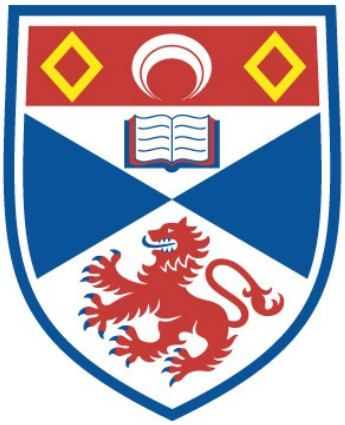
MECHANISTIC STUDIES ON GLUTAMATE DECARBOXYLASE AND SERINE HYDROXMETHYLTRANSFERASE

Janet Elizabeth Rose

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



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MECHANISTIC STUDIES ON GLUTAMATE DECARBOXYLASE AND SERINE HYDROXMETHYLTRANSFERASE

a thesis presented by

Janet Elizabeth Rose

to the

UNIVERSITY OF ST. ANDREWS

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St. Andrews

September 1992

DECLARATION

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

Signed .... Date 18th September 1992

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

Signed . ..... Date 18th September 1992

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

Signature of supervisor

Date ..... 18 Sept 1992

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TO

MY PARENTS AND RICHARD

Acknowledgements

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Finally I must thank Richard for his never ending support, for coming to St. Andrews with me, and for completing the last year of his physics Ph.D in a room surrounded by organic chemists.

ABSTRACT

(2S)- and (2R)-Serine O-sulphate have been synthesised and shown to inactivate glutamate decarboxylase (GAD) from *E. Coli.* Novel methodology was developed to enable the stereospecific synthesis of (2S) and (2R)-α-deuteriated serine in order to probe the mechanism of inactivation. The rates of inactivation of glutamate decarboxylase by (2S)-, (2S)-[2-^{2}H]-, (2R)- and (2R)-[2-^{2}H]-serine O-sulphate have been measured for each of the isotopomers at a range of concentrations. From the data obtained the deuterium isotope effects were determined for each enantiomer. The inactivation by the (2S)-enantiomer was shown to involve C^{α}-H bond cleavage while inactivation by the (2R)-isomer involves C^{α}-decarboxylation. Both processes were shown to occur on the 4'-*re*-face of the coenzyme, the opposite face to that utilised in the physiological decarboxylation reaction.

The methodology developed for the synthesis of the deuteriated serines involved the regiospecific introduction of deuterium to the C-6 centre of (3R)- and (3S)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine. Schollkopf chemistry was then exploited for the stereospecific alkylation at C-6 of the dihydropyrazines. This chemistry was versatile and enabled the synthesis of other α-deuteriated α-amino acids. For example (2S)-[2-^{2}H]-phenylalanine, (2S)-[2-^{2}H]-allylglycine and (2S)-[2-^{2}H]-aspartic acid were synthesised using this chemistry.

The decarboxylation of 2-aminomalonic acid by cytosolic serine hydroxymethyltransferase (SHMT) was studied. Contrary to previous reports, the reaction was found to be stereospecific and the newly introduced hydrogen was shown to occupy the 2-*pro*-S position of the glycine product.

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Abbreviation	Meaning
AAT	aspartate aminotransferase
c. AAT	cytosolic aspartate aminotransferase
m. AAT	mitochondrial aspartate aminotransferase
AET	aminoethylisothiuronium bromide
BOC	benzyloxycarbonyl
n-BuLi	*normal*-butyllithium
DDC	dopa decarboxylase
DOPA	dihydrophenylalanine
DMSO	dimethylsulphoxide
E2	bimolecular elimination
EC	Enzyme Catalogue
E. coli	Escherichia coli
e.e.	enantiomeric excess
FH_4	tetrahydrofolate
5,10-CH_2FH_4	5,10-methylenetetrahydrofolate
5-methyl-FH_4	5-methyltetrahydrofolate
5-hydroxymethyl-FH_4	5-hydroxymethyltetrahydrofolate
F-MOC	9-fluorenylmethyloxycarbonyl
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
GAD	glutamate decarboxylase
HDC	histidine decarboxylase
I.E.P.	Isoelectric point
k_{app}	apparent first order rate constant
K_M	Michaelis constant

K_i	enzymic inhibition constant
k_{cat}	enzymic catalytic constant/turnover no.
k_{inact}	catalytic constant for inhibition
$k_{inact(H)}/k_{inact(D)}$	ratio of k_{inact}'s for protiated compound and deuteriated compound
K_H/K_D	ratio of inhibition constants for protiated compound and deuteriated compound
M_r	relative molecular mass
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NaTMS	3-(trimethylsilyl)propionic-2,2,3,3-$^2H_4$ sodium salt
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
PO-	phosphate
PQQ	pyrroloquinone quinoline
SAM	S-adenosylmethionine
SDM	site-directed mutagenisis
SDS	sodium dodecylsulphate
SHMT	serine hydroxymethyltransferase
TBDMSCl	^{t}Butyldimethylsilylchloride

THF	tetrahydrofuran
tlc	thin layer chromatography
TMS	tetramethylsilane
V_{max}	maximum rate of substrate turnover at saturation
v	enzymic reaction rate
v_{inact}	inactivation rate
V/K	first order rate constant for enzymic reaction at low substrate concentration
^{D}V	V_H/V_D
$^{D}(V/K)$	$(V_H/V_D)/(K_H/K_D)$
uv	ultra violet

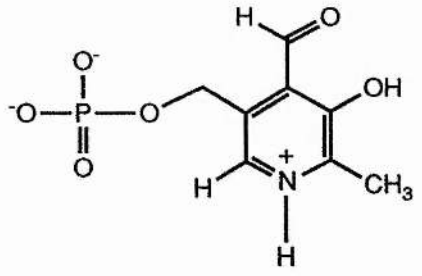
Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr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

CHAPTER ONE

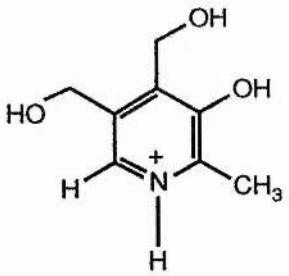
INTRODUCTION TO PYRIDOXAL 5'-PHOSPHATE ENZYMES

1.0 Pyridoxal 5'-phosphate dependent enzymes

Enzymes requiring pyridoxal 5'-phosphate (PLP) (**1**) as a coenzyme are ubiquitous in nature, and have been isolated from bacterial and mammalian sources. PLP, the biologically active form of vitamin B_6 (pyridoxol) (**2**), serves as a cofactor for a myriad of enzyme catalysed reactions, many of which are essential for amino acid metabolism. In the mid 1940's, Snell[1] identified PLP as the coenzyme for transaminases, and since then PLP has been established as the coenzyme for over 20 different types of enzyme catalysed reactions including decarboxylases.



(**1**)

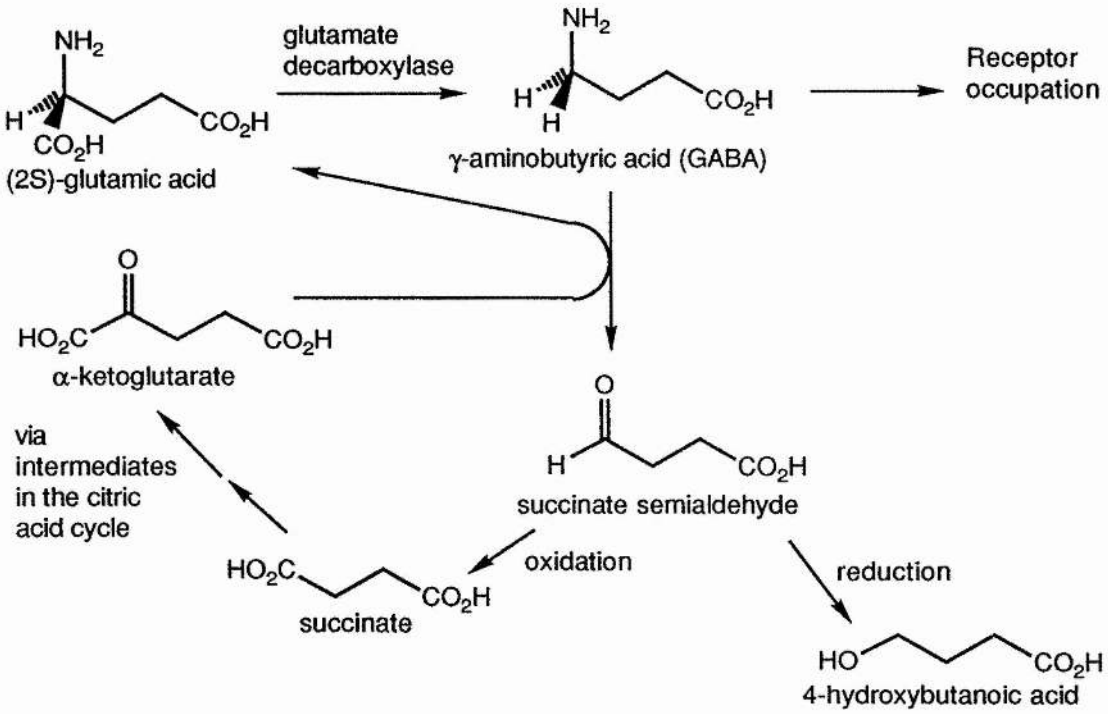


(**2**)

1.1 The metabolic role of PLP dependent enzymes

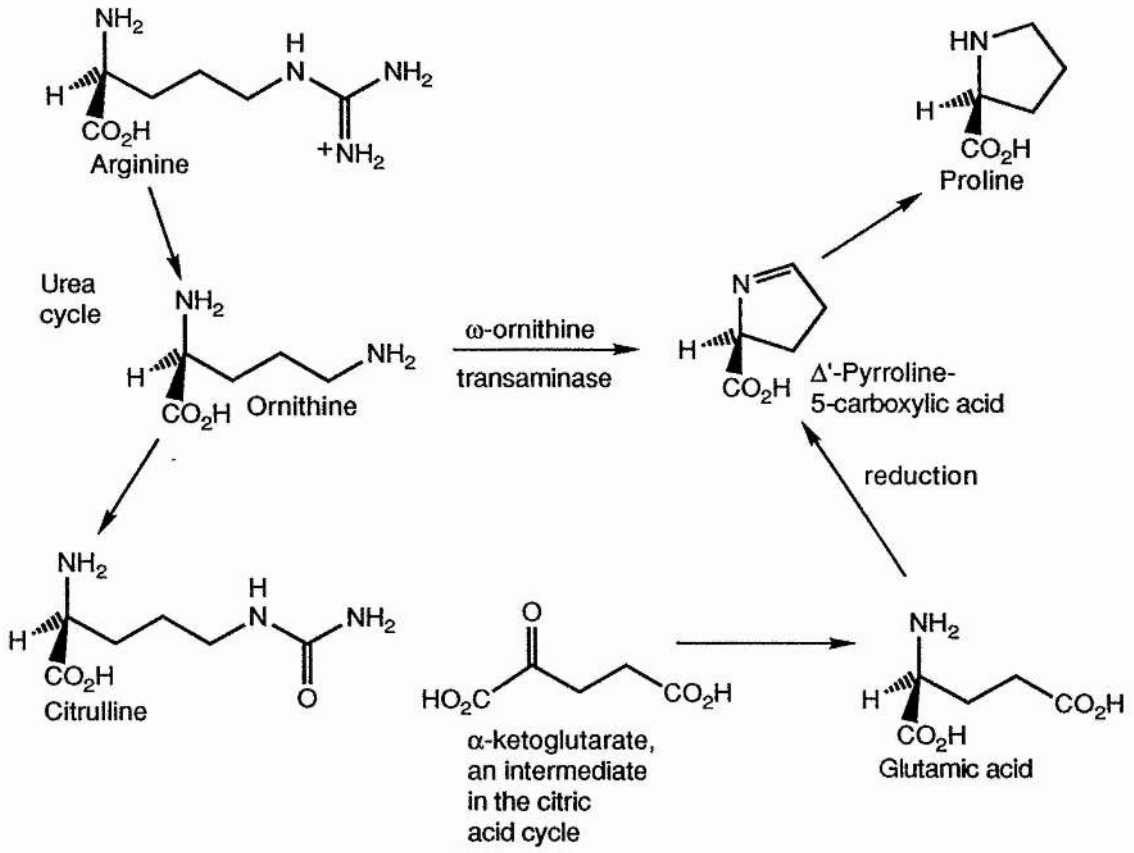
The mechanisms of action of the PLP dependent enzymes are of great scientific interest. The PLP dependent enzymes play a major role in the regulation of physiological processes. Some of the more important processes are detailed below.

Two important transaminases are γ-aminobutyric acid transaminase (GABA-T) and ω-ornithine transaminase. GABA-T is a key enzyme involved in the regulation of the GABA-ergic system (Scheme 1.1) in the mammalian central nervous system.



Scheme 1.1. The GABA-ergic system.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter and there is much evidence to suggest that high cerebral concentrations prevent convulsions.[2] ω-Ornithine transaminase not only interconnects the citric acid cycle and the urea cycle but also couples ornithine to proline metabolism (Scheme 1.2).

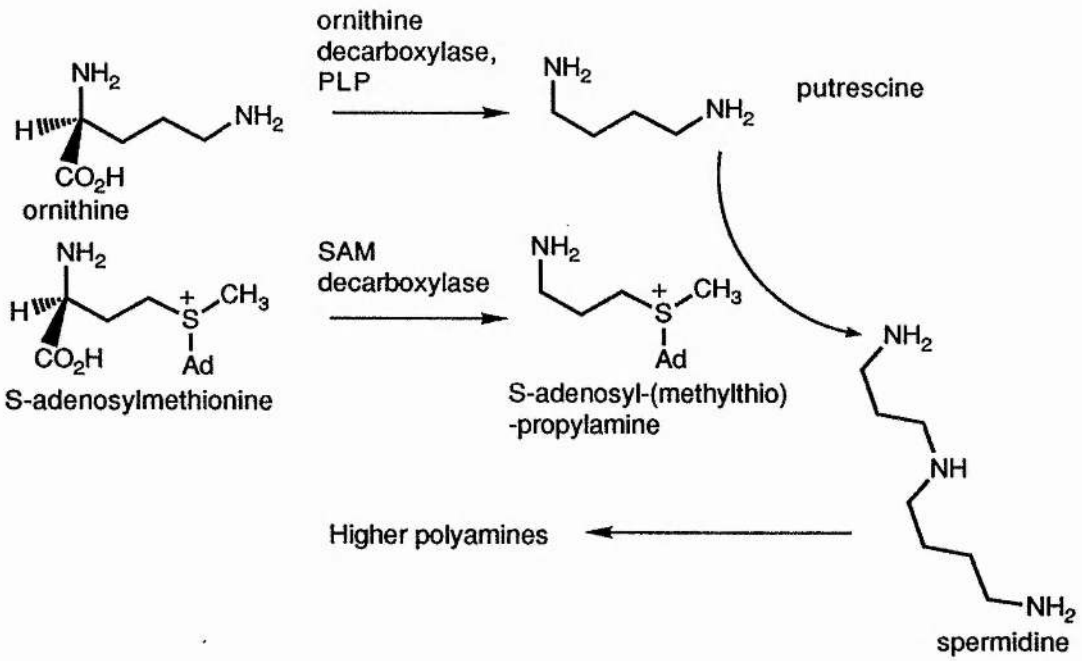


Scheme 1.2. The metabolic significance of ornithine transaminase.

The decarboxylases are one of the most important classes of PLP dependent enzymes. The physiological role of the amino acid decarboxylases in bacteria is not fully understood, but inducible decarboxylases for glutamate, arginine and lysine have been purified to homogeneity.[3] One of the most important physiological roles of the decarboxylases in mammals is the conversion of amino acids to pharmacologically active amines. The formation of dopamine, histamine, GABA, and putrescine all depend on PLP-dependent decarboxylation of their respective parent amino acids. The biosynthesis of the higher polyamines, spermine and spermidine, from putrescine involves the pyruvate-dependent decarboxylation of S-adenosylmethionine (SAM) (Scheme 1.3).

Mammalian brain glutamate decarboxylase is directly responsible for the biosynthesis

of GABA in the GABA-ergic system (Scheme 1.1). Knowledge of the mechanism of action will enable the synthesis of inhibitors to control the level of GABA in the brain. Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the biosynthetic pathway leading to putrescine and higher polyamines (Scheme 1.3).[4] The activity of the enzyme *in vivo* increases dramatically in response to cellular stimulation which promotes regeneration and replication. Cellular levels of the enzyme are high during growth, and low at other stages.[5] The association of high polyamine levels with rapid cellular proliferation, and protein biosynthesis, led to the idea that polyamines may be required for RNA/DNA biosynthesis.[6] The enzyme has been identified as a target for cancer chemotherapy.[4]

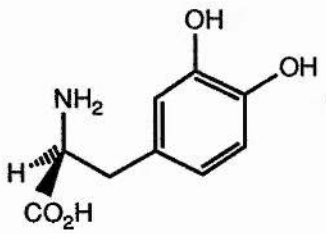


Scheme 1.3. The synthesis of higher polyamines.

Histidine decarboxylase (HDC) catalyses the decarboxylation of histidine to give histamine. In mammals, histidine is important as the major receptor agonist.[7] Overproduction of histamine is associated with many biological responses including gastric secretion, allergic and hypersensitivity reactions.[3] The development of

antihistamines, antagonists for the receptors, has been an important area in medicinal chemistry. Histamine is also active as a regulator of peripheral blood circulation.[3]

The aromatic amino acid decarboxylases catalyse the formation of many pharmacologically important amines. For example, DOPA decarboxylase (DDC) is able to convert many aromatic substrates to the corresponding amines including, phenylalanine to phenyethylamine, tyrosine to tyramine, tryptophan to tryptamine, histidine to histamine, 3,4-dihydroxyphenylalanine (**3**) to 3,4-dihydroxy-phenylethylamine (dopamine) and 5-hydroxytryptophan (**4**) to serotonin.[8] Dopamine and serotonin act on smooth muscles and the cardiovascular system. Dopamine is a precursor in the biosynthesis of adrenaline, and serotonin is itself neuroactive.[3]



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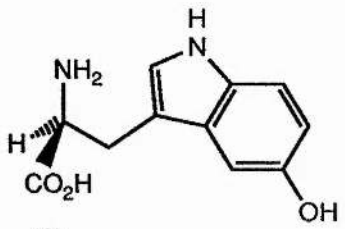
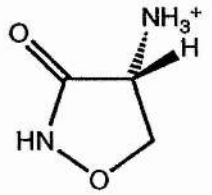


(**4**)

Serine hydroxymethyltransferase (SHMT) catalyses the formation of 5,10-methylenetetrahydrofolic acid and glycine from tetrahydrofolic acid and (2S)-serine. The enzyme is a key enzyme in single carbon (C_1) metabolism at all oxidation levels and will be discussed further in Chapter 3.

Another important class of PLP dependent enzymes, the racemases, catalyse the interconversion of (2R)- and (2S)-amino acids. These enzymes are common in prokaryotes and several are involved in the biosynthesis of peptidoglycan in bacterial cell walls.[9] Alanine racemase is an important enzyme in peptidoglycan biosynthesis

and as such the enzyme is a target for the action of many antibacterial agents,[10] including (2R)-*cyclo*serine (**5**),[11] O-carbamyl-(2R)-serine (**6**)[11] and phosphoalanine (**7**).[10]



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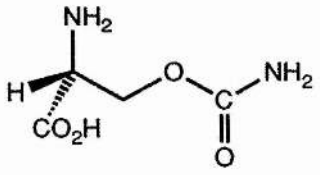

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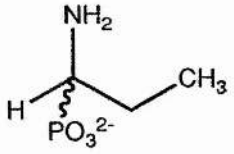


(7)

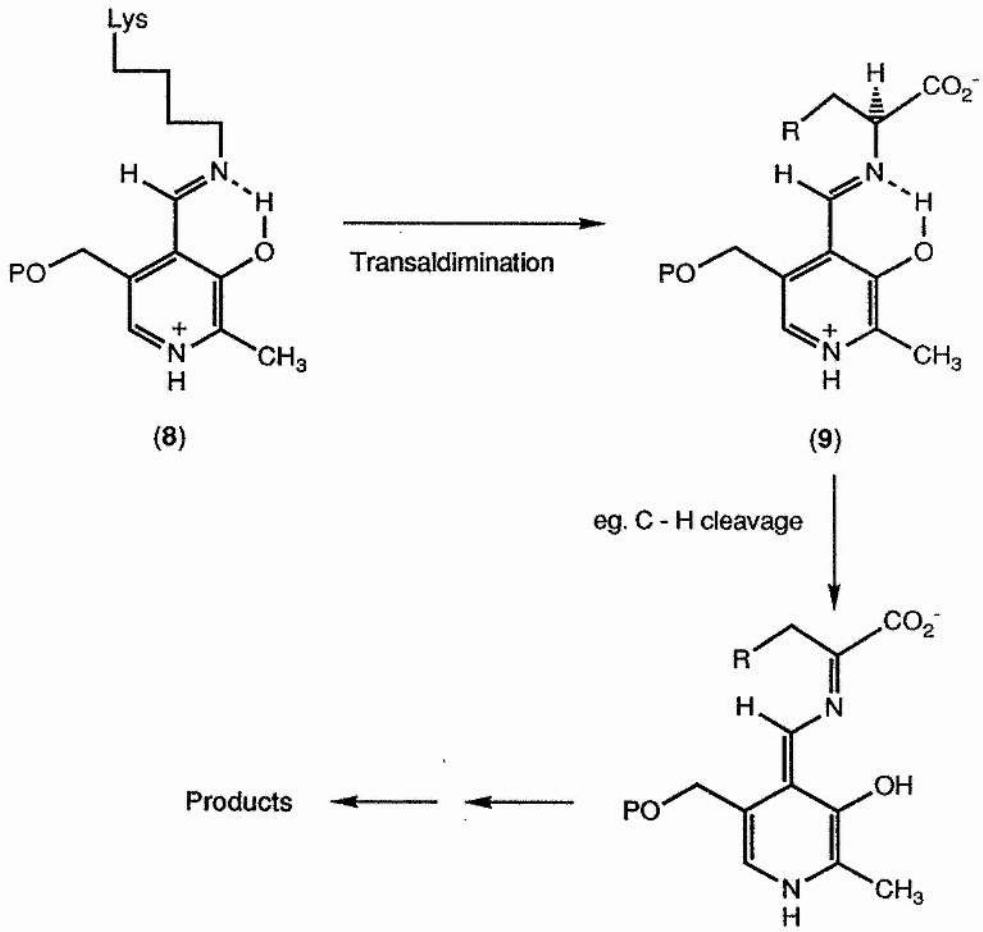
1.2 Structure and mechanism of PLP dependent enzymes

The coenzyme, PLP, is bound at the enzyme active site as a Schiff's base complex with the ε-amino group of an active site lysine residue (Scheme 1.4, **8**).[12-14] The nature of the coenzyme binding at the active site has been shown by chemical and X-ray crystallographic studies. Introduction of the substrate leads to a transaldimination reaction which frees the active-site lysine residue and leads to the formation of another Schiff's base (Scheme 1.4, (**9**)). When the Schiff's base is bound at an enzyme active site it can undergo a range of reactions as a result of labilisation of the C^{α} bonds.

For some enzymic systems the transaldimination reaction causes a large conformational change. After the formation of the substrate aldimine (**9**) specific cleavage of one of the C^{α} bonds of the amino acid occurs.

Dunathan[15] proposed that the bond to be cleaved is orientated perpendicularly to the plane of the conjugated pyridinium ring system by the apoenzyme, so that the maximal orbital overlap is achieved between the developing negative charge at C^{α} and the

extended π-electron system.

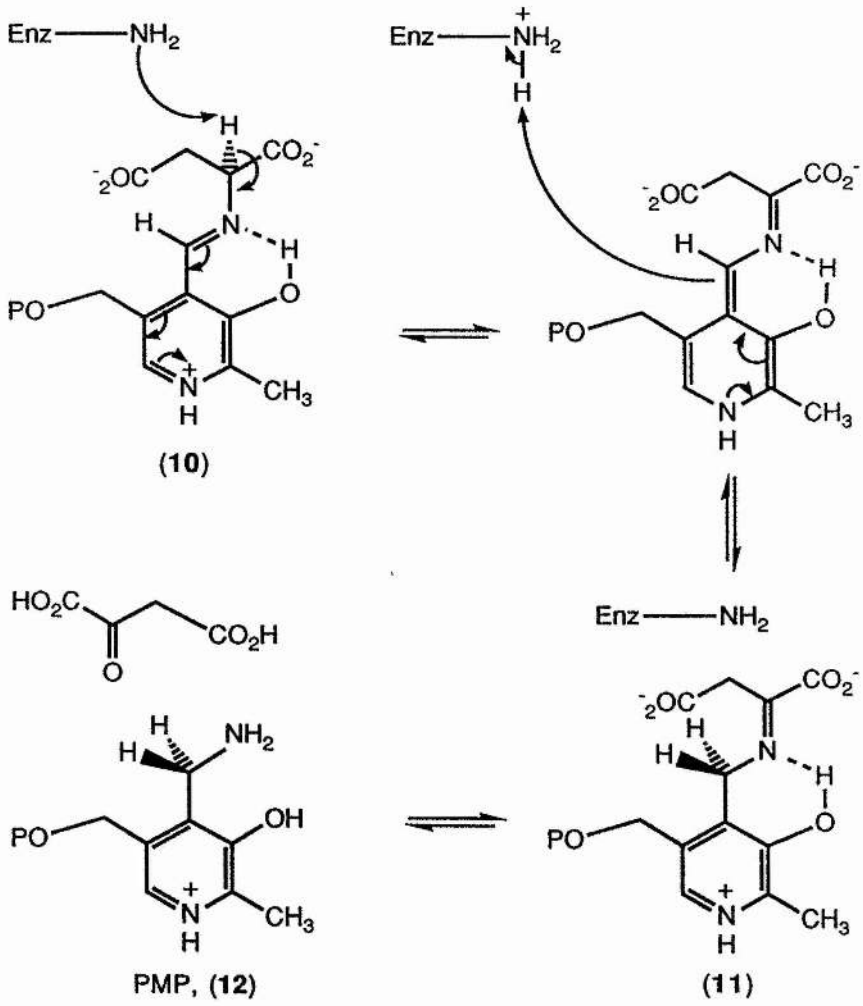


(8) (9)

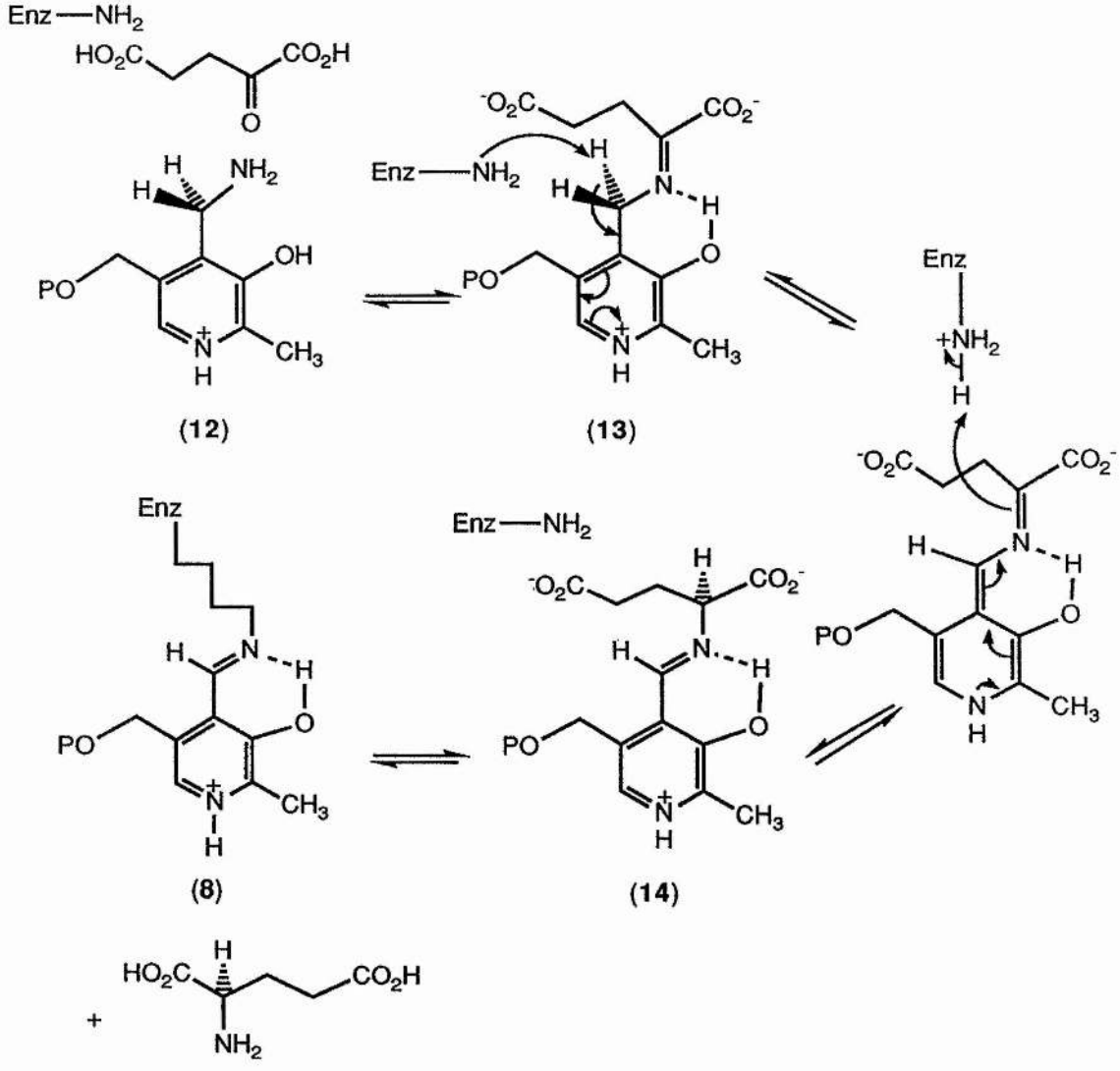
Scheme 1.4. PLP and substrate at the enzyme active site.

1.2.1 Transamination

Transaminases (or aminotransferases) are the best studied of the PLP-dependent enzymes. They catalyse the reversible interconversion of amino acids and α-ketoacids using the coenzyme as an ammonia carrier. In the first half-reaction the C^{α} proton of the aldimine (**10**) is transferred to the ketimine (**11**) on the C-4'-*si*-face of the coenzyme giving pyridoxamine 5'-phosphate (PMP) (**12**) and an α-ketoacid (Scheme 1.5a). When a new α-ketoacid is added to the solution it condenses with the PMP (**12**) to give a second ketimine (**13**). 1,3-suprafacial proton transfer from C-4' to C^{α} on the 4'-*si*-face of the coenzyme gives the aldimine (**14**) which undergoes transaldimination to yield a new (2S)-amino acid and the internal PLP-aldimine (**8**) (Scheme 1.5b).



Scheme 1.5a. The first half-reaction catalysed by aspartate aminotransferase.



Scheme 1.5b. The second half-reaction catalysed by aspartate aminotransferase.

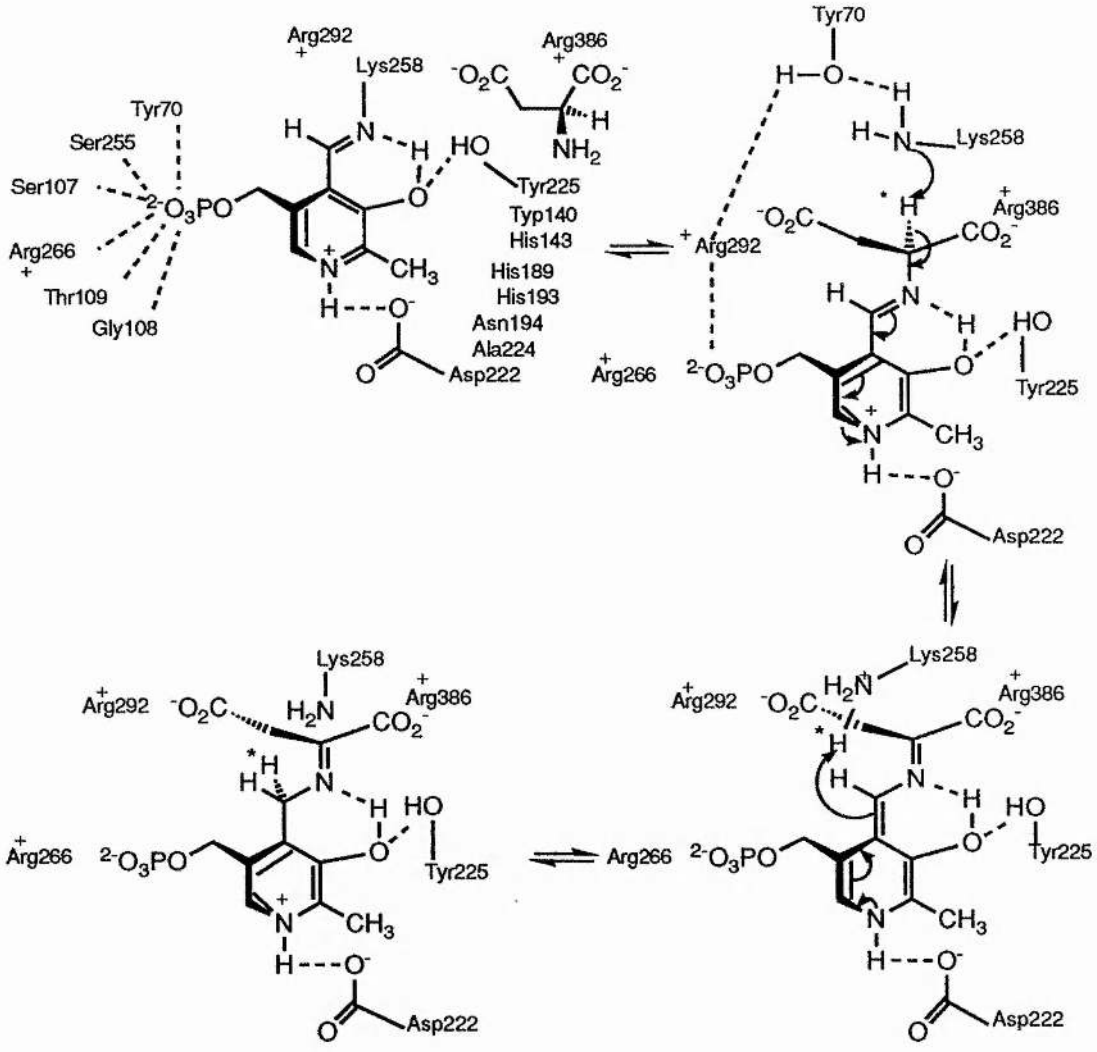
Of all the PLP-dependent enzymes, aspartate aminotransferase (AAT) has been studied the most intensely.[16-19] X-ray structures of the active holoenzyme-substrate complex have been published for chicken mitochondrial AAT,[20] pig cytosolic AAT,[21] and chicken heart cytosolic AAT[22] at 2.8Å. More recently a crystal structure of *E. coli* AAT has been elucidated,[23] and the overall structure was found to be similar to that of higher animals.

Arigoni and Besmer,[13] and Dunathan *et al.*[14,24] have independently studied the aminotransferase reaction. It was discovered that pyridoxal was a coenzyme for the reaction, but that it did not bind to the apoenzyme as tightly as PLP. Both groups of researchers demonstrated that the removal of the proton from C-4' occurs from the 4'-*si*-face of the pyridoxamine. For the transamination catalysed by pyridoxamine pyruvate transaminase it was shown that direct internal hydrogen transfer occurred between C^{α} of (2S)-alanine and C-4' of the coenzyme suggesting that the protonation and deprotonation are suprafacial processes mediated by a single base.

X-ray crystallographic studies of mitochondrial chicken heart AAT has allowed the identification of the active-site residues that are important for binding PLP and the substrate (Scheme 1.6). The protonated nitrogen of the pyridinium ring forms a hydrogen bond with Asp222, whilst the deprotonated phenolic hydroxyl is hydrogen bonded to Tyr225. The 2-methyl group lies in a pocket defined by eight amino acids. The 5'-phosphate ester is hydrogen bonded within a hydrophilic pocket to six amino acids, including Ser255 and Arg266. Arg266 neutralises the dianionic phosphate. The proximal and distal carboxylate groups of the physiological substrates are bound by Arg386 and Arg292 respectively. These studies have improved the understanding of the conformational changes which occur in the protein binding.[12]

The protein consists of two identical subunits each consisting of two domains. The coenzyme is bound to the larger domain and is situated in an open pocket near the subunit interface which is made up of residues from both subunits. The two carboxylate groups of the dicarboxylic acid substrates are bound to Arg386 and Arg292 from adjacent subunits, and the substrate specificity is determined by these interactions. The charge stabilisation provided by these interactions not only positions the substrate correctly for efficient catalysis but also shifts the conformational equilibrium to the closed form which brings Arg386 3Å closer to the coenzyme which is rigidly positioned in the smaller domain. Transaldimination of the ε-amino group of Lys258 by the α-amino group of the substrate to form the external aldimine is accompanied by a tilting of the coenzyme by ≈30°, Scheme 1.6. The released ε-amino group of Lys258 then serves as a proton donor/acceptor in the 1,3-prototropic shifts accompanying transamination. At this stage or after formation and hydrolysis of the initial ketimine the coenzyme rotates back by ≈20°. Throughout this process, the pyridinium ring is hydrogen bonded to the β-carboxylate group of Asp222.

Two genetically distinct isoenzymes exist in animal tissue, cytosolic (cAAT) and mitochondrial (mAAT).[25] The primary structures of a number of a number of AAT's have been completely or partly elucidated.[26] The amino acid sequence of mAAT's from chicken, pig, rat,[26] mouse,[27] horse,[28] and human[29] and of cAAT from chicken, pig,[26] mouse,[27] and horse[28] are known as well as those from *E. coli* B[30] and *E. coli* K12.[31] In general the mAAT's from different sources show ≈85% sequence homology.



Scheme 1.6. The active site of chicken mitochondrial aspartate aminotransferase.

Similar homology is also found between cAAT's. However the sequence homology between two isozymes of the same species is only about 45%.[32] It should be noted that in all the above examples there is almost 100% homology for the regions of the protein corresponding to substrate or coenzyme binding sites. The active enzyme, as stated above is a dimer of two identical sub-units (M_r 45 000 Daltons)[33] and contains two independent active sites.[34,35] A comparison of the amino acid sequence for selected regions of AAT isoenzymes[36] and the amino acid sequences for the Schiff's base forming region of several AATs[37] are shown in Tables 1.1. and 1.2.

Table 1.1. Comparison of the amino acid sequences for selected regions of AAT isozymes.

Isoenzyme Source	Amino acid residue number 70	108	140	190	222
	*	*	*	*	*
Human (mit.)	EYL	SGTG	TWGNH	LHACAHNPTG	FFDMAYQGF
Chicken (mit.)	EYL	SGTG	SWGNH	LHACAHNPTG	YFDMAYQGF
Pig (mit.)	EYL	SGTG	SWGNH	LHACAHNPTG	FFDMAYQGF
Rat (mit.)	EYL	SGTG	SGGNH	LHACAHNPTG	FFDMAYQGF
Chicken (cyt.)	EYL	GGTG	TWENH	LHACAHNPTG	FFDSAYQGF
Pig (cyt.)	EYL	GGTG	TWENH	LHACAHNPTG	FFDSAYQGF
E. coli	NYL	GGTG	SWPNH	FHGCCHNPTG	LFDFAYQGF

Isoenzyme Source	258	266	292	360	386
	*	*	*	*	*
Human (mit.)	QSYAKN	ERV	IRP	MFC	GRI
Chicken (mit.)	QSYAKN	ERA	IRP	MFC	GRI
Pig (mit.)	QSYAKN	ERV	IRP	MFC	GRI
Rat (mit.)	QSYAKN	ERV	IRP	MFC	GRI
Chicken (cyt.)	QSFSKN	ERV	VRT	MFS	GRI
Pig (cyt.)	QSFSKN	ERV	VRV	MFS	GRI
E. coli	SSYSKN	ERV	IRA	DFS	GRV

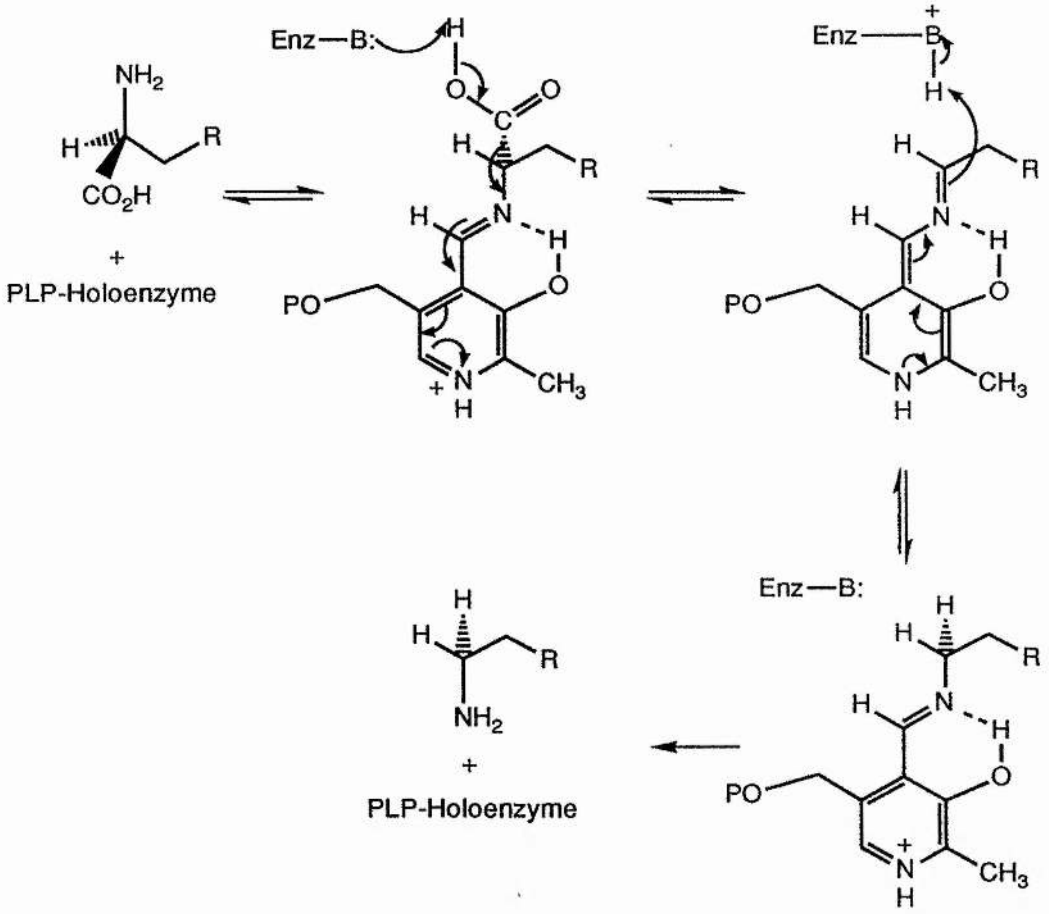
* Corresponds to the numbered residue, the residues are numbered according to the sequence for cytsolic pig AAT.

Table 1.2. The Schiff's base forming regions of several AAT isozymes.

Isoenzyme Source	Amino acid sequence
E. coli	I V A S S Y S **K** N F G L Y
Chicken (mitochondrial)	V L S Q S Y A **K** N M G L Y
Turkey (mitochondrial)	V L S Q S Y A **K** N M G L Y
Pig (mitochondrial)	C L C Q S Y A **K** N M G L Y
Rat (mitochondrial)	C L C Q S Y A **K** N M G L Y
Human (mitochondrial)	C L C Q S Y A **K** N M G L Y
Chicken (cytosolic)	F C A Q S F S **K** N F G L Y
Pig (cytosolic)	F C A Q S F S **K** N F G L Y

1.2.2 Decarboxylation

Specific α-decarboxylases are known for more than ten amino acids. For example glutamate, histidine, tyrosine, lysine, ornithine, arginine, methionine, valine, DOPA and phenylalanine decarboxylase. In each case the α-carboxyl group is cleaved to give CO_2 and an amine as the products (Scheme 1.7). All of the above decarboxylases require PLP as a coenzyme. (For the physiological roles of the decarboxylase enzymes see section 1.1).

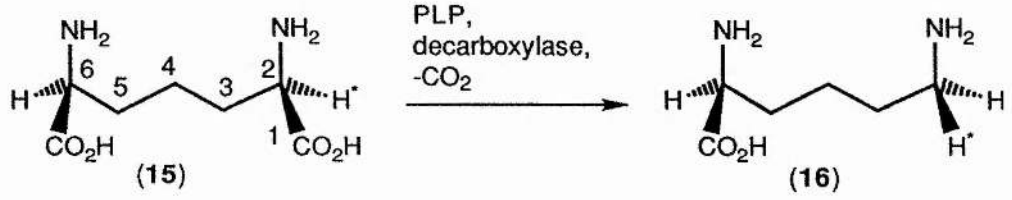


Scheme 1.7. Mechanism of decarboxylation.

However only one β-decarboxylase is known. Aspartate β-decarboxylase catalyses the α-decarboxylation of aminomalonic acid[38] and the β-decarboxylation of (2S)-aspartic acid to give (2S)-alanine and carbon dioxide.[39] Enzymes also exist which catalyse the condensation of an amino acid with an electrophile and subsequent decarboxylation of the product. Almost all of these enzymes require PLP as a coenzyme. Two exceptions are *Lactobacillus* histidine decarboxylase[40,41] and *E. coli* S-adenosylmethionine decarboxylase,[5,42] as these require pyruvate as a prosthetic group.

All of the α-amino acid decarboxylases studied to date, except two, show retention of stereochemistry at C^{α}. These include glutamate,[43-46] lysine,[47-49] tryptophan,[50,51] tyrosine,[52] phenylalanine,[53] histidine,[54] methyldopa,[55] α-methylglutamic acid,[45]

ornithine,[48,56-59] and arginine.[48,56,59] The two decarboxylases which show inversion of configuration are wheat[60] and bacterial[61] *meso*-α,ω-diaminopimelic acid decarboxylases which decarboxylate at the (R)-amino acid centre of the substrate (**15**) to give (2S)-lysine (**16**) with inversion of stereochemistry at C^{α}, Scheme 1.8.



Scheme 1.8. The decarboxylation of α,ω-diaminopimelic acid (**15**).

Meso-α,ω-diaminopimelic acid decarboxylase is the only known α-amino acid decarboxylase which acts on a D-amino acid. However only the (2R,6S)-isomer is a substrate for the enzyme from *Bacillus sphaericus*, the (2R,6R)- and (2S,6S)-isomers are not substrates or inhibitors.[62] There is evidence that the conformation of the coenzyme-substrate complex in L-specific decarboxylases is controlled by binding of distal groups in the extended side chain of the substrates.[63] For the decarboxylases studied it has been shown that PLP binds to the protein in the presence of the substrate with the C-4'-*si*-face of the coenzyme exposed to the solvent.[12]

It was reported in the literature[64] that aminomalonate decarboxylase [an activity of serine hydroxymethyltransferase (SHMT)] catalysed the non-stereospecific decarboxylation of aminomalonate. We have shown[65] that the glycine formed in the earlier experiments[64] was racemic, however the racemic nature of the glycine was not due the enzyme catalysed reaction but due to a non-enzymic exchange of the α-hydrogen which occurred prior to the decarboxylation reaction (see Chapter 3 for more details).

In addition to the α-decarboxylation of aminomalonic acid[38] and the β-decarboxylation of (2S)-aspartic acid,[39] the enzyme aspartate β-decarboxylase also catalyses the desulphination of (2S)-cysteine sulphinate, β-elimination of (2S)-3-chloroalanine, and a variety of transaminations between several (2S)-amino acids and α-ketoglutarate, pyruvate and oxaloacetate.[66] The decarboxylation of aminomalonate occurs with retention of configuration.[38,67] However the stereochemical course of β-decarboxylation of (2S)-aspartate occurred with inversion of configuration at C-3.[68] A single base mechanism seems unlikely due to the fact that when the enzyme was incubated with C^{α}-tritiated (2S)-aspartic acid 17% of the ^{3}H from the α position of aspartate was incorporated at the C-4' *pro*-S position of the PMP, whereas less than 1% was found in the alanine. A two base mechanism[69] has now been proposed for the enzyme (Figure 1.1). The two base mechanism would explain the stereochemistry of the aminomalonate decarboxylation, and the unusual observation that aspartate-β-decarboxylase, modified by the use of N-methyl-PLP, can decarboxylate (2R)-aspartate at a significant rate to give (2S)-alanine.[70]

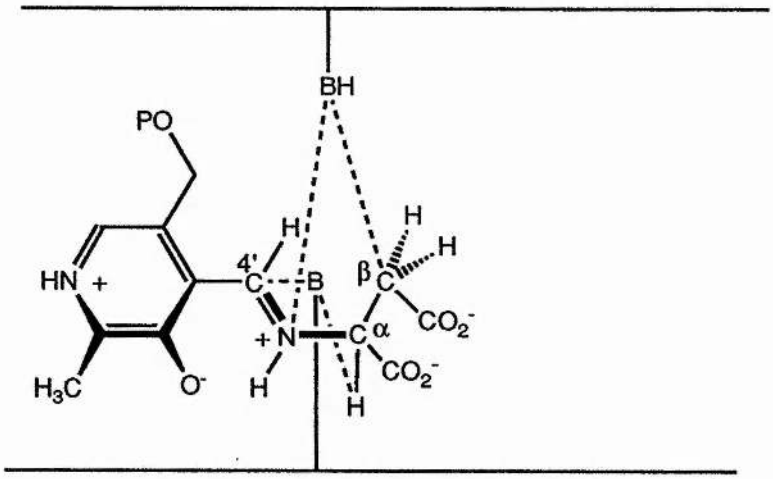


Figure 1.1. The two base mechanism for aspartate β-decarboxylase.

1.3 Mechanism-based inhibitors of PLP enzymes

Many mechanism-based or suicide inhibitors have been prepared and tested for specific time-dependent inhibition of PLP-dependent enzymes. There has been a wide interest in such inactivators as potential drug candidates, because of the pharmacological and therapeutic utility of inhibiting specific PLP enzymes (Table 1.3). The suicide inhibitors are substrate analogues and as such are useful tools for investigating the mechanism of specific enzymes because they act at the active site of the enzyme.

Table 1.3. Some therapeutically important target enzymes dependent on PLP.

Enzyme	Potential Effect
bacterial alanine racemase	antibacterial
brain GABA transaminase	antiepileptic
mammalian ornithine decarboxylase	antineoplastic
mammalian DOPA decarboxylase	antihypertensive

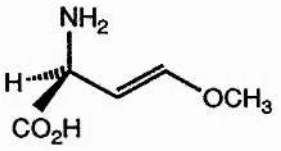
In general, enzymes that require PLP, catalyse some chemical change at the α-, β-, or γ-carbon of the common α- or γ-amino acids. In every case the role of the PLP moiety is to stabilise the carbanionic intermediates that develop during the catalytic process. The suicide inhibitors all have latent functional groups that become activated by their proximity to the site of an enzyme-generated carbanion. Such an intermediate can breakdown to yield a reactive species that may react with an active-site amino acid side chain, or with tightly bound PLP coenzyme. Each of these processes leads to the inactivation of the enzyme, in the first case the active site is blocked by an unreactive

species and in the second the coenzyme is no longer available to bind to the substrate.

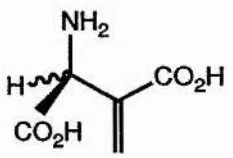
Mechanism-based inactivating functional groups in substrates that inhibit PLP enzymes include, acetylenic, olefinic, β-halo substituents and other leaving groups, nitriles, aryl sulphoxides, dihydroaromatics and phosphonoamino acids.[71] The most common functional groups used for suicide inhibitors, and the mechanisms of inactivation are presented in the next three sections.

1.3.1 Transaminase inhibitors

In the catalysis of transamination reactions, there are two half-reactions for each complete catalytic cycle (see Section 1.2). In the first half-reaction catalysis proceeds through the substrate-PLP anion and the product α-imino acid-PMP enzyme complex (Scheme 1.5a, (**11**)). Both of these intermediates are species allowing for activation of latent functional groups in suicide substrates. Activation by net oxidation to the ketoacid equivalent is observed in both olefinic and acetylenic amino acid analogues. The olefin analogues include vinyl glycine (**17**),[72,73] (E)-methoxy-vinyl glycine (**18**),[74] and β-methylene aspartate (**19**).[75] The generally accepted inactivation mechanism is as outlined in Scheme 1.9.

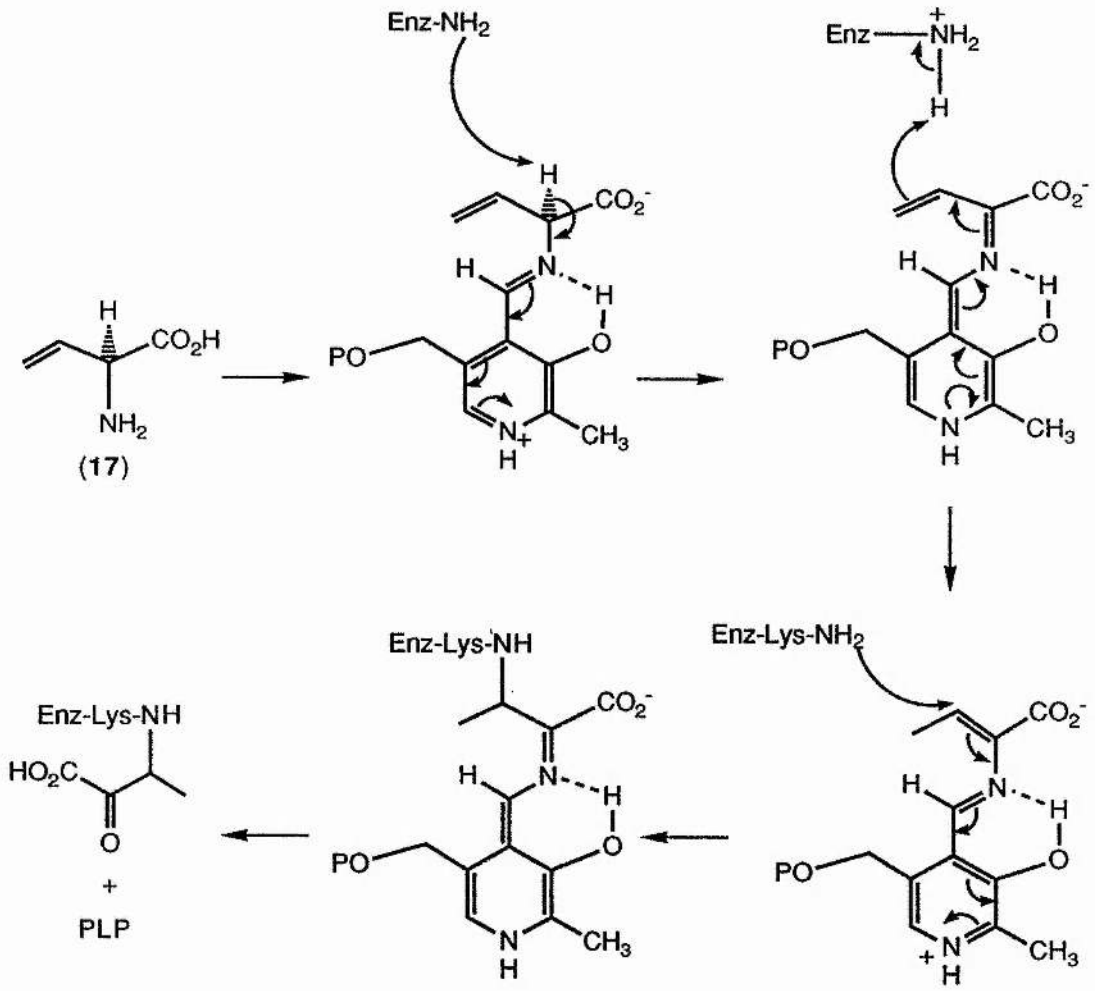


(**18**)

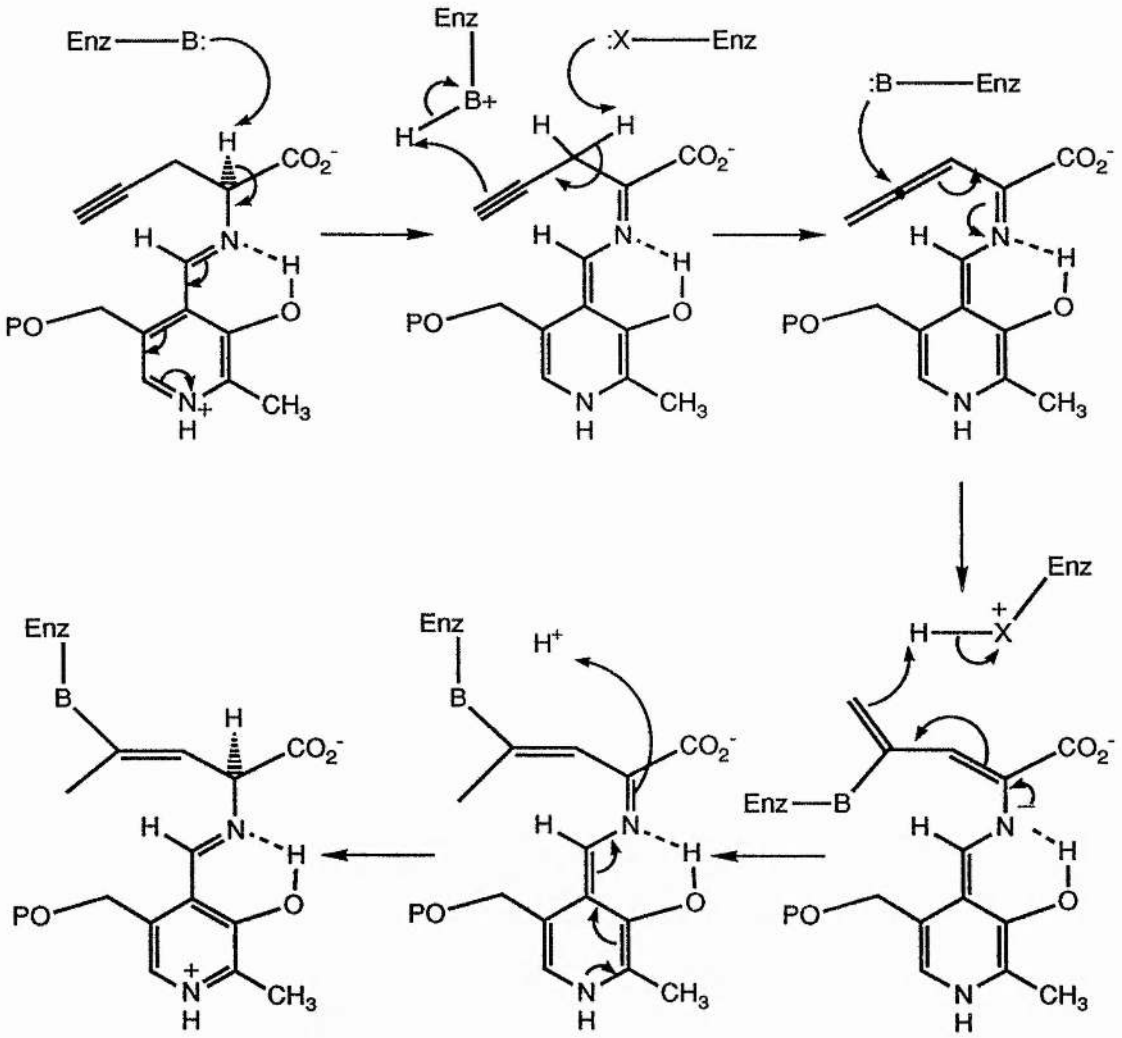


(**19**)

The acetylenic analogues include the natural product (2S)-propargyl glycine,[76] and the mechanism of inactivation is depicted in Scheme 1.10.

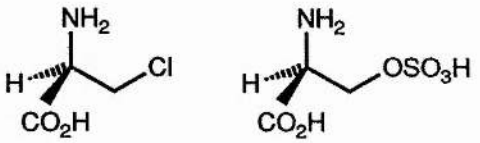


Scheme 1.9. The mechanism of inactivation of AAT by vinylglycine (**17**).



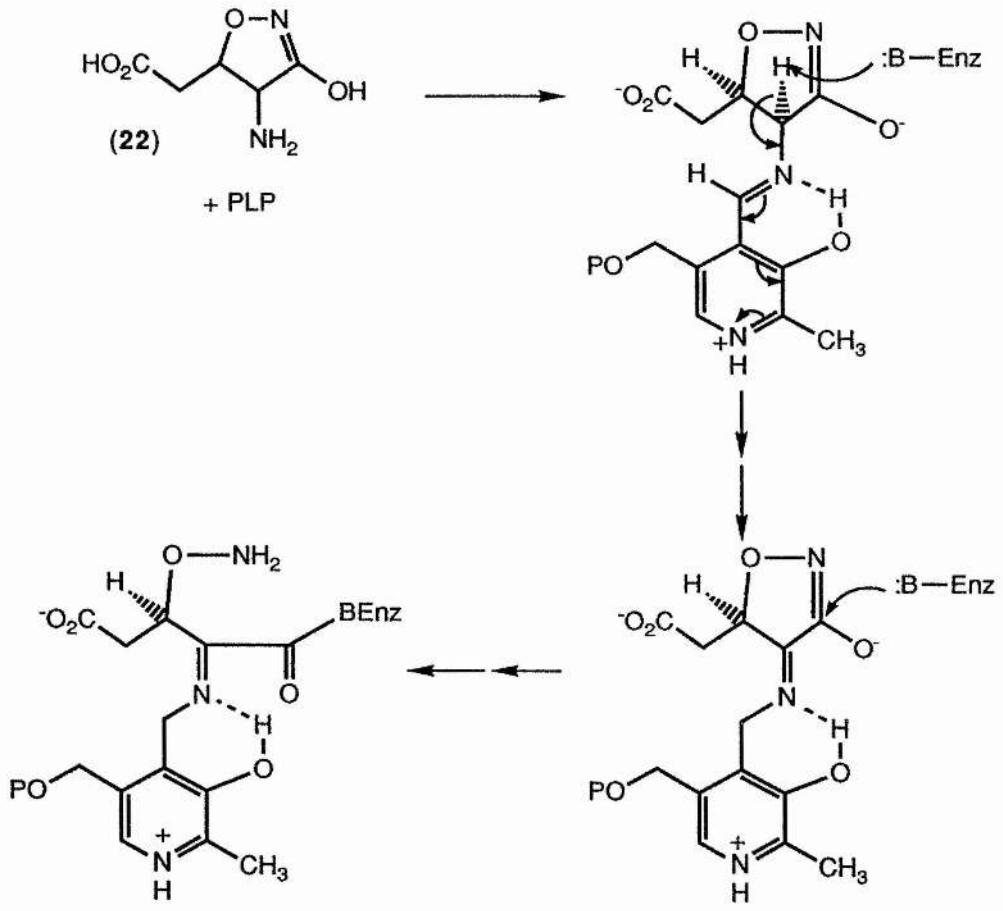
Scheme 1.10. Mechanism of inactivation of AAT by (2S)-propargylglycine.

Some of the more common types of inhibitor contain good leaving groups at C^{β}. These compounds are able to undergo facile elimination to generate olefinic intermediates. These include, 3-chloro-(2S)-alanine (**20**)[77,78] an inhibitor of alanine aminotransferase, and (2S)-serine O-sulphate (**21**)[79] which inhibits aspartate aminotransferase and glutamate decarboxylase (see Chapter 2 for more details).

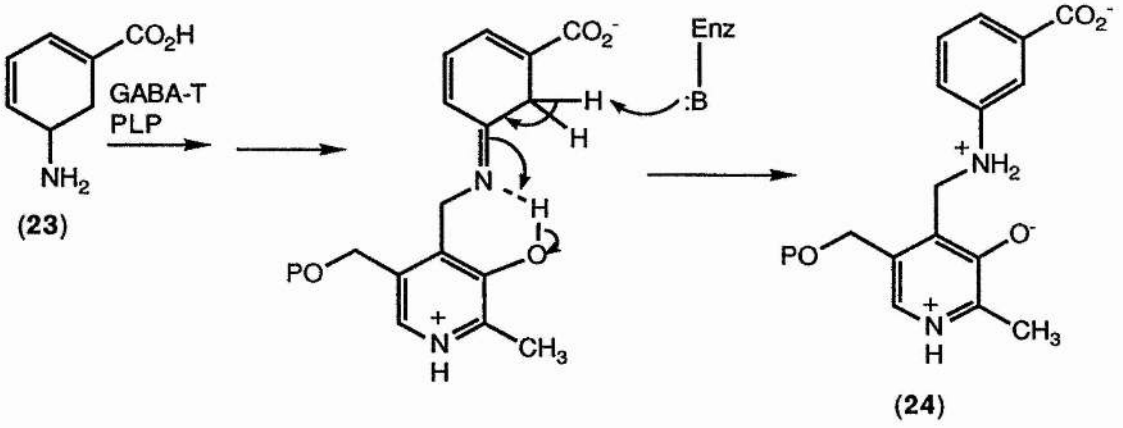


(20) **(21)**

The accepted mechanism had been that the killing species was the aminoacryl-PLP intermediate, which undergoes a putative Michael reaction with an enzymic nucleophile. Although a Michael reaction does occur with some enzymes (aspartate-β-decarboxylase, serine hydroxymethyl-transferase(SHMT)), Metzler and coworkers[80] have shown that the inactivation of aspartate aminotransferase is not due to the occurrence of a Michael reaction. Bright and coworkers[81] have used the nitro group of 3-nitroalanine as an inactivator of aspartate aminotransferase and alanine aminotransferase.[82] The nitroalanine undergoes α-proton abstraction followed by β-nitro elimination. Alanine aminotransferase is also inhibited by *cyclo*serine (**5**),[83] while aspartate aminotransferase is inhibited to varying extents by α- and γ-*cyclo*glutamic acids.[84-86] α-*Cyclo*glutamate[84,86] (Scheme 1.11, (**22**)) is believed to acylate an active-site bound nucleophile to give a stable inactivated complex. γ-*Cyclo*glutamate is thought to form an oxime of β-aminooxyglutamate with PLP by ring opening of the isoxazolidone ring. Various analogues of GABA inhibit GABA-transaminase. These include β-chloro, 3-phenyl, γ-acetylenic and γ-vinyl GABA and ethanolamine O-sulphate.[87] Finally the mammalian GABA transaminase is inhibited by the naturally occurring product gabaculine (**23**) via an interesting aromatisation mechanism to give an aromatic PMP adduct[88,89] (Scheme 1.12, (**24**)).



Scheme 1.11. Mechanism of inactivation of AAT by α-*cyclo*glutamate.

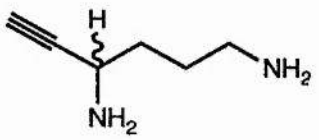


Scheme 1.12. Mechanism of inactivation of GABA-T by gabaculine (**23**).

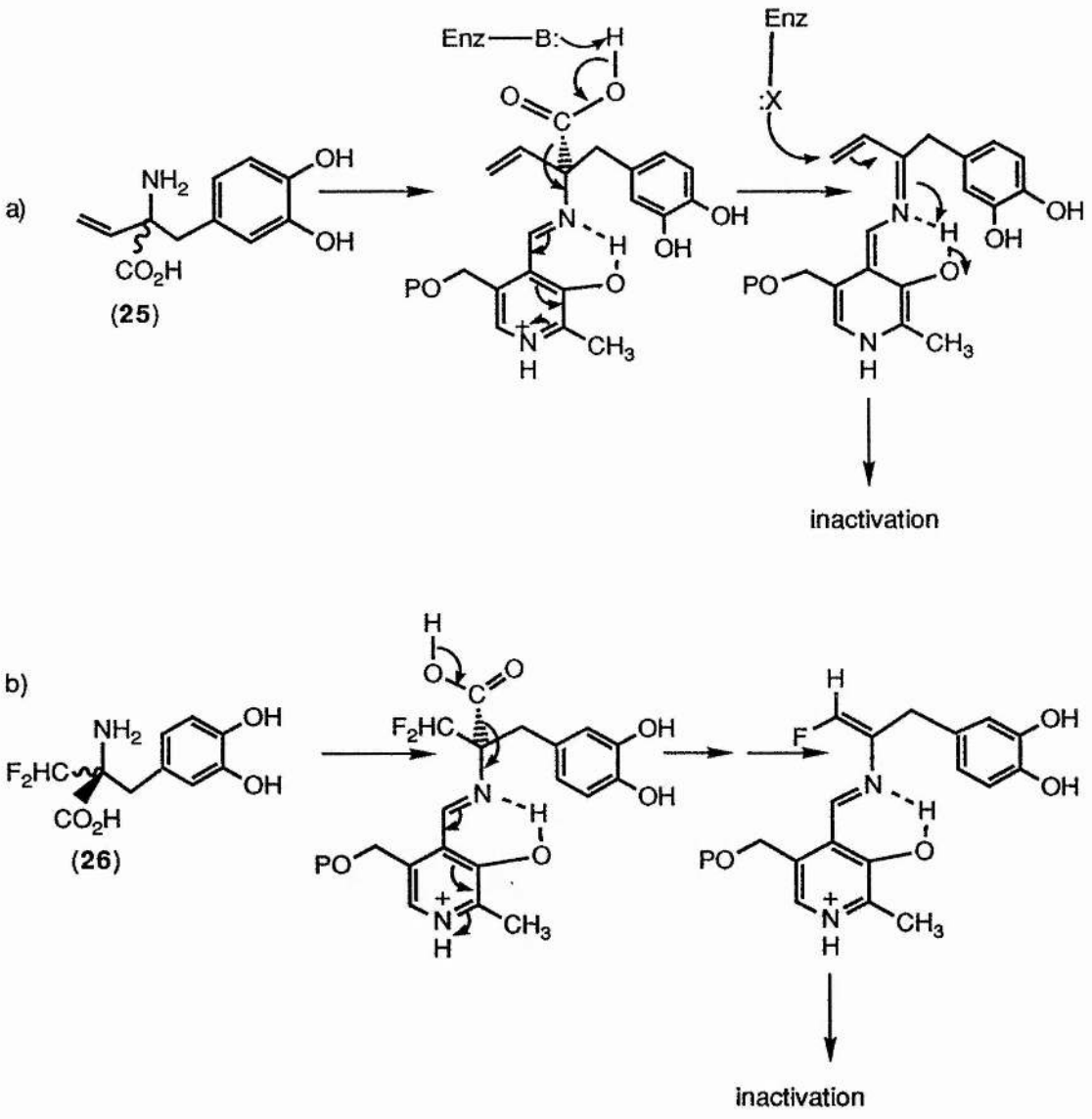
1.3.2 Decarboxylase Inhibitors

These inhibitors are particularly important because of the role of the decarboxylases in the biosynthesis of many pharmacologically active amines (see section 1.1). Many examples with alkynyl, olefinic, and mono-, di-, or trihalomethyl groups have been reported. Decarboxylases bind C-2 alkylated α-amino acids in the active-site pocket and the decarboxylation step occurs as for the natural substrate. When the alkyl groups are α-ethynyl, α-vinyl, α-fluoromethyl, and α,α-difluoromethyl amino acids, loss of CO_2 generates a reactive intermediate which can react with an active-site nucleophile or remain bound to the PLP and so these compounds act as suicide substrates. The inactivation of DOPA decarboxylase by α-vinyl DOPA (**25**)[90,91] and α,α-difluoromethyl DOPA (**26**)[92] are shown in Scheme 1.13.

5-Hexyne-1,4-diamine (**27**), a putrescine analogue, inactivates ornithine decarboxylase;[93] in view of the microscopic reversibility principle, presumably via a similar mechanism to that for α-ethynylornithine.

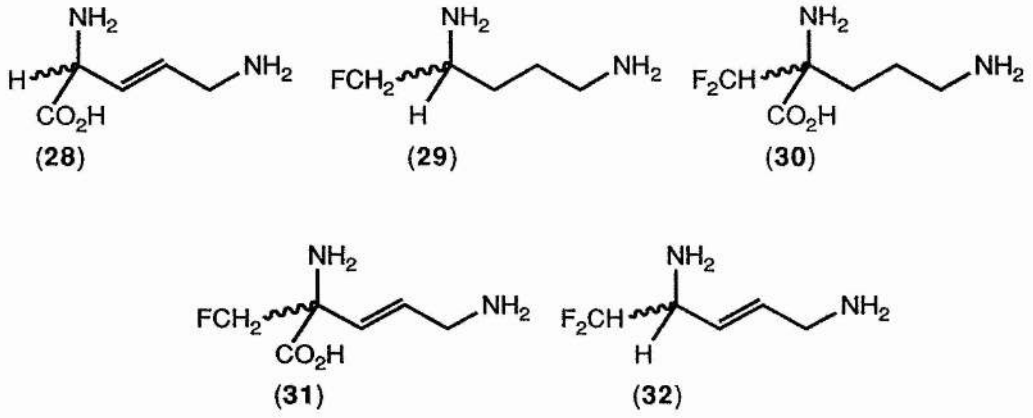


(27)



Scheme 1.13. The inactivation of DOPA decarboxylase by a) α-vinyl DOPA (**25**) and b) α,α-difluoromethyl DOPA (**26**).

Inhibitors of ornithine decarboxylase include β,γ-dehydroornithine (**28**),[94] α-fluoromethylputrescine (**29**)[95] and α,α-difluoromethylornithine (**30**).[93] Halomethyl and olefinic groups have been incorporated into a single molecule by Bey and collegues.[96] α-Fluoromethyldehydroornithine (**31**) and α,α-difluoromethyldehydroputrescine (**32**) have been tested as ornithine decarboxylase inhibitors. The dehydroornithine is more effective as an inhibitor than the saturated analogues and its K_i is 30 times lower.



1.3.3 Other PLP Inhibitors

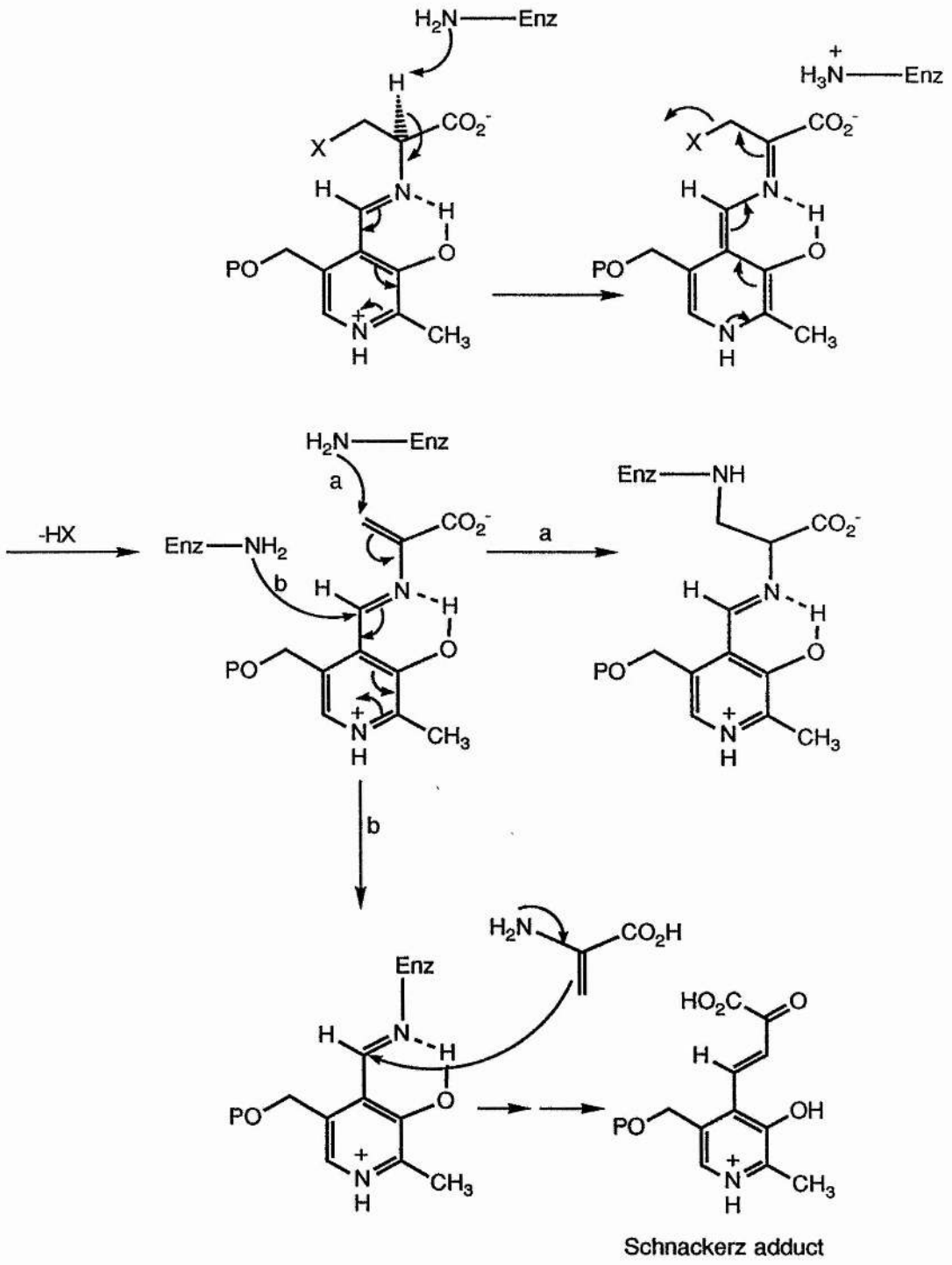
Other types of PLP inhibitors include those for racemases, and for enzymes that catalyse reactions at the β-carbon.

PLP-dependent racemase enzymes, in particular alanine and glutamate racemase are important because they are involved in the biosynthetic pathway leading to peptidoglycan (see section 1.1). Several suicide substrates, both natural and synthetic are known, including β-substituted alanines[97-99] (for alanine racemase) and phosphoalanine[100,101] (for gram-positive alanine racemase only). In the latter case the inactivation mechanism is thought to involve the initial reversible formation of a weak complex (competitive inhibition) which slowly isomerises to a stoichiometric complex which then dissociates extremely slowly.[101] The complex is not reducible by borohydride and shows a non-perturbed fluorescence spectrum for the bound coenzyme.

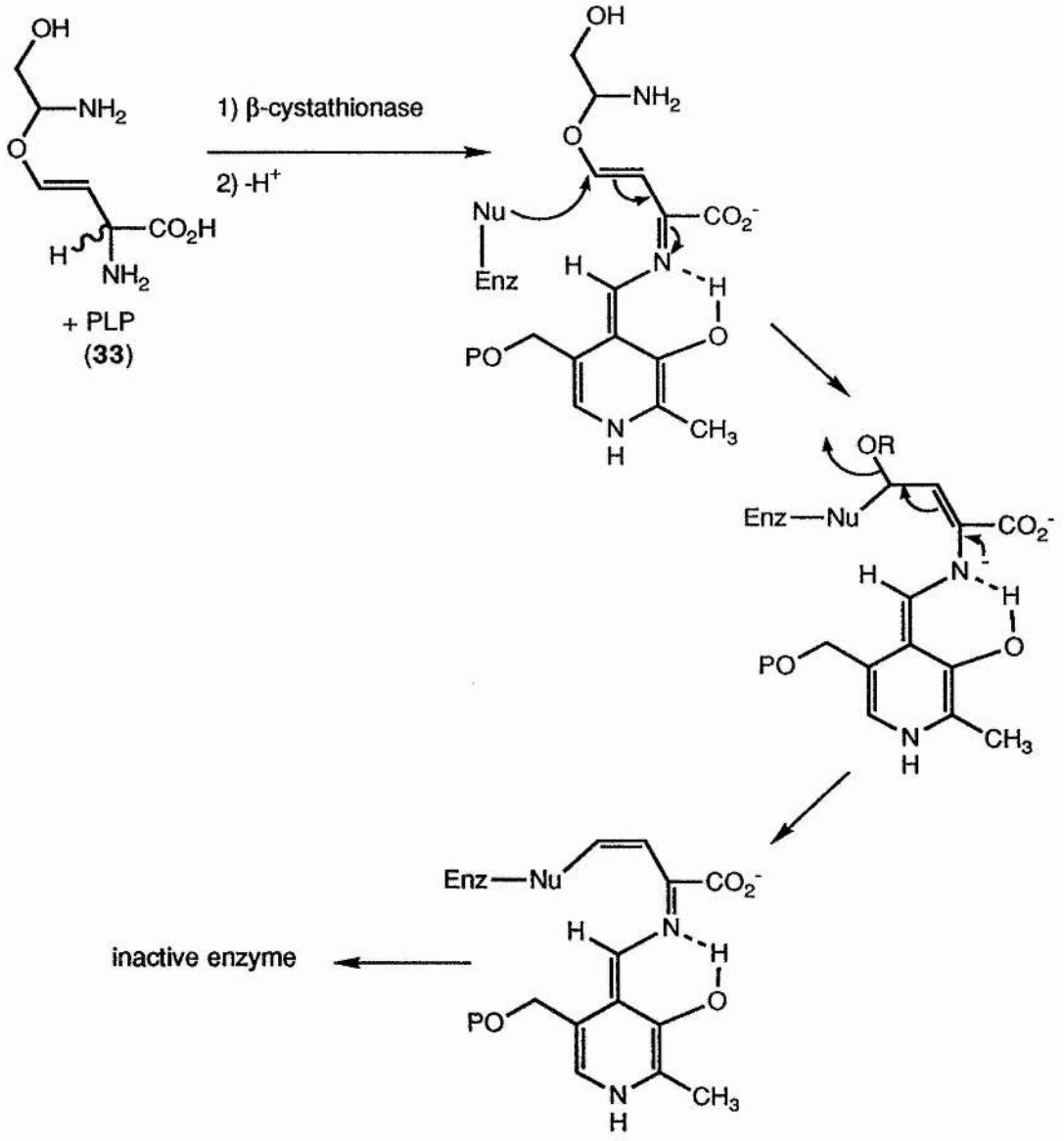
Enzymes that catalyse α,β-elimination are not susceptible to inhibition initiated by many of the typical suicide substrates containing halogens, acetylenic or olefinic functionalities. However, occasionally an abortive reaction occurs with a physiological

substrate. For example, threonine deaminase is very slowly inactivated by (2S)-serine (and also by 3-choroalanine[102]) about once in every 10^4 turnovers.[103] However (2S,3R)-threonine does not cause inactivation, presumably because the intermediate is less reactive to Michael addition or enamine condensation (which gives the inhibited Schnackerz-type product[104]) (Scheme 1.14).

E. coli tryptophanase is irreversibly inactivated by trifluoroalanine.[105] The hypothesis is that the difluoroaminoacrylyl-PLP-aldimine adduct is more susceptible to nucleophilic attack at C-3 by an enzyme bound base than hydrolysis at the aldimine carbon atom. The enzyme β-cystathionase is irreversibly inactivated by the fungal toxin rhizobiotoxin[106] (Scheme 1.15, (**33**)). The alkoxyvinylglycine analogue, rhizobiotoxin, may undergo initial C^{α}-H abstraction, and then an addition and elimination sequence, similar to that for (E)-methoxyvinylglycine with aspartate transaminase. β-Cystathionase is also irreversibly inhibited by trifluoroalanine.[107] Tryptophan synthase is also irreversibly inhibited by trifluoroalanine, trichloro- and dichloroalanine.[107] *E. coli* tryptophan synthase also reacts with cyanoglycine, causing reversible inactivation on dialysis or gel-filtration. The inactivated coenzyme is presumed to be a stable α-aminomalomononitrile anion.[108]



Scheme 1.14. Possible mechanisms for the inactivation of enzymes that catalyse α,β-elimination reactions.

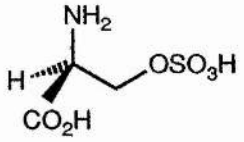


Scheme 1.15. The inactivation of β-cystathionase by rhizobiotoxin.

1.4 Research objectives

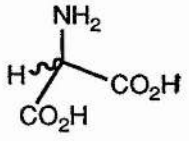
The aim of our work was to clarify two reports in the literature. These reports were inconsistent with the current models for reactions catalysed by PLP dependent enzymes.

The first report[109] concerned the inactivation of GAD by (2S)-serine O-sulphate (**21**). The mechanism of which did not involve the loss of CO_2 from the serine O-sulphate (Chapter 2).



(**21**)

The second[64] reported that the decarboxylation of 2-aminomalonic acid (**34**) catalysed by SHMT appeared to be non-stereospecific (Chapter 3).



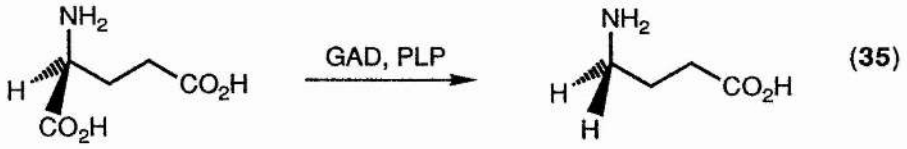
(**34**)

CHAPTER TWO

GLUTAMATE DECARBOXYLASE

2.0 Glutamate Decarboxylase

Glutamate decarboxylase (GAD) (EC 4.1.1.15) is one of the most widely studied of the PLP-dependent decarboxylases. The enzyme catalyses the conversion of (2S)-glutamic acid to γ-aminobutyric acid (GABA) (**35**) (Scheme 2.1).



(**35**)

Scheme 2.1. The physiological reaction catalysed by glutamate decarboxylase (GAD).

2.1 Properties and Structure of GAD

2.1.1 Properties and Structure of mammalian GAD

Mammalian GAD is of considerable physiological importance. The enzyme has been isolated from several species, including mouse,[110] rat,[111] human[112] and pig.[113] The most common source of mammalian GAD is from brain tissue. The enzyme has also been cloned from fruit-fly[114] and cat.[115]

The mouse enzyme was found to be unstable with respect to the conditions used for the purification processes that were originally developed. Stabilisation of the enzyme preparation with PLP and aminoethylisothiuronium bromide (AET) enabled the completion of a 200-fold purification process in 1966.[116] In 1973 a 700-fold purification of the mouse enzyme was achieved using a combination of ammonium sulphate fractionation, size exclusion, calcium phosphate gel and DEAE-Sephadex chromatography.[110] The pure mouse GAD is stable for 1-2 hours at 30-40 °C, but at

higher temperatures a rapid loss of activity occurs.[117]

The determination of the molecular weight of the various mammalian glutamate decarboxylases has been a contentious issue as there are multiple forms of the enzyme in several species including rat[118] and pig.[119] The apparent molecular weight of native rat brain GAD has been determined to be 110 000 ± 10 000 Daltons by size exclusion chromatography.[111] SDS-polyacrylamide gel electrophoresis of the purified enzyme indicated a subunit molecular weight of 60 000 Daltons,[120] and SDS-polyacrylamide gel electrophoresis of the purified enzyme after cross linking with dimethylsuberimidate indicated a dimeric subunit structure.[120] The omission of mercaptoethanol from the SDS cocktail enabled the molecular weight to be determined using SDS-polyacrylamide gel electrophoresis under non reducing conditions. The molecular weight of the enzyme determined in this manner was found to be 120 000 Daltons.[121] The results of all three studies suggested that the enzyme is dimeric. The results from parallel studies indicated that human and pig brain GAD are also dimeric. Human brain GAD has a subunit molecular weight of 67 000 ± 5 000 Daltons.[112] Pig brain GAD has a subunit molecular weight of 60 000 Daltons, and the native enzyme molecular weight is 120 000 Daltons.[122]

Due to the problems encountered during the purification of the mammalian enzymes, the amount of kinetic data available is limited. The Michaelis constant, K_M, for the natural substrate, (2S)-glutamic acid, has been determined for the three forms of pig brain GAD.[122] The relative rates of the abortive decarboxylation-transamination reaction (see section 2.1.6) leading to the formation of inactive enzyme catalysed by the three forms of porcine brain GAD have also been determined.[122] The value of V_{max} for the three forms of pig brain GAD have been determined to be 0.337, 0.73 and 0.7

unit/mg for the α-, β- and γ-forms respectively. The effect of pH on the value of V_{max} of the three forms of enzyme was found to be quite similar. In each case the highest relative value occurred at pH 6.2-6.5. The rates of the abortive decarboxylation-transamination reaction of the three forms of porcine enzyme with the pseudo substrate (2S)-aspartic acid have also been studied.[123]

2.1.2 Properties and Structure of plant GAD

GAD activity has been detected in various plants including squash,[124] tea leaves,[125] potato tubers,[126] barley,[127] radish leaves,[128] wheat[129] and lupin seeds.[130] However, there are only a few reports of the purification of the enzyme from higher plants, for example, from radish,[128] wheat[129] and lupin seeds.[130] Efficient purification has not been achieved largely due to instability and the resulting loss of enzyme activity during the purification process. Prior to 1985 the only report of homogeneous GAD from a higher plant, a 220-fold purification in 10% yield from squash tissue came from the laboratory of Melius.[131] However the subunit structure and the PLP content of the enzyme were not determined. At that time the first molecular weight for a plant GAD was determined, 310 000 Daltons for barley root GAD.[132] The molecular weight of the potato tuber isozyme[126] was found to be 91 000 Daltons where SDS-polyacrylamide gel electrophoresis gave a single band of 43 000 Daltons, consistent with a dimeric structure. More recent work with squash GAD[133] indicates that the enzyme is homohexameric with subunits of 58 000 Daltons. Apparently, at pH 7.2, the enzyme dissociates to active dimers which appear to be less stable than the hexamer isolated at pH 5.8.

2.1.3 Properties and Structure of Bacterial GAD

The discovery of the bacterial protein enabled the first detailed study of the enzyme mechanism.[134] GAD has been isolated from *E. coli*[135] and *Clostridium perfringens*.[136] The *Clostridium perfringens* enzyme has a M_r of 290 000 Daltons, an unknown subunit structure and contains 2 molecules of PLP. The *E. coli* enzyme has been extensively studied and is a hexameric species of M_r 300 000 Daltons which is readily purified, and contains one molecule of PLP per subunit. It shows a high degree of stability and has a relatively high turnover number ($k_{cat} \approx 1.875 \times 10^3\ s^{-1}$ at pH 4.6).[137] In several cases the mechanistic and stereochemical features of some suicide inactivation processes have been difficult to rationalise within the context of the known properties of pyridoxal dependent systems. In certain cases bonds connected to C^{α} were apparently cleaved on the wrong and unexpected 4'-*re*-face of the coenzyme.[36] Some of these observations have now been rationalised (see section 2.1.7).

2.1.4 Glutamate Decarboxylase as a chemotherapeutic target

The mammalian brain enzyme is directly responsible for the biosynthesis of GABA in the GABA-ergic system (section 1.1).[138] GABA, the product of the decarboxylase reaction is a major neurotransmitter and there is much evidence to suggest that high cerebral concentrations prevent convulsions.[2] Reduced levels of GABA not only trigger convulsive attacks but are also associated with a variety of maladies including Parkinson's Disease, Alzheimer's Disease and Huntington's Chorea.

2.1.5 Substrate and Reaction Specificity

(2S)-Glutamic acid is the only naturally occurring amino acid that is decarboxylated by GAD at a significant rate. The Michaelis constant (K_M) for *E. coli* GAD was determined to be 0.5-1.0 mM at pH 4.6 by Shukuya and Schwert in 1960.[139]

Several pseudo substrates, for example glutaric acid, (2S)-aspartic acid, α-methyl-(2RS)-glutamic acid and (2S)-serine O-sulphate, have been incubated with the enzyme. They were all found to inhibit the enzyme. (2S)-Aspartic acid and α-methyl-(2RS)-aspartic acid were decarboxylated but at 10^3 times and 10^4 times more slowly than the natural substrate. GAD is substrate and reaction specific unlike SHMT (see Chapter 3).

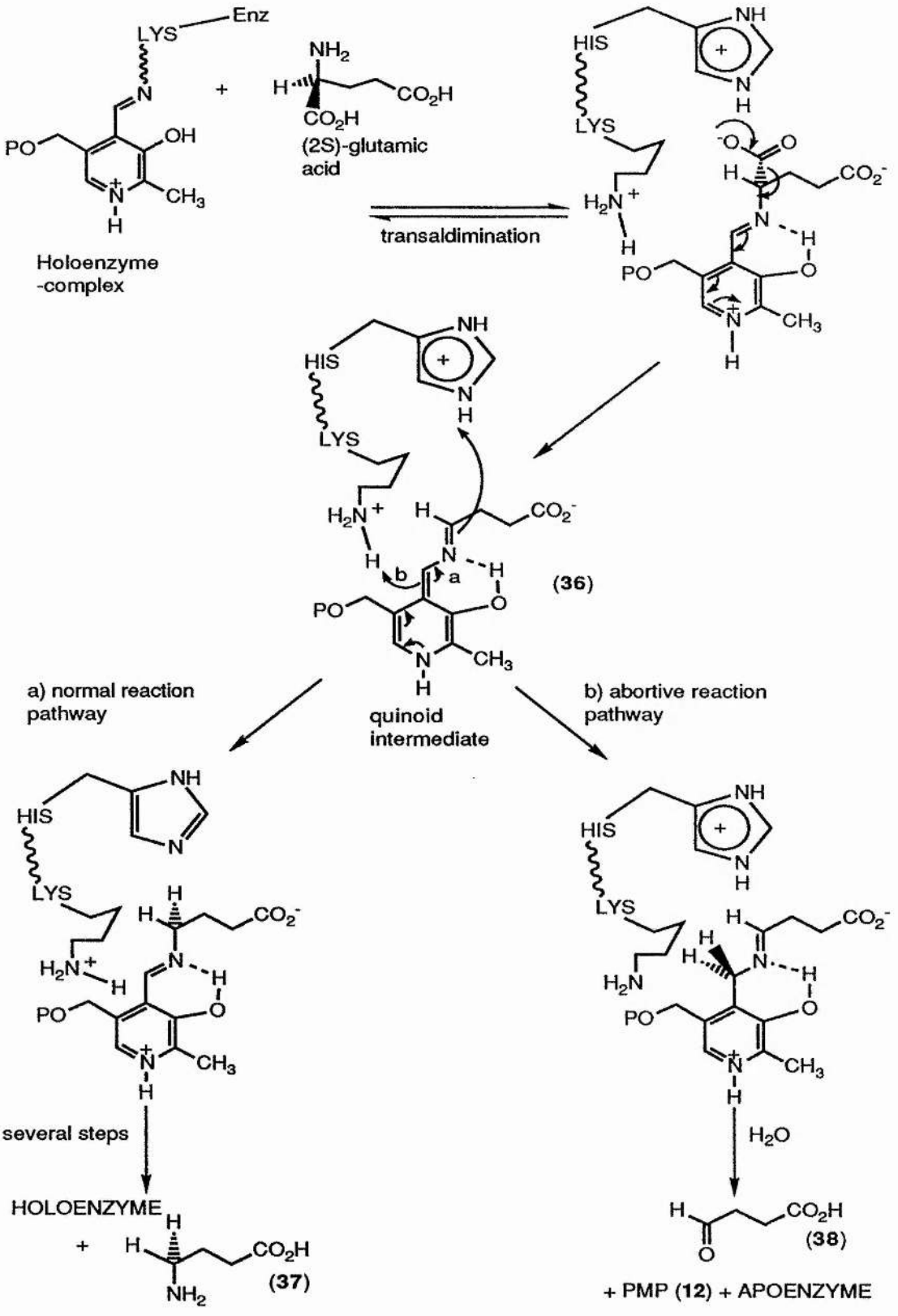
2.1.6 Mechanistic features of decarboxylation

During decarboxylation there are two competing reactions that occur (Scheme 2.2). Following the transaldimination of the internal aldimine by the enzyme-bound substrate, the protein orients the substrate so that the C^{α}-CO_2H bond is orthogonal to the plane of the pyridinium ring, the optimal geometry for the delocalisation of the developing negative charge.[140] Loss of the carboxylate group leads to the formation of the quinoid intermediate (**36**), which is reprotonated at C^{α} on the 4'-*si*-face of the coenzyme to give the corresponding amine (**37**) and PLP (Scheme 2.2, a). The second reaction occurs when the quinoid (**36**) is occasionally reprotonated at C-4', GAD is one of the few enzymes that catalyse this unusual reaction.[141-145] This second reaction leads to the formation of an aldehyde (**38**) and PMP (**12**) (Scheme 2.2, b). The second reaction pathway is called an abortive decarboxylative-transamination.

The abortive reaction results in the inactivation of the enzyme as the PLP is converted to PMP. Tilley[146] has studied the pH dependence of the abortive reaction and found that at pH 4.6 approximately 1 in every 30 000 turnovers leads to an abortive reaction.

It is known that when substrate analogues, for example, (2S)-aspartic acid, are decarboxylated, pyridoxamine phosphate (PMP) (**12**) is produced as a result of the abortive decarboxylation-transamination reaction (see above). The process leads to inactivation of the enzyme.

The mechanism of inactivation by (2S)-serine O-sulphate (**21**) is more complex and is discussed in detail below (Section 2.2).

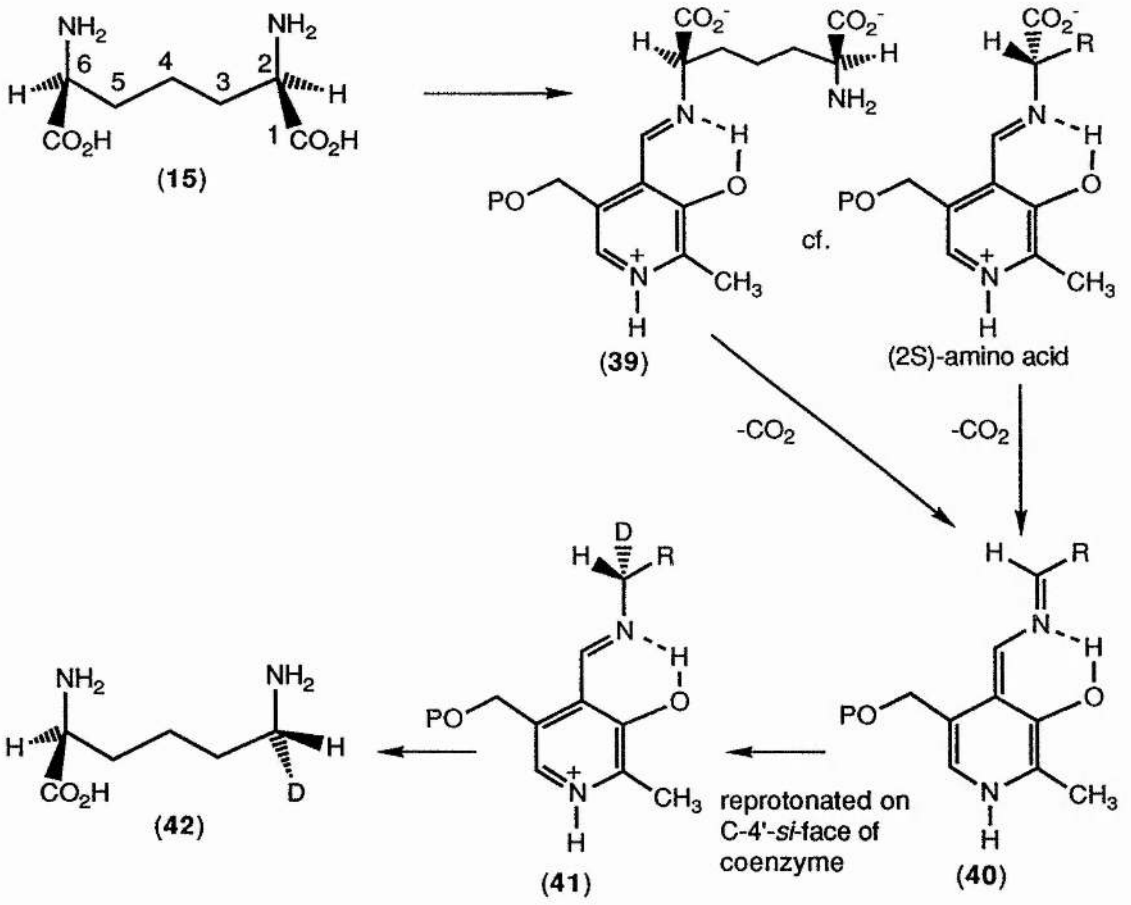


Scheme 2.2. The mechanism of GAD showing a) the normal reaction pathway and b) the abortive reaction pathway.

2.1.7 Stereochemical Studies

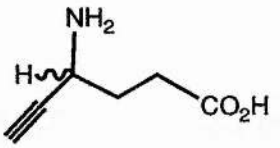
The decarboxylation of (2S)-glutamic acid has been shown[43,45,147] to occur with retention of configuration at C^{α}. Indeed this stereochemical outcome has been noted for the reactions of most other decarboxylases that have been studied.

One exception is α,ω-diaminopimelic acid (**15**) decarboxylase which decarboxylates the (2R)-centre of the substrate with an inversion of configuration at C^{α}. The inversion can be rationalised by the following hypothesis. In the external aldimine form of the enzyme the Schiff's base binds to the protein with the C-4'-*si* face exposed to the solvent and the active-site lysine. The (2R)-amino acid α-centre of α,ω-diaminopimelic acid binds to the active site of the enzyme with the C^{α}-H and the side chain occupying similar positions to those in (2S)-amino acid decarboxylases (Scheme 2.3, (**39**)). This binding mode disposes the α-carboxylate group on the C-4'-*re*-face of the coenzyme (Scheme 2.3). The α-carboxylate group is perpendicular to the π-electron system and so decarboxylation is stereoelectronically assisted.[140] The resulting ketimine (**40**) is identical to that produced by (2S)-amino acid decarboxylation and can be protonated from the C-4'-*si* face of the coenzyme in the normal fashion. Hence (2S)-lysine (**42**) is formed via a mechanism involving decarboxylation with inversion at C-6 of the substrate (**15**).



Scheme 2.3. The decarboxylation of α,ω-diaminopimelic acid (**15**).

4-Aminohex-5-ynoic acid (acetylenic GABA) (**43**) inactivates both *E. coli* and mammalian GAD.[44,148]



(**43**)

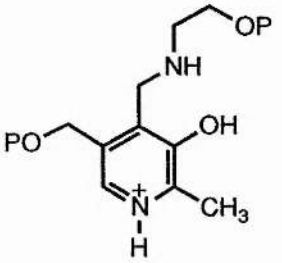
The bacterial enzyme is inhibited by the (4R)-enantiomer[148] while the rat brain enzyme is inhibited by the (4S)-enantiomer.[148] In the physiological reaction both enzymes catalyse decarboxylation in a retention mode and thus inhibition must be initiated via different mechanisms. Tilley *et al*[149] have carried out stereochemical investigations to define the conformations of substrates and inhibitors and hence, the positions of the

distal binding groups at the active site of GAD from *E. coli*.

The stereospecificity of the protonation of the quinoid intermediate (**36**) (in the abortive transamination reaction) at C-4' was determined using the natural substrate, (2S)-glutamic acid.[149] An earlier study had employed racemic 2-methylglutamic acid.[141] This substrate was unsuitable for use in simple stereochemical correlations in view of its very low V_{max} value and racemic nature.[141] In Tilley's work [4'-^{3}H]-PLP was incubated with the enzyme in the presence of (2S)-glutamic acid. The abortive transamination product, [4'-^{3}H]-PMP, was separated and treated with alkaline phosphatase to give 4'-tritiated pyridoxamine. The absolute configuration at C-4' was determined by incubation with apoaspartate aminotransferase which is known to exchange the 4'-*pro*-S hydrogen of pyridoxamine with the solvent. None of the tritium was exchanged while 50% of the label in a synthetic racemic sample was lost to the solvent. The retention of the tritium indicated that the sample was (4'R)-[4'-^{3}H]-PMP and that a proton is transferred to the 4'-*si*-face of the coenzyme during the transamination catalysed by the decarboxylase. This result is in accord with the results obtained for methionine decarboxylase[150] and aspartate aminotransferase.[13,14]

The stereospecificity and fidelity of the decarboxylation and reprotonation at C^{α} of the quinoid intermediate derived from a "loose-fit" substrate, (2S)-aspartic acid, was investigated.[149] The results indicated that the decarboxylation reaction occurred stereospecifically and with retention of configuration at C^{α} of the substrate. The high chiral integrity of the decarboxylation products is in keeping with the notion that either a single acid or two acids operate on the 4'-*si*-face of the coenzyme. This result mirrors those obtained for methionine decarboxylase.[151]

The regeneration of active holoenzyme from apoenzyme and $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate (**44**) had not been reported for *E. coli* GAD, but it had been demonstrated for the porcine brain enzyme.[144]



$N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate
(**44**)

Tilley *et al.*[149] prepared $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate (**44**) by reduction with $NaBH_4$ of the imine formed by the condensation of PLP and ethanolamine phosphate. The $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate (**44**) was incubated with *E. coli* apoGAD and active holoenzyme was regenerated. In order to study the mechanism of the regeneration, the C-2 deuteriated isotopomers of (**44**) were prepared and were incubated with the apoenzyme. Incubation of (2S)-[2-^{2}H]-$N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate with *E. coli* apoGAD gave reactivation rates identical to those obtained for the unlabelled compound. The (2R)-antipode gave rates close to those obtained for the dideuteriated compound which revealed that the enzyme removes the 2-*pro*-R hydrogen from the phosphoethyl group of $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5'-phosphate during the reactivation process.

The collective results of Tilley's work indicate that enzyme catalysed proton transfers occur on the 4'-*si*-face of the coenzyme at C-4' and C^{α} and that the distal binding groups of substrates and analogues occupy similar positions on the 3'-OH side of the coenzyme. As a result of these findings it is postulated that the unexpected reactions

observed for GAD with the suicide substrates do not result from alternative binding modes, but may be promoted by the enhanced acidity of the methylene protons in the derived aldimines due to the flanking multiply bonded moieties, and in actual fact, the enzymes may not provide a base on the 4'-*re*-face of the coenzyme at all.

A recent review by Smith *et al.*[37] compares PLP dependent decarboxylases and transaminases at a molecular level. The amount of stereochemical, mechanistic and structural information available for the decarboxylases at the start of the work was very limited. However the active-site peptide sequences for some decarboxylases were known, and the stereochemical courses of decarboxylation had been reported for some systems.[36,152] From these studies it was evident that, in general, PLP dependent decarboxylases catalyse the decarboxylation of (2S)-amino acid substrates with retention of configuration at C^{α}. Transaminases appear to catalyse proton transfers on the C-4'-*si*-face of the coenzyme only. Other PLP dependent enzymes also utilise the C-4'-*si*-face of the coenzyme, including SHMT. (2S)-Methionine decarboxylase from *Dryopteris filix-mas*[150] and *Streptomyces* sp.[153] were used for chemical studies due to their wide substrate structure tolerance and stabilities. It was demonstrated that both enzymes catalysed the decarboxylation of methionine with retention of configuration at C^{α}.[150,153,154] The enzymes also decarboxylated other straight chain and branched chain hydrophobic amino acids with retention of configuration even at extreme pH. For the fern enzyme, Stevenson *et al.* demonstrated that the coenzyme was protonated from the C-4'-*si*-face of the coenzyme during an essentially irreversible abortive decarboxylative reaction.[150] Stevenson *et al.* carried out stereochemical and kinetic analysis of the fern enzyme, and observed that a proton was transferred to the C-4'-*si*-face of the coenzyme during the abortive transamination.[150] In order to determine the identity of the proton donor for the C-4' and C^{α} of the quinoid intermediate, Stevenson

et al. determined the deuterium solvent isotope effects on V_{max} and V/K for (2S)-methionine, valine and leucine over the pH range 4.0-7.5. The results indicated that the proton donors for C^{α} and C-4' of the quinoid intermediate are distinct. For the fern enzyme, Akhtar *et al.* established that the proton donor for the quinoid intermediate at C^{α} is probably the imidazolium side chain of a histidine residue, and the proton donor at C-4' is probably the ε-ammonium group of the active-site lysine residue.[151] Tilley[149] performed a similar stereochemical and kinetic analysis with *E. coli* GAD using (2S)-glutamic acid as the substrate. A proton was transferred to the C-4'-*si*-face of the coenzyme during the abortive transamination reaction in the same way as for the fern enzyme.[150] Strausbauch[155] had previously established that borohydride reduction of the holoenzyme gave a pyridoxyllysine peptide, and so it appeared that the proton donor was the ε-ammonium group of the lysine residue. For the *E. coli* enzyme, Tilley[149] demonstrated that the large isotope effect of 5.0 observed for ^{D}V was due, in part, to partially rate limiting protonation of the quinoid intermediate at C^{α}. These findings were in accord with the operation of a monoprotic conjugate acid. Thus it appeared that for fern methionine decarboxylase and *E. coli* GAD, the lysyl ε-ammonium and the histidyl imidazolium groups are disposed on the C-4'-*si*-face of the coenzyme. It appears that these conjugate acids are responsible for the specific protonation of the quinoid intermediate at C-4' and C^{α}, respectively.

2.1.8 Active-site structures of PLP-dependent decarboxylases

In view of the similarities discussed above, the reported active-site peptide sequences for the PLP-dependent decarboxylases were examined and aligned with regions of the active-site sequence of chicken mitochondrial AAT. Each residue deemed to be functionally important was completely conserved (Table 2.1).

Table 2.1. Active-site residues of several AAT isozymes

Isoenzyme Source	Amino acid sequence
E. coli[30]	I V A S S Y S **K** N F G L Y
Chicken (mitochondrial)[156]	V L S Q S Y A **K** N M G L Y
Turkey (mitochondrial)[157]	V L S Q S Y A **K** N M G L Y
Pig (mitochondrial)[158]	C L C Q S Y A **K** N M G L Y
Rat (mitochondrial)[159]	C L C Q S Y A **K** N M G L Y
Human (mitochondrial)[29]	C L C Q S Y A **K** N M G L Y
Chicken (cytosolic)[160]	F C A Q S F S **K** N F G L Y
Pig (cytosolic)[161]	F C A Q S F S **K** N F G L Y

Analysis of the regions of AAT which interact with the coenzyme indicated that a tetrapeptide unit -S-X-X-K- contained two completely conserved residues, a serine (S255) which forms a side-chain hydrogen bond with the 5'-phosphate ester group of PLP, and the active-site lysine (K258) which forms the internal aldimine with the coenzyme and also serves to shuttle protons between C^{α} and C-4'.

The decarboxylases were examined in the light of the findings for the AAT's, in many the equivalent residue to AAT Ser255 was a Ser, Asn, or Thr residue, all of which are hydrogen bond donors (Table 2.2). In particular the active-site tetrapeptides for the decarboxylases were well conserved, and contained the sequence -S(N,T)-X-H-K-, unless there were rationalisable mechanistic reasons for why they should not be conserved.

Table 2.2. Schiff's base forming region of PLP-dependent decarboxylase enzymes.

Enzyme Source	Amino acid residues												
Arginine (*E. coli*)[162]		A	T	H	S	T	H	**K**	L	L	N	A	L
Glutamate (*E. coli*)[155]	S	I	S	A	S	G	H	**K**	F				
Histidine (*Morganella morganii*)[163]	S	I	G	V	S	G	H	**K**	M	I	G	S	P
Lysine (*E. coli*)[164]	Y	E	T	E	S	T	H	**K**	L	L	A	A	F
Lysine (*Hafnia alvei*)[165]	Y	E	T	Q	S	T	H	**K**	L	L	A	A	F
Ornithine (*E. coli*)[166]						V	H	**K**	Q	Q	A	G	Q
Dopa (*Drosophila*)[167]	S	F	N	F	N	P	H	**K**	W	M	L	V	N
Dopa (Pig)[168]			N	F	N	P	H	**K**	W				
Glutamate (Feline)[115]	S	V	T	W	N	P	H	**K**	M	M	G	V	L
Glycine (Chicken)[169]	V	S	H	L	N	L	H	**K**	T	F	C	I	P
SHMT (*E. coli*)[170]	V	V	T	T	T	T	H	**K**	T	L	A	G	P
SHMT (Rabbit, Cyt.)[171]	V	V	T	T	T	T	H	**K**	T	L	R	G	C
SHMT (Rabbit, mit.)[171]	V	V	T	T	T	T	H	**K**	T	L	R		

2.1.9 The optical properties of bacterial glutamate decarboxylase

The electronic absorption spectrum of *E. coli* glutamate decarboxylase has been widely studied. The solution was found to be yellow in acid solution, but colourless at neutral pH values. These properties are similar to those exhibited by several PLP enzymes and by Schiff's bases which are formed from PLP and peptides and some proteins.[172-177]

The catalytically active form of the enzyme existing in solution at pH <5.0 has a spectral absorption maximum at 415 nm. As the pH is increased to 6.5, the intensity of the peak

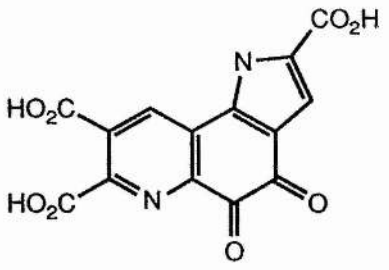
at 415 nm reduces and a new absorption maximum appears at pH 340 nm. The form of the enzyme exhibiting the absorption maximum at 340 nm is no longer active. The spectra at pH 5.1 and 4.6 are experimentally identical, so it is presumed that the structure which gives rise to the changes in the spectrum is fully protonated at pH 5.1. Anderson and Chang[178] concluded, on the basis of experiments with sodium borohydride, that the absorption maximum at 415 nm arises from the formation of a Schiff's base between the PLP and an active-site lysine residue (in common with many other PLP dependent enzymes), in which the hydrogen of the phenolic hydroxyl of pyridoxal is hydrogen bonded to the imino nitrogen of the Schiff base. The identity of the structure responsible for the absorption at 340 nm has not been clearly established. However there are a number of compounds formally derived from Schiff bases of PLP and pyridoxal in which various groups or molecules are added across the imine double bond resulting in saturation. These include the reduced aldimines, the addition compounds with cysteine, histidine, 3,4-dihydroxyphenylalanine and related substances,[179] and aminoacetal and carbinolamine derivatives.[177] These materials characteristically show an absorption band around 330 nm in neutral solution. Christensen[175] suggests that the unknown compound is more likely to be a zwitterionic aldimine, where the phenolic hydroxyl group has lost its proton and the pyridinium nitrogen has remained protonated.

In the presence of an excess of substrate, (2S)-glutamic acid, at pH 4.6 a spectral change was observed. There is an instantaneous reduction of absorbance at 420 nm and an increase at 330 nm. As the substrate is exhausted, the spectrum returns to the original position within several seconds. Shukuya and Schwert[180] made no attempt to suggest the nature of the species responsible for the absorption at 340 nm. In an attempt to discover the nature of the intermediates responsible for the above spectral

shifts, Sukhareva and Torchinsky[181] employed α-methyl glutamate, a substrate analogue that acts as an inhibitor of the enzyme.[182] The changes observed in the spectrum were similar to those that had been seen to occur with the natural substrate but at a considerably slower rate. The drop in absorbance at 415 nm required at least 30 min for completion, but was not reversed on further incubation. The enzyme-quasisubstrate complex was found to be inactive. Activity was not restored with the addition of (2S)-glutamic acid, but the addition of PLP (4 mM) restored the activity to 73% of the initial value. During later work with α-methyl glutamate, Sukhareva and Braunstein[141] detected and characterised successive intermediate forms and complexes of the enzyme which were formed during the inactivation reaction. The inactive form with the absorption maximum at 340 nm was found to be the PMP form of the enzyme. However the identities of the short-lived intermediates with an absorption maximum at 340 nm, for both the natural substrate and α-methyl glutamate, were not discovered.

2.1.10 Pyrroloquinoline quinone (PQQ)

The electronic absorption spectra of the PLP enzymes are well documented (section 2.1.9). However in some cases there is an unidentifiable chromophore in the spectrum of active enzyme in the 330-340 nm range. Enzyme samples have been treated to remove the excess PLP, but the chromophore is not removed. It has been suggested that the chromophore is due to the presence of covalently bound pyrroloquinoline quinone (PQQ) (**45**).



(45)

On the basis of such data it was suggested that rat liver ornithine aminotransferase, (2S)-lysine 6-aminotransferase and mammalian GABA-T contain PQQ.[183] Since then evidence has been provided to show that mammalian DOPA decarboxylase,[184] methylamine dehydrogenase,[185] *E. coli* (2S)-glutamate decarboxylase[186] and soybean lipoxygenase-1[187] contain tightly bound PQQ. However, more recently it has been reported that dopamine β-hydroxylase[188] and soybean lipoxygenase-1[189] are not PQQ dependent enzymes.

Recent work has been published for *E. coli* glutamate decarboxylase[190] demonstrating that the only cofactor present is PLP and describing the origins of the unidentifiable chromophore. The spectrum of *E. coli* GAD prepared in the absence of added PLP shows only one chromophore absorbing at wavelengths above those expected for the aromatic amino acids. The absorption maximum of the band at 417 nm is consistent with it being an imine of PLP with the ε-amino group of lysine. The conversion to a 330 nm absorbing species by treatment with α-methylglutamate confirms that the enzyme has undergone a decarboxylation dependent transamination to the PMP form. In this form the cofactor can be completely removed from the enzyme to leave a protein with no trace of any chromophore above 280 nm. Choi and Churchich[191] have studied pig brain GAD and concluded that the holoenzyme is not a PQQ protein, and that the catalytic mechanism of action involves the participation of only one cofactor i.e. PLP. However, free PQQ is a strong inhibitor of GAD (K_i 13 μM) and the reaction with the

protein results in spectral changes resembling those of polylysine treated with PQQ. It is suggested that if the concentration of free PQQ in some regions of the brain reaches the micromolar level, then PQQ may play a role in the regulation of GAD activity. In the light of this recent information the status of many proteins that have previously been identified as containing PQQ should be reconsidered.

2.2 Inhibition of Glutamate Decarboxylase by (2S)-serine O-sulphate

2.2.1 Introduction

In the late 1960's Sukhareva and Braunstein[141] discovered that (2S)-serine O-sulphate (**21**) inactivates glutamate decarboxylase. It was established that at 10 mM concentrations the inhibition suppressed activity by 54% after 1 hour. Incubation of the enzyme with (2S)-serine O-sulphate lowered the PLP content from 6 to 2.4 moles/mole of enzyme. The decrease in PLP content was concomitant with a decrease in the absorption at 420 nm and the appearance of a new band at 330 nm due to the conversion of PLP to a new unknown product.

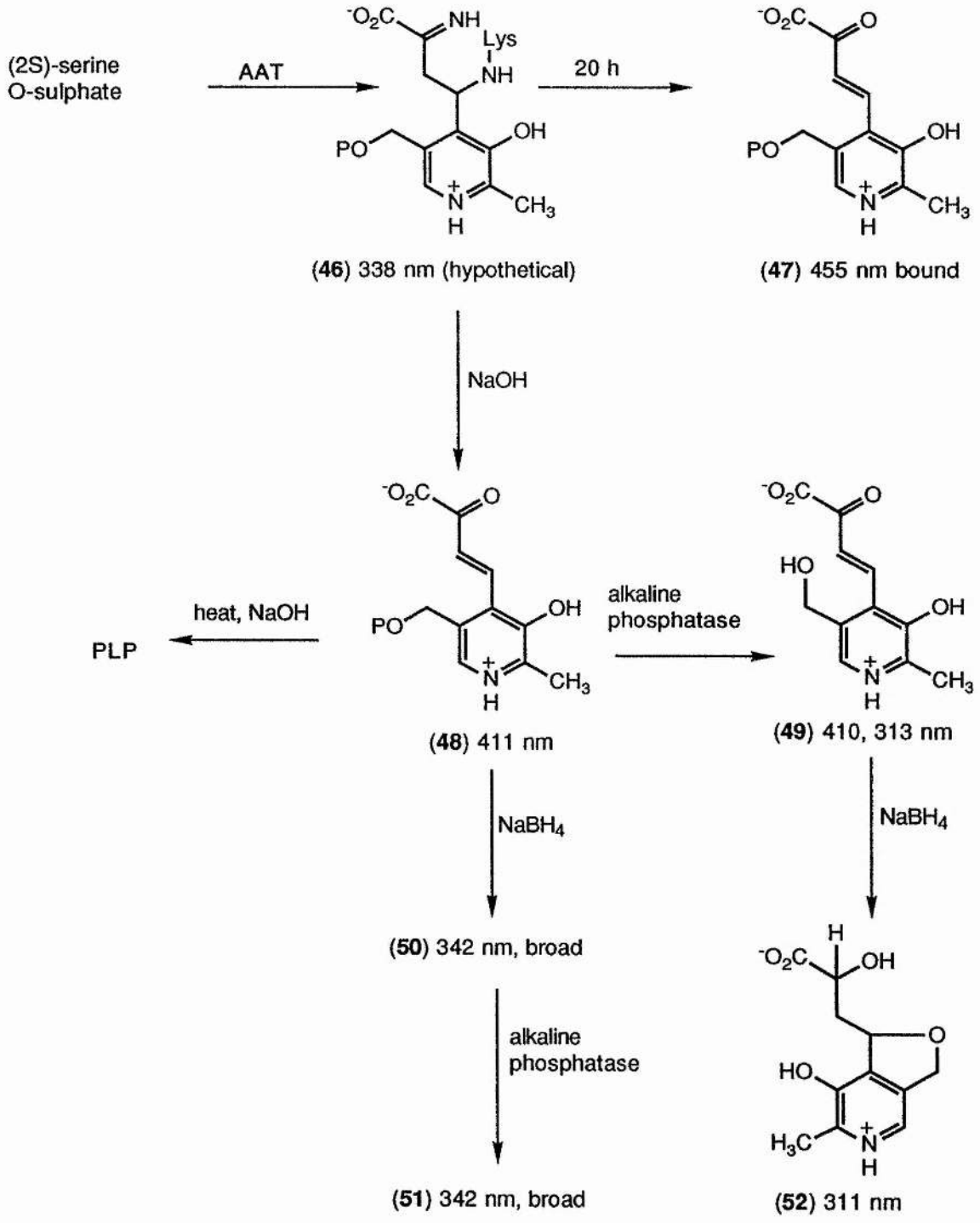
More recently Ueno, Likos and Metzler[80] discovered that (2S)-serine O-sulphate caused the irreversible inactivation of cytosolic aspartate aminotransferase (AAT) from pig heart. The enzyme was inactivated over a 10 minute period, and the absorption maximum at pH 5.4 shifted from 430 to 338 nm (**46**). On prolonged standing (20 h), the peak shifts again to 455 nm (**47**). Morino[78] reported the same behaviour for the inactivation of AAT by β-chloroalanine in the presence of 3 M formate.

When the pH of the AAT enzyme solution was raised to pH >11, a yellow diffusible compound (**48**) was released from the protein. Compound (**48**), as well as its dephosphorylation and reduction products (**49-52**), was isolated and studied by ultra-violet and proton NMR spectroscopy (Scheme 2.4).

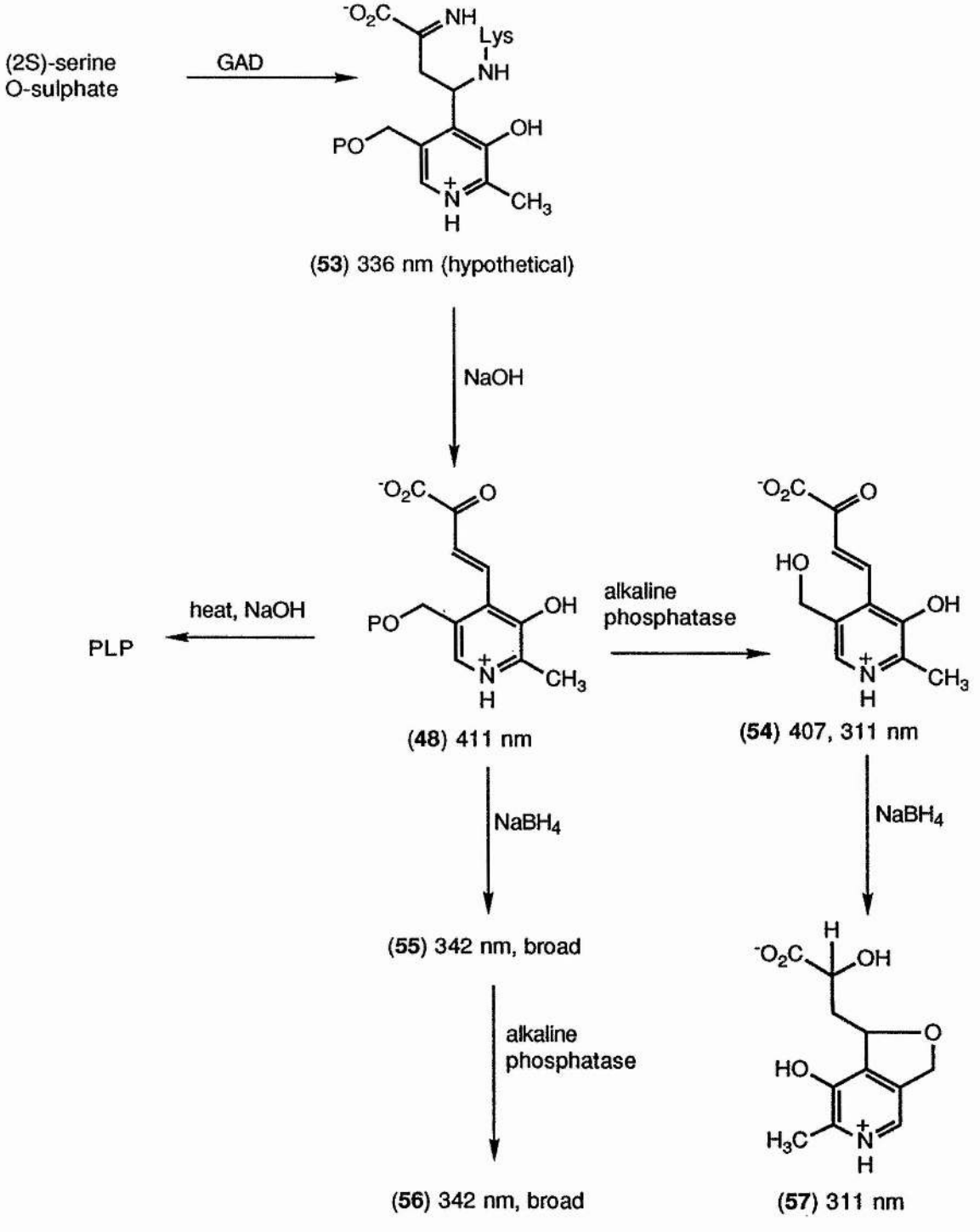
Compound (**48**) was found to be identical with a compound formed from (2S)-serine O-sulphate and glutamate decarboxylase by a similar reaction sequence.[109] The uv data

obtained on the compound (**48**) and its dephosphorylation and reduction products (**54-57**) are shown in Scheme 2.5.

The compound (**48**) isolated from the inactive enzyme is the product of an aldol condensation between pyruvate and pyridoxal 5'-phosphate. The compound (**48**) has been previously synthesised by Schnackerz,[104] and can also be synthesised from oxalacetic acid and PLP via a Knoevagel condensation reaction. Compound (**48**) is often called the Schnackerz adduct.

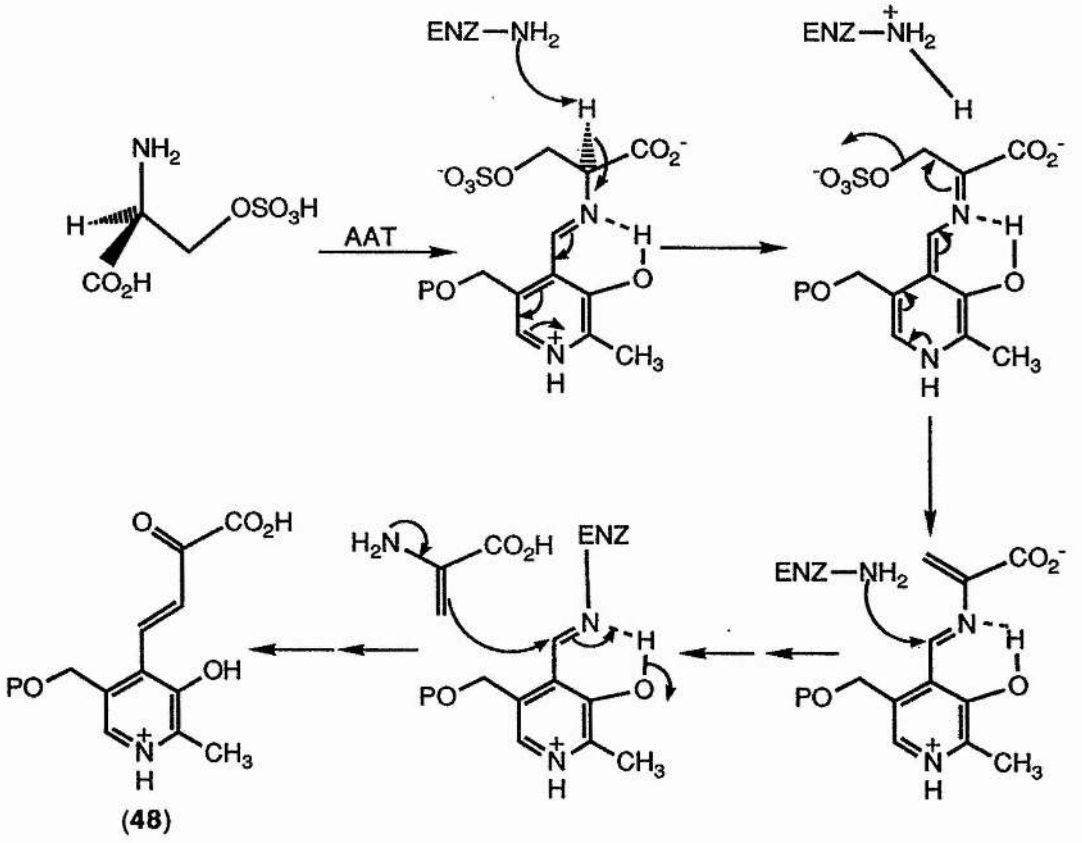


Scheme 2.4. The compounds isolated from the inactivation of AAT by (2S)-serine O-sulphate.

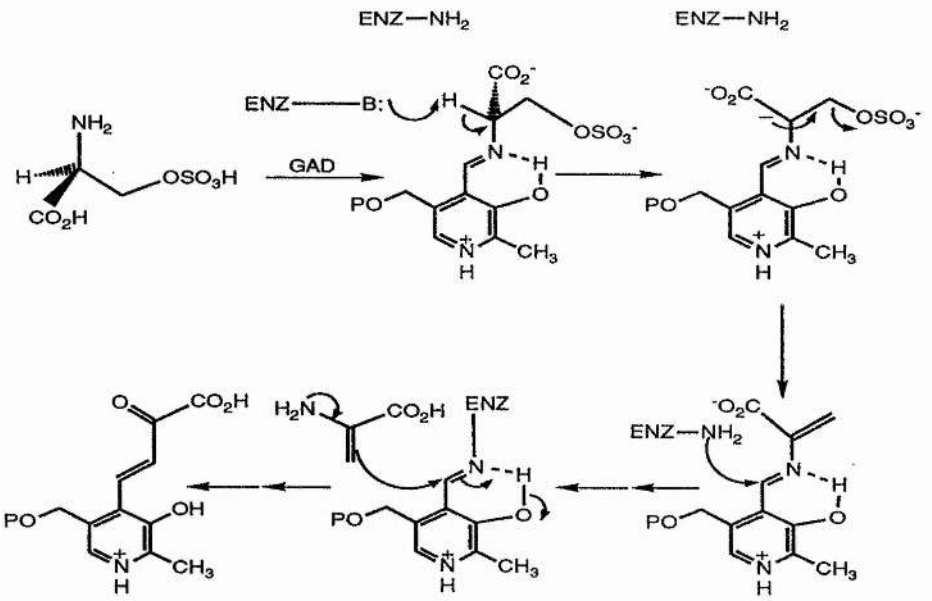


Scheme 2.5. The compounds isolated from the inactivation of GAD by (2S)-serine O-sulphate.

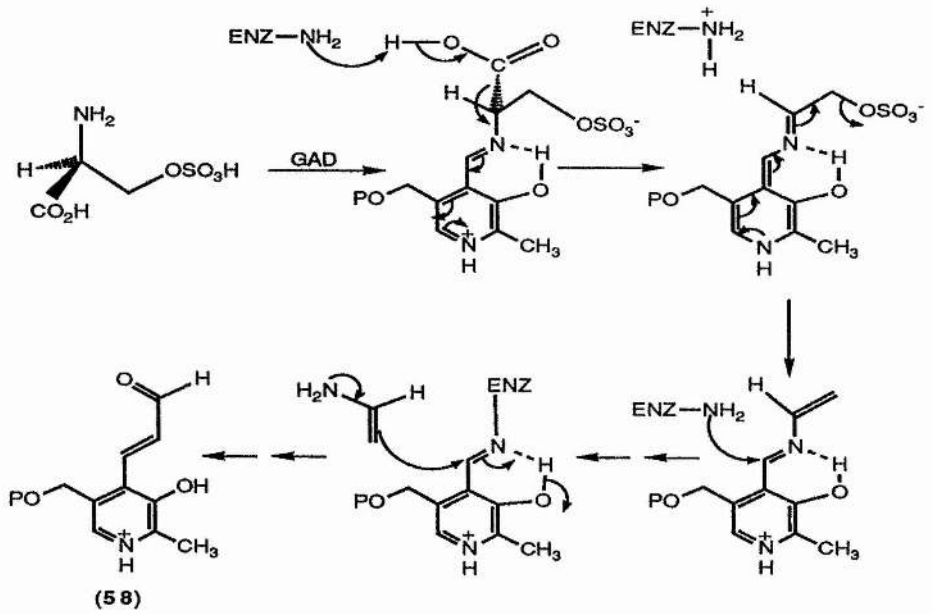
Mechanisms for the formation of the Schnackerz adduct have been proposed for both enzymes (Scheme 2.6, 2.7). The proposed mechanism in the AAT case is straightforward (Scheme 2.6). However for glutamate decarboxylase, if the inactivation occurs by a similar mechanism to give the adduct (**48**), the first step in the reaction sequence would be a transaldimination reaction (Scheme 2.7). The second chemical step would be the removal of a proton from the C-4'-*re*-face of the coenzyme and not the loss of carbon dioxide from the C-4'-*si*-face. Loss of CO_2 from the C-4'-*si*-face of the coenzyme would lead to the formation of a different PLP adduct (Scheme 2.8, (**58**)). If the pseudo substrate binds in the expected manner[149] the sulphate group would occupy the same site as the β-acid group of the natural substrate, (2S)-glutamic acid (Figure 2.1).



Scheme 2.6. The mechanism of inactivation of AAT by (2S)-serine O-sulphate.



Scheme 2.7. The proposed mechanism of inactivation of GAD by (2S)-serine O-sulphate.



(58)

Scheme 2.8. The expected mechanism of inactivation of GAD by (2S)-serine O-sulphate.

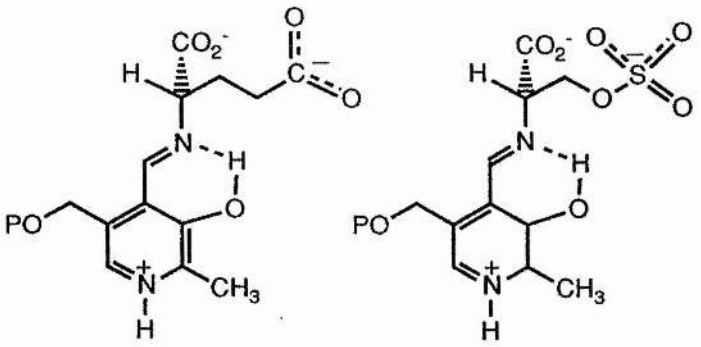
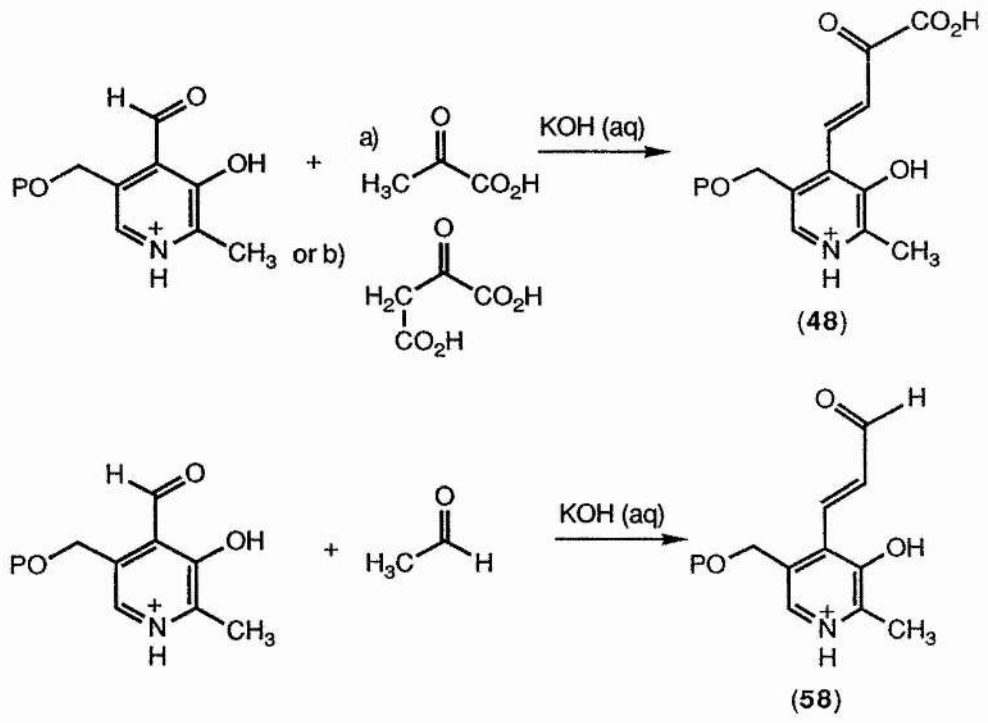


Figure 2.1. The PLP-Schiff's base of (2S)-glutamic acid and (2S)-serine O-sulphate.

Initially we wanted to prepare (2S)-serine O-sulphate (**21**), and other β-hydroxy-α-amino acid sulphate esters and incubate them with the enzyme to verify that the inactivation occurred for (2S)-serine O-sulphate and discover if any inactivation occurred with (2R)-serine O-sulphate (**59**), (2S,3R)-threonine O-sulphate (**60**) and (2S,3S)-threonine O-sulphate (**61**). Metzler's theory was based on comparing the proton NMR spectrum of products isolated from the active site of the inactivated enzyme with the proton NMR spectrum of the synthetic Schnackerz adduct (**35**).[109] However the proton NMR spectrum of the product (**58**) of the condensation reaction between acetaldehyde and PLP would be expected to be quite similar to that of the Schnackerz adduct (**48**), although the carbon NMR spectrum of the decarboxylated compound (**58**) contains one less carbon and so the carbon NMR spectra should be distinguishable. The carbon NMR spectrum published by Metzler[109] for the product (**48**) from the inactivation of GAD by (2S)-serine O-sulphate clearly shows a signal at ≈165 ppm which has been assigned to the carboxylic acid. Attempts to prepare the products of the two condensation reactions were largely unsuccessful due to problems in separating the products. The synthetic approach (Scheme 2.9) to solving the mechanism of inactivation was discontinued. We envisaged the possibility of determining whether CO_2 was being given off during the inactivation process by the use of ^{14}C labelled (2S)-serine O-sulphate.



Scheme 2.9. The attempted synthesis of PLP condensation products.

2.2.2 Synthesis of O-sulphate esters

At the start of the project, the preparation of the O-sulphate esters (**21**, **59-61**) was attempted according to the method of Tudball.[192] This method was successful, but time consuming. The amino acid was added to concentrated sulphuric acid and the mixture stirred *in vacuo* until the amino acid had completely dissolved. The solution was diluted by the addition of ice, and the excess sulphuric acid destroyed by the addition of saturated barium hydroxide. The precipitated barium sulphate was removed by centrifugation, and the aqueous solution concentrated to dryness. The product was purified using an ion exchange column. The maximum yield achieved was 50%. For (2S)-serine O-sulphate, the proton NMR spectrum contained a triplet at 3.53 ppm for the α-proton and a multiplet at 4.18 ppm for the methylene protons. The carbon NMR signals at 178.4, 71.5, 47.7 ppm correspond to the acid carbonyl, 2CH and $3CH_2$ respectively. Both the proton and the carbon NMR spectra were run in D_2O/NaOD. The

$[\alpha]_D$ was found to be -10.25° (c. 1 in H_2O) compared to the literature value of -9.5° (c. 5.0 in H_2O).[192] A more recent paper by Previero and coworkers[193] described an alternative method. Accordingly, the amino acid was dissolved in trifluoroacetic acid and chlorosulphonic acid was added dropwise using a syringe. The O-sulphate ester precipitated as the reaction occurred. When the addition was complete, the mixture was allowed to stand for 20 min to allow for further precipitation. The excess chlorosulphonic acid was destroyed by the addition of ethanol, and ether was added to complete the precipitation. The solid was filtered, washed with diethyl ether, and recrystallised from water/ethanol.

In our hands we did not obtain the high yields quoted in the paper (92%) but the yields of pure product were 60-65%, slightly better than those using the method of Tudball.[192] The method was however much faster than Tudball's synthesis and for this reason the method was adopted for all the large scale synthesis. Previero found that the isolated product was the hydrochloride salt, however when the product was isolated in our laboratories, microanalysis indicated that the product was the free base (or zwitterion). The proton and carbon NMR spectra and optical rotation measurements were in agreement with those obtained for the product using Tudball's method.

The O-sulphate esters of (2R)-serine, (2S,3R)- and (2S,3S)-threonine were prepared in an identical manner to (2S)-serine O-sulphate using the method of Tudball[192] in 30-50% yield. The proton NMR spectrum of (2R)-serine O-sulphate contained a triplet at 3.55 ppm for the α-proton and a multiplet at 4.2 ppm for the C-3 methylene protons. The optical rotation was found to be +6.3° (c. 1 in H_2O). The proton NMR spectrum of (2S,3R)-threonine O-sulphate exhibited a doublet at 1.38 ppm for the C-4 protons, a doublet at 3.22 and a double quartet at 4.55 ppm for the α-proton and C-3 proton

respectively. The optical rotation was determined as -20° (c. 9.7 in H_2O). The proton NMR spectrum of (2S,3S)-threonine O-sulphate exhibited a doublet at 1.1 ppm for the C-4 protons, a doublet at 3.32 and a double quartet at 4.05 ppm for the α-proton and C-3 proton respectively. The optical rotation was determined as +0.8° (c. 0.56 in H_2O). All of the above proton NMR were obtained in basic D_2O.

2.2.3 Preliminary studies with glutamate decarboxylase

In previous studies the inactivation reaction of GAD and AAT[80,109] by (2S)-serine O-sulphate had been followed by electronic absorption spectroscopy. On addition of (2S)-serine O-sulphate to the enzyme the absorption maximum at 420 nm was reduced in intensity and a new band at 336 nm appeared. Although this method is easy to perform, it requires large quantities of GAD to detect the spectral bands and the activity of the enzyme is not measured directly. We have also had difficulty obtaining good uv/vis spectra of the glutamate decarboxylase purchased from Sigma.

Since we wanted to measure the enzyme activity directly we chose to incubate the inhibitor with a known amount of enzyme and measure the enzyme activity at various time intervals using a radiochemical method (Chapter 4).

Experiments in our laboratory showed that (2S)-serine O-sulphate (**21**) inactivated GAD over several hours at 2-10 mM concentrations. The values of the kinetic parameters, K_i and k_{inact}, were not determined at this stage. (2R)-Serine O-sulphate (**59**) was also found to inhibit the enzyme albeit at a slower rate than the (2S)-antipode, this was very interesting and merited further investigation. The O-sulphate esters of (2S,3R)-threonine (**60**) and (2S,3S)-threonine (**61**) were incubated with the

enzyme at concentrations up to 50 mM, no inactivation of the enzyme was observed.

The inactivation of GAD by (2R)-serine O-sulphate (**59**) was an unexpected result, however it was possible that the (2R)-serine O-sulphate was contaminated with (2S)-serine O-sulphate and that the inactivation was due to the contaminant. The optical rotations of the (2S)- and (2R)- enantiomers suggested that the (2R)-isomer was pure, but the purity was further verified through the synthesis of the (1'S,4'R)-camphanamides of each enantiomer of serine O-sulphate (**62,63**). Each enantiomer of serine O-sulphate was reacted with freshly prepared camphanoyl chloride under Schotten-Bauman conditions.[194] Due to the highly polar nature of the serine O-sulphate, it was not possible to extract the camphanamide into the organic layer (even at low pH), so the aqueous layer was concentrated to dryness *in vacuo* to yield the serine O-sulphate camphanamide contaminated with inorganic salts. Inspection of the proton NMR spectrum in D_2O/NaOD at 200 MHz clearly showed that the signal corresponding to the α-proton in the (2S)-serine O-sulphate (1'S,4'R)-camphanamide (**62**) occurs at 4.55 ppm. In the proton NMR spectrum for the (1'S,4'R)-camphanamide of (2R)-serine O-sulphate (**63**), the equivalent signal occurred at 4.47 ppm. Comparison of the two proton NMR spectra indicated that there was no contamination. A proton NMR spectrum of a mixture of the two samples clearly showed two independent signals (Figure 2.2).

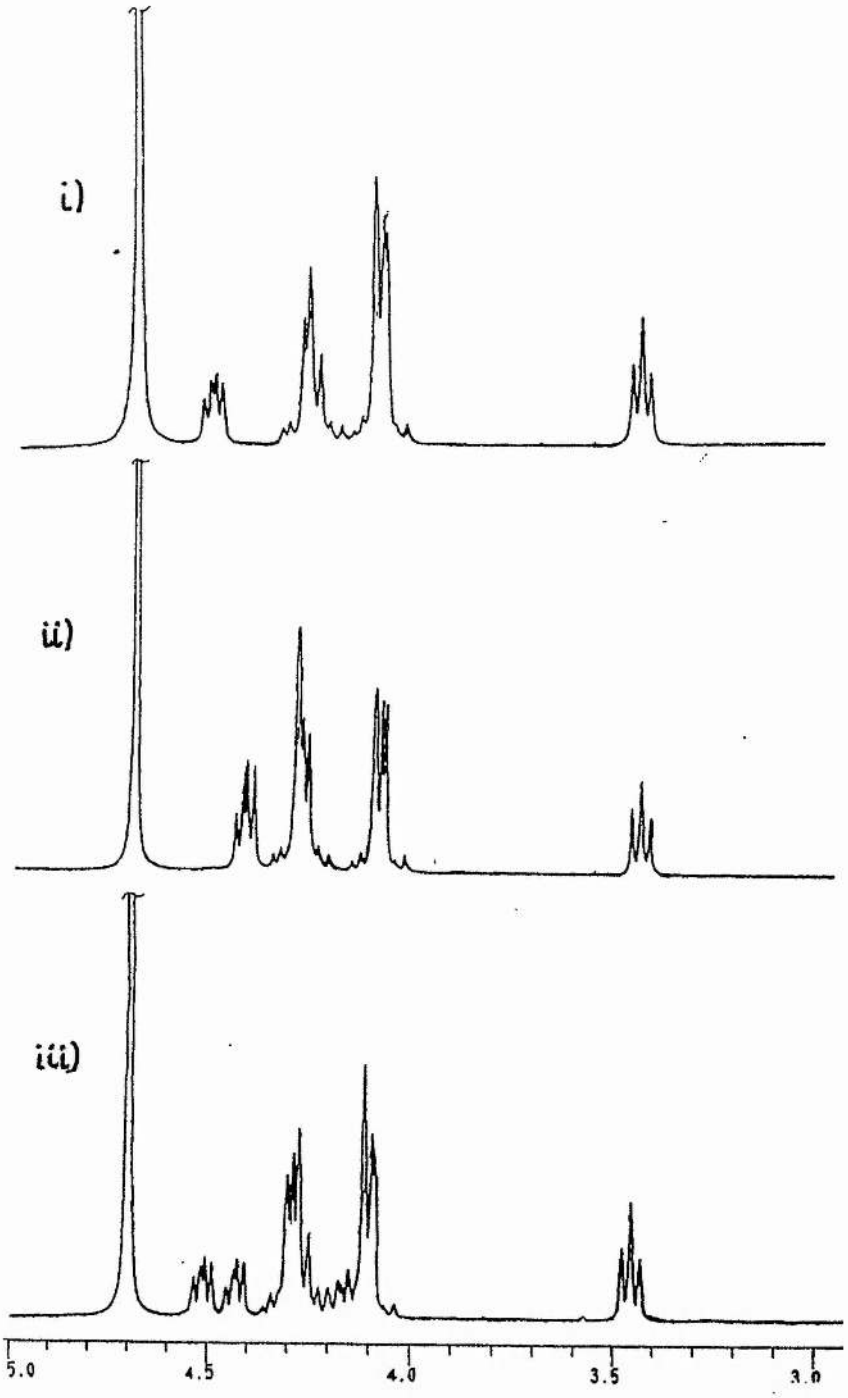
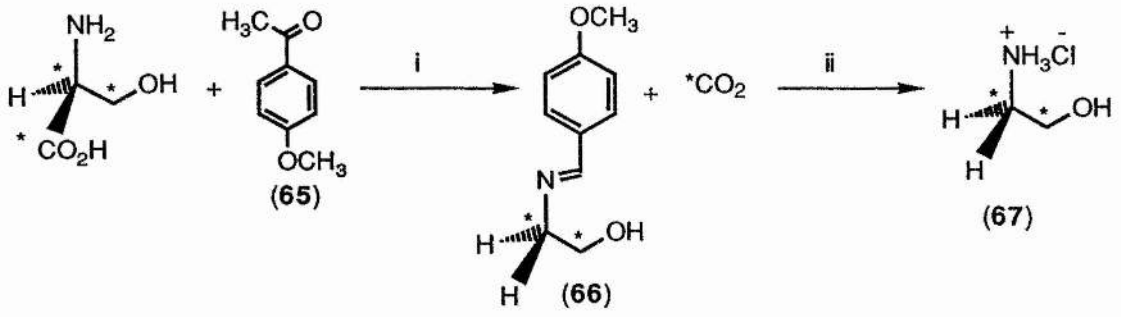


Figure 2.2. The 200 MHz proton NMR spectra in D_2O/NaOD of i) (2S)-serine O-sulphate (1'S,4'R)-camphanamide (**49**), ii) (2R)-serine O-sulphate (1'S,4'R)-camphanamide (**50**) and iii) a mixture of (2S)-serine O-sulphate (1'S,4'R)-camphanamide and (2R)-serine O-sulphate (1'S,4'R)-camphanamide.

2.2.4 Studies with (2S)-serine O-sulphate

In order to probe the mechanism of the inactivation further we needed to discover whether any carbon dioxide was liberated during the inactivation process. Carbon dioxide is known to react with barium hydroxide to form insoluble barium carbonate as a white precipitate. Initially the inactivation experiment was carried out so that any gas liberated was passed through barium hydroxide solution. No cloudiness was seen, but the quantity of gas liberated may not have been enough to form a precipitate visible to the naked eye. To circumvent the problem, radiolabelled carbon dioxide could be used. The radioactive carbon dioxide could be trapped in barium hydroxide and the amount of radioactivity measured using a scintillation counter. (2S)-[U-^{14}C]-Serine was available commercially, and was converted to (2S)-[U-^{14}C]-serine O-sulphate (**64**) using methods described in section 2.2.2. Before the inactivation experiment was performed it was necessary to verify that the (2S)-[U-^{14}C]-serine was uniformly labelled. The (2S)-[U-^{14}C]-serine was degraded following the method of Gani,[195] by heating it with 4-methoxyacetophenone (**65**) (Scheme 2.10) in a stream of nitrogen. The 4-methoxyacetophenone reacts with the (2S)-serine to form an imine which decarboxylates to yield a second imine (**66**). Refluxing with conc. HCl hydrolyses the decarboxylated imine to yield ethanolamine.HCl (**67**) and regenerate the 4-methoxyacetophenone (**52**). The effluent gas was passed through barium hydroxide. The amount of radioactivity in the carbon dioxide and ethanolamine was detected using a scintillation counter. It was found that the amount of radioactivity in the ethanolamine corresponded to twice that found in the carbon dioxide proving that the serine was indeed uniformly labelled.



Reagents: i) N_2, 160-190 °C;
ii) HCl (aq), reflux, 2 h.

Scheme 2.10. The chemical decarboxylation of (2S)-serine.

(2S)-[U-^{14}C]-Serine O-sulphate (**64**) was incubated with a solution of the enzyme in a Warburg apparatus in such a way that any gas liberated was passed through a solution of barium hydroxide. It was found that there was no increase in the level of radioactivity in the barium hydroxide solution. There was also no detectable decrease in the radioactivity level of the enzyme incubation solution. As no carbon dioxide was liberated, the C^{α}-CO_2H bond is not broken during the inactivation reaction. Therefore a different group must be lost, most plausibly a proton, thus adding weight to Metzler's theory.[109]

2.3 Deuteriated (2S)-serine O-sulphate as a mechanistic probe

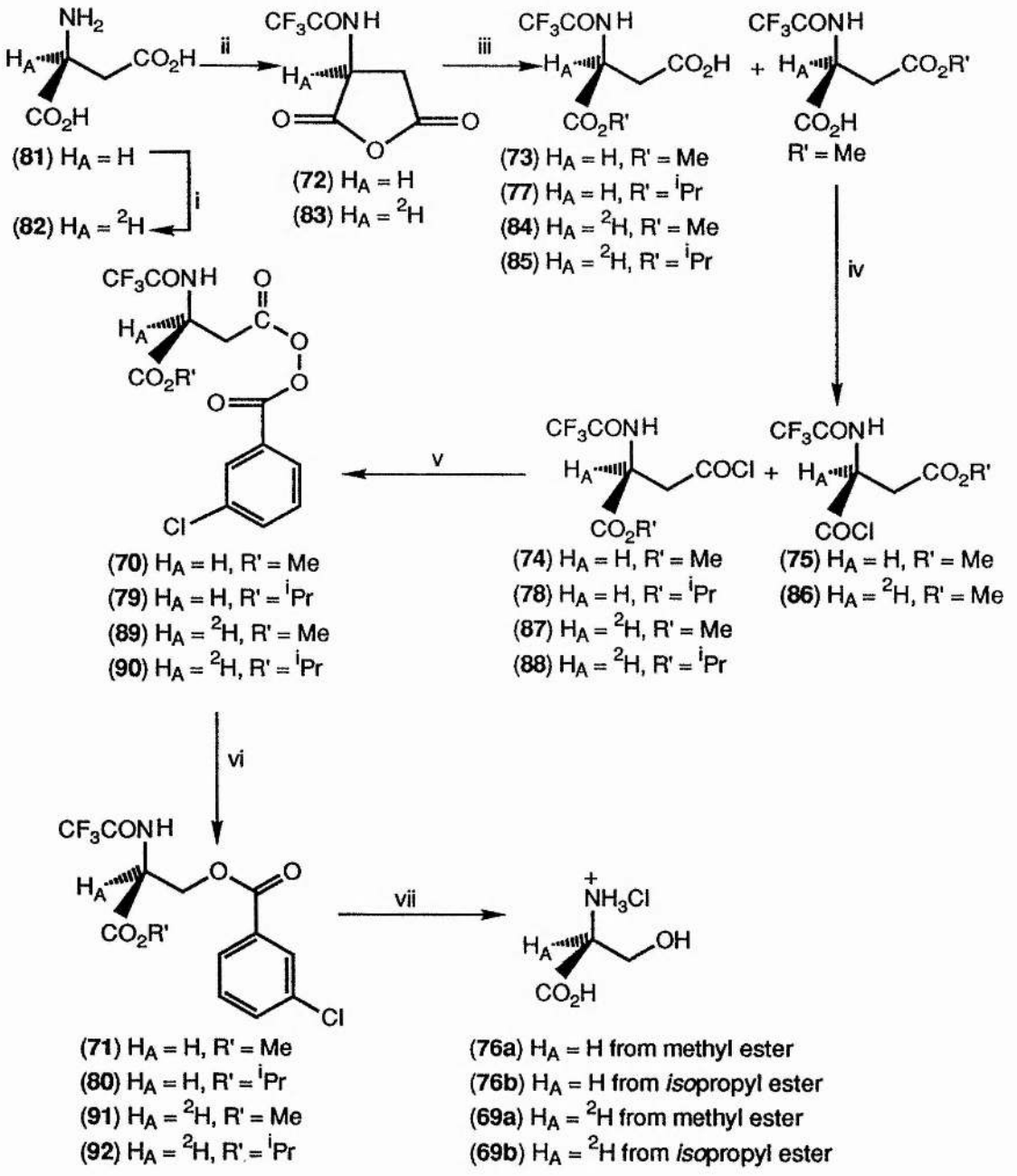
If Metzler's[109] mechanistic theory and the structure for the inactivated product were correct, the inactivation reaction would proceed via the elimination of H_2SO_4. It is possible that the proton abstraction would be the rate-limiting step in the inactivation process. As the activation energy required to break a C-H bond is lower than that for C-D bond cleavage this possibility could be tested using kinetic isotope effect techniques. A synthesis of (2S)-[2-^{2}H]-serine O-sulphate (**68**) was, therefore, required. The conversion of (2S)-serine to (2S)-serine O-sulphate had already been achieved and

so we needed to prepare (2S)-[2-^{2}H]-serine (**69**) for conversion to the required O-sulphate ester.

2.3.1 Synthesis of (2S)-[2-^{2}H]-serine O-sulphate from (2S)-aspartic acid

(2S)-Serine had been prepared from (2S)-aspartic acid by Young[196,197] in the 1980's. This route (Scheme 2.11) could be used for the synthesis of deuteriated (2S)-serine as the deuterium could be introduced into the α-centre of the (2S)-aspartic acid by an enzymic exchange reaction.[198] Incubation of (2S)-aspartic acid with the enzyme aspartate aminotransferase in deuterium oxide with pyridoxal 5'-phosphate at 37 °C for 3 days caused the exchange of the α-proton for deuterium with retention of configuration and ≈90% incorporation of deuterium. The reaction progress was followed by proton NMR spectroscopy, where integration of the signal corresponding to the α-proton (4.4 ppm in acidic D_2O) decreased with respect to time. The mechanism involves the simple deprotonation-deuteriation of the C^{α} position of (2S)-aspartic acid within the chiral environment provided by the protein, see section 1.2.1.

The important step in the reaction pathway (step v, scheme 2.11) published by Young[197] is the formation of a diacyl peroxide (peroxy-anhydride) (**70**). Heating this compound causes a radical cage rearrangement to occur, with loss of carbon dioxide from the peroxy-anhydride (**70**) to form (2S)-methyl N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**71**).



Reagents: i) ^2H_2O, PLP, AAT, pH 7.25, 37 °C, 3 days, 72%; ii) TFAA (10 eq), THF, 0 °C to room temp, 2 h; iii) a) MeOH, 0 °C to room temp, 20 min, 85% of mixture of α and β esters over 2 steps or b) ^{i}PrOH, 37 °C, 12 h, 55% of a ester over 2 steps; iv) $SOCl_2$, reflux, 2 h, 10-99%; v) *m*CPBA, pyridine (1.5 eq), ether, 0 °C, 4 h, 40%; vi) CCl_4, 6 days, 20%; vii) 5 M HCl, 2 h, 90%.

Scheme 2.11. The synthesis of (2S)-[2-2H]-serine from (2S)-aspartic acid (**81**).

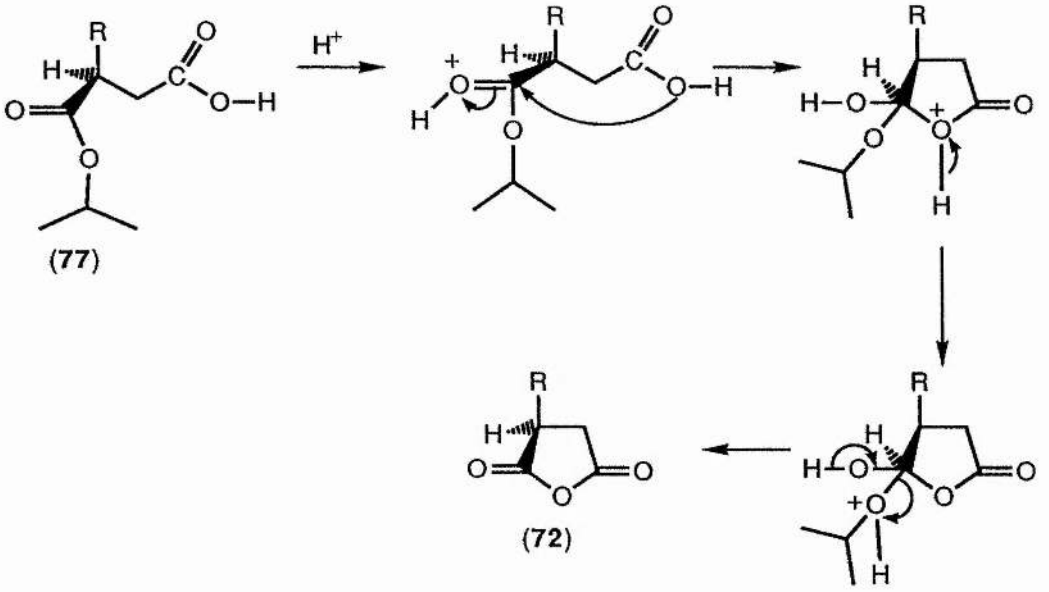
The initial part of the synthesis was repeated successfully. (2S)-Aspartic acid was treated with trifluoroacetic anhydride, and the intermediate cyclic anhydride (**72**) was treated with methanol. The cyclic anhydride was not fully characterised. The infrared spectrum showed an absorption at 1780 cm^{-1} corresponding to the anhydride carbonyl stretching frequency. The proton NMR spectrum in DMSO showed an ABX system with the signal for the methylene protons centred at 3.25 ppm and the α-proton signal centred at 5.05 ppm. The methylene protons of (2S)-aspartic acid in basic deuterium oxide were at 3.48 ppm in the proton NMR spectrum. The proton NMR spectrum of the product (**73**) of the reaction of the anhydride (**72**) with methanol showed two distinct methoxy singlets (the α-signal at 3.85 ppm and the β-signal at 3.7 ppm), and the signal for the methylene protons at 3.02 ppm. The α-ester accounted for about 60% of the total mixture. The monoesters (**73**) were converted into a mixture of the corresponding acid chlorides (**74,75**) which could be separated by fractional recrystallisation from diethyl ether, although this was not a reliable process. At this stage a sample of the pure α-methyl ester was obtained by hydrolysis of the pure β-acid chloride.

Young's published work for the later part of the synthesis used the ethyl ester.[197] It was decided not to use the ethyl esters as they are much harder to crystallise than the methyl esters.[199] As the methyl ester had been successfully crystallised it was decided to continue the synthesis with the methyl esters. The next step was the formation of the diacyl peroxide (**70**). The acid chloride (**74**) from (2S)-α-methyl N-trifluoroacetyl-aspartate (**73**) was reacted with *m*CPBA to yield the diacyl peroxide (**70**). The proton NMR spectrum of the product (**70**) contained a multiplet in the aromatic region corresponding to the *m*-chlorobenzoyl fragment. The signals for the hydrogen atoms on C-3 which are part of an ABX system can be differentiated.

The published rearrangement step[197] was complete after reflux in toluene for 3 hours, however this was not found to be the case for the methyl ester. In attempts to overcome the problem, several other solvents with varying boiling points were tried, and also varying the length of the reaction time. We found that for the methyl ester diacyl peroxide (**70**), the rearrangement reaction occurred smoothly on refluxing in tetrachloromethane for 6 days. The product (**71**) was purified by column chromatography on silica (to remove a small amount of rearranged product that had been hydrolysed). Hydrolysis of (2S)-methyl N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**71**) in hydrochloric acid yielded (2S)-serine (**76a**). Studies carried out by Young and coworkers[197] concluded that this was the first reported decarboxylative rearrangement of a diacyl peroxide in which a chiral primary carbon centre migrates non-stereospecifically. This supports a radical cage mechanism for the process and is in keeping with previous studies using ^{18}O.[200-202] It is evident that chirality cannot be maintained within the cage whereas, in the ionic mechanism,[203,204] secondary diacyl peroxides rearrange with retention of stereochemistry.

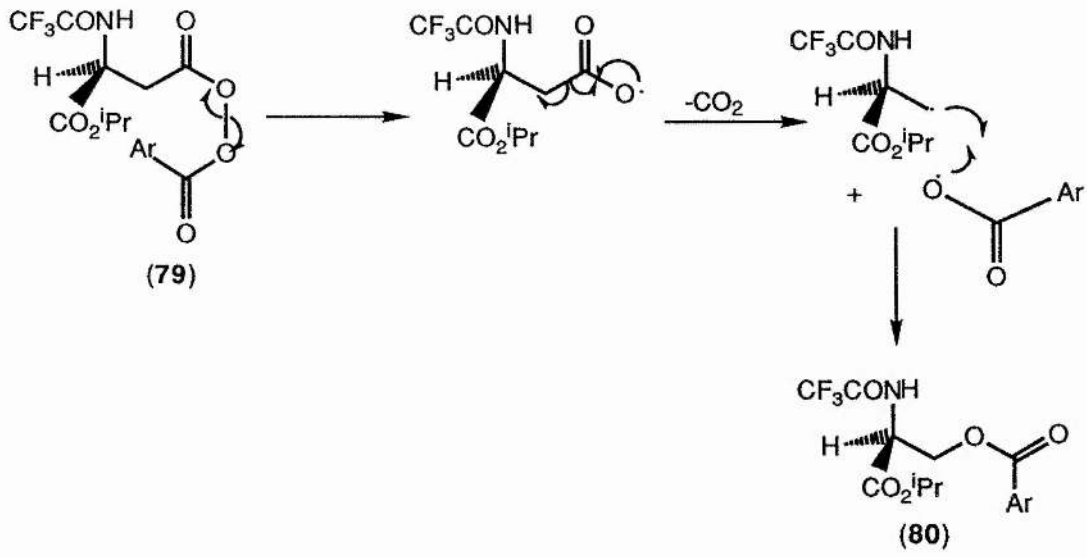
The difficult step of separating the mixture of acid chlorides (**74,75**) needed to be overcome as acid chlorides are inherently unstable. They are easily hydrolysed in traces of acid and so any recrystallisation step could be difficult. One possible approach was to open the cyclic anhydride (**72**) regioselectively. *Iso*propanol was tried and the reaction was found to be regiospecific as only one set of signals for the *iso*propyl group were seen in the proton NMR spectrum, at 1.27 ppm for the methyl protons and at 5.11 ppm for the *iso*propyl C-2 proton. The α-carbonyl group is more susceptible to nucleophilic attack due to the electron withdrawing effect of the trifluoroacetyl protecting group and the extra bulk of the *iso*propanol makes it a more selective nucleophile. The α-*iso*propyl ester N-trifluoroacetyl β-aspartic acid (**77**) was

converted to the acid chloride (**78**) by refluxing in excess thionyl chloride for one hour. However, this step was not reliable as the presence of traces of acid in the thionyl chloride caused the reformation of the cyclic anhydride (**72**) (Scheme 2.12). Several other methods were tried to improve this step. Attempts to form the acid chloride using, for example, phosphorus pentachloride, phosphorus trichloride, oxalyl chloride, triphenyl phosphine in carbon tetrachloride, shorter reaction times, lower temperatures, reaction in an inert atmosphere, higher purity thionyl chloride, and smaller equivalents of thionyl chloride in other solvents, gave mixed results. The most successful of these was using 1.1 equivalents of thionyl chloride in toluene at reflux for 6 hours. This gave a mixture of products, but yields of 70% of the required acid chloride were deemed to be sufficient as the product could be purified after the next step. Since this work was finished another member of the group has discovered a reliable route for the synthesis of the acid chloride (**78**).[205] α-*Iso*propyl N-trifluoroacetyl β-(2S)-aspartic acid (**77**) was dissolved in dry ether, and the solution cooled to 0 °C. One equivalent of phosphorous pentachloride was added to the solution. After 2 hours, the product was precipitated by the addition of petroleum ether. Recrystallisation from diethyl ether and petroleum ether gave the pure acid chloride (**78**) in 80% yield. The infrared spectrum of the acid chloride contained a band at 1810 cm^{-1} which was assigned as the acid chloride carbonyl stretching frequency. The methylene signal in the proton NMR spectrum had shifted to 3.5-3.73 ppm, compared with 2.93-3.25 ppm for the same protons in the acid (**77**).



Scheme 2.12. The formation of the cyclic anhydride (**72**) from the ester (**77**) in the presence of traces of acid.

The diacyl peroxide (**79**) was formed by stirring the acid chloride with one equivalent of pyridine and *m*CPBA in dry ether at 0 °C for 4 hours. The signal for the methylene protons in the NMR spectrum occurs as a multiplet at 3.1-3.4 ppm, and the aromatic protons occur as a multiplet at 7.4-8.05 ppm. The rearrangement step was not straightforward, as the reaction was slower than for the methyl ester (**70**). A mixture of products was formed and only a small amount was the required (2S)-α-*iso*propyl-N-trifluoroacetyl (m-chlorobenzoyl)-serinate (**80**), the rest of the mixture being α-*iso*propyl-N-trifluoroacetyl-(2S)-serine, and unreacted starting material. The extra bulk of the *iso*propyl group compared to the methyl group may cause the rearrangement reaction (Scheme 2.13) to occur at a slower rate. As some hydrolysed product is formed it would appear that under the reaction conditions the small amount of rearranged product (**80**) is hydrolysed.



Scheme 2.13. The rearrangement of the diacyl peroxide (**79**).

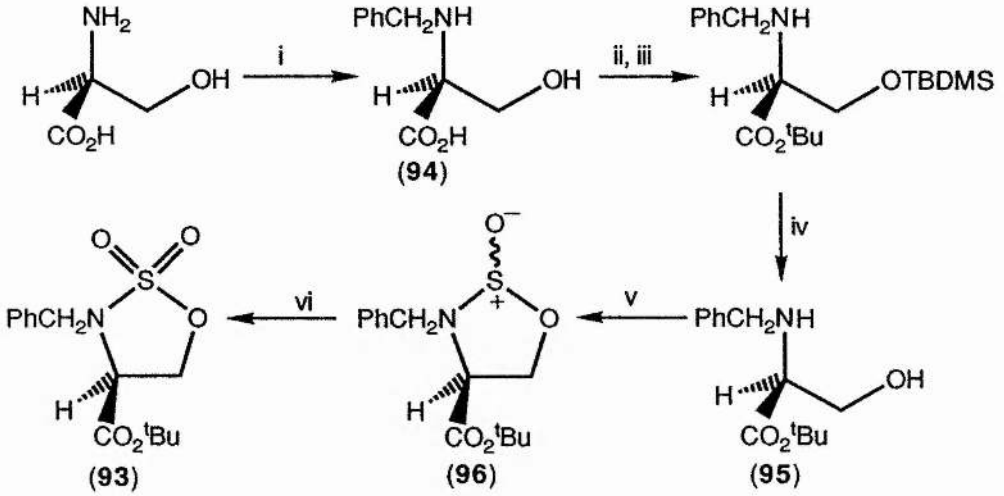
A small amount of the required product (**80**) was isolated by column chromatography (7:3 petroleum ether: ethyl acetate), and hydrolysed with 5 M hydrochloric acid to yield (2S)-serine hydrochloride (**76b**). The free base was obtained by refluxing the hydrochloride salt in ethanol and propylene oxide for 15 min. The product co-ran with authentic serine by tlc. The proton NMR spectrum in D_2O/NaOD showed the presence of an ABX system with the signal for the methylene protons centred at 3.65 ppm and the α-proton signal centred at 3.32 ppm.

A small amount of (2S)-[2-^{2}H]-serine (**69a**) was prepared via the methyl esters of (2S)-aspartic acid (**81**) but the problems encountered and the low yields prevented us from using this route for the large scale synthesis of (2S)-[2-^{2}H]-serine (**69**).

2.3.2 An alternative route to deuteriated serines via cyclic sulphamidates

Baldwin *et al.*[206] have prepared cyclic sulphamidates from serine as new synthetic precursors for β-functionalised α-amino acids. It was hoped that we could make use of these cyclic sulphamidates to prepare α-deuteriated serines in a stereoselective synthesis.

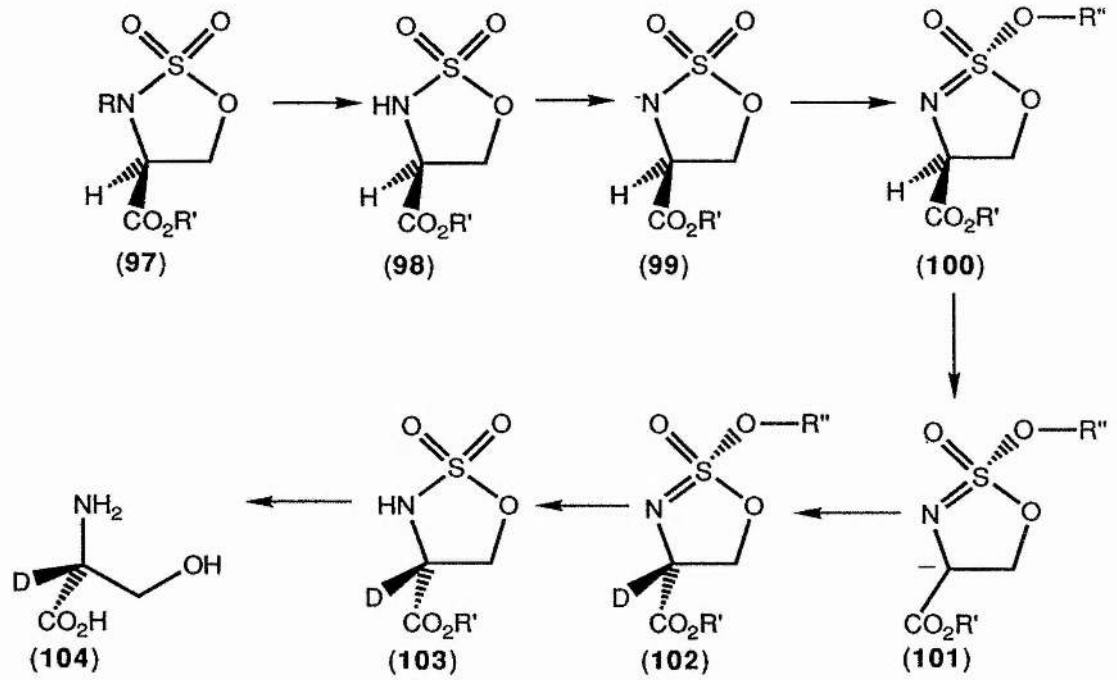
Baldwin[206] prepared N-benzyl-2,2-dioxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *tert*-butyl ester (**93**) from (2S)-serine in 6 steps with 32% overall yield (Scheme 2.14). In the synthesis described, N-benzyl serine (**94**) was prepared according to the method of Ohfune *et al.*[207] by a one pot reaction with benzaldehyde followed by sodium cyanoborohydride reduction of the intermediate imine in 73% yield. Protection of the alcohol with TBDMSCl, followed by reaction with $CCl_3C(=NH)O^tBu$ and removal of the protecting group with tetrabutyl ammonium fluoride yielded (2S)-N-benzyl serine *tert*-butyl ester (**95**) in 62% yield from N-benzyl-(2S)-serine. The cyclisation of (**95**) was effected by reaction with thionyl chloride (1 eq) and triethylamine (2 eq) in toluene in 82% yield. The mixture of oxo-oxathiazolidines (**96**) was oxidised to the dioxo-oxathiazolidine (**93**) with sodium periodate and $RuCl_3$ as a catalyst in 91% yield. The key signals in the proton NMR spectrum of the sulphamidate (**93**) are a double doublet at 3.94 ppm for the C-4 proton (the α-proton in the serine moiety), and a double doublet at 4.59 and 4.64 ppm, one for each of the C-5 protons (the methylene group from the serine moiety).



Reagents: i) Benzaldehyde (1.1 eq), $NaBH_3CN$ (0.7 eq), MeOH, rt, 18 h, 73%;
ii) $^tBuMe_2SiCl$ (1.1 eq), imidazole (2.2 eq), DMF, rt, 4 days;
iii) $\{CCl_3C(=NH)O^tBu\}$ (10 eq), $BF_3.Et_2O$ (cat.), DCM:cyclohexane (2:1), rt, 18 h, 76%; iv) $^nBu_4N^+F^-$ (1.0 M in THF, 1.5 eq), THF, O °C, 1 h, 82%;
v) $SOCl_2$ (1.0 eq), Et_3N (2.0 eq), toluene, 0 °C, 92%; vi) $NaIO_4$ (1.0 eq), $RuCl_3.H_2O$ (cat.), MeCN:H_2O (1:1), rt, 91%.

Scheme 2.14. Baldwins synthesis of the sulphamidate (**93**).

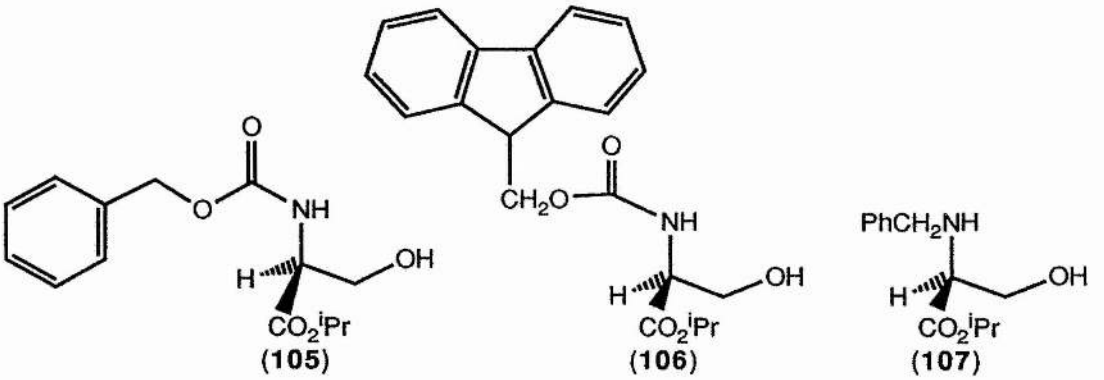
It was hoped that the deuteriated serine could be formed from the N-protected dioxo-oxathiazolidine carboxylic acid ester (**97**) by the following sequence of reactions (Scheme 2.15). First, the removal of the N-protecting group from (**97**) without disruption of the ring structure to yield (**98**). Second, the deprotonation of (**98**) at the nitrogen giving (**99**) followed by an electrophilic reaction at one of the sulphur oxygens with a suitable electrophile introducing a second chiral centre to the system forming (**100**). Third, removal of the α-proton from (**100**) resulting in the formation of a planar trigonal centre at C-4 (**101**). Fourth, stereoselective reprotonation/deuteriation on the opposite face to the alkylated oxygen forming (**102**). Removal of the oxygen protecting group yielding (**103**) followed by ring opening to yield the required serine (**104**).



Scheme 2.15. Theoretical synthesis of (2R)-[2-^{2}H]-serine from a cyclic sulphamidate (**97**).

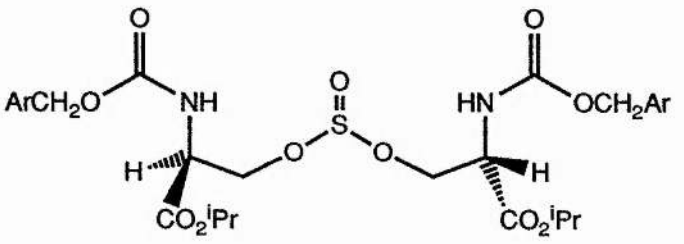
We wanted to be able to remove the N-protecting group from N-protected dioxo-oxathiazolidine carboxylic acid ester (**97**) easily. We decided to prepare *iso*propyl esters, by direct reaction with *iso*propanol and thionyl chloride, and avoid the need to protect the -OH group. To this end N-benzyloxycarbonyl-(2S)-serine *iso*propyl ester (**105**), N-(9-fluorenylmethyloxycarbonyl)-(2S)-serine *iso*propyl ester (**106**) and N-benzyl-(2S)-serine *iso*propyl ester (**107**) were prepared from (2S)-serine. N-BOC-(2S)-Serine *iso*propyl ester (**105**) and N-FMOC-(2S)-serine *iso*propyl ester (**106**) were prepared from (2S)-serine *iso*propyl ester (**108**) by reaction with triethylamine and benzylchloroformate, and 9-fluorenylmethyloxycarbonylchloride respectively. N-Benzyl-(2S)-serine *iso*propyl ester (**107**) was prepared from N-benzyl-(2S)-serine (**94**) by reaction in *iso*propanol with 5 equivalents of thionyl chloride under reflux for 3 days. An easier method was found for the preparation of N-benzyl-(2S)-serine (**94**),[208] in which the reduction step was carried out with sodium borohydride instead of sodium cyanoborohydride. The reaction was complete after 4 hours and the product was

isolated in 75% yield. The proton NMR spectrum contained a triplet at 3.61 ppm for the α-proton, a double doublet at 3.86 ppm for the serine methylene protons, a singlet at 4.18 and 4.20 ppm for the benzyl methylene protons, and a singlet at 7.38 ppm for the aromatic protons.



(105) **(106)** **(107)**

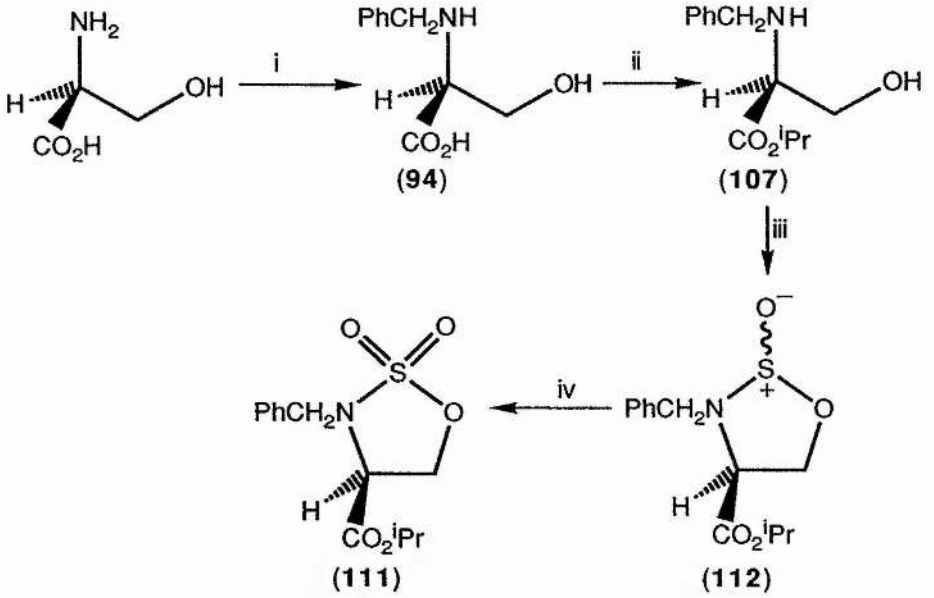
Attempts to cyclise the BOC and F-MOC derivatives (**105,106**) under the conditions of Baldwin resulted in the formation of bis sulphite esters (**109,110**) (dimers of the starting materials). These compounds exhibited peaks for $[M+H]^+$ at 609 and 785 respectively.



(109) Ar = C_6H_5
(110) Ar = $C_{13}H_9$

The N-benzyl-(2S)-serine *iso*propyl ester (**107**) was converted to N-benzyl-2,2-dioxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *iso*propyl ester (**111**) via the diastereomeric mixture of oxo-oxathiazolidines (**112**) following the method of Baldwin[206] in 60% yield

(Scheme 2.16). The key signals in the proton NMR spectrum are for an ABX system with the signal for the C-4 proton centred at 4.02 ppm and the signal for the C-5 methylene protons centred at 4.65 ppm.



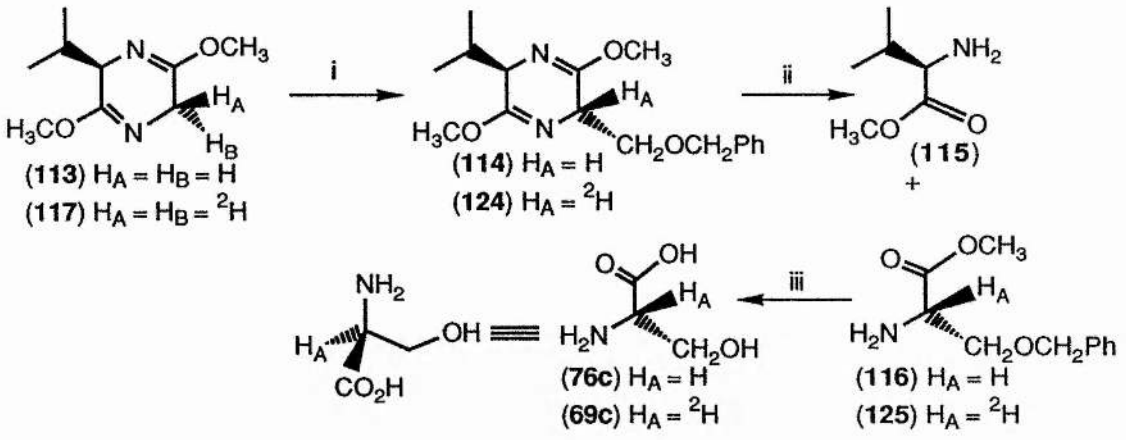
Reagents: i) 2M NaOH, benzaldehyde (2.0 eq), $NaBH_4$ (0.6 eq), 4 °C, 3 h, 75%; ii) $SOCl_2$ (5eq), 0 °C for 30 mins then reflux 3 days, 40%; iii) $SOCl_2$ (1.0 eq), Et_3N (2.0 eq), toluene, 0 °C, 74%; iv) $NaIO_4$ (1.0 eq), $RuCl_3.H_2O$ (cat.), $MeCN:H_2O$ (1:1), rt, 81%.

Scheme 2.16. The synthesis of N-benzyl-2,2-dioxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *iso*propyl ester (**111**).

This methodology was not developed any further after the discovery that the work by Schollkopf, described below (Section 2.3.3), could be exploited to give the required deuteriated serines.

2.3.3 Synthesis of (2S)-[2-^{2}H]-serine O-sulphate using Schollkopf methodology

Schollkopf had discovered that chiral 2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazines can be stereospecifically alkylated at C-6 on the opposite face to the bulky *iso*propyl group at C-3.[209,210] A stereospecific synthesis of (2R)-serine had been published (Scheme 2.17 shows the same synthesis of (2S)-serine).[209] The bis-lactim ether (**113**) formed from (2R)-valine and glycine can be deprotonated at C-6 using *n*-butyl lithium at -80 °C and the anion stereospecifically alkylated by the addition of benzylchloromethylether on the opposite face to the bulky *iso*propyl side chain of the (2R)-valine leading to (S)-stereochemistry at the C-6 centre of the product (**114**). Acid hydrolysis of the alkylated dihydropyrazine (**114**) results in the regeneration of (2R)-valine as the methyl ester (**115**) and the formation of (2S)-O-benzyl serine methyl ester (**116**). Separation of the methyl esters (**115,116**) was effected by flash column chromatography on silica gel. Acid hydrolysis of the (2S)-O-benzyl serine methyl ester (**116**) resulted in the formation of (2S)-serine.HCl. The free base (**76c**) was obtained by refluxing the HCl salt in ethanol and propylene oxide.



Reagents: i) *n*BuLi (1 eq) , THF, -90°C to -60°C, then $PhCH_2OCH_2Cl$ (1.5 eq), THF, -90°C, stir, 16 h, 60%; ii) 0.2 M HCl (2 eq), rt, stir, 16 h then separation on flash silica 19:1 Et_2O:EtOH, 70%; iii) 5 M HCl, reflux, 3 h then EtOH, propylene oxide, reflux, 15 min, 55%.

Scheme 2.17. The synthesis of (2S)- and (2S)-[2-^{2}H]-serine using Schollkopf chemistry.

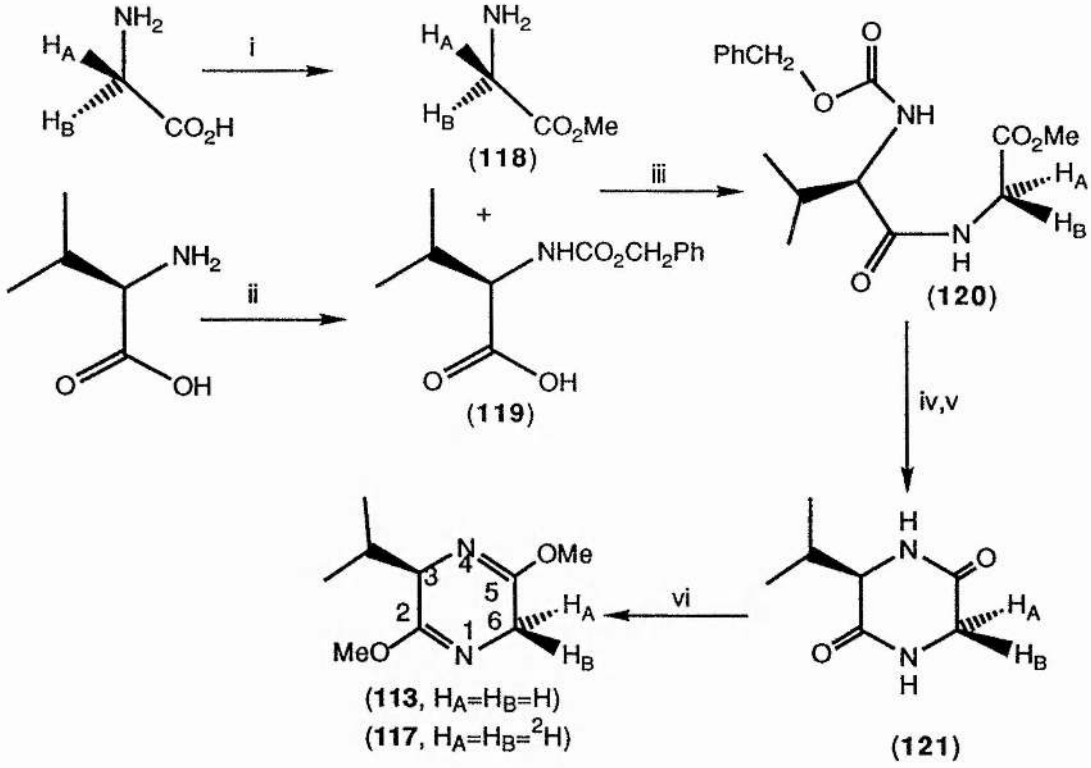
Although the methodology had been developed for the stereoselective synthesis of serine, a method was required for the introduction of deuterium at C-6. The dideuteriated dihydropyrazine (**117**) could be synthesised from dideuteriated glycine and valine. Initially (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) was prepared from glycine and (2R)-valine in up to a maximum overall yield of 62% (from glycine) (Scheme 2.18). Glycine was converted to the methyl ester (**118**) by reflux in methanol with thionyl chloride in dry methanol in 100% yield, the proton NMR spectrum contained a singlet for the methyl ester at 3.76 ppm and a singlet for the methylene protons at 3.86 ppm. (2R)-valine was N-protected with benzyl chloroformate in 88% yield, and the resulting N-carbobenzoxy-(2R)-valine (**119**) was reacted with glycine methyl ester (**118**) to form the dipeptide N-carbobenzoxy-(2R)-valine-glycine methyl ester (**120**) in 87% yield. This compound exhibited the required proton NMR spectrum and had infrared absorptions at 1645 and 1535 cm^{-1} indicative of the presence of an amide bond. The mass spectrum of the compound gave the $[M+H]^+$ at 323. Removal of the nitrogen protecting group was effected by hydrogenation with 10% Pd/C in dry

methanol:dichloromethane 1:3, this caused partial cyclisation to *cyclo*-((2R)-valine-glycine) (**121**) (a diketopiperazine). The cyclisation was completed by refluxing the mixture of products from the hydrogenation step for 12 hours in dry toluene, the precipitated product was obtained by filtration and dried thoroughly. The proton NMR spectrum showed a doublet at 0.87 and 0.96 ppm with *J* 7 Hz for the valine methyl groups, a double septet at 2.2 ppm for the proton adjacent to the valine methyl groups, a doublet at 3.88 ppm, *J* 3 Hz for the ring C-3 proton and a double doublet at 3.9 and 4.09 ppm for the C-6 protons. The conversion of the diketopiperazine (**121**) to the bis-lactim ether (**113**) with trimethyloxonium tetrafluoroborate in dry dichloromethane gave the most variable yields (66-89%) and was wasteful of trimethyloxonium tetrafluoroborate. It was impractical to synthesise large quantities of the bis-lactim ethers and so they were purchased commercially. Synthesis of the deuteriated analogues in this manner would require large quantities of deuteriated glycine, and would be very costly.

Several methods for introducing deuterium at C-6 of the commercially purchased bis-lactim ethers were considered using the known kinetic preference for C-6 proton abstraction.[209,210] Methods based upon repeatedly quenching the *n*BuLi generated anion of the dihydropyrazine (**113**) with ^2H_2O or CH_3O^2H were quickly abandoned due to the formation of an array of by-products after the first cycle. This method also suffered from the potential problem of low selectivity for removing protium from the singly deuteriated compound in the second cycle of anion formation.

Stirring compound (**113**) in ^2H_2O in the presence of KOH under a variety of conditions also proved to be of little or no utility. At room temperature, the exchange of the C-6 protons was almost undetectable after 3 hours, and at higher temperatures, several

by-products were formed. However, under optimised conditions, CH_3O^2H:^2H_2O (10:1, v/v), refluxing in the presence of 1 equivalent of KO^2H, the C-6 deuteriation of a 1 M solution of compound (**113**) proceeded smoothly, without the formation of side products or C-3 deuteriated material, and was complete within 3 h, (Scheme 2.19, Figure 2.3).



Reagents: i) $SOCl_2$ (1.2 eq), MeOH, 0°C, then reflux 30 min, 100%;
ii) $PhCH_2OCOCl$ (1.1 eq), $NaHCO_3$ (3.5 eq), H_2O, rt, 18 hours, 88%;
iii) isobutylchloroformate (1 eq), N-methylmorpholine (2 eq),
EtOAc/DMF, stir, rt, 16 h, 87%; iv) H_2, Pd/C, DCM/MeOH (3:1); v) PhMe,
reflux, 16 h, 45% over 2 steps; vi) $[Me_3O]^+BF_4^-$ (3.5 eq), DCM, 66%.

Scheme 2.18. The synthesis of (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine .

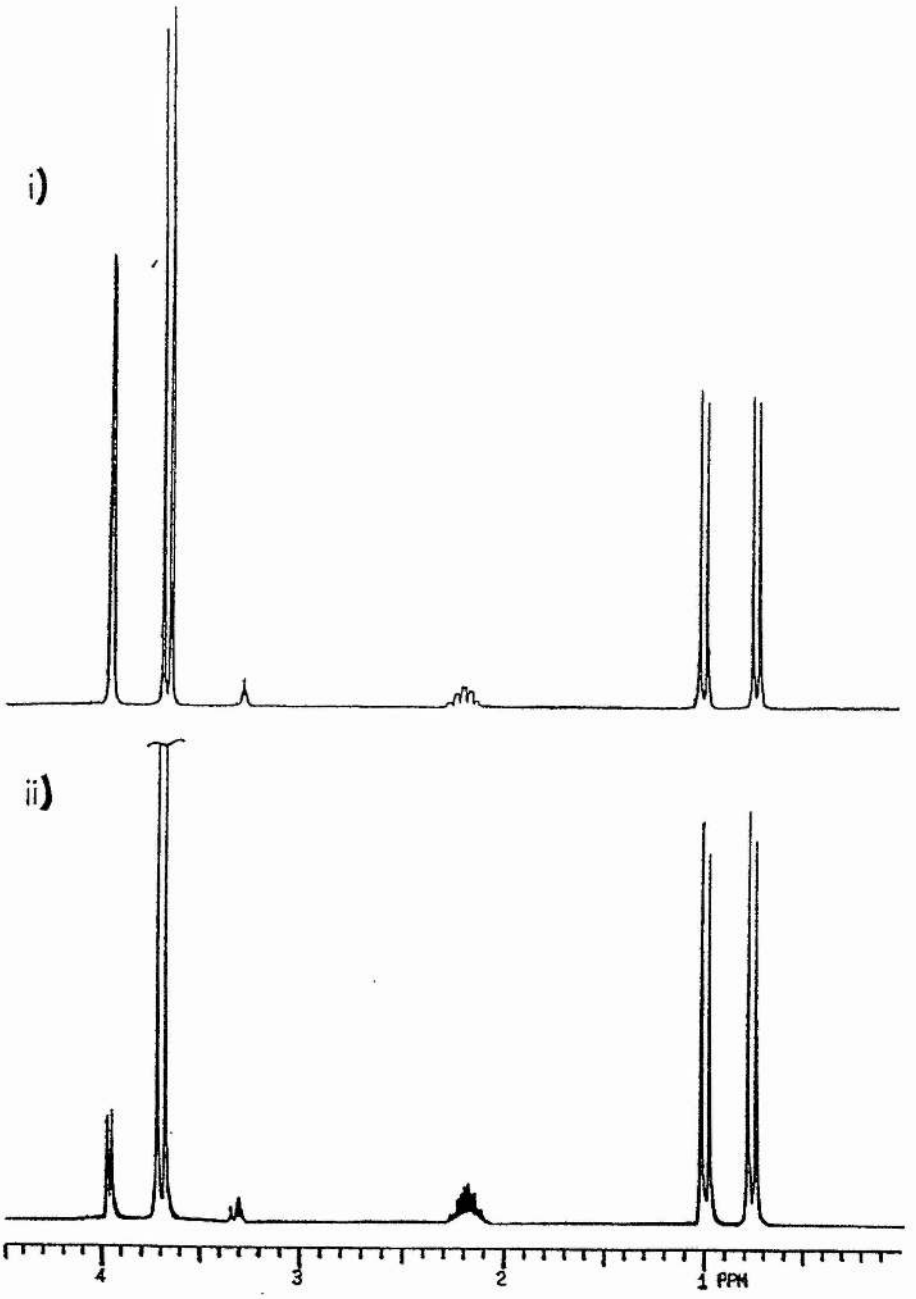
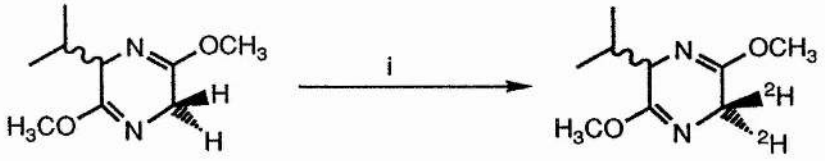


Figure 2.3. i) The 200 MHz proton NMR spectra in CD_3OD of (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**). The signals at 4.02 ppm are due to the C-3 proton (1H, d, *J* 3.8 Hz) and the 6-C protons (2H, s), at 3.7 for the 2 and 5 methyloxy protons (6H, 2s), 2.15 for the *iso*propyl C-H proton and at 1.0 and 0.75 for the *iso*propyl CH_3's (6H, 2d, *J* 6.8 Hz). ii) The 200 MHz proton NMR spectra in CD_3OD of (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**). The signal at 4.02 ppm is due to the C-3 proton (1H, d, *J* 3.8 Hz).



(**113**) (3R)-
(**126**) (3S)-

(**117**) (3R)-
(**127**) (3S)-

Reagents: i) CH_3O^2H-^2H_2O (10:1 v/v), KOH (1 eq), reflux, 3 h, 80%.

Scheme 2.19. The synthesis of C6 dideuteriated dihydropyrazines.

This pleasing selectivity for C-6 anion formation over C-3 anion formation was further probed by refluxing compound (**113**) under the optimised exchange conditions for 6 h, twice as long as that required for exchange at C-6. Very little deuterium (<10 atom %) was incorporated at C-3 as judged by proton NMR spectroscopy and mass spectrometry. It was noted that C-6 alkylated derivatives of the dihydropyrazine (**113**) were resistant to base catalysed deuteriation at C-6 under the same conditions.

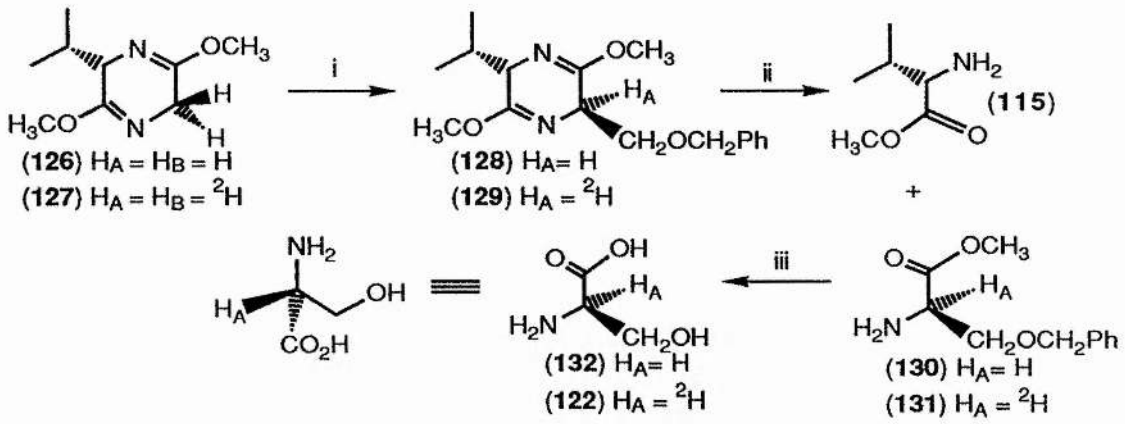
To confirm that the chiral centre at C-3 of the dideuteriated material (**117**) was intact, the dihydropyrazine ring was cleaved (0.1 M HCl, 16 h) to give the valine and glycine methyl esters. These were separated, the valine methyl ester was hydrolysed (5 M HCl, reflux, 3 h) and converted to its free base form with propylene oxide. The optical rotation of the recrystallised valine was -24.2° (c. 1.01, 5 M HCl) which compared favourably to that of an authentic sample of (2R)-valine [-24.95° (c. 1.09, 5 M HCl)], indicative of an enantiomeric excess of ≥97%. Thus, the deuteriation had proceeded highly selectively and without disturbing the stereogenic centre at C-3.

2.4 (2R)-Serine O-sulphate as a mechanistic probe

(2R)-Serine O-sulphate (**59**) was also found to be an inhibitor of GAD (section 2.2.3), however the mechanism of inactivation was not known. Initially we wanted to determine whether CO_2 was being liberated from the enzyme during the inactivation process. (2R)-[U-^{14}C]-Serine was not commercially available so we decided to investigate the mechanism by determining whether there was a kinetic isotope effect on the rate of inactivation.

2.4.1 Synthesis of (2R)-[2-^{2}H]-serine O-sulphate

The synthesis of (2R)-[2-^{2}H]-serine (**122**) would not have been possible using the route from aspartic acid (section 2.3.1) as (2R)-aspartic acid is not a substrate for the enzyme AAT. Therefore (2R)-[2-^{2}H]-serine O-sulphate (**123**) was synthesised from (3S)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**126**) in an identical manner to the (2S)-antipode in 5% overall yield (Scheme 2.20).



Reagents: i) *n*BuLi (1 eq) , THF, -90°C to -60°C, then $PhCH_2OCH_2Br$ (1.5 eq), THF, -90°C, stir, 16 h, 56%; ii) 0.2 M HCl (2 eq), rt, stir, 16 h then separation on flash silica 19:1 Et_2O:EtOH, 70%; iii) 5 M HCl, reflux, 3 h then EtOH, propylene oxide, reflux, 15 min, 89%.

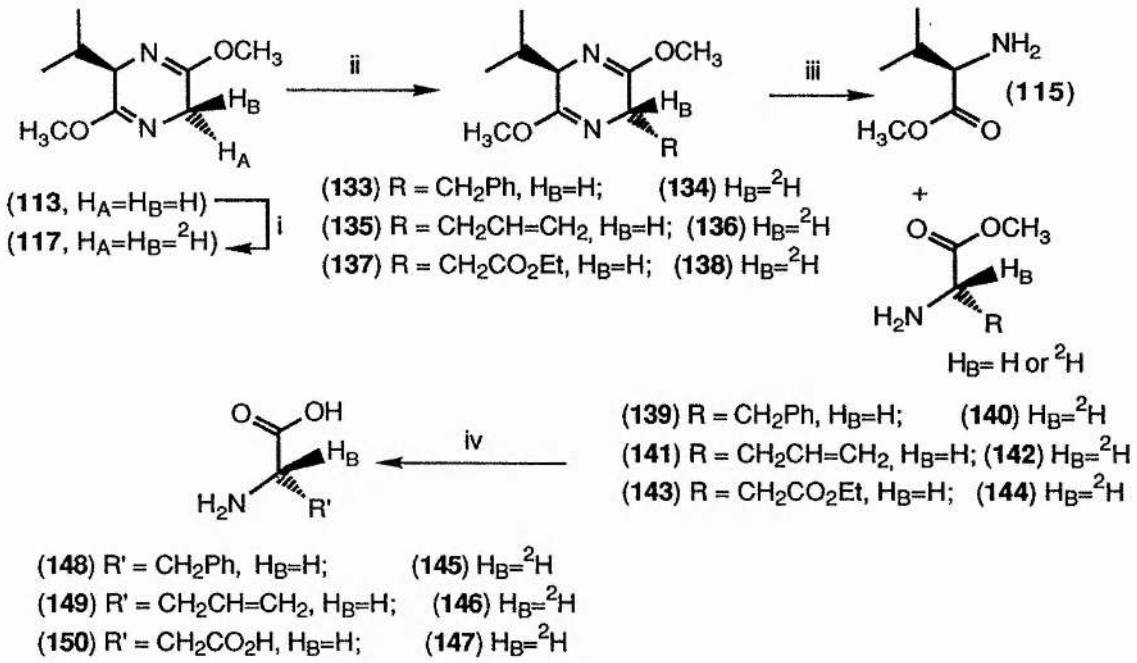
Scheme 2.20. The synthesis of (2R)- and (2R)-[2-^{2}H]-serine using Schollkopf chemistry.

2.5 Synthesis of other deuteriated α-amino acids

The availability of labelled amino acids in enantiomerically pure form is of prime importance in many biosynthetic and metabolic studies and also in the delineation of enzyme mechanism. Access to some C^{α}-deuteriated and tritiated (2S)-α-amino acids, for example, chiral glycines, (2S)-aspartic acid and (2S)-glutamic acid, has been facilitated through the use of enzymes in appropriately labelled buffer solution.[154,195] The preparation of labelled (2R)-amino acids, however, has been much more difficult. Some (2R)-α-amino acids (as well as the (2S)-antipodes) have been prepared through the acylase catalysed kinetic resolution of racemic mixtures of N-acetylated amino acids.[211] Here, deuterium or tritium can be introduced by exchanging the acidic hydrogen of the azlactone intermediates during acetylation.[154] Unfortunately, the differential rates for the deacylation vary considerably for the two antipodes of different amino acids, so the enantiomeric excesses are variable. Furthermore, the technique is not applicable in several instances.

Schollkopf's bis-lactim ether methodology has proved to be of enormous utility in the preparation of a wide range of (2R)- and (2S)-α-amino acids[209,210] and the chiral dihydropyrazine precursors are now commercially available as discussed in section 2.3.3. The chemistry developed in the synthesis of the deuteriated serines can be applied to other amino acids (Scheme 2.21).

The anion of the dideuteriated dihydropyrazine (**117**) can be alkylated with a wide variety of alkylating agents, e.g. benzyl bromide, allyl bromide, ethyl bromoacetate, yielding (**133-138**). It was found that the alkylation proceeded smoothly in most cases, long straight chain halides required longer reaction times (up to 24 hours) than benzylchloromethylether (16 hours) whereas ethyl bromoacetate only needed 5 hours. Acid hydrolysis (0.2 M HCl) to the mixture of methyl esters (**115,139-144**) did not present any problems. The limiting step in this route to a wide variety of amino acids is the separation of the methyl esters. There must be a significant difference in the side chains to allow the complete separation of the esters, either by chromatography or distillation. This methodology is probably not suitable for the synthesis of leucine or *iso*leucine. (2S)-[2-^{2}H]-Phenylalanine (**145**), (2S)-[2-^{2}H]-allylglycine (**146**), and (2S)-[2-^{2}H]-aspartic acid (**147**) were all prepared successfully from (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) using this method. Inspection of the proton NMR spectra of the alkylated (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazines showed that the signal for the remaining C-3 proton had become coupled to another proton and shifted from 4 ppm to 4.1-4.4 ppm dependent on the alkylating agent. The proton NMR spectrum also contained new signals due to the protons from the new side chain at C-6. Full details are described in the experimental section (Chapter 4), overall yields and optical rotations of each of the obtained amino acids are as shown in Table 2.3.



Reagents: i) CH_3O^2H-^2H_2O (10:1, v/v), KOH (1 eq), reflux, 3 h;
ii) *n*BuLi (1 eq), THF, -90°C to -60°C, then RBr (1.5 eq), THF,
-90°C, stir, 5-20 h; iii) 0.2 M HCl (2 eq), rt, stir,16 h; then separation
(chromatography for R=$CH_2CH{=}CH_2$, CH_2CO_2Et, distillation for R=CH_2Ph);
iv) 5 M HCl, reflux, 3 h then ethanol, propylene oxide, reflux, 15 min.

Scheme 2.21. Synthesis of α-deuteriated amino acids using Schollkopf chemistry.

Table 2.3. Amino acids synthesised using Schollkopf chemistry.

Compound[a] (Stereochemistry and Label at C[a])		Yield (%)[b]	$[\alpha]_D$[c]	$[\alpha]_D$ Theory
Serine				
76c	(S-, H)	25	+13.2° (c. 1.00, 1 M HCl)	+14.5° (c. 1.0, 1 M HCl)[212,213]
69c	(S-, ^{2}H)	20	+12.7° (c. 1.01, 1 M HCl)	
132	(R-, H)	20	-13.6° (c. 1.02, 1 M HCl)	-14.3° (c. 1.0, 1 M HCl)[212,213]
122	(R-, ^{2}H)	35	-13.5° (c. 1.03, 1 M HCl)	
Phenylalanine				
148	(S-, H)	30	-30.9° (c. 2.04, H_2O)	-32.5° (c. 2.0, H_2O)[214]
145	(S-, ^{2}H)	35	-28.2° (c. 1.50, H_2O)	
Allylglycine				
149	(S-, H)	15	-5.7° (c. 2.00, 5 M HCl)	+5.7° (c. 2.0, 5 M HCl) for R-(24)[215]
146	(S-, ^{2}H)	20	-4.2° (c. 1.87, 5 M HCl)	
Aspartic Acid				
150	(S-, H)	15	+21.8° (c. 0.495, 5 M HCl)	+25.2° (c 2.0, 5 M HCl)[213]
147	(S-, ^{2}H)	15	+19.5° (c. 0.495, 5 M HCl)	

[a]All compounds and intermediates gave the expected spectral and analytical data.

[b]Yield from R- or S- (**110**) or (**123**).

[c]Values determined at 23-25 °C.

2.6 Mathematical analysis of the data from the inactivation of GAD by serine O-sulphates

For an enzyme-substrate reaction, the substrate binds to the enzyme to form the complex ES. The reaction then occurs and ES breaks down to give enzyme and products. This can be written as shown below.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

At a particular time each of E, S and ES are present at the following concentrations e_t - x, s and x respectively and where e_t is the total concentration of free enzyme.

The rate of change of the concentration of ES is dx/dt and this can be written as:-

$$\frac{dx}{dt} = k_1 (e_t - x)s - k_2x - k_3x = k_1 e_t s - (k_1 s + k_2 + k_3)x \qquad (1)$$

If steady state conditions are assumed i.e. that the concentration of ES remains constant, then dx/dt = 0. Equation (1) can then be solved for x, giving:-

$$x = \frac{k_1 e_t s}{k_1 s + k_2 + k_3} \qquad (2)$$

The rate of reaction of the enzyme is first order with respect to the concentration of ES. Hence we have that:-

$$\text{rate, } v = k_3 x \qquad (3)$$

Substituting equation (2) in equation (3) gives:-

$$v = \frac{k_3 k_1 e_t s}{k_1 s + k_2 + k_3}$$

$$= \frac{k_3 e_t s}{s + (k_2 + k_3)/k_1} \qquad (4)$$

If the substrate is present in large quantities compared to the enzyme, then $s \approx$ constant and the enzyme is said to be saturated. The rate v, is then directly proportional to e_t. We can then write:-

$$k_{app} = \frac{k_3 s}{s + (k_2 + k_3)/k_1} \qquad (5)$$

where k_{app} is the apparent first order rate constant for the enzyme reaction.

Taking reciprocals of both sides of the rate equation (4) yields:-

$$\frac{1}{v} = \frac{1}{k_3 e_t} + \frac{k_2 + k_3}{k_1 k_3 e_t s} \qquad (6)$$

Equation (6) can be written in the more general form:-

$$\frac{1}{v} = \frac{1}{V} + \frac{K_M}{Vs} \qquad (7)$$

where $V = k_3 e_t$ and $K_M = (k_2 + k_3)/k_1$. From equation (7) it can be seen that a plot of 1/v

against 1/s will result in a straight line graph that intercepts the x-axis at $-1/K_M$ and the y-axis at 1/V. The slope of the line equal to K_M/V. One problem with this method of graphical analysis is that points of small v and s become over emphasised.

In the analysis of the enzyme-substrate reaction data the numerical values of the rate (v) and substrate concentration (s) are fitted to Michaelis-Menten kinetics using the computer program Enzfitter[216] which carries out a direct non linear fit and so avoids the over emphasis of small v and s, this yields the parameters K_M and k_{cat} ($k_{cat}=V_{max}/e_t$).

In the above analysis it was assumed that the steady state assumption was valid. For an inactivation reaction $dx/dt \neq 0$ and so the mathematics become more complicated. If we model the inactivation reaction on the same system as for the enzyme-substrate reaction, the equation can be written as shown below.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} E' \text{ (inactive enzyme)}$$

At a particular time each of E, I and EI are present at the following concentrations $e_t - x$, i and x respectively and where e_t is the total concentration of free enzyme.

The rate of change of the concentration of EI is dx/dt and this can be written as:-

$$\frac{dx}{dt} = k_1 (e_t - x)i - k_2x - k_3x = k_1e_ti - (k_1i + k_2 + k_3)x \qquad (8)$$

This expression can be integrated by the method of separation of variables (as $dx/dt \neq 0$) and after evaluating the constant of integration, the following expression is obtained.

$$t = [-1/(k_1 i+k_2+k_3)] \ln \{1-[(k_1 i+k_2+k_3)x/k_1 e_t i]\} \quad (9)$$

Dividing both sides by $-1/(k_1 i+k_2+k_3)$ gives:-

$$-(k_1 i+k_2+k_3)t = \ln \{1-[(k_1 i+k_2+k_3)x/k_1 e_t i]\} \quad (10)$$

Taking the exponential of both sides gives the expression (11) which can then be solved for x to give equation (12).

$$\exp [-(k_1 i+k_2+k_3)t] = 1-[(k_1 i+k_2+k_3)x/k_1 e_t i] \quad (11)$$

$$x = \frac{k_1 e_t i\{1-\exp[-(k_1 i+k_2+k_3)t]\}}{k_1 i+k_2+k_3} \quad (12)$$

The rate of inactivation of the enzyme is first order with respect to the concentration of EI. Hence we have that:-

$$\text{rate of inactivation, } v_{inact} = k_3 x \quad (13)$$

Equation (12) can be substituted into equation (13) to give an expression for the rate of inactivation of the enzyme (14). The following expressions can then be obtained for k_{app} and $1/k_{app}$.

$$v_{inact} = \frac{k_3 k_1 e_t i\{1-\exp[-(k_1 i+k_2+k_3)t]\}}{k_1 i+k_2+k_3} \quad (14)$$

At saturating i, v_{inact} is first order with respect to e_t, i.e. $v_{inact}=k_{app} e_t$ and k_{app} is as shown overleaf.

$$k_{app} = \frac{k_3\{1-\exp[-(k_1 i+k_2+k_3)t]\}}{1+(k_2+k_3)/k_1 i} \qquad (15)$$

Taking reciprocals of both sides gives equation (16).

$$\frac{1}{k_{app}} = \frac{1}{k_3\{1-\exp[-(k_1 i+k_2+k_3)t]\}} + \frac{(k_2+k_3)}{k_1 k_3 i\{1-\exp[-(k_1 i+k_2+k_3)t]\}} \qquad (16)$$

$$= \frac{1}{k_{inact}} + \frac{K_i}{k_{inact} i}$$

k_{app} is measured as the enzyme activity decreases, it is an apparent rate constant, but is effectively the same as a reaction velocity, v. Plots of ln (GAD activity) versus time yield straight lines with a gradient equal to $-k_{app}$ and an intercept on the y axis equal to the enzyme activity at t=0. Thus plots of ln (GAD activity) versus time at different inhibitor concentrations will give different slopes, but they will intercept at the same point on the y axis when the initial activity is the same. k_{inact} and K_i can then be determined graphically by plotting the negative of the gradient obtained (k_{app}) against the inhibitor concentrations. k_{inact} is the number of moles of inhibitor turned over per mole of enzyme per minute and K_i is the inhibitor concentration at which the enzyme is inactivated at half the theoretically maximum rate ($V_{max}/2$). These points can be fitted to Michaelis-Menten kinetics using Enzfitter[216] or transformed into straight lines by plotting the reciprocal of the gradient against the reciprocal of the inhibitor concentration.

Accordingly a number of incubations were prepared with a variety of different serine O-sulphate concentrations (1, 2, 3.5 and 7 mM for the (2S)-isomers and 2, 3.5, 5 and 7

mM for the (2R)-isomers). Aliquots were removed after various time intervals (15 seconds to 7 hours) and the GAD activity determined using a radiochemical assay and the results analysed as above.

2.7 Results and interpretation of the inactivation experiments

2.7.1 (2S)-Serine O-sulphates

Once the experiments had been completed for the (2S)-serine O-sulphate inhibitors the information was depicted in two ways. The first was to plot the apparent first order rate constant (k_{app}) against the concentration of inhibitor [I] and fit to the Michaelis-Menten curve (Figure 2.4) (see section 2.6). The second was to transform the Michaelis-Menten fit to a straight line, $1/k_{app}$ against 1/[I] (Figure 2.5) (see section 2.6). K_i and k_{inact} can be obtained from these graphs, the intercept of the 1/[I] axis is equal to $-1/K_i$ and the intercept of the $1/k_{app}$ axis is equal to k_{inact}. The values obtained for the direct non linear fit of the data using Enzfitter[216] are shown in Table 2.4:-

Table 2.4. Values of K_i and k_{inact} for the inactivation of GAD by (2S)- and (2S)-[2-^{2}H]-serine O-sulphate.

Inhibitor	K_i (mM)	k_{inact} (min^{-1})
(2S)-serine O-sulphate	2.57 ± 0.519	0.01076 ± 0.00098
(2S)-[2-^{2}H]-serine O-sulphate	7.59 ± 2.225	0.01368 ± 0.00264

The graphs obtained from the data for the inactivation of GAD by (2S)- and (2S)-[2-^{2}H]-serine O-sulphate are shown in Figures 2.4 and 2.5.

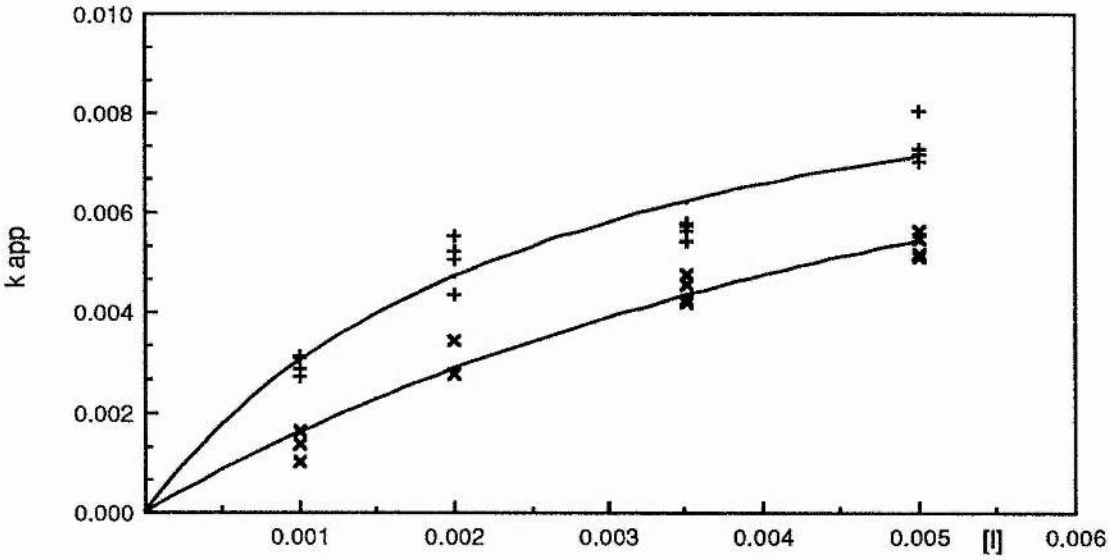


Figure 2.4. k_{app} against [I] for the inhibition of GAD by + (2S)-serine O-sulphate and x (2S)-[2-^{2}H]-serine O-sulphate.

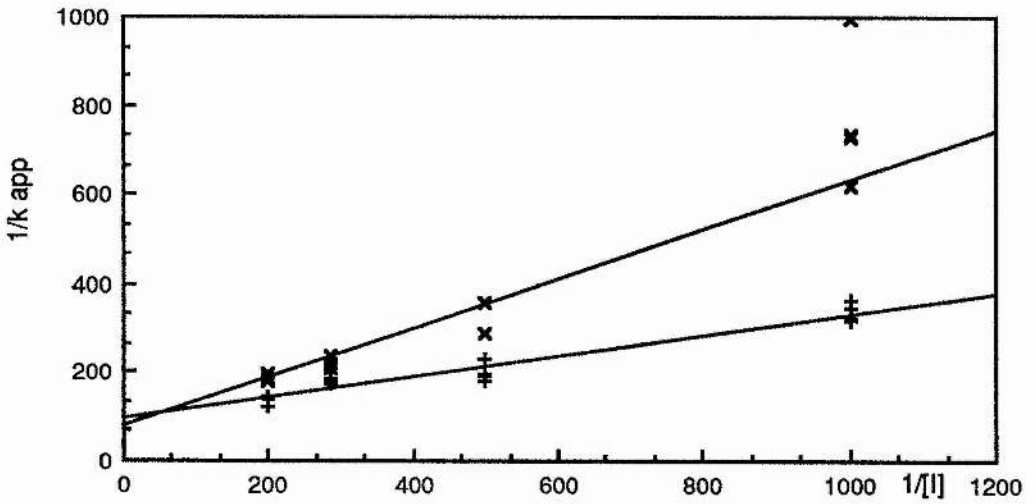


Figure 2.5. $1/k_{app}$ against 1/[I] for + (2S)-serine O-sulphate and x (2S)-[2-^{2}H]-serine O-sulphate inhibition of GAD.

From the data in Table 2.4, it can be seen that k_{inact} is approximately equal for both of the inhibitors, and so at constant enzyme concentration ^{D}V (or V_H/V_D) was about 1. However the values for K_i are significantly different and $^{D}(V/K)$ [or $(V_H/V_D)/(K_H/K_D)$] was 2.3. The value $^{D}(V/K)$ indicates that C^{α}-H bond cleavage does, indeed, occur during the inactivation of GAD. The large size of $^{D}(V/K)$ indicates that the reaction commitments to C-H bond cleavage are not large and that C-H bond cleavage is not the most significant transition state in the inactivation process.[217] If the C-H bond cleavage was the rate determining step then there would also be a significant isotope effect on V as V_{max} would be decreased for the deuteriated inhibitor and this is clearly not the case. After the C^{α} bond has broken, the intermediates are identical and therefore have identical reaction rates. Hence the rate determining step is prior to the first irreversible step (the C-H bond breakage). Binding to the enzyme active site and transaldimination of the inhibitor with the active-site lysine bound to the PLP have both occurred before the C-H bond breaks. It is possible that either of these two processes is rate determining.

For (2S)-serine O-sulphate we have shown that a kinetically significant step in the inactivation process is the breakage of the C^{α}-H bond which supports the mechanism proposed by Metzler.[109] An incubation of (2S)-serine O-sulphate with GAD in 0.1 M pyridine/DCl buffer at pH 4.6 was studied by proton NMR spectroscopy. No exchange of the α-proton occurred after several days. This observation implies that the anion formed on the loss of the α-proton is not stabilised, and almost immediately the C-C double bond is formed and HO_3SO^- is eliminated from the enzyme active site. Hence, the inactivation process has been shown to occur via the removal of a proton from the C-4'-*re*-face of the coenzyme and the data is most consistent with an E2 type elimination reaction.

2.7.2 (2R)-Serine O-sulphates

At all concentrations studied for (2R)-serine O-sulphate, the deuteriated (2R)-serine O-sulphate inactivated the enzyme faster than the unlabelled isotopomer, so there is an inverse isotope effect. The averaged apparent values rates of inactivation for the two inhibitors (V_H and V_D) and the ratio of rates (V_H/V_D) are shown in Table 2.5.

Table 2.5. The average rates of inactivation of GAD by (2R)- and (2R)-[2-^{2}H]-serine O-sulphate.

Concentration (mM)	V_H (x 10^{-3})	V_D (x 10^{-3})	V_H/V_D
2.0	0.921	1.419	0.649
3.5	1.321	2.598	0.509
5.0	1.560	3.891	0.411
7.0	1.861	5.821	0.320

The isotope effect is clearly inverse, but ^DV could not be determined accurately from the narrow range of inhibitor concentrations amenable to kinetic analysis, as the rate of inactivation at points near V_{max} was too fast to be measured using the radiochemical method. Nevertheless, the existence of an inverse isotope effect indicates that C^{α}-CO_2^- bond cleavage occurs. The C^{α}-CO_2^- bond must be disposed on the 4'-*re*-face of the coenzyme as the binding position of the distal sulphate group is identical for all substrates and inhibitors,[149] and so the reaction occurs on the C-4'-*re*-face of the coenzyme. The bond cleavage step is rate-limiting, and the observed isotope effect values could represent secondary α-isotope effects where the C^{α}-H bond is stiffer in the transition state than in the product external aldimine.[218] However, the magnitude of the measured inverse isotope effect is too large to be due a secondary α-isotope effect and a more likely explanation is that there are alternative reaction pathways which do

not lead to inactivation of the enzyme which are suppressed on the addition of deuterium. This would make the rate of inactivation faster for the deuteriated inhibitor than the non deuteriated one, this is an induced isotope effect. $^{D}(V/K)$ for the inactivation reaction was 1.0 indicating that there is a large reaction commitment to the first isotopically sensitive step and that the suicide substrate is extremely sticky,[217] in contrast to the (2S)-enantiomer. The data is consistent with an early C-OSO_3^- bond ionisation.

A proton NMR experiment with (2R)-serine O-sulphate (0.226 mmol) and GAD (8.467 nmoles) in 1 ml of 0.1 M pyridine/DCl buffer at pD 4.2 was carried out. No new signals were detected for times up to 113 hours. However, the maximum amount of Schnackerz adduct produced would only be about 1% of the amount of (2R)-serine O-sulphate present at t=0, and so would not be seen. The rate of the alternative reaction pathway cannot be slower than the inactivation or the introduction of deuterium would not result in the increased rate of the pathway leading to inactivation. It is likely that the two rates are similar and, therefore, it is hardly surprising that no new signals were seen in the proton NMR spectrum. It was impractical to attempt to isolate the products of the inactivation reaction as a very large amount of enzyme would be required to form a realistic amount of the Schnackerz adduct.

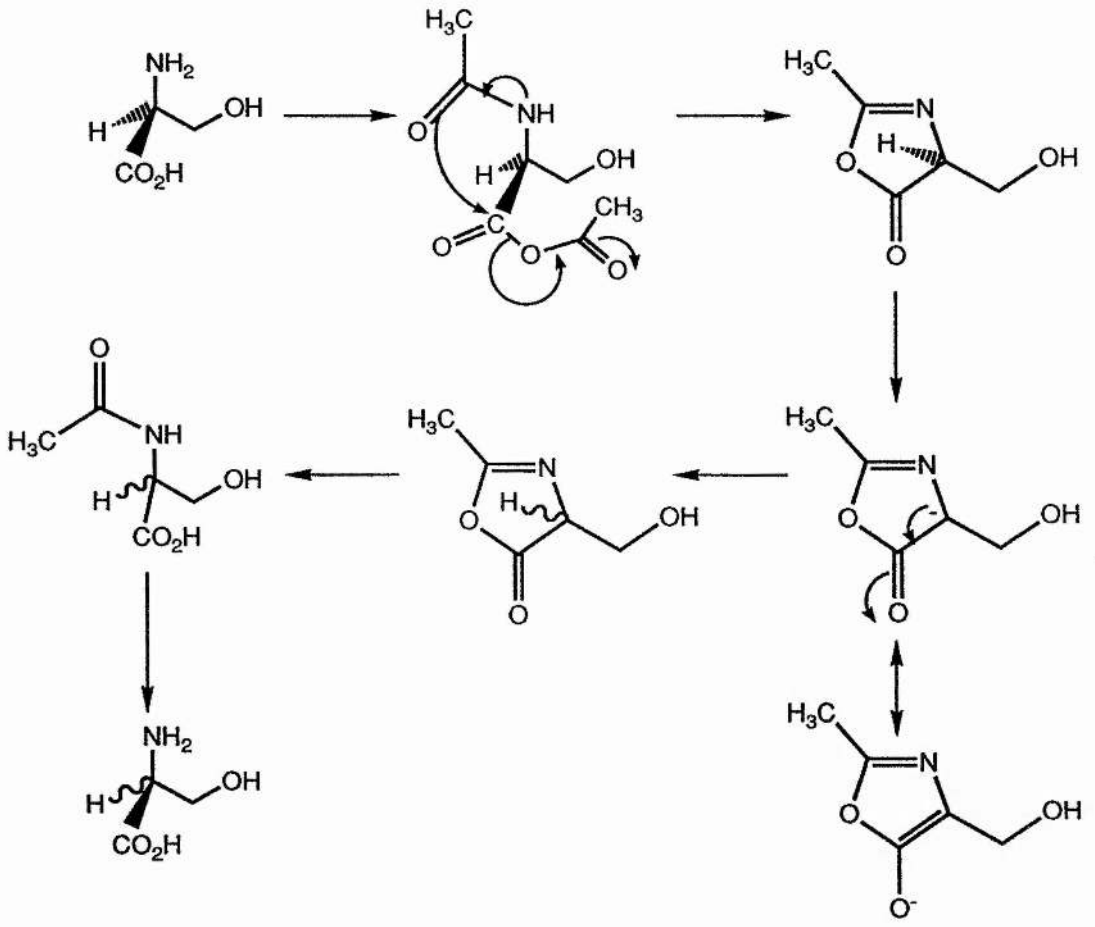
In order to verify our conclusion we wanted to inactivate GAD with (2R)-[U-^{14}C]-serine O-sulphate. This study had not been carried out previously due to the lack of availability of (2R)-[U-^{14}C]-serine (section 2.4). In order to obtain a sample of (2R)-[U-^{14}C]-serine O-sulphate, (2S)-[U-^{14}C]-serine would need to be racemised, followed by the resolution of the racemate to yield (2R)-[U-^{14}C]-serine. The (2R)-[U-^{14}C]-serine could then be converted into (2R)-[U-^{14}C]-serine O-sulphate. The synthesis of (2R)-[U-

^{14}C]-serine O-sulphate in this manner would be time consuming due to the resolution step. The possibility of incubating (2RS)-[U-^{14}C]-serine O-sulphate (**151**) with the enzyme was therefore investigated. Any $^{14}CO_2$ evolved would be from the (2R)-[U-^{14}C]-serine O-sulphate as (2S)-[U-^{14}C]-serine O-sulphate does not liberate $^{14}CO_2$ during the inactivation process (section 2.2.4).

N-Acetyl-(2RS)-serine (**152**) was prepared by reaction of (2S)-serine with acetic anhydride and pyridine in water.[219] Racemisation occurs when the acidic hydrogen of the azlactone intermediate exchanges with the solvent (Scheme 2.22). Hydrolysis of the N-acetyl-(2RS)-serine (**152**) in 2 M HCl, followed by treatment with propylene oxide in ethanol gave the required (2RS)-serine (**153**) in 54% yield with an $[\alpha]_D$ of +0.15° compared to 11.41° for pure (2S)-serine (c. 2.0 in 1 M HCl). (2RS)-[U-^{14}C]-Serine (**154**) was prepared in an identical manner to the non labelled racemate (**153**) in 77% yield {$[\alpha]_D$ +3°, (c. 2.0 in 1 M HCl)}.

The (2RS)-[U-^{14}C]-serine (**154**) was converted to (2RS)-[U-^{14}C]-serine O-sulphate (**155**) using the method of Tudball.[192] Incubation of a 10 mM solution of (2RS)-[U-^{14}C]-serine O-sulphate (**155**) (400 µl, 20.956 nCi) in 0.1 M pyridine/HCl buffer with the enzyme (1 mg) at pH 4.6 and 37 °C was carried out, so that any $^{14}CO_2$ liberated would be trapped in barium hydroxide solution (10 ml). The amount of radioactivity remaining in the solutions, after the inactivation, was determined using a scintillation counter. Radioactivity (≈400 molar equivalents) was lost from the serine O-sulphate solution although an increase was not detected in the barium hydroxide solutions. The inactivation experiment was repeated using larger quantities of enzyme (3.5 mg) attempting to trap the CO_2 in a smaller volume (2 ml) of barium hydroxide. Again the radioactivity of the incubation solution decreased but much lower quantities of $^{14}CO_2$

were detected in the barium hydroxide solution.



Scheme 2.22. Racemisation of (2S)-serine.

To verify that this effect was due to the (2R)-[U-^{14}C]-serine O-sulphate, we decided to attempt to resolve the racemate. Resolution of the racemate involved the preparation of N-acetyl-(2RS)-serine (**152**) which was then incubated with pig liver acylase for 24 hours. The acylase catalyses the hydrolysis of N-acetyl-(2S)-serine at a much faster rate than hydrolysis of the (2R)-antipode. The (2S)-serine and N-acetyl-(2R)-serine (**156**) can be separated by ion exchange chromatography and the resolution is achieved. The optical rotation of the (2R)-serine (**157**) from the resolution process was 1.75 ° compared to +7.87 ° for an authentic sample (c. 2.0 in H_2O). The (1'S, 4'R)-camphanamides of authentic (2R)- and (2S)- serine (**158,159**) were synthesised and

the chemical shifts for the three serine protons were indistinguishable. These camphanamides (**158,159**) were converted to methyl esters (**160,161**) by reaction with diazomethane, and proton (Figure 2.6 i and ii) and carbon NMR spectra obtained at 400 and 100 MHz respectively. The proton NMR spectrum of the (1'S,4'R)-camphanamide methyl ester of (2R)-serine (**162**) isolated from the resolution process was obtained (Figure 2.6. iii) and the enzymically derived (2R)-serine found to be a mixture of 58 % (2R)-serine and 42 % (2S)-serine. The resolution step was therefore not viable and so this route was not explored with the radioactive material.

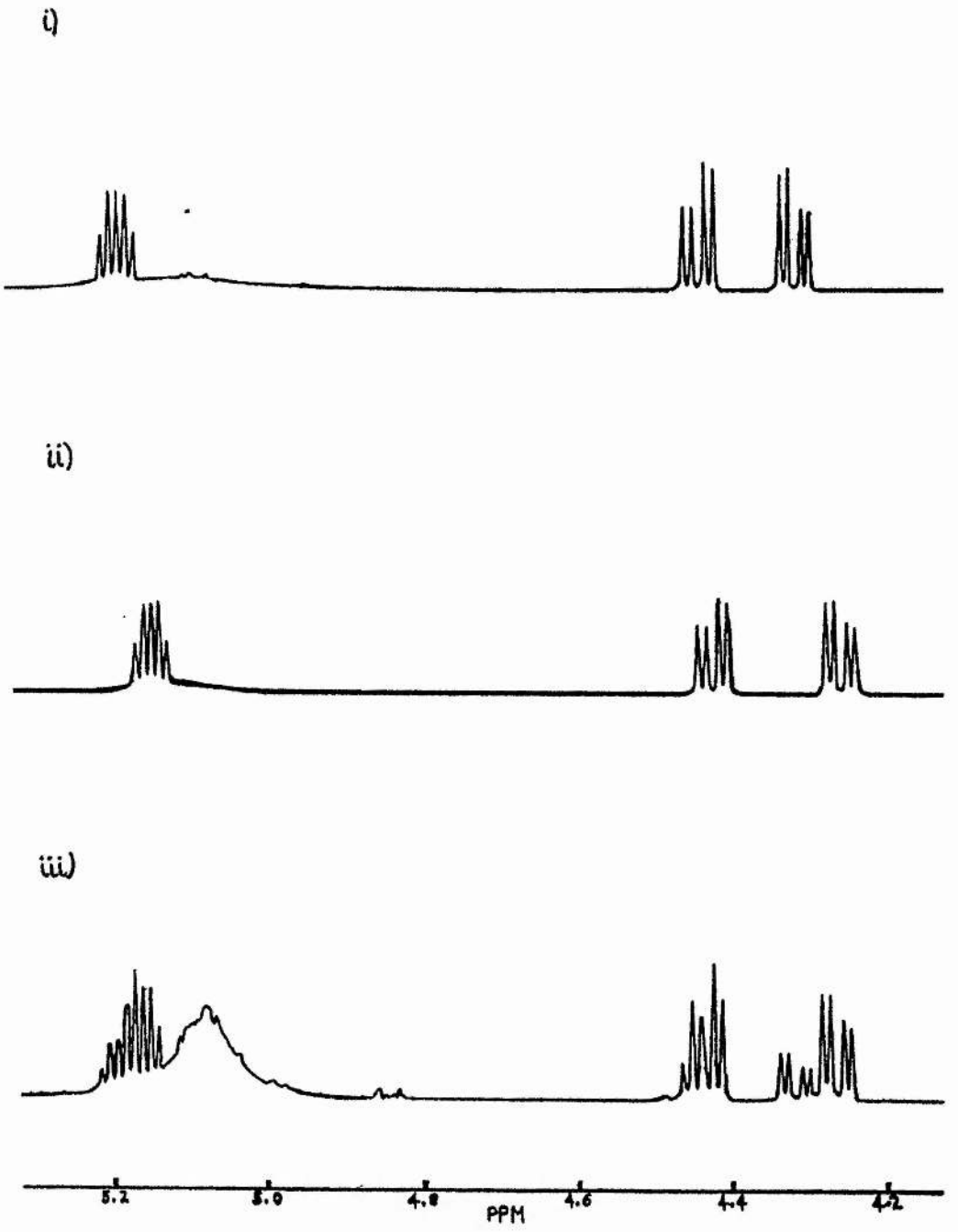
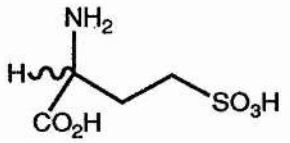


Figure 2.6. The 400 MHz proton NMR spectra in C_6D_5N of i) (2S)-serine camphanamide methyl ester (**160**), ii) (2R)-serine camphanamide methyl ester (**161**) and iii) the camphanamide methyl ester of (2R)-serine after resolution of N-acetyl-(2RS)-serine with acylase (**162**).

2.7.3 Incubations of (2S)- and (2R)-homocysteic acid with GAD

Homocysteic acid (2-aminobutanoic acid-4-sulphonic acid) (**163**) is isosteric with glutamic acid and serine O-sulphate. The side chains of glutamic acid, homocysteic acid and serine O-sulphate are - $CH_2CH_2CO_2H$, -$CH_2CH_2SO_3H$ and -CH_2OSO_3H respectively. The conformations adopted by each of the side chains in the presence of GAD are therefore expected to be similar. Both enantiomers of homocysteic acid were incubated with the enzyme. The (2S)-antipode was found to form a GABA analogue, 3-aminopropane sulphonic acid, whereas the (2R)-antipode was unchanged after the same incubation period. As (2R)-glutamic acid and (2R)-homocysteic acid do not react with GAD, the inactivation reaction with (2R)-serine O-sulphate must be due to the presence of the sulphate group as a good leaving group.



(163)

Equivalent amounts of 10 mM (2S)-glutamic acid and (2S)-homocysteic acid (0.5 ml of each) in 0.1 M pyridine/DCl buffer at pH 4.6 were mixed together and glutamate decarboxylase (0.2 mg) was added. The reaction was followed by proton NMR spectroscopy; after 40 minutes all of the glutamic acid had been converted to GABA, but it was several days before there was any appreciable conversion of the (2S)-homocysteic acid to its GABA analogue. From the large difference in reaction rates we can deduce that V/K for the α-decarboxylation of (2S)-homocysteic acid is ≈1/250 of V/K for (2S)-glutamic acid.

2.7.4 Uv/vis measurements on the inactivation mixture

No experiments to trap intermediates in the inactivation path for the (2S)- and (2R)-serine O-sulphate have been carried out. It was discovered that the amount of $^{14}CO_2$ given off during the inactivation of the GAD by (2R)-serine O-sulphate (**59**) was equivalent to ≈500 turnover events. We wanted to determine whether any pyruvic acid or acetaldehyde was released during the inactivation process. Alcohol dehydrogenase and lactate dehydrogenase reduce acetaldehyde and pyruvic acid to ethanol and lactate respectively. These enzymes require β-nicotinamide adenine dinucleotide, reduced form (NADH), for their activity. On reduction of a substrate the cofactor is oxidised. NADH has an absorption maximum at 340 nm (ε 6220 dm^3 mol^{-1} cm^{-1}). As the NADH is oxidised to NAD^+ the magnitude of the absorption at 340 nm is reduced. This property of NADH allows the reactions of alcohol dehydrogenase and lactate dehydrogenase to be studied by uv/visible spectroscopy. An aliquot (10 μl) of each inactivation mixture was added to 1 ml of a 100 mM potassium phosphate buffer solution at pH 7.0 containing NADH (150 mM) and alcohol dehydrogenase (0.1-0.2 mg) or lactate dehydrogenase (0.1-0.2 mg). Control experiments showed that the presence of acetaldehyde or pyruvic acid in the inactivation solution would be detected by a decrease in the absorption maximum at 340 nm. However acetaldehyde and pyruvic acid were not detected in the inactivation solution of either (2S)- or (2R)-serine O-sulphate (**21,59**).

2.8 Conclusions

E. coli GAD is inactivated by both enantiomers of the suicide inhibitor serine O-sulphate; inactivation by the (2S)-antipode appears to involve C^{α}-H bond cleavage while inactivation by the (2R)-isomer involves C^{α}-decarboxylation. If the distal $-OSO_3^-$ binding group occupies the same position in each of the external aldimines of the two suicide substrates then both of the inactivation events involve the loss of electrofuges from the 4'-*re*-face of the coenzyme. The fact that (2S)-homocysteic acid is a decarboxylation substrate for GAD whereas the (2R)-antipode is not is in accord with this analysis. The torsional geometry of the distal $-OSO_3^-$ group which leaves during each reaction should be *anti*- to the 4'-*re*-face electrofuge. In these conformations the activation energies for the concerted processes could be lower than those for the alternative stepwise (stabilised carbanion) 4'-*si*-face processes which are expected for pyridoxal systems[220] but which are not observed here.

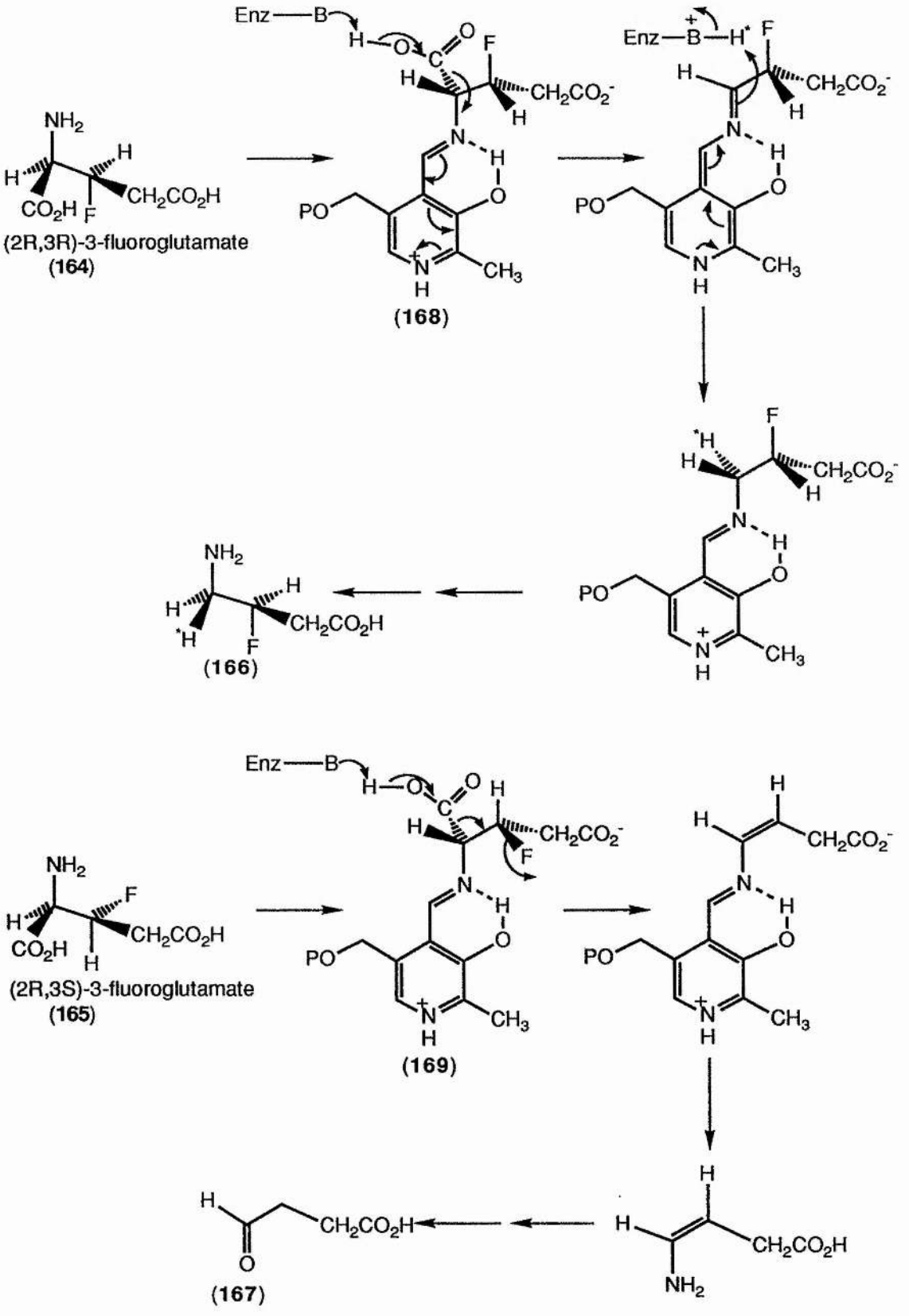
Tilley's work[149] has indicated that the distal binding groups of substrates and inhibitors of GAD occupy similar positions at the active site on the 3'-phenolic group side of the coenzyme.

Marquet[221] has studied the interaction of (2R,3R)- and (2R,3S)-3-fluoroglutamate (**164,165**) with *E. coli* GAD. Both isomers were substrates: the (2R,3R)-isomer was decarboxylated into optically active 4-amino-3-fluorobutyrate (**166**), whereas the (2R,3S)-isomer lost the fluorine atom during the reaction yielding succinic semialdehyde (**167**). The difference between the two isomers demonstrates that the external aldimine is present at the active site under a rigid conformation. The C^{α}-CO_2^- bond must be perpendicular to the plane of the pyridinium ring.[24] In order for β-

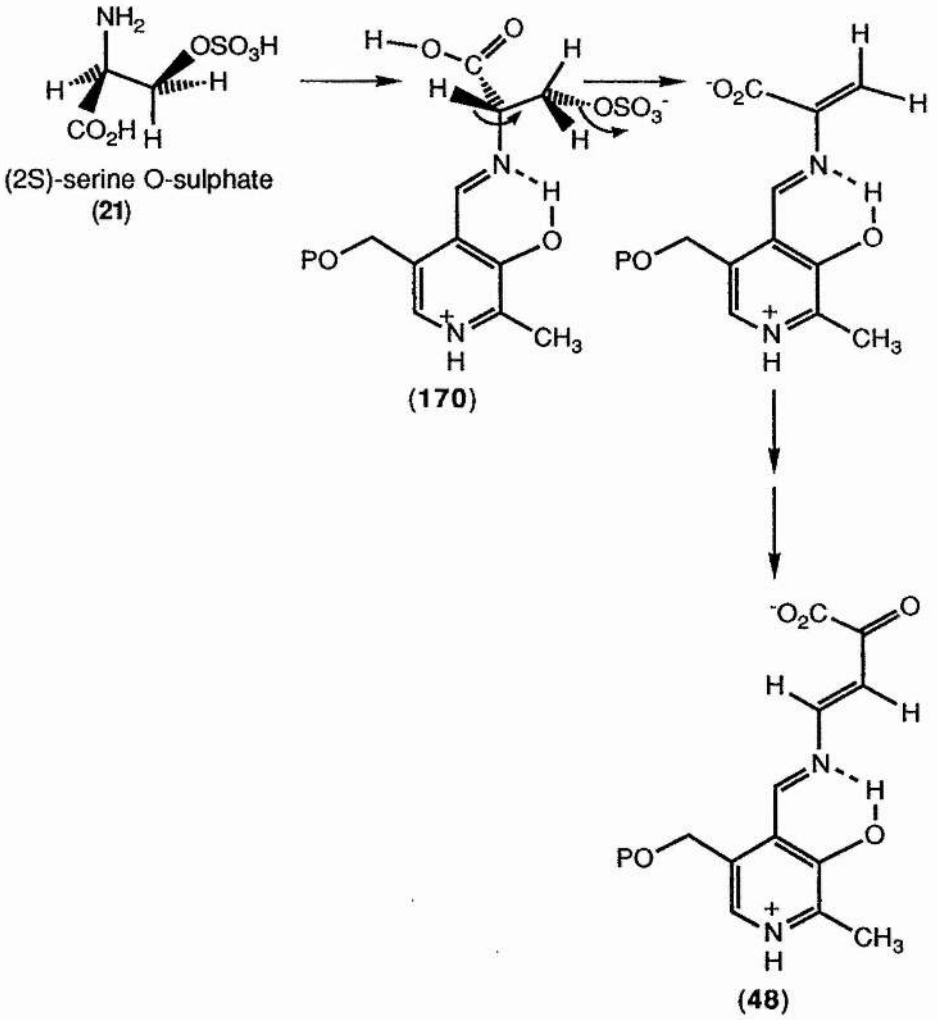
elimination to occur the C^{β}-F bond must be coplanar with the C^{α}-CO_2^- bond. It is most likely that the C^{β}-F bond will be trans to the C^{α}-CO_2^- bond resulting in *anti*-elimination, as is depicted in Scheme 2.23. The external aldimines for the (2R,3R)- and (2R,3S)-3-fluoroglutamates (**168,169**) are shown with the distal $CH_2CO_2^-$ group occupying the same space. It can be seen that the C^{β}-F bond is correctly positioned for elimination in the external aldimine (**169**) but this is not the case for the aldimine from (2R,3R)-3-fluoroglutamic acid (**168**).

Therefore we would expect the Schiff's base for each of the serine O-sulphate inhibitors at the active site of GAD (**170,171**) to be held in a rigid conformation (Scheme 2.24, 2.25). The conformational preferences of the (2S)-and (2R)-serine O-sulphate were assessed using the Monte Carlo simulation. The trans and *anti*-conformations were found to be highly populated.

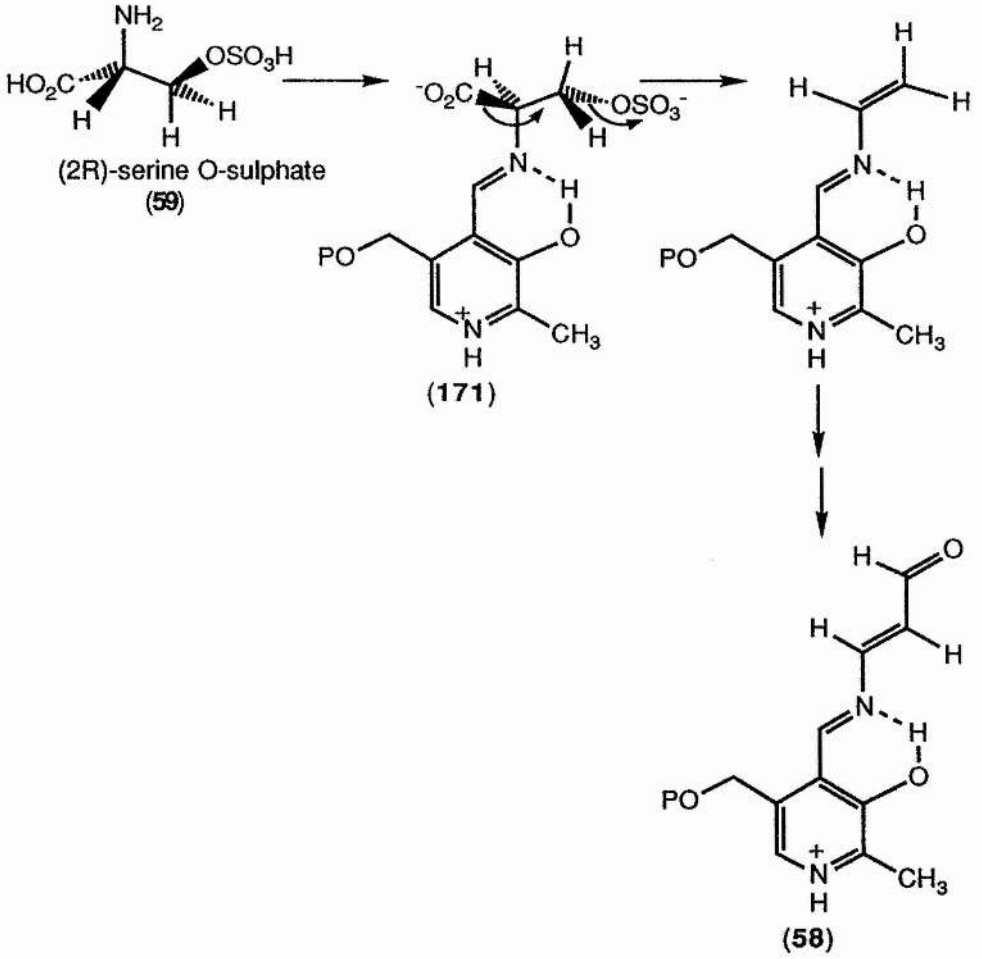
For both the (2S)- and the (2R)-serine O-sulphate the cleavage of the C^{α}-H and C^{α}-CO_2^- bonds on the C-4'-*re*-face of the coenzyme occurs in preference to the expected reaction on the C-4'-*si*-face of the coenzyme due to the position of the OSO_3^- leaving group enabling a facile elimination to occur. These events lead to the inactivation of the enzyme, without decarboxylation for (2S)-serine O-sulphate and with decarboxylation for (2R)-serine O-sulphate.



Scheme 2.23. Reaction of GAD with (2R,3R)- and (2R,3S)-3-fluoroglutamate.



Scheme 2.24. Inactivation of GAD by (2S)-serine O-sulphate, showing the rigid conformation of the Schiff's base.



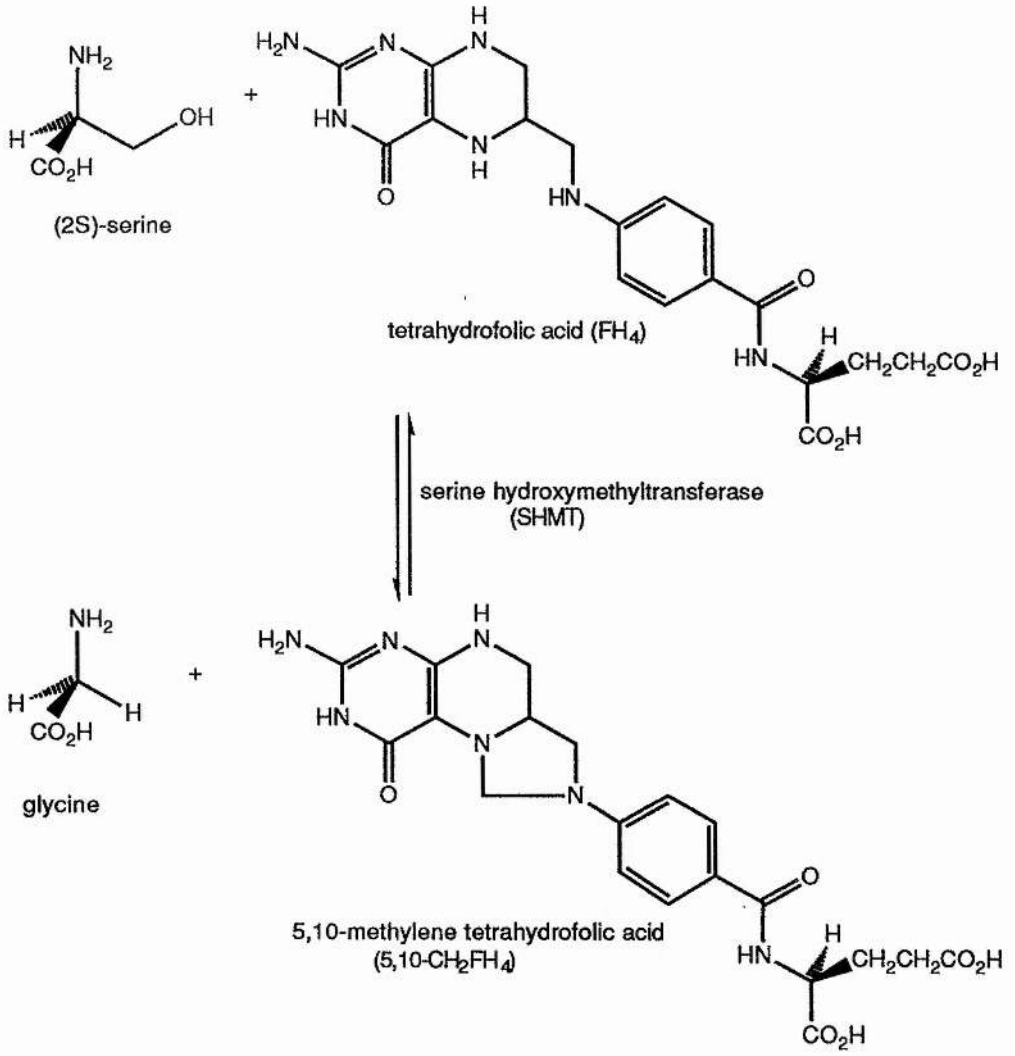
Scheme 2.25. Inactivation of GAD by (2R)-serine O-sulphate, showing the rigid conformation of the Schiff's base.

CHAPTER THREE

SERINE HYDROXYMETHYLTRANSFERASE

3.0 Serine hydroxymethyltransferase (SHMT)

Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1)[222] is a pyridoxal 5'-phosphate (PLP) dependent enzyme which catalyses the reversible retro-aldol cleavage of (2S)-serine to glycine and formaldehyde. Under physiological conditions, tetrahydrofolic acid (FH_4) traps the nascent formaldehyde and is converted to 5,10-methylene tetrahydrofolic acid (5,10-CH_2FH_4) (Scheme 3.1).



Scheme 3.1. The physiological reaction catalysed by SHMT.

Considerable indirect evidence, from feeding labelled substrates to whole animals, has suggested that the physiological role of SHMT is the generation of one-carbon groups in the form of 5,10-methylene-FH_4. These units are required in the biosynthesis of many primary metabolites including purines, thymidylate, and methionine.

The one-carbon units transferred to the tetrahydrofolic acid from serine by SHMT are also used in the formation of secondary metabolites. The enzyme, therefore, performs a key role, linking primary and secondary metabolism. SHMT is widely distributed in nature and has been studied in, and purified from, prokaryotic[223] and eukaryotic systems (vertebrate,[224] invertebrate[225] and plant[226]).

3.1 Properties and structure of SHMT

3.1.1 Properties and structure of mammalian SHMT

Mammalian SHMT has been purified to homogeneity from rat,[227] rabbit,[228] beef,[229] and sheep liver.[230] The enzyme is known to be present in mammalian brain, kidney, uterus, prostate and lymphocyte tissue. SHMT exists in both the cytosol and the mitochondria of eukaryotes. The cytosolic and mitochondrial isoenzymes have been purified to homogeneity from rabbit[231] and from rat liver.[227] The mammalian isoenzymes possess different amino acid sequences and molecular weights (Table 3.1) indicating that they are the products of different genes.[171]

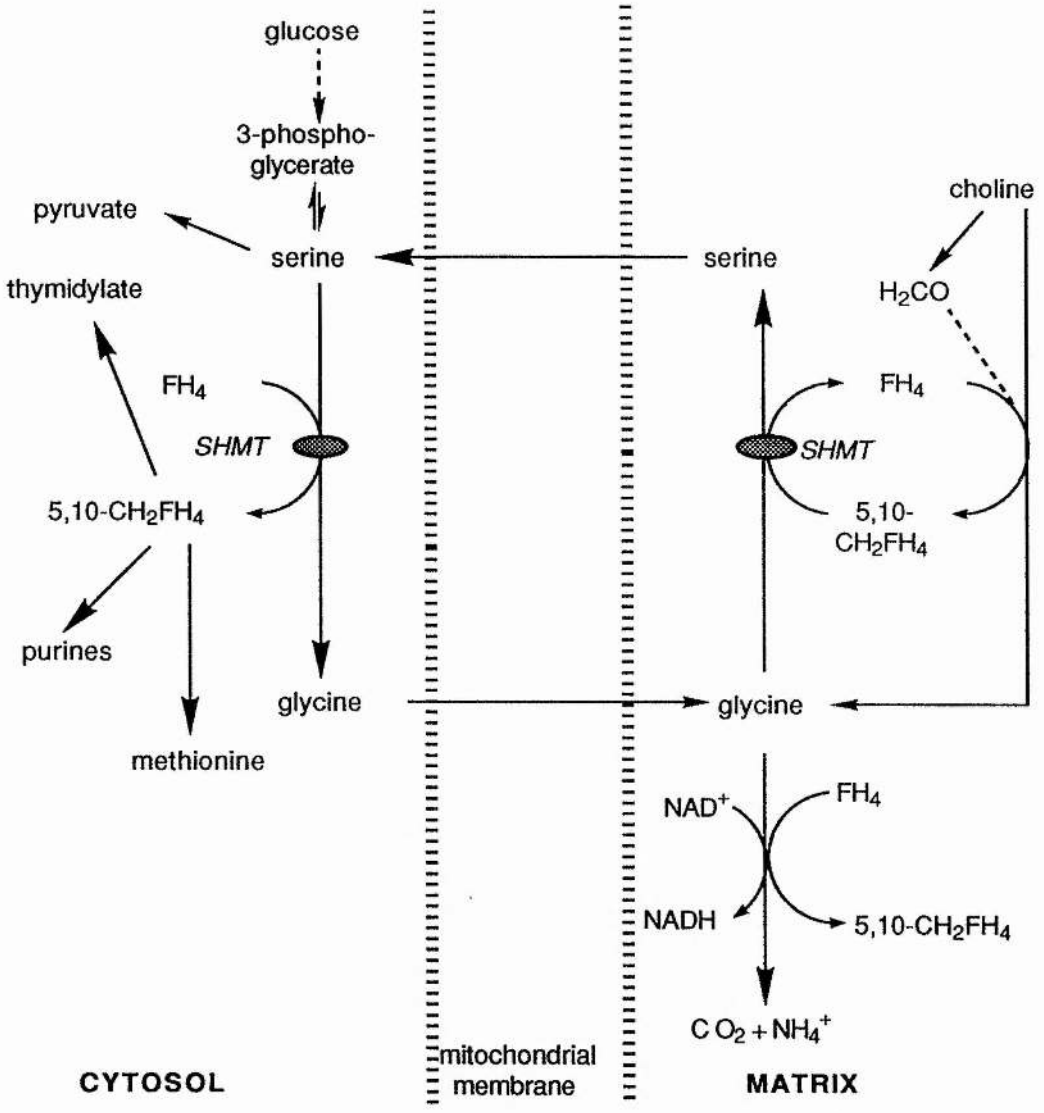
Table 3.1. SHMT protein properties.

Source	M_r (Daltons)	Subunit (Daltons)	No. Cys (per subunit)	I.E.P.
rabbit liver (cyt.)	215000	55000	8	7.2
rabbit liver (mit.)	210000	53000	6	7.2
rat liver (cyt.)	216000	56000	3	4.95
rat liver (mit.)	204000	54000	3	5.30
E. coli	95000	47000	3	<6.0[A]
F. methylotroph (S)[232]	100000	100000		B
F. methylotroph (M)[232]	200000	50000		C

A 30% homology with rabbit liver (cyt.), B activated by Ca^{2+}, K^+, Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+}, C activated by Ca^{2+}, K^+, Na^+.

It has been proposed[233] that the cytosolic and mitochondrial enzymes participate in a shuttle system to link one-carbon metabolism in the two cellular compartments. The mitochondrial membrane is impermeable to tetrahydrofolic acid, but serine and glycine are readily transported through the membrane. SHMT could, therefore, act as a major control enzyme in balancing the two isolated tetrahydrofolic acid pools (Scheme 3.2).

SHMT has also been found in nerve tissue,[234] where it is proposed to be important in either supplying methyl groups, via 5,10-methylene-FH_4, required for the biosynthesis of various neurotransmitters (e.g. histidine and tryptophan), or in regulating the glycine pool. Note that glycine is a major inhibitory neurotransmitter.



Scheme 3.2. The location of SHMT in mammailian systems.

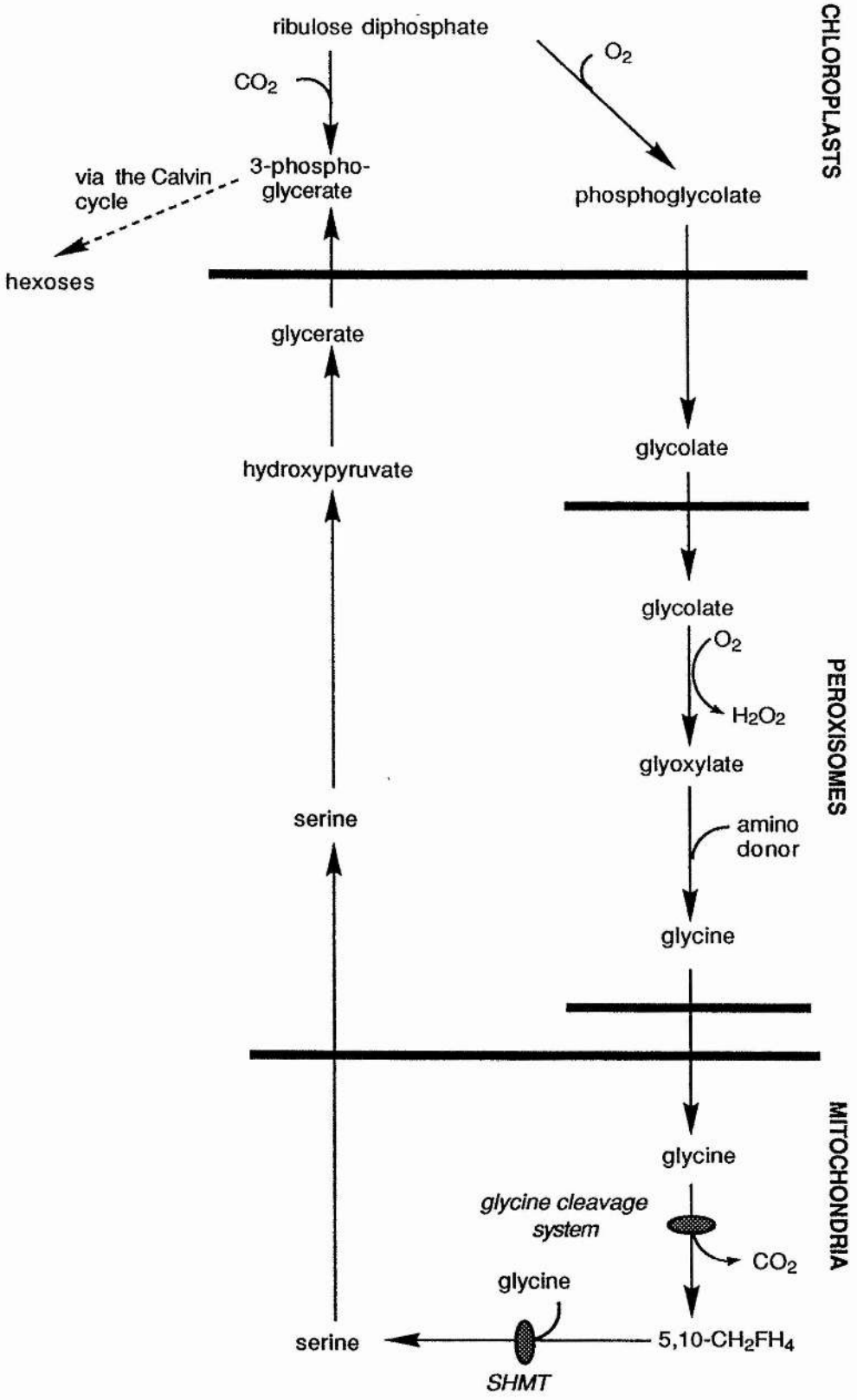
3.1.2 Properties and structure of plant SHMT

SHMT activity has also been found in plant tissue. In higher plants the activity is restricted to the mitochondrial matrix.[235] The enzyme has been partially purified from cauliflower where it was found to utilise PLP and FH_4 as coenzymes. The addition of the potassium ion was found to stimulate the enzyme catalysed reaction.[236] A report on the purification of SHMT from mung bean (*Vigna radiata*)[237] to homogeneity claimed that the enzyme did not require added PLP for full catalytic activity. The mung bean

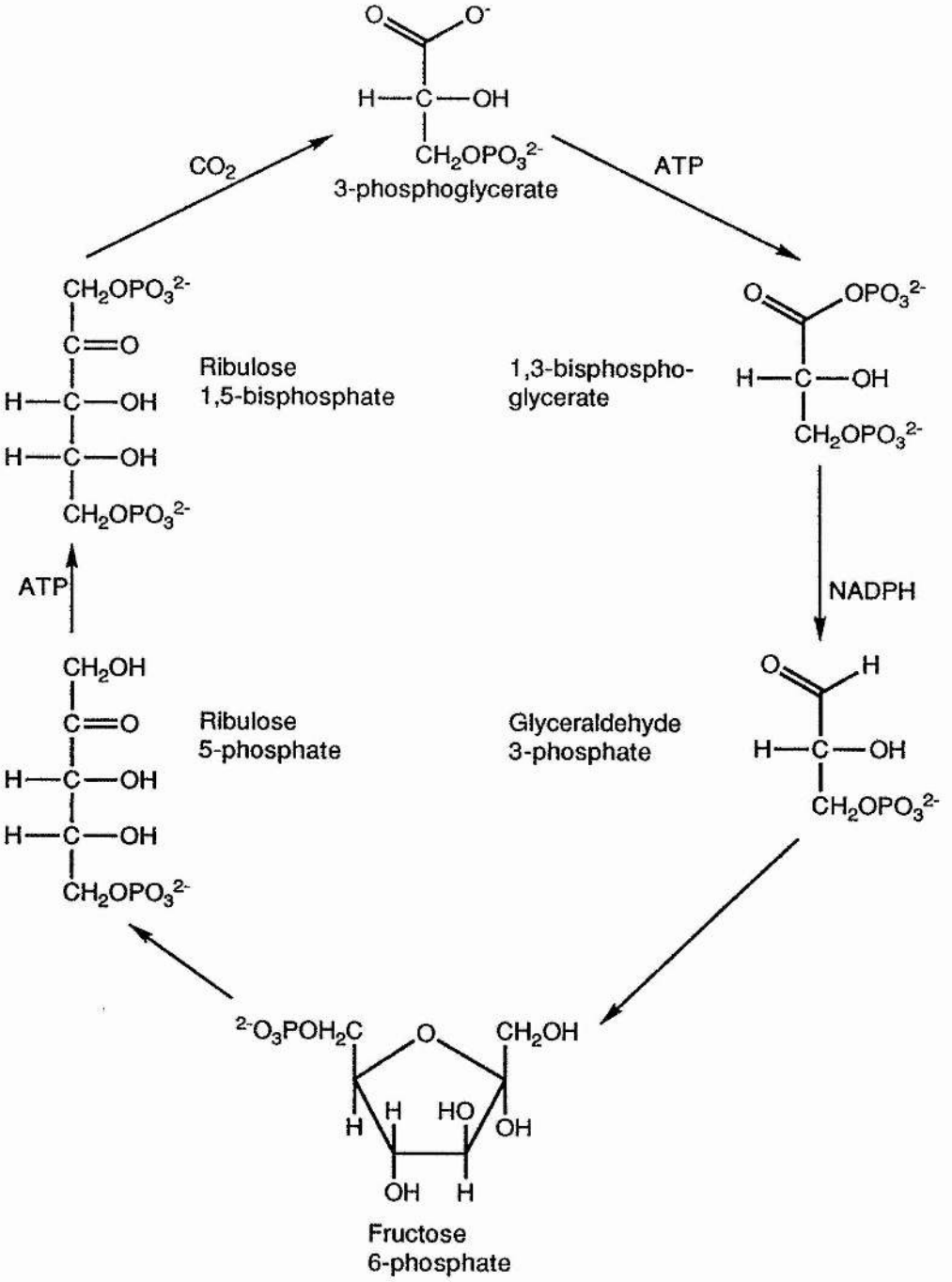
enzyme is, to date, the only known non PLP-dependent SHMT and therefore PLP may not be involved in the reaction catalysed by this enzyme. A study of the mung bean enzyme active site and the catalytic mechanism of action of this enzyme may give an insight into the role of PLP. In plants the metabolism of glycine and (2S)-serine is closely linked with the glycolate pathway and photorespiration as shown in Scheme 3.3.

A primary role of plant SHMT is as a catalyst for the formation of (2S)-serine. The (2S)-serine is converted by a series of reactions to 3-phosphoglycerate, which is utilised in the Calvin cycle, Scheme 3.4.

SHMT has also been studied in baker's yeast (*Saccharomyces cerevisiae*) where it is involved in the synthesis of methionine and S-adenosylmethionine.[238]



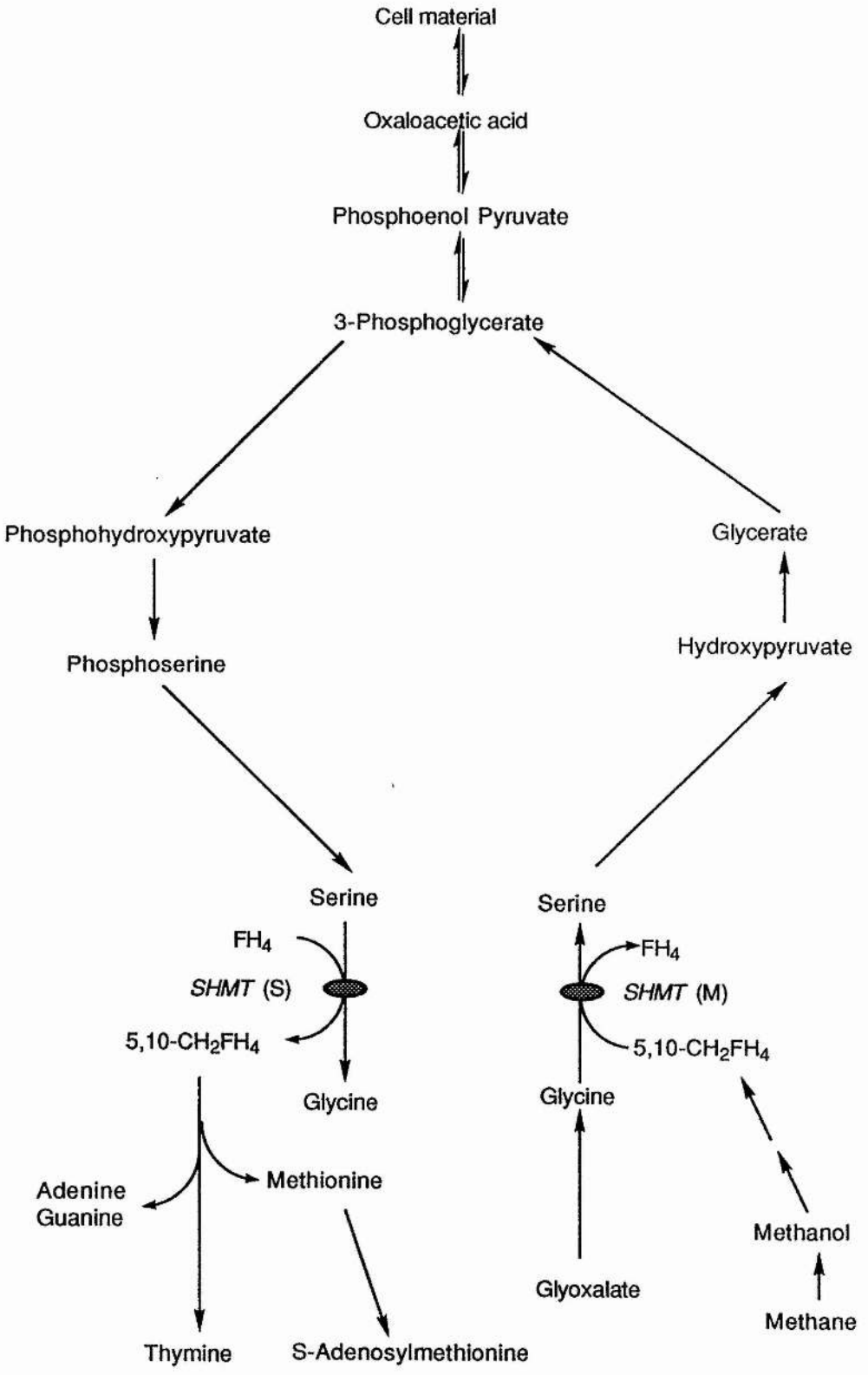
Scheme 3.3. The role of (2S)-serine and glycine in photorespiration and the glycolate pathway.



Scheme 3.4. The Calvin cycle.

3.1.3 Properties and structure of bacterial SHMT

SHMT has been purified to homogeneity from both *E. coli*[223] and a facultative methylotrophic bacterium.[232] For both microorganisms, the enzyme's major function is to provide glycine for protein and purine synthesis and 5,10-methylenetetrahydrofolic acid for the one-carbon pool. In the case of microorganisms which use reduced one-carbon compounds as the sole source of carbon, the biosynthesis of serine is utilised by the microorganism as a energy generating pathway. The problem these organisms face in regulating two pathways which require the same chemical conversions has been tackled by the expression and use of two different isoenzymes. For a methylotrophic bacterium, one isoenzyme predominates when the organism is grown on methane or methanol, and this was found to be activated by glyoxalate. When the organism is grown on succinate, a different isoenzyme, which is activated by Mg^{2+}, Mn^{2+}, or Zn^{2+}, is produced.[232] Some pathways involving SHMT in methylotrophic bacteria are shown in Scheme 3.5.



Scheme 3.5. Pathways involving SHMT in methylotrophic bacteria.

3.1.4 Serine hydroxymethyltransferase as a chemotherapeutic target

Methionine has been observed to exacerbate the symptoms of schizophrenia in some patients. The observation has suggested that elevated levels of SHMT may, in part, be a cause of this disease.[239] As there is some evidence that in both prokaryotic and in eukaryotic cells, methionine levels regulate SHMT, by an as yet unknown mechanism, it is possible that the level of activity of brain and liver SHMT in schizophrenic subjects is abnormal. The levels of activity of liver SHMT were discovered to be higher in schizophrenic patients compared with non-schizophrenic patients after methionine loading.[240] However until the method of control exerted by (2S)-methionine on SHMT is understood, this result is of little therapeutic potential.

Rapidly proliferating tissues require an increased supply of one-carbon groups. These one-carbon groups are used for the synthesis of the purine and pyrimidine bases required in the biosynthesis of DNA. It would, therefore, be expected that these tissues should have elevated levels of SHMT. The level of SHMT is found to be elevated in some types of tumour cell. Patients with chronic lymphocytic leukaemia have raised levels of SHMT,[241] whilst malignant mouse L1210 cells were found to contain SHMT with altered kinetic properties.[242] (2R)-*Cyclo*serine (**172**) (Figure 3.1) has been shown to inhibit the growth of Friend leukaemia cells in mice,[241] and tetrahydrohomofolic acid[241] (**173**) (Figure 3.1) inhibits SHMT from L1210 cells. (2R)-*Cyclo*serine (**172**) and tetrahydrohomofolic acid are not specific for SHMT and so their cytotoxicity is not clearly linked to the inhibition of SHMT. The reason for their inhibitory effects requires further study.

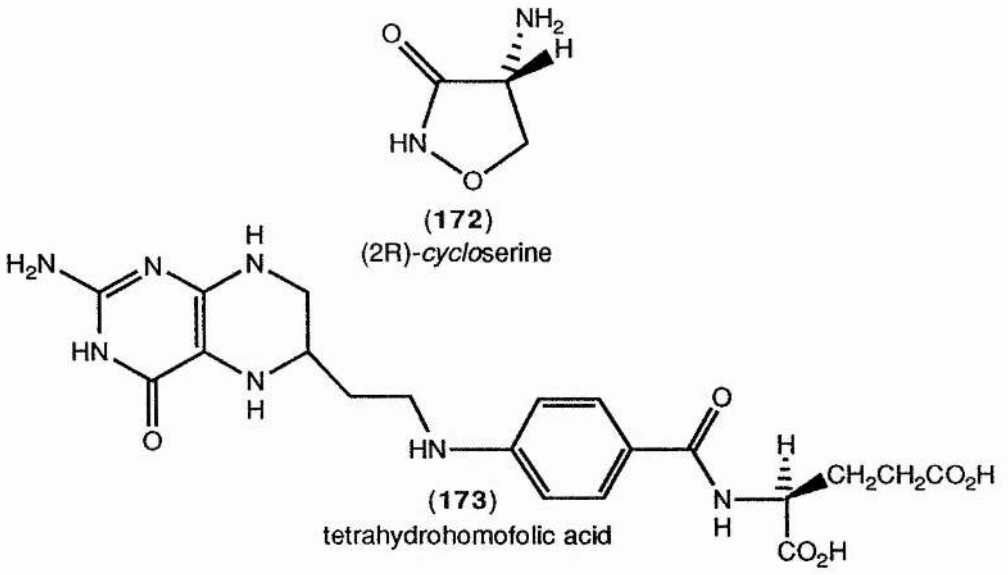
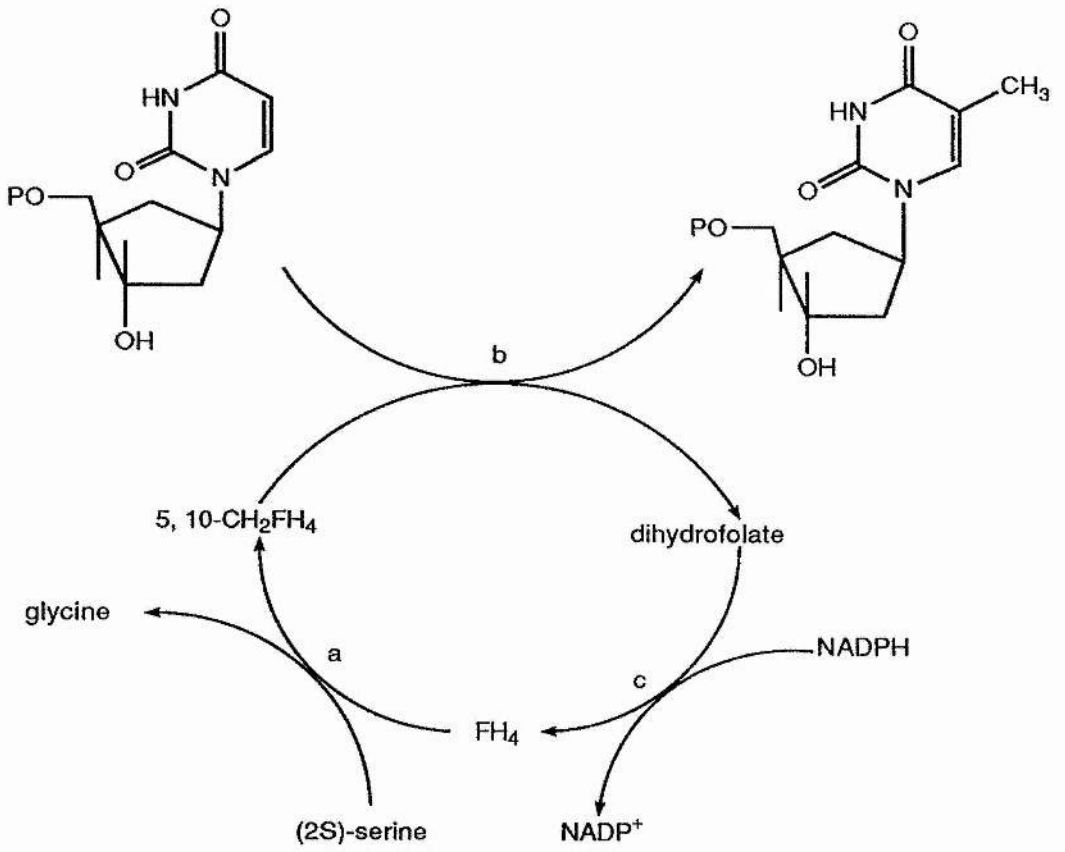
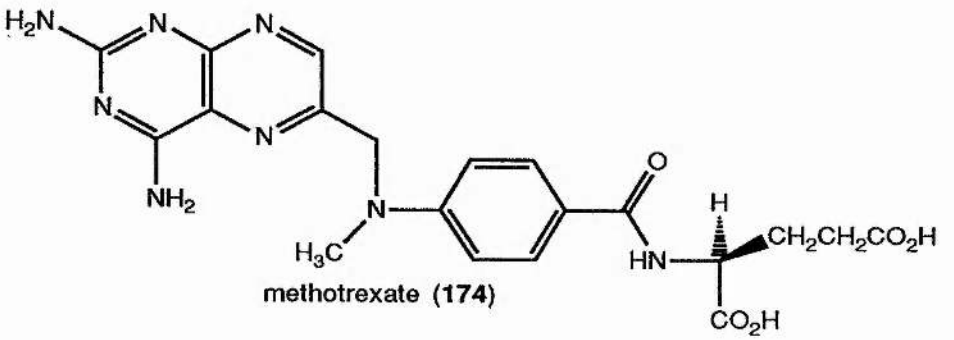


Figure 3.1. Inhibitors of SHMT.

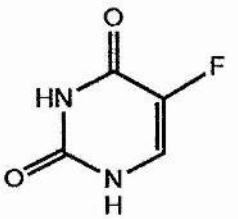
Inhibiting SHMT interferes with the supply of one-carbon units for thymidylate synthetase. However SHMT has serious disadvantages as a chemotherapeutic target compared to thymidylate synthetase [inhibited by 5-fluorouracil (**174**)], or dihydrofolate reductase [inhibited by methotrexate (**175**)][243] (Scheme 3.6), due to its wide tissue distribution and probable regulatory role in nervous tissue.



a) serine hydroxymethyltransferase (SHMT)
b) thymidylate synthetase
c) dihydrofolate reductase



methotrexate (**174**)



5-fluorouracil (**175**)

Scheme 3.6. The synthesis of thymidylate.

3.1.5 Substrate and reaction specificity

SHMT has been shown to exhibit a broad substrate and reaction specificity (Table 3.2). As well as catalysing the aldolase activity (reactions 1-8[244-249] below) in which a 3-hydroxyamino acid undergoes a reversible retro-aldol cleavage, the enzyme has also been shown to catalyse several other reactions. These reactions include a slow (10^{-3} times the rate of reaction with (2S)-serine/FH_4) half transamination reaction with (2R)-alanine (reaction 13),[228] the condensation of a number of 2-ketoacids with pyridoxamine 5'-phosphate (PMP) in the reverse of this reaction,[250] and a fast decarboxylation reaction with aminomalonate[64] (three times the rate of reaction with (2S)-serine/FH_4) (reaction 18). SHMT has also been shown to catalyse exchange of the *pro*-S hydrogen of glycine (but not the *pro*-R hydrogen) (reaction 9),[251] the exchange of the α-hydrogen of several (2S)-amino acids (reactions 11 and 12),[252] and most recently the racemisation of alanine.[253] SHMT demonstrates the catalytic activity of several classes of pyridoxal 5'-phosphate dependent enzymes and so an in-depth study of the structure-function relationships in SHMT has been made.

Table 3.2. The reaction and substrate specificity of SHMT.

ALDOLASE REACTION			Ref.
1.	(2S)-serine + FH_4	$\rightleftharpoons$ glycine+5,10-CH_2FH_4	244
2.	(2S)-α-methylserine + FH_4	$\longrightarrow$ (2R)-alanine + 5,10-CH_2FH_4	245
3.	(2S,3S)-threonine	$\rightleftharpoons$ glycine + acetaldehyde	246
4.	(2S,3R)-threonine	$\longrightarrow$ glycine + acetaldehyde	246
5.	(2S,3R)-3-phenylserine	$\rightleftharpoons$ glycine+benzaldehyde	247
6.	(2S,3S)-3-phenylserine	$\longrightarrow$ glycine+benzaldehyde	247
7.	(2S,3R)-3-phenylserine methyl ester	$\longrightarrow$ glycine methyl ester benzaldehyde	247

8. ε-trimethyl-3-hydroxy-lysine $\longrightarrow$ glycine + γ-butyrobetaine aldehyde 248

also a substrate; (2S,3S)-3-phenylcysteine (NT29)

α-PROTON EXCHANGE:

(2S)-proton

9. (2S)-[^{3}H]-glycine + H_2O $\rightleftharpoons$ glycine + ^3H_2O 251

10. (2R)-[^{3}H]-alanine + H_2O $\rightleftharpoons$ (2R)-alanine + ^3H_2O 228

(2R)-proton

11. (2S)-[^{3}H]-alanine + H_2O $\rightleftharpoons$ (2S)-alanine + ^3H_2O 252

12. (2S)-phenylalanine + ^2H_2O $\rightleftharpoons$ (2S)-[2-^{2}H]-phenylalanine 252

also substrates; (2S)-tryptophan, (2S)-cyanoglycine, (2S)-leucine, (2S)-*iso*leucine and (2S)-valine

TRANSAMINATION:

13. (2R)-alanine + PLP $\rightleftharpoons$ pyruvate + PMP 228

14. (2S)-alanine + PLP $\rightleftharpoons$ pyruvate + PMP 252

15. glyoxalate + PMP $\rightleftharpoons$ glycine + PLP 250

16. fluoropyruvate + PMP $\rightleftharpoons$ (2R)-3-fluoroalanine 250

not transaminated; (2S)-serine, glycine, (2R)-serine, (2R)-threonine, and (2R)-phenylalanine.

RACEMISATION:

17. (2S)-alanine $\rightleftharpoons$ (2R)-alanine 253

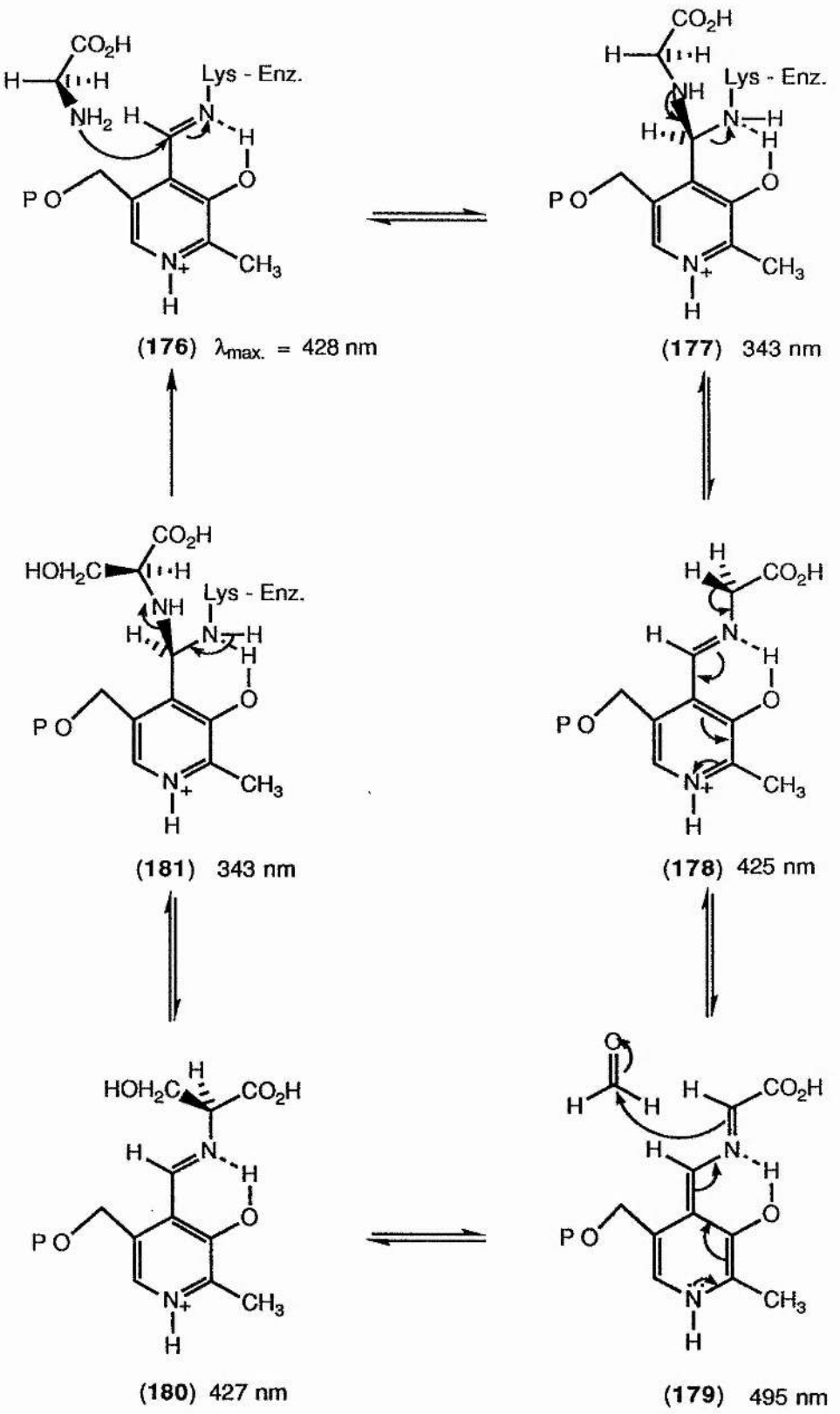
DECARBOXYLATION:

18. aminomalonic acid $\longrightarrow$ glycine + CO_2 64

3.1.6 Role of pyridoxal 5'-phosphate

Initial mechanistic studies on SHMT took advantage of the fact that the conjugated π-system of the PLP-Schiff's base could be used as a uv active probe to investigate the catalytic events of the physiological reaction. The holoenzyme has an absorption maximum at 428 nm due to the internal aldimine (Scheme 3.6, (**176**)). The addition of saturating levels of glycine to the incubation mixture resulted in the appearance of three new bands at 343, 425 and 495 nm. The order of appearance of these new bands was determined using rapid reaction kinetics, and allowed structural assignments for these bands to be made [structures (**177**), (**178**) and (**179**)].[254] High concentrations of (2S)-serine produced absorption maxima at 495, 427 and 343 nm. The use of a combination of stopped-flow and temperature-jump kinetic studies[255] enabled the assignment of these absorptions to the structures (**179**), (**180**) and (**181**) as shown (Scheme 3.7).

The holoenzyme (**176**) undergoes nucleophilic attack at C-4' by the amino group of the substrate to give a geminal diamine (**177**). A synchronous proton transfer from the glycyl nitrogen to the ε-amino group of the active-site lysine group also occurs. After the proton transfer the lysine ε-amino group leaves concertedly with the formation of another imine at C-4'. This completes the transaldimination reaction and gives the external aldimine (**178**). As yet SHMT is the only enzyme for which the geminal diamine (**177**) has been observed, suggesting that for SHMT there may be a large conformational change in the protein on formation of the external aldimine (**178**).



Scheme 3.7. The proposed mechanism for the reaction catalysed by SHMT with assigned uv absorption maxima.

Following the formation of the external aldimine (**178**) (Scheme 3.7.) a base on the enzyme then removes the *pro*-S hydrogen of glycine moiety of the external aldimine to form the quinoid intermediate (**179**). The quinoid intermediate has several contributing resonance structures which give rise to the characteristic absorption maximum at 495 nm. In the absence of an aldehyde the reaction, which is thus far completely reversible, stops and an equilibrium distribution of complexes (**176**) to (**179**) can be observed.

(2S)-Serine is the substrate for the retro-aldol cleavage reaction. The retro-aldol reaction involves a similar series of events, culminating in the cleavage of the C^{α}-C^{β} bond. Studies on the retro-aldol reaction[255] have shown that the enzyme can cleave (2S)-serine to glycine and formaldehyde in the absence of tetrahydrofolic acid, but the formaldehyde remains at the active site preventing further reaction unless tetrahydrofolic acid is added.

Kallen *et al.*[230,256] studied the pre-steady state and steady state kinetics of the conversion of (2S)-3-phenylserine to glycine and benzaldehyde, using the enzyme from lamb liver. In the absence of FH_4 a detectable concentration of the quinoid intermediate (**179**), absorbing at 505 nm, was formed. Stopped-flow experiments have indicated that the cleavage of (2S)-3-phenylserine is followed by an ordered release of products, benzaldehyde and then glycine. In deuterium oxide the release of glycine shows an isotope effect of 6 on V_{max}, suggesting that addition of the proton to the quinoid intermediate (**179**) is the rate determining step in the release of glycine.

3.1.7 Role of tetrahydrofolic acid

The observation that only reactions 1 and 2 (Table 3.2) have a requirement for FH_4 suggests that the involvement of formaldehyde in these two reactions introduces constraints which are not imposed by the other aldehydes. Formaldehyde exists almost totally as the hydrate in aqueous solution and so the primary role of FH_4 is probably to transport formaldehyde to and from the active site in a chemically useful form. High concentrations of formaldehyde have been found to inhibit SHMT.[251] The inhibition suggests that the hydrated form of formaldehyde is a competitive inhibitor.

The addition of FH_4 to incubations of (2S)-serine and SHMT has been shown to increase the overall rate of the retro-aldol cleavage reaction by a factor of 160. In the reverse direction FH_4 increases the equilibrium concentration of the quinoid intermediate (**179**) 25-fold.[254] It was first thought that these increases were due to either N^{10}, or more likely N^5 acting as a base. However studies using analogues of 5,10-methylene-FH_4 in which N^5 or N^{10} is blocked (i.e. N^{10}-formyl, N^5-methyl),[257,258] have shown similar increases in the reaction rate, implying that FH_4 does not participate directly in formation of the quinoid intermediate. Temperature jump experiments, in the absence of FH_4 and formaldehyde, have shown that the rate of formation of the quinoid intermediate from glycine is two orders of magnitude greater than the rate of exchange of the α-proton of glycine with those of the solvent. Addition of FH_4 accelerates the proton exchange reaction by more than two orders of magnitude, possibly due to greater exposure of the base to the solvent, while the rate of formation of the quinoid intermediate is increased less than fourfold. The changes in the presence of FH_4 are thought to be due to the FH_4 slowing down the step in which reprotonation of the quinoid intermediate occurs.

In the absence of FH_4, the rate determining steps in the very slow reversible conversion of glycine and formaldehyde to (2S)-serine, have been shown to be either the slow addition of formaldehyde at the active site in a reactive form (serine synthesis), or the slow release of formaldehyde from the active site (serine breakdown).

Indeed the presence or absence of FH_4 changes the kinetic mechanism of serine hydroxymethyltransferase.[259] In the absence of FH_4, the binding of substrates/debinding of products is sequentially ordered.

However, the addition of FH_4 causes the binding of substrates/debinding of products to change to a random process. The change of mechanism suggests that addition of FH_4 not only makes the addition/removal of formaldehyde from the active site less rate determining, but also causes the protein to form an 'open' conformation which allows glycine or (2S)-serine to bind in the presence of FH_4 or 5,10-methylene-FH_4. 5-Methyl-FH_4 and 5,10-methylene-FH_4 are both non-competitive inhibitors of (2S)-serine, suggesting that the one-carbon binding site can accommodate two one-carbon units simultaneously without steric hindrance.

In a series of elegant experiments, Chen *et al.*[251] showed that FH_4 is converted to 5,10-methylene-FH_4 which acts as a one-carbon carrier for the enzyme. It was established that the one-carbon moiety did not leave the active site as either HCHO, or 5-hydroxymethyl-FH_4. It was also showed that the formation of 5,10-methylene-FH_4 from HCHO and FH_4 was not catalysed by SHMT.

There are still many questions concerning the involvement of tetrahydrofolic acid in the

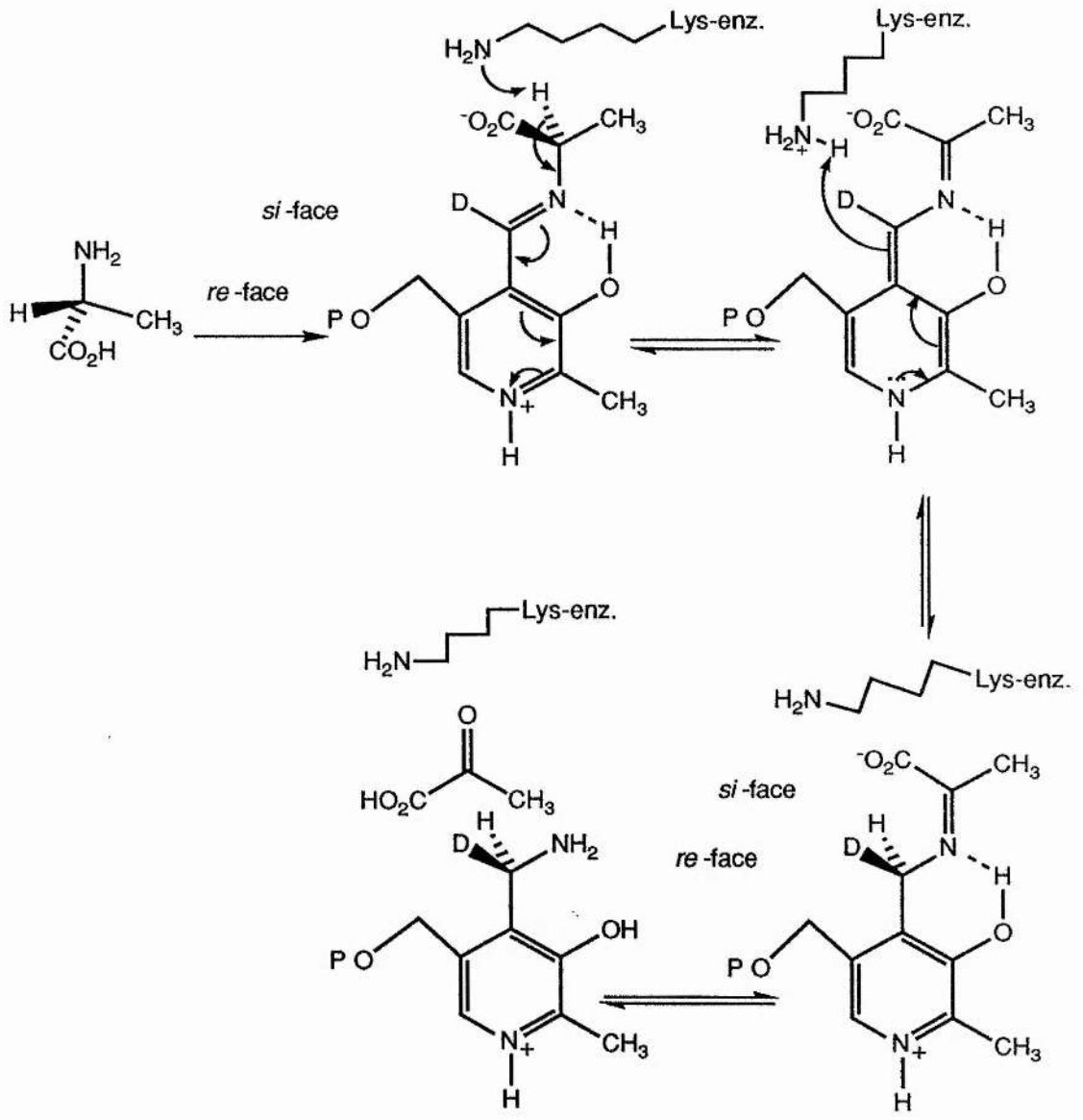
reaction catalysed by SHMT that remain unanswered. These include, how the one-carbon unit is transferred from serine to FH_4, and also what the distance and orientation of the FH_4 binding pocket are with respect to the external aldimine. It is possible that the binding of FH_4 and analogues causes a large conformational change in the protein, possibly revealing a concealed active-site base which is used to mediate the transfer of the hydroxymethyl group.

3.1.8 Stereochemical studies

The reversible retro-aldol cleavage catalysed by SHMT is specific for (2S)-3-hydroxy amino acids. SHMT catalyses the reaction with both *threo*- and *erythro*-diastereoisomers. (2S,3R)-Threonine and (2S,3S)-threonine are substrates,[246] as are (2S,3S)- and (2S,3R)-3-phenylserines.[247] For the retro-aldol cleavage, all pure SHMT enzymes so far studied show higher V_{max} and lower K_M values for the (2S,3R)-isomer. The SHMT catalysed condensation of glycine and acetaldehyde gives 98% (2S,3S)-threonine and 2% (2S,3R)-threonine.[64,251]

The irreversible inactivation of SHMT by (2R)-alanine has been shown to be due to a slow transamination reaction in which (2R)-alanine is converted to pyruvate, and the active-site PLP is converted to PMP (Scheme 3.8). The PMP readily dissociates from the active site, leaving the inactive apoenzyme. This reaction could be predicted by Dunathan's postulate[24] for stereoelectronically assisted cleavage. If one assumes that each amino acid is bound with the carboxyl group in the same position, the α-proton of (2R)-alanine occupies the same position as the hydroxymethyl group of (2S)-serine. Using the same postulate, Dunathan predicted that SHMT would catalyse the removal of only one of the α-protons of glycine, that in the *pro*-S position. This has since been

confirmed.[260-262] The external aldimine in the transamination of (2R)-alanine is deprotonated/protonated at both C^{α} and C-4' from the 4'-*si*-face of the coenzyme (Scheme 3.8).[15,263] This indicates that the 4'-*si*-face of the coenzyme is exposed to the solvent. In all (2S)-amino acid transaminases that have been studied the aldimine is also protonated from the 4'-*si*-face of the coenzyme. This has been interpreted as evidence for an evolutionary relationship among PLP dependent enzymes. It has also been suggested that all vitamin B_6 dependent enzymes have evolved from a common precursor enzyme, from which the orientation of the pyridoxyl ring has been preserved. A difference between transaminases and SHMT is that for a transaminase the glycine *pro*-R proton is transferred to C-4', whereas in SHMT it is the glycine *pro*-S proton that is transferred. This difference illustrates that the amino acid substrates are bound differently in the external aldimine of these enzymes.



Scheme 3.8. The stereochemical course of the transamination of (2R)-alanine catalysed by SHMT.

There are three reactions described in the literature for which the reported stereochemical courses were not in accord with our current understanding of the mechanism of SHMT. First, the rapid decarboxylation of aminomalonic acid[64] was reported to occur without stereochemical preference for either the carboxyl bond cleavage step, or the quinoid intermediate reprotonation step. Second, the exchange of the α-proton of (2S)-phenylalanine and several other amino acids[247,252] with deuterium. Third, the racemisation of alanine.[253] A rationalisation for the

aminomalonate decarboxylation reaction is presented in section 3.2.2.

Investigation of the exact mechanism of the cleavage of the C^{α}-C^{β} bond of (2S)-serine, using stereochemical probes, has also produced some puzzling results. Benkovic *et al.*[264] investigated the stereochemical course of one-carbon transfer using samples of (2S)-serine stereospecifically labelled with tritium at C-3, and found that the reaction was only partially stereospecific. If free formaldehyde was produced, it should be able to freely rotate at the active site and hence produce 5,10-methylene-FH_4 labelled equally in both positions. If however, the formaldehyde was covalently bound at the active site as a hemithioacetal, for example, one might expect either retention or inversion of configuration of the C-3 moiety in the transfer to 5,10-methylene-FH_4. They determined the stereochemical purity of the labelled 5,10-methylene-FH_4 using an enzymic method in which the 5,10-methylene-FH_4 was oxidised to 5,10-methenyl-FH_4 with 5,10-methenyltetrahydrofolate dehydrogenase and $NADP^+$. Starting with (2S)-[3-^{3}H] serine, 76% of the label was retained in the 5,10-methenyl-FH_4. When (3R)-[3-^{3}H] serine was used, the 5,10-methenyl-FH_4 contained 24% of the tritium. These results were in accord with earlier experimental findings[265] with rat liver slices, which had shown that [^{3}H]-formate was converted to (2S)-[3-^{3}H]-serine with 72% of the label in the 3-*pro*-S position. From these findings it was impossible to determine whether formaldehyde transfer was via an intermediate thiohemiacetal.

Relevant to this work, Akhtar *et al.*[266] discovered that acetaldehyde produced from the SHMT catalysed retro-aldol cleavage of (2S,3R)-[3-^{18}O]-threonine contained ^{18}O. This result eliminated the possibility that the acetaldehyde could form an intermediate imine with the active-site lysine residue after it had undergone the C^{α}-C^{β} bond cleavage, since formation of the imine would involve the loss of ^{18}O from the acetaldehyde.

3.1.9 Active-site structure

No X-ray crystal structure for SHMT from any source has been reported although crystals of the rabbit liver mitochondrial enzyme have been produced.[222] Attempts to identify the amino acid residues present at the active site have, therefore, relied upon a combination of chemical and molecular biological techniques.

The first active-site amino acid to be identified was the lysine residue which forms an internal Schiff's base complex with PLP. The reduction of the imine with sodium borohydride followed by acid hydrolysis of the enzyme resulted in the isolation of ε-pyridoxyllysine.[228] A similar procedure using tritiated sodium borohydride, followed by proteolytic digestion, allowed the isolation and identification of a nonapeptide containing the ε-pyridoxyllysine residue.[267] Through the use of a combination of such techniques and gene sequencing, it has since been shown that the decapeptide encompassing the active-site lysine is VVTTTTHKTL and the sequence is identical for the cytosolic and mitochondrial enzyme from rabbit liver and from *E. coli*.[170] The sequence contains the motif S(N,T)-X-H-K which is common in many PLP dependent enzymes which display decarboxylase activity including *E.coli* glutamate decarboxylase (see section 2.1.8 for a table of active-site structures).

Although there are X-ray crystal structures available for some decarboxylases,[268] they are not refined enough to determine the role of individual amino acids. Some insight into the role of the individual amino acids has been gained from examination of the active-site structures of aspartate aminotransferase (AAT) and tryptophan synthase, the only PLP-dependent enzymes for which good high-resolution X-ray crystal structures are available (see section 2.1.8). Attention has been focused on the possible catalytic

role of the histidine residue that is adjacent to the Schiff's base forming lysine residue. The 3-hydroxy group of the serine residue (Ser257) that occupies an analogous position in AAT is known to form a hydrogen bond with one of the negatively charged oxygens of the PLP 5'-phosphate group.

Extensive kinetic studies on (2S)-methionine decarboxylase from *Dryopteris filix-mas*,[150] and glutamate decarboxylase from *E. coli*[149] have shown that there are (at least) two distinct bases involved in the catalytic mechanism of these enzymes (see section 2.1.7). These catalytically important histidine and lysine bases are also conserved in SHMT.

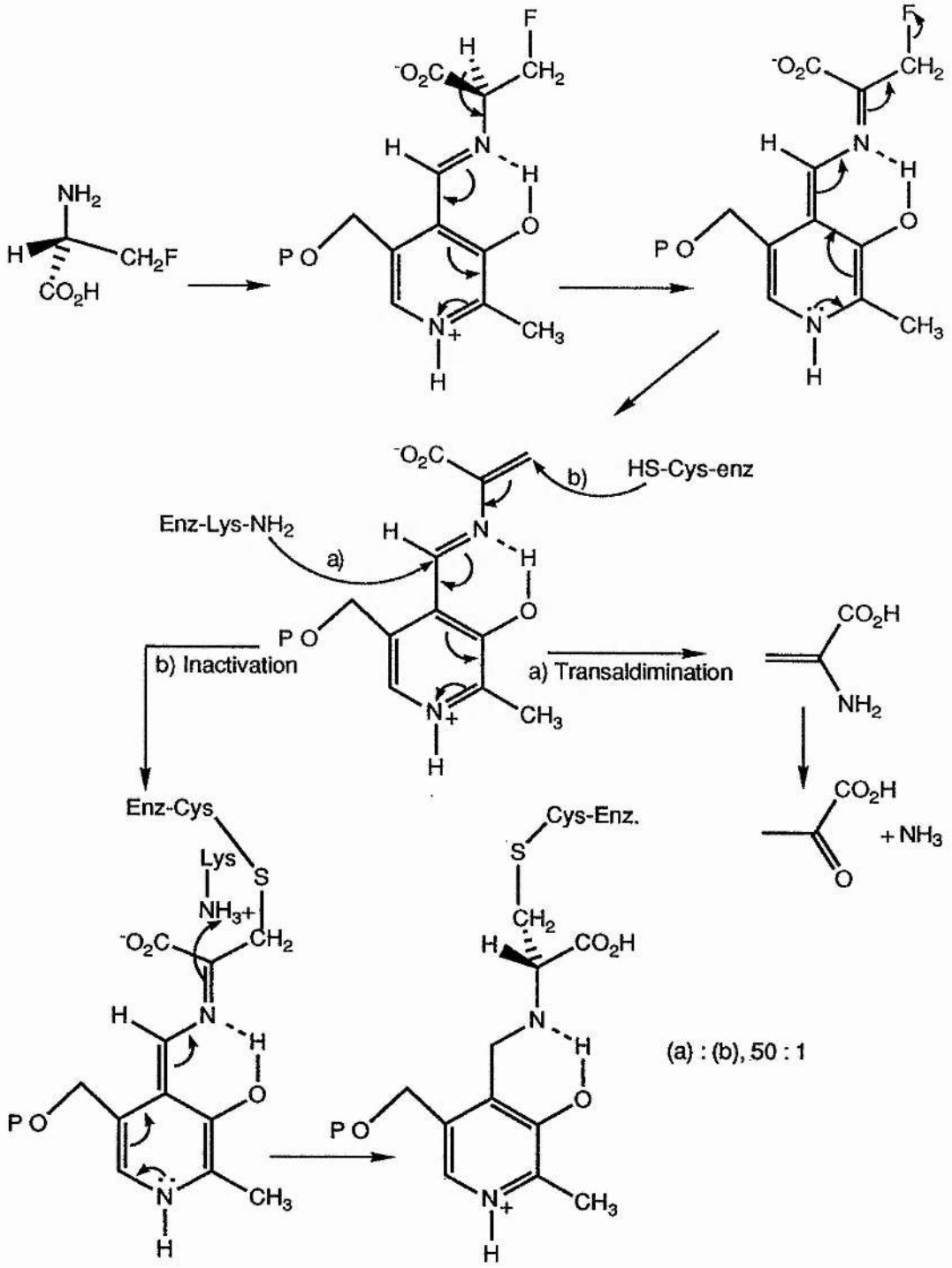
Evidence for several ionisable groups at the active site of SHMT has been produced using a variety of kinetic and spectral studies. A cationic group of pK_a 6.2, which binds anions that are competitive inhibitors of the normal substrates has been found.[269] This cationic group is not at the binding site for the substrate amino acid α-carboxyl group in the external aldimine form as the K_M for (2S,3S)-threonine is invariant between pH 5.8 and 7.6. The uv spectrum of the enzyme-glycine complex varies as a function of pH as the different forms of the complex are obtained. Studies of this variation have concluded that the spectral shifts are due to the dissociation of a single proton from the enzyme-glycine complex with a pK_a of 6.9. For the rabbit liver mitochondrial enzyme, it is possible to observe that a group of pK_a 9.0 affects the concentration of the quinoid intermediate. This ionisable group is probably the α-proton of the external glycine-Schiff's base complex.[231]

Pre-steady state kinetic techniques have been used to investigate the cleavage of (2S,3R)-3-phenylserine by lamb liver SHMT. Ching and Kallen[230] observed a group of

pK_a 7.1 which was involved in the transaldimination process (compared with the group of pK_a 6.9 in the rabbit liver enzyme and of pK_a 6.2 in decarboxylases). Catalytically important groups of pK_a 6.9 and 7.7 were also found, which were thought to be involved in the formation of the quinoid intermediate. The group of pK_a 7.7 was proposed to be involved in the reprotonation of the group of pK_a 6.9.

From these studies it was proposed that the conserved histidine residue was the group which possessed a pK_a of 6.2, which was involved in the transaldimination and quinoid reprotonation steps. Site-directed mutagenesis (SDM) experiments were carried out on SHMT from *E. coli*, with His228 being replaced by an asparagine residue.[270]

The use of sulphydryl attacking reagents has suggested that there are one or more cysteine residues at the active site of SHMT from rabbit liver or *E.coli*.[271] One of the cysteine residues was found to be involved in the mechanism-based inactivation of the cytosolic rabbit liver enzyme by (2S)-3-fluoroalanine (Scheme 3.9).[272,273]

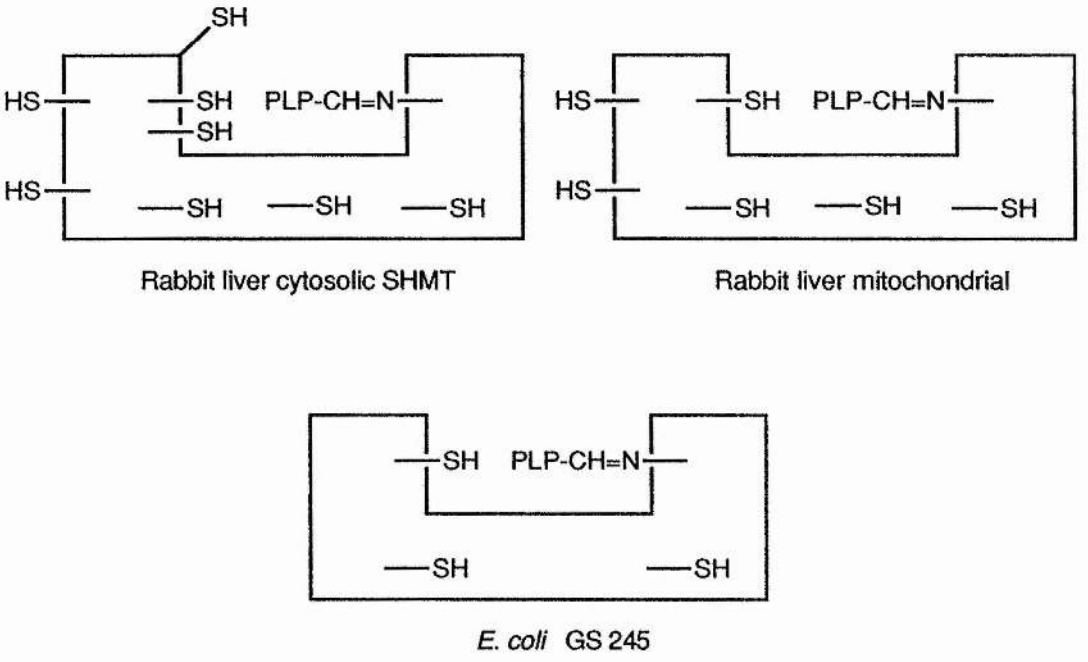


Scheme 3.9. The inactivation of SHMT by (2S)-3-fluoroalanine.

In the presence of tetrahydrofolic acid and (2S)-3-fluoroalanine, the enzyme was found to partition between transamination and irreversible inactivation. The inactivation reaction was shown to proceed via the attack of an active-site cysteine side chain at C-3 of the nascent aminoacrylate, to form a covalently attached inhibitor (Scheme 3.9). Interestingly, this same sulphydryl group was the putative acceptor group for transfer of nascent formaldehyde to serine and tetrahydrofolic acid via an intermediate hemithioacetal. Studies have now shown that this cysteine residue can be selectively blocked with methyl methanethiosulphonate to form a methyl disulphide without affecting the catalytic activity.[271]

Lamb liver SHMT is also inactivated by (2S)-3-fluoroalanine, however rabbit liver SHMT is not inhibited by this compound. It would be anticipated that the enzyme from *E. coli* would not be inhibited as it has no analogous cysteine residue. This raises the question of whether this cysteine residue is involved in a regulatory role, rather than a catalytic one.

A second active-site cysteine which is protected by PLP and which can only be chemically modified by denaturing the enzyme (6 M urea), or by forming the apoenzyme (through reaction of the holoenzymes with (2R)-alanine or (2S)-cysteine) has been discovered. Chemical modification of this group with iodoacetate prevents reassociation of the apoenzyme with PLP.[272] Blocking the group as a methyl disulphide, formed using methyl methanethiosulphonate, results in the formation of an inactive holoenzyme. This result suggests that the cysteine residue may have a role in the catalytic mechanism of the enzyme. The residue is conserved in both the rabbit liver mitochondrial and *E. coli* enzymes (see Figure 3.2)



Rabbit liver cytosolic SHMT

Rabbit liver mitochondrial

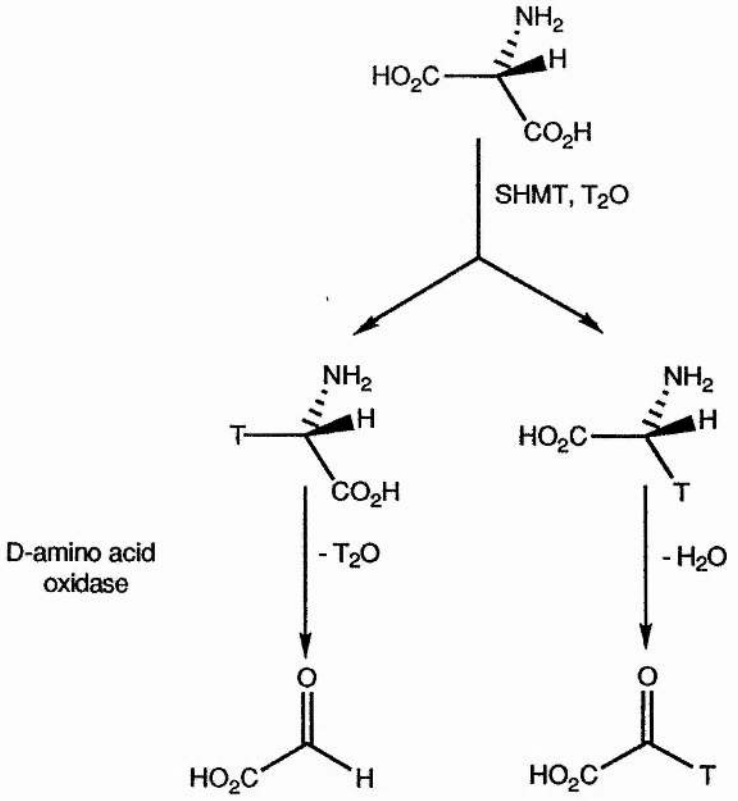
E. coli GS 245

Figure 3.2. The disposition of cysteine residues in three forms of SHMT.

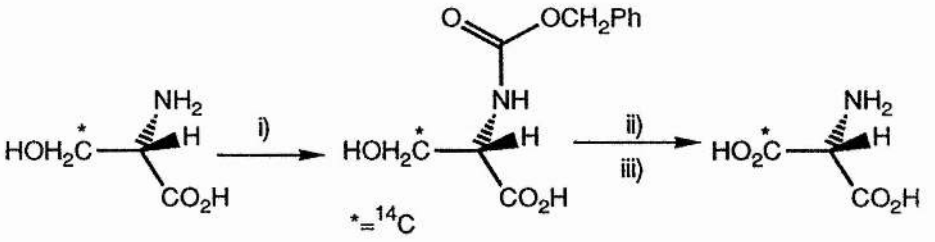
3.2 The decarboxylation of aminomalonic acid by SHMT

The properties and reactions of serine hydroxymethyltransferase have been discussed in section 3.1. The report by Palekar, Tate and Meister[64] that SHMT catalysed the non-stereospecific decarboxylation of aminomalonic acid, was of interest to previous workers in the group, since the claim was the first and only reported example of a non-stereospecific decarboxylation reaction catalysed by a PLP dependent enzyme. The non-stereospecific nature of the reaction was established on the basis of two experiments. In the first, 2-aminomalonic acid was incubated with SHMT and PLP in tritiated sodium phosphate buffer for 30 min. at 37 °C. The reaction was quenched by the precipitation of the enzyme with ethanol after 40% of the substrate had been consumed. The unreacted 2-aminomalonate was separated from the tritiated glycine formed in the reaction and the tritium content of the glycine was measured. The glycine was then oxidised to pyruvic acid using D-amino acid oxidase, a process known to

remove the *pro*-S hydrogen of glycine selectively.[262] Approximately 48% of the tritium label was released, suggesting that reprotonation of the quinoid intermediate formed during the decarboxylation was non-stereospecific (Scheme 3.10). In the second, incubation of (2R)-[1-^{14}C]-aminomalonic acid, synthesised in three steps from (2R)-[3-^{14}C]-serine (Scheme 3.11), with SHMT and PLP in potassium phosphate buffer (pH 6.5) for 30 min. at 25 °C. Half of the radiolabel was lost as $^{14}CO_2$ and half was retained in the glycine product (Scheme 3.12), again suggesting that the decarboxylation reaction was non-stereospecific.

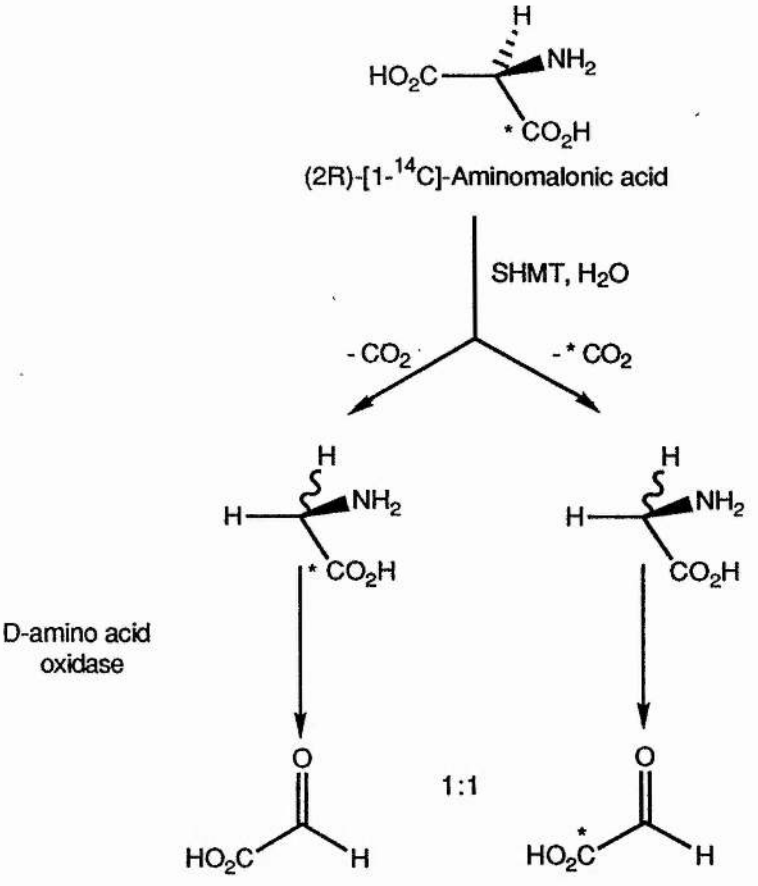


Scheme 3.10. Determination of the stereochemical course of reprotonation for the decarboxylation of 2-aminomalonate catalysed by SHMT.



Reagents: i) $PhCH_2OCOCl$, $NaHCO_3$, H_2O
ii) $KMnO_4$, KOH, H_2O; iii) H_2 / Pd / C.

Scheme 3.11. The synthesis of (2R)-[1-^{14}C]-aminomalonic acid.

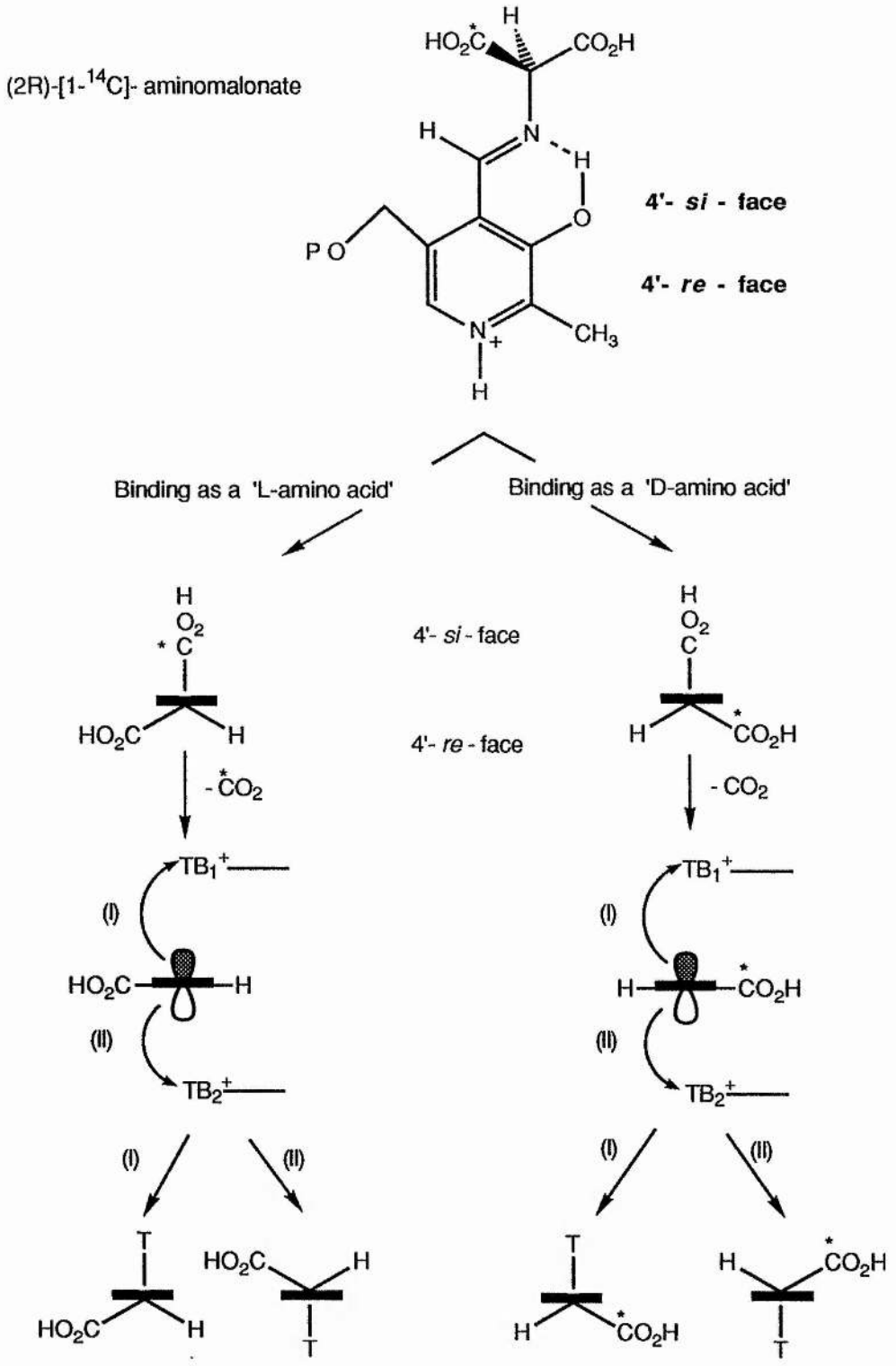


Scheme 3.12. Determination of the stereochemical course of the carboxyl cleavage step of the decarboxylation of (2R)-[1-^{14}C]-aminomalonic acid catalysed by SHMT.

The conclusion that SHMT catalysed a completely non-stereoselective decarboxylation reaction, therefore, seemed to be irrefutable. However the result appeared to contradict Dunathan's proposals regarding stereoelectronic control in PLP dependent enzymes.[36]

Palekar suggested that the substrate, 2-aminomalonic acid, might be able to bind in two equally populated conformations at the active site of the enzyme, such that each of the two carboxyl groups was positioned correctly for decarboxylation at 90° to the plane of the coenzyme. If the decarboxylation and subsequent protonation steps occurred stereospecifically for each form, then the observed results would be obtained (Scheme 3.13). This proposal is supported by the fact that both (2R)- and (2S)-antipodes of several amino acid substrates bind to and are processed by the enzyme,[250] although the K_M values for the (2R)-antipodes are tenfold larger than for the (2S)-amino acids.

However, if racemisation of the substrate occurred prior to the decarboxylation, the same observations might have been expected. Prior racemisation it appeared could be ruled-out in view of Palekar's earlier finding; that the PLP-dependent enzyme, aspartate β-decarboxylase, catalysed the stereospecific decarboxylation of aminomalonate.[67]



Scheme 3.13. (2R)-[1-^{14}C]-Aminomalonic acid binding at the active site of SHMT in two distinct conformations.

3.2.1 Re-examination of the decarboxylation of 2-aminomalonic acid by SHMT

Recent work in our laboratories with mammalian cytosolic and *E. coli* SHMT, using the chiral ^{13}C-labelled isotopomers of the very slow decarboxylation substrate 2-amino-2-methylmalonic acid,[274,275] had shown that cleavage of the *pro-R* carboxy group of the substrate occurred with retention of configuration to give (2R)-alanine. Thus, in the context of the earlier work, either the bulkier substrate was only able to bind to the enzyme in one of the two conformations available to 2-aminomalonic acid, or Palekar's explanation for non-stereospecificity with 2-aminomalonic acid as the substrate was incorrect.

In order to test these possibilities, Thomas[276] repeated Palekar's[64] experiments with 2-aminomalonic acid and used chemical assays for the chirality of the product glycine.

2-Aminomalonic acid (30 mM, 3 times K_M) was incubated with SHMT and PLP in deuteriated buffer under the exact conditions described by Palekar *et al.*[64] but, on a 20-fold larger scale, and the decarboxylation products were obtained. The glycine was treated with (1S,4R)-camphanoyl chloride to give the (1'S,4'R)-camphanamide derivative (**182**) which was examined by NMR spectroscopy. The unlabelled camphanamide derivative (**183**) and the 2-dideuterio isotopomer (**184**) were known to display well separated AB-type signals for the diastereotopic C-2 hydrogens of the glycine moiety in the proton and deuterium NMR spectra, H_R and H_S occur at δ 4.05 and 4.16, respectively in the 360 MHz proton NMR spectrum.[195,277] Hence, on the basis of the earlier work[64] the spectra of the derivatives of the decarboxylation products were expected to show the incorporation of ≈50 atom% solvent derived hydrogen in

each of the C-2 positions. In actuality the incorporation of solvent hydrogen was close to 100 atom% for each of the C-2 positions as judged by the proton and deuterium NMR and mass spectrum of the camphanamide derivative (**182**). Thus, it was evident that either α-hydrogen exchange had occurred prior to decarboxylation or that the product glycine had racemised.

As Palekar[64] had used tritium for his experiments and analysed the chirality of the tritiated glycine product using (2R)-amino acid oxidase, which is known to transfer the 2-*pro*-S hydrogen of glycine to the solvent, their apparent results were identical with those obtained by Thomas.[276] However, the result of Thomas[276] had shown that too much solvent derived hydrogen had been incorporated into the glycine.

In order to determine whether non-enzymic α-hydrogen exchange between the substrate and the solvent could occur during the enzymic decarboxylation reaction, Thomas[276] measured the rates of α-deuterium atom incorporation into the unlabelled substrate in a vast excess of deuterium oxide under pseudo-first order conditions over the pD range 5.0-8.0 at 20 °C. The reaction was followed by proton NMR spectroscopy. It was found that for pD 5.0-6.5, the half-lives for the exchange reactions were 15-20 min, whilst those for pD 7.0-8.0 were 35, 55 and 95 min respectively. Under Palekar's[64] conditions, pH 6.0 and 37 °C, the half-life for exchange would have been ≈5 min, a considerably shorter period than the duration of the experiments upon which Palekar performed the original stereochemical analyses.

3.2.2 Re-examination of the decarboxylation reaction in the light of the findings of Thomas

It was now evident that a substantial amount of the 2-aminomalonic acid substrate had undergone α-hydrogen exchange or had been racemised prior to the actual enzyme catalysed decarboxylation reaction in the original experiments and it was absolutely clear that SHMT, in fact, had not been shown to catalyse decarboxylation non-stereospecifically. From the kinetic analysis of the competition for the unlabelled substrate between the exchange/racemisation reaction, a pseudo-first order process, and the enzyme-catalysed decarboxylation, an effectively first order process with respect to substrate at $[S]>K_M$ (the standard conditions at the start of the reactions) it seemed expedient to reassess the stereochemical course in incubations containing much larger amounts of enzyme.

2-Aminomalonic acid (**34**) was prepared by heating diethyl aminomalonate hydrochloride in potassium hydroxide solution, and was purified by ion exchange chromatography as recorded by Thomas.[278] It was planned that SHMT would be obtained from Verne Schirch (Virginia Commonwealth University) as a dry powder and that on arrival at St. Andrews the activity would be regenerated by dialysis. However problems were encountered in obtaining fully active enzyme. The maximum activity measured on assaying the enzyme for (2S,3S)-threonine aldolase activity was 0.5 unit/mg (where 1 unit is the amount of enzyme required to convert 1 μmole of (2S,3S)-threonine to glycine and acetaldehyde per minute at 37 °C), the expected activity was ≈15 unit/mg. In the presence of glycine and 5-formaldehyde-tetrahydrofolic acid, the active enzyme should exhibit a uv absorption of about 0.4-0.5 absorbance units at 500 nm. In our hands the cytosolic enzyme showed a small amount of (2S,3S)-threonine

aldolase activity and had an absorption of 0.216 units at 500 nm. The *E. coli* enzyme had no detectable (2S,3S)-threonine aldolase activity and did not have a measurable absorption signal at 500 nm. There was no doubt that the cytosolic enzyme was not very active but, fortunately, we had enough enzyme to carry out the necessary decarboxylation reactions.

The cytosolic enzyme (≈25 units) was dissolved in deuterium oxide (5 ml) and the pL was adjusted to 7.0. 2-Aminomalonate (6 mg) (**34**) was added in two portions at five minute intervals and the solution incubated at 37 °C for a total of 15 min. Denaturation of the enzyme was accomplished by the addition of an equal volume of ethanol, and the precipitated enzyme removed by filtration. The mixture of 2-aminomalonate and glycine was purified by preparative tlc (^{i}PrOH:NH_3:H_2O 26:6:5) and the glycine was isolated and converted to its (1'S,4'R)-camphanamide derivative (**185**).

Deuteriated 2-aminomalonate (**186**), prepared by the exchange reaction in deuterium oxide at 37 °C, was incubated with the enzyme in protium oxide. The glycine product was isolated and derivatised as a camphanamide (**187**) under identical conditions to those described for the non-deuteriated 2-aminomalonate (**34**).

Examination of the proton and deuterium NMR spectra and mass spectra of the glycine camphanamide derivative (**185**) from the non-deuteriated aminomalonate (**34**) indicated that very little dideuterioglycine (<5%) had been formed and that the monodeuteriated glycine was of (2S)-configuration only. For the [2-^{2}H]-2-aminomalonic acid (**186**), the spectral analyses of the derivatised glycine (**187**) indicated that only (2R)-glycine had been formed. The proton NMR spectra and the mass spectra for the enzymically derived (2R)- and (2S)-[2-^{2}H]-glycine-(1'S,4'R)-

camphanamide (**187,185**) along with the spectra for glycine-(1'S,4'R)-camphanamide (**183**) and [2-$^{2}H_{2}$]-glycine-(1'S,4'R)-camphanamide (**184**) are shown in Figures 3.3, 3.4a and 3.4b. From these results it is clear that SHMT catalyses the stereospecific decarboxylation of 2-aminomalonic acid.

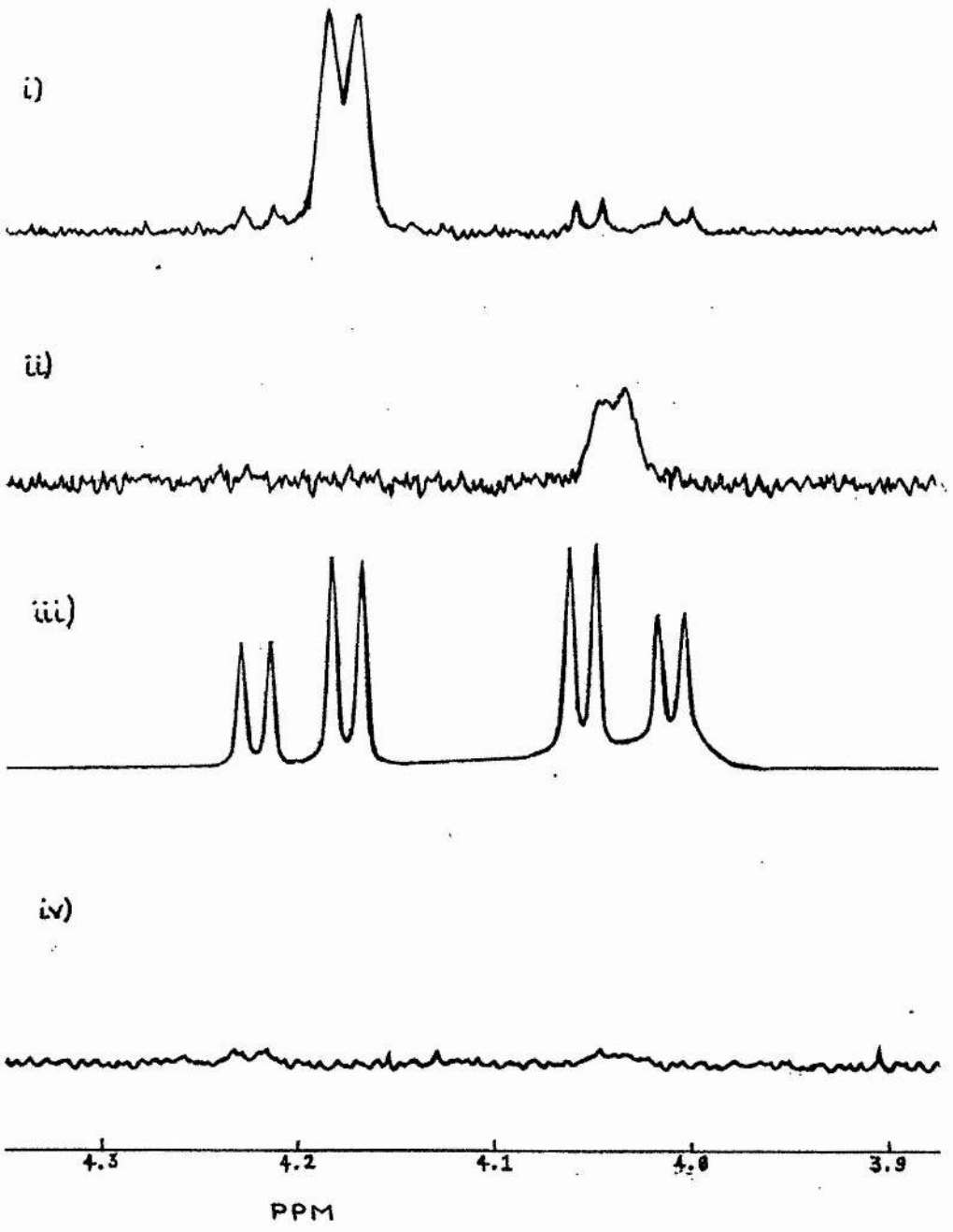


Figure 3.3. The 400 MHz proton NMR spectra in $CDCl_3$ of i) the (1'S,4'R)-camphanamide of (2R)-[2-^{2}H]-glycine (**187**), ii) the (1'S,4'R)-camphanamide of (2S)-[2-^{2}H]-glycine (**185**), iii) the (1'S,4'R)-camphanamide of glycine (**183**) and iv) the (1'S,4'R)-camphanamide of [2-$^2H_2$]-glycine (**184**).

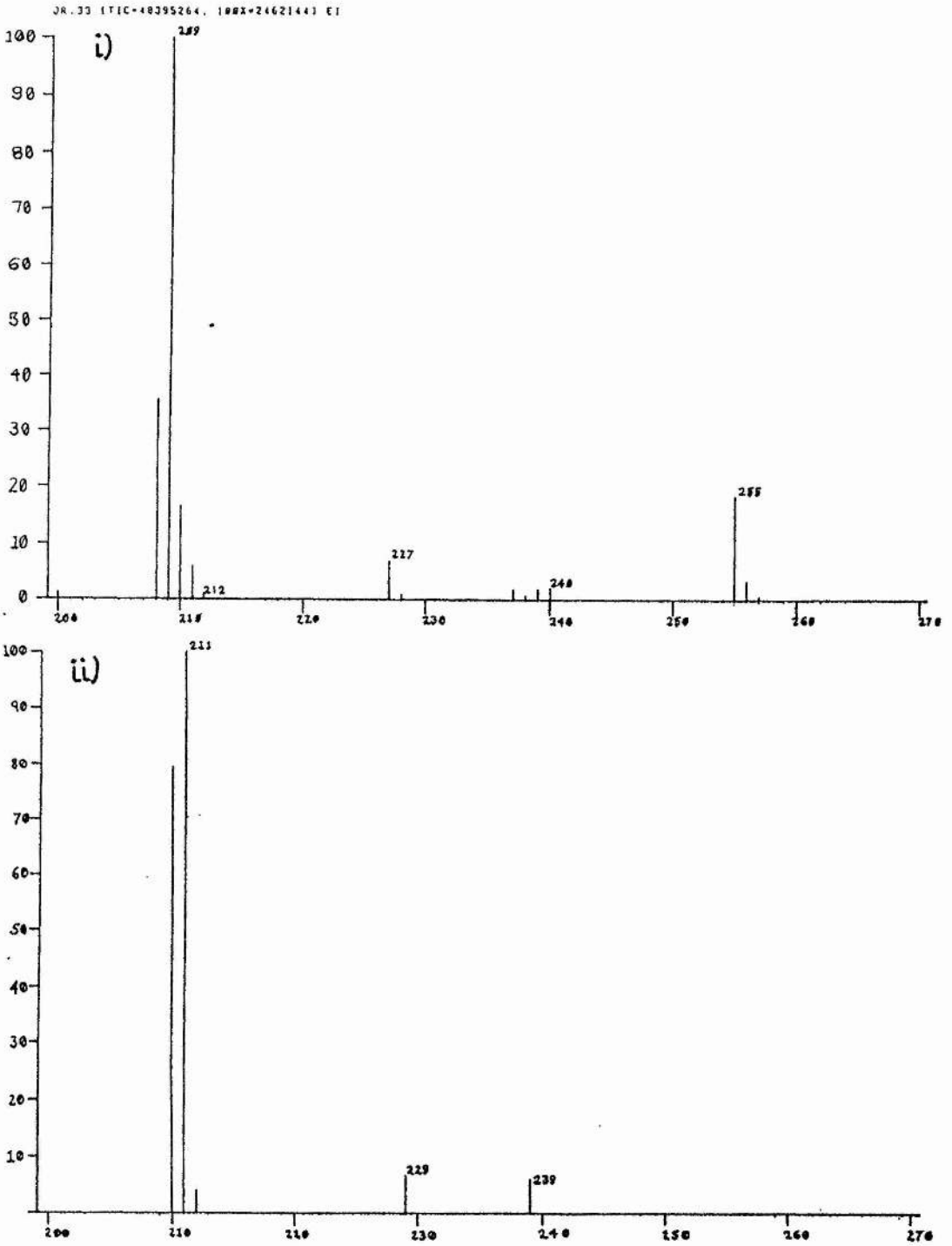


Figure 3.4a. The mass spectra of i) the (1'S,4'R)-camphanamide of glycine (**183**) and ii) the (1'S,4'R)-camphanamide of [2-$^{2}H_{2}$]-glycine (**184**).

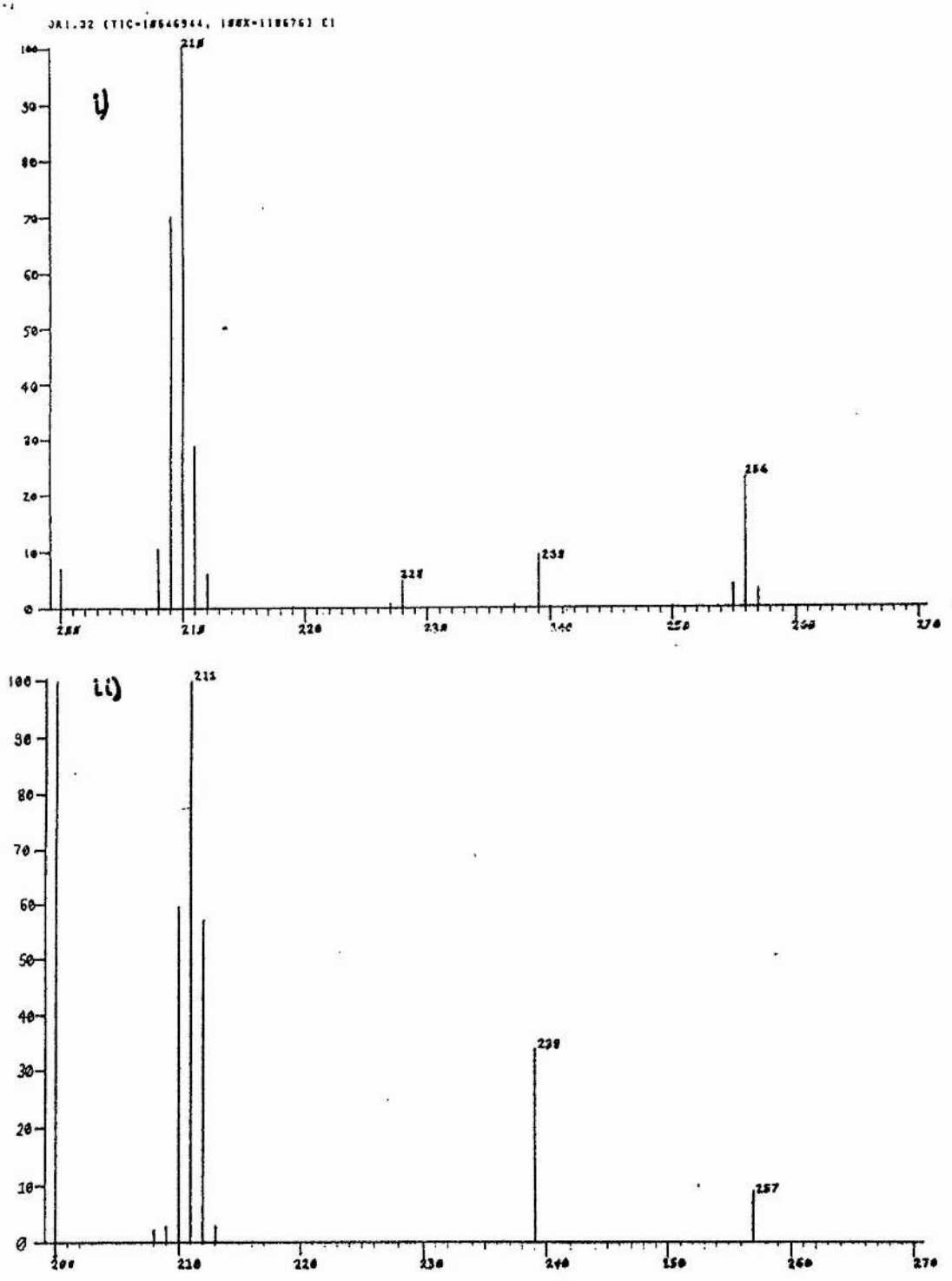
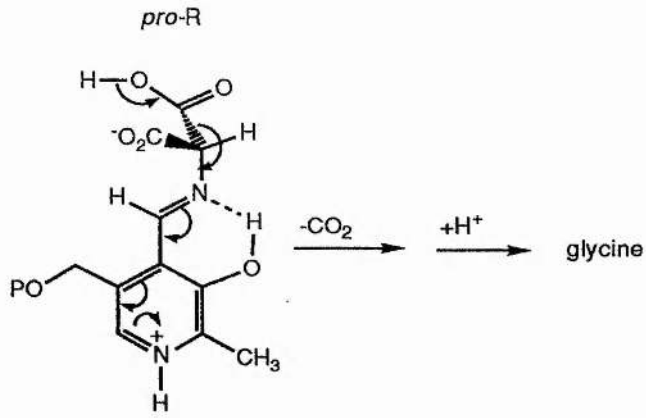


Figure 3.4b. i) The mass spectra of the (1'S,4'R)-camphanamide of (2S)-[2-^{2}H]-glycine (**185**) and ii) the (1'S,4'R)-camphanamide of (2R)-[2-^{2}H]-glycine (**187**).

3.3 Conclusions

In the light of earlier work carried out in Professor Gani's group with 2-methyl-2-aminomalonic acid[274] and studies with the enzyme (2S)-methionine decarboxylase,[150,151] the work described here indicates that cleavage of the *pro*-R carboxy group of 2-aminomalonic acid occurs with retention of configuration. Hence protonation introduces the 2-*pro*-S hydrogen of glycine, and all of this chemistry occurs on the 4'-*si*-face of the coenzyme (Scheme 3.14).



Scheme 3.14. The decarboxylation of 2-aminomalonic acid on the C-4'-*si*-face of the coenzyme.

These results support the notion that there is an evolutionary relationship between (2S)-amino acid transaminases, (2S)-α-amino acid decarboxylases and SHMT.

A recent report[279] states that an enzyme from *Alcaligenes bronchisepticus* KU 1201 decarboxylates α-methyl-α-phenylmalonic acid to give (2R)-phenylpropionic acid. The result indicates that the *pro*-R carboxyl group of α-methyl-α-phenylmalonic acid is removed to form (2R)-phenylpropionic acid with inversion of configuration. This is in sharp contrast to the situation discussed above for SHMT. Note that the

decarboxylation of S-methylmalonyl-CoA by malonyl-CoA decarboxylase[280] also occurs with retention of configuration.

CHAPTER FOUR

EXPERIMENTAL

4.0 Experimental

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

Elemental analyses were carried out in the departmental microanalytical laboratory.

Optical rotations were measured at room temperature using an AA 1000 polarimeter at St. Andrews University and an AA 100 polarimeter at Southampton University. 10 cm or 20 cm path-length cells were used.

Infrared spectra were recorded on a Perkin-Elmer 1710 IR FT spectrometer, a Perkin-Elmer 1330 IR spectrometer, or a Perkin-Elmer 1500 series FT IR spectrometer. The spectra were recorded as thin films between sodium chloride discs. The absorption maxima are given in wavenumbers (cm^{-1}) relative to polystyrene standard.

^1H Nuclear magnetic resonance spectra were recorded at 90 MHz on a Jeol FX90Q, at 200 MHz on a Varian Gemini-200, at 270 MHz on a Jeol JNM-GX270, at 300 MHz on a Bruker AM-300, and at 360 MHz on a Bruker AM-360 instrument. The ^1H nuclear magnetic resonance spectra recorded at 400 MHz were obtained from the SERC NMR service at Warwick University. All NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position (δ_H), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-double doublet, sp-septet and br-broad), coupling constant (*J* Hz), and assignment (numbering according to IUPAC nomenclature for the compound).[281]

^{13}C Nuclear magnetic resonance spectra were recorded at 22.6 MHz on a Jeol FX90Q, at 50.30 MHz on a Varian Gemini-200, at 67.8 MHz on a Jeol JNM-GX270, at 75.47 MHz on a Bruker AM-300, and at 90.56 MHz on a Bruker AM-360 instrument. The ^{13}C nuclear magnetic resonance spectra recorded at 100.61 MHz were obtained from the SERC NMR service at Warwick University.

^{2}H Nuclear magnetic resonance spectra were recorded at 61.44 MHz by the SERC NMR service at Warwick University and are quoted in ppm relative to $CDCl_3$ at 7.27 ppm.

$CHCl_3$ was used as an internal standard for $CDCl_3$ spectra. HOD was used as an internal standard for D_2O, DCl and NaOD spectra referenced at 4.7 ppm. For ^{13}C spectra run in D_2O, DCl and NaOD, dioxane (67.3 ppm) and methanol (49.9 ppm) were used as internal standards.

Mass spectra and accurate mass measurements were recorded at Southampton University on a VG 70 250 SE, at St. Andrews on a Kratos MS 50, or by the SERC service at Swansea using a VG ZAB E. Major fragments are given as percentages of the base peak intensity (100%). Fast atom bombardment (FAB) spectra were recorded using glycerol as a matrix.

Electronic absorption spectra were obtained using a Pye-Unicam SP8-500 uv/vis spectrometer.

"Flash" chromatography was performed according to the procedure of Still[282] using Sorbsil C60 silica gel.

Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (MN SIL G/UV_{254}) or on 0.1 mm precoated cellulose plates (CEL MN 300-10 UV_{254}), and compounds were visualised by uv fluorescence, iodine vapour, phosphomolybdic acid in ethanol, aqueous potassium permanganate or ninhydrin.

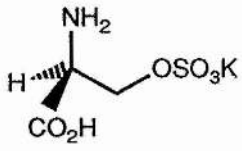
Solvents were dried and purified according to the methods of Perrin and Armarego.[283]

Enzymes other than aspartate aminotransferase (AAT) and serine hydroxymethyltransferase were obtained from Sigma Chemical Co., Poole, UK. and were used without further purification.

Aspartate aminotransferase (AAT) [Glutamate-oxaloacetate transaminase (GOT)] was obtained from Boehringer Mannheim UK, Lewes, UK. and was used without further purification.

Serine hydroxymethyltransferase was donated by Professor Verne Schirch of Virginia Commonwealth University and was reconstituted prior to use.

Potassium (2S)-serine O-sulphate (21)

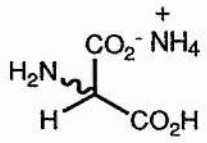


(2S)-Serine (1 g, 9.6 mmol) was added at room temperature to conc. sulphuric acid (sp. gr. 1.84, Analar, 2 ml). The mixture was stirred *in vacuo* until the serine had fully dissolved (about 2 h). Ice cold water (10 ml) was added, and the pH adjusted to 7.25 with an aqueous solution of barium hydroxide (saturated). The precipitated barium sulphate was removed by centrifugation and the clear supernatant was concentrated *in vacuo* to a small volume (approx 2 ml) at 38 °C. This solution was passed through an Amberlite IR-120(H) ion exchange column and eluted with water. Fractions (10 ml) were collected and checked by tlc (using ^{i}PrOH:NH_3:H_2O 26:6:5 and visualised with ninhydrin) for the presence of the product. The product containing fractions were combined, adjusted to pH 7.8 with 5% aq. KOH and concentrated to dryness *in vacuo* at 38 °C. The white residue was dissolved in the minimum amount of cold water and precipitated by the dropwise addition of ice cold ethanol. The (2S)-serine O-sulphate was filtered off and dried overnight (550 mg, 30%), m.p. 150 °C (dec.); (Found: C, 16.16; H, 2.56; N, 6.16; M^+, 223.0981. Calc. for $C_3H_6NO_6SK$: C, 16.14; H, 2.71; N, 6.26%; *M*, 223.0936); $[\alpha]_D$ -10.25° (c. 1.0 in H_2O) [lit.,[192] $[\alpha]_D^{16}$ -9.5° (c. 5 in H_2O)]; ν_{max} (nujol) 3400-3100 (OH) and 1650 cm^{-1}(amino acid C=O); δ_H (90 MHz, D_2O/NaOD) 3.53 (1H, t, *J* 5Hz, 2 C<u>H</u>) and 4.18 (2H, m, 3 C<u>H</u>$_2$); δ_C (67.5 MHz, D_2O/NaOD/dioxane) 47.7 (3 <u>C</u>H_2), 71.5 (2 <u>C</u>H) and 178.4 (<u>C</u>O_2H).

(2S)-Serine O-sulphate (21a)

Chlorosulphonic acid (1 ml, 15 mmol) was added dropwise to a stirred solution of (2S)-serine (1.05 g, 10 mmol) in trifluoroacetic acid (10 ml). As the chlorosulphonic acid was added a white solid precipitated. Once the addition was complete, the mixture was left to stand at room temperature for 20 min. The excess chlorosulphonic acid was destroyed by the addition of ethanol (1 ml). Four volumes of diethyl ether (40 ml) were added to complete the precipitation. The solid was filtered off and dried. The crude product was recrystallised from water and ethanol (1.38 g, 62%), m.p. 228-230 °C; (Found: C, 19.72; H, 3.65; N, 7.57. Calc. for $C_3H_7NO_6S$: C, 19.46; H, 3.81; N, 7.56%); *m/z* (Found: $[M+H]^+$ 186.0072. $C_3H_8O_6NS$ requires, 186.0072); $[\alpha]_D$ +10.03° (c. 3.4 in 1 M HCl) [lit.,[193] $[\alpha]_D^{20}$ +9.8° (c. 3.4 in 1 M HCl)]; $[\alpha]_D$ -2.96° (c. 1.145 in 5% KOH); ν_{max} (nujol) 3200 (NH/OH str), 2800-3000 (CH str and nujol) and 1755 cm^{-1} (acid CO_2H monomer); δ_H (200 MHz, D_2O/DCl) 4.1-4.35 (m, 2 C<u>H</u> and 3 C<u>H</u>$_2$); δ_C (50 MHz, D_2O/DCl/dioxane) 54.81 (3 <u>C</u>H_2), 68.95 (2 <u>C</u>H) and 171.01 (<u>C</u>O_2H).

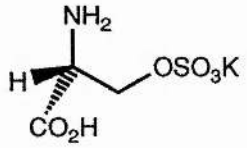
Aminomalonate monoammonium salt (34)



Diethyl aminomalonate hydrochloride (11.11 g, 52.5 mmol) was dissolved in 2 M KOH (110 ml) and the resulting solution refluxed for 15 min. The solution was cooled in ice and adjusted to pH 6.0 using 30% acetic acid solution. Two volumes of 95% ethanol were added, the resulting precipitate filtered off and washed with ethanol. This precipitate was taken up in the minimum amount of water (approx 100 ml) and applied to a column (30 x 2 cm) of Amberlite IRA 400 anion exchange resin (acetate form). The

column was washed with water to remove inorganic salts, and the desired product was displaced using 2 M ammonium acetate. Fractions 2-33 (ninhydrin positive) were concentrated to dryness *in vacuo*, recrystallised from 2 M ammonium hydroxide and ethanol giving a white solid (3.64 g, 51%), m.p. 208 °C (lit.,[277] 208 °C); (Found: C, 26.58; H, 5.60; N, 20.80. Calc. for $C_3H_8N_2O_4$: C, 26.47; H, 5.92; N, 20.58%); δ_H (200 MHz, $CDCl_3$) 4.1 (1H, s, 2 C<u>H</u>); δ_C (50 MHz, $CDCl_3$) 62.04 (2 C), 173.08 (acid carbons); *m/z* (EI) 75 ($[M+H-CO_2NH_4]^+$, 5%), 44 (80, $[CO_2H]^+$) and 30 (100, $[CH_2NH_2]^+$).

Potassium (2R)-serine O-sulphate (59)



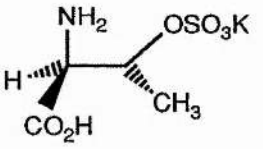
This was prepared in an identical manner to the (2S)-antipode (**21**) starting from (2R)-serine in 30% yield. M.p. 164-166 °C; (Found: C, 16.28; H, 2.67; N, 6.22; M^+, 223.0954. Calc. for $C_3H_6NO_6SK$: C, 16.14; H, 2.71; N, 6.26%; *M*, 223.0936); $[\alpha]_D$ 6.3° (c. 1 in H_2O); ν_{max} (nujol) 3400-3100 (OH), 1650 (amino acid C=O), 1609 (NH_2 bend), 1210 (S-O) and 794 cm^{-1} (C-O-S); δ_H (90 MHz, D_2O/NaOD) 3.55 (1H, t, *J* 4.3 Hz, 2 C<u>H</u>), and 4.2 (2H, m, 3 C<u>H</u>$_2$). δ_C (22.5 MHz, D_2O/MeOH) 52.06 (3 <u>C</u>H_2), 64.52 (2 C) and 168.63 (<u>C</u>O_2H).

(2R)-Serine O-sulphate (59a)

This was prepared in an identical manner to (2S)-serine O-sulphate (**21a**) using (2R)-serine following the method of Previero[163] in 70% yield. M.p. 188-190 °C; (Found: C, 19.37; H, 3.58; N, 7.51. Calc. for $C_3H_7NO_6S$: C, 19.46; H, 3.81; N, 7.56%); $[\alpha]_D$ -9° (c. 3.4 in 1 M HCl) [lit.,[163] $[\alpha]_D^{20}$ +9.8° (c. 3.4 in 1 M HCl for (2S)-isomer)]; $[\alpha]_D$ +3.5° (c.

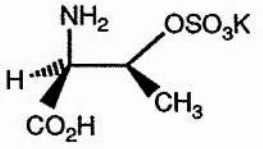
1.085 in 5% KOH); ν_{max} (nujol) 3200 (NH/OH str), 2800-3000 (CH str and nujol) and 1760 cm^{-1} (acid CO_2H monomer); δ_H (200 MHz, D_2O/NaOD) 4.29 (1H, m, 2 C<u>H</u>) and 4.39 (2H, m, 3 C<u>H</u>$_2$); δ_C (50 MHz, D_2O/NaOD/dioxane) 54.94 (3 <u>C</u>H_2), 68.52 (2 <u>C</u>H) and 171.35 (<u>C</u>O_2H).

Potassium (2S,3R)-threonine O-sulphate (60)



This was prepared in an identical manner to potassium (2S)-serine O-sulphate (**21**) using (2S,3R)-threonine in 51% yield. M.p. 150 °C (dec.); (Found: C, 18.61; H, 3.74; N, 5.35. Calc. for $C_4H_8NO_6SK.H_2O$: C, 18.82; H, 3.95; N, 5.50); $[\alpha]_D$ -20°(c. 9.7 in H_2O) [lit., [192] $[\alpha]_D^{18}$ -20.6° (c. 9.7 in H_2O)]; ν_{max} (nujol) 2800-3500 (OH) and 1640 cm^{-1} (amino acid C=O); δ_H (90 MHz, D_2O/NaOD) 1.38 (3H, d, *J* 5 Hz, 4 C<u>H</u>$_3$), 3.22 (1H, d, *J* 6 Hz, 2 C<u>H</u>) and 4.55 (1H, d of q, 3 C<u>H</u>); δ_C (67.5 MHz, D_2O/NaOD/dioxane) 18.2 (4 <u>C</u>H_3), 61.7 (3 <u>C</u>H_2), 79.4 (2 C) and 178.6 (<u>C</u>O_2H); *m/z* (FAB) 238 [*M*+H]$^+$.

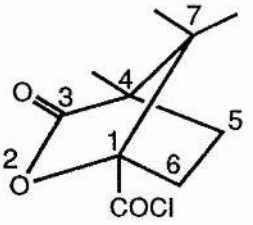
Potassium (2S,3S)-threonine O-sulphate (61)



This was prepared in an identical manner to potassium (2S)-serine O-sulphate (**21**) using (2S,3S)-threonine in 30% yield. M.p. 224-226 °C; $[\alpha]_D$ +0.8° (c. 0.56 in H_2O); ν_{max} (nujol) 3400-3000 (NH/OH str), 3000-2800 (nujol and CH str) and 1650 cm^{-1} (amino

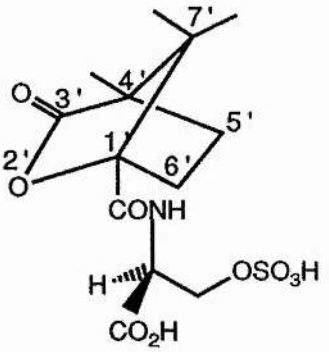
acid C=O); δ_H (90 MHz, D_2O/NaOD) 1.10 (3H, d, *J* 6.4 Hz, 4 C<u>H</u>$_3$), 3.32 (1H, d, *J* 5.5 Hz, 2 C<u>H</u>) and 4.05 (1H, d of q, 3 C<u>H</u>); δ_C (75 MHz, D_2O/NaOD/dioxane) 15.45 (4 <u>C</u>H_3), 59.58 (3 <u>C</u>H), 77.15 (2 <u>C</u>H) and 175.53 (<u>C</u>O_2H); *m/z* (FAB) 238 [*M*+H]$^+$.

(1S)-(-)-Camphanoyl chloride



Distilled thionyl chloride (10 ml) was added to the (1S)-(-)-camphanic acid monohydrate (0.84 g, 3.88 mmol), and the solution refluxed for 2 h. The thionyl chloride was removed *in vacuo* and the resulting solid left to dry *in vacuo* (0.84 g, 100%), m.p. 71-73 °C; ν_{max} (nujol) 1800 (acid chloride C=O) and 1780 cm^{-1} (ester C=O).

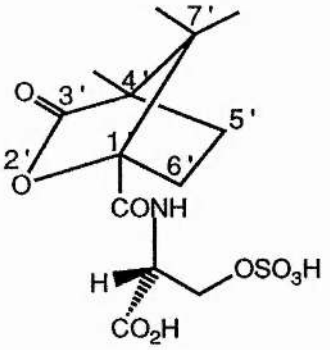
(1'S,4'R)-Camphanamide of (2S)-serine O-sulphate (62)



Camphanoyl chloride (0.3 g, 1.37 mmol) was dissolved in toluene (5 ml). (2S)-Serine O-sulphate (0.44 g, 1.97 mmol) and potassium hydroxide (0.22 g, 3.94 mmol) were dissolved in water (5 ml). The pH of the aqueous layer was checked (pH > 9). The two

solutions were mixed and stirred vigorously overnight. The mixture was extracted with dichloromethane (3 x 10 ml), and the aqueous layer concentrated to dryness *in vacuo*. The product could not be isolated in an organic solution due to the polar nature of the compound. δ_H (200 MHz, D_2O) 0.95 (3H, s, 4" C<u>H</u>$_3$), 1.08, 1.13 (6H, 2s, 2 x 7" C<u>H</u>$_3$), 1.7-2.5 (4H, 3m, 5' C<u>H</u>$_2$ and 6' C<u>H</u>$_2$), 4.37-4.55 (2H, m, 3 C<u>H</u>$_2$) and 4.9 (1H, t, *J* 4.8 Hz, 2 C<u>H</u>); δ_C (100 MHz, D_2O/Na TMS salt) 9.26 (4" <u>C</u>H_3), 16.29, 16.54 (2 x 7" <u>C</u>H_3), 28.99, 29.95 (5' and 6' <u>C</u>H_2), 53.49, 55.21, 56.25 (2 <u>C</u>H, 4' C, 7' C), 67.43 (3 <u>C</u>H_2), 94.22 (1' C), 169.75, 172.29 and 182.58 (ester, amide and acid <u>C</u>=O).

(1'S,4'R)-Camphanamide of (2R)-serine O-sulphate (63)



This was prepared in an identical manner to the (1'S,4'R)-camphanamide of (2S)-serine O-sulphate (**62**). δ_H (200 MHz, D_2O) 0.95 (3H, s, 4" C<u>H</u>$_3$), 1.08, 1.13 (6H, 2s, 2 x 7" C<u>H</u>$_3$), 1.7, 2.08, 2.5 (4H, 3m, 5' C<u>H</u>$_2$ and 6' C<u>H</u>$_2$), 4.45 (2H, m, 3 C<u>H</u>$_2$) and 4.78 (1H, t, *J* 4.9 Hz, 2 C<u>H</u>); δ_C (100 MHz, D_2O/ NaTMS salt) 9.30 (4" <u>C</u>H_3), 16.33, 16.64 (2 x 7" <u>C</u>H_3), 29.04, 30.10 (5' and 6' <u>C</u>H_2), 54.18, 55.33, 56.32 (2 <u>C</u>H, 4' C, 7' C), 67.50 (3 <u>C</u>H_2), 94.27 (1' C), 169.83, 172.58 and 182.66 (ester, amide and acid <u>C</u>=O).

(2S)-[U-^{14}C]-Serine O-sulphate (64)

(2S)-[U-^{14}C]-Serine (24.5 μCi) in aqueous ethanol was added to (2S)-serine (50 mg, 0.22 mmol) in a small round bottomed flask. The solution was lyophilised. The (2S)-[U-^{14}C]-serine O-sulphate was prepared in an identical manner to the non-labelled material. Fractions (10 ml) from the ion exchange column were collected and checked for the presence of compound by tlc (visualising with ninhydrin) and scintillation counting of a small portion of each fraction. The product containing fractions were combined and the pH adjusted to 7.8 by the addition of aqueous 5% potassium hydroxide solution. The product accounted for 4 μCi (109 mg, 16%).

Ethanolamine hydrochloride (67a)

(2S)-Serine (100 mg, 0.95 mmol) and 4-methoxyacetophenone (**65**) (200 mg, 1.33 mmol) were ground together. The mixture was heated in a stream of nitrogen from 160-190 °C. The effluent gas was passed through barium hydroxide solution. When the solution no longer became cloudy (approx 1 h) the reaction mixture was cooled. The red/brown mixture was refluxed with conc. HCl (5 ml) for 2 h and cooled. The residue was added to cold water (5 ml) and extracted five times with chloroform. The aqueous layer was decolourised with charcoal (50 mg). The charcoal was filtered off and the solution concentrated to dryness *in vacuo* yielding a yellow solid (70 mg, 75%), m.p. 50 °C (lit.,[284] 75-77 °C); ν_{max} (nujol) 3300-2200 (H bonding) and 1599 cm^{-1} (NH_3^+ bending); δ_H (90 MHz, D_2O) 2.9 (2H, t, *J* 6 Hz, C$\underline{H}_2$-N) and 3.55 (2H, t, *J* 6 Hz, C$\underline{H}_2$-O); δ_C (22.5 MHz, D_2O/dioxane) 42.49 (<u>C</u>H_2-N) and 58.76 (<u>C</u>H_2-O); *m/z* (EI) 61 ([*M*-HCl]$^+$, 10%), 36 (45, [HCl]$^+$) and 30 (100, [*M*-HCl-CH_2O]$^+$).

[U-^{14}C]-Ethanolamine hydrochloride (67)

(2S)-Serine (100 mg) was added to (2S)-[U-^{14}C]-serine (0.1 ml, 5 μCi) in aqueous ethanol. The solution was lyophilised. 4-Methoxyacetophenone (**65**) (200 mg) was added to the (2S)-[U-^{14}C]-serine and the powders ground together. The procedure was followed as for ethanolamine.HCl (**67a**) omitting the decolourisation step.

(2S)-[2-^{2}H]-Serine O-sulphate (68)

This was prepared in an identical manner to (2S)-serine O-sulphate (**21**) using (2S)-[2-^{2}H]-serine (**69**) using the method of Previero[193] in 55% yield. M.p. 220 °C; (Found: C, 19.37; H, 3.21; N, 7.47. Calc. for $C_3H_6NO_6DS$: C, 19.36; H, 3.25; N, 7.52%); $[\alpha]_D$ -0.92° (c. 1.035 in 5% KOH); ν_{max} (nujol) 2800-3000 (CH str and nujol), 1766 (acid CO_2H monomer), 1216 and 724 cm^{-1} (C-O-S); δ_H (D_2O/NaOD) 3.35 (0.05H, t, *J* 4.5 Hz, 2 C<u>H</u> non deuteriated), 4.00 (1H, d, *J* 10 Hz, 3 C<u>H</u>$_a$) and 3.97 (1H, d, *J* 10 Hz, 3 C<u>H</u>$_b$); δ_C (D_2O/NaOD/NaTMS salt) 55.4 (t, 2 <u>C</u>D), 72.34 (3 <u>C</u>H_2), and 180.0 (<u>C</u>O_2H); δ_D (D_2O/NaOD) 3.47 (2 C<u>D</u>).

(2S)-[2-^{2}H]-Serine hydrochloride (69a)

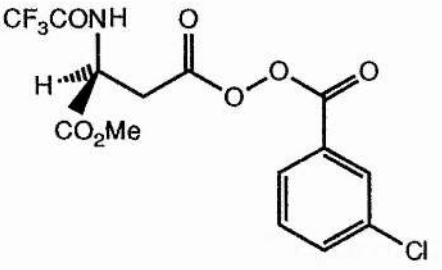
This was prepared in an identical manner to (2S)-serine hydrochloride (**76a**) using (2S)-[2-^{2}H]-methyl-N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**91**) in 100% yield. Spectral data agreed with the proposed structure and tlc spot co-ran with an authentic sample of (2S)-serine hydrochloride.

(2S)-[2-^{2}H]-Serine (69c)

This was prepared in an identical manner to (2S)-serine (**76c**) using (2S)-[2-^{2}H]-O-benzyl serine methyl ester (**125**) in 72% yield (95% deuteriated by proton NMR). M.p. 220-222 °C; (Found: C, 34.24; H, 5.95; N, 13.37; *M*$^{+}$, 106.0489. Calc. for $C_3H_6NO_3D$: C,

33.96, H, 5.70; N, 13.20%; *M*, 106.0489); $[\alpha]_D$ +12.72° (c. 1 in 1 M HCl) [lit.,[212,213] $[\alpha]_D^{23}$ +14.5° (c. 1.0 in 1 M HCl) for (2S)-serine)]; ν_{max} (nujol) 3400 (NH_2 str), 3000-2800 (CH str) and 1597 cm^{-1} (CO_2^-); δ_H (400 MHz, D_2O/NaOD) 3.51, 3.33 (2H, 2d, *J* 11 Hz, 3 C$\underline{H}_2$); δ_C (100 MHz, D_2O/NaOD/dioxane) 59.10 (t, *J* 20.85 Hz, 2 $\underline{C}$D), 66.19 (3 $\underline{C}H_2$) and 182.32 ($\underline{C}$=O); δ_D (61.4 MHz, H_2O/NaOH) 3.24 (2 C$\underline{D}$); *m/z* (CI) 107 ([*M*+H]$^+$, 100%), 61 (80, [*M*-CO_2H]$^+$) and 45 (30, [CO_2H]$^+$).

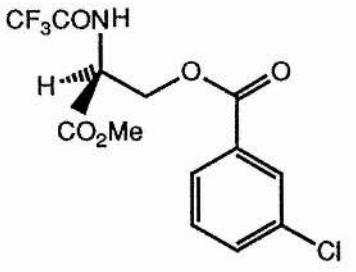
(2S)-α-Methyl-N-trifluoroacetyl-β-aspartyl-m-chlorobenzoyl peroxide (70)



The acid chloride (**74**) (1.5 g, 6 mmol) and 85% *meta*-chloroperbenzoic acid (1.03 g, 6 mmol) were added to dry diethyl ether (45 ml) and the solution was stirred under nitrogen in an ice-salt bath. Pyridine (800 µl, 9 mmol) in dry diethyl ether (3 ml) was added to the mixture dropwise and the stirring continued for 4 h at 0 °C after the addition was complete. The solution was filtered, washed with water and aqueous 1 M sodium carbonate, and dried over sodium sulphate. The solvent was removed *in vacuo* to yield a white solid which was recrystallised from diethyl ether/petroleum ether yielding the pure product (0.5 g, 21%), m.p. 106-107 °C; (Found: C, 42.46; H, 2.53; N, 3.53. Calc. for $C_{14}H_{11}ClNF_3O_7$: C, 42.28; H, 2.79; N, 3.52%); $[\alpha]_D$ +68° (c. 0.69 in chloroform); ν_{max} (nujol) 3269 (amide NH), 1795, 1758 (peroxy-anhydride C=O), 1751 (ester C=O) and 1714 cm^{-1} (trifluoroacetyl C=O); δ_H (90 MHz, $CDCl_3$) 3.25 (2H, m, 3 C$\underline{H}_2$), 3.88 (3H, s, OMe), 5.00 (1H, m, 2 C$\underline{H}$) and 7.35-8.00 (5H, aromatic protons and

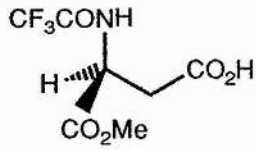
NH br); δ_C (22.5 MHz, $CDCl_3$) 32 (3 $\underline{C}H_2$), 48 ($O\underline{C}H_3$), 54 (2 $\underline{C}H$) and 128-135 (aromatic carbons); *m/z* (CI), 415 ([*M*+NH_4]$^+$, 5%), 371 (6, [*M*+NH_4-CO_2H]$^+$), 261 (100, [*M*-$C_7H_4O_3$]$^+$), 217 (30, [*M*-$C_8H_4O_5$]$^+$) and 35 (60, [Cl]$^+$).

(2S)-Methyl N-trifluoroacetyl-O-(m-chlorobenzoyl)serinate (71)



(2S)-α-Methyl N-trifluoroacetyl-β-aspartyl m-chlorobenzoylperoxide (**70**) (500 mg, 1.2 mmol) was heated to reflux in carbon tetrachloride (40 ml) under nitrogen for 6 d. The solvent was removed *in vacuo* yielding a gummy solid. Purification by column chromatography on silica (petrol:ethyl acetate 7:3) yielded the product which was recrystallised from diethyl ether/petrol to give a white crystalline solid (110 mg, 30%), m.p. 77-78 °C; (Found: C, 44.25; H, 2.93; N, 3.76. Calc. for $C_{13}H_{11}ClF_3NO_5$: C, 44.15; H, 3.13; N, 3.96%); $[\alpha]_D$ +50.3° (c. 0.295 in chloroform); ν_{max} (nujol) 3245 (NH str), 1740 (aliphatic ester C=O), 1730 (aromatic ester C=O) and 1700 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 3.85 (3H, s, $CO_2C\underline{H}_3$), 4.75 (2H, m, 3 $C\underline{H}_2$), 4.97 (1H, m, 2 $C\underline{H}$) and 7.35-8.00 (>5H, m, aromatic protons and N$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 52.87 (2 $\underline{C}H$), 54 ($CO_2\underline{C}H_3$), 64.19 (3 $\underline{C}H_2$) and 128-134 (aromatic carbons); *m/z* (CI) 371 ([*M*+NH_4]$^+$, 100%), 307 (50, [*M*-CO_2H-H]$^+$), 261 (38, [*M*-C_6H_4O]$^+$) and 35 (50, [Cl]$^+$).

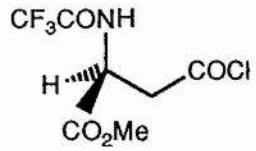
α-Methyl N-trifluoroacetyl-(2S)-aspartate (73)



Trifluoroacetic acid anhydride (25 g, 120 mmol) was added to a stirred suspension of (2S)-aspartic acid (1.6 g, 12 mmol) in dry tetrahydrofuran (50 ml) at 0 °C over 30 min under nitrogen. The reaction mixture was allowed to warm to room temperature and stirred for a further 2 h. After this time the dissolution was complete. The solvent was removed *in vacuo* and the solid thoroughly dried. The anhydride intermediate (**72**) was treated with dry methanol (25 ml) at 0 °C and the solution warmed to room temperature. The solvent was removed *in vacuo* yielding an off white solid, a mixture of the α- and β-methyl esters (2:1 α:β) (2.49 g, 85%). δ_H (90 MHz, $CDCl_3$) 3.02 (m, 3 C$\underline{H}_2$), 3.7 (s, β-methyl ester), 3.85 (s, α-methyl ester), 4.85 (m, 2 C$\underline{H}$) and 7.5 (N$\underline{H}$, broad).

The pure α-methyl ester was obtained by hydrolysis overnight in water at room temperature of the pure β-acid chloride in 96% yield. M.p. 101-103 °C; $[\alpha]_D$ +10.9° (c. 0.623 in chloroform:methanol 9:1) [lit.,[196] $[\alpha]_D^{35}$ +27.9° (c. 0.623 in chloroform:methanol 9:1)]; ν_{max} (nujol) 1745 (ester C=O), 1730 (acid C=O) and 1700 cm^{-1} (trifluoroacetyl C=O); δ_H (90 MHz, $CDCl_3$) 3.3 (2H, m, 3 C$\underline{H}_2$), 4.05 (3H, s, OC$\underline{H}_3$) and 5.1 (1H, m, 2 C$\underline{H}$); δ_C (22.5 MHz, $CDCl_3$/TMS) 35.2 (3 $\underline{C}H_2$), 48.86 (2 $\underline{C}$H), 53.9 (O$\underline{C}H_3$) and 188.0 ($\underline{C}O_2H$); *m/z* (CI), 261 ([*M*+NH_4]$^+$, 100%) and 35 (35, [Cl]$^+$).

α-Methyl N-trifluoroacetyl-(2S)-aspartyl-β-acid chloride (74)



The mixture of monoesters (**73**) (2 g, 8.2 mmol) was refluxed in redistilled thionyl chloride (25 ml) for 1 h. The solution was cooled and the solvent removed *in vacuo* yielding a yellow/white solid which was recrystallised from diethyl ether and petroleum ether yielding the pure β-acid chloride, a white crystalline solid (2.02 g, 99%), m.p. 116-117 °C; $[\alpha]_D$ +53.4° (c. 1.49 in chloroform) [lit.,[196] $[\alpha]_D^{27}$ +62.6° (c. 1.830 in chloroform)]; ν_{max} (nujol) 1798 (acid chloride C=O), 1740 (ester C=O) and 1709 cm^{-1} (trifluoroacetyl C=O); δ_H (90 MHz, $CDCl_3$) 3.65 (2H, dd, 3 C$\underline{H}_2$), 3.85 (3H, s, methyl ester) and 4.79 (1H, m, 2 C$\underline{H}$); δ_C (22.5 MHz, $CDCl_3$) 47.6 (3 $\underline{C}H_2$), 49.7 (O$\underline{C}H_3$) and 54.3 (2 $\underline{C}$H); *m/z* (CI), 279 ([*M*+NH_4]$^+$, 35%), 261 (40, *M*$^+$), 260 (100, [*M*-H]$^+$), 243 (65, [*M*+NH_3-Cl]$^+$) and 35 (35, [Cl]$^+$).

(2S)-Serine hydrochloride (76a)

(2S)-Methyl N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**71**) (63.7 mg, 0.18 mmol) was refluxed in 5 M HCl (10 ml) for 2 h under nitrogen. The solution was cooled and washed with chloroform (3 x 10 ml) and the aqueous layer lyophilised to give a white solid (10 mg, 32%), m.p. 140 °C (dec.); $[\alpha]_D$ +11.5° (c. 0.5 in H_2O) [lit.,[197] $[\alpha]_D^{20}$ +7.2° (c. 1.2 in H_2O)]; ν_{max} (nujol) 3815 (NH), 3500-3000 (OH), 3000-2800 (CH), 1760 (acid CO_2H monomer) and 1590 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 3.32 (1H, t, *J* 5 Hz, 2 C$\underline{H}$) and 3.65 (2H, m, 3 C$\underline{H}_2$); δ_C (50 MHz, D_2O/NaTMS salt) 57.67 (3 $\underline{C}H_2$), 62.27 (2 $\underline{C}$H) and 173.22 ($\underline{C}O_2H$); *m/z* (CI) 106 (100%, [*M*-Cl]$^+$), 88 (30, [*M*-OH]$^+$), 60 (50, [*M*-CO_2H_2]$^+$) and 44 (50, [CO_2]$^+$).

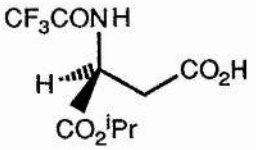
(2S)-Serine hydrochloride (76b)

This was prepared in an identical manner to (2S)-serine hydrochloride (**76a**) using (2S)-*iso*propyl N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**80**) in 75% yield. Spectral data agreed with the proposed structure and the tlc spot co-ran with an authentic sample of (2S)-serine.

(2S)-Serine (76c)

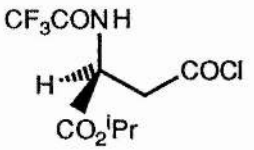
(2S)-O-Benzyl serine methyl ester (**116**) (0.36 g, 1.72 mmol) was added to 6 M HCl (10 ml) and the mixture refluxed for 3 hours. The acid was removed *in vacuo*, and the residue dried over phosphorus pentoxide. The residue was dissolved in dry ethanol (30 ml) and propylene oxide (30 ml) and refluxed for 15 min. The solvents were removed *in vacuo*, and the product recrystallised from water and ethanol to give a white crystalline solid (0.1 g, 55%), m.p. 214 °C (dec.); (Found: C, 34.20; H, 6.39; N, 13.03. Calc. for $C_3H_7NO_3$: C, 34.29; H, 6.71; N, 13.33%); $[\alpha]_D$ +13.2° (c. 1.0 in 1 M HCl) [lit.,[212,213] $[\alpha]_D^{23}$ +14.5° (c. 1.0 in 1 M HCl)]; ν_{max} (nujol) 3400 (NH_2 str), 3000-2800 (CH str) and 1590 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 3.19 (1H, t, *J* 5 Hz, 2 C<u>H</u>) and 3.57 (2H, m, 3 C<u>H</u>$_2$); δ_C (75 MHz, D_2O/NaOD/MeOH) 58.58 (2 <u>C</u>H), 65.76 (3 <u>C</u>H_2) and 181.65 (<u>C</u>=O); *m/z* (CI) 106 ([*M*+H]$^+$, 100%), 88 (35, [*M*-OH]$^+$) and 44 (50, [CO_2]$^+$).

α-*Iso*propyl N-trifluoroacetyl-(2S)-aspartic acid (77)



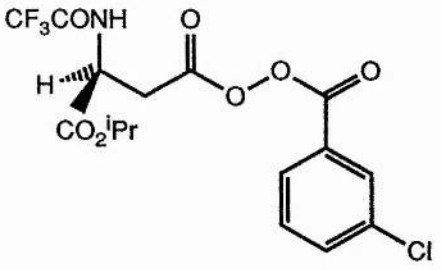
To a suspension of (2S)-aspartic acid (3 g, 22.5 mmol) in dry THF (60 ml) at 0 °C in an atmosphere of nitrogen was added trifluoroacetic anhydride (27 ml, 190 mmol) dropwise over 30 min. The reaction mixture was left to stir for 2 h at 0 °C during which time the dissolution was complete. The solvent was removed *in vacuo* to yield a white solid which was dried at reduced pressure. The dry material was dissolved in dry *iso*propanol (25 ml) and the stoppered reaction flask was kept at 37 °C for 12 h. The solvent was removed *in vacuo* to give the crude *iso*propyl ester. Traces of *iso*propanol were removed by washing the crude compound with cold diethyl ether, recrystallisation from diethyl ether and petrol gave a white solid (5.8 g, 95%), m.p. 88-90 °C; (Found: C, 39.65; H, 4.58; N, 5.14. Calc. for $C_9H_{12}F_3NO_5$: C, 39.86; H, 4.46; N, 5.16%); $[\alpha]_D$ +41.7° (c. 0.5 in chloroform); ν_{max} (nujol) 3320 (NH str), 2800-3000 (CH str and nujol), 1760 (ester C=O), 1745 (acid C=O) and 1710 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 1.27 (6H, dd, *J* 6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.17 and 2.98 (2H, ABX, J_{AB} 17.6 Hz, J_{AX}=J_{BX}=4.4 Hz, 3 C$\underline{H}_2$), 4.79 (1H, m, 2 C$\underline{H}$), 5.11 (1H, sp, *J* 6 Hz, ^{i}Pr C$\underline{H}$) and 7.45 (1H, br s, N$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 21.38, 21.52 (2 x ^{i}Pr $\underline{C}H_3$), 32.21 (3 $\underline{C}H_2$), 48.85 (2 $\underline{C}$H), 70.97 (^{i}Pr $\underline{C}$H), 168.39 and 175.43 ($\underline{C}O_2{}^i$Pr and $\underline{C}O_2$H); *m/z* (EI) 226 ([*M*-CO_2H]$^+$, 10%), 212 (25, [*M*-OCH$(CH_3)_2$]$^+$), 184 (60, [*M*-CO_2H-CH$(CH_3)_2$]$^+$), 166 (50, [*M*-CO_2CH$(CH_3)_2$-OH_2]$^+$), 139 (60, [*M*-CO_2CH$(CH_3)_2$-CO_2H]$^+$) and 43 (100, [CH$(CH_3)_2$]$^+$).

α-*Iso*propyl N-trifluoroacetyl-(2S)-aspartyl-β-acid chloride (78)



Thionyl chloride (20 ml) was added to α-*iso*propyl N-trifluoroacetyl-(2S)-aspartic acid (**77**) (2 g, 7.38 mmol) and the mixture refluxed for 1 h. The solvent was removed *in vacuo* to yield the product (2.07 g, 92%), m.p. 123-125°; (Found: C, 37.26; H, 3.30; N, 4.73. Calc. for $C_9H_{11}F_3NO_4Cl$: C, 37.31; H, 3.63; N, 4.83%); $[\alpha]_D$ +65.8° (c. 0.63 in chloroform); ν_{max} (nujol) 3320 (NH str), 2800-3000 (CH str and nujol), 1810 (acid chloride C=O), 1740 (ester C=O) and 1715 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 1.3 (6H, dd, *J* 6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.62 (2H, m, 3 C$\underline{H}_2$), 4.7 (1H, m, 2 C$\underline{H}$) and 5.15 (1H, m, ^{i}Pr C$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 21.46, 21.56 (2 x ^{i}Pr $\underline{C}H_3$), 47.4 (3 $\underline{C}H_2$), 49.4 (2 $\underline{C}$H) and 71.65 (^{i}Pr $\underline{C}$H); *m/z* (CI) 307 ([*M*+NH_4]$^+$, 100%), 288 (75, [*M*-H]$^+$) and 271 (25, [*M*-Cl+OH]$^+$).

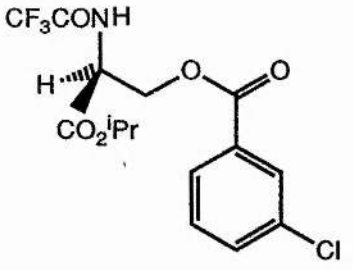
(2S)-α-*Iso*propyl-N-trifluoroacetyl-β-aspartyl-m-chlorobenzoyl peroxide (79)



This was prepared in an identical manner to (2S)-α-methyl-N-trifluoroacetyl-β-aspartyl m-chlorobenzoylperoxide (**70**) from α-*iso*propyl N-trifluoroacetyl-(2S)-aspartyl-β-acid chloride (**78**) in 49% yield. M.p. 110-112 °C; (Found: C, 44.93; H, 3.38; N, 3.71. Calc.

for $C_{16}H_{15}NClF_3O_7$: C, 45.17; H, 3.55; N, 3.29%); $[\alpha]_D$ +67.5° (c. 0.54 in chloroform); ν_{max} (nujol) 3300 (NH str), 2800-3000 (CH str and nujol), 1805, 1770 (peroxy anhydride C=O), 1750 (ester C=O) and 1710 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 1.3 (6H, d, *J* 6.3 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.1-3.4 (2H, m, 3 C$\underline{H}_2$), 4.9 (1H, m, 2 C$\underline{H}$), 5.15 (1H, sp, *J* 6.3 Hz, ^{i}Pr C$\underline{H}$) and 7.4-8.05 (5H, m, aromatic protons and N$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 21.33, 21.48 (2 x ^{i}Pr $\underline{C}H_3$), 31.97 (3 $\underline{C}H_2$), 48.92 (2 $\underline{C}$H), 71.36 (^{i}Pr $\underline{C}$H), 126.47-135.07 (aromatic carbons) and 156.80-167.32 (4 $\underline{C}$=O and $\underline{C}F_3$); *m/z* (CI) 443 ([*M*+NH_4]$^+$, 100%) and 289 (40, [*M*-$C_7H_4O_3$]$^+$).

(2S)-*Iso*propyl N-trifluoroacetyl-O-(m-chlorobenzoyl) serinate (80)



This was prepared in an identical manner to (2S)-methyl N-trifluoroacetyl-O-(m-chlorobenzoyl)serinate (**71**) from (2S)-α-*iso*propyl-N-trifluoroacetyl-β-aspartyl m-chlorobenzoyl peroxide (**79**) in 4% yield. M.p. 74-75 °C; *m/z* (Found: [*M*+NH_4]$^+$, 399.0935. $C_{15}H_{19}O_5N_2ClF_3$ requires 399.0935); $[\alpha]_D$ +61.5° (c. 0.335 in chloroform); ν_{max} (nujol) 3240 (NH str), 2800-3000 (CH str and nujol), 1730 (ester C=O) and 1700 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 1.24-1.39 (6H, 2d, *J* 6.4 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 4.75 (2H, d, *J* 5 Hz, 3 C$\underline{H}_2$), 4.9 (1H, m, 2 C$\underline{H}$), 5.15 (1H, sp, *J* 6.4 Hz, ^{i}Pr C$\underline{H}$) and 7.3-8.0 (5H, aromatic protons and N$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 21.47, 21.55 (2 x ^{i}Pr $\underline{C}H_3$), 52.49 (2 $\underline{C}$H), 63.69 (3 $\underline{C}H_2$), 71.17 (^{i}Pr $\underline{C}$H), 127.67, 129.66, 129.84, 130.50, 133.58, 134.69 (aromatic carbons), 164.72 and 167.22 ($\underline{C}O_2{}^i$Pr, $CF_3\underline{C}$ONH).

(2S)-[2-^{2}H]-Aspartic acid (82)

(2S)-Aspartic acid (3 g, 22.5 mmol) was added to deuterium oxide (50 ml) and the pH adjusted to 7.25 with ammonia solution. Pyridoxal phosphate (5 mg, 20 μmol) and aspartate aminotransaminase (AAT) (1 mg, 300 unit) were added and the solution incubated at 37 °C, the reaction progress was monitored by proton NMR spectroscopy. The reaction was found to be complete after 72 h. The pH was readjusted to 7.25 after 24 h (if necessary). The solution was boiled, filtered, and concentrated to dryness *in vacuo*. The off white solid was purified by crystallisation from hot water (pH 4) and ethanol, yielding the free amino acid (2.19 g, 72%), m.p. >300 °C; $[\alpha]_D$ +21.05° (c. 0.95 in 6 M HCl); ν_{max} (nujol) 3000-2500 (OH stretching), 1688 and 1643 (carboxylate anion) and 1586 cm^{-1} (N-H deformations); δ_H (270 MHz, D_2O/DCl) 3.12 (2H, d, *J* 2.7 Hz, 3 C$\underline{H}_2$) and 4.4 (1/13H, t, 2 C$\underline{H}$, 7% undeuteriated amino acid); δ_C (67.5 MHz, D_2O/dioxane) 34.32 (3 $\underline{C}H_2$), 49.68 (2 $\underline{C}D$), 171.44 and 173.80 (carboxylate carbons); *m/z* (FAB), 135 ([*M*+H]$^+$, 70%), 134 (30, [*M*]$^+$) and 74 (52, [*M*-HCl-CO_3]$^+$).

α- and β-Methyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartate (84)

This was prepared in an identical manner to that described for α-methyl N-trifluoroacetyl-(2S)-aspartate (**73**) using (2S)-[2-^{2}H]-aspartic acid (**82**) in 100% yield. δ_H (200 MHz, $CDCl_3$) 2.85-3.25 (m, 3 C$\underline{H}_2$ in α- and β-esters), 3.75 (s, ß-methyl ester), 3.85 (s, α-methyl ester), 7.45 and 7.55 (two broad singlets, N-H of both esters).

α-*Iso*propyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartate (85)

This was prepared in an identical manner to that described for α-*iso*propyl N-trifluoroacetyl-(2S)-aspartate (**77**) using (2S)-[2-^{2}H]-aspartic acid (**82**) in 55% yield. M.p. 85-87 °C; $[\alpha]_D$ +52.9° (c. 0.48 in chloroform); ν_{max} (nujol) as for the non-deuteriated sample (**77**); δ_H (200 MHz, $CDCl_3$) 1.25 (6H, 2d, *J* 6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.05 (2H, q, *J* 18

Hz, 3 C$\underline{H}_2$), 5.1 (1H, sp, *J* 6 Hz, ^{i}Pr C$\underline{H}$) and 7.35 (1H, br s, N$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 21.26, 21.39 (2 x ^{i}Pr $\underline{C}H_3$), 35.02 (3 $\underline{C}H_2$), 48.56 (t, *J* 21 Hz, 2 $\underline{C}D$), 70.86 (^{i}Pr $\underline{C}H$), 115.44 (q, *J* 285 Hz, $\underline{C}F_3$), 156.98 (q, *J* 38 Hz, $CF_3\underline{C}O$), 168.31 and 175.15 ($\underline{C}O_2{}^i$Pr, $\underline{C}O_2H$); *m/z* (EI) 227 ([*M*-CO_2H]$^+$, 5%), 213 (15, [*M*-$OCH(CH_3)_2$]$^+$), 185 (48, [*M*-CO_2H-$CH(CH_3)_2$]$^+$), 167 (22, [*M*-$CO_2CH(CH_3)_2$-OH_2]$^+$), 140 (40, [*M*-$CO_2CH(CH_3)_2$-CO_2H]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

α-Methyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartyl-β-acid chloride (87)

This was prepared in an identical manner to α-methyl N-trifluoroacetyl-(2S)-aspartyl-β-acid chloride (**74**) using α-methyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartate (**84**) in 93% yield. M.p. 112-113 °C; $[\alpha]_D$ +66.3° (c. 0.395 in chloroform); δ_H (200 MHz, $CDCl_3$) 3.62 (2H, d, *J* 5.2 Hz, 3 C$\underline{H}_2$), 3.9 (3H, s, CO_2CH_3) and 7.25 (1H, br s, N$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 47.83 ($\underline{C}H_2COCl$), 54.32 ($CO_2\underline{C}H_3$); *m/z* (EI) 227 ([*M*-Cl]$^+$, 10%), 203 (70, [*M*-CO_2CH_3]$^+$) and 199 (35, [*M*-COCl]$^+$).

α-*Iso*propyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartyl-β-acid chloride (88)

α-*Iso*propyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartate (**85**) (1 g, 3.69 mmol) was suspended in dry toluene (70 ml) and the mixture heated under nitrogen until the dissolution was complete. Thionyl chloride was added and the mixture refluxed for 6 h. The solvent was removed *in vacuo* yielding an off white solid (1.06 g, 100%); δ_H (200 MHz, $CDCl_3$) 1.3 (6H, dd, *J* 6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.62 (2H, m, 3 C$\underline{H}_2$), and 5.15 (1H, m, ^{i}Pr C$\underline{H}$).

(2S)-[2-^{2}H]-α-Methyl-N-trifluoroacetyl-β-aspartyl-m-chlorobenzoyl peroxide (89)

This was prepared in an identical manner to (2S)-α-methyl-N-trifluoroacetyl-β-aspartyl m-chlorobenzoyl peroxide (**70**) using α-methyl N-trifluoroacetyl-(2S)-aspartyl-β-acid chloride (**87**) in 40% yield. M.p. 102-103 °C; $[\alpha]_D$ +49.5° (c. 0.66 in chloroform); ν_{max} (nujol) 3260 (NH), 1790, 1770 (peroxy-anhydride C=O), 1750 (ester C=O) and 1710 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 3.25 (2H, dd, *J* 16Hz, 3 C$\underline{H}_2$), 3.9 (3H, s, $CO_2C\underline{H}_3$) and 7.4-8.1 (5H, m, 4 aromatic protons and N$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 32.58 ($\underline{C}H_2CO$), 49.35 (t, *J* 21 Hz, $\underline{C}D$), 54.21 ($CO_2\underline{C}H_3$), 126.98, 128.36, 130.27, 130.78, 135.20, 135.67 (aromatic carbons), 157.28, 162.08, 166.65 and 168.97 (4 x $\underline{C}$=O); *m/z* (CI) 416 ([*M*+NH_4]$^+$, 100%) and 262 (35, [*M*-$C_7H_4O_3$]$^+$).

(2S)-[2-^{2}H]-α-*iso*propyl-N-trifluoroacetyl-β-aspartyl-m-chlorobenzoyl peroxide (90)

This was prepared in an identical manner to (2S)-α-*iso*propyl-N-trifluoroacetyl-β-aspartyl m-chlorobenzoyl peroxide (**79**) using α-*iso*propyl N-trifluoroacetyl-(2S)-[2-^{2}H]-aspartyl-β-acid chloride (**88**) in 21% yield. M.p. 104-105 °C; *m/z* (Found: [*M*+NH_4]$^+$, 444.0896. $C_{16}H_{18}N_2O_7ClF_3FD$ requires 444.0896); $[\alpha]_D$ +42.2° (c. 0.54 in chloroform); ν_{max} (nujol) 3300 (NH str), 2800-3000 (CH str and nujol), 1790, 1760 (peroxy anhydride C=O), 1740 (ester C=O) and 1700 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 1.3 (6H, d, *J* 5 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.18, 3.3 (2H, dd, *J* 17.5 Hz, 3 C$\underline{H}_2$), 5.15 (1H, sp, *J* 5 Hz, ^{i}Pr C$\underline{H}$) and 7.4-8.0 (5H, m, aromatic protons and N$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 21.43, 21.58 (2 x ^{i}Pr $\underline{C}H_3$), 31.97 (3 $\underline{C}H_2$), 48.97 (2 $\underline{C}D$), 71.42 (^{i}Pr $\underline{C}H$), 126.53-135.15 (aromatic carbons) and 156.89-167.41 (4 $\underline{C}$=O and $\underline{C}F_3$); *m/z* (CI) 444 ([*M*+NH_4]$^+$, 2%) and 290 (100, [*M*-$C_7H_4O_3$]$^+$).

(2S)-[2-^{2}H]-Methyl-N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (91)

This was prepared in an identical manner to (2S)-methyl-N-trifluoroacetyl-O-(m-chlorobenzoyl)-serinate (**71**) using (2S)-[2-^{2}H]-α-methyl-N-trifluoroacetyl-β-aspartyl m-chlorobenzoyl peroxide (**89**) in 27% yield. M.p. 73-74 °C; $[\alpha]_D$ +79.3° (c. 0.295 in chloroform); ν_{max} (nujol) 3245 (NH str), 3000-2800 (CH str and nujol), 1750 (aliphatic ester C=O), 1730 (aromatic ester C=O) and 1705 cm^{-1} (trifluoroacetyl C=O); δ_H (200 MHz, $CDCl_3$) 3.88 (3H, s, $CO_2C\underline{H}_3$), 4.72 (2H, s, 3 $C\underline{H}_2$), 7.25 (1H, br s, N$\underline{H}$) and 7.4-7.95 (aromatic protons); δ_C (75 MHz, $CDCl_3$) 52.1 (t, *J* 20 Hz, $\underline{C}D$), 53.6 ($CO_2\underline{C}H_3$), 63.7 ($\underline{C}H_2O$), 115.5 (q, *J* 285 Hz, $\underline{C}F_3$), 127.8, 129.95, 129.79, 130.6, 133.7, 134.8 (aromatic carbons), 157.1 (q, *J* 38 Hz, $\underline{C}F_3CO$), 164.9, 168.3 ($\underline{C}O_2{}^iPr$ and $CF_3\underline{C}ONH$); *m/z* (CI) 372 ([*M*+NH_4]$^+$, 100%).

N-Benzyl (2S)-serine (94)

(2S)-Serine (2.1 g, 20 mmol) was dissolved in 2 M NaOH (10 ml) and freshly distilled benzaldehyde (2.04 ml, 20 mmol) was added. After 30 min of vigorous stirring, sodium borohydride (226 mg, 6 mmol) was added portionwise keeping the temperature of the reaction mixture below 15 °C. After 30 min a further equivalent of benzaldehyde, followed by sodium borohydride was added as above. The mixture was stirred for a further 2 h. The solution was washed with diethyl ether (3 x 50 ml) and the pH of the aqueous layer was adjusted to 6 by the addition of 6 M HCl. The product precipitated from solution and was isolated by filtration (2.96 g, 75%), m.p. 216-217 °C (lit.,[207] 220-222 °C); *m/z* (Found: [*M*+H]$^+$, 196.0974. $C_{10}H_{14}NO_3$ requires 196.0974); $[\alpha]_D$ +5.9° (c. 1.0 in 2 M HCl) [lit.,[207] $[\alpha]_D$ +6.8° (c. 1.0 in 2 M HCl)]; ν_{max} (nujol) 3200-3300 (NH str and OH str), 2800-3000 (CH str and nujol), 1630 (acid CO_2H), 1575 (CO_2^-) and 1540 cm^{-1} (RNH_2 deformations); δ_H (200 MHz, D_2O) 3.61 (1H, t, *J* 4.2 Hz, 2 C$\underline{H}$), 3.86 (2H, 2d, *J* 4.2 and 1.4 Hz, 3 $C\underline{H}_2$), 4.18, 4.20 (2H, 2 s, 4' $C\underline{H}_2$) and 7.38 (5H, s, aromatic protons);

δ_C (75 MHz, D_2O/dioxane) 51.01 (2 $\underline{C}H$), 60.42 (3 $\underline{C}H_2$), 63.69 (benzyl $\underline{C}H_2$), 130.29, 130.70, 131.01, 131.69 (aromatic carbons) and 172.32 ($\underline{C}O_2H$).

N-Benzyloxycarbonyl (2S)-serine *iso*propyl ester (105)

(2S)-Serine *iso*propyl ester hydrochloride salt (**108**) (2 g, 10.8 mmol) was suspended in dry THF (80 ml) and triethylamine (3.44 ml, 24 mmol) was added, this mixture was cooled to 0°C and benzylchloroformate (1.716 ml, 12 mmol) was added dropwise, the mixture was warmed to room temperature and stirred overnight. Water (50 ml) and diethyl ether (50 ml) were added, the mixture shaken and the diethyl ether layer isolated, washed with water (20 ml), dried ($MgSO_4$), and concentrated *in vacuo* to dryness yielding a clear oil. The oil was crystallised from ethyl acetate and petroleum ether yielding a white solid (2.31 g, 76%), m.p. 74-75 °C; (Found: C, 60.07; H, 6.83; N, 4.97. Calc. for $C_{14}H_{19}NO_5$: C, 59.78; H, 6.81; N, 4.98%); $[\alpha]_D$ +11.4° (c. 0.9 in dichloromethane); ν_{max} (nujol) 3480, 3380 (NH and OH str), 2800-3000 (CH str and nujol) and 1715 cm^{-1} (ester and urethane C=O); δ_H (200 MHz, $CDCl_3$) 1.25 (6H, d, *J* 7.5 Hz, 2 x ^{i}Pr $C\underline{H}_3$), 1.9 (1H, br s, O$\underline{H}$), 3.98 (2H, d, *J* 3.6 Hz, 3 $C\underline{H}_2$), 4.4 (1H, t, *J* 3.6 Hz, 2 $C\underline{H}$), 5.0-5.2 (3H, s and sp, ^{i}Pr $C\underline{H}_3$, benzyl $C\underline{H}_2$), 5.7 (1H, br s, N$\underline{H}$) and 7.35 (5H, s, aromatic protons); δ_C (50 MHz, $CDCl_3$) 21.7 (2 x ^{i}Pr $\underline{C}H_3$), 56.2 (3 $\underline{C}H_2$), 63.2 (2 $\underline{C}H$), 67.3 (benzyl $\underline{C}H_2$), 128.1, 128.2, 128.5, 136.1 (aromatic carbons), 156.2 (urethane $\underline{C}$=O) and 169.9 (ester $\underline{C}$=O); *m/z* (EI) 281 ([*M*]$^+$, 5%), 108 (30, $[C_6H_5CH_2OH]^+$), 91 (100, $[C_6H_5CH_2]^+$) and 43 (40, $[CH(CH_3)_2]^+$).

N-(9-Fluorenylmethyloxycarbonyl)-(2S)-serine *iso*propyl ester (106)

(2S)-Serine *iso*propyl ester hydrochloride salt (**108**) (1 g, 5.5 mmol) was suspended in dry THF (40 ml) and triethylamine (1.68 ml, 12 mmol) was added. The mixture was cooled to 0 °C and 9-fluorenylmethyloxycarbonylchloride (**105**) (1.55 g, 6 mmol)

added. The product was isolated in the same manner as the benzyloxycarbonyl analogue (1.56 g, 77%), m.p. 94-95 °C; (Found: C, 67.99; H, 6.26; N, 3.76. Calc. for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.79%); $[\alpha]_D$ +2.9° (c. 0.324 in chloroform); ν_{max} (nujol) 3340, 3360 (OH and NH str), 2800-3000 (CH str and nujol), 1730 (ester C=O) and 1665 cm^{-1} (urethane C=O); δ_H (200 MHz, $CDCl_3$) 1.3 (6H, d, *J* 7.5 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 1.9 (1H, br s, O$\underline{H}$), 3.95 (2H, s, 3 C$\underline{H}_2$), 4.25 (1H, t, *J* 7.5 Hz, 2 C$\underline{H}$), 4.4 (2H, s, FMOC C$\underline{H}_2$), 4.45 (1H, s, FMOC C$\underline{H}$), 5.1 (1H, sp, *J* 7.5 Hz, ^{i}Pr C$\underline{H}$), 5.72 (1H, br d, N$\underline{H}$) and 7.28-7.8 (8H, m, FMOC aromatic protons); δ_C (75 MHz, $CDCl_3$) 21.57, 21.59 (2 x ^{i}Pr $\underline{C}H_3$), 46.99 (FMOC $\underline{C}$H), 56.09 (3 $\underline{C}H_2$), 63.31 (2 $\underline{C}$H), 67.04 (FMOC $\underline{C}H_2$), 69.72 (^{i}Pr $\underline{C}$H), 119.85, 124.93, 126.94, 127.59, 141.15, 143.54 (aromatic carbons of FMOC), 156.14 (urethane $\underline{C}$=O) and 169.81 (ester $\underline{C}$=O); *m/z* (CI) 370 ([*M*+H]$^+$, 25%).

N-Benzyl (2S)-serine *iso*propyl ester (107)

N-Benzyl (2S)-serine (**94**) (500 mg, 2.5 mmol) was suspended in dry *iso*propanol (15 ml) and the mixture cooled in an ice bath. Thionyl chloride (935 µl, 12.8 mmol) was added dropwise. The mixture was stirred in an ice bath for 2 h and refluxed for 3 d. The solution was cooled, and triethylamine added (1.4 ml, 10 mmol). Water (25 ml) was added, and the product extracted into dichloromethane (3 x 25 ml). The organic layer was dried ($MgSO_4$), and concentrated *in vacuo*. The pure product was obtained by recrystallisation from diethyl ether and petrol (220 mg, 36%), m.p. 54-55 °C; (Found: C, 63.69; H, 8.18; N, 5.66. Calc. for $C_{13}H_{19}NO_3.1/2H_20$: C, 63.39; H, 8.18; N, 5.69%); *m/z* (Found: [*M*+H]$^+$, 238.1443; $C_{13}H_{20}NO_3$ requires 238.1443); $[\alpha]_D$ -44.65° (c. 1.0 in dichloromethane); ν_{max} (nujol) 3500-3000 (OH and NH), 3000-2800 (CH str and nujol) and 1720 (ester C=O); δ_H (200 MHz, $CDCl_3$) 1.27, 1.28 (6H, 2d, *J* 6.2 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.43 (2H, br s, O$\underline{H}$ and N$\underline{H}$), 3.41 (1H, ABX, J_{AX} 4.5 Hz, J_{BX} 6.3 Hz, 2 C$\underline{H}$), 3.59-3.86 (2H, ABX, J_{AB} 10.6 Hz, J_{AX} 4.5 Hz, J_{BX} 6.3 Hz, 3 C$\underline{H}_2$), 3.78, 3.93 (2H, 2d, *J* 12.5 Hz,

PhC$\underline{H}_2$), 5.08 (1H, sp, *J* 6.2Hz, ^{i}Pr C$\underline{H}$) and 7.28-7.36 (6H, m, aromatic protons and N$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 21.65, 21.79 (2 x ^{i}Pr $\underline{C}H_3$), 51.99 (2 $\underline{C}$H), 61.88 (3 $\underline{C}H_2$), 62.26 (Ph$\underline{C}H_2$), 69.18 (^{i}Pr $\underline{C}$H), 127.54, 128.46, 128.61, 138.58 (aromatic carbons) and 171.99 (ester $\underline{C}$=O); *m/z* (EI) 238 ([*M*+H]$^+$, 15%), 206 (70, [*M*-H$(CH_3)_2$]$^+$), 164 (73, [*M*-CH$(CH_3)_2$-CHOH]$^+$), 150 (90, [*M*-CO_2CH$(CH_3)_2$]$^+$), 106 (80, [$C_6H_5CH_2NH$]$^+$), 91 (100, [$C_6H_5CH_2$]$^+$) and 43 (100, [CH$(CH_3)_2$]$^+$).

(2S)-Serine *iso*propyl ester hydrochloride salt (108)

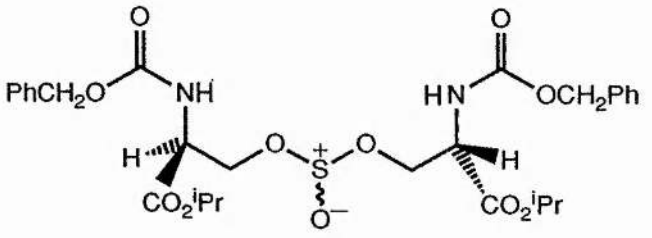
(2S)-Serine (500 mg, 4.75 mmol) was suspended in dry *iso*propanol (40 ml) and the suspension cooled to 0 °C. Thionyl chloride (760 µl, 10.45 mmol) was added dropwise, the resulting suspension was refluxed for 5 h and cooled. The solution was poured into dry diethyl ether (100 ml) and after a few minutes white crystals were formed. The crystals were filtered off and dried (640 mg, 92%), m.p. 141-142 °C (lit.,[285] 142-143 °C); (Found: C, 39.42; H, 7.23; N, 7.63. Calc. for $C_6H_{14}ClNO_3$: C, 39.24; H, 7.68; N, 7.63%); $[\alpha]_D$ -7.5° (c. 2 in H_2O); ν_{max} (nujol) 3370 (NH str and OH str), 2800-3000 (CH str and nujol) and 1730 cm^{-1} (ester C=O); δ_H (200 MHz, D_2O) 1.23 (6H, d, *J* 7.5 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 4.1 (3H, m, 2 C$\underline{H}$ and 3 C$\underline{H}_2$) and 5.1 (1H, sp, *J* 7.5 Hz, ^{i}Pr C$\underline{H}$); δ_C (50 MHz, D_2O/NaTMS salt) 23.72 (2 x ^{i}Pr $\underline{C}H_3$), 57.76 (3 $\underline{C}H_2$), 62.20 (2 $\underline{C}$H), 75.37 (^{i}Pr $\underline{C}$H) and 170.83 (ester $\underline{C}$=O); *m/z* (EI) 148 ([*M*-Cl]$^+$, 100%) and 106 (100, [$C_3H_8NO_3$]$^+$).

(2S)-Serine *iso*propyl ester (108a)

Dry THF (10 ml) was added to the hydrochloride salt of (2S)-serine *iso*propyl ester (**108**) (250 mg, 1.36 mmol) and triethylamine (200 µl, 1.36 mmol) was added. The mixture was stirred overnight and the solid was removed by filtration. The solution was concentrated *in vacuo* to give a clear colourless oil (190 mg, 97%). δ_H (200 MHz, $CDCl_3$) 1.25 (6H, d, *J* 7.6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.8 (3H, br s, O$\underline{H}$ and N$\underline{H}_2$), 3.5-3.9 (3H, m, 2

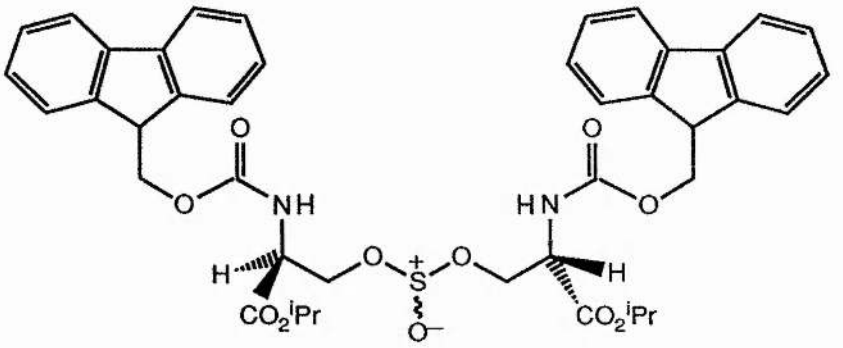
C<u>H</u> and 3 C<u>H</u>$_2$) and 5.07 (1H, sp, *J* 7.6 Hz, ^{i}Pr C<u>H</u>); δ_C (50 MHz, $CDCl_3$) 21.61 (2 x ^{i}Pr <u>C</u>H$_3$), 55.91 (3 <u>C</u>H$_2$), 63.66 (2 <u>C</u>H), 68.72 (^{i}Pr <u>C</u>H) and 173.09 (<u>C</u>O$_2{}^i$Pr).

N-Benzyloxycarbonyl-(2S)-serine-*iso*propyl ester bis sulphite ester (109)



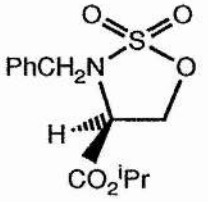
N-Benzyloxycarbonyl-(2S)-serine *iso*propyl ester (**105**) (300 mg, 1.07 mmol) was suspended in dry toluene (25 ml) and cooled to 0 °C over an ice/salt bath. Triethylamine (327 µl, 2.35 mmol) in toluene (5 ml) and thionyl chloride (85 µl, 1.17 µmol) in toluene (5 ml) were added dropwise successively over 15 min. The mixture was warmed to room temperature and stirred for 1.5 h. Water (10 ml) and ether (10 ml) were added. The diethyl ether layer was isolated and concentrated to dryness *in vacuo*, and after several washes with small portions of diethyl ether yielding a pale brown solid. The solid was recrystallised from ethyl acetate and petrol to yield a white solid (0.3 g, 92%), m.p. 106-108 °C; (Found: C, 55.48; H, 6.04; 4.61. Calc. for $(C_{14}H_{17}NO_5)_2SO$: C, 55.25; H, 5.96; N, 4.60 %); $[\alpha]_D$ +20.7° (c. 1.15 in dichloromethane); ν_{max} (nujol) 3280 (NH str), 2800-3000 (CH str and nujol), 1730 (ester C=O) and 1670 cm^{-1} (urethane C=O); δ_H (200 MHz, $CDCl_3$) 1.25 (12H, d, *J* 5 Hz, 4 x ^{i}Pr C<u>H</u>$_3$), 4.1-4.65 (6H, ABX, 2 C<u>H</u> and 3 C<u>H</u>$_2$), 4.9-5.2 (6H, sp and s, benzylic C<u>H</u>$_2$ and ^{i}Pr C<u>H</u>) and 7.3 (10H, s, aromatic protons); δ_C (50 MHz, $CDCl_3$) 21.5 (4 x ^{i}Pr <u>C</u>H$_3$), 53.7 (2 <u>C</u>H), 62.6 (3 <u>C</u>H$_2$), 67.0 (benzyl <u>C</u>H$_2$), 70.2 (^{i}Pr CH), 127.9, 128.1, 128.4, 135.9 (aromatic carbons), 155.63 (urethane <u>C</u>=O), and 168.1 (ester <u>C</u>=O); *m/z* (FAB) 609 ([*M*+H]$^+$, 55%), 281 ([$C_{13}H_{32}NO_5$]$^+$, 100) and 238 (95, [$C_{11}H_{12}NO_5$]$^+$).

N-(9-Fluorenylmethyloxycarbonyl)-(2S)-serine-*iso*propyl ester bis sulphite ester (110)



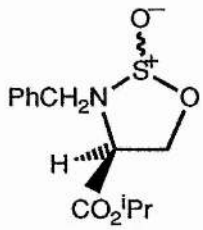
The F-MOC bis sulphite ester (**110**) was prepared from N-(9-fluorenylmethyloxycarbonyl)-(2S)-serine *iso*propyl ester (**106**) in an identical manner to that described for the BOC analogue in 64% yield. M.p. 104-105 °C; $[\alpha]_D$ +9.5° (c. 0.25 in chloroform); ν_{max} (nujol) 3300 (NH str), 2800-3000 (CH str and nujol), 1740, 1730 (C=O ester and urethane) and 1690 cm^{-1} (aromatic C=C); δ_H (200 MHz, $CDCl_3$) 1.29 (12 H, d, *J* 6.2 Hz, 4 x ^{i}Pr C$\underline{H}_3$), 4.15-4.7 (12H, m, 2 C$\underline{H}$, 3 C$\underline{H}_2$, FMOC C$\underline{H}$ and C$\underline{H}_2$), 5.15 (2H, sp, ^{i}Pr C$\underline{H}$), 6.85 (2H, N$\underline{H}$) and 7.2-7.9 (16H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 21.62, 21.69 (4 x ^{i}Pr $\underline{C}H_3$), 46.99 (FMOC $\underline{C}$H), 53.88 (3 $\underline{C}H_2$), 62.30 (2 $\underline{C}$H), 67.36 (FMOC $\underline{C}H_2$), 70.4 (^{i}Pr $\underline{C}$H), 119.95, 125.09, 127.05, 127.69, 141.23, 143.70 (aromatic carbons), 155.73 (urethane $\underline{C}$=O) and 168.70 (ester $\underline{C}$=O); *m/z* (FAB) 785 ([*M*+H]$^+$, 5%) and 179 (100, $[C_{13}H_9CH_2]^+$).

N-Benzyl-2,2-dioxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *iso*propyl ester (111)



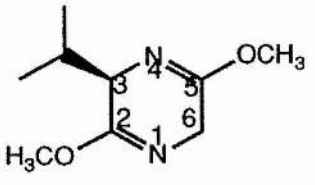
The mixture of diastereoisomers of N-benzyl-oxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *iso*propyl ester (**112**) (0.83 g, 2.93 mmol) was dissolved in dry acetonitrile (15 ml) and cooled to 0 °C over ice. Ruthenium (III) chloride monohydrate (<1 mg) was added followed by sodium periodate (0.69 g, 3.223 mmol). Water (15 ml) was added. The mixture was stirred for 30 min at 4 °C and for 1 h at room temperature, a thick white precipitate appeared. The solution was diluted with diethyl ether (75 ml). The phases were separated, the aqueous phase re-extracted with diethyl ether (3 x 30 ml), and the combined organic fractions washed with saturated aqueous sodium bicarbonate (50 ml) and brine (50 ml), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to yield an oil (0.71 g, 81%); *m/z* (Found: $[M+H]^+$, 300.0906. $C_{13}H_{18}O_5NS$ requires 300.0906); $[\alpha]_D$ -30.6° (c. 1.2 in dichloromethane); ν_{max} (neat) 2980 (CH str), 1730 (C=O), 1340-1360 and 1180 cm^{-1} (sulphate modes); δ_H (200 MHz, $CDCl_3$) 1.25, 1.26 (6H, 2d, *J* 6.4 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 4.02 (1H, ABX, J_{AX} 3 Hz, J_{BX} 9.2 Hz, 4 C$\underline{H}$), 4.52 (2H, d, *J* 2.2 Hz, C$\underline{H}_2$Ph), 4.65 (2H, ABX, J_{AB} 6.6 Hz, J_{AX} 3 Hz, J_{BX} 9.2 Hz, 5 C$\underline{H}_2$), 5.06 (1H, sp, *J* 6.4 Hz, ^{i}Pr C$\underline{H}$), and 7.3-7.5 (5H, m, aromatic protons); δ_C (75 MHz, $CDCl_3$) 21.45, 21.50 (2 x ^{i}Pr $\underline{C}H_3$), 50.21 ($\underline{C}H_2$Ph), 58.12 (4 $\underline{C}$H), 67.36 (5 $\underline{C}H_2$), 70.63 (^{i}Pr $\underline{C}$H), 128.54, 128.74, 129.03, 133.58 (aromatic carbons) and 167.18 (ester $\underline{C}$=O); *m/z* (CI) 317 ($[M+NH_4]^+$, 100%), 300 (20, $[M+H]^+$),108 (30, $[PhCH_2OH]^+$) and 91 (20, $[PhCH_2]^+$).

N-Benzyl-(2RS)-oxo-1,2,3-oxathiazolidine-(4S)-carboxylic acid *iso*propyl ester (112)



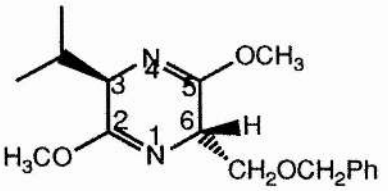
To a solution of N-benzyl (2S)-serine *iso*propyl ester (**107**) (0.94 g, 3.97 mmol) in dry toluene (20 ml) at 0 °C in an ice-salt bath was added dropwise, successively, triethylamine (1.22 ml, 8.734 mmol), followed by freshly distilled thionyl chloride (0.315 ml, 4.367 mmol) over a period of 15 min. The temperature was allowed to rise to room temperature and after a further hour, diethyl ether (75 ml) was added and the mixture extracted with water (2 x 10 ml). The organic layer was dried ($MgSO_4$), filtered, and evaporated to dryness *in vacuo* to yield an oil (diastereomeric ratio 5:1 by proton NMR spectroscopy) (0.83 g, 74%). ν_{max} (neat) 2960 (CH str), 1725 (C=O ester) and 1300-1100 cm^{-1} (S-O); δ_H (400 MHz, $CDCl_3$) major isomer 1.24 (6H, t, *J* 6.6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 4.08 (1H, dd, 4 C$\underline{H}$), 4.27 and 4.47 (2H, ABq, *J* 13.8 Hz, C$\underline{H}_2$Ph), 4.62 (1H, dd, 5 C$\underline{H}_a$), 4.95 (1H, dd, 5 C$\underline{H}_b$), 5.05 (1H, sp, *J* 6.3 Hz, ^{i}Pr C$\underline{H}$), 7.28-7.53 (5H, m, aromatic protons), minor isomer 1.24 (6H, t, *J* 6.6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 3.92 (1H, dd, 4 C$\underline{H}$), 4.27 and 4.47 (2H, ABq, *J* 13.8 Hz, C$\underline{H}_2$Ph), 4.56 (1H, dd, 5 C$\underline{H}_a$), 4.98-5.10 (2H, m, ^{i}Pr C$\underline{H}$ and 5 C$\underline{H}_b$) and 7.28-7.53 (5H, m, aromatic protons); δ_C (100 MHz, $CDCl_3$) major isomer 21.56 (2 x ^{i}Pr $\underline{C}H_3$), 49.07 ($\underline{C}H_2$Ph), 60.35 (4 $\underline{C}$H), 69.88 (^{i}Pr $\underline{C}$H), 71.96 (5 $\underline{C}H_2$), 128.08, 128.58, 129.02, 135.54 (aromatic carbons) and 168.80 (ester $\underline{C}$=O); *m/z* (CI) 284 ([*M*+H]$^+$, 40%), 196 (47, [*M*-$CO_2CH(CH_3)_2$]$^+$) and 91 (100, [$PhCH_2$]$^+$).

(3R)-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (113)



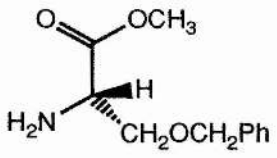
A suspension of *cyclo*-((2R)-val-gly) (**121**) (400 mg, 2.56 mmol) and trimethyloxonium tetrafluoroborate (950 mg, 6.41 mmol) in dry dichloromethane was stirred vigorously for 3 d with a further portion of trimethyloxonium tetrafluoroborate (380 mg, 2.56 mmol) being added after 24 h. To the resulting mixture a solution of disodium hydrogen phosphate (8.5 g) and dihydrogen sodium phosphate (2.25 g) in water (40 ml) was added. The organic phase was separated, and the aqueous phase extracted with dichloromethane (3 x 10 ml). The organic portions were combined and dried ($MgSO_4$), the solvent was removed *in vacuo* yielding a yellow oil (0.31 g, 66%). *m/z* (Found: *M*$^+$, 184.1212. $C_9H_{16}N_2O_2$ requires 184.1212); $[\alpha]_D$ -120.7° (c. 1.0 in ethanol) [lit.,[210] $[\alpha]_D^{20}$ 106.3° (c. 1.0 in ethanol) for the (6S)-isomer]; ν_{max} (neat) 2940 (CH) and 1680 cm^{-1} (C=N); δ_H (200 MHz, $CDCl_3$) 0.75, 1.03 (6H, 2 d, *J* 7 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.22 (1H, dsp, ^{i}Pr C$\underline{H}$), 3.68, 3.71 (6H, 2s, 2 and 5 OC$\underline{H}_3$) and 4.0 (3H, br s, 3 C$\underline{H}$ and 6 C$\underline{H}_2$); δ_C (75 MHz, $CDCl_3$) 16.9, 18.2 (2 x ^{i}Pr $\underline{C}H_3$), 32.42 (^{i}Pr $\underline{C}$H), 46.54 (6 $\underline{C}H_2$), 52.47, 52.51 (2 and 5 O$\underline{C}H_3$), 61.02 (3 $\underline{C}$H), 162.28 and 164.85 (2 and 5 C); *m/z* (EI) 185 ([*M*+H]$^+$, 30%) and 171 (100, [*M*-CH]$^+$).

(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6 dihydropyrazine (114)



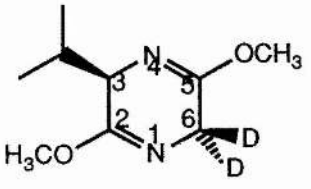
*n*BuLi (2 M in Hexane) (750 µl, 1.474 mmol) was added to a stirred solution of (3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (0.25 g, 1.34 mmol) in dry THF (3.5 ml) at -80 °C. The mixture was stirred until the solution warmed to -60 °C to allow for the formation of the anion. A solution of benzylchloromethylether (0.315 g, 2.01 mmol) in dry THF (750 µl) at -70 °C was added dropwise to the solution containing the anion and the mixture was stirred at -70 °C overnight. The solvent was removed *in vacuo* and the crude product shaken with 100 mM potassium phosphate solution (pH 7, 5 ml). The aqueous phase was extracted with diethyl ether (4 x 5 ml), dried ($MgSO_4$), and the volatile products removed *in vacuo*. The product was purified using flash chromatography (petrol:ethyl acetate 9:1), the fractions with $R_f \approx 0.3$ (when visualised in iodine and uv light) yielded a pale yellow oil (250 mg, 60%). (Found: C, 67.24; H, 8.10; N, 9.52. Calc. for $C_{17}H_{24}N_2O_3$: C, 67.08; H, 7.95; N, 9.20%); *m/z* (Found: $[M+H]^+$ 305.18651. $C_{17}H_{25}N_2O_3$ requires 305.18651); $[\alpha]_D$ +2.6° (c. 0.8 in dichloromethane); ν_{max} (neat) 2800-3000 (CH str), 1685 (C=N), 1230, 1065 (=C(R)OMe) and 1120 cm^{-1} (CH_2OCH_2Ph); δ_H (200 MHz, $CDCl_3$) 0.71, 1.08 (6H, 2d, *J* 6.8 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.32 (1H, m, ^{i}Pr C$\underline{H}$), 3.72 (6H, 2s, 2 and 5 OC$\underline{H}_3$), 3.7-4.2 (4H, m, 3' C$\underline{H}_2$, 3 C$\underline{H}$, 6 C$\underline{H}$), 4.55 (2H, s, PhC$\underline{H}_2$O) and 7.3 (5H, s, aromatic protons); δ_C (75 MHz, $CDCl_3$) 16.47, 19.02 (2 x ^{i}Pr $\underline{C}H_3$'s), 31.37 (^{i}Pr $\underline{C}$H), 52.53, 52.41 (2 O$\underline{C}H_3$ and 5 O$\underline{C}H_3$), 56.78 (6 $\underline{C}$H), 60.69 (3 $\underline{C}$H), 71.29, 73.08 ($\underline{C}H_2O\underline{C}H_2Ph$), 127.29, 128.11, 138.45 (aromatic carbons), 161.49 and 164.81 (2 and 5 C); *m/z* (EI) 305 ($[M+H]^+$, 100) and 91 (30, $[PhCH_2]^+$).

(S)-O-Benzyl serine methyl ester (116)



(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**114**) (0.19 g, 0.625 mmol) was added to 0.1 M hydrochloric acid (12.5 ml) and the mixture stirred at room temperature overnight. The aqueous solution was extracted with diethyl ether (3 x 10 ml). The aqueous portion was evaporated *in vacuo*. The residue was dissolved in water (2 ml) and aqueous 25% ammonia solution (0.2 ml) added. The mixture was extracted with diethyl ether. The diethyl ether extract was dried ($MgSO_4$) and concentrated to dryness *in vacuo*. The mixture of (2R)-valine methyl ester and the (S)-O-benzyl serine methyl ester were separated by flash silica chromatography (diethyl ether:ethanol 19:1), the uv active fractions (Fr 6-11) were combined and concentrated to dryness *in vacuo* to yield the pure (S)-O-benzyl serine methyl ester (40 mg, 31%). *m/z* (Found: $[M+H]^+$, 210.113. $C_{11}H_{16}NO_3$ requires 210.1130); ν_{max} (nujol) 3200 (NH str), 2800-3000 (CH str and nujol) and 1730 cm^{-1} (C=O); $[\alpha]_D$ +1.24 (c. 1.208 in dichloromethane); δ_H (200 MHz, $CDCl_3$) 1.95 (2H, br s, $N\underline{H}_2$), 3.64-3.74 (6H, m, $CO_2C\underline{H}_3$, 2 C$\underline{H}$, 3$C\underline{H}_2$), 4.53 (2H, s, $OC\underline{H}_2Ph$) and 7.32 (5H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 52.09 (2 $\underline{C}H$), 54.80 ($CO_2\underline{C}H_3$), 71.83, 73.20 (3 $\underline{C}H_2$ and 3' $\underline{C}H_2$), 127.55, 127.67, 128.33, 137.76 (aromatic carbons) and 174.07 (ester $\underline{C}$=O).

(3R)-[6-$^2H_2$]-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (117)



(3R)-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (1.09 g, 5.9 mmol) was added to CD_3OD (5 ml) and KOH (5.9 mmol) in H_2O (0.5 ml). The solution was refluxed under nitrogen for 3 h and cooled. Water (10 ml) containing one equivalent of HCl was added. The product was extracted into dichloromethane (3 x 10 ml), dried ($MgSO_4$), and concentrated *in vacuo* (0.8 g, 80%). $[\alpha]_D$ -74.2° (c. 1.016 in ethanol); ν_{max} (neat) 2800-3000 (CH str) and 1697 cm^{-1} (C=N); δ_H (200 MHz, $CDCl_3$) 0.78, 1.01 (6H, 2d, *J* 7 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.1-2.3 (1H, m, ^{i}Pr C$\underline{H}$), 3.69, 3.73 (6H, 2s, 2 and 5 OC$\underline{H}_3$) and 3.97 (1H, d, *J* 3.8 Hz, 3 C$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 16.91, 18.96 (2 x ^{i}Pr $\underline{C}H_3$), 32.37 (^{i}Pr $\underline{C}$H), 52.42 (2 and 5 O$\underline{C}H_3$), 60.99 (3 $\underline{C}$H), 162.26 and 164.81 (2 and 5 C); δ_D (61.4 MHz, $CHCl_3$) 3.97 and 3.93 (6 $C\underline{D}_2$); *m/z* (CI) 187 (M^+, 100%).

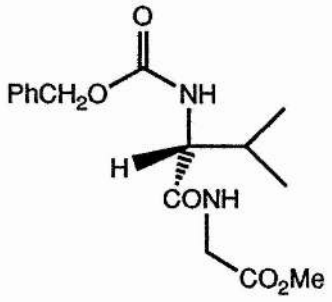
Glycine methyl ester hydrochloride (118)

Glycine (7 g, 90.9 mmol) was suspended in dry MeOH (70 ml) and cooled in ice. Thionyl chloride (7.7 ml, 105.5 mmol) was added dropwise. The resulting solution was refluxed for 30 min, cooled, and concentrated to dryness *in vacuo.* A hygroscopic white solid was obtained (12.3 g,100%), m.p. 176-178 °C [lit.,[286] 174 °C (dec.)]; ν_{max} (nujol) 2500-3300 (NH, CH str and nujol) and 1740 cm^{-1} (ester C=O); δ_H (200 MHz, D_2O) 3.76 (3H, s, OC$\underline{H}_3$), 3.86 (2H, s, 2 C$\underline{H}_2$); δ_C (50 MHz, D_2O/MeOH) 41.1 (2 $\underline{C}H_2$), 54.4 (O$\underline{C}H_3$) and 169.7 ($\underline{C}$=O); *m/z* (EI) 89 (M^+, 10%) and 30 (100, $[CH_2NH_2]^+$).

N-Carbobenzoxy-(2R)-valine (119)

Sodium bicarbonate (12.55 g, 150 mmol) was suspended in water (100 ml) and (2R)-valine (5 g, 42.7 mmol) added with vigorous stirring. Benzylchloroformate (8 g, 47 mmol) was added in 5 portions over 30 min. The mixture was stirred overnight, and extracted with diethyl ether (25 ml) and the aqueous layer adjusted to pH 2 using 5 M HCl, this was extracted with ethyl acetate (3 x 25 ml), the ethyl acetate portions were combined, dried ($MgSO_4$) and concentrated *in vacuo* yielding an oil (9.4 g, 88%) which was carried on to the next step without further purification. δ_H (200 MHz, $CDCl_3$) 0.9, 1.0 (6H, 2d, *J* 8 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.25 (1H, m, ^{i}Pr C$\underline{H}$), 4.38 (1H, dd, *J* 5 Hz, 2 C$\underline{H}$), 5.51 (2H, s, 2' C$\underline{H}_2$) and 7.35 (5H, s, aromatic protons); δ_C (50 MHz, $CDCl_3$) 17.3, 18.9 (2 x ^{i}Pr), 31.0 (^{i}Pr $\underline{C}$H), 58.8 (2 $\underline{C}H_2$), 67.16 (2' $\underline{C}$H), 128.08, 128.18, 128.48, 136.06 (aromatic carbons), 156.42 (urethane $\underline{C}$=O) and 176.48 (acid $\underline{C}$=O).

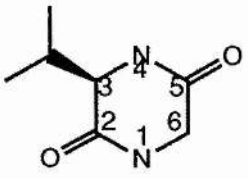
N-Carbobenzoxy-(2R)-valine glycine methyl ester (120)



N-Carbobenzoxy-(2R)-valine (**119**) (9.4 g, 37.4 mmol) was dissolved in dry THF (125 ml) and cooled to -5 °C in an ice/salt bath. This was treated with N-methyl morpholine (4.1 ml, 37.4 mmol), isobutylchloroformate (4.8 ml, 37.4 mmol) and left to stir for 3 minutes. A suspension of glycine methyl ester hydrochloride salt (**118**) (4.675 g, 37.4 mmol) in DMF (20 ml) containing N-methylmorpholine (4.8 ml, 37.4 mmol) was added. After stirring for 2 h a further portion of DMF (45 ml) was added. The reaction mixture

was stirred overnight. Addition of water (100 ml) caused the suspension to dissolve, the solution was concentrated slightly *in vacuo* yielding a white precipitate, this was filtered off at the pump washed with water and dried. Recrystallisation from dichloromethane and petrol yielded the pure product (10.44 g, 87%), m.p. 139-140 °C (lit.,[287,288] 130-132, 151-153 °C); $[\alpha]_D$ +6.8° (c. 1.0 in dichloromethane); ν_{max} (nujol) 3290 (NH str), 2800-3000 (CH str and nujol), 1750 (ester C=O), 1685 (urethane C=O), 1645 and 1535 cm^{-1} (amide); δ_H (200 MHz, $CDCl_3$) 0.93, 0.97 (6H, 2d, *J* 7 Hz, 2 x ^{i}Pr CH_3), 2.1 (1H, m, *J* 7 Hz, ^{i}Pr CH), 3.7 (3H, s, 1 OCH_3), 3.9-4.3 (3H, m, 2' CH and 2 CH_2), 5.1 (2H, s, $PhCH_2$), 5.6 and 6.9 (2 x NH) and 7.35 (5H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 17.69, 19.12 (2 x ^{i}Pr CH_3), 31.04 (^{i}Pr CH), 41.01 (2 CH_2), 52.26 (1 OCH_3), 60.21 (2' CH), 67.00 ($PhCH_2$), 127.92, 128.1, 128.47, 136.18 (aromatic carbons), 156.18 (urethane C=O), 170.10 and 171.74 (ester and amide C=O); *m/z* (FAB) 323 ($[M+H]^+$, 100%).

***Cyclo*-((2R)-val-gly) (121)**



N-Carbobenzoxy-(2R)-valine glycine methyl ester (**120**) (2.21 g, 6.8 mmol) was dissolved in dry methanol (12 ml) and dry dichloromethane (36 ml), and 10% Pd/C (100 mg) was added. Hydrogen gas was bubbled through the solution until the starting material was no longer detected by tlc (ethyl acetate) (time varies from 3 h-3 d). The solution was filtered through Celite to remove the catalyst, concentrated to dryness *in vacuo*, and the residue was refluxed in dry toluene (35 ml) for 12 h to complete the cyclisation. The resulting suspension was cooled to 0 °C to allow for the precipitation of

the product, the white solid was filtered off, washed with diethyl ether, and recrystallised from hot water (0.48 g, 45%), m.p. 257-259 °C; $[\alpha]_D$ -25.3° (c. 0.9 in H_2O); ν_{max} (nujol) 3180 (NH str), 2800-3000 (CH str and nujol) and 1670 cm^{-1} (amide C=O's); δ_H (200 MHz, D_2O) 0.87, 0.96 (6H, dd, *J* 7 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.2 (1H, 2sp, *J* 7Hz, ^{i}Pr C$\underline{H}$), 3.88 (1H, d, *J* 3 Hz, 3 C$\underline{H}$), 3.9 (1H, dd, *J* 17 and 3 Hz, 6 C$\underline{H}$) and 4.09 (1H, dd, *J* 17 and 3 Hz, 6 C$\underline{H}$); δ_C (75 MHz, D_2O/NaTMS salt) 18.47, 20.70 (2 x ^{i}Pr $\underline{C}H_3$), 35.72 (^{i}Pr $\underline{C}$H), 46.54 (6 $\underline{C}H_2$), 62.79 (3 $\underline{C}$H), 171.72 and 173.13 ($\underline{C}$=O's); *m/z* (CI) 157 ([*M*+H]$^+$, 100%) and 114 (82.5, [*M*-CH$(CH_3)_2$+H]$^+$).

(2R)-[2-^{2}H]-Serine (122)

This was prepared in an identical manner to (2S)-serine (**73**) using (2R)-[2-^{2}H]-O-benzyl serine methyl ester (**128**) in 89% yield (95% deuteriated by proton NMR spectroscopy). M.p. 194 °C; (Found: C, 33.95; H, 5.98; N, 13.05; *M*$^+$, 106.0489. Calc. for $C_3H_6NO_3D$: C, 33.96; H, 5.70; N, 13.20%; *M*, 106.0489); $[\alpha]_D$ -13.5° (c. 1.025 in 1 M HCl) [lit.,[212,213] $[\alpha]_D^{23}$ +14.5°(c. 1.0 in 1 M HCl) for (2S)-serine]; ν_{max} (nujol) 3200-2400 (NH_2, OH and CH str) and 1661 cm^{-1} (amino acid CO_2H); δ_H (400 MHz, D_2O) 3.47, 3.29 (2H, 2d, *J* 11 Hz, 3 C$\underline{H}_2$) and 3.04 (0.05H, m, 2 C$\underline{H}$ not deuteriated); δ_C (100 MHz, D_2O/NaOD/dioxane) 58.81 (t, *J* 20 Hz, 2 $\underline{C}$D), 65.89 (3 $\underline{C}H_2$) and 182.02 ($\underline{C}$=O); δ_D (61.4 MHz, H_2O/NaOH) 3.21 (2 C$\underline{D}$); *m/z* (CI) 107 ([*M*+H]$^+$, 100%), 61 (60, [*M*-CO_2H]$^+$) and 45 (20, [CO_2H]$^+$).

(2R)-[2-^{2}H]-Serine O-sulphate (123)

This was prepared in an identical manner to (2S)-serine O-sulphate (**21**) using (2R)-[2-^{2}H]-serine (**122**) using the method of Previero[193] in 17% yield. M.p. 228 °C; (Found: C, 19.25; H, 3.20; N, 7.53. Calc. for $C_3H_6NO_6DS$: C,19.36; H, 3.25; N, 7.52%); $[\alpha]_D$ +0.9° (c. 0.995 in 5% KOH); ν_{max} (nujol) 2800-3000 (CH str and nujol), 1766 (acid CO_2H

monomer), 1216 and 724 cm^{-1} (C-O-S); δ_H (400 MHz, D_2O/NaOD) 3.32 (0.05 H, t, *J* 4.7 Hz, 2 C<u>H</u> non deuteriated), 3.94 (1H, d, *J* 10 Hz, 3 C<u>H</u>$_a$) and 3.97 (1H, d, *J* 10 Hz, 3 C<u>H</u>$_b$); δ_C (100 MHz, D_2O/NaOD/NaTMS salt) 72.03 (3 <u>C</u>H_2); δ_D (H_2O/NaOH) 3.46 (2 C<u>D</u>).

(3R,6S)-[6-^{2}H]-2,5-Dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (124)

This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**114**) using (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**) in 60% yield. *m/z* (Found: [*M*+H]$^+$, 306.193. $C_{17}H_{24}N_2O_3D$ requires 306.1928); $[\alpha]_D$ +2.52° (c. 0.396 in dichloromethane); ν_{max} (neat) 2800-3000 (CH str), 1670 (C=N) and 1230 cm^{-1} (C(R)OMe); δ_H (200 MHz, $CDCl_3$) 0.70, 1.01 (6H, 2d, *J* 6.8 Hz, 2 x ^{i}Pr C<u>H</u>$_3$), 2.15-2.4 (1H, m, ^{i}Pr C<u>H</u>), 3.6-3.9 (8H, s and dd, 2 OC<u>H</u>$_3$, 5 OC<u>H</u>$_3$ and C<u>H</u>$_2$OC<u>H</u>$_2$Ph), 4.02 (1H, d, *J* 3.4 Hz, 3 C<u>H</u>), 4.54 (2H, s, C<u>H</u>$_2$Ph) and 7.2-7.4 (5H, m, aromatic protons); δ_C (75 MHz, $CDCl_3$) 16.54, 19.13 (2 x ^{i}Pr <u>C</u>H$_3$), 31.41 (^{i}Pr <u>C</u>H), 52.43, 52.54 (2 and 5 O<u>C</u>H$_3$), 56.40 (t, *J* 21 Hz, 6 <u>C</u>D), 60.75 (3 <u>C</u>H), 71.3, 73.16 (<u>C</u>H$_2$O<u>C</u>H$_2$Ph), 127.36, 128.19, 138.53 (aromatic carbons), 161.54 and 164.95 (2 and 5 C); δ_D (61.4 MHz, $CHCl_3$) 4.08 (6 C<u>D</u>).

(2S)-[2-^{2}H]-O-Benzyl serine methyl ester (125)

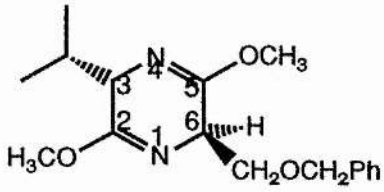
This was prepared in an identical manner to (S)-O-benzyl serine methyl ester (**116**) from (3R,6S)-[6-^{2}H]-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**124**) in 51% yield. *m/z* (Found: [*M*+H]$^+$, 211.1193. $C_{11}H_{15}NO_3D$ requires 211.1193); $[\alpha]_D$ +0.83° (c. 1.022 in dichloromethane); ν_{max} (neat) 2800-3000 (CH str) and 1737 cm^{-1} (C=O); δ_H (200 MHz, $CDCl_3$) 1.95 (br s, N<u>H</u>$_2$), 3.7 (5H, m, C<u>H</u>$_2$ and CO$_2$C<u>H</u>$_3$), 4.55 (2H, s, C<u>H</u>$_2$Ph) and 7.3 (5H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 51.82 (O<u>C</u>H$_3$), 54.22

(t, *J* 20.55 Hz, 2 C**D**), 71.59, 72.95 (3 CH_2 and CH_2Ph), 127.34, 127.45, 128.12, 137.59 (aromatic carbons) and 173.9 (C=O); δ_D (61.4 MHz, $CHCl_3$) 3.58 (2C**D**).

(3S)-[6-$^2H_2$]-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (127)

This was prepared in an identical manner to (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**) using (3S)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**126**) in 94% yield. $[\alpha]_D$ +65.6° (c. 1.38 in ethanol); ν_{max} (neat) 2800-3000 (CH str) and 1697 cm^{-1} (C=N); δ_H (360 MHz, $CDCl_3$) 0.75, 1.03 (6H, 2d, *J* 7 Hz, 2 x ^{i}Pr C**H**$_3$), 2.23 (1H, m, ^{i}Pr C**H**), 3.68, 3.72 (6H, 2s, 2 and 5 OC**H**$_3$) and 3.99 (1H, d, *J* 3.8 Hz, 3 C**H**); δ_C (90 MHz, $CDCl_3$) 16.92, 18.92 (2 x ^{i}Pr C**H**$_3$), 32.31 (^{i}Pr C**H**), 46.23 (t, *J* 21.42 Hz, 6 C**D**), 52.43, 52.37 (2 and 5 OC**H**$_3$), 61 (3 C**H**), 162.27 and 164.84 (2 and 5 C); *m/z* (CI) 187 ([*M*+H]$^+$, 100%).

(3S,6R)-2,5-Dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (128)



This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**114**) from (3S)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**126**) in 56% yield. *m/z* (Found: [*M*+H]$^+$, 305.187. $C_{17}H_{25}N_2O_3$ requires 305.1865); $[\alpha]_D$ -3° (c. 0.7 in dichloromethane); ν_{max} (neat) 2900-3000 (CH str), 1690 (C=N) and1230 cm^{-1} (C(R)OMe); δ_H (200 MHz, $CDCl_3$) 0.7, 1.08 (6H, 2d, *J* 7.5 Hz, 2 x ^{i}Pr C**H**$_3$), 2.3 (1H, dsp, ^{i}Pr C**H**), 3.7 (6H, s, 2 and 5 OC**H**$_3$), 3.8 (2H, m, C**H**$_2$OC**H**$_2$Ph), 4.03, 4.08 (2H, 2m, 3 C**H** and 6 C**H**), 4.55 (2H, s, OC**H**$_2$Ph) and 7.3 (5H,

m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 16.54, 19.11 (2 x ^{i}Pr $\underline{C}H_3$), 31.43 (^{i}Pr $\underline{C}H$), 52.39, 52.48 (2 and 5 $O\underline{C}H_3$'s), 56.87 (6 $\underline{C}H$), 60.76 (3 $\underline{C}H$), 71.36, 73.15 ($\underline{C}H_2O\underline{C}H_2Ph$), 127.35, 128.18, 138.53 (aromatic carbons), 161.55 and 164.88 (2 and 5 C).

(3S,6R)-[6-^{2}H]-2,5-Dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (129)

This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**114**) using (3S)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**127**) in 54% yield. $[\alpha]_D$ -2.7° (c. 0.748 in dichloromethane); ν_{max} (neat) 2800-3000 (CH str), 1698 (C=N) and 1240 cm^{-1} (C(R)OMe); δ_H (360 MHz, $CDCl_3$) 0.67, 1.07 (6H, 2d, *J* 7 Hz, 2 x ^{i}Pr $C\underline{H}_3$), 2.27 (1H, m, ^{i}Pr $C\underline{H}$), 3.81 (6H, 2s, 2 and 5 $OC\underline{H}_3$), 3.7-3.85 (2H, dd, $C\underline{H}_2OCH_2Ph$), 4.0 (1H, d, *J* 1.6 Hz, 3 $C\underline{H}$), 4.53 (2H, s, $C\underline{H}_2Ph$) and 7.22-7.34 (5H, m, aromatic protons); δ_C (90 MHz, $CDCl_3$) 16.5, 19.1 (2 x ^{i}Pr $\underline{C}H_3$), 31.4 (^{i}Pr $\underline{C}H$), 52.4 (2 and 5 $O\underline{C}H_3$), 56.4 (t, *J* 21 Hz, 6 $\underline{C}D$), 60.80 (3 $\underline{C}H$), 71.3, 73.2 ($\underline{C}H_2O\underline{C}H_2Ph$), 127.4, 128.2, 139 (aromatic carbons), 161.5 and 165 (2 and 5 C); *m/z* (EI) 306 ([*M*+H]$^+$, 100%).

(R)-O-Benzyl serine methyl ester (130)

This was prepared in an identical manner to (S)-O-benzyl serine methyl ester (**116**) using (3S,6R)-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**128**) in 61% yield. *m/z* (Found: [*M*+H]$^+$, 210.1130. $C_{11}H_{16}NO_3$ requires 210.1130); $[\alpha]_D$ -2.22° (c. 0.9 in dichloromethane); ν_{max} (neat) 3600 (br NH_2 str), 2800-3000 (CH str) and 1742 cm^{-1} (CO_2Me); δ_H (200 MHz, $CDCl_3$) 2.15 (2H, br s, $N\underline{H}_2$), 3.7 (6H, m, $OC\underline{H}_3$, 2 $C\underline{H}$, 3 $C\underline{H}_2$), 4.5 (2H, s, $PhC\underline{H}_2O$) and 7.3 (5H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 51.92 ($O\underline{C}H_3$), 54.56 (2 $\underline{C}H$), 71.58 (3 $\underline{C}H_2$), 73.00 ($Ph\underline{C}H_2$), 127.38,

127.51, 128.16, 137.59 (aromatic carbons) and 173.9 (C=O).

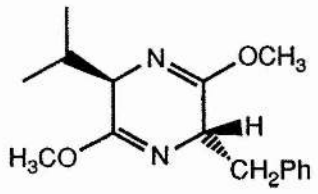
(2R)-[2-^{2}H]-O-Benzyl serine methyl ester (131)

This was prepared in an identical manner to (S)-O-benzyl serine methyl ester (**116**) from (3S,6R)-[6-^{2}H]-2,5-dimethoxy-3-*iso*propyl-6-benzyloxymethyl-3,6-dihydropyrazine (**129**) in 70% yield. *m/z* (Found: $[M+H]^+$, 211.1193. $C_{11}H_{15}NO_3D$ requires 211.1193); $[\alpha]_D$ -0.32° (c. 0.932 in dichloromethane); ν_{max} (neat) 2800-3000 (CH str) and 1740 cm^{-1} (C=O); δ_H (250 MHz, $CDCl_3$) 1.56 (br s, NH_2), 3.7 (5H, m, CH_2 and CO_2CH_3), 4.53 (2H, s, CH_2Ph) and 7.35 (5H, m, aromatic protons); δ_C (75 MHz, $CDCl_3$) 51.97 (OCH_3), 54.34 (t, *J* 21 Hz, 2 CD), 71.68, 73.08 (3 CH_2 and CH_2Ph), 127.43, 127.56, 128.22, 137.65 (aromatic carbons) and 174 (C=O).

(2R)-Serine (132)

This was prepared in an identical manner to (2S)-serine (**76**) from (R)-O-benzyl serine methyl ester (**130**) in 20% yield. M.p. 221-223 °C (dec.); (Found: C, 34.41; H, 6.80; N, 13.10. Calc. for $C_3H_7NO_3$: C, 34.29; H, 6.71; N, 13.33%); *m/z* (Found: $[M+H]^+$, 106.050. $C_3H_8NO_3$ requires 106.050); $[\alpha]_D$ -13.6° (c. 1.02 in 1 M HCl) [lit.,[212,213] $[\alpha]_D^{23}$ +14.5 (c. 1.0 in 1 M HCl) for (2S)-serine]; ν_{max} (nujol) 3459 (NH/OH str), 2800-3000 (CH str and nujol) and 1597 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 3.22 (1H, t, *J* 5 Hz, 2 CH) and 3.60 (2H, m, 3 CH_2); δ_C (50 MHz, D_2O/NaOD/MeOH) 58.12 (2 CH), 64.17 (3 CH_2) and 178.75 (CO_2H); *m/z* (CI) 106 ($[M+H]^+$, 100%), 60 (42, $[M-CO_2H]^+$) and 44 (12, $[CO_2]^+$).

(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-benzyl-3,6-dihydropyrazine (133)

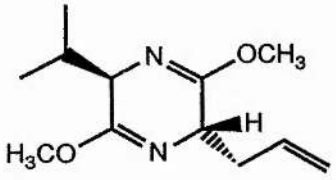


(3R)-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (250 mg, 1.36 mmol) was dissolved in dry THF (3 ml) and cooled to -80 °C. To this was added dropwise 2 M *n*BuLi (0.68 ml, 1.36 mmol). After 30 min a solution of benzyl bromide (349 mg, 2.04 mmol) in dry THF (3 ml) was added and the mixture was stirred at -80 °C overnight. The resulting solution was concentrated to dryness *in vacuo*. Potassium phosphate buffer (20 ml, 100 mM, pH 7) was added and the suspension extracted with diethyl ether (3 x 20 ml). The diethyl ether layer was dried ($MgSO_4$) and concentrated *in vacuo* to yield the crude product. This was purified by flash chromatography (petroleum ether:diethyl ether 9:1). The product was uv and I_2 active with an $R_f \approx 0.46$. The product containing fractions were combined and concentrated *in vacuo* to yield the pure material as a colourless oil (260 mg, 70%), *m/z* (Found: $[M+H]^+$, 275.176. $C_{16}H_{23}N_2O_2$ requires 275.1756); $[\alpha]_D$ +65.4° (c. 1.6 in dichloromethane); ν_{max} (neat) 2944, 2871 (CH str), 1698 (C=N) and 1495 cm^{-1} (Ph); δ_H (200 MHz, $CDCl_3$) 0.61, 0.95 (6H, 2d, *J* 6.8 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.15 (1H, dsp, ^{i}Pr C$\underline{H}$), 3.10 (2H, d, *J* 4.6 Hz, C$\underline{H}_2$Ph) 3.27 (1H, dd, *J* 3.4 Hz, 3 C$\underline{H}$), 3.70, 3.73 (6H, 2s, 2 and 5 OC$\underline{H}_3$), 4.35 (1H, 2d, *J* 4.6 Hz, 6 C$\underline{H}$), and 7.07-7.23 (5H, m, aromatic protons); δ_C (75 MHz, $CDCl_3$) 16.39, 18.98 (2 x ^{i}Pr $\underline{C}H_3$), 31.1 (^{i}Pr $\underline{C}$H), 39.95 ($\underline{C}H_2$Ph), 52.07, 52.31 (2 and 5 O$\underline{C}H_3$), 56.59 (6 $\underline{C}$H), 60.16 (3 $\underline{C}$H), 126.24, 127.76, 129.95, 137.26 (aromatic carbons), 162.38 and 163.91 (2 and 5 C); *m/z* (EI) 275 ($[M+H]^+$, 100%), 183 (20, $[M-PhCH_2]^+$) and 141 (30, $[M-PhCH_2-CH(CH_3)_2+H]^+$).

(3R,6S)-[6-^{2}H]-2,5-Dimethoxy-3-*iso*propyl-6-benzyl-3,6-dihydropyrazine (134)

This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-6-benzyl-3,6-dihydropyrazine (**133**) using (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**) in 88% yield. *m/z* (Found: $[M+H]^+$, 276.1822. $C_{16}H_{22}N_2O_2D$ requires 276.1822); ν_{max} (neat) 2944 (CH str), 1696 (C=N) and 1496 cm^{-1} (Ph); $[\alpha]_D$ +40.4° (c. 1.556 in dichloromethane); δ_H (200 MHz, $CDCl_3$) 0.64, 0.97 (6H, 2d, *J* 6.8 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.18 (1H, dsp, ^{i}Pr C$\underline{H}$), 3.11 (2H, s, C$\underline{H}_2$Ph) 3.28 (1H, d, *J* 3.2 Hz, 3 C$\underline{H}$), 3.70, 3.74 (6H, 2s, 2 and 5 OC$\underline{H}_3$) and 7.1-7.3 (5H, m, aromatic protons); δ_C (50 MHz, $CDCl_3$) 16.31, 18.95 (2 x ^{i}Pr $\underline{C}H_3$), 30.98 (^{i}Pr $\underline{C}$H), 39.81 ($\underline{C}H_2$Ph), 51.98, 52.23 (2 and 5 O$\underline{C}H_3$), 56.0 (t, *J* 22 Hz, 6 $\underline{C}$H), 60.07 (3 $\underline{C}$H), 126.19, 127.70, 129.89, 137.17 (aromatic carbons), 162.38 and 163.31 (2 and 5 C); δ_D (61.4 MHz, $CHCl_3$) 4.31 (6 C$\underline{D}$); *m/z* (CI) 276 ($[M+H]^+$, 100), 184 (20, $[M-PhCH_2]^+$) and 142 (32, $[M-PhCH_2-CH(CH_3)_2+H]^+$).

(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-allyl-3,6-dihydropyrazine (135)



(3R)-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (250 mg, 1.36 mmol) was dissolved in dry THF (3 ml) and cooled to -80 °C. To this was added dropwise 1.9 M *n*BuLi (0.72 ml, 1.36 mmol). After 30 min a solution of allyl bromide (329 mg, 2.72 mmol) in dry THF (3 ml) was added and the mixture was stirred at -80 °C overnight. The resulting solution was concentrated to dryness *in vacuo*. Potassium phosphate buffer (20 ml, 100 mM, pH 7) was added and the suspension extracted with diethyl ether (3 x 20 ml). The diethyl ether layer was dried ($MgSO_4$) and concentrated *in vacuo*

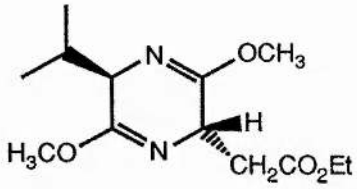
to yield the crude product. This was purified by flash chromatography (petroleum ether:ethyl acetate 7:3). The product was I_2 active with an R_f ≈0.82. The product containing fractions were combined and concentrated to yield the pure material as a colourless oil (220 mg, 73%), *m/z* (Found: M^+, 224.1525. $C_{12}H_{20}N_2O_2$ requires 224.1525); $[\alpha]_D$ +13.9° (c. 2.8 in dichloromethane); ν_{max} (neat) 3078, 2945, 2872 (CH str), 1699 (C=N) and 1642 cm^{-1} (C=C); δ_H (200 MHz, $CDCl_3$) 0.68, 1.05 (6H, 2d, *J* 7.6 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.27 (1H, dsp, ^{i}Pr C$\underline{H}$), 2.55 (2H, m, 3' C$\underline{H}_2$), 3.7 (6H, 2s, 2 and 5 OC$\underline{H}_3$), 3.9 (1H, t, *J* 3.4 Hz, 3 C$\underline{H}$), 4.1 (1H, dd, 6 C$\underline{H}$), 5.0-5.15 (2H, m, 1' C$\underline{H}_2$) and 5.7 (1H, m, 2' C$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 16.47, 19.03 (2 x ^{i}Pr $\underline{C}H_3$), 31.56 (^{i}Pr $\underline{C}$H), 38.43 (3' $\underline{C}H_2$), 52.25, 52.39 (2 and 5 O$\underline{C}H_3$), 55.41 (6 $\underline{C}$H), 60.65 (3 $\underline{C}$H), 117.77 (1' $\underline{C}H_2$), 133.78 (2' $\underline{C}H_2$), 163.06 and 163.77 (2 and 5 C); *m/z* (EI) 225 ([*M*+H]$^+$, 100%), 183 (50, [*M*-CH_2CHCH_2]$^+$) and 141 (43, [*M*-CH_2CHCH_2-$CH(CH_3)_2$+H]$^+$).

(3R,6S)-[6-^{2}H]-2,5-Dimethoxy-3-*iso*propyl-6-allyl-3,6-dihydropyrazine (136)

This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-6-allyl-3,6-dihydropyrazine (**135**) using (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**) in 65% yield. *m/z* (Found: M^+ 225.1590. $C_{12}H_{19}N_2O_2D$ requires 225.1586); $[\alpha]_D$ +11.11° (c. 2.88 in dichloromethane); ν_{max} (neat) 3000-2800 (CH str), 1696 (C=N) and 1641 cm^{-1} (C=C); δ_H (200 MHz, $CDCl_3$) 0.68, 1.02 (6H, 2d, *J* 6.8 Hz, 2 x ^{i}Pr C$\underline{H}_3$), 2.25 (1H, dsp, ^{i}Pr C$\underline{H}$), 2.52 (2H, d, *J* 7 Hz, 3' C$\underline{H}_2$), 3.7 (6H, 2s, 2 and 5 OC$\underline{H}_3$), 3.92 (1H, d, *J* 3.2 Hz, 3 C$\underline{H}$), 4.99-5.1 (2H, m, 1' C$\underline{H}_2$) and 5.7 (1H, m, 2' C$\underline{H}$); δ_C (50 MHz, $CDCl_3$) 16.48, 19.06 (2 x ^{i}Pr $\underline{C}H_3$), 31.54 (^{i}Pr $\underline{C}$H), 38.37 (3' $\underline{C}H_2$), 52.28, 52.43 (2 and 5 O$\underline{C}H_3$), 55.4 (t, *J* 20 Hz, 6 $\underline{C}$D), 60.65 (3 $\underline{C}$H), 117.83 (1' $\underline{C}H_2$), 133.79 (2' $\underline{C}H_2$), 163.09 and 163.85 (2 and 5 C); δ_D (61.4 MHz, $CHCl_3$) 4.06 (6 C$\underline{D}$); *m/z* (CI) 226 ([*M*+H]$^+$, 100%), 212 (35, [*M*-CH_2+H]$^+$), 184 (120, [*M*-CH_2CHCH_2]$^+$) and 142 (15, [*M*-

CH_2CHCH_2-$CH(CH_3)_2$+H]$^+$).

(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-dihydropyrazine-6-acetic-acid ethyl ester (137)



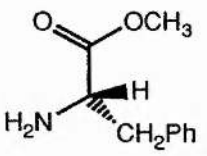
(3R)-2,5-Dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (1 g, 5.49 mmol) was dissolved in dry THF (25 ml) and cooled to -80 °C. To this was added dropwise 2.06 M *n*BuLi (2.67 ml, 5.49 mmol). When the mixture had warmed to -60 °C, the temperature was reduced to -90 °C and a cold solution of ethyl bromoacetate (0.92 g, 5.49 mmol) in dry THF (2 ml) was added and the mixture was stirred at -90 °C for 5 h. The resulting solution was concentrated to dryness *in vacuo*. Potassium phosphate buffer (20 ml, 100 mM, pH 7) was added and the suspension extracted with diethyl ether (3 x 20 ml). The diethyl ether layer was dried ($MgSO_4$) and concentrated *in vacuo* to yield the crude product. Purification using flash column chromatography in various solvent systems was unsuccessful. The proton NMR contained a quartet in the 3.5-4.0 ppm region characteristic of the β aspartic acid protons. The crude material was taken straight on to the next step.

(3R,6S)-[6-^{2}H]-2,5-Dimethoxy-3-*iso*propyl-dihydropyrazine-6-acetic-acid ethyl ester (138)

This was prepared in an identical manner to (3R,6S)-2,5-dimethoxy-3-*iso*propyl-dihydropyrazine-6-acetic-acid ethyl ester (**137**) using (3R)-[6-$^2H_2$]-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**117**). The proton NMR contained a quartet in the 3.5-4.0 ppm region characteristic of the β aspartic acid protons for a deuteriated sample.

The crude material was taken straight on to the next step.

(2S)-Phenylalanine methyl ester (139)



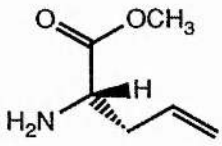
(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-benzyl-3,6-dihydropyrazine (**133**) (750 mg, 2.74 mmol) was added to 0.1 M HCl (55 ml) and stirred at room temperature overnight. The mixture was extracted with diethyl ether to remove unreacted starting material. The aqueous layer was concentrated *in vacuo* and dissolved in a small amount of water. The pH of the solution was adjusted to 8-10 with concentrated ammonia solution. The solution was extracted with diethyl ether (3 x 30 ml), dried ($MgSO_4$) and concentrated *in vacuo*. The mixture of (2R)-valine methyl ester and (2S)-phenylalanine methyl ester were separated by distillation under reduced pressure, (2S)-phenylalanine methyl ester having the lower boiling point (0.64 g, 70%), used for the next step without further purification. δ_H (200 MHz, $CDCl_3$) 1.76 (2H, br s, $N\underline{H}_2$), 2.87 (1H, ABX, J_{AB} 13.4 Hz, J_{AX} 5.2 Hz, benzyl $C\underline{H}_a$), 3.11 (1H, ABX, J_{AB} 13.4 Hz, J_{BX} 7.9 Hz, benzyl $C\underline{H}_b$), 3.7-3.8 (4H, m, $OC\underline{H}_3$ and 2 $C\underline{H}$) and 7.15-7.4 (5H, m, aromatic protons); δ_C (75 MHz, $CDCl_3$) 40.97 (3 $\underline{C}H_2$), 51.92 (1' $\underline{C}H_3$), 55.71 (2 $\underline{C}H$), 126.77, 128.49, 129.19, 137.13 (aromatic carbons) and 175.26 ($\underline{C}O_2Me$).

(2S)-[2-^{2}H]-Phenylalanine methyl ester (140)

This was prepared in an identical manner to (2S)-phenylalanine methyl ester (**139**) using (3R,6S)-[6-^{2}H]-2,5-dimethoxy-3-*iso*propyl-6-benzyl-3,6-dihydropyrazine (**134**) in 82% yield, used for the next step without further purification. δ_H (200 MHz, $CDCl_3$) 1.76

(2H, br s, $N\underline{H}_2$), 2.87 (1H, d, *J* 14 Hz, benzyl $C\underline{H}_a$), 3.11 (1H, d, *J* 14 Hz, benzyl $C\underline{H}_b$), 3.73 (3H, s, $OC\underline{H}_3$) and 7.1-7.35 (5H, m, aromatic protons).

(2S)-Allylglycine methyl ester (141)

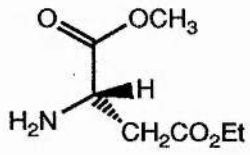


(3R,6S)-2,5-Dimethoxy-3-*iso*propyl-6-allyl-3,6-dihydropyrazine (**135**) (1.3 g, 5.8 mmol) was added to 0.2 M HCl (58 ml) and the suspension stirred at room temperature overnight. The mixture was extracted with diethyl ether (20 ml) to remove unreacted starting material. The aqueous layer was concentrated *in vacuo* and dissolved in a small amount of water (5 ml). The pH of the solution was adjusted to 8-10 with concentrated ammonia solution. This solution was extracted with diethyl ether (3 x 30 ml), dried ($MgSO_4$) and concentrated *in vacuo* yielding a mixture of (2R)-valine methyl ester and (2S)-allyl glycine methyl ester. The two products were separated using flash column chromatography on silica (diethyl ether and a drop of ammonia solution) (valine methyl ester R_f 0.38, allyl glycine methyl ester R_f 0.22) to give the required compound as a clear oil (0.2 g, 39%). *m/z* (Found: $[M+H]^+$, 130.087. $C_6H_{12}NO_2$ requires 130.0868); $[\alpha]_D$ +3.2° (c. 0.8 in dichloromethane); ν_{max} (neat) 3382 (NH_2 str), 3079 (C=C-H str), 2800-3000 (CH str), 1740 (CO_2CH_3) and 1642 cm^{-1} (C=C); δ_H (200 MHz, $CDCl_3$) 1.60 (2H, s, $N\underline{H}_2$), 2.36 (2H, m, 3 $C\underline{H}_2$), 3.5 (1H, 2d, *J*5 Hz, 2 C$\underline{H}$), 3.66 (3H, s, $OC\underline{H}_3$), 5.04 (1H, d, *J*1.2 Hz, 5 C$\underline{H}$ cis), 5.11 (1H, d, *J*5.8 Hz, 5 C$\underline{H}$ trans) and 5.6 (1H, m, 4 C$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 38.9 (3 $\underline{C}H_2$), 51.69 ($O\underline{C}H_3$), 53.66 (2 $\underline{C}$H), 118.36 (5 $\underline{C}H_2$), 133.17 (4 $\underline{C}$H) and 175.37 ($\underline{C}$=O); *m/z* (EI) 130 ($[M+H]^+$, 100%) and 70 (58, $[M-CO_2CH_3]^+$) 44 (20, $[CO_2]^+$).

(2S)-[2-^{2}H]-Allylglycine methyl ester (142)

This was prepared in an identical manner to (2S)-allyl glycine methyl ester (**141**) using (3R,6S)-[6-^{2}H]-2,5-dimethoxy-3-*iso*propyl-6-allyl-3,6-dihydropyrazine (**136**) in 40% yield. *m/z* (Found: $[M+H]^+$, 131.093. $C_6H_{11}NO_2D$ requires 131.0931); $[\alpha]_D$ +2.6° (c. 0.93 in dichloromethane); ν_{max} (neat) 3381 (NH_2 str), 3079 (C=C-H str), 2800-3000 (CH str), 1735 (CO_2CH_3) and 1642 cm^{-1} (C=C); δ_H (200 MHz, $CDCl_3$) 1.55 (2H, s, N$\underline{H}_2$), 2.4 (2H, m, 3 C$\underline{H}_2$), 3.67 (3H, s, OC$\underline{H}_3$), 5.1 (2H, m, 5 C$\underline{H}_2$) and 5.7 (1H, m, 4 C$\underline{H}$); δ_C (75 MHz, $CDCl_3$) 38.86 (3 $\underline{C}H_2$), 51.68 (O$\underline{C}H_3$), 53.34 (t, *J* 21.6 Hz, 2 $\underline{C}$D), 118.35 (5 $\underline{C}H_2$), 133.19 (4 $\underline{C}$H) and 175.41 ($\underline{C}$=O); δ_D (61.4 MHz $CHCl_3$) 3.53 (2 C$\underline{D}$); *m/z* (EI) 131 ($[M+H]^+$, 100%) and 71 (36, $[M-CO_2CH_3]^+$).

(2S)-Aspartic acid-α-methyl-β-ethyl ester (143)



Crude (3R,6S)-2,5-dimethoxy-3-*iso*propyl-dihydropyrazine-6-acetic-acid ethyl ester (**137**) (1.85 g, 6.9 mmol) was stirred overnight in 0.2 M HCl (69 ml, 13.8 mmol). The solution was extracted with diethyl ether (15 ml) and the diethyl ether discarded. The aqueous layer was concentrated to dryness *in vacuo*. It was dissolved in water (2 ml) and the pH adjusted to ≈8-10 with 25% ammonia solution. The basic solution was extracted with diethyl ether (3 x 10 ml). The diethyl ether extracts were combined, dried ($MgSO_4$), and concentrated to dryness *in vacuo* to yield a mixture of the required product and (2R)-valine methyl ester. The esters were separated by flash column chromatography (19:1 diethyl ether:ethanol with a drop of ammonia) and the products visualised with ninhydrin. The required product was obtained as a clear colourless oil

contaminated with (2R)-valine methyl ester (0.32 g, 39%). δ_H (200 MHz, $CDCl_3$) 1.25 (3H, t, *J* 7.6 Hz, ethyl C$\underline{H}_3$), 1.85 (2H, br s, N$\underline{H}_2$), 2.7 (2H, ABX, J_{AB} 12.6 Hz, J_{AX} 3 Hz, J_{BX} 9.6 Hz, 3 C$\underline{H}_2$), 3.7 (3H, s, OC$\underline{H}_3$), 3.8 (1H, ABX, J_{AX} 3 Hz, J_{BX} 9.6 Hz, 2 C$\underline{H}$) and 4.15 (2H, q, *J* 7.6 Hz, ethyl C$\underline{H}_2$); δ_C (50 MHz, $CDCl_3$) 14.05 (ethyl $\underline{C}H_2$), 38.88 (3 $\underline{C}H_2$), 51.08 (O$\underline{C}H_3$), 52.24 (2 $\underline{C}$H), 60.72 (ethyl $\underline{C}H_2$), 171.07 and 174.59 (ester $\underline{C}$=O).

(2S)-[2-^{2}H]-Aspartic acid-α-methyl-β-ethyl ester (144)

This was prepared in an identical manner to (2S)-aspartic acid-α-methyl-β-ethyl ester (**143**) using (3R,6S)-[6-^{2}H]-2,5-dimethoxy-3-*iso*propyl-dihydropyrazine-6-acetic-acid ethyl ester (**138**) in 41% yield. δ_H (200 MHz, $CDCl_3$) 1.25 (3H, t, *J* 7.6 Hz, ethyl C$\underline{H}_3$), 1.85 (2H, br s, N$\underline{H}_2$), 2.7 (2H, dd, *J* 17.7 Hz, 3 C$\underline{H}_2$), 3.7 (3H, s, OC$\underline{H}_3$) and 4.15 (2H, q, *J* 7.6 Hz, ethyl C$\underline{H}_2$).

(2S)-[2-^{2}H]-Phenylalanine (145)

This was prepared in an identical manner to (2S)-phenylalanine (**148**) using (2S)-[2-^{2}H]-phenylalanine methyl ester (**140**) in 60% yield. M.p. 264 °C (lit.,[289] 283°C (dec) for (2S)-phenylalanine); (Found: C, 64.83; H, 5.68; N, 8.23. Calc. for $C_9H_{10}NO_2D$: C, 65.04; H, 6.02; N, 8.43%); *m/z* (Found: [*M*+H]$^+$, 167.0931 $C_9H_{11}NO_2D$ requires 167.0931); $[\alpha]_D$ -28.2° (c. 1.5 in H_2O) [lit.,[214] $[\alpha]_D^{20}$ -32.5° (c. 2.0 in H_2O) for (2S)-phenylalanine]; ν_{max} (nujol) 2800-3000 (CH str and nujol) and 1559 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 2.69 (1H, d, *J* 13.2 Hz, 3 C$\underline{H}_a$), 2.84 (1H, d, *J* 13.2 Hz, 3 C$\underline{H}_b$) and 7.1-7.3 (5H, m, aromatic protons); δ_C (75 MHz, D_2O/NaOD/MeOH) 41.79 (3 $\underline{C}H_2$), 58.55 (2 $\underline{C}$H), 127.68, 129.66, 130.50, 139.48 (aromatic carbons), and 183.52 ($\underline{C}O_2H$); δ_D (61.4 MHz, H_2O/NaOH) 3.36 (2 C$\underline{D}$).

(2S)-[2-^{2}H]-Allylglycine (146)

This was prepared in an identical manner to (2S)-allyl glycine (**149**) using (2S)-[2-^{2}H]-allylglycine methyl ester (**142**) in 59% yield. M.p. 246-248 °C (dec.); (Found: C, 51.42; H, 6.74; N, 11.94. Calc. for $C_5H_8NO_2D$: C, 51.71; H, 6.94; N, 12.06%); *m/z* (Found: $[M+H]^+$, 117.077. $C_5H_9NO_2D$ requires 117.0774); $[\alpha]_D$ -4.2° (c. 1.87 in 6 M HCl)[lit.,[215] $[\alpha]_D^{24}$ +5.7 (c. 2.0 in 5 M HCl) for (2R)-allylglycine]; ν_{max} (nujol) 3200-2600 (nujol, CH str and OH str) and 1581 cm^{-1} (CO_2^-); δ_H (300 MHz, D_2O) 2.49 (1H, d, *J* 4 Hz, 3 C$\underline{H}_a$), 2.52 (1H, d, *J* 3 Hz, 3 C$\underline{H}_b$), 5.15 (2H, m, 5 C$\underline{H}_2$) and 5.6 (1H, m, 4 C$\underline{H}$); δ_C (75 MHz, D_2O/dioxane) 35.43 (3 $\underline{C}H_2$), 54.36 (t, *J* 22 Hz, 2 $\underline{C}$D), 121.20 (5 $\underline{C}H_2$), 132.03 (4 $\underline{C}$H) and 174.73 ($\underline{C}$=O); δ_D (61.4 MHz, H_2O) 3.54 (2 C$\underline{D}$); *m/z* (EI) 117 ($[M+H]^+$, 100%) and 71 (20, $[CO_2H]^+$).

(2S)-[2-^{2}H]-Aspartic acid (147)

This was prepared in an identical manner to (2S)-aspartic acid (**150**) using (2S)-[2-^{2}H]-aspartic acid-α-methyl-β-ethyl ester (**144**) in 12% yield from the initial bis-lactim ether. M.p. >300 °C; (Found: C, 35.59; H, 4.54: N, 10.32. Calc. for $C_4H_6NO_4D$: C, 35.82; H, 4.51; N, 10.44%); *m/z* (Found: $[M+H]^+$, 135.0516. $C_4H_7NO_4D$ requires 135.0516); $[\alpha]_D$ +19.5° (c. 0.495 in 5 M HCl); ν_{max} (nujol) 3000-2800 (CH str, NH str and nujol), 1690, 1643 (CO_2^-) and 1591 cm^{-1}(NH_3^+ deformations); δ_H (200 MHz, D_2O/NaOD) 2.24 (1H, d, J_{AB} 16 Hz, 3 C$\underline{H}_a$) and 2.54 (1H, d, J_{AB} 16 Hz, 3C$\underline{H}_b$); δ_C (100 MHz, D_2O/NaOD/dioxane) 43.91 (3 $\underline{C}H_2$), 54.41 (t, *J* 20.7 Hz, 2 $\underline{C}$D), 180.93 and 183.09 (1 and 4 $\underline{C}O_2H$); δ_D (61.4 MHz, H_2O/NaOH) 3.46 (2 C$\underline{D}$).

(2S)-Phenylalanine (148)

(2S)-Phenylalanine methyl ester (**139**) (0.82 g, 4.6 mmol) was suspended in 2 M HCl (50 ml) and refluxed under nitrogen for 2 h. The solvent was removed *in vacuo*, the

residue was dried over phosphorus pentoxide. The crude hydrochloride salt was added to dry ethanol (20 ml) and propylene oxide (20 ml) and the mixture refluxed for 15 min. The product was filtered, dried and recrystallised from water and ethanol to yield white crystals (0.76 g, 60%), m.p. 275 °C [lit.,[289] 283 °C (dec.)]; (Found: C, 65.66; H, 6.40; N, 8.48. Calc. for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48 %); *m/z* (Found: $[M+H]^+$, 166.0868. $C_9H_{12}NO_2$ requires 166.0868); $[\alpha]_D$ -30.9° (c. 2.035 in H_2O) (lit.,[214] $[\alpha]_D^{20}$ -32.5° (c. 2.0 in H_2O)); ν_{max} (nujol) 2800-3000 (CH str and nujol) and 1559 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 2.7 (1H, ABX, J_{BX} 7.4 Hz, J_{AB} 13.4 Hz, 3 C$\underline{H}_b$), 2.85 (1H, ABX, J_{AX} 5.5 Hz, J_{AB} 13.4 Hz, 3 C$\underline{H}_a$), 3.36 (1H, ABX, 2 C$\underline{H}$) and 7.1-7.3 (5H, m, aromatic protons); δ_C (75 MHz, D_2O/NaOD/MeOH) 41.79 (3 $\underline{C}H_2$), 58.46 (2 $\underline{C}H$), 127.63, 129.59, 130.43, 139.32 (aromatic carbons) and 183.41 ($\underline{C}O_2H$); *m/z* (EI) 166 ($[M+H]^+$, 100%) and 120 (20, $[M-CO_2H]^+$).

(2S)-Allylglycine (149)

(2S)-Allylglycine methyl ester (**141**) (0.27 g, 2.09 mmol) was added to 2 M HCl (10 ml) and the mixture refluxed for 2 h. The solvent was removed *in vacuo* and the residue dried over phosphorus pentoxide. Dry ethanol (10 ml) and propylene oxide (10 ml) were added and the mixture refluxed under nitrogen for 15 min. The suspension was cooled and the solid filtered off and dried (0.2 g, 83%), m.p. 254°C (dec.); (Found: C, 52.21; H, 8.12; N, 12.14. Calc. for $C_5H_9NO_2$: C, 52.16; H, 7.88; N, 12.17%); *m/z* (Found: $[M+H]^+$, 116.071. $C_5H_{10}NO_2$ requires 116.0712); $[\alpha]_D$ -5.7° (c. 2.0 in 5 M HCl) [lit.,[215] $[\alpha]_D^{24}$ +5.7° (c. 2.0 in 5 M HCl) for (2R)-allylglycine)]; ν_{max} (nujol) 3200-2600 (nujol, CH str and OH str), 1586 (CO_2^-); δ_H (300 MHz, D_2O) 2.5 (2H, m, 3 C$\underline{H}_2$), 3.7 (1H, dd, 2 C$\underline{H}$), 5.15 (2H, m, 5 C$\underline{H}_2$) and 5.7 (1H, m, 4 C$\underline{H}$); δ_C (75 MHz, D_2O/dioxane) 35.55 (3 $\underline{C}H_2$), 54.66 (2 $\underline{C}H$), 121.21 (5 $\underline{C}H_2$), 132.06 (4 $\underline{C}H$) and 174.69 ($\underline{C}$=O); *m/z* (EI) 116 ($[M+H]^+$, 100%) and 70 (15, $[CO_2H]^+$).

(2S)-Aspartic acid (150)

(2S)-Aspartic acid-α-methyl-β-ethyl ester (**143**) (0.32 g, 2.67 mmol) was refluxed in 5 M HCl (10 ml) for 2 h. The solvent was removed *in vacuo*. The product was dried over phosphorus pentoxide. The product was dissolved in dry ethanol (10 ml) and propylene oxide (10 ml) and the solution refluxed for 15 min. The amino acid was filtered off and recrystallised from water and ethanol yielding a white crystalline solid (20 mg, 11% from the initial bis-lactim ether), m.p. 290 °C (dec.) (lit.,[290] 270-271 °C); (Found: C, 36.48; H, 5.73; N, 10.16. Calc. for $C_4H_7NO_4$: C, 36.09; H, 5.30; N, 10.52%); *m/z* (Found: $[M+H]^+$, 134.046. $C_4H_8NO_4$ requires 134.0453); $[\alpha]_D$ +21.8° (c. 0.495 in 5 M HCl) [lit.,[213] $[\alpha]_D^{23}$ +24.1° (c. 0.56 in 5 M HCl)]; ν_{max} (nujol) 3000-2800 (CH, NH and nujol), 1692, 1644 (CO_2^-) and 1599 cm^{-1} (NH_3^+ deformations); δ_H (200 MHz, D_2O/NaOD) 2.21 (1H, ABX, J_{AX} 9.9 Hz, J_{AB} 15.8 Hz, 3 C$\underline{H}_a$), 2.55 (1H, ABX, J_{BX} 3.5 Hz, J_{AB} 15.8 Hz, 3 C$\underline{H}_b$) and 3.48 (1H, ABX, J_{AX} 9.9 Hz, J_{BX} 3.5 Hz, 2 C$\underline{H}$); δ_C (D_2O/NaOD/dioxane) 42.46 (3 $\underline{C}H_2$), 54,30 (2 $\underline{C}H$), 180.32 and 181.13 (1 and 4 $\underline{C}O_2H$); *m/z* (CI) 134 ($[M+H]^+$, 100%) and 44 (53, $[CO_2]^+$).

Kinetics of the pyrazine deuteriation reaction

(3R)-2,5-dimethoxy-3-*iso*propyl-3,6-dihydropyrazine (**113**) (0.5 g, 2.72 mmol) was added to CD_3OD (10 ml). To this was added a solution of KOH (0.15 g, 2.72 mmol) in D_2O (1 ml). The mixture was refluxed under nitrogen. Aliquots (0.7 ml) were removed at t=0 and at intervals of 30 min until t=300 min. To each aliquot was added 5 M HCl (34 µl) to quench the reaction. A proton NMR spectrum was obtained for each aliquot. The aliquots were filtered (to remove precipitated KCl), and each diluted to 2 ml and the optical rotation measured for each sample. The samples were combined and concentrated *in vacuo*.

N-Acetyl-(2RS)-serine (152)

(2S)-Serine (2 g, 19 mmol) was dissolved in water (20 ml) and pyridine (1.56 ml, 19 mmol). Acetic anhydride (6 ml) was added to the solution dropwise. The resulting solution was stirred at room temperature overnight. The solution was poured onto an Amberlite IR-120(H) column. The column was eluted with water and fractions (25 ml) collected until an alkaline reaction to Congo Red indicator was obtained. The acidic fractions were pooled and concentrated to dryness *in vacuo* yielding an oil (2.1 g, 90%). ν_{max} (neat) 3348 (br, NH and OH str), 2977 (CH str) 1732 (acetyl C=O) and 1652 cm^{-1} (CO_2^-); δ_H (200 MHz, D_2O/NaOD) 1.9 (3H, s, acetyl C$\underline{H}_3$), 3.75 (2H, m, 3 C$\underline{H}_2$) and 4.35 (1H, m, 2 C$\underline{H}$); δ_C (50 MHz, D_2O/NaOD/MeOH) 22.7 (acetyl $\underline{C}H_3$), 55.84 (3 $\underline{C}H_2$), 62.07 (2 $\underline{C}$H) and 174.52, 175.39 (acid and acetyl $\underline{C}O_2H$); *m/z* (CI) 148 ([*M*+H]$^+$, 100%), 130 (50, [*M*-OH]$^+$), 102 (20, [*M*-CO_2H]$^+$) and 60 (20, [*M*-CO_2H-CH_3CO+H]$^+$). The N-acetyl-(2RS)-serine was taken on to the next step without further purification.

(2RS)-Serine (153)

N-Acetyl-(2RS)-serine (**152**) (2 g, 13.6 mmol) was dissolved in 2 M HCl (80 ml) and refluxed under nitrogen for 2 h. The solution was cooled, concentrated to dryness *in vacuo*, and dried over phosphorus pentoxide. The serine hydrochloride salt was dissolved in dry ethanol (20 ml) and propylene oxide (20 ml). The mixture was refluxed for 15 min. The solid was filtered, and recrystallised from water and ethanol to yield a white crystalline solid (0.86 g, 60%), m.p. 235-237 °C (dec); $[\alpha]_D$ +0.15° (c. 2 in 1 M HCl); ν_{max} (nujol) 3200-2400 (NH_2, OH and CH str) and 1661 cm^{-1} (amino acid CO_2H); δ_H (200 MHz, D_2O/NaOD) 3.49 (1H, t, *J* 4.6 Hz, 2 C$\underline{H}$) and 3.72 (2H, m, 3 C$\underline{H}_2$); δ_C (50 MHz, D_2O/NaOD/dioxane) 57.09 (2 $\underline{C}$H), 60.89 (3 $\underline{C}H_2$) and 173.05 ($\underline{C}O_2H$); *m/z* (CI) 106 ([*M*+H]$^+$, 100%) and 60 (35, [*M*-CO_2H]$^+$).

(2RS)-[U-^{14}C]-Serine (154)

(2S)-[U-^{14}C]-Serine (5 μCi) in aqueous ethanol was added to (2S)-serine (200 mg, 1.89 mmol) and lyophilised. The racemate (**154**) was prepared in an identical manner to (2RS)-serine (**153**) in 77.5% overall yield. $[\alpha]_D$ +3.0° (36 % (2R)-, 64 % (2S)-[U-^{14}C]-serine).

(2RS)-[U-^{14}C]-Serine O-sulphate (155)

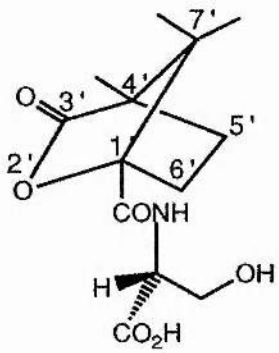
(2RS)-[U-^{14}C]-Serine (**154**) (0.1551 g, 1.47 mmol) was converted into (2RS)-[U-^{14}C]-serine O-sulphate (**155**) using the method of Tudball[192] in 18% yield.

(2R)-Serine (157)

N-Acetyl-(2RS)-serine (**152**) (1 g, 6.75 mmol) was dissolved in water (60 ml). The pH was adjusted to 7.2 with aq. NH_3. Pig liver acylase I (8 mg) was added and the mixture incubated at 37 °C for 24 h. The solution was poured onto an Amberlite IR-120(H) column and eluted with water until an alkaline reaction to Congo Red indicator was obtained. The acidic fractions containing N-acetyl-(2R)-serine (**156**) were pooled and concentrated to dryness *in vacuo*. The column was further eluted with 2 M HCl until a negative reaction to ninhydrin was obtained. The (2S)-serine containing fractions were pooled and concentrated. N-Acetyl-(2R)-serine (**156**) (190 mg, 1.28 mmol) was dissolved in 2 M HCl (10 ml) and refluxed under nitrogen for 2 h. The hot solution was decolourised with charcoal, concentrated to dryness *in vacuo* and dried over phosphorus pentoxide. The oil was dissolved in dry ethanol (10 ml) and propylene oxide (10 ml). The mixture was refluxed for 15 min, and the solid filtered off and dried (40 mg, 11%), m.p. 237-239 °C (dec); $[\alpha]_D$ + 1.75° (c. 0.8 in 1 M HCl) {$[\alpha]_D$ +7.87° (c. 0.8 in 1 M HCl) for authentic (2R)-serine}; δ_H (200 MHz, D_2O/NaOD) 3.75 (1H, br s, 2 C$\underline{H}$) and 3.9 (2H, br s, 3 C$\underline{H_2}$); δ_C (50 MHz, D_2O/NaOD/dioxane) 57.08 (2 $\underline{C}$H), 60.8 (3 $\underline{C}H_2$)

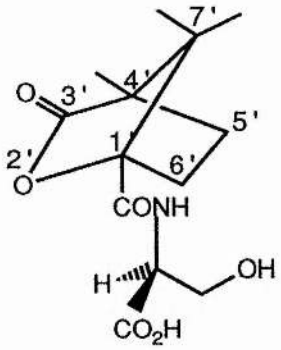
and 173.05 ($\underline{C}O_2H$); *m/z* (CI) 106 ([*M*+H]$^+$, 100%) and 60 (35, [M-CO_2H]$^+$).

(N)-(-)-Camphanoyl-2-amino-(2R)-3-hydroxypropanoic acid (158)



This was prepared in an identical manner to (N)-(-)-camphanoyl-2-amino-(2S)-3-hydroxypropanoic acid (**159**) using (2R)-serine in 63% yield. M.p. 133-135 °C; $[\alpha]_D$ -32.6° (c. 0.73 in 0.1 M NaOH); ν_{max} (nujol) 3408 (NH stretch), 3295 (broad, OH stretch), 1790 (lactone C=O), 1755 (acid C=O) and 1650 cm^{-1} (amide C=O or NH bend); δ_H (300 MHz, D_2O) 0.98 (3H, s, 4" C$\underline{H}_3$), 1.08 and 1.13 (6H, 2s, 2 x 7" C$\underline{H}_3$), 1.72-2.5 (4H, 3m, 5' C$\underline{H}_2$ and 6' C$\underline{H}_2$), 4.0 (2H, d, *J* 8.5 Hz, 3 C$\underline{H}_2$) and 4.62 (1H, t, *J* 8.5 Hz, 2 C$\underline{H}$); δ_H (300 MHz, C_5D_5N) 1.05, 1.15, 1.24 (9H, 3s, 4" C$\underline{H}_3$ and 2 x 7" C$\underline{H}_3$), 1.5-2.75 (4H, 3m, 5' C$\underline{H}_2$ and 6' C$\underline{H}_2$), 4.41 (1H, dd, J_{AB} 11 Hz, J_{AX} 3.6 Hz, 3 C$\underline{H}_a$), 4.62 (1H, dd, J_{AB} 11 Hz, J_{BX} 4.1 Hz, 3 C$\underline{H}_b$) and 5.28 (1H, m, 2 C$\underline{H}$); δ_C (100 MHz, C_5D_5N) 9.83 (4" $\underline{C}H_3$), 16.75 (2 x 7" $\underline{C}H_3$), 29.12, 30.61 (5' and 6' $\underline{C}H_2$), 54.17, 55.45, 55.78 (2 $\underline{C}$H, 4' C, 7' C), 62.46 (3 $\underline{C}H_2$), 92.82 (1' C), 167.45, 173.38 and 178.25 (ester, acid and amide $\underline{C}$=O); *m/z* (CI) 286 ([*M*+H]$^+$,100%).

(N)-(-)-Camphanoyl-2-amino-(2S)-3-hydroxypropanoic acid (159)



(2S)-Serine (200 mg, 1.97 mmol) was dissolved in water (5 ml) and potassium carbonate (530 mg, 3.94 mmol) was added. The pH of the solution was checked and adjusted to >9 (if necessary). The basic solution was added to a suspension of camphanoyl chloride (300 mg, 1.38 mmol) in toluene (5 ml). The mixture was stirred vigorously overnight. The aqueous phase was isolated, washed with chloroform (2 x 20 ml), acidified to pH 2 with 6 M HCl, extracted with chloroform (3 x 10 ml). The chloroform extracts were combined and dried with sodium sulphate. The solvent was removed *in vacuo*, yielding a white solid (190 mg, 48%), m.p. 179-181 °C; $[\alpha]_D$ -28.1° (c. 0.75 in 1 M NaOH); ν_{max} (nujol) 3400 (N-H stretch), 3250 (broad, OH stretch), 1775 (lactone C=O), 1755 (acid C=O) and 1635 cm^{-1} (amide C=O or N-H bend); δ_H (300 MHz, D_2O) 0.92 (3H, s, 4" C$\underline{H}_3$), 1.05 and 1.12 (6H, 2s, 2 x 7" C$\underline{H}_3$), 1.7, 2.08, 2.5 (4H, 3m, 5' C$\underline{H}_2$ and 6' C$\underline{H}_2$), 3.9 (2H, m, 3 C$\underline{H}_2$) and 4.55 (1H, m, 2 C$\underline{H}_2$); δ_H (300 MHz, C_5D_5N) 1.07, 1.09, 1.15 (9H, 3s, 4" C$\underline{H}_3$ and 2 x 7" C$\underline{H}_3$), 1.5-2.7 (4H, 3m, 5' C$\underline{H}_2$ and 6' C$\underline{H}_2$), 4.44 (1H, dd, J_{AB} 10.9 Hz, J_{AX} 3.5 Hz, 3 C$\underline{H}_a$), 4.61 (1H, dd, J_{AB} 10.9 Hz, J_{BX} 4.1 Hz, 3 C$\underline{H}_b$) and 5.26 (1H, broad s, 2 C$\underline{H}$); δ_C (100 MHz, C_5D_5N) 9.81 (4" $\underline{C}H_3$), 16.62, 16.75 (2 x 7" $\underline{C}H_3$), 29.09, 30.61 (5' and 6' $\underline{C}H_2$), 53.86, 55.32, 55.85 (2 $\underline{C}$H, 4' C, 7' C), 62.68 (3 $\underline{C}H_2$), 92.77 (1' C), 167.28, 173.39 and 178.15 (ester, acid and amide $\underline{C}$=O); *m/z* (CI) 286 ([*M*+H]$^+$, 100%).

(1'S,4'R)-Camphanamide of (2R)-serine methyl ester (160)

This was prepared in an identical manner to (1'S,4'R)-camphanamide of (2S)-serine methyl ester (**161**) from (1'S,4'R)-camphanamide of (2R)-serine (**158**) in 70% yield. δ_H (400 MHz, C_5D_5N) 1.06, 1.12, 1.15 (9H, 3s, 4" and 2 x 7" C$\underline{H}_3$), 1.51-1.6, 1.81-1.88, 2.59-2.68 (4H, 3m, 5' and 6' C$\underline{H}_2$), 3.62 (3H, s, $CO_2C\underline{H}_3$), 4.23-4.26 (1H, ABX, J_{AB} 11.16 Hz, J_{AX} 4.44 Hz, 3 C$\underline{H}_a$), 4.39-4.43 (1H, J_{AB} 11.16 Hz, J_{BX} 3.92 Hz, 3 C$\underline{H}_b$), 5.12-5.16 (1H, ABX, J_{AX} 4.44 Hz, J_{BX} 3.92 Hz, 2 C$\underline{H}$), 8.46 (1H, br d, J 8.12 Hz, N$\underline{H}$); δ_C (100 MHz, C_5D_5N) 9.89 (4" $\underline{C}H_3$), 16.68, 16.79 (2 x 7" $\underline{C}H_3$), 29.16, 30.66 (5' and 6' $\underline{C}H_2$), 52.16 ($CO_2\underline{C}H_3$), 54.29, 55.44 (4' and 7' C), 55.49 (2 $\underline{C}$H), 61.99 (3 $\underline{C}H_2$), 92.72 (1' C), 167.76, 171.45, 178.24 (3 $\underline{C}$=O carbons).

(1'S,4'R)-Camphanamide of (2S)-serine methyl ester (161)

(1'S,4'R)-Camphanamide of (2S)-serine (**159**) (100 mg, 0.35 mmol) was dissolved in water (5 ml) and dichloromethane (5 ml). The solution was cooled to 0 °C in an ice-salt bath. Diazomethane was added dropwise until a slight yellow colour remained. The solution was stirred for a further hour at room temperature. A stream of nitrogen was bubbled through the solution to remove the excess diazomethane. The solution was concentrated to dryness *in vacuo* yielding a white solid (70 mg, 70%). δ_H (400 MHz, C_5D_5N) 1.00, 1.05, 1.11 (9H, 3s, 4" and 2 x 7" C$\underline{H}_3$), 1.5-1.6, 1.78-2.0, 2.58-2.65 (4H, 4m, 5' and 6' C$\underline{H}_2$), 3.61 (3H, s, $CO_2C\underline{H}_3$), 4.29-4.32 (1H, ABX, J_{AB} 11.12 Hz, J_{AX} 4.6 Hz, 3 C$\underline{H}_a$), 4.41-4.45 (1H, ABX, J_{AB} 11.12 Hz, J_{BX} 4.02 Hz, 3 C$\underline{H}_b$), 5.16-5.21 (1H, ABX, J_{AX} 4.6 Hz, J_{BX} 4.02 Hz, 2 C$\underline{H}$), 8.57 (1H, br d, J 8.12 Hz, N$\underline{H}$); δ_C (100 MHz, C_5D_5N) 9.86 (4" $\underline{C}H_3$), 16.63, 16.79 (2 x 7" $\underline{C}H_3$), 29.14, 30.71 (5' and 6' $\underline{C}H_2$), 52.19 ($CO_2\underline{C}H_3$), 53.99, 55.38 (4' and 7' C), 55.52 (2 $\underline{C}$H), 62.29 (3 $\underline{C}H_2$), 92.67 (1' C), 167.67, 171.39, 178.17 (3 $\underline{C}$=O carbons).

Chirality assay for the (2R)-serine isolated from the acylase resolution (162)

(2R)-Serine (**157**) (200 mg, 1.89 mmol) from the acylase resolution procedure was converted into the (1'S,4'R)-camphanamide methyl ester in an identical manner to that used to prepare the (1'S,4'R)-camphanamide methyl ester of authentic (2R)-serine (**158**). Proton and carbon NMR spectra were obtained and compared with those for the pure materials. Comparison of the region 4.2-4.5 ppm in the proton spectrum showed that the sample had been derived from a mixture of 57.4 % (2R)- and 42.6 % (2S)-serine.

(1'S,4'R)-(-)-N-Camphanoyl glycine (183)

Glycine (225 mg, 3 mmol) was dissolved in 1.0 M NaOH (3 ml) and the solution was shaken with freshly produced (1S,4R)-camphanoyl chloride (500 mg, 2.3 mmol) in toluene (2 ml) for 15 min. The resulting mixture was stirred vigorously for a further 2 h, diluted with water (5 ml) and dichloromethane (5 ml). The organic phase was separated and discarded, and the aqueous phase was adjusted to pH 2 using 5 M HCl. The solution was extracted with dichloromethane (3 x 10 ml), the combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to yield a thick oil. Addition of a few drops of water to the thick oil caused crystallisation. The solid was recrystallised from diethyl ether/petrol to give white crystals (357 mg, 70%), m.p. 69-70 °C (lit.,[276] 73.5-74.5 °C); (Found: C, 49.71; H, 7.23; N, 4.75. Calc. for $C_{12}H_{17}NO_5.2H_2O$: C, 49.48; H, 7.27; N, 4.81%); $[\alpha]_D$ -20.9° (c. 1.55 in methanol) [lit.,[276] $[\alpha]_D^{20}$ -19.7° (c. 1.55 in methanol)]; ν_{max} (nujol) 3520 (OH), 3340 (NH), 2800-3000 (CH str), 1775 (lactone C=O), 1705 (acid C=O), 1665 and 1550 cm^{-1} (amide C=O and NH); δ_H (200 MHz, $CDCl_3$) 0.95 (3H, s, 4" C$\underline{H}_3$), 1.10 and 1.15 (6H, 2s, 2 x 7" C$\underline{H}_3$), 1.60-2.68 (4H, m, 5' and 6' C$\underline{H}_2$), 4.15 (2H, ABX, J_{AB} 16.5 Hz, J_{AX}=J_{BX} 6.5 Hz, 2 C$\underline{H}_2$), 7.3 (>1H,t br,

NH) and 8.45 (1H, s br, OH); δ_C (50 MHz, $CDCl_3$) 9.62 (4" $\underline{C}H_3$), 16.42 and 16.62 (2 x 7" $\underline{C}H_3$), 28.94 and 30.14 (5' and 6' $\underline{C}H_2$), 40.67 (2 $\underline{C}H_2$), 54.27 (7' C), 55.36 (4' C), 92.47 (1' C), 167.81, 172.75 and 178.67 (3 x $\underline{C}$=O carbons); *m/z* (EI) 255 (M^+, 100%) and 209 ([M-CO_2H-H]$^+$, 20).

(1'S,4'R)-(-)-N-Camphanoyl dideuterio glycine (184)

This was prepared in an identical manner to (1S,4R)-(-)-N-camphanoyl glycine (**183**) on a small scale and the following data was obtained for the crude product. δ_H (400 MHz, $CDCl_3$) 0.9-1.15 ($C\underline{H}_3$ camphanic acid and camphanamide), 1.65-2.5 ($C\underline{H}_2$ camphanic acid and camphanamide) and 7.0 (N$\underline{H}$); δ_D (60 MHz, $CDCl_3$) 4.15 and 4.00 (CD_2 of dideuteriated glycine camphanamide); *m/z* (EI) 211 ([M-CO_2H-H]$^+$, 100%).

Deuteriated aminomalonate monoammonium salt (186)

Aminomalonate monoammonium salt (60 mg, 29.4 mmol) was dissolved in deuterium oxide (1 ml) in an NMR tube, dioxane was added as an internal standard. After 19.5 h at 37 °C the product was 89% deuteriated as judged by proton NMR spectroscopy.

Studies with Glutamate Decarboxylase (GAD)

Glutamate Decarboxylase Activity Assay (standard radioassay technique)

The enzyme activity was determined at pH 4.6 in 0.1 M pyridine/HCl buffer at pH 4.6. Each vial contained 200 µl of solution, 88% of 18 mM (2S)-glutamic acid, 10% (2S)-[1-^{14}C]-glutamic acid (5 µCi in 2.3 ml) and 2% 50 mM PLP. The enzymic reaction was initiated by the addition of a 10 µl aliquot of a buffered GAD solution (2 units/ml) to each of seven assay vials at t=0. At t=0, 5, 10, 15, 20, 30, 40 min respectively the incubations were quenched by the addition of 6 M H_2SO_4 (200 µl). After allowing for the complete liberation of carbon dioxide (about 30 min), scintillation fluid (3 ml) was added to each vial. Residual ^{14}C label was counted using a scintillation counter and the results represented graphically. Each assay was carried out in duplicate.

Inactivation of GAD by (2S)-serine O-sulphate

(2S)-Serine O-sulphate solutions (3.7, 10 mM) in 0.1 M pyridine/HCl buffer at pH 4.6 (1.2 ml) containing 0.2 mg GAD were prepared. A reference solution containing 0.2 mg enzyme in buffer (1.2 ml) was also prepared. The solutions were assayed in duplicate for enzyme activity using the standard radioassay technique, at t=30 min, 4.5 h, 24 h, 72 h.

Inactivation of GAD by (2S,3R)-threonine O-sulphate

(2S,3R)-Threonine O-sulphate solutions (3.7, 10 mM) in 0.1 M pyridine/HCl buffer at pH 4.6 (1.2 ml) containing 0.2 mg GAD were prepared. A reference solution containing 0.2 mg enzyme in buffer (1.2 ml) was also prepared. The solutions were assayed in duplicate for enzyme activity using the standard radioassay technique, at t=30 min, 4.5 h, 24 h, 72 h.

Inactivation of GAD by (2S,3S)-threonine O-sulphate

(2S,3S)-Threonine O-sulphate solutions (10, 25, 50 mM) in pyridine/HCl buffer at pH 4.6 (1.2 ml) containing GAD (0.2 mg) were prepared. A reference solution was also prepared containing enzyme (0.2 mg) in buffer solution (1.2 ml). These solutions were assayed for enzyme activity at t=0, 5, 24 h.

Inactivation of GAD by (2R)-serine O-sulphate

(2R)-Serine O-sulphate solutions (10, 25, 50 mM) in a 0.1 M pyridine/HCl buffer (1.2 ml) at pH 4.6 containing GAD (0.2 mg) were prepared. A reference solution was also prepared containing enzyme (0.2 mg) in buffer (1.2 ml) was also prepared. The solutions were assayed for enzyme activity using the standard method at t=0, 5, 24.5 and 29 h.

Inactivation of GAD by (2S)-[U-^{14}C]-serine O-sulphate

This reaction was carried out in a Warburg apparatus. (2S)-[U-^{14}C]-Serine O-sulphate solution (5.3 mM) in 0.1 M potassium acetate buffer solution at pH 4.6 was prepared. An aliquot (400 µl, 0.1 µCi) was added to the enzyme (1.2 mg, 24 nmol) in one of the side arms of the Warburg apparatus. The main chamber of the apparatus contained barium hydroxide solution (25 ml) and there was a tube from the neck at the top to another vessel containing barium hydroxide solution (25 ml). The remaining opening to the vessel was sealed with a stopper. After a 26 h incubation time several aliquots of the enzyme solution, the internal barium hydroxide solution and the external barium hydroxide solution were removed and counted mechanically for the presence of ^{14}C.

Kinetic studies of GAD inactivation by (2S)-serine O-sulphates

(2S)-Serine O-sulphate and (2S)-[2-^{2}H]-serine O-sulphate (1, 2, 3.5, 5 mM) solutions in pyridine/HCl buffer (pH 4.6, 0.6 ml) containing GAD (0.1 mg) were prepared. A reference solution was also prepared containing enzyme (0.1 mg) in buffer solution (0.6 ml). These solutions were incubated at 37 °C and assayed for enzyme activity using the method given below at t=15 s, 45 min 15 s, 2 h 15 s, 4 h 15 s, 7 h 15 s after the addition of the enzyme.

Kinetic studies of GAD inactivation by (2R)-serine O-sulphates

(2R)-Serine O-sulphate and (2R)-[2-^{2}H]-serine O-sulphate (2, 3.5, 5, 7 mM) solutions of in pyridine/HCl buffer (pH 4.6, 0.6 ml) containing GAD (0.1 mg) were prepared. A reference solution was also prepared containing enzyme (0.1 mg) in buffer solution (0.6 ml). These solutions were incubated at 37 °C and assayed for enzyme activity using the method given below at t=15 s, 45 min 15 s, 2 h 15 s, 4 h 15 s, 7 h 15 s after the addition of the enzyme.

Modified Assay for for kinetic studies of GAD activity

The assay was carried out by adding a portion of the preincubation solution (60 µl) to the assay mixture (this dilution quenches the inactivation reaction). The assay mixture has an initial volume of 1.5 ml and contains 88% 18 mM (2S)-glutamic acid (in 0.1 M pyridine/HCl buffer solution, pH 4.6), 10% (2S)-[1-^{14}C]-glutamic acid (5 µCi in 2.3 ml), and 2% buffer solution. At t=5, 10, 15, 20, 25 min after the addition of the preincubation solution, aliquots (200 µl) are removed and added to 6 M H_2SO_4 (200 µl), which stops the conversion of (2S)-glutamic acid to GABA. After 30 min, scintillation fluid is added to each vial and the sample is counted for the amount of residual radioactivity.

Incubation of (2RS)-[U-^{14}C]-serine O-sulphate with GAD

(2RS)-[U-^{14}C]-Serine O-sulphate solution (10 mM) in 0.1 M pyridine/HCl buffer at pH 4.6 was prepared. An aliquot (400 µl) of this solution was added to GAD (1.1 mg) in the side arm of a Warburg apparatus. The main body of the Warburg apparatus contained barium hydroxide solution (10 ml). The side arm was added to the main body and incubated at 37 °C for 24 h. Aliquots of the inactivated enzyme solution, and the barium hydroxide solution were removed and checked for the amount of radioactivity present compared to the initial amount in the (2RS)-[U-^{14}C]-serine O-sulphate solution and the background level of radioactivity in the barium hydroxide solution. It was found that the amount of radioactivity in the inactivated solution was less than in the starting solution, but a corresponding increase in the barium hydroxide solution was not discovered.

NMR experiments with GAD

(2R)-Serine O-sulphate incubation with GAD

(2R)-Serine O-sulphate (50 mg, 0.226 mmol) was added to 0.1 M pyridine/DCl buffer (pD 4.2, 1 ml), GAD was added (2.54 mg, ≈50 units) and the solution incubated at 37 °C. Proton and carbon NMR spectra were obtained at t=0, 17, 32.5, 113 h and compared. It was found that no exchange or formation of new products had occurred.

(2S)-Serine O-sulphate incubation with GAD

(2S)-Serine O-sulphate (5 mM in 0.1 M pyridine/DCl buffer, pD 4.2) was added to GAD (0.1 mg, approx 4 units) and the solution incubated at 37 °C. Proton NMR spectra were obtained at t= 0, 60, 120, 180, 240, 300, 510, 1795, 4410 min and compared. It was found that no exchange or rearrangement of the carbon skeleton had occurred.

(2R)-Aspartic acid incubation with GAD

(2R)-Aspartic acid (50 mg, 0.37 mmol) was dissolved in pyridine/DCl buffer (pD 4.25, 0.1 M, 4 ml). An aliquot (0.7 ml) was put into an NMR tube and GAD was added (50 units). The proton and carbon NMR spectra were obtained at t=0 min. The solution was incubated at 37 °C for one month and a proton NMR spectrum was obtained at weekly intervals. No exchange had taken place.

(2R)-Glutamic acid incubation with GAD

(2R)-Glutamic acid (50 mg, 0.34 mmol) was dissolved in pyridine/DCl buffer (pD 4.39, 0.1 M, 4 ml). An aliquot (0.7 ml) was put into an NMR tube and GAD was added (50 units). The proton and carbon NMR spectra were obtained at t=0 min. The solution was incubated at 37 °C for one month and a proton NMR spectrum was obtained at weekly intervals. No exchange had taken place.

Incubation of (2S)-homocysteic acid with GAD

GAD (2.5 mg) was dissolved in 0.1 M pyridine/HCl buffer pH 4.6. To this was added 54 mM (2S)-homocysteic acid (100 µl). The solution was incubated at 37 °C overnight. The solution was concentrated to dryness *in vacuo*, and proton and carbon NMR spectra obtained. δ_H (200 MHz, D_2O/pyridine buffer) 2.0 (2H, m, 2 C$\underline{H}_2$), 2.8-3.2 (4H, m, 1 and 3 C$\underline{H}_2$'s); δ_C (100 MHz, D_2O/pyridine buffer/dioxane) 23.23 (2 $\underline{C}H_2$), 39.07, 48.71 (1 and 3 $\underline{C}H_2$'s).

Incubation of (2R)-homocysteic acid with GAD

The incubation was carried out in an identical manner to that for (2S)-homocysteic acid. Inspection of the proton NMR spectrum showed that no reaction had occurred.

Incubation of (2S)-homocysteic acid and (2S)-glutamic acid with GAD

A solution containing (2S)-glutamic acid (34 mM) and (2S)-homocysteic acid (34 mM) was prepared in 0.1 M pyridine/DCl buffer, pL 4.6. GAD (0.2 mg) was added to an aliquot of the above solution (700 µl) and the solution shaken. The solution was incubated at 37 °C for several days. The reaction progress was followed by proton NMR spectroscopy.

Ultra-violet/visible studies of the GAD reactions

Inactivation reaction of GAD with (2R)-serine O-sulphate

GAD (5 mg) and (2R)-serine O-sulphate (19.6 mg, 0.88 mmol) were added to 0.1 M pyridine/HCl buffer, pH 4.6. The mixture was incubated at 37 °C for 7 h. Solutions containing NADH (150 mM) and either alcohol dehydrogenase (0.1-0.2 mg) or lactate dehydrogenase (0.1-0.2 mg) in 100 mM potassium phosphate buffer at pH 7.0 were prepared and equilibrated to 37 °C prior to the experiment. Aliquots (10 µl) of the GAD solution were added to the alcohol dehydrogenase solution and the lactate dehydrogenase solution in duplicate. Any change in absorption at 340 nm was noted.

Studies with serine hydroxymethyl transferase (SHMT)

Preparation of SHMT

E. coli and cytosolic SHMT were obtained from Verne Schirch (Virginia Commonwealth University) in the form of the enzyme-glycine-5-formaldehyde tetrahydrofolic acid ternary complex, this needed regeneration to regain enzyme activity. Half (about 25 mg enzyme) of each of the powders were dissolved in 20 mM potassium phosphate buffer (1.5 ml) at pH 7.0, containing 5 mM mercaptoethanol and 20 mM (2S)-serine and dialysed overnight against 1 litre of the same buffer at 4 °C, the enzyme solutions were dialysed for a further 8 h at 4 °C against 1 litre 20 mM potassium phosphate at pH 7.0 containing 5 mM mercaptoethanol. The enzyme solutions were diluted to 2 ml with H_2O, half was frozen (12.5 mg enzyme) and the remainder was lyophilised and then frozen.

SHMT assay

(2S,3S)-Threonine aldolase activity[246]

SHMT (10 µl) was added to a 1 ml cuvette containing (2S,3S)-threonine (1.33 mg, 10 mM), dithiothreitol (0.15 mg,1 mM), PLP (2.65 µg, 10 µM), NADH (0.21 mg, 0.3 mM) and yeast alcohol dehydrogenase (35 units) which had been equilibrated at 30 °C. The amount of enzyme activity was determined by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH (λ_{max} 340 nm, ε 6220 $dm^3\ mol^{-1}\ cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme required to convert one micromole of (2S,3S)-threonine to acetaldehyde and glycine per minute.

uv assay

The provided mixture of glycine and 5-formaldehyde-tetrahydrofolic acid was dissolved in 20 mM potassium phosphate buffer pH 7.0 (1 ml) containing 5 mM mercaptoethanol.

A portion of this solution (20 µl) was added to a 0.5 mg/ml solution of the enzyme. A uv spectrum was then obtained.

SHMT Incubations

Cytosolic SHMT

1. In deuterium oxide (**185**).

The freeze dried solid from the cytosolic SHMT enzyme preparation (approx 12.5 mg enzyme) was dissolved in D_2O (1 ml), a small portion was removed and its activity was determined to be approx 2 unit/mg. The remaining enzyme solution was diluted with D_2O to 5 ml, and the pH of this solution was checked and found to be 7.19. 2-Aminomalonate (6 mg, 44.1 µmol) was added in two equal aliquots at t=0 and t=5 min and the solution incubated at 37 °C for 15 min. The protein was denatured by the addition of ethanol (5 ml), and removed by centrifugation. The solution was concentrated to dryness *in vacuo* and rewashed with water/ethanol until no further precipitation occurred. The final solution was concentrated once more to yield a solid (6.96 mg) containing glycine and no aminomalonate as judged by tlc and proton NMR spectroscopy. The solid was dissolved in water and purified by preparative tlc (^{i}PrOH:NH_3:H_2O 26:6:5), 15 mg of solid was obtained but there was some contamination with cellulose. It was assumed that this sample contained glycine (6 mg, 0.0789 mmol). The glycine/cellulose mixture was suspended in 2 M sodium hydroxide solution and residual cellulose removed. Freshly prepared camphanoyl chloride (25 mg, 0.118 mmol) was dissolved in toluene. The two solutions were mixed and shaken for 15 min and stirred vigorously for 2 h. The organic layer was separated and discarded. The pH of the aqueous layer was adjusted to 2 with 5 M HCl, and extracted with dichloromethane (3 x 10 ml), these washings were combined, dried ($MgSO_4$), and concentrated to dryness *in vacuo*. δ_H (400 MHz, $CDCl_3$) 0.9-1.15 ($C\underline{H}_3$ camphanic acid

and camphanamide), 1.65-2.5 (C<u>H</u>$_2$ camphanic acid and camphanamide), 4.03 (br d, C<u>H</u> of monodeuteriated glycine camphanamide) and 7.0 (N<u>H</u>); δ_D (60 MHz, $CDCl_3$) 4.2 (CD of monodeuteriated glycine camphanamide); *m/z* (EI) 256 (M^+, 25%) and 210 (100, [*M*-CO_2H-H]$^+$).

2. In protium oxide (**187**).

The frozen aqueous solution from the c. SHMT enzyme preparation (approx 12.5 mg enzyme) was defrosted and a small portion was removed and its activity determined to be approx 2 unit/mg. The remaining enzyme solution was diluted with H_2O (5 ml). The pH was determined and adjusted to 7.0. Freshly prepared deuteriated aminomalonate was added in two aliquots of 50 µl, the first at t=0, and the second at t=5 min. The solution was incubated at 37 °C for 15 min, the protein was denatured, the product purified and converted into the camphanamide derivative (16 mg solid isolated). δ_H (400 MHz, $CDCl_3$) 0.9-1.15 (C<u>H</u>$_3$ camphanic acid and camphanamide), 1.65-2.6 (C<u>H</u>$_2$ camphanic acid and camphanamide), 4.24 (br d, C<u>H</u> of monodeuteriated glycine camphanamide) and 7.0 (N<u>H</u>); δ_D (60 MHz, $CDCl_3$) 3.99 (CD of monodeuteriated glycine camphanamide); *m/z* (EI) 257 (10%, [*M*+H]$^+$), 239 ([*M*-OH]$^+$, 30) and 210 ([*M*-CO_2H]$^+$, 60).

APPENDIX

Publications

1. Decarboxylation of 2-Aminomalonic Acid Catalysed by Serine Hydroxymethyltransferase is, in fact, a Stereospecific Process. N.R. Thomas, J.E. Rose, D. Gani, *J. Chem. Soc, Chem. Commun.*, 1991, 908.

2. Regiospecific Deuteriation of Chiral 3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazines in the Stereospecific Synthesis of α-Deuteriated α- Amino Acids. J.E. Rose, P.D. Leeson, D. Gani, *J. Chem. Soc., Perkin Trans.1*, 1992, 1563.

3. Mechanisms and Stereochemistry of the Activation of (2S)- and (2R)-Serine O-Sulphate as Suicide Inhibitors for *E. coli* Glutamic Acid Decarboxylase. J.E. Rose, P.D. Leeson, D. Gani, accepted for publication.

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