernatant was added 12 ml buffer (200 mM $MgCl_2.6H_2O$, 200 mM NH_4Cl, 200 mM mesaconate, pH 7.6 with potassium hydroxide). The supernatant was divided into four portions and shaken in a water bath for 10 minutes at 52 °C. The debris was removed by centrifugation (48 400 g, 40 minutes).

3. To the supernatant was added, over 15 minutes, protamine sulphate (1 % solution) in potassium phosphate buffer (100 ml, 50 mM, pH 7.6) at 0 °C. The mixture was left to stir for a further 15 minutes, at 4 °C. The debris was removed by centrifugation (48 400 g, 45 minutes).

4. To the supernatant was added, slowly, precrushed solid ammonium sulphate to 50 % saturation, at 4 °C. This was left stirring for 30 minutes. The precipitated protein was removed by centrifugation (48 400 g, 30 minutes).

5. The supernatant was collected and ammonium sulphate added slowly to a final saturation of 75 %. The mixture was stirred for a further 30 minutes, at 4 °C. The supernatant was removed by centrifugation (48 400 g, 30 minutes).

6. The precipitate from above was suspended in potassium phosphate buffer (10 ml, 50 mM, pH 7.6) and dialysis conducted overnight in potassium phosphate buffer (6 l, 50 mM, pH 7.6) at 4 °C. The solution was centrifuged (3 020 g, 15 minutes) to remove any debris and gave 3-methylaspartase solution in a final volume of 29.9 ml, with an activity of 91 units / ml.

Assay to Determine 3-Methylaspartase Activity

To assay buffer (3 ml) containing Tris (0.5 M), magnesium chloride hexahydrate (2 mM), potassium chloride (1 mM) and 3-methylaspartic acid (4 mM), adjusted to pH 9.0 with sodium hydroxide was added 3-methylaspartase solution (20 µl), diluted if necessary. The change in absorbance at 240 nm was measured. One unit of enzyme catalyses the formation of 1 µmol of mesaconic acid min^{-1} at pH 9.7 and 25 °C, under assay conditions[9].

Detection of Glutamate Mutase Activity

To an incubation containing Tris buffer (1 ml, 1 M, pH 8.2), sodium glutamate (6.5 ml, 1.36 mM), potassium chloride (0.2 ml, 1 M), magnesium chloride hexahydrate (0.1 ml, 0.2 M), pre-incubated partially purified enzyme solution (10 ml) and unincubated partially purified enzyme extract (2 ml) was added adenosyl cobalamin (0.2 ml, 6 mM) to initiate the reaction. (The pre-incubated partially purified enzyme solution consisted of partially purified enzyme extract (2 ml) in aqueous mercaptoethanol (2 ml, 0.1 M) and potassium phosphate buffer (6 ml, 30 mM, pH 8.2) incubated at 25 °C for 15 minutes). Aliquots (1 ml) were removed at various time intervals. The protein was denatured at 100 °C and removed by centrifugation. The supernatant was concentrated *in vacuo*, the residue taken up in deuterium oxide / deuterium chloride and ^1H NMR spectra recorded. The conversion of L-glutamate to mesaconate was detected. The presence of ammonia was also detected using Nessler's reagent[250].

^1H NMR spectra of aliquots from a similar incubation containing 3-methylaspartate as substrate and calcium chloride (0.1 ml, 0.05 M) to inhibit 3-methylaspartase, showed a small amount of glutamate was produced.

Attempted Purification of Component S

1. Sonication

Clostridium tetanomorphum cell paste (50 g) was added to sodium phosphate buffer (100 ml, 20 mM, pH 7.6, 2 mM mercaptoethanol) and stirred until thawed. The mixture was sonicated (power setting: 40), in bursts, for 10 minutes, keeping the temperature as close to 4 °C as possible. The mixture was then centrifuged (10 000 g, 30 minutes) to remove cell debris. The protein concentration of the supernatant was estimated by UV absorption at 280 nm, at 40 mg / ml, volume 130 ml.

2. Isoelectric Precipitation

The supernatant was diluted with distilled water to give a protein concentration of 10 mg / ml. The pH was lowered to 4.60 with 5 M acetic acid and the precipitate removed by centrifugation (15 000 g, 30 minutes). The pH of the supernatant was taken back up to 7.60 with concentrated ammonia.

3. Ammonium Sulphate Precipitation

Pre-crushed ammonium sulphate was added slowly to the supernatant to give 80 % saturation. The solution was then stirred for a further 30 minutes and the proteins spun down to give a pellet (15 000 g, 30 minutes). The supernatant was discarded.

4. Desalting by Gel Filtration

The precipitated pellets were redissolved in a minimum volume of sodium phosphate buffer (20 mM, pH 7.50, 5 mM mercaptoethanol, 1 mM EDTA). This was applied to a Sephadex G-50 column (5 x 10 cm), which was eluted with the above buffer at a flow rate of 50 ml / hour. This yielded various desalted protein fractions which were pooled into 4 portions and each subjected to further purification.

5a. Ion-exchange Chromatography - Low Pressure Chromatography

The portions were separately applied to a DEAE-52 column (5 x 10 cm) at a flow rate of 25 ml / hour. The column was washed with sodium phosphate buffer (10 mM, pH 7.50, 5 mM mercaptoethanol, 1 mM EDTA) for one column volume. Then, a linear gradient of 10 to 200 mM sodium phosphate buffer (pH 7.50, 5 mM mercaptoethanol, 1 mM EDTA) (total volume 500 ml) was applied. Component S was expected to elute at about 50 mM salt concentration.

5b. Ion Exchange Chromatography - FPLC

The high resolution FPLC equivalent column, DEAE-5PW was also used. Before application the fractions were re-concentrated by ammonium sulphate precipitation and dissolved in a minimum volume of buffer. The protein solution was desalted on small (10 ml) Sephadex G-50 or G-25 columns, or, dialysed and then re-concentrated by ultrafiltration using a YM-10 filter.

6. FPLC gel exclusion column

Fractions eluted at 50 mM ionic strength were pooled, concentrated by ultrafiltration and applied to the FPLC gel exclusion column. The protein

was eluted with potassium phosphate buffer (10 mM, pH 6.5, 1 mM mercaptoethanol). Molecular weight standards were also applied to the column for calibration.

7. SDS-PAGE

The molecular weight of isolated proteins was determined by gel electrophoresis (SDS-PAGE).

Attempted Purification of Component E

1. Sonication

Clostridium tetanomorphum cell paste (50 g) was added to sodium phosphate buffer (100 ml, 10 mM, pH 7.6, 2 mM mercaptoethanol) and stirred until thawed. Charcoal (20 g) was added and the mixture sonicated (power setting: 40), in bursts, for 10 minutes, keeping the temperature as close to 4 °C as possible. The mixture was centrifuged (13 000 g, 30 minutes) to remove cell debris.

2. Nucleic Acid Removal

To the supernatant was added, over 15 minutes, protamine sulphate (1 % solution) in potassium phosphate buffer (100 ml, 40 mM, pH 7.0) at 0 °C. The mixture was left to stir for a further 15 minutes, at 4 °C and the debris removed by centrifugation (13 000 g, 30 minutes).

3. Ammonium Sulphate Precipitation (1)

To the supernatant was added potassium phosphate buffer (20 ml, 1 M, pH

7.0) and EDTA (0.5 ml, 1 M). Precrushed solid ammonium sulphate was added, slowly, at 4 °C, to give 45 % saturation. The solution was stirred for a further 30 minutes and the precipitated protein removed by centrifugation (48 400 g, 30 minutes).

4. Ammonium Sulphate Precipitation (2)

The supernatant was collected and ammonium sulphate added slowly to a final saturation of 70 %. The mixture was stirred for a further 30 minutes, at 4 °C. The supernatant was removed by centrifugation (48 400 g, 30 minutes).

5. Dialysis (1)

The precipitated protein was suspended in potassium phosphate buffer (5 ml, 10 mM, pH 7.0) and dialysis conducted overnight in potassium phosphate buffer (6 l, 2 mM, pH 6.5) at 4 °C.

6. Dialysis (2)

The solution was redialysed against potassium phosphate buffer (6 l, 10 mM, pH 8.0) containing mercaptoethanol (10 mM).

7. Ion-exchange Chromatography

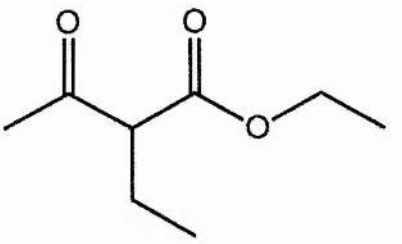
The protein solution was applied to a DEAE-52 column, which had been prewashed with potassium phosphate buffer (0.3 M, pH 8.0) containing mercaptoethanol (10 mM) and then potassium phosphate buffer (10 mM, pH 8.0) containing mercaptoethanol (10 mM). The column was eluted with potassium phosphate buffer (10 mM, pH 8.0) containing mercaptoethanol (10 mM), followed by potassium phosphate buffer (10 mM, pH 6.65)

containing mercaptoethanol (10 mM) and finally potassium phosphate buffer (50 mM, pH 6.7) containing mercaptoethanol (10 mM). Any fractions containing protein were concentrated by ammonium sulphate precipitation (80 % saturation) and centrifugation (14 000 g, 25 minutes). The protein pellet was dissolved in a minimum volume of distilled water and dialysed against sodium acetate buffer (6 l, 5 mM, pH 5.2) containing mercaptoethanol (5 mM).

Incubations of (2S,3S)-3-Ethylaspartic Acid with Crude Enzyme Extract

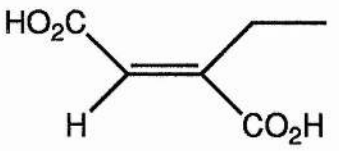
(2S,3S)-3-Ethylaspartic acid (100 mg) was added to an incubation containing enzyme solution from the partial purification of 3-methylaspartase (2 ml), potassium phosphate buffer (0.8 ml, 0.3 M, pH 7.6; 0.38 M ammonium chloride), adenosyl cobalamin (0.2 ml, 6 mM) and a preincubated enzyme solution (2 ml) in potassium phosphate buffer (1 ml, 0.18 M, pH 7.6; 0.2 M mercaptoethanol). Aliquots were removed at various time intervals and concentrated *in vacuo*. The residue was dissolved in deuterium oxide and ^{1}H NMR spectra recorded at 500 MHz.

2-Ethylacetoacetate Ethyl Ester (119 a)



Sodium ethoxide solution was prepared by dissolving sodium (9.6 g, 0.42 mol) in anhydrous ethanol (150 ml). Ethyl acetoacetate (47.75 g, 36.7 mmol) was added dropwise with stirring. After 5 minutes further stirring, bromoethane (53.7 g, 0.49 mol) was added slowly dropwise. The reaction mixture was then refluxed for 2 hours and allowed to cool. The solution was poured into water (200 ml) and extracted with diethyl ether (3 x 200 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a yellow oil (48.97 g, 84.5 %) which was distilled under reduced pressure to give 2-ethyl acetoacetate ethyl ester as a colourless oil (46.09 g, 79.5 %), b.p. 103-104 °C / 10 mmHg; υ_{max} (neat)/cm^{-1} 3000 (C-H), 1725 (CO ester) and 1715 (CO ketone); δ_H (90 MHz; $CDCl_3$) 0.91 (3H, t, *J* 7.0 Hz, 2'-CH_3), 1.25 (3H, t, *J* 7.0 Hz, -$OCH_2\underline{C}H_3$), 1.86 (2H, qn, *J* 7.0 Hz, 1'-CH_2), 2.20 (3H, s, 4-CH_3), 3.30 (1H, t, *J* 7.0 Hz, 2-CH) and 4.18 (2H, q, *J* 7.0 Hz, -$OC\underline{H}_2CH_3$); δ_C (67.8 MHz; $CDCl_3$) 12.35 (2'-CH_3), 14.57 (-$OCH_2\underline{C}H_3$), 22.07 (1'-CH_2), 23.87 ($\underline{C}H_3CO$), 29.28 (CH), 61.87 (-$O\underline{C}H_2CH_3$), 170.29 (-CO_2-) and 173.02 (CO); *m/z* (EI) 158 (M^+, 2 %), 130 (13, $[M - C_2H_4]^+$), 116 (68, $[M - C_3H_6]^+$), 101 (55, $[M - C_3H_5O]^+$), 73 (69, $[M - C_5H_9O]^+$), 43 (100, $C_2H_3O^+$) and 29 (59, $C_2H_5^+$).

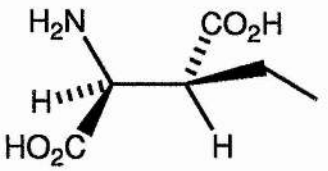
Ethylfumaric Acid (122)



Bromine (18.0 g, 113 mmol) was added slowly to a vigorously stirred solution of 2-ethyl acetoacetate ethyl ester (9.0 g, 56.7 mmol) in dry diethyl ether (75 ml). The resulting solution was refluxed for 3 hours. The solvent and hydrogen bromide were removed *in vacuo* to give the dibromide as a yellow oil. This was added slowly to rapidly stirred ethanol (60 ml) containing powdered potassium hydroxide (18.0 g, 0.32 mol). The mixture was refluxed for 30 minutes.

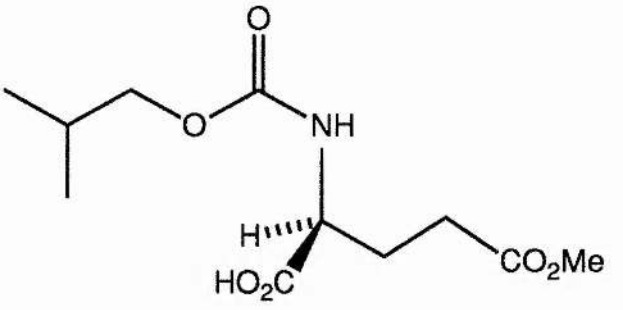
Steam distillation was performed until 250 ml of distillate had been collected. The undistilled material was acidified with concentrated hydrochloric acid and extracted with diethyl ether (4 x 150 ml). The pooled organic extracts were dried ($MgSO_4$). The solvent was removed *in vacuo* to give an orangey-yellow solid. This was recrystallized from diethyl ether / petroleum ether to give ethylfumaric acid as an off white solid (2.9 g, 36 %), m.p. 198-200 °C (lit.,[255] 194-195 °C); υ_{max} (nujol)/cm^{-1} 2400-3500 (COOH) and 1740 (CO); δ_H (270 MHz; D_2O/NaOD) 0.86 (3H, t, *J* 7 Hz, CH_3), 2.31 (2H, q, *J* 7 Hz, CH_2) and 6.20 (1H, s, C=CH); δ_C (75.47 MHz; D_2O/NaOD) 13.77 (CH_3), 22.77 (CH_2), 128.01 (C-3), 148.01 (C-2), 174.37 and 175.44 (CO_2Hs); *m/z* (EI) 126 ([*M* - H_2O]$^+$, 48 %) and 98 (100, [*M* - CH_2O_2]$^+$).

(2S,3S)-3-Ethylaspartic Acid (118)



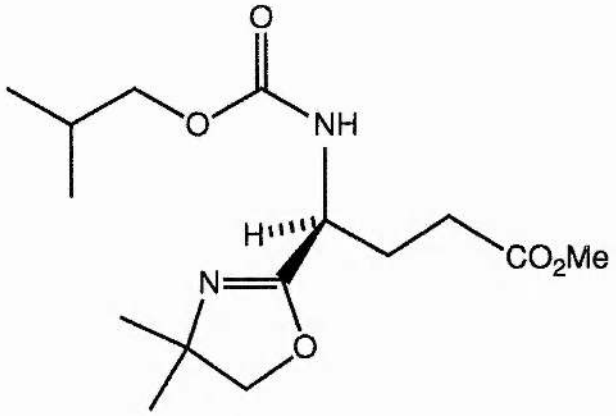
Ethylfumaric acid (1.44 g, 10 mmol) was suspended in water (15 ml) and the pH adjusted to 9.0 with concentrated ammonia solution. The solution was reduced *in vacuo* to give the diammonium salt. This was redissolved in water (15 ml) and potassium chloride (6 mg, 3.9 mmol) and magnesium chloride hexahydrate (30 mg, 7.5 mmol) added. The pH was readjusted to 9.0 with concentrated ammonia solution. 3-Methylaspartase (100 µl, 10 units) was added and the absorbance of the solution at 240 nm was measured. The solution was then incubated at 30 °C until absorbance measurements showed no further decrease in absorbance. The protein was denaturated by heating to 80 °C for 2 minutes and removed by filtration. The filtrate was acidified to pH 1.0 with concentrated hydrochloric acid and extracted with diethyl ether (3 x 15 ml). The aqueous layer was adjusted to pH 4.0 and the water removed *in vacuo*. The resulting solid was recrystallized from water / ethanol to give a white solid (1.22 g, 76 %), m.p. > 240-242 °C (lit.,[241] 245-246 °C); $[\alpha]_D$ +13.9 ° (c 1.0 in 6 M HCl) (lit.,[241] +15.0 ° (c 0.6 in 6 M HCl)); υ_{max} (nujol)/cm^{-1} 2400-3500 (COOH) and 1630 (CO); δ_H (270 MHz; D_2O/NaOD) 0.70 (3H, t, *J* 7 Hz, $-CH_2CH_3$), 1.22 (2H, m, *J* 7 Hz, $-CH_2CH_3$), 2.34 (1H, m, *J* 4 Hz, 3-H) and 3.38 (1H, d, *J* 5 Hz, 2-H); δ_C (50.3 MHz, D_2O/NaOD) 13.5 (CH_3), 22.1 (CH_2), 50.8 (C-3), 57.6 (C-2), 174.9 and 182.0 (CO_2Hs); *m/z* (EI) 116 ([*M* - CHO_2]$^+$, 12 %).

N-Isobutyloxy Carbonyl (2S)-Glutamic Acid-5-Methyl Ester (128)



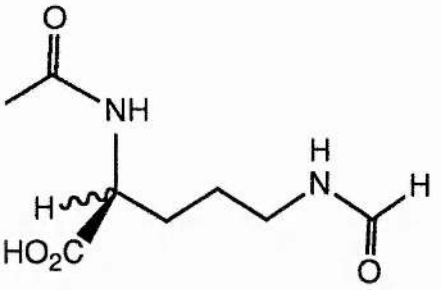
(2S)-Glutamic acid-γ- methyl ester (2.0 g, 12.4 mmol) was dissolved in water (25 ml) and toluene (30 ml) and the mixture stirred very vigorously. *Iso*-butylchloroformate (5.2 g, 38 mmol) was added slowly and the pH of the reaction mixture maintained above 7.5 by the periodic addition of sodium bicarbonate. Stirring was continued for 90 minutes. The reaction mixture was extracted with chloroform (3 x 60 ml). The aqueous layer was adjusted to pH 2.0 with hydrochloric acid and re-extracted with chloroform (3 x 25 ml). The pooled chloroform extracts were dried ($MgSO_4$) and evaporated to dryness to give N-isobutyloxy carbonyl (2S)-glutamic acid-5-methyl ester as a hygroscopic white solid (2.6 g, 84 %), m.p. 35-37 °C; δ_H (270 MHz; $CDCl_3$) 0.92 (6H, d, *J* 7.9 Hz, $(C\underline{H}_3)_2CH$-), 1.92 and 2.06 (2H, sp and qn, *J* 5.6 Hz, 3-CH_2), 2.28 (1H, m, *J* 4.5 Hz, $(CH_3)_2C\underline{H}$-), 2.48 (2H, m, *J* 6.2 Hz, 4-CH_2), 3.67 (3H, s, CO_2CH_3), 3.87 (2H, d, *J* 7.9 Hz, $(CH_3)_2CHC\underline{H}_2$-), 4.40 (1H, m, *J* 4.9 Hz, 2-CH) and 5.40 (1H, d, *J* 9.8 Hz, NH); δ_C (22.6 MHz, $CDCl_3$) 19.13 ($(\underline{C}H_3)_2CH$-), 27.53 (C-3), 28.07 ($(CH_3)_2\underline{C}H$-), 30.25 (C-4), 52.10 ($CO_2\underline{C}H_3$), 53.28 (C-2), 71.77 ($(CH_3)_2CH\underline{C}H_2$-), 156.82 ($CO_2NH$), 173.73 ($\underline{C}O_2CH_3$) and 176.05 ($CO_2H$); *m/z* (CI) 262 ([*M* + H]$^+$, 100 %), 216 (15, [*M* - CO_2H]$^+$), 205 (8, [*M* + H - C_4H_9]$^+$) and 116 (12, [*M* - $C_6H_9O_4$]$^+$).

2'-(N-Isobutyloxy Carbonyl (2'S)-Glutamic Acid-5'-Methyl Ester)-4,4-Dimethyl-2-Oxazoline (129)



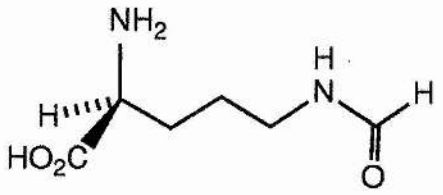
N-Isobutyloxy carbonyl (2S)-glutamic acid-5-methyl ester (0.7 g, 2.68 mmol) was dissolved in toluene (40 ml). *p*-Toluenesulfonic acid (40 mg, 0.2 mmol) and 2-amino-2-methyl-1-propanol (0.26 g, 2.92 mmol) were added. The reaction mixture was refluxed with azeotropic removal of water for 36 hours. The toluene and unreacted alcohol was removed *in vacuo* to give 2'-(N-isobutyloxy carbonyl (2'S)-glutamic acid-5'-methyl ester)-4,4-dimethyl-2-oxazoline as a colourless oil (0.83 g, 99 %); υ_{max} (nujol)/cm^{-1} 3300 (N-H), 1715 (CO), 1675 (CN) and 1250 (CO); δ_H (270 MHz; $CDCl_3$) 0.90 (6H, d, *J* 8.0 Hz, $(C\underline{H}_3)_2CH$-), 1.30 (6H, s, $(C\underline{H}_3)_2C(N)CH_2$-), 1.92 and 2.05 (2H, m, *J* 6.4 Hz, 3'-CH_2), 2.25 (1H, m, *J* 8.0 Hz, $(CH_3)_2C\underline{H}$-), 2.46 (2H, m, *J* 6.4 Hz, 4-CH_2), 3.65 (3H, s, CO_2CH_3), 3.85 (2H, d, *J* 8.0 Hz, $(CH_3)_2CHC\underline{H}_2$-), 4.0 (2H, s, $(CH_3)_2C(N)C\underline{H}_2$-), 4.45 (1H, m, *J* 6.0 Hz, 2'-CH) and 5.45 (1H, d, *J* 10 Hz, NH); δ_C (67.8 Hz; $CDCl_3$) 18.93 ($(\underline{C}H_3)_2CH$-), 23.37 ($NC(\underline{C}H_3)_2$), 27.81 (C-3'), 28.23 ($(CH_3)_2\underline{C}H$-), 29.54 (C-4'), 32.90 ($N\underline{C}(CH_3)$, 51.60 ($CO_2\underline{C}H_3$), 54.16 (C-2'), 67.05 ($O\underline{C}H_2C(CH_3)_2$), 71.14 ($(CH_3)_2CH\underline{C}H_2$-), 156.64 (OCN), 164.50 (CO_2NH) and 173.74 ($\underline{C}O_2CH_3$); *m/z* (CI) 315 ([*M* + H]$^+$, 100 %), 241 (8, [*M* - C_4H_9O]$^+$) and 90 (37, $C_4H_{12}NO^+$).

α-N-Acetyl-δ-N-Formyl-(2S/R)-Ornithine (134)



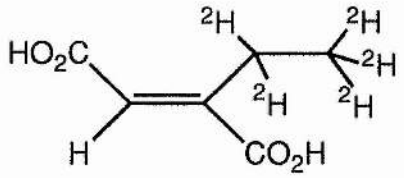
α-N-Acetyl-(2S/R)-ornithine (200 mg, 1.15 mmol) was dissolved in glacial acetic acid. To this solution, stirred at 8 °C, was added formic acetic anhydride (0.3 g, 3.45 mmol). Stirring was continued at this temperature for 40 minutes and then for a further 80 minutes at room temperature. The solvent and excess reagent was removed *in vacuo* yield a white solid which was recrystallized from ethanol / ethyl acetate, to give α-N-acetyl-δ-N-formyl-(2S/R)-ornithine (0.16 g, 69 %), m.p. 141-143 °C; (Found: *m/z* $[M + H]^+$ 203.103. $C_8H_{15}N_2O_4$ requires 203.103); δ_H (270 MHz, D_2O) 1.40 (2H, m, *J* 9.2 Hz, 4-CH_2), 1.52 and 1.65 (2H, ms, *J* 8.2 and 5 Hz, 3-CH_2), 1.80 (3H, s, CH_3CO), 3.00 (2H, t, *J* 8.2 Hz, 5-CH_2), 4.10 (1H, dd, *J* 5 Hz, 2-CH) and 7.80 (1H, s, NHCOH); δ_C (67.8 MHz, D_2O) 22.36 ($\underline{C}H_3CO$), 25.73 (C-4), 28.78 (C-3), 38.16 (C-5), 53.36 (C-2), 164.93 ($CH_3\underline{C}O$), 175.06 and 176.55 (CHO and CO_2H); *m/z* (CI) 220 ($[M + NH_4]^+$, 14 %), 203 (100, $[M + H]^+$), 185 (69, $[M - OH]^+$), 157 (20, $[M - CO_2H]^+$) and 98 (10, $[M - C_3H_6NO_3]^+$).

δ-N-Formyl-(2S)-Ornithine (132)



α-N-Acetyl-δ-N-formyl-(2S/R)-ornithine (70 mg, 0.35 mmol) was dissolved in water (5 ml) and the pH adjusted to 6.80 with dilute sodium hydroxide solution. Acylase (1 mg, 2300 units) was added and the reaction mixture incubated at 25 °C for 24 hours. Charcoal was added and the solution filtered through celite to remove the enzyme. Concentration *in vacuo* gave α-N-Acetyl-δ-N-formyl-(2R)-ornithine and δ-N-formyl-(2S)-ornithine (62 mg, 99 %). Attempts to separate these two compounds were unsuccessful; δ_H (270 MHz, D_2O) 1.42 and 1.75 (8H, 2m, *J* 9.2 and 5.0 Hz, 3,4-CH_2), 1.80 (3H, s, CH_3CO), 3.05 (4H, t, *J* 9.2 Hz, 5-CH_2), 3.88 (1H, t, *J* 7.4, 2-CH), 4.10 (1H, dd, *J* 5 Hz, 2-CH) and 7.80 (2H, 2s, NHCOHs); δ-N-formyl-L-ornithine; δ_C (67.8 MHz, D_2O) 25.06 (C-4), 28.14 (C-3), 37.98 (C-5), 53.18 (C-2), 172.51 (CO_2H) and 176.55 (CHO).

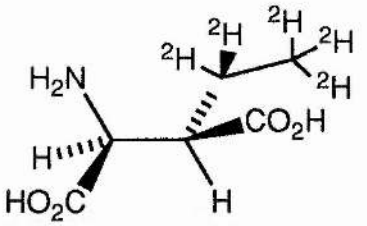
[$^2H_5$]-Ethylfumaric Acid (139)



Ethyl acetoacetate (1.3 g, 8.28 mmol) was added dropwise to a stirred solution of sodium ethoxide in anhydrous ethanol (2.54 ml, 2.8 M, 7.1 mmol). After 5 minutes further stirring, [$^2H_5$]-iodoethane (1.0 g, 6.2 mmol) was added slowly dropwise. The reaction mixture was then refluxed for 2 hours and allowed to cool. The solution was poured into water (3.4 ml) and extracted with diethyl ether (3 x 3.5 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give [$^2H_5$]-2-ethylacetoacetate ethyl ester as a yellow oil (0.83 g, 82 %).

Bromine (5.0 g, 31.3 mmol) was added slowly to a vigorously stirred solution of the [$^2H_5$]-2-ethylacetoacetate ethyl ester (0.83 g, 5.2 mmol) in dry diethyl ether (20 ml). The resulting solution was refluxed for 3 hours. The solvent and hydrogen bromide were removed *in vacuo* to give the dibromide as a yellow oil. This was added slowly to rapidly stirred ethanol (17 ml) containing powdered potassium hydroxide (5 g, 87.7 mmol). The mixture was refluxed for 30 minutes and then acidified with concentrated hydrochloric acid and extracted with diethyl ether (4 x 35 ml). The pooled organic extracts were dried ($MgSO_4$). The solvent was removed *in vacuo* to give a yellow solid. This was recrystallized from diethyl ether / petroleum ether to give [$^2H_5$]-ethylfumaric acid as an off white solid (0.275 g, 36 %); m.p. 196-199 °C; υ_{max} (nujol)/cm^{-1} 2400-3500 (CO<u>OH</u>) and 1740 (CO); δ_H (270 MHz; D_2O/NaOD) 6.59 (1H, s, -CH); δ_D (61.4 MHz; D_2O/NaOD) 0.94 (3^2H, s, C^2H_3) and 2.53 (2^2H, s, C^2H_2); δ_C (100.61 MHz; D_2O/NaOD) 12.36 (m, C^2H_3), 21.15 (m, C^2H_2), 127.17 (C-3), 148.98 (C-2), 171.10 and 172.23 (<u>C</u>O_2Hs); *m/z* (EI) 130 ([*M* - H_3O]$^+$, 77 %), 102 (97, [*M* - CH_3O_2]$^+$), 57 (48, $C_2HO_2^+$), 45 (38, CHO_2^+) and 28 (100, CO^+).

(2S,3S)-[$^2H_5$]-3-Ethylaspartic Acid (140)



[$^2H_5$]-Ethylfumaric acid (0.20 g, 1.3 mmol) was suspended in water (2 ml) and the pH adjusted to 9.0 with concentrated ammonia solution. The solution was reduced *in vacuo* to give the diammonium salt. This was redissolved in water (2 ml) and potassium chloride (1 mg, 0.5 mmol) and magnesium chloride hexahydrate (4 mg, 1 mmol) added. The pH was readjusted to 9.0 with concentrated ammonia solution. 3-Methylaspartase (10 µl, 1.5 units) was added and the absorbance of the solution at 240 nm was measured. The solution was then incubated at 30 °C until subsequent absorbance measurements showed no further decrease in absorbance. The protein was denaturated by heating to 80 °C for 2 minutes and removed by filtration. The filtrate was acidified to pH 1.0 with concentrated hydrochloric acid and extracted with diethyl ether (3 x 2 ml). The aqueous layer was adjusted to pH 4.0 and the water removed *in vacuo*. The resulting solid was recrystallized from water / ethanol to give (2S,3S)-[$^2H_5$]-3-ethylaspartic acid as a white solid (0.12 g, 53 %), m.p. 239-242 °C; (Found: (*M* + H)$^+$ 167.108. $C_6H_7O_4ND_5$ requires 167.108); $[\alpha]_D$ +10.85 ° (c 1.0 in 0.5 M HCl); υ_{max} (nujol)/cm^{-1} 2400-3500 (COOH) and 1630 (CO); δ_H (400 MHz; D_2O/NaOD) 2.71 (1H, d, *J* 3.7 Hz, 3-CH) and 3.90 (1H, d, *J* 3.7 Hz, 2-CH); δ_D (61.4 MHz; D_2O/NaOD) 0.81 (3^2H, s, C^2H_3) and (2^2H, d, *J* 9.2 Hz, C^2H_2); δ_C (100.61 MHz; D_2O/NaOD) 11.18 (qn, C^2H_3), 20.05 (qn, C^2H_2), 48.33 (C-3), 56.12 (C-2), 173.40 and 179.97 (CO_2Hs); *m/z* (CI) 167 ([*M* + H]$^+$, 100 %), 121 (9, [*M* - CHO_2]$^+$), 77 (20, [*M* - C_2HO_4]$^+$) and 58 (9, [*M* - $C_2H_6NO_4$]$^+$).

Propionyl Chloride (162)

Propanoic acid (0.5 g, 6.75 mmol) was dissolved in chloroform (20 ml) and phosphorus pentachloride (1.41 g, 6.75 mmol) was added. The mixture was stirred at reflux for 24 hours. The propionyl chloride and solvent were co-distilled to give propionyl chloride in chloroform (estimated yield 0.3 g, 48 %); υ_{max} ($CHCl_3$)/cm^{-1} 1790 (CO); δ_H (200 M Hz; $CDCl_3$) 1.22 (3H, t, *J* 5.3 Hz, CH_3) and 2.93 (2H, q, *J* 5.3 Hz, CH_2); δ_C (75.47 MHz; $CDCl_3$) 9.26 (CH_3), 41.30 (CH_2) and 174.85 (COCl); *m/z* (GCMS, EI) 92 (M^+, 1 %), 57 (38, $[M - Cl]^+$) and 29 (70, $[M - COCl]^+$).

1-Diazobutan-2-one (163)

Propionyl chloride (0.5 g, 5.4 mmol)) in chloroform (20 ml) was added slowly dropwise to a stirred ethereal solution of diazomethane (11 mmol, as determined by titration) which was cooled in ice. Stirring was continued for 2 hours, allowing the solution come up to room temperature. The diazoketone was obtained on removal of the solvent *in vacuo* as a yellow oil (0.42 g, 80 %); υ_{max} (neat)/cm^{-1} 2105 (NN) and 1740 (CO); δ_H (200 MHz; $CDCl_3$) 1.12 (3H, t, *J* 7.5 Hz, CH_3), 2.35 (2H, q, *J* 7.5 Hz, CH_2) and 5.23 (1H, s, CHN_2); δ_C (50.3 MHz; $CDCl_3$) 9.55 (CH_3), 34.48 (CH_2), 54.46 (CHN_2) and 196.36 (CO); *m/z* (EI) 98 (M^+, 32 %), 68 (27, $[M - N_2H_2]^+$), 57 (79, $[M - CHN_2]^+$), 42 (27, $CH_2N_2^+$) and 30 (100, $N_2H_2^+$).

Butan-2-one (164)

1-Diazobutan-2-one (1.0 g, 10.2 mmol) was dissolved in dichloromethane (30 ml) and hydroiodic acid (55 %, 3 ml, 12.8 mmol) was added slowly to the stirred diazoketone solution. The reaction mixture was left stirring for 10 minutes to ensure complete reaction. The mixture was then washed with 50 % aqueous sodium thiosulphate (5 x 30 ml), until complete decolourization had occurred. The organic layer was washed with water (2 x 5 ml) and dried

($NaSO_4$). The filtrate distilled to give butan-2-one in dichloromethane (0.34 g, 46 %); υ_{max} (CH_2Cl_2)/cm^{-1} 1710 (CO); δ_H (200 MHz; $CDCl_3$) 1.05 (3H, t, *J* 7.5 Hz, $C\underline{H}_3CH_2$), 2.14 (3H, s, CH_3CO) and 2.45 (2H, q, *J* 7.5 Hz, CH_2); δ_C (75.47 MHz; $CDCl_3$)7.05 ($\underline{C}H_3CH_2$), 27.52 ($CO\underline{C}H_3$), 35.23 ($CH_3\underline{C}H_2$) and 207.64 (CO); *m/z* (GCMS, EI) 73 ([*M* + H]$^+$, 14 %), 58 (25, [*M* + H - CH_3]$^+$) and 31 (100, $C_2H_3O^+$).

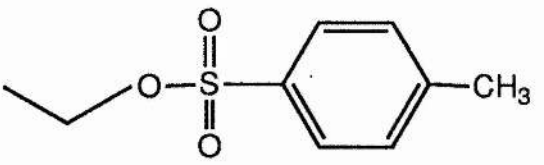
Ethyl Acetate (165)

Butan-2-one (1.0 g, 13.5 mmol) was dissolved in dry 1,1,2-trichloroethane (30 ml) and 3-chloroperbenzoic acid (50 %, 10 g, 28 mmol) was added. The mixture was stirred at 50 °C for 48 hours. The reaction mixture was allowed to cool and the precipitated acid was filtered off. The solvent and ethyl acetate were co-distilled, to give ethyl acetate in 1,1,2-trichloroethane (1.21 g, 99 %); υ_{max} (1,1,2-trichloroethane)/cm^{-1} 2950 (CH) and 1740 (CO); δ_H (200 MHz; $CDCl_3$) 1.27 (3H, t, *J* 7.5 Hz, $C\underline{H}_3CH_2$), 2.05 (3H, s, CH_3CO_2) and 4.13 (2H, q, *J* 7.5 Hz, CH_2); δ_C (50.3 MHz; $CDCl_3$) 9.16 ($\underline{C}H_3CH_2$), 27.21 ($CH_3\underline{C}H_2$), 49.96 ($\underline{C}H_3CO_2$) and 173.27 (CO); *m/z* (GCMS, EI) 88 (*M*$^+$, 7 %), 73 (5, [*M* - CH_3]$^+$), 61 (15, [*M* - C_2H_3]$^+$), 43 (100, $C_2H_3O^+$), 29 (12, $C_2H_5^+$) and 15 (5, CH_3^+).

Ethanol (166)

Ethyl acetate (1.0 g, 11 mmol) was dissolved in dioxane (5 ml) and hydrazine hydrate (34 mmol, 1.10 g) was added slowly dropwise. The reaction was stirred at 50 °C for 24 hours. Phthalic anhydride (0.81 g, 5.5 mmol) was added to destroy the excess hydrazine. The ethanol and dioxane were co-distilled off from the acetate by-product, to give ethanol in dioxane (0.47 g, 90 %); υ_{max} (dioxane)/cm^{-1} 3400 br (OH); δ_H (200 MHz; $CDCl_3$) 1.20 (3H, t, *J* 7.5, CH_3) and 3.70 (2H, q, *J* 7.5 Hz, CH_2); δ_C (50.3 Hz; $CDCl_3$) 18.32 (CH_3) and 57.95 (CH_2); *m/z* (GCMS, EI) 45 ([*M* - H]$^+$, 100 %), 31 (100, CH_3O^+) and 15 (10, CH_3^+).

Ethyl Tosylate (167)



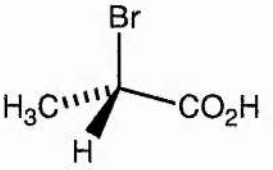
Ethanol (0.5 g, 11 mmol) in dioxane (8 ml) was dried ($MgSO_4$) and dry pyridine (3.5 ml, 43.3 mmol) added. The mixture was cooled in ice. Tosyl chloride (5.03 g, 26 mmol) was added in small portions, whilst maintaining the temperature below 10 °C. The reaction mixture was stirred for a further 4 hours. It was, then, tipped into ice water (22 ml) containing hydrochloric acid (6.6 ml). Trituration gave solid ethyl tosylate, which was filtered off. The product was dissolved in diethyl ether (10 ml) and the resulting solution dried ($MgSO_4$). Concentration *in vacuo* gave the product as a white solid. It was recrystallized from diethyl ether / petroleum ether, to give ethyl tosylate (1.8 g, 83 %), m.p. 29-32 °C (lit.,[263] 33-34 °C); υ_{max} (nujol)/cm^{-1} 1590 (Ar C-C) and 1180 (SO); δ_H (200 MHz; $CDCl_3$) 1.30 (3H, t, *J* 7.5 Hz, $C\underline{H}_3CH_2$), 2.43 (3H, s, $C\underline{H}_3Ar$), 4.10 (2H, q, *J* 7.5 Hz, $CH_3C\underline{H}_2$), 7.35 (2H, d, *J* 7.5 Hz, Ar) and 7.80 (2H, d, *J* 7.5 Hz, Ar); δ_C (75.47 MHz; $CDCl_3$) 14.5 ($CH_2\underline{C}H_3$), 21.4 (Ar-$\underline{C}H_3$), 66.6 ($\underline{C}H_2CH_3$), 127.7 and 129.7 (Ar), 133.2 (<u>Ar</u>-CH_3) and 144.5 (Ar-S-); *m/z* (EI) 200 (M^+, 51 %), 172 (23, $[M - C_2H_4]^+$), 155 (77, $[M - C_2H_5O]^+$), 91 (100, $[M - C_7H_7]^+$), 65 (45, $[M - C_5H_5]^+$) and 28 (21, $C_2H_2^+$).

2-Ethylacetoacetate Ethyl Ester (119 b)

Sodium (1.28 g, 55.7 mmol) was dissolved in dry ethanol (20 ml) to give sodium ethoxide, a portion (1.54 ml, 4.3 mmol) of which was placed in the reaction flask and ethyl acetoacetate (0.35 ml, 3.8 mmol) added. After 5 minutes ethyl tosylate (1.0 g, 5.0 mmol), dissolved in a minimum volume of dry ethanol was added. The reaction mixture was refluxed for 5 hours. Water (10 ml) was added and the mixture extracted with diethyl ether (3 x 30

ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo*, to give an oil which was purified by column chromatography on silica gel (15 % ethyl acetate / petroleum ether) to give 2-ethylacetoacetate ethyl ester (0.32 g, 40 %), b.p. 103-104 °C / 10 mmHg; υ_{max} (neat)/cm^{-1} 3000 (CH), 1725 (CO ester) and 1715 (CO ketone); δ_H (200 MHz; $CDCl_3$) 0.91 (3H, t, *J* 7.5 Hz, 2'-CH_3), 1.25 (3H, t, *J* 7.5 Hz, -$OCH_2C\underline{H}_3$), 1.86 (2H, qn, *J* 7.5 Hz, 1'-CH_2), 2.20 (3H, s, 4-CH_3), 3.30 (1H, t, *J* 7.5 Hz, 2-CH) and 4.18 (2H, q, *J* 7.5 Hz, -$OC\underline{H}_2CH_3$); δ_C (50.3 MHz; $CDCl_3$) 12.35 (C-2'), 14.57 ($OCH_2\underline{C}H_3$), 22.07 (C-1'), 23.87 ($\underline{C}H_3CO$), 29.28 (CH), 61.87 ($O\underline{C}H_2CH_3$), 170.29 (-CO_2-) and 173.02 (CO); *m/z* (EI) 158 (*M*$^+$, 2 %), 130 (13, [*M* - C_2H_4]$^+$), 116 (68, [*M* - C_3H_6]$^+$), 101 (55, [*M* - C_3H_5O]$^+$), 73 (69, [*M* -C_5H_9O]$^+$), 43 (100, $C_2H_3O^+$) and 29 (59, $C_2H_5^+$).

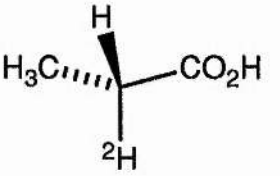
(2S)-2-Bromopropanoic Acid (160)



(2S)-Alanine (4.0 g, 45 mmol) was added to a saturated solution of potassium bromide (10 ml), followed by the dropwise addition of hydrogen bromide (15 ml of a 48 % solution). The resulting solution was, then, cooled to 0 °C and sodium nitrite (6.21 g, 90 mmol) was added over 1 hour. The reaction mixture was maintained below 5 °C for a further hour and then allowed to warm to room temperature. The resulting solution was extracted with diethyl ether (3 x 25 ml) and the combined ether extracts were then dried ($MgSO_4$) and concentrated *in vacuo* to give a pale yellow oil (6.67 g, 97 %). This was distilled under reduced pressure to give pure (2S)-2-bromopropanoic acid, b.p. 68-70 °C / 0.1 mmHg; $[\alpha]_D$ - 28.6° (c 2.0 in $CHCl_3$) (lit.,[306] -45.2 ° (neat)); υ_{max} (neat)/cm^{-1} 3000 br (CO$\underline{O}$H) and 1710 ($\underline{C}$OOH); δ_H (300 MHz; $CDCl_3$) 1.85 (3H, d, *J* 7 Hz, 3-CH_3), 4.40 (1H, q, *J* 7 Hz, 2-CH) and 9.65 (1H, s br, 1-CO_2H); δ_C (75 MHz; $CDCl_3$) 21.26 (C-3), 39.34 (C-2)

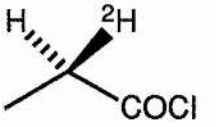
and 175.88 (CO_2H); *m/z* (EI) 153 (M^+, 25 %), 152 (26, $[M - H]^+$), 108 (56, $[M - CO_2H]^+$), 73 (51, $[M - Br]^+$), 55 (22, $[M - BrOH]^+$), 45 (73, CO_2H^+) and 28 (100, $[M - BrCO_2H]^+$).

(2R)-[2-^{2}H]-Propanoic Acid (161)



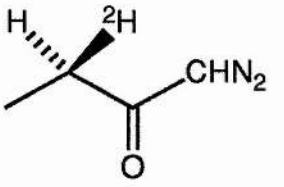
A solution of 1 M Superdeuteride in THF (14 ml, 14 mmol) was added to (2S)-2-bromopropanoic acid (0.76 g, 5 mmol) dissolved in anhydrous THF (5 ml), under nitrogen at 0 °C. The mixture was heated under reflux for 2 hours. The solution was allowed to cool and, then, the excess deuteride was decomposed by the addition of water (5 ml). The mixture was acidified (pH 1.0) and extracted with diethyl ether. The ether was removed *in vacuo* and the residue thus obtained treated with water (7 ml), followed by a 1:1 mixture of hydrogen peroxide (100 vols) and 2 M sodium hydroxide, added until efferverscence ceased (approximately 30 ml). The reaction mixture was then stirred at room temperature for 15 hours. The resulting alkaline solution was extracted with diethyl ether (5 x 20 ml) and then the aqueous portion saturated with Na_2PO_4 and sodium chloride and the pH adjusted to 6.0 with 1 M sulphuric acid. The saturated solution was extracted with diethyl ether (6 x 20 ml). The organic extract was dried ($MgSO_4$) and the excess diethyl ether removed carefully *in vacuo* to give (2R)-[2-^{2}H]-propanoic acid (0.36 g, 99 %) and triethyl borate; υ_{max} (neat)/cm^{-1} 3000 br (CO<u>O</u>H); δ_H (200 MHz; $CDCl_3$) 1.16 (3H, d, *J* 7.5 Hz, CH_3) and 2.40 (1H, m, *J* 2.5 Hz, C<u>H</u>^{2}H); δ_C (50.3 MHz; $CDCl_3$) 9.10 (CH_3), 27.91 (CH^2H) and 179.90 (CO_2H); *m/z* (GCMS, EI) 75 (M^+, 100 %), 58 (33, $[M - OH]^+$), 45 (58, CO_2H^+) and 28 (100, CO^+).

(2R)-[2-^{2}H]-Propionyl Chloride (168)



(2R)-[2-^{2}H]-Propanoic acid (0.36 g, 5 mmol) was dissolved in chloroform (20 ml) (which had been passed down an alumina column immediately prior to use) and phosphorus pentachloride (1.15 g, 5.5 mmol) was added. The mixture was stirred at reflux for 24 hours. The chloroform and propionyl chloride were co-distilled off, giving (2R)-[2-^{2}H]-propionyl chloride (0.45 g, 99 %) in chloroform; υ_{max} ($CHCl_3$)/cm^{-1} 1790 (CO); δ_H (200 MHz; $CDCl_3$) 1.22 (3H, d, *J* 7.5 Hz, CH_3) and 2.93 (1H, m, *J* 3.0 and 7.5 Hz, C<u>H</u>^{2}H); δ_C (75.47 MHz; $CDCl_3$) 9.10 (CH_3), 46.34 (CH^2H) and 174.90 (COCl); *m/z* (GCMS, EI) 93 (M^+, 1 %), 58 (41, [M - Cl]$^+$) and 30 (76, [M - COCl]$^+$).

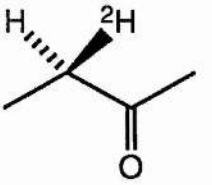
(3R)-[3-^{2}H]-1-Diazobutan-2-one (169)



(2R)-[2-^{2}H]-Propionyl chloride (0.45 g, 4.8 mmol) in chloroform (25 ml) was distilled off N,N-dimethyl aniline, to remove any phosphorus oxychloride and hydrogen chloride present. The solution was added slowly dropwise to a stirred ethereal solution of diazomethane (16.5 mmol, as determined by titration) cooled in ice. Stirring was continued for 16 hours, allowing the solution to warm up to room temperature. Potassium carbonate (0.35 g, 2.5 mmol) was added to the solution and the solvent removed *in vacuo*. The residue was dissolved in diethyl ether and the solid potassium carbonate filtered off. The diazoketone was obtained by removal of solvent, *in vacuo*,

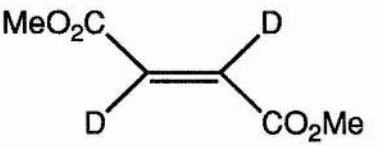
as a yellow oil (0.42 g, 76 %); υ_{max} (neat)/cm^{-1} 2105 (NN) and 1740 (CO); δ_H (200 MHz; $CDCl_3$) 1.10 (3H, d, *J* 7.5 Hz, CH_3), 2.30 (1H, m, C<u>H</u>^{2}H) and 5.22 (1H, s, CHN_2); δ_C (50.3 MHz; $CDCl_3$) 9.46 (CH_3), 54.72 (CHN_2) 34.43 (CH^2H) and 196.20 (CO); *m/z* (EI) 99 (M^+, 29 %), 69 (23, [M - N_2H_2]$^+$), 58 (82, [M - CHN_2]$^+$), 42 (26, CH_2N_2]$^+$) and 30 (100, $N_2H_2^+$).

<u>(3R)-[3-^{2}H]-Butan-2-one</u> <u>(170)</u>



(3R)-[3-^{2}H]-1-Diazobutan-2-one (0.42 g, 3.6 mmol) was dissolved in dichloromethane (5 ml). Hydroiodic acid (55 %, 2.4 ml) was added dropwise to the diazoketone solution, with shaking (care: pressure). After 5 minutes, the reaction mixture was washed with 50 % aqueous sodium thiosulphate (5 x 5 ml), until complete decolourization had occurred. The organic layer was dried ($NaSO_4$). 1,1,2-Trichloroethane was added to the filtrate to facilitate co-distillation of the butanone. Distillation gave a mixture of (3R)-[3-^{2}H]-butan-2-one and unlabelled butan-2-one (0.25 g, 95 %) in dichloromethane / 1,1,2-trichloroethane; υ_{max} (CH_2Cl_2 / 1,1,2-trichloroethane)/cm^{-1} 1710 (CO); δ_H (200 MHz; $CDCl_3$) 1.05 (3H, d, *J* 7.5 Hz, C<u>H</u>$_3$CH^{2}H), 2.14 (3H, s, $COCH_3$) and 2.44 (1H, m, *J* 7.5 and 2.5 Hz, C<u>H</u>^{2}H); δ_C (50.3 MHz; $CDCl_3$) 7.1 (<u>C</u>H$_3$CH^{2}H), 27.6 (CO<u>C</u>H$_3$) 35.2 (CH^{2}H) and 207.5 (CO); *m/z* (GCMS, EI) 73 and 74 ([M + H]$^+$, 12 %), 58 and 59 (26, [M + H - CH_3]$^+$) and 31 (100, $C_2H_3O^+$).

Dimethyl Dideuterofumarate (193)

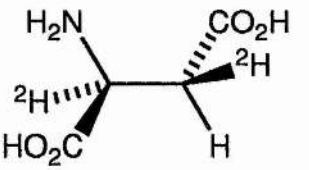


To a stirred solution of dimethyl acetylene dicarboxylic acid (5 g, 35 mmol) in anhydrous THF (72 ml) containing deuterium oxide (0.7 g, 35 mmol) was added dropwise a solution of triphenylphosphine (9.3 g, 35 mmol) in anhydrous THF (72 ml). The reaction temperature was maintained below 5 °C during the addition. The reaction mixture was then allowed to attain room temperature and heated at reflux for 5 hours. The resultant solution was dried ($MgSO_4$) and concentrated *in vacuo* to give a red-brown solid which was sublimed twice (80-100 °C / 1 mmHg) to give dimethyl dideutero-fumarate as white crystals (2.11 g, 41 %), m.p. 103-104 °C (lit.,[289] 104-105 °C); υ_{max} (nujol)/cm^{-1} 2260 (C^2H), 1730 (CO) and 1610 (CC); δ_H (270 MHz; $CDCl_3$) 3.81 (6H, s, CO_2CH_3s); δ_C (75.47 MHz; $CDCl_3$) 52.10 ($CO_2\underline{C}H_3$s), 133.12 ($\underline{C}(^2H)CO_2CH_3$s) and 165.14 ($\underline{C}O_2CH_3$s); *m/z* (EI) 146 ($M^+$, 12 %), 115 (100, $[M - CH_3O]^+$), 87 (95, $[M - C_2H_3O_2]^+$), 59 (91, $C_2H_3O_2^+$) and 28 (86, CO^+).

Dideuterofumaric Acid (194)

Dimethyl dideuterofumarate (2.0 g, 13.7 mmol) was added to 12 % aqueous sodium hydroxide solution (13 ml) and stirred at room temperature for 72 hours. The pH of the solution was adjusted to 2.0 using 3 M HCl, causing dideuterofumarate to precipitate out of solution. The solid was filtered off and recrystallized from 8 % aqueous sodium hydroxide solution with 3 M HCl to give dideuterofumaric acid as a white solid (1.19 g, 66 %), m.p. 286-287.6 °C; υ_{max} (nujol)/cm^{-1} 3400-2400 (OH), 2260 (C^2H) and 1680 (CO); δ_C (67.8 MHz; D_2O) 158 (CO_2H); *m/z* (EI) 118 (M^+, 4 %), 101 (11, $[M - OH]^+$), 73 (17, $[M - CHO_2]^+$), 57 (22, $C_2HO_2^+$) and 44 (41, CO_2^+).

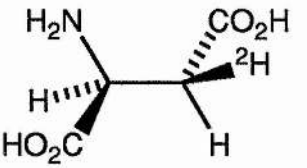
(2S,3S)-[2,3-^{2}H]-Aspartic Acid (195)



Dideuterofumaric acid (1.0 g, 8.5 mmol) was suspended in water (10 ml) and the pH adjusted to 9.0 with concentrated ammonia solution. The resulting solution was concentrated *in vacuo* to give diammonium dideuterofumarate. This was redissolved in water (10 ml) and magnesium chloride hexahydrate (20 mg, 0.01 mmol) and potassium chloride (4 mg, 0.05 mmol) was added. The pH was readjusted to 9.0 with concentrated ammonia solution. 3-Methylaspartase (900 µl, 40 units) was added and the absorbance at 240 nm measured. The reaction mixture was incubated at 30 °C until the absorbance at 240 nm remained constant.

The protein was denatured by heating at 100 °C for two minutes and removed by filtration. The filtrate was acidified to pH 1.0 with 12 M HCl and extracted with diethyl ether (2 x 7 ml). The aqueous layer was adjusted to pH 4.0 and the addition of ethanol caused the precipitation of (2S,3S)-[2,3-^{2}H]-aspartic acid. The solid was filtered off and recrystallized from hot water / ethanol (60 : 40) to give (2S,3S)-[2,3-^{2}H]-aspartic acid as a white solid (0.52 g, 51 %), m.p. >300 °C; (Found: (*M* + H)$^+$ 136.0579. $C_4H_6NO_4D_2$ requires 136.0577); $[\alpha]_D$ +25.2 ° (c 0.5 in 6 M HCl); υ_{max} (nujol)/cm^{-1} 2600 (OH) and 1700 (CO); δ_H (270 MHz; D_2O/DCl) 2.85 (1H, s, 3-C<u>H</u>^{2}H); δ_C (75.47 MHz; D_2O/DCl) 33.76 (C-3), 49.56 (C-2), 171.19 and 173.50 (CO_2Hs); *m/z* (CI) 136 ([*M* + H]$^+$, 92 %) and 45 (100, CHO_2^+).

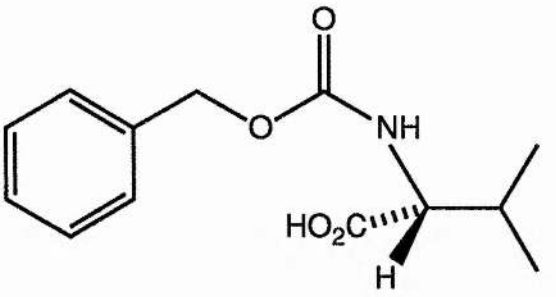
(2S,3S)-[3-^{2}H]-Aspartic acid (190)



(2S,3S)-[2,3-^{2}H]-Aspartic acid (0.75 g, 5.5 mmol) was dissolved in water (10 ml) and the pH of the solution adjusted to 7.0 with concentrated ammonia solution. Pyridoxal 5-phosphate (1 mg, 4 μmol) was added and the solution incubated with aspartate aminotransferase (1 ml, 2000 units) for 48 hours.

The enzyme was denatured by heating at 100 °C for two minutes and removed by filtration. The filtrate was acidified to pH 1.0 with 12 M HCl and the addition of ethanol caused the precipitation of (2S,3S)-[3-^{2}H]-aspartic acid. Recrystallization from hot water / ethanol (60 : 40) gave (2S,3S)-[3-^{2}H]-aspartic acid as a white solid (0.38 g, 51 %), m.p. >300 °C; (Found: (*M* + H)$^+$ 135.0518. $C_4H_7NO_4D_1$ requires 135.0514); $[\alpha]_D$ + 19.7 ° (c 1.0 in 6 M HCl); υ_{max} (nujol)/cm^{-1} 2600 (OH) and 1700 (CO); δ_H (300 MHz; D_2O/DCl) 2.85 (1H, d, *J* 2.4 Hz, 3-C$\underline{H}^2$H) and 4.12 (1H, d, *J* 2.4 Hz, 2-CH); δ_C (75.47 MHz; D_2O/DCl) 33.80 (C-3), 49.53 (C-2), 171.11 and 173.44 (CO_2Hs); *m/z* (CI) 135 ([*M* + H]$^+$, 100 %), 88 (7, [*M* - CH_2O_2]$^+$) and 45 (34, CHO_2]$^+$).

N-Carbobenzoxy-(2R)-Valine (180)

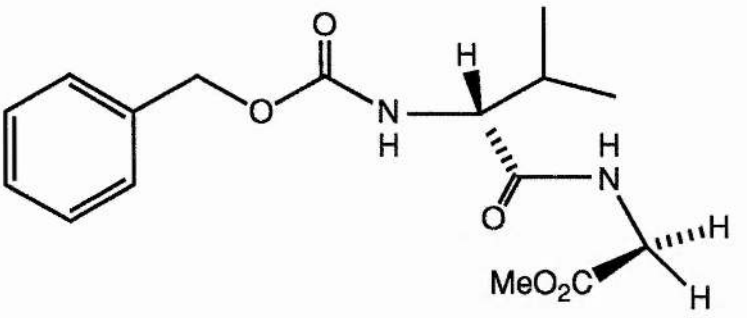


Sodium bicarbonate (12.55 g, 0.15 mol) was suspended in water (100 ml) and (2R)-valine (5.0 g, 42.7 mol) added with vigorous stirring. Benzyl chloroformate (8.0 g, 47 mol) was added in five portions over 30 minutes and the resulting mixture left stirring overnight. The mixture was extracted once with diethyl ether (25 ml). The aqueous fraction was acidified to pH 2 using 5 M HCl and extracted with ethyl acetate (3 x 25 ml). The pooled organic extracts were dried ($MgSO_4$) and the ethyl acetate removed *in vacuo* at 40 °C, to give a colourless oil which was recrystallized from diethyl ether / petroleum ether. N-Carbobenzoxy-(2R)-valine was obtained as a white solid (2.0 g, 93 %), m.p. 63-65 °C; $[\alpha]_D$ +4.2 ° (c 1.0 in acetic acid); υ_{max} (nujol)/cm^{-1} 3400 (NH), 1725 (OCOH), 1660 (OCONH) and 1550 (OCONH); δ_H (300 MHz; $CDCl_3$) 0.93 (3H, d, *J* 8 Hz, 4-CH_3), 1.02 (3H, d, *J* 8 Hz, 4-CH_3), 2.25 (1H, m, *J* 8 Hz, 3-CH), 4.38 (1H, dd, *J* 5 Hz, 2-CH), 5.13 (2H, s, PhCH_2), 5.42 (1H, d, *J* 8 Hz, 2-NH) and 7.35 (5H, s, Ph); δ_C (50.3 MHz; $CDCl_3$) 17.3 and 18.9 (CH(CH_3)$_2$), 31.0 (CH(CH_3)$_2$), 58.80 (PhCH_2) 67.16 (C-2), 128.08, 128.18, 128.48 and 136.06 (Ar), 156.42 (HNCO) and 176.48 (CO_2H); *m/z* (EI) 251 (M^+, 1 %), 126 (9, [M - $C_7H_8O_2$]$^+$), 108 (100, $C_7H_8O^+$), 91 (81, $C_7H_7^+$), 79 (89, $C_6H_7^+$), 43 (49, $C_3H_7^+$) and 28 (32, CO^+).

Glycine Methyl Ester (181)

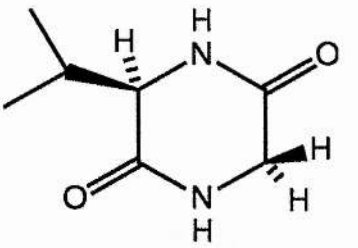
Glycine (7.0 g, 94.5 mmol) was suspended in dry methanol (70 ml) and cooled in ice. Thionyl chloride (7.7 ml, 0.105 mol) was added dropwise. The resulting solution was refluxed for 30 minutes and then allowed to return to room temperature. The excess methanol and thionyl chloride were removed *in vacuo*, leaving the required ester hydrochloride as a hygroscopic white solid (2.62 g, 78.5 %), m.p. 175 °C; υ_{max} (nujol)/cm^{-1} 1735 (CO_2Me); δ_H (300 MHz; $CDCl_3$) 3.85 (3H, s, CO_2CH_3) and 3.97 (2H, s, 2-CH_2); δ_C (50.3 MHz; D_2O) 41.1 (C-2), 54.4 ($CO_2\underline{C}H_3$) and 169.7 ($\underline{C}O_2CH_3$); *m/z* (EI) 89 (M^+, 11 %), 59 (2, $CO_2CH_3^+$) and 30 (100, CH_4N^+).

N-Carbobenzoxy-(2R)-Valine-Glycine Methyl Ester (179)



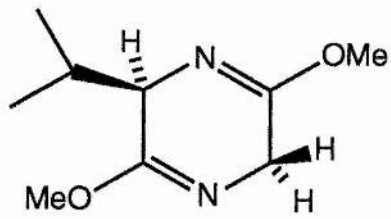
N-Carbobenzoxy-(2R)-valine (10.0 g, 40 mmol) was dissolved in THF (150 ml) and cooled to -15 °C. N-Methyl morpholine (4.35 ml, 40 mmol) was added, followed by *iso*- butyl chloroformate (5.5 ml, 40 mmol). The resulting mixture was left to stir for 3 minutes before glycine methyl ester hydrochloride (5.0 g, 40 mmol) suspended in DMF (25 ml) containing N-methyl morpholine (4.35 ml, 40 mmol) was added. Stirring was continued for 2 hours, before a further 45 ml DMF was added. The reaction mixture was stirred for a further 15 hours. Water (100 ml) was added, which caused the precipitation of N-carbobenzoxy-(2R)-valine-glycine methyl ester, which was filtered and washed with further water. Recrystallization of the white solid from dichloromethane / petroleum ether, gave N-carbobenzoxy-(2R)-valine-glycine methyl ester (10.19 g, 77 %), m.p. 151-153 °C; (Found: (*M* + H)$^+$ 323.1607. $C_{16}H_{23}N_2O_5$ requires 323.1601); $[\alpha]_D$ +8.32 ° (c 1.0 in CH_2Cl_2) (lit.,[307] +6.8 ° (c 1.0 in CH_2Cl_2); υ_{max} (nujol)/cm^{-1} 3290 (NH), 1750 (OCOCH$_3$), 1690 (OCONH), 1650 and 1530 (OCNH); δ_H (300 MHz; $CDCl_3$) 0.95 and 1.00 (6H, 2d, *J* 7 Hz, 4'-CH_3), 2.04 (1H, m, *J* 6 Hz, 3'-CH), 3.73 (3H, s, 1-OCH_3), 4.05 (1H, m, *J* 7 Hz, 2'-CH), 5.10 (2H, s, 5'-CH_2), 5.63 and 6.90 (1H, d, *J* 9 Hz, NH) and 7.35 (5H, s, 5'-Ph); δ_C (75.47 MHz; $CDCl_3$) 18.61 and 19.99 (C-4's), 31.94 (C-3'), 41.88 (C-2), 53.14 (-CO_2CH_3), 61.07 (C-2'), 67.87 (ArCH_2-), 128.80, 128.98 and 129.34 (2-,3-,4-Ar), 137.04 (Ar-1), 157.33 (-OCONH-), 170.98 and 172.63 (CO_2CH_3 / -CONH-); *m/z* (CI) 323 (*M*$^+$, 100 %), 279 (15, [*M* - CO_2]$^+$), 215 (14, [*M* - $C_7H_8O^+$), 108 (15, $C_7H_8O^+$) and 91 (10, $C_7H_7^+$).

Cyclo -(3R)-Valine-Glycine (178)



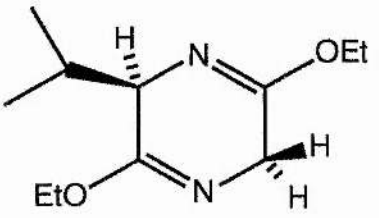
N-Cbz-(2R)-Valine-glycine methyl ester (5 g, 15.5 mmol) was dissolved in methanol / dichloromethane (25 ml / 75 ml) and 10 % Pd on charcoal (125 mg) added. The resulting suspension was stirred under an atmosphere of hydrogen, until there was no evidence of starting material remaining (as judged by tlc). The palladium on charcoal was removed by filtering through celite. The filtrate was concentrated *in vacuo* to give a white solid which was found to be a mixture of the dipeptide methyl ester and the *cyclo*-dipeptide. Cyclisation was completed by refluxing the residue in toluene (180 ml), in which it did not fully dissolve, for 12 hours. The flask was cooled to 0 °C to ensure complete precipitiation of the required product. The solid was filtered off and washed with cold diethyl ether (30 ml) to remove the toluene. The *cyclo* -(2R)-valine-glycine was recrystallized from water, and dried *in vacuo* at 60 °C for 3 days to give *cyclo* -(3R)-valine-glycine (1.91 g, 79 %), m.p. 149 °C (decomp.); $[\alpha]_D$ -27.2 ° (c 0.9 in H_2O) (lit.,[294] (3S)-isomer, + 20.2 ° (c 0.9 in H_2O), lit.,[295] (3R)-isomer, - 24.6 ° (c 0.9 in H_2O)); υ_{max} (nujol)/cm^{-1} 3180, 3040 (NH) and 1660 (OCNH); δ_H (300 MHz; D_2O) 0.74 (3H, d, *J* 8Hz, 3-CH(CH_3)$_2$), 0.82 (3H, d, *J* 8 Hz, 3-CH(CH_3)$_2$), 2.07 (1H, dsp, *J* 4 Hz and 3 Hz, 3-CH(CH_3)$_2$), 3.73 (1H, d, 3-H), 3.76 (1H, dd, *J* 19 Hz and 2.5 Hz, 6-H) and 3.95 (1H, dd, *J* 19 Hz and 2.5 Hz, 6-H); δ_C (75.47 MHz; D_2O) 18.39 and 20.71 (3-CH(CH_3)$_2$), 35.73 (3-CH(CH_3)$_2$), 51.67 (C-3), 62.74 (C-6), 171.65 and 173.09 (C-2 and C-4); *m/z* (EI) 156 (*M* $^+$, 7 %), 114 (88, [*M* - C_3H_8]$^+$), 85 (20, $C_5H_9O^+$), 72 (70, $C_4H_{10}N^+$) and 57 (100, $C_2H_3NO^+$).

(3R)-2,5-Dimethoxy-3-Isopropyl-3,6-Dihydropyrazine (182 a)



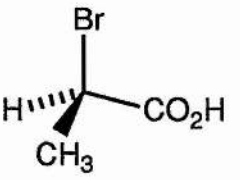
A suspension of *cyclo*- (3R)-valine-glycine (1.5 g, 9.6 mmol) and trimethyloxonium tetrafluoroborate (3.54 g, 24 mmol) in dry dichloromethane (60 ml) was vigorously stirred for 3 days, with further trimethyloxonium tetrafluoroborate (1.41 g, 9.6 mmol) being added after 24 hours. To the resulting mixture, a solution of disodium hydrogen phosphate (31.8 g) and dihydrogen sodium phosphate (8.4 g) in water (200 ml) was added. The layers were separated and the aqueous phase was extracted with dichloromethane (3 x 30 ml). The combined organic phases were dried ($MgSO_4$) and the solvent removed *in vacuo* to give an oil, which was purified by short path distillation under reduced pressure, to give (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (1.42 g, 80 %), b.p. 100-102 °C / 20 mmHg; $[\alpha]_D$ -102.6 ° (c 1.0 in ethanol) (lit.,[294] (3S)-isomer + 106.3 ° (c 1.0 in ethanol)); υ_{max} (neat)/cm^{-1} 2920 (CH) and 1680 ($N\underline{C}O\underline{C}H_3$); δ_H (300 MHz; $CDCl_3$) 0.75 and 1.03 (6H, 2d, *J* 8Hz, 3-$CH(C\underline{H}_3)_2$), 2.22 (1H,dsp, *J* 3 Hz and 8 Hz, 3-$C\underline{H}(CH_3)_2$), 3.69 and 3.73 (6H, 2s, 2,5-OCH_3) and 4.00 (3H, s, 3,6-H); δ_C (75.47 MHz; $CDCl_3$) 16.9 and 18.2 (3-$CH(\underline{C}H_3)_2$), 32.42 (3-$\underline{C}H(CH_3)_2$), 46.54 (C-6), 52.47 and 52.51 (2,5-OCH_3), 61.02 (C-3), 162.28 and 164.85 (C-2, C-5); *m/z* (EI) 184 (M^+, 2.8 %) and 169 (100, $[M - CH_3]^+$).

(3R)-2,5-Diethoxy-3-Isopropyl-3,6-Dihydropyrazine (182 b)



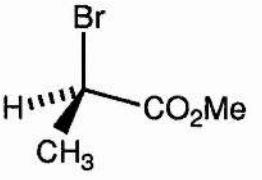
Cyclo - (3R)-valine-glycine (0.5 g, 3.2 mmol) was dissolved in dry dichloromethane (10 ml). The flask was flushed with argon and heated to a temperature of 33-35 °C. Triethyloxonium tetrafluroborate (9.6 ml of a 1 M solution in dichloromethane, 9.6 mmol) was added dropwise, with vigorous stirring. The reaction was stirred at this temperature for 10 hours. Further triethyloxonium tetrafluoroborate solution (4.8 ml, 4.8 mmol) was added and stirring at this temperature continued for 14 hours. Another portion of triethyloxonium tetrafluoroborate solution (4.8 ml, 4.8 mmol) was added and stirring at 33-35 °C continued for a further 24 hours. The solution was then added slowly dropwise to a vigorously stirred solution of sodium bicarbonate buffer (200 mM, pH 8.5, 50 ml). The pH was kept between 7.0 and 7.5 with additions of sodium hydroxide solution (10 M). The layers were then separated and the aqueous layer extracted with dichloromethane (3 x 30 ml). The combined organic layers were dried ($NaSO_4$) and the solvent removed *in vacuo* to give a brown oil which was distilled by short path distillation (40-42 °C / 15 mmHg) to give pure (3R)-2,5-diethoxy-3-isopropyl-3,6-dihydropyrazine (0.13 g, 19.6 %); (Found: (*M* + H)$^+$ 213.1603. $C_{11}H_{21}N_2O_2$ requires 213.1598); $[\alpha]_D$ -74.5 ° (c 1.0 in ethanol); υ_{max} (neat)/cm^{-1} 2920 (CH) and 1680 (N$\underline{C}\underline{O}CH_2CH_3$); δ_H (200 MHz; $CDCl_3$) 0.76 and 1.02 (6H, 2d, *J* 7 Hz, -CH(C$\underline{H}_3$)$_2$), 1.28 (6H, dt, *J* 6 Hz, C$\underline{H}_3$CH$_2$), 2.23 (1H, dsp, *J* 8 Hz, -C$\underline{H}$(CH$_3$)$_2$), 3.96 (3H, s, 3,6-H) and 4.13 (4H, m, OC$\underline{H}_2$CH$_3$); δ_C (75.47 MHz; $CDCl_3$) 14.3 ($\underline{C}H_3CH_2$), 17.1 and 19.0 (-CH($\underline{C}$H$_3$)$_2$), 32.9 ($\underline{C}$H(CH$_3$)$_2$), 46.8 (C-6), 60.7 (CH$_3\underline{C}$H$_2$O), 61.1 (C-3), 161.9 and 164.4 (OCNs); *m/z* (EI) 213 ([*M* + H]$^+$, 11 %), 169 (35, [*M* + H - CO_2]$^+$), 141 (40, $C_7H_{13}N_2O^+$), 113 (83, $C_5H_{10}NO^+$), 85 (100, $C_5H_9O^+$ / $C_4H_7NO^+$) and 56 (96, $C_2H_2NO^+$).

(2R)-2-Bromopropanoic Acid (185)



(2R)-Alanine (4.0 g, 45 mmol) was added to a saturated solution of potassium bromide (10 ml), followed by the dropwise addition of hydrogen bromide (15 ml of a 48 % solution). The resulting solution was then cooled to 0 °C and sodium nitrite (6.21 g, 90 mmol) was added over 1 hour. The reaction mixture was maintained below 5 °C for a further hour and then allowed to warm to room temperature. The resulting solution was then extracted with diethyl ether (3 x 25 ml) and the combined ether extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a pale yellow oil (6.67 g, 97 %). This was distilled by short path distillation, under reduced pressure to give pure (2R)-2-bromopropanoic acid, b.p. 68-70 °C / 0.1 mmHg; $[\alpha]_D$ +28.6 ° (c 2.0 in $CHCl_3$) (Lit.,[306,307] + 24 °, + 44.2 ° (neat)); υ_{max} (neat)/cm^{-1} 3000 br (COOH) and 1710 (COOH); δ_H (300 MHz; $CDCl_3$) 1.85 (3H, d, *J* 7 Hz, 3-CH_3), 4.40 (1H, q, *J* 7 Hz, 2-CH) and 9.65 (1H, s br, CO_2H); δ_C (75.47 MHz; $CDCl_3$) 21.26 (C-3), 39.34 (C-2) and 175.88 (CO_2H); *m/z* (EI) 153 (M^+, 25 %), 152 (26, $[M - H]^+$), 108 (56, $[M - CO_2H]^+$), 73 (51, $[M - Br]^+$), 55 (22, $[M - BrOH]^+$), 45 (73, CO_2H^+) and 28 (100, $[M - BrC_2H]^+$).

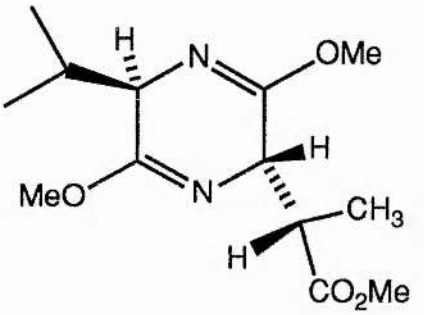
(R)-Methyl-2-Bromopropanoate (183)



To an ethereal solution of (2R)-bromopropanoic acid (5.0 g, 33 mmol) was slowly added an ethereal solution of diazomethane until the solution

remained permanently yellow. The excess diazomethane and solvent were removed *in vacuo* to give a pale yellow oil which was distilled from calcium hydride to give (R)-methyl-2-bromopropanoate as a colourless oil (4.18 g, 77 %); $[\alpha]_D$ +69.1 ° (neat) (lit.,[272] + 68.6 ° (neat)); υ_{max} (neat)/cm^{-1} 2920 (CH) and 1730 (OCOCH$_3$); δ_H (300 MHz; $CDCl_3$) 1.80 (3H, d, *J* 8 Hz, 3-CH$_3$), 3.75 (3H, s, 1-CO$_2$CH$_3$) and 4.35 (1H, q, *J* 8 Hz, 2-CH); δ_C (75.47 MHz; $CDCl_3$) 21.69 (3-CH$_3$), 39.76 (C-2) and 52.98 (CO$_2$CH$_3$); *m/z* (EI) 166 (M^+, 5 %), 135 (3, [M - CH_3O]$^+$), 107 (27, [M - $C_2H_3O_2$]$^+$), 87 (41, [M - Br]$^+$), 59 (39, $C_2H_3O_2^+$), 43 (20, $C_2H_3O^+$), 27 (100, $C_2H_3^+$) and 15 (70, CH_3^+).

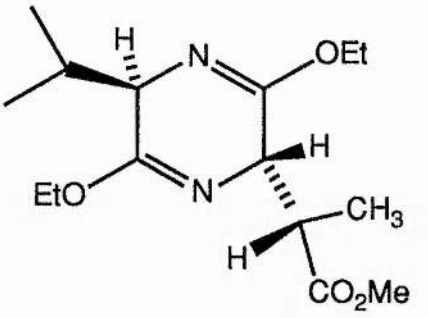
(3S,6R)-2,5-Dimethoxy-3-((2'R)-Methoxycarbonylethyl)-6-Isopropyl-3,6-Dihydropyrazine (186 a)



(3R)-2,5-Dimethoxy-3-isopropyl-3,6-dihydropyrazine (0.5 g, 2.71 mmol) was placed in a two necked flask with side arm and dissolved in anhydrous THF (2 ml). Methyl (2R)-bromopropanoate (0.68 g, 4.07 mmol) in anhydrous THF (0.5 ml) was placed in the side arm. The apparatus was flushed with argon and the solution cooled to -95 °C, using an ethanol / liquid nitrogen bath with cryocool assistance. *n*- Butyl lithium (1.55 ml of a 2.0 M solution in hexane, 3.1 mmol) was added dropwise and the solution temperature was allowed to rise to -65 °C, causing the generation of the yellow *bis*- lactim ether anion. The solution was then cooled to -100 °C and the solution of methyl (2R)-bromopropanoate in THF added to the *bis* -lactim ether anion by turning the side arm. The resulting solution was stirred for 7 hours at -100 °C and was then allowed to warm up to room temperature. The solvent was removed *in*

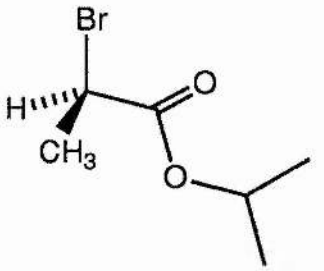
vacuo, and the residual oil was partitioned between diethyl ether (10 ml) and potassium phosphate buffer (100 mM, pH 7.0; 10 ml). The organic phase was separated, and the aqueous phase extracted with diethyl ether (2 x 10 ml). The combined organic phases were dried ($MgSO_4$) and then concentrated *in vacuo* to give a yellow oil containing both the required (3S,6R)-2,5-dimethoxy-3-((2'R)-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine and the 2,5-dimethoxy-3-propionyl-6-isopropylpyrazine (0.81 g, 99 %). This material was partially purified by eluting from a short column of triethylamine-pretreated silica with 10 % diethyl ether / petroleum ether, to give an essentially pure mixture of the two major reaction products (0.53 g, 72 %). Column chromatography, on silica gel, eluting with 25 % diethyl ether / petroleum ether, or on neutral alumina, eluting with 10 % diethyl ether / petroleum ether gave pure (3S,6R)-2,5-dimethoxy-3-((2'R)-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine as a colourless oil (0.25 g, 34 %), (Found: M^+ 270.1581. $C_{13}H_{22}N_2O_4$ requires 270.1580); $[\alpha]_D$ +23.5 ° (c 1.0 in $CHCl_3$) (lit.,[295] + 24.6 ° (c 1.0 in $CHCl_3$); υ_{max} (neat)/ cm^{-1} 2970 (CH), 1734 (CO) and 1696 (CN); δ_H (300 MHz; $CDCl_3$) 0.70 and 1.03 (6H, 2d, *J* 7 Hz, 6 -CH($C\underline{H}_3$)$_2$), 1.10 (3H, d, *J* 7 Hz, 3-(CH($C\underline{H}_3$)CO_2CH_3)), 2.25 (1H, dsp, *J* 3 and 7 Hz, 6-C$\underline{H}$(CH_3)$_2$), 2.99 (1H, dq, *J* 3 and 7 Hz, 3-(C$\underline{H}$(CH_3)CO_2CH_3)), 3.67 (9H, s br, 2,5-OCH_3, and 3-(CH(CH_3)$CO_2C\underline{H}_3$), 3.96 (1H, t, *J* 3 Hz, 6-H) and 4.40 (1H, t, *J* 3 Hz, 3-H); δ_C (75.47 MHz; $CDCl_3$) 11.88 and 17.13 (6-CH($\underline{C}H_3$)$_2$), 19.46 (3-(CH($\underline{C}H_3$)CO_2CH_3)), 32.39 (6-$\underline{C}$H(CH_3)$_2$), 43.74 (3-($\underline{C}$H(CH_3)CO_2CH_3), 49.98 (3-(CH(CH_3)$CO_2\underline{C}H_3$), 52.09 and 52.89 (2,5-OCH_3), 58.26 (C-6), 61.39 (C-3), 162.38 and 165.04 (2,5-OCN) and 174.30 ($\underline{C}O_2CH_3$); *m/z* (EI) 271 (M^+, 55 %), 239 (8, [M - CH_3O]$^+$), 227 (80, [M - CH(CH_3)$_2$]$^+$), 183 (28, [M - $C_4H_7O_2$]$^+$) and 167 (100, [M - $C_5H_{11}O_2$]$^+$).

(3S,6R)-2,5-Diethoxy-3-((2'R)-Methoxycarbonylethyl)-6-Isopropyl-3,6-Dihydropyrazine (186 b)



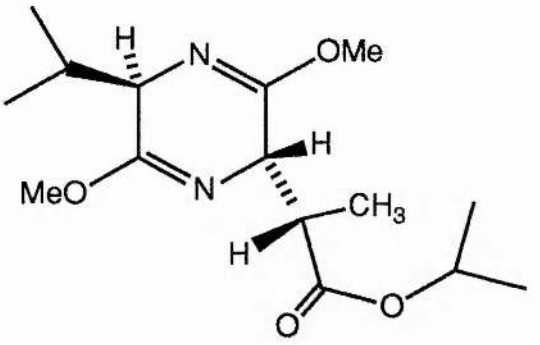
This was prepared in an identical manner to (3S,6R)-2,5-dimethoxy-3-((2'R)-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine (186 a) using the ethyl analogue (3R)-2,5-diethoxy-3-isopropyl-3,6-dihydropyrazine (182 b) (0.95 g, 5.1 mmol) as starting material, to give a yellow oil (1.25 g, 86 %) which was purified to give pure (3S,6R)-2,5-diethoxy-3-((2'R)-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine as a colourless oil (0.29 g, 20 %), (Found: (M + H)$^+$ 299.1971. $C_{15}H_{27}N_2O_4$ requires 299.1964); $[\alpha]_D$ +16.0 ° (c 1.0 in $CHCl_3$); δ_H (200 MHz; $CDCl_3$) 0.65 and 0.95 (6H, 2d, J 8 Hz, 6-CH($C\underline{H}_3$)$_2$), 1.00 (3H, d, J 7 Hz, 3 -(CH($C\underline{H}_3$)CO_2CH_3)), 1.19 (6H, 2t, J 7 Hz, 2,5-$OCH_2C\underline{H}_3$), 2.19 (1H, dsp, J 4 and 7 Hz, 6-C$\underline{H}$(CH_3)$_2$), 2.89 (1H, dq, J 4 and 7 Hz, 3-(C$\underline{H}$(CH_3)CO_2CH_3), 3.60 (3H, s, 3-(CH(CH_3)$CO_2C\underline{H}_3$), 3.86 (1H, t, J 4 Hz, 6-H), 4.05 (4H, m, J 7 Hz, 2,5-$OC\underline{H}_2CH_3$) and 4.35 (1H, t, J 4 Hz, 3-H); δ_C (75.47 MHz; $CDCl_3$) 9.98 and 13.11 (6-CH($\underline{C}H_3$)$_2$), 13.28 (CH($\underline{C}H_3$)CO_2CH_3)), 15.79 and 17.96 (2,5-$OCH_2\underline{C}H_3$), 31.09 (6-$\underline{C}$H(CH_3)$_2$), 42.55 (3-($\underline{C}$H(CH_3)CO_2CH_3), 50.53 (3-(CH(CH_3)$CO_2\underline{C}H_3$), 56.61 (C-6), 59.77 (2,5-$O\underline{C}H_2CH_3$), 59.96 (C-3), 160.43 and 163.02 (2,5-OCN) and 172.90 ($\underline{C}O_2CH_3$); *m/z* (CI) 299 ([M - H]$^+$, 100 %), 271 (4, [M - C_2H_3]$^+$), 255 (8, [M - CH(CH_3)$_2$]$^+$), 211 (4, [M - $C_4H_7O_2$]$^+$), 195 (5, [M - $C_5H_{11}O_2$]$^+$).

Isopropyl (2R)-2-Bromopropanoate (212)



(2R)-2-Bromopropanoic acid (3.0 g, 19.62 mmol) was dissolved in dry isopropanol (10 ml) and the solution cooled to 0 °C. Thionyl chloride (1.425 ml, 58.86 mmol) was added slowly dropwise. The reaction was stirred at room temperature for 5 hours. This gave isopropyl bromopropanoate, and a chloro- or hydroxy-isopropyl ester of propanoic acid, in isopropanol. The excess isopropanol was removed by careful distillation, and the remaining traces of isopropanol were removed by column chromatography on silica gel, eluting with 10 % diethyl ether / petroleum ether. This gave isopropyl (2R)-2-bromopropanoate, which was inseparable from the chloro- or hydroxy- compound (2.57 g, 67 %); δ_H (200 MHz; $CDCl_3$) 1.27 (6H, d, *J* 8 Hz, $(C\underline{H}_3)_2CH$), 1.80 (3H, d, *J* 8 Hz, 3-CH_3), 4.33 (1H, q, *J* 8 Hz, 2-CH) and 5.05 (1H, q, *J* 8 Hz, $(CH_3)_2C\underline{H}$); δ_C (50.3 MHz; $CDCl_3$) 22.02 ($(\underline{C}H_3)_2CH$), 24.09 (3-CH_3), 68.73 (2-CH) and 70.06 ($(CH_3)_2\underline{C}H$).

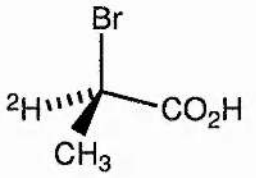
(3S,6R)-2,5-Dimethoxy-3-((2'R)-Isopropoxycarbonylethyl)-6-Isopropyl-3,6-Dihydropyrazine (213)



This was synthesized from (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (0.25 g, 1.36 mmol) in an identical manner to (3S,6R)-2,5-dimethoxy-3-((2'R)-methoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine (186 a), using isopropyl (2R)-2-bromopropanoate as the alkylating agent, to give a yellow oil containing both the required (3S,6R)-2,5-dimethoxy-3-((2'R)-isopropoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine and the 2,5-dimethoxy-3-propionyl-6-isopropylpyrazine (0.38 g, 95 %). This material was purified by column chromatography using triethylamine pretreated silica, eluting with 25 % diethyl ether / petroleum ether, to separate the required the (3S,6R)-2,5-dimethoxy-3-((2'R)-isopropoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine from the 2,5-dimethoxy-3-propionyl-6-isopropylpyrazine. Further column chromatography on silica gel, eluting with 10 % diethyl ether / petroleum ether, separated the unreacted isopropylbromopropanoate and isopropylchloro- or hydroxy- propanoate from the (3S,6R)-2,5-dimethoxy-3-((2'R)-isopropoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine, to give (3S,6R)-2,5-dimethoxy-3-((2'R)-isopropoxycarbonylethyl)-6-isopropyl)-3,6-dihydropyrazine as a colourless oil (0.05 g, 12 %), (Found: (M + H)$^+$ 299.1971. $C_{12}H_{27}N_2O_4$ requires 299.1964); $[\alpha]_D$ -35.5 ° (c 1.0 in $CHCl_3$): δ_H (200 MHz; $CDCl_3$) 0.71 and 0.93 (6H, 2d, J 8.5 Hz, 6-CH($C\underline{H}_3$)$_2$), 1.07 (3H, d, J 8.5 Hz, 3-(CH($C\underline{H}_3$)CO_2CH_3), 1.25 and 1.26 (6H, 2d, J 8.5 Hz, CO_2CH($C\underline{H}_3$)$_2$), 2.27 (1H, dsp, J 4 and 8.5 Hz, 6-C$\underline{H}$(CH_3)$_2$), 3.01 (1H, dq, J 4 and 8.5 Hz,

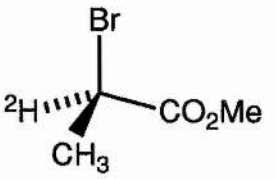
3-($CH(CH_3)CO_2CH_3$), 3.64 and 3.74 (6H, 2s, 2,5-OCH_3s), 4.00 (1H, t, *J* 4 Hz, 6-H), 4.58 (1H, t, *J* 4 Hz, 3-H) and 5.10 (1H, m, *J* 8.5 Hz, $CO_2C\underline{H}(CH_3)_2$); δ_C (75.47 MHz; $CDCl_3$) 9.89 and 16.57 (6-$CH(\underline{C}H_3)_2$), 18.90 (3-($CH(\underline{C}H_3)CO_2CH_3$)), 21.61 and 21.75 ($CO_2CH(\underline{C}H_3)_2$), 31.82 (6-$\underline{C}H(CH_3)_2$), 42.23 (3-($\underline{C}H(CH_3)CO_2CH(CH_3)_2$), 52.32 (2,5-$OCH_3$), 57.35 (C-6), 60.81 (C-3), 67.46 ($CO_2\underline{C}H(CH_3)_2$), 162.01 and 163.94 (2,5-OCN) and 173.36 (CO); *m/z* (CI) 299 ([*M* + H]$^+$, 100 %), 255 (10, [*M* - $CH(CH_3)_2$]$^+$), 239 (7, [*M* - $CH(CH_3)_2O$]$^+$), 183 (12, [*M* - $C_6H_{11}O_2$]$^+$) and 167 (7, [*M* - $C_5H_{11}O_2$]$^+$).

(2R)-[2-^{2}H]-2-Bromopropanoic Acid (203)



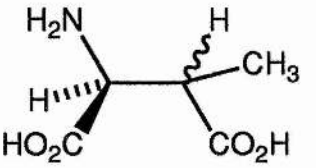
This was prepared in an indentical manner to the unlabelled (2R)-2-bromopropanoic acid (185) using (2R)-[2-^{2}H]-alanine (1.0 g, 11.3 mmol) as starting material. (2R)-[2-^{2}H]-2-Bromopropanoic acid was obtained as a pale yellow oil (1.38 g, 81 %) which was distilled by short path distillation, under reduced pressure to give pure (2R)-[2-^{2}H]-2-bromopropanoic acid, b.p. 68-70 °C / 0.1 mmHg; $[\alpha]_D$ +26.84 ° (c 2.0 in $CHCl_3$); υ_{max} (neat)/cm^{-1} 3000 br (CO$\underline{O}$H) and 1710 ($\underline{C}$OOH); δ_H (200 MHz; $CDCl_3$) 1.85 (3H, s, 3-CH_3); δ_C (75.47 MHz; $CDCl_3$) 21.28 (C-3), 39.36 (C-2) and 175.90 (CO_2H); *m/z* (EI) 154 (*M*$^+$, 30 %), 153 (29, [*M* - H]$^+$), 109 (60, [*M* - CO_2H]$^+$), 74 (55, [*M* - Br]$^+$), 56 (24, [*M* - BrOH]$^+$), 45 (72, CO_2H^+) and 29 (100, [*M* - $BrCO_2H$]$^+$).

(2R)-Methyl-[2-^{2}H]-2-Bromopropanoate (196)



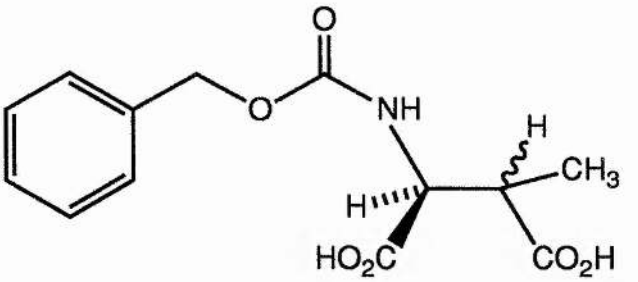
This was prepared in an indentical manner to the unlabelled (2R)-methyl-2-bromopropanoate (183) using (2R)-[2-^{2}H]-2-bromopropanoic acid (1.3 g, 8.4 mmol) as starting material. (2R)-methyl-[2-^{2}H]-2-bromopropanoate was obtained as a pale yellow oil which was distilled from calcium hydride to give a colourless oil (1.09 g, 77 %); $[\alpha]_D$ +8.74 ° (c 2.0 in $CHCl_3$); υ_{max} (neat)/cm^{-1} 1730 ($\underline{O}\underline{C}OCH_3$); δ_H (200 MHz; $CDCl_3$) 1.82 (3H, s, 3-CH_3) and 3.78 (3H, s, 1-CO_2CH_3); δ_C (75.47 MHz; $CDCl_3$) 21.67 (3-CH_3), 39.74 (C-2) and 52.96 ($CO_2\underline{C}H_3$); *m/z* (EI) 167 (M^+, 5 %), 136 (2, [M - CH_3O]$^+$), 108 (25, [M - $C_2H_3O_2$]$^+$), 88 (43, [M - Br]$^+$), 59 (37, $C_2H_3O_2^+$), 43 (20, $C_2H_3O^+$), 27 (100, $C_2H_3^+$) and 15 (73, CH_3^+).

(2S,3S/R)-3-Methylaspartic Acid (213)



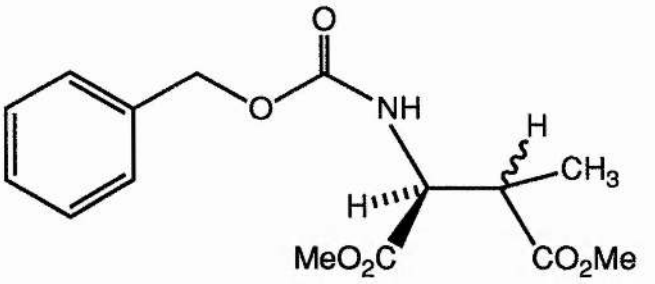
Mesaconic acid (5 g, 38.5 mmol) was dissolved in water (5 ml) and ammonia solution added to adjust the pH to 9.0. The water was removed *in vacuo* to give the ammonium salt. This was redissolved in buffer (50 ml) containing 0.2 M ammonium chloride, 20 mM magnesium chloride hexahydrate and 10 mM potassium chloride, and the pH readjusted to 9.0. 3-Methylaspartase (0.5 ml, 50 units) was added and the mixture incubated at 30 °C. The reaction was monitored by removing 0.5 ml aliquots at various time intervals, which were concentrated *in vacuo*, redissolved in deuterium oxide and their ^{1}H NMR spectra recorded. Further aliquots of enzyme were added as required, until approximately half the 3-methylaspartate produced was in the L-*erythro* -form. The enzyme was, then, denaturated by heating at 80 °C for several minutes, and filtered off. The filtrate was acidified to pH 1.0 with concentrated hydrochloric acid and extracted with diethyl ether (2 x 30 ml). The aqueous layer was concentrated *in vacuo* to give (2S,3S/R)-3-methylaspartic acid and buffer salts (3.98 g, 85.6 %); δ_H (200 MHz; D_2O) 1.20 (3H, d, *J* 7.6 Hz, (2S,3S)-CH_3), 1.31 (3H, d, *J* 7.6 Hz, (2S,3R)-CH_3), 3.01 (2H, m, *J* 7.6 and 5.0 Hz, 3-H), 3.81 (1H, d, *J* 5.0 Hz, (2S,3R)-2-H) and 4.06 (1H, d, *J* 5.0 Hz, (2S,3S)-2-H).

N-Carbobenzoxy-(2S,3S/R)-3-Methylaspartic Acid (214)



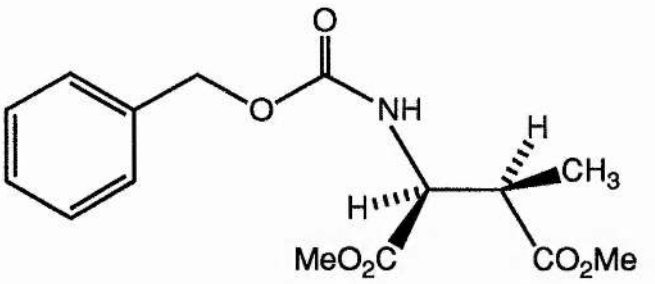
(2S,3S/R)-3-Methylaspartic acid (3.98 g, 27 mmoles) was dissolved in potassium carbonate solution (10 %, 100 ml). N-(Benzyloxycarbonyloxy)-succinimide (13.5 g, 54 mmol) was added, and the reaction stirred for 16 hours. The reaction mixture was extracted with dichloromethane (2 x 100 ml) and the aqueous layer acidified to pH 2.0 and extracted with ethyl acetate (5 x 100 ml). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil (7.53 g, 99 %), (Found: [*M* + NH_4 - H_2O]$^+$, 281.1137. [$C_{13}H_{15}NO_6$ + NH_4 - H_2O]$^+$ requires 281.1137); υ_{max} ($CHCl_3$)/cm^{-1} 3100 (CO<u>OH</u>) and 1720 (<u>CO</u>OH); δ_H (200 MHz; $CDCl_3$) 1.22 and 1.28 (6H, 2d, *J* 7.6 Hz, 3-CH_3), 3.00 (1H, m, *J* 5.0 Hz, (2S,3S)-3-H), 3.33 (1H, m, *J* 5.0 Hz, (2S,3R)-3-H), 4.58 (2H, m, *J* 3.6 Hz, 2-H), 5.07 and 5.10 (4H, 2s, Ar<u>C</u>H_2) 6.71 (2H, 2 br d, *J* 10.0 Hz, NHs) and 7.30 (10H, s, Ars); δ_C (50.3 MHz; $CDCl_3$) 13.77 and 14.03 (3-CH_3s), 41.65 ((2S,3S)-C-3), 42.52 ((2S,3R)-C-3), 55.87 and 56.04 (C-2s), 67.98 (Ar<u>C</u>H_2s), 128.54, 128.67 and 129.04 (Ars), 136.40 (Ar<u>C</u>-1), 157.12 and 157.71 (OCONHs), 175.09, 175.66 and 178.73, 179.19 (CO_2Hs); *m/z* (EI) 263 ([*M* - H_2O]$^+$, 7 %), 108 (47, $PhCH_2OH^+$), 107 (51, $PhCH_2O^+$), 91 (100, $PhCH_2^+$), 44 (37, CO_2^+); *m/z* (CI) 281 ([*M* + NH_4 - H_2O]$^+$, 60 %), 263 (14, [*M* + H - H_2O]$^+$), 192 (16, [*M* - C_2O_4H]$^+$) and 57 (6, [*M* + H - $PhC_4H_4O_6$]$^+$).

N-Carbobenzoxy-(2S,3S/R)-3-Methylaspartic Acid Dimethyl Ester (215)



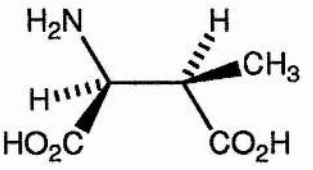
N-Carbobenzoxy-(2S,3S/R)-3-methylaspartic acid (7.53 g, 27 mmoles) was dissolved in diethyl ether (50 ml) and an ethereal solution of diazomethane was added slowly dropwise, until the solution remained permanently yellow. The excess diazomethane was removed by purging the solution with nitrogen and the solvent concentrated *in vacuo* to give a yellowish oil (8.12 g, 98 %), (Found: M^+ 309.1212. $C_{15}H_{19}NO_6$ requires 309.1212); υ_{max} ($CHCl_3$)/cm^{-1} 3420 (NH) and 1730 (CO); δ_H (200 MHz; $CDCl_3$) 1.22 (3H, d, *J* 7.5 Hz, (2S,3S)-CH_3), 1.27 (3H, d, *J* 7.5 Hz, (2S,3R)-CH_3), 3.00 (1H, m,*J* 4.0 Hz, (2S,3S)-3-H), 3.30 (1H, m, *J* 4.0 Hz, (2S,3R)-3-H), 3.67 and 3.74 (2 x 6H, 2s, CO_2CH_3s), 4.58 (1H, m, *J* 4 Hz, (2S,3R)-2-H), 4.70 (1H, m, *J* 4 and 5 Hz, (2S,3S)-2-H), 5.12 and 5.18 (2 x 2H, 2s, Ar$C\underline{H}_2$), 5.55 (1H, br d, *J* 7.6 Hz, (2S,3S)-NH), 5.70 (1H, br d, *J* 10.0 Hz, (2S,3R)-NH) and 7.35 (10H, s, Ar); δ_C (50.3 MHz; $CDCl_3$) 12.29 ((2S,3S)-3-CH_3), 14.19 ((2S,3R)-3-CH_3), 41.62 ((2S,3S)-C-3), 41.80 ((2S,3R)-C-3), 51.76 and 52.22 ((2S,3S)-$CO_2\underline{C}H_3$s), 52.62 and 53.15 ((2S,3R)-$CO_2\underline{C}H_3$), 55.44 ((2S,3S)-C-2), 56.34 ((2S,3R)-C-2), 66.70 ((2S,3S)-Ar$\underline{C}H_2$), 67.67 ((2S,3R)-Ar$\underline{C}H_2$), 127.70, 127.79, 128.12 and 135.83 ((2S,3S)-Ar), 128.53, 128.69, 129.02 and 136.66 ((2S,3R)-Ar), 155.56 ((2S,3S)-OCONH), 157.13 ((2S,3R)-OCONH), 170.67 and 173.08 ((2S,3S)-$\underline{C}O_2CH_3$s), 171.70 and 174.67 ((2S,3R)-$\underline{C}O_2CH_3$s); *m/z* (EI) 309 (M^+, 8 %), 250 (19, [M - $C_2H_3O_2$]$^+$), 206 (31, [M - $C_3H_3O_4$]$^+$) 174 (7, [M - $PhCH_2CO_2$]$^+$), 108 (41, $PhCH_2OH^+$) and 91 (100, $PhCH_2^+$).

N-Carbobenzoxy-(2S,3R)-3-Methylaspartic Acid Dimethyl Ester (217)



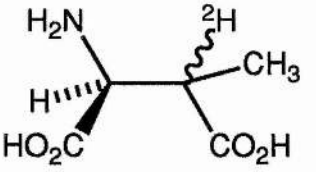
N-Carbobenzoxy-(2S,3S/R)-3-methylaspartic acid dimethyl ester (1 g, 3.4 mmoles) was dissolved in diethyl ether and adsorbed onto silica (tlc grade), this was applied to a silica chromatography column and eluted with 50 % diethyl ether / petroleum ether. N-Carbobenzoxy-(2S,3R)-3-methylaspartic acid dimethyl ester was eluted first (0.35 g, 35 %); (Found: M^+ 309.1212. $C_{15}H_{19}NO_6$ requires 309.1212); υ_{max} ($CHCl_3$)/cm^{-1} 3420 (NH) and 1730 (CO); δ_H (200 MHz; $CDCl_3$) 1.27 (3H, d, *J* 7.5 Hz, 3-CH_3), 3.30 (1H, m, *J* 4.0 Hz, 3-H), 3.67 and 3.74 (2 x 3H, 2s, CO_2CH_3s), 4.58 (1H, m, *J* 4 Hz, 2-H), 5.18 (2H, s, Ph$C\underline{H}_2$), 5.70 (1H, br d, *J* 10.0 Hz, NH) and 7.35 (5H, s, Ar); δ_C (50.47 MHz; $CDCl_3$) 14.19 (3-CH_3), 41.80 (C-3), 52.62 and 53.15 ($CO_2\underline{C}H_3$), 56.34 (C-2), 67.67 (Ar$\underline{C}H_2$), 128.53, 128.69, 129.02 and 136.66 (Ar), 157.13 (OCONH), 171.70 and 174.67 ($\underline{C}O_2CH_3$s); *m/z* (EI) 309 (M^+, 8 %), 250 (19, [M - $C_2H_3O_2$]$^+$), 206 (31, [M - $C_3H_3O_4$]$^+$) 174 (7, [M - $PhCH_2CO_2$]$^+$), 108 (41, $PhCH_2OH^+$) and 91 (100, $PhCH_2^+$).

(2S,3R)-3-Methylaspartic Acid (171)



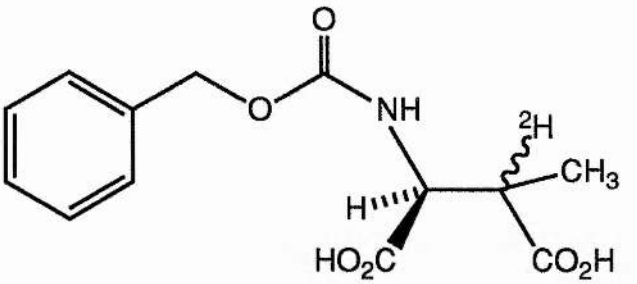
N-Carbobenzoxy-(2S,3R)-3-methylaspartic acid dimethyl ester (0.72 g, 2.33 mmol) was refluxed in concentrated hydrochloric acid / glacial acetic acid (1 : 1) (20 ml) for 2 hours. The solvent was removed *in vacuo* and the residue redissolved in water (20 ml), this was removed *in vacuo* to give the hydrochloride salt, free from excess hydrochloric acid. The residue was dissolved in the minimum volume of water and ammonia solution (0.11 ml, 2.3 mmol) was added. Addition of ethanol gave (2S,3R)-3-methylaspartic acid in the free amine form (0.19 g, 55 %), m.p. 257-259 °C (decomp.); (Found: C, 40.59; H, 6.44; N, 9.66. $C_5H_9NO_4$ requires C, 40.81; H, 6.17; N, 9.52.); (Found: $(M + H)^+$ 148.0610. $C_5H_{10}NO_4$ requires 148.0610); $[\alpha]_D$ +36.3 ° (c 1.0 in 5 M HCl) (lit.,[285] +38.7 ° (c 1.83 in 5 M HCl); lit.,[288] +32.9 ° (c 0.8 in 5 M HCl)); δ_H (200 MHz; D_2O) 1.26 (3H, d, *J* 7.5 Hz, 3-CH_3), 3.13 (1H, m, *J* 7.5 and 5.0 Hz, 3-H) and 3.95 (1H, d, *J* 5.0 Hz, 2-H); δ_C (50.3 MHz; D_2O) 15.72 (CH_3), 42.78 (C-3), 58.99 (C-2), 175.25 and 180.13 (CO_2Hs); *m/z* (CI) 148 ($[M + H]^+$, 100 %), 102 (20, $[M - CO_2H]^+$) and 58 (53, $[M - C_2O_4H]^+$).

(2S,3S/R)-[3-^{2}H]-3-Methylaspartic Acid (218)



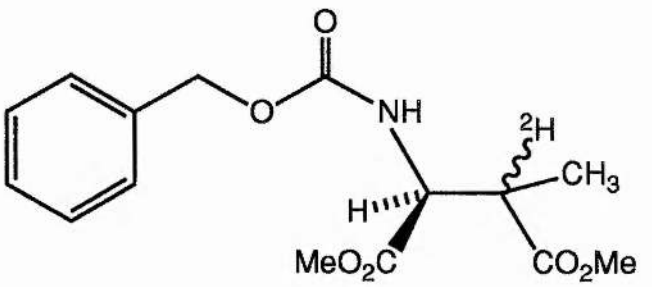
Mesaconic acid (5 g, 38.5 mmol) was dissolved in deuterium oxide (5 ml) and ammonia solution added to adjust the pD to 8.6. The deuterium oxide was removed *in vacuo* to give the ammonium salt. The buffer salts (ammonium chloride (0.535 g, 10 mmol), magnesium chloride hexahydrate (0.203 g, 1 mmol) and potassium chloride (37 mg, 0.5 mmol)) were dissolved in deuterium oxide and concentrated *in vacuo* twice before being redissolved in deuterium oxide (50 ml). The mesaconate diammonium salt was redissolved in the deuteriated buffer (50 ml, 0.2 M ammonium chloride, 20 mM magnesium chloride hexahydrate and 10 mM potassium chloride) and the pD readjusted to 8.6 with sodium deutoxide. A portion of 3-methylaspartase enzyme solution (0.5 ml, 50 units) was diluted with deuterium oxide (2 ml) and lyophilysed. The residue was dissolved in deuterium oxide (0.5 ml) and added to the buffered substrate solution. The mixture was incubated at 30 °C. The reaction was monitored by ^{1}H NMR spectroscopy of 0.5 ml aliquots removed at various time intervals. Further aliquots of enzyme solution were added as required, until approximately one third of the [3-^{2}H]-3-methylaspartic acid produced was present as the (2S,3R)-isomer. The enzyme was then denaturated by heating at 80 °C for several minutes, and filtered off. The filtrate was acidified to pD 1.0 with concentrated hydrochloric acid and extracted with diethyl ether (2 x 30 ml). The aqueous layer was concentrated *in vacuo* to give (2S,3S/R)-[3-^{2}H]-3-methylaspartic acid (97 % deuteriated) and buffer salts (4.61 g, 81.1 %); δ_H (200 MHz; D_2O) 1.20 (3H, s, (2S,3S)-CH_3), 1.31 (3H, s, (2S,3R)-CH_3), 3.81 (1H, s, (2S,3R)-2-H) and 4.06 (1H, s, (2S,3S)-2-H).

N-Carbobenzoxy-(2S,3S/R)-[3-^{2}H]-3-Methylaspartic Acid (229)



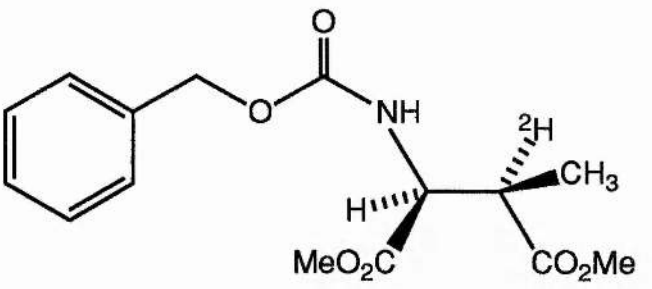
This was synthesized in an identical manner to that described for N-carbobenzoxy-(2S,3S/R)-3-methylaspartic acid (214), to give a yellow oil (8.75 g, 99.6 %), (Found: [*M* + NH_4 - H_2O]$^+$ 282.1200. [$C_{13}H_{15}NO_6$ + NH_4 - H_2O]$^+$ requires 282.1200); υ_{max} ($CHCl_3$)/cm^{-1} 3100 (CO<u>OH</u>) and 1720 (CO); δ_H (200 MHz; $CDCl_3$) 1.22 and 1.28 (6 H, 2s, 3-CH_3s), 4.60 and 4.65 (2H, 2d, *J* 5.0 Hz, 2-Hs), 5.08 and 5.11 (4H, 2s, Ar<u>C</u>H_2s) 6.91 and 6.98 (2H, 2d, *J* 5.0 Hz, NHs) and 7.30 (10H, s, Ars); δ_C (50.3 MHz; $CDCl_3$) 13.71 and 13.96 (3-CH_3s), 56.00 (C-2s), 67.98 (Ar<u>C</u>H_2s), 128.54, 128.77 and 129.04 (Ars), 136.32 (Ar-C-1), 157.01 and 157.70 (OCONHs), 175.02 and 175.65, 178.71 and 179.21 (CO_2Hs); *m/z* (CI) 282 ([*M* + NH_4 - H_2O]$^+$, 10 %), 264 (20, [*M* + H - H_2O]$^+$), 220 (12, [*M* + H - CH_2O_3]$^+$), 108 (100, $PhCH_2OH$), 91 (83, $PhCH_2$), 58 (6, [*M* + H - $PhC_4H_5O_6$]$^+$).

N-Carbobenzoxy-(2S,3S/R)-[3-^{2}H]-3-Methylaspartic Acid Dimethyl Ester (219)



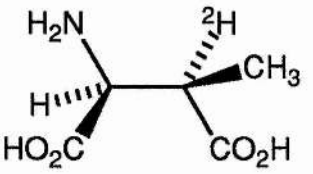
This was synthesized in an identical manner to that described for N-carbobenzoxy-(2S,3S/R)-3-methylaspartic acid dimethyl ester (215), to give a yellowish oil (7.42 g, 78 %), (Found: [*M* + H]$^+$ 311.1353. $C_{15}H_{19}DNO_6$ requires 311.1353); υ_{max} ($CHCl_3$)/cm^{-1} 3420 (NH) and 1730 (CO); δ_H (200 MHz; $CDCl_3$) 1.22 (3H, s, (2S,3S)-CH_3), 1.27 (3H, s, (2S,3R)-CH_3), and 3.76 (2 x 6H, 2s, CO_2CH_3s), 4.58 (1H, d, *J* 10 Hz, (2S,3R)-2-H), 4.70 (1H, d, *J* 10 Hz, (2S,3S)-2-H), 5.12 and 5.17 (2 x 2H, 2s, Ar$\underline{C}H_2$), 5.53 (1H, br d, *J* 10 Hz, (2S,3S)-NH), 5.71 (1H, br d, *J* 10 Hz, (2S,3R)-NH) and 7.35 (10H, s, Ar); δ_C (50.3 MHz; $CDCl_3$) 13.06 ((2S,3S)-3-CH_3), 14.12 ((2S,3R)-3-CH_3), 52.66 and 53.14 ((2S,3S/R)-$CO_2\underline{C}H_3$s), 56.27 ((2S,3S/R)-C-2), 65.15 ((2S,3S)-Ar$\underline{C}H_2$), 67.66 ((2S,3R)-Ar$\underline{C}H_2$), 128.58, 128.70, 129.02 and 136.59 ((2S,3S/R)-Ar), 156.29 ((2S,3S)-OCONH), 157.13 ((2S,3R)-OCONH), 171.43 and 171.71 ((2S,3S/R)-$\underline{C}O_2CH_3$s); *m/z* (EI) 311 ([*M* + H]$^+$, 8 %), 267 (9, [*M* + H - CO_2]$^+$), 251 (5, [*M* - CO_2CH_3]$^+$), 207 (15, [*M* - $C_3H_3O_4$]$^+$) 174 (9, [*M* - $PhCH_2CO_2$]$^+$), 108 (11, $PhCH_2OH$) and 91 (100, $PhCH_2$); *m/z* (CI) 311 ([*M* + H]$^+$, 100 %), 267 (59, [*M* + H - CO_2]$^+$), 251 (7, [*M* - CO_2CH_3]$^+$), 207 (27, [*M* - $C_3H_3O_4$]$^+$), 108 (20, $PhCH_2OH$) and 91 (77, $PhCH_2$).

N-Carbobenzoxy-(2S,3R)-[3-^{2}H]-3-Methylaspartic Acid Dimethyl Ester (230)



This was prepared in an identical manner to N-carbobenzoxy-(2S,3R)-3-methylaspartic acid dimethyl ester (217) to give N-carbobenzoxy-(2S,3R)-[3-^{2}H]-3-methylaspartic acid dimethyl ester (0.40 g, 40 %), (Found: [*M* + H]$^+$ 311.1353. $C_{15}H_{19}DNO_6$ requires 311.1353); υ_{max} ($CHCl_3$)/cm^{-1} 3420 (NH) and 1730 (CO); δ_H (200 MHz; $CDCl_3$) 1.27 (3H, s, 3-CH_3), 3.70 and 3.76 (2 x 3H, 2s, CO_2CH_3s), 4.58 (1H, d, *J* 10 Hz, 2-H), 5.17 (2H, s, ArCH_2), 5.71 (1H, br d, *J* 10 Hz, NH) and 7.35 (5H, s, Ar); δ_C (50.3 MHz; $CDCl_3$) 14.12 (3-CH_3), 52.66 and 53.14 (CO_2CH_3s), 56.27 (C-2), 67.66 (ArCH_2), 128.58, 128.70, 129.02 and 136.59 (Ar), 157.13 (OCONH), 171.43 and 171.71 (CO_2CH_3s); *m/z* (EI) 311 ([*M* + H]$^+$, 8 %), 267 (9, [*M* + H - CO_2]$^+$), 251 (5, [*M* - CO_2CH_3]$^+$), 207 (15, [*M* - $C_3H_3O_4$]$^+$) 174 (9, [*M* - $PhCH_2CO_2$]$^+$), 108 (11, $PhCH_2OH$) and 91 (100, $PhCH_2$); *m/z* (CI) 311 ([*M* + H]$^+$, 100 %), 267 (59, [*M* + H - CO_2]$^+$), 251 (7, [*M* - CO_2CH_3]$^+$), 207 (27, [*M* - $C_3H_3O_4$]$^+$), 108 (20, $PhCH_2OH$) and 91 (77, $PhCH_2$).

(2S,3R)-[3-^{2}H]-3-Methylaspartic Acid (191)



This was prepared in an identical manner to (2S,3R)-3-methylaspartic acid (171) to give (2S,3R)-[3-^{2}H]-3-methylaspartic acid in the free amine form (0.47 g, 34 %), m.p. 258-259 °C (decomp.) (Found C, 40.10; H, 6.46; N, 9.77. $C_5H_8NO_4D$ requires C, 40.54; H, 6.80; N, 9.46) (Found: [*M* + H]$^+$ 149.0673. $C_5H_9DNO_4$ requires 149.0670); $[\alpha]_D$ +30.5 °; δ_H (200 MHz; D_2O) 1.25 (3H, s, 3-CH_3) and 3.92 (1H, s, 2-H); δ_C (50.3 MHz; D_2O) 15.63 (CH_3), 58.91 (C-2), 175.27 and 180.15 (CO_2Hs); *m/z* (CI) 149 ([*M* + H]$^+$, 100 %), 131 (6, [*M* - OH]$^+$), 103 (9, [*M* - CO_2H]$^+$) and 59 (21, [*M* - C_2HO_4]$^+$).

Determination of Kinetic Parameters

Values were obtained for K_m and V_{max} for (2S,3R)-3-methylaspartic acid and (2S,3R)-[3-^{2}H]-3-methylaspartic acid at 1 mM and 50 mM potassium ion concentrations with the enzyme 3-methylaspartase.

An aliquot (20 µl) of 3-methylaspartase solution was added to assay buffer (total volume 3 ml, pH 9.0) containing (2S,3S)-3-methylaspartic acid (4 mM), Tris (0.5 M), magnesium chloride hexahydrate (20 mM) and potassium chloride (1 mM) in a 3 ml quartz cuvette (10 mm pathlength) at 30 ± 0.1 °C. The increase in absorbance at 240 nm due to the production of mesaconic acid (ε 3850 M^{-1} cm^{-1}; pH 9.0) was monitored. One unit of enzyme is defined as the amount of enzyme that converts 1 µmole of (2S,3S)-3-methylaspartic acid to 1µmole of mesaconic acid in 1 minute[9].

Solutions containing Tris (0.5 M, pH 9.0), magnesium chloride hexahydrate (20 mM), potassium chloride (1 mM) and (2S,3R)-3-methylaspartic acid (2.5, 8.0, 12.5, 20.0, 25.0, 35.0, 50.0 mM) were pre-equilibrated to 30 ± 0.1 °C. A 0.3 ml aliquot was removed and placed in a quartz cell (path length 1 mm). 3-Methylaspartase (*ca.* 0.35 units) was added to initiate the reaction which was followed spectrophotometrically at 240 nm, recording OD at 10 minute intervals. Each rate determination was performed in duplicate. Reactions were also followed using 1 ml aliquots in quartz cuvettes of 10 mm path length and *ca.* 2.3 units of enzyme. These reactions were followed continuously.

Similar reactions were monitored at 50 mM potassium ion concentration for (2S,3R)-3-methylaspartic acid concentrations between 1 and 10 mM and for (2S,3R)-[3-^{2}H]-3-methylaspartic acid at 1 and 50 mM potassium ion concentrations. The rate data was analysed by a non-linear regression program[308] and by Lineweaver-Burk plots.

Table 5.1 - Rate Data for the Deamination of (2S,3R)-3-Methylaspartic Acid at 1 mM Potassium Chloride Concentration

Substrate concentration (mM)	Rate (mol dm^{-3} min^{-1})
50	1.722×10^{-5}
50	1.692×10^{-5}
35	1.296×10^{-5}
35	1.344×10^{-5}
25	1.319×10^{-5}
20	9.536×10^{-6}
20	9.536×10^{-6}
12.5	7.185×10^{-6}
12.5	7.451×10^{-6}
8	6.901×10^{-6}
8	7.067×10^{-6}
2.5	1.597×10^{-6}
2.5	1.597×10^{-6}

Table 5.2 - Rate Data for the Deamination of (2S,3R)-3-Methylaspartic Acid at 50 mM Potassium Chloride Concentration

Substrate concentration (mM)	Rate (mol dm^{-3} min^{-1})
10	2.573×10^{-5}
10	2.677×10^{-5}
8	2.079×10^{-5}
8	2.881×10^{-5}
6	2.484×10^{-5}
6	2.228×10^{-5}
4	1.734×10^{-5}
4	1.766×10^{-5}
2	1.167×10^{-5}
2	1.137×10^{-5}
1	5.832×10^{-6}
1	5.536×10^{-6}

Table 5.3 - Rate Data for the Deamination of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid at 1 mM Potassium Chloride Concentration

Substrate Concentration (mM)	Rate (mol dm^{-3} min^{-1})
10	8.359×10^{-7}
10	8.230×10^{-7}
8	6.726×10^{-7}
8	7.389×10^{-7}
6	4.873×10^{-7}
6	7.071×10^{-7}
4	4.981×10^{-7}
4	4.292×10^{-7}
2	1.903×10^{-7}
2	1.642×10^{-7}
1	1.026×10^{-7}
1	1.162×10^{-7}

Table 5.4 - Rate Data for the Deamination of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid at 50 mM Potassium Chloride Concentration

Substrate Concentration (mM)	Rate (mol dm^{-3} min^{-1})
10	4.613×10^{-6}
10	4.675×10^{-6}
8	4.236×10^{-6}
8	4.188×10^{-6}
6	3.879×10^{-6}
6	4.110×10^{-6}
4	3.641×10^{-6}
4	3.379×10^{-6}
2	2.234×10^{-6}
2	2.261×10^{-6}
1	1.432×10^{-6}
1	1.336×10^{-6}

Following the Deamination of (2S,3R)-3-Methylaspartic Acid and (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid by ^{1}H NMR Spectroscopy

(2S,3R)-3-Methylaspartic acid (5 mg, 34 mmol) was dissolved in imidazole buffer (1 ml, 50 mM, pH 9.0) containing magnesium chloride hexahydrate (20 mM) and potassium chloride (1 mM) and concentrated *in vacuo*. The residue was dissolved in deuterium oxide (1 ml), the pD readjusted to 8.6 and the solution reconcentrated *in vacuo*. The residue was once again dissolved in deuterium oxide (1 ml). A ^{1}H NMR spectrum was recorded at T = 0 and 3-methylaspartase solution (*ca.* 5 units) added. Spectra were recorded at one hourly time intervals for 15 hours. One reaction was also performed in Tris buffer to confirm that there was no buffer effect.

Similar experiments were performed with (2S,3R)-[3-^{2}H]-3-methylaspartic acid (5 mg, 34 mmol).

A competition experiment containing deuteriated material (5 mg, 34 mmol) and non-deuteriated material (5 mg, 34 mmol) in imidazole buffer, as above, was also performed.

Equilibrium Measurements

(2S,3R)-3-Methylaspartic acid (0.25 mM solution in 0.5 M Tris buffer at pH 9.0, containing 20 mM magnesium chloride and 1 mM potassium chloride, 3 ml) was incubated with 3-methylaspartase (4 units), at 30 °C, until equilibrium was reached. Similar incubations were also carried out at 50 mM potassium chloride concentration. The experiment was repeated with (2S,3R)-[3-^{2}H]-3-methylaspartic acid. The equilibrium position for all four incubations was identical, within experimental error, at 0.95.

Double Isotope Fractionation Experiments

(2S,3R)-3-Methylaspartic acid (50 mg) was incubated in buffer solution (0.5 M Tris at pH 9.0 containing 20 mM magnesium chloride and 1 mM potassium chloride, 2 ml) with 3-methylaspartase (22 units). The reaction was followed by removing aliquots (20 µl) at various time intervals. These were added to buffer solution (0.5 M Tris at pH 9.0 containing 20 mM magnesium chloride and 1 mM potassium chloride, 3 ml) and the optical density at 240 nm measured. When 20 % conversion to mesaconic acid had occurred the reaction was quenched by the addition of concentrated hydrochloric acid (120 µl). The incubations were performed in a closed system to prevent escape of ammonia. Once the ammonium chloride produced in the quenching reaction had been absorbed into the solution, the samples were transferred to vials and freeze dried. The samples were then sent to Prof. A Jackson at the University of Southampton who distilled the ammonia produced by the deamination of (2S,3R)-3-methylaspartic acid into dilute sulphuric acid. The ammonia was then oxidised to dinitrogen using hypobromite. The ^{15}N / ^{14}N ratio was determined by mass spectrometry.

The ^{15}N / ^{14}N ratio at the beginning of the reaction was determined by Kjeldahl digestion of a sample of the substrate. To (2S,3R)-3-methylaspartic acid (10-15 mg) was added potassium sulphate (0.3 g), concentrated sulphuric acid (1.2 ml), mercury sulphate (0.3 ml) (made by dissolving red mercuric oxide (2.5 g) in concentrated sulphuric acid (3 ml) diluted to 25 ml) and anti-bumping granules. The reaction mixture was boiled for 2 hours, during which time it went clear. The solution was then cooled to room temperature and water (5 ml) added, after re-cooling, zinc dust (0.12 g) was added. The mixture was left for 30 minutes. The zinc was then filtered off and the resulting solution concentrated by freeze drying. These samples were sent to Prof. Jackson, who again extracted the ammonia, oxidised it to dinitrogen and measured the nitrogen isotope ratio.

The experiments were repeated with (2S,3R)-[3-^{2}H]-3-methylaspartic acid.

Table 5.5 ^{15}N Isotope Data from Kjeldahl Digestions and Incubations of (2S,3R)-3-Methylaspartic Acid

Sample	Atoms % XS (relative to standard 0.3663)	f	$^{15}(V/K)$
Kjedahl digestion 1	-0.0045		
Kjedahl digestion 2	-0.0025		
Kjedahl digestion 3	+0.0010		
Kjedahl digestion 4	+0.0009		
Kjedahl digestion 5	+0.0004		
Kjedahl digestion 6	+0.0003		
Incubation 1	-0.0050	0.250	1.0048
Incubation 2	-0.0022	0.207	0.9960
Incubation 3	-0.0015	0.214	1.0076
Incubation 4	-0.0011	0.207	1.0063
Incubation 5	-0.0001	0.192	1.0014
Incubation 6	+0.0002	0.208	1.0005

Table 5.6 ^{15}N Isotope Data from Kjeldahl Digestions and Incubations of (2S,3R)-[3-^{2}H]-3-Methylaspartic Acid

Sample	Atoms % XS (relative to standard 0.3663)	f	^{15}(V/K)
Kjedahl digestion 1	+0.0016		
Kjedahl digestion 2	+0.0001		
Kjedahl digestion 3	-0.0015		
Kjedahl digestion 4	-0.0018		
Kjedahl digestion 5	-0.0017		
Kjedahl digestion 6	-0.0033		
Incubation 1	-0.0020	0.195	1.0087
Incubation 2	-0.0019	0.193	1.0084
Incubation 3	-0.0028	0.198	1.0035
Incubation 4	-0.0025	0.192	1.0026
Incubation 5	-0.0026	0.175	1.0003
Incubation 6	-0.0013	0.165	0.9964

The ^{15}(V/K) effects for the deamination of (2S,3R)-3-methylaspartic acid and (2S,3R)-[3-^{2}H]-3-methylaspartic acid were calculated using the following equation, where R is the isotope ratio in the ammonia after the fraction of the reaction, f, has occurred (approx. 20 %) and R_0 is the isotope ratio in the substrate.

$$^{15}(V/K) = \log (1 - f) / \log [1 - (fR / R_0)]$$

The following nitrogen isotope effects are the averaged values. For the deamination of (2S,3R)-3-methylaspartic acid, ^{15}(V/K) = 1.0028 ± 0.0040, and for the deamination of (2S,3R)-[3-^{2}H]-3-methylaspartic acid ^{15}(V/K) = 1.0033 ± 0.0043

Prolonged Incubation of Fumaric Acid with 3-Methylaspartase

Fumaric acid (1 g, 3.2 mmol) was dissolved in deuterium oxide (5 ml) and ammonia solution added to adjust the pD to 8.6. The deuterium oxide was removed *in vacuo* to give the ammonium salt. The buffer salts (ammonium chloride (0.21 g, 4 mmol), magnesium chloride hexahydrate (81 mg, 0.4 mmol) and potassium chloride (15 mg, 0.2 mmol)) were dissolved in deuterium oxide and concentrated *in vacuo* twice before being redissolved in deuterium oxide (20 ml). The diammonium fumarate was redissolved in the deuteriated buffer (20 ml, 0.2 M ammonium chloride, 20 mM magnesium chloride hexahydrate and 10 mM potassium chloride) and the pD readjusted to 8.6 with sodium deutoxide. A portion of 3-methylaspartase solution (0.1 ml, 10 units) was diluted with deuterium oxide (1 ml) and lyophilysed. The residue was dissolved in deuterium oxide (0.1 ml) and added to the buffered substrate solution. The mixture was incubated at 30 °C for many days. ^{1}H NMR spectra were recorded for 0.5 ml aliquots of the incubation mixture at various time intervals.

Prolonged Incubation of Ethylfumaric Acid with 3-Methylaspartase

Ethylfumaric acid (100 mg, 0.7 mmol) was dissolved in water (5 ml) and concentrated ammonia solution added to adjust the pH to 9.0. The water was removed *in vacuo* to give the ammonium salt. This was redissolved in buffer (5 ml) containing ammonium chloride (50 mg, 0.93 mmol), magnesium chloride (20 mg, 0.1 mmol) and potassium chloride (3.75 mg, 0.05 mmol) and the pH readjusted to 9.0 with concentrated ammonia solution. 3-Methylaspartase (0.1 ml, 10 units) was added and the mixture incubated at 30 °C for many days. ^{1}H NMR spectra were recorded on 0.5 ml aliquots of the incubation mixture, which had been concentrated *in vacuo* and redissolved in deuterium oxide, at various time intervals.

REFERENCES

1. H. A. Barker, *Enzymologia*, 1937, **2**, 175.
2. H. A. Barker, in 'The Bacteria,' ed. I. C. Gunsalus and R. Y. Stanier, Academic Press, New York,1961, vol. 2, Ch. 3.
3. H. Ohmori, H. Ishitani, K. Sato, S. Shimizu and S. Fukui, *Biochem. Biophys. Res. Comm.*, 1971, **43**, 156.
4. H. Ohmori, H. Ishitani, K. Sato, S. Shimizu and S. Fukui, *Agr. Biol. Chem.*, 1974, **38**, 359.
5. M. Roehr, *Naturwissenschaften*, 1961, **48**, 478.
6. M. E. Maragoudakis, Y. Sekizawa, A. Baich, T. E. King and V. H. Cheldin, *Chim. Chronika*, 1963, **28**, 33.
7. S. Greenfield and G. W. Claus, *J. Bacteriol.*, 1972, **112**, 1295.
8. H. A. Barker, V. Rooze, F. Suzuki and A. A. Iodice, *J. Biol. Chem.*, 1964, **239**, 3260.
9. H. A. Barker, R. D. Smyth, R. M. Wilson and H. Weissbach, *J. Biol. Chem.*, 1959, **234**, 320.
10. C. C. Wang and H. A. Barker, *J. Biol. Chem.*, 1969, **244**, 2516.
11. W. Buckel and A. Bobi, *Eur. J. Biochem.*, 1976, **64**, 255.
12. J. T. Wachsman and H. A. Barker, *J. Biol. Chem.*, 1955, **217**, 695.
13. J. T. Wachsman, *J. Biol. Chem.*, 1956, **223**, 19.
14. A. Munch-Petersen and H. A. Barker, *J. Biol. Chem.*, 1958, **230**, 649.
15. H. A. Barker, in 'Reflections on Biochemistry,' ed. A. Kornberg, B. L. Horecker, L. Cornudella and J. Oro, Academic Press, New York, 1976, p. 75.
16. H. A. Barker, R. D. Smyth and R. M. Wilson, *Fed. Proc.*, 1958, **17**, 185.
17. H. A. Barker, R. D. Smyth and H. J. Bright, *Biochem. Prep.*, 1961, **8**, 89.
18. H. R. Whiteley, *J. Bacteriol.*, 1957, **74**, 324.
19. D. F. Horler, D. W. S. Westlake and W. B. McConnell, *Can. J. Microbiol.*, 1966, **12**, 1247.
20. W. M. Johnson and D. W. S. Westlake, *Can. J. Biochem.*, 1969, **47**, 1103.
21. W. Buckel and S. L. Miller, *Eur. J. Biochem.*, 1987, **164**, 565.
22. W. Buckel and H. A. Barker, *J. Bacteriol.*, 1974, **117**, 1248.
23. J. G. Hauge, T. E. King and V. H. Cheldin, *J. Biol. Chem.*, 1955, **214**, 11.

24. Y. Sekizawa, M. E. Maragoudakis, S. S. Kerwar, M. Flikke, A. Baich, T. E. King and V. E. Cheldelin, *Biochem. Biophys. Res Commun.*, 1962, **9**, 361.
25. M. E. Maragoudakis, Y. Sekizawa, T. E. King and V. H. Cheldin, *Biochemistry*, 1966, **5**, 2646.
26. K. Kato, M. Hayashi and T. Kamikubo, *Biochim. Biophys. Acta*, 1968, **165**, 233.
27. R. Mazumder and S. Ochoa, *Methods Enzymol.*, 1969, **13**, 198.
28. H.-F. Kung, S. Cederbaum, L. Tsai and T. C. Stadtman, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **65**, 978.
29. H. A. Barker, *Methods Enzymol.*, 1985, **113**, 121.
30. G. Brendel, J. Retey, D. M. Ashworth, K. Reynolds, F. Willenbrock and J. A. Robinson, *Angew. Chem. Int. Ed. Engl.*, 1988, **27**, 1089.
31. H. A. Lee Jr. and R. H. Abeles, *J. Biol. Chem.*, 1963, **238**, 2367.
32. K. L. Smiley and M. Sobolov, *Arch. Biochem. Biophys.*, 1962, **97**, 538.
33. C. Bradbeer, *J. Biol. Chem.*, 1965, **240**, 4675.
34. T. C. Stadtman and P. Renz, *Arch. Biochem. Biophys.*, 1968, **125**, 226.
35. E. E. Dekker and H. A. Barker, *J. Biol. Chem.*, 1968, **243**, 3232.
36. T. C. Stadtman and L. Tsai, *Biochem. Biophys. Res. Commun.*, 1967, **28**, 290.
37. J. K. Dyer and R. N. Costilow, *J. Bacteriol.*, 1970, **101**, 77.
38. Y. Tsuda and H. C. Friedman, *J. Biol. Chem.*, 1970, **245**, 5914.
39. J. M. Poston, *J. Biol. Chem.*, 1976, **251**, 1859.
40. E. Vitiols, C. Brownson, W. Gardiner and R. L. Blakley, *J. Biol. Chem.*, 1967, **242**, 3035.
41. D. Dolphin, in 'B_{12}', ed. D. Dolphin, Wiley-Interscience, 1982, p. v.
42. W. P. Murphy and G. R. Minot, *J. Am. Med. Assoc.*, 1926, **87**, 470.
43. E. L. Rickes, N. G. Brink, F. R Koniuszy, T. R. Wood and K. Folkes, *Science*, 1948, **107**, 396.
44. E. L. Smith and L. F. J. Parker, *Biochem. J.*, 1948, **43**, viii
45. D. C. Hodgkin, J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, J. G. White, R. Bonnet, J. R. Cannon, A. W. Johnson, I. Sutherland, A. Todd, E. L. Smith, *Nature,* 1955, **176**, 325.
46. H. A. Barker, H. Weissbach and R. D. Smyth, *Proc. Nat. Acad. Sci.*

U.S.A., 1958, **44**, 1093.

47. H. A. Barker, R. D. Smyth, H. Weissbach, A. Munch-Petersen, J. I. Toohey, J. N. Ladd, B. E. Volcani and R. M. Wilson, *J. Biol. Chem.*, 1960, **235**, 181.
48. P. G. Lenhert and D. C. Hodgkin, *Nature*, 1961, **192**, 937.
49. B. M. Babior, *Acc. Chem. Res.*, 1975, **8**, 376.
50. J. M. Pratt, *Chem. Soc. Rev.*, 1985, 161.
51. R. H. Abeles and D. Dolphin, *Acc. Chem. Res.*, 1976, **9**, 114.
52. H. P. C. Hogenkamp, *Biochemistry*, 1966, **5**, 417.
53. J. Retey, *Angew. Chem. Int. Ed. Engl.*, 1990, **29**, 355.
54. J. Halpern, *Science*, 1985, **227**, 869.
55. J. E. Valinsky, R. H. Abeles and J. A. Fee, *J. Am. Chem. Soc.*, 1974, **96**, 4709.
56. S. A. Cockle, H. A. O. Hill, R. J. P. Williams, S. P. Davies and M. A. Foster, *J. Am. Chem. Soc.*, 1972, **94**, 275.
57. B. M. Babior and D. C. Gould, *Biochem. Biophys. Res. Commun.*, 1970, **34**, 441.
58. B. M. Babior, T. H. Moss and D. C. Gould, *J. Biol. Chem.*, 1972, **247**, 4389.
59. G. R. Buettner and R. E. Coffman, *Biochim. Biophys. Acta*, 1977, **480**, 495.
60. Y. Zhao, P. Such and J. Retey, *Angew. Chem. Int. Ed. Engl.*, 1992, **31**, 215.
61. U. Leutbecher, S. P. J. Albracht and W. Buckel, *FEBS Lett.*, 1992, **307**, 144.
62. B. M. Babior, T. H. Moss, W. H. Orme-Johnson and H. Beinert, *J. Biol. Chem.*, 1974, **249**, 4537.
63. J. E. Valinsky, R. H. Abeles and A. S. Mildvan, *J. Biol. Chem.*, 1974, **249**, 2751.
64. P. A. Frey and R. H. Abeles, *J. Biol. Chem.*, 1966, **241**, 2732.
65. B. M. Babior, *J. Biol. Chem.*, 1969, **244**, 449.
66. O. W. Wagner, H. A. Lee Jr., P. A Frey and R. H. Abeles, *J. Biol. Chem.*, 1966, **241**, 1751.
67. B. M. Babior, T. J. Carty and R. H. Abeles, *J. Biol. Chem.*, 1974, **248**, 1689.

68. M. Flavin, P. J. Ortiz and S. Ochoa, *Nature*, 1955, **175**, 823.
69. M. Flavin and S. Ochoa, *J. Biol. Chem.*, 1957, **229**, 965.
70. J. Retey, in 'B_{12}', ed. D. Dolphin, Wiley-Interscience, 1982, vol. 2, ch. 13, p. 359.
71. S. H. G. Allen, R. W. Kellermeyer, R. Stjernholm and H. G. Wood, *J. Bacteriol.*, 1964, **87**, 171.
72. H. G. Wood, H. Lochmuller, C. Riepertinger and F. Lynen, *Biochem. Z.*, 1963, **337**, 247.
73. J. J. B. Cannata, A. Focesi Jr., R. Mazumder, R. C. Warneer and S. Ochoa, *J. Biol. Chem.*, 1965, **240**, 3249.
74. B. Zagalak, J. Retey and H. Sund, *Eur. J. Biochem.*, 1974, **44**, 529.
75. R. W. Kellermeyer, S. H. G. Allen, R. Stjernholm and H. G. Wood, *J. Biol. Chem.*, 1964, **239**, 2562.
76. R. Mazumder and S. Ochoa, *Methods Enzymol.*, 1969, **13**, 198.
77. H. Eggerer, P. Overath, F. Lynen and E. R. Stadtman, *J. Am. Chem. Soc.*, 1960, **82**, 2643.
78. R. W. Swick, *Proc. Nat. Acad. Sci. U. S. A.*,1962, **42**, 288.
79. R. W. Kellermeyer and H. G. Wood, *Biochemistry*, 1962, **1**, 1124.
80. E. F. Phares, M. V. Long and S. F. Carson, *Biochem. Biophys. Res. Commun.*, 1962, **8**, 142.
81. M. Sprecher, M. Y. Clark and D. B. Sprinson, *Biochem. Biophys. Res. Commun.*, 1964, **15**, 581.
82. J. Retey, E. H. Smith and B. Zagalak, *Eur. J. Biochem.*, 1978, **83**, 437.
83. J. Retey and B. Zagalak, *Angew. Chem. Int. Ed. Engl.*, 1973, **12**, 671.
84. H. A. Barker, *Ann. Rev. Biochem.*, 1972, **41**, 55.
85. R. L. Switzer, in 'B_{12}', ed. D. Dolphin, Wiley-Interscience, 1982, vol. 2, ch. 11, p. 291.
86. H. A. Barker, F. Suzuki, A. A. Iodice and V. Rooze, *Ann. N. Y. Acad. Sci.*, 1964, **112**, 644.
87. R. L. Switzer and H. A. Barker, *J. Biol. Chem.*, 1967, **242**, 2658.
88. F. Suzuki and H. A. Barker, *J. Biol. Chem.*, 1966, **241**, 878.
89. U. Leutbecher, R. Bocher, D. Linder and W. Buckel, *Eur. J. Biochem.*, 1992, **205**, 759.
90. E. N. G. Marsh and D. E. Holloway, *FEBS Lett.*, 1992, **310**, 167.
91. D. E. Holloway and E. N. G. Marsh, *FEBS Lett.*, 1993, **317**, 44.

92. M. Brecht, J. Kellermann and A. Pluckthun, *FEBS Lett.*, 1993, **319**, 84.
93. A. G. Lezius and H. A. Barker, *Biochemistry*, 1965, **4**, 510.
94. J. I. Toohey, D. Perlman and H. A. Barker, *J. Biol. Chem.*, 1969, **236**, 2119.
95. Y. Uchida, M. Hayashi and T. Kamikubo, *Vitamines*, 1975, **49**, 19.
96. M. Sprecher, R. L. Switzer and D. B. Sprinson, *J. Biol. Chem.*, 1966, **241**, 864.
97. R. G. Eagar, B. G. Baltimore, M. M. Herbst, H. A. Barker and J. H. Richards, *Biochemistry*, 1972, **11**, 253.
98. R. L. Switzer, B. G. Baltimore and H. A. Barker, *J. Biol. Chem.*, 1969, **244**, 5263.
99. G. Hartrampf and W. Buckel, *FEBS Lett.*,1984, **171**, 73.
100. H. F. Kung and T. C. Stadtman, *J. Biol. Chem.*, 1971, **246**, 3378.
101. L. Tsai and E. R. Stadtman, *Methods Enzymol.*, 1971, **18**, 233.
102. C. Michel, G. Hartrampf and W. Buckel, *Eur. J. Biochem.*, 1989, **184**, 103.
103. C. Michel and W. Buckel, *FEBS Lett.*, 1991, **281**, 108.
104. G. Hartrampf and W. Buckel, *Eur. J. Biochem.*, 1986, **156**, 301.
105. R. H. Abeles, A. M. Brownstein and C. H. Randles, *Biochem. Biophys. Acta*, 1960, **41**, 531.
106. T. Toraya, T. Shirakashi, T. Kosuga and S. Fukui, *Biochem. Biophys. Res. Commun.*, 1976, **69**, 475.
107. T. Toraya, E. Krodel, A. S. Mildvan and R. H. Abeles, *Biochemistry*, 1979, **18**, 417.
108. T. Toraya, K. Ushio, S. Fukui and H. P. C. Hogenkamp, *J. Biol. Chem.*, 1977, **252**, 963.
109. A. A. Poznznskaja, K. Tanizawa, K. Soda, T. Toraya and S. Fukui, *Arch. Biochem. Biophys.*, 1979, **194**, 379.
110. M. K. Essenberg, P. A. Frey and R. H. Abeles, *J. Am. Chem. Soc.*, 1971, **93**, 1242.
111. T. Toraya, M. Uesaka, M. Kondo and S. Fukui, *Biochem. Biophys. Res. Commun.*, 1973, **52**, 350.
112. T. Toraya, M. Uesaka and S. Fukui, *Biochemistry*, 1974, **13**, 3895.
113. T. Toraya and S. Fukui, *Biochem. Biophys. Res. Commun.*, 1969, **36**, 469.

114. T. Toraya, M. Kondo, Y. Isemura and S. Fukui, *Arch. Biochem. Biophys.*, 1979, **194**, 379.
115. T. Toraya, Y. Sugimoto, Y. Tamao, S. Shimizu and S. Fukui, *Biochemistry*, 1971, **10**, 3475.
116. R. G. Eagar Jr., W. W. Bachovchin and J. H. Richards, *Biochemistry*, 1975, **14**, 5523.
117. W. W. Bachovchin, R. G. Eagar Jr., K. W. Moore and J. H. Richards, *Biochemistry*, 1977, **16**, 1082.
118. B. Zagalak, P. A. Frey, G. L. Karabatsos and R. H. Abeles, *J. Biol. Chem.*, 1966, **241**, 3028.
119. P. A. Frey, G. L. Karabatsis and R. H. Abeles, *Biochem. Biophys. Res. Commun.*, 1965, **18**, 551.
120. A. M. Brownstein and R. H. Abeles, *J. Biol. Chem.*, 1961, **236**, 1199.
121. J. Retey, A. Umani-Rochi, J. Seibl and D. Argoni, *Experimentia*, 1966, **22**, 502.
122. J. E. Valinsky and R. H. Abeles, *Arch. Biochem. Biophys.*, 1975, **166**, 608.
123. Y. Tamao, Y. Morikawa, S. Shimizu and S. Fukui, *Biochem. Biophys. Acta*, 1968, **151**, 260.
124. T. Toraya, K. Ohashi, H. Ueno and S. Fukui, *Bioinorg. Chem.*, 1975, **4**, 245.
125. T. Toraya and R. H. Abeles, *Arch. Biochem. Biophys.*, 1980, **203**, 174.
126. G. N. Schrauzer and J. W. Sibert, *J. Am. Chem. Soc.*, 1970, **82**, 1022.
127. P. A. Frey, M. K. Essenberg, R. H. Abeles and S. S. Kerwar, *J. Am. Chem. Soc.*, 1970, **92**, 4488.
128. C. Bradbeer, *J. Biol. Chem.*, 1965, **240**, 4669.
129. B. H. Kaplan and E. R. Stadtman, *J. Biol. Chem*, 1968, **243**, 1787.
130. B. H. Kaplan and E. R. Stadtman, *J. Biol. Chem*, 1968, **243**, 1794.
131. O. C. Wallis, A. W. Johnson and M. F. Lappert, *FEBS Lett.*, 1979, **97**, 196.
132. B. M. Babior and T. K. Li, *Biochemistry*, 1969, **8**,154.
133. J. Retey, C. J. Suckling, D. Arigoni and B. M. Babior, *J. Biol. Chem.*, 1974, **249**, 6359.
134. T. J. Carty, B. M. Babior and R. H. Abeles, *J. Biol. Chem.*, 1974, **249**, 1683.

135. P. Diziol, H. Haas, J. Retey, S. W. Graves and B. M. Babior, *Eur. J. Biochem.*, 1980, **106**, 221.
136. S. W. Graves, J. S. Krouwer and B. M. Babior, *J. Biol. Chem.*, 1980, **255**, 7444.
137. D. A. Weisblat and B. M. Babior, *J. Biol. Chem.*, 1971, **246**, 6064.
138. S. W. Graves, J. A. Fox and B. M. Babior, *Biochemistry*, 1980, **19**, 3630.
139. M. R. Hollaway, H. A White, K. N. Joblin, A. W. Johnson, M. F. Lappert and O. C. Wallis, *Eur. J. Biochem.*, 1978, **82**, 143.
140. B. M. Babior, *Biochim. Biophys. Acta*, 1968, **167**, 456.
141. B. M. Babior, *J. Biol. Chem.*, 1970, **245**, 6125.
142. P. Y. Law, D. G. Brown, E. L. Lein, B. M. Babior and J. M. Wood, *Biochemistry*, 1971, **10**, 3428.
143. B. Babior, in 'B_{12}', ed. D. Dolphin, Wiley-Interscience, 1982, vol. 2, ch. 10, p. 282.
144. T. P. Chirpich, V. Zappia, R. N. Costilow and H. A. Barker, *J. Biol. Chem.*, 1970, **245**, 1778.
145. V. Zappia and H. A. Barker, *Biochem. Biophys. Acta*, 1970, **207**, 505.
146. J. J. Baker and T. C. Stadtman in 'B_{12}', ed. D. Dolphin, Wiley-Interscience, 1982, vol. 2, ch. 8, p. 206.
147. R. M. Petrovich, F. J. Ruzicka, G. H. Reed and P. A. Frey, *J. Biol. Chem.*, 1991, **266**, 7656.
148. C. G. D. Morley and T. C. Stadtman, *Biochemistry*, 1970, **9**, 4890.
149. J. J. Baker, C. van der Drift and T. C. Stadtman, *Biochemistry*, 1973, **12**, 1054.
150. J. J. Baker, *Fed. Proc.*, 1973, **32**, 2126.
151. T. C. Stadtman, *Adv. Enzymol.*, 1973, **38**, 413.
152. W. J. McGahren, B. A. Hardy, G. O. Morton, F. M. Lovell, N. A. Pekinson, R. T. Hargreaves, D. B. Borders and G. A. Ellestad, *J. Org. Chem.*, 1981, **46**, 792.
153. M. L. Moss and P. A. Frey, *J. Biol. Chem.*, 1990, **265**, 18112.
154. P. A. Frey, *Chem. Rev.*, 1990, **90**, 1343.
155. M. D. Ballinger, P. A. Frey and G. H. Reed, *Biochemistry*, 1992, **31**, 10782.
156. D. J. Aberhart, S. J. Gould, H.-J. Lin, T. K. Thirubengadam and B. H.

Weiller, *J. Am. Chem. Soc.*, 1983, **105**, 5461.
157. T. C. Stadtman and P. Renz, *Fed. Proc.*, 1967, **26**, 343.
158. J. M. Poston, *Science*, 1977, **195**, 301.
159. J. M. Poston and B. A. Hemmings, *J. Bacteriol.*, 1979, **140**, 1013.
160. K. U. Ingold, in 'Organic Free Radicals', ed. W. A. Prior, Am. Chem. Soc. Symposium Series 69, 1978, ch. 11, p. 198.
161. A. G. Davies, B. T. Golding, R. S. Hay-Motherwell, S. Mwesigye-Kibende, D. N. R. Rao and M. C. R. Symons, *J. Chem. Soc. Chem. Comm.*, 1988, 378.
162. P. Dowd, M. Shapiro and J. Kang, *Tetrahedron*, 1984, **40**, 3069.
163. P. Dowd and M. Shapiro, *Tetrahedron*, 1984, **40**, 3063.
164. S. Wollowitz and J. Halpern, *J. Am. Chem. Soc.*, 1988, **110**, 3112.
165. J. N. Lowe and L. L. Ingraham, *J. Am. Chem.Soc.*, 1971, **93**, 3801.
166. H. Flohr, W. Pannhorst and J. Retey, *Angew. Chem. Int. Ed. Engl.*, 1976, **15**, 561.
167. P. Dowd, S.-C. Choi, F. Duah and C. Kaufman, *Tetrahedron*, 1988, **44**, 2137.
168. Y. Murakami, Y. Hisaeda and T. Ohno, *Chem. Lett.*, 1987, 1357.
169. Y. Murakami, Y. Hisaeda and T. Ohno, *J. Chem. Soc. Chem. Commun.*, 1988, 856.
170. Y. Murakami, *Trends Biotech.*, 1992, **10**, 170.
171. 'Enzyme Nomenclature', International Union of Biochemistry, Academic Press, 1984, p 428.
172. J. M. Merrick and S. Roseman, *J. Biol. Chem.*, 1960, **325**, 1274.
173. N. Tudball and J. G. O'Neill, *Microbios.*, 1975, **13**, 217.
174. A. J. L. Cooper and A. Meister, *Biochem. Biophys. Res. Commun.*, 1973, **55**, 780.
175. T. J. Carty, B. M. Babior and R. H. Abeles, *J. Biol. Chem.*, 1971, **246**, 6313.
176. R. N. Costilo and L. Laycock, *J. Biol. Chem.*, 1971, **246**, 6655.
177. J. C. Rabinowitz and W.E. Pricer, *J. Am. Chem. Soc.*, 1956, **78**, 5702.
178. E. R. Vagelos, J. M. Earl and E. R. Stadtman, *J. Biol. Chem.*, 1959, **234**, 490.
179. E. Y. Levin and D. L. Coleman, *J. Biol. Chem.*, 1967, **242**, 4248.
180. N. J. MacLeod and J. B. Pridham, *J. Biol. Chem.*, 1963, **246**, 45.

181. A. I. Virtenen and J. Tarnanen, *Biochem. Z.*, 1932, **250**, 193.
182. H. Tabor, A. H Meyer, O. Hayaishi and J. White, *J. Biol. Chem.*, 1952, **196**, 121.
183. J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, **236**, 2692.
184. R. P. Cook and B. Woolf, *Biochemical J.*, 1928, **22**, 474.
185. Y. Kurata, *Exp. Cell Res.*,1962, **28**, 424.
186. F. Salvatore, V. Zappia and C. Costa, *Comp. Biochem. Physiol.*, 1965, **16**, 303.
187. S. Sazuki, J. Yamagushi and M. Tokushige, *Biochem. Biophys. Acta*, 1973, **321**, 369.
188. J. S. Takagi, N. Ida, M. Tokushige, M. Sakamoto and Y. Shimura, *Nucl. Acids Res.*, 1985, **13**, 2063.
189. T. F. Emery, *Biochemistry*, 1963, **2**, 1041.
190. W. E. Karsten, R. B. Gates and R. E. Viola, *Biochemistry*, 1986, **25**, 1299.
191. V. R. Williams and D. J. Lartigue, *Methods Enzymol.*, 1969, **13**, 354.
192. R. H. Depue and A. G. Moat, *J. Bacteriol.*, 1961, **82**, 383.
193. K. Mizuta and M. Tokushige, *Biochem. Biophys. Acta*, 1975, **403**, 221.
194. N. Ida and M. Tokushige, *J. Biochem.*, 1984, **96**, 1315.
195. N. Ida and M. Tokushige, *J. Biochem.*, 1985, **98**, 793.
196. I. I. Nuiry, J. D. Hermes, P. D. Weiss, C-Y Chan and P. J. Cook, *Biochemistry*, 1984, **23**, 5168.
197. D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, 1980, **255**, 4772.
198. S. J. Field and D. W. Young, *J. Chem. Soc. Perkin Trans. I*, 1983, 351.
199. N. W. Cornell and C. A. Villee, *Comp. Biochem. Physiol.*, 1968, **27**, 603.
200. T. G. Lessie and F. C. Neidhart, *J. Bacteriol.*, 1967, **93**, 1800.
201. K. R. Hanson and E. A. Havir, 'The Enzymes', ed. P. D. Boyer, Academic Press, NY, 1972, **7**, p. 75-163.
202. M. M. Rechler, *J. Biol. Chem.*, 1969, **244**, 551.
203. A. Peterkofsky, *J. Biol. Chem.*, 1962, **237**, 787.
204. T. A. Smith, F. H. Cordelle and R. H. Abeles, *Arch. Biochem. Biophys.*, 1967, **120**, 724.
205. R. B. Wickner, *J. Biol. Chem.*, 1969, **244**, 6550.
206. I. Givot, T. Smith and R. Abeles, *J. Biol. Chem.*, 1969, **244**, 6341.

207. T. Furata, H. Takahashi and Y. Kasuya, *J. Am. Chem. Soc.*, 1990, **112**, 3633.
208. T. Furata, H. Takahashi, H. Shibasaki and Y. Kasuya, *J. Biol. Chem.*, 1992, **267**, 12600.
209. M. R. Young, G. H. N. Towers and A. C. Neish, *Can. J. Botany*, 1966, **44**, 341.
210. R. J. Bandoni, K. Moore, P. V. Stubba Rao and G. H. N. Towers, *Phytochemistry*, 1968, **7**, 205.
211. K. Hahlbrock and D. Scheel, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1989, **40**, 347.
212. E. A. Havir and K. R. Hanson, *Biochemistry*, 1973, **12**, 1583.
213. E. A. Havir and K. R. Hanson, *Biochemistry*, 1975, **14**, 1620.
214. E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1904.
215. E. A. Havir and K. R. Hanson, *Methods Enzymol.*, 1970, 575.
216. K. R. Hanson, E. A. Havir and C. Ressler, *Biochemistry*, 1979, **18**, 1431.
217. N. Amrhein and K. H. Godeke, *Plant Sci. Lett.*, 1977, **8**, 313.
218. B. Laber, H. H. Kiltz and N. Amrhein, *Z. Naturforsch.C: Biosci.*, 1986, **41**, 9.
219. J. Zon and N. Amrhein, *Liebigs Ann. Chem.*, 1992, 625.
220. K. R. Hanson and E. A. Havir, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 1969, **28**, 602.
221. K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1.
222. J. Zon and B. Laber, *Phytochemistry*, 1988, **27**, 711.
223. W. W. Cleland, *Bioorganic Chemistry*, 1987, **13**, 217.
224. O. Adachi, K. Matsushita, E. Shinagawa and M. Ameyama, *Agric. Biol. Chem.*, 1990, **54**, 2839.
225. M. W. Hsiang and H. J. Bright, *J. Biol. Chem.*, 1967, **242**, 3079.
226. M. W. Hsiang and H. J. Bright, *Methods Enzymol.*, 1969, **13**, 347.
227. M. W. Hsiang and H. J. Bright, *Fed. Proc.*, 1967, **26**, 605.
228. W. T. Wu, Ph. D. Thesis, Louisiana State University, 1968.
229. M. A. Cohen, Ph, D. Thesis, Southampton University, 1989.
230. W. T. Wu and V. R. Williams, *J. Biol. Chem.*, 1968, **243**, 5644.
231. S. K. Goda, N. P. Minton, N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 10747.

232. V. R. Williams and W. Libano, *Biochim. Biophys. Acta*, 1966, **118**, 144.
233. H. J. Bright, *Biochemistry*, 1967, **6**, 1191.
234. H. J. Bright and L. L. Ingraham, *Biochim. Biophys. Acta*, 1960, **44**, 586.
235. H. J. Bright and R. Silverman, *Biochim. Biophys Acta*, 1964, **81**, 175.
236. H. J. Bright, *J. Biol. Chem.*, 1965, **240**, 1198.
237. G. A. Fields and H. J. Bright, *Biochemistry*, 1970, **9**, 3801.
238. H. Bright, *J. Biol. Chem.*, 1964, **239**, 2307.
239. C. Walsh, in 'Enzymatic Reaction Mechanisms', W. H. Freeman, San Francisco, 1979, Ch. 18.
240. M. F. Winkler and V. R. Williams, *Biochim. Biophys. Acta*, 1967, **140**, 287.
241. M. Akhtar, N. P. Botting, M. A. Cohen and D. Gani, *Tetrahedron*, 1987, **43**, 5899.
242. M. Akhtar, M. A. Cohen and D. Gani, *J. Chem. Soc. Chem. Commun.*, 1986, 1290.
243. N. P. Botting, M. Akhtar, M. A. Cohen and D. Gani, *Biochemistry*, 1988, **27**, 2953.
244. N. P. Botting, M. A. Cohen, M. Akhtar and D. Gani, *Biochemistry*, 1988, **27**, 2956.
245. N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 1509.
246. N. P. Botting, A. A. Jackson and D. Gani, *J. Chem. Soc. Chem. Commun.*, 1989, 1583.
247. N. P. Botting and D. Gani, unpublished results.
248. N. P. Botting, M. Akhtar, C. H. Archer, M. A. Cohen, N. R. Thomas, S. Goda, N. P. Minton and D. Gani, in 'Molecular Recognition: Chemical and Biochemical Problems II', ed. S. M. Roberts, RSC, 1992.
249. H. A. Barker, R. D. Smyth, E. J. Wawszkiewicz, M. N. Lee and R. M. Wilson, *Arch. Biochem. Biophys.*, 1958, **78**, 468.
250. H. A. Barker, in 'Enzymes', ed. P. D. Boyer, 3rd ed. vol. 6, p. 509.
251. C. C. Wang and H. A. Barker, *J. Biol. Chem.*, 1969, **244**, 2527.
252. A. I. Vogel, in 'A Textbook of Quantitative Inorganic Analysis, Including Elementary Instrumental Analysis', Longmans, 1961, 3rd Edition., p. 784.
253. U. K. Laemmeli, *Nature*, 1970, **227**, 680.

254. B. Hartzoulakis, personal communication.
255. P. Walden, *Chem. Ber.*, 1891, **24**, 2035.
256. C. Rappe, *Acta Chem. Scand.*, 1963, **17**, 2766.
257. O. E. Edwards and M. Lesage, *Can. J. Chem.*, 1963, **41**, 1592.
258. H. C. Brown and G. Zweifel, *J. Am. Chem. Soc.*, 1960, **82**, 3222.
259. A. I. Meyers and D. L. Temple, *J. Am. Chem. Soc.*, 1970, **92**, 6644.
260. K. Barlos, P. Mamos, D. Papaioannou, S. Patrianakou, *J. Chem. Soc., Chem. Commun.*, 1987, 1583.
261. K. Barlos, D. Papaioannou and D. Theodoropoulos, *J. Org. Chem.*, 1982, **47**, 1324.
262. I. Muramatsu, M. Murakami, T. Yoneda and A. Hagitani, *Bull. Chem. Soc. Jpn.*, 1965, **38**, 244.
263. Dictionary of Organic Compounds, ed. J. R. A. Pollock and R. Stevens, Chapman and Hall, New York, 1982, 5th Edition, vol. 4, p. 3749.
264. F. A. Loewus, F. H. Westheimer and B. Vennesland, *J. Am. Chem. Soc.*, 1953, **75**, 5018.
265. D. Arigoni and E. L. Eliel, in 'Topics in Stereochemistry', ed. N. L. Allinger and E. L. Eliel, Interscience, New York, 1969, vol 4, p. 155.
266. H. Weber, PhD Dissertation No. 3591, ETH, Zurich, Switzerland, 1965.
267. H. Weber, J. Seibl and D. Arigoni, *Helv. Chim. Acta*, 1966, **49**, 741.
268. A. Steitwieser and M. R. Granger, *J. Org. Chem.*, 1967, **32**, 1528.
269. H. R. Levy, F. A. Loewus and B. Vennesland, *J. Am. Chem. Soc.*, 1959, **79**, 2949.
270. H. Gunther, M. A. Alizade, M. Kellner. F. Biller and H. Simon, *Z. Naturforsch.*, 1973, **28**, 241.
271. W. A. Cowdrey, E. D. Hughes and C. K. Ingold, *J. Am. Chem. Soc.*, 1937, **59**, 1208.
272. H. C. Brown and S. Krishnamurthy, *J. Am. Chem. Soc.*, 1973, **95**, 1669.
273. W. L. F. Armarego, B. A. Milloy and W. Pendergast, *J. Chem. Soc. Perkin Trans 1*, 1976, 2229.
274. S. Brandange and L. Morch, *Acta Chem. Scand,. Ser. B*, 1979, **33**, 776.

275. D. J. Prescott and J. L. Rabinowitz, *J. Biol. Chem.*, 1968, **243**, 1551.
276. L. F. Fieser and M. Fieser, in 'Reagents for Organic Synthesis', John Wiley and Sons, New York, 1967, vol. 1.
277. Dictionary of Organic Compounds, ed. J. R. A. Pollock and R. Stevens, Chapman and Hall, New York, 1982, vol. 5.
278. Vogel's Textbook of Practical Organic Chemistry, ed. B. S. Furniss, A. J. Hannaford. P. W. G. Smith and A. R. Tatchell, Longman Scientific and Technical, 1989, 5th Edition, p. 692.
279. J. B. Lee, *J. Am. Chem. Soc.*, 1966, **88**, 3440.
280. Vogel's Textbook of Practical Organic Chemistry, ed. B. S. Furniss, A. J. Hannaford. P. W. G. Smith and A. R. Tatchell, Longman Scientific and Technical, 1989, 5th Edition, p. 1073.
281. W. Kirmse and L. Horner, *Ann.*, 1959, **625**, 34.
282. Dictionary of Organic Compounds, ed. J. R. A. Pollock and R. Stevens, Chapman and Hall, New York, 1982, vol. 3.
283. Vogel's Textbook of Practical Organic Chemistry, ed. B. S. Furniss, A. J. Hannaford. P. W. G. Smith and A. R. Tatchell, Longman Scientific and Technical, 1989, 5th Edition, p. 887.
284. N. P. Botting, unpublished observations.
285. D. Seebach and D. Wasmuth, *Angew. Chem. Int. Ed. Engl.*, 1981, **20**, 971.
286. U. Schollkopf, *Topics in Curr. Chem.*, 1983, **109**, 65.
287. U. Schollkopf, J. Nozulak and M. Grauert, *Synthesis*, 1985, 55.
288. S. Hanessian, K. Sumi and B. Vanasse, *Syn. Lett.*, 1992, 33.
289. E. M. Richards, J. C. Tebby. R. S. Ward and D. H. Williams, *J. Chem. Soc. (C)*, 1969, 1542.
290. V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, 1932, **98**, 295.
291. S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, 1952, **194**, 455.
292. M. Wilchek and A. Patchornik, *J. Org. Chem.*, 1963, **28**, 1874.
293. G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Org. Chem.*, 1967, **89**, 5013.
294. U. Schollkopf, U. Groth and C. Deng, *Angew. Chem. Int. Ed. Engl.*, 1981, **20**, 798.
295. N. R. Thomas, Ph. D. Thesis, University of Southampton, 1990.

296. A. Paquet, *Can. J. Chem.*, 1982, **60**, 976.
297. A. Fersht, in 'Enzyme Structure and Mechanism,' W. H. Freeman, New York, 1985, 2nd Edition.
298. W.-B. Chiao and W. H. Saunders, *J. Am. Chem. Soc.*, 1977, **99**, 6699.
299. Y.-T. Tao and W. H. Saunders, *J. Am. Chem. Soc.*, 1983, **105**, 3183.
300. M. H. O'Leary, in 'Isotope Effects on Enzyme-Catalysed Reactions,' eds. W. W. Cleland, M. H. O'Leary and D. B. Northrop, University Park Press, Baltimore, 1977, pp. 233-251.
301. R. H. Burris, *Methods Enzymol.*, 1957, **4**, 355.
302. P. Fresenius, 'Organic Chemical Nomenclature', John Wiley and Sons, New York, 1988.
303. W. C. Still, M. Kahn and A. J. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.
304. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.
305. D. D. Perrin, W. L. F. Armarego and D. R. Perrin, 'Purification of Laboratory Chemicals', Permagon Press, Oxford, 1980, 2nd Edition.
306. E. Fischer, *Chem. Ber.*, 1907, **40**, 489.
307. J. E. Rose, Ph. D. Thesis, University of St. Andrews, 1992.
308. R. J. Leatherbarrow, Enzfitter, A non-linear regression data analysis program (from the Chemistry Department, Imperial College of Science, Technology and Medicine, London, SW7 2AY, U. K.) published and distributed by Biosoft, Cambridge, CB2 1JP, U. K., 1987.

Appendix 1

Recombinant 3-methylaspartase was used, when it became available, as the preparations had a higher specific activity. The recombinant enzyme was prepared, by Dr Mahmoud Akhtar, utilizing the method below.

Grow Up and Purification of Recombinant 3-Methylaspartase

L-Broth (3 l) was autoclaved and allowed to cool. L-Broth (1 l) contains sodium chloride (10 g), Bacto-yeast extract (5 g) and Bacto-tryptone (10 g) and has a pH of 7.5.

Ampercillin was added to a concentration of 50 mg/l and the broth innoculated with cells containing plasmid, pSG4. The mixture was shaken at 37 °C for 16 hours. The cells were then harvested.

Cell paste (10 g) was suspended in 50 mM potassium phosphate buffer (20 ml, pH 7.6) containing mercaptoethanol (1 mM) and the protease inhibitor phenylmethanesulphonyl fluoride (PMSF) (200 µl of a 34 mg ml^{-1} solution in *iso*- propanol). The solution was sonicated (140 W, 20 kHz) in eight 30 second bursts at 0-5 °C. Acetone (25 ml), precooled to -20 °C, was added with stirring over 5 minutes and stirring continued at 4 °C for a further 10 minutes. The cell debris and precipitated protein were removed by centrifugation at 0 °C (37 000 g, 30 min.) to give a crude extract. A further portion of precooled acetone (30 ml) was added and, after being stirred for 10 minutes, the precipitated protein was collected by centrifugation at 0 °C (37 000 g, 20 min.). The protein pellet was resuspended in 50 mM potassium phosphate buffer (2.5 ml, pH 7.6) with swirling at 4 °C and the insoluble material was removed by centrifugation at 10 °C (37 000 g, 10 min.).

The protein solution (2.85 ml) was applied to a column of Sephadex G-150 (1.3 x 170 cm), pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.6) containing 10 mM mercaptoethanol at 4 °C. The protein was eluted with

the same buffer at a rate of 6 ml h^{-1}, collecting 6 ml fractions. The active fractions (25 - 39) were pooled and the protein solution was concentrated to 3 ml by ultrafiltration (Amicon cell fitted with a YM10 filter), at 4 °C. Distilled water (15 ml) was added to the ultrafiltration cell to dilute the salt and the protein solution was again concentrated to 3 ml.

A 1 ml aliquot of the protein solution was applied to an LKB-Pharmacia TSK DEAE-5PW column (2.15 x 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.6). The column was washed with the same buffer at a flow rate of 4 ml min^{-1}, collecting 4 ml fractions and a salt gradient of 0 - 500 mM KCl in the same buffer was applied. The active enzyme eluted in a volume of 24 ml at approximately 80 mM KCl. The pooled active fractions were concentrated to 1 ml by ultrafiltration as before and the potassium ion concentration was reduced to 10 mM by adding distilled water and reconcentrating the protein to about 1 ml, as above. The resulting solution was reapplied to the TSK DEAE-5PW FPLC column, pre-equlibrated as before and the active enzyme was collected in three 4 ml fractions after application of the salt gradient. The protein was obtained in 22 % yield.

Appendix 2 - Publications

1. N. P. Botting, M. Akhtar, C. H. Archer, M. A. Cohen, N. R. Thomas, S. Goda, N. P. Minton and D. Gani, in 'Molecular Recognition: Chemical and Biochemical Problems II', ed. S. M. Roberts, RSC, 1992.

2. C. H. Archer and D. Gani, *J. Chem. Soc., Chem.Commun.,* 1993, 140.

3. C. H. Archer, N. T. Thomas and D. Gani, *Tetrahedron Asymmetry,* 1993, in press.