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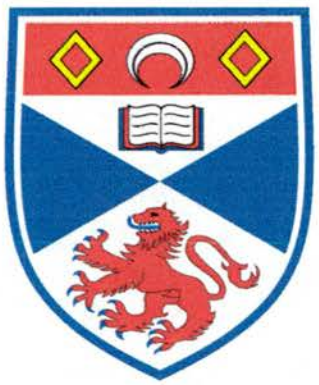

STUDIES ON THE SYNTHESIS OF NOVEL AMINO ACIDS AS INHIBITORS OF KYNURENINASE

A thesis presented for the degree of
Doctor of Philosophy
to the
University of St. Andrews
on 31st May 2002
by Karen Christina O'Shea

Supervisor: Dr. Nigel P. Botting

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ABBREVIATIONS

AD	Alzheimer's disease
AIDS	Acquired Immune Deficiency Syndrome
Boc	(*tert*-butoxycarbonyl)
b.p.	boiling point
Cbz	carbobenzyloxy
CNS	central nervous system
CSF	cerebrospinal fluid
d.e.	diastereomeric excess
DEAD	diethyl azodicarboxylate
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FI	fluorescence intensity
GABA	γ-amino-butyric acid
3-HAO	3-hydroxyanthranilic acid oxygenase
HD	Huntington's disease
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
IC_{50}	concentration of inhibitor to produce 50% inhibition
K_{app}	apparent K_m
KAT	kynurenine Aminotransferase
k_{cat}	enzyme catalytic constant/ turnover number
k_{eq}	equilibrium constant
K_i	enzyme inhibition constant
K_m	Michaelis constant
m.p.	melting point
NAD^+	nicotine adenine dinucleotide (oxidised form)
NADH	nicotine adenine dinucleotide (reduced form)

$NADP^+$	nicotine adenine dinucleotide phosphate (oxidised form)
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
Nu^-	nucleophile
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
PMSF	phenylmethylsulfonyl fluoride
TBDMSCl	*tert*-butyldimethylsilyl chloride
TBDMS	*tert*-butyldimethylsilyl
THF	tetrahydrofuran
tlc	thin layer chromatography
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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STUDIES ON THE SYNTHESIS OF NOVEL AMINO ACIDS AS INHIBITORS OF KYNURENINASE

Kynureninase (EC 3.7.1.3) is a pyridoxal 5'-phosphate (PLP) dependent enzyme on the tryptophan metabolic pathway. The enzyme catalyses the β,γ-hydrolytic cleavage of two substrates, L-kynurenine and 3-hydroxy-L-kynurenine, to give anthranilic acid and 3-hydroxyanthranilic acid, respectively, as well as L-alanine. Quinolinic acid, an important metabolite on this pathway is a known neurotoxin which is implicated in the aetiology of a number of neurodegenerative diseases most particularly AIDS and Huntington's disease. In contrast, a second metabolite kynurenic acid is a neuroprotectant and anticonvulsant. Kynureninase is a potential therapeutic target for modulating the biosyntheses of quinolinic acid and kynurenic acid. Studies have been carried out to prepare inhibitors of kynureninase.

To date inhibition studies on kynureninase have been limited to the use of bacterial kynureninase and rat liver kynureninase. Recently, within this group recombinant human kynureninase has been successfully expressed and isolated in quantities which allow detailed inhibition studies to be conducted. In this study a number of monocyclic and bicyclic substrate analogues were prepared, as racemic mixtures, in order to obtain information on the specificity of the human enzyme and interactions at the active site. 3'-Hydroxydesaminokynurenine was found to be the most potent inhibitor of kynureninase to date. The derivative exhibited K_i values of 5 nM, 20 nM and 25 nM when examined against human, rat liver and bacterial kynureninase. Comparative studies with 3'-methoxydesaminokynurenine showed that the hydroxyl group at the 3-position of the ring was an important recognition element for the human enzyme. A bicyclic 5-hydroxy-1-tetralone derivative was also shown to be less effective as an inhibitor of the enzyme exhibiting a K_i value of 57 μM when tested against recombinant human kynureninase.

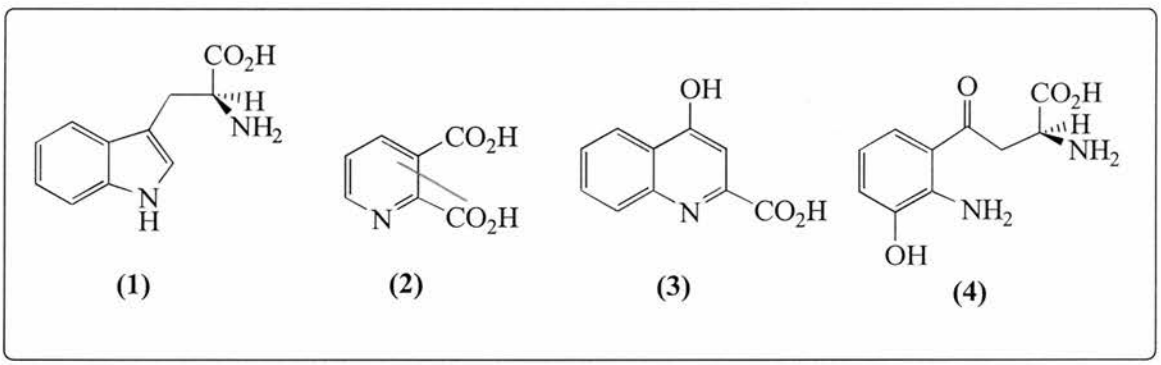
A series of substrate analogues containing a γ-hydroxy group, designed as putative transition state mimics, were also synthesised and their interaction with kynureninase examined. Dihydroxydesaminokynurenine was shown to be a potent competitive inhibitor of kynureninase exhibiting K_i values of 100 nM, 130 nM, and 10 μM when examined against human, rat liver and bacterial kynureninase. Comparative studies with the γ-hydroxy-3'-methoxydesaminokynurenine derivative again showed that methylation at the 3-position of the ring resulted in a decrease in the potency. A bicyclic 1,5-dihydroxytetralone derivative was found to be less potent as an inhibitor of human kynureninase exhibiting a *K*i of 66 μM. A number of synthetic strategies towards 2*S* kynurenines were also examined.

CHAPTER 1

1 INTRODUCTION

1.1 Background

The synthesis of the essential cofactor nicotinamide adenine dinucleotide (NAD) from the amino acid tryptophan **(1)**, involves a series of enzymes and the formation of a number of metabolites collectively known as kynurenines.[1] The kynurenine pathway has been recognised as the main route for tryptophan metabolism since the 1940's with over 95% of dietary tryptophan metabolised to kynurenines.[2,3] Initial studies on the pathway led to the isolation and identification of kynurenine metabolites, with kynurenic acid and quinolinic acid reported in 1904 and 1946, respectively.[4,5] Subsequent interest in the kynurenine pathway centred on its contribution to the synthesis of nicotinamide and its nucleotide conjugates as well as its response to vitamin B_6 deficiency, since a number of enzymes on the pathway depend on pyridoxal 5'-phosphate (PLP) as a cofactor. It is the main pathway affected by vitamin B_6 deficiency.[6-8] However, reports in the late 1970's, on convulsant and other behavioural effects as a result of systemic administration of kynurenines prompted a new focus on the functional role of these metabolites in the central nervous system.[9]



Since that time a number of kynurenines have been cited as having a marked effect on neuronal activity. The most studied of these is quinolinic acid **(2)** which activates selectively the *N*-methyl-D-aspartate (NMDA)-sensitive subpopulation of glutamate receptors.[10] Glutamate receptors in the brain are thought to mediate neuronal damage occurring in a number of neurodegenerative disorders. Neurotoxicity induced by quinolinic acid **(2)** is thought to occur through overstimulation of these receptors.[11]

Quinolinic acid **(2)** is increasingly implicated in the pathogenesis of AIDS related dementia[12] and Huntington's disease,[13] as well as Lyme disease.[14] The mechanisms of neuronal damage by quinolinic acid, in addition to a direct excitoxic effect on neurones, include mytochondrial dysfunction as well as the formation of reactive oxygen species.[15]

Kynurenic acid **(3)** is another neurologically active metabolite and functions as an antagonist of a number of subpopulations of glutamate receptors including those sensitive to NMDA. It behaves as a high affinity antagonist of the strychnine insensitive glycine coagonist site on the NMDA receptor.[16] This property has led to the development of kynurenates as glutamate antagonists for the treatment of some neurodegenerative disorders. Numerous analogues of kynurenic acid have recently been reported with potential therapeutic value as neuroprotectants and anticonvulsants.[17] Brain kynurenic acid **(3)** levels were observed to be high in a number of studies in relation to Huntington's disease,[18] while elevated levels of kynurenic acid **(3)** have also been observed in patients with AIDS,[19] Downs Syndrome[20] and Alzheimer's disease (AD).[21] Since kynurenic acid **(3)** inhibits NMDA receptors involved in cognitive function, increased levels may represent a pathogenic factor resulting in the cognitive deterioration observed in AD patients. The dysfunction of kynurenic acid **(3)** synthesis is thought to be an important factor in neuronal degeneration.

3-Hydroxykynurenine **(4)** is also thought to induce neuronal cell death. Neurological damage by 3-hydroxykynurenine is mediated by the formation of free radicals, resulting in oxidative stress and apoptotic or necrotic cell death.[22] Elevated levels of 3-hydroxykynurenine have been observed in cases of HIV infection[23] and hepatic encephalopathy.[24]

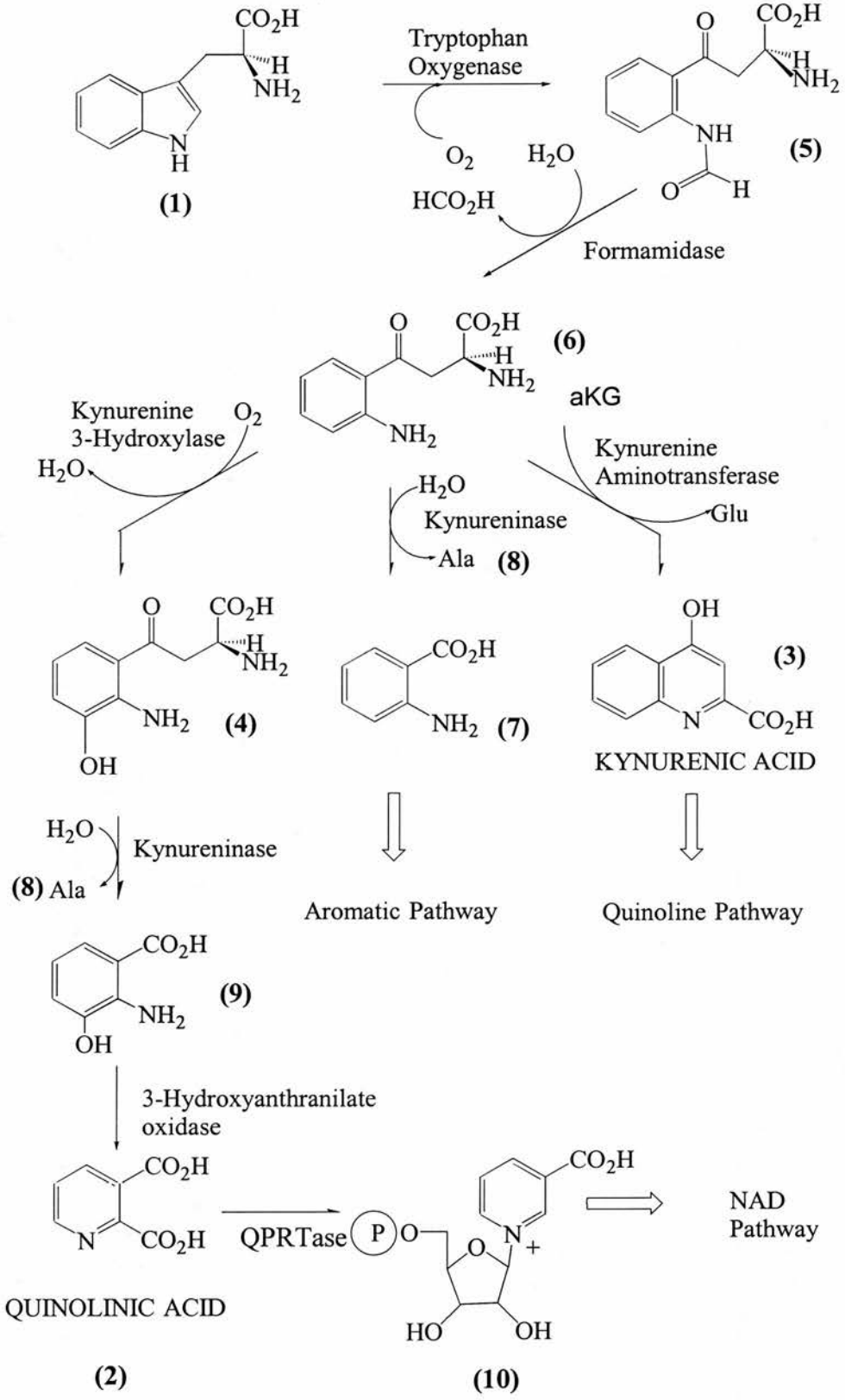
As a number of kynurenine metabolites exhibit significant neurological activity, the synthesis of selective inhibitors of their biosynthesis has become important both in elucidating their physiological role and in the development of potential therapeutic agents in treating neurological diseases.

1.2 The tryptophan metabolic pathway

Interest in tryptophan pharmacology has long concentrated on its role as precursor to the important neurotransmitter serotonin (5-hydroxytryptamine).[25] However, less than 1% of tryptophan is metabolised *via* the serotonin pathway.[3] The kynurenine pathway is not only the main pathway for tryptophan catabolism but is important as a source of the nicotinamide required for synthesis of the coenzymes NAD and nicotinamide adenine dinucleotide phosphate (NADP) which are essential to basic cellular function.

The first step on the kynurenine pathway (Scheme 1.1) is the irreversible oxidative cleavage of the indole ring of tryptophan **(1)** to afford *N*-formylkynurenine **(5)**. This cleavage is catalysed by one of two enzymes; tryptophan 2,3-dioxygenase (EC 1.13.11) or indolamine 2,3-dioxygenase (EC 1.13.11.17), both of which are haem dependent. Rapid hydrolysis of formylkynurenine **(5)** by a formamidase enzyme (EC 3.5.1.9) gives L-kynurenine **(6)**. At least three different enzymes metabolise L-kynurenine **(6)** in mammalian tissue. Kynureninase (EC 3.7.1.3) catalyses the hydrolytic cleavage of L-kynurenine **(6)** to afford anthranilic acid **(7)** and L-alanine **(8)**. Anthranilic acid **(7)** is a precursor in the biosynthesis of aromatic amino acids. Alternatively, transamination of L-kynurenine **(6)** catalysed by kynurenine aminotransferase (KAT, EC 2.6.1.7) results in the formation of kynurenic acid **(3)** and this metabolite is often employed in the biosynthesis of quinolines as well as being an important neurologically active compound. Finally, 3-hydroxykynureninase (EC 1.14.13.9) catalyses the hydroxylation of the benzene ring of L-kynurenine **(6)** to afford 3-hydroxy-L-kynurenine **(4)**. Hydrolytic cleavage of 3-hydroxy-L-kynurenine **(4)** also by kynureninase, results in the formation of 3-hydroxyanthranilic acid **(9)** and alanine **(8)**. 3-Hydroxyanthranilate oxidase then oxidatively cleaves **(9)** and subsequent rearrangement gives the most neurologically important compound, quinolinic acid **(2)**. Finally, quinolinate phosphoribosyl transferase (QPRTase, EC 2.4.1.19) catalyses the conversion of **(2)** to nicotinic acid mononucleotide **(10)**. Further transformations to nicotinamide coenzymes may then occur.

TRYPTOPHAN METABOLIC PATHWAY

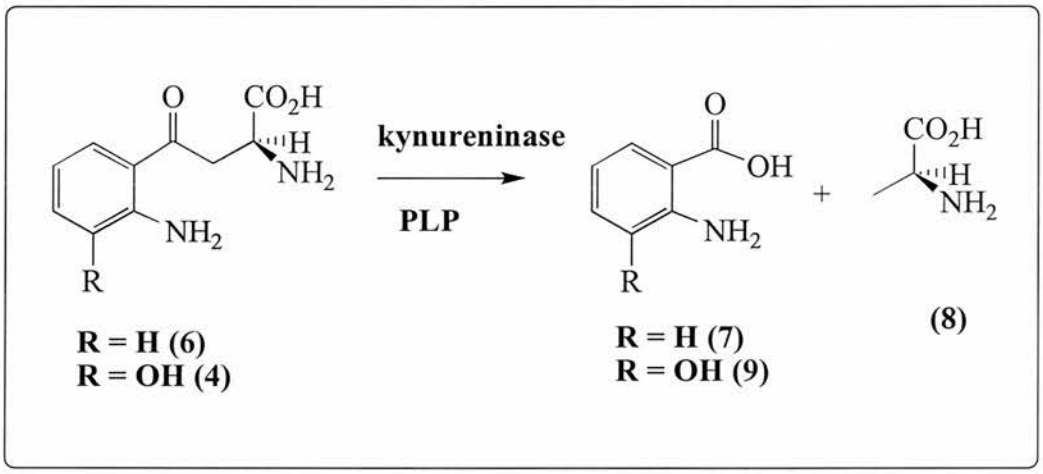


Scheme 1.1

1.3 Kynureninase

1.3.1 Enzyme characterisation

The kynureninase (EC 3.7.1.3) enzyme catalyses a unique reaction, the β,γ-hydrolytic cleavage of aryl-substituted γ-keto-α-amino acids. L-Kynurenine **(6)** and 3-hydroxy-L-kynurenine **(4)** are substrates for the enzyme and are cleaved to afford anthranilic acid **(7)** or 3-hydroxyanthranilic acid **(9)** respectively as well as L-alanine **(8)** (Scheme 1.2).



Scheme 1.2

Kynureninase requires pyridoxal 5'-phosphate as a coenzyme for the reaction. Studies on pyridoxine deficient rats indicated a decrease in the activity of the enzyme in the absence of the cofactor.[26] Kynureninase has been isolated from mammalian sources (human liver,[27] rat liver[26] and porcine liver[28]) as well as fungus (*Neurospora crassa*[29]) and bacteria (*Pseudomonas fluorescens*[30,31] formerly known as *Pseudomonas marginalis*).

The enzyme was purified and crystallised from *Pseudomonas fluorescens* and a partial sequence of kynureninase from this source was also reported.[31,32] More recently it has been fully cloned.[33] The absorption spectrum of kynureninase exhibited maxima at 280, 337 and 430 nm.[31] The absorption at 430 nm was attributed to the coenzyme pyridoxal 5'-phosphate bound through a Schiff's base to a lysine residue on the protein. The bacterial enzyme consists of two subunits with molecular weight of 50,000. Initial reports that one mole of pyridoxal 5'-phosphate was bound per mole of enzyme were later refuted when

kinetic evidence implying that both subunits were catalytically active indicated the presence of two moles of pyridoxal 5'-phosphate, one bound to each of the subunits. [34]

The amino acid sequence of the rat liver kynureninase has been reported and recently the enzyme has been cloned and expressed. [35,36] The mammalian enzyme is a single protein with a dimeric structure containing two identical subunits. The rat enzyme subunit consists of 464 amino acids and the molecular weight of the kynureninase subunit was determined to be 54 kDa.

Alberati-Giani *et al.* recently reported the isolation and expression of a cDNA clone encoding for human kynureninase.[37] The cDNA clone which contained 1651 base pairs encoded for a protein consisting of 456 amino acids with a theoretical mass of 52 kDa. Comparison of the predicted amino acid sequence of the human enzyme with that of the rat enzyme showed a high degree of similarity. Both indicated a lysine residue, Lys276, as the residue responsible for covalently binding the pyridoxal 5'-phosphate coenzyme. The rat and human recombinant kynureninase enzymes hydrolysed 3-hydroxy-L-kynurenine **(4)** faster than L-kynurenine **(6)**. Expression of human recombinant kynureninase in human embryonic kidney (HEK-293) cells and determination of kinetic parameters indicated that catalytic efficiency of the human enzyme was two hundred times higher for 3-hydroxy-L-kynurenine **(4)** than for L-kynurenine **(6)**. The Michaelis constants determined were K_m values of 13.2 μM and 671 μM for 3-hydroxy-L-kynurenine **(4)** and L-kynurenine **(6)**, respectively. Recently, a more successful method for the expression and isolation of recombinant human kynureninase, in significant quantities, was developed within this group using a baculovirus/insect cell expression system (see Section 3.2).[38] This expression system enabled the first detailed kinetic studies to be conducted on potential inhibitors of the human enzyme. A purification protocol was also developed and a detailed characterisation of recombinant human kynureninase was possible (see Section 3.3).[39] As a homodimer, the monomeric molecular weight was determined to be 52.4 kDa and was >99.9% of the predicted molecular mass. The enzyme also displayed a pH optimum of 8.25. In contrast to the study by Alberati-Giani *et al.*,[37] this study revealed that the human enzyme was specific for 3-hydroxykynurenine, exhibiting a K_m of 3.0 ± 0.1 μM. No activity was observed with L-kynurenine and it was observed to be an inhibitor of the human enzyme, exhibiting competitive inhibition at low

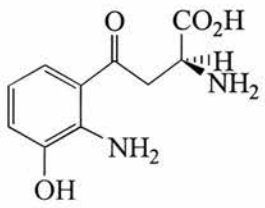
substrate concentrations and non-competitive inhibition at high concentrations. This is in contrast to the bacterial enzyme from *Pseudomonas fluorescens*, where L-kynurenine **(6)** was hydrolysed five times faster then 3-hydroxy-L-kynurenine **(4)**. K_m values of 35 μM and 200 μM for **(6)** and **(4)** were obtained, respectively. The enzyme displays differences in hydrolysis rates between L-kynurenine **(6)** and 3-hydroxy-L-kynurenine **(4)** which appear to be dependent on the source of the enzyme.

1.3.2 Substrate specificity

To elucidate the mechanism of action and structure of kynureninase the susceptibility of several analogues of L-kynurenine **(6)** to β,γ-hydrolytic cleavage by the bacterial enzyme was studied. A report by Tanizawa and Soda showed that introduction of a hydroxyl group, **(4)** or **(11)**, or a methyl group, **(12)** into the aromatic ring decreased reactivity towards kynureninase while fluorine substituted analogues **(13)** and **(14)** were quite susceptible to hydrolysis (Table 1.1).[40]

A qualitative analysis of the relative rates of hydrolysis of the analogues suggested inductive and resonance effects by *ortho*-substituents and an inductive effect by *meta*-substituents.[41] The reactivities of *N*-formyl-L-kynurenine **(5)** and desamino-L-kynurenine **(15)** were low, suggesting a key role for the *ortho*-amino group. The absence of the *ortho*-amino group did not significantly affect the affinity of desamino-L-kynurenine **(15)** for the enzyme, demonstrated by a low K_m, suggesting that the amino group enhances the reactivity of substrate to hydrolysis but does not reduce binding to the enzyme. The γ-carbonyl group is not essential for reactivity since the dihydro-L-kynurenine analogue, 2-amino-4-hydroxy-4-phenylbutyric acid **(16)** analogue, was hydrolysed to L-alanine **(8)** and *o*-aminobenzaldehyde **(17)**.

Relative reactivities of kynurenine analogues.[a 40]



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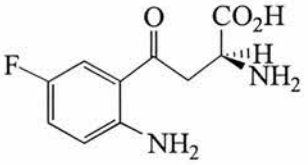

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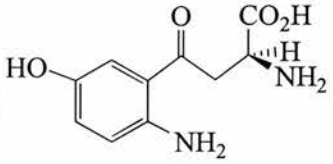

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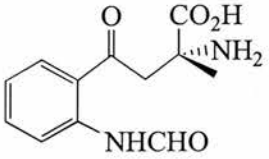

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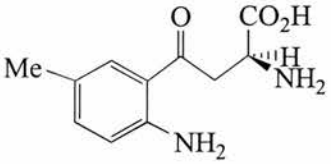

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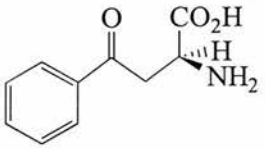

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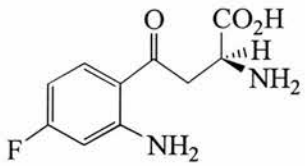

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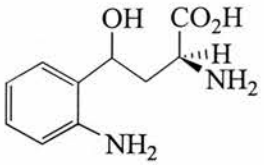

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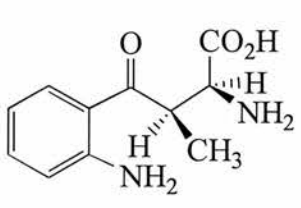
[a] Relative reactivities are expressed as amounts of alanine formed when they were examined with kynureninase from *Pseudomonas fluorescens*.[40]

Table 1.1

The diastereomeric composition of the dihydro-L-kynurenine **(16)** analogue reported by Tanizawa and Soda was not known. However, Phillips and Dua subsequently reported the synthesis and chiral resolution of the compound. [42] The reaction of the two dihydro-L-kynurenine diastereomers with kynureninase was then examined. The (4*R*,2*S*)-dihydrokynurenine **(16a)** was hydrolysed while the (4*S*,2*S*)-dihydrokynurenine **(16b)** showed no significant reactivity in the presence of kynureninase. Both diastereomers were also competitive inhibitors of the enzyme, the 4*S* isomer being the more potent exhibited a K_i of 0.3 μM while the 4*R* exhibited a K_i of 1.3 μM. These results supported a postulated general base mechanism for kynureninase which involves formation of a *gem*-diolate intermediate (see Section 1.3.4).

Recently, examples of β-substituted kynurenines which behave both as substrates and inhibitors of kynureninase have been reported.[43] The diastereomers of β-methyl-L-kynurenine, (2*S*,3*S*)-*erythro*-β-methylkynurenine **(18)** and (2*S*,3*R*)-*threo*-β-methylkynurenine **(19)** were synthesised by ozonolysis from the respective parent β-methyl-L-trytophan **(20)** which had been resolved enzymatically. Reaction of the β-methyl-L-kynurenines with kynureninase showed that (2*S*,3*S*)-*erythro*-β-methylkynurenine **(18)** was a slow substrate for the enzyme. Kinetic analysis indicated a thousand-fold decrease in catalytic efficiency of the enzyme in the presence of the *erythro* β-methyl group. The (2*S*,3*R*)-*threo*-β-methylkynurenine **(19)** showed no reaction with enzyme when analysed by UV, however, some reaction was observed using a fluorescence assay. The reaction of the β-methyl-L-kynurenines with kynureninase is highly diasterospecific for the *erythro* analogue which reacts 390-fold faster than the *threo*.



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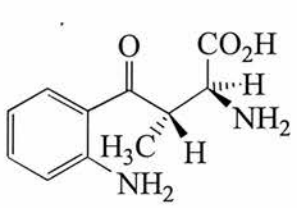

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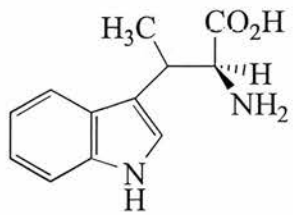

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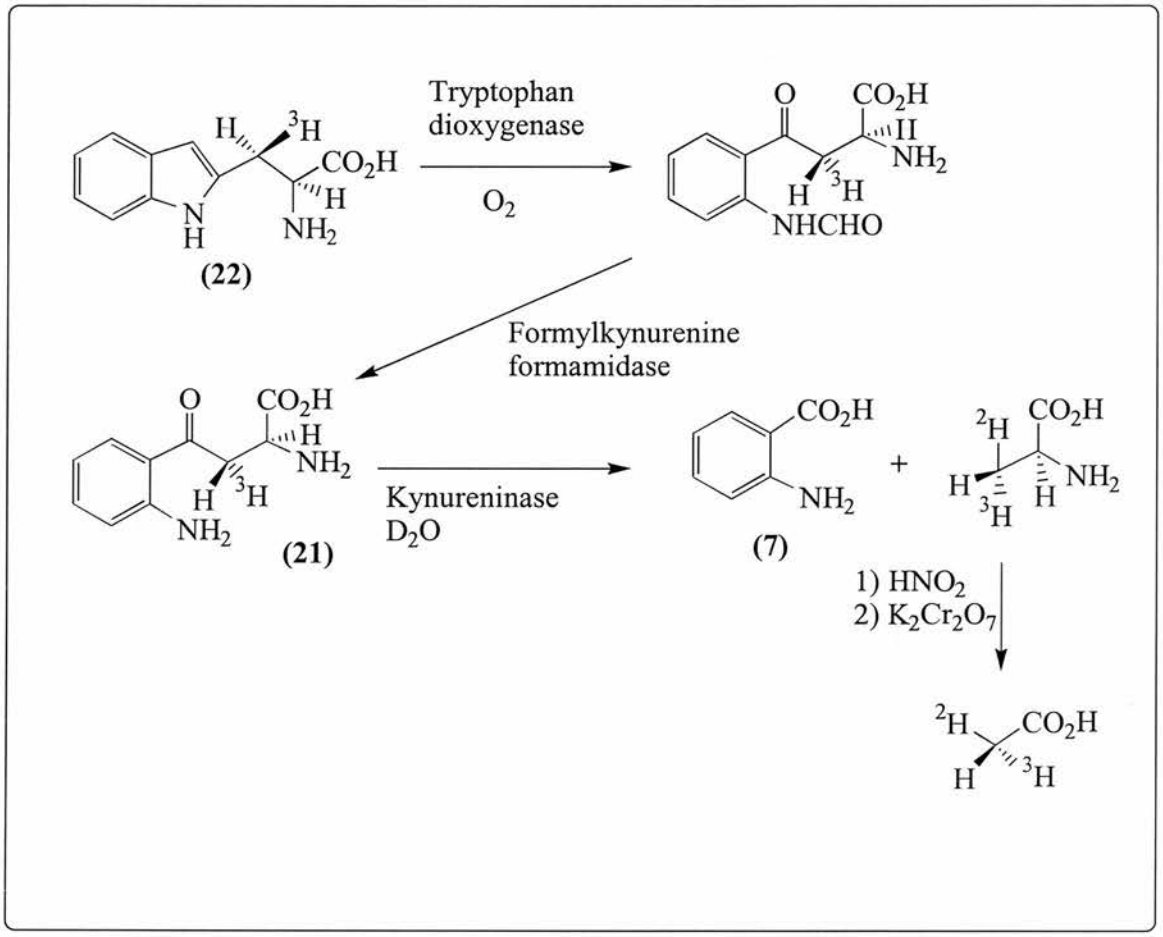
1.3.3 Stereochemistry of the kynureninase reaction

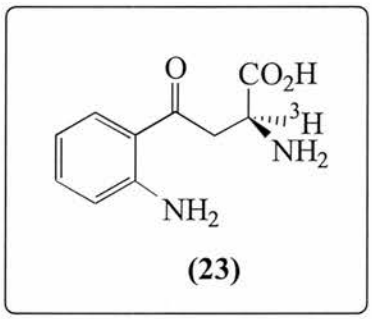
The steric course for the replacement of the anthranilyl group by hydrogen in the kynureninase reaction was determined by Soda and Floss (Scheme 1.3). [44]



Scheme 1.3

S-[2-^{3}H]-Kynurenine **(21)** was converted into alanine enzymatically in the presence of D_2O to determine the stereochemical course of the kynureninase reaction. *S*-[2-^{3}H]-Kynurenine, tritiated stereospecifically at the β-carbon, was generated *in situ* from (2*S*,3*S*)-[3-^{3}H]-tryptophan **(22)** under conditions which prevented enolisation of the isotopically labelled chiral centre. The labelled alanine product was subsequently converted to acetic acid, by diazotisation and dichromate oxidation, for configurational analysis of the methyl group, to determine the steric course of the replacement. The reaction was shown to proceed with retention of configuration with the proton adding to the same side from which the anthranilate group leaves.

Soda and Floss also reported that when kynurenine was tritiated at the α-carbon, *S*-[2-^{3}H]-kynurenine **(23)** was converted into alanine and the tritium was distributed between the α- and β-carbons of alanine which suggested the α-H of kynurenine **(6)** was recycled. This finding together with retention of configuration supported a single base mechanism for the reaction.



(23)

1.3.4 Mechanism of action

The mechanism for the kynureninase reaction has been the source of much speculation. However, recently, Koushik *et al.* conducted a series of detailed studies on the kynureninase reaction which together with evidence from other groups supported the mechanism given in Scheme 1.4.[45,46] Effects of pH and isotopic substitution on steady state and presteady state kinetics, as well as rapid-scanning stopped-flow spectrophotometry of the reaction catalysed by bacterial kynureninase from *Pseudomonas fluorescens*, afforded detailed evidence for the proposed mechanism (Scheme 1.4).[45,46]

In common with other PLP dependent enzymes the mechanism involves initial binding of the pyridoxal 5'-phosphate coenzyme to the apoenzyme *via* the ε-amino group of a lysine residue to form an internal aldimine. L-Kynurenine **(6)** then binds to the enzyme and a transaldimination reaction takes place with L-kynurenine **(6)** displacing the lysine residue forming a Schiff's base with the coenzyme **(24)**. The C_α-H of the Schiff's base is then cleaved to afford the L-kynurenine quinonoid intermediate **(25)**. Rapid scanning stopped-flow spectrophotometry indicated rapid formation of this intermediate which absorbed at 494 nm. The pH dependence of k_{cat}/K_m obtained for both L-kynurenine and 3-hydroxykynurenine fitted to only a single pK_a in the acidic limb indicating that a single

base is responsible for deprotonation and reprotonation in the reaction mechanism. This was also supported by the observation by Palcic *et al.* of recycling of the α-H back into the C-α position of the alanine product.[44] It has been postulated that the orientation of the C_α-H bond perpendicular to the π-plane of the substrate-pyridoxal 5'-phosphate favours cleavage of this bond because of maximal overlap between the C_α-H bond and the extended π-system of the cofactor according to the Dunathan postulate.[47]

Subsequent protonation at C-4' of the PLP cofactor results in formation of the ketimine intermediate **(26)** with concomitant regeneration of the free base. Rapid chemical quenching techniques confirmed the formation of a large amount of pyridoxamine 5'-phosphate, PMP, in a time frame comparable to the decay of the quinonoid absorption at 494 nm. Isotopic substitution studies also showed there was no observed k_{cat}/K_m isotope effect when α-[^{2}H]-L-kynurenine was reacted with the enzyme at various pH values, from 5.86 to 8.60, in both H_2O and D_2O. This indicated that all of the steps up to formation of the ketimine intermediate were in fast equilibrium.

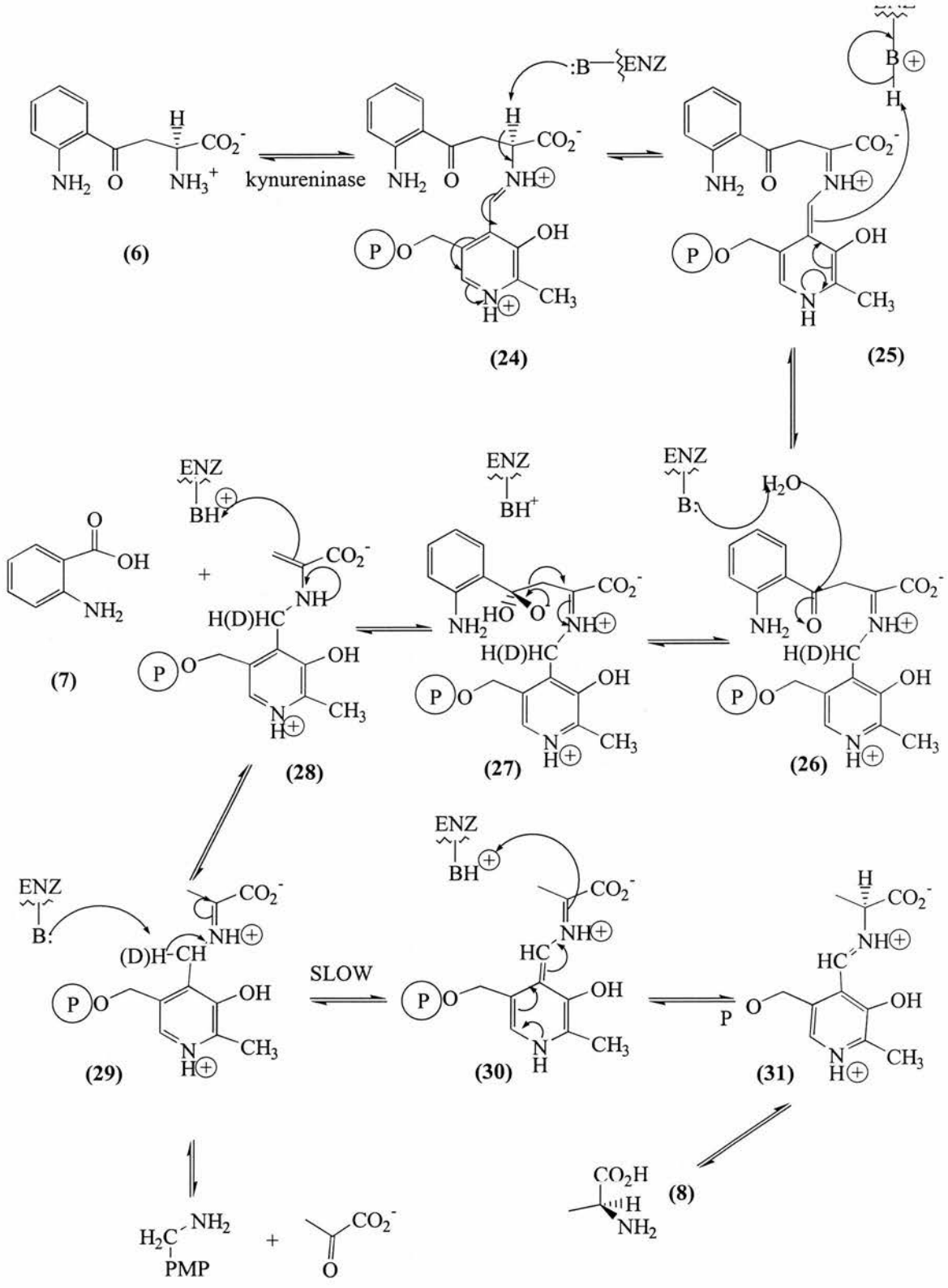
In the mechanism proposed by Koushik *et al.* an enzyme bound water molecule is attacked by the enzymic base generating a hydroxide anion. Subsequent attack by the anion on the γ-carbonyl results in formation of a *gem*-diolate intermediate **(27)**. Cleavage of the β,γ C-C bond then occurs with release of anthranilate **(7)** and the formation of an aminoacrylate intermediate **(28)**. The nature of the attacking nucleophile at the γ-carbonyl is however still uncertain and direct attack by an enzymic nucleophile is also possible. Both postulates are discussed below. Elimination of anthranilate was shown by rapid quench techniques to be fast (54 s^{-1}) and irreversible. Reprotonation at the β carbon of the aminoacrylate then occurs to give the pyruvate ketimine **(29)**, a Schiff's base between the PMP and alanine. From the studies by Palcic *et al.* protonation of the β carbon is stereospecific and occurs with retention of configuration. [44]

The rate determining step of the kynureninase reaction is C-4' deprotonation of the pyruvate pyridoxamine 5'-phosphate ketimine intermediate **(29)** to form the alanine quinonoid intermediate **(30)**. This was supported by isotopic substitution studies in which a large solvent isotope effect was observed with k_{cat} = 6.56 when the reaction was carried out in D_2O. These findings, together with neither a normal solvent isotope effect nor a

substrate kinetic isotope effect on k_{cat}/K_m, showed that the rate determining step involved proton transfer and that this step occurs after the first irreversible step in the reaction. The proton inventory showed that only a single proton is transferred in the rate determining step. The observation of a large solvent isotope effect was supported by isotopic studies conducted within this group in which solvent isotope effects were observed, $^{D}V = 4.4$ and $^{D}(V/K) = 4.6$. [48,49] The formation of the alanine quinonoid **(30)** was also supported by observation of an absorption at 500 nm using rapid-scanning stopped-flow spectrophotometry.

Reprotonation of the quinonoid **(30)** gives rise to the L-alanine aldimine **(31)**. This also explains the observed internal transfer of label from the C-α of α-[^{3}H]-kynurenine to the alanine product as reported by Palcic *et al.*, as the original α-proton would be partially transferred to C-4' then returned to C-α. However, the distribution of the labelled tritium isotope between the α and β carbons of the alanine also reported by Palcic *et al.* remains unexplained by the proposed mechanism. L-Alanine is then released in the final step of the reaction. Reaction of L-alanine with kynureninase gives rise to both the external aldimine **(31)** and alanine quinonoid **(30)**, as observed by rapid-scan stopped-flow spectrophotometry with absorptions at 420 nm and 500 nm, respectively.

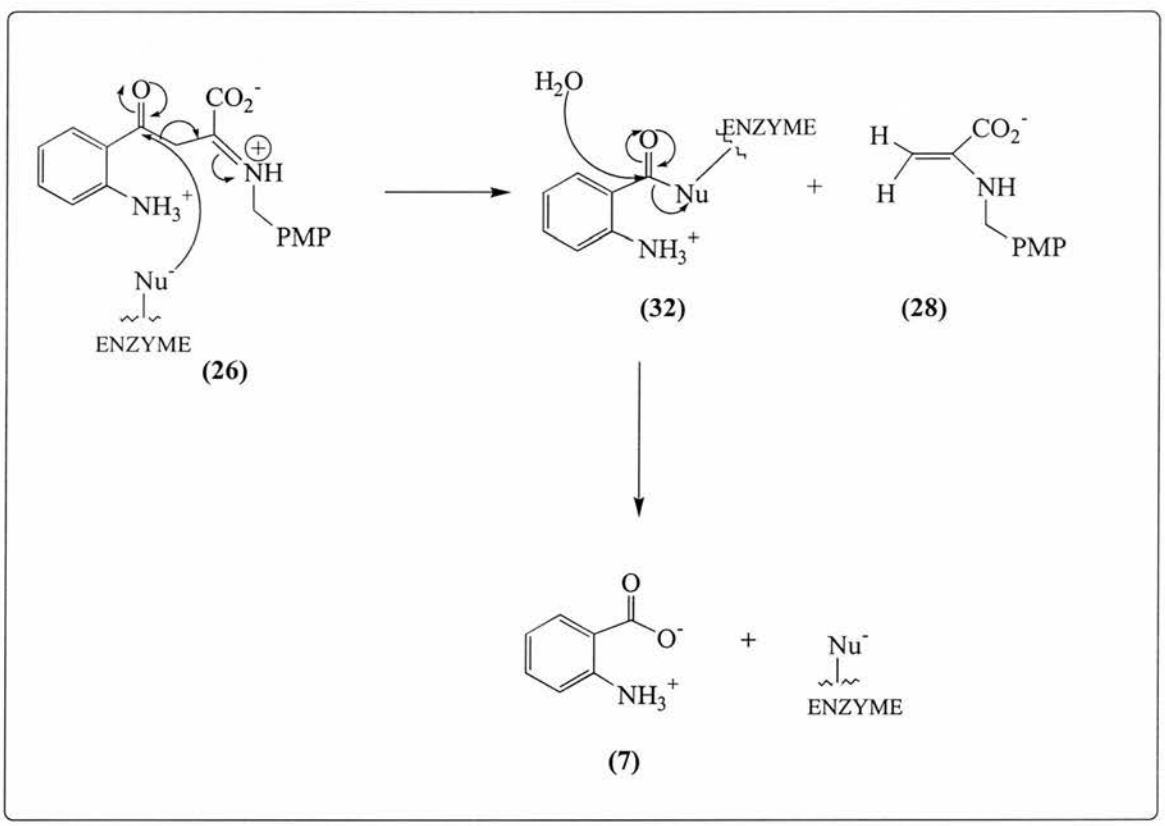
Although the nature of the base which mediates the reaction is not known the ε-amino group of a lysine residue has been proposed.[44] However, a study by Kishore also identified an active site carboxylate group, alkylated when investigating mechanism based inhibitors of kynureninase, as having a possible role in α-hydrogen abstraction.[50]



Scheme 1.4

The identity of the nucleophile which attacks the γ-carbonyl during hydrolysis has not been established. The reaction is thought to be mediated either by an enzymic nucleophile or by a water molecule.

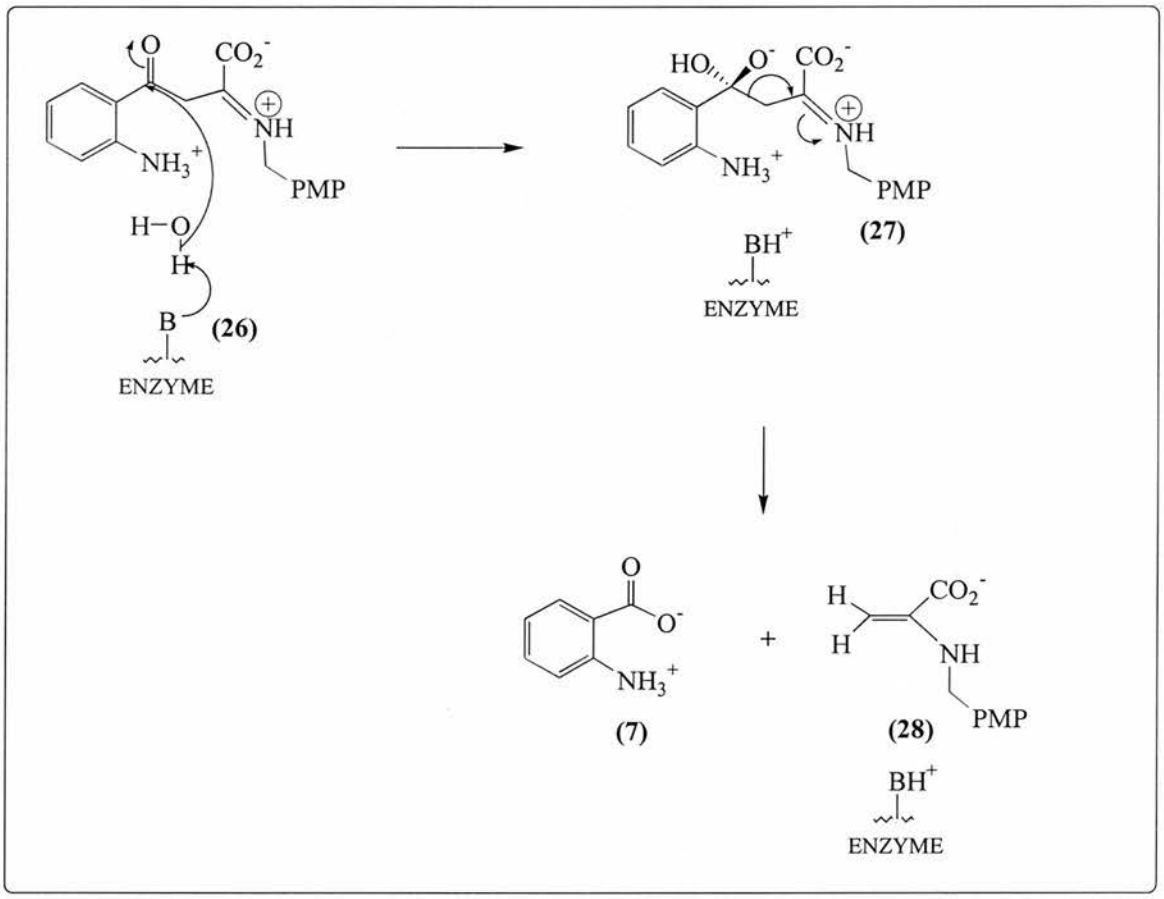
The first postulate involving reaction mediated by an enzymic nucleophile would involve two steps. An initial attack by the nucleophile on the γ-carbonyl of the ketimine **(26)** (Scheme 1.5) resulting in β,γ-cleavage with attendant formation of an acyl-enzyme intermediate **(32)** and the α-aminoacrylate derivative **(28)**. Then a subsequent hydrolysis step is required to release the anthranilate product **(7)** and the enzymic nucleophile. However, there is no evidence for an enzymic nucleophile mediated hydrolysis as yet. Indeed, kinetic studies by Phillips and Dua offer evidence for the operation of a bound water mediated mechanism.



Scheme 1.5

The bound water mediated mechanism requires a water molecule be bound to the enzyme within the active site in a position which facilitates direct attack on the γ-carbonyl of the ketimine **(26)**. A catalytic base in the active site removes a proton from the water

generating a hydroxyl anion. Subsequent attack by the anion on the γ-carbonyl results in the formation of a tetrahedral intermediate **(27)**. The breakdown of this species results in the formation of the α-aminoacrylate intermediate **(28)** and release of the anthranilate product **(7)**, (Scheme 1.6). Evidence supporting this mechanism was demonstrated when the diastereomers of dihydro-L-kynurenine, compounds **(16a,16b)**, were found to be potent inhibitors of kynureninase.[42] This suggested these compounds mimicked the *gem*-diolate intermediate **(27)** and functioned as transition state analogue inhibitors since the tetrahedral intermediate can be used as a model for transition state structure. Stereochemical studies on these analogues also supported the bound water mediated mechanism.

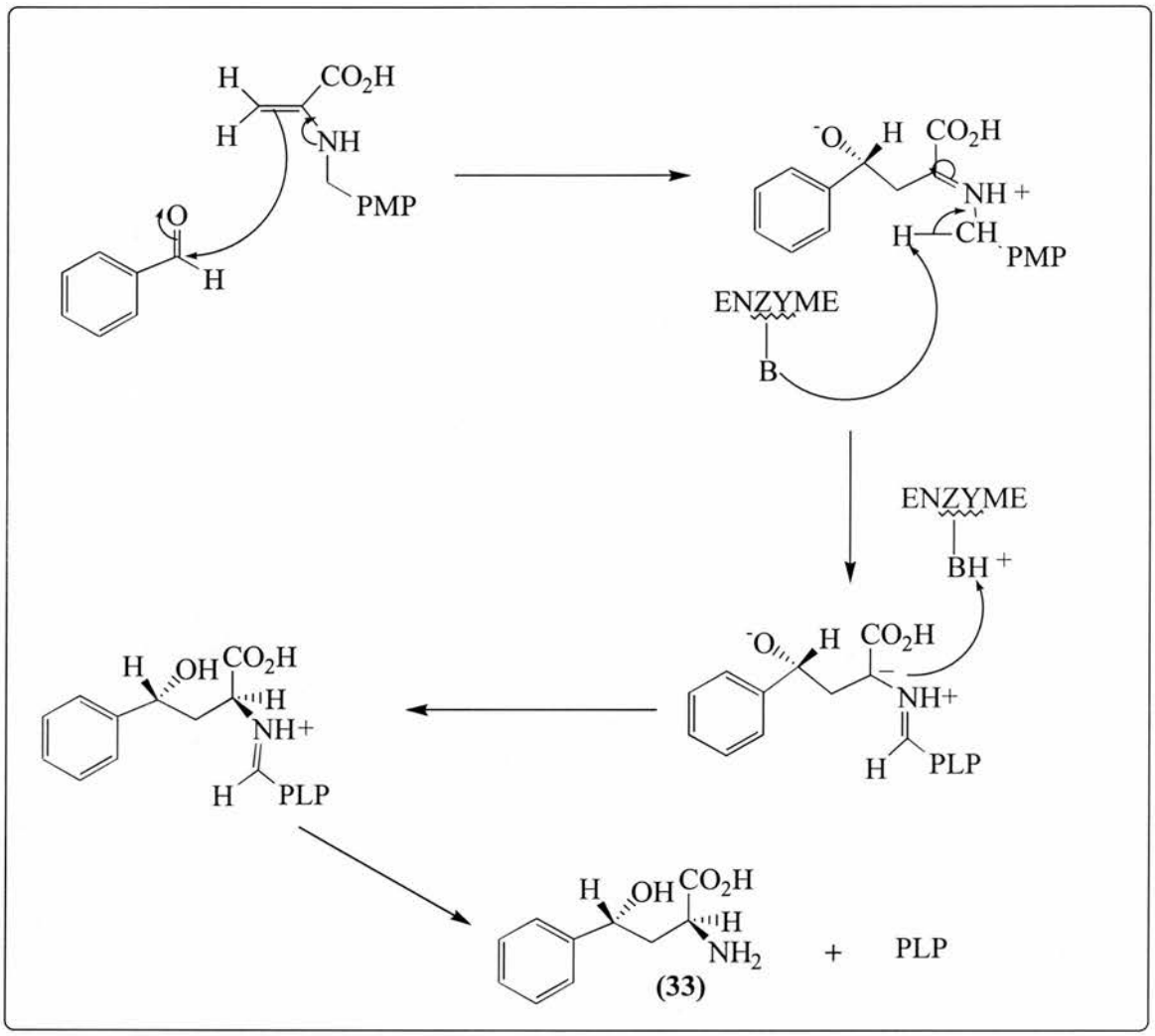


Scheme 1.6

The formation of an α-aminoacrylate intermediate during the kynureninase reaction was supported by a number of trapping experiments reported by Bild and Morris.[51] The γ-hydroxy-α-L-amino acid, 2-amino-4-hydroxy-4-phenylbutyric acid **(33)** was obtained

from the enzymatic hydrolysis of L-kynurenine **(6)** in the presence of benzaldehyde (Scheme 1.7). The reaction was later reported to be stereospecific, forming the (4*R*,2*S*) isomer. Examination of several alkyl and aryl aldehydes confirmed the reaction as specific for aromatic aldehydes which suggests that they bind to the site usually occupied by the aromatic ring of the substrate or the anthranilate product. Radioactive labelling experiments established that the L-alanine moiety originated from L-kynurenine **(6)**.

The trapping of the α-aminoacrylate intermediate also indicates that its lifetime is long enough to allow the product anthranilate to depart and the aldehyde to bind and undergo reaction. The use of L-alanine **(8)** instead of L-kynurenine **(6)** as a precursor to the aminoacrylate intermediate was examined. However, while reaction did occur it was catalytic only at 1% the rate at which L-kynurenine **(6)** reacted. The enzyme was also shown to be inactivated by saturating amounts of benzaldehyde.



Scheme 1.7

1.3.5 Kynureninase in organic synthesis

The enzymatic synthesis of 2-amino-4-hydroxy-4-phenylbutyric acid **(33)** from the hydrolytic cleavage of L-kynurenine **(6)** in the presence of benzaldehyde prompted a study by Ida *et al.* on the use of kynureninase as a synthetic tool in the synthesis of γ-hydroxy-α-L-amino acids. [52]

Several aromatic and heteroaromatic aldehydes (Table 1.2) underwent a transaldol reaction to afford γ-hydroxy-α-L-amino acids in low to moderate yields. The reaction produced mixtures of diastereomers but was stereoselective for the formation of the (4*R*, 2*S*) isomer, exhibiting a diastereomeric excess (d.e.) of >90% for most of the substrates

examined. All of the substrates which reacted contained an sp^2 or an sp hybridised carbon at the α-position. A number of aromatic aldehydes however did not undergo reaction. This was thought to be due to the presence of sp^3 hybridised carbon atoms adjacent to the aldehyde which hindered their ability to bind at the active site. However a number of aldehydes with sp^2 hybridised carbons at the α-position were also unsuccessful these included *p*-dimethylaminobenzaldehyde, 1-methylindole-3-carboxaldehyde and cinnamic aldehyde.

All of the reactions were carried out with the aldehyde present in excess. A study by Bild and Morris showed that kynureninase was inactivated by excess amounts of benzaldehyde.[51] Therefore the synthetic feasibility of the enzyme catalysed reaction may also be limited by the apparent inactivation of the enzyme by large amounts of aldehyde. This may also explain the low yields obtained in the reactions studied.

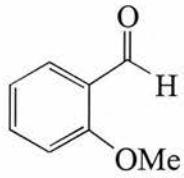
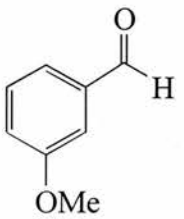
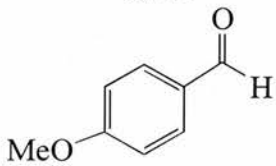
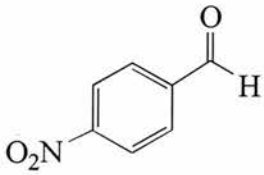
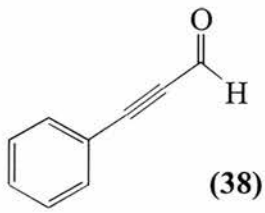
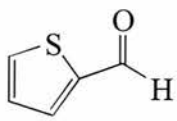
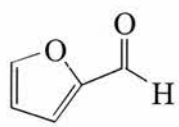
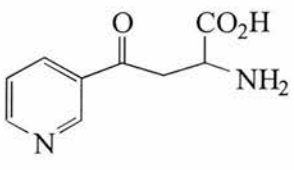
	Diastereomeric ratio R / S	% yield
 (34)	96 : 4	46
 (35)	96 : 4	69
 (36)	96 : 4	50
 (37)	88 : 12	52
 (38)	63 : 37	20
 (39)	95: 5	46
 (40)	96 : 4	55

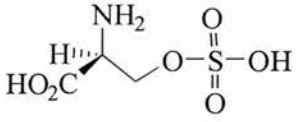
Table 1.2

1.3.6 Inhibition of kynureninase

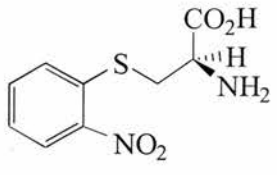
Athough the first inhibitor of kynureninase, nicotinylalanine **(41)**, was reported in the 1960's, studies on the inhibition began in earnest in the 1980's to probe the mechanism in more detail.[53] Kynureninase is among a small group of pyridoxal 5'-phosphate dependent enzymes, together with L-aspartate β-decarboxylase and L-selenocysteine β-lyase, which are unique in that they catalyse electrophilic displacement reactions at their β-carbon. The β-substituted amino acid β-chloroalanine was observed to be an inhibitor of L-aspartate β-decarboxylase which prompted the examination of β-substituted amino acids as possible mechanism based inhibitors of kynureninase.[50] A number of compounds; β-chloroalanine, L-serine-*O*-sulphate **(42)** and *O*-acetyl-L-serine, were found to be inhibitors of the enzyme. The most potent inhibitor, however, was *S*-(*ortho*-nitrophenyl)-L-cysteine **(43)** exhibiting a K_i of 0.1 mM. This was thought to be due to strong interactions between the aromatic moiety of the aryl amino acid and the active site of kynureninase. A β-elimination reaction catalysed by the enzyme results in formation of an aminoacrylate–pyridoxal 5'- phosphate complex **(44)** (Scheme 1.8). It was suggested that subsequent attack on the β-carbon of the aminoacrylate intermediate by an enzymic nucleophile results in formation of a covalent bond inactivating the enzyme.



(41)



(42)



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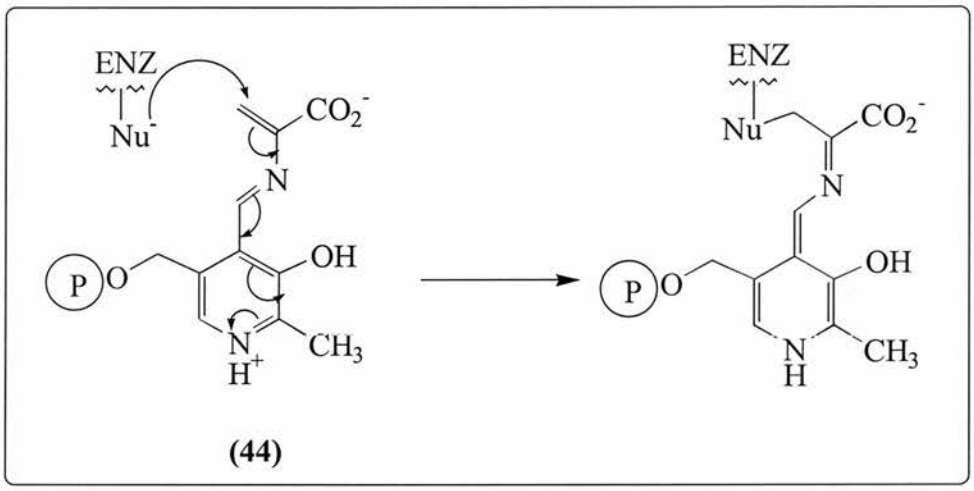
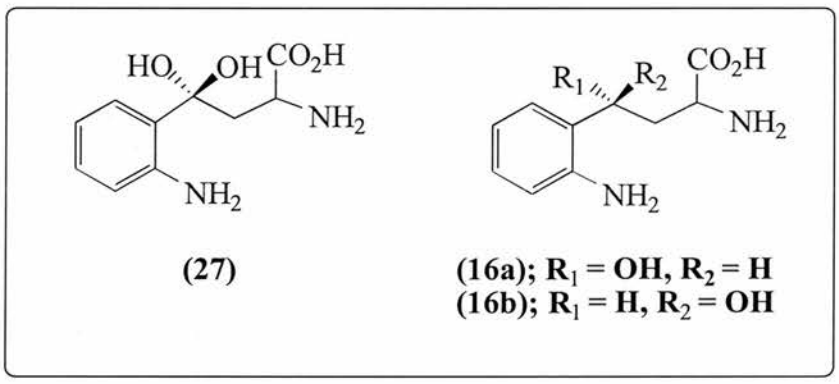


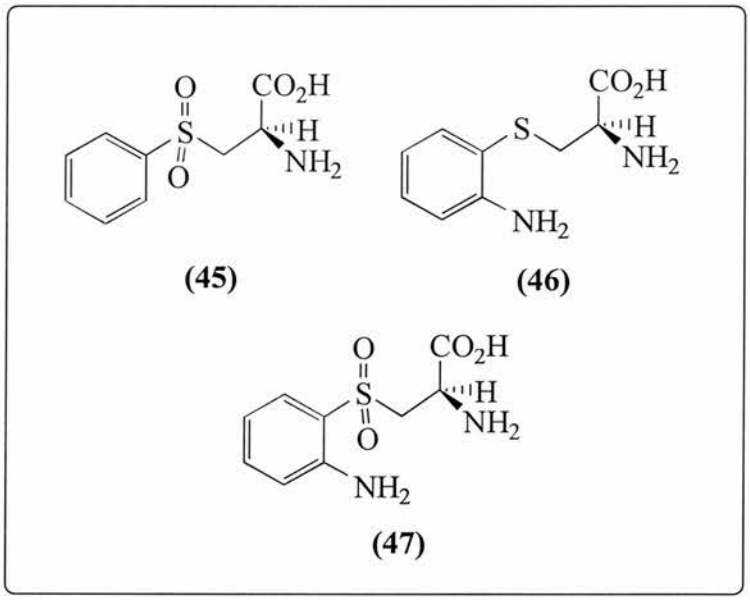
Scheme 1.8

The postulated bound water mediated mechanism for hydrolytic cleavage in the kynureninase reaction gives rise to a tetrahedral intermediate **(27)**, which can be used as a model for the transition state. Several groups have used this tetrahedral intermediate to design and synthesise analogues as selective inhibitors of kynureninase.

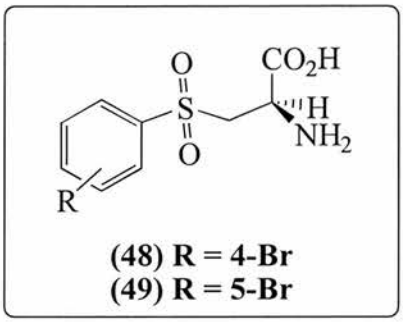


The (4*R*,2*S*) **(16a)** and (4*S*,2*S*) **(16b)** dihydrokynurenines reported by Phillips and Dua were observed to be competitive inhibitors of the enzyme.[42] The (4*R*,2*S*) **(16a)** isomer exhibited a K_i of 1.4 μM but was also found to be a substrate of the enzyme undergoing a retro-aldol cleavage reaction to produce *o*-aminobenzaldehyde. The (4*S*,2*S*) **(16b)** isomer was a more potent inhibitor, K_i = 0.3 μM, but was not a substrate for the enzyme.

A number of *S*-aryl-L-cysteine *S,S*-dioxides, which are structural analogues of the postulated tetrahedral intermediate **(27)**, have also been reported as inhibitors of kynureninase.[34] *S*-(Phenyl)-L-cysteine *S,S*-dioxide **(45)** and *S*-(2-aminophenyl)-L-cysteine **(46)** were synthesised and their interaction with bacterial kynureninase showed that they were competitive inhibitors of the enzyme. Testing of *S*-(phenyl)-L-cysteine against the enzyme showed it to be a weak inhibitor with a K_i of 700 μM. *S*-(Phenyl)-L-cysteine was then oxidised at sulfur to afford *S*-(phenyl)-L-cysteine S,S-dioxide **(45)** which exhibited a K_i value of 3.9 μM against the enzyme, a decrease of 180-fold in the K_i value. The substitution by an *ortho* amino group in *S*-(2-aminophenyl)-L-cysteine **(46)** also resulted in a 318-fold decrease in K_i value to 2.5 μM. The presence of both of these structural features in **(47)** increased the potency with *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide **(47)** giving a K_i of 70 nM.

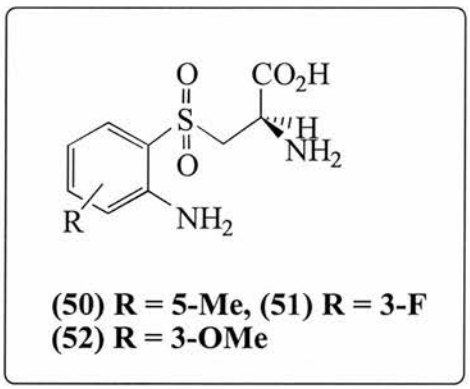


A lower K_i value for *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide **(47)** of 27 nM was recently reported by Phillips and Anderson when comparing its inhibition of bacterial kynureninase with the inhibition exhibited by a number of brominated S-aryl-L-cysteine *S,S*-dioxides.[54] The study also showed that the brominated compounds S-(4-bromo-2-aminophenyl)-L-cysteine *S,S*-dioxide **(48)** and S-(5-bromo-2-aminophenyl)-L-cysteine *S,S*-dioxide **(49)** were less potent inhibitors than the non-brominated compounds, exhibiting K_i values of 301 nM and 372 nM, respectively.



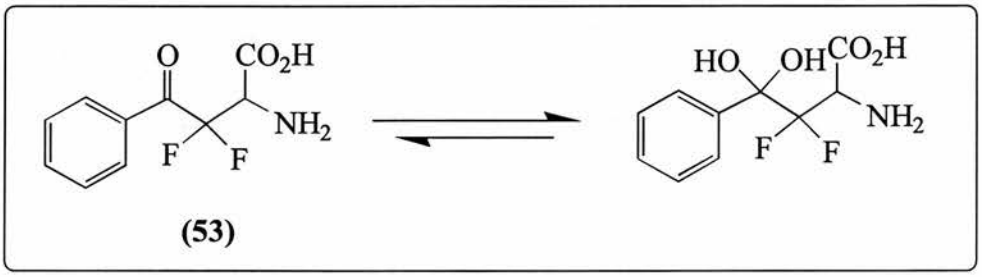
(48) R = 4-Br
(49) R = 5-Br

Drysdale and Reinhard also reported the synthesis of a series of 2-amino-*S*-aryl-L-cysteine *S,S*-dioxides which were observed to be selective inhibitors of kynureninase.[55] The most effective of these inhibitors was 2-amino-5-methyl-*S*-phenyl-L-cysteine *S,S*-dioxide **(50)**. The compounds were tested against the rat liver enzyme. The study showed that compounds which had substituents on the 3 or 5 positions of the ring were stronger inhibitors of the mammalian enzyme than non substituted compounds, exhibiting IC_{50} values of 11μM, 20 μM and 29 μM for the 5-Me **(50)**, 3-F **(51)** and 3-OMe **(52)** and derivatives, respectively.



(50) R = 5-Me, (51) R = 3-F
(52) R = 3-OMe

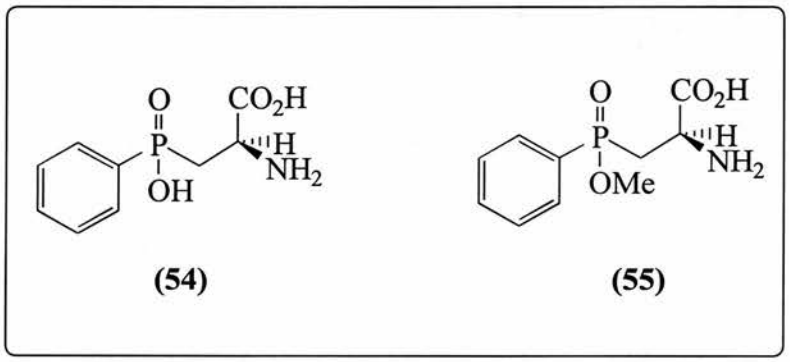
Fluoroketones have often been used as mimics for tetrahedral transition states in protease reactions. This is because under aqueous conditions they are readily hydrated to give the *gem*-diol. α-Amino-β,β-difluoro-γ-oxobenzenebutanoic acid **(53)**, which may inhibit kynureninase by mimicking the tetrahedral intermediate **(27)**, has been synthesised although no biological data has been obtained as yet.[56] In aqueous solution the difluoro substituted analogue of desaminokynurenine will exist predominantly as the hydrate and so behave as a transition state analogue (Scheme 1.9).



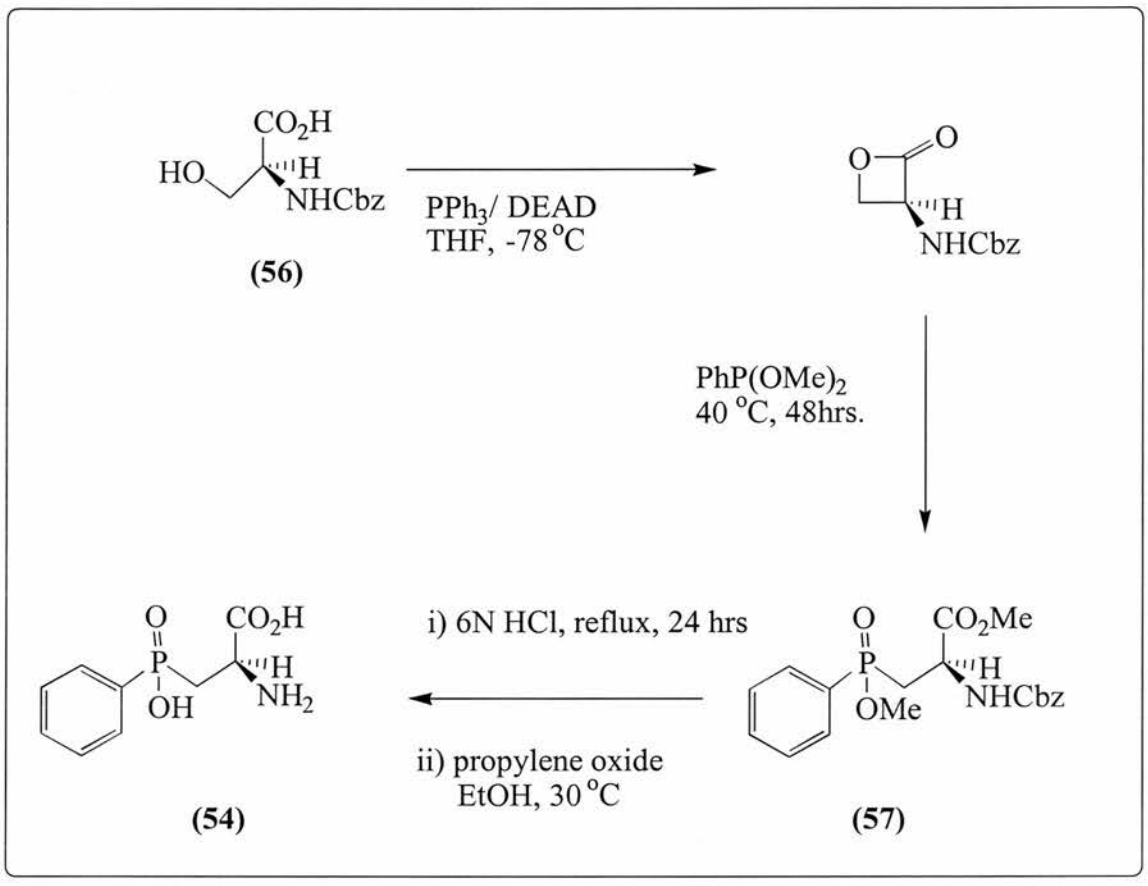
(53)

Scheme 1.9

Phosphinic acid based transition state analogues of the kynureninase reaction have been synthesised at St Andrews. Compounds **(54)** and **(55)** were prepared using the β-lactone methodology originally developed by Vederas.[57,58]



(54) **(55)**



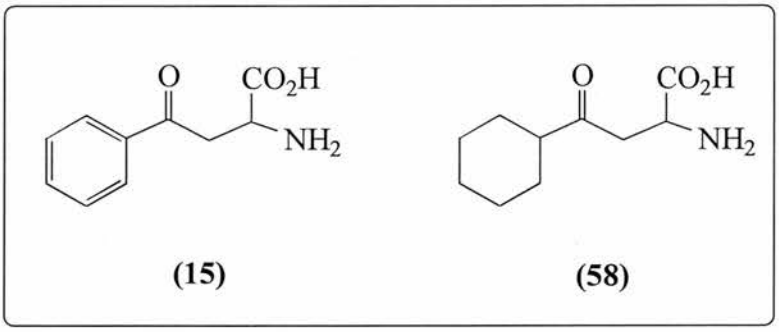
Scheme 1.10

Lactonisation of the *N*-protected serine **(56)** was carried out using triphenylphosphine and diethyl azodicarboxylate (DEAD) to afford the strained β-lactone (Scheme 1.10). The lactone was then ring-opened using a suitable nucleophile. Formation of the methyl ester **(57)** results from migration of one of the methyl groups from the phosphonite onto the carboxylate produced on ring opening, in an Arbusov type reaction.

Kinetic studies using the phosphinic acid analogue **(54)** showed that it behaved as a competitive inhibitor of the enzyme. However, its binding to the active site was observed to be poor, $K_i = 4.28$ mM, while the K_m for the substrate was 25.6 μM. This was thought to be due to pH effects and the formation of a negatively charged phosphinate ion. Destablising interactions between the negative charge on the phosphinate and the other groups at the active site may result in reduced binding. The methyl phosphinate analogue **(55)** was also synthesised and further kinetics showed a fivefold increase in the binding of this inhibitor relative to the free acid, (K_i 0.88 mM). The increase in binding was attributed

to the removal of the negative charge. Both inhibitors were observed to bind more weakly than kynurenine itself.

Several other monocyclic and bicyclic analogues of kynurenine have been synthesised within this group as inhibitors of kynureninase. Desaminokynurenine **(15)** and the cyclohexyl derivative **(58)** were observed to be inhibitors of bacterial kynureninase. Desaminokynurenine was a mixed inhibitor behaving as both a substrate and inhibitor of the enzyme and exhibited a K_i value of 11.6 μM. The cyclohexyl derivative was observed to be a competitive inhibitor of the enzyme exhibiting a K_i of 422 μM.



(15) **(58)**

The recent successful expression of recombinant human kynureninase using the baculovirus/insect cell system within this group allowed a comparative study with bacterial kynureninase from *Pseudomonas fluorescens* with regard to their inhibition by a number of bicyclic compounds.[38] The tetralone **(59)**, indanone **(60)**, chromanone **(61)** and naphthyl **(62)** derivatives were all observed to be competitive reversible inhibitors of kynureninase (Table 1.3). The rationale for the design of the bicyclic inhibitors **(59)-(61)** was that they should be capable of undergoing the early stages of the kynureninase reaction, as far as cleavage of the β,γ-carbon bond. However, the presence of the new carbon chain linking the alanine moiety to the 2-position of the benzene ring would mean both parts of the molecule remain covalently bonded together (Scheme 1.11). This may prevent release from the active site and improve inhibition. It was observed that alteration of the size of the second ring of the inhibitor affected the K_i values of the compounds for both enzymes. There was a 5-fold reduction in the observed K_i when the ring size was altered from a 6- to a 5-membered one. It is thought that the increased conformational flexibility of the 6-membered ring may explain the decrease in binding if only one of its

conformers were to fit appropriately into the active site. In the case of the chromanone derivative **(61)**, in which an oxygen atom was introduced into the ring, there was little effect on binding to the bacterial enzyme but more potent inhibition of the human enzyme was observed. This may be due to additional hydrogen bonding between the ring oxygen and groups at the active site which may usually bind the hydroxyl group of 3-hydroxykynurenine, the preferred substrate for the human enzyme.

The naphthyl derivative was observed to be the most potent inhibitor for both enzymes with K_i values of 5 μM and 22 μM for the bacterial and human enzyme, respectively. Thus, extending the benzene ring of kynurenine to give a larger planar aromatic system resulted in increased binding. This indicated the presence of some additional binding site, a hydrophobic pocket, which can bind the second benzene ring.

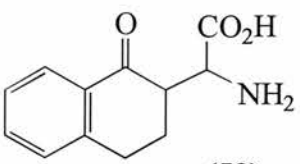
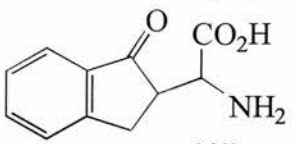
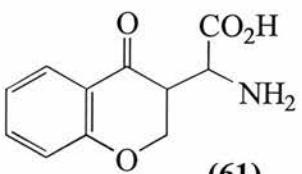
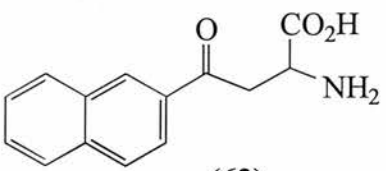
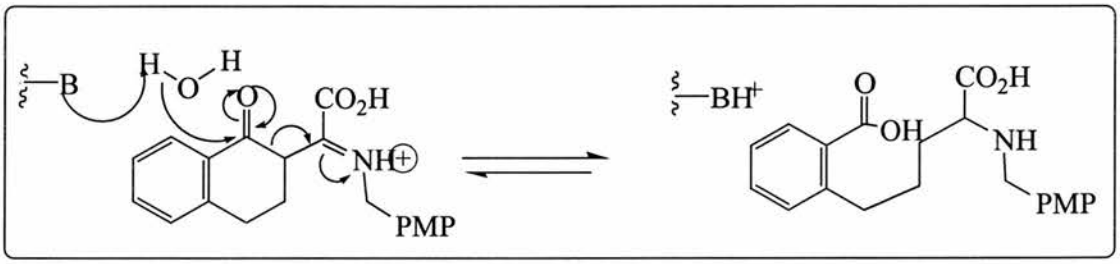
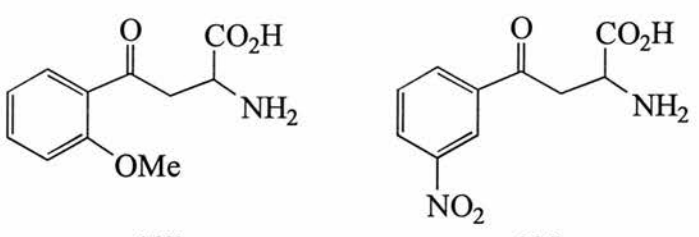
COMPOUND	BACTERIAL K_i /μM	HUMAN K_i/μM
 **(59)**	170 ± 24	227 ± 47
 **(60)**	34.9 ± 10	45 ± 12
 **(61)**	162 ± 31	77 ± 23
 **(62)**	5 ± 2	22 ± 6

Table 1.3



Scheme 1.11

Several non-specific inhibitors of kynureninase have been synthesised which also inhibit other enzymes on the kynurenine pathway (see Section 1.4.1). A number of benzoyl alanine derivatives which were mono substituted at the *ortho*, *meta* and *para* positions of the phenyl ring were examined as inhibitors against rat liver kynureninase.[59,60] The most potent inhibitor of the enzyme was *o*-methoxybenzoylalanine **(63)** which exhibited an IC_{50} of 3 μM while *m*-nitrobenzolyalanine **(64)** was less potent, (IC_{50} = 120 μM). *Para* substituted (bromo-, chloro-, methyl, methoxy- and nitro-) benzoyl alanines were the least potent inhibitors of the enzyme with IC_{50} values of 1000, 150, 800, >100 and 300 μM, respectively. Subsequent testing of **(63)** and **(64)** *in vivo* resulted in anticonvulsant effects, which were thought to be due to increased levels of the neuroprotectant kynurenic acid.

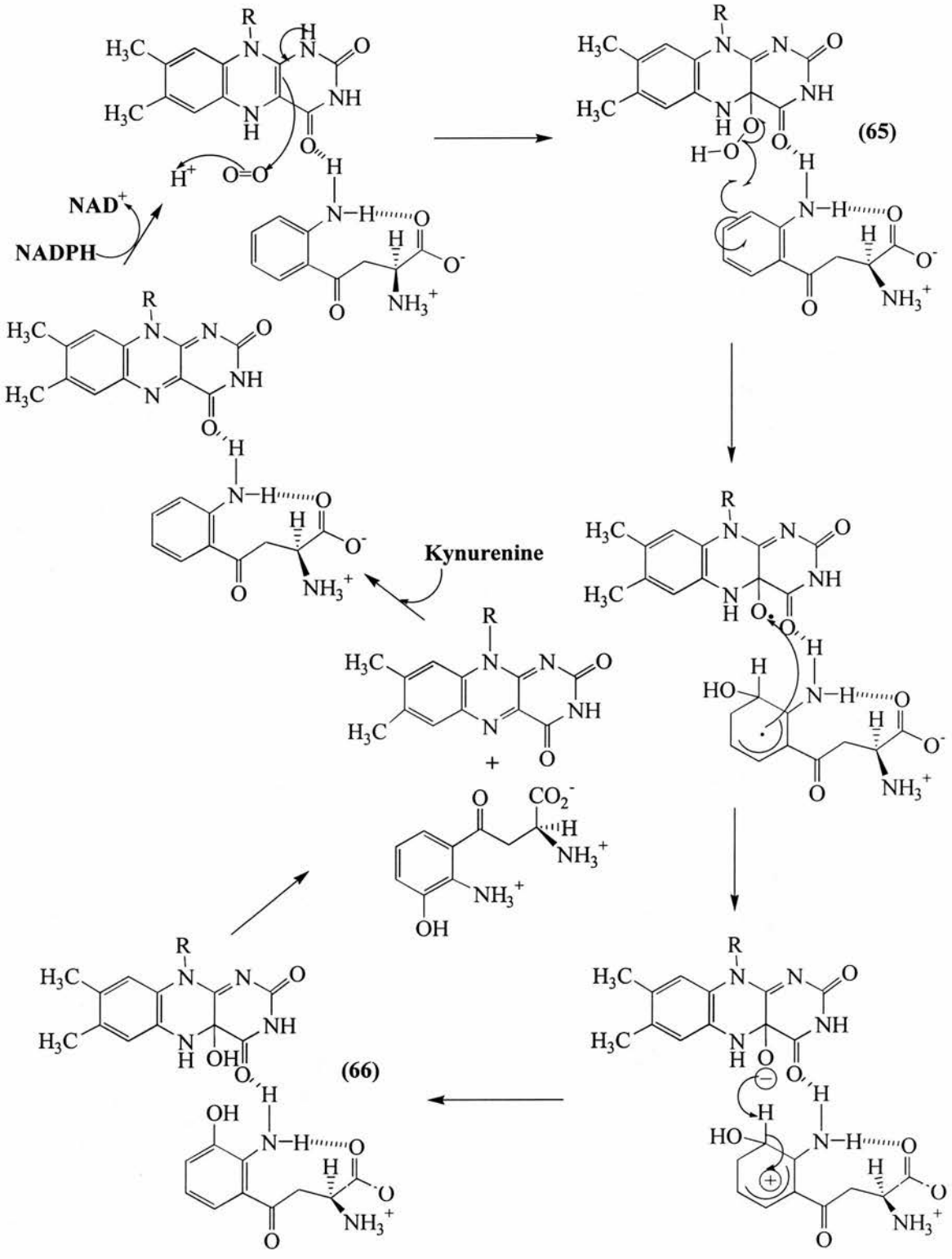


(63) **(64)**

1.4 Kynurenine-3-hydroxylase

Kyurenine-3-hydroxylase (EC 1.14.13.9) catalyses the oxidation of L-kynurenine **(6)** to 3-hydroxy-L-kynurenine **(4)**. The monooxygenase incorporates oxygen from molecular oxygen into the product. It requires flavin adenine dinucleotide (FAD) as a cofactor and requires NADH or NADPH as a coreductant. The enzyme is predominantly a mitochondrial enzyme being located in the brain mitochondria of human, macaque, gerbil, rat and mouse.[61] This is in contrast to the other enzymes on the kynurenine pathway which are localised largely in the brain cytosol. Kynurenine-3-hydroxylase has also been found in other organs such as the liver, kidney, spleen and intestine.

A catalytic cycle for kynurenine-3-hydroxylase has been postulated based on cumulative kinetic and spectroscopic data (Scheme 1.12).[62] The cycle begins with a reduced enzyme/kynurenine complex which reacts with molecular oxygen to give a C(4a)-hydroperoxyflavin intermediate **(65)**. Electrophilic attack from the hydroperoxy group on the aromatic ring of kynurenine is followed by an electron transfer reaction and deprotonation of the cationic substrate. Simultaneous protonation of the isoalloxazine anionic group then affords the intermediate **(66)**. Subsequent dehydration of the hydroxyflavin moiety liberates the product, 3-hydroxykynurenine **(4)**. A second molecule of kynurenine then binds to the enzyme. Completion of the catalytic cycle occurs when the enzyme/kynurenine complex binds to NADPH, resulting in flavin reduction and the release of NAD^+.

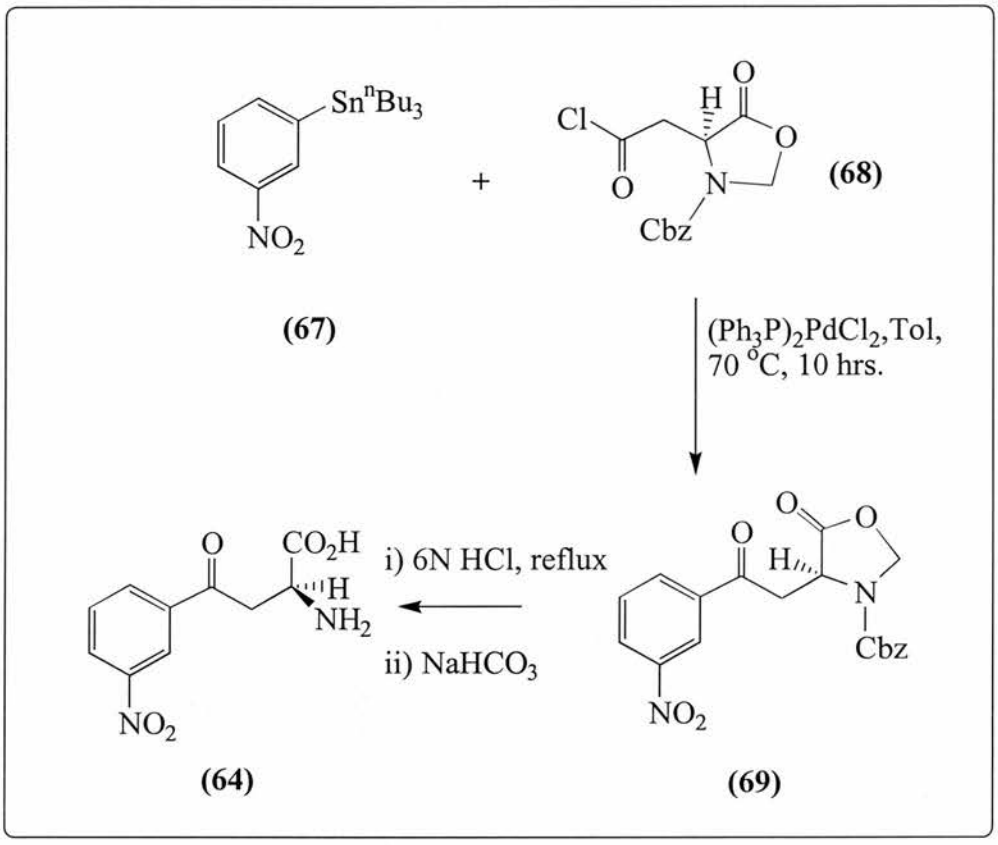


Scheme 1.12

1.4.1 Inhibitors of kynurenine-3-hydroxylase

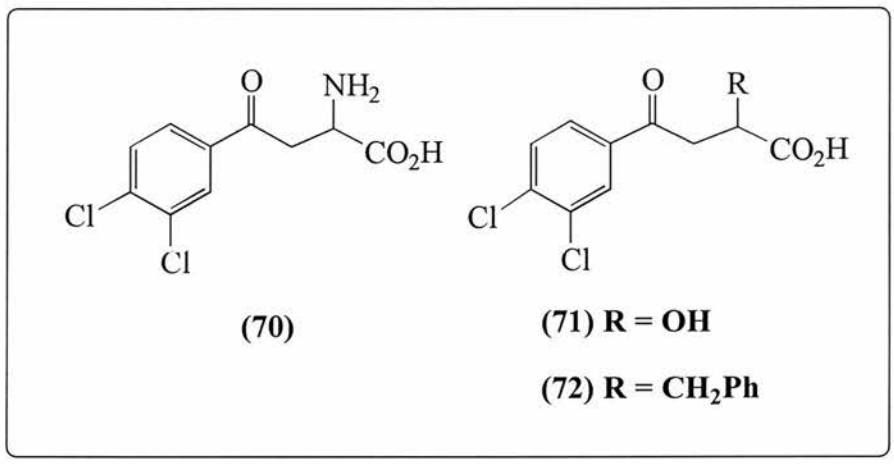
Studies on the design and synthesis of potential inhibitors of kynurenine-3-hydroxylase have been carried out, often in parallel with studies on kynureninase with the aim of identifying new compounds which can inhibit the synthesis of quinolinic acid and produce increased levels of kynurenic acid.

The kynurenine analogue (2*S*)-*m*-nitrobenzoyl alanine **(64)** was shown to be a potent inhibitor of the kynurenine-3-hydroxylase. Initial inhibition studies using the racemic compound indicated that *m*-nitrobenzoyl alanine was probably a competitive inhibitor of the enzyme, exhibiting a K_i of 0.8 μM.[59,61,63] Subsequently a stereoselective synthesis was undertaken (Scheme 1.13). 3-Nitrophenyl-tributyl-stannane **(67)** was reacted with (*S*)-3-carbobenzyloxy-5-oxo-4-oxazolidinyl acid chloride **(68)** in the presence of bis(triphenylphosphine)palladium(II) chloride to afford the (*S*)-*m*-nitrobenzoyl alanine **(64)** *via* the oxazolidinyl-acetyl nitrobenzene **(69)**. The *R* isomer was synthesised in the same manner. Competitive inhibition studies indicated that the *S* isomer was the active constituent in the racemic mixture. The inhibitor was also tested *in vivo* and an increase in kynurenate levels was observed with concomitant anticonvulsant effects.



Scheme 1.13

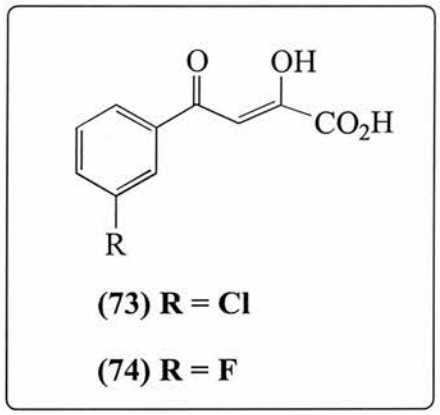
The compound 2,4-dichlorobenzoyl alanine **(70)** was also reported as a competitive inhibitor of the enzyme.[64] The compound exhibited a K_i of 95 nM. 4-Phenyl-4-oxo-butanoic acid derivatives in which the 2-position was substituted by various groups yielded compounds which displayed significant inhibition when tested against the enzyme.[65] The most potent of these were 2-hydroxy-4-(3,4-dichlorophenyl)-4-oxo-butanoic acid **(71)** and 2-benzyl-4-(3,4-dichlorophenyl)-4-oxo-butanoic **(72)** acid with IC_{50} values of 0.3 μM and 0.18 μM, respectively, when tested against rat brain enzyme. Values of 1.4 μM and 2.9 μM were obtained with rat liver enzyme. Enantiospecific synthesis and chiral resolution of **(71)** allowed the (*S*) and (*R*) enantiomers of both compounds to be tested separately. In both cases the (*S*) enantiomers proved to be the stronger inhibitors exhibiting IC_{50} values of 0.42 μM and 2.3 μM, respectively, when tested against rat kynurenine-3-hydroxylase.



(70) **(71) R = OH**

(72) R = CH_2Ph

The synthesis and examination of a series of 4-aryl-2-hydroxy-4-oxobut-2-enoic acids and their ester derivatives afforded a number of potent inhibitors of the enzyme.[66] 4-(3-Chlorophenyl)-2-hydroxy-4-oxobut-2-enoic acid **(73)** and 4-(3-fluorophenyl)-2-hydroxy-4-oxobut-2-enoic acid **(74)** exhibited IC_{50} values of 0.32 μM and 0.58 μM, respectively. They were also shown to prevent interferon-γ-induced synthesis of the neurotoxin quinolinic acid in human blood macrophages.

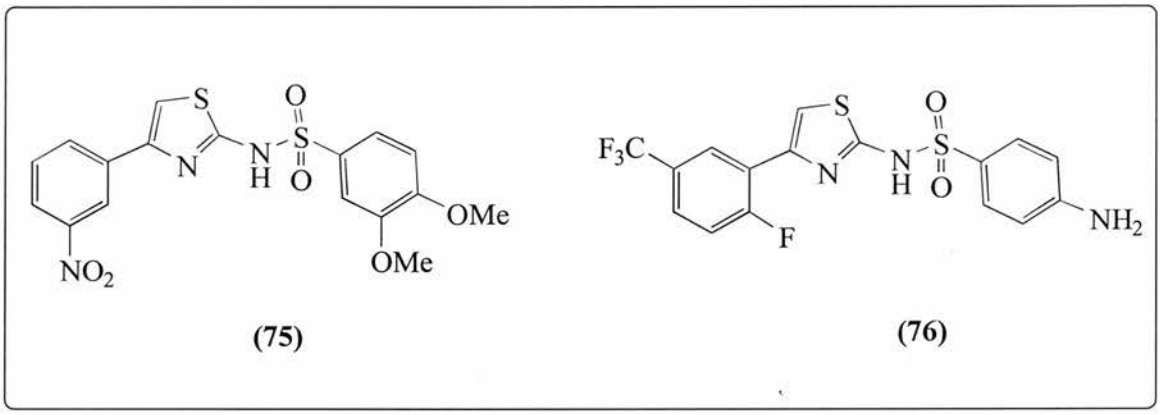


(73) R = Cl

(74) R = F

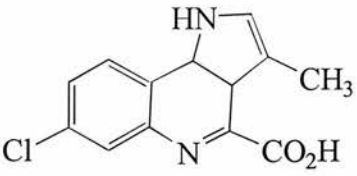
A new class of inhibitors, namely a series of *N*-(4-phenylthiazol-2-yl)benzenesulfonamides was also synthesised and yielded the most potent kynurenine-3-hydroxylase inhibitors known thus far.[67] 3,4-Dimethoxy-*N*-[4-(3-nitrophenyl) thiazol-2yl]

benzenesulfonamide **(75)** and 4-amino-*N*-[4-[2-fluoro-5-(trifluoromethyl)phenyl]thiazol-2yl]benzenesulfonamide **(76)** were shown to be high affinity inhibitors, exhibiting IC_{50} values of 37 nM and 19 nM, respectively. These values can be compared with those obtained for *m*-nitrobenzoyl alanine **(64)** and 2,4-dichlorobenzoyl alanine **(70)** which were significantly higher at 774 nM and 237 nM, respectively. The sulfonamide functional group is a classical carboxylic acid bioisostere.



(75) **(76)**

The synthesis and evaluation of a series of pyrrolo[3,2-*c*]quinolene derivatives as inhibitors of selected enzymes on the kynurenine pathway has recently been reported.[68] 7-Chloro-3-methyl-1*H*-pyrrolo[3,2-*c*]-quinoline-4-carboxylic acid **(77)** was found to be a relatively potent and highly selective inhibitor of kynurenine-3-hydroxylase. The compound exhibited an IC_{50} value of 24 μM. The study indicated that the carboxylic acid group was essential to the inhibitory activity. While compound **(77)** was less potent than previously reported inhibitors, its rigid structure made it an interesting tool and a molecular modelling study was performed to mimic a mechanism for kynurenine-3-hydroxylase inhibition. The superimposition of **(77)** and kynurenine indicated that the amino group of kynurenine and the pyrrolo NH were pointing in the same direction and may hydrogen bond with the same residue in the active site of the enzyme. However, this hydrogen bond does not appear to be critical for strong inhibitory action.

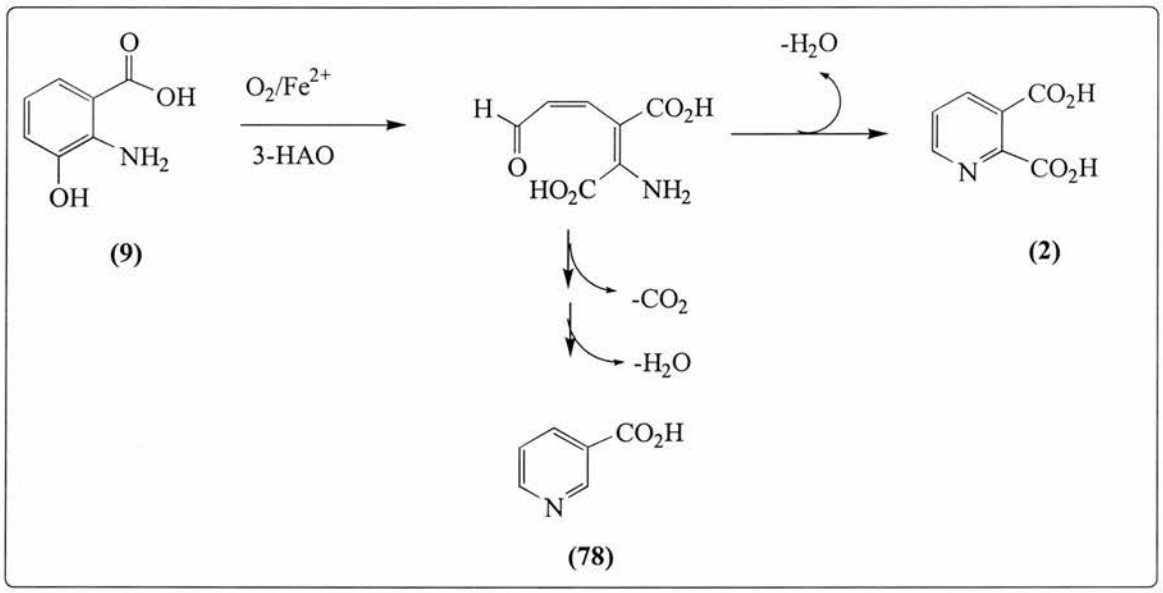


(77)

1.5 3-Hydroxyanthranilic acid oxidase

3-Hydroxyanthranilic acid oxidase (3-HAO, EC.13.11.6) is the final enzyme involved in the synthesis of quinolinic acid **(2)**. The enzyme has been found in the liver and kidney of several species including rat, calf and ox.[69] It is a monomeric enzyme found in the cytosol and it belongs to a family of intramolecular dioxygenases which contain non haem Fe^{2+}.[70,71,72] The enzyme has been found in rat and human brain.[73,74] The rat brain enzyme has been purified and characterised and was observed to be a single-unit protein with a molecular mass of 38,000. The enzyme was also observed to be localised in the astroglial cells throughout the brain. Molecular cloning and expression of human liver enzyme has also been successful.[74] The predicted amino acid sequence for the human enzyme exhibited 94% similarity to the partial rat sequence of the rat enzyme. The molecular weight for the human enzyme was determined to be 32,000.

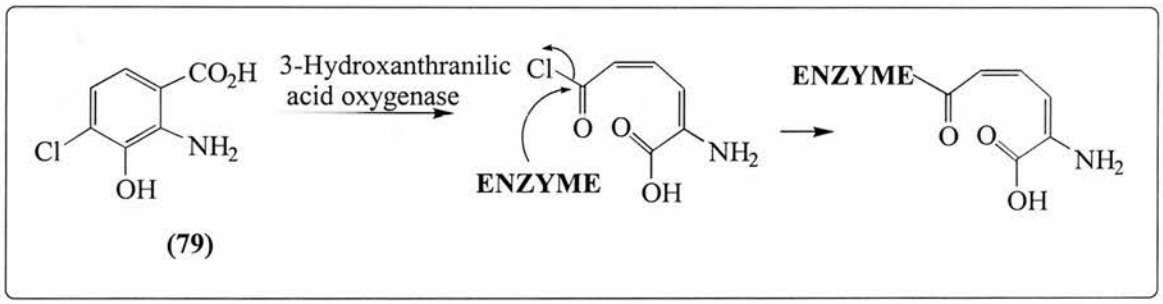
The enzyme catalyses the oxidative cleavage of 3-hydroxyanthranilic acid **(9)** to form an unstable intermediate, α-amino-β-carboxymuconic acid semialdehyde (Scheme 1.14).[70,71] This intermediate can then spontaneously rearrange to form quinolinic acid **(2)** or be converted to picolinic acid **(78)** after decarboxylation. The rate limiting reaction in its spontaneous disappearence is possibly an isomerisation of the double bond to place the carboxyl groups in *cis* configuration. This would then be followed by a rapid condensation reaction to form the stable pyridine ring. In a study by Mehler, the rapid formation of the semialdehyde intermediate was observed spectrophotometrically at 360 nm.[70] In a separate study the substrate, 3-hydroxyanthranilate **(9)**, which absorbs at 317 nm was observed to decrease in the presence of 3-HAO.[75]



Scheme 1.14

1.5.1 Inhibitors of 3-hydroxyanthranilic acid oxidase

4-Halo-3-hydroxyanthranilic acids were investigated as possible inhibitors of 3-hydroxy anthranilic acid oxygenase. Initial examination of 4-chloro-3-hydroxyanthranilic acid **(79)** *in vivo* by systemic administration to mammals indicated an increase in levels of excreted 3-hydroxyanthranilic acid and therefore decreased enzyme activity.[76] Subsequent studies *in vitro* suggested that inhibition occurs when oxidation of 4-chloro-3-hydroxy anthranilic acid **(79)** by the enzyme affords a product which then binds irreversibly to the enzyme (Scheme 1.15).[77] This binding was thought to cause non-competitive inhibition of the enzyme.



Scheme 1.15

However, a later study on the inhibition of 4-chloro-, fluoro- and bromo-3-hydroxy anthranilic acids revealed that all three inhibitors were reversible competitive inhibitors of

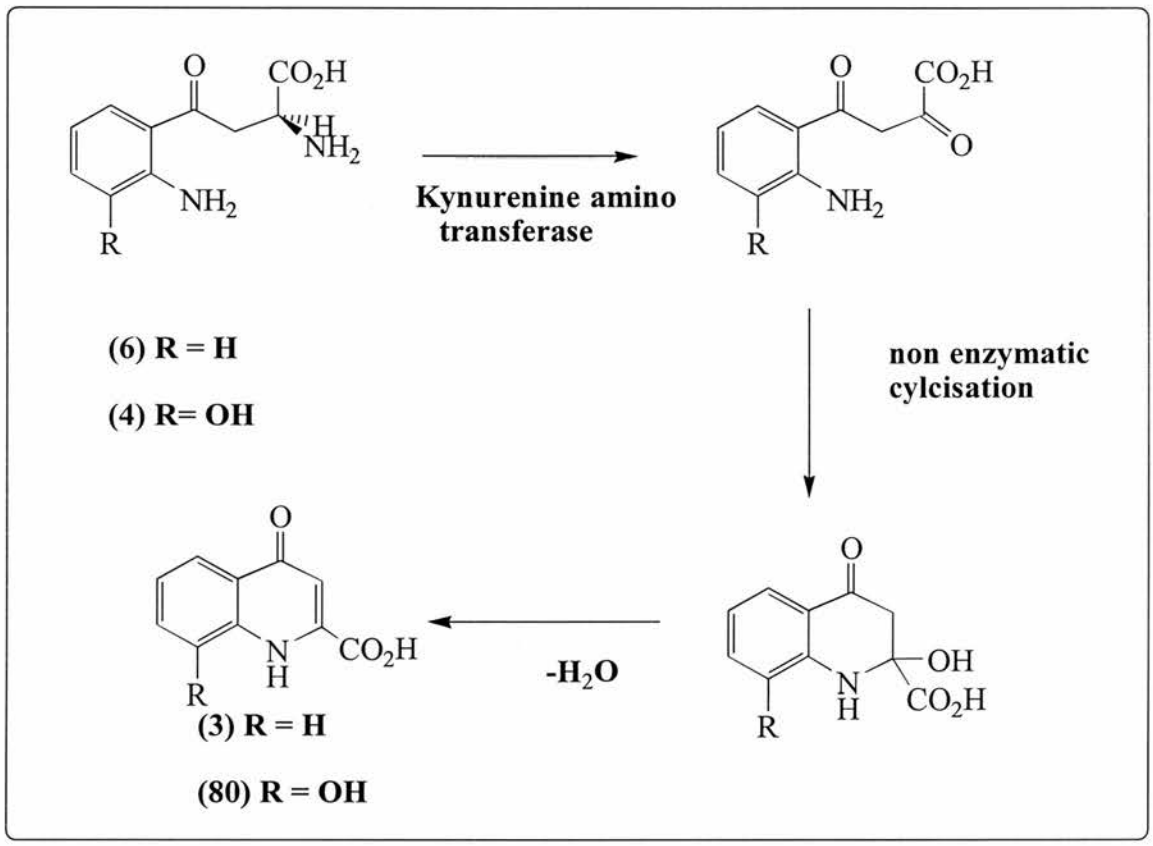
the enzyme.[78] Using conditions which could differentiate between reversible and irreversible binding to the enzyme, the three compounds were determined to be tight binding inhibitors exhibiting K_i values of 190, 6 and 4 nM for the 4-chloro-, fluoro- and bromo-derivatives, respectively. It was also suggested that the compounds were also substrates for the enzyme. However, no evidence for the formation of the expected 6-hydroxyquinolinic acid products were observed.

1.6 Kynurenine Aminotransferase (KAT)

Kynurenine aminotransferase (EC 2.6.1.7) catalyses the irreversible transamination of L-kynurenine to kynurenic acid **(3)**. The initial transamination product undergoes a rapid non enzymatic cyclisation reaction followed by dehydration and tautomerisation to afford kynurenic acid **(3)** (Scheme 1.16).[79] The reaction is irreversible because of the aromatic nature of the product which confers stability, driving reaction to completion. 3-Hydroxy-L-kynurenine **(4)** can also be a substrate for the enzyme resulting in formation of xanthurenic acid **(80)**. While α-ketoglutarate is the main amino acid acceptor in the reaction a number of other oxo acids can also participate. Kynurenine aminotransferase requires pyridoxal 5'-phosphate as a cofactor and is located almost exclusively in the mitochondria.

The neuroprotective qualities associated with kynurenic acid have resulted in significant interest in the isolation and characterisation of mammalian KAT enzymes. Schwarcz *et al.* isolated kynurenine aminotransferase from rat brain.[80] It was found to be identical with kynurenine aminotransferase isolated from rat kidney. The enzyme was characterised by a sensitive assay in which [^{3}H]kynurenine was converted irreversibly to [^{3}H]kynurenic acid in the presence of physiological levels of L-kynurenine substrate. The enzyme consists of a single protein which uses both α-ketoglutarate and pyruvate as amino acid acceptors. The enzyme was found to be highly substrate specific, 3-hydroxykynurenine was found to be a poor substrate for the enzyme in the presence of kynurenine while both tryptophan and α-aminoadipate were effective substrates. The enzyme exhibited a pH optimum of 8.6.

In human brain, two kynurenine aminotransferases have been identified, KAT I and KAT II, which are responsible for the production of kynurenic acid from its precursor L-kynurenine.[81] KAT I was determined to be a dimer consisting of two identical subunits each of 60 kDa. KAT I and KAT II were shown to exhibit distinct catalytic



Scheme 1.16

characteristics. While KAT I has a very high and sharp pH optimum of 9.5-10.0, KAT II displays a shallow pH optimum in the physiological range. KAT I was inhibited by a number of common amino acids such as L-glutamine, L-phenylalanine and L-tryptophan while KAT II was not inhibited. KAT I also showed a high specificity for amino acid acceptors, showing a high activity with 2-oxoisocaproate while KAT II shows no preference for amino acid acceptors. An immunological assessment of the enzyme using anti-KAT I antibodies showed a clear distinction between KAT I and KAT II. Studies also showed that kynurenic acid production under physiological conditions may be catalysed primarily by KAT II whereas KAT I may be of importance in pathological situations. Studies on KAT I and KAT II activities in the brain of Huntington's disease (HD) patients showed a dysfunction of kynurenic acid metabolism with apparent decreases

in the activites of both enzymes in most areas of the brain with the exception of the putamen where activity increased.[18] Human heart KAT has also been isolated and showed distinct differences to both KAT I and KAT II.[82] The enzyme exhibited a shallow pH optimum between 8.9 and 9.0, high cosubstrate specificity for 2-oxoglutarate and a lack of sensitivity to inhibition by L-glutamine, L-phenylalanine and L-tryptophan.

Although KAT enzymes have a primary function in the transamination of kynurenine to kynurenic acid a number of other PLP dependent transaminases have been identified which are capable of catalysing kynurenic acid formation with a variety of amino acceptors. In rat liver four such enzymes were found.[83] Amino acid acceptors used included α-ketoglutarate, glycoxylate and pyruvate. The four proteins exhibited molecular weights ranging between 79,000 and 100,000. One such enzyme was found to be identical to α-aminoapidate aminotransferase, when it had the same elution pattern on all column chromatographies.[84] In human liver a single protein was found to catalyse transamination reactions for a number of amino acid donor acceptor pairs including kynurenine/glycoxylate, alanine/glycoxylate and serine/pyruvate.[85]

1.7 Endogenous neurotoxins from tryptophan

The resurgence of interest in the kynurenine pathway of tryptophan metabolism in the last twenty years is due mainly to the implication of kynurenines in neurological disorders. The metabolites which have elicited greatest attention are quinolinic acid **(2)** and kynurenic acid **(3)**. Both metabolites are present in the mammalian brain.[86] To date quinolinic acid and kynurenic acid remain the only two endogenous compounds known to respectively activate and block the NMDA subpopulation of glutamate receptors selectively.[87] The NMDA subpopulation of excitatory amino acid receptors have an important role within the central nervous system (CNS). They are involved not only in synaptic transmissions within the CNS but also in long term neuronal activity. NMDA receptors are also involved in the determination of cell viability by activating destructive enzymes such as phospholipidases, proteases and protein kinases. NMDA receptors in particular are thought to mediate the excitotoxic response of many neurons to excessive stimulation by glutamate and related compounds which leads to neuronal cell death in a number of degenerative injuries to the CNS.[88] The action of both quinolinic acid and kynurenic acid at these receptors has resulted in their implication in the aetiology of a number of neurological diseases. While the balance between these two metabolites may also be important in maintaining the activity of the NMDA receptor.

More recently attention has begun to focus on other mechanisms of neuronal damage, in particular the role of oxidative stress as a causative factor in neuronal cell death.[89] Oxidative stress refers to the cytotoxic consequences which result from the formation of reactive oxygen species such as the superoxide anion $O_2^{\cdot}$, hydroxyl radical $OH^{\bullet}$ and hydrogen peroxide. Pertinent to this is the emergence of another kynurenine, 3-hydroxykynurenine **(4)** as a putative neurotoxin in a number of neurodegenerative diseases.

1.7.1 Quinolinic acid

Quinolinic acid **(2)**, which functions as selective agonist of the glutamate subpopulation of NMDA receptors in the CNS, also discriminates between the two major types of NMDA receptor subunits which exist within the brain.[90,91] The subunits have different distribution patterns within the brain which are in broad agreement with observed regional variations of quinolinate acid evoked excitation.[87,92]

Quinolinic acid **(2)** within the human CNS is limited to the brain cortex and cerebrospinal fluid (CSF) at levels which rarely exceed 1 μM.[93] Small amounts of quinolinic acid are also found in the striatum, cerebellum and hippocampus of rats, guinea pigs and rabbits. Quinolinic acid levels are found to increase during the ageing process in rat, guinea pig and rabbit.[94] Unlike the tryptophan metabolites, L-kynurenine **(6)** and 3-hydroxykynurenine **(4),** which are readily taken up into the brain through the blood brain barrier by the large neutral amino acid carrier, quinolinic acid does not readily pass through.[95] Peripheral quinolinic acid is only thought to enter the CNS through passive diffusion across the blood brain barrier. Under normal circumstances brain and CSF quinolinic acid levels are maintained below those in the blood by extrusion *via* a probenecid-sensitive carrier system. About 70% of quinolinic acid present in CSF under normal conditions is thought to originate in the blood.[96]

The enzymes responsible for the biosynthesis and degradation of quinolinic acid **(2)** are 3-hydroxyanthranilic acid oxidase (3-HAO) and quinolinic acid phosphoribosyltransferase (QPRTase) respectively. Both enzymes are present in human and rat brain.[97,98] In both species the brain exhibits a higher capacity for synthesis than break down, displaying V_{max} values of 80 and 100 times higher for 3-HAO compared with QPRTase for the rat and human brain, respectively.[99,100] The regulation of quinolinic acid metabolism *in vivo* is pertinent to its role as a potent neurotoxin. Quinolinic acid as an endogenous excitant at NMDA receptors is thought to mediate neuronal damage by overstimulation of these receptors.[10]

The implication of quinolinic acid **(2)** in the aetiology of Huntington's disease has come from studies demonstrating the excitotoxic effects of quinolinic acid.[101] Systemic

administration of quinolinic acid in nanomolar amounts produced axon sparing lesions in rat brain which were similar to those obtained from administration of exogenous excitatory amino acids such as kainate and mimicked those observed in Huntington's disease.[11] Beal *et al.* also reported that quinolinic acid lesions caused a depletion in neurotransmitters such as GABA and substance P from striatal spiny cells in a pattern which closely resembled that of Huntington's disease.[102] Both Haik and Rosillo produced rodent models of Huntingtons disease where both behavioural and neuroanatomical alterations in rodents were observed upon administration of quinolinic acid.[13,103] Both studies found impairments in learning ability and balance as well as striatal neuronal loss.

Studies on 3-HAO and QPRTase activity in relation to Huntington's disease found that the activity of both enzymes is increased. Although an increase in 3-HAO activity supports increased quinolinic acid **(2)** levels as an aetiological factor in Huntington's disease, the increased activity of QPRTase has not been explained.[104] It is thought that elevated levels of QPRTase may be due to a change in quinolinic acid metabolism and a response to abnormally high tissue levels of quinolinic acid. While elevation of quinolinic acid levels in cases of Huntington's disease have been disputed, the integrity of the quinolinic acid hypothesis is retained by the finding that normal physiological levels of quinolinic acid can induce neuronal cell death.[105]

In contrast, QPRTase activity was found to decrease in epileptic brain tissue and this was cited as a possible mechanism for the pathological accumulation of quinolinic acid in some cells in the epileptic focus.[106] However quinolinic acid levels were in general not elevated in the brain tissues of patients with epilepsy.[107,108] This may be explained by the finding that quinolinic acid has different pharmacological properties in different brain regions, discriminating between NMDA receptor subtypes. It behaves as a potent agonist, and therefore potential excitoxin, in some areas of the brain while being a weak agonist in other regions.[109]

Elevated levels of quinolinic acid **(2)**, up to 300-fold increased, have been found in the brain tissue and CSF of humans infected with HIV.[12] Similar increases were observed in retrovirus infected macaques.[110] In conditions of immune activation local synthesis of quinolinic acid within the CNS is the primary source of quinolinic acid rather than entry from the blood. Quinolinic acid formation from L-tryptophan in immune specific

macrophage cells was demonstrated by Heyes *et al.*[111] The incubation of macrophages with L-[$^{13}C_6$]-tryptophan, and subsequent stimulation by interferon-γ, resulted in the formation of [$^{13}C_6$]-quinolinic acid. Therefore increased levels of quinolinic acid in HIV, as well as other inflammatory diseases such as poliovirus, are thought to be due to interferon-γ stimulated production of the neurotoxin from L-tryptophan within the CNS.

In addition to a direct excitotoxic effect on neurones other mechanisms of damage by quinolinic acid includes induction of progressive mitochondrial dysfunction which may contribute to neuronal cell death.[112] The formation of reactive oxygen species may also be a factor in the neurotoxicity of quinolinic acid. This was demonstrated by the attenuation of neuronal damage by free radical scavengers and spin trap reagents.[113]

1.7.2 Kynurenic acid

Kynurenic acid **(3)** is an antagonist of the three glutamate receptors sensitive to NMDA, quisqualic acid and kainic acid.[114-117] It has a high affinity for the strychnine insensitive glycine receptor on the NMDA subpopulation of receptors.[16] Kynurenic acid **(3)** was found to be a potent antagonist in the presence of both glutamate and quinolinic acid **(2)** and as a result interest in this metabolite has centred on its role as a potential neuroprotectant and anticonvulsant. [118]

The anticonvulsant effects of kynurenic acid were examined both in mice and rats. Systemic administration of kynurenic acid and quinolinic acid in a molar ratio of 3:1 prevented nerve cell loss in the striatal and hippocampal areas of the brain examined. However, it was found that larger than equimolar amounts of kynurenic acid are required to prevent quinolinic acid neurotoxicity.

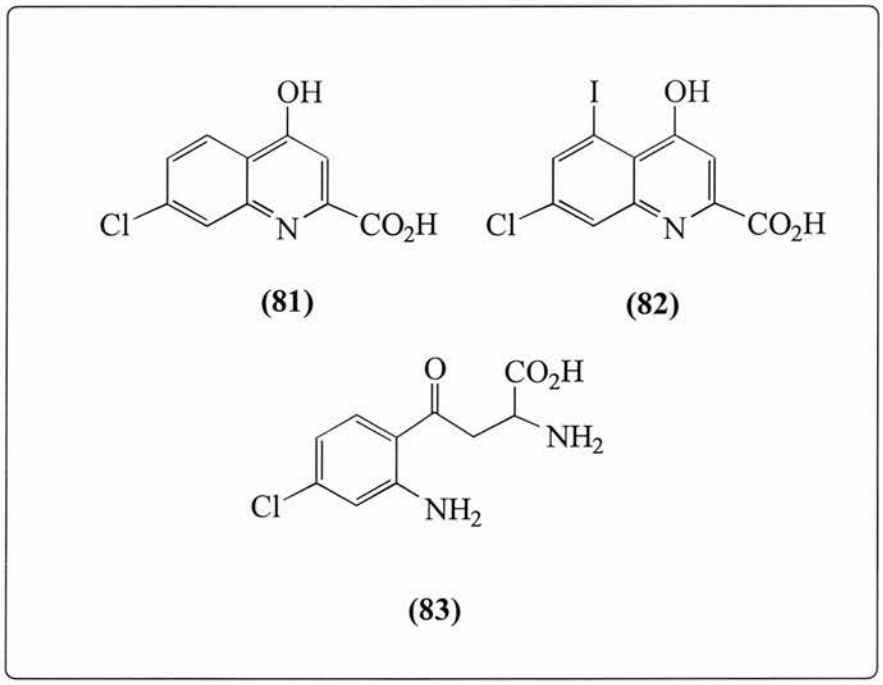
Schwarcz *et al.* reported that kynurenic acid **(3)** was found in the human brain at levels of approximately 1μM,[119] however, much lower concentrations of 150 pM have been found by other groups.[120] It is also found in the brain tissue of rat, mouse and guinea pig as well as in other organs such as the liver, kidney, intestine and heart.[121,122] It was found that both striatal and peripheral application of its biosynthetic precursor, L-kynurenine **(6)**, to rats resulted in a significant increase in kynurenic acid levels in the brain.[123,124] Although

kynurenic acid cannot pass through the blood brain barrier, except by passive diffusion, under normal physiological conditions kynurenine is readily taken up into the CNS by a large neutral amino acid carrier. Inhibition of the biosynthetic enzyme for kynurenic acid, KAT, which is located in brain mitochondria, resulted in a reduction in kynurenic acid biosynthesis. Increasing brain tissue levels of 2-oxoacids, such as pyruvate and α-ketoisocaproate, known to behave as cosubstrates in the KAT catalysed transamination of kynurenine to kynureninc acid were also shown to increase levels of the metabolite.[122] Therefore under normal physiological conditions kynurenic acid is synthesised locally within the CNS from peripheral kynurenine transported across the blood brain barrier. Kynurenic acid is synthesised primarily in astroglial brain cells then exported and released into the extracellular fluid.[119,125] Although its synthesis appears to be region specific it appears to be present in all brain areas of rat, mouse, guinea pig and rabbit brain. Kynurenic acid levels in rat brain also appear to increase as a result of the ageing process.[126]

Significant changes in kynurenic acid **(3)** levels have been observed in the brain tissues of patients suffering from a number of neurological diseases such as Alzheimers disease,[21,127] HIV-1 associated dementia,[19] Downs syndrome[20] and Huntington's disease.[18,108] While both increased and decreased levels of kynurenic acid have been reported in cases of Huntington's disease, significantly elevated levels of kynurenic acid were observed in the patients with Alzheimer's disease, HIV-1 associated dementia and Downs syndrome. This was coupled with an increase in the activity of KAT I in both HIV-1 associated dementia and Alzheimer's disease. However, KAT I activity was reduced in cases of Downs syndrome. In all cases, KAT II activity appeared unchanged. The increased activity in KAT I indicates that kynurenic acid increases under pathological conditions are due largely to localised synthesis within the brain. There is evidence that the integrity of the blood brain barrier is compromised under pathological conditions which may lead to increased levels of peripheral kynurenine, its biosynthetic precursor, within the CNS. While elevated kynurenic acid **(3)** levels in cases of HIV associated dementia may attenuate the neurotoxic effect of increased quinolinic acid **(2),** its function in cases of Alzheimer's disease is less clear since increased quinolinic acid levels are not associated with the disease. Kynurenic acid is implicated in the pathogenesis of Alzheimer's disease in its role as a glutamate receptor antagonist causing impairment of cognitive function and

memory loss. Increased levels of kynurenic acid are also a feature in neonatal asphyxia where levels are increased by over 200%, although, KAT activity was unchanged. Increased levels of α-keto acid cosubstrates for kynurenine transamination to kynurenic acid were observed however, and may explain increased levels of kynurenic acid.[128] Aberrant Kynurenic acid metabolism is thought to play a role in sudden infant death syndrome.

The ability of kynurenic acid **(3)** to attenuate the neurotoxicity of a number of endogenous excitotoxins has led to the synthesis of a number of kynurenic acid analogues as antagonists for use in the treatment of neurological disease.[17]A number of mono and disubstituted halo-kynurenic acid derivatives have been synthesised which are potent antagonists of NMDA receptor sites. The 7-chlorokynurenic acid analogue **(81)** was shown to be a strong antagonist of the glycine site of NMDA receptor complex exhibiting an IC_{50} value of 0.56 μM.[129] The most potent antagonist reported, however, was 5-iodo-7-chlorokynurenic acid **(82)** which exhibited an IC_{50} of 32 nM.[130] However, the efficacy of these compounds is affected the limitations of blood brain barrier permeability. 7-Chlorokynurenic acid is unable to penetrate the CNS in concentrations high enough to have a therapeutic effect. This has led to the synthesis of a number of compounds which can behave as prodrugs.[17,131] Synthesised for their ability to cross the blood brain barrier they are converted within the CNS to neuroactive kynurenic acid derivatives. 4-Chloro-kynurenine **(83)**, a neutral amino acid, was found to be a good substrate for transportation across the blood brain barrier *via* an amino acid carrier and may be a potential prodrug for 7-chlorokynurenic acid.



(81) **(82)**

(83)

In conclusion, kynurenic acid functions as a potent antagonist of the glycine site of the NMDA receptor. It has neuroprotective qualities attenuating the neurotoxicity of glutamate and quinolinic acid. Dysfunction in kynurenic acid metabolism may implicate it in a number of neurological diseases and injuries.

1.7.3 3-Hydroxykynurenine

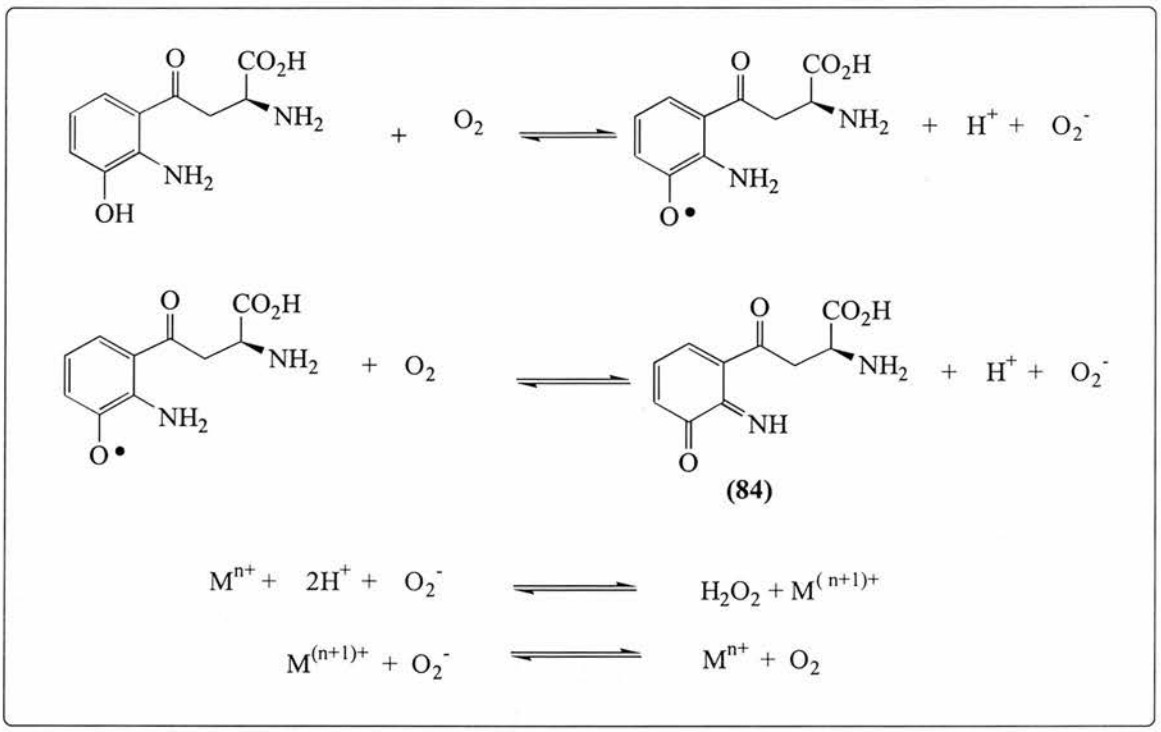
3-Hydroxykynurenine **(4)** is a metabolite of tryptophan **(1)** obtained from the oxidation of kynurenine by kynurenine-3-hydroxylase. Kynurenine-3-hydroxylase is found in human, rat and gerbil brain.[61] However, local production of 3-hydroxykynurenine within the brain may not be its only source,[61] and it may also be taken up into the brain by the large neutral amino acid carrier of the blood brain barrier since it was shown to competitively inhibit uptake of the neutral amino acid leucine.[95]

The activity of kynurenine-3-hydroxylase has been shown to increase in inflammatory conditions or after immune stimulation.[1,61] 3-Hydroxykynurenine **(3)** levels are increased in both Huntington's disease and HIV-1 associated dementia as well as in the neurological deficits associated with Vitamin B_6 deficiency.[23,132-134] Exposure of neuronal cells to 3-hydroxykynurenine **(4)** at concentrations similar to those found in the CNS of rats with

vitamin B_6 deficiency showed it to be toxic to almost all of the cells. Comparison with other kynurenine metabolites such as kynurenine **(6)**, xanthurenic acid **(80)** and quinolinic acid **(2)** showed that only 3-hydroxykynurenine and xanthurenic acid induced a toxic response. This suggested the presence of phenolic functionality as an important factor in toxic potency. The characteristics of apopotic neural cell death upon application of 3-hydroxykynurenine were also similar to the type observed in Huntington's disease.[132]

Huntington's disease pathology is restricted to regional neuronal cell death in the caudate and putamen of the brain. In studies on Huntington's disease, however, a general increase in levels of 3-hydroxykynurenine was found throughout the brain.[23] It is thought that increased levels of 3-hydroxykynurenine in Huntington's disease may result in neuronal cell death in some regions of the brain while other areas remain unaffected. Research has shown region specific neuronal death when brain tissue is exposed to small (1-10 μM) levels of 3-hydroxykynurenine. Certain areas of brain, therefore, appear to be less susceptible to the neurotoxic effects of 3-hydroxykynurenine. This may be explained by the fact that uptake of 3-hydroxykynurenine by neurons is thought to be facilitated by large neutral amino acid transporters, therefore neurons insensitive to 3-hydroxykynurenine toxicity may have a poor ability to take up large neutral amino acids. Increased levels of 3-hydroxykynurenine were limited to the frontal cortex of the brain in patients with HIV.[133]

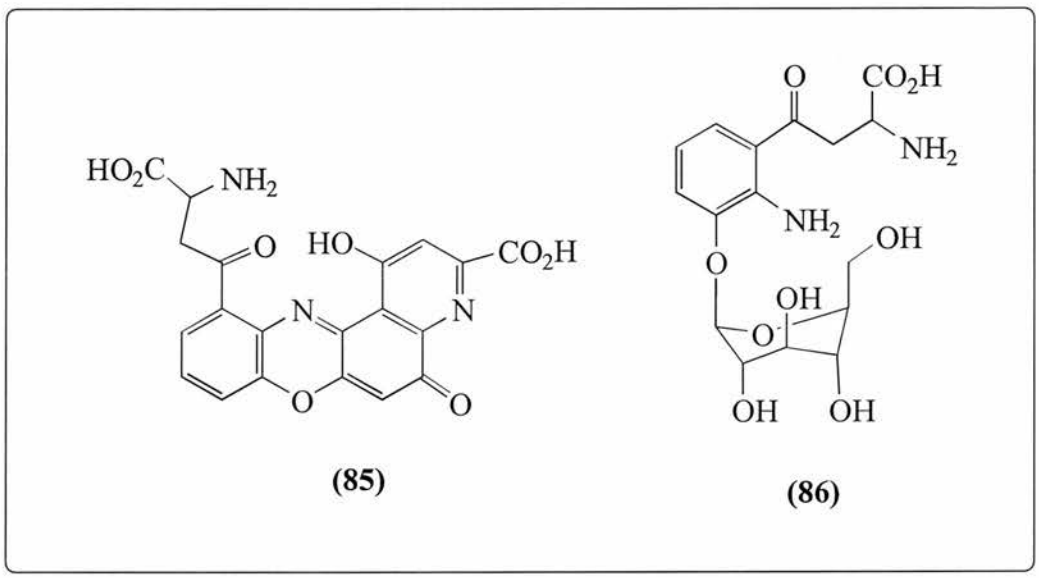
The neurotoxicity of 3-hydroxykynurenine is thought to be mediated by hydrogen peroxide (H_2O_2) formation and the generation of reactive oxygen species within the brain resulting in oxidative stress with concomitant neuronal cell death.[79,134-136] The *o*-aminophenol structure of 3-hydroxykynurenine is thought to undergo oxidation to form a quinoimine **(84)** with concomitant formation of H_2O_2 (Scheme 1.17). Studies carried out by Okudo *et al.* indicated intracellular accumulation of H_2O_2 which occurred in tandem with increases in applied levels of 3-hydroxykynurenine.[136] Neuronal cell death is thought to occur through lipid peroxidation of polyunsaturated fatty acids present in neuronal membranes. Attenuation of 3-hydroxykynurenine toxicity was achieved by inhibition of the intracellular oxidatitive enzyme xanthine oxidase. The formation of H_2O_2 during oxidation of 3-hydroxykynurenine is thought to be a transition metal mediated reaction (Scheme 1.17).[137]



Scheme 1.17

The trapping of a 3-hydroxykynurenine derived radical formed during its oxidation in the presence of H_2O_2 and horseradish peroxidase has also been reported.[138] The product of the reaction, xanthommatin **(85)**, is also found in the human lens and is implicated in the formation of senile cataracts. Since the fluorescent O-β-D-glucopyranoside of 3-hydroxykynurenine **(86)** is present in the lens of humans and plays an important role in retinal protection as a UV filter, pathological changes associated with senile cataract formation may involve dysfunction in the formation of the glucopyranoside conjugate or in its breakdown by enzymes.[139,140]

3-Hydroxykynurenine has thus emerged as a potent neurotoxin present in the mammalian brain whose mechanism of neuronal damage involves the formation of reactive oxygen species resulting in oxidative stress and neuronal cell death.



(85) **(86)**

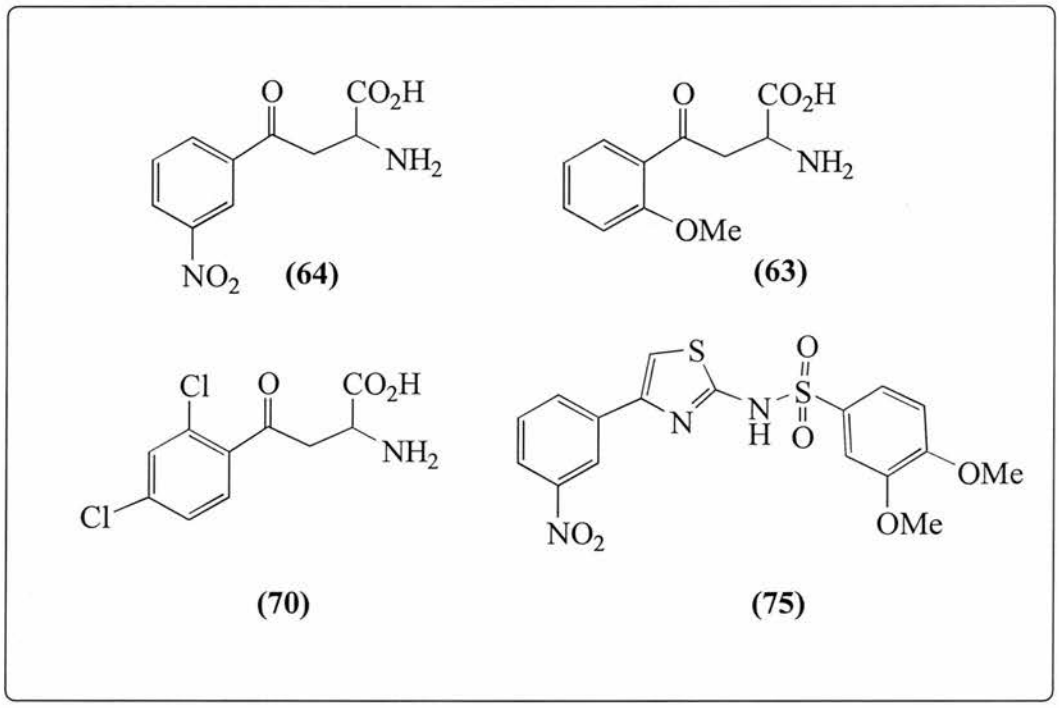
1.7.4 Modulation of the kynurenine pathway

A possible therapeutic approach for treating neurological diseases may be modulation of the biosyntheses of quinolinic acid **(2)**, 3-hydroxykynurenine **(4)** and kynurenic acid **(3)** *in vivo*. The viability of this method was demonstrated when it was shown that the co-administration of kynurenine **(6)**, probenecid (an inhibitor of organic acid transport) and nicotinylalanine (an inhibitor of both kynurenine–3-hydroxylase and kynureninase enzymes) increased levels of kynurenic acid *in vivo*, had sedative effects and stopped convulsions induced by quinolinic acid in rats.[141,142,143] Previous studies had shown that administration of kynurenine with probenecid can result in increased levels of kynurenic acid.[144] Nicotinylalanine augments its synthesis by inhibiting enzymes on the kynurenine pathway leading to quinolinic acid and drives metabolism of kynurenine towards the synthesis of kynurenic acid. These studies have raised the possibility of using inhibitors of the kynurenine pathway to reduce excitotoxic effects.

A number of benzoylalanine derivatives synthesised as inhibitors of kynurenine-3-hydroxylase and kynureninase were found to increase cerebral kynurenate levels and have sedative and anticonvulsant effects on rats and mice.[59,60] *m*-Nitrobenzoylalanine **(64)** and *o*-methoxybenzoylalanine **(63)**, inhibitors of kynurenine–3-hydroxylase and kynureninase respectively, when administered to rats caused an increase in kynurenine and kynurenate levels in the blood, brain, liver and kidneys. *m*-Nitrobenzoylalanine **(64)** was more potent

than *o*-methoxybenzoylalanine **(63)** in increasing kynurenate levels and the effect was additive when they were co-administered. The effects included reduced locomotor activity in rats and the prevention of convulsions in mice.

Another benzoylalanine derivative, 2,4-dichlorodibenzoylalanine **(70)**, which is an even more potent inhibitor of kynurenine-3-hydroxylase was found to increase brain and plasma kynurenine and kynurenic acid levels 10-fold and 80-fold respectively after a single injection to rats. It also attenuated nerve cell in a gerbil model of ischemia.[64]



The novel class of *N*–(4-phenylthiazol-2-yl)benzenesulfonamides behave as potent inhibitors of kynurenine-3-hydroxylase.[67] 3,4-Dimethoxy-*N*-[4-(3-nitrophenyl)thiazol-2-yl]-benzenesulfonamide **(75)** is the most potent inhibitor of kynurenine-3-hydroxylase known to date, exhibiting an IC_{50} of 37 nM. It inhibited the enzyme *in vivo* and produced a 7.5-fold increase in brain kynurenate levels after oral administration to rats, and was more potent than 2,4-dichlorobenzoylalanine **(69)** at the same dosage. Studies have also shown that the compound reduced convulsions and induced coma in rats exposed to hyperbaric oxygen (HBO).[145] Hyperbaric oxygen therapy is used in a number of clinical disorders such as carbon monoxide poisoning and severe burns but is limited by the toxic

effects of high pressure oxygen. In conclusion it can be seen that modulation of the kynurenine pathway to prevent formation of neurotoxic quinolinic acid **(2)** and 3-hydroxykynurenine **(4)** and to augment kynurenic acid **(3)** levels represents a feasible therapeutic approach for the treatment of many neurological disorders.

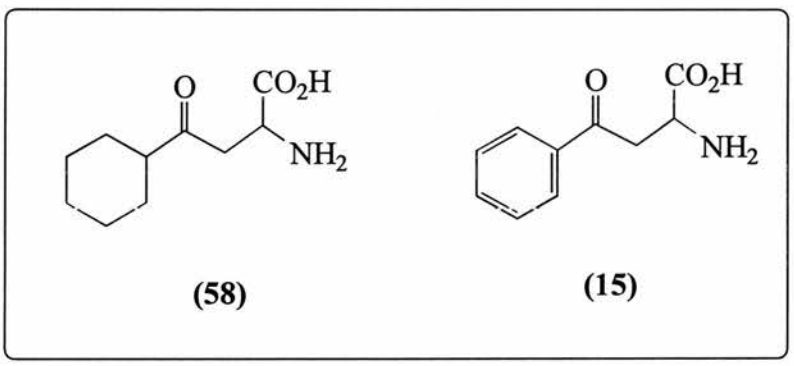
CHAPTER 2

2 SYNTHESIS OF KYNURENINE ANALOGUE INHIBITORS OF HUMAN RECOMBINANT KYNURENINASE

2.1 Inhibition studies at St. Andrews

Modulation of the kynurenine pathway as discussed in Chapter 1 is a possible therapeutic approach for the treatment of neurological disease and has resulted in significant interest in the synthesis of compounds as inhibitors of key enzymes on the pathway.[17] L-Kynurenine **(6)** as a substrate of both kynureninase and kynurenine-3-hydroxylase plays an important role on the kynurenine pathway. Therefore, the synthesis of kynurenine analogues which may compete with the substrate for the active site of these enzymes has formed the basis of much of the work.

Previous work within this group has involved the design and synthesis of several kynurenine analogues as inhibitors of kynureninase. A number of racemic kynurenine analogues were synthesised which were observed to inhibit the enzyme as discussed in Section 1.3.6. The cyclohexyl **(58)** derivative was a competitive inhibitor when tested against bacterial kynureninase, exhibiting a K_i value of 422 ± 47 μM. Desaminokynurenine **(15)** was found to be a mixed inhibitor of the enzyme, K_i = 11 ± 3 μM, and also acted as a substrate for the enzyme.[49]



(58) **(15)**

The use of kynureninase inhibition in the modulation of the kynurenine pathway as a possible treatment of neurological disorders requires inhibition studies that are reflective

of conditions *in vivo*. To date most of the studies have relied on a bacterial source of the enzyme, namely *Pseudomonas fluorescens*. This is not ideal since bacterial and mammalian kynureninase have different substrate specificities with bacterial kynureninase hydrolysing L-kynurenine faster than 3-hydroxy-L-kynurenine while mammalian kynureninase shows a much higher specificity for 3-hydroxy-L-kynurenine.[26,35] Although some work has been carried out on the rat enzyme, human kynureninase has not been available in quantities sufficient to carry out comprehensive kinetic studies. Recently human recombinant kynureninase has been successfully isolated and expressed within our group (see Chapter 3).

A number of bicyclic analogues have previously been synthesised within the group which have been tested against both human and bacterial sources of kynureninase.[38] All of the compounds were observed to behave as competitive inhibitors of the enzyme. The tetralone **(59)**, indanone **(60)** and naphthyl **(62)** derivatives were found to inhibit bacterial kynureninase more potently than human recombinant kynureninase (Table 2.1). In contrast the chromanone derivative **(61)** inhibited the human enzyme more potently than the bacterial with K_i values of 77 ± 23 μM and 162 ± 31 μM, respectively. The naphthyl derivative was the most potent of all of the bicyclic derivatives tested. Since all of these compounds were synthesised as racemates the K_i values obtained in each case may be halved as only L-amino acids are recognised by the enzyme active site. As mammalian kynureninase hydrolyses 3-hydroxy-L-kynurenine **(4)** preferentially it was our aim to synthesise several racemic analogues in which the 3-position of aryl ring was hydroxylated, such inhibitors ought to be more specific for human recombinant kynureninase.

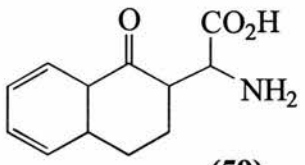
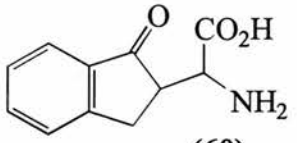
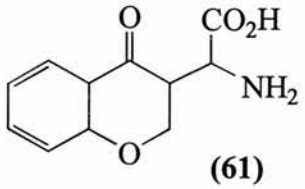
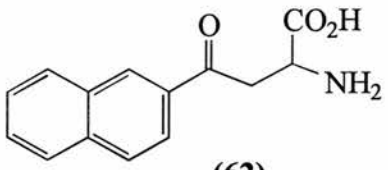
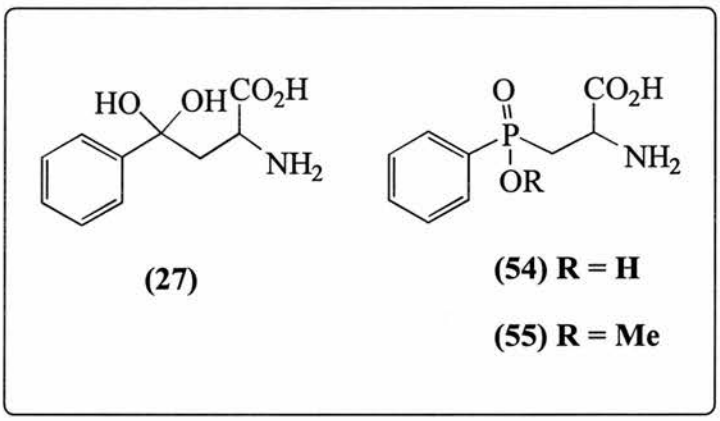
COMPOUND	BACTERIAL K_i /μM	HUMAN K_i/μM
 **(59)**	170 ± 24	227 ± 47
 **(60)**	34.9 ± 10	45 ± 12
 **(61)**	162 ± 31	77± 23
 **(62)**	5 ± 2	22 ± 6

Table 2.1

The postulated bound water mediated mechanism for the kynureninase reaction involves formation of a *gem*-diolate intermediate **(27)** which can be used as a model for the transition state.[42] This has led to the design and synthesis of inhibitors of kynureninase which mimic the tetrahedral intermediate but which are chemically stable and therefore potentially tight binding competitive inhibitors. Since the intermediate can be used as a model for the transition state, these compounds can be thought of as acting as transition state analogues. Both the phosphinic acid analogue **(54)** and its methyl phosphinate derivative **(55)** were synthesised and examined as transition state analogue inhibitors of bacterial kynureninase.[49] Both compounds were found to be weak competitive inhibitors of the enzyme with K_i values of 4.28 ± 0.1 mM and 0.88 ± 0.05 mM, respectively. Both compounds bound more weakly to the enzyme than kynurenine itself, which exhibited a K_m of 25.6 ± 0.6 μM.

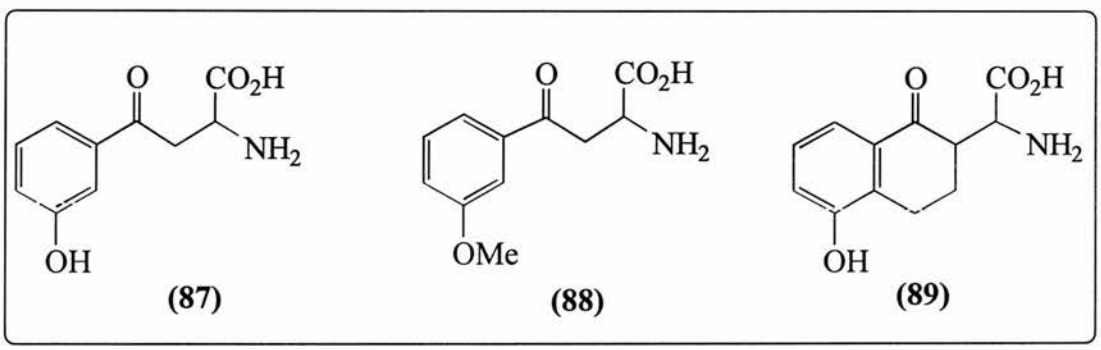


(27) **(54) R = H** **(55) R = Me**

It was our aim to synthesise a number of analogues of 3-hydroxykynurenine which would mimic the *gem*-diolate intermediate **(27)** and behave as transition state analogue inhibitors of human recombinant kynureninase.

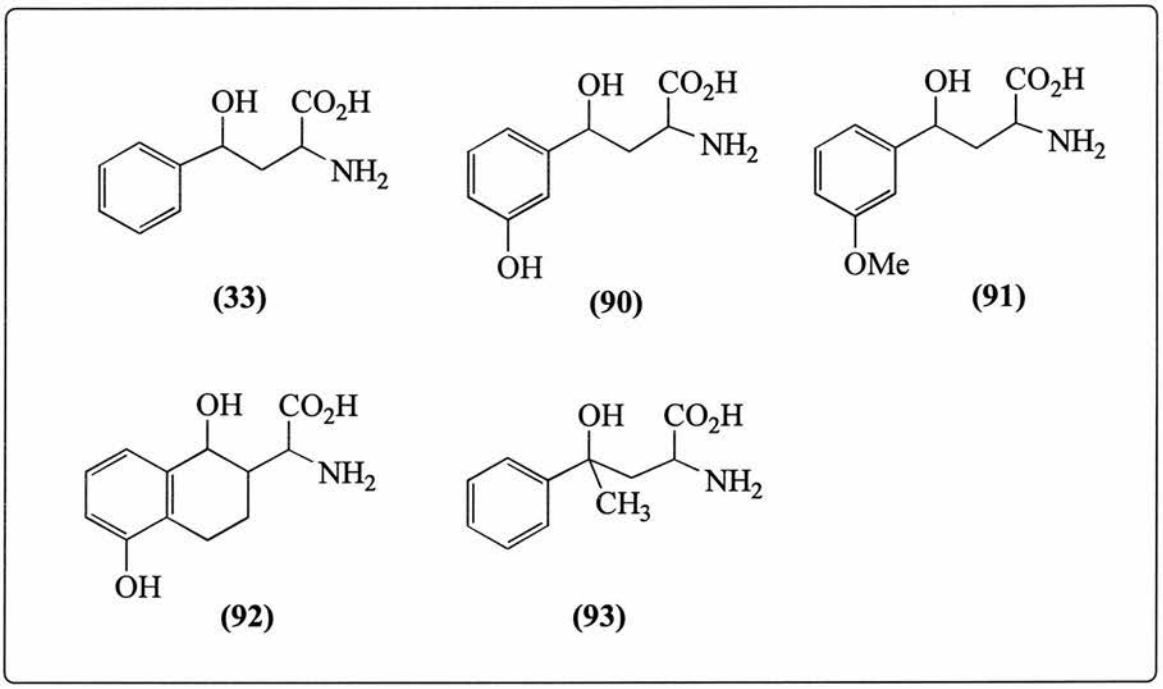
2.2 Synthetic targets

The first aim was to synthesise racemic analogues of 3-hydroxykynurenine and examine their inhibition of human recombinant kynureninase. It was our aim to synthesise both monocyclic and bicyclic compounds **(87)-(89)** and to determine whether a hydroxyl group or a methoxy group at the 3-position of the aryl ring affects interactions with the active site of the enzyme.



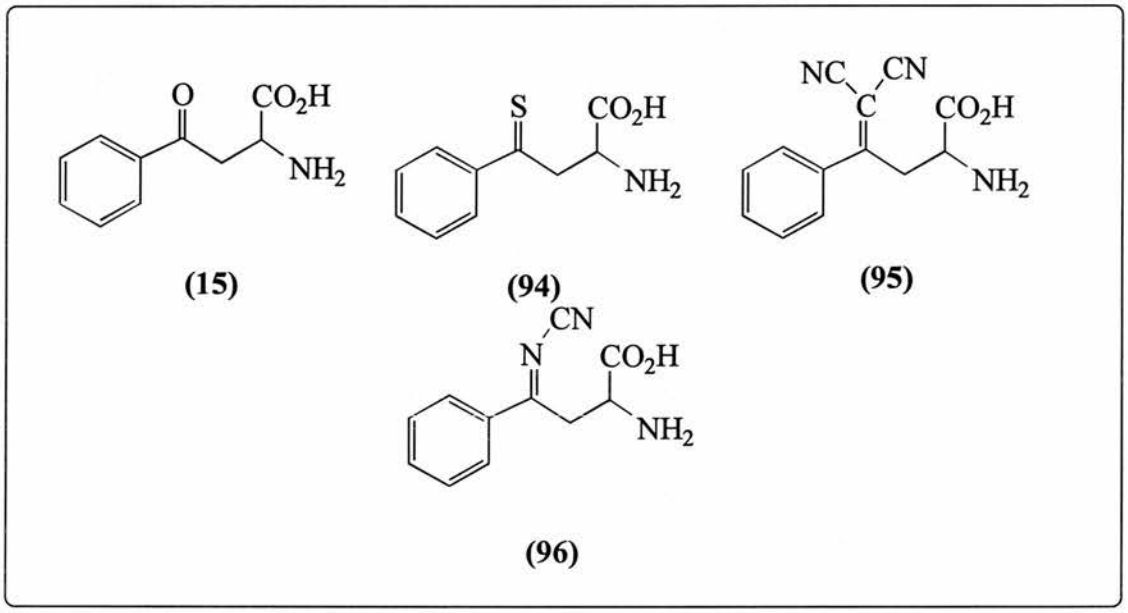
(87) **(88)** **(89)**

It was also intended to synthesise derivatives of kynurenine and 3-hydroxykynurenine **(33), (90)-(93)** in which the γ-carbonyl group was reduced, as transition state analogues.

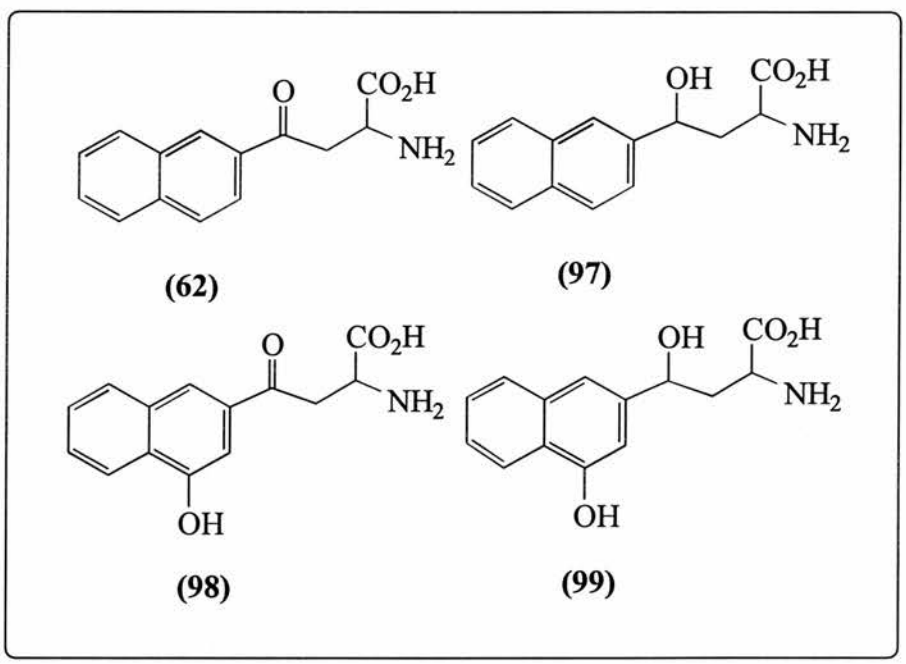


Compound **(90)** had previously been synthesised as a minor product in an attempted synthesis of **(87)**.[146] Therefore a strategy which allowed discrete synthesis of **(87)** and **(90)** in high yield was required.

A third objective was the synthesis of a group of analogues of desaminokynurenine **(15)** in which the γ-carbonyl was replaced by other atoms such as sulfur or molecular fragments such as malononitrile and cyanamide. In medicinal chemistry such functional groups have been used as bioisosteres for the carbonyl group.[147] Bioisosteres have similar physical and chemical properties and produce similar biological effects. Such compounds may be potent and novel inhibitors of the enzyme.



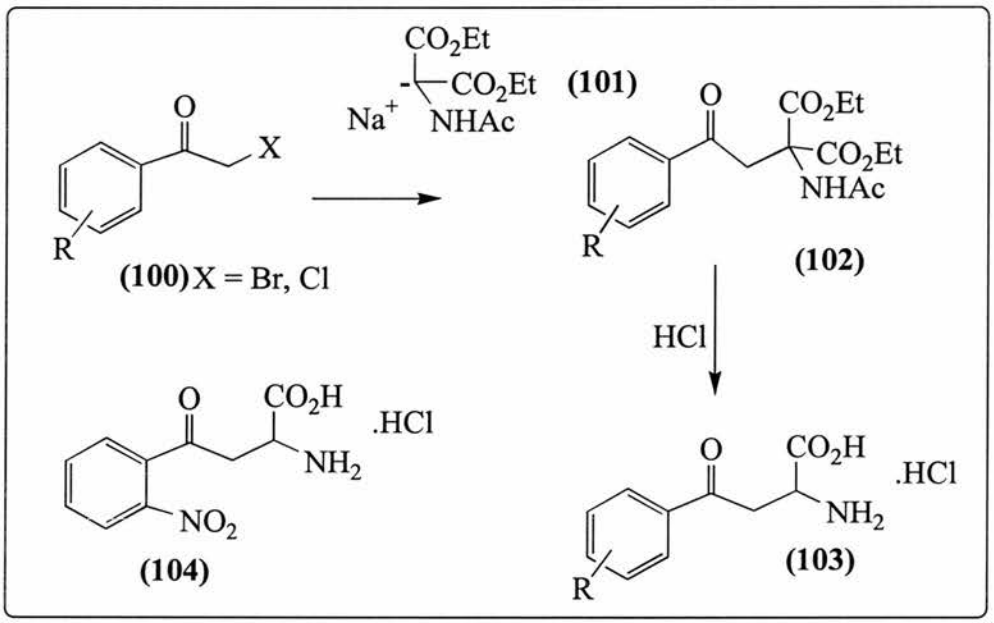
The potent inhibition of both bacterial and human recombinant kynureninase by the naphthalene derivative **(62)** warranted further investigation and the synthesis of a number of naphthalene analogues of kynurenine **(97)-(99)** was also investigated.



2.3 Synthetic routes to racemic kynurenine analogues

2.3.1 Literature synthesis

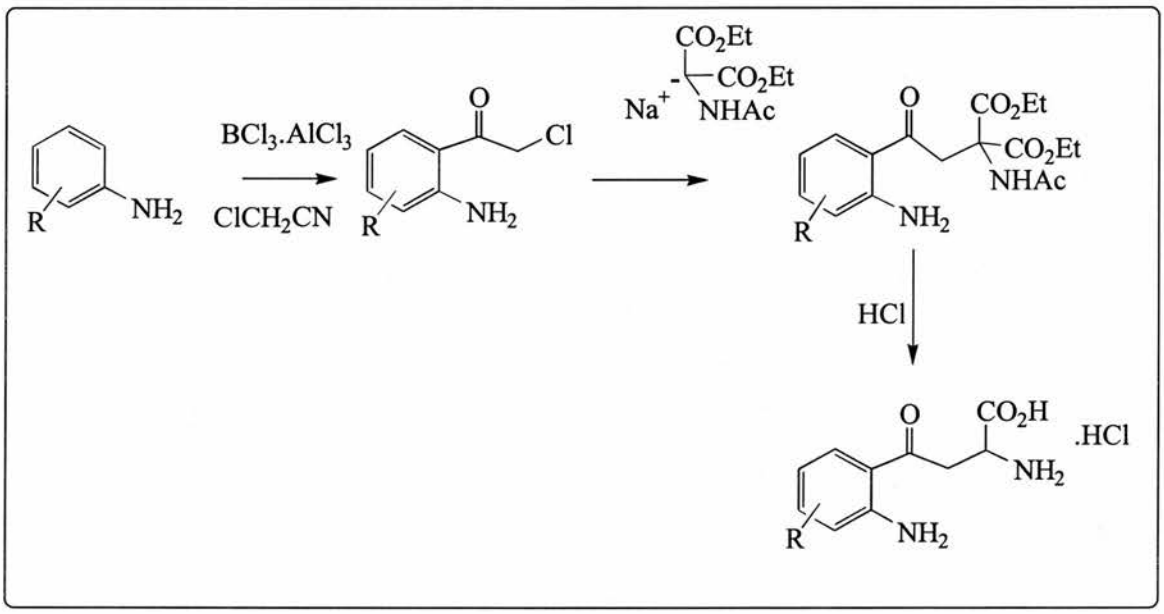
A number of synthetic strategies towards kynurenine analogues have been reported in the literature.[62,148] All of the synthetic routes reported involved reaction of an α-haloketone **(100)** with the preformed anion of diethyl acetamidomalonate **(101)** to afford a diethyl arylacylacetamidomalonate derivative **(102)**. Subsequent acid hydrolysis then afforded a racemic amino acid product **(103)** (Scheme 2.1).



Scheme 2.1

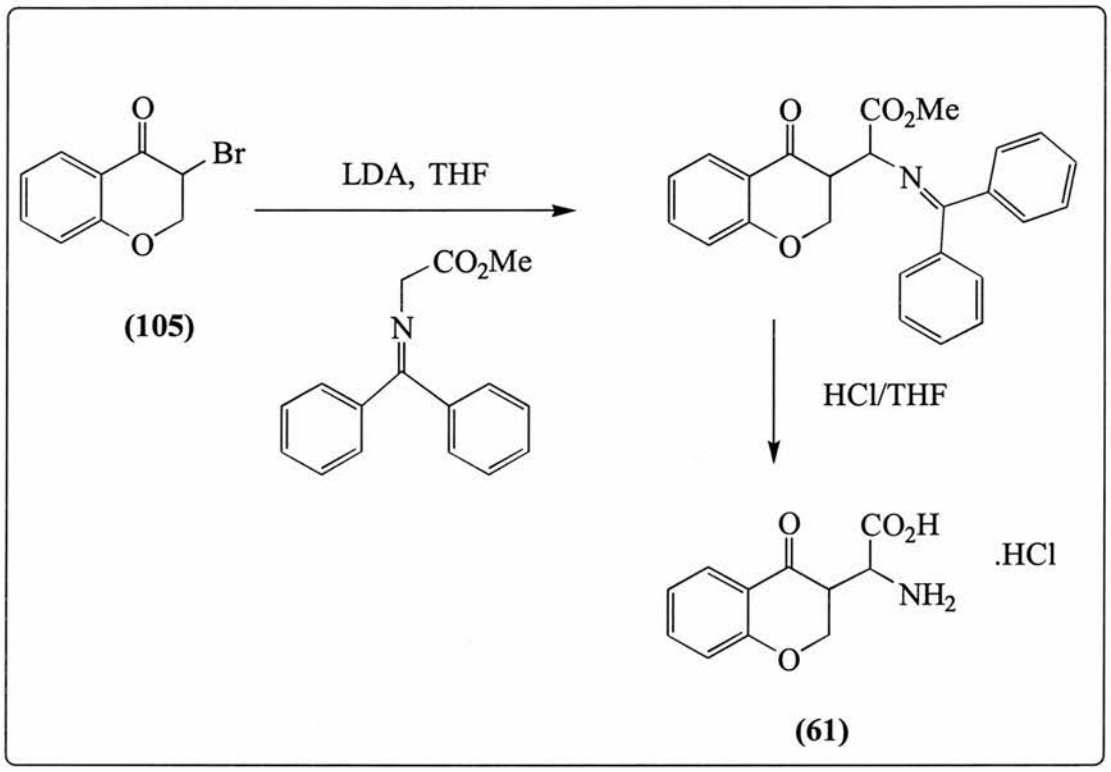
A number of benzoylalanine analogues of kynurenine, synthesised as inhibitors of kynureninase and kynurenine-3-hydroxylase, were reported by Pellicari *et al.*[62] The *o*-methoxy- **(63)**, *o*-nitro- **(104)** and *m*-nitrobenzoylalanine **(64)** analogues were synthesised by reaction of the α-bromoketone with the anion of acetamidomalonate **(101)** generated by reaction with sodium hydride in DMF at 0 °C. The hydrochloride salt of the amino acid was obtained following acid hydrolysis in HCl. Varasi *et al.* also reported a similar synthetic route to kynurenine analogue inhibitors of KAT.[148] Substituted anilines were converted to their α-chloroacetophenone derivatives under Houben-Hoesch conditions.

Subsequent reaction with the anion of diethyl acetamidomalonate and acid hydrolysis afforded the racemic kynurenines (Scheme 2.2).



Scheme 2.2

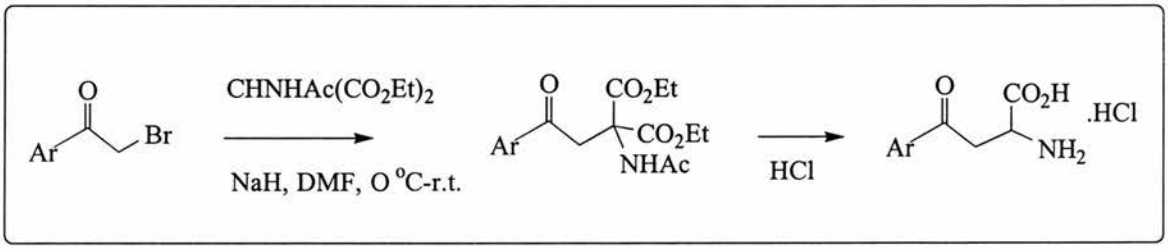
The bicyclic analogue **(61)** reported by Muirhead was synthesised by a number of routes.[38,149] The strategy which afforded the highest yield involved reaction of α-bromochromanone **(105)** with O'Donnell's reagent which was used as a glycine equivalent.[150] Acid hydrolysis afforded the amino acid in 40% yield (Scheme 2.3).



Scheme 2.3

2.3.2 General strategy towards synthetic targets

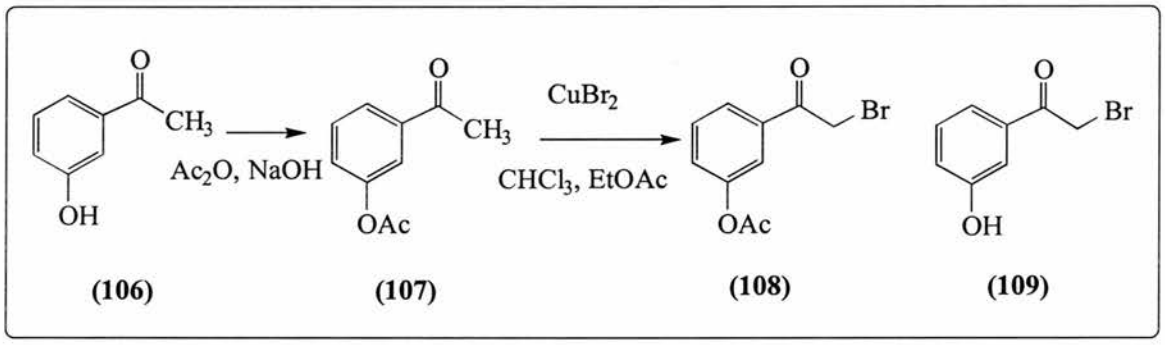
The method reported by Pellicari *et al.* was adopted and modified for the synthesis of substrate analogues as potential inhibitors of human recombinant kynureninase. The anion of diethyl acetamidomalonate was formed from reaction with sodium hydride in DMF. Subsequent addition of the corresponding α-bromoketone resulted in formation of a diethyl arylacylacetamidomalonate derivative. Deprotection of the coupled product by acid hydrolysis resulted in formation of the racemic amino acid as a hydrochloride salt (Scheme 2.4).



Scheme 2.4

2.3.3 Synthetic strategies towards 3-hydroxykynurenine analogues

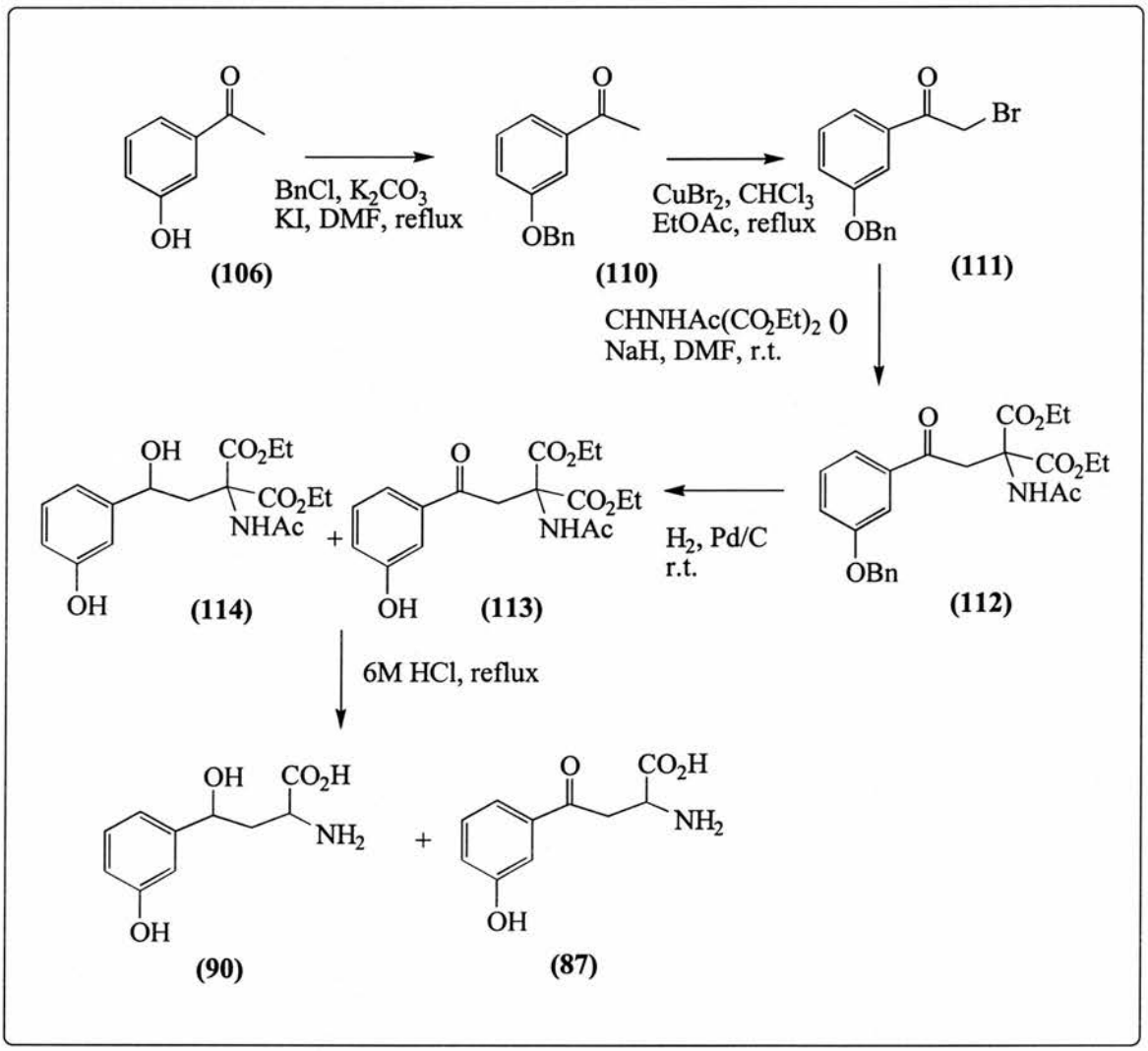
A number of strategies have been adopted in previous attempts to synthesise 3'-hydroxydesaminokynurenine **(87)** within the group.[146] In preparations of 3-hydroxykynurenine analogues protection of the free 3'-hydroxyl group is essential to prevent reaction at this site in subsequent coupling procedures. An initial method involved protection of 3'-hydroxyacetophenone **(106)** with an acetyl group (Scheme 2.5). However, bromination of the acetylated compound **(107)** using bromine in methanol resulted in deprotection of the 3'-hydroxyl group as well as bromination of the benzene ring. A more selective bromination procedure using cupric bromide was thus employed.[151,152,153] While the α-bromoketone was formed selectively partial deprotection occurred to give a mixture of compounds **(108)** and **(109)**.



Scheme 2.5

A strategy for improving the synthesis involved using a benzyl protecting group, which proved to be more stable under the bromination conditions.[146] The benzylated acetophenone **(110)** was generated and brominated successfully to afford **(111)** (Scheme 2.6). Coupling with the anion of diethyl acetamidomalonate **(101)** afforded the protected precursor **(112)** of 3'-hydroxydesaminokynurenine **(87)**. Attempts to deprotect **(112)** by acid hydrolysis in 6M HCl were unsuccessful as the large protecting groups made the compound insoluble in the acid. The benzyl protecting group was thus removed *via* catalytic hydrogenation to afford a mixture of compounds **(113)** and **(114)**. Subsequent deprotection in 6M HCl afforded 1,3-dihydroxydesaminokynurenine **(90)** in 29% yield. Some evidence for the presence of 3'-hydroxydesaminokynurenine **(87)** was observed by NMR spectroscopy but the compound could not be isolated. An improved synthetic

strategy which would allow discrete synthesis of 3'-hydroxy desaminokynurenine **(87)** was thus investigated.



Scheme 2.6

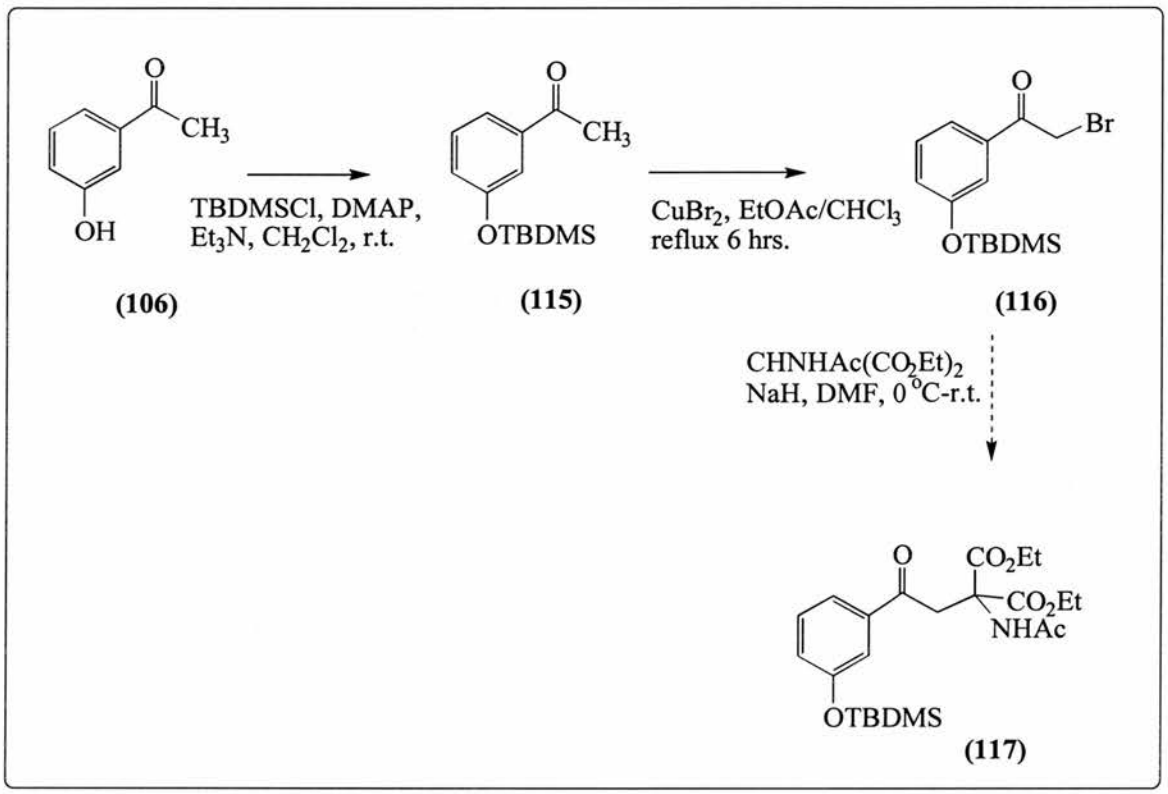
2.3.3.1 Synthesis of 2-amino-4-oxo-4-(3-hydroxy-phenyl)-butyric acid (3'-hydroxydesaminokynurenine) (87)

The strategy for improving the synthesis of 3'-hydroxydesaminokynurenine **(87)** initially involved using a *tert*-butyldimethylsilyl group to protect the 3'-hydroxy acetophenone. *Tert*-Butyldimethylsilyl protecting groups are stable under a variety of reaction conditions and can be removed in a more selective manner without reduction of the γ-carbonyl group.[154,155,156] 3'-Hydroxyacetophenone **(106)** was thus reacted with *tert*-butyldimethylsilyl chloride (TBDMSCl), DMAP and triethylamine in dry dichloromethane at room temperature for twenty four hours (Scheme 2.7). Purification by column chromatography gave the protected acetophenone **(115)** as a colourless oil in 78% yield. Resonances observed in the ^{1}H NMR spectrum at 0.22 ppm and 0.99 ppm were attributed to the methyl and *tert*-butyl groups, respectively, from the TBDMS protecting group.

The bromide **(116)** was then synthesised from **(115)** in 53% yield using cupric bromide in refluxing ethyl acetate with chloroform as a cosolvent. After heating under reflux for six hours the reaction mixture was cooled, filtered to remove copper salts and decolourised. Removal of the solvent under reduced pressure afforded a yellow oil which was used without further purification. The ^{1}H NMR spectrum of **(116)** displayed a resonance at 4.45 ppm assigned to the brominated methylene group. The mass spectrum also showed peaks of almost equal intensity at 330 and 328 *m/z* units for the molecular ions respectively, as expected for the ^{81}Br and ^{79}Br isotopes of a monobrominated species.

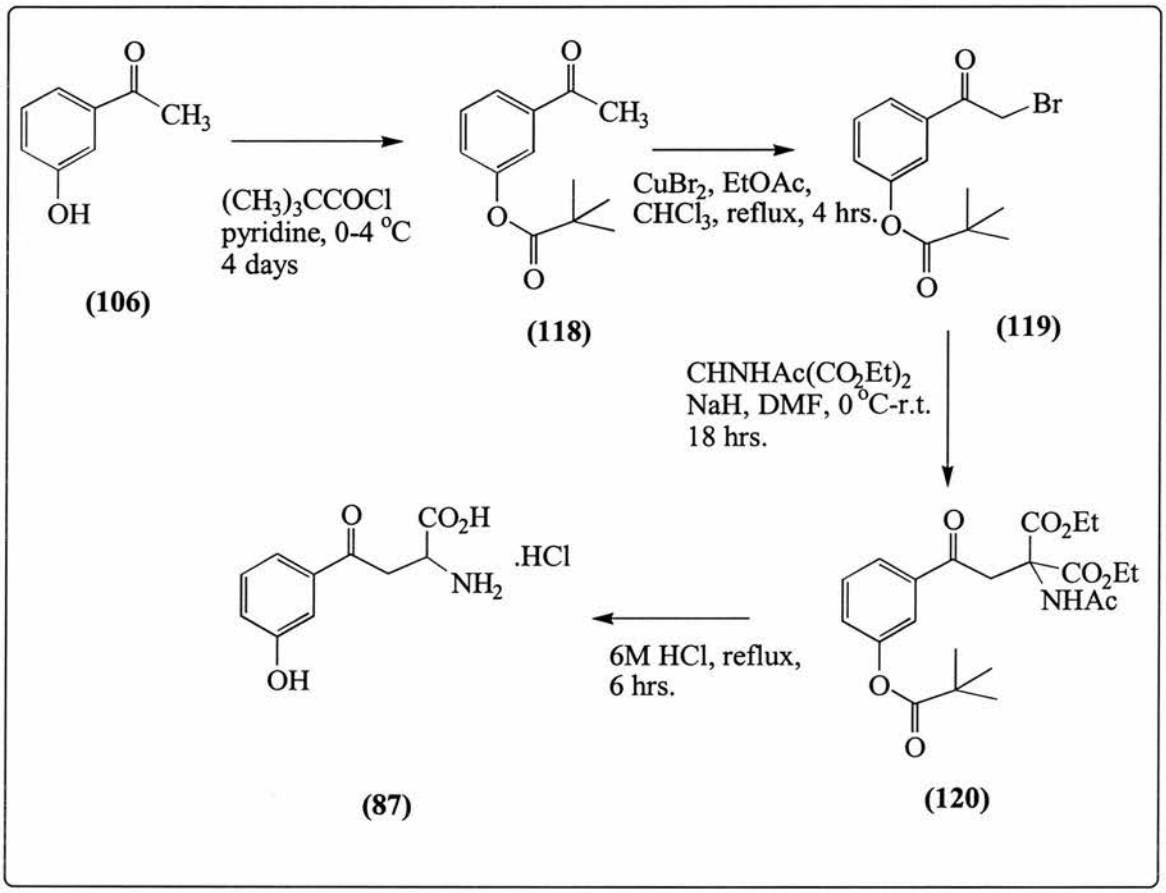
The bromide **(116)** was then coupled to diethyl acetamidomalonate. The anion of diethyl acetamidomalonate **(101)** was formed as a pale yellow solution in dry redistilled DMF with NaH at 0 °C over three hours. Slow addition of the bromide over one hour resulted in a yellow solution which was stirred overnight. After quenching with dilute acid the reaction mixture was extracted in diethyl ether and the solvent removed to give a yellow oil. Analysis of the compound by NMR spectroscopy was inconclusive. Unreacted diethyl acetamidomalonate subsequently precipitated from the oil as verified by NMR spectroscopy. Repetition of the reaction in which the mode of addition was reversed and the preformed anion **(101)** was added to the brominated acetophenone **(116)** resulted in reaction as verified by tlc. However, there was a large number of products present and

purification by column chromatography using ethyl acetate and petroleum ether as eluents afforded trace fractions too small to characterise.



Scheme 2.7

The synthesis of **(87)** (Scheme 2.8) was modified to employ a pivaloate ($OCOC(CH_3)_3$) protecting group, which was thought to be more stable under reaction conditions. Pivaloate esters can be cleaved *via* acid hydrolysis and should be stable to the conditions required for the coupling of **(118)** with diethyl acetamidomalonate **(101)**.[157] The pivaloate ester of 3'-hydroxyacetophenone was synthesised using a method reported by Mengel *et al.*,[157] by reaction of 3'-hydroxyacetophenone **(106)** with pivaloyl chloride in dry pyridine at 4 °C for ninety six hours. This gave the pivaloate ester as a clear oil **(118)** in 79% yield. A resonance observed in the ^1H NMR spectrum at 1.35 ppm was attributed to the *tert*-butyl group of the pivaloate ester. New resonances in the ^{13}C NMR spectrum observed at 27.04, 39.08 and 177.05 ppm were assigned respectively to the methyl, quarternary and carbonyl carbons of the pivaloate group. Bromination of **(119)** using established methodology then afforded the α-brominated compound **(119)** as a yellow oil in 63% yield. The ^1H NMR spectrum exhibited a resonance at 4.42 ppm, integrating for two protons, which was assigned to the brominated methylene group.



Scheme 2.8

Coupling of the bromide **(119)** with diethyl acetaminomalonate was performed under standard conditions. After further purification **(120)** was obtained as a white crystalline solid in 57% yield. Resonances in the ^{1}H NMR spectrum at 1.24, 1.35 and 2.02 ppm can be assigned respectively to the methyl protons of the ethyl ester, pivaloyl group and amido groups. Low resolution mass spectrometry of the compound showed a molecular ion peak $[M]^+$ at the expected 435 *m/z* units.

Acid hydrolysis of **(120)** in 6M HCl under reflux conditions for six hours followed by an ethyl acetate wash to remove any unreacted starting materials and removal of solvent under reduced pressure afforded the target compound, 3'-hydroxy desaminokynurenine **(87)** as an off-white solid in 76% yield. The formation of **(87)** was confirmed by NMR spectroscopy and mass spectrometry. In the ^{1}H NMR spectrum a doublet at 3.73 and a

triplet at 4.39 were assigned to the β-CH_2 and α-CH hydrogens respectively. CI Mass spectrometry exhibited a $[M + H]^+$ peak at 210 *m/z* units. The compound was pure by microanalysis and a crystal structure of **(87)** was obtained.

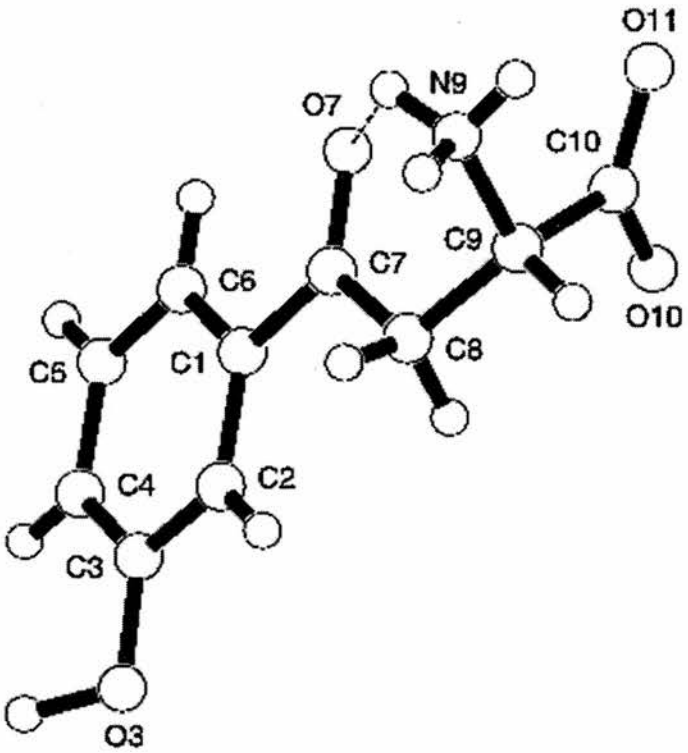


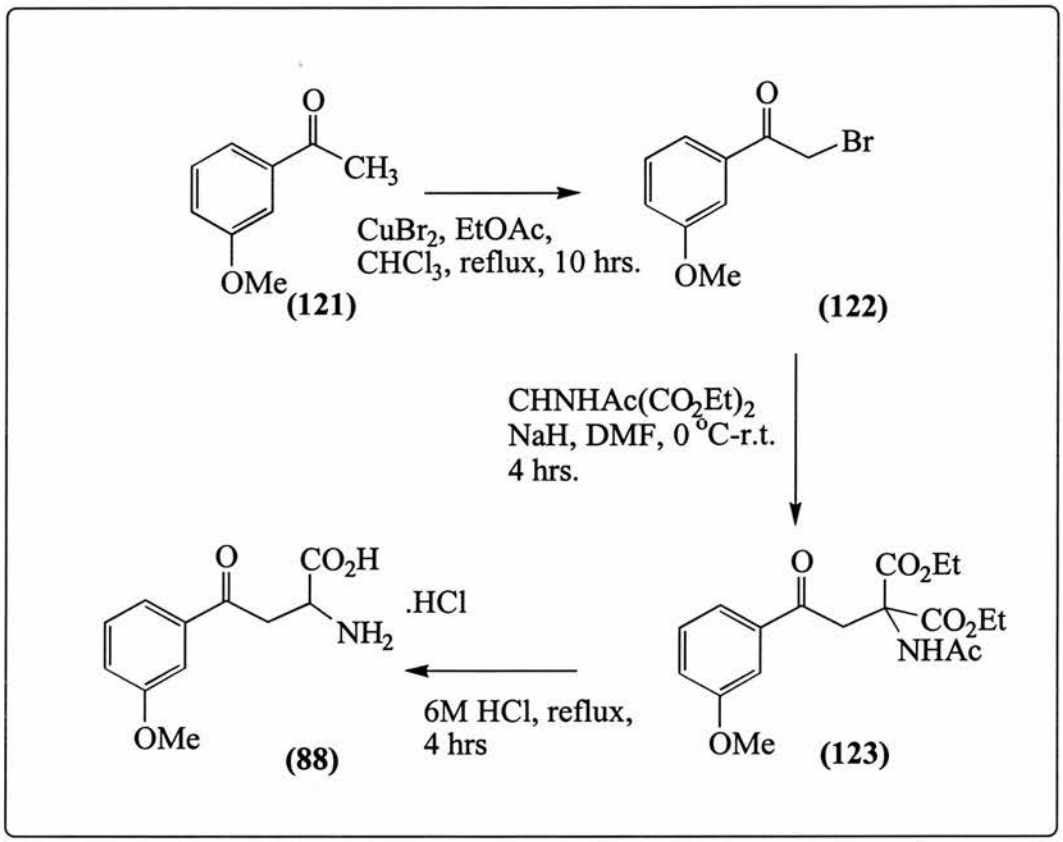
Figure 2.1 Crystal structure of 3'-hydroxydesaminokynurenine (87)

3'-Hydroxydesaminokynurenine **(87)** crystallised as its zwitterion from deuterium oxide. The collection of data and the structure solution were carried out by Dr. Alex Slawin at St. Andrews. Crystal data and relevant structure solution data are given in the appendix (Crystal data set A.). An intramolecular hydrogen bond is observed to occur between the oxygen atom of the γ-carbonyl and a hydrogen on the ammonium group. The C(10)-O(10) and C(10)-O(11) bond distances for the carboxylate group of 1.251(3)Å and 1.259(3)Å are very similar and indicate the significant delocalisation of the negative charge. These distances are comparable to the C(7)-O(7) bond distance of 1.229Å observed for the γ-carbonyl. The N-H distances are estimated to be 0.94Å, 0.91Å and 0.83Å, while the bond length of the hydroxyl group, O(3)-H(3), is estimated to be 1.07Å.

2.3.4 Synthesis of 2-amino-4-oxo-(3-methoxy-phenyl)-butyric acid (3'-methoxy desaminokynurenine) (88)

The synthesis of 3'-methoxydesaminokynurenine **(88)** was also undertaken. This was to investigate the importance of hydrogen bonding by the hydroxyl group at the active site of kynureninase. The target compound offered synthetic advantages to 3'-hydroxydesaminokynurenine, requiring fewer protection and deprotection steps.

3'-Methoxyacetophenone **(121)** was heated under reflux with cupric bromide in ethyl acetate and chloroform for ten hours to afford the α-bromoketone **(122)** as a yellow sticky solid in 92% yield (Scheme 2.9). Resonances observed in the ^{1}H NMR spectrum at 3.83 and 4.43 ppm were assigned to the methoxy methyl and brominated methylene groups respectively. The mass spectrum showed molecular ion peaks, of equal intensity, at 228 and 230 *m/z* units indicative of the presence of bromine isotopes. The bromide **(122)** was added to the anion of diethyl acetamidomalonate as before. The coupled product **(123)** was obtained by crystallisation from diethyl ether as a white crystalline solid in 45% yield. The compound was pure by microanalysis. The ^{1}H NMR spectrum showed resonances at 1.23 and 4.25 ppm due to the methyl and methylene hydrogens, respectively of the ethyl ester group, and at 1.96 ppm assigned to the methyl hydrogens of the acyl group. Deprotection of compound **(123)** in 6M HCl and further purification by preparative cellulose tlc using water, isopropyl alcohol and ammonia as eluents afforded 3'-methoxydesaminokynurenine **(88)** in 79% yield as a white solid. The ^{1}H NMR spectrum showed a doublet at 3.80 ppm and a triplet at 4.47 ppm due to the β-CH_2 and α-CH hydrogens, respectively. Mass spectroscopy also displayed a molecular ion peak at 224 *m/z* units. Analytical HPLC (C_{18} reverse phase silica, flow rate 3ml/min, 1% acetic acid, 20% methanol) showed the presence of a single peak (Figure 2.2).



Scheme 2.9

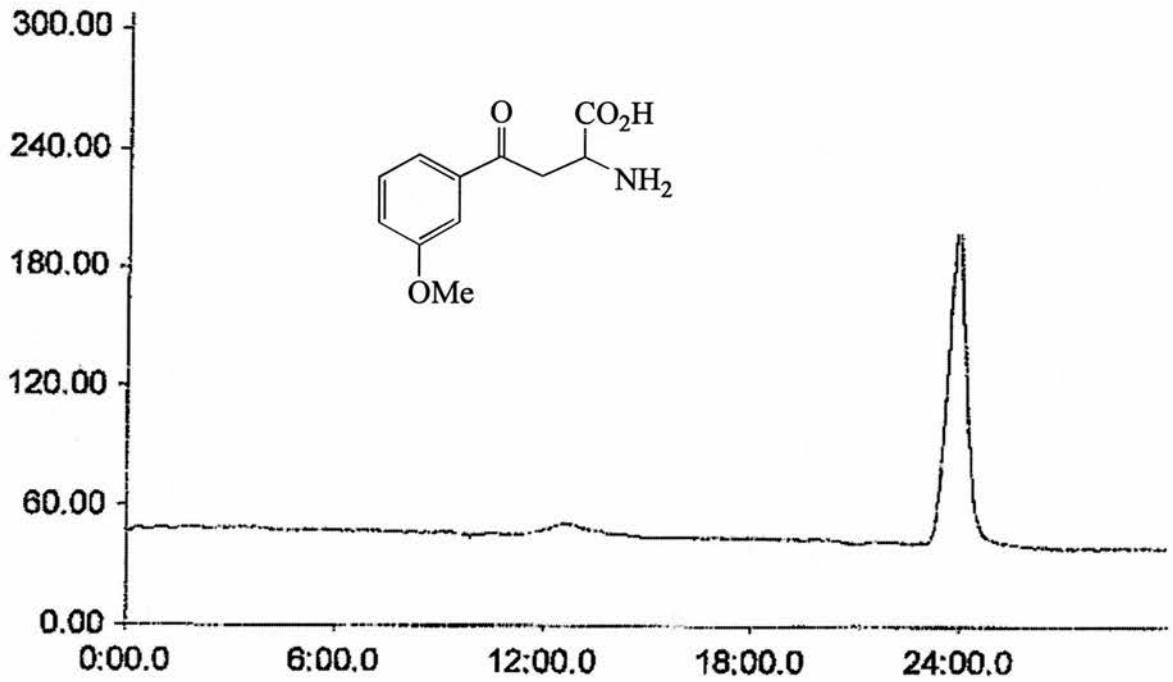


Figure 2.2

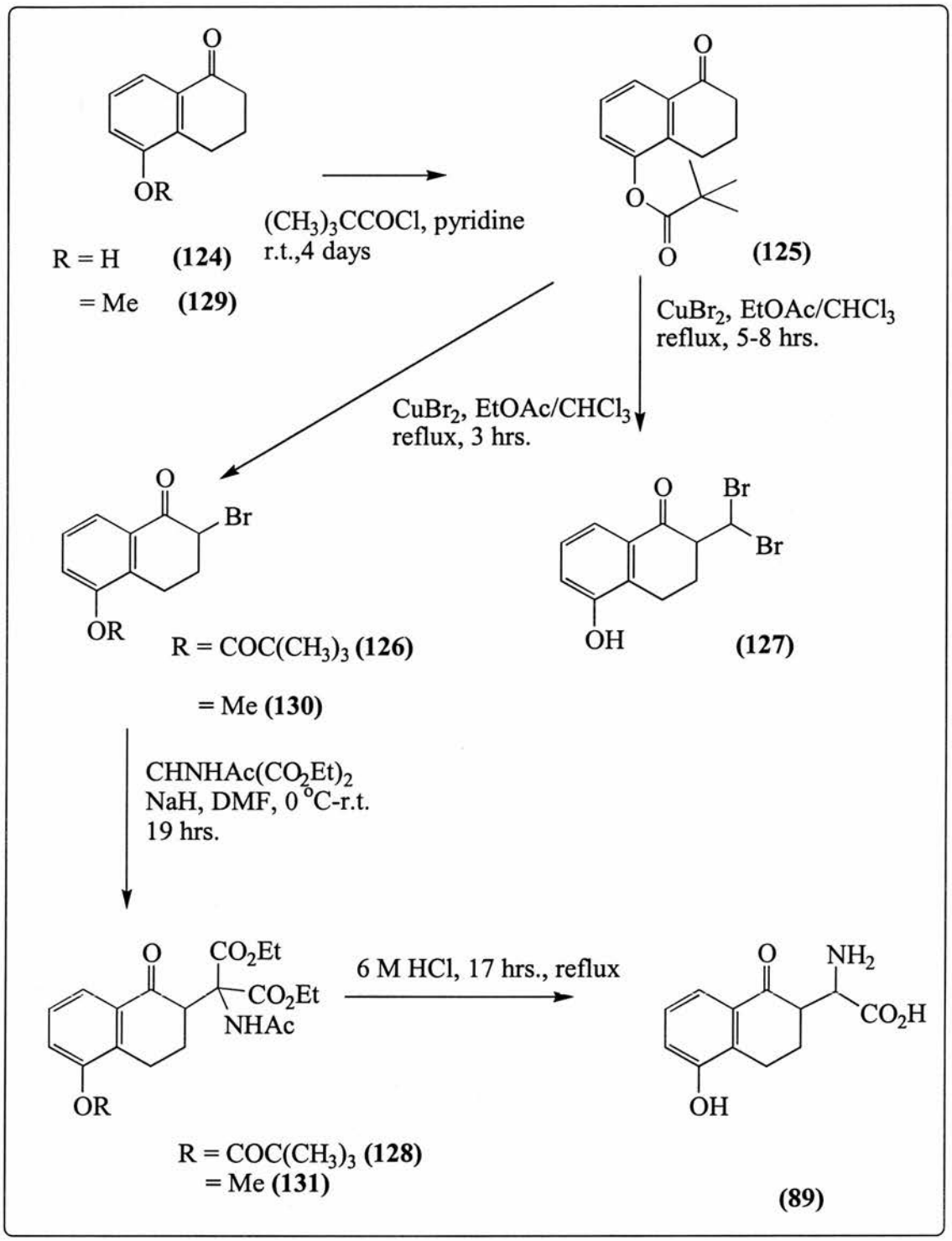
2.3.5 Synthesis of amino-(5-hydroxy-1-oxo-1, 2, 3, 4-tetrahydro-naphthalen-2-yl)-acetic acid (5-hydroxy-1-tetralone derivative) (89)

The strategy used in the preparation of 3'-hydroxydesaminokynurenine **(87)** was applied to the synthesis of the tetralone derivative **(89)** (Scheme 2.10). 5-Hydroxyl-1-tetralone **(124)** was reacted with pivaloyl chloride in dry pyridine over four days to afford the pivaloate ester **(125)** as a colourless oil in 71% yield. The compound was pure by microanalysis. The low resolution mass spectrum exhibited the molecular ion peak at 246 *m/z* units. A new resonance in the ^{1}H NMR spectrum observed at 1.39 ppm was assigned to the methyl protons of the pivaloate ester group ($OCOC(CH_3)_3$). Resonances in the ^{13}C NMR spectrum at 27.18, 39.24 and 176.71 ppm were attributed respectively to the methyl carbons, quaternary carbon and carbonyl carbon of the pivaloate ester. Subsequent bromination of **(125)** to afford the α-brominated compound **(126)** was initially unsuccessful. Reaction of **(125)** with cupric bromide in refluxing ethyl acetate with chloroform as a cosolvent for five hours resulted in incomplete reaction verified by tlc. However, continuation of the reaction for a further three hours resulted in the formation of the dibrominated compound **(127)**. This was confirmed by low resolution mass spectrometry which exhibited molecular ions at 402, 404 and 406 *m/z* units, an isotope pattern indicative of the presence of a dibrominated species **(127)**. The ^{13}C NMR spectrum also displayed a resonance at 183.71 ppm which is attributed to a carbonyl carbon adjacent to the bromine groups. Reduction of the reaction time to five hours resulted in formation of a mixture of both the mono and dibrominated tetralone derivatives. The bromination reaction was difficult to follow by tlc because both the mono and dibrominated compounds had very similar R_f values. Subsequent reduction of the reaction time to three hours resulted in formation of the monobrominated compound **(126)** in 49% yield. The microanalytically pure oil exhibited resonances in the ^{1}H NMR spectrum at 49.58 and 190.01 ppm assigned to the brominated methylene carbon and adjacent carbonyl. These can be compared with resonances observed for the dibrominated compound **(127)** at 66.17 and 183.17 ppm of the methylene carbon and carbonyl, respectively. The low resolution mass spectrum also showed M+1 peaks for the brominated product at 325 and 323 *m/z* units.

The protected tetralone derivative **(128)** was synthesised by reaction of **(126)** with the preformed anion of diethyl acetamidomalonate **(101)** in DMF at room temperature for nineteen hours. Further purification by column chromatography using ethyl acetate and petroleum ether as eluents afforded a white solid in 35% yield. Characterisation by microanalysis, NMR spectroscopy and mass spectrometry confirmed the structure of **(128)**. The precursor **(128)** was deprotected *via* acid hydrolysis in refluxing 6M HCl for seventeen hours which was monitored by tlc. The poor solubility of the precursor necessitated a considerable increase in reaction time. Upon cooling, an off-white solid precipitate from the acid was filtered off and dried under vacuum. The 5-hydroxy-1-tetralone amino acid derivative **(89)** was obtained in a low yield of 24%. A doublet at 4.37 ppm due to the α-CH hydrogen was observed in the ^{1}H NMR spectrum. There was no evidence for the presence of diastereomers by ^{1}H NMR spectroscopy. Only a single band was observed by tlc using reverse phase silica and a number of water/acetonitrile mixtures as eluents.

In order to compare the reactivity of the pivaloate protected tetralone derivative **(125)** under bromination conditions with other tetralone compounds, 5-methoxy-1-tetralone **(129)** was brominated under the same conditions. The reaction afforded the monobrominated derivative **(130)** as a yellow oil in 98% yield with no evidence for the formation of the dibrominated compound. The microanalytically pure compound was characterised by NMR spectroscopy and mass spectrometry. Subsequent coupling of **(130)** with diethyl acetamidomalonate afforded the protected 5-methoxy tetralone derivative **(131)** in 13% yield as a brown solid. The compound was characterised by microanalysis, NMR spectroscopy and mass spectrometry.

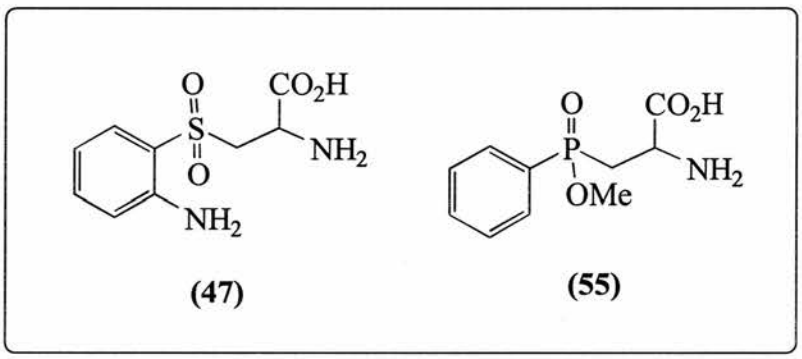
The evidence from the bromination reactions of both tetralone derivatives **(125)** and **(129)** suggested that the pivaloate derivative is more reactive to bromination conditions than the methoxy derivative and that the monobrominated pivalate ester **(126)** is more reactive than the pivaloate ester prescursor **(125)** to bromination. This may be due to the pivalate ester **(125)** being less soluble in the reaction solvent than the methoxy tetralone **(129)**. This would mean that less of the pivaloate ester **(125)** would be in solution to react with the cupric bromide resulting in dibromination.


O
OR
R = H (124)
= Me (129)
(CH3)3CCOCl, pyridine
r.t.,4 days
O
O
O
(125)
CuBr2, EtOAc/CHCl3
reflux, 5-8 hrs.
CuBr2, EtOAc/CHCl3
reflux, 3 hrs.
O
Br
OR
R = COC(CH3)3 (126)
= Me (130)
O
Br
Br
OH
(127)
CHNHAc(CO2Et)2
NaH, DMF, 0 °C-r.t.
19 hrs.
O
CO2Et
CO2Et
NHAc
OR
R = COC(CH3)3 (128)
= Me (131)
6 M HCl, 17 hrs., reflux
O
NH2
CO2H
OH
(89)

Scheme 2.10

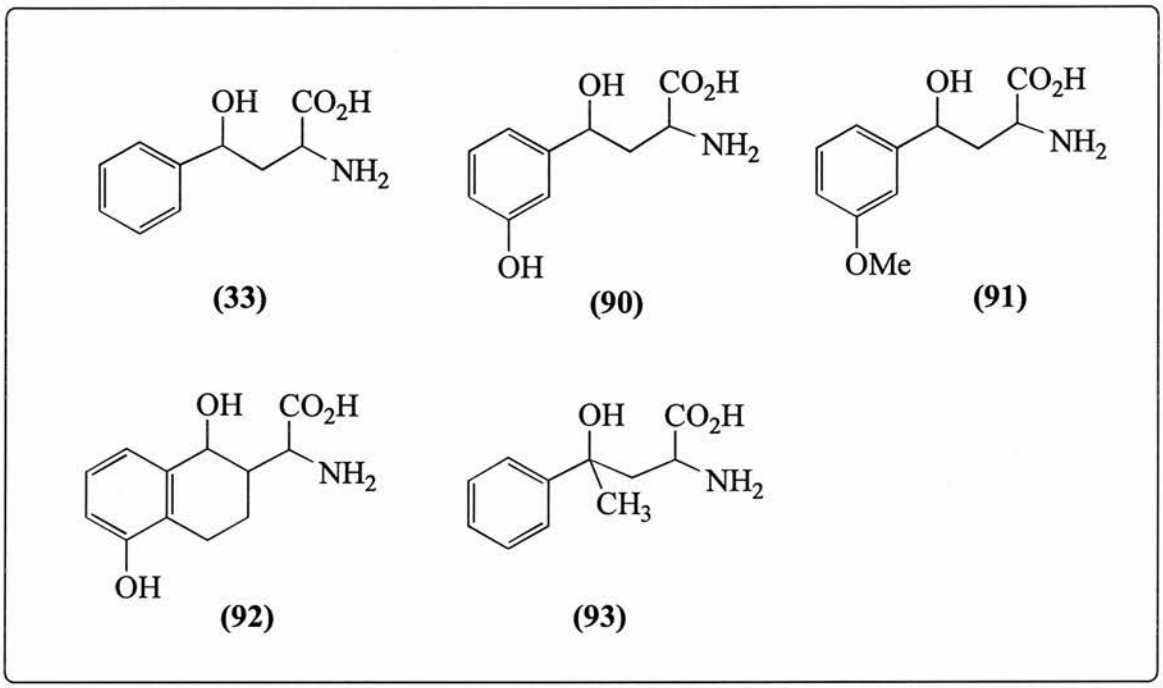
2.4 Kynurenine analogues modified at the γ-position

Transition state analogues, which mimic the postulated gem diolate intermediate **(27)** and therefore serve as models for the transition state may behave as potent competitive inhibitors of kynureninase binding tightly to the enzyme active site. This hypothesis is partly based on the competitive inhibition observed for the (4*R*,2*S*) **(16a)** and (4*S*,2*S*) **(16b)** dihydrokynurenines exhibiting K_i values of 1.3 and 0.3 μM respectively, when tested against bacterial kynureninase. As a result of this postulate several novel analogues of kynurenine such as **(47)** and **(55)** have been reported as transition state analogue inhibitors of the enzyme, as discussed in Section 1.3.6. However, there have been no other reported examples of analogues of the dihydrokynurenines (**(16a)**, **(16b)**), where the γ-carbonyl is reduced to an alcohol.

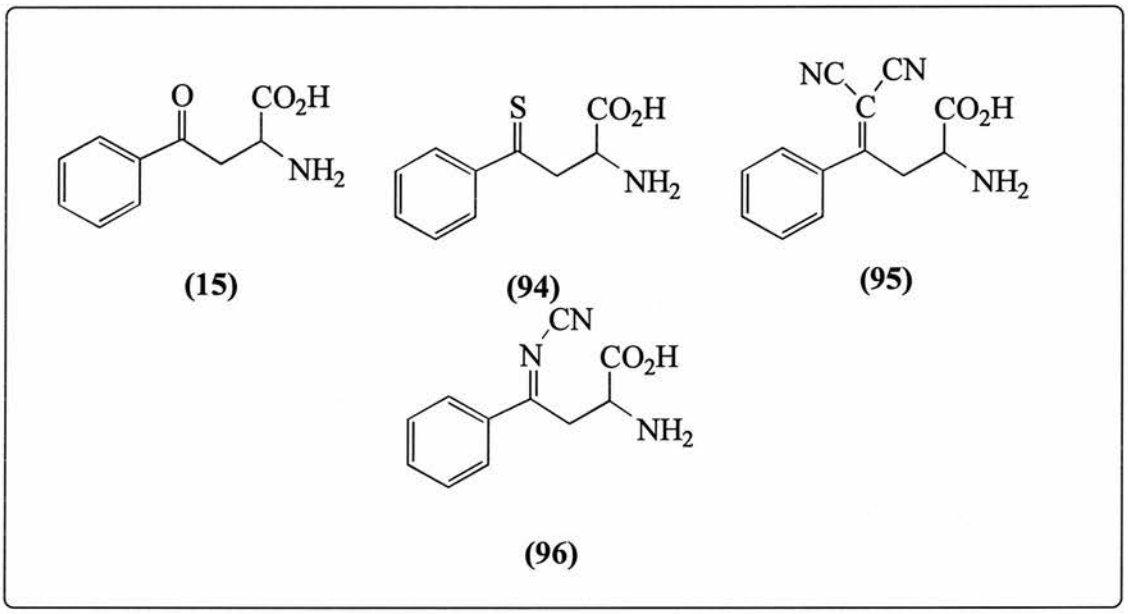


(47) **(55)**

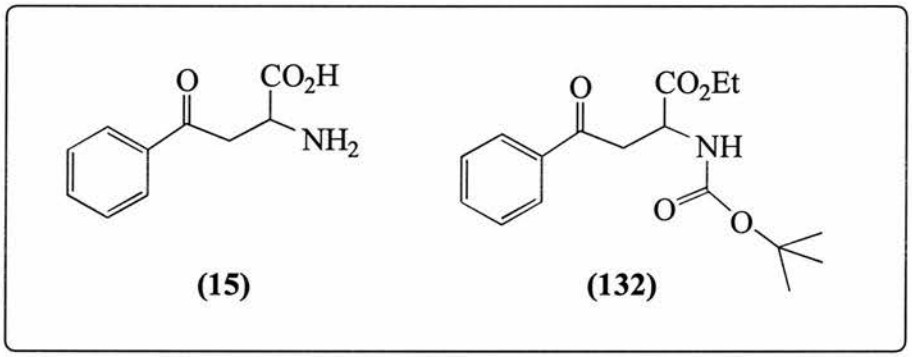
The synthesis of several racemic analogues of the dihydrokynurenines (**(16a)**, **(16b)**), compounds **(33)** and **(90)-(93)**, in which the γ-carbonyl was reduced and which contained a substituent on the 3-position of the ring was thus undertaken. Dihydroxydesaminokynurenine **(90)** had been obtained as a side product in the synthesis of 3'-hydroxydesaminokynurenine **(87)** and observed to be a very potent inhibitor of human recombinant kynureninase. Therefore, a preparative route which allowed discrete synthesis of **(90)** in high yield was required.



In the design and synthesis of new analogues of kynurenine, which may function as potent inhibitors of kynureninase, the synthesis of analogues in which the γ-carbonyl was replaced by a number of bioisosteres was also investigated. Bioisosteres are atoms or molecules which have chemical and physical similarities producing broadly similar biological properties.[147] The use of bioisosteres in drug design and in the synthesis of lead compounds is increasingly popular since bioisosteric replacement allows molecular modifications which are subtle, altering some properties to improve in this case potency while retaining other properties, for example, solubility. In this regard three bioisosteres of the carbonyl group; a thiocarbonyl group, a malononitrile moiety and cyanamide moiety were investigated as replacements for the γ-carbonyl in desaminokynurenine **(15)**.



Synthesis of reduced kynurenines **(33)** and **(93)** as well as the modification of the γ-carbonyl using bioisosteres, required the synthesis of desaminokynurenine **(15)** as well as a protected derivative, such as ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate **(132)**.

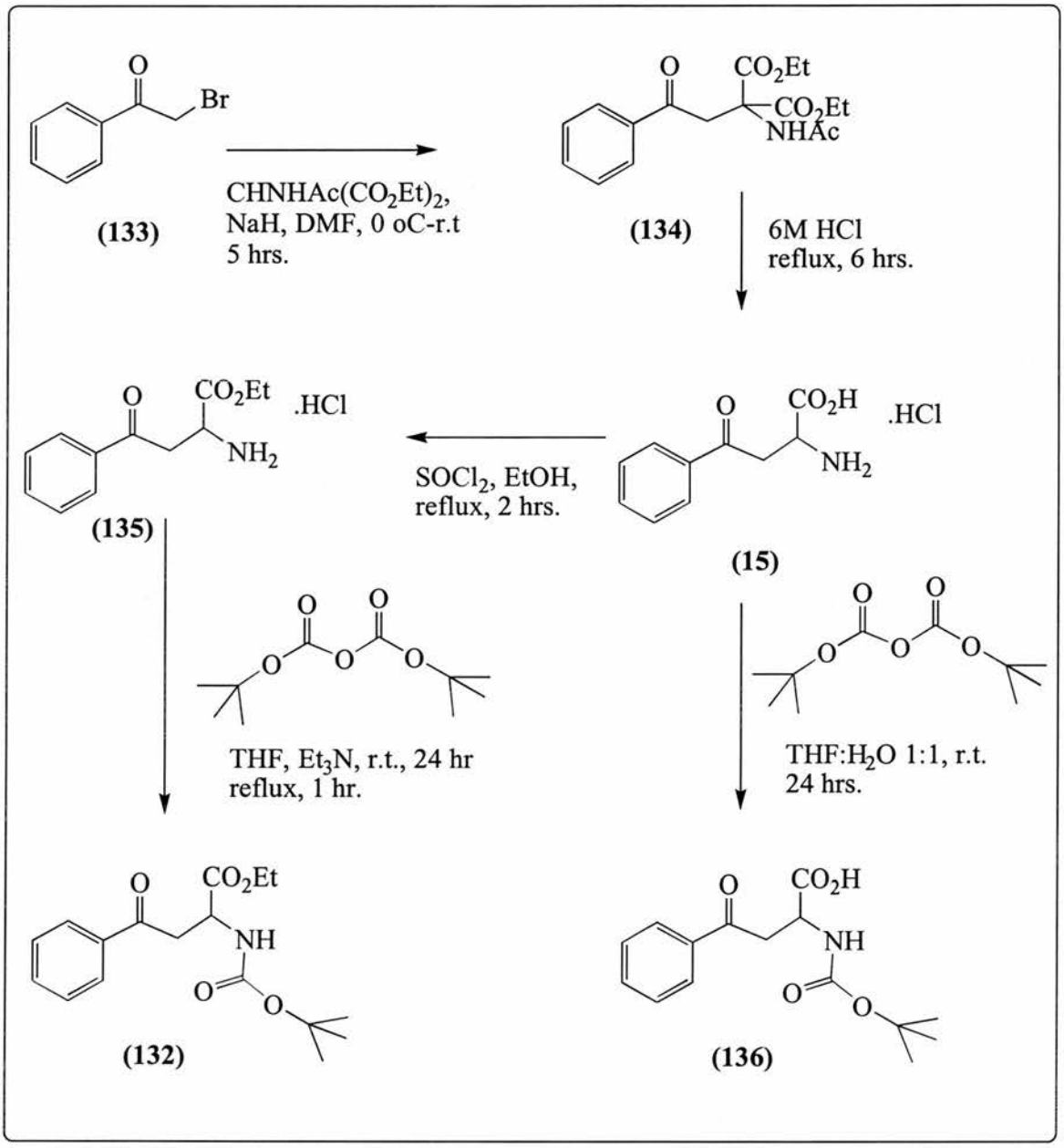


2.4.1 Synthesis of 2-amino-4-oxo-4-phenyl-butyric acid (desaminokynurenine) (15) and ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (132)

The synthetic strategy towards desaminokynurenine **(15)** was the same as that used in the synthesis of 3'-methoxydesaminokynurenine **(88)**. The anion of diethyl acetamidomalonate **(101)** was formed by reaction with sodium hydride in DMF at 0 °C for three hours. A solution of α-bromoacetophenone **(133)** was then added and the resulting orange reaction mixture stirred at room temperature for five hours. After extraction the

residue was recrystallised from diethyl ether to afford the protected desaminokynurenine **(134)** as a white crystalline solid in 65% yield. The compound was pure by microanalysis and its structure was confirmed by NMR spectroscopy and mass spectrometry. Deprotection was accomplished by refluxing in 6M HCl. Upon cooling the organic impurities were removed by washing with ethyl acetate and the solvent removed under reduced pressure to afford an off-white crystalline solid. Further washing with diethyl ether afforded the microanalytically pure desaminokynurenine **(15)** as the hydrochloride salt in 82% yield. The ethyl ester **(135)** was then obtained in 86% yield from reaction of **(134)** with thionyl chloride in refluxing ethanol for two hours. Subsequent recrystallisation from ethanol afforded the ester **(135)** as a white crystalline solid. The structure was verified by observable resonances at 1.28 and 4.19 ppm due to the methyl and methylene hydrogens from the ester in the ^{1}H NMR spectrum.

The amino group of 2-amino-4-oxo-4-phenyl-butyric acid ethyl ester **(135)** was then protected *via* a non-aqueous *tert*-butoxycarbonylation, a novel method for the protection of amino acids reported by Ponnusamy *et al.*[158] The ester **(135)** was reacted with di-*tert*-butylpyrocarbonate in dry THF under reflux for one hour. The solvent was removed under reduced pressure and subsequent purification by column chromatography (silica gel, ethyl acetate: petroleum ether 1:3) afforded the ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate **(132)** as a white sticky solid in 37% yield. The compound was analytically pure. NMR spectroscopy and mass spectrometry confirmed the structure (Scheme 2.11).



Scheme 2.11

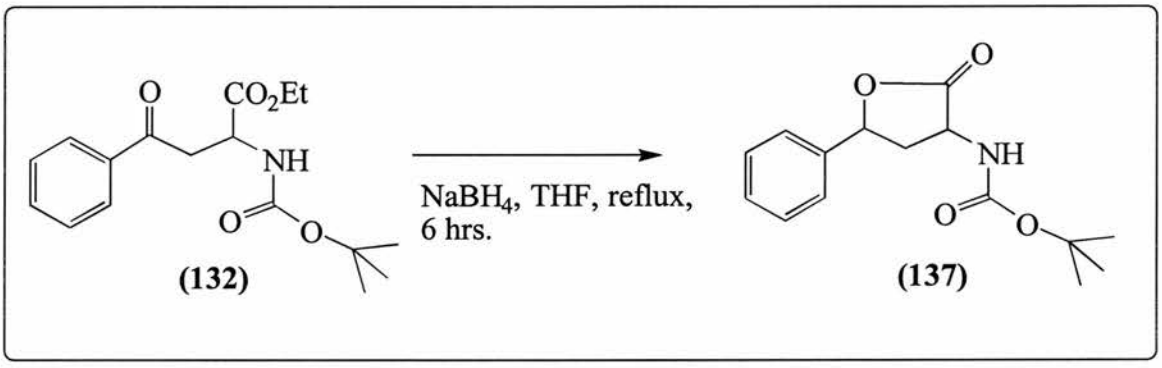
An alternative method for the preparation of **(132)** was also examined. Desaminokynurenine **(15)** was reacted with di-*tert*-butylpyrocarbonate and sodium bicarbonate in a mixture of THF and water (1:1) at room temperature. The reaction showed some evidence by NMR spectroscopy for formation of the protected amino acid **(136)**. Subsequent reaction with thionyl chloride in ethanol, however, resulted in loss of the *tert*-butoxycarbonyl (Boc) group and reformation of desaminokynurenine. The first method used for *N*-protection was thus more efficient and was used in further syntheses.

2.4.2 Synthesis of 4-hydroxy-4-phenyl-butyric acid (γ-hydroxydesaminokynurenine) (33)

The synthesis of **(33)** was initially attempted by reduction of the γ-carbonyl of desaminokynurenine using the method reported by Phillips and Dua for the reduction of the dihydrokynurenines (**(16a), (16b)**). Desaminokynurenine **(15)** was dissolved in water to which an excess of sodium borohydride was added the reaction mixture was allowed to stir at room temperature overnight. The reaction was then quenched with acid and the solvent removed under reduced pressure to afford a white residue. Analysis by NMR spectroscopy, however, proved inconclusive. The reduction of the γ-carbonyl of the protected precursor of desaminokynurenine, 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester **(134)** was thus attempted instead. Reduction in dry methanol using excess sodium borohydride at room temperature overnight followed by quenching did not result in reduction of the γ-carbonyl and NMR analysis showed only the presence of starting material. Reaction of **(134)** with excess sodium borohydride in refluxing THF for three hours was then tried, however no reaction was observed to occur. As a comparison, reduction of the 3'-methoxy analogue, 2-acetylamino-2-[2-(3-methoxy-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester **(123)** with sodium borohydride in refluxing THF was also attempted, again without success. Ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate **(132)** was then reacted with an equimolar amount of sodium borohydride in dry THF under reflux conditions for six hours. The reaction mixture was then poured onto an ammonium chloride solution and crushed ice. Extraction into diethyl ether and subsequent removal of solvent under reduced pressure afforded a yellow oil. Analysis by tlc and NMR spectroscopy confirmed the formation of a new compound, which appeared to be a mixture of diastereomers and was tentatively identified as the lactone, 3-*N*-(*tert*-butoxycarbonyl)-5-phenyl-2(3,4,5-*H*)-furanone **(137)** (Scheme 2.12). A doubling of resonances was observed in both the ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR spectrum showed two resonances at 1.43 and 1.45 ppm each of which integrated for nine hydrogens. Doubling of resonances was also observed for the α-CH, NH and aromatic hydrogens. However, the yield was low at 50%, therefore another method was sought.

The lack of reduction of the protected precursors by sodium borohydride may have been due to the hydride anion behaving as a base and possibly removing the hydrogen on the

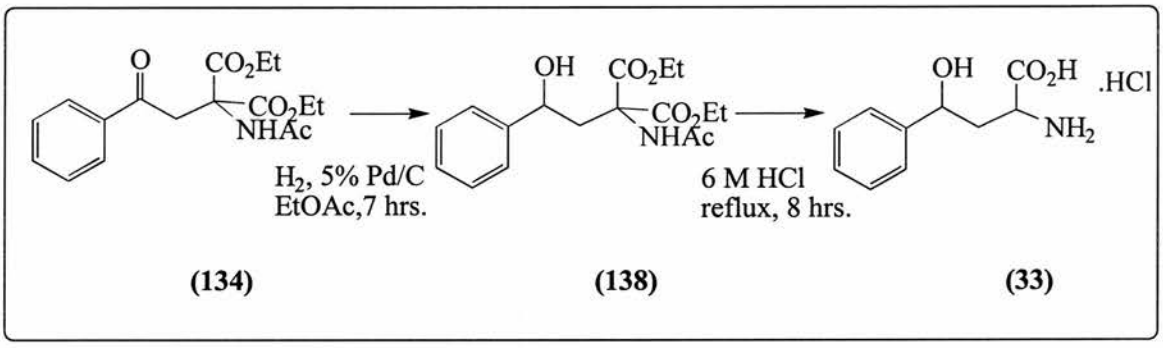
amide nitrogen. When the reaction was then quenched with acid it resulted in reformation of the starting material. In contrast, the hydrogen on the nitrogen of the urethane may be less acidic which allowed reduction at the γ-carbonyl by the hydride anion.



Scheme 2.12

An alternative route to the reduced kynurenine 4-hydroxy-4-phenyl-butyric acid **(33)** using catalytic hydrogenation of the protected 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester **(134)** with a Pd/C catalyst and subsequent deprotection using acid hydrolysis proved to be a more successful strategy.

A solution of 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester **(134)** in ethyl acetate was stirred with a catalytic amount of Pd/C under an atmosphere of hydrogen for twenty-four hours. After filtration through celite to remove the palladium catalyst, the solvent was removed under reduced pressure to afford the reduced 2-acetylamino-2-[2-hydroxy-2-phenyl-ethyl)]malonic acid diethyl ester **(138)** as a white solid in 94% yield. The compound was microanalytically pure and further characterised by NMR spectroscopy and mass spectrometry. A resonance in the ^{1}H NMR spectrum at 4.77 ppm was due to the new γ-CH. The aromatic hydrogens also collapsed from a multiplet in the starting material to give a broad singlet in the reduced compound. The formation of a new stereogenic centre at the γ-position resulted in the β-CH_2 hydrogens becoming diastereotopic with an AB system being observed at 2.72 and 2.81 ppm. Deprotection by refluxing in 6M HCl afforded the target compound 4-hydroxy-4-phenyl-butyric acid **(33)** as a white solid in 52% yield (Scheme 2.13).



Scheme 2.13

The amino acid product **(33)** was closely examined by NMR spectroscopy and HPLC. The reduction produces a new stereogenic centre at the γ-position and it was necessary to determine what mixture of diastereomers had been produced. Analysis by HPLC (C_{18} reverse phase silica, 5% methanol, 1% acetic acid) showed that the diastereomeric pairs were present in a ratio of 1:2 (Figure 2.3). The data from NMR spectroscopy (Figure 2.4) were then analysed by comparison with related data from an earlier study by Phillips and Dua (Figure 2.5).

In the earlier study Phillips and Dua synthesised 4-hydroxy-4-phenyl-butyric acid **(33)** enzymatically by reaction of L-kynurenine **(6)** with kynureninase in the presence of benzaldehyde. The enzyme catalysed reaction was found to be selective for the (4*R*,2*S*) isomer present in a ratio of 4:1 with the (4*S*,2*S*) isomer. The major isomer was identified as (4*R*,2*S*) by comparison of its spectrum with those of (*R*) and (*S*)-mandelic acids. The diastereotopic β-CH_2 hydrogens of the (4*R*,2*S*) isomer were observed as an overlapping multiplet (Figure 2.5, spectrum A). This spectrum was then used to identify the diastereomers of dihydro-L-kynurenine synthesised by reduction of L-kynurenine with sodium borohydride and separated by HPLC. The diastereotopic β-CH_2 hydrogens of the (4*R*,2*S*)-4-hydroxy-phenyl-butyric acid **(33)** agreed closely with the unresolved multiplet observed for one of the dihydrokynurenines at 2.85 ppm. This was thus assigned (4*R*,2*S*)-dihydrokynurenine **(16a)** (Figure 2.5, spectrum B). In contrast the β-CH_2 hydrogens of the second dihydrokynurenine were observed as resolved multiplets at 2.50 and 3.05 ppm, this was assigned (4*S*,2*S*)-dihydrokynurenine **(16b)** (Figure 2.5, spectrum C). Thus, the stereochemical changes at the 4-position of the dihydrokynurenine analogues resulted in differences in the chemical shift and coupling patterns of the β-CH_2 hydrogens. These

differences were used to determine which pairs of enantiomers of 4-hydroxy-4-phenyl-butyric acid **(33)** were present as the minor and major isomers in our preparation.

The minor pair of enantiomers obtained in our reaction were identified as being (4*R*,2*S*) and (4*S*,2*R*)-4-hydroxy-4-phenyl-butyric acid **(33a)**. In this enantiomeric pair the diastereotopic β-CH_2 hydrogens were observed as an overlapping multiplet at 2.93 ppm. This compared closely with the multiplet observed by Phillips and Dua for (4*R*,2*S*)-4-hydroxy-4-phenyl-butyric acid **(33)** as well as the multiplet attributed to the β-CH_2 hydrogens of the (4*R*,2*S*)-dihydro-L-kynurenine **(16a)**, at 2.85 ppm (Figure 2.5, spectra A and B, respectively). The α-CH and γ-CH hydrogens of **(33a)** were observed at 4.55 and 6.00 ppm respectively and were similar to those observed in both (4*R*,2*S*)-4-hydroxy-4-phenyl-butyric acid **(33)** and (4*R*,2*S*)–dihydro-L-kynurenine **(16a)**. The major pair of enantiomers were identified as (4*S*,2*S*) and (4*R*,2*R*)-4-hydroxy-4-phenyl-butyric acid **(33b)**. In this enantiomeric pair the diastereotopic β-CH_2 hydrogens were well-resolved multiplets at 2.46 and 3.19 ppm respectively. These were similar to the multiplets observed in the (4*S*,2*S*)-dihydro-L-kynurenine **(16b)** at 2.50 and 3.05 ppm (Figure 2.5, spectrum C).

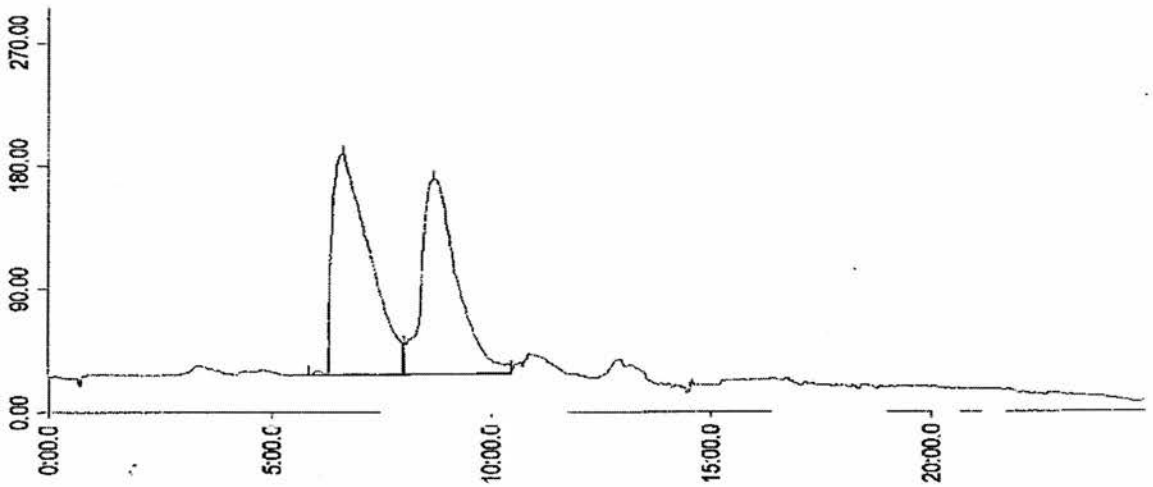


Figure 2.3

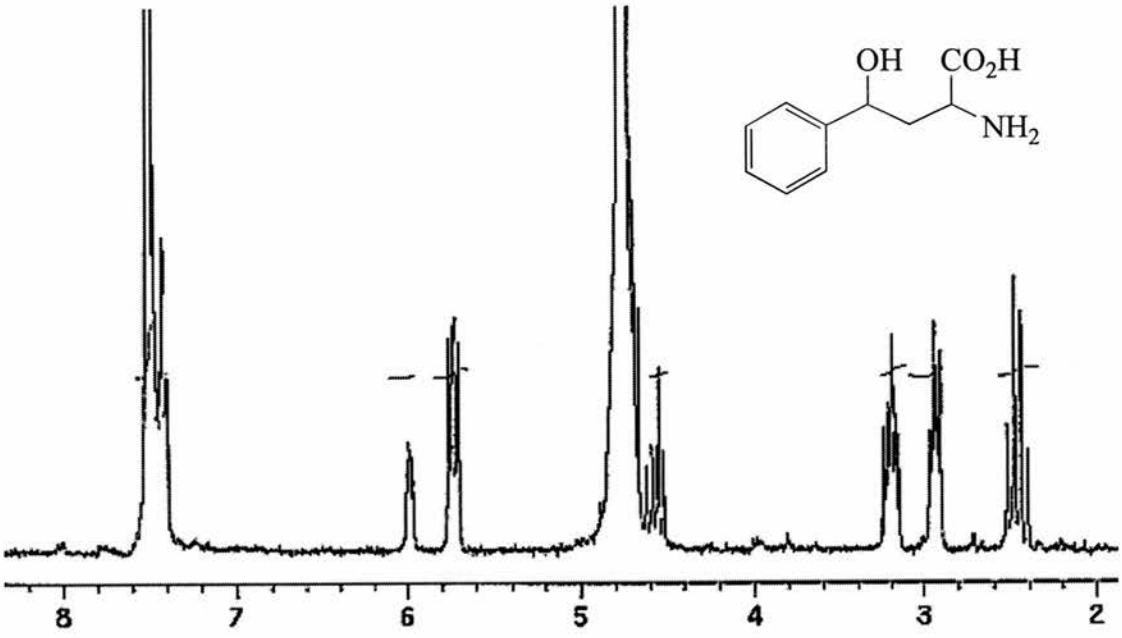


Figure 2.4

Diastereomeric mixture of 4-hydroxy-4-phenyl-butyric acid (33).

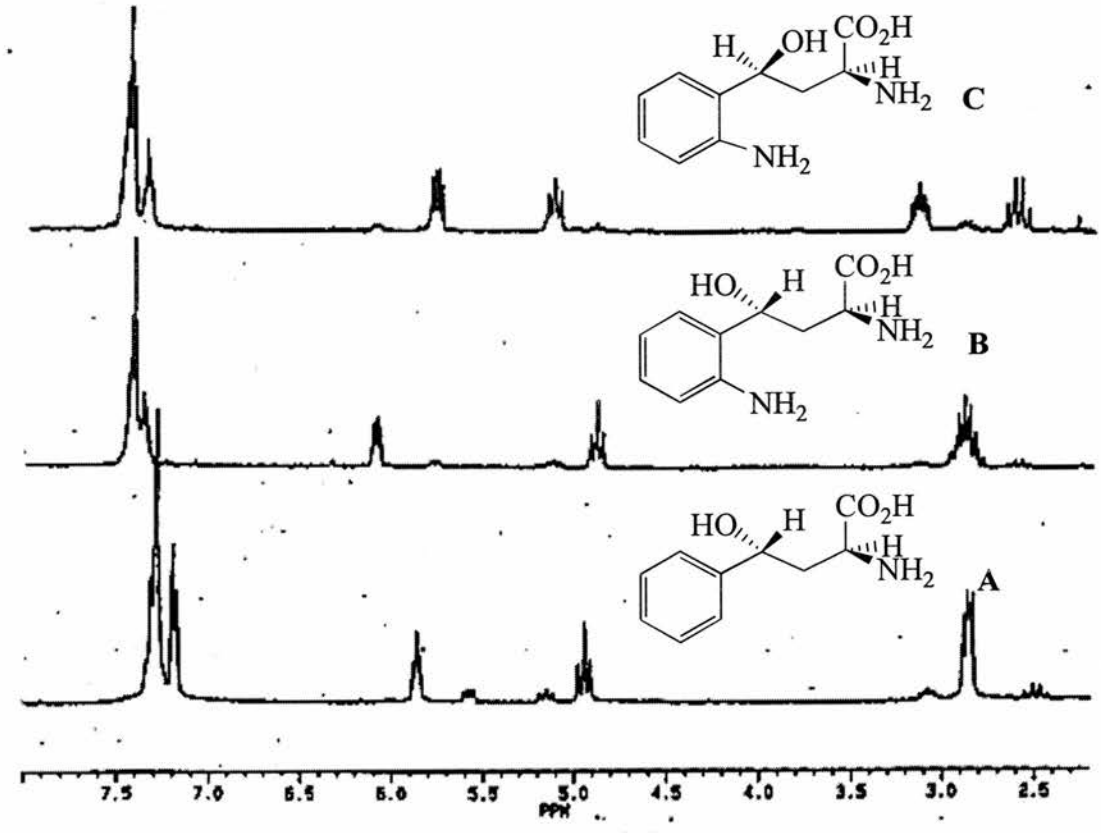


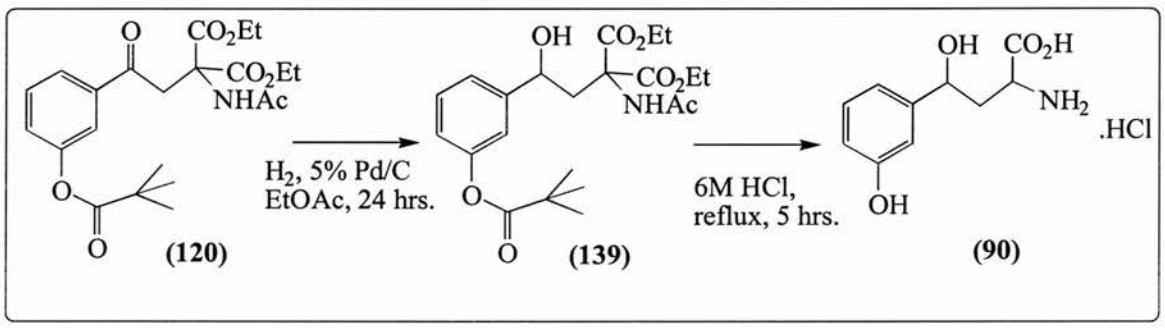
Figure 2.5

Spectrum A: (4*R*,2*S*)-4-hydroxy-4-phenyl-butyric acid, Spectrum B: (4*R*,2*S*)-dihydrokynurenine. Spectrum C: (4*S*,2*S*)-dihydrokynurenine.

(From the study by R.S Phillips and R.K. Dua)[42]

2.4.3 Synthesis of 2-amino-4-hydroxy-4-(3-hydroxy-phenyl)-butyric acid (dihydroxydesaminokynurenine) (90)

The desired synthesis of dihydroxydesaminokynurenine **(90)** in high yield was successful. It was prepared in an analogous manner to γ-hydroxydesaminokynurenine **(33)** (Scheme 2.14). The catalytic hydrogenation of the protected precursor, 2-acetylamino-2-{2-[3-(2,2-dimethyl-propionyloxy)-phenyl]-2-oxo-ethyl}-malonic acid diethyl ester **(120)** as a solution in ethyl acetate under a hydrogen atmosphere for twenty-four hours afforded the γ-reduced derivative **(139)** in 66% yield.



Scheme 2.14

The microanalytically pure compound was obtained as a white solid. Deprotection of **(139)** was achieved by refluxing in 6M HCl for five hours. After washing with ethyl acetate the solvent was removed under reduced pressure to afford an off-white solid. Recrystallisation from ethanol gave the dihydroxy derivative **(90)** as a white solid in 98% yield. Analysis by NMR spectroscopy indicated the presence of a single diastereomer of the final product as a racemic mixture. The diastereotopic β-CH_2 appeared as well resolved multiplets in the ^{1}H NMR spectrum at 2.31 and 3.06 ppm, respectively (Figure 2.6). These resonances compare closely with the resonances observed for the β-CH_2 hydrogens in (4*S*,2*S*)-dihydrokynurenine **(16b)**, which were resolved multiplets at 2.50 and 3.05 ppm (Figure 2.5, spectrum C). They are in contrast to the (4*R*,2*S*)-dihydrokynurenine **(16a)** analogue where the diastereotopic β-CH_2 hydrogens overlap at 2.85 ppm, as well as the overlapping multiplet observed for (4*R*,2*S*)-4-hydroxy-4-phenyl-butyric acid **(33)** (Figure 2.5, Spectra B and A, respectively) reported by Phillips and Dua.[42] 2-Amino-4-hydroxy-4-(3-hydroxy-phenyl)-butyric acid **(90)** can thus be identified as the (4*S*,2*S*) and (4*R*,2*R*) enantiomeric pair.

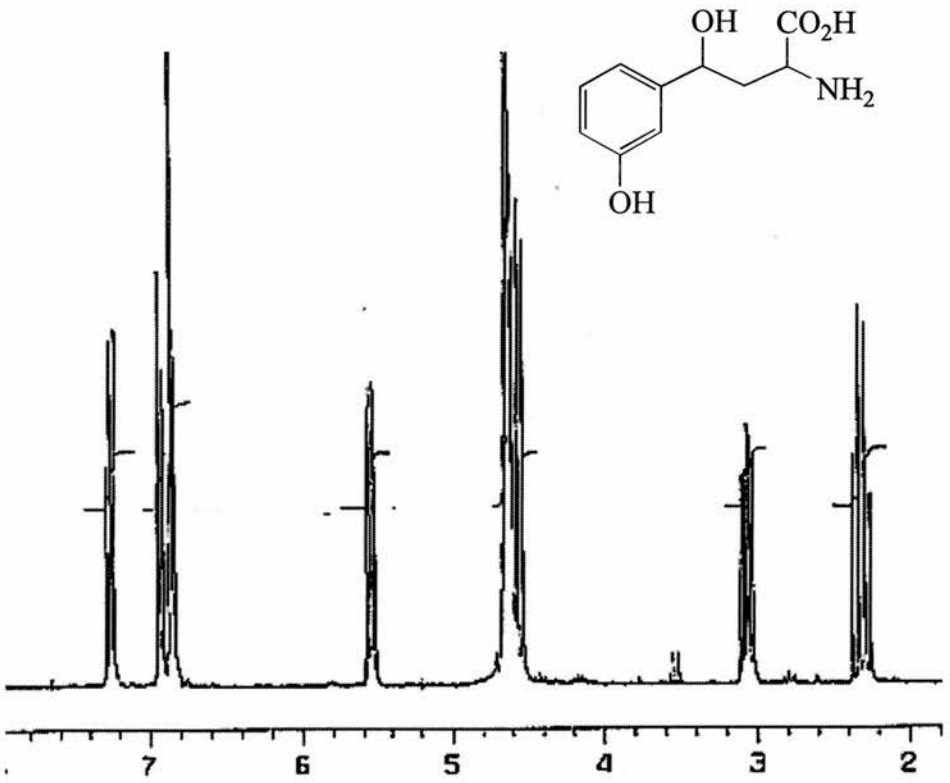
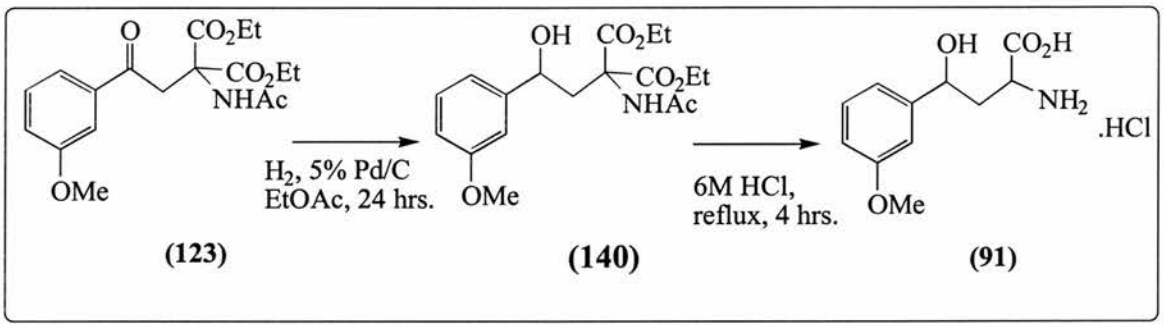


Figure 2.6

2.4.4 Synthesis of 2-amino-4-hydroxy-4-(3-methoxy-phenyl)-butyric acid (γ-hydroxy-3'-methoxydesaminokynurenine) (91)

The γ-hydroxy-3'-methoxydesaminokynurenine **(91)** was prepared in an analogous manner to the dihydroxydesaminokynurenine **(90)**. 2-Acetylamino-2-[2-(3-methoxy-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester **(123)** as a solution in ethyl acetate was subjected to catalytic hydrogenation in the presence of a palladium catalyst for twenty-four hours. The product **(140)** was obtained in 94% yield as a sticky clear oil and its structure was confirmed by NMR spectroscopy and mass spectrometry. The diastereomeric β-CH_2 hydrogens were observed as doublets of doublets at 2.68 and 2.80 ppm and the γ-CH was observed as a doublet of doublets at 4.67 ppm. Deprotection of **(140)** was achieved by refluxing in 6M HCl for four hours. Upon cooling a white solid precipitated from solution. This was redissolved in water, washed with ethyl acetate and the solvent removed under reduced pressure. The resulting white solid was recrystallised from water and γ-hydroxy-3'-methoxydesaminokynurenine **(91)** was obtained in 64% yield (Scheme 2.15). The ^{1}H NMR spectrum and analytical HPLC chromatogram (C_{18}

reverse phase silica, 20% methanol, 1% acetic acid) indicated the presence of diastereomers. The diastereomers were observed to be present in a ratio of 1:2 (Figure 2.7).



Scheme 2.15

The minor isomer was identified as the (4*R*,2*S*) and (4*S*,2*R*) **(91a)** pair of enantiomers. The corresponding diastereotopic β-CH_2 hydrogens were identified as the overlapping multiplet at 2.81 ppm, (Figure 2.8) and compared closely with the overlapping β-CH_2 observed at 2.85 ppm in (4*R*,2*S*)-dihydrokynurenine **(16a)** and that observed for (4*R*,2*S*)-4-hydroxy-4-phenyl-butyric acid **(33)** in study reported by Phillips and Dua (Figure 2.5, Spectra B and A, respectively). The major isomer was identified as the (4*S*,2*S*) and (4*R*,2*R*) **(91b)**, pair of enantiomers as they exhibited diastereotopic β-CH_2 hydrogens as well resolved multiplets at 2.34 and 3.09 ppm similar to those observed for the (4*S*,2*S*)-dihydrokynurenine **(16b)** at 2.50 and 3.05 ppm (Figure 2.5, Spectrum C).

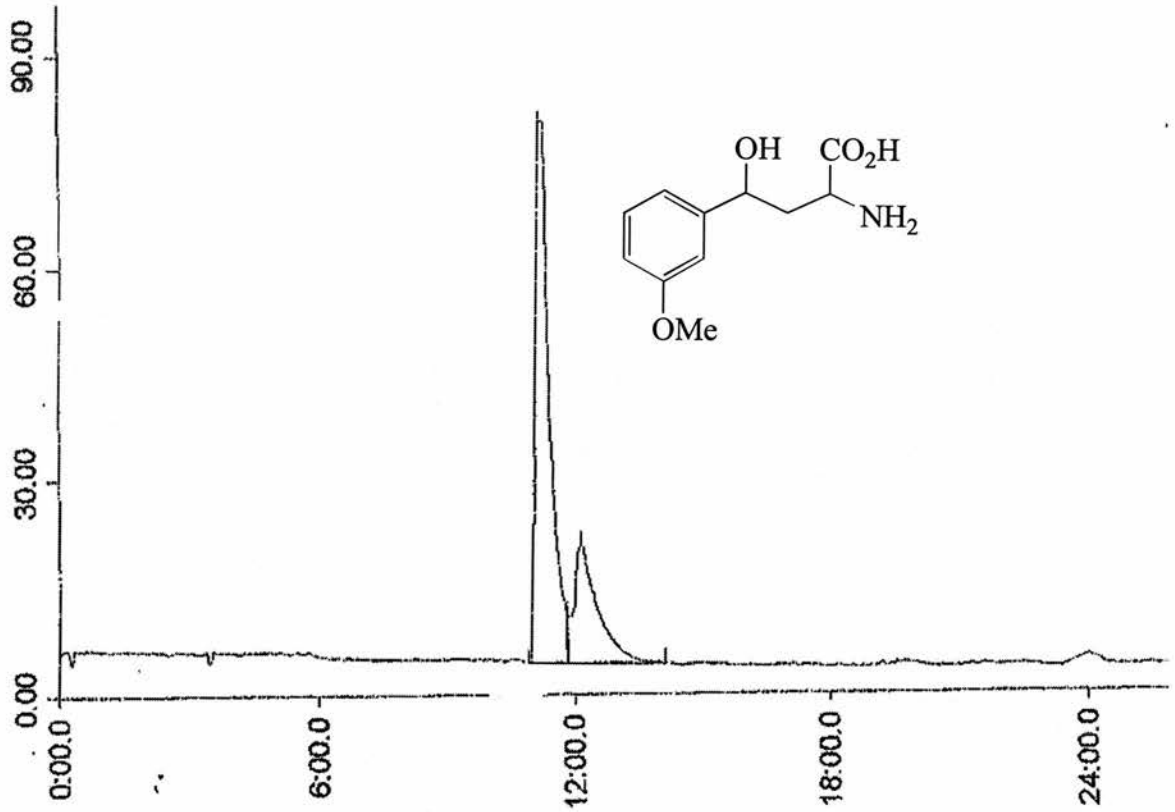


Figure 2.7

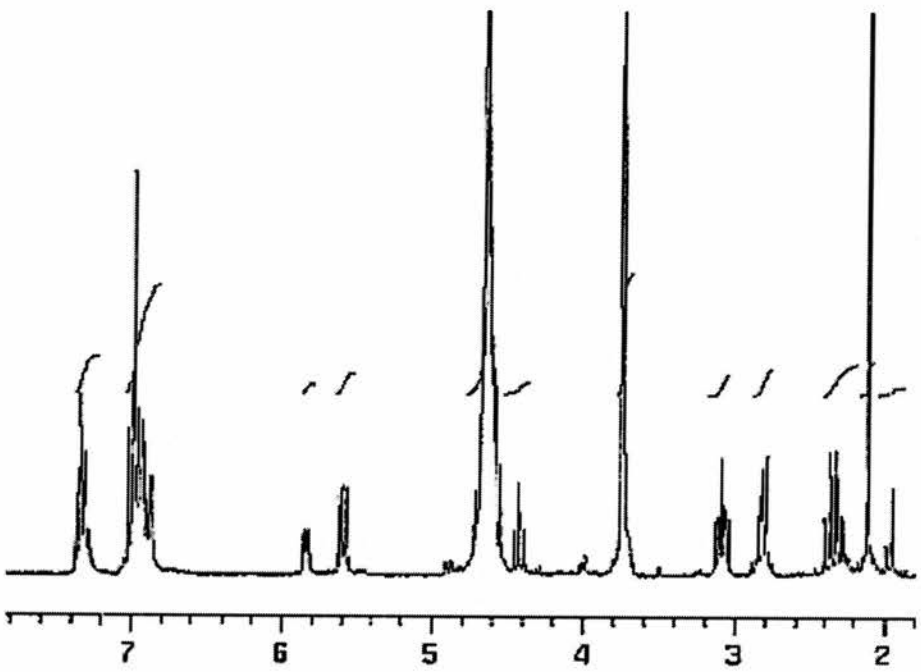
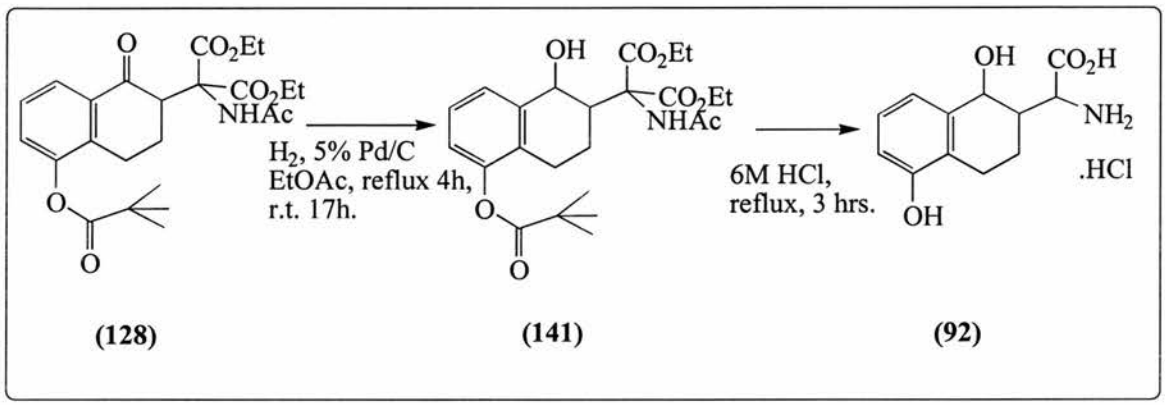


Figure 2.8

2.4.5 Synthesis of amino-(1,5-dihydroxy-1, 2, 3, 4-tetrahydro-naphthalen-2-yl)-acetic acid (1,5-dihydroxytetralone derivative) (92)

Preparation of the bicyclic 1,5-dihydroxytetralone derivative **(92)** required synthesis of the γ-reduced protected precursor **(141)**. Initial attempts to reduce the γ-carbonyl *via* catalytic hydrogenation were unsuccessful. A solution of **(128)** in ethyl acetate in the presence 5% Pd/C catalyst was stirred under an atmosphere of hydrogen for twenty-four hours. After filtration through celite and subsequent removal of the solvent under reduced pressure, NMR analysis indicated no reaction had occurred. An increase in the reaction time to three days also failed to cause reaction. The reaction of **(128)** with sodium cyanoborohydride in dry THF heated under reflux for three hours was also unsuccessful, analysis by NMR spectroscopy showed only the presence of starting materials. As an alternative to using the hydrogenation apparatus the reaction was repeated using a hydrogen balloon. The reaction mixture was heated under reflux for three hours and was monitored by tlc, however no reaction occurred. Repetition of the reaction with an increase in reflux time to four hours and subsequently stirring the reaction mixture at room temperature for a further seventeen hours was successful and the reduced derivative **(141)** was finally obtained as a white sticky solid in 37% yield. The compound was pure by microanalysis and the ^{1}H NMR spectrum of the major isomer exhibited a resonance at 5.62 ppm due to the γ-CH. The minor isomer exhibited a resonance at 5.42 ppm. The isomers were present in a ratio of 1:10. The compound was deprotected *via* acid hydrolysis in refluxing 6M HCl for three hours (Scheme 2.16). Some decomposition was seen to occur during reaction which was monitored by tlc. Upon cooling the reaction mixture was filtered to eliminate any decomposed material the solvent was then removed under reduced pressure to afford a yellow oil. This was freeze dried to remove water and a pale yellow solid was obtained. The compound was pure by microanalysis. However initial attempts to characterise the compound by NMR analysis in D_2O resulted in decomposition of the compound. The compound was found to be stable in deuterated, methanol, however and the ^{13}C NMR spectrum confirmed the structure of the compound. Resonances were observed at 65.61 and 79.55 ppm due to the α-CH and 4-CHOH carbons. The ^{1}H NMR spectrum was difficult to analyse as there appeared to be a number of isomers present, however, some of the resonances were assigned tentatively.



Scheme 2.16

2.4.6 Attempted synthesis of 4-hydroxy-4-methyl-4-phenyl-butyric acid (93)

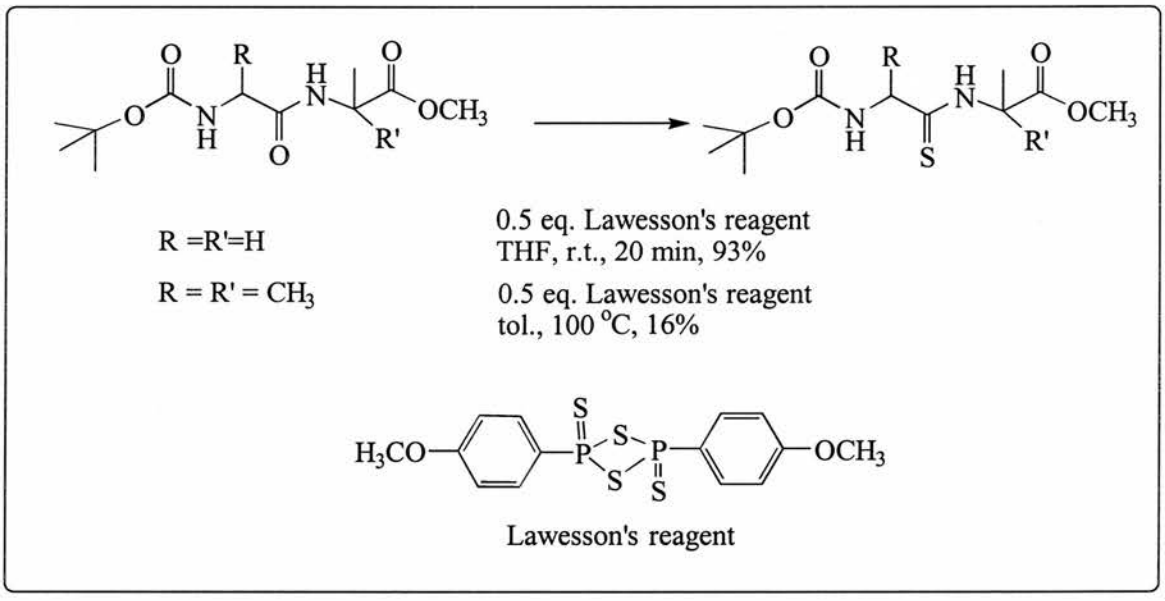
The strategy for the synthesis of 4-hydroxy-4-methyl-4-phenyl-butyric acid **(93)** involved alkylation of ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate **(132)** *via* a Grignard reagent methylmagnesium bromide to afford the methylated derivative. Subsequent deprotection by acid hydrolysis would then afford the amino acid **(93)**. Methylmagnesium bromide was added to a solution of **(132)** in dry THF and stirred at room temperature for four hours and then the solution refluxed for a further ten hours. Upon cooling the solution was poured onto ammonium chloride and crushed ice to quench the reaction. After extraction into diethyl ethyl ether the solvent was removed under reduced pressure to afford a yellow oil. However, subsequent purification by column chromatography and analysis by NMR spectroscopy indicated that mostly starting material had been recovered. A minor product was formed but NMR analysis was inconclusive, as was mass spectrometry.

An alternative method using anhydrous cerium chloride as a catalyst was also attempted. Cerium chloride is known to promote nucleophilic addition of Grignard reagents to ketones to afford alcohols.[159,160] Anhydrous cerium chloride is thought to suppress side reactions which often accompany Grignard reactions such as enolisation, conjugate addition and condensation reactions. Pre-dried cerium chloride in dry THF was thus reacted with methylmagnesium bromide under argon at room temperature with subsequent addition of **(132)**, the reaction mixture was then heated under reflux for six hours.

However, only starting materials were recovered after work-up, with no evidence by tlc or NMR spectroscopy for any reaction having occurred.

2.4.7 Attempted thiation of ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenylbutyrate (132)

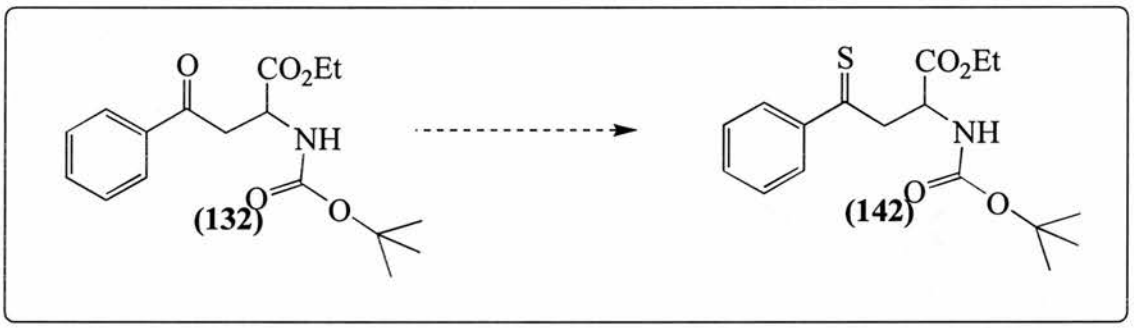
The strategy for synthesis of the thiocarbonyl analogue of desaminokynurenine **(15)** involved initial thiation of the protected precursor **(132)** followed by deprotection *via* acid hydrolysis. The conversion of the γ-carbonyl to the thiocarbonyl was attempted using a method reported by Lawesson *et al.* for the preparation of monothiated oligopeptides in high yield.[161] This method allowed selective thiation of the amide group of an oligopeptide in the presence of ester and urethane protecting groups using Lawesson's reagent [162-165] (Scheme 2.17).



Scheme 2.17

Urethanes and esters will only react with Lawesson's reagent at temperatures greater than 110 °C and 140 °C, respectively. It was thus thought that the γ-carbonyl in ethyl *N*-(*tert*-butylcarbonyloxy)-2-amino-4-oxo-4-phenyl-butyrate **(132)** would be selectively thiated in the presence of protecting groups. Therefore compound **(132)** was reacted with Lawesson's reagent in dry THF at room temperature and under reflux (Scheme 2.18). However, subsequent purification of the reaction mixture by column chromatography afforded only starting materials. This was verified by tlc and NMR analysis. When

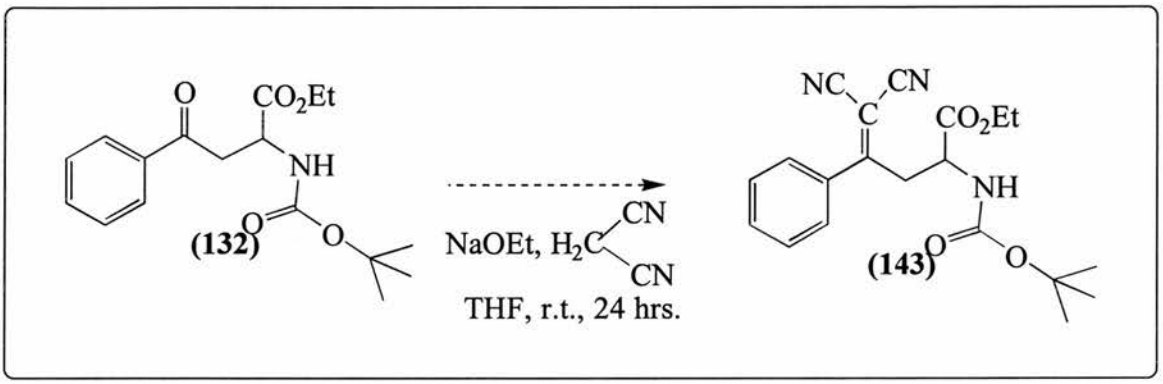
conditions were altered and the reaction was carried out in toluene at reflux temperature some reaction took place but no evidence for the formation of **(142)** was observed. A minor product obtained after workup and subsequent purification by column chromatography as a yellow solid could not be identified by NMR spectroscopy or mass spectrometry. However, the formation of an adduct between the starting material and fragments of Lawesson's reagent is possible as such byproducts have previously been reported.[166]



Scheme 2.18

2.4.8 Attempted reactions of ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenylbutyrate (132) with malononitrile and cyanamide

The Knoevenagel type reaction of malononitrile with carbonyl compounds in which the carbonyl oxygen is replaced by a malononitrile moiety has been reported in the literature.[167,168] The synthesis of compound **(143)** (Scheme 2.19) was thus attempted by reaction of **(132)** with a preformed malononitrile anion in diethyl ether at room temperature. Unfortunately, there was no evidence for the formation of the desired product. NMR spectroscopy showed only the presence of starting material and infrared spectroscopy showed only very weak absorption at 2093 cm^{-1}. Again the carbonyl group appeared to be unreactive.



Scheme 2.19

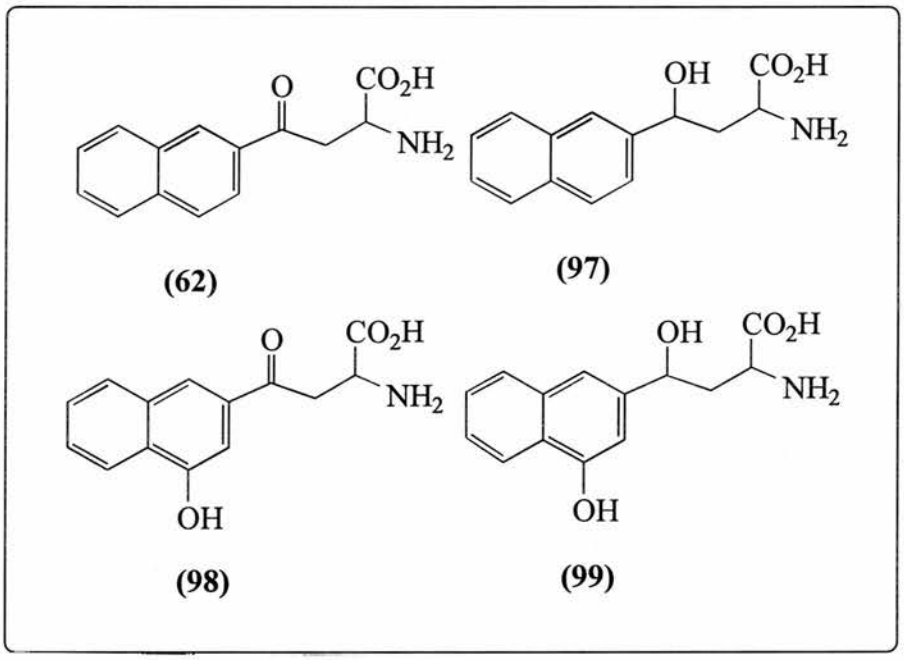
Due to its amphoteric nature cyanamide (H_2N-CN) can behave both as a nucleophile and an electrophile. The use of cyanamide in nucleophilic displacement reactions, in which the carbonyl oxygen atom or thiocarbonyl sulfur atom atom of β-keto esters, thiono esters and xanthates have been replaced by the cyanamide moiety N-CN, has previously been reported in the literature.[169] However, reaction of cyanamide with a solution of **(132)** in THF at room temperature for forty eight hours was unsuccessful and only starting materials were recovered after workup and purification.

2.4.9 Synthetic problems in modifying the γ-carbonyl of kynurenine analogues

The apparent lack of reactivity of the γ-carbonyl of the protected kynurenine analogue **(132)** to alkylation or attack by a variety of other nucleophiles such as cyanamide, malononitrile or dithiophosphine ylid may indicate that the protecting groups are too bulky and prevent nucleophilic attack. The β-CH_2 hydrogens may also be too acidic resulting in the methyl anion and other nucleophiles behaving as bases, causing enolisation of the γ-carbonyl, rather than acting as nucleophiles. However, there was evidence for the reduction of the γ-carbonyl to a lactone (Section 2.4.2) using sodium borohydride, which indicates that it is in fact steric bulk and the increased size of attacking nucleophiles rather than the basicity of the β-CH_2 groups which may be preventing the modification of the γ-carbonyl. As such the use of alternative protecting groups should be considered, for example, using an acyl group to protect the nitrogen rather than the bulky BOC group and forming a methyl ester rather than ethyl ester.

2.5 Naphthalene analogues

The synthesis of the naphthyl derivatives **(97)-(99)** was of interest because they contain features such as the naphthyl group and hydroxyl group both of which conferred potent inhibitory activity on other analogues examined within the group. Naphthyl analogues of kynureninase were thought to be promising candidates as potent inhibitors of kynureninase. The naphthalene analogue **(62)** was previously shown to be a potent competitive inhibitor of human recombinant kynureninase exhibiting a K_i of 22 μM. This result implied that there may be an additional hydrophobic pocket in the active site which could accommodate the extra benzene ring. A series of synthetic targets **(97)-(99)** was devised to exploit this result. It was intended to combine the additional binding of the naphthyl group with hydroxyl groups at the 4' and/or γ position.



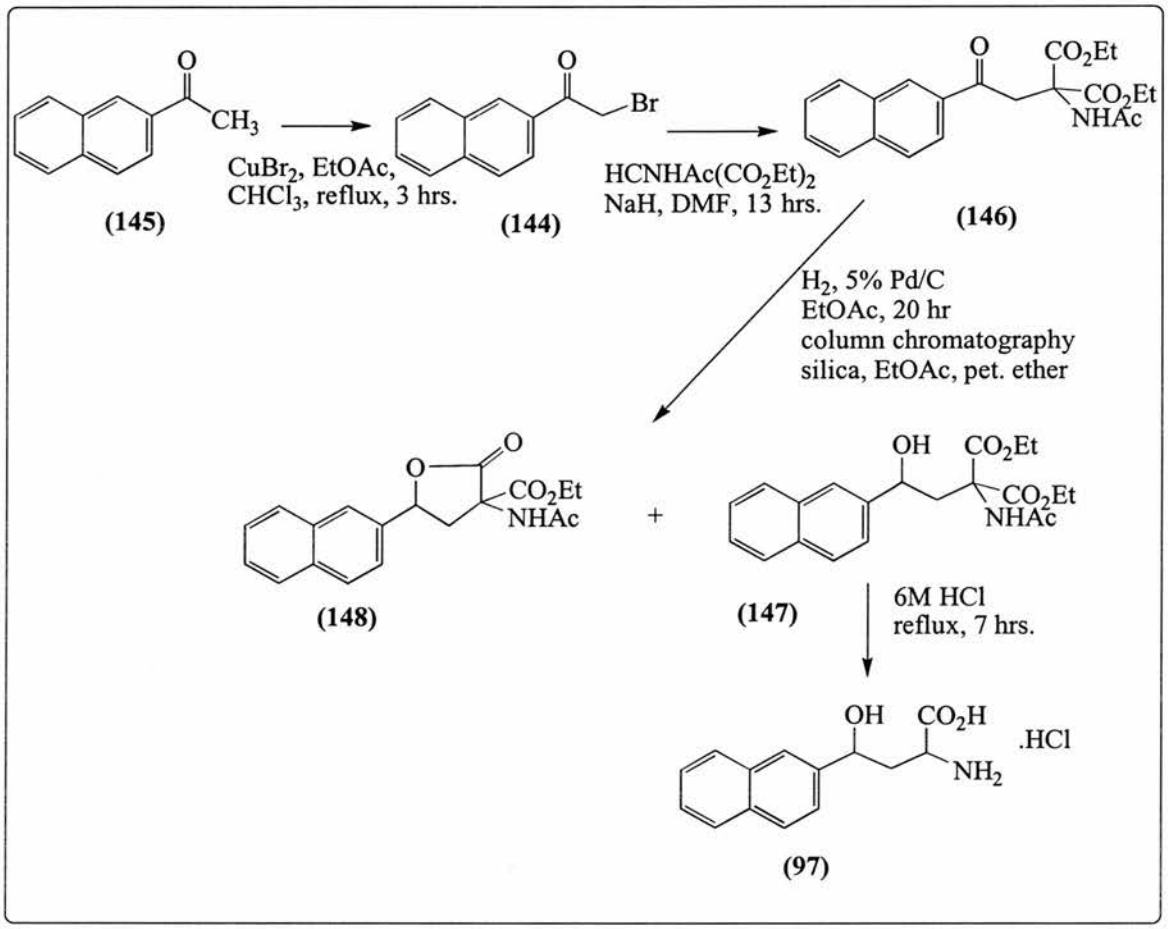
2.5.1 2-Amino-4-hydroxy-4-naphthyl-butyric acid (γ-hydroxynaphthalene derivative) (97)

The strategy for the synthesis of the γ-hydroxynaphthalene analogue **(97)** was the same as that applied to the synthesis of the reduced kynurenine analogues (Scheme 2.20). The α-bromoacetonaphthone **(144)** was synthesised by reaction of 2-acetonaphthone **(145)** with cupric bromide in refluxing ethyl acetate with chloroform as a cosolvent in 93% yield as a yellow sticky solid. The brominated α-CH_2 group was observed as a singlet at 4.68 ppm in the ^{1}H NMR spectrum. Molecular ion peaks were observed at 250 and 248 *m/z* units indicating the presence of single bromine atom. The compound was used without further purification.

Coupling with diethyl acetamidomalonate gave the protected precursor **(146)** as colourless crystals in 68% yield. This was characterised by NMR spectroscopy and mass spectrometry and compared with reported spectral data.

Catalytic hydrogenation of **(146)** was achieved by reaction in degassed ethyl acetate with 5% palladium on carbon catalyst under an atmosphere of hydrogen for twenty hours to afford the crude product. Subsequent purification by column chromatography however, afforded two compounds which eluted together but which were successfully separated by recrystallisation from diethyl ether. The reduced naphthalene **(147)** was obtained as a sticky white solid in 10% yield. The second product was identified as the lactone **(148)** and was obtained as a white precipitate in 20% yield. Formation of the lactone **(148)** had occurred by cyclisation during column chromatography, presumably catalysed by the acidic silica gel. The structure of both compounds was confirmed by NMR spectroscopy. The reduced naphthalene exhibited a resonance at 5.01 ppm due to the γ-CH hydrogen. A similar resonance was observed further downfield at 5.92 ppm for the lactone. Formation of the lactone was also confirmed by microanalysis and mass spectrometry. Though deprotection of the lactone *via* acid hydrolysis would also yield the γ-hydroxynaphthalene derivative the hydrogenation reaction was repeated and the reduced naphthalene **(147)** was reacted on without purification.

Deprotection of **(147)** was achieved under the normal acidic conditions to afford the γ-hydroxynaphthalene derivative **(97)** as a white precipitate in 38% yield. Analytical HPLC (Figure 2.9) and ^{1}H NMR spectroscopy (Figure 2.10) implied the presence of a single diastereomer present as a racemic mixture.



Scheme 2.20

The ^{1}H NMR spectrum of **(97)** contained only a single set of resonances due to the α-CH and γ-CH hydrogens which indicated the presence of a single diastereomer. The spectrum was compared with those of the dihydrokynurenines reported by Phillips and Dua (Figure 2.5). The β-CH_2 hydrogens were observed as well resolved multiplets at 2.69 and 3.15 ppm which compare with those observed for the (4*S*,2*S*)-dihydrokynurenine **(16b)** at 2.50 and 3.05 ppm (Figure 2.5, Spectrum C). The spectral data for **(97)** were collected using DMSO as a solvent since the compound was only partially soluble in D_2O or CD_3OD. As a result, the multiplet at 2.69 ppm is partially obscured by the DMSO peak, therefore it was not possible to determine all of the coupling constants for the β-CH_2 hydrogens. The

single diastereomer obtained in the synthesis of **(97)** is a racemic mixture of the (4*S*,2*S*) and (4*R*,2*R*) pair of enantiomers.

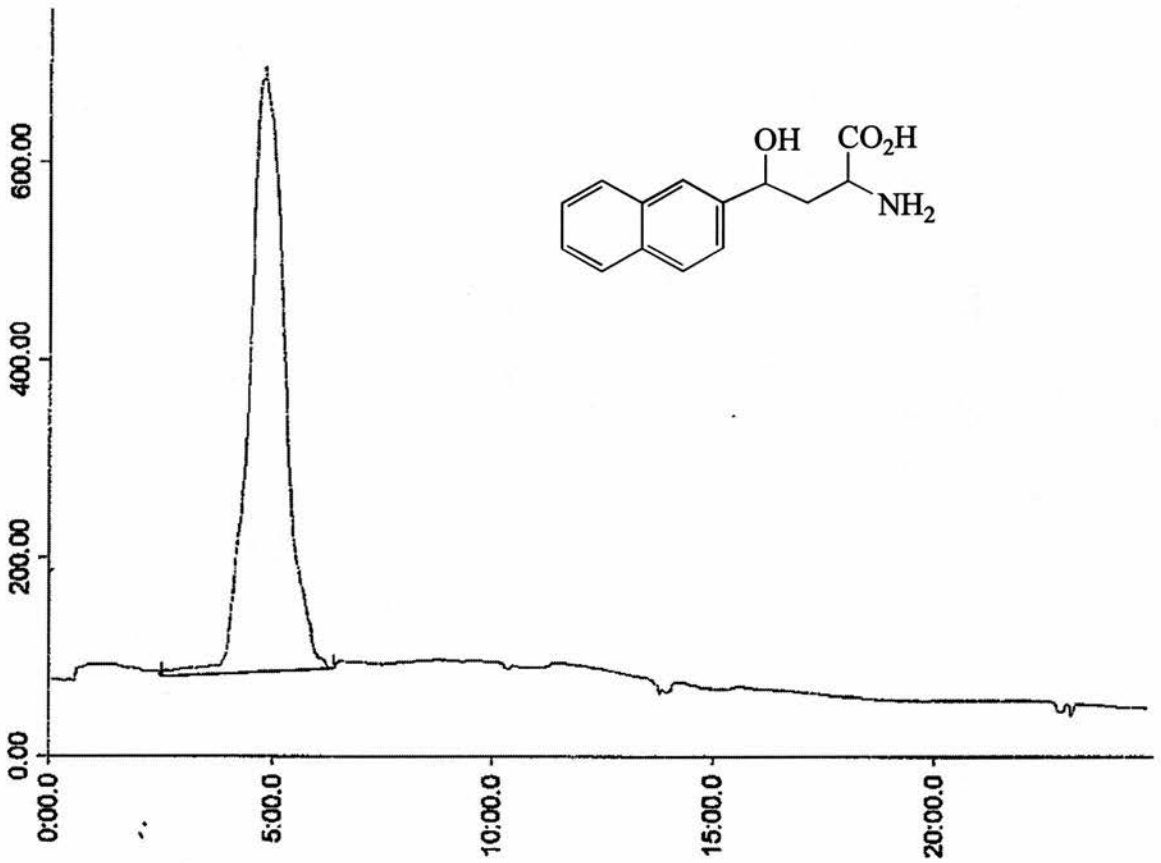


Figure 2.9

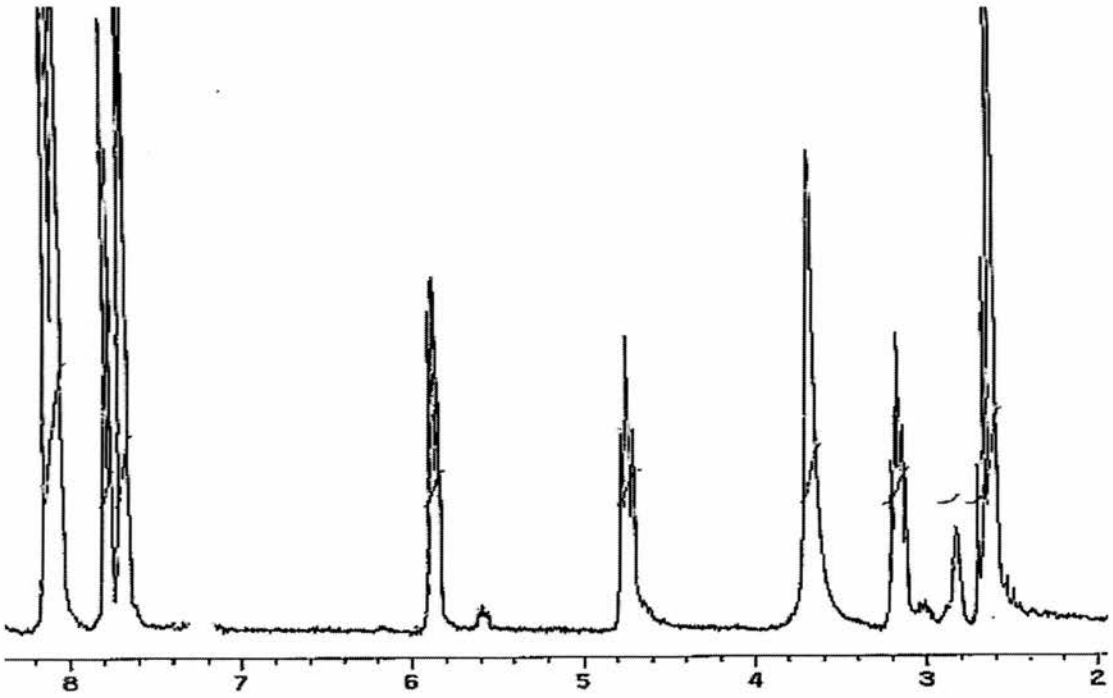
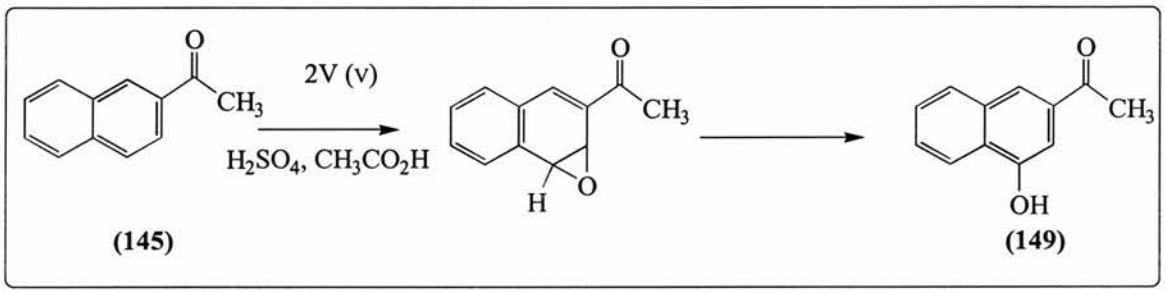


Figure 2.10

2.5.2 Oxidation of acetonaphthone

Employing the established synthetic strategy to the naphthyl derivatives **(98)** and **(99)** required the synthesis of the appropriate starting material 4-hydroxynaphthone **(149)**. The synthesis of this polysubstituted naphthalene is difficult because of the opposing directing effects of the hydroxy and acetyl groups. The only literature synthesis for **(149)** was reported by Behera *et al.* 4-Hydroxy-2-acetonaphthone **(149)** was synthesised in a kinetic study on the use of vanadium (V) as an oxidant in the oxidation of 1- and 2-acetyl naphthalenes.[170] The study reported no yield or purification for the 4-hydroxy-2-acetonaphthone **(149)**, obtained by oxidation of 2-acetylnaphthalene **(145)** with vanadium pentoxide in a mixture of acetic acid (37.5% v/v) and catalytic amounts of 2.2M sulphuric acid at a range of temperatures (Scheme 2.21).



(145) (149)

Scheme 2.21

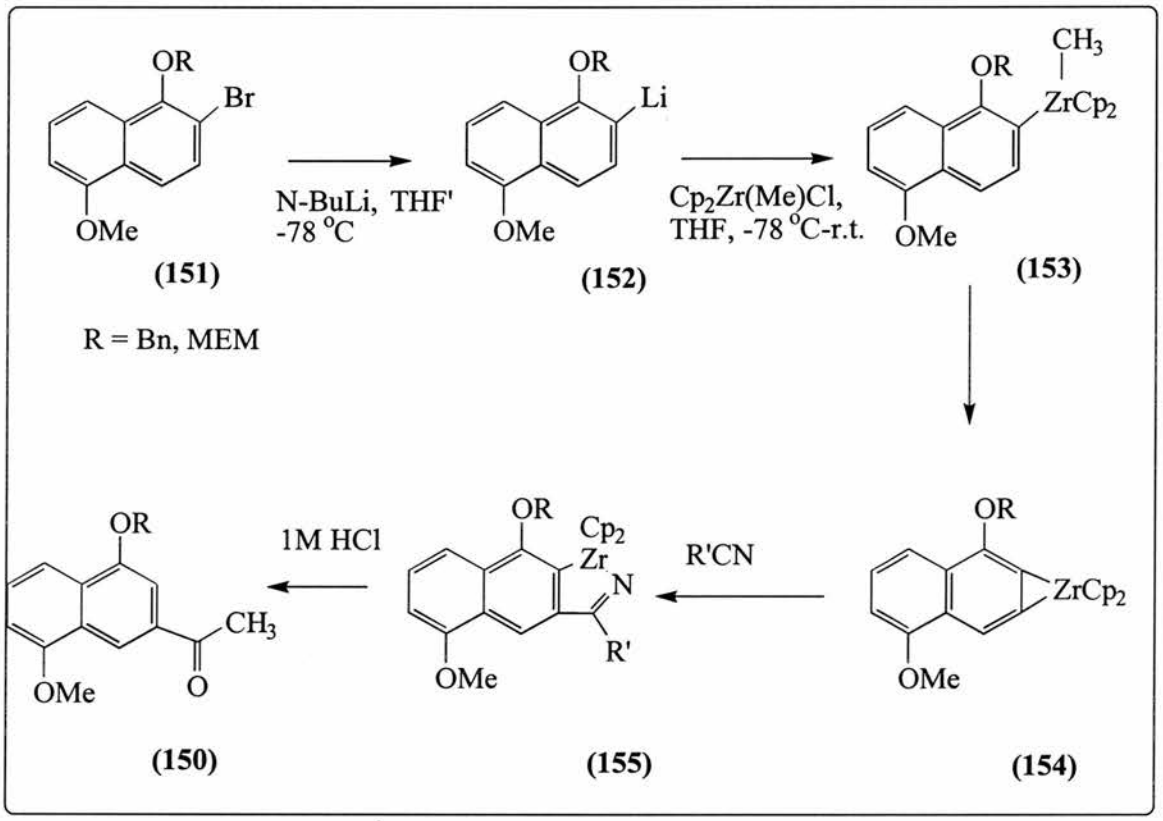
The reported method was employed in an attempt to synthesise 4-hydroxynaphthone **(149)**. 2-Acetylnaphthone **(145)** was reacted, in a molar ratio of 7.5:1.0, with vanadium pentoxide, V_2O_5, in acetic acid (37.5% v/v) with a catalytic amount of sulphuric acid. The suspension was heated initially to 50 °C for four hours and the reaction monitored by tlc. Although a faint band was observed to form by tlc continuation of the reaction under reflux conditions for a further twenty four hours did not result in further reaction. After cooling and filtration through celite to remove the vanadium salts the aqueous layer was extracted into diethyl ether and the solvent removed under reduced pressure to afford an orange solid, which was confirmed by NMR spectroscopy to be starting material. Alteration of the reaction conditions including the use of more concentrated acetic acid solutions, increased amounts of vanadium pentoxide and sulphuric acid as well as longer reaction times did not result in reaction.

Although formation of 4-hydroxy-2-acetonaphthone **(149)** may have been observed under kinetic conditions there was no evidence that oxidation of 2-acetonaphthone **(145)** by vanadium pentoxide was a feasible synthetic route to the desired product.

2.5.3 Regiospecific synthesis of 4-hydroxy-2-acetonaphthone *via* a zirconocene complex

A regiospecific method for the preparation of polysubstituted naphthalenes was reported by Buchwald *et al.* [171,172] A one pot synthesis, conducted under a nitrogen atmosphere with standard Schlenk techniques, was used to prepare a number of *o*-benzylated and *o*-methoxyethoxymethylated, 8-methoxy-4-hydroxy-2-acetonaphthones **(150)** (Scheme 2.22). Reaction of the protected 8-methoxy-4-hydroxy-2-bromo naphthol **(151)** with n-

butyl lithium in THF at -78 °C resulted in *ortho* lithiation of the protected naphthols. The lithiated product was then added to a solution of methylchlorodicyclopentadienylzirconium, $Cp_2Zr(Me)Cl$ in THF at -78 °C with displacement of the lithium by zirconium to form intermediate **(153)**. Loss of methane from intermediate **(153)** resulted in the formation of a nascent complex **(154)**. Addition of a nitrile then resulted in formation of a metallacycle **(155)**. Subsequent acid hydrolysis of **(155)** with dilute HCl afforded the substituted 2-acetonaphthones **(150)** in high yield.

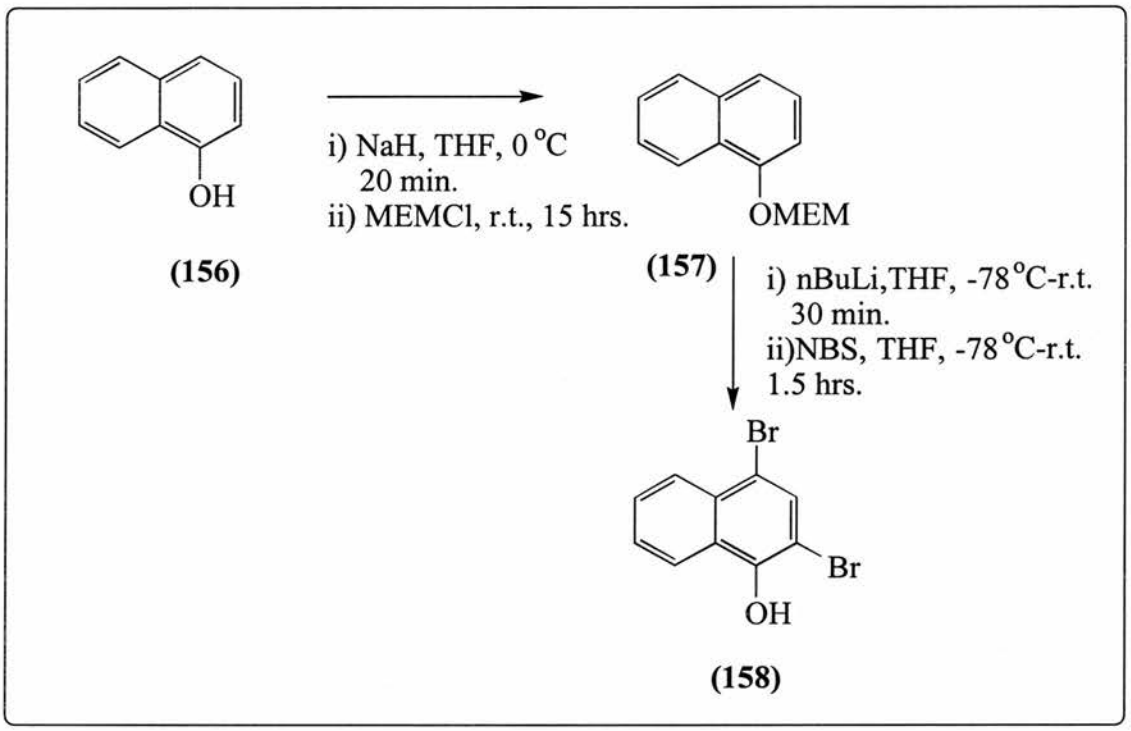


Scheme 2.22

The method was employed in an attempt to synthesise 4-hydroxy-2-acetonaphthone **(149)**. The strategy involved initial protection of the hydroxyl group of the 1-naphthol **(156)** starting material. A methoxyethoxymethyl protecting group may facilitate *ortho* lithiation by coordination of the lithium metal to the ethoxy oxygen. Therefore synthesis of 1-(2-methoxy-ethoxymethyl)-naphthalene **(157)** was undertaken. The method used was that reported by Corey *et al.* [173] for the protection of hydroxyl groups. 1-Naphthol, recrystallised from methanol as a solution in THF was added to a suspension of sodium

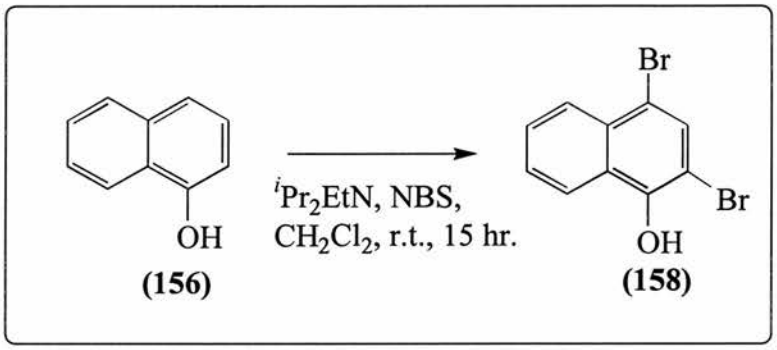
hydride in THF at 0 °C. The formation of the anion of 1-naphthol was monitored by tlc and was completed within twenty minutes, initially a pale green solution formed and then a white precipitate. Methoxyethoxymethylchloride (MEMCl) was added slowly to the anion of 1-naphthol a white suspension formed which was allowed to stir overnight at room temperature. The suspension was then poured onto water, extracted into diethyl ether and the solvent removed under reduced pressure to give an orange oil. Further purification by column chromatography (silica, ethyl acetate: petroleum ether 1:3) afforded **(157)** as a yellow oil in 41% yield. The compound was characterised by NMR spectroscopy and mass spectrometry.

ortho Bromination of **(157)** was attempted by reaction of 1-(2-methoxy-ethoxymethyl)-naphthalene with *N*-bromosuccinimide in the presence of n-butyllithium. 1-(2-Methoxy-ethoxymethyl)-naphthalene **(157)** was dissolved in THF cooled to -78 °C and n-butyl lithium added slowly. The solution was allowed to warm to room temperature and stirred for thirty minutes. The pale green solution was then recooled to -78 °C and *N*-bromosuccinimide was added, resulting in the formation of a yellow precipitate. The mixture was warmed to room temperature and stirred for a further hour. The reaction mixture was then poured onto methylene chloride which dissolved the yellow solid and precipitated succinimide. After filtration and removal of the solvent an orange oil was obtained. Further purification by column chromatography afforded 2,4-dibromo-1-naphthol **(158)** as the main product (Scheme 2.23). The structure was confirmed by NMR spectroscopy and mass spectrometry. The resonances due to the methoxyethoxymethyl group were absent and a resonance due to the naphthol OH was observed at 6.07 ppm.



Scheme 2.23

A selective method for the *ortho* bromination of phenols reported by Fugisaki *et al.* was also employed in an attempt to synthesise 2-bromo-1-naphthol **(159)**.[174] This could then be used to synthesise the *o*-brominated methoxyethoxymethyl naphthol compound by reaction of 2-bromo-1-naphthol **(159)** with methoxyethoxyethoxy chloride as described above. 1-Naphthol **(156)** and diisopropylethylamine were stirred at room temperature in methylene chloride for thirty minutes before the slow addition of *N*-bromosuccinimide. The reaction was stirred overnight before being quenched with acid. After workup and further purification by column chromatography, however, 2,4-dibromo-1-naphthol **(158)** was obtained as the major product (Scheme 2.24). This was verified by NMR spectroscopy and mass spectrometry.



Scheme 2.24

Since *ortho* bromination was not successful direct *ortho* metalation was attempted. Direct *ortho* metalation is a well known synthetic strategy in the preparation of polysubstituted aromatics. Using Schlenck techniques 1-(2-methoxy-ethoxymethyl)-naphthalene **(157)** was added to n-butyllithium in THF at -78 °C then warmed to room temperature and stirred for twenty minutes. The reaction mixture was recooled to -78 °C and to this a solution of $Cp_2Zr(Me)Cl$ in THF was added. This was stirred for fifteen minutes and then warmed to room temperature. Acetonitrile was then added and the reaction mixture stirred at room temperature for seventeen hours. This was followed by acid hydrolysis and subsequent work-up to afford an orange oil. Analysis by NMR spectroscopy showed only the presence of starting materials. The reaction was repeated with the mode of addition reversed, ie. the zirconocene complex was added to the solution of 1-(2-methoxy-ethoxymethyl)-naphthalene **(157)** and n-butyllithium in THF, but no reaction was observed to occur.

Attempts to trap the anion of 1-(2-methoxy-ethoxymethyl)-naphthalene **(157)** did not succeed. 1-(2-Methoxy-ethoxymethyl)-naphthalene **(157)** was reacted with n-butyllithium in THF at -78 °C and methyl iodide then added to the solution. The reaction mixture was stirred at -78 °C for one hour and at room temperature for a further three hours. However, after workup, ^{1}H NMR spectroscopy confirmed the presence of starting materials. Trapping the anion using benzaldehyde under the same conditions was also unsuccessful.

The above examination of *ortho* metalation of 1-naphthol indicates that formation of a lithium stabilised anion is difficult. The use of an alternative protecting group may facilitate successful *ortho* metalation and subsequent acylation of 1-naphthol. There are a number of protecting groups which may be of greater synthetic utility. Methoxymethyl

(MOM) is a similar protecting group which is known to be more successful when compared with the methoxyethoxymethyl (MEM) group as a directing group in *ortho* metalation reactions.[175] A benzyl group may also be an alternative, being used extensively as a protecting group in the synthesis of polysubstituted naphthalenes.[172] It may also afford the protected 1-naphthol in higher yield requiring a less difficult synthesis.

2.6 Conclusion

A number of kynurenine analogues were synthesised in high yield and the use of a pivaloyl protecting group proved successful for the synthesis of analogues containing a hydroxyl group *meta* to the butyric acid moiety. Reduced kynurenines which contained a hydroxyl group at the γ-position were obtained as diastereomeric mixtures with the exception of dihydroxydesaminokynurenine **(90)** and the γ-hydroxynaphthalene derivative **(97)** (Table 2.2). Following final recrystallisation from either ethanol or water respectively, spectral data for both compounds indicated the presence of a single diastereomer of the final product as the racemic mixture, (4*S*,2*S*) and (4*R*,2*R*). In contrast, γ-hydroxydesaminokynurenine **(33)** and γ-hydroxy-3'-methoxydesaminokynurenine **(91)** were each obtained as a diastereomeric mixture, ratio 1:2, with the major isomer being the (4*S*,2*S*) and (4*R*,2*R*) pair of enantiomers. Both compounds were recrystallised from methanol. The predominance of one diastereomer, for both dihydroxydesaminokynurenine **(90)** and the γ-hydroxynaphthalene **(97)** derivative, may be due to differences in the solubility of different isomers in the solvents used for crystallisation. The dihydroxytetralone derivative **(92)** was obtained as a complex mixture of diastereomers due to the presence of three stereogenic centres. As a result the ^{1}H NMR spectrum of **(92)** was very difficult to analyse.

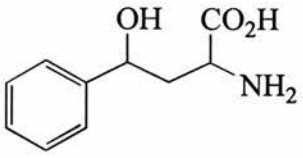
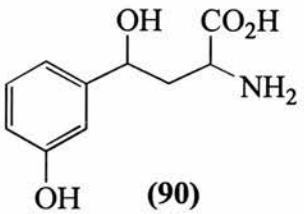
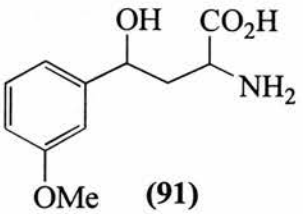
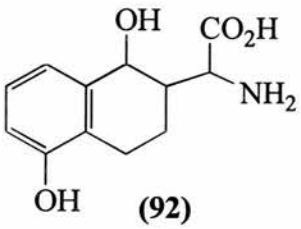
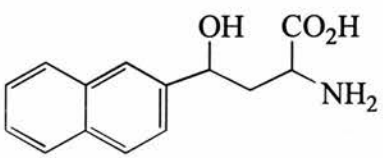
COMPOUND	Major diastereomer/ pair of enantiomers	Minor diastereomer/ pair of enantiomer	ratio
 **(33)**	(4*S*,2*S*), (4*R*,2*R*)	(4*R*,2*S*), (4*S*,2*R*)	2:1
 **(90)**	(4*S*,2*S*), (4*R*,2*R*)	–	–
 **(91)**	(4*S*,2*S*), (4*R*,2*R*)	(4*R*,2*S*), (4*S*,2*R*)	2:1
 **(92)**	–	–	–
 **(97)**	(4*S*,2*S*), (4*R*,2*R*)	–	–

Table 2.2

CHAPTER 3

3 BIOLOGICAL STUDIES WITH HUMAN RECOMBINANT KYNURENINASE

3.1 Background

Inhibitors of kynureninase may be used as modulators of the kynurenine pathway to increase levels of neuroprotective kynurenic acid **(3)** while preventing the accumulation of neurotoxic quinolinic acid **(2)**. Thus far, most inhibition studies on kynureninase have used the bacterial enzyme isolated from *Pseudomonas fluorescens*. However, as discussed in Chapter one the bacterial and mammalian enzymes exhibit different substrate specificities. The bacterial enzyme hydrolyses L-kynurenine faster than 3-hydroxy-L-kynurenine while the mammalian enzyme hydrolyses 3-hydroxy-L-kynurenine much faster than L-kynurenine.[26] Differences in active site specificity may result in inhibition studies which are not reflective of conditions *in vivo* and therefore affect the assessment of potential inhibitors of the enzyme. Up until now rat liver kynureninase has been used as a source of mammalian enzyme. Although human recombinant kynureninase was successfully isolated and expressed in human embryonic kidney cells by Aberati-Giani *et al.*, the quantities of enzyme isolated were too small to allow either proper characterisation of the human enzyme or its use in kinetic studies.[37] Recently, within this group, human recombinant kynureninase has been expressed and isolated in quantities which allow comprehensive studies to be carried out on the inhibitory properties of synthesised kynurenine analogues.[38] Successful purification of the enzyme has also allowed detailed characterisation of the human form of kynureninase.[39] As part of this project human recombinant kynureninase was expressed and isolated and used to determine the inhibitory properties of the substrate analogues discussed in Chapter two.

3.2 Cloning and expression of human recombinant kynureninase

Human recombinant kynureninase was successfully cloned and expressed in this group by Dr. Deirdre Fitzgerald see Section 6.2.[38] The cDNA clone encoding human liver kynureninase was isolated and cloned into the eukaryotic expression vector pBC/CMV. The 'Bac to Bac' baculovirus expression system (GibcoBRL) was then used to express kynureninase in *Spodoptera frugiperda* (*sf*9) insect cells using the protocols described by the manufacturer's manual. A number of batches of human recombinant kynureninase were prepared for this project using the above protocol and used in the examination of the inhibitory properties of the substrate analogues discussed in Chapter two.

3.3 Purification and characterisation of human recombinant kynureninase

Recombinant human kynureninase was successfully purified to homogeneity within this group by Dr. Harold Walsh.[39] The purity of the enzyme was determined by SDS-PAGE analysis and the molecular weight verifed by MALDI-TOF mass spectrometry. Human kynureninase was found to be a homodimer with a monomeric molecular mass of approximately 52.4 kDa. It shares an amino acid sequence homology of 85% with rat liver kynureninase which has also been successfully cloned and expressed.[36] The UV absorption spectrum of the purifed enzyme showed a peak at 432 nm due to the presence of the PLP co-factor. This is similar to that reported by Kishore for bacterial kynureninase from *Pseudomonas fluorescens* (formerly known as *Pseudomonas marginalis*).[50] The human enzyme exhibits a pH optimum of 8.25 and the enzyme activity was found to be strongly dependent on the ionic strength of the buffer. All activity assays for human kynureninase were monitored spectrofluorometrically according to the method of Shetty and Gaertner, (see Sections 3.4.1 and 6.2).[177] The assays were also carried out at pH 7.9. Although the enzyme exhibits a pH optimum of 8.25 no difference in enzyme activity was observed between the two pH's.

Kinetic characterisation of the human kynureninase showed that the enzyme was specific for 3-hydroxykynurenine exhibiting a K_m of 3.0 ± 0.1 μM. This is lower than previously reported values in this laboratory,[38] K_m = 9.4 ± 1.8 μM as well as that reported by Alberati-

Giani *et al.*, K_m = 13.2 ± 2.0 μM.[37] The value is also lower than the average K_m value of 5.7 ± 0.3 μM, determined for a combination of a number of batches of the enzyme prepared for inhibition studies in this project (see section 3.4.1). The K_m value is similar to that reported by Shetty and Gaertner for fungal kynureninase (*P. Roqueforti*), K_m = 4.0 μM.[177] The enzyme showed no activity towards L-kynurenine in contrast to the report by Aberati-Giani *et al.* where a K_m value of 671 ± 37 μM was demonstrated.[37] L-kynurenine was in fact shown to be a competitive inhibitor of the enzyme at low concentrations, K_i = 20 μM, while exhibiting mixed inhibition at higher substrate concentrations, K_i = 55 μM. In contrast other mammalian sources of kynureninase such as rat liver, exhibited substrate activity for L-kynurenine. This is thought to be due to the preparation of crude cell homogenates resulting in additional adventitious enzyme activity. The human enzyme was also shown to be inhibited by D-kynurenine with K_i of 12 μM. Kinetic analysis of recombinant human kynureninase also revealed that the enzyme appeared to be subject to cooperative modulation by the substrate 3-hydroxykynurenine. This was based on the observation of sigmoidal plots obtained in the absence and presence of the potent inhibitor dihydroxdesaminokynurenine **(90)**, K_i = 100 nM. This inference of a second regulatory binding site on the human enzyme was further corroborated by the observation of mixed inhibition by the analogue **(90)** which produced a reduction in V_{max} and increase in K_m. The low K_m value obtained for the purified enzyme was also ascribed to an enzyme displaying sigmoidal kinetics so that the K_m value is not the same as the Michaelis constant K_m but rather a sigmoidal constant K_s which incorporates interaction factor(s) and therefore is not the substrate concentration at 50% V_{max}. The evident specificity of recombinant human kynureninase for 3-hydrokynurenine was used in the design and synthesis of a number of inhibitors as discussed Chapter two

3.4 Assay of kynureninase activity

The activity of kynureninase was determined spectrofluorometrically at 37 °C using the method reported by Shetty and Gaertner.[177] The rate of formation of the product 3-hydroxyanthranilate was measured at wavelengths of excitation at 330 nm and emission at 410 nm. The reaction mixture contained 20 μM PLP, 10 mM Tris-base buffer (pH 7.9), the substrate DL-3-hydroxykynurenine and the enzyme (10-20 μl). The reaction rate was measured in fluorescence intensity units/min (FI/min).

3.4.1 Determination of K_m for 3-hydroxykynurenine

The K_m and V_{max} for each batch of human enzyme were determined by conducting a series of assays over a substrate range of 2.5-20.0 μM. The change in fluorescence intensity (FI/min) for each assay was then corrected for one unit of enzyme activity to eliminate variations in activity from different batches of enzyme (See Section 6.3). Each assay was carried out in triplicate and the best two or three values (± 5%) were averaged. The kinetic parameters, K_m and V_{max}, were then calculated by fitting the initial rate data to the Michaelis Menten equation using non linear regression with the GraphPad Prism 3 software package on a PC.

The average K_m value from four batches of enzyme was determined to be 5.7 ± 0.3 μM (see Section 6.3, Table 6.2). This is somewhat higher than the K_m value obtained for the purified enzyme of 3.0 ± 0.1 μM while being smaller than the previously reported value in this laboratory of 9.4 ± 1.8 μM. The average V_{max} was determined to be 7.7 ± 0.6 nmol/min/mg.

The substrate velocity plots for the four enzyme batches were found to obey Michaelis Menten kinetics in the substrate range examined.

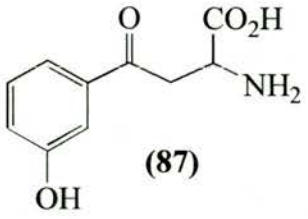
3.5 Inhibition studies on substrate analogues

All of the 3-hydroxykynurenine analogues discussed in chapter two were examined against the crude recombinant human kynureninase to determine their inhibitory properties.

The inhibition of recombinant human kynureninase by the substrate analogues was examined by assaying the activity of the enzyme at various concentrations of 3-hydroxykynurenine, 2.5 to 20 μM, in the presence of appropriate concentrations of inhibitor using the method described in Sections 3.4. and 6.3. The range of inhibitor concentrations used for each analogue was determined by preliminary screening, assessing the % inhibition of enzyme activity at a fixed concentration of 3-hydroxykynurenine (25 μM). In order to determine the inhibitory properties of the analogues, the kinetic parameters, K_m and V_{max}, were then re-determined, at various concentrations of the inhibitor, by fitting the initial rate data to the Michaelis-Menten equation using non linear regression with the GraphPad Prism 3 software package on a PC. The effect on the kinetic parameters then gave a measure of the strength and type of inhibition.

K_i values for some inhibitors were determined by linear regression plots of K_{app} versus [I], where the X intercept of this plot gives $-K_i$. The K_i value is the dissociation constant for an inhibitor enzyme complex, therefore the lower the K_i value the more tightly the inhibitor binds to the enzyme. Lineweaver-Burk plots were also drawn to illustrate the type of inhibition exhibited by each inhibitor.

3.5.1 2-Amino-4-oxo-4-(3-hydroxy-phenyl)-butyric acid (3'-hydroxy desaminokynurenine) (87)



(87)

Since the human enzyme selectively hydrolyses 3-hydroxykynurenine and not kynurenine, 3'-hydroxydesaminokynurenine **(87)** was examined as an inhibitor of kynureninase. This inhibitor thus possesses a hydroxyl function in the 3-position of the ring but no amino group at the 2-position. The kinetic analysis of 3'-hydroxydesaminokynurenine **(87)** showed it to be the most potent inhibitor of recombinant human kynureninase synthesised to date. Preliminary testing to establish the appropriate inhibitor concentration range for inhibition studies indicated potent inhibition of the enzyme with 60% inhibition occurring at 100 nM (Figure 3.1). The kinetic data obtained with recombinant human kynureninase for 3'-hydroxy desaminokynurenine **(87)** are given in Table 3.1. Each rate was measured in triplicate and then the average value of two or three consistent values (± 5%) were obtained and plotted (Velocity versus [Substrate]) using non-linear regression analyses to determine the values of K_m and V_{max}.

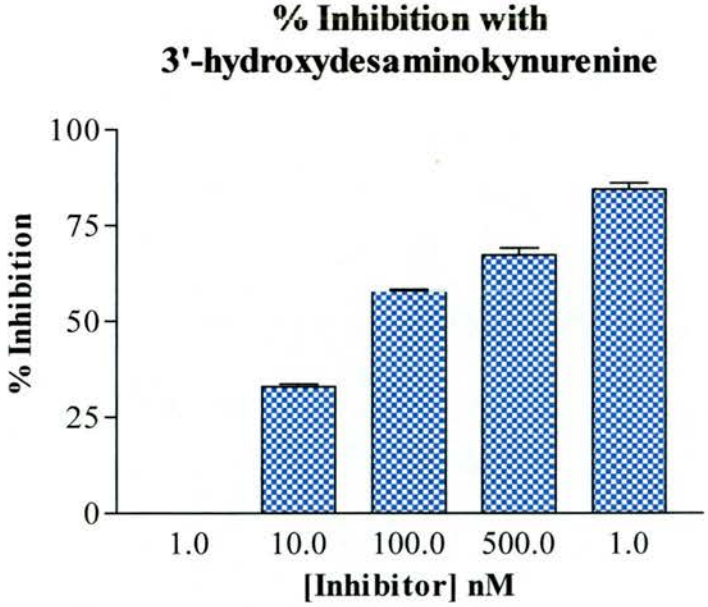


Figure 3.1

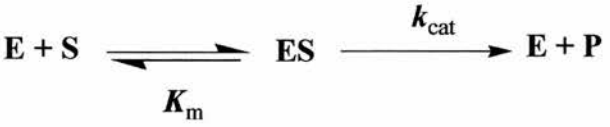
[Substrate] μM	10 nM [I] rate (FI/min)	20 nM [I] rate (FI/min)	40 nM [I] rate (FI/min)
2.5	4.24	2.68	1.73
5.0	6.66	4.76	3.08
10.0	11.83	9.09	6.29
15.0	14.69	13.38	8.41
20.0	18.91	14.84	10.72
V_{max} nmol/min/mg	7.61 ± 1.15	7.86 ± 1.87	7.84 ± 1.29
K_m μM	13.6 ± 3.66	19.3 ± 6.43	31.8 ± 6.51

Table 3.1 Substrate and velocity data for 3'-hydroxydesaminokynurenine (87)[I]

The type of inhibition exhibited by **(87)** can be assessed by comparing the apparent change in K_m and V_{max} with the values obtained for the substrate in the absence of inhibitor. The average K_m and V_{max} determined for the substrate 3-hydroxykynurenine **(87)** were 5.71 ±0.3 μM and 7.70 ± 0.6 nmol/min/mg, respectively. 3'-Hydroxy desaminokynurenine **(87)** was shown to be a competitive inhibitor of kynureninase, giving an increase in K_m with increasing inhibitor concentration while V_{max} remained unchanged within experimental error (Table 3.1).

A competitive inhibitor binds to the free enzyme in a manner which prevents the substrate from binding. Therefore the enzyme can bind to either the inhibitor or the substrate but not both. Thus the inhibitor competes with the substrate to bind to the active site of the enzyme and diminishes the rate of catalysis by reducing the amount of enzyme available to bind to the substrate.

A simple model for a single substrate enzyme reaction was developed by Michaelis-Menten in which a substrate (S) binds to the active site of an enzyme E to form an enzyme-substrate (ES) complex. This complex may break down to release the substrate or result in reaction and the subsequent release of the product.

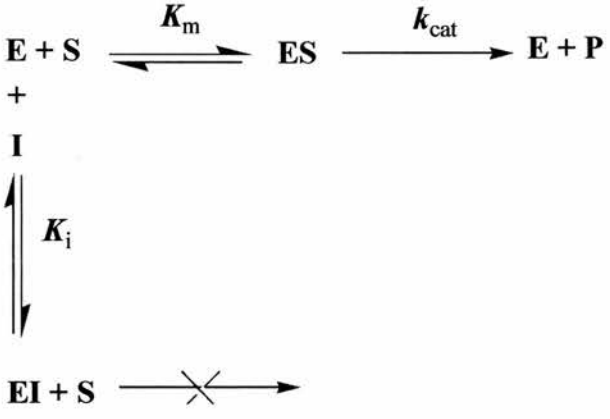


The first step of the catalytic reaction involves formation of the enzyme substrate complex (ES), which is a rapid and reversible process. Chemical reaction occurs in the second step with subsequent release of the product. This reaction is first order with a rate constant k_{cat}.

This system is described by the rate equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

However in the presence of a competitive inhibitor the reaction becomes more complicated with the enzyme distributed between three species; free enzyme (E), the enzyme substrate complex (ES) and the enzyme inhibitor complex (EI).



The equation is then modified to give

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]}$$

Thus in the case of competitive inhibition the apparent K_m is altered by a factor of $(1 + [I]/K_i)$ and is known as K_{app}. Therefore the K_{app} of the substrate increases in the presence of a competitive inhibitor. However, V_{max} remains unchanged as at infinite substrate concentration the effect of the inhibitor is competed out by substrate.

A Lineweaver-Burk Plot can also be used to illustrate the type of inhibition observed. The Michaelis-Menten equation can be rewritten in reciprocal form

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

The plot of $1/v$ (velocity) versus $1/[S]$ affords a straight line in which the intercept at the Y axis is $1/V_{max}$ while the intercept at the X axis is $-1/K_m$. The plot thus gives a good pictorial representation of changes in V_{max} and K_m. However, it is a very poor model for calculating accurate values of the kinetic parameters. This is because being a double reciprocal plot it overemphasizes errors in low concentration data and underemphasizes errors in high concentration data. The Lineweaver-Burk plot for **(87)** is shown in Figure 3.2.

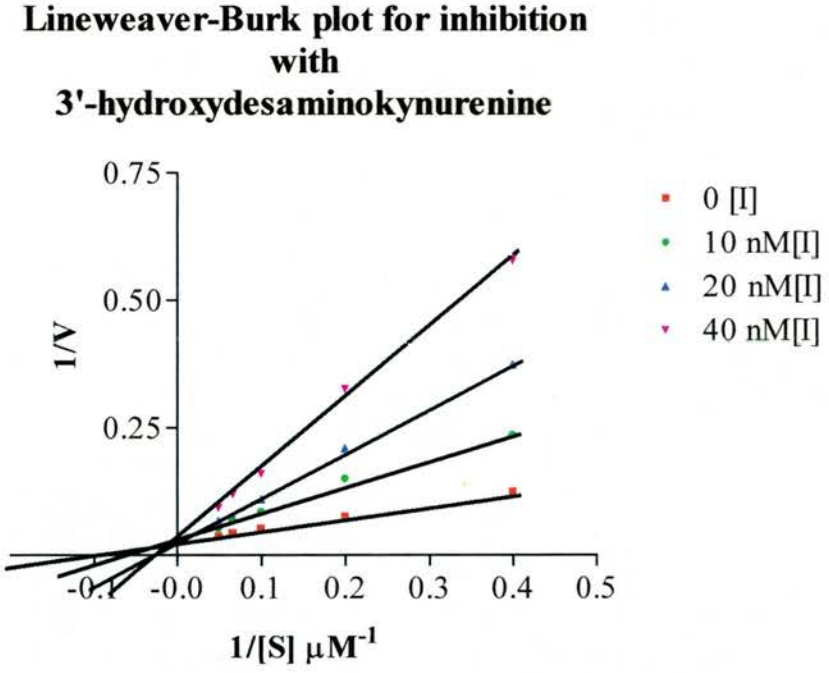


Figure 3.2

The Lineweaver-Burk plot for 3'-hydroxydesaminokynurenine **(87)** shows that the compound is indeed a competitive inhibitor of recombinant human kynureninase. The K_i value for an inhibitor can be determined from

$$K_{app} = K_m (1 + [I]/K_i)$$
$$= K_m[I]/K_i + K_m$$

Therefore a plot of the K_{app} versus inhibitor concentration gives an intercept at the X-axis which is $-K_i$. The value of K_i is a measure of the potency of an inhibitor. The K_i of 3'-hydroxydesaminokynurenine **(87)** was obtained from such a plot (Figure 3.3). Linear regression analysis was employed to determine the best straight line and errors were obtained from the regression analyses.

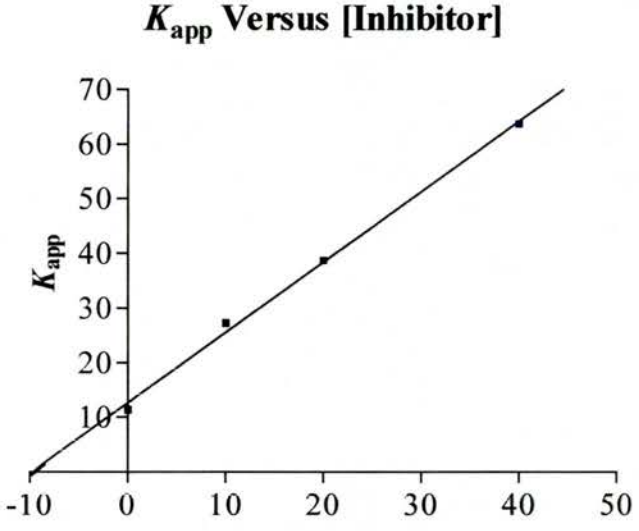
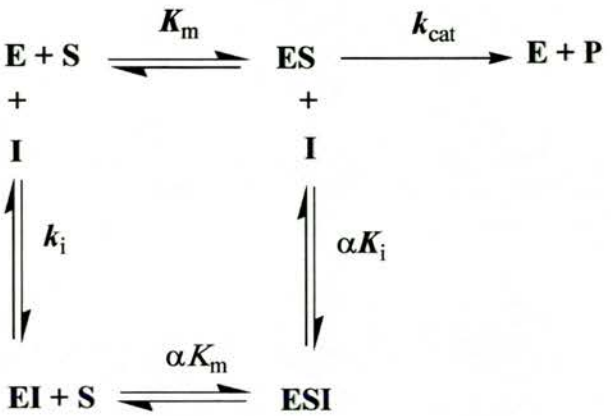
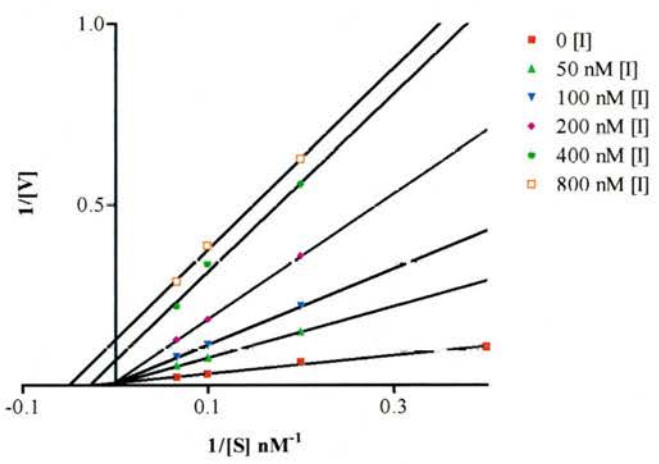


Figure 3.3

The K_i value for 3'-hydroxydesaminokynurenine **(87)** was determined to be 5.0 ± 0.5 nM. This is the most potent inhibitor of kynureninase to date. As a result of the potency of the inhibition of recombinant human kynureninase by **(87)** it was decided to carry out a comparative study with both rat liver kynureninase and bacterial kynureninase from *Pseudomonas fluorescens*. This study was carried out by Dr. Harold Walsh. When tested against both the rat liver enzyme and the bacterial enzyme, 3'-hydroxydesaminokynurenine **(87)** was shown to be a mixed inhibitor giving changes in both V_{max} and K_m with increasing inhibitor concentration. The data are displayed in Lineweaver-Burk plots for the rat liver and bacterial enzyme respectively (Figures 3.4 and 3.5). Inhibition of the rat enzyme appears to be non-competitive at low concentrations and

competitive at high concentrations. Linear mixed inhibitors can bind to the ES complex while the substrate can bind to the EI complex and both the inhibitor and substrate bind reversibly. The ESI complex is, however, catalytically inactive. The presence of the inhibitor on the enzyme changes the dissociation constant of S from K_m to αK_m. The dissociation constant for I, K_i, is changed by the same factor to αK_i.





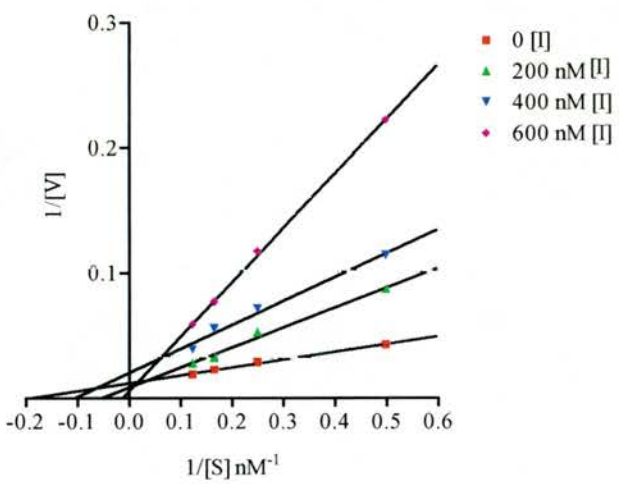


Figure 3.4 **Figure 3.5**

Lineweaver-Burk plots for 3'-hydroxydesaminokynurenine (87) with rat and bacterial kynureninase, respectively

The K_i value for the rat enzyme was obtained from a replot of slope (from the Lineweaver-Burk plot where slope = K_m/V_{max}) versus inhibitor concentration (Figure 3.6). The K_i value for the bacterial enzyme was obtained from a plot of 1/V versus inhibitor concentration (Figure 3.7). The intercept at the X axis is $-K_i$. The values for 3'-hydroxy desaminokynurenine were 20 nM and 25 nM respectively for the rat and bacterial enzyme. This is the most potent inhibtion of both the rat and bacterial enzyme to date.

In summary, 3'-hydroxydesaminokynurenine **(87)** is the most potent inhibitor of kynureninase thus far. It behaves as a highly potent competitive inhibitor of recombinant human kynureninase exhibiting a K_i of 5 nM. 3'-Hydroxydesaminokynurenine **(87)** also exhibits mixed inhibition towards rat liver kynureninase and bacterial kynureninase. It is the most potent inhibitor so far of these two sources of kynureninase exhibiting K_i values of 20 nM and 25 nM, respectively. These values are much lower than the most potent inhibitor of bacterial kynureninase reported in the literature to date, K_i = 70 nM, for *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide **(47)**

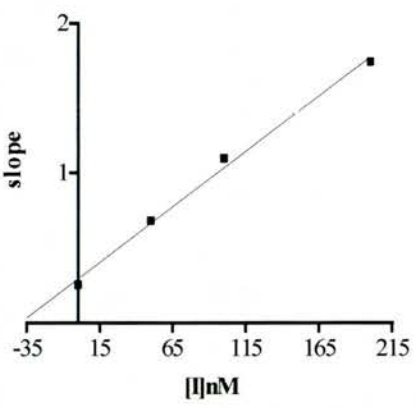


Figure 3.6

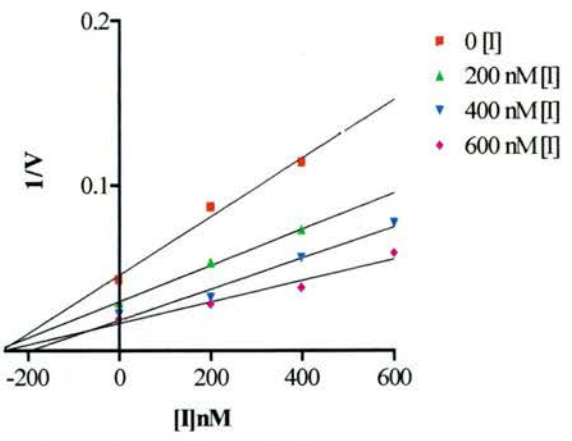
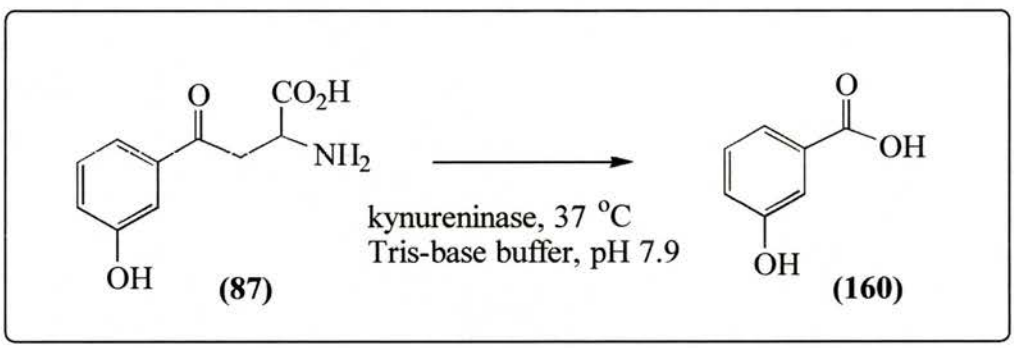


Figure 3.7

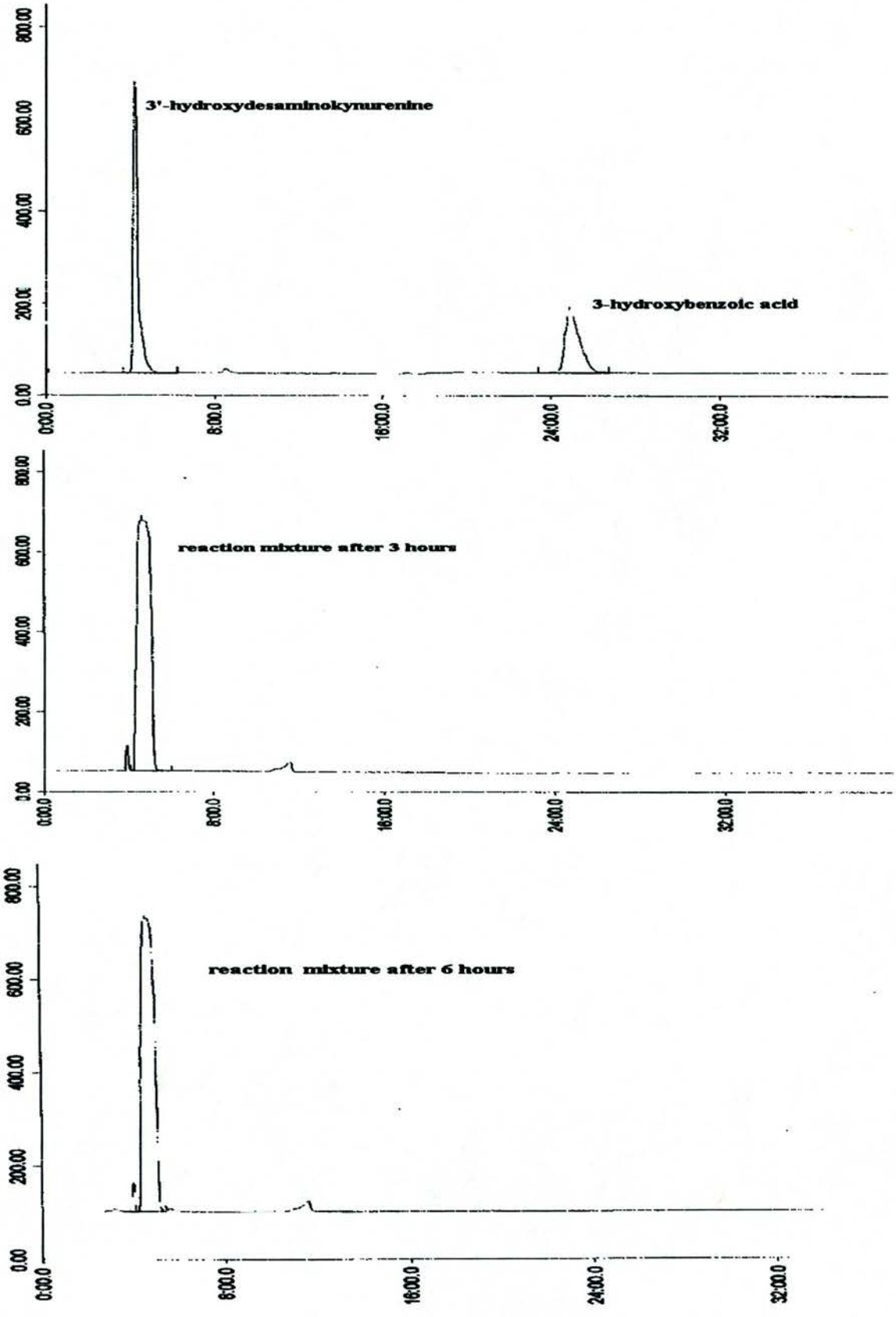
K_i determination for 3'-hydroxydesaminokynurenine (87) with rat and bacterial kynureninase, respectively

3.5.1.1 Turnover experiment for 3'-hydroxydesaminokynurenine (87)

A turnover experiment was performed to determine whether 3-hydroxydesaminokynurenine **(87)** was hydrolysed by kynureninase under standard conditions (Tris-base buffer, 10 mM (pH 7.9), 37 °C), to afford the hydrolysis product, 3-hydroxybenzoic acid (Scheme 3.1). The reaction was monitored by analytical HPLC (chromatograms shown on following pages, Figure 3.8). Thus, 3'-hydroxydesaminokynurenine **(87)** was incubated with kynureninase at 37 °C for three hours and then an aliquot of the reaction mixture was removed. The enzyme was precipitated using trichloroacetic acid, the mixture filtered and the solvent removed under reduced pressure. The resulting white residue was then analysed by HPLC. There was no evidence for reaction having occurred when compared with a standard containing 3'-hydroxydesaminokynurenine **(87)** and 3-hydroxybenzoic acid **(160)**. Two further additions of kynureninase were made and the reaction analysed after six and twenty seven hours. However, analysis of the reaction mixture after workup with trichloroacetic acid again showed no evidence for hydrolysis having occurred.



Scheme 3.1


3'-hydroxydesaminokynurenine
3-hydroxybenzoic acid
reaction mixture after 3 hours
reaction mixture after 6 hours
800.00
600.00
400.00
200.00
0.00
0:00.0
8:00.0
16:00.0
24:00.0
32:00.0

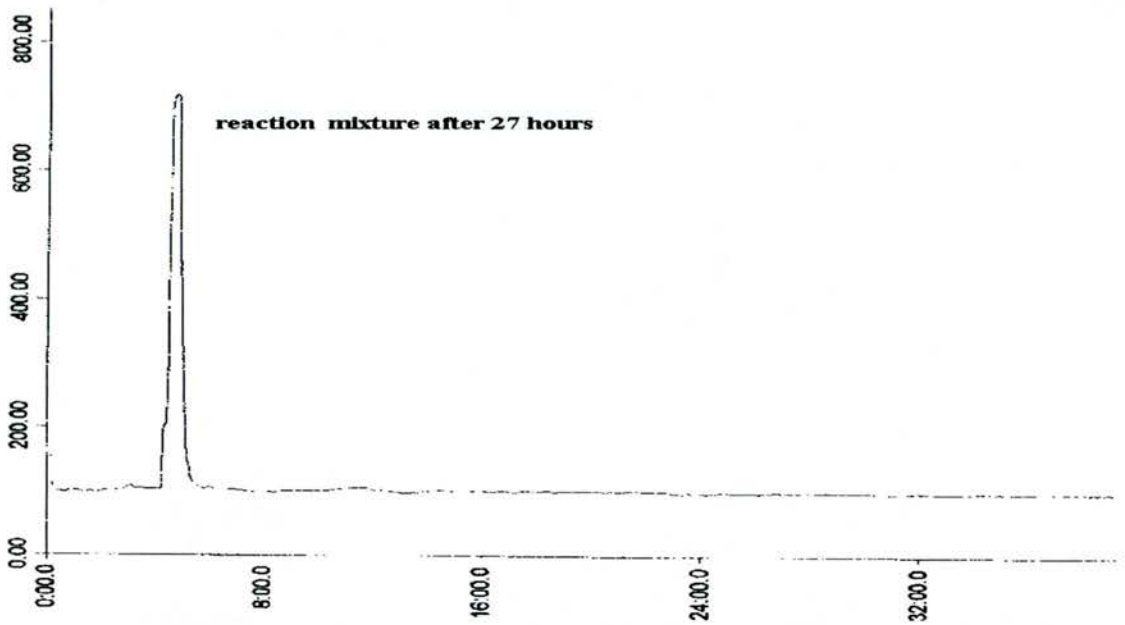
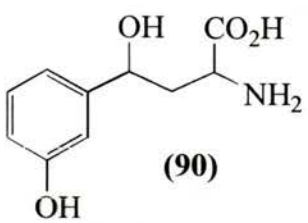


Figure 3.8 HPLC chromatograms for 3'-hydroxydesminokynurenine (87) turnover experiment

3.5.2 2-Amino-4-hydroxy-4-(3-hydroxy-phenyl)-butyric acid (dihydroxy desaminokynurenine) (90)



(90)

In the reaction mechanism proposed by Phillips and Dua, the β,γ-hydrolytic cleavage of kynureninase is mediated by a water molecule, which attacks the γ-carbonyl of the substrate to form a tetrahedral gem diolate intermediate **(27)**.[42] This can be used as a model for the transition state for the reaction. Some evidence for this mechanism arose from inhibition studies using the dihydrokynurenines **(16a)** and **(16b)** which were observed to be potent competitive inhibitors of the enzyme. It was proposed that the tight binding arose because both alcohols were able to mimic the tetrahedral intermediate **(27)**, thus behaving as transition state analogues. The design and synthesis of a number of inhibitors using the tetrahedral intermediate **(27)** as a model for the transition state have been reported. 2-Amino-4-hydroxy-4-(3-hydroxy-phenyl)-butyric acid **(90)**, which contains a γ-hydroxy group thus mimicking the *gem*-diolate intermediate was synthesised

as a transition state analogue inhibitor of kynureninase (see paper in appendix). The compound also contains a hydroxyl group at the 3-position of the aryl ring which should function as a recognition element for the human enzyme.

A detailed kinetic study on the inhibition properties of the dihydroxy desaminokynurenine **(90)** was carried out by Dr. Harold Walsh in this laboratory. The inhibitory properties of **(90)** were examined against recombinant human kynureninase, rat liver kynureninase and bacterial kynureninase. The inhibitor was assayed spectrofluorometrically using similar conditions to those dicussed in Section 3.3. The compound was observed to be a potent inhibitor of mammalian kynureninase exhibiting K_i values of 100 nM and 130 nM for the human and rat enzyme respectively. It was found to be almost 100-fold less potent as an inhibitor of bacterial kynureninase, exhibiting a K_i value of 10 μM. The inhibition observed with human kynureninase was mixed with competitive inhibition occurring at low substrate concentrations while the inhibition became non-competitive at higher substrate concentrations. The mixed inhibition is thought to due to the presence of a second regulatory binding site on the human enzyme as discussed in Section 3.3. The Lineweaver-Burk plots for inhibition studies against both human and rat kynureninase, respectively, are shown in Figure 3.9 and Figure 3.10.

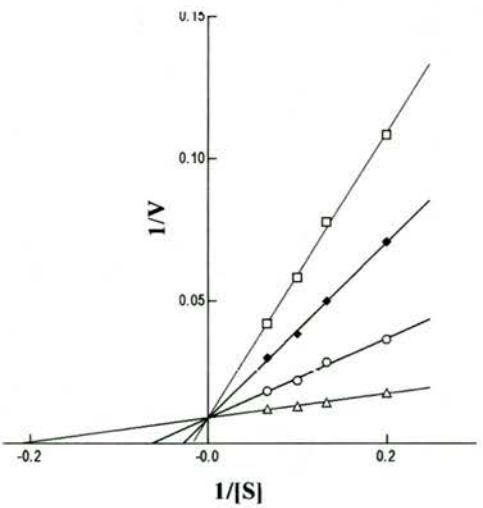


Figure 3.9

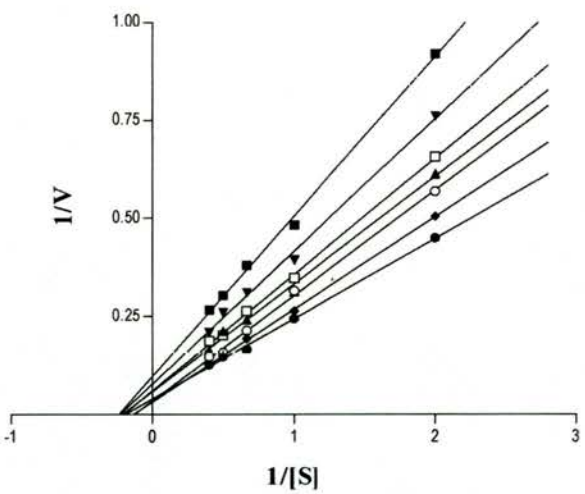


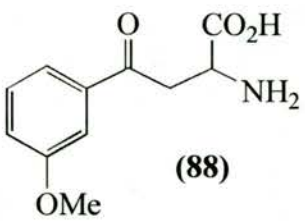
Figure 3.10

Lineweaver-Burk plots for dihydroxydesaminokynurenine (90) with human and rat kynureninase, respectively

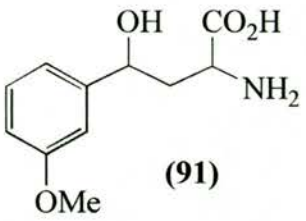
The potency of the inhibition of mammalian kynureninase by dihydroxy desaminokynurenine **(90)** and 3'-hydroxydesaminokynurenine **(87)**, K_i 5 nM, indicates that the presence of a hydroxy function on the 3-position is important as a recognition element for the mammalian enzyme. However, 3'-hydroxy desaminokynurenine **(87)** was also an exceptionally potent inhibitor of bacterial kynureninase exhibiting a K_i of 25 nM while dihydroxydesaminokynurenine **(90)**, K_i 10 μM, was a much weaker inhibitor by an order of 400-fold. This may imply that the presence of the γ-carbonyl is a more important recognition factor for bacterial kynureninase than for the mammalian enzyme. It was thought that dihydroxy desaminokynurenine **(90)** would be a better inhibitor because it mimicked the transition state but this does not appear to be the case. The absence of the *ortho* amino group from the analogues does not appear to significantly affect binding to the enzyme. However, 3'-hydroxydesaminokynurenine **(87)** was not hydrolysed indicating that the amino group may be important in promoting β,γ-hydrolytic cleavage. This is supported in a study on substrate specificity by Tanisawa and Soda, which showed that the absence or modification of the *ortho* amino group of kynurenine analogues resulted in lower reactivity towards hydrolysis.[40] It was also found that while compounds such as *N*-formyl-L-kynurenine **(5)** and desamino-L-kynurenine **(15)** exhibited low reactivities towards kynureninase binding to the enzyme was not reduced.

3.5.3 3'-Methoxy substituted inhibitors of kynureninase

The hydroxyl group at the 3-position on the aryl ring of compounds **(87)** and **(90)** appears to serve as a recognition element for the mammalian enzyme. This may involve hydrogen bonding of the hydroxyl group at the active site. To further investigate this, the hydroxyl group was replaced by a methoxy group, and the compounds 3'-methoxydesaminokynurenine **(88)** and γ-hydroxy-3'-methoxydesaminokynurenine **(91)** were examined as inhibitors of the enzyme to compare with 3'-hydroxydesaminokynurenine **(87)** and dihydroxy desaminokynurenine **(90)**, respectively.



(88)



(91)

3.5.3.1 2-Amino-4-oxo-4-(3-methoxy-phenyl)-butyric acid (3'-methoxy desaminokynurenine) (88)

The preliminary inhibition study using 3'-methoxydesaminokynurenine **(88)** in Figure 3.11 showed that inhibition of recombinant human kynureninase by 3'-methoxydesaminokynurenine **(88)** occurred over a very narrow inhibitor concentration range, 5-50 μM, with virtually no inhibition occurring below 5 μM and 100% inhibition occurring above 50 μM. Detailed kinetic rate data were thus obtained for three inhibitor concentrations of 10 μM, 20 μM and 40 μM. Each rate was determined in triplicate and then the average value of two or three consistent values (± 5%) was obtained and plotted. Non-linear regression analysis afforded the values of K_m and V_{max} (Table 3.2).

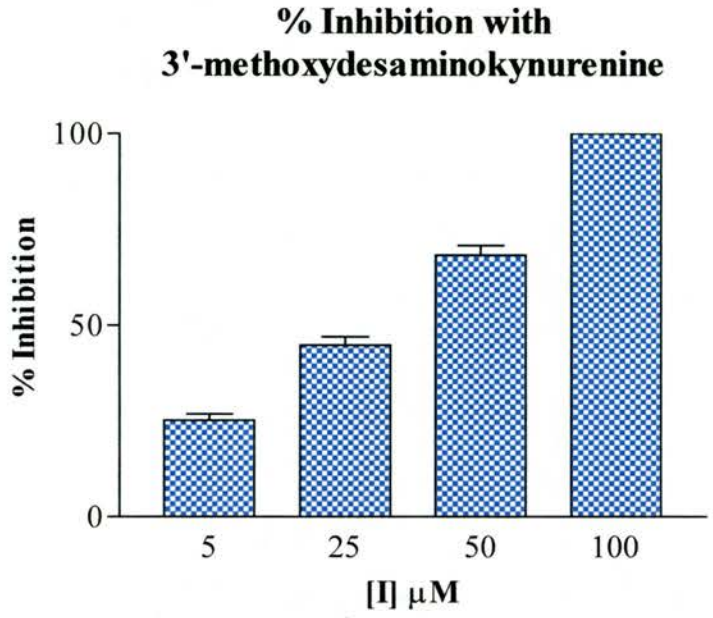


Figure 3.11

[Substrate] μM	10 μM [I] rate FI/min	20 μM [I] rate FI/min	40 μM [I] rate FI/min
2.5	5.87	3.13	2.29
5.0	10.83	8.65	6.97
10.0	19.09	15.63	12.98
15.0	22.71	19.74	17.21
20.0	26.52	20.76	18.67
V_{max} nmol/min/mg	8.65 ± 0.62	7.55 ± 1.67	8.15 ±2.28
K_m μM	8.6 ± 1.09	9.75 ± 3.71	13.87 ± 5.52

Table 3.2 Substrate versus velocity data for 3'-methoxydesaminokynurenine (88)[I]

The V_{max} values were observed to vary slightly with increasing inhibitor concentration with respect to V_{max} in the absence of inhibitor (7.70 ± 0.6 nmol/min/mg). However, the variation in V_{max} was small. The apparent K_m was observed to increase with increasing inhibitor concentration with respect to the K_m in the absence of inhibitor, (5.71 ± 0.3 μM). The apparent increase in K_m while V_{max} remains constant indicates that 3'-methoxy desaminokynurenine **(88)** exhibits competitive inhibition with recombinant human kynureninase. This is clearly seen in the Lineweaver-Burk plot for this data (Figure 3.12).

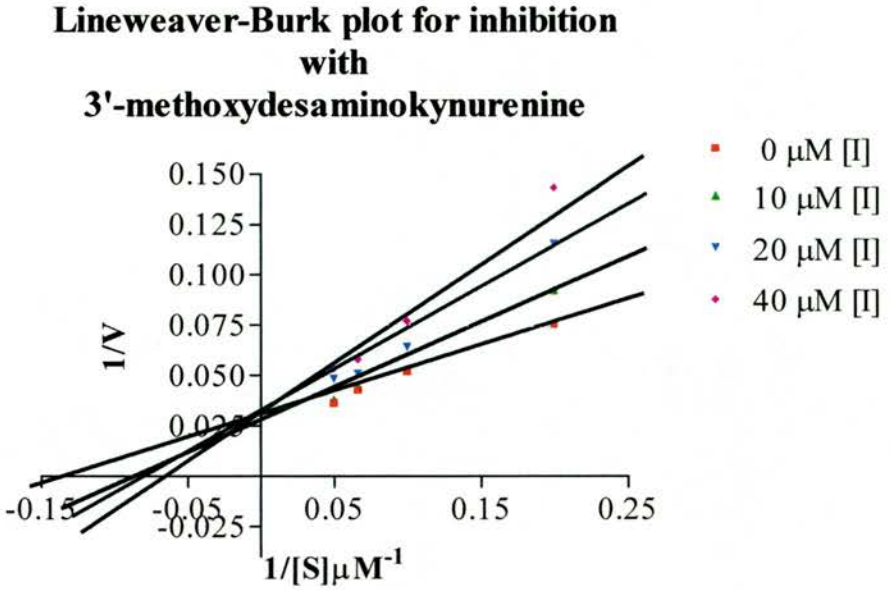


Figure 3.12

The K_i value for 3'-methoxydesaminokynurenine **(88)** was determined to be 15 ± 0.9 μM (Figure 3.13). This value indicates that replacing the hydroxy function in the 3-position of the ring with a methoxy function appears to significantly reduce binding to the enzyme. 3'-Hydroxydesaminokynurenine **(87)**, which exhibits a K_i of 5 nM, is a more potent inhibitor of kynureninase by over three orders of magnitude compared with 3'-methoxydesaminokynurenine **(88)**. This indicates that the presence of the hydroxy group in the 3-position of the ring is critical in the design of potent inhibitors of recombinant human kynureninase. This is probably due to hydrogen bonding at the active site. It is not clear whether the hydroxy group is acting as a hydrogen bond donor or acceptor, as methylation could interfere with both types of interaction. In the first case the methyl group obviously replaces the hydrogen atom, in the second it could exert a steric effect.

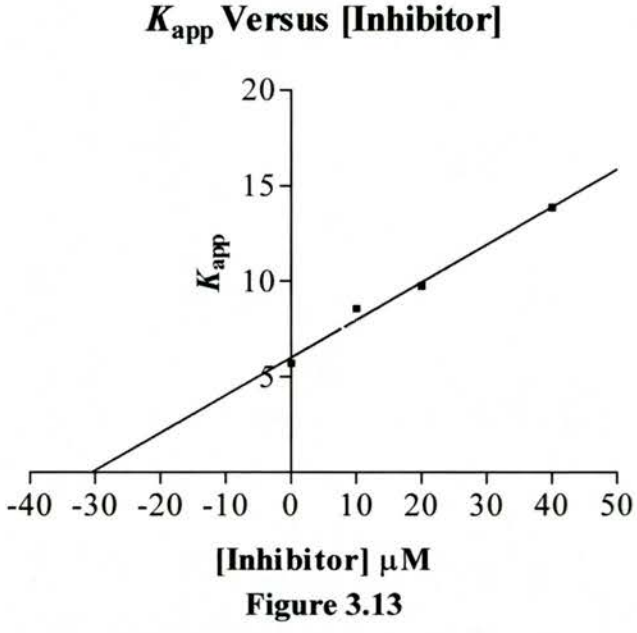


Figure 3.13

Comparative studies against both rat liver kynureninase and bacterial kynureninase were carried out by Dr. Harold Walsh. The inhibition against rat liver kynureninase again occurred over a narrow range of inhibitor concentrations. Only two inhibitor concentrations were examined. 3'-Methoxydesaminokynurenine **(88)** was observed to exhibit mixed inhibition when tested against the rat liver kynureninase. This was demonstrated by variation in both V_{max} and K_m with increasing inhibitor concentration. This is illustrated in the Lineweaver-Burk plot (Figure 3.14). The K_i value for **(88)** was determined from a replot of inhibitor concentration versus the slope (obtained from 1/[S] versus 1/v plot for each inhibitor concentration, slope = K_m/V_{max}) (Figure 3.15). The K_i was determined to be 88 μM. This is far less potent than 3'-hydroxydesaminokynurenine **(87)**, which exhibited a K_i of 20 nM against rat liver kynureninase.

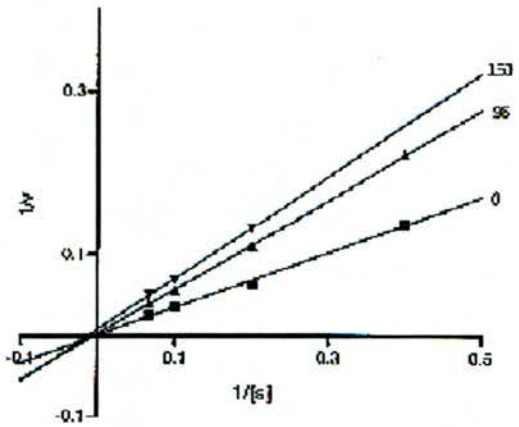


Figure 3.14

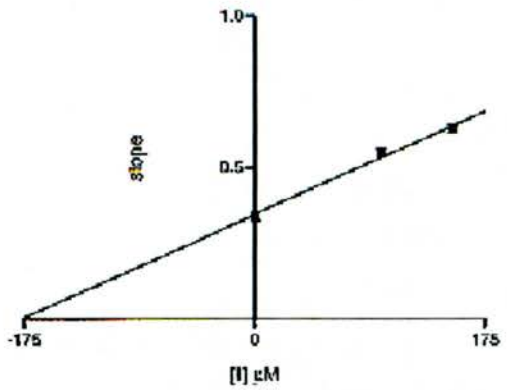
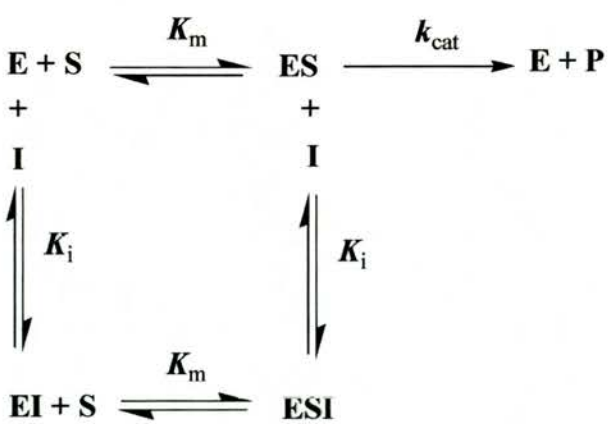


Figure 3.15

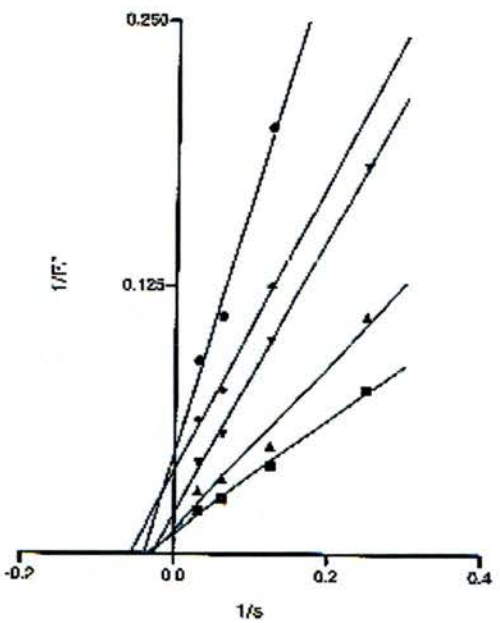
Lineweaver-Burk plot and K_i determination plot for 3'-methoxydesaminokynurenine with rat enzyme

3'-Methoxydesaminokynurenine **(88)** showed non-competitive inhibition against bacterial kynureninase. The V_{max} was observed to decrease with increasing inhibitor concentration while K_m remained largely unchanged. Non-competitive inhibitors can bind to the enzyme substrate complex ES as well as to free enzyme while the substrate can bind to both the EI complex and the free enzyme. However, the catalytic activity of the resulting ESI complex is reduced. Thus, at infinitely high substrate concentration not all of the enzyme will be in the ES form and V_{max} will decrease. Since the substrate can bind equally to both E and EI, K_m remains unchanged in the presence of a non-competitive inhibitor.



The inhibition observed with bacterial kynureninase is depicted in a Lineweaver-Burk plot (Figure3.16). The K_i value for **(88)** was determined to be 3 µM from a replot of inhibitor

concentration versus Y-intercept (obtained from 1/[S] versus 1/v data for each inhibitor concentration, Y-intercept = $1/V_{max}$ for a noncompetitive inhibitor) (Figure 3.17). Interestingly 3'-methoxy desaminokynurenine **(88)** appears to be a more potent inhibitor of bacterial kynureninase than mammalian kynureninase exhibiting K_i values of 3 μM, 15 μM and 88 μM for bacterial, human and rat kynureninase, respectively. 3'-Hydroxy desaminokynurenine **(87)** is still significantly more potent as an inhibitor of all three sources of enzyme, exhibiting K_i values of 5 nM, 20 nM and 25 nM respectively, for the human, rat and bacterial enzyme. Therefore introduction of a methoxy group at the 3-position of the ring significantly reduces binding to the enzyme for all three enzyme sources although there appears to be less of a detrimental effect on bacterial inhibition. This may be explained by the fact that for the bacterial enzyme kynurenine is a better substrate than 3-hydroxykynurenine therefore a hydroxy function at the 3-position may not be such an important recognition factor. Consequently, the loss of hydrogen bonding has less effect on inhibition.



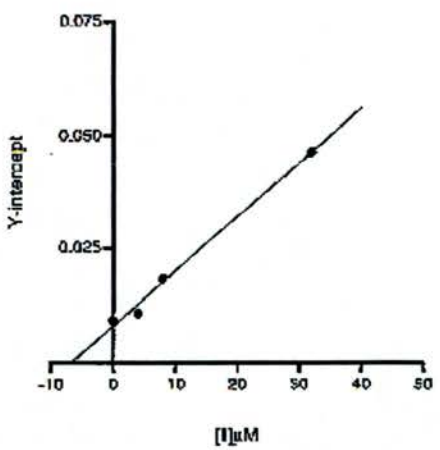


Figure 3.16 **Figure 3.17**

Linweaver-Burk plot and K_i determination plot for 3'-methoxydesminokynurenine (88) with bacterial enzyme

3.5.3.2 2-Amino-4-hydroxy-4-(3-methoxy-phenyl)-butyric acid (γ-hydroxy- 3'-methoxy desaminokynurenine) (91)

Preliminary inhibition studies using γ-hydroxy-3'-methoxydesaminokynurenine **(91)** indicated no inhibition of recombinant human kynureninase below 25 μM of inhibitor while 100% inhibition was observed above 100 μM concentrations of the inhibitor (Figure 3.18). Non linear regression analyses were used to afford values of K_m and V_{max} from initial rate data obtained for **(91)** with recombinant human kynureninase (Table 3.3). Initial rates were determined in triplicate, over a range of substrate concentrations for each inhibitor concentration and the average value of two or three consistent values (± 1.0%) was plotted.

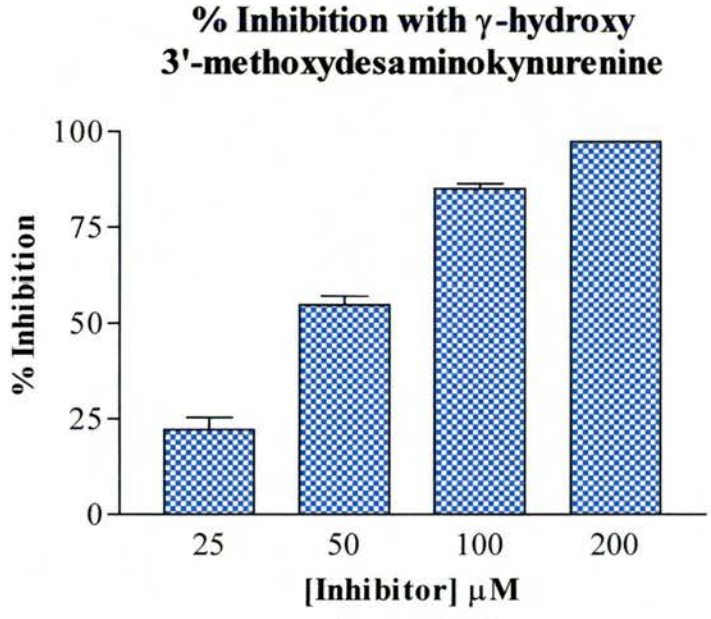


Figure 3.18

[Substrate] μM	25 μM [I] rate FI/min	50 μM [I] rate FI/min	75 μM [I] rate FI/min
2.5	6.04	5.52	4.50
5.0	10.57	9.27	5.89
10.0	18.19	13.46	10.67
15.0	19.50	18.97	14.99
20.0	25.61	22.47	17.55
V_{max} nmol/min/mg	7.70 ± 1.24	7.88 ± 0.98	7.42 ±1.42
K_m μM	7.9 ± 2.35	10.3 ± 2.16	14.12 ± 4.10

Table 3.3 Substrate and Velocity data for γ-hydroxy-3'-methoxy desaminokynurenine (91) [I]

The kinetic analysis shows that **(91)** behaves as a competitive inhibitor of the human enzyme. The V_{max} does not change with increasing concentrations of inhibitor while the apparent K_m is observed to increase. This is clearly observed in the Lineweaver-Burk plot (Figure 3.19).

Lineweaver-Burk plot for inhibition with γ-hydroxy-3'-methoxy desaminokynurenine

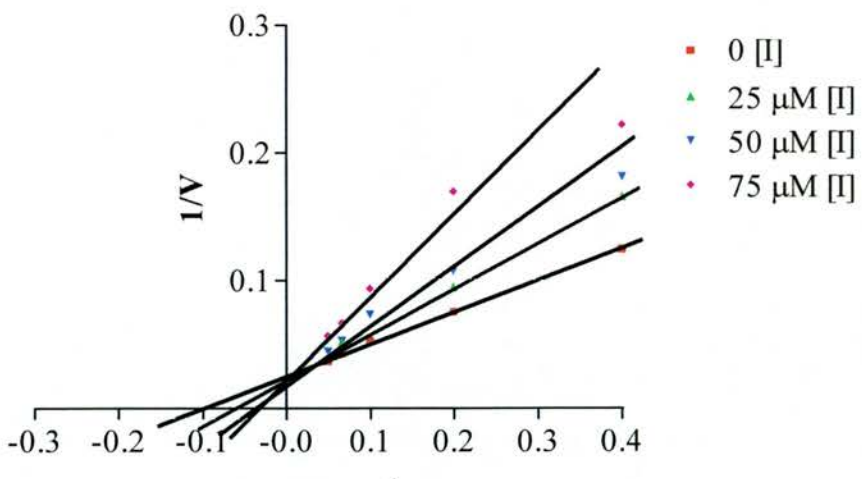


Figure 3.19

The K_i value for **(91)** of 240 ± 46 nM was determined from a plot of K_{app} versus inhibitor concentration where the intercept at the X-axis is $-K_i$ (Figure 3.20). Linear regression analysis was employed to determine the best straight line and the errors are determined from the regression analyses. The K_i value obtained for dihydroxydesaminokynurenine **(90)** in which there is a hydroxy function both at the γ-position and the 3-position of the aromatic ring results in potent inhibition, K_i 100 nM, of the human enzyme. Comparison of these values indicates that replacement of the 3-hydroxyl group with a methoxy function reduces the affinity of the inhibitor for the enzyme. The effect of the methoxy function in reducing binding to the human enzyme is much greater in the non-reduced 3'-methoxydesminokynurenine **(88),** which exhibited a K_i of 15 μM compared with a K_i of 5 nM for 3'-hydroxy desaminokynurenine **(87)**. The reduced γ-hydroxy-3'-methoxydesaminokynurenine **(88)** is a more potent inhibitor of human kynureninase compared with 3'-methoxydesaminokynurenine **(88)** by an order of 70-fold while dihydroxydesaminokynurenine **(90)** is somewhat less potent as an inhibitor than 3'-hydroxydesaminokynurenine **(87)** by an order of 20-fold. This indicates that transition state analogues can function as powerful inhibitors of mammalian enzyme. However, it is the presence of a hydroxyl group in the 3-position of the ring which is critical in conferring potency. Therefore, γ-hydroxy-3'-methoxydesaminokynurenine **(91)** is a competitive inhibitor of human kynureninase which illustrates that the incorporation of a methoxy function into the 3-position of the ring greatly reduces the potency of inhibition of recombinant human kynureninase.

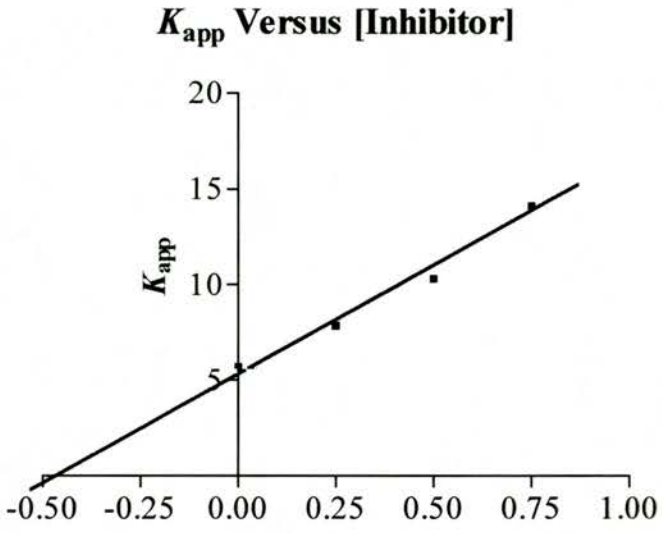


Figure 3.20

3.5.4 2-Amino-4-hydroxy-4-phenyl-butyric acid (γ-hydroxydesaminokynurenine) (33)

Preliminary % inhibition studies on γ-hydroxy desaminokynurenine **(33)** were carried out to determine the appropriate inhibitor concentration range with which to carry out detailed kinetic studies (Figure 3.21).

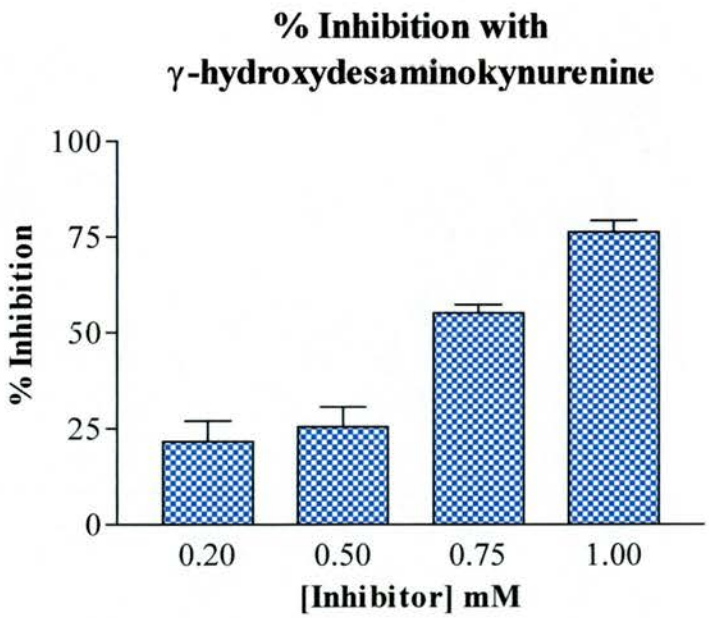


Figure 3.21

The substrate and velocity data are given in Table 3.4 and the velocity versus substrate plot is shown in Figure 3.22. Each rate was determined in triplicate and the two or three most consistent values (± 1.6 %) were then averaged using non linear regression analyses. The K_m and V_{max} values produced from non linear regression analyses were exceptionally high and could not be used to explain the type of inhibition observed for γ-hydroxydesaminokynurenine **(33)**. However, the Lineweaver-Burk plot for **(33)** is shown in Figure 3.23 which indicates that **(33)** displays mixed type inhibition. The K_m and V_{max} values were estimated from the Linweaver Burk plot using linear regression analyses. Both the K_m and V_{max} were observed to change with increasing inhibitor concentration and are shown in Table 3.4.

[Substrate] μM	**250 μM [I] rate FI/min**	**500μM [I] rate FI/min**	**750μM [I] rate FI/min**
2.5	5.60	3.30	2.20
5.0	9.01	5.60	3.27
10.0	15.84	9.83	6.21
15.0	23.71	19.58	13.11
20.0	23.25	23.25	14.74
V_{max} nmol/min/mg	8.39	12.28	5.78
K_m μM	9.68	26.001	18.46

Table 3.4 Substrate and Velocity data for γ-hydroxydesaminokynurenine (33) [I]

Velocity versus substrate plot for inhibition with γ-hydroxy desaminokynurenine

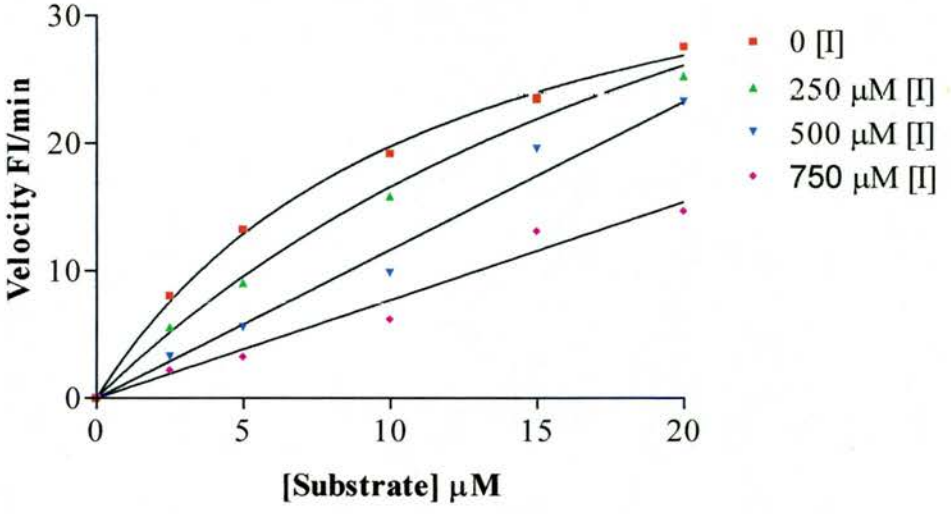


Figure 3.22

Lineweaver-Burk plot for inhibition with γ-hydroxy desaminokynurenine

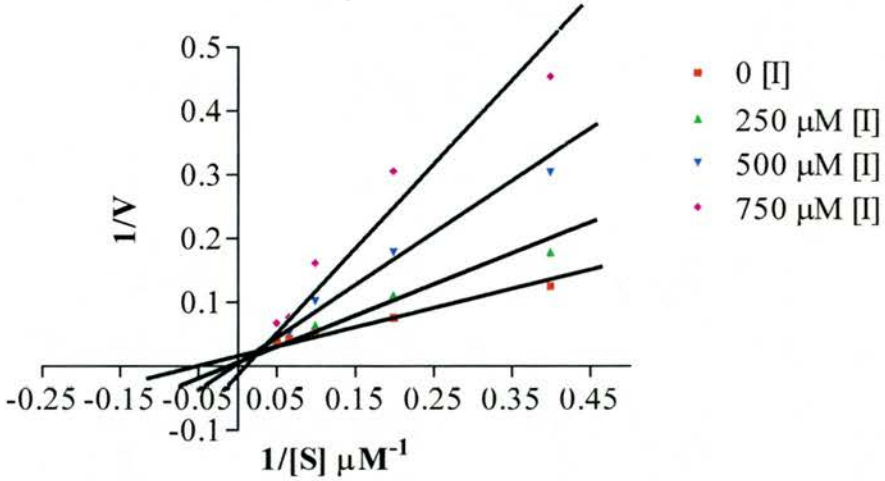


Figure 3.23

A K_i value of 79 ± 29 μM was obtained from a Dixon plot of the slope (obtained from 1/v versus 1/[S] at each inhibitor concentration, slope = K_m/V_{max}) versus inhibitor concentration using linear regression analysis (Figure 3.24). The mixed inhibition displayed by γ-hydroxydesaminokynurenine **(33)** may be explained by the presence of the

second regulatory site on the human enzyme. The inhibitor also illustrates the importance of the hydroxyl group at the 3-position of the ring as a recognition element for the human enzyme since it is a significantly weaker inhibitor than dihydroxydesaminokynurenine **(90)** which exhibits a K_i of 100 nM against the human enzyme. This inhibitor also exhibited mixed inhibition against the enzyme. γ-Hydroxydesaminokynurenine **(33)** is also weaker than the dihydrokynurenines, **(16a)** and **(16b)**, reported by Phillips and Dua. Both behaved as competitive inhibitors of bacterial kynureninase and exhibited K_i values of 1.3 and 0.3 μM, respectively.[42] Dihydroxydesaminokynurenine **(90)** exhibited a K_i of 10 μM against the bacterial enzyme.

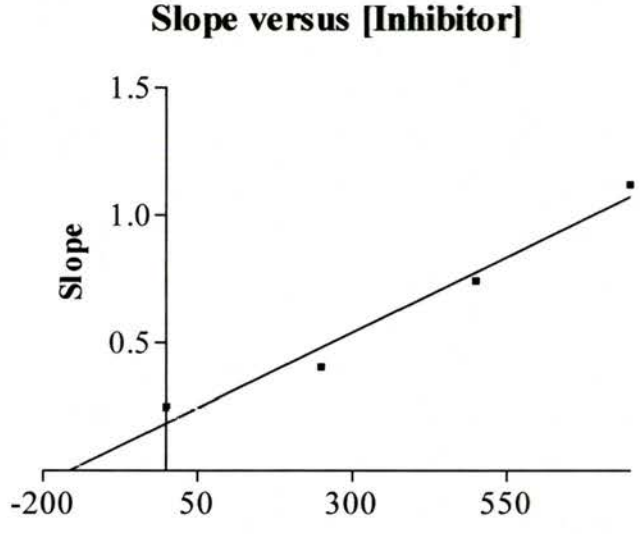
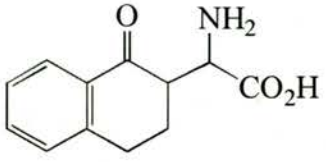


Figure 3.24

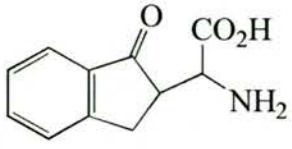
3.5.5 Tetralone derivative inhibitors of kynureninase.

The rationale for the design of the bicyclic inhibitors, as discussed in Section 1.3.6, was that they should be capable of undergoing the early steps of the kynureninase catalysed reaction involving cleavage of the β,γ carbon bond. However, the presence of the carbon chain linking the alanine fragment to the 2-position of the benzene ring means that following cleavage the two products remain covalently attached together. The bicyclic tetralone **(59)**, indanone **(60)** and chromanone **(61)** synthesised within the group were all

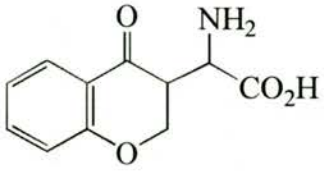
shown to inhibit human kynureninase in the μM region. They behaved as competitive inhibitors, exhibiting K_i values of 227 μM, 45 μM and 77 μM, respectively.[38]



(59)

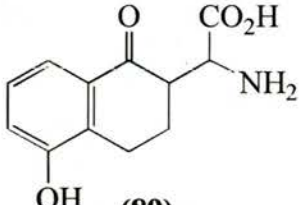


(60)

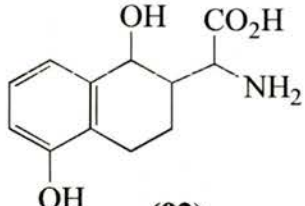


(61)

In the present study, the 5-hydroxy-1-tetralone derivative **(89)** was examined in order to determine the effect upon inhibition of a hydroxyl group at the 5-position of the benzene ring. As a recognition element for the human enzyme the hydroxyl group on the benzene ring was hoped to increase the inhibitory potency of the tetralone derivative. The 1,5-dihydroxytetralone derivative **(92)** was also examined as a possible transition state analogue inhibitor of the enzyme. This derivative would behave as a mimic of the *gem*-diolate intermediate **(92)** formed upon attack by a hydroxyl group on the γ-carbonyl of the substrate in the proposed mechanism for the reaction (Section 1.3.4).



(89)



(92)

3.5.5.1 2-Amino-(5-hydroxy-1-oxo-1,2,3,4-tetrahydro-naphthalene-2-yl)-acetic acid (5-hydroxy-1-tetralone derivative) (89)

The preliminary inhibition study using **(89)** indicated no inhibition of recombinant human kynureninase occurred below 0.1 mM of inhibitor while 100% inhibition was observed above 1 mM concentrations of the inhibitor (Figure 3.25). Detailed kinetic data for **(89)** were obtained, as discussed for other inhibitors, and are given in Table 3.5. Initial rates

were determined in triplicate and the average of two or three consistent values (± 12%) were then plotted using non-linear regression analyses to afford the values of K_m and V_{max}.

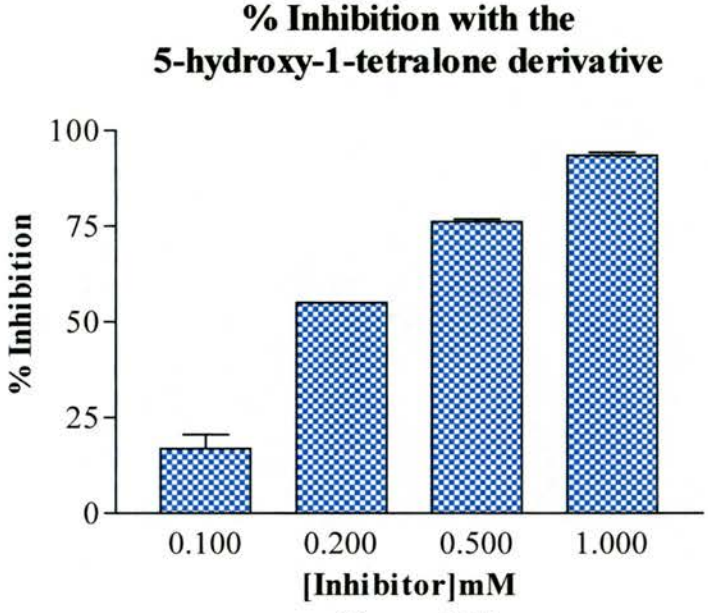


Figure 3.25

[Substrate] μM	100μM [I] rate FI/min	200μM [I] rate FI/min	400μM [I] rate FI/min
2.5	4.38	4.31	1.84
5.0	7.25	4.98	3.22
10.0	12.27	10.82	3.44
15.0	13.23	12.03	6.96
20.0	17.71	13.00	5.62
V_{max} nmol/min/mg	5.29 ± 0.91	3.71 ± 0.66	1.71 ± 0.66
K_m μM	7.9 ± 2.53	5.92 ± 2.19	5.79 ± 2.96

Table 3.5 Substrate and velocity data for 5-hydroxy-1-tetralone amino acid (89) [I]

The V_{max} for the substrate was observed to decrease with increasing inhibitor concentration, while the apparent K_m did not vary greatly from the K_m of 5.71 ± 0.3 μM for the substrate in the absence of inhibitor. The decrease in V_{max} while the K_m remains unchanged as inhibitor concentration is increased is characteristic of non-competitive

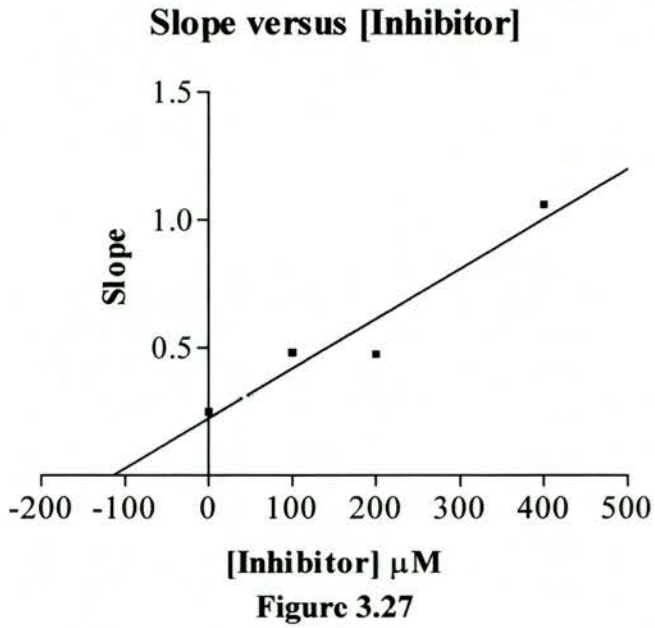


Figure 3.27

3.5.5.2 2-Amino-(1,5-dihydroxy-1,2,3,4-tetrahydro-naphthalene-2-yl)-acetic acid (1,5-dihydroxytetralone derivative) (92)

There was no inhibition of recombinant human kynureninase below 100 μM when **(92)** was examined in preliminary inhibition studies though some inhibition was observed above 1 mM concentrations of the inhibitor (Figure 3.28). Initial rate data obtained over a range of substrate concentrations for each inhibitor concentration of **(92)** are given in Table 3.6. Values for K_m and V_{max} were obtained from non linear regression analyses of this data. Each rate was determined in triplicate and the average value of two or three consistant values (± 1%) was plotted

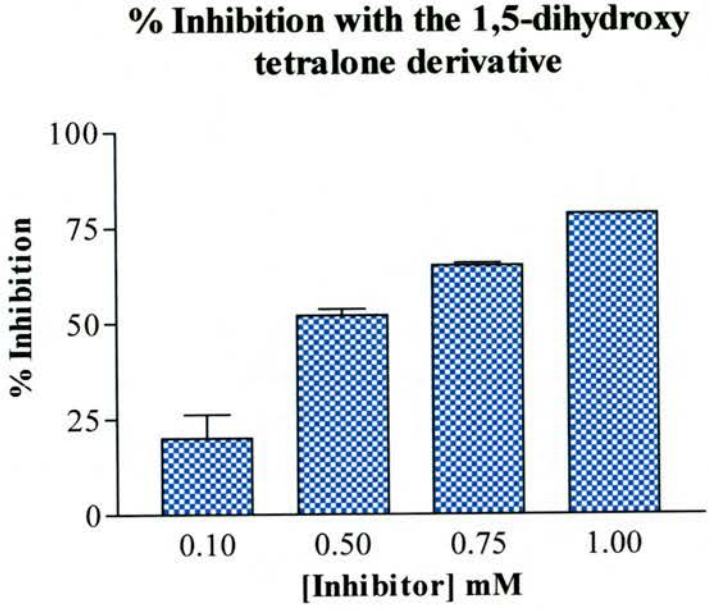


Figure 3.28

[Substrate] μM	125μM [I] rate FI/min	250μM [I] rate FI/min	500μM [I] rate FI/min
2.5	5.96	4.24	3.32
5.0	12.96	9.74	4.13
10.0	16.82	15.48	9.83
15.0	21.10	19.37	11.88
20.0	26.44	22.83	15.38
V_{max} nmol/min/mg	7.65 ± 1.19	7.89 ± 0.72	8.23 ± 3.00
K_m μM	7.2 ± 2.15	9.8 ± 1.51	20.9 ± 10.4

Table 3.6 Substrate and Velocity data for 1,5-dihydroxytetralone derivative (92) [I]

The apparent K_m of the substrate was observed to increase with increasing inhibitor concentration while the V_{max} remained largely unchanged. The 1,5-dihydroxy tetralone derivative **(92)** thus appears to exhibit competitive inhibition against recombinant human kynureninase. The Lineweaver-Burk plot for **(92)** (Figure 3.29) clearly illustrates this competitive inhibition.

Lineweaver-Burk plot for inhibition with the 1,5-dihydroxy tetralone derivative

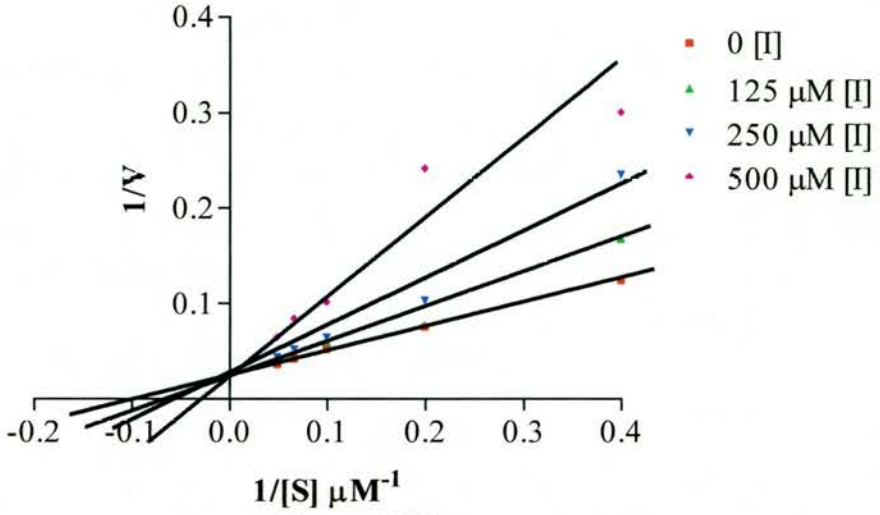
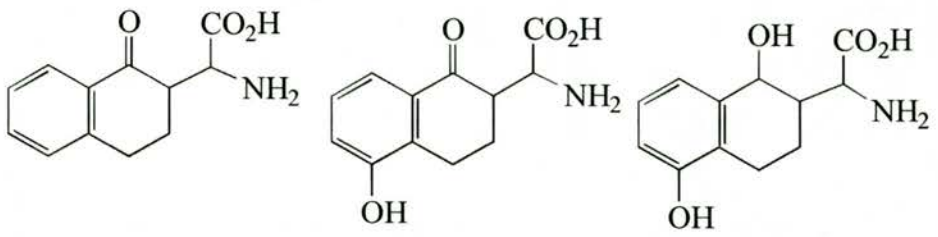


Figure 3.29

The K_i value for **(92)** was determined from a plot of K_{app} versus inhibitor concentration (Figure 3.30) and found to be 66 ± 26 μM. When compared with the 5-hydroxy-1-tetralone derivative **(89)** which exhibited a K_i of 57 ± 22 μM there appears to be no significant difference in the potency of the inhibition by both compounds however the type of inhibition was different with the 1,5-dihydroxytetralone **(92)** exhibiting competitive inhibition while the 5-hydroxy-1-tetralone derivative **(89)** exhibited non-competitive inhibition.



(59) $K_i = 227 \pm 47\ \mu M$

(89) $K_i = 57 \pm 22\ \mu M$

(92) $K_i = 66 \pm 26\ \mu M$

Interestingly the unsubstituted tetralone **(59)** reported by Botting *et al.* was also a competitive inhibitor. The difference in the type of inhibition found for the 5-hydroxy-1-tetralone **(89)** may be due to a combination of structural elements of the compound which allow it to bond to other sites on the enzyme. The 1,5-dihydroxytetralone derivative **(92)** is a more potent inhibitor compared with **(59)** which supports the inference that the presence of a hydroxyl function on the aromatic ring of the bicyclic compound *meta* to the carbonyl group increases binding to the enzyme. Compared with the monocyclic systems the bicyclic compounds appear to be much less effective as inhibitors of human kynureninase. This may be explained by steric effects which may arise as a result of the carbon link at the 2-position of the benzene ring.

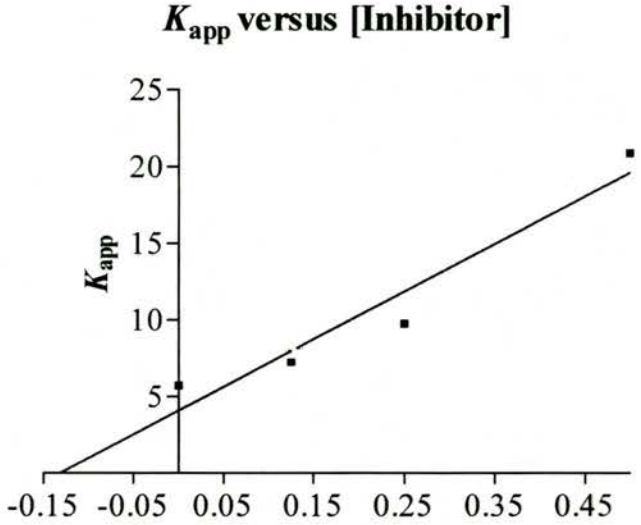
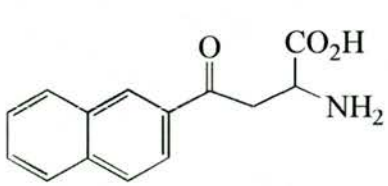


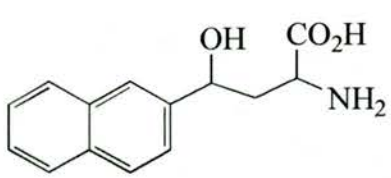
Figure 3.30

3.5.6 2-Amino-4-hydroxy-4-naphthyl-butyric acid (γ-hydroxy naphthalene derivative) (97)

The naphthyl analogue, 2-amino-4-oxo-4-naphthyl-butyric acid **(97)**, was previously reported as a potent inhibitor of both human and bacterial kynureninase exhibiting K_i values of 22 ± 6 μM and 5 ± 2 μM respectively. As discussed previously the extension of the benzene ring of kynurenine is thought to result in an increase in binding. The aromatic system is thought to fill a hydrophobic pocket at the enzyme active site. In order to examine the effect of a reduced centre at the γ-position, 2-amino-4-hydroxy-4-naphthyl-butyric acid **(97)** was examined as a possible transition state analogue inhibitor of kynureninase. It was hoped that the γ-hydroxy group would further increase the potency of the naphthyl analogue.



(62)



(97)

However, a detailed kinetic analysis of the γ-hydroxy naphthalene analogue **(97)** was not possible. Preliminary inhibition studies were conducted which showed that no inhibition occurred below 10 μM while 100% inhibition occurred at concentrations above 100 μM (Figure 3.31).

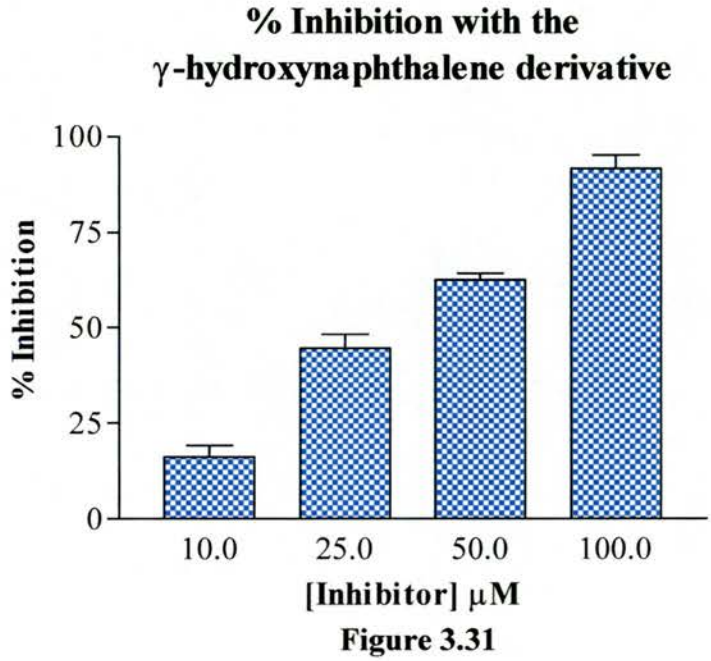


Figure 3.31

When detailed kinetic analyses were carried out for each inhibitor concentration over a range of substrate concentrations, 2.5-20 μM, the rate measured by fluorescence was observed to decrease with increasing substrate concentration as shown in (Figure 3.32), rather than reach the maximum as expected.

Velocity versus substrate plot for inhibition with the γ-hydroxynaphthalene derivative

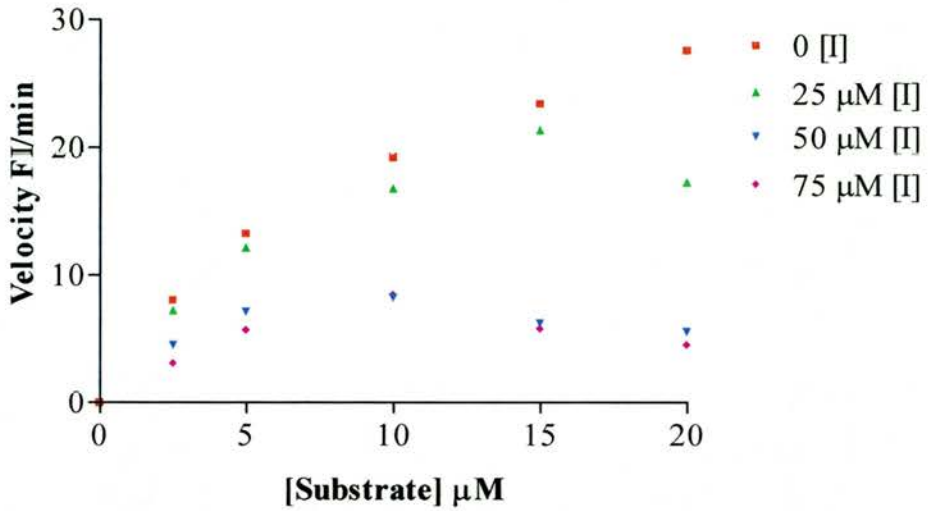
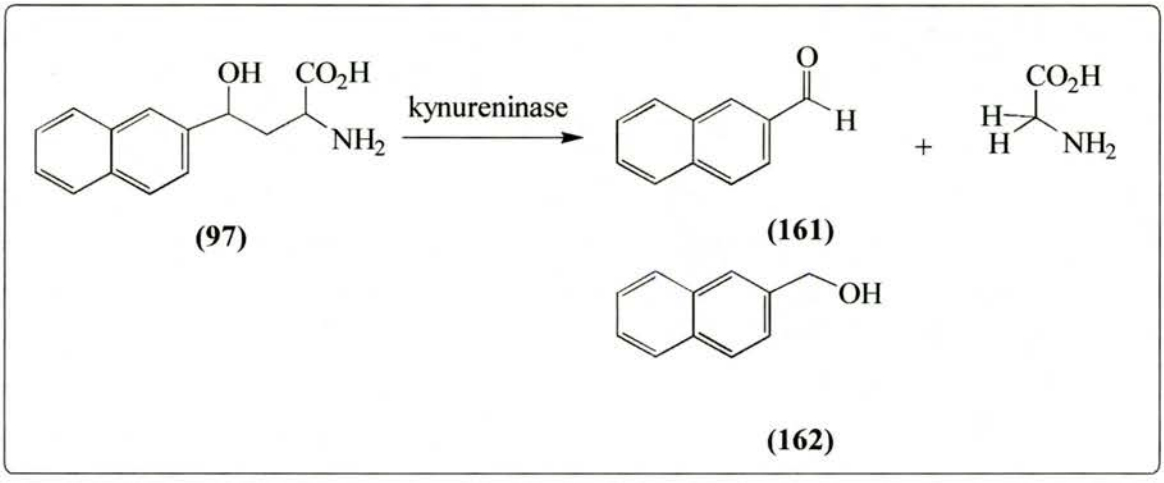


Figure 3.32

It was thought that quenching of the fluorescence of 3-hydroxyanthranilate **(9)**, the product of hydrolysis of 3-hydroxykynurenine, might be taking place due to the presence of the inhibitor, γ-hydroxy naphthalene **(97)** or the product of hydrolysis of **(161)** (Scheme 3.2). To examine this possibility, another similar compound, namely, 2-naphthalene methanol **(162)** was also added to the reaction mixture to examine its effect. A series of assays of **(162)** (75 μM) were carried out employing the same conditions used over the standard substrate concentration range, 2.5-20 μM. 2-Naphthalene methanol **(162)** was observed to completely quench the fluorescence of 3-hydroxyanthranilate **(9)** over the substrate range examined. Fox *et al.* have reported that some naphthyl based chromophores, which exhibit characteristic naphthalene fluorescence at 335 nm, self quench *via* non radiative pathways as their concentration in solution increases.[178] It may thus be possible that the fluorescence of 3-hydroxyanthranilate **(9)** can be quenched by some intermolecular interaction in the assays as both the inhibitor and substrate concentrations are increased. The non-reduced naphthalene derivative **(62)** was successfully tested against human kynureninase using a fluorescence assay.[38] This may be due to the electronic nature of this derivative as it has a mesomeric electronic withdrawing effect. In contrast, 2-naphthalene methanol **(162)** has only very a slight inductive electron withdrawing effect

through the sigma bonds which could cause a difference in fluorescence and therefore have an affect on the assay. Unfortunately, this means that an accurate estimate of the inhibitory properties of the γ-hydroxy naphthalene analogue **(97)** cannot be obtained. This would be seen to require an alternative assay method. The non-reduced naphthalene **(62)** derivative was also tested successfully against bacterial kynureninase using a UV assay and so testing **(97)** against bacterial kynureninase is a possibility.



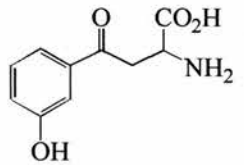
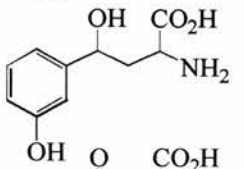
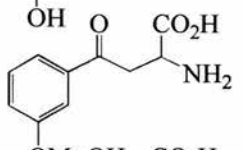
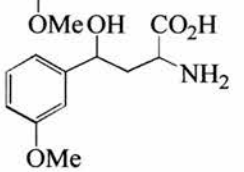
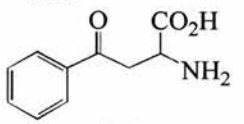
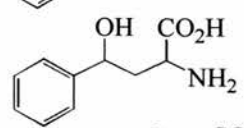
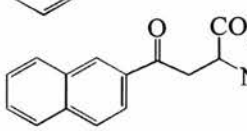
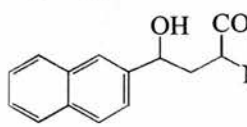
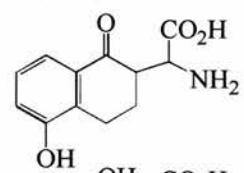
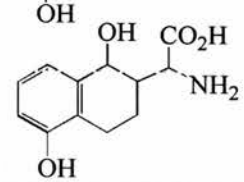
(97) **(161)** **(162)**

Scheme 3.2

3.6 Conclusion

A detailed kinetic study of several substrate analogue inhibitors of kynureninase has been undertaken (Table 3.7). The analogue 3'-hydroxydesaminokynurenine **(87)** was identified as the most potent inhibitor of kynureninase to date. The compound exhibited K_i values of 5 nM, 20 nM and 25 nM when tested against human, rat liver and bacterial kynureninase. It was observed to be a competitive inhibitor of the human enzyme while exhibiting mixed inhibition against both the rat and bacterial enzyme. These results indicated that a hydroxyl group at the 3-position of the aryl ring was an important recognition element for the enzyme. This was further supported by inhibition studies on 3'-methoxydesaminokynurenine **(88)** where K_i values of 15 μM, 88 μM and 3 μM were determined for human, rat and bacterial

K_i values for substrate analogues

	Compound	Human	Rat	Bacterial[a]
(87)		5 nM	20 nM	25 nM
(90)		100 nM	130 nM	10 μM
(88)		15 μM	88 μM	3 μM
(91)		240 nM	N/D	N/D
(15)		11 μM [49]	N/D	N/D
(33)		79 μM	N/D	N/D
(62)		22 μM[38]	N/D	5 μM[38]
(97)		-	N/D	N/D
(89)		57μM	N/D	N/D
(92)		66 μM	N/D	N/D

[a]Bacterial kynureninase from *Pseudomonas fluorescens.*

N/D –not done, no inhibition study carried out.

Table 3.7

kynureninase respectively. Inhibition appears to be significantly diminished for all three sources of enzyme upon introduction of a methoxy function at the 3-position indicating hydrogen bonding has a significant effect on binding. Methylation at the 3-position was not as detrimental in the case of bacterial kynureninase, which indicates that a loss of hydrogen bonding here is not as important as for the mammalian enzyme. This agrees with the observation that L-kynurenine is a better substrate for the bacterial enzyme than 3-hydroxy-L-kynurenine. 3'-Hydroxydesaminokynurenine **(87)** was not turned over by the enzyme, which agrees with other studies[40] that indicate that the *ortho* amino group, is more important for turnover, than substrate recognition. The transition state analogues, which contained a hydroxyl group at the γ-position, were all observed to inhibit human kynureninase (Table 3.7) with the exception of the naphthalene derivative for which a K_i value could not be obtained due to problems with the assay. The introduction of a hydroxyl group at the γ-position did not appear to increase binding with respect to the non-reduced analogue in the case of dihydroxydesaminokynurenine **(90)**. However, binding was found to increase for both the γ-hydroxy-3'-methoxydesaminokynurenine **(91)** and the 1,5-dihydroxytetralone **(92)** derivatives when compared with their non-reduced analogues. This seems to indicate that the presence of a hydroxyl group again seems to be important in conferring potency in the case of mammalian kynureninase. However, transition state analogues which mimic the *gem*-diolate intermediate can function as potent inhibitors of kynureninase.

In a study by Phillips and Dua the diastereomers of dihydrokynurenine, (4*R*,2*S*) **(16a)** and (4*S*,2*S*) **(16b)** were observed to be competitive inhibitors of bacterial kynureninase exhibiting K_i values of 1.3 μM and 0.3 μM, respectively. In contrast, dihydroxydesaminokynurenine **(90)** obtained in this work as the (4*S*,2*S*) and (4*R*,2*R*) pair of enantiomers, was observed to be a weaker inhibitor of the bacterial enzyme, exhibiting a K_i of 10 μM. This small decrease in affinity could be attributed to the absence of the 2-amino group. The dihydroxydesaminokynurenine **(90)** was however a very potent inhibitor of mammalian enzyme with K_i values of 100 nM and 130 nM for the human and rat enzyme, respectively. These results seem to indicate that a hydroxyl group at the 3-position of the ring does not appear to confer potency with regard to the bacterial enzyme. The transition analogues γ-hydroxydesaminokynurenine **(33)** and the dihydroxytetralone **(92)** derivatives are both weaker inhibitors of kynureninase compared with the

dihydrokynurenines while γ-hydroxy-3'-methoxydesaminokynurenine **(91)** was more potent. However, these inhibitors were tested against the human enzyme only and testing against the bacterial would be required before a comprehensive comparison can be made.

The bicyclic analogues appeared to be less effective as inhibitors than the monocyclic compounds. The non-reduced and reduced tetralone inhibitors **(89)** and **(92)** exhibited the highest K_i values in this study. However, the low K_i value of 22 μM, reported by Muirhead *et al.* for the naphthalene derivative **(62)** indicates there is a pocket within the active site of the enzyme which can accommodate an added benzene ring.[38] Unfortunately problems with quenching of the fluorometric assay precluded a proper examination of the inhibitory properties of **(97)**. However, it would be interesting to carry out further study of naphthalene derivatives which have a hydroxyl group at the 3-position.

Finally, most of the substrate analogues examined exhibited competitive inhibition of the human enzyme. 3'-Hydroxydesaminokynurenine **(87)**, 3'-methoxydesaminokynurenine **(88)**, γ-hydroxy-3'-methoxydesaminokynurenine **(91)** and the 1,5-dihydroxytetralone derivative **(92)** were all competitive inhibitors of the human enzyme. However, dihydroxydesaminokynurenine **(90)** and γ-hydroxydesaminokynurenine **(33)** behaved as mixed inhibitors while the 5-hydroxy-1-tetralone derivative **(89)** was a non-competitive inhibitor. The presence of a second regulatory site on the human enzyme as discussed in Section 3.2 may explain the observed mixed inhibition since these analogues may contain a combination of structural features, which facilitates their binding to this site. The analogues may bind to either or both the regulatory site and the active site resulting in different types of inhibition. Compounds **(87)** and **(90)** were also mixed inhibitors of the rat and bacterial enzyme while **(88)** exhibited mixed inhibition of the rat enzyme and was a non-competitive inhibitor of the bacterial enzyme. Again, these analogues may possess structural features which allow them to bind to other sites on the rat and bacterial enzyme.

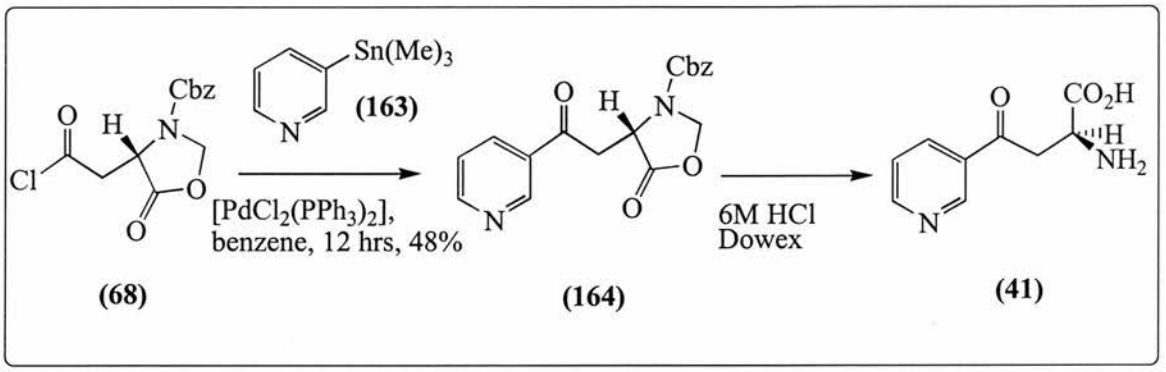
CHAPTER 4

4 CHIRAL KYNURENINE ANALOGUES

Kynureninase exhibits substrate specificity for (2*S*)-amino acids. Therefore the synthesis of substrate analogues which may behave as potent inhibitors of the enzyme ideally requires the synthesis of enantiomerically pure (2*S*)-substrate analogues. All of the kynurenine analogues discussed in Chapter two were synthesised as racemic mixtures in order to determine their inhibitory properties against kynureninase. Another objective of this project was to investigate synthetic routes which would allow synthesis of those analogues as enantiomerically pure (2*S*)-amino acids.

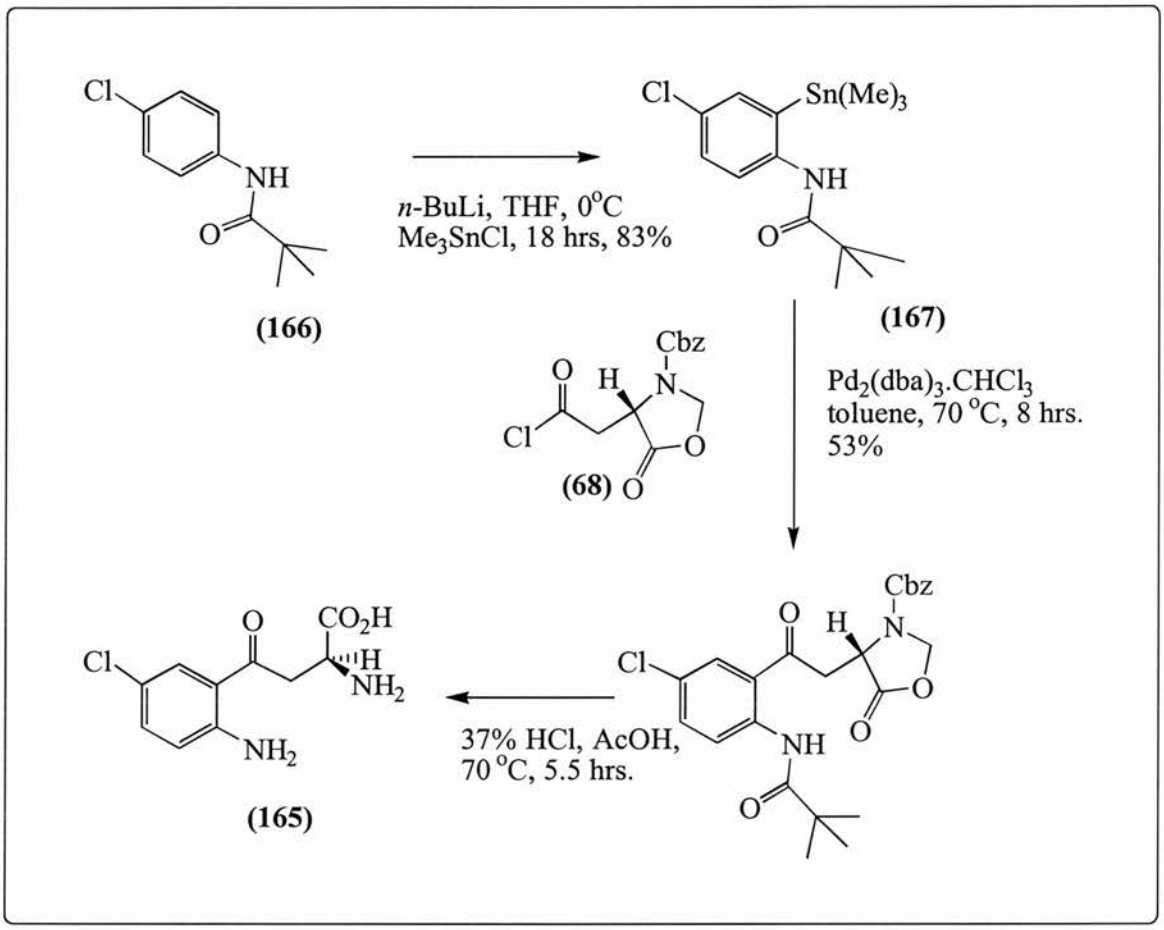
4.1 Literature synthesis of (2*S*)-kynurenine analogues

A number of synthetic routes to enantiomerically pure kynurenine analogues have been reported in the literature. The Stille palladium coupling reaction has been adopted and modified by a number of groups in the synthesis of substrate analogue inhibitors. A stereospecific synthesis of the enantiomers of nicotinylalanine **(41)** first reported in the early 1960's as an inhibitor of kynureninase was reported by Pellicari *et al.* (Scheme 4.1).[142] A mixture of 3-trimethylstannylpyridine **(163)** and *S*-(3-benzlyoxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)**, was reacted in the presence of dichlorobis(triphenylphosphine)palladium II, [$PdCl_2(PPh_3)$] to give the corresponding pyridylketone **(164)** which was converted to (2*S*)-nicotinylalanine **(41)** in an overall yield of 44% by deprotection in 6M HCl followed by purification using Dowex ion exchange chromatography. The corresponding (2*R*) enantiomer was synthesised by the same method using *R*-(3-benzlyoxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride.



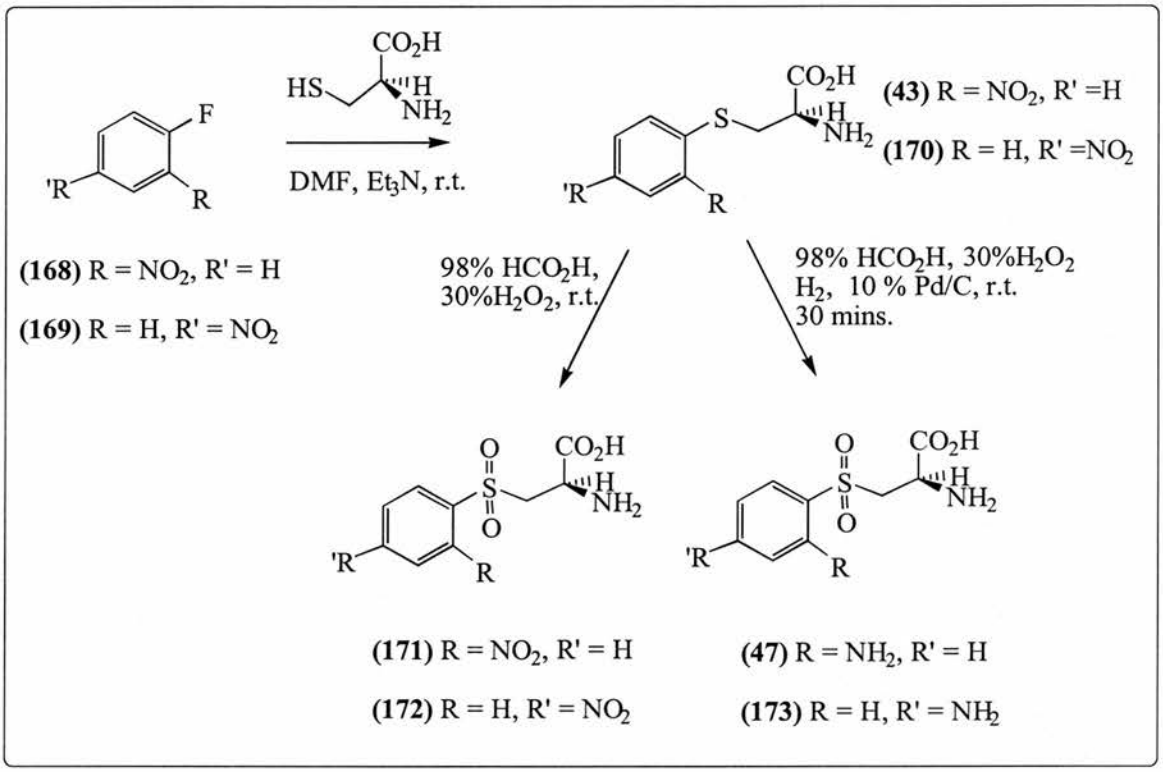
Scheme 4.1

The same methodology was also applied to synthesis of (2*S*)-*m*-nitrobenzoylalanine **(64)**. The racemic compound was found to exhibit inhibitory properties when examined against both kynureninase and kynurenine-3-hydroxylase.[63] However, synthesis and further inhibition studies with the two pure enantiomers indicated that the inhibition was due to the *S* enantiomer.[63] A modification of the above synthetic strategy reported by Varasi *et al.* was used to prepare (2*S*)-5-chlorokynurenine **(165)**[153] (Scheme 4.2). The racemic compound was found to be an inhibitor of the KAT enzyme responsible for converting L-kynurenine to kynurenic acid. The enantiospecific synthesis involved *ortho* lithiation and subsequent stannylation of *N*-pivoylated 4-chloroalanine **(166)** to afford the intermediate **(167)**. Subsequent reaction of **(167)** with *S*-(3-benzlyoxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)** followed by deprotection in 37% v/v HCl and acetic acid afforded (2*S*)-5-chlorokynurenine **(165)** in 51% yield. Further inhibition studies indicated the (2*S*) enantiomer to be far more potent as an inhibitor than the (2*R*) enantiomer.



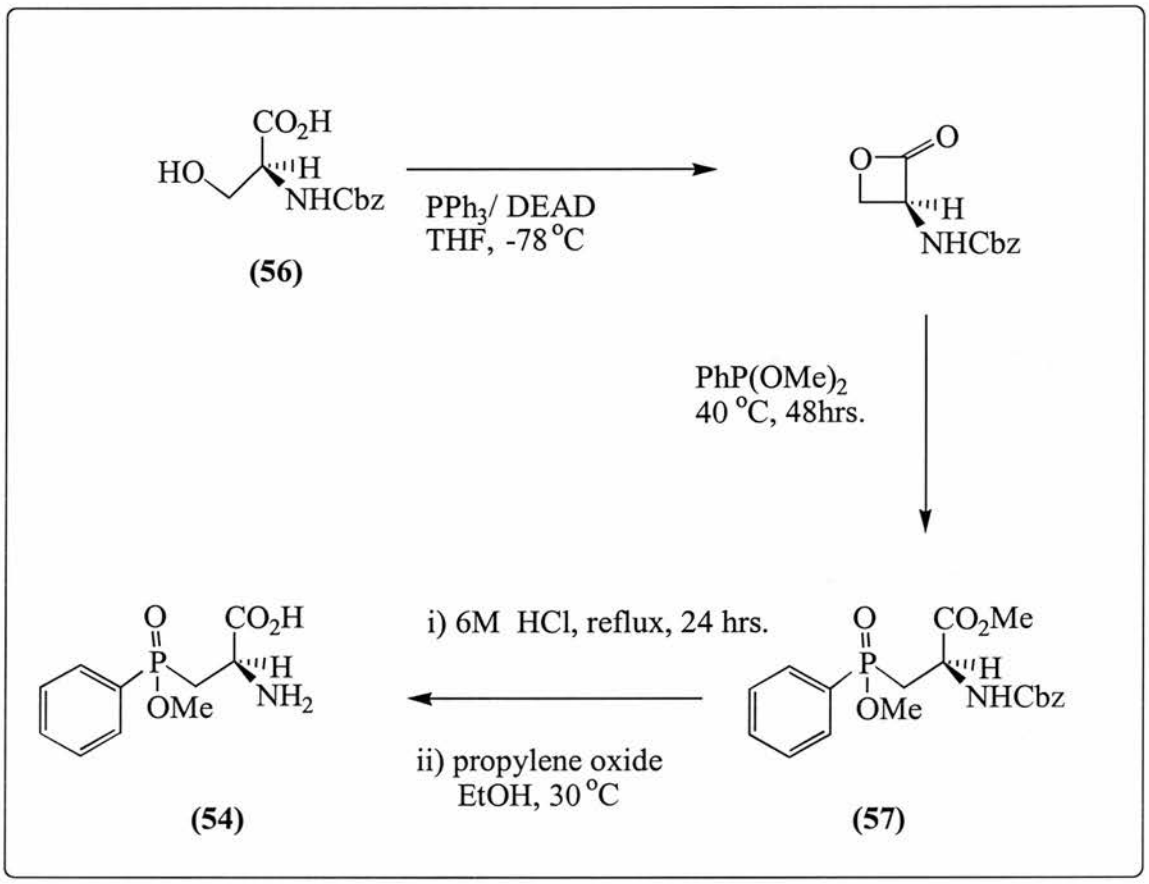
Scheme 4.2

Nucleophilic aromatic substitution reactions have also been used in the synthesis of enantiopure kynurenine analogues.[54,55] The *ortho* and *para* substituted halobenzenes *o*-nitrofluorobenzene **(168)** and *p*-nitrofluorobenzene **(169)** underwent nucleophilic substitution of fluorine with L-cysteine in the presence of a base, triethylamine, to afford *S*-(2-nitrophenyl)-L-cysteines **(43)** and *S*-(4-nitrophenyl)-L-cysteines **(170)** in yields of 69% and 65% respectively (Scheme 4.3). The *S*-(2-nitrophenyl)-L-cysteine *S,S*-dioxide **(171)** and *S*-(4-nitrophenyl)-L-cysteine *S,S*-dioxide **(172)** were then synthesised by oxidation of the sulfur atom in the presence of formic acid and hydrogen peroxide. Subsequent reduction of the nitro group by reaction with zinc dust afforded the *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxides **(47)** and *S*-(4-aminophenyl)-L-cysteine *S,S*-dioxides **(173)** in high yield. Similar *S*-aryl-L-cysteine and *S*-aryl-L-cysteine *S,S*-dioxides were also synthesised in a modification of the method using *N*-acetylcysteine in the substitution reaction followed by subsequent deprotection in acid to afford the free amino acid.



Scheme 4.3

The synthesis of the phosphinic acid analogue **(54)** utilized the β-lactone methodology, reported by Vederas *et al.*, in which Mitsunobu conditions were employed to synthesise the *N*-protected β-lactone of L-serine (Scheme 4.4).[58] Subsequent reaction of the strained β-lactone with phenyl dimethyl phosphonite resulted in nucleophilic attack of the phosphorus at the β-position of the lactone. Opening up of the lactone resulted in an Arbusov type reaction in which a methyl group was transferred from a methoxy group to the carboxylate oxygen anion to afford the methyl phosphinate methyl ester **(57).** Subsequent deprotection *via* acid hydrolysis to give the amino acid **(54)** in 39% yield.



Scheme 4.4

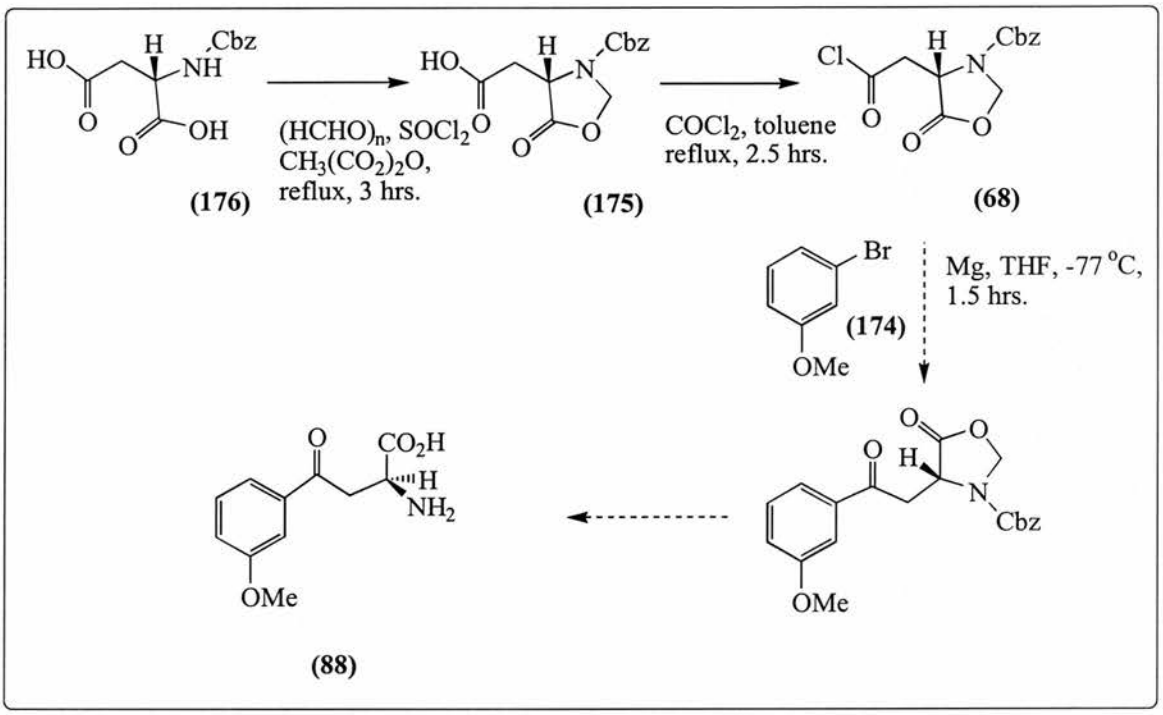
4.2 Grignard coupling reactions

The synthesis of 3'-methoxydesaminokynurenine **(88)** as the single (2*S*) enantiomer using a Grignard coupling reaction was attempted. The general strategy involved reaction of the protected aspartic acid chloride, *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)**, with the Grignard reagent formed *in situ* from reaction of 3-bromoanisole **(174)** with magnesium turnings in THF (Scheme 4.5). In general the reaction of acid halides with Grignard reagents has not been considered as a general route to ketone synthesis because of the formation of tertiary and/or secondary alcohols and other reagents such as organo-zinc,[179] cadmium,[180] copper [181] and rhodium[182] compounds have been used in preference for ketone synthesis. However, studies by Eberle *et al.*[183] and Sato *et al.*[184] described the preparation of ketones in high yield by direct reaction of Grignard reagents with acid chlorides in THF at low temperature. The reaction temperature was maintained below -67 °C during addition of the acid chloride and then the reaction mixture was

allowed to warm to room temperature. A similar reaction by Sato *et al.* reported the synthesis of aromatic as well as primary, secondary and tertiary ketones in high yield by addition of a Grignard reagent to the corresponding acid chloride in THF where the temperature was maintained at -78 °C during addition and then subsequently allowed to warm to room temperature. Therefore, the methods reported above were adopted and modified in an attempt to synthesise the (2*S*) enantiomer of 3'-methoxydesaminokynurenine **(88)** (Scheme 4.5).

The protected aspartic acid derivative, *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetic acid **(175)** was synthesised from the reaction of *N*-carbobenzyloxy-L-aspartic acid **(176)** with paraformaldehyde, thionyl chloride and acetic anhydride in acetic acid under reflux for three hours using a method reported by Itoh.[185] Upon cooling, the solvent was removed under reduced pressure to afford an orange residue. This was dissolved in ethyl acetate and then extracted into sodium bicarbonate. Upon acidification of this solution a white suspension formed which was re-extracted into ethyl acetate, and washed successively with water and brine to remove aqueous soluble impurities. Further purification by column chromatography using ethyl acetate and methylene chloride as eluents afforded the product as a sticky white solid in 33% yield. The methylene hydrogens of the oxazolidine ring were observed as a broad singlet at 5.47 ppm in the ^{1}H NMR spectrum.

The corresponding acid chloride **(68)** was formed by heating a solution of **(175)** in toluene and an excess of oxalyl chloride under reflux for two and half hours. The excess oxalyl chloride was removed as an azeotrope with toluene to afford **(68)** as an orange oil in 91% yield. The structure of **(68)** was verified by NMR spectroscopy and mass spectrometry. The acyl chloride was then reacted on without further purification.

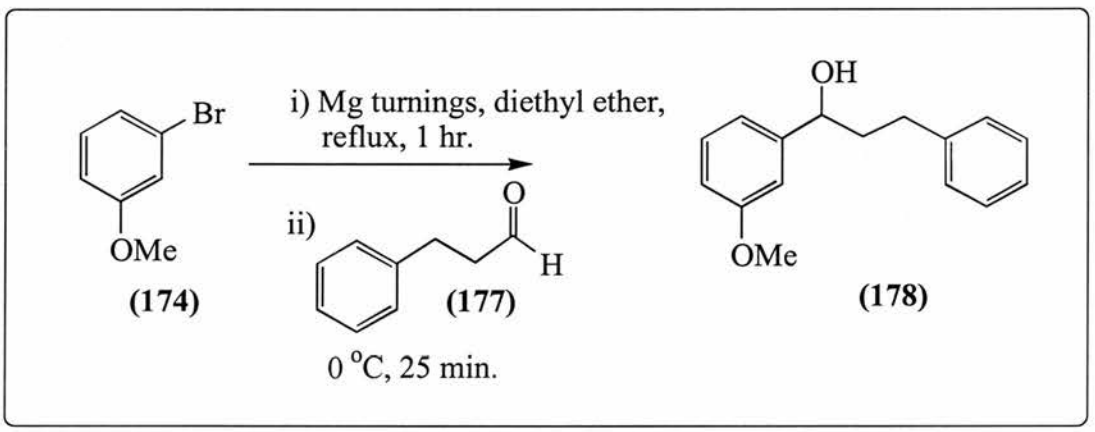


Scheme 4.5

3-Bromoanisole **(174)** was added to magnesium turnings in THF and heated under reflux over two hours during which a pale yellow solution formed. The solution was then cooled to -77 °C and a solution of **(68)** in THF was added slowly, this was stirred for a further one and half hours before the reaction was warmed to room temperature and the reaction quenched by pouring onto an ammonium chloride solution. However, after extraction into diethyl ether analysis of the reaction by NMR spectroscopy indicated no reaction had occurred. The reaction was repeated again using diethyl ether as a solvent however no reaction was found to occur. Reversing the mode of addition and adding the Grignard reagent to the acid chloride **(68)** was also unsuccessful. The conditions were altered and magnesium turnings in diethyl ether together with a single crystal of iodine were heated under reflux for twenty five minutes during which a red solution began to form this was cooled to 0 °C and a solution of **(68)** in THF was added and the reaction mixture stirred at room temperature for one hour. However, analysis by tlc and NMR spectroscopy indicated that no reaction had occurred.

In order to test whether the Grignard reagent was forming *in situ* a test reaction was carried out (Scheme 4.6). The reaction was thus repeated and to the solution of the

Grignard reagent was added 3-phenylpropionaldehyde **(177)**. The pale yellow reaction mixture formed was then stirred for a further twenty five minutes at 0 °C. The reaction was quenched by pouring the mixture onto a solution of ammonium chloride with subsequent extraction into diethyl ether. Removal of the solvent under reduced pressure and then further purification using column chromatography with ethyl acetate and petroleum ether as eluents afforded the coupled product 1-(3-methoxy-phenyl)-2-phenyl-ethanol **(178)** as a pale yellow oil in 56% yield. NMR spectroscopy and mass spectrometry confirmed the structure of the compound. In the ^{1}H NMR spectrum a triplet at 4.66 ppm due to the methine CHOH hydrogen was observed while the low resolution mass spectrometry exhibited a molecular ion peak at 242 *m/z* units.



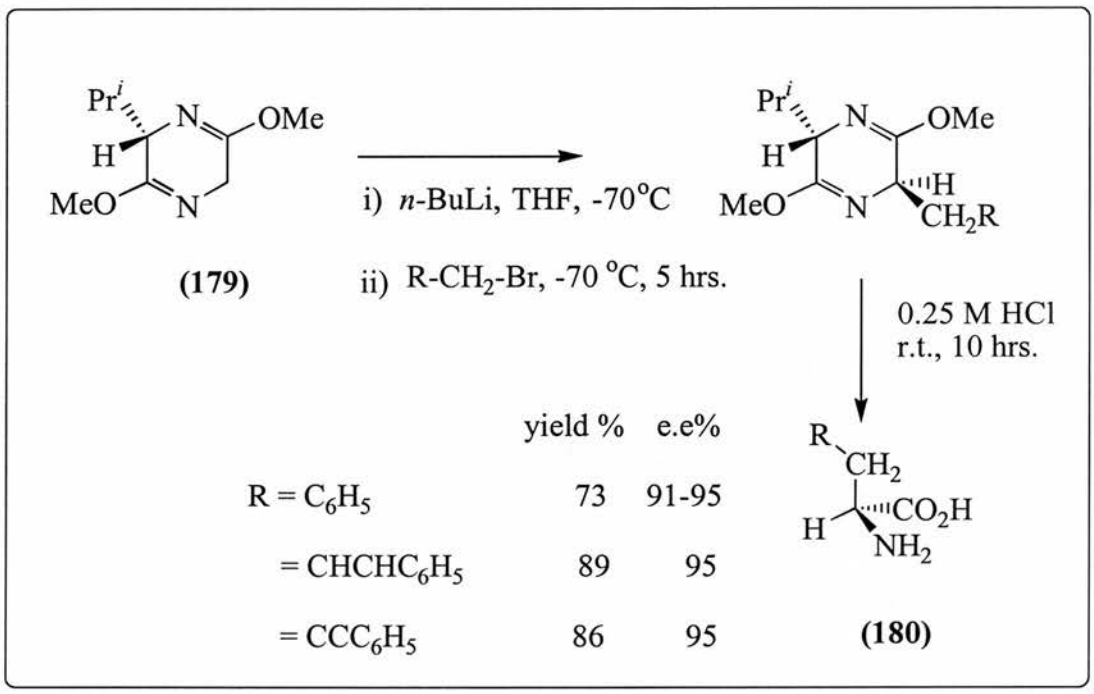
Scheme 4.6

As the formation of the Grignard reagent had been confirmed it was decided to change the acyl chloride, *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)** to the anhydride thereby providing a better leaving group for the attacking anisole anion. *S*-(3-Benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)** as a solution in THF was added to a premixed solution of *o*-anisic acid and triethylamine in THF at -13 °C and stirred for two and half hours at room temperature during which time a white suspension formed. NMR spectroscopic and tlc analysis of this compound indicated the formation of the anhydride and the compound was used without further purification. Reaction of the anhydride with the Grignard reagent at -78 °C, then warming to room temperature gave no evidence for the formation of the protected ketone.

The investigation into the use of a Grignard coupling reaction to prepare the (2*S*) enantiomer of 3'-methoxykynurenine **(88)** was not exhaustive. While it was possible to generate and trap the Grignard reagent using 3-phenylpropionaldehyde **(177)**, reaction with the acetyl chloride **(68)** or its corresponding anhydride derivative were not successful. One possible explanation may be the continued presence of excess oxalyl chloride which may react with the Grignard before the acyl halide, however, there was no evidence for such a reaction having occurred.

4.3 Asymmetric α-amino acid synthesis using Schollkopf chemistry

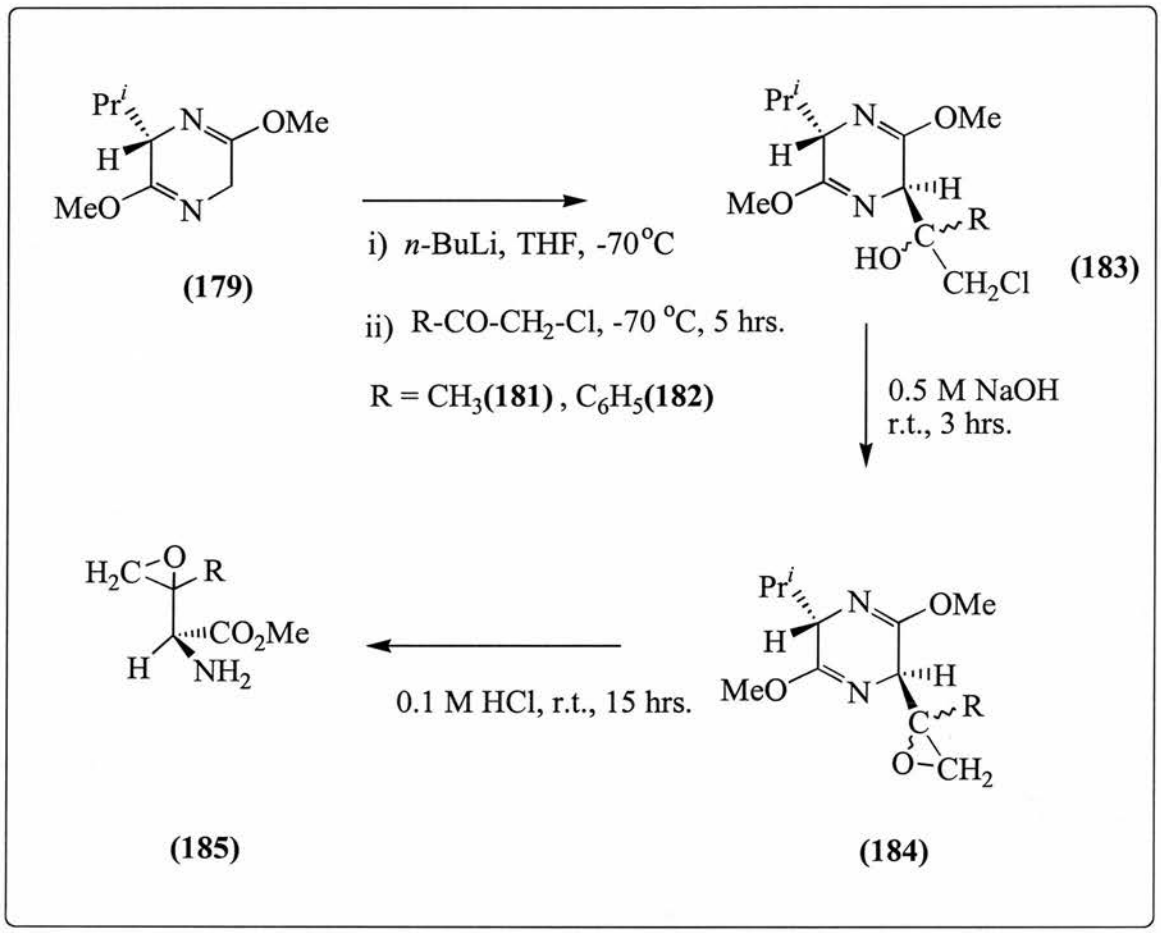
A method for enantioselective synthesis of α-amino acids which involved base induced alkylation of lactim ethers was pioneered by Schollkopf.[186,187] The method was used initially to synthesise α-methyl amino acids by reaction of the lithiated bislactim ether of *cyclo*-(L-ala-L-ala) with electrophiles. The chemistry was then developed to use the mixed bislactim ether, (3*S*)-3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine **(179)**, obtained from *cyclo*-(L-val-gly) to synthesise several (2*R*)-α-amino acids, α-H amino acids, in high enantiomeric excess.[188] The bislactim ether (3*S*)-3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine **(179)** is metalated regiospecifically at the glycine moiety by butyllithium. The lithiated compound was then reacted with a number of alkyl and aryl bromides with subsequent deprotection *via* acid hydrolysis to afford the (2*R*)-α-amino acids **(180)** in high yield and with a high degree of enantioselectivity (Scheme 4.7).



Scheme 4.7

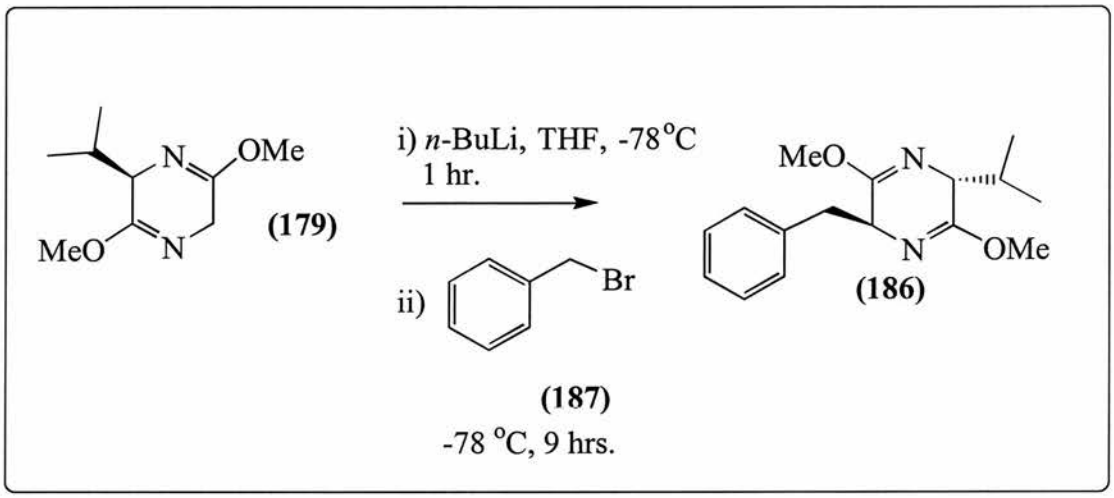
The bislactim ether method was recently used in the synthesis of α-deuterated α-amino acids by Gani *et al.*[189] The reaction of the bislactim ether **(179)** in deuterated aqueous methanol, MeO^{2}H – ^{2}H$_2$O and deuterated potassium hydroxide KO^{2}H under reflux conditions afforded a bislactim ether in which the glycine moiety was dideuterated. This was coupled with a number of alkyl and aryl bromides followed by deprotection *via* acid hydrolysis to afford α-deuterated α-amino acids in moderate yield with a high degree of enantioselectivity. Both (3*R*) and (3*S*) bislactim ethers were used, giving both (2*S*) and (2*R*) α-deuterated α-amino acids.

The bislactim ether method was also applied to the asymmetric synthesis of amino acid esters with a β,γ-epoxy function.[190] Both chloroacetone **(181)** and α-chloroacetophenone **(182)** were reacted with the lithiated (3*S*)-(3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine which attacked the carbonyl group to afford the corresponding hydroxy derivative **(183)**. Subsequent reaction with sodium hydroxide resulted in formation of the corresponding epoxide **(184)** and acid hydrolysis afforded amino acid ester **(185)**. The reactions gave virtually only the (3*R*)-addition products (Scheme 4.8).



Scheme 4.8

The synthesis of the (2*S*) enantiomer of desaminokynurenine, (2*S*)-4-oxo-4-phenyl-butyric acid **(15)** was attempted using the Schollkopf method. Initially, the synthesis of (3*R*,6*S*)-6-benzyl-3-isopropyl-2,5-dimethoxy-3,6-dihydroxypyrazine **(186)** was undertaken to check the methodology (Scheme 4.9). A slight excess of n-butyllithium was added to a solution of (3*R*)-(3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine **(179)** in THF at -78 °C and the reaction mixture stirred for one hour before benzyl bromide **(187)** was added to the preformed anion. The reaction mixture was then stirred at -78 °C for nine hours before being warmed to room temperature and the solvent removed under reduced pressure. After addition of potassium phosphate buffer and extraction into diethyl ether the solvent was removed under reduced pressure. Further purification of the yellow oil obtained by column chromatography afforded the product as a clear oil in 22% yield. The structure was verified by NMR spectroscopy and mass spectrometry and agreed with the literature characterisation.



Scheme 4.9

The enantioselective synthesis of (2*S*)-4-oxo-4-phenyl-butyric acid **(15)**, (Figure 4.1), was then attempted using similar conditions. Formation of the lithiated anion of the bislactam ether was monitored by tlc. Although the starting bislactim ether **(179)** is not U.V. active, upon removal of a proton by n-butyllithium a pseudo aromatic species forms which is visible by UV.

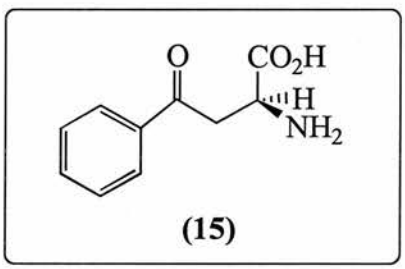


Figure 4.1

Once formation of the anion was complete a solution of α-bromoacetophenone **(133)** in THF was added and the reaction mixture stirred at -78 °C for eight hours. Two equivalents of the α-haloketone were used in order to improve upon the low yield obtained for the reaction with aryl halide. After addition of potassium phosphate buffer and extraction into diethyl ether the solvent was removed under reduced pressure. The orange residue obtained was further purified by column chromatography using ethyl acetate and petroleum ether as eluents. The oxirane, (3-bromomethyl-3-phenyloxiranyl)-phenyl-methanone **(188)**, was obtained as the major product, a white solid in 33% yield. The

structure of the compound was verified by NMR spectroscopy, mass spectrometry and a X-ray crystal structure was also obtained (Figure 4.1). Complete crystallographic data is given in the appendix (Crystal data set B.).

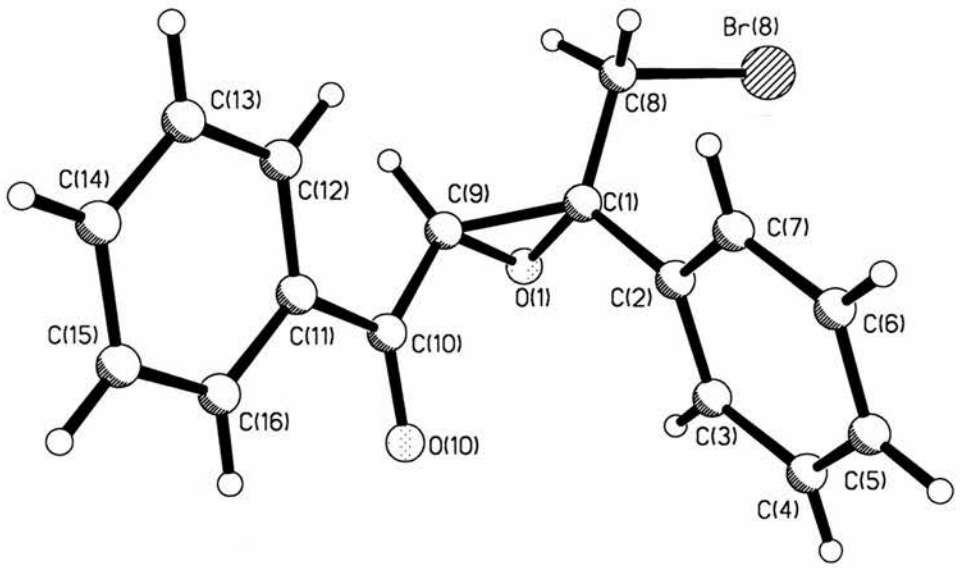
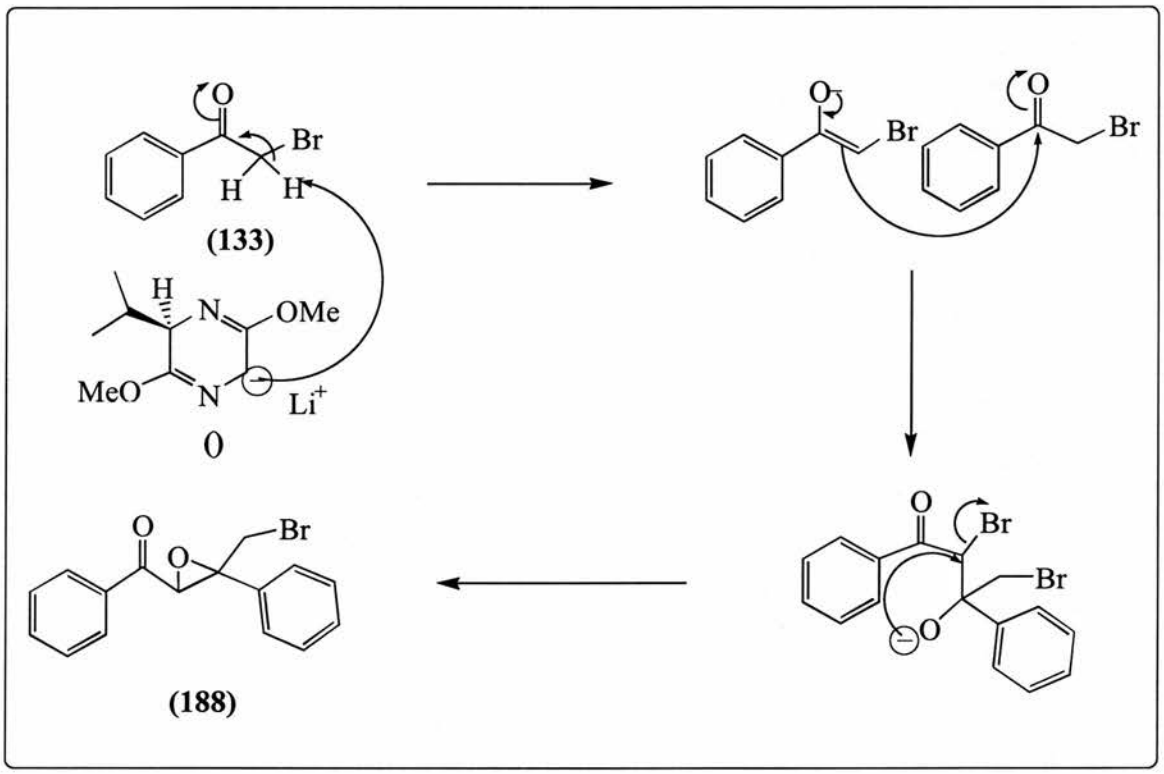


Figure 4.2

Crystal structure of (3-bromomethyl)-3-phenyl-oxiranyl)-phenyl-methanone (188)

It was hoped that the bislactim ether would displace the bromine to form the protected precursor of (2*S*)-4-oxo-4-phenyl-butyric acid **(15)**. Instead the lithiated bislactim ether behaved as a base removing one of the α-hydrogens from α-bromoacetophenone (Scheme 4.10). The newly formed anion then attacked the carbonyl group of another α-bromoacetophenone molecule. Cyclisation of this anion resulted in formation of the oxiranyl functionality with concomitant loss of a bromide anion. There was also no evidence in this reaction for any addition of the bislactim ether anion to the carbonyl group of **(133)** to form a hydroxy function. This is in contrast to the reaction of α-chloroacetophenone **(182)** discussed previously in which the bislactim ether anion attacked the carbonyl group to form an addition product **(183)** in 91% yield. It was thought that the desired reaction would occur because a bromide ion is a better leaving group than a chloride ion which might favour attack at the α-carbon over the carbonyl. However, a change seems to occur in the pKa of the α-hydrogens which makes them readily removable.

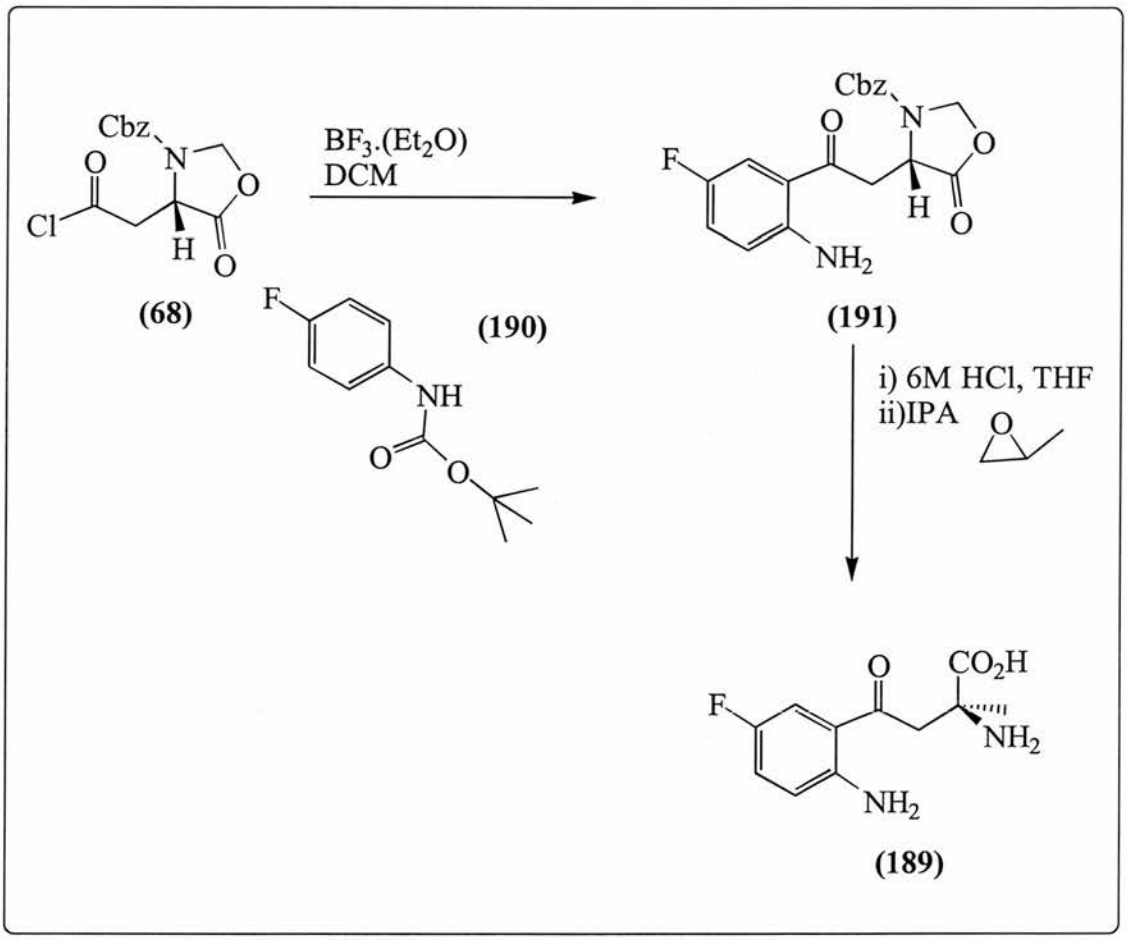


Scheme 4.10

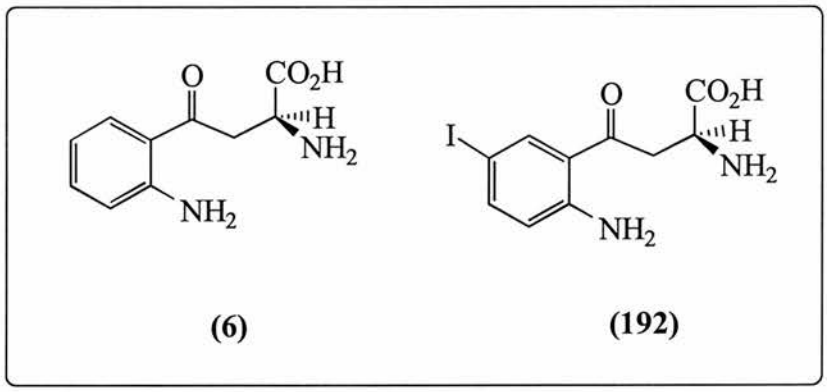
In conclusion the reaction of α-haloketones with bislactim ethers does not appear to offer a route to kynurenine analogues. The reaction either gives alcohols *via* attack at the carbonyl of the α-chloroketones or epoxide formation following α-hydrogen abstraction in the case of α-bromoketones.

4.4 Friedel-Crafts acylation reactions

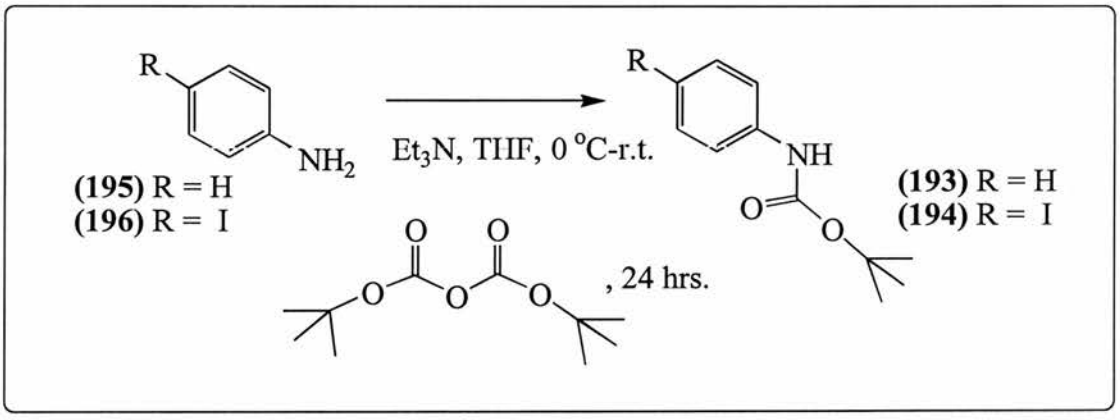
The successful synthesis of 5-fluoro-L-kynurenine **(189)** using a Friedel-Crafts acylation reaction was recently reported by Muirhead (Scheme 4.11).[149] The reaction of *N*-boc protected 4-fluoroaniline **(190)** with *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)** in the presence of boron trifluoride diethyl etherate resulted in formation of **(191)** in 63% yield. Subsequent deprotection *via* acid hydrolysis gave 5-fluoro-L-kynurenine **(189)** in 33% yield. Since this method appeared to offer a novel and short synthetic route to (2S)-kynurenines the syntheses of both L-kynurenine **(6)** and 5-iodo-L-kynurenine **(192)** were attempted using this method. Iodokynurenine **(192)** may be utilized in the synthesis of other kynurenines by substituting the iodine for other functionalities.



Scheme 4.11



The *N*-Boc protected anilines **(193)** and **(194)** were synthesised using the method reported by Ponnusamy *et al.* (Scheme 4.12).[158] A solution of the aniline **(195)** and triethylamine in THF was cooled to 0 °C and di-*tert*-butylpyrocarbonate was added slowly. The reaction mixture was stirred at room temperature overnight after which time the solvent was removed under reduced pressure. Further purification by column chromatography afforded the *N*-Boc protected aniline **(193)** as a white solid in 79% yield. The molecular ion peak was observed at 193 *m/z* units in the mass spectrum while the ^{1}H NMR exhibited a resonance at 1.64 ppm due to the methyl hydrogens of the *t*-butyl group. 4-Iodoaniline **(194)** was protected in a similar manner and was obtained after purification by column chromatography as a white solid in 35% yield. NMR spectroscopy and mass spectrometry confirmed the structure of **(194)**. Resonances in the ^{1}H NMR spectrum at 1.66 and 6.57 ppm were assigned to the methyl hydrogens of the *t*-butyl group and the urethane N-H, respectively.



Scheme 4.12

Reaction of the protected anilines with *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride **(68)** in the presence of boron trifluoride diethyl etherate however did not result in reaction. *N*-Boc protected aniline **(193)** as a solution in methylene chloride was added to a solution of the acid chloride **(68)** and stirred overnight at room temperature. The reaction was monitored by tlc. The protected aniline **(193)** was not visible by tlc and a number of bands were observed to form. The reaction was stopped and the solvent removed under reduced pressure. Analysis by NMR was inconclusive but there was no evidence for acylation having occurred. Reaction of 4-iodo-*N*-Boc protected aniline **(194)** was attempted under the same conditions and the reaction was monitered by tlc. Although two new bands were evident by tlc and there was evidence for the disappearance of the starting material **(194)** analysis by NMR spectroscopy of the crude reaction mixture provided no evidence for acylation having occurred. The reaction of 4-iodo-*N*-Boc protected aniline **(194)** was repeated using tin (IV) chloride as the Lewis acid catalyst instead of boron trifluoride diethyl etherate. The reaction was carried out at room temperature in methylene chloride and after stirring overnight the reaction was stopped. Analysis by tlc again indicated loss of starting materials. The reaction mixture was poured onto aqueous acid extracted into methylene chloride and the solvent removed under reduced pressure to afford a yellow oil. After further purification by column chromatography the main band a yellow oil was analysed by NMR spectroscopy. Acylation had not occurred but there was some evidence for loss of the protecting group from the amine **(194)**.

Although Friedel-Crafts acylation was successful in the synthesis of 5-fluoro-L-kynurenine **(189)** using boron trifluoride diethyl etherate as a Lewis acid catalyst it does not appear to catalyse Friedel-Crafts acylation reactions for the *N*-Boc protected anilines examined. The use of tin (IV) chloride as a Lewis acid in the Friedel-Crafts reaction gave evidence for the loss of the boc protecting group but no evidence for acylation having occurred. However, the *N*-Boc protected aniline **(193)** and 4-iodo aniline **(194)** would have been more reactive to the Friedel-Crafts reaction conditions which may have resulted in loss of the protecting group from nitrogen to reform the aniline starting materials as well as other side reactions.

CHAPTER 5

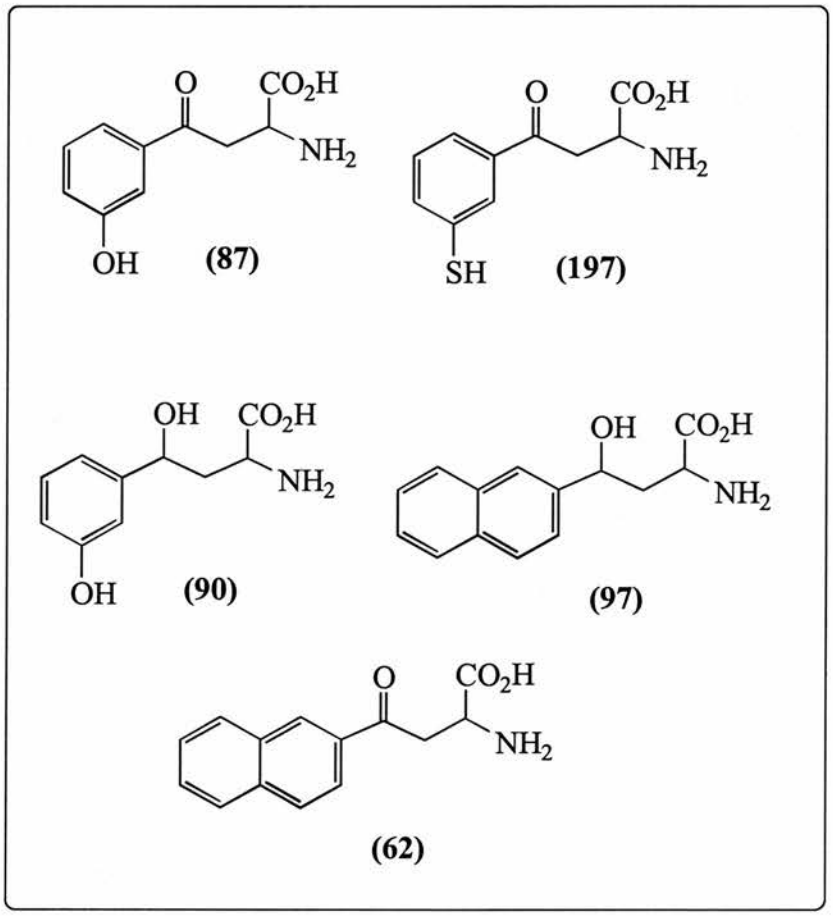
5 FUTURE WORK

A series of kynurenine analogues was prepared and examined as inhibitors of recombinant human kynureninase. The work described in this thesis offers a detailed examination of the specificity of the human enzyme to give a greater understanding of interactions at the active site. 3'-Hydroxydesaminokynurenine **(87)** was determined to be the most potent inhibitor of kynureninase to date. The compound exhibited K_i values of 5 nM, 20 nM and 25 nM when tested against human, rat liver and bacterial kynureninase, respectively. This indicated that a hydroxy group at the 3-position of the aryl ring is an important recognition element for the enzyme and is probably due to hydrogen bonding at the active site. Further work is warranted in replacing the hydroxyl group with other substituents, such as a thiol (SH) **(197)**, to investigate whether potency can be increased further. There is also scope for further work in the synthesis of the enantiopure 2*S*-isomer of **(87)**. Although attempts to synthesise 2*S*-amino acids were unsuccessful, the development of a synthetic route to chiral kynurenines in high yield is desirable.

The synthesis of transition state analogue inhibitors which contain a hydroxy group at the γ-position was also undertaken. The most potent of these was dihydroxydesaminokynurenine **(90)** which exhibited K_i values of 100 nM, 130 nM and 10 μM when tested against human, rat liver and bacterial kynureninase respectively. The synthesis of other transition state analogue inhibitors containing a γ-hydroxy group is worthy of further investigation as they can function as potent inhibitors of kynureninase.

The monocyclic analogues were observed to be better inhibitors of the human enzyme when compared with the bicyclic 5-hydroxy-1-tetralone **(89)** and 1,5-dihydroxy tetralone derivatives **(92)**. These compounds exhibited K_i values of 57 μM and 66 μM, respectively. This indicated that the presence of a carbon linker between the alanine moiety and benzene ring did not confer potency. Though problems with the assay precluded a study with the γ-hydroxynapthalene derivative **(97)** the non-reduced analogue **(62)**[38] was successfully tested using a fluorescence assay. The naphthalene derivative **(62)** was observed to be a strong inhibitor of the enzyme exhibiting K_i values of 22 μM and 5

μM against human and bacterial kynureninase, respectively. This showed that a hydrophobic pocket exists within the active site, which can accommodate a second benzene ring, and so further investigation of other naphthalene analogues is merited, as long as problems with the enzyme assay can be overcome.



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.

Infrared spectra were recorded on a Paragon 2000 FT IR spectrometer. The samples were prepared as nujol mulls or as thin films between NaCl plates. Absorption maxima are given in wavenumbers (cm^{-1}) (vs-very strong, s-strong, sh-shoulder, m-medium, w-weak, vw-very weak, b-broad).

NMR spectra were recorded on a Varian Gemini f.t. spectrometer (^{1}H, 300 MHz; ^{13}C, 74.76 MHz) and a Varian Gemini f.t. spectrometer (^{1}H, 200 MHz, ^{13}C, 50.31 MHz). ^{1}H NMR spectra were referenced to chloroform, TMS, methanol or DMSO. NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as positions (δ_H or δ_C), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-doublet of doublets, dt-doublet of triplets, and b-broad), coupling constant ($J_{X,Y}$ Hz if applicable) and assignment.

EI and CI mass spectra and accurate mass measurements were recorded on a VG AUTOSPEC and MALDI-TOF spectra were recorded on a TofSpec 2E Micromass instrument. Major fragments are given as percentages of the base peak intensity.

Flash chromatography was performed using the procedure of Still[191] using Sorbisil C60 (40-60 MM) silica gel, Kieselgel 60 and BakerBond C_{18} reverse phase silica gel.

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, aqueous potassium permanganate, bromocresol green in ethanol or ninhydrin.

Analytical HPLC was carried out on a CECIL 1200 VW instrument using phenosphere C_{18} reverse phase columns, 3 or 5μ, with internal guard column. All columns were purchased from Phenomenex.

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

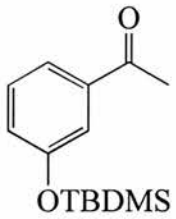
Centrifugation was carried out in a Kontron Centrikon T-124 centrifuge.

Assays were carried out using a Kontron Instruments UVICON 932 spectrophotometer and a Perkin Elmer Luminescence spectrophotometer (Model LS50B).

Solvents were dried and purified according to the methods of Perrin and Armarego.[192]

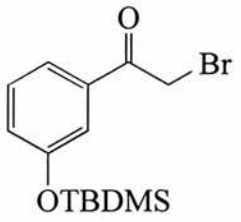
6.1 Inhibitors of kynureninase

3'-*tert*-Butyldimethylsilyloxyacetophenone (115)



To a solution of 3'-hydroxyacetophenone (5.54 g, 40.7 mmol) in dry CH_2Cl_2 (125 ml), were added DMAP (1.13 g, 9.3 mmol) and triethylamine (6.7 ml, 48.0 mmol). The mixture was cooled to 0 °C and *tert*-butyldimethylsilyl chloride (6.14 g, 40.7 mmol), was added slowly and the mixture stirred at room temperature for twenty-four hours. The reaction mixture was washed with water (100 ml). The aqueous layer was then extracted with ethyl acetate (2 × 40 ml) and the ethyl acetate layers combined, dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Further purification using column chromatography (silica gel, ethyl acetate: petroleum ether 1:2) afforded a colourless oil (7.97 g, 78%); δ_H (200 MHz, C^2HCl_3), 0.21 (6H, s, $Si(C\underline{H}_3)_2C(CH_3)_3$), 0.99 (9H, s, $Si(CH_3)_2C(C\underline{H}_3)_3$), 2.57 (3H, s, $COC\underline{H}_3$), 7.05 (1H, dd, *J* 8.0, 2.0 Hz, 4'-H), 7.32 (1H, t, *J* 8.0 Hz, 5'-H), 7.41 (1H, t, *J* 2.0 Hz, 2'-H), 7.54 (1H, m, 6'-H); δ_C (50.31 MHz, C^2HCl_3) 14.70 (s, $Si\underline{C}(CH_3)_3$), 18.68 (s, $Si(\underline{C}H_3)_2C(CH_3)_3$), 26.13 (s, $C(\underline{C}H_3)_3$), 27.26 (s, $CO\underline{C}H_3$), 119.95 (s, 2'-C), 122.06 (s, 6'-C), 125.47 (s, 4'-C), 130.05 (s, 5'-C), 139.04 (s, 1'-C), 156. 43 (s, 3'-C), 198.45 (s, $\underline{C}OCH_3$); *m/z* (EI) 250 ($[M]^+$, 47%), 193 (100, $[M\text{-}C(CH_3)_3]^+$, 135 (7, $[M\text{-}Si(CH_3)_2C(CH_3)_3]^+$), 43 (25, $[CH_3CO]^+$).

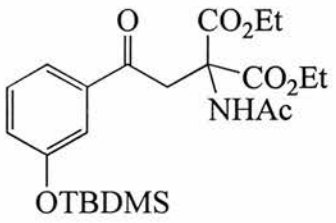
2-Bromo-(3'-*tert*-butyldimethylsiloxy)acetophenone (116)



Cupric bromide (5.45 g, 24.4 mmol) was suspended in ethyl acetate (45 ml) and heated to reflux with stirring. 3'-*tert*-Butyldimethylsilyloxyacetophenone (3.05 g, 12.2 mmol) in chloroform (8 ml) was then added and the reaction mixture heated under reflux for six hours. The CuBr and $CuBr_2$ residues were filtered off and the filtrate decolourised with activated charcoal. The activated charcoal was removed by filtration through celite, which was washed with ethyl acetate (4 × 25 ml). The solvent was then removed under reduced pressure to afford a yellow oil (2.12 g, 53%), which was used without further purification; δ_H (200 MHz, C^2HCl_3) 0.22 (6H, s, $Si(C\underline{H}_3)_2C(CH_3)_3$), 0.99 (9H, s, $SiC(C\underline{H}_3)_3(CH_3)_2$), 4.45 (2H, s, $COC\underline{H}_2Br$), 7.1 (1H, m, 4'-H), 7.35 (1H, t, *J* 8 Hz, 5'-H), 7.44 (1H, t, *J* 2.1 Hz, 2'-H), 7.53 (1H, m, 6'-H); δ_C (50.31 MHz, C^2HCl_3) 18.70 (s, $Si(\underline{C}H_3)_2C(CH_3)_3$), 26.12 (s, $C(\underline{C}H_3)_3$), 31.83 (s, $CO\underline{C}H_2Br$), 115.89 (s, 2'-C), 122.66 (s, 6'-C), 126.60 (s, 4'-C), 130.45 (s, 5'-C), 135. 69 (s, 1'-C), 157.14 (s, 3'-C), 192.29 (s, $\underline{C}OCH_2Br$). *m/z* (EI) 330, 328 ($[M]^+$, 19), 273 (29, $[M+2\text{-}C(CH_3)_3]^+$), 271 (28, $[M\text{-}C(CH_3)_3]^+$), 193 (84, $[(CH_3)_2HSiOC_6H_4CO]^+$), 75 (100, $[(CH_3)_3SiOH]^+$).

Reaction of 2-bromo-(3'-*tert*-butylsilyloxy)acetophenone (116) with diethyl acetamidomalonate

Procedure 1

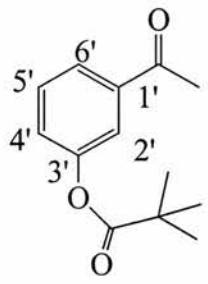


To a solution of diethyl acetamidomalonate (4.78 g, 22.0 mmol) in dry DMF (35 ml) sodium hydride (60% wgt in oil, 0.32 g, 22.0 mmol) was added at 0 °C and under nitrogen. The reaction mixture was allowed to stir for three hours. To this 2-bromo-(3'-*tert*-butyldimethylsilyloxy)acetophenone (7.27 g, 22.0 mmol) was added slowly over one hour and the reaction mixture allowed to stir overnight. The reaction mixture was then poured onto water (350 ml) and acidified with 3% HCl. The off white suspension was extracted into diethyl ether (5 × 150 ml), dried ($MgSO_4$) and the solvent removed to afford a yellow oil. The NMR spectra were inconclusive.

Procedure 2

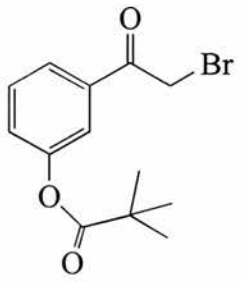
To diethyl acetamidomalonate (1.32 g, 6.1 mmol) in dry DMF (20 ml), sodium hydride (60% weight in oil, 0.24 g, 6.1 mmol), was added at 0 °C and under nitrogen and the reaction mixture allowed to stir for three hours. The resulting yellow solution was the added slowly over one hour to a solution of 2-bromo-(3'-*tert*-butylsilyloxy)acetophenone (2.00 g, 6.11 mmol) in DMF and allowed to stir at room temperature overnight. The dark orange solution was then added to water (350 ml). The off white suspension was acidified with 3% HCl, extracted into ethyl acetate (3 × 150 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Analysis by tlc showed the presence of several bands and further purification via column chromatography (silica gel, ethyl acetate: petroleum ether 1:3) afforded only trace amounts too small to characterise.

2,2-Dimethyl-propionic acid 3-(2-acetyl)-phenyl ester (118)



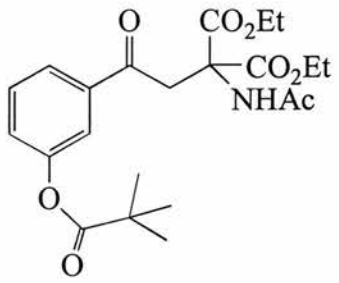
To a solution of 3'-hydroxyacetophenone (3.46 g, 25.5 mmol) in dry pyridine (50 ml) was added pivaloyl chloride (4.60 g, 38.2 mmol) at 0 °C and under nitrogen. The reaction mixture was allowed to warm to 4 °C and stirred for ninety-six hours. The solvent was then removed under reduced pressure and the off-white residue dissolved in a minimum of chloroform (75 ml), washed with water (2 × 75 ml) and sodium hydrogen carbonate (75 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. This was further purified by column chromatography (silica gel, ethyl acetate: petroleum ether 1:3) afforded a clear oil (4.48 g, 79%); ν_{max} (nujol)/cm^{-1} 1752 (CO, ester), 1688 (CO); δ_H (300 MHz, C^2HCl_3) 1.35 (9H, s, $OCOC(C\underline{H}_3)_3$), 2.57 (3H, s, $COC\underline{H}_3$), 7.34 (1H, ddd, *J* 7.8, 2.1, 1.2 Hz, 6'-H), 7.45 (1H, t, *J* 7.8 Hz, 5'-H), 7.62 (1H, bm, 2'-H), 7.78 (1H, ddd, *J* 7.8, 2.1, 1.2 Hz, 4'-H); δ_C (75.4 MHz, C^2HCl_3) 26.61 (s, $NHCOC\underline{H}_3$), 27.04 (s, $OCOC(\underline{C}H_3)_3$), 39.08 (s, $OCO\underline{C}(CH_3)_3$), 121.50 (s, 2'-C), 125.73 (s, 6'-C), 126.52 (s, 4'-C). 129.67(s, 5'-C), 138.64 (s, 3'-C), 151.52 (s, 1'-C), 177.05 (s, $O\underline{C}OC(CH_3)_3$), 197.26 (s, $\underline{C}OCH_3$); *m/z* (EI) 220 ($[M]^+$, 19), 136 (52, $[OC_6H_5COCH_3]^+$), 121 (74, $[HOC_6H_5CO]^+$), 77, (6, $[C_6H_5]^+$), 65 (10, $[C_5H_5]^+$), 57 (100, $[(CH_3)_3C]^+$).

2,2-Dimethyl-propionic acid 3-(2-bromoacetyl)-phenyl ester (119)



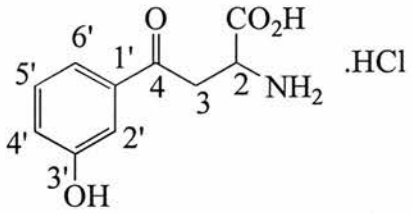
Cupric bromide (6.86 g, 30.0 mmol) was suspended in ethyl acetate and heated to reflux with stirring. To this was added **(118)** (3.38 g, 15.0 mmol) in chloroform (15 ml) and the reaction mixture heated under reflux for four hours. The CuBr and $CuBr_2$ residues were filtered off and the filtrate decolourised with activated charcoal. The activated charcoal was removed by filtration through celite and the solvent removed under reduced pressure to afford a yellow oil (2.83 g, 63%), which was used without further purification; (Found: C, 52.66; H, 5.05 $C_{13}H_{15}BrO_3$ requires: C, 52.19; H, 5.05%); ν_{max} (nujol)/cm^{-1} 1750 (CO, ester), 1692 (CO); δ_H (300 MHz, C^2HCl_3) 1.35 (9H, s, $OCOC(C\underline{H}_3)_3$), 4.42 (2H, s, $COC\underline{H}_2Br$), 7.29 (1H, ddd, *J* 8.0, 2.0, 1.2 Hz, 6'-H), 7.47 (1H, t, *J* 8.0 Hz, 5'-H), 7.65 (1H, t, *J* 2.0 Hz, 2'-H), 7.81 (1H, ddd, *J* 8.0, 2.0, 1.2 Hz, 4'-H); δ_C (75.4 MHz, C^2HCl_3) 27.06 (s, $OCOC(\underline{C}H_3)_3$), 30.71 (s, $CO\underline{C}H_2Br$), 39.31 (s, $OCO\underline{C}(CH_3)_3$), 122.92 (s, 2'-C), 126.60 (s, 6'-C), 127.94 (s, 4'-C), 129.66 (s, 5'-C), 135.47 (s, 3'-C), 151.69 (s, 1'-C), 176.68 (s, $O\underline{C}OC(CH_3)_3$), 190.59 (s, $\underline{C}OCH_2Br$); *m/z* (EI) 300, 298 ($[M]^+$, 4) 205 (7, $[(CH_3)_3COCOC_6H_4CO]^+$), 121 (53, $[OC_6H_4CO]^+$), 85 (39, $[C_5H_9O]^+$), 57 (100, $[(CH_3)_3C]^+$).

2-Acetylamino-2-{2-[3-(2,2-dimethyl-propionyloxy)-phenyl]-2-oxo-ethyl}-malonic acid diethyl ester (120)



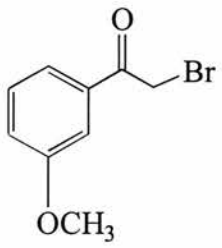
To a stirred solution of diethyl acetamidomalonate (2.07 g, 9.00 mmol) in dry DMF (60 ml), was added sodium hydride (60% weight in oil, 0.38 g, 9.00 mmol) at 0 °C and under an atmosphere of nitrogen. The reaction mixture was warmed to room temperature and stirred for three hours before adding of 2,2-dimethyl-propionic acid 3-(2-bromoacetyl)-phenyl ester (2.86 g, 9.00 mmol). The resulting orange solution was stirred overnight before pouring the mixture over water (600 ml), acidifying with 1M HCl, extracting into diethyl ether (5 × 150 ml), drying ($MgSO_4$) and removing the solvent under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel; ethyl acetate: petroleum ether 1:2) afforded a white crystalline solid (2.38 g, 57%), m.p. 84 °C; (Found: C, 60.02; H, 7.16; N, 3.09 $C_{22}H_{29}NO_8$ requires: C, 60.68; H, 6.71; N, 3.22%); ν_{max} (nujol)/cm^{-1} 3358 (br, NH), 1742 (CO, ester), 1681 (CO); δ_H (300 MHz, C^2HCl_3) 1.24 (6H, m, 2 × $OCH_2C\underline{H}_3$), 1.35 (9H, s, $OCOC(C\underline{H}_3)_3$), 2.02 (3H, s, $COC\underline{H}_3$), 4.24 (6H, m, 2 × $OC\underline{H}_2CH_3$, β-$C\underline{H}_2$), 7.09 (1H, s, $N\underline{H}COCH_3$), 7.27 (1H, dd, *J* 8.0, 2.0 Hz, 4'-H), 7.46 (1H, t, *J* 8.0 Hz, 5'-H), 7.6 (1H, bs, 2'-H), 7.81 (1H, d, *J* 8.0, Hz, 6'-H); δ_C (75.4 MHz, C^2HCl_3) 13.83 (s, 2 × $OCH_2\underline{C}H_3$), 22.88 (s, $NHCO\underline{C}H_3$), 27.06 (s, $OCOC(\underline{C}H_3)_3$), 39.08 (s, $OCO\underline{C}(CH_3)_3$), 42.37 (s, β-$\underline{C}H_2$), 62.94 (s, $CH_2\underline{C}NHAc(CO_2Et)_2$), 63.93(s, 2 × $O\underline{C}H_2CH_3$), 121.46 (s, 2'-C), 125.91 (s, 6'-C), 127.25 (s, 4'-C), 129.83 (s, 5'-C), 137.52 (s, 1'-C), 151.56 (s, 3'-C), 167.37 (s, $NH\underline{C}OCH_3$), 169.67 (s, $O\underline{C}OCH_2CH_3$), 171.29 (s, $O\underline{C}OCH_2CH_3$), 177.07 (s, $O\underline{C}OC(CH_3)_3$), 196.15 (s, γ-CO); *m/z* (EI) 435 ($[M]^+$, 36), 390 (13, $[M\text{-}OCH_2CH_3]^+$), 362 (60, $[M\text{-}OCOCH_2CH_3]^+$), 205 (100, $[(CH_3)_3COCOC_6H_4CO]^+$), 121 (98, $[HOC_6H_4CO]^+$), 93 (8, $[OC_6H_5]^+$), 85 (11, $[(CH_3)_3CCO]^+$), 57 (78, $[(CH_3)_3C]^+$).

2-Amino-4-oxo-4-(3-hydroxy-phenyl)-butyric acid hydrochloride (3'-hydroxydesaminokynurenine (87)



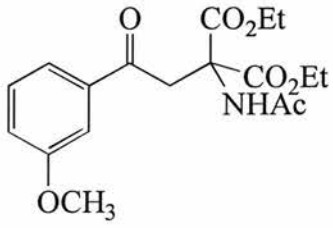
A stirred solution of 2-acetylamino-2-{2-[3-(2,2-dimethyl-propionyloxy)-phenyl]-2-oxo-ethyl}-malonic acid diethyl ester (2.03 g, 4.65 mmol) in diethyl ether (5 ml) and HCl (6M, 60 ml) was heated under reflux for six hours. The reaction mixture was cooled to room temperature and washed with ethyl acetate (3 × 50 ml) and the solvent removed under reduced pressure to afford an off white precipitate (0.87 g, 76%), dec. 188 °C; (Found: C, 48.61; H, 5.00; N, 5.63 $C_{10}H_{12}ClNO_4$ requires C, 48.89; H, 4.92; N, 5.69%); ν_{max} (nujol)/cm^{-1} 3383 (NH), 1739 (CO, acid), 1660 (CO); δ_H (300 MHz, ^2H_2O) 3.73 (2H, d, $J_{3,2}$ 5.0 Hz, 3-C$\underline{H}_2$), 4.39 (1H, t, $J_{2,3}$ 5.0 Hz, 2-C$\underline{H}$), 7.06 (1H, dd, J 2.4, 1.0 Hz, 4'-$\underline{H}$), 7.26 (1H, m, 5'-$\underline{H}$), 7.30 (1H, m, 2'-$\underline{H}$), 7.41 (1H, dd, J 7.8, 1.0 Hz, 6'-$\underline{H}$); δ_C (75.4 MHz, ^2H_2O) 38.21 (s, 3-$\underline{C}H_2$), 48.88 (s, 2-$\underline{C}$H), 114.47 120.78 (s, 4'-$\underline{C}$), 121.90 (s, 2'-$\underline{C}$), 121.90 (s, 6'-$\underline{C}$), 130.51 (s, 5'-$\underline{C}$), 136.56 (1'-$\underline{C}$), 156.10 (s, 3'-$\underline{C}$), 171.77 (s, O$\underline{C}$OH), 199.07 (s, 4-$\underline{C}$O); *m/z* (CI) 210 ([M + H]$^+$, 13), 195 (100, ([M+H-NH]$^+$), 177 (18, [$C_{10}H_8O_3$ +H]$^+$), 164 (17, [M-(CO_2H)$^+$]), 149 (35, [$C_9H_9O_2$]$^+$), 43 (95, [CH_2CNH_2]$^+$). For crystallographic data see appendix (Crystal data set A.).

2-Bromo-1-(3-methoxy-phenyl)-ethanone (122)



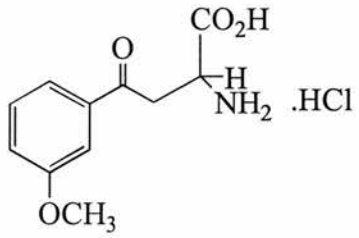
Cupric bromide (14.0 g, 65.9 mmol) was suspended in ethyl acetate (45 ml) and heated to reflux with stirring. 3'-methoxyacetophenone (4.52 g, 32.90 mmol) in chloroform (40 ml) was then added and the reaction mixture heated under reflux for ten hours. The CuBr and $CuBr_2$ residues were filtered off and the filtrate decolourised with activated charcoal. The activated charcoal was removed by filtration through celite, which was washed with ethyl acetate (4 × 25 ml). The solvent was removed under reduced pressure to afford a yellow solid (6.96 g, 92%), which was used without further purification; ν_{max} (nujol)/cm^{-1} 1650 (CO); δ_H (300 MHz, C^2HCl_3) 3.83 (3H, s, $OC\underline{H}_3$), 4.43 (2H, s, $COC\underline{H}_2$ Br), 7.13 (1H, dt, *J* 8, 2 Hz, 4'-H), 7.38 (1H, t, *J* 8 Hz, 5'-H), 7.49 (1H, t, *J* 2 Hz, 2'-H), 7.53 (1H, dt, *J* 8, 2 Hz, 6'-H); δ_C (75.4 MHz, C^2HCl_3) 30.92 (s, $CO\underline{C}H_2Br$), 55.50 (s, $O\underline{C}H_3$), 113.22 (s, 2'-C), 120.61 (s, 4'-C), 121.60 (s, 6'-C), 129.95 (s, 5'-C), 135.43 (s, 1'-C), 160.17 (s, 3'-C), 190.6 (s, $\underline{C}OCH_2Br$); *m/z* (EI) 230, 228 ($[M]^+$, 12, 11 %), 150 (8, $[M\text{-}Br]^+$), 135 (100, $[M\text{-}CH_2Br]^+$), 107 (15, $[C_6H_4OCH_3]^+$), 92 (11, $[C_6H_4O]^+$), 77 (15, $[C_6H_5]^+$).

2-Acetylamino-2-[2-(3-methoxy-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester (123)



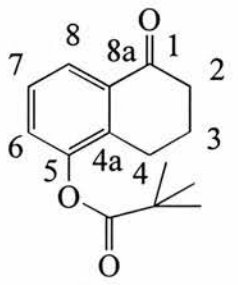
To a stirred solution of diethyl acetamidomalonate (3.01 g, 13.9 mmol) in dry redistilled DMF (50 ml), was added sodium hydride (60% weight in oil, 0.55 g, 13.9 mmol) at 0 °C under an atmosphere of nitrogen. The mixture was stirred for three hours before adding 2-bromo-1-(3-methoxy-phenyl)-ethanone (3.20 g, 13.9 mmol). The orange solution was stirred for a further 4 hours before pouring the mixture onto distilled water (500 ml), acidifying with 1M HCl, extracting into diethyl ether (3 × 150 ml) and drying ($MgSO_4$). The solvent was removed under reduced pressure and the yellow residue recrystallised from diethyl ether to afford a white crystalline solid (2.29 g, 45%) m.p. 72-76 °C; (Found C, 59.41; H, 6.33; N, 3.78 $C_{18}H_{23}NO_7$ requires C, 59.17; H 6.35; N, 3.83%); ν_{max} (nujol)/cm^{-1} 3332 (NH), 1762 (CO, ester), 1650 (CO, amide), δ_H (200 MHz, C^2HCl_3) 1.23 (6H, t, *J* 7 Hz, $OCH_2C\underline{H}_3$), 1.96 (3H, s, $COC\underline{H}_3$), 3.82 (3H, s, $OC\underline{H}_3$), 4.25 (6H, m, $OC\underline{H}_2CH_3$, β-$C\underline{H}_2$), 7.13 (1H, d, *J* 7 Hz, 4'-H), 7.35 (1H, t, *J* 7 Hz, 5'-H), 7.43 (1H, t, *J* 2 Hz, 2'-H), 7.56 (1H, m, 6'-H); δ_C (50.31 MHz, C^2HCl_3) 14.39 (s, $CO_2CH_2\underline{C}H_3$), 23.43 (s, $NHCO\underline{C}H_3$), 42.86 (s, β-$\underline{C}H_2$), 55.92 (s, $O\underline{C}H_3$), 63.37 (s, $O\underline{C}H_2CH_3$), 112.49 (s, 2'-$\underline{C}$), 121.01 (s, 4'-$\underline{C}$), 121.49 (s, 6'-$\underline{C}$), 130.22 (s, 5'-$\underline{C}$), 137.83 (s, 1'-$\underline{C}$), 160.31 (s, 3-$\underline{C}$), 167.93 (s, $NH\underline{C}OCH_3$), 169.14 (s, $O\underline{C}OCH_2CH_3$), 169.75 (s, $O\underline{C}OCH_2CH_3$), 176.13 (s, $O\underline{C}OC(CH_3)_3$),199.75 (s, $\underline{C}O$) ; *m/z* (EI) 365 ($[M]^+$,10%), 292 (18, $[M\text{-}CO_2CH_2CH_3]^+$), 250 (23, $[CH_3O\text{-}PhCOCH_2C(CO_2Et)CH_3]^+$), 135 (100, $[CH_3O\text{-}PhCO]^+$), 107 (10, $[C_6H_4OCH_3]^+$), 77 (7, $[C_6H_5]^+$).

2-Amino-4-oxo-4-(3-methoxy-phenyl)-butyric acid hydrogen chloride (3'-methoxydesaminokynurenine) (88)



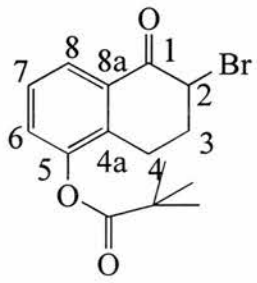
A solution of **(123)** (1.05 g, 2.87 mmol) in 6M HCl (100 ml) was heated under reflux for four hours. Upon cooling the solution was washed with ethyl acetate (2 × 25 ml) and the water removed by freeze drying to afford a white hygroscopic solid. Further purification by preparative tlc (cellulose; H_2O: isopropyl alcohol: NH_3 10:6:3) afforded a white hygroscopic solid (0.58 g, 79%), dec. 164 °C; ν_{max} (nujol)/cm^{-1} 3378 (NH), 1738 (CO, acid), 1681 (CO); δ_H (200 MHz, ^2H_2O), 3.77 (3H, s, $OC\underline{H}_3$), 3.80 (2H, d, $J_{3,2}$ 5 Hz, 3-$C\underline{H}_2$), 4.47 (1H, t, $J_{2,3}$ 5 Hz, 2-C$\underline{H}$), 7.17 (1H, dt, J 8, 2.8 Hz, 4'-H), 7.39 (2H, m, 2', 5'-H), 7.53 (1H, m, 6'-H); δ_C (50.31 MHz, ^2H_2O), 41.26 (s, 3-$\underline{C}H_2$), 51.82 (s, 2-$\underline{C}$H), 58.48 (s, O$\underline{C}H_3$), 115.72 (s, 2'-C), 123.64 (s, 4'-C), 124.35 (s, 6'-C), 133.22 (s, 5'-C), 139.06 (s, 1'-C), 162.06 (s, 3'-C), 174.46 (s, $\underline{C}O_2H$), 201.66 (s, 4-$\underline{C}$O); *m/z* (CI) 224.0913 M+H $C_{11}H_{14}NO_4$ requires 224.0922, 224 ($[M+H]^+$, 80), 209 (100, ($[M+H\text{-}CH_3]^+$), 191 (49, [HO-$C_6H_4COCH_2CHCO_2HNH_2$-$H_2O]^+$), 178 (73, $[M+H\text{-}C_2H_2O_2]^+$), 135 (18, $[CH_3OC_6H_4CO]^+$). Analytical HPLC of **(88)** (C_{18}, reverse phase silica, 3μ, flow rate 3 ml/min, 1% acetic acid, 20% methanol) showed the presence of a single peak.

2,2-Dimethyl-propionic acid 5-oxo-5,6,7,8-tetrahydro-naphthalen-1-yl ester (125)



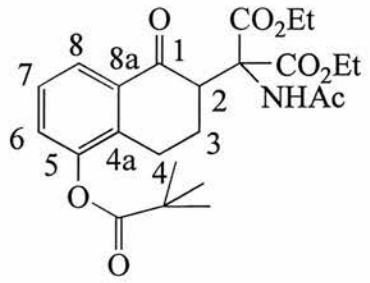
To a stirred solution of 5-hydroxy-1-tetralone (5.00 g, 30.8 mmol) in dry pyridine (60 ml) was added pivaloyl chloride (5.65 ml, 45.9 mmol) at 0 °C and under an atmosphere of nitrogen. The reaction mixture was allowed to warm to 4 °C and stirred for ninety-six hours. The solvent was removed under reduced pressure the residue dissolved in CH_2Cl_2 (300 ml) washed with water (3 × 100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a colourless oil (4.87 g, 71%); (Found: C, 72.82; H, 7.13 $C_{15}H_{18}O_3$ requires: C, 73.15; H, 7.36%); ν_{max} (nujol)/cm^{-1} 1751 (CO, ester), 1689 (CO); δ_H (300 MHz, C^2HCl_3) 1.39 (9H, s, OCOC($C\underline{H}_3$)$_3$), 2.13 (2H, t, *J* 6.0 Hz, 2-$C\underline{H}_2$), 2.63 (2H, m, 3-$C\underline{H}_2$), 2.77 (2H, t, *J* 6.0 Hz, 4-$C\underline{H}_2$), 7.18 (1H, dd, *J* 7.8, 1.0 Hz, 6-$\underline{H}$), 7.31 (1H, t, *J* 7.8 Hz, 7-$\underline{H}$), 7.94 (1H, d, *J* 7.8 Hz, 8-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3) 22.26 (s, 4-$\underline{C}H_2$), 23.15 (s, 3-$\underline{C}H_2$), 27.18 (s, OCOC($\underline{C}H_3$)$_3$), 38.59 (s, 2-$\underline{C}H_2$), 39.24 (s, OCO$\underline{C}$(CH_3)$_3$), 124.92 (s, 6-$\underline{C}$), 126.98 (s, 8-$\underline{C}$), 127.02 (s, 7-$\underline{C}$),134.22 (s, 4a-$\underline{C}$), 136.66 (s, 8a-$\underline{C}$), 148.81 (s, 5-$\underline{C}$), 176.71 (s, O$\underline{C}$OC(CH_3)$_3$), 197.68 (s, 1-$\underline{C}$O); *m/z* (EI) 246 ([*M*]$^+$, 17), 162 (45, [$HOC_{10}H_9O$]$^+$), 134 (17, [$HOC_{10}H_9O$-CO]$^+$), 85 (27, [$(CH_3)_3CO$]$^+$), 57 (100, [$(CH_3)_3C$]$^+$).

2,2-Dimethyl-propionic acid 6-bromo-5-oxo-5, 6, 7, 8-tetrahydro-naphthalen-1-yl ester (126).



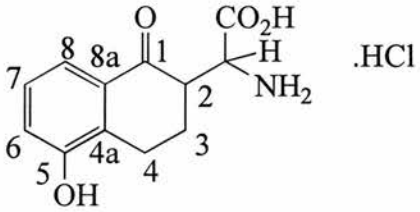
A stirred suspension of cupric bromide (8.84 g, 39.0 mmol) in ethyl acetate (45 ml) was heated under reflux. To this 2,2-dimethyl-propionic acid 5-oxo-5,6,7,8-tetrahydro-naphthalen-1-yl ester (4.87 g, 19.0 mmol) in $CHCl_3$ (15 ml) was added and the orange reaction mixture heat under reflux for three hours. The CuBr and $CuBr_2$ salts were removed by filtration and the filtrate decolourised with activated charcoal. The activated charcoal was removed by filtration through celite, the filtrate dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil (3.00 g, 49%); (Found: C, 52.19; H, 4.88 $C_{15}H_{17}BrO_3$ requires: C, 52.40; H, 5.26%); δ_H (300 MHz, C^2HCl_3) 2.49 (2H, m, 4-C$\underline{H}_2$), 2.78 (2H, m, 3-C$\underline{H}_2$), 4.70 (1H, t, $J_{2,3}$ 4.5 Hz, 2-C$\underline{H}$), 7.27 (1H, dd, *J* 7.8 1.5 Hz, 6-$\underline{H}$), 7.36 (1H, t, *J* 7.8 Hz, 7-$\underline{H}$), 7.97 (1H, dd, *J* 7.8, 1.5 Hz, 8-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3) 20.16 (s, 4-$\underline{C}H_2$), 27.16 (s, OCOC($\underline{C}H_3)_3$), 30.84 (s, 3-$\underline{C}H_2$), 39.29 (s, OCO$\underline{C}(CH_3)_3$), 49.58 (s, 2-$\underline{C}$HBr), 126.25 (s, 8-$\underline{C}$), 127.56 (s, 6-$\underline{C}$), 128.17 (s, 7-$\underline{C}$), 131.42 (s, 4a-$\underline{C}$), 135.25 (s, 8a-$\underline{C}$), 148.69 (s, 5-$\underline{C}$), 176.55 (s, O$\underline{C}$OC$(CH_3)_3$), 190.01 (s, $\underline{C}$OCH$_2$Br); *m/z* (EI) 325, 323 ($[M]^+$, 2), 240 (10, [*M*- COC$(CH_3)_3]^+$), 161 (11, $[OC_{10}H_9O]^+$), 85 (42, $[COC(CH_3)_3]^+$, 57 (100, $[(CH_3)_3C]^+$).

2-Acetylamino-2-[5-(2,2-dimethyl-propionyloxy)-1-oxo-1, 2, 3, 4-tetrahydro-naphthalen-2-yl]-malonic acid diethyl ester (128)



To a stirred solution of diethyl acetamidomalonate (5.86 g, 27.0 mmol) in dry DMF (60 ml), was added sodium hydride (60% weight in oil, 1.08 g, 27.0 mmol) at 0 °C and under an atmosphere of nitrogen. The reaction mixture was stirred for three hours before the addition of **(126)** (8.77 g, 27.00 mmol). The resulting orange solution was stirred for nineteen hours before pouring the mixture over water (600 ml), acidifying with 1M HCl, extracting into diethyl ether (5 × 150 ml), drying ($MgSO_4$) and removing the solvent under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel; ethyl acetate: petroleum ether 1:2) gave a sticky white solid (4.33 g, 35%); (Found: C, 62.62; H, 6.80; N, 2.93 $C_{24}H_{31}NO_8$ requires: C, 62.46; H, 6.76; N, 3.03%); ν_{max} (nujol)/cm^{-1} 1757 (CO, ester), 1693 (CO); δ_H (300 MHz, C^2HCl_3) 1.24 (6H, t, *J* 7.2 Hz, 2 × $OCH_2C\underline{H}_3$), 1.37 (9H,s, $OCOC(C\underline{H}_3)_3$), 1.80 (1H, m, H_b 4-$C\underline{H}_2$), 1.99 (3H, s, $NHCOC\underline{H}_3$), 2.90 (3H, m, 3-$C\underline{H}_2$, H_a 4-$C\underline{H}_2$), 3.93 (1H, dd, *J* 14.0, 3.6 Hz, 2-C<u>H</u>), 4.24 (4H, m, 2 × $OC\underline{H}_2CH_3$), 6.83 (1H, s, $N\underline{H}COCH_3$), 7.20 (1H, dd, *J* 8.0, 1.2 Hz, 6-<u>H</u>), 7.30 (1H, t, *J* 8.0 Hz, 7-<u>H</u>), 7.84 (1H, dd, *J* 8.0, 1.2 Hz, 8-<u>H</u>); δ_C (75.4 MHz, C^2HCl_3) 13.71 (s, 2 × $OCH_2\underline{C}H_3$), 23.01 (s, 4-$\underline{C}H_2$), 23.63 (s, $NHCO\underline{C}H_3$), 25.55 (s, 3'-$\underline{C}H_2$), 27.13 (s, $OCOC(\underline{C}H_3)_3$), 39.25 (s, $OCO\underline{C}(CH_3)_3$), 55.27 (s, 2-<u>C</u>H), 62.27 (s, <u>C</u>$(CO_2Et)NHAc$), 65.94 (s, 2 × $O\underline{C}H_2CH_3$), 124.98 (s, 6-<u>C</u>), 127.14 (s, 8-<u>C</u>), 127.48 (s, 7-<u>C</u>), 133.85 (s, 4a-<u>C</u>), 136.10 (s, 8a-<u>C</u>), 148.88 (s, 5-<u>C</u>), 166.15 (s, $NH\underline{C}OCH_3$), 168.50 (s, O<u>C</u>OEt), 169.87 (s, O<u>C</u>OEt), 176.18 (s, $O\underline{C}OC(CH_3)_3$), 197.25 (s, 1-<u>C</u>O); *m/z* (EI) 461 ($[M]^+$, 14), 416 (9, $[M\text{-}OCH_2CH_3]^+$), 217 (97, $[C(CO_2Et)_2NHAc]^+$), 171 (97, $[C(CO_2Et)_2NHAc\text{-}OCH_2CH_3]^+$), 91 (32, $[C_7H_7]^+$), 57 (100, $[(CH_3)_3C]^+$).

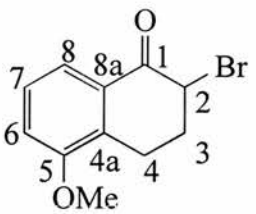
Amino-(5-hydroxy-1-oxo-1, 2, 3, 4-tetrahydro-naphthalen-2-yl)-acetic acid hydrogen chloride (5-hydroxy-1-tetralone derivative) (89)



.HCl

A suspension of **(128)** (1.12 g, 2.42 mmol) in HCl (6M, 60 ml) was heated under reflux for seventeen hours. The reaction mixture was cooled to room temperature and the white suspension which formed was filtered in air to afford an off cream solid. (0.21 g, 24%), dec. 200 °C; ν_{max} (nujol)/cm^{-1} 1730 (CO, acid), 1681 (CO); δ_H (300 MHz, ^2H_2O) 2.09-2.34 (2H, m, 4-C$\underline{H}_2$), 3.15-3.21 (2H, m, 3-C$\underline{H}_2$), 3.58-63 (1H, m, 2-C$\underline{H}$), 4.37 (1H, d, *J* 4.0 Hz, α-C$\underline{H}$), 7.15 (1H, d, *J* 8.0 Hz, 8-$\underline{H}$) 7.27 (1H, m, 6-$\underline{H}$), 7.45 (1H, t, *J* 8.0 Hz, 7-$\underline{H}$); δ_C (75.4 Hz, ^2H_2O) 22.73 (s, 4-$\underline{C}$H), 25.25 (s, 3-$\underline{C}$H), 48.38 (s, 2-$\underline{C}$H), 66.1 (s, α-$\underline{C}$H), 119.14 (s, 6-$\underline{C}$), 121.29 (s, 7-$\underline{C}$), 127.69 (s, 8-$\underline{C}$), 131.98 (s, 4a-$\underline{C}$), 132.51 (s, 8a-$\underline{C}$), 153.49 (s, 5-$\underline{C}$), 173.24 (s, O$\underline{C}$OH), 199.01 (s, 1-$\underline{C}$O); *m/z* (Maldi-TOF) 258 ([*M*+Na], 48%) (CI) 221 ([*M*+H-NH]$^+$, 8), 163 (17, [HOC$_{10}$H$_9$O+H]$^+$, 91 (58, [C$_7$H$_7$]$^+$), 46 (100, [CO$_2$H+H]$^+$).

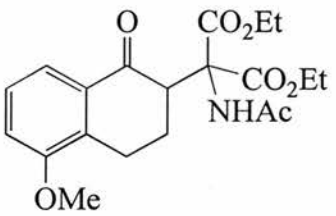
2-Bromo-5-methoxy-1-tetralone (129)



Cupric bromide (10.14 g, 45.0 mmol) was suspended in ethyl acetate (45 ml) and heated to reflux with stirring. 3-methoxy-1-tetralone (4.00 g, 22.0 mmol) in chloroform (25 ml) was then added and the reaction mixture heated under reflux for five hours. The CuBr and $CuBr_2$ residues were filtered off and the filtrate decolourised with activated charcoal. The activated charcoal was removed by filtration through celite, which was washed with ethyl acetate (4 × 25 ml). The solvent was removed under reduced pressure to afford a yellow

oil (5.61 g, 98%), (Found; C, 51.84; H, 4.34 $C_{11}H_{11}BrO_2$ requires; C, 51.78; H, 4.34%); ν_{max} (nujol)/cm^{-1} 3346 (NH), 1750 (CO, ester), 1721 (CO, amide), 1670 (CO); δ_H (300 MHz, C^2HCl_3) 2.45 (2H, dd, *J* 6.0, 2.1 Hz, 4-C$\underline{H}_2$), 3.00 (2H, t, *J* 6.0 Hz, 3-C$\underline{H}_2$), 4.69 (1H, t, *J* 2.1 Hz, 2-C$\underline{H}$Br), 7.04 (1H, dd, *J* 8.1, 1.2 Hz, 6-$\underline{H}$), 7.28 (1H, dd, *J* 8.1, 7.8 Hz, 7-$\underline{H}$), 7.68 (1H, dd, *J* 8.1, 1.2 Hz, 8-$\underline{H}$); δ_C (75.4 Hz, C^2HCl_3) 19.84 (s, 4-$\underline{C}H_2$), 31.09 (s, 3-$\underline{C}H_2$), 50.48 (s, 2-$\underline{C}$HBr), 55.99 (s, OCH_3), 115.14 (s, 8-$\underline{C}$), 120.15 (s, 6-$\underline{C}$), 127.31 (7-$\underline{C}$), 130.92 (s, 4a-$\underline{C}$), 132.18 (s, 8a-$\underline{C}$), 156.80 (s, 5-$\underline{C}$), 191.05 (s, $\underline{C}OCH_2Br$); *m/z* (EI) 256, 254 ([*M*]$^+$, 88, 90), 175 (45, [*M*-Br]$^+$), 148 (100, $[CH_3OC_6H_3CH_2CO]^+$), 120 (45, $[CH_3OC_6H_5CH_2]^+$), 77 (30, $[C_6H_5]^+$).

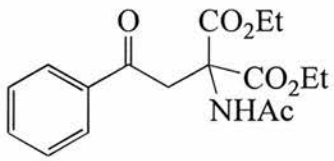
2-Acetylamino-2-[5-methyoxy-1-oxo-1, 2, 3, 4-tetrahydro-naphthale-2-yl]-malonic acid diethyl ester (130)



To a stirred solution of diethyl acetamidomalonate (4.77 g, 22.0 mmol) in dry DMF (60 ml), was added sodium hydride (60% weight in oil, 0.88 g, 22.0 mmol) at 0 °C and under an atmosphere of nitrogen. The reaction mixture was stirred for three hours before adding 2-bromo-5-methoxy-1-tetralone **(129)** (3.87 g, 22.0 mmol). The resulting orange solution was stirred overnight before pouring the mixture over water (600 ml), acidifying with 1M HCl, extracting into diethyl ether (5 × 150 ml), drying ($MgSO_4$) and removing the solvent under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel; ethyl acetate: petroleum ether 1:2) to afford a brown solid (1.00 g, 13%), m.p. 116-120 °C; (Found; C, 61.69; H, 6.44; N, 3.54 $C_{20}H_{25}NO_7$ requires: C, 61.53; 6.19; N, 3.58%); ν_{max} (nujol)/cm^{-1} 3340 (NH), 1752 (CO, ester), 1721 (CO, amide), 1664 (CO); δ_H (300MHz, C^2HCl_3) 1.21 (6H, m, 2 × $OCH_2C\underline{H}_3$), 1.77 (1H, m, H_a 3-C$\underline{H}_2$), 1.98 (3H, s, $NHCOC\underline{H}_3$), 2.75 (2H, m, H_a,H_b 4-C$\underline{H}_2$) 3.13 (1H, m, H_b 3-C$\underline{H}_2$), 3.89 (1H, dd, *J* 14.0, 3.6 Hz, 1-C$\underline{H}$), 4.23 (4H, m, 2 × $OC\underline{H}_2CH_3$), 6.84 (1H, m, N$\underline{H}$COCH$_3$), 6.99 (1H, dd, J 8.0, 1.0 Hz, 6-$\underline{H}$), 7.23 (1H, t, J 8.0 Hz, 7-$\underline{H}$), 7.24 (1H, dd,

8.0, 1.0 Hz 8-H); δ_C (75.4 MHz, C^2HCl_3) 13.76 (s, 4-CH_2), 13.87 (s, 2 × OCH_2CH_3), 23.05 (s, 3-CH_2), 23.31 ($NHCOCH_3$), 25.71 (s, 2-CH), 55.50 (s, OCH_3), 62.63 (s, 2 × OCH_2CH_3), 66.06 (s, $C(CO_2Et)_2NHAc$), 114.72 (s, 8-C), 118.80 (s, 6-C), 127.01 (s, 7-C), 133.68 (s, 4a-C), 137.86 (s, 8a-C), 157.04 (s, 5-C), 166.08 (s, $NHCOCH_3$), 168.65 (s, $OCOCH_2CH_3$), 19.77 (s, $OCOCH_2CH_3$), 176.72, 198.32 (s, 1-CO); *m/z* (EI) 391 ($[M]^+$, 14), 346 (9, $[M\text{-}OCH_2CH_3]^+$), 276 (42, $[CH_3OC_{10}H_8OC(CO_2Et)NH_2]^+$), 217 (72, $[C(OHOEt)(CO_2Et)NHAc]^+$, 175 (100, $[CH_3OC_{10}H_8O]^+$, 91 (7, $[C_7H_7]^+$).

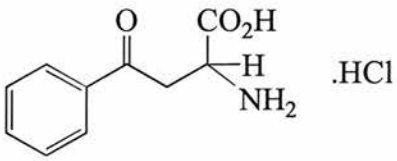
2-Acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester (134)



Diethyl acetamidomalonate (12.24 g, 56.35 mmol) in dry redistilled DMF (75 ml) was added to sodium hydride (60% weight in oil, 2.25 g, 56.35 mmol) at 0 °C under an atmosphere of nitrogen. The mixture was stirred for 3 hours before adding a solution of α-bromoacetophenone (10.00 g, 50.25 mmol) in DMF (25 ml). The dark orange solution was stirred for a further five hours before pouring the mixture onto distilled water (750 ml), acidifying with 1M HCl, extracting into diethyl ether (5 x 150 ml), washing with brine (250 ml) and drying over magnesium sulfate. The solvent was removed under reduced pressure and the residue recrystallised from diethyl ether to afford a white crystalline solid (11 g, 65%), m.p. 106-109 °C (lit.[193] 118-119 °C); ν_{max} (nujol)/cm^{-1} 3272 (NH), 1740 (CO, ester), 1687 (CO), 1643 (CO, amide); δ_H (300 MHz, C^2HCl_3) 1.24 (6H, t, *J* 7.5 Hz, 2 × OCH_2CH_3), 1.97 (3H, s, $NHCOCH_3$), 4.26 (6H, m, 2 × OCH_2CH_3, β-CH_2), 7.10 (1H, s, $NHCOCH_3$), 7.48 (2H, m, 3'-H, 5'-H), 7.6 (1H, t, *J* 7.5 Hz, 4'-H), 7.9 (2H, d, *J* 7.5 Hz, 2'-H, 6'-H); δ_C (75.4 MHz, C^2HCl_3) 13.76 (s, OCH_2CH_3), 22.49 (s, $NHCOCH_3$), 42.19 (s, β-CH_2), 62.85 (s, $CO_2CH_2CH_3$), 63.97 (s, α-C), 128.29 (s, 3'-C, 5'-C), 128.78 (s, 2'-C, 6'-C), 133.82 (s, 4'-C), 136.18 (s, 1'-C), 167.44 (s, 2 × $CO_2CH_2CH_3$), 169.61 (s,

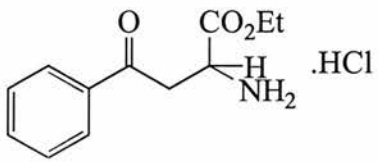
$\underline{C}OCH_3$), 197.13 (s, $\underline{C}$=O); *m/z* (EI) 335 ($[M]^+$, 19%), 290 (7, $[M\text{-}OCH_2CH_3]^+$), 262 (54, $[M\text{-}CO_2Et]^+$), 105 (100, $[PhCO]^+$), 77 (38, $[C_6H_5]^+$).

2-Amino-4-oxo-4-phenyl-butyric acid hydrogen chloride (desaminokynurenine) (15)



A suspension of **(134)** (9.46 g, 28.21 mmol) in 6M HCl (100 ml) was heated under reflux for six hours. Upon cooling the mixture was washed with ethyl acetate (3 × 50 ml) and the solvent removed under reduced pressure. The off white residue was then washed again diethyl ether to afford a white crystalline solid (5.31 g, 82%), dec. 183 °C (lit[194] 190-200 °C); (Found; C, 52.30; H, 5.34; N, 6.15. $C_{10}H_{12}ClNO_3$ requires C, 52.30; H, 5.27; N, 6.09%); ν_{max} (nujol)/cm^{-1} 1730 (CO, acid), 1681 (CO); δ_H (200 MHz, ^2H_2O), 3.78 (2H, d, *J* 5 Hz, 3-C$\underline{H}_2$), 4.42 (1H, t, $J_{2,3}$ 5 Hz, 2-C$\underline{H}$), 7.44 (2H, t, $J_{3,2}$ 8 Hz 3'-$\underline{H}$, 5'-$\underline{H}$), 7.60 (1H, t, *J* 8 Hz, 4'-$\underline{H}$), 7.87 (3H, d, *J* 8 Hz, 2'-$\underline{H}$, 6'-$\underline{H}$); δ_C (50.31 MHz, ^2H_2O) 41.00 (s, 3-$\underline{C}H_2$), 51.66 (s, 2-$\underline{C}$H), 131.20 (s, 3'-$\underline{C}$, 5'-$\underline{C}$), 131.72 (S, 2'-$\underline{C}$, 6'-$\underline{C}$), 137.62 (s, 1'-$\underline{C}$, 4'-$\underline{C}$), 174.44 (s, $\underline{C}O_2H$), 202.00 (s, $\underline{C}$=O). *m/z* (CI) 194 ([*M*+H], 67), 148 (7, $[M\text{-}CO_2H]$), 43 (100, $[C_2H_5N]$).

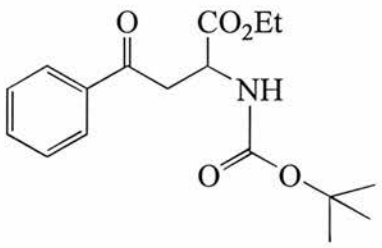
2-Amino-4-oxo-4-phenyl-butyric acid ethyl ester hydrochloride (135)



To a solution of 2-amino-4-oxo-4-phenylbutyric acid (4.01 g, 21.39 mmol) in dry redistilled ethanol (70 ml), thionyl chloride (1.39 ml, 19.22 mmol) was added at 0 °C and under nitrogen. The reaction mixture was allowed to reach room temperature then heated under reflux for two hours. The reaction mixture was cooled to room temperature and the

solvent removed at reduced pressure. Recrystallisation from ethanol afforded a white crystalline solid (3.91g, 86%), m.p. 148-152 °C; ν_{max} (nujol)/cm^{-1} 1746 (CO,ester), 1672 (CO); δ_H (300 MHz, ^2H_2O), 1.28 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 3.83 (2H, dd, *J* 5.0, 5.0 Hz, β-$C\underline{H}_2$), 4.19 (2H, q, *J* 7.0 Hz $OC\underline{H}_2CH_3$), 4.48 (1H, t, *J* 5.0 Hz, α-C<u>H</u>), 7.47 (2H, t, *J* 8.0 Hz, 3'-<u>H</u>, 5'-<u>H</u>), 7.66 (1H, t, *J* 8.0 Hz 4'-<u>H</u>), 7.91 (2H, d, *J* 8.0 Hz, 2'-<u>H</u>, 6'-<u>H</u>); δ_C (75.4 MHz, ^2H_2O) 13.00 (s, $OCH_2\underline{C}H_3$), 38.04 (S, β-$\underline{C}H_2$), 48.63 (s, α-$\underline{C}$H), 63.76 (s, $O\underline{C}H_2CH_3$), 128.44 (s, 3'-<u>C</u>, 5'-<u>C</u>), 129.13 (s, 2'-<u>C</u>, 6'-<u>C</u>), 135.01 (s, 1'-<u>C</u>, 4'-<u>C</u>), 169.82 (s, $\underline{C}O_2CH_2CH_3$), 199.37 (s, <u>C</u>=O); *m/z* (EI) 175 ([$PhCOCH_2CHNH_2CO$]$^+$, 9%), 148 (67, [$PhCOCH_2CHNH_2$]$^+$), 105 (100, [PhCO]$^+$), 77 (32, [C_6H_5]$^+$).

Ethyl *N*- (*tert*-Butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (132)



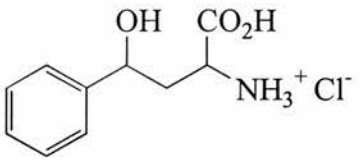
To a solution of **(135)** (1.22 g, 4.73 mmol) in dry THF (35 ml), di-*tert*-butylpyrocarbonate (0.63 g, 2.86 mmol) and dry redistilled triethylamine (0.87 ml, 6.28 mmol) was added at 0 °C under nitrogen. The reaction mixture was allowed to reach room temperature, left to stir for twenty-four hours then heated under reflux for one hour. The solvent was removed under reduced pressure to afford a yellow residue. This was partitioned between diethyl ether (150 ml) and water (150 ml). The aqueous layer was washed further with diethyl ether (2 × 100 ml). The organic layers were added together and then washed successively with 3% HCl, 5% $NaHCO_3$ and brine (150 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a pale yellow oil. Subsequent purification was carried out by column chromatography (silica, ethyl acetate: petroleum ether, 1:3) afforded the product as a white solid (0.56 g, 37%), m.p. 46-48 °C; (Found: C, 63.54; H, 7.33; N, 4.32 $C_{17}H_{23}NO_5$ requires C, 63.54; H, 7.21; N, 4.36%); ν_{max} (nujol)/cm^{-1} 3401 (NH), 1741 (CO, ester), 1695 (CO), 1678 (CO, urethane); δ_H (300 MHz, C^2HCl_3) 1.24 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 1.40 (9H, s, $OC(C\underline{H}_3)_3$), 3.52 (1H, dd, *J* 15.0, 4.2 Hz, β-$C\underline{H}_2$ H_a), 3.70 (1H,

dd, *J* 15.0, 4.2 Hz, β-C$\underline{H}_2$ H_b), 4.20 (2H, q, *J* 7.0 Hz OC$\underline{H}_2$CH$_3$), 4.65 (1H, t, *J* 4.2 Hz, α-C$\underline{H}$), 5.61 (1H, bm, N$\underline{H}$CO$_2$C(CH$_3$)$_3$), 7.47 (2H,t, *J* 8.0 Hz, 3'-$\underline{H}$, 5'-$\underline{H}$), 7.59 (1H, t, *J* 8.0 Hz, 4'-$\underline{H}$), 7.93 (2H, d, *J* 8.0 Hz, 2'-$\underline{H}$, 6'-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3), 14.00 (s, OCH$_2\underline{C}$H$_3$), 28.27 (s, OCOC($\underline{C}$H$_3$)$_3$), 40.87 (s, β-$\underline{C}$H$_2$), 49. 72 (s, α-$\underline{C}$H), 61.64 (s, O$\underline{C}$H$_2$CH$_3$), 79.94 (s, O$\underline{C}$(CH$_3$)$_3$, 128.25 (s, 3'-$\underline{C}$, 5'-$\underline{C}$), 128.83 (s, 2'-$\underline{C}$, 6'-$\underline{C}$), 133.79 (s, 4'-$\underline{C}$), 136.33 (s, 1'-$\underline{C}$), 155.80 (s, NH$\underline{C}$O$_2$C(CH$_3$)$_3$), 171.65 (s, $\underline{C}$O$_2$CH$_2$CH$_3$), 198.04 (s, $\underline{C}$=O); *m/z* (CI) 322 ([MH]$^+$, 88%), 266 (100, [MH-C(CH$_3$)$_2$CH$_2$]$^+$), 248 (29, [MH-CO$_2$Et]$^+$), 222 (76, [MH-OCOC(CH$_3$)$_3$]$^+$, 105 (17, [PhCO]$^+$), 57 (37, [C(CH$_3$)$_3$]$^+$).

Attempted protection of 2-amino-4-oxo-4-phenyl-butyric acid hydrochloride (15) with di-*tert*-butylpyrocarbonate and subsequent esterification using thionyl chloride

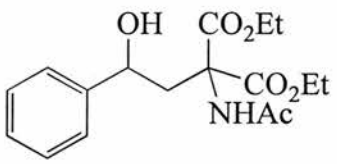
To di-*tert*-butylpyrocarbonate (2.01 g, 9.2 mmol) in THF: H_2O (1:1) (50 ml) was added sodium hydrogen carbonate (2.36 g, 22.3 mmol) and 2-amino-4-oxo-4-phenyl butyric acid hydrochloride (1.277 g, 5.6 mmol). The white suspension was allowed to stir at room temperature for twenty-four hours. The reaction mixture was then acidified with dilute HCl and the solvent removed under reduced pressure to afford a white residue. Analysis of the crude compound showed some reaction had occurred. δ_H (300 MHz ^2H_2O) 1.15 (9H, s, OC(C$\underline{H}_3$)$_3$), 3.40 (2H, d, *J* 9.0 Hz, β-C$\underline{H}_2$), 4.25 (1H, t, *J* 4.2 Hz, α-C$\underline{H}$), 7.40 (2H, t, *J* 8.0 Hz, 3'-$\underline{H}$, 5'-$\underline{H}$), 7.60 (1H,t, *J* 8.0 Hz, 4'-$\underline{H}$), 7.85 (2H, d, 8 Hz, 2'-$\underline{H}$, 6'-$\underline{H}$). To a stirred suspension of crude *N*-(*tert*-butoxycarbonyl)-4-oxo-4-phenyl butyric acid (1.63 g, 5.58 mmol) in dry ethanol (60 ml) thionyl chloride (0.44 g, 6 mmol) was added and the reaction mixture heated under reflux for 1 hour. The solvent was removed under reduced pressure to afford an off white residue. This was washed with ethyl acetate (2 × 50 ml) to afford a white residue. (2.03 g, crude) Analysis by NMR spectroscopy showed it to be 2-amino-4-oxo-4-phenyl butyric acid; δ_H (200 MHz, ^2H_2O), 3.82 (2H, m, β-C$\underline{H}_2$), 4.40 (1H, t, *J* 5.3 Hz, α-C$\underline{H}$), 7.44 (2H, t, *J* 7.0 Hz 3'-$\underline{H}$, 5'-$\underline{H}$), 7.60 (1H, t, *J* 7.0 Hz, 4'-$\underline{H}$), 7.87 (3H, d, *J* 7.0 Hz, 2'-$\underline{H}$, 6'-$\underline{H}$); δ_C (50.31 MHz, ^2H_2O) 40.88 (s, β-$\underline{C}$H$_2$), 51.69 (s, α-$\underline{C}$H), 131.07 (s, 3'-$\underline{C}$, 5'-$\underline{C}$), 131.41 (S, 2'-$\underline{C}$, 6'-$\underline{C}$), 137.54 (s, 1'-$\underline{C}$, 4'-$\underline{C}$), 174.53 (s, $\underline{C}$O$_2$H), 201.99 (s, $\underline{C}$=O).

Attempted reduction of 2-amino-4-oxo-4-phenyl-butyric acid hydrogen chloride (15) using sodium borohydride



To a stirred solution of desaminokynurenine hydrogen chloride (0.25 g, 1.07 mmol) in H_2O (45 ml), sodium borohydride (0.10 g, 2.67 mmol) was added slowly over 30 minutes and the reaction mixture allowed to stir overnight. The reaction was quenched upon addition of 3% HCl and the solvent removed under reduced pressure to afford a white solid. The NMR spectroscopic data were inconclusive.

Attempted reduction of 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester (134) using sodium borohydride



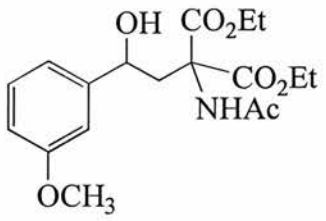
To a stirred solution of 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester (0.31 g, 0.94 mmol) in dry redistilled MeOH (30 ml), sodium borohydride (0.4 g, 1.03 mmol) was added at 0 °C and under nitrogen. The reaction mixture was allowed to stir overnight at room temperature. The reaction was then quenched with 3% HCl and the product was extracted into diethyl ether (3 × 50 ml) dried ($MgSO_4$) and the solvent removed to afford a white crystalline product. This was shown by tlc and NMR spectroscopy to be starting material.

Procedure 2

To 2-acetylamino-2-(2-oxo-2-phenyl-ethyl)-malonic acid diethyl ester (0.36 g, 1.07 mmol) and sodium borohydride (0.01 g, 0.29 mmol) was added dry THF (25 ml). The reaction

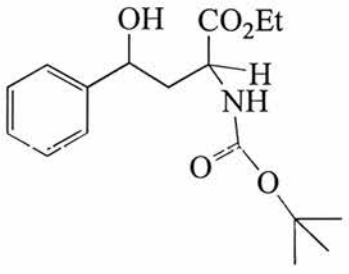
mixture was set up to reflux for three hours under an atmosphere of nitrogen. The reaction was quenched with 1.0 M HCl and the solvent removed under reduced pressure to afford a yellow residue. This was shown by tlc and NMR spectroscopy to be starting material.

Attempted reduction of 2-acetylamino-2-[2-(3-methoxy-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester (123)



To 2-acetylamino-2-[2-(3-methoxy-phenyl)-2-oxo-ethyl]-malonic acid diethyl ester (0.54 g, 1.48 mmol), sodium borohydride (0.02 g, 0.40 mmol) was added dry THF (35 ml). The reaction mixture was heated under reflux in an atmosphere of nitrogen for three hours. The reaction was quenched with 1.0 M HCl and the solvent removed under reduced pressure to afford a yellow residue. This was shown by tlc and NMR spectroscopy to be starting material.

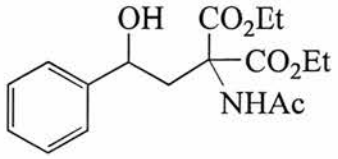
Attempted reduction of ethyl *N*- (*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (132) with sodium borohydride



To ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (0.32 g, 1.0 mmol) and sodium borohydride (0.04 g, 1.0 mmol) was added dry THF (40 ml). The reaction mixture was heated under reflux for six hours in an atmosphere of nitrogen. The reaction

was quenched by pouring the cooled reaction mixture onto 1.0 M NH_4Cl (20 ml) on crushed ice. Extraction into diethyl ether (3× 50 ml), drying ($MgSO_4$) and removal of solvent under reduced volume afforded a yellow oil. (0.14 g) which appeared to be a mixture of diastereomers (diast.); δ_H (300 MHz, C^2HCl_3) 1.43 (9H, s, $OC(C\underline{H}_3)_3$, diast. 1), 1.45 (9H, s, $OC(C\underline{H}_3)_3$, diast. 2), 3.62 (2H, d, *J* 6.6 Hz, β-$C\underline{H}_2$), 3.71 (1H, m, γ-C<u>H</u>), 4.74 (1H, dd, *J* 4.5, 4.2 Hz, α-C<u>H</u>, diast. 1), 4.80 (1H, dd, *J* 4.5, 4.2 Hz, α-C<u>H</u>, diast. 2), 5.17 (1H, bm, $N\underline{H}CO_2C(CH_3)_3$, diast. 1), 5.24 (1H,bm, $N\underline{H}CO_2C(CH_3)_3$, diast. 2), 7.32 (5H, s, 2',3',4',5',6'-H, diast. 1), 7.34 (5H, s, 2',3',4',5',6'-H, diast. 2); δ_C (75.4 MHz, C^2HCl_3) 28.33 (s, $OC(\underline{C}H_3)_3$), 41.14, (s, β-$\underline{C}H_2$), 42.20 (s, β-$\underline{C}H_2$), 49.95 (s, α-C<u>H</u>), 50.71 (s, α-<u>C</u>H), 70.53 (s, γ-<u>C</u>), 71.59 (s, γ-<u>C</u>), 79.89 (s, $O\underline{C}(CH_3)_3$), 80.23 (s, $O\underline{C}(CH_3)_3$), 125.69, 125.79 (s, 3'-<u>C</u>, 5'-<u>C</u>, diast. 1 & 2), 127.41, 127.78 (s, 2'-<u>C</u>, 6'-<u>C</u>, diast. 1 & 2), 128.53, 128.68 (s, 4'-<u>C</u>, diast. 1 & 2), 144.29, 144.45 (s, 1'-<u>C</u>, diast. 1 & 2); *m/z* (CI) 282, 250, 226, 208, 104, 60.

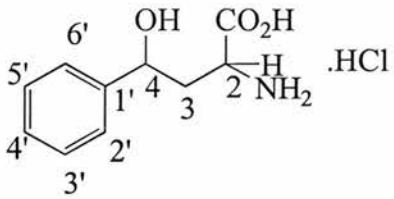
2-Acetylamino-2-[2-hydroxy2-phenyl-ethyl)]malonic acid diethyl ester (138)



To a stirred suspension of palladium catalyst (5% on carbon, 0.30 g) in dry ethyl acetate (10 ml) was added a solution of **(134)** (1.22 g, 3.65 mmol) in dry ethyl acetate (25 ml). After degassing of the solution the reaction mixture was stirred at room temperature under an atmosphere of hydrogen for seven hours. The palladium catalyst was removed by filtration through celite which was washed with ethyl acetate (3 × 50 ml). The filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a white solid (1.61 g, 94%), 96-98 °C; (Found: C, 60.50; H, 6.93; N, 4.14 $C_{17}H_{23}NO_6$ requires C, 60.52; H, 6.87; N, 4.15%); ν_{max} (nujol)/cm^{-1} 3460 (γ-OH), 3380 (NH), 1737 (CO, ester), 1660 (CO, amide); δ_H (300 MHz, C^2HCl_3) 1.21 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 1.28 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 2.72 (1H, dd, *J* 15.0, 10.0 Hz, H_a β-$C\underline{H}_2$), 2.81 (1H, dd, *J* 10.0, 3.3 Hz, H_b β-$C\underline{H}_2$), 4.17 (2H, q, *J* 7.0 Hz, $OC\underline{H}_2CH_3$), 4.26 (2H, q, *J* 7.0 Hz, $OC\underline{H}_2CH_3$), 4.73 (1H,

dd, *J* 15.0, 3.3 Hz, C<u>H</u>OH), 7.03 (1H, s, N<u>H</u>COCH$_3$), 7.30 (5H, s, aromatic-<u>H</u>); δ_C (75.4 MHz, C^{2}HCl$_3$) 13.85 (s, 2 × OCH$_2$<u>C</u>H$_3$) 23.05 (s, NHCO<u>C</u>H$_3$), 41.14 (s, β-<u>C</u>H$_2$), 62.32 (s, 1 × O<u>C</u>H$_2$CH$_3$), 62.82 (s, 1 × O<u>C</u>H$_2$CH$_3$), 65.04 (s, <u>C</u>(CO$_2$Et)$_2$NHAc), 70.70 (s, <u>C</u>HOH), 126.02 (s, 2', 6'-<u>C</u>), 127.75 (s, 3', 5'-<u>C</u>), 128.78 (s, 4'-<u>C</u>), 144.00 (s, 1'-<u>C</u>), 167.97 (s, NH<u>C</u>OCH$_3$), 169.18 (s, O<u>C</u>OCH$_2$CH$_3$), 169.65 (s, O<u>C</u>OCH$_2$CH$_3$); *m/z* (EI) 291 ([*M*-HOCH$_2$CH$_3$]$^+$), 217 (32, [C(COHOCH$_2$CH$_3$)(CO$_2$Et)NHAc]$^+$), 204 (76, [C$_6$H$_5$CHOHCH$_2$C(CO$_2$Et)-H$_2$]$^+$), 105 (63, [PhCO]$^+$), 91 (20, [C$_7$H$_7$]$^+$), 77 (29, [C$_6$H$_5$]$^+$), 43 (100, [C$_2$H$_3$N]$^+$).

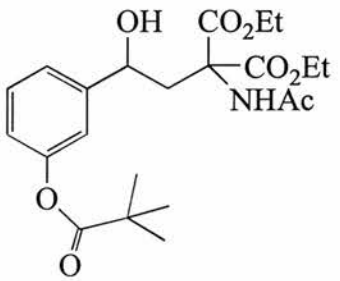
2-Amino-4-hydroxy-4-phenyl-butyric acid hydrochloride (γ-hydroxy desminokynurenine) (33)



A suspension of **(138)** (1.04 g, 3.09 mmol) in 6M HCl (60 ml) was heated under reflux for eight hours. Upon cooling the mixture was washed with ethyl acetate (3 × 50 ml) and the solvent removed under reduced pressure afford a white crystalline solid (0.37 g, 52%), **(33)** was obtained as a mixture of diastereomers, dec 210 °C; ν_{max} (nujol)/cm^{-1} 1735 (CO, acid), 1678 (CO); δH (300 MHz, ^{2}H$_2$O), (4*S*,2*S*),(4*R*,2*R*) pair of enantiomers; 2.50 (1H, m, $J_{3a,3b}$12.6 $J_{3a,2}$ 12.0 Hz, 3a-<u>H</u>), 3.05 (1H, m, $J_{3b,3a}$ 12.6, $J_{3b,2}$ 8.7, $J_{3b,4}$ 5.4 Hz, 3b-<u>H</u>), 4.69 (1H, dd, $J_{2,3a}$ 12.0, $J_{2,3b}$ 8.7 Hz, 2-C<u>H</u>), 5.72 (1H, dd, $J_{4,3a}$, $J_{4,3b}$ 5.4 Hz, 4-C<u>H</u>OH), 7.50 (5H, s, aromatic-<u>H</u>), (4*R*,2*S*), (4*S*,2*R*) pair of enantiomers; 2.93 (2H, m, 3-C<u>H</u>$_2$), 4.55 (1H, m, 2-C<u>H</u>), 6.00 (1H, m, 4-C<u>H</u>OH); δ_C (75.4 MHz, ^{2}H$_2$O) 35.02 (s, 3-<u>C</u>H$_2$), 50.03 (s, 2-<u>C</u>H), 80.47 (s, 4-<u>C</u>HOH), 125.36 (s, 4'-<u>C</u>), 126.52 (s, 2', 6'-<u>C</u>) 129.23 (s, 3', 5'-<u>C</u>), 136.66 (s, 1'-<u>C</u>), 173.64 (s, O<u>C</u>OH); *m/z* (CI) 178 (34, [*M*+H-H$_2$O]$^+$), 133 (100, [C$_6$H$_5$CHCH$_2$NH$_2$ - CO$_2$H]$^+$, 105 (9, [PhCO]$^+$), 74 (56, [CHNHCOHOH]$^+$). Analytical HPLC of **(33)** (C$_{18}$ reverse phase silica column, 3μ, flow rate 4 ml/min, 5% MeOH, 1% AcOH) afforded a

chromatogram showing two peaks indicating the presence of diastereomers, (4*S*,2*S*), (4*R*,4*R*) and (4*R*,2*S*), (4*S*,2*R*), in a ratio of 2:1.

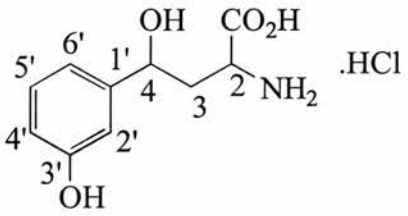
2-Acetylamino-2-{3-[3-(2,2-dimethyl-propionyloxy)-phenyl]-2-hydroxy-ethyl}-malonic acid diethyl ester (139)



To a stirred suspension of palladium catalyst (5% on carbon, 0.45 g) in dry ethyl acetate (20 ml) was added a solution of 2-acetylamino-2-{2-[3-(2,2-dimethyl-propionyloxy)-phenyl]-2-oxo-ethyl}-malonic acid diethyl ester (2.38 g, 5.47 mmol) in dry ethyl acetate (15 ml). After degassing of the solution the reaction mixture was stirred under an atmosphere of hydrogen for twenty-four hours. The palladium catalyst was removed by filtration through celite and washed with ethyl acetate (3 × 50 ml), the filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a white precipitate (1.59 g, 66%), 94-96 °C; (Found: C, 60.07; H, 7.23; N, 3.21 $C_{22}H_{31}NO_8$ requires: C, 60.40; H, 7.41; N, 3.20%); ν_{max} (nujol)/cm^{-1} 3452 (γ-OH), 3383 (NH), 1742 (CO, ester), 1668 (CO); δ_H (300 MHz, C^2HCl_3) 1.25 (6H, m, 2 × $OCH_2C\underline{H}_3$), 1.33 (9H, s, $OCOC(C\underline{H}_3)_3$), 2.01 (3H, s, $NHCOC\underline{H}_3$), 2.67 (1H, dd, *J* 15.0, 10.0 Hz, H_a β-$C\underline{H}_2$), 2.82 (1H, dd, *J* 15.0, 3 Hz, H_b β-$C\underline{H}_2$), 4.16 (2H, m, 2 × $OC\underline{H}_2CH_3$), 4.73 (1H, dd, *J* 10.0, 3 Hz, γ-C<u>H</u>OH), 6.92 (1H, m, 4'-<u>H</u>), 7.02 (2H, bs, N<u>H</u>$COCH_3$, 2'-<u>H</u>), 7.14 (1H, d, *J* 7.8 Hz, 6'-<u>H</u>), 7.31 (1H, t *J* 7.5 Hz, 5'-<u>H</u>), δ_C (75.4 MHz, C^2HCl_3) 13.87 (s, 2 × $OCH_2\underline{C}H_3$), 22.87 (s, $NHCO\underline{C}H_3$), 27.08 (s, $OCOC(\underline{C}H_3)_3$), 41.11 (s, β-$\underline{C}H_2$), 62.79 (s, $CH_2\underline{C}NHAc(CO_2Et)_2$), 64.97 (s, 2 × $O\underline{C}H_2CH_3$), 70.00 (s, γ-<u>C</u>HOH), 118.97 (s, 2'-<u>C</u>), 120.80 (s, 4'-<u>C</u>), 123.08 (s, 6'-<u>C</u>), 129.50 (S, 5'-<u>C</u>), 145.90 (s, 1'-<u>C</u>), 151.36 (s, 3'-<u>C</u>), 167.87 (s, NH<u>C</u>OCH_3), 169.04 (s, 1 × $O\underline{C}OCH_2CH_3$), 169.74 (s, $O\underline{C}OCH_2CH_3$), 177.33 (s, NH<u>C</u>OCH_3); *m/z* (EI) 419 (11,

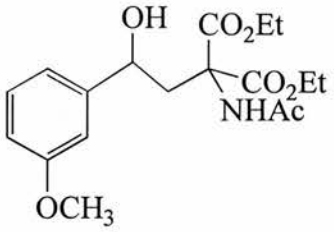
[M-H_2O]$^+$), 391 (8, [*M*-C_2H_4]$^+$, 217 (71, [$OC_6H_4COCNHAc(CO_2Et)_2$]$^+$), 85 (13, [$(CH_3)_3CO$]$^+$), 57 (100, [$(CH_3)_3C$]$^+$).

2-Amino-4-hydroxy-4-(3-hydroxy-phenyl)-butyric acid hydrogen chloride (dihydroxydesaminokynurenine) (90)



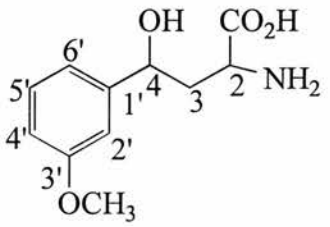
A stirred solution of **(139)** (4.45 g, 10.10 mmol) in HCl (6M, 60 ml) was heated under reflux for five hours. The reaction mixture was cooled to room temperature washed with ethyl acetate (3 × 50 ml) and the solvent removed under reduced pressure to afford an off-white precipitate. This was recrystallised from ethanol to afford a white precipitate (2.10 g, 98% crude yield), a single diastereomer obtained as a racemic mixture (4*S*,2*S*) and (4*R*,2*R*), dec. 220 °C; ν_{max} (nujol)/cm^{-1} 1790 (CO, acid); δ_H (300 MHz, ^2H_2O) 2.31 (1H, m, $J_{3a,3b}$ 12.7, $J_{2,3a}$ 12.0 and $J_{3a,4}$ 10.7 Hz, 3a-H), 3.06 (1H, ddd, $J_{3a,3b}$ 12.7, $J_{2,3b}$ 8.2 and $J_{3b,4}$ 4.9 Hz, 3b-H), 4.57 (1H, dd, $J_{2,3a}$ 12.0, $J_{2,3b}$ 8.2 Hz, 2-CH), 5.54 (1H, dd, $J_{3a,4}$ 10.7, $J_{3b,4}$ 5.4 Hz, 4-CHOH), 6.83 (1H, dd, $J_{2',4'}$ 2.0, $J_{4',5'}$ 7.0 Hz, 4'-H), 6.85 (1H, d, $J_{2',4'}$ 2.0 Hz, 2'-H), 6.93 (1H, d, $J_{5',6'}$ 7.0 Hz, 6'-H), 7.29 (1H, t, $J_{5',6'}$ 7.0 Hz, 5'-H); δ_C (75.4 MHz, ^2H_2O) 34.97 (s, 3-CH_2), 49.92 (s,2-CH), 80.03 (s, 4-CHOH), 113.1 (s, 2'-C), 116.7 (s, 4'-C), 118.5 (s, 6'-C), 130.7 (s, 5'-C), 138.6 (s, 1'-C), 156.1 (s, 3'-C), 173.6 (s, OCOH) *m/z* (CI) 194 ([*M*+H-H_2O]$^+$, 100), 149 (33, [$HOC_6H_4CHCHNH_2$]$^+$), 121 (8, [HOC_6H_4CO]$^+$), 116 (20, [$(CO_2H)NHCHCO$]$^+$), 74 (61, [$(CO_2H)NHCHCH_2$]$^+$).

2-Acetylamino-2-[2-hydroxy-2-(3-methoxy-phenyl)-ethyl]-malonic acid diethyl ester (140)



To a stirred suspension of palladium catalyst (5% on carbon, 0.13 g) in dry ethyl acetate (10 ml) was added a solution of **(123)** (0.57 g, 1.56 mmol) in dry ethyl acetate (25 ml). After degassing of the solution the reaction mixture was stirred at room temperature under an atmosphere of hydrogen for twenty-four hours. The palladium catalyst was removed by filtration through celite which was washed with ethyl acetate (3 × 50 ml). The filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a colourless oil (0.54 g, 94%); ν_{max} (nujol)/cm^{-1} 3331 (γ-OH), 1737 (CO, ester), 1651 (CO, amide); δ_H (300 MHz, C^2HCl_3) 1.21 (3H, t, *J* 7.2 Hz, $OCH_2C\underline{H}_3$), 1.27 (3H, t, *J* 7.2 Hz, $OCH_2C\underline{H}_3$), 2.00 (3H,s, $NHCOC\underline{H}_3$), 2.68 (1H, dd, *J* 15.0, 10.0 Hz, H_a β-$C\underline{H}_2$), 2.80 (1H, dd, *J* 15.0, 3.3 Hz, H_b β-$C\underline{H}_2$), 3.77 (3H, s, $OC\underline{H}_3$), 4.16 (2H, q, *J* 7.2 Hz, $O\underline{C}H_2CH_3$), 4.26 (2H, q, *J* 7.2 Hz, $O\underline{C}H_2CH_3$), 4.67 (1H, dd, *J* 10.0, 3.3 Hz, γ-$\underline{C}$HOH), 6.77 (1H, ddd, *J* 8.0, 2.4, 1.2 Hz, 4'-$\underline{H}$), 6.78 (2H, m, $N\underline{H}COCH_3$, 6'-$\underline{H}$), 7.06 (1H, s, 2'-$\underline{H}$), 7.22 (1H, t, *J* 8.0 Hz, 5'-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3) 13.80 (s, 2 × $OCH_2\underline{C}H_3$), 22.91 (s, $NHCO\underline{C}H_3$), 41.17 (s, β-$\underline{C}H_2$), 55.24 (s, $O\underline{C}H_3$), 62.43 (s, $O\underline{C}H_2CH_3$), 62.79 (s, $O\underline{C}H_2CH_3$), 65.07 (s, $\underline{C}(CO_2Et)_2NHAc$), 70.48 (s, $\underline{C}$HOH), 111.37 (s, 2'-$\underline{C}$), 112.48 (s, 6'-$\underline{C}$), 118.21 (s, 4'-$\underline{C}$), 129.66 (s, 5'-$\underline{C}$), 145.90 (s, 1'-$\underline{C}$), 159.96 (s, 3'-$\underline{C}$), 167.93 (s, $NH\underline{C}OCH_3$), 169.14 (s, $O\underline{C}OCH_2CH_3$), 169.75 (s,$O\underline{C}OCH_2CH_3$), 169.75 (s, $O\underline{C}OCH_2CH_3$). *m/z* (EI) 367 ($[M]^+$, 6), 349 (16, $[M-H_2O]^+$), 217 (100, $[C(NHAc)CO_2EtCOHOEt]^+$), 171 (93, $[C(NHAc)CO_2EtCOHOEt - HOCH_2CH_3]^+$), 135 (30, $[MeOC_6H_4CO]^+$), 77 (12, $[C_6H_5]^+$).

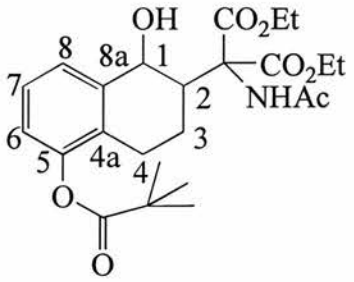
2-Amino-4-hydroxy-4-(3-methoxy-phenyl)-butyric acid hydrogen chloride (γ-hydroxy-3'-methoxydesasminokynurenine) (91)



.HCl

A suspension of **(140)** (1.65 g, 4.5 mmol) in 6M HCl (50 ml) was heated under reflux four hours. Upon cooling a white suspension formed which was dissolved in distilled water (150 ml), washed with ethyl acetate (3 × 75 ml) and the solvent removed under reduced pressure to afford a white solid which was then recrystallised from water (0.75 g, 64%), **(91)** was obtained as a mixture of diastereomers; δ_H (300MHz, ^2H_2O) (4*S*,2*S*), (4*R*,2*R*) pair of enantiomers; 2.34 (1H, m, $J_{3a,3b}$ 12.6, $J_{3a,2}$ $J_{3a,4}$ 10.8 Hz, 3a-C<u>H</u>), 3.09 (1H, m, $J_{3b,3a}$ 12.6, $J_{3b,2}$ 8.7, $J_{3b,4}$ 5.4 Hz 3b-C<u>H</u>), 3.75 (3H, s, OC<u>H</u>$_3$), 4.59 (1H, dd, $J_{2,3a}$ 10.8, $J_{2,3b}$ 8.7 Hz, 2-C<u>H</u>), 5.62 (1H, dd, $J_{4,3a}$ 10.8, $J_{4,3b}$ 5.4 Hz, 4-C<u>H</u>OH), 6.99 (4H, m, aromatic-<u>H</u>); (4*R*,2*S*), (4*S*,2*R*) pair of enantiomers; 2.81 (2H, m, 3-C<u>H</u>$_2$), 4.42 (1H, t, $J_{2,3a,3b}$ 10.0 Hz, 2-C<u>H</u>), 5.84 (1H, m, 4-C<u>H</u>OH); δ_C (75.4 MHz, ^2H_2O) 35.53 (s, 3-<u>C</u>H$_2$), 49.65 (s, 2-<u>C</u>H), 55.76 (s, O<u>C</u>H$_3$), 79.28 (s, 4-<u>C</u>HOH), 112.66 (s, 2'-<u>C</u>), 115.20 (s, 4'-C), 119.36 (s, 6'-<u>C</u>), 130.55 (s, 5'-<u>C</u>), 140.15 (s, 1'-<u>C</u>), 160.14 (s, 3'-<u>C</u>), 173.05 (s, O<u>C</u>OH); *m/z* (CI) 208 (100, [*M*+H-H_2O]$^+$), 163 (24, [$CH_3OC_6H_4CHCHNH_2$]$^+$), 135 (48, $CH_3OC_6H_4CO$]$^+$), 116 (18, [$H_2NCH(CO_2H)CH_2CO$]$^+$, 74 (29, [C(COHOC)NH_2]$^+$. Analytical HPLC of **(91)** (C_{18}, reverse phase silica, 3μ, flow rate 4 ml/min, 1% acetic acid, 20% methanol) showed the presence of two peaks indicating the presence of diastereomers, (4*S*,2*S*),(4*R*,2*R*) and (4*R*,2*S*), (4*S*,2*R*), in a 2:1 ratio.

2-Acetyl-2-[5-(2,2-dimethyl-propionyloxy)-1-hydroxy-1, 2, 3, 4-tetrahydro-naphthalen-2-yl]-malonic acid diethyl ester (141)



Procedure 1

To a stirred suspension of palladium catalyst (5% on carbon, 0.17 g) in dry ethyl acetate (15 ml) was added a solution of **(128)** (3.15 g, 6.8 mmol) in dry ethyl acetate (25 ml). After degassing of the solution the reaction mixture was stirred at room temperature under an atmosphere of hydrogen for twenty-four hours. The palladium catalyst was removed by filtration through celite which was washed with ethyl acetate (3 × 50 ml), the filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a white sticky solid. Analysis by NMR spectroscopy indicated the presence of starting material.

Procedure 2

Procedure as in 1 with stirring at room temperature for 3 days. Palladium catalyst (5% on carbon, 0.19 g), **(128)** (0.665 g, 1.44 mmol) and dry ethyl acetate (40 ml). Analysis by NMR spectroscopy indicates the presence of starting materials.

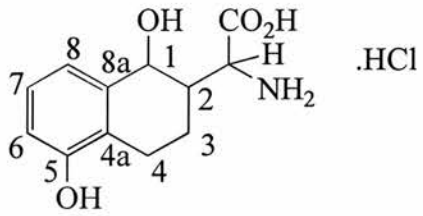
Procedure 3

To a stirred solution of **(128)** (0.26 g, 0.57 mmol) in dry THF (25 ml), was added sodium cyanoborohydride (1M solution in THF, 0.57 ml, 0.57 mmol) and the reaction mixture heated under reflux for three hours under an atmosphere of nitrogen. The reaction was stopped by addition of 1M HCl, extracted into diethyl ether (2 × 50 ml) dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a white solid. Analysis by NMR spectroscopy indicated the presence of starting materials.

Procedure 4

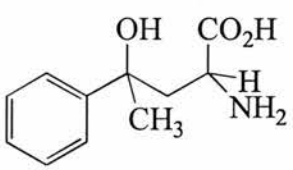
To a stirred suspension of palladium catalyst (5% on carbon, 0.50 g) in dry ethyl acetate (15 ml) was added a solution of **(128)** (3.00 g, 6.5 mmol) in dry ethyl acetate (55 ml). After degassing of the solution the reaction mixture was heated under reflux under an atmosphere of hydrogen for four hours and allowed to stir at room temperature for a further seventeen hours. The palladium catalyst was removed by filtration through celite and washed with ethyl acetate (3 × 50 ml), the filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a white sticky solid (1.13 g, 37%); (Found: C, 62.13; H, 6.90; N, 3.11 $C_{24}H_{33}NO_8$ requires: C, 62.19; H, 7.17; N, 3.02%); ν_{max} (nujol)/ cm^{-1} 1754 (CO,ester); δ_H (300 MHz, C^2HCl_3) 1.24 (6H, t, *J* 1.2 Hz, 2× $OCH_2C\underline{H}_3$), 1.34 (9H,s, $OCOC(C\underline{H}_3)_3$), 1.97 (2H, m, 4-$C\underline{H}_2$) 2.03 (3H, s, $NHCOC\underline{H}_3$), 2.77 (2H, m, 3-$C\underline{H}_2$), 4.23 (4H, m, 2 × $OC\underline{H}_2CH_3$), 5.62 (1H, d, *J* 9.3 Hz, 1-C<u>H</u>OH), 6.72 (1H, m, 6-<u>H</u>), 6.84 (1H, m, $N\underline{H}COCH_3$), 6.90 (1H, m, 7-<u>H</u>), 7.24 (1H, s, 8-<u>H</u>); δ_C (75.4 MHz, C^2HCl_3) 13.89 (s, $OCH_2\underline{C}H_3$), 21.99 (s, 4-$\underline{C}H_2$), 23.05 (s, 3-$\underline{C}H_2$), 27.14 (s, $OCOC(\underline{C}H_3)_3$), 39.20 (s, $OCO\underline{C}(CH_3)_3$), 49.62 (s, 2-<u>C</u>H), 62.75 (s, $\underline{C}(CO_2Et)_2NHAc$), 65.70 (s, 2 × $O\underline{C}H_2CH_3$), 80.92 (s, 1-<u>C</u>HOH), 119.17 (s, 6-<u>C</u>), 120.84 (s, 8-<u>C</u>), 121.34 (s, 7-<u>C</u>), 127.26 (s, 4a-<u>C</u>), 137.86 (s, 8a-<u>C</u>), 149.35 (s, 5-<u>C</u>), 168.85 (s, $NH\underline{C}OCH_3$), 170.18 (s, O<u>C</u>OEt), 170.74 (s, O<u>C</u>OEt), 176.72 (s, $O\underline{C}OCH(CH_3)_3$); *m/z* (EI) 416, (14, $[M\text{-}H_2O\text{-}C_2H_5]^+$), 246 (27, $[C_{15}H_{19}O_3]^+$), 217 (70, $[C(CO_2Et)_2NHAc]^+$), 85 (13, $[(CH_3)_3CCO]^+$), 57 (100, $[(CH_3)_3C]^+$).

Amino-(1,5-dihyroxy-1, 2, 3, 4-tetrahydro-naphthalen-2-yl)-acetic acid hydrogen chloride (1,5-dihyroxy tetralone derivative) (92)



A suspension of 2-acetyl-2-[5-(2,2-dimethyl-propionyloxy)-1-hydroxy-1, 2, 3, 4-tetrahydro-naphthalen-2-yl]-malonic acid diethyl ester (1.00 g, 2.16 mmol) in 6M HCl (60 ml) was heated under reflux for three hours. The reaction mixture was filtered to remove black decomposition formed and the solvent removed under reduced pressure to afford a yellow oil which upon freeze drying afforded a pale yellow solid (0.27 g, 47%), dec. 146 °C; (Found: C, 52.80; H, 5.70; N, 4.96 $C_{12}H_{16}ClNO_4$ requires C, 52.66; H, 5.89; N, 5.12%);ν_{max} (nujol)/cm^{-1} 1736 (CO, acid), 1684 (CO); δ_H (300 MHz, $C^2H_3O^2H$); δ_H (300 MHz, $C^2H_3O^2H$) 1.07-1.28 (2H, m, 3-C$\underline{H}_2$) 2.01-2.49 (1H, m, 2-C$\underline{H}$), 2.57-2.63, (2H, m, 4-C$\underline{H}_2$), 5.39 (1H, d, *J* 4.2 Hz, 1-C$\underline{H}$OH), 5.73 (1H, d, *J* 8.1 Hz, 1-C$\underline{H}$OH diasteomer), 6.50-7.05 (m, aromatic-$\underline{H}$'s, diastereomers); δ_C (75.4 MHz, $C^2H_3O^2H$), 22.13 (s, 3-$\underline{C}$H), 24.73 (s, 4-$\underline{C}$H), 59.15 (s, 2-$\underline{C}$H), 65.61 (s, α-$\underline{C}$H), 79.22 (s, 1-$\underline{C}$HOH), 117.14 (s, 6-$\underline{C}$), 120.32 (s, 8-$\underline{C}$), 122.14 (s, 7-$\underline{C}$), 127.80 (s, 4a-$\underline{C}$), 131.81 (s, 8a-$\underline{C}$), 140. 33 (s, 8a-$\underline{C}$), 155.43 (s, 5-$\underline{C}$), 170.68 (s, O$\underline{C}$OH). *m/z* (Maldi-TOF) 242 ([*M*+Na-H_2O], 80%). The compound was obtained as a complex mixture of diastereomers therefore it was not possible to assign all of the resonances in the ^{1}H NMR.

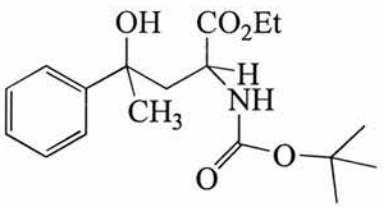
Attempted alkylation of ethyl *N*- (*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (132) using methyl magnesium bromide



To a stirred solution of ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (0.31 g, 0.90 mmol) in dry THF (50 ml) was added 3.0 M methyl magnesium bromide

(0.33 ml, 0.90 mmol). The reaction mixture was stirred at room temperature for four hours under an atmosphere of nitrogen then heated under reflux for a further ten hours. The resulting yellow solution was cooled to room temperature, poured onto 1M NH_4Cl (20 ml) on crushed ice. This was extracted into diethyl ether (4 × 25 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Further purification by column chromatography (silica gel, ethyl acetate: petroleum ether 1:2) afforded two bands, $R_f = 0.7$ and 0.6 respectively. The major band was shown to be starting material by tlc and NMR spectroscopy; δ_H (300 MHz, C^2HCl_3) 1.22 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 1.48 (9H, s, $OC(C\underline{H}_3)_3$), 3.50 (1H, dd, *J* 15.0, 4.0 Hz, β-$C\underline{H}_2$ H_a), 3.71 (1H, dd, *J* 15.0, 4.0 Hz, β-$C\underline{H}_2$ H_b) 4.18 (2H, q, *J* 7.0 Hz $OC\underline{H}_2CH_3$), 4.65 (1H, t, *J* 4.0 Hz, α-C<u>H</u>), 5.64 (1H, bm, $N\underline{H}CO_2C(CH_3)_3$), 7.45 (2H, t, *J* 8.0 Hz, 3'-<u>H</u>, 5'-<u>H</u>), 7.58 (1H, t, *J* 7.0 Hz, 4'-<u>H</u>), 7.92 (2H, d, *J* 7.0 Hz, 2'-<u>H</u>, 6'-<u>H</u>); δ_C (75.4 MHz, C^2HCl_3), 14.00 (s, $OCH_2\underline{C}H_3$), 28.25 (s, $OCOC(\underline{C}H_3)_3$), 41.89 (s, β-$\underline{C}H_2$), 49. 72 (s, α-<u>C</u>H), 61.64 (s, $O\underline{C}H_2CH_3$), 79.94 (s, $O\underline{C}(CH_3)_3$, 128.25 (s, 3'-<u>C</u>, 5'-<u>C</u>), 128.83 (s, 2'-<u>C</u>, 6'-<u>C</u>), 133.79 (s, 4'-<u>C</u>), 136.33 (s, 1'-<u>C</u>), 155.82 (s, $NH\underline{C}OC(CH_3)_3$), 171.65 (s, $\underline{C}O_2CH_2CH_3$), 198.04 (s, <u>C</u>=O). The minor product with Rf = 0.7 was (0.03 g) *m/z* (CI) $[MH]^+$ = 393 *m/z*. The NMR data were inconclusive.

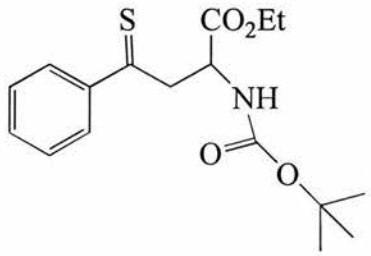
Attempted alkylation of ethyl *N*- (*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (132) using methyl magnesium bromide in the presence of a $CeCl_3$ catalyst



To pre-dried $CeCl_3$ (1.63 g, 4.38 mmol) was added dry THF (30 ml) and the suspension stirred at room temperature and under an atmosphere of argon for two hours. Methyl magnesium bromide (3.0 M solution in THF) (1.46 ml, 4.38 mmol) was added and the reaction mixture stirred at room temperature for a further 1.5 hours. To this a solution of ethyl *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyrate (1.08 g, 3.37 mmol) in THF (10 ml) was added and the reaction mixture allowed to stir at room temperature for fourteen hours under an atmosphere of argon. This was heated under reflux for a further

six hours and then cooled to room temperature. Ammonium Chloride solution (1.0 M) was then added and the solvent removed under reduced pressure. The residue was redissolved in diethyl ether (100 ml), washed with water and filtered through celite. The solvent was removed under reduced pressure to afford a pale yellow precipitate. Analyses by NMR spectroscopy and tlc indicated the presence of starting materials.

Attempted thiation of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyric acid ethyl ester (132)



Procedure 1

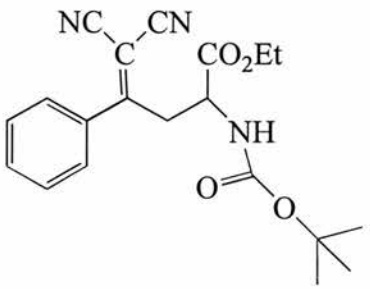
To a stirred solution of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenylbutyric acid ethyl ester (0.17 g, 0.52 mmol) in dry THF (35ml), Lawesson's Reagent (0.15 g, 0.37 mmol) was added under nitrogen. The reaction mixture was allowed to stir at room temperature for 24 hours. The solvent was then removed under reduced pressure to afford an off white residue. This was partitioned between ethyl acetate (100 ml) and water (100 ml) and the aqueous layer washed with ethyl acetate (2 × 40 ml). The ethyl acetate washings were combined and dried over magnesium sulfate. The solvent was removed under reduced pressure to give a pale yellow oil. Examination by NMR spectroscopy and tlc showed only starting materials present.

Procedure 2

To a stirred solution of *N*-(*tert*-butoxycarbonyl)-2-oxo-2-phenyl-butyric acid ethyl ester (0.20 g, 0.63 mmol) in dry toluene (30 ml), Lawesson's reagent (0.13 g, 0.31 mmol) was added and the reaction mixture heated under reflux over two days. The solvent was then removed and further purification by column chromatography (silica gel, ethyl acetate:

petroleum ether 1:2) afforded two bands with R_f = 0.6 and 0.80 respectively. The major band with R_f = 0.6 was shown to be starting material by tlc and NMR spectroscopy; δ_H (200 MHz, C^2HCl_3) 1.20 (3H, t, *J* 7.0 Hz, $OCH_2C\underline{H}_3$), 1.40 (9H, s, $OC(C\underline{H}_3)_3$), 3.52 (1H, dd, *J* 18.0, 4.0 Hz, β-$C\underline{H}_2$ -H_A), 3.75 (1H, dd, 18.0, 4.0 Hz, β-$C\underline{H}_2$-H_B), 4.30 (2H, m, $OC\underline{H}_2CH_3$), 4.60 (1H, bm, α-C<u>H</u>), 5.61 (1H, bm, $N\underline{H}CO_2C(CH_3)_3$), 7.47 (2H,t, *J* 7.5 Hz, 3'-<u>H</u>, 5'-<u>H</u>), 7.54 (1H, t, *J* 7.5 Hz, 4'-<u>H</u>), 7.97 (2H, d, *J* 7.5 Hz, 2'-<u>H</u>, 6'-<u>H</u>); δ_C (75.4 MHz, C^2HCl_3), 14.55 (s, $OCH_2\underline{C}H_3$), 28.78 (s, $OCOC(\underline{C}H_3)_3$), 41.37 (s, β-$\underline{C}H_2$), 50.14 (s, α-<u>C</u>H), 62.13 (s, $O\underline{C}H_2CH_3$), 80.40 (s, $O\underline{C}(CH_3)_3$, 128.62 (s, 3'-<u>C</u>, 5'-<u>C</u>), 128.91 (s, 2'-<u>C</u>, 6'-<u>C</u>), 134.16 (s, 4'-<u>C</u>), 136.58 (s, 1'-<u>C</u>), 155.80 (s, $NH\underline{C}O_2C(CH_3)_3$), 171.99 (s, $\underline{C}O_2CH_2CH_3$), 198.31 (s, <u>C</u>=O) A minor product with R_f = 0.8 was also obtained. (0.04g), molecular ion peak $[MH]^+$ = 392 *m/z*. The NMR data were inconclusive.

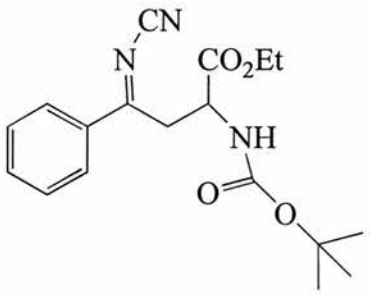
Attempted reaction of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyric acid ethyl ester (132) with malononitrile ($CH_2(CN)_2$)



Sodium metal (0.019 g, 0.80 mmol) was dissolved in dry redistilled ethanol (20 ml) and allowed to stir at room temperature until all of the sodium had reacted. The sodium ethoxide solution was then added to malononitrile (0.056 g, 0.84 mmol) and the reaction mixture allowed to stir at room temperature for one hour. A solution of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenylbutyric acid ethyl ester (0.26 g, 0.8 mmol) in dry diethyl ether (25 ml) was added to the pre-formed malononitrile anion and the reaction mixture allowed to stir under nitrogen at room temperature for a further twenty-four hours. The solution was then acidified with 3% HCl, the solvent removed under reduced pressure and the residue partitioned between diethyl ether (100 ml) and water (100 ml). The aqueous layer was washed with diethyl ether (2 × 50 ml), the organic layers combined, dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil

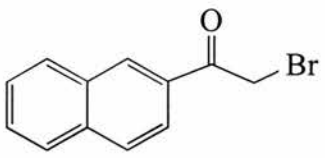
(0.04 g). Characterisation by NMR spectroscopy showed only the presence of starting materials; δ_H (300 MHz, C^2HCl_3), 1.24 (3H, t, *J* 9 Hz, $OCH_2C\underline{H}_3$), 2.03 (9H, s, $OC(C\underline{H}_3)_3$), 3.54 (1H, dd, *J* 18.0, 3.0 Hz, β-$C\underline{H}_2$ H_A), 3.61 (1H, dd, *J* 18.0, 3.0 Hz, β-$C\underline{H}_2$ H_B), 4.12 (2H, m, $OC\underline{H}_2CH_3$), 4.67 (1H, bm, α-C<u>H</u>), 5.67 (1H, bm, $N\underline{H}CO_2C(CH_3)_3$), 7.47 (2H, t, *J* 6.9 Hz, 3'-<u>H</u>, 5'-<u>H</u>), 7.59 (1H, m, 4'-<u>H</u>), 7.94 (2H, d, *J* 5.4 Hz, 2'-<u>H</u>, 6'-<u>H</u>); ν_{max} (nujol)/cm^{-1} 2985.8 (s) (C-H aliphatic), 2093.4 (vw) (C≡N), 1743 (vs) (C=O)

Attempted reaction of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenyl-butyric acid ethyl ester (132) with cyanamide (H_2NCN)



To a stirred solution of *N*-(*tert*-butoxycarbonyl)-2-amino-4-oxo-4-phenylbutyric acid ethyl ester (0.34 g, 1.06 mmol) in dry THF (15 ml) was added cyanamide (0.04 g, 1,06 mmol). The reaction mixture was allowed to stir for forty-eight hours at room temperature under an atmosphere of nitrogen. The solvent was concentrated under reduced pressure and the black reaction mixture purified by column chromatography (silica, ethyl acetate: petroleum ether 1:2) to afford a clear oil. Examination of the oil by NMR spectroscopy, mass spectrometry and tlc showed only starting materials present.

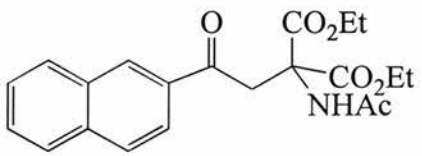
α-Bromo-acetonaphthone (144)



Cupric bromide (12.95 g, 58.00 mmol) was heated to reflux in ethyl acetate (45 ml) with stirring. 2-acetonaphthone (5.00 g, 29.00 mmol) as a solution in chloroform (15 ml) was then added and the reaction mixture heated under reflux for three hours. The CuBr and

$CuBr_2$ salts were filtered off and the filtrate decolourised with activated charcoal. This was removed by filtration through celite which was washed with ethyl acetate (3 × 50 ml). The solution was dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to afford a pale yellow solid (6.71 g, 93%), m.p. 82-84 °C (lit.[191], 83 °C) which was used in the next step without further purification; ν_{max} (nujol)/cm^{-1} 1660 (CO); δ_H (300 MHz, C^2HCl_3) 4.68 (2H, s, $COC\underline{H}_2Br$), 7.66-7.77 (2H, m, 6', 7'-$\underline{H}$), 8.00 (2H, t, *J* 8.0 Hz 5', 8'-$\underline{H}$), 8.07 (1H, d, *J* 8.0 Hz, 4'-$\underline{H}$), 8.13 (1H, dd, *J* 3.0, 8.0 Hz, 3'-$\underline{H}$), 8.61 (1H, bs, 1'-$\underline{H}$); δ_C (74.5 MHz, C^2HCl_3) 31.34 (s, $COC\underline{H}_2Br$), 124.5 (s, 3'-C), 125.03 (s, 7'-C), 127.11 (s, 5'-C), 127.41 (s, 4'-C), 128.80 (s, 6'-C), 129.38 (s, 8'-C), 130.04 (s, 1'-C), 131.63 (s, 10'-C), 132.74 (s, 9'-C), 136.22 (s, 2'-C), 191.58 (s, $\underline{C}OCH_2Br$); *m/z* (EI) 250, 248 ($[M]^+$, 11), 155 (100, $[C_{10}H_7CO]^+$), 141 (15, $[C_{10}H_7CCH_2]^+$),127 (58, $[C_{10}H_7]^+$]).

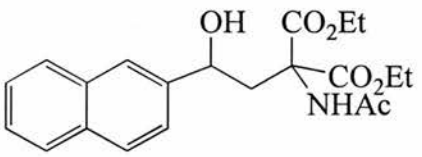
2-Acetylamino-2-[(2-naphthyl)-2-oxo-ethyl)]malonic acid diethyl ester (146)



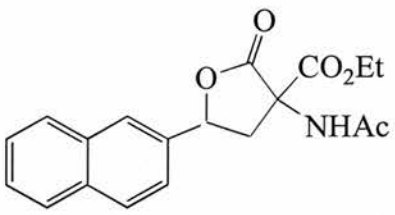
To a stirred solution of diethyl acetamidomalonate (5.12 g, 24.00 mmol) in dry DMF (60 ml) at 0 °C and under nitrogen was added sodium hydride (60% weight in oil, 0.96 g, 24.00 mmol). The solution was stirred for three hours before α-bromoacetonaphthone (6.00 g, 24.00 mmol) was added as a solution in DMF (25 ml) and the reaction mixture stirred at room temperature overnight. The reaction mixture was poured onto water (600 ml), acidified to pH 1 with 6M HCl, extracted into diethyl ether (5 × 150 ml) and washed with brine (100 ml). The combined organic layers were dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Further purification by column chromatography (silica gel, ethyl acetate : petroleum ether 1:3) afforded colourless crystals (6.28 g, 68%), m.p. 86-89 °C; ν_{max} (nujol)/cm^{-1} 3331 (NH), 1739 (CO, ester), 1685 (CO), 1650 (CO, amide); δ_H (300 MHz, C^2HCl_3) 1.37 (6H, t, *J* 7.0 Hz, 2 × $OCH_2C\underline{H}_3$), 2.08 (3H, s, $NHCOC\underline{H}_3$), 4.40 (4H, q, 7.0 Hz, 2 × $OC\underline{H}_2CH_3$), 4.52 (3H, s, β-$C\underline{H}_2$), 7.27 (1H, s, $N\underline{H}COCH_3$), 7.72 (2H, m, 3', 4'-$\underline{H}$), 7.97 (2H, m, 5', 8'-$\underline{H}$), 8.08 (2H, m, 6',7'-$\underline{H}$), 8.63 (1H, s, 1'-$\underline{H}$); δ_C (74.5 MHz, C^2HCl_3) 14.31 (s, 2 × $OCH_2\underline{C}H_3$), 23.34 (s,

NHCOCH$_3$), 42.66 (s, β-CH$_2$), 63.26 (s, 2 × OCH$_2$CH$_3$), 64.45 (s, C(CO$_2$Et)$_2$NHAc), 123.74 (s, 3'-C), 127.29 (s,7'-C), 128.05 (s, 5'-C), 128.88 (s, 4'-C), 129.15 (s, 6'-C), 130.04 (s, 8'-C), 130.80 (s, 1'-C), 132.77 (s, 10'-C), 133.72 (s, 9'-C), 136.16 (s, 2'-C); *m/z* (EI) 385.1518 $C_{21}H_{23}NO_6$ requires 385.1525, 385 ([*M*]$^+$, 10), 312 (23, ([*M*- CO_2Et]$^+$), 270 (23, ([*M*-C_2H_2O]$^+$), 155 (100, [$C_{10}H_7CO$]$^+$), 127 (38, [$C_{10}H_7$]$^+$).

2-Acetylamino-2-[(2-naphthyl)-2-hydroxy-ethyl)]malonic acid diethyl ester (147)



(147)



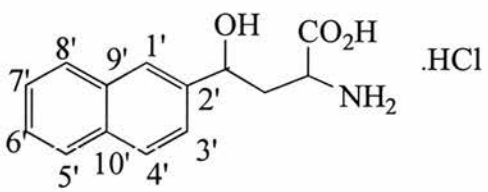
(148)

To a solution of **(146)** (3.00 g, 7.78 mmol) in ethyl acetate was added palladium catalyst (10 % on carbon, 1.00 g). The suspension was degassed and stirred under an atmosphere of hydrogen for twenty hours. The palladium was removed by filtration through celite and washed with ethyl acetate (3 × 50 ml). The organic layers were added together dried $MgSO_4$ and the solvent removed under reduced pressure to afford a white sticky solid. Further purification by column chromatography (silica gel, diethyl ether: petroleum ether 2:1) afforded two compounds with the same R_f = 0.5, separation of these compounds by recrystallisation from diethyl ether. The lactone **(148)** precipitated from the ether solution first and was filtered off before removal of the diethyl ether to afford the product as a sticky clear oil **(147)** (0.33 g, 10%), 96-98 °C; ν_{max} (nujol)/cm^{-1} 3373 (γ-OH), 1749 (CO, ester), 1657 (CO,amide); δ_H (300 MHz, C^2HCl_3) 1.33 (3H, t, *J* 8.0 Hz, OCH$_2$CH$_3$), 1.42 (3H, t, *J* 8.0 Hz, OCH$_2$CH$_3$), 2.14 (3H, s, NHCOCH$_3$), 2.91 (1H, dd, *J* 15.0, 10.0 Hz, H$_a$ β-CH$_2$), 3.03 (1H, dd, *J* 15.0, 3.3 Hz, H$_b$ β-CH$_2$), 4.28-4.37 (4H, m, 2 × OCH$_2$CH$_3$), 5.01 (1H, d, *J* 10.0 Hz, γ-CHOH), 7.27 (1H, s, NHCOCH$_3$), 7.52-7.59 (2H, m, 1', 3'-H), 7.65-7.69 (1H, m, 4'-H), 7.87 (2H, dd, *J* 8.7, 4.5 Hz, 6', 7'-H), 7.93-8.10 (2H, m, 5', 8'-H); δ_C (75.4 MHz, C^2HCl_3) 14.29 (s, 2 × OCH$_2$CH$_3$), 23.39 (s, NHCOCH$_3$), 41.52 (s, β-CH$_2$), 62.71 (s, OCH$_2$CH$_3$), 63.19 (s, OCH$_2$CH$_3$), 65.44 (s, C(CO$_2$Et)$_2$NHAc), 70.98 (s, γ-

CHOH), 124.29 (s, 3'-C), 124.62 (s, 7'-C), 126.27 (s, 5'-C), 126.54 (s, 4'-C), 127.98 (s, 6'-C), 128.24 (s, 8'-C), 128.65 (s, 1'-C), 133.30 (s, 10'-C), 133.53 (s, 9'-C), 141.56 (s, 2'-C); *m/z* (EI) 387.1674 M^+ $C_{21}H_{25}NO_6$ requires 387.1681;387 ([*M*]$^+$, 6), 369 (5, [*M*-H_2O]$^+$), 341 (44, [*M*-OCH_2CH_3-H]$^+$), 217 (59, [C(COHOEt)(CO_2Et)NHAc]$^+$), 155 (58, [$C_{10}H_7CO$]$^+$), 130 (100, [$HOCH_2CH_2CCO_2Et$]$^+$).

Compound **(148)** the γ-lactone, was obtained as a white precipitate (0.54 g, 20%); (Found: C, 66.89; H, 5.73; N, 4.07 $C_{19}H_{19}NO_5$ requires C, 66.85; H, 5.61; N, 4.10%); ν_{max} (nujol)/cm^{-1}3399 (NH), 1774 (CO, lactone), 1736 (CO, ester), 1674 (CO, amide); δ_H (300 MHz, C^2HCl_3) 1.53 (3H, t, *J* 7 Hz, OCH_2CH_3), 2.23 (3H, s, $NHCOCH_3$), 3.11 (2H, dd, *J* 3, 9 Hz, β-CH_2), 4.51 (2H, q, *J* 7 Hz, OCH_2CH_3), 5.92 (1H, dd, *J* 7, 9 Hz, γ-CH), 6.92 (1H, s, $NHCOCH_3$), 7.62 (2H, m, 6', 7'-H), 7.89 (1H, m, 5', 8'-H), 7.96 (1H, m, 4'-H), 7.98 (1H, m, 3'-H), 8.04 (1H, bs, 1'-H); δ_C (74.5 MHz, C^2HCl_3) 14.42 (s, OCH_2CH_3), 23.28 (s, $NHCOCH_3$), 41.32 (s, β-CH_2), 64.22 (s, OCH_2CH_3), 65.20 (s, C(CO_2Et)NHAc), 80.64 (s, CHOH), 124.09 (s, 3'-C), 126.47 (s, 7'-C), 126.79 (s, 5'-C), 126.93 (s, 4'-C), 128.13 (s, 6'-C), 128.45 (s, 8'-C), 129.47 (s, 1'-C), 133.26 (s, 10'-C), 133.95 (s, 9'-C), 135.93 (s, 2'-C), 167.91 (s, $NHCOCH_3$), 169.90 (s, $OCOCH_2CH_3$), 170.1 (s, $OCOCH_2CH_3$); *m/z* (EI) 341 ([*M*]$^+$, 55), 282 (12, [*M*-$NHCOCH_3$-H]$^+$), 209 (100, [*M*-$NHCOCH_3$-CO_2Et-H]$^+$), 155 (55, [$C_{10}H_7CO$]$^+$), 127 (32, [C_{10},H_7]$^+$).

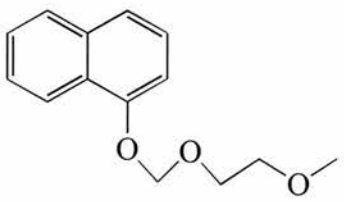
2-Amino-4-hydroxy-4-naphthyl-butyric acid (97)



A suspension of **(147)** (0.88 g, 2.24 mmol) in 6M HCl (50 ml) was heated under reflux for seven hours. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure to afford a white solid. This was recrystallised from methanol to afford a white precipitate (0.24 g, 38%), a single diastereomer obtained as a racemic mixture (4*S*,2*S*) and (4*R*,2*R*), dec 266 °C; ν_{max} 3370 (OH), 1737 (CO, acid); δ_H

(300 MHz, DMSO) 2.61 (1H, m, $J_{2,3a}$ 12.0 Hz, 3a-H), 3.15 (1H, m, $J_{3a,3b}$ 12.0, $J_{2,3b}$ 8.7, $J_{4,3b}$ 6.0 Hz, 3b-H), 4.73 (1H, dd, , $J_{3a,2}$ 12.0, $J_{3b,2}$ 8.7 Hz, 2-H), 5.85 (1H, dd, $J_{3a,4}$ 11.0, $J_{3b,4}$ 6.0 Hz, 4-CH), 7.65 (2H, dd, *J* 6.3, 3.3 Hz, 6', 7'-H), 7.76 (1H, d, *J* 8.7 Hz, 4'-H), 8.02-8.07 (2H, m, 5', 8'-H), 8.10-8.12 (2H, m, 1', 3'-H); δ_C (75.4 MHz, DMSO) 35.89 (s, 4-CH_2), 50.12 (s, 2-CH), 79.85 (s, 4-CH), 124.73 (s, 3'-C), 126.74 (s, 7'-C), 127.46 (s, 5'-C), 127.52 (s, 4'-C), 128.47 (s, 6'-C), 128.80 (s, 8'-C), 129.38 (s, 1'-C), 133.27 (s, 10'-C), 133.81 (s, 9'-C), 136.10 (s, 2'-C); *m/z* (CI) 228 ([*M*+H-H_2O]$^+$, 100), 84 (10, [*M*+H-H_2O-CO_2]), 155 (18, [$C_{10}H_7CO$]$^+$), 74 (21, [$CHCO_2HNH_2$]). Analytical HPLC of **(97)** (C_{18} reverse phase silica, 3μ, flow rate 4 ml/min; CH_3CN, H_2O 1:1) afforded a chromatogram showing a single peak.

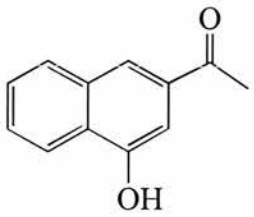
1-(2-methoxy-ethoxymethoxy)-naphthalene (157)



A solution of 1-naphthol (5.00 g, 34.6 mmol) in THF (20 ml) was added to a suspension of sodium hydride (1.66 g, 41.5 mmol) in dry THF (40 ml) over 20 minutes at 0 °C and under an atmosphere of nitrogen. To this was added methoxyethoxymethyl chloride (8.54 ml, 69.3 mmol) and the white suspension which resulted was stirred at room temperature overnight. The suspension was poured onto water (100 ml), extracted into diethyl ether (2 × 50 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. This was further purified by column chromatography (silica gel; ethyl acetate: petroleum ether 1:3) to afford a pale yellow oil (3.29 g, 41%); ν_{max} neat/ cm^{-1} 1053 (vs, br, ether); δ_H (300 MHz, C^2HCl_3) 3.51 (3H, s, OCH_3), 3.70 (2H, dd, *J* 3.0, 4.5 Hz, $OCH_2CH_2OCH_3$), 4.04 (2H, dd, *J* 3.0, 4.5 Hz, $OCH_2CH_2OCH_3$), 5.62 (2H, s, OCH_2O), 7.28 (1H, d, *J* 10 Hz, 2-H), 7.50 (1H, t, *J* 10 Hz, 3-H), 7.62 (3H, m, 4, 5, 8-H), 7.94 (1H, m, 7-H), 8.39 (1H, m, 6-H); δ_C (74.5 MHz, C^2HCl_3) 59.40 (s, OCH_3), 68.72 (s, OCH_2CH_2O), 72.02 (s, OCH_2CH_2O), 94.15 (s, OCH_2O), 108.28 (s, 2-C), 121.74 (s, 8-C), 122.26 (s, 4-C), 125.69 (s, 3-C), 126.27 (s, 5-C), 126.65 (s, 6-C), 127.92 (s, 7, 9-C), 134.89 (s, 10-C), 153.20 (s, 1-C); *m/z* (EI) 232.1106 M^+ $C_{14}H_{16}O_3$, requires 232.1099, 232

([M]$^+$,25), 157 (8, ([M- $OCH_2CH_2OCH_3$]$^+$), 144 (26, [$C_{10}H_7OH$]$^+$), 127 (14, [$C_{10}H_7$]$^+$), 89 (72, [$CH_3OCH_2CH_2OCH_2$]$^+$), 59 (100, [$CH_3OCH_2CH_2$]$^+$).

Attempted synthesis of 4-hydroxynaphthone (149)

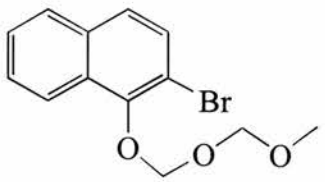


To a solution of 1-(2-methoxy-ethoxymethoxy)-naphthalene (0.62 g, 2.66 mmol) in THF (15 ml) at -78 °C under an atmosphere of nitrogen was added n-butyllithium (2.5 M in hexanes) (1.17 ml, 2.92 mmol). The solution was warmed to room temperature and stirred for 20 min and recooled to -78 °C. This was added slowly to a solution of methylchlorodicyclopentadienylzirconium ($Cp_2Zr(Me)Cl$) (1.00 g, 3.43 mmol) in THF (15 ml) and stirred for an additional 15 minutes at -78 °C. The reaction mixture was then warmed to room temperature and acetonitrile (0.15 ml, 2.92 mmol) was added. The reaction mixture was stirred at room temperature for seventeen hours after which 1M HCl (3.5 ml) was added and the reaction mixture was stirred under nitrogen for a further seventeen hours. The reaction mixture was then diluted with diethyl ether (100 ml) washed with water (100 ml) and brine (50 ml). The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. NMR spectroscopy showed only unreacted starting material.

Procedure 2

To a stirred suspension of 2-acetylnaphthone in acetic acid (37.5%, 50 ml) and H_2SO_4 (2.2 M, 5 ml) was added divanadium pentoxide V_2O_5. The reaction mixture was heated at 50 °C for four hours and heated under reflux overnight. The vanadium salt was removed by filtration through celite. The aqueous layer was then washed with diethyl ether (4 × 50 ml) dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. Analysis by tlc and NMR spectroscopy showed only the presence of starting material.

Attempted *ortho* bromination of 1-(2-methoxy-ethoxymethoxy)-naphthalene.



Procedure 1

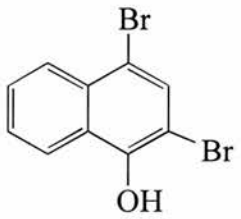
To a stirred solution of 1-(2-methoxyethoxymethoxy)-naphthalene (0.24 g, 1.01 mmol) in THF (10 ml) at -78 °C under an atmosphere of nitrogen was added n-butyllithium (2.5 M in hexanes) (0.44 ml, 1.11 mmol). The solution was warmed to room temperature and stirred for 30 minutes. The solution was then cooled to –78 °C and *N*-bromosuccinimide (0.59 g, 3.33 mmol) added. The solution was allowed to warm up to room temperature slowly and stirred for 3 hours. The reaction mixture was then poured onto CCl_4 (50 ml), filtered to remove succinimide, dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a brown solid. NMR spectroscopic and mass spectrometric data were inconclusive.

Prodedure 2

To a solution of 1-(2-methoxy-ethoxymethoxy)-naphthalene (1.32 g, 5.69 mmol) in THF (10 ml) was added n-butyllitium (2.5 M in hexanes) (2.5 ml, 6.26 mmol) at -78 °C under nitrogen. The solution was warmed to room temperature and stirred for 30 minutes. The green solution was recooled to -78 °C and *N*-bromosuccinimide (1.11 g, 6.26 mmol) as a solution in THF (10 ml) was added. The yellow precipitate which formed was stirred at -78 °C for 30 minutes and at room temperature for one hour. The reaction mixture was then poured onto dichloromethane (100 ml) filtered to remove succinimide and the solvent removed under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel, ethyl acetate: petroleum ether 1:3) afforded a white preciptate. NMR spectroscopy and mass spectrometry indicated the loss of the methoxyethoxymethyl protecting group and formation of 2,4-dibromo-1-naphthol **(158)**; δ_H (300 MHz, C^2HCl_3) 6.07 (1H, s, 1'-O<u>H</u>), 7.73 (2H, m, 5', 8'-<u>H</u>), 7.91 (1H, s, 3'-<u>H</u>), 8.36 (2H, s, 6', 7'-<u>H</u>); δ_C

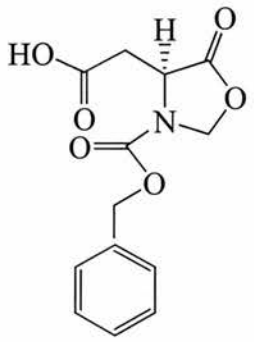
(75.4 MHz, C^2HCl_3) 103.52 (s, 2'-C), 113.75 (s, 4'-C), 122.52 (s, 8'-C), 122.62 (s, 9'-C), 123.13 (s, 5'-C), 127.25 (s, 6'-C), 304, 302, 300 ([*M*]$^+$, 52,100, 58), 222 (42, [*M*+2-Br]$^+$), 220 (44, [*M*-Br]$^+$), 195 (29, [$C_9H_5{}^{81}Br$]$^+$), 193 (31, [$C_9H_5{}^{79}Br$]$^+$), 113 (52, $(C_9H_5)^+$).

Attempted *ortho* bromination of 1-naphthol



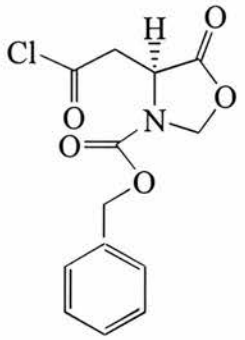
To a stirred solution of 1-naphthol (1.24 g, 8.63 mmol) and diisopropylethylamine (0.12 ml, 8.63 mmol) in methylene chloride (10 ml) was added a solution of *N*-bromosuccinimide (1.53 g, 8.63 mmol) as a solution in methlyene chloride (60 ml) slowly over 30 minutes under nitrogen and at room temperature. The reaction mixture was allowed to stir overnight. The reaction mixture was then acidified with 2.2 M H_2SO_4 and washed with water (2 × 25 ml). The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a brown solid. This was further purified (silica gel diethyl ether: petroleum ether 1:10) to afford a white solid. NMR spectroscopy and mass spectrometry showed the formation of 2,4-dibromo-1-naphthol **(158)** (0.63 g, 24%); δ_H (300 MHz, C^2HCl_3) 6.06 (1H, s, 1'-OH), 7.71 (2H, m, 5', 8'-H), 7.90 (1H, s, 3'-H), 8.30 (2H, m, 6',7'-H); δ_C (74.5 MHz, C^2HCl_3) 103.53 (s, s, 2'-C), 113.7 (s, 4'-C), 121.68 (s, 8'-C), 123.13 (s, 9'-C), 126.44 (s, 5'-C), 127.47 (s, 6'-C), 127.92 (s, 7'-C), 128.47 (s, 10'-C), 131.43 (s, 3'-C), 148.50 (s, 1'-C); *m/z* (EI) 301.8758 M^+ $C_{10}H_6Br_2O$ requires 301.8764, 304, 302, 300 ([*M*]$^+$, 48, 100, 50), 222, 220 (9, 10, [*M*-Br]$^{+)}$, 195 (19, [$C_9H_5{}^{81}Br$]$^+$), 193 (21, [$C_9H_5{}^{79}Br$]$^+$), 113 (30, [C_9H_5]$^+$).

***S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetic acid (175)**



To glacial acetic acid (85 ml) was added *N*-carbobenzyloxy-L-aspartic acid (5.22 g, 19.0 mmol), paraformaldehyde (1.76 g, 58.0 mmol), acetic anhydride (3.87 ml, 38.0 mmol) and thionyl chloride (0.29 ml, 4.0 mmol). The suspension was heated under reflux for three hours. The solvent was removed under reduced pressure to afford an orange residue. This was dissolved in ethyl acetate (50 ml) extracted into 5% $NaHCO_3$ (100ml). The pH was reduced to 1 with 6M HCl to afford a white precipitate. This was re-extracted into ethyl acetate (2 × 100 ml) washed with water (2 × 100 ml), brine (100 ml) and dried ($MgSO_4$). The solvent was removed under reduced pressure to afford a pale yellow oil. Further purification by column chromatography (silica gel dichloromethane: ethyl acetate 3:1) to afford a white sticky solid, (1.76 g, 33%); ν_{max} (nujol)/cm^{-1} 1820 (CO, oxazolidinone), 1734 (CO, acid), 1669 (CO, amide); δ_H (300 MHz, C^2HCl_3) 3.03 (2H, dd, *J* 15.0, 2.7 Hz, β-C$\underline{H}_2$), 4.34 (1H, bs, α-C$\underline{H}$), 5.18 (2H, bs, $C_6H_5C\underline{H}_2O$), 5.28 (1H, bs, $NC\underline{H}_2O$), 5.47 (1H, bs, $NC\underline{H}_2O$) 7.34 (5H, s, aromatic-H); δ_C (75.4 MHz, C^2HCl_3) 34.86 (s, β-$\underline{C}H_2$), 51.35 (s, α-$\underline{C}H$), 68.27 (s, $O\underline{C}H_2C_6H_5$), 78.45 (s, $N\underline{C}H_2O$), 128.51 (s, 2', 4', 6'-$\underline{C}$), 128.89 (s, 3', 5'-$\underline{C}$), 135.25 (s, 1'-$\underline{C}$), 152.94 (s, $N\underline{C}O$), 171.66 (s, $CH\underline{C}O_2CH_2$), 175.30 (s, $CH_2\underline{C}O_2H$); *m/z* (EI) 279 ($[M]^+$, 2), 235 (19, ($[M\text{-}CO_2]^+$), 144 (12, ($[M\text{-}C_7H_7]^+$), 126 (13, $[C_6H_6NO_6\text{-}H_2O]^+$), 91 (100, $[C_7H_7]^+$), 77 (10, $[C_6H_5]^+$), 65 (28, $[C_5H_5]^+$).

***S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride (68)**



To a stirred solution of *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazodinyl)-acetic acid (1.09 g, 3.91 mmol) in toluene (10 ml) was added oxalyl chloride (11.7 ml, 13.3 mmol). The reaction mixture was heated under reflux for two hours and the solvent was then removed under reduced pressure. The residue was washed several times with toluene to remove excess oxalyl chloride and afford an orange oil (1.06 g, 91%), which was used without further purification; δ_H (200 MHz, C^2HCl_3) 3.62 (2H, bs, β-C$\underline{H}_2$), 4.43 (1H, bs, α-C$\underline{H}$), 5.31 (2H, s, $C_6H_5C\underline{H}_2O$), 5.35 (1H, bs, $NC\underline{H}_2O$), 5.44 (1H, bs, $NC\underline{H}_2O$), 7.48 (5H, s, aromatic-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3) 46.66 (s, β-$\underline{C}H_2$) 51.95 (s, α-$\underline{C}H$), 68.73 (s, $O\underline{C}H_2C_6H_5$), 78.77 (s, $N\underline{C}H_2O$), 128.76 (s, 2',4',6'-$\underline{C}$), 129.14 (s, 3', 5'-$\underline{C}$), 135.33 (s, 1'-$\underline{C}$), 152.87 (s, N$\underline{C}$O), 170.51 (s, CH$\underline{C}O_2CH_2$), 172.09 (s, $CH_2\underline{C}OCl$); *m/z* (CI) 300, 298 ($[M+H]^+$, 4, 8), 256, 254 (11, 29 $[M+H\text{-}CO_2]^+$), 192 (59, $[CH_3CHN(CH_2)CO_2CH_2C_6H_5]^+$), 164 (8, $[N(CH_2)CO_2CH_2C_6H_5]^+$), 91 (100, $[C_7H7]^+$.

Attempted Grignard coupling of *S*-(3-benzoyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride

Procedure 1

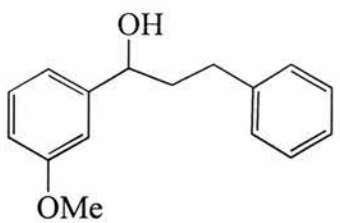
To magnesium turnings (0.26 g, 30.6 mmol) in THF (20 ml) was added 3-bromoanisole (0.40 ml, 3.23 mmol) under a nitrogen atmosphere. The reaction mixture was heated under reflux for two hours during which time a pale yellow solution had formed. This was added slowly over 15 minutes to a solution of *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride in THF at -77 °C under nitrogen. The orange solution was stirred at -77 °C for 1.5 hours then warmed to room temperature and poured onto 2M NH_4Cl (15 ml). This was extracted into diethyl ether (2 × 50 ml) washed with $NaHCO_3$

sat. (2 × 25 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Analysis by NMR spectroscopy showed only the presence of stating materials.

Procedure 2

To magnesium turnings (0.14 g, 5.86 mmol) in diethyl ether (7 ml) was added 3-bromoanisole (0.20 ml, 1.66 mmol) and a single crystal of iodine. This was heated under reflux for 25 minutes under an atmosphere of nitrogen until bubbling occurred and a red solution began to form. The reflux was stopped and the reaction continued until no more magnesium was present. The solution was cooled to 0 °C and to this a solution of *S*-(3-benzlyoxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride in THF (10 ml) was added. A pale yellow suspension formed which was stirred at room temperature for one hour poured onto 2M NH_4Cl (15 ml). This was extracted into diethyl ether (2 × 50 ml) washed with $NaHCO_3$ sat. (2 × 25 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Analysis by tlc and NMR spectroscopy indicated no reaction had occurred.

1-(3-Methoxy-phenyl)-2-phenyl-ethanol (178)



To magnesium turnings (0.33 g, 13.5 mmol) in diethyl ether (7 ml) was added 3-bromoanisole (1.72, 13.5 mmol) and a single crystal of iodine. The reaction mixture was heated under reflux for one hour during which time a red solution formed. The red solution was stirred under nitrogen until no more magnesium was present. The solution was then cooled to 0 °C and 3-phenylpropionaldehyde (1.77 ml, 13.5 mmol) was added slowly. The pale yellow reaction mixture was then stirred for a further 25 minutes 0 °C, poured onto crushed ice and acidified to pH 1 with 1 M H_2SO_4 (15 ml). The reaction

mixture was then extracted into diethyl ether (2 × 25 ml) dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a yellow oil. Further purification by column chromatography (silica gel ethyl acetate: petroleum ether 1:5) afforded a pale yellow oil (1.83 g, 56 %); ν_{max} (nujol)/cm^{-1} 3409 (br, OH), 1263 (C-O-CH_3), 1043 (C-O-CH_3); δ_H (300 MHz, C^2HCl_3) 1.99-2.11 (2H, m, $CH_2C\underline{H}_2CHOH$) 2.65-2.78 (2H, m, $C\underline{H}_2CH_2CHOH$), 4.66 (1H, t, *J* 7.0 Hz, $CH_2C\underline{H}OH$), 6.82-6.85 (1H, m, 4-H), 6.93-6.94 (2H, m, 2, 6-H), 7.17-7.31 (6H,m, 5-H and aromatics-C_6H_5); δ_C (75.4 MHz, C^2HCl_3) 32.03 (s, $\underline{C}H_2CHOH$), 40.05 (s, $\underline{C}H_2CH_2CHOH$), 55.24 (s, $O\underline{C}H_3$), 73.83 (s, $CH_2\underline{C}HOH$), 111.56 (s, 1-C), 113.19 (s, 4-C), 114.04 (s, 5-C), 119.85 (s, 6-C), 126.00 (s, 2', 6'-C), 128.54 (s, 4'-C), 129.60 (s, 3', 5'-C), 141.98 (s, 1'-C), 146.54 (s, 1-C), 160.03 (s, 3-C); *m/z* (EI) 242 ($[M]^+$, 100), 224 (13, ($[M-H_2O]^+$), 137 (70, $[CH_3OC_6H_4CHOH]^+$), 91 (44, $[C_7H_7]^+$), 77 (26, $[C_6H_5]^+$), 65 (10, $[C_5H_5]^+$).

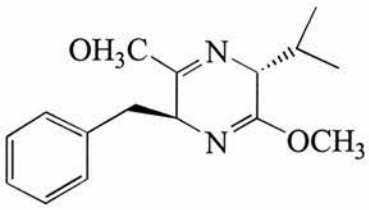
***In situ* preparation of o-anisic acid anhydride of *S*-(3-benzoyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride and subsequent Grignard reaction with 3-bromoanisole**

To a stirred solution of *S*-(3-benzyloxycarbonyl-5-oxo-4-oxazolidinyl)-acetyl chloride (0.34 g, 1.14 mmol) in THF (20 ml) at –13 °C and under a nitrogen atmosphere was added a premixed solution of *o*-anisic acid (0.173 g, 1.14 mmol) and triethylamine (0.16 ml, 1.14 mmol) in THF (10 ml). The reaction mixture was stirred at -13 °C for a further 15 minutes and at room temperature for two hours during which time a white suspension formed. This was used without further purification; δ_H (200 MHz, C^2HCl_3) 3.31 (2H, bs, β-$C\underline{H}_2$), 3.87 (3H, s, $OC\underline{H}_3$), 4.44 (1H, bs, α-C<u>H</u>), 5.22 (2H, s, $C\underline{H}_2C_6H_5$), 5.38 (1H, s, $NC\underline{H}_2O$), 5.51 (1H, s, $NC\underline{H}_2O$), 7.18 (2H, m, 2', 4'-H), 7.38 (5H, s, aromatic-H C_6H_5), 7.59 (1H, t, *J* 7.5 Hz, 5'-H), 8.02 (1H, d, *J* 7.5); δ_C (75.4 MHz, C^2HCl_3) 35.39 (s, β-$\underline{C}H_2$), 51.79 (s, α-$\underline{C}H$), 56.76 (s, $O\underline{C}H_3$), 68.65 (s, $O\underline{C}HC_6H_5$), 78.95 (s, $N\underline{C}H_2O$), 112.77 (s, 2-C), 112.87 (s, 6-C), 121.35 (s, 4- C), 128.67 (s, 2', 4', 6'-C), 129.03 (s, 3', 5'-C), 133.71 (s, 3-C), 135.52 (s, 5-C), 136.83 (s, 1'-C), 153.17 (s, N<u>C</u>O), 161.41 (s, 1-C), 166.24 (s, $MeOC_6H_5\underline{C}OOCOCH_2$), 167.37 (s, $MeOC_6H_5COO\underline{C}OCH_2$), 171.90 (s, $CH\underline{C}OCH_2$).

To magnesium turnings (0.04, 1.66 mmol) in THF (10 ml) was added 3-bromoanisole (0.21 ml, 1.66 mmol) and a single crystal of iodine. The solution was heated under reflux until the magnesium disappeared and the solution went clear. This solution was added to

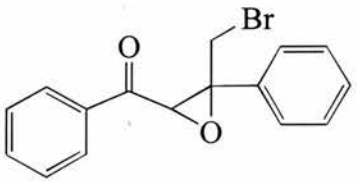
a solution of the anhydride at -78 °C and under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stir for 40 minutes before being poured onto 1M NH_4Cl (15 ml). This was extracted into diethyl ether (2× 50 ml), washed with brine (50 ml), dried $MgSO_4$ and the solvent removed under reduced pressure to afford a yellow oil. Analysis by NMR spectroscopy showed no evidence for the formation of the desired ketone.

(3*R*, 6*S*)-6-Benzyl-3-isopropyl-2,5-dimethoxy-3,6-dihydroxypyrazine (186)



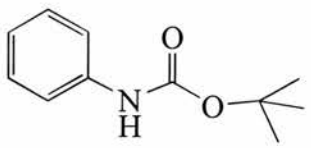
To a stirred solution of (3*R*)-(3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine (0.24 ml, 1.35 mmol) in THF (10 ml) was added n-butyllithium (2.5 M in hexanes)(0.65 ml, 1.62 mmol) at -78 °C and under an atmosphere of nitrogen. This was stirred for one hour before the addition of benzyl bromide (0.16 ml, 1.36 mmol). The reaction mixture was stirred at -78 °C for nine hours then warmed to room temperature and the solvent removed under reduced pressure. To the residue was added 100 mM potassium phosphate buffer (20 ml). The white suspension formed was extracted into diethyl ether (3 × 20 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a pale yellow oil. Further purification by column chromatography (silica gel; diethyl ether; petroleum ether 1:9) to afford a clear oil. (82 mg, 22 %); δ_H (300 MHz, C^2HCl_3) 0.73 (3H, d, *J* 7.0 Hz, ^{i}PrC$\underline{H}_3$), 1.06 (3H, d, *J* 7.0 Hz, ^{i}PrC$\underline{H}_3$), 2.25 (1H, sept, ^{i}PrC$\underline{H}$), 3.21 (2H, d *J* 4.8 Hz, C$\underline{H}_2$Ph), 3.39 (1H, t, *J* 3.3, 3-C$\underline{H}$), 3.79 (3H, s, OC$\underline{H}_3$), 3.84 (1H, s, OC$\underline{H}_3$), 4.44 (1H, dd, *J* 4.8, 1.5 Hz, 6-C$\underline{H}$), 7.19-7.41 (5H, m, aromatic-$\underline{H}$); δ_C (75.4 MHz, C^2HCl_3) 16.80 (s, ^{i}Pr$\underline{C}H_3$), 19.36 (s, ^{i}Pr$\underline{C}H_3$), 31.51 (s, ^{i}Pr$\underline{C}$H), 40.34 (s, $\underline{C}H_2$Ph), 52.48 (s, O$\underline{C}H_3$), 52.72 (s, O$\underline{C}H_3$), 56.96 (s, 3-$\underline{C}$H), 60.56 (s, 6-$\underline{C}$H), 126.61 (s, 4'-$\underline{C}$H), 128.13 (s, 3',5'-$\underline{C}$H), 130.31 (s, 2', 6'-$\underline{C}$H), 138.01 (s,1'-$\underline{C}$H), 162.11 (s, 2-C), 164.23 (s, 5-C); *m/z* (EI) 274 ([*M*]$^+$, 12), 231 (7, [^{i}Pr]$^+$), 183 (48, [*M*-C_7H_7]$^+$), 141 (100, [$CH_3OCNCH_2CN(OCH_3)CH_2$]$^+$), 91 (30, [C_7H_7]$^+$).

(3-Bromomethyl)-3-phenyl-oxiranyl)-phenyl-methanone (188)



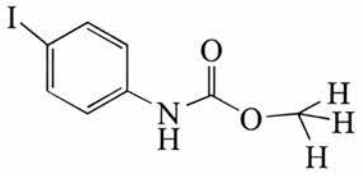
To a stirred solution of (3*R*)-(3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazine (0.45 ml, 2.51 mmol) in dry THF (5 ml) under an atmosphere of nitrogen and at -78 °C was added n-butyllithium (2.5 M solution in hexanes) (1.20 ml, 3.01 mmol). The bright yellow solution was stirred for 30 minutes and α-bromoacetophenone (1.00 g, 5.02 mmol) was added as a solution in THF (10 ml) and the reaction mixture stirred at -78 °C for eight hours. The reaction was stopped and (100 mM) potassium phosphate buffer (20 ml) pH 7.0 was added. The mixture was extracted into diethyl ether (3 × 50 ml) dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel; ethyl acetate: petroleum ether 1:5) gave one main band as a white solid, (0.54 g, 33%) m.p. 202-204 °C; δ_H (300 MHz, C^2HCl_3) 3.813 (1H, d, 11 Hz, H_b-C$\underline{H}_2$), 3.97 (1H, d, *J* 11 Hz, H_a- C$\underline{H}_2$), 4.56 (1H, s, C$\underline{H}$), 7.5-8.15 (10, m, aromatic-$\underline{H}$); δ_C (74.5 MHz, C^2HCl_3) 31.69 (s, $\underline{C}H_2$), 65.44 (s, 3-$\underline{C}$), 67.31 (s, 4-$\underline{C}$), 126.52 (s, 2', 4', 6'-$\underline{C}$), 128.79 (s, 3', 5'-$\underline{C}$), 129.158 (s, 2'', 6''- 3'',5''-$\underline{C}$), 134.57 (4''-$\underline{C}$), 135.99 (s, 1''-$\underline{C}$), 136.89 (s, 1'-$\underline{C}$); *m/z* (EI) 319,317 ($[M]^+$, 2,2), 223 (100, ($[M$-$CH_2Br]^+$), 105 (40, $[C_6H_5CO]^+$), 77 (29, $[C_7H_7]^+$). For crystallographic data see appendix (Crystal data set B).

***N*-(Boc)-aniline (193)**



To a solution of aniline (5 ml, 54.0 mmol) in THF (20 ml) was added triethylamine (7.52 ml, 54.0 mmol) under an atmosphere of nitrogen. The solution was cooled to 0 °C and di-*tert*-butylpyrocarbonate (11.97 g, 54.0 mmol) was added slowly as a solution in THF (20 ml). The reaction mixture was stirred overnight and then the solvent removed under reduced pressure to afford a white solid. Further purification by column chromatography (silica gel, ethyl acetate: petroleum ether 1:3) afforded a white solid (8.22 g, 79%), m.p. 126-128 °C; ν_{max} (nujol)/cm^{-1} 3308 (NH), 1643 (CO, urethane); δ_H (300 MHz, C^2HCl_3) 1.64 (9H, s, $OCOC(C\underline{H}_3)_3$, 6.63 (1H, s, $N\underline{H}CO_2C(CH_3)_3$), 7.14 (1H, t, *J* 7.0 Hz, 4-H), 7.28 (2H, t, *J* 7.0 Hz, 3, 5-H), 7.34 (2H, d, *J* 7.0 Hz, 2, 6-H); δ_C (75.4 MHz, C^2HCl_3) 28.72 (s, $OCOC(\underline{C}H_3)_3$), 80.84 (s, $OCO\underline{C}(CH_3)_3$), 118.91 (s, 2, 6-C), 123.38 (s, 4-C), 129.33 (s, 3, 5-C), 138.72 (s, 1-C), 153.15 (s, $NH\underline{C}O_2C(CH_3)_3$); *m/z* (EI) 193 ($[M]^+$, 17), 137 (52, $[C_6H_5NHCO_2H]^+$), 93 (68, $[C_6H_5NH_2]^+$), 57 (100, $[OCOC(CH_3)_3]^+$).

***N*-(Boc)-4-iodo-aniline (194)**



To a stirred solution of 4-iodoaniline (7.0 g, 32.0 mmol) in THF (20 ml) was added triethylamine (4.45 ml, 32.0 mmol) under an atmosphere of nitrogen. This was cooled to 0 °C and di-*tert*-butylpyrocarbonate (6.97 g, 32.0 mmol) in THF (20 ml) was added slowly over 15 minutes. The reaction was stirred overnight poured onto 1M HCl (30 ml). The solution was extracted into methylene chloride (2 × 50 ml), the organic layers combined, dried ($MgSO_4$) and the solvent removed under reduced pressure to afford an orange oil. Further purification by column chromatography (silica gel ethyl acetate: petroleum ether 1:1) afforded a white solid (3.56 g, 35%), mp 140-142 °C; ν_{max} (nujol)/cm^{-1} 3382 (NH), 1701 (CO, urethane); δ_H (300 MHz, C^2HCl_3) 1.66 (9H, s, $OCOC(C\underline{H}_3)_3$), 6.57 (1H, bs, $N\underline{H}CO_2C(CH_3)_3$), 7.26 (2H, d, *J* 9.0 Hz, 2, 6-<u>H</u>), 7.68 (2H, d, *J* 9.0 Hz, 3, 5-<u>H</u>); δ_C (75.4 MHz, C^2HCl_3) 28.68 (s, $OCOC(\underline{C}H_3)_3$), 81.29 (s, 4-<u>C</u>), 120.75 (s, 2, 6-<u>C</u>), 138.18 (s, 1, 3, 5-<u>C</u>), 152.79 (s, $O\underline{C}OC(CH_3)_3$); *m/z* (EI) 319.0061 $C_{11}H_{14}INO_2$ requires 319.0069, 319 ($[M]^+$, 40), 263 (100, $[M\text{-}C(CH_3)_3]^+$), 219 (42, $[IC_6H_4NH_2]^+$), 91 (5, $[C_7H_7]^+$), 57 (52, $[(CH_3)_3C]^+$).

6.2 Cloning and Expression of human kynureninase in baculovirus/insect cell system

The protocol for the expression of human kynureninase in insect cells was developed by Dr. Deirdre Fitzgerald. The cDNA clone encoding human liver kynureninase, was a gift from Dr. Andrea Cesura, Hofmann la Roche Laboratories, Basel Switzerland. The cDNA was isolated by the method of Alberati-Giani *et al.*[37] and cloned into the eukaryotic expression vector pBC/CMV.[195] The 1600 base pair (bp) kynureninase clone was inserted into the Sma site of the multiple cloning site and was supplied, in this form to this laboratory. The Sma site is flanked on either side by an EcoR 1 site and BamH1 site. The 1600 bp cDNA encoding kynureinase was isolated from an agarose gel of a double digest, with these two restriction enzymes.

The 'Bac to Bac' Baculovirus Expression System (GibcoBRL) was used to express kynureinase in *sf*9 insect cells. The protocols used were those described in the manufacturers manual. Kynureninase cDNA was cloned into pFastBac donor plasmid, which was then transformed into DH10Bac cells for transposition to the bacmid. *sf*9 cells were then transfected with recombinant bacmid DNA. To determine the optimal conditions for expression of kynureninase, preliminary studies were conducted in *sf*9 cells grown on 6-well plates, where both the duration of infection and the concentation of baculovirus were varied. Expression of kynureninase was monitored by SDS–PAGE analysis and by measuring enzyme activity on resulting cell sonicates.

Expression and isolation of Recombinant human kynureninase in SF9 insect cells

Preparing insect cells.

A tube of Foetal calf serum (100 ml) was preheated to 28 °C in a waterbath together with a bottle of TC-100 medium (500 ml). Foetal calf serum (37 ml) was then added to the TC-100 to produce complete medium. *sf*9 Insect cells (100 ml) in suspension were added to a spinner bottle in 10 ml aliquots. To this was added complete medium (400 ml) and the suspension stirred at 28 °C for 48-72 hours.

To check cell confluence a 10 ml suspension was added to a small petri dish and incubated at 28 °C for two hours then checked using light microscopy.
Cells (2 ×100 ml) grown for three days were then added to complete medium (2 × 400 ml) in (2 ×500 ml) flasks and allowed to grow for a further three days.

Insect cell infection with Baculovirus.

Baculovirus stock was preheated to 28 °C together with TC-100. The cell suspensions were then centrifuged in for 4-6 min at 1500 rpm. The cell pellets were resuspended in baculovirus (4 × 20 ml) and incubated at 28 °C for two hours. These were then divided between (2 × 500 ml) bottles and and made up to 500 ml with TC-100. Infection was allowed to proceed for 96 hours.

Harvesting kynureninase from infected *sf*9 cells.

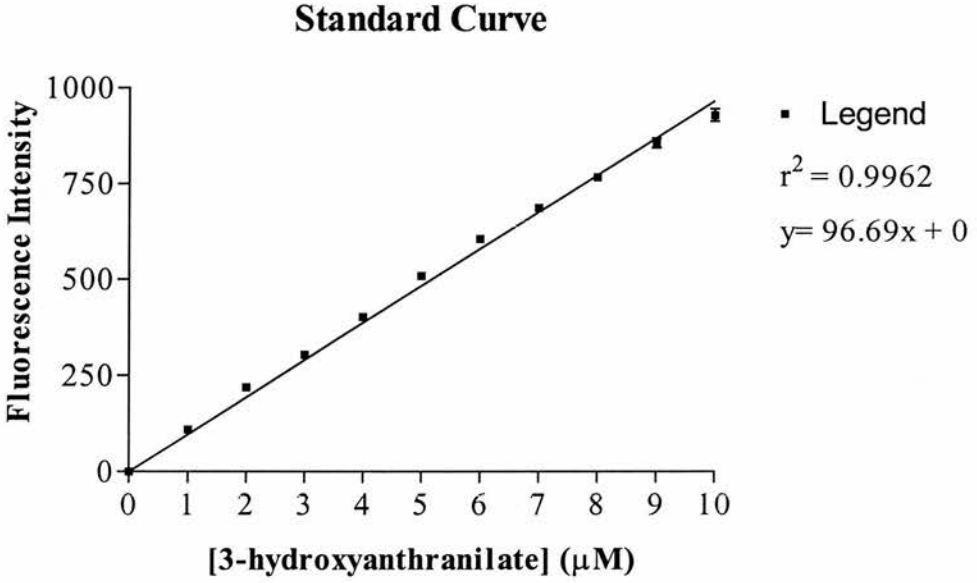
The infected *sf*9 cells were the harvested by centrifugation of 50 ml aliquots at 1500 rpm for 4-6 minutes. A portion of the supernatant which contained the baculovirus was transferred to a new tube and centrifuged as above. This was sterile filtered and 40 mls transferred to a new tube and stored in the dark at -4 °C. The pelleted cells were then resuspended in cold buffer (15 ml) consisting of 20 mM Tris-base buffer (pH 7.5) containing; 0.25 M sucrose, 1 mM DTT; 0.5 mM EDTA, 100 μM PLP, 100 μM PMSF, 2 μg/ml aprotonin, 1 μg/ml pepstatin and 1μg/ml leupeptin. This was sonicated on ice, with ten twenty second bursts with twenty seconds between bursts. The sonicated fraction was then transferred to a 50 ml centrifuge tube and centrifuged at 16,400 rpm for 20 minutes at 4 °C. The supernatant was then assayed for kynureninase activity, aliquoted and stored at -80 °C.

6.3 Inhibition studies on kynurenine analogues

Assay of kynureninase activity

The activity of kynureninase was determined fluorimetrically at 37 °C, according to the method of Shetty and Gaertner[177] using a Perkin Elmer luminescence spectrometer (MODEL LS50B) connected to a GRANT circulating water bath. The rate of formation of product 3-hydroxyanthranilate was measured at wavelengths of excitation and emission at 330 nm and 410 nm. The wavelengths for excitation and emission for anthranilate are at 310 nm and 417 nm. The reaction mixture contained 20 μM pyridoxal 5'-phosphate, 10 mM Tris-base buffer (pH 7.9) the substrate *DL*-3-hydroxykynureine and an appropriate amount of enzyme (10-20 μl). The reaction was initiated by the addition of the enzyme.

The rate of reaction was measured in fluorescence intensity units/min (FI/min) For kynureninase one unit activity was that amount of enzyme required to convert 1 nmol of substrate per minute under standard conditions. A standard curve relating fluorescence intensity to amount of product formed was constructed.



Conversion of unit activity to fluorescence intensity/min

1 unit of activity = 1 nmol/min

the assay volume was 3ml

Therefore rate for 1 unit of activity = 0.333 nmol/min/ml

= 0.333 μmol/min/1000 ml

= 0.333 μM/min

From the standard curve

Slope= $\delta F/\delta C$= 96.69FI/μM

Therefore one unit enzyme activity corresponds to

Fluorescence FI/min = 0.333 (μM/min) × 96.69 (FI/μM)

= 32.197 FI/min

K_m and V_{max} determination for 3-hydroxykynurenine.

A number of batches of enzyme were prepared for this study. To compare with previous work the kinetic parameters, K_m and V_{max}, were determined by assaying for the activity of the enzyme at various concentrations of 3-hydroxykynurenine, 2.5-20 μM. The reaction was linear over the time course studied and the rates were measured in duplicate or triplicate. To calculate the kinetic parameters, initial rate data were then fitted to the Michaelis-Menten equation using non linear regression with the GraphPad Prism 3 software package on a PC.

Inhibition studies and the determination of kinetic parameters.

The inhibition of kynureninase by a number of compounds was examined by assaying the activity of the enzyme at various concentrations of 3-hydroxykynurenine, 2.5 to 20 μM, at appropriate concentrations of inhibitor. The range of inhibitor concentrations was determined by preliminary screening of each compound, assessing the % inhibition of enzyme activity at a fixed concentration of the substrate 3-hydroxykynurenine (25 μM). Inhibition studies for each compound were then carried out at three inhibitor concentrations. The reactions were linear over the time course measured and the rate was

measured in duplicate or triplicate. Kinetic parameters, K_m and V_{max}, were calculated by fitting initial rate data to the Michaelis-Menten equation using non linear regression with the GraphPad Prism 3 software package on a PC. K_i values were determined using linear regression plots of K_{app} versus [Inhibitor]. A Lineweaver-Burk Plot was also drawn for illustrative purposes.

Each inhibitor was compared to a standard assay of 3-hydroxykynurenine from 2.5 to 20 μM. Before each run an assay was carried out so that the fluorescence change could be corrected for one unit of enzyme activity and eliminate variations in activity from different batches of enzyme.

For example; Full data for a standard assay of 3-hydroxykynurenine from 2.5 to 20 μM is shown below (Table 6.1) together with the preliminary assay at 25 μM of 3-hydroxykynurenine to correct for one unit of enzyme activity.

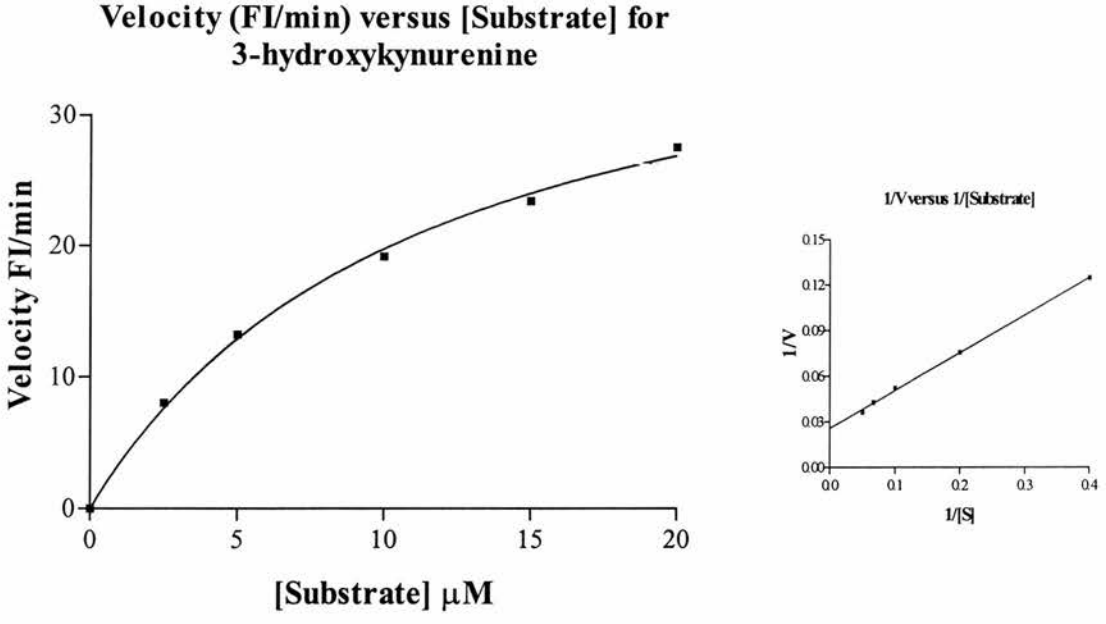
Fluorescence change for 25μM 3-hydroxykynureine = 29.03 FI/min
1 unit enzyme activity = 32.197 FI/min
Fluorescence change corrected for one unit enzyme activity = 32.197/29.03
Correction value = 1.109
Corrected fluorescence change for one unit enzyme activity = ΔFluorescence (FI/min) × 1.109

Measurement of K_m and V_{max} for 3-hydroxykynurenine

[Substrate] μM	ΔFluorescence (FI/min) for 3-hydroxykynurenine	ΔFluorescence (FI/min) for 3-hydroxykynureine corrected for unit enzyme activity	Average ΔFluorescence (FI/min) for 3-hydroxykunrenine Corrected for one unit enzyme activity
2.5	7.436	8.247	8.030
	7.051	7.820	
5.0	11.989	13.296	13.246
	11.844	13.136	
10.0	17.154	19.025	19.188
	17.449	19.352	
15.0	20.950	23.656	23.445
	21.330	23.235	
20.0	25.988	28.823	27.583
	23.751	26.342	

Table 6.1

This data was then fitted to the Michaelis-Menten rate equation using GraphPad Prism 3 software to determine the K_m and V_{max}. The values of 1/V were plotted against the reciprocal of substrate concentration 1/[S] in a Lineweaver-Burk Plot.



The K_m for this set of data was calculated as 5.67 ± 0.65 μM while V_{max} was calculated as 7.39 ± 0.40 nmol/min/mg.

The velocity versus [substrate] curve exhibits three distinct regions where the velocity changes with respect to substrate concentration. At very low [S] the velocity versus [substrate] curve is essentially linear so that the velocity is directly proportional to substrate concentration. As [S] increases this relationship breaks down and the increase in velocity is no longer proportional to [S]. At very high concentrations of [S] the reaction rate is essentially independent of [S] and reaches a maximum V_{max}. The relationship between reaction rate and [S] is described by the Henri-Michaelis-Menten equation.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

K_m, the Michaelis constant is defined as the substrate concentration at $1/2V_{max}$.
The average K_m for human kynureninase was calculated as 5.71±0.3 μM (Table 6.2) from a combination of all sets of data at zero inhibitor concentration.
The average V_{max} was determined to be 43.98 FI/min. V_{max} is expressed as nmol/min/mg which is obtained by converting the change in fluorescence to nmol of product and dividing by the protein concentration (mg/ml) of the enzyme as determined by Bradford Assay.

ΔFluorescence (FI/min) corrected for 1one unit activity enzyme = 43.98 ± 3.77 FI/min

Converting to nmol product/min using the slope of the standard curve

43.98 (FI/min) = 0.455 ± 0.04 μM/min
96.69 (FI/μM)

= 0.455 ±0.04 (μmoles/1000ml)/min

= 0.455 ±0.04 nmoles/min/ml

each assay had a total volume of 3 ml

= 0.455 ± 0.04 × 3 nmoles/min

= 1.365 ± 0.12 nmoles/min

10 μl of enzyme was used (0.01ml)

$$= \frac{1.365 \pm 0.12}{0.01} \text{ nmoles/min/ml}$$

$$= 136.5 \pm 12 \text{ nmol/min/ml}$$

protein concentration = 17.7 mg/ml

$$V_{max} = \frac{136.5 \pm 12 \text{(nmol/min/ml)}}{17.7 \text{ (mg/ml)}}$$

$$= 7.71 \pm 0.6 \text{ nmol/min/mg}$$

Protein Determination

Protein was determined by the method of Bradford[196] using bovine serum albumin as a standard (0-10 μg in 0.1 ml). Bradford reagent 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 standard curve was plotted and the protein concentration (mg/ml) determined for the crude enzyme harvested from *sf*9 cells.

K_m Determination for human kynureninase.

K_m (μM)	error	V_{max} (FI/min)	error	V_{max} nmol/min/mg	error
5.75	**±1.89**	**42.25**	**±6.65**	**7.40**	**±1.16**
5.67	**±0.65**	**42.20**	**±2.31**	**7.39**	**±0.40**
6.42	**±1.19**	**39.97**	**±3.69**	**7.00**	**±0.65**
5.01	**±1.88**	**51.53**	**±8.72**	**9.03**	**±1.52**

Table 6.2

Average $K_m = 5.71 \pm 0.3$ μM

Average $V_{max} = 7.70 \pm 0.6$ nmol/min/mg

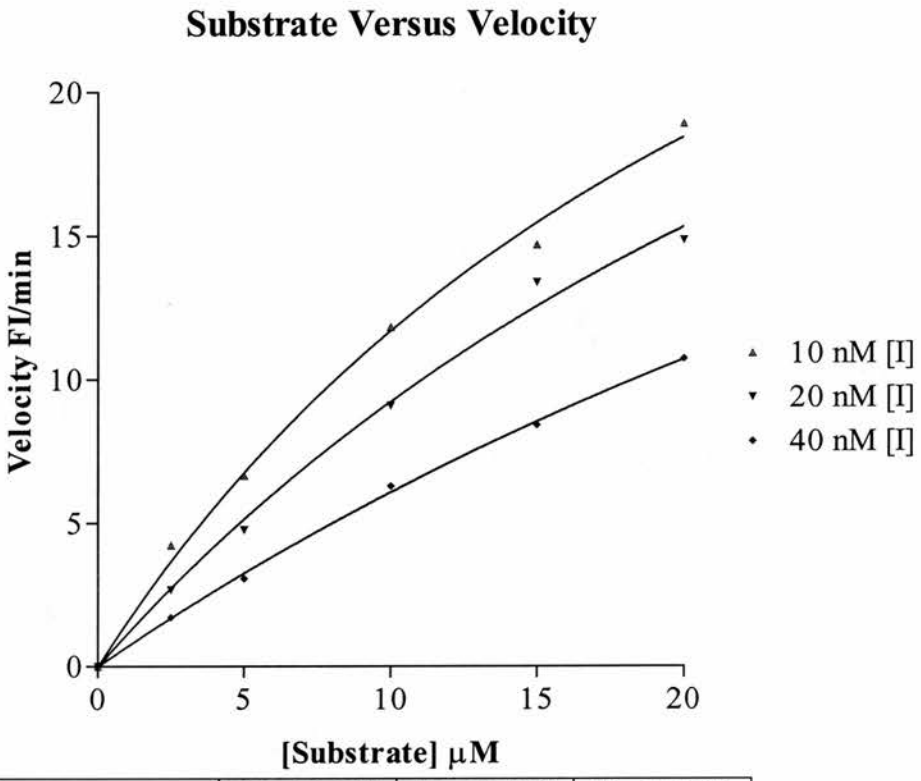
Turnover experiment

A turnover experiment was performed to determine whether the, inhibitor 3'-hydroxydesaminokynurenine **(87)** (K_i = 5 nmol), was hydrolysed by kynureninase under standard conditions, Tris-base buffer (10 mM, pH 7.9, 37 °C), to afford the hydrolysis product, 3-hydroxybenzoic acid. The reaction was monitored by analytical HPLC (C_{18}, 5μ, 6 ml/min, acetic acid, methanol, water 1:20:79). 3'-Hydroxydesaminokynureine (50 mg) was dissolved in Tris-base buffer (10mM, pH 7.9) (1 ml) and incubated at 37 °C with kynureninase (20 μl) for three hours. An aliquot (100 μl) of the reaction mixture was removed and trichloroacetic acid (1M, 100 μL) added to precipitate the enzyme. The solution was filtered and the solvent removed under reduced pressure to afford a white residue. This was dissolved in the HPLC eluent (acetic acid, methanol, water 1:20:79) and analysed by HPLC. There was no evidence for reaction when compared with a chromatogram of a mixture of 3-hydroxykynureine and 3-hydroxybenzoic acid, and only 3-hydroxy desaminokynurenine was observed. A further addition of kynureninase (50μl) was made and the reaction continued for a further three hours. Analysis by HPLC again showed no evidence for the presence of 3-hyroxybenzoic acid. A final addition kynureninase (50μl) was made and the reaction mixture allowed to incubate overnight. Analysis of the reaction mixture after workup with trichloroacetic acid again showed no evidence of hydrolysis having occurred.

Inhibition studies

The raw data for studies on the inhibition of human recombinant kynureninase by inhibitors synthesised in this work are given below.

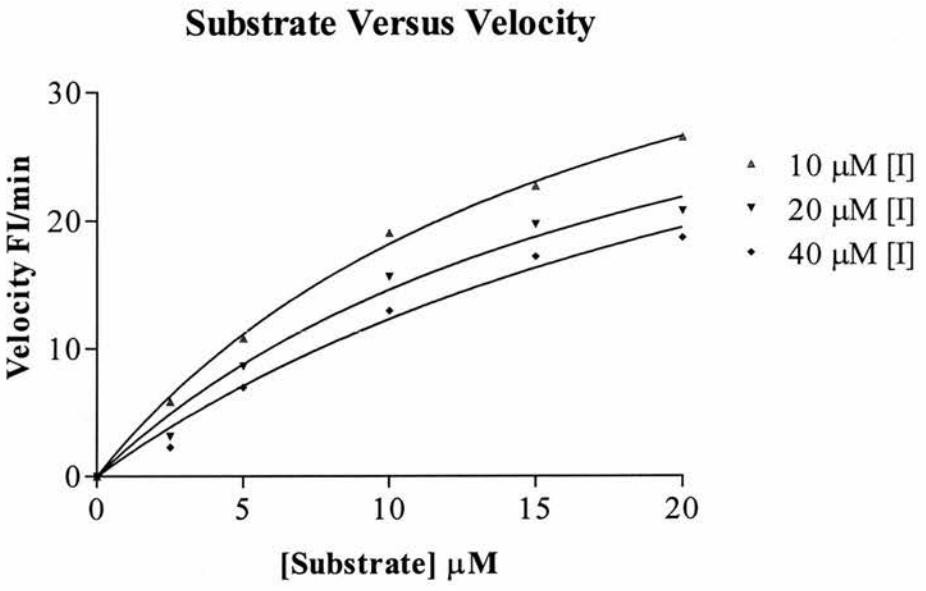
3'-Hydroxydesaminokynurenine (87)



[S] μM	10 nM [I][a] FI/min	20 nM [I][a] FI/min	40 nM [I][a] FI/min
2.5	4.24	2.68	1.73
5.0	6.66	4.76	3.08
10.0	11.83	9.09	6.29
15.0	14.69	13.38	8.41
20.0	18.91	14.84	10.72
V_{max} /nmol/min/mg	7.61 ± 1.15	7.86 ± 1.87	7.84 ± 1.29
K_m /μM	13.6 ± 3.66	19.3 ± 6.43	31.8 ± 6.51

[a] Fluorescence intensity values corrected for unit enzyme activity

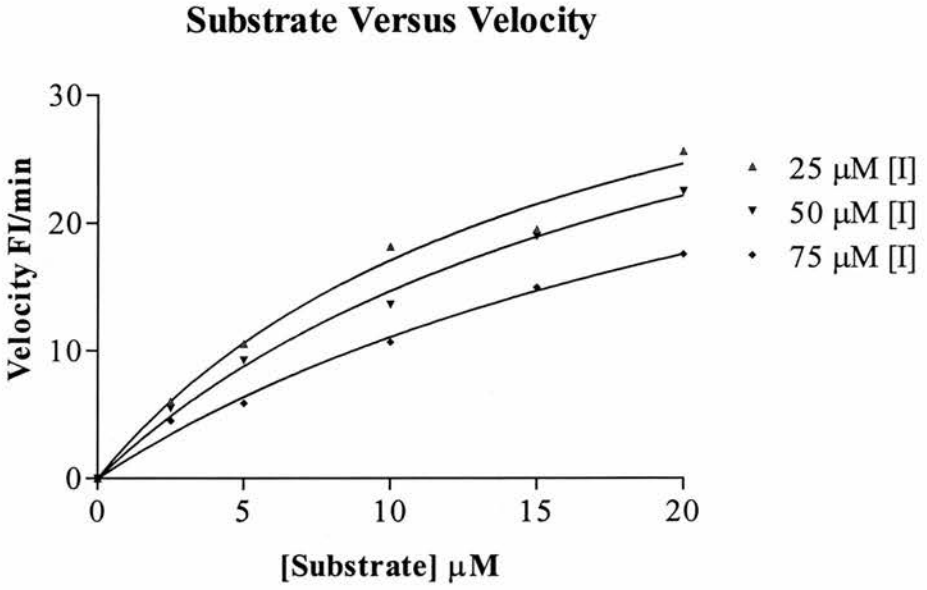
3'- Methoxydesaminokynurenine (88).



[S] μM	10 μM [I][a] FI/min	20 μM [I][a] FI/min	40 μM [I][a] FI/min
2.5	5.87	3.13	2.29
5.0	10.83	8.65	6.97
10.0	19.09	15.63	12.98
15.0	22.71	19.74	17.21
20.0	26.52	20.76	18.67
V_{max} /nmol/min/mg	8.65 ± 0.62	7.55 ± 1.67	8.15 ±2.28
K_m /μM	8.6 ± 1.09	9.75 ± 3.71	13.87 ± 5.52

[a] Fluorescence intensity values are corrected for unit enzyme activity.

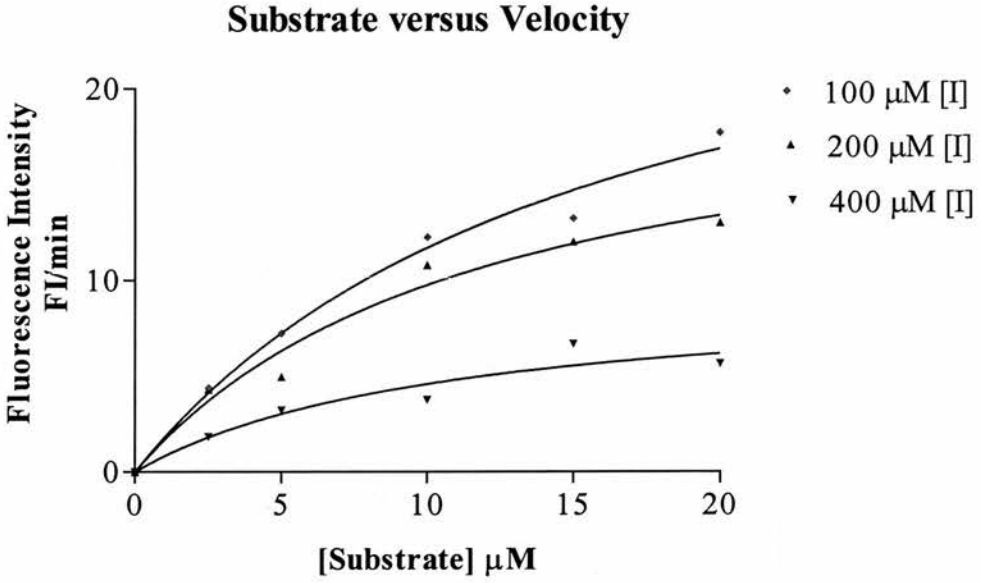
γ-hydroxy-3'-methoxy-desaminokynurenine (91)



[S] μM	25 μM [I][a] FI/min	50 μM [I][a] FI/min	75 μM [I][a] FI/min
2.5	6.04	5.52	4.50
5.0	10.57	9.27	5.89
10.0	18.19	13.46	10.67
15.0	19.50	18.97	14.99
20.0	25.61	22.47	17.55
V_{max} /nmol/min/mg	7.70 ± 1.24	7.88 ± 0.98	7.42 ±1.42
K_m /μM	7.9 ± 2.35	10.3 ± 2.16	14.12 ± 4.10

[a]Fluorescence intensities corrected for one unit enzyme activity

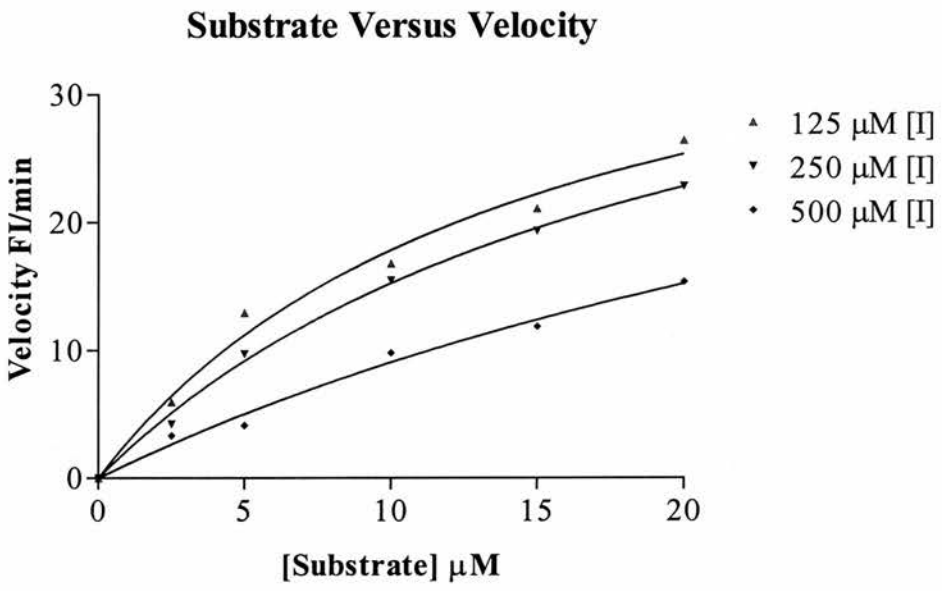
5-Hydroxy-1-tetralone derivative (89)



[S] μM	100μM [I][a] FI/min	200μM [I][a] FI/min	400μM [I][a] FI/min
2.5	4.38	4.31	1.84
5.0	7.25	4.98	3.22
10.0	12.27	10.82	3.44
15.0	13.23	12.03	6.96
20.0	17.71	13.00	5.62
V_{max} /nmol/min/mg	5.29 ± 0.91	3.71 ± 0.66	1.71 ± 0.66
K_m /μM	7.9 ± 2.53	5.92 ± 2.19	5.79 ± 2.96

[a]Fluorescence intensities corrected for one unit enzyme activity.

1,5-dihydroxytetralone derivative (92)



[S] μM	125μM [I][a] FI/min	250μM [I][a] FI/min	500μM [I][a] FI/min
2.5	5.96	4.24	3.32
5.0	12.96	9.74	4.13
10.0	16.82	15.48	9.83
15.0	21.10	19.37	11.88
20.0	26.44	22.83	15.38
V_{max} nmol/min/mg	7.65 ± 1.19	7.89 ± 0.72	8.23 ± 3.00
K_m /μM	7.2 ± 2.15	9.8 ± 1.51	20.9 ± 10.4

[a]Fluorescence intensities corrected for one unit enzyme activity.

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APPENDIX

EXPERIMENTAL DETAILS

A. Crystal Data

Empirical Formula	$C_{10}H_{11}NO_4$
Formula Weight	209.20
Crystal Color, Habit	clear, plate
Crystal Dimensions	0.12 X 0.12 X 0.07 mm
Crystal System	monoclinic
Lattice Type	Primitive
No. of Reflections Used for Unit	
Cell Determination (2θ range)	18 (15.3 - 25.0°)
Omega Scan Peak Width	
at Half-height	0.29°
Lattice Parameters	a = 10.509(6)Å b = 8.298(3) Å c = 10.767(5) Å β = 98.16(4)°
	V = 929.5(7) $Å^3$
Space Group	$P2_1/a$ (#14)
Z value	4
D_{calc}	1.495 g/cm^3
F_{000}	440.00
μ(CuKα)	9.90 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku AFC7S

Radiation	CuKα (λ = 1.54178 $\AA$) graphite monochromated
Attenuator	Ni foil (factor = 9.06)
Take-off Angle	6.0°
Detector Aperture	9.0 mm horizontal 13.0 mm vertical
Crystal to Detector Distance	400 mm
Voltage, Current	0kV, 0mA
Temperature	25.0°C
Scan Type	ω
Scan Rate	16.0°/min (in ω) (up to 4 scans)
Scan Width	(1.10 + 0.35 tan θ)°
$2\theta_{max}$	120.2°
No. of Reflections Measured	Total: 1586 Unique: 1497 (R_{int} = 0.035)
Corrections	Lorentz-polarization Absorption (trans. factors: 0.9627 - 1.0000) Decay (0.23% decline) Secondary Extinction (coefficient: 1.28368e-05)

C. Structure Solution and Refinement

Structure Solution	Direct Methods (SIR92)
Refinement	Full-matrix least-squares
Function Minimized	$\Sigma w(\|Fo\| - \|Fc\|)^2$
Least Squares Weights	$w = \frac{1}{\sigma^2(Fo)} = [\sigma_c^2(Fo) + \frac{p^2}{4}Fo^2]^{-1}$
p-factor	0.0000
Anomalous Dispersion	All non-hydrogen atoms
No. Observations (I>3.00σ(I))	1197
No. Variables	137

Reflection/Parameter Ratio	8.74
Residuals: R; Rw	0.038 ; 0.031
Goodness of Fit Indicator	3.87
Max Shift/Error in Final Cycle	0.01
Maximum peak in Final Diff. Map	0.19 $e^-/\AA^3$
Minimum peak in Final Diff. Map	-0.19 $e^-/\AA^3$

Table 1. Atomic coordinates and B_{iso}/B_{eq}

atom	x	y	z	B_{eq}
O(3)	0.5300(2)	0.3391(2)	1.2041(2)	4.82(5)
O(7)	0.5953(2)	0.0504(2)	0.7109(1)	3.92(4)
O(10)	0.6231(1)	0.4038(2)	0.5916(2)	3.57(4)
O(11)	0.5745(2)	0.2199(2)	0.4404(1)	3.68(4)
N(9)	0.3647(2)	0.1156(2)	0.5284(2)	3.21(5)
C(1)	0.5935(2)	0.1413(3)	0.9188(2)	2.90(5)
C(2)	0.5383(2)	0.2401(3)	1.0006(2)	3.15(6)
C(3)	0.5850(2)	0.2378(3)	1.1277(2)	3.33(6)
C(4)	0.6871(2)	0.1380(3)	1.1731(2)	3.58(6)
C(5)	0.7412(2)	0.0396(3)	1.0912(2)	3.80(6)
C(6)	0.6953(2)	0.0397(3)	0.9647(2)	3.50(6)
C(7)	0.5475(2)	0.1409(3)	0.7819(2)	3.04(5)
C(8)	0.4427(2)	0.2559(3)	0.7284(2)	2.94(5)
C(9)	0.4265(2)	0.2645(3)	0.5866(2)	2.81(5)
C(10)	0.5527(2)	0.2972(3)	0.5358(2)	3.03(6)
H(2)	0.4688	0.3090	0.9696	3.7766
H(3o)	0.5381	0.2827	1.2943	6.1640
H(4)	0.7197	0.1373	1.2600	4.2995
H(5)	0.8108	-0.0290	1.1225	4.5608
H(6)	0.7328	-0.0289	0.9092	4.2049
H(8a)	0.3639	0.2213	0.7531	3.5292
H(8b)	0.4633	0.3605	0.7612	3.5292
H(9a)	0.3660	0.1133	0.4519	6.1640
H(9b)	0.4084	0.0229	0.5617	6.1640

Table 1. Atomic coordinates and B_{iso}/B_{eq} (continued)

atom	x	y	z	B_{eq}
H(9)	0.3704	0.3520	0.5616	3.3696
H(9c)	0.2810	0.1159	0.5401	6.1640

$$B_{eq} = \frac{8}{3}\pi^2(U_{11}(aa^*)^2 + U_{22}(bb^*)^2 + U_{33}(cc^*)^2 + 2U_{12}aa^*bb^*\cos\gamma + 2U_{13}aa^*cc^*\cos\beta + 2U_{23}bb^*cc^*\cos\alpha)$$

Table 2. Anisotropic Displacement Parameters

atom	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
O(3)	0.086(1)	0.060(1)	0.038(1)	0.022(1)	0.011(1)	-0.002(1)
O(7)	0.060(1)	0.050(1)	0.039(1)	0.014(1)	0.0070(9)	-0.0038(9)
O(10)	0.041(1)	0.046(1)	0.050(1)	-0.0059(9)	0.0076(8)	-0.0044(9)
O(11)	0.056(1)	0.051(1)	0.036(1)	0.0015(9)	0.0144(8)	-0.0013(9)
N(9)	0.038(1)	0.043(1)	0.041(1)	0.000(1)	0.0045(9)	-0.005(1)
C(1)	0.041(1)	0.036(1)	0.034(1)	-0.003(1)	0.005(1)	0.001(1)
C(2)	0.044(2)	0.040(1)	0.035(1)	0.003(1)	0.005(1)	0.003(1)
C(3)	0.052(2)	0.039(1)	0.036(1)	-0.003(1)	0.011(1)	-0.003(1)
C(4)	0.051(2)	0.046(2)	0.037(1)	-0.005(1)	0.000(1)	0.003(1)
C(5)	0.048(2)	0.050(2)	0.045(2)	0.005(1)	0.000(1)	0.003(1)
C(6)	0.047(2)	0.043(2)	0.043(1)	0.005(1)	0.006(1)	-0.001(1)
C(7)	0.043(1)	0.036(1)	0.038(1)	-0.005(1)	0.009(1)	0.000(1)
C(8)	0.039(1)	0.038(1)	0.035(1)	0.000(1)	0.008(1)	-0.001(1)
C(9)	0.036(1)	0.035(1)	0.036(1)	0.000(1)	0.004(1)	-0.001(1)
C(10)	0.040(1)	0.039(1)	0.036(1)	0.006(1)	0.004(1)	0.007(1)

The general temperature factor expression:

$$\exp(-2\pi^2(a^{*2}U_{11}h^2 + b^{*2}U_{22}k^2 + c^{*2}U_{33}l^2 + 2a^*b^*U_{12}hk + 2a^*c^*U_{13}hl + 2b^*c^*U_{23}kl))$$

Table 3. Bond Lengths($\AA$)

atom	atom	distance	atom	atom	distance
O(3)	C(3)	1.361(3)	O(7)	C(7)	1.229(3)
O(10)	C(10)	1.251(3)	O(11)	C(10)	1.259(3)
N(9)	C(9)	1.492(3)	C(1)	C(2)	1.389(3)
C(1)	C(6)	1.396(3)	C(1)	C(7)	1.485(3)
C(2)	C(3)	1.387(3)	C(3)	C(4)	1.388(3)
C(4)	C(5)	1.381(3)	C(5)	C(6)	1.379(3)
C(7)	C(8)	1.508(3)	C(8)	C(9)	1.513(3)
C(9)	C(10)	1.529(3)			

Table 4. Bond Lengths($\AA$)

atom	atom	distance	atom	atom	distance
O(3)	H(3o)	1.07	N(9)	H(9a)	0.83
N(9)	H(9b)	0.94	N(9)	H(9c)	0.91
C(2)	H(2)	0.95	C(4)	H(4)	0.95
C(5)	H(5)	0.95	C(6)	H(6)	0.95
C(8)	H(8a)	0.95	C(8)	H(8b)	0.95
C(9)	H(9)	0.95			

Table 5. Bond Angles(°)

atom	atom	atom	angle	atom	atom	atom	angle
C(2)	C(1)	C(6)	120.0(2)	C(2)	C(1)	C(7)	121.6(2)
C(6)	C(1)	C(7)	118.4(2)	C(1)	C(2)	C(3)	119.7(2)
O(3)	C(3)	C(2)	117.8(2)	O(3)	C(3)	C(4)	121.9(2)
C(2)	C(3)	C(4)	120.3(2)	C(3)	C(4)	C(5)	119.7(2)
C(4)	C(5)	C(6)	120.8(2)	C(1)	C(6)	C(5)	119.5(2)
O(7)	C(7)	C(1)	121.0(2)	O(7)	C(7)	C(8)	119.3(2)
C(1)	C(7)	C(8)	119.7(2)	C(7)	C(8)	C(9)	112.8(2)
N(9)	C(9)	C(8)	111.4(2)	N(9)	C(9)	C(10)	110.1(2)
C(8)	C(9)	C(10)	113.0(2)	O(10)	C(10)	O(11)	126.0(2)
O(10)	C(10)	C(9)	116.0(2)	O(11)	C(10)	C(9)	118.0(2)

Table 6. Bond Angles(°)

atom	atom	atom	angle	atom	atom	atom	angle
C(3)	O(3)	H(3o)	107.0	C(9)	N(9)	H(9a)	111.8
C(9)	N(9)	H(9b)	110.9	C(9)	N(9)	H(9c)	108.2
H(9a)	N(9)	H(9b)	106.6	H(9a)	N(9)	H(9c)	107.0
H(9b)	N(9)	H(9c)	112.3	C(1)	C(2)	H(2)	120.1
C(3)	C(2)	H(2)	120.2	C(3)	C(4)	H(4)	120.2
C(5)	C(4)	H(4)	120.2	C(4)	C(5)	H(5)	119.6
C(6)	C(5)	H(5)	119.6	C(1)	C(6)	H(6)	120.3
C(5)	C(6)	H(6)	120.2	C(7)	C(8)	H(8a)	108.7
C(7)	C(8)	H(8b)	108.7	C(9)	C(8)	H(8a)	108.6
C(9)	C(8)	H(8b)	108.6	H(8a)	C(8)	H(8b)	109.5
N(9)	C(9)	H(9)	107.3	C(8)	C(9)	H(9)	107.3
C(10)	C(9)	H(9)	107.3				

Table 7. Non-bonded Contacts out to 3.60 $\AA$

atom	atom	distance	ADC	atom	atom	distance	ADC
O(3)	O(11)	2.708(2)	55601	O(3)	C(5)	3.265(3)	45504
O(3)	C(8)	3.442(3)	66703	O(3)	C(10)	3.562(3)	55601
O(3)	C(4)	3.577(4)	45504	O(7)	N(9)	3.003(2)	65603
O(7)	O(11)	3.170(2)	65603	O(10)	N(9)	2.725(3)	4
O(10)	C(10)	3.276(3)	66603	O(10)	C(9)	3.354(3)	66603
O(10)	O(10)	3.419(3)	66603	O(10)	C(9)	3.488(3)	4
O(10)	C(4)	3.569(3)	65702	O(11)	N(9)	2.866(3)	65603
O(11)	C(4)	3.334(3)	55401	O(11)	N(9)	3.353(3)	4
O(11)	C(3)	3.388(3)	55401	N(9)	C(10)	3.370(4)	45504
N(9)	N(9)	3.554(4)	65603	C(2)	C(6)	3.438(4)	65703
C(4)	C(7)	3.466(4)	65703	C(10)	C(10)	3.592(5)	66603

The ADC (atom designator code) specifies the position of an atom in a crystal. The 5-digit number shown in the table is a composite of three one-digit numbers and one two-digit number: TA (first digit) + TB (second digit) + TC (third digit) + SN (last two digits). TA, TB and TC are the crystal lattice translation digits along cell edges a, b and c. A translation digit of 5 indicates the origin unit cell. If TA = 4, this indicates a translation of one unit cell length along the a-axis in the negative direction. Each translation digit can range in value from 1 to 9 and thus ± 4 lattice translations from the origin (TA=5, TB=5, TC=5) can be represented.

The SN, or symmetry operator number, refers to the number of the symmetry operator used to generate the coordinates of the target atom. A list of symmetry operators relevant to this structure are given below.

For a given intermolecular contact, the first atom (origin atom) is located in the origin unit cell and its position can be generated using the identity operator (SN=1). Thus, the ADC for an origin atom is always 55501. The position of the second atom (target atom) can be generated using the ADC and the coordinates of the atom in the parameter table. For example, an ADC of 47502 refers to the target atom moved through symmetry operator two, then translated -1 cell translations along the a axis, +2 cell translations along the b axis, and 0 cell translations along the c axis.

An ADC of 1 indicates an intermolecular contact between two fragments (eg. cation and anion) that reside in the same asymmetric unit.

Symmetry Operators:

(1)	X,	Y,	Z	(2)	1/2-X,	1/2+Y,	-Z
(3)	-X,	-Y,	-Z	(4)	1/2+X,	1/2-Y,	Z

EXPERIMENTAL DETAILS

B. Crystal Data

Empirical formula	C16 H13 Br O2	
Formula weight	317.17	
Temperature	293(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)/c	
Unit cell dimensions	a = 10.19(2) Å	α= 90°.
	b = 11.79(3) Å	β= 108.85(2)°.
	c = 11.95(3) Å	γ = 90°.
Volume	1360(5) $Å^3$	
Z	4	
Density (calculated)	1.549 Mg/m^3	
Absorption coefficient	3.017 mm^{-1}	
F(000)	640	
Crystal size	.21 x .1 x .02 mm^3	
Theta range for data collection	2.11 to 23.29°.	
Index ranges	-11<=h<=11, -9<=k<=13, -13<=l<=13	
Reflections collected	5527	
Independent reflections	1925 [R(int) = 0.1100]	
Completeness to theta = 23.29°	98.0 %	
Absorption correction	Sadabs	
Max. and min. transmission	1.00000 and 0.634354	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	1925 / 0 / 173	
Goodness-of-fit on F^2	0.946	
Final R indices [I>2sigma(I)]	R1 = 0.0494, wR2 = 0.0750	
R indices (all data)	R1 = 0.1327, wR2 = 0.0978	
Extinction coefficient	0.0052(9)	
Largest diff. peak and hole	0.350 and -0.376 $e.Å^{-3}$	

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for ksnb2. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
O(1)	1299(4)	1388(3)	7882(4)	42(1)
C(1)	1212(6)	1855(5)	6759(5)	30(2)
C(2)	1688(6)	3053(5)	6775(6)	28(2)
C(3)	2403(6)	3587(6)	7834(6)	36(2)
C(4)	2877(6)	4686(7)	7833(7)	49(2)
C(5)	2633(7)	5262(6)	6807(8)	52(2)
C(6)	1932(7)	4748(7)	5753(7)	57(2)
C(7)	1450(6)	3645(6)	5730(6)	43(2)
C(8)	1566(5)	1039(5)	5947(5)	37(2)
Br(8)	3567(1)	905(1)	6312(1)	53(1)
C(9)	-55(6)	1572(5)	7082(5)	37(2)
C(10)	-895(6)	2506(6)	7357(6)	39(2)
O(10)	-484(4)	3025(4)	8279(4)	55(1)
C(11)	-2275(6)	2745(5)	6471(6)	31(2)
C(12)	-2722(7)	2251(5)	5366(6)	43(2)
C(13)	-4017(7)	2506(6)	4590(6)	50(2)
C(14)	-4869(7)	3241(6)	4898(8)	57(2)
C(15)	-4427(7)	3739(5)	5993(8)	58(2)
C(16)	-3138(7)	3486(6)	6770(6)	47(2)