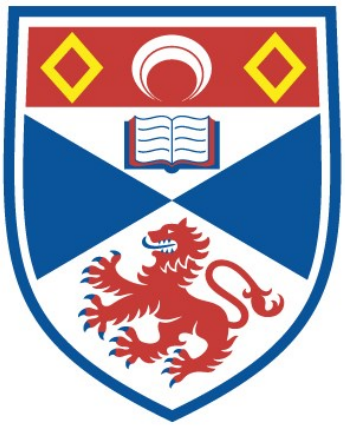
# MECHANISTIC STUDIES ON GLUTAMATE MUTASE

Basil Hartzoulakis

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



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# MECHANISTIC STUDIES ON GLUTAMATE MUTASE

a thesis presented by

Basil Hartzoulakis

to the

University of St. Andrews

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St. Andrews March 1994

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I, Basil Hartzoulakis, hereby certify that this thesis has been composed by myself, that 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 17th March 1994

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

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TO THE MEMORY OF MY FATHER

TO MY MOTHER

*For Their*
*Will, Integrity, Courage*
*and Love*

"It is not our part to master all the tides of the world,
but to do what is in us for the succour
of those years wherein we are set,
uprooting the evil in the fields that we know,
so that those who live after may have a clean earth to till.
What weather they shall have is not ours to rule."

J.R.R.Tolkien

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Finally, a million thanks to my mother and sister. Thought of them kept me going through all of the difficulties I came up against.

## Abstract

The coenzyme $B_{12}$-dependent enzyme glutamate mutase (E.C. 5.4.99.1) catalyses the rearrangement of (2S)-glutamic acid to (2S,3S)-3-methylaspartic acid. Each of the two components of the enzyme was purified to homogeneity using a combination of low and high performance chromatographic techniques. Component E and S displayed molecular weights of 53 KDa and 13 KDa respectively as determined by gel electrophoresis, contrary to literature reports.

A large number of glutamate and 3-methylaspartate analogues were synthesised and tested as substrates for the enzyme from *Clostridium tetanomorphum*. No rearrangement products could be detected for (2S,3R)-3-methylaspartic acid, (2S,3S)-3-ethylaspartic acid, 3-methylglutamic acid, (2S,3R)-3-methylsuccinic acid or *N*-methyl-3-methylaspartic acid. Five inhibitors were discovered for the enzyme. Four of them were typical competitive inhibitors: (2S,3S)- and (2S,3R)-3-methylglutamates ($K_i$ = 1.0 mM and $K_i$ = 1.5 mM respectively); (2S)-homocysteic acid, $K_i$ = 5 mM; and 1-bromo-*cis*-1,2-cyclopropanedicarboxylic acid ($K_i$= 2.2±0.2 mM). Finally 1-bromo-*trans*-1,2-cyclopropanedicarboxylic acid prevented the enzyme from processing (2S)-glutamic acid for periods of times proportional to its concentration. Our results support a radical mechanism with a protein bound glycyl radical as an intermediate, and provide evidence for the existence of two distinct conformations of the holoenzyme, prior to and after the activation of the cofactor.

(2S,3R)-3- and (2S,3S)-3-Methylglutamic acids were synthesised stereospecifically by extending Schollkopf's *bis*-lactim ether methodology. The attack of various carbon anions at C-5 of isopropyl *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-carboxylate S,S-dioxide was not a versatile pathway. Nevertheless, the reaction of the oxathiazolidone with an allylmagnesium lithium cuprate complex gave some promising results, but more research is necessary to optimise certain problematic steps. Several different routes were evaluated for the preparation of 1-amino-1,2-cyclopropanedicarboxylic acid, but either low yields or instability of intermediates thwarted any attempts to achieve this goal. Finally 1-bromo-*cis*- and *trans*-1,2-cyclopropanedicarboxylic acids were synthesised by reacting methyl acrylate with methyl dibromoacetate in the presence of sodium hydride. The two pairs of enantiomers, *cis*- ((2S,3S) and (2R,3R)) and *trans*- ((2R,3S) and (2S,3R)) were separated by selective ester formation.

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## List of Schemes

## Abbreviations

| | |
|---|---|
| $[\alpha]_D$ | optical rotation at 25 °C |
| APS | ammonium sulphate |
| ATP | adenosine 5'-triphosphate |
| *n*-BuLi | *normal*-butyllithium |
| BDE | bond dissociation energy |
| b.p. | boiling point |
| bp | base pairs |
| BSA | bovine serum albumin |
| CD | circular dichroism |
| DMF | dimethylformamide |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DTT | D,L-dithiothreitol |
| E. coli | *Escherichia coli* |
| E.C. | Enzyme Catalogue |
| EDTA | ethylenediaminetetraacetic acid |
| EXAFS | Extended X-ray absorption fine structure spectroscopy |
| FPLC | Fast protein liquid chromatography |
| GTP | Guanidine 5'-triphosphate |
| HMPA | hexamethylphosphonamide |
| ITP | Inosine 5'-triphosphate |

| | |
|---|---|
| $K_i$ | enzyme inhibition constant |
| $K_m$ | Michaelis-Menten constant |
| ITP | 5'-triphosphate |
| LDA | lithium di-*iso*-propyl amide |
| m.p. | melting point |
| $M_r$ | relative molecular mass |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| PAGE | polyacrylamide gelelectrophoresis |
| p.p.m | parts per million |
| r.p.m. | revolutions per minute |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulphate |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| Tris | tris(hydroxymethyl)aminomethane |
| UTP | uridine 5'-triphosphate |
| UV | ultraviolet |

**The one and three letter codes for the amino acids**

| Amino acid | Three letter code | Single letter code |
|---|---|---|
| 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 1

# Introduction to $B_{12}$-Dependent Enzymes & Glutamate Mutase

## 1.0 Introduction

Nature's ability to accomplish an immense variety of catalytic events relies primarily on two evolutionary achievements. The development of a large number of alternative protein structures and the use of cofactors.

The conformations of polypeptide chains, selected from the pool of an infinite possible number, are necessary to establish the vital element of selectivity. In the cases where binding of the transition state (TS) is sufficient for catalysis, a protein (or even an RNA molecule[1]) can be synthesised by the cell that will perform the task. However, many processes in living organisms would be impossible to realise with the intervention of a protein structure alone. Many "difficult" key transformations, oxygen transport, and energy transfer require a cofactor.

In this context, the term "cofactor" includes metals (Zn, Mg) , metal clusters (Fe-S) and coenzymes. Coenzymes are defined as "small" thermostable molecules necessary for enzymatic activity ( CoA, NADH, NAD, ATP, flavins *etc.*). One family of coenzymes is characterised by a tetrapyrole macrocycle with a metal coordinated in its centre and includes haem (Fe), chlorophylls (Mg), coenzyme F430 (Ni) and coenzyme $B_{12}$ (Co).[2] The larger and structurally more complicated member of this family is 5'-deoxyadenosylcobalamin (adenosylcobalamin, AdoCbl) (Fig. 1.1). Its *de novo* synthesis is restricted to bacteria. The concentrations of the coenzyme in the environment are very low ([AdoCbl] $\leq$ 1nM), so all the higher mammals have evolved very sophisticated systems in order to absorb, store and utilise it. The task of unravelling the secrets of coenzyme $B_{12}$ chemistry has been a target for scientists for the last 50 years. While the elucidation of the biosynthetic pathway to vitamin $B_{12}$ has almost been completed,[2] chemists are not in a position to claim sufficient understanding of the mechanistic details of its enzymatic action. The consensus is that it serves as a reversible carrier of free radicals in the same way that myoglobin/haemoglobin can bind and release molecular oxygen. Homolysis of the unique $\sigma$ Co-C bond affords cob(II)alamin and 5'-adenosyl radical. The latter gives rise to a range of new radicals derived from the appropriate substrates. The special properties of the coenzyme that make this process possible will be discussed. The structural and mechanistic aspects of the protein's

involvement in the individual steps will be evaluated. Finally the chemical models of the rearrangement process will be reviewed.

### 1.0.1 Discovery of Cobalamins

Living organisms contain, in addition to the biosynthetic intermediates, four related cobalamin structures, namely 5'-adenosyl-, cyano-, methyl-, and hydroxyl-cobalamin. The corresponding group occupies in each case the β-axial position (Fig. 1.1).[3] Cyanocobalamin functions as the metabolic precursor for AdoCbl, and MeCbl. The two latter molecules are the only ones that are used as cofactors in various biochemical systems. There is evidence to support the existence of specific enzymes that can add or remove the axial cobalamin substituents.[4] However, our understanding in this area is poor.

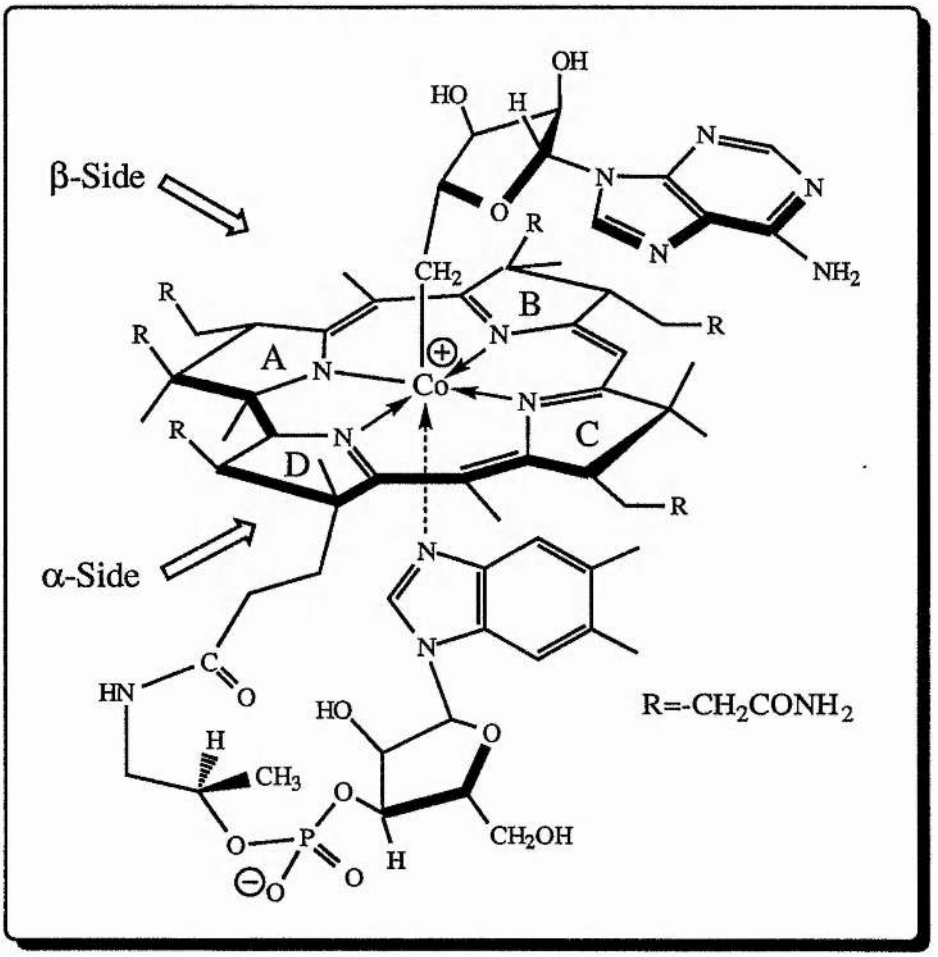



*Figure 1.1 5'-Adenosylcobalamin (Coenzyme $B_{12}$)*

The first cobalamin to be isolated[5] and characterised[6] was cyanocobalamin. AdoCbl (Fig. 1.1) was crystallised in 1959 by Barker *et al.*,[7] and characterised by Lenhert and Hodgkin[8] in 1961. The discovery of the stable C-Co bond in AdoCbl provoked a considerable amount of research. Nevertheless, the mode(s) of action of cobalamins still provoke much speculation.

### 1.0.2 Biological Function of Cobalamins

Methyl- and 5'-adenosyl-cobalamin serve as coenzymes for a variety of unusual catalytic processes:

1) Methyl group transfers (synthesis of methionine).

2) Isomerisations.

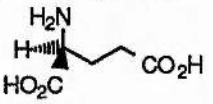
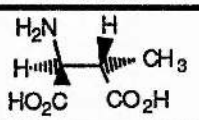
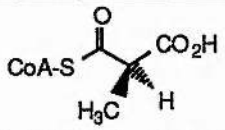
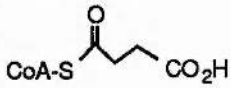
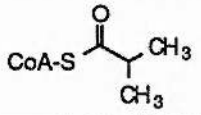
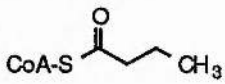
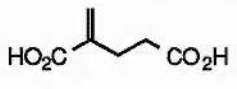
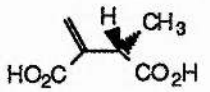
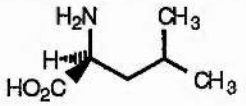
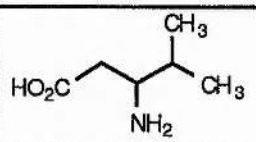
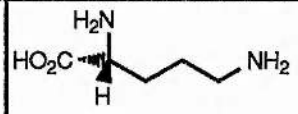
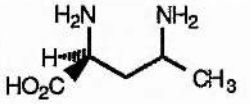
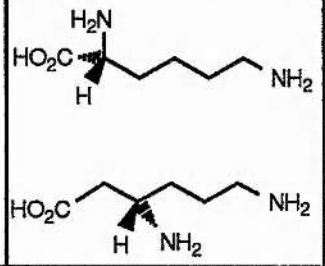
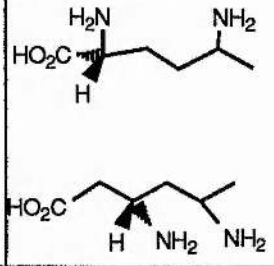
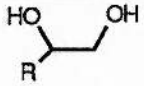
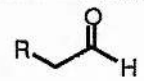
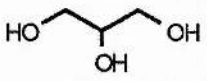
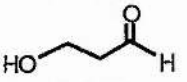
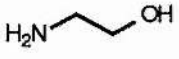
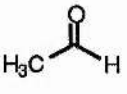
3) Reduction of ribonucleotides to deoxyribonucleotides.

The ten apoenzymes which require AdoCbl, in order to catalyse the rearrangements, are listed in Table 1.

There is a small number of systems which catalyse reactions which appear to be similar to $B_{12}$-dependent isomerisations. For example lysine 2,3-aminomutase (E.C. 5.4.3.2) is not AdoCbl dependent but catalyses the interconversion of (S)-lysine to (3S)-β-lysine. This reaction is very similar to the lysine 5,6-aminomutase reaction which is dependent on the coenzyme (entry 7, Table 1.1). For the 2,3-aminomutase iron, cobalt(III), S-adenosylmethionine, and pyridoxal phosphate are necessary for activity. It is believed that a radical is generated on an iron/sulphur cluster, and then transferred to the adenine part of S-adenosylmethionine.[9] The role of the Co(III) ions is yet to be established.

Another case worthy of mention is ribonucleotide reductase.[10] This enzyme uses different cofactors in different species, either an ion cluster or AdoCbl. Ribonucleotide reductases are important enzymes which play a central role in DNA biosynthesis in catalysing the conversion of GTP, ATP, ITP, and UTP to the corresponding deoxyribonucleotides. The protein from *E. coli* is the prototype for the enzyme present in mammals, yeast and herpes simplex virus. Mechanistic studies have shown that the adenosyl radical abstracts a hydrogen from an amino acid at the active site and is thus how the protein itself is involved in the chemical changes during the catalytic cycle.

***Table 1.*** *$B_{12}$ - Dependent enzymes catalysing rearrangements*

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

* Reaction reversibility under physiological conditions is also indicated.

Ribonucleotide reductase is characterised by the presence of two catalytically significant thiol groups in the active site.[10] This is another common feature of all coenzyme $B_{12}$-dependent enzymes (see § 1.13).[11,12]

## 1.1 Structure of Coenzyme $B_{12}$

Coenzyme $B_{12}$ belongs to a specific group of corrinoids called cobalamins. A nitrogen containing macrocycle known as a corrin forms the core of the coenzyme (Fig. 1.2.**a**). There are two main differences between corrins and the more widely distributed porphyrins (Fig. 1.2.**b**). First, corrins exist in a highly reduced state, there are only six double bonds instead of the eleven present in porphyrins. Corrins also incorporate a characteristic linkage between rings A and D. Halpern[13] provided evidence to support the view that corrins, display greater flexibility than porphyrins and therefore respond more efficiently to steric pressures induced by bulky ligands. The rigid structure of the fully aromatic porphyrins does not allow such flexibility. It has to be stressed, however, that despite the ability of the corrin to change its conformation, it is, on the basis of X-ray crystal data, extremely reluctant to do so. Nevertheless, severe interactions with the axial ligands, (Fig.1.1), are able to push the cobalt atom out of the corrin plane and distort the ring.[14]

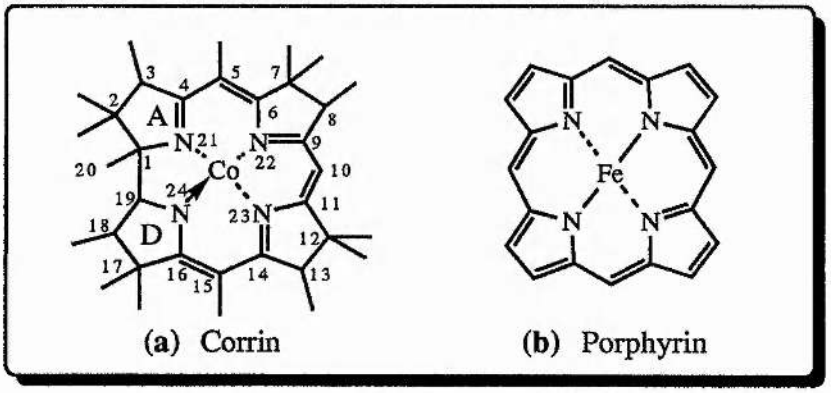



***Figure 1.2** The corrin and the porphyrin nucleus*

Seven amide chains are attached to the corrin ring. One of them, a propanoate side-chain on ring D (C-17), is bonded through amidation with a (2R)-1-aminopropan-2-ol phosphodiester, to the 3'-hydroxyl group of a 5,6-dimethyl-

benzimidazolyl $\alpha$-ribofuranoside. The 5,6-dimethylbenzimidazole is coordinated through N-3 to the cobalt's $\alpha$-axial position (Fig. 1.1) and thus forms a loop.

This highly functionalized ring D to Co(III) loop bears many groups that can interact with the environment of the active site. In fact, recent data[15] point to a significant role for this loop during the initial catalytic steps when the radical species are formed. In solution the coenzyme exists in an equilibrium in which 5,6-dimethylbenzimidazole is either coordinated (base-on) or not (base-off) at the $\alpha$-position of the coenzyme. A water molecule occupies the $\alpha$-axial position in the base-off form to provide a yellow[16] rather than a red-orange colour. There is no firm evidence supporting the involvement of the base-off form in the catalytic events. The other chains attached to the corrin ring interact with the environment of the active site and together with eleven methyl substituents stabilise the important Co-C bond by preventing the approach of other molecules.[16]

The Co(III) atom is octahedrally coordinated in the centre of the macrocycle. It is very tightly bound in this position such that any efforts to remove it, result in the destruction of the corrinoid ring.[17] Its coordination sphere is slightly, but significantly distorted due to the direct linkage between rings A and D. The field provided by the unsaturated nitrogen-containing ligand induces very strong changes in the energy levels of cobalt's orbitals.[18] This affects the nature of the Co-C bond and the stability of intermediates. For example cob(II)alamin radical is stable and has been crystallised and fully characterised.[15]

The $\sigma$ Co-C bond is stable to water and oxygen, and was the first stable organometallic bond to be discovered.[8] The ability of cobalt to form this thermodynamically and kinetically stable bond is not matched by any other metal.[14,19,20] The bond dissociation energy (BDE) for the Co-C bond was calculated from kinetic data[20] to be 109±8 kJ mol$^{-1}$. Finke[21] reported a slightly higher value of 126±8 kJ mol$^{-1}$. The "base-off" form in models has a higher BDE by about 17 kJ mol$^{-1}$. The stability of the bond depends on electronic as well as steric factors. Indeed the BDE is substantially affected by the nature (*e.g.* electron donating or neutral) of the ligand on the $\alpha$-position (this is the so called "*trans* effect"[16]) and the steric interactions between the adenosine and the substituents on the corrin ring.[14]

## 1.2 3-Dimensional Structure of Coenzyme $B_{12}$ and Analogues

The major features of the three dimensional structure of the coenzyme are the σ Co-C bond, the loop connecting ring D with the 5,6-dimethylbenzimidazolyl ligand, the extended substitution on the corrin ring, and the ribose moiety which neatly caps the Co-atom.

The length of the Co-C is 2.03 Å, slightly longer than the Co-CN bond in vitamin $B_{12}$ (1.92 Å). The steric compression that occurs between the adenosine and the corrin substituents is revealed by the distorted (Co-$CH_2$-C) bond angle of 124.4°, 15° larger than normal.[22]

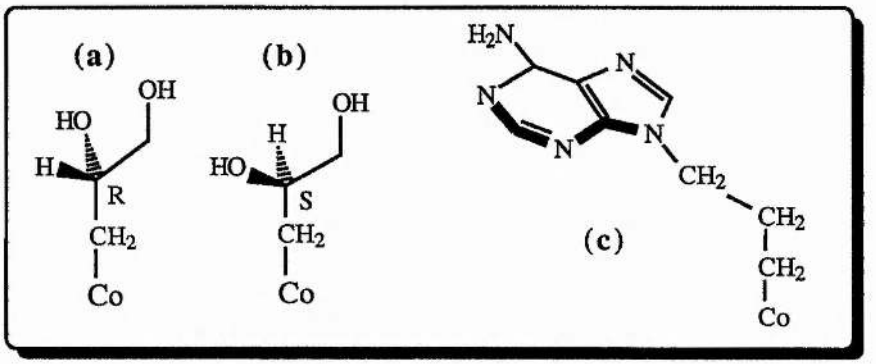



*Figure 1.3 Analogues of AdoCbl*

The number of coenzyme $B_{12}$ analogues in which the adenosine moiety is replaced by another group has been only recently extended sufficiently to allow comparative studies. Golding *et al.*[23] analysed the structure of (R)- and (S)-dihydroxypropyl-cobalamin (Fig. 1.3.**a** and **b**). Careful examination confirmed that the (R)-analogue could not accommodate itself very well between the substituents on the corrin ring. On the other hand the (S)-analogue (Fig. 1.3.**b**) positions its hydroxymethyl group between rings C and D (the same position AdoCbl nests the ribose ring oxygen, (Fig. 1.1)) and forms a hydrogen bond, with one of the acetamido ligands releasing thereby much of the strain. The (Co-$CH_2$-C) bond angle in this case is only 113°. In a similar study Marzilli and Glusker[24] presented data on adeninylpropyl-cobalamin (Ade$(CH_2)_3$-cobalamin) (Fig 1.3.**c**). By comparison, no important differences were found in the conformation of corrin

nucleus in the analogue, and in AdoCbl and MeCbl. The most important structural properties for some analogues are summarised in Table 1.2.

***Table 1.2.*** *Structural characteristics of AdoCbl analogues with various β-axial substituents R (cobalamin as in Fig. 1.1)*

| R | Co-C (Å) | Co-N(ax) (Å) | Co-N(eq) (Å) | Co-Ca-Cb (°) |
|---|---|---|---|---|
| 1) Ado[22] | 2.03 | 2.24 | 1.87-1.91 | 124.4 |
| 2) Ade-$(CH_2)_3$-[24] | 1.96 | 2.12 | 1.85-1.88 | 119 |
| 3) Me[25] | 1.99 | 2.19 | 1.88-1.97 | - |
| 4) (R)-2,3-dihydroxypropyl[23] | 2.00 | 2.27 | 1.84-1.95 | 120 |
| 5) (S)-2,3-dihydroxypropyl[23] | 2.08 | 2.36 | 1.89-1.98 | 114 |
| 6) CN[26] | 1.91 | 2.03 | 1.91-1.94 | 175.0 |

## 1.3 Coenzyme $B_{12}$ Models

Many groups have attempted to use model systems to gain an understanding of the chemistry of AdoCbl. Typically, a model for the coenzyme is constructed when large, electron rich (nitrogen or oxygen containing) molecules, are complexed with a Co(III)-atom. The two remaining axial positions of the Co(III) atom are then available to coordinate with an alkyl group at the β-axial position, or Lewis bases at the $\alpha$-axial position. The most popular ligand for cobalt's equatorial coordination sphere has been *bis*-(dimethyl glyoximato), $(DH)_2$, and a number of its substituted derivatives (Fig. 1.4.**a**, X=H, $CH_3$, Ph ). The chemistry of these systems has been extensively reviewed.[17,19,27,28] Other coenzyme models include the mixed oxime-Schiff bases, $[(DO)(DOH)_{pn}]$ (Fig. 1.4.**b**), bis(acetylacetone)ethylenediimine (BEA) (Fig. 1.4.**c**), and bis-(salicylaldehydo hydroethylenediimine) (SALEN) (Fig. 1.4.**d**).

Cobaloximes (Fig. 1.4.**a**) have been successfully used to support predictions concerning the properties of cobalamins made nearly three decades ago.[29] An increase in the bulkiness of the R or the L ligand would elongate the Co-C bond and decrease the BDE. For example $pyCo(DH)_2CH_3$ has a BDE of 143±4 kJ $mol^{-1}$. This value is significantly higher than the 89.5±8 kJ $mol^{-1}$ obtained for the $pyCo(DH)_2{}^{i}Pr$ analogue. Additionally the presence of any steric compression is typically reflected in the C-$CH_2$-C-5' bond angle. For example the introduction of a

methylene bridge (Fig. 1.4.**b**, oxime-Schiff base) reduced the flexibility of the equatorial ligand and subsequently a value of 130° for the Co-$CH_2$-C bond angle was observed, when R= $CH_2C(CH_3)_3$. The distortion of the Co-$CH_2$-C bond angle is even more pronounced when the other axial ligand B is also bulky. The importance of this kind of distortion in the enzymatic catalysed activation of the Co-C bond is, however, ambiguous since obviously it is not connected with the stability of the Co-C bond. Neopentyl cobaloximes with a distorted angle of 130° are particularly stable.

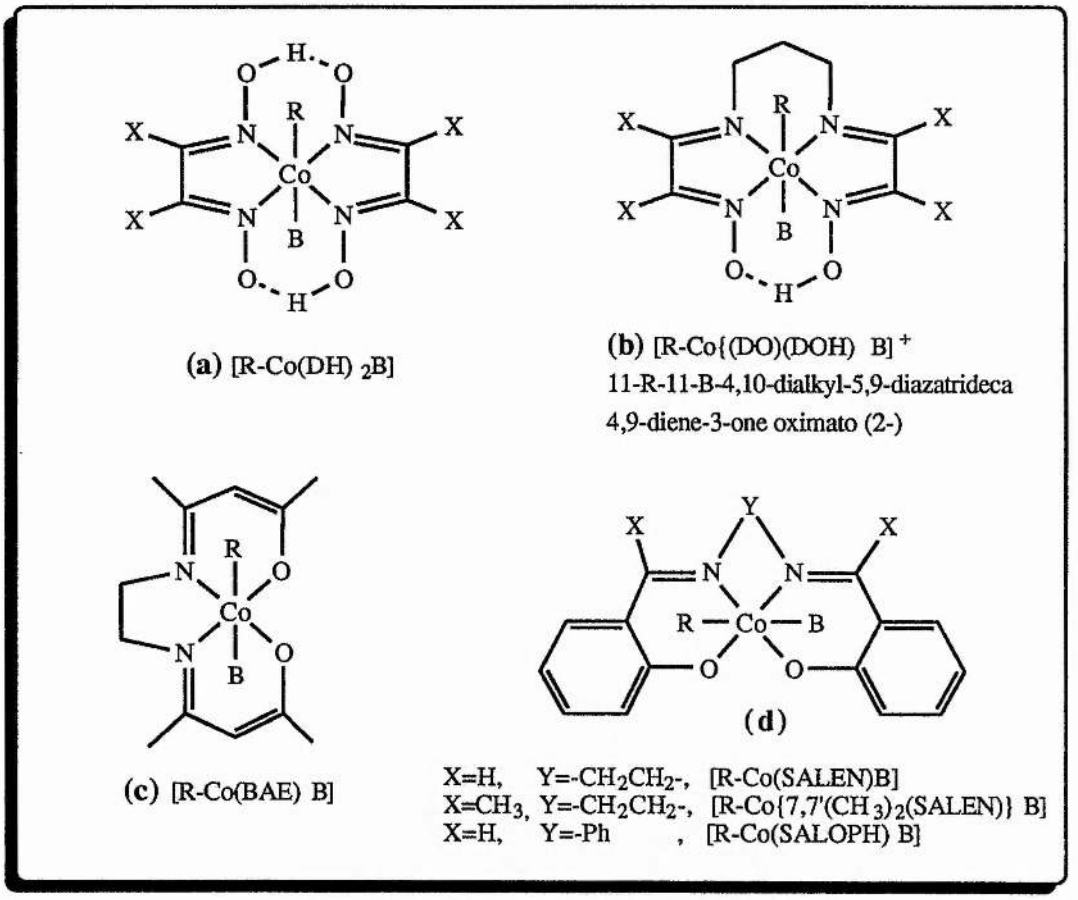



***Figure 1.4** Models for coenzyme $B_{12}$*

When a bulky, "electron rich", Schiff base (Fig. 1.4.**c**,**d**) is the equatorial ligand donor, the complexes display Co-R distances more similar to cobalamins than the previously discussed models. These large complexes, in contrast with the "electron poor" cobaloximes, have similar properties to corrins, but are difficult to prepare

and study. All the models mentioned above display similar trends in the dependency of the BDE on steric and electronic factors.[19]

A new of generation of model compounds, with structural characteristics very similar to cobalamins, has recently appeared in the literature (Fig. 1.5).[30] Whether these structures will be of any practical importance remains to be seen.

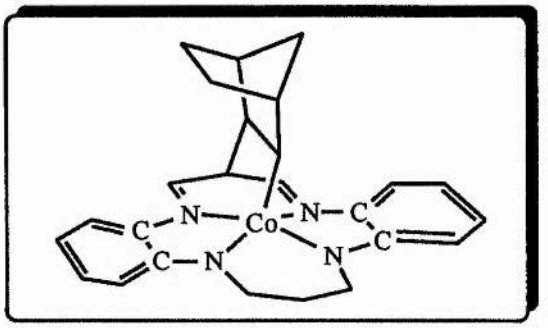



*Figure 1.5 Model with a Co-C-equatorial ligand bridge*

## 1.4 Properties of Cobalamins

Cobalamins owe their unique properties to cobalt. Co is the only metal that can form stable σ M-Co(III) bonds and the same time retain easy access to its range of oxidation states (Co(I), Co(II)).[14] Therefore, not only can the Co-C bond be synthesised and preserved efficiently in biochemical systems, but more importantly it can be manipulated during catalysis to afford the kinetically competent species (5'-adenosyl radical and cob(II)alamin). This interplay of oxidation states offers the means for observing coenzyme $B_{12}$-dependent reactions in real time, since there are very characteristic differences between the U.V./ visible spectra of cob(I)-, cob(II)- and cob(III)-alamin.[31]

Cyclic voltametry, and other spectroelectrochemical methods are usually applied to study the redox properties of AdoCbl and its analogues.[32,33] These methods can simplify (in an electrochemical cell) comparisons between models and the naturally occurring cobalamins. In some cases important findings have emerged, some of which allowed the identification of compounds with potentially useful properties.[34]

The tetrapyrrole ring and the side chains of cobalamins have been derivatised or transformed using a variety of chemical means.[35] The products of these reactions are coenzyme analogues that provided useful information about the interactions between the side chains and the protein enviroment of the active site (see § 1.7). The formation of the Co-C bond is another important reaction. This can be easily achieved by reacting alkyl halides with Co(I) or Co(II)[35,36] species. Many organocobalt complexes have been synthesised in this manner.

Once formed the Co-C bond is rather inert under a variety of conditions. Heterolytic cleavage was achieved only under relatively forcing conditions[37] *(e.g.* $CN^-$, $H^+$, $HO^-$, $H_2$), and formation of degradation products (cob(I)alamin, hydroxycob(III)alamins, elimination products of the adenosyl moiety) was commonly observed. The mild homolytic cleavage of the Co-C bond seems to be the only alternative left to the apoenzyme as the first step of the coenzyme activation.

## 1.5 Radical Intermediates; Structure and Properties

Abeles and coworkers suggested in 1972 the involvement of highly reactive radical intermediates in the mechanism of the coenzyme dependent dehydration of ethanediol by diol dehydrase.[38] The presence of enzyme bound radical species in the AdoCbl-dependent rearrangements is now supported by a large body of evidence (see §1.8).[18,39]

Since cob(II)alamin radical appears to be the active form of the coenzyme during catalysis, its structural characteristics are of particular interest. Recent EXAFS studies[40] of cob(II)alamin showed that the axial Co-N-3 bond becomes shorter while the equatorial Co-N distances display only minimal changes, implying that upon homolysis the cobalt atom moves towards the $\alpha$-axial nitrogen. This movement is not very important in solution due to the very low energy barrier for the recombination of the Co(II) and the organic radical. In the active site, however, maintaining the distance between the Co(II) radical and the enzyme bound 5'-adenosyl radical may be necessary for the propagation of the reaction. One of the enzyme's tasks must be to prevent the immediate reverse reaction (eq. 1, Fig. 1.6) by holding the two radicals as far apart as possible.

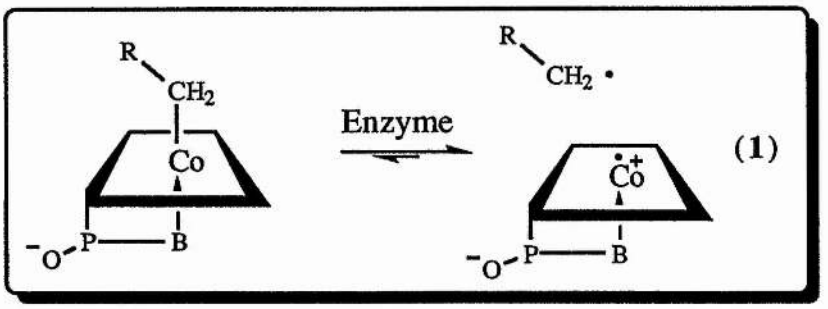



***Figure 1.6** Activation of the coenzyme*

Krautler and coworkers[15] published a crystal structure of cob(II)alamin which was found to be remarkably similar to the intact coenzyme. An upward movement of the substituents on ring D, including the functionalized, ribonucleotide containing loop, were the only differences observed. These two conformational changes are probably related to the repositioning of Co(II) towards the 5,6-dimethyl-benzimidazolyl base.

Many studies have probed the mechanism of alkylcobalamins photolysis. Elaboration of radical-trapping experiments afforded methods to measure the BDE of organocobalt complexes free in solution[41] and bound by enzymes.[42]

Lowe *et al.* reported in 1978 that photolysis of AdoCbl in aqueous propan-1,2-diol produced a small cob(II)alamin signal detectable by EPR.[43] Upon warming up and re-freezing the samples, without further irradiation, the EPR signal increased substantially up to six times. A likely explanation seemed to be that the coenzyme, after irradiation either in solution or in the active site, existed in a peculiar state of "incipient "homolysis. This state might have arisen from either i) a heterolytic cleavage of the Co-C bond to form diamagnetic products, which on warming up afforded the radicals, or, ii) from a state in which the electrons of the bond were unpaired but EPR-silent as a result of dipole-dipole interactions in the triplet state. A similar behaviour was observed for the coenzyme complexed with diol dehydrase/holoenzyme (§ 1.7.3). Although the coenzyme was EPR silent, irreversible deactivation of the enzyme system was observed.[44] It was clear that the Co-C bond was not cleaved, but the coenzyme was not in the ground state either.

## 1.6 Radicals In Biological Systems

The fact that Nature uses radical intermediates to achieve a remarkable variety of tasks is well documented.[10] Photosynthetic $O_2$-evolving systems, isopenicillin N synthase, and lipoxygenases are just three cases, chosen from a wide variety of available examples. In biological systems radicals are commonly employed in redox reactions or as intermediates in "difficult" transformations. Radicals can be generated by the photo irradiation of semiquinones, from Fe-S clusters, or by the "manipulation" of AdoCbl. Over the last few years the generation and use of high energy intermediates in enzymatic systems has been the focus of considerable research activity.[10]

The study of enzyme bound radical intermediates is complicated by the fast appearance and disappearance of the species involved. EPR or stopped-flow visible spectroscopy are the methods of choice. If, however, the concentration of the active species is not high enough then "spin traps", can be useful, if the enzyme allows the spin trap access in the active site. In the case of the $B_{12}$-dependent rearrangements (and MeCbl mediated methyl transfers) all of these methods have been used to try to elucidate the details of the catalytic steps.[9,12]

It is generally accepted that in the first catalytic event the apoenzyme binds the coenzyme and the other cofactors necessary for catalysis *e.g.* a metal for diol dehydrase, or pyridoxal phosphate for the aminomutases. The protection of the Co-C bond in the holoenzyme is an important task of the protein. The binding of the appropriate substrate triggers the homolytic cleavage of the Co-C bond. All the highly reactive radical intermediates are protected by the protein and after the rearrangement of the substrate to the product the coenzyme is regenerated. The Co-C bond activation step is not fully understood despite the extended literature on the subject. Finke and coworkers[45] found that diol dehydrase and ethanolamine ammonia lyase accelerate the homolysis of the AdoCbl Co-C bond by a factor of $10^{12\pm1}$! For such a high acceleration to be rationalised two major issues have to be dealt with. First, what tertiary and quaternary structures can establish the ability of these enzymes to control so many remarkable steps and second how is the binding energy converted to catalysis along the Co-C bond cleavage co-ordinate profile?

## 1.7 Co-C Bond Homolysis Induced by the Protein

Considerations on how the protein facilitates the Co-C bond activation should take into account the following useful facts.

1) Modifications of the amide side chains of the coenzyme caused partial or complete loss of the coenzymatic activity in several AdoCbl-dependent systems, including ribonucleotide reductase,[46] diol dehydrase,[47] ethanolamine ammonia lyase,[47] and glutamate mutase.[48] The EPR signal for the inactive coenzyme analogues was, in the presence of the protein, either missing or reduced (see § 1.21).

2) The adenine moiety is necessary for activity, although small alterations can be tolerated.[49] Ichikawa and Toraya synthesised a series of coenzyme analogues (Fig. 1.7) incorporating various changes at the ribose ring.[50]

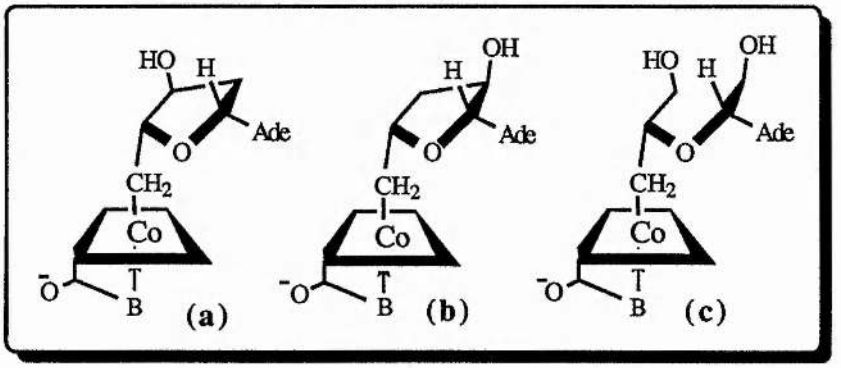



*Figure 1.7 Analogues of AdoCbl; importance of the two hydroxyl groups and the ribose ring*

The coenzyme analogues (**a**, **b**, Fig. 1.7) lacking the 2' and 3' hydroxyl group respectively, retained a percentage of activity with diol dehydrase (**a**, 31%; **b**, 19%). The open-ring form (**c**) was inactive.[50] The importance of the nitrogens on the adenine, the two hydroxyl groups and the ribose ring[51] probably arise from their ability to keep the 5'-adenosyl radical in a very specific and restricted orientation in the active site, thus minimising harmful side reactions.

3) The Co-C bond seems to be under severe strain even without the presence of substrate. An interesting finding by Krouwer *et al.*[52] was that longer alkyl chain AdoCbl analogues, (Fig. 1.3.**c**, p. 7) despite being inactive and photostable, showed, in the presence of ethanolamine ammonia lyase, changes in their CD

spectra. On the other hand AdoCbl is photolabile and no changes were observed in its corresponding CD spectrum after binding on the enzyme. More elaborate studies have dismissed the suggestion that the changes in the CD spectra were due to changes in the conformation of the corrin ring (§ 1.2).[15] Alternatively it seemed that the adenine group in the analogues adopted a conformation quite different to that of AdoCbl, clearly due to the increased flexibility of the longer alkyl chain. The authors concluded that the large relief of conformational strain observed for the analogues should be involved in the cleavage of the Co-C bond.[52]

### 1.7.1 Conformational Changes Induce the Cleavage of the Co-C bond

A number of working hypotheses have been formulated in order to explain these results and highlight the particular first event between the coenzyme and the apoenzyme that leads to Co-C labilisation, and eventual cleavage.

i) The suggestion that the protein induces changes in the conformation of the corrin ring is probably the earliest of them all. An upward or downward (butterfly-type) distortion that increases the steric interaction between the corrin nucleus and the adenosyl moiety, thus labilising the Co-C bond, has been very popular and is supported by model studies (see §1.3).[16,19]

ii) Alternatively the enzyme can further distort the Co-$CH_2$-C-5' angle and elongate the Co-C bond by means of tight binding of the adenosyl group. In view of studies on model systems the probability of contribution of this type is low (see § 1.3).[28]

iii) Finally, alterations of the position of 5,6-dimethylbenzimidazolyl base, induced by the appropriate binding of the groups on the loop between ring D and the Co(III), should also be considered.[15] Pratt[53] used U.V. data to argue that in the case of ribonucleotide reductase, the Co(III)-N benzimidazolyl bond is broken during catalysis affording the so-called base-off form of the coenzyme. This report, however, has not been confirmed by any other researchers.

In 1985 Pratt[54] summarised the above views. "The metallo-enzymes emphasise the ability of the protein to 'activate' the metal by changing an equilibrium constant....instead of increasing a rate constant by lowering the energy of the TS."

### 1.7.2 Stabilisation of the Transition State (TS)

The suggestion that the activation of the Co-C bond is achieved by altering the conformation of the coenzyme in the active site was challenged by Krautler *et al.*[15] and Nie *et al.*[55] Both groups argued that the TS hypothesis should be used to interpret older and more recent literature. The protein could stabilise the TS between the coenzyme and the product radicals - a "trick" widely utilised in biochemical systems. Krautler's argument was based on the crystal structure of cob(II)alamin. Since the structure of the intact coenzyme and the cob(II)alamin are so similar (§ 1.5) it did not seem likely that the protein would choose the distortion of the corrin ring as the initial event of the activation process. Less dramatic and probably more effective could be a differential binding between the two radical homolysis products and the intact AdoCbl. In other words the enzyme stabilises the TS.

Nie and Marzilli also supported the TS hypothesis but their argument was based on a larger collection of data concerning the cleavage of the Co-C bond. Elegant studies on the BDEs of coenzyme models[55,56] showed that many discrepancies could be explained on the assumption that the outcome of the cleavage of the Co-C bond was dependent on differences in the free energies of the products (and hence in the TS) rather than in differences of the ground states (intact coenzyme).

Finally, all the available data on the behaviour of coenzyme models are consistent with the stabilisation of the TS hypothesis.

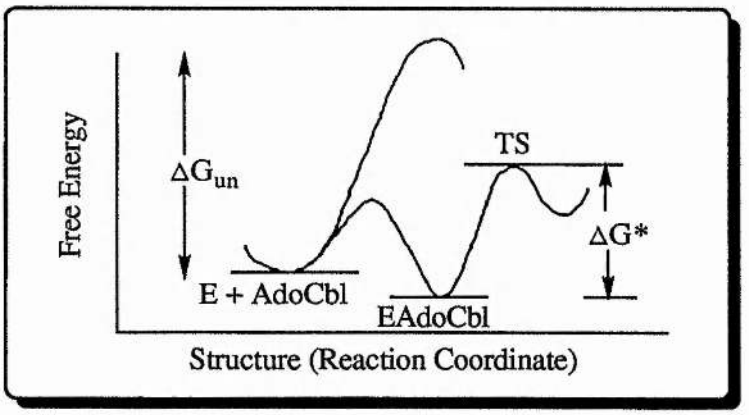



***Figure 1.8** Free energy diagram for Co-C bond cleavage*

Accordingly, the "relief of strain" mentioned above (§ 1.7) was the binding energy, transformed into $\Delta G^{\neq}$, the Gibbs energy of activation, and used by the enzyme to break the Co-C bond (Fig. 1.8). No conformational distortions of the coenzyme have to be evoked. The protein does *not* force the coenzyme to adopt a new conformation, it just binds the cob(II)alamin and the 5'-adenosyl radical more efficiently than the intact coenzyme.

### 1.8 Coenzyme $B_{12}$-Dependent Reactions and EPR Spectra

The ten AdoCbl-dependent enzymes mentioned above (§1.2, p. 3) catalyse twelve rearrangements that fall into three categories. The first one includes the dehydration of ethanediol, propanediol, glycerol and the deamination of ethanolamine. (R)-ornithine-, (S)-β-lysine-, (R)-α-lysine- and (S)-leucine-2,3-aminomutase fall into a second class, in which the migrating group in each case is an $-NH_2$. The third group includes glutamate, methylmalonyl-CoA, isobutyryl-CoA, and α-methyleneglutarate mutase.

From the preceding discussion it is clear that homolytic cleavage of the Co-C bond is by far the most favourable and controllable mild reaction that the coenzyme can undergo under enzymatic control. Additionally the presence of highly reactive radical intermediates offers a framework which explains more satisfactorily, though not completely, the rearrangement step. The "hard" evidence for the existence of kinetically competent radical intermediates is that i) the appearance of paramagnetic species took place only after the addition of the appropriate substrate, and that ii) the species observed, were not due to any kind of interaction between substrates and AdoCbl, or related with any possible degradation products.[12]

EPR signals have been observed so far for diol dehydrase,[57] ethanolamine ammonia lyase, methylmalonyl CoA mutase,[58] ribonucleotide reductase[59] and more recently for glutamate mutase[60] (Fig. 1.9, p. 19). These spectra, after the appropriate mathematical treatment,[61] can provide information not only about the radical cob(II)alamin but also about the exchange coupling and the geometry of the magnetic dipole-dipole and spin-spin coupling with other species (presumambly carbon centered radicals) present in the active site.

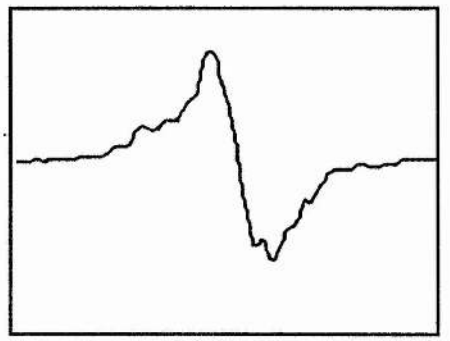
Initial attempts to obtain a interpretable EPR spectra using slow freezing techniques were unsuccessful. When this problem was overcome[62] a number of efforts were made to interpret the results. Coffman *et al.*[63,64] and Boas *et al.*[65,66,67] developed methods that explained thoroughly and convincingly the spectra obtained. The two teams performed computer simulations of the low spin Co(II) $3d^7$ system with an unpaired electron. The solutions had to fit the simpler spectrum obtained at 9 MGHz (Fig. 1.9.a) as well as the more complicated one obtained at 35 MGHz (Fig. 1.9.b). The large Co(II) separation is due to the presence of another radical at a distance of 9.9 Å from Co(II) at a position approximately 34° off the principal axis, which is the axis of the C-N-3 bond. The implication is that the catalytic activity in AdoCbl-dependent enzymes does not take place in the Co(II) coordination sphere and that following the homolytic cleavage of the Co-C bond, the 5'-adenosyl radical is positioned (towards the substrate binding pocket) at a distance of about 10 Å, where the rearrangement and hydrogen transfers occur.

The similarity of the EPR spectrum of glutamate mutase and ribonucleotide reductase is striking (Fig. 1.9.a, and 1.9.e.A) for systems that catalyse such different reactions. This fact, however, only strengthens the hypothesis outlined above to account for the observations concerning the initial catalytic steps.

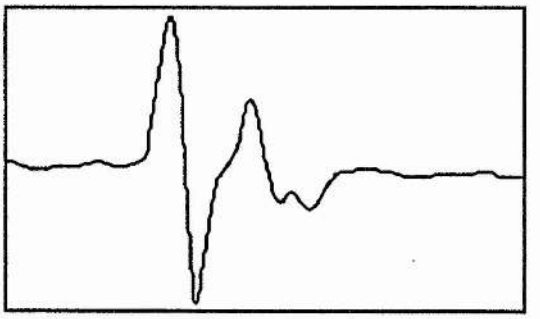
Kinetically competent radical intermediates were observed only after the addition of substrate. There was, however, evidence that in some cases binding of the coenzyme was sufficient to "activate" but not cleave the Co-C bond. This would explain the slow deactivation of the apoenzyme-coenzyme complex observed for diol dehydrase,[44] and ribonucleotide reductase.[46]

## 1.9 Mechanistically Plausible Pathways

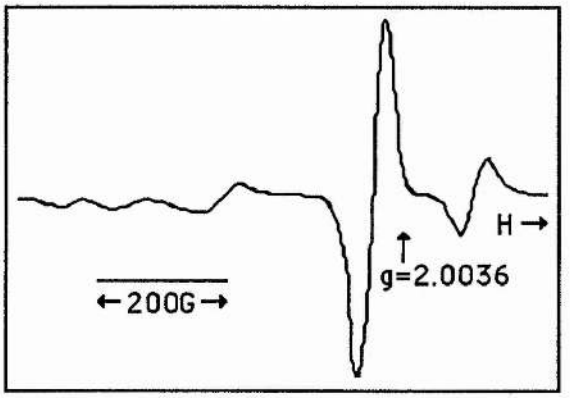
After the homolytic cleavage of the Co-C bond the 5'-adenosyl radical is likely to react directly with the substrate. The product of this step had been the subject of much controversy.[68] A number of experiments with isotopically labelled substrates,[12] revealed two important mechanistical features. First, the coenzyme serves as an intermediate hydrogen carrier at $CH_2$-5', and second, 5'-deoxyadenosine is an intermediate in the catalytic process.[12,69]



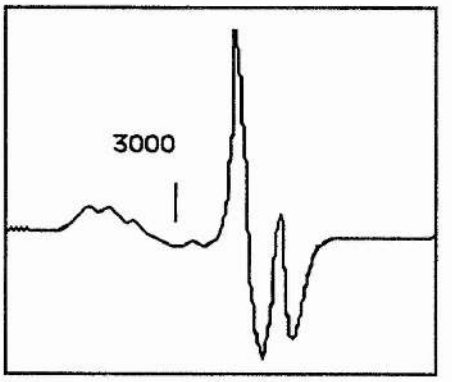
9 GHz EPR spectrum of r. reductase



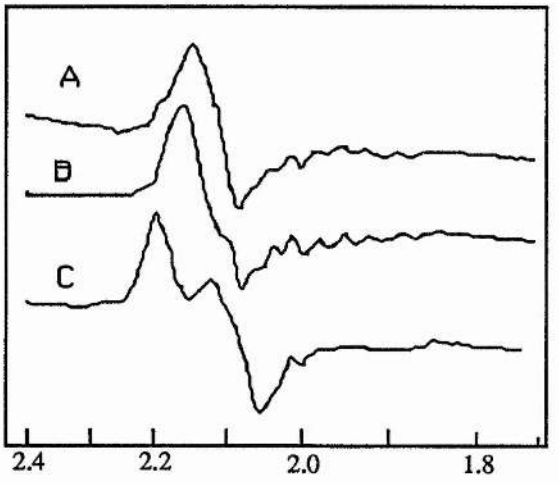
35 GHz EPR Spectrum of r. reductase



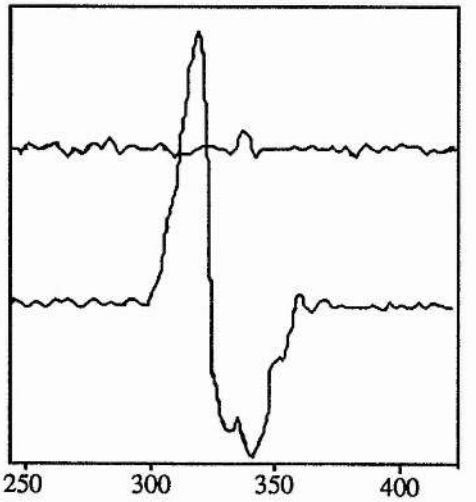

9 GHz EPR spectrum of diol dehydrase




9 GHz EPR spectrum of ethanolamine ammonia lyase




9 GHz spectrum for glutamate mutase (A)




9 GHz spectrum of methylmalonyl-CoA mutase

***Figure 1.9** EPR spectrums of $B_{12}$ - dependent enzymes*

In accordance with these results the reaction of the 5'-adenosyl radical with the substrate gives rise to the substrate radical S•. The fate of this species is still a matter of debate. S• can interact with the protein or cob(II)alamin, to give (Scheme 1.1) the charged (**b**,**c**) or other (**d**, organocobalt) species that actually rearrange.

Pathway (**a**) has become popular for the carbon skeleton rearrangements since all data seem to support non charged intermediates.[70] With the exception of methylmalonyl-CoA mutase, for which pathway (**c**) has also been postulated (Scheme 1.3, p. 28), all the other carbon skeleton rearrangments can be envisaged to proceed through radical intermediates.

Pathway (**b**) may be of some importance for the reactions involving the rearrangement of hydroxyl- and amino- group. Golding and Radom[71] used *ab initio* molecular orbital calculations to demonstrate that [1,2]-shifts in hydroxy- or amino-containing radicals might be facilitated by protonation of the migrating group (Fig. 1.10). An amino acid in the active site could provide the necessary proton.

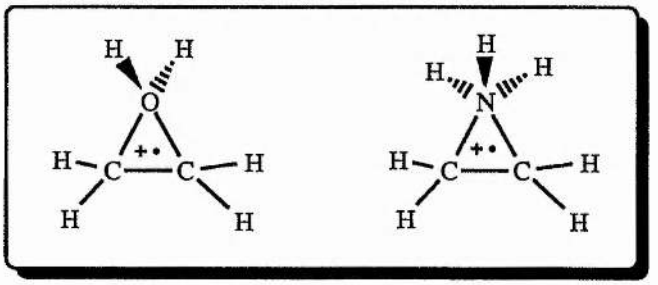
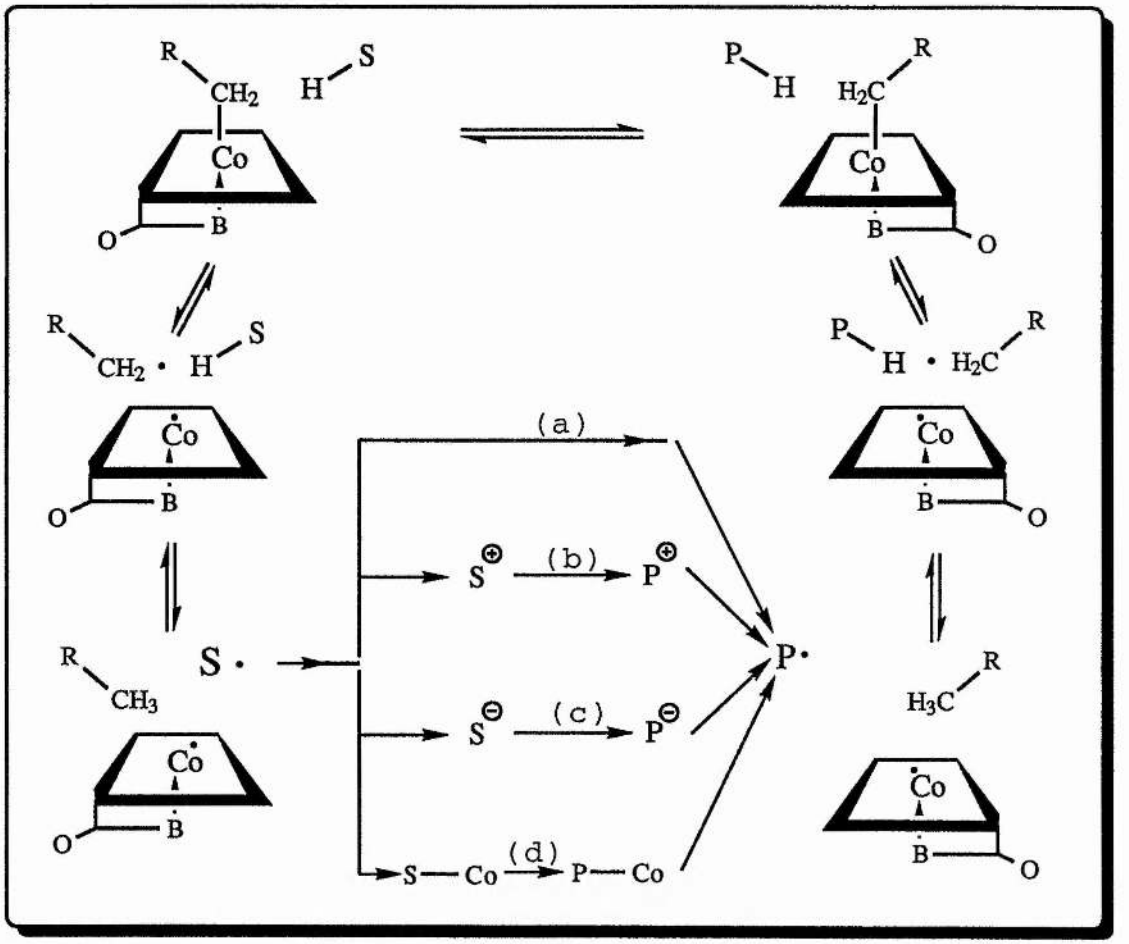


***Figure 1.10** Postulated charged intermediates*

Pathway (**c**) would involve the transformation of the radical species present in the active site, to a negative charged substrate intermediate, $S^-$. Speculations about the formation of $S^-$ in the active site exist,[72,73] but they are not supported by any experimental evidence.

Despite substantial early consideration,[74,75] route (**d**) is now regarded with scepticism. Many experimental efforts to prove that the cobalt actually *can* form complexes with radical intermediates and induce [1,2]-migrations have been inconclusive, although there were studies providing evidence for putative Co-alkyl complex formation.[75,76] Unfortunately, even if these data are reproduced, no link

exists between studies of cobalt complexes in solution and the enzyme catalysed reactions. Pratt's suggestion[14] that a long range (in order to explain the EPR signals, § 1.6.3) orbital coupling produces "a cobalt-promoted carbonium ion" was interesting but lacked experimental support. The large distance between the radicals present in the active site (cob(II)alamin and the organic radical) and the discovery of certain chemical models for the rearrangement (§ 1.14) steps argue strongly against the participation of Co(II) in the crucial step.




***Scheme 1.1*** *Possible Pathways for the Organical Radical S•*

Another aspect emerging from the inspection of pathways (**a**,**b**,**c**,**d**) is the importance of two hydrogen transfers. A hydrogen atom, initially abstracted from the substrate, equilibrates with the other two protons on C-5' of the ribose (R-$CH_2$•, Scheme 1.1) and is returned after the rearrangement to the product radical P•. The

isotope effect for these two steps has been measured and reported for most of the enzymes studied so far (diol dehydrase,[77] ethanolamine ammonia lyase,[78] methylmalonyl-CoA mutase,[79] glutamate mutase[80]). The values for the primary isotope effect were in the expected range (between 3.5-10) and indicated that the initial hydrogen abstraction (A in Scheme 1.1) was actually the rate limiting step. In the case of ethanolamine ammonia lyase a large tritium effect ($k_H/k_T$ 160) was observed.[77] One explanation offered by Cleland[81] to fit the experimental data was the presence of another hydrogen carrier in the active site closely located to the adenosyl group. Additional proof has been delivered[82] but is yet to be confirmed by other researchers.

## 1.10 Oxo-Forming Reactions

The overall stoichiometry of these irreversible reactions (Fig. 11, eqs. 2-3) points to a deceptively simple dehydration or deamination picture. Early studies proved that they all proceed *via* an 1,2-interchange of a hydrogen and a -OH or an $-NH_2$ group.[83]

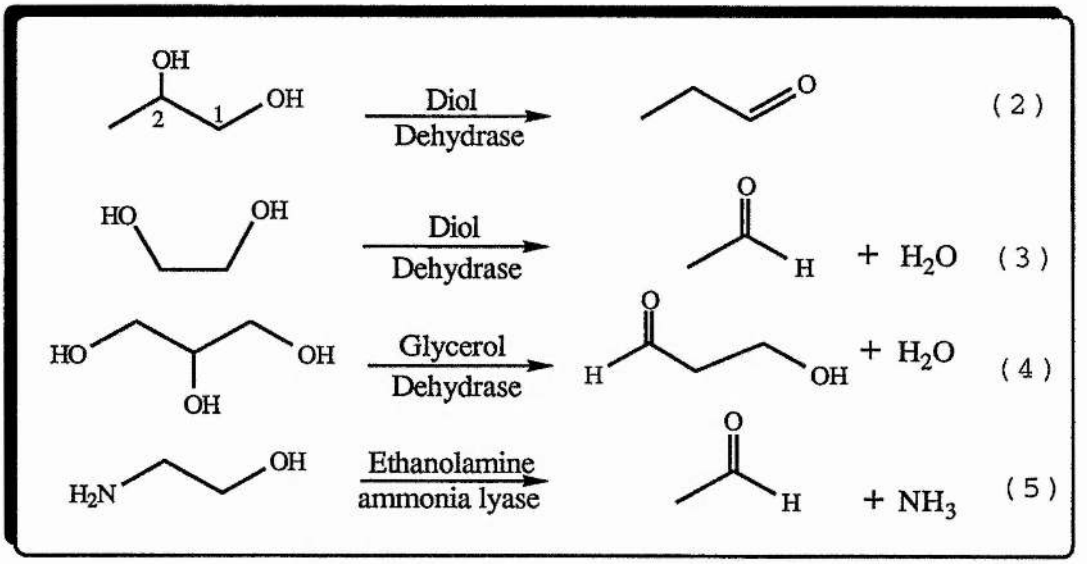



*Figure 1.11 $B_{12}$ -Dependent dehydrations*

### 1.10.1 Diol Dehydrase

### 1.10.1 Diol Dehydrase

Diol dehydrase (E.C. 4.2.1.28) is probably the most extensively studied enzyme of the group[84] and catalyses the conversion of 1,2-ethanediol, and 1,2-propanediol to acetaldehyde and propionaldehyde respectively (Fig. 11, eqs. 2,3). The enzyme was isolated and purified from extracts of *Klebsiella pneumoniae* and needs a monovalent cation for activity ($K^+$ or $NH_4^+$).

The actual composition of the active apoenzyme, finally elucidated[85,86] in the early 1980's, revealed a complicated structure in which four subunits ($M_r$ 60,000, 51,000, 29,000, and 15,000) combine to create a large protein of $M_r$ 259,000. The large subunit, ($M_r$ 60,000), is acidic, and contains sensitive sulphydryl groups. No functional assignment has been reported for any of the subunits.

The lack of substrate stereospecificity is rather impressive. 1,2-Ethanediol, (R)- and (S)-1,2-propanediol, 1,2-butanediol, 2,3-butanediol, (R)- and (S)-glycerol, 2-methyl-1,2-propanediol, 2-fluoro-1,2-propanediol are all substrates for the enzyme. All the rearrangement steps are stereospecific. The enzyme essentially catalyses the migration of the hydroxyl group from C-2 to C-1. The shift of a hydrogen atom from C-1 to C-2 proceeds with inversion of configuration on C-2. Which of the two prochiral hydrogens on C-1 migrates surprisingly depends on which isomer of propanediol is processed by the enzyme. The final dehydration of the diol to the aldehyde is not enzyme catalysed.

Diol dehydrase is the only system for which the Co participation question has been tackled with success.[87] Finke and coworkers synthesised the acetal protected $HOCH_2CH(OH)$-cob(III)alamin (Fig. 1.12), which has been postulated to be an intermediate of the catalytic process.[88] Upon deprotection and generation of the corresponding radicals no rearrangement products were detected under a variety of conditions. Moreover, the formation of highly reactive cob(I)alamin species proved the incompatibility of this kind of intermediate with a protein environment.

### 1.10.2 Glycerol Dehydrase

Glycerol dehydrase (E.C. 4.2.1.30) is not very well studied.[84] Nothing is known about the stereochemistry of the reaction (Fig. 1.11, eq. 4)

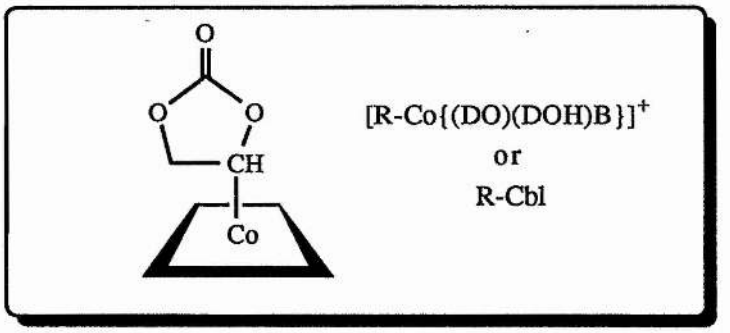


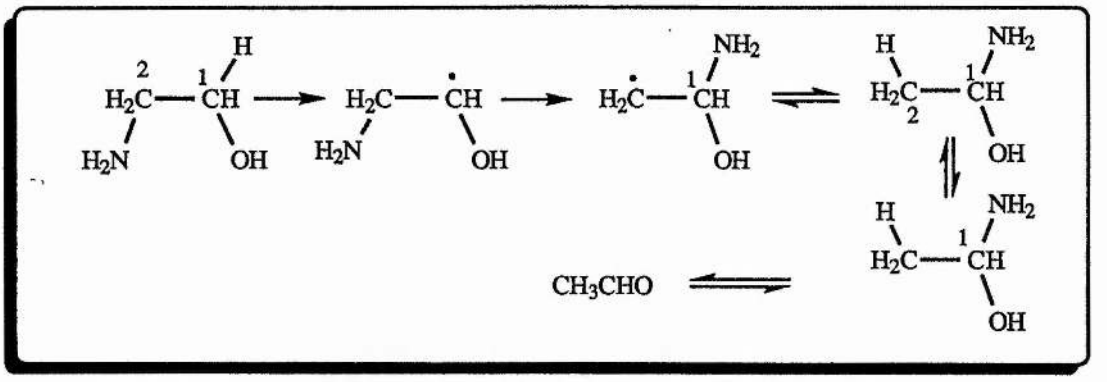

***Figure 1.12*** *Postulated intermediate for Co-participation mechanism*

### 1.10.3 Ethanolamine Ammonia Lyase

Ethanolamine ammonia lyase (E.C. 4.3.1.7) is a widely distributed enzyme.[89] It has been identified in many bacteria including *E. coli.*, but was originally isolated in large amounts from *Clostridium*. The enzyme catalyses the formation of acetaldehyde and propionaldehyde from ethanolamine and 2-aminopropanol respectively (Fig. 1.11, eq. 5) and, unlike diol dehydrase (§ 1.10.1), no other substrates are known.

Only the clostridial enzyme needs a monovalent cation for activity ($K^+$ or $NH_4^+$). Both the clostridial and *E. coli* enzymes are very big proteins with a molecular weight of 520,000 and 560,000 respectively. The huge protein complex can actually be "seen" using electron microscopy. It is composed of at least two types of subunits and contains catalytically important sulphydryl groups. Early kinetic studies reported two active sites[90] but the value was revised later to six using more advanced methods.[91] These findings supported an $I_6II_6$ subunit structure. The coenzyme is not dissociated by the apoenzyme during the purification procedure due to very strong binding.

The only important stereochemical information was reported by Gani *et al.*[92] The pro-S hydrogen on C-1 migrates to C-2. Any stereochemical information on C-2 is finally lost due to racemisation occuring during the last two reversible steps of the overall mechanism (Scheme 1.2).[93] The behaviour of ethanolamine ammonia lyase with compounds of the type $Ado(CH_2)_nCb$ has been discussed previously (see § 1.7).



*Scheme 1.2 Ethanolamine ammonia lyase - reversible steps*

## 1.11 Interconversion between Amines - Aminomutases

The AdoCbl-dependent aminomutases catalyse the migration of an amino group from and to various positions on amino acid skeletons (Fig. 1.13, eqs. 6-9).

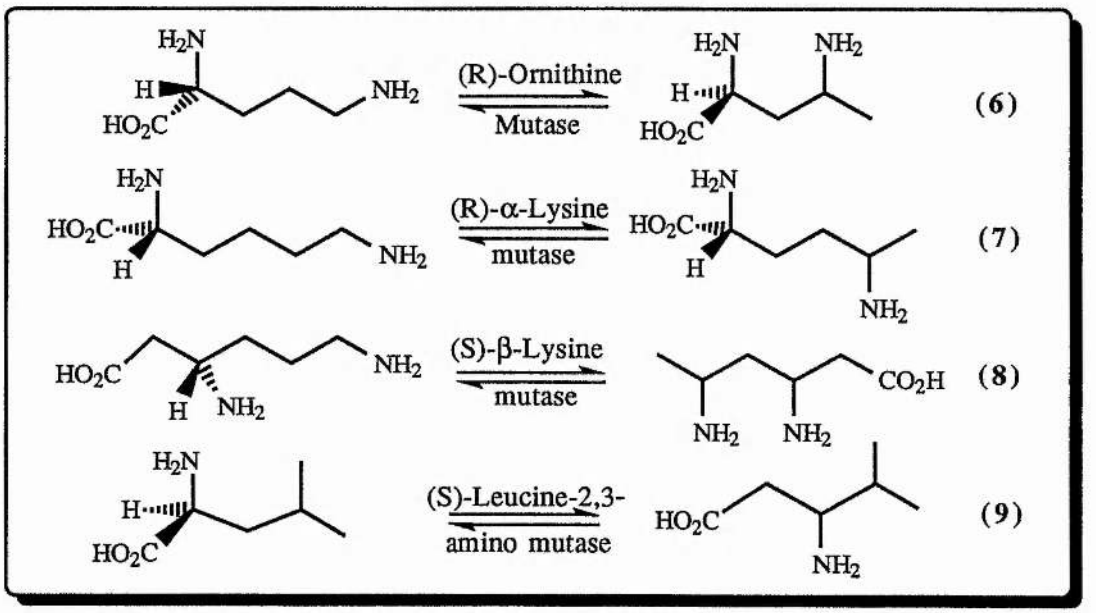



*Figure 1.13 Coenzyme $B_{12}$-dependent amino mutases*

### 1.11.1 (R)-Ornithine- and (R)-α-Lysine-aminomutase

(R)-Ornithine- (E.C. 5.4.3.5) and (R)-α-lysine-((S)-β-lysine)-aminomutase (E.C. 5.3. 3.3) (Fig. 1.13, eq. 6 and 7,8 respectively) were isolated by bacteria of the genus *Clostridia*, and their biological role is to provide intermediates that can be easily metabolised by the bacteria. No studies are available on their mode of action

and the most recent review was published in 1981.[94] The enzymes have similar molecular weights (200,000 and 170,000 respectively) but different subunit structures. Ornithine mutase: 90,000 and 12,800, $(E_1E_2)_2$ and lysine mutase: 51,000, 30,000, 12,800, $(E_1E_2E_3)_2$. The small subunit seems to be identical in both apoenzyme complexes and protects the system from fast deactivation. A monovalent and a divalent cation are necessary for activity. Pyridoxal phosphate and ATP are not essential but enhance activity.

### 1.11.2 L-Leucine-2,3-aminomutase

L-Leucine-2,3-aminomutase (E.C. 5.4.3.7) is probably the most widely distributed amino-mutase. Its physiological role in humans is probably the conversion of β- to α-leucine (Fig. 1.13, eq. 9).[94] Despite the possible medical interest, the enzyme has been studied only in crude extracts. since it is extremely unstable.

## 1.12 Carbon Skeleton Rearrangements

The carbon skeleton rearrangements.(Fig. 1.14, eqs. 10-13) are [1,2] intramolecular shifts in which the bond between the most heavily substituted C-2 and C-3 is cleaved and a new bond between C-2 and C-4 is formed. The four enzymes of the group require nothing but the cofactor to promote the transformation.

### 1.12.1 Glutamate Mutase

Glutamate mutase (E.C. 5.4.99.1) catalyses the interconversion of (S)-glutamate to (2S,3S)-3-methylaspartate (Fig. 1.14, eq. 10) and will be discussed separately later (§ 1.16).

### 1.12.2 Methylmalonyl-CoA Mutase

Methylmalonyl-CoA mutase (E.C. 5.4.99.2) catalyses the rearrangement of 3-carboxy-propionyl-CoA to (2R)-methylmalonyl-CoA (Fig. 1.14, eq. 11) as part of the propionic acid metabolism. The enzyme can be found in most mammals and is important for the metabolism of branched-chain fatty acids and isoleucine. Some

bacteria, such as *P. shermanii* use the system in the opposite direction i.e. to produce propionate from succinate.[95]

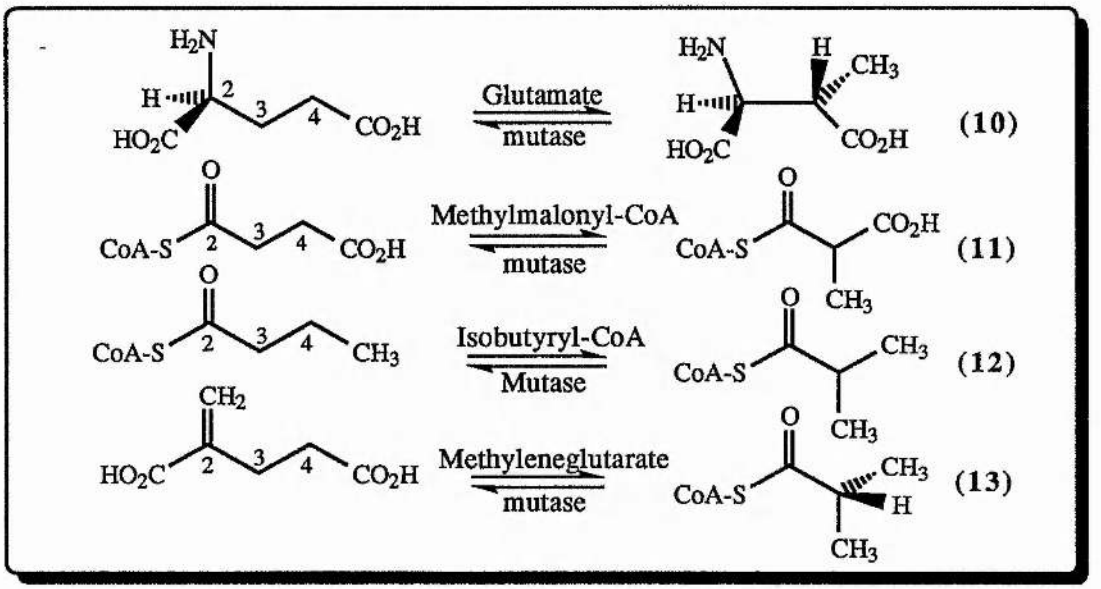
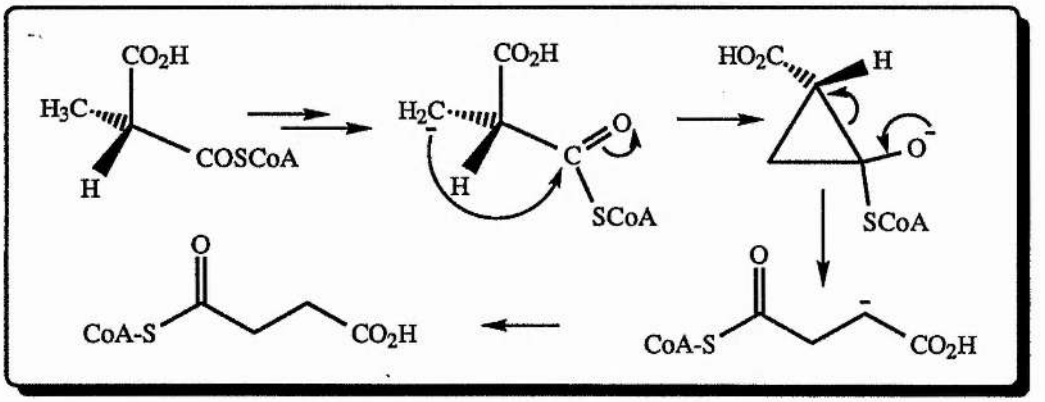



***Figure. 1.14*** *Coenzyme $B_{12}$-Dependent carbon skeleton rearrangements*

Two components ($M_r$ 79,000, and 67,000) were identified by Leadlay and coworkers[96] amending earlier results by Zagalak *et al.*[97] The characterisation of the genes containing the enzyme from *P. shermanii* three years later[98] provided the first sequence for a protein of this group ($M_r$ 80,147, and 69,465).

Methylmalonyl-CoA mutase is the only AdoCbl-dependent carbon skeleton rearrangement for which an anionic mechanism has been postulated to be operational (Scheme 1.3).[99] However, the recent discovery of an EPR signal[57] (Fig. 1.9.f) confirmed the involvement of radicals known to occur in other $B_{12}$ dependent systems. The stereochemical course of the migration step was established using suitably labelled compounds.[100,101] The reaction at C-2 proceeds with retention of configuration. Elucidating the stereochemical course of the reaction at C-3 was rather more complicated but retention of configuration was found to prevail. Most of the stereochemical problems in the reaction catalysed by methylmalonyl-CoA were resolved in a recent publication by Retey and coworkers.[102] Finally a coenzyme A analogue in which the sulphur atom had been replaced by a methylene group *was*

also reported to be turned over by the enzyme in similar efficiency to the natural substrate.




*Scheme 1.3 Rearrangement involving charged intermediates*

### 1.12.3 Isobutyryl-CoA Mutase

Isobutyryl-CoA mutase (E.C. 5.4.99.3) is the most recently discovered coenzyme $B_{12}$-dependent enzyme.[103,104] No information is available for the transformation catalysed (Fig. 1.14, eq. 12).

### 1.12.4 α-Methyleneglutarate Mutase

The transformation of 2-methyleneglutarate to (R)-3-methylitaconate (Fig. 1.14, eq. 13) catalysed by methyleneglutarate mutase (E.C. 5.4.99.4) is a step in the fermentation of nicotinate by *Clostridium barkeri.* [105]

The protein has been only partially purified recently[106] and no information is available about the structure of the apoenzyme. Experiments performed using crude preparations of the enzyme established that the reaction is proceeding with inversion of configuration at the α-carbon.[107] 1-Methyl-*cis*- and 1-methyl-*trans*-cyclopropane-dicarboxylate have been shown not to be substrates for the enzyme.

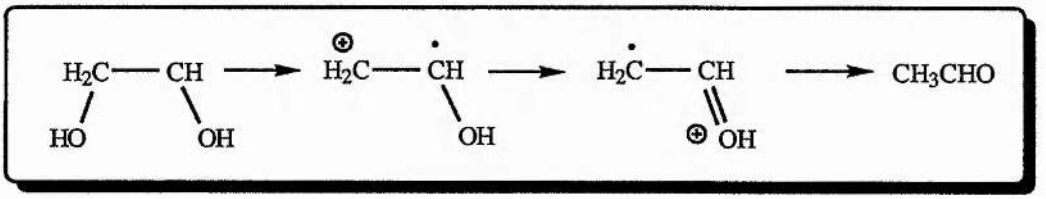
## 1.13 Sulphydryl groups

All the coenzyme $B_{12}$ dependent enzymes studied so far have been found to be sensitive to reagents that react specifically with -SH groups.[10,12,108] Usually the enzyme is completely inactive when the sensitive sulphydryl groups are in the

oxidized form. In some cases the presence of the cofactor or the substrate protects the system against this kind of inactivation. The role of the -SH groups in the catalytic process is yet to be reconciled with the present knowledge on the $B_{12}$-dependent rearrangements.

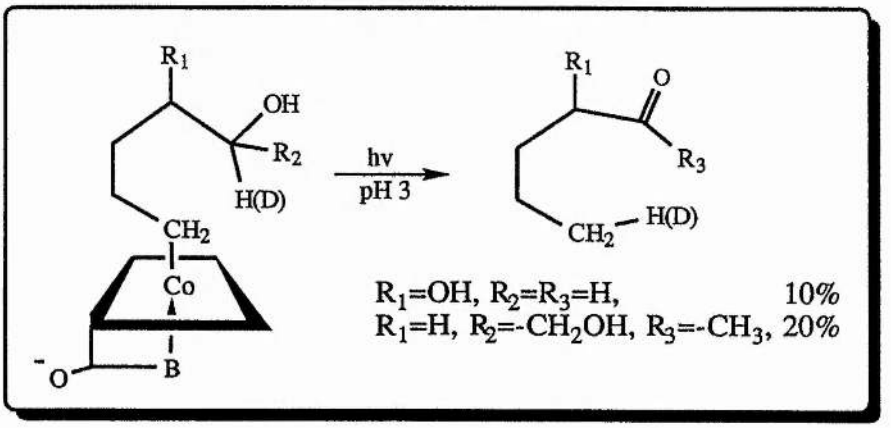
### 1.14 Model Systems

An integral part of the effort to elucidate the mechanistic details of the isomerisations presented above has been the development of appropriate chemical models. Carefully designed molecules can test the feasibility of various mechanistic pathways under a variety of experimental conditions. The oxo-forming reactions were the easiest to model. For example generation of a radical on ethylenediol yielded acetaldehyde in a straightforward manner (Scheme 1.4).[109]



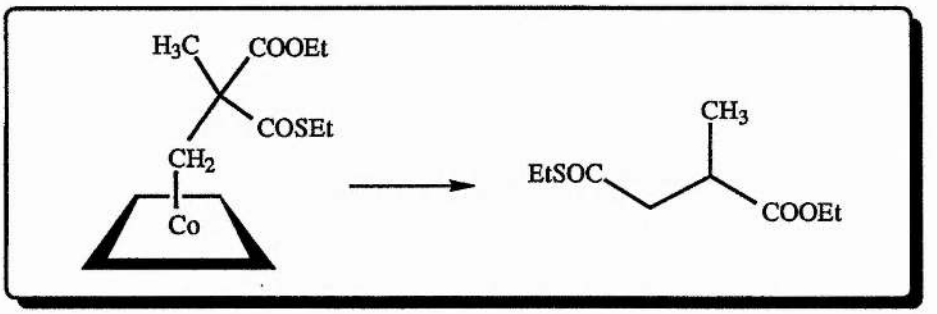

*Scheme 1.4 Chemical model for eqs. 2-4*

Other more elaborate models that incorporate a hydrogen abstraction step were developed by Golding and others. (Scheme 1.5).[99] In this case the model mimicked both the radical generation (homolytic cleavage of the Co-C bond), and the abstraction of a hydrogen by a methylene radical.



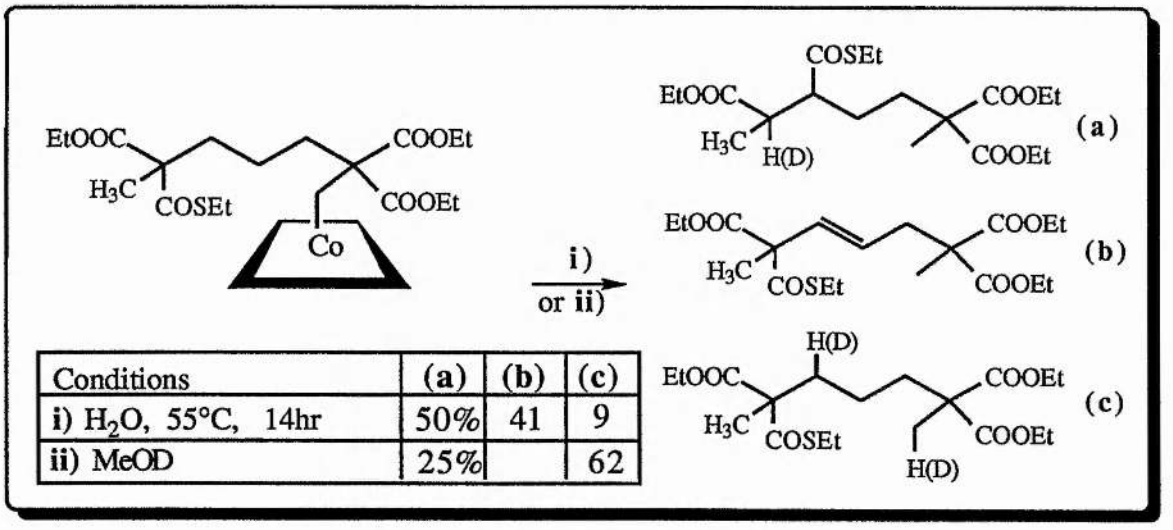

*Scheme 1.5 Advanced model reaction for diol dehydrase*

For methylmalonyl mutase, Scott and Kang[110] developed a successful model in 1977. After irradiation induced homolytic cleavage of the Co-C bond, the thioester migrated in 70% yield (Scheme 1.6).




***Scheme 1.6** Model reaction or methylmalonyl-CoA mutase*

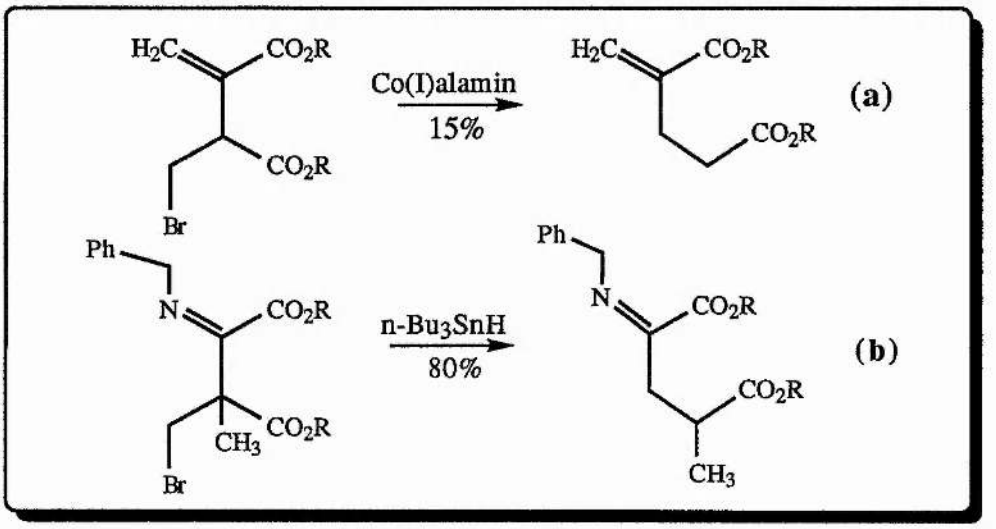
An elegant model for methylmalonyl-CoA mutase was recently reported by Dowd and coworkers.[111] The initial Co-C bond homolytic cleavage was followed by an intramolecular hydrogen abstraction. The radical thus formed rearranged to the products (Scheme 1.7). Rearrangement products could also be detected after treatment of the corresponding bromide with tributyltin hydride. Both models, however, have to be extended in order to incorporate Retey's discovery[112], however, that the succinyl-$CH_2$CoA is also a good substrate for the enzyme (§ 1.9.2).




| Conditions | (a) | (b) | (c) |
|---|---|---|---|
| i) $H_2O$, 55°C, 14hr | 50% | 41 | 9 |
| ii) MeOD | 25% | | 62 |

***Scheme 1.7** Advanced model for methylmalonyl-CoA mutase*

Models for the methyleneglutarate mutase catalysed rearrangement were developed by Dowd during unsuccessful efforts to model the action of glutamate mutase.[113] The migration of an $sp^2$-hybridised carbon was found to be easily reproduced by chemical systems, although the yields were usually low (Scheme 1.8.**a**).




*Scheme 1.8 Model reaction for methyleneglutarate mutase*

One could characterise the rearrangement of the bromoester (Scheme 1.8.**a**) as "metal catalysed" if it was not known that a similar bromoaspartate analogue (Scheme 1.8.**b**) rearranged when treated with tributyltin hydride without the presence of Co(III) species. The radical species generated clearly isomerised without difficulty.

## 1.15 Main Characteristics of The Systems Reviewed

The data presented so far suggest that the protein is completely in charge of the rearrangement step. This does not imply that substrate radicals initially generated do not have the intrinsic tendency to isomerize but that the enzyme is controlling the reaction in a way that it will be fast, stereochemically reliable, and of course with no by-products that can be very harmful to the environment of the active site. Finke and Golding in two recent reviews arrive at the same conclusion.[39,99] Especially Finke, who claims that the term "bound radical mechanism" describes

accurately what is happening at the active site. Under this term the authors suggested a mechanistic scheme in which the enzyme is the conductor of the rearrangement by employing strategically positioned groups around the substrate. These groups interact (protonate, hydrogen bond, *etc.*) with the substrate and promote the reaction. The discovery of chemical models just points out once more the fruitful interplay taking place in bioorganic chemistry, without offering any proof against the "bound radical mechanism".

Three main points are clear from the previous discussion.

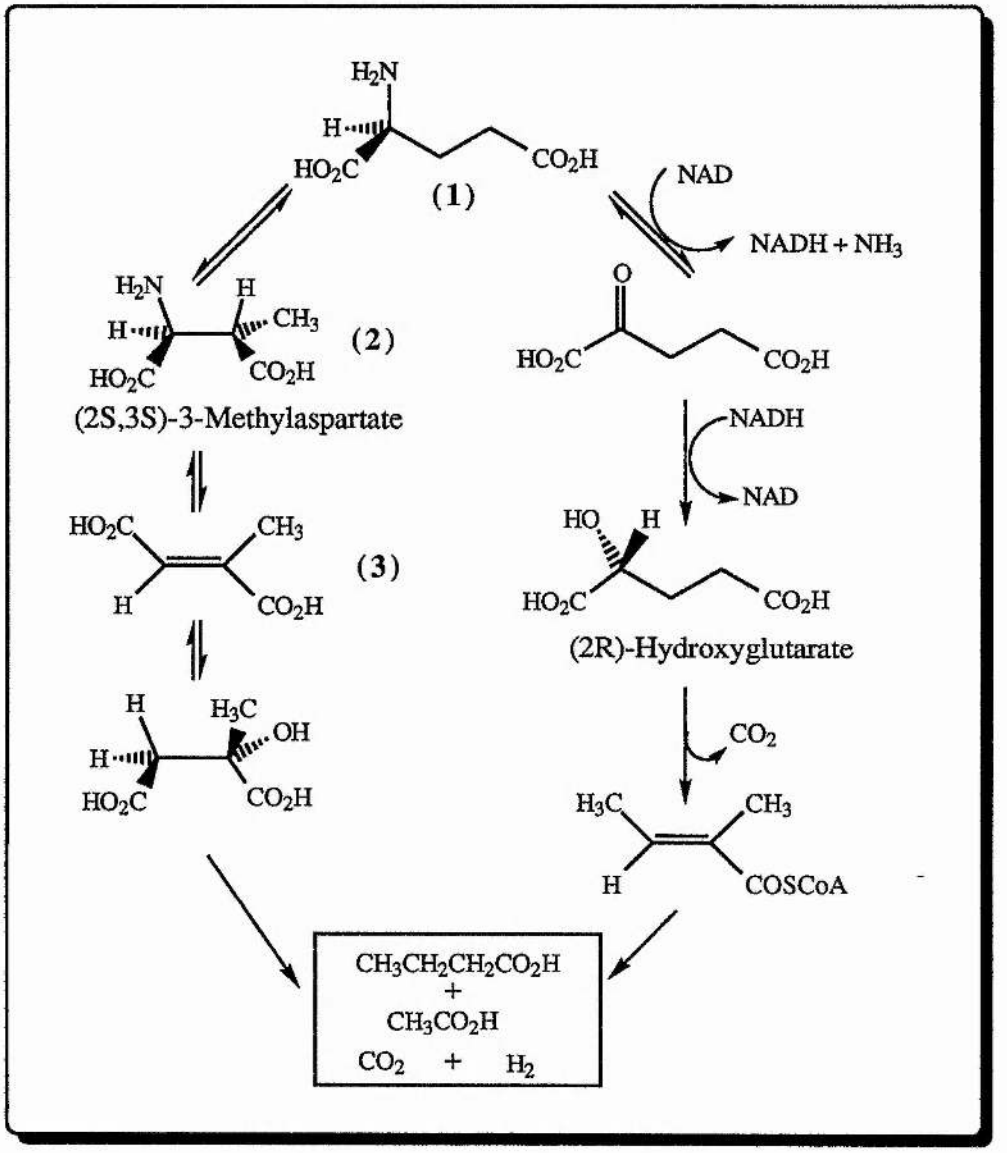
1) Coenzyme $B_{12}$ is a stable organometallic molecule evolved in biological systems in order to serve as a radical "lender". AdoCbl-dependent enzymes, in the presence of the appropriate substrate, activate the cofactor and generate cob(II)alamin and 5'-deoxyadenosyl radicals.

2) All the $B_{12}$-dependent enzymes are large proteins ( $M_r \geq 130,000$) with a complicated subunit structure, obviously necessary to perform the tasks described previously (see § 1.6.).

3) The uncertainty about the rearrangement step persists. Chemical models developed so far do not fill the gaps of our understanding. Moreover, as long as there are no kinetic studies and X-ray structures for any of these proteins, nobody will be able to claim firm understanding of the coenzyme $B_{12}$-dependent rearrangements.

## 1.16 Biological Role and Discovery of Glutamate Mutase

A significant number of bacteria ferment (2S)-glutamic acid through two distinct energy yielding pathways (Scheme 1.9). The final products in both cases are acetate, butyrate, $CO_2$, and ammonia. The methylaspartic acid pathway is used only by species of the genus *Clostridium* , whereas the (R)-2-hydroxyglutarate pathway is used by several other genera.[114]




*Scheme 1.9 Fermentation of glutamate in bacteria*

The elucidation of the methylaspartate pathway was reported in 1958 by Barker *et al.*[115] as a result of fermentation studies on the bacterium *Clostridium tetanomorphum*, strain H1. Glutamate mutase catalyses the first step of the pathway, converting (2S)-glutamic acid to (2S,3S)-3-methylaspartic acid. Growing the bacterium with glutamate as the sole carbon source results in the overproduction of all the enzymes involved in the pathway, including the mutase.

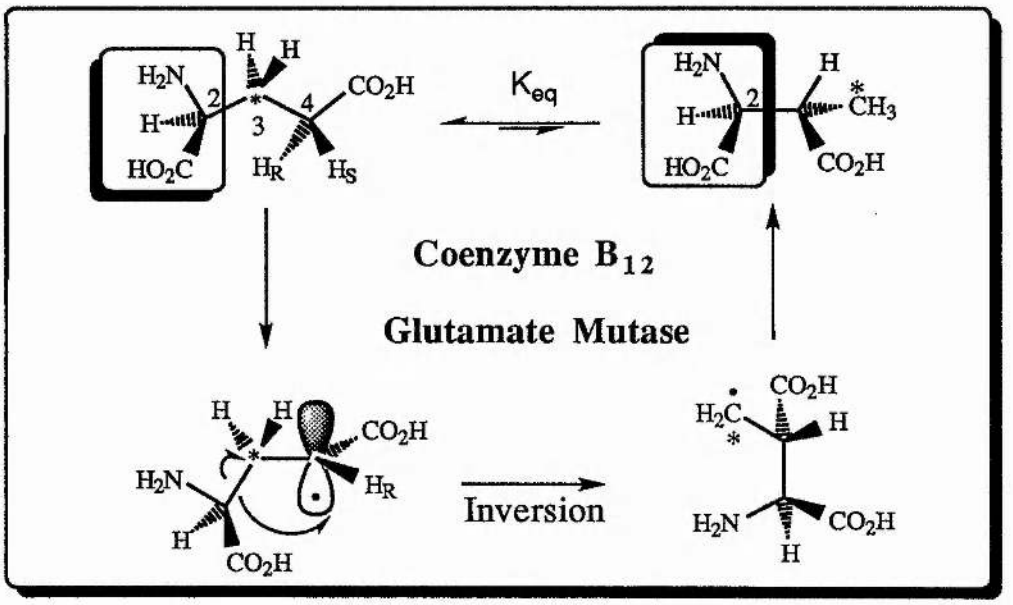
Charcoal treatment of the crude cell extracts inhibited the consumption of glutamate while ammonia could still be added to mesaconate in order to form a rather rare amino acid, soon identified as (2S,3S)-3-methylaspartic acid.[115] The existence of an enzyme that actually catalyses the interconversion of glutamate to methylaspartate was established in 1961.[116] Complete loss of activity on addition of alkaline cyanide and the pink colour of active protein fractions were clues implying the presence of corrinoids. When protein of satisfactory purity was available the dependence on coenzyme $B_{12}$ was demonstrated.[117] In 1967 full purification protocols and some preliminary kinetic results were published.[118,119] Since then only one paper[80] appeared in the literature until 1992. Despite the involvement of many groups with other AdoCbl catalysed rearrangements, glutamate mutase remained out of the main stream of interest. Clostridial cultures are expensive, require large fermentation facilities, and the yield of glutamate mutase for the overall isolation is not high.

In contrast, β-methylaspartase, the second enzyme of the pathway, is stable and easier to purify. Its availability in large quantities (overexpressed in *E. coli* ) in our laboratory, has allowed its use as a synthetic tool in the study of glutamate mutase.

### 1.17 Reaction Catalysed and Assays

The formal [1,2]-migration of C-2 of glutamic acid from C-3 to C-4 (Scheme 1.10), promoted by the enzyme, is the only rearrangement known in which all the three carbon atoms involved, are saturated. Glutamate and methylaspartate are the *only* substrates known for the enzyme, with apparent $K_m$ values of 1.35 (see § 3.5) and 0.5 mM respectively.[119] Some of the compounds tested for the mutase as substrates with negative results were (R)-glutamate, (RS)-2-methylglutamate, (2S,3R)-3-methylaspartate, and 2-methyleneglutarate (see Tables 1.6, p 44, and

3.4, p. 103).[115-118] The reaction is reversible. The equilibrium established, in the presence of the enzyme between the two substrates, at pH 8.2, favours glutamic acid. The $K_{eq}$ is approximately 10.7. This corresponds to a $\Delta F^{\circ}$ between glutamate and methylaspartate of 6 kJ $mol^{-1}$. Molecules such as methacrylic, propionic, pyruvic, 2-ketoglutaric acid, glycine or ammonia have been excluded as free intermediates using the appropriately labelled compounds.[117] Accordingly the reaction proceeds in $^2H_2O$ without the incorporation of deuterium.




***Scheme 1.10*** *The Glutamate mutase reaction*

Two assays have been developed to measure the activity of the mutase.[120] The first is based on the conversion of glutamate to mesaconate by the coupled action of glutamate mutase and β-methylaspartase (Scheme 1.9). The double bond on mesaconate **(3)** allows a convenient measurement of the absorbance at 240nm. The method, however, has a disadvantage. Some methylaspartate analogues which could give valuable information for glutamate mutase are also substrates for β-methylaspartase, therefore interfering with the assay. The anaerobic assay relies on the conversion of methylaspartic acid to glutamic acid under strict anaerobic conditions. The amount of 3-methylaspartic acid left is calculated by enzymatic or chemical means. Unfortunately this modification does not cancel the restriction mentioned above.

### 1.18 Structure of the Holoenzyme

The active form of glutamate mutase, is a complex of two subunits, both necessary for activity. Barker *et al.* reported their size as 128 kDa and 17 KDa for E and S respectively,[118,119] but recent studies by us and others[121,122,123] dismissed part of these results. The correct molecular weights for the two components are 53 and 14 KDa.

The number of AdoCbl molecules per molecule of active apoenzyme and the effect of the coenzyme concentration on the affinity of the two components for each other and for the substrate have been studied using typical saturation experiments. When the amount of component E was kept constant, an increase in the concentration of component S decreased the $K_m$ of the coenzyme (Table 1.3), and *vice versa*. In a similar manner high concentrations of coenzyme increased the affinity of the two components for each other.[119]

***Table 1.3*** *Effect of the E/S molar ratio on the $K_m$ of the coenzyme*

| S/E molar ratio | $K_m$ for AdoCbl |
|---|---|
| 0.35 | 1.29 |
| 3.6 | 0.12 |
| 17.8 | 0.31 |

When the S/E molar ratio was increased further the activity of the system remained unaffected but the $K_m$ still decreased. An explanation for this unusual behaviour "would appear to require a mechanism in which the catalytic function of component S is *distinct* from its function in enhancing coenzyme binding".[119]

It is well established that the coenzyme binds on component E[118] but the number of molecules per molecule of component E is still uncertain. Analysis of kinetic data indicated a value of 1 mole coenzyme per mole of component E (after correcting for the molecular weight).[118] When tritium labelled coenzyme was incubated with the enzyme the label was transferred to the product amino acids. From the percentage of the radioactivity transferred the ratio of coenzyme molecules per molecule of component E was calculated as 3.[124] This discrepancy cannot be easily explained on the basis of the present knowledge on the system.

## 1.19 Purification and Physical Properties of the Enzyme

The two purification procedures reported so far for component E are summarised and compared in Table 1.4. In the original method, reported by Barker,[118,119] the overall yield was 18%. The partially purified protein was estimated to be 75% homogeneous and displayed a specific activity of 3.6 units $mg^{-1}$. The procedure recently reported by Buckel and coworkers afforded the pure protein with higher specific activity (6.57 units $mg^{-1}$) despite the low overall yield (6%).[121] A small amount of component S (5-10%) was still present.

The presence of sodium glutamate or β-methylaspartate had been shown to protect component E against deactivation in cationic buffers (*e.g.* Tris).[118] These findings suggest that the "pocket" for the substrate is located on component E. This protective effect could arise either from exclusion of solvent molecules from the active site or adoption by the protein of a more stable conformation.

***Table 1.4*** *Purifications of component E of glutamate mutase*

| Barker's purification (*C. tetanomorphum*)* | Activity Units(%) | Units/ $mg^{-1}$ | Buckel's Purification (*C. cochlearium*)* | Activity Units(%) | Units/ $mg^{-1}$ |
|---|---|---|---|---|---|
| Sonic vibration | 261(100) | 0.02 | Sonic vibration | 434(100) | 0.09 |
| Charcoal/protamine | 254 (97) | 0.06 | Ammonium Sulphate | 414 (95) | 0.12 |
| Ammonium Sulphate | 240 (92) | 0.12 | DEAE-Sephacel | 168 (39) | 0.41 |
| DEAE cellulose and G-100 | 144 (55) | 1.10 | Superdex 200 | 131 (30) | 1.28 |
| Carboxymethyl cellulose | 51 (20) | 3.20 | Mono Q | 78 (18) | 3.10 |
| Brushite | 46 (18) | 3.60 | Phenyl-Superose | 25 (6) | 6.57** |

* Data adjusted for 100gr of cell paste

** More recently Buckel reported briefly that he improved his purification by achieving a specific activity of 12.4 units $mg^{-1}$.[60]

The primary structure of component E contains seven cysteines. None of the sulphydryl groups seems to be catalytically important, since treatment with iodoacetate or 2-mercaptoethanol had no effect on the activity of the holoenzyme.[118] Nevertheless, the presence of mercaptoethanol in the storage buffers quickly deactivated the protein.

AdoCbl was shown to have no effect on the stability of component E, but the experiments were performed in the absence of component S. Subsequently, no comparison can be made with other systems, like for examble diol dehydrase, known to lose their activity faster in the presence of the coenzyme.[39]

Component S is more stable than component E and has been purified 350-fold over the crude extract in 25-30% yield to give preparations of much higher specific activities (28-35 units $mg^{-1}$). An effective early step in Barker's original purification was an isoelectric precipitation at pH 4.6. Stability under these denaturing conditions implies a rather simple tertiary structure.

Exposure to oxygen results in the formation of intra- and inter-molecular S-S bridges. Activity was lost but the affinity of component S for component E remained unaffected.[119] Incubation with reducing agents like 2-mercaptoethanol or dithiothreitol restored activity. The protein is marginally more stable during storage, in its oxidized state than the reduced one. Under optimum conditions (T<-20 °C, 0.1 M $K_2HPO_4$, pH 7.4) no activity losses were observed for months.[115]

The complicated behaviour of the sulphydryl groups prompted a more careful study by Switzer and Barker.[119] Underestimating the accuracy of their results, they suggested the presence of five or six cysteines in the polypeptide chain instead the four actually present (their experimental value was 4.4). Reaction with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) under denaturing conditions showed that only three of them could be exposed to the solvent. One reacted with labelled iodoacetate resulting in a 65-70% loss of activity.[115] At the same time complete inhibition by arsenite implied the presence of two vicinal -SH groups that could form a S-S bridge and both were necessary for full activity. An interesting picture emerged. One of the -SH groups is deeply buried in the protein and very unreactive, a second one is on the surface and can be readily oxidized or reduced but it is not involved in the catalytic activity. The last two are closely located (maybe cysteines 71 & 78, Fig. 1.16) and both have to be reduced for the protein to attain full activity. Whether the thiol groups are somehow involved in the catalytic activity, or, more likely, are important for binding, is still not clear.

## 1.20 Amino Acid Sequences

The sequence of component E and S were reported recently by Marsh and Hollaway[122,123] and Brecht *et al.*[125] The two genes are located just before the gene of β-methylaspartase (Fig. 1.15).[126]

Component E consists of 485 amino acids, ($M_r$ 53,708). Comparison of its deduced amino acid sequence (Fig. 1.16) with other proteins revealed no homologies with AdoCbl-dependent enzymes.[122] A possible explanation is that the size of the coenzyme causes a "dilution" of the amino acids necessary for its binding and activation. Similarities must be conserved in the structural level and will be established when three-dimensional structures are available.

Component S is located further upstream and consists of 137 amino acids ($M_r$ 14,748) (Fig. 1.16). An unusual finding by Hollaway and Marsh was that the two sequences are not adjacent to each other on the chromosome (Fig. 1.15). Between E and S lies a 1500 bp piece of DNA designated as L.[122] Its expression ($M_r$ 50,171) together with S, E, and β-methylaspartase as one operon seems to be almost certain. The deduced amino acid sequence for L suggested no involvement in the glutamate fermentation pathway or any other biological function.

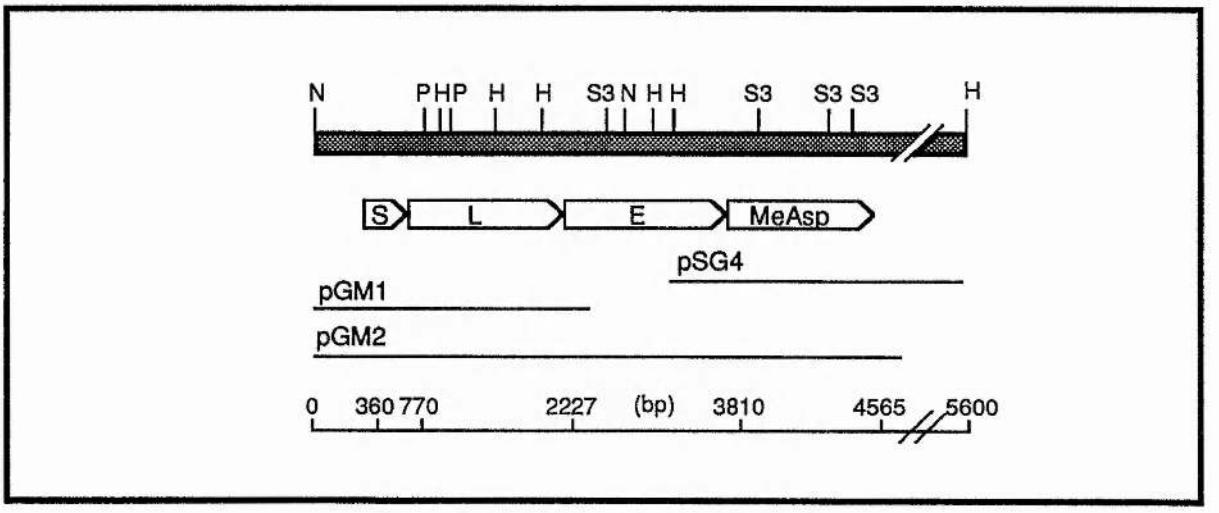



**Figure 1.15** The gene containing S, L, E, and β-3-methylaspartase. Restriction sites by different nucleases are indicated. pGM1 an pGM2 are plasmids sequenced in ref 122, and pSG4 is the plasmid sequenced in ref. 126. Restriction enzymes are H, *Hind*III; N, *Nco*I; P, *Pst*I; S3, *Sau*3AI.

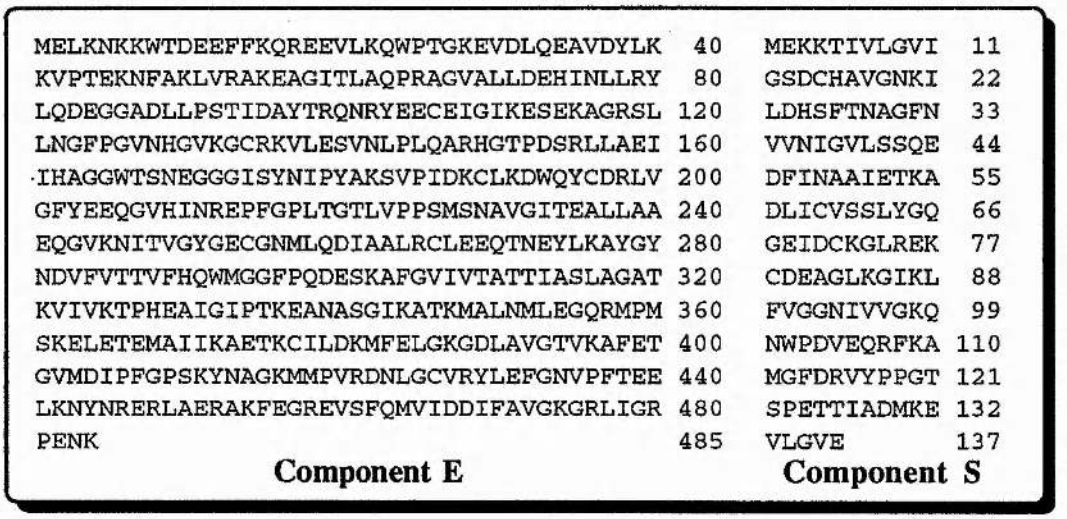



***Figure 1.16** Amino acid sequences of component E and S*

While further research is needed (site-specific mutagenesis or X-ray structures) to establish the role of specific amino acid residues, the possibility of post translational modifications as the means for studying the reaction is now not far in the future.

## 1.21 Catalytic Properties

The activation of the coenzyme and the breaking of the Co-C bond by the protein have been discussed (see § 1.7). Modified or incomplete corrinoids are inactive with glutamate mutase.[48] Additionally some of these modified, inactive derivatives at the Co-5'-deoxyadenosyl moiety acted as very strong competitive inhibitors with respect to AdoCbl.

The only type of change that seemed to be tolerated was on the sixth $\alpha$-axial position, where the enzyme could accept ligands such as 5-hydroxymethyl-benzimidazole, adenine (the main natural form occurring in *C. tetanomorphum*), benzimidazole *etc.* (Table 1.5). It was interesting that the coenzyme with the bulkier base at the $\beta$-axial position, 5,6-dimethylbenzimidazole (entry 1, Table 1.5) had a similar $V_{max}$ but higher $K_m$ than the benzimidazolyl coenzyme (entry 6). The benzimidazolyl coenzyme could fulfil its coenzyme duties in much lower concentrations. The size of the base affected the binding but not the catalytic activity. The activation of the Co-C bond did not appear to be directly related with

the tight binding of the intact coenzyme in the active site. This observation is in accordance with previous discussion concerning the cleavage of the Co-C bond by the protein (see § 1.7.2).

***Table 1.5*** *Kinetic properties of different coenzymes with glutamate mutase*

| Cobamide Coenzyme | $K_m$ M x $10^{-7}$ | $V_{max}$ ΔA/min | Relative molar act. |
|---|---|---|---|
| 1) 5,6-dimethylbenzimidazolyl | 128.0 | 0.27 | 1.00 |
| 2) Diaminopurinyl | 16.4 | 0.27 | 8.8 |
| 3) Adenilyl* | 12.4 | 0.29 | 10.0 |
| 4) 5-Nitrobenzimidazolyl | 5.3 | 0.32 | 30.0 |
| 5) 5-Methylbenzimidazolyl | 4.3 | 0.40 | 43.0 |
| 6) Benzimidazolyl | 2.3 | 0.36 | 85.0 |

* The natural occurring coenzyme in *C. tetanomorphum*

Exposure of the reaction mixtures to oxygen resulted in at least partial inhibition of the reaction.[117] Maximal activities could only be obtained when the reaction mixture was treated so as to remove dissolved oxygen. This sensitivity towards oxygen is probably related with the existence of the catalytically competent radical species.

Two different EPR signals have been obtained from glutamate mutase reaction mixtures. Partially purified preparations of component E displayed a spectrum[121] with very similar hyperfine splitting as the one observed during deactivation of ribonucleotide reductase[127] in the presence of the coenzyme (Fig. 1.17).

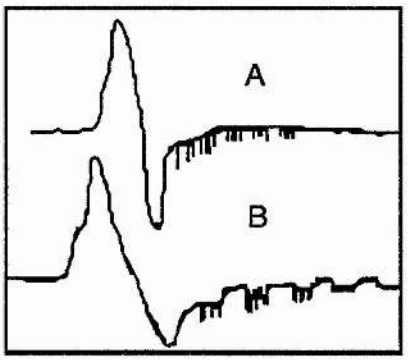



***Figure 1.17*** *A) EPR: Signal from inactivated glutamate mutase*
*B) EPR: Signal from inactivated ribonucleotide reductase*

A second EPR signal due to kinetically competent species, was reported by Buckel and coworkers.[60] (Fig. 1.18.A). The similarities with other AdoCbl-dependent enzymes have already been discussed. Surprisingly the assignment of the signal (Fig. 1.18.A) was wrong.

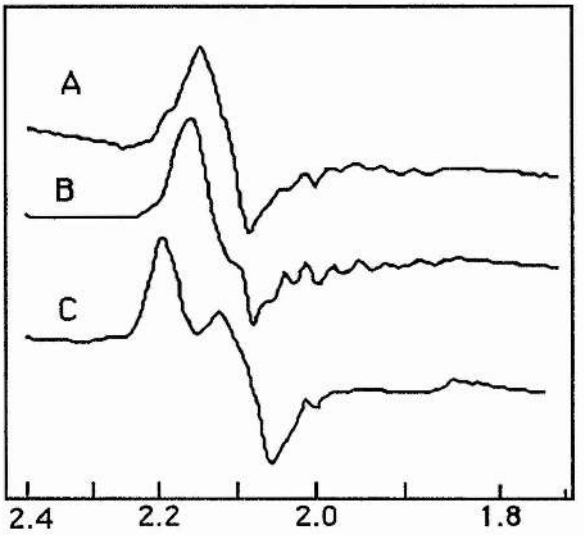



*Figure 1.18 EPR Spectrum of glutamate mutase*

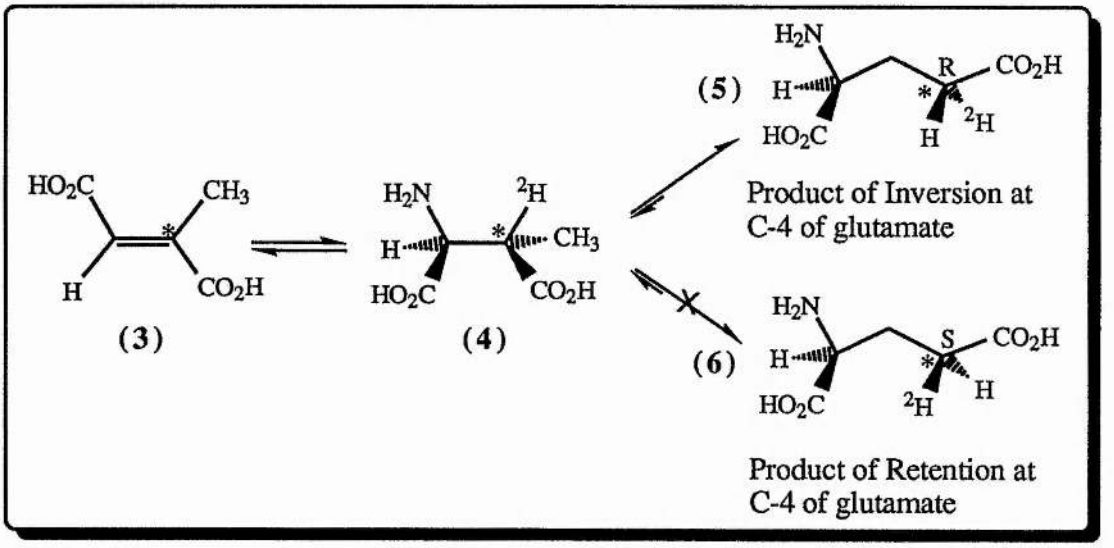
Buckel based his interpretation on an early publication by Coffman *et al.*,[63] and claimed[60] that the spectra implied the presence of two Co(II) species in a strongly distorted 6-fold coordination. Coffman and coworkers, however, in a followup study,[64] which superseded their initial findings, reported (see discussion in §1.8) (Fig. 1.9) that the hypothesis of a 5-coordinated Co(II) radical coupled by an organic radical at a distance of around 10 Å, explained all the available data and therefore was more reliable.[65]

### 1.22 Substrates and The Rearrangement Step

The 5'-deoxyadenosyl radical, after its generation, occupies a new position ~10 Å away from the cobalt and close to the substrate, already bound on the enzyme. The part of the active site where the rearrangement and the hydrogen transfers take place is inaccessible to water or oxygen molecules (see § 1.17). However, this may not be the case for the location where the cob(II)alamin is kept, thus explaining why the enzyme is so easily inactivated by oxygen during the reaction.

The adenosyl radical abstracts the *pro*-S hydrogen from C-4 of glutamic acid and the [1,2] shift of the glycine moiety takes place. The product radical retrieves its proton back from the C-5' of adenosine.[128] We do not know if the Co-C bond is reformed before the start of another catalytic cycle. It is conceivable that the enzyme can control the flux of substrate molecules without allowing solvent molecules to enter the active site. In this way we could have the adenosyl radical working in a catalytic cycle before it recombines with cob(II)alamin. For ribonucleotide reductase it has been shown that 1000 catalytic events take place before the two radicals recombine.[11]

Incubation of [4-$^{14}$C]-glutamic acid with the mutase and β-methylaspartase[129] afforded mesaconate **(3)** labelled on C-2 (Scheme 1.11). This observation was sufficient to establish the identity and the destination of the migrating carbon. Sprecher *et al.*[128] synthesised (2S,3S)-[3-$^{2}$H]-3-methylaspartic acid **(4)**. Incubation with the enzyme afforded (2S,4R)-[4-$^{2}$H]-glutamic acid **(5)**. A careful inspection of the result revealed that the new bond is formed in a $S_N2$ fashion. In this aspect the enzyme is similar with α-methyleneglutarate mutase.




***Scheme 1.11** Stereochemical course of the rearrangement*

If the relative position between the substrate and the adenosyl radical does not change drastically after the migration, it is most likely that inversion occurs also on C-3 of glutamic acid. Evidence, however, is difficult to obtain due to the reversibility

of the reaction and the torsiosymmetry of the product methyl group. Hence, when Hartrampf and Buckel[130] incubated (2S,3S)-[3-$^2$H]-glutamic acid with the enzyme, the equilibration of the hydrogens on the methyl group could not be prevented.

Tritium labelled coenzyme at C-5', in the presence of appropriate amount of protein, exchanged more than 90% of the label with glutamate and methylaspartate.[131] Eagar *et al.* in 1972 published data concerning the isotope effect of the hydrogen transfers.[80] Their elegant but complicated analysis showed that the hydrogens on 5'-deoxyadenosyl radical become equivalent. The kinetic isotope effect for hydrogen abstraction was 7.5±1.0. Although this value $k_H/k_D$ was not the actual microscopic isotope effect, it strongly suggested that the abstraction of the hydrogen was the rate-determining step. Another point which emerged from their research was the existence of an intermediate between 3-methylaspartic acid and glutamic acid. The value of the intermediate's partitioning ratio was calculated 1.7 in favour of glutamate. It seems that the TS from intermediate, to reactant or product, reflects in some degree the relative ground-state energies of reactant and product (see § 1.17).

A variety of derivatives of glutamic acid and (2S,3S)-methylaspartic acid did not rearrange. Some of the compounds cited in older literature and their interaction with the enzyme is presented in Table 1.6.

***Table 1.6*** *Some substrate analogues and their effect*

| Compound* | Substrate | Inhibitor | $K_m$ or $K_i$ |
|---|---|---|---|
| (2S)-Glutamic acid **(1)** | + | | 1.35 |
| (R)-Glutamate **(7)** | - | - | - |
| (2S,3S)-3-methylaspartic acid **(2)** | + | † | 0.5 |
| (RS)-2-Methylglutamate **(8)** | - | † | - |
| 2-Ketoglutarate **(9)** | - | † | - |
| (2R,3R)-3-methylaspartic acid **(10)** | - | † | - |

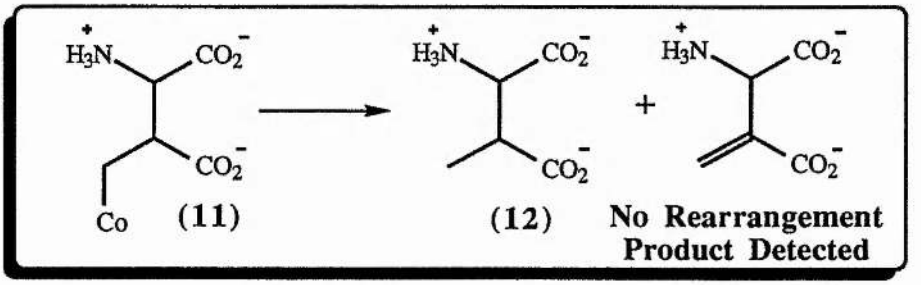
* All compounds tested by Barker, refs 118, 119. See also Table 3.4, p. 103.

† No data available

## 1.23 Model Systems

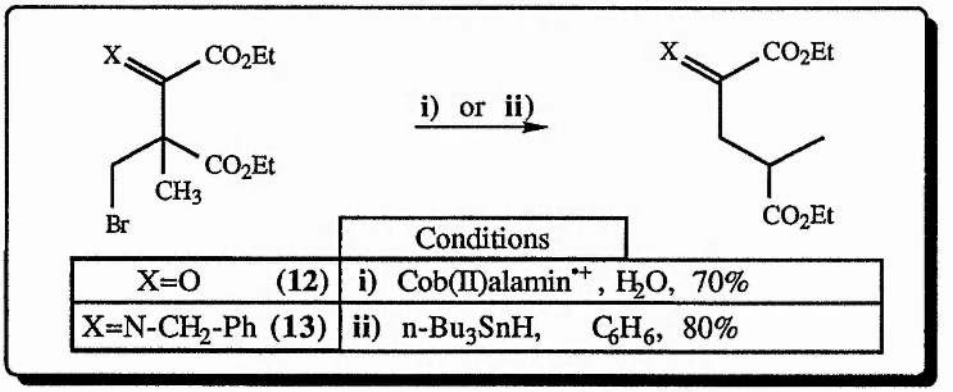
The discovery of a good model for glutamate mutase proved to be rather elusive,[69] reflecting the absence of similar known organic reactions.[113] The groups of Dowd and Murarakami have performed most of the work in this field.

3'-Methylaspartylcobalamins **(11)** were prepared by Dowd and coworkers, by reacting cob(I)alamin with the appropriate bromide.[113b] Irradiation of alkylcobalamin **(11)** generated the radicals, thought to be the intermediates in the glutamate mutase reaction. Unfortunately under a variety of conditions no rearrangement product was formed (Scheme 1.12). Inconclusive results were obtained when the corresponding 3-methylaspartate esters were tested as alternative substrates with cob(I)alamin.[113a]




*Scheme 1.12 Dowd's unsuccessful model*

Similar models were more successful and high yielding when C-2 was activated as the keto- or imine-analogues, **(12)** and **(13)** respectively (Scheme 1.13).[113b]




| | Conditions |
|---|---|
| X=O (12) | i) Cob(II)alamin$^{\bullet+}$, $H_2O$, 70% |
| X=N-$CH_2$-Ph (13) | ii) n-$Bu_3SnH$, $C_6H_6$, 80% |

*Scheme 1.13 Rearrangement of (12) and (13)*

The first successful model for glutamate mutase was reported six years ago by Murakami *et al.*[132] A highly modified corrin ring, [Cob(I)7$C_3$ester]$ClO_4$ (Fig. 1.16), was reacted with diethyl 3'-bromomethyl-[3-$^2$H]-(RS)-aspartate to prepare the lipophilic fully esterified alkylcobalamin **(14)**. The molecule was then incorporated into an intra-membrane domain formed by synthetic lipid molecules of the type $Me_3N^+(CH_2)_5CHONCH(CH_3)HCON\text{-}(C_{16}H_{33})_2$.

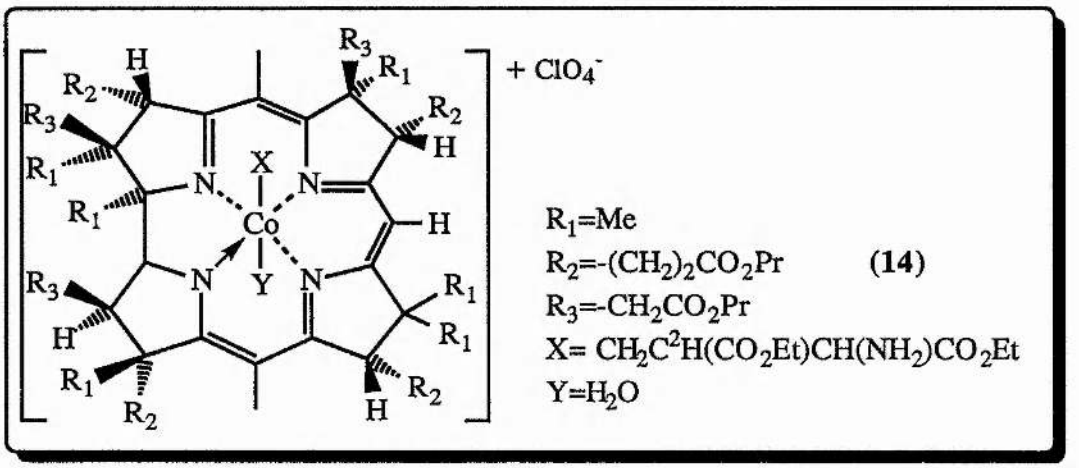
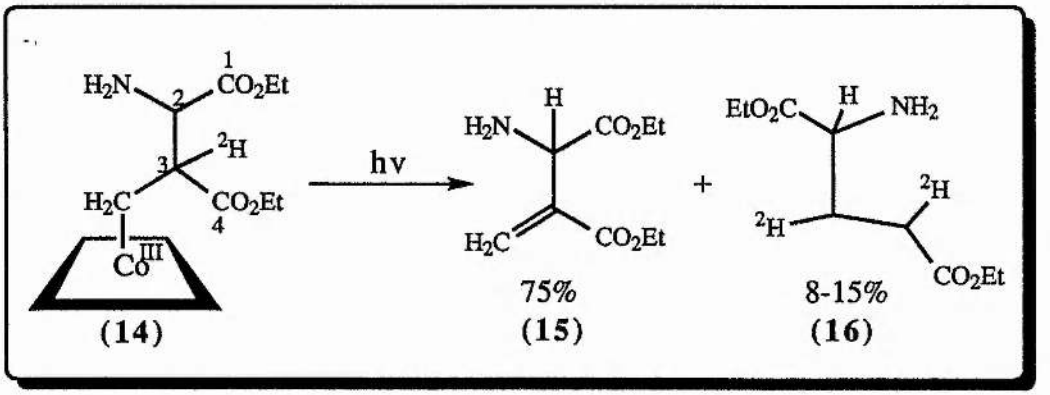



***Figure 1.19** Murakami's model compound (14)*

After irradiation, glutamate **(16)** was indeed detected in low yields (8-15%) (Scheme 1.14). Diethyl glutamate **(16)** can be formed by the shift either of C-2 or C-4. Labelling experiments established that most of the product (70-83%) is formed *via* the desired biomimetic rearrangement of C-2.[133] The discovery was important because it established the feasibility of the rearrangement in an environment that suppresses molecular motion and where hydrophobic interactions were dominant.[134] A variant of the system was able to serve as a model for methylmalonyl-CoA mutase but the yield of the product succinate was even lower (9%).

The differences between Murakami's experiments and Dowd's unsuccessful attempts have to be emphasised. The corrin ring and its substituents were not important. The modifications performed on the corrin by Murakami were necessary just to make the species soluble in the lipid layer. Esterification of the carboxyl groups of the 3-methylaspartatylcobalamin **(14)**, in comparison with the

unprotected alkylcobalamin **(11)**, probably did not effect the electronic properties of C-3 (no interference with the rearrangement step).




***Scheme 1.14** Product after generation of radicals by irradiation*

The most important difference, however, was the environment in which the radical was generated. The lipid side chains were obviously more inert to radical attack and offered more time to the structure to rearrange. Another subtle point emerging from this comparison was the certain non involvement of the cobalt in the migration step. If cobalt participation was the key mechanistic feature of the rearrangement (see §1.9), Dowd's model should have provided some glutamate. Subsequently the success of Murakami's model focused our attention onto the environment created by the amino acids in the active site and demonstrated that the enzyme catalyses an unusual but not "impossible" reaction.

### Summary

It is clear that studying a system like glutamate mutase can further our understanding in a number of areas. Both mechanistical enzymology and radical chemistry have something to learn from Nature's approach to a problem of this kind. Some of the questions that have to be addressed concern the structure of the TS of the uncatalysed (Murakami's model) reaction and its relationship with the enzymatic catalysed reaction. Finally, the means by which the protein protects the high energy intermediates present in the active site, and manipulates the substrate radical to form the product, have to be elucidated.

# Chapter 2

# Synthesis of Substrates

## 2.0 Introduction

The transformation catalysed by glutamate mutase is a uni-uni reaction. (2S)-Glutamic acid **(1)** and (2S,3S)-3-methylaspartic acid **(2)** are the only known substrates. The high selectivity of the enzyme for these two compounds,[117] severely restricts the physico-chemical methods that can be used in order to delineate details for the rearrangement. The discovery of appropriate molecular probes, substrates or inhibitors, which might interact with the enzyme and provide information about the mechanism and the radical chemistry involved, was therefore of highest priority.

The structures initially identified as possible mechanistical probes were (2S,3S)-3-methylaspartic acid **(2)**, (2S,3R)- **(17)** and (2S,3S)-3-methylglutamic acid **(18)**, and 1-amino-1,2-cyclopropanedicarboxylic acid (2,3-methanoaspartic acid) **(19)**. Our efforts towards these synthetic targets are discussed in this chapter.

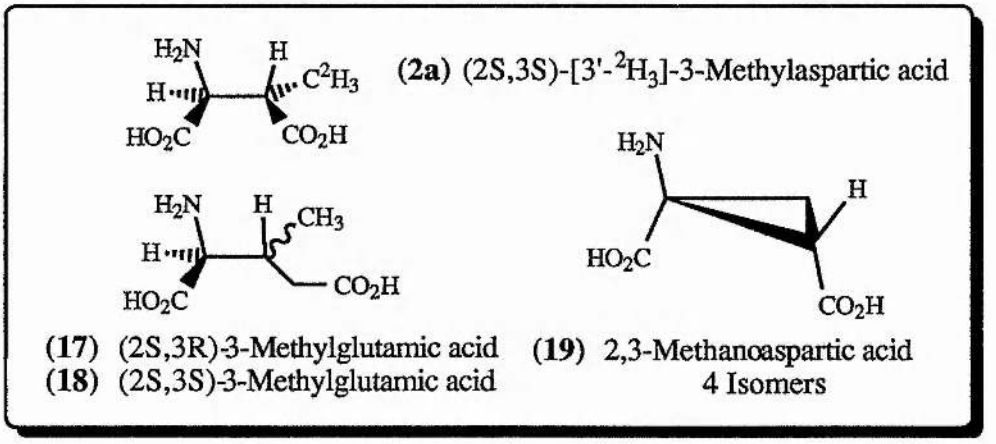
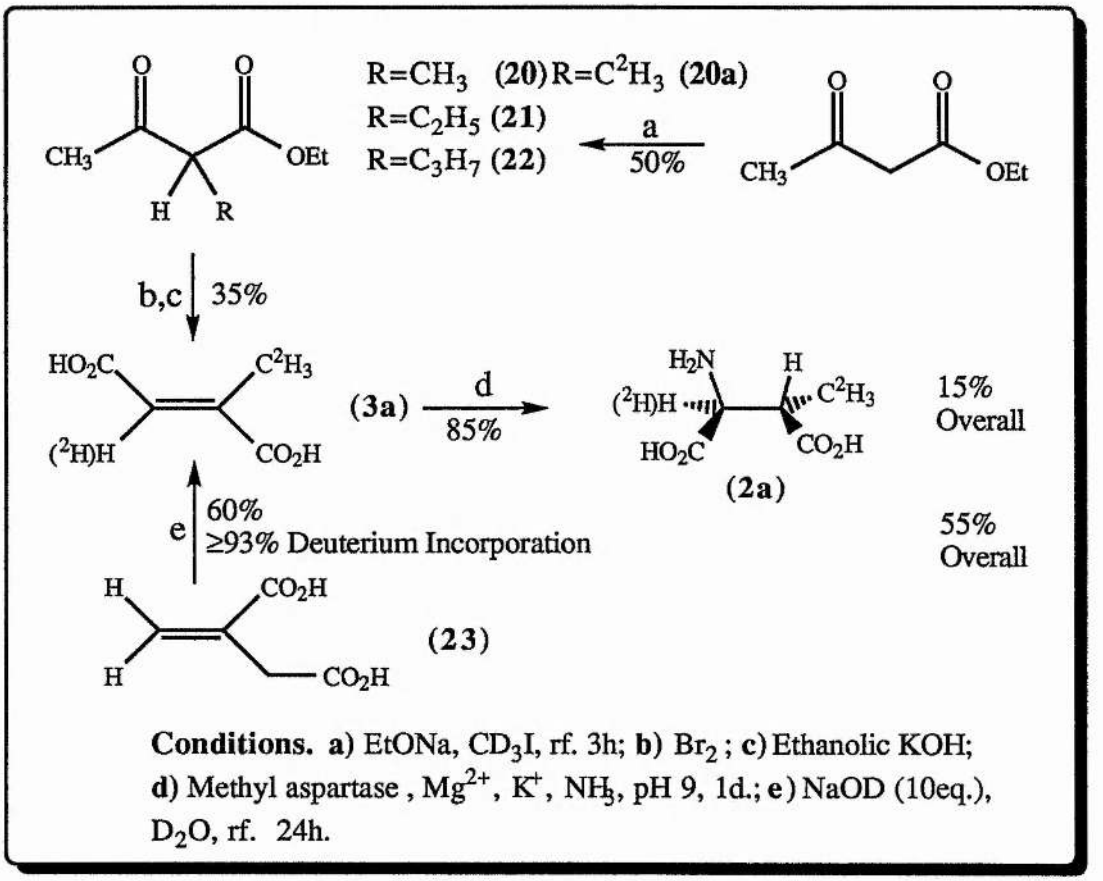



*Figure 2.1 Mechanistic probes for the enzyme glutamate mutase*

## 2.1 (2S,3S)-[3',3',3'-$^2H_3$]-3-Methylaspartic Acid (2a)

(2S,3S)-[3',3',3'-$^2H_3$]-3-Methylaspartic acid **(2a)** can be synthesised *via* the stereoselective addition of ammonia to deuteriated mesaconic acid **(3a)** catalysed by β-methylaspartase (see § 1.16). Two routes were considered for the preparation of the diacid **(3a)**. Treatment of ethyl acetoacetate with sodium ethoxide followed by alkylation with [$^2H_3$]-iodomethane, affords ethyl 2-methylacetoacetate **(20a)**. Reaction of the ester **(20a)** with bromine and subsequent treatment with base

(sodium ethoxide) would give mesaconic acid **(3a)**. Previous work in our laboratory showed that the overall yield over the three steps was acceptable (~30-35%) when R=Et or Pr, **(21)** and **(22)** respectively.[135] In the case of R=$CH_3$ **(20)**, however, we found that the corresponding overall yield was ~15% (Scheme 2.1 a,b,c). The main problem encountered was the high level of dimethylation occurring during the first step (Scheme 2.1 a).




***Scheme 2.1.*** *Synthesis of (2S,3S)-[3'-$^2H_3$]-3-methylaspartate (2a)*

A method used by Barker *et al.*,[124] to prepare (2S,3S)-[3'-$^3H_3$]-3-methylaspartate, was adapted in order to obtain the deuteriated analogue. Itaconic acid **(23)** was refluxed in NaO$^2$H/ $^2H_2O$ for 12-24 hr. The base promoted isomerisation of itaconate to mesaconate **(3a)** in 50% yield, was accompanied by high levels of deuterium incorporation. When care was taken to exclude all the protium present in the original reaction mixture, the deuterium incorporation, as judged by $^1$H-NMR, and accurate mass spectrometry, was higher than 95%.

## 2.2 (2S,3R)- and (2S,3S)-Methylglutamic Acids (17) and (18)

Despite the importance of (2S)-glutamic acid and its analogues in many physiological pathways, the C-3 alkyl derivatives have received very little attention. Ring opening of a β-lactone,[136] Evan's chiral auxiliary methodology,[137] or other methods[138] can be used to obtain 3-alkylglutamates. However, no stereocontrolled syntheses of the enantiomerically pure (2S,3S)- and (2S,3R)- diastereomers have been reported so far. Only very recently Paz and Sardina[139] managed to obtain the versatile dimethyl 3,4-di-dehydroglutamate derivative **(24)** which can be attacked by $Me_2CuLi$ to afford the fully protected versions of 3-methylglutamates **(17)** and **(18)**. Despite the absence of any stereoselectivity during the C-C bond formation it was claimed that the two diastereomers were separated after selective pyrrolidone formation.

(24)

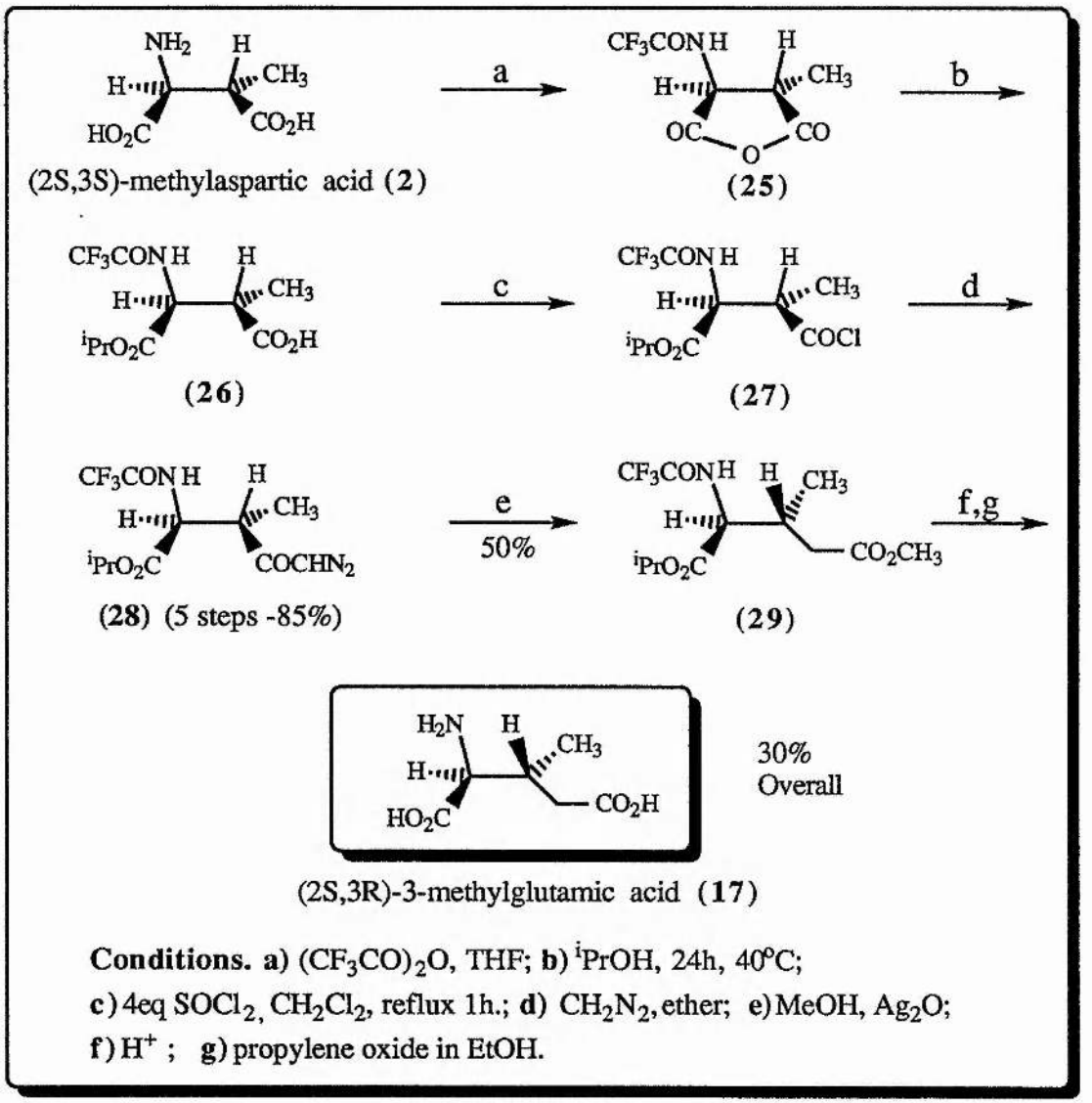
(2S,3R)-3-Methylglutamic acid **(17)** could also be synthesised *via* an Arndt-Eistert homologation of (2S,3S)-3-methylaspartic acid **(2)** which was available in gram quantities in our laboratory.

### 2.2.1 Homologation of (2S,3S)-3-Methylaspartic Acid

The *N*-protected anhydride **(25)** was synthesised in quantitative yield by treatment of 3-methylaspartic acid **(2)** with trifluoroacetic anhydride in THF. Alcoholysis of the anhydride ring with cold methanol gave a mixture 3:1 of the C-1:C-4 esters.[140] The higher electrophilicity of the α-carbonyl was more pronounced when isopropanol was used, and the 1-isopropyl ester **(26)** was exclussively formed (Scheme 2.2). In the $^1$H-NMR spectrum of the product **(26)** only one ester group could be detected.

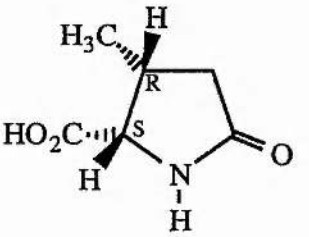
Refluxing the 4-carboxylic acid **(26)** in thionyl chloride afforded a mixture of compounds including the desired acid chloride **(27)** in 40% yield.[141] Recrystallization removed most of the impurities but the sensitivity of the product to

moisture routinely resulted in the contamination of the chloride **(27)** by the free acid **(26)**. The optimum conditions for the formation of 1-isopropyl 3-methylaspartic acid chloride **(27)** were established as 1hr. reflux of **(26)** in dry dichloromethane in the presence of 4eq. of $SOCl_2$. The acid chloride **(27)** was furnished in quantitative yield, and after thorough removal of the volatile material under reduced pressure, it was treated with an excess of dry diazomethane. The diazoketone **(28)** was obtained in 96% yield from the free acid **(26)** as a clear yellow oil which upon freezing to 0 °C solidified as large bright yellow crystals (m.p. 22-24 °C).




*Scheme 2.2 Homologation of (2S,3S)-3-methylaspartic acid*

The Wolff rearrangement, promoted either by irradiation under a medium pressure mercury lamp or by heating in methanol in the presence of $Ag_2O$, afforded the 1-isopropyl 5-methyl 3-methylglutamate **(29)**. The yields after irradiation or treatment with silver oxide in methanol were similar (~50%), although in the latter case a small amount of 1-isopropyl 4-methyl methylaspartate **(26)** was formed (5-10%), probably due to decomposition of the diazoketone prior to rearrangement. The protected 3-methylglutamate **(29)** was purified by flash chromatography on silica gel (10% ethyl acetate in hexane). (2S,3R)-3-Methylglutamic acid **(17)** was obtained after hydrolysis of the protecting groups in 6 M hydrochloric acid. In low pH all the three protons of 3-methylglutamate (2 x 4-H, 3-H) have similar shifts in their $^1$H-NMR spectrum (2.4 -2.6 ppm) (Fig. 2.3, B). Hydrochloric acid (6 M) or water:ethanol mixtures were not very effective in obtaining the pure, salt-free amino acid. Treatment of the hydrochloride salt with propylene oxide in ethanol, centrifugation and prolonged drying under vacuum at 50 °C afforded the pure amino acid. 3-Methylglutamic acid **(17)** cyclised readily under basic conditions to form the (4R,5S)-2-oxo-4-methyl-tetrahydropyrrole-5-carboxylate **(30)**. The characteristic ABX quartet of the two C-4 methylene protons could be more reliably identified in the $^1$H-NMR spectrum of the cyclic derivative **(30)** (Fig. 2.2). The homologation was concluded in 30% overall yield without encountering any major difficulties (Scheme 2.2).




(30)

## 2.3 Synthesis of (2S,3S)-3-Methylglutamic acid (18)

### 2.3.1 1,2,3-Oxathiazolidine S,S-Dioxide Methodology

The absence of any stereospecific route leading to the desired (2S,3S)-diastereomer (see § 2.2) prompted us to investigate the 1,2,3-oxathiazolidine S,S-dioxide methodology.

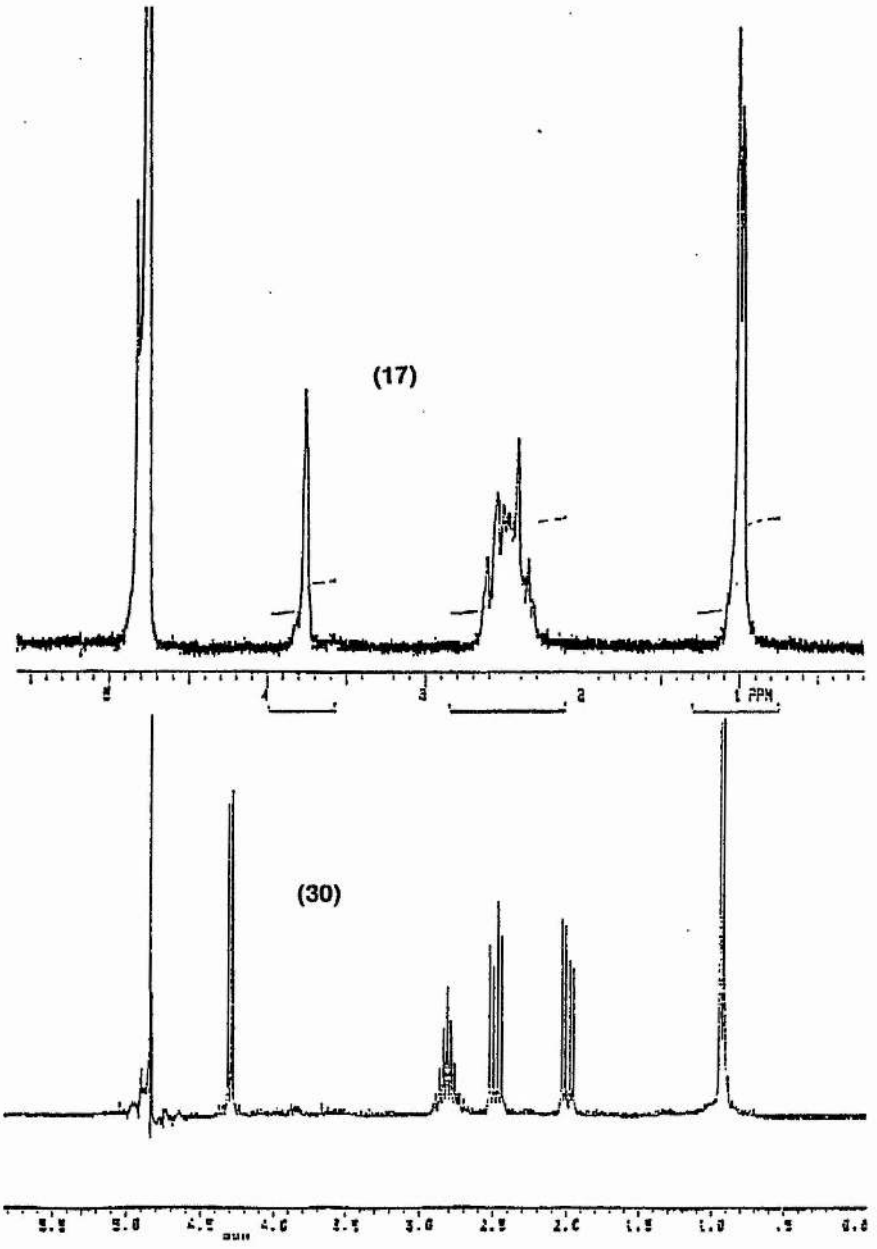
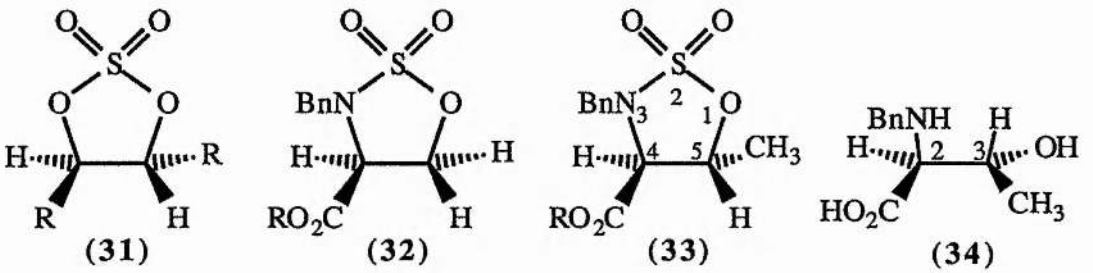



***Figure 2.2*** *Spectrum of (2S,3R)-3-methylglutamate (17) and its cyclised derivative (30)*

This approach was originally developed by Gao and Sharpless, who showed in 1988 that cyclic sulphates **(31)** were synthetic equivalents to epoxides. Opening of the five membered ring was facile, regioselective and did not require the presence of a Lewis acid.[142,143] Baldwin *et al.*[144] replaced the diols used by Sharpless with serine, to form the 1,2,3-oxathiazolidine S,S-dioxide (sulphamidate) **(32)**. In preliminary experiments it was found that the attack of diethyl malonate anion at C-5 of the sulphamidate **(32)** proceeded in 70% yield. In a similar fashion, the attack

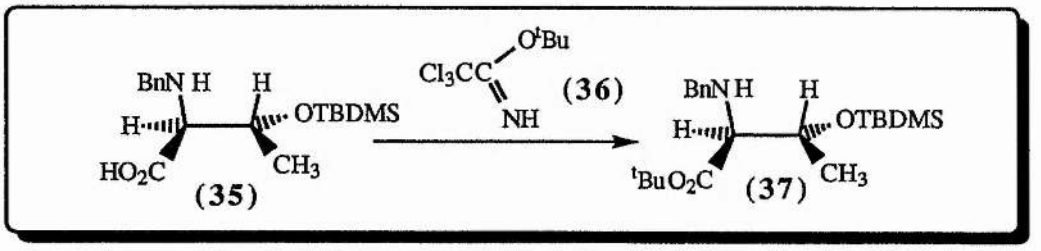
of diethyl malonate anion at the corresponding threonine derivative **(33)** would furnish the carbon skeleton of 3-methylglutamate. The 1,2,3-oxathiazolidine S,S-dioxide **(33)**, originating either from threonine or *allo*-threonine would provide the desired glutamate **(17)** or **(18)** respectively.




An extended study of the opening of these ring systems could also be performed in an effort to evaluate other possible synthetic applications.

(2S,3R)-Threonine was treated with benzaldehyde in the presence of potassium hydroxide. The imine formed, was further reduced with $NaBH_4$ to afford *N*-benzyl protected threonine **(34)** in 85%.[145] The reaction was reliable and high yielding in contrast to a more recent protocol which employs sodium cyanoborohydride as the reducing agent and gave compound **(34)** in only 30% yield.[146,147]

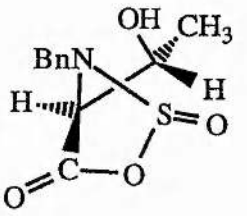
A bulky group protecting the ester can often prevent the removal of the acidic $\alpha$-H in the presence of a strong base, so Baldwin and coworkers[144] chose to protect the acid functionality by forming the *tert*-butyl ester. Armstrong *et al.*[148] developed a method for synthesising this hindered ester which employed the trichloroacetamidate **(36)**[149] in the presence of $BF_3{\cdot}Et_2O$ as the catalyst.




***Scheme 2.3** Formation of ᵗbutyl N-benzyl-O-TBDMS-threoninate*

Accordingly, after we protected the alcohol functionality of *N*-benzyl-threonine **(34)**, by forming the O-TBDMS derivative **(35)**, we tried to esterify the carboxyl group (Scheme 2.3). The yields, however, of the fully protected threonine **(37)** were remarkably low (15-20%), probably due to the insolubility of the free acid **(36)** in the dichloromethane:hexane mixtures used.

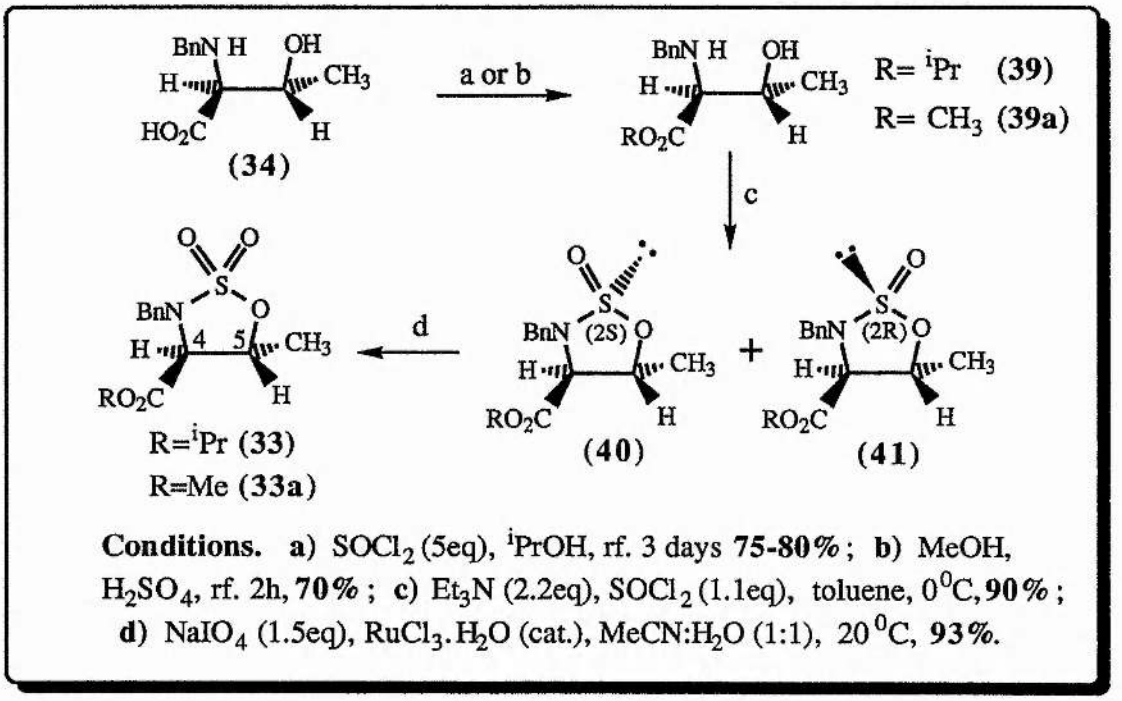
Treatment of *N*-benzyl-threonine **(34)** with isobutene in a pressure cell in the presence of $H_2SO_4$, afforded no ester either. *N*-Benzyl-threonine was again completely insoluble in dicloromethane, which was used as the solvent for the esterification. Searching for a convenient way to form the less hindered isopropyl ester we refluxed *N*-benzyl-threonine **(34)** in isopropanol in the presence of thionyl chloride. The clean product formed for periods of reflux less than 10 hr. had a very characteristic $^1$H-NMR spectrum. The downfield shift of the $\alpha$-H and the absence of any hydrogen atom on the amine were in agreement with the structure of the sulphamidite anhydride **(38)**. Alcoholysis of the five-membered ring of sulphamidite **(38)** to furnish the desired ester was achieved only after prolonged periods of reflux. Typically, isopropyl *N*-benzyl-threoninate **(39)** was formed in good yield (~75%), by refluxing protected threonine in dry isopropanol for three days in the presence of 4 eq of $SOCl_2$. The ester **(39)** was purified by flash chromatography on silica gel ($R_f$ 0.25 in 10% ethyl acetate in petrol ether) and its identity was secured by all the spectroscopic data collected.




**(38)**

The methyl ester analogue **(39a)** was formed in MeOH in the presence of $H_2SO_4$. Treatment of the protected threonine **(39)** with $SOCl_2$ and $Et_3N$ resulted in the formation of the two diastereomeric 1,2,3-oxathiazolidone S-oxides (epimeric sulphamidites) **(40)** and **(41)** as a 1:3 mixture, which was separated by flash chromatography on silica gel ($R_f$ 0.25 and 0.30 respectively in 10% ethyl acetate in hexane) (Scheme 2.4). The two sulfamidites (2S) and (2R), **(40)** and **(41)**

respectively, were isolated in similar amounts after the chromatography on silica gel. Clearly half of the (2R) isomer **(41)**, present in the crude mixture, partially decomposed on the silica gel.




***Scheme 2.4** Synthesis of 1,2,3-Oxathiazolidine S,S-dioxide (33)*

The differences between the $^1$H-NMR spectra (Fig. 2.3) of the two sulphamidites allowed assignment of their absolute stereochemistry according to the analysis of Lowe and Reed.[144c] The proximity between the oxygen and the methyl group in the (2R)-epimer **(41)** caused a characteristic shift downfield (0.2 ppm) in the $^1$H-NMR of the methyl group. A similar effect was observed for the 5-H (0.5 ppm difference between the two corresponding hydrogens) (Fig. 2.3).

The mixture of the pure 1,2,3-oxathiazolidine S-oxides **(40** and **41)** was smoothly oxidized under carefully controlled Sharpless conditions,[142] (sodium metaperiodate in the presence $RuCl_3.H_2O$), to afford the 1,2,3-oxathiazolidine S,S-dioxide **(33)** in high yield (90%). Satisfactory microanalysis and accurate mass confirmed the synthesis of the sulphamidate **(33)** in four steps, and in 50% overall

yield from threonine, improving by far the previously reported sequence for serine.[144]

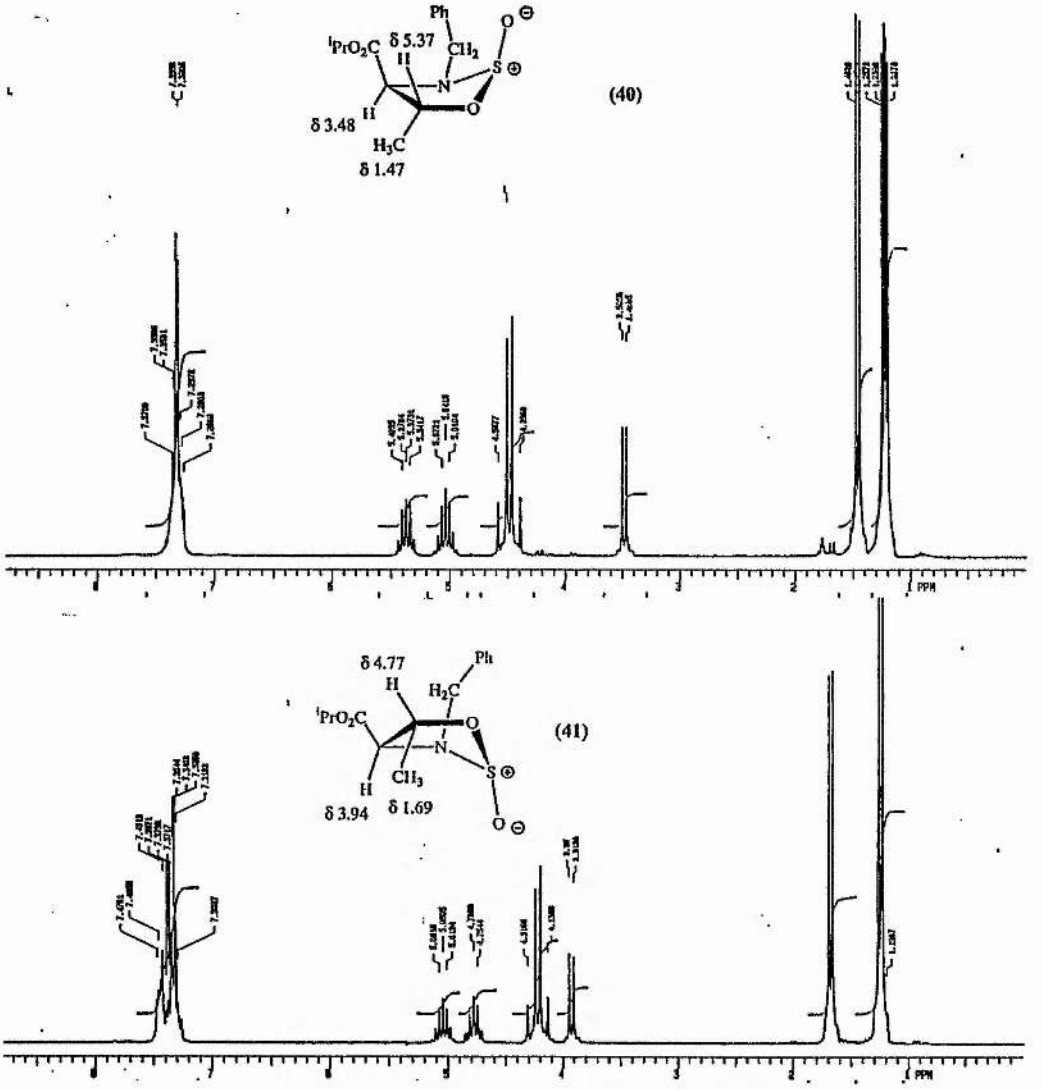



*Figure 2.3 $^{1}$H-NMR of the epimeric sulphamidites (40) and (41)*

The reaction between the diethyl malonate **(42)** and *tert*-butyl acetate **(43)** anions with the oxathiazolidine **(33)** could now be studied. Both anions were generated at -78°C by LDA in THF, and sulphamidate **(33)** was added to the reaction mixture last. Careful isolation of the products was necessary because during standard work-up, either with saturated ammonium chloride or acetic acid in THF, the sulphonate group was not always removed. If the desired attack had taken place (Fig. 2.4) the two hydrogens on the newly introduced 4-methylene group on

glutamate **(44)** would show a characteristic ABX signal (Fig. 2.3, B), not present in the starting materials. Unfortunately the alkene **(45)** emerged as the main product when either of the anions **(42** or **43)** was reacted with the oxathiazolidone **(33)**. No $S_N2$ attack product **(44)** could be detected under a variety of conditions. For temperatures lower than -70°C the recovery of starting material was usually higher than 50% and no elimination product **(45)** was obtained. Formation of the alkene was always observed at elevated at temperatures, higher than -50°C. Prolonged reaction times at -70°C had no effect on the outcome of the reaction. At room temperature the isopropyl 2-amino-but-2-enoate **(45)** was the exclusive product with a yield of 75%. The alkene **(45)** can be also synthesised by stirring oxathiazolidine **(33)** in a mixture of LiOH, MeOH, and $H_2O$ for 24 hr.

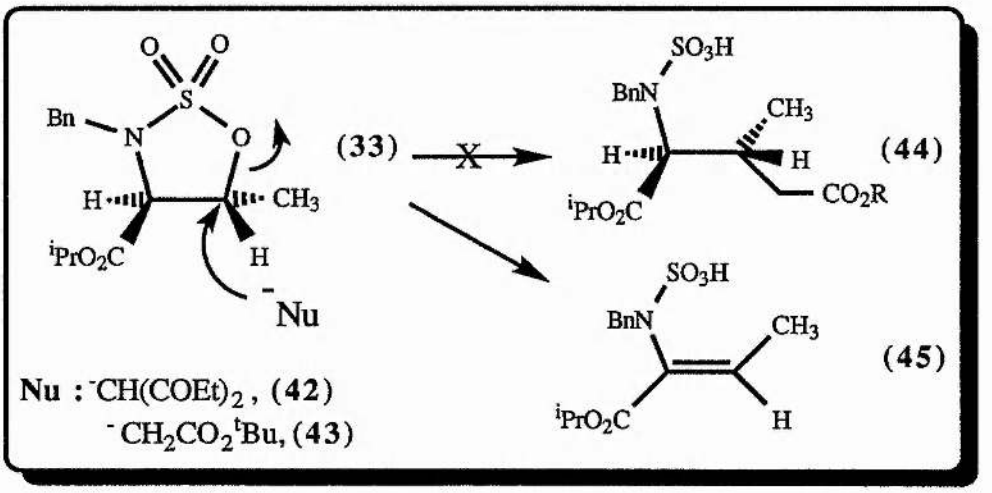
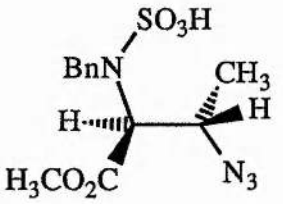



*Figure 2.4 Attack on the oxathiazolidine (33)*

The use of THF:HMPA mixtures, apart from introducing complications in the work-up, had no effect on the mode of the attack. Finally no improvement was detected when the nucleophile **(43)** was reacted with the dioxide **(33)** in the presence of Lewis acids, namely titanium(IV)isopropoxide and $BF_3{\cdot}Et_2O$. In the latter case, in an HMPA:THF (50% mixture) with 0.5 eq. of the Lewis acid present, the characteristic ABX multiplet of the two 4-methylene protons was observed in the $^1$H-NMR spectrum of the crude product. Unfortunately the yields of the glutamate **(44)** were very low (≤5%). It was clear that in the case of the sulphamidate **(33)**

the elimination was competing successfully with the more hindered attack on the secondary carbon centre.

At this point we decided to evaluate the vulnerability at C-5 of the sulphamidate **(33)** using the well documented[143] mild nucleophiles azide or thiocyanate, which react in an $S_N2$ fashion with a variety of similar electrophiles. The reaction with the azide was followed by TLC and the 3-azido-2-amino-butanoate **(46)** was indeed formed after two days of stirring in aqueous acetone. For comparison the reaction times necessary for completion of the azide attack were reported to be 12 hr. for serine **(32)**,[144] and just 1 hr. for the cyclic sulphates **(31)**.[142] The azide **(46)** was thoroughly dried under reduced pressure and then recrystallized from ethyl acetate/petrol ether to afford **(46)** as highly hygroscopic white crystals in 80% yield. All the spectroscopic data collected were in accordance with structure **(46)**.



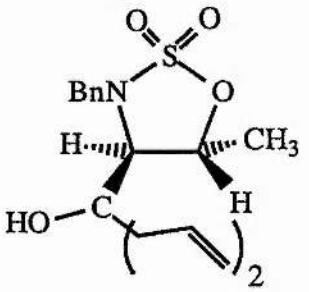

**(46)**

The above result prompted us to extend the range of the attacking species. The obvious choice was a large variety of organometallic complexes known for their mildness and selectivity.[150,151,152] Copper Grignards,[153] various types of organocuprates,[154] and titanium complexes[155] are by far the most widely used and have been employed to achieve a variety of transformations.[156,157]

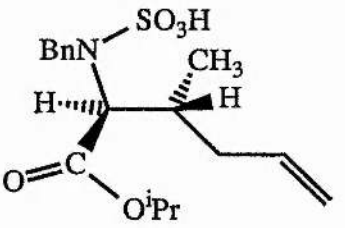
The mild and very selective $RTiCl_3$ reagent (formed by the addition of $TiCl_4$ to a solution of allyltrimethylsilane[158]) did not react with oxathiazolidine **(33)**. Mori *et al.*[159] reported in 1984 the displacement of a primary O-tosyl group by a methyl group using the complex $(CH_3)_2Cu(CN)Li_2$. The same methodology was not as successful in promoting the sulphamidate ring opening. The mono- and di-alkylcyanocuprates (entries 2,3, Table 2.1) were prepared and reacted with the sulphamidate **(33)** but the desired mode of attack was still elusive. One of the problems encountered in the case of the copper complexes was the short half-lives

of these reagents. For reaction times less than 4 hr. (the useful reaction time for cuprate complexes)[154] starting material was often recovered.

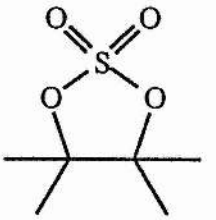
Two alternative organocopper complexes were employed. The more reactive organocopper-Lewis acid species (entry 4, Table 2.1) afforded a mixture of unidentifiable by-products. The Grignard·Cu complex RMgBr/Cu(I) (1.2eq.), prepared by adding the appropriate volume (0.1 molar equivalents) of a $Li_2CuCl_2$ solution in THF to a cooled solution of the Grignard reagent, reacted cleanly and efficiently with the oxathiazolidine **(33)** over a variety of temperatures to give a mixture of starting material **(33)** and the tertiary alcohol **(47)**. Interestingly when one molar equivalent of $Li_2CuCl_2$ was used[160] the desired 1-isopropyl 2-amino-3-methyl-hex-5-ene **(48)** was found by mass spectroscopy to represent ~10% of the crude product (Fig. 2.5). The structure of the alcohol **(47)** was also verified by 2D-NMR and accurate mass spectrometry. Varying the equivalents of the cuprate complex did not improve the yield of the ester **(48)**.




(47) M.W. 337




(48) M.W. 355




(31)

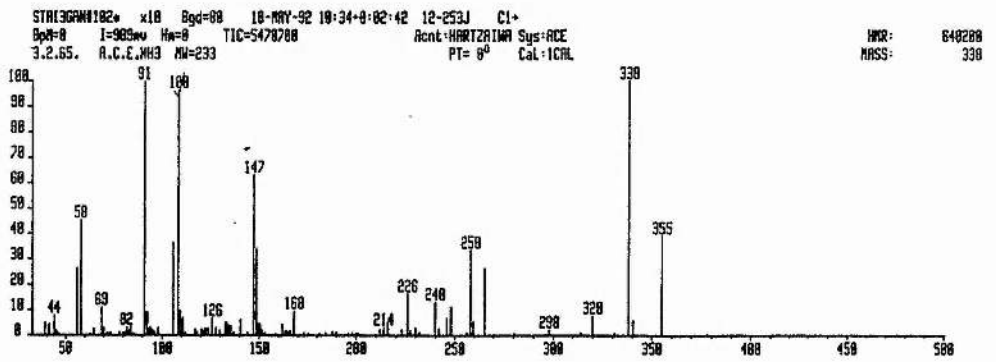



*Figure 2.5 Mass spectrum of the crude mixture of (47) and (48).*

Unfortunately the ability of the Grignard·Cu reagent to open the ring was accompanied by a non-selectivity, towards the ester. Transformation of the carboxylate to an oxazolidone or an ortho-ester can protect it from Grignard attack[161] but this line of investigation was not pursued further.

In Table 2.1 are listed the reagents used in increasing order of "hardness". The allyl group was usually employed as R since the double bond could be later efficiently transformed to a methyl ester *via* an ozone cleavage.[162]

***Table 2.1*** *Organometallic reagents used for attacking C-5 of the sulphamidate (33)*

| Ent. | Organometallic Complex | Result* | R |
|---|---|---|---|
| 1 | $RSiMe_3+TiCl_4$ | No Reaction | Allyl |
| 2 | $R_2Cu(CN)Li_2$ | No reaction | Allyl or $^nBu$ |
| 3 | $RCu(CN)Li_2$ | No reaction | Allyl or $^nBu$ |
| 4 | $RCu{\cdot}BF_3$ | Mixture | Allyl |
| 5 | RMgBr/Cu(I) | **(47)** + **(48)**† | Allyl |

* Under the conditions specified in the text.

† Yield for **(48)** ~10%.

Finally the *tert*-butylhydroperoxide anion (*tert*-$BuOO^-$) generated by BuLi[163] at -70 °C was reacted with the oxathiazolidone **(33a)**. The $^1$H-NMR spectrum of the products suggested ring opening but no peroxy ether could be detected. Obviously the nucleophile attacked the sulphur. This behaviour had been also observed by Berridge *et al.*,[164] when the phenoxide anion was used to open various sulphuryl pyranosides **(31)**. Whether the displacement took place on the sulphur or on the carbon, depended on the 3-dimensional structure of the sulphate **(31)**.

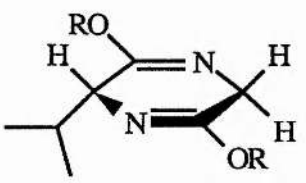
The experiments described so far underline the importance of steric factors in the opening of similar ring systems. In the case of sulphamidate **(33)** the secondary centre reduced the rate of the nucleophilic displacement at C-5,[165] and subsequently, when "hard" carbon anions were used, the formation of the alkene **(45)** became dominant. The Grignard copper reagent seemed to be sufficiently

reactive to open the ring. Unfortunately its selectivity towards the ester was very low. The rest of the organometallic complexes were too mild to interact with the electrophile.

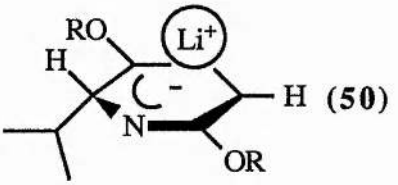
Only if a short and good yielding protocol is developed to incorporate a Grignard resistant protection on the carboxylate, will the above methodology become of practical importance for forming C-C bonds. Nevertheless the 1,2,3-oxathiazolidone S,S-dioxides **(32** and **33)** can be conveniently employed to functionalise C-3 of serine and threonine with a variety of mild nucleophiles.

### 2.3.2 Bis-Lactim Ether Methodology

Preliminary experiments showed that (2S,3R)-3-methylglutamic acid **(17)** was not a substrate for the glutamate mutase catalysed rearrangement. We were, therefore, keen to obtain the (2S,3S)-diastereomer **(18)** in order to compare the behaviour of the two (2S)-3-methylglutamic acids. Schollkopf's bis-lactim ether methodology had been used successfully in our laboratory to prepare a wide variety of $\alpha$-amino acid analogues.[166,167]




**(49)**




**(50)**

The anion **(50)**, generated from the *bis*-lactim ether of cyclo-(D-Val-Gly) **(49)** and BuLi, can attack various nucleophiles including bromides **(51)** and alkenoates **(52)** in order to give the alkylated dihydropyrazines **(53)** and **(54)** respectively (Fig. 2.6).

The utility of the methodology originates from the facile establishment of the stereochemistry at C-2', since the attack takes place almost exclusively from the face opposite to the bulky isopropyl group of valine. When (2R)-valine has been incorporated in the bis-lactim ether ring **(50)**, the $\alpha$-amino acid centre formed after the attack has the (S) stereochemistry.[168] The enantiomeric excess is typically higher than 97%.

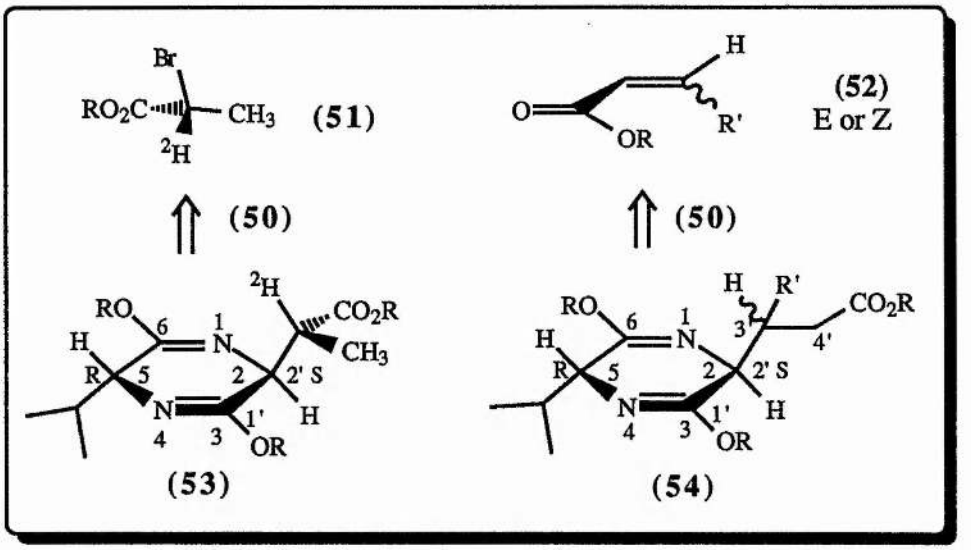
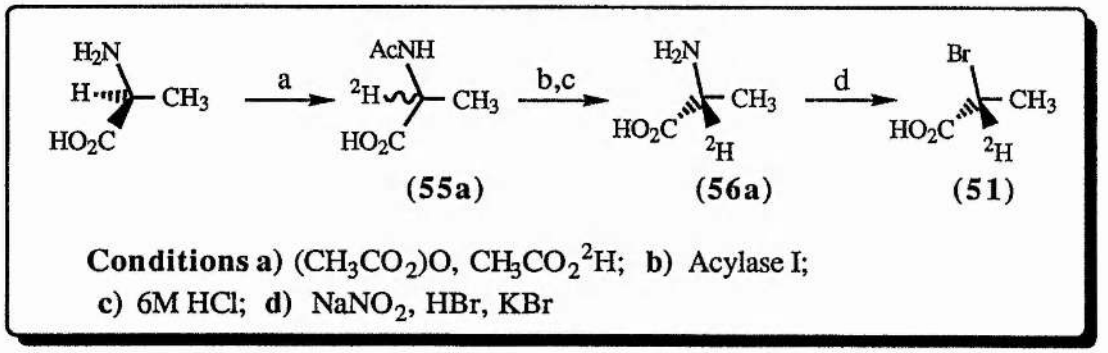



***Figure 2.6*** *Alkylating reagents and alkylation products in bis-lactim ether methodology*

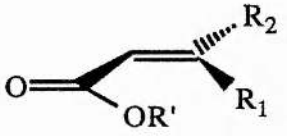
The attack on the bromide **(51)** was the key step in an effort to construct analogues of 3-methylaspartic acid. (S)-alanine was treated with a mixture of acetic anhydride / acetic acid-$^2$H to produce racemised *N*-acetyl-alanine **(55a)** (Scheme 2.5). Acylase I de-acetylated only the (S) isomer. Free (S)-alanine was easily separated from the *N*-acetyl-(R)-alanine, which was subsequently hydrolysed in 6 M hydrochloric acid to afford (R)-[2-$^2$H]-alanine **(56a)**. Treatment of compound **(56)** with $NaNO_2$/HBr/KBr furnished, with retention of configuration, the 2-bromopropanoic acid **(51)** which was further reacted with the anion **(50)** to generate the 2-alkyl-dihydropyrazine **(53)** (Fig. 2.6). Hydrolysis liberated (2S,3S)-[3-$^2$H]-3-methylaspartic acid.[167]




***Scheme 2.5*** *Synthesis of (2R)-[2-$^2$H]-bromopropanoic acid*

Alkylated dihydropyrazines **(54)** can be prepared *via* a Michael type addition of the anion **(50)** to the double bond of various crotonate derivatives **(52)**. Hydrolysis of the product pyrazine **(54)** would secure (2S)-3-alkylglutamate and (2R)-valine (Scheme 2.6, p. 66).

Schollkopf and coworkers performed the above reaction with various alkenoates **(52)** to obtain a number of C-3' substituted dihydropyrazines-2-propanoic acid methyl esters **(54)**.[169] Only compound **(54)**, with R'=Ph, had its stereochemistry at C-3' assigned by comparison to known structures. The rest of the pyrazines that were synthesised, were characterised using $^1$H-NMR or chromatographic methods. For dihydropyrazine-2-[3'-methyl]-propanoic acid methyl ester **(60)** (R=$CH_3$ on **(54)**), only a moderate diastereomeric excess was reported (3:1). More importantly the means used to calculate the ratio of the two diastereomeric pyrazines (**(60)** and **(61)**, see p. 66) was not specified.

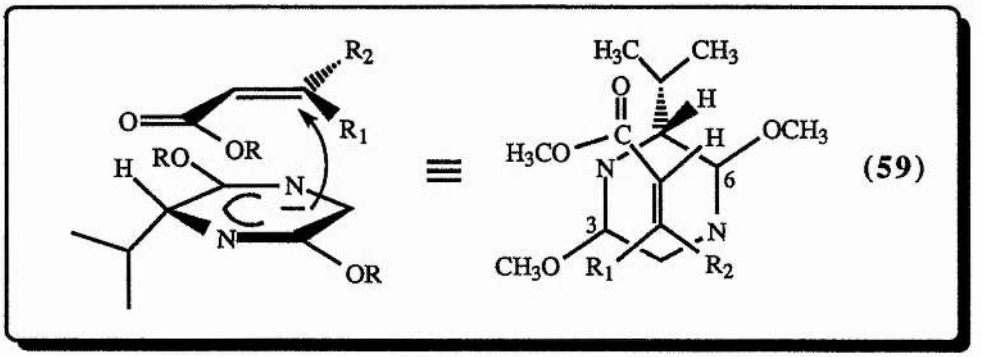



**(57)** *E* $R_1$=H, $R_2$=$CH_3$
**(58)** *Z* $R_1$=$CH_3$, $R_2$=H

The stereoselectivity observed was rationalised by postulating a transition state in which the two 2 π-systems (the π-system from the diazapentadienyl anion **(50)** and the π-system of the 2-alkenoate, **(57)** or **(58)**) form a π-complex **(59)** which is stabilised by HOMO-LUMO interactions (Fig. 2.7). Because of the nature of the reactants there is only one orientation that minimises the electrostatic interactions and allows the attack to occur smoothly. Only when the carboxyl group of the but-2-enoate ester **(57)** is positioned over N-4 of the ring, the electrostatic repulsions between the oxygens are minimised. A top view of the transition state suggests that the *trans* alkenes **(57)** will accommodate themselves slightly better over the ring.

When the complex **(59)** is formed between the anion **(50)** and the *cis* isomer **(58)** an additional steric interaction between $R_1$ and the methoxy group on C-3, might affect the yield but it cannot cancel the strong repulsion which is present between the carboxyl group of the alkenoate and the methoxy group on C-6. In this manner the outcome of the reaction as far as it concerns the stereochemistry on C-

3' is defined by the nature of the alkenoate (*E* or *Z*). According to this rationale, reaction of the anion **(60)** with the *trans* crotonate **(57)** would provide the (2S,3S)-3-methylglutamate **(18)** carbon skeleton. The reported 3:1 excess[169] was satisfactory for some preliminary experiments with the enzyme; so we carried out the alkylation of pyrazine **(49)** using *trans* crotonate **(52)** as the Michael acceptor.



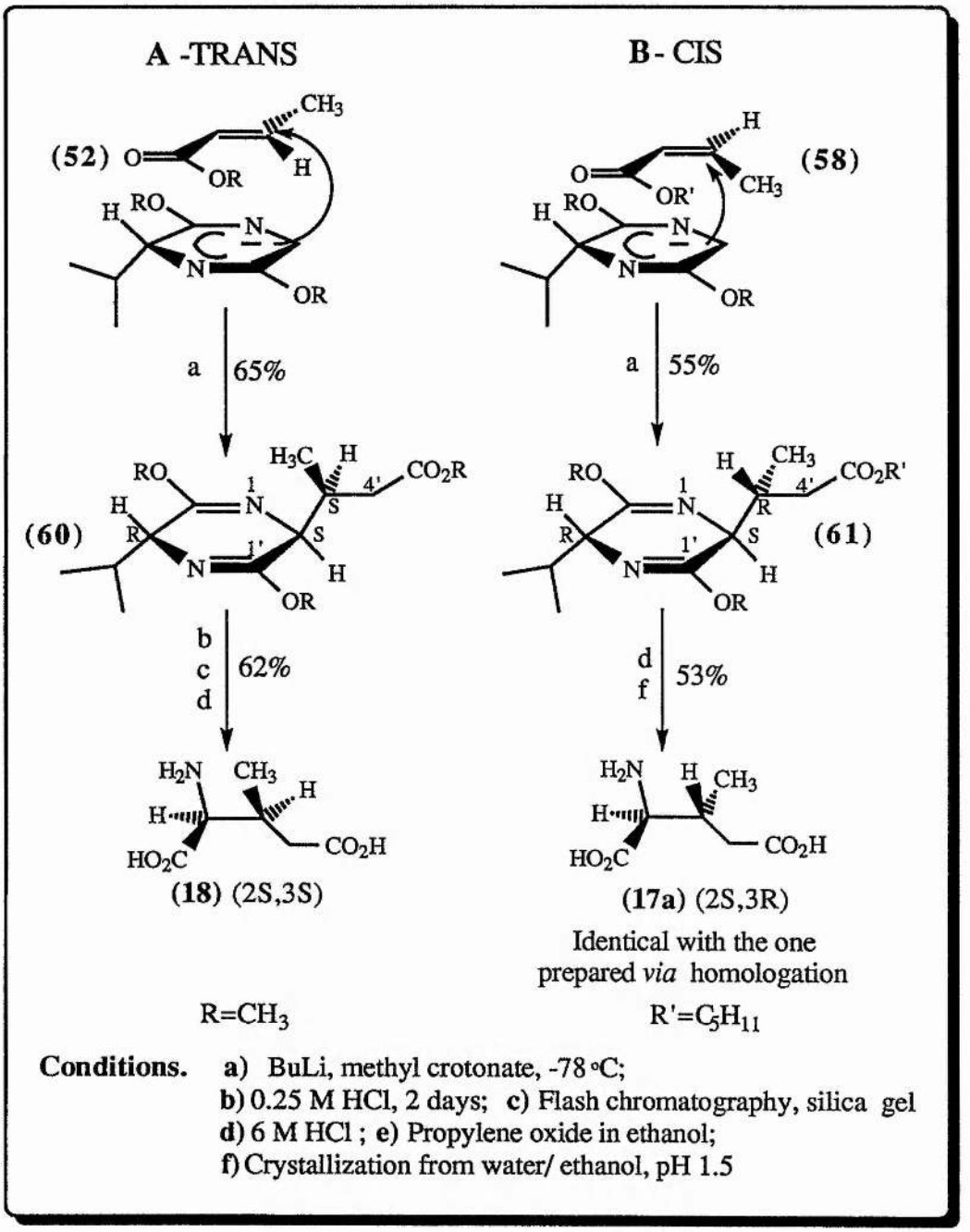

**(59)**

***Figure 2.7** Transition state of the attack*

(5R,2S)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazine-2-propanoate **(60)**, (Scheme 2.6 A), as judged by $^1$H-NMR spectroscopy, was the main product but it could not be easily purified. Despite Schollkopf's reports[169] distillation did not remove any of the impurities. Finally dihydropyrazine **(60)** was carefully flash chromatographed twice on silica gel (1:20 ethyl acetate:hexane) and obtained pure in moderate yields (60%); the only way to actually visualise the compound **(60)** on a TLC plate was by phosphomolybdic acid (10% in ethanol), but even this procedure was successful only when concentrated solutions of the product were used. The characteristic yellow spot obtained was very different from the deep green colour of the impurities (probably N-alkylated compounds). The $^1$H- and $^{13}$C-NMR spectra of the pure alkylpyrazines, at 300 MHz and 75 MHz respectively, offered no indication for the existence of two diastereomers. Either the selectivity of the reaction was higher than reported[168] or the pyrazines **(60)** and **(61)** could not be differentiated by NMR spectroscopy.

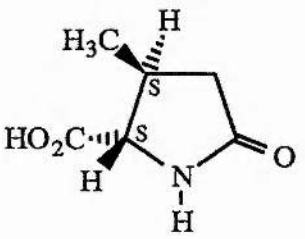
Hydrolysis of the dihydropyrazine **(60)** in 6 M hydrochloric acid gave a mixture of 3-methylglutamic acid **(18)** and valine **(64)**. Whereas (2S)-glutamic acid and valine can be easily separated using an anion exchange column (*e.g.* amberlite),

(2S,3S)-3-methylglutamic acid **(18)** cyclised readily under basic conditions to give the (4S,5S)-4-methyl-pyrroglutamate **(62),** which could not be separated from valine. Selective crystallization of 3-methylglutamic acid **(18)** at low pH was one alternative, but the method was not ideal for two reasons.

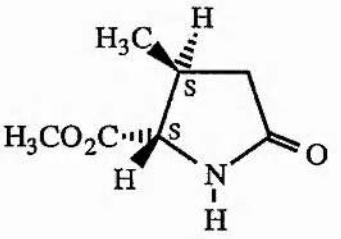



***Scheme 2.6** Synthesis of 3-methylglutamic acids*

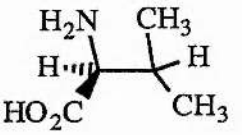
First, salts co-crystallized and second, the amount of the amino acid that could be obtained was only about 50% of the total product. The residual 3-methylglutamate could not be separated from valine. Alternatively stirring the pyrazine **(60)** in 0.20 M hydrochloric acid for two days afforded the methyl pyrroglutamate **(63)** and valine methyl ester. The two compounds were easily separated on silica gel. Hydrolysis of the methyl (4S,5S)-4-methyl-tetrahydropyrrole-5-carboxylate **(63)** in 6 M hydrochloric acid and crystallization in ethanol using propylene oxide furnished 3-methylglutamic acid **(18)** in reasonable overall yield; 42% from the pyrazine **(49)**. Satisfactory microanalysis was obtained for (2S,3S)-3-methylglutamic acid and the melting point (166-167°C) was identical to that of 3-methylglutamate **(17)** prepared *via* homologation.



**(62)**



**(63)**

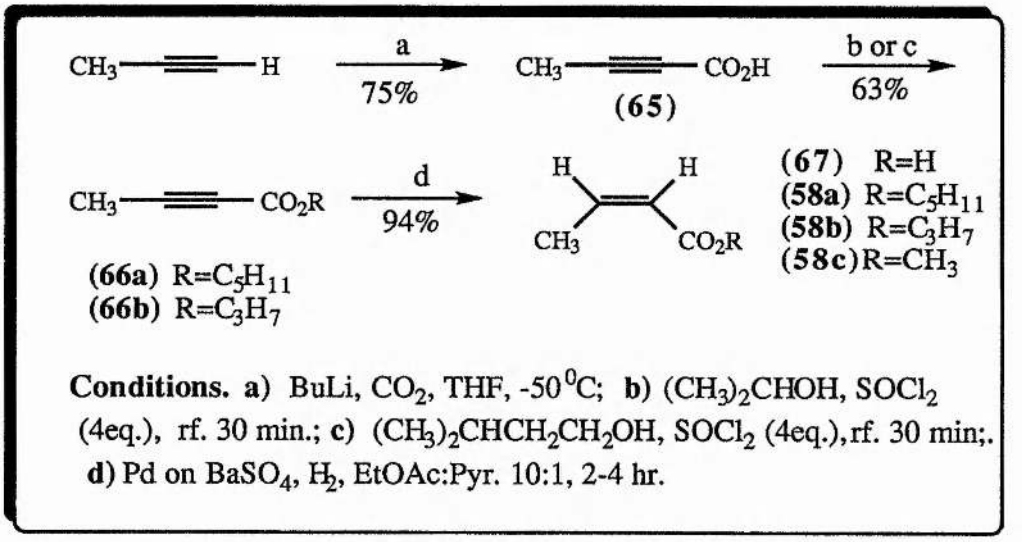


**(64)**

The optical rotation values of (2S,3S)- **(18)** and (2S,3R)-3-methylglutamic acid **(17)** (prepared *via* homologation) (Table 2.2, p. 69) were substantially different. This encouraging result, however, was not sufficient to establish the diastereomeric excess of the reaction. Only when the (2S,3R) diastereomer **(17a)** was obtained *via* the same route and compared with our standard (2S,3R)-3-methylglutamic acid **(17)** (prepared by homologation (Scheme 2.2)), were we be able to clearly comment on the stereoselectivity of the attack (see p. 64). To this end, *cis*-crotonate **(58)** had to be used as the alkylating agent.

The first reliable method for the synthesis of *cis*-crotonic acid **(67)** was reported in 1956. A mixture of *trans*- **(57)** and *cis*-but-2-enoic acid **(67)** (1:5) was obtained after hydrogenation of but-2-ynoic acid **(65)** over Lindlar catalyst.[170] Separation of the two isomers was easily achieved since the two acids have very different physical properties. Accordingly the expensive but-2-ynoic acid **(65)** was

synthesised in multigram quantities by saturating a solution of BuLi in THF with methylacetylene and then purging the reaction mixture with carbon dioxide at -50 °C. The crude acid **(65)** was distilled and recrystallized twice. Hydrogenation of but-1-ynoic acid **(65)** over Lindlar catalyst in MeOH for 1 hr. afforded *cis*-crotonic acid **(67)** including 10% of the *trans* isomer. The regioselectivity of the hydrogenation improved markedly when a mixture of ethyl acetate:pyridine (9:1)[171] was used as the solvent mixture. No *trans* isomer could be detected but ironically the reaction times for the completion of the reaction increased to 2-3 days. Finally the *cis* isomer **(67)** was quantitatively formed in less than 3 hr. when freshly prepared[172] Pd on $BaSO_4$ was employed as the catalyst. The two acids can be easily identified by their very characteristic $^1$H-NMR spectra. The chemical shift for the 2-H methylene proton is 6.9 ppm and 6.3 ppm, for the *trans* and the *cis* isomer respectively.




***Scheme 2.7** Synthesis of cis-crotonate esters*

*cis*-But-2-enoic acid **(67)** isomerised or even decomposed under the usual esterification conditions (acid or $SOCl_2$ in the presence of the alcohol). *cis*-Crotonic acid methyl ester **(58c)** was obtained after treatment with diazomethane but its low b.p. (~35 °C) prevented easy isolation. Therefore, the but-2-ynoate esters **(66 a,b)** were prepared first, and further reduced to the corresponding but-2-enoates **(58a** and **58b)**. (Scheme 2.7, d). Both esters were easily purified on silica gel ($R_f$ 0.6 in

5% ethyl acetate in petrol ether) but only isoamyl *cis*-crotonate **(58a)** (b.p. 135°) was isolated in sufficient quantities.

With the *cis* alkene **(58a)** in hand, we turned to the preparation of the pyrazine **(61)** (Scheme 2.6). Treatment with the diazapentadienyl anion **(50)** furnished the pure dihypropyrazine **(61)** which was isolated in the anticipated (see p. 64) slightly lower yield (45%), compared to the diastereomeric pyrazine **(60)** (55-60%). The $^{1}$H-NMR spectra of the two diastereomeric molecules **(60** and **61)** revealed no substantial differences. The $^{13}$C-NMR at 75 MHz, however, showed small but detectable differences in the shifts of C-3' and C-4'. The (2S,3R)-3-methylglutamic acid **(17a)** could not be isolated in the same manner as the (2S,3S)-isomer **(18)**, because during the mild hydrolysis of the pyrazine **(61)** the corresponding (4R,5S)-3-methylpyroglutamate was not readily formed (see p. 65). So the pure 3-methylglutamic acid **(17a)** was simply crystallized out from water:ethanol at pH 1.5. One additional re-crystallisation was necessary for the product to give satisfactory microanalysis and melting point. The optical rotation values (Table 2.2) verified the high stereospecificity of the attack (≥ 90%) hinted by our previous NMR spectroscopy experiments. Therefore the method was considered to be suitable for obtaining 3-alkylglutamates of high optical purity.

***Table 2.2*** *Optical rotation values for (2S)-3-methylglutamic acids*

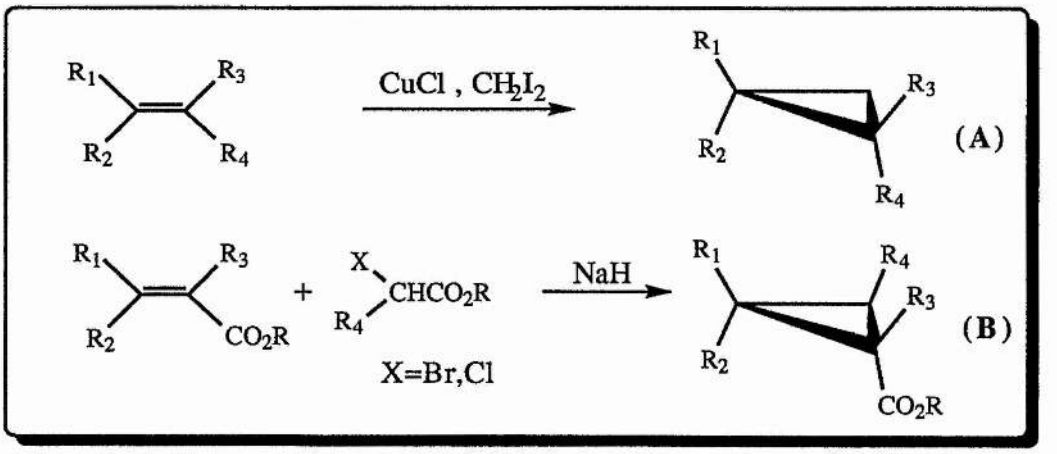
| 3-Methylglutamates | $[\alpha]_D^{23}$ (c 1 in 6M HCl) | m.p.°C |
|---|---|---|
| (2S,3R)-Methylglutamate **(17)** (homologation) | +22.0 | 169-171 |
| (2S,3R)-Methylglutamate **(17a)** (attack on *cis*-crotonate) | +23.7 | 167-169 |
| (2S,3S)-Methylglutamate **(18)** (attack on *trans*-crotonate) | +36.8 | 166-167 |

We cannot speculate about the reason Schollkopf and coworkers reported such a low stereoselectivity for this particular attack. According to our findings no mixture of diastereomers could be detected by NMR spectroscopy at 300 MHz ($^{1}$H) or 75 MHz ($^{13}$C) for the pyrazines **(60)** and **(61)** and the diastereomeric excess seems

to be at least 90%. One additional advantage of the method described above is that labels can be incorporated in literally every desirable position by using the appropriate starting materials.

## 2.4 Synthesis of 1,2-Cyclopropanedicarboxylic Acids

1-Amino-1,2-cyclopropanedicarboxylic acid **(19)** was a molecule of considerable importance, due to its possible structural resemblance to the transition state for the rearrangement catalysed by glutamate mutase. A number methods exist for the preparation of cyclopropane ring bearing carboxyl substituents. The Simmons-Smith[173] cyclopropanation of olefins (Scheme 2.8, A), and the use of halo-acetates and acrylate analogues, as developed by McCoy *et al.*[174] and McDonald and Reitz (Scheme 2.8, B),[175] are the best known approaches. More recently the dipolar addition of diazo compounds (*e.g.* $N_2CHCO_2Et$) to dehydro alanine derivatives was developed as an alternative route.[176]



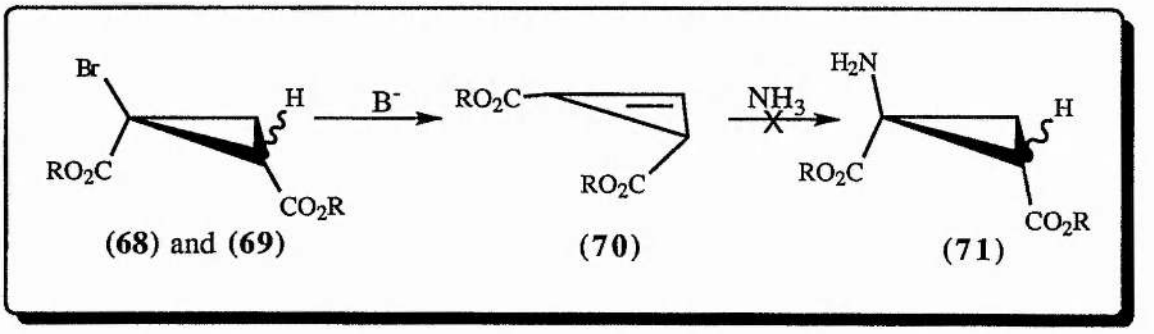

***Scheme 2.8** General methods for the preparation of functionalized cyclopropane rings*

One synthesis of the amino acid **(19)** was published by Kraus *et al.*[177] in 1990. The bromo diester **(68)** was treated with potassium bis-(trimethylsilyl)amide to give the cyclopropenedicarboxylate intermediate **(70)** (Scheme 2.9). Regioselective addition of ammonia to the double bond furnished dimethyl 2,3-methanoaspartate

**(71)** in yields between 30-50%. The *cis* and the *trans* isomers **(71)** were separated and hydrolysed to afford the corresponding free amino acids **(19)**. Despite the low yield the method seemed to be short and reliable.

In the same year, however, Stammer reported[178] that only the amino protected version of 2,3-methanoaspartic acid **(19)** could be synthesised in his laboratory. Upon deprotection, the free amine formed the imine with concomitant destruction of the three-membered ring. Finally only the corresponding keto compound was isolated.

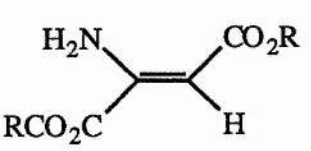
Our efforts to reproduce Kraus synthesis afforded only mixtures of starting material **(68** and **69)** often partially deprotected. A large number of protocols was evaluated including different bases (NaH, LDA, $EtO^-$) and a spectrum of temperatures. Lastly the reaction was performed in a pressure cell which allowed the reaction to be warmed up to room temperature without lowering the concentration of ammonia. No amino acid **(71)** could be unequivocally detected by NMR spectroscopy under any of the above conditions. There was no doubt that the cyclopropene intermediate **(70)** was formed since addition of the base caused a characteristic change to the colour of the reaction mixture to brown, due to the release of bromine. The addition of ammonia to the double bond seemed to be the problematic step. Our failure to obtain any reasonable amounts of product seems to confirm Stammers report on the instability of the free amine.[178]



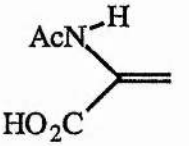

***Scheme 2.9** Unsuccessful synthesis of 1-amino 1,2-cyclopropanedicarboxylate*

The bromide **(68** or **69)** was also heated in concentrated ammonia in the presence of silver nitrate in an effort to promote an $S_N1$ type of nucleophilic substitution. Only starting material was isolated from the reaction mixture.

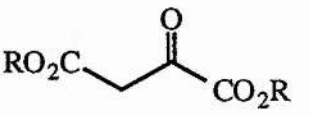
Obtaining methanoaspartic acid **(19)** *via* an amino protected precursor would allow us to evaluate the claims concerning its stability. All the possible synthetic routes to the amino acid **(19)**, briefly mentioned above (see p. 69) could provide the desired compound **(19)** in its fully protected form. Two molecules were required as starting materials. According to the Simmons-Smith approach, dimethyl (2,3)-methanoaspartate **(71)** could be constructed by the reaction of amino fumarate **(72)** with diiodomethane. Alternatively, 2-aminoacrylate **(73)** could be employed; its reaction with bromo acetic acid methyl ester or a diazo compound (Scheme 2.8, B) would furnish the protected cyclopropane amino acid **(71)**.




(72)



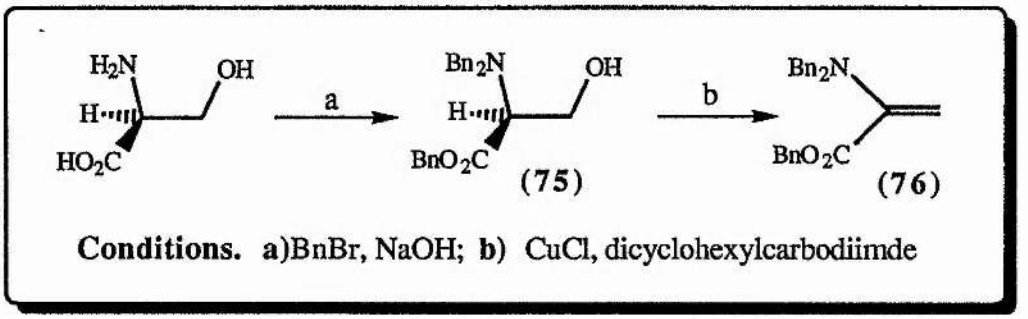

(73)




(74)

Domschke[179] synthesised dimethyl aminofumarate **(72)** by refluxing dimethyl oxaloacetate **(74)** in toluene under an ammonia atmosphere in 40% yield. The reaction was repeated twice but no fumarate **(72)** was isolated. NMR spectroscopy showed that ammonolosysis of the esters took place. At the same time the commercially available version of aminoacrylate **(73)** proved to be sensitive under usual esterification procedures (*e.g.* acid , thionyl chloride). It usually decomposed rather quickly, even before the ester was formed. Alternatively to prepare the fully protected amino-acrylate derivative **(76)**, serine was fully benzylated in one step under the conditions described by Velluz and Amiard.[180] The dehydration reaction using $CuCl_2$ and dicyclohexylcarbodiimide in dry $CH_2Cl_2$[181] proceeded smoothly to afford the benzyl *N,N*-dibenzyl-2-aminoacrylate **(76)** in good yield. The identity of the product was verified by its characteristic $^1$H-NMR spectrum (Fig. 2.8). Unfortunately all our efforts to remove residual cooper and carbodiimide were unsuccessful. The acrylate **(76)** decomposed on silica gel, or during Kugelrohr distillation (60-70 °C) to afford a red polymer. The crude material was treated with sodium hydride and bromoacetate (Scheme 2.8B) but no cyclopropane ring formation was detected by NMR spectroscopy.

sodium hydride and bromoacetate (Scheme 2.8B) but no cyclopropane ring formation was detected by NMR spectroscopy.




***Scheme 2.10*** *Synthesis of benzyl N,N-dibenzyl-2-aminoacrylate*

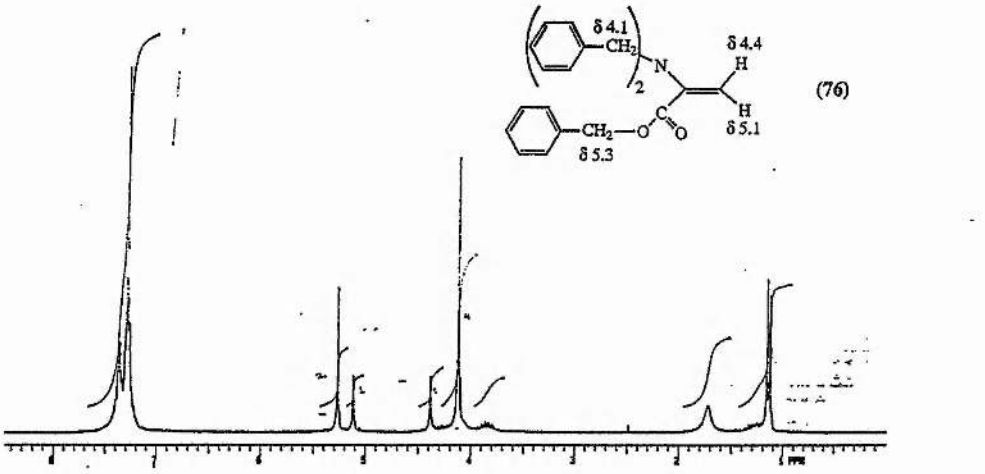



***Figure 2.8*** *$^1$H-NMR spectrum of benzyl N,N-dibenzyl-2-aminoacrylate (76)*

Since the amino dicarboxylate **(19)** seemed to be more difficult to obtain than originally thought, a sample of dimethyl 1-bromo-cyclopropane-1,2-dicarboxylate (mixture of 9:1 *cis* and *trans* **(68)** and **(69)** respectively) was hydrolysed in 6 M hydrochloric acid to give the free acid **(80)** (Scheme 2.11). The bromo diacid was recrystallized from ethyl acetate/hexane and incubated with the enzyme. Its inhibitory action prompted us to synthesise the *trans* isomer **(81)** and compare their behaviour.

McCoy studied the synthesis of substituted cyclopropane rings bearing two carboxyl groups (Scheme 2.8, B) and reported that the stereochemistry of the cyclised products depended on the co-operative or opposing nature of two

"steric effect" simply means that the TS conformation is established by the equal distribution of the bulk of the substituents, to each side of the ring under formation, thus favouring structure **(78)** (Fig. 2.9). When the two effects co-operate the result is clear cut, otherwise mixtures of the isomers are obtained. Accordingly the use of toluene (Scheme 2.11) resulted in an internal solvation effect, thus favouring structure **(77)** and finally the *cis* product **(68)** was almost exclusively formed (10:1). When DMF was used together with elevated temperatures, TS **(78)** was stabilised by the solvent. Subsequently the weak steric effect became operational and the *trans*- isomer **(69)** emerged as the main product (3:2).

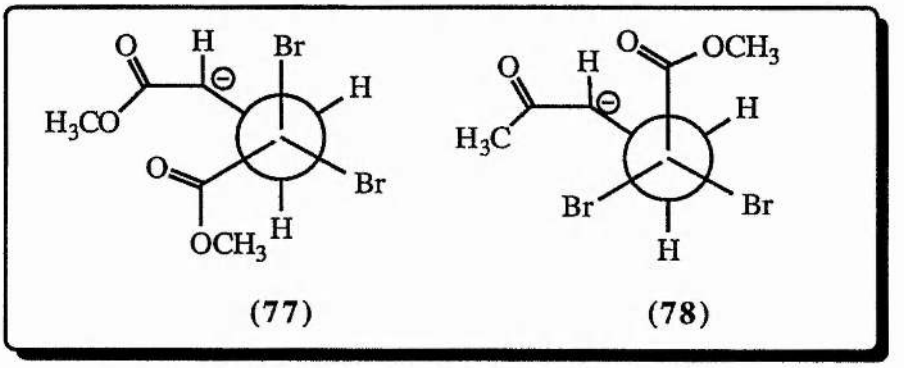
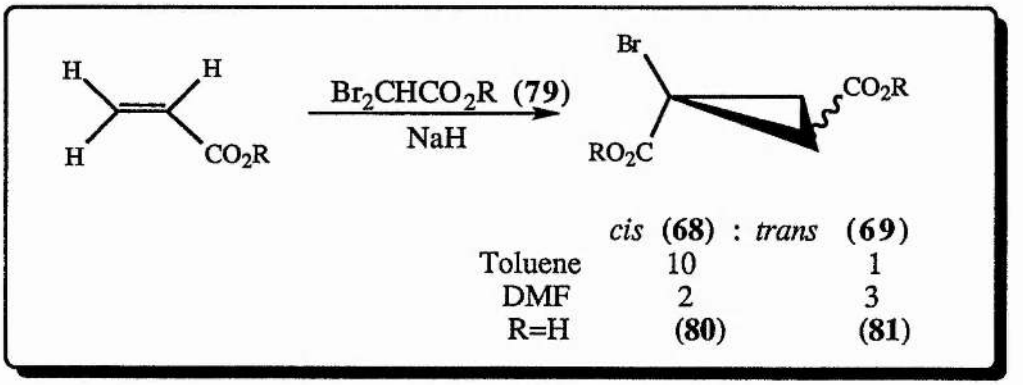



***Figure 2.9*** *Transition sates leading to dimethyl cis and trans-1-bromo-1,2-cyclopropane-dicarboxylates*

Isolation of the pure dimethyl 1-bromo *cis*-cyclopropanedicarboxylate **(68)** (reaction in toluene) was achieved by flash chromatography on silica gel (5% ethyl acetate in hexane). The small amount of the *trans* isomer initially present was easily removed under these conditions. Unfortunately this was not the case for the *trans*-dicarboxylate **(69)** (reaction in DMF). When the 2:3 mixture of the isomers was subjected to flash chromatography only a small amount of compound **(69)** was obtained completely pure. Therefore, we decided to take advantage of the proximity of the two carboxyl groups on the *cis*-isomer.

The mixture of the esters **(68)** and **(69)** was hydrolysed and re-esterified using isopropanol and $H_2SO_4$ for 8 hr. without reflux. Under these condition the *cis* isomer was only partially esterified. Indeed, normal work up, afforded the *trans* dicarboxylate more than 90% pure. Further purification was effected on silica gel.

Hydrolysis of the diester **(69)** afforded the pure 1-bromo-*trans*-1,2-cyclopropane dicarboxylate **(81)** (Fig. 2.10) in 20% overall yield from the dibromoacetate **(79)** (Scheme 2.11). Forming the anhydride of the *cis* isomer is an alternative method for separating the two dicarboxylates **(81** and **81)**. Each of the pure *cis* and *trans* isomers is a 50% mixture of two enantiomers. Specifically bromo-*cis*-dicarboxylate **(80)** is a mixture of the (1S,2R)- and (1R,2S)- enantiomers; bromo-*trans*-dicarboxylate **(81)** is a mixture of the (1R,2R)- and (1R,2R)- enantiomers.




***Scheme 2.11*** *Synthesis of cis and trans 1-bromo-1,2-cyclopropane-dicarboxylic acids*

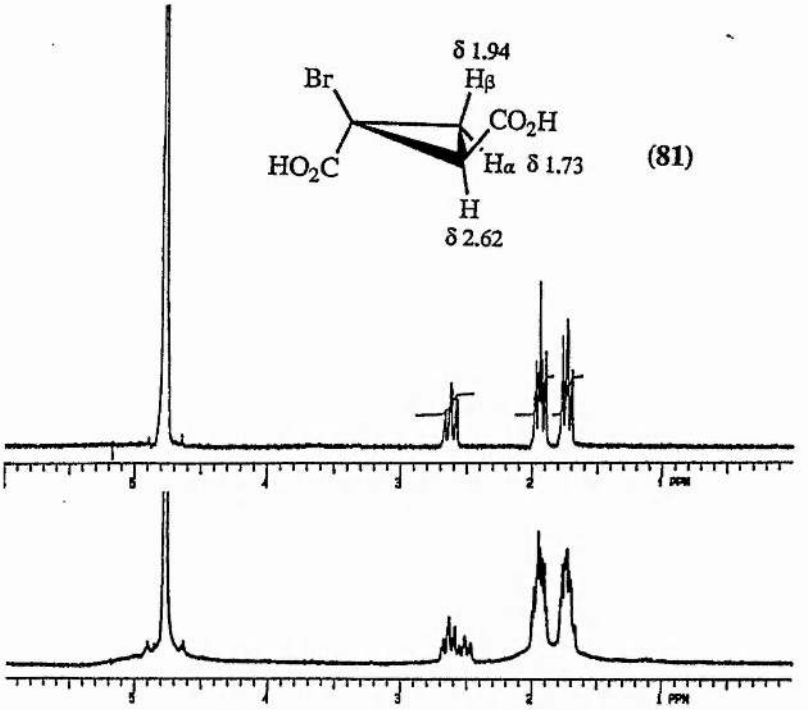



***Figure 2.10*** *$^1$H-NMR spectrum of pure 1-bromo trans-1,2-cyclopropanedicarboxylate (81) and the mixture*

The synthesis of the substituted cyclopropane dicarboxylates proved to be quite problematic. Many methods exist in the literature for their preparation but are neither very selective nor good yielding; if they work at all. Our failure to obtain 1-amino-1,2-cyclopropanedicarboxylate **(19)**, by addition of ammonia on the cyclopropene intermediate **(70)** was puzzling, but in accordance with recent reports on its instability. Synthesising these compound in a stereoselective manner not only will be of great importance for the study of glutamate mutase but seems to be a demanding synthetic challenge in its own right.

# Chapter 3

# Purification

# and

# Mechanistic Studies

## 3.0 Protein Purification

Early studies on glutamate mutase in our laboratory[183] indicated that experiments with crude preparations can be ambiguous or even misleading. In order to circumvent this problem and take advantage of the large number of potential molecular probes that had been synthesised by the group, the enzyme had to be purified in reasonable amounts, following reproducible protocols. Possible substrates or inhibitors could then be evaluated and various kinetic experiments performed. Finally, homogeneous samples of the two components E and S of glutamate mutase would allow N-terminal sequencing of the two proteins and subsequent elucidation of their primary structure.

Glutamate mutase was purified by Barker and coworkers in the sixties.[118,119] The source of the enzyme was frozen cell paste of *Clostridium tetanomorphum*, fermented on glutamic acid as the only source of carbon. An overview of Barker's purification made clear that the two components of the enzyme (see § 1.18,1.19) had to be isolated separately. It was also evident that the old-fashioned techniques would have to be replaced by modern high performance chromatography steps in order to purify each component to homogeneity. The purification protocols developed for component E and S are reported below.

### 3.1 Purification of Component E

The cell paste was thawed, sonicated and treated with protamine sulphate to precipitate the nucleic acids.[118,119] After centrifugation, the protein content of the supernatant liquid, was fractionated to 45% saturation with ammonium sulphate. The precipitate was discarded and the supernatant saturated to 85%. The solution was centrifuged and the pellet formed was dissolved in the appropriate phosphate buffer. The resulting solution contained more than 95% of the mutase activity and was used for the following steps.

The activity measurements were initially performed according to Barker *et al.*[117] A sample of the crude enzyme solution was added to the assay buffer (50 mM TrisCl, pH 8.4) which contained (2S)-glutamic acid **(1)**, 2-mercaptoethanol, and β-methylaspartase. Upon addition of coenzyme $B_{12}$, mesaconic acid **(3)** is produced

(see Scheme 1.9 and § 1.17). The rate of mesaconic acid formation can be monitored at 240 nm.

The crude protein mixture obtained after the ammonium sulphate fractionation, displayed only minimal amounts of mutase activity. This worrying finding verified similar observations reported by Buckel and Barker,[117] but it was in contrast with Barker's original purification information (see Table 1.4).[118] The reasons for this discrepancy were not obvious, but impurities with inhibitory action or the presence of other enzymes that react with mesaconic acid (see § 1.16) were the most probable explanations. Initial concern about the quality of the cell paste of *C. tetanomorphum* provided by the UEA fermentation facilities were dismissed, when during the course of the purification it was realised that mutase of higher purity was substantially more active.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely employed to evaluate the protein content of the various fractions isolated during the purification. The chromatogram of the crude protein mixture (Fig. 3.1.A.4) showed that only one of the 6 different major protein components qualified for the high molecular weight component E. Using gels of lower density (7.5% acrylamide) the molecular weight for this particular protein was calculated ($M_r$ 110,000±2000) (Fig. 3.1.B.1-2). This value was considerably lower than the one reported by Barker ($M_r$ 126,000).[116] The strong band at the low region was attributed to the presence of component S (Fig. 3.1.A.1 and 4). The amount of component E was estimated at 5-8% of the total protein content.

Typically the crude protein mixture after sonication, and ammonium sulphate fractionation had to be dialysed and the ionic impurities removed. The use of ion-exchange chromatography on DEAE-52 cellulose equilibrated at pH 7.5 was reported not only to achieve partial purification but also to remove most of the β-methylaspartase activity.[115] Our efforts to reproduce the separation between the mutase and β-methylaspartase activity were not successful. A shallow linear salt gradient was applied but no improvement on the separation was observed.

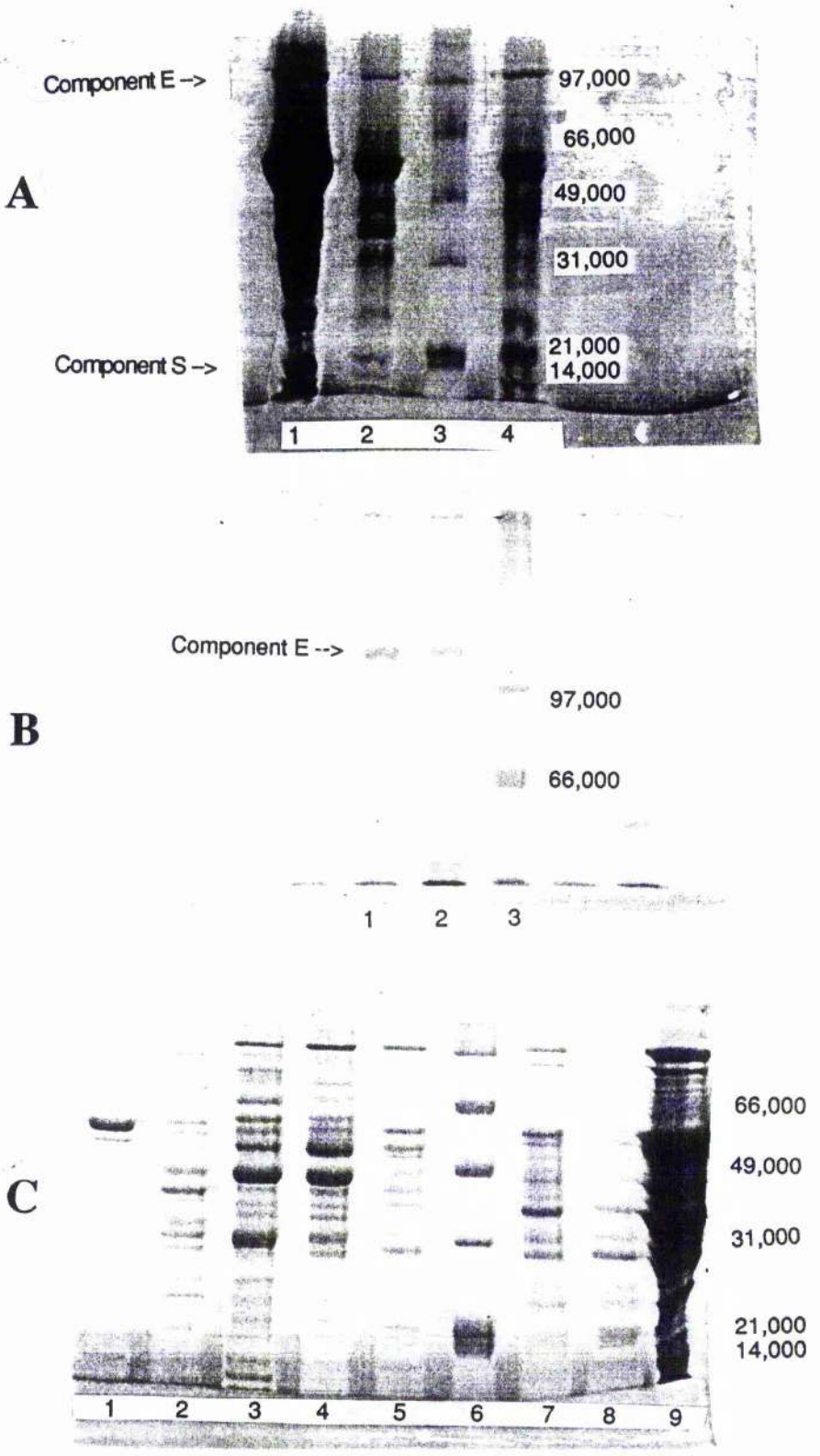



***Figure 3.1*** *Gels from early purification steps of glutamate mutase*

The inadequacy of the DEAE-52 cellulose for this particular separation was further highlighted by the SDS-PAGE chromatogram of the major peaks (Fig. 3.1.C.2-7). Nevertheless, the DEAE-52 was routinely used to fractionate the useful enzymes from the ionic impurities present in the mixture.

The crude material was applied on the DEAE-cellulose and the total protein content was eluted with 80 mM of potassium phosphate at pH 6.8. Altering the conditions (pH, eluting salt *etc.*) usually resulted in lower yields. The fractions containing the mutase (recovery of activity was usually higher than 80%) could be concentrated either by ultrafiltration or by saturation with $(NH_4)_2SO_4$. The latter method was usually followed in large scale preparations when some of the material had to be stored in the pellet form at -80 °C for future use. This partially purified material was still too crude to be applied directly to an FPLC system.

Partial separation of the mutase component E and β-methylaspartase ($M_r$ 110,000 and 92,000 respectively) activities was achieved on an exclusion chromatography column packed with G-150-Sephadex or Superfine G-100 gel. β-Methylaspartase eluted as the dimer and subsequently the difference between the molecular weights was not sufficient enough to effect complete separation. The absorbance of the eluent at 254 nm was recorded over the period of 30 hr. necessary to fractionate all the proteins contained in the sample (Fig. 3.2). Interestingly, the protein eluting between 200-250 ml from the G-150 column showed mutase activity (without addition of component S) and therefore both components must have been present. If Barker's information was accurate components E and S should have been separated on the column. Clearly the interaction between the two subunits are stronger than originally thought.[113]

The activity containing fractions (Fig. 3.2, 200-270 ml) from the previous step were concentrated and applied on an ion exchange 5-PW DEAE FPLC system, eluting with 0.5 M KCl (Fig. 3.3). Electrophoretic analysis of the proteins eluting in the main peaks revealed two features. First, the intensity of the band assigned to the high molecular weight component E had diminished substantially, and second, no separation could be detected between component E and residual β-methylaspartase (Fig. 3.4.A.1-9).

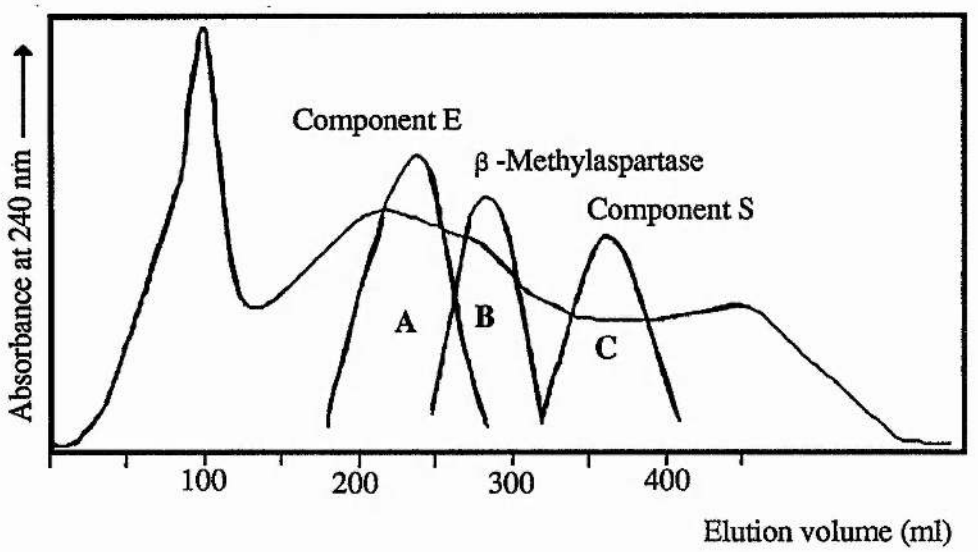



***Figure 3.2** Protein elution trace from the G-150 exclusion chromatography column*

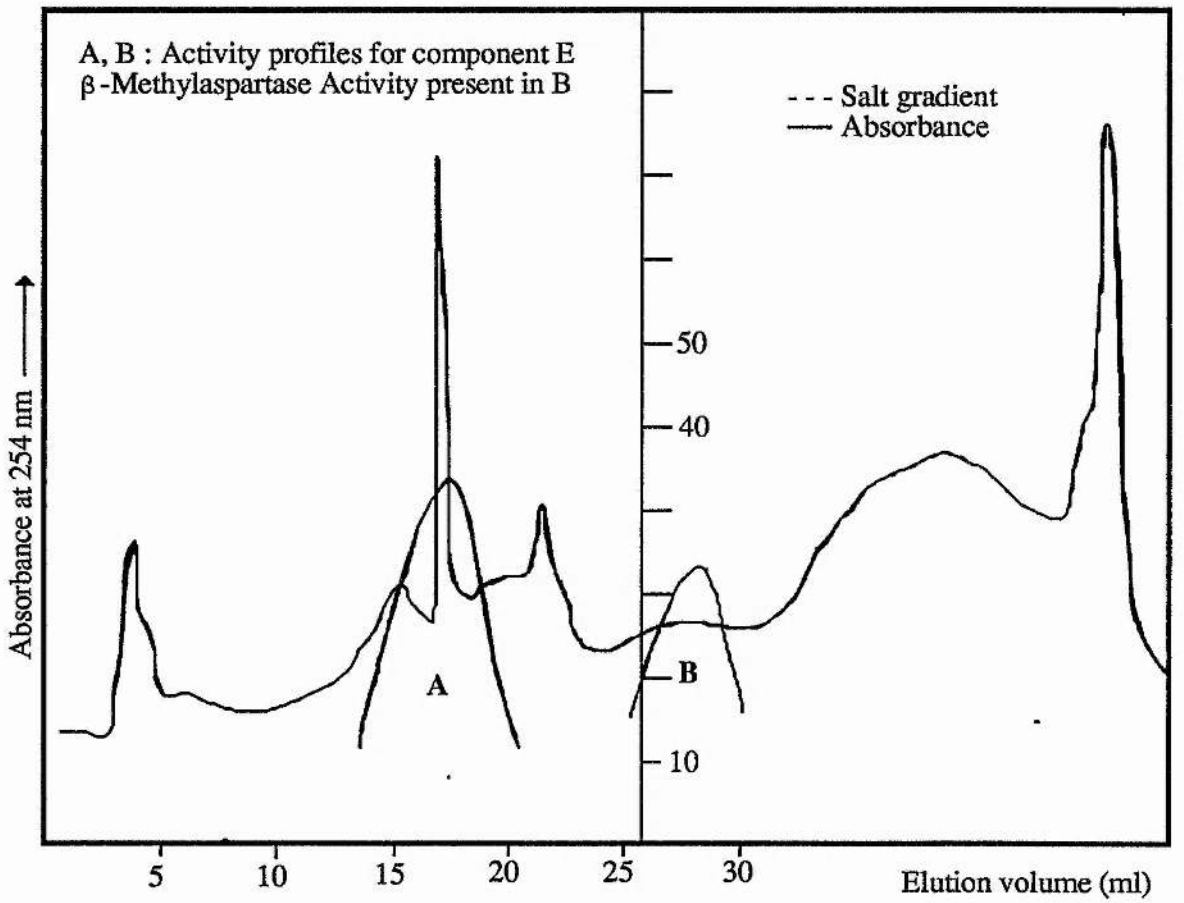



***Figure 3.3** FPLC trace of component E on the TSK DEAE-PW. For details see text*

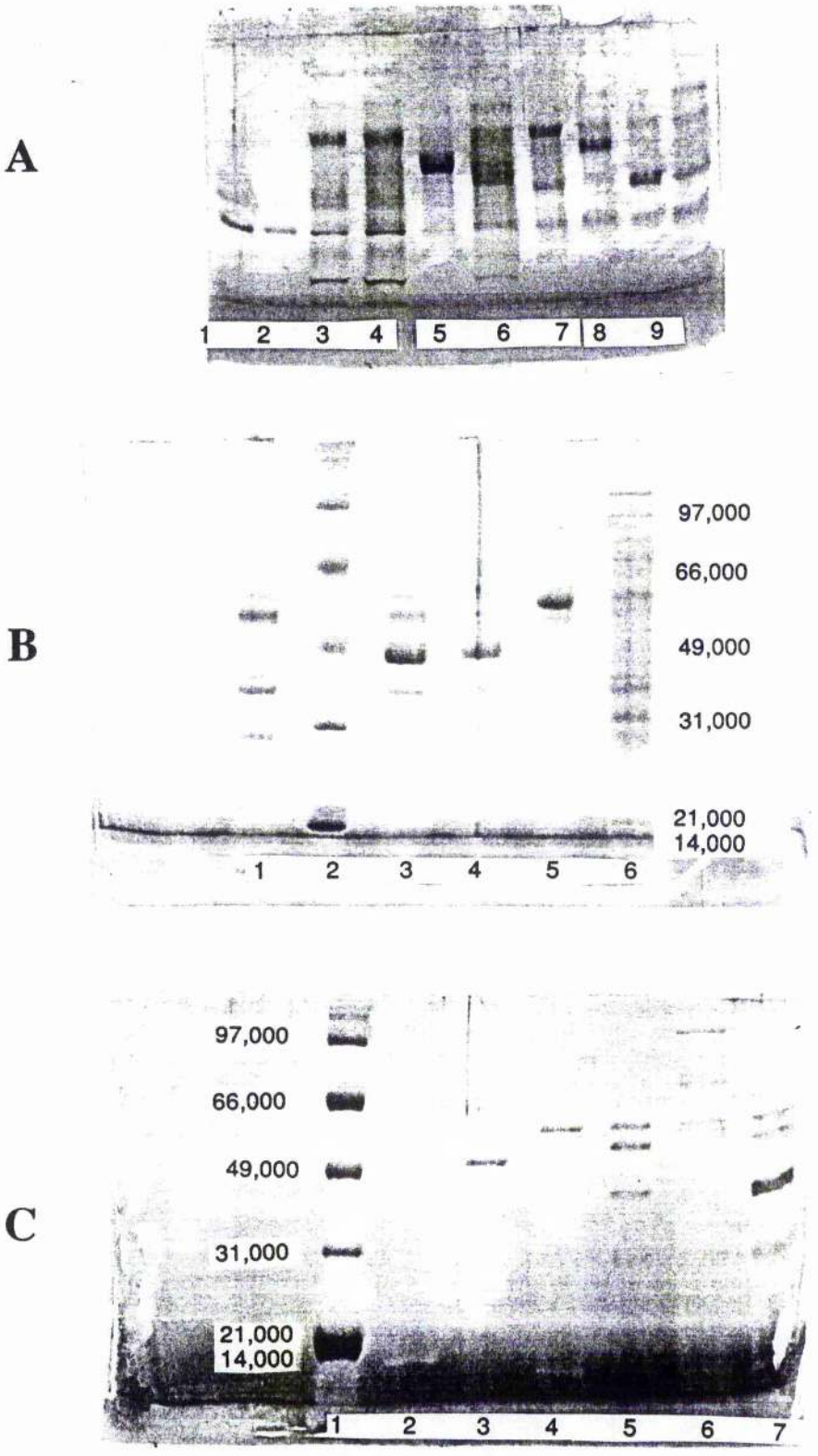



***Figure 3.4** Gels from later steps during the purification of glutamate mutase*

Re-application of the enriched in component E fractions (Fig. 3.4.A.3-4) on the ion exchange FPLC column resulted in complete loss of component E (Fig. 3.4.B.1,3-5). It was evident that the protein was dissociating as the purification proceeded.

A more rigorous approach, involving the use of the assay, was obviously necessary to solve the problem of the "unstable" component E. Essentially, an extension of the assay protocol discussed above, would allow component E to be located by its activity rather than its molecular weight.

Component S activity, free from component E, was obtained when the late fractions of the G-150 exclusion chromatography column (Fig. 3.2, 350-400 ml) were concentrated and assayed in combination with the mutase active fractions that had eluted earlier. The mutase activity previously measured in the early fractions (Fig. 3.2, 200-250 ml, p. 79) increased many fold when reduced component S was added.

With the activity of component S in hand we could assay all the fractions collected from the ion exchange FPLC system. It was discovered that a fraction of the total component E activity was eluting at 18 ml and a second at 25 ml (Fig. 3.3). Moreover the SDS-PAGE revealed that each of the above fractions contained the active component E as a protein of different molecular weight. The fraction that eluted sharply first (Fig. 3.3, 15-18 ml, 8%, 40 mM KCl) (Fig. 3.4.C.4), contained a protein with a molecular weight of ~53 KDa, whereas the later fractions (Fig 3.3, B, 12%, 60 mM KCl) (Fig. 3.4 C.5-6) contained a small amount of the initially observed dimer ($M_r$ 110 KDa, Fig. 3.1.B). It was now clear that the dimeric form of component E eluted later during the gradient, together with β-methylaspartase, thus explaining the failure of the low performance DEAE-cellulose step to separate them.

The TSK DEAE-PW ion-exchange column provided us consistently with reasonable and sufficiently pure amounts of component E. The yield, however, over the step was not satisfactory. The protein typically lost 50-60% of its activity on the column after every FPLC run. No conditions could be developed to prevent or reduce the amount of deactivation. Component E was also applied to a hydrophobic interactions column (TSK Phenyl-PW) but no activity could be detected in the fractions collected.

Component E after one high performance step (80% homogeneous) (Fig. 3.4.B.5) was used for kinetic studies and incubations. A pure sample of the protein was obtained after a second run on the FPLC system (Fig. 3.5). Finally, the total activity of component E isolated from 50 g of cell paste, after one FPLC run, was ~10 units (1.27 units $mg^{-1}$) in 2 ml of 50 mM phosphate buffer, pH 7.0. The half-life of the activity at -30 °C is one month, but for temperatures between 0-5 °C is only ~12 hr. When the concentration exceeded 2 mg/ml the solution was slightly pink, due to small amounts of corrinoid coenzymes bound to the protein.

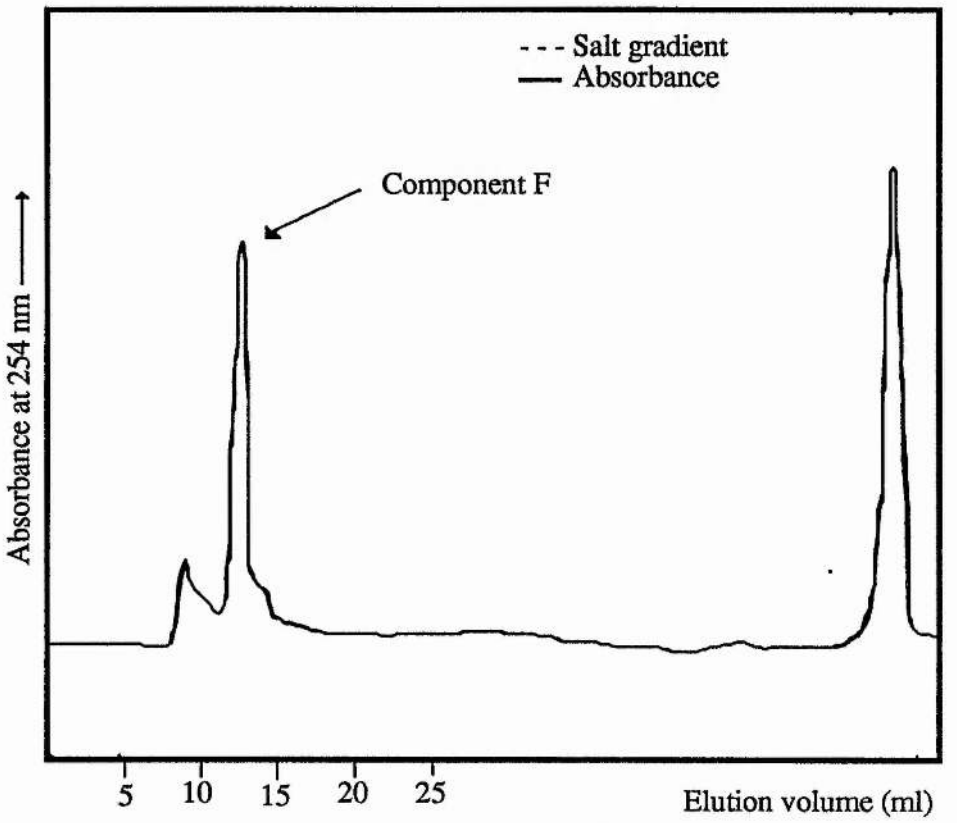



*Figure 3.5 Purified Component E - Final FPLC*

### 3.2 Purification of Component S

Barker's protocol[117] for component S was reproduced but the activities we obtained were much lower, compared to the values in the original report.[119] In our protocol the late fractions from the G-150 column (Fig. 3.2, 380-500 ml), contained more than 70% of the total component S activity, so they were combined, concentrated and applied on an FPLC system. The protein was deactivated on the

ion exchange 5-PW DEAE column eluting with 0.5 M KCl. On the other hand the TSK Phenyl-PW hydrophobic interactions column proved to be ideal (Fig. 3.6, A). The small protein was eluting at 35% ammonium sulphate ( 0.35 M, 43-48 ml).

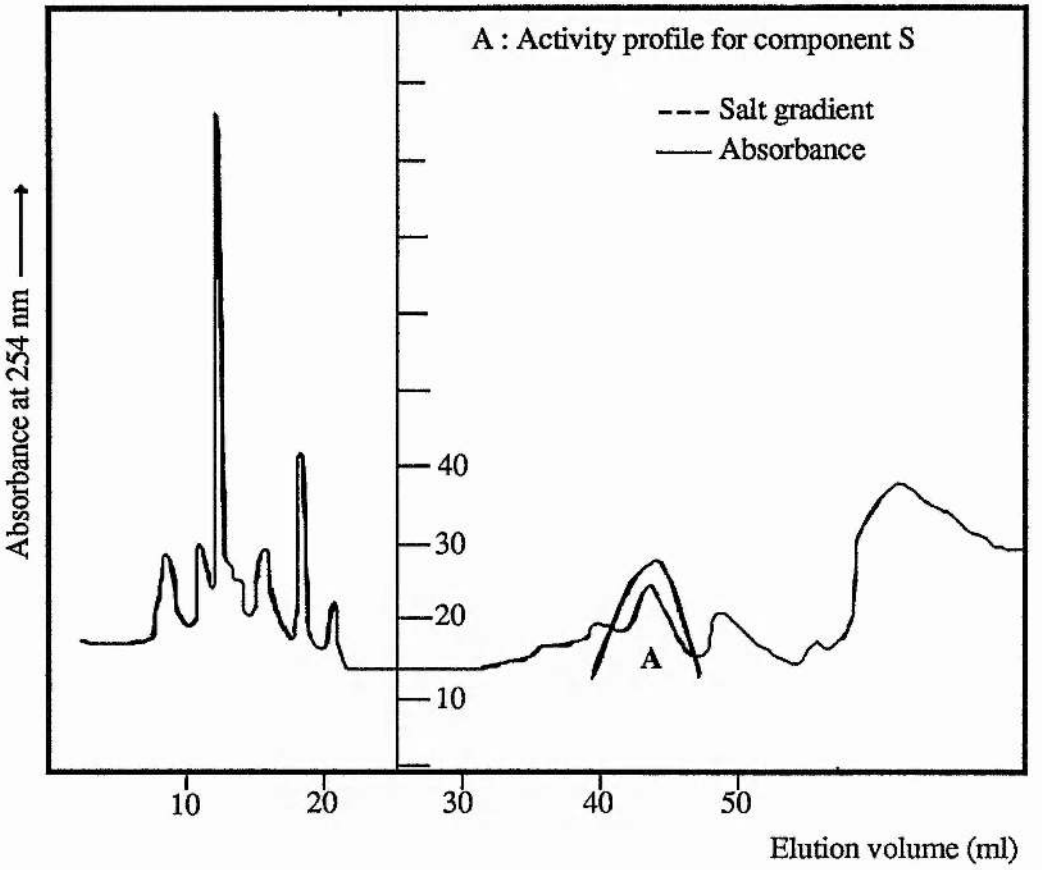



*Figure 3.6 FPLC trace for component S on Phenyl-PW*

A ~30% loss of activity was observed after every run. Unlike component E, component S can be purified directly from the material after the DEAE-cellulose step (see p. 80). The Phenyl-PW column seems to be able to fractionate the protein mixture eluting from the DEAE-cellulose very successfully. From 50 g of cell paste 15-20 units of pure component S (3.6 units $mg^{-1}$) were isolated. While the protein is less sensitive than component E on the high performance columns, its half-life during storage at -30 °C, is shorter (~15 days). Two FPLC runs were again required to obtain homogenous protein (Fig. 3.7.1). The molecular weight of component S could not be calculated accurately because of its position very low on the gel, but a fair estimation was 13,000±1000 KDa.

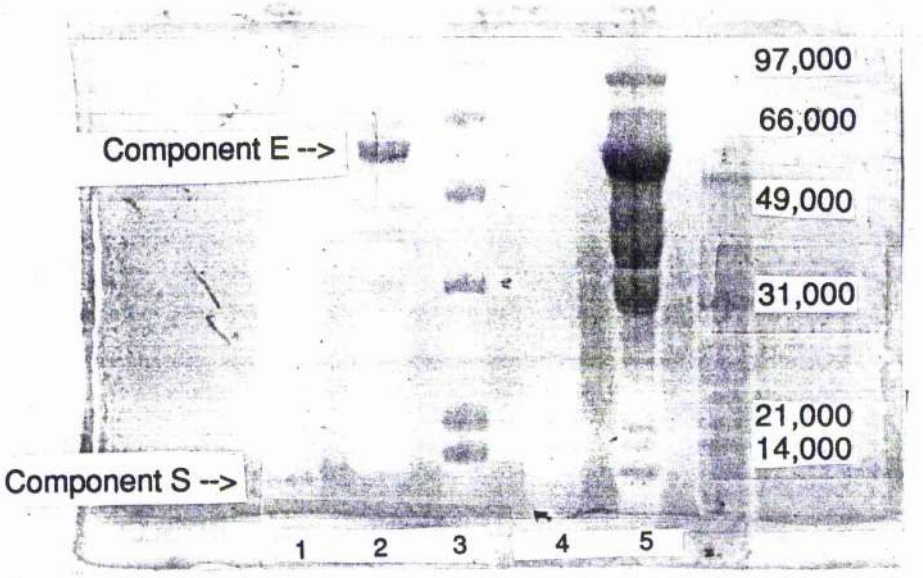



***Figure 3.7** SDS PAGE of pure component s E and S*

### 3.3 Discussion of the Purification - Function of Components E and S

The purification of glutamate mutase from *C tetanomorphum* was undoubtedly a tedious and time consuming task. The half-life of the activity in the phosphate buffers used, at 4 °C, was ~1 day. Therefore, all manipulations of the protein took place fast, without unnecessary delays, until each component had been subjected to one FPLC purification step. One week was typically required to isolate 10-15 units of each component per 50 g of cell paste. While developing our protocol it was necessary to compromise the yield for the purity. Later, the optimised purification procedures had to be followed methodically to minimise any losses of activity. Undoubtedly there was room for improvement, but at this stage the purity and quantity of enzyme was sufficient for the immediate purposes.

One of the advantages of the purification described above is that both components were isolated from the same cell paste. In Barker's purification,[119] component E was essentially treated as an impurity and removed during the drastic isoelectric precipitation step (§ 1.17 Table 1.4, p. 37). In comparison with Barker's our protocols were simpler and more reproducible. The availability of the FPLC systems offered the opportunity to isolate the homogeneous components.

Despite the failure of the DEAE-cellulose to separate component E from β-methylaspartase, Barker's experimental details were very useful during the initial stages (Table 3.1). For example all the buffers described in the original purification publications were found to be optimised. No improvement could be devised, despite our systematic efforts.

***Table 3.1** Purification of glutamate mutase - initial steps*

| STEP | (ml) | Protein (mg/ml) | Activity (units) | Specific Activity | Yield % |
|---|---|---|---|---|---|
| 1. Sonication (50g cell paste) | 149 | 15.3 | 94±1* | 0.041 | 100 |
| 2. Protamine Sulphate | 189 | 5.0 | 41.5 | 0.044 | 44 |
| 3. Ammonium sulphate fract. (50-80%) | 43 | 12 | ** | ** | ** |
| 4. Dialysis | 53 | 10.1 | 18.2 | 0.035 | 28 |
| 5. Fractionation on DEAE-Cellulose | 9 | 31.4 | 17.0 | 0.59 | 31 |

* Value not reproducible-Mean of three measurements

** Value could not be obtained reliably

The low mutase activities measured during the initial steps was another puzzling finding, especially in relation with Barker's early results. Buckel and Barker in 1972 made similar observations[112] but unfortunately they did not comment on this discrepancy. The most probable explanation, as suggested by an overview of the data available, is that various impurities acted in an inhibitory manner towards glutamate mutase. In many instances the total amount of activity increased two or even three fold after the protamine sulphate cut or the DEAE-cellulose fractionation. Finally, a ~70% loss of activity, over the five initial steps, was typical. It has to be emphasised, however, that yields mentioned in Table 3.1, most probably underestimate the amount of mutase activity actually present.

This became apparent after every gel exclusion chromatography column (p. 82), when the activities, detected for both components, increased at least 2-fold (Table 3.2). After application on the ion exchange (5-PW DEAE) FPLC system, component E was isolated in similar specific activities as in Barker's purification.[118]

The protein was homogenous and had a specific activity of 3.8 units $mg^{-1}$ (Table 3.2).

***Table 3.2** Purification of Component E - Final Steps*

| STEP | Volume (ml) | Protein (mg) | Activity (units) | Specific activity | Yield* % |
|---|---|---|---|---|---|
| 1. G-150 | 3 | 62.5 | 45.0 | 0.72 | 50 |
| 2. 1st FPLC on PW-DEAE | 1.9 | 12.4 | 15.8 | 1.27 | 17.5 |
| 3. 2nd FPLC on PW-DEAE | 1.8 | 1.1 | 4.2 | 3.8 | 5 |

* Values use 90 units as the total component E activity - See table 3.1

Similar amounts of both components of glutamate mutase were obtained. The total amount of component S activity typically isolated, was slightly higher than that of component E (15-20 units per 50 g of cell paste). Unfortunately the small protein has a shorter half-life under the specific storage conditions. Barker's accomplishment in isolating ten times more units of component S than component E, could not be duplicated. Component S was purified to homogeneity (Fig. 3.7); the overall yields seemed to be consistent, and reproducible but much lower than these of Barker's (Table 3.3).

***Table 3.3** Purification of component S- final steps*

| STEP | Volume (ml) | Protein (mg) | Activity (units) | Specific activity | Yield % |
|---|---|---|---|---|---|
| 1. G-150 | 3.0 | 120 | 39.6 | 0.33 | 44 |
| 2. 1st FPLC on PW-DEAE | 1.9 | 20.0 | 17.5 | 0.87 | 19 |
| 3. 2nd FPLC on PW-DEAE | 1.2 | 2.7 | 9.7 | 3.6 | 10 |

While the final details of our purification were being established, and the pure components were to be subjected to N-terminal sequencing, Buckel *et al.*[121] published the purification of glutamate mutase from *Clostridium cochlearium*, a bacterium of the same species as *C. tetanomorphum*. The German group encountered a number of difficulties similar to ours (*e.g.* gradual deactivation, loss of activity on FPLC systems *etc.*). Component E was purified on a hydrophobic

interactions column and in accordance with our results lost at least ~50% of its activity during this kind of manipulation (Table 2.2, p. 37).

The high specific activities reported (6 units $mg^{-1}$, Table 1.4, p. 37) could be attributed either to the source of the protein (*C. cochlearium*), or the different FPLC columns employed. A comparison between the purification tables (Table 4.1 and 2.2, p. 38), however, revealed that preparations from *C tetanomorphum* or *C. cochlearium* do not actually contain different amounts of mutase activity. For example, after the common exclusion chromatography step (see Tables 3.2 and 2.2, p. 37), we obtained 90 units of component E (from 100 g of cell paste), whereas Buckel using a much faster gel (the separation lasted only of few hours instead of days), obtained 130 units. Although the claim that component E isolated from *C. cochlearium* was more stable than that from *C. tetanomorphum* was not rigorously supported by the available data, a more thorough comparison has to be performed, especially if component E is to be cloned and overexpressed.

Our estimations concerning the molecular weights of components E and S, were also confirmed by Buckel.[119] Advances in the molecular biology area support the notion that the large component is produced by the cell as a monomer with a molecular weight of 53 KDa.[122,123] The value of 128 KDa reported by Barker, was an artefact caused by the use of native gels. The above value for component E almost certainly represented an inactive complex with a composition of $E_2S$.

It is not clear at all if the discovery of the monomer solved all the problems concerning the composition of the apoenzyme. If $E_2$ was a simple dimer, due to the presence of weak interactions between two monomers, it should not have been observed in the first place under the denaturing conditions of the SDS-PAGE electrophoresis. A similar example is β-methylaspartase, which is known to be active as a dimer, but only the monomer can be detected on the gels (Fig. 3.4.C.3). An overview of our experiments suggested that the integrity of the $E_2$ complex depended on both the presence of component S and the overall progress of the purification. One satisfactory explanation can be that the two monomers are connected with an S-S bridge which is somehow protected when E and S are complexed but finally breaks down after prolonged exposure to 2-mercaptoethanol. Interestingly Buckel and coworkers who used the more powerful reducing agent,

1,2-dithiothreitol, throughout their purification do not mention observing any of the dimer. There was some difficulty in establishing a consistent relation between the dissociation of the dimer and loss of activity for component E. The reason for that is that the dimer is always complexed with various amounts of component S, so it is not possible to attribute the apparent increased stability of the dimer to either the presence of component S or to the existence of the dimer itself.

A few months after Buckel's report, the amino acid sequence of glutamate mutase was published by Marsh *et al.* (Fig. 1.16, p. 40).[122,123] This knowledge can be useful if significant homologies between the protein under inspection and other better studied enzymes were found. In the case of glutamate mutase a region with significant local similarities (Fig. 3.8) between component E and β-methylaspartase was discovered and reported by Marsh and Hollaway.[122] This observation can quite possibly reveal the substrate (3-methylaspartic acid) binding site. Ser-173 from β-methylaspartase,[126] thought to be involved in the deamination mechanism, is indeed included in this region. This local homology is also in agreement with the early experimental evidence that suggested the location of the glutamate binding site on component E.[118]

| | 170 190 210 |
|---|---|
| β-MeAsp | **•• • •• * • ••**•• • • ***•• ••*•*• • • *•** *•** *• |
| | GAEINAVPVFAQSGDDRYDNVDKMIIKEADVLPHALINNVEE KLGLKG EKLLEYVK |
| Comp.E | GADLLPSTIDAYTRQNRYEECE IGIKESEKAGRSLLNGFPGVNHGVKGCRKVLESVN |
| | 90 120 140 |

***Figure 3.8*** *Homologies between comp.E and MeAsp. Identical residues are denoted by "*"; conserved by "•".*

The small subunit (14 KDa) on the other hand shows an appreciable amount (40%) of conserved positions with methionine synthase and methylmalonyl-CoA mutase from a number of sources (Fig. 3.9). Based on these data a possible role for component S during catalysis was suggested.[123] The small protein may be important for the crucial binding of the coenzyme from the α-side (chapter 1). The fact that oxidation of the -SH bond did not affect the affinity for component E but

reduced activity,[118] implied an important binding role specifically for the cysteines of component S in connection with the coenzyme.

| | ††† | † * | * ** | †† † * |
|---|---|---|---|---|
| Methionine Synth. | QGKTNGKMVI | ATVKGDVHDI | GKNIVGVVLQ | CNNYEIVDLG |
| Hum. Methylmal.CoA | REGRRPRLLV | AKMGQDGHDR | GAKVIATGFA | DLGFDVDIGP |
| Mous.Methylmal.CoA | REGRRLGLLV | AKMGKDGHDR | GAKVIATGFA | DLGFDVDIGP |
| *P.Sh.*Methylmal.CoA | AEGRRPRILL | AKMGQDGHDR | GQKVIATAYA | DLGFDVDVGP |
| Component S | MEKKTIVL | GVIGSDCHAV | GNKILDHSFT | NAGFNVVNIG |

| * † | † † †** | †*†* † | † ††† | † †* † | †** † |
|---|---|---|---|---|---|
| VMVPAEKILR | TAKEVNADLI | GLSGLITPSL | DEMVNVAKEM | ERQGF.TIPL | LIGGATTSKA |
| LFQTPREVAQ | QAVDADVHAV | GVSTLAAGHK | TLVPELIKEL | NSLGRPDILV | MCGGVIPPQD |
| LFQTPREVAH | DAVDADVHAV | GVSTHAAGHK | TLVPELIKEL | TALGRPDILV | MCGGVIPPQD |
| LFQTPEETAR | QAVEADVHVV | GVSSLAGGHL | TLVPALRKEL | DKLGRPDILI | TVGGVIPEQD |
| VLSSQEDFIN | AAIETKADLI | CVSSLYGQGE | IDCKGLREKC | DEAGLKGIKL | FVGGNIVVGK |
| | † †† | *††* | † | | |
| HTAVKIEQNY | SGPT...VYV | QNAS.R..TV | GVVAALLSDT | QRDDFVARTR | |
| YEF.....L | FEVGVSNVFG | PGTRIPKAAV | QVLDDIEKCL | EKKQQSV | |
| YEF.....L | YEVGVSNVFG | PGTRIPRAAV | QVLDDIEKCL | AEKQQSV | |
| FDE.....L | RKDGAVEIYT | PGTVIPESAI | SLVKKLRASL | DA | |
| QNWPDVEQRF | KAMGFDRVYP | PGTSPETTIA | DMKEVLGVE | | |

***Figure 3.9*** *Homologies between of component S and other coenzyme dependent enzymes; identical amino acids denoted by "*"; conserved by "†"*

Although more data have been available recently, the question of the composition of the active form of the apoenzyme is still a matter of speculation. Typically, purified component E contains substantial amounts of inactive protein which makes the calculation of the molecular ratio between E:S rather difficult. Barker's kinetic studies in combination with the correct values for the molecular weights allowed an estimation of 0.85 for the molar ratio between E and S.[115] According to this calculation the most likely structure of the active holoenzyme complex is $E_2S_2$.

Finally the stability of the apoenzyme was studied in the presence and absence of coenzyme. For other enzymes of the group the effect varied, and in some cases the presence of AdoCbl induced an irreversible fast deactivation of the system (see § 1.10.1). This observation implied the presence of activated species in the active site even before the addition of substrate. This was not the case for glutamate mutase. Two identical mixtures of component E and S were incubated in the dark at 35 °C in a 50 mM potassium phosphate buffer, pH 7.0. One of the mixture contained 20 mM AdoCbl. Aliquots were taken and their activity measured every hour for around 10 hr. The percentage of inactivation detected during that period was very similar in both cases. This result dismissed the presence of any activated form of the coenzyme in the active site prior to the addition of the substrate.

### 3.4 Assay Conditions - Limitations and Improvements

The standard method developed by Barker[117] was used for routine assays. The principal and its applications were discussed above (see 1.17, and 3.1 p. 75). The activities measured are usually slightly lower (10-20%) than those obtained under strict anaerobic conditions. Nevertheless, the values can be accurate and reproducible, even suitable for kinetic analysis provided that the buffers are degassed prior to use. Additionally the solutions of AdoCbl and (2S)-glutamic acid were prepared fresh every 3-4 days.

Modifications in several instances were necessary to increase efficiency and reproducibility or to fulfil certain requirements. Component S was found to be reduced rapidly and attain full activity by incubation with 3 mM of 2-mercaptoethanol for 6-10 min in 37 °C. Component E and the coenzyme were added last. Longer incubation times of component S with 20 mM 2-mercaptoethanol, as suggested by Barker,[119] usually resulted in much lower activities.

Some effort was spent to develop an assay that will measure the activity from the direction of 3-methylaspartic acid **(2)** to glutamate **(1)**. Glutamate dehydrogenase could be used to remove glutamic acid from the equilibrium. The reaction would be monitored by the changes in the absorbance of NADH at 340

nm. Unfortunately the activities obtained using glutamate dehydrogenase were much lower than these obtained under the classic method, which utilizes β-methylaspartase. Further elaboration on the conditions was probably necessary.

### 3.5. Catalytic Constant

The availability of pure enzyme and the knowledge of the exact molecular weight allowed us to calculate with accuracy the $K_m$ for glutamic acid as 1.35±0.05 mM, and the $K_{cat}$, catalytic constant (turnover number) of the enzyme as 300±100 $s^{-1}M^{-1}$. This value was calculated from the Michealis-Menten equation 3.1 ($M_r$ 53 KDa for component E).

$$v = \frac{kcat}{K_M}[E]_0[S] \qquad (3.1)$$

The catalytic constant represents the number of catalytic events that take pace at every active site per second. For the active holoenzyme, assuming a structure of $E_2S_2$, the turnover will be ~$10^3$ $s^{-1}M^{-1}$. Glutamate mutase is indeed a slow enzyme.

### 3.6 Mechanistic Studies

With the protein available in sufficient amounts the mechanistic studies could be initiated. The a long list of structures that might interact with the enzyme (Fig. 3.10) included (2S,3S)-3-ethylaspartic acid **(82)**, (2S)-homocysteic acid **(83)**, (3S)-methylsuccinic acid **(84)**, (2S,3S)- **(17)** and (2S,3R)-3-methylglutamic acid **(18)**, (2S,3R)-3-methylaspartic acid **(85)**, *N*-methyl-(2S,3S)-3-methylaspartic acid **(86)**, 1-bromo-*cis*-cyclopropane-1,2-dicarboxylic acid **(80)**, 1-bromo-*trans*-cyclopropane-1,2-dicarboxylic acid **(81)**. Unfortunately the synthesis of 2,3-methanoaspartic acid **(19)** was not concluded (see chapter 2). The interactions discovered between some of these molecules and the protein are reported below. The mechanistic implications suggested by our findings are discussed later in this chapter.

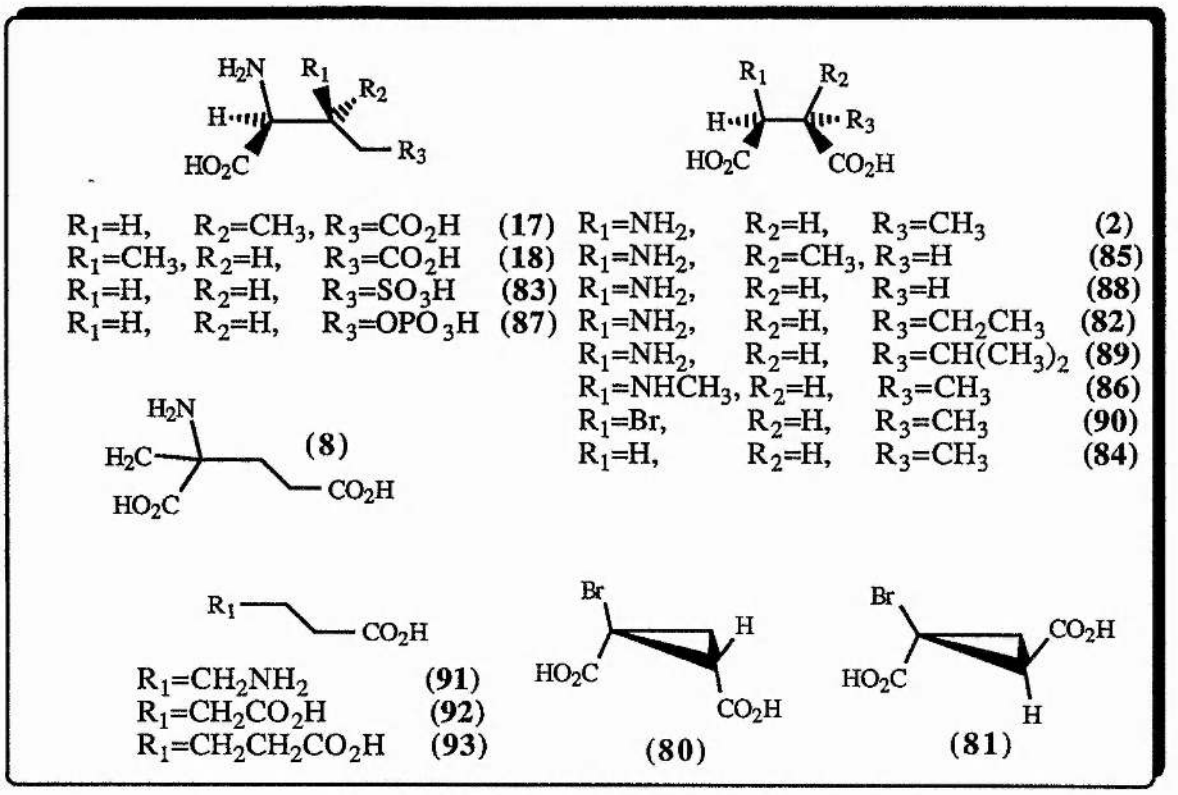



***Figure 3.10** Molecules studied with glutamate mutase*

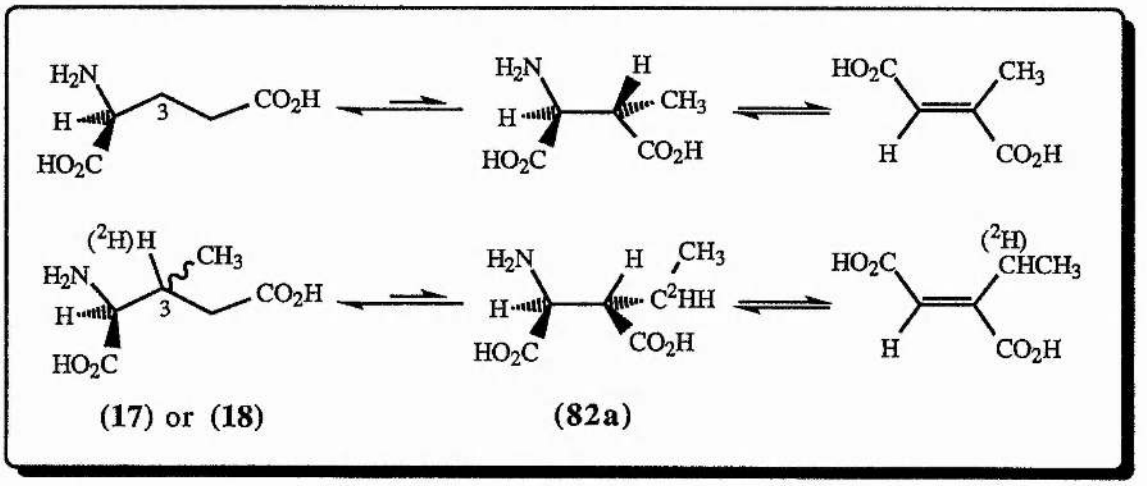
### 3.6.1 (2S,3S)-3-Methylaspartate Analogues as Inhibitors or Substrates

(2S,3R)-Methylaspartic acid **(85)**, N-methyl-(2S,3S)-methylaspartic acid **(86)**, (3S)-methylsuccinic acid **(84)**, and aspartic acid **(88)** were tested with glutamate mutase as inhibitors. No interaction seemed to take place between them and the active holoenzyme. The first three methylaspartic acid analogues were also incubated with the enzyme but no rearrangement products detected. (2S,3S)-2-Bromo-3-methylsuccinic acid **(90)** was another promising structure but it was found to react quickly with 2-mercaptoethanol present in the incubation and assay mixtures.

Defining the stereochemical course of the reaction at C-3 of glutamic acid was a long pursued target of our research. As discussed earlier (§ 2.7) the stereochemical course of the rearrangement at C-3 cannot be easily defined due to the torsiosymmetry of the -$CH_3$ group formed on 3-methylaspartic acid. So we had to design a molecule that on rearrangement would preserve the stereochemical

information introduced by the enzyme. This can be done by replacing one of the three protons with a methyl group and a second with a deuterium, thus forming the specifically labelled 3-ethylaspartic acid **(82a)** (Scheme 3.1). During the initial stages of the project, we were fairly confident that the presence of an additional methyl group on the substrate apart from reducing the rate of the reaction, it would not interfere with the fundamental aspects of the rearrangement. Additionally the methyl group could also "lock" the methylene group of 3-ethylaspartic acid **(82a)** in such an orientation that only one of the two prochiral protons present would be removed. The identity of the proton abstracted and the stereochemistry at the C-3 of the 3-methylglutamic acid product (Scheme 3.1, **(17** or **18)**) would be sufficient to establish if the rearrangement proceeds *via* an inversion or retention of configuration.

Although (2S,3S)-ethylaspartic acid **(82)** was available in our laboratory crude preparations of the mutase could not be incubated with the compound for two reasons. First, the impure enzyme loses its activity quickly, usually after 2-3 hr. This means that a slow substrate, would require large amounts of enzyme to give any detectable quantities of product.




***Scheme 3.1*** *Plan for defining the stereochemical course of the reaction at C-3 of glutamic acid.*

Additionally the equilibrium is shifted to the direction of ethyl fumarate (Scheme 3.1) because the substrate **(82)** is processed by β-methylaspartase present in the

crude mixture. It is possible to inhibit β-methylaspartase using $Ca^{2+}$ ions but the inhibition was bound to be inefficient for slow substrates for the rearrangement.

The incubation of ethylaspartic acid **(82)** with the mutase was repeated a few months later, when protein free of β-methylaspartase activity was available. Using substantial amounts of enzyme (1-2 units) no 3-methylglutamic acid could be detected by $^1$H-NMR spectroscopy in the incubation mixtures.

Although it was clear that 3-ethylaspartic acid was not a substrate for glutamate mutase, no satisfactory explanation was available at this stage. The key information missing was whether or not the holoenzyme in the presence of 3-ethylaspartic acid, activates the coenzyme and subsequently generates the 5'-deoxyadenosyl radical. If the 5'-adenosyl radical was formed then a proton would be most certainly abstracted from the molecule of 3-ethylaspartic acid occupying the active site. In other words the lack of rearrangement products was not conclusive as far as it concerned any hydrogen exchange between ethylaspartic acid and the coenzyme.

To investigate this possibility a number of deuteriated ethylaspartates **(82 b,c,d)** were synthesised in our laboratory by S. Gulzar and K. Badiani, and incubated with unlabelled 3-ethylaspartic acid or (2S)-glutamic acid in several different molar ratios. (2S,3S)-[3'-$H_3$]-3-methylaspartic acid **(2a)** (§ 3.1) was also incubated with the enzyme in the presence of unlabelled 3-ethylaspartatic acid. A combination of $^1$H- and $^2$H-NMR spectroscopy was then employed to detect any exchange of deuterium.

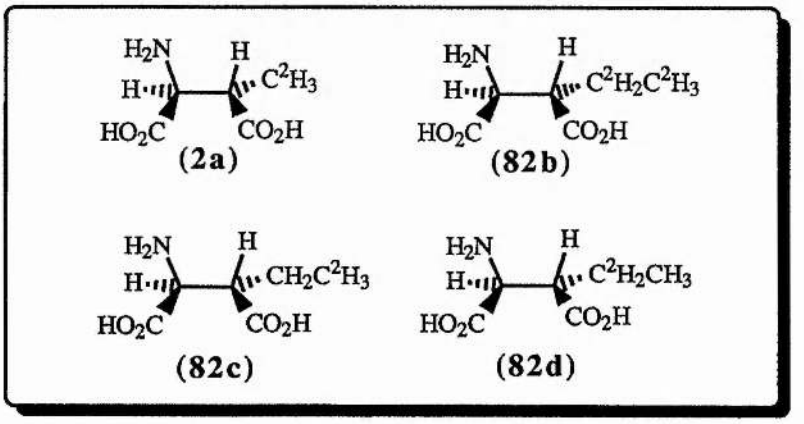
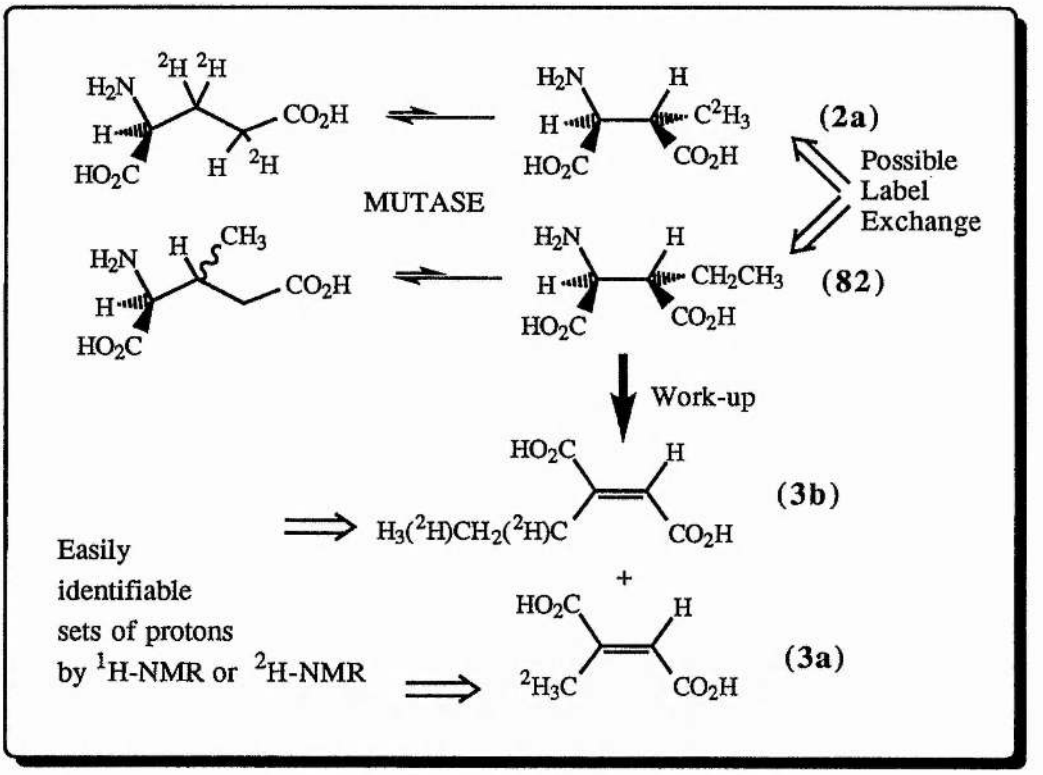



*Figure 3.11 Deuteriated ethylaspartic acids employed*

For example, a mixture of [3'-$^2H_3$]-3-methylaspartic acid **(2a)** and unlabelled ethylaspartic acid **(17)** was incubated with the pure mutase. After a few hours β-methylaspartase was added, the alkyl aspartic acids present were transformed into alkyl fumarates **(3a)**, **(3b)**, and extracted into ether. If any deuterium label had been transferred from [3'-$^2H_3$]-methylaspartic acid to ethylaspartic acid **(82)** (Scheme 3.2) it would have been easily detected by means of $^2H$-NMR spectroscopy, since now all three sets of protons present in the product mixture were different. Complete lack of deuterium exchange confirmed that the holoenzyme could not abstract hydrogens from either the 3'- or the 4'-position of 3-ethylaspartic acid **(82)**.




***Scheme 3.2** Label Exchange experiments with glutamate mutase*

The $^1H$-NMR spectra of the experiment described above is shown in Fig. 3.12. After the incubation (Fig. 3.12, C), the aspartic acids were deaminated and the $^2H$-NMR indicated no label exchange. The 4-H of [3'-$^2H_2$]-(2S)-glutamic acid produced by the enzyme can be seen at 2.2 ppm (Fig. 3.12, B).

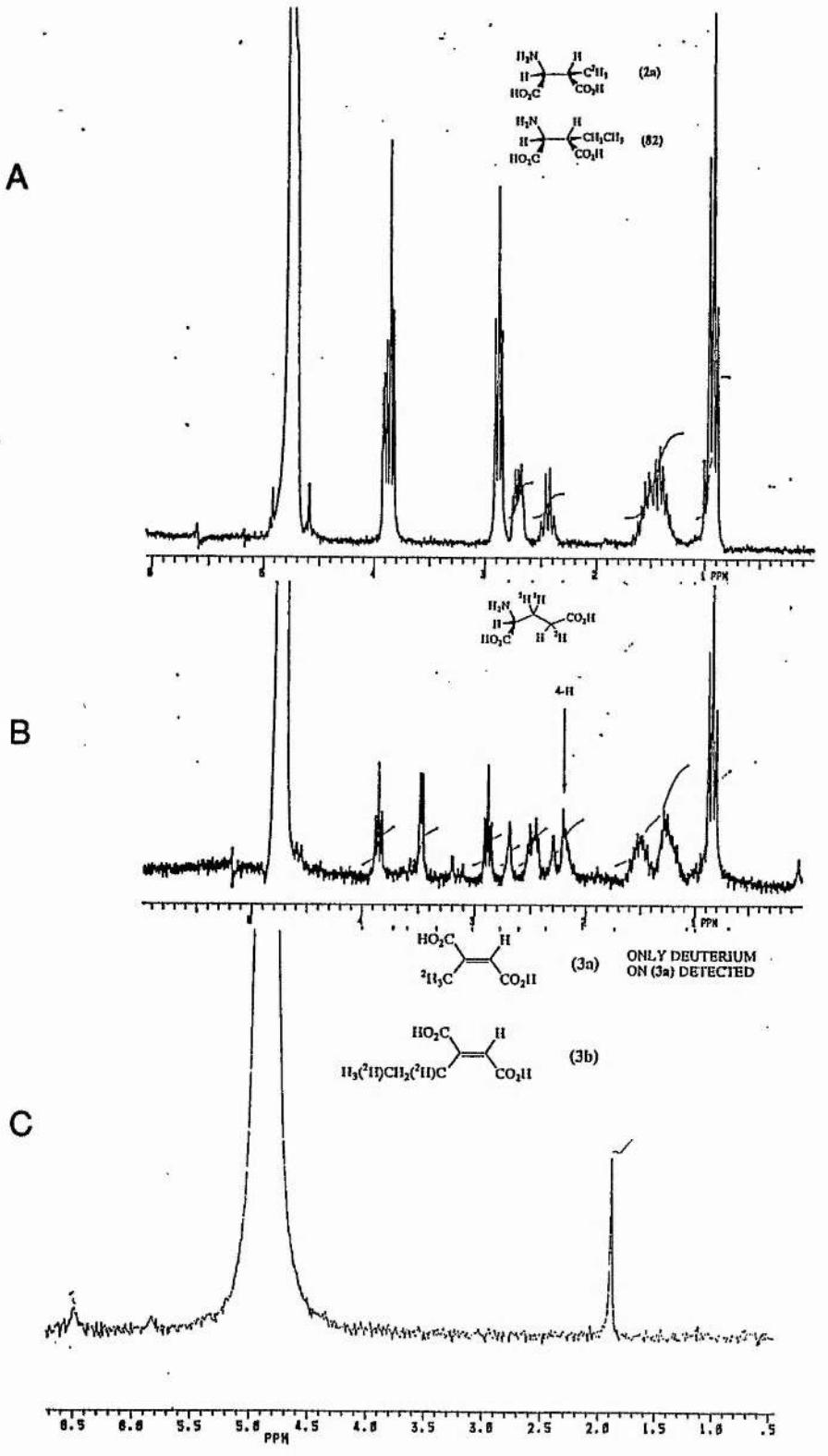



*Fig 3.12 NMR spectra of incubations containing 3-methylaspartic acid (**2a**) and 3-ethylaspartic acid (**82**). For details see text, p. 97*

### 3.6.2 3-Methylglutamic Acids

The inability of the enzyme to tolerate any additional groups on the C-3' of 3-methylaspartic acid, did not exclude the possibility of 3-methylglutamic acids being substrates for the rearrangement. The compact structure of 3-ethylaspartic acid **(82)** could be more difficult to be accommodated in the active site than the relatively more flexible 3-methylglutamate structure. If any of the two diastereomers on C-3 **(17** and **18)** (see § 2.3) was a substrate, interesting stereochemical information could still be deduced. An additional practical advantage of investigating the reaction from the direction of 3-methylglutamic acid to ethylaspartic acid (Scheme 3.11), is that β-methylaspartase can be used to deaminate any product 3-ethylaspartic acid. Even if the rearrangement of 3-methylglutamic acid to ethylaspartic acid was an favourable equilibrium, some product ethyl fumarate would have been easily detected (Scheme 3.11), in the presence of β-methylaspartase. Unfortunately neither of the two diastereomers, **(17)** or **(18)**, rearranged at any detectable rate under the conditions of the incubations (0.5-1 unit of enzyme present).

While 3-ethylaspartic acid cannot be reliably evaluated as an inhibitor because it is a substrate for β-methylaspartase, the two methylglutamic acids **(17)** and **(18)** were inhibitors for glutamate mutase (Figs. 3.13, 3.14, and Fig. 3.15 respectively).

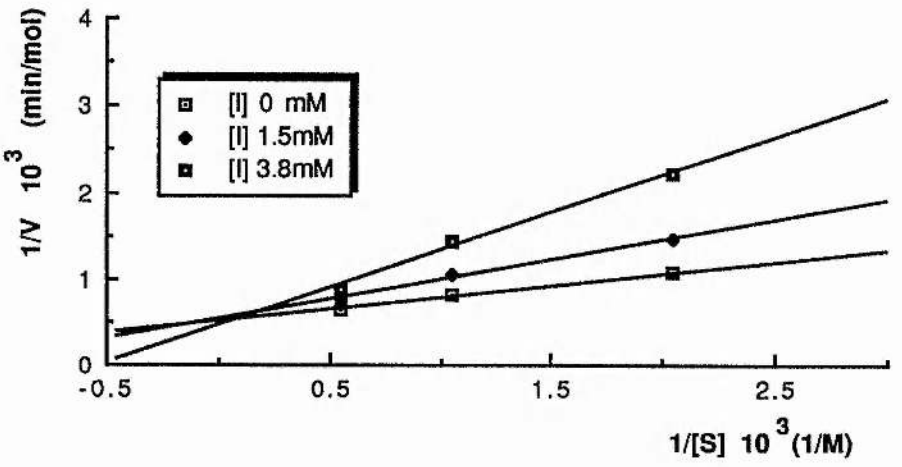



*Figure 3.13 Lineweaver-Burk plot for (2S,3R)-3-methylglutamate, characteristic of competitive inhibition. The lines coincide on the Y-axis*

Typically the plot (1/v) vs. (1/s), for **(17)** verifies the competitive nature of the inhibition (Fig. 3.13). The $K_i$ values indicate that both diastereomers bind in a similar unproductive manner the holoenzyme complex, competing with the substrate for the active site. The difference in the $K_i$ values for the two diastereomeric 3-methylglutamic acids probably reflected the preference of the enzyme to accommodate the (2S,3S)-isomer **(18)** better in the active site.

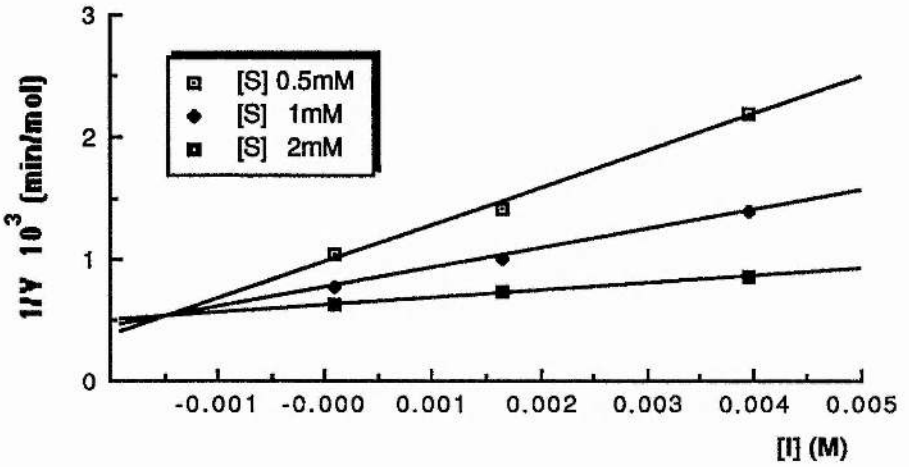



*Figure 3.14 Inhibition by (2S,3R)-3-methylglutamic acid [$K_i$ = 1.5 mM]*

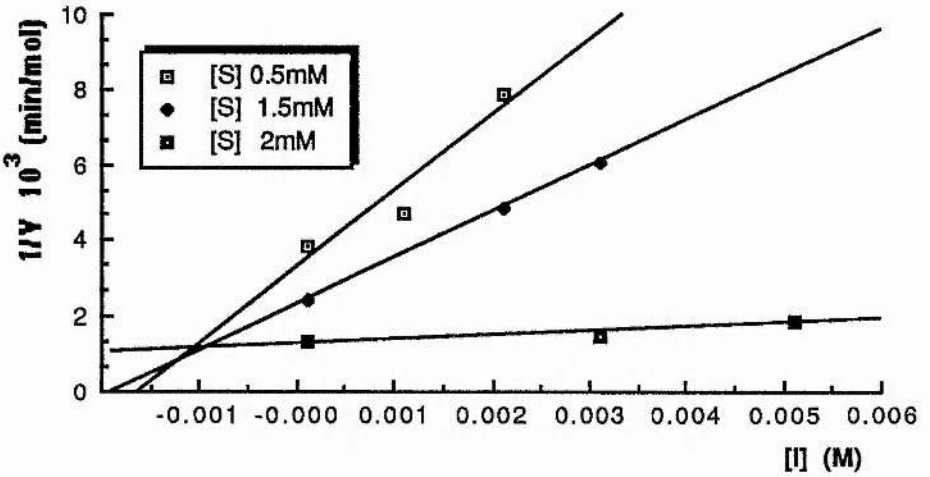



*Figure 3.15 Inhibition by (2S,3S)-3-methylglutamic acid [$K_i$ = 1 mM]*

### 3.6.3 Homocysteic acid (83) and Phosphohomoserine (87)

Replacing the carboxyl group of glutamic acid with $-SO_3$ or $-OPO_3$ leads to the structure of (2S)-homocysteic acid **(83)** and phosphohomoserine **(87)**

respectively. The former is commercially available and the latter had been synthesised in our group.[184] Homocysteic acid was a competitive inhibitor for glutamate mutase ($K_i$ 5 ± 0.5mM) (Fig. 3.17).

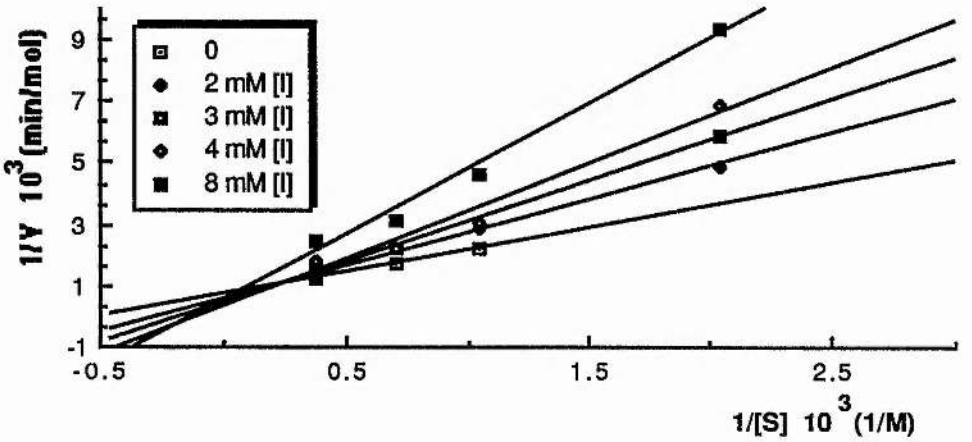



*Figure 3.16 Lineweaver-Burk plot , for (2S)-homocysteic acid*

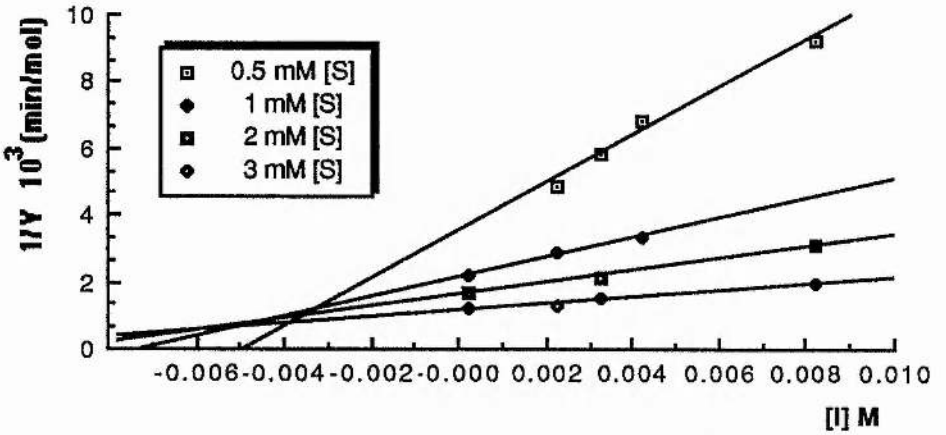



*Figure 3.17 Inhibition by (2S)-homocysteic acid, $K_i$ = 5 ± 0.5mM*

The high $K_i$ value for (2S)-homocysteic acid **(83)** was somewhat a surprise, since the only essential difference between **(83)** and (2S)-glutamic acid is the slightly larger volume of the sulphonate in comparison with the carboxylate. It was expected that the the molecule would bind better in the active site. At the same time the inability of the enzyme to process homocysteic acid suggested that changes in the electronic properties of C-4 (the carbon atom is attached to a sulphur instead of a carbon) cannot be tolerated.

Phosphohomoserine **(87)** has a longer side chain (1 additional oxygen atom ) and the expected lack of interaction with the enzyme was verified. No inhibitory action could be observed.

### 3.6.4 Straight Chain Analogues

4-Aminobutyric (GABA) **(91)**, 1,5-pentadicarboxylic **(92)**, and adipic acid **(93)** were not substrates and no inhibitory action could be detected at concentrations up to 20 mM, with the substrate at 0.5 mM.

### 3.6.5 1-Bromo-*trans*- and *cis*-cyclopropanedicarboxylic acid, (80) and (81)

The precursors of 2,3-methanoaspartic acid **(19)**, pure 1-bromo-*trans*- and *cis*-cyclopropanedicarboxylic acids, **(80)** and **(81)** (see discussion § 2.4) were the only molecules available containing the cyclopropane skeleton. The two acids, each one a mixture of two enantiomers, were tested with glutamate mutase as inhibitors and substrates. It was immediately clear that the cyclopropane diacids absorbed at the region of 240 nm. After the necessary alterations in the assay conditions to accommodate this feature, it was found that both *cis* and *trans* **(80)** and **(82)**, interacted with the enzyme. The mode of their interaction, however, was completely different. The *cis* isomer **(80)** was a typical example of competitive inhibitor with a $K_i$ of 2.2±0.2 mM. No other effects on the kinetic behaviour of the enzymatic system were detected when the diacid **(80)** was present in the assay solution.

On the other hand the behaviour of the bromo-*trans*-1,2-cyclopropane-dicarboxylate **(81)** was far more complicated. Under the specific assay conditions, discussed previously in detail (p. 77), the enzyme reaches its maximum activity 15-20 seconds after the addition of AdoCbl. When the diacid **(81)** is present in the solution no activity was observed for several minutes. After this period, formation of mesaconic acid **(3)** was observed. The performance of the enzyme kept increasing for several minutes. The time-delay for the appearance of activity, and the final rate attained depended on the concentration of the *trans* isomer. At higher concentrations of the diacid **(81)** the system stayed inactive for longer and the final

rates achieved by the enzyme were lower. Unfortunately, because initial rates could not be measured, accurate $K_i$ determination was not possible. However, a fair estimate of $K_i$ would be ~3 mM.

A first evaluation of the data accumulated hinted that the *trans* diacid **(81)** might be a substrate for glutamate mutase. Unfortunately no rearrangement products were detected by $^1$H-NMR spectroscopy when either the *trans* **(81)**, or the *cis* **(80)** cyclopropanediacids were incubated with the mutase.

## 3.7 Discussion

All the important compounds that have been used as probes for the glutamate mutase system in our laboratory or by others are listed in Table 4. The variety of structural characteristics incorporated in these structures allows a thorough evaluation of the substrate requirements for glutamate mutase. Unfortunately, none of these molecules is chemically modified in the presence of the holoenzyme.

Since no information is available on the three dimensional structure of glutamate mutase, our knowledge about the overall shape of the active site is bound to be sketchy. Nevertheless the data collected can be used to address three important issues. First, is the design of novel structures to probe the mechanism of the rearrangement in relation with the shape of the active site. A second issue is highlighting the role of the different functional groups on (2S)-glutamate (and (2S,3S)-3-methylaspartic acid). Finally, the various mechanistical aspects of the actual rearrangement step can be evaluated and a satisfactory working hypothesis will be proposed.

### 3.7.1 Mapping of the Active Site

The remarkable selectivity of glutamate mutase is probably due to the unique characteristics and requirements of the rearrangement step. The pocket reserved for (2S)-glutamic acid is clearly very tight. (R,S)-2-Methylglutamic acid **(91)** and *N*-methyl-(2S,3S)-3-methylaspartic acid **(86)** did not interact with the enzyme in any detectable manner, implying that there is no free space neither towards the *re*-face of the $\alpha$-amino acid centre nor in the pocket that binds the amino group (Fig. 3.18, region A). Phosphohomoserine **(87)**, with an additional oxygen atom on its

skeleton, but acceptable electronic properties was not an inhibitor. The size of each group and the length of the molecule (5 atom units) is important.

***Table 3.4** Compounds studied with glutamate mutase*

| Compound | Substrate | Inhibitor* | $K_m$ or $K_i$ | Ref. |
|---|---|---|---|---|
| (2S)-Glutamic acid **(1)** | + | | 1.35 | 119 |
| (2S,3S)-3-Methylaspartic acid **(2)** | + | - | 0.5 | 119 |
| (2S,3R)-3-Methylaspartic acid **(85)** | - | - | - | √ |
| (2S,3S)-3-Ethylaspartic acid **(82)** | - | - | - | √ |
| N-Methyl-(2S,3S)-3-methylaspartic acid **(86)** | - | - | - | √ |
| (2S)-Aspartic acid **(88)** | - | - | - | √ |
| (2S,3R)-3-Methylglutamic acid **(17)** | - | + | 1.5±0.1 | √ |
| (2S,3S)-3-Methylgutamic acid **(18)** | - | + | 1±0.1 | √ |
| (2S)-Homocysteic acid **(83)** | - | + | 5±0.5 | √ |
| 1-Bromo-cyclopropane-*cis*-1,2-diacid **(80)** | - | + | 2.2±0.2 | √ |
| 1-Bromo-cyclopropane-*trans*-1,2-diacid **(81)** | - | + | ~3mM | √ |
| (RS)-2-Methylglutamic acid **(8)** | - | - | - | √ |
| (2S,4S)-4-Fluoroglutamic acid | - | + | 0.07 | 121 |
| 2-Methyleneglutaric acid | - | + | 0.4 | 121 |
| (S)-3-Methylitaconic acid | - | + | 0.1 | 121 |
| Itaconic acid | - | + | 1.2 | 121 |

* All the inhibitors discovered in our laboratory were competitive. The behaviour of **(81)** has not been accurately evaluated (see § 3.6.5). For the inhibitors discovered by Buckel no information was reported on the mode of inhibition.

√ Our work.

The situation with the 3-alkylglutamic acids, **(17)**, **(18)**, is slightly different. Although they are good inhibitors, implying a firm occupation of the active site, they do not rearrange. These results are related with the inability of the enzyme to process 3-ethylaspartic acid **(82)** or to exchange any labels with the deuteriated analogues **(82 a,b,c,d)**. It seems logical to suggest that when (2S)-glutamic acid

**(1)** is present in the active site the pocket accommodating the methyl group (Fig. 3.18, region D) is almost empty. On formation of 3-methylaspartic acid, however, this pocket gets neatly occupied by the newly formed methyl group. The good competitive but non productive binding of 3-methylglutamic acids, and especially of the (2S,3S)-isomer **(18)** was probably due to the fact that region D was occupied by the additional methyl group (Fig. 3.18). Subsequently there is no space for an additional methylene group to develop.

This hypothesis by itself cannot account for the lack of any label exchange in our detailed experiments with 3-ethylaspartic acid **(82)** and its deuteriated analogues **(82 a,b,c,d)** (see p. 94). It could be envisioned, however, that the addition of the extra methyl group on 3-methylaspartic acid disturbed the geometry of the active site so much that even if the 5'-adenosyl radical was generated, the substrate **(82)** might not have been appropriately positioned to have its protons abstracted. Of course the other alternative would be that the conformational changes induced by the extra methyl group resulted in complete obstruction of the radical generation process.

As far as it concerns the role of the two carboxyl- and the amino- group two results were of particular interest. Both *cis*- **(80)** and *trans*- **(81)** bromocyclopropanedicarboxylic acids, were inhibitors of glutamate mutase. The ability of the bromo diacids **(80)** and **(81)** to interact with the enzyme suggests an amount of flexibility for the binding of the C-5 carboxyl group. Additionally Buckel and coworkers observed, using EPR, the presence of radicals during incubation of methyleneglutaric and 3-methylitaconic acids with the enzyme (Table 3.4), although no rearrangement products were observed. Compounds lacking either of the carboxyl groups, for example 4-aminobutyrate (GABA) **(91)** were completely inactive. (2S)-Homocysteic acid **(83)** bears all the necessary for binding groups and it was a competitive inhibitor. Whereas the carboxyl groups are important for "anchoring" the substrate in the active site, the amine can clearly be replaced by another group of similar size. This substitution can be tolerated by the enzyme and does not affect the binding properties of a substrate or even the ability to induce the generation of radicals species.

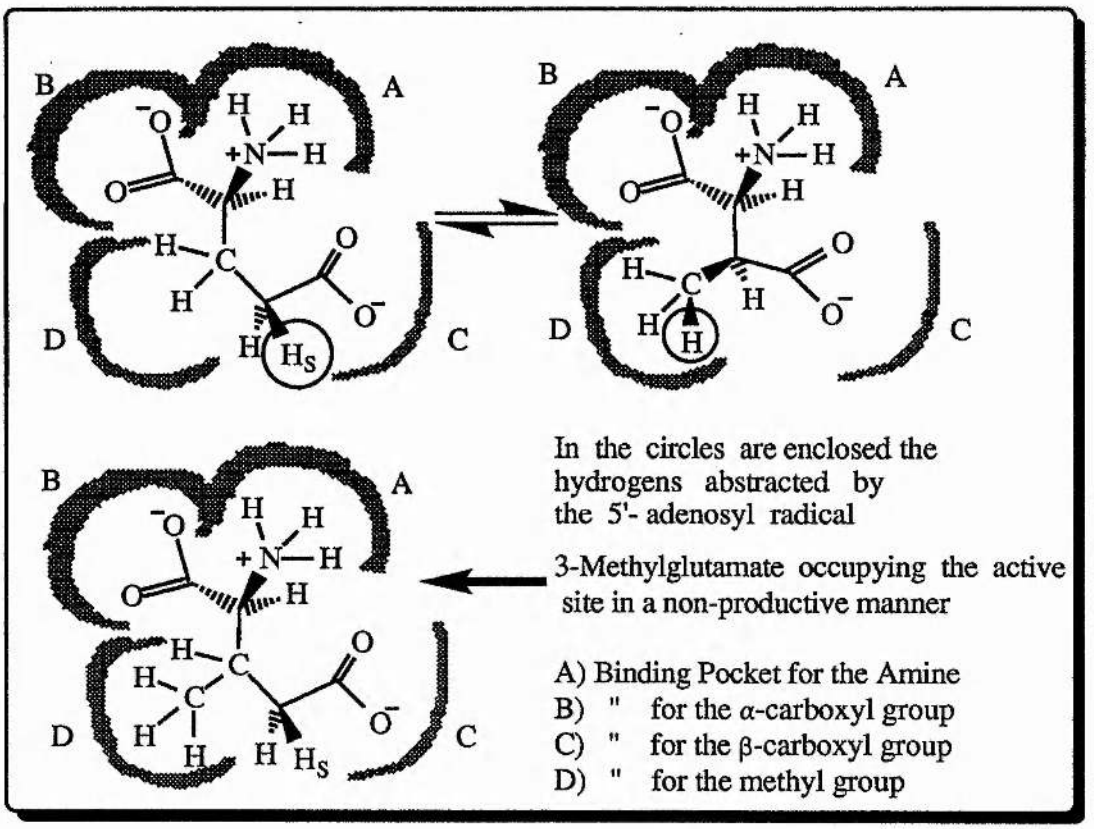



***Figure 3.18** Main characteristic of the active site and non-productive binding of 3-methylglutamic acids*

The binding of the two substrates (glutamic acid or methylaspartic acid) takes place in a highly selective binding site. The enzyme recognises every part of the molecule and does not tolerate any additions on the basic carbon skeleton. The two carboxyl groups and the five atom long chain are responsible for "anchoring" the substrate in the active site. The amine, or a group of similar size (bromo, or methylene), is necessary to "steer" the molecule in the correct orientation. The methyl group is probably involved in the radical generation process. The enzyme seems to rely, for the success of the whole process, on the adoption of a very strict conformation during the actual rearrangement step.

### 3.7.2 Cyclopropane Containing Inhibitors

The usefulness of rigid inhibitors for probing active conformations of enzyme substrates is well documented.[185] Their binding in the active site is entropically favoured, and they can offer reliable information about the arrangement of the

protein residues. Cyclopropane containing structures possess a number of advantages over unstrained inhibitors. For a start they do not contain any additional atom(s) that will prevent their entrance into the active site. Additionally, it was conceivable that the enzyme might be "fooled" and abstract a proton from the substrate inducing destruction of the cyclopropane ring. Finally these compounds have a rigid structure that should mimic, at least structurally, the TS of the rearrangement.

If the enantiomerically pure bromo-cyclopropane diacids were available, we would be able to comment on the exact conformation of the substrate in the active site prior to, and after the rearrangement. Nevertheless, compounds **(80)** and **(81)** proved to be quite useful. Most certainly the Br occupied the space reserved for the amino group (radius of -Br and $-NH_3^+$, 1.14 Å and 1.00 Å respectively) so the molecules can fit in the active site without any problems. In their racemic form, the cyclopropane diacids **(80)** and **(81)** were both inhibitors of the enzyme, but their mode of action followed a completely different pattern. On the one hand is the *cis* isomer **(80)** represented a simple case of competitive inhibition, and on the other the *trans* isomer **(80)** prevented the enzyme from turning over any substrate for several minutes (see § 3.6.5). The two molecules were neither substrates nor inactivators.

Since bromo-*trans*-1-cyclopropane-1,2-dicarboxylic acid **(81)** was not a substrate, but the nature of its interaction with the enzyme, from t=0 (enzyme not active) to t=10min (activity present) *changed*, it was clear that the enzyme existed in two significantly different conformations. The first (ground state) binds the *trans* isomer **(81)** very tightly, resulting in the inability of (2S)-glutamic acid to access the active site. Subsequently the holoenzyme complex remained inactivated. Because the binding of acid **(81)** in the active site was reversible, glutamic acid **(1)** slowly activated the system which adopted a new conformation (excited conformation). The new shape of the active site did not favour the binding of compound **(81)**, which from then on acted only as weak inhibitor (3 mM). In other words our suggestion is that the *trans* bromo diacid "locks" the enzyme in an inactive conformation, until the substrate manages to slowly replace the inhibitor and activate the system.

The ability of the holoenzyme complex to adopt two distinct conformations is more or less expected according to the discussion in chapter 1, concerning the activation of the Co-C bond. In the excited conformation, the 5'-adenosyl radical is located close to the substrate, 10 Å away from the cob(II)alamin radical. This assumption can also explain the facile deactivation of the system by oxygen, only after substrate is added in the enzyme solution (§ 1.21). Only in the excited conformation, triggered by (2S)-glutamic acid, the oxygen is harmful since it can react with the radicals in the active site and destroy the important Co-C bond.

### 3.7.3 Energetics of the Rearrangement

The rearrangement catalysed by glutamate mutase is energetically a highly unfavourable reaction. The activation energy for such a transformation is very high. In solution the enzyme has to perform two energy consuming tasks. One is to assemble the reactive complex (desolvation, entropy *etc.*) and the second to form the enzymatic transition state. In the case of glutamate mutase and other $B_{12}$ dependent enzymes this energy undoubtedly originates from the binding of the intricate cofactor and the substrate. Energetics of this kind have been analysed in detail by Schowen[186] and shown to fit perfectly the fundamentalist view of "transition state stabilisation."[187]

## 3.8 The Mechanism of the Rearrangement

Glutamate mutase is stabilising transition state intermediates whose exact nature are yet to be established. Although the literature on the subject is limited, three different routes can be envisaged, on the basis of chemically plausible alternatives, existing rearrangement models (§ 1.10.2) and theoretical studies.[188]

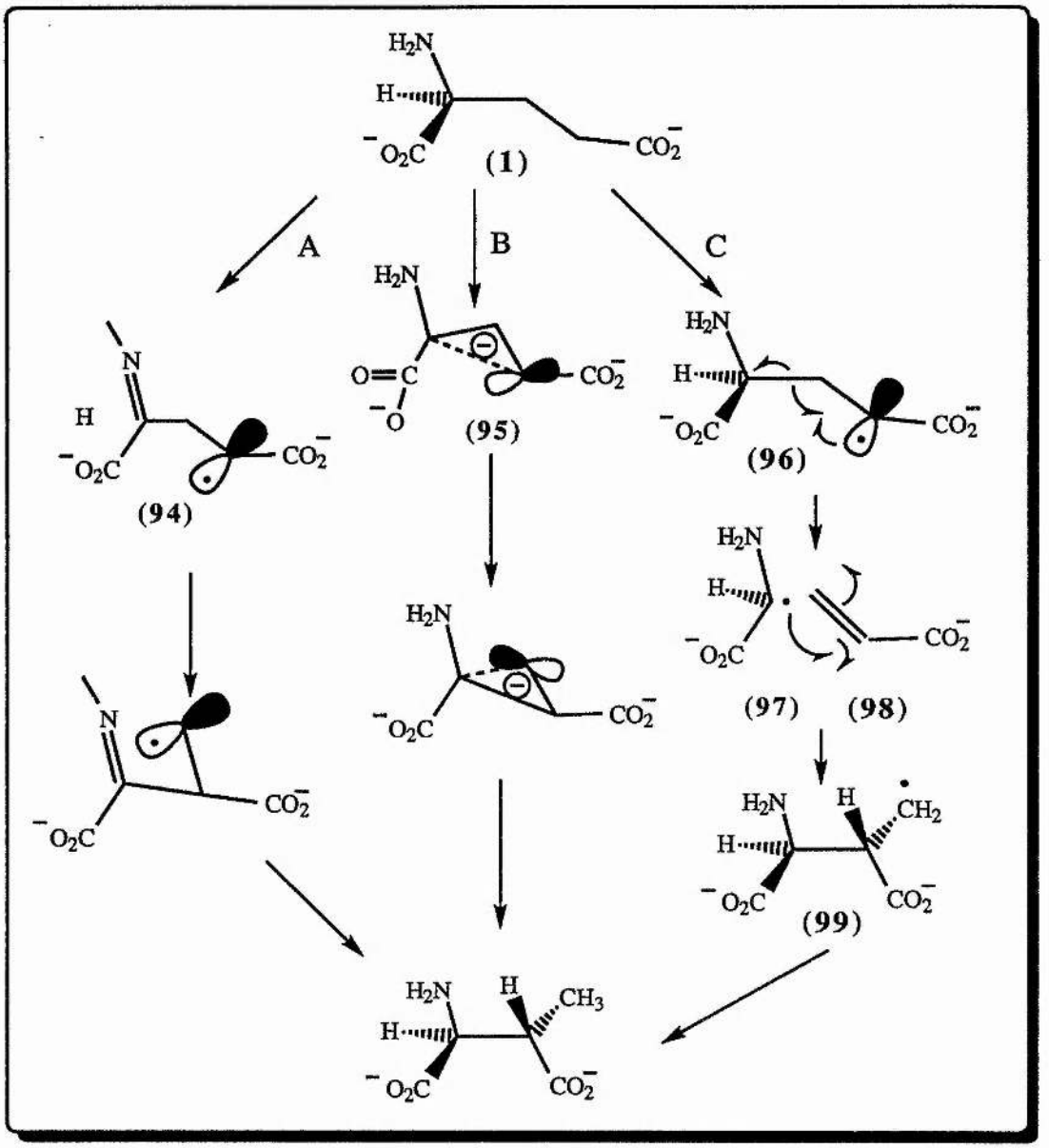
The involvement of a Schiff's base **(94)** (Scheme 3.3) was one of the first suggestions[189] concerning glutamate mutase. It was dismissed in the sixties when it was established that the enzyme does not require pyridoxal phosphate for activity. The presence an N-terminal pyruvoyl anhydro serine residue in the active site of was another possibility. This assumption, however, is not supported by the recent molecular biology studies performed on the system.[125]

The possibility of charged intermediates to be operational has already been discussed for methylmalonyl-CoA (see § 1.12.2). Merkelbach and coworkers[187] in a theoretical study extended this mechanism to include the case of glutamate mutase. Their suggestion, that the rearrangement can proceed through an enolate cyclopropane anion **(95)**, has some interesting features, as for example, control of stereochemistry by electronic instead of steric factors. One of the weak points, however, of this pathway is lack of any reasonable suggestion concerning the generation of the anion **(95)**. Although the ability of enzymes to generate enolates is well established,[190] it is very difficult to envision a link between the generation of the 5'-adenosyl radical and the abstraction of a $H^+$ to afford anion **(95)**.[72,73]

In a more general context both these mechanism are not compatible with Murakami's findings concerning the dimethyl methylaspartylcobalamin **(14)** (see § 2.8). His discovery that the 3-methylaspartic acid carbon skeleton has the intrinsic tendency to rearrange, even in low yields, in the absence of any factors that would promote Schiff's base formation or enolisation, poses a severe problem for the proponents of either of these two alternatives.

The third suggestion, which has not been presented in this form elsewhere, involves the abstraction of a proton by the 5'-adenosyl radical to form the radical **(96)** on C-4 of glutamic acid (Scheme 3.3, C). This initial abstraction is supported through many lines of evidence discussed previously (§ 1.6, 1.9). The radical **(96)** breaks down to two intermediates, namely the glycyl radical **(97)** and acrylic acid **(99)**. These two intermediates are very strongly bound on the enzyme and cannot leave the active site in this form (see § 1.17). The radicals **(97)** and **(98)** are probably very short lived. In the special environment created by the active site residues, with all the side reactions suppressed, recombination is probably the only alternative.

Glycyl radicals like **(97)** are considered to be very stable due to the combined action of an electron withdrawing ($CO_2^-$) and electron-releasing group $NH_2$.[191,192] This well documented phenomenon is called *captodative effect,* and the pair of the amine and the carboxyl group is one of the best stabilising pairs known. In other words a radical on the $\alpha$-centre of an amino acid **(97)**, is much more stable than the radical originally generated on C-4 of glutamic acid **(96)**.




*Scheme 3.3 Intermediates for the rearrangement step*

Finally we could comment that the third pathway is very sensitive to any steric perturbations. Any alteration of the substrate structure will either prevent the intermediates **(97** and **98)** from being generated or will destroy the geometry and orientation of the radical species involved. The enzyme would not have been so selective for its substrates if any of the other two mechanisms was operating.

### 3.9 Proposal about the overall mechanism

Essentially the enzyme performs three tasks. First it binds the coenzyme and the substrate in the correct conformation, second, second it triggers the homolytic cleavage of the C-Co bond every time a compound with the correct conformation occupies the active, and third, the enzyme adopts a conformation that keeps the 5'-adenosyl radical away from cob(II)alamin and promotes the formation of 3-methylaspartic acid ("excited" state).

Components E and S form the active apoenzyme complex. The affinity of the two components for each other is not very high, which is reasonable if the coenzyme is complexed in a region between the two subunits. The increase of $K_m$ for the coenzyme (see § 1.18) at high concentrations of component S supports the notion that the enzyme must have a large flexible domain which will allow the sizeable cofactor to be enclosed. In glutamate mutase this domain seems to be component S. After the holoenzyme is formed, (2S)-glutamic acid is confined in a highly selective sub-region of the active site, on component E (Fig. 3.19, p. 112) adopting the conformation shown in Fig. 3.19, **A**. This kind of binding will afford the product 3-methylaspartic acid in a *trans*-like and less strained conformation, with the two carboxyl groups as far away from each other as possible (Fig. 3.19, **D**). The 5'-adenosyl radical is located above the plane defined by the glutamic acid skeleton and probably does not move during the catalysis. The radical is attached on a highly functionalised ribose ring, whose rigidity had been shown to be of vital importance (§ 1.7). The enzyme is likely to exist in two different conformations, the ground state conformation-radical off, and the excited state conformation-radical on. The latter probably favours the binding of the more compact structure of 3-methylaspartic acid to that of (2S)-glutamic acid. Glutamate binding induces the necessary changes on the protein backbone for the Co-C bond to be cleaved and the adenosyl radical to be generated. After the abstraction of the *pro*-S 4-H, and formation of the acrylate and the glycyl radical, the enzyme must use the binding region C (Fig. 3.19) of the C-5 carboxyl group in order to push the acrylic acid in the methyl group binding pocket. The ($NH_2$) / ($CO_2H$) captodative pair stabilises the glycyl radical during the time necessary for the enzyme to adjust the acrylate in a conformation, that favours the formation of the new bond between C-2 and C-4.

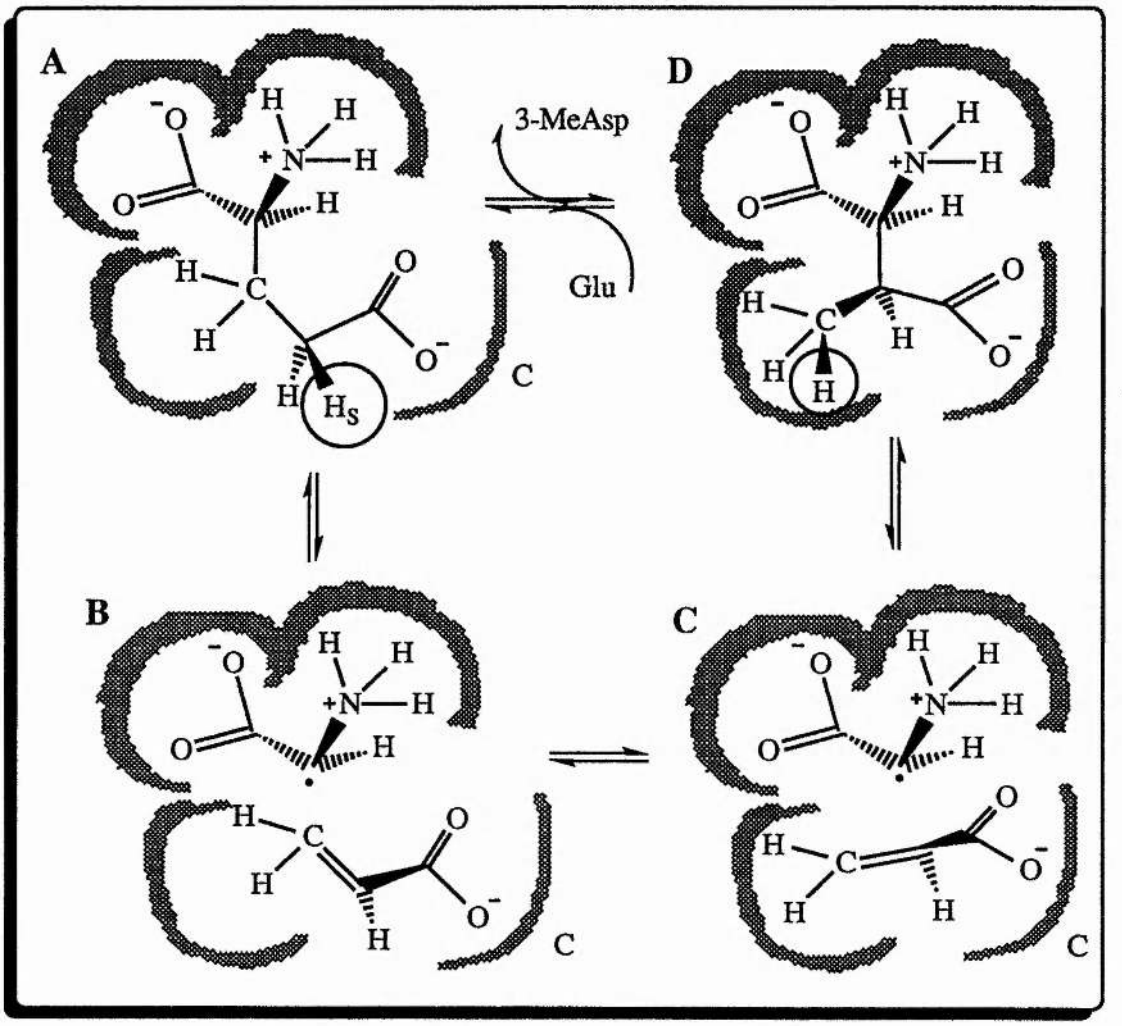


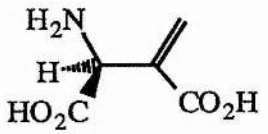

***Figure 3.19*** *Possible shape of the active site of glutamate mutase. The 5'-deoxyadenosyl radical is located above the page*

## 3.10 Conclusions

Glutamate mutase is an intriguing enzymatic system. Research on the mechanistic details of its action has been hampered by the low yielding purification procedures and the high stereospecificity of the enzyme for its substrates. Recent advances by Buckel[121] and Marsh[122] contributed in obtaining the primary structure of the protein but no additional understanding on the mechanistic details of the rearrangement emerged. Our approach was more fundamental since we tried to elucidate the basic structure of the reaction transition state and the interactions

between the substrate and the protein. Achieving these two targets is essential in order to understand the mechanism of such a complicated process. The enzyme by orienting the substrate accurately in the active site and manipulating the coenzyme $B_{12}$, manages to conduct the very reactive intermediates and promote the isomerisation. The information presented above will be soon integrated with more stereochemical and kinetic data.

For a molecule to be chemically modified by the enzyme three requirements have to be fulfilled. First , the compound has to fit in the active site; second, it must generate the 5'-deoxyadenosyl radical; and third it should be able to adopt a conformation that will allow proton abstraction. Compounds that fulfil all three requirements like other cyclopropane intermediates, 2,3-methanoaspartate **(19)** and (2S)-3-methyleneaspartate **(100)** are currently under synthesis in our laboratory. The exact mode of interaction with 1-bromo *trans*-1,2-cyclopropanedicarboxylate **(81)** has also to be elucidated.




**(100)**

Additionally the isotope effect from the direction of (2S)-glutamic acid to (2S,3S)-3-methylaspartic acid will be measured by employing [4-$^2H_2$]-(2S)-glutamic acid. The compound was synthesized very recently and the isotope effect is about be measured.

When the research described above is coupled with E.S.R and stop-flow kinetic studies, this complex enzymatic system will reveal most of the details of its action, including the nature of the cobalt intermediate and its relation to the reaction coordinate profile. Elucidation of the three dimensional structure could finally provide a unique opportunity of answering the fundamental question of how a proteinic structure manipulates all the highly reactive intermediates known to participate in the process.

# Chapter 4

# Experimental

**A) Synthesis of Substrates and Inhibitors**

Elemental microanalyses were performed in the departmental microanalytical laboratory.

NMR spectra were recorded on a Bruker AM-300 (300 MHz; FT. $^{1}$H-NMR, and 74.76 MHz; $^{13}$C-NMR.), or a Varian gemini 200 (200 MHz; FT. $^{1}$H-NMR and 50.31 MHz; $^{13}$C-NMR) spectrometers. $^{1}$H-NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position ($\delta_H$ or $\delta_C$), relative integral, multiplicity (s.-singlet, d.-doublet, t.-triplet, q.-quartet, d.d.-double of doublets, sep.-septet, m.-multiplet, and br.-broad), coupling constant (Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). $^{1}$H-NMR were referenced internally on $^{2}$HOH (4.68 p.p.m.), $CHCl_3$ (7.27 p.p.m.) or DMSO (2.47 p.p.m.). $^{13}$C-NMR were referenced on $CH_3OH$ (49.9 p.p.m.), $C^{2}HCl_3$ (77.5 p.p.m.), or DMSO (39.70 p.p.m.).

I.R. spectra were recorded on a Perkin-Elmer 1710 FT IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies ($\upsilon$) as absorption maxima are given in wavenumbers ($cm^{-1}$) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE, a Kratos MS-50 or by the SERC service at Swansea using a VG AZB-E. Fast atom bombardment spectra were recorded using glycerol as a matrix. Major fragments were given as percentages of the base peak intensity (100%). UV spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectro-photometers.

Flash chromatography was performed according to the method of Still *et al.*[193] using Sorbsil C 60 (40-60 μm mesh) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Macherey-Nagel SIL g/UV254) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, or ninhydrin.

Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 23 °C on a Optical Activity AA-100 polarimeter using 10 or 20 cm path length cells.

The solvents used were either distilled or of analar quality and light petrol ether refers to that portion boiling between 40 and 60° C. Solvents were dried according to literature procedures. Ethanol and methanol were dried using magnesium turnings. Isopropanol, isopentanol, DMF, toluene, $CH_2Cl_2$, acetonitrile, diisopropylamine, triethylamine, and pyridine were distilled over $CaH_2$. THF and diethylether were dried over sodium / benzophenone and distilled under nitrogen. Thionyl chloride was distilled over sulphur, and the initials fractions were always discarded. BuLi was titrated according to the method of Lipton.[194]

**Diazomethane:**

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

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**Acetic acid-$^2$H:**[195]

Freshly distilled acetic anhydride (36.7 g, 36 mol), zinc powder (1 g), and $CCl_4$ (1 ml) together with a trace of $P_2O_5$ were stirred under vacuum for 3 hr. in a three necked flask provided with a thermometer. The solution was cooled to 0 °C and $^2H_2O$ (7.4 g, 0.37 mol) added slowly over 30 min. under $N_2$. The temperature was gradually raised to 60 °C for 2 hr. and stirring continued overnight at room temperature $CH_3CO_2{}^2H$ was distilled in a trap cooled to -50 °C; (39 g, 88%);. b.p. 117 °C (lit.,[196] 117.9 °C). The acid prepared in this manner contained ≥ 98% $^2$H as judged by $^1$H-NMR spectroscopy, using the methyl group as internal integration reference.

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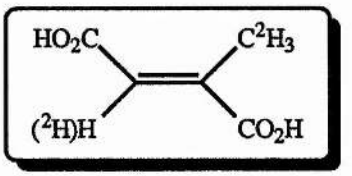
**[2'-$^2H_3$]-Mesaconic acid (3a):**[124]




Methylenesuccinic acid **(29)** (2 g, 20 mmol) and NaOH (8 g, 200 mmol) were dissolved in deteuterim oxide (18 ml, 0.9 mol) and the solvent was removed *in vacuo*. The process was repeated twice. Deteuterim oxide (18 ml, 0.9 mol) was added and the resulting solution was refluxed for 24 hr. under nitrogen. After cooling to room temperature the pH of the solution was adjusted to 1.5 with 6 M HCl. Water (40 ml) was added and mesaconic acid was extracted with diethyl ether (3 x 100 ml), the organic phases pooled, dried ($MgSO_4$) and reduced *in vacuo* to give [2'-$^2H_3$]-mesaconic acid **(3a)** as white residue which was crystallized twice from ether/ petrol ether (1.3 g, 43%), m.p. 196-197 °C (lit.,[196] 204-205 °C ); (Found: C, 45.9; H, 4.3. Calc. for $C_5H_6O_4$: C, 46.1; H, 4.6%); *m/z* (Found: $[M + NH_4]^+$ 152.086. $C_5H_2{}^2H_4O_4$ requires 152.086); $\delta_H$ (200 MHz; $^2H_2O$) 1.9 (s, residual $CH_3$), and 6.4 (s, C=CH); $\delta_C$ (50 MHz; $^2H_2O$, $NaO^2H$) 16.20 (m, $C^2H_3$), 123.21 ($\underline{C}(C^2H_3)CO_2H$), 140.08 ($\underline{C}HCO_2H$), 177.3 (C-1) and 177.7 (C-4); *m/z* (EI) 135 ($M^+$, 4%), 115 (42), 87 (39), 70 (22) and 45 (100). Dimethyl mesaconate was prepared by treatment of mesaconic acid **(3a)** with diazomethane. The sample contained ≥ 95% $^2H$ as judged by $^1H$-NMR spectroscopy, using the methyl ester group as internal integration reference.

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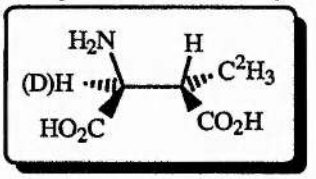
**[3'-$^2H_3$]-(2S,3S)-3-Methylaspartic acid (2a):**[197]




Mesaconic acid **(3a)** (1 g, 7.5 mmol) was dissolved in water (10 ml), and the pH of the solution was adjusted to 9 with concentrated aqueous ammonia solution. Magnesium chloride hexahydrate (0.1 g, 0.5 mmol), potassium chloride (20 mg,

0.27 mmol), and β-methylaspartase (~80units) were added and the solution was incubated at 30 °C. The absorbance at 240 nm was monitored. Periodically the pH was readjusted to 9 using concentrated ammonia solution. When the absorbance at 240 nm stopped decreasing (usually after 2 days) the solution was boiled for 1 min., then allowed to cool and the denatured protein was removed by filtration through a celite pad. The solvent was removed under reduced pressure and the residue was recrystallised from the minimum amount of hot water to afford [3'-$^2H_3$]-(2S,3S)-3-methylaspartic acid **(2a)** as white crystals (0.73 g, 65%), m.p. 266-267 °C (lit.,[196] 276-278 °C); (Found: C, 40.3; H, 6.1; N 9.4. Calc. for $C_5H_9NO_4$: C, 40.4; H, 6.15; N, 9.50%.); *m/z* (Found: [*M* +H]$^+$ 152.0861. $C_5H_5{}^2H_4O_4N$ requires 152.0861); $[\alpha]_D$ -10.2° (c 0.4 in $H_2O$), (lit.,[197] -10±2 (c 0.42 in $H_2O$)); $\delta_H$ (200 MHz; $^2H_2O$) 3.24 (1H, m, β-H) and 4.35 (1H, d, α-H); $\delta_C$ (50 MHz; $^2H_2O$, $NaO^2H$), 12.35 ($CH_3$), 42.35 (C-3), 57.63 (C-2), 175.48 (C-4) and 182.69 (C-1); *m/z* (EI) , 148 (*M*$^+$, 1.3%), 147 (2.7%), 102 (68.2), 84 (62), 57 (84), 45 (89) and 28 (100). The sample contained ≥ 95% $^2H$ as judged by $^1H$-NMR spectroscopy, using the 3-H as internal integration reference.

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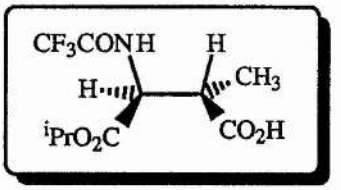
**1-Isopropyl *N*-trifluoroacetyl-(2S,3S)-3-methylaspartic acid (26):**[140]




Trifluoroacetic anhydride (50 g, 0.24 mol) was added to a stirred suspension of 3-methylaspartic acid **(2)** (4 g, 27 mmol) in dry tetrahydrofuran (50 ml) at 0 °C over 10 min. under nitrogen. The reaction mixture was then allowed to warm up to room temperature and stirred until dissolution was complete (approximately 1.5 hr.). The solvent was then removed *in vacuo* and the residue dried for 3-4 hr. under high vacuum. Dry $^i$PrOH (40 ml) was then added and the solution left at 30 °C overnight. $^i$PrOH was removed *in vacuo* to give 1-isopropyl N-trifluoroacetyl-(2S,3S)-3-methylaspartic acid **(26)** which was recrystallized from ether / petrol ether as a

white solid (6.6 g, 85%), m.p. 113-115 °C; (Found: C, 42.0; H, 4.8; N, 4.8. Calc for $C_{10}H_{14}O_5F_3N$: C, 42.1; H, 4.95; N, 4.9%); $[\alpha]_D$ +11.8° (c 0.53 in $CH_2Cl_2$); $\upsilon_{max}$ ($CHCl_3$)/$cm^{-1}$ 3410 (NH), 2987-3155 ($CO_2$-H) and 1705, 1732 (2 x CO); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.26, 1.30 (6H, 2 d, *J* 2 Hz, $(C\underline{H}_3)_2CH$), 1.36 (3H, d, *J* 3.6 Hz. $CH_3$), 3.08 (1H, dq., $J_{3',3}$ 3.6 Hz and $J_{2,3}$ 2 Hz, 3-H), 4.79 (1H, dd., $J_{3,2}$ 2 Hz and $J_{NH,2}$ 4 Hz, 2-H), 5.11 (1H, sep., *J* 3 Hz, $(CH_3)_2C\underline{H}$), 7.23 (1H, d, NH) and 8.3 (1H, s, COOH); $\delta_C$ (50 MHz; $C^2HCl_3$), 13.71 ($CH_3$), 21.99, 22.06 ($(\underline{C}H_3)_2CH$), 42.01 (C-3), 54.68 (C-2), 71.44 ($(CH_3)_2\underline{C}H$), 118.75 ($CF_3$), 159.10 (CONH), 168.75 ($\underline{C}O_2CH$) and 177.80 ($\underline{C}O_2H$); *m/z* (EI) 240 (5.5%), 226 (31), 198 (74), 102 (68), 153 (76), 69 (41) and 43 (100). No 4-isopropyl 3-methylaspartic acid could not be detected in the crude product by $^1$H-NMR spectroscopy at 200 MHZ.

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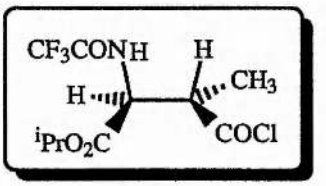
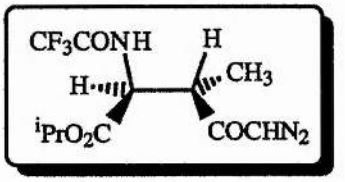
**1-Isopropyl *N*-trifluoroacetyl-(2S,3S)-3-methylaspartic acid chloride (27):**[140]




1-Isopropyl *N*-trifluoroacetyl-3-methylaspartate **(26)** (5.5g, 19 mmol) was dissolved in dry $CH_2Cl_2$ (30 ml) and the solution was cooled in an ice bath. $SOCl_2$ (9.0 g, 5.65 ml, 4 eq) was added dropwise over 5 min. The solution was then heated to reflux for 1 hr. Removal of the volatile material under reduced pressure gave 1-isopropyl 3-methylaspartic acid chloride **(27)** as an off white solid, which was recrystallized from dry ether / petrol ether (1.76 g, 83%), m.p. 67-69 °C; *m/z* (Found: *M*$^+$ 304.0562. $C_{10}H_{13}O_4NF_3Cl$ requires 304.0563); $\upsilon_{max}$ ($CHCl_3$)/$cm^{-1}$ 3326 (NH), 2987, 1780 (COCl) and 1727 (CO); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.30 (6H, 2d, $(C\underline{H}_3)_2CH$), 1.43 (3H, d, $CH_3$), 3.48 (1H, dq, 3-H), 4.88 (1H, dd, 2-H), 5.14 (1H, sep., $(CH_3)_2C\underline{H}$) and 7.12 (1H, d, NH); $\delta_C$ (50 MHz; $C^2HCl_3$) 14.33 ($CH_3$), 22.01, 22.07 ($(\underline{C}H_3)_2CH$), 53.58 (C-3), 54.49 (C-2), 68.47 ($(CH_3)_2\underline{C}H$), 113.04 ($CF_3$), 157.27 (CONH), 168.05 ($\underline{C}O_2CH$) and 175.08 (COCl); *m/z* (CI) 303 (*M*$^+$, 92%) and 240 ([M-COCl]$^+$, 10%).

**1-Isopropyl (2S,3S)-5-diazo-4-oxo-*N*-trifluoroacetylisoleucinate (28):**[140]




The crude 3-methylaspartic acid chloride **(27)** (5.8 g, 18.5 mmol) was dissolved in dry THF (10 ml). The resulting cloudy solution was added dropwise over 10 min. to an etheral solution of dry diazomethane (2.66 g, 150 ml, 63 mmol) cooled in an ice-water bath. After 1 hr. at room temperature the solution was purged with nitrogen to remove the excess of diazomethane and filtered. The volatile material was removed under reduced pressure to afford the diazoketone **(28)** as a bright yellow solid (5.75g, 96% from **(26)**), m.p. 23-25 °C; (Found: C, 42.45; H, 4.9; N, 13.4. Calc. for $C_{11}H_{14}N_3O_4F_3$: C, 42.70; H, 4.6 N, 13.58%); $\upsilon_{max}$ ($CHCl_3$)/$cm^{-1}$, 3326 (N-H), and 2113 ($CHN_2$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.19 (6H, d, $(C\underline{H}_3)_2CH$), 1.26 (3H, d, $CH_3$), 3.01 (1H, m, 3-H), 4.52 (1H, m, 2-H), 5.05 (1H, sep., $(CH_3)_2C\underline{H}$), 5.37 (1H, s, $CHN_2$) and 7.24 (1H, d, $N\underline{H}$); $\delta_C$ (50 MHz; $C^2HCl_3$) 14.65 ($CH_3$), 22.03, 22.08 $(\underline{C}H_3)_2CH$), 46.30 (C-3), 55.14 (C-2), 67.54 ($CHN_2$), 70.92 ($(CH_3)_2\underline{C}HO$), 113 ($CF_3$), 169.9 ($\underline{C}O_2{}^iPr$), 179 ($\underline{C}OCHN_2$) and 183 ($CF_3\underline{C}O$); *m/z* (EI) 308 (*M*+,1%), 239 (13), 166 (26), 154 (36), 126 (71), 69 (100) and 43 (92).

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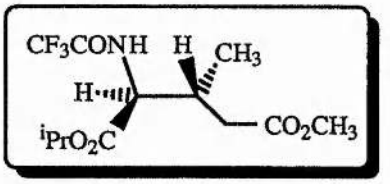
**1-Isopropyl 5-methyl *N*-trifluoroacetyl-(2S,3R)-3-methylglutamate (29):**




The diazoketone **(28)** (5.5 g, 18.6 mmol) was dissolved in dry MeOH (50 ml) and the solution was flushed with nitrogen. Silver oxide (0.5 g) was added and the solution was heated in a water bath at 50 °C for 30 min. The solution was filtered through a celite pad and the solvents were removed *in vacuo*. The resulting dark oil was subjected to flash chromatography (10% ethyl acetate in petrol ether) to give

the 3-methylglutamate diester **(29)** as a clear oil (3.1 g, 53%), b.p. 115 °C (15 mmHg); (Found: C, 46.1; H, 6.2; N, 4.8. Calc. for $C_{12}H_{18}O_4NF_3$: C, 46.0; H, 5.8; N, 4.5%); *m/z* (Found: *M* + 313.1137. $C_{12}H_{18}O_5NF_3$ requires 313.1137); $[\alpha]_D$ +32.1° (c 1.15 in $CH_2Cl_2$); $\delta_H$ (200 MHz; $C^2HCl_3$), 0.96 (3H, d, *J* 3.4 Hz, $CH_3$), 1.29 (6H, d, $(C\underline{H}_3)_2CH$), 2.11-2.52 (2H, ABX q, $J_{3,4}$ 3.4 Hz, 4-$H_2$), 2.66 (1H, m, $J_{3',3}$ 3.4 Hz and $J_{2,3}$ 1.4 Hz, 3-H), 3.69 (3H, s, $OCH_3$), 4.7 (1H, dd, 2-H), 5.10 (1H, sep., $(CH_3)_2C\underline{H}$) and 7.38 (1H, d., *J* 4 Hz, NH); $\delta_C$ (50 MHz; $C^2HCl_3$) 15.73 ($CH_3$), 22.14 (($CH_3)_2$), 33.23 (C-4), 37,94 (C-3), 52.38 ($OCH_3$), 56.42 (C-2), 70.86 ( $CH_3)_2\underline{C}H$), 112.86 ($CF_3$), 169.91 ($\underline{C}O_2{}^iPr$) and 173.02 ($CO_2CH_3$), 179.7 ($CF_3\underline{C}$); *m/z* (EI) 313 (*M*+, 2%), 271 (5, [*M* - $C_3H_7$]+), 226 (67, [*M* - $C_4H_7O_2$]+), 194 (69, [*M* - $C_7H_{10}O_3$]+), 166 (85, [*M* - $C_3H_7$]+), and 43 (100); ($R_f$ 0.35, 20% ethyl acetate in petrol ether).

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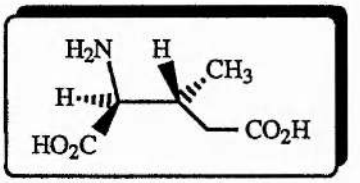
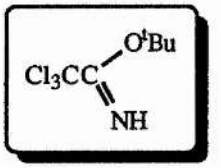
**(2S,3R)-3-Methylglutamic acid (17):**




The *N*-protected glutamate diester **(29)** (0.4 g, 1.27 mmol) was refluxed in 6 M HCl (20 ml) for 2 hr. The solvent was removed *in vacuo* and the residue was treated twice with water to remove the excess acid. The residue was thoroughly dried under high vacuum, dissolved in dry ethanol (10 ml) and propylene oxide (4 ml) was added. The mixture was refluxed for 30 min., cooled to room temperature, centrifuged, and the solvent decanted. (2S,3R)-3-Methylglutamic acid **(17)** was dried for 2 days under reduced pressure (120 mg, 58%), m.p. 166-168 °C; (Found: C, 44.47; H, 6.7; N, 8.7. Calc. for $C_6H_{11}O_4N$: C, 44.7; H, 6.9; N, 8.7%); *m/z* (Found: [*M* + H]+ 162.0766. $C_6H_{12}O_4N$ requires 162.0766); $[\alpha]_D$ +22.6 (c 1.03 in 6 M HCl); $\delta_H$ (200 MHz; $^2H_2O$) 0.93 (3H, s, $CH_3$), 2.26 (1H, m, 3-H), 2.38-2.43 (2H, m, 4-$CH_2$), and 3.66 (1H, s, 2-H); $\delta_C$ (50 MHz; $^2H_2O$) 17.11 ($CH_3$), 34.00 (C-3), 41.86 (C-4), 61.56 (C-2) and 177.8, 179.9 (2 x $CO_2H$); *m/z* (FAB), 162 ([*M* + H]+, 23%), 144 (32, [*M* - $H_2O$+H]+, and 98 (40, [*M* - $CH_4O_2N$]+).

**_tert_-Butyl-(2,2,2)-trichloroacetimidate (36):**[149]




The solution prepared by the dissolution of sodium (1 g, 43 mmol) in *tert*-butanol (20 ml) was added dropwise (0.5 ml per min.) to a vigorously stirred solution of trichloroacetonitrile (18 g, 12.5 ml, 124 mmol) and *tert*-butanol (18.5 g, 250 mmol). The reaction mixture became a clear yellow-orange and its temperature increased slightly. When the colour of the solution changed to brownish the addition was stopped. Stirring was continued for 30 min. and the product was directly distilled under vacuum. The white distillate quickly solidified and was identified as *tert*-butyl-trichloacetamidate **(36)** (19 g, 70%); b.p. 64-66 °C/ 40 mmHg; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.55 (9H, s, 3 x $CH_3$) and 8.25 (1H, s, NH).[149]

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**_N_-Benzyl-(2S,3S)-threonine (34):**[145]

L-Threonine (11.9 g, 0.1 mol) was dissolved in 2 M NaOH (50 ml). Freshly distilled benzaldehyde (10.1 ml, 0.11 mol) was added to the solution under vigorous stirring. After the solution had become homogeneous (20-30 min.), sodium borohydride (1.14 g, 30 mmol) was added slowly and in small portions over 30 min. The temperature, during the addition, was maintained at 5 °C with an ice-water bath. After an additional 30 min. of stirring, benzaldehyde and sodium borohydride (identical quantities) were added in exactly the same manner at 5 °C. The solution was finally left for 3 hr. at room temperature, diluted with water (20 ml), and extracted with ether (2 x 30 ml). On acidification of the aqueous layer to pH 6-7 the product *N*-benzyl-(2S,3S)-threonine **(34)** crystallized out. The solution was kept at 2 °C for 24 hr. and filtered. The white crystalls were washed with cold water and dried at 100 °C under vacuum (18.45 g, 88%), m.p. 236-238 °C; $[\alpha]_D$ -28.5° (c 1 in 2 M NaOH); (Found: C, 62.9; H. 7.2; N, 6.55. Calc. for $C_{11}H_{15}O_3N$: C, 63.1; H, 7.2; 6.7%); *m/z* (Found: $[M + H]^+$ 210.1130. $C_{11}H_{15}O_3N$ requires 210.1130); $\delta_H$ (200 MHz; $^2H_2O$, $NaO^2H$), 1.1 (3H, d, $CH_3$), 2.9 (1H, d, 2-H), 3.40 -4.55 (2H, ABX,

PhC$\underline{H}_2$), 3.5 (1H, m, 3H) and 7.2 (5H, s, $C_6H_5$); $\delta_C$ (50 MHz; $^2H_2O$), 20.10 ($CH_3$), 52.65 (Ph$\underline{C}H_2$), 69.62 (C-3), 70.57 (C-2), 128.4 &129.7 (aromatic), 140.1 (ipso-C) and 181 ($CO_2H$); *m/z* (CI) 210 ([*M* + H]$^+$, 100%); *m/z* (EI) 165 (5%), 120 (9), 91 (100), 74 (23), 65 (22), and 45 (7).

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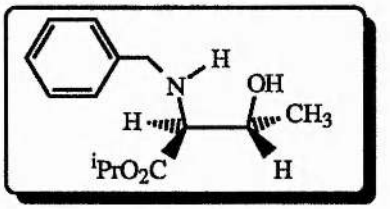
**Isopropyl *N*-benzyl-(2S,3R)-threoninate (39):**




*N*-Benzyl-threonine **(34)** (3 g, 4.8 mmol) was suspended in dry isopropanol (90 ml). Thionyl chloride (1.87 ml, 24 mmol, 5 eq) was added to the suspension over 10 min. at 0 °C. The cloudy solution was allowed to warm up to room temperature and refluxed for 3 days. Triethylamine (4.8 g, 2.6 ml, 48 mmol) was added, the solution was diluted with water (40 ml) and extracted with $CH_2Cl_2$ (2 x 50 ml). The organic phases were pooled, washed with brine (2 x 50 ml), dried ($MgSO_4$) and removed under reduced pressure to give isopropyl N-benzyl-(2S,3R)-threoninate **(39)** as a clear yellow oil, which was further purified by flash chromatography on silica gel (20% ethyl acetate in petrol ether) (2.7 g, 75%), b.p. 217 °C; *m/z* (Found: [*M* + H]$^+$ 252.1600. $C_{14}H_{21}O_3N$ requires 252.1599); $[\alpha]_D$ -56.5 (c 1.35, in $CHCl_3$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.20 (3H, d, $CH_3$), 1.25 (6H, d, ($C\underline{H}_3$)$_2$CH), 2.85 (1H, br., OH), 2.97 (1H, d, 2-H), 3.65 (1H, m., 3-H), 3.75 (2H, ABX, 2H, PhC$\underline{H}_2$), 5.06 (1H, sep., ($CH_3$)$_2$C$\underline{H}$) and 7.27-7.32 (5H, aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 19.76 ($CH_3$), 22.25, 22.41 (($\underline{C}H_3$)$_2$CH), 53.20 (Ph$\underline{C}H_2$), 68.02 (C-2), 68.51 (C-3), 69.27 (($CH_3$)$_2\underline{C}$H), 127.83, 128.81, 128.98 (aromatic), 139.58 (ipso-C) and 173.6 ($\underline{C}O_2$); *m/z* (EI) 252 ([*M* + H]$^+$, 18%), 210 (64, [*M* - $C_4H_7O_2$]$^+$), 120 (21) and 91 (100, $PhCH_2^+$).

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**Methyl *N*-benzyl-(2S,3R)-threoninate (39a):**

*N*-Benzyl-threonine **(34)** (0.5 g, 4.8 mmol) was suspended in absolute methanol (20 ml). Concentrated $H_2SO_4$ (0.5 ml) was added and the solution was refluxed for 2 hr. The acid was neutralised by the addition of saturated $NaHCO_3$ (15 ml), and methanol was removed *in vacuo*. The water phase was extracted with ethyl acetate (3 x 50 ml), the organic phases were pooled, washed with brine (2 x 50 ml), dried ($MgSO_4$) and removed under reduced pressure to give methyl N-benzyl-(2S,3R)-threoninate **(39a)** as a yellow oil which was further purified by flash chromatography on silica gel (20% ethyl acetate in hexane) (2.7 g, 65%); *m/z* (Found: $[M + H]^+$ 224.128. $C_{12}H_{18}O_3N$ requires 224.129); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.20 (3H, d, $CH_3$), 3.04 (1H, d, 2-H), 3.65-3.87 (2H, ABX, $C\underline{H}_2Ph$), 3.68 (1H, m, 3-H), 3.72 (3H, s, $OCH_3$), and 7.31 (5H, m, aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 19.29 ($CH_3$), 51.96 ($OCH_3$), 52.66 ($\underline{C}H_2Ph$), 67.30 (C-2), 67.99 (C-3), 127.38-128.47 (aromatic), 139.08 (ipso) and 174.55 ($CO_2$); *m/z* (EI) 224 ($[M + H]^+$, 100%), 206 (8), 120 (5) and 91(41).

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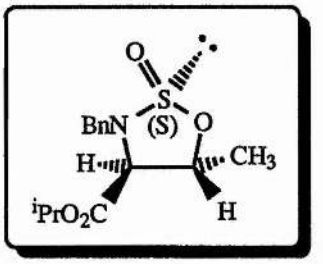
**Isopropyl *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-carboxylate S-oxide (40) and (41):**[142]

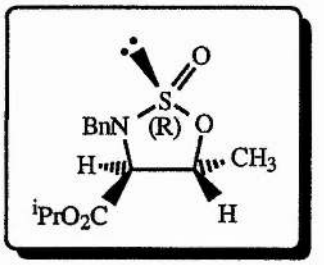
Isopropyl *N*-benzyl-threoninate **(39)** (2.0 g, 10.7 mmol) was dissolved in dry toluene (40 ml) and the solution was cooled to 0 °C under nitrogen. Freshly distilled triethylamine (2.4 g, 23.6 mmol, 2.2 eq) and thionyl chloride (11.8 mmol, 0.92 ml 1.1eq) were added succesively over 20 min. The solution was then stirred for 1hr. at 0 °C and then for 1 hour at 20 °C. Ether (150 ml) was added, and the organic phase was washed with water (2 x 50 ml), dried ($MgSO_4$) and removed *in vacuo* to afford *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone S-oxide **(40)** and **(41)** as a 3:2 mixture of the two diastereomers on S-2. The two compounds **(40)** and **(41)** were seperated and purified by flash chromatography on silica gel using 10% ethyl acetate in hexane (2.11 g, 89%); *m/z* (Found: $[M + H]^+$ 298.1113. $C_{14}H_{19}O_4NS$ requires 298.1113); *m/z* (CI) 298 ($[M + H]^+$, 100%), 252 (56), 210 (62), and 146 (34).

**(2S,4S,5R)-isomer (40):**




$R_f$ 0.25 (10% ethyl acetate in hexane); $[\alpha]_D$ -65.8 (c 0.61 in $CHCl_3$); $\upsilon_{max}$ (neat)/$cm^{-1}$ 2986, 1741, 1279.5, 1205, 1168, 1103, and 1028; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.23 (6H, dd, *J* 1.8 Hz, $(C\underline{H}_3)_2CH$), 1.47 (3H, d, *J* 3 Hz, $CH_3$), 3.48 (1H, d, *J* 3.7 Hz, 4-H), 4.49 (2H, ABX, *J* 4 Hz, $PhC\underline{H}_2$), 5.04 (1H, sept., $(CH_3)_2C\underline{H}$), 5.37 (1H, dt., *J* 3 Hz, 5-H) and 7.26-7.37 (5H, m, aromatic); $\delta_C$ (200 MHz; $C^2HCl_3$) 19.46 ($CH_3$), 22.13, 22.17 ($(\underline{C}H_3)_2CH$), 49.71 ($Ph\underline{C}H$), 67.59 (C-4), 70.32 ($(CH_3)_2\underline{C}H$), 82.33 (C-5), 128.67, 129.13, 129.30 (aromatic), 136.00 (quat. aromatic) and 168.76 (CO); *m/z* (EI), 298 ([*M* + H]$^+$, 1%) 233 (57), 210 (73), 146 (82), 104 (37), 91 (100) and 43 (100).

**(2R,4S,5R)-Isomer (41):**




$R_f$ 0.30 (10% ethyl acetate in hexane); $[\alpha]_D$ +21.6 (c 0.65 in $CHCl_3$); $\upsilon_{max}$ (neat)/$cm^{-1}$ 2986, 1741.5, 1277, 1208, 1157, 1103, 1030; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.25 (6H, d, $(C\underline{H}_3)_2CH$)), 1.69 (3H, d, *J* 3 Hz, $CH_3$), 3.93 (1H, d, *J* 3.8 Hz, 4-H), 4.23 (2H, ABX, $PhC\underline{H}_2$), 4.79 (1H, m, $J_{3',3}$ 3.8 Hz, 5-H), 5.05 (1H, sept., $(CH_3)_2C\underline{H}$), and 7.30-7.47 (5H, m, aromatic); $\delta_C$ (200 MHz; $C^2HCl_3$) 22.14, 22.18 ($(\underline{C}H_3)_2CH$), 22.58 ($CH_3$), 49.99 ($Ph\underline{C}H$), 67.58 (C-4), 70.54 ($(CH_3)_2\underline{C}H$), 86.23 (C-5), 128.70, 128.12, 128.92 (aromatic), 135.78 (quat. aromatic), and 168.76 (CO); *m/z* (EI), 298 ([*M* + H]$^+$, 1%) 233 (57), 210 (73), 146 (82), 104 (37), 91 (100) and 43 (100).

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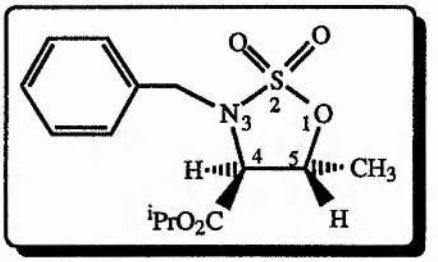
**Isopropyl *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-carboxylate S,S-dioxide (33):**[142]




The mixture of *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone S-oxides **(40+41)** (2 g, 6.7 mmol) was dissolved in dry acetonitrile (30 ml) and the solution was cooled to 0 °C in an ice-water bath. Ruthenium(III) chloride monohydrate (8 mg), sodium periodate (2.15 g, 5 mmol, 1.5 eq), and water (40 ml) were then added successively. The solution was stirred for 30 min. at 0 °C and then allowed to warm up to room temperature. After 1hr. the solution was extracted with ether (3x100 ml), and the combined organic phases were pooled, washed with water (30 ml), brine (30 ml), dried ($MgSO_4$), filtered through a silica pad and removed under reduced pressure to give a clear oil which solidified on standing. Recrystallization from ethyl acetate / hexane gave large transparent crystals of the isopropyl *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-carboxylate S,S-dioxide **(33)** (1.82 g, 80%), m.p. 59-61 °C; (Found: 53.3; H, 6.4; N 4.4. Calc. for $C_{14}H_{19}O_5NS$: C, 53.6; H, 6.1; N, 4.5%); *m/z* (Found: $[M + NH_4]^+$ 331.1328. $C_{14}H_{19}O_5NS$ requires 331.1327.); $[\alpha]_D$ -18.9° (c 0.62 in $CH_2Cl_2$); $\upsilon_{max}$ ($CHCl_3$)/$cm^{-1}$ 2986. 1747, 1356, 1160, 1103; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.19-1.24 (6H, 2d, $(C\underline{H}_3)_2CH$), 1.55 (3H, d, $CH_3$), 3.66 (1H, d., 4-H), 4.47 (2H, s, $PhC\underline{H}_2$), 4.88 (1H, m, 5-H), 5.00 (1H, m, $(CH_3)_2C\underline{H}$) and 7.32-7.40 (5H, m, aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 19.28 ($CH_3$), 21.46, 21.52 (2 x $CH_3$), 50.38 ($Ph\underline{C}H_2$), 65.15 (C-4), 70.57 ($(CH_3)_2\underline{C}H$), 77.52 (C-5), 128.7-129.3 (ar-C), 133.59 (ipso-C) and 166.76 (CO); *m/z* (CI) 331 ($[M + H]^+$, 100%), 314 (35), 205 (11), 148 (8), 108 (30) and 91 (22).

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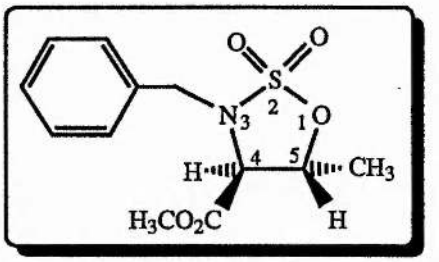
**Methyl *N*-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-carboxylate S,S-dioxide (33a):**




Methyl oxathioazolidine-carboxylate S,S-dioxide **(33a)** was prepared from methyl *N*--benzyl-threoninate **(39a)** (2g, 9.5 mmol) in an identical manner to that described for **(39)** (2 steps, 1.95 g, 72%), m.p. 57-58 °C; *m/z* (Found: $[M + NH_4]^+$ 303.1015. $C_{12}H_{15}O_5NS$ requires 303.1014.); $[\alpha]_D$ -17.5 (c 0.64 in $CH_2Cl_2$); $\upsilon_{max}$ ($CHCl_3$)/$cm^{-1}$ 2986. 1747, 1356, 1160, and 1103; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.53 (3H, d, $CH_3$), 3.65 (3H, s, $OCH_3$), 3.73 (1H, d, 4-H), 4.47 (2H, s, $PhC\underline{H}_2$), 4.90 (1H, m, 5-H) and 7.35-7.40 (5H, m, aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 19.79 ($CH_3$), 51.15 ($OCH_3$), 53.61 ($Ph\underline{C}H_2$), 65.62 (C-4), 78.11 (C-5), 129.1-129.7 (aromatic C), 134.01 (ipso-C) and 167.9 ($CO_2$); *m/z* (EI) 286 ($[M + H]^+$, 100%), 210 (25), 146 (18) and 91 (100).

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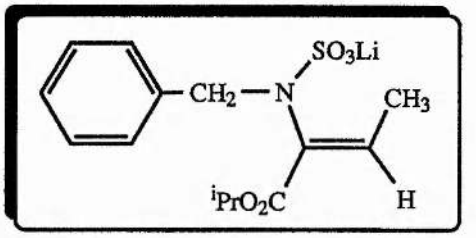
**1-Isopropyl Lithium *N*-Benzyl-*N*-sulpho-2-amino-*trans*-but-2-enoate (45):**




Oxathiazolidone S,S-doxide **(33)** (0.2 g, 0.6 mmol) was treated with lithium hydroxide (2 eq.) in water:ethanol (50% v/v, 10ml) for 24 hr. The solvents were removed *in vacuo* and the residue was recrystallized from water / ethanol to give 1-isopropyl *N*-benzyl-2-amino-trans-but-2-enoate **(45)** (0.12 g, 62%); *m/z* (Found: $[M + H]^+$ 322.1814. $C_{14}H_{18}O_5NSLi$ requires 322.1814); $\delta_H$ (200 MHz; $^2H_6$-DMSO) 1.21 (6H, d, $(C\underline{H}_3)_2CH$), 1.62 (3H, m, $CH_3$), 4.41-4.21 (2H, ABX q, $PhC\underline{H}_2$), 4.95 (1H,

sep., $(CH_3)_2C\underline{H}$), 6.73 (1H, q, $C=C\underline{H}(CH_3)$), and 7.35-7.40 (5H, m, aromatic); $\delta_C$ (50 MHz; $^2H_6$-DMSO) 14.43 ($CH_3$), 21.63 ($\underline{C}H_3)_2CH$), 52.01 ($Ph\underline{C}H_2$), 68.49 $(CH_3)_2\underline{C}H$), ($CH_3O$), 126.8-129.1 (aromatic C), 132.38 (C-3), 138.03 (ipso-C), 143.19 (C-2), and 172.1 ($CO_2$); *m/z* (CI) 322 ([M + H]$^+$, 100%), 234 (63, [*M* - $SO_3Li$]$^+$), 196 (92), 144 (12), 108 (54), 91 (14).

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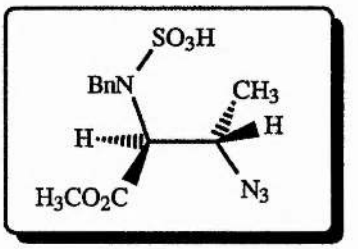
**Isopropyl *N*-benzyl-*N*-sulpho-(2S,3S)-2-amino-3-azido-butanoate (46):**




Methyl oxathiazolidone-carboxylate S,S-dioxide **(33a)** (100 mg, 0.32 mmol) was dissolved in water / acetone (50% v/v, 5 ml). $NaN_3$ (50 mg, 0.64 mmol) was added and the solution was stirred until no starting material could be detected by TLC (2 days). The solvents were removed *in vacuo* and the residue was dissolved in acetonitrile. The solution was filtered and the solvent was removed. The white residue was dried under vacuum for 4 hr. and then recrystallized from ethyl acetate / hexane to give isopropyl N-benzyl-2-amino-3-azido-butanoate **(46)** as white crystals (75 mg, 65%); *m/z* (Found: [*M* -$SO_3$]$^+$ 249.135. $C_{12}H_{16}O_2N$ requires 249.135.); $[\alpha]_D$ -35.35° (c 0.52 in $CH_2Cl_2$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.03 (3H, d, $CH_3$), 3.67 (1H, m, 2-H), 3.71 (3H, s, $OCH_3$), 4.05 (1H, m, 2-H), 4.35 (2H, ABX, $PhC\underline{H}_2$) and 7.35-7.40 (5H, m, aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 17.22 ($CH_3$), 52.51 ($Ph\underline{C}H_2$), 53.18 ($OCH_3$), 58.22 (C-3), 64.39 (C-2), 129.1-129.6 (aromatic C), 138.03 (ipso-C) and 173.2 ($CO_2$); *m/z* (EI) 249 ([*M* - $SO_3$]$^+$, 10%), 206 (12), 178 (37) and 91 (100).

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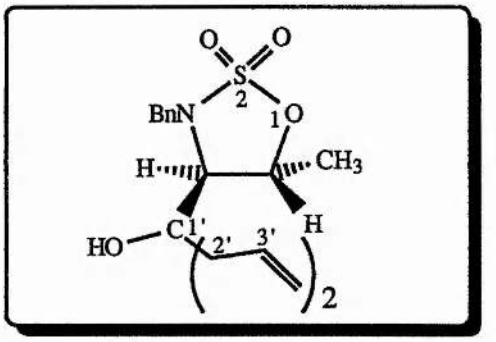
**_N_-benzyl-(4S,5R)-1,2,3-oxathiazolidone-5-methyl-4-[1'-allyl]-but-3'-en-1'-ol S,S-dioxide (47):**




Isopropyl *N*-benzyl-oxathiazolidone-carboxylate S,S-dioxide **(33)** (260 mg, 0.78 mmol) was dissolved in dry tetrahydrofuran (10 ml). Dilithium tetrachlorocuprate (II), as a solution in THF, (0.15 ml of 0.1 M solution, 0.02 eq.) was added and the solution was cooled to -78 °C. An etheral solution of allyl-magnesium bromide (1.5 mmol, 1.5 ml of 1.0 M solution in ether) was added and the solution was stirred overnight. at -78 °C. The reaction mixture was allowed to warm up to -20 °C, acetic acid (1 ml) was added and the solvents removed *in vacuo*. The residue was partitioned between water/ ethyl acetate (50:50 v/v, 20 ml). The aqueous phase was washed with ethyl acetate (2 x 10 ml), and the organic phases were pooled, dried ($MgSO_4$), and removed *in vacuo*. The off white residue was flash chromatographed on silica gel (50% ethyl acetate: DCM) to give the oxathiazolidone-4-buten-1'-ol **(47)** (0.2 g, 77%); *m/z* (Found: [*M* + H]$^+$ 338.1425 $C_{17}H_{23}O_4NS$ requires 338.1425); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 (3H, d, $CH_3$), 2.1-2.5 (4H, m., ($C\underline{H}_2CH=CH_2)_2$), 3.31 (1H, d, 5-H), 4.52 (2H, ABX, $PhC\underline{H}_2$), 4.87 (1H, m., 6-H), 5.02-5.23 (4H, m., 2 x $CH_2CH=C\underline{H}_2$), 5.60-5.96 (2H, m., 2 x $CH_2C\underline{H}=CH_2$) and 7.29-7.48 (5H, m., aromatic); $\delta_C$ (50 MHz; $C^2HCl_3$) 21.02 ($CH_3$), 39.98, 41.34 ($\underline{C}H_2CH=CH_2$), 55.20 ($Ph\underline{C}H_2$), 70.75 (C-4), 77.73 (C-5), 120.17, 120.73 ($CH_2\underline{C}H=CH_2$), 129.19-130.20 (aromatic), 132.51, 132.54 ($CH_2CH=\underline{C}H_2$), 135.09 (quat. aromatic) and 183.5 (CO); *m/z* (CI) 338 ([M + H]$^+$, 100%), 258 (32), 168 (11), 147 (64), 108 (96), 91 (100), 58 (45).

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***N*-Acetyl-(R,S)-alanine (55):**[198]

(S)-Alanine (2.5 g, 28 mmol) was dissolved in acetic acid (25 ml, 26 g, 0.44 mol) and the mixture was brought to the boil. After cooling for 2 min. at room temperature, distilled acetic anhydride (4.85 g, 47 mmol) was added in portions, the solution was brought again to the boil and reflux was continued for 2 min. The solution was allowed to cool and the volatile materials were removed *in vacuo.* Racemic *N*-acetyl-alanine **(55)** was recrystallised from hot ethyl acetate (3.1 g, 83%), m.p. 134-5 °C (lit.,[198] 134-136 °C); $\delta_H$ (300 MHz; $^2H_6$-DMSO) 1.35 (3H, d, $CH_3$), 1.97 (3H, s, $CH_3CO$), 4.3 (1H, q, 2-H), 8.24 (1H, d, NH) and 12.4 (1H, br., $CO_2H$), $\delta_C$ (50 MHz; $^2H_6$-DMSO) 17.3 ($CH_3$), 22.5 ($\underline{C}H_3CO$), 47.7 ($\alpha$-C), 169.3 (CONH) and 174.56 (CO).

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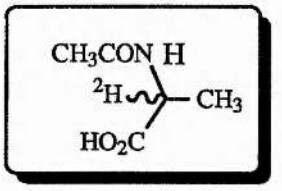
***N*-Acetyl-[2-$^2$H]-(R,S)-alanine (55a):**[198]




(S)-Alanine (2 g, 22 mmol) was dissolved twice in hot $^2H_2O$ (5 ml) and the solvent removed *in vacuo.* The amino acid was redissolved in acetic acid-$^2$H (26.7 g, 25 ml, 0.44 mol) and treated as described previously for **(55)**, under a stream of nitrogen. The solvents were removed under reduced pressure. The residue was dissolved with heating in $^2H_2O$ (2 ml) and the solvent was again removed *in vacuo.* After drying for several hours *N*-acetyl-[2-$^2$H]-(R,S)-alanine **(55a)** was recrystallized from hot ethyl acetate, and dried at 60 °C (2.4 g, 81%), m.p. 134-135 °C (lit.,[198] 134-136 °C); (Found: C, 45.7; H, 6.75; N, 10.4. Calc. for $C_3H_6DO_2N$: C, 45.8; H, 6.9; N, 10.7%); (Found: [*M* + H]$^+$ 133.073. $C_5{}^1H_8{}^2HO_3N$ requires 133.073); $\delta_H$ (300 MHz; $^2H_6$-DMSO) 1.23 (3H, s, $CH_3$), 1.83 (3H, s, $CH_3CO$), 8.15 (1H, s, NH) and 12.4 (1H, br., $CO_2H$), $\delta_C$ (50 MHz; $^2H_6$-DMSO) 17.32 ($CH_3$), 22.54 ($\underline{C}H_3CO$), 47.69 (C-2), 169.31 (CONH) and 174.56 ($CO_2H$). The title compound contained ≥

93% $^{2}H$ as judged by $^{1}H$-NMR, using the methyl group as internal integration reference.

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**(R)-Alanine (56):**[198]

*N*-Acetyl-(R,S)-alanine **(55)** (1.78 g, 13.5 mmol) was dissolved in water (80 ml) and the pH of the solution was adjusted to 7.3 with conc. ammonia solution. Acylase I (4 mg) was added and the solution was incubated in a water-bath at 38 °C. After 24 hr. acetic acid (1 ml) was added and the solution was heated to reflux for 1 min. The digested protein was filtered off through a celite pad and the volume was reduced *in vacuo* to 10 ml. On addition of alcohol (70 ml) and cooling at 5 °C, (S)-alanine precipitated as a white solid (0.44 g, 72%), m.p. 303-305 °C (lit.,[198] 314 °C (dec.)); $[\alpha]_D$ +13.3° (c 1 in 6 M HCl ) (lit.,[198] +13.6° (c 1 in 6 M HCl).

Washings and filtrates were pooled and removed under reduced pressure. Water (10 ml) was added and the pH of the solution was adjusted to 3 using 3 M $H_2SO_4$. The solvent was again removed *in vacuo*. *N*-Acetyl-(R)-alanine was extracted from the residue using hot acetone (5 x 20 ml). The pooled organic extracts were filtered and removed *in vacuo* to give a yellow syrup, which was suspended in 6 M HCl (20 ml). The suspension was refluxed for 2 hr., and the solvent was removed under reduced pressure. Excess acid was removed by treating the residue several times with water. Finally the crude amino acid was taken up in water (3 ml), and the pH of the solution adjusted to 4 with few drops of concentrated ammonia. On addition of ethanol (15 ml) (R)-alanine **(56)** precipitated rapidly. Recrystallisation from hot water / ethanol gave the pure amino acid (0.39 g, 65%), m.p. 299-301 °C (lit.,[196] 314 °C); $[\alpha]_D$ -13.0° (c 1 in 6 M HCl) (lit.,[198] -13.6° (c 1 in 6 M HCl)); $\delta_H$ (300 MHz; $^{2}H_2O$) 1.25 (3H, d, $CH_3$) and 3.55 (1H, q, 2-H); $\delta_C$ (74 MHz; $^{2}H_2O$) 19.45 ($CH_3$), 53.20 (C-2) and 168.2 ($CO_2H$).

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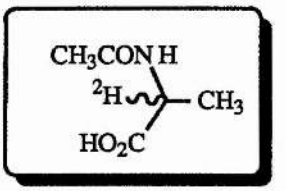
**[2-$^2$H]-(R)-Alanine (56a)**




(R)-[2-$^2$H]-Alanine **(56a)** was synthesized in an identical manner to **(56)** from **(55a)** (0.55 g, 62%), m.p. 291-293 °C, (lit.,[198] 300 °C (dec.).); (Found: C, 40.4; H, 8.02; N, 15.5. Calc. for $C_3H_6DO_2N$: C, 40.4; H, 7.9; N, 15.7%); (Found: [*M* + H]$^+$ 91.062±0.001. $C_3H_6DO_2N$ requires 91.063); [$\alpha_D$] -13.1° (c 1 in 6 M HCl) (lit.,[198] -13.6° (c 1 in 6 M HCl)); $\delta_H$ (300 MHz; $^2H_2O$) 1.22 (H, s, $CH_3$); $\delta_C$ (50 MHz; $^2H_2O$) 17.58 (-$CH_3$), 51.38 (C-2) and 178.23 (-$CO_2H$); *m/z* (CI) 91 (*M*$^+$, 100%), 75 (5), and 45 (20); The title compound contained ≥ 93% $^2$H as judged by $^1$H-NMR, using the methyl group as internal integration reference..

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**But-2-ynoic acid (65)**

BuLi (0.22 mol, 140 ml, 1.55 M in hexanes) was cooled to -50 °C and added *via* cannula to THF (400 ml) at -78 °C under a nitrogen atmosphere. The solution was purged with propyne for 10 min., strirred for 5 min., and then purged with carbon dioxide. Soon the solution became cloudy and a yellowish precipitate appeared. As soon as the colour changed to orange, the $CO_2$ flow was stopped and the solution was stirred for an additional 10 min. at room temperature. The reaction was quenched with saturated $NH_4Cl$ solution (200 ml), and the THF was removed under reduced pressure. The pH of the aqueous phase was adjusted to 1.5 (conc. HCl) and extracted with ether (3 x 200 ml). The organic phases were pooled, dried ($MgSO_4$) and removed *in vacuo* to afford a brown oil which, after distillation under reduced pressure, solidified on standing. Recrystallization from ethyl acetate / hexane gave but-2-ynoic acid **(65)** as large white crystalls (14.5 g, 80%), m.p. 69-71 °C (lit.,[196] 70-72 °C); b.p. 115-120 °C/ 0.1mmHg; (Found: C, 57.3; H, 4.9. Calc. for $C_5H_6$: C 57.1 H 4.9%); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.95 (3H, s, $CH_3$), and 10.0 (1H, br., $CO_2H$).

**Isopentyl but-2-ynoate (66a)**

But-2-ynoic acid **(65)** (3 g, 35 mmol) was dissolved in dry isoamyl alcohol (40 ml) and $SOCl_2$ (8 ml, 0.1 mol, 3 eq.) was added over 5 min. at 0 °C. The reaction mixture was heated to 50 °C for 30 min. After 3 hr. at room temperature isoamyl alcohol was distilled off at 35 °C under reduced pressure (10 mmHg). The desired ester distilled between 60-64 °C. The purity of the but-2-ynoate at this stage was 70% since an unidentified impurity with similar b.p. was co-distilling. Chromatography of the distillate on silica gel (5% ethyl acetate in hexane) afforded isopentyl but-2-ynoate **(66a)** as a clear oil of ~85% purity (2.8 g, 64%), b.p. 60-64 °C/ 10 mmHg; *m/z* (Found: $[M + H]^+$ 155.1071. $C_9H_{14}O_2$ requires 155.1072); $\delta_H$ (200 MHz; $C^2HCl_3$) 0.92 (6H, d, 2 x $CH_3$), 1.55 (2H, m, $OCH_2C\underline{H}_2CH$), 1.68 (1H, m, $OCH_2CH_2C\underline{H}$), 1.97 (3H, s, ($C\underline{H}_3C{\equiv}C$) and 4.25 (2H, t, $OC\underline{H}_2CH_2CH$); $\delta_C$ (50 MHz; $C^2HCl_3$) 4.23 ($\underline{C}H_3C{\equiv}C$), 22.8 ($\underline{C}H_3)_2CH$), 25.58 $(CH_3)_2\underline{C}H$), 37.5 ($OCH_2\underline{C}H_2CH$), 64.9 ($O\underline{C}H_2CH_2CH$) 85.7 ($CH_3\underline{C}{\equiv}C$) 117.3 ($CH_3C{\equiv}\underline{C}$) and 154.3 ($CO_2$); *m/z* (CI) 155 ($[M + H]^+$, 12%), 103 (10) and 58 (3); $R_f$ 0.35 (5% ethyl acetate in hexane).

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**Isopropyl but-2-ynoate (66b)**

But-2-ynoic acid **(65)** (3 g, 35 mmol) was dissolved in dry isopropanol (40 ml) and $SOCl_2$ (0.1 mol, 8 ml, 3eq.) was added over 5 min. at 0 °C. The solution was left at room temperature for 10 min. and then refluxed for 30 min. The volume was reduced *in vacuo* and the crude isopropyl but-2-ynoate **(66b)** was purified by flash chromatography on silica gel (ethyl acetate/ hexane 1:20) (2.2 g, 50%), b.p. 64-66 °C/ 15 mmHg; *m/z* (Found: $[M + H]^+$ 127.0759. $C_7H_{10}O_2$ requires 127.07589); $\upsilon_{max}$ (nujol)/$cm^{-1}$ 2985, 2253 (C≡C), 1701 (CO), 1271, 1106, and 1072; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.25 (6H, d, $(C\underline{H}_3)_2CH$), 1.97 (3H, s, $C\underline{H}_3C{\equiv}C$) and 5.05 (1H, s, $(CH_3)_2C\underline{H}$); $\delta_C$ (50 MHz; $C^2HCl_3$) 4.27 ($\underline{C}H_3C{\equiv}C$), 22.1 ($\underline{C}H_3)_2CH$), 70.13 ($(CH_3)_2\underline{C}H$), 85.36 ($CH_3\underline{C}{\equiv}C$), 117.7 ($CH_3C{\equiv}\underline{C}$) and 153.8 (CO); *m/z* (EI) 127 ($[M + H]^+$, 55%), 67 (7) and 58 (5). $R_f$ 0.3 (5% ethyl acetate in hexane).

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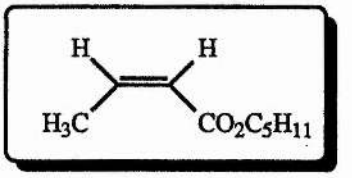
**Isopentyl *cis*-but-2-enoate (58a)**




Isopentyl but-2-ynoate **(66a)** (2 g, 16 mmol) was dissolved in ethyl acetate / dry pyridine (9:1), palladium on barium sulphate (5% palladium content, 10% per weight of the ester) was added and the solution was purged with $H_2$ for 4 hr. After filtration through a celite pad the solution was partitioned between ethyl acetate (30 ml) and 1 M HCl solution (100 ml). The aqeous phase was extracted with ethylacetate (2 x 20 ml), the organic phases were pooled, dried ($MgSO_4$) and removed *in vacuo* to afford a yellow oil which was chromatographed on silica gel (ethyl acetate/ hexane 1:9) to give isopentyl *cis*-but-2-enoate **(58a)** as a clear oil (1.9 g, 77%); *m/z* (Found: $[M + H]^+$ 157.1229. $C_9H_{16}O_2$ requires 157.1228); $\delta_H$ (200 MHz; $C^2HCl_3$) 0.92 (6H, d, $(C\underline{H}_3)_2CH$), 1.52 (2H, m, $OCH_2C\underline{H}_2CH$), 1.69 (1H, m, $OCH_2CH_2C\underline{H}$), 2.11 (3H, s, $C\underline{H}_3C{\equiv}C$), 4.13 (2H, t, $OC\underline{H}_2CH_2CH$), 5.79 (1H, dd, $CH_3CH{=}C\underline{H}$) and 6.20 (1H, dq, $CH_3C\underline{H}{=}CH$); $\delta_C$ (50 MHz; $C^2HCl_3$) 15.8 ($\underline{C}H_3CH{=}CH$), 22.9 $(\underline{C}H_3)_2CH$), 25.58 $(CH_3)_2\underline{C}H$), 37.9 ($OCH_2\underline{C}H_2CH$), 63.3 ($O\underline{C}H_2CH_2CH$) 121.2 ($CH_3\underline{C}H{=}CH$) 145.3 ($CH_3CH{=}\underline{C}H$) and 167.2 ($CO_2$); *m/z* (EI) 157 ($[M + H]^+$, 2%), 121 (18), 103 (57), 70 (100) and 55 (73). $R_f$ 0.55 (10% ethyl acetate in hexane).

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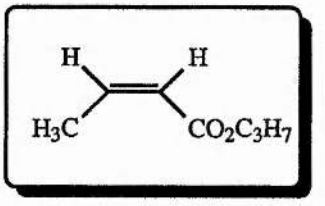
**Isopropyl *cis*-but-2-enoate (58b)**




But-2-enoic acid isopropyl ester **(58b)** was prepared from the isopropyl but-2-ynoate **(66b)** (2 g, 15 mmol) in an identical manner to that described for **(58a)** (1.73 g, 84%), *m/z* (Found: $[M + H]^+$ 129.091. $C_7H_{12}O_2$ requires 129.091); $\delta_H$ (200

MHz; $C^2HCl_3$) 1.22 (6H, d, $(C\underline{H}_3)_2CH$), 2.13 (3H, dd, $C\underline{H}_3CH=CH$), 5.02 (1H, s, $(CH_3)_2C\underline{H}$) 5.74 (1H, dd, $CH_3CH=C\underline{H}$), and 6.27 (1H, dq, $CH_3C\underline{H}=CH$); $\delta_C$ (50 MHz; $C^2HCl_3$) 15.7 ($\underline{C}H_3CH=CH$), 22.4 ($\underline{C}H_3)_2CH$), 67.43 $(CH_3)_2\underline{C}HO$), 121.6 ($CH_3CH=\underline{C}H$), 144.95 ($CH_3\underline{C}H=CH$), and 166.6 ($CO_2$); *m/z* (CI) 155 ([*M* + H]$^+$, 12%), 103 (10), and 58 (3). $R_f$ 0.6 (10% ethyl acetate in hexane).

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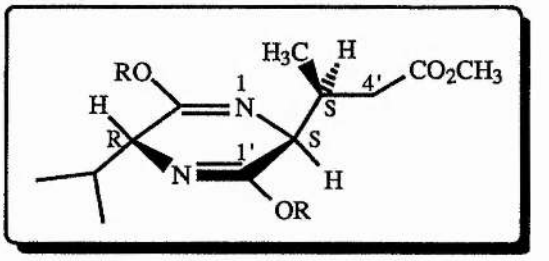
**Methyl (5R,2S)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine-2-[(3'S)-3'-methyl]-propanoate (60)**




BuLi (1.86 ml, 2.9 mmol, 1.6 M, 1.1 eq) in hexane was added (under nitrogen *via* syringe) to a solution of the *bis*-lactim ether **(49)** (0.5 g, 2.7 mmol) in dry THF (10 ml) cooled at -78 °C. After 15 min. a solution of methyl *trans*-crotonate **(52)** (0.4 g, 4 mmol, 1.5 eq) in THF (5 ml) was added. After 3 hr. a solution of acetic acid (0.16 g, 2.7 mmol, 1 eq.) in THF (2 ml) was added, and the mixture was allowed to warm up to room temperature The solvent was removed *in vacuo*, and the residue was partitioned between ethyl acetate / water (50% v/v, 20 ml). The water phase was re-extracted with ethyl acetate (2 x 20 ml). The organic phases were pooled, dried ($MgSO_4$) and concentrated to afford the crude 2,5-dihydropyrazine-2-[(3'S)-3'-methyl]-propanoate **(60).** The sample was applied on a silica gel coloumn and washed with neat hexane (1/2 the bed volume) in order to improve separation. Elution with a mixture of 4% ethyl acetate in hexane gave dihydropyrazin **(60)** (0.47 g, 62%); *m/z* (Found: [*M* + H]$^+$ 285.1814. $C_{14}H_{24}O_4N_2$ requires 285.1814); $[\alpha]_D$ +18.3 (c 0.61 in EtOH), $\upsilon_{max}$ ($CHCl_3$)/cm$^{-1}$ 3154, 2960, 1702 (ester), 1644 (C=N), and 1485; $\delta_H$ (300 MHz; $C^2HCl_3$) 0.65 (3H, d, $C\underline{H}_3CH(CH_3)$), 1.01 (3H, d, $CH_3CH(C\underline{H}_3)$), 1.05 (3H, d, $CH_3$), 2.02-219 (2H, m, $C\underline{H}_2CO_2CH_3$), 2.20-2.25 (1H, m, 3'-C$\underline{H}$), 3.62 (3H, s, $CH_2CO_2C\underline{H}_3$), 3.65, 3.66 (6H, s, 2 x $OCH_3$). and 3.9 (2H, m, 2

and 5-H); $\delta_C$ (75 MHz; $C^2HCl_3$) 16.38 ($CH(\underline{C}H_3)_2$), 18.85 ($-CH_3$), 31.53 ($\underline{C}H(CH_3)_2$), 33.76 (C-3'), 36.56 (C-4'), 51.25 ($CO_2\underline{C}H_3$), 52.18, 52.22 (2 x $OCH_3$), 59.46 (C-5), 60.46 (C-2), 162.49 (C-6), 163.81 (C-3) and 173.27 ($CO_2$); *m/z* (EI) 285 ([*M* + H]$^+$, 50%), 241 (84, [*M* - $C_3H_7$]$^+$), 209 (100, [*M* - $C_4H_{10}O$]$^+$), 184 (63, [*M*- $C_5H_9O_2$]$^+$), 167 (96), and 141 (92); ($R_f$ 0.32 in 10% ethyl acetate in hexane).

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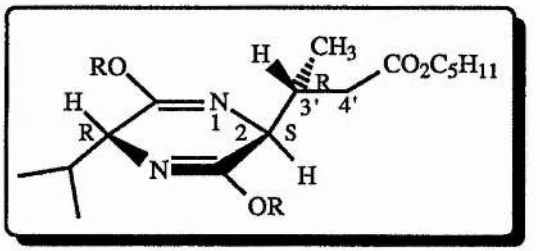
**Isopentyl (5R,2S)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine-2-[(3'R)-3'-methyl]-propanoate(61).**




Alkylpyrazine **(61)** was prepared and purified in an identical manner to that described for the dihydropyrazine-2-propanoate **(60)**, starting from the *bis*-lactim ether **(49)** and the isopentyl *cis*-but-2-enoate **(58a)** (0.25 g, 1.36 mmol), (0.20 g, 44%), *m/z* (Found: [*M* + H]$^+$ 341.2440. $C_{18}H_{32}O_4N_2$ requires 341.2440); $[\alpha]_D$ +14.3 (c 0.78 in EtOH); $\upsilon_{max}$ ($CHCl_3$)/cm$^{-1}$ 2961, 2873, 1738 (C=N), 1644 (ester) and 1485; $\delta_H$ (300 MHz; $C^2HCl_3$) 0.68 (3H, d, $C\underline{H}_3CH(CH_3)$), 0.92 (6H, d, ester $(C\underline{H}_3)_2CHCH_2$), 1.04 (3H, d, $CH_3CH(C\underline{H}_3)$), 1.08 (3H, d, $CH_3$), 1.51 (2H, d. of t., $OCH_2C\underline{H}_2CH(CH_3)_2$), 1.68 (1H, m, ester $(CH_3)_2C\underline{H}CH_2$), 1.98-2.15 (2H, m, $C\underline{H}_2CO_2R$), 2.24-2.28 (1H, m, $C\underline{H}$-3'), 2.64 (1H, m, ester $(CH_3)_2C\underline{H}CH_2$), 3.689, 3.696 (6H, 2s, 2 x $OCH_3$)), 3.93 (2H, m, 3 and 6-H) and 4.08 (2H, t, $OCH_2$); $\delta_C$ (75 MHz; $C^2HCl_3$) 16.40, 16.48 (ring $CH(\underline{C}H_3)_2$), 19.02 ($CH_3$), 22.48 (ester $((\underline{C}H_3)_2CHCH_2$), 25.05 (ester $(CH_3)_2\underline{C}HCH_2$), 31.69 (ring $\underline{C}H(CH_3)_2$), 33.94 (C-3'), 36.99 (C-4'), 37.32 (ester $(CH_3)_2CH\underline{C}H_2$), 52.38, 52.36 (2 x $OCH_3$), 59.73 (C-5), 60.61 (C-2), 162.49 (C-6), 163.81 (C-3) and 173.27 ($CO_2$); *m/z* (EI) 285 ([*M* + H]$^+$, 50%), 241 ([*M* - $C_3H_7$]$^+$, 84), 209 ( [*M* - $C_4H_{10}O$]$^+$, 100), 184 ([*M* - $C_5H_9O_2$]$^+$, 63), 167 (96) and 141 (92)

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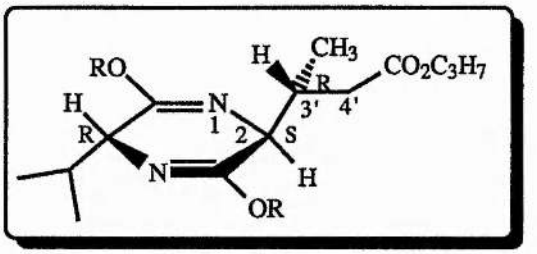
**Isopropyl (5R,2S)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine-2-[(3'R)-3'-methyl]-propanoate (61a)**




Alkylpyrazine **(61a)** was prepared and purified in an identical manner as for the dihydropyrazine-2-propanoate **(60)**, starting from the *bis*-lactim ether **(49)** and the isopropyl *cis*-but-2-enoate **(58b)** (0.20 g, 1.1 mmol), (140 mg, 40%), *m/z* (Found: $[M + H]^+$ 313.2127. $C_{16}H_{28}O_4N_2$ requires 313.2127); $\upsilon_{max}$ ($CHCl_3$) / $cm^{-1}$ 2961, 2873, 1738 (C=N), 1644 (ester) and 1485; $\delta_H$ (300 MHz; $C^2HCl_3$) 0.68 (3H, d, ring $C\underline{H}_3CH(CH_3)$), 1.04 (3H, d, ring $CH_3CH(C\underline{H}_3)$), 1.06 (3H, d, 3'-$CH_3$), 1.22 (6H, dd, ester $(C\underline{H}_3)_2CH$), 1.98-2.15 (2H, ABX, $C\underline{H}_2CO_2R$), 2.24-2.28 (1H, m, ring $(CH_3)_2C\underline{H}$), 2.64 (1H, m, 3'-H), 3.68, 3.69 (6H, 2s, 2 x $OCH_3$), 3.93 (2H, m, 3 and 6-H) and 5.01 (1H, s, ester $(CH_3)_2C\underline{H}$); $\delta_C$ (75 MHz; $C^2HCl_3$) 16.28, 16.44 (ring $(\underline{C}H_3)_2CH$), 19.02 ($CH_3$), 21.71, 21.81 (ester $((\underline{C}H_3)_2CH$), 31.58 (ring $(CH_3)_2\underline{C}H$), 33.89 (C-3'), 37.15 (C-4'), 52.31 (2 x $OCH_3$), 59.72 (C-5), 60.49 (C-2), 67.38 (ester $(CH_3)_2\underline{C}H$), 162.5 (C-6), 163.8 (C-3) and 172.5 ($CO_2$); *m/z* (EI) 313 ($[M + H]^+$, 16%), 269 (48), 209 (77), 167 (42), 141 (82), 127 (79) and 43 (100).

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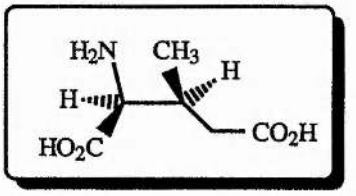
**(2S,3S)-3-Methylglutamic acid (18)**




A suspension of the pyrazine **(60)** (300 mg, 1.05 mmol) was stirred for 2 days in 0.25 M HCl (8 ml, 2 eq). The aqueous solution was extracted with ether (2 x 10 ml) and the organic phases discarded. The pH was adjusted to 9-10 with

concentrated ammonia and the aqueous phase extracted with ether (3 x 10 ml). The organic phases were pooled, dried ($MgSO_4$), and removed *in vacuo*. The residual oil, which was a mixture of the methyl pyrrolidone-2-carboxylate **(63)** and valine methyl ester **(64)**, was separated on a silica gel using 10% ethyl acetate in hexane. Only pyrrolidone **(63)** elutes ($R_f$ 0.6) (120 mg, 58%). The clear oil was refluxed in 6 M HCl for 2 hr. The solvent was removed *in vacuo* and the residue was dried thouroughly under high vacuum. Ethanol (5 ml) and propylene oxide (2 ml) were added and the resulting solution was refluxed for 30 min. The white precipitate was centrifuged, the supernatant decanted, and the crystals of (2S,3S)-3-methylglutamic acid **(18)** were dried for 2 days *in vacuo* at 50 °C, (87 mg, 51%), m.p. 169-171 °C; (Found: C, 41.5; H, 6.6; N, 7.8. Calc. for $C_6H_{12}O_4N \cdot 0.75\ H_2O$: C, 41.24; H, 6.9; N, 8.0%); *m/z* (Found: $[M + H]^+$ 162.0766. $C_6H_{12}O_4N$ requires 162.0766); $[\alpha]_D$ +12.4° (c 0.34 in 6 M HCl); $\delta_H$ (300 MHz; $^2H_2O$) 0.93 (3H, s, $CH_3$), 2.26 (1H, m , 3-H), 2.38-2.43 (2H, m, 4-$CH_2$) and 3.66 (1H, s, 2-H); $\delta_C$ (75 MHz; $^2H_2O$) 16.76 ($CH_3$), 33.29 (C-3), 40.16 (C-4), 60.54 (C-2) and 175.07, 179.11 (2 x $CO_2$); *m/z* (FAB) 162 ($[M + H]^+$, 23%), 144 (32, $[M - H_2O+H]^+$) and 98 (40, $[M - CH_4O_2N]^+$).

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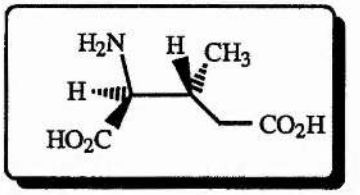
**(2S,3R)-3-Methylglutamic acid (17a)**




Alkylpyrazine **(61)** (180 mg, 0.53 mmol) was refluxed in 6 M HCl (5 ml) for 2 hr. The solvent was removed and the solution treated twice with water to remove the excess of the acid. The residue was dissolved in water (2 ml), the pH adjusted to 1.5 and ethanol added (~70 ml) until the solution became cloudy. The mixture was kept at 5 °C for 2 days and then filtered to afford a white solid, which was recrystallized from water ethanol, dried for 2 days at 50 °C *in vacuo*, and identified as (2S,3R)-3-methylglutamic acid **(17a)** (36 mg, 42%), m.p. 166-168 °C; (Found:

C, 40.05; H, 7.5; N, 7.6. Calc. for $C_6H_{11}O_4N \cdot H_2O$: C, 40.22; H, 7.31; N, 7.82%) *m/z* (Found: [*M* - $H_2O$]$^+$ 144.0661. $C_6H_{11}O_4N$ requires 144.0661); $[\alpha]_D$ +10.2° (c 0.43 in 6 M HCl); $\delta_H$ (300 MHz; $^2H_2O$) 1.04 (3H, d, $CH_3$), 2.30-2.65 (3H, m, C<u>H</u>($CH_3$)$CH_2$) and 3.80 (1H, d, 2-H); $\delta_C$ (75 MHz; $^2H_2O$), 17.11 ($CH_3$), 33.63 (C-3), 40.50 (C-4), 60.89 (C-2) and 175.44, 179.49 (2 x $CO_2H$); *m/z* (EI), 162 ([*M* + H]$^+$, 8%), 98 (100) and 55 (95).

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**Methyl dibromoacetate (79)**

Dibromoacetic acid (10g, 45 mmol) was dissolved in dry methanol 100 ml, concentrated $H_2SO_4$ (5 ml) was added and the solution was left stirring overnight. Saturated $Na_2CO_3$ (100 ml) was added, methanol was removed *in vacuo* and the ester was extraced from the aqueous phase with ether (2 x 150 ml). The organic phases were pooled, dried ($MgSO_4$), and removed *in vacuo* to give methyl dibromoacetate **(79)** (8.7 g, 82%); b.p. 179-181 °C (lit.,[196] 182-183 °C); $\delta_H$ (200 MHz; $C^2HCl_3$) 4.85 (3H, s, $OCH_3$) and 5.82 (1H, s, $CHBr_2$); $\delta_C$ (50 MHz; $C^2HCl_3$) 31.7 ($CH_3$), 54.1 ($CHBr_2$) and 165.0 ($CO_2$).

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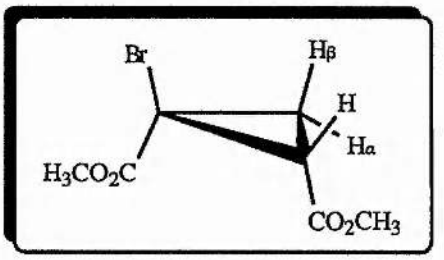
**Dimethyl 1-Bromo-*cis*-cyclopropane-1,2-dicarboxylate (68)**[174]




A mixture of the dibromoacetate **(79)** (10 g, 43 mmol) and methyl acrylate (4.8 g, 56 mmol, 1.3 eq) was added to a suspension of NaH (1.9 g, 47 mmol, 1.1 eq, 60% in oil) in dry toluene. After 1 hr. at room temperature the solution was refluxed for 24 hr. The excess hydride was destroyed by the addition of water (5 ml). The volatile material was removed *in vacuo* and the oily residue was partitioned between water and ethyl acetate (1:1 v/v, 100 ml). After furter extraction with ethyl

acetate (3 x 50 ml) the organic phases were pooled, dried ($MgSO_4$), and removed to give a yellow oil. The crude oil was a 10:1 mixture of the 1-bromo-*cis*- **(68)** and 1-bromo-*trans*-cyclopropanedicarboxylate dimethyl ester **(69)**. The *cis* isomer was purified with flash chromatography on silica gel (5% ethyl acetate in hexane) (5.6 g, 55%); $\upsilon_{max}$ (neat)/$cm^{-1}$ , 3450, 3100, 1740, 1385, 1307, 1279, 1225, 1186, and 1106; $\delta_H$ (200 MHz; $C^2HCl_3$), 1.69 (1H, dd, $H_\beta$), 2.12 (1H, dd., $H_\alpha$), 2.44 (1H, t, $CHCO_2$), and 3.60, 3.73 (6H, 2s, 2 x $OCH_3$); $\delta_C$ (50 MHz; $C^2HCl_3$) 22.59 ($CH_2$), 29.35 ($\underline{C}HCO_2$), 30.88 ($\underline{C}BrCO_2$), 52.55 ($CHCO_2\underline{C}H_3$), 53.51 ($CBrCO_2\underline{C}H_3$), and 167.80, 169.08 (2 x $CO_2$). ($R_f$ 0.33, 5% ethyl acetate in hexane). The *cis* isomer **(69)** is a mixture of two enantiomers, namely the (1R,2S)- and (1S,2R)-bromo-dicarboxylates.

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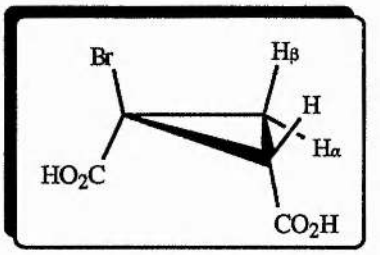
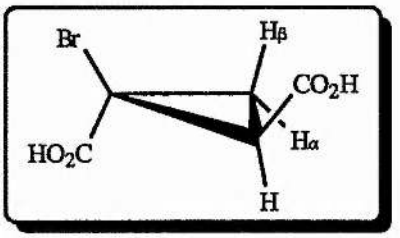
**1-Bromo-*cis*-cyclopropane-1,2-dicarboxylic acid (80)**[174]




Dimethyl 1-bromo-*cis*-dicarboxylate **(79)** (0.5 g, 2.1 mmol) was suspended in 6 M HCl (20 ml) and the solution was refluxed for 2 hr. The solution was basified with potassium hydroxide (2 M) until pH 9-10. The aqueous phase was washed with dichloromethane (2 x 20 ml), acidified to pH 1 (6 M HCl), and extracted with ethyl acetate (3 x 20 ml). The organic phases were pooled, dried ($MgSO_4$) and removed *in vacuo* to give 1-bromo-*cis*-cyclopropane-1,2-dicarboxylic acid **(80)** as a white solid which was recrystallized from ethyl acetate / petrol ether (290 mg, 67%), m.p. 139-141 °C; *m/z* (Found: $[M + NH_4]^+$ 225.9715. $C_5H_5O_4Br$ requires 225.9715); $\upsilon_{max}$ (nujol)/$cm^{-1}$ 1700 (b), 1302, 1263 and 1211; $\delta_H$ (200 MHz; $^2H_2O$), 1.80 (1H, dd, $H_\beta$) 2.02 (1H, dd, $H_\alpha$) and 2.60 (1H, t, CHBr); $\delta_C$ (75 MHz; $^2H_2O$, $CH_3OH$) 23.48 ($CH_2$), 31.62 (CBr), 32.41 (CH) and 171.86, 173.67 ($2CO_2$), *m/z* (CI) 249 ($[M + NH_4]^+$, 38%), 148 (100), 105 (17), 62 (15), 52 (12) and 39 (18).

**1-Bromo-*trans*-cyclopropane-1,2-dicarboxylic acid (81)**




A mixture of dibromoacetate **(82)** (3.5 g, 15 mmol) and methyl acrylate (1.7 g, 20 mmol, 1.3eq) was added to a suspension of NaH (0.66 g, 47 mmol, 1.1 eq, 60% in oil) in dry dimethylformamide (25 ml). The solution was heated to 80 °C for 5 hr. The excess of hydride was destroyed by the addition of water (20 ml). The resulting solution was partitioned between water and ethyl acetate (1:1 v/v 100 ml). After further extraction with ethyl acetate (3 x 50 ml) the organic phases were pooled, dried ($MgSO_4$), and removed to give a yellow oil. The crude oil was a 2:3 mixture of 1-bromo-*cis*- **(68)** and 1-bromo-*trans*-cyclopropanedicarboxylate dimethyl ester **(69)** (both isomers 2.80 g, 79%); An analytical sample of the 1-bromo-*trans*-diester **(69)** was obtained by collecting only the initial fractions after silica gel chromatography (5% ethyl acetate in hexane); ($R_f$ 0.36, 5% ethyl acetate in hexane); $\upsilon_{max}$ (neat)/$cm^{-1}$ 3462, 3104, 1749, 1377, 1316, 1283 and 1225; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.85-2.05 (2H, m, $H_\beta$ and $H_\alpha$), 2.60 (1H, t, $CHCO_2$) and 3.77, 3.80 (6H, 2s, 2 x $OCH_3$); $\delta_C$ (50 MHz; $C^2HCl_3$) 23.58 ($CH_2$), 29.93 ($\underline{C}HCO_2$), 30.43 ($\underline{C}BrCO_2$), 52.69 ($CHCO_2\underline{C}H_3$), 34.09 ($CBrCO_2\underline{C}H_3$) and 166.76, 168.67 (2 x $CO_2$).

The above mixture (1.5 g, 6.3 mmol) was suspended in 6 M HCl (20 ml) and refluxed for 2 hr. The pH of the solution was adjusted to 9-10 with potassium hydroxide (2 M). The aqueous phase washed twice with dichloromethane, acidified again, and re-extracted with ethyl acetate (3 x 20 ml). The organic phases were pooled, dried ($MgSO_4$), filtered, and removed to give a mixture of the free bromodiacids **(80), (81)** (0.95g, 72%).

The mixture of the free acids from the previous step (0.95 g, 4.5 mmol) was dissolved in dry isopropanol (20 ml), concentrated $H_2SO_4$ (0.2 ml) was added, and the solution was stirred for 8 hr. ar room temperature. After addition of saturated

$Na_2CO_3$ (5 ml) the solution was extracted with dichloromethane (2 x 10 ml). The aqueous phase was then acidified, and re-extracted with ethyl acetate (3 x 30 ml). The organic phases were pooled, dried ($MgSO_4$), and removed to give an oily residue which contained the *trans* and *cis* bromo-cyclopropane-dicarboxylate isopropyl diesters in a ratio of 8:1. Flash cromatography on silica gel (5% ethyl acetate in hexane) gave diisopropyl 1-bromo-*trans*-1,2-isopropylcyclopropane-dicarboxylate **(69).** Hydrolysis and work-up in an identical manner to that described for **(80)**, afforded, after recrystallization, the pure *trans* diacid **(81)** (140 mg, 38%). *m/z* (Found: $[M + NH_4]^+$ 225.9715. $C_5H_5O_4Br$ requires 225.9715); $\upsilon_{max}$ (nujol)/$cm^{-1}$ 1699 (b), 1290, 1242, and 1139; $\delta_H$ (200 MHz; $^2H_2O$) 1.73, 1.94 (2H, dd, $CH_2$) and 2.61 (1H, t, CHBr); $\delta_C$ (50 MHz; $^2H_2O$) 25.68 ($CH_2$), 32.21 (CH), 34.70 (CBr) and 174.80, 174.90 (2 x $CO_2$), *m/z* (CI) 249 ($[M + NH_4]^+$, 226, 38%), 148 (100), 105 (17), 62(15), 52 (12) and 39 (18). The *trans* isomer **(81)** is a mixture of two enantiomers, namely the (1R,2R)- and (1S,2S)-bromo-cyclopropane-1,2-dicarboxylates.

**B) Purification Protocols**

The cells used in this study were cultured in the Department of Biological Sciences at the University of East Anglia. The fermentation service provided batches of 450 g of frozen cell paste of *Clostridium tetanomorphum* (Strain H1) from a 150 L fermentation.

For the purification of the two components of glutamate mutase four types of chromatographic techniques were used. Anion exchange chromatography of the crude protein extract was performed on DEAE-52 cellulose. The chromatographic media was dissolved in a 10 mM $K_2PO_4$, pH 7.0, left for 24 hours, packed (300 x 26 mm) and used as such during the whole study.

Gel exclusion chromatography was performed using Sephadex G-150-120. The gel was suspended in the appropriate buffer and allowed to settle several times to remove fine particles. The column was repacked (150 x 26 cm) every time before use to achieve maximum flow rates (20-25 ml $hr.^{-1}$).

Anion exchange FPLC chromatography was performed on a TSK DEAE-5PW (75 x 8 mm). A hydrophobic interactions TSK Phenyl-PW (75 x 8 mm) high performance column was employed for the purification of component S.

SDS-PAGE was performed following the procedure of Laemli[199] on a discontinuous medium. A Mini-Protean II Dual Slab Cell apparatus was used.

The centrifugations were carried out on a CENTRICON C-124 centrifuge. Pooled fractions from various steps (see below) were concentrated by ultrafiltration. Ultrafiltration was performed using an AMICON apparatus (43 or 76 mm diameter), through a YM10 or a YM30 membrane, under $N_2$.

The purification was carried out between 0-4 °C. The FPLC steps were conducted at room temperature. All materials used were of AR quality. All procedures involving coenzyme $B_{12}$ were performed under reduced light or in dark room.

Finally the amount of protein present was determined by the method of Lowry *et al.*[200] Appropriately diluted samples of the protein solution was mixed with Coomasie blue dye reagent (100 mg of Coomasie brilliant blue dissolved in 50 ml of 95 % ethanol to which 100 ml of 85 % of phosphoric acid has been added and

the whole diluted to 1 L with water). After 5 min. the absorbance was measured at 595 nm. Standard curves were prepared by using bovine serum albumin.

**Buffers for the Purification of Glutamate Mutase**

1) Sonication buffer: 50 mM potassium phosphate, pH 7.6.

2) Protamine sulphate solution: 50 mM potassium phosphate pH 7.0, 1% protamine sulphate.

3) Dialysis Buffer: 2 mM Potassium phosphate, pH 7.6, 10 mM 2-mercaptoethanol, 2 mM EDTA.

4) Ion Exchange Chromatography - DEAE-52 cellulose.

Equilibration Buffer: 10 mM potassium phosphate, pH 7.6, 10 mM 2-mercaptoethanol, 2 mM EDTA. Elution Buffer: 80 mM potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 2 mM EDTA.

5) G-150 gel exclusion chromatography column. Elution buffer: 50 mM Potassium phosphate, pH 5.8, 10 mM 2-mercaptoethanol, 2 mM EDTA

6) Ion exchange TSK DEAE-5PW

Low Salt: 10 mM potassium phosphate, pH 7.6;

High Salt: 10 mM potassium phosphate, 0.5 M KCl, pH 7.6

7) Hydrophobic interactions TSK Phenyl-PW

Low Salt : 10 mM potassium phosphate pH 7.4;

High Salt : 10 mM potassium phosphate, 1 M ammonium sulphate, pH 7.4;

**Purification of Component E and S: Steps 1-6**

**Step 1: Sonication**

Frozen *Clostridium Tetanomorphum* cell paste (HI strain) (50 g), was thawed in 100 ml of 50 mM potassium phosphate buffer, pH 7.6. The suspension was sonicated for 15 min. at <6 °C, in bursts of 1 min. using a Ultrasonic W-220F sonicator (150W). The resulting suspension was centrifuged (20,000 g, 30 min.). The precipitate was discarded and the suspension including a slimy grey layer was used immediately for the next step.

**Step 2: Protamine Sulphate**

To the 145 ml of the sonic extract (15.3 mg/ ml) was added 50 ml of 1% protamine sulphate solution in 50 mM potassium phosphate buffer, pH 7.0 at 4 °C. After stirring for an additional 10 min. the solution was centrifuged (20,000 g, 30 min.) and the precipitate discarded.

**Step 3: Ammonium Sulphate Fractionation**

To 170 ml of the protamine extract (5 mg/ml) was added precrushed ammonium sulphate to 45% saturation at 4 °C. After stirring for an additional 20 min., the precipitate was separated by centrifugation (20,000 g, 30 min.) and discarded. Ammonium sulphate was added again to a final saturation of 85%. The resulting precipitate was separated by centrifugation (30,000 g, 45 min.) and then dissolved in 36 ml of 10 mM potassium phosphate, pH 7.6.

The resulting solution was then dialysed for 6-8 hr against 5 L of 2 mM potassium phosphate, pH 6.8 containing 10 mM 2-mercaptoethanol and 2 mM EDTA. The conductivity of the dialysate was checked to be less than that of the equilibration buffer for the DEAE-cellulose.

**Step 4: DEAE-Cellulose Fractionation**

The above solution was applied to a column of DEAE-cellulose equilibrated with 10 mM potassium phosphate pH 7.6. The column was then eluted successively with 400 ml of 10 mM pH 7.6, and 500 ml of 80 mM potassium phosphate pH 6.8 at a flow rate of 3 ml $min.^{-1}$. 15 ml fractions of were collected and assayed. The fractions containing active protein (13-20, ~120 ml) were pooled and concentrated (6-8 ml) using ultrafiltration. Alternatively the enzyme was precipitated by saturation to 80% in the presence of 2 mM EDTA. The precipitate was recoverd as a pellet by centrifugation (30,000 g) and stored at -80 °C.

**Step 5: Sephadex-G-150**

The cloudy concentrated solution from the previous step was centrifuged (10,000 g, 10 min.) and then applied to a G-150 column equilibrated with 50 mM potassium phosphate, pH 6.8. The protein was eluted with the same buffer at a flow rate of 20 ml $hr.^{-1}$ (fractions of 10 ml were collected). The major peaks were those containing component E, β-methylaspartase, and component S.

Fractions containing component E (35-48, 200-300 ml), and component S (70-90, 320-420 ml) activity were concentrated respectively to a volume of 2-3 ml by ultrafiltration. Residual 2-mercaptoethanol was removed by addition of 20 mM potassium phosphate buffer pH 7.0 (10 ml) for component E and of 50 mM potassium phosphate buffer pH 7.0 (10 ml) for component S. The volume was again reduced by ultrafiltration and the solutions containing component E and S were stored at -80 °C in aliquots of 0.7 ml.

**Step 7: FPLC for Component E:**

An aliquot from the above step was thawed, diluted with water to 2 ml, filtered through a 2 μm filter, and applied to TSK DEAE-PW ion exchange column equilibrated with a 10 mM potassium phosphate buffer, pH 7.6. The protein was eluted with a gradient of 0-500 mM KCl in the same buffer at a flow rate of 1 ml $min.^{-1}$, and fractions of 1.5 ml were collected. Gradient: 0% (5 ml); 0-8% (20 ml); 8-20% (40 ml); 20-100% (5 ml); 100% (5 ml); 100-0% (5 ml). The fractions (15-18, 15-20 ml), which contained component E without any β-methylaspartase activity, were concentrated to 2 ml. Potassium phosphate buffer (50 mM, pH 7.4, 5 ml) was added, the volume reduced again to 1 ml. The resulting protein solution was assayed (4 units $ml^{-1}$) and used for kinetic experiments and incubations. The half-life of this preparation at -25 °C is ~20 days.

To obtain a completely homogenous sample of component E, the fractions from two consecutive runs were pooled, concentrated and immediately reapplied on the FPLC system. Gradient: 0% (5 ml); 0-20% (60 ml); 20-100% (5 ml); 100% (5 ml); 100-0% (5 ml). Fractions containing the pure monomer form of component E (18-22) were concentrated (1 ml) and stored at -80 °C. Typically the activities obtained were 1.5-2 units $ml^{-1}$

**Step 6: FPLC for Component S**

An aliquot from Step 5 was thawed, diluted with water to 2 ml, brought to 800 mM ammonium sulphate, filtered and applied to a TSK Phenyl-5PW hydrophobic interaction column, which had been equilibrated with 1 M ammonium sulphate in 10 mM potassium phosphate pH 7.6. The protein was eluted with a gradient of 1 -0 M $(NH_4)_2SO_4$ (1 ml $min.^{-1}$) and fractions of 1.5 ml were collected. Gradient: 100% (10 ml); 100-0% (60 ml); 0 (10 ml); The fractions containing component S activity.(48-52), were concentrated by ultrafiltration (2 ml), potassium phosphate buffer (50 mM, pH 7.0, 5 ml) was added, and the volume reduced to afford a protein solution that contained ~10 units $ml^{-1}$ (1 unit $mg^{-1}$) of component S activity. The half-life of this preparation at -25 °C was ~30 days. Completely homogeneous protein was obtained with re-application of the above solution on the column.

**C) Experiments with Glutamate Mutase**

**Assay Buffers**

1 M Tris-Cl , pH 8.3

0.5 M Sodium (2S)-glutamate, pH 8.3

0.5 M KCl, 0.1 M $MgCl_2$

0.3 M β-mercaptoethanol

3 mM AdoCbl

Solution of β-methylaspartase 500 units $ml^{-1}$

2-Mercaptoethanol and glutamate solutions were prepared always fresh. The rest were stocked at 4 °C, and fresh aliquots used every 4-5 days.

**Procedure**

In a 1 ml quartz cell (10 mm path) were added the following reagents: 50 μl Tris buffer, 10 μl (2S)-glutamate solution, 10 μl KCl, $MgCl_2$, and 10 μl 2-mercaptoethanol. The solution was incubated at 37 °C with the appropriate amount of component S (10-50 μl, depending on concentration and purpose), together with a precalculated volume of water. The solution was usually equilibrated for 7-10 minutes to allow component S to be fully reduced. Component E (10-50 μl,

depending again on concentration and purpose) was added. 30 Seconds later the reaction was initiated by adding 5 μl of coenzyme solution (total volume of the assay solution 1 ml). The absorption at 240 nm was followed at 37 °C for 2-3 min. One unit of mutase activity causes a 3.85 change in absorbance units per minute at 240 nm, corresponding to 1 μmol of mesaconate per minute.

In order to obtain reproducible results it was necessary to degas the water used to dilute the reagents. For the early stages of the purification the reduction of component S seems to be not reproducible, resulting in the problems discussed previously (see § 4.7).

**Inhibition Buffers**

All the inhibitors were prepared as 40 mM solutions at pH 8.2 (sodium hydroxide was used to adjust pH when necessary). The solution was then filtered through 2 μm filter, and stored at 4 °C. Appropriate volume of the inhibitor solution was used to obtain concentrations ranging from 0.5 mM (12.5 μl) to 20 mM (500 μl).

**Inhibition studies**

The inhibition studies were performed under the same conditions as the assay described above. The appropriate amount of inhibitor solution was incubated with component S and the precalculated volume of water (total volume 1 ml). The amount of protein used was typically ~0.02 units. Two or three assay were always performed initially to establish the range of concentrations to be used and the amount of time necessary to fully reduce the specific batch of component S, since there obviously were different amounts of the oxidised form in every preparation.

In the case of the 1-bromo-cyclopropane-1,2-diacids (the molecules absorb at 240 nm), 5 mm path cells (1 ml) were used. The assays performed for 1-bromo-cyclopropane-*trans*-1,2-dicarboxylic were followed for a longer period of time, usually 10-12 min. The compounds not identified as inhibitors were incubated with the enzyme with concentrations up to 20 mM (substrate concentration 1 or 0.5 mM).

The values of the rate used for kinetic analysis were always corrected (divided by the units of the enzyme present in the assay solution).

**Incubation Studies - General method**

The possible substrate (2-3 mg, 1-2 x $10^{-5}$ mol) the appropriate volume of water, and potassium phosphate buffer (50 μl, 1 M, pH 8.3) were added in small (5 ml) pyrex test tube and sealed. The solution (~1.5 ml) was flushed with nitrogen through a syringe for 5 min. 2-Mercaptoethanol (20 μl, 0.3 M), and component S (0.3-0.8 units) was added and the resulting solution was incubated at 37 °C for 10 min. Component E (0.3-0.8 units), and coenzyme (20 μl) were added last. A typical assay was always performed using 20 μl of the resulting incubation solution to verify the presence of the expected activity. Incubation times ranged from 8-15 hr. The solvents were removed *in vacuo* and the residue analysed by $^{1}$H-NMR spectroscopy.

**Incubations Containing Labelled Compounds**

A typical incubation containing labelled 3-alkylaspartic acids (see p. 97) proceeded as follows: A mixture of [3'-$^{2}H_3$]-(2S,3S)-3-methylaspartic acid **(2a)** (2 mg, 1.36 x $10^{-5}$ mol) and 3-(2S,3S)-ethylaspartic acid **(82)** (5 mg, 3 x $10^{-5}$ mol) together with the appropriate volume of water, and potassium phosphate buffer (50 μl, 1 M, pH 8.3) were sealed in small (5 ml) pyrex test tube. The solution (~1.5 ml) was flushed with nitrogen through a syringe for 5 min. 2-Mercaptoethanol (20 μl, 0.3 M), and component S (0.3-0.8 units) were added and the resulting solution was incubated at 37 °C for 10 min. Component E (0.3-0.8 units), and coenzyme (20 μl) were added last. Incubation times ranged from 8-15 hr. The solvents were removed *in vacuo* and the residue was analysed by $^{1}$H-NMR spectroscopy (see Fig. 3.12, p. 98). The above experiment was performed using different ratios of the two alkylaspartates.

Other 3-alkylaspartates mixtures that were treated with glutamate mutase in a similar manner are listed below.

i) (2S,3S)-3-methylaspartic acid **(2)** / [3',4'-$^{2}H_5$]-(2S,3S)-3-ethylaspartate **(82).**

ii) (2S,3S)-3-methylaspartic acid **(2)** / [4'-$^{2}H_3$]-(2S,3S)-3-ethylaspartate **(82c).**

iii) (2S,3S)-3-methylaspartic acid **(2)** / [3'-$^{2}H_2$]-(2S,3S)-3-ethylaspartate **(82d).**

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