KYNURENINASE: SYNTHESIS OF SUBSTRATE ANALOGUES AND MECHANISTIC STUDIES

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

1997

Kynureninase: Synthesis of Substrate Analogues and Mechanistic Studies

A thesis presented for the degree of
Doctor of Philosophy
to the
University of St. Andrews
on the 20 th December 1996
by
Fiona C. Ross B. Sc. (Hons)

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ACKNOWLEDGEMENTS

Firstly I must thank Dr. Nigel Botting, my supervisor, for all his help, advice and enthusiasm during this project and for the excellent meals and cakes. I must also thank my industrial supervisors, Dr. Paul Leeson, for his help and advice and interest in the project whilst he was at Merck Sharp and Dohme and Dr. Steve Fletcher.

A huge thank you to all the people in Lab. 414, especially Nicola Davidson and Neil Anderson who helped make the lab a happy and productive place to work and introduced 'silly hour'. Thank you also to Neil Anderson for letting me use his desk after I lost mine. Thank you also to everyone at Merck Sharp and Dohme for making my stay enjoyable and profitable, especially Sylvie Bourrain , Roy Penguilly and Bodil Van Niel for all their help in the lab and useful discusssions. A big thank you to Steve Thomas for all his help in interpreting spectra and for running all the NMRs and Mass Spectra I sent by post.

Thank are also due to the technical staff of the School of Chemistry at the University of St. Andrews: Melanja Smith (NMR), Colin Millar (MS), Sylvia Williamson (Microanalysis), Colin Smith (Glassblowing), Bobby Cathcart and Jim Rennie (Workshop). Also, at Merck Sharp and Dohme, thank you to Hugh Verrier (HPLC).

For financial support during this project I would like to thank the EPSRC and Merck Sharp and Dohme (CASE award).

I would like to thank Marc Payne for all the support, encouragement and for all the diagrams and typing he did (and for braving the cold room). Denise Lawlor for the emergency biscuits and cheerful chatter at all times. Finally, I would like to thank Neil Anderson, again, for his wonderful photocopying skills and help in submitting.

ABBREVIATIONS

AIDS	Aquired Immune Deficiency Syndrome
AMPA	(*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATCC	American *Type Culture* Collection
^{t}Boc	*tert*-butoxycarbonyl
b.p.	boiling point
Cbz	benzyloxycarbonyl
CNS	central nervous system
COSY	correlated spectroscopy
DCC	dicyclohexylcarbodiimide
DEAD	diethyl azodicarboxylate
DEAE	diethylaminoethyl
DIBAL	diisobutylaluminium hydride
DMAD	dimethyl azodicarboxylate
DMAP	dimethylaminopyridine
DMF	dimetylformamide
DMSO	dimethyl sulphoxide
ε	extinction coefficient
EC	Enzyme Commission
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Enz	enzyme
FAD	flavin adenine dinucleotide
$FMNH_2$	flavin mononucleotide (reduced form)
Fmoc	fluorenylmethyloxycarbonyl
GC	gas chromatography

GTK	Glutamine transaminase K
3HAO	3-Hydroxyanthranilic acid 3,4 dioxygenase
HIV	Human Immunodificiency Virus
HPLC	high performance liquid chromatography
IC_{50}	Concentration of inhibitor to produce 50% inhibition
IDO	Indoleamine 2,3-dioxygenase
IPA	isopropanol
IR	infrared
K_{app}	apparent K_M
KAT	Kynurenine Aminotransferase
k_{cat}	enzyme catalytic constant / turnover number
K_{eq}	equilibrium constant
KHDMS	potassium bis(trimethylsilyl)amide
K_I	enzyme inhibition constant
K_M	Michaelis constant
m.p.	melting point
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
Nu	nucleophile
p.	page
pD	-log[$^2H^+$], equivalent to (pH - 0.4)
PLP	pyridoxal 5' phosphate
PMP	pydidoxamine 5' phosphate

PRPP	5-phosphoribosyl-1-pyrophosphate
QPRTase	Quinolinic phosphoribosyl transferase
S_N2	nucleophilic bimolecular substitution
TDO	Tryptophan 2,3 dioxygenase
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tlc	thin layer chromatography
TMS	trimethylsilyl
Tris	tris(hydroxymethyl)amino methane
UV	ultra-violet
$^D(V)$	V_H/V_D
$^D(V/K)$	$(V_H/V_D) / (K_H/K_D)$
V_{max}	maximum rate of substrate turnover at saturation

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KYNURENINASE: SYNTHESIS OF SUBSTRATE ANALOGUES AND MECHANISTIC STUDIES.

Kynureninase (EC.3.7.1.3) is an unusual pyridoxal 5'-phosphate (PLP) dependent enzyme which catalyses the β-γ hydrolytic cleavage of kynurenine to give anthranilic acid and alanine. The enzyme is a potential therapeutic target for controlling the levels of metabolites, quinolinic acid and kynurenic acid, that are important in a variety of neurodegenerative and inflammatory disorders, such as Alzheimer's disease, AIDS, Lyme disease and poliovirus. Studies have been carried out to elucidate the mechanism of the enzyme.

A phosphinic acid analogue of kynurenine, designed as a putative transition state mimic for the reaction, has been prepared. This was found to be a competitive inhibitor, with a K_I of 4.19 mM when its interaction with kynureninase, isolated from *Pseudomonas fluorescens*, was examined. The corresponding methyl phosphinate was also prepared. This was found to be a more potent inhibitor, with a K_I of 0.88 mM. It is proposed that methylation of the acid removes a destabilising interaction between the negative charge of the ionised phosphinic acid and some group of the active site.

A number of analogues of kynurenine were prepared in order to obtain information on the specificity of the enzyme and the interactions at the active site. A racemic mixture of desaminokynurenine was found to have a K_I of 23.2 μM. Cyclohexyl and naphthyl amino acid derivatives prepared by the same method were found to be weak competitive inhibitors of the enzyme with K_I values of 844 μM and 207 μM repectively. Desaminokynurenine was found to be a substrate with a rate of reaction twenty times slower than that of kynurenine. The naphthyl amino acid derivative was also found to be a substrate. The results suggested that interactions with the aromatic ring are important and that the naphthyl amino acid derivative may be too large for the active site.

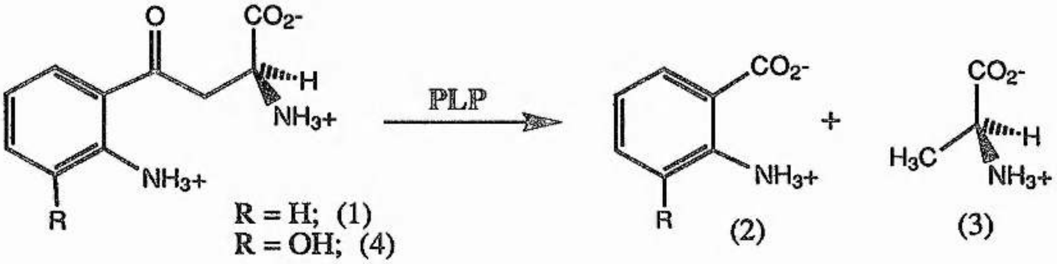
The synthesis of 2*S*-[2-^{2}H]-kynurenine is described using two different routes. Diacetylation / racemisation of racemic kynurenine in deuterium oxide followed by acylase catalysed resolution is the most direct route. The alternative is to prepare 2*S*-[2-^{2}H]-tryptophan by a similar procedure and then to convert this to 2*S*-[2-^{2}H]-kynurenine *via* ozonolysis. This compound has been used in isotope studies to gain an understanding of the mechanism of the enzyme catalysed reaction. The primary deuterium isotope effect, $^DV = 0.98$ and $^D(V/K) = 3.6$, showed that for the kynureninase catalysed reaction, α-H abstraction is partially rate limiting. A solvent isotope effect, $^DV = 4.4$ and $^D(V/K) = 4.6$ was also determined, showing that a proton transfer is also partially rate limiting.

CHAPTER 1

1. INTRODUCTION.

1.1 BACKGROUND.

Kynureninase (EC 3.7.1.3) is a pyridoxal 5' phosphate (PLP) dependent enzyme which catalyses the β,γ-hydrolytic cleavage of *2S*-kynurenine (1) to give anthranilic acid (2) and *2S*-alanine (3)[1] (Scheme 1.1). The enzyme has been purified from a number of sources including *Pseudomonas marginalis*[2] *Neurospora crassa* ,[3] rat liver[4] and porcine liver.[5]

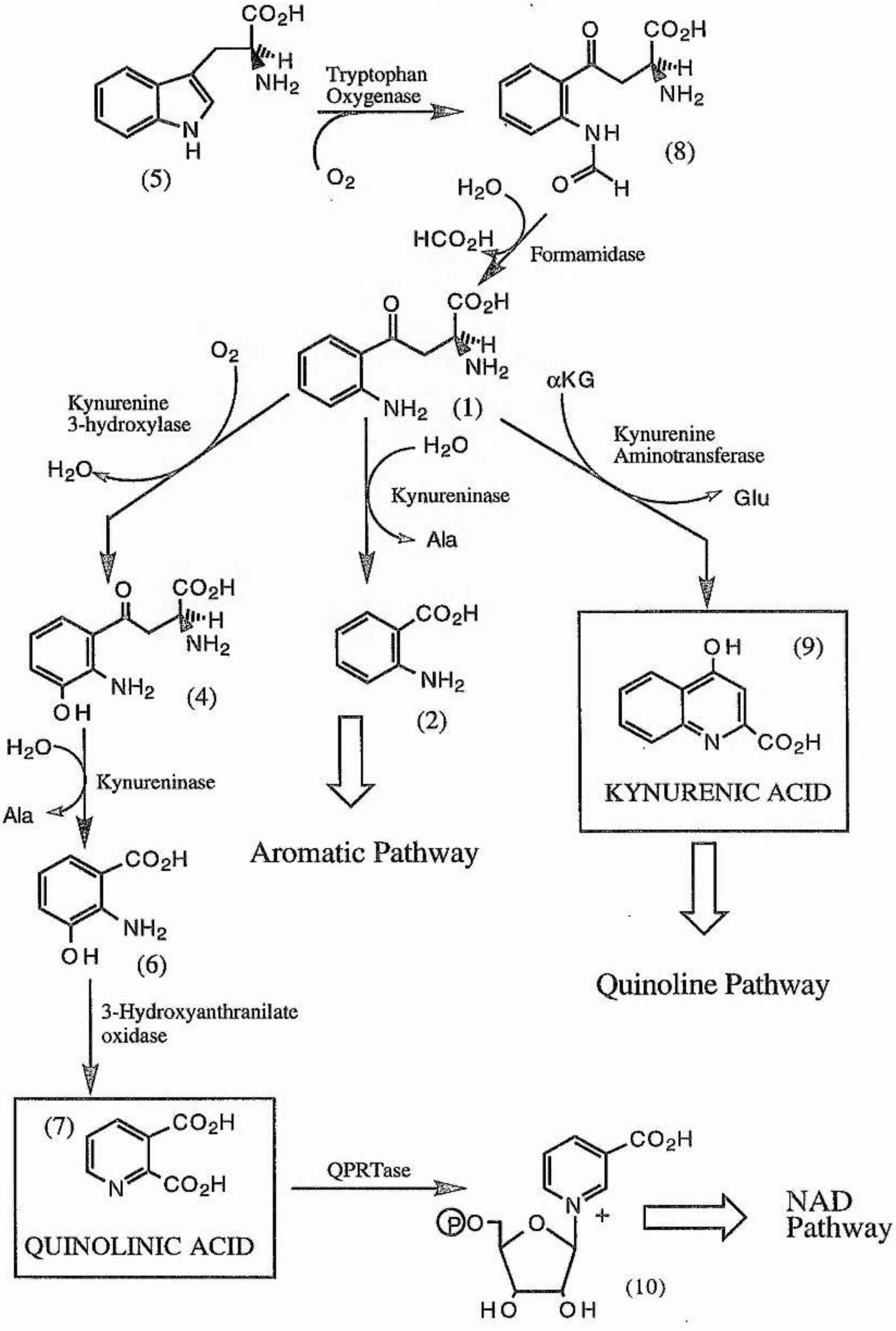


Scheme 1.1

The enzyme also catalyses the hydrolysis of 3-hydroxykynurenine (4) and the relative rates of the two substrates have been observed to vary with enzyme preparations from different sources. For example, the pseudomonad enzyme hydrolyses kynurenine five times faster than 3-hydroxykynurenine,[6] while the liver enzyme hydrolyses the hydroxy derivative twice as rapidly as kynurenine.[4]

Kynurenine is a major metabolite of tryptophan (5), on one of its three major pathways of metabolism (Scheme 1.2) (The other two are the serotonin and the tryptamine pathways). The metabolites on the kynurenine pathway include *2S*-kynurenine (1), 3-hydroxykynurenine (4), 3-hydroxyanthranilic acid (6), anthranilic acid (2), quinolinic acid (7), picolinic acid, xanthurenic acid, nicotinic acid and nicotinamide to mention those that have been investigated most.[7]

TRYPTOPHAN METABOLIC PATHWAY



Scheme 1.2

Although kynurenic acid was recognised long ago as a tryptophan metabolite, it was not until about 1947 that the kynurenine pathway was recognised as a major route for the conversion of tryptophan to nicotinamide and its nucleotide conjugates.[8] It was some years later that quinolinic acid was accepted as an intermediate. Interest in the kynurenine pathway was long centred around its importance as a source of nicotinamide and as a major disturbed pathway in cases of vitamin B6 deficiency,[9-11] being an essential cofactor for several of the kynurenine enzymes.[12] However, more recently interest has been reawakened due to the discovery of the neurological importance of some metabolites on the pathway.[8]

1.2 THE TRYPTOPHAN METABOLIC PATHWAY.

The kynurenine pathway is the major route of tryptophan catabolism. However, as it provides the pyridine nucleus of the nicotinamide nucleotide coenzymes it must also be regarded as an important biosynthetic pathway.

The first step of the pathway is oxidative cleavage of the heterocyclic ring of tryptophan by either tryptophan 2,3-dioxygenase (EC 1.13.11.11) or by indolamine 2,3-dioxygenase (EC 1.13.11.17). The immediate product of this is *N*'-formyl kynurenine (8) which is rapidly hydrolysed to kynurenine (1) by a formamidase enzyme (EC 3.5.1.9). In mammals, fungi and many micro-organisms kynurenine has three fates. It can undergo hydrolysis to anthranilic acid (2) catalysed by kynureninase (EC 3.7.1.3). The anthranilic acid is used as a precursor for the synthesis of aromatic amino acids. Alternatively, it can undergo transamination to kynurenic acid (9) which is the biosynthetic precursor of some of the quinolines. This is catalysed by the enzyme kynurenine aminotransferase (KAT, EC 2.6.1.7). Finally, it can undergo hydroxylation to give 3-hydroxykynurenine (4) catalysed by kynurenine 3-hydroxylase (EC 1.14.13.9). This last is the major fate of kynurenine arising from the oxidation of physiological amounts of tryptophan and leads into the pathway of nicotinamide nucleotide synthesis.[13] Kynureninase is also able to hydrolyse 3-

hydroxykynurenine to give 3-hydroxyanthranilic acid (6). Rearrangement then yields quinolinic acid, the most neurologically important metabolite on the pathway. This is catalysed by 3-hydroxyanthranilate oxidase (EC 1.13.11.6). Quinolinic acid is then converted to nicotinic acid mononucleotide (10) by quinolinate phosphoribosyl transferase (QPRTase, EC 2.4.2.19). Further transformations may then follow, leading to the nicotinamide coenzymes.

1.2.1 Oxidative Cleavage of Tryptophan.

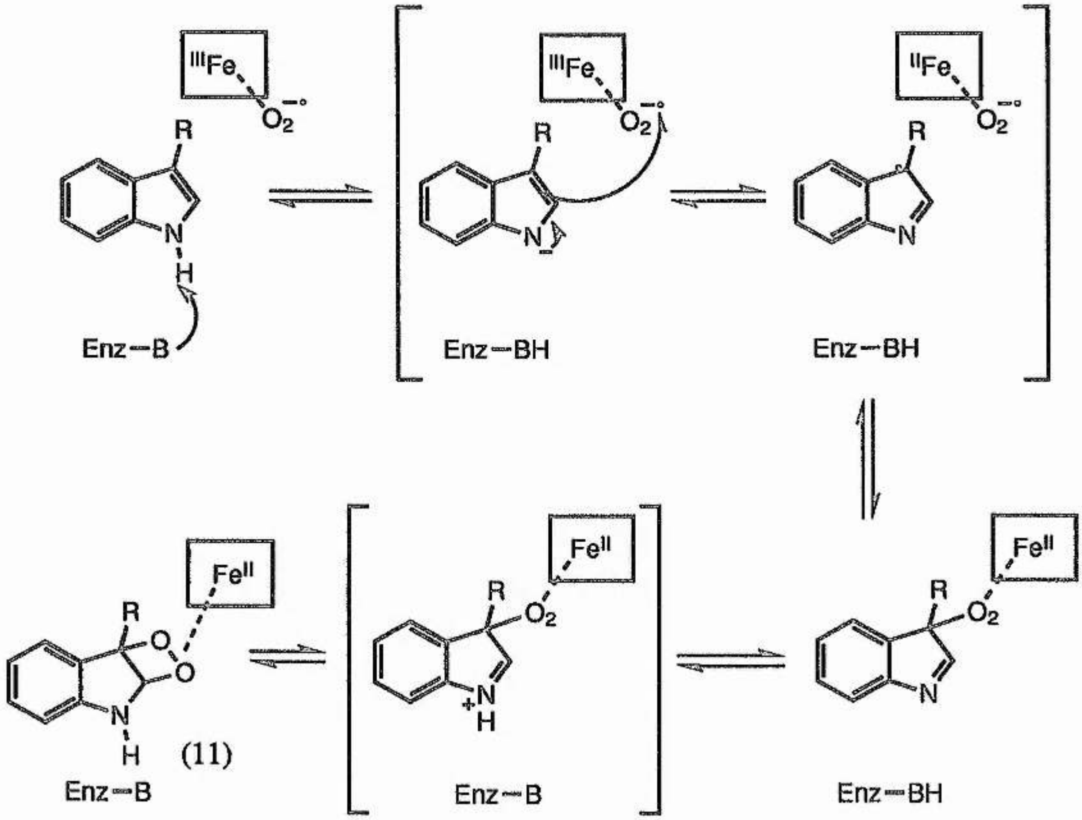
There appear to be two distinct enzymes which catalyse the oxidative cleavage of the indole ring of tryptophan and they are both haem dependent. Tryptophan dioxygenase (EC 1.13.11.11) in the liver and indolamine 2,3-dioxygenase (EC 1.13.11.17) in most other tissues.[13]

1.2.1.1 Tryptophan 2,3-Dioxygenase.

Tryptophan dioxygenase is rate limiting for the entry of tryptophan into the irreversible pathway of catabolism of what is, for mammals, an essential amino acid. The mammalian enzyme has a short half life (of the order of two hours) and is subject to regulation by several different mechanisms.[13]

The enzyme from fungi and bacteria appears to be essentially the same as that from mammalian liver. Both are tetramers with a unit molecular weight of 103 000 and two haem groups per tetramer.[13] The enzyme is haem dependent. Tryptophan binds to the activated form of the holo-enzyme (enzyme-haem complex) and the complex then binds oxygen. The enzyme activity is increased by tryptophan and a number of tryptophan analogues such as α-methyl tryptophan.[14] These act by stabilising the holo-enzyme and promote conjugation of the apo-enzyme with the haem complex. They do not compete with tryptophan at the catalytic site but bind to a regulatory site which is distinct from the catalytic site.[15] Ascorbate is used as the reductant *in vitro* to increase activity although it is possible that the ultimate reductant *in vivo* is superoxide.[13]

Wiseman *et al.* carried out isotope effect studies on tryptophan dioxygenase and proposed a mechanism from the results (Scheme 1.3).[16]



Scheme 1.3

A primary solvent deuterium isotope effect of 4.4 at pH 7.0 implied that abstraction of the indole proton was at least partially rate limiting. An inverse secondary isotope effect of 0.96 at pH 7.0 was observed indicitive of a change in hybridisation from sp^2 to sp^3 at position 2. This is consistent with C-O bond formation at C-2. Since the secondary isotope effect increased with pH as the solvent isotope effect decreased, proton transfer and bond formation at C-2 must represent different steps in the reaction mechanism. Furthermore, the proton transfer step must precede C-O formation. Variation of oxygen concentration did not significantly effect the secondary isotope effect and this can be taken as indication of a predominantly ordered binding pattern with oxygen binding first. Stepwise rather than concerted formation of the oxetane (11) was proposed as the initial deprotonation of the indole would make the concerted pathway energetically inaccessible. The final product arises *via* decomposition of the oxetane, consistent with labelling studies that show both of the new oxygen atoms in the product are derived from molecular oxygen.[16]

The activity of tryptophan dioxygenase is readily and rapidly modified by substrate availability. The enzyme is sensitive to induction by both glucocorticoid hormones and glucagon whereas insulin prevents induction.[8] High concentrations of the nicotinamide nucleotide coenzymes, especially NADPH both inhibit preformed tryptophan synthase and repress its synthesis by binding at the regulatory site of the enzyme.[17]

Seifert [18] described a tryptophan dioxygenase assay which used liver slices as the source of the enzyme and simultaneous HPLC determination of the formyl kynurenine and kynurenine formed from tryptophan. This assay has a major advantage of being able to discriminate between formyl kynurenine and kynurenine in defining tryptophan dioxygenase activity as both products could be detected spectroscopically after their separation on a reversed-phase C_{18} column.

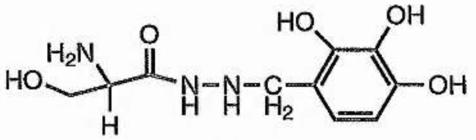
1.2.1.2 Indolamine 2,3-Dioxygenase.

Indolamine dioxygenase is a monomeric haem containing glycoprotein with a molecular weight of 41 000. Unlike tryptophan dioxygenase this enzyme is a superoxide dependent enzyme which requires the presence of free radical generating systems, such as ascorbate and methylene blue, for activity *in vitro*. *In vivo* the role of the dye is thought to be assumed by either a flavin (eg $FMNH_2$) or tetrahydrobiopterin, for which there is likely to be a specific binding site.[15] Superoxide dismutase inhibits the enzyme and superoxide is the oxygenating agent. The enzyme has a broad specificity and acts on *2S*- and *2R*- tryptophan, *2S*- and *2R*-5-hydroxytryptophan, tryptamine, serotonin and melatonin.[19] Indolamine dioxygenase is widely distributed in mammalian tissues, having been identified not only in the distial ileum, but also in the stomach, lung and brain. However it is not present in either liver or kidney. The human enzyme is found in a variety of tissues with especially high activity in lung, small intestine and placenta. The enzyme is not induced by the same mechanisms as tryptophan dioxygenase and contains no activating site for tryptophan analogues. There are few mechanistic data on the reaction and no isotope effects have been measured. However, a similar mechanism to that of tryptophan dioxygenase is likely as they are both haem-dependent enzymes.[13]

Whereas tryptophan dioxygenase is induced by a number of physiological factors as discussed above, indolamine dioxygenase appears to respond to pathological stress and it is induced by γ-interferon. Induction of the enzyme results in depletion of available tryptophan, which is the least abundant of the essential amino acids required for cellular integrity. Such a restriction of available tryptophan would more severely effect rapidly dividing cells, such as microbial pathogens and tumour cells, as tryptophan is required for replication. As a result the enzyme has apparent antimicrobial and antitumour properties.[20] Nitric oxide, the product of nitric oxide synthase initiated arginine degradation, is a regulator of haem enzymes. It has been found that both exogenous and endogenous NO is capable of inhibiting indolamine dioxygenase activity in γ-interferon primed mononuclear phagocytes *in vitro*. A plausible mechanism by which NO inhibits indolamine dioxygenase is *via* interaction with the haem iron present at the active site of the enzyme because NO has a high affinity for haem iron. Such binding may interfere with the conversion of ferric to ferrous iron required for indolamine dioxygenase catalysis.[21] Oxidative arginine and tryptophan metabolism in γ-interferon primed mononuclear phagocytes are therefore functionally related. This provides an insight into how cells may use these methods to regulate their antimicrobial and anti-tumor activities.

1.2.1.3 Inhibitors of Dioxygenases.

Bender [22] has studied the effect of three drugs on tryptophan metabolism in the rat. Benserazide (12), Carbidopa (13) and Isoniazid (14) are potent inhibitors of tryptophan oxygenase and kynureninase.



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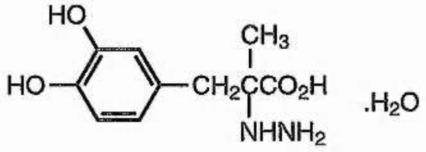

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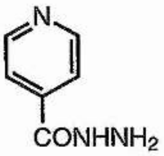
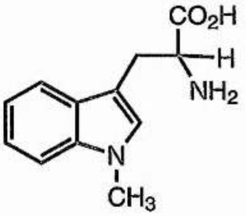


(14)

As inhibitors of tryptophan oxygenase they would be expected to reduce the entry of tryptophan into the pathway of nicotinamide nucleotide synthesis. The anti-Parkinsonian drugs Benserazide and Carbidopa are thought to be inhibitors because their phenolic nature allows them to interact with the haem binding site. Isoniazid is not phenolic but it is possible that it is capable of interacting with the regulatory site of tryptophan oxygenase.[17] It has been shown that a number of hydrazine derivatives of tryptophan analogues are more potent inhibitors of tryptophan oxygenase than are the parent compounds and suggested that the hydrazines can interact with the haem binding site of the enzyme, thus preventing activation of apo-tryptophan oxygenase.[22] A number of indole derivatives and β-carboline derivatives have also been tested with both enzymes. Peterson[23] has prepared a number of derivatives and analogues of tryptophan to elucidate the structural requirements of tryptophan dioxygenase. Cady and Sono [24] tested 1-methyl-*2RS*-tryptophan (15), β-(3-benzofuranyl)-*2RS*-alanine (16, the oxygen analogue of tryptophan) and β-(3-benzo(*b*)thienyl)-*2RS*-alanine (17, the sulphur analogue of tryptophan), each of which was found to be a competitive inhibitor for tryptophan dioxygenase. However, none of these compounds acted as substrates, indicating that the free form of the indole nitrogen is an important physical and / or electronic structural requirement for tryptophan to be metabolised by the enzyme.



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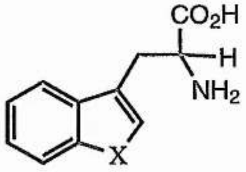
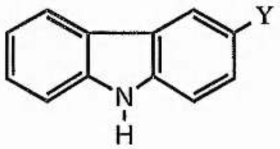


X = O (16)
X = S (17)

Eguchi[19] and Peterson [25] have tested a number of β-carboline derivatives including norharman (18). Norharman is the most potent inhibitor known for both enzymes from mammalian sources. It is a non-competitive inhibitor of indoleamine dioxygenase[26] and competitive inhibitor of tryptophan dioxygenase.[19] Peterson [25] has reported 3-butyl-β-carboline (19) as the most potent inhibitor of indoleamine dioxygenase with a K_I of 3.3 μM (the K_M of 2*S*-tryptophan is 13 μM).

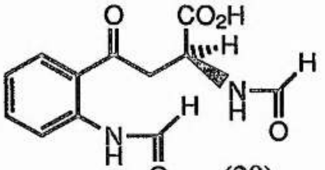


Y= H (18)
Y= ⁿBu (19)

1.2.2 Kynurenine Formamidase.

The immediate product of oxidative cleavage of the indole ring of tryptophan, catalysed by either tryptophan dioxygenase or indoleamine dioxygenase is *N'*-formyl kynurenine. This is rapidly hydrolysed to kynurenine by kynurenine formamidase (EC 3.5.1.9) with release of formate. The enzyme from all sources is essentially the same, however there are some differences in substrate specificity and sensitivity to inhibitors. Kynurenine formamidase has a relatively broad substrate specificity and will hydrolyse a number of aryl formylamines, including *2S*- and *2R*-*N'*-formyl kynurenine, *N'*-formyl hydroxykynurenine and *N'*-formyl anthranilate.

Kynurenine formamidase will not hydrolyse *N*-formyl kynurenine and this forms the basis of an assay for the enzyme. If *N'*, *N*-diformyl kynurenine (20) is employed as the substrate the product is *N*-formyl kynurenine which accumulates in the medium and its concentration can be monitored.

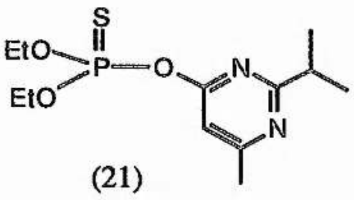


(20)

1.2.2.1 Inhibitors of Kynurenine Formamidase.

Penwim and Seifert[27] have shown that kynurenine formamidase is inhibited very strongly by organophosphorus and methylcarbamate insecticides. These compounds altered the formation of several *2S*-tryptophan metabolites associated with the *2S*-kynurenine pathway in mice. Liver kynurenine formamidase was inhibited almost completely by Diazinon (21), which is one of its most potent inhibitors *in vivo*.[28] Diazinon and related compounds show toxicity due to cholinesterase inhibition but also have a degree of non-cholinergic toxicity.

The structural requirements for optimal kynurenine formamidase inhibition were studied by testing a number of organophosphorus compounds (22).[27]



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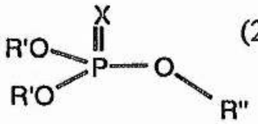
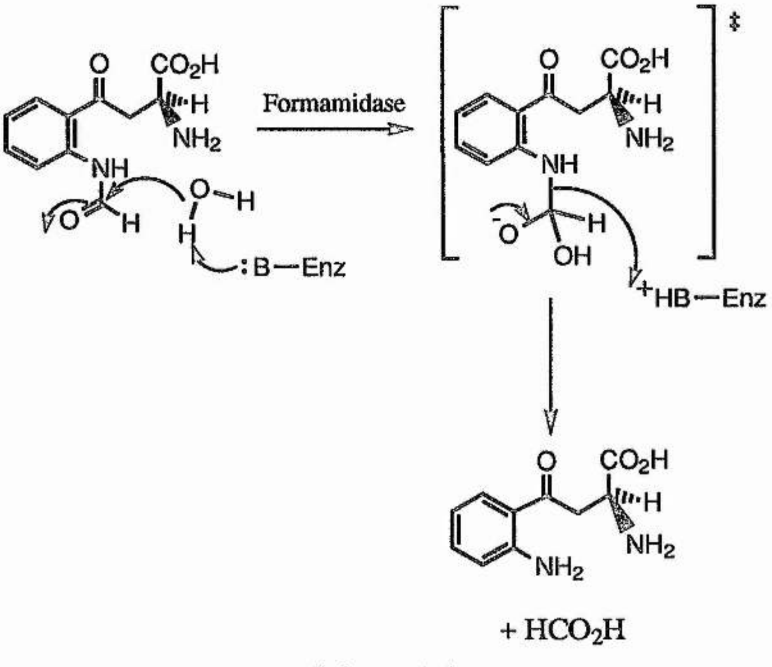


(22)

R' = Alkyl, X= S or O
R'' = Alkyl or Aryl

Pyrimidinyl phosphorothioates followed by crotonamide phosphates were the most potent compounds. Replacement of sulphur by oxygen in the phosphorothioate Diazinon reduced *in vivo* liver kynurenine fomamidase inhibition. The aryl phosphorothioates may be thought of as transition state mimics for the hydrolysis reaction and therefore bind tightly to the enzyme active site thus explaining the potent inactivation (Scheme 1.4).[29]

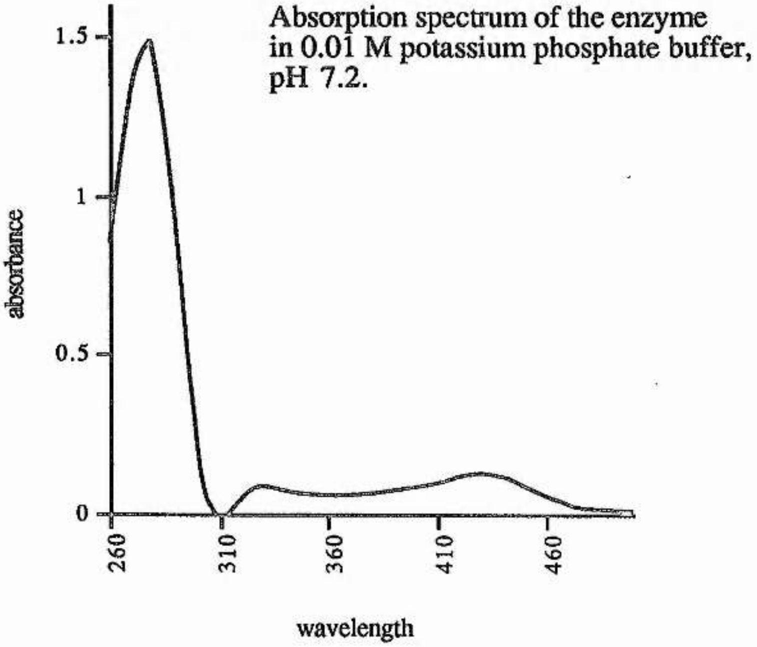


Scheme 1.4

1.2.3. Kynureninase.

1.2.3.1 Studies on kynureninase.

Moriguchi, Yamamoto and Soda have purified and crystallised kynureninase from *Pseudomonas marginalis*.[2] The enzyme is composed of two identical subunits and the molecular weight determined by electrospray ionisation mass spectrometry was 45 484 per subunit for kynureninase isolated from *Pseudomonas fluorescens* [30] Note that *Pseudomonas fluorescens* was formerly known as *Pseudomonas marginalis*.[31] The partial amino acid sequence has also been determined from this source as well as the sequence from rat liver kynureninase.[32] In addition, Alberatigiani *et al.* have isolated human kynureninase.[33] One mole of pyridoxal phosphate is bound per mole of enzyme. The enzyme exhibits absorption maxima at 280, 337 and 430 nm (see Graph 1.1).

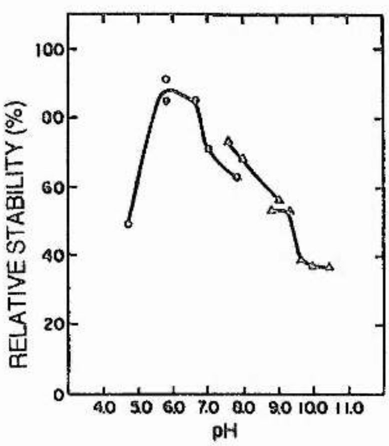


Absorption spectrum of the enzyme in 0.01 M potassium phosphate buffer, pH 7.2.

Graph 1.1

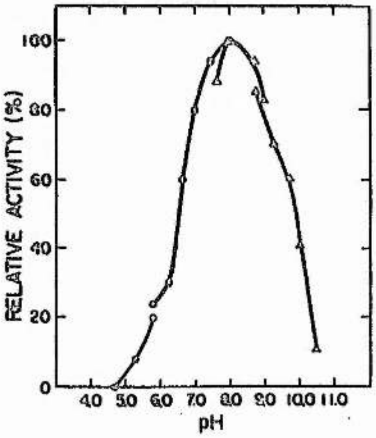
Moriguchi *et al.* [34] obtained a UV spectrum indicative of a bound pyridoxal phosphate for pig liver kynureninase. A peak in the 430 range is characteristic of a pyridoxal phosphate bound to the enzyme as a Schiff's base with an amino group. This characteristic spectrum and also the isolation of ε-*N*-pyridoxyllysine from the hydrolysate of borohydride reduced enzyme show that PLP is bound to an ε-amino group of a lysine residue of the protein through an aldimine linkage.

The enzyme was found to be stable in the pH range 5.8-8.0 when heated at 50 °C for five minutes (see graph 1.2). The enzyme has an optimum reactivity at about pH 8, when examined in the presence of Tris-HCl buffer and potassium phosphate buffer (see graph 1.3).[34]



Graph 1.2: Effect of pH on enzyme stability

The buffers used were acetate (●) for pH 4.7-5.8, potassium phosphate (O) for pH 5.8-7.8,Tris-HCl (Δ) for pH 7.6-9.0 and glycine-NaOH (▲) for pH 8.8-10.5.



Graph 1.3: Effect of pH on kynureninase activity

The buffers used were acetate (●) for pH 4.7-5.8, potassium phosphate (O) for pH 5.8-8.0, Tris-HCl (▲) for pH 7.6-9.0 and glycine-NaOH (Δ) for pH 8.8-10.5.

A paper by Hayaishi and Stanier stated that the activity of kynureninase may be measured conveniently either by spectrophotometric or manometric methods. Manometric assays of kynureninase were made by measuring carbon dioxide evolution resulting from acid production in bicarbonate buffer in an atmosphere of 5% carbon dioxide and 95% nitrogen at 30 °C.[35] All the spectrophotometeric methods involved measurement of the decrease in absorbance at 360 nm (ε= -4500 $M^{-1}cm^{-1}$) upon the conversion of kynurenine to anthranilic acid at 25 °C. However, different buffers have been employed. The standard assay system of Moriguchi *et al.* consisted of 200 µmol of tris(hydroxymethyl)amino methane (pH 8.0), 1 µmol of 2*S*-kynurenine sulphate, 0.2 µmol of PLP and enzyme in a final volume of 3.1 ml.[34] The assay buffers used by Phillips and Dua contained 0.4 mM 2*S*-kynurenine in 0.04 M potassium phosphate (pH 7.8) with 40 µM PLP.[36]

1.2.3.2 Substrate Specificity.

A number of kynurenine analogues have been prepared to obtain information on kynureninase structure and mechanism. Although the enzyme from *Pseudomonas fluorescens* and *Pseudomonas marginalis* is identical, different results have been obtained by different groups.[1, 6, 37] This could be due to the different methods of detection employed. These include measuring the alanine formed by paper chromatography or determining anthranilic acid derivatives formed fluorometrically or determining the hydrolysis of the substrate spectophotomerically. The results (summarised in table 1.1) show that the α–amino group is essential and that only the 2*S*-enantiomers are reactive. When the aromatic *ortho*-amino group of kynurenine is blocked with a formyl group (23), replaced by a nitro group (24) or moved to the *para*-position (25) the activity disappears. The derivatives in which the aromatic amino group is substituted by a hydroxy group (26), or removed (27), show some activity with the enzyme, although the rate of reaction is considerably decreased. The introduction of a hydroxyl group to the aromatic ring (28) significantly decreases the reaction rate but the fluoro compound (29) has high reactivity.

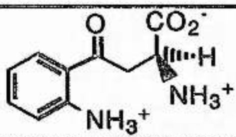
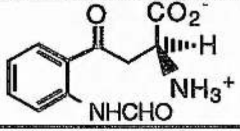
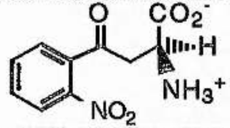
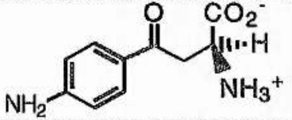
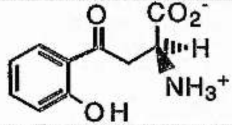
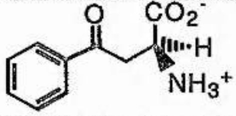
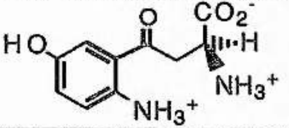
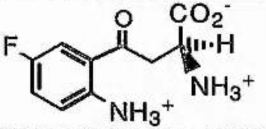
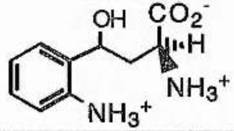
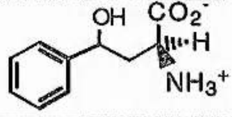
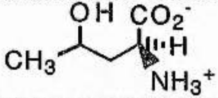
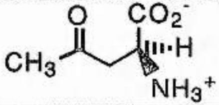
		Relative reactivity (%), kynureninase of		
		Rat liver[a]	Pseudomonas fluorescens[b]	Pseudomonas marginalis[c]
(1)		++	100	100
(23)			0	14.9
(24)		-	0	
(25)			0	
(26)			13.4	
(27)		++	6.0	2.7
(28)			55.0	1.2
(29)				91.2
(30)				65.1
(31)		++		
(32)		+		
(33)		+		

Table 1.1

a-Relative reactivities are expressed as relative amounts of alanine formed.[37]

b-Relative reactivities were determined by measuring spectrophotometrically the substrate hydrolysed.[6]

c-data from reference.[38]

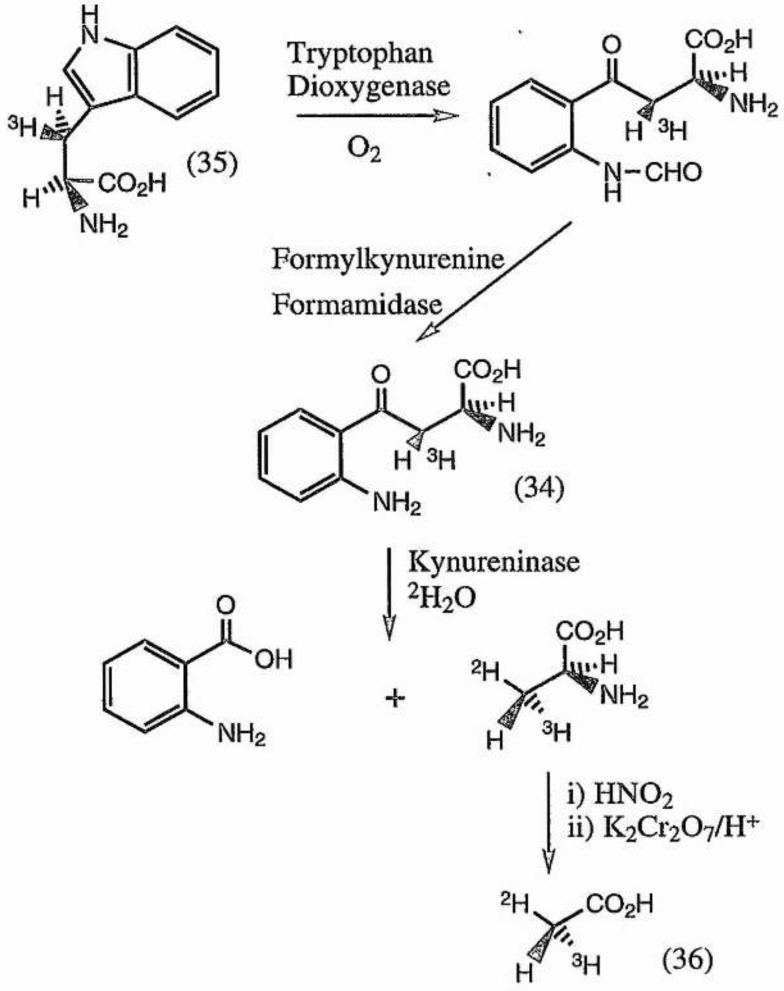
The occurrence of γ-carbonyl group is not essential in substrates because γ-hydroxy derivatives (30, 31, 32) are also cleaved (see 1.2.3.5 Further Mechanistic Studies). Compounds (32) and (33) were also found to be substrates, suggesting that an aryl group is not necessarily required (Table 1.1).

The results show that the aromatic amino group at the *ortho*-position of kynurenine has a significant influence on the reactivity. The relative rates of hydrolysis of the analogues may be explained, although not quantitatively, by the inductive and resonance effects of substituents (ie. NH_2, NO_2, OH etc) and by only the inductive effect of *meta*-hydroxyls as suggested by Hayaishi.[6]

However, substituents in the aromatic moiety of kynurenine have an insignificant influence on the affinity of the enzyme for the substrate. 2-Amino-4-oxo-4-phenyl-butyric acid (27) has a relatively low K_M value (1.6×10^{-4} M) but is hydrolysed very slowly.[38]

1.2.3.3 Stereochemistry.

The stereochemical course of the reaction with regard to C-3 has been elegantly determined (Scheme 1.5) by Floss, Soda and co-workers.[31]

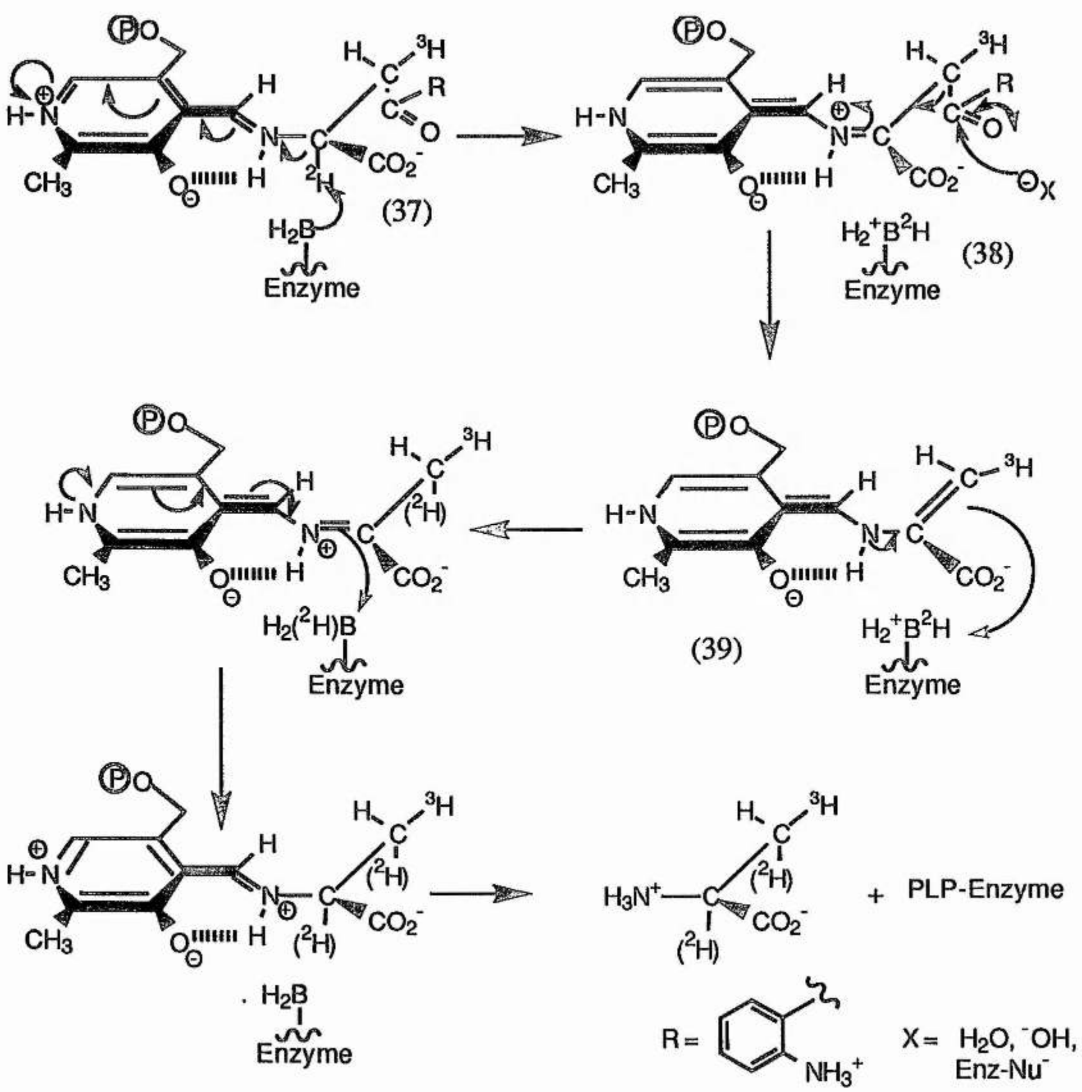


Scheme 1.5

(2*S*,3*R*)-Kynurenine and (2*S*,3*S*)-[3-^{3}H] kynurenine (34) were first prepared from the corresponding stereospecifically tritiated tryptophan species (35) by enzymic methods. They were then converted into alanine using kynureninase in deuterium oxide. Alanine can be converted into acetic acid (36), by diazotisation and dichromate oxidation, for chiral analysis of the methyl group by the method of Cornforth,[39] to determine the steric course of the replacement of the anthranilyl group by hydrogen. The reaction was found to occur with retention of configuration i.e. the proton is added on the same side from which the anthranilyl group has departed.

1.2.3.4 Proposed mechanism.

Of all the mechanisms proposed the following seems the most reasonable and accounts for the above results (Scheme 1.6).[31]



Scheme 1.6

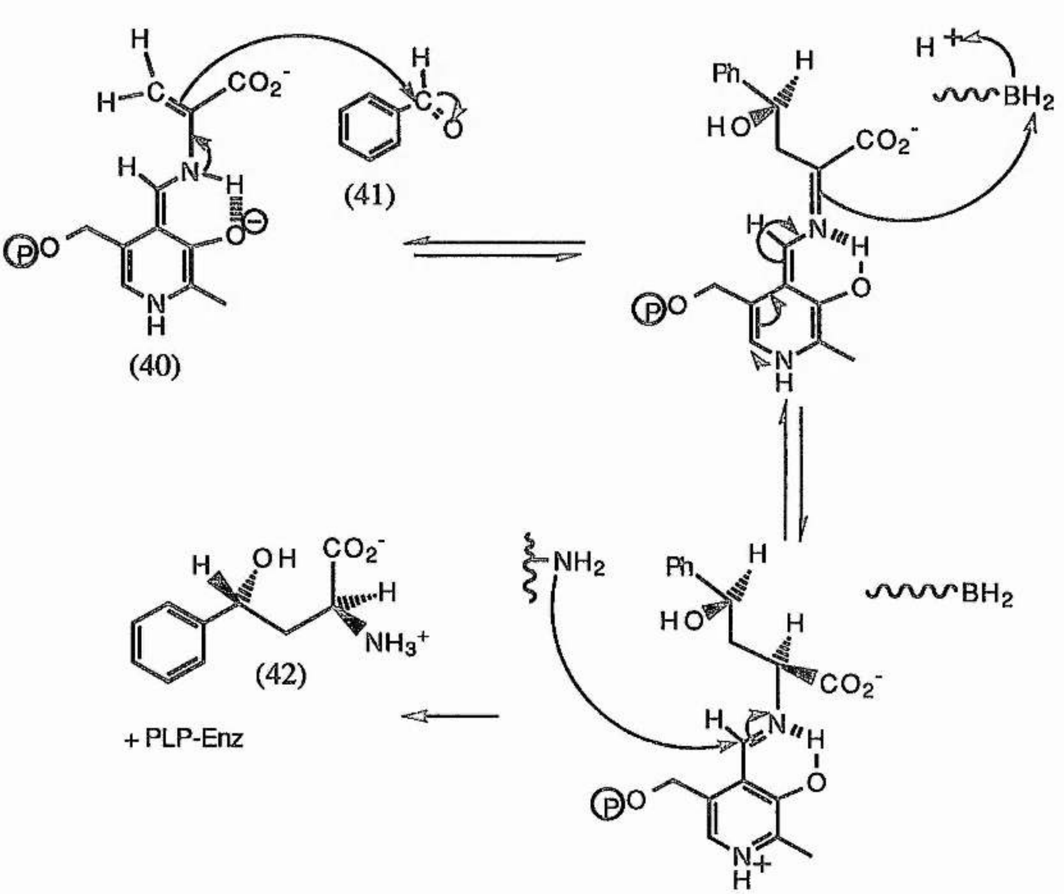
The PLP binds to the apoenzyme via the ε–amino group of an active site lysine residue to give a Schiff's base. This is termed the internal aldimine and is common to most other pyridoxal phosphate dependent enzymes. Kynurenine then binds to the enzyme and a transaldimination reaction takes place, displacing the active site lysine and forming a Schiff's base between the PLP and kynurenine (37), the external aldimine. Cleavage of the Cα-H bond then generates the ketimine intermediate (38), a resonance-stabilised carbanionic species. As suggested by Dunathan,[40] orientation of the Cα-H bond perpendicular to the Π plane of the substrate–pyridoxal phosphate Schiff's base should favour cleavage of this bond by maximising orbital overlap between the electrons of the σ(Cα–H) bond and the extended Π system of the cofactor. Attack of an enzymic nucleophile, water molecule or hydroxide ion (X) then takes place at the γ–carbonyl group of the quinoid intermediate. The β,γ-carbon-carbon bond is cleaved to give anthranilate and an α-aminoacrylate derivative (39). For the above stereoelectronic reasons the Cβ,Cγ bond should be oriented perpendicular to the plane of the PLP π–system. However it may lie on the same or the opposite side to the Cα-H bond. The stereochemical results show that protonation of the α-aminoacrylate is stereospecific and therefore enzyme mediated, and that it occurs with retention. Therefore, the departing anthranilyl group and the base delivering a proton must be disposed on the same face of the pyridoxal phosphate-substrate complex. The finding of internal proton recycling from the α-proton of the substrate kynurenine, not only to the α-position but also to the β-position of the product alanine, indicates that proton abstraction / addition at Cα and protonation at Cβ are mediated by the same base. This and the observed retention of stereochemistry, support an active site conformation of the pyridoxal phosphate-substrate complex in which the α–hydrogen and the anthranilyl group at Cβ are *syn* oriented. It was assumed but not proven, that they were both on the *si* face relative to C4' of the coenzyme, as in all other PLP dependent enzymes so far examined.[41] The identity of the active site base has not been confirmed. Kishore[42] observed alkylation of an active site carboxyl group using mechanism based inhibitors of kynureninase, implying that this was involved. However the observed partitioning of the tritium between Cα and Cβ of alanine also suggests that the base mediating these proton transfers is polyprotic such as the ε-amino group of a lysine residue.

Although the stereochemistry is known for the reaction, the identity of X in the hydrolysis step is unknown. The hydrolysis could be mediated by a water molecule bound at the active site using general base catalysis from the enzyme, or alternatively, by an enzymic nucleophile. This is currently one of the main areas of investigation and several putative inhibitors have been designed in an attempt to distinguish between the two alternatives (see Chapter 2).

1.2.3.5 Further mechanistic studies

Trapping of the α-aminoacrylate intermediate (40) with added aromatic aldehydes was observed by Bild and Morris.[43] The characteristics of the novel reaction product produced from benzaldehyde (41) and kynurenine, in the presence of kynureninase and pyridoxal phosphate, are consistent with the identity of the compound as 2-amino-4-hydroxy-4-phenylbutyric acid (42). Phillips and Dua demonstrated that the reaction was stereospecific and that only the (*2S*,*4R*) isomer was formed.[36] The aromatic ring is derived from benzaldehyde, as shown by the dependence of the reaction on the presence of benzaldehyde and the observation that the substitution of other aromatic aldehydes for benzaldehyde produced a predictable variation in the R_f value on thin layer chromatography plates. Radioactive labelling demonstrated that the side chain of kynurenine was incorporated into the product.

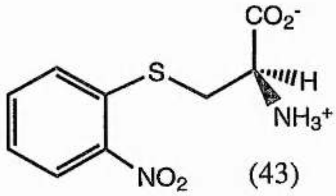
It therefore appears that the α–aminoacrylate intermediate, once formed, can partition in the presence of benzaldehyde to form either alanine or 2-amino-4-hydroxy-4-phenylbutyric acid (Scheme 1.7). The reaction is specific to aromatic aldehydes, which is presumably a consequence of the ability of aromatic electrophiles to bind to the site normally occupied by either anthranilic acid or the aromatic portion of kynurenine. The observed product would seem to suggest that the α-aminoacrylate intermediate has a sufficiently long lifetime to enable the anthranilic acid to debind from the active site, and the benzaldehyde bind and undergo reaction. It would seem feasible that 2-amino-4-hydroxy-4-phenylbutyric acid could be formed equally as well from alanine or kynurenine. It was, however, determined that the reaction proceeded only approximately 1% as rapidly with alanine. This was shown not to be due to an inactive enzyme conformation which would have been reconverted into an active conformation by the addition of anthranilate or an anthranilate analogue. It was, therefore, reasoned that formation of the α-aminoacrylate intermediate (40) from alanine may be thermodynamically less favourable than from kynurenine.[43]



Scheme 1.7

1.2.3.6 Inhibitors of kynureninase.

A number of compounds have been observed to act as mechanism based inhibitors of kynureninase. These have all been β-substituted amino acids, a class of compounds known to inhibit PLP dependent enzymes.[42] Examples include β-chloro-2*S*-alanine and 2*S*-serine-*O*-sulphate. Interestingly the *ortho*- amino group, as observed in kynurenine is not necessary for activity since 2-amino-4-oxo-4-phenyl-butyric acid is a substrate for the enzyme and β–chloro-alanine and analogues are inhibitors. The most potent, however, was 2*S*-S-(ortho-nitrophenyl)-cysteine (43) presumably as a result of the strong binding interaction between the aromatic moiety and the active site.



(43)

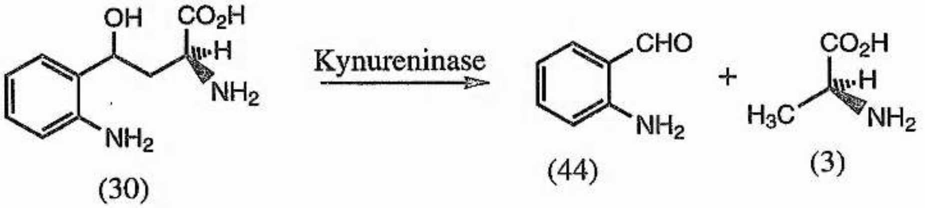
The effects of various aromatic compounds on kynureninase activity have also been investigated.[38] *O*-Aminophenyl compounds having a carbonyl group, especially a formyl group are most inhibitory. *O*-Aminobenzaldehyde, *o*-aminoacetophenone and benzaldehyde inhibit about 80-40% of the activity, while *o*-nitrobenzaldehyde and anthranilic acid are much less inhibitory, and aniline, benzoic acid and acetophenone are not inhibitory at all. The inhibition by *o*-aminobenzaldehyde is non-competitive with the substrate. This could be due to *o*-aminobenzaldehyde binding only to the enzyme aminoacrylate complex and as this does not compete with the binding of kynurenine to the enzyme non-competitive inhibition results (Scheme 1.8)

$$\mathrm{E + Ky} \rightleftharpoons \mathrm{E.Ky} \rightleftharpoons \mathrm{E.A.B} \xrightleftharpoons{\mathrm{-A}} \overset{*}{\mathrm{E.B}} \rightleftharpoons \mathrm{E.Ala} \rightleftharpoons \mathrm{E + Ala}$$

E = enzyme, Ky = Kynurenine, A = anthranilate, B = aminoacrylate, Ala = alanine.

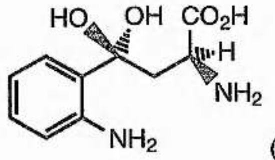
Scheme 1.8

Phillips and Dua[36] examined both diastereomers of 2-amino-4-hydroxy-4-phenylbutyric acid (30, dihydro-2*S*-kynurenine) as inhibitors of kynureninase from *Pseudomonas fluorescens*. The (4*R*,2*S*) diastereomer was a potent competitive inhibitor with a K_I of 1.4 μM but was also a substrate for the enzyme, undergoing a retro-aldol cleavage reaction (Scheme 1.9)[1] to give 2-aminobenzaldehyde (44) and 2*S*-alanine (3). The (4*S*,2*S*) diastereomer was an even more potent competitive inhibitor with a K_I of 0.3 μM but did not act as a substrate.



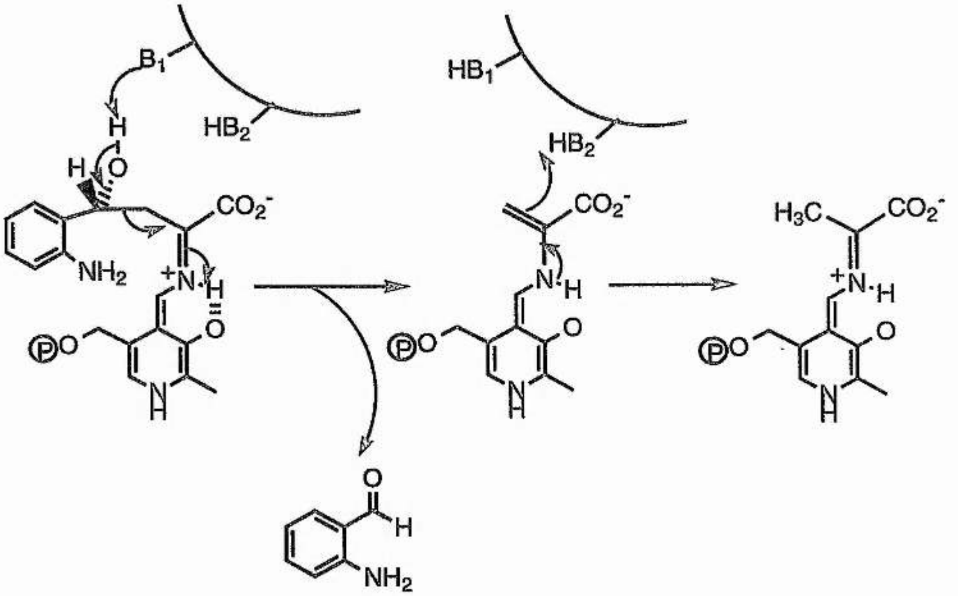
Scheme 1.9

Both dihydro-2*S*-kynurenines are similar in structure to the tetrahedral intermediate (45) formed if the reaction mechanism involves hydrolysis using a bound water molecule. This intermediate is a model of the transition state and the potent competitive inhibition exhibited by these compounds suggests that they are transition state analogues.



(45)

It was previously shown for kynureninase that reaction proceeds with retention of configuration at Cβ.[31] This result, along with the demonstration of stereospecific recycling of the α-hydrogen of the substrate to the cofactor and / or the β-carbon of the product, suggested that reaction took place on one side of a planar coenzyme-substrate complex and was catalysed by a single base in the enzyme active site. However, from the study of the dihydro-2*S*-kynurenine diastereomers Phillips and Dua[36] proposed that there must be a second base present to remove the proton from the alcohol to facilitate the retro aldol cleavage (Scheme 1.10).



Scheme 1.10

The observed stereospecificity is then due to the fact that the (4*S*,2*S*) diastereomer of dihydro-2*S*-kynurenine would have the OH oriented projecting away from the catalytic base and is not cleaved since proton abstraction cannot occur.

The majority of PLP dependent enzymes which catalyse β replacement reactions and α,β-elimination reactions have, like kynureninase, been shown to react with retention of configuration e.g. tryptophan synthetase, tyrosine phenol lyase and 2*S*-selenocysteine β-lyase.[44]

1.2.3.7 Related PLP Dependent Enzymes.

Kynureninase has been classified in the same group as aspartate β–decarboxylase[45] and 2*S*-selenocysteine β-lyase,[46] both of which have been studied and are described below. These three enzymes, which catalyse β-elimination reactions, also catalyse transamination reactions, although more slowly.[47]

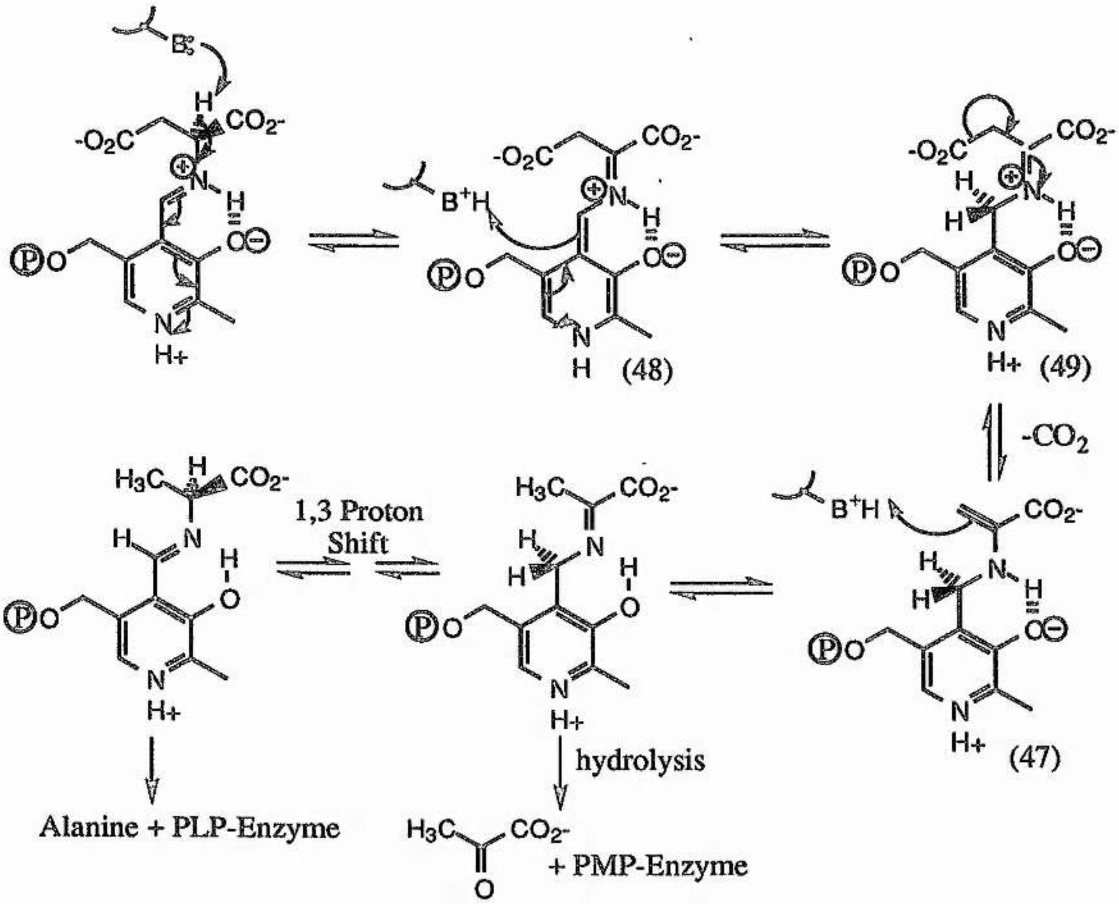
Aspartate β-decarboxylase catalyses the decarboxylation of 2*S*-aspartic acid (46) to alanine (Scheme 1.11).

Scheme 1.11

O'Leary, Floss and coworkers studied the stereochemical course of the β-decarboxylation of 2*S*-aspartic acid by incubating C-β tritiated 2*S*-aspartic acid with the enzyme in ^2H_2O which gave samples of 2*S*-alanine containing chiral methyl groups. Determination of the stereochemistry of the labelled alanine revealed that β-decarboxylation had occurred predominantly with inversion of configuration at C-β of aspartate. This is therefore different from all related PLP dependent enzymes that have been studied.[44, 48] They concluded that there was one base mediating deprotonation and protonation at Cα and optionally protonating C-4' on the *si* face. A second enzymic base at C-β was proposed to cover the C-4' *re* face and be responsible for protonating at C-β of aspartate. Their evidence against a single base came from studying the transamination reaction. Incubation of the enzyme with Cα-tritiated 2*S*-aspartic acid led to 17% incorporation of the label at C-4' of pyridoxamine 5' phosphate (PMP), but only 1% appears in 2*S*-alanine. Since protonation at C-β must precede protonation at C-4' a single base charged with tritium from Cα should deliver more of this tritium at C-β than at C4'.[48]

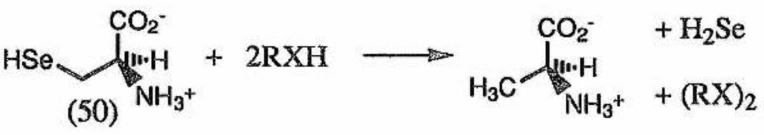
However, an alternative possibility exists that transfer of hydrogen from C-α of aspartate to C4' of the coenzyme occurs in both the normal β-decarboxylation and abortive transamination, before further reaction. β-Decarboxylation would then yield the enamine (47) which would be protonated from the C-4' *si* face of the coenzyme by the enzyme bound base to give the ketimine with inversion of configuration. As most of the label present at C-α of aspartate would have already been transfered to C-4' of the coenzyme, the remaining label associated with the base would not be expected to result in a large enrichment at C-β of the ketamine. Solvent exchange must account for the small enrichment observed on the back transfer of 4'-H atoms to C-α as otherwise both one and two base mechanisms would predict significant incorporation at C-α. Rosenberg and O'Leary proposed this mechanism from their results that showed that α- and β- hydrogen / solvent hydrogen exchange are faster than the decarboxylation of 2*S*-aspartic acid and that decarboxylation is not entirely rate limiting.[49]

Thus, α-hydrogen abstraction produces a quinoid intermediate (48) which undergoes a hydrogen transfer to give the ketimine intermediate (49). Decarboxylation then yields the corresponding enamine (47) which leads to the product and PLP as described above (Scheme 1.12).[49]



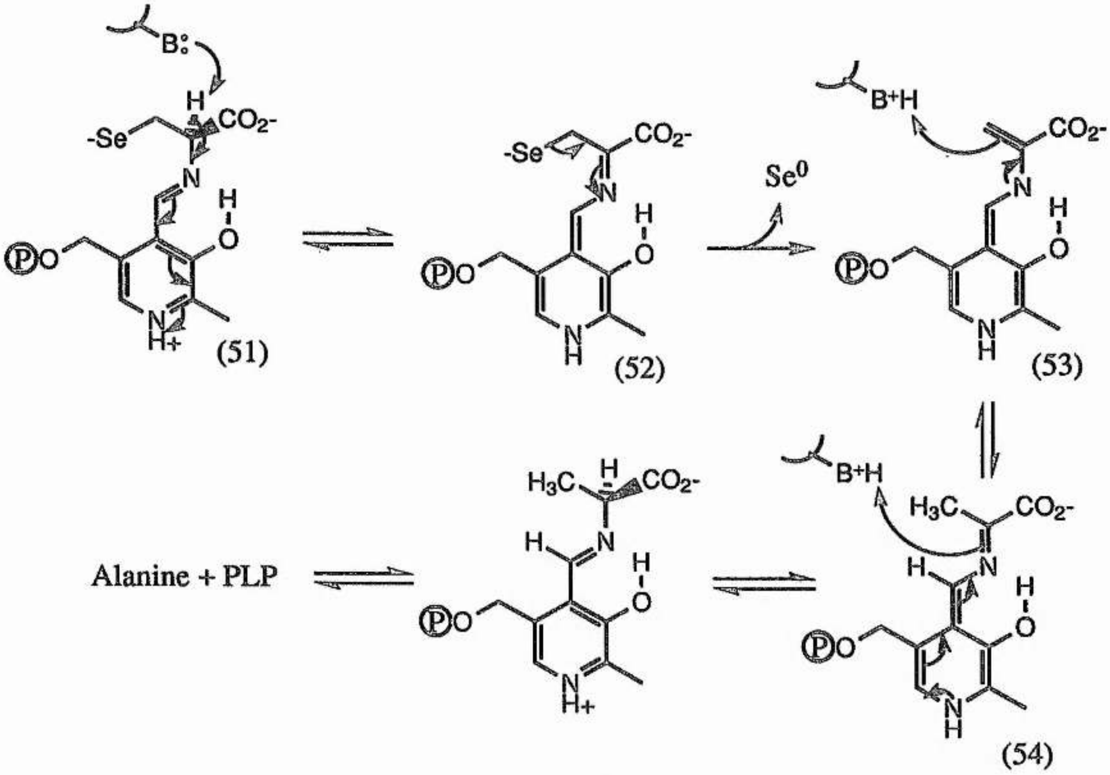
Scheme 1.12

Selenocysteine β-lyase is a unique PLP dependent enzyme that catalyses the β-elimination of 2*S*-selenocysteine (50) into 2*S*-alanine (Scheme 1.13). The enzyme is found in mammalian tissues and in bacteria. Elemental selenium is released enzymatically from selenocysteine (a lyase reaction) and then reduced nonenzymatically to H_2Se with selenocysteine or other reductants in the reaction mixture.



Scheme 1.13

The following mechanism has been proposed (Scheme 1.14) where the selenocysteine PLP complex (51) is converted into a quinoid intermediate (52) through α-hydrogen abstraction. Selenium is released in elemental form from this intermediate and an aminoacrylate intermediate (53) is formed.

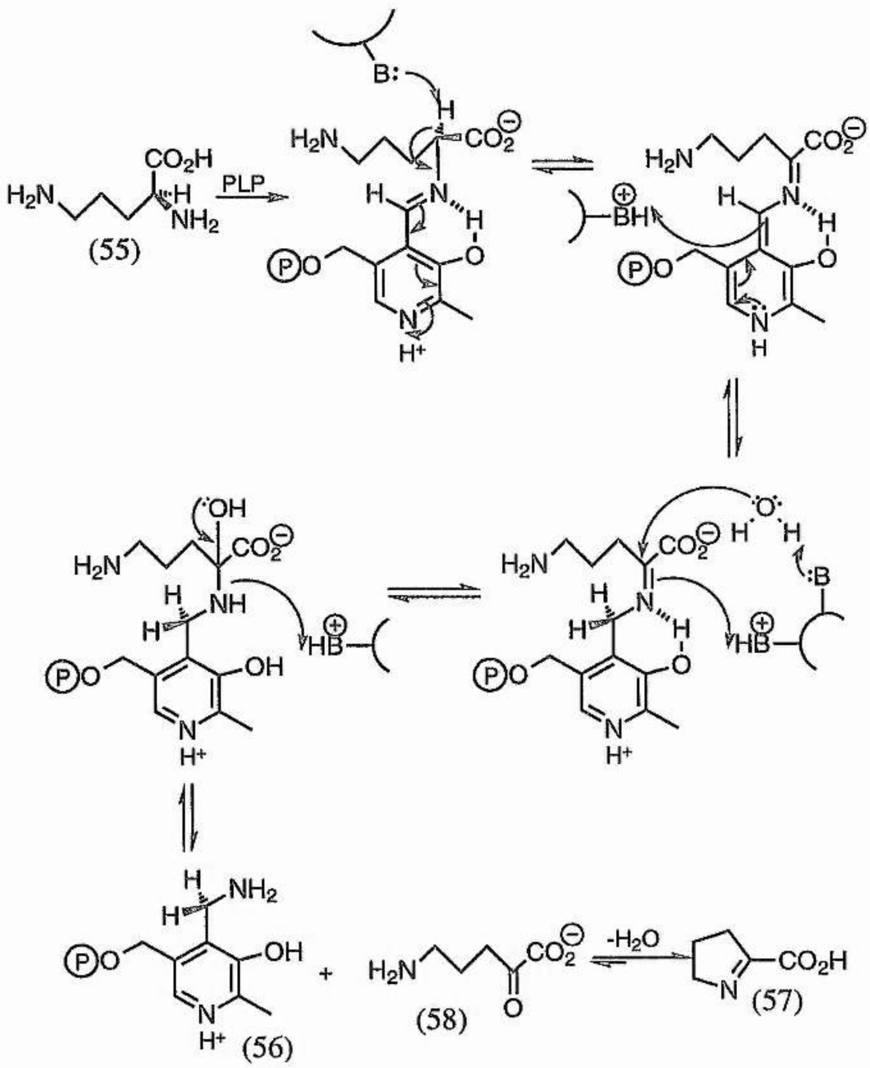


Scheme 1.14

The ^{1}H and ^{13}C NMR spectra of alanine produced from selenocysteine in deuterium oxide indicated the enzymatic formation of [β-$^2H_1$] and [β-$^2H_2$] alanines. Therefore, in addition to the incorporation of one deuterium atom into the β position of alanine after removal of elemental selenium one of the two β-hydrogen atoms of selenocysteine is exchanged with a solvent deuterium. Since the enzyme catalyses no hydrogen exchange at α and β positions of alanine with a solvent deuterium, [β-$^2H_2$] alanine is probably formed from selenocysteine through the rapid reversible reaction between intermediates (53) and (54). A two-base mechanism was proposed for the enzyme reaction where the α-protonation and deprotonation is performed by one base and the other base, which is probably in its protonated form in order to function catalytically mediates the β-protonation.[47] Evidence for this was provided by the fact that when reaction was carried out in ^2H_2O the α-hydrogen of selenocysteine was fully retained in the product alanine. For kynureninase, experiments were carried out to probe for internal transfer of tritium from the α-position of kynurenine to the product alanine. These indicated that 40% of the tritium from the α-position of the substrate is transferred to the β-position of the product alanine and 60% to the α-position of the product. Indicating that proton abstraction / addition at Cα and protonation at Cβ are mediated by the same base.[31] Thus, a one base mechanism has been proposed for kynureninase and selenocysteine β-lyase and aspartate β-decarboxylase probably follows a one base mechanism. For kynureninase, a second base has been proposed as a result of studies on dihydro-2*S*-kynurenine (See 1.2.3.6 Inhibitors of Kynureninase).[1, 36] In the physiological reaction, the role of this second base could be removal of a proton from a water molecule to assist in attack of the carbonyl group.

1.2.3.8 Transamination Catalysed by Kynureninase.

It has also been shown that kynureninase is inactivated by 2*S*-alanine a reaction product, or by 2*S*-ornithine (55). The activity is restored by the addition of PLP. Spectrophotometric studies on the inactivation indicate that the addition of 2*S*-alanine or 2*S*-ornithine to the holoenzyme leads to loss of peaks at 337 and 430 nm and appearance of a new peak at 325 nm. Therefore, inactivation is due to formation of the bound PMP (56) from the bound PLP by transamination with 2*S*-alanine or 2*S*-ornithine. The product from 2*S*-ornithine was identified as Δ'-pyrroline-2-carboxylic acid (57), the dehydrated form of α-keto-γ-aminovaleric acid (58) (Scheme 1.15). It is thought that the enzyme's activity as a highly substrate specific α-aminotransferase is important as a regulator of enzyme activity *in vivo*, by interconversion of the coenzyme from PLP to PMP.[50]



Scheme 1.15

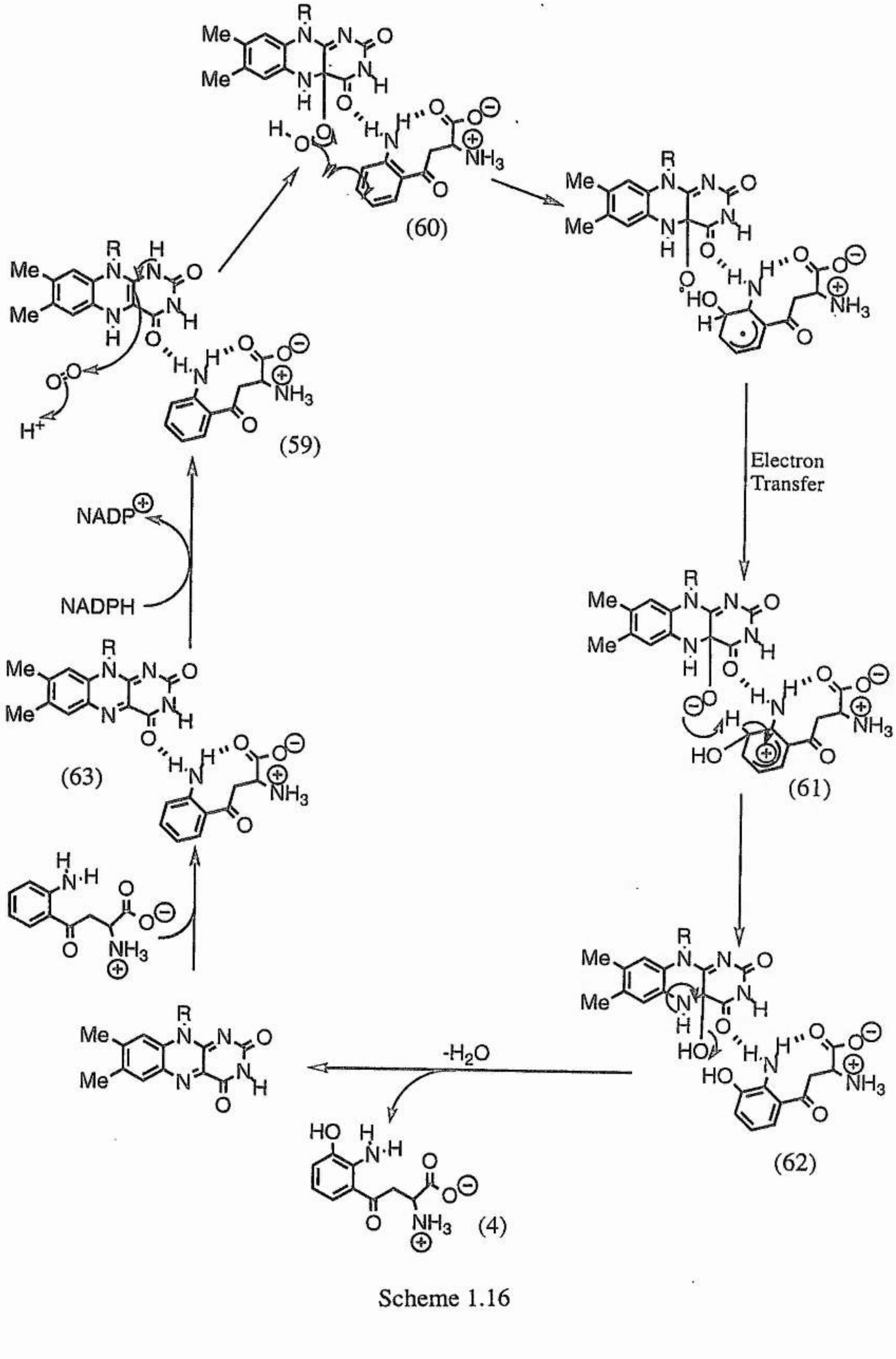
1.2.4 3-Hydroxykynureninase.

3-Hydroxykynureninase (EC 3.7.1.3) catalyses the hydrolysis of *2S*-3-hydroxykynurenine to 3-hydroxyanthranilic acid and *2S*-alanine. PLP is the required enzyme cofactor. The enzyme although catalysing a similar reaction to kynureninase is not inducible by tryptophan and functions primarily in the biosynthesis of NAD from tryptophan. Kynureninase is inducible by tryptophan and is involved in the catabolism of tryptophan.[38] Two types of kynureninase were isolated from *Neurospora crassa*. One of them was independent of the presence of tryptophan in the growth medium. The molecular weight and optimum pH values of both enzymes are very similar. However, the constitutive enzyme shows much higher activity and affinity for 2*S*-3-hydroxykynurenine than for 2*S*-kynurenine suggesting that the enzyme functions as a 3-hydroxykynureninase.[51]

1.2.5 Kynurenine 3-hydroxylase

Kynurenine 3-hydroxylase (EC 1.14.13.9) is an external flavoprotein monoxygenase located in the outer mitochondrial membrane which incorporates oxygen into kynurenine to give 3-hydroxykynurenine. It requires FAD and either NADH or NADPH as co-reductant. The purified enzyme is an oligomer containing four moles of FAD per monomeric unit. The monomer has only about 10% of the catalytic activity of the dimer.[13] The catalytic cycle has been proposed based on available kinetic and spectroscopic data[52] (Scheme 1.16).

The cycle begins with a reduced enzyme / kynurenine complex (59) which reacts with molecular oxygen to give the corresponding C(4a)-hydroperoxyflavin intermediate (60). Electrophilic attack from the hydroperoxy group to the aromatic ring of kynurenine, followed by electron transfer, deprotonation of the cationic substrate (61) and protonation of isoalloxazine anionic group leads to (62). This then undergoes dehydration of the hydroxyflavin moiety and liberation of 3-hydroxykynurenine. A new molecule of kynurenine binds to the enzyme and the catalytic cycle is completed when the enzyme / kynurenine complex (63) reacts with NADPH reducing the flavin and liberating $NADP^+$.

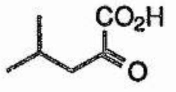


Scheme 1.16

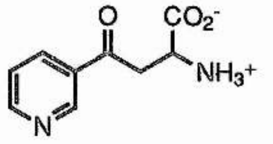
1.2.5.1 Inhibitors of kynurenine-3-hydroxylase.

Kynurenine-3-hydroxylase from *Saccharomyces carlsbergensis* is inhibited by the α-oxoacids of branched chain amino acids. In particular, α-oxo-isocaproate (64), derived from leucine, is a competitive inhibitor with respect to both kynurenine (K_I = 4.2 mM) and NADPH (K_I = 8.3 mM).[13]

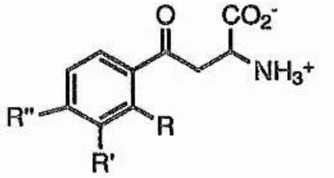
Pellicciari *et al.*[52, 53] have carried out the synthesis of nicotinylalanine (65), *o*-nitrobenzoyl-(66), *m*-nitrobenzoyl-(67), *p*-nitrobenzoyl-alanine (68), all analogues of kynurenine, and reported their evaluation as inhibitors of kynureninase and kynurenine-3-hydroxylase. Nicotinylalanine with an IC_{50} of 800 ± 120 μM for kynureninase and 900 ± 180 μM for kynurenine-3-hydroxylase, showed similar but weak activities toward both enzymes. Of the nitro analogues, *m*-nitrobenzoylalanine, with an IC_{50} of 100 ± 12 μM for kynureninase and 0.9 ± 0.1 μM for kynurenine-3-hydroxylase, proved to be a potent and selective inhibitor for the latter enzyme.



(64)



(65)



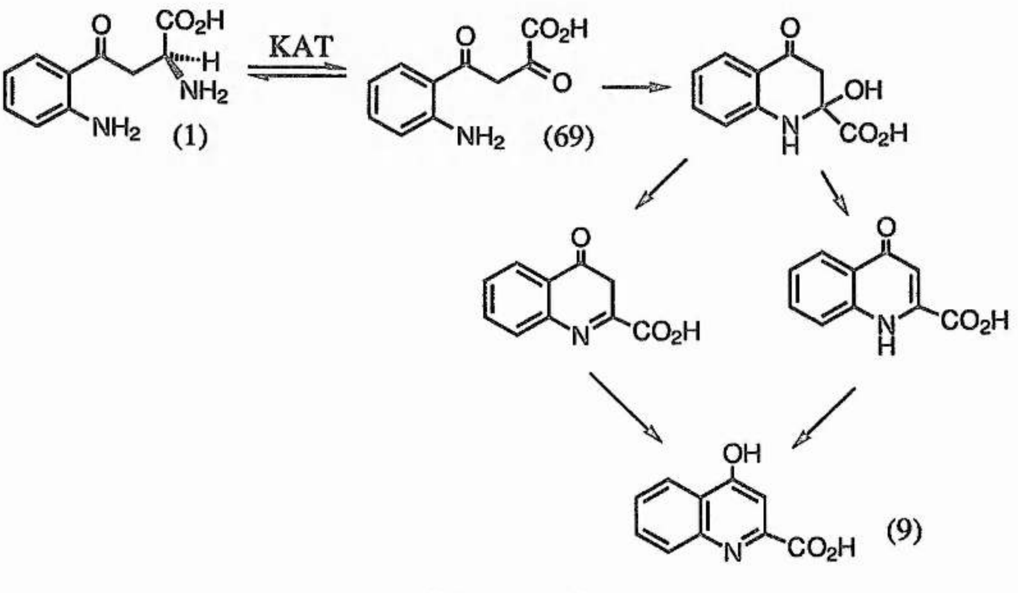
(66) *o*- R= NO_2, R'= H, R"= H
(67) *m*- R= H, R'= NO_2, R"= H
(68) *p*- R= H, R'= H, R"= NO_2

In order to account for the selectivity observed molecular modelling studies were carried out with the three nitro analogues. In *m*-nitrobenzoylalanine one of the oxygen atoms of the nitro group was shown to mimic closely the oxygen atom of molecular oxygen involved in the hydrogen bonding network, thus accounting for the potent inhibition observed.

As a continuation of this work Pellicciari *et al.* reported an enantioselective synthesis of (2S)-*m*-(nitrobenzoyl)-alanine along with its inhibition of kynureninase and kynurenine 3-hydroxylase.[54] *2S-m*-Nitrobenzoylalanine with an IC_{50} of 0.5 μM proved to be the active constituent of the previously reported racemic mixture.

1.2.6 Kynurenine Aminotransferase.

Kynurenine aminotransferase (KAT, EC 2.6.1.7) catalyses the transamination of kynurenine. This reaction is irreversible as the transamination product (69) undergoes cyclisation, dehydration and tautomerisation to give kynurenic acid (9), which is a stable aromatic product (Scheme 1.17).[55]



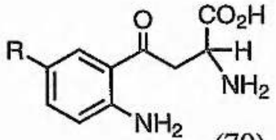
Scheme 1.17

Kynurenic acid is in fact an antagonist at the glycine site of N-methyl-D-aspartate receptors and increased levels of kynurenic acid may exert a neuroprotective action in some pathological conditions (See **1.3 NEUROLOGICAL IMPORTANCE OF KYNURENINES**). Since several PLP dependent aminotransferases with broad substrate specificity are able to catalyse this reaction, the identification of the isoenzyme responsible for the biosynthesis of cerebral kynurenic acid is of importance for clarifying the role of this metabolic pathway in pathophysiology.

The enzyme has been isolated from various sources including yeasts, bacteria and mammals. Takeuchi *et al.* isolated an enzyme from rat liver mitochondria which was found to have identical elution patterns on all column chromatographies to α-aminoadipate aminotransferase (EC 2.6.1.39).[56] Alberti-Giani *et al.* and Mosca *et al.* have found a single enzyme which accounts for both KAT and cysteine conjugate β-lyase (also known as, glutamine transaminase K, GTK, EC 2.6.1.64) activities in rat kidney cytosol.[57-59] Buckberry reports a similar enzyme in human kidney.[60] Malherbe *et al.* have isolated an enzyme in rat brain which is identical to the soluble form but also carries an additional stretch of thirty two amino acids at its NH_2 terminus. Several structural features of this sequence resemble those of leader peptides for mitochondrial import.[61] By using physiological concentrations of the substrate kynurenine and by determining the irreversible conversion of $[^3H]$-kynurenine to $[^3H]$-kynurenic acid as a measure of KAT activity, a novel, simple and sensitive assay was developed which permitted the detailed characterisation of the enzyme.[62] A single enzyme is responsible for kynurenic acid production in the rat brain and is found mainly in mitochondria. This is in contrast to the human brain which contains two distinct KAT proteins, KAT 1 and KAT II, capable of kynurenic acid biosynthesis.[63] Schwarcz *et al.* have confirmed the characterisation by Baran using $[^3H]$-kynurenine as a substrate.[64] KAT I was found to be a dimer consisting of two identical subunits of approximately 60 kDa. Kinetic analyses of the pure enzyme revealed an absolute K_M of 2.0 mM and 10.0 mM for 2*S*-kynurenine and pyruvate respectively. Unlike KAT II activity, which displays a shallow pH optimum in the physiological range and does not appear to be inhibited by common amino acids, KAT I has a sharp pH optimum at pH 9.5-10 and is potentially blocked by glutamine, tryptophan or phenylalanine. This has led to the conclusion that KAT II may be preferentially responsible for the production of kynurenic acid under physiological conditions, whereas KAT I may become of particular importance in pathological situations.

1.2.6.1 Inhibitors of kynurenine aminotransferase.

Aminooxyacetic acid, a non specific transaminase inhibitor blocked KAT activity, from rat brain, with an apparent K_I of 5 μM.[62] Amphetamine is a competitive inhibitor of kynurenine aminotransferase and also kynureninase. It has been suggested that the inhibitory effect was due to the formation of a Schiff's base with pyridoxal phosphate, causing a reduction in the level of free pyridoxal phosphate, which is a co-factor for both enzymes.[65] Schwarcz *et al.* reported the synthesis of a number of substituted kynurenines, both racemic and enantiomers (70) and their testing with a partially purified enzyme from rat brain.[66, 67]



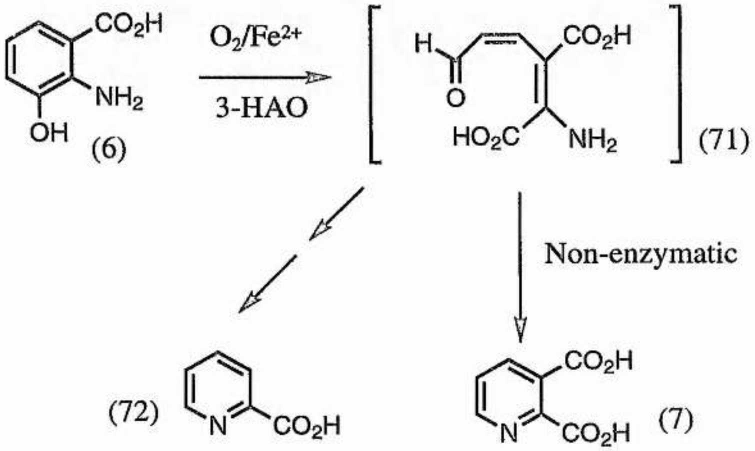
(70)

R= Cl, Br, cyclohexyl,
Me, Et, ^{n}Pr, ^{i}Pr

The most potent of these was 5-chloro-kynurenine with most of the activity residing in the *2S* enantiomer. Kinetic analysis gave a K_I for the *2S* enantiomer of 5.4 μM (K_M for kynurenine is 28 μM). Reduction or elimination of the keto group or decarboxylation of the amino acid moiety yielded compounds which were completely inactive as enzyme inhibitors. This could mean that a H-bond acceptor residue in the enzyme's active site is important. It was also shown that chlorination in positions 3 and 4 produced compounds with a relatively low inhibitory effect, therefore the existence of a lipophilic pocket in the active site of the enzyme able to bind the substituent at the 5-position of kynurenine, is likely to play a role in enzyme inhibition.

1.2.7 3-Hydroxyanthranilic Acid Dioxygenase.

3-Hydroxyanthranilic acid-3,4-dioxygenase (3HAO, EC 1.13.11.6) is a monomeric cytosolic protein belonging to the family of intramolecular dioxygenases containing non-haem ferrous iron. 3HAO catalyses the cleavage of the benzene ring of 3-hydroxyanthranilic acid (6). The product of the reaction is an unstable intermediate, α-amino-β-carboxymuconic acid ω-semialdehyde (71) which can either spontaneously rearrange to quinolinic acid (7) or be converted to picolinic acid (72) after decarboxylation (Scheme 1.18).[68] The rate limiting step in its spontaneous disappearance is probably isomerisation of the double bond to place the carboxyl groups in the *cis* position. This would be followed by a rapid condensation to form the stable pyridine ring. The enzyme that converts the intermediate to picolinic acid is much slower in its activity so the bulk of the intermediate goes spontaneously to quinolinic acid.



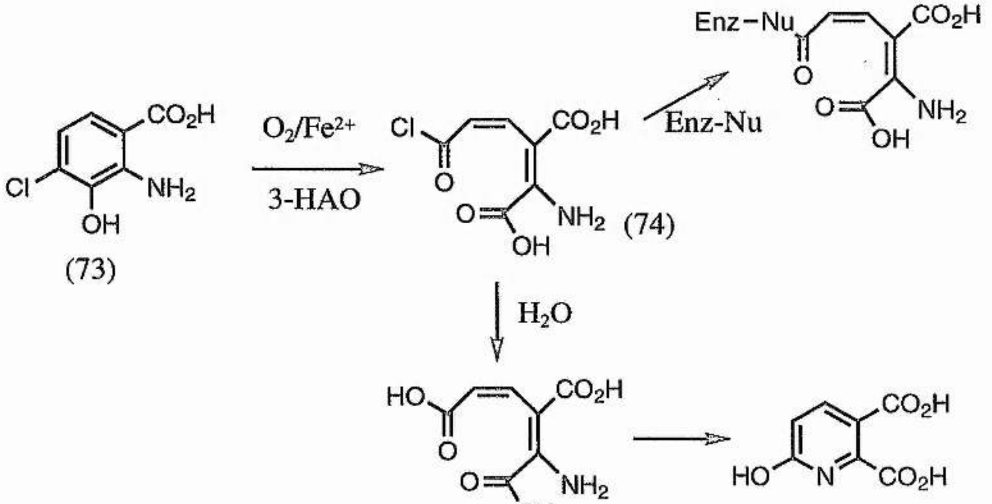
Scheme 1.18

The reaction has been followed spectroscopically by Bokman *et al.* An absorption maximum occured at 360 nm with the simultaneous disappearance of 3-hydroxyanthranilate (absorption maximum 315-320 nm). The product was then observed from the appearance of an absorption maximum at 268 nm, which indicated that the metabolism of 3-hydroxyanthranilate resulted in the formation of an intermediate before quinolinic acid was formed.[69] Conversion of the intermediate to quinolinic acid was proposed to be spontaneous as theoretical amounts of quinolinic acid were produced from decomposition of the intermediate in aqueous or ethanolic solutions after removal of the protein with 83-92% ethanol.[70]

3HAO is widely distributed in peripheral organs such as liver and kidney and is also present in small amounts in the brain where the enzyme is localised in astrocytes.[71] The enzyme has been isolated from rat liver, rat brain [72] and a human enzyme has been cloned.[73] The human enzyme is a protein with 286 amino acid residues and a molecular mass of approximately 32 kDa. The enzyme has a K_M for 3-hydroxyanthranilic acid of approximately 2 μM.

1.2.7.1 Inhibitiors of 3-Hydroxyanthranilic acid Dioxygenase.

Using either liver homogenate or purified 3HAO 4-chloro-3-hydroxyanthranilic acid (73) was found to inhibit enzyme activity. Initially it was thought that the inhibition occurred when the 4-chloro-3-hydroxyanthranilic acid was oxidised by the enzyme to produce an electrophilic product (74) that can react with an active site nucleophile and irreversibly inhibit 3HAO (Scheme 1.19).[74]



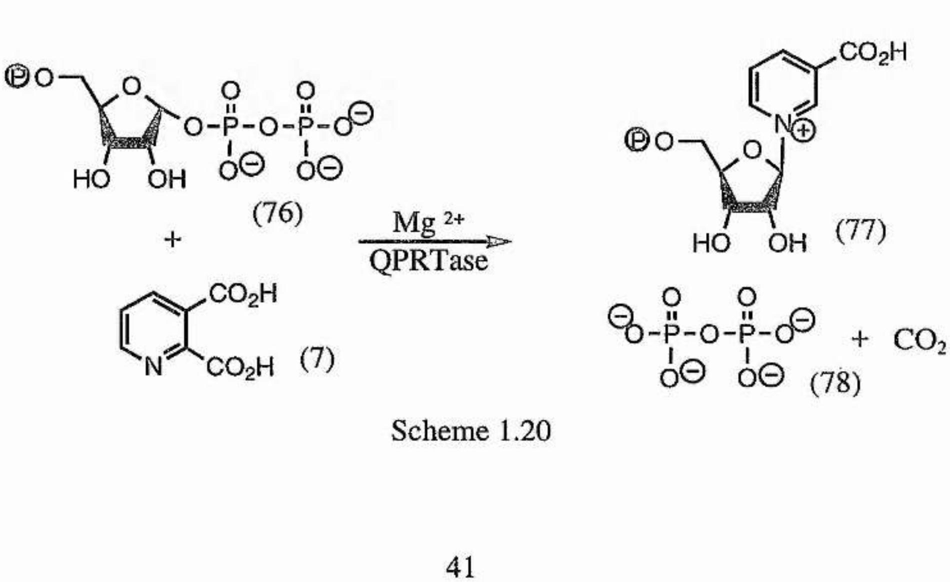
Scheme 1.19

In vitro experiments indicated that this binding resulted in non-competitive inhibition of the enzyme. It is difficult to distinguish between competitive and non-competitive enzyme inhibition when dealing with drugs which bind very tightly to the protein and also difficult to differentiate between reversible and irreversible inhibitors. Walsh *et al.* who also tested the fluoro- and bromo-derivatives actually detected reversibility after warming the complexes at 37 °C. This result was confirmed when the physical association between enzyme and inhibitors was examined by dialysis.[75]

Further examination showed the inhibition was in fact competitive in nature and gave apparent K_I values of 190 nM, 6 nM and 4 nM for the fluoro-, chloro- and bromo- derivatives respectively. Since all three inhibitors are substrate analogues they may be metabolised in an identical manner as 3-hydroxyanthranilic acid itself and yield 6-hydroxyquinolinic acid (75), although this has not been identified.[75]

1.2.8 Quinolinate Phosphoribosyl transferase (QPRTase)

Quinolinate phosphoribosyl transferase (QPRTase, EC 2.4.2.19) is an enzyme of the *de novo* NAD biosynthetic pathway. The enzyme catalyses the reaction between quinolinic acid (7) and 5-phosphoribosyl-1-pyrophosphate (76, PRPP) in the presence of Mg^{2+} to give nicotinic acid mononucleotide (77), pyrophosphate (78) and CO_2 (Scheme 1.20).[76]

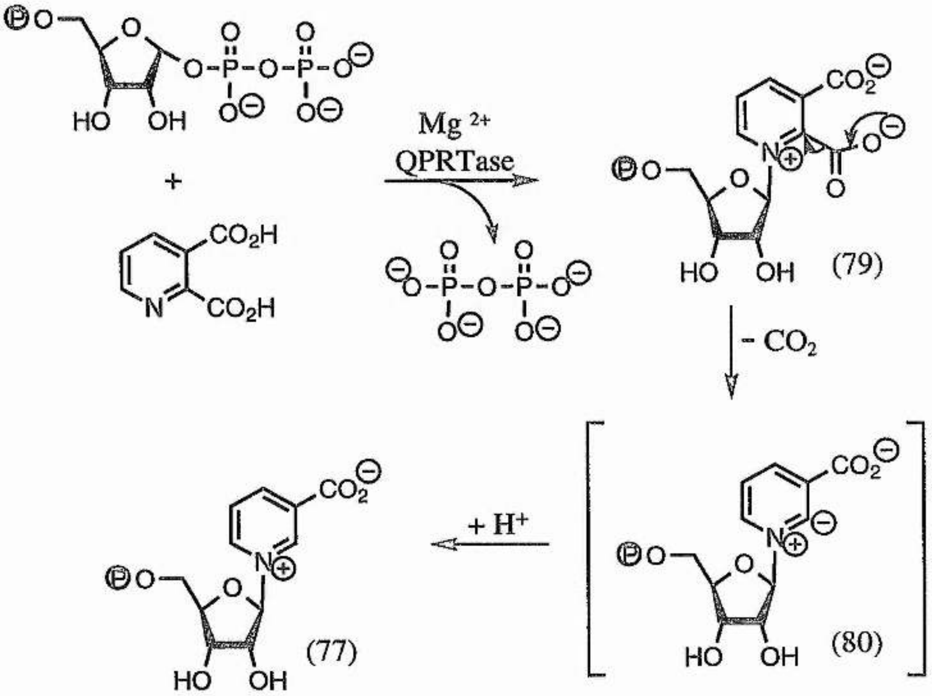


Scheme 1.20

Although there are at least three different pathways to the production of quinolinic acid in various organisms, the formation of nicotinic acid mononucleotide and its subsequent conversion to NAD follows the same sequence of biosynthetic steps in all biological systems including micro-organisms, plants and animals.

The enzyme has been isolated from several sources including *E. coli*,[76, 77] a soil pseudomonad,[78] *Alcaligens eutrophus*,[79] pig kidney[80] and pig liver.[81] The enzymes have a varying number of subunits depending on the source, although the monomer molecular weight is 32 to 35 kDa. Although Mn^{2+} can be used by the enzyme Mg^{2+} is the preferred metal ion. The enzyme is specific for quinolinate and no reaction with nicotinate is detectable.

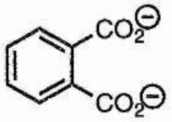
The chemical mechanism of decarboxylation and phosphoribosyl transfer is unknown. In terms of the phosphoribosyl transfer the PRPP and quinolinic acid may both bind simultaneously giving a ternary complex and the transfer reaction then takes place in one step.[78] Alternatively, there could be a two step process where PRPP binds first and reaction takes place to eliminate pyrophosphate and leave a reactive phosphoribosyl intermediate at the active site. The quinolinic acid then binds and reacts with this intermediate to give the mononucleotide (79). From their kinetic studies Bhatia and Calvo have shown that there is an ordered binding mechanism where the magnesium ion complex of PRPP binds first followed by quinolinate. Based on inhibitor studies Bhatia and Calvo have therefore proposed a two step reaction for the enzyme in which quinolinic acid displaces pyrophosphate from PRPP to form quinolinic acid mononucleotide (79).[77] This intermediate then undergoes decarboxylation to yield a nitrogen ylid intermediate (80) that is protonated to give the final product (77) (Scheme 1.21). This type of mechanism has been proposed for orotidine-5'-monophosphate.[82] However, on the metabolism of orotic acid the sequence of phosphoribosyl transferase and decarboxylation is catalysed by two separate enzymes, whereas for quinolinic acid only a single protein, QPRTase, is involved.

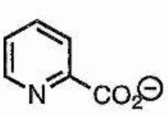


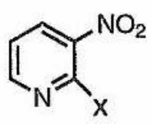
Scheme 1.21

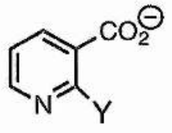
1.2.8.1 Inhibitors of QPRTase.

Phthalic acid (81) was a good inhibitor for QPRTase from a variety of sources.[79, 83] Picolinic acid (82) on the other hand is a potent inhibitor of enzymes from some sources[79] but a poor inhibitor of enzymes from others.[83] With the enzyme from *Alcaligens eutrophus* both picolinic and phthalic acid showed competitive inhibition. Picolinic acid methyl ester and phthalic acid butyl ester did not have much inhibitory effect. These results suggest that the 2-carboxyl group of quinolinic acid plays a very important role in the binding of substrate to the active site of the enzyme.[79]


(81)


(82)


X = SH, OH
(83)


Y = SH, OH
(84)

Kalikin and Calvo tested the activity of some quinolinic acid analogues (83, 84).[84] The 2-hydroxy (and 2-mercapto) pyridines exist mostly in a tautomeric form where the oxygen (or sulphur) is present as a carbonyl and the ring is really an unsaturated lactam (or thiolactam). All the compounds are inhibitors of the enzyme with the 3-nitro compounds (83) being the most potent. The general effect of the nitro substituent at the 3-position of pyridine is to increase the amount of hydroxyl or thiol form relative to lactam or thiolactam. Therefore, the degree of inhibition can be correlated with the degree of ionisation of the group at C-2, implying that a negative charge is an important contributor to tight binding of the enzyme. The pH dependence of the inhibition also supports this hypothesis. Kalikin and Calvo suggested that the inhibition observed by these pyridines with tautomeric thiolates or alcoholates at the 2-position might be due to the fact that these compounds resemble the intermediate ylid that was formed immediately following decarboxylation. However, on the substrate the charge is two atoms away from the aromatic ring and the ylid has the charge on the ring. The inhibitors have the charge one atom away from the ring so could equally well mimic the substrate and not the ylid.

1.3 NEUROLOGICAL IMPORTANCE OF KYNURENINES.

In recent years there has been a resurgence of interest in the kynurenine pathway due to its neurological importance. Two metabolites of tryptophan, quinolinic acid (7) and kynurenic acid (8) have pronounced affects on neuronal activity. Quinolinic acid is a selective agonist for the N-methyl-D-aspartate (NMDA) population of receptors. Conversely kynurenic acid is a selective antagonist, at low concentrations, at the strychnine-resistant glycine modulatory site associated with the NMDA receptor[85] and may serve as the endogenous neuroprotective antidote to quinolinic acid.[86] Thus the balance between these two metabolites may be very important in maintaining the activity of the NMDA receptor.

Of the kynurenines, quinolinic acid may be the most relevant to neurodegenerative diseases, since the dicarboxylic acid has been implicated in the aetiology of a number of neurologic disorders, including Huntington's disease, temporal lobe epilepsy, glutaric aciduria, hepatic encephalopathy and coma.[87] Neurotoxicity induced by quinolinic acid occurs preferentially in the neocortex, striatum and hippocampus, sites in the brain particularly affected by these disorders.

Early studies of the biochemistry of the kynurenines were concerned primarily with metabolism in peripheral tissues. Although tryptophan-2,3-dioxygenase activity was reported in brain, it was not until 1977[8] that the first reports appeared of the definitive presence of any products of this pathway in the CNS. Since then, several of the key enzymes of the kynurenine pathway, including indoleamine 2,3-dioxygenase, kynureninase, kynurenine hydroxylase, kynurenine aminotransferase, 3-hydroxyanthranilic oxygenase and quinolinate phosphoribosyltransferase have been demonstrated to occur in brain tissue.[88] Using the incorporation of tritium label into various tryptophan metabolites Guidetti *et al.* provided evidence for a functional kynurenine pathway in the normal rat brain and for an increase in metabolism in lesioned tissue.[89] Using radiolabelled tryptophan it can be seen that 40% of the 2*S*-kynurenine detected within the CNS is synthesised in that tissue. This implies that 60% is derived by uptake from plasma kynurenines, synthesised in peripheral tissues.[90] It has been shown by Schwarcz *et al.* that 2*S*-kynurenine is readily taken up into brain by the large neutral amino acid carrier of the blood brain barrier.[91] The same carrier may also mediate the brain uptake of 3-hydroxykynurenine. They also showed that for 3-hydroxyanthranilic acid, anthranilic acid, kynurenic acid and quinolinic acid uptake appeared to be mediated by passive diffusion. This occurred at a significant rate for anthranilic acid and at far lower rates for the other metabolites.

1.3.1 Quinolinic acid.

Quinolinic acid can cause neurological damage by overstimulation of the NMDA receptor and it has been shown to be capable of discriminating between the NMDA receptor subtypes.[92] In rat brain this results in a selective pattern of nerve cell death, the neuropathology of which is very similar to that found in human diseases such as Huntington's disease and temporal lobe epilepsy.[93] Therefore increased endogenous levels of quinolinic acid have been proposed to be an important etiological factor in these diseases. Recently, it has been demonstrated, using newly developed analytical procedures and labelling studies,[94] that [$^{13}C_6$] kynurenic acid is synthesised from *2S*-[$^{13}C_6$] tryptophan *in vivo*.[95]

It is interesting to note that the maximal enzymatic rate for 3-hydroxyanthranilate oxygenase is approximately 100 fold higher than that for QPRTase, although the Michaelis Menten constant is about the same. Thus an increased flux of tryptophan into the kynurenine pathway could increase levels of quinolinic acid which QPRTase might be unable to remove. The levels of tryptophan are precisely regulated, human serum albumin binds tryptophan and its availability to cross the blood-brain barrier is determined by the amount of plasma free tryptophan. In addition, it is controlled by a specific large neutral amino acid transport system.

An excess of the tryptophan metabolite quinolinic acid in the brain has been hypothetically related to the pathogenesis of Huntington's disease.[96, 97] Other recent studies show conflicting evidence[98, 99] and it has been suggested that there is a decrease in the amount of kynurenic acid rather than an increase in quinolinic acid.[100] The activity of 3-hydroxyanthranilate oxygenase has also been observed to be increased in Huntington's disease brains as compared to control brains.[97]

In epileptic human brain tissue Feldblum *et al.* showed a reduction of QPRTase activity which accounts for the pathological accumulation of quinolinic acid as it is not degraded.[101]

High levels of quinolinic acid have also been observed in the brains of patients with various inflammatory neurological diseases including AIDS,[102] lyme disease[8] and poliovirus.[103] Up to two thirds of AIDS patients suffer from neurological problems including dysfunction of cognition movement and sensation.[102] Some very detailed studies have shown that the degree of damage may be correlated with the level of quinolinic acid.[104] It seems likely that there is a general increase in kynurenine pathway activity which accounts for the observed increase in 3-hydroxykynurenine and 3-hydroxykynureninase activity.[105] It has also been postulated that the HIV virus activates macrophage cells of the immune system to biosynthesise the quinolinic acid.[102, 104]

Studies have also shown that infection and immune activation are associated with accumulations of quinolinic acid. Heyes *et al.* examined the cerebrospinal fluid quinolinic acid concentrations in children with infections of the central nervous system, hydrocephalus, tumors or haemorrhage.[106] Extremely high concentrations of quinolinic acid were found in patients with bacterial infections of the central nervous system. These high concentrations are attributed to increased metabolism of 2*S*-tryptophan through the kynurenine pathway.[107] 2*S*-Kynurenine levels increased in parallel to the accumulation of quinolinic acid, which is consistent with increased activity of indoleamine-2,3-dioxygenase.[106] Heyes *et al.* concluded that γ-interferon is an important mediator between immune stimulation and indoleamine-2,3-dioxygenase induction following CNS inflammation.[108]

In the human brain 3-hydroxyanthranilic oxygenase and quinolinic acid phosphoribosyltransferase (QPRTase) are located in morphologically distinct compartments in the brain. In view of this differential localisation, the conclusion seems inescapable that quinolinic acid must be synthesised in one population of glial cells and must presumably exit those cells to be metabolised by QPRTase in a separate population of QPRTase containing glial cells and neurones. This raises the interesting possibility that quinolinic acid must exist in the extracellular fluid and under some circumstances may accumulate sufficiently to activate or otherwise modify, for example, by potentiation or desensitisation, the NMDA sensitive population of glutamate receptors.

The striatum at least does not appear to possess mechanisms either for the rapid removal of quinolinic acid or for its metabolic degradation in the extracellular space by significant QPRTase or other metabolising enzymes. On the basis of this lack of uptake and extracellular metabolism, it therefore seems unlikely that quinolinic acid is a neurotransmitter in the classical sense.[109]

Interestingly, quinolinic acid has recently been shown to increase permeability of the blood-brain barrier. Intracerebroventricular injections of quinolinic acid increased the penetration of magnesium from plasma into brain. It is not clear whether this effect involved activation of NMDA receptors, a secondary change of extracellular osmolarity or pH effects.[8]

1.3.2 Kynurenic acid.

2S-Kynurenine can cross the blood-brain barrier as a result of transport by the large neutral amino acid carrier.[110, 111] Kynurenic acid, on the other hand has been considered essentially unable to cross the barrier in normal animals. The latter is important because in many of the pathological states in which kynurenines may be implicated (encephalopathies, AIDS-related brain damage etc.) there is likely to be a significant weakening of the barrier's integrity. The barrier is known to be compromised, for example, in Alzheimer's dementia, raising the possibility that enhanced penetration by kynurenic acid may occur in such conditions.

Most of the normal extracellular kynurenic acid in brain is probably of glial origin.[112] Since release of kynurenic acid into the extracellular medium persisted in the absence of external calcium, it is probably the result of diffusional efflux rather than an active exocytotic mechanism.[91]

It has been argued that quinolinic acid and kynurenic acid cannot be of physiological or pathological relevance because their normal extracellular concentrations are in the nanomolar range, at least three orders of magnitude lower than those required to act at NMDA receptors.[113] However one possibility is that both quinolinic acid and kynurenic acid may be present in very high concentrations locally at some sites in the brain that cannot be reflected in mean extracellular levels. A second possibility is that there may be a subpopulation of NMDA receptors that possess a glycine modulatory site with a much higher sensitivity to kynurenic acid making them more susceptible to fluctuations of endogenous kynurenic acid levels.[8]

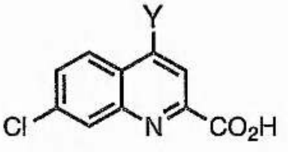
1.3.3 NMDA Receptors.

The NMDA sensitive population of receptors is involved not only with the process of synaptic transmission in many parts of the CNS but also in long term aspects of neuronal activity. NMDA receptors are also involved in the plastic processes concerned in the targeting of neurones and synapses and their adjustment during development, especially in the visual system of vertebrates and invertebrates. They are also involved in the determination of cell viability and can activate destructive enzymes such as phospholipases, proteases and protein kinases. This may be of importance in explaining some cell death occurring with ageing and loss of neurones that occur in dementing disorders such as Alzheimer's disease. It may also explain related neurodegenerative disorders such as Huntington's disease and epilepsy as well as the widespread neuronal damage that results from cerebral infarctions.[85]

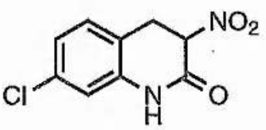
1.4 MODULATION OF THE KYNURENINE PATHWAY.

Further information regarding the regulation of this metabolic pathway is required to fully elucidate the neurological roles of the various metabolites. Two main strategies aimed at favourably altering the kynurenic and quinolinic acid balance have so far been followed.

The first strategy focused on the development of more selective and potent kynurenic acid analogues. Leeson *et al.* investigated substituents at the 5, 7 and 5 and 7 positions of kynurenic acids and evaluated their *in vivo* antagonist activity at the glycine modulatory site of NMDA.[114, 115] 7-Chloro kynurenic acid (85) had the greatest increase in antagonist activity over kynurenic acid itself. Moroni *et al.* showed similar activity with the thiokynurenate (86).[116] Further studies [117, 118] showed that the 3-nitro-3,4-dihydro-2(1*H*)-quinolone (87) is the most potent combined glycine / NMDA-AMPA antagonist yet reported, *in vivo*, and may prove to be a useful pharmacological tool.

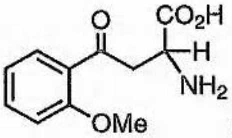


Y = OH (85)
Y = SH (86)



(87)

The second strategy is aimed either at blocking quinolinic acid production or at increasing the kynurenic acid production by inhibiting key enzymes of the kynurenine pathway. Kynurenine loading has been shown to increase kynurenic acid levels and has been shown to exert antiepileptic effects.[119] Inhibition of kynureninase should prevent quinolinic acid synthesis and accumulation, as should inhibition of kynurenine hydroxylase. Nicotinylalanine, an inhibitor of kynureninase and kynurenine hydroxylase, has been shown to increase the cerebral kynurenic acid content in rat brain.[120] It has also been shown to inhibit the accumulation of quinolinic acid, following the administration of bacterial endotoxins.[121] Naritsin *et al.* found that 4-chloro-3-hydroxyanthranilate (73), an inhibitor of 3-hydroxyanthranilate-3,4-dioxygenase, decreased the accumulation of quinolinic acid in the brain following immune activation. This was accompanied by an increase in kynurenic acid concentration and a decrease in kynurenine concentration. On the other hand, 6-chloro-2*S*-tryptophan decreased the formation of quinolinic acid, but also decreased the formation of kynurenine and kynurenic acid.[95] 6-Chloro-2*S*-tryptophan may be an effective pro drug for the delivery of 7-chloro-kynurenic acid (see above) as it is a substrate for indoleamine-2,3-dioxygenase and the 4-chlorokynurenine produced is a substrate for kynurenine aminotransferase. Chiarugi *et al.* have compared the inhibitory properties of *m*-nitrobenzoylalanine (67) as an inhibitor of kynurenine hydroxylase and *o*-methoxybenzoylalanine (88) as an inhibitor of kynureninase.[122]



(88)

It has been shown that inhibiting either of these enzymes results in an increase in kynurenic acid in the brain. In addition, kynurenine hydroxylase inhibition resulted in a larger increase of kynurenine and kynurenic acid in the brain of rats than kynureninase inhibition. However a selective inhibition of kynureninase may result in the accumulation of 3-hydroxykynurenine and there is evidence from studies in cell culture that this is also neurotoxic.[113] This finding has not as yet been duplicated *in vivo*, however, and 3-hydroxykynurenine toxicity seems to be related to the generation of free radicals rather than a direct action on excitatory amino acid receptors. Thus 3-hydroxykynurenine may play a role in brain pathology without functioning as a neuroactive brain metabolite under physiological conditions.

It can thus be seen that there is still a need for further research into the mechanisms of the enzymes to enable more active and selective inhibitors to be developed. Such compounds may be employed to investigate in greater detail the neurological consequences of inhibiting the tryptophan pathway. Eventually, this may then lead to the production of drugs suitable for the treatment of these neurological disorders.

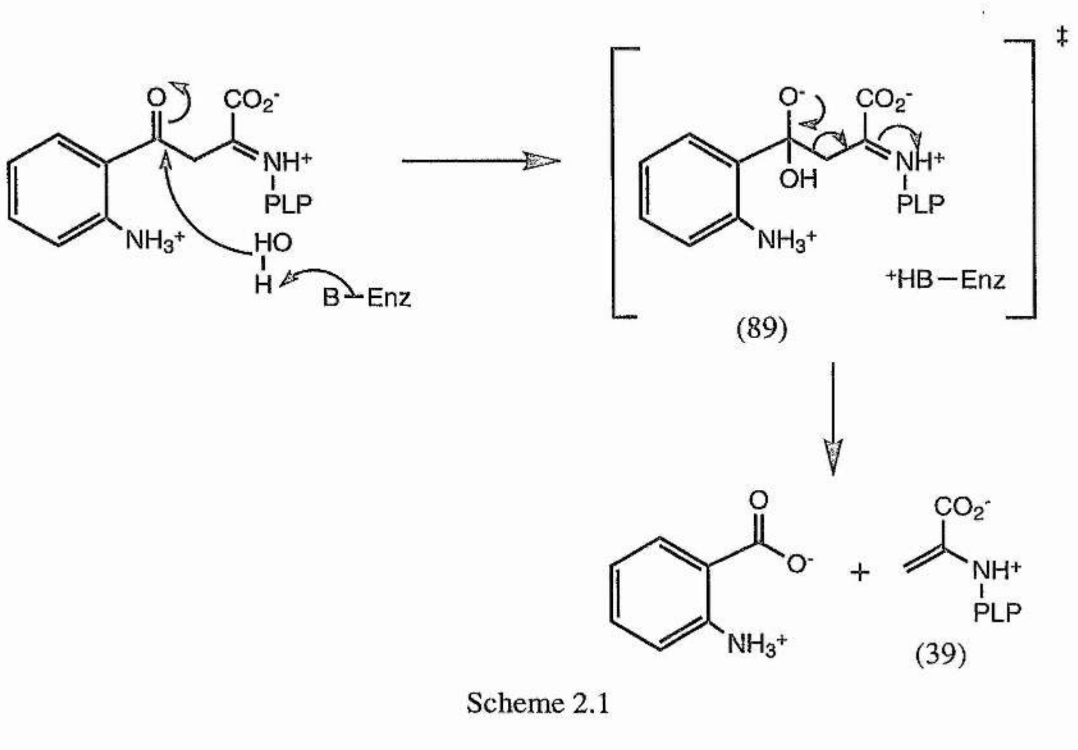
CHAPTER 2

2. INVESTIGATION OF HYDROLYSIS REACTION AND DESIGN OF INHIBITORS.

As discussed in the introduction (1.2.3.4 Proposed mechanism) one of the major questions concerning the chemical mechanism of kynureninase is the mode of the hydrolysis reaction. This could be mediated by a water molecule bound at the active site or by an enzymic nucleophile. One way to probe this part of the reaction is to use novel inhibitors of kynureninase which are designed by making assumptions concerning the mechanism. The relative efficiencies of the inhibitors will then provide circumstantial evidence concerning the mechanism.

2.1 THE BOUND WATER MEDIATED MECHANISM.

The first possible mechanism involves a water molecule bound at the active site. General base catalysis, by an active site amino group, then generates hydroxide ion which attacks at the γ-carbonyl group of the substrate. This mechanism is given in Scheme 2.1.



Scheme 2.1

Such a reaction must proceed *via* a tetrahedral intermediate (89), which then breaks down to give an α-aminoacrylate derivative (39) and anthranilate.

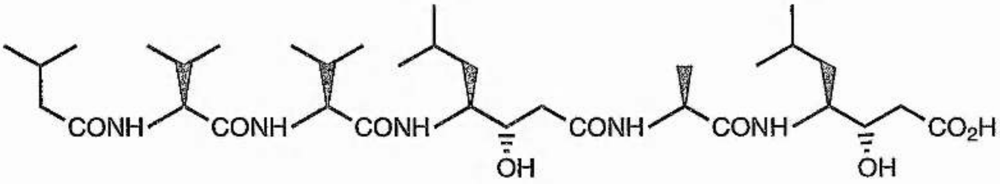
A tetrahedral intermediate is a high energy species on the reaction co-ordinate pathway and as a result, it can be used as a model for the transition state structure. Designing a stable analogue of this structure provides a useful method for designing inhibitors for the enzyme. Indeed if such an inhibitor bound tightly to kynureninase it would be evidence that such a bound water mechanism was in operation. There is already some evidence from stereochemical studies that implies that a bound water mediated reaction is most likely. Also in agreement are the inhibitory properties of the dihydro-2*S*-kynurenines (30). Both dihydro-2*S*-kynurenines are similar in structure to this proposed tetrahedral intermediate. As this intermediate is a model of the transition state, the potent competitive inhibition observed by these compounds suggests that they may also act as transition state analogues.[36]

2.2 TRANSITION STATE ANALOGUES.

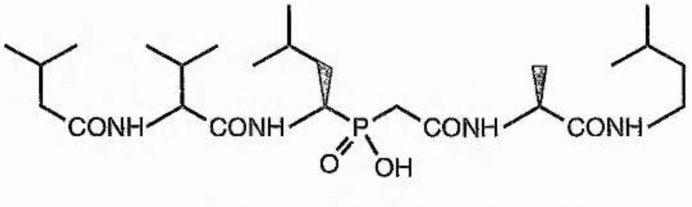
The concept of transition state mimics has been particularly successful for the design of potent enzyme inhibitors for peptidase enzymes.[123, 124] The rationale for this approach is the recognition that additional, favourable binding interactions develop between an enzyme and the substrate as their complex approaches its transition-state conformation. Such extra binding interactions, which are not available in the ground-state complex, assist in lowering the energy of activation of the reaction and therefore are an important mechanism for enzymatic rate enhancement. Transition-state analogue inhibitors seek to take advantage of these extra binding interactions by incorporating key structural elements of the unstable, transition-state structure of the substrate in the stable structure of the inhibitor.

For the zinc and aspartic peptidases a number of effective strategies have been developed based on the ability of phosphorous containing amino acid analogues to mimic the unstable tetrahedral intermediates involved in peptide hydrolysis.[125]

For the aspartic peptidases incorporation of a tetrahedral species into an oligopeptide, as a mimic of the transition state has led to effective inhibitors. The most potent inhibitors for the aspartic peptidases have been analogues of the naturally occurring pepsin inhibitor pepstatin (90) in which the unusual amino acid statine occupies the P_1 and P_1' positions and presents a secondary hydroxyl group to the catalytic residues in the enzyme active site. Bartlett and Kezer synthesised and evaluated a number of 'phosphastatine' derivatives (91) as pepsin inhibitors.[126] The 2*S*-derivative of Iva-Val-Sta^P-Ala-Iaa shows very high affinity and is a slow binding inhibitor of the aspartic peptidase pepsin with a K_I of <0.07 nM.

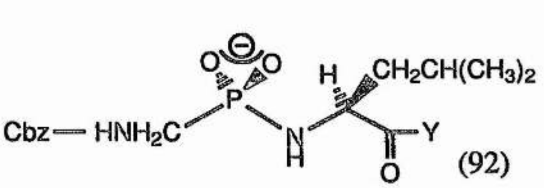


Iva-Val-Val-2*S*-Sta-Ala-2*S*-Sta (90)



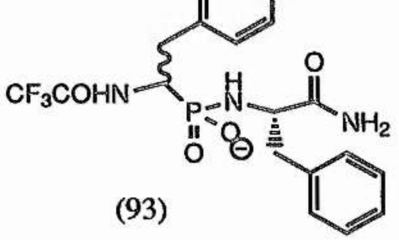
Iva-Val-2*S*-Sta^P-Ala-Iaa (91)

Bartlett and Marlowe have also synthesised a series of phosphonamidate peptide derivatives (92) as transition state analogues for the zinc peptidase thermolysin. The most potent inhibitor, where Y= Leu, had a K_I of 9.1 nM.[123]



(92)

A number of HIV-1 (human immunodeficiency virus type-1) proteinase inhibitors have also been studied. The HIV-1 proteinase is a member of the family of aspartyl retroviral proteinases and plays an essential role in viral replication. One strategy to inhibit the proteinase has been replacement of the scissile peptide bond at the substrate cleavage site with non-hydrolysable transition state analogue moieties. McLeod *et al.* synthesised some phosphonamidate dipeptide analogues (93, 94) which showed modest potency as inhibitors with K_I values of 45 μM and 52 μM respectively.[127]



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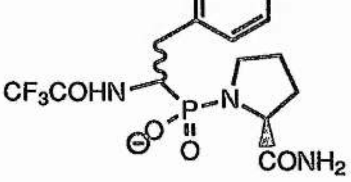
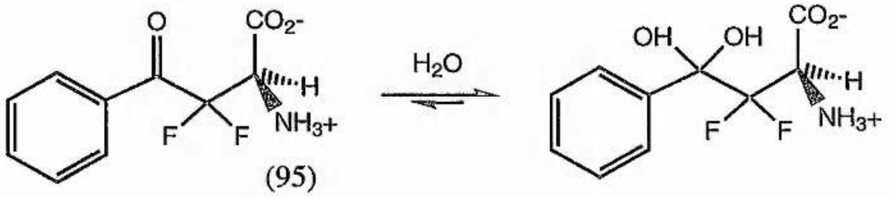


(94)

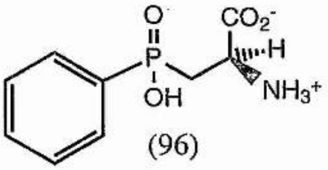
2.2.1 Design of Proposed Transition State Analogue.

Prior to our work McCarthy *et al.* had devised convenient synthetic access to 2,2-difluoro-2-benzoyl-alanine (95)[128] as a potential new inhibitor of kynureninase. Fluoroketone containing peptides are proteolytic enzyme inhibitors. These compounds form stable hydrates or hemiketals which are thought to inhibit proteolytic enzymes by mimicking the tetrahedral transition state of the amide bond cleavage. On the basis of this precedent, the difluoro analogue is an attractive target however, as yet, there are no biological data.



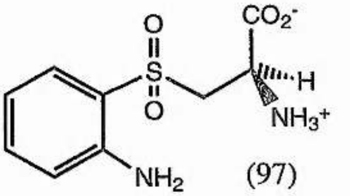
(95)

However it was decided to employ the phosphorous based approach to transition state mimics. The target compound was thus the phosphinic acid (96). This has the required tetrahedral arrangement of groups, but should be chemically stable. It, like the transition state, has two oxygens present and should show similar hydrogen bonding. The P-O bond length is midway between the C=O bond length and the C-O bond length. The transition state bonds are thought to be of a similar intermediate length, in which only partial bonding has developed between the carbon atom and the incoming water molecule. Therefore the phosphinic acid should be a very good mimic. If this compound acts as a transition state mimic it should bind very tightly to the active site. This would be an example of competitive reversible inhibition.



(96)

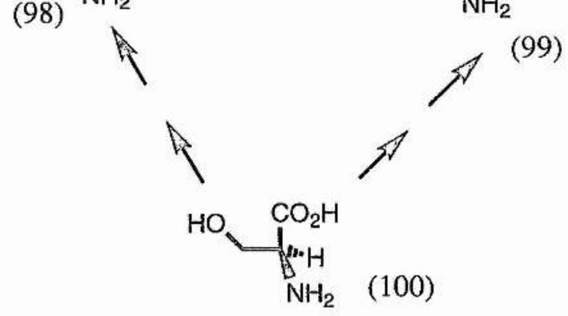
While our work was in progress Phillips and Dua prepared a series of *S*-aryl-2*S*-cysteines and their corresponding *S,S*-Dioxides [129] and evaluated their interaction with kynureninase. The most potent of these compounds *S*-(2-aminophenyl)-2*S*-cysteine *S,S*-dioxide (97) has a K_I value of 70 nM. These results suggested that the sulphone group may be generally useful in the design of potent inhibitors of enzymes which proceed via hydrated tetrahedral intermediates.



(97)

2.3 SYNTHESIS OF TRANSITION STATE ANALOGUES.

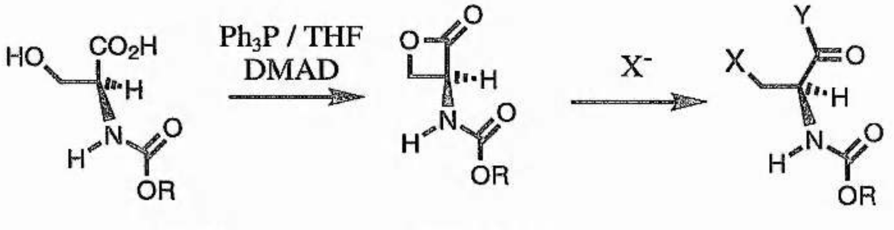
The desired phosphorous analogue (96) is an example of a β-substituted alanine. Various synthetic routes can be envisaged to the target molecule. The two general routes to β-substituted alanines require either a cation (98) or an anion (99) equivalent. A number of synthons are available for these fragments, most of which are prepared from the amino acid serine (100). As both enantiomeric forms of serine are available in high optical purity, at relatively low expense, they are especially attractive chiral starting materials. A number of approaches used in the literature are outlined below.



2.3.1 Alanine β-Cation Equivalents.

2.3.1.1 β-Lactone Methodology.

Arnold, Kalantar and Verderas[130] have reported the conversion of serine to stereochemically pure β-substituted α-amino acids via β-lactones (Scheme 2.2). Pure enantiomers of *N*-(benzyloxycarbonyl)-serine (*N*-Cbz-serine, 101a) and *N*-(*tert*-butoxycarbonyl)-serine (*N*-^{t}Boc serine, 101b) were cyclised without racemisation to give the corresponding *N*-protected α-amino β-lactones (102a, 102b) in 60 and 72% yield respectively, using modified Mitsunobu conditions, (Ph_3P and dimethyl azodicarboxylate, DMAD).



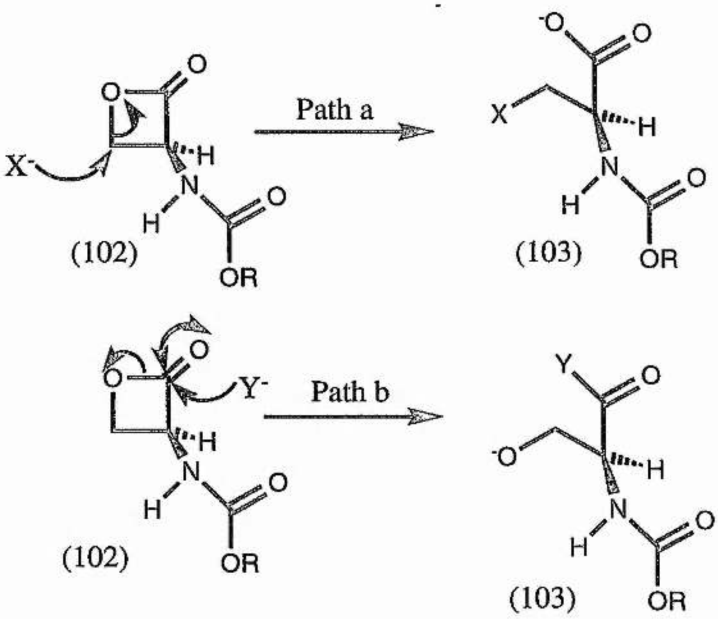
(101a) $R=CH_2Ph$
(101b) $R=C(Me)_3$

(102a) 60%
(102b) 72%

(103)

Scheme 2.2

Ring opening of the β-lactone with a variety of halogen, oxygen, sulphur, or nitrogen nucleophiles then produced pure enantiomers of *N*-protected β-substituted alanines (103) in high yield (Scheme 2.3). Attack occurred exclusively at the β-carbon (Path a, Scheme 2.3). The product was obtained in a stereochemically pure form and deprotection was carried out by conventional methods. Only relatively hard nucleophiles, like ammonia and methoxide, attacked the carbonyl to give acyl oxygen cleavage (Path b, Scheme 2.3) (see Table 2.1).



X^-= Hal, O, S, nucleophiles
Y^-= OMe, NH_3

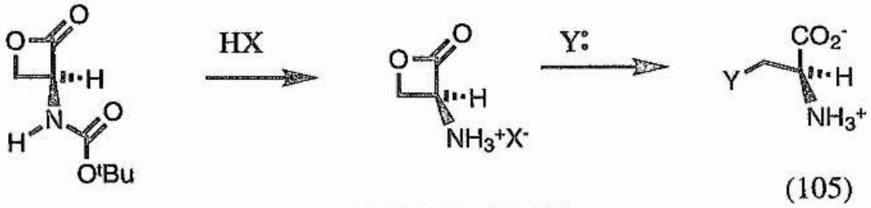
Scheme 2.3

β-lactone (102a, 102b)	**Nucleophile**	**X(103)**	**Y(103)**	**Config.**	**Yield (%)**
102a	$MgBr_2$-Et_2O Et_2O, 22 °C	-Br	-OH	2*S*	67
102b	$PhCH_2S^-Na^+$ DMF, 22 °C	-SCH_2Ph	-OH	2*R*	65
102a	$NH_3(g)$/MeCN 0 °C	-OH	-NH_2	2*S*	77
102a	NaOMe	-OH	-OMe	2*RS* [a]	88

[a] Racemised.

Table 2.1

A complimentary β-lactone system that directly provides access to the corresponding free amino acid has also been described (Scheme 2.4).[131]



(102b)

(104a) X=CF_3CO_2
(104b) X=OTs

(105)

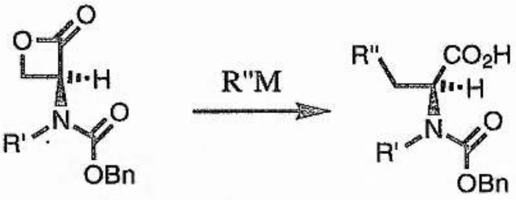
Scheme 2.4

Removal of the ^{t}butoxycarbonyl protecting group on the amine with acid gave the corresponding amine salts (104a, 104b). The tosylate salt was reported to be crystalline, stable and amenable to storage. These interesting compounds readily added even very weak nucleophiles (such as trifluoroacetic acid, TFA) at the β-carbon, directly forming the free amino acids (105). The authors noted that some functionalities, such as azido, introduced using the protected β-lactones were labile to the conditions required to remove the nitrogen protecting groups. Therefore, the salts allowed the preparation of even relatively labile amino acids in a very simple manner. The yields were generally good and the diversity of the nucleophiles impressive (Table 2.2). It is also significant that some of the addition reactions (for example, with sulphur nucleophiles) could be performed in water near pH 5.

Nucleophilic reagent (Y:)	**Conditions**	**Y(105)**	**Yield (%)**
$H_2NCH_2CH_2SH$-HCl	pH 5.5	$-SCH_2CH_2NH_2$	85
LiSH	MeCN/THF	-SH	88
$CF_3CO_2^-$	TFA	$-OCOCF_3$	87
K_2HPO_4	18-crown-6, DMF	$-OPO_3H_2$	87
conc. HCl	30 min	-Cl	92
^{n}-Bu_4NCN	DMF, 10 °C	-CN	84
NaN_3	DMF	$-N_3$	96

Table 2.2

The addition of cuprates to serine β-lactone (Scheme 2.5) was also shown to provide a practical and convenient method for introducing carbon functionality at the β-carbon.[132]

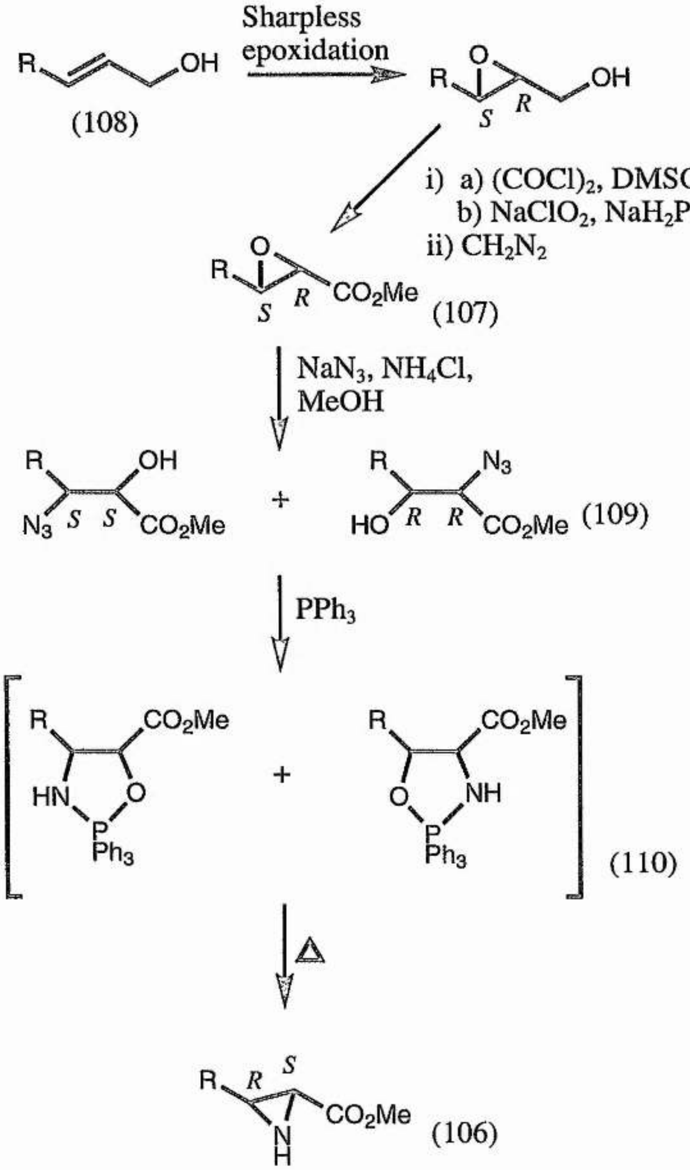


Scheme 2.5

2.3.1.2 Aziridine-2-carboxylic acid derivatives.

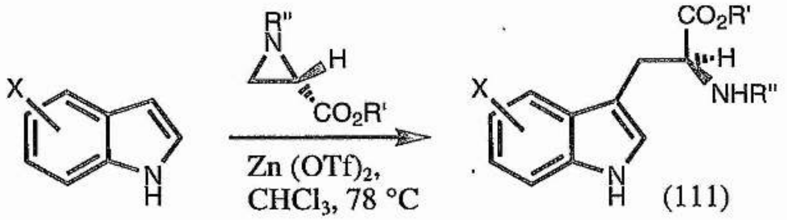
Aziridine-2-carboxylic acid derivatives (106) are another class of α-amino acid β-cation equivalents. They may be considered simultaneously as α- or β- amino acid derivatives. Most of the syntheses of aziridinecarboxylic acids are non-stereospecific and are generally not applicable to *N*-unsubstituted derivatives. Zwanenburg and co-workers were particularly interested in *N*-unsubstituted examples because they can be incorporated into a peptide chain and serve therein as an electrophilic moiety.[133]

Optically active glycidic esters (107) were prepared from allylic alcohols (108) by the Sharpless epoxidation. The glycidic esters were then treated with sodium azide. In the case of alkyl substituted glycidic esters this S_N2 type reaction invariably led to a mixture of the isomeric azido alcohols (109). The azido alcohols were treated with triphenylphosphine to give the corresponding 1,3,2-oxazaphospholidines (110), which could be isolated when reaction was carried out in ether. Thermal elimination of triphenylphosphine oxide was then effected by Kügelrohr distillation to yield the aziridinecarboxylic esters in moderate yields. When however, the Staudinger reaction was performed in DMF or acetonitrile, stirring at room temperature followed by heating at 80 °C for a few hours afforded the aziridines (106) in good yields without isolation of the intermediate oxazaphospholidines (Scheme 2.6).



Scheme 2.6

Applications of these optically active aziridinecarboxylic acid derivatives include the preparation of tryptophan derivatives and nucleophilic ring opening. The possibility of preparing optically pure tryptophan derivatives (111) from various substituted indoles and (2*R*)- or (2*S*)-2-aziridinecarboxylates has been examined (Scheme 2.7, Table 2.3). Sato *et al.* found that zinc triflate was the only Lewis acid capable of bringing about this reaction in moderate yields.[134]



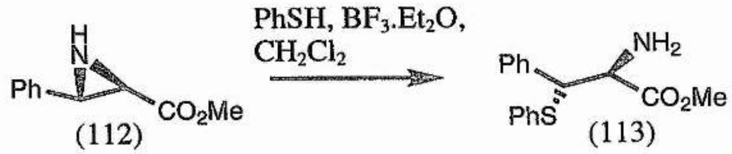
Scheme 2.7

X	R'	R''	Yield (%)
H	Bn	Cbz	44
H	Me	Boc	30
5-Me	Bn	Cbz	46
4-OMe	Bn	Cbz	33
6-Cl	Bn	Cbz	12
4-NO_2	Bn	Cbz	3

Table 2.3

Indole derivatives containing electron releasing groups, for example 5-methyl and 4-methoxy, reacted under these conditions to afford the desired tryptophan products. Although, indoles with an electron withdrawing substituent, for example 6-chloro and 4-nitro, gave rise to little if any of the expected tryptophan products. Thus the chemistry described offers a useful method for gaining access to optically pure tryptophan derivatives only in certain instances.

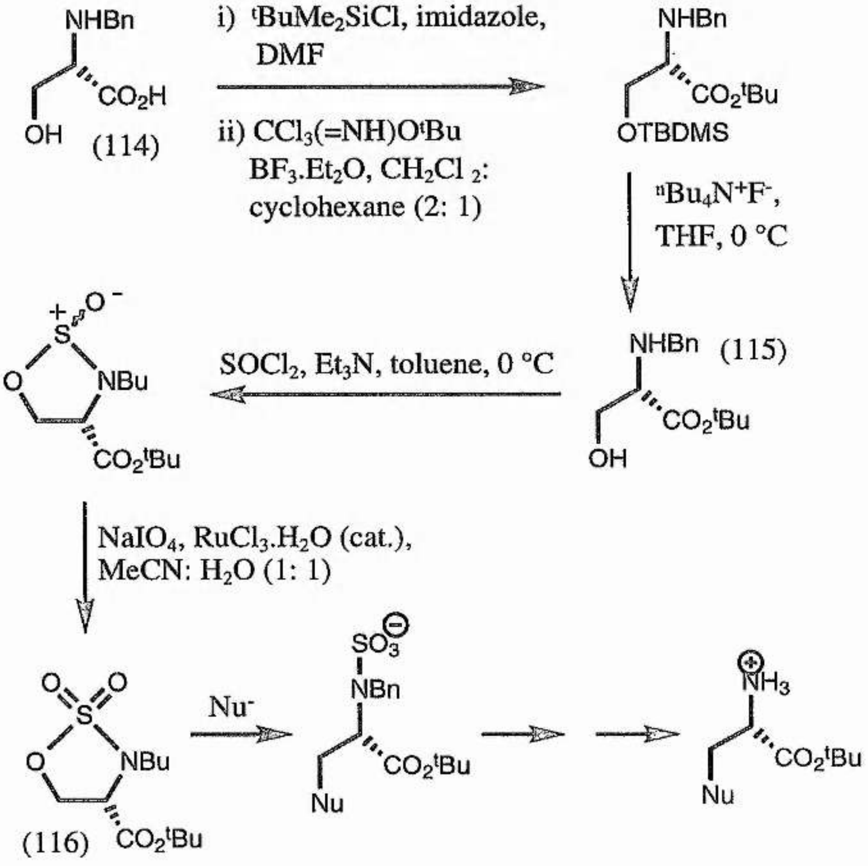
Other nucleophiles have also been employed, for example, ring opening of 3-aryl aziridine ester (112) with thiophenol in the presence of one equivalent of boron trifluoride etherate affords the 3-phenylthio amino ester (113) in 67% yield as a single diastereomer (Scheme 2.8).[133]



Scheme 2.8

2.3.1.3 Serine sulphamidates.

Baldwin and co-workers used the serine sulphamidate as a substitute for aziridinecarboxylate in substitutions with a variety of nucleophiles including cyanide, pyrazole and malonate (See Table 2.4). They hypothesised that a cyclic sulphamidate derived from serine might simultaneously activate the β-position to nucleophilic attack and partially protect the amino group and thus provide a useful 'β-alanyl cation' synthon for the synthesis of α-amino acids.



Scheme 2.9

The sulphamidate was synthesised from *N*-benzyl serine (114). The acid was protected as the ^{t}butyl ester (115), using ^{t}butyl trichloroacetimidate in the prescence of a catalytic amount of boron trifluoride etherate.[135] Formation of the cyclic sulphamidate (116) was then achieved by reaction with thionyl chloride and oxidation was carried out using sodium periodate with catalytic ruthenium (Scheme 2.9).[136]

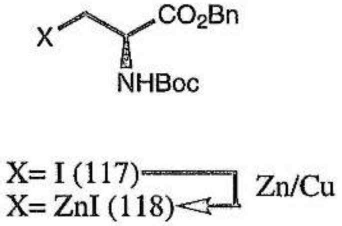
Nucleophile	**Reaction conditions**	**Yield (%)**
H_2O	2 M HCl: dioxane (1: 1), 14 h, 0-20 °C	63
N_3^-	NaN_3, acetone: water (1: 1), 12 h, 20 °C	93
SCN^-	NH_4SCN, DMF, 12 h, 20 °C	91
pyrazole	pyrazole, DMF, 11 h, 60 °C	55
CN^-	NaCN, DMF, 12 h, 20 °C	82

Table 2.4

Although the protected amino acids were prepared in good to excellent yields, the authors did not provide any details of the deprotection and isolation of the free amino acid.

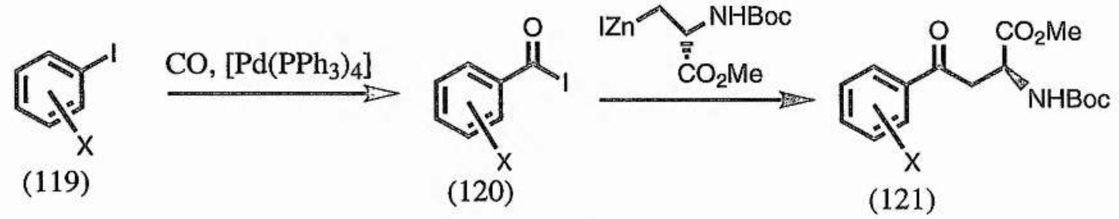
2.3.2 Alanine β-Anion Equivalents.

The polarity of the serine side chain can be reversed from electrophilic to nucleophilic by the preparation of β-iodoalanine (117) and the derived organozinc reagent (118) as reported by Jackson *et al.*[137] The latter is prepared by treatment of protected iodoalanine with activated zinc.[138]



X= I (117)
X= ZnI (118)

This organozinc reagent (119) can undergo carbonylative cross coupling with functionalised aryl iodides (120) using palladium catalysis to give protected 4-aryl-4-oxo α-amino acids (121) (Scheme 2.10, Table 2.5). In fact, 2*S*-kynurenine has been successfully prepared using this method.



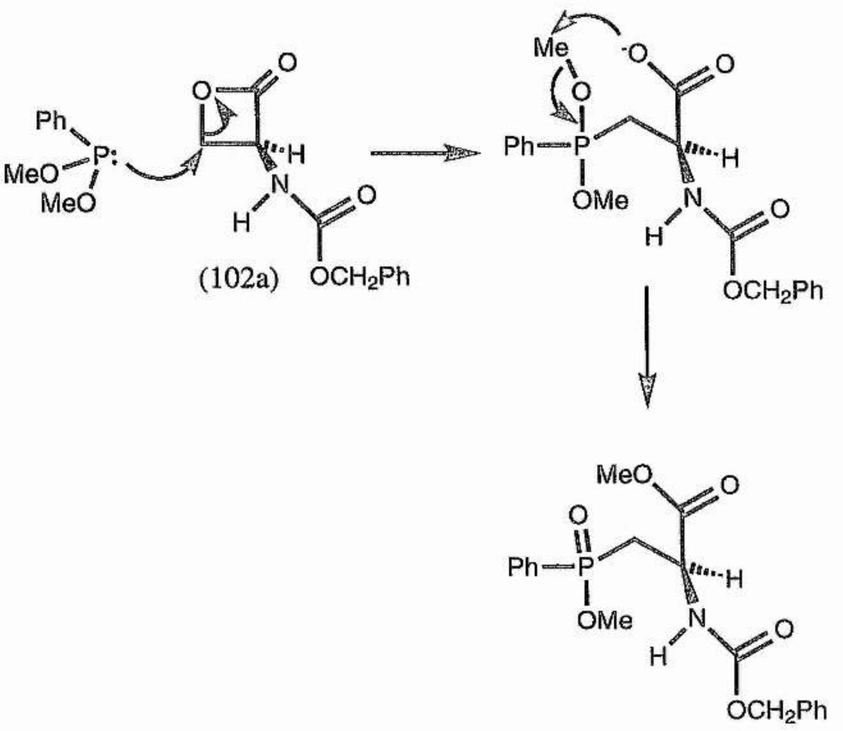
Scheme 2.10

Aryl iodide	Yield (%)
Iodobenzene	60
1-Napthyl Iodide	54
4-Iodoanisole	58
4-Iodotoluene	59
2-Iodoanaline	52

Table 2.5

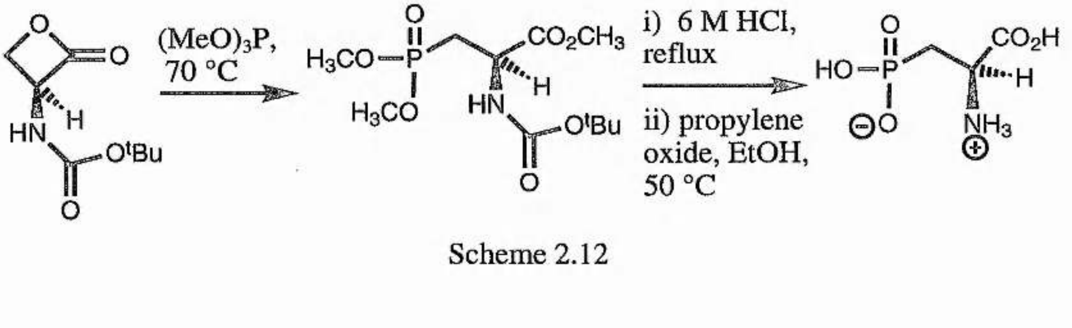
2.3.4 Conclusions.

It was decided to employ the β-lactone methodology [130] for the synthesis of the phosphinic acid using phenyl dimethylphosphonite as the nucleophile. Direct access to the free amino acid via the β-lactone salt [131] is unnecessary as the methyl ester should be formed on reaction with the nucleophile in an Arbusov type reaction (Scheme 2.11). Therefore, a final hydrolysis step is inevitable.



Scheme 2.11

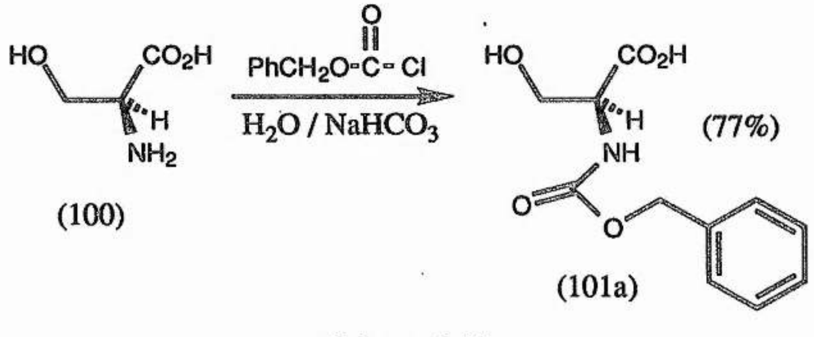
A similar reaction has been carried out by Smith *et al.* using trimethyl phosphite as the nucleophile (Scheme 2.12).[139] This provided more evidence for the viability of this route.



Scheme 2.12

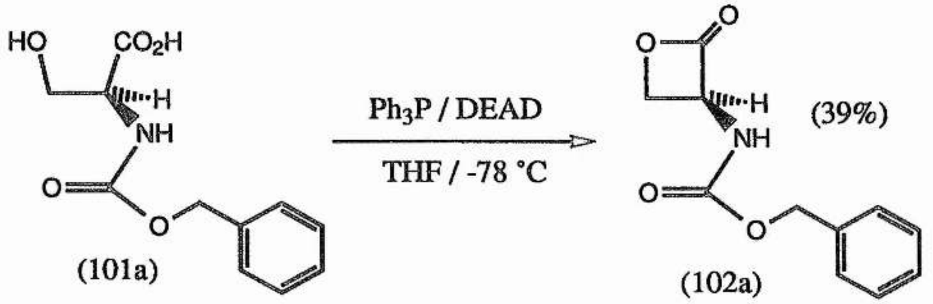
2.4 SYNTHESIS OF THE PHOSPHINIC ACID ANALOGUE.

Serine was first protected as the *N*-Benzyloxycarbonyl (Cbz) derivative. Reaction was carried out under aqueous conditions using potassium bicarbonate and benzylchloroformate (Scheme 2.13). The product was isolated after acidification of the reaction solution and extraction into ether in 77% yield. The identity of the product was confirmed by ^{1}H NMR spectroscopy which showed the methylene protons of the Cbz group at 5.05 ppm.



Scheme 2.13

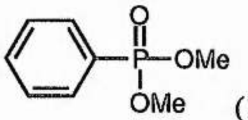
Serine β-lactone (102a) was then prepared by cyclisation of *N*-Cbz serine (101a) under modified Mitsunobu conditions. Triphenylphosphine and diethyl azodicarboxylate were dissolved in THF and cooled to -78 °C (Scheme 2.14).



Scheme 2.14

The scale of this reaction was limited as the solution had a tendency to solidify as the *N*-Cbz serine solution was being added. This problem was minimised by periodical shaking of the reaction mixture. The product was purified by column chromatography (silica, ethyl acetate: petroleum ether; 45: 55). Solubility problems necessitated preabsorption of the product onto silica to obtain optimum results. The lactone and the hydrazine ran very close together on silica under these conditions, with triphenylphosphine oxide running close to the solvent front and unreacted Cbz serine close to the baseline. The hydrazine was readily visualised by staining with potassium permanganate, while the other compounds were visualised using bromocrescol green. The product and hydrazine were thus easily separated from these other components. A second column was then carried out with a larger excess of silica to give white needles of the pure product which were observed to directly precipitate out of solution in the column fractions. The optimised yield of pure β-lactone was 39%.

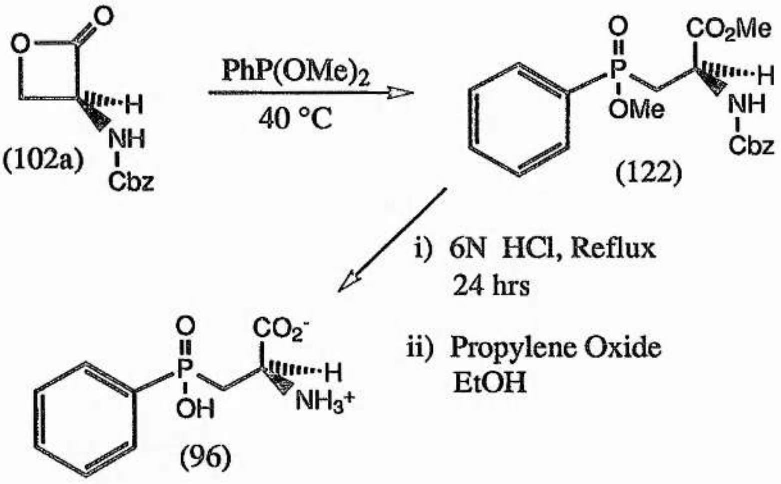
The β-lactone was then ring opened with a suitable phosphorous nucleophile. *N*-Cbz-serine β-lactone (102a) was heated in excess phenyl dimethylphosphonite at 40 °C. The phosphonite was employed both as the solvent and the nucleophile. After distillation the product (122) was obtained in 85% yield. The methyl ester obtained as a result of the Arbusov reaction was observed in the ^{1}H NMR spectrum at 3.55 ppm. The phosphinate ester was observed at 3.75 ppm as a doublet due to coupling with the phosphorous, with a value of J_{PH} 11 Hz. Partial purification was carried out using column chromatography (silica, dichloromethane: IPA, 95: 5) and the product used without further purification. Although attempts were made to purify the product further these were unsuccessful due to decomposition of the product. The main impurity was discovered to be the diester (123).



(123)

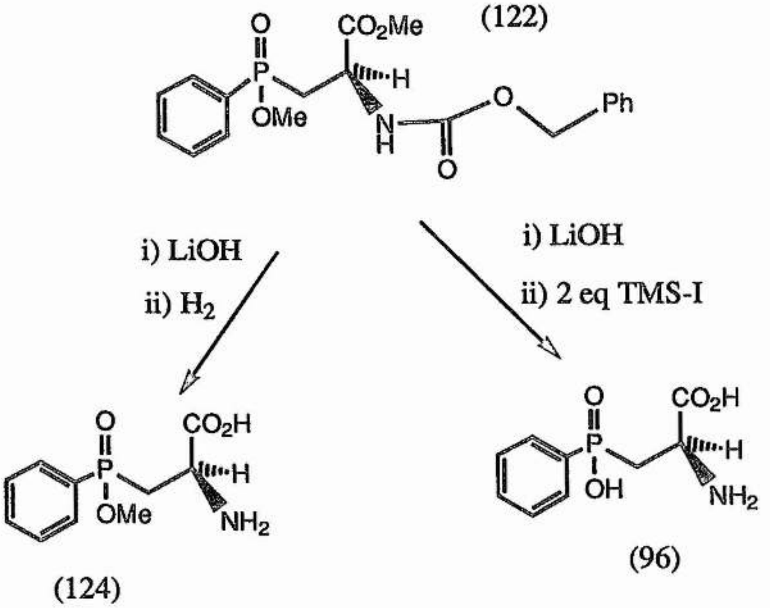
It was therefore decided to carry the material through and leave further purification until the end of the synthesis. Deprotection was achieved by heating at reflux in 6M HCl. Isolation of the free amino acid was carried out by heating with ethanol and propylene oxide to neutralise the hydrochloride salt[139] (Scheme 2.15). Further purification was initially attempted by ion exchange chromatography (on IR 120 (H)) although only a very small amount of pure material was recovered in 13% yield. Preparative tlc (cellulose, IPA: NH_3:

H_2O; 36: 6: 10) gave two ninhydrin positive broad bands which were extracted with (methanol: water; 1: 1). The product band (Rf 0.3-0.5) was identified by NMR spectroscopy. Although the white crystals produced in 87% yield appeared to be pure by ^1H NMR spectroscopy, microanalysis revealed that recrystallisation was required and this was attempted from ethanol / water. At this stage the quantity was too small for repeated recrystallisation and therefore an alternative to preparative tlc was sought.



Scheme 2.15

An alternative strategy involved removal of the protecting groups consecutively rather than simultaneously. In the synthesis of kynurenine[140] (see <u>4.3.2.1 Synthesis of Kynurenine</u>) the protecting groups were removed consecutively allowing purification of each individual intermediate, eliminating impurities in stages rather than in one attempt. Thus lithium hydroxide could be used to hydrolyse the methyl ester, TMS-iodide or hydrogenation to remove the *N*-Cbz group and finally cleavage of the phosphinate ester with TMS-iodide. In this way access to a new compound (124) should also be available (Scheme 2.16).

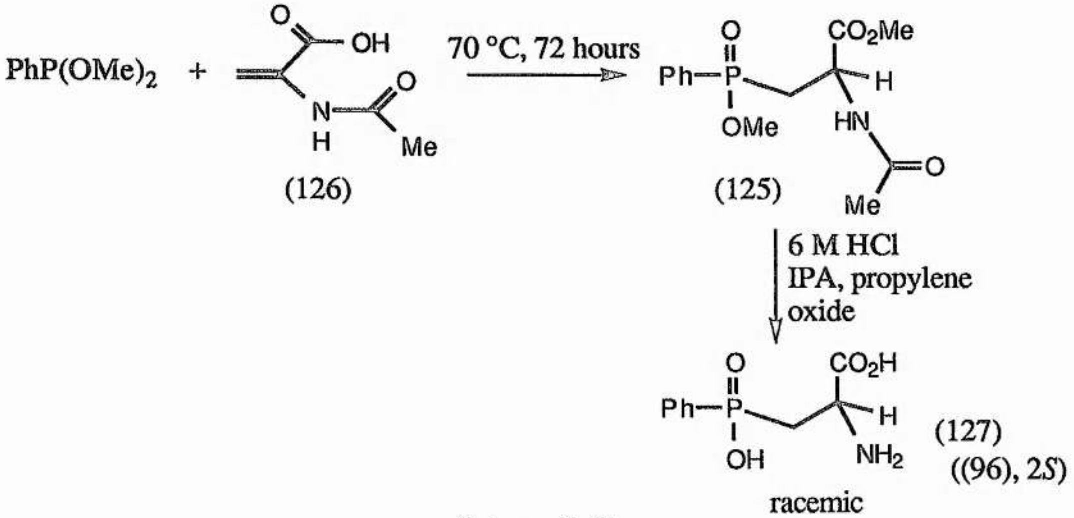


Scheme 2.16

The (2*S*)-methyl *N*-Cbz-2-amino-3-(*O*-methyl phenylphosphino)-propanoate was dissolved in (THF: water; 1: 1), lithium hydroxide monohydrate added and the solution warmed at 70 °C. After reaction the solution was poured into 1 M HCl and extracted with ethyl acetate. ^{1}H NMR spectroscopy showed that both the carboxylate methyl ester (3.55 ppm) and the phosphinate methyl ester (3.75 ppm) had been removed. However, slow addition of the lithium hydroxide gave a mixture where the desired product was obtained in addition to the compound with both esters removed. This mixture was difficult to separate by column chromatography so it was deprotected in this form.

An alternative procedure for neutralisation of the amino acid hydrochloride was employed using propylene oxide and isopropanol, as in the synthesis of kynurenine, to replace the use of propylene oxide and ethanol. This method did not require any heating so it was hoped that these milder conditions would aid in the isolation of a pure free amino acid. This was tried on the product from the partial deprotection using lithium hydroxide and was found to give a solid product. At this stage it was not clear if the use of propylene oxide and isopropanol was sufficient to produce a solid product, or if this was helped by the partial deprotection using lithium hydroxide. To clarify this point it was necessary to deprotect the phosphorous compound using acid and then isolate the amino acid using propylene oxide and isopropanol. For a comparison partial deprotection using lithium hydroxide and then isolation of the amino acid using this method was necessary. These two alternatives could then be evaluated. A racemic version of the protected phosphorous compound (125) was prepared on a large scale to help to predict the viability of this procedure.

Thus, 2-acetamidoacrylic acid (126) was dissolved in excess phenyl dimethylphosphonite and heated to 70 °C for seventy two hours (Scheme 2.17).



Scheme 2.17

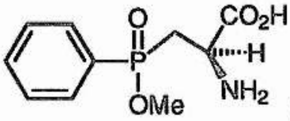
Excess phenyl dimethylphosphonite was removed by Kügelrohr distillation. Washing with 5% sodium bicarbonate and extraction into ethyl acetate removed any residual phenyl dimethylphosphonite still remaining. This protected compound showed four stereoisomers in the ^1H NMR which can be seen from the four singlets present due to the hydrogens of the methyl group of the *N*-acetyl at 1.44, 1.51, 1.68 and 1.72 ppm. Deprotection was carried out without further purification, by dissolving in 6 M HCl and refluxing for twelve hours. The solution was concentrated at reduced pressure and taken up in isopropanol containing a small amount of 2*RS*-dithiothreitol. This was then neutralised with propylene oxide and a white solid precipitated out of solution. Diethyl ether was added and more white solid precipitated out of solution. This was filtered under nitrogen and recrystallised from acetone / water to give the pure amino acid in 54% yield. The spectral data were identical to those obtained for the product from the β-lactone route.

As a result of this experiment, the same method was then applied to the chiral protected phosphorous compound and this also resulted in a microanalytically pure sample.

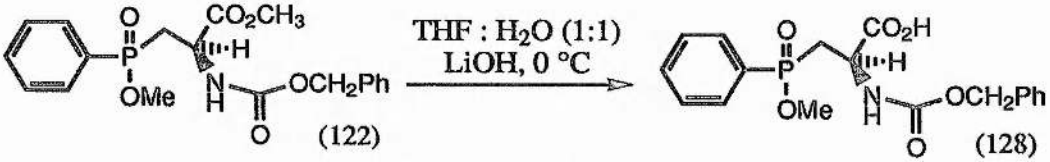
Preliminary investigation of the inhibition of kynureninase, isolated from *Pseudomonas fluorescens*[2], by the phosphinic acid showed poor inhibition. (Full details are reported in Section **2.6.1 Phosphinic acid**). A possible reason for this poor inhibition was sought. The pK_a of the phosphinic acid is very low (ca. 3 to 3.5)[141] and so at the pH of the assays it will be negatively charged. This negative charge may produce destabilising interactions with groups at the active site of the enzyme. There is precedent for this phenomenon in work on aspartyl proteases where studies of related phosphinic acid analogues showed that the inhibitory proficiency of the compounds increased with decreasing pH, from which it was concluded that the proteinase has a preference for the uncharged form of the phosphinic acid.[124] It is believed that these effects result from repulsive interactions that develop between an active site aspartate carboxylate and the negatively charged phosphonamidate as it ionises with increasing pH.[125]

2.5 SYNTHESIS OF METHYL PHOSPHINATE ANALOGUE.

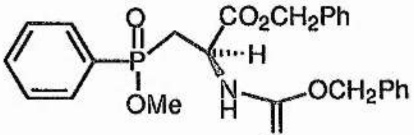
It was hypothesised above that the negative charged phosphinate group may produce destabilising interactions with groups at the active site of the enzyme. In order to test this hypothesis it was decided to prepare the methyl phosphinate analogue. Examination of the inhibitory properties of the methyl phosphinate should then allow the importance of the negative charge to be assessed.


(124)

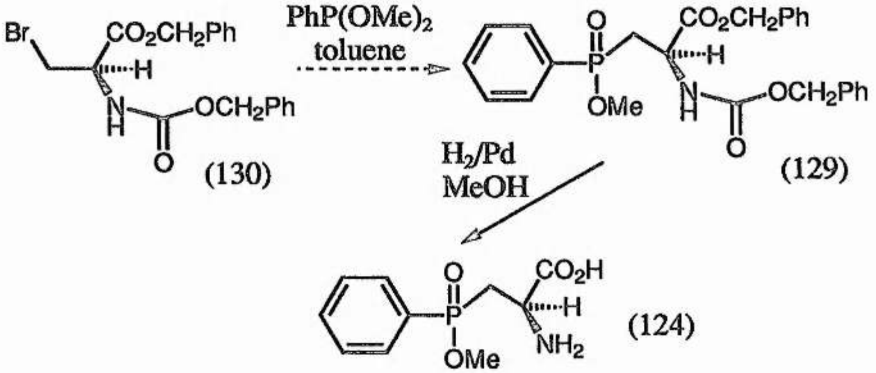
As the dimethyl ester (122) had been previously prepared via the β-lactone route, further attempts were made to selectively hydrolyse the carboxylate ester using lithium hydroxide (Scheme 2.18). Previous attempts had involved heating the mixture at 70 °C and so milder conditions were employed. Treatment with one equivalent of lithium hydroxide in a 1: 1 mixture of THF and water at 0 °C gave a product that appeared to be correct as mass spectrometry showed a peak at 379 and ^{1}H NMR spectroscopy showed a doublet due to the phosphinate ester at 3.75 ppm (*J* 11 Hz, PH). However, the compound proved to be difficult to isolate and purify.


Scheme 2.18

An alternative route to the product was devised, using a different protecting group strategy. This would furnish a compound amenable to purification by column chromatography. A final deprotection step using hydrogenation could then be employed to give the final product.

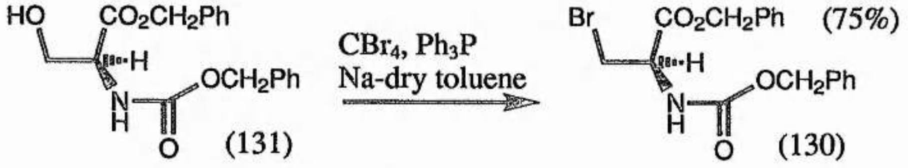

(129)

The proposed route involved reaction of *N*-Cbz 2-bromoalanine benzyl ester (130) with phenyl dimethylphosphonite. This protected intermediate (129) could then be hydrogenated to give the methyl phosphinate analogue (Scheme 2.19).



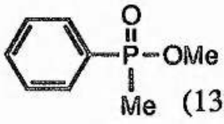
Scheme 2.19

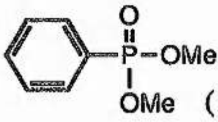
Starting with *N*-Cbz serine benzyl ester (131), the alcohol was converted to the bromide using carbontetrabromide and triphenylphosphine in toluene (Scheme 2.20).[142] The triphenyl phosphine oxide by-product was removed by filtration through celite and column chromatography (ethyl acetate: 2: 8) gave a white crystalline solid in 75% yield. Mass spectrometry confirmed the identity of the product and the optical rotation was identical to the literature value.[143]



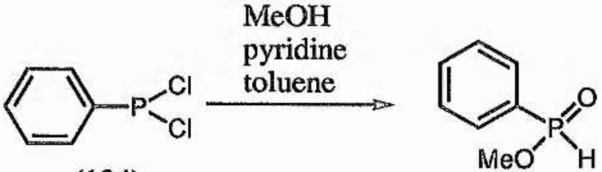
Scheme 2.20

Reaction of *N*-Cbz 2-bromoalanine benzyl ester (130) with phenyl dimethylphosphonite was then attempted (See Scheme 2.19). Reaction at reflux at 120 °C in toluene for seventy two hours gave no product on distillation. The number of equivalents of phenyl dimethylphosphonite was increased from 2.2 to five. The compounds were also heated without toluene in a 1: 1 ratio as a melt, however, still no reaction was seen. Along with starting material the only compounds isolated were (132) and (123). It therefore, seemed necessary to explore some other alternative routes.


(132)


(123)

An alternative reagent to dimethyl phenylphosphonite was methyl phenylphosphinate (133). This was prepared according to the method of DeBriun[144] (See Scheme 2.21). Thus, a solution of methanol and pyridine in toluene was added dropwise over one and a half hours to a stirred, cooled solution of dichlorophenylphosphine (134) in toluene. The pyridinium hydrochloride, formed within one hour, was vacuum filtered from solution, and water was added dropwise to the filtrate. The organic layer was extracted with saturated sodium bicarbonate and water and then the aqueous phase was back extracted twice with dichloromethane. The combined organic phases were dried and concentrated and distillation gave 43% of the pure product. This reaction was found to be most successful when carried out on as large a scale a possible. The ^{1}H NMR spectrum clearly showed the proton attached to the phosphorus at 7.55 ppm. This was a doublet due to coupling with phosphorus, giving *J* 565 Hz which is in good agreement with the literature value (*J* 560 Hz, PH).[144]

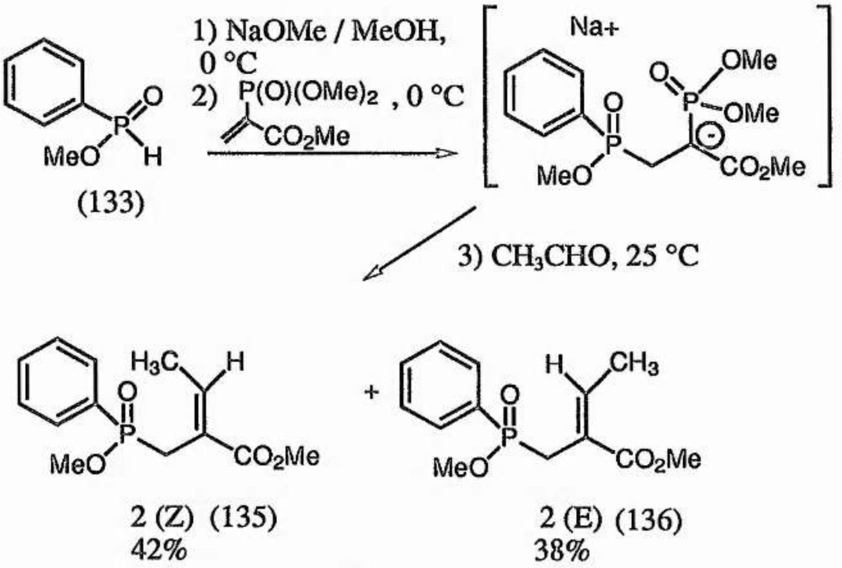


(134)

(133)
43%

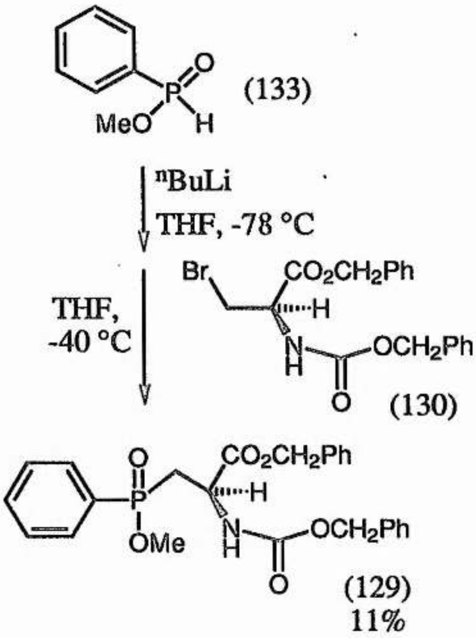
Scheme 2.21

Methyl phenylphosphinate was then reacted with *N*-Cbz-2-bromo-alanine benzyl ester (130) in a procedure based on the method of Parsons,[145] who was interested in 3-substituted-2-phosphomethyl acrylates, (135) and (136), both as inhibitors and synthetic intermediates. The syntheses of these compounds were described using this easily obtainable phosphorous nucleophile (See Scheme 2.22).



Scheme 2.22

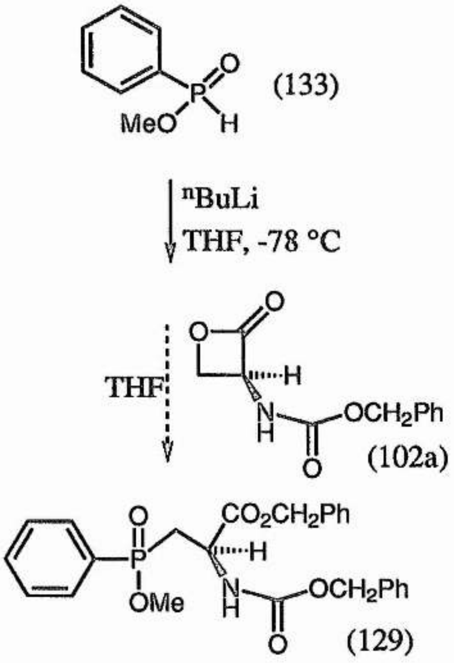
For our purposes, methanolic sodium methoxide (2 M) was initially used as the base. This proved to be unsuccessful even in alternative solvents such as THF and DMF. A variety of different bases were also employed such as sodium hydride and ^{n}butyl lithium. ^{n}Butyl lithium was found to be the most promising and some product was formed in the reaction. Thus ^{n}butyl lithium was added dropwise to methyl phenylphosphinate (133) in THF at -78 °C under nitrogen (See Scheme 2.23).



Scheme 2.23

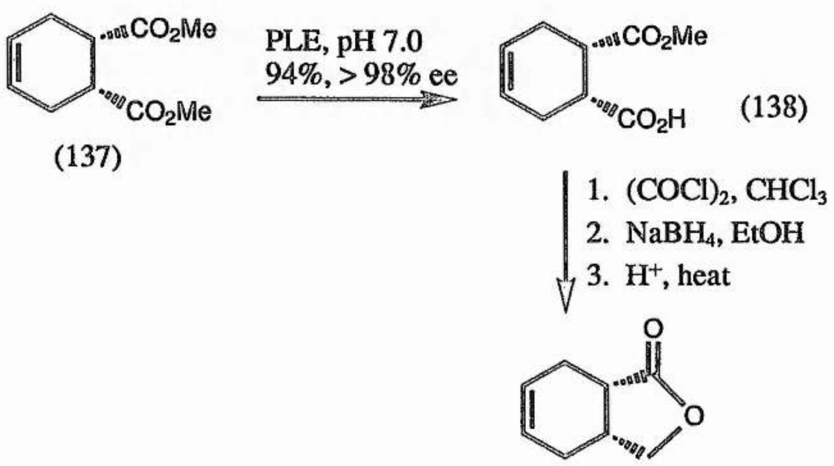
After stirring for thirty minutes the bromo compound (130), which had previously been dried under vacuum, was added at -40 °C in THF. A tlc examination (silica, ethyl acetate) of the reaction showed seven spots and mass spectrometry was carried out on each of these to identify the desired product. Separation was then achieved by column chromatography using a gradient solvent system (silica, initially ethyl acetate: petroleum ether, 1: 1, then 3: 1, then pure ethyl acetate) giving pure product (129) but in only 4% yield. Incomplete reaction between methyl phenylphosphinate and the ^{n}butyl lithium was thought to be the reason, leaving excess base to react with the bromo compound. The alkene product can be seen in the ^{1}H NMR spectrum and is characterised by two singlets at δ= 5.85 ppm and 6.28 ppm, the geminal coupling is very small and is not observed in the spectrum. In an attempt to alleviate this problem, the reaction mixture was allowed to warm to room temperature after the addition of ^{n}butyl lithium and stirred for thirty minutes before being cooled to -40 °C. A lower temperature of -50 °C resulted in the isolation of less product. As a strong base is employed in this reaction a low temperature was desirable to reduce the possibility of racemisation. In addition a few crystals of 2,2-dipyridyl were added which gave the solution a red colour if excess ⁿbutyl lithium was added. These improvements however, only increased the yield to 11%. Nevertheless hydrogenation could be carried out to allow isolation of the desired product in 38% yield. Due to the fact that this reaction gave such a crude mixture of products further alternatives were investigated in the hope that these may give purer products.

The first of these was the reaction of the phosphinate with serine β-lactone (102a) (See Scheme 2.24), this was carried out in a similar manner as with the bromo compound, however, no product was isolated.



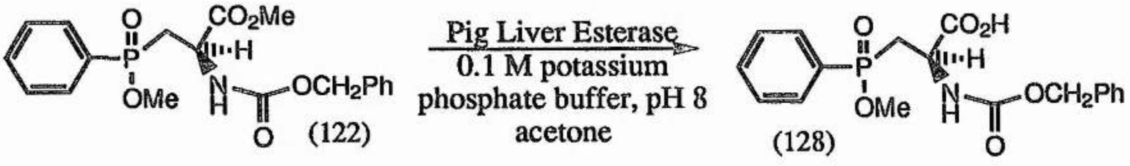
Scheme 2.24

Attention was then returned to the selective hydrolysis of the carboxylate ester. One approach to obtaining such selectivity is to use enzymic methods. For example, pig liver esterase is a serine type of esterase that catalyses the stereoselective hydrolysis of a wide variety of esters.[146] It is relatively cheap and does not require the use of cofactors. In particular pig liver esterase has been widely used in the enantioselective hydrolysis of symmetrical diesters. Most of these transformations, such as the example by Lukas[147] were carried out in aqueous solution (Scheme 2.25). Thus hydrolysis of *meso*-tetrahydrophthalic anhydride (137) was carried out using pig liver esterase in pH 7 buffer at ambient temperature and resulted in almost exclusive hydrolysis of the (1*R*)-ester group to afford the enantiomerically pure (1*S*,2*R*)-monoester (138). The optical purity of the monoester was analysed by conversion to the lactone and analysis by chiral GC. This compound has been widely used as an important chiral synthon in the preparation of some complex natural products.[148, 149]



Scheme 2.25

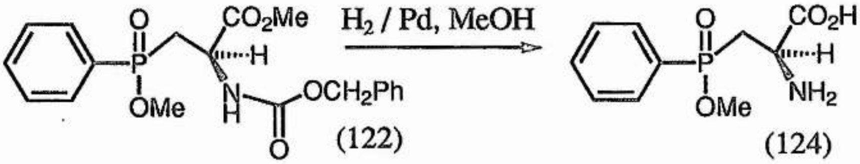
The (2*S*)-*N*-(benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (122) was poorly soluble in aqueous solution, however it was noted that Ohno *et al.* had used 10% acetone to solubilise their starting material.[150] A similar solvent system was therefore employed for our substrate (See Scheme 2.26).



(122) (128)

Scheme 2.26

Pig liver esterase was added to a solution of the diester (122) in 10% acetone and 0.1 M potassium phosphate buffer (pH 8). Initially incubation was carried out at 25 °C but no product was observed by mass spectrometry of aliquots removed from the reaction mixture. The temperature was thus increased to 33 °C and the amount of esterase increased. After twelve hours some reaction was observed. This was further increased to an optimum temperature of 38 °C and approximately five units of pig liver esterase per milligram of compound. Incubation was continued until ^{1}H NMR spectroscopy showed reaction to be complete, with the disappearance of the methyl ester at 3.55 ppm and the phosphinate ester remaining at 3.70 ppm. Mass spectrometry also confirmed that one of the methyl groups had been selectively removed. The reaction mixture was acidified and the product extracted. Chromatography was not successful so the compound was deprotected in this form. Deprotection was achieved by hydrogenation at atmospheric pressure in methanol using a palladium catalyst (Scheme 2.27). The product (124) was obtained in 67% yield after recrystallisation from acetone / water. The product was observed to be a mixture of diastereomers in a 2: 1 ratio by NMR spectroscopy, the ^{31}P NMR spectrum showed two peaks and the clearest indication in the ^{13}C NMR was due to two doublets of the β-carbon at 29.63 and 31.61 ppm (J_{PC} 100 Hz).



(122) (124)

Scheme 2.27

2.6 INVESTIGATION OF THE INTERACTION OF THE PUTATIVE INHIBITORS WITH KYNURENINASE.

Kinetic studies were carried out to examine the interaction of the two phosphorus based transition state analogues, the phosphinic acid (96) and methyl phosphinate (124), with kynureninase. The enzyme was partially purified from *Pseudomonas fluorescens* (see Chapter 5). The kinetic studies were carried out under the same conditions as employed for the assay of kynureninase activity. Incubation was carried out at 25 °C and the assay buffer contained 0.4 mM kynurenine, 40 μM PLP in 0.04 M potassium phosphate at pH 7.0. The reaction was followed by UV spectophotometry by measuring the rate of decrease in absorbance at 360 nm due to conversion of kynurenine to anthranilic acid (see Experimental).

Using commercial kynurenine, obtained from Sigma as the sulphate salt, the kinetic data for the substrate were determined. K_M and V_{max} data were determined by non linear regression using the ENZFITTER[151] programme. In order to determine the inhibitory properties of the phosphinic acid analogues the kinetic parameters were re-determined in the prescence of varying concentrations of the inhibitors. The effect on the kinetics then gave a measure of the strength and type of inhibition.

For kynurenine the values used were the average values obtained i.e. $K_M = 25.5 \pm 0.6\ \mu M$, in excellent agreement with Palfreyman *et al.* ($K_M = 25\ \mu M$)[140] and $V_{max} = (1.07 \pm 0.01) \times 10^{-3}$ mol dm^{-3} min^{-1} (corrected for one unit of enzyme activity). (The full data are in the Appendix).

2.6.1 Phosphinic Acid (96).

The data obtained for the inhibition of kynureninase by the phosphinic acid analogue are given in tables 2.6, 2.7, 2.8 and shown graphically in graph 2.1. Each rate was determined two or three times until the values were consistent (± 3%) and the average values (± 1% from regression analyses) are shown.

0.5 mM Phosphinic Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.77	0.265
0.20	0.50	4.17	0.240
0.30	0.333	4.29	0.233
0.45	0.222	4.56	0.219
0.50	0.20	4.60	0.217

Table 2.6

1 mM Phosphinic Acid

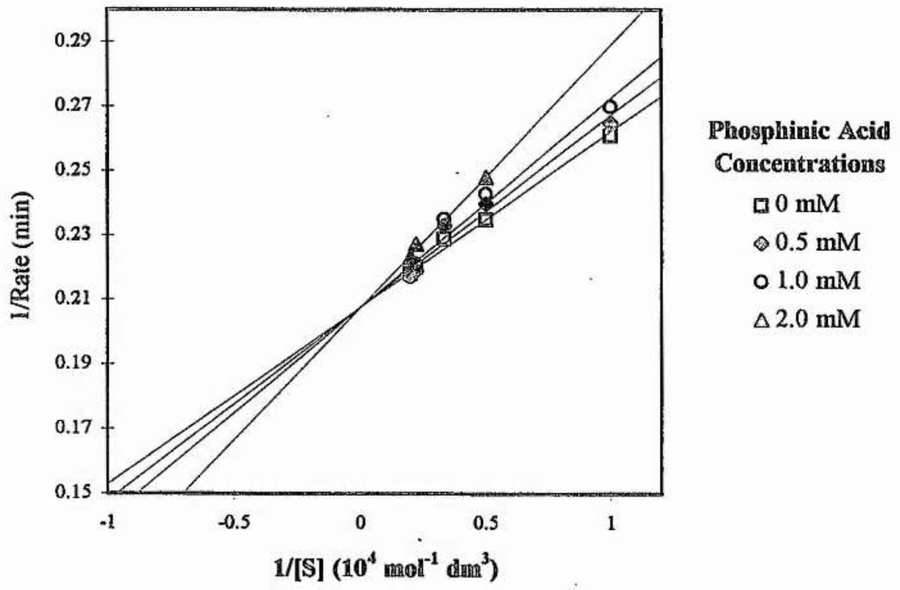
[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.70	0.270
0.20	0.50	4.12	0.243
0.30	0.333	4.25	0.235
0.45	0.222	4.53	0.221
0.50	0.20	4.61	0.217

Table 2.7

2 mM Phosphinic Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.20	0.50	4.04	0.248
0.45	0.222	4.41	0.227
0.50	0.20	4.49	0.223

Table 2.8



Graph 2.1: Lineweaver-Burk plot for kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of phosphinic acid.

For the phosphinic acid it can be seen that V/K varies while V_{max} is constant which implies that the phosphinic acid is a competitive inhibitor. Competitive inhibition occurs when the inhibitor competes with the substrate to bind at the active site, therefore, the enzyme can bind substrate or inhibitor but not both. The substrate is thereby prevented from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate.

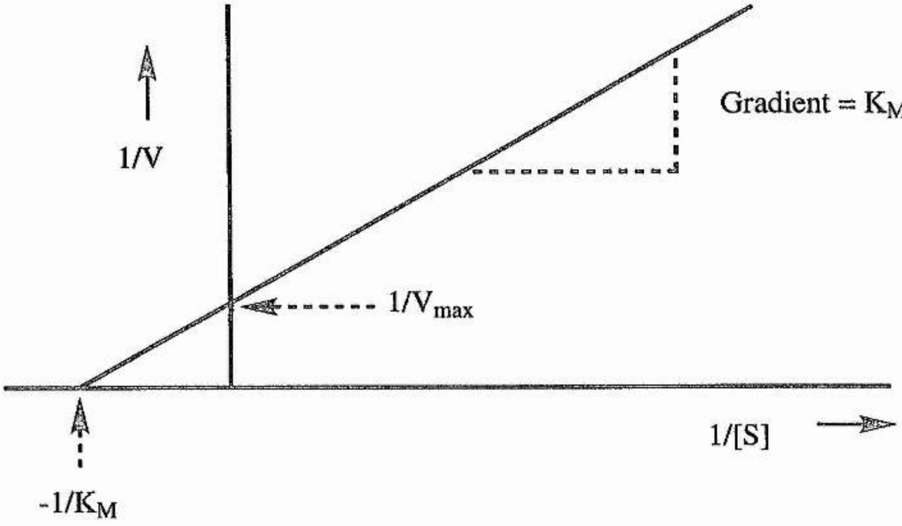
In order to show the type of inhibition it is often preferable to display it using the Lineweaver-Burk plot.

$$V = \frac{V_{max}\,[S]}{[S]\ + K_M}$$

therefore,

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \circ \frac{1}{[S]}$$

A plot of 1/V versus 1/[S], called a Lineweaver-Burk plot, yields a straight line whose intercept with the x axis is $-1/K_M$ and the intercept with the y axis is $1/V_{max}$ and the gradient is equal to K_M/V_{max}.



Lineweaver-Burk plot

In competitive inhibition, the intercept of the plot 1/V versus 1/[S] is the same in the prescence and absence of inhibitor, although the slope is different. This reflects the fact that V_{max} is not altered by a competitive inhibitor. The hallmark of competitive inhibition is that it can be overcome by a sufficiently high concentration of substrate. At a sufficiently high concentration, virtually all the active sites are filled with substrate, and the enzyme is fully operative. The increase in the slope of the 1/V versus 1/[S] plot indicates the strength of binding of the competitive inhibitor.

Michaelis and Menten proposed a simple model to account for the kinetic characteristics of single substrate reactions.

$$E + S \underset{K_M}{\rightleftharpoons} ES \xrightarrow{k_{cat}} \text{Products}$$

The catalytic reaction is divided into two processes. The enzyme and the substrate first combine to give an enzyme substrate complex, ES. This step is assumed to be rapid and reversible with no chemical changes taking place; the enzyme and the substrate are held together by physical forces. The chemical processes then occur in a second step with a first order rate constant, k_{cat} (the turnover number). For competitive inhibition an additional equilibrium must be considered.

$$\begin{tikzcd}
E + S \arrow[r, shift left] \arrow[d, shift left, "I"] & ES \arrow[l, shift left, "K_M"] \arrow[r, "k_{cat}"] & \text{Products} \\
EI + S \arrow[u, shift left, "K_I"] \arrow[r, "\times" marking] & {} &
\end{tikzcd}$$

The modified equation is:

$$V = \frac{V_{max}\,[S]}{[S] + K_M\,(1 + [I]/K_I)}$$

Therefore,

K_M is apparently increased by a factor of $(1+[I]/K_I)$.

This is denoted K_{app} and this equation can be used to determine the value of K_I.

$$K_{app} = K_M\left(1 + \frac{[I]}{K_I}\right)$$

$$\text{i.e. } K_{app} = \frac{K_M\,[I]}{K_I} + K_M$$

Thus a plot of K_{app} versus [I] gives a graph whose gradient is equal to K_M/K_I.

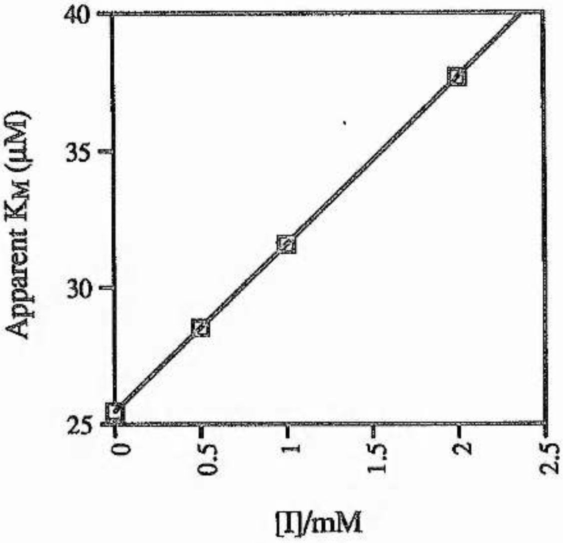
K_{app} is in fact, the value determined in the presence of varying concentrations of the inhibitor.

The data and plot for K_{app} versus [I] for the kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of the phosphinic acid analogue are shown in table 2.9 and graph 2.2 respectively. Thus from the gradient of this graph we can determine K_I as K_M is already known. Linear regression analyses was employed to determine the best straight line and errors were obtained from the regression analyses.

Inhibitor	**Inhibitor concentration (mM)**	**V_{max}^{a} (x 10^{-3}) (mol dm^{-3} min^{-1})**	**K_{app} (μM)^a**
No inhibitor	0 mM	1.07 ± 0.01	25.5 ± 0.6
Phosphinic acid	0.5 mM	1.07 ± 0.02	28.6 ± 0.4
Phosphinic acid	1.0 mM	1.07 ± 0.02	31.6 ± 0.5
Phosphinic acid	2.0 mM	1.07 ± 0.01	37.7 ± 0.4

^{a}V_{max} and K_{app} were obtained from fitting the data to the Michaelis-Menten equation by non linear regression using the ENZFITTER [151] programme.

Table 2.9



graph 2.2: Plot of K_{app} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Phosphinic Acid Analogue.

From the graph,

The K_I for the phosphinic acid analogue = **4.19 ± 0.1 mM**

2.6.2 Methyl Phosphinate (124).

The data obtained for the inhibition of kynureninase by the methyl phosphinate analogue is given in tables 2.10, 2.11, 2.12, 2.13 and shown graphically in graph 2.3. Each rate was determined two or three times until the values were consistent (± 3%) and the average values (± 1.2% from regression analyses) are shown.

2 mM Methyl Phosphinate (preliminary study)

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	2.79	0.358
0.20	0.50	3.77	0.265
0.30	0.333	4.25	0.235
0.45	0.222	4.44	0.225
0.50	0.20	4.59	0.218

Table 2.10

0.5 mM Methyl Phosphinate

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.50	0.286
0.20	0.50	4.14	0.242
0.30	0.333	4.29	0.233
0.45	0.222	4.46	0.224
0.50	0.20	4.57	0.219

Table 2.11

1 mM Methyl Phosphinate

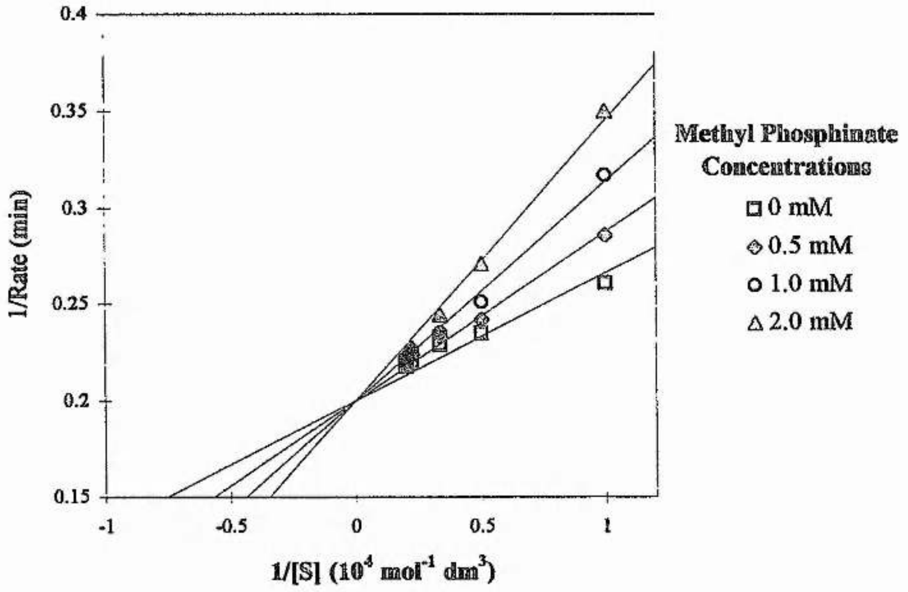
[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.15	0.317
0.20	0.50	3.98	0.251
0.30	0.333	4.25	0.235
0.45	0.222	4.43	0.226
0.50	0.20	4.50	0.222

Table 2.12

2 mM Methyl Phosphinate

[Substrate] (mM)	1/[Substrate] ($\times 10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	2.86	0.350
0.20	0.50	3.69	0.271
0.30	0.333	4.10	0.244
0.45	0.222	4.41	0.227
0.50	0.20	4.48	0.223

Table 2.13



Graph 2.3: Lineweaver-Burk plot for Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Methyl Phosphinate.

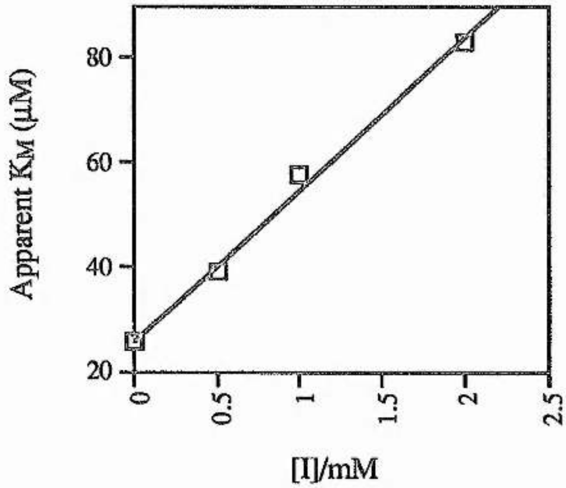
For the methyl phosphinate it can be seen that K_M varies while V_{max} is constant which implies that the methyl phoshinate may also be a competitive inhibitor.

The data and plot for K_{app} versus [I] for the kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of the methyl phosphinate analogue are shown in table 2.14 and graph 2.4 respectively. Thus from the gradient of this graph we can determine K_I as K_M is already known. Linear regression analyses was employed to determine the best straight line and errors were obtained from the regression analyses.

Inhibitor	**Inhibitor concentration (mM)**	**V_{max}^{a} (x 10^{-3}) (mol dm^{-3} min^{-1})**	**K_{app} (μM)^a**
No inhibitor	0 mM	1.07 ± 0.01	25.5 ± 0.6
Methyl phosphinate	0.5 mM	1.09 ± 0.01	39.0 ± 0.3
Methyl phosphinate	1.0 mM	1.12 ± 0.02	57.6 ± 0.4
Methyl phosphinate	2.0 mM	1.16 ± 0.03	83.1 ± 0.9

$^a V_{max}$ and K_{app} were obtained from fitting the data to the Michaelis-Menten equation by non linear regression using the ENZFITTER[151] programme.

Table 2.14



graph 2.4: Plot of K_{app} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Methyl Phosphinate Analogue.

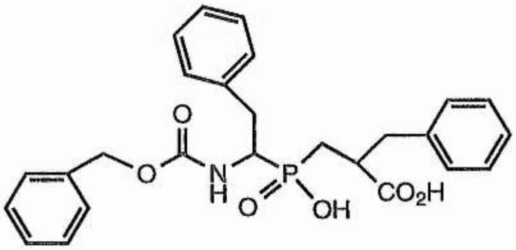
From the graph,

The K_I for the methyl phosphinate analogue = **0.88 ± 0.05 mM**

This implied that the methyl ester improved binding relative to the free acid, by almost five fold.

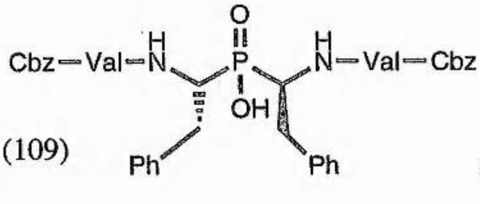
2.6.3 Discussion of Enzyme Results.

It can therefore be seen that preparation of the phosphinate ester does improve the inhibitory properties, presumably by removing the negative charge and hence its interaction with the active site, as discussed earlier. Phosphinic acid inhibitors of pepsin are amongst the most potent reported.[126] However, initial attempts to get similar results with HIV-1 proteinase were disappointing. This is possibly due to the anionic nature of the phosphinate at the assay pH of 6.0 (pK_a 3-3.5). The assay pH of pepsin was 3.5 thus, more protonated phosphinate should be present which could account for greater inhibition as there is precedent for the repulsion of negatively charged species away from a negative aspartate.[125] This theory is backed up by an experiment with the HIV-1 proteinase and its interaction with the phosphinic acid isostere of the dipeptide Cbz-Phe-Phe (139). This was found to be a moderately potent inhibitor of HIV-1 proteinase with a K_I of 26 μM at pH 6.5 and 1 μM at pH 4.5. The inhibition increases at lower pH possibly due to the presence of the more protonated phosphinate.[124]



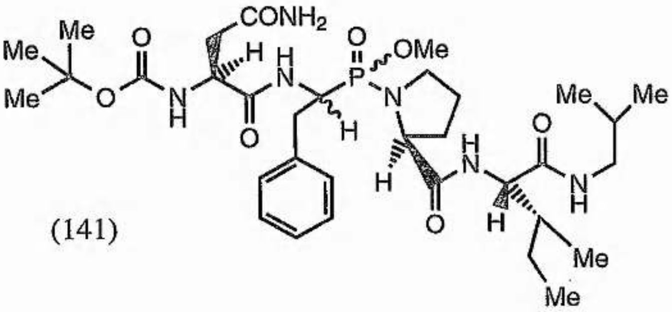
(139)

A potent phosphinic acid inhibitor, SB 204144 (140), has been reported by Abdel-Meguid and co-workers which had a K_I of 2.8 nM at pH 6.0.[152] Their pH analysis failed to unambiguously answer the question of whether the inhibitor would be more potent at lower pH, however, crystallographic results indicated the presence of a protonated phosphinate.



(109) (140)

Gani et al. have synthesised stereochemically defined and epimeric phosphonamidate methyl ester containing peptide analogues (141, and related structures) of the HIV-1 proteinase.[153]

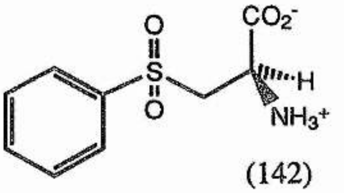


(141)

They reasoned that the lack of potency of phosphonamidate inhibitors for the HIV-1 proteinase stems from their negatively ionised nature at the pH optimum for the enzyme which results in repulsive interactions between the inhibitor and the negatively charged active site carboxylate group. This problem of ionisation is not inherent in the phosphonamidate methyl ester analogues and consequently these neutral compounds have proved to be better inhibitors with IC_{50} values from 1-100 μM. However, Gani *et al.*[153] and McLeod *et al.*[127] noticed only small differences in the potencies of the phosponamidates and the corresponding methyl esters as inhibitors for HIV-1 proteinase.

It is interesting that neither of our phosphorus compounds are as potent inhibitors of kynureninase as the sulphone transition state analogue, *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide (97), prepared by Phillips and Dua, which had a K_I of 70 nM.[129] The reduced binding may in part result from the absence of the *ortho*-amino group. The K_I for racemic desaminokynurenine under our conditions was 23.2 ± 3.1 μM (see Chapter 3) and the rate of turnover was approximately twenty times less than that of kynurenine, implying that the k_{cat} is reduced twenty times. As noted by Bartlett and Marlowe the inhibition constant K_I for a transition state analogue should be related to the K_M/k_{cat} value of the corresponding substrate.[123] Thus it might be predicted that the *ortho*-amino substituted analogue of the methyl phosphinate would have a K_I of approximately 44 μM, still about six hundred times greater than that of the sulphone. Interestingly, with the *S,S*-dioxide an *ortho*-amino group gave a fifty five times increase in the observed K_I value.[129] The K_I for *S*-(2-aminophenyl)-2*S*-cysteine *S,S*-dioxide (142) was 0.07 μM whereas the K_I for *S*-(phenyl)-2*S*-cysteine *S,S*-dioxide (142) was 3.9 μM.

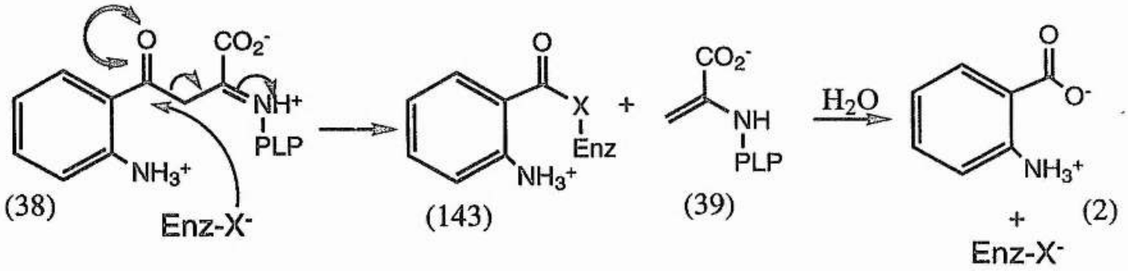
One reason for the poor binding is that the methyl ester may be too large for the active site. However, as there is no crystal structure of kynureninase at present it is difficult to be sure of this. Whether the poor binding of the phosphinic acid transition state analogues has any implications for the proposed general base catalysed mechanism of kynureninase is as yet unclear. This mechanism will also be discussed in relation to the results of the solvent isotope effect studies (see Chapter 4).



(142)

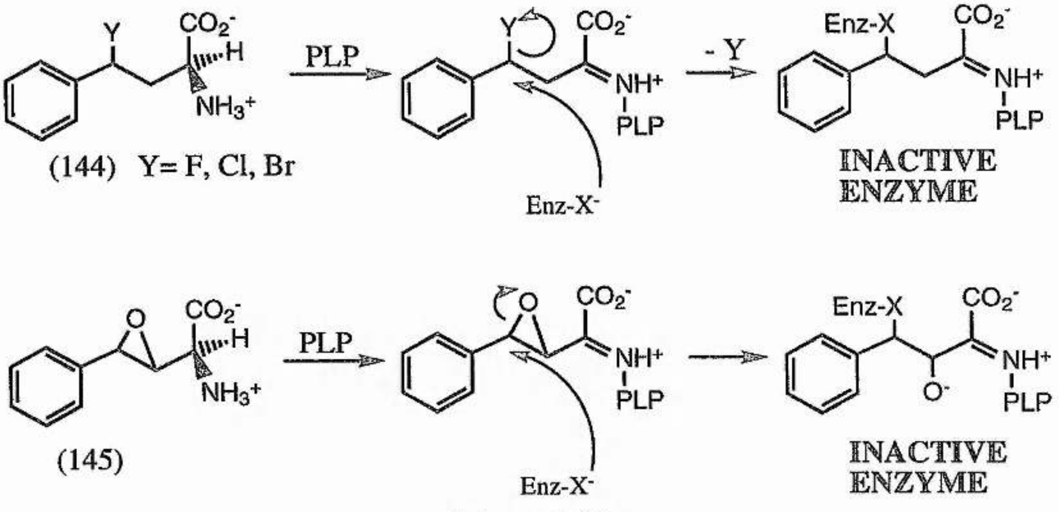
2.7 THE ENZYMIC NUCLEOPHILE MEDIATED REACTION.

As mentioned previously the hydrolysis of kynurenine could also be mediated by an enzymic nucleophile (Scheme 2.28). In this case the ketimine intermediate (38) is attacked by an enzymic nucleophile at the γ-carbonyl group. Cleavage of the β,γ-carbon-carbon bond then takes place, thus giving an α-aminoacrylate derivative (39) and a covalent intermediate (143). Reaction of this with water gives anthranilate (2) and regenerates the enzymic nucleophile.



Scheme 2.28

One method to probe for the nucleophile mediated mechanism is to design inhibitors to alkylate the putative active site nucleophile. Examples of these would be the fluoro, chloro and bromo derivatives (144) and the epoxide (145). These compounds should form the Schiff's bases with the coenzyme but when attacked by the nucleophile they will become covalently attached to the enzyme. This is an example of irreversible inhibition (Scheme 2.29). If the inhibitors do indeed become irreversibly attached to the protein they could then be prepared in a radioactively labelled form. Covalent attachment to the protein and digestion of the inactive enzyme should then produce a number of labelled peptides. Purification and analysis of the peptides would then allow identification of amino acid residues at the active site. This would be the first structural information regarding the active site of kynureninase.

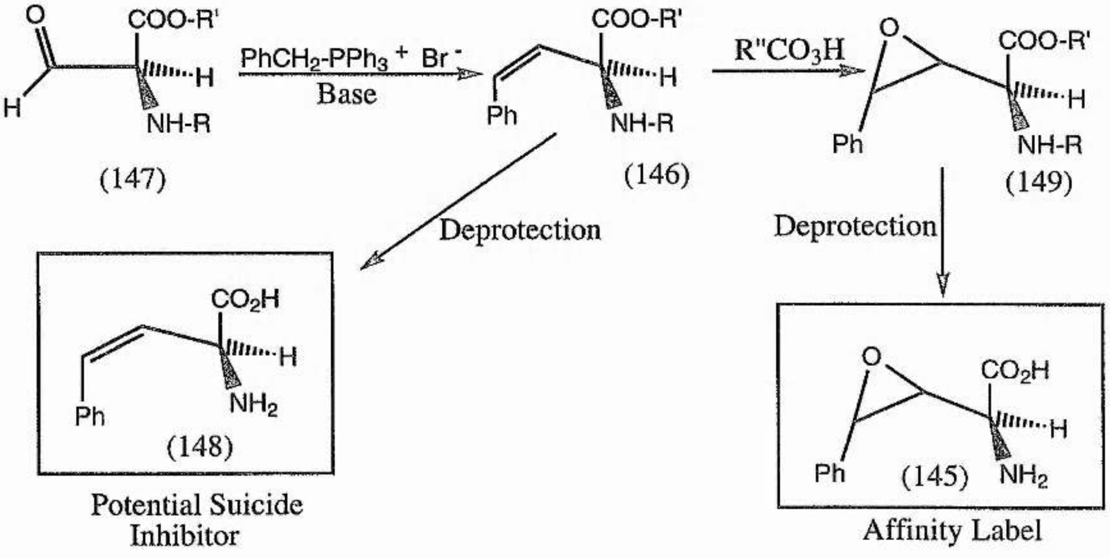


Scheme 2.29

Synthetic studies towards the preparation of the epoxide analogue (145) were undertaken.

2.7.1 Design of Proposed Epoxide Analogue.

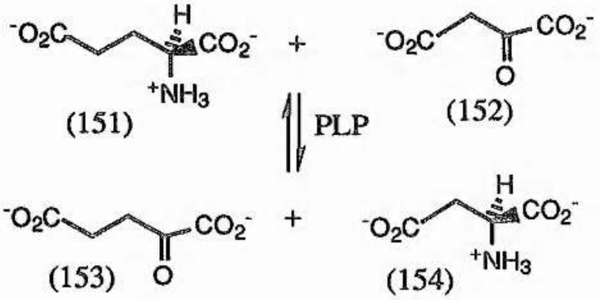
A possible synthetic route to the epoxide (145) is given in Scheme 2.30.

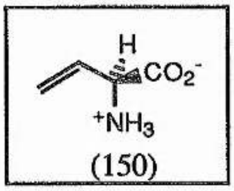


Scheme 2.30

The key intermediate is the alkene (146). The alkene could be prepared by using Wittig chemistry with the serine aldehyde (147) and the ylid derived from the alkyltriphenylphosphonium salt. The alkene (148) is itself a potential suicide inhibitor of kynureninase. Reaction of the alkene with meta-chloroperbenzoic acid should then give the epoxide (149). Theoretically it should be possible to carry out the deprotection before or after this epoxidation step.

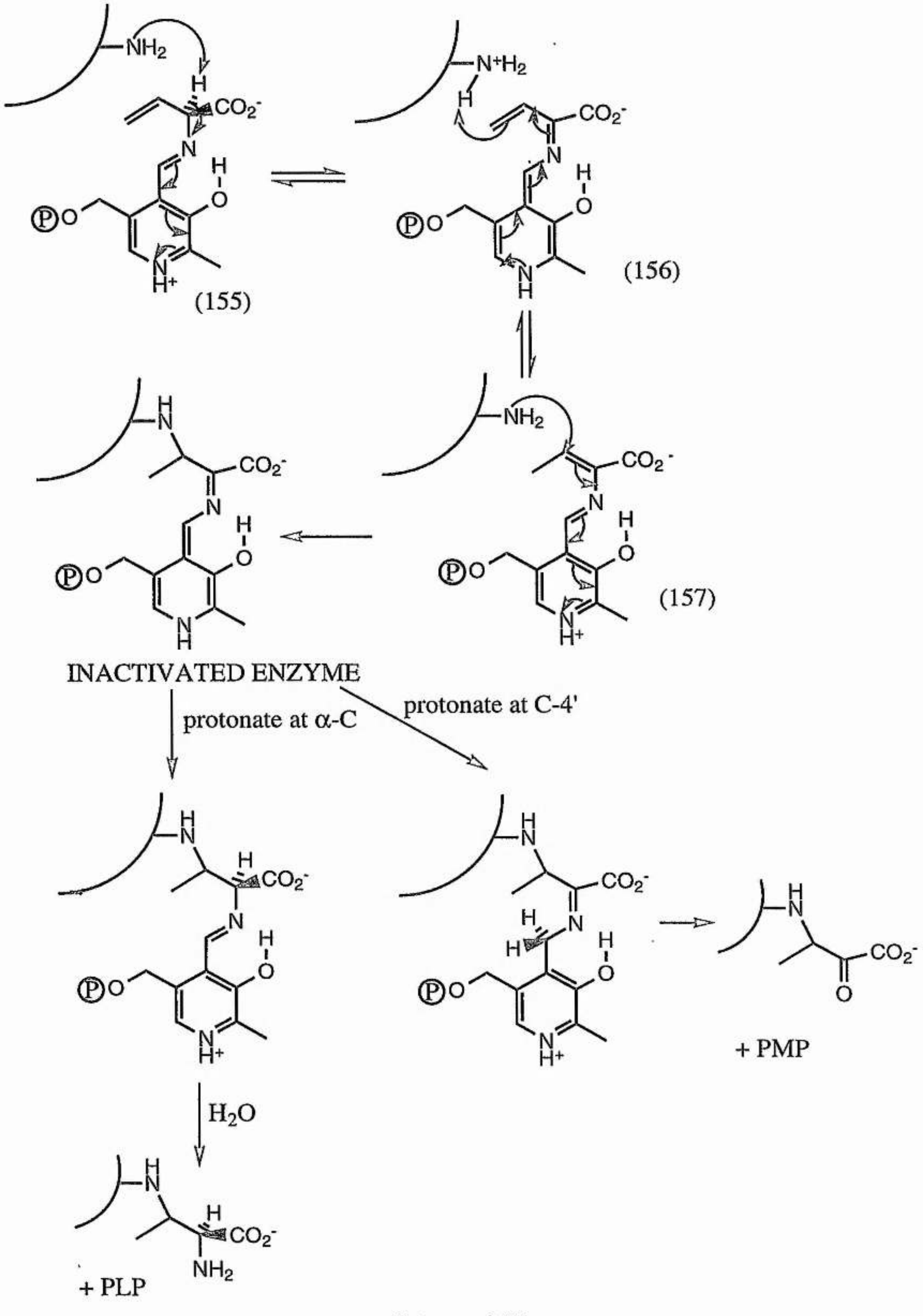
There are many examples of alkene inhibitors of PLP dependent enzymes, such as vinyl glycine (150) an inhibitor of aspartate aminotransferase.[154] Aspartate amino transferase catalyses the amino transfer from glutamate (151) to oxaloacetic acid (152) to give α-ketoglutaric acid (153) and aspartate (154) (Scheme 2.31).





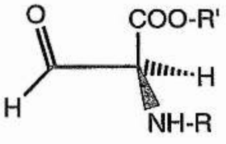
Scheme 2.31

The vinyl glycine PLP complex (155) fits in the same orientation as the glutamate PLP complex and after α-hydrogen abstraction gives the quinoid intermediate (156). Protonation then occurs at the double bond, instead of at C-4' as in the normal enzyme catalysed reaction. This results in the formation of a Michael acceptor (157) which undergoes conjugate addition producing inactivated enzyme. Either protonation at the α-C or at C-4' furnishes a product which is irreversibly attached to the enzyme (Scheme 2.32).


(155)
(156)
(157)
INACTIVATED ENZYME
protonate at α-C
protonate at C-4'
H₂O
+ PMP
+ PLP

Scheme 2.32

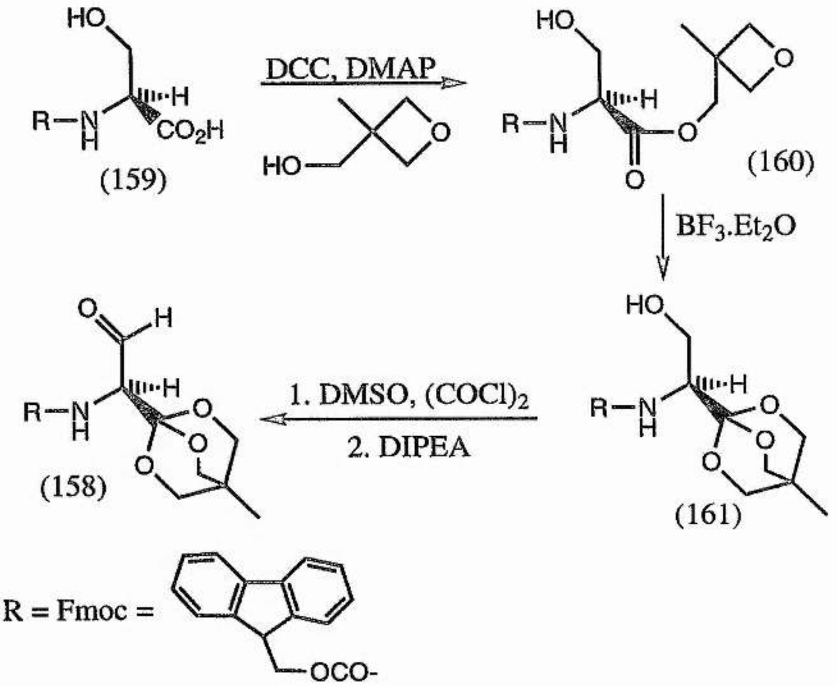
Therefore, the first target on the route to the epoxide analogue is a chiral serine aldehyde synthon. Literature syntheses of some routes to suitable compounds are outlined below.



(147)

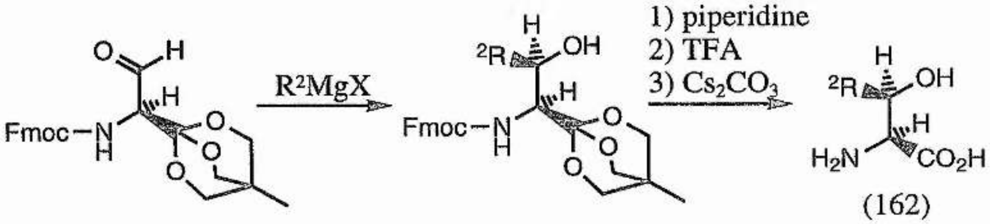
2.7.1.1 *N*-(9-Fluorenylmethyloxycarbonyl)-(1*S*)-1-amino-2-hydroxyethyl-4-methyl-2,6,7-trioxabicyclo[2.2.2]octane (Fmoc-*S*-Serine Ortho ester).

Blaskovich and Lajoie[155] prepared a base stable cyclic-*ortho* ester (158) serine aldehyde equivalent. The reason for the *ortho*-ester protecting group can be explained as follows. If the protecting group was, for example, a methyl ester, this would mean that the α-proton would be quite acidic due to the electron withdrawing effects of both protecting groups. This would mean that the α-centre would racemise under Wittig conditions, that is, in the presence of base the stereochemistry would be lost. In contrast, with the less electron withdrawing *ortho*-ester as protecting group, the α-proton is much less acidic and thus should alleviate the problem. Thus the amine group was protected as the N^{α}-9-fluorenyl-methoxycarbonyl (Fmoc) (159) derivative. The oxetane ester (160) was prepared from Fmoc-2*S*-serine and 3-methyl-3-(hydroxymethyl)-oxetane using dicyclohexylcarbodiimide (DCC), and dimethylaminopyridine (DMAP) to effect the coupling. The oxetane ester was then converted to the *ortho*-ester (161) using boron trifluoride etherate and oxidation under Swern conditions gave the aldehyde (158) (Scheme 2.33).



Scheme 2.33

The *ortho*-ester has been reacted with Grignard reagents and used in a Reformatsky reaction, as shown below. Thus Grignard addition with MeMgBr at room temperature resulted in protected 2*S*-threonine in 77% yield. Similarly reaction with PhMgBr at room temperature provided protected 2*S*-phenylserine in 85% yield (Scheme 2.34, Table 2.15). The resulting β-hydroxy intermediates were deprotected with piperidine. After evaporation the free amino derivative was reacted with aqueous trifluoroacetic acid (TFA) to give the dihydroxyester. Finally hydrolysis with Cs_2CO_3 gave the free amino acid (162).

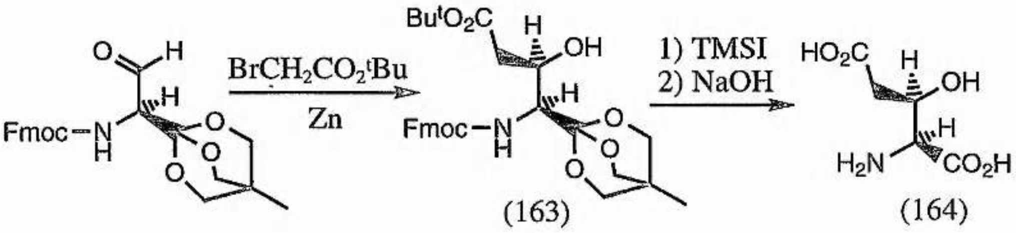


Scheme 2.34

R	**Yield (162, %)**	**2*S*,3*R*: 2*S*,3*S***	**e.e. (%)**
Me	77	84: 16	98
Ph	85	83: 17	98

Table 2.15

Reformatsky reaction of the aldehyde with the organozinc compound derived from ᵗbutyl bromoacetate gave a β-hydroxyglutamic acid derivative (163) in 73% yield as a 92: 8 mixture of (2*S*,3*R*: 2*S*,3*S*) diastereomers. Deprotection with trimethylsilyl iodide removed all the protecting groups to give (2*S*,3*R*)-β-hydroxyglutamic acid (164) in good yield with >98% e.e. (Scheme 2.35).

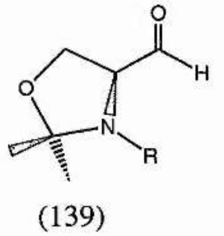


(163) (164)

Scheme 2.35

2.7.1.2 The Garner Aldehyde.

The most popular serine aldehyde equivalent is the Garner aldehyde. The acetonides (165) were introduced by Garner in 1984 and are noted for their synthetic versatility.[156] They have been prepared with various nitrogen protecting groups (165 a to d).[157]

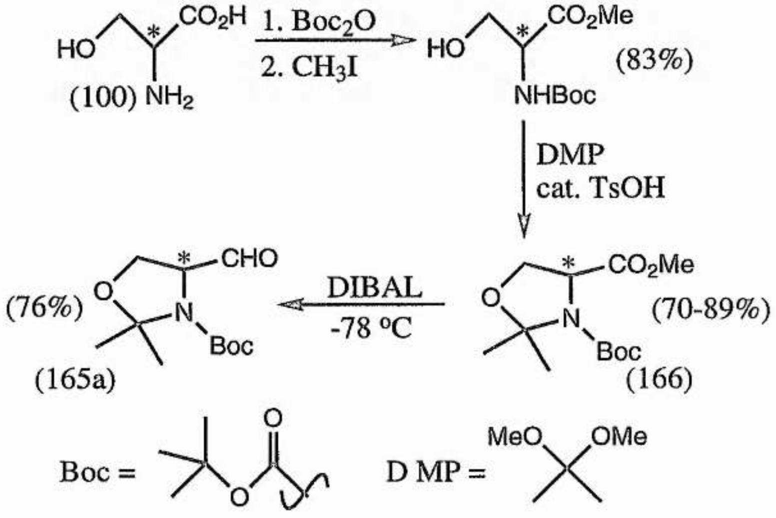


(139)

a) R: $CO_2{}^tBu$
b) R: CO_2CH_2Ph
c) R: CO_2CH_3
d) R: CH_2Ph

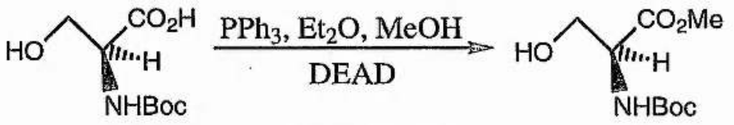
It should be noted that with this synthon, the carboxylic acid of serine becomes the aldehyde. On deprotection, what was originally the alcohol becomes the carboxylic acid of the new amino acid derivative and so as a result 2*R*-serine is eventually converted to the 2*S*-amino acid derivative.

The synthesis begins with protection of the amino group of serine (100) with di-*tert*-butyldicarbonate and formation of the ester using iodomethane and potassium carbonate in DMF. The oxazolidine (166) was then prepared by reaction with 2,2-dimethoxypropane and a catalytic amount of *p*-toluenesulphonic acid monohydrate. Reduction of the ester with diisobutylaluminium hydride (DIBAL) gave the desired ester. The product (165a) was obtained in good yield and was determined to be 95% enantiomerically pure (Scheme 2.36).



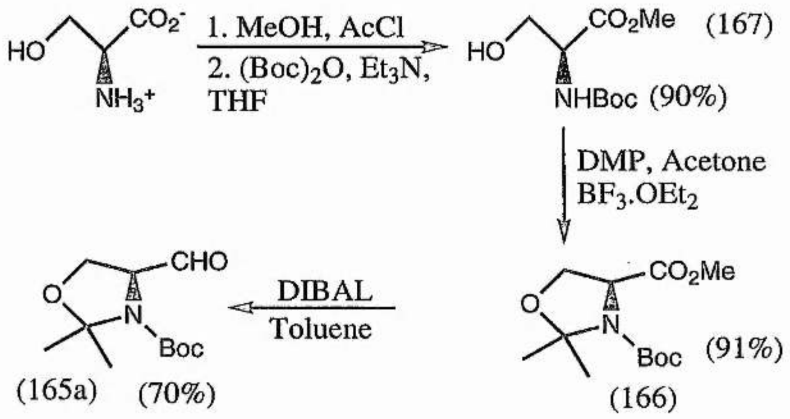
Scheme 2.36

Alternative syntheses of this intermediate have been developed, for example Branquet *et al.* overcame the limitation of using diazomethane by using the Mitsunobu reaction (Scheme 2.37).[158]



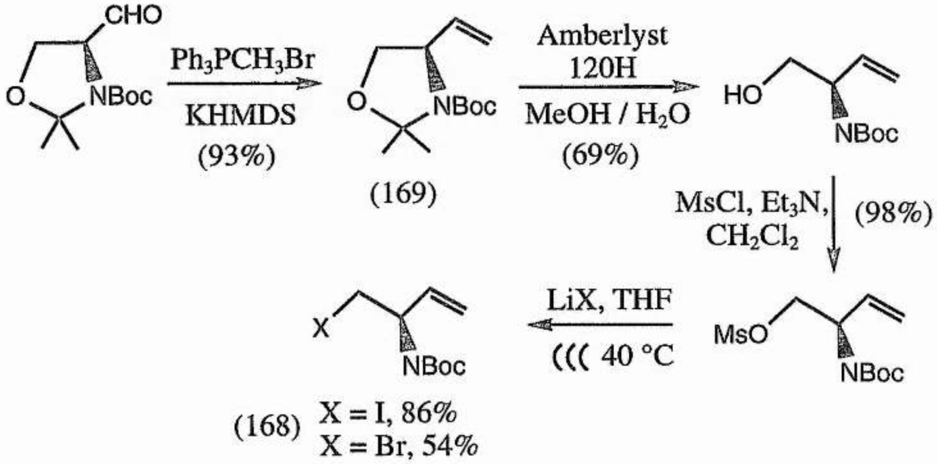
Scheme 2.37

McKillop and co-workers carried out the esterification of serine by reaction with methanol and acetyl chloride. The ester was then converted into the carbamate (167) by using di-*tert*-butyldicarbonate in THF and triethylamine. Formation of the oxazolidine (166) was achieved by the use of 2,2-dimethoxypropane in acetone at room temperature with boron trifluoride etherate as the catalyst. The ester was then reduced with DIBAL following Garner's procedure (165a) (Scheme 2.38).[159]



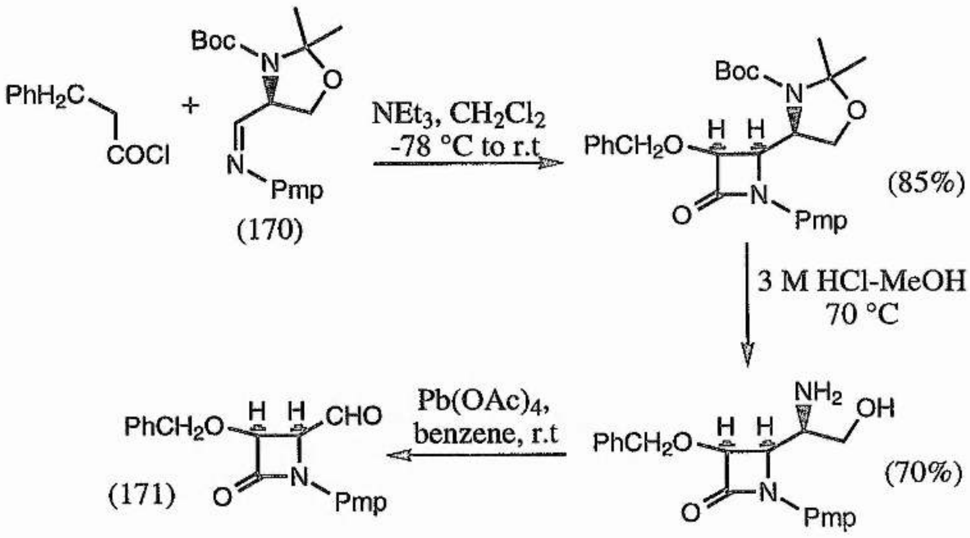
Scheme 2.38

The Garner aldehyde has been used in the synthesis of *N*-Boc halides (168), which are required for the synthesis of naturally occurring antibiotics.[160] Wittig olefination using potassium bis(trimethylsilyl)amide (KHDMS) was carried out. Hydrolysis of the acetonide (169) using Amberlyst 120H resin then gave vinyl glycinol. Mesylation of the alcohol followed by displacement using lithium iodide or lithium bromide in THF with ultrasonication gave the desired *N*-Boc halides (168) (Scheme 2.39).[159]



Scheme 2.39

The Garner Aldehyde has also been used in the stereoselective synthesis of monocyclic β-lactams. This can be achieved by cycloaddition between benzyloxyacetyl chloride and the imine derived from N,O-diprotected 2*S*-serinal (170).[161, 162] The product was isolated in 85% as a single diastereomer which could be converted into the formyl derivative (171). This was achieved by simultaneous N,O deprotection of both aminoketal and Boc groups to afford the β-lactam in 70% yield. The amino alcohol was then cleaved by treatment with a slight excess of lead tetraacetate at room temperature in benzene to give the formyl derivative in almost quantitative yield (Scheme 2.40).[163]

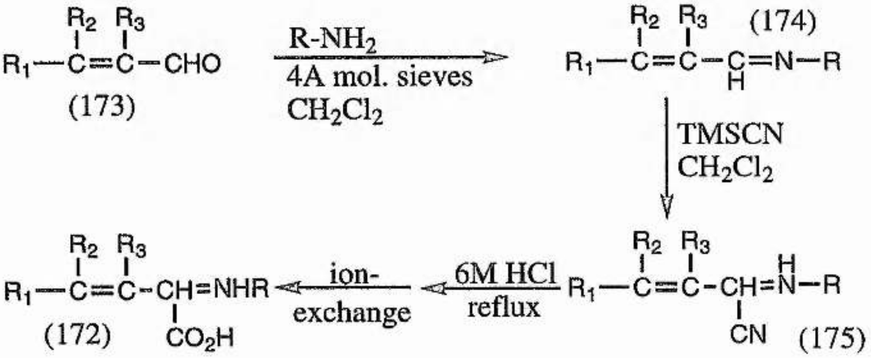


Pmp = *p*-methoxyphenyl

Scheme 2.40

2.7.1.3 The Strecker Synthesis.

Another possibility is to investigate the racemic synthesis of the β,γ-unsaturated amino acid (172). This would provide a good comparison with the stereospecific alkene (148), synthesised *via* the chiral serine aldehyde derivative, when these compounds are incubated with the enzyme. A method has been devised by Greenlee[164] which utilises a Strecker reaction.



Scheme 2.41

Treatment of α,β-unsaturated aldehydes (173) with primary amines in the presence of molecular sieves afforded the corresponding imine (174). These were then subjected to the action of trimethylsilyl cyanide, which, by 1,2-addition, produced β,γ-unsaturated aminonitriles (175). Direct treatment with aqueous HCl (6M) provided, after ion-exchange chromatography, the β,γ-unsaturated amino acids in moderate yields (Scheme 2.41).

When 4,4'-dimethoxybenzhydrylamine (176) was used as the amine component the dimethoxybenzhydryl group was cleaved under acidic hydrolysis which allowed isolation of the free amino acid (see table 2.16).

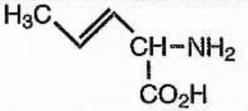
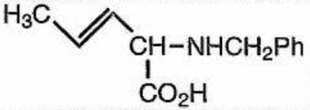
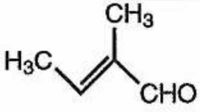
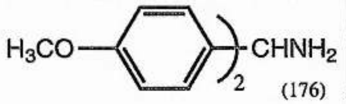
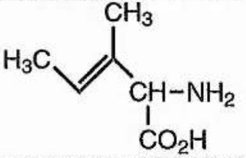
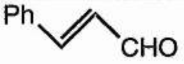
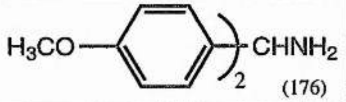
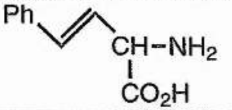
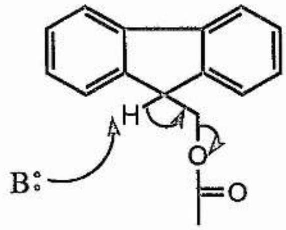
Aldehyde	**Amine**	**Product**	**Yield**
	 (176)		48
	$PhCH_2NH_2$		43
	 (176)		44
	 (176)		15

Table 2.16

2.8 ATTEMPTED SYNTHESIS OF SERINE ALDEHYDE EQUIVALENT.

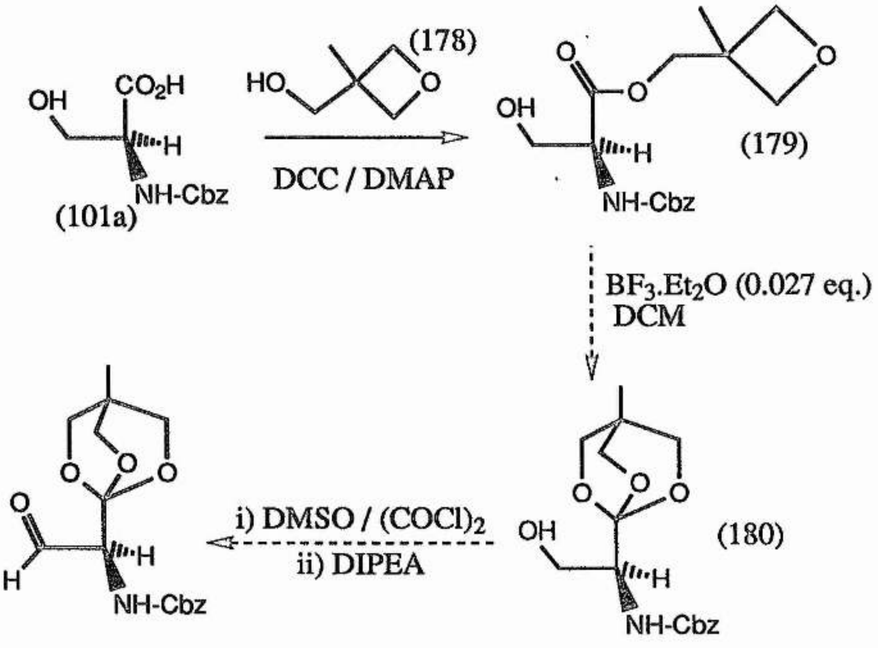
The *ortho*-ester as described by Blaskovich and Lajoie [155] was chosen as the most suitable synthon for our purposes. In this case, however, Cbz was used to protect the amino group instead of Fmoc (177). As the Fmoc group is removed under basic conditions (Scheme 2.42) and a base would also be required for the Wittig reaction, this protecting group is unsuitable for this route.



(177)

Scheme 2.42

The first step in the synthesis (Scheme 2.43) involved the preparation of Cbz-serine (101a) from serine (100) as described in section **2.4 (SYNTHESIS OF THE PHOSPHINIC ANALOGUE)** and was carried out in 77% yield. This was then added to a stirred solution of DCC, DMAP and oxetane alcohol (178) at 0 °C under nitrogen. As a different protecting group for the nitrogen, was used, Cbz instead of Fmoc, the starting material was not as soluble in dichloromethane. After the reaction was complete the solution was reduced in volume and dicyclohexylurea, the biproduct of the reaction, removed by filtration. The product was purified by column chromatography (silica, ethyl acetate: petroleum ether; 2: 1). Recrystallisation was carried out using ethyl acetate which helped remove the remaining dicyclohexylurea giving the product in 57% yield. The identity of *N*-Cbz serine oxetane ester (179) was confirmed by NMR and this could be achieved by comparing the product with oxetane alcohol. In oxetane alcohol the methylene group next to the alcohol is seen at δ = 3.45 ppm as a singlet and the ring methylene protons as two doublets at δ = 4.20 and 4.35 ppm (*J* 6 Hz, CH). In the product the singlet disappears and the six methylene protons can be seen as a multiplet at δ = 4.50 ppm. The methyl protons are also shifted from δ = 1.10 ppm in the alcohol to δ = 1.30 ppm in the product.



Scheme 2.43

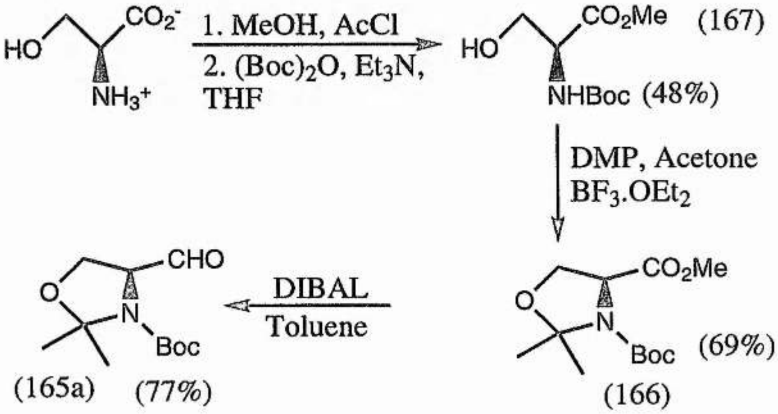
The next step was the preparation of the *ortho*-ester using boron trifluoride etherate. However, the formation of the *ortho*-ester proved to be problematic. Blaskovich and Lajoie stated that in order to isolate high yields of the *ortho*-ester, it was essential to maintain the concentration of boron trifluoride at less than 0.1 equivalent. The boron trifluoride was therefore added as a dilute solution in dichloromethane. However, the stability of this solution was under some doubt. A fresh stock of solution was prepared using freshly distilled boron trifluoride before each addition. Unfortunately, this did not improve the reaction. It was thought that an extended reaction time was also detremental as there seemed to be an equilibrium between the product and the starting material. This was investigated by reducing the reaction time but, this did not improve the yield of the product. As isolation of the *ortho*- ester was proving to be extremely difficult, it seemed reasonable to investigate whether the Cbz protecting group was causing any problem.

Therefore the literature method was repeated using the Fmoc protecting group on the nitrogen. Unfortunately, the formation of the *ortho*-ester proved to be as much of a problem, and was low yielding and difficult to purify.

At this stage other aspects of the project were thought to be more fruitful and work was discontinued. However, it was thought that the alternative route using the Garner aldehyde[157] would be worth pursuing.

2.8.1 Synthesis of Serine Aldehyde Equivalent.

Indeed further work in the group[165] has resulted in the synthesis of the aldehyde in 26% overall yield using the McKillop route.[159]

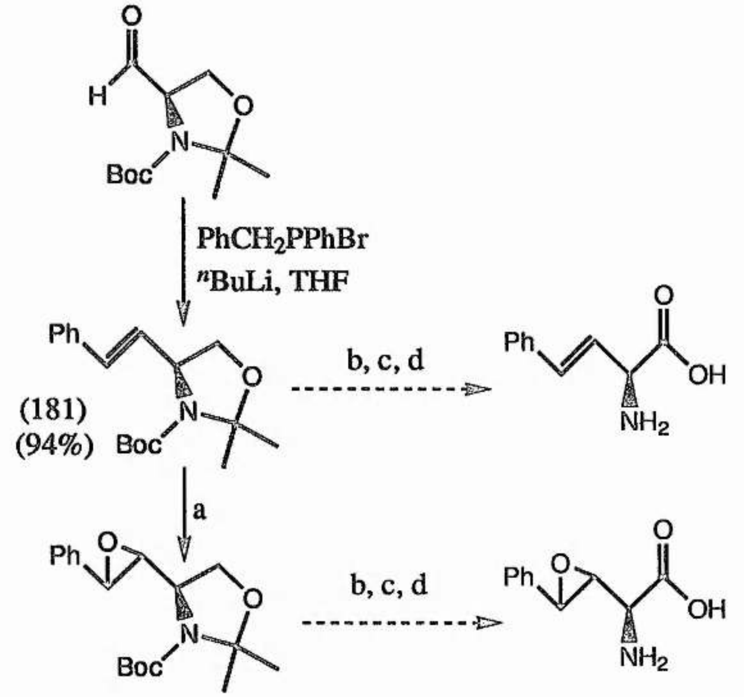


Scheme 2.44

Serine was refluxed with acetyl chloride in methanol to give methyl serinate hydrochloride with a yield of 82% and this was then used without further purification to produce the protected methyl ester using di-*tert*-butyldicarbonate in THF / triethylamine in 59% yield. The formation of the oxazolidine methyl ester was carried out by the use of 2,2-dimethoxypropane with boron trifluoride etherate as catalyst, distillation under reduced pressure gave the product in 69% yield. The aldehyde was then formed by reducing the ester with DIBAL and distillation under reduced pressure gave the product in 77% yield (Scheme 2.44).

2.8.1.1 Synthesis of Epoxide Analogue.

The alkene (181) was produced in good yield after optimisation of the reaction conditions, using Wittig chemistry, as a 3: 1 mixture of *E* and *Z* isomers which could be separated using column chromatography.



a) *m*CPBA, DCM; b) Amberlite IR-120, MeOH/H_2O;
c) CrO_3-H_2SO_4, Acetone/H_2O, 0 °C - RT; d) HCl, EtOAc, RT

Scheme 2.45

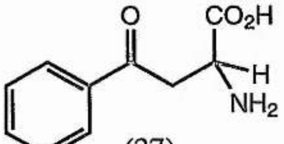
The epoxidation of the fully protected alkene was investigated and preliminary results indicated that *meta*-chloroperbenzoic acid was effective. The deprotection and oxidation steps now need to be optimised (Scheme 2.45). The deprotection conditions are very likely to cleave the epoxide. Therefore, it may be better to deprotect the alkene by hydrolysis of the aminoketal group, oxidise the alcohol to the acid and then epoxidise the double bond. Finally, removal of the Boc protecting group should give the epoxide target. Full deprotection and epoxidation of the free vinyl amino acid is also a possibility.

When the epoxide has been obtained its interaction with kynureninase will be examined kinetically in order to investigate the mechanism of hydrolysis in the enzyme catalysed reaction. The synthesis will obviously give a mixture of diastereomers of the epoxide. If inhibition is observed, then further information about the structure of the active site may be obtained by examining the relative inhibitory activities of the separated diastereomers. However, separation and / or stereospecific synthesis is probably only worthwhile if inhibition is observed with the mixture.

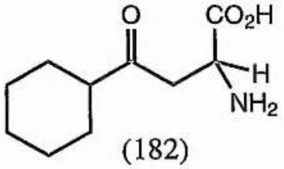
CHAPTER 3

3 ANALOGUES OF KYNURENINE.

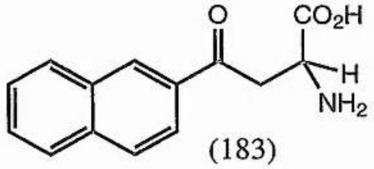
It was decided to prepare a number of kynurenine analogues and examine their activity with kynureninase in order to obtain information on the specificity of the enzyme and the important interactions at the active site. The compounds identified were the cyclohexyl (182) and naphthyl (183) amino acid derivatives as well as desaminokynurenine (27).



(27)



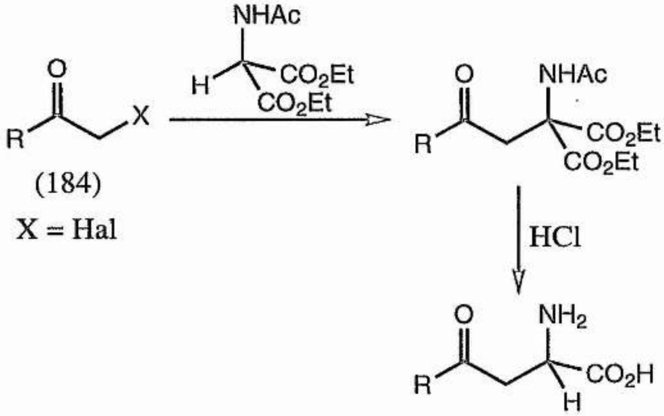
(182)



(183)

3.1 LITERATURE SYNTHESES OF RACEMIC KYNURENINE ANALOGUES.

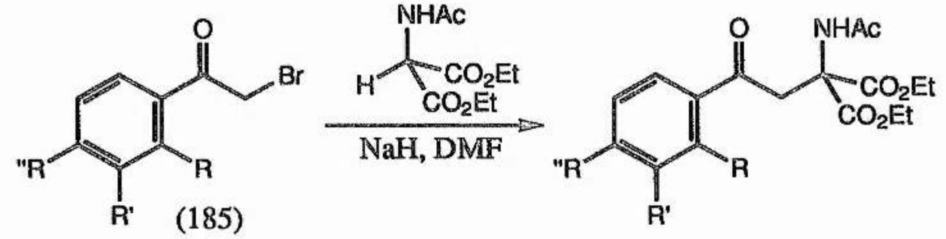
In the literature, essentially the same basic method has been employed for the synthesis of all racemic kynurenines. This involves the addition of diethyl acetamidomalonate to an appropriate α-haloacetophenone derivative (184), followed by decarboxylative hydrolysis (Scheme 3.1).



(184)
X = Hal

Scheme 3.1

Pellicciari *et al.* synthesised *ortho, meta* and *para* nitrobenzoyl alanines (66, 67 and 68 respectively).[52] Condensation of the nitrophenylacyl bromide (185) with diethyl acetamidomalonate was carried out in DMF at room temperature using sodium hydride to give the anion (Scheme 3.2).



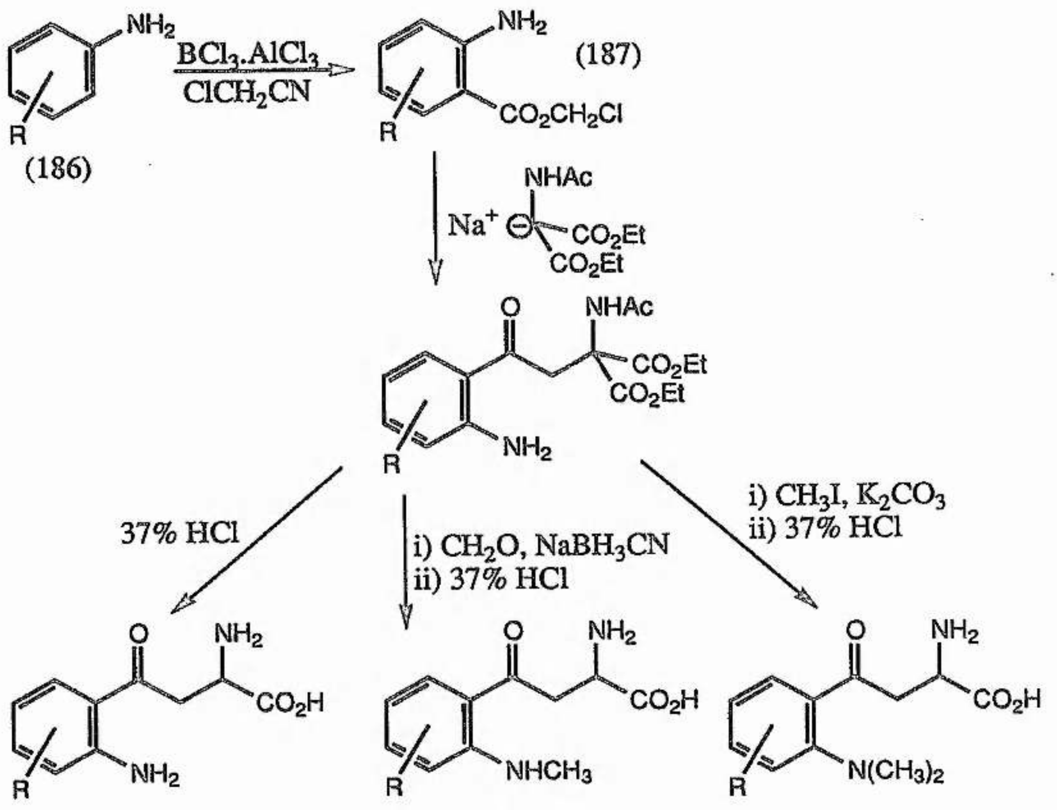
(185)

$R = NO_2; R' = H; R'' = H$
$R = H; R' = NO_2; R'' = H$
$R = H; R' = H; R'' = NO_2$

Scheme 3.2

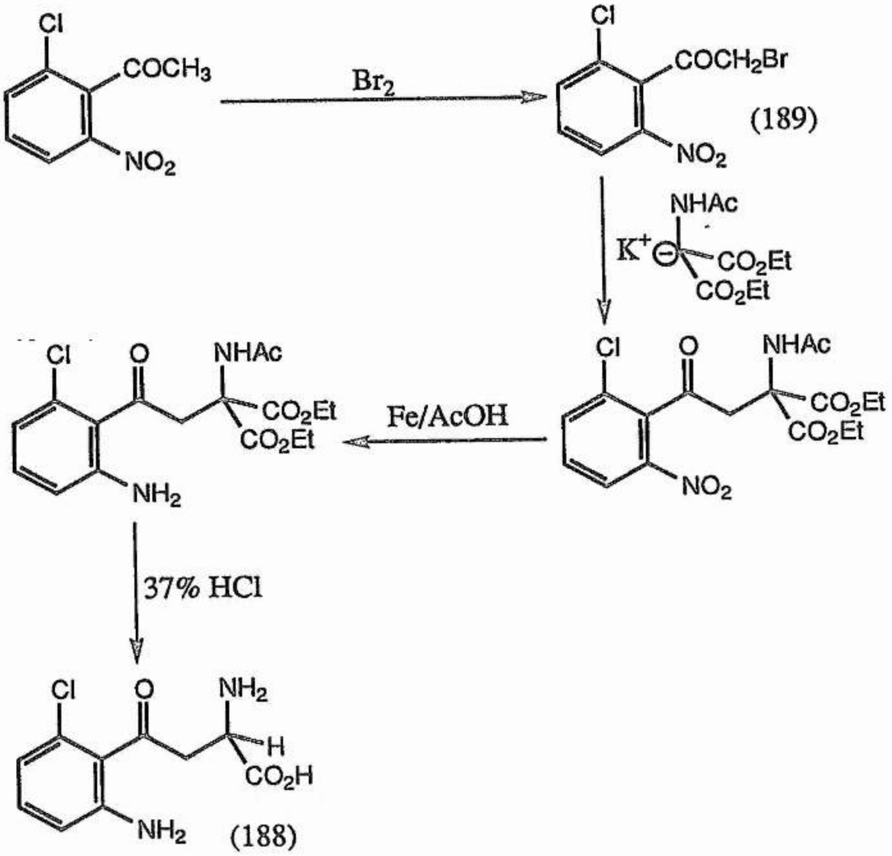
Hydrolysis was then achieved with 6M HCl followed by neutralisation with sodium hydrogen carbonate to give the product as a free base.

Varasi *et al.* prepared a number of kynurenine analogues which were substituted on the 3-, 4-, 5- and 6- positions of the benzene ring.[67] The syntheses began with the corresponding substituted alanines (186) which were converted to α-chloroacetophenones (187) under Houben-Hoesch conditions according to the procedure of Sugasawa.[166] The anion of diethylacetamidomalonate was prepared using sodium ethoxide in ethanol at 45 °C for one hour. After cooling a solution of the α-chloroacetophenone in ethanol was then added dropwise under nitrogen. The product was then refluxed in 37% HCl to afford the amino acid. Mono- or dimethylation of the aromatic amino group was achieved by treatment of formaldehyde / sodium cyanoborohydride or iodomethane / potassium carbonate before the hydrolytic step (Scheme 3.3).



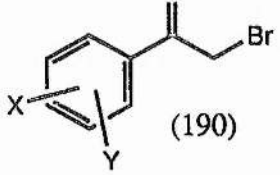
Scheme 3.3

6-Chlorokynurenine (188) was prepared from the corresponding 6-chloro-2-nitro-α-bromoacetophenone (189). Condensation with diethyl acetamidomalonate was followed by Fe / AcOH reduction of the nitro group and hydrolysis (Scheme 3.4).[67]



Scheme 3.4

Similar syntheses have also been carried out by Varasi[167] *et al.* starting from the substituted α-bromoacetophenone (190) and Schwarcz[66] *et al.* have prepared kynurenine derivatives starting from 4-substituted aniline.



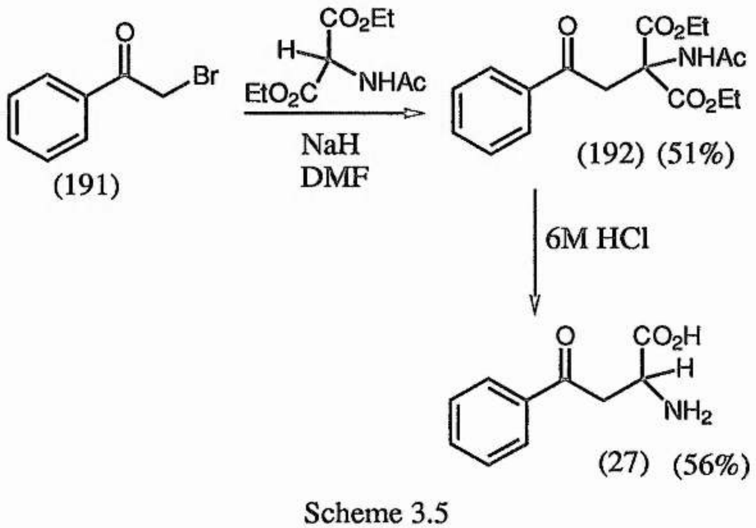
(190)

3.2 SYNTHESIS OF KYNURENINE ANALOGUES.

The method of Pellicciari was adopted and modified for the various analogues as described below.

3.2.1 Preparation of 2-amino-4-oxo-4-phenyl-butyric acid (desaminokynurenine, 27).

Diethyl acetamidomalonate in redistilled DMF was added to sodium hydride at 0 °C under a nitrogen atmosphere. Stirring was continued for three hours, a solution of the α-bromoacetophenone (191) in redistilled DMF was added and the resulting mixture stirred overnight. After extraction the residue was triturated with diethyl ether: dichloromethane, 3: 1 to give pure diethyl (phenacyl) acetamidomalonate (192) in 51% yield. Mass spectrometry confirmed the disappearance of bromine and the melting point (118-122 °C) agreed well with the literature value (118-119 °C).[168] Deprotection was then accomplished by refluxing in 6 M HCl. Upon cooling, the organic impurities were removed by washing with ethyl acetate. Trituration with acetone and neutralisation with 5% sodium hydrogen carbonate afforded the free base (27) as a solid. Recrystallisation from acetone / water yielded the microanalytically pure product in 56% yield (Scheme 3.5).

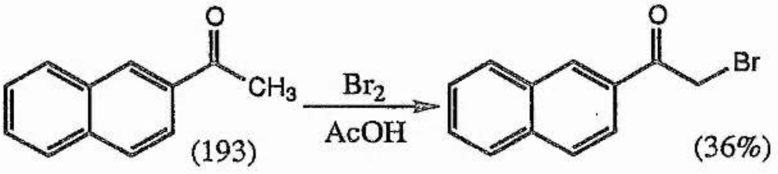


Scheme 3.5

The identity of the product was confirmed by mass spectrometry and the melting point (189-191 °C, decomposes 200 °C) agreed well with the literature value (190-200 °C, with decomposition).[169]

3.2.2 Bromination of methyl ketones.

For the preparation of the cyclohexyl and naphthyl analogues the corresponding bromo ketone was required. Bromination of 2-acetyl naphthalene (193) was initially attempted using the method of Vogel[170] (Scheme 3.6). Thus, 2-acetyl naphthalene was dissolved in glacial acetic acid and bromine was added dropwise very slowly over thirty minutes. After stirring for two hours the flask was cooled in an ice salt bath and the precipitated product filtered and washed with a little cold glacial acetic acid, followed by a small volume of water. Recrystallisation was then carried out from ethanol giving the product in only 36% yield. A small amount of the dibromo product was seen which could be identified by the appearance of the remaining α-proton at 6.10 ppm. In the desired product the methylene protons were observed at 4.60 ppm. As this reaction was low yielding an alternative was sought.



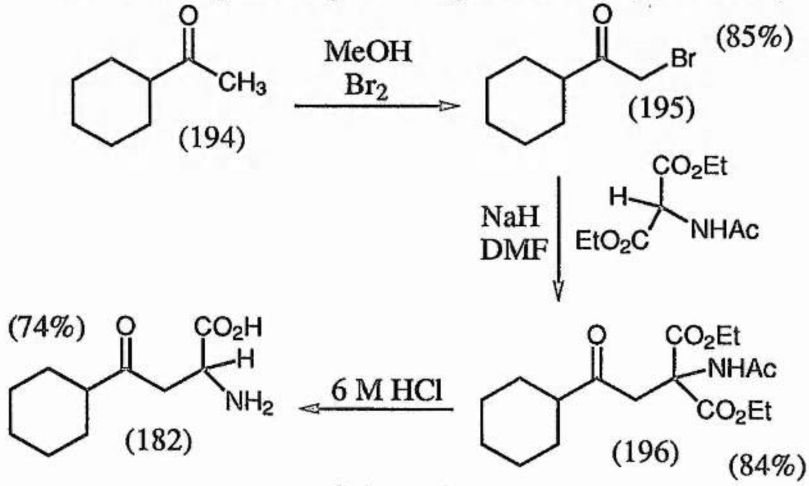
Scheme 3.6

Reaction using bromine in methanol, was then attempted as it is reported that dibromination is very slow in methanol. Indeed, the crude reaction products appeared to contain only traces of dibromoketones. Cyclohexyl methyl ketone (194) had also previously been brominated using this method.[171]

3.2.3 Preparation of 2-amino-4-oxo-4-cyclohexyl-butyric acid (cyclohexyl amino acid derivative, 182).

The first step was preparation of bromo-methyl cyclohexyl ketone (195). Thus, bromine was added slowly to cyclohexyl methyl ketone (194) in redistilled methanol. The reaction mixture was maintained at 5-6 °C during the reaction and was warmed to room temperature once the red colour had disappeared. After extraction the product was purified by distillation and gave a colourless liquid in 85% yield. ^1H NMR spectroscopy showed the appearance of the methylene protons which gave a signal at 3.9 ppm. There was a peak at 2.40 ppm in the ^1H NMR spectrum which could correspond to the protons that would be shifted with bromination occurring at 1'C on the ring, however this only appeared to be approximately 4% of the product.

In the second step, diethylacetamidomalonate in redistilled DMF was added to sodium hydride at 0 °C under a nitrogen atmosphere. Stirring was continued for three hours after which a solution of the bromo-methyl cyclohexyl ketone in redistilled DMF was added and the resulting mixture stirred overnight. After extraction the residue was triturated with diethyl ether: dichloromethane, 3: 1 to give pure diethyl (cyclohexane carbonyl) acetamidomalonate (196) in 84% yield. Mass spectrometry and NMR confirmed the structure of the product. Finally deprotection was accomplished by refluxing in 6 M HCl (Scheme 3.7).

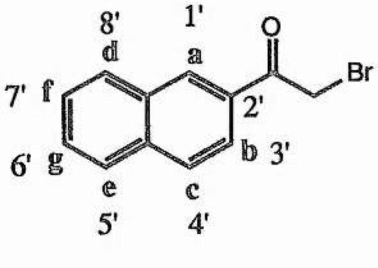


Scheme 3.7

Upon cooling the impurities were removed by washing with ethyl acetate and then trituration with acetone afforded the free base after neutralisation with 5% sodium hydrogen carbonate. This was recrystallised twice from acetone / water to give microanalytically pure product (182) in 74% yield.

3.2.4 Preparation of 2-amino-4-oxo-4-naphthoyl-butyric acid (naphthyl amino acid derivative, 183).

A similar method was used for the preparation of the naphthyl amino acid derivative (183), the first step being bromination of 2-acetyl naphthalene (193) (See Scheme 3.8). The 2-acetyl naphthalene precipitated out of solution when the reaction mixture was cooled, although it was seen to redissolve as the reaction proceeded, some starting material was also isolated. By increasing the amount of methanol the precipitation of the starting material was prevented and the reaction went smoothly to completion. Recrystallisation from ethyl acetate gave the pure product (197) in 91% yield. ^{1}H NMR spectroscopy showed the appearance of the methylene protons which gave a signal at 4.6 ppm. A ^{1}H-^{13}C COSY spectrum allowed the identification of all the carbon signals (Pages 123 and 124).



(197)

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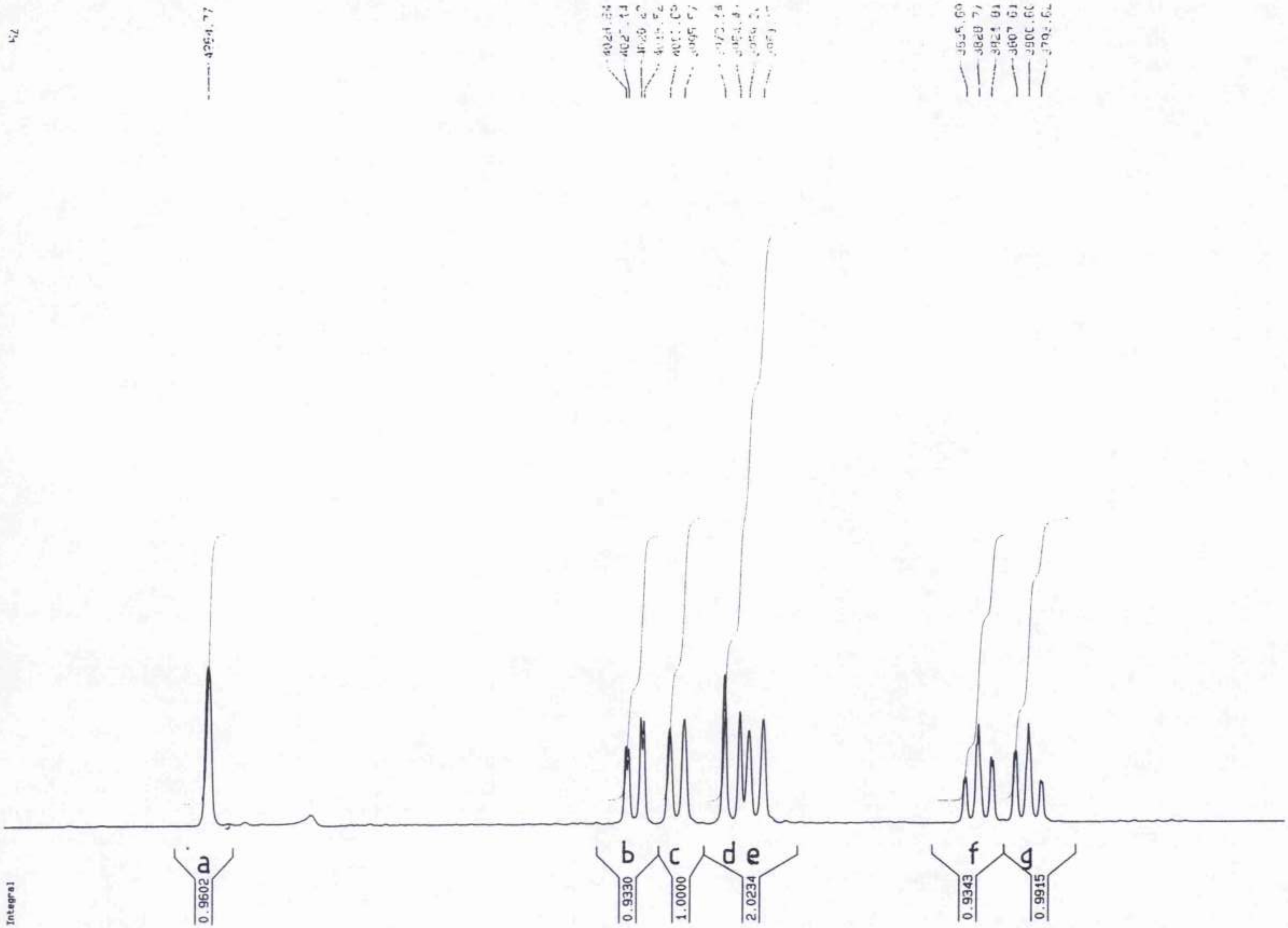

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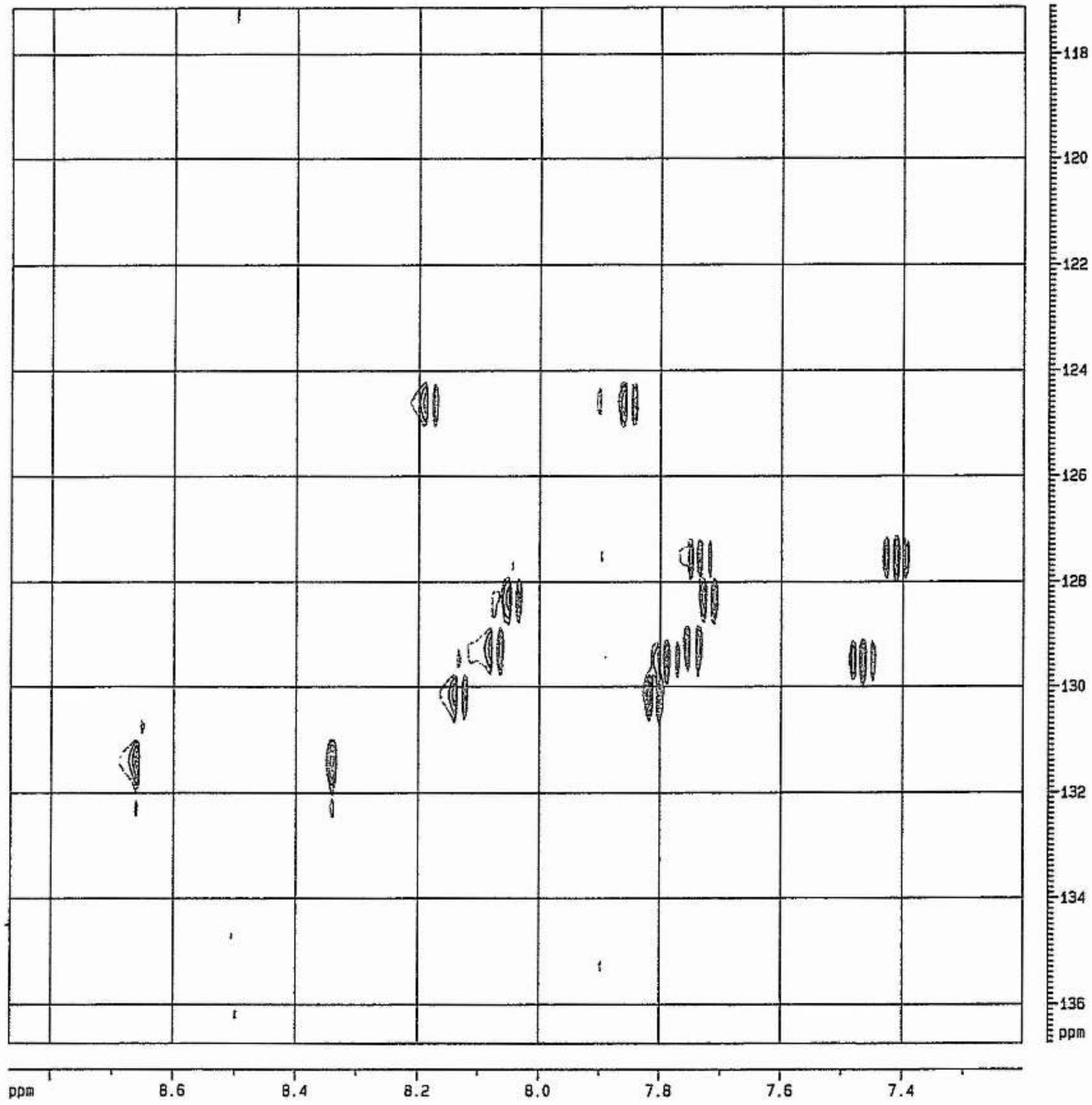

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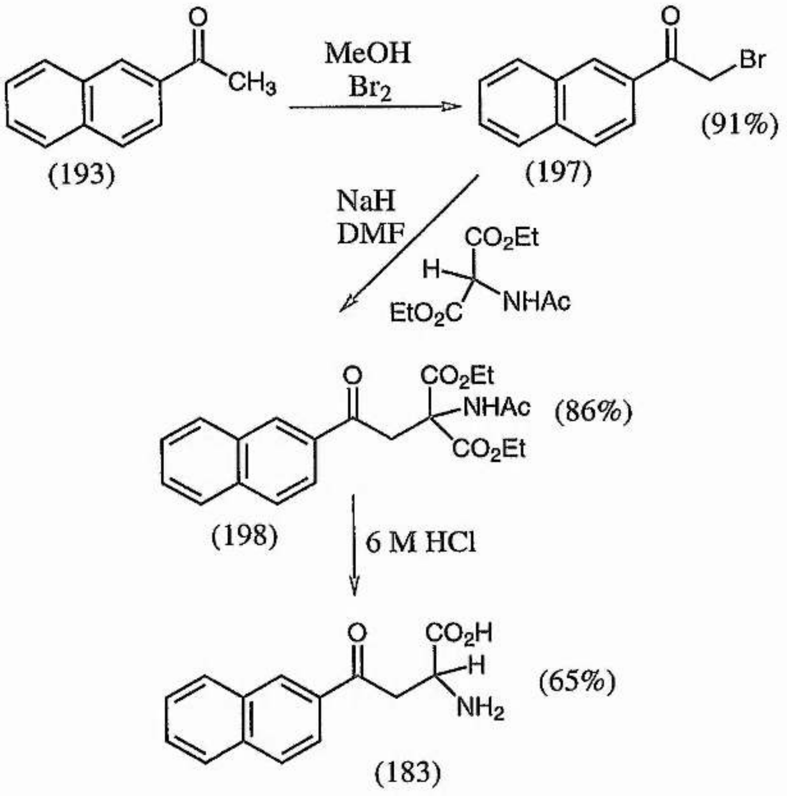
F2PHI              7.204
F1PLO            136.725
F1PHI            117.092
```

Number	Signal in ^{13}C spectrum (ppm)	Signal in ^{1}H spectrum (ppm)	Assignment in ^{1}H spectrum[A]
3'	124.1	8.05	b
6'	127.0	7.59	g
7'	127.9	7.92	e
5'	128.8	7.95	d
8'	129.0	7.65	f
4'	129.6	8.00	c
1'	130.9	8.52	a

[A] Spectrum shown on page 123 .

Table 3.1

The α-bromoketone was then reacted with diethyl acetamidomalonate as before to give diethyl (2-naphthoyl) acetamidomalomnate (198) which was purified by column chromatography (silica, ethyl acetate: hexane; 45: 55) to give a pale yellow solid in 86% yield. Mass spectrometry and NMR spectroscopy confirmed the structure of the product. Deprotection was carried out by refluxing in 6 M HCl. The impurities were removed by washing with ether as ethyl acetate was found to be too polar and trituration with acetone afforded the hydrochloride salt which was neutralised with 5% sodium hydrogen carbonate to give the free base. This was recrystallised from methanol to give microanalytically pure product in 65% yield (Scheme 3.8).



Scheme 3.8

3.3 TESTING OF ANALOGUES AS INHIBITORS OF KYNURENINASE.

Once the three kynurenine analogues had been successfully prepared, kinetic studies were carried out to examine their interaction with kynureninase. Initially work was focused on their action as inhibitors of the enzyme catalysed reaction. Thus the kinetic parameters for kynurenine (K_M and V_{max}) were determined in the presence of fixed concentrations of each analogue. The kinetic parameters of kynurenine without putative inhibitor were the average values calculated ($K_M = 25.5 \pm 0.6$ μM and $V_{max} = (1.07 \pm 0.01) \times 10^{-3}$ mol dm^{-3} min^{-1}, see experimental). The same experimental conditions were employed as for the normal assay of enzyme activity. Incubation was carried out at 25 °C and the assay buffer contained 0.4 mM kynurenine, 40 μM PLP in 0.04 M potassium phosphate at pH 7.0. The reaction was followed by UV spectophotometry by measuring the rate of decrease in absorbance at 360 nm due to conversion of kynurenine to anthranilic acid (see Experimental for full details). The analogues had limited solubility and sonication was often required to aid dissolution.

It was found that all three analogues acted as inhibitors of kynureninase to various degrees.

3.3.1. 2-amino-4-oxo-4-cyclohexyl-butyric acid (cyclohexyl amino acid derivative, 182).

The data obtained for the inhibition of kynureninase by the cyclohexyl amino acid is given in tables 3.2, 3.3, 3.4 and shown graphically in graph 3.1 using a Lineweaver-Burk plot. Each rate was determined two or three times until the values were consistent (± 3%) and the average values (± 1.7% from regression analyses) are shown. For the cyclohexyl amino acid K_M varies and V_{max} is constant which is possibly due to competitive inhibition.

0.05 mM Cyclohexyl Amino Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.72	0.269
0.20	0.50	4.02	0.249
0.30	0.333	4.24	0.236
0.45	0.222	4.40	0.227
0.50	0.20	4.49	0.223

Table 3.2

0.1 mM Cyclohexyl Amino Acid

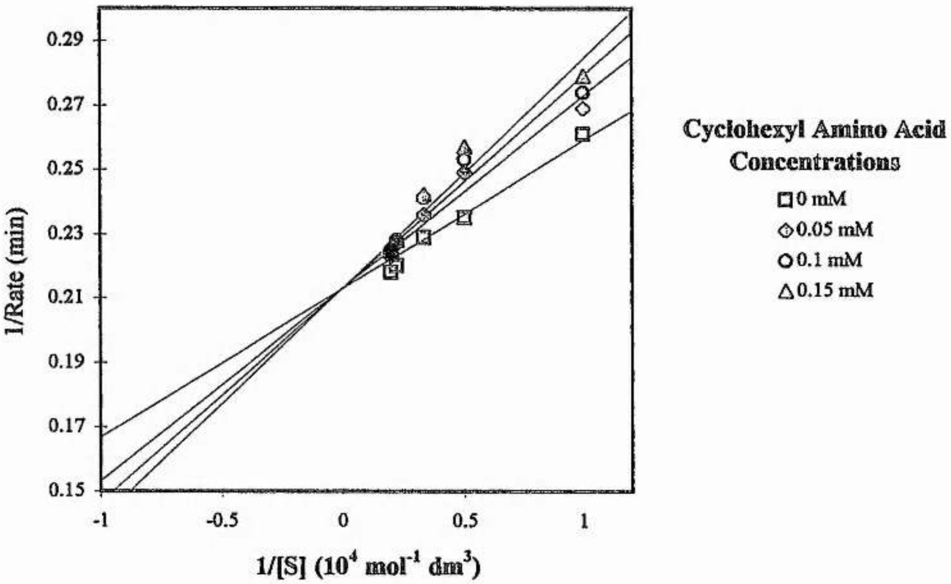
[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.65	0.274
0.20	0.50	3.96	0.253
0.30	0.333	4.15	0.241
0.45	0.222	4.39	0.228
0.50	0.20	4.44	0.225

Table 3.3

0.2 mM Cyclohexyl Amino Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.58	0.279
0.20	0.50	3.89	0.257
0.30	0.333	4.13	0.242
0.45	0.222	4.38	0.228
0.50	0.20	4.42	0.226

Table 3.4



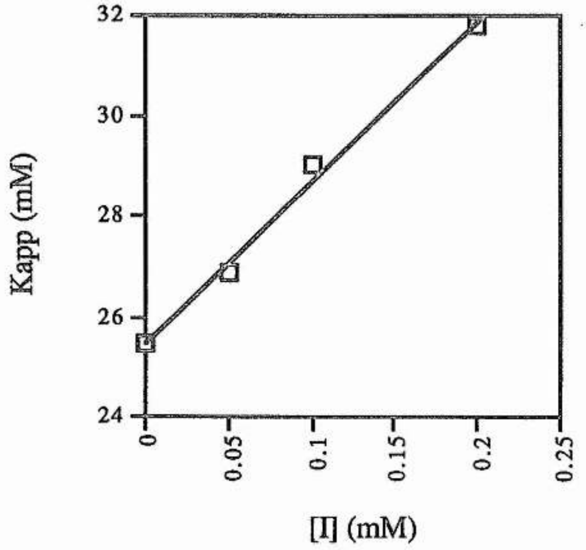
Graph 3.1: Lineweaver-Burk Plot for Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Cyclohexyl Amino Acid.

The data and plot for K_{app} versus [I] for the kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of cyclohexyl amino acid are shown in table 3.5 and graph 3.2 respectively. Thus from the gradient of this graph we can determine K_I as K_M is already known. Linear regression analyses was employed to determine the best straight line and errors were obtained from the regression analyses.

Inhibitor	**Inhibitor concentration (mM)**	**V_{max}^{a} (x 10^{-3}) (mol dm^{-3} min^{-1})**	**K_{app} (μM)**
No inhibitor	0	1.07 ± 0.01	25.5 ± 0.6
Cyclohexyl Amino Acid	0.05	1.04 ± 0.02	26.9 ± 3.6
Cyclohexyl Amino Acid	0.1	1.03 ± 0.02	29.0 ± 4.9
Cyclohexyl Amino Acid	0.2	1.03 ± 0.02	31.8 ± 5.4

[a] V_{max} and K_{app} were obtained from fitting the data to the Michaelis-Menten equation by non linear regression using the ENZFITTER[151] programme.

Table 3.5



graph 3.2: Plot of K_{app} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Cyclohexyl Amino Acid.

From the graph,

The K_I for the cyclohexyl amino acid derivative = 844 ± 47 μM

3.3.2. 2-amino-4-oxo-4-naphthoyl-butyric acid (naphthyl amino acid derivative, 183).

The data obtained for the inhibition of kynureninase by the naphthyl amino acid is given in tables 3.6, 3.7, 3.8 and shown graphically in graph 3.3. Each rate was determined two or three times until the values were consistent (± 3%) and the average values (± 1.1% from regression analyses) are shown. For the naphthyl amino acid K_M varies and V_{max} is constant which is possibly due to competitive inhibition.

0.05 mM Naphthyl Amino Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.82	0.262
0.20	0.50	4.27	0.234
0.30	0.333	4.36	0.229
0.45	0.222	4.67	0.214
0.50	0.20	4.70	0.213

Table 3.6

0.1 mM Naphthyl Amino Acid

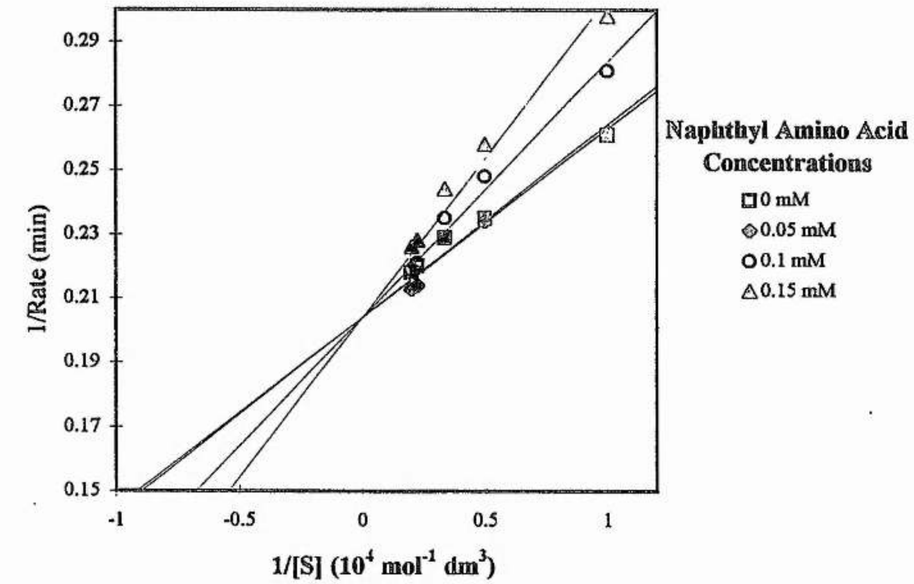
[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.56	0.281
0.20	0.50	4.04	0.248
0.30	0.333	4.26	0.235
0.45	0.222	4.52	0.221
0.50	0.20	4.56	0.219

Table 3.7

0.15 mM Naphthyl Amino Acid

[Substrate] (mM)	1/[Substrate] ($x10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	3.36	0.298
0.20	0.50	3.88	0.258
0.30	0.333	4.09	0.244
0.45	0.222	4.38	0.228
0.50	0.20	4.42	0.226

Table 3.8



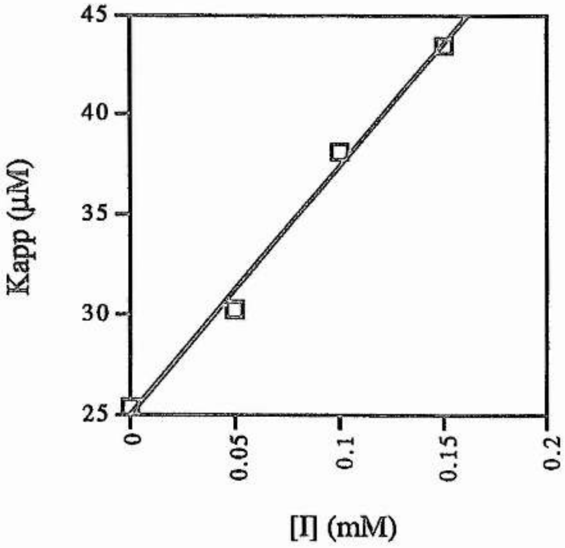
Graph 3.3: Lineweaver-Burk Plot for Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Naphthyl Amino Acid.

The data and plot for K_{app} versus [I] for the kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of the naphthyl amino acid are shown in table 3.9 and graph 3.4 respectively. Thus from the gradient of this graph we can determine K_I as K_M is already known. Linear regression analyses was employed to determine the best straight line and errors were obtained from the regression analyses.

Inhibitor	**Inhibitor concentration (mM)**	**V_{max}^{a} (x 10^{-3}) (mol dm^{-3} min^{-1})**	**K_{app} (μM)**
No inhibitor	0	1.07 ± 0.01	25.5 ± 0.6
Naphthyl Amino Acid	0.05	1.10 ± 0.02	30.2 ± 4.4
Naphthyl Amino Acid	0.1	1.08 ± 0.02	38.1 ± 3.5
Naphthyl Amino Acid	0.15	1.06 ± 0.02	43.4 ± 3.6

$^{a}V_{max}$ and K_{app} were obtained from fitting the data to the Michaelis-Menten equation by non linear regression using the ENZFITTER[151] programme.

Table 3.9



Graph 3.4: Plot of K_{app} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Naphthyl Amino Acid.

From the graph,

The K_I of the naphthyl amino acid derivative = **207 ± 16 μM**

3.3.3. 2-amino-4-oxo-4-phenyl-butyric acid (desaminokynurenine, 27).

The data obtained for the inhibition of kynureninase by the desaminokynurenine is given in tables 3.10, 3.11, 3.12 and shown graphically in graph 3.5. Each rate was determined two or three times until the values were consistent (± 5%) and the average values (± 4.6% from regression analyses) are shown.

0.05 mM Desaminokynurenine

[Substrate] (mM)	1/[Substrate] (x10^4 mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	0.891	1.12
0.20	0.50	0.983	1.02
0.30	0.333	1.30	0.769
0.45	0.222	1.35	0.741
0.50	0.20	1.47	0.680

Table 3.10

0.2 mM Desaminokynurenine

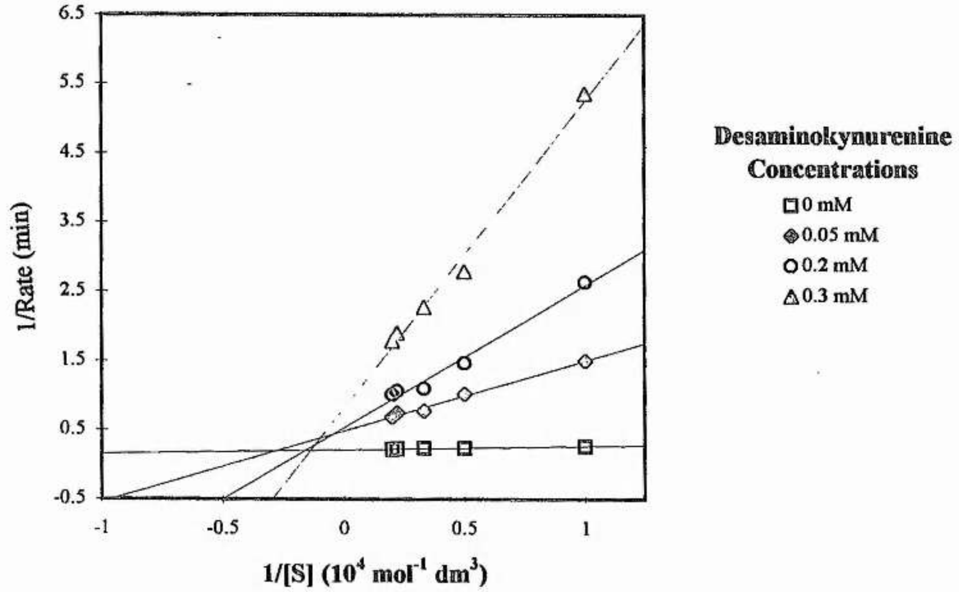
[Substrate] (mM)	1/[Substrate] (x10^4 mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	0.380	2.63
0.20	0.50	0.681	1.47
0.30	0.333	0.908	1.10
0.45	0.222	0.947	1.06
0.50	0.20	0.992	1.01

Table 3.11

0.3 mM Desaminokynurenine

[Substrate] (mM)	1/[Substrate] ($\times 10^4$ mol^{-1} dm^3)	Rate (min^{-1})	1/Rate (min)
0.10	1.0	0.187	5.35
0.20	0.50	0.360	2.78
0.30	0.333	0.441	2.27
0.45	0.222	0.526	1.90
0.50	0.20	0.561	1.78

Table 3.12



Graph 3.5: Lineweaver-Burk Plot for Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Desaminokynurenine.

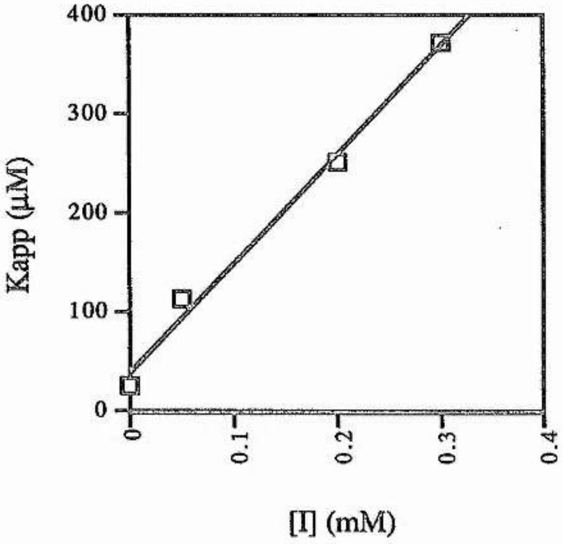
The K_M and V_{max} for the kynureninase catalysed reaction of kynurenine in the presence of varying concentrations of desaminokynurenine are shown in table 3.13.

Inhibitor	**Inhibitor concentration (mM)**	**V_{max}^{a} (x 10^{-3}) (mol dm^{-3} min^{-1})**	**K_{app} (μM)**
No inhibitor	0	1.07 ± 0.01	25.5 ± 0.6
Desaminokynurenine	0.05	0.39 ± 0.04	113 ± 38
Desaminokynurenine	0.2	0.34 ± 0.04	252 ± 78
Desaminokynurenine	0.3	0.22 ± 0.02	371 ± 62

[a] V_{max} and K_{app} were obtained from fitting the data to the Michaelis-Menten equation by non linear regression using the ENZFITTER[151] programme.

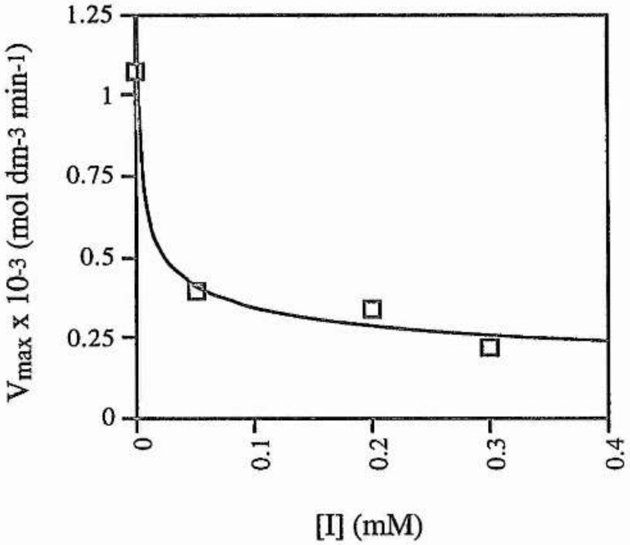
Table 3.13

Data for inhibition by desaminokynurenine does not show a simple competitive pattern. Both K_{app} and V_{max} vary. However, K_{app} does show a linear relationship with inhibitor concentration which gives a value of K_I as 23.2 ± 3.1 μM (Graph 3.6).



Graph 3.6: Plot of K_{app} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Desaminokynurenine.

On the other hand, the variation of V_{max} with inhibitor concentration is not linear (Graph 3.7).



Graph 3.7: Plot of V_{max} versus [I] for the Kynureninase Catalysed Reaction of Kynurenine in the Presence of Varying Concentrations of Desaminokynurenine.

An IC_{50} value of 17.6 μM can be estimated, however, which is similar to the K_I calculated. Both of these values are similar to the K_M determined for desaminokynurenine (see 2.5.1 Study of Reaction of Desaminokynurenine by UV Spectrophotometry).

This mixed inhibition may be a consequence of the activity of desaminokynurenine as a substrate for the normal kynureninase catalysed reaction or possibly as a substrate for transaldimination.

The K_I values obtained are summarised below:

KYNURENINE ANALOGUE	K_I (μM)
Desaminokynurenine	23.2 ± 3.1
Cyclohexyl amino acid derivative	844 ± 47
Naphthyl amino acid derivative	207 ± 16

The K_I values above are for the racemic mixtures of the amino acid. However, as previous work has shown that the 2*R* isomer is not a substrate or inhibitor,[34] it might be implied that in this case only the 2*S* enantiomers are binding. The K_I values for the 2S enantiomers would be half those experimentally determined, i.e.

Desaminokynurenine	$K_I = 11.6 \pm 3.1$ μM
Cyclohexyl amino acid derivative	$K_I = 422 \pm 47$ μM
Naphthyl amino acid derivative	$K_I = 103 \pm 16$ μM

3.5 EXAMINATION OF SUBSTRATE ACTIVITY OF ANALOGUES.

In order to determine whether the analogues could act as substrates they were incubated with kynureninase and the reactions monitored by tlc.

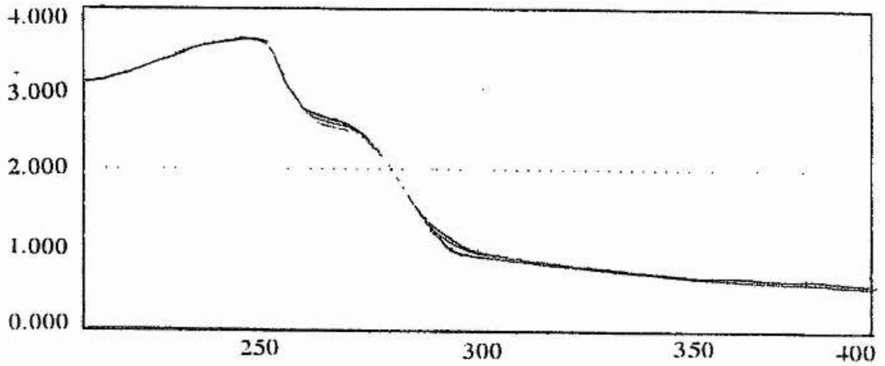
3.5.1 Reactions Monitored by tlc.

Using cellulose as the stationary phase and a mixture of IPA, conc. NH_3 and water (36: 6: 10) as eluant it was possible to separate desaminokynurenine (Rf 0.63, brown when visualised with ninhydrin), alanine (Rf 0.33, purple when visualised with ninhydrin) and PLP (baseline, lilac when visualised with ninhydrin). Desaminokynurenine at 0.8 mM concentration (1ml) was incubated with (120 μl, 0.6 units) enzyme at 25 °C in buffer containing 0.4 mM kynurenine, 40 µM PLP in 0.04 M potassium phosphate, pH 7.0. After eighteen hours tlc examination, on visualisation with ninhydrin, showed 2*S*-alanine in the enzyme mixture which implied that desaminokynurenine was a substrate for the enzyme. Under these same conditions it was possible to separate the cyclohexyl amino acid (Rf 0.71, brown when visualised with ninhydrin), the naphthyl amino acid (Rf 0.68, brown when visualised with ninhydrin), alanine (Rf 0.41, purple when visualised with ninhydrin) and PLP (baseline, lilac when visualised with ninhydrin). Using the same procedure no reaction was observed on incubation of the cyclohexyl and naphthyl derivative with kynureninase. However, if the amount of enzyme was doubled then tlc, on visualisation with ninhydrin, the naphthyl amino acid mixture showed the presence of some alanine. This implied that the naphthyl amino acid was a substrate for the enzyme. Even with another equivalent of enzyme no reaction was seen for the cyclohexyl amino acid under these conditions.

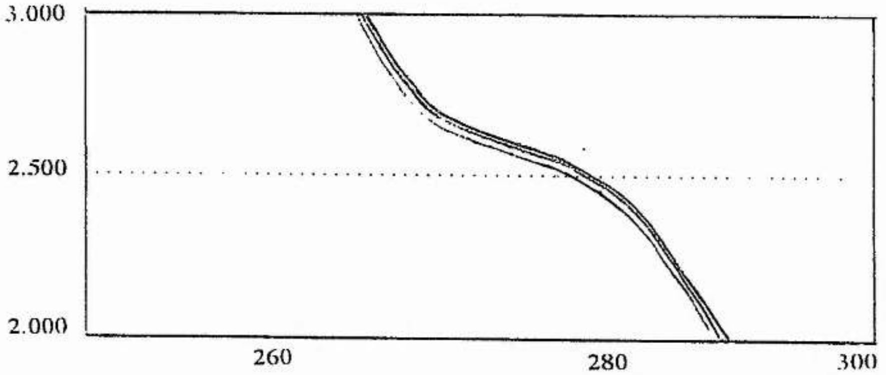
3.5.2 Study of Reaction of Desaminokynurenine (27) by UV Spectophotometry.

As desaminokynurenine seemed to be the most active substrate its reaction was further investigated by UV spectophotometry. This should allow the kinetic parameters to be determined.

The UV spectrum of desaminokynurenine (1 ml, 0.4 mM, 25 °C in buffer containing 0.4 mM kynurenine, 40 µM PLP in 0.04 M potassium phosphate, pH 7.0.) was first determined. An aliquot of kynureninase (30 µl, 0.15 units) was then added and the spectrum recorded again at five minute intervals. When these were superimposed it was possible to see a decrease in absorbance at 278 nm.

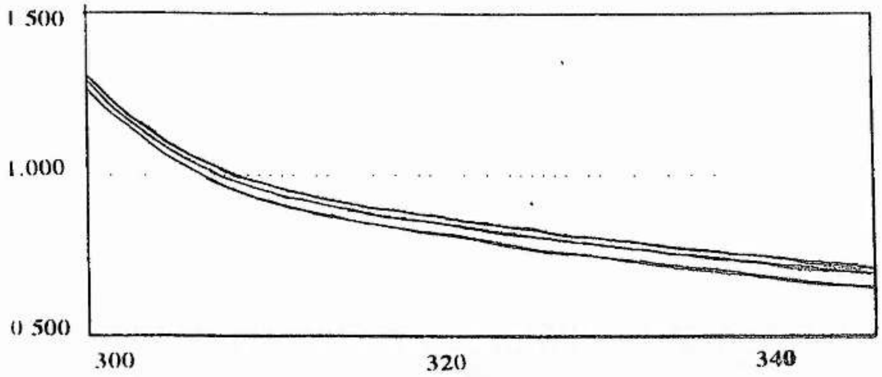


UV spectra of desaminokynurenine from 200-400 nm.



UV spectra of desaminokynurenine-showing decrease in absorbance at 278 nm. Spectra recorded at 5 minute intervals.

The UV spectrum of benzoic acid (0.4 mM) the product of the reaction, was recorded under identical conditions to ensure that there was no interference at this wavelength due to product formation. This was confirmed to be the case. However, the reaction was found to be very slow compared to that of the natural substrate. The decrease in absorbance was very small, even at high substrate concentrations. It was therefore decided that this wavelength (278 nm) was unsuitable for kinetic studies. Further examination of the spectral changes revealed an alternative wavelength (330 nm) which was more suitable.



UV spectra of desaminokynurenine-showing increase in absorbance at 330 nm.

The absorbance at this wavelength was seen to increase as reaction took place and was probably due to the formation of benzoic acid. This was confirmed by an increase in absorbance at this wavelength with increasing concentrations of benzoic acid. The increase in absorbance was monitored with time and the initial rates gave a linear increase in absorbance. The assays could therefore be carried out at this wavelength and were recorded overnight. The rates are shown (See table 3.14).

Substrate concentration (mM)	**Rate x 10^{-3} (ΔA min^{-1})**
7	3.33
8	3.47
9	4.12
10	4.23

Table 3.14

Non linear regression analysis gave the values of V_{max} and K_M. V_{max} was 15.2 x 10^{-3} min^{-1} and K_M was 25.5 μM. The value of V_{max} can be used to determine the rate of reaction for desaminokynurenine, if we divide this value by the extinction coefficient for benzoic acid. This was determined in 0.04 M potassium phosphate buffer to be 2883 M^{-1} cm^{-1}.

Therefore, **rate for desaminokynurenine** = $\dfrac{15.2 \times 10^{-2}}{2883}$

$= \mathbf{5.27 \times 10^{-5}\ mol\ dm^{-3}\ min^{-1}}$.

This can be compared with **kynurenine,**

which has a rate of $\mathbf{1.07 \times 10^{-3}\ mol\ dm^{-3}\ min^{-1}}$.

Thus desaminokynurenine is hydrolysed twenty times slower than kynurenine implying that the *ortho*-amino group is important for substrate activity. This is in good agreement with Tanizawa and Soda who determined the reactivity of desaminokynurenine to be 6% that of kynurenine, i.e. sixteen times slower, by measuring the relative amounts of 2*S*-alanine formed.[38]

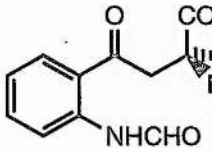
The K_M determined from this experiment was 25.5 μM in good agreement with the K_I determined for desaminokynurenine of 23.2 μM as described earlier, providing further evidence for the validity of this result.

3.6 CONCLUSIONS.

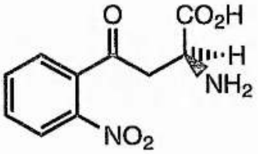
It can thus be seen that the cyclohexyl and naphthyl analogues were competitive inhibitors of the enzyme kynureninase to varying extents and desaminokynurenine showed mixed inhibition. The relative K_I values determined were $K_I = 11.6 \pm 3.1$ μM (for desaminokynurenine), $K_I = 422 \pm 47$ μM (for the cyclohexyl amino acid derivative) and $K_I = 103 \pm 16$ μM (for the naphthyl amino acid derivative) implying that the cyclohexyl derivative binds very poorly to the enzyme. Similarly, the naphthyl amino acid has a poor interaction with the enzyme, however, desaminokynurenine has a K_I of a similar magnitude to the natural substrate. It was shown by tlc that both the naphthyl amino acid and desaminokynurenine are substrates of the enzyme. Rough kinetic data was determined for desaminokynurenine by UV spectophotometry and the rate of reaction was found to be twenty times slower than that of kynurenine.

These results reveal some information about binding interactions at the active site of kynureninase. The *ortho*-amino group is important and when this is absent the binding is reduced by twenty times. The cyclohexyl amino acid shows exceptionally poor binding to the enzyme suggesting that interactions with the aromatic ring are important. Hydrogen bonding interactions between amide groups and aromatic rings have been proposed as important factors in determining the three-dimensional structure and recognition properties of proteins. Structural evidence for interactions in which the π-electrons of an aromatic ring act as a H-bond acceptor comes from crystal structures of small molecules and proteins.[172] This would imply that the active site is not just a simple hydrophobic pocket or if it is, the cyclohexyl ring may be too large. The naphthyl amino acid shows poor binding compared to desaminokynurenine and this indicates that this molecule is probably too large for the active site.

Soda and Tanizawa reported that the *ortho*-amino group was indispensable in substrates. Compounds in which the amino group was blocked with a formyl group (23), replaced by a nitro (24) or moved to a *para* position (25) are not reactive as substrates.



(23)



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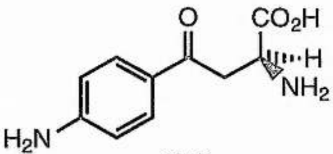
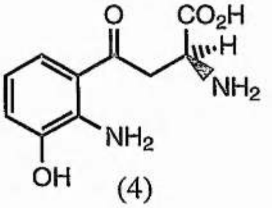


(25)

The K_I values were determined for various compounds using bacterial kynureninase from *Pseudomonas fluorescens*, *N'*-formyl-2*S*-kynurenine (23) was 2.2 mM showing very weak activity towards the enzyme and 2*S*-3-hydroxykynurenine (4) was 200 μM displaying weak activity. The K_M of kynurenine in these experiments was 35 μM.[38]

Kishore reported the K_I for 2*S*-S-(ortho-nitrophenyl)-cysteine (43) to be 0.1 mM, thus, also showing weak activity towards the enzyme.[42]



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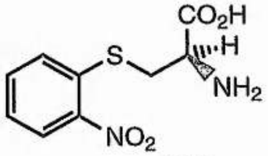
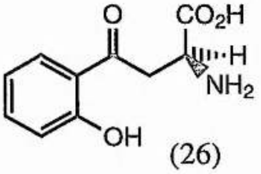
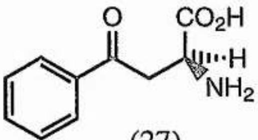


(43)

Soda and Tanizawa also reported that derivatives in which the aromatic amino group was replaced by a hydroxyl group (26) or removed (27) are susceptible to the enzyme, however, their reactivity was found to be considerably decreased.[38] The relative reactivities reported to be 13.4% and 6%, respectively, were determined by measuring spectophotomerically the substrate hydrolysed.

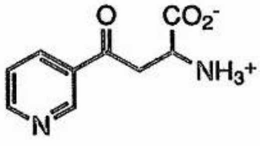


(26)

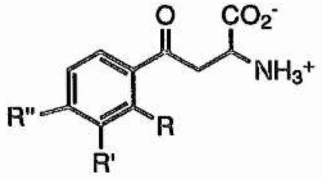


(27)

Pellicciari *et al.* [52, 53]have carried out the synthesis of nicotinylalanine (65), *o*-nitrobenzoyl-(66), *m*-nitrobenzoyl-(67), *p*-nitrobenzoyl-alanine (68), all analogues of kynurenine, and reported their evaluation as inhibitors of kynureninase.



(65)



(66) *o*- R= NO_2, R'= H, R"= H
(67) *m*- R= H, R'= NO_2, R"= H
(68) *p*- R= H, R'= H, R"= NO_2

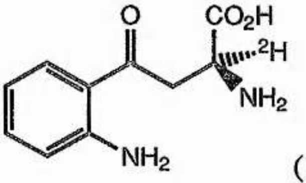
Nicotinylalanine (65) with an IC_{50} of 800 ± 120 μM for kynureninase showed weak activity towards the enzyme. Of the nitro analogues *m*-nitrobenzoylalanine (67) and *o*-nitrobenzoylalanine (66) showed similar, but moderate activity with IC_{50} values of 100 ± 12 μM and *p*-nitrobenzoylalanine (68) showed weak activity with an IC_{50} values of 900 ± 12 μM.

CHAPTER 4

4. STUDIES ON SYNTHESIS OF α-DEUTERIATED KYNURENINE AND MEASUREMENT OF ISOTOPE EFFECTS.

4.1 (2*S*)-[2-^{2}H] KYNURENINE.

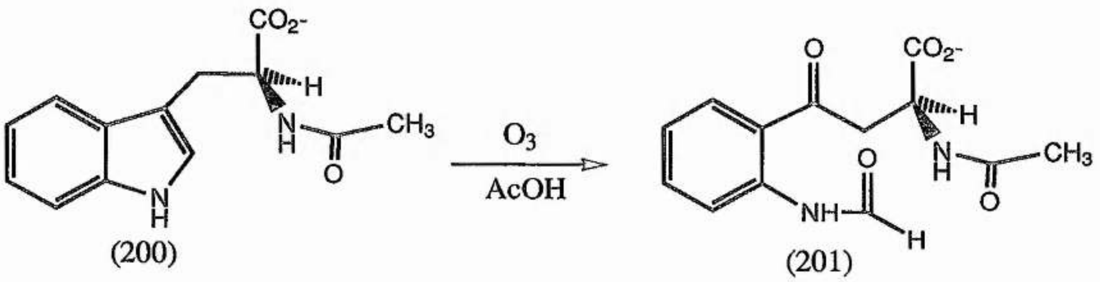
Specifically deuteriated kynurenine derivatives are required for kinetic isotope effect studies on the enzyme catalysed reaction. For example, α-deuteriated kynurenine (199) would allow measurement of the α-deuterium kinetic isotope effect for the Cα-H bond cleavage.



(199)

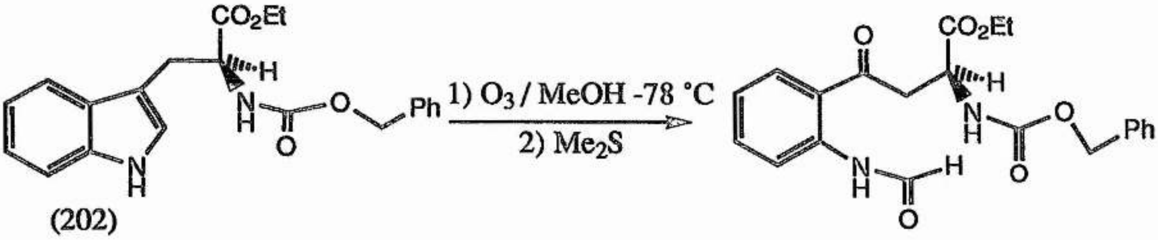
4.2 LITERATURE SYNTHESES OF KYNURENINE

The original literature synthesis of kynurenine involved the ozonolysis of *N*-acetyl tryptophan (200) in glacial acetic acid (Scheme 4.1). Oxidative cleavage of the indole double bond readily takes place giving *N'*-formyl, *N*-acetyl kynurenine (201). Acidic hydrolysis of the *N'*-formyl and *N*-acetyl groups was then carried out in 20-50% yield to provide kynurenine.[173, 174]



Scheme 4.1

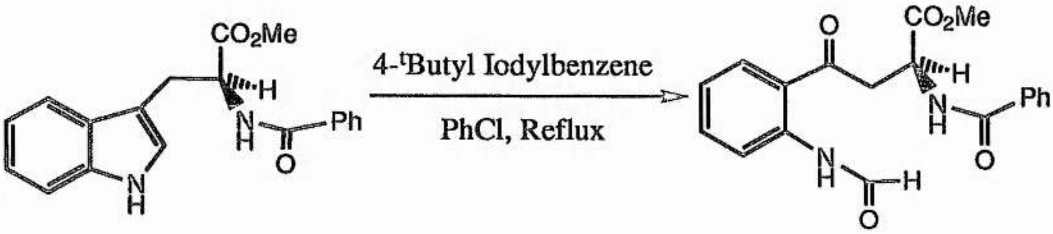
This method is not very clean and gives poor yields, as has been found in our laboratory.[175] However, several modifications have since appeared in the literature. Palfreyman *et al.*, in their synthesis of 4,6-disubstituted kynurenines from 4,6-disubstituted tryptophans, [140] used tryptophan protected with the *N*-benzyloxycarbonyl group (*N*-Cbz) instead of *N*-acetyl. They also used the ethyl ester (202) rather than the free carboxylic acid. The reaction was carried out in methanol at -78 °C and quenched with dimethylsulphide (Scheme 4.2). The yield for ozonolysis of the 4, 6-dichloro compound was reported to be 96%, an improvement on the ozonolysis of *N*-acetyl tryptophan. The three deprotection steps were carried out in 76% overall yield.



(202)

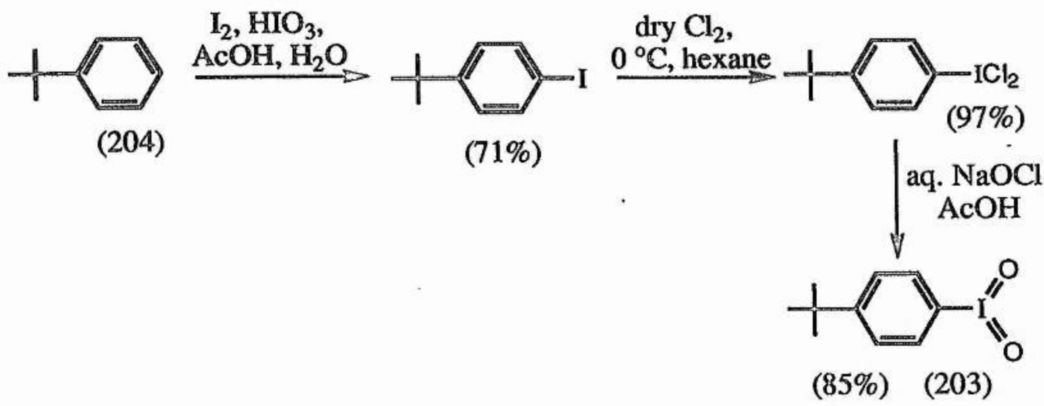
Scheme 4.2

Ranganathan, used a similar diprotected tryptophan derivative, but in this case the oxidative cleavage was carried out using 4-^{t}butyl iodylbenzene in refluxing chlorobenzene, instead of ozone (Scheme 4.3). The protected kynurenine was produced in 70% yield.[176]



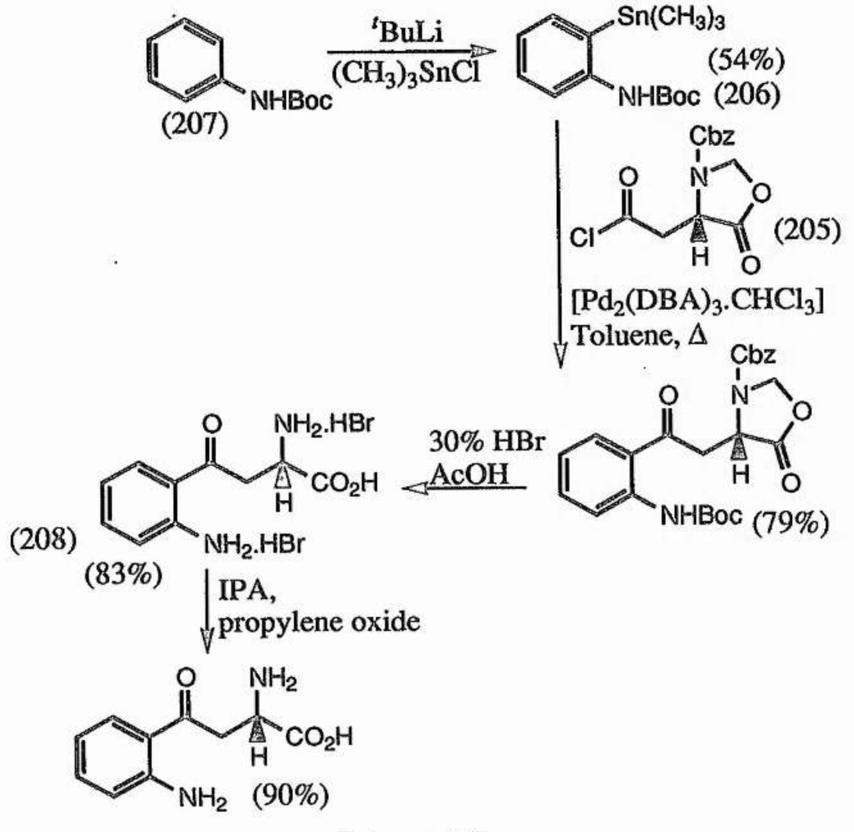
Scheme 4.3

However, it is necessary to prepare the 4-^{t}butyl iodylbenzene (203), which is potentially explosive. This can be carried out in three steps from 4-^{t}butyl benzene (204) (Scheme 4.4).



Scheme 4.4

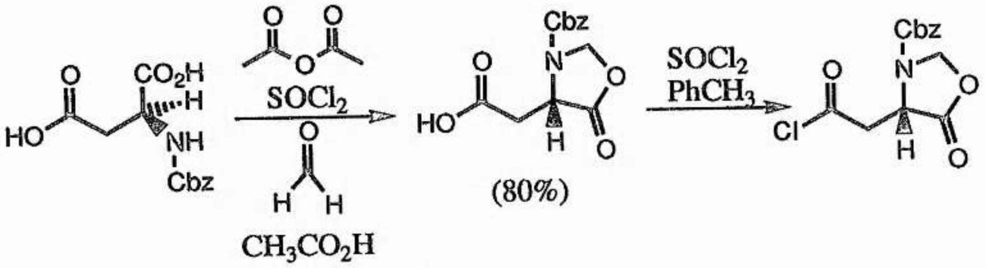
Synthesis of kynurenine can also be achieved by building up the molecule from an amino acid synthon and a suitable aromatic side chain. For example, Salituro and McDonald coupled (2*S*)-*N*-Cbz-5-oxo-4-oxazolidineacetyl chloride (205) with *N*-^{t}Boc-2-(trimethylstannyl) aniline (206) using palladium (0) catalysis (Scheme 4.5).



Scheme 4.5

Thus, *ortho*-lithiation of *N*-^{t}Boc aniline (207) and quenching with trimethylstannyl chloride gave *N*-^{t}Boc-2-(trimethylstannyl) aniline (205) in 54% yield.[177] The coupling was then carried out in toluene at 70 °C using 0.5 mol% of the chloroform adduct of tris(dibenzylideneacetone) dipalladium(0) ($[Pd_2(DBA)_3].CHCl_3$)[178] as catalyst precursor, giving the product in 79% yield. Complete deprotection was achieved in one step with 30% hydrobromic acid in acetic acid at room temperature for fifteen to twenty minutes followed by addition of diethyl ether which precipitated the bis(dihydrobromide) salt (208). The free amino acid was then isolated using propylene oxide and isopropanol in 38% overall yield.

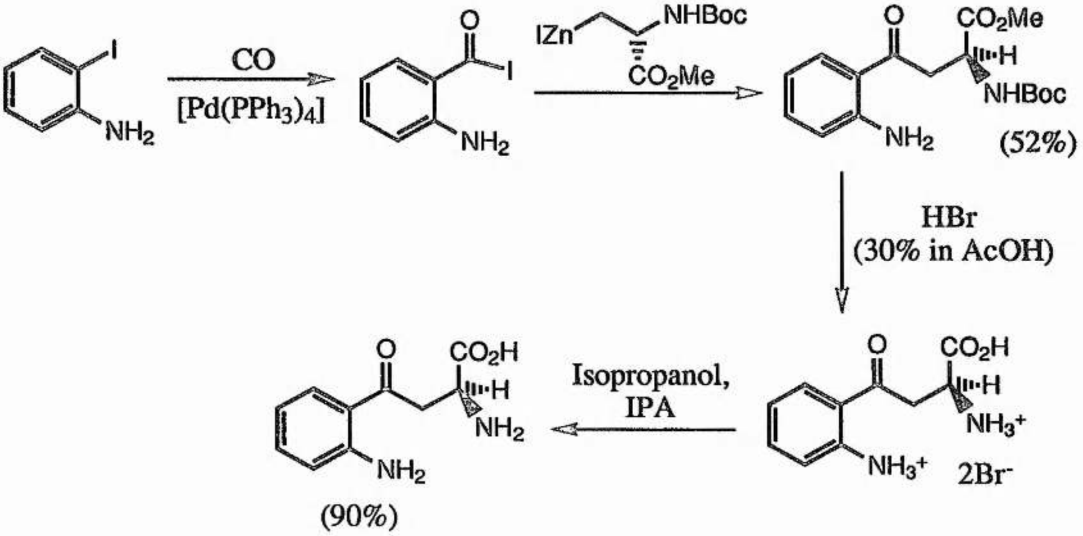
The (2*S*)-*N*Cbz-5-oxo-4-oxazolidineacetyl chloride (205) was prepared from the corresponding acid by use of an excess of thionyl chloride in toluene. The acid was prepared according to the method of Itoh from N-Cbz (2*S*)-aspartic acid, paraformaldehyde, acetic anhydride and thionyl chloride which were heated at 100 °C for four hours (Scheme 4.6).[179]



Scheme 4.6

A similar method was employed by Varasi *et al.* for the synthesis of 2*S*-5-chlorokynurenine.[67] Pelliciari *et al.*[53] and Natalini *et al.*[54] used the same strategy for the stereospecific synthesis of (2*S*)-nicotinylalanine and (2*S*)-*m*-nitrobenzoyl alanine, respectively, although the catalyst employed was dichlorobis(triphenylphosphine) palladium(II) ($[PdCl_2(PPh_3)_2]$).

Jackson *et al.*[137] employed a similar palladium (0) catalysed reaction of a serine derived organozinc reagent with 2-iodoaniline (209). This coupling was achieved at room temperature in THF under a carbon monoxide atmosphere using (tetrakis) triphenylphosphine palladium (0) [Pd $(PPh_3)_4$] as catalyst, giving *N*-^{t}Boc kynurenine methyl ester (210) in 52% yield. Removal of the protecting groups, according to the procedure of Salituro and McDonald,[177] was achieved by treatment with 30% hydrogen bromide in acetic acid to give the corresponding bis(hydrobromide) salt (211) which was then converted to the free amino acid using propylene oxide in 90% overall yield (Scheme 4.7).



Scheme 4.7

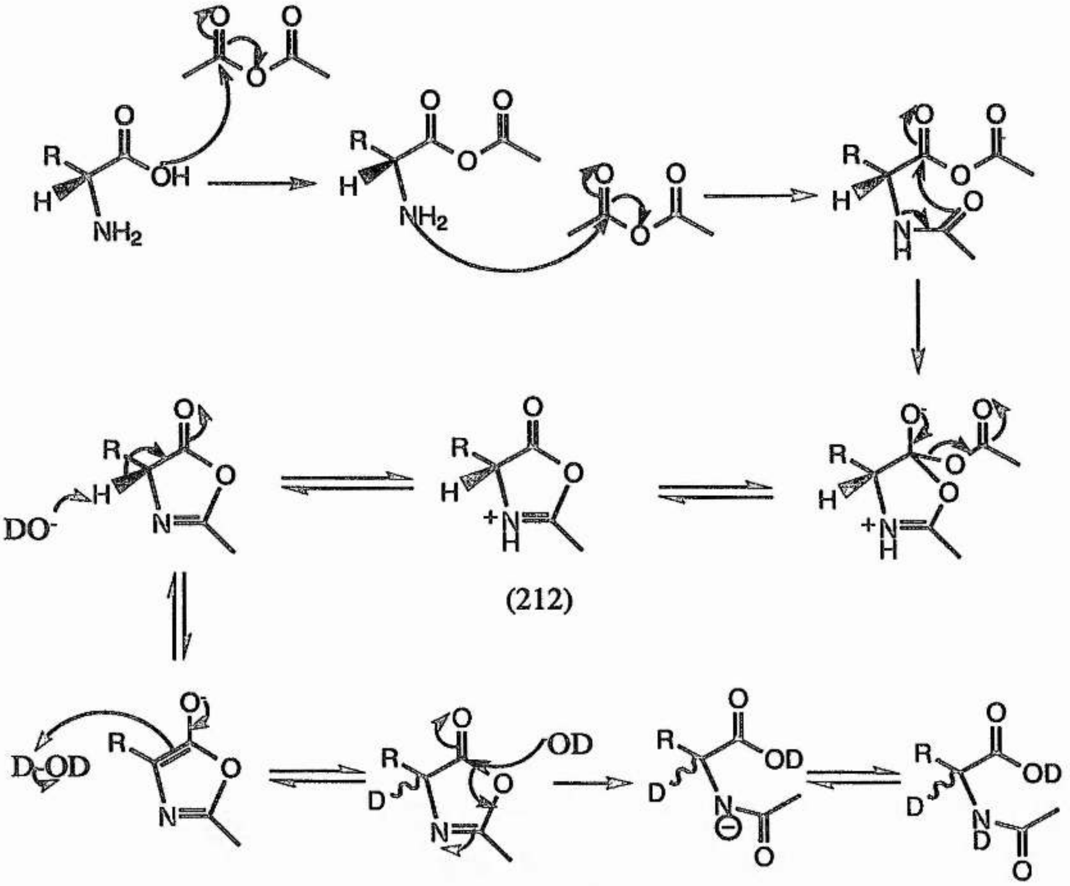
An interesting alternative strategy for the synthesis of kynurenine is to employ biocatalysis. Thus, Bild *et al.* synthesised ^{14}C labelled 2*S*-kynurenine from ^{14}C labelled tryptophan using crude extracts of tryptophan-adapted cells of *Pseudomonas marginalis*.[180] The synthesis was based on the selective, rapid inactivation of kynureninase by 3-chloro-2*S*-alanine, a potent suicide inhibitor. Therefore, the crude cell extracts were first incubated with 3-chloro-2*S*-alanine, before ^{14}C labelled 2*S*-tryptophan was added. The reaction was then monitored spectroscopically by observing the increase in absorption at 360 nm due to kynurenine formation. After sixty minutes of incubation under these conditions, approximately 89% conversion, the reaction was quenched by the addition of 2 M HCl and the product isolated by ion-exchange chromatography. The overall yield of ^{14}C labelled kynurenine (specific activity 35.2 μCi $mmol^{-1}$) was 76% of theoretical.

4.3 SYNTHESIS OF (2*S*)-[2-^{2}H] KYNURENINE.

Two potential routes were investigated for the synthesis of (2*S*)-[2-^{2}H] kynurenine (199). The first involved the acetylation and racemisation of kynurenine in deuterium oxide and resolution of the product. The second involved a similar procedure, using tryptophan, which is considerably less expensive, followed by conversion of the deuteriated tryptophan to kynurenine.

4.3.1 Direct Synthesis from (2*RS*)-Kynurenine.

A common procedure for the incorporation of deuterium into the α-position of amino acids is to carry out an acetylation / racemisation reaction in deuterium oxide.[181] The mechanism for this reaction involves the acetylation of both the acid and amino functionality of the amino acid using acetic anhydride. This is carried out in sodium deuteroxide and deuterium oxide, i.e. under basic conditions. The acetylated intermediate forms an azalactone (212) with the elimination of acetic acid. The α-hydrogen is now highly acidic due to the combined electron withdrawing effects of the carbonyl and the imine and therefore, there is rapid exchange with a solvent deuterium. Basic hydrolysis then affords the racemic α-deuteriated product which retains the *N*-acetyl group (Scheme 4.8).



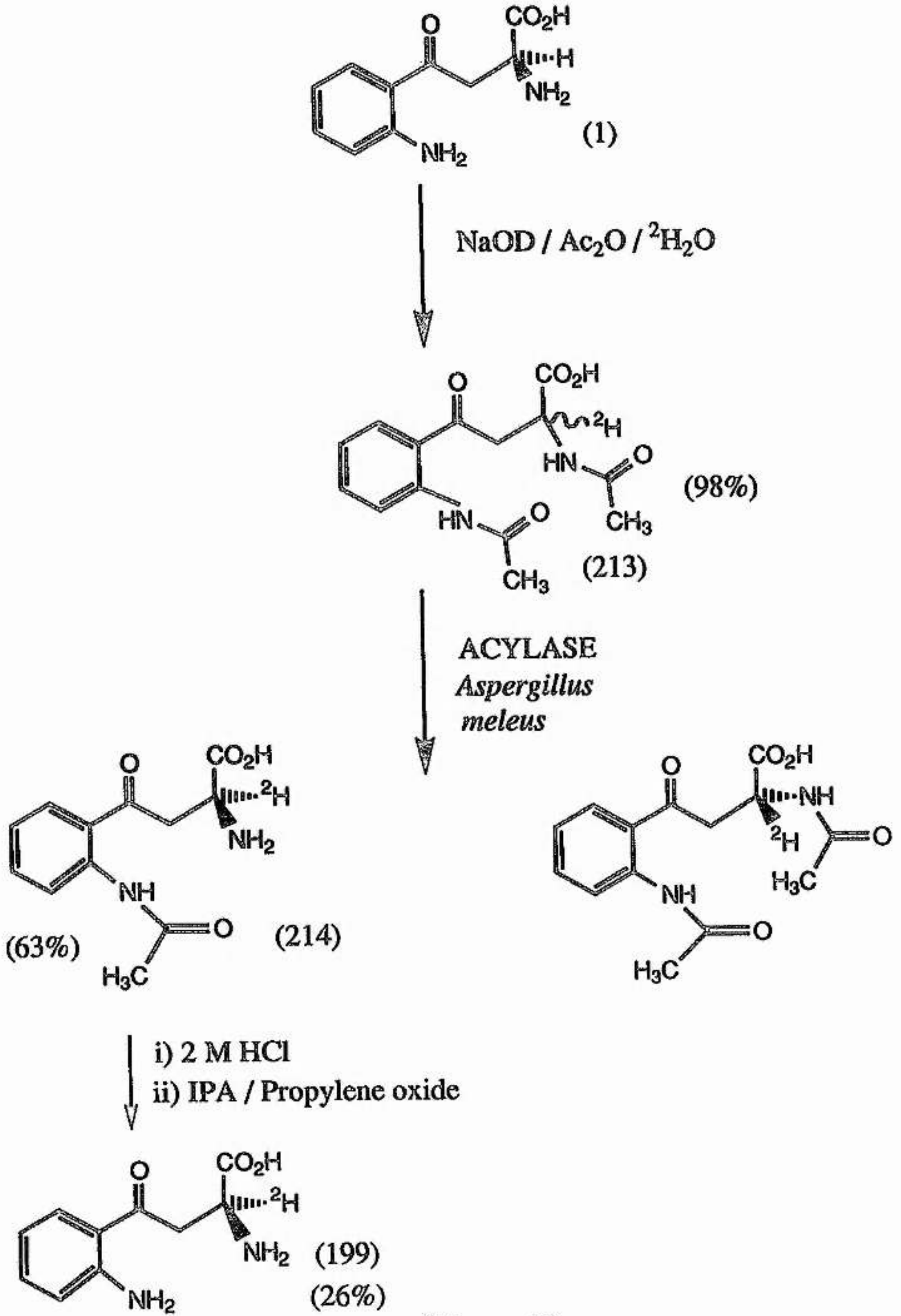
Scheme 4.8

The required (2*S*)-amino acid can then be obtained using an acylase which specifically hydrolyses the 2*S*-enantiomer.[181, 182] It is then possible to separate the two chemically different compounds. This general method has been used for a number of amino acids. However, kynurenine, unlike many other amino acids has two amino groups. It was, therefore, unclear how the aromatic amino group would affect the process.

(2*RS*)-Kynurenine, as its free base, was reacted with acetic anhydride in sodium deuteroxide and deuterium oxide. Spectral data implied that the compound had been diacetylated (213). The mass spectrum gave a molecular ion at 292, while the NMR spectrum showed the aromatic *N*'-acetyl protons at $\delta = 2.0$ ppm and the other *N*-acetyl protons at $\delta = 1.8$ ppm (from comparisons with the NMR spectrum of *N*-acetyl tryptophan from Sigma). Extraction was initially attempted using ethyl acetate, however the yields were poor and the material difficult to handle. When this was changed to dichloromethane a crystalline solid was produced in 98% yield.

Selective hydrolysis was then attempted using acylase from *Aspergillus meleus*. Acylase enzymes isolated from *Aspergillus meleus* were reported to be much more effective in the hydrolysis of *N*-acetyl aromatic amino acids than the pig liver acylase normally used.[182] Incubation was carried out for eighteen hours at 38 °C in aqueous solution, which was brought to pH 7.5 with concentrated ammonia. Examination by tlc (cellulose; IPA: conc. aq NH_3: H_2O, 36: 6: 10) gave no indication of the progress of the reaction, therefore, NMR spectroscopy was used to determine when reaction was complete by examination of the acetyl region for the disappearance of the *N*-acetyl protons at $\delta = 1.8$ ppm. The product was purified by ion exchange chromatography (DOWEX 1) and obtained in 63% yield. NMR of the purified product showed the aromatic *N*-acetyl protons at $\delta = 2.0$ ppm implying that this group was still present. Therefore, the acylase was specific for the removal of the acetyl group from the α-amino group.

Deprotection was initially attempted by refluxing in a mixture of dioxan and water with 0.5 M HCl.[183] This method required extensive periods of reflux and resulted in decomposition of the product. The acetyl kynurenine (214) was then dissolved in 2 M HCl and the solution was refluxed for one hour to remove the acetyl group. The free amino acid was initially isolated using propylene oxide and ethanol at reflux, however, this method did not result in pure material. Subsequently, the use of propylene oxide and isopropanol resulted in a solid product which became an oil on filtration. Lyophilisation was then carried out to obtain a pure solid product (scheme 4.9). This was found to be the correct product by NMR


(1)
NaOD / Ac$_2$O / ^{2}H$_2$O
(98%)
(213)
ACYLASE
Aspergillus meleus
(63%)
(214)
i) 2 M HCl
ii) IPA / Propylene oxide
(199)
(26%)

Scheme 4.9

The only disadvantage of this procedure is the high cost of kynurenine, 50% of which is by necessity lost during the reaction. Note that the yields quoted were corrected for a maximum yield of 50%.

4.3.2 Proposed Synthesis *via* Tryptophan.

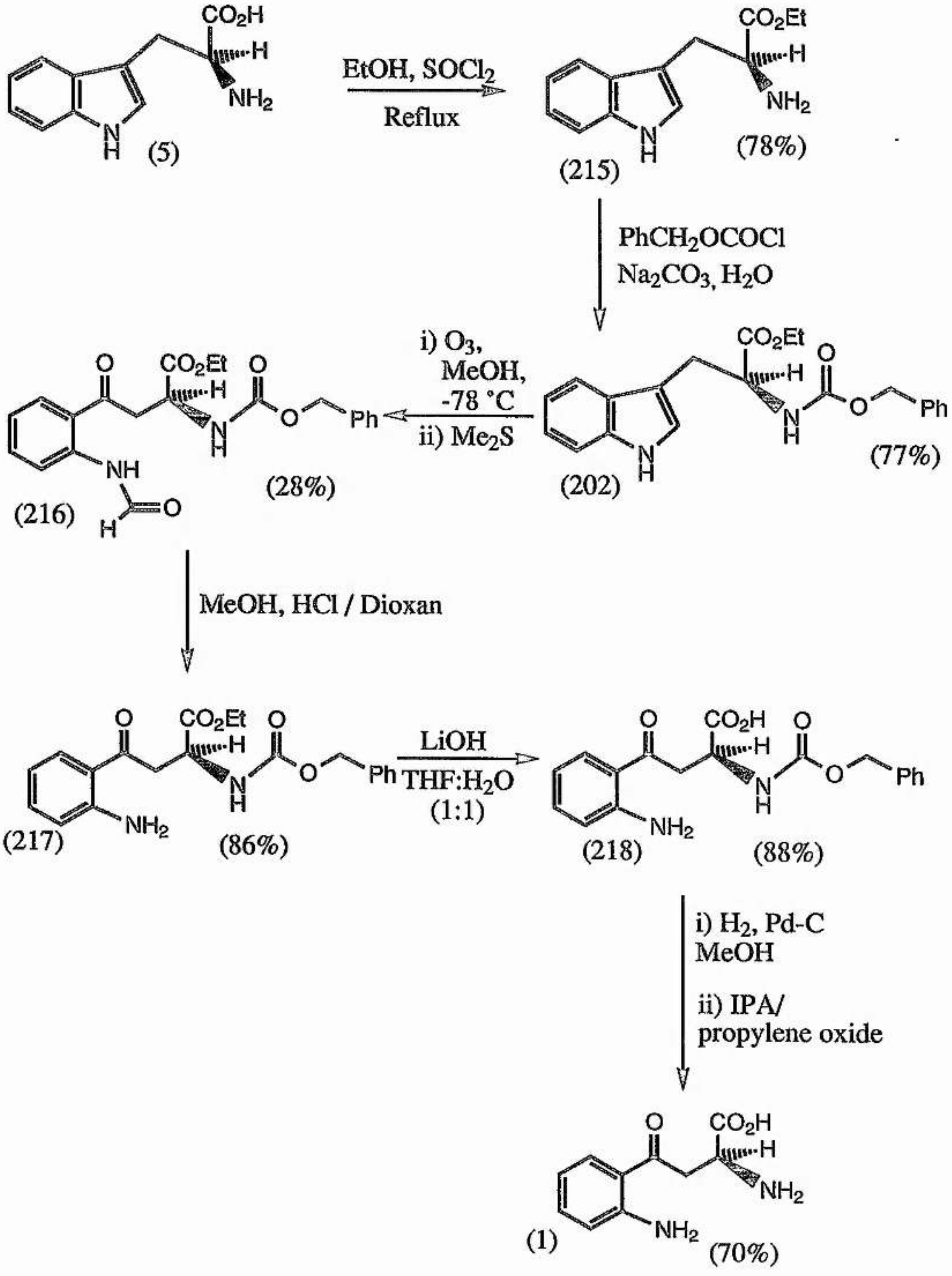
The alternative synthesis of α-deuteriated kynurenine involved initial preparation of α-deuteriated (2*S*)-tryptophan, by a similar method to that for kynurenine and then conversion of this through to α-deuteriated kynurenine. Before this procedure could be implemented it was necessary to examine the synthesis of unlabelled kynurenine and optimise the reaction conditions.

4.3.2.1 Synthesis of Kynurenine.

It was proposed to synthesise kynurenine from tryptophan by the method of Palfreyman,[140] as outlined in Scheme 4.10. However, this is reported in a Patent and there are few experimental details.

Firstly, tryptophan ethyl ester was prepared by refluxing tryptophan (5) in redistilled ethanol and thionyl chloride. The product was then recrystallised from ethanol and obtained in 72% yield. NMR analysis showed the ester at $\delta = 1.10$ and 4.10 ppm and the melting point of the product (226-227 °C) was in good agreement with the literature value (225-226 °C).[184] The amino group was protected as the Cbz derivative (202) by treatment of tryptophan ethyl ester (215) with a slight excess of benzylchloroformate and sodium carbonate. This reaction was particularly successful due to the fact that 2*S*-tryptophan ethyl ester was soluble in water whereas the product, *N*-Cbz tryptophan ethyl ester, was not. Therefore by carrying out this reaction in water, the pure product precipitated out of solution and could be filtered off. The concentration was important to obtain a crystalline product because if the solution was too concentrated the product precipitated as an oil. ^{1}H NMR spectroscopy showed this to be the correct product and that no further product remained in the filtrate. The characteristic peak for the $C\underline{H}_2Ph$ of the Cbz group was observed at δ = 5.05 ppm and the melting point (84 °C) was very similar to the literature value (84-85 °C).[185] *N*-Cbz tryptophan ethyl ester was then converted to kynurenine in its protected form (216) by ozonolysis. The protected tryptophan was dissolved in redistilled methanol and ozone was bubbled through the solution at -78 °C. The reaction was quenched with dimethylsulphide to give the desired product which was purified by column chromatography and recrystallised from ethyl acetate in 28% yield. The ester protons could be seen at δ = 1.10 and 4.10 ppm, the $C\underline{H}_2Ph$ of the Cbz group at δ = 5.05 ppm and the formyl group at δ = 8.50 ppm. This reaction was fairly low yielding. However, it was observed that some *N*-Cbz kynurenine ethyl ester was also isolated which could be used in the remaining deprotection steps. This implies that the formyl group may be hydrolysing on the column. Attempts were made to improve the yield of the ozonolysis by quenching the reaction using trimethyl phosphite, however this gave the purified product in a poorer yield of 15%.

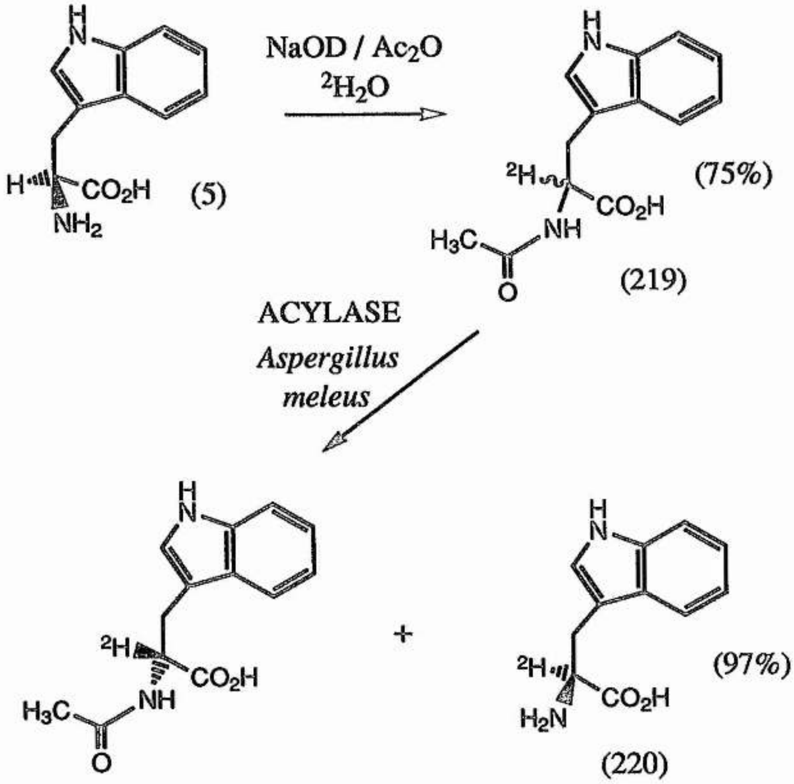
The next stage was removal of the protecting groups and this was carried out sequentially. The formyl group was removed by stirring the protected kynurenine in 4M HCl and dioxan in methanol which gave the product in 86% yield. The disappearance of the peak at δ = 8.50 ppm confirmed the loss of the formyl group. The product and starting material were difficult to distinguish by tlc (silica; petroleum ether: ethyl acetate, 2: 1) so the reaction was monitored by NMR spectroscopy. The *N*-Cbz kynurenine ethyl ester (217) was then dissolved in a 1: 1 mixture of THF and water and warmed to 70 °C for four hours giving *N*-Cbz kynurenine (218) in 88% yield. To ensure complete reaction the *N*-Cbz kynurenine ethyl ester was initially dissolved in THF and the lithium hydroxide dissolved in water. The disappearance of the peaks at δ = 1.10 and 4.10 ppm confirmed the loss of the ethyl group. Finally the amino group was deprotected by hydrogenation over palladium on charcoal to afford the product in 70% yield. This was found to be a much cleaner method than the TMS-iodide initially tried. Iodine precipitated out of solution when the product was dissolved in water.


(5)
EtOH, SOCl$_2$
Reflux
(215)
(78%)
PhCH$_2$OCOCl
Na$_2$CO$_3$, H$_2$O
(202)
(77%)
i) O$_3$, MeOH, -78 °C
ii) Me$_2$S
(216)
(28%)
MeOH, HCl / Dioxan
(217)
(86%)
LiOH
THF:H$_2$O (1:1)
(218)
(88%)
i) H$_2$, Pd-C MeOH
ii) IPA/ propylene oxide
(1)
(70%)

Scheme 4.10

4.3.2.2 Synthesis of (2*S*)-[2-^{2}H]-Tryptophan.

(2*S*)- Tryptophan (5) was reacted with sodium deuteroxide and acetic anhydride in deuterium oxide (Scheme 4.11) under similar conditions to those employed for kynurenine. This time however, the product precipitated out of solution and after refrigeration was filtered and washed with cold water. The residue was suspended in 0.2 M HCl, cooled, filtered and washed until free of chlorides, then dried.[181] The deuteriated *N*-acetyl tryptophan (219) was obtained in 75% yield. Selective hydrolysis was then carried out using the acylase from *Aspergillus meleus*, again as for kynurenine. The solubility of the *N*-acetyl tryptophan was much poorer than the diacetylated kynurenine and as it dissolved the pH decreased. However, this was countered by periodically adding concentrated aqueous ammonia. Gentle heating also helped the dissolution. Incubation was carried out for several days until NMR showed the reaction to be complete. Centrifugation proved to be helpful to remove the acylase. The product was then purified by ion exchange chromatography (DOWEX 1) to give a microanalytically pure product (220) which precipitated out of solution once collected. This was obtained in 97% yield. This material was analysed by chiral hplc and shown to have an enantiomeric excess of 99%.



Scheme 4.11

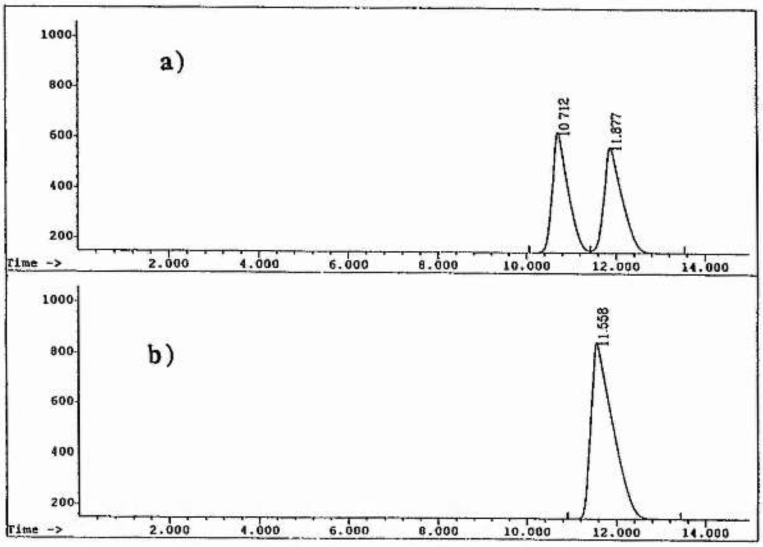


Figure 4.1: HPLC analysis of (2*S*)-[2-^{2}H]-Tryptophan. Conditions, Crownpak CR (+) (150 mm x 4 mm id, 5μm) using 2.5% methanol in aqueous HClO$_4$ at pH 2 (premixed) at 1 ml min $^{-1}$ and 50 °C.

a) Commercial *RS*-tryptophan (Sigma Chemical Co. Ltd.)

b) (2*S*)-[2-^{2}H]-Tryptophan. Ratio of peak areas gives ee at 99%.

4.3.2.3 Conversion of (2*S*)-[2-^{2}H]-Tryptophan to (2*S*)-[2-^{2}H]-Kynurenine.

The synthesis of (2*S*)-[2-^{2}H]-kynurenine was then carried out using the procedure outlined in Section 4.3.2.1. As before, the carboxylic acid and the amino groups were protected as the ethyl ester and *N*-Cbz derivatives, respectively, in 60% overall yield. Ozonolysis was then performed in methanol solution at -78 °C for one hour to give the protected *N*'-formyl 2*S*-[2-^{2}H]-kynurenine (223) in 33% isolated yield. Removal of the three protecting groups was carried out sequentially. The *N*'-formyl group was removed by stirring at room temperature in 4 M HCl and dioxan in methanol in 98% yield. The ethyl ester was hydrolysed using lithium hydroxide in a 1: 1 mixture of THF and water on warming to 70 °C for four hours. The product was obtained in 45% yield. Finally the amino group was deprotected by hydrogenation over palladium on charcoal. Isolation of the free amino acid was achieved using isopropanol and propylene oxide (Scheme 4.12). The material was identical in all respects to that obtained from the first method, with an enantiomeric excess of 98%.

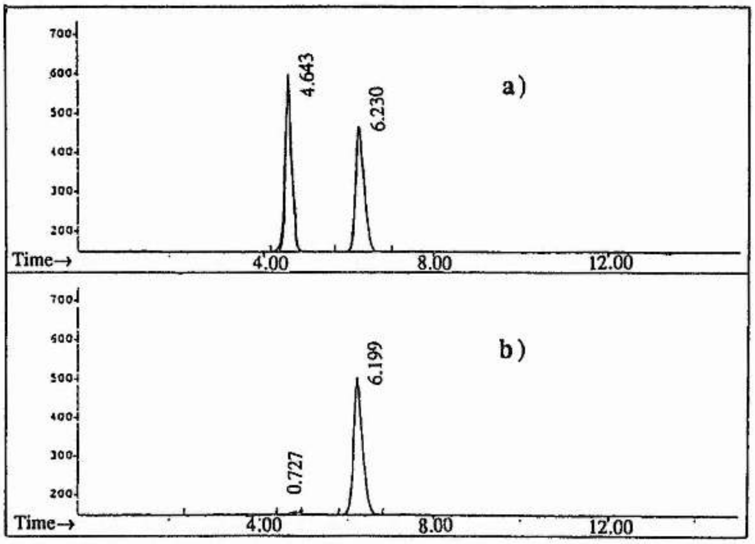
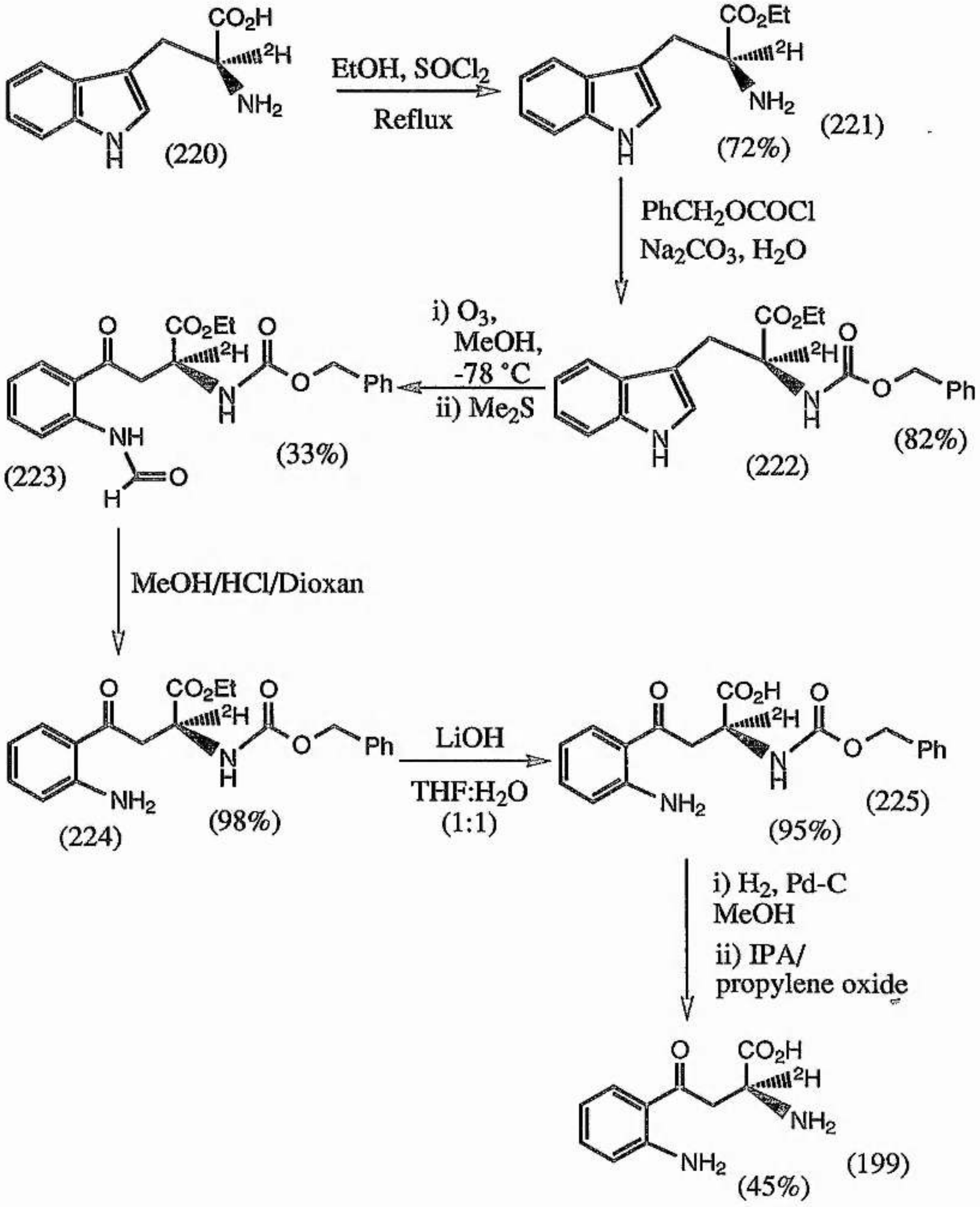


Figure 4.2: HPLC analysis of (2*S*)-[2-^{2}H]-Kynurenine. Conditions, Crownpak CR (+) (150 mm x 4 mm id, 5µm) using 2.5% methanol in aqueous $HClO_4$ at pH 2 (premixed) at 1 ml min $^{-1}$ and 50 °C.

a) Commercial *RS*-Kynurenine (Sigma Chemical Co. Ltd.)

b) (2*S*)-[2-^{2}H]-Kynurenine. Ratio of peak areas gives ee at 97.4%.


(220)
EtOH, SOCl2
Reflux
(221)
(72%)
PhCH2OCOCl
Na2CO3, H2O
(222)
(82%)
i) O3,
MeOH,
-78 °C
ii) Me2S
(223)
(33%)
MeOH/HCl/Dioxan
(224)
(98%)
LiOH
THF:H2O
(1:1)
(225)
(95%)
i) H2, Pd-C
MeOH
ii) IPA/
propylene oxide
(199)
(45%)

Scheme 4.12

4.3.2.4 Comparison of Synthetic routes to (2*S*)-[2-^{2}H]-kynurenine.

Both of these routes are complementary and give material of comparable purity and enantiomeric excess. The direct synthesis from racemic kynurenine is the shorter route, giving an overall yield of 8%, however, the starting material is expensive. Although, the synthesis of kynurenine from tryptophan is longer, it begins from the much cheaper amino acid tryptophan. The overall yield is 8% from 2*S*-[2-^{2}H]-tryptophan, but 6% from 2*S*-tryptophan. The second route offers the additional advantage that it produces a protected compound from which the three protecting groups can be removed selectively.

The α-deuteriated kynurenine prepared using the second route was used to measure the primary deuterium isotope effects for the kynureninase catalysed reaction The solvent isotope effect was also determined.

4.4 KINETIC ISOTOPE EFFECTS.

Changes in the reaction rate which are brought about by isotopic substitution, i.e. substitution of a heavy atom for a lighter one, are known as *kinetic isotope effects* and carry mechanistic information. The most obvious piece of information that can be provided by isotope effects is the identity of the rate limiting step for a reaction. It is also possible to distinguish stepwise reactions from concerted reactions and for stepwise reactions the order of the steps and the nature of the intermediates can often be determined. The following types of isotope effect are distinguished:

a) *primary*, in which the bond to the isotopic atom is broken in the rate determining step.

b) *secondary*, in which the bond to the isotopic atom(s) remains intact throughout the reaction.

c) *solvent isotope effects*, which result from isotopic differences in the medium.[186]

A kinetic isotope effect is the ratio of the reaction rates for molecules containing the light and the heavy atoms i.e. k_H/k_D. Using the nomenclature originally developed by Northrop such an isotope effect is then written as ^Dk.[187] When the value of an isotope effect is greater than one it is known as a normal isotope effect, but when it is less than one, it is an inverse isotope effect.

Kinetic isotope effects can be determined for enzyme catalysed reactions on the fundamental kinetic parameters V_{max} (V) and V_{max}/K_M (V/K), the apparent first order rate constant for the reaction at low substrate concentration. Using the Northop nomenclature they are written as ^DV and $^DV/K$ respectively. Thus V and V/K are separately measured for both the labelled and unlabelled substrate and comparison of the results will directly provide the isotope effects on V and V/K.[188]

4.4.1 The Observed Isotope Effect.

An enzyme catalysed reaction usually consists of a large number of separate reaction steps of similar energy, the rate of only one of which is affected by isotopic substitution. Therefore, it is rare for the isotopically sensitive step to be cleanly rate limiting. For example, the binding and debinding of substrate(s) and product(s), further chemical steps and possibly conformational changes in the protein may all be partially rate limiting. It is possible, however, to derive an equation to express the observed isotope effect on either V/K or V.

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

$$^D(V) = \frac{^Dk + c_{vf} + {}^DK_{eq}\, c_r}{1 + c_{vf} + c_r}$$

Where, ${}^{D}k$ is the intrinsic isotope effect on the isotopically sensitive step in the forward direction. The intrinsic isotope effect is the isotope effect that would be observed if the isotopically sensitive step was completely rate limiting. ${}^{D}K_{eq}$ is the equilibrium isotope effect for the overall reaction. The constants c_f and c_r are known as the reaction commitments, in the forward and reverse directions respectively. 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. The term c_{vf} in the equation for ${}^{D}V$ is in fact not a commitment but a special constant found only in this equation. This is the sum of the ratios of the net constant for the isotopically sensitive step to all net rate constants in the forward direction multiplied by a precatalytic proportion factor.

For example, for a general enzyme catalysed reaction with a single substrate (A) and two products (P and Q) (Scheme 4.13). If the isotopically sensitive steps are k_5 and k_6. Then c_f, c_r and c_{vf} are as shown.

$$E + A \xrightleftharpoons[k_2]{k_1} EA \xrightleftharpoons[k_4]{k_3} EA^* \xrightleftharpoons[k_6]{k_5} EPQ^* \xrightleftharpoons[k_8]{k_7} EPQ \xrightarrow{k_9} EQ \xrightarrow{k_{11}} E$$

Scheme 4.13

$$c_f = (k_5 / k_4)\ [1 + (k_3 / k_2)]$$
$$c_r = (k_6 / k_7)\ [1 + (k_8 / k_9)]$$

$$c_{vf} = \frac{k_5}{(1 + (k_4 / k_3))}\left[\frac{1}{k_3} + \left(\frac{1}{k_7}\right)\left(1 + \frac{k_8}{k_9}\right) + \frac{1}{k_9} + \frac{1}{k_{11}}\right]$$

An interesting comparison can be seen from ${}^{D}(V/K)$ and ${}^{D}(V)$. ${}^{D}(V/K)$ compares steps near the isotopically sensitive one, however, for ${}^{D}(V)$, which contains the parameter c_{vf}, a wider range of steps in the enzyme catalysed reaction are considered.

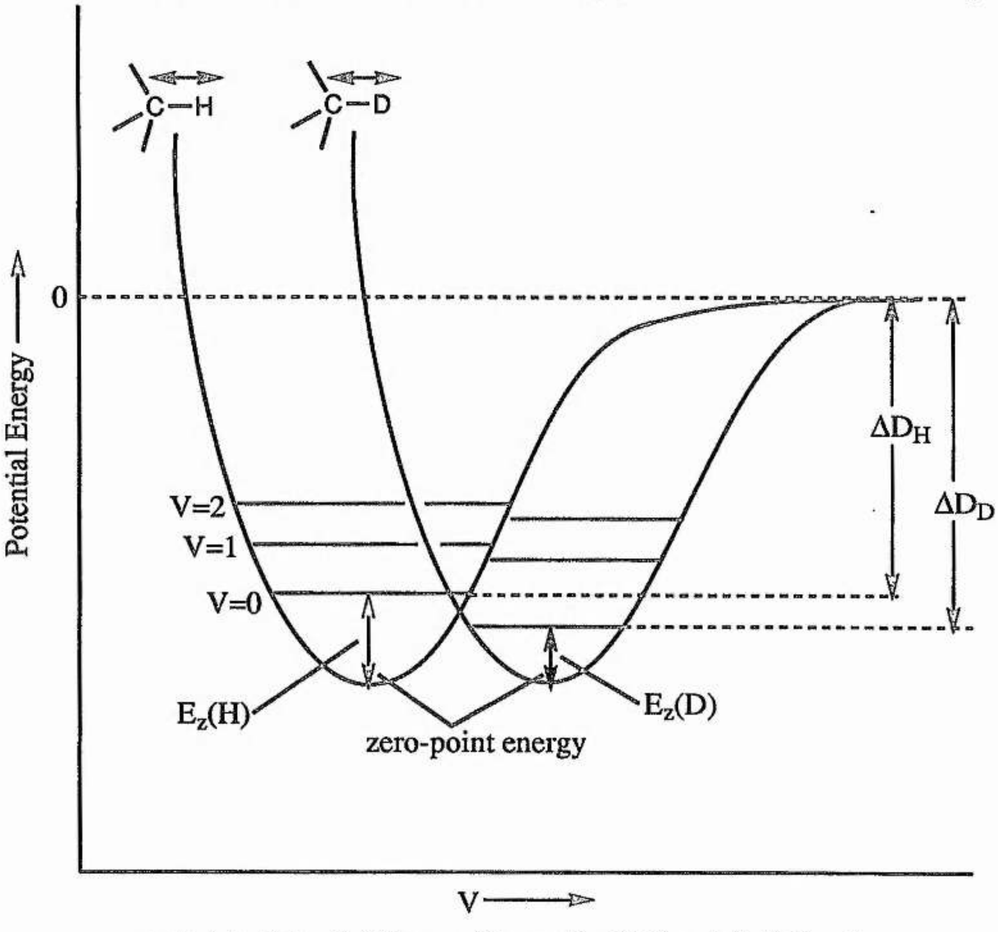
The precatalytic proportion factor is $1/[1+(k_4+k_3)]$. This is the proportion at equilibrium of complexes prior to the isotopically sensitive step that are reversibly connected and available to undergo the isotopically sensitive step. The net rate constants that are being compared with k_5 are k_3, $k_7(1+k_8/k_9)$, k_9 and k_{11} and if any of these are smaller than k_5, then c_{vf} will be large and the values of ^DV will approach one.

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 of the forward and reverse commitments. Thus, slow release of the substrate, i.e. when c_f is large, commits the reaction to take place and suppresses the isotope effect, so that $^D(V/K)$ will approach one. Slow product release or slow steps after the isotopically sensitive step but prior to product release i.e. c_r is large, brings the chemical step to equilibrium so that $^D(V/K)$ will approach $^DK_{eq}$. Only when both c_f and c_r are small will the observed isotope effect approach the intrinsic isotope effect, ^Dk.

The isotope effect on V is different, in that slow steps following release of the first product suppress the observed isotope effect. In the example, if k_{11} is small then the value of c_{vf} will be increased and as a result ^DV will approach one. There is no corresponding effect on $^DV/K$.

4.4.2 The Primary Kinetic Isotope Effect.

Vibrational energy will usually change during the course of a reaction or between reactant and transition state, since some bonds are in the course of being broken or made and their associated frequencies will be effected. Isotopic substitution should, therefore, effect reaction rates. The potential functions for C-H and C-D bond stretching are essentially identical but the distribution of vibrational energy levels in each bond differs. Those of the C-D bond lie at a lower energy than those of the C-H bond for a given value of V because the vibrational frequency of D is lower due to its greater mass. The ground vibrational state is defined by V=0, this is known as the *zero point energy* (vibrational energy remaining even at 0 K) and lies lower for the C-D bond (Graph 4.1). It is clear, then, that more energy is expended in breaking a C-D bond than a C-H bond since it was originally at the lower potential. In general, the dissociation energy of a bond to a heavy isotope is greater than that to a light isotope of the same element and is associated with a slower rate. Therefore, the thermal dissociation of a C-H bond should proceed faster than that of a C-D bond under similar conditions since the activation energies are simply the bond dissociation energies.[186]



graph 4.1: Potential Energy Curves for C-H and C-D Bonds

4.4.2.1 Determination of the Primary Isotope Effect for (2*S*)-[2-^{2}H]-kynurenine.

The purity of (2*S*)-[2-^{2}H]-kynurenine was confirmed by agreement of the extinction coefficients which were determined for both kynurenine and α-deuteriated kynurenine (average values are shown below.) ^{1}H NMR showed that there was 13% hydrogen in the α-position of the compound.

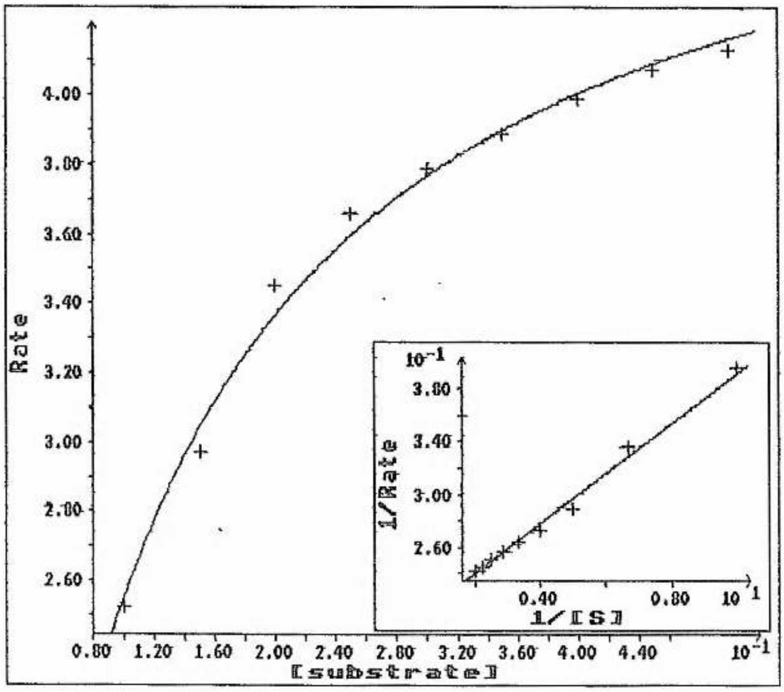
	Kynurenine	**α-Deuteriated kynurenine**
ε	4497 M^{-1} cm^{-1} (lit. 4500 M^{-1} cm^{-1})[42]	4516 M^{-1} cm^{-1}

Kinetic measurements using (2*S*)-[2-^{2}H]-kynurenine were carried out under identical conditions to those used for determination of the K_M and V_{max} for kynurenine. Thus the rate was determined at increasing concentrations of (2*S*)-[2-^{2}H]-kynurenine. Each rate was determined two or three times until the values were consistent and the average value is quoted. Incubation was carried out at 25 °C and the assay buffer contained 0.4 mM kynurenine, 40 μM PLP in 0.04 M potassium phosphate at pH 7.0. The reaction was followed by UV spectophotometry by measuring the rate of decrease in absorbance at 360 nm due to conversion of kynurenine to anthranilic acid (see Experimental for full details). The kinetic parameters of kynurenine without (2*S*)-[2-^{2}H]-kynurenine were the average values calculated ($K_M = 25.5 \pm 0.6$ μM and $V_{max} = 4.78 \pm 0.06$ min^{-1}, see experimental).

The averaged results are shown in table 4.1 and the Michealis-Menten and Lineweaver-Burk plots are shown in graph 4.2.

[Substrate] (mM)	**Rate (min^{-1})**
0.10	2.52
0.15	2.97
0.20	3.45
0.25	3.66
0.30	3.79
0.35	3.89
0.40	3.99
0.45	4.08
0.50	4.13

Table 4.1



Graph 4.2

The K_M and V_{max} values obtained are shown below along with the corresponding values for kynurenine and from these results ^{D}V and $^{D}(V/K)$were calculated.

	Kynurenine	**α-Deuteriated kynurenine**
V_{max}	$(1.07 \pm 0.01) \times 10^{-3}$ $mol\ dm^{-3}\ min^{-1}$	$(1.09 \pm 0.02) \times 10^{-3}$ $mol\ dm^{-3}\ min^{-1}$
K_M	25.5 μM ± 0.6 μM	93.6 μM ± 5.3 μM

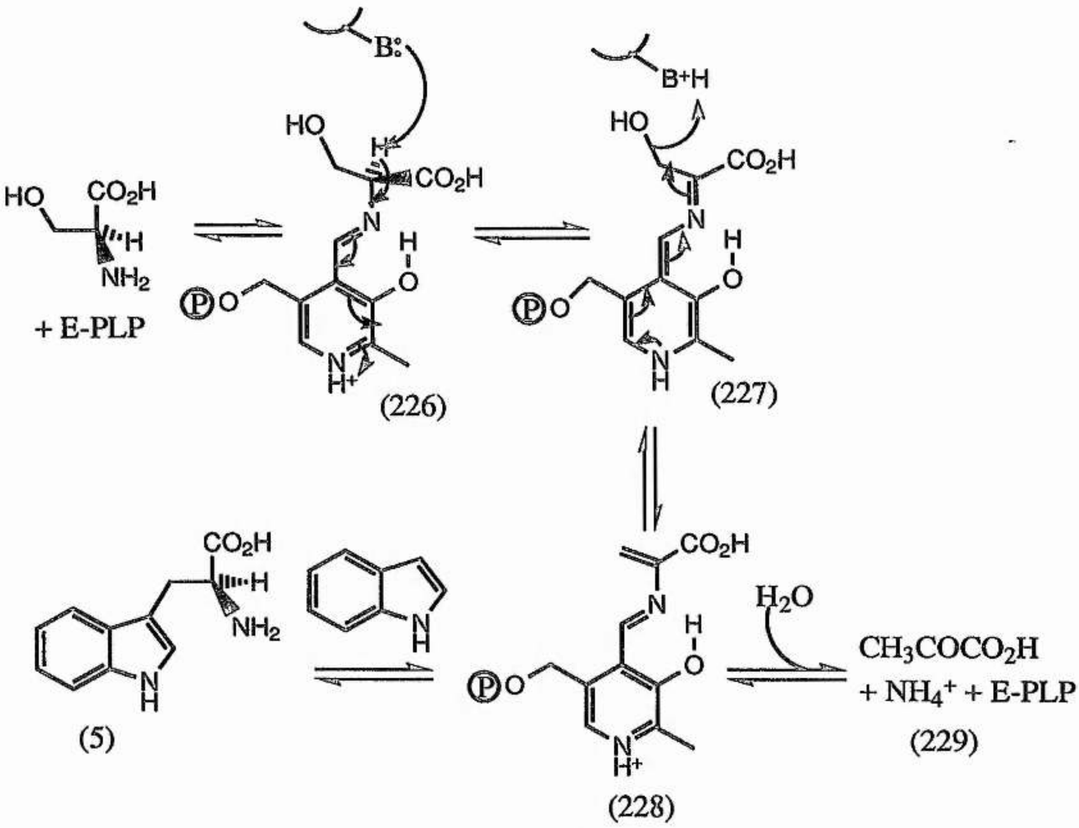
$$^{D}V = 0.98, \quad ^{D}\left(V/K\right) = 3.6$$

As an isotope effect is observed this implies that α-H cleavage is at least partially rate limiting. These results are interesting when we compare them to those obtained by Miles and McPhie who studied the α-H abstraction in the reaction catalysed by the β2 subunit of tryptophan synthase.[189]

4.4.2.2 The Primary Isotope Effect for Tryptophan Synthase β2 Subunit.

The enzyme tryptophan synthase is a multienzyme complex with an α2β2 subunit structure. The α-subunit catalyses the cleavage of 3-indole-2*R*-glycerol 3' phosphate to 2*R*-glyceraldehyde-3-phosphate and indole. The β-subunit catalyses the condensation of indole with 2*S*-serine to form tryptophan. The β-subunit also catalyses the dehydration of serine to pyruvate in the absence of indole. Both of these reactions involves an α-β-elimination and the intermediate formation of an aminoacrylate derivative with PLP.

The reaction of the tryptophan synthase β2 complex with 2*S*-serine involves formation of an enzyme substrate Schiff's base (226), abstraction of the α-proton of 2*S*-serine produces a quinoid intermediate (227) and the loss of hydroxide ion forms the aminoacrylate intermediate (228). Indole then adds to the product to give tryptophan as the product or, in the absence of indole, hydrolysis occurs to give pyruvate and ammonia (229) as the products (Scheme 4.14).



Scheme 4.14

Miles and McPhie studied the rate of proton abstraction for the conversion of 2*S*-serine to pyruvate and ammonia. Substitution of deuterium for the α-hydrogen had no effect on the K_M but increased the V_{max} four fold. They reasoned that this kinetic isotope effect indicated that cleavage of the α-CH bond of 2*S*-serine is the rate determining step in the formation of pyruvate under these conditions.

4.4.2.3 Comparison of Primary Isotope Effects.

The results found for the tryptophan synthase β2 complex were $^{D}V = 4.0$ and $^{D}(V/K) = 4.0$. However, for kynureninase values of $^{D}V = 0.98$ and $^{D}(V/K) = 3.6$ have been determined. As has been discussed the values of ^{D}V and $^{D}(V/K)$ can be very different. The rate limiting step for V/K is simply the one with the highest barrier on the free energy profile for the reaction. When this step comes before or after the isotope sensitive step, then c_f or c_r is large and the size of the isotope effect is reduced. The rate limiting step for V, however, may not necessarily be the same. It may be limited by steps that follow the first irreversible one or steps that precede the addition of the variable substrate.

In both cases, $^DV/K$ is quite large implying that c_f and c_r are not very big, from the equation:

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

For kynureninase, however, ^DV is approximately equal to one implying that c_{vf} is large from the equation:

$$^D(V) = \frac{^Dk + c_{vf} + {}^DK_{eq}\, c_r}{1 + c_{vf} + c_r}$$

This suggests that α-hydrogen abstraction is only partially rate limiting as the effect on ^DV is suppressed by the commitments and a later slower step.

$$c_f = (k_5 / k_4)\ [1 + (k_3 / k_2)]$$

$$c_r = (k_6 / k_7)\ [1 + (k_8 / k_9)]$$

$$c_{vf} = \frac{k_5}{(1 + (k_4 / k_3))}\left[\frac{1}{k_3} + \left(\frac{1}{k_7}\right)\left(1 + \frac{k_8}{k_9}\right) + \frac{1}{k_9} + \frac{1}{k_{11}}\right]$$

This also suggests that one of the later steps in the reaction is more rate limiting as the value of c_{vf} takes into account the rate of these steps, for example, k_{11} in the general example. In the kynureninase catalysed reaction this may correspond to protonation of the aminoacrylate derivative, product debinding etc. In contrast, the effect on ^DV is not suppressed for tryptophan synthase as the α-hydrogen abstraction step is cleanly rate limiting and there are no other slower steps following it.

In order to compare the results with literature observations, further examples of primary isotope effects will be discussed later, along with the results for the solvent isotope effect studies. The solvent isotope effect for kynurenine was, therefore, measured as a further mechanistic probe.

4.4.3 The Solvent Isotope Effect.

With most isotope effect measurements only a single site in the substrate is substituted with the heavy atom and only one reaction step is sensitive to this change. However, solvent kinetic isotope effects are much more global in nature. When an enzyme catalysed reaction is carried out in ^2H_2O rather than H_2O all the acidic hydrogens in both the enzyme and the substrate will be exchanged for deuterium. The consequences of so many substitutions, possibly hundreds of atoms, may cause problems in the interpretation of the observed isotope effect.[190] Nevertheless, solvent isotope effects have been used extensively in the investigation of enzyme catalysed reactions.[191-194]

A solvent effect will be observed whenever a reaction involves changes in the bonding of any water molecule or exchangeable hydrogen. The effect is primary if the hydrogen is transferred during reaction. The magnitude of the observed primary solvent isotope effect is greatest when the hydrogen is in flight between water (or an exchangeable position) and a carbon atom. If hydrogen is transferred between two atoms such as oxygen or nitrogen smaller isotope effects are observed depending on the transition state structure.[190]

4.4.4 Determination of Solvent Isotope Effect for Kynurenine.

4.4.4.1 Preparation of Buffers.

In order to obtain accurate results all the labile hydrogens must be exchanged for deuterium in all the buffers. To achieve this the buffer solutions were lyophilised from ^2H_2O two or three times. This did not effect the concentrations or stability of PLP or other buffer constituents, as a comparable assay with normal buffer gave the same result. During the preparation of the buffers the ^2H_2O solutions were not exposed to atmospheric moisture to avoid re-exchange. Measurement of the pH (pD) of the solution was carried out using a normal glass pH electrode. The electrode was soaked in a small amount of ^2H_2O prior to measuring the pD of a ^2H_2O buffer solution. To determine pD for buffers in ^2H_2O an allowance was made for the isotope effect on the electrode response. Thus,

pD = meter reading + 0.4[190]

A stock solution of NaO^2H was prepared for the pD adjustment of the buffers employed. This was prepared by adding sodium, which had been washed in ethanol, petroleum ether and ^2H_2O, to a small amount of ^2H_2O. This was stored under nitrogen until required.

4.4.4.2 Effects of ^2H_2O on Enzyme Structure.

The effect of ^2H_2O on the structure and stability of proteins has been an area of much concern in the interpretation of solvent isotope effects. Incomplete exchange of all the acidic enzyme protons in ^2H_2O has been proposed as a source of error in isotope effect measurements.[190] Control experiments were therefore carried out to investigate this possibility. Stock solutions of enzyme were prepared in equivalently buffered H_2O and ^2H_2O and then the isotope effect measured using each stock solution.

4.4.4.3 Preparation of kynureninase in ^2H_2O

One method of obtaining the enzyme in ^2H_2O involves lyophilising the partially purified protein from ^2H_2O two or three times so that all the labile hydrogens will be exchanged for deuterium. However, when this was carried out using kynureninase the enzyme activity was found to be decreased by about forty percent after only one lyophilisation. The protein also appeared to be less stable and began to precipitate out of solution during the assay.

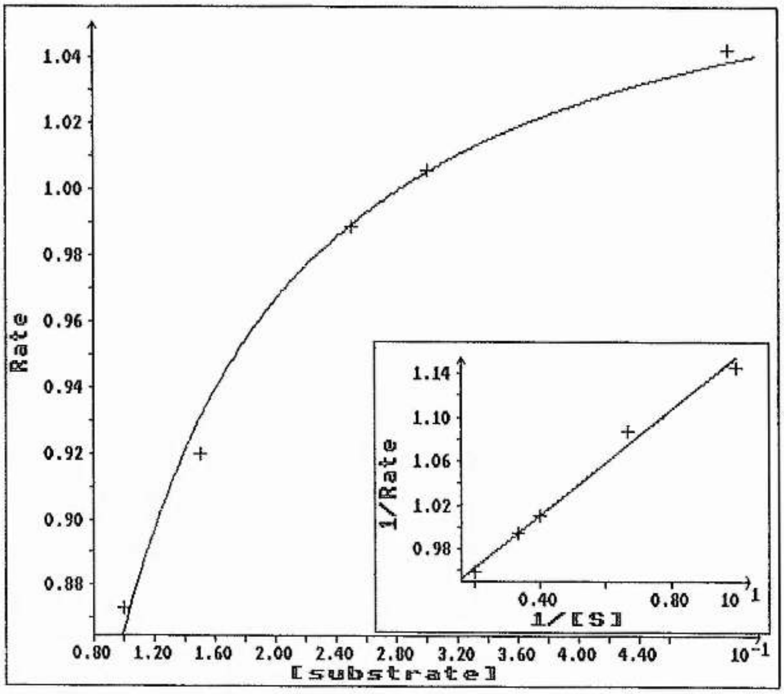
A less harsh method involved the addition of ^2H_2O whilst carrying out the ultrafiltration step in the purification. Several portions of ^2H_2O were added to ensure that all the labile hydrogens were exchanged for deuterium. The ultrafiltration apparatus was cooled with cold water as opposed to ice due to the higher melting temperature of ^2H_2O compared with H_2O. The enzyme once obtained was used immediately although a portion was also stored in the freezer (-78 °C) overnight. The enzyme isolated and purified had been split into two batches. Half of it was in H_2O based buffer and the other half in ^2H_2O based buffer. The enzyme activity of the two portions was compared and it was found that negligible activity was lost in the ^2H_2O solution compared to the normal buffer solution.

4.4.4.4 Measurement of Solvent Isotope Effects.

The kinetic parameters for kynurenine were then determined in the ^2H_2O buffer solution. Thus the rate was determined at increasing concentrations of kynurenine. Each rate was determined two or three times until the values were consistent and the average value is quoted. Incubation was carried out at 25 °C and the assay buffer contained 0.4 mM kynurenine, 40 µM PLP in 0.04 M potassium phosphate at pH 7.0. The reaction was followed by UV spectophotometry by measuring the rate of decrease in absorbance at 360 nm due to conversion of kynurenine to anthranilic acid (see Experimental for full details). Assays were carried out using both stock solutions of kynureninase in H_2O and in ^2H_2O. In each case, an aliquot of enzyme solution (30µl) was added to the assay buffer (1ml) to commence reaction. An example is shown in table 4.2 and graph 4.3 and the full results are found in the Appendix.

[Kynurenine] (mM)	**Rate (min^{-1})**
0.10	0.873
0.15	0.920
0.25	0.989
0.30	1.01
0.50	1.04

Table 4.2



Graph 4.3

A summary of the results obtained are shown below.

V_{max} (mol dm^{-3} min^{-1})	**K_M (μM)**
$(0.24 \pm 0.01) \times 10^{-3}$ [a]	27.0 ± 0.946 [a]
$(0.24 \pm 0.02) \times 10^{-3}$ [b]	26.2 ± 1.72 [b]
$(0.24 \pm 0.007) \times 10^{-3}$ [b]	26.2 ± 0.667 [b]

[a] Enzyme in H_2O, [b] enzyme in ^2H_2O.

The average K_M and V_{max} values obtained are shown (table 4.3) along with the corresponding values for kynurenine and from these results ^DV and $^D(V/K)$ were calculated.

	Kynurenine in H_2O	**Kynurenine in ^2H_2O**
V_{max}	$(1.07 \pm 0.01) \times 10^{-3}$ mol dm^{-3} min^{-1}	$(0.24 \pm 0.01) \times 10^{-3}$ mol dm^{-3} min^{-1}
K_M	25.5 μM ± 0.6 μM	26.5 μM ± 0.8 μM

Table 4.3

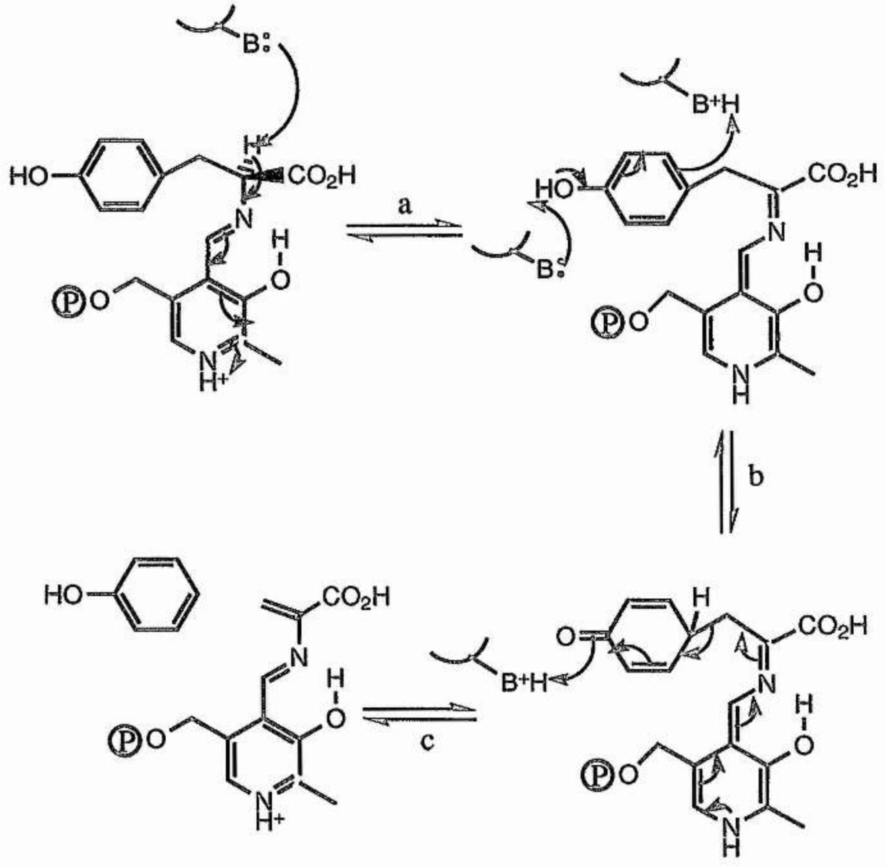
$$^DV = 4.4,\ ^D\left(V/K\right) = 4.6$$

From the results it can be seen that changing the solvent from H_2O to ^2H_2O has no effect on the K_M but decreases the V_{max} four fold. The kinetic parameters are identical whether or not the kynureninase is pre-incubated in ^2H_2O. This implies that exchange is faster than reaction with kynurenine. The values were also found to be consistent during the enzyme assays implying that there are no slowly exchanging sites on the enzyme which can affect the isotope effect.

The results can be compared with literature values for similar enzymes. For example, Faleev *et al.* have studied the isotope effects of tyrosine phenol lyase with suitable substrates.[194]

4.4.4.5 Isotope Effects for Tyrosine Phenol Lyase.

Tyrosine phenol lyase has a broad substrate specificity and catalyses the α,β-elimination of 2*S*-tyrosine and its ring substituted analogues to give phenols and ammonium pyruvate. The generally accepted mechanism of catalysis by tyrosine phenol lyase includes three principle chemical transformations of the substrate. The first being abstraction of the α-proton in the external aldimine (a), the second being tautomerisation of the aromatic moiety to convert it into a good leaving group (b) and finally β-elimination of the leaving group (c) (Scheme 4.15). The relative significance of these three stages has been elucidated for the reaction with 2*S*-tyrosine and 3-fluoro-2*S*-tyrosine and their deuteriated equivalents.



Scheme 4.15

For the α-hydrogen abstraction it was found that ^{D}V was 2.9 for tyrosine and ^{D}V was 3.4 for 3-fluoro-tyrosine which decreased to 2.0 in $^{2}H_2O$.

A reaction mechanism was suggested where the tautomerisation stage contributes significantly to the total free energy barrier although the highest maximum on the free energy profile corresponds to the subsequent β-elimination stage. The α-kinetic isotope effects may seem to be too high to be compatible with this concept. However, if the α-proton abstraction is an equilibriated stage the kinetic isotope effect reflects the isotope effect on the steady state concentration of the quinoid intermediate [EX] which is given by $\frac{k_a}{k_a + k_r + k_f}$.

From:

$$\mathrm{ES} \underset{k_r}{\overset{k_a}{\rightleftharpoons}} \mathrm{EX} \xrightarrow{k_f} \mathrm{P}$$

where:

k_a = Rate of isotope sensitive α-proton abstraction

k_r = Rate of reprotonation

k_f = Rate of subsequent steps

The considerable values of observed α-kinetic isotope effects mean that the following conditions are satisfied:

(i) k_a is considerably less than $k_r + k_f$

and (ii) kr is not sensitive to α deuteriation of the substrate.

This is possible when the degree of internal return of the abstracted proton is negligible due to fast exchange with the solvent. In this case the decrease in the α-kinetic isotope effect on going from water to ^2H_2O may be a result of a decrease in the k_r value, which partially compensates the α-kinetic isotope effect on k_a.

Changing the solvent from water to ^2H_2O should mainly influence the rate of tautomerisation of the phenolic ring into a cyclohexadienone ring. This process consists of protonation of the C1 atom of the ring by a general acid catalyst and deprotonation of the phenolic hydroxy group by the other base. The protons of both the group performing protonation and the phenolic hydroxy groups are exchangable with the solvent; consequently the process should be slowed down in ^2H_2O.

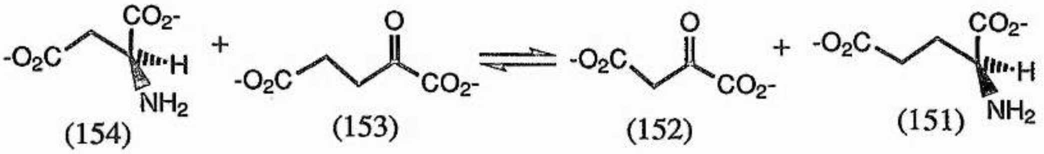
For the solvent isotope effect in ^2H_2O,

$^DV = 1.66$ for tyrosine and $^DV = 1.7$ for 3-fluoro-tyrosine.

The values of solvent and α-kinetic isotope effect are practically the same for tyrosine and 3-fluoro-tyrosine. Based on this result and the low absolute solvent kinetic isotope effect it may be assumed that the tautomerisation stage is at equilibrium.

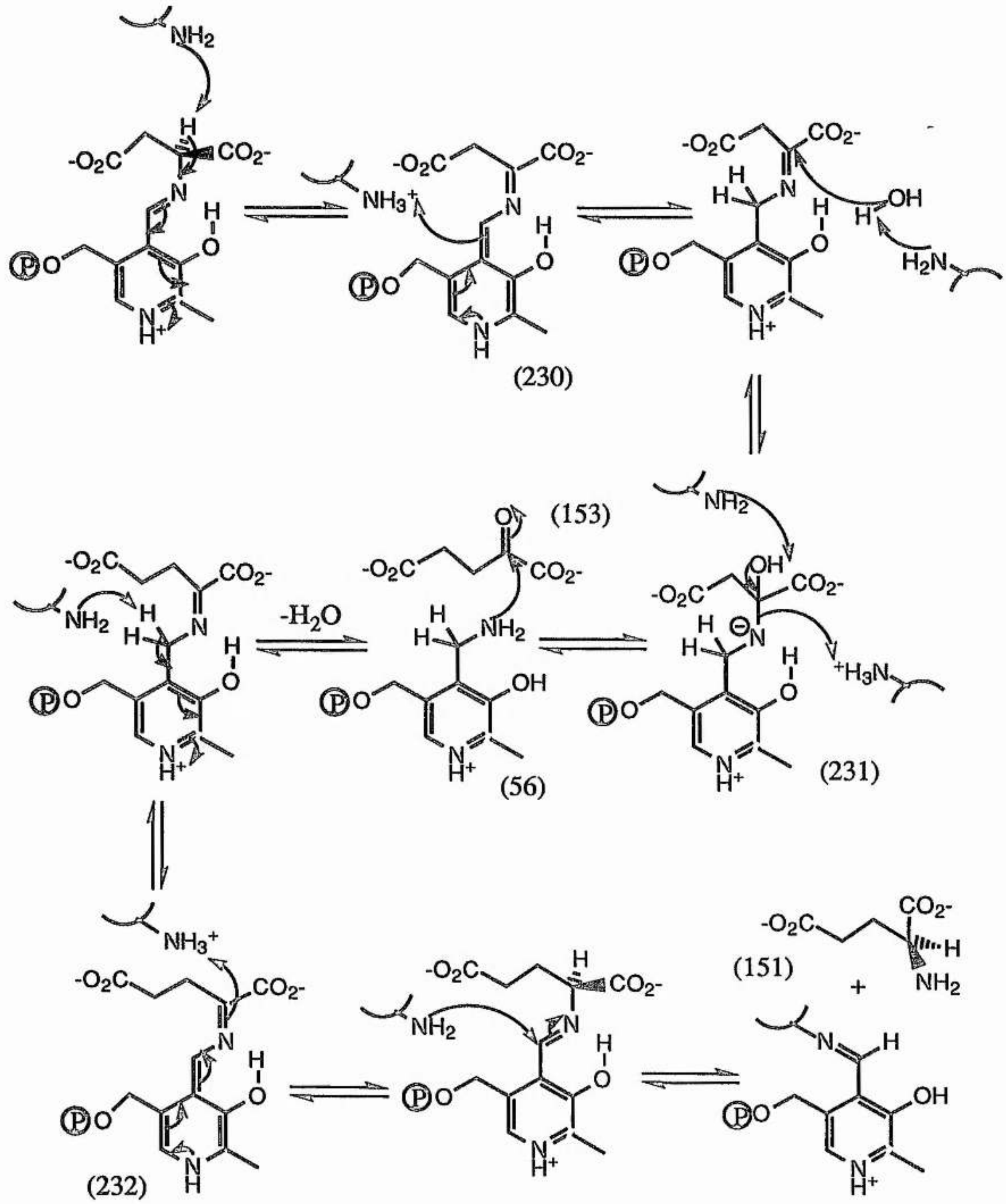
4.4.4.6 Isotope Effects for Aspartate Aminotransferase.

Aspartate aminotransferase is another PLP dependent enzyme whose kinetic isotope effects have been studied.[195] Aspartate aminotransferase catalyses the reversible exchange of the α-amino group between 2*S*-aspartate (154) and α-ketoglutarate (153) (Scheme 4.16). The amino group of aspartate is transferred to an active site PLP with the subsequent release of oxaloacetate (152) and this is followed by the transfer of the amine to α-ketoglutarate to give 2*S*-glutamate (151).



Scheme 4.16

α-Hydrogen abstraction takes place to form a ketimine intermediate (230) which then undergoes protonation at C4'. The general base then accepts a water proton to facilitate the nucleophilic attack by water and then aids in collapse of the resultant carbinolamine (231) to the PMP enzyme. The PMP (56) and α-ketoglutarate (153) then form a complex which is deprotonated at C4' to give the quinoid intermediate (232). Protonation then occurs at the α-position and on release from the PLP 2*S*-glutamate (151) is formed and the holoenzyme regenerated (Scheme 4.17).



Scheme 4.17

The α-kinetic isotope effects have been determined for aspartate and glutamate.

For aspartate, $^{D}V = 1.4$ and $^{D}(V/K) = 1.7$,

for glutamate, $^{D}V = 1.5$ and $^{D}(V/K) = 2.0$.

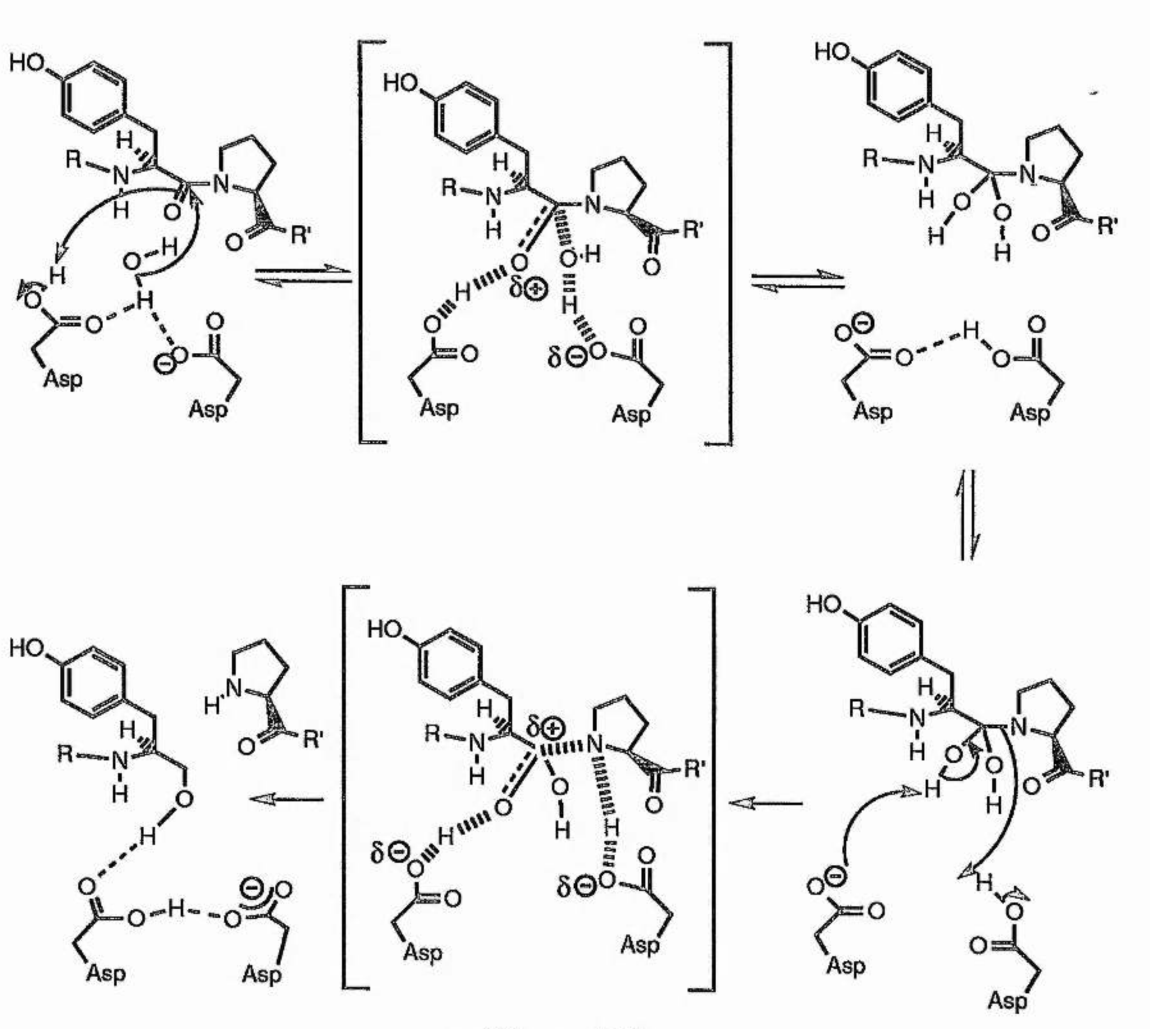
$^{D}(V/K)$ reflects the rate limitation on the first half reaction and ^{D}V is a function of both half reactions. As a result of the effects on (V/K) in both reaction directions, the abstraction of the α-proton is at least partially rate determining. In addition, the lower value of ^{D}V compared to $^{D}(V/K)$ suggests that the second half reaction also limits at saturating reactant concentrations.

The solvent isotope effects have also been determined for aspartate and were found to be:
$^{D}V= 2.21$ and $^{D}(V/K)= 1.71$

The observed solvent isotope effects suggest that a step in the aspartate amino transferase reaction has a proton in flight in the transition state. The larger value of the solvent effect compared to the substrate effect suggests that this step is more rate limiting than α-hydrogen abstraction.

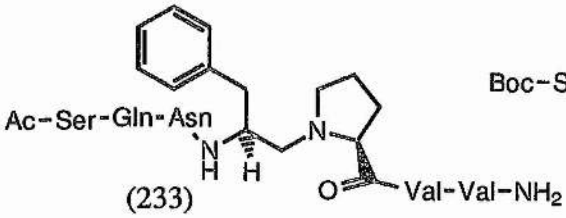
4.4.4.7 Isotope Effects for HIV-1 Proteinase.

The chemical mechanism of HIV-1 proteinase is another example which involves a water molecule. For HIV-1 proteinase, each ninety nine amino acid subunit of the C-2 symmetric homodimer contributes a glycine rich loop that constitutes in part the substrate binding site of the enzyme and one of two active site aspartyl residues. The active-site aspartyl residues exist on opposite sides of the C-2 axis of symmetry present in the homodimeric protein. It is believed that the active-site aspartyl groups assume opposite roles in general acid-general base catalysis, in which deprotonation of the lytic water is performed by the unprotonated aspartyl residue and protonation of the scissile carbonyl oxygen is achieved by the protonated aspartyl residue. A proposed chemical mechanism for this is shown in scheme 4.18.

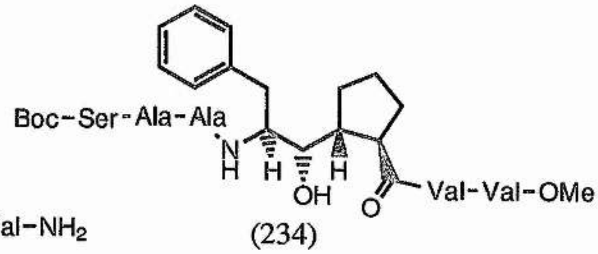


Scheme 4.18

The solvent isotope effect was determined for two substrates (233, 234) over a range of pH.



(233)



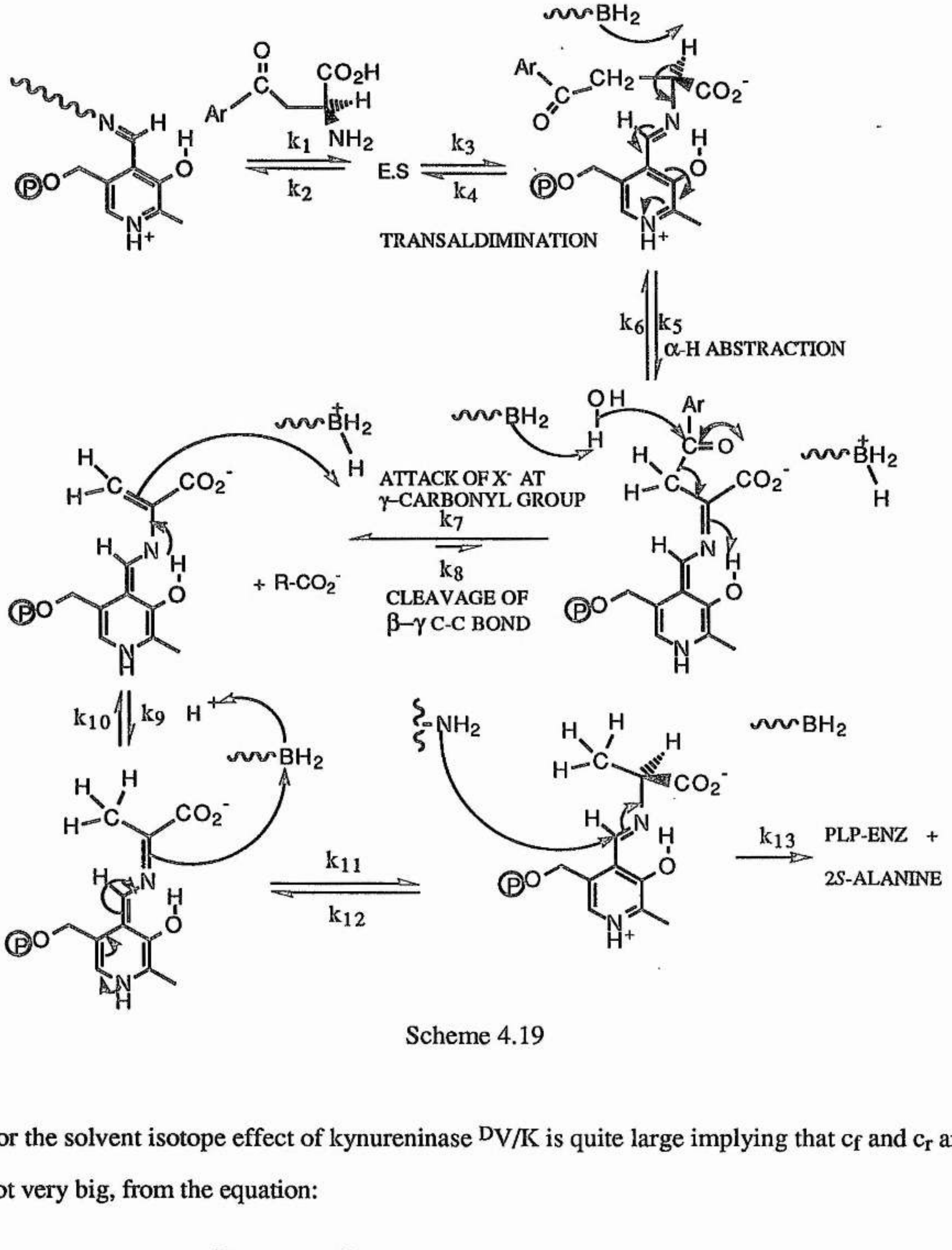
(234)

The value of $^{D}(V/K)$ was 1.0 and pH independent while ^{D}V decreased from 2.2-3.2 to 1.5-1.7 as the pH was decreased. This was consistent with a change in rate limiting step from a chemical one at high pH to product release or enzyme isomerisation being partially rate limiting at low pH.[196]

4.4.4.8 Comparisons of Isotope Effects.

The putative mechanism of kynureninase is shown in Scheme 4.19.

For aspartate aminotransferase, the values of the solvent isotope effect were the same for ^{D}V and $^{D}(V/K)$, i.e. $^{D}V= 2.21$ and $^{D}(V/K)= 1.71$. For kynureninase this was also found to be the case where, $^{D}V= 4.4$ and $^{D}(V/K)= 4.6$. This result was interpreted as a proton in flight in the rate determining step. It can therefore be implied that kynureninase also has a proton in flight in the rate determining step. Interestingly, for the HIV-1 proteinase the value of $^{D}(V/K)$ was 1.0 and pH independent while ^{D}V decreased from 2.2-3.2 to 1.5-1.7 as the pH was decreased. The lack of effect on $^{D}(V/K)$ results from a high forward commitment. Also the attenuation of the forward commitment is highly pH dependent. The peptide substrates are thought to be able to bind only to the correctly protonated form and chemical steps cannot be made fully rate limiting at a non optimal value of pH(D). The values of ^{D}V indicate that also in this case a proton transfer is at least partially rate limiting for these substrates. The rate limiting proton transfer step occurs either in the formation or breakdown of the enzyme bound tetrahedral adduct or substrate and water.



Scheme 4.19

For the solvent isotope effect of kynureninase $^{D}V/K$ is quite large implying that c_f and c_r are not very big, from the equation:

$$^{D}(V/K) = \frac{^{D}k + c_f + {}^{D}K_{eq}\, c_r}{1 + c_f + c_r}$$

^{D}V is also quite large implying that c_{vf} is also quite small from the equation:

$$^{D}(V) = \frac{^{D}k + c_{vf} + {}^{D}K_{eq}\, c_r}{1 + c_{vf} + c_r}$$

The solvent isotope effect could arise as a result of changes in the rate of a number of different steps. These include formation of the external aldimine (k_3), attack of water on the γ-carbonyl group (k_7), reprotonation at C-β (k_9) and hydrolysis to produce the product. The magnitude of the observed solvent isotope effect is high for transfer of a hydrogen atom between oxygen and nitrogen atoms (usually 2-4). However, for transfer to or from carbon higher values can be obtained (up to 8).

As the effect on ^{D}V is not suppressed, this implies that there are no subsequent slow steps insensitive to changes in the solvent i.e. product debinding.

One possibility for the solvent sensitive step is reprotonation of the aminoacrylate intermediate (k_9). It is known[43] that this intermediate has sufficient lifetime to be trapped out by added aromatic aldehydes. This is consistent with slow protonation of the intermediate, which could be responsible for suppression of the isotope effect for α-H abstraction. One interesting question is the degree of reversibility of this step. Experiments to produce, and trap out, the aminoacrylate from alanine were unsuccessful.[43] This would imply that abstraction of the β-proton from alanine does not take place.

Attack of a water molecule on the γ-carbonyl would also be expected to be a solvent sensitive step. This occurs too soon after α-H cleavage to be responsible for suppression of ^{D}V. The rate constant k_7 also appears in the term for c_r and if it were small would increase c_r and therefore reduce $^{D}(V/K)$ aswell. The reversibility of this step is also open to question. There is no evidence to show trapping of the aminoacrylate intermediate with aromatic acids, only the more reactive aldehydes. Indeed if k_9 is proposed to be the slow step then in order for it to appear only in C_{vf} and not C_r, for the α-deuterium isotope effect, this would require k_7 to be the first irreversible step in the reaction.

However, as yet the evidence provided here is not complete and further experiments are required to elucidate the mechanism in more detail. Firstly, it is important to determine both isotope effects at different pH's to establish if they are dependent or independent of pH. A pH dependence may arise because the commitments have two parts. Firstly, there is the internal commitment which involves rate constants for steps other than reactant release. This does not vary with the levels of other substrates and is independent of pH for most mechanisms. Secondly, there is an external commitment involving rate constants for reactant release steps. The external protonation may become very small at pH values where V/K is decreasing by a factor of about ten per pH unit. This is because incorrect protonation reduces rate constants for the chemical reaction but not rate constants for reactant release. Thus it is possible to eliminate the external commitments by changing the pH and so enhance the observed isotope effect. Thus if the observed V/K isotope effect increases when the pH is changed from the optimum and hence V/K decreases, it implies that the overall commitment was essentially caused by the external commitment.

It would also be necessary to carry out the measurement of the α-kinetic isotope effect in ^2H_2O. This should determine whether the two isotope effects are independent or mutually dependent. If they are independent then the two processes are performed in the same step and the reaction is concerted. On the other hand, if the α-kinetic isotope effect and solvent kinetic isotope effect are mutually dependent this provides evidence for a stepwise mechanism and the result may give some information on the order of these steps.

Also, the preparation and testing of the β,β-dideuteriated kynurenine should complete the isotope effect measurements. For example, Faleev *et al.* determined the isotope effect for β,β-dideuteriated 3-fluoro-2*S*-tyrosine to determine the relative significance of the β-elimination stage of tyrosine phenol lyase.[194] When interpreting the β-kinetic isotope effect the two stages involved in the β-elimination process were considered.

(i) The β-elimination stage leading to α-aminoacrylate formation and

(ii) Subsequent protonation of this intermediate which begins the aminoacrylate hydrolysis.

The rate of the later stage should be the same for all substrates of tyrosine phenol lyase. The elimination stage is accompanied by rehybridisation of the β-carbon atom from sp^3 to sp^2 and consequently is slowed down on passing from the normal to the β,β-dideuteriated substrate. The observed value of the β-kinetic isotope effect (1.1) is comparable with the normally observed secondary kinetic isotope effect in other reactions with sp^3 to sp^2 hybridisation changes and therefore it was concluded that for β,β-dideuteriated 3-fluoro-2*S*-tyrosine, β-elimination is partially rate limiting.

A similar aminoacrylate formation has been proposed in the reaction catalysed by kynureninase so the comparison should be useful.

4.4.4.9 Conclusion.

The isotope effect measurements have shown that for the kynureninase catalysed reaction α-H abstraction is partially rate limiting. The observation of ${}^{D}V = 0.98$, whereas ${}^{D}(V/K) = 3.6$ implying that there is a subsequent slow step in the mechanism, which suppresses the isotope effect on V. The solvent isotope effect was ${}^{D}V= 4.4$ and ${}^{D}(V/K)= 4.6$. These show that a proton transfer is also partially rate limiting. In this case there is almost no suppression of the value of ${}^{D}V$.

The results have begun to shed some light on the chemical mechanism and an indication of which chemical steps are important to the rate but further experiments are required to get a detailed understanding of the mechanism of the enzyme catalysed reaction.

CHAPTER 5

5 PARTIAL PURIFICATION OF KYNURENINASE FROM *PSEUDOMONAS FLUORESCENS.*

5.1 Purification Method.

Kinetic studies were carried out with partially purified kynureninase from *Pseudomonas fluorescens* (Strain ATCC 11250), which was obtained as a freeze dried sample from NCIMB (the National Collection of Industrial and Marine Bacteria). The method for the isolation and purification of the enzyme outlined by Moriguchi *et al.* was followed and as there were few experimental details given each step of the procedure was optimised.[2]

As the enzyme is on the tryptophan metabolic pathway the bacteria are grown in a medium containing a high percentage of tryptophan. The bacteria must metabolise this as a food source to grow and this, therefore, encourages expression of the enzymes on the pathway.

Initially resuscitation of the freeze dried culture was unsuccessful in minimal media and so it was first incubated in nutrient broth which was richer in nutrients. This contained 'Lab.-Lemco' powder 0.1%, yeast extract 0.2%, peptone 0.5% and NaCl 0.5% at pH 7.4. Small bottles of nutrient broth were sterilised and the freeze dried culture divided amongst them. After incubation at 28 °C for eighteen hours these suspensions were kept refrigerated. This was then sub-cultured into the minimal media which contained 0.1% 2*S*-tryptophan, 0.2% peptone, 0.1% glycerin, 0.05% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4.7H_2O$ at pH 7.0. The flasks were completely cooled before inoculation, which was carried out using a sterilised wire loop. Each flask was inoculated twice. The cultures were then grown at 28 °C for eighteen hours under aeration. If the flasks are not shaken then only approximately five per cent growth resulted.

The harvested cells were washed twice with 0.85% sodium chloride solution and subsequently with 0.01 M potassium phosphate buffer (pH 7.2). All buffers used contained 20 μM PLP and 0.01% 2-mercaptoethanol. The yield of cells was approximately 4g (wet weight) per litre of the medium. The washed cells were mixed in a small amount of buffer and sonication was carried out in eight thirty second bursts. The resultant slurry was centrifuged in order to separate the cell-free extract from the cellular debris. It was possible to judge by eye if sonication had been completely successful as the slurry became thinner and darker in colour. This was confirmed by running an assay to check for kynureninase activity. The pellet obtained on centrifugation could be re-extracted into potassium phosphate buffer and showed no kynureninase activity if sonication was complete. Any pellets that were kept to be checked for kynureninase activity were stored at -78 °C until required.

1% Protamine sulphate in 0.01 M potassium phosphate buffer (pH 7.2) was added to the cell free extract with stirring to remove nucleic acids. The mixture was centrifuged and the bulky inactive precipitate was discarded. The protamine sulphate was added slowly and efficient stirring maintained throughout the addition. It was observed that if the addition was too rapid then complete precipitation of the protein resulted. It was possible to re-extract the protein into potassium phosphate buffer (pH 7.2) and repeat the procedure, however this was not as efficient. This process could readily be followed by monitoring for kynureninase activity.

The enzyme solution was brought to 20% saturation with ammonium sulphate and the precipitate removed by centrifugation. The ammonium sulphate was added slowly in portions which were allowed to dissolve before a further portion was added. This required efficient but not too vigorous stirring as the protein could be denatured (shown by the presence of white froth). Ammonium sulphate was then added to the supernatant to 55% saturation. The precipitate, collected by centrifugation, was dissolved in 0.01 M potassium phosphate buffer (pH 7.2) and the resulting enzyme solution was dialysed overnight against one hundred volumes of the same buffer to remove the ammonium sulphate. The dialysed enzyme solution was centrifuged to remove particulates. It was important to assay for kynureninase activity and also to determine the protein content at this stage so that the efficiency of the column chromatography could be evaluated.

5.2 Assay for Kynureninase Activity.

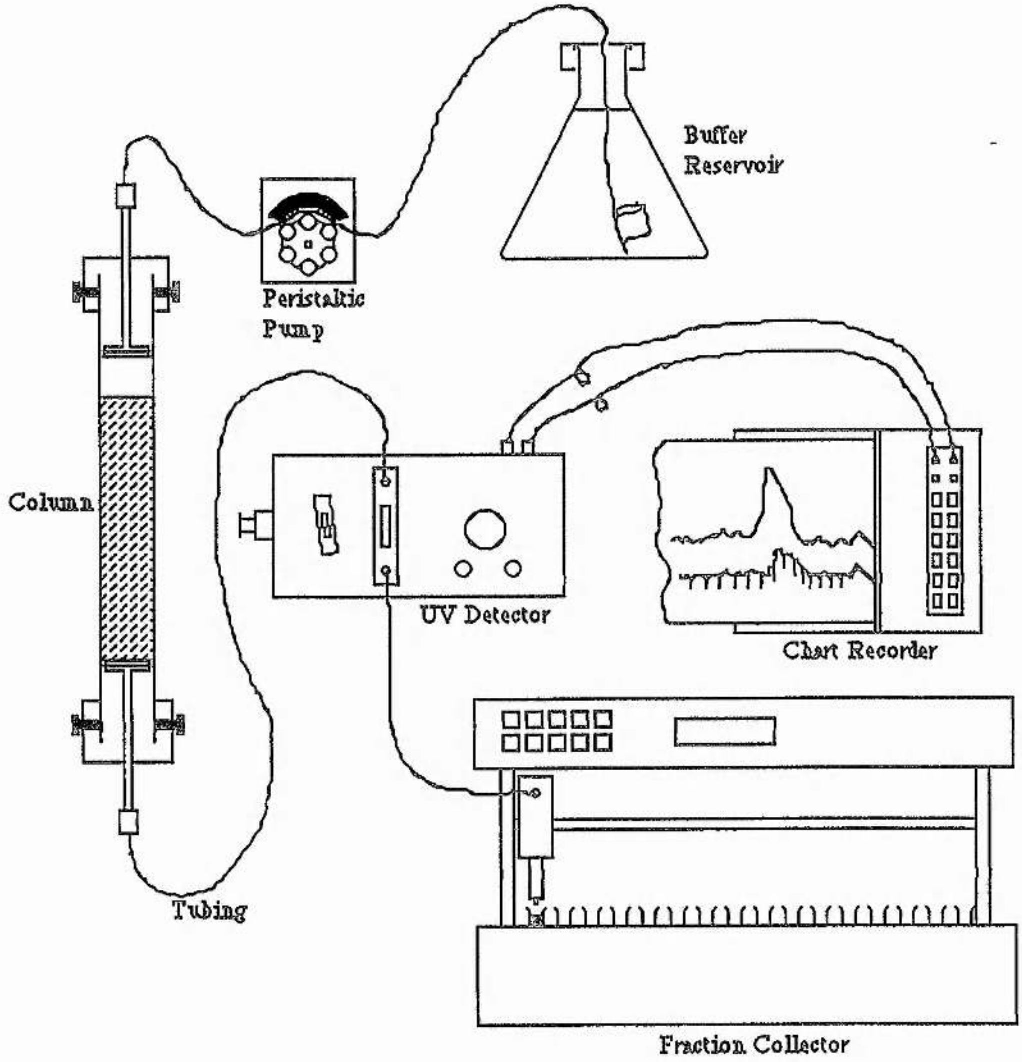
Kynureninase activity was measured from the decrease in absorbance at 360 nm (ε= -4500 M^{-1} cm^{-1}) upon the conversion of kynurenine to anthranilic acid. The reaction mixture contained 0.4 mM (2*S*)-kynurenine in 0.04 M potassium phosphate, pH 7.0 containing 40 μM PLP at 25 °C.[129]

5.3 Determination of Kinetic Parameters.

Determination of inhibition of kynureninase by various compounds was performed by variation of the concentration of (2*S*)-kynurenine at several fixed values of the inhibitor concentration. K_M and V_{max} values were calculated by fitting of initial rate data to the Michaelis-Menten equation with the ENZFITTER[151] programme on a PC.

5.4 Ion Exchange Chromatography of Enzyme Solution.

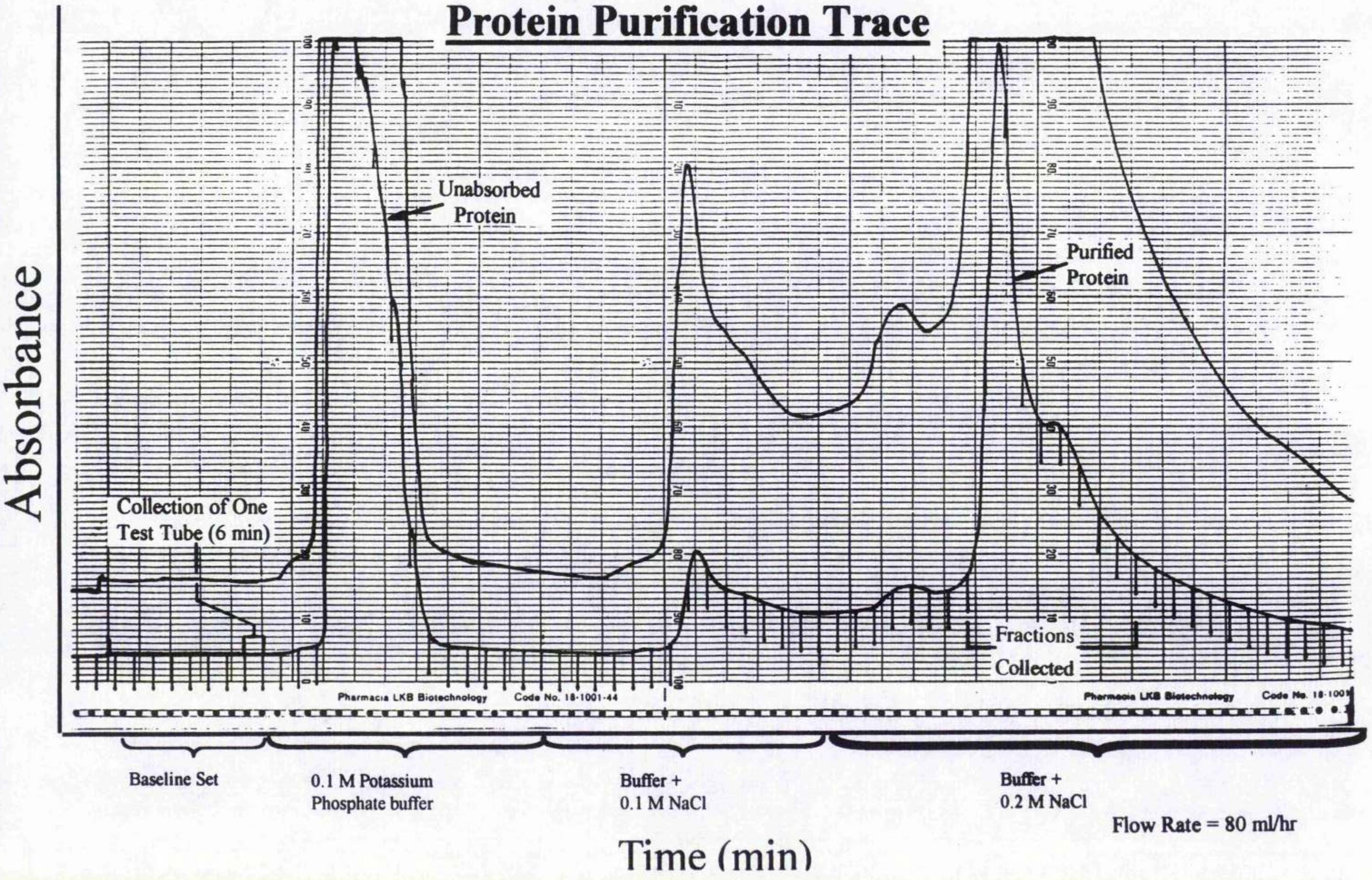
The dialysed enzyme solution was purified by DEAE-cellulose chromatography using the apparatus illustrated overleaf.



Protein purification apparatus

Prior to running the column chromatography, test tube experiments were carried out using aliquots of enzyme and beads. It was hoped to demonstrate that enzyme was indeed absorbed onto the beads removing the activity from the solution. Several washes were then carried out to remove the protein from the beads. Therefore, enzyme solution (3.4 units in 10 ml) was diluted with 0.01 M potassium phosphate buffer (pH 7.2) and ion exchange beads (1.5 g) were added. This was stirred and allowed to settle. When the supernatant was checked for kynureninase activity, none was observed, confirming that the enzyme did bind to the beads. The supernatant was poured off and replaced with the same buffer containing 0.1 M, 0.2 M and 0.5 M sodium chloride respectively. The ion exchange beads were washed a few times with each buffer and the supernatant checked for activity. Unfortunately, there was no sharply defined point when activity was released into the supernatant. Therefore, careful monitoring of the fractions eluted from the column was required.

The DEAE cellulose was mixed with potassium phosphate buffer and the column was packed under gravity and then connected to the pump. Buffer was run through the column at eighty ml per hour to compress the column. The UV detector was zeroed using distilled water which was run through the system (minus the column) until the trace on the chart recorder was stabilised. The baseline was set using 0.01 M potassium phosphate buffer (pH 7.2). The complete system was reconnected and the enzyme solution was applied *via* the peristaltic pump to the top of the column. The column was washed with approximately two bed volumes of 0.01 M potassium phosphate buffer (pH 7.2) to remove any unabsorbed protein. It was then washed with the same buffer containing 0.1 M sodium chloride for one and a half bed volumes. The enzyme was eluted using the buffer supplemented with 0.2 M sodium chloride and the trace was recorded (see example p 194). From the trace of absorbance at 280 nm peaks due to proteins being eluted from the column were observed. The fractions containing protein were assayed for kynureninase activity and the active fractions pooled and brought to seventy percent saturation with ammonium sulphate. The precipitate was collected by centrifugation and suspended in 0.01 M potassium phosphate buffer (pH 7.2). Finally, ultrafiltration using an AMICON stirred cell (50 ml) fitted with a filter (YM 10, 43 mm) was used to concentrate the sample to a few millilitres, which was stored in Epindorph tubes (1.5 ml) at -78 °C until required.



Protein Purification Trace

From the early column purifications it was found that the protein obtained from the 0.1 M elution had negligible kynureninase activity when analysed. This confirmed that some purification had taken place as an inactive protein was removed from the sample. The column length was optimised at 20-25 cm in length and 2.5 cm in diameter with an enzyme loading of 10 ml (40-45 units of activity).

The activity and protein content was calculated for each step in the procedure (See Table 5.1).

From 19.9 g of cells

Step of Procedure	Activity (units)	Protein Content (mg)	Specific Activity (units mg^{-1})	% Purification[a]
Sonication	81.5	1522	0.0535	0 (0)
1% Protamine Sulphate	76.0	1458	0.0521	6.7 (2)
20% Ammonium Sulphate Cut	72.0	1155	0.0623	12 (10)
Dialysis	47.1	812	0.0580	42 (-)[b]
Column Chromatography	20.2	131	0.154	75 (79)

[a] Figures in brackets denote literature values.[2]

[b] Literature value not quoted.

Table 5.1

The literature procedure shows comparable purity to the method outlined above for the final partially purified enzyme. Some steps showed slightly greater purity than the literature values possibly because these were easier to monitor and control on a small scale.

5.5 Determination of Kinetic Parameters for Kynurenine.

The partially purified enzyme was used to determine the kinetic parameters for kynurenine under the standard conditions (0.4 mM (2*S*)-kynurenine in 0.04 M potassium phosphate, pH 7.0 containing 40 µM PLP at 25 °C), K_M was found to be 25.5 µM and Vmax was 1.07 x

10^{-3} mol dm^{-3} min^{-1} (see Appendix). The K_M is in excellent agreement with literature data i.e. K_M is 25 μM.[140, 36] The enzyme was then used in further kinetic studies and in the evaluation of new compounds as substrates and inhibitors.

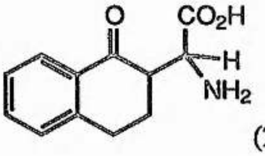
FURTHER WORK.

The work described in the thesis has allowed some preliminary investigation into the elucidation of the enzyme catalysed reaction.

The synthesis of a novel phosphinic acid transition state analogue has been carried out and its interaction with kynureninase has been determined. It was found to be a poor competitive inhibitor of the enzyme, with a K_I of 4.19 mM. Preparation of the corresponding methyl ester of the phosphinic acid improved the inhibitory properties and this compound had a K_I of 0.88 mM. It is proposed that methylation of the acid removes a destabilising interaction between the negative charge of the ionised phosphinic acid and some group at the active site. Due to the poor inhibition observed the separation of diastereomers or synthesis of the compounds with the addition of the *ortho*-amino group are not of primary importance at this stage.

Synthetic routes towards an epoxide analogue, a putative irreversible inhibitor of kynureninase, were examined. Examination of the inhibitory properties of this compound would provide a useful comparison with those of the phosphinic acid analogues. This inhibitor is designed to alkylate the putative active site nucleophile and probe for the nucleophile mediated mechanism. If the inhibitors do become irreversibly attached to the protein, preparing them in radioactively labelled form and digestion of the inactive enzyme formed would give some structural information on the active site.

A number of kynurenine analogues have also been prepared in order to obtain information on the specificity of the enzyme and these have begun to give an understanding of the interactions at the active site. A racemic mixture of desaminokynurenine was found to have a K_I of 23.2 μM. Cyclohexyl and naphthyl amino acid derivatives prepared by the same method were found to be weak competitive inhibitors of the enzyme with K_I values of 844 μM and 207 μM, respectively. Further analogues are required in order to gain more information on the specificity of the enzyme. Introducing the *ortho*-amino group into both the cyclohexyl and naphthyl amino acid derivatives may allow further examination of these compounds as substrates of kynureninase. A further interesting analogue (235) would be that prepared from α-tetralone. If this binds at the active site cleavage of the β,γ-carbon carbon bond could take place. However, there is now an extra covalent linkage between the aromatic ring and the alanine fragment. This may result in improved inhibition.



(235)

Stereospecific syntheses and / or methods of separation of the enantiomers, for all the analogues prepared maybe useful for further study in this area.

α-Deuteriated kynurenine has been prepared for the first time by two complementary synthetic routes. Diacetylation / racemisation of racemic kynurenine in deuterium oxide followed by acylase catalysed resolution is the most direct route. This labelled compound has then been used in isotope effect studies to gain an understanding of the mechanism of the enzyme catalysed reaction. The primary deuterium isotope effect, $^{D}V = 0.98$ and $^{D}(V/K) = 3.6$, showed that for the kynureninase catalysed reaction, α-H abstraction is partially rate limiting. The solvent isotope effect, $^{D}V = 4.4$ and $^{D}(V/K) = 4.6$, showed that a proton transfer is also partially rate limiting. Further work is required to elucidate the mechanism in more detail. Double kinetic isotope effect studies of the primary and solvent isotope effects should distinguish between stepwise and concerted proton transfers. The preparation and testing of the β,β-dideuteriated kynurenine would then complete the isotope effect measurements. This would give the secondary deuterium isotope effect and assess the kinetic importance of carbon-carbon bond cleavage. Finally, all of the measurements need to be carried out at different pHs to establish whether each isotope effect is dependent or independent of pH.

CHAPTER 6

6. EXPERIMENTAL.

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

Elemental analyses were carried out in the departmental microanalytical laboratory.

Optical rotations were measured at room temperature using an Optical Activity Ltd. AA 1000 polarimeter. 10 cm path-length cells were used.

Infrared spectra were recorded on a Perkin-Elmer series 1420 IR spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. Absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard.

NMR spectra were recorded on a Bruker AM-300 f.t. spectrometer (^1H, 300 MHz; ^{13}C, 74.76 MHz; ^{31}P, 121.49 MHz) and a Varian Gemini f.t. spectrometer (^1H, 200 MHz; ^{13}C, 50.31 MHz). They were also obtained at Merck Sharp and Dohme Research Laboratories using a Bruker AC-250 f.t. spectrometer (^1H, 250 MHz), a Bruker AM-360 f.t. spectrometer (^1H, 360 MHz; ^{13}C, 90.52 MHz) and a Bruker AMX-500 f.t. spectrometer (^1H, 500 MHz; ^{13}C, 125.70 MHz). ^1H NMR spectra were referenced on chloroform, TMS, methanol or d^6-DMSO, ^{13}C NMR spectra were referenced on chloroform, methanol or d^6-DMSO 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 (δH or δC), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-doublet of doublets and b-broad), coupling constant ($J_{X,Y}$Hz if applicable) and assignment.

Mass spectra and accurate mass measurements were recorded on a Kratos MS50, obtained on an EPSRC service basis at the University of Swansea using a VG ZAB E and at Merck Sharp and Dohme Research Laboratories using a VG Quattro. Major fragments are given as percentages of the base peak intensity. Fast atom bombardment (FAB) spectra were recorded using glycerol as a matrix.

Flash chromatography was performed according to the procedure of Still[197] using Sorbisil C60 (40-60 mm) silica gel and Kieselgel 60.

Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Whatman SIL G/UV$_{254}$) or on 0.1 mm precoated cellulose plates (CEL Macherey-Nagel 300/UV$_{254}$). Compounds were visualised by UV fluorescence, iodine vapour, aqueous potassium permanganate, bromocresol green in ethanol or ninhydrin.

Ozonolysis was carried out using a Fischer Ozon Ozon-generator 500.

Protein concentrations were measured by the method of Bradford.[198]

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

Centrifugation was carried out in a Kontron Centrikon T-124 centrifuge.

Assays were carried out using a UVICON 932 spectrophotometer.

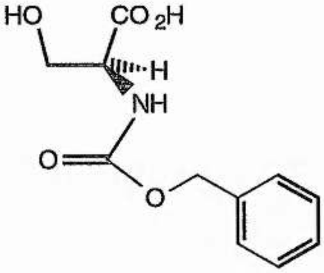
All equipment used in protein purification (peristalic pumps, UV detectors, fraction collector etc.) were purchased from Pharmacia.

Solvents were dried and purified according to the methods of Perrin and Armarego.[199]

Pseudomonas fluorescens (Strain ATCC 11250) was obtained from NCIB in Aberdeen.

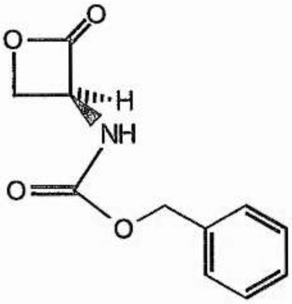
6.1 COMPOUNDS SYNTHESISED.

N-(Benzyloxycarbonyl)-(2S)-serine (101a).



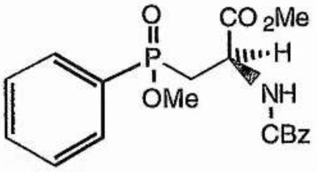
A solution of 2*S*-serine (10 g, 93 mmol) in water (100 ml) containing potassium bicarbonate (23.5 g, 0.24 mol) was stirred vigorously. Benzylchloroformate (15 ml, 0.105 mol) was added in small portions over fifteen minutes. The reaction was then stirred for three hours. The solution was extracted with diethyl ether (2 x 100 ml). The aqueous layer was then acidified with conc. HCl to pH 2 and extracted with diethyl ether (3 x 100 ml). The diethyl ether extracts were washed with brine (50 ml) and dried (Na_2SO_4). Concentration under reduced pressure yielded a white solid which was recrystallised from ethyl acetate/petroleum ether to give (22.73 g, 77%) of white crystals, m.p. 116-118 °C (lit.,[200] 117-119 °C); $[\alpha]_D^{20}$ +2.6° (c 3 in MeOH) (lit.,[200] $[\alpha]_D^{26}$ +5.8° (c 6 in AcOH)); υ_{max} (nujol)/cm^{-1} 3450-3200 (OH and NH), 1750 (CO carbamate), 1695 (CO acid); δ_H (200 MHz; d^6-DMSO), 3.65 (2H, d, *J* 5.6 Hz, $C\underline{H}_2$), 4.08 (1H, m, α-$\underline{H}$), 5.05 (2H, s, $OC\underline{H_2}Ph$), 6.95 (d, *J* 7.5 Hz, $N\underline{H}$), 7.40 (5H, s, aromatic); δ_C (50.31 MHz; d^6-DMSO), 56.34 (s, α-$\underline{C}$), 62.01 (s, β-$\underline{C}H_2$), 66.15 (s, $\underline{C}H_2$-Ph), 128.42 and 129.03 (s, aromatic), 137.65 (s, quat. aromatic), 156.73 (s, $NH\underline{C}O$), 172.86 (s, $\underline{C}O_2H$); *m/z* (CI) 257 ($[M+NH_4]^+$, 100%), 240 (53, $[M+H]^+$), 149 (95, $[M+H-CH_2Ph]^+$, 108 (36, $[PhCH_2OH]^+$).

N-(Benzyloxycarbonyl)-(2S)-serine β-lactone (102a).



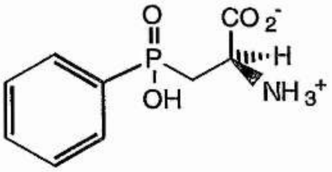
A solution of recrystallised triphenylphosphine (2.2 g, 8.4 mmol) in dry THF (40 ml) was cooled to -78 °C using solid carbon dioxide/acetone and stirred under nitrogen. Diethyl azodicarboxylate (DEAD) (1.46 g, 1.64 ml, 8.4 mmol) was added dropwise over ten minutes. The reaction was left stirring for ten minutes then *N*-(Benzyloxycarbonyl)-(2*S*)-serine (2 g, 8.2 mmol) in dry THF (40 ml) was added dropwise over one hour, with shaking, to prevent the solution from solidifying. The reaction mixture was left stirring for twenty minutes at -78 °C and then allowed to warm to room temperature and stirred overnight (18 hours). The solvent was evaporated under reduced pressure and the product was purified by column chromatography (silica, ethyl acetate: petroleum ether, 45: 55). The product was obtained as white needles (0.70 g, 39%), m.p. 127-128 °C (lit.,[130] 133-134 °C); $[\alpha]_D^{20}$ -26° (c 1 in CH_3CN) (lit., [130] -26.6° (c 1 in CH_3CN)); υ_{max} (nujol)/cm^{-1} 3380 (NH), 1840 (CO lactone), 1690 (CO carbamate); δ_H (200 MHz; C^2HCl_3), 4.45 (2H, d, *J* 7.5 Hz, C$\underline{H}_2$), 5.15 (3H, m, OC$\underline{H}_2$Ph, α$\underline{H}$), 5.62 (1H, br d, *J* 7.5 Hz, N$\underline{H}$), 7.24-7.50 (5H, m, aromatic); δ_C (50.31 MHz; C^2HCl_3), 60.04 (s, α–$\underline{C}$), 66.81 (s, $\underline{C}H_2$Ph), 68.25 (s, $\underline{C}H_2$), 128.87, 129.03, 129.14 (3x s, aromatic), 135.94 (s, quat. aromatic), 155.83 (s, $\underline{C}$O lactone), 169.58 (s, $\underline{C}O_2CH_2Ph$); *m/z* (CI) 239 ([M+NH_4]$^+$, 100%), 222 (8, [M+H]$^+$), 178 (7, [M-CO_2H]$^+$), 108 (48, [$PhCH_2OH$]$^+$).

(2*S*)-Methyl *N*-(Benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (122).



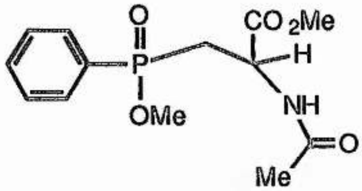
Serine β-lactone (0.5 g, 2.44 mmol) was dissolved in phenyldimethylphosphonite (5 ml, 29.4 mmol) and the solution heated at 40-50 °C under nitrogen for forty eight hours. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether; 1: 1). On completion of reaction, excess solvent was distilled off at reduced pressure on the Kügelrohr apparatus at 80 °C (0.1 mmHg). The product was obtained as a pale yellow oil (0.81 g, 85%) and was partially purified by column chromatography (silica, isopropanol: dichloromethane, 5: 95). Two diastereomers can be seen, δ_H (200 MHz; C^2HCl_3) 2.50 (2H, m, β–$C\underline{H}_2$), 3.55 (3H, s, $CO_2C\underline{H}_3$), 3.75 (3H, 2x d, *J* 11 Hz, $POC\underline{H}_3$), 4.60 (1H, m, α–$\underline{H}$), 5.05 (2H, s, $OC\underline{H}_2Ph$), 6.10 and 6.25 (1H, d, *J* 7.5 Hz $\underline{N}H$, diastereomers), 7.20-7.85 (10H, m, aromatic); δ_C (50.31 MHz; C^2HCl_3), 31.49 (d, β–$\underline{C}$, J_{PC} 100 Hz), 49.84 (s, α–$\underline{C}$), 51.87 (s, $CO_2\underline{C}H_3$), 53.16 (d, $PO\underline{C}H_3$, J_{PC} 4.6 Hz), 67.30 (s, $O\underline{C}H_2Ph$), 128.32-133.27 (aromatic), 129.31 (d, *ortho*-aromatic, J_{PC} 12.6 Hz), 132.03 (d, *meta*-aromatic, J_{PC} 10 Hz), 132.27 (s, *para*-aromatic), 134.99 (quat. aromatic, J_{PC} 172 Hz), 156.16 (s, $NH\underline{C}O$), 171.86 (s, $\underline{C}O_2Me$); *m/z* (EI) 392 ($[M+H]^+$, 10%), (391 (28, M^+), 332 (24, $[M-CO_2Me]^+$), 256 (15, $[M-PhCH_2OCO]^+$), 108 (19, $[PhCH_2OH]^+$), 77 (100, $[Ph]^+$).

(2*S*)-2-Amino-3-(phenyl phosphino)-propanoic acid (96).



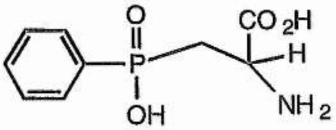
The partially purified (2*S*)-methyl *N*-(benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (0.1 g, 0.26 mmol) was heated at reflux in 6 M HCl (10 ml) for twelve hours. After cooling, the mixture was evaporated under reduced pressure and then taken up in isopropanol (1 ml) containing a small amount of RS-dithiothreitol. This was neutralised with propylene oxide (0.07 g, 1.28 mol). Diethyl ether was added and a white solid precipitated out of solution. This was then filtered under nitrogen and recrystallised from acetone/water to give a white solid (0.03 g, 50%), m.p. 210-212 °C; (Found: C, 47.45; H, 5.45; N, 6.03; $[M+H]^+$, 230.0573. $C_9H_{13}NO_4P$ requires C, 47.17; H, 5.28; N, 6.11%; $[M+H]^+$, 230.0582); $[\alpha]_D^{20}$ +10.1° (c 0.4 in H_2O); υ_{max} (nujol)/cm^{-1} 1680 (CO acid), 1130, 1150 (P=O, $R_2(OH)P=O$); δ_H (200 MHz; ^2H_2O) 2.00 (1H, m, β–C<u>H</u> (Half of ABX system)) and 2.30 (1H, m, β–C<u>H</u> (Half of ABX system)), 3.70 (1H, m, α–<u>H</u>), 7.50 (5H, m, aromatic); δ_P (121.49 MHz; ^2H_2O) 29.43; δ_C (74.76 MHz; ^2H_2O) 31.68 (d, β–C<u>H</u>$_2$, J_{PC} 90 Hz), 50.07 (s, α–<u>C</u>), 129.50 (d, *ortho*-aromatic, J_{PC} 12 Hz), 131.63 (d, *meta*-aromatic, J_{PC} 10 Hz), 132.69 (s, *para*-aromatic), 135.17 (d, quat. aromatic, J_{PC} 130 Hz), 172.46 (s, <u>C</u>O$_2$H); *m/z* (FAB) 230 ($[M+H]^+$, 12%).

(2*RS*)-Methyl *N*-(Acetyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (125).



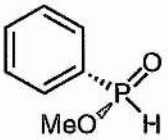
Phenyl dimethylphosphonite (21.76 g, 128 mmol) and 2-acetamidoacrylic acid (1.90 g, 14.8 mmol) were combined and heated at 70 °C for seventy two hours. The product was distilled at reduced pressure on the Kügelrohr to give a pale yellow oil (4.10 g, 94%). The oil was dissolved in diethyl ether (50 ml) and washed with 5% sodium bicarbonate (2 x 50 ml) to remove any residual phenyl dimethylphosphonite. The aqueous phase was then evaporated at reduced pressure, acidified with HCl to pH 3 and extracted into ethyl acetate (3 x 50 ml). The product was obtained as a pale yellow oil and used without further purification. Data is given for the impure mixture of diastereomers and some traces of phenyl dimethylphosphonite are also visible in the spectrum (2.26 g, 51%), υ_{max} (nujol)/cm^{-1} 1725 (CO ester), 1210 (P=O, $R_2(R'O)P=O$); δ_H (200 MHz; C^2HCl_3), 1.60 (3H, 4 x s, $C\underline{H}_3CON$), 2.55 (2H, m, β-$C\underline{H}_2$), 3.25, 2.60 (3H, s, $CO_2C\underline{H}_3$), 3.45 (3H, 2 x d, *J* 11 Hz, $POC\underline{H}_3$), 4.80 (1H, m, α-$\underline{H}$), 7.00 (1H, dd, *J* 7.5Hz, $N\underline{H}$), 7.70 (5H, m, aromatic); δ_P (121.49 MHz; C^2HCl_3) 43.56, 43.30 (1:1 mixture of diastereomers); δ_C (50.31 MHz; C^2HCl_3), 23.51 (s, $NHCO\underline{C}H_3$), 30.68 (d, β-$C\underline{H}_2$, J_{PC} 101Hz), 48.45 (s, α-$\underline{C}$), 51.74 (s, $CO_2\underline{C}H_3$), 53.27 (d, $PO\underline{C}H_3$, J_{PC} 5.6 Hz), 128.52-133.51 (aromatic), 170.45, 170.57 (s, $\underline{C}OCH_3$ and s, $\underline{C}O_2Me$); *m/z* (EI) 299 (M^+, 8%), 240 (87, $[M\text{-}CO_2Me]^+$), 155 (91, $[PhPOOCH_3]^+$), 140 (21, $[PhPOO]^+$), 77 (84, $[Ph]^+$), 43 (97, $[CH_3CO]^+$).

(2*RS*)-2-Amino-3-(phenyl phosphino)-propanoic acid (127).



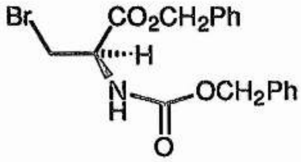
(2*RS*)-Methyl *N*-(acetyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (1.1 g, 3.72 mmol) was dissolved in 6 M HCl (73 ml) and heated under reflux for twelve hours. The solution was concentrated at reduced pressure and taken up in isopropanol (8.5 ml) containing a small amount of RS-dithothreitol. This was then neutralised with propylene oxide (1.00 g, 18.6 mmol) and a white solid precipitated out of solution. Diethyl ether was added and more white solid precipitated out of solution. This was then filtered under nitrogen and washed with dry diethyl ether. Recrystallisation from acetone/water to give (0.34 g, 54% yield) of a white solid, m.p. 211-213 °C (decomp.); (Found: C, 47.46; H, 5.21; N, 6.05; $[M+H]^+$ 230.0566. $C_9H_{12}NO_4P$ requires C, 47.17; H, 5.28; N, 6.11% ; $[M+H]^+$, 230.0582); υ_{max} (nujol)/cm^{-1} 1680 (CO acid), 1125, 1145 (P=O, $R_2(HO)P=O$); δ_H (200 MHz; ^2H_2O), 1.80 (1H, m, β-C$\underline{H}$ (Half of ABX system)), and 2.10 (1H, m, β-C$\underline{H}$ (Half of ABX system)), 3.30 (1H, m, α-$\underline{H}$), 7.60 (5H, m, aromatic); δ_P (121.49 MHz; ^2H_2O) 29.83; δ_C (74.76 MHz; ^2H_2O / ^2HCl) 28.85 (d, β-$\underline{C}H_2$, J_{PC} 90 Hz), 47.20 (s, α-$\underline{C}$), 126.74 (d, *ortho*-aromatic, J_{PC} 12 Hz), 128.87 (d, *meta*-aromatic, J_{PC} 10 Hz), 130.00 (s, *para*-aromatic), 132.15 (d, quat. aromatic, J_{PC} 129 Hz), 169.59 (s, $\underline{C}O_2H$); *m/z* (FAB) 230 ($[M+H]^+$, 100%), 252 (25, $[M+ Na]^+$).

Methyl phenylphosphinate (133).



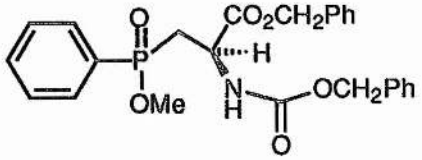
A solution of methanol (6 ml, 0.25 mol) and pyridine (12 g, 12.4 ml, 0.152 mol) in toluene (24 ml) was added dropwise over one and a half hours to a stirred solution of dichlorophenylphosphine (21.48 g, 16.4 ml, 0.12 mol) in toluene (140 ml) cooled to 0 °C. The pyridinium hydrochloride, formed within one hour, was vacuum filtered from solution and water (30 ml) was added dropwise to the filtrate. The organic layer was extracted with saturated sodium bicarbonate (50 ml) and water (50 ml) and the aqueous layer was back extracted twice with dichloromethane (2 x 30 ml). The combined organic layers were dried (Na_2SO_4) and concentrated at reduced pressure. The product was purified by distillation to give a colourless liquid (8.24 g, 43%), bp 82 °C/ 0.2 mmHg, (lit.,[144] 72 °C/ 0.1 mmHg); υ_{max} (C^2HCl_3)/cm^{-1} 2380 (PH), 1230 (P=O); δ_H (250 MHz; C^2HCl_3) 3.80 (3H, d, *J* 12 Hz, POC$\underline{H_3}$), 7.42-7.85 (5H, m, aromatic), 7.55 (1H, d, *J* 565 Hz, P-$\underline{H}$); δ_C (90.18 MHz; C^2HCl_3) 52.00 (d, O$\underline{C}H_3$, J_{PC} 6.4 Hz), 128.74 (d, *ortho*-aromatic, J_{PC} 14 Hz), 130.86 (d, *meta*-aromatic, J_{PC} 12 Hz), 132.43 (d, quat. aromatic, J_{PC} 126 Hz), 133.13 (s, *para*-aromatic); *m/z* (EI) 157 ([*M*+H]$^+$, 90%), 313 (100, [*M*+*M*+H]$^+$).

Benzyl-(2*S*)-*N*-(Benzyloxycarbonyl)-2-bromoalanine benzyl ester (130).



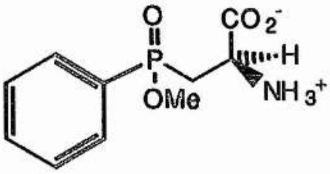
Carbon tetrabromide (1.47 g, 4.4 mmol) and triphenyl phosphine (1.16 g, 4.4 mmol) were dried in a desiccator over P_2O_5 for twelve hours. They were then dissolved in Na-dry toluene (20 ml) and *N*-CBz serine benzyl ester (1 g, 3 mmol) was added in several portions over thirty minutes. This was then left to stir overnight. Precipitated triphenyl phosphine oxide was removed by filtration through celite and purification was carried out by column chromatography (silica, ethyl acetate: dichloromethane 2: 8) to give (0.89 g, 75%) of white crystals, m.p. 85-87 °C (lit.,[143] 84-85 °C); $[\alpha]_D^{20}$ -18.1° (c 1 in MeOH) (lit.,[143] $[\alpha]_D$ -18.1° (c 1 in MeOH)); υ_{max} (nujol)/cm^{-1} 1730 (CO ester), 1680 (CO amide), 695 (C-Br); δ_H (200 MHz; C^2HCl_3) 3.80 (2H, m, $C\underline{H}_2$-ABX system), 4.85 (1H, m, α-$\underline{H}$), 5.10 (2H, s, $C\underline{H}_2$ of Cbz), 5.20 (2H, s, $C\underline{H}_2$ of ester), 5.70 (1H, d, *J* 7.5 Hz, $N\underline{H}$), 7.35 (10H, s, 2 x aromatic), δ_C (50.31 MHz; C^2HCl_3), 34.3 (s, β-$\underline{C}H_2$), 55.0 (s, α-$\underline{C}$), 67.8, 68.5 (benzyl $\underline{C}H_2$'s), 128.7, 129.0, 129.1, 129.2, (aromatics), 135.3, 135.5 (quat. aromatics), 156.0 ($\underline{C}O$ Cbz), 169.3 ($\underline{C}O$ ester), *m/z* (EI) 409, 411 ($[M+NH_4]^+$, 95, 100%), 392, 394 (14, 38, $[M+H]^+$).

Benzyl-(2*S*)-*N*-(benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (129).



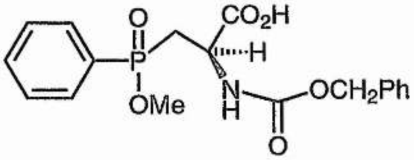
^{n}Butyl lithium (2.41 mmol, 1.1 eq, 0.96 ml, 2.5 M solution in hexanes) was added dropwise to methyl phenylphosphinate (343 mg, 2.19 mmol) in THF (4 ml) at -78 °C under nitrogen. This was then stirred at -78 °C for thirty minutes. The *N*-CBz-2-bromo alanine benzyl ester was then added (0.85 g, 2.19 mmol) in THF (5 ml) at -40 °C. This was stirred at -40 °C for one hour and then allowed to warm to room temperature. The reaction was quenched with a saturated solution of ammonium chloride (8 ml) and extracted into dichloromethane (3 x 20 ml). The solvent was then removed at reduced pressure. Purification was carried out using column chromatography and a gradient solvent system (silica, initially ethyl acetate: petroleum ether, 1: 1, then 3: 1 then pure ethyl acetate). It was therefore possible to separate a small amount of pure product (100 mg, 11%), υ_{max} (nujol)/cm^{-1} 1720 (CO ester), 1680 (CO amide), 1225 (P=O, $R_2(R'O)P=O$); δ_H (250 MHz; C^2HCl_3) 2.60 (2H, m, β-C$\underline{H}_2$), 3.65 (3H, d, *J* 12 Hz, POC$\underline{H}_3$), 4.60 (1H, m, α-$\underline{H}$), 5.05 (4H, m, OC$\underline{H}_2$Ph), 6.18 and 6.25 (1H, d, *J* 7.5 Hz, N$\underline{H}$), 7.24-7.80 (15H, m, aromatic); δ_C (74.76 MHz; C^2HCl_3), 30.99 (d, β-$\underline{C}$, J_{PC} 100 Hz), 49.67 (s, α-$\underline{C}$), 51.25 (d, $POCH_3$, J_{PC} 5.4 Hz), 66.96, 67.15 (2 x s, O$\underline{C}H_2$Ph), 128.13-132.94 (aromatics), 128.70 (d, *ortho*-aromatic J_{PC} 15 Hz), 132.00 (d, *meta*-aromatic J_{PC} 11 Hz), 132.94 (s, *para*-aromatic), 134.06 (quat. aromatic J_{PC} 170 Hz), 135.43, 136.42 (quat. aromatic), 156.00 (s, NH$\underline{C}$O), 170.69 (s, $\underline{C}O_2CH_2$Ph ester), *m*/*z* (EI) 468 ([*M*+H]$^+$, 100%), 506 (26, [*M*+K]$^+$).

(2*S*)-2-Amino-3-(*O*-methyl-phenyl phosphino)-propanoic acid (124).



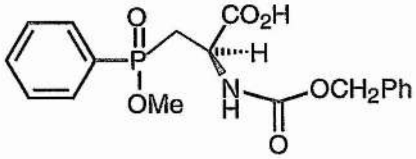
Benzyl-(2*S*)-*N*-(benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (100 mg, 0.24 mmol) was dissolved in methanol (10 ml) and Pd/C (100 mg) was added. Hydrogenation was then carried out overnight at atmospheric pressure using the hydrogenation apparatus. The catalyst was removed by filtration through a celite bed and the methanol removed under reduced pressure. This gave the desired product (23 mg, 38%), m.p. 188 °C (decomp.); $[\alpha]_D^{20}$ -6.6° (c 0.25 in H_2O); υ_{max} (nujol)/cm^{-1} 3320 (OH acid), 1690 (CO acid), 1225 (P=O, $R_2(R'O)P{=}O$); δ_H (200 MHz; ^2H_2O) 2.50 (2H, m, β-C$\underline{H_2}$), 3.60 (3H, d,, *J* 10 Hz POC$\underline{H_3}$), 4.10 (1H, m, α-$\underline{H}$), 7.35-7.80 (5H, m, aromatic); δ_P (121.49 MHz; ^2H_2O) 48.58, 47.94 (2:1 mixture of diastereomers), δ_C (74.76 MHz; ^2H_2O) 29.63 (d, β-$\underline{C}$, J_{PC} 100 Hz), 49.45 (s, α-$\underline{C}$), 52.57 (d, $POCH_3$, J_{PC} 5.6 Hz), 128.74 (d, *ortho*-aromatic, J_{PC} 12 Hz), 130.83 (d, *meta*-aromatic, J_{PC} 10 Hz), 131.76 (s, *para*-aromatic), 133.06 (d, quat. aromatic, J_{PC} 169 Hz), 172.27 (s, $\underline{C}O_2H$), *m*/*z* (CI) 244 ([*M*+H]$^+$, 100%).

(2*S*)-*N*-(Benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (128).



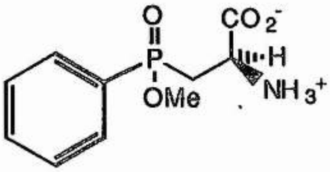
(2*S*)-Methyl *N*-(benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (210 mg, 0.54 mmol) was dissolved in a mixture of THF: water (1: 1, 10 ml) with stirring at 0 °C. This was then treated with lithium hydroxide monohydrate (0.023 g, 0.54 mmol) in THF: water (10 ml) which was added dropwise at 0 °C. This was then stirred for three hours and followed by mass spectrometry. The solution was then poured into 1M HCl (5 ml) and extracted into ethyl acetate (3 x 20 ml). The ethyl acetate extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give a colourless oil (40.6 mg, 20%); δ_H (250 MHz; C^2HCl_3) 2.70 (2H, m, β-C$\underline{H}_2$), 3.75 (3H, d,, *J* 11 Hz, POC$\underline{H}_3$), 4.60 (1H, m, α-$\underline{H}$), 5.05 (2H, m, OC$\underline{H}_2$Ph), 5.95 and 6.11 (1H, d, *J* 7.5 Hz, N$\underline{H}$), 7.25-7.84 (10H, m, aromatic); *m/z* (EI) 378 ([*M*+H]$^+$, 60%), 187 (100, [PhP(O)OMe$_2$]$^+$).

(2*S*)-*N*-(Benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (128).



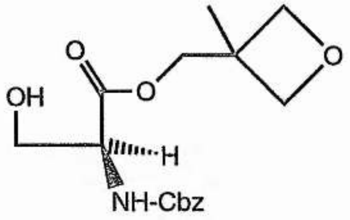
(2*S*)-2-Amino-3-(*O*-methyl-phenyl phosphino)-propanoic acid (244 mg, 0.622 mmol) was dissolved in acetone (6 ml) and 0.1M potassium phosphate buffer (60 ml, pH 8) was added. (This had previously been prepared using di-potassium hydrogen orthophosphate which was buffered to pH 8 using dilute phosphoric acid). Then pig liver esterase[150] (5 mg in 3.2 M $(NH_4)_2SO_4$, 1150 units) was added and the mixture incubated at 38 °C. Once NMR showed the reaction to be complete the mixture was acidified to pH 4 with 2 M HCl and extracted with ethyl acetate (3 x 15 ml). The organic layer was washed with water, dried ($MgSO_4$) and concentrated at reduced pressure to give a pale yellow oil (104 mg, 44%), υ_{max} (nujol)/cm^{-1} 1690 (CO acid), 1680 (CO amide), 1225 (P=O, $R_2(R'O)P=O$); δ_H (200 MHz; ^2H_2O) 2.60 (2H, m, β-C<u>H</u>$_2$), 3.70 (3H, d,, J 10 Hz, POC<u>H</u>$_3$), 4.60 (1H, m, α-<u>H</u>), 5.05 (2H, m, OC<u>H</u>$_2$Ph), 6.05 and 6.20 (1H, d, J 7.5 Hz, N<u>H</u>), 7.20-7.90 (10H, m, aromatic); δ_C (50.31 MHz; ^2H_2O) 29.53 (d, β-<u>C</u>, J_{PC} 108 Hz), 49.50 (s, α-<u>C</u>), 52.68 (d, PO<u>C</u>H$_3$, J_{PC} 5.3 Hz), 67.02 (2x s, O<u>C</u>H$_2$Ph), 127.87-133.06 (aromatics), 128.48 (d, *ortho*-aromatic, J_{PC} 10 Hz), 132.26 (d, *meta*-aromatic, J_{PC} 9 Hz), 132.30 (d, quat. aromatic, J_{PC} 129 Hz), 132.65 (s, *para*-aromatic), 156.39 (s, NH<u>C</u>O), 173.40 (s, <u>C</u>O$_2$H), *m/z* (CI) 395 ($[M+NH_4^+]^+$, 21%), 378 (11, $[M+H]^+$).

(2S)-2-Amino-3-(*O*-methyl-phenyl phosphino)-propanoic acid (124).



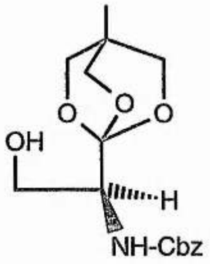
(2*S*)-*N*-(Benzyloxycarbonyl)-2-amino-3-(*O*-methyl phenyl phosphino)-propanoate (150 mg, 0.40 mmol) was dissolved in methanol (10 ml) and Pd/C (100 mg) was added. Hydrogenation was then carried out overnight at atmospheric pressure using the hydrogenation apparatus. The catalyst was removed by filtration through a celite bed and the methanol removed under reduced pressure. The yellow oil isolated was dissolved in water and washed with diethyl ether (2 x 10 ml) and ethyl acetate (2 x 10 ml). The aqueous phase was then lyopholised to give a pale yellow solid and recrystallised from acetone / water (60 mg, 67%), m.p. 188 °C, $[\alpha]_D^{20}$ -6.6° (c 0.25 in H_20); υ_{max} (nujol)/cm^{-1} 3320 (OH acid), 1690 (CO acid), 1225 (P=O, $R_2(R'O)P{=}O$); δ_H (200 MHz; $^2H_20$) 2.50 (2H, m, β-C$\underline{H_2}$), 3.60 (3H, d, *J* 10 Hz POC$\underline{H_3}$), 4.10 (1H, m, α-$\underline{H}$), 7.35-7.80 (5H, m, aromatic); δ_P (121.49 MHz, $^2H_20$), 48.58, 47.94 (2:1 mixture of diastereomers), δ_C (74.76 MHz; $^2H_20$) 29.63 (d, β-$\underline{C}$, J_{PC} 100 Hz), 49.45 (s, α-$\underline{C}$), 52.57 (d, $POCH_3$, J_{PC} 5.6 Hz), 128.74 (d, *ortho*-aromatic, J_{PC} 12 Hz), 130.83 (d, *meta*-aromatic, J_{PC} 10 Hz), 131.76 (s, *para*-aromatic), 133.06 (d, quat. aromatic, J_{PC} 169 Hz), 172.27 (s, $\underline{C}O_2H$), *m/z* (EI) 244 ($[M{+}H]^+$, 100%).

N-(Benzyloxycarbonyl)-(2*S*)-serine 3-methyl-3-(hydroxymethyl) oxetane ester (179).



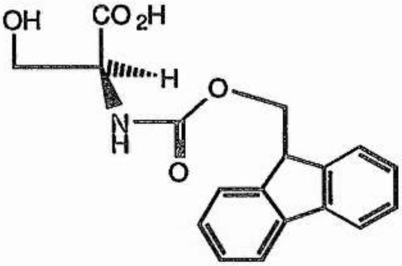
N-CBz serine (1.13 g, 4.47 mmmol) was dissolved in dichloromethane (125 ml) and added dropwise over three hours to a stirred solution of DCC (1.47 g, 7.11 mmol, 1.5 equiv.), DMAP (29.0 mg, 0.237 mmol, 0.05 equiv.) and 3-methyl-3-(hydroxymethyl)-oxetane (9.68 g, 94.8 mmol, 20 equiv.) cooled to 0 °C under a nitrogen atmosphere. The solution was allowed to warm to room temperature and stirred for a further twelve hours. The solution was then filtered to remove dicyclohexylurea (DCU). The filtrate was washed with 1% NH_4Cl (2 x 125 ml), 5% $NaHCO_3$ (1 x 125 ml), dried ($MgSO_4$) and evaporated under reduced pressure, yielding a white solid. The product was purified by column chromatography (silica, ethyl acetate/petroleum ether; 2: 1), to give a white solid (0.88 g, 57%). (The aqueous fractions were saved for recovery of oxetane alcohol[155]), m.p. 84-85 °C; *m/z* (Found: $[M+H]^+$ 324.1441. $C_{16}H_{21}NO_6$ requires 324.1447); υ_{max} (nujol)/cm^{-1} 3310-3410 (OH and NH), 1675 (CO); δ_H (200 MHz; C^2HCl_3), 1.30 (3H, s, $C\underline{H}_3$), 3.20 (1H, br s, $O\underline{H}$), 3.90 (1H, m, α–$\underline{H}$), 4.10 (2H, d, *J* 10 Hz, $C\underline{H}_2OH$), 4.50 (6H, m, $C\underline{H}_2$ oxetane), 5.05 (2H, s, $OC\underline{H}_2Ph$), 5.85 (1H, d, *J* 7.5 Hz, $N\underline{H}$), 7.35 (5H, s, aromatic); δ_C (50.31 MHz; d^6-DMSO), 21.25 (s, oxetane $\underline{C}H_3$), 40.10 (s, quat. oxetane), 56.88 (s, α–$\underline{C}$), 63.81 (s, oxetane $\underline{C}H_2OCO$), 67.65 (s, $\underline{C}H_2Ph$), 69.39 (s, β-$\underline{C}$), 79.97 (s, oxetane $\underline{C}H_2O$), 128.63, 128.75, 129.05 (s, aromatic), 136.60 (s, quat. aromatic), 156.73 (s, $NH\underline{C}O$), 171.24 (s, CO_2-oxetane); *m/z* (CI) 324 ($[M+H]^+$, 27%), 306 (10, $[M+H-H_2O]^+$), 280 (8, $[M+H-CO_2]^+$), 233 (100, $[M+H-CH_2Ph]^+$), 216 (72, $[M+H-HOCH_2Ph]^+$).

Attempted preparation of *N*-(benzyloxycarbonyl)-(1*S*)-1-amino-2-hydroxyethyl-4-methyl-2,6,7-trioxabicyclo[2.2.2]octane, *N*-Cbz-ortho ester (180).



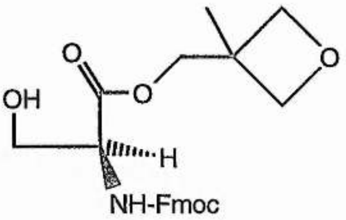
The oxetane ester (0.43 g, 1.32 mmol) was dissolved in freshly distilled (off CaH_2) dichloromethane (8.2 ml) and cooled to 0 °C under nitrogen. A solution of $BF_3.Et_2O$ (22 μL of a 20% (v/v) solution in CH_2Cl_2, 0.033 mol, 0.027 equiv.) was then added. The solution was stirred and allowed to warm to room temperature. After twenty four hours, tlc (silica, ethyl acetate/petroleum ether; 2: 1) showed reaction to be incomplete and a further portion of $BF_3.Et_2O$ (22 μL of a 20% (v/v) solution in CH_2Cl_2, 0.033 mol, 0.027 equiv.) was added. The reaction was left to stir for a further twenty four hours, then Et_3N (108 μL, 0.78 mmol, 0.58 equiv.) was added and the solution evaporated to dryness under reduced pressure. The product was (0.379 g, 88%) of a white solid which was found to be starting material.

N-(9-Fluorenylmethyloxycarbonyl)-(2*S*)-serine (159).



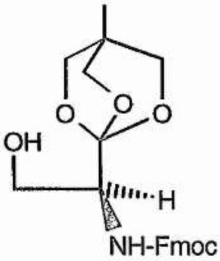
Fmoc chloride (1.45 g, 5.25 mmol) in dioxane (5 ml) was added dropwise over thirty minutes to a cooled stirred solution of serine (0.525 g, 5 mmol) dissolved in a mixture of dioxane (5 ml) and 10% Na_2CO_3 (13.5 ml). The reaction was then stirred for forty eight hours. The dioxane was evaporated at reduced pressure and water (62.5 ml) was added to the remaining aqueous solution. Extraction was carried out with ether (3 x 75 ml). On acidification of the aqueous layer with HCl the product precipitated and was extracted into ethyl acetate (3x40ml). The combined organic extracts were washed with brine (50ml) and dried (Na_2SO_4). The ethyl acetate was evaporated at reduced pressure and recrystallisation was carried out using ethyl acetate/petroleum ether giving pale yellow crystals (1.23 g, 71.3%), m.p. 84-90 °C (lit.,[155] 74-86 °C); $[\alpha]^{20}_D$ +16.1° (c 1 in MeOH), (lit.,[201] $[\alpha]^{20}_D$ +13.3° (c 1 in EtOAc); υ_{max} (nujol)/cm^{-1} 3310 (OH and NH), 1670 (CO); δ_H (200 MHz; d^6-DMSO); 3.75 (2H, m, β–C$\underline{H}_2$), 4.08 (1H, m, Fmoc C$\underline{H}$CH$_2$O), 4.30 (2H, m, Fmoc CHC$\underline{H}_2$O), 5.04 (1H, br m, α–$\underline{H}$), 7.30-7.94 (8H, m, Fmoc=C$\underline{H}$); δ_C (50.31 MHz; d^6-DMSO); 46.87 (s, Fmoc $\underline{C}$HCH$_2$), 56.93 (s, α-$\underline{C}$), 64.03 (s, β-$\underline{C}$), 66.0 (s, Fmoc $\underline{C}$H$_2$O), 120.39, 125.58, 127.37, 127.92 (s, Fmoc=$\underline{C}$H), 144.11, 145.46 (s, Fmoc=$\underline{C}$=), 156.35 (s, $\underline{C}$ONH), 172.44 (s, $\underline{C}$O$_2$H); *m/z* (CI) 345 ([*M*+NH$_4$]$^+$, 44%), 328 (30, [*M*+H]$^+$), 179 (76, [$C_6H_4CH(CH_2)C_6H_4$]$^+$).

***N*-(9-Fluorenylmethyloxycarbonyl)-(2*S*)-serine 3-methyl-3-(hydroxy-methyl) oxetane ester** (160).



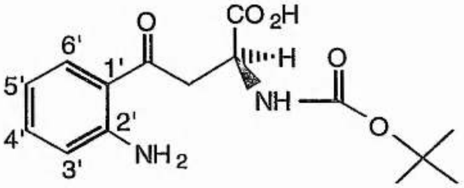
Fmoc-(2*S*)-serine (1.23 g, 2.96 mmol) was dissolved in CH_2Cl_2 (31 ml) and added dropwise over an hour to a stirred solution of DCC (0.92 g, 4.44 mmol,1.5 eq), DMAP (18.0 mg, 0.148 mmol, 0.05 eq) and oxetane alcohol (6.04 g, 59.2 mmol, 20 eq) cooled to 0 °C under nitrogen. The reaction solution was allowed to warm up to room temperature and stirred for a further twelve hours. The solution was then filtered to remove dicyclohexylurea (DCU). The filtrate was washed with 1% NH_4Cl (2 x 125 ml), 5% $NaHCO_3$ (1 x 125 ml), dried ($MgSO_4$) and evaporated under reduced pressure to dryness. The product was purified by column chromatography (silica, ethyl acetate/petroleum ether; 2: 1), to give a pale yellow foam (1.05 g, 86%), m.p. 104-106 °C (lit.,[155] 106-107 °C); υ_{max} (nujol)/cm^{-1} 3320 (OH and NH), 1675 (CO); δ_H (200 MHz; C^2HCl_3); 1.28 (3H, s, C$\underline{H}_3$ oxetane), 3.65 (1H, br s, O$\underline{H}$), 3.86 (1H, m, β–C$\underline{H}$H), 4.10 (2H, m, α–C$\underline{H}$, β–CH$\underline{H}$), 4.20 (1H, t; *J* 5 Hz, Fmoc C$\underline{H}$CH$_2$), 4.30-4.60 (8H, m, Fmoc C$\underline{H}_2$O, 3 oxetane C$\underline{H}_2$O), 6.12 (1H, br d, *J* 5 Hz, N$\underline{H}$), 7.24-7.79 (8H, m, Fmoc=C$\underline{H}$); δ_C (50.31 MHz; C^2HCl_3); 21.34 (s, oxetane C$\underline{C}$H$_3$), 39.88 (s, oxetane $\underline{C}$CH$_3$), 47.55 (s, Fmoc $\underline{C}$HCH$_2$), 56.93 (s, α–$\underline{C}$), 69.43 (s, β–$\underline{C}$), 67.57 (s, Fmoc $\underline{C}$H$_2$O), 69.45 (s, oxetane $\underline{C}$H$_2$OCO), 79.93 (s, oxetane $\underline{C}$H$_2$O), 120.46, 125.63, 127.57, 128.21 (s, Fmoc=$\underline{C}$H), 141.72, 144.21, 144.33 (s, Fmoc=$\underline{C}$=), 156.89 (s, $\underline{C}$ONH), 171.38 (s, $\underline{C}$O$_2$-oxetane); *m/z* (CI) 429 ($[M+NH_4]^+$, 3%), 412 (12, $[M+H]^+$), 179 (30, $[C_6H_4CH(CH_2)C_6H_4]^+$).

Attempted preparation of *N*-(9-Fluorenylmethyloxycarbonyl)-(1*S*)-1-amino-2-hydroxyethyl-4-methyl-2,6,7-trioxabicyclo[2.2.2]octane, Fmoc-*S*-Serine Ortho ester (161).



This was prepared in an identical manner to that described for the *N*-Cbz ortho ester, however ^{13}C NMR showed that only starting material was isolated.

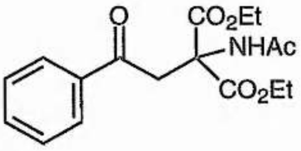
***N*-(*tert*-Butoxycarbonyl)-(2*S*)-kynurenine.**



Di-*tert*-butylpyrocarbonate (0.38 g, 1.738 mmol) was added to a solution of 2*S*-kynurenine (0.48 g, 1.58 mmol) and sodium bicarbonate (0.44 g, 5.21 mmol) in THF: water, (1: 1, 50 ml). The solution was then stirred at room temperature for twenty four hours after which time tlc examination (cellulose, IPA: conc. aq NH_3: H_2O; 3: 6: 10) showed reaction to be incomplete. A further (0.19 g, 0.869 mmol) di-*tert*-butylpyrocarbonate was added and the solution stirred for a further eight hours. Hydrochloric acid (0.145 ml, 1.738 mmol, 12 M) was then added. The solvent was then evaporated under reduced pressure and recrystallisation carried out from ethanol / water to give (0.2 g, 41%) of yellow crystals, m.p.

170 °C; υ_{max} (nujol)/cm^{-1} 1685 (CO acid), 1660 (CO amide); δ_H (200 MHz; d^6-DMSO), 1.38 (9H, s, ^{t}Bu), 3.32 (2H, m, β-C$\underline{H}_2$), 4.48 (1H,m, α–$\underline{H}$), 6.55 (1H, t, J 10 Hz, 5'$\underline{H}$), 6.78 (1H, d, J 10 Hz, 3'$\underline{H}$), 6.95 (1H, d, J 7.5 Hz, N$\underline{H}$), 7.25 (3H, m, 4'$\underline{H}$,N$\underline{H}_2$), 7.75 (1H, d, J 7.5 Hz, 6'$\underline{H}$); δ_C (74.76 MHz; d^6-DMSO), 28.40 (s, $\underline{C}$H$_3$'s), 41.69 (s, $\underline{C}$-3), 49.92 (s, $\underline{C}$-2), 78.70 (s, $\underline{C}$(CH$_3$)$_3$), 115.00 (s, 3'$\underline{C}$), 116.81 (s, 1'$\underline{C}$), 117.33 (s, 5'$\underline{C}$), 131.37 (s, 6'$\underline{C}$), 134.64 (s, 4'$\underline{C}$), 136.99 (s, 2'$\underline{C}$), 151.29 (s, $\underline{C}$O$_2$C(CH$_3$)$_3$), 173.70 (s, $\underline{C}$-1), 198.78 (s, $\underline{C}$-4); m/z (CI) 309 ([M+H]$^+$, 32%), 265 (9, [M+H-CO$_2$]$^+$), 253 (100, [M+H-CH$_2$=C(CH$_3$)$_2$]$^+$), 192 (15, M+H-(CH$_3$)$_3$COCONH$_2$]$^+$), 57 (20, [(CH$_3$)$_3$C]$^+$).

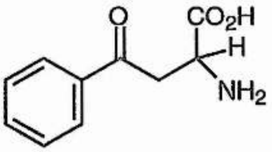
2-Acetylamino-2-(2-oxo-2-phenyl-ethyl) malonic acid diethyl ester (192).



Diethylacetamidomalonate (12.2 g, 56.16 mmol) in redistilled DMF (74 ml) was added to sodium hydride (60% weight in oil) (2.25 g, 56.25 mmol) at 0 °C under a nitrogen atmosphere. Stirring was continued for three hours after which a solution of α-bromoacetophenone (10 g, 50.24 mmol) in redistilled DMF (22 ml) was added, and the resulting dark mixture was stirred for a further five hours. The reaction was then poured into distilled water (740 ml), acidified with 1 M HCl, extracted with diethyl ether (5 x 150 ml), washed with brine (250 ml) and dried (MgSO$_4$). The solvent was then removed under reduced pressure and the residue triturated four times with diethyl ether/dichloromethane (3: 1) to give a white crystalline solid (8.56 g, 25.53 mmol, 51%); m.p. 118-122 °C (lit.,[168] 118-119 °C); υ_{max} (nujol)/cm^{-1} 3200 (NH), 1750 (ester), 1725 (C=O), 1680 (CO amide); δ_H (200 MHz; C^{2}HCl$_3$) 1.25 (6H, t, J 7.5 Hz, 2 x CO$_2$CH$_2$C$\underline{H}_3$), 2.00 (3H, s, COC$\underline{H}_3$), 4.25

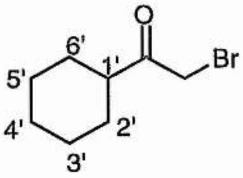
(6H, m, 2 x $CO_2C\underline{H}_2CH_3$, β-$C\underline{H}_2$), 7.10 (1H, s, N$\underline{H}$), 7.50 (3H, m, 3'$\underline{H}$, 4'$\underline{H}$, 5'$\underline{H}$), 7.90 (2H, d, *J* 7.5 Hz, 2'$\underline{H}$, 6'$\underline{H}$); δ_C (50.31 MHz; C^2HCl_3) 14.39 (s, $CO_2CH_2\underline{C}H_3$), 23.40 (s, $NHCO\underline{C}H_3$), 42.76 (s, β-$\underline{C}$), 63.34 (s, $CO_2\underline{C}H_2CH_3$), 64.43 (s, α-$\underline{C}$), 128.67 (3'$\underline{C}$, 5'$\underline{C}$), 129.17 (2'$\underline{C}$, 6'$\underline{C}$), 134.21 (4'$\underline{C}$), 136.49 (1'$\underline{C}$), 167.74 (s, $\underline{C}O_2Et$), 169.98 (s, $\underline{C}OCH_3$), 197.35 (s, $\underline{C}OCH_2$); *m/z* (CI) 336 ([*M*+H]$^+$, 1%), 262 (19, [*M*-CO_2Et]$^+$), 105 (100, $[PhCO]^+$).

(*RS*)-2-amino-4-oxo-4-phenyl-butyric acid (27).



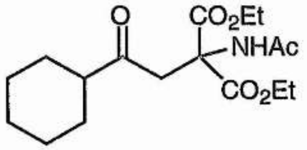
A suspension of diethyl (phenacyl) acetamidomalonate (8 g, 23.86 mmol) in 6 M HCl (225 ml) was heated at reflux for five hours. Upon cooling, the mixture was washed with ethyl acetate (2 x 50 ml), concentrated under reduced pressure. Trituration with acetone gave the pure hydrochloride salt which was then neutralised with 5% sodium hydrogen carbonate to afford the free base. Recrystallisation from acetone / water gave a white crystalline solid (2.6 g, 56%); m.p. 189-191 °C, (lit.,[169] 190-200 °C); (Found: C, 61.88; H, 5.65; N, 7.15. Calc. for $C_{10}H_{11}NO_3$: C, 62.17; H, 5.74; N, 7.25%); δH (300 MHz; ^2H_2O) 3.50 (2H, m, β-$C\underline{H}_2$), 4.15 (1H, m, α–$\underline{H}$), 7.50 (2H, m, 3'$\underline{H}$, 5'$\underline{H}$), 7.35 (1H, m, 4'$\underline{H}$), 7.70 (2H, d, *J* 7.5 Hz, 2'$\underline{H}$, 6'$\underline{H}$); δ_C (50.31 MHz; ^2H_2O) 55.09 (s, α-$\underline{C}$), 131.02 (s, 3'$\underline{C}$, 5'$\underline{C}$), 131.64 (s, 2'$\underline{C}$, 6'$\underline{C}$), 136.93 (s, 4'$\underline{C}$), 138.83 (s, 1'$\underline{C}$), 184.14 (s, $\underline{C}O_2H$), 205.45 (s, $\underline{C}OCH_2$); *m/z* (CI) 194 ([*M*+H]$^+$, 100%). The compound was poorly soluble in a wide variety of NMR solvents; thus it was not possible to see all the signals in the NMR spectrum.

2-Bromo-1-cyclohexylethanone (195).



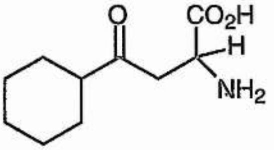
A solution of bromine (2 ml, 37 mmol) in redistilled methanol (5 ml) was added slowly to cyclohexylethanone (4.25 g, 33.7 mmol) which was dissolved in redistilled methanol (20 ml) and cooled to 0 °C. The reaction mixture was maintained at 5-6 °C during the reaction. Once the red colour disappeared, distilled water (10 ml) was added and the mixture stirred at room temperature overnight. Distilled water (30 ml), was then added and the reaction mixture extracted with diethyl ether (4 x 30 ml). The organic layers were combined and washed with 10% potassium carbonate (15 ml), distilled water (2 x 15 ml) and then dried (Na_2SO_4). The solvent was removed under reduced pressure to give (6.58 g, 96%) of a pale yellow liquid which was purified by distillation to give a colourless liquid (5.83 g, 85%), b.p. 90 °C/ 0.25 mmHg (lit.,[202] 78 °C/ 0.05 mmHg); υ_{max} (C^2HCl_3)/cm^{-1} 2670 (cyclohexane); 1706 (C=O); δ_H (250 MHz; C^2HCl_3) 1.10-1.90 (10H, 2 x m, 2'C$\underline{H}_2$, 3'C$\underline{H}_2$, 4'C$\underline{H}_2$, 5'C$\underline{H}_2$, 6'C$\underline{H2}$), 2.70 (1H, m, 1'$\underline{CH}$), 3.90 (2H, s, C$\underline{H}_2$Br); δ_C (50.31 MHz; C^2HCl_3) 25.95 (s, 3'$\underline{C}$, 5'$\underline{C}$), 26.1 (s, 4'$\underline{C}$), 29.2 (s, 2'$\underline{C}$, 6'$\underline{C}$), 34.1 (1'$\underline{C}$), 48.5 ($\underline{C}H_2$Br), 205.1 ($\underline{C}OCH_2$Br), *m/z* (EI) 205, 207 ([*M*+H]$^+$, 16, 12%), 226, 228 (25, 23, [*M*+Na]$^+$).

2-Acetylamino-2-(2-oxo-2-cyclohexyl-ethyl) malonic acid diethyl ester (196).



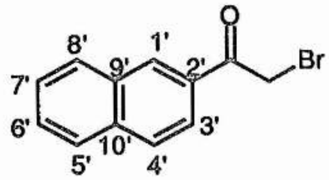
Diethylacetamidomalonate (2.44 g, 11.2 mmol) in dried redistilled DMF (15 ml) was added to sodium hydride (60% weight in oil, 0.45 g, 11.2 mmol) in DMF (2 ml) at 0 °C under a nitrogen atmosphere. Stirring was continued for three hours, after which time a solution of 2-bromo-1-cyclohexylethanone (2.14 g, 10.48 mmol) in redistilled DMF (4 ml) was added and the resulting mixture stirred overnight. The reaction mixture was poured into distilled water (15 ml), acidified with 1M HCl, extracted into diethyl ether (5 x 20 ml), washed with brine (25 ml) and dried ($MgS0_4$). The solvent was then removed at reduced pressure and the residue triturated four times with diethyl ether/ dichloromethane (3: 1) to give a white crystalline solid (3.14 g, 84%), m.p. 232 °C; υ_{max} (C^2HCl_3)/cm^{-1} 3350 (NH), 1750 (C=O), 1725 (CO ester), 1680 (CO amide); δ_H (250 MHz; C^2HCl_3) 1.20 (11H, m, 2 x $CO_2CH_2C\underline{H}_3$ + 5 cyclohexyl $\underline{H}$'s), 1.70 (5H, m, 5 cyclohexyl $\underline{H}$'s), 1.95 (3H, s, $COC\underline{H}_3$), 2.30 (1H, m, 1'$\underline{H}$), 3.70 (2H, s, β-$C\underline{H}_2$), 4.20 (4H, q, *J* 7 Hz, 2 x $CO_2C\underline{H}_2CH_3$), 7.00 (1H, s, N$\underline{H}$); δ_C (90.52 MHz; C^2HCl_3) 13.86 (s, $CO_2CH_2\underline{C}H_3$), 22.92 (s, $NHCO\underline{C}H_3$), 25.40 (s, 3'$\underline{C}$, 5'$\underline{C}$), 25.69 (s, 4'$\underline{C}$), 28.01 (s, 2'$\underline{C}$, 6'$\underline{C}$), 43.92 (s, β-$\underline{C}$), 50.64 (s, 1'$\underline{C}$), 62.76 (s, $CO_2\underline{C}H_2CH_3$), 63.72 (s, α-$\underline{C}$), 167.16 (s, $\underline{C}O_2Et$), 169.29 (s, $\underline{C}OCH_3$), 211.14 (s, $\underline{C}OCH_2$); *m/z* (EI) 342 ([*M*+H]$^+$, 100%).

(*RS*)-2-amino-4-oxo-4-cyclohexyl-butyric acid (182).



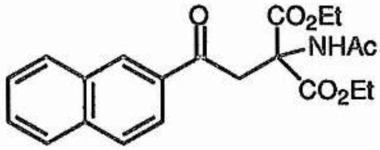
2-Acetylamino-2-(2-oxo-2-cyclohexyl-ethyl) malonic acid diethyl ester (1.663 g, 5.0 mmol) was refluxed for five hours in 6 M HCl (48 ml). Upon cooling the solution was washed with ethyl acetate (2 x 20 ml) and then concentrated under reduced pressure. Trituration with acetone gave a the hydrochloride salt which was neutralised with 5% sodium hydrogen carbonate solution to afford the free base. This was then recrystallised twice from acetone/ water to give white crystals (0.73 g, 74%), m.p. 194 °C; (Found: C, 60.23; H, 8.45; N, 6.81. Calc. for $C_{10}H_{17}NO_3$: C, 60.28; H, 8.60; N, 7.03%); υ_{max} (nujol)/cm^{-1} 2670 (cyclohexane), 1690 (CO acid); δ_H (250 MHz; ^2H_2O) 1.20 (5H, m, 5 cyclohexyl H's), 1.75 (5H, m, 5 cyclohexyl H's), 2.50 (1H, m, 1'H), 3.20 (2H, d, *J* 6 Hz, β-CH_2), 3.90 (1H, t, *J* 6 Hz, α–H); δ_C (74.76MHz; ^2H_2O) 25.00 (s, 3'C, 5'C), 25.29 (s, 4'C), 27.91 (s, 2'C, 6'C), 39.87 (s, β-C), 49.49 (s, 1'C), 50.28 (s, α-C), 172.77 (s, CO_2H), 215.88 (s, $COCH_2$); *m/z* (FAB) 200 ([*M*+H]$^+$, 100%).

2-Bromo-1-acetonaphthone (197).



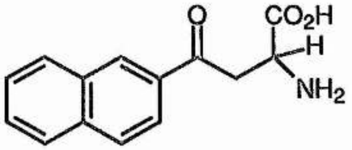
2-Acetonaphthone (5.74 g, 33.7 mmol) was dissolved in redistilled methanol (70 ml) and cooled to 0 °C. Bromine (2 ml, 37 mmol) was added slowly as a solution in methanol (5 ml). The reaction mixture was maintained at 2-3 °C during the reaction. This was then stirred for two hours below 10 °C until the reaction mixture was a pale yellow colour. Distilled water (10 ml) was then added and the mixture stirred at room temperature overnight. To the solution was added distilled water (30 ml). This was extracted with diethyl ether (4 x 30 ml). The organic layers were combined and washed with 10% potassium carbonate (15 ml), distilled water (2 x 15 ml) and then dried (Na_2SO_4). The solvent was removed under reduced pressure and the product recrystallised from ethyl acetate to give (7.65 g, 91%) of a pale brown solid, m.p. 80-81 °C (lit.,[203] 80-81 °C); υ_{max} (nujol)/cm^{-1} 1650 (C=O); δ_H (250 MHz; C^2HCl_3) 4.60 (2H, s, $C\underline{H}_2Br$), 7.30 (2H, m, 6'$\underline{H}$, 7'$\underline{H}$), 7.66 (4H, m, 3'$\underline{H}$, 4'$\underline{H}$, 5'$\underline{H}$, 8'$\underline{H}$), 8.50 (1H, s, 1'$\underline{H}$); δ_C (90.52 MHz; C^2HCl_3) 30.89 (s, $\underline{C}H_2Br$), 124.16 (s, 3'$\underline{C}$), 127.07 (s, 6'$\underline{C}$), 127.85 (s, 7'$\underline{C}$), 128.82 (s, 5'$\underline{C}$), 129.04 (s, 8'$\underline{C}$), 129.69 (s, 4'$\underline{C}$), 130.95 (s, 1'$\underline{C}$), 131.31 (s, 10'$\underline{C}$), 132.40 (s, 9'$\underline{C}$), 135.89 (s, 2'$\underline{C}$) 191.28 (s, $\underline{C}OCH_2Br$); *m/z* (EI) 249, 251 ([*M*+H]$^+$, 95,100%), 171 (90, [*M*+H]$^+$-Br).

2-Acetylamino-2-(2-oxo-2-naphthyl-ethyl) malonic acid diethyl ester (198).



Diethylacetamidomalonate (2.44 g, 11.2 mmol) in redistilled DMF (15 ml) was added to sodium hydride (60% weight in oil, 0.45 g, 11.2 mmol) at 0 °C under a nitrogen atmosphere. Stirring was continued for three hours after which time a solution of 2-bromo-1-acetonaphthone (2.61 g, 10.48 mmol) in redistilled DMF (8 ml) was added and the resulting mixture stirred overnight. The reaction mixture was poured into distilled water (15 ml), acidified with 1M HCl, extracted into diethyl ether (5 x 30 ml), washed with brine (50 ml) and dried ($MgS0_4$). The solvent was then removed under reduced pressure and the residue triturated four times with diethyl ether/ dichloromethane (3: 1), giving a dark brown oil (3.98 g, 99%). The product was purified by column chromatography (ethyl acetate: hexane; 45: 55) to give a pale yellow solid (3.45 g, 86%), m.p. 89-90 °C; υ_{max} (nujol)/cm^{-1} 1730 (C=O ester), 1680 (C=O amide); δ_H (250 MHz; C^2HCl_3) 1.28 (6H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 1.98 (3H, s, $COC\underline{H}_3$), 4.34 (2H, q, *J* 7 Hz, $CO\underline{C}\underline{H}_2CH_3$), 4.42 (2H, s, β-$C\underline{H}_2$), 7.20 (1H, s, N$\underline{H}$), 7.60 (2H, m, 6'$\underline{H}$, 7'$\underline{H}$), 7.92 (4H, m, 3'$\underline{H}$, 4'$\underline{H}$, 5'$\underline{H}$, 8'$\underline{H}$), 8.52 (1H, s, 1'$\underline{H}$); δ_C (90.52 MHz; C^2HCl_3) 13.87 (s, $CO_2CH_2\underline{C}H_3$), 22.90 (s, $NHCO\underline{C}H_3$), 42.25 (s, β-$\underline{C}$), 62.86 (s, $CO_2\underline{C}H_2CH_3$), 64.06 (s, α-$\underline{C}$), 123.36 (s, 3'$\underline{C}$), 126.91 (s, 6'$\underline{C}$), 127.70 (s, 7'$\underline{C}$), 128.51 (s, 5'$\underline{C}$), 128.79 (s, 8'$\underline{C}$), 129.67 (s, 4'$\underline{C}$), 130.43 (s, 1'$\underline{C}$), 132.39 (s, 10'$\underline{C}$), 133.34 (s, 9'$\underline{C}$), 135.79 (s, 2'$\underline{C}$), 167.31 (s, $\underline{C}O_2Et$), 169.48 (s, $\underline{C}OCH_3$), 196.77 (s, $\underline{C}OCH_2$); *m/z* (EI) 386 ($[M+H]^+$, 100%), 403 (20, $[M+H]^+ + NH_4$), 771 (20, $[M+M+H]^+$).

(*RS*)-2-amino-4-oxo-4-naphthyl-butyric acid (183).

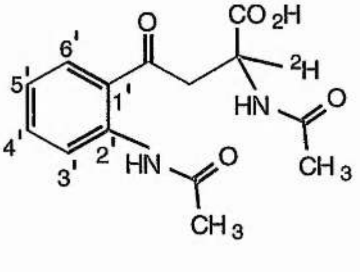


2-Acetylamino-2-(2-oxo-2-naphthyl-ethyl) malonic acid diethyl ester (1.00 g, 2.68 mmol) was refluxed for eight hours in 6 M HCl (28 ml) and dioxan (20 ml). Upon cooling the solution was washed with diethyl ether (2 x 20 ml) and then concentrated under reduced pressure. This was then triturated with acetone to give the hydrochloride salt which was neutralised with 5% sodium hydrogen carbonate solution to afford the free base. This was recrystallised from methanol to give off white crystals (0.42 g, 65%), m.p. 201 °C; (Found: C, 69.00; H, 5.55; N, 5.78. Calc. for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76%); υ_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (C=O acid), 1580 (aromatic); δ_H (200 MHz; $C^2H_3O^2H$), 3.95 (2H, m, β-C$\underline{H}_2$), 4.50 (1H, m, α–$\underline{H}$), 7.65 (2H, m, 6'$\underline{H}$, 7'$\underline{H}$), 8.05 (4H, m, 3'$\underline{H}$, 4'$\underline{H}$, 5'$\underline{H}$, 8'$\underline{H}$), 8.68 (1H, s, 1'$\underline{H}$); δ_C (74.76 MHz; $C^2H_3O^2H$) 39.42 (s, β-$\underline{C}$), 50.01(s, α-$\underline{C}$), 124.44 (s, 3'$\underline{C}$), 128.34 (s, 6'$\underline{C}$), 128.96 (s, 7'$\underline{C}$), 129.89 (s, 5'$\underline{C}$), 130.33 (s, 8'$\underline{C}$), 130.95 (s, 4'$\underline{C}$), 131.87 (s, 1'$\underline{C}$), 134.08 (s, 10'$\underline{C}$), 134.31 (s, 9'$\underline{C}$), 137.62 (s, 2'$\underline{C}$), 171.45 (s, $\underline{C}O_2H$), 197.82 (s, $\underline{C}OCH_2$); *m/z* (FAB) 266 ([*M*+Na]$^+$, 25%), 244 (65, [*M*+H]$^+$).

Preparation of (2*S*)-[2-^{2}H]-kynurenine.

i) Acetylation / deuteriation.

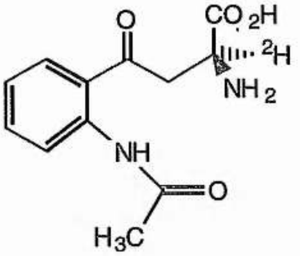
***N*, *N*'-Diacetyl-[2-^{2}H]-kynurenine** (213).



(2*RS*)-Kynurenine (1 g, 4.8 mmol) was dissolved in deuterium oxide (10 ml). Sodium (0.46 g, 0.02 mol) was added carefully after washing in petroleum ether and ethanol. Acetic anhydride (3 ml, 31.8 mmol) was added slowly, and the solution was shaken vigorously. The solution was then stirred at room temperature for three hours. Sulphuric acid (1 M, 10 ml) was added and the solution concentrated at reduced pressure. The residue was extracted with dichloromethane (4 x 50 ml). The combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a pale yellow solid (1.17 g, 98%), m.p. 194 °C, υ_{max} (nujol)/cm^{-1} 3320 (OH), 1675 (CO acid), 1645 (CO amide); δ_H (200 MHz; d^6-DMSO) 1.85 (3H, s, NHOC$\underline{H}_3$), 2.15 (3H, s, NHOC$\underline{H}_3$ aromatic), 4.70 (1H, d, *J* 7.5 Hz, N$\underline{H}$, aromatic), 7.25 (1H, t, *J* 10 Hz, 4'$\underline{H}$), 7.62 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 8.00 (1H, d, *J* 10 Hz, 6'$\underline{H}$), 8.21 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$), 8.28 (1H, s, N$\underline{H}$); δ_C (50.31 MHz; d^6-DMSO) 22.64 (s, NHCO$\underline{C}H_3$), 24.96 (s, NHCO$\underline{C}H_3$ aromatic), 41.57 (s, β-$\underline{C}$), 48.27 (s, α-$\underline{C}$), 121.26, (s, 3'$\underline{C}$), 123.42 (s, 5'$\underline{C}$), 124.96 (s, 1'$\underline{C}$), 130.78 (s, 6'$\underline{C}$), 134.28 (s, 4'$\underline{C}$), 138.85 (s, 2'$\underline{C}$), 169.08, 169.42 (NH$\underline{C}$OCH$_3$), 173.20 (s, $\underline{C}O_2H$); *m/z* (CI) 310 ([*M*+NH4]$^+$, 17%), 293 (100, [*M*+H]$^+$), 275 (24, [*M*+H-H$_2$O]$^+$), 162 (5, [*M*+H-CH$_2$C(CO$_2$H)(^{2}H)NHCOCH$_3$]$^+$).

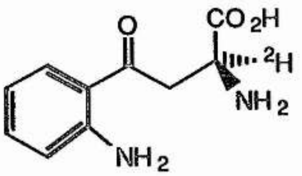
ii) Resolution / deacetylation.

N'-Acetyl-(2*S*)-[2-^{2}H]-kynurenine (214).



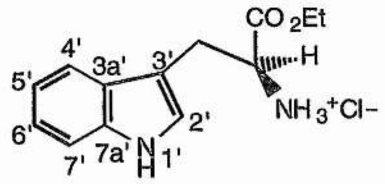
N, N' Diacetyl-[2-^{2}H]-(2*RS*)-kynurenine (0.2 g, 0.68 mmol) was added to water (10 ml) and solution was effected by adjusting the pH to 7.5 with concentrated NH_4OH. Acylase from *Aspergillus meleus* (100 mg, 0.47 units/mg) was added and the solution incubated at 38 °C for eighteen hours. Examination by tlc (cellulose, IPA: conc. aq NH_3: H_2O; 3: 6: 10) gave no indication if the reaction was complete. A further portion of acylase (100 mg) was added and the reaction incubated for eighteen hours. As the two tlcs were comparable an NMR was run which showed that the reaction had occured to completion. The acylase and other protein fragments were filtered off and the product evaporated to approximately one ml. Ion exchange column chromatography was carried out using strongly basic anion exchanger (DOWEX 1). The product was obtained as a pale yellow solid (0.045 g, 62.5%); m.p. 156 °C; *m/z* (Found: $[M+H]^+$ 252.1102. $C_{12}H_{13}O_4N_2{}^2H$ requires 252.1095); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1680 (CO acid), 1650 (CO amide); δ_H (200 MHz; ^2H_2O) 2.05 (3H, s, NHCOC$\underline{H}_3$), 3.55 (2H, s, β-C$\underline{H}_2$), 7.18 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 7.45 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.70 (2H, 2 x d, *J* 10 Hz, 6'3'$\underline{H}$); δ_C (50.31 MHz; ^2H_2O) 26.42 (s, NHCO$\underline{C}H_3$ aromatic), 42.91 (s, β-$\underline{C}$), 53.21 (s, α-$\underline{C}$), 125.93 (s, 3'$\underline{C}$), 127.91 (s, 5'$\underline{C}$), 128.94 (s, 1'$\underline{C}$), 133.10 (s,6'$\underline{C}$), 137.44 (s, 4'$\underline{C}$), 139.29 (s, 2'$\underline{C}$), 175.66 (s, $\underline{C}O_2H$); *m/z* (FAB) 252 ($[M+H]^+$, 100%), 274 (50.5, $[M+Na]^+$).

iii) (2*S*)-[2-^{2}H]-Kynurenine (199).



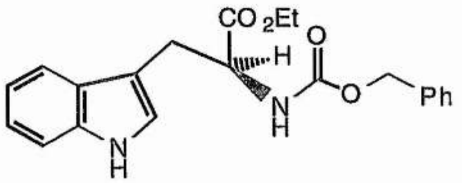
*N'*Acetyl-(2S)-[2-^{2}H]-kynurenine (0.14 g, 0.56 mmol) was dissolved in 2 M HCl (15 ml) The solution was heated under reflux for one hour then evaporated to dryness under reduced pressure. Isopropanol (1.25 ml) was added containing a small amount of *RS*-dithiothreitol. The dark orange solid which resulted was neutralised with propylene oxide (0.15 g, 2.75 mmol). Diethyl ether was added and a pale yellow solid precipitated out of solution. After filtration more solid was seen to precipitate out of the diethyl ether. On filtering this became a yellow oil and was taken up in water and the resulting pale yellow solution was freeze dried to give (0.05 g, 42%) of a yellow solid; m.p. 188 °C; *m/z* (Found: $[M+H]^+$ 210.0986. $C_{10}H_{11}N_2O_3{}^2H$ requires 210.0989); $[\alpha]^{20}_D$ +25.5° (c 0.5 in H_2O); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1730 (CO), 1650 (CO acid); δ_H (200 MHz; ^2H_2O) 3.75 (2H, s, β–$C\underline{H}_2$), 7.30 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$), 7.40 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.62 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 8.02 (1H, d, *J* 7.5 Hz, 6'$\underline{H}$); δ_C (74.76 MHz; ^2H_2O), 36.95 (s, β–$\underline{C}$), 47.98 (t, α-$\underline{C}$), 117.78 (s, 3'$\underline{C}$), 117.89 (s, 5'$\underline{C}$), 129.53 (s, 6'$\underline{C}$), 133.80 (s, 4'$\underline{C}$), 171.07 (s, $\underline{C}O_2H$), 197.73 (s, $\underline{C}OCH_2$); *m/z* (FAB) 210 ($[M+H]^+$, 100%), 232 (35, $[M+Na]^+$). Chiral hplc showed an enantiomeric excess of 97.4%.

(2*S*)-Tryptophan ethyl ester hydrochloride (215).



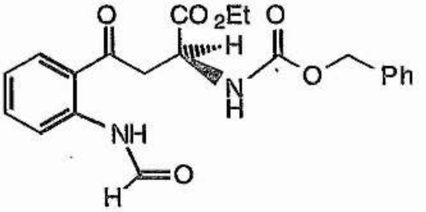
Tryptophan (5 g, 24.5 mmol) was dissolved in dry redistilled ethanol (100 ml) cooled in ice. Redistilled thionyl chloride (1.9 ml, 1.1 eq) was added and the solution was heated at reflux for one hour. The solvent was evaporated under reduced pressure to obtain a white solid which was recrystallised from ethanol to give white crystals (4.43 g, 78.3%), m.p. 226-227 °C (lit.,[184] 225-226 °C); (Found: C, 58.24: H, 6.19; N, 10.52. Calc. for $C_{13}H_{16}N_2O_2Cl$: C, 58.25, H, 5.97, N, 10.46%); $[\alpha]^{20}_D$ +11.8° (c 1 in MeOH) (lit.,[184] $[\alpha]^{20}_D$ +12° (c 1 in MeOH)); υ_{max} (nujol)/cm^{-1} 3300 (indole NH) and 1730 (CO_2Et); δ_H (200 MHz; d^6–DMSO) 1.10 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.20 (2H, dd, *J* 7 Hz, β–$C\underline{H}_2$), 4.10 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 4.30 (1H, m, α-$\underline{H}$), 7.05 (2H, 2x t, *J* 7 Hz, 5'$\underline{H}$, 6'$\underline{H}$), 7.38 (1H, d, *J* 7 Hz, 7'$\underline{H}$), 7.50 (1H, s, 2'$\underline{H}$), 7.54 (1H, d, *J* 7 Hz, 4'$\underline{H}$), 8.50 (3H, s, indole N$\underline{H}$); δ_C (50.31 MHz; d^6–DMSO) 13.97 (s, $CO_2CH_2\underline{C}H_3$), 26.40 (s, β–$\underline{C}$), 52.92 (s, α-$\underline{C}$), 61.85 (s, $CO_2\underline{C}H_2CH_3$), 106.68 (s, 3'$\underline{C}$), 111.78 (s, 7'$\underline{C}$), 118.28 (s, 5'$\underline{C}$), 118.79 (s, 6'$\underline{C}$), 121.38 (s, 4'$\underline{C}$), 125.20 (s, 2'$\underline{C}$), 127.19 (s, 7a'$\underline{C}$), 136.41 (s, 3a'$\underline{C}$), 169.61 (s, $\underline{C}O_2Et$); *m/z* (CI) 268 ($[M+H]^+$, 36%), 130 (100, $[CH_2$-indole$]^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-tryptophan ethyl ester** (202).



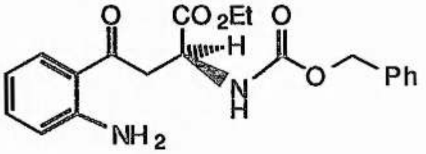
Sodium carbonate (2.2 eq, 32.33 mmol, 7.9 g) and benzylchloroformate (1.2 eq, 17.6 mmol, 2.6 g, 2.17 ml) were added to tryptophan ethyl ester hydrochloride (3.4 g, 14.7 mmol) in water (566 ml). The solution was left to stir overnight at room temperature. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 3: 7). The white product which had precipitated out of solution was filtered off. The product was obtained as white crystals (4.11 g, 77%), m.p. 84 °C (lit.,^{185} 84-85 °C); (Found; C, 68.96; H, 6.07; N, 7.70. Calc. for $C_{21}H_{22}N_2O_4$: C, 68.84; H, 6.05; N; 7.65%); $[\alpha]^{20}_D$ -8.8° (c 1 in EtOH) (lit.,^{185} $[\alpha]^{25}_D$ -6.7° (c 2 in EtOH); υ_{max} (nujol)/cm^{-1} 3310 (indole NH), 1720 (ester CO) and 1690 (carbamate CO); δ_H (200 MHz; d^6–DMSO) 1.10 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.10 (2H, m, β–$C\underline{H}_2$), 4.05 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 4.30 (1H, m, α-$\underline{H}$), 5.00 (2H, s, $C\underline{H}_2Ph$), 7.00 (1H,t, *J* 7 Hz, 5'$\underline{H}$), 7.10 (1H, t, *J* 7 Hz, 6'$\underline{H}$), 7.20 (1H, s, 2'$\underline{H}$), 7.30 (5H, m, aromatic), 7.50 (1H, d, *J* 7 Hz, 7'$\underline{H}$), 7.55 (1H, d, *J* 7 Hz, 4'$\underline{H}$); δ_C (74.76 MHz; d^6–DMSO) 14.06 (s, $CO_2CH_2\underline{C}H_3$), 27.06 (s, β–$\underline{C}$), 55.26 (s, α-$\underline{C}$), 60.63 (s, $CO_2\underline{C}H_2CH_3$), 65.54 (s, $\underline{C}H_2Ph$), 109.74 (s, 3'$\underline{C}$), 111.59 (s, 7'$\underline{C}$), 118.12 (s, 5'$\underline{C}$), 118.58 (s, 6'$\underline{C}$), 121.11 (s, 4'$\underline{C}$), 123.98 (s, 2'$\underline{C}$), 127.18 (s, 7a'$\underline{C}$), 127.74, 127.92, 128.47 (3x s, aromatic), 136.26 (s, 3a'$\underline{C}$), 137.05 (s, quat. aromatic), 156.11 ($PhCH_2O\underline{C}O$), 172.36 (s, $\underline{C}O_2Et$); *m/z* (EI) 367 ($[M+H]^+$, 3%), 258 (5, $[M-PhCH_2OH]^+$), 215 (1, $[M-PhCH_2OCONH_2]^+$), 130 (99, $[CH_2\text{-indole}]^+$), 108 (5, $[PhCH_2OH]^+$), 77 (11, $[Ph]^+$).

N'Formyl, N-(benzyloxycarbonyl)-(2S)-kynurenine ethyl ester (216).



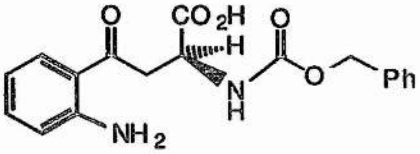
N-(Benzyloxycarbonyl)-(2S)-tryptophan ethyl ester (0.88 g, 2.4 mmol) was dissolved in dry redistilled methanol (110 ml). The solution was cooled to -78 °C with stirring and then treated with ozone for one hour, the reaction was then quenched by the addition of dimethyl sulphide (10 ml). The reaction was checked by tlc (silica, ethyl acetate: dichloromethane, 1: 9) and left to stir for a further hour at -78 °C. The solution was then evaporated to a dark orange oil. The product was dissolved in diethyl ether (25 ml) and washed with brine (2 x 50 ml) and water (2 x 50 ml). The solution was then dried ($MgSO_4$) and the solvent removed at reduced pressure to give an orange oil. Purification was carried out by column chromatography (silica, ethyl acetate: petroleum ether, 1: 2). The product was recrystallised from ethyl acetate giving a pale yellow solid (0.26 g, 28%), m.p. 111 °C; (Found: C, 63.31; H, 5.57; N, 7.03. Calc. for $C_{21}H_{22}N_2O_6$: C, 63.10; H, 5.47; N, 6.91%; $[\alpha]^{20}_D$ +3.4° (c 1 in MeOH); υ_{max} (nujol)/cm^{-1} 3305 (indole NH), 1725 (ester CO), and 1680 (amide CO); δ_H (200 MHz; d^6–DMSO) 1.20 (3H, t, J 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.50 (2H, d, J 7 Hz, β–$C\underline{H}_2$), 4.10 (2H, q, J 7 Hz, $CO_2C\underline{H}_2CH_3$), 4.60 (1H, m, α-$\underline{H}$), 5.10 (2H, s, $C\underline{H}_2Ph$), 7.20 (1H,t, J 7 Hz, 5'H); 7.50 (5H, m, aromatic), 7.65 (1H,t, J 7 Hz, 4'H), 7.80 (1H, d, J 7 Hz, 3'H), 8.05 (1H, d, J 7 Hz, 6'H), 8.50 (1H, m, $N\underline{H}COH$), δ_C (74.76 MHz; d^6–DMSO) 13.91 (s, $CO_2CH_2\underline{C}H_3$), 41.32 (s, β–$\underline{C}$), 50.02 (s, α-$\underline{C}$), 60.85 (s, $CO_2\underline{C}H_2CH_3$), 65.62 (s, $\underline{C}H_2Ph$), 121.24 (s, 3'$\underline{C}$), 123.40 (s, 5'$\underline{C}$), 123.90 (s, 1'$\underline{C}$), 127.64, 127.81, 128.32 (3x s, aromatic), 130.83 (s, 6'$\underline{C}$), 134.27 (s, 4'$\underline{C}$), 136.88 (s, quat. aromatic), 137.96 (s, 2'$\underline{C}$), 155.87 (s, $PhCH_2O\underline{C}O$), 161.16 (s, $NH\underline{C}OH$), 171.39 (s, $\underline{C}O_2Et$), 200.13 (s, $Ph\underline{C}OCH_2$); m/z (CI) 416 ($[M+NH_4]^+$, 20%), 399 (78, $[M+H]^+$), 371 (8, $[M+H\text{-}CO]^+$), 355 (22, $[M\text{-}NHCO]^+$), 308 (86, $[M+H\text{-}CH_2Ph]^+$), 291 (84, $[M\text{-}O_2CH_2Ph]^+$), 108 (35, $[PhCH_2OH]^+$).

N-(Benzyloxycarbonyl)-(2*S*)-kynurenine ethyl ester (217).



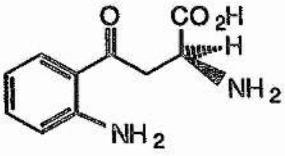
N'Formyl, *N*-(benzyloxycarbonyl)-(2*S*)-kynurenine ethyl ester (1.54 g, 3.87 mmol) was dissolved in methanol (150 ml). 4 M HCl (16 ml, 7.7 mmol) and dioxane (45 ml) were added. The solution was then stirred at room temperature for one hour. After this time it was poured into saturated $NaHCO_3$ (300 ml) and extracted into ethyl acetate (250 ml). The organic extracts were then washed with water. They were dried ($MgSO_4$), filtered and evaporated under reduced pressure to obtain a yellow oil (1.28 g, 86%) which crystallised on standing, m.p. 58 °C; (Found: C, 64.61; H, 6.11; N, 7.57. Calc. for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56%); υ_{max} (nujol)/cm^{-1} 1725 (ester CO) and 1680 (amide CO); δ_H (200 MHz; d^6–DMSO) 1.20 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.40 (2H, m, β–$C\underline{H}_2$), 4.10 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 4.65 (1H, m, α–$\underline{H}$), 5.10 (2H, s, $C\underline{H}_2Ph$), 6.65 (1H, t, *J* 7 Hz, 5'$\underline{H}$), 6.80 (1H, d, *J* 7 Hz, 6'$\underline{H}$), 7.20 (2H, s, $N\underline{H}_2$), 7.30 (1H, t, *J* 7 Hz, 4'$\underline{H}$), 7.40 (5H, m, aromatic), 7.70 (2H, m, $N\underline{H}$, 3'$\underline{H}$); δ_C (74.76 MHz; d^6–DMSO); 13.85 (s, $CO_2CH_2\underline{C}H_3$), 39.97 (s, β-$\underline{C}$), 50.04 (s, α–$\underline{C}$), 60.58 (s, $CO_2\underline{C}H_2CH_3$), 65.46 (s, $\underline{C}H_2Ph$), 114.40 (s, 3'$\underline{C}$), 116.27 (s, 1'$\underline{C}$), 116.93 (s, 5'$\underline{C}$), 127.54, 127.69, 128.23 (3x s, aromatic), 131 02 (s, 6'$\underline{C}$), 134.24 (s, 4'$\underline{C}$), 136.89 (s, quat. aromatic), 151.15 (s, 2'$\underline{C}$), 155.77 (s, $PhCH_2O\underline{C}O$), 171.68 (s, $\underline{C}O_2Et$) 197.68 ($Ph\underline{C}OCH_2$); *m/z* (CI) 371 ($[M+H]^+$, 100%), 263 (95, $[M\text{-}OCH_2Ph]^+$), 108 (42, $[PhCH_2OH]^+$).

N-(Benzyloxycarbonyl)-(2*S*)-kynurenine (218).



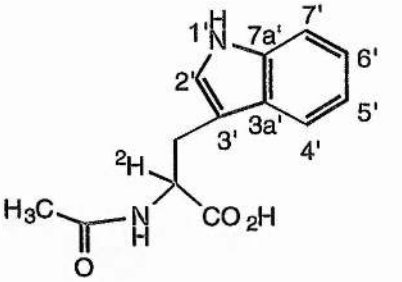
N-(Benzyloxycarbonyl)-(2*S*)-kynurenine ethyl ester (1.28 g, 3.22 mmol), was dissolved in a 1: 1 mixture of THF and water (60 ml) and treated with lithium hydroxide monohydrate (378 mg, 9.0 mmol). The solution was warmed at 70 °C for four hours. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 1: 2). The solution was poured into 1M HCl (30 ml) and extracted into ethyl acetate (150 ml). It was dried ($MgSO_4$), filtered and the solvent removed at reduced pressure to give a bright yellow solid (0.96 g, 88%); mp 169 °C; υ_{max} (nujol)/cm^{-1} 1720 (ester CO) and 1680 (amide CO); δ_H (200 MHz; d^6–DMSO) 3.35 (2H, m, β–C$\underline{H_2}$), 4.50 (1H, m, α–$\underline{H}$), 5.00 (2H, s, C$\underline{H_2}$Ph), 6.50 (1H, t, *J* 7 Hz, 5'$\underline{H}$), 6.70 (1H, d, *J* 7 Hz, 6'$\underline{H}$), 7.30 (1H, t, *J* 7 Hz, 4'$\underline{H}$), 7.40 (5H, m, aromatic), 7.70 (1H, d, 3'$\underline{H}$); *m/z* (CI) 343 ([*M*+H]$^+$, 100%), 325 (18, [*M*+H-H_2O]$^{+)}$, 252 (18, [*M*+H-CH_2Ph]$^+$), 235 (55, [*M*-PhCH_2O]$^+$).

(2*S*)-Kynurenine (1).



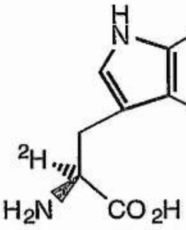
N-(Benzyloxycarbonyl)-(2*S*)-kynurenine (359 mg, 1.05 mmol) was dissolved in 15 ml methanol and 5% Pd/C (359 mg) was added. This was hydrogenated overnight at atmospheric pressure using the hydrogenation apparatus then filtered through a celite bed. The methanol was evaporated under reduced pressure to afford kynurenine (153 mg, 70%). The pale yellow solid was taken up in water and freeze dried; mp 188 °C (lit.,[174] 191 °C); $[\alpha]^{20}_{D}$ -29.5° (c 0.5 in H_2O) (lit.,[174]$[\alpha]^{25}_{D}$ -30.5° (c 1 in H_2O)); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1720 (CO), 1650 (acid CO); δ_H (200 MHz; ^2H_2O) 3.60 (2H, d, *J* 10 Hz, β–C$\underline{H}_2$), 4.05 (1H, m, α–$\underline{H}$), 6.50 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 6.70 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 7.50 (1H, d, *J* 7.5 Hz, 6'$\underline{H}$), 7.70 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$); δ_C (50.31 MHz; d^6–DMSO) 41.70 (s, β–$\underline{C}$), 53.19 (s, α–$\underline{C}$), 120.67 (s, 3'$\underline{C}$), 120.93 (s, 5'$\underline{C}$), 121.39 (s, 6'$\underline{C}$), 134.12 (s, 4'$\underline{C}$), 138.43 (s, 1'$\underline{C}$), 151.51 (s, 2'$\underline{C}$), 176.57 (s, $\underline{C}O_2H$), 202.59 (s, $CH_2\underline{C}OPh$); *m/z* (FAB) 209 ($[M+H]^+$, 66%).

N-Acetyl (2*SR*)-[2-^{2}H]-tryptophan (219).



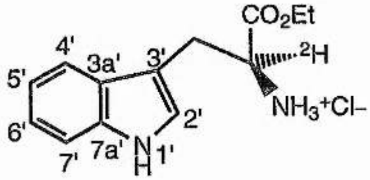
Tryptophan (5 g, 24.5 mmol) was added to ^2H_2O (50 ml) with stirring. Sodium (2.34 g, 105.6 mmol) was added carefully after washing in petroleum ether and ethanol. Acetic anhydride (15 ml) was added to the solution in 5 ml portions, shaking after each addition. The resulting dark orange solution was left stirring for two hours, after which time a white solid had precipitated from solution. A further amount of ^2H_2O (5ml) was added and the solution was left to stir overnight. The resulting solution was cooled in the fridge for several hours and then the crystals were filtered off and washed with cold water. The residue was suspended in 0.2 M HCl, cooled, filtered and washed with cold water until free of the chloride ion. The pale pink crystals obtained (4.55 g, 75%) were dried, m.p. 278 °C; υ_{max} (nujol) /cm^{-1} 3380 (indole NH), 1710 (CO ester), 1610 (CO amide); δ_H (200 MHz; d^6-DMSO) 1.85 (3H, s, NHCOC$\underline{H}_3$), 3.05 (2H, dd, *J* 15 Hz, β-C$\underline{H}_2$), 6.95 (2H, m, 5'$\underline{H}$, 6'$\underline{H}$), 7.15 (1H, s, 2'$\underline{H}$), 7.30 (1H, d, *J* 7.5 Hz, 7'$\underline{H}$), 7.50 (1H, d, *J* 7.5Hz, 4'$\underline{H}$), 8.25 (1H, s, indole N$\underline{H}$); δ_C (50.31 MHz; d^6-DMSO) 22.62 (s, $\underline{C}H_3$), 27.27 (s, β-$\underline{C}$), 52.94 (t, α–$\underline{C}$), 110.15 (s, 3'$\underline{C}$), 111.65 (s, 7'$\underline{C}$), 118.37 (s, 5'$\underline{C}$), 118.65 (s, 6'$\underline{C}$), 121.20 (s, 4'$\underline{C}$), 123.78 (s, 2'$\underline{C}$), 127.40 (s, 7a'$\underline{C}$), 136.32 (s, 3a'$\underline{C}$), 169.70 (s, CH$_3\underline{C}$O), 173.81 (s, $\underline{C}O_2H$); *m/z* (CI) 265 ([*M*+NH$_4$]$^+$,12%), 248 (100, [*M*+H]$^+$), 230 (53, [*M*+H-H$_2$O]$^+$), 130 (100, [CH$_2$ ind]$^+$).

(2*S*)-[2-^{2}H]-Tryptophan (220).



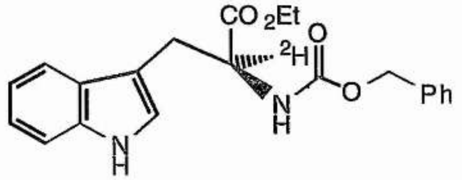
N-Acetyl (2*SR*)-2-[^{2}H]-tryptophan (5.14 g, 20.8 mmol) was added to water (200 ml) and the pH adjusted to 7.5 using concentrated ammonia. Acylase from *Aspergillus meleus* (520 mg, 0.47 units/mg) was added and the solution incubated at 38 °C. After a few days it was seen to have reacted to completion by ^{1}H NMR. The acylase was filtered off and the solution reduced in volume. Ion exchange chromatography (DOWEX 1) was carried out and the product fractions freeze dried to give a white solid (2.08 g, 97%), m.p. 232 °C (lit.,[Sigma L-Tryptophan sample] 228 °C); (Found: C, 64.60; H, 5.5.72; N, 13.61. Calc. for $C_{11}H_{11}N_2O_2{}^2H$: C, 64.375; H, 5.89; N, 13.65%); $[\alpha]^{20}_D$ -10.4° (c 0.5 in H_2O) (lit.,^{204} -33.4° (c 1 in EtOH); υ_{max} (nujol) /cm^{-1} 3370 (indole NH), 1645 (CO ester); δ_H (200 MHz; ^2H_2O) 3.20 (2H, dd, *J* 15 Hz, β-C$\underline{H}_2$), 7.00 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.15 (2H, m, 2'$\underline{H}$, 6'$\underline{H}$), 7.40 (1H, d, *J* 7.5 Hz, 7'$\underline{H}$), 7.60 (1H, d, *J* 7.5 Hz, 4'$\underline{H}$); δ_C (74.76 MHz; d^6-DMSO) 24.40 (s, β-$\underline{C}$), 53.19 (s, α-$\underline{C}$), 105.62 (s, 3'$\underline{C}$), 110.03 (s, 7'$\underline{C}$), 116.54 (s, 5'$\underline{C}$), 117.55 (s, 6'$\underline{C}$), 120.22 (s, 4'$\underline{C}$), 123.10 (s, 2'$\underline{C}$), 124.76 (s, 7a'$\underline{C}$), 134.43 (s, 3a'$\underline{C}$), 172.59 (s, $\underline{C}O_2H$); *m/z* (FAB) 433 ([2*M*+Na]$^+$, 2%), 411 (9, [2*M*+H]$^+$), 228 (14, [*M*+Na]$^+$), 206 (100, [*M*+H]$^+$), 160 (16, [*M*-CO$_2$]$^+$), 130 (53, [CH$_2$-indole]$^+$).

(2*S*)-[2-^{2}H]-Tryptophan ethyl ester hydrochloride (221).



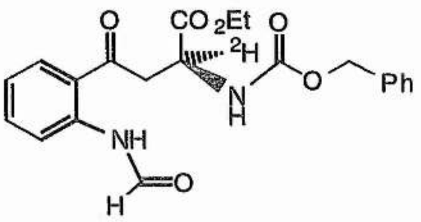
(2*S*)-[2-^{2}H]-Tryptophan (3.12 g, 15.16 mmol) was dissolved in dry redistilled ethanol (60 ml) cooled in ice. Redistilled thionyl chloride (1.2 ml, 1.1 eq) was added and the solution was refluxed for one hour at approximately 100 °C. The solvent was concentrated in vacuo and recrystallisated from ethanol to give an off white solid (2.53 g 72%), m.p. 224 °C; $[\alpha]^{20}_D$ +12.25° (c 1 in MeOH); υ_{max} (nujol)/cm^{-1} 3250 (indole NH) and 1725 (CO ester); δ_H (200 MHz; d^6–DMSO) 1.10 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.20 (2H, m, β–$C\underline{H}_2$), 4.10 (2H,q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 7.10 (3H, m, 2'$\underline{H}$, 5'$\underline{H}$, 6'$\underline{H}$), 7.60 (1H, d, *J* 7 Hz, 7'H), 7.80 (1H, d, *J* 7 Hz, 4'$\underline{H}$), 8.70 (1H, s, indole N$\underline{H}$); δ_C (50.31 MHz; d^6–DMSO) 13.96 (s, $CO_2CH_2\underline{C}H_3$), 26.32 (s, β–$\underline{C}$), 61.85 (s, $CO_2\underline{C}H_2CH_3$), 106.69 (s, 3'$\underline{C}$), 111.79 (s, 7'$\underline{C}$), 118.28 (s, 5'$\underline{C}$), 118.81 (s, 6'$\underline{C}$), 121.39 (s, 4'$\underline{C}$), 125.18 (s, 2'$\underline{C}$), 127.20 (s, 7a'$\underline{C}$), 136.44 (s, 3a'$\underline{C}$), 169.56 (s, $\underline{C}O_2Et$); *m/z* (EI) 233 ([*M*+H]$^+$, 10%), 130 (100, [CH$_2$-indole]$^+$) 103 (13, [*M*+H-CH$_2$-indole]$^+$).

N-(Benzyloxycarbonyl)-(2S)-[2-^{2}H]-tryptophan ethyl ester (222).



(2*S*)-Tryptophan ethyl ester hydrochloride (3.54 g, 15.2 mmol) was dissolved in water (80 ml). Sodium carbonate (2.2 eq, 33.40 mmol, 3.52 g) was added followed by benzylchloroformate (1.2 eq, 18.24 mmol, 2.6 g, 2.7 ml) The solution was left to stir overnight at room temperature. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 3: 7). A pale brown precipitate was seen and the solid was filtered off to give (4.54 g, 82%) of an off white solid, m.p. 76-78 °C; $[\alpha]^{20}_D$ -9.0° (c 1 in EtOH); υ_{max} (nujol)/cm^{-1} 3300 (indole NH), 1720 (ester CO) and 1680 (amide CO); δ_H (200M Hz; d^6–DMSO) 1.10 (3H, m, $CO_2CH_2C\underline{H}_3$), 3.10 (2H, m, β–$C\underline{H}_2$), 4.05 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.05 (2H, s, $C\underline{H}_2Ph$), 7.05 (2H, m 5'$\underline{H}$, 6'$\underline{H}$), 7.15 (1H, s, 2'$\underline{H}$), 7.35 (6H, m, 7'$\underline{H}$, aromatic), 7.55 (1H, d, *J* 7 Hz, 4'$\underline{H}$) 7.80 (1H, s, N$\underline{H}$ indole); δ_C (50.31 MHz; d^6–DMSO) 14.20 (s, $CO_2CH_2\underline{C}H_3$), 27.11 (s, β–$\underline{C}$), 60.72 (s, $CO_2\underline{C}H_2CH_3$), 65.66 (s, $\underline{C}H_2Ph$), 109.84 (s, 3'$\underline{C}$), 111.71 (s, 7'$\underline{C}$), 118.24 (s, 5'$\underline{C}$), 118.68 (s, 6'$\underline{C}$), 121.23 (s, 4'$\underline{C}$), 124.11 (s, 2'$\underline{C}$), 127.30 (s, 7a'$\underline{C}$), 127.88, 128.05, 128.60 (3x s, aromatic), 136.37 (s, quat. aromatic), 137.19 (s, 3a'$\underline{C}$), 157.22 (s, $COCH_2Ph$), 172.49 (s, $\underline{C}O_2Et$); *m/z* (EI) 367 (M^+, 28%), 216 (10, $[M\text{-}PhCH_2OCONH_2]^+$), 130 (100, $[CH_2\text{-indole}]^+$), 77 (15, $[Ph]^+$).

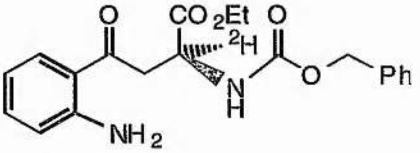
N'Formyl, N-(benzyloxycarbonyl)-(2S)-[2-^{2}H]-kynurenine ethyl ester (223).



N-(Benzyloxycarbonyl)-(2S)-[2-^{2}H]-kynurenine ethyl ester (2.2 g, 2.0 mmol) was dissolved in dry distilled methanol (100 ml). The solution was cooled to -78 °C with stirring and then treated with ozone for one hour, the reaction was then quenched with dimethyl sulphide (10 ml). The reaction was checked by tlc (silica, ethyl acetate: dichloromethane, 1: 9) and left to stir for a further hour at -78 °C. The solution was then evaporated to a dark orange oil. The product was dissolved in diethyl ether (50 ml) and washed with brine (2 x 100 ml) and water (2 x 100 ml). The solution was then dried ($MgSO_4$) and evaporated under reduced pressure to an orange oil. Purification was carried out by column chromatography (silica, ethyl acetate: petroleum ether, 1: 2) giving the product (786 mg, 33%), m.p. 111-112 °C; (Found: C, 63.35; H, 5.81; N, 6.96; $[M+H]^+$ 400.1619. $C_{21}H_{21}N_2O_6{}^2H$ requires C, 63.15; H, 5.55; N, 7.01%; $[M+H]^+$ 400.1613); $[\alpha]^{20}_D$ +3.2° (c 1 in MeOH); υ_{max} (nujol)/cm^{-1} 3305 (indole N$\underline{H}$), 1725 (ester CO), and 1680 (amide CO); δ_H (200 MHz; d^6–DMSO) 1.15 (3H, t, J 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.45 (2H, s, β–$C\underline{H}_2$), 4.10 (2H, q, J 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.05 (2H, s, $C\underline{H}_2Ph$), 7.25 (1H,t, J 7 Hz, 5'$\underline{H}$); 7.35 (5H, m, aromatic), 7.65 (1H,t, J 7 Hz, 4'$\underline{H}$), 7.75 (1H, s, N$\underline{H}$COH), 7.80 (1H, d, J 7 Hz, 3'$\underline{H}$), 8.00 (1H, d, J 7 Hz, 6'$\underline{H}$), 8.45 (1H, m, NHCO$\underline{H}$); δ_C (74.76 MHz; d^6–DMSO) 14.03 (s, $CO_2CH_2\underline{C}H_3$), 41.16 (s, β–$\underline{C}$), 60.91 (s, $CO_2\underline{C}H_2CH_3$), 65.64 (s, $\underline{C}H_2Ph$), 121.25 (s, 3'$\underline{C}$), 123.45 (s, 5'$\underline{C}$), 123.87 (s, 1'$\underline{C}$), 127.82, 127.94, 128.43 (3x s, aromatic), 130.94 (s, 6'$\underline{C}$), 134.37 (s, 4'$\underline{C}$), 136.93 (s, quat. aromatic), 137.99 (s, 2'$\underline{C}$), 155.92 (s, $PhCH_2O\underline{C}O$), 161.34 (s, NH$\underline{C}$OH), 171.57 (s, $\underline{C}O_2Et$), 200.07 (s, $Ph\underline{C}OCH_2$); m/z (CI) 417 ($[M+NH_4]^+$, 100%), 400 (55, $[M+H]^+$), 372

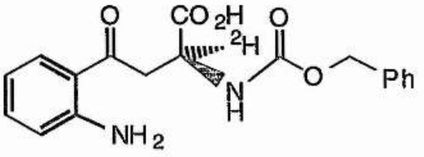
(6, [*M*+H-CO]$^+$), 356 (10, [*M*-NHCO]$^+$), 309 (32, [*M*+H-CH_2Ph]$^+$), 292 (12, [*M*-O_2CH_2Ph]$^+$), 108 (19, [$PhCH_2OH$]$^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester** (224).



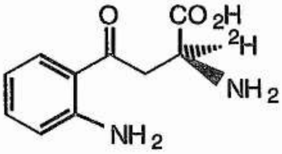
The protected kynurenine (362 mg, 0.91 mmol) was dissolved in methanol (45 ml). 4 M HCl (4 ml, 3.44 mmol) and dioxane (15 ml) were added to the solution. The solution was stirred at room temperature for one hour. It was poured into saturated $NaHCO_3$ (120 ml) and extracted into ethyl acetate (150 ml). The organic extracts were then washed with water (2 x 50ml). They were dried ($MgSO_4$), filtered and evaporated at reduced pressure to obtain a yellow oil (33 mg, 98%) which crystallised on standing, m.p. 60 °C; υ_{max} (nujol)/cm^{-1} 1725 (ester CO) and 1680 (carbamate CO); δ_H (300 MHz; d^6–DMSO) 1.15 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.40 (2H, m, β–$C\underline{H}_2$), 4.10 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.10 (2H, s, $C\underline{H}_2Ph$), 6.65 (1H, t, *J* 7 Hz, 5'$\underline{H}$), 6.80 (1H, d, *J* 7 Hz, 6'$\underline{H}$), 7.15 (2H, s, $N\underline{H}_2$), 7.30 (1H, t, *J* 7 Hz, 4'$\underline{H}$), 7.35 (5H, m, aromatic), 7.55 (1H, s, N$\underline{H}$) 7.70 (1H, d, *J* 7 Hz, 3'$\underline{H}$); δ_C (74.76 MHz; d^6–DMSO) 13.91 (s, $CO_2CH_2\underline{C}H_3$), 60.63 (s, $CO_2\underline{C}H_2CH_3$), 65.50 (s, $\underline{C}H_2Ph$), 114.46 (s, 3'$\underline{C}$), 116.30 (s, 1'$\underline{C}$), 116.98 (s, 5'$\underline{C}$), 127.62, 127.76, 128.30 (3x s, aromatic), 131 09 (s, 6'$\underline{C}$), 134.33 (s, 4'$\underline{C}$), 136.95 (s, quat. aromatic), 151.22 (s, 2'$\underline{C}$), 155.83 (s, $PhCH_2O\underline{C}O$), 171.77 (s, $\underline{C}O_2Et$), 197.75 ($Ph\underline{C}OCH_2$); *m/z* (CI) 372 ([*M*+H]$^+$, 100%), 264 (57, [*M*-$PhCH_2O$]$^+$), 108 (15, [$PhCH_2OH$]$^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine** (225).



N-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester (291 mg, 0.78 mmol), was dissolved in a 1: 1 mixture of THF and water (14 ml) and treated with lithium hydroxide monohydrate (91 mg, 2.18 mmol). The solution was warmed at 70 °C for four hours. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 1: 2). The solution was poured into 1 M HCl (7 ml) and extracted into ethyl acetate (125 ml). It was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow oil. This was then dissolved in ethanol and concentrated under reduced pressure to give a bright yellow solid (267 mg, 95%); m.p. 169-170 °C; $[\alpha]^{20}_{D}$ + 44.8° (c 0.13 in MeOH); υ_{max} (nujol)/ cm^{-1} 1725 (ester CO) and 1680 (amide CO); δ_H (300 MHz; d^6–DMSO) 3.40 (2H, m, β–C$\underline{H}_2$), 5.05 (2H, s, C$\underline{H}_2$Ph), 6.55 (1H, t, *J* 7 Hz, 5'$\underline{H}$), 6.75 (1H, d, *J* 7 Hz, 6'$\underline{H}$), 7.10 (2H, br s, N$\underline{H}_2$), 7.25 (1H, t, *J* 7 Hz, 4'$\underline{H}$), 7.35 (5H, m, aromatic), 7.40 (1H, m, N$\underline{H}$), 7.70 (1H, d, *J* 7 Hz, 3'$\underline{H}$); δ_C (74.76 MHz; d^6–DMSO) 65.58 (s, $\underline{C}H_2$Ph), 114.65 (s, 3'$\underline{C}$), 116.51 (s, 1'$\underline{C}$), 117.11 (s, 5'$\underline{C}$), 127.73, 127,87, 128.43 (3x s, aromatic), 131 22 (s, 6'$\underline{C}$), 134.42 (s, 4'$\underline{C}$), 137.06 (s, quat. aromatic), 151.28 (s, 2'$\underline{C}$), 156.01 (s, PhCH$_2$O$\underline{C}$O), 173.40 (s, $\underline{C}O_2H$), 198.21 (Ph$\underline{C}$OCH$_2$), *m/z* (EI) 343 (M^+, 5%), 235 (5, [*M*-PhCH$_2$OH]).

(2*S*)-[2-^{2}H]-Kynurenine (199).



N-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine (235 mg, 0.69 mmol) was dissolved in methanol (15 ml) and 5% Pd/C (235 mg) was added. This was hydrogenated overnight at atmospheric pressure and filtered through a celite bed. The methanol was evaporated under reduced pressure and isopropanol (1.50 ml) was added containing a small amount of *RS*-dithiothreitol. The dark orange solid which resulted was neutralised with propylene oxide (0.18 g, 3.39 mmol). Diethyl ether was added and a pale yellow solid precipitated out of solution. After filtration more solid was seen to precipitate out of the diethyl ether. On filtering this became a yellow oil and was taken up in water and the resulting pale yellow solution was freeze dried to give (0.05 g, 42%) of a yellow solid (65 mg, 45%); m.p. 188 °C; $[\alpha]^{20}_D$ -25.5° (c 0.5 in H_2O); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1720 (CO), 1650 (acid CO); δ_H (200 MHz; ^2H_2O) 3.60 (2H, d, *J* 10 Hz, β–C$\underline{H}_2$), 4.05 (1H, m, α–$\underline{H}$), 6.50 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 6.70 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 7.50 (1H, t, *J* 7.5 Hz, 6'$\underline{H}$), 7.70 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$); δ_C (50.31 MHz; d^6–DMSO) 41.70 (s, β–$\underline{C}$), 53.19 (s, α–$\underline{C}$), 120.67 (s, 3'$\underline{C}$), 120.93 (s, 5'$\underline{C}$), 121.39 (s, 6'$\underline{C}$), 134.12 (s, 4'$\underline{C}$), 138.43 (s, 1'$\underline{C}$), 151.51 (s, 2'$\underline{C}$), 176.57 (s, $\underline{C}O_2H$), 202.59 (s, $CH_2\underline{C}OPh$); *m/z* (FAB) 209 ([*M*+H]$^+$, 66%). The material was identical in all respects to that obtained from the first method, with an enantiomeric excess of 98%.

6.2 Partial Purification of *Pseudomonas fluorescens*[2]

The freeze dried culture of *Pseudomonas fluorescens* was grown in nutrient broth containing 'Lab. Lemco' powder (meat extract) 0.1%, yeast extract 0.2%, peptone 0.5% and sodium chloride 0.5% (0.26 g in 20 ml distilled water). The cultures were grown at 28 °C for eighteen hours under aeration and then kept refrigerated until required. This was then sub-cultured to a media containing 0.1% 2*S*-tryptophan, 0.2% peptone, 0.1% glycerin, 0.05% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4.7H_2O$ (3 l). The cultures were grown at 28 °C for eighteen hours under aeration.

The harvested cells were washed twice with 0.85% sodium chloride solution (10 ml) and subsequently with 0.01 M potassium phosphate buffer (10 ml, pH 7.2). All operations were carried out at 4 °C. All buffers used contained 20 µM PLP and 0.01% 2-mercaptoethanol. After each wash, centrifugation was carried out at 6 500 g and 4 °C.

Step 1

The washed cells (28.8 g) were mixed with 0.01 M potassium phosphate buffer (pH 7.2) (35 ml) and sonication was carried out in eight thirty second bursts with two minutes between bursts. The resultant slurry was then centrifuged (32 000 g for twenty minutes at 4 °C).

Step 2

To the cell free extract (35 ml), 1 ml of 1% protamine sulphate in 0.01 M potassium phosphate buffer (pH 7.2) was added per 100 mg of protein (17 ml) with stirring over ten minutes. The mixture was then stirred for a further thirty minutes and centrifuged (32 000 g for twenty minutes at 4 °C) and the bulky inactive precipitate was discarded.

Step 3

The enzyme solution (60 ml) was brought to 20% saturation with ammonium sulphate (6.84 g) added over twenty minutes and then stirred for a further thirty minutes. The precipitate was removed by centrifugation (32 000 g for twenty minutes at 4 °C).

Step 4

Ammonium sulphate (13.5 g) was added to the supernatant to 55% saturation over approximately thirty minutes and stirring was continued for thirty minutes. The precipitate was collected by centrifugation (32 000 g for twenty minutes at 4 °C).

Step 5

The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.2) (10 ml) and then dialysed overnight against 100 volumes (1000 ml) of the same buffer. After dialysis the enzyme solution was centrifuged (32 000 g for twenty minutes at 4 °C).

Step 6

The dialysed enzyme solution (10 ml, 25-30 units activity) was placed on a DEAE-cellulose column, (2.5 x 20 cm) and equilibriated with 0.01 M potassium phosphate buffer (pH 7.2). The column was washed thoroughly at a flow rate of 80 ml per hour (120 ml) with the same buffer followed by the buffer containing 0.1 M sodium chloride (120 ml). The enzyme was eluted with the buffer supplemented with 0.2 M sodium chloride. The active fractions were pooled (55 ml) and brought to 70% saturation with ammonium sulphate (25.96 g) added over about thirty minutes and then stirred for a further thirty minutes. The precipitate was collected by centrifugation (32 000 g for twenty minutes at 4 °C).

Step 7

The precipitate was suspended in 0.01 M potassium phosphate buffer (pH 7.2) (10 ml) and concentrated by ultrafiltration (4.5 ml) using a filter (AMICON, YM 10, 43 mm). This was then stored in the freezer ready for use.

6.2.1 Preparation of dialysis tubing.

Dialysis tubing was prepared by boiling for five to ten minutes in distilled water (200 ml) containing EDTA (0.2 g). Prepared dialysis tubing was kept in a solution of ethanol: water (1: 1) and rinsed with distilled water before use.

6.2.2 Regeneration of DEAE cellulose.

The DEAE cellulose was washed with hydrochloric acid (0.5 M, 1000 ml) followed by distilled water (1500 ml) five times or until the pH was the same as distilled water. The DEAE cellulose was washed with sodium hydroxide (0.5 M, 1000 ml) followed by distilled water (1500 ml) ten times or until the pH was the same as distilled water. Most of the water was then removed and 0.01 M potassium phosphate buffer added. This was decanted and more buffer added in order to pack the column.

6.3 ASSAY OF KYNURENINASE ACTIVITY.

The activity of kynureninase was determined by measuring the rate of decrease in absorbance at 360 nm (ε= -4500 mol^{-1} dm^{3} cm^{-1}) due to the conversion of kynurenine to anthranilic acid. Incubation was carried out at 25 ± 0.1 °C in the thermostated housing of the UV spectrophotometer using a 1 cm path length cuvette (1.2 ml). Reaction was initiated by the addition of 30 µl of enzyme solution to the assay buffer (1 ml). The assay buffer contained 0.4 mM kynurenine, 40 µM PLP in 0.04 M potassium phosphate and was brought to pH 7.0 using 4 M potassium hydroxide. Enzyme activity was measured in units where one unit is defined as the amount of enzyme required to convert one μmol of substrate per minute under standard conditions.

From the Beers law,

$A = \varepsilon cl$, therefore $c = A/\varepsilon l$

volume = 1 ml + 30 μl

= 1.03 ml

The rate for one unit under assay conditions is

$$\frac{dc}{dt} = 1 \ \mu\text{mol} \ \text{x} \frac{1000}{1.03}$$

$$= 9.709 \text{ x } 10^{-4} \text{ mol dm}^{-3} \text{ min}^{-1}$$

This is then converted to rate in absorbance units per minute for one unit.

$$\frac{\partial A}{\partial t} = 9.709 \text{ x } 10^{-4} \text{ x } 1 \text{ x } -4500$$

$$= -4.369$$

The number of units of enzyme activity can, therefore, be calculated.

$$\text{no. of units} = \frac{\text{absorbance per minute from assay}}{\text{absorbance per minute for one unit}}$$

$$\text{no. of units} = \frac{\text{gradient}}{4.369}$$

The number of units per ml can then be calculated.

6.3.1 Determination of Kinetic Parameters.

Determination of inhibition of kynureninase by various compounds was performed by variation of the concentration of (2*S*)-kynurenine, from 0.1 to 0.5 mM, at several fixed values of the inhibitor concentration ranging from 0.05 mM to 2.0 mM. The reactions were linear over the time course measured and the rate was measured in duplicate or in triplicate. K_M and V_{max} values were calculated by fitting of initial rate data to the Michaelis-Menten equation via non linear regression with the ENZFITTER[151] programme on a PC. The analysis for each compound was carried out at three inhibitor concentrations. Each compound was compared to a standard assay of kynurenine from 0.1 to 0.5 mM (For example, see Table 6.1 and 6.2).

Change in Absorbance for 0.4 mM Kynurenine (min $^{-1}$)	**Kynurenine concentration (mM)**	**Change in Absorbance for Standard Assay (min $^{-1}$)**	**Average Change in Absorbance for Standard Assay (min $^{-1}$)**
0.2403	0.1	0.2107 0.2110	0.21085
0.2346	0.2	0.2280 0.2290 0.2288	0.2286
0.20685	0.3	0.2065 0.2063	0.2064
0.2000	0.45	0.2078 0.2083 0.2076	0.2079
0.1970	0.5	0.1881 0.1880	0.18805

Table 6.1

Before each run an assay was carried out in normal assay buffer (0.4 mM kynurenine) so that the change in absorbance, of the standard assay, could be corrected for one unit of enzyme activity.

Change in Absorbance for 0.4 mM Kynurenine (min^{-1})	**Number of units of enzyme activity**	**Average Change in Absorbance for Standard Assay (min^{-1})**	**Average Change in Absorbance corrected for one unit of enzyme activity (min^{-1})**
0.2403	0.0550	0.21085	3.83
0.2346	0.0537	0.2286	4.26
0.2068	0.0473	0.2064	4.36
0.0458	0.0458	0.2079	4.54
0.1790	0.0410	0.18805	4.59

Table 6.2

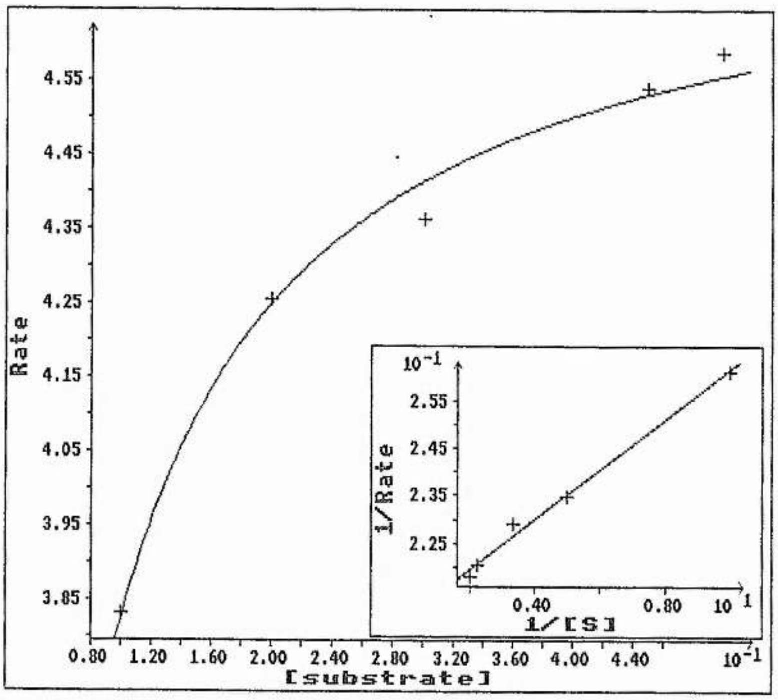
The data was then fitted to the Michaelis-Menten equation by non-linear regression using the ENZFITTER[151] programme. This provided values of K_M and V_{max} with calculated errors. A Lineweaver-Burk plot was also drawn (Graph 6.1).

The rate generally follows saturation kinetics with respect to the concentration of the substrate. At sufficiently low [S], V increases linearly with [S] but as [S] is increased, this relationship breaks down and V increases less rapidly than [S] until, at sufficiently high or saturating [S], V tends towards a limiting value termed V_{max}. This is expressed quantitatively in the Michaelis-Menten equation, the basic equation of enzyme kinetics.

$$V = \frac{[E]_0\ [S]\ k_{cat}}{[S]\ + K_m} \quad \textbf{Michaelis-Menten equation}$$

where, $k_{cat}\ [E]_0 = V_{max}$.

The concentration of substrate at which $V = 1/2\ V_{max}$ is termed K_M, the Michaelis constant.



Graph 6.1

For kynurenine K_M was calculated as 25.1 μM and V_{max} as 4.78 min^{-1}. This V_{max} can be converted to standard units by dividing by the molar extinction coefficient. Therefore V_{max} is equal to 1.06×10^{-3} mol dm^{-3} min^{-1}.

The complete set of results are shown in the Appendix.

6.4 Protein determination.

Protein was determined by the method of Bradford[198] using bovine serum albumin as a standard (0-10 μg in 0.1 ml). Bradford reagent (1 ml) was added to each sample (0.1 ml) and the mixture left to react for thirty minutes before the optical density was recorded at 595 nm. A calibration curve was plotted and used to determine the amount of protein in each sample.

6.4.1 Specific activity.

Specific activity is expressed as units per milligram of protein and is defined as:

$$\text{specific activity} = \frac{\text{units of activity}}{\text{mass of protein}}$$

6.5 Determination of Substrate Properties.

Determination of the substrate properties of compounds was performed by tlc (cellulose, IPA: conc. NH_3: H_2O; 36: 6: 10).

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APPENDIX

K_M Determination for Kynurenine.

Number	**K_M (μM)**	**Error (μM)**	**V_{max} (min^{-1})**	**Error ($x10^{-2}$ min^{-1})**
1[a]	25.26	±1.17	4.79	±2.34
2[a]	25.03	±0.597	4.80	±1.20
3[a]	25.22	±9.80	4.82	±19.8
4[a]	26.12	±8.70	4.88	±17.6
5[a]	26.13	±10.1	4.83	±20.2
6[a]	25.15	±6.52	4.80	±13.1
7[a]	25.91	±7.28	4.85	±14.7
8[a]	25.39	±1.60	4.79	±3.20
9[b]	25.10	±1.01	4.78	±2.03

[a] Buffer at pH 7.0

[b] Buffer at pH 7.8

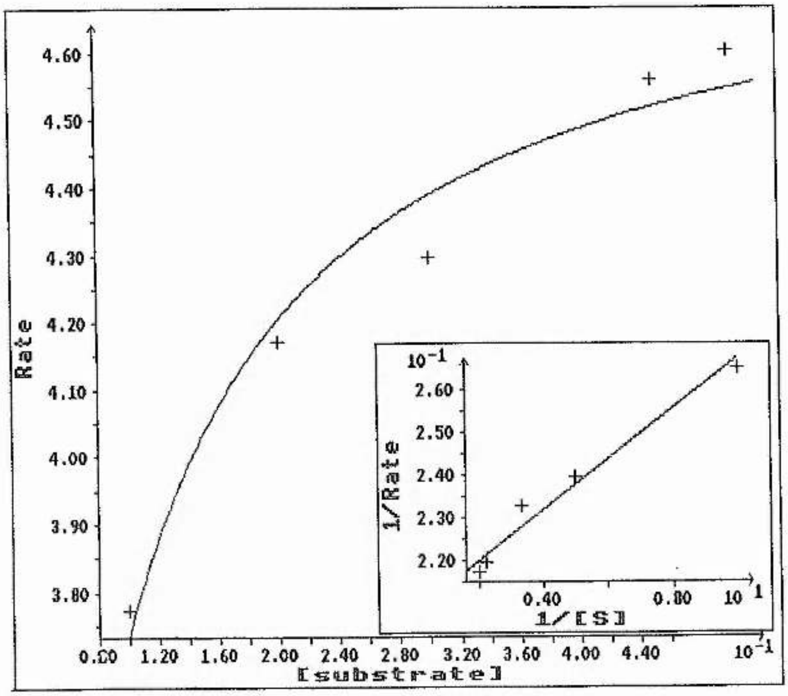
Table 7.1

Average $K_M = 25.48 \pm 0.6$ μM

Average $V_{max} = 4.82 \pm 0.06$ min^{-1}

Phosphinic Acid Analogue. 0.5 mM Phosphinic Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.77
0.20	4.17
0.30	4.29
0.45	4.56
0.50	4.60

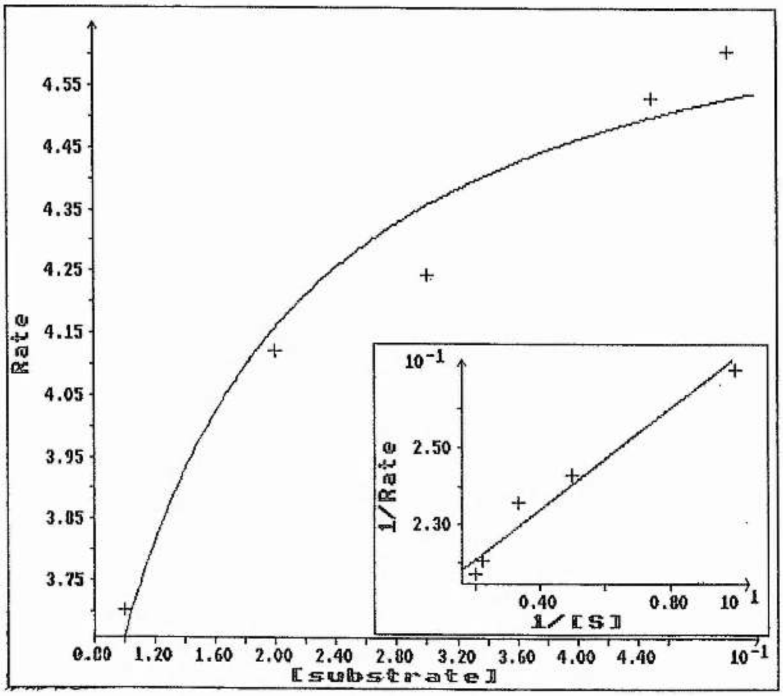


Kinetic Parameters for 0.5 mM Phosphinic Acid

Variable	Value	Error
V_{max} (min^{-1})	4.81	±0.0739
K_M (μM)	28.6	±3.78

Phosphinic Acid Analogue. 1 mM Phosphinic Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.70
0.20	4.12
0.30	4.25
0.45	4.53
0.50	4.61

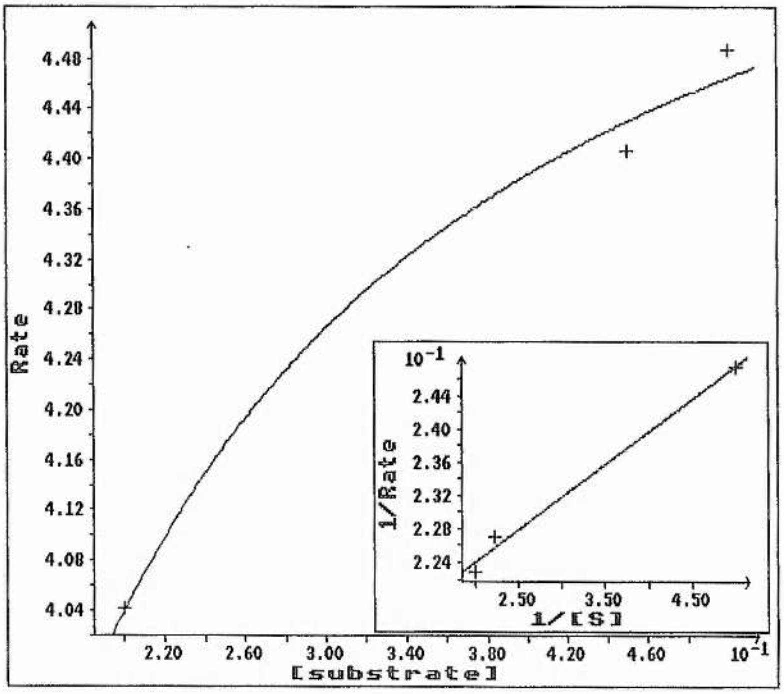


Kinetic Parameters for 1 mM Phosphinic Acid

Variable	Value	Error
V_{max} (min^{-1})	4.82	±0.0905
K_M (μM)	31.6	±4.73

Phosphinic Acid Analogue. 2 mM Phosphinic Acid

[Substrate] (mM)	Rate (min^{-1})
0.20	4.04
0.45	4.41
0.50	4.49

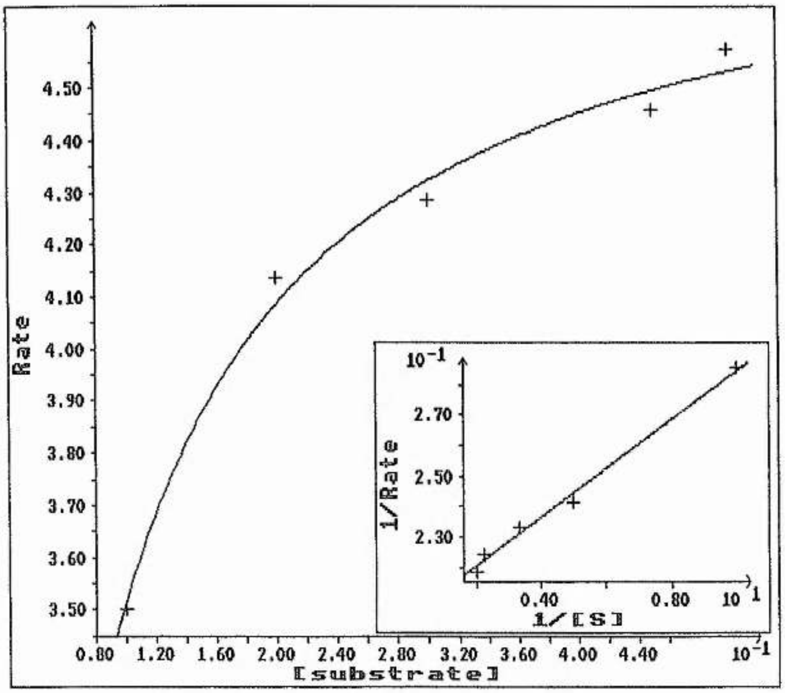


Kinetic Parameters for 2 mM Phosphinic Acid

Variable	Value	Error
V_{max} (min^{-1})	4.81	±0.0576
K_M (μM)	37.7	±4.27

Methyl Phosphinate Analogue. 0.5 mM Methyl Phosphinate

[Substrate] (mM)	Rate (min^{-1})
0.10	3.50
0.20	4.14
0.30	4.29
0.45	4.46
0.50	4.57

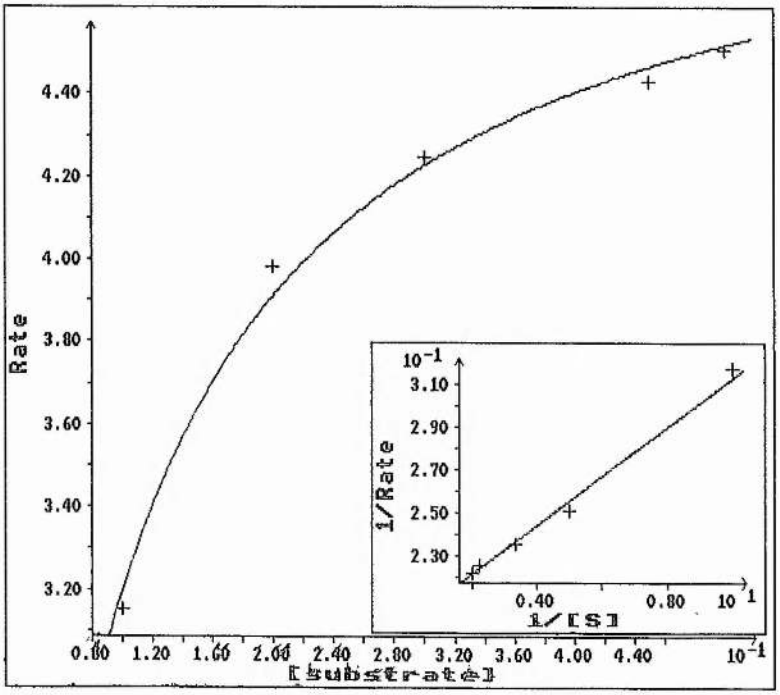


Kinetic Parameters for 0.5 mM Methyl Phosphinate

Variable	Value	Error
V_{max} (min^{-1})	4.89	±0.0522
K_M (μM)	39.0	±2.84

Methyl Phosphinate Analogue. 1 mM Methyl Phosphinate

[Substrate] (mM)	Rate (min^{-1})
0.10	3.15
0.20	3.98
0.30	4.25
0.45	4.43
0.50	4.50

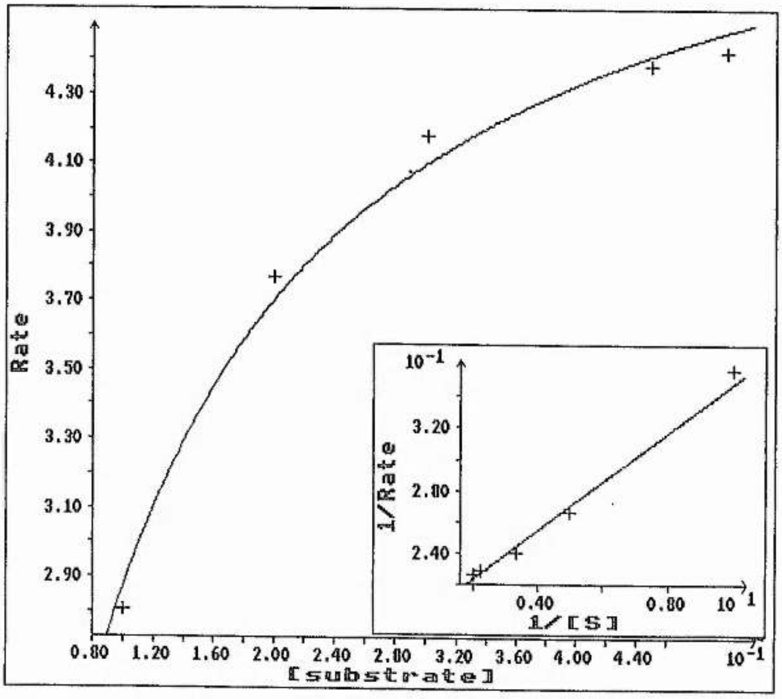


Kinetic Parameters for 1 mM Methyl Phosphinate

Variable	Value	Error
V_{max} (min^{-1})	4.89	±0.0522
K_M (μM)	39.0	±2.84

Methyl Phosphinate Analogue. 2 mM Methyl Phosphinate

[Substrate] (mM)	Rate (min^{-1})
0.10	2.86
0.20	3.69
0.30	4.10
0.45	4.41
0.50	4.48

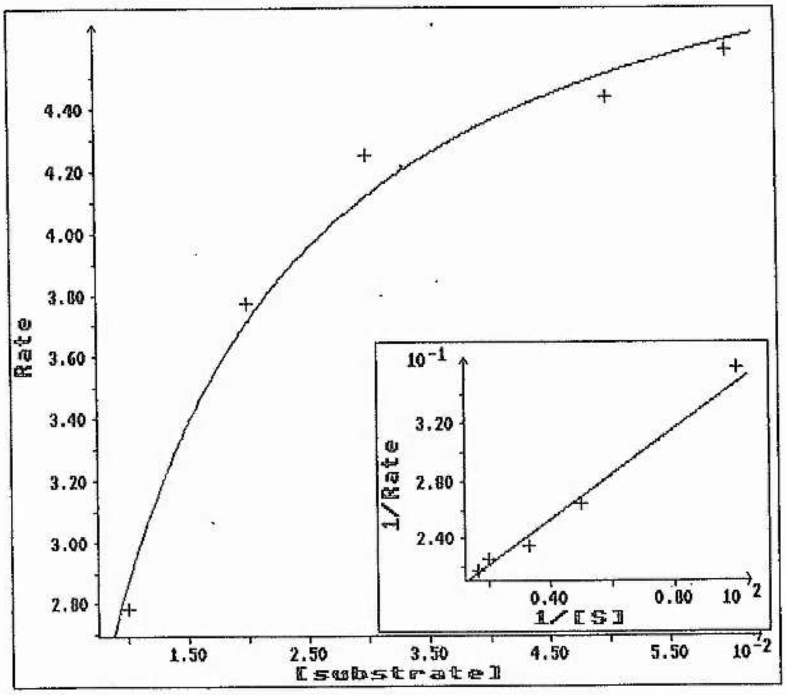


Kinetic Parameters for 2 mM Methyl Phosphinate

Variable	Value	Error
V_{max} (min^{-1})	5.23	±0.131
K_M (μM)	83.1	±8.66

Methyl Phosphinate Analogue. 2 mM Methyl Phosphinate

[Substrate] (mM)	Rate (min^{-1})
0.10	2.79
0.20	3.77
0.30	4.25
0.50	4.44
0.60	4.59

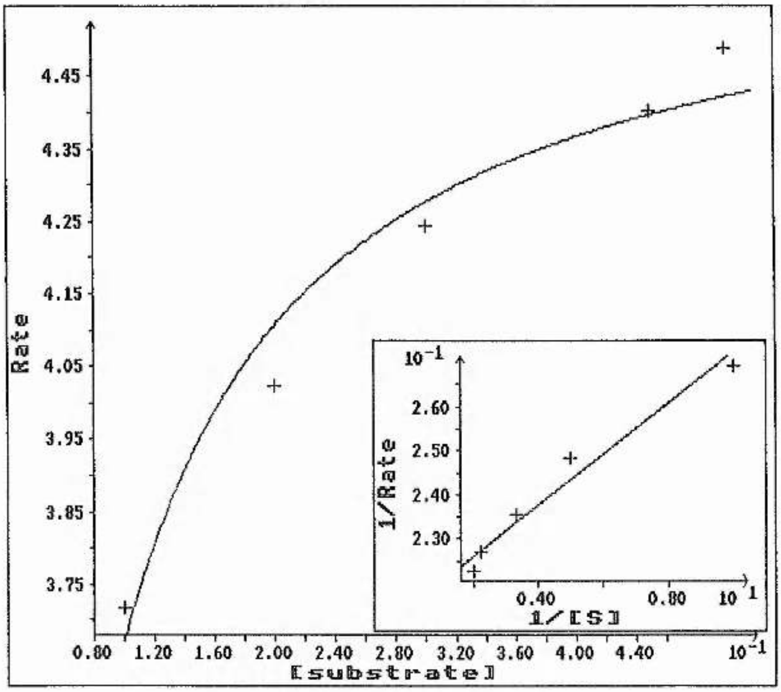


Kinetic Parameters for 2 mM Methyl Phosphinate

Variable	Value	Error
V_{max} (min^{-1})	5.27	±0.143
K_M (μM)	83.7	±9.66

Substrate Analogue. 0.05 mM Cyclohexyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.72
0.20	4.02
0.30	4.24
0.45	4.40
0.50	4.49

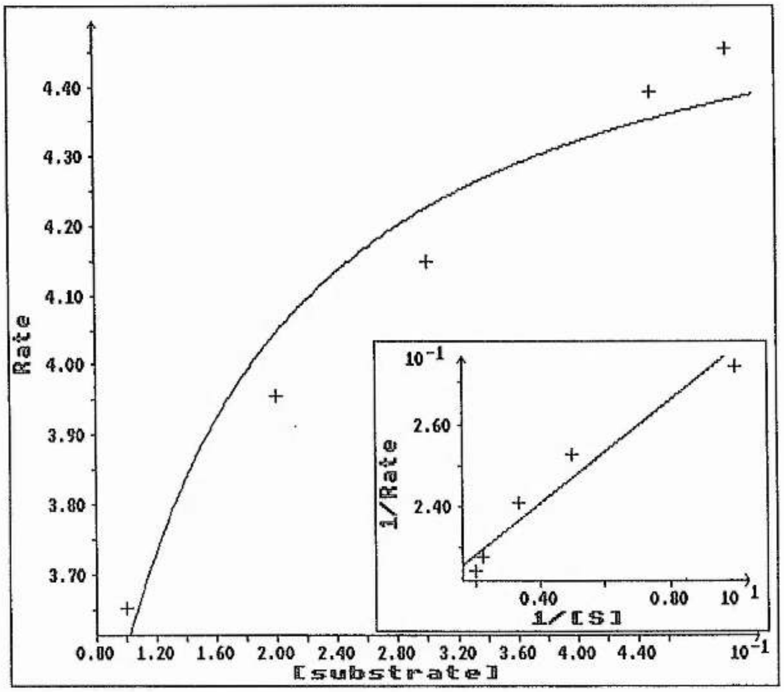


Kinetic Parameters for 0.05 mM Cyclohexyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.66	±0.0703
K_M (μM)	26.9	±3.66

Substrate Analogue. 0.1 mM Cyclohexyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.65
0.20	3.96
0.30	4.15
0.45	4.39
0.50	4.45

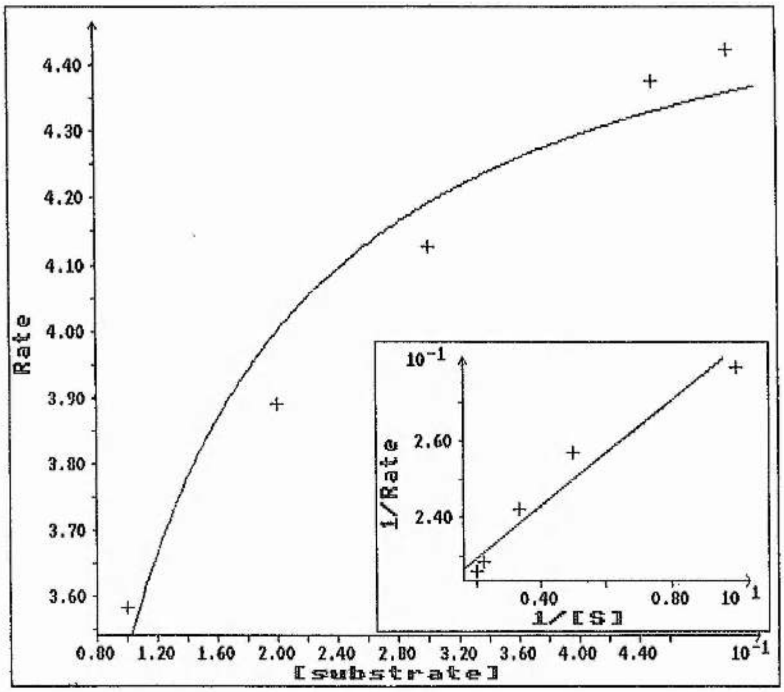


Kinetic Parameters for 0.1 mM Cyclohexyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.64	±0.0927
K_M (μM)	29.0	±4.93

Substrate Analogue. 0.2 mM Cyclohexyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.58
0.20	3.89
0.30	4.13
0.45	4.38
0.50	4.42

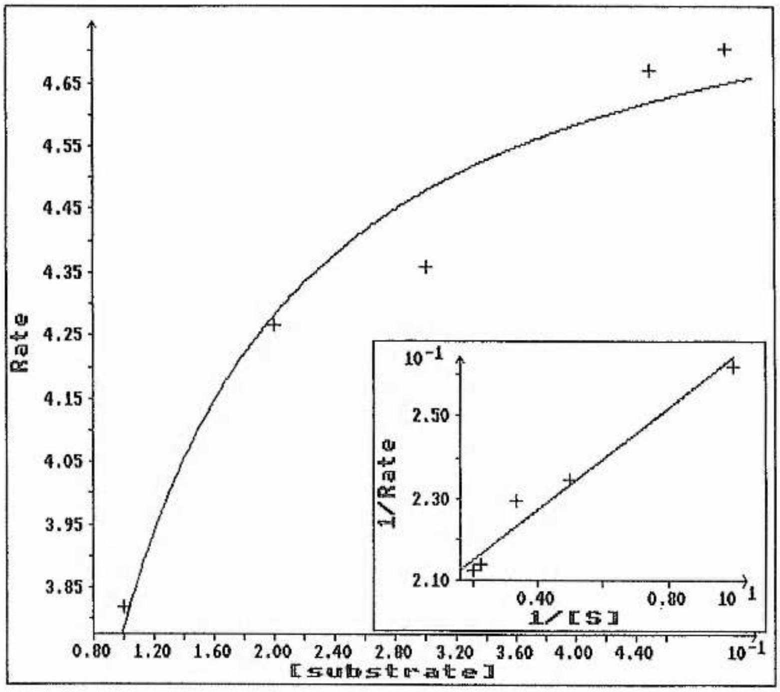


Kinetic Parameters for 0.2 mM Cyclohexyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.64	±0.0985
K_M (μM)	31.8	±5.36

Substrate Analogue. 0.05 mM Naphthyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.82
0.20	4.27
0.30	4.36
0.45	4.67
0.50	4.70

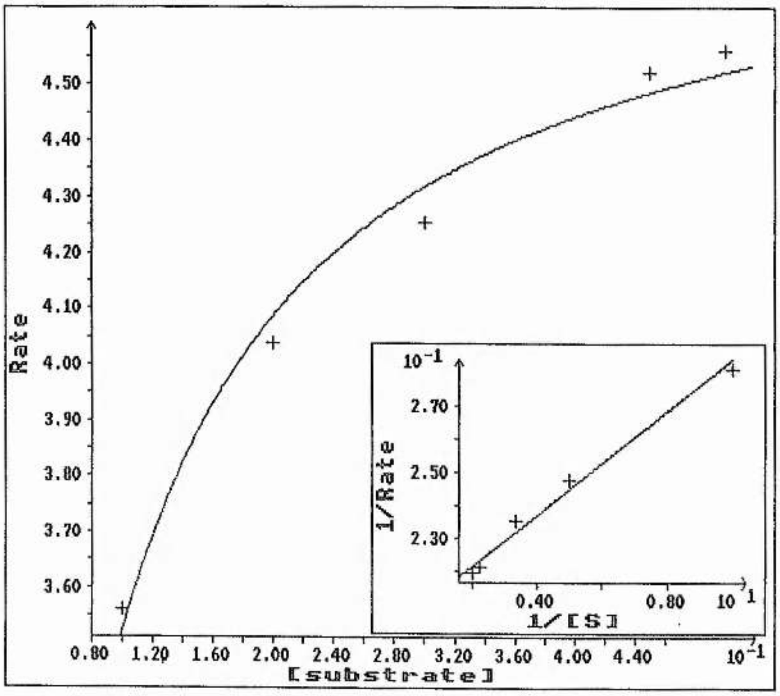


Kinetic Parameters for 0.05 mM Naphthyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.93	±0.0876
K_M (µM)	30.2	±4.42

Substrate Analogue. 0.1 mM Naphthyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.56
0.20	4.04
0.30	4.26
0.45	4.52
0.50	4.56

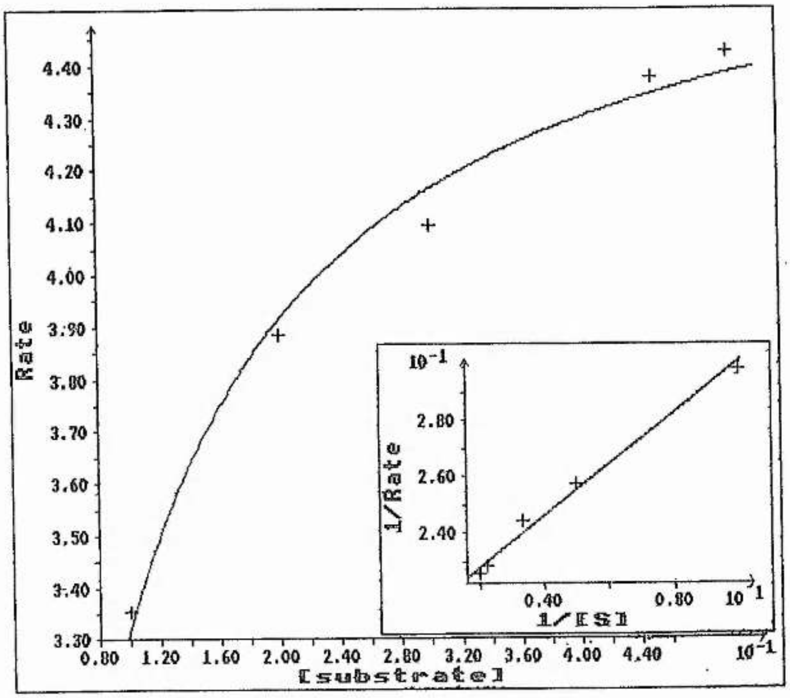


Kinetic Parameters for 0.1 mM Naphthyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.87	±0.0651
K_M (μM)	38.1	±3.54

Substrate Analogue. 0.15 mM Naphthyl Amino Acid

[Substrate] (mM)	Rate (min^{-1})
0.10	3.36
0.20	3.88
0.30	4.09
0.45	4.38
0.50	4.42

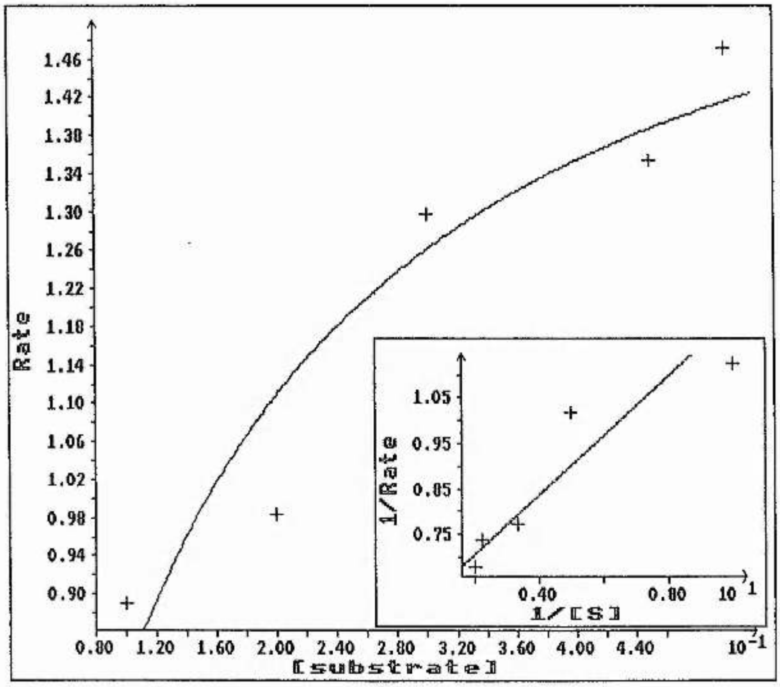


Kinetic Parameters for 0.15 mM Naphthyl Amino Acid

Variable	Value	Error
V_{max} (min^{-1})	4.77	±0.0625
K_M (μM)	43.4	±3.60

Substrate Analogue. 0.05 mM Desaminokynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.891
0.20	0.983
0.30	1.30
0.45	1.35
0.50	1.47

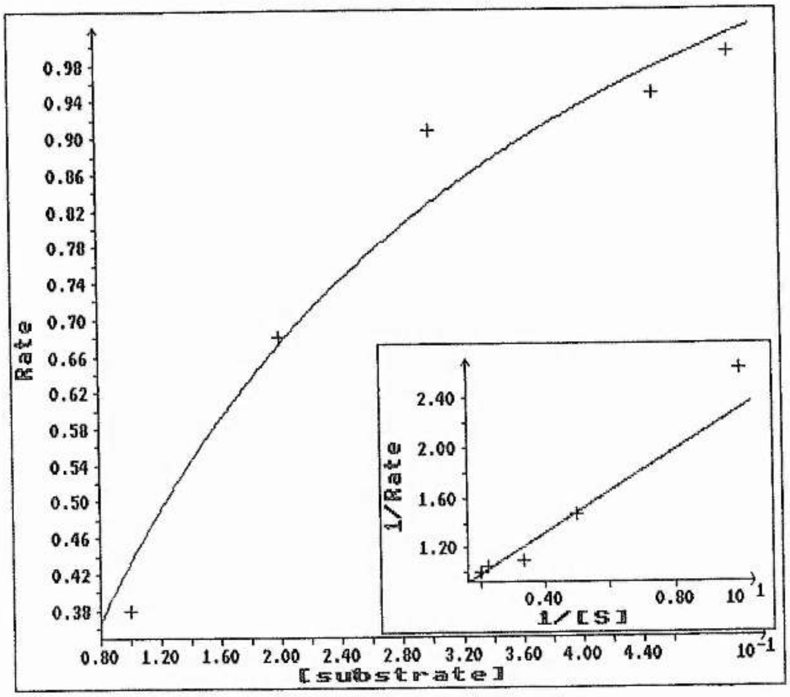


Kinetic Parameters for 0.05 mM Desaminokynurenine

Variable	Value	Error
V_{max} (min^{-1})	1.74	±0.169
K_M (μM)	113	±38.3

Substrate Analogue. 0.2 mM Desaminokynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.380
0.20	0.681
0.30	0.908
0.45	0.947
0.50	0.992

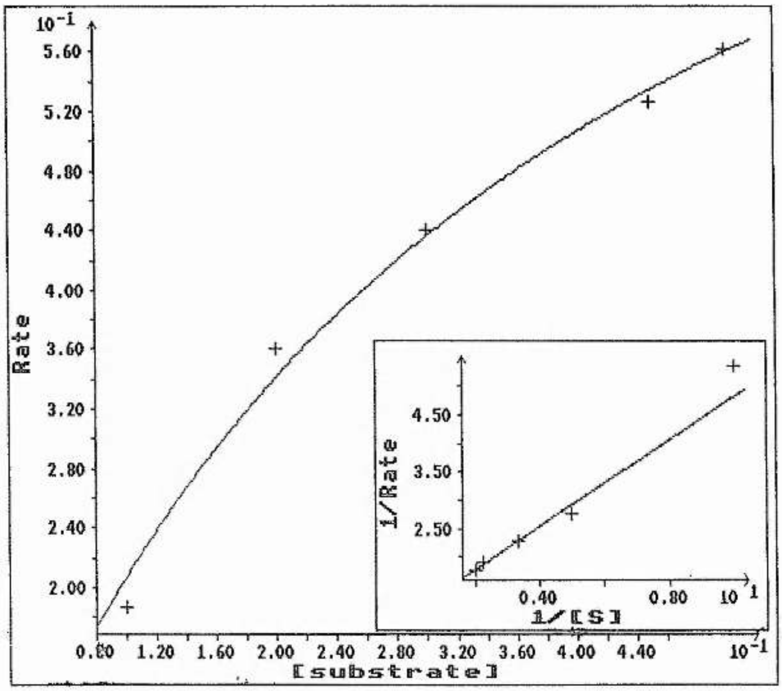


Kinetic Parameters for 0.2 mM Desaminokynurenine

Variable	Value	Error
V_{max} (min^{-1})	1.52	±0.205
K_M (μM)	252	±78.5

Substrate Analogue. 0.3 mM Desaminokynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.187
0.20	0.360
0.30	0.441
0.45	0.526
0.50	0.561

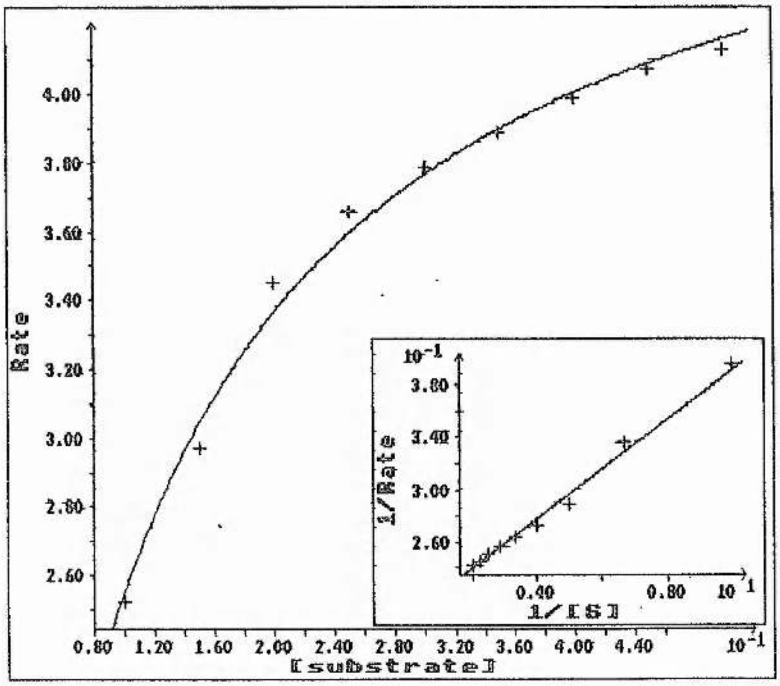


Kinetic Parameters for 0.3 mM Desaminokynurenine

Variable	Value	Error
V_{max} (min^{-1})	0.976	±0.838
K_M (μM)	371	±62.0

Primary Isotope Effect Studies on α-Deuteriated Kynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	2.52
0.15	2.97
0.20	3.45
0.25	3.66
0.30	3.79
0.35	3.89
0.40	3.99
0.45	4.08
0.50	4.13

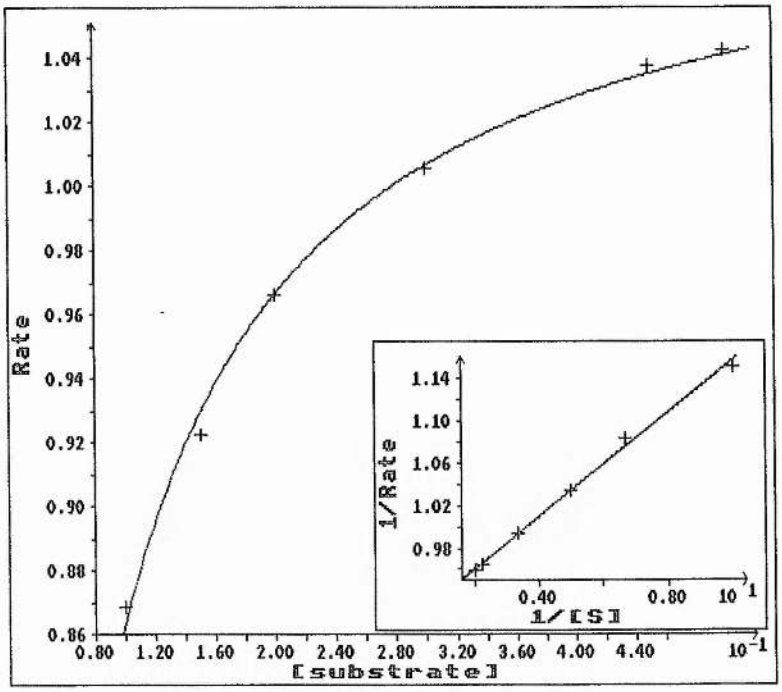


Kinetic Parameters for 0.4 mM α-Deuteriated Kynurenine

Variable	Value	Error
V_{max} (min^{-1})	4.94	±0.0726
K_M (μM)	93.6	±5.32

Solvent Isotope Effect Studies on Kynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.869
0.15	0.923
0.20	0.966
0.30	1.01
0.45	1.04
0.50	1.04

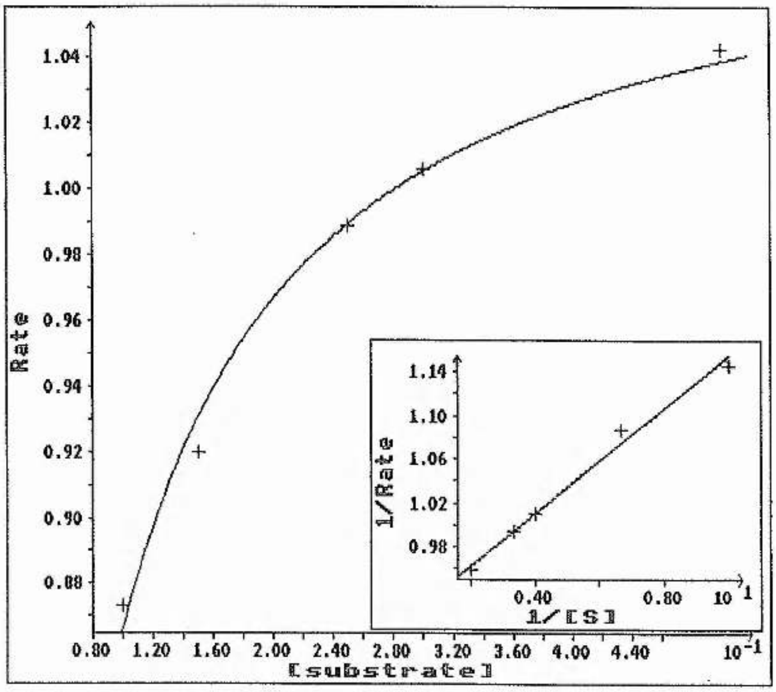


Kinetic Parameters for 0.4 mM Kynurenine

Variable	Value	Error
V_{max} (min^{-1})	1.10	±0.0456
K_M (μM)	27.0	±0.946

Solvent Isotope Effect Studies on Kynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.873
0.15	0.920
0.25	0.989
0.30	1.01
0.50	1.04

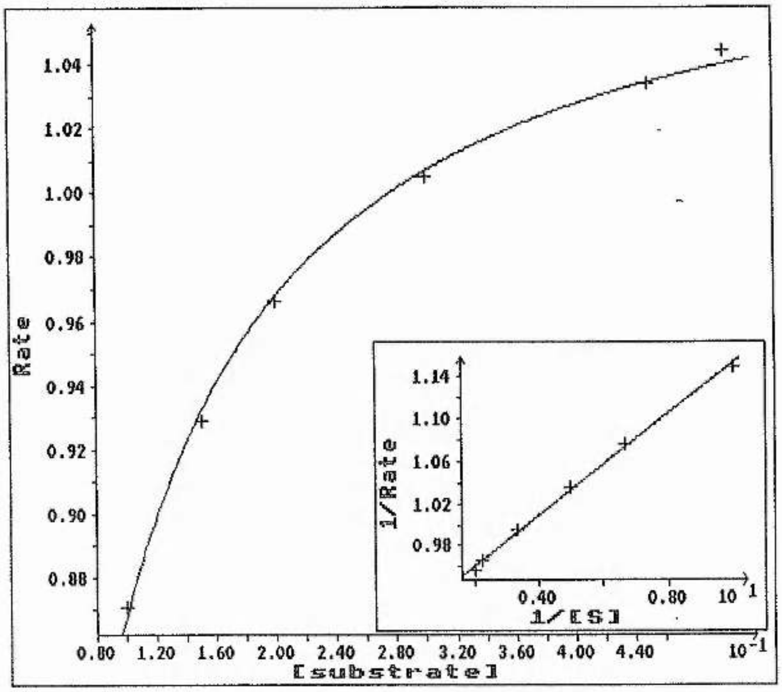


Kinetic Parameters for 0.4 mM Kynurenine

Variable	Value	Error
V_{max} (min^{-1})	1.09	±0.0877
K_M (μM)	26.2	±1.72

Solvent Isotope Effect Studies on Kynurenine

[Substrate] (mM)	Rate (min^{-1})
0.10	0.871
0.15	0.929
0.20	0.966
0.30	1.01
0.45	1.03
0.50	1.04



Kinetic Parameters for 0.4 mM Kynurenine

Variable	Value	Error
V_{max} (min^{-1})	1.09	±0.0323
K_M (μM)	26.2	±0.667