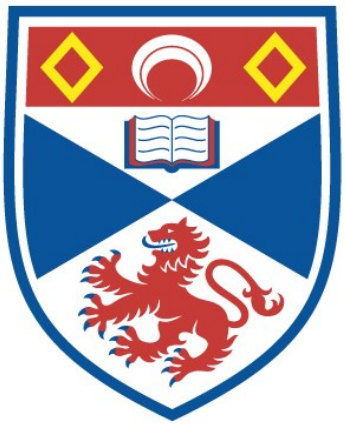
# MECHANISTIC STUDIES ON THE THREONINE SYNTHASE

Fiona Barclay

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



1995

# MECHANISTIC STUDIES ON THREONINE SYNTHASE.

a thesis presented by

Fiona Barclay

to the

UNIVERSITY OF ST. ANDREWS

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

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# DECLARATION

I, Fiona Barclay, 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 fulfiment of any other degree or professional qualification.

Signed Date 16/11/94

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 1st Oct 1991 and as a candidate for the degree of Ph.D. on 1st Oct 1991

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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 17/11/94

## Copyright

**Acknowledgements.**

I would like to thank my supervisor Professor David Gani for his enthusiasm and help. I would also like to thank him for his excellent choice in conference, and for being such good company on our trip to Capri. Thanks also to the SERC and Zeneca Agrochemicals for their financial help, and to Dr. Ewan Chrystal for his supervision and hospitality.

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Finally and most importantly I would like to thank my family for being so understanding and supportive. It has not been an easy time for any of us, and their support has been far above anything I deserve.

TO MY PARENTS

AND BROTHER

## ABSTRACT

Threonine synthase catalyses the conversion of (2*S*)-*O*-phosphohomoserine to (2*S*,3*R*)-threonine with the elimination of phosphate. A novel and efficient synthesis of (2*S*)-*O*-phosphohomoserine has been developed starting from (2*S*)-aspartic acid. Using this methodology the singly labelled isotopomers (2*S*,3*S*)-[3-$^{2}H_{1}$]-, (2*S*,3*R*)-[3-$^{2}H_{1}$]-, and (2*S*)-[2-$^{2}H_{1}$]-*O*-phosphohomoserine have also been synthesised from labelled (2*S*)-aspartic acid and used to probe the mechanism of the enzyme. Measurement of the kinetic deuterium isotope effects for the labelled substrates showed that both the $C^{\alpha}$-H and $C^{\beta}$-H proton removal steps in *E. coli* threonine synthase display primary deuterium isotope effects, and that cleavage of the C-3-(*pro*-S)-H bond is at least partially rate limiting. This confirms data already available for the yeast enzyme, which indicated that the 3-*pro*-S proton is removed in the course of the reaction. The kinetic isotope effect for the removal of the 3-*pro*-S proton indicates a high forward commitment for the elimination of phosphate.

Threonine synthase from *E. coli* was also shown to be activated in the presence of *S*-adenosyl methionine. This activation has been documented for the plant enzyme, but this is the first time such an activation has been seen for the bacterial enzyme.

Threonine synthase for the study was partially purified from *E. coli* K12 Tir8, using novel methodology.

## Contents

## List of Figures

| Abbreviation | Meaning |
|---|---|
| AAT | aspartate aminotransferase |
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| BOC | *t*-butoxycarbonyl |
| BSA | bovine serum albumin |
| CBZ | benzyloxycarbonyl |
| CNS | central nervous system |
| DDC | DOPA decarboxylase |
| DMAP | 4-dimethylaminopyridine |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethylsulfoxide |
| DOPA | 3,4-dihydroxyphenylalanine |
| DTT | dithiothreitol |
| EC | Enzyme Catalogue |
| EDTA | ethylenediaminetetraacetic acid |
| *E. coli* | Escherichia coli |
| GABA | γ-aminobutyric acid |
| GABA-T | γ-aminobutyric acid transaminase |
| HDC | histidine decarboxylase |
| HEPES | (*N*-2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid |
| Hom | (2*S*) -homoserine |
| $k_{app}$ | apparent first order rate constant |
| $K_M$ | Michaelis constant |
| $k_{cat}$ | enzymic catalytic constant/turnover number |
| $K_H/K_D$ | deuterium isotope effect for a dissociation or association constant |
| $K_i$ | enzymic inhibition constant |

| | |
|---|---|
| *m*-CPBA | *m*-chloroperbenzoic acid |
| $M_r$ | relative molecular mass |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NADP+ | nicotinamide adenine dinucleotide phosphate (oxidised form) |
| NMDA | *N*-methyl-D-aspartate |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| ODC | ornithine decarboxylase |
| Pi | inorganic phosphate |
| PHS | (2*S*)-*O*-phosphohomoserine |
| PLP | pyridoxal 5'-phosphate |
| PMP | pyridoxamine 5'-phosphate |
| PPi | inorganic pyrophosphate |
| SAM | *S*-adenosylmethionine |
| SDS | sodium dodecylsulfate |
| THF | tetrahydrofuran |
| tlc | thin layer chromatography |
| TMS | tetramethylsilane |
| Tris | tris(hydroxymethyl)aminoethane |
| TS | threonine synthase |
| $V_{max}$ | theoretical maximum rate of enzymic reaction at saturating substrate concentration |
| v | initial enzymic reaction velocity |
| 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 |

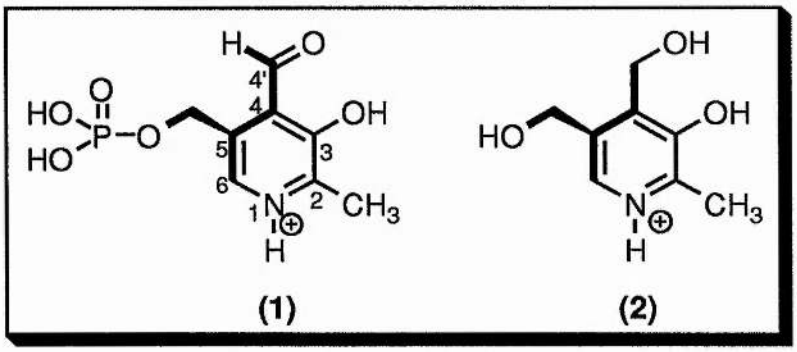
| | |
|---|---|
| Z-APPA | (2*S*)-*Z*-2-amino-5-phosphonopent-3-enoic acid |

| Amino acid | Three letter code abbreviation | Single letter code abbreviation |
|---|---|---|
| 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 |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |
| Unknown or other | - | X |

# INTRODUCTION

## 1.0 Pyridoxal 5'-Phosphate Dependent Enzymes.

Several hundred enzymes use pyridoxal 5'-phosphate (PLP) (**1**) as a cofactor. These include the transaminases, the decarboxylases and the racemases among others.



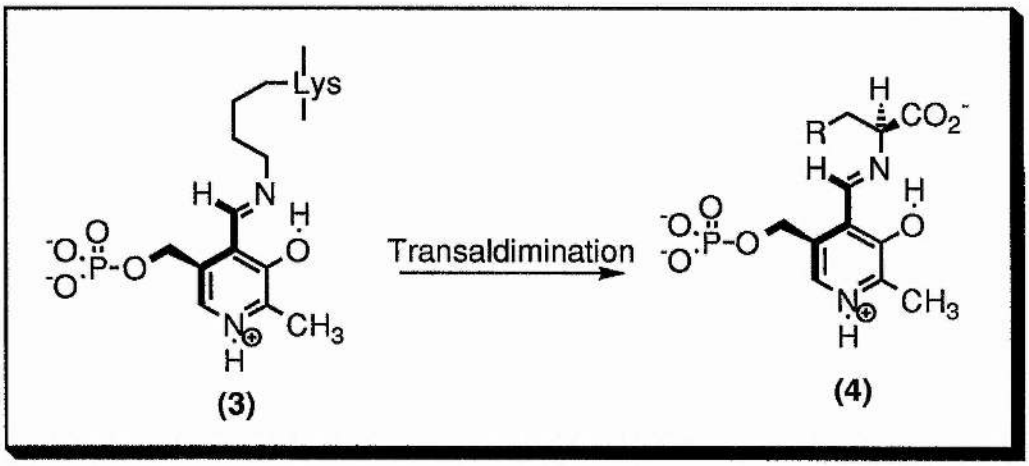

Paul György first discovered the nutritional factor vitamin $B_6$ (pyridoxol) (**2**), of which PLP is a metabolite, in 1934.[1] When purified preparations of thiamine and riboflavin became available in the early 1930's it became apparent that another heat stable vitamin, later called vitamin $B_6$, was required to prevent the development of a florid dermatitis (acrodynia) in rats. This compound was isolated independently by five laboratories in 1938, and its structure and synthesis were reported in 1939 when it was named pyridoxine.[2] Snell discovered that vitamin $B_6$ occurs in multiple free forms, and by studying non-enzymic transaminations between pyridoxal and amino acids he provided the first clues to the function of the vitamin.[3]

PLP was eventually recognised as probably nature's most versatile coenzyme, playing a crucial role in a number of important processes. PLP is the required cofactor for enzymes integral to amino acid metabolism and in connecting the carbon and nitrogen cycles. The coenzyme is also involved in the formation of biogenic amines and in providing entry into the "one-carbon pool". The fact that

PLP dependent enzymes occur in four of the six EC classes reflects its versatility.[4] Experimental $B_6$ deficiency in animals results in vitamin $B_6$ pellagra, characterized by loss of hair, edema and red, scaly skin. Deficiency inhibits the degradation of (2*S*)-tryptophan, and excretion of xantherenic acid (a product of tryptophan degradation) is used as an index of vitamin $B_6$ deficiency.

**Structure and Mechanism of PLP Enzymes.**

The general theory of the PLP mediated amino acid reactions was developed independently by Braunstein and Snell in 1953-54.[5,6] PLP binds to these apoenzymes through the 4-aldehyde group to yield the holoenzyme (**3**). In all known PLP enzymes studied to date this Schiff's base - the so-called internal aldimine - is derived from the ε-amino group of an active-site lysine residue.

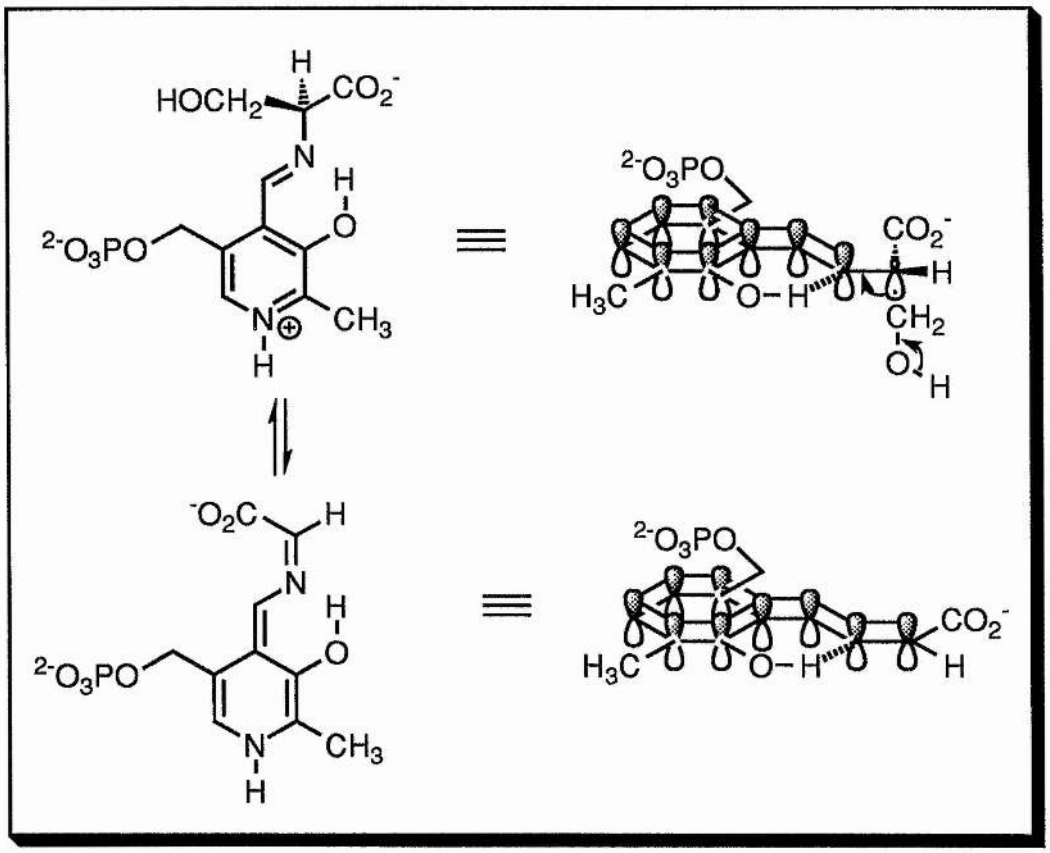



**Scheme 1.01:** *PLP and substrate at the enzyme active site.*

Upon the formation of the holoenzyme, the amino acid substrate can bind to give the Michaelis complex. The internal aldimine can undergo a transaldimination (sometimes called transimination) reaction with the bound amino acid, which now

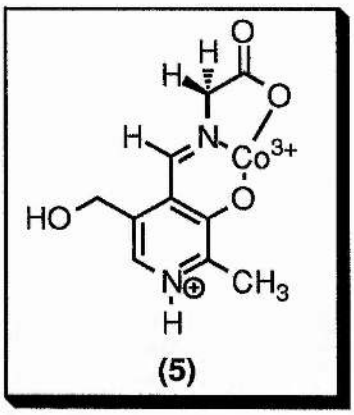
leads to the formation of a Schiff's base substrate aldimine or external aldimine complex (**4**) (Scheme 1.01). The structure of the aldimine (**4**) was first put forward by Braunstein and Snell as a possible solution to the mechanistic problem of how to effect $C^{\alpha}$-H bond cleavage in generating a stabilised intermediate such that the resulting carbanion was not too basic to be kinetically competent. Thus, it was suggested that $C^{\alpha}$-H bond cleavage would be stereoelectronically assisted by the geometry in the external aldimine, as the charge on the pyridinium nitrogen could be transmitted *via* the conjugated $\pi$-system to the substrate $\alpha$-carbon atom. The lability of the bonds to the $C^{\alpha}$-atom is therefore increased due to the electron withdrawal from $C^{\alpha}$.

According to the hypothesis of Dunathan, these cleavages are stereoelectronically assisted by disposing the bond to be broken perpendicular to the plane of the pyridinium ring, thereby maximising orbital overlap between the developing negative charge and the conjugated, electron deficient $\pi$-system, Scheme 1.02.[7,8] Indeed, quantum mechanical calculations have shown that the strength of a $\sigma$ bond between $C^{\alpha}$ of the Schiff's base and the electrofuge is least when the bond lies orthogonal to the pyridinium ring.[9]



**Scheme 1.02:** *Preferential cleavage of the bond orthogonal to the plane of the pyridinium ring, as predicted by the Dunathan hypothesis. This example shows $C^{\alpha}$-H cleavage, but $C^{\alpha}$-$CO_2^-$ and $C^{\alpha}$-$CH_2OH$ bond cleavage (i.e. decarboxylation and retro-aldol reaction respectively) can also occur.*

Experimental support for the idea that the lability of the $C^{\alpha}$-R bond is enhanced when it is held orthogonal to the $\pi$-system has been provided by Abbot.[10] In a complex between cobalt (III) and PLP-glycine Schiff's base (**5**), the two $C^{\alpha}$-hydrogens are held in distinct conformations relative to the $\pi$-system, and apparently exchange at different rates, as measured by $^1$H-NMR spectroscopy.




**(5)**

The structure of the external aldimine (**4**) at the active site has yet to be fully established in most cases, but a *trans* configuration at the imine double bond would be sterically favourable, avoiding the interactions that would occur if the C-5' substituent was in the *cis* coplanar conformation.[11] A *trans* conformation would also allow stabilising hydrogen bonding between the imine nitrogen and the hydroxyl group of the pyridinium ring.

The major function of the enzyme seems to be to enhance certain of the catalytic routes available to the Schiff's base, and to suppress others. The enzyme imposes substrate specificity and orientates the $C^{\alpha}$-R bond (where R is any of the groups attached to the $C^{\alpha}$-atom) determining reaction specificity by the positioning of specific substate binding sites and catalytically active residues.

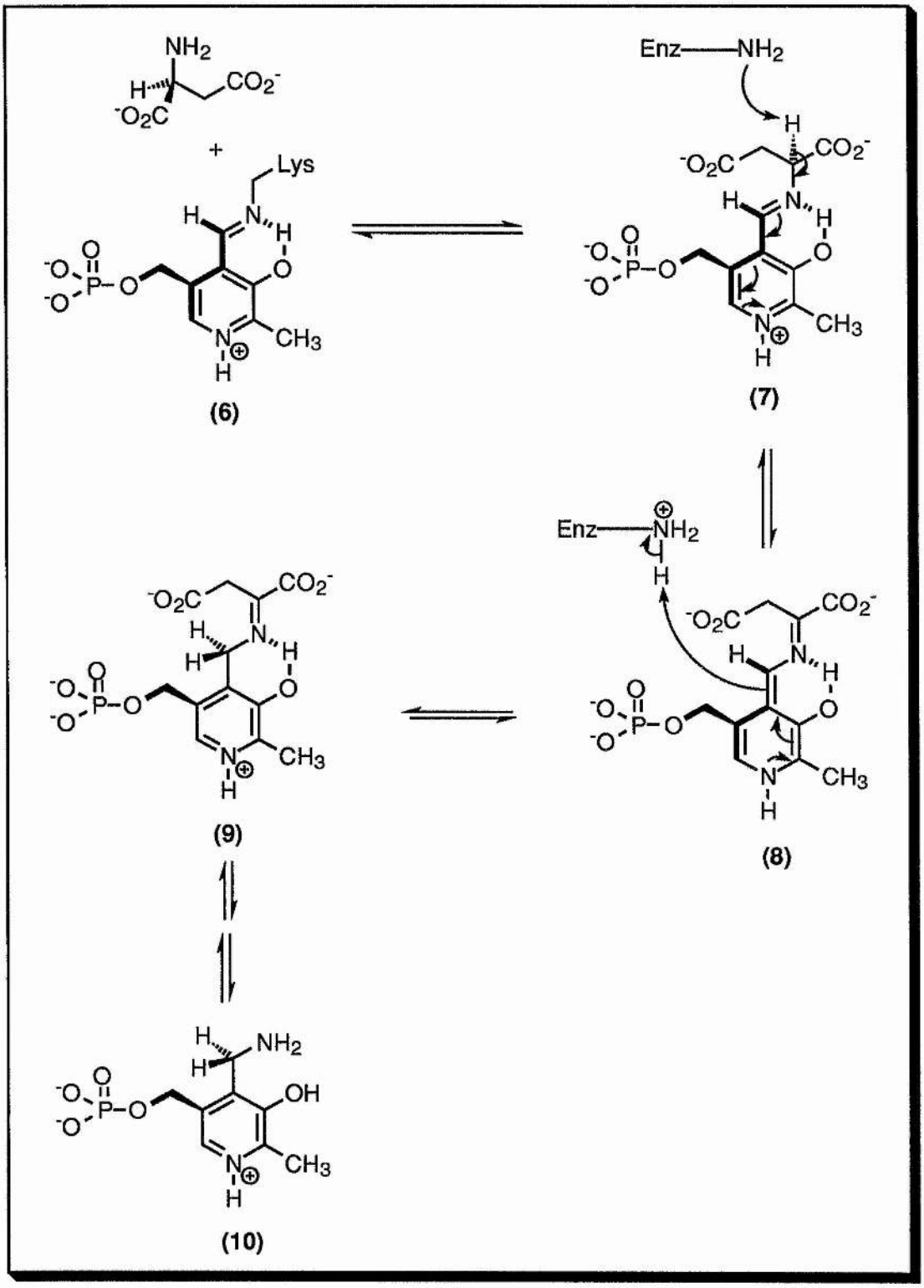
Snell and Metzler postulated that the catalytic abilities of PLP dependent systems are, to an exaggerated degree, those of the coenzyme; and that enzymic and non-enzymic reactions proceed by similar mechanisms.[6] Floss and Vederas suggest that the apoenzyme could bind the relatively rigid PLP-cofactor *via* the pyridinium nitrogen atom and phosphate group.[11] Attachment to one of the $C^{\alpha}$ functional groups of the substrate (probably the carboxyl group), would then result in the three

point binding of the substrate aldimine (**4**), fixing a particular conformation of the $C^{\alpha}$-N bond.[11]

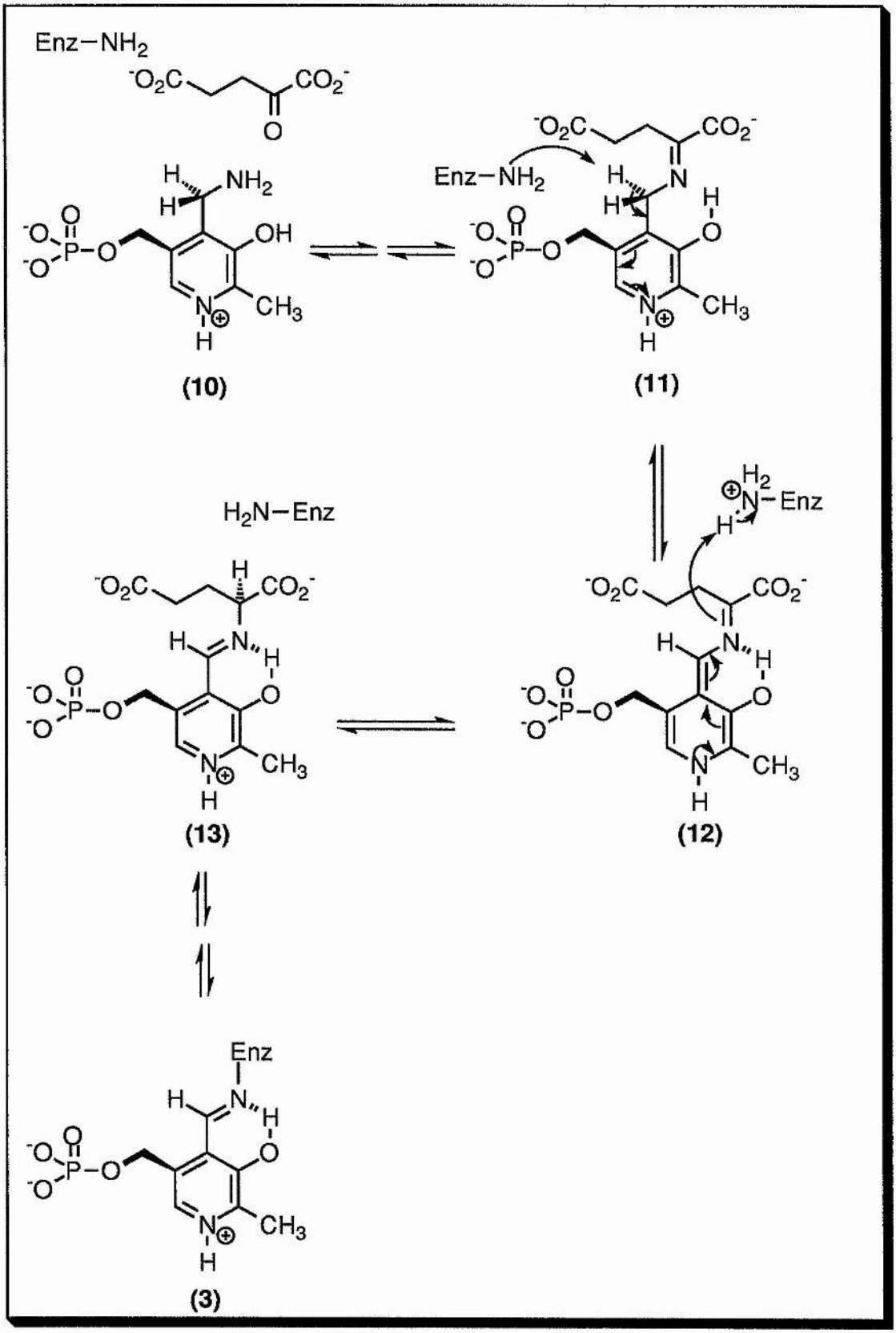
## An Example of a PLP Dependent Enzyme: Aspartate Aminotransferase

Aspartate aminotransferase (AAT) is the best understood PLP enzyme.[12-15] X-ray crystal structures are available of the active holoenzyme complex of chicken mitochondrial AAT,[16] pig cytosolic AAT,[17] and chicken heart cytosolic AAT,[18] among others.

All the aspartate aminotransferases are extremely similar in structure. The protein consists of two identical subunits each of which possesses two domains. The coenzyme is bound to the larger domain, and is situated in an open pocket near the subunit interface. After conversion of the Michaelis complex (**6**) to the aldimine (**7**) the $C^{\alpha}$ proton of the aldimine (**7**) is transferred *via* the conjugated enamine (**8**) to ketimine (**9**) on the C-4'-*si* face of the coenzyme. This yields pyridoxamine 5'-phosphate (PMP) (**10**) and an $\alpha$-ketoacid (Scheme 1.03). When a new $\alpha$-ketoacid becomes available, it condenses with the PMP (**10**) to give a second ketimine (**11**). 1,3-suprafacial proton transfer from C-4' to $C^{\alpha}$ on the 4'-*si*-face of the coenzyme gives the aldimine (**13**) which undergoes transaldimination to yield (2*S*)-glutamic acid, and the internal PLP-aldimine (**3**) (scheme 1.04).




**Scheme 1.03:** *The first half-reaction of aspartate aminotransferase.*

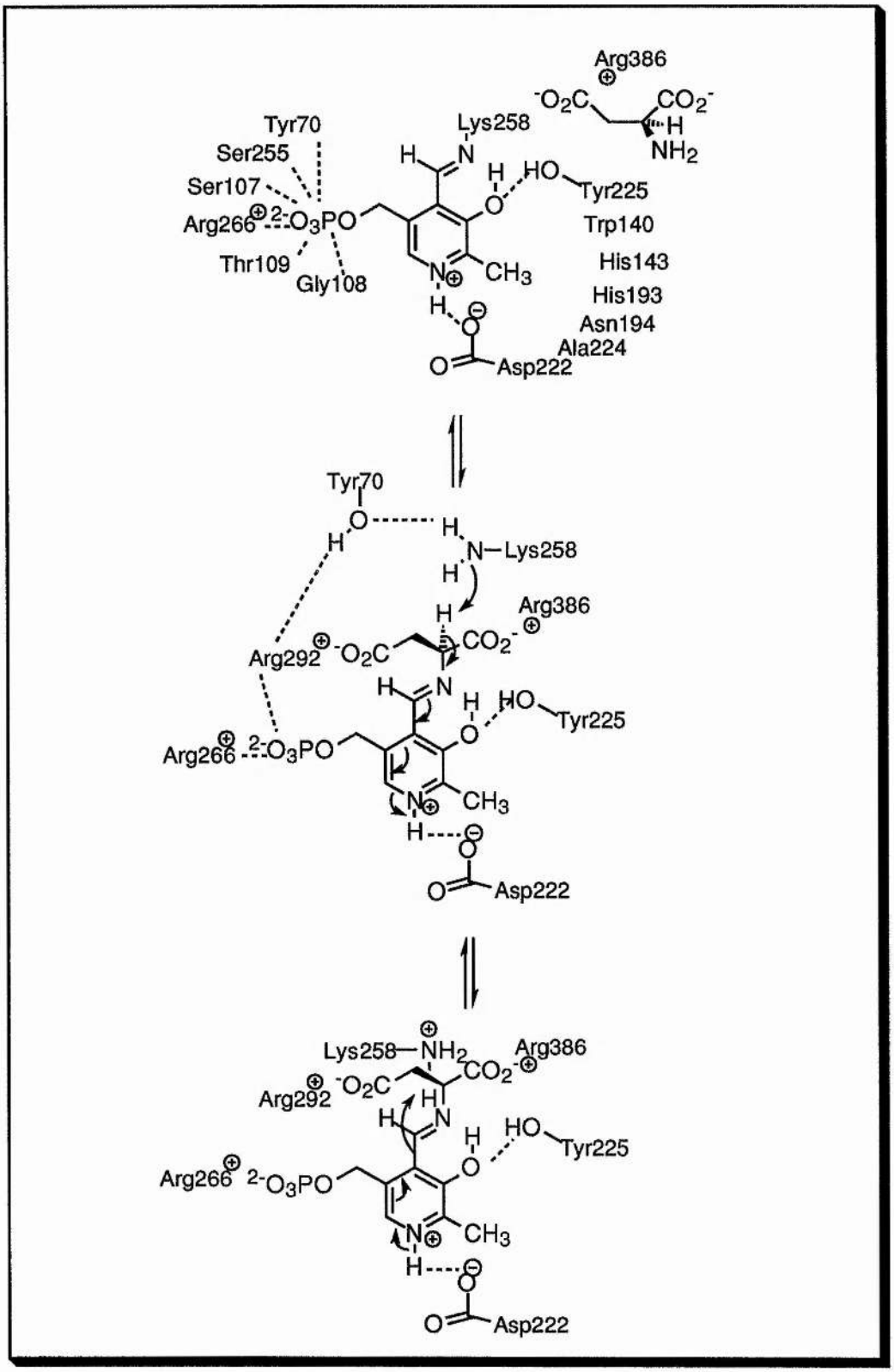



**Scheme 1.04:** *The second half reaction of aspartate aminotransferase.*

X-ray crystallographic studies of mitochondrial chicken heart AAT have also allowed the active site residues that are important for binding to the PLP and to the subtrate to be identified. The protonated nitrogen atom of the pyridinium ring forms a hydrogen bond with Asp222, whilst the deprotonated phenolic hydroxyl moiety is hydrogen bonded to the hydroxyl of Tyr225. The 2-methyl group lies in a pocket defined by eight amino acids, and 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 (Scheme 1.05).[19]

Two genetically distinct isoenzymes of aspartate aminotransferase exist in animal tissue: cytosolic (cAAT) and mitochondrial (mAAT).[20] The primary structures of a number of AATs have been completely or partially elucidated.[21] The amino acid sequence from chicken, pig, rat, mouse,[22] horse[23] and human mAAT[24] and the cAAT from chicken,[21] pig, mouse[22] and horse[23] are known, as well as those from *E. coli* B.[25] and *E. coli* K12.[26]

The aspartate aminotransferases show almost 100% homology for the regions of the protein corresponding to substrate or coenzyme binding sites. A comparison of the amino acid sequence for selected regions of AAT isoenzymes[27] and the amino acid sequences for the Schiffs base forming region of several AATs[28] are shown in Tables 1.06 and 1.07 respectively.




**Scheme 1.05:** *Residues at the active site of chicken heart mitochondrial AAT.*

| Source | 70 | 108 | 140 | 190 | 222 | 258 | 266 | 292 | 360 | 386 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human (mit) | EYL | SGTG | TWGNH | LHACAHNPTG | FFDMAYQGF | QSYAKN | ERV | IRP | MFC | GRI |
| Chicken (mit) | EYL | SGTG | SWGNH | LHACAHNPTG | YFDMAYQGF | QSYAKN | ERA | IRP | MFC | GRI |
| Pig (mit) | EYL | SGTG | SWGNH | LHACAHNPTG | FFDMAYQGF | QSYAKN | ERV | IRP | MFC | GRI |
| Rat (mit) | EYL | SGTG | SGGNH | LHACAHNPTG | FFDMAYQGF | QSYAKN | ERV | IRP | MFC | GRI |
| Chicken (cyt) | EYL | GGTG | TWENH | LHACAHNPTG | FFDSAYQGF | QSFAKN | ERV | VRT | MFS | GRI |
| Pig (cyt) | EYL | GGTG | TWENH | LHACAHNPTG | FFDSAYQGF | QSYAKN | ERV | VRV | MFS | GRI |
| *E. coli* | NYL | GGTG | SWPNH | LHACAHNPTG | LFDFAYQGF | SSYSKN | ERV | IRA | DFS | GRV |

**Table 1.06:** *Amino acid sequence for selected regions of AAT isoenzymes. The underlined residues correspond to the residue numbers given for cytosolic pig AAT. (mit = mitochondrial, cyt = cytosolic)*

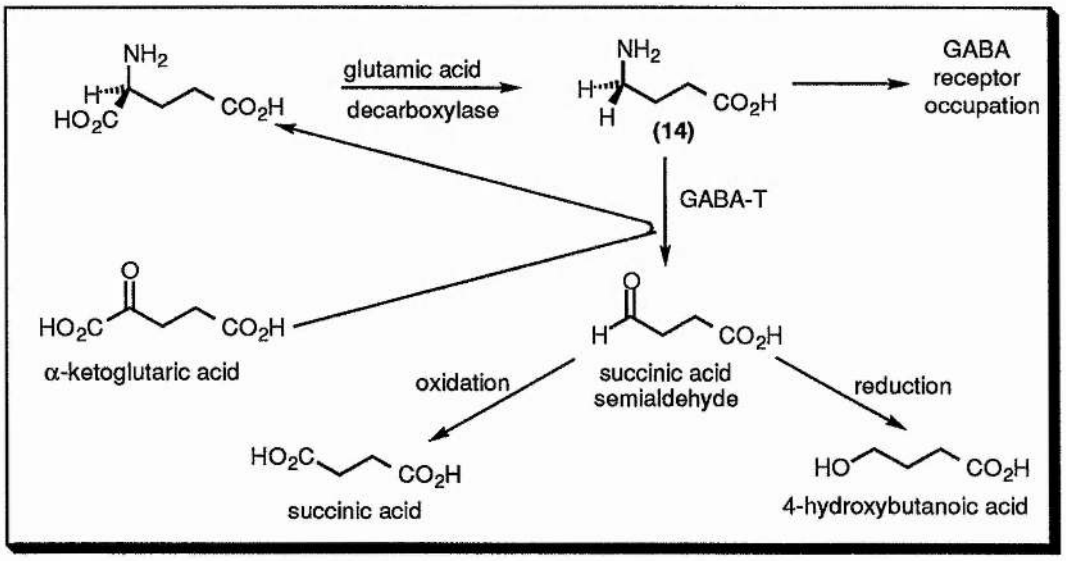
| Source | Sequence |
|---|---|
| Chicken (mit) | VLSQSYAKNMGLY |
| Turkey (mit) | VLSQSYAKNMGLY |
| Pig (mit) | VLSQSYAKNMGLY |
| Rat (mit) | VLSQSYAKNMGLY |
| Human (mit) | VLSQSYAKNMGLY |
| Chicken (cyt) | VLSQSYAKNMGLY |
| Pig (cyt) | VLSQSYAKNMGLY |
| *E. coli* | VLSQSYAKNMGLY |

**Table 1.07:** *Amino acid sequences for the Schiff's base forming region of several AATs.*

## 1.1 The Metabolic Role of PLP dependent Enzymes.

PLP dependent enzymes are ubiquitous in nature, and occur in all species. The mechanisms of action of PLP dependent enzymes are of great scientific interest because of the integral role they play in many important physiological processes. This section includes examples which underline this fact, and emphasise the crucial part that pyridoxal 5'-phosphate dependent enzymes have to play in metabolism.

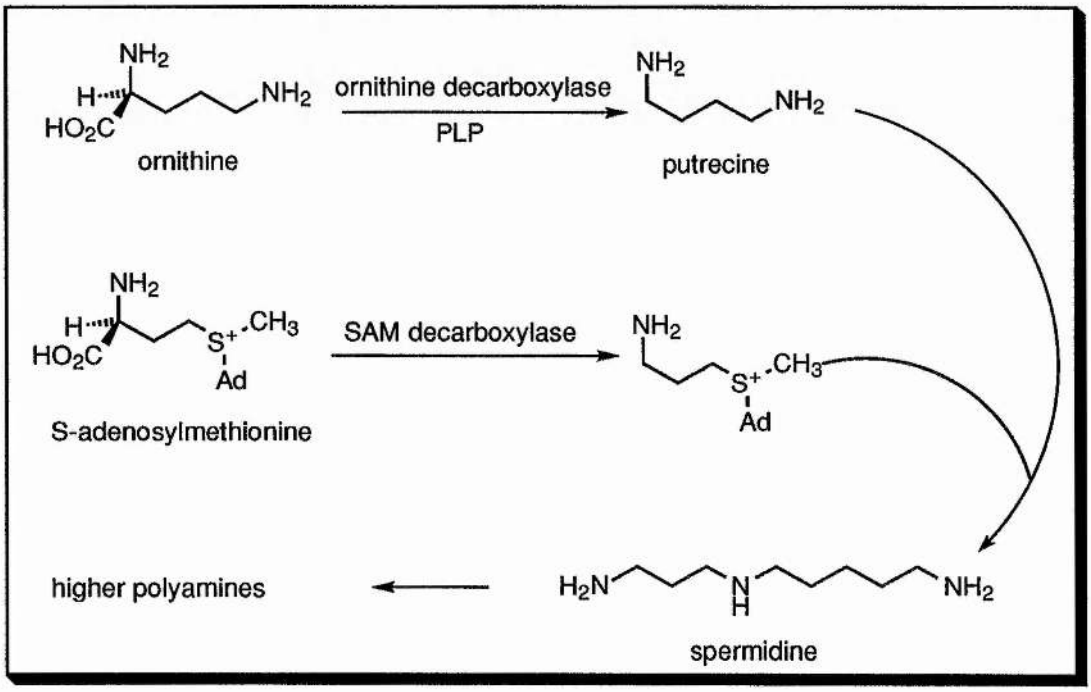
γ-Aminobutyric acid transaminase (GABA-T) is a key PLP dependent enzyme involved in the regulation of the GABA-ergic system,[29] an important pathway in the mammalian central nervous system (CNS) (scheme 1.08). γ-Aminobutyric acid (GABA) (**14**) is a major inhibitory neurotransmitter and there is much evidence to suggest that high cerebral concentrations of this compound prevent convulsions, by depressing the firing of neurons throughout the CNS.




**Scheme 1.08:** *The GABA-ergic system.*

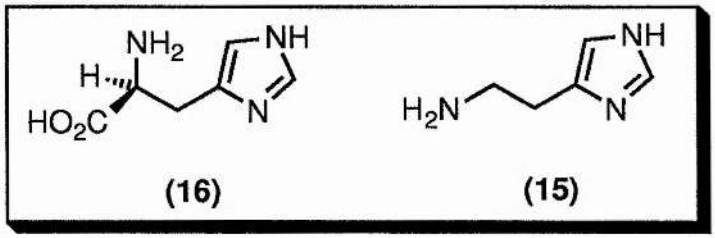
Mammalian brain decarboxylase is directly responsible for the production of GABA in the GABA-ergic system. This is also a PLP dependent enzyme.

Ornithine decarboxylase (ODC) is the first and rate limiting enzyme in the biosynthetic pathway to putrecine and higher polyamines (Scheme 1.09).[30] Cellular stimulation increases the activity of ODC dramatically, and promotes regeneration and replication. The enzyme is present at high cellular levels during protein biosynthesis and cell growth, but low at most other times.[31] Given that ODC is so closely associated with growth and cell proliferation it seemed likely that polyamines may be required for nucleic acid biosynthesis.[32] The enzyme has been identified as a target for cancer chemotherapy.




**Scheme 1.09:** *The biosynthesis of higher polyamines.*

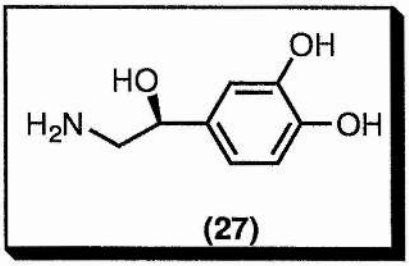
Histidine decarboxylase (HDC) catalyses the formation of histamine (**15**) (a major receptor agonist and regulatory factor for peripheral blood circulation) from histidine (**16**).[33] Many biological reponses are associated with the overproduction of histamine. These include gastric secretion, allergic and hypersensitivity reactions.[32] The development of antagonists for histamine (antihistamines) has been an important area in medicinal chemistry.



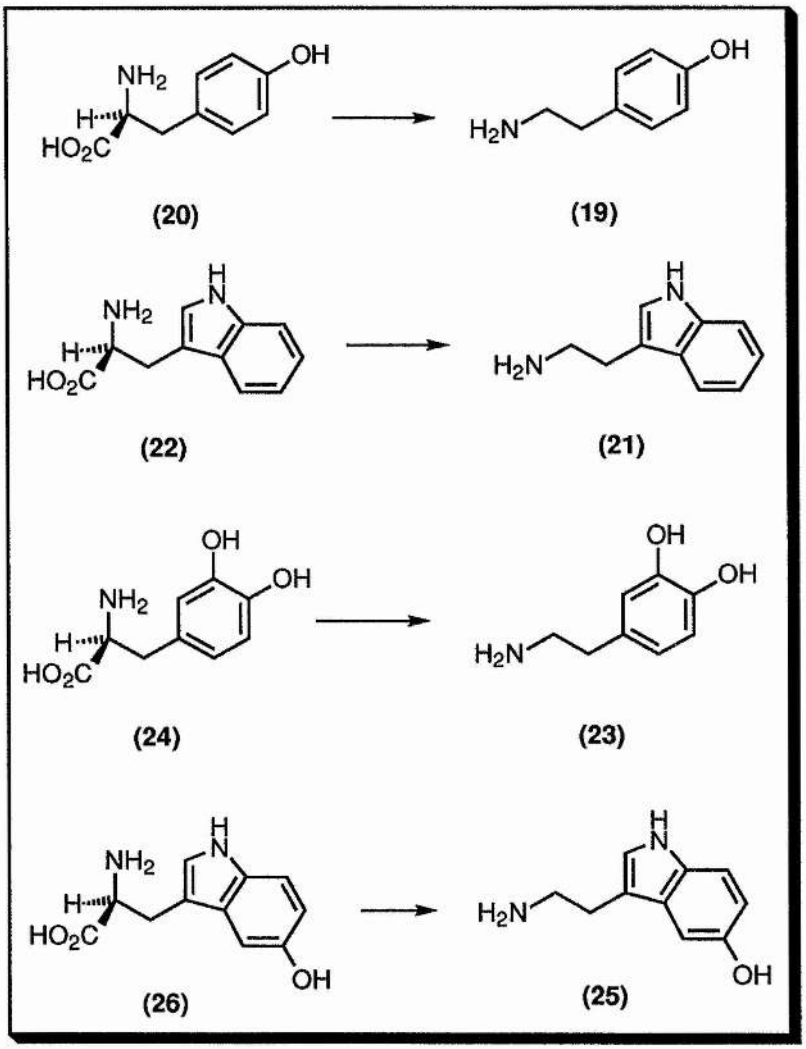

**(16)** **(15)**

DOPA decarboxylase (DDC) catalyses the conversion of many aromatic substrates to their corresponding amines (Scheme 1.10). For example, the formation of phenylethylamine (**17**) from phenylalanine (**18**), tyramine (**19**) from tyrosine (**20**), tryptamine (**21**) from tryptophan (**22**), histamine (**15**) from histidine (**16**), 3,4-dihydroxyphenylethylamine (dopamine) (**23**) from 3,4-dihydroxyphenylalanine (DOPA) (**24**) and serotonin (**25**) from 5-hydroxytryptophan (**26**).[34]

Dopamine is a precursor in adrenaline (**27**) biosynthesis, and serotonin is a neurotransmitter. Both compounds also act on smooth muscle tissue and the cardiovascular system.

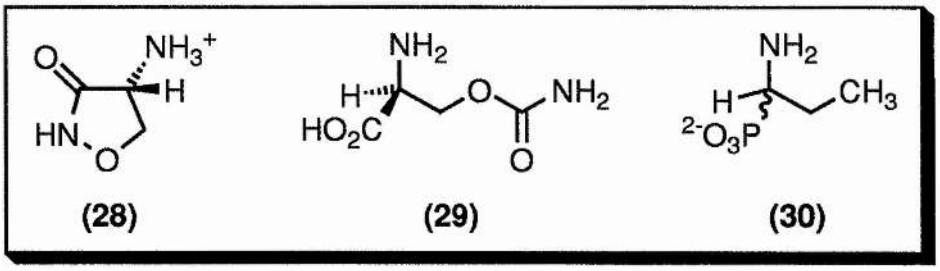



**(27)**




**Scheme 1.10:** *Reactions catalysed by DOPA decarboxylase.*

The racemases are an important group of PLP enzymes, catalysing the interconversion of (2*S*)- and (2*R*)-amino acids. They are common in most prokaryotes where they play a fundamental role in the biosynthesis of peptidoglycan (a cell wall component).[35] The racemases present a useful target for chemotherapy, and alanine racemase in particular is the target of many antibacterial agents,[36] such as (2*R*)-*cyclo*serine (**28**),[37] *O*-carbamyl-(2*R*)-serine (**29**)[36] and phosphoalanine (**30**).[37]

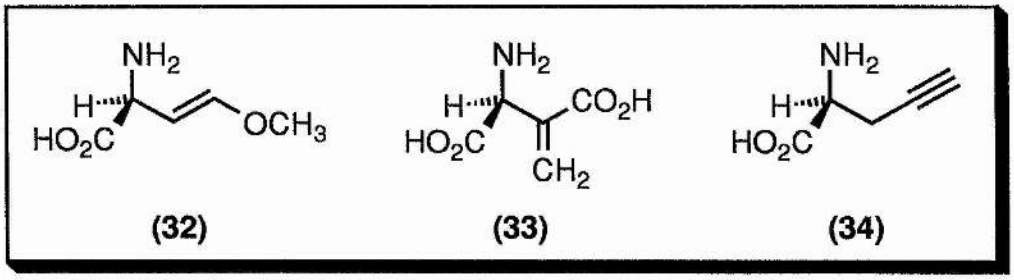



**(28)** **(29)** **(30)**

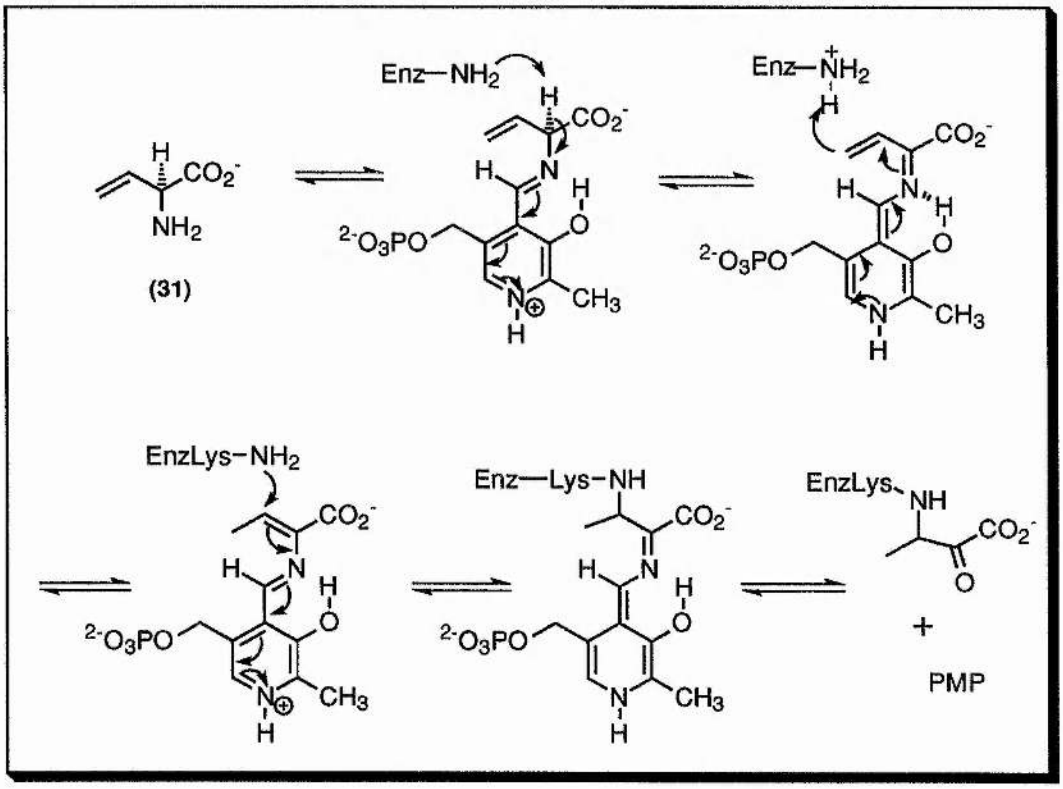
## 1.2 Mechanism Based Inhibitors of PLP dependent enzymes.

Mechanism based or suicide inhibitors of PLP dependent enzymes are potential drug candidates due to the pharmacological and therapeutic utility of inhibiting specific PLP enzymes. These inhibitors are substrate analogues and can also be useful tools in investigating the mechanism of specific enzymes. As already mentioned (section 1.0) the role of PLP is to stabilise carbanionic intermediates that form during the catalytic process. Inhibitors that possess functional groups which become activated by their proximity to an enzyme generated carbanion can breakdown to yield a reactive species that may react with an active site amino acid side chain, or with tightly bound PLP coenzyme. Either of these processes will lead to the inactivation of the enzyme, in the first instance blocking the active site with an unreactive species; or in the second case causing the coenzyme to become unavailable to bind substrate. Functional groups commonly used in the design of such inhibitors include acetylenic, olefinic, β-halo substituents and other leaving groups, nitriles, aryl sulfoxides, dihydroaromatics and phosphonoamino acids.[38]

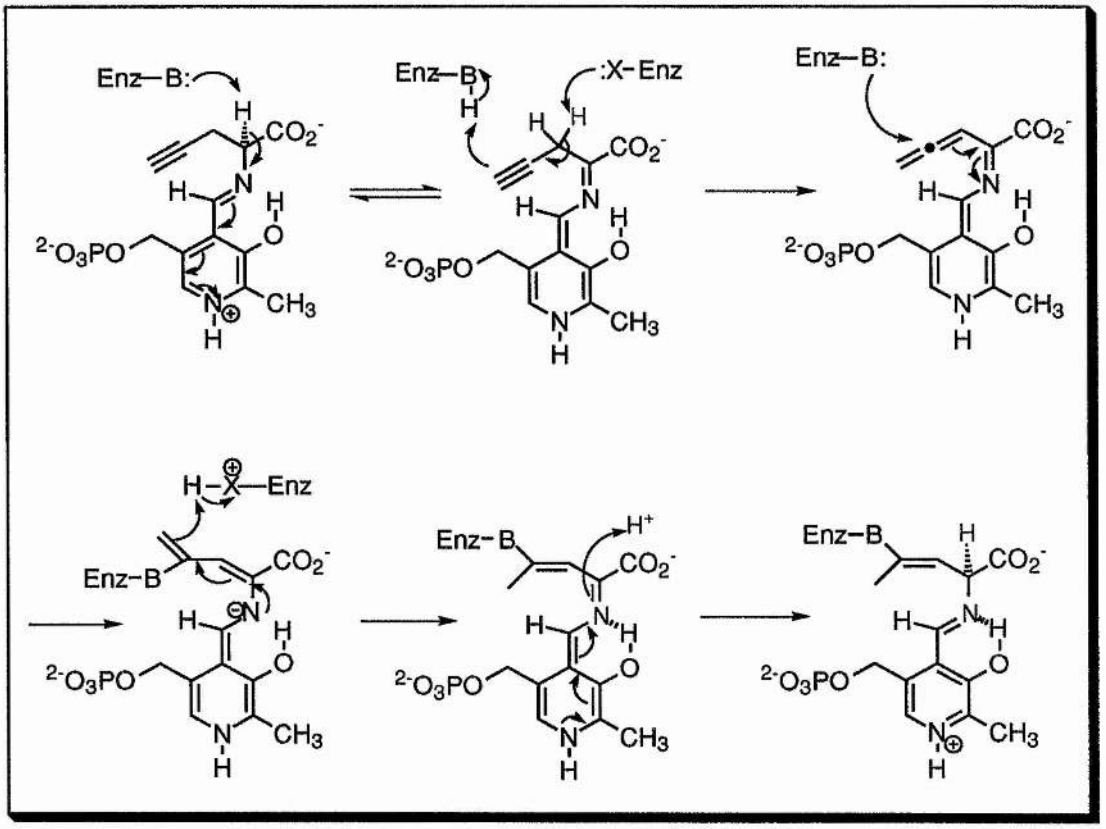
In the first half reaction of transamination (Scheme 1.04) catalysis proceeds through the substrate-PLP anion and the product α-amino acid-PMP enzyme complex (**10**). Both of these intermediates have the potential to be activated as suicide substrates. This is indeed what is observed in both olefinic and acetylenic amino acid analogues. The olefinic analogues include vinyl glycine (**31**),[39] (*E*)-methoxy vinyl glycine (**32**)[40] and β-methylene aspartate (**33**).[41] The accepted mechanism for inhibition of AAT by vinyl glycine (**31**) is shown in Scheme 1.11.




The acetylenic analogues include the natural product (2*S*)-propargyl glycine (**34**).[42] The mechanism of inactivation is shown in Scheme 1.12.

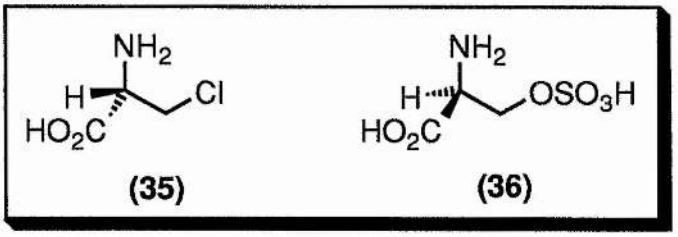



**Scheme 1.11:** *The inhibition of AAT by vinylglycine.*

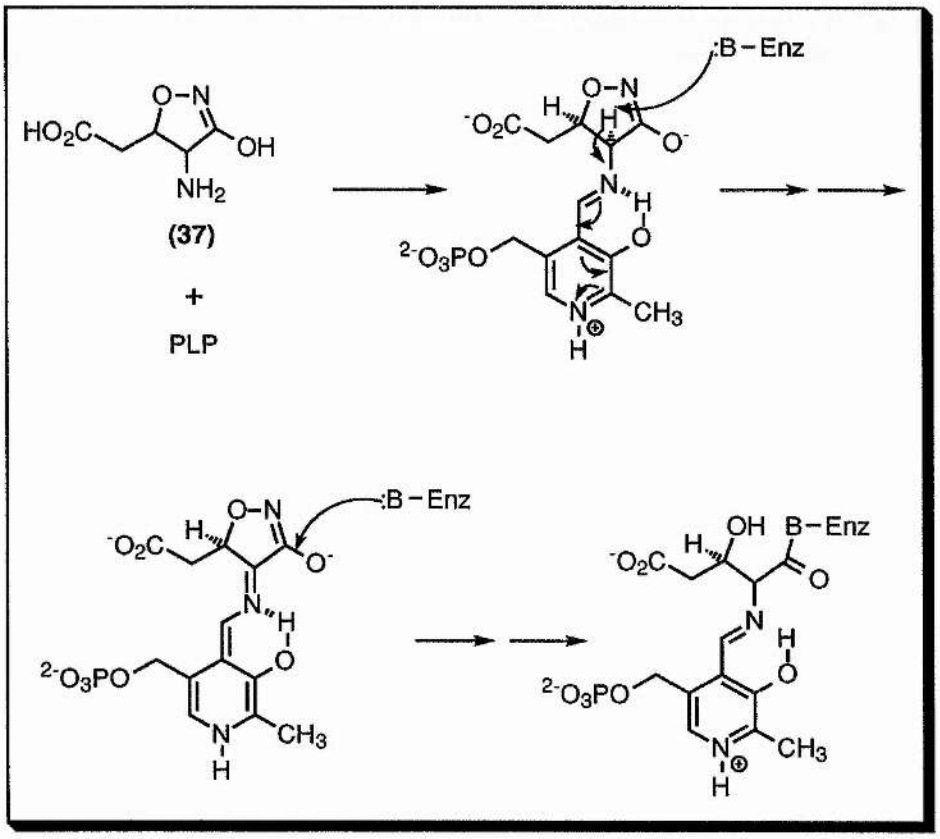



**Scheme 1.12:** *The inhibition of AAT by (2S)-propargyl glycine.*

Suicide substrates which possess a good leaving group at $C^{\beta}$ are some of the most common. These compounds can undergo a facile elimination reaction to generate an olefinic intermediate. Examples of this class of inhibitor are (2*S*)-3-chloroalanine (**35**)[43,44] which inhibits alanine aminotransferase, and (2*S*)-serine-*O*-sulfate (**36**) which inhibits aspartate aminotransferase.[45]

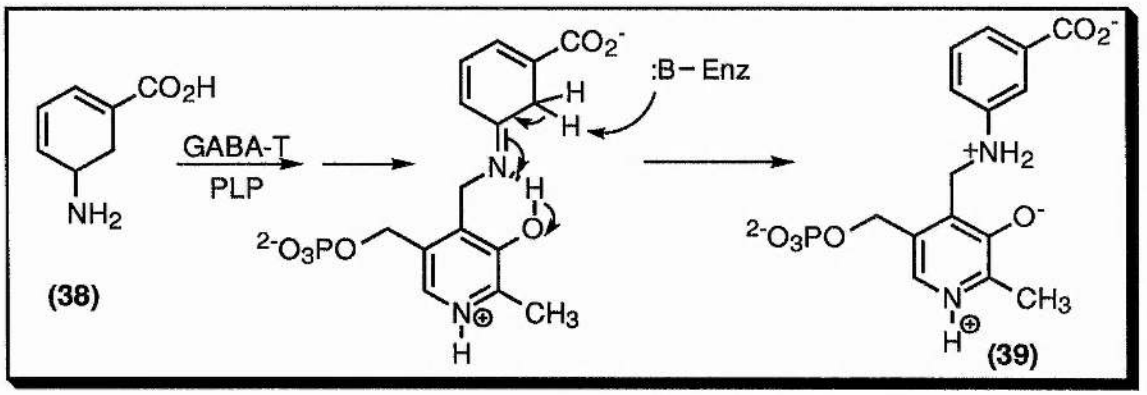


It was originally thought that the inactivating species was the aminoacryl-PLP intermediate, which would undergo a putative Michael reaction with an enzyme bound nucleophile. Although this is the case with some enzymes, such as serine hydroxymethyltransferase, Metzler and his coworkers have shown that the inhibition of aspartate aminotransferase is not due to the occurrence of a Michael reaction.[46] Bright and coworkers have used the nitro group of 3-nitroalanine as an inactivator of aspartate aminotransferase and alanine aminotransferase.[47,48] Alanine aminotransferase is also inhibited by *cyclo*serine (**28**),[49] while aspartate aminotransferase is inhibited to varying extents by α- and γ-*cyclo*glutamic acids.[50-52] α-*Cyclo*glutamate (Scheme 1.13, (**37**)) is believed to acylate an active site bound nucleophile to give a stable inactivated complex, whilst γ-*cyclo*glutamate is thought to form an oxime of β-aminooxyglutamate with PLP.




**Scheme 1.13:** *Mechanism of inactivation of AAT by α-cycloglutamate.*

Various analogues of GABA inhibit GABA-transaminase. These include β-chloro-, 3-phenyl, γ-acetylenic and γ-vinyl GABA as well as ethanolamine *O*-sulfate.[53] Mammalian GABA-transaminase is inhibited by the naturally occurring product gabaculine (**38**) *via* an interesting aromatisation mechanism to give the conjugated PMP adduct (Scheme 1.14, (**39**)).[54-55]

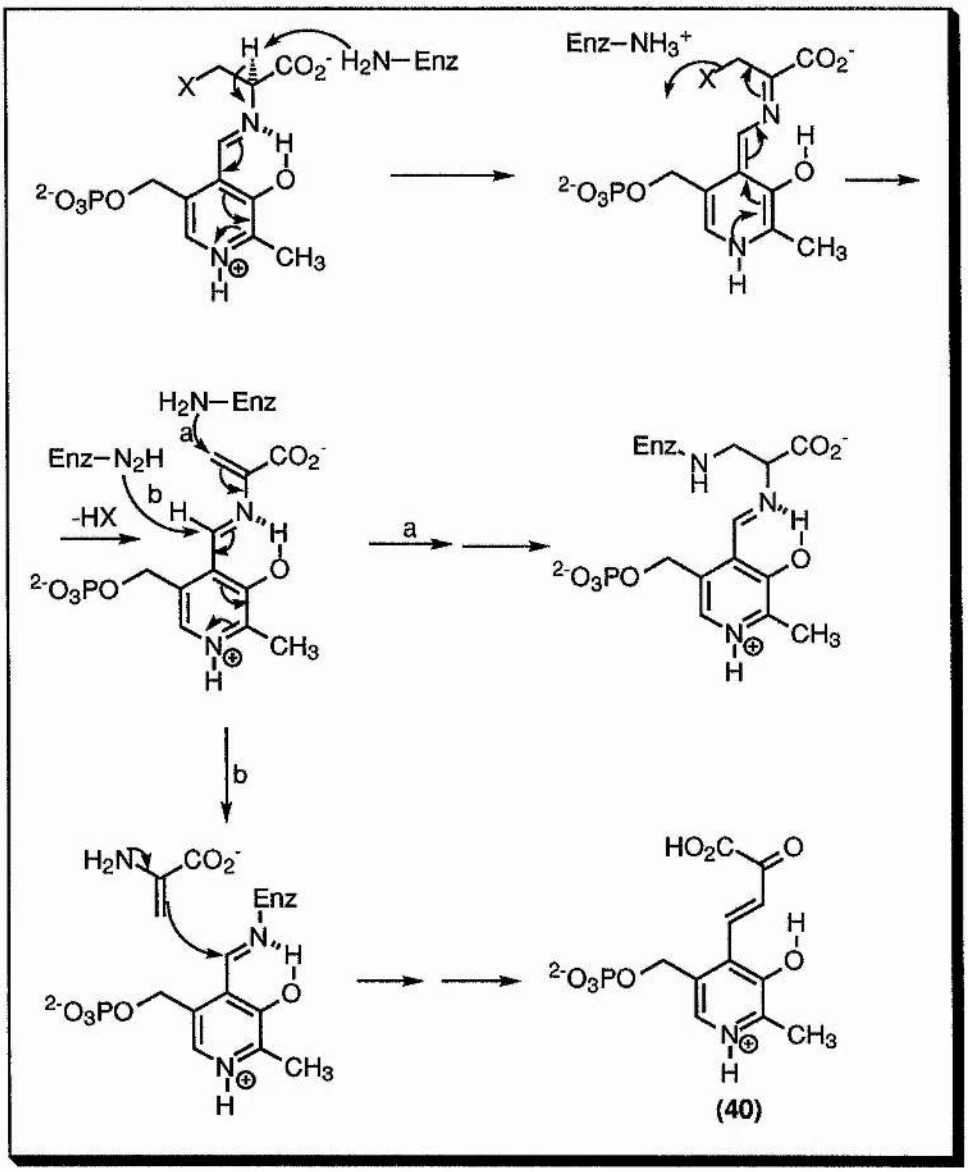



**Scheme 1.14:** *Mechanism of inactivation of GABA-transaminase by gabaculine (38).*

PLP dependent racemase enzymes are important because they are involved in the biosynthetic pathway leading to peptidoglycan. Many suicide substrates are known for the racemases, including β-substituted alanines for alanine racemase[56-58] and phosphoalanine (for gram positive alanine racemase only).[59,60] Phosphoalanine is thought to inhibit alanine racemase *via* an initial reversible formation of a weak complex which slowly isomerises to a stoichiometric complex that dissociates extremely slowly. The complex is not reducible with borohydride.[73]

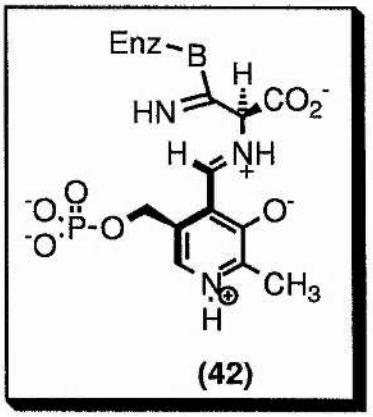
Enzymes which catalyse α,β-elimination occasionally undergo an abortive reaction with a physiological substrate. For example, threonine deaminase is very slowly inactivated by (2*S*)-serine approximately once every $10^4$ turnovers,[61] note that a

similar situation occurs with 3-chloroalanine.[62] In contrast, (2*S*,3*R*)-threonine does not cause inactivation, presumably as the intermediate formed is less susceptible to Michael addition or enamine condensation to give the Schnackerz type product (**40**) (Scheme 1.15).[63]

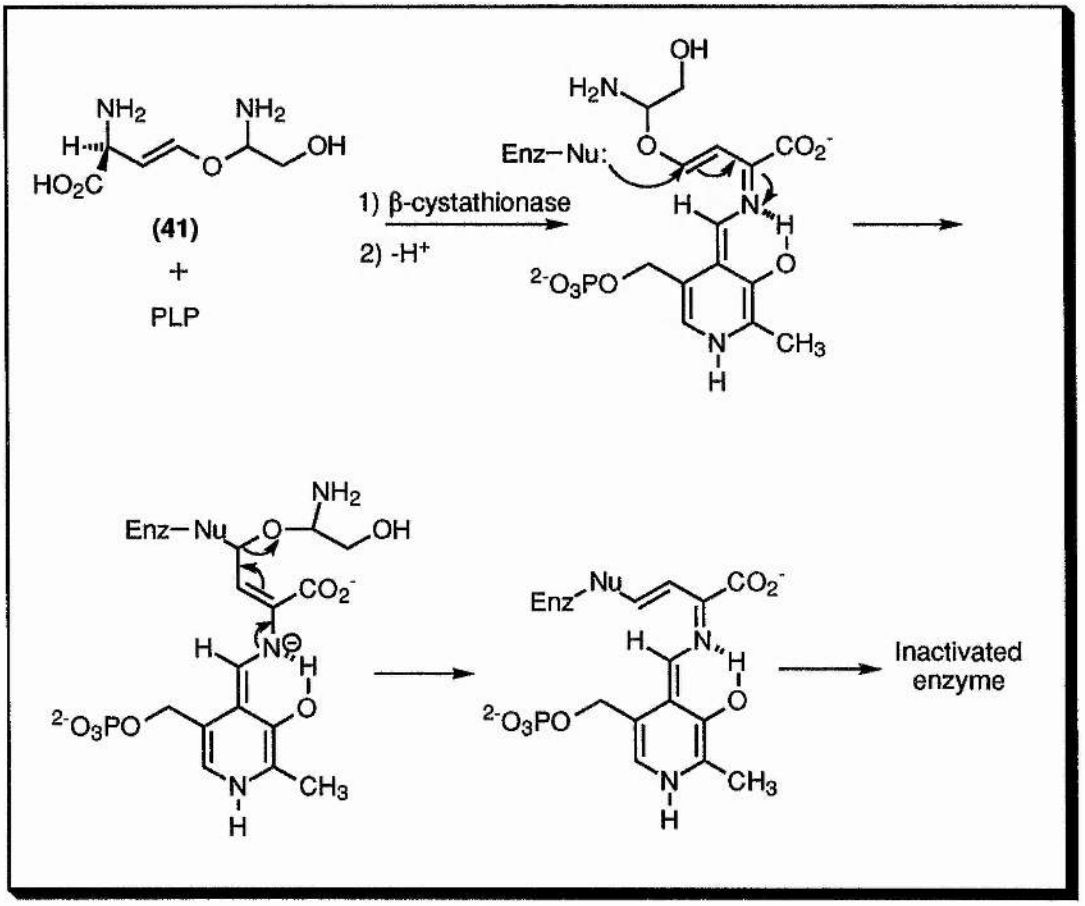



**Scheme 1.15:** *Two possible mechanisms for the inactivation of enzymes that catalyse α,β-elimination reactions. Route a causes the modified coenzyme to be covalently bound to the enzyme, and route b leads to the Schnackerz adduct (**40**).*

*E. coli* tryptophanase is irreversibly inhibited by trifluoroalanine.[64] It is proposed that the difluoroaminoacrylyl-PLP-aldimine complex is more susceptible to nucleophilic attack at C-3 by an enzyme bound base, than to hydrolysis at the aldimine carbon atom. The enzyme β-cystathionase is irreversibly inactivated by the fungal toxin rhizobiotoxin (Scheme 1.16, (**41**)).[65] The alkoxyvinylglycine analogue rhizobiotoxin may undergo initial $C^{\alpha}$-H abstraction, and then an addition and elimination sequence, similar to that for (*E*)-methoxyvinylglycine (**32**) with aspartate aminotransferase. β-Cystathionase is also irreversibly inhibited by trifluoroalanine, and tryptophan synthase is subject to suicide inhibition by trifluoro-, trichloro- and dichloroalanine.[66] *E. coli* tryptophan synthase also reacts with cyanoglycine, causing inhibition which is reversible upon dialysis or gel-filtration. The inactivated coenzyme is presumed to be a stable β-imine PLP adduct (**42**).[67]



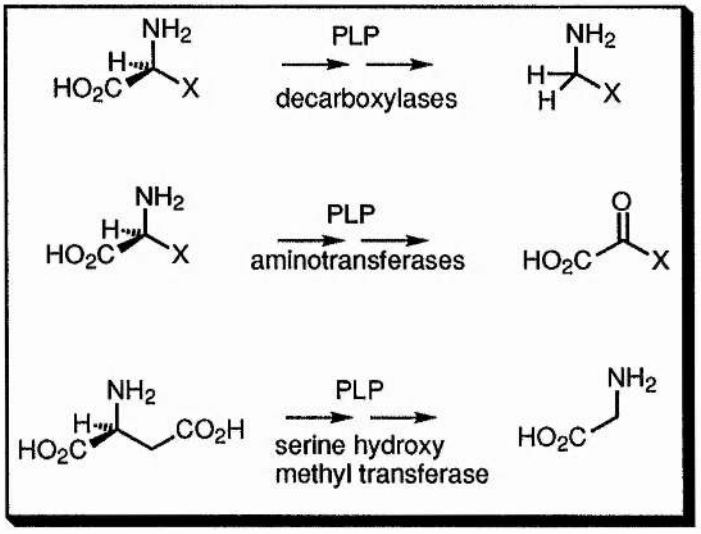

**(42)**




**Scheme 1.16:** *The inactivation of β-cystathionase by rhizobiotoxin (**41**).*

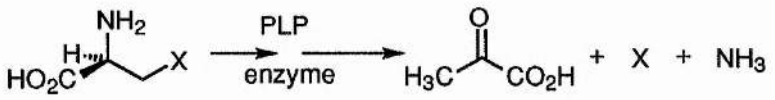
## 1.3 The Classification of PLP Enzymes.

In 1994, P. Christen and his coworkers proposed that most PLP dependent enzymes can be assigned to one of three different "families" of homologous proteins.[68] The relationships between the different PLP enzymes were deduced by alignment and comparison of their amino acid sequences and 3D structures. By also comparing the reactions catalysed by these enzymes (Scheme 1.17), Christen deduced that the regiospecificity of the enzymes was directly related to the membership of one of these "families". Accordingly the enzymes catalysing reactions at $C^{\alpha}$ were assigned to the so-called $\alpha$-family; those acting at $C^{\beta}$ to the $\beta$-family; and those acting at $C^{\gamma}$ to the $\gamma$-family. There are some interesting exceptions to this rule, and indeed some PLP dependent enzymes do not seem to belong to any of the groups.




**Scheme 1.17a:** *Some types of reaction catalysed by PLP dependent enzymes.*

a)




i. tryptophanase X = indole

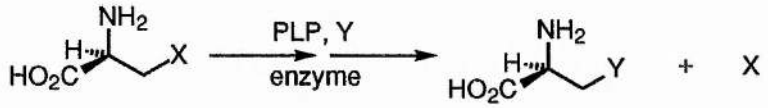
ii. tyrosine phenol-lyase X = phenol

iii. threonine dehydratase X = -OH

iv. tryptophan dehydratase X = indole

v. cystathionine β-synthase X = homocysteine

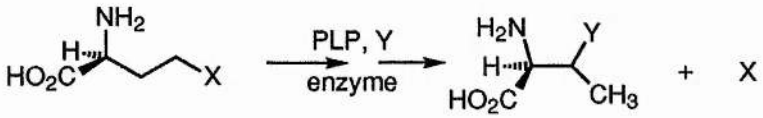
b)




i. tryptophan synthase X = -OH, Y = indole

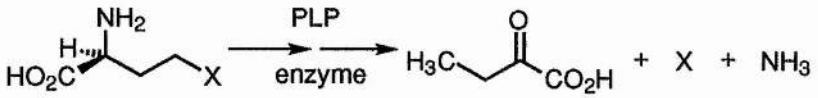
ii. cysteine synthase X = $^{-}O_2CCH_3$, Y = -SH

c)



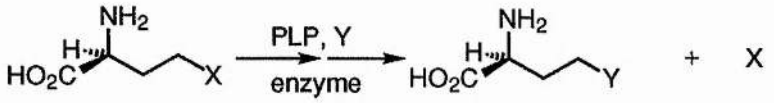

i. threonine synthase X = $-OPO_3^{2-}$, Y = -OH

d)




i. cystathionine γ-synthase X = -cysteine

e)




i. *O*-succinylhomoserine(thiol)-lyase X =-$O_2CCH_2CH_2CO_2H$, Y = -cysteine

ii. *O*-acetylhomoserine(thiol)-lyase X = -$O_2CCH_3$, Y = methanethiol

**Scheme 1.17b:** *Some other types of reaction catalysed by PLP dependent enzymes.*

**The α-Family of PLP Dependent Enzymes.**

The α-family has, by far, the most members of all the PLP dependent groups (Table 1.18), including the aminotransferases and the group II decarboxylases. Many of these enzymes have 3D structures available for comparison, and the structural similarities can be seen clearly between different types of enzyme.

| |
|---|
| Most aminotransferases (excluding branched chain and D-amino acid)[69,70] |
| Group II amino acid decarboxylases |
| Tryptophanase |
| Tyrosine phenol-lyase[71] |
| Glycine hydroxymethyltransferase |
| 1-Aminocyclopropane-1-carboxylate synthase[72] |
| 2-Amino-6-caprolactam racemase |
| Glutamate-1-semialdehyde 2,1-aminomutase |
| Isopenicillin N-epimerase |
| 2,2-dialkyl decarboxylase[73] |
| 4-Amino-4-deoxychorismate synthase[74,75] |
| Glycine-C-acetyltransferase |
| 5-Aminolevulinate synthase |
| 8-Amino-7-oxononanoate synthase |
| The gene product of cobC (cobalamin synthesis) |
| The gene product of nifS (nitrogen fixation) |
| The gene product of malY (abolishes induction of the maltose system) |

**Table 1.18 :** *PLP dependent enzymes belonging to the α-family.*

In the α-family the PLP binding lysine residue occurs between residues 209 and 256 of the various members. The most intensely studied members of the α-family are the aminotransferases (or transaminases), which have already been discussed (page 6 and Scheme 1.17a), as have examples of the decarboxylases (Scheme 1.17a). Serine hydroxymethyltransferase catalyses the decarboxylation of aminomalonate as shown in Scheme 1.17a. Tryptophanase and tyrosine phenol-lyase catalyse reactions of type a) (Scheme 1.17b).

**The β-Family of PLP Dependent Enzymes.**

The β-family has seven members, and includes threonine synthase (Table 1.19). Parsot first postulated the evolutionary connection of these enzymes in 1987.[76,77] These suggestions were later upheld by Bork and Rohde.[78] If these enzymes are related in an evolutionary context, then they may be distantly related to an archaic enzyme with very broad specificity.

| |
|---|
| (2S)-Serine dehydratase |
| (2R)-Serine dehydratase |
| Threonine dehydratase |
| Threonine synthase |
| Tryptophan dehydratase |
| Tryptophan synthase (β-subunit) |
| Cysteine synthase (isoenzyme A and B) |

**Table 1.19:** *The β-family of PLP dependent enzymes.*

The β-family has the PLP binding lysine lying between amino acid residues 41 and 118. This is clearly very different from the α-family, and may reflect the alternative PLP arrangement needed for the enzymes to carry out reactions at the $C^{\alpha}$-centre rather than at the $C^{\beta}$-atom. The serine dehydratases, threonine dehydratase and tryptophan dehydratase catalyse reactions of type a (Scheme 1.17b). Tryptophan synthase and cysteine synthase catalyse reactions of type b, and threonine synthase catalyses a reaction of type c.

**The γ-Family of PLP Dependent Enzymes.**

This is the smallest "family" of enzymes with only four members (Table 1.20). Interestingly Bork and Rohde have shown significant sequence similarities between threonine synthase and *O*-acetylserine sulfhydrolase.[78] This may suggest that the β- and γ- families are more closely related than Christen's work would suggest on first examination. On the other hand, the fact that threonine synthase also catalyses a change at the $C^{\gamma}$-atom of its substrate may account for this similarity.

| |
|---|
| *O*-Succinylhomoserine(thiol)-lyase |
| *O*-Acetylhomoserine(thiol)-lyase |
| Cystathionine β-lyase |
| Cystathionine γ-lyase |

**Table 1.20:** *The γ-family of PLP dependent enzymes.*

In the γ-family PLP-Lys occurs in the same sequence segment as in the α-family, thus hinting at a possible evolutionary relationship between the two. This could be further investigated by comparison of the 3D structures of α- and γ- family members, when the structure of a γ-family member becomes available. *O*-succinylhomoserine(thiol)-lyase and *O*-acetylhomoserine(thiol)-lyase catalyse similar reactions of type e (Scheme 1.17b). Cystathionine β-synthase catalyses a reaction of type a (acting at the β-carbon as the name suggests), and the closely related cystathionine γ-synthase catalyses the similar reaction d.

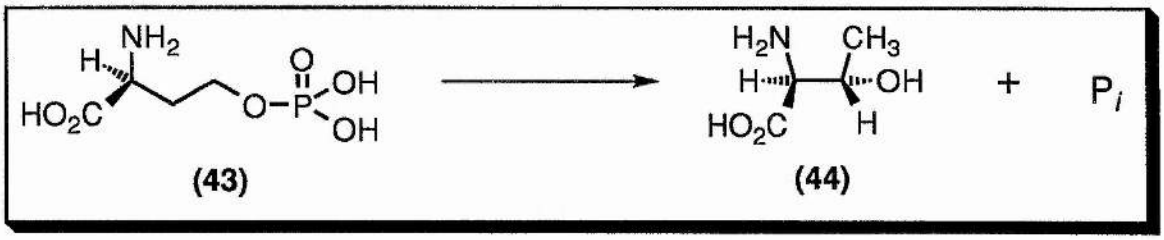
**Exceptions to the "Family Rule".**

As already mentioned, a few PLP dependent enzymes do not belong to the family that would be suggested by their regioselectivity. Tryptophanase, tyrosine phenol-lyase and 4-amino-4-deoxychorismate synthase are all members of the α-family by sequence, yet catalyse β-elimination reactions; whereas 1-aminocyclopropane-1-carboxylate synthase, another α-family enzyme, catalyses an α,γ-replacement. Similarly, threonine synthase which as we have seen assigned to the β-family, catalyses a β,γ-replacement reaction; and cystathionine β-lyase (a γ-family member) catalyses a β-elimination.

With increased access to sequence searching and alignment software, the evolutionary history of threonine synthase and other PLP dependent enzymes is gaining more attention. Using up to date computer technology it may be possible to reliably fit related enzyme sequences to already solved structures to increase our understanding of these enzymes.

## 1.4 Introduction to Threonine Synthase.

Pyridoxal 5'-phosphate enzymes had been known about for over two decades (see section 1.0) when, in 1960, Flavin and Slaughter reported a new and quite different pyridoxal 5'-phosphate dependent enzyme. In their words, the enzyme "...which will be called threonine synthetase, catalysed an elimination of orthophosphate coupled to isomerisation from α- to β- hydroxy compound, to yield threonine from *O*-phosphohomoserine.".[79] Now more commonly referred to as threonine synthase (EC 4.2.99.2), the enzyme is known to stereospecifically catalyse the conversion of (2*S*)-*O*-phosphohomoserine (**43**) to (2*S*)-threonine (**44**) and inorganic phosphate in the final step of threonine biosynthesis in plants (Scheme 1.21).[2]




**Scheme 1.21:** *The reaction catalysed by threonine synthase.*

### The Biosynthesis of Threonine from Aspartic Acid.

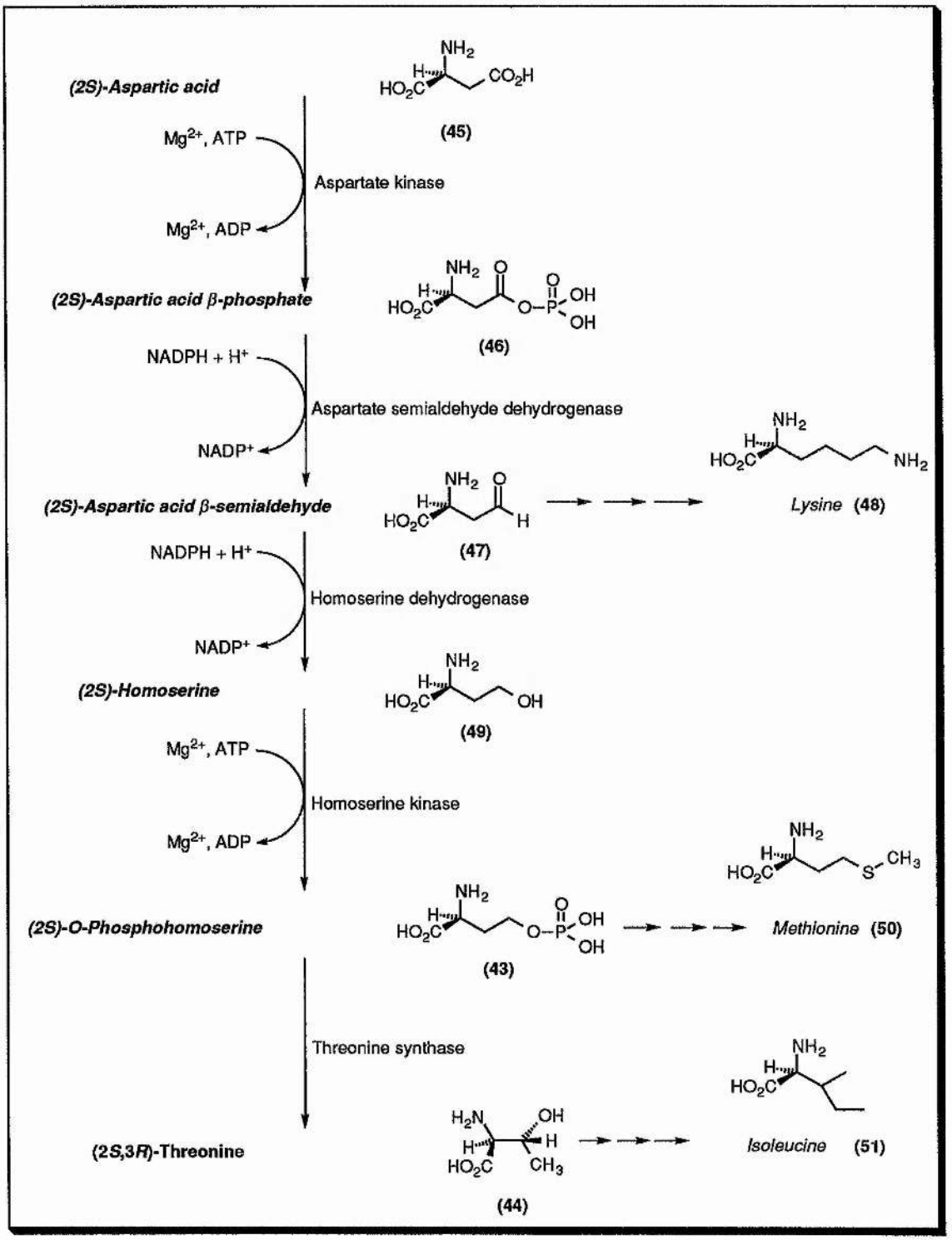
For man, (2*S*)-threonine (**44**) is one of the nutritionally essential amino acids (Table 1.22), and must be supplied in the diet. An inadequate supply leads to a negative nitrogen balance by inhibiting protein synthesis. This occurs when the ribosome-mRNA nascent polypeptide complex suspends its operation at the point where a missing amino acid should be incorporated. Other amino acids then

accumulate and are shunted into degradative metabolic pathways, causing a net loss in nitrogen.

Threonine is biosynthesised in five steps from aspartic acid (Scheme 1.23). These steps also have a role in the biosynthesis of lysine, methionine and isoleucine.[80] (2*S*)-Aspartic acid (**45**) is phosphorylated by aspartate kinase at the expense of ATP to give (2*S*)-aspartic acid β-phosphate (**46**). The enzyme aspartate semialdehyde dehydrogenase reduces this activated compound to (2*S*)-aspartic acid β-semialdehyde (**47**). (2*S*)-aspartic acid β-semialdehyde (**47**) is an intermediate in the biosynthesis of lysine (**48**), however in this case the amino acid (**47**) is reduced by homoserine dehydrogenase to yield (2*S*)-homoserine (**49**). Phosphorylation by homoserine kinase yields (2*S*)-*O*-phosphohomoserine (**43**), which is converted to threonine (**44**) by the action of threonine synthase. Phosphohomoserine (**43**) is also a precursor of methionine (**50**), and (2*S*,3*R*)-threonine (**44**) lies on the isoleucine (**51**) biosynthetic pathway.

| | Ile | Leu | Lys | Phe | Met | Cys | Thr | Trp | Val |
|---|---|---|---|---|---|---|---|---|---|
| **Child** | 90.0 | - | 90.0 | 90.0 | 85.0 | - | 60.0 | 30.0 | 85.0 |
| **Man** | 10.4 | 9.9 | 8.8 | 4.3 | 1.3 | 11.6 | 6.5 | 2.9 | 8.8 |
| **Woman** | 5.2 | 7.1 | 3.3 | 3.1 | 4.7 | 0.5 | 3.5 | 2.1 | 9.2 |
| **WHO*** | 3.0 | 3.4 | 3.0 | 2.0 | 1.6 | 1.4 | 2.0 | 3.0 | - |

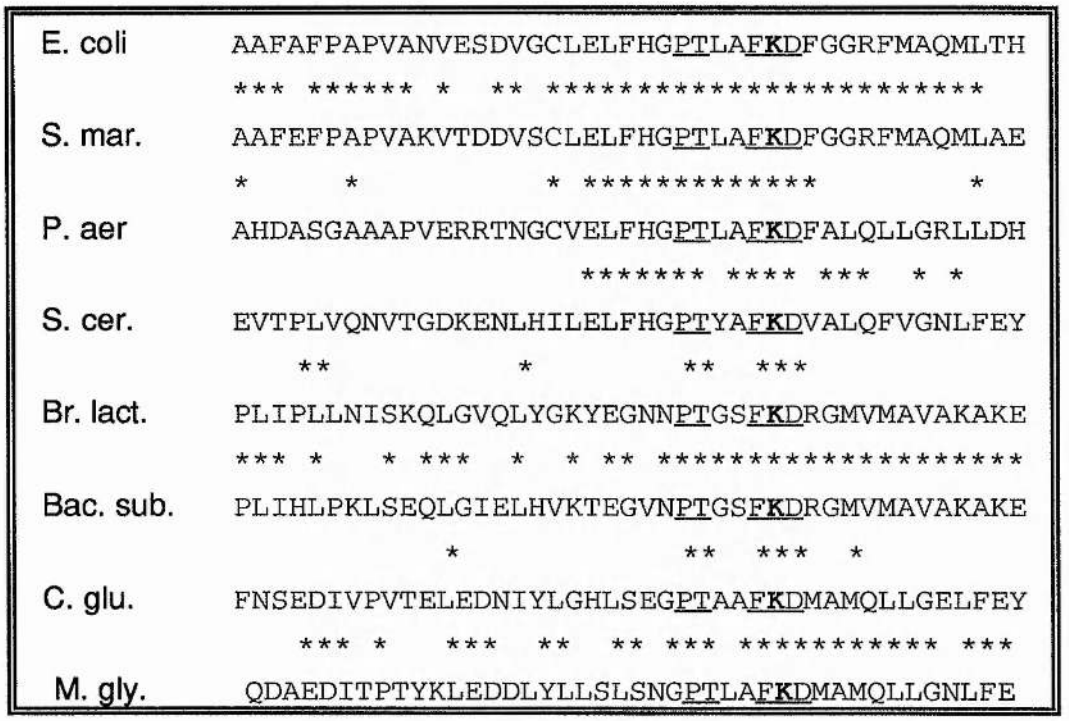
**Table 1.22:** *The minimal requirements of human beings for essential amino acids in mg $kg^{-1}$ $day^{-1}$.*[81] * *World Health Organisation norms.*




**Scheme 1.23:** *The biosynthesis of threonine from aspartic acid.*

**Threonine Synthase from Bacterial Sources.**

The gene for threonine synthase has been isolated and sequenced from several sources including yeast,[82] *Brevibacterium lactofermentum*,[83] *E. coli*,[84] *Bacillus subtilis*,[85,86] *Corynebacterium glutamicum*,[87] *Serratia marcesens*[88] and *Pseudomonas aeruginosa*.[89] When the amino acid sequences of the enzyme from various sources were compared, the enzymes showed remarkable homology, especially for the residues close to the active site (Table 1.24). The lysine residue (bold and underlined) that forms the internal aldimine with pyridoxal 5'-phosphate is conserved in all cases, as are the phenylalanine and aspartic acid residues flanking it. The positions 5 and 4 residues upstream of the Lys-PLP are occupied by a proline residue and a threonine residue respectively. In all sequences published to date these amino acids are conserved.




| | | | |
|---|---|---|---|
| E. coli | *Escherichia coli* | S. mar. | - *Serratia marcesens* |
| Bac sub. | *Bacillus subtilis* | P. aer. | - *Pseudomonas aeruginosa* |
| Br. lact. | *Brevibacterium flavum* | M. gly. | -*Methylobacillus glycogenes* |
| C. glu. | *Corynebacterium glutamicum* | S. cer. | -*Saccharomyces cerevisiae* |

**Table 1.24:** *Sequence homology of threonine synthase from bacterial sources. Stars indicate conserved residues between sequences.*

Threonine synthase was first purified 500-fold from*Neurospora* in 1960,[79] through a series of acetone and ammonium sulfate fractionations, followed by ion exchange chromatography. A crude preparation of the enzyme was described from *Brevibacterium flavum* in 1968.[90] There were no reports of a purification of threonine synthase to homogeneity until Parsot and his co-workers described a preparation of the enzyme from *E. coli* K12 strain Tir $8^{83}$ (a constitutive mutant for the enzyme).[91] The purification was carried out by a variety of ion-exchange

chromatography steps. Threonine synthase from *E. coli* was found to have a molecular weight of between 46 000 and 48 000 Daltons as determined by SDS-PAGE, and of 47 060 as deduced from the amino acid sequence. Han and coworkers purified the enzyme from *Corynebacterium glutamicum*. The molecular weight of the protein was reported to be 54 000 Daltons as determined by SDS-PAGE and amino acid sequence.

**Threonine Synthase from Plant Sources.**

There is substantial interest in the aspartate pathway of plant species due to man's desire to improve the nutritional qualities of some crops. Cereals are deficient in lysine and methionine, and legume seeds exhibit low levels of methionine.[92] In addition, an understanding of the aspartate pathway and particularly of threonine synthase in plant tissues may be of help in the design and synthesis of effective and selective herbicides. Wallsgrove and coworkers have shown that the enzymes involved in the aspartate pathway are located in the chloroplasts of plants.[93]

Various groups have succeeded in purifying threonine synthase (TS) from plant sources and these are summarised below (Table 1.25). The enzyme was first detected in sugar beet and radish leaves,[94] and has since been partially purified from *Lemna minor*,[95] pea,[96] barley,[97] *Lemna pausicostata*,[98] and soybean.[99]

In all plants examined to date, threonine synthase shows an almost absolute requirement for *S*-adenosylmethionine (SAM). SAM appears to act as an allosteric activator, with a Hill coefficient of 2.0-2.5. Half maximal activation occurs between 40 and 200 μM. Plant TS has a pH optimum of around 8.0 and no isoenzymes have yet been detected.

| Source | | $K_M$ (μM) | $V_{max}$ | Hill coefficient |
|---|---|---|---|---|
| Sugar beet | | 2 700 | 38.2 nmoles/h | |
| | +SAM | 2 200 | 534.3 nmoles/h | - |
| Pea | | 2 200 | | 2.1 |
| | +SAM | 670 | 4 fold increase | |
| *Lemna* | +SAM | 5.1 | 47.2 pmol/40 min | 2.5 |

**Table 1.25:** *Kinetic properties of threonine synthase from higher plants. The Hill Coefficient is a measure of cooperativity, where the binding of ligand (in this case SAM) to protein is at 50% saturation.*

**Threonine Synthase Expression in Mammalian Systems.**

Non-ruminant diet can be significantly improved by feeding rations supplemented with the essential amino acids, and especially lysine and threonine. The ability to introduce pathways for the biosynthesis of these amino acids into animal cells would eliminate the need for this supplementation.[100] Rees and Hay recently described the expression of the aspartokinase and threonine synthase gene in mouse 3T3 cells.[101] These cells were then able to grow on threonine deficient medium containing (2*S*)-homoserine.

There is no known case of threonine synthase or homoserine kinase occurring naturally in mammalian systems, however, it has been noted that (2*S*)-*O*-phosphohomoserine (**43**) is an antagonist of *N*-methyl-D-aspartate (NMDA) in rat brain.[102,103] Phosphohomoserine has been the subject of a patent for its use in conjunction with the drug levemopamil. The administration of these compounds together is used to treat damage to neurons in patients with AIDS related dementia,

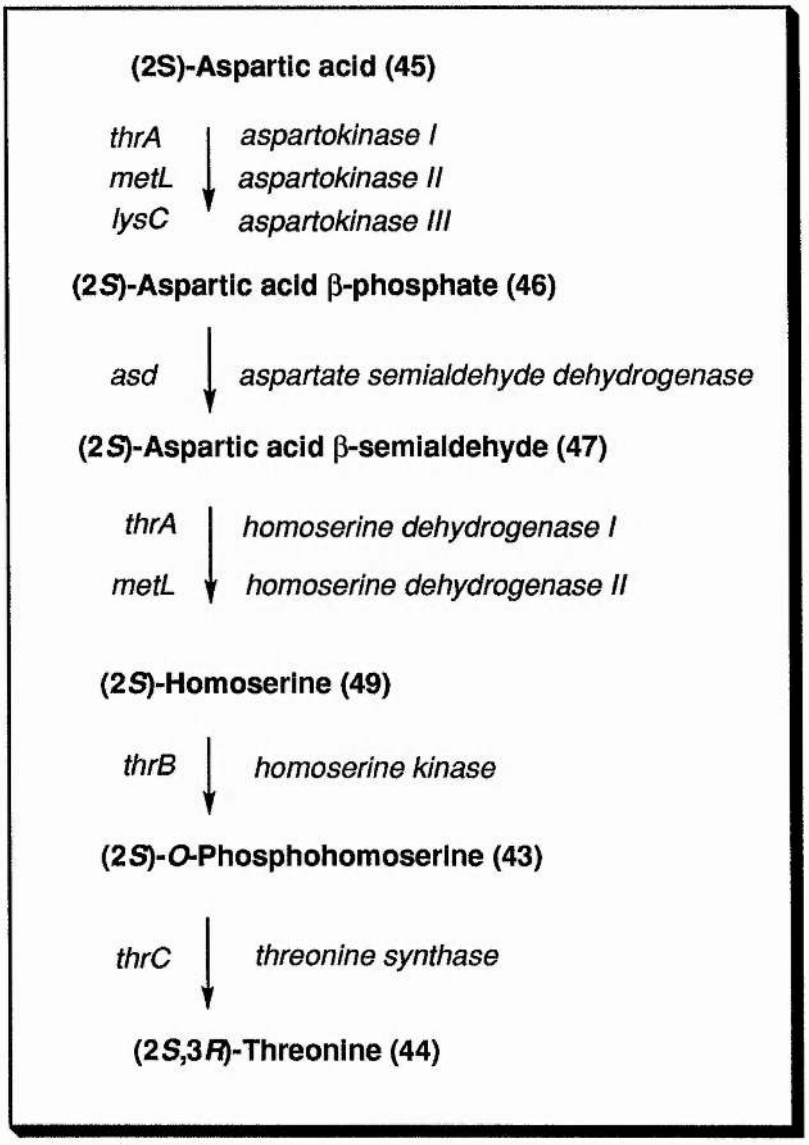
myelopathy, peripheral neuropathy and vision loss, by reduction of the gp120 responsive rise in calcium ions.[104]

**The Threonine Operon of *E. coli*.**

Studies over the past 40 years have shown that amino acid biosynthesis in bacteria is regulated by controlling the catalytic efficiency of certain key enzymes, and by controlling the levels of these enzymes *via* gene expression.[105] Threonine biosynthesis is no exception, and the method of control has been studied in yeast,[106] *Corynebacterium glutamicum*,[86,107] *Brevibacterium flavum*,[108] *Serratia marcescens*,[87] *Pseudomonas aeruginosa*,[88] *Bacillus subtilis*[41] and *Brevibacterium lactofermentum*.[109] By far the best understood is the control of threonine biosynthesis in *E. coli*. This is discussed here.

It is established that (2*S*,3*R*)-threonine is biosynthesised in five steps from aspartic acid (scheme 1.23). It has been found that the genes encoding most of the enzymes that catalyse these steps exist as an operon (Scheme 1.26).

The *thrA* gene encodes the threonine-sensitive bifunctional aspartate kinase I - homoserine dehydrogenase I.[110,111] *E. coli* K12 contains two additional aspartate kinases and one additional homoserine dehydrogenase. The biosynthesis of the bifunctional aspartate kinase II - homoserine dehydrogenase II is regulated by methionine.[112] The activity and biosynthesis of aspartate kinase III is regulated by lysine.[113]




**Scheme 1.26:** *Enzymes of the threonine biosynthetic pathway.*

The *thrB* gene encodes homoserine kinase, which catalyses the phosphorylation of (2*S*)-homoserine to yield (2*S*)-*O*-phosphohomoserine (**43**). It has an $\alpha_2$-structure, each subunit having a mass of around 29 000 Daltons.[114] The purified protein requires $Mg^{2+}$, and $K^+$ stimulates activity.[115] *ThrB* has been sequenced, and the amino acid sequence deduced.[116] Homoserine kinase is competitively inhibited by threonine with an apparent $K_i$ of 0.6 mM.[114] Product inhibition is not observed,

and the threonine analogues (2*R*,3*R*)-threonine, glycylthreonine, 3- and 4-hydroxybutyrate, 3-aminobutyrate and *O*-methyl threonine display no inhibitory effect even at high concentrations.[114]

The coordinate regulation of the threonine biosynthetic enzymes was first demonstrated in *Salmonella typhinurium* and *E. coli* by Freundlich in 1963.[117] It was observed that both threonine and isoleucine were required for attenuation. The structural genes encoding the threonine biosynthetic enzymes have all been identified and mapped.[118-121] The *thr* operon of *E. coli* consists of *thrA*, *thrB* and *thrC* at 0 min on the linkage map. The *asd* gene which encodes aspartate semialdehyde dehydrogenase lies at 75 minutes.[122]

The order of transcription: *thrA*, *thrB*, *thrC* has been established by analysis of nonsense mutations and phage Mu insertions in the *thr* cluster,[119] and by analysis of a λ phage insertion. When λ phage insertion was directed to the *thr* regulatory region it was found that it lay to the left of *thrA* (that is *trpR*-λ-*thrA*), and pleiotropically affected the expression of both *thrA* and *thrB*. Deletion analysis confirmed these findings, and the genetic fine structure of the *thr* operon has been deduced by deletion mapping.[123] Complementation analysis of the *thrB* and *thrC* regions showed that these genes consisted of single cistrons, whilst *thrA* was composed of two cistrons specifying a single polypeptide chain. The findings are in accord with the bifunctional character of the *thrA* gene product.

Various regulatory mutants of *E. coli* have been studied,[124-128] and have identified a regulatory region that precedes the *thr* operon structural genes. Mutation in this region causes pleiotropic effects on *thr* expression. It was originally thought that these mutants were classical operator mutants, in an analogous manner to those isolated in the *trp* and *lac* operons.[121,124] However, because no apo-repressor mutants have been isolated, and because all mutations analysed to date lie in the

*thr* attenuator; it does not appear that the *thr* operon of *E. coli* is regulated by a repressor-operator mechanism.

The *thr* operon promoter lies approximately 230 nucleotides upstream of *thrA*.[127] Regulation of transcription termination is a common regulatory mechanism,[129] and can either be rho-dependent or rho-independent, depending upon the *in vitro* termination efficiency. As in other rho-dependent terminators the *thr* regulatory region contains extensive dyad symmetry and the characteristics of being G-C rich, followed by a stretch of A-T base pairs at -63 to -30 bases. When described into RNA, the terminators give rise to secondary structure containing a stem and loop with a run of uridine residues at the 3'-end (necessary for efficient termination (figure 1.27)).[130,131] *In vitro* transcription studies found the termination efficiency of the *thr* attenuator to be around 90%.[127]

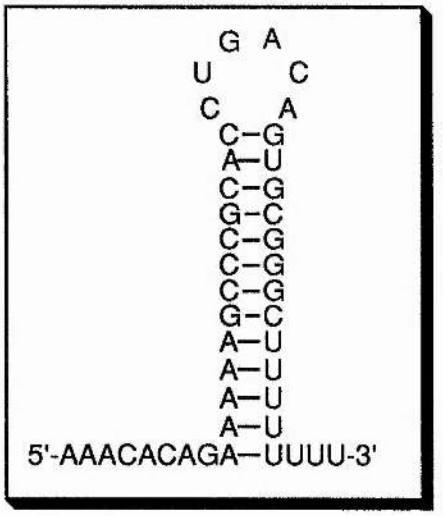



**Figure 1.27:** *The secondary structure of DNA from the thr regulatory region from -71 to -27 bases, presented in the most stable conformation to maximise base pairing in the RNA termination structure.*

In addition to the promoter and attenuator, the *thr* regulatory region contains two features relevant to attenuation: a sequence encoding a leader peptide, and a pause site for RNA polymerase. Positions -147 to -82 encode the putative leader peptide of 21 amino acids. This peptide contains eight threonine and four isoleucine residues. Eleven of these twelve codons occur consecutively, and their replacement by histidine and tyrosine codons produced mutants showing a loss of regulation by threonine and isoleucine.[132]

The *thr* regulatory region contains several regions of overlapping rotational symmetry. The first lies in the attenuator region at -63 to -30 bases, the second at -93 to -49 bases, overlapping the attenuator. This allows the formation of two mutually exclusive secondary structures; formation of the first causing termination, and formation of the second blocking the attenuator region allowing transcription to proceed through into the structural genes. Incomplete regions of rotational symmetry include -99 to -90 bases and direct repeats -130 to -121 and -112 to -103. A pause site for RNA polymerase has been shown to lie within the region -90 to -80 bases.[133] The significance of this site is not known, but may be to facilitate a link between the transcription and translation of the leader region.

A model has been proposed for the multivalent regulation of *thr* operon expression involving coupled transcription and translation of the leader region and the consequences of this coupling on RNA secondary structure formation and transcription termination.[124] This is a similar model to those proposed for the other amino acid biosynthetic operons.[134-142] The RNA encoded by the *thr* regulatory region from -147 to -27 bases has four possible secondary structures (Figures 1.28a and 1.28b) reflecting a) attenuation; b) transcription through the attenuator; c) and d) no translation of the leader RNA.

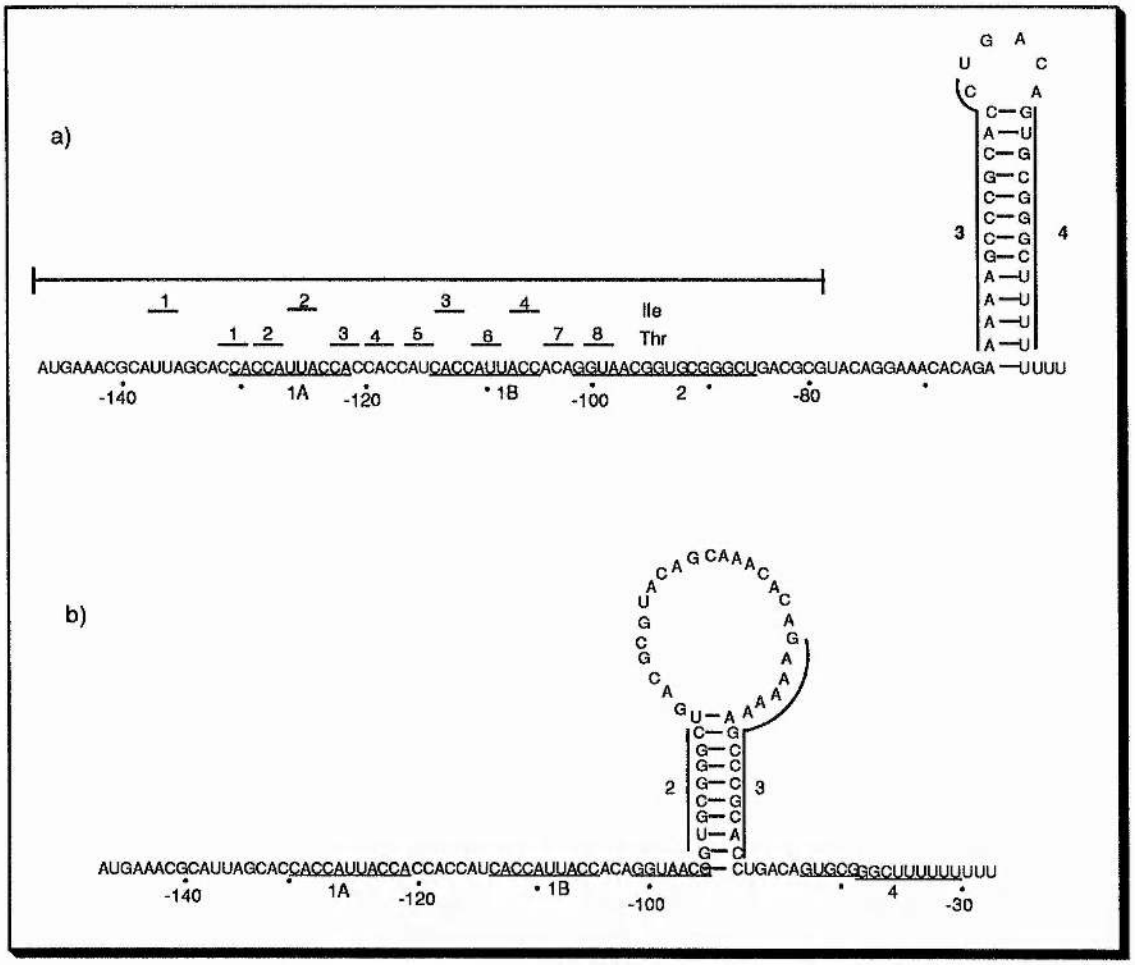



**Figure 1.28a:** *Control of thr operon expression by attenuation. Model a) shows attenuation and b) shows transcription through the attenuator. The bar in a) indicates the coding region for the leader peptide. The numbers above the sequence denote the positions of the threonine and isoleucine codons in the leader peptide. The boxed areas indicate regions involved in secondary structure formation.*

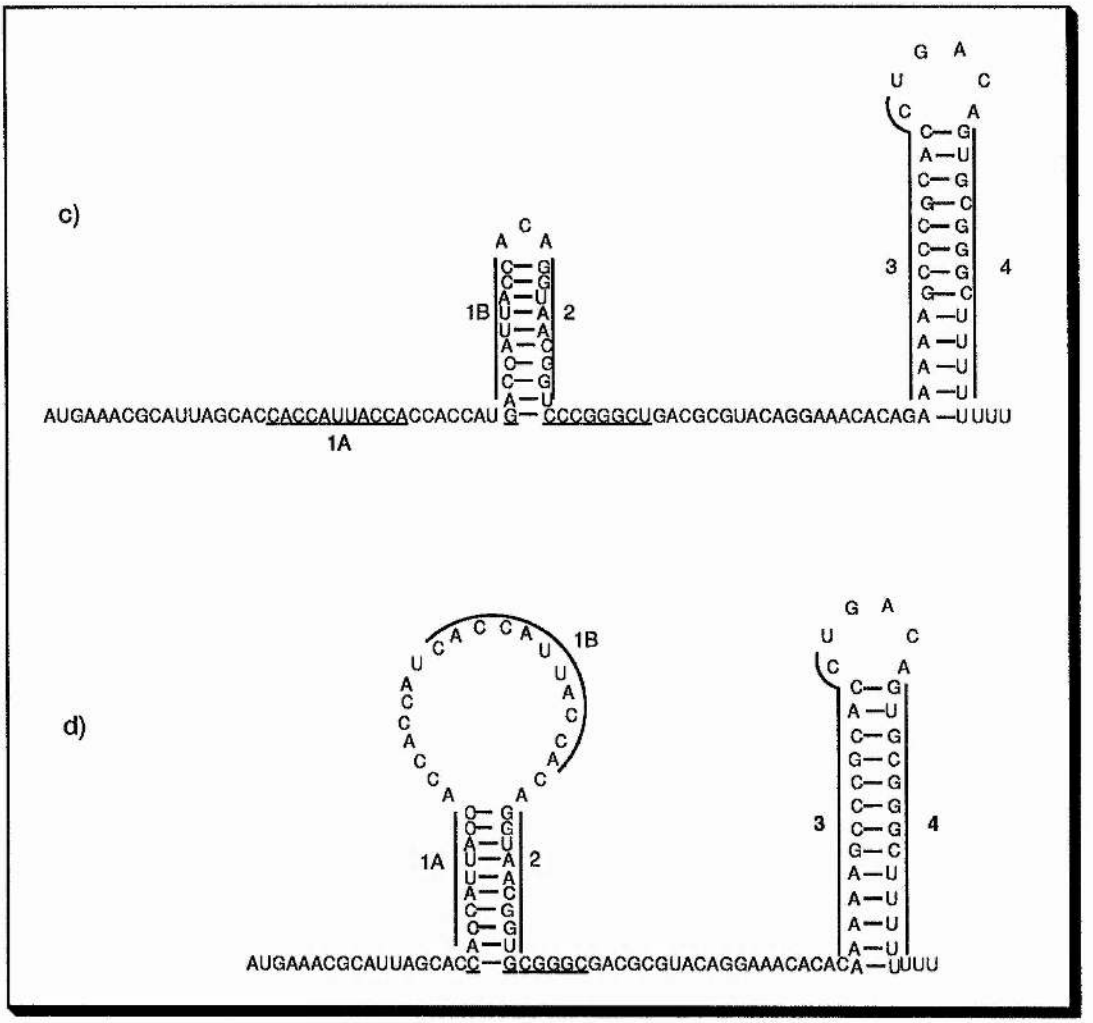



**Figure 1.28b:** *Control of thr operon expression by attenuation. Models c) and d) show poor or no translation of the leader RNA.*

Under conditions of threonine and isoleucine excess, transcription initiating at the *thr* promoter is coupled to the translation of the leader peptide terminating at the UGA codon at -82. The formation of the RNA termination structure (stem 3-4, a) is favoured due to ribosomal blocking of the pairing bases -58 to -49 and -93 to -84.

If threonine and isoleucine become growth limiting, the ribosomes slow down or stall in the region of consecutive threonine and isoleucine codons. This allows the formation of stem 2-3 (b), precluding the formation of the RNA termination structure. The *thr* structural genes are therefore expressed.

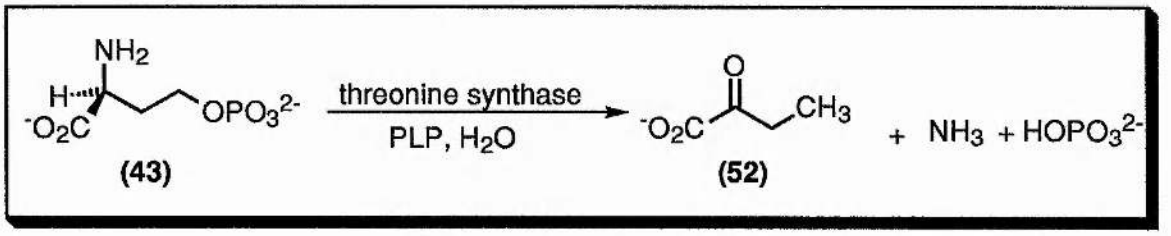
When the leader RNA is not translated, transcription is efficiently terminated by formation of RNA secondary structures stem 1A-2 (c) or 1B-2 (d), allowing formation of the termination structure. Evidence for this postulate has been obtained in both the *trp*[143] and *his*[144] operons.

A G-C rich region of dyad symmetry followed by a stretch of six uridine residues occurs 21 base pairs after the stop codon of *thrC*.[83] This region could form a stable stem and loop secondary structure, a characteristic rho-dependent transcription termination site, and could be the *thr* operon termination region.

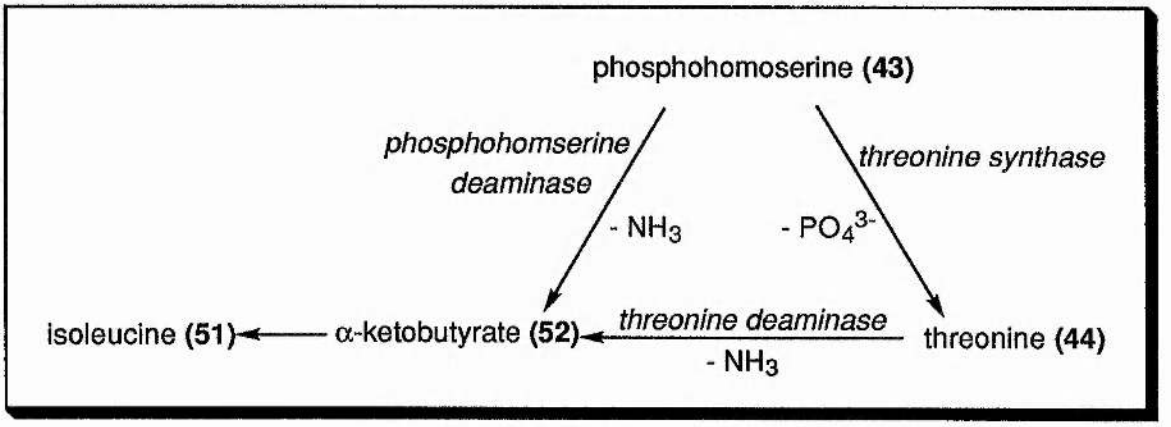
Amplification of the *thr* operon has been carried out by various groups,[145-147] and has been the subject of patents.[148,149] An understanding of the mechanisms of control of the *thr* operon is allowing the application of molecular biology to form threonine accumulating mutants of microorganisms.

**Threonine Synthase Has an Associated Dehydratase Activity.**

It has been shown that threonine synthase of *Bacillus subtilis* can also catalyse the deamination of (2*S*)-*O*-phosphohomoserine (**43**) to yield α-ketobutyrate (**52**).

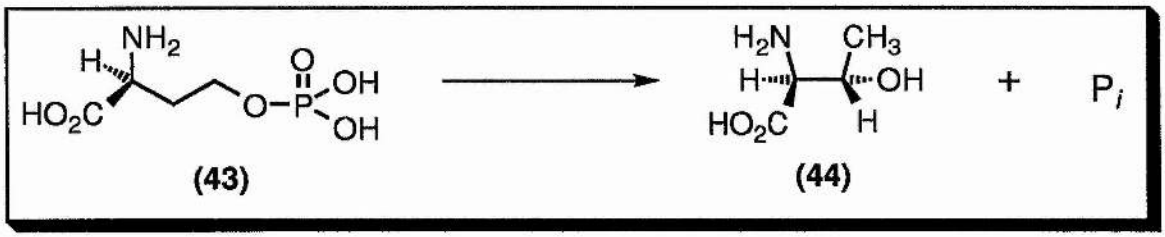



This is a distinct activity from the usual route to α-ketobutyrate *via* threonine deaminase, and has been shown not to pass through a threonine intermediate.



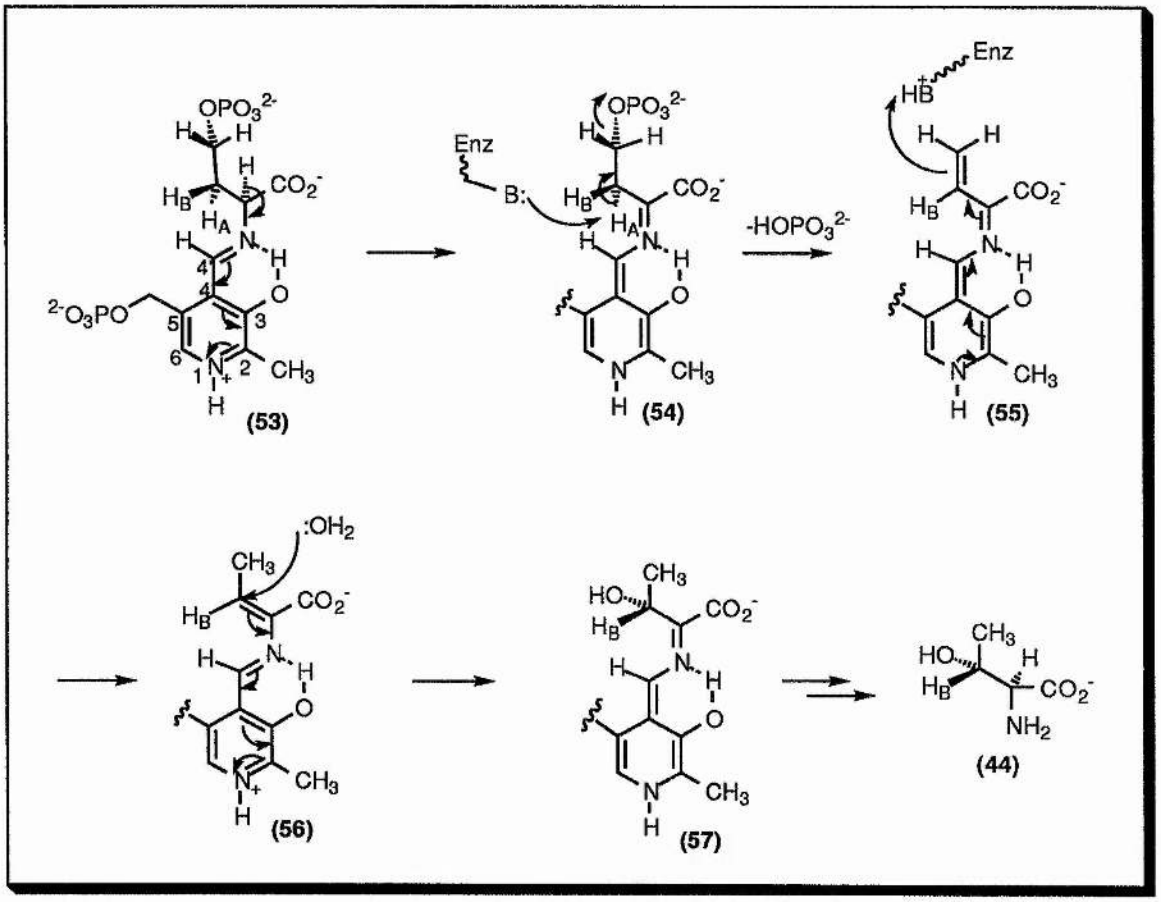

This phophohomoserine deaminase activity was shown to be a lesser activity of threonine synthase, as the two activities are shown to co-purify, to be of the same molecular weight, to co-inactivate with heat, and to display the same $K_M$ for the substrate phosphohomoserine (**43**).[150] Mutational evidence was also cited.[85,151] The associated deaminase activity has not been reported in any other organism to date.

**The Postulated Mechanism of Threonine Synthase.**




The first mechanistic studies into threonine synthase were carried out by Flavin and Slaughter in 1960 on the *Neurospora* enzyme.[152] When the reaction catalysed by threonine synthase was carried out in $^{18}O$-labelled water, $^{18}O$-label was incorporated into threonine, but not into the eliminated phosphate. Phosphate is therefore released by a non-hydrolytic elimination, with cleavage of the C-O bond of the phosphate ester. When the reaction was carried out in deuteriated or tritiated water, one non-exchangeable solvent hydrogen was incorporated into the γ-position of threonine. This indicates that the reaction proceeds through an intermediary derivative of vinylglycine (**31**). A second solvent hydrogen was introduced at $C^{\alpha}$ of threonine. From the incubations conducted in deuterium oxide, it was evident that threonine synthase introduced two atoms of deuterium into the product. It could therefore be deduced that no significant internal proton transfer occurred.

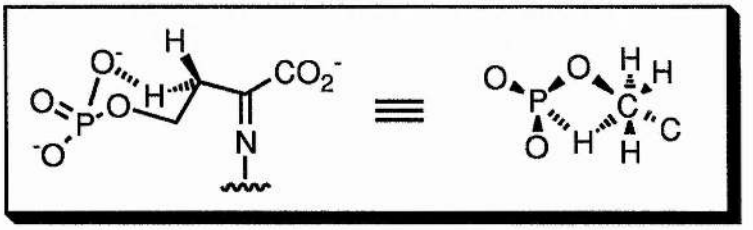
Fuganti later investigated the reaction of threonine synthase with phosphohomoserines stereospecifically labelled with tritium at each of the C-3 positions.[153] He found that the 3-*pro*-S hydrogen was removed, and that the 3-*pro*-R hydrogen was retained in the threonine product. This small amount of mechanistic information allows the following mechanism for the conversion of (2*S*)-*O*-phosphohomoserine (**43**) to (2*S*,3*R*)-threonine (**44**) (Scheme 1.29) to be proposed.




**Scheme 1.29:** *The proposed mechanism of threonine synthase.*

The conversion of the external aldimine (**53**) to the quinonoid intermediate (**54**) increases the acidity of the 3-*pro*-S proton of the substrate such that the β,γ-elimination of phosphoric acid can occur. If threonine synthase shows the same stereochemical imperative as other PLP-dependent enzymes in utilising the 4'-*si*-face of the coenzyme for its reactions the elimination would occur in a *syn*-fashion to give the conjugated enamine (**55**). Protonation at C-4 of the imino acid moiety of (**56**) would allow attack by water at C-3 on the 4'-*si*-face of the coenzyme to give product quinonoid (**57**). Protonation at C-2 would then furnish the product aldimine, which upon transaldimination would yield (2*S*,3*R*)-threonine (**44**).

The β,γ-elimination of phosphoric acid may happen in a sequential or concerted manner. It has also been postulated that if this elimination does occur in a concerted manner, then the phosphate may act as the base removing the C-3 proton.[154] This would give a plausible six-membered intermediate.



There are many questions that arise from this postulated mode of action, and these will be considered in the discussion.

**The Inhibition of Threonine Synthase.**

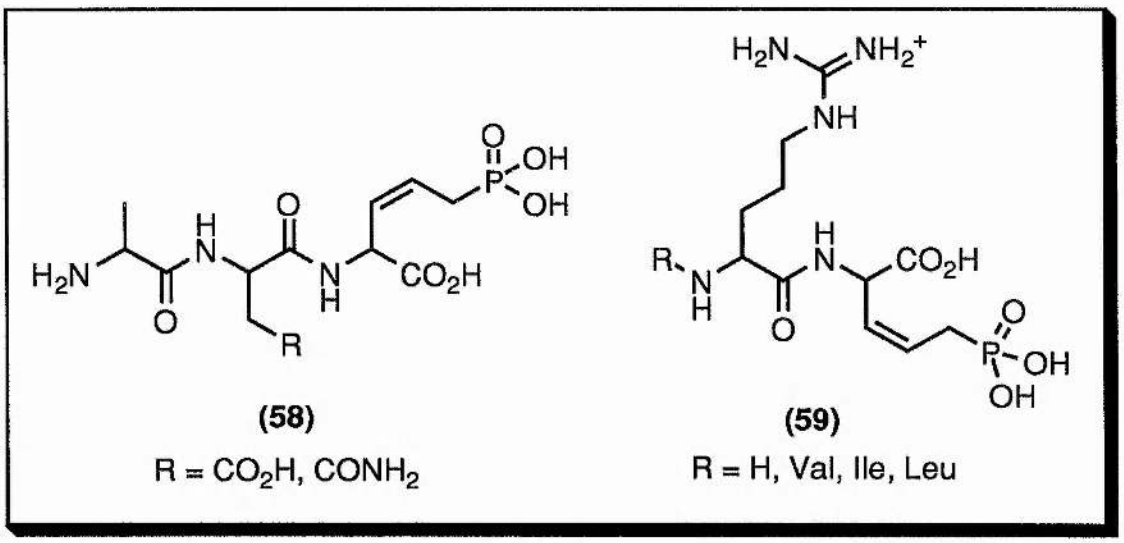
Compounds that can block the action of threonine synthase may be of herbicidal, bactericidal or fungicidal potential. These compounds may also be able to yield mechanistic information about the enzyme. Some amino acids inhibit threonine synthase as part of the control of enzyme activity. Cysteine shows strong inhibition of TS from soybean,[98] sugar beet[93] and *Brevibacterium flavum*;[89] but no inhibition of TS from other sources such as barley[155] and *Lemna*.[97] Methionine shows inhibitory effects for the barley enzyme,[155] whilst glutathione inhibits in *Brevibacterium flavum*.[89] Other natural amino acid inhibitors are listed in Table 1.30.

| | Beet | Barley | *Lemna* | Soy bean | *Brev. flavum* |
|---|---|---|---|---|---|
| cysteine | √ | x | x | √ | √ |
| methionine | - | √ | - | - | - |
| glutathione | - | - | - | - | √ |
| alanine | - | - | - | - | √ |
| threonine | - | - | - | - | √ |
| isoleucine | - | - | - | - | √ |
| lysine | - | - | - | - | √ |
| phosphothreonine | - | - | √ | - | - |
| phosphoserine | - | - | √ | - | - |

**Table 1.30:** *The inhibition of threonine synthase from various sources by naturally occuring amino acids.*

It has been reported that AMP is a potent and stucturally specific competitive inhibitor of threonine synthase of *Lemna.*[156] Phosphate is also an inhibitor of the enzyme in *Lemna*, but this has not been reported for any other source of the enzyme.[156] Interestingly threonine synthase from *Lemna* does not seem to be inhibited by propargylglycine (**34**) or gabaculine (**38**). Furthermore vinylglycine (**31**) inhibits the *Lemna* enzyme only very weakly.

The plumbemycins (**58**)[157,158] and rhizocticins (**59**)[159] are di- and tripeptides that possess bactericidal, fungicidal or acaricidal activity depending on the nature of the peptide.



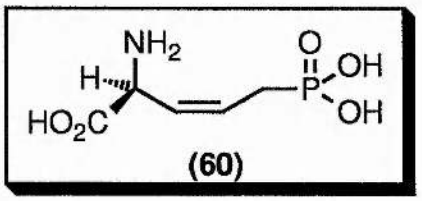
**(58)**

R = $CO_2H$, $CONH_2$

**(59)**

R = H, Val, Ile, Leu

(2*S*)-*Z*-2-Amino-5-phosphonopent-3-enoic acid, or Z-APPA (**60**), is the active component of these two groups of polypeptides. This compound has been shown to decrease the activity of threonine synthase by acting as a suicide inhibitor.[160] It has also been reported as having herbicidal activity.[158,161,162].



**(60)**

Recently the synthesis and investigation of novel inhibitors of threonine synthase has been attracting considerable attention.[163-166] The inhibitors depicted below are effective (Figure 1.31).

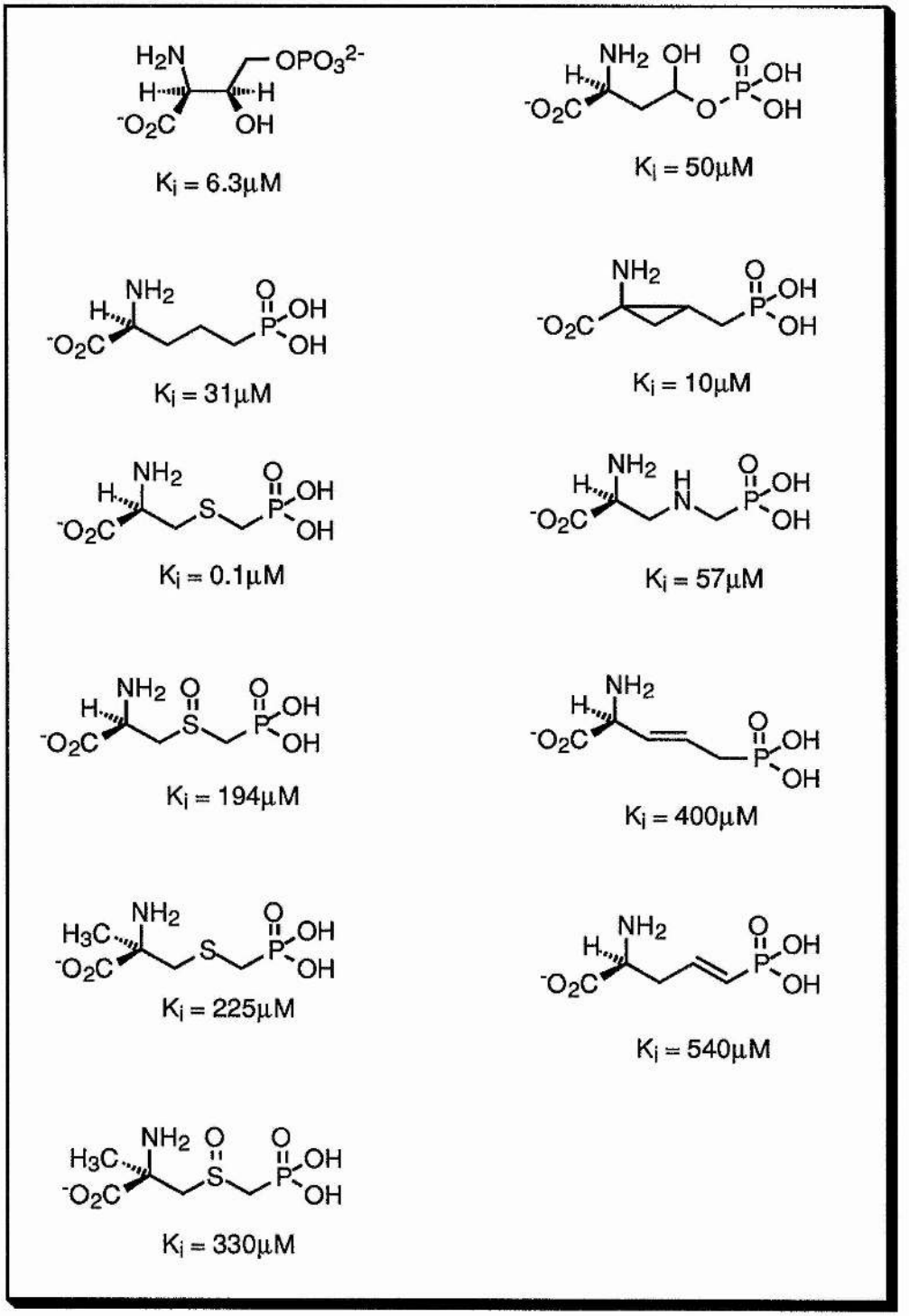



**Figure 1.31:** *Some inhibitors of threonine synthase.*

# DISCUSSION

## 2 Introduction.

As already mentioned (section 1.4) there is considerable interest in the structure and mechanism of threonine synthase.[84] Such information is needed to underpin the rational design of potential inhibitors of the enzyme, which may be useful as herbicides, fungicides or bactericides. In general terms, specific inhibitors would be of particular importance to the agrochemical industry where targeting an enzyme that only occurs in bacterial and plant biosynthetic pathways is highly desirable. Clearly, inhibitors of this type may cause no adverse affects on animal species, and, therefore, potentially represent a strategy for minimal environmental impact. Furthermore, and specifically, it is known that blocking amino acid biosynthesis is a highly effective method of inhibiting growth in plants and bacteria.[167]

Although there is much literature on the sequence and mechanisms of control of threonine synthase, details of its mode of action remain sparse. In order to obtain as much information as possible about the kinetics and mechanism of the enzyme, it was important to first find a suitable source of the enzyme, and method for its purification. It was also important to develop an efficient and convenient synthesis of the substrate (2*S*)-*O*-phosphohomoserine (**43**), which would be flexible enough to allow for the synthesis of anologues and isotopomers.

### The Postulated Mechanism of Threonine Synthase.

In spite of the fact that there was only a small amount of information available in the literature on the mechanism of the threonine synthase system, by drawing analogies to other PLP dependent enzymes it was possible to suggest the

mechanism shown in Scheme 2.00 (see also section 1.4). The mechanism provided a framework for our thinking and the design of all of the experiments described in this thesis.

After the substrate binds to holoenzyme, transaldimination occurs to give the external aldimine (**53**). It is believed that the conversion of the external aldimine (**53**) to the quinonoid intermediate (**54**) increases the acidity of the C-3 protons of the substrate by creating an allylic environment. Thus the β,γ-elimination of phosphoric acid can occur to give the conjugated enamine (**55**).[79,153] It is known that in yeast the 3-*pro*-S proton is removed in this step, as shown in Scheme 2.00. Protonation at C-4 of the imino acid moiety of (**55**) would give the enimine (**56**). Attack by water at C-3 of the enimine (**56**) would then give the product quinonoid (**57**). Protonation at C-2 of the quinonoid (**57**) would then furnish the product aldimine, which upon transaldimination would yield (2*S*,3*R*)-threonine (**44**).

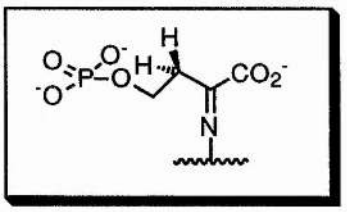
This mechanism, although plausible, still leaves many questions to be answered:

i) How kinetically important to the overall mechanism is the removal of the $C^{\alpha}$-proton to give the quinonoid intermediate (**54**)? In other words, is it kinetically more difficult to remove the $C^{\alpha}$-H atom than the C-3 proton?

ii) In the *E. coli* enzyme, is it the 3-*pro*-S proton that is removed? When Fuganti determined the stereochemistry of this step he used the yeast enzyme,[153] and although one would expect the stereochemistry to be the same in this case, this cannot be assumed. Yeast is a eukaryotic organism, and has a far more complex metabolic system than prokaryotes such as *E. coli*.

iii) The removal of the C-3 proton and the elimination of phosphate from the quinonoid intermediate (**54**) may be sequential or concerted. If the enzyme does

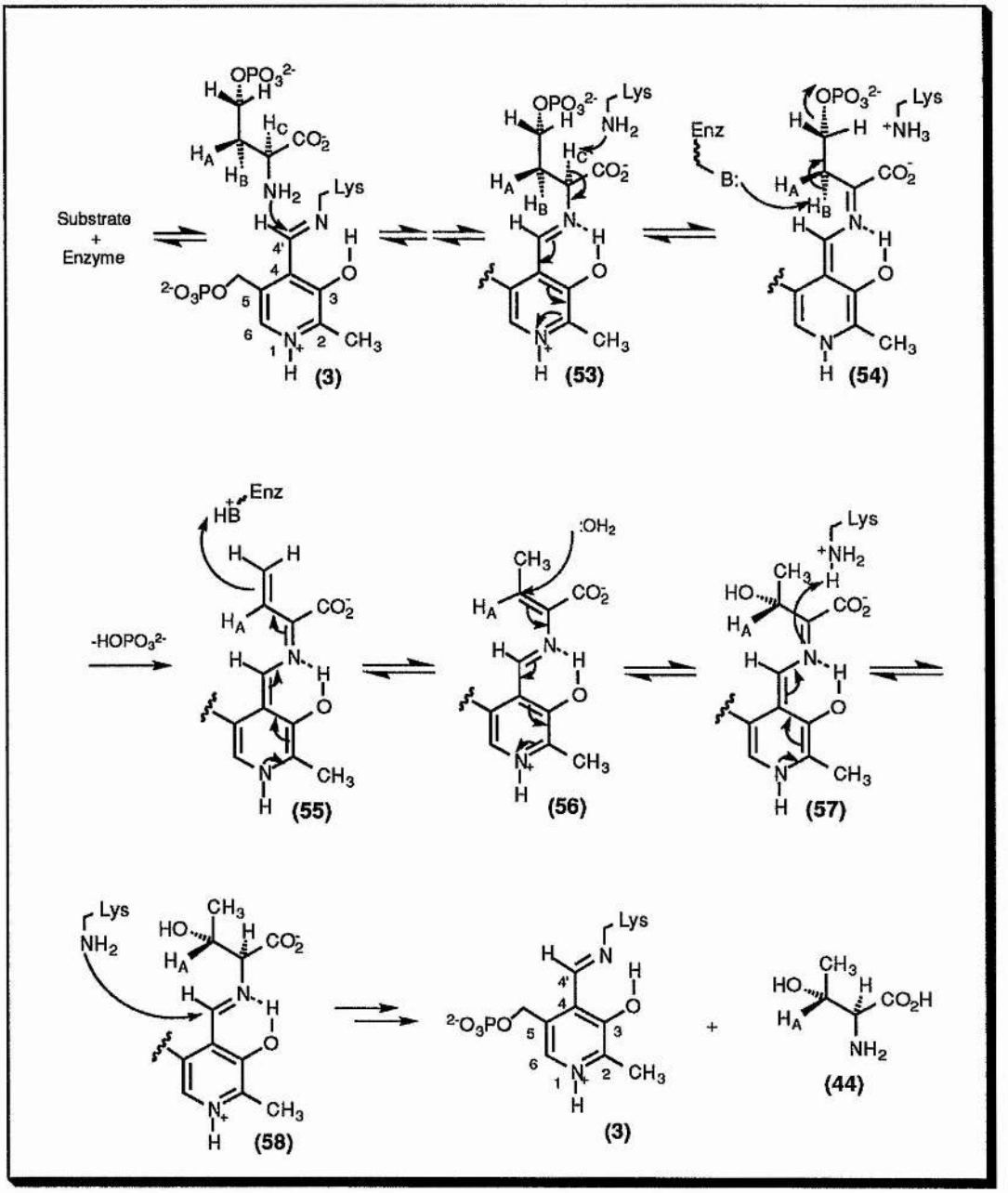
indeed show the same stereochemical imperative as other PLP dependent enzymes in utilising the 4'-*si*-face, then the elimination would occur in a *syn* fashion to give conjugated enamine (**55**), as shown in Scheme 2.00. It has also been postulated that if this elimination does occur in a concerted manner, then the phosphate may act as the base removing the C-3 proton.



It is also possible that the proton may be abstracted by the ε-amino group of the transaldiminated lysine residue. Also, we do not know which face of the coenzyme the phosphate leaves from.

iv) Does reprotonation of the enamine (**55**) to give the imino acid moiety (**56**) occur with retention of configuration, and which side of the coenzyme does the protonation occur from? Is this step reversible? Is the proton attacking at C-4 derived from the same base that removed the C-3 proton, *i.e.* is there internal proton transfer when the substrate is converted to product?

v) The attack of water at C-3 of (**56**) is defined by the stereochemistry of C-3 of threonine (that is 3*R*), but the geometry of the attack is not known with respect to the coenzyme. The water molecule could attack from either the 4'-*si*- or 4'-*re*-face of the coenzyme.




**Scheme 2.00:** *The postulated mechanism of threonine synthase.*

From the mechanism outlined above it was evident that we needed to develop a synthesis for the substrate (2*S*)-*O*-phosphohomoserine (**43**) which be amenable to isotopic substitution at various positions on the substrate. For instance, by labelling phosphohomoserine with deuterium or tritium it may be possible to address the

questions raised earlier in points i), ii) and iii).

In designing the best route for the synthesis of the substrate (2*S*)-*O*-phosphohomoserine (**43**), it was important to keep in mind the questions we would like to answer. The investigation of point i) the kinetic importance of $C^{\alpha}$-H removal could be addressed by the inclusion of a deuterium atom in this position, allowing the measurement of the primary deuterium isotope effect. The confirmation of Fuganti's finding's[153] regarding the stereochemistry of C-3 proton removal (point ii) could be investigated by the stereospecific labelling of phosphohomoserine at each of the C-3 positions. If the isotope incorporated into the substrate was deuterium this would facilitate the assessment of the kinetic importance of this step, also by measurement of the kinetic isotope effect. If we wanted to alter the substrate in these ways, it would be necessary that the synthesis was amenable to this.

If the transfer of the deuterium from the C-3 position to the C-4 position was examined by $^1$H-NMR spectroscopy, it would be possible to comment on whether the same base was responsible for reprotonation of C-4 (point iv). Flavin and Slaughter saw no internal proton transfer of this sort, when they assessed tritium transfer from the substrate to the product.[152] However if the base-bound proton is readily exchangeable with solvent protons, then only a very small amount may be transferred, and careful analysis of the isotope content of the threonine methyl group from very short-time experiments would be required

The synthesis of phosphohomoserine analogues stereospecifically altered at each of the C-4 positions, would allow us to deduce the geometry of the reaction at C-4. For example, (2*S*,4*S*)- and (2*S*,4*R*)-methyl-*O*-phosphohomoserine could be synthesised. If either diastereomer was a substrate, then we may be able to infer the geometry of the active site with respect to C-4.

It was decided to commence our studies by first tackling the stereochemistry of the C-3 proton removal and its kinetic importance to the overall mechanism, together with the importance of the $C^{\alpha}$-H removal. This, we decided, would be possible by measuring the primary deuterium isotope effects for the turnover of deuterium labelled (2*S*)-*O*-phosphohomoserine isotopomers.

A rough alignment of threonine synthase from *E. coli*[84] and yeast[82] was carried out to ascertain the similarity of the two proteins' sequence (Figure 2.01). If the sequence was very similar, especially close to the active site, then there would be no reason for the stereochemistry of the mode of action of these enzymes to be dissimilar. As we can see from Figure 2.01, there is a fair degree of homology around the active site, but there appears to be very little similarity in the outlying sequence.

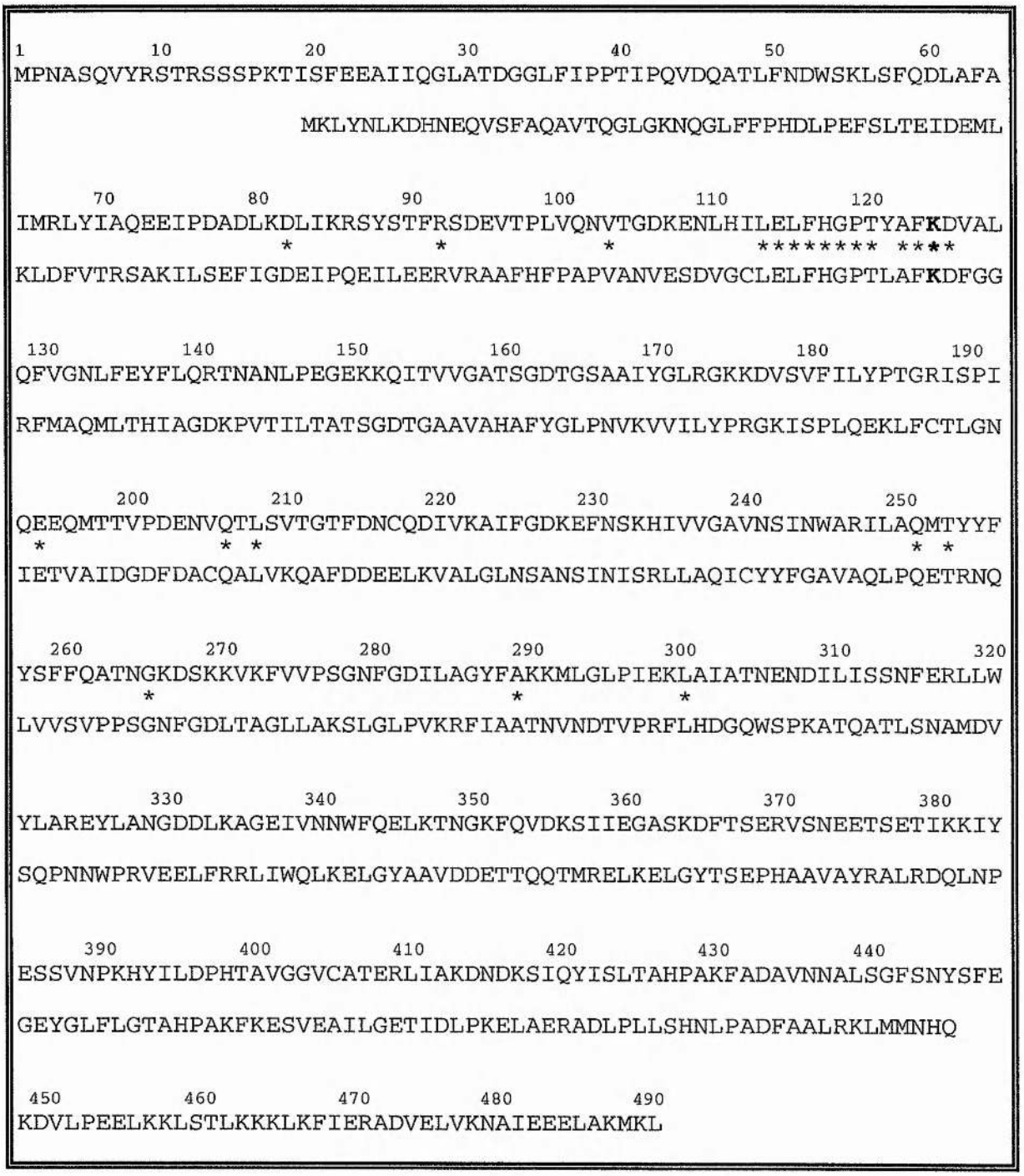



**Figure 2.01:** *Sequence alignment of threonine synthase from yeast (top) and E. coli (bottom). Numbering is with respect to the yeast sequence. Stars indicate conserved residues. The lysine residue at 124 emphasised in bold is involved in transaldimination.*

**Introduction to Kinetic Isotope Effects.**

One of the most informative ways to study enzymes and their mechanisms is by looking at their kinetic properties.[168] When coupled to the study and comparison of isotopically labelled substrate, a very much more detailed understanding of the system can be gained. Indeed, isotope effects provide a powerful tool for determining kinetic mechanisms and kinetically rate limiting steps, even in complex mechanisms.[169]

Isotope effects occur when an isotope (normally a heavier isotope) is substituted for an abundant isotope in a substrate. For example, one may substitute deuterium or tritium for protium, $^{13}C$ for $^{12}C$, $^{15}N$ for $^{14}N$, or $^{18}O$ for $^{16}O$. A substitution of this sort may alter the equilibrium constant for the reaction (an equilibrium isotope effect), or the rate constants for one or more steps in the reaction (a kinetic isotope effect). Isotope effects are referred to as primary when bonds are made or broken to the isotopic atom during the reaction; and secondary when they are not.

In simple terms, an isotope effect arises because the vibrational frequencies of the isotopic atom are different in the product and/ or in the transition state, to their vibrational frequencies in the substrate. For example, in a reaction where a C-H or a C-$^{2}$H bond is broken in the transition state, more energy is required to bring the C-$^{2}$H bond to the transition state from the ground state than for the lighter C-H bond system.[170] Therefore an isotope effect for the reaction can be measured by comparing the kinetic properties of the labelled and unlabelled compound. Exactly the same reasoning holds for enzyme catalysed reactions but here binding and debinding steps can suppress isotope effects.

In view of the fact that we wished to examine the cleavage of C-H bonds, it was first necessary to label the substrate with deuterium in the $C^{\alpha}$-, 3-*pro*-S and 3-*pro*-R

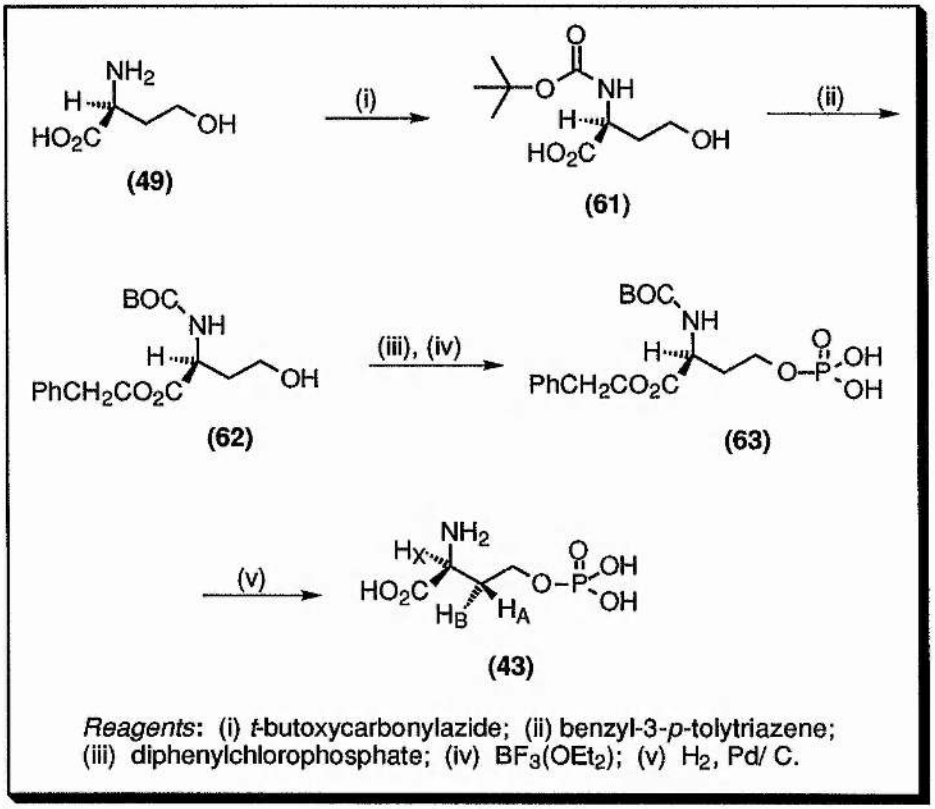
positions. Accordingly, syntheses of (2*S*,3*R*)-[3-$^{2}H_{1}$]-, and (2*S*,3*S*)-[3-$^{2}H_{1}$]- and (2*S*)-[2-$^{2}H_{1}$]-*O*-phosphohomoserine: (**43a**), (**43b**) and (**43c**) respectively, were devised.

## 2.1 Synthesis of (2*S*)-*O*-Phosphohomoserine.

Several approaches to the synthesis of (2*S*)-*O*-phosphohomoserine (**43**) are reported in the literature. [79,85,171-174]

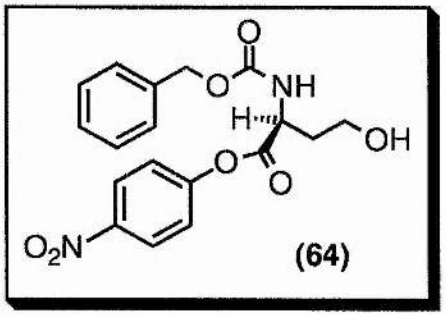
Ågren reported the isolation of phosphohomoserine from trichloroacetic acid extracts of *Lactobacillus casei*. A yield of 55 mg of the substrate was obtained from 650 g (dry weight) of cells.[171] A few years later, Watanabe and co-workers reported the synthesis of phosphohomoserine by the treatment of homoserine with a yeast preparation containing homoserine kinase.[173]

Ten years later, in 1973, Fickel and Gilvarg reported the first synthesis of phosphohomoserine starting from (2*S*)-homoserine (**49**) in 17% yield (Scheme 2.02).[172] *N*-Protection with *t*-butoxycarbonylazide to yield the *N*-*t*-butoxycarbonylhomoserine (**61**) followed by esterification with benzyl 3-*p*-tolytriazene gave α-benzyl-*N*-*t*-butoxycarbonylhomoserine (**62**). Phosphorylation with diphenylchlorophosphate, and removal of the butoxycarbonyl and benzyl protecting groups with boron trifluoride and hydrogenolysis respectively afforded phosphohomoserine (**43**). The authors reported that almost 20% racemisation had occurred in the final product.



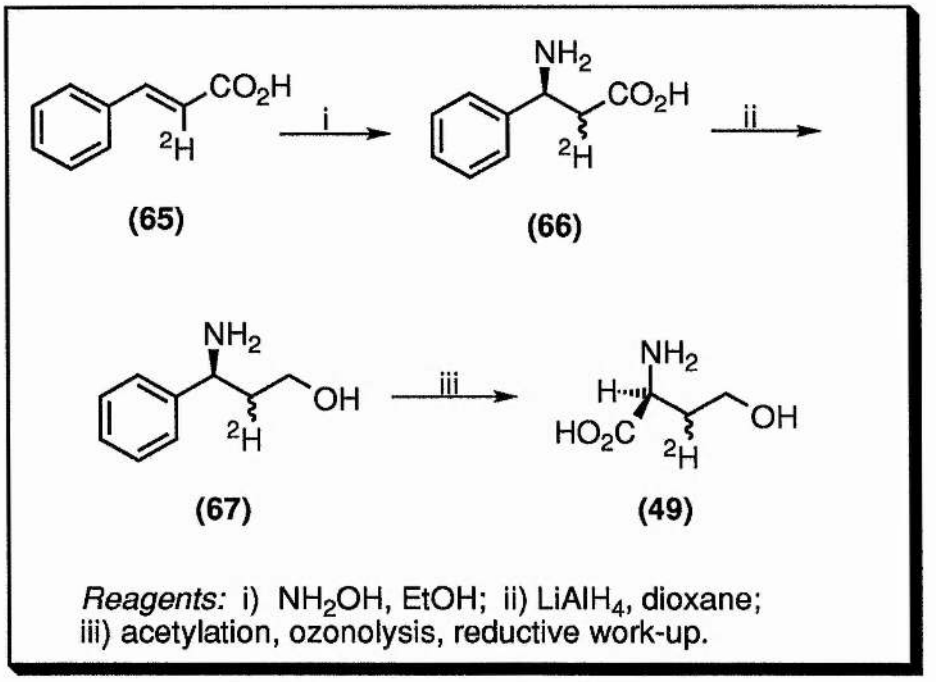

**Scheme 2.02:** *Fickel and Gilvarg's synthesis of (2S)-O-phosphohomoserine.*

Schnyder and Rottenburg reported a more efficient synthesis two years later starting from α-*p*-nitrobenzyl-*N*-benzyloxycarbonyl-(2*S*)-homoserine (**64**).[174] Phosphorylation again with diphenylchlorophosphate, followed by hydrogenolysis gave the desired (2*S*)-*O*-phosphohomoserine (**43**) in 62% yield.



Skarstedt and Greer have used purified aspartokinase to phosphorylate (2*S*)-homoserine (**49**) at the expense of ATP in 90% yield.[85] The enzyme was purified from a threonine synthase deficient strain of *Bacillus subtilis*, and the authors reported that the product, after purification by ion exchange chromatography, was completely free of phosphate containing contaminants.

A synthesis of phosphohomoserine labelled at the each of the C-3 positions with either tritium or deuterium had already been carried out by Fuganti's group (Scheme 2.03).[175] Addition of hydroxylamine in ethanol to *E*-[$\alpha$-$^2H_1$]-cinnamic acid (**65**) yielded a racemic mixture of 3-amino-3-phenyl-[2-$^2H_1$]-propionic acid (**66**). After resolution the propionic acids (**66**) were reduced in boiling dioxan with $LiAlH_4$ to give the corresponding alcohols (**67**). Ozonolysis of the alcohols followed by oxidative work-up and acid hydrolysis gave the corresponding labelled (2*S*)-homoserine (**49**). The preparation of C-4 labelled racemic homoserine in an analogous manner was also described. However no yields are quoted in the communication, and despite comprehensive searching of the literature, no full paper could be located.

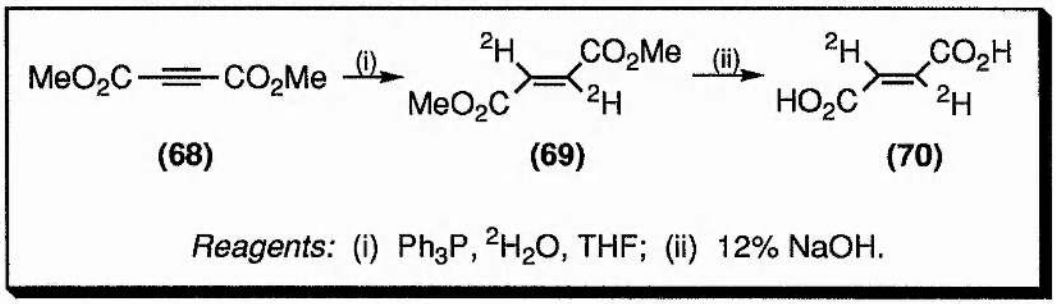



**Scheme 2.03:** *Fuganti's synthesis of labelled homoserine.*

All of the above methods were judged to be too lengthy or impractical for our needs. We would require substantial quantities of the substrate and its labelled isotopomers ((2*S*,3*R*)-[3-$^2H_1$]-, (2*S*,3*S*)-[3-$^2H_1$]- and (2*S*)-[2-$^2H_1$]-*O*-phosphohomoserine) to allow us to determine the stereochemistry and kinetic importance of $C^\beta$-proton abstraction from the substrate, and the relative importance of $C^\alpha$-H abstraction in the mechanism of threonine synthase.

As it was already possible, using a well established method developed previously in our laboratories, to incorporate deuterium selectively into the 3-*pro*-R and 3-*pro*-S and $C^\alpha$-positions of (2*S*)-aspartic acid (**45**),[176-178] we decided to utilise this as our starting material. Hence a route for the conversion of (2*S*)-aspartic acid to phosphohomoserine would allow the introduction of a deuterium atom at any of the proton sites on the molecule. This would constitute a biomimetic synthesis.

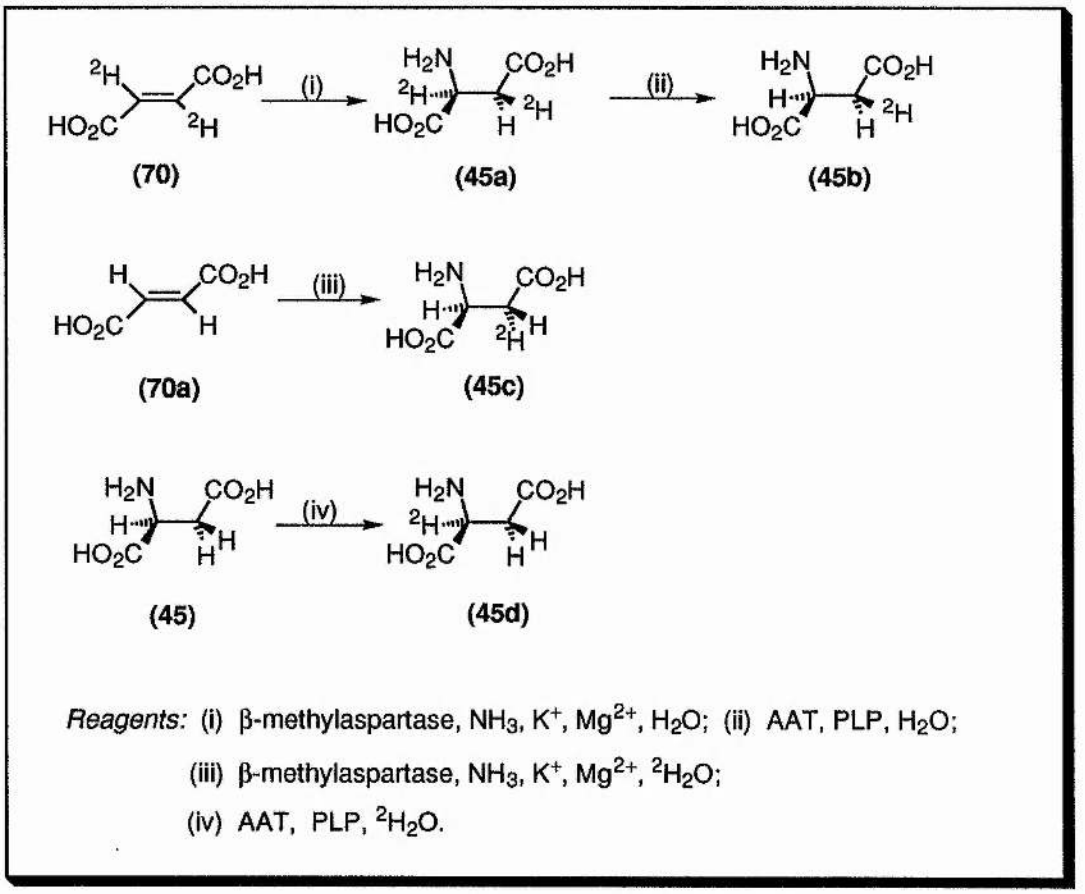
Dimethyl acetylenedicarboxylate (**68**) was reacted with triphenylphosphine in deuterium oxide to yield dimethyl dideuteriofumarate (**69**) as a dark red tar in moderate yield (Scheme 2.04) according to the method of Richards *et al.*.[176] Removal of triphenylphosphine oxide by sublimation gave the dideuteriofumarate (**69**) as a pale yellow solid which was recrystallised from methanol to give the pure product. The $^1$H-NMR spectrum showed only one signal at 3.81 ppm due to the methyl esters. By scrupulously ensuring that water was excluded from the reaction, the deuterium incorporation into the product was judged to be greater than 97% (as judged by $^1$H-NMR and mass spectroscopy). Saponification readily converted the diester into the free dideuteriofumaric acid (**70**) in good yield .




**Scheme 2.04:** *The synthesis of dideuteriofumaric acid (**70**).*

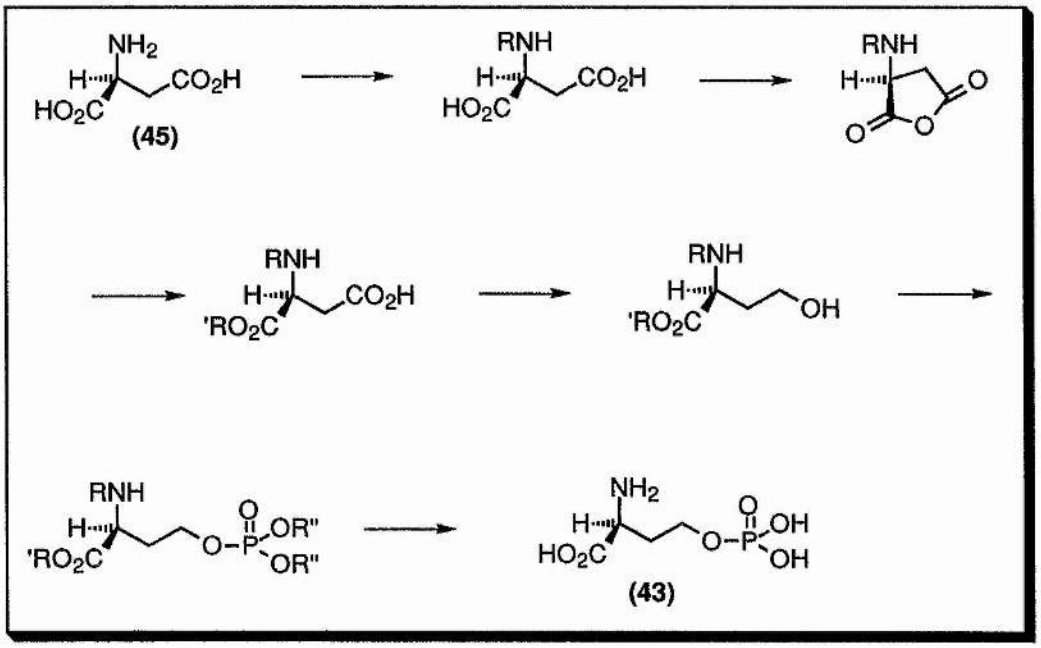
Dideuteriofumaric acid (**70**) was converted in a *trans* manner to (2*S*,3*S*)-[2-$^2$H$_1$,3-$^2$H$_1$]-aspartic acid (**45a**) according to a modification of the method of Gani and Young,[177] published by Akhtar *et al.* using the enzyme β-methylaspartase in 60% yield (Scheme 2.05).[178] This compound showed only one signal at 2.51 ppm in the $^1$H-NMR spectrum, corresponding to the C$^\beta$-H atom. Further reaction with PLP dependent aspartate aminotransferase gave the desired (2*S*,3*S*)-[3-$^2$H$_1$]-aspartic acid (**45b**), as illustrated by the appearance of the C$^\alpha$-H atom at 3.50 ppm. This enzyme is known to catalyse the reversible exchange of the C-2 hydrogen of aspartic acid with solvent hydrogen.[179]

(2*S*,3*R*)-[3-$^2H_1$] Aspartic acid (**45c**) was synthesised by incubating fumaric acid (**70a**) with β-methylaspartase in deuterium oxide (Scheme 2.05) (*m/z* Found: $M^+$, 135.0498, $C_4H_6{}^2HNO_4$ requires 135.0516). (2*S*)-[2-$^2H_1$]-Aspartic acid (**45d**) was prepared *via* deuterium oxide exchange with the $C^{\alpha}$-proton of (2*S*)-aspartic acid using a commercial preparation of aspartate aminotransferase (Scheme 2.05).




**Scheme 2.05:** *The synthesis of deuterium labelled isotopomers of (2S)-aspartic acid.*

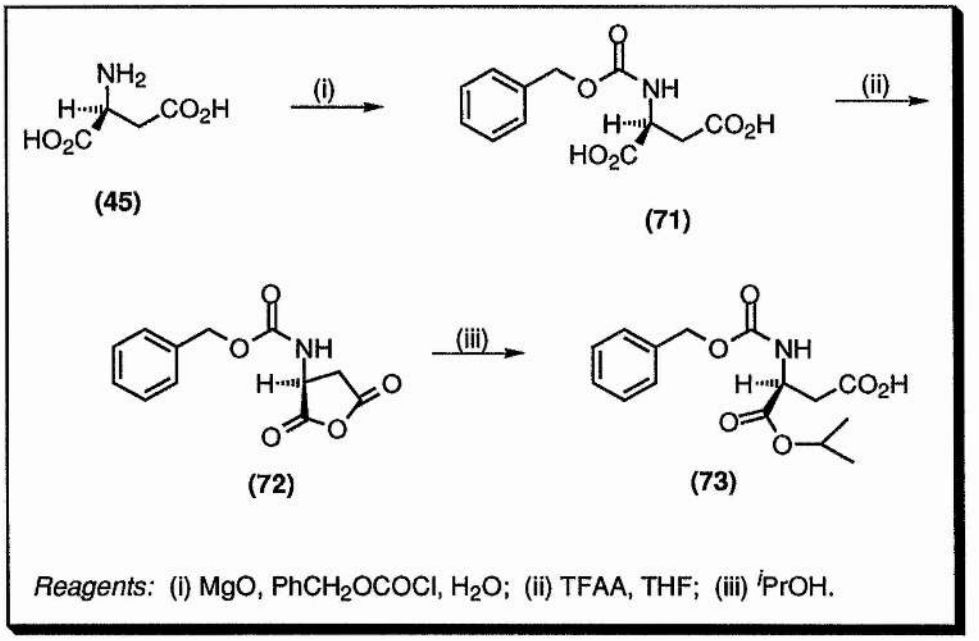
It was envisaged that the most direct route for the synthesis of phosphohomoserine (**43**) would be a simple phosphorylation of a protected homoserine derivative prepared by reducing the β-carboxyl group of aspartic acid (Scheme 2.06). It would be neccessary to protect the amine functionality of aspartic acid before carrying out this procedure. The α-carboxyl group would also have to be selectively protected.




**Scheme 2.06:** *The strategy of the synthesis of (2S)-O-phosphohomoserine (43).*

*t*-Butoxycarbonyl protection of aspartic acid was unsuccessful under a variety of conditions. Benzyloxycarbonyl (Z) protection using benzyl chloroformate and sodium hydroxide under Schotten-Baumann conditions only resulted in 50% isolation of the product.[180] Carrying out the Z-protection under Bergmann and Zervas' conditions where (2*S*)-aspartic acid (**45**) is suspended in water with 3 equivalents of magnesium oxide, followed by the addition of benzyl chloroformate; resulted in the formation of *N*-carbobenzyloxy (2*S*)-aspartic acid (**71**) in a 62%

yield after recrystallisation.[181] The product (**71**) was treated with trifluoroacetic anhydride in tetrahydrofuran to give the Z-protected cyclic anhydride (**72**) in excellent yield. Selective opening of the cyclic anhydride was attempted using a variety of alcohols, including isopropanol, *p*-nitrophenol, and 2,4-dinitrophenol. Alcoholysis with isopropanol gave the best selectivity, and α-isopropyl Z-aspartic acid (**73**) was isolated in 55% yield (Scheme 2.07).

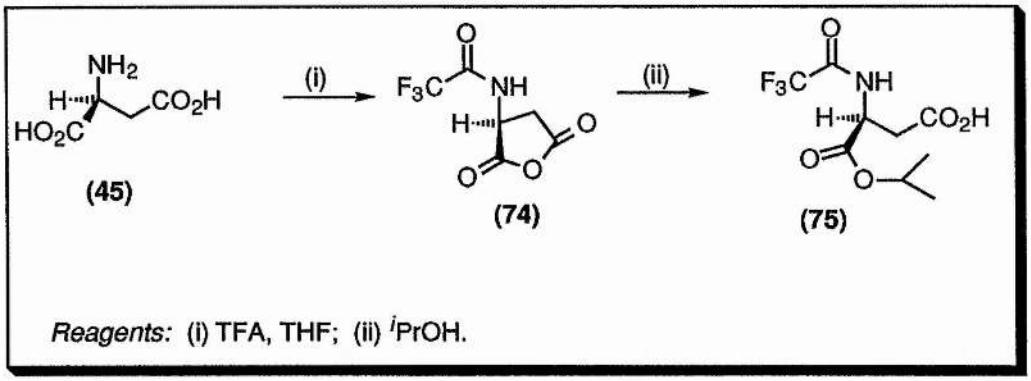



**Scheme 2.07:** *The formation of α-isopropyl N-carbobenzyloxy (2S)-aspartic acid (73).*

As the desired β-free acid (**73**) was only obtained in moderate yield, we decided to investigate the use of the trifluoracetyl N-protecting group in order to improve the overall yield of the synthesis. This would have the advantage of both N-protection and formation of the protected cyclic anhydride in a one pot reaction (Scheme 2.08) using the procedure described by Gani and Young.[182] (2*S*)-Aspartic acid was therefore treated with 5 equivalents of trifluoracetic anhydride in anhydrous THF with cooling. The reaction mixture became homogeneous over a period of

around 2 h. Removal of the solvent and careful removal of excess trifluoracetic acid under vacuum allowed the isolation of N-trifluoracetyl (2*S*)-aspartic anhydride (**74**) as a white solid in excellent yield ($\nu_{max}$ Nujol/ $cm^{-1}$ 1805 (C=O) and 1685 (C=O)). Although it was possible to recrystallise the cyclic anhydride from dry ether and petroleum ether, the hygroscopic nature of the compound meant that it was more efficient to take the anhydride through to the next step without further purification.

The selective protection of the α-carboxyl group through treatment of the anhydride with methanol had been described in the past and was reported to yield 75-80% of the desired α-methyl ester β-acid.[182] The remainder was the unwanted α-acid β-ester. Performing the reaction at temperatures down to -78 °C did not improve the ratio in favour of the α-ester, nor did treatment with ethanol. However, treatment of the anhydride with isopropanol at 0 °C gave the desired α-isopropyl ester β-acid (**75**) in 92% yield after one recrystallisation from ether and petroleum ether (Found: C, 39.90; H, 4.65: N, 5.25. $C_9H_{12}NO_5F_3$ requires C, 39.90; H 4.45; N, 5.15%).[183] Examination of the C-3 proton signals in the $^1$H-NMR spectrum of the crude material (before purification) indicated that the regioselectivity of attack at the α-carbonyl group was better than 25:1. The highly selective nature of this reaction is thought to be facilitated by the electron withdrawing effect of the *N*-trifluoracetyl protection, which enhances the electrophilicity of the α-carboxyl carbon.

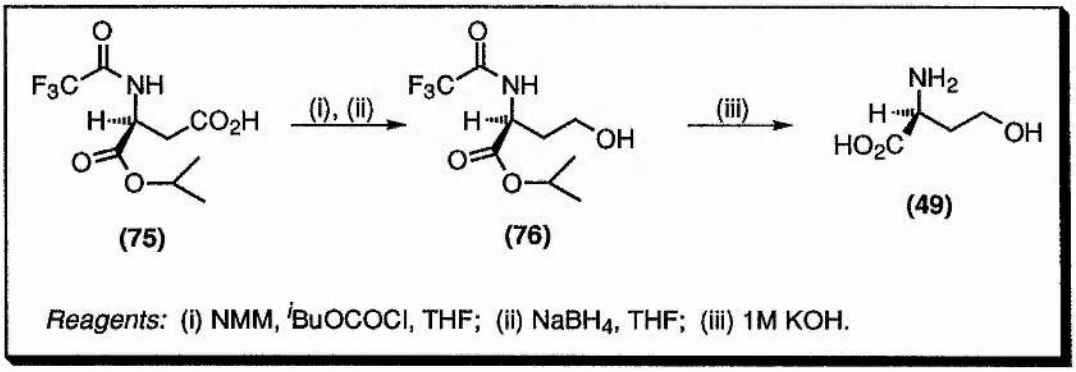



**Scheme 2.08:** *The formation of α-isopropyl N-trifluoracetyl (2S)-aspartic acid.*

Having protected the α-carboxylic acid as the isopropyl ester (**75**), a range of methods and conditions for reducing the β-carboxyl group to the corresponding alcohol were examined. The reduction of the free β-carboxylic acid group was not a trivial problem. The use of $LiAlH_4$ was precluded due to the protecting groups already in place. It is always necessary to activate carboxylic acids before reduction to an alcohol by borohydride. Reduction of the the β-carboxyl group was attempted by first forming the mixed β-aspartic isobutylcarbonic anhydride from the acid (**75**) by treatment with an equivalent of *N*-methyl morpholine and isobutylchloroformate at -40 °C, following the reaction by tlc. After removal of the precipitated salts, the mixed anhydride was treated with a solution of aqueous sodium borohydride. This procedure was found to be unsuccessful and none of the required alcohol was formed. The amount of borohydride used in the reduction was varied; however this did not improve the situation. Changing the solvent used for the reduction allowed the isolation of some of the desired alcohol product. After several further trial experiments in THF, isopropanol and DCM, it was found that carrying out the reaction in anhydrous THF yielded the desired *N*-trifluoroacetylhomoserine isopropyl ester (**76**) in excellent yield (Scheme 2.09). Ishizuma and coworkers describe a similar procedure using ethyl chloroformate to

form the mixed anhydride.[184] Purification of the alcohol by flash silica chromatography eluting with diethyl ether/ petroleum ether/ ethyl acetate (2:4:4) containing a small amount of acetic acid gave α-isopropyl *N*-trifluoracetyl (2*S*)-homoserine (**76**) as a pale yellow oil in yields of up to 93%. The $^{13}$C-NMR spectrum clearly showing the loss of the carbonyl signal at 175.9 ppm, and the appearance of a new signal at 59.2 ppm corresponding to the $CH_2OH$. Note that an ideal tlc visualisation stain for the protected (2*S*)-homoserine was not found during the course of this work. Nevertheless, a dilute solution of potassium permanganate allowed the visualisation of the compound.

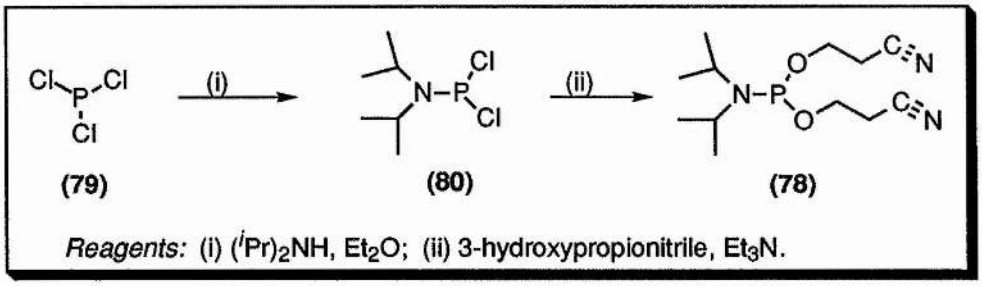
Base catalysed hydrolysis of the trifluoroacetyl and isopropyl protecting groups of alcohol (**76**) in aqueous ethanol afforded (2*S*)-homoserine (**49**) in 95% yield, representing an overall yield of 85% from (2*S*)-aspartic acid (**45**) after ion-exchange chromatography (Scheme 2.9). This compares well with other syntheses from aspartic acid.[185] No loss of chiral integrity occurred at C-2 during the saponification {$[\alpha]_D$ -24.4 (*c* 10 in water) and lit.,[185] $[\alpha]_D$ -24.5 (*c* 10 in water)} and the sample was in all respects identical to an authentic sample.




**Scheme 2.09:** *The synthesis of (2S)-homoserine (**49**).*

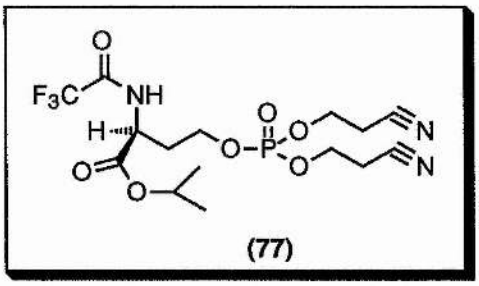
In order to convert the alcohol (**76**) into its phosphate ester derivative, a range of phosphorylating reagents were considered. As phosphate esters are labile under acidic conditions, we chose to form a phosphate ester in which the protecting groups could be removed under basic or neutral conditions. We had already established that the *N*-trifluoroacetyl and isopropyl protection could be removed under alkaline conditions without loss of chiral integrity and, therefore, opted first to form the base-labile *bis*cyanoethyl phosphate triester (**77**) which should cleave *via* β-elimination to give the phosphate moiety.

Accordingly, the alcohol (**76**) was treated with *N,N*-diisopropyl *bis*cyanoethyl phosphoramidite (**78**) to give the phosphate triester which was oxidised *in situ* using *m*-CPBA in dichloromethane. The phosphoramidite reagent (**78**) has been previously used to phosphitylate the primary 5'-hydroxyl group of oligonucleotides[186] and the primary 6-hydroxyl group of carbohydrates.[187] The phosphoramidite (**78**) was readily prepared in two steps from phosphorus trichloride (**79**) (Scheme 2.10).[188] The addition of two equivalents of diisopropylamine to phosphorus trichloride in diethyl ether afforded the *N,N*-diisopropyldichlorophosphoramidite (**80**) in moderate yield (60%). Esterification *via* nucleophilic displacement of chloride with excess 3-hydroxypropionitrile in the presence of triethylamine yielded the phosphoramidite (**78**), which could be purified by silica gel column chromatography in 61% yield (*m/z* Found: $M^+$, 271.1462. $C_{12}H_{22}N_3O_2P$ requires 271.1460).



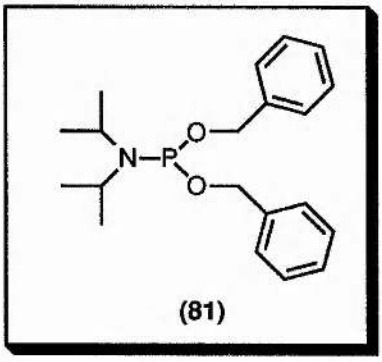

**Scheme 2.10:** *The synthesis of N,N-diisopropyl biscyanoethylphosphoramidite.*

The addition of 1.5 equivalents of the phosphoramidite (**78**) to the protected homoserine (**76**), in the presence of tetrazole, resulted in an almost quantitative yield of the phosphite triester. Removal of the solvent followed by $^{1}$H-NMR spectroscopy confirmed the phosphitylation had been successful. The phosphite was treated without further purification with 1.5 equivalents of *m*-CPBA in DCM as described by Corrie and Trentham.[189] The resulting phosphate triester (**77**) was obtained in excellent yield (87%) but proved difficult to purify. The $^{13}$C-NMR spectrum clearly showed phosphorus splitting of the signals. {$\delta_C$ (50.3 MHz; $C^2HCl_3$) 20.0 (d, *J* 7.3, POC), 22.1 ($^{i}$PrCH$_3$), 32.2 (d, $J_{PC}$ 5.3, C$^{\beta}$), 50.1 (C$^{\alpha}$), 63.4 (t, *J* 5.1, -CCN), 65.0 (d, $J_{PC}$ 5.8, C$^{\gamma}$), 71.2 ($^{i}$PrCH), 116 (q, $J_{FC}$ 290, F$_3$C), 117.2 (CN), 158.2 (q, $J_{FC}$ 38, F$_3$CCO) and 170.3 (CO$_2{}^{i}$Pr).} Attempts to deprotect the crude material directly under a variety of basic conditions met with limited success. Although (2*S*)-*O*-phosphohomoserine (**43**) could be purified from the hydrosylate by ion-exchange chromatography, it was evident that much of the product was (2*S*)-homoserine (**49**). Most of the phosphate group had been hydrolysed under the reaction conditions, and the pseudoquartet signal provided by phosphate splitting of the γ-hydrogens was absent. Using diphenylphosphorochloridate as phosphorylating agent did not improve the retention of the phosphate group.



(77)

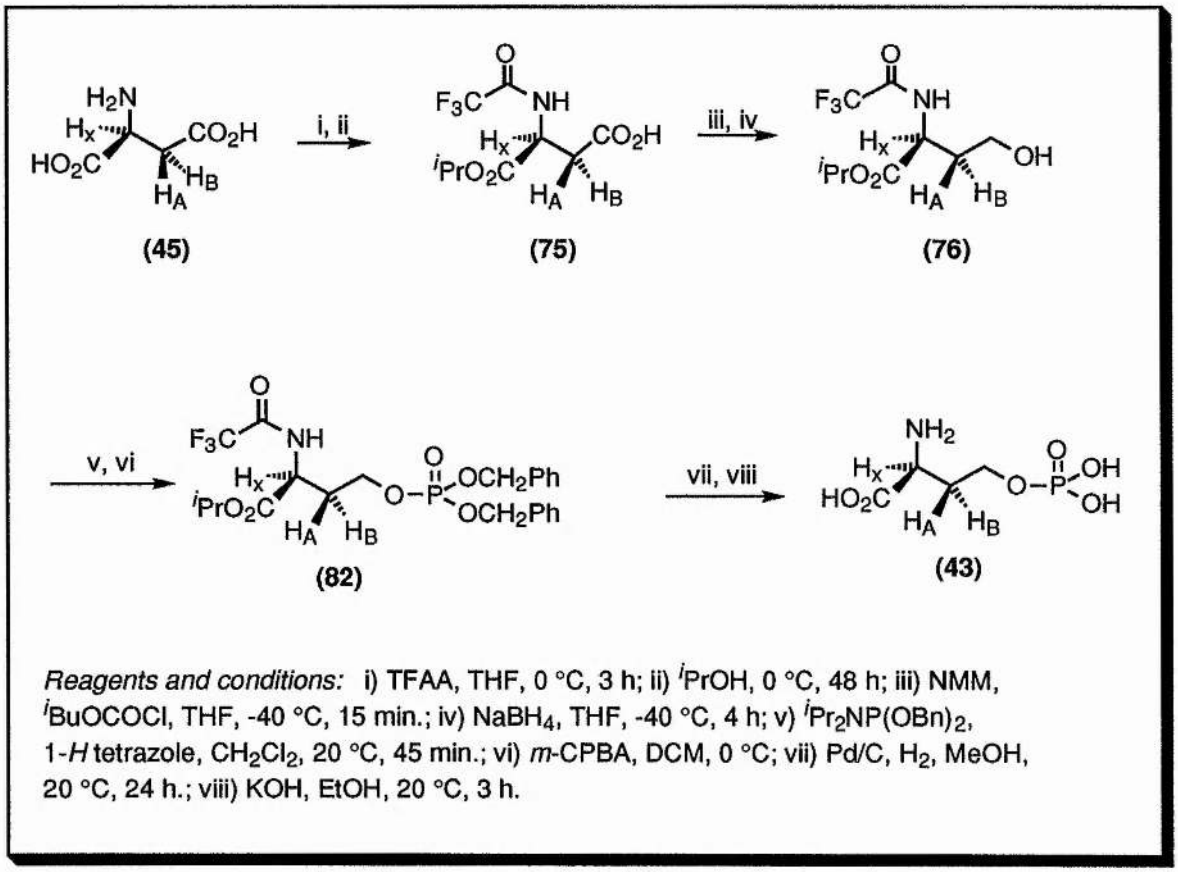
In order to prevent the hydrolysis of the phosphate moiety by base, we opted to use benzyl protecting groups for the phosphate triester and to carry out the deprotection in two stages. The phosphoramidite (**81**) was synthesised in a similar manner to that described for (**78**) above, using benzyl alcohol instead of 3-hydroxypropionitrile in 72% yield (*m/z*, Found: $[M + H]^+$, 346.1936. $C_{20}H_{29}NO_2P$ requires 346.1936). Thus, the alcohol (**76**) was treated with *N,N*-diisopropyl dibenzyl phosphoramidite (**81**) and the intermediate phosphite was oxidised with *m*-CPBA as described by Yu and coworkers.[190] This phosphorylation reaction proved to be very effective after optimisation of the reaction conditions. Using only one equivalent of phosphoramidite resulted in the phosphate triester (**82**) being obtained in 92% yield (*m/z*, Found: $[M + H]^+$ 518.1556. $C_{23}H_{27}NO_7PF_3$ requires 518.1555).



(81)

The dibenzyl triester was then smoothly converted in two steps, *via* catalytic hydrogenolysis and then base catalysed hydrolysis of the protecting groups, to (2*S*)-*O*-phosphohomoserine (**43**) Purification by cation-exchange chromatography (Dowex 50-W ($H^+$) form) eluting with water gave the pure phosphohomoserine in

90% yield over the two steps. (2*S*)-*O*-Phosphohomoserine obtained *via* this reaction strategy results in an overall yield of 66% from aspartic acid (**45**), Scheme 2.11. *m/z* (Found: $[M + H]^+$ 200.0329, $C_4H_{11}NO_6P$ requires 200.0324); m.p. 170 °C; $\delta_H$ (200 MHz, $^2H_2O$) 4.12 (1H, dd, $J_{AX}$ 12.4, $J_{BX}$ 4.9 Hz, 2-H), 4.01 (2H, dt, $J_{HP}$ 5.49, $J$ 5.7 Hz, 4-$H_2$) and 2.23 (2H, m, 3-$H_2$); $\delta_C$ (50.3 MHz, $^2H_2O$) 186.2 ($CO_2H$), 64.3 (d, $J_{PC}$ 4.9 Hz, 4-C), 56.3 (2-C) and 38.7 (d, $J_{PC}$ 7.4 Hz, 3-C); $\delta_P$ (121.5 MHz, $^2H_2O$) 0.5; $[\alpha]_D$ + 4.19 (*c* 2.4 in water) {lit.,[174] $[\alpha]_D$ 4.21 (*c* 2.4 in water)}.




**Scheme 2.11:** *The synthesis of phosphohomoserine (**43**) from aspartic acid.*

In order to prepare the chirally deuteriated enantiomers (**43a**, $H_A$ = H, $H_B$= $^2$H), (**43b**, $H_A$ = $^2$H, $H_B$ = H) and (**43c**, $H_X$ = $^2$H, $H_A$ = $H_B$ = H); (2*S*,3*R*)-; (2*S*,3*S*)-[3-$^2H_1$]- and (2*S*)-[2-$^2H_1$]-aspartic acids were subjected to the same reaction sequence as the unlabelled material, as outlined in Scheme 2.11. Each of the chirally deuteriated (2*S*)-*O*-phosphohomoserines was obtained in ~60 % overall yield from aspartic acid and showed the expected omissions in the $^1$H-NMR spectra. In each case, the signal corresponding to the deuterium atom was missing and other signals showed a corresponding simplification. The $^{13}$C-NMR spectra showed a splitting for each carbon atom attached to a deuterium atom.

## 2.2 An Assay for the Detection of Threonine Synthase Activity.

In order to carry out any meaningful studies on threonine synthase, it was important for us to develop an accurate and sensitive assay for the reaction catalysed by threonine synthase. In most purifications and kinetic studies to date, turnover of a radiolabelled substrate has been detected by scintillation counting, or the products determined by a colourimetric assay.[80] A colourimetric assay was judged to be potentially the most user friendly technique.

For some time inorganic phosphate has been detected colourimetrically by the reduction of phosphomolybdate to molybdenum blue.[191] Some basic dyes form a complex with phosphomolybdate, increasing the sensitivity of the reaction.[192] The most effective of these complexes is that of Malachite green in acidic solution. Itaya and Ui found that levels of phosphate as low as 0.05 μg/ ml could be detected using this reagent.[192]

An improvement on this method was reported in 1979 by Lanzetta *et al.*.[193] They found that they could detect nanomolar amounts of phosphate by monitoring the adsorption at 660 nm of a sample treated with Malachite-molybdate complex. When tested this technique proved reproducible and convenient. The reagent was simply prepared by the addition of ammonium molybdate to a solution of Malachite green in hydrochloric acid. After filtration the reagent could be stored in the dark for periods of up to one month.

Addition of a sample (100 μl) containing phosphate to Malachite reagent (900 μl) formed a green solution. After the colour had been allowed to develop for 20 min, the absorption in the visible spectrum at 660 nm was measured. A standard curve was set up by measuring the absorptions of known concentrations of phosphate in

triplicate, and then plotting the absorption as a function of phosphate concentration. Over the range 0-200 mM, the relationship was linear. At very high concentrations of phosphate it was sometimes neccessary to increase the ratio of reagent to phosphate containing sample.

## 2.3 The Purification of Threonine Synthase.

*E. coli* was deemed the most convenient source of the threonine synthase enzyme for our needs. It was neccessary to find a constitutive mutant for the enzyme, because, as explained in detail in the introduction, the enzyme is only expressed under certain conditions, see page 38. There were literature reports of just such a constitutive mutant: *E. coli* K12 strain Tir8. Unfortunately this strain was not commercially available, and was therefore kindly donated to us by Prof. C. Parsot of the Institut Pasteur, Paris.

*E. coli* cells were grown on L-broth[194] in 6 l batches. Harvesting of the cells was carried out by centrifugation once the culture had achieved the optimum cell density ($OD_{660nm} \approx 9$) after approximately 17 h. The cells were efficiently lysed by sonication and the cell debris was removed by centrifugation. The nucleic acids were then precipitated. Streptomycin sulfate and protamine sulfate were both tested for their efficacy in this process, and it was decided to use streptomycin sulfate in view of its superior solubility.

The first step in the purification of the protein lysate was an ammonium sulfate fractionation. Parsot and his coworkers used a 40-60% ammonium sulfate fraction.[84] Suitability of this cut for our use was tested by a series of trial fractionations. The 40-60% saturation fraction was indeed found to have most activity (Table 2.12).

| % sulfate saturation | relative activity |
|---|---|
| 30-40 | 15% |
| 40-60 | 100% |
| 60-70 | 10% |
| 70-80 | 5% |

**Table 2.12:** *Relative threonine synthase activity of varying ammonium sulfate cuts of E. coli lysate.*

The next step in the purification was size exclusion chromatography. Threonine synthase from *E. coli* has a reported molecular weight of around 37 000 daltons.[84] It was decided, therefore, that a G150 Sephadex column would give a satisfactory separation. A column of 80 x 3 cm was used with a relatively slow flow rate of 30 ml/ h. Fractions (15 ml) were collected from the column (Figure 2.13), and were assayed for threonine synthase activity, using the malachite assay described above. The active fractions were pooled and ultrafiltrated to around 3 ml. The protein content of the concentrated fractions was found to be around 50 mg/ ml when measured by the Bradford method,[195] and the activity was 2 800 nmoles $min^{-1}$ $mg^{-1}$. The size exclusion column had to be repacked before each use to ensure optimum flow rates.

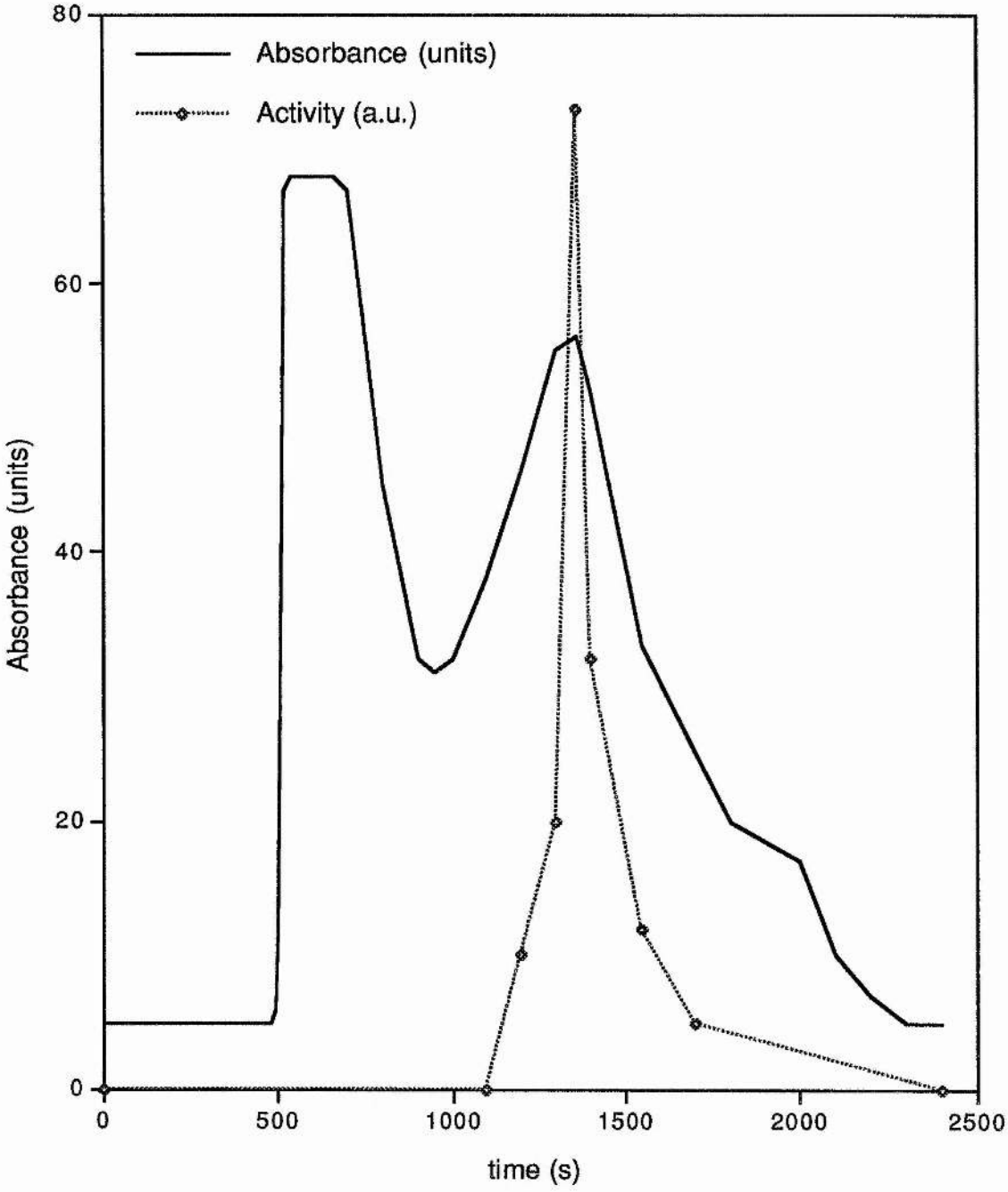



**Figure 2.13:** *G150 column trace showing threonine synthase activity. a.u. stands for arbitrary units.*

Although the activity of threonine synthase was high enough from the G150 column to allow us to carry out initial kinetic studies, we were keen to purify the protein further. Ion-exchange chromatography was attempted with DEAE Sephacel and DEAE TSK 5PW FPLC, eluting with a KCl gradient. Unfortunately in both cases, no active protein was found. These attempts were repeated lowering the pH in an attempt to combat the protein sticking to the ion-exchange material very tightly and being careful to assay the unbound fractions. It quickly became clear that the protein was in some way deactivating on the column. The experiments were repeated, adding threonine to the buffer in an attempt to stabilise the protein's active site. Sadly, this did not help. SDS-PAGE was carried out on the fractions to try and detect threonine synthase. Protein of the correct molecular weight was present, and this fraction was concentrated 100 fold to around 1 ml by ultrafiltration. The protein was assayed and found to be inactive.

We were beginning to suspect that the lack of phosphate in the buffer was causing the enzyme to inactivate in some way. Parsot had successfully purified the enzyme using the same DEAE medium, although the column was eluted with a phosphate buffer.[84] It was decided to repeat this work, although the column would have to be run "blind", that is the fractions could not be assayed directly from the column. Instead, protein peaks eluted from the column were pooled (Figure 2.14) and the buffer exchanged for HEPES using a squat Sephadex G25 column (7 cm x 2.5 cm).

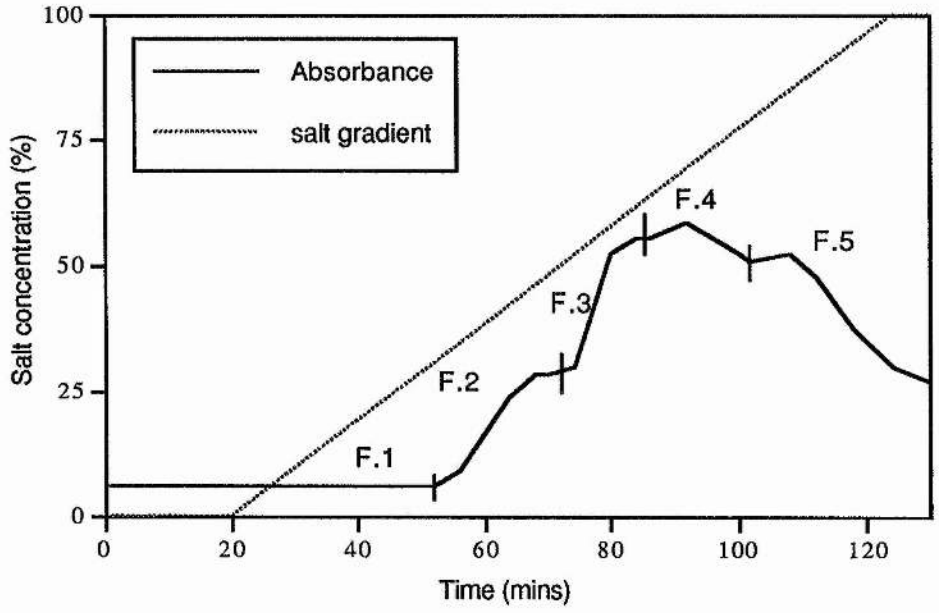



**Figure 2.14:** *DEAE Sephacel column trace.*

Each pooled, buffer exchanged fraction was assayed, and indeed activity was found in fraction 4. An SDS-PAGE was carried out on the fractions, and a protein of the correct molecular weight was indeed found in fraction 4. Fraction 4 had a protein content of 0.5 mg/ ml. Attempts to purify the protein further were abandoned due to pressures of time, and the very labour intensive protocol. All subsequent kinetic experiments were therefore carried out using threonine synthase from the G150 column.

The amount of background non-specific phosphatase activity associated with this preparation of threonine synthase was determined. Tlcs of small threonine synthase assays were run in a solvent that distinguished between threonine, homoserine and phosphohomoserine ($^t$butanol/ methyl ethyl ketone/ $H_2O$/ $NH_3$ 4:3:2:1). No homoserine was detected when the tlcs were developed with ninhydrin.

By carrying out sequential dilutions of homoserine and threonine and detecting their presence with ninhydrin, we could see that each of the compounds had an approximately equal lower limit of detection using this technique. This lower limit of detection was in the region of 1-10 nanomolar. It was judged that the amount of non-specific phosphatase activity was much less than 5% of threonine synthase activity. This indicated that kinetic assays carried out would only be affected to a small extent by other phosphatase activities.

## 2.4 Determination of Kinetic Parameters.

### Introduction

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 concentrations of $e_t - x$, s and x respectively, where $e_t$ is the total concentration of free enzyme.

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

$$\frac{dx}{dt} = k_1(e_t-x)s - k_2x - k_3x = k_1e_ts - (k_1s+k_2s+k_3)x \qquad (1)$$

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:-

$$x = \frac{k_1e_ts}{k_1s+k_2+k_3} \qquad (2)$$

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

$$\text{rate, } v = k_3x \qquad (3)$$

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 enzyme is present in large quantities compared to the enzyme, then s ≈ 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 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 will be equal to $K_M/V$. One drawback to this method is that points of small v and s become overemphasised.

In the analysis of the enzyme-substrate reaction data the numerical values of the rate (v) and substrate concentration (s) were fitted to the Michaelis-Menten kinetics using the computer program Enzfitter,[196] which carries out a direct non-linear fit and so avoids the overemphasis of small values of v and s, thus yielding the parameters $K_M$ and $k_{cat}$ ($k_{cat} = V_{max}/e_t$).

The kinetic isotope effect is the ratio of the reaction rates for molecules containing the light and heavy atoms, *i.e.* $k_H/k_D$, and is written as $^Dk$ (using Northrop notation). An isotope effect can also be observed on the equilibrium constant for the reaction, giving an equilibrium isotope effect, written as $^DK_{eq}$. Isotope effects can be measured on both V and V/K, *i.e.* one can examine the difference in rates ($^DV$) and the ratio of the apparent first order rate constant ($^D(V/K)$). Thus, V and V/K are separately measured for both the labelled and the unlabelled substrate and comparison of the results will directly provide the isotope effects on V and V/K. This is the only method to determine an isotope effect on V as well as V/K, and requires a high level of isotopic substitution.

The observed values of the isotope effects on $^D(V/K)$ and $^DV$ are given by:-

$$^D(V/K) = \frac{^Dk + c_f + c_r{}^DK_{eq}}{1 + c_f + c_r}$$

$$^DV = \frac{^Dk + c_{vf} + {}^DK_{eq}c_r}{1 + c_{vf} + c_r}$$

In this equation $^Dk$ is the intrinsic isotope effect in the forward direction, and $^DK_{eq}$ is the equilibrium isotope effect. The constants $c_f$ and $c_r$ are commitments to the reaction in forward and reverse directions. A commitment is a ratio of the rate

constant for the isotopically sensitive step to a net rate constant that refers to the release of a particular species from the enzyme. For example, a high forward reaction commitment indicates that it is considerably easier for the reaction to carry on in the forward direction, than to go back through earlier steps in the mechanism. Finally, the constant $c_{vf}$ in the equation for $^{D}V$ is, in fact, not a commitment, but a special constant found only in this equation.

The symmetry of the equation for $^{D}(V/K)$ means that dividing it by $^{D}K_{eq}$ produces the comparable equation in the reverse direction. In general terms, it can be seen from the above equations that the observed isotope effect for an enzyme-catalysed reaction will vary depending on the values for the forward and reverse commitments. Thus when the forward commitment is large, the isotope effect is supressed, and $^{D}(V/K)$ will approach unity.

**Kinetic Experiments.**

The values of $K_M$ and $V_{max}$ were measured for the conversion of phosphohomoserine to threonine by threonine synthase both with and without the reported allosteric activator *S*-adenosyl methionine. Initial rates were obtained in triplicate for the release of phosphate at several concentrations of the substrate (2*S*)-*O*-phosphohomoserine (**43**) (20, 30, 50, 100, 200 and 500 μM) without activator and in the presence of 0.4 mM SAM at 37 °C. The reactions were initiated by the addition of 0.5 mg of threonine synthase. The data collected were fitted using the computer program Enzfitter[196] and are presented graphically.

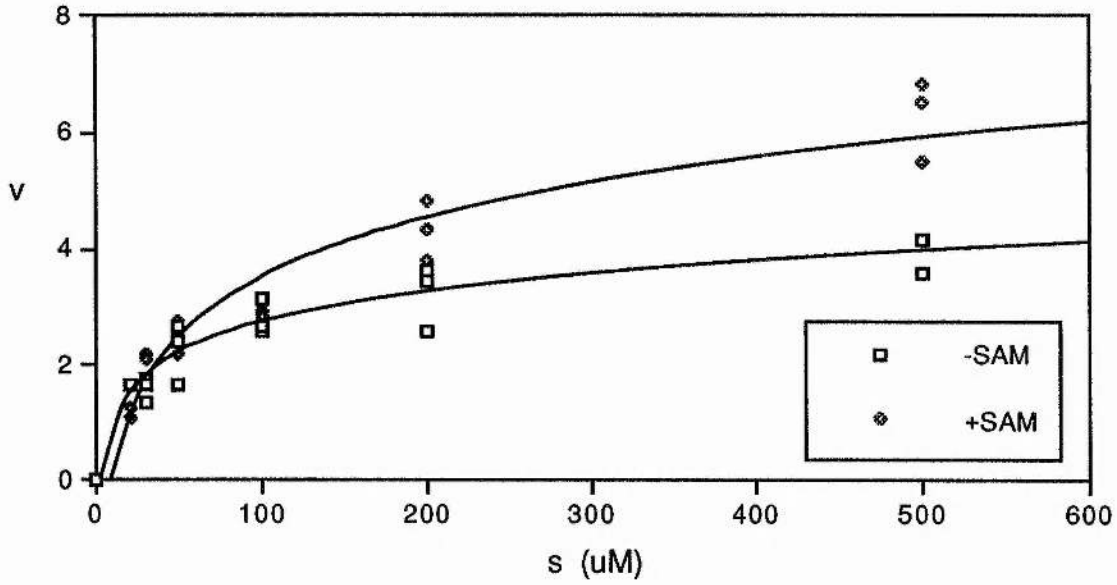



**Figure 2.15:** *The effect of S-adenosyl methionine on the initial rate of threonine synthase. Data fitted by non-linear regression using Enzfitter.*

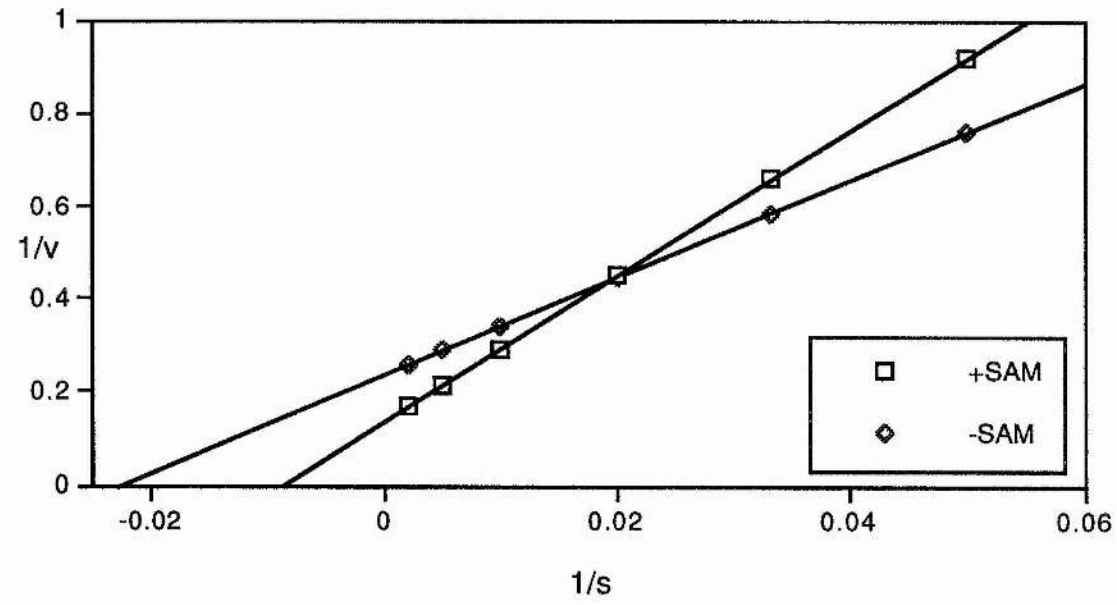



**Figure 2.16:** *Double reciprocal plot with and without S-adenosyl methionine. Theoretical data points are derived from the best fit curves shown in Figure 2.15*

Examination of the plots of initial rates (Figure 2.15) and their reciprocals (Figure 2.16) shows that *S*-adenosyl methionine is clearly exercising some effect on threonine synthase. The $K_M$ for threonine synthase in the presence of the activator is almost doubled, and the $V_{max}$ has increased almost three-fold (Table 2.17). This is in accord with the results reported for threonine synthase from plants.[156] Since in the presence of high concentrations of activators species binding and debinding processes can become the kinetically dominant steps, the kinetic isotope effects of the labelled isotopomers were conducted in the absence of SAM.

| | $K_M$ (μM) | ± error | $V_{max}$ (μM $min^{-1}$) | ± error |
|---|---|---|---|---|
| -SAM | 43.5 | 7.82 | 4.18 | 0.22 |
| +SAM | 115.1 | 7.15 | 7.34 | 0.18 |

**Table 2.17:** *The $V_{max}$ and $K_M$ for threonine synthase with and without SAM*

The next experiment to be carried out was a comparison of the kinetic parameters of unlabelled phosphohomoserine with the (2*S*,3*R*)-[3-$^2H_1$]-isotopomer. If threonine synthase form *E. coli* showed the same stereochemistry of C-3 proton removal as the yeast enzyme, then we would not expect to see a kinetic isotope effect for the labelled substrate.[153] We can see both from the graphical portrayals of the rates (Figure 2.19 and 2.20), and from the kinetic parameters (Table 2.18) deduced from these that the substitution of (2*S*,3*R*)-[3-$^2H_1$]-*O*-phosphohomoserine for the unlabelled substrate has no effect within experimental error. $^DV$ has a value of 1.0 and $^D(V/K)$ is 1.2.

| | $K_M$ (μM) | ± error | $V_{max}$ (μM $min^{-1}$) | ± error |
|---|---|---|---|---|
| PHS | 43.5 | 7.82 | 4.18 | 0.22 |
| RPHS | 52.1 | 8.56 | 4.09 | 0.22 |

**Table 2.18:** *Kinetic data for (2S)-O-phosphohomoserine and (2S,3R)-[3-$^2H_1$]-O-phosphohomoserine.*

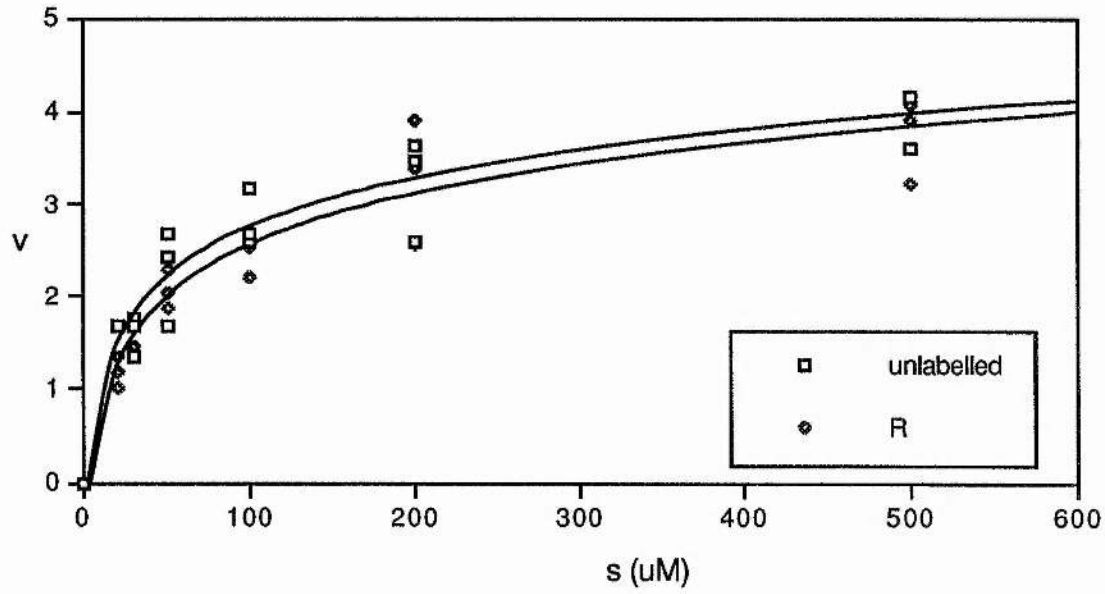



**Figure 2.19:** *A comparison of the initial rates of threonine synthase turnover of (2S)-O-phosphohomoserine and (2S,3R)-[3-$^2H_1$]-O-phosphohomoserine. Data fitted by non-linear regression using Enzfitter*

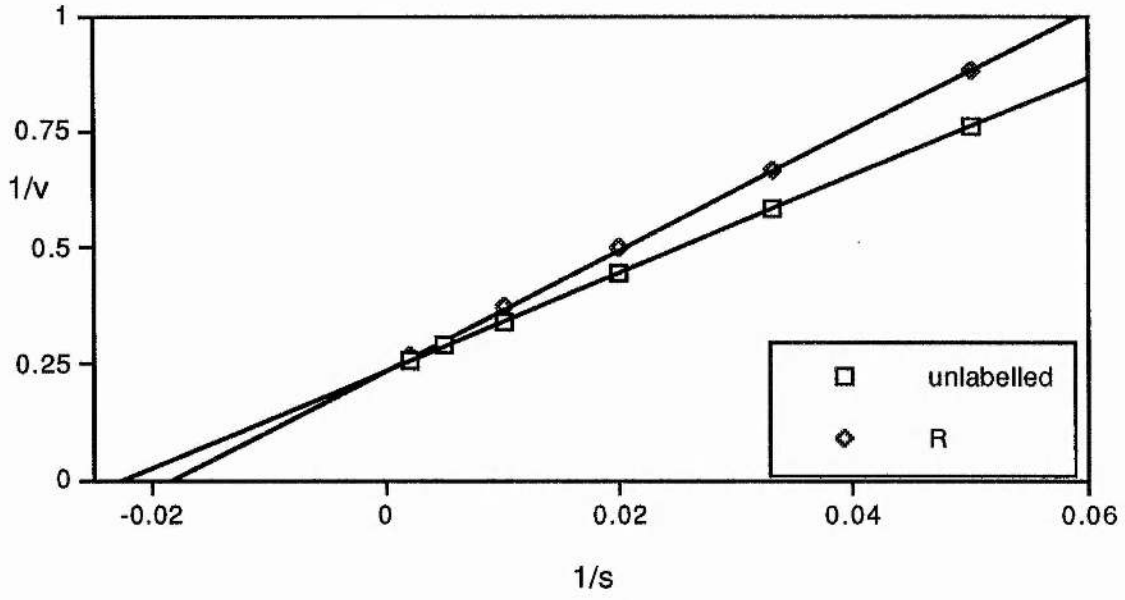



**Figure 2.20:** *The double reciprocal plot of threonine synthase activity with (2S)-O-phosphohomoserine and (2S,3R)-[3-$^2H_1$]-O-phosphohomoserine. Theoretical data points are derived from the best fit curves shown in Figure 2.18.*

When (2*S*,3*S*)-[3-$^2H_1$]- and (2*S*)-[2-$^2H_1$]-labelled phosphohomoserine were compared with the unlabelled substrate, significant changes in the kinetic parameters were observed (Table 2.21 and Figures 2.22 and 2.23).

| | $K_M$ (μM) | ± error | $V_{max}$ (μM $min^{-1}$) | ± error |
|---|---|---|---|---|
| PHS | 43.5 | 7.82 | 4.18 | 0.22 |
| SPHS | 24.2 | 3.91 | 2.40 | 0.10 |
| αPHS | 35.69 | 4.79 | 3.50 | 0.14 |

**Table 2.21:** *Kinetic data for (2S)-O-phosphohomoserine, (2S,3S)-[3-$^2H_1$]-O-phosphohomoserine and (2S)-[2-$^2H_1$]-O-phosphohomoserine.*

We can see that the (2*S*,3*S*)-[3-$^2H_1$]-isotopomer displays isotope effects of 1.74 and 1.0 for $^DV$ and $^D(V/K)$ respectively. This data reveals that the 3-*pro*-S hydrogen is removed from the substrate during the elimination process and is an identical result to that obtained for the yeast enzyme using tritiated substrates.[153] Furthermore, the magnitude of the $V_{max}$ isotope effect indicates that the elimination process is slow compared to other steps in the catalysed reaction. The finding that the value of $^D(V/K)$ is 1.0 shows that there are large forward reaction commitments and that the substrate cannot readily debind from the enzyme *via* reverse steps once the quinoid intermediate (**55**) has formed.

When (2*S*)-[2-$^2H_1$]-*O*-phosphohomoserine was incubated with the enzyme and the rate data were processed, isotope effects of 1.2 and 1.0 were obtained for $^DV$ and $^D(V/K)$ respectively. This result indicates that the removal of the $C^{\alpha}$-H from the substrate is kinetically significant but is not rate-limiting

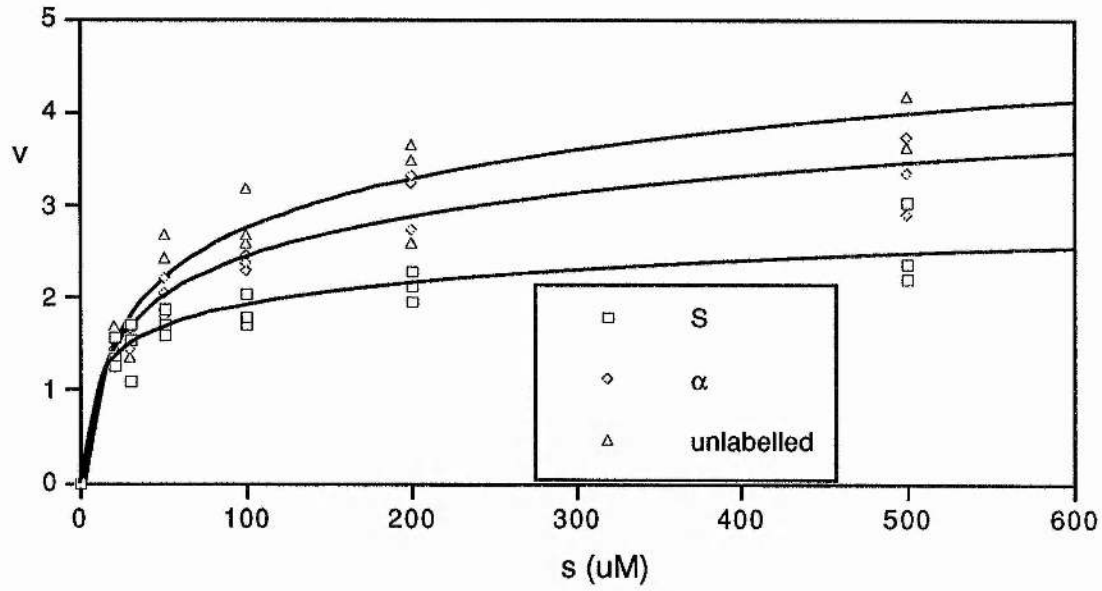



**Figure 2.22:** *A comparison of the initial rates of threonine synthase turnover of (2S)-O-phosphohomoserine, (2S,3S)-[3-$^2H_1$]-O-phosphohomoserine and (2S)-[2-$^2H_1$]-O-phosphohomoserine.*

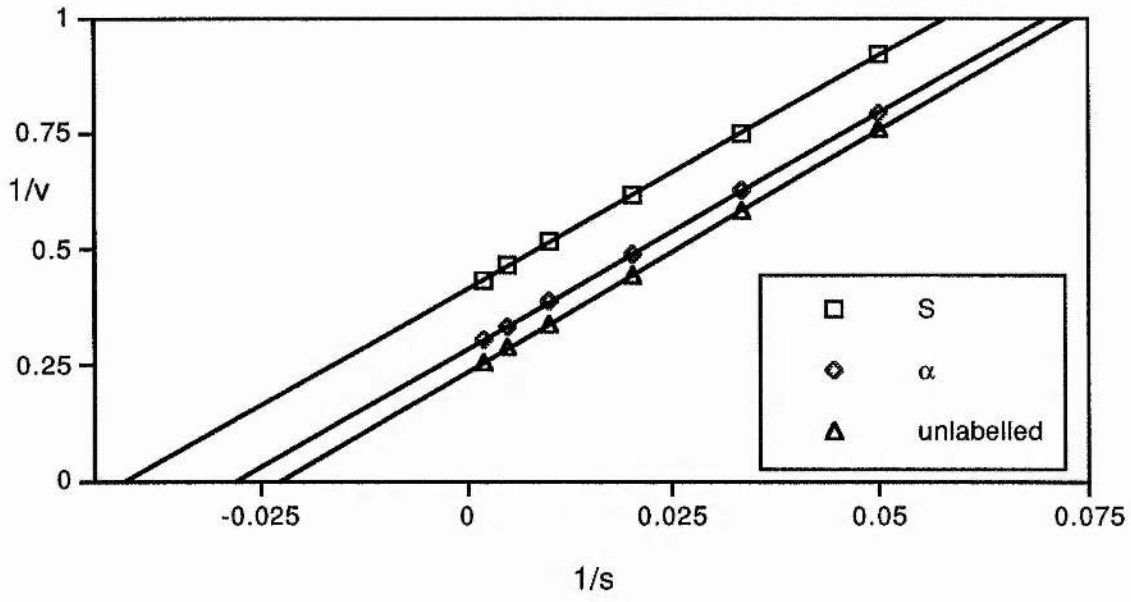



**Figure 2.23:** *The double reciprocal plot of threonine synthase activity with (2S)-O-phosphohomoserine, (2S,3S)-[3-$^2H_1$]-O-phosphohomoserine and (2S)-[2-$^2H_1$]-O-phosphohomoserine. Points are derived from the best-fit curves shown in Figure 2.22.*

**Conclusions.**

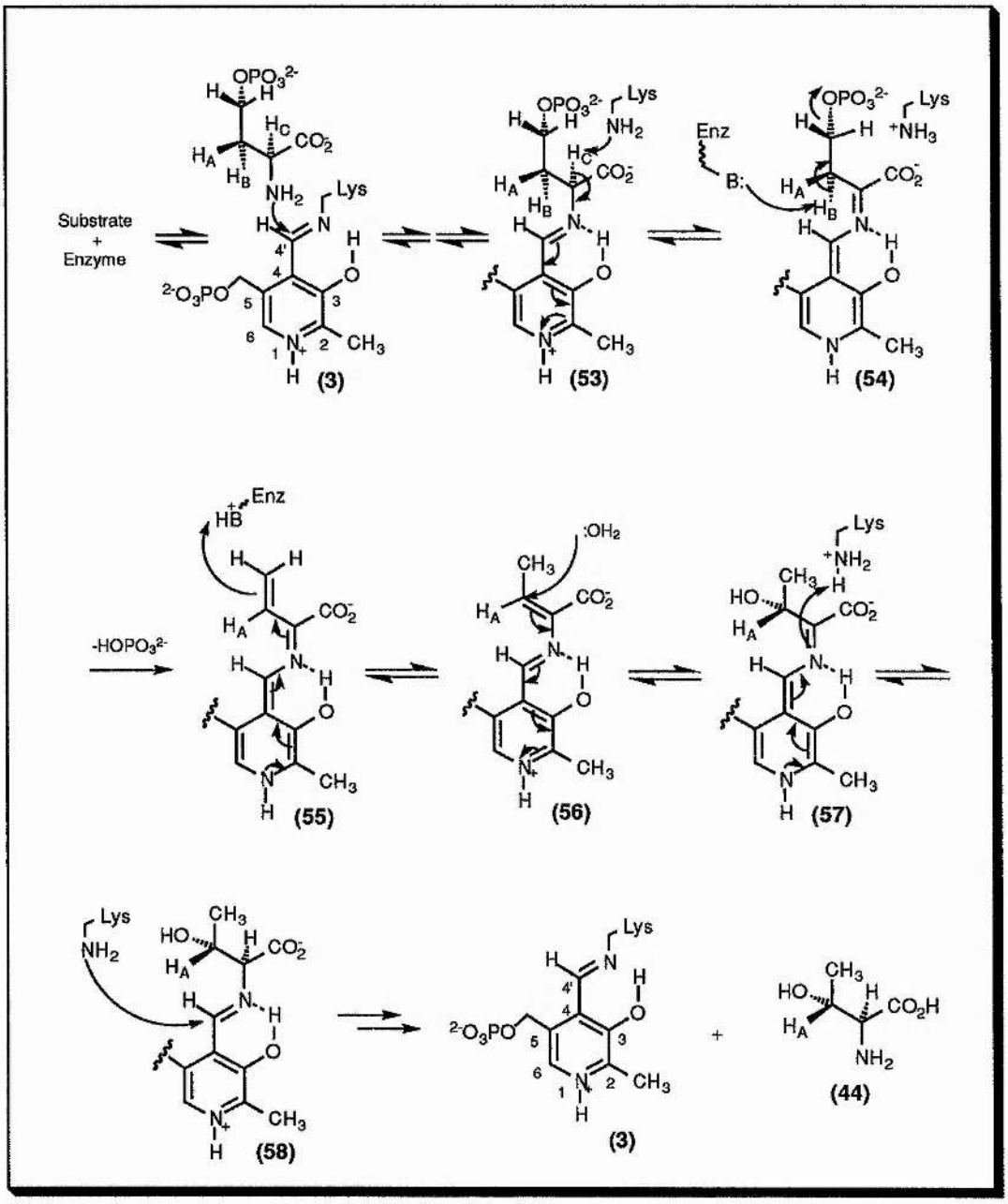
The above experiments reveal that both the $C^{\alpha}$-H and $C^{\beta}$-H proton removal steps in the *E. coli* threonine synthase mechanism display primary deuterium isotope effects, and that cleavage of the C-3-(*pro*-S)-H bond is at least partially rate limiting.

The removal of the $C^{\alpha}$-H proton from the external aldimine (**53**) to give the quinonoid intermediate (**54**) exhibits a small but significant kinetic isotope effect on V (Scheme 2.24). This indicates that the reaction is kinetically significant but is not slow compared to other steps in the reaction. As already stated, the value of $^{D}$(V/K) for removal of the $C^{\alpha}$-proton was unity. The fact that the value of $^{D}$V is small but larger than the value of $^{D}$(V/K) for the C-2 deuteriated compound strongly suggests that the high forward commitments to both the $C^{\alpha}$-H and $C^{\beta}$-H proton removal steps) occur because transaldimination in the reverse reaction direction and/ or substrate debinding is very slow.

The β,γ-elimination of phosphoric acid from the quinonoid intermediate (**54**) to give the conjugated enamine (**55**) was confirmed to take place with the loss of the 3-pro-S proton. This confirms that the *E. coli* enzyme has the same stereochemical path as the yeast enzyme at this point.[153] The kinetic isotope effect on the value of $^{D}$V of 1.75 suggests that this step in the mechanism of threonine synthase is at least partially rate-limiting. The value of $^{D}$(V/K) for the (2*S*,3*S*)-[3-$^{2}H_{1}$]-*O*-phosphohomoserine was unity, again pointing towards a high forward commitment to the reaction. This suggests that the steady-state concentration of the quinoid (**54**) is quite high.

In addition, this work confirms that *S*-adenosyl methionine behaves as an activator of *E. coli* threonine synthase. This is, to our knowledge, the first documented report of a prokaryotic threonine synthase being activated in this way. A full kinetic

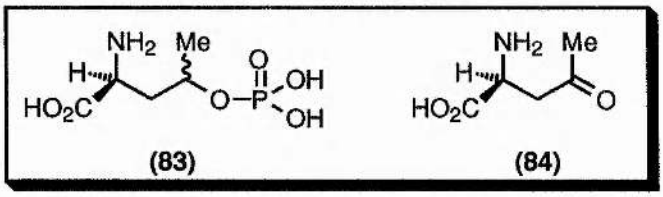
analysis of the activation of TS from *E. coli* by SAM, determining the Hill coefficient for the activation would confirm that the bacterial enzyme behaves in the same way as that of plants.




**Scheme 2.24:** *The proposed mechanism of E. coli threonine synthase.*

## 2.5 Phosphohomoserine Analogues.

In order to further investigate the mechanism of threonine synthase especially with reference to C-4, it was neccessary to synthesise 4-methyl phosphohomoserine (**83**). If either the (4*S*)- or (4*R*)-methyl phosphohomoserines were turned over as substrates, we would be able to infer the spatial availability around C-4 of the substrate and perhaps the geometry of the reactions occurring at C-4. The protected oxopentanoate (**84**) was envisaged as a possible synthetic intermediate for the methyl analogues.



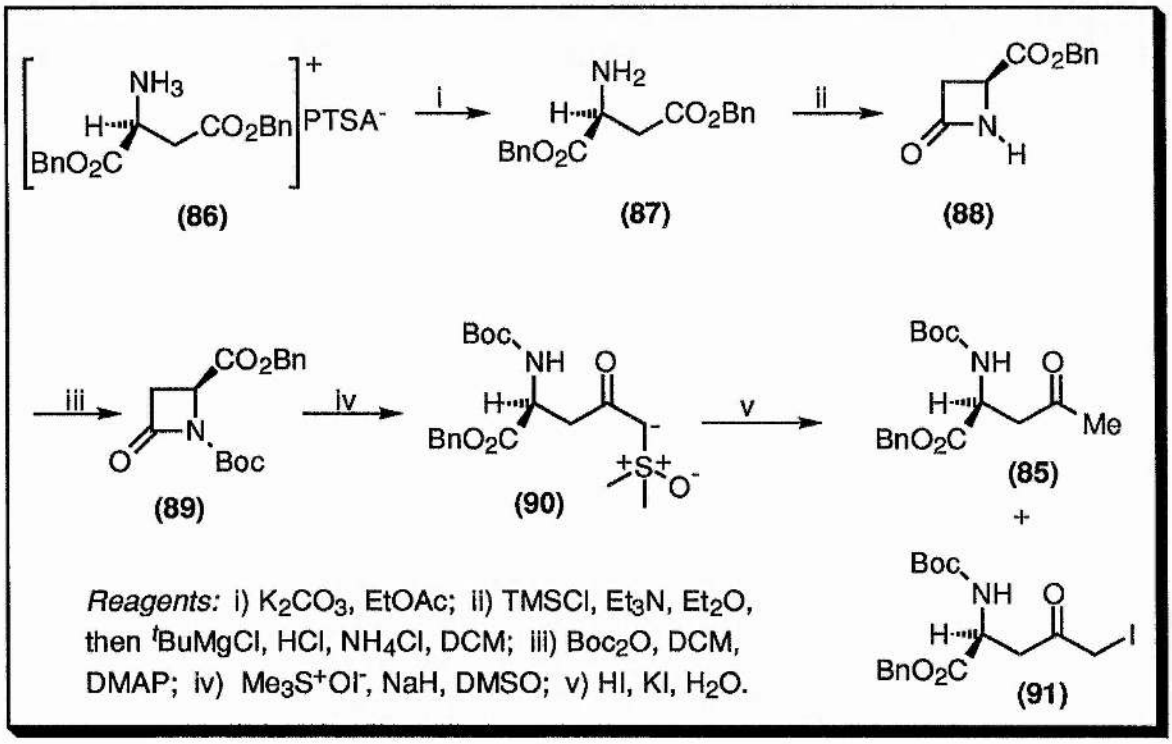

(**83**) (**84**)

Baldwin *et al.* have reported the synthesis of of (2*S*)-benzyl 2-(*t*-butyloxycarbonylamino)-4-oxopentanoate (**85**) from (2*S*)-aspartic acid dibenzyl ester *p*-toluenesulfonate (**86**) in 5 steps.[197-199] We decided to repeat this work in order to obtain substantial amounts of the starting material.

(2*S*)-Aspartic acid dibenzyl ester *p*-toluenesulfonate (**86**) was extracted into ethyl acetate from a solution of $K_2CO_3$ to provide the free amine (**87**). Cyclisation to the β-lactam involved the formation of (2*S*)-*N*-(trimethylsilyl)dibenzyl aspartate by reaction with TMSCl and $Et_3N$ in diethyl ether. Treatment of this species *in situ* with *t*-butyl magnesium chloride led to the formation of the azetidinone (**88**) in good yield, showing the β-lactam C=O IR signal at 1780 $cm^{-1}$. N-protection of the azetidinone with Boc anhydride and a catalytic amount of DMAP yielded the fully protected azetidinone (**89**), the $CH_3$'s of the Boc group clearly visible in the $^1H$-NMR spectrum at 1.44 ppm. This protected azetidinone was reacted with

trimethylsulfoxonium iodide and sodium hydride in DMSO to give the sulfoxonium ylide (**90**) in almost quantitative yield.

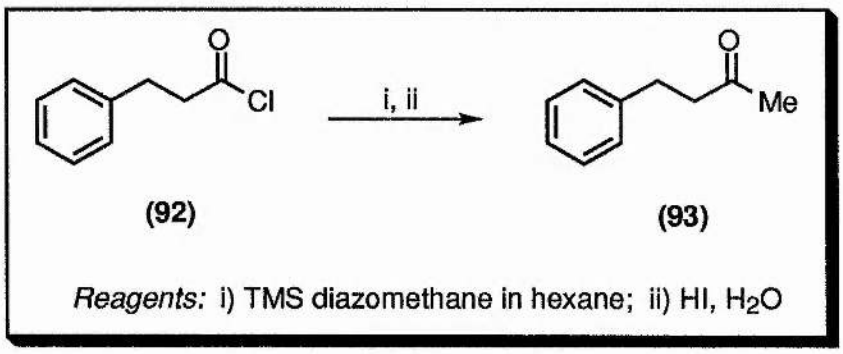
The sulfoxonium ylide (**90**) thus obtained was a relatively stable species and was carried forward to the next step without further purification. Reduction of the ylide with hydrogen iodide gave the desired oxopentanoate (**85**) only in a moderate yield of 48% ($\delta_H$ 2.12 ppm C(O)C$\underline{H}_3$). In an attempt to improve the yield of the desired oxopentanoate (**85**) an equivalent of potassium iodide was added to the reaction mixture. This, however, lead to the additional formation of the iodomethyl ketone (**91**) ($\delta_H$ 3.78 ppm C(O)$CH_2$I). The synthesis of protected oxopentanoate (**84**) by this route was of interest, but was not suitable for our needs (Scheme 2.25). The introduction of several extra steps was our main concern.




**Scheme 2.25:** *Baldwin's synthesis of 2-amino-4-oxopentanoate (**85**).*

We decided that a more efficient route would be to treat a suitably activated derivative of the already synthesised α-isopropyl *N*-trifluoracetyl aspartic acid (**75**) with diazomethane to form the diazoketone, and to reduce this to the methyl ketone *via* treatment with hydrogen iodide. Thus the use of TMS-diazomethane as a safe alternative to diazomethane and its performance as a methylating agent was investigated.

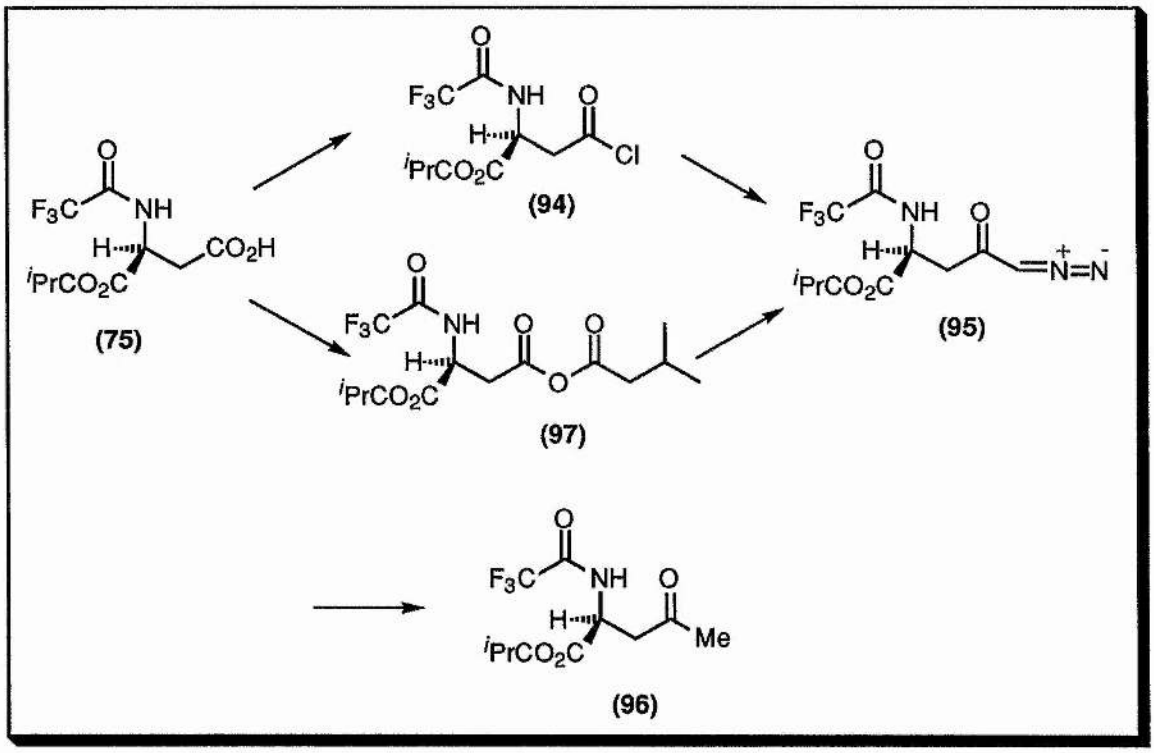
Using the simple and readily available hydrocinnamic acid chloride (**92**) as a model substrate, this was reacted with a solution of TMS-diazomethane in hexane for two hours at 0 °C, Scheme 2.26. Reduction of the diazoketone was carried out *in situ* by addition of excess hydrogen iodide. It was thought that this reduction of the ketone would also result in the loss of the trimethylsilyl group. Although some of the desired benzylacetone (**93**) had indeed been formed, there were also several rearrangement products, as well as the chloromethyl ketone. It was evident that although the reagent TMS-diazomethane could be used as a safe substitute for distilled diazomethane, in our case it was far from ideal.




**Scheme 2.26:** *The synthesis of benzyl acetone using TMS-diazomethane.*

The above strategies were therefore discarded in favour of a more direct route using methodology already developed in the group.[200] It was envisaged that *N*-

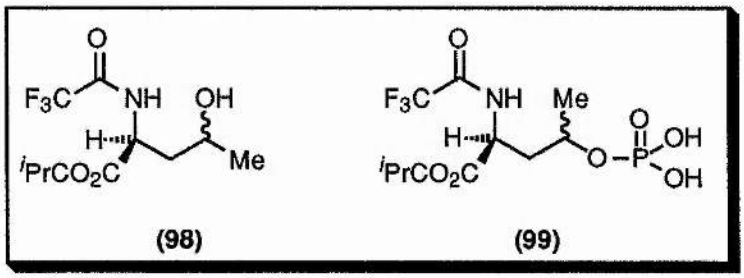
trifluoroacetyl α-isopropyl aspartic acid (**75**) would readily yield the protected acid chloride (**94**) on treatment with thionyl chloride. This would then be reacted with diazomethane to furnish the diazoketone (**95**), which could be reduced with hydrogen iodide to give oxopentanoate (**96**).[200] The formation of the methyl ketone could also be envisaged by forming the mixed anhydride derivative of the acid (**97**) as before, and treating this with diazomethane followed by treatment with iodide (Scheme 2.27).




**Scheme 2.27:** *Strategy for the synthesis of 4-methylphosphohomoserine*

Reduction of the ketone to the diastereomeric alcohols (**98**) with borohydride would allow esterification to the protected methyl analogue of phosphohomoserine (**99**) using the methodology developed earlier, *i.e.* by treatment with *N,N* diisopropyl bisbenzylphosphoramidite and tetrazole, followed by oxidation with *m*-CPBA. Deprotection of the phosphate triester by hydrogenolysis and treatment

with base would then allow the isolation of 4-methyl phosphohomoserine (**83**).




**(98)** **(99)**

# EXPERIMENTAL

Elemental microanalyses were performed in the departmental microanalytical laboratory.

NMR Spectra were recorded on a Bruker AM-300 (300 MHz; f.t. $^{1}$H-NMR, 74.76 MHz; $^{13}$C-NMR and 121.5 MHz; $^{31}$P-NMR), a Varian Gemini 200 (200 MHz; $^{1}$H-NMR, and 50.3 MHz; $^{13}$C-NMR) and a JEX GX 400 (400 MHz; f.t. $^{1}$H-NMR, 100.6 MHz; $^{13}$C-NMR) spectrometers. $^{1}$H-NMR spectra were referenced on $^{2}$HOH (4.68 ppm), $CHCl_3$ (7.27 ppm) or DMSO (2.47 ppm). $^{13}$C spectra were referenced on $CH_3OH$ (49.9 ppm), $CHCl_3$ (77.5 ppm) or DMSO (39.70 ppm) and $^{31}$P spectra on external $H_3PO_4$. NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position ($\delta_H$ or $\delta_C$), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-doublet of doublets, sp-septet and br-broad), coupling constant ($J_{X,Y}$ Hz if applicable) and assignment.

Infrared spectra were taken on a Perkin-Elmer 1420 recording spectrometer and a Perkin Elmer 1710 f.t. I.R. spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. The frequencies ($\upsilon$) as absorption maxima are given in wavenumbers ($cm^{-1}$) relative to a polystyrene standard. Mass spectra were recorded on a Kratos MS50, a JEOL DX 303, a VG TRIO 1 and obtained on an SERC service basis at the University of Swansea using a VG ZAB E. Major fragments are given as percentages of the base peak intensity. GC/MS spectra were recorded on a Hewlett Packard 5890A 6C. U.V./ VIS. optical intensities were measured on a Cam Spec M302 spectrophotometer.

Flash chromatography was performed according to the procedure of Still *et al.*[201] using Sorbisil C60 (40-60 μm mesh) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica plates (Macherey-Nagel 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, ethanolic phosphomolybdic acid, aqueous potassium permanganate, acidic

palladium chloride or ninhydrin.

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

The solvents used were either distilled or of analar quality and light petroleum refers to that portion boiling between 40 ° and 60 °C. Solvents were dried according to literature procedures.[202] Ethanol and methanol were dried using magnesium turnings. Isopropanol, DMF, $CH_2Cl_2$, acetonitrile, diisopropylamine, triethylamine and pyridine were distilled over $CaH_2$. THF and diethylether were distilled under nitrogen from the sodium ketal of benzophenone directly before use. N-Methylmorpholine was distilled from ninhydrin before use.

Anion exchange chromatography was performed on DEAE Sephacel unless otherwise stated. The chromatographic media was dissolved in the appropriate buffer, left for 24 h, packed (100 x 15 mm) and used as such during the whole study.

Gel exclusion chromatography was performed using Sephadex G-150. The gel was suspended in the appropriate buffer and allowed to settle several times to remove fine particles. The column was repacked (80 x 3 cm) before each use to achieve maximum flow rates.

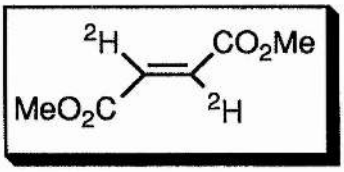
SDS-PAGE was performed following the procedure of Laemmli on a discontinuous medium.[203] A Mini-Protean II Dual Slab Cell apparatus was used.

Centrifugations were carried out on a CENTRIKON C-124 centrifuge. Pooled fractions from protein purification steps were concentrated by ultrafiltration using an AMICON apparatus (43 or 76 mm diameter), through a YM30 membrane under $N_2$.

All protein purification steps were carried out at 0-4 °C, and all materials used were of AR quality. Protein concentrations were determined by the method of Bradford.[195] Appropriately diluted samples of protein solution were mixed with Coomasie blue dye reagent (100 mg of Coomasie brilliant blue dissolved in 50 ml of 95 % ethanol to which 100 ml of 85 % of phosphoric acid has been added and the whole diluted to 1l with water, and after 5 min the absorbance was measured at 595 nm. Standard curves were prepared using bovine serum albumin.

*E. coli* Tir 8 were the kind gift of Prof. C. Parsot of the Institut Pasteur, Paris. The cells were stored in 10 ml aliquots of 10 % glycerol solution at -80 °C. The innoculae were destroyed after 10 usages, to prevent spontaneous reversion to wild type after repeated freeze-thaw cycles.

**Dimethyl dideuteriofumarate (69).**



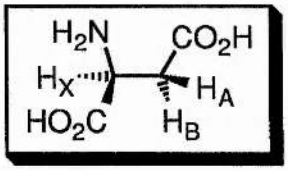

To a stirred solution of dimethyl acetylenedicarboxylate (**68**) (5 g, 35 mmol) in dry THF (70 ml) containing deuterium oxide (0.7 g, 35 mmol) was added dropwise triphenylphosphine (9.3 g, 35 mmol) in THF (70 ml). The reaction temperature was not allowed to exceed 5 °C during the addition. The reaction mixture was then allowed to warm to room temperature, before being refluxed for 5 h. The resultant solution was dried ($MgSO_4$), filtered, and the filtrate washed with dry THF. The solvent was removed under reduced pressure. The red/ brown tar was dried under vacuum and then sublimed at 80-100 °C to yield yellowish white crystals. Recrystallisation from methanol yielded the product (**69**) as white crystals (2.11 g, 41%) of m.p. 98-100 °C (lit.,[176] 98-101 °C); (Found: C, 49.45; H, 5.15. $C_6H_6{}^2H_2O_4$ requires C, 49.30; H, 5.15 %); $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 2260 (C-$^2$H), 1730 (ester C=O) and 1610 (C=C); $\delta_H$ (200 MHz; $^2H_2O$, 5% $NaO^2H$) 3.81 (6H, s, 2 x $CH_3$); $\delta_C$ (50.3 MHz, $^2H_2O$; 5% $NaO^2H$) 51.6 ($OCH_3$), 137.0 (t, *J* 12, alkenic C) and 177.49 ($\underline{C}O_2Me$); *m/z* (EI) 146 ($M^+$, 2%) and 59 (75, $CO_2Me^+$).

**Dideuteriofumaric acid (70).**

Dimethyl dideuteriofumarate (**69**) (2 g, 13.7 mmol) was added to 12 % NaOH (13 ml) with stirring for 72 h at room temperature. The pH was adjusted to 2 with 3 M HCl and the acid precipitated out. The product was recrystallised from 8% NaOH using 3 M HCl to regenerate the un-ionized acid (**70**) (1.19 g, 66 %) of m.p. 297-300 °C, (lit.,[176] 299-300 °C); (Found: C, 39.50; H, 3.40. $C_4H_2{}^2H_2O_4$ requires

C, 40.70; H, 3.40 %); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 2400-3400 (acid OH), 2260 (C-$^2$H) and 1680 (acid C=O); $\delta_C$ (50.3 MHz; $^2H_2O$, 5% NaO$^2$H) 133.10 (t, *J* 25, C=C) and 172.88 ($CO_2H$); *m/z* (EI) 118 (*M*$^+$, 38%) and 101 (41, [*M* - OH]$^+$).

**(2*S*,3*S*)-[2-$^2H_1$, 3-$^2H_1$]Aspartic acid (45a) {$H_X$=$H_A$=$^2$H}.**




Dideuteriofumaric acid (**70**) (2 g, 16.9 mmol) was suspended in water (20 ml) and the pH adjusted to 9 with concentrated ammonia solution. The solution was then concentrated under reduced pressure. The diammonium fumarate was redissolved in water (20 ml), and magnesium chloride hexahydrate (40 mg, 10 mmol), and potassium chloride (7 mg, 4.5 mmol) were added. The pH was again adjusted to 9 with concentrated ammonia, 3-methylaspartase (30 units) was added and the reaction was incubated at 30 °C until no further decrease in the absorbance at 240 nm occurred (about 3 days).

The solution was heated at 100 °C for 2 min and filtered through a Celite pad. The filtrate was acidified to pH 1 with 12 M HCl and extracted with ether (2 x 10 ml). The aqueous layer was adjusted to pH 4 and the aspartic acid (**9a**) crystallised out on addition of ethanol. The product was recrystallised from hot water/ ethanol to give the product as white crystals (1.39 g, 60 %) of m.p. 300 °C (decomp.); $[\alpha]_D$ +24.7 (*c* 0.6 in 6 M HCl) {lit.,[204] $[\alpha]_D$ +25.2 (*c* 0.6 in 6 M HCl)}; $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3425 (N-H), 2656 (C-H), 2260 (C-$^2$H), 2081 (C-$^2$H) and 1688 (C=O); $\delta_H$ (200 MHz; $^2H_2O$, 5% NaO$^2$H) 2.51 (1H, s, Cβ-H); $\delta_C$ (50.3 MHz; $^2H_2O$, 5% NaO$^2$H) 46.5

(t, *J* 20, Cβ), 56.0 (t, *J* 19, Cα), 183.21 (β-$CO_2H$) and 185.33 (α-$CO_2H$); *m/z* (EI) 135 ($M^+$, 3%), 44 (100, $CO_2^+$).

**(2*S*,3*S*)-[3-$^2H_1$]Aspartic acid (45b) {$H_A$=$^2H$}.**

(2*S*,3*S*)-[3-$^2H_1$]Aspartic acid (**45a**) (2.66 g, 20 mmol) was dissolved in water (50 ml) and the pH adjusted to 7.25 with concentrated ammonia solution. aspartate aminotransferase (AAT) (200 units) and pyridoxal 5'-phosphate (5 mg) were added and the reaction incubated at 37 °C. The progress of the reaction was then followed by NMR. On completion of the reaction (approx. 72 h), the enzyme was denatured by boiling the solution for 2 min, and the solution filtered and concentrated under reduced pressure. The solid residue was dissolved in water (10 ml) and crystallised using 6 M HCl to yield the product (**45b**) as fine white crystals (2.09 g, 78 %), m. p. >300 °C; (Found: C, 35.80; H, 5.25; N, 11.30. $C_4H_6{}^2HNO_4$ requires C, 35.80; H, 5.25; N, 10.45 %); $[\alpha]_D$ +24.1 (*c* 0.6 in 6 M HCl) {lit.,[204] 23.9 (*c* 0.6 in 6 M HCl)}; $\delta_H$ (200 MHz; $^2H_2O$, 5% $NaO^2H$) 2.58 (1H, d, *J* 2.4, Cβ-H) and 3.50 (1H, d, *J* 2.4, Cα-H); $\delta_C$ (50.3 MHz; $^2H_2O$, 5% $NaO^2H$) 45.8 (t, *J* 17, Cβ), 56.8 (Cα), 183.2 (β-$CO_2H$) and 185.3 (α-$CO_2H$).

**(2*S*,3*R*)-[3-$^2H_1$]Aspartic acid (45c) {$H_B$=$^2H$}.**

This was prepared from fumaric acid (2 g, 17.2 mmol), as described above using deuterium oxide as the solvent. (2*S*,3*R*)-[3-$^2H_1$] Aspartic acid was obtained as fine white crystals (1.55 g, 67 %); m.p. 290 °C (decomp.); (Found: C, 35.90; H, 5.25; N, 10.90; $M^+$, 135.0498. $C_4H_6{}^2HNO_4$ requires: C, 35.80; H, 5.25; N, 10.45 %; $M^+$, 135.0516); $[\alpha]_D$ +23.9 (*c* 0.6 in 6 M HCl); $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 2740 (C-H), 2180 (C-$^2H$), 1691 (C=O) and 1600 (N-H); $\delta_H$ (200 MHz; $^2H_2O$, 5% $NaO^2H$) 2.35 (2H, br m,

Cβ-H) and 3.41 (1H, d, *J* 6.6, Cα-H); $\delta_C$ (50.3 MHz; $^2H_2O$, 5% $NaO^2H$) 45.0 (t, *J* 15, Cβ), 56.47 (Cα), 182.79 (β-$CO_2H$) and 184.10 (α-$CO_2H$); *m/z* (FAB) 269 (2 x [*M* + H]+, 10%), 135 (100, [*M* + H]+).

**(2*S*)-[2-$^2H_1$]Aspartic acid (45d) {$H_X$=$^2H$}.**

This was prepared in an identical manner to that described for compound (**45b**) using (2*S*)-aspartic acid (3 g, 22.5 mmol) as the starting amino acid and carrying out the enzyme incubation in deuterium oxide. Recrystallisation from water/ 6 M HCl gave (2*S*)-[2-$^2H_1$]-aspartic acid as white crystals (2.19 g, 72 %); m.p. > 300 °C (decomp.); $[\alpha]_D$ +21.02 (*c* 0.95 in 6 M HCl) [lit.,[183] +21.05 (*c* 0.95 in 6 M HCl)]; $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 3000-2500 (OH stretching), 1688 & 1643 (carboxylate anion) and 1586 (NH deformations); $\delta_H$ (200 MHz; $^2H_2O$, 5% $NaO^2H$) 2.6 (2H, dd, *J* 1.3, *J* 15.3, Cβ-H); $\delta_C$ (50.3 MHz; $^2H_2O$, 5% $NaO^2H$) 46.1 (Cβ), 56.5 (t, *J* 17.4, Cα), 183.1 (β-$CO_2H$) and 185.3 (α-$CO_2H$); *m/z* (FAB) 135 ([*M* + H]+, 70 %) and 134 (30, *M*+).

**Carbobenzyloxy (2*S*) aspartic acid (71).**

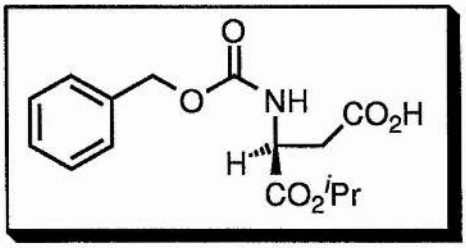
(2*S*)-Aspartic acid (2.63 g, 20 mmol) was dissolved in water (50 ml), overlaid with ether (10 ml). To this was added to MgO (2.42 g, 60 mmol) and the solution was stirred vigorously at 0 °C. Benzyl chloroformate (6.82 g, 40 mmol) was added in five portions over 30 min, and the reaction left to stir for four days. The solution was acidified to pH 2 with 1 M HCl, and then extracted with ethyl acetate (3 x 25 ml). The organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to yield a white crystalline solid. Recrystallisation from ethyl acetate/ petroleum ether gave the product (**71**) as white crystals (3.26 g, 62%); m.p. 114-

115 °C (lit.,[181] 116 °C); $[\alpha]_D$ +4.5 (*c* 7.1 in acetic acid) [lit.,[181] +9.6 (in acetic acid)]; $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 3360 (amide NH), 1720 (C=O) and 1540 (N-H stretch); $\delta_H$ (200 MHz; $d_6$-DMSO) 2.65 (2H, ABX, $J_{BA}$ 16.5, $J_{XA}$ 5.5, $J_{XB}$ 7.7, C$\beta$-$H_2$), 4.37 (1H, dd, *J* 7.7, *J* 16.5, C$\alpha$-H), 5.06 (2H, s, benzyl $CH_2$), 7.35 (5H, m, aromatic) and 7.65 (1H, d, *J* 8.4); $\delta_C$ (50.3 MHz; $d_6$-DMSO) 36.26 (C$\beta$), 45.63 (C$\alpha$), 65.76 ($PhCH_2$), 128.40 & 128.64 (aromatic), 137.16 (4° aromatic), 156.16 (urethane C=O) and 171.95 & 172.99 ($CO_2H$).

**Carbobenzyloxy (2*S*) aspartic anhydride (72).**

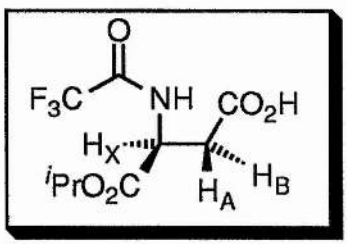
Trifluoroacetic acid anhydride (0.84 g, 4 mmol) was added to a stirred suspension of carbobenzyloxy-(2*S*)-aspartic acid (**71**) (3.7 mmol, 0.97 g) in dry THF (30 ml) over 30 min. The reaction was then left stirring for an additional 30 min. The solvent was removed under reduced pressure to yield a white crystalline solid which was recrystallised from ether/ petroleum ether to give pure (**72**) as a white solid (0.89 g, 96 %), m.p. 69-70 °C; $\upsilon_{max}$ (Nujol)/ $cm^{-1}$ 3410 (amide NH), 1805 (C=O), 1685 (C=O) and 1535 (N-C=O); $\delta_H$ (200 MHz; $C^2HCl_3$) 3.25 (2H, ABX, C$\beta$-$H_2$), 4.5 (1H, m, C$\alpha$-H), 5.15 (2H, s, $PhCH_2$), 5.6 (1H, m, NH) and 7.35 (5H, s, aromatic); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 34.8 (C$\beta$), 68 (C$\alpha$), 128 (benzyl), 136 (4° aromatic), 156 (C=O) and 168 & 175 ($CO_2H$); *m/z* (EI) 249 ($M^+$, 10%) and 107 (54, $C_6H_5CH_2O^+$).

**α-Isopropyl carbobenzyloxy (2*S*) aspartate (73).**




The anhydride (**72**) (1.99 g, 8 mmol) was dissolved in cold dry isopropanol (30 ml) at 0 °C. The reaction was allowed to warm to room temperature and then left for 15 h to complete dissolution. Isopropanol was removed under reduced pressure to leave a clear colourless oil which crystallised on standing. Recrystallisation from diethyl ether/ petroleum ether gave the product (**73**) as white crystals (1.36 g, 55%); m.p. 112 °C; $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3420 (N-H), 1730 (C=O) and 1170 (C-O); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.25 & 1.27 (6H, 2 x $^i$PrC$H_3$), 3.00 (2H, m, C$\beta H_2$), 4.15 (1H, m, C$\alpha$), 5.15 (2H, m, Ph$CH_2$), 5.95 (1H, d, *J* 6.5, NH), 7.35 (5H, s, aromatic) and 8.18 (1H, s, $CO_2H$); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 14.6 & 21.5 ($^i$Pr$\underline{C}H_3$), 37.0 (C$\beta$), 51.0 (C$\alpha$), 61.16 ($^i$Pr$\underline{C}$H), 67.7 (Ph$\underline{C}H_2$), 128.7 (aromatic), 136.1 (4° aromatic), 156.2 (urethane C=O), 169.21 ($CO_2{}^i$Pr) and 172.33 ($CO_2$H); *m/z* (EI) 309 (*M*$^+$, 1%) and 264 (10, [*M* - $CO_2$H]$^+$).

**α-Isopropyl *N*-trifluoroacetyl (2*S*)-aspartate (75).**



Trifluoroacetic anhydride (80 g, 381 mmol) was added dropwise over 30 min to a stirred suspension of (2*S*)-aspartic acid (6 g, 45.1 mmol) in dry THF (150 ml) at 0 °C under an atmosphere of nitrogen. The reaction was allowed to warm to room temperature over 2 h when the solution became homogeneous. The solvent was removed under reduced pressure and the resulting white solid was thoroughly dried. Cold dry isopropanol (50 ml) was added and the reaction was allowed to warm to room temperature. The reaction was stirred for a further 48 h and the solvent was removed under reduced pressure to yield a clear oil which solidified on standing. Recrystallisation from ether/ petroleum ether yielded the product (**75**) as white crystals (11.2 g, 92 %), m.p. 98 °C lit.,[183] 98 °C; (Found: C, 39.90; H, 4.65; N, 5.25. $C_9H_{12}NO_5F_3$ requires C, 39.90; H, 4.45; N, 5.15 %); $[\alpha]_D$ -40.7 (*c* 1.0 in methanol); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3311 (N-H), 1740 ($F_3CC{=}O$), 1708 & 1707 (superimposed $CO_2{}^iPr$ and $CO_2H$), 1280 (C-O) and 1185-1106 ($CF_3$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.27 (6H, dd, *J* 4.4, *J* 17.5, 2 x $^iPrCH_3$), 3.23 (2H, ABX, $J_{AX} = J_{BX}$ 4.3, $J_{AB}$ 17.7, C$\beta$-$H_2$), 4.77 (1H, dd, *J* 3.9, *J* 7.6, C$\alpha$-H), 5.11 (1H, sp, *J* 4.5, $^i$PrCH) and 7.39 (1H, d, *J* 7.6, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 21.8 & 22.0 ($^iPrCH_3$), 35.7 (C$\beta$), 49.4 (C$\alpha$), 71.5 ($^i$PrCH), 116.0 (q, *J* 287, $F_3C$), 157.6 (q, *J* 37.9 , $F_3C\underline{C}O$), 169.0 ($CO_2{}^iPr$) and 175.86 ($\beta$-$CO_2H$); *m/z* (EI) 272 ([*M* + H]$^+$, 1%), 96 (21, $F_3CCO^+$) and 43 (100, $[(CH_3)_2CHOH]^+$).

### $\alpha$-Isopropyl *N*-trifluoroacetyl (2*S*,3*R*)-[3-$^2H_1$]aspartate (75a).

This was prepared in an identical manner to that described for compound (**75**) using (2*S*,3*R*)-[3-$^2H_1$]aspartic acid (**45c**) (1.0 g, 7.5 mmol) as starting amino acid in 90 % yield (1.84 g), m.p. 97-98 °C; $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3311 (N-H), 1740 ($F_3CC{=}O$), 1708 & 1707 (superimposed $CO_2{}^iPr$ and $CO_2H$), 1280 (C-O) and 1185-1106 ($CF_3$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.28 (6H, t, *J* 6.0, 2 x $^iPrCH_3$), 3.16 (1H, d, *J* 4.3, C$\beta$-H), 4.83 (1H, dd, *J* 3.9, *J* 6.2, C$\alpha$-H), 5.11 (1H, m, *J* 6.3, $^i$PrCH), 7.56 (1H, d, *J* 7.7, NH) and

10.21 (1H, br s, $CO_2H$); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 21.8 & 22.0 ($^iPrCH_3$), 35.7 (t, $J_{C^2H}$ 6.6, Cβ), 49.3 (Cα), 71.4 ($^iPrCH$), 123.1 (q, *J* 288, $F_3C$), 158.0 (q, *J* 37.8, $F_3C\underline{C}O$), 169.0 ($CO_2{}^iPr$) and 176.0 (β-$CO_2H$); *m/z* (EI) 227 ([*M* - $CO_2H$]$^+$, 7%), 213 (21, [*M* - $OCH(CH_3)_2$]$^+$), 140 (45, [*M* - $CO_2CH(CH_3)_2$]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

### α-Isopropyl N-trifluoroacetyl (2*S*,3*S*)-[3-$^2H_1$]aspartate (75b).

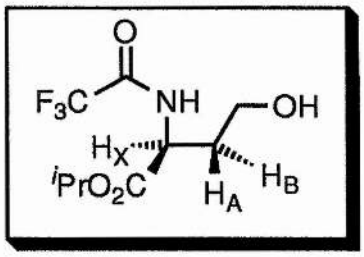
This was prepared in an identical manner to that described for compound (**75**) using (2*S*,3*S*)-[3-$^2H_1$]aspartic acid (**45b**) (1.50 g, 11.2 mmol) as starting amino acid in 91 % yield (2.78 g), m.p. 96-98 °C; $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 3311 (N-H), 1740 ($F_3CC{=}O$), 1708 & 1707 (superimposed $CO_2{}^iPr$ and $CO_2H$), 1280 (C-O) and 1185-1106 ($CF_3$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.28 (6H, t, *J* 6.0, 2 x $^iPrCH_3$), 2.93 (1H, d, *J* 4.3, Cβ-H), 4.82 (1H, dd, *J* 3.9, *J* 7.7, Cα-H), 5.12 (1H, m, *J* 6.3, $^iPrCH$), 7.60 (1H, d, *J* 7.7, NH) and 8.43 (1H, br s, $CO_2H$); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 21.84 & 21.96 ($^iPrCH_3$), 49.40 (t, *J* 5.6, Cβ), 71.47 ($^iPrCH$), 116.0 (q, *J* 287, $F_3C$), 157.61 (q, *J* 37.9, $F_3C\underline{C}O$), 169.04 (α-$CO_2{}^iPr$) and 175.86 (β-$CO_2H$); *m/z* (EI) 227 ([*M* - $CO_2H$]$^+$, 10%), 213 (25, [*M* - $OCH(CH_3)_2$]$^+$), 185 (40, [*M* - $CO_2H$ - $CH(CH_3)_2$]$^+$), 167 (22, [*M* - $CO_2CH(CH_3)_2$ - $OH_2$]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

### α-Isopropyl *N* trifluoroacetyl (2*S*)-[2-$^2H_1$]aspartate (75c).

This was prepared in an identical manner to that described for compound (**75**) using (2*S*)-[2-$^2H_1$]aspartic acid (**45d**) (1.15 g, 8.58 mmol) as starting amino acid in 87 % yield (2.03 g), m.p. 95-87 °C; $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 3311 (N-H), 1740 ($F_3CC{=}O$), 1708 & 1707 (superimposed $CO_2{}^iPr$ and $CO_2H$), 1280 (C-O) and 1185-1106 ($CF_3$); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 & 1.25 (6H, d, $^iPrCH_3$), 3.04 (2H, q, *J* 18.7, Cβ-$H_2$), 5.07 ( 1H, sp, *J* 5.9, $^iPrCH$), 7.55 (1H, d, *J* 7.9, NH) and 10.08 (1H, br s, $CO_2H$); $\delta_C$ (50.3

MHz; $C^2HCl_3$) 21.65 & 21.79 ($^iPrCH_3$), 35.6 (t, *J* 9.2, Cα), 49.2 (Cβ), 71.5 ($^iPrCH$), 116 (q, *J* 287, $F_3C$), 157.6 (q, *J* 38.1, $F_3C\underline{C}O$), 169.0 ($CO_2{}^iPr$) and 175.9 ($CO_2H$); *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 (45, [*M* - $CO_2CH(CH_3)_2$]$^+$) and 43 (100, $[CH(CH_3)_2]^+$).

**α-Isopropyl *N*-trifluoroacetyl (2*S*) homoserine (76).**




The α-isopropyl ester (**75**) (1 g, 3.9 mmol) was dissolved in dry THF (15 ml) and the solution cooled to -50 °C. *N*-Methylmorpholine (0.39 g, 3.9 mmol) and isobutyl chloroformate (0.506 g, 3.9 mmol) were then added. A white precipitate formed immediately and after 5 min the solution was filtered into a solution of $NaBH_4$ (0.10 g, 2.7 mmol) in THF (10 ml) at -20 °C. The reaction was left to stir for 4 h, after which time water was added to destroy any excess borohydride. The solution was evaporated under reduced pressure until water began to distill off, and then the aqueous phase was extracted with ether (3 x 15 ml). The ethereal layer was washed with brine, dried ($MgSO_4$) and then evaporated to dryness under reduced pressure. The crude pale yellow oil was purified by flash chromatography [silica, eluting with ethyl acetate, petroleum ether and diethyl ether; (40:40:20) containing a few drops of glacial acetic acid] to yield the pure alcohol (**76**) as a clear oil (0.86 g, 92 %); (Found C, 42.45; H, 5.15; N, 5.45. Calc. for $C_9H_{14}NO_4F_3$ C: 42.05; H, 5.50; N, 5.45 %); *m/z* (Found: [*M* - $C_3H_7O$]$^+$ 198.0363. Calc. for $C_6H_7NO_3F_3$: 198.0378); $[\alpha]_D$ -48.2 (*c* 1.75 in diethyl ether); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3400-3000 (OH)

and 1720 (ester C=O); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.28 (6H, m, 2 x $^iPrCH_3$), 1.9 (1H, m, C$\beta$-$H_B$), 2.17 (1H, m, C$\beta$-$H_A$), 4.67 (1H, dd, $J_{XA}$ 4.32, $J_{XB}$ 7.7, C$\alpha$-H), 5.08 (1H, sp, *J* 6.27, $^i$PrCH) and 7.64 (1H, d, *J* 2.1, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 20.0 & 20.1 ($^iPrCH_3$), 34.5 (C$\beta$), 51.6 (C$\alpha$), 59.2 (C$\gamma$), 70.8 ($^i$PrCH), 126.0 (q, *J* 287, $F_3C$), 158.0 (q, *J* 37.9 Hz, $F_3CCO$) and 170.8 ($CO_2{}^iPr$); *m/z* (CI) ([*M* + $NH_4$]$^+$, 60 %), (100, [*M* - $C_3H_7OH$]$^+$).

## α-Isopropyl *N*-trifluoroacetyl (2*S*,3*R*)-[3-$^2H_1$]homoserine (76a).

This was prepared in an identical manner to that described for compound (**76**) using (2*S*,3*R*)-[3-$^2H_1$]protected aspartate (**75a**) (0.57 g, 2.09 mmol) as starting material in 86 % yield (.469 g); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3400-3000 (OH) and 1720 (ester C=O); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.18 (6H, m, 2 x $^iPrCH_3$), 1.93 (1H, m, C$\beta$-$H_B$), 3.67 (2H, m, C$\gamma$-$H_2$), 4.57 (1H, t, *J* 7.3, C$\alpha$-H), 4.98 (1H, sp, *J* 6.3, $^i$PrCH) and 8.06 (1H, d, *J* 6.9, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.0 & 22.1 (2 x $^iPrCH_3$), 33.6 (t, *J* 4.7, C$\beta$), 51.6 (C$\alpha$), 59.2 (C$\gamma$), 70.7 ($^i$PrCH), 126.0 (q, *J* 287, $F_3C$), 158.0 (q, *J* 37.9, $F_3CCO$) and 170.6 ($CO_2{}^iPr$); *m/z* (EI) 259 ([*M* + H]$^+$, 45 %), 241 (15, [*M* - OH]$^+$), 199 (70, [*M* - $OCH(CH_3)_2{}^+$], 171 (35, [*M* - $CO_2CH(CH_3)_2$]$^+$), 141 (60, [*M* - $CO_2CH(CH_3)_2$ - HCOH]$^+$), 57 (75, [$OCH(CH_3)_2$]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

## α-Isopropyl *N*-trifluoroacetyl (2*S*,3*S*)-[3-$^2H_1$]homoserine (76b).

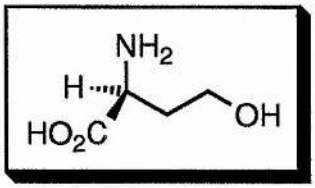
This was prepared in an identical manner to that discussed for compound (**76**) using (2*S*,3*R*)-[3-$^2H_1$]protected aspartate (**75a**) (0.65 g, 2.34 mmol) as starting material in 75 % yield (0.462 g); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3400-3000 (OH) and 1720 (ester C=O); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.18 (6H, m, 2 x $^iPrCH_3$), 2.17 (1H, m, C$\beta$-$H_A$), 3.67 (2H, m, C$\gamma$-$H_2$), 4.57 (1H, dd, *J* 4.2, *J* 6.4, C$\alpha$-H), 5.09 (1H, sp, *J* 6.3, $^i$PrCH) and

7.71 (1H, d, *J* 6.9, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.1 & 22.2 (2 x $^iPrCH_3$), 33.6 (t, *J* 7.2, Cβ), 51.6 (Cα), 59.2 (Cγ), 70.7 ($^iPrCH$), 126.3 (q, *J* 287, $F_3C$), 158.0 (q, *J* 37.8, $F_3CCO$) and 171.2 ($CO_2{}^iPr$); *m/z* (EI) 259 ([*M* + H]$^+$, 40 %), 241 (18, [*M* - OH]$^+$), 199 (60, [*M* - $OCH(CH_3)_2$]$^+$), 171 (45, [*M* - $CO_2CH(CH_3)_2$]$^+$), 141 (50, [*M* - $CO_2CH(CH_3)_2$ - HCOH]$^+$), 57 (80, [$OCH(CH_3)_2$]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

**α-Isopropyl *N*-trifluoroacetyl (2*S*)-[2-$^2H_1$]homoserine (76c).**

This was prepared in an identical manner to that described for compound (**76**) using (2*S*,3*R*)-[3-$^2H_1$]protected aspartate (**75a**) (0.83 g, 3.05 mmol) as starting material in 75 % yield (0.59 g); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3400-3000 (OH) and 1720 (ester C=O); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.17 (6H, m, 2 x $^iPrCH_3$), 1.93 (2H, m, Cβ-$H_2$), 3.67 (2H, m, Cγ-$H_2$), 5.11 (1H, sp, *J* 6.3, $^iPrCH$) and 7.75 (1H, d, *J* 6.9, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.0 & 22.1 (2 x $^iPrCH_3$), 33.7 (Cβ), 51.7 (t, *J* 4.7, Cα), 59.2 (Cγ), 70.7 ($^iPrCH$), 125.9 (q, *J* 287, $F_3C$), 158.0 (q, *J* 37.8, $F_3CCO$) and 170.7 ($CO_2{}^iPr$); *m/z* (EI) 259 ([*M* + H]$^+$, 35 %), 241 (25, [*M* - OH]$^+$), 199 (65, [*M* - $OCH(CH_3)_2$$^+$], 171 (45, [*M* - $CO_2CH(CH_3)_2$]$^+$), 141 (75, [*M* - $CO_2CH(CH_3)_2$ - HCOH]$^+$), 57 (60, [$OCH(CH_3)_2$]$^+$) and 43 (100, [$CH(CH_3)_2$]$^+$).

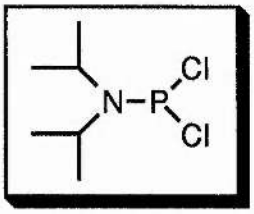
**(2*S*)-Homoserine (49).**




α-Isopropyl *N*-trifluoracetyl (2*S*) homoserine (**76**) (1g, 3.89 mmol) was dissolved in ethanol (20 ml). On addition of 1 M KOH (20 ml), the solution immediately warmed

and became a deep, clear yellow colour. After 2 h the solution was neutralized with 1 M HCl and the solvent removed under reduced pressure. The resultant yellow material was applied to a column of Dowex 50W ($H^+$ form). After washing with water (100 ml), the column was eluted with 0.1 M KOH. The ninhydrin positive fractions were pooled and concentrated under reduced pressure. The residue was recrystallised from water/ ethanol yielding (**49**) as a white crystalline solid (0.43 g, 95%), m.p. 202-203 °C (decomp.), [lit.,[205] 203 °C (decomp.)]; $[\alpha]_D$ +21.2 (*c* 0.5 in 1 M HCl), {lit.,[5] +20.4 (*c* 0.5 in 1 M HCl)}; $\upsilon_{max}$ (Nujol)/$cm^{-1}$ 3420 (NH), 3400-2400 (acid OH), 1640 (acid C=O) and 1230 (C-O); $\delta_H$ (200 MHz; $^2H_2O$) 1.62 (2H, m, C$\beta$-$H_2$), 3.14 (1H, dd, C$\alpha$-H) and 3.49 (2H, t, C$\gamma$-$H_2$); $\delta_C$ (50.3 MHz; $^2H_2O$) 34.57 (C$\beta$), 56.12 (C$\alpha$), 61.40 (C$\gamma$) and 177.18 ($CO_2H$).

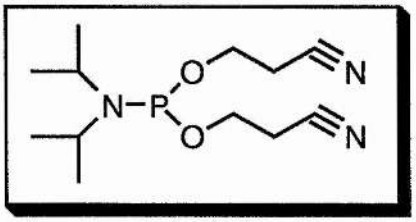
### *N,N*-Diisopropyldichlorophosphoramidite (80).




To a vigorously stirred solution of $PCl_3$ (21.3 ml, 0.155 mol) in anhydrous diethyl ether (75 ml), at -20 °C, under an atmosphere of nitrogen, was added dropwise dry diisopropylamine (31.8 ml, 0.315 mol) in anhydrous diethyl ether (75 ml). After stirring for 2 h at -20 °C, the reaction was allowed to warm to room temperature and stirred for a further 1 h. The precipitated salts were removed by filtration, and the solvent removed under reduced pressure The residue was fractionally distilled to give the product as a colourless liquid (18.8 g, 60%), b.p. 80-81 °C/ 8 mm Hg; (Found C: 34.90; H, 7.00; N, 6.65. $C_6H_{14}NPCl_2$ requires C, 35.65; H, 7.00; N, 6.95%); *m/z* (Found: $[M + H]^+$, 201.0240. $C_6H_{14}NPCl_2$ requires 201.0241); $\delta_H$ (200

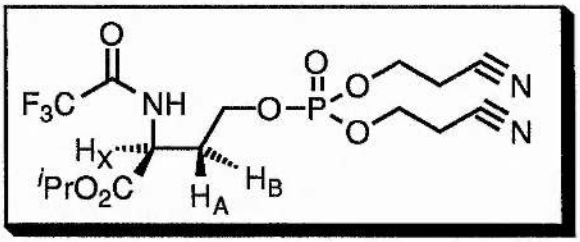
MHz; $C^2HCl_3$) 1.25 (12 H, d, $J_{MeH}$ 6.3, 4 x $^iPrCH_3$) and 3.9 (2H, d sp, $J_{PH}$ 12.5, $J_{Me\,H}$ 6.3, 2 x $^iPrCH$); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 23.9 (d, $J_{PC}$ 8.5, $^iPr\underline{C}H_3$) and 47.7 (d, $J_{PC}$ 14, $^iPr\underline{C}H$); $\delta_P$ (121.5 MHz; $C^2HCl_3$), 169; *m/z* (EI) 203 & 201 ($M^+$, chlorine isotopes, 7 & 14 %), 186 (64, $[M - CH_3]^+$), 166 (47, $[M - Cl]^+$), 144 (49, $Cl_2P^iPr^+$) and 88 (93, $PN^iPr^+$).

**_N,N_-Diisopropylbis(β-cyanoethyl)phosphoramidate (78).**



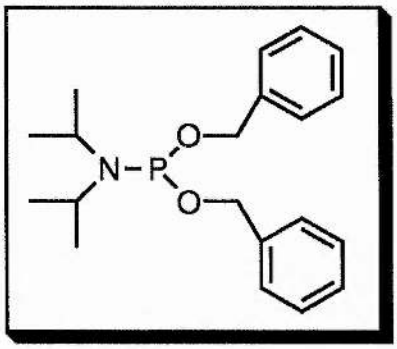

To diisopropyldichlorophosphoramidite (**80**) (5.05 g, 25 mmol), in anhydrous dichloromethane (20 ml), at -10 °C under a nitrogen atmosphere was added a solution of 3-hydroxypropionitrile (4.6 g, 65 mmol) and anhydrous triethylamine (6.6 g, 65 mmol) in anhydrous dichloromethane (20 ml). The reaction was stirred at -10 °C for 30 min, and then warmed to room temperature and stirred for a further 4 h. The reaction was washed with 100 mM phosphate buffer, brine, dried ($MgSO_4$) and the solvent removed under reduced pressure. The product was purified on a basified silica column using dichloromethane, to yield a clear oil (3.9 g, 61 %); *m/z* (Found, $M^+$, 271.1462. $C_{12}H_{22}N_3O_2P$ requires 271.1460); $\upsilon_{max}$ (neat)/$cm^{-1}$ 3000 s, 2280 w (CN), 1030 vs, 730 s; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.20 (12H, d, $J_{Me\text{-}H}$ 7, 4 x $^iPrCH_3$), 2.63 (4H, t, $J_{1,2}$ 6.4, 2 x $CH_2CN$) and 3.68 (6H, m, 2 x $^iPrCH$ & 2 x $POCH_2$); $\delta_C$ (50 MHz; $C^2HCl_3$) 20.5 (d, $J_{P,POC}$ 6.7, $\underline{C}H_2CN$), 24.6 (d, $J_{PC}$ 8.1, $^iPr\text{-}CH_3$), 43.5 (d, $J_{PC}$ 13.4, $^iPrC$), 58.6 (d, $J_{PC}$ 18.8, POC) and 118.0 (CN); $\delta_P$ (36.2 MHz; $C^2HCl_3$) 139; *m/z* (EI) 271 ($M^+$, 7%), 256 (35, $[M - CH_3]^+$), 201(25, $[M - O(CH_2)_2CN]^+$), 171 (95, $[P(O(CH_2)_2CN)_2]^+$) and 70 (15, $[O(CH_2)_2CN]^+$).

**α-Isopropyl *N*-trifluoroacetyl (2*S*)-phosphohomoserine biscyanoethyl ester (77).**



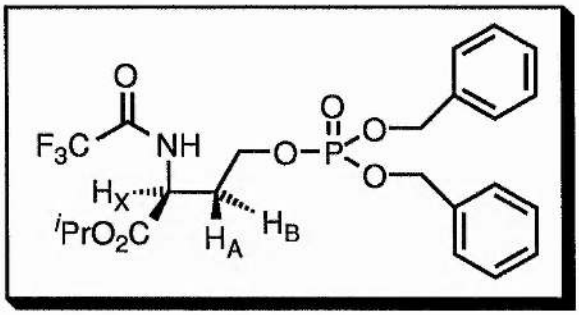
α-Isopropyl *N*-trifluoracetyl (2*S*)-homoserine (**76**) (100 mg, 0.4 mmol) and 1*H* - tetrazole (63 mg, 0.9 mmol) were added to THF (3 ml) under argon. Bis-(2-cyanoethyl) diisopropyl phosphoramidite (136 mg, 0.5 mmol) was then added to the solution and the reaction was followed by tlc (50:50 ethyl acetate/ petrol; developing with iodine). After 1 h the solution was cooled in an ice bath and *m*-CPBA (114 mg, 0.66 mmol) in dichloromethane (2 ml) was added dropwise. The reaction was stirred for 45 min, and then diluted with ether (5 ml), and washed with 10% $Na_2SO_3$, 1 M HCl, saturated $NaHCO_3$ and 100 mM phosphate buffer pH7 in succession. The organic fraction was dried with ($Na_2SO_4$), filtered and the solvent removed under reduced pressure to leave the product (**77**) as a clear gum (154 mg, 87 %); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 (6H, m, 2 x $^i$PrC$H_3$), 2.23 (2H, m, C$\beta$-$H_2$), 2.78 (4H, t, *J* 7.5, $H_2$CCN), 4.26 (6H, m, C$\gamma$-$H_2$ & 2 x POC$H_2$), 4.66 (1H, dd, $J_{XA}$ 4.3, $J_{XB}$ 7.7, C$\alpha$-H), 5.05 (1H, sp, *J* 6.7, $^i$PrCH) and 7.5 (1H, d, *J* 2.1, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 20 (d, *J* 7.3, POC), 22 ($^i$PrC$H_3$), 32 (d, $J_{PC}$ 5.3, C$\beta$), 50 (C$\alpha$), 63 (t, *J* 5.1, 2 x -CCN), 65 (d, $J_{PC}$ 5.8, C$\gamma$), 71 ($^i$PrCH), 116 (q, $J_{FC}$ 290, $F_3$C), 117 (CN), 158 (q, $J_{CF}$ 38, $F_3$CCO) and 170 ($CO_2{}^i$Pr).

***N,N* Diisopropyldibenzylphosphoramidate (81).**



This was prepared in an identical manner to that described for compound (**78**) using benzyl alcohol instead of cyanoethanol in 65 % yield. The crude phosphoramidite was purified by silica chromatography using 30% ethyl acetate in petroleum ether as the eluant to give the pure compound (**81**) as a clear oil (5.2 g, 72 %); *m/z* (Found: [*M* + H]$^+$ 346.1936. $C_{20}H_{29}NO_2P$ requires 346.1936); $\upsilon_{max}$ (neat)/cm$^{-1}$ 3000 (CH), 1600 (aromatic), 1010 (P-O), 750 and 700 (CH aromatic out of plane); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.3 (12H, d, *J* 6.8, 4 x $^i$PrC$\underline{H}_3$), 3.8 (2H, m, 2 x $^i$PrCH), 4.8 (4H, t, *J* 6.8, 2 x PhC$H_2$) and 7.4 (10H, m, aromatic); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 25.1 (d, *J* 7.3, 4 x $^i$PrC$H_3$), 43.6 (d, *J* 12.2, 2 x $^i$PrCH), 65.9 (d, $J_{CP}$ 18.1 Hz, 2 x PhC$H_2$), 127.51, 127.7 & 128.8 (aromatic CH) and 140.1 (aromatic 4° C); $\delta_P$ (121.5 MHz; $C^2HCl_3$) 148.1; *m/z* (CI) 346 ([*M* + H]$^+$, 100 %), 238 (5, [*M* - BnO]$^+$) and 91 (10, Bn$^+$).

**α-Isopropyl *N*-trifluoroacetyl (2*S*)-phosphohomoserine dibenzyl ester (82).**



To a mixture of α-isopropyl *N*-trifluoracetyl (2*S*)-homoserine (**76**) (100 mg, 0.4 mmol) and 1-*H* tetrazole (84 mg, 1.2 mmol) in anhydrous dichloromethane (10 ml) was added *N,N* diisopropyldibenzylphosphoramidite (**81**) (127 mg, 0.4 mmol). The reaction was stirred at room temperature for 2 h before being cooled to -40 °C, and *m*-CPBA (138 mg, 0.8 mmol) in dichloromethane (5 ml) was added dropwise. The resulting solution was stirred at 0 °C for 45 min before being diluted with dichloromethane (40 ml), washed with $Na_2SO_3$ (10 %, 2 x 20 ml), $NaHCO_3$ (2 x 15 ml), water (15 ml) and brine (15 ml). The crude product obtained was purified by flash silica chromatography using 25 % ethyl acetate in petroleum ether as the eluant to yield the pure phosphate triester (**82**) as a clear oil (190 mg, 92 %); *m/z* (Found: $[M + H]^+$, 518.1556. $C_{23}H_{27}NO_7PF_3$ requires 518.1555); $[\alpha]_D$ +3.04 (*c* 2.7 in diethyl ether); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 (6H, m, 2 x $^i$PrCH$_3$), 2.18 (2H, m, C$\beta$-H$_2$), 4.0 (2H, q*, *J* 6.3, C$\gamma$-H$_2$), 4.33 (1H, dd, $J_{AX}$ 6.6, $J_{BX}$ 13.5, C$\alpha$-H), 5.0 (5H, m, $^i$PrCH and 2 x benzyl CH$_2$), 7.34 and 7.35 (10H, aromatic CH) and 7.63 (1H, d, $J_{NH,X}$ 7.6, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.0 & 22.1 ($^i$PrCH$_3$), 31.8 (d, $J_{PC}$ 27, C$\beta$), 50.7 (C$\alpha$), 64.1 (d, $J_{PC}$ 22, C$\gamma$), 70.0 & 70.1 (benzyl CH$_2$), 70.8 ($^i$PrCH), 116 (q, $J_{CF}$ 290, F$_3$C), 128.5, 129.1 & 129.2 (aromatic CH), 143.2 (benzyl 4° C), 158.1 (q, $J_{CF}$ 38, F$_3$C<u>C</u>O) and 169.9 (CO$_2$$^i$Pr); $\delta_P$ (121.5 MHz; $C^2HCl_3$) -0.55 .

q* = pseudo quartet arising from the phosphorus splitting of a triplet.

**α-Isopropyl *N*-trifluoroacetyl (2*S*,3*R*)-[3-$^2H_1$]phosphohomoserine dibenzyl ester (82a).**

This was prepared in an identical manner to that discussed for compound (**82**) using (2*S*,3*R*)-[3-$^2H_1$]α-isopropyl *N*-trifluoracetyl homoserine (**76a**) (0.50 g, 1.94 mmol) as alcohol in 85 % yield (0.855 g); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.24 (6H, m, 2 x $^i$PrC$H_3$), 2.16 (1H, m, Cβ-H), 4.15 (2H, m, Cγ-$H_2$), 4.55 (1H, t, *J* 7.2, Cα-H), 5.0 (5H, m, $^i$PrCH and 2 x benzyl $CH_2$), 7.33 & 7.36 (10H, aromatics) and 7.94 (1H, d, *J* 7.7, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.1 & 22.0 ($^i$PrC$H_3$), 31.5 (t, $J_{C^2H}$ 4.6, Cβ), 50.5 (Cα), 64.2 (d, $J_{PC}$ 21, Cγ), 70.0 & 70.1 (benzyl $CH_2$), 70.7 ($^i$PrCH), 115.0 (q, $J_{FC}$ 288, $F_3C$), 128.6, 129.1 & 129.2 (aromatic CH), 142.9 (benzyl 4° C), 159.0 (q, $J_{FC}$ 39, $F_3C\underline{C}O$) and 169.8 ($CO_2{}^i$Pr); $\delta_P$ (121.5 MHz; $C^2HCl_3$) -0.57.

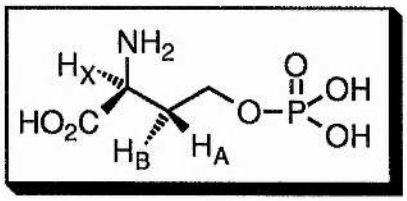
**α-Isopropyl *N*-trifluoroacetyl (2*S*,3*S*)-[3-$^2H_1$]phosphohomoserine dibenzyl ester (82b).**

This was prepared in an identical manner to that described for compound (**82**) using α-isopropyl *N*-trifluoracetyl (2*S*,3*S*)-[3-$^2H_1$]homoserine (**76b**) (0.425 g, 1.65 mmol) as alcohol in 87 % yield (0.742 g); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.24 (6H, m, 2 x $^i$PrC$H_3$), 2.16 (1H, m, Cβ-H), 4.55 (1H, t, *J* 7.2, Cα-H), 5.0 (5H, m, $^i$PrCH and 2 x $PhCH_2$), 7.33 & 7.36 (10H, aromatics) and 7.94 (1H, d, *J* 7.7, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.1 & 22.0 ($^i$PrC$H_3$), 31.5 (t, *J* 4.6, Cβ), 50.5 (Cα), 64.2 (d, $J_{PC}$ 21, Cγ), 70.0 & 70.1 ($PhCH_2$), 70.7 ($^i$PrCH), 115.0 (q, $J_{FC}$ 289, $F_3C$), 128.6, 129.1 & 129.2 (aromatic CH), 143.0 (aromatic 4° C), 158.8 (q, $J_{FC}$ 39, $F_3C\underline{C}O$) and 170.1 ($CO_2{}^i$Pr); $\delta_P$ (121.5 MHz; $C^2HCl_3$) -0.53 .

**α-Isopropyl *N*-trifluoroacetyl (2*S*)-[2-$^2H_1$]phosphohomoserine dibenzyl ester (82c).**

This was prepared in an identical manner to that described for compound (**82**) using α-isopropyl *N*-trifluoracetyl (2*S*)-[2-$^2H_1$]homoserine (**76c**) (0.675 g, 2.6 mmol) as alcohol in 76 % yield (1.030 g); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.23 (6H, m, 2 x $^i$PrC$H_3$), 2.16 (1H, m, Cβ-H), 4.04 (2H, dq, *J* 7.1, *J* 9.3, Cγ-$H_2$), 5.03 (5H, m, $^i$PrCH and 2 x Ph$CH_2$), 7.32, 7.34 & 7.36 (10H, aromatic) and 7.65 (1H, d, *J* 6.9, NH); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 22.1 & 22.0 ($^i$PrC$H_3$), 31.5 (Cβ), 50.5 (t, *J* 4.8, Cα), 64.2 (d, $J_{PC}$ 21, Cγ), 70.0 & 70.1 (Ph$CH_2$), 70.7 ($^i$PrCH), 115.0 (q, $J_{FC}$ 288, $F_3C$), 128.6, 129.1 & 129.2 (aromatic), 142.9 (benzyl 4° C), 159.0 (q, $J_{FC}$ 39, $F_3C\underline{C}O$) and 169.8 ($CO_2{}^i$Pr); $\delta_P$ (121.5 MHz; $C^2HCl_3$) -0.54.

**(2*S*)-*O*-Phosphohomoserine (43).**




To α-isopropyl *N*-trifluoracetyl phosphohomoserine dibenzyl ester (**82**) (500 mg, 10 mmol) dissolved in methanol (15 ml) was added 10 % palladium on charcoal (50 mg). The mixture was purged with dihydrogen gas, and dihydrogen was bubbled through the stirring suspension 6 h. The mixture was filtered through a Celite pad, evaporated to dryness and taken up in ethanol (10 ml). On addition of 1 M KOH (10 ml), the solution immediately turned yellow, and was left stirring for 3 h. The solution was concentrated under reduced pressure and the resulting yellowish solid was subjected to ion-exchange chromatography (Dowex 50-W 200-

400 mesh, 8 % cross-linked) eluting with water. The fractions were analysed by tlc [isopropanol:water:concentrated ammonia solution; (26:6:5)] developing with ninhydrin. The fractions containing the product (**43**) were lypholized to yield a white solid (155 mg, 80%), m.p. 170 °C; *m/z* (Found: $[M + H]^+$, 200.0328. $C_4H_{11}NO_6P$ requires 200.03240); $[\alpha]_D$ +4.19 (*c* 2.4 in $H_2O$) {lit.,[174] $[\alpha]_D$ + 4.21 (*c* 2.4 in water)}; $\delta_H$ (200 MHz; $^2H_2O$) 2.23 (2H, m, C$\beta$-$H_2$), 4.01 (2H, t, $J_{AB,X}$ 11.4, d, $J_{AB,P}$ 5.49, C$\gamma H_2$) and 4.12 (1H, dd, $J_{AX}$ 12.4, $J_{BX}$ 4.9, C$\alpha$-H); $\delta_C$ (50.3 MHz; $^2H_2O$) 38.7 (d, $J_{PC}$ 7.4, C$\beta$), 56.3 (C$\alpha$), 64.3 (d, $J_{PC}$ 4.9, C$\gamma$) and 186.2 ($CO_2H$); $\delta_P$ (121.5 MHz; $^2H_2O$) 0.245; *m/z* (FAB) 200 ($[M + H]^+$, 20 %).

**(2*S*,3*R*)-[3-$^2H_1$]*O*-Phosphohomoserine (43a).**

This was prepared in an identical manner to that described for compound (**43**) using α-isopropyl *N*-trifluoracetyl (2*S*,3*R*)-[3-$^2H_1$]phosphohomoserine dibenzyl ester (**82a**) (0.785 g, 1.5 mmol) as starting material in 83 % yield (0.250 g), m.p. 168-169 °C; $\delta_H$ (200 MHz; $^2H_2O$) 2.03 (2H, m, C$\beta$-H) and 4.0 (3H, m, C$\alpha$-H and C$\gamma$-$H_2$); $\delta_C$ (50.3 MHz; $^2H_2O$) 34.3 (t, $J_{C^2H}$ 4.7, C$\beta$), 55.3 (C$\alpha$), 65.0 (d, $J_{PC}$ 5.0, C$\gamma$) and 181.3 ($CO_2H$); $\delta_P$ (121.5 MHz; $^2H_2O$) 0.251.

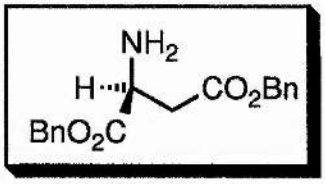
**(2*S*,3*S*)-[3-$^2H_1$]*O*-Phosphohomoserine (43b).**

This was prepared in an identical manner to that described for compound (**43**) using α-isopropyl *N*-trifluoracetyl (2*S*,3*S*)-[3-$^2H_1$]phosphohomoserine dibenzyl ester (**82b**) (0.685g, 0.13 mmol) as starting material in 81 % yield (0.213 g), m.p. 167-169 °C; $\delta_H$ (200 MHz; $^2H_2O$) 2.34 (1H, m, C$\beta$-H), 4.06 (2H, m, C$\gamma$-$H_2$) and 4.16 (1H, t, *J* 4.5, C$\alpha$-H); $\delta_C$ (50.3 MHz; $^2H_2O$) 34.2 (t, $J_{C^2H}$ 4.6, C$\beta$), 54.9 (C$\alpha$), 64.8 (d, $J_{PC}$ 5.0, C$\gamma$) and 182.4 ($CO_2H$); $\delta_P$ (121.5 MHz; $^2H_2O$) 0.240.

**(2*S*)-[2-$^2H_1$]*O*-Phosphohomoserine (43c).**

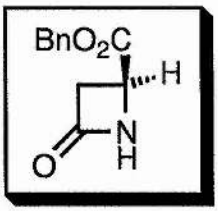
This was prepared in an identical manner to that described for compound (**43**) using α-isopropyl *N*-trifluoracetyl (2*S*)-[2-$^2H_1$]phosphohomoserine dibenzyl ester (**82c**) (0.855 g, 1.6 mmol) as starting material in 78 % yield (0.257 g), m.p. 168-169 °C; $\delta_H$ (200 MHz; $^2H_2O$) 2.34 (2 H, m, Cβ-H) and 4.06 (2H, m, Cγ-$H_2$); $\delta_C$ (50.3 MHz; $^2H_2O$) 34.2 (Cβ), 54.9 (t, *J* 4.8, Cα), 64.8 (d, $J_{PC}$ 5.0, Cγ) and 182.4 ($CO_2H$); $\delta_P$ (121.5 MHz; $^2H_2O$) 0.239.

**(2*S*)-Dibenzyl aspartate (87).**



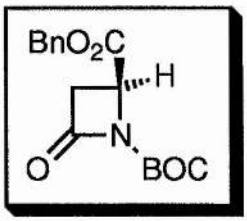

(2*S*)-Aspartic acid dibenzyl ester *p*-toluenesulfonate (**86**) (40.98 g, 84.5 mmol) and potassium carbonate (34.97 g, 253 mmol) were dissolved in distilled water (500 ml) and the solution extracted with ethyl acetate (3 x 250 ml). The organic layer was washed with saturated brine (500 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to yield free amine (**87**) as a clear oil (26.24 g, 90%); *m/z* (Found: [*M* - $CO_2Bn$]$^+$, 178.0859. $C_{10}H_{12}NO_2$ requires 178.0860); $\upsilon_{max}$ (neat)/ $cm^{-1}$ 1740 (s, C=O), 1500 (w), 1455 (w) and 1170 (m); $\delta_H$ (400 MHz; $C^2HCl_3$) 2.05 (2H, br s, $NH_2$), 2.73-2.96 (2H, m, 3-H), 3.82-3.92 (1H, m, 2-H), 5.13 & 5.16 (2H, s, PhC$\underline{H}_2$) and 7.33-7.37 (10H, m, aromatic); $\delta_C$ (125 MHz; $C^2HCl_3$) 38.7 (3-C), 51.2 (2-C), 66.6 & 67.1 ($PhCH_2$), 127.0-128.7 (Ph), 135.6 & 135.7 (Ph *ipso* C), and 171.2 & 174.2 (C=O).

**(4*S*) Benzyl azetidin-2-one-4-carboxylate (88).**



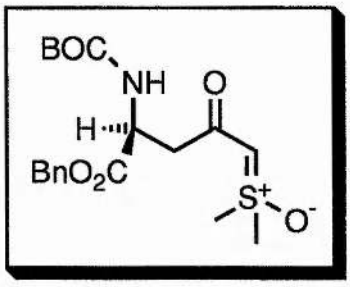

To a solution of (2*S*)-dibenzyl aspartate (**87**) (12.6 g, 40 mmol) in diethyl ether (55 ml) at 0 °C was added trimethylchlorosilane (5.8 ml, 46 mmol). After 15 min triethylamine (6.4 ml, 46 mmol) was added and a white precipitate immeadiately formed. After 1 h the resulting suspension was filtered under an inert atmosphere, and the filtrate diluted with diethyl ether (100 ml). Dropwise addition of *t*-butylmagnesium chloride (23.0 ml of a 2 M solution in diethyl ether, 46 mmol), over 45 min yielded a suspension in a pale yellow oil. This suspension was allowed to warm to room temperature and stirred overnight. The mixture was again cooled to 0 °C and 2 M HCl saturated with $NH_4Cl$ (80 ml) was carefully added, the organic layer was separated. The aqueous phase washed with DCM (2 x 60 ml) and the combined organic portions were then washed with saturated $NaHCO_3$ (100 ml), brine (100 ml) and dried ($MgSO_4$). Removal of the solvent under reduced pressure yielded a white dispersion in a pale yellow oil, which crystallised from DCM to give (**88**) as white flakes (5.60 g, 69 %), m.p. 138-139 °C (lit,[199] 138-139 °C); $R_F$ 0.5 (EtOAc:DCM 4:1); *m/z* (Found: [*M* - $CH_2(OC)$]$^+$, 163.0625. $C_9H_9NO_2$ requires 163.063323); $\upsilon_{max}$ (Nujol)/cm$^{-1}$ 3450 (m), 1780 (s, β-lactam C=O), 1748 (m, ester C=O), 1350 (w), 1265 (m) and 1045 (w); $\delta_H$ (400 MHz; $C^2HCl_3$) 3.09 (1H, ddd, *J* 2, 3, 15, 3-H), 3.35 (1H, ddd, *J* 1, 6, 15, 3-H), 4.22 (1H, dd, *J* 3, 6, 4-H), 5.22 (2H, s, PhC$\underline{H}_2$), 6.11 (1H, br s, NH) and 7.34-7.42 (5H, m, aromatic); $\delta_C$ (125 MHz; $C^2HCl_3$) 43.3 (3-C), 47.2 (4-C), 67.4 (Ph-$\underline{C}H_2$-O), 128.6-128.8 (Ph), 125.1 (Ph *ipso* C), and 166.8 & 171.2 (2 x C=O); *m/z* [DCI($NH_3$)] 223 ([*M* + $NH_4$]$^+$, 100 %), 2.6 (43, [*M* + H]$^+$), 178 (47), 108 (36) and 91 ($C_7H_7^+$,54).

**(4*S*)-Benzyl N-(*t*-butoxycarbonyl) azetidin-2-one-4-carboxylate (89).**



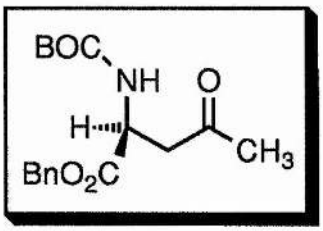

(4*S*)-Benzyl azetidin-2-one-4-carboxylate (**88**) (2.00 g, 9.7 mmol), di-*t*-butyl dicarbonate (4.25 g, 19.5 mmol), and DMAP (12.2 mg, 0.1 mmol) were stirred in acetonitrile (20 ml) for 24 h. The solvent was removed under reduced pressure and the residue was redissolved diethyl ether (50 ml). The resulting solution was then washed with 1 M potassium hydrogensulfate (2 x 60 ml), saturated $NaHCO_3$ (40 ml), brine (60 ml), and dried ($MgSO_4$), and the solvent removed under reduced pressure to yield a pale brown oil which was purified by flash chromatography [silica, eluting with diethyl ether/ hexane; (1:1)] to afford (**89**) as a colourless oil (2.51 g, 85 %), $R_F$ 0.6 ($Et_2O$); *m/z* (Found: $[M - C_4H_8]^+$, 249.06229. $C_{12}H_{11}NO_5$ requires 249.055169); $\upsilon_{max}$ (neat)/$cm^{-1}$ 1822 (s, β-lactam C=O), 1745 (m, ester C=O), 1375 (m), 1340 (s), 1155 (m) and 1050 (m); $\delta_H$ (400 MHz; $C^2HCl_3$) 1.44 [9H, s, $CO_2C(CH_3)_3$], 2.96 (1H, dd, *J* 3 and 12, 3-H), 3.24 (1H, dd, *J* 6 and 16, 3-H), 4.41 (1H, dd, *J* 3 and *J* 6, 4-H), 5.20 & 5.25 (2H, ABq, $J_{AB}$ 12, $PhC\underline{H}_2$) and 7.29-7.31 (5H, m, aromatics); $\delta_C$ (125 MHz; $C^2HCl_3$) 27.7 [$CO_2C(\underline{C}H_3)_3$], 41.2 (3-C), 49.5 (4-C), 67.5 ($Ph\underline{C}H_2O$), 84.0 [$CO_2\underline{C}(CH_3)_3$], 128.3-128.8 (Ph-C), 134.9 (Ph *ipso* C), 147.0 ($N\underline{C}O_2t$-Bu), and 162.5 & 169.4 (C=O); *m/z* [DCI($NH_3$)] 323 ($[M + NH_4]^+$, 43%), 306 (7, $[M + H]^+$), 223 (100, $[(M\text{-}CO_2C_4H_8) + H]^+$), 108 (21), and 91 (27, $C_7H_7^+$).

**(2*S*)-Benzyl 2-[(*t*-butyloxycarbonyl)amino]-5-(dimethylsulfoxonium methylide)-4-oxobutanoate (90).**




To sodium hydride (0.274 g of a 50% dispersion) and trimethylsulfoxonium iodide (1.48 g, 6.76 mmol) was added anhydrous DMSO (20 ml). After the initial effervescence the resulting mixture was stirred for 1 h. A solution of azetidinone (**89**) (1.52 g, 5.2 mmol) in DMSO (10 ml) was then added. The resulting deep yellow solution was stirred for exactly 15 min before being diluted with ethyl acetate (100 ml) and washed with water (100 ml). The resulting aqueous portion was re-extracted with ethyl acetate (3 x 50 ml) and the combined organic portions were further washed with water (2 x 200 ml), and brine (200 ml). After drying ($MgSO_4$), the solvent was removed under reduced pressure to yield (**90**) as an orange oil (1.96 g, 95 %) that required no further purification, $R_F$ 0.2 (EtOAc); $\upsilon_{max}$ (neat/ $cm^{-1}$) 3362 (m), 3065 (m), 3010 (m), 3010 (m), 2977 (m), 1717 (s, C=O), 1570 (s), 1491 (s), 1453 (s), 1247 (s), and 1045 (s); $\delta_H$ (400 MHz; $C^2HCl_3$) 1.51 (9H, s, $CO_2C(CH_3)_3$), 2.75-2.90 (2H, ABX, $J_{AB}$ 22 Hz, $J_{AX}$ 4, $J_{BX}$ 6, 3-H), 3.21 & 3.32 (2 x 3H, 2 x s, $S(CH_3)_2$), 4.31 (1H, s, 5-H), 4.50 (1H, m, 2-H), 5.18 (2H, ABq, *J* 15, $CO_2C\underline{H}_2Ph$), 5.95 (1H, d, *J* 11, NH), and 7.28-7.38 (5H, m, aromatic); $\delta_C$ (125 MHz; $C^2HCl_3$) 28.35 ($CO_2C(\underline{C}H_3)_3$), 41.20 (3-C), 41.87 & 42.04 ($S(\underline{C}H_3)_2$), 51.10 (2-C), 66.81 ($CO_2\underline{C}H_2Ph$), 70.36 (5-C), 79.57 ($CO_2\underline{C}(CH_3)_3$) 128.16, 128.21 & 128.48 (Ph-C), 135.86 (Ph *ipso* C), 155.73 ($NH\underline{C}O_2t$-Bu), 172.06 ($\underline{C}H$=CO), and 186.19 (C=O); *m/z* (EI) 398 ($M^+$, 15 %), 324 (10), 177 (30), 128 (25), 119 (100), 91 (90, $C_7H_7^+$), and 57 (90, $C_4H_9^+$).

**(2*S*)-Benzyl 2-(*t*-butyloxycarbonylamino)-4-oxopentanoate (85)**

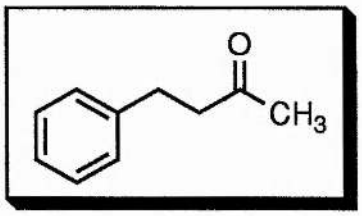



To a solution of (2*S*)-benzyl 2-(*t*-butyloxycarbonylamino)-5-(dimethyl sulfoxonium methylide)-4-oxobutanoate (**90**) (122 mg, 0.3 mmol) in DMF (5 ml), in a brown glass vessel, was added hydroiodic acid (46 ml of a 57 % aqueous solution, 0.34 mmol). The resulting solution was stirred for 48 h before being diluted with ethyl acetate (10 ml) and washed with water (3 x 15 ml), 0.1 M sodium thiosulfate (10 ml), and brine (15 ml). After drying ($Na_2SO_4$) the solvent was removed under reduced pressure to give a yellow oil which was purified by flash silica chromatography [eluting with diethyl ether:hexane; (1:1)] to yield methyl ketone (**85**) (45 mg, 48 %) as a colourless oil, $R_F$ 0.15 [$Et_2O$/ Hexane (1:1)]; $\upsilon_{max}$ (neat)/ $cm^{-1}$ 3366 (m), 2978 (m), 2933 (m), 1718 (s, C=O), 1500 (s), 1393 (s), 1368 (s), 1167 (s), and 1054 (m); $\delta_H$ (400 MHz; $C^2HCl_3$) 1.43 (9H, s, $CO_2C(C\underline{H}_3)_3$), 2.12 (3H, s, $C(O)C\underline{H}_3$), 2.90-3.24 (2H, ABX, $J_{AB}$ 18, $J_{AX}$ 4, $J_{BX}$ 4, 3-H), 4.50-4.55 (1H, m, 2-H), 5.15 (2H, s, $CO_2CH_2Ph$), 5.51 (1H, d, *J* 11, NH) and 7.30-7.36 (5H, m, aromatic); $\delta_C$ (125 MHz; $C^2HCl_3$) 28.10 ($CO_2C(\underline{C}H_3)_3$), 29.71 ($C(O)\underline{C}H_3$), 45.21 (3-C), 49.51 (2-C), 67.25 ($CO_2\underline{C}H_2Ph$), 80.01 ($CO_2\underline{C}(CH_3)_3$), 128.26, 128.45 & 128.66 (Ph-C), 135.40 (Ph *ipso* C), 155.95 ($NH\underline{C}O_2t$-Bu), 171.47 ($\underline{C}O_2CH_2Ph$) and 206.13 (C=O).

**Iodomethyl ketone** (**91**); $\delta_H$ (200 MHz; $C^2HCl_3$) 1.45 (9H, s, $CO_2C(CH_3)_3$), 2.90-3.24 (2H, ABX, $J_{AB}$ 18, $J_{AX}$ 4, $J_{BX}$ 4, 3-H), 3.78 (2H, s, $CH_2I$), 4.50-4.55 (1H, m, 2-H), 5.15 (2H, s, $CO_2CH_2Ph$), 5.43 (1H, d, *J* 11, NH) and 7.30-7.36 (5H, m, aromatic); *m/z* (EI) 391 ([*M* - $C_4H_8$]$^+$, 10 %), 312 (50, [*M* - $CO_2CH_2Ph$]$^+$), 91 (90, $PhCH_2^+$) and 57 (100, $^tBu^+$); *m/z* (CI) 465 ([*M* + $NH_4$]$^+$, 30 %), 448 (10, [*M* + H]$^+$),

409 (100, [*M* - $C_4H_8$ + $NH_4$]$^+$).

**Benzylacetone (93).**




To TMS-diazomethane (4 ml of a 2 M solution in hexane, 7 mmol) at 0 °C was added slowly a solution of hydrocinnamic acid chloride (**92**) (0.5 ml, 3.4 mmol) in diethyl ether (5 ml). The solution was allowed to stir for 2 h before the careful and slow addition of 98% HI (1.5 ml). The resulting mixture was allowed to stir for a further 2 h before being diluted with ethyl acetate (20 ml) and washed with 0.1 M sodium thiosulfate (2 x 20 ml), water (20 ml), brine (20 ml) and dried ($Na_2SO_4$). Removal of the solvent under reduced pressure resulted in a yellow oil which was purified by flash silica chromatography [eluting with diethyl ether:hexane; (1:4)] to yield benzylacetone (**93**) as a colourless oil (237 mg, 47 %); *m/z* (Found: 148.0879. $C_{10}H_{12}O$ requires 148.0888); $\delta_H$ (270 MHz; $C^2HCl_3$) 2.15 (3H, s, $C(O)CH_3$), 2.75-2.95 (4H, m, $PhC\underline{H_2}C\underline{H_2}$), and 7.10-7.35 (5H, m, aromatics); *m/z* (EI) 148 (*M*$^+$, 70%), 133 (22, [*M* - Me]$^+$), 105 (95, [*M* - $CH_3CO$]$^+$), 91 (87, $C_7H_7^+$) and 43 (100, $CH_3CO^+$).

**Purification of threonine synthase from *E. coli* Tir 8.**

| Buffer A: | Buffer B: |
|---|---|
| 50 mM HEPES | 20 mM $KPO_4H_3$ |
| 1 mM DTT | 1 mM DTT |
| 2 mM EDTA | 2 mM EDTA |
| 0.25 mM PLP | 0.25 mM PLP |
| | 2 mM (2*S*)-threonine |

both adjusted to pH 7.5 with KOH solution.

**1)** *E. coli* strain Tir 8 was grown in 4l L-Broth[194] incubating for 16 h to allow harvest in late log phase.

**2)** The cells were harvested by centrifugation at 8,000 rpm for 20 min at 4 °C, to yield 37 g of wet cell paste. The cell paste was resuspended in buffer (40 ml) and sonicated at <6 °C, for 15 min in bursts of 2 min using an Ultrasonic W-220F sonicator (150 W). The lysed cell debris was removed by centrifugation at 18,000 rpm for 20 min. The resultant precipitate was discarded, and the supernatant was used immeadiately in the next step.

**3)** Streptomycin sulfate was added to the sonic extract to a final concentration of 3% w/v, and stirred for 30 min. The precipitated nucleic acids were spun out at 18,000 rpm for 30 min and discarded.

**4)** The streptomycin sulfate extract (45 ml) was brought to 40% ammonium sulfate saturation by addition of 10.94 g precrushed ammonium sulfate in portions over 30 min at 4 °C. The suspension was allowed to stir at 0 °C for a further 30 min before

spinning down the precipitated proteins at 18,000 rpm for 30 min and discarding the precipitate.

**5)** The 40 % supernatant was adjusted to 60 % ammonium sulfate saturation by addition of a further 6.34 g of ammonium sulfate over a 20 minute period. After stirring this suspension for 30 min the precipitated protein was spun down at 18,000 rpm for 30 min and the supernatant discarded. The protein pellet (3.69 g) was resuspended in buffer A to a final volume of 7 ml (50 mg/ml). This solution was spun at 18,000 rpm for 30 min to remove any debris before the next purification step.

**6)** The protein solution was loaded onto a Sephadex G150 column (80 x 3 cm) which had been pre-equilibrated with buffer A. The column was eluted at a flow rate of 30 ml/h collecting 50 x 15 ml fractions. Each fraction was assayed for SAM enhanced phosphatase activity, and the fractions containing threonine synthase were pooled, and their volume reduced to approx. 3 ml by ultrafiltration, (52 mg/ml).

**7)** The cloudy concentrated solution from the previous step was centrifuged (18,000 rpm, 10 min). A portion of the protein solution (0.25 ml) was diluted to 1 ml with $H_2O$, and the solution applied to a DEAE Sephacel column (10 cm x 1.5 cm) pre-equilbrated with buffer B. The protein was eluted with a linear gradient of 0 - 250 mM KCl in the same buffer at a flow rate of 1 ml/min, and fractions (2 ml) were collected. The fractions were pooled by peak, relating to the absorbance at 280 nm. This yielded 5 fractions (Figure 2.14) each of which were reduced to approx. 1 ml by ultrafiltration.

**8)** The fractions collected were all subjected to a G 25 column (10 cm x 2.5 cm) pre-equilibrated with buffer A. The column was eluted with buffer A at a flow rate of

30 ml/ h and fractions (15 min) collected. All protein containing fractions were pooled and ultrafiltrated to approximately 1 ml (2 mg/ ml).

## Colourimetric Assays.

### Reagent.

Colourimetric assay reagent: Malachite green (1.5 g) was dissolved in hydrochloric acid (25 ml, 5 M), and diluted with water (750 ml). To this solution was added ammonium molybdate (10.5 g) in hydrochloric acid (225 ml, 5 M), and the solution stirred at room temperature for 10 minutes. The solution was filtered by gravity, and stored in the dark for periods of up to one month.

### Threonine synthase assay.

The following crude assay was used to assess the progress of the purification of threonine synthase. Each vial contained 500 µl of solution, and a concentration of 1 mM (2*S*)-*O*-phosphohomoserine at pH 7.5 in buffer A. The reaction was initiated started by addition of 10 µl enzyme. The assay samples were incubated at 37 °C for a suitable period of time, and the reaction quenched by addition to the malachite reagent (0.1 ml aliquot was added to 0.9 ml colorimetric assay reagent). The colour was allowed to develop for 20 min, and the optical adsorption at 660 nm was measured. Comparison of the optical adsorption between the sample, a blank and a sample containing 0.4 mM SAM allowed threonine synthase activity to be assessed.

### Determination of $K_M$ and $V_{max}$ for threonine synthase.

Several solutions of (2*S*)-*O*-phosphohomoserine (20 - 500 µM) in buffer A (990 µl) were prepared. Enzyme (0.5 mg from G150 step) was added to each solution, and

aliquots (100 µl) were taken every minute over a period of 9 min. Malachite reagent (900 µl) was added to each aliquot immeadiately to quench the reaction, and the colour was allowed to develop for 20 min. The optical adsorption at 660 nm was measured as described above. Known concentrations of phosphate were made up in buffer A and were assayed in the same way, to give a standard phosphate concentration curve (Figure 3.1) from which accurate concentrations of phosphate could be calculated. The entire experiment was carried out in triplicate.

**Determination of $K_M$ and $V_{max}$ for threonine synthase activated by SAM.**

Experiments similar to those described above were performed in buffer A containing SAM (0.4 mM). A blank was also prepared containing enzyme (0.5 mg) in buffer A containing SAM (0.4 mM) to a final volume of 1 ml. The solutions were assayed as described above.

**Determination of $K_M$ and $V_{max}$ for the action of threonine synthase on (2*S*,3*R*)-[3-$^2H_1$]-*O*-phosphohomoserine.**

Experiments similar to those described above for (2*S*)-*O*-phosphohomoserine were performed in buffer A, but using (2*S*,3*R*)-[3-$^2H_1$]-*O*-phosphohomoserine as substrate. A blank was also prepared containing enzyme (0.5 mg) in buffer A to a final volume of 1 ml. The solutions were assayed as described above.

**Determination of $K_M$ and $V_{max}$ for the action of threonine synthase on (2*S*,3*S*)-[3-$^2H_1$]-*O*-phosphohomoserine.**

Experiments similar to those described above for (2*S*)-*O*-phosphohomoserine were performed in buffer A, but using (2*S*,3*S*)-[3-$^2H_1$]-*O*-phosphohomoserine as substrate. A blank was also prepared containing enzyme (0.5 mg) in buffer A to a final volume of 1 ml. The solutions were assayed as described above.

**Determination of $K_M$ and $V_{max}$ for the action of threonine synthase on (2*S*)-[2-$^2H_1$]-*O*-phosphohomoserine.**

Experiments similar to those described above for (2*S*)-*O*-phosphohomoserine were performed in buffer A, but using (2*S*)-[2-$^2H_1$]-*O*-phosphohomoserine as substrate. A blank was also prepared containing enzyme (0.5 mg) in buffer A to a final volume of 1 ml. The solutions were assayed as described above.

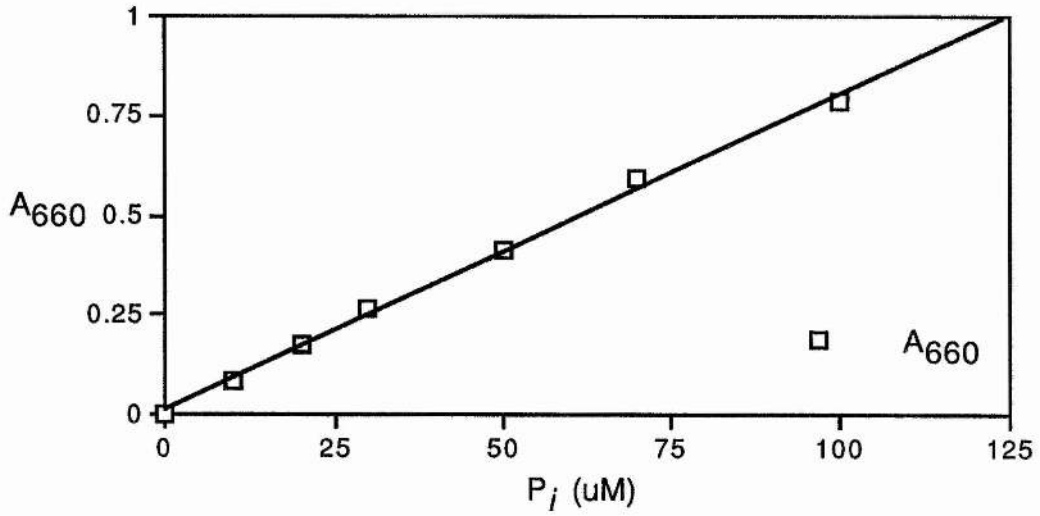



**Figure 3.1:** *Phosphate standards.*

**Appendix 1: Raw data from kinetic assays.**

| [s] (μM) | -SAM | +SAM | RPHS | SPHS | αPHS |
|---|---|---|---|---|---|
| 500 | 3.611 | 6.833 | 3.222 | 3.0330 | 3.731 |
| | 4.167 | 6.500 | 3.900 | 2.188 | 2.883 |
| | 4.167 | 5.500 | 4.070 | 2.357 | 3.333 |
| 200 | 3.625 | 4.833 | 3.391 | 2.289 | 3.222 |
| | 3.472 | 4.330 | 3.900 | 2.120 | 3.307 |
| | 2.580 | 3.833 | 2.459 | 1.950 | 2.713 |
| 100 | 3.167 | 3.083 | 2.544 | 2.035 | 2.289 |
| | 2.580 | 2.917 | 2.628 | 1.696 | 2.459 |
| | 2.667 | 2.833 | 2.205 | 1.781 | 2.374 |
| 50 | 2.667 | 2.500 | 2.035 | 1.583 | 2.205 |
| | 2.417 | 2.167 | 2.289 | 1.865 | 2.035 |
| | 1.667 | 2.750 | 1.865 | 1.696 | 1.781 |
| 30 | 1.750 | 2.167 | - | 1.102 | 1.696 |
| | 1.667 | 2.083 | 1.696 | 1.526 | 1.441 |
| | 1.333 | 1.677 | 1.442 | 1.696 | 1.611 |
| 20 | 1.667 | 1.250 | 1.017 | 1.357 | 1.441 |
| | 1.667 | 2.083 | 1.187 | 1.526 | 1.441 |
| | - | 1.250 | 1.336 | 1.272 | 1.357 |

Values given are μmoles phosphate formed per minute.

## Appendix 2: Purification of β-methyl aspartase from *Clostridium tetanomorphum.*

*Clostridium tetanomorphum* strain H1 (ATCC 15920) (obtained from the American Type Culture Collection) was grown according to the method of Barker,[206] using a modification of literature procedures, by Prof. C. Greenwood and Mr. A Thompson, University of East Anglia.

**1)** Frozen *Clostridium tetanomorphum* H1 cell paste (42 g) was added to potassium phosphate buffer (110 ml, 5 mM, pH 6.8). After the cell paste had thawed, charcoal (10 g) was added and the mixture sonicated in bursts for 10 min at 4 °C on a power setting of 40. The cell debris was spun down at 14,000 rpm for 50 min.

**2)** To the supernatant from above was added 12 ml of buffer (200 mM $MgCl_2.6H_2O$, 200 mM $NH_4Cl$ and 200 mM mesaconate, pH 6.8) and the supernatant was divided into four portions, shaken in a water bath for 10 min at 52 °C and the debris removed by centrifugation (40 min, 18,000 rpm).

**3)** To the supernatant from above was slowly added 100 ml of 1% protamine sulphate in potassium phosphate buffer (50 mM, pH 6.8) at 0 °C over 15 min. After addition, the mixture was left to stir for 15 min at 4 °C, and the debris was removed by centrifugation (45 min, 18,000 rpm).

**4)** To the supernatant from above was added potassium phosphate buffer (10 ml, 1 M, pH 7.6) followed by slow addition of ammonium sulphate to 50 % saturation at 4 °C. This was left stirring for 30 min, and the precipitated protein was removed by centrifugation (30 min, 18,000 rpm). The supernatant was collected and

ammonium sulphate was slowly added to a final saturation of 75 % and the mixture stirred for a further 30 min at 4 °C after addition. The supernatant was removed by centrifugation (30 min, 18,000 rpm).

**5)** The precipitate from above was suspended in potassium phosphate buffer (10 ml, 50 mM, pH 7.6) and dialysed overnight against 6 l of the same buffer. The solution was centrifuged (15 min, 5000 rpm) to remove any debris.

**Assay of β-methyl aspartase activity.**

The assay buffer consisted of Tris (0.5 M), $MgCl_2.6H_2O$ (2 mM), KCl (1 mM) and 3-methylaspartic acid (4 mM), adjusted to pH 9 with NaOH. A total volume of 3 ml was used and the change of absorbance at 240 nm over 1 cm was monitored. Typically the above crude preparation gave an activity of around 40 units/ml.

## Appendix 3: Publications

1. Synthesis of L-*O*-Phosphohomoserine and its C-3 Chirally Deuteriated Isotopomers: Probes for the Pyridoxal Phosphate Dependent Threonine Synthase Reaction. F. Barclay, E. Chrystal and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1994, 815-816

2. Synthesis of L-*O*-Phosphohomoserine and its C-3 Chirally Deuteriated Isotopomers: Probes for the Pyridoxal Phosphate Dependent Threonine Synthase Reaction. F. Barclay, D. Gani and E. Chrystal, Proceedings of the 9th Meeting on Vitamin B6 and Carbonyl Catalysis and the 3rd Symposium on PQQ and Quinoproteins, Capri, Italy, 1994.

3. Sterochemical Course and Primary Deuterium Isotope Effects for the *E. coli* Threonine Synthase Reaction. F. Barclay, E. Chrystal and D. Gani, *J. Chem. Soc., Chem. Commun.*, submitted for publication

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