UNIVERSITY OF ST. ANDREWS

SCHOOL OF CHEMISTRY

STUDIES ON THE SYNTHESIS OF NOVEL INHIBITORS AND SUBSTRATES FOR KYNURENINASE

A thesis presented for the degree of

Doctor of Philosophy

to the

University of St.Andrews

on 26th June 2000

by

Karen M Muirhead

Supervisor: Dr. Nigel Botting

I, Karen Muirhead, hereby certify that this thesis, which is approximately 51 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date 26 June 2000 signature of candidate

I was admitted as a research student in October 1996 and as a candidate for the degree of PhD in August 1997; the higher study for which this is a record was carried out in the University of St. Andrews between 1996 and 2000.

date 26 June 2000 signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

date 26th June 2000 signature of supervisor

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any *bona fide* library or research worker.

date 26 June 2000 signature of candidate

ACKNOWLEDGEMENTS

The course of true love and PhDs never did run smooth and I am grateful to the following for their support, both in the academic sense and for their friendship and support. Firstly my thanks go to my supervisor Dr. Nigel Botting for his unfailing optimism, fantastic birthday cakes, meals and gossip. I must also thank Dr. Deirdre Fitzgerald for conducting the enzyme studies with the compounds synthesised in this thesis.

The present Botting Group: Deirdre, Harold, Tara, Mark, Matt, Karen, Quique and Humphrey have made the lab a friendly place to work and the regular panini trips are greatly missed. Past members of the Botting Group also deserve thanks, especially Jacqui and Pauline for their company at the Cellar Bar. I have met many people over my four years in St. Andrews, and although many have moved on to bigger things, they all remain friends. This page is not large enough to do them all justice but I thank Mark, Sue, Janette, Claire, Dave, Mike, Colin, Arwel and Doug especially for their support, friendship and advice.

Thanks are also due to the technical staff of the School of Chemistry at the University of St. Andrews: Melanja Smith (Nmr), Colin Millar (MS), Sylvia Williamson (Microanalysis) and Colin Smith (Glassblowing). Also, a big thank you to Mel and the gang for introducing me to benchball and a little exercise.

For financial support during this project I would like to thank the BBSRC and the University of St. Andrews.

None of this would have been possible without the support of my family, and I thank my Dad, Susan and Jean for their enthusiasm and encouragement especially when I was feeling at my lowest. My thanks also go to my Mum and Chris.

I would also like to thank Dr. Alan Aitken for putting me in touch with the right people and subsequently for finding me a job, although I don't think my parents were as pleased as he was when they heard I was moving to Stuttgart. Finally I would like to thank Mark Oldfield and Tara Fryatt again for the help they have given from afar in submitting this thesis.

ABBREVIATIONS

3-HAO	3-hydroxyanthranilic acid oxygenase
3-HK	3-hydroxykynurenine
5-HT	5-hydroxytryptamine, serotonin
AIDS	acquired immunodeficiency syndrome
αKG	α-ketoglutarate
Ala	alanine
Boc	(*tert*-butyloxycarbonyl)
CAG	cytosine-adenine-guanine
CBz	carbobenzyloxy
CNS	central nervous system
COSY	correlation spectroscopy
$^{D}(V/K)$	$(V_H/V_D)/(K_H/K_D)$
DCC	1,3-dicyclohexylcarbodiimide
DEAD	diethylazodicarboxylate
DIBAL-H	diisobutylaluminium hydride
^{D}K	(K_H/K_D)
DMAP	4-(dimethylamino)pyridine
DMF	*N,N'*-dimethylformamide
DMP	2,2-dimethoxypropane
DNA	deoxyribonucleic acid
^{D}V	(V_H/V_D)
EAA	excitatory amino acid
FAD	flavin adenine dinucleotide
Fmoc	N^{α}-9-fluorenyl-methoxycarbonyl
HD	Huntington's Disease
HIV	human immunodeficiency virus
IC_{50}	concentration of inhibitor to produce 50% inhibition

IDO	indolamine 2,3-dioxygenase
KAT	kynurenine aminotransferase
k_{cat}	enzyme catalytic constant / turnover number
KHMDS	potassium bis(trimethylsilyl)amide
K_i	enzyme inhibition constant
K_m	Michaelis constant
LDA	lithium diisopropylamide
LiBSBA	lithium (*tert*-butyldimethylsilyl)-*tert*-butylamide
MBA	(methoxybenzoyl)alanine
mCPBA	*meta*-chloroperoxybenzoic acid
MRS	magnetic resonance spectroscopy
MTPA	α-methoxy-α-trifluoromethylphenylacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NaHMDS	sodium bis(trimethylsilyl)amide
NBA	(nitrobenzoyl)alanine
NMDA	*N*-methyl-D-aspartate
nmr	nuclear magnetic resonance
PLP	pyridoxal 5'-phosphate
QPRTase	quinolinate phosphoribosyltransferase
ROS	reactive oxygen species
TDO	tryptophan 2,3-dioxygenase
THF	tetrahydrofuran
tlc	thin layer chromatography
UV	ultraviolet
V_{max}	maximum rate of substrate turnover at saturation

CONTENTS

STUDIES ON THE SYNTHESIS OF NOVEL INHIBITORS AND SUBSTRATES FOR KYNURENINASE

Kynureninase (EC 3.7.1.3) is a PLP dependent enzyme which catalyses the hydrolytic cleavage of its natural substrates 2*S*-kynurenine and 3-hydroxykynurenine to 2*S*-alanine and their respective anthranilic acids. The major metabolites of kynurenine, quinolinic acid and kynurenic acid have pronounced effects on neuronal activity and have been implicated as an etiological factor in a range of neurodegenerative diseases such as Huntington's disease, Lyme disease and temporal lobe epilepsy. As such the pathway has been identified as an important target for drug action for effective treatment of these conditions.

It has been our aim to synthesise a series of analogues of kynurenine and kynurenic acid to act as inhibitors of both the bacterial and mammalian forms of kynurenine. A series of bicyclic kynurenine analogues were synthesised and tested as racemates as inhibitors of kynureninase. The most promising of these was an indanone derivative which gave K_i values of 35 ± 9.6 μM and 45 ± 12 μM for bacterial and human kynureninase respectively. These results were five fold higher than those observed for the tetralone derivative (170 μM and 227 μM respectively). A chromanone derivative also showed promising results with the human enzyme (77 μM) although the K_i value observed for the bacterial enzyme was similar to that for the tetralone derivative.

A series of irreversible enzyme inhibitors was also synthesised. These alkenes however, were found to be competitive inhibitors at low concentrations and only irreversible at concentrations of over 0.5 mM. Both the phenyl alkene and nitrophenyl alkene synthesised were found to form a Schiff's base complex with PLP.

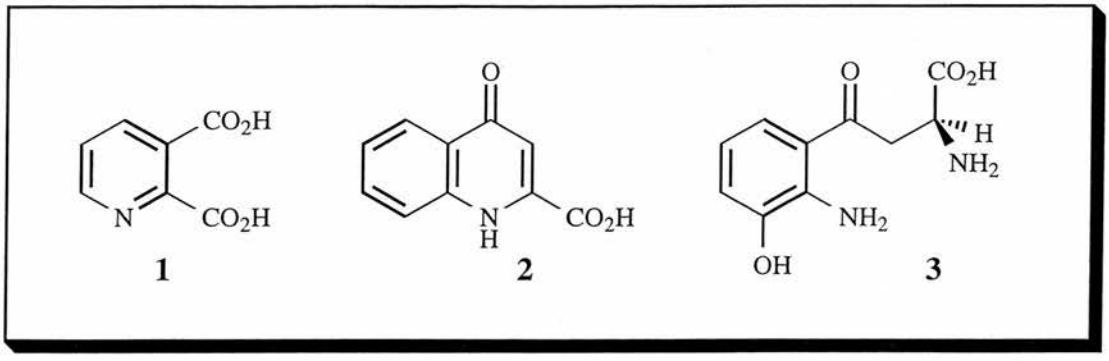
Finally, 5-fluorokynurenine and 6-fluorokynurenic acid were synthesised in order to monitor the conversion of kynurenine into kynurenic acid in the reaction catalysed by kynurenine aminotransferase. Initial ^{19}F-nmr studies showed that the fluorokynurenine synthesised was indeed a substrate for the enzyme kynureninase and was converted to fluoroanthranilic acid.

CHAPTER 1

1 INTRODUCTION

1.1 BACKGROUND

The kynurenine pathway was first recognised as a major route for the conversion of tryptophan to nicotinamide and its nucleotide derivatives in 1947.[1] Initial interest in this pathway was centred on the importance of nicotinamide and its role in vitamin B_6 deficiency. The discovery of cellular release of 5-hydroxytryptamine (5-HT) shifted research to concentrate on the importance of tryptophan as a precursor of this neurotransmitter. However, further research showed that only 1% of dietary tryptophan was converted into 5-HT[2] and over 95% is metabolised to kynurenines.[3] Although discovered in 1904 and 1964 respectively, research into kynurenic acid[4] and quinolinic acid[5] as neurotransmitters was not prominent until the early 80s. The high concentrations of these metabolites found in the liver had also shown a pronounced effect on neuronal activity and in particular at central neurone excitatory amino acid (EAA) receptors.[6] The resurgence in interest for this pathway is mainly due to interest in the role of EAA receptors in neurotransmission, memory, learning, excitotoxicity and neurodegenerative disorders. The implication of quinolinic acid as an etiological factor in a range of neurodegenerative diseases including Huntington's disease, AIDS related dementia, Lyme disease and temporal lobe epilepsy[7] and of kynurenic acid as a neuroprotective agent[8] has stimulated research into this pathway. Mechanistic studies on the enzymes involved in the pathway have provided information for the design of specific and effective inhibitors and the kynurenine pathway remains an important target for drug action in the design of effective treatments for neurodegenerative disorders.



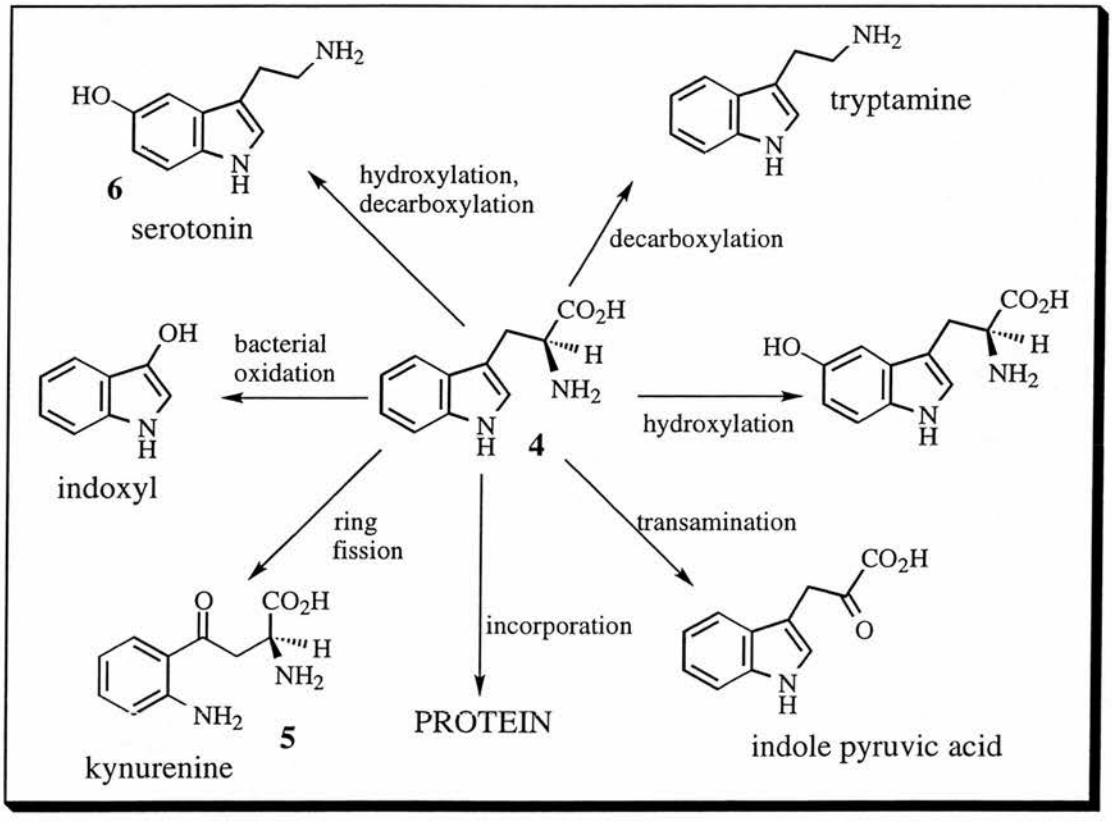
To date the two most important metabolites of kynurenine have been quinolinic acid and kynurenic acid. Both have been shown to have pronounced neuronal activity. Quinolinic acid (**1**) is a selective agonist of the *N*-methyl-D-aspartate (NMDA) population of receptors, causing neuronal damage by over stimulation of the receptor.[9] Quinolinic acid causes a selective pattern of cell death in rat brain which has similarities to the neuropathology of Huntington's disease and temporal lobe epilepsy in human brain.[10] High levels of quinolinate have also been observed in inflammatory brain diseases such as AIDS related dementia, polio virus and Lyme disease. It has also been demonstrated that quinolinate increases the permeability of the blood-brain barrier, it is not clear however, whether this is due to activation of the NMDA receptor, pH effects or extracellular osmolarity.[11]

In contrast, kynurenic acid (**2**) is a selective antagonist of the strychnine-insensitive glycine modulatory site related to the NMDA receptor[11] and has been shown to act as a neuroprotective antidote to quinolinic acid toxicity in brain.[12] Kynurenic acid cannot cross the blood-brain barrier in normal animals. It is important that this distinction is made as significant weakening of the blood-brain barrier is observed in many of the pathological states in which kynurenines are implicated. The barrier is known to be compromised in Alzheimer's disease, raising the possibility that increased kynurenate penetration may contribute in these conditions.[11] The balance of these two metabolites is important in maintaining the activity of the NMDA receptor. More recently research into 3-hydroxykynurenine (3-HK, **3**) and its role in neurodegenerative disorders[13] has implied

that 3-HK is a more potent neurotoxin than quinolinic acid,[14] and the hypothesis that its toxicity is mediated by oxidative stress is well supported.[15]

1.2 THE TRYPTOPHAN METABOLIC PATHWAY

Tryptophan (**4**) is an amino acid essential in mammalian systems, yet unlike lower organisms humans are unable to synthesise tryptophan biologically. As the relative amount of tryptophan *in vivo* is small, our needs are met entirely by dietary intake.[16] The only other amino acid found in comparably low concentrations in tissue and plasma is methionine. Tryptophan is a precursor of many substances and it is metabolised by a series of complicated pathways, the serotonin, tryptamine and kynurenine pathways.[7]



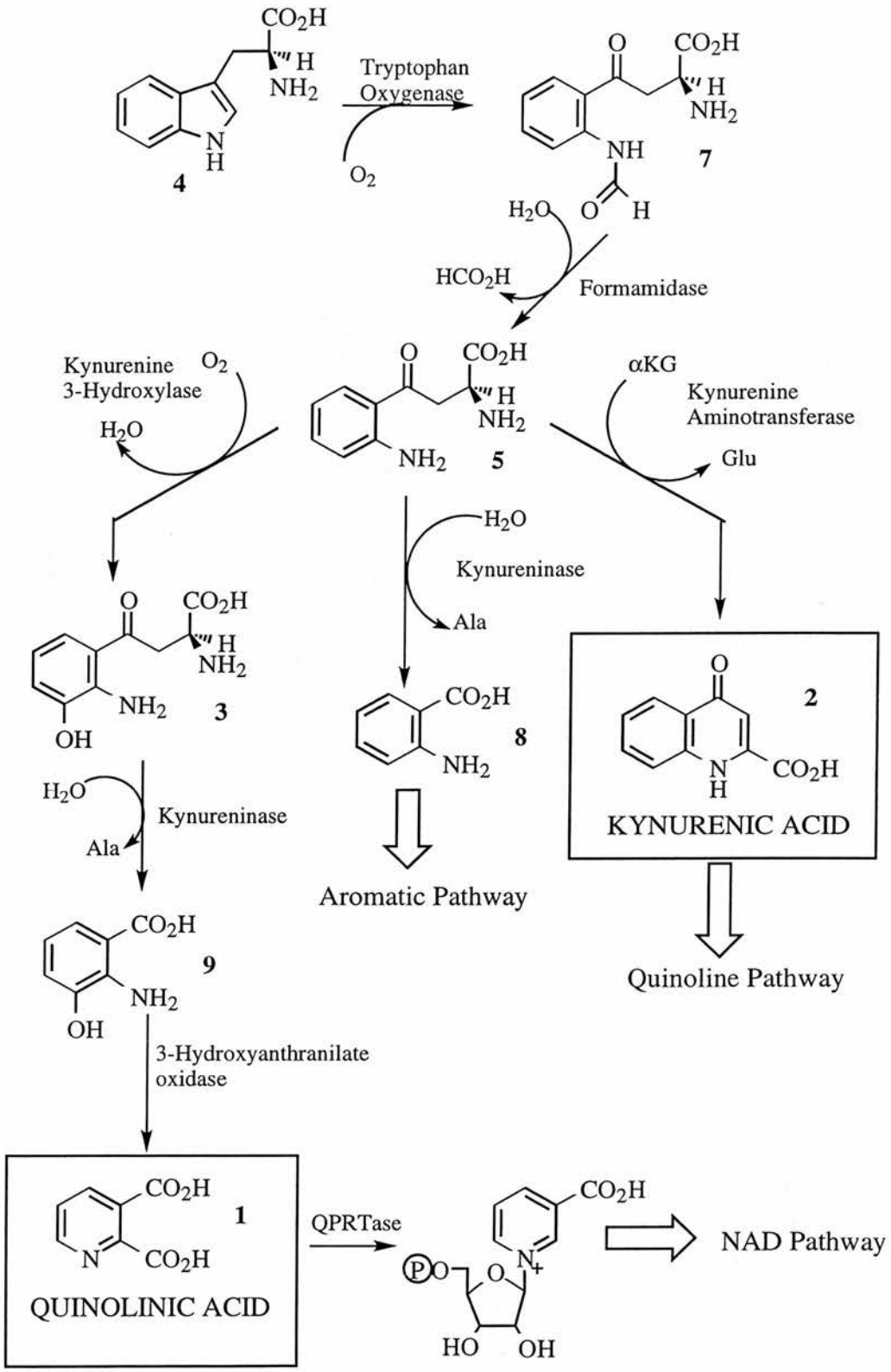
Scheme 1.1: Pathways of Tryptophan Metabolism

Tryptophan is present largely bound to plasma albumin but only free tryptophan is able to cross the blood-brain barrier. As albumin binding sites are saturatable, free tryptophan concentrations can be elevated by increasing dietary intake. Also, tryptophan is transported across the blood-brain barrier by a sodium dependent large neutral amino acid transport system. Thus plasma tryptophan levels are also dependent on levels of other large neutral amino acids such as tyrosine, phenylalanine, valine and isoleucine.[17] Products of the metabolism of tryptophan such as kynurenine (**5**) and 3-hydroxykynurenine (**3**) have also been shown to cross the blood-brain barrier using the same transport system.[14]

Tryptophan has been employed in the treatment of several neuropsychiatric disorders, such as depression, insomnia and obesity, as an oral dietary supplement.[17] This was as a result of its role as a precursor of 5-hydroxytryptamine (5-HT, **6**, serotonin).[18,19] 5-HT is synthesised almost exclusively in the brain[20] and neurovisceral functions such as sleep, appetite, motor function and thermoregulation are said to be controlled by neurones containing serotonin.[21] However, tryptophan loading was later found instead to increase levels of various kynurenines and their neurotoxic metabolites in brain, as the kynurenine pathway is the major route of tryptophan catabolism. As it also provides the pyridine nucleus of the nicotinamide and nucleotide coenzymes it must also be regarded as an important biosynthetic pathway (Scheme 1.2).[22]

The initial step in the kynurenine pathway is the oxidative cleavage of the heterocyclic ring of tryptophan (**4**) by one of two haem dependent enzymes, tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) or indolamine 2,3-dioxygenase (IDO, EC 1.13.11.17).[11] The product of this reaction, *N*-formyl kynurenine (**7**) is an intermediate which rapidly undergoes hydrolysis to form kynurenine (**5**), which is catalysed by a formamidase enzyme (EC 3.5.1.9). The fate of this intermediate (**5**) in mammals, fungi and many micro-organisms is threefold. Firstly it can undergo hydrolysis to anthranilic acid (**8**) as catalysed by kynureninase (EC 3.7.1.3). Anthranilic acid is used as a precursor for the

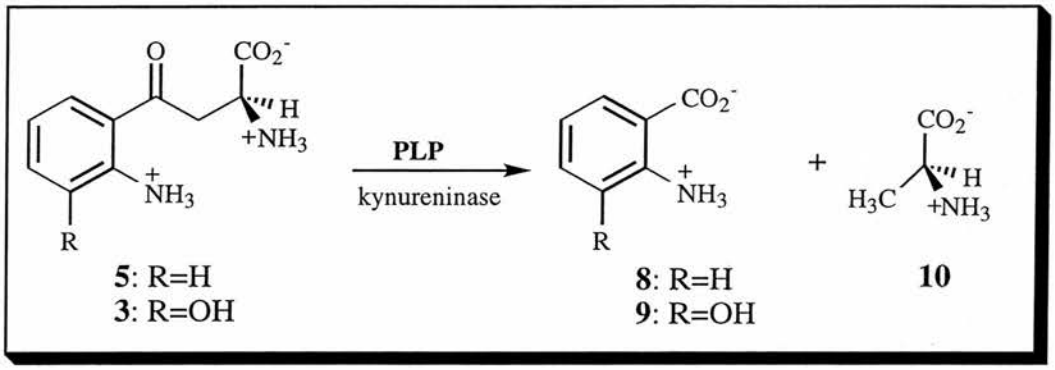
synthesis of aromatic amino acids.[11] Secondly it can undergo transamination to kynurenic acid (**2**), a neuroprotective agent, as catalysed by kynurenine aminotransferase (KAT, EC 2.6.1.7). Kynurenic acid is the biosynthetic precursor of some of the quinolines. Thirdly it can undergo hydroxylation to 3-hydroxykynurenine (**3**) which is catalysed by kynurenine 3-hydroxylase (EC 1.14.13.19). 3-Hydroxykynurenine is in itself an important metabolite of tryptophan.[15] This last is the major fate of kynurenine and leads into the pathway of nicotinamide nucleotide synthesis.[23] Kynureninase also catalyses the conversion of 3-hydroxykynurenine (**3**) to 3-hydroxyanthranilic acid (**9**). Rearrangement of this yields quinolinic acid (**1**) the most neurologically important metabolite on the pathway. Further transformations lead to nicotinic acid mononucleotide and to the nicotinamide coenzymes.



Scheme 1.2: The Tryptophan Metabolic Pathway

1.3 KYNURENINASE

Kynureninase (EC 3.7.1.3) is an unusual pyridoxal 5'-phosphate (PLP) dependent enzyme on the tryptophan metabolic pathway. It catalyses the β,γ-hydrolytic cleavage of aryl substituted γ-keto-α-amino acids. The two natural substrates for this reaction are 2*S*-kynurenine (**5**) and 3-hydroxykynurenine (**3**) and they are hydrolysed by kynureninase to 2*S*-alanine (**10**) and anthranilic acid (**8**) and 3-hydroxyanthranilic acid (**9**) respectively, as shown in Scheme 1.3.[24] Other PLP dependent enzymes which catalyse similar β,γ-cleavage reactions include L-aspartate-β-decarboxylase and L-selenocysteine-β-lyase.



Scheme 1.3: The Reaction Catalysed by Kynureninase

The enzyme has been isolated and purified from many sources including *Pseudomonas fluorescens*[25] (previously *P. marginalis*),[26] rat,[27] suncus[28] and pig liver [29] as well as human liver.[30] A partial amino acid sequence has also been determined for *P. marginalis* as well as the sequence for rat liver kynureninase.[31] No crystal structure for any kynureninase has as yet been determined. The highest kynureninase activity was observed in the liver for both human, rat and suncus models. The suncus liver shows higher activity than other mammalian livers,[28] showing activity 50-fold higher than that of pig liver,[29] 100 times that of rat liver and 40 times that of human liver. Activity of kynureninase in human liver is three times greater than in rat liver, and high human kynureninase activity was also detected in spleen, kidney and lung. Work by Moriguchi *et al.* suggested that the *Pseudomonas marginalis* enzyme contained only one mole of PLP

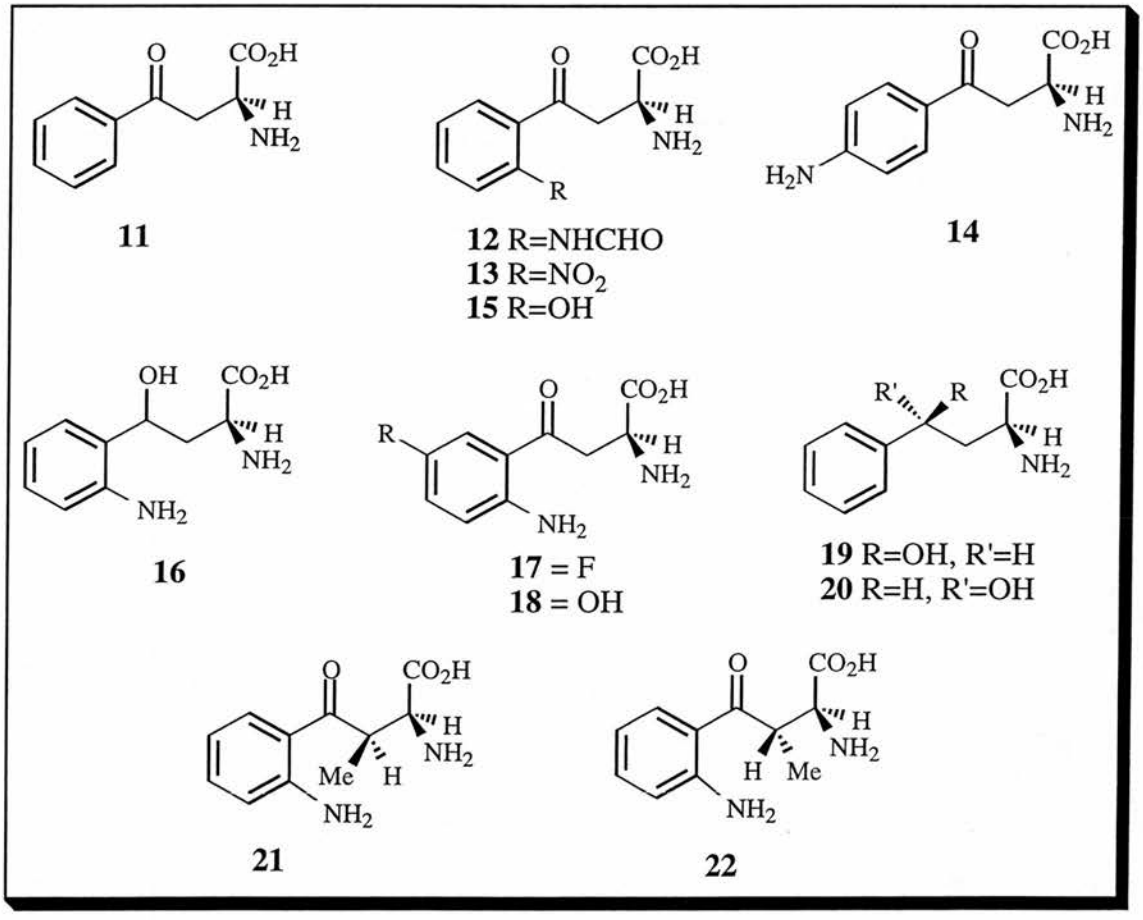
per mole of dimeric enzyme,[32] Dua *et al.* found however, that for the *P. fluorescens* enzyme one PLP is bound to each subunit.[33] This finding was important as they also later showed that one molecule of inhibitor is bound to each 50 kDa subunit.

The rate of hydrolysis of the substrates 2*S*-kynurenine (**5**) and 3-hydroxykynurenine (**3**) has been observed to vary with enzyme preparations from different sources. The enzyme from bacterial sources such as *Pseudomonas fluorescens* hydrolyses kynurenine preferentially and 5 times faster than the 3-hydroxy derivative. Mammalian liver enzymes hydrolyse 3-hydroxykynurenine twice as rapidly as kynurenine. The enzyme isolated from *P. fluorescens* was shown to be comprised of two identical subunits of molecular weight 45 484 per subunit. The rat enzyme was estimated to have a molecular weight of 95 000 and also consisted of two identical subunits. Michaelis constants for the rat enzyme were calculated as 240 μM for 2*S*-kynurenine (**5**) and 13 μM for 3-hydroxykynurenine (**3**). The human enzyme was shown to be of mass 130 000 and the activity ratio for 3-hydroxykynurenine (77 μM) and kynurenine (1.0 mM) was 15:1.[34] These characteristics are similar to the rat enzyme. This is because the mammalian form of the enzyme is constitutive whilst the bacterial enzyme is inducible in tryptophan rich growth media. The enzyme catalysing the conversion of 3-hydroxykynurenine (**3**) to 3-hydroxyanthranilic acid (**9**) is also termed 3-hydroxykynureninase.

1.3.1 SUBSTRATE SPECIFICITY

A number of kynurenine analogues have been prepared in order to obtain information on kynureninase structure and mechanism. Specificity studies with the *P. fluorescens* enzyme demonstrated that it would tolerate some substitutions in the benzene ring of the substrate and retain activity. The *ortho*-amino group, as shown by desaminokynurenine (**11**) is not essential as desaminokynurenine was still a substrate, although at a much reduced rate. If the *ortho*-amino group is formylated (**12**), replaced by a nitro group (**13**) or moved to the *para* position (**14**) activity is lost. Substitution of the aromatic amino

group with a hydroxyl group (**15**) gave a substrate with reduced activity. Reduction of the γ-carbonyl to a hydroxyl (**16**) and introduction of a fluorine (**17**) or a hydroxy group (**18**) at the 5'-position of the ring gave compounds active with the *Pseudomonas marginalis* enzyme. The fluoro analogue (**17**) was the most active of these. The presence of the *ortho*-amino group has a significant effect on reactivity. Hayaishi suggested that the relative rates of hydrolysis of the analogues could be explained by inductive and resonance effects of amino, nitro and hydroxyl groups in the *o*-position and by inductive effects in *meta*-hydroxyls.[35] The γ-carbonyl group has also been shown not to be essential as γ-hydroxy derivatives are also cleaved. The enzyme is however, only reactive to (2*S*)-amino acids.

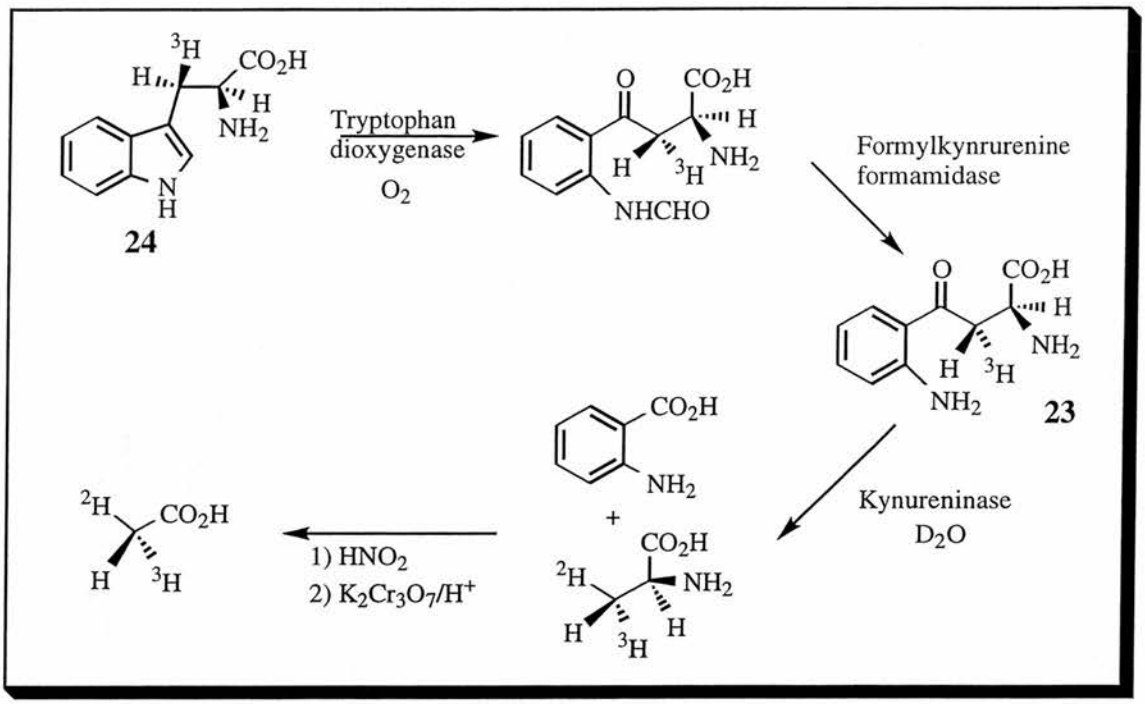


Work by Phillips and Dua showed that the (4*S*) and (4*R*)-isomers of dihydrokynurenine (**19**, **20**) had different interactions with bacterial kynureninase. The (4*R*)-isomer (**20**) is a substrate for a retro-aldol cleavage reaction, while the (4*S*)-isomer (**19**) is a potent

competitive inhibitor with a K_i value of 0.3 μM.[36] The potent inhibitory effect of (*4S*)-dihydrokynurenine (**19**) is thought to be due to the similarity of this compound to the postulated *gem*-diolate intermediate discussed later in this chapter. The synthesis of a series of *S*-aryl-L-cysteine-*S,S*-dioxides as inhibitors of kynureninase was undertaken to probe this hypothesis (see Modulation of the Kynurenine Pathway). Phillips also synthesised *threo* and *erythro* diastereomers of β-methyl-2*S*-kynurenine (**21**, **22**) and examined them as substrates and inhibitors to probe the *gem*-diolate theory.[37] They found that (2*S*, 3*S*)-*erythro*-β-methyl-2*S*-kynurenine (**22**) is a slow substrate for kynureninase (k_{cat}/K_m = 0.1% that of kynurenine) and that (2*S*, 3*R*)-*threo*-β-methyl-2*S*-kynurenine (**21**) is about 390-fold less reactive than the *erythro* compound. Analysis by rapid-scanning stopped flow spectrophotometry showed that β-methyl substitution affects the rate of α-deprotonation of the L-kynurenine Schiff's base.[38] This demonstrates that β-substituted kynurenines can be substrates for kynureninase and this may be useful in the design of mechanism-based inhibitors.

1.3.2 STEREOCHEMISTRY

In 1985 Floss, Soda and co-workers elegantly determined the stereochemical course of the replacement of the anthranilyl group of kynurenine by hydrogen.[39] This was achieved by converting (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]kynurenine (**23**) into alanine with kynureninase in deuterium oxide. The tritiated kynurenines were prepared enzymatically *in situ* from the corresponding stereospecifically tritiated tryptophan species (**24**). Subsequent diazotisation and dichromate oxidation of the alanine produced in the hydrolysis made chiral analysis of the methyl group possible (Scheme 1.4).

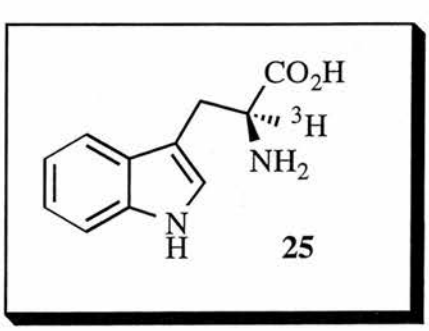


Scheme 1.4: Generation of Stereospecific Tritiated Kynurenine

The reaction was found to occur with retention of configuration where the proton is added on the same side from which the anthranilyl group leaves.

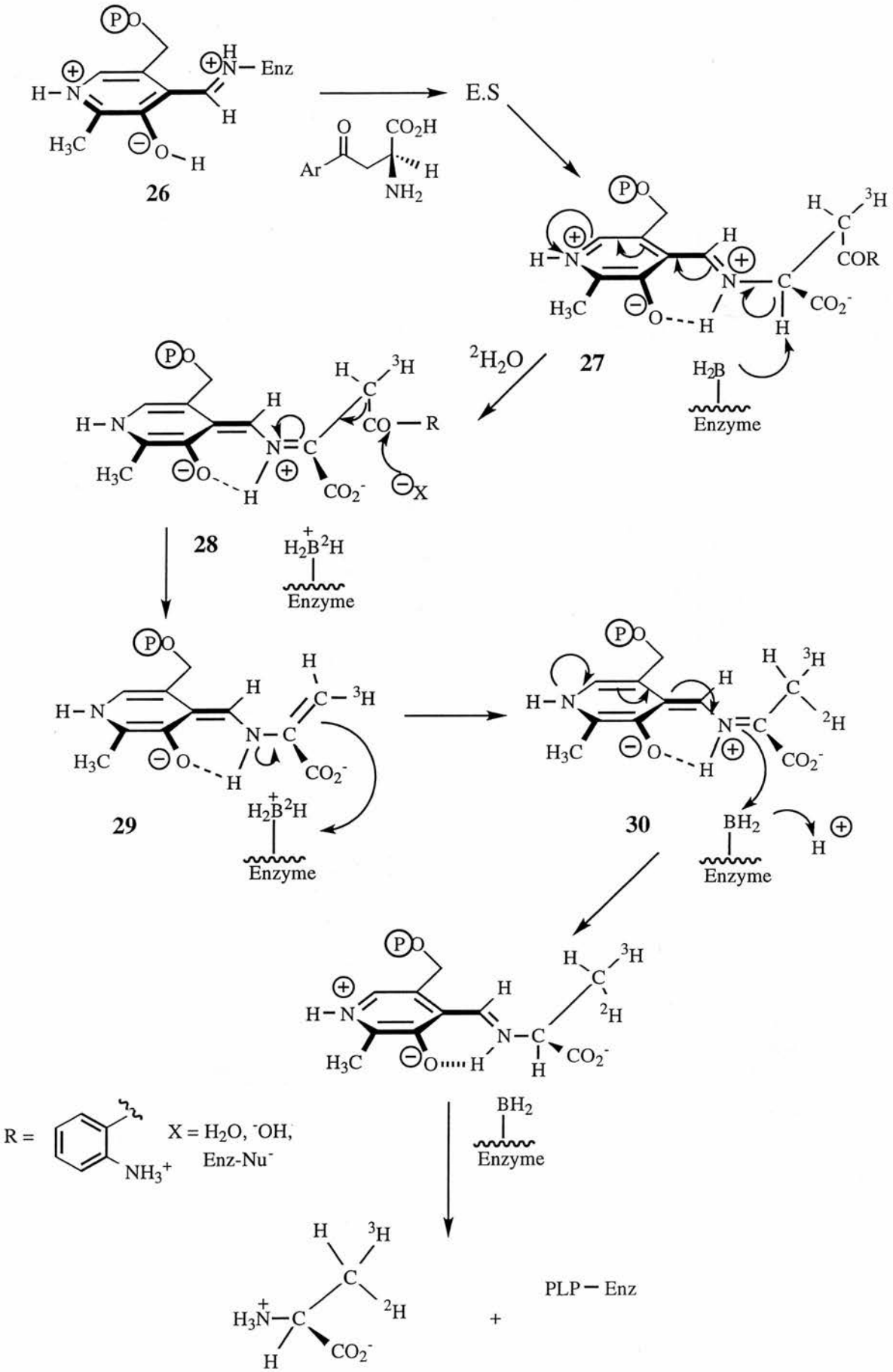
1.3.3 PROPOSED MECHANISM

Floss and Soda also carried out experiments to probe for internal transfer of tritium from the α-position of kynurenine to the product alanine. Using 2*S*[2-^{3}H]tryptophan (**25**) they showed that 40% of the tritium from the α-position of the substrate is transferred to the β-position of the product and 60% to the α-position of the product.



Many mechanisms have been proposed for this reaction but the one in Scheme 1.5 seems most feasible and accounts for both the retention of configuration and the proton transfer previously described.

A Schiff's base is formed when PLP binds to the apoenzyme *via* the ε-amino group of an active site lysine residue (**26**). This internal aldimine is common to most PLP dependent enzymes. The substrate, kynurenine then binds to the enzyme and a transaldimination reaction takes place. This displaces the active site lysine and another Schiff's base, the external aldimine (**27**) is formed between PLP and kynurenine. The α-CH bond is then cleaved to generate a ketimine intermediate (**28**), which is resonance stabilised. The Dunathan postulate states that the π-system of a Schiff's base is extended by loss of a group from the α-carbon of the amino acid. The increase in delocalisation energy in the π-system can aid the bond breaking process if the geometry of the transition state places the bond to be broken in the plane perpendicular to that of the pyridoxal imine system thus maximising σ-π overlap.[40] Attack at the γ-carbonyl of the quinoid intermediate by an enzymic nucleophile, water molecule or hydroxide ion (X), followed by immediate cleavage of the β,γ-carbon—carbon bond then yields anthranilate and an α-aminoacrylate derivative (**29**). Protonation at the β-carbon of this derivative is stereospecific and therefore enzyme mediated and occurs with retention of stereochemistry (**30**). This suggests that the anthranilyl group and the base delivering a proton must be disposed on the same face of the pyridoxal phosphate-substrate complex. For the same stereoelectronic reasons the β,γ-carbon—carbon bond should be oriented perpendicular to the plane to the π-system of PLP to minimise the energy required for bond cleavage. However, it may lie on the same or opposite face of the α-CH bond. The finding that the internal α-proton of kynurenine is recycled to both the α- and β-positions of the product, indicates that proton abstraction and reprotonation at α-C and protonation at β-C are mediated by the same base.

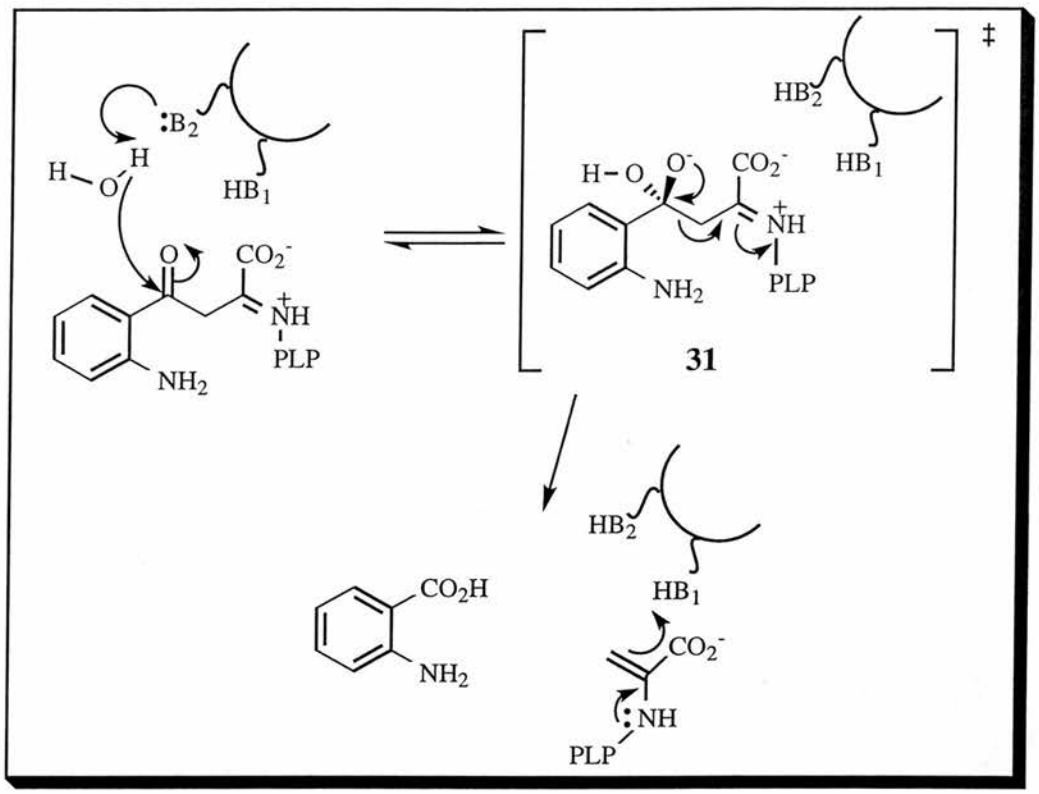


Scheme 1.5: Stereochemistry of the Kynureninase Reaction

Retention of stereochemistry and recycling of internal protons to both α- and β-positions supports an active site conformation of the pyridoxal phosphate–substrate complex in which the α-hydrogen and β-carbon anthranilyl group are *syn* oriented. Although not proven it was assumed that both bonds are displayed in the *Si* face in relation to C4' of the cofactor, which is a feature common to all PLP dependent enzymes studied so far. As yet the active site nucleophile or base has not been identified but the observed partitioning of tritium between α- and β-carbons of alanine suggests that the base mediating these proton transfers is polyprotic, such as the ε-amino group of a lysine residue.[39] Kishore also suggested that an active site carboxyl group may be involved in deprotonation as mechanism based inhibitors of kynureninase caused alkylation of this group.[41]

1.3.3.1 BOUND WATER MEDIATED HYDROLYSIS

In the hydrolysis step the exact identity of X is still unknown. One hypothesis favours hydrolysis by an enzymic active site nucleophile as described in the next section, whilst the reaction could also be mediated by a water molecule at the active site. This employs general base catalysis by an active site amino group which generates a hydroxide ion which attacks the γ-carbonyl of the substrate as shown in Scheme 1.6. The high energy tetrahedral intermediate (**31**) can be used as a model for the transition state structure.



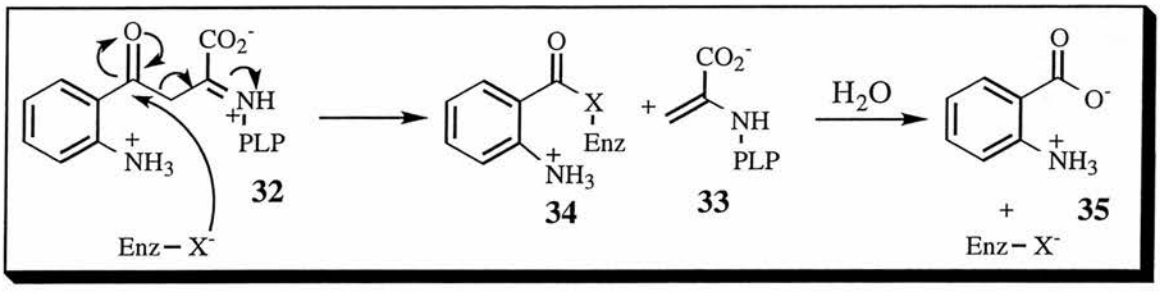
Scheme 1.6: Water Mediated Hydrolysis of Kynurenine

Both dihydro-2*S*-kynurenines (**19**, **20**) are similar in structure to the *gem*-diolate intermediate (**31**) and the potent inhibition exhibited by these compounds suggests that they are transition-state analogue inhibitors. Other analogues of the transition state, including phosphinic acid analogues,[42] 2,2-difluoro-2-benzoyl-alanine[43] and *S*-aryl-2*S*-cysteines and their corresponding *S,S*-dioxides[33] will be discussed later (Section 1.6.1).

1.3.3.2 THE ENZYMIC NUCLEOPHILE MEDIATED MECHANISM

As mentioned previously, the hydrolysis of kynurenine may also be mediated by attack by an enzymic nucleophile (Scheme 1.7). The ketimine intermediate (**32**) is attacked by an enzymic nucleophile at the γ-carbonyl group. Cleavage of the β,γ-carbon—carbon bond releases the α-aminoacrylate derivative (**33**) described earlier and gives an enzyme bound covalent intermediate (**34**). Release of anthranilate (**35**) upon reaction with water

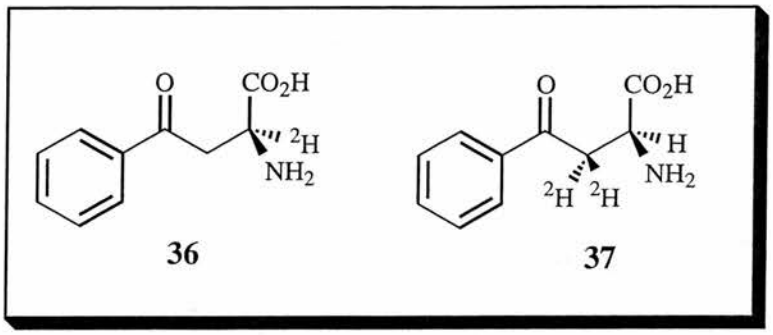
regenerates the enzymic nucleophile. At present there is no evidence for this mechanism over the bound water mediated mechanism. However, alkylation of the putative active site nucleophile could be used to probe for the enzymic nucleophile mediated reaction. Identification of the covalent intermediate could also be used to probe for evidence of the enzyme mediated reaction.



Scheme 1.7: Enzymic Nucleophile Mediated Reaction

1.3.4 STUDIES ON ISOTOPE EFFECTS

α-Deuteriated kynurenine (**36**) has been synthesised at St. Andrews by two complementary methods (from *R,S*-kynurenine and 2*S*-(2-^{2}H)-tryptophan) and this has been used in isotope effect studies in order to gain an understanding of the enzyme catalysed reaction.[44] The solvent isotope effect has shown proton transfer to be partially rate limiting (^DV = 4.4 and $^D(V/K)$ = 4.6) where K_m remains unchanged but V_{max} shows a four-fold decrease after changing the solvent from H_2O to D_2O. α-H Abstraction has also been shown to be partially rate limiting by the primary deuterium isotope effect (^DV = 0.98 and $^D(V/K)$ = 3.6). This is in direct opposition to the findings of Koushik *et al.* described below. However, similar effects were observed using deuteriated solvents.



36 **37**

At present these primary isotope effects have only been carried out at pH 7. These need to be carried out over a range of pHs to determine whether the isotope effects are dependent or independent of pH. Double kinetic isotope effect studies of the primary and solvent isotope effects should distinguish between stepwise and concerted proton transfers in the active site. The preparation of β,β-dideuteriated kynurenine (**37**) would complete isotope effect measurements by establishing the kinetic importance of carbon—carbon bond cleavage in the reaction and would give the secondary deuterium effect.[45]

Work by Koushik *et al.*, using steady-state and pre-steady state kinetics showed a bell-shaped pH dependence for kynureninase with p*K*a's of 6.25 ± 0.05 on the acidic limb and 8.9 ± 0.1 on the basic limb.[38] They also showed that there is no significant isotope effect on either k_{cat} or k_{cat}/K_m for α-[^{2}H]-2*S*-kynurenine (**36**) as substrate but a large kinetic isotope effect ($^D K = 6.56$) on k_{cat} in D_2O. The release of anthranilate formed in the pre-steady state of the kynureninase reaction was not affected by D_2O. This indicated that:

i) the rate-limiting step in the kynureninase reaction occurs after the first irreversible step (anthranilate release)

ii) that the reaction is base catalysed

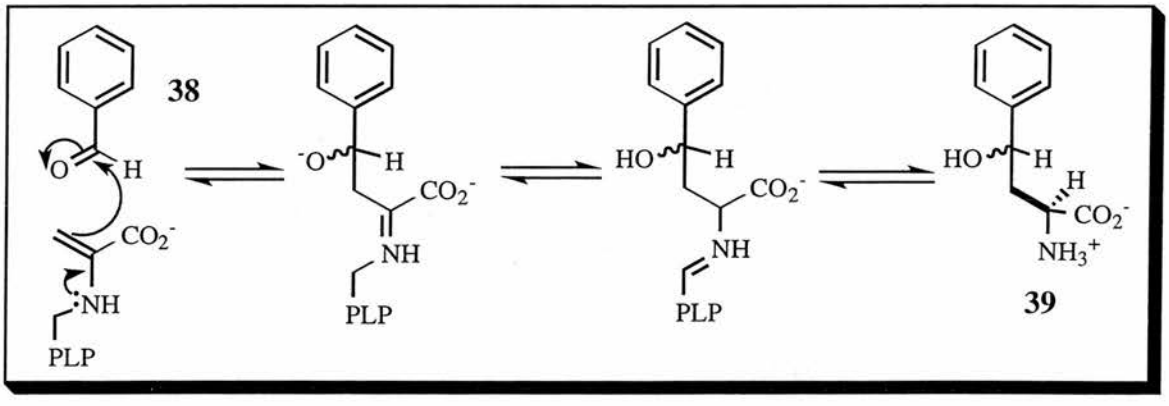
iii) and that it involves the transfer of only one proton.

A mechanism was proposed from these observations: the rate-limiting step in the reaction of kynureninase is proton abstraction from C-4' of the pyruvate pyridoxamine 5'-phosphate ketimine intermediate. Later studies by the same group supported this

proposed reaction mechanism and demonstrated that both quinoid and ketimine intermediates are kinetically competent in this reaction.[37] Studies showed that the transient quinoid species decays by proton transfer from a solvent exchangeable acid, the two basic sites available for this protonation are at α-C and C-4'. Since protonation at α-C leads back to the external aldimine, protonation is more likely to occur at C-4' to give a kynurenine ketimine. The rapid formation of both these intermediates supports the conclusion that all species up to the kynurenine ketimine are in fast equilibrium based on the lack of a k_{cat}/K_m isotope effect with α-[^{2}H]-2*S*-kynurenine (**36**).[37]

1.3.5 KYNURENINASE IN ORGANIC SYNTHESIS

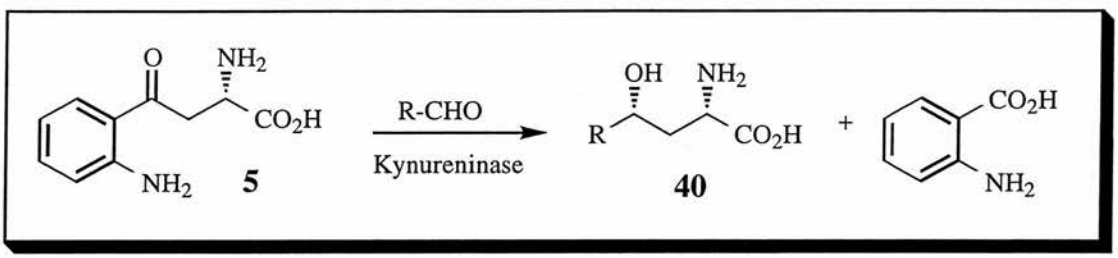
Kynureninase has also been shown to catalyse aldol-type condensations. The α-aminoacrylate intermediate formed following kynurenine hydrolysis can be trapped out using benzaldehyde (**38**) to give 2-amino-4-hydroxy-4-phenylbutanoic acid (**39**) as shown in Scheme 1.8.[46]



Scheme 1.8: Aldol Condensation of Benzaldehyde and 2S-Alanine

Although the stereochemistry at the 4-position was not determined it was postulated that only a single isomer was formed. This transaldol reaction between benzaldehyde (**38**) and kynurenine (**5**) has formed the basis for research into kynureninase as a means of synthesising γ-hydroxy-α-2*S*-amino acids (**40**). Kynureninase was found to tolerate many other aromatic aldehydes and propargyl aldehydes as substrates to yield γ-hydroxy-

α-2*S*-amino acids which, along with β-hydroxy-α-2*S*-amino acids from 2*S*-threonine aldolase, are useful building blocks for the synthesis of many kinds of bioactive compounds. Scheme 1.9 below gives a general reaction.



Scheme 1.9: Formation of γ-Hydroxy-α-2S-amino Acids

The stereochemistry of the newly formed γ-chiral centre was determined to be the *R*-configuration and the stereochemistry at the α-position was confirmed by enzymatic oxidation with *R*- and *S*-amino acid oxidases. The reaction was compatible with aldehydes with sp^2- as well as *sp*-carbons at the α-position (Table 1.1). Also the introduction of oxygen or nitrogen functional groups into the aldehydes did not effect the progress of the reaction.[47] Although kynureninase has been shown to be a useful synthetic tool, it is worth noting that the substrate, kynurenine is required for all of these experiments and is relatively expensive. Kynureninase does not catalyse an aldol-type reaction between benzaldehyde (**38**) and 2*S*-alanine in the presence of PLP so this could be a deciding factor in its use as a tool for synthesis.[48]

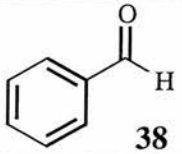
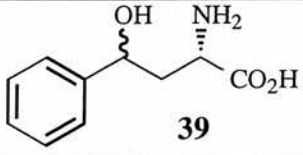
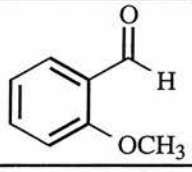
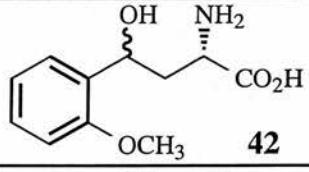
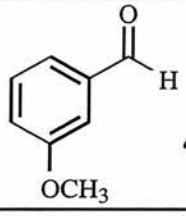
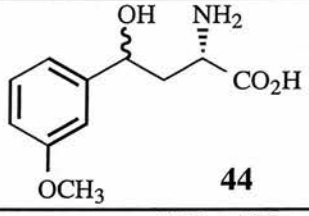
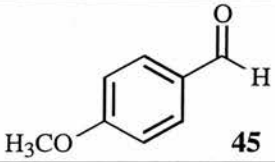
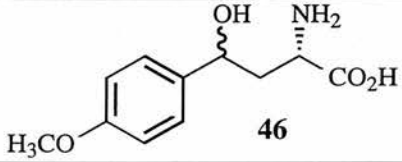
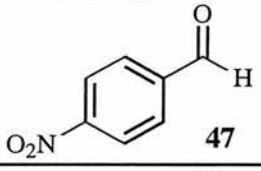
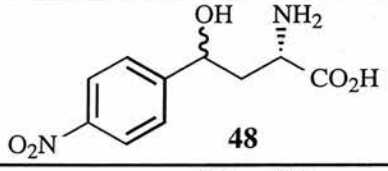
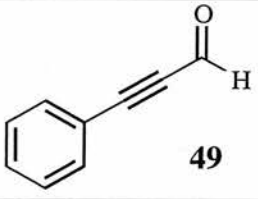
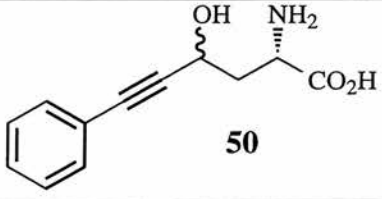
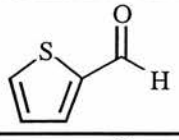
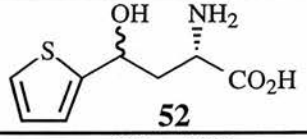
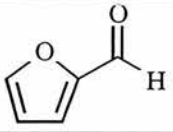
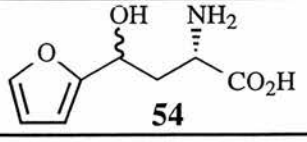
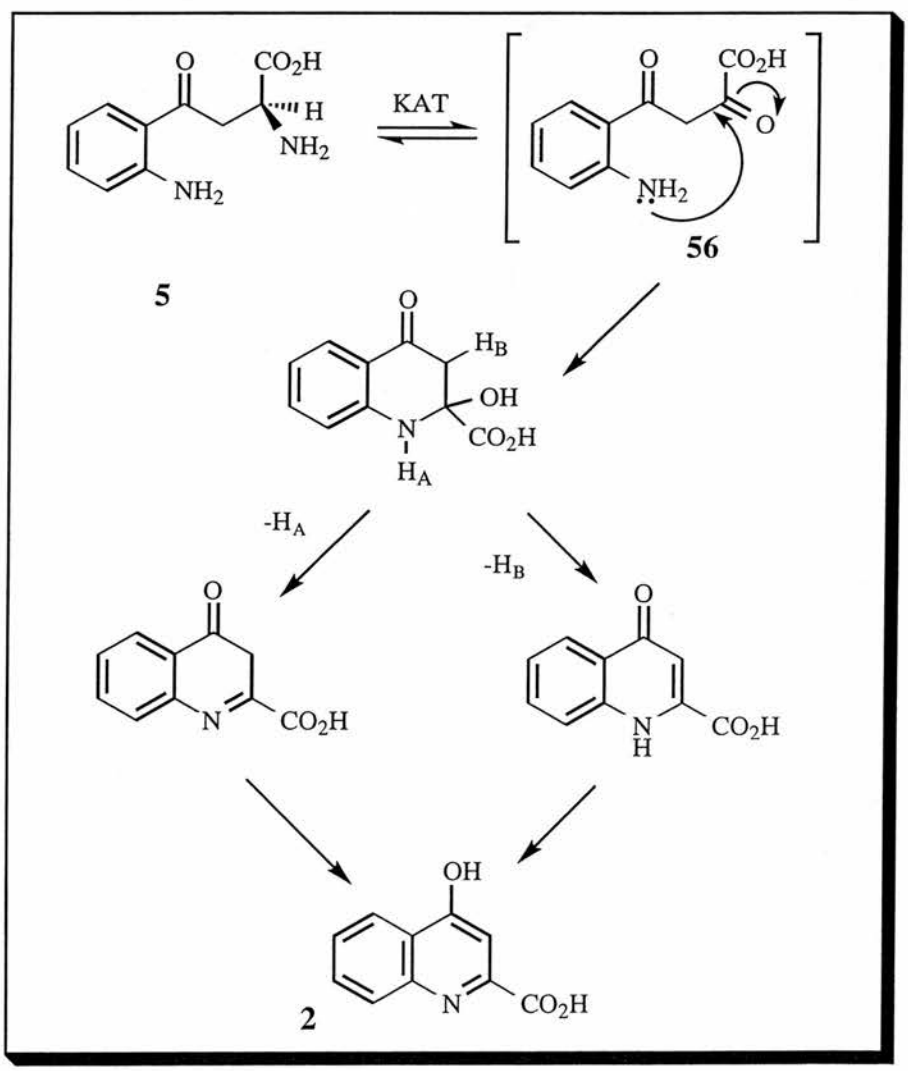
Substrate	Product	Diastereo -ratio *R:S*	Chemical Yield
 **38**	 **39**	91 : 9	50%
 **41**	 **42**	96 : 4	46%
 **43**	 **44**	96 : 4	69%
 **45**	 **46**	96 : 4	50%
 **47**	 **48**	88 : 12	52%
 **49**	 **50**	63 : 37	20%
 **51**	 **52**	95 : 5	46%
 **53**	 **54**	96 : 4	55%

Table 1.1 Substrate-Specificity of Aldol Reactions Catalysed by Kynureninase

1.4 KYNURENINE AMINOTRANSFERASE

The formation of kynurenic acid (**2**) is catalysed by kynurenine aminotransferase (KAT). KAT is a PLP dependent enzyme which catalyses the transamination of kynurenine (**5**) and 3-hydroxykynurenine (**3**) to kynurenic acid (**2**) and xanthurenic acid (**55**), respectively. The reaction is unusual for an aminotransferase in that it is irreversible.[22] The α-ketoglutarate (**56**) formed in the first step of the reaction undergoes rapid cyclisation *via* attack of the aromatic amino group on the newly formed carbonyl. Loss of water yields the very stable aromatic species, kynurenic acid (**2**). The stability of kynurenic acid drives the reaction to the right preventing a reverse of the initial transamination step (Scheme 1.10).



Scheme 1.10: Reaction Catalysed by KAT

The identity of the enzyme catalysing the above reaction has not always been clear. For instance in 1977 the enzyme kynurenine aminotransferase (EC 2.6.1.7) was found to be identical with 2-aminoadipate aminotransferase (EC 2.6.1.39) from rat kidney[49,50] and more recent work has shown that several different enzymes are capable of catalysing the transamination between kynurenine (**5**) and several 2-oxoacids to form kynurenic acid (**2**) in the mammalian system.[51] In rat liver four separate isozymes of kynurenine aminotransferase have been reported and classified by their substrate specificity, pH optimum and isoelectric point. Table 1.2 below summarises these.

Isozyme	pI	pH optimum	Identical with
1	5.2	9.0 - 9.3	glutamine-oxo-acid AT (EC 2.6.1.15) histidine-pyruvate AT 2 (EC 2.6.1.-)
2	6.6	6.0 - 6.5	2-amino adipate AT (EC 2.6.1.39)
3	8.0	8.0 - 8.5	histidine-pyruvate AT 1 (EC 2.6.1.-) serine-pyruvate AT (EC 2.6.1.51) alanine-glyoxalate AT (EC 2.6.1.44)
4	9.4	8.0 - 8.5	m-aspartate-2-oxoglutarate AT (EC 2.6.1.1) m-tyrosine-2-oxoglutarate AT (EC 2.6.1.5)

Table 1.2 Isozymes of Rat Kynurenine Aminotransferase
AT = aminotransferase
m- = mitochondrial

Both KAT and kynureninase have similar affinities for PLP but KAT is not affected by the onset of vitamin B_6 deficiency. This is because KAT is localised at the inner mitochondrial membrane which protects it from vitamin B_6 deficiency. Kynureninase, however, is a cytosolic enzyme and therefore more vulnerable. Recently human brain has been studied[52] and in contrast to rat brain,[53] human brain has two kynurenine aminotransferases arbritrarily termed KAT I and KAT II. Both are capable of producing

the neuroinhibitory brain metabolite, kynurenic acid (**2**) from 2*S*-kynurenine (**5**) in human brain tissue.

KAT I was found to be a dimer consisting of two identical subunits of 60 KDa. Kinetic analysis of the pure enzyme revealed an absolute K_m of 2.0 mM and 10.0 mM for kynurenine and pyruvate respectively. KAT I activity has a sharp pH optimum at pH 9.5-10 and is inhibited by common amino acids such as tryptophan, glutamine and phenylalanine.[52] KAT II has a shallow pH optimum in the physiological range and does not appear to be inhibited by common amino acids. Antibodies raised to KAT I protein are also immunologically distinct to those for KAT II. KAT I is also significantly larger than other kynurenine aminotransferases. It has therefore been proposed that KAT II is primarily responsible for the synthesis of kynurenic acid (**2**) under normal physiological conditions and that KAT I may become particularly important in pathological situations. It is believed that KAT I plays a role in neuropsychiatric diseases, such as Huntington's disease which have abnormal brain kynurenic acid synthesis and dysfunctional glutamine metabolism. Also there is a possible link between kynurenines and excitotoxic brain diseases and brain damage. Abnormal KAT activity causes the hyper- or hypofunction of kynurenic acid biosynthesis and may therefore be considered to be an etiological factor in pathological phenomena related to dysfunction of exictatory amino acid receptors in the brain.[54]

Work carried out by Schwarcz *et al.* led to the development of a sensitive assay which has allowed the detailed characterisation of the enzyme.[53] It was shown to be highly substrate-specific and to be heterogeneously distributed between the nine regions of rat brain studied.

More recently, kynurenine aminotransferase was also discovered in heart. It has been shown that blocking of EAA receptors by kynurenic acid (**2**) affects cardiac function.[55] Heart KAT was found to have a shallow pH optimum between 8 and 9, the highest

activity being shown in the presence of 2-oxoglutarate. Unlike KAT I, heart KAT activity was not inhibited by an excess of tryptophan, glutamine or phenylalanine at pH 9.6, 8 or 7.4. It was shown that heart KAT could synthesise kynurenic acid from low concentrations of kynurenine selectively. The role of kynurenic acid is discussed later in this chapter.

1.5 KYNURENINE 3-HYDROXYLASE

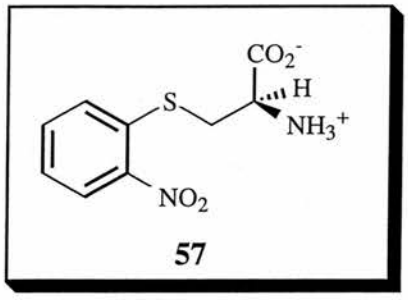
Kynurenine 3-hydroxylase (EC 1.14.13.9) catalyses the oxidation of 2*S*-kynurenine at the 3-position of the ring to give 3-hydroxykynurenine. It is FAD dependent and employs either NADH or NADPH as an electron donor. The oxygen incorporated into the kynurenine is derived from molecular oxygen. The purified enzyme is an oligomer with 4 moles of flavin per monomeric unit. Assays using 2*S*-[^{3}H]-kynurenine demonstrated that there was sequential binding of the nicotinamide cofactor and kynurenine. Stereospecifically deuteriated NADH and NADPH was used to determine the stereochemistry of the hydride transfer. A large primary deuterium isotope effect was observed only when the *pro-R* hydrogen was labelled. The isotope effect was dependent on the concentration of kynurenine which indicated that the NADH was binding before the kynurenine.[56]

1.6 MODULATION OF THE KYNURENINE PATHWAY

1.6.1 INHIBITORS OF KYNURENINASE

PLP dependent enzymes such as kynureninase are known to be inhibited by many β-substituted amino acids,[41] which act as mechanism based inhibitors. β-Chloro-L-alanine was among the first of these inhibitors to be described for L-aspartate-β-decarboxylase.[57] The same compound inhibits kynureninase and approximately 1 in 500 turnovers result in the formation of the inactive enzyme complex. L-Serine-*O*-sulfate inhibits by a similar

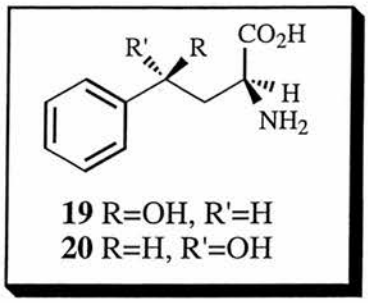
mechanism. The mechanism of inactivation appears to involve nucleophilic addition of a carboxylate group at the active site to the β-carbon of the aminoacrylate complex (see Mechanism of Kynureninase). The most potent of these inhibitors is *S*-(*o*-nitrophenyl)-L-cysteine (**57**) a suicide substrate which binds strongly, presumably as a result of the interaction between the aromatic moiety and the active site.[41]



57

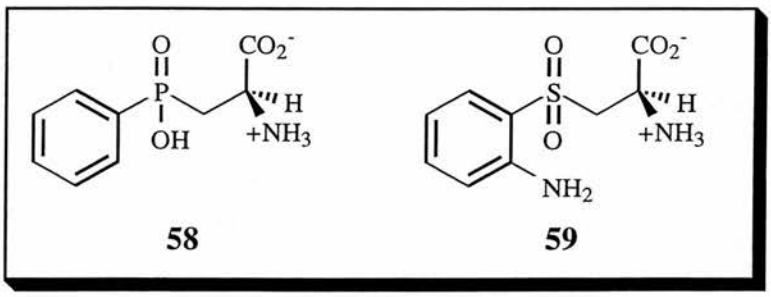
Until recently few selective inhibitors of kynureninase had been reported. The non-selective inhibitors in the literature also inactivated other PLP dependent enzymes. One strategy for inhibition is the synthesis of kynurenine analogues which mimic the tetrahedral intermediate in the water mediated reaction described below. Evidence, although not conclusive, indicates that kynureninase most likely catalyses reaction *via* a pathway involving general base catalysed hydrolysis of the substrate/PLP ketimine (see Scheme 1.6).

The dihydrokynurenines mentioned previously are good mimics of the *gem*-diolate transition state proposed by Philips and Dua. The (*4R*) isomer (**20**) is a substrate, but the (*4S*) isomer (**19**) is not. Compound **19** is a more potent inhibitor than the (*4R*) isomer, with a K_i of 0.3 μM.[36]



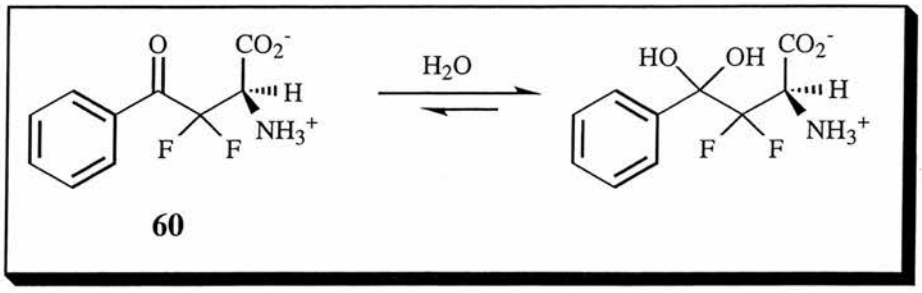
19 R=OH, R'=H
20 R=H, R'=OH

Other compounds synthesised include the phosphinic acid[42] (**58**) and several *S*-aryl-L-cysteines and their corresponding *S,S*-dioxides,[33] the most potent of which is *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide (**59**) this is synthesised enzymatically from thiophenol and L-serine. The results for this compound, with a K_i value of 70 nM, suggest that the sulfone group may be generally useful in the design of potent inhibitors of enzymes which proceed via hydrated tetrahedral intermediates. The strategy behind the synthesis of (**59**) was that *S*-phenyl-L-cysteine was only a weak inhibitor. However, oxidation at sulphur gave an 180-fold decrease in K_i. Substitution by an *ortho*-amino group gave a further 318-fold decrease in K_i. The combination of both these structural features gave rise to *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide (**59**) which turned out to be the most potent competitive inhibitor of kynureninase known to date.[33]



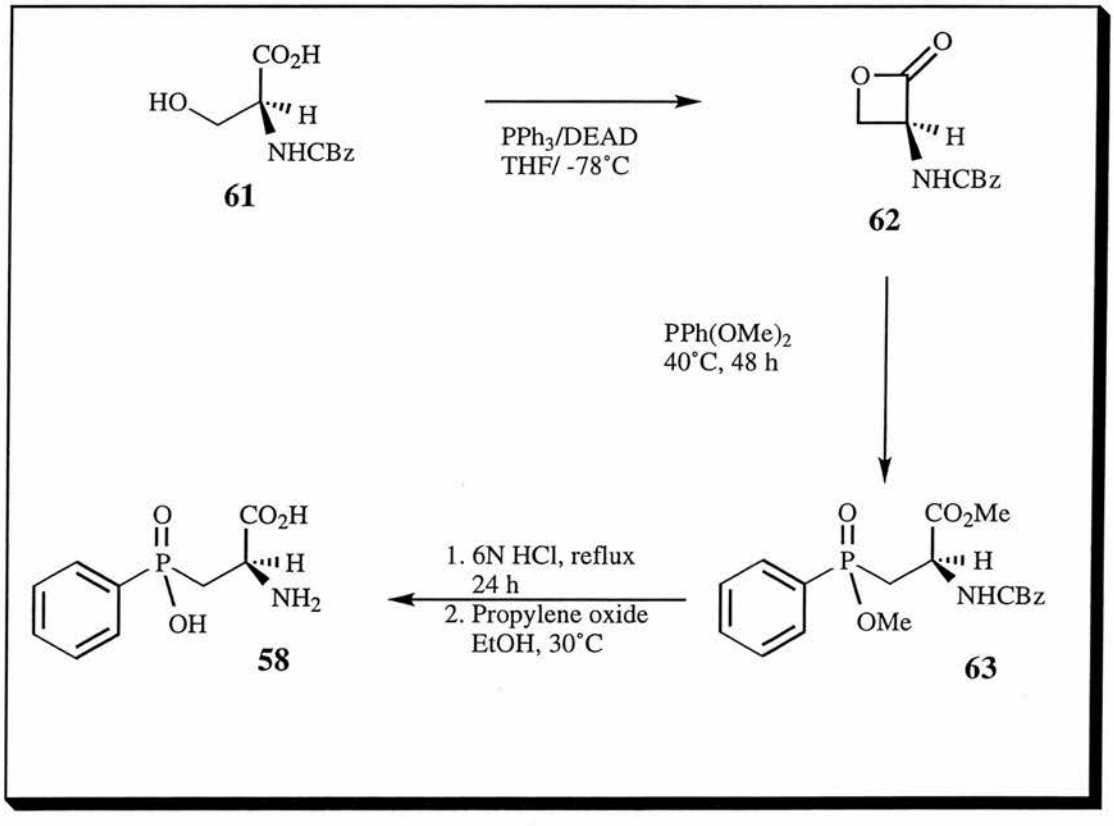
58 **59**

Also McCarthy *et al.* have developed a convenient synthetic route to β,β-difluoro-γ-keto-α-amino acids (**60**).[43] Difluoroketone containing peptides form stable hydrates or hemiketals which mimic the tetrahedral transition state.



60

Work has been carried out within the Botting group on the synthesis of competitive inhibitors. The phosphinic acid analogue (**58**) was designed, synthesised and tested as a transition state mimic.[42] As it was known that the *ortho*-amino group was not essential for activity so this was omitted in order to simplify synthesis. The original β-lactone methodology as developed by Vederas[58] was employed in the synthesis of the phosphinic acid analogue (**58**) (Scheme 1.11).

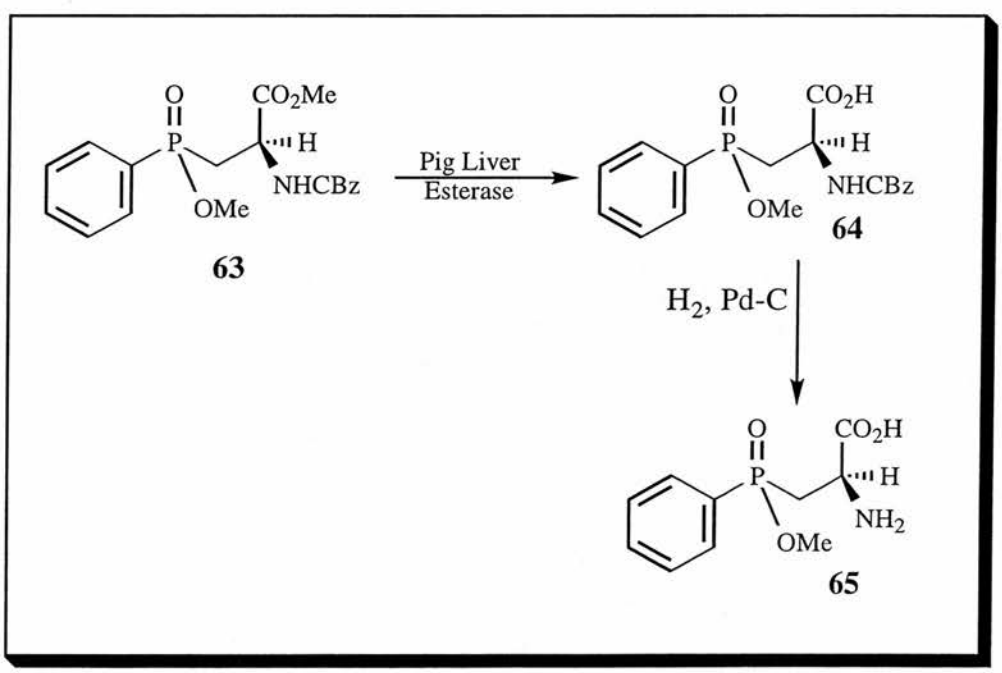


61 **62** **58** **63**

Scheme 1.11: Synthesis of Phosphinic Acid Analogue

Cyclisation of *N*-protected serine (**61**) with triphenylphosphine and diethylazodicarboxylate (DEAD) yields the strained β-lactone (**62**) which was opened using a suitable nucleophile. The methyl ester (**63**) is formed, as one of the methyl groups from phenyl dimethylphosphonite migrates on to the carboxylate. This was the first β-lactone ring opening in the literature to use a phosphinate nucleophile.

Inhibition by the phosphinic acid (**58**) was assessed and kinetic studies showed it to be a competitive inhibitor of kynureninase. The binding constant, K_i of the phosphinic acid (4.28 ± 0.1 mM) shows poor binding for the compound in comparison with the K_m for the substrate (2.56 ± 0.6 μM). In theory the phosphinic acid analogue should, as a transition state mimic, show good inhibition. It is believed that the binding affinity is reduced because at the pH of the assays the phosphinic acid is negatively charged and this causes destabilising interactions at the active site.



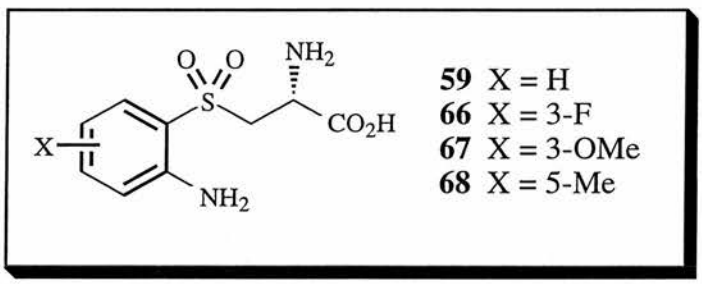
Scheme 1.12: Synthesis of Methyl Phosphinate Inhibitor

The methyl phosphinate analogue was also prepared to test this theory. This was achieved enzymatically. The methyl phosphinate methyl ester (**63**) was selectively hydrolysed

using pig liver esterase to give **64** and hydrogenation of the CBz group yielded (**65**) (Scheme 1.12).

As there was no stereochemical control at the phosphorus atom, the analogue was tested as a mixture of diastereomers. The methyl phosphinate was also a competitive inhibitor and showed a five-fold increase in binding relative to the free acid (K_i = 0.88 ± 0.05 mM). This was however, still bound more weakly than kynurenine itself.[42]

More recent work by Drysdale[59] has improved on the *S,S*-dioxides synthesised by Dua. The series below were synthesised and tested on mammalian kynureninase. Compound **59** showed a K_i of 18 μM, considerably lower than those reported with bacterial kynureninase (0.07 μM), this however is consistent with the different substrate preference of the mammalian enzyme. The introduction of substituents in the 3-position such as **66** and **67** gave IC_{50}'s of 20 μM and 29 μM respectively.

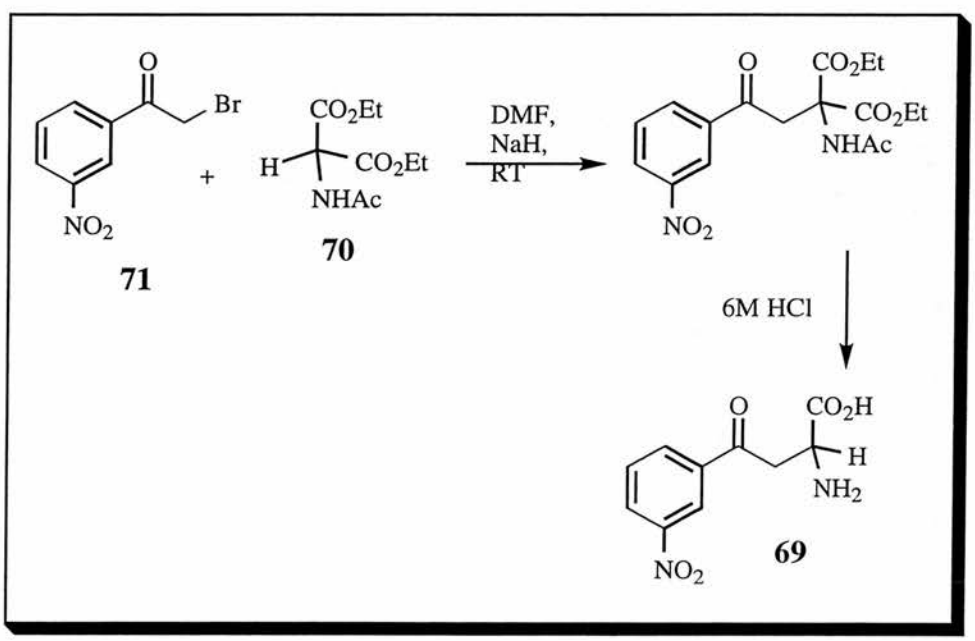


59 X = H
66 X = 3-F
67 X = 3-OMe
68 X = 5-Me

The most potent of these inhibitors was 2-amino-5-methyl-*S*-phenyl cysteine *S,S*-dioxide (**68**) with an IC_{50} of 11 μM. The effect of this compound on interferon γ-induced quinolinc acid synthesis was also tested, as it inhibits the formation of quinolinate by this route it may also have a utility in spinal cord injury.[59]

1.6.2 INHIBITORS OF KYNURENINE 3-HYDROXYLASE

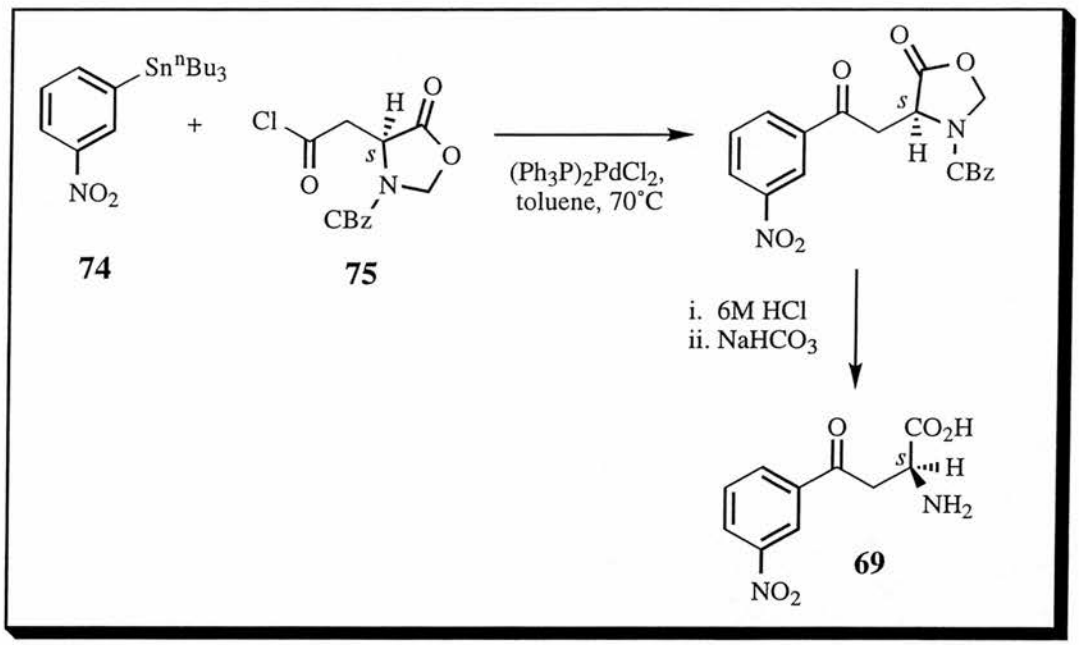
Inhibitors of kynurenine 3-hydroxylase prevent the formation of 3-hydroxykynurenine and thus of quinolinic acid. Kynurenine (**5**) is then processed either to anthranilic acid (**8**) or to kynurenic acid (**2**). Pellicciari *et al.* synthesised *o*-(nitrobenzoyl)alanine (m-NBA, **69**), an inhibitor of kynurenine 3-hydroxylase, as a racemate by coupling diethyl acetamidomalonate (**70**) with *o*-nitrophenacyl bromide (**71**) and deprotecting in acid (Scheme 1.13).[60] Both *ortho*- (**72**) and *para*- (**73**) derivatives were synthesised similarly from the corresponding nitrophenacyl bromides.



Scheme 1.13: Synthesis of m-NBA

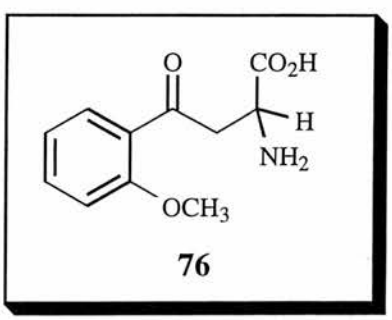
p-NBA (**73**) showed low potency and selectivity for both kynureninase and kynurenine 3-hydroxylase. *o*-NBA (**72**) gave an IC_{50} of 100 ± 12 μM for kynureninase and 2000 μM for kynurenine 3-hydroxylase resulting in a fairly potent inhibitor of kynureninase. *m*-NBA (**69**) was by far the most potent inhibitor of kynurenine 3-hydroxylase with an IC_{50} of 0.9 ± 0.1 μM, its IC_{50} for kynureninase was 100 ± 12 μM.[60]

Pellicciari and Natalini set about an enantioselective synthesis of *S*-(+)-*meta*-nitrobenzoylalanine (**69**) based on a Stille cross coupling reaction of the stannane derivative (**74**) and a protected aspartyl chloride (**75**) as shown in Scheme 1.14.[61]



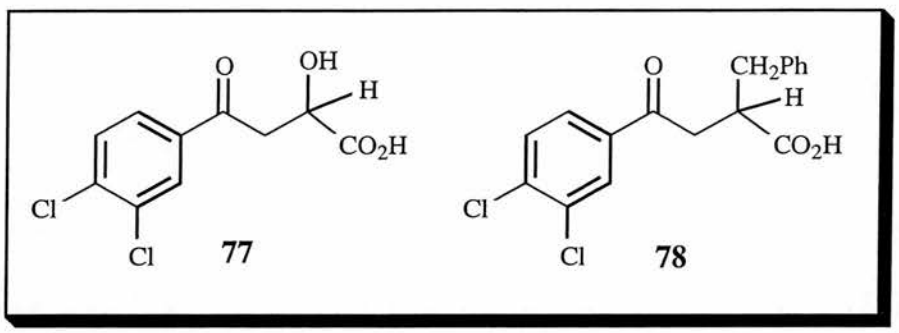
Scheme 1.14: Racemic Synthesis of m-NBA

The *S*(+) isomer of *m*-NBA (**69**) gave an IC_{50} of 0.5 μM for kynurenine 3-hydroxylase, and at higher concentrations was also shown to inhibit kynureninase (IC_{50} 60 μM in rat liver preparations).[61] Molecular modelling showed that one of the oxygen atoms of the nitro group of *m*-NBA closely mimics the oxygen atom involved in the hydrogen bonding network, thus accounting for the high selectivity and potent inhibition of this compound.



A similar strategy to that for racemic *m*-(nitrobenzoyl)alanine (**69**) was employed in the synthesis of *o*-(methoxybenzoyl)alanine (*o*-MBA, **76**) which was shown to inhibit kynureninase,[62] showing an IC_{50} of approximately 10 μM.[63]

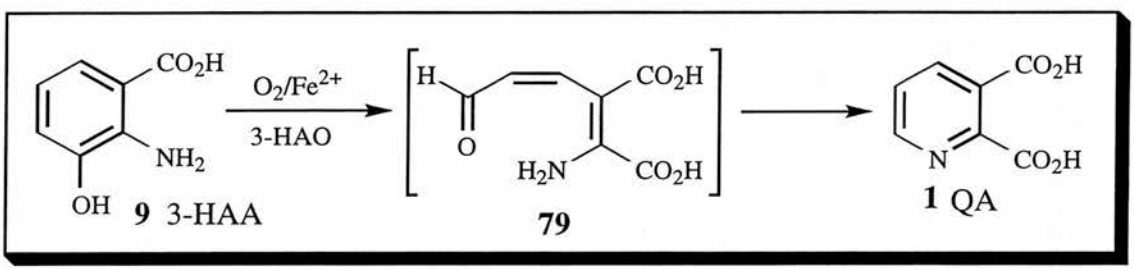
Other inhibitors of kynurenine 3-hydroxylase include 4-phenyl-4-oxo-butanoic acid derivatives.[64] The two most important of these being the 2-hydroxy- (**77**) and 2-benzyl- (**78**) derivatives.



These were tested on rat liver and rat brain kynurenine 3-hydroxylase with the following results; **77** showed an IC_{50} of 1.4 ± 0.3 μM in rat liver and 0.3 ± 0.06 μM in rat brain and **78** showed an IC_{50} of 2.9 μM in liver and 0.18 ± 0.01 μM in brain. Enantiospecific synthesis of the *S*(-) and *R*(+)-isomers of both the 2-hydroxy (**77**) and 2-benzyl (**78**) compounds revealed that the *S*(-)-isomers were the more potent inhibitors. It was postulated that the substituents at the 2-position increased the potency. The hydroxy group may possibly be involved in a hydrogen bond with an enzymic amino acid residue. The phenyl moiety occupies a different spatial region from the hydroxy group and may be ascribed to a hydrophobic interaction with a suitable amino acid residue.[64]

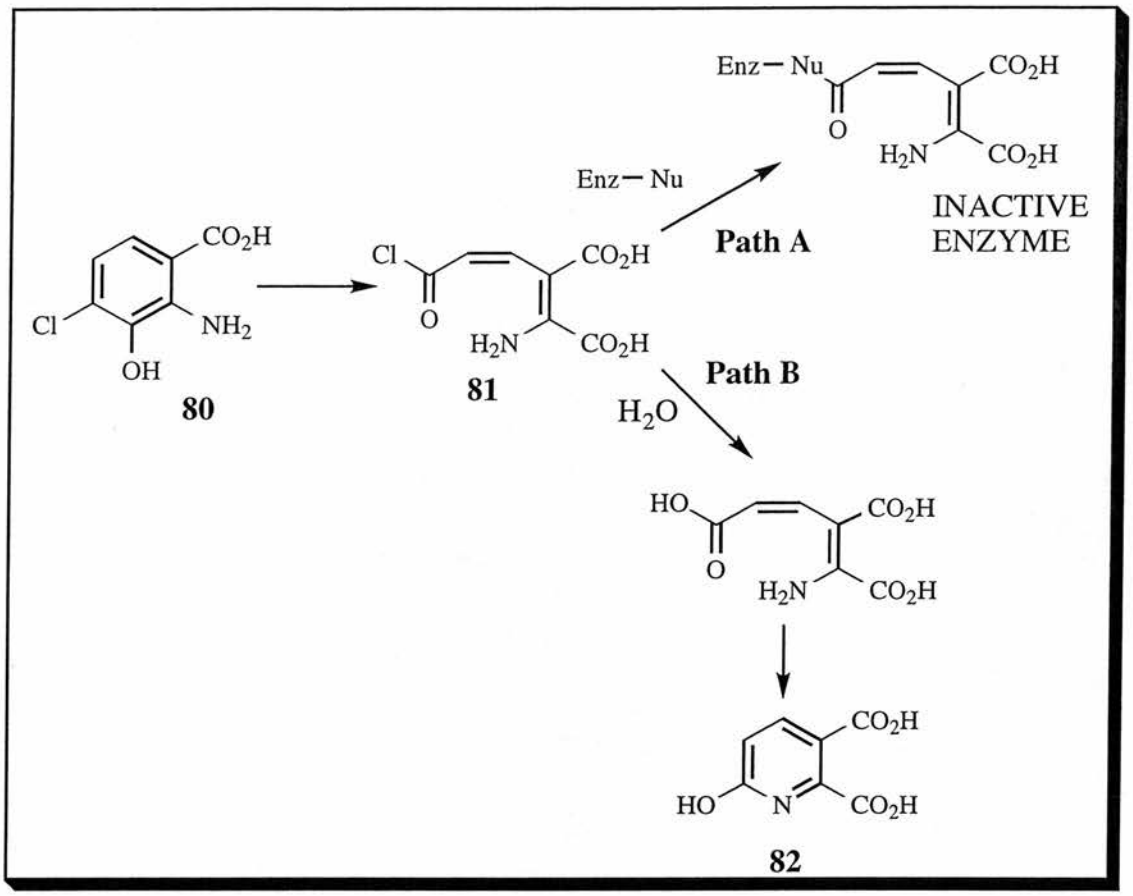
1.6.3 INHIBITORS OF 3-HYDROXYANTHRANILIC ACID OXIDASE

3-Hydroxyanthranilic acid oxidase (3-HAO) is dependent on both iron and sulfhydryl groups for its activity and quinolinic acid (QA, **1**) in mammals is exclusively synthesised by the action of this enzyme. It is an important target for drug action and provides the most direct method of reducing QA levels. The initial product of the reaction is α-amino-β-carboxymuconic acid ω-aldehyde (**79**) and the spontaneous cyclisation of this product to quinolinic acid (**1**) is non-enzymic (Scheme 1.15).



Scheme 1.15: Reaction Catalysed by 3-Hydroxyanthranilic Acid Oxidase

Halogenated derivatives of 3-hydroxyanthranilic acid (**9**) have been found to be very potent inhibitors of 3-HAO. One example is 4-chloro-3-hydroxyanthranilic acid (**80**). Initially the inhibition was thought to be irreversible and it was proposed that the inhibitor was converted to the acid chloride (**81**) by 3-HAO, which then acylated the enzyme causing inactivation (Scheme 1.16, Path A).[65] Later work revealed that the inhibition was in fact reversible and competitive.[66] It was proposed that the inhibitor was metabolised by the enzyme to give 6-hydroxyquinolinic acid (**82**) (Scheme 1.16, Path B) as the final product, although this was not identified. 4-Fluoro, 4-chloro and 4-bromo analogues gave K_i values of 190, 6 and 4 nmol l^{-1} respectively.



Scheme 1.16: Inactivation of 3-Hydroxyanthranilic Acid Oxidase

1.7 EXCITATORY AMINO ACID RECEPTORS

2*S*-Glutamate and 2*S*-aspartate are excitatory neurotransmitters in the mammalian CNS[67] and glutamate stimulates three distinctly different receptor types (the NMDA, kainate and quisqualate receptors) which are divided according to their recognition sites. Quinolinic acid is a potent agonist on the NMDA receptor. NMDA receptors are able to couple to other regulatory systems and receptor sites[68] and glycine, for example amplifies the NMDA response. Studies by Kemp showed some compounds to be non-competitive antagonists of the NMDA receptor and showed it to be equipped with allosteric binding sites.[69] It is generally accepted that EAA receptors have chemically gated ion channels. NMDA receptors are permeable to both Na^+, K^+ and Ca^{2+} ions. Intracellular calcium is important in many physiological processes but excessive influx of Ca^{2+} may contribute to

the damage of neurones by overstimulation of normal processes. The figure below shows a block representation of the NMDA receptor complex (Figure 1.1).[70]

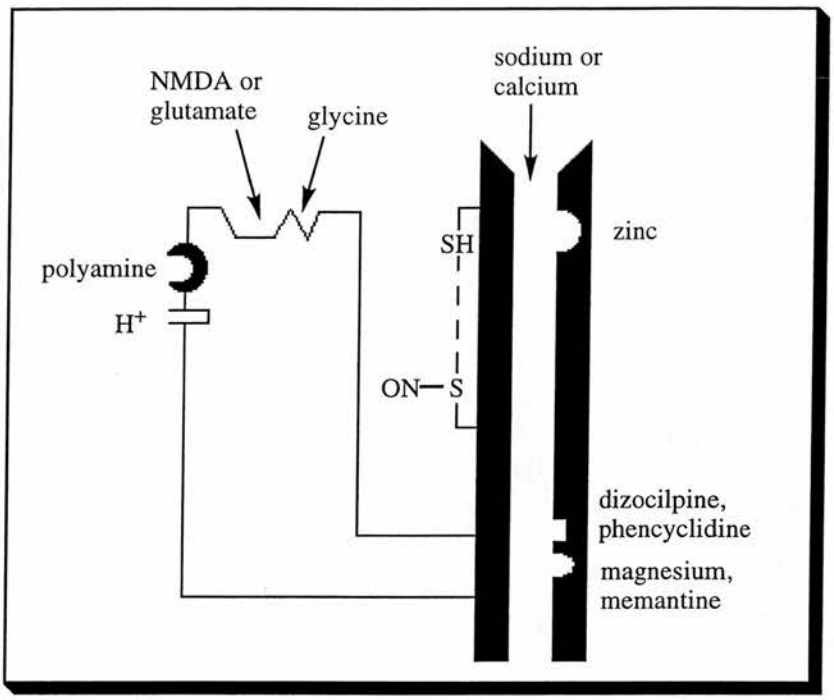


Figure 1.1: The NMDA Receptor–Channel Complex

The receptor complex is stimulated by NMDA and glutamate with glycine acting as a co-agonist. Cation influx (Na^+, Ca^{2+}) is regulated by modulatory sites (Mg^{2+} and various drugs). There is also a pH sensitive region, zinc- and polyamine-binding sites and a redox site, where one or more thiol groups may react to form an *S*-nitrosothiol.

Excessive stimulation of the NMDA receptor produces nitric oxide and superoxide which may react together to form peroxynitrite, a species which causes neuronal death.[71] Where $NO^{\bullet}$ causes cell death, the nitrosonium ion (NO^+) has been shown to decrease NMDA receptor activity and afford neuroprotection from overstimulation.[71]

Excitotoxicity occurs when a compound which normally stimulates EAA receptors is present for prolonged periods of time in synaptic clefts and begins to kill the cells for which it is normally a neurotransmitter. The autosomal inherited neurodegenerative

condition, Huntington's Disease (HD) is believed to have glutamate excitotoxicity as its pathogenic factor[72] and proton MRS suggests disordered glutamate metabolism in HD.[73] Glutamate-mediated neurotoxicity is also believed to be stimulated by calcium overload in NMDA receptor neurones.[74,75]

Much research however, concentrates on quinolinic acid as an etiological factor in HD. One indication shows that quinolinic acid excitotoxicity is brought about by activation of one of the receptor subtypes of the NMDA receptor by non-competitive antagonists.[76] A second indication for quinolinic acid as an etiological factor in HD is 3-hydroxyanthranilic acid oxygenase activity which is dramatically increased in HD patients.[77]

The NMDA sensitive population of receptors are involved in long term neuronal activity and with synaptic transmission in the CNS. They also play a central role in the plastic processes involved in the targeting of neurones and synapses during development, especially in the visual systems of vertebrates and invertebrates. NMDA receptors are also involved in the determination of cell viability and can activate destructive enzymes such as phospholipases, proteases and protein kinases.[78] The cell death occurring with the ageing process, and subsequent loss of neurones as seen in Alzheimer's disease may be explained to some extent by the activity of this population of receptors. Related neurodegenerative disorders such as Huntington's disease, epilepsy, as well as neuronal damage resulting from cerebral infarctions may also be explained by NMDA-receptor activation.

1.7.1 QUINOLINIC ACID

Quinolinic acid (**1**) has been recognised as a naturally occurring compound for many years[5] and is an intermediate in the synthesis of NAD from tryptophan (**4**). As described elsewhere, Lapin showed that quinolinic acid, as well as other metabolites of kynurenine, have stimulant and convulsant effects in rats.[79] Quinolinic acid causes neuronal damage by overstimulation of the NMDA receptor and had been shown to discriminate between receptor subtypes.[9] The selective pattern of cell death caused by quinolinic acid is reminiscent of those observed in many neurodegenerative disorders.[80] NMDA receptors are located in the cerebral cortex, basal ganglia and hippocampus and are involved in synaptic transmission in the CNS as well as in neuronal plasticity, cell viability and learning and memory. It is thought that high concentrations of quinolinic acid induce neuronal dysfunction at the NMDA receptor.[70]

Although it has been shown that quinolinic acid is present in both human and rat brain[81,82] its source is as yet undefined.[82] The enzymes of the kynurenine pathway are found primarily in the liver and quinolinic acid is unable to cross the blood-brain barrier under physiological conditions. Quinolinic acid uptake is thus presumably mediated by passive diffusion.[83] The enzyme 3-hydroxyanthranilic acid oxygenase (3-HAO) and 3-hydroxyanthranilic acid (**9**) are both present in brain and these represent the most likely source of quinolinic acid (**1**). Quinolinate phosphoribosyltransferase (QPRTase) is also present in human brain. Both of these enzymes however, have been shown to be localised in different areas of the brain. 3-HAO is localised primarily in the frontal cortex, striatum and hippocampus and almost entirely in the soluble fraction of brain homogenates in rat brain. The areas show little detectable QPRTase which is localised in glial cells such as astrocytes.[84] Only the olfactory bulb, an area of high neuronal turnover, has equal activities of 3-HAO and QPRTase. Quinolinic acid is present in both cellular and extra-cellular components of brain. The conclusion being that quinolinic acid is metabolised by cerebral mechanisms. This suggests that quinolinic acid is synthesised in one population

of glial cells by 3-HAO and then metabolised by QPRTase in a separate population of glial cells and neurones.[11] Bender also argues that the distinct compartmentation of QPRTase and 3-HAO is more due to the formation of nicotinamide nucleotides.[85] An important feature of the kynurenine pathway is its role in the formation of nicotinamide nucleotides and this raises the question that the main function of QPRTase is not the catabolism of quinolinic acid rather the biosynthesis of nicotinamide in cells requiring NAD as a cofactor.

3-HAO is 80-fold more active than QPRTase[86,87] and administration of 3-hydroxyanthranilic acid (**9**) promotes the formation of quinolinic acid (**1**) showing that 3-HAO is saturated with substrate.[88] The relative activities of 3-HAO and QPRTase may not be important factors in quinolinic acid concentrations, but they do imply that the activity of QPRTase is rate limiting.

Quinolinic acid has a lower affinity for the NMDA receptor than NMDA itself, but it has been suggested that two populations of NMDA receptor exist within the CNS.[89,90] The second of these, which is contained in brain, is sensitive to both quinolinic acid and NMDA. Quinolinic acid is a more potent neurotoxin than its affinity for the NMDA receptor suggests. One theory is that concentrations of quinolinic acid at synapses are higher and these levels are not reflected in mean levels of quinolinic acid.[11] Low concentrations of quinolinic acid in brain have led to the development of sensitive assays[91,92] and although there are elevated levels of quinolinic acid in inflammatory brain disease, in Huntington's disease Heyes showed that there was no significant increase.[93] Although Heyes' work conflicts with that of other groups,[80] Schwarcz, who showed that quinolinic acid caused the same nerve cell death as observed in Huntington's disease, argues that Heyes' research does not invalidate the quinolinic acid hypothesis for Huntington's disease as metabolically compromised neurones could be vulnerable to normal levels of quinolinic acid.[92]

The fact that quinolinic acid concentrations have been shown to increase upon administration of tryptophan (**4**)[88] and quinolinate synthesis is also stimulated by elevation of 3-hydroxyanthranilic acid (**9**) suggests that quinolinic acid (**1**) levels are elevated in HD. Activity of the enzyme 3-HAO which catalyses this conversion, is significantly increased in the brain of patients suffering from Huntington's disease.[77] Elevated concentrations of quinolinic acid have been shown in epilepsy.[94] Quinolinic acid does not act as a classic neurotransmitter as it is not released on depolarisation and no mechanism exists to remove it from extracellular space. A reduction in the amount of endogenous neuroprotection as supplied by kynurenic acid (**2**) has also been implicated in this pathological state.[8]

The inability of quinolinic acid (**1**) to penetrate the blood-brain barrier suggests that it is synthesised intracerebrally. Prolonged stimulation of excitatory amino acid receptors (EAA) causes cell degeneration.[69] Quinolinic acid concentrations increase during the ageing process.[95] Moroni showed in rats that levels approached 10 μM in brain and if these were maintained over several days they resulted in neurotoxicity. The same group also studied brain samples from Alzheimer's diseased patients, but no correlation between neurodegeneration and quinolinic acid levels in the cerebral cortex were observed.[96]

Inflammatory neurological diseases such as AIDS related dementia, Lyme disease and polio virus also show increased levels of quinolinate. Detailed studies have shown that an overall increase in activity throughout the kynurenine pathway accounts for increased levels of the quinolinic acid precursor, 3-hydroxyanthranilic acid.[97] It has also been postulated that the HIV virus itself activates the macrophage cells of the immune system to synthesise quinolinic acid.[98] Paradoxically, it has also been shown in rats that a tryptophan-free diet, which should in theory lower quinolinic acid levels in brain, also results in increased quinolinate levels.[99] One explanation for this is that quinolinic acid (**1**) can also be synthesised *via* a different pathway. It has already been shown that some bacteria and plants synthesise quinolinic acid from the condensation of aspartic acid and

dihydroxyacetone.[23] This has also been shown in sufferers of pellagra who show CNS symptoms including hallucinations and dementia following tryptophan-deficient diets.

Quinolinic acid neurotoxicity is dependent on the presence of QPRTase. It is less effective as a neurotoxin in areas where there is a high QPRTase activity (e.g., cerebellum, hypothalamus and substantia nigra) in comparison to areas of low activity (e.g., cerebral cortex, striatum and hippocampus). An increase in 3-HAO activity is often responsible for elevated quinolinic acid levels.

1.7.2 KYNURENIC ACID

Kynurenic acid (**2**) was one of the first metabolites of tryptophan to be isolated and characterised in mammals.[4] The role of 2*S*-kynurenine (**5**) as its precursor was described later by Heidelberger.[100] Interest in kynurenic acid centres around its possible involvement in several neurodegenerative diseases with excitotoxic etiologies.[101,102] Kynurenic acid is a broad spectrum antagonist of EAA receptors and can antagonise electrophysiological responses to NMDA and kainic acid receptor agonists.[103] Recent evidence has shown that kynurenic acid acts both at the glycine allosteric site and the agonist recognition site of the NMDA receptor as well as at non-NMDA receptors.[104,105] Kynurenic acid is also potentially relevant in the physiological regulation of excitatory synaptic transmission mediated by endogenous EAAs.[106]

Unlike its precursor, 2*S*-kynurenine, kynurenic acid cannot cross the blood-brain barrier under normal physiological conditions. As with quinolinic acid, it is probably due to its polar nature that kynurenic acid does not enter the brain from the periphery to a significant extent.[83] Kynurenic acid is the only known endogenous inhibitor of EAA receptors in the mammalian CNS. Its presence in the CNS was demonstrated in 1988 by Moroni, but it is still unclear what role it plays.[107] It may take on a modulatory role in EAA

neurotransmission under physiological conditions or it could be involved in the pathogenesis of neurological disease.

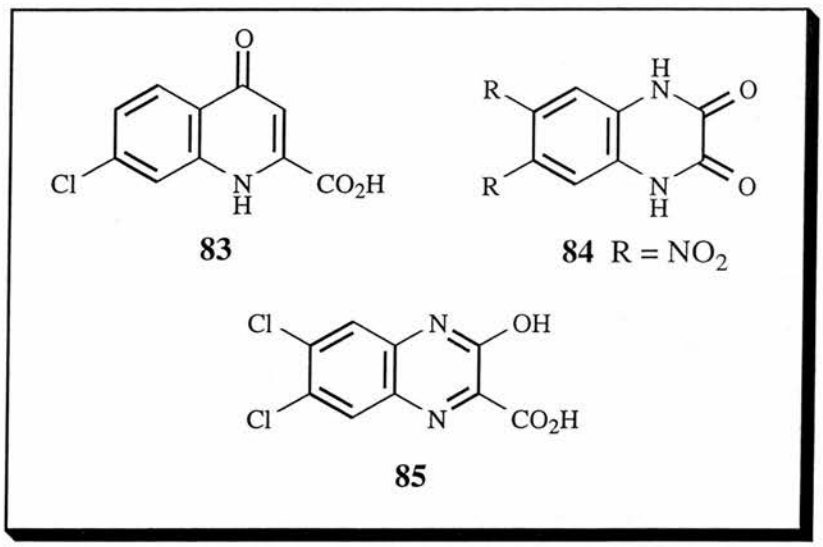
For this to be established Swartz *et al.* showed that kynurenic acid (**2**) was synthesised in the CNS and not transported from peripheral tissues, and that it could be released into extracellular fluid in concentrations that could significantly effect EAA receptors.[108] They also showed that intracerebral infusions of 2*S*-tryptophan did not increase levels of kynurenic acid where 2*S*-kynurenine did. This suggests that kynurenic acid is synthesised from kynurenine and not tryptophan which has crossed the blood-brain barrier. Further studies ruled out transportation of kynurenic acid across the blood-brain barrier by blocking kynurenine aminotransferase with inhibitors after peripheral administration of kynurenine. This again showed that kynurenic acid is synthesised from kynurenine in the CNS.

The concentration of kynurenic acid in rat brain is shown to increase with ageing.[107] A rapid increase in kynurenic acid content occurs during the first 80 days of life, with 2 month old animals having concentrations 20-fold greater than those of 7 day old animals. Kynurenic acid content in brain increases much more slowly after sexual maturity is reached, but 18 month old animals still have a threefold greater concentration of kynurenic acid in brain than three month old animals. This change in brain kynurenic acid concentrations may have functional implications.[107] The importance of EAA-mediated neurotransmission in the different phases of development and the ageing process has already been established.[109] The lack of kynurenic acid in new born animals could be useful to allow glutamate and other endogenous agonists of glutamate receptors to play their roles in the regulation of neuronal growth and development and neurone loss during maturation of the CNS.[110] The low levels of kynurenic acid observed in the first period of life could also explain in part the susceptibility to glutamic acid neurotoxicity and convulsions in the brains of newborn animals. In later life the accumulation of kynurenic acid in the brains of aged rats could explain some age-related pathophysiological changes.

EAA antagonists affect learning and memory and increased kynurenic acid levels have been implicated in memory loss. Interestingly kynurenic acid levels also change at various times of the day with levels twice as high during the day than at night. This suggests a possible role in biological rhythms and sleep induction and maintenance for kynurenic acid.

The mechanism of action by which kynurenic acid antagonises the EAA receptor has been extensively studied.[111] It is generally accepted that 10 μM concentrations of kynurenic acid displace glycine from the strychnine-insensitive binding sites and antagonise, in a competitive manner, glycine actions on the NMDA receptor–ion-channel complex.[112] On average kynurenic acid (**2**) is approximately 100-times less concentrated than quinolinic acid (**1**) and 5-hydroxytryptamine (**6**) in the brain and kynurenic acid is localised more in the brain stem than in other brain areas.[11] This again suggests compartmentation of quinolinate, kynurenate and serotonin molecules. Concentrations of kynurenic acid outside the brain are significantly higher with high concentrations being detected in the liver, kidney, heart and blood. Kynurenic acid is present in micromolar concentrations in the human brain whereas concentrations are 50-times lower in rat brain.

Increased neuronal activity and depolarisation, induced for example by veratridine or potassium can decrease kynurenic acid concentrations in brain.[113] Although the mechanism is unknown it is possible that neuronal depolarisation may result in the release of a factor which suppresses kynurenic acid efflux/synthesis in astroglial cells. This is supported by evidence that shows that veratridine does not directly inhibit kynurenine aminotransferase in rat brain.[114] Also, potassium depolarisation does not suppress kynurenate production in the absence of external calcium. This suggests that a neuronally derived agent requiring calcium for release is responsible for decreases in kynurenate synthesis.[115] Glutamate has been proposed as this agent[114] and evidence supporting this theory shows glutamate does reduce kynurenic acid production in lesions.[116]



83 **84** R = NO_2 **85**

It has recently been reported that analogues of kynurenic acid,[117] such as 7-chlorokynurenic acid[118] (**83**), quinoxaline-2,3-diones (**84**) and 6,7-dichloroquinoxalic acid (**85**) act as pure antagonists at the glycine modulatory site of NMDA. Derivatives of kynurenic acid have also been shown to inhibit the binding of nerve growth factor to the low-affinity p75 receptor.[119]

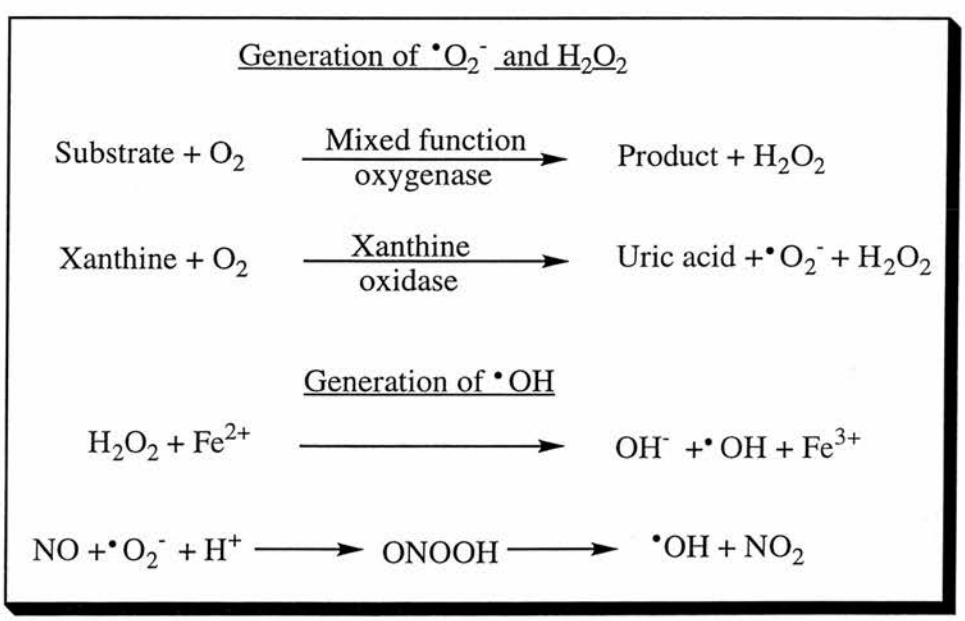
In summary, kynurenic acid is an important neuroprotective agent protecting against the excitotoxicity of quinolinic acid and reducing neuronal damage after anoxic and ischemic brain injury. It is a non-competitive antagonist at excitatory amino acid receptors and is present in the mammalian CNS.

1.7.3 3-HYDROXYKYNURENINE

3-Hydroxykynurenine (3-HK, **3**) is a tryptophan metabolite whose concentrations in brain are markedly elevated in Huntington's disease[54] and HIV infected brain.[97] It is known to be both carcinogenic and neurotoxic.[120] Increased levels of 3-HK are also seen in Parkinson's disease.[121] Although it has been shown that 3-HK exhibits neurotoxic properties[13] the argument that it is involved in brain pathogenesis can be dismissed. This is because the concentration at which 3-HK is toxic in brain is 100 fold higher than the

levels found naturally in brain even under pathological conditions.[13] However, it has been shown[15] that low concentrations of 3-HK can cause cell death in certain neurone populations and that increased concentrations of 3-HK in brain are relevant in neurodegenerative diseases.

Research by Okuda *et al.*[15] showed that the neurotoxicity of 3-HK (**3**) was not blocked by superoxide dismutase suggesting that hydrogen peroxide and hydroxyl radicals are involved in toxicity. Recent evidence[122] has indicated that oxidative stress caused by reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radicals may be an important factor in neuronal death (Scheme 1.17).[14] Cellular defence mechanisms eliminate ROS generated by normal metabolism in cells. Cumulative damage caused by an imbalance in these protective mechanisms leads to cell death as characterised in several neurodegenerative disorders. It is believed that the imbalance is due more to increased ROS production than reduced cellular defences. Okuda suggests that 3-HK may be one of the factors that contribute to ROS generation in brain.[15]



Scheme 1.17: Free Radical Reactions Leading to the Formation of ROS

The toxicity of 3-HK is mediated by hydrogen peroxide produced by the oxidation of 3-HK.[15] It is interesting to note that 3-HK mediated damage corresponds to the pathological features seen in Huntington's diseased brain. Research into the toxicity and potency of kynurenines by Okuda[14,15] on cultured striatal neurones showed that kynurenine (**5**), quinolinic acid (**1**) and xanthurenic acid (**55**) were not toxic at concentrations of 1–100 μM. The result for quinolinic acid is in direct contrast to evidence from studies by Schwarcz[123] and Beal.[80] The finding that only 3-HK (**3**) and its metabolite, 3-hydroxyanthranilic acid (**9**) were neurotoxic indicates that the *o*-aminophenol structure is essential for toxicity.[14] This structural feature has also been shown to cause autoxidation generating ROS.[120] This theory was strengthened by the finding that anthranilic acid (**8**) also showed no neurotoxicity at similar concentrations. For completeness, Okuda[14,15] also showed that ROS scavengers such as α-tocopherol, its water-soluble analogue Trolox and *N*-acetylcysteine, among others prevented neurotoxicity of 3-HK at 1–10 μM concentrations. At higher concentrations only *N*-acetylcysteine showed any neuroprotection. Their research concluded that 3-HK is a much more potent neurotoxin than quinolinic acid.

1.8 IMPORTANCE OF KYNURENINES IN DISEASE

As previously mentioned the kynurenine pathway plays an important role in disease. Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder affecting cognitive ability, movement and emotional control. It affects approximately 1 in 10,000 people in most western countries and both men and women have an equal chance of inheriting the adult form of the disease. Generally the disease progresses to death over 15 to 20 years from complications such as choking or pneumonia.[124]

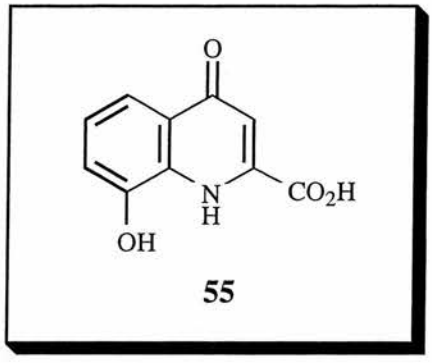
The gene causing Huntington's disease was discovered in 1993[125] previous work having shown a linkage between the HD gene and a DNA marker that mapped to human chromosome 4.[126] A region of the IT15 (HD) gene has an expanded and unstable CAG

trinucleotide repeat. The non-mutated gene has between 11 and 35 CAG repeats but in HD patients this increases to 36-80 repeats, longer repeats (upto 120) are associated with the juvenile form of the disorder and more severe disease.[124] HD shows selective neuronal loss in the basal ganglia causing the symptoms of disease.[127] Work by several research groups has suggested a variety of tryptophan metabolites to be responsible for HD, and loss of neuroprotection and increased levels of agonists are equally likely to be involved.

In Alzheimer's diseased brain, levels of kynurenine (**5**) and 3-hydroxykynurenine (**3**) are seen to decrease, but brain concentrations of kynurenic acid (**2**) are shown to be significantly increased.[128] The weakening of the blood-brain barrier in this disease state allows for greater kynurenate penetration and changes in kynurenic acid metabolism may account for neurone loss and cell death in this dementing disorder.

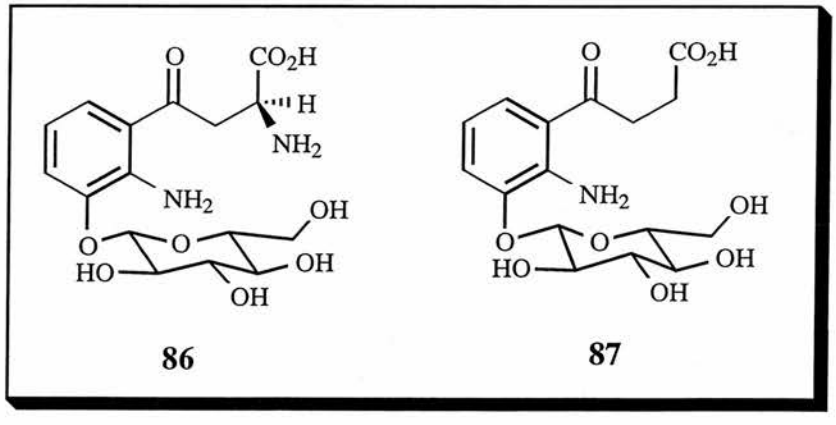
Temporal lobe epilepsy and seizures are also affected by kynurenines, with 2*S*-kynurenine, quinolinic acid and kynurenic acid all acting as convulsants. Increased permeability of the blood-brain barrier and thus increased penetration of kynurenines in to the brain from the liver are observed in epileptic patients who suffer severe seizures.[129] Kynurenic acid has been shown to block such seizures.[130]

Dursun[131] *et al.* showed that sufferers of Tourettes syndrome have increased levels of plasma kynurenine and that these elevated concentrations were due to a decrease in the activity of kynureninase and kynurenine 3-hydroxylase. Patients with Downs syndrome were shown to have a threefold increase in levels of kynurenic acid in specific regions of the brain and decreased levels of brain kynurenine aminotransferase I. The cause of this increase in unclear but in these cases, as the blood-brain barrier has not been compromised, it cannot be due to changes in the metabolism of kynurenic acid.[132] Another metabolite of 3-hydroxykynurenine, xanthurenic acid (**55**) has recently been shown to be an inducer of malaria development in mosquitoes.[133]



55

The role of the NMDA receptor in the development of the visual systems of vertebrates has already been mentioned, but the role of endogenous tryptophan-derived UV filters in ageing lenses and in human cataract is becoming of increasing interest.[134] The lenses of many vertebrates contain low molecular weight fluorescent compounds which act as UV filters, protecting the retina and possibly the lens from UV radiation. Kynurenine, 3-hydroxykynurenine and the *O*-β-D-glucopyranosides of 3-hydroxykynurenine (**86**) and 2-amino-3-hydroxy-γ-oxobutanoic acid (**87**) fulfil such a role in human lenses.[135]

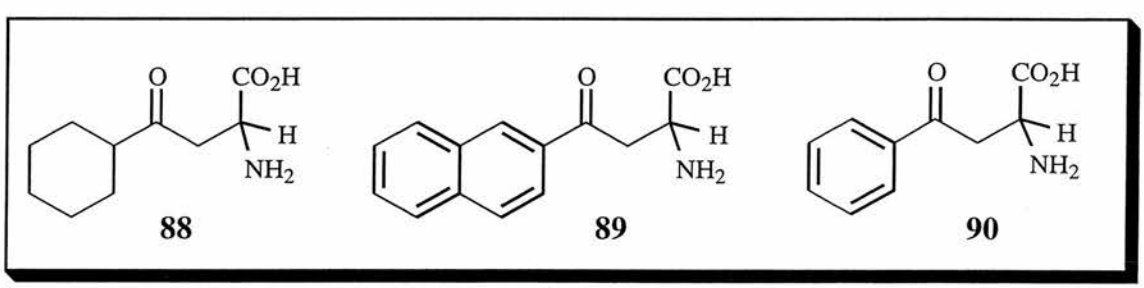


86 **87**

CHAPTER 2

2 ANALOGUES OF KYNURENINE AS COMPETITIVE INHIBITORS OF KYNURENINASE

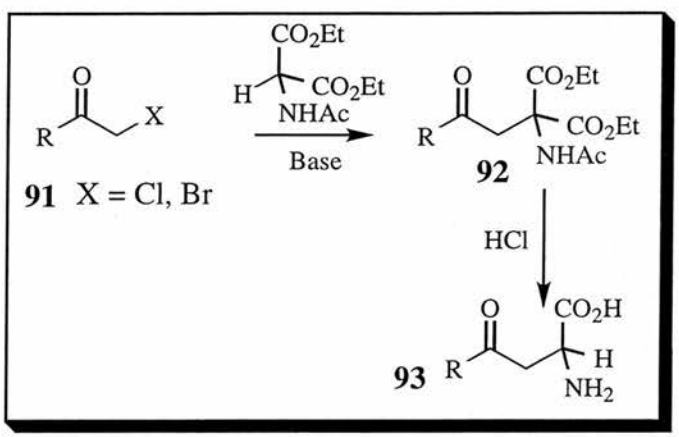
As described in Chapter 1, there are few known inhibitors of kynureninase. The most active of these inhibitors mimic the *gem*-diolate tetrahedral transition state postulated by Phillips and Dua.[36] It was our aim to synthesise a series of racemic competitive inhibitors which could compete for the binding sites and cofactor. The racemic synthesis of *o*-, *p*- and most importantly *m*-nitrobenzoylalanine (**72**, **73**, and **69**) as inhibitors of kynurenine 3-hydroxylase has been the starting strategy for much of this work.[60] Previously within the group cyclohexyl (**88**) and naphthyl (**89**) derivatives have been tested for activity against kynureninase isolated from *Pseudomonas fluorescens*. The naphthyl derivative showed a K_i of 85 μM and the cyclohexyl derivative was a weaker inhibitor with a K_i of 844 ± 47 μM.[45]



Desaminokynurenine (**90**) gave a K_i of 23.2 ± 3.1 μM, but also showed mixed inhibition indicating it may be a substrate for the reaction normally catalysed by kynureninase.[45]

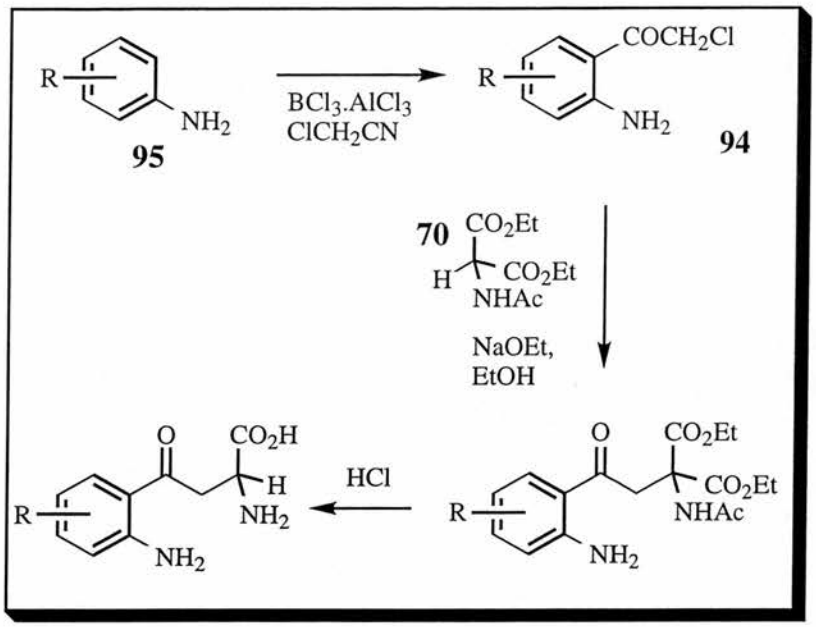
2.1 LITERATURE SYNTHESIS OF KYNURENINE ANALOGUES

A number of racemic kynurenine analogues have been synthesised using the same basic method. In these, an α-haloketone (**91**) is added to diethyl acetamidomalonate and the resulting compound (**92**) is subsequently deprotected and decarboxylated by heating in aqueous acid to give the free amino acid (**93**) (Scheme 2.1).



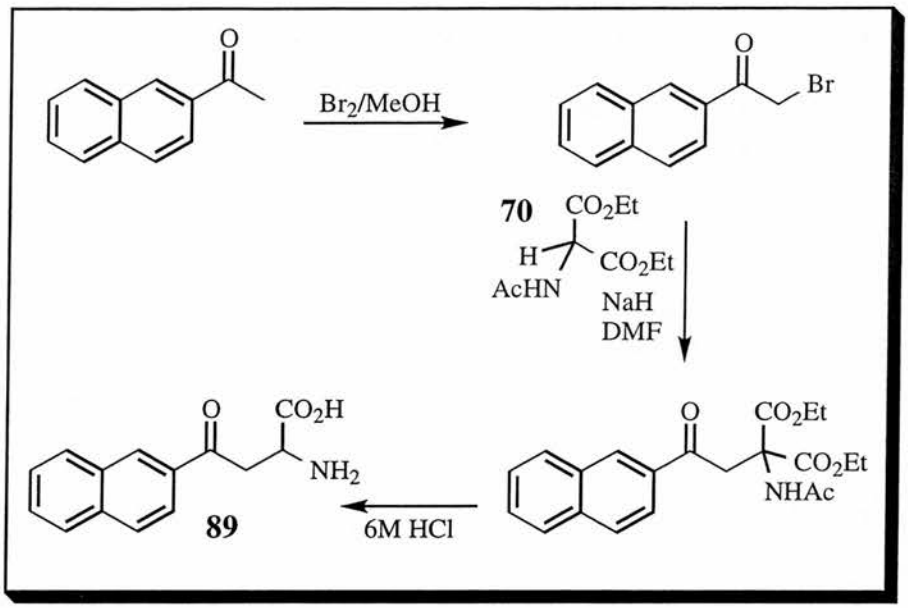
Scheme 2.1: Racemic Synthesis of Kynurenine Analogues

Pellicciari *et al.* used this method to couple diethyl acetamidomalonate (**70**) to nitrophenacyl bromides to form *o*-, *p*- and *m*-nitrobenzoylalanines as shown previously in Scheme 1.13.[60] The conversion of α-chloroacetophenones (**94**) from substituted anilines (**95**) was employed by Varasi *et al.* to afford substituted kynurenines (Scheme 2.2).[136]



Scheme 2.2: Synthesis from α-Chloroacetophenones

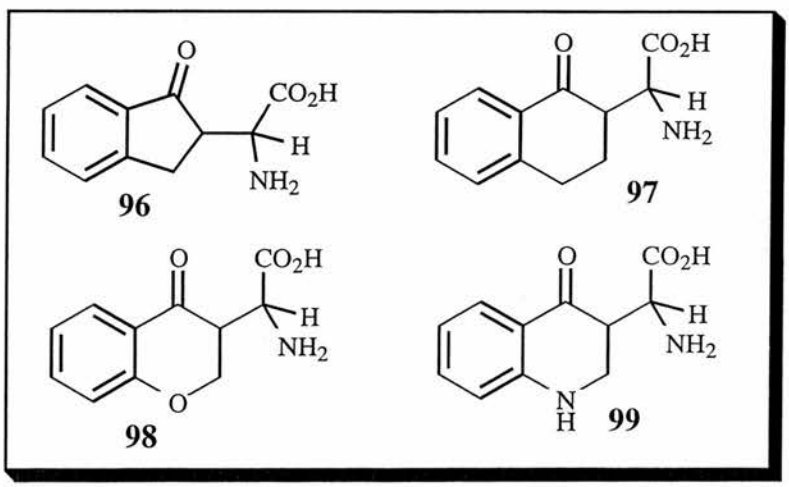
Other methods have been used to prepare variously substituted kynurenines from substituted anilines. Ross was the first to prepare a naphthyl analogue of kynurenine (**89**) in the manner described below (Scheme 2.3).[45] Bromination was facilitated by either bromine in acetic acid or more selectively by using bromine in methanol. The bromide was then added to the preformed sodium anion of diethyl acetamidomalonate which was deprotected by decarboxylative hydrolysis in 6M hydrochloric acid to give the desired product as a racemate. This route was also applied for the synthesis of desaminokynurenine (**90**) and the cyclohexyl derivative (**88**) described above.



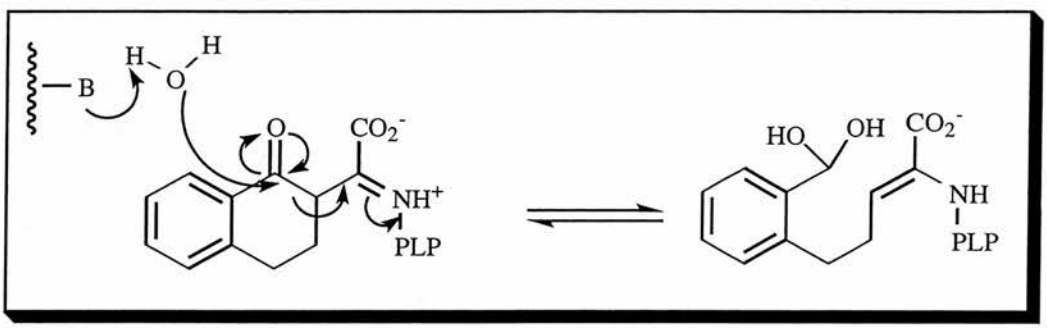
Scheme 2.3: Synthesis of Bicyclic Analogues

2.2 AIMS

It was our aim to synthesise a number of bicyclic analogues (**96–99**). An indanone (**96**) and tetralone derivative (**97**) were to be synthesised in order to probe the effect of ring size on inhibition at the active site. Heteroatoms would then be introduced to mimic the *ortho*-amino group present in kynurenine. This might also affect the configuration at the active site.



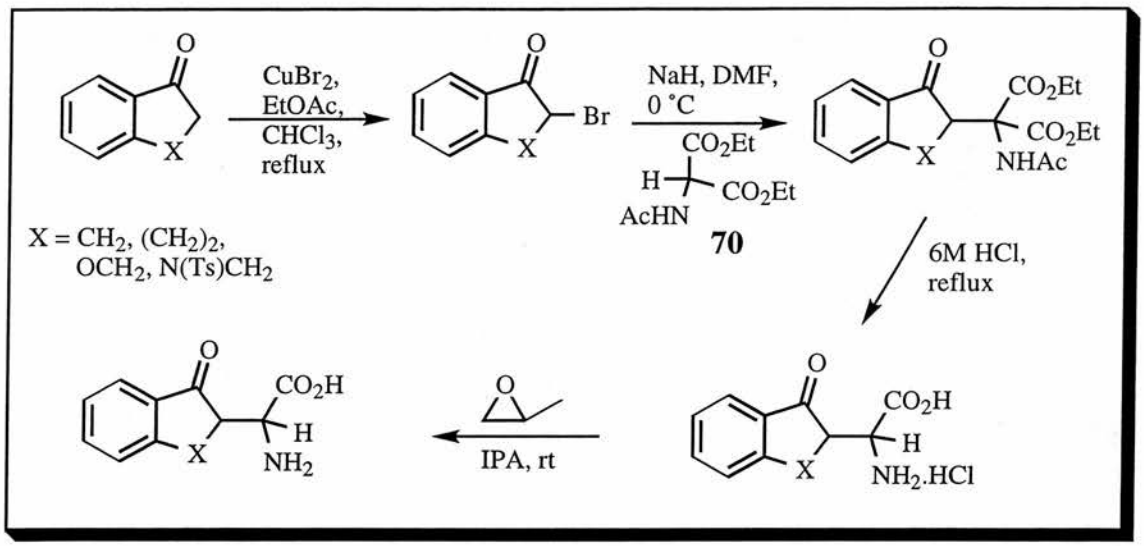
All the analogues were synthesised as racemates with the intention that if a racemate exhibited exceptional inhibition it could be synthesised enantiospecifically at a later stage. It is worth noting however that only the two (2*S*)-isomers should have any effect at the active site, this is because kynureninase only reacts with (2*S*)-amino acids. The other feature of the bicyclic analogues is that following the normal kynureninase catalysed reaction the side chain would still be attached to the amino acid fragment, (Scheme 2.4). This may improve their binding to the active site.



Scheme 2.4: Postulated Reaction of Bicyclic Analogues

2.2.1 GENERAL STRATEGY FOR THE SYNTHESIS OF BICYCLIC ANALOGUES

The yield of the initial bromination step when carried out using bromine in methanol or bromine in acetic acid was quite poor and in some cases dibromination alpha to the ketone occurred. A more-selective mono-bromination can be facilitated by using cupric bromide in ethyl acetate.[137,138] The use of chloroform as a co-solvent greatly increased the yield without causing over bromination at the α-carbon. The bromide is then coupled to the anion of diethyl acetamidomalonate. The isolated coupled product is then deprotected by decarboxylative hydrolysis in acid and the free base obtained by stirring in propylene oxide and isopropyl alcohol (Scheme 2.5).



Scheme 2.5: General Route to Bicyclic Analogues

2.2.1.1 Preparation of the Indanone Derivative [Amino-(1-oxo-2,3-(1*H*)-dihydroinden-2-yl)-acetic acid, 96]

The first step in the scheme was the bromination of indanone alpha to the ketone. This was achieved by heating cupric bromide in ethyl acetate under reflux. The indanone was then added in the co-solvent, chloroform and the reaction heated at reflux for a further five hours. After cooling the reaction mixture was filtered, decolourised and the solvent was removed to give the product (**100**) as an orange oil in 95% yield. The ^{1}H-nmr spectrum showed three distinct signals at $\delta = 3.43$, 3.85 and 4.48 ppm representing the two protons at the 3-position of the ring and the 2-CH respectively. Analysis by mass spectrometry showed that only the mono-brominated product had been formed.

The bromide was then coupled to diethyl acetamidomalonate. The anion of diethyl acetamidomalonate (**70**) was formed in dry DMF using sodium hydride at 0 °C for three hours. The solution became yellow once the anion had been fully formed. A solution of 2-bromo-1-indanone (**100**) in DMF was then added at 0 °C and the solution stirred overnight at room temperature. After quenching in dilute acid, the product was extracted into diethyl ether and the solvent removed to give a brown oil. This oil was purified by

column chromatography to yield the pure product (**101**) in 44% yield as an orange oil. The ^{1}H-nmr spectrum showed characteristic peaks for the ethyl ester group at δ = 1.3 and 4.2 ppm and for the acetate group at δ = 2.15 ppm. The signals for the protons at positions 2 and 3 of the ring were seen as multiplets and were assigned using ^{1}H–^{13}C-COSY experiments. The ^{13}C-nmr spectrum confirmed the structure of the product (**101**).

Deprotection of the coupled product was afforded by dissolving the protected indanone derivative (**101**) in 1,4-dioxane and adding 6M hydrochloric acid. The mixture was heated under reflux for 7 hours and the reaction monitored by thin layer chromatography. After cooling, the solution was washed with ethyl acetate and the aqueous phase concentrated to a yellowish liquid. Trituration of this with acetone gave a white solid which was difficult to dry. The free amine was obtained in 22% yield by dissolving in isopropyl alcohol and stirring with propylene oxide. Analysis by nmr spectroscopy confirmed the structure of the product (**96**) as a mixture of diastereomers in the ratio 1.43:1; major to minor. The 2-CH was seen as a multiplet at δ = 3.45 ppm, whilst the α-proton was seen as two doublets at δ = 4.38 ppm and 4.30 ppm, major to minor, respectively. The protons at position 3 were seen as a multiplet. The signals in the carbon spectrum also showed two signals for the diastereomers. The α-CH showed peaks at δ = 51.7 and 53.8 ppm with a coupling constant of *J* 153 Hz. The mass spectrum and accurate mass measurement further confirmed the structure of the product.

2.2.1.2 Preparation of the Tetralone Derivative [Amino-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-acetic acid, 97]

The tetralone derivative was prepared in an analogous manner to the indanone derivative with the bromide (**102**) being formed in 90% yield as an orange oil. The nmr signals for the protons at the 3-position of the ring were split into a triplet at δ = 2.93 ppm and a multiplet at δ = 3.3 ppm for H_A and H_B. The signal for the protons at position 4 were not

affected by bromination at the 2-position although all the protons shifted slightly. Mass spectrometry confirmed the presence of the mono-brominated product.

The coupling reaction occurred in 37% yield after column chromatography on silica using diethyl ether as eluent. The orange oil was shown by nmr spectroscopy to be a single product (**105**). In the ^{13}C-nmr spectrum the carbon alpha to the ketone shifted from 51 ppm in the bromide to 63 ppm in the diethyl acetamidomalonate coupled product. Mass spectrometry confirmed the molecular weight at 361.

The deprotection/decarboxylation step was again carried out in 6M hydrochloric acid and 1,4-dioxane. The low solubility of both the tetralone and indanone derivatives required a co-solvent to afford deprotection. The reaction time for the tetralone was 8 hours and the solution was washed with ethyl acetate to remove any traces of starting material or byproducts. The brown liquid obtained upon concentrating the aqueous phase was triturated with acetone to give a grey solid. Once again this was stirred in propylene oxide and isopropyl alcohol over night and after concentration gave an off white solid in 15% yield. The product was pure by microanalysis and was shown by nmr spectroscopy to be the product (**97**) as a mixture of diastereomers in the ratio 3:1 (major: minor). The protons at positions 3 and 4 were seen as a doublet and a triplet respectively whilst the α-proton was seen as two multiplets at 4.0 ppm and 4.3 ppm. The 2-CH signal overlapped and it was not possible to see the diastereomers.

2.2.1.3 Preparation of the Chromanone Derivative [Amino-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetic acid, 98]

The bromination of 4-chromanone was facilitated as described above in 81% yield using cupric bromide. The pale yellow oil (**104**) was used without further purification. The proton nmr signals for the methylene at position 2 and the methine at position 3 were seen as a multiplet at δ = 4.65 ppm. Prior to bromination the protons at 2- and 3-positions

were seen as double doublets at $\delta = 2.81$ and 4.51 ppm. As before the coupling reaction was facilitated by reacting the preformed anion of diethyl acetamidomalonate (**70**) with the bromide (**104**). After extraction into diethyl ether, 55% of a crude brown oil was obtained. Purification by column chromatography on silica with diethyl ether as eluent gave a sticky orange oil (**105**) in 31% yield. The ^{1}H-nmr spectrum showed characteristic peaks at $\delta = 1.25$ and 2.01 ppm corresponding to the methyl protons of the esters and the acetate protecting groups. The signal for the methylene of the esters was a multiplet at $\delta =$ 4.16–4.35 ppm which overlapped with the signal for the protons at position 2 of the chromanone. The yield of this coupling reaction was significantly lower than those of the previous ring systems synthesised and this led to alternative routes to the target compound as discussed later.

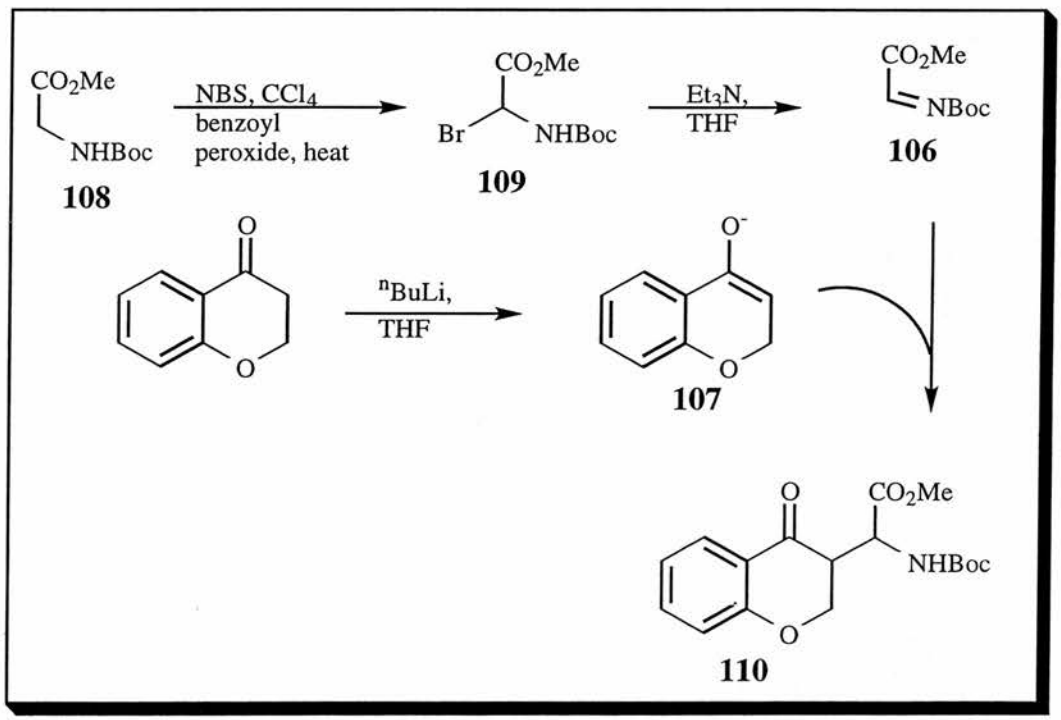
Attempts to deprotect the coupled chromanone described above were hindered by the apparent insolubility of the compound. Deprotection of the indanone and tetralone derivatives (**101**, **103**) was facilitated by heating in 1,4-dioxane and aqueous acid, this resulted in decomposition when employed in the deprotection of the chromanone derivative (**105**). However, the chromanone derivative could be decarboxylatively deprotected by dissolving in the minimum of THF and heating in aqueous acid. The free base (**98**) was then formed by stirring the red solid obtained above in isopropyl alcohol and propylene oxide. The pink solid was obtained in only 5% yield. The ^{1}H-nmr spectrum showed multiplets for the 3-methine and the α-proton at $\delta = 3.2$–3.5 ppm and δ = 3.9–4.1 ppm, respectively. The methylene group at position 2 however, was seen as a doublet at $\delta = 4.27$ ppm. Different synthetic routes however gave identical spectra.

Preparative cellulose tlc was used to try and improve the purification procedure. A mobile phase of 4:1 ammonia: methanol was employed. Two bands were clearly visible on the cellulose plate, each probably containing two diastereomers. The ^{1}H-nmr spectra for both the brown oils obtained in this manner showed similarities to those obtained previously. However, yields achieved by this method were very low and preparative tlc is both time

consuming and limited in the amount that can be purified on each plate. As this purification step did not improve yield or purity it was felt that another method of coupling should be sought.

2.3 ALTERNATIVE GLYCINE EQUIVALENTS AND THEIR USE IN THE SYNTHESIS OF THE CHROMANONE DERIVATIVE

As the overall yield for the chromanone derivative (**98**) by the initially planned route was low, alternative coupling reactions and alternatives to diethyl acetamidomalonate (**70**) were sought. The use of a cationic glycine equivalent such as the imine (**106**) negates the need for an α-halide. Instead the enolate anion of chromanone (**107**) would be used to form the carbon—carbon bond to the imine (**106**) (Scheme 2.6).

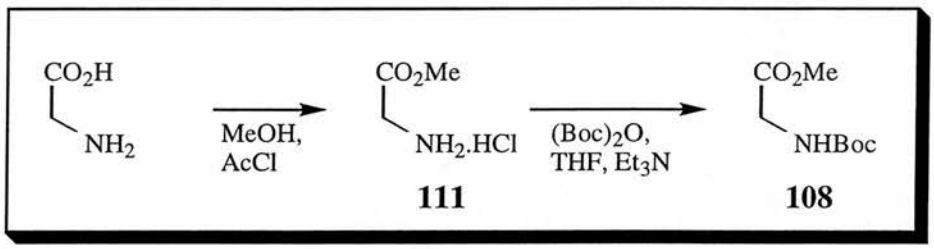


Scheme 2.6: Synthesis and Use of Glycine Imine

A possible route thus involves bromination of the *N*-Boc-glycine methyl ester (**108**) using *N*-bromosuccinimide in carbon tetrachloride with a catalytic amount of benzoyl peroxide.

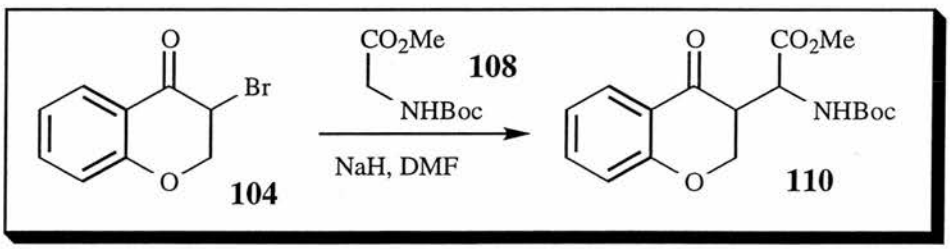
The imine (**106**) is then formed by dehydrobromination with triethylamine. Finally reaction of the enolate (**107**) with the imine (**106**) as shown above would give the protected product (**110**, Scheme 2.6).[139-141]

Alternatives to diethyl acetamidomalonate include (**108**), synthesised from glycine methyl ester hydrochloride (**111**) protecting the nitrogen with a Boc group as shown in Scheme 2.7 below.



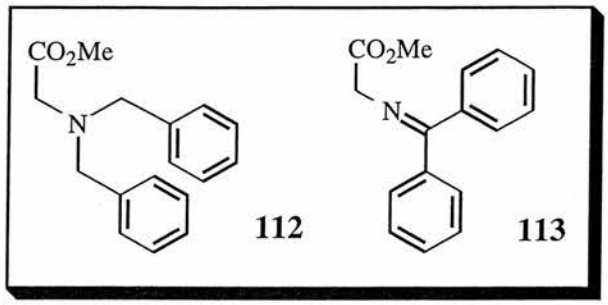
Scheme 2.7: Synthesis of Methyl N-Boc Glycinate

This could then be treated as before with sodium hydride in DMF to give the anion which could be coupled to the α-bromochromanone (**104**) to give compound (**110**), Scheme 2.8.

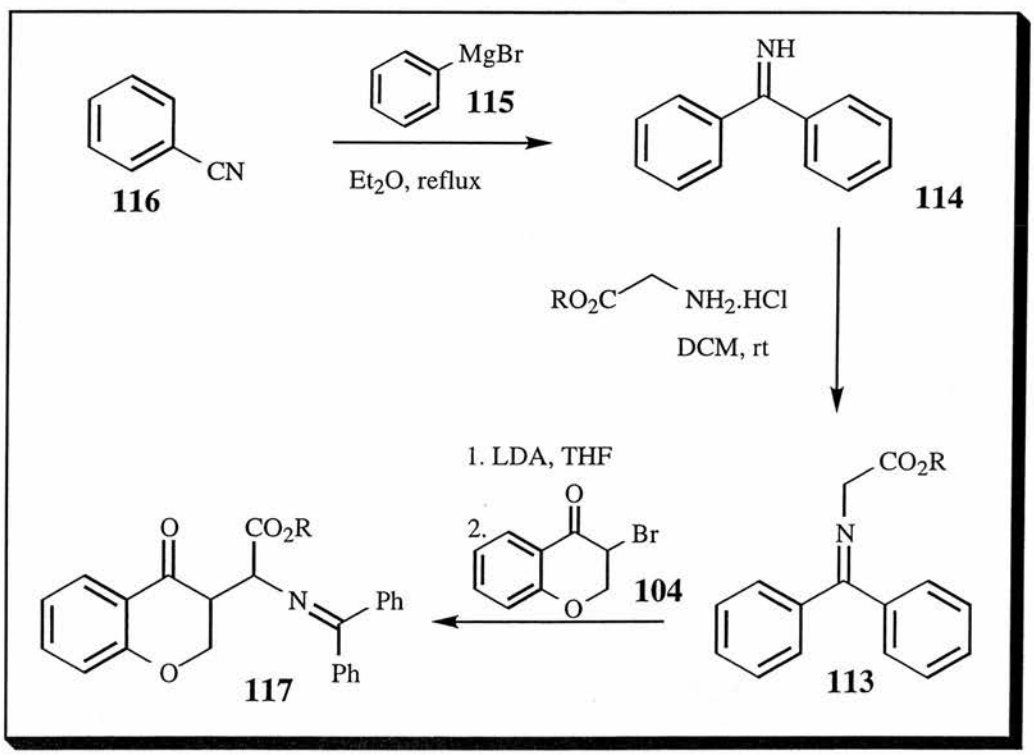


Scheme 2.8: Coupling with Methyl N-Boc Glycinate

Other anion equivalents include the dibenzylated glycine methyl ester (**112**). Compound (**112**) could be easily prepared from glycine methyl ester hydrochloride (**111**) and benzyl bromide in acetone under basic conditions.



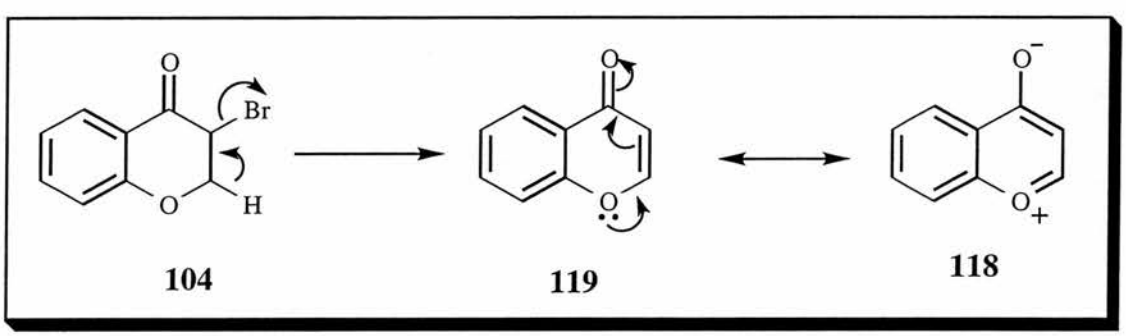
An alternative would be to use O'Donnell's reagent (**113**).[142,143] The benzophenone imine (**114**) from which it is synthesised is commercially available but it can be easily prepared according to the method of Pickard and Tolbert as shown in Scheme 2.9.[144] The Grignard of bromobenzene (**115**) is formed in the initial step of the reaction and reacted *in situ* with benzonitrile (**116**) to give diphenyl ketimine (benzophenone imine, **114**). This is then stirred with the hydrochloride salt of a glycine ester to give O'Donnell's reagent (**113**).[143] The product after recrystallisation could be coupled to the α-bromide of chromanone using LDA and THF to give compound (**117**) which can be deprotected in acid to yield the target compound (**98**).



Scheme 2.9: Synthesis and Use of O'Donnell's Reagent

Both the dibenzylated glycine methyl ester (**112**) and O'Donnell's reagent (**113**) have the added advantage that only one equivalent of base is required to form the anion thus increasing the yield and lowering the possibility of competing reactions.

All of the above provide possible solutions to the poor yield obtained in the coupling reaction with diethyl acetamidomalonate (**70**). It was believed at first that the poor yield was due an excess of base being required for the reaction to go to completion. However, a more likely possibility is that the α-bromide (**104**) undergoes elimination to give an aromatic species. As this is not possible with the other derivatives it could explain why the indanone and tetralone derivatives were obtained in more reasonable yields.



104 **119** **118**

Scheme 2.10: Aromatisation of Chromanone Derivative

2.3.1 Synthesis Using Cationic Glycine Equivalent (106)

Methyl 2-bromo-2-*N*-(*tert*-butoxycarbonyl) glycinate (**109**) was formed by the method of Steglich *et al.*[139-141] by suspending *N*-bromosuccinimide, methyl *N*-(*tert*-butoxycarbonyl) glycinate (**108**) and benzoyl peroxide in carbon tetrachloride and, after initial stirring at room temperature, heating under reflux for 3 hours. The reaction was then filtered and the solvent removed under reduced pressure to give a yellow syrup in 78% yield which was stored in the refrigerator. Spectral data confirmed the structure.

The use of the cationic glycine equivalent required completely different reaction conditions. The enolate of chromanone was preformed by stirring in THF in the presence of ^{n}butyl lithium at -78 °C under a nitrogen atmosphere. The imine (**106**) was formed by

dissolving bromoglycine (**109**) in THF at -78 °C under a nitrogen atmosphere and adding dry triethylamine. Once both the enolate (**107**) and the imine (**106**) had been formed, the enolate was added by cannula to the imine and the solution stirred for 4 hours at -78 °C. The reaction was quenched in aqueous acid and extracted to give the product as a yellow oil. The oil was purified by column chromatography in 44% yield. ^{1}H-Nmr spectroscopy of the compound showed it to be similar to compound (**110**) obtained by a different method, but further analysis by thin layer chromatography showed that there were still several byproducts present.

2.3.2 Synthesis from N-Boc Glycine Methyl Ester

N-Boc glycine methyl ester (**108**) was synthesised by suspending glycine methyl ester hydrochloride in dry THF and triethylamine and then adding a solution of Boc-anhydride to the reaction at 0 °C. The product was obtained as a pale yellow oil in 72% yield and required no further purification.

An attempt was made to couple methyl *N*-(*tert*-butoxycarbonyl) glycinate (**108**) to α-bromochromanone (**104**) using THF and butyl lithium as solvent and base respectively. This would negate the need for the special work-up procedures needed for DMF and the solvent would be more easily removed. The coupling was carried out in a similar manner to that above but the orange oil obtained was neither starting material or product by ^{1}H-nmr spectroscopy.

The coupling was eventually achieved by first stirring the glycine derivative (**108**) in the presence of sodium hydride in dry DMF to form the anion as described for the other analogues. Addition of α-bromochromanone (**104**) in DMF gave an orange-red solution which was stirred overnight, quenched in dilute acid and extracted into diethyl ether. Concentration of the organic phases gave a semi-crystalline yellow oil (**110**) in 79% yield. Analysis by ^{1}H-nmr spectroscopy showed characteristic peaks at δ = 1.45 ppm for the Boc protons and δ = 3.89 ppm for the methyl ester. Signals for the methylene at

position 2, the methine at position 3 and the α-proton were all seen as multiplets which could not be assigned further. However, thin layer chromatography in diethyl ether revealed that more than one compound was present in the mixture. Attempts were not made to purify the product at this stage as it was felt that removal of the methyl ester in the next step would reduce the amount of byproducts.

The methyl ester was removed by dissolving the mixture of products in aqueous potassium hydroxide and THF and stirring the reaction overnight. The basic solution was washed with diethyl ether and the aqueous phase acidified and extracted into diethyl ether. This process removed both unreacted methyl ester as well as byproducts from the coupling. The organic layers were concentrated under reduced pressure to an orange oil in 64% yield. ^{1}H-Nmr spectroscopy again showed the characteristic peak for the Boc methyl groups at δ = 1.42 ppm. The signals for the protons at positions 2 and 3 were still seen as a multiplet at δ = 3.86–4.01 ppm but the signal for the α-proton was a broad singlet at δ = 5.12 ppm. ^{13}C-Nmr spectroscopy and mass spectrometry confirmed the structure as the Boc protected free acid (**120**).

The Boc protection was removed by dissolving the free acid in ethyl acetate and bubbling HCl gas through the solution. A solid was formed which was filtered off but later found not to be the product. The reaction was then concentrated under reduced pressure and the residue partitioned between ethyl acetate and water. The aqueous layer was washed again with chloroform and then freeze dried to give the product (**98**) as a hygroscopic pale yellow solid in 11% yield. The ^{1}H-nmr spectrum was identical to that seen for the compound synthesised from diethyl acetamidomalonate, with signals at δ = 3.3 ppm for the methine at position 3, δ = 4.3 ppm for 2-methylene and a multiplet at approximately δ = 4 ppm for the α-proton of the amino acid. The resolution however, was poor and some impurities were evident.

2.3.3 Synthesis Using O'Donnell's Reagent (113)

O'Donnell's reagent was synthesised as described in Scheme 2.9 by reacting bromobenzene and benzonitrile together under Grignard conditions to give diphenylmethanimine (**114**) as described by Pickard and Tolbert.[144] After removing the solvents under reduced pressure the residue was distilled using a Vigreux column and the product obtained in 67.5% yield. Spectral data confirmed the presence of the imine. In the second step the benzophenone imine (**114**) described above was dissolved in dry DCM and glycine ethyl ester hydrochloride was added. The mixture was stirred overnight under nitrogen and then filtered. The filtrate was concentrated under reduced pressure and the solid recrystallised from hexane/diethyl ether to give the product (**113**) as white crystals in 55% yield. The structure was confirmed by mass spectrometry and nmr data were similar to those given by O'Donnell.[143]

Coupling was facilitated by dissolving O'Donnell's reagent (**113**) in dry THF at -78 °C under nitrogen and adding LDA to form the anion. After stirring at -78 °C for 2 hours a solution of the bromide (**104**) was then added and the solution stirred for a further hour at -78 °C then overnight at room temperature. As before the reaction was quenched with dilute acid, extracted and the resulting orange oil purified by column chromatography to give a yellow syrup (**117**) in 16% yield. The ^{1}H-nmr spectrum of this product showed signals for the ethyl ester at δ = 1.24 and 4.18 ppm. The 2-methylene was a doublet of doublets at δ = 4.75 ppm, the 3-methine a doublet of triplets at δ = 3.68 ppm and the α-proton was seen as a doublet at δ = 4.84 ppm. The carbon nmr spectrum was assigned using DEPT and confirmed the structure of the compound.

Attempts were made to increase the yield of this coupling reaction by varying the temperature (cooling to 0 °C, heating under reflux) and by employing a phase transfer catalyst.[142] None of these alterations made any difference to the yield and so the compound was carried forward.

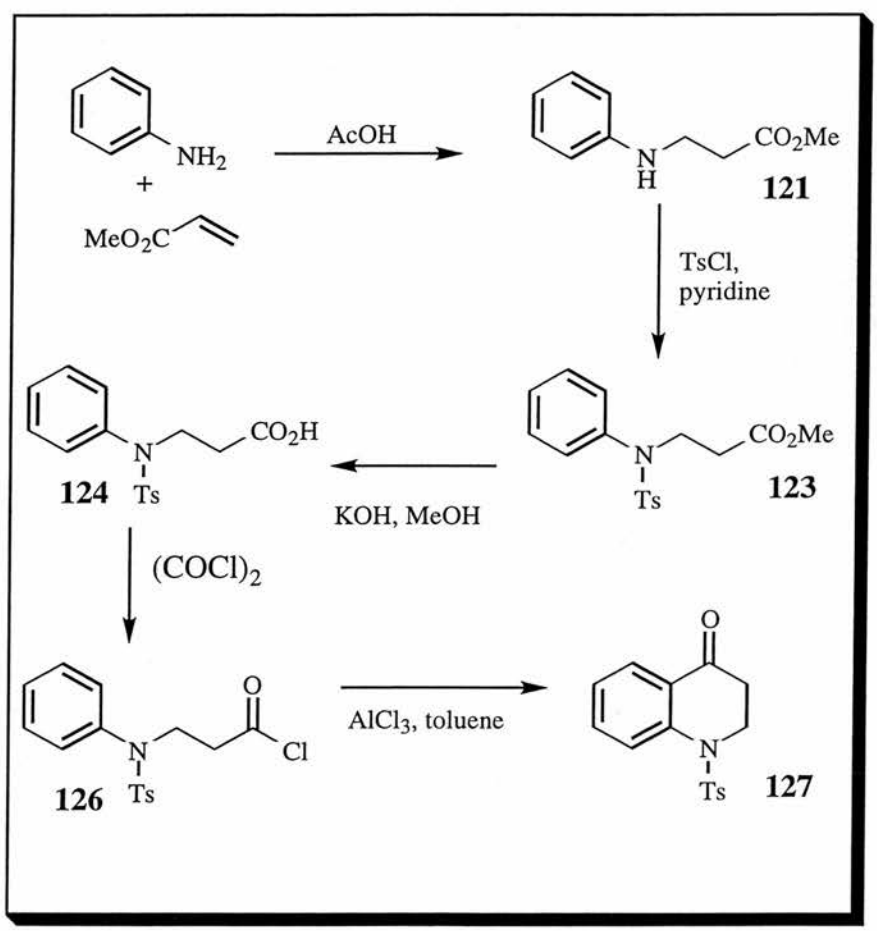
Deprotection of the imine protected chromanone (**117**) was afforded by heating in 6M hydrochloric acid and THF for 5 hours. After washing with ethyl acetate the aqueous phase was reduced and the resultant solid treated with isopropyl alcohol and propylene oxide. Removal of the solvent under reduced pressure gave a pink solid (**98**) in 40% yield which was purified further by column chromatography on reverse phase silica using acetonitrile/water (80:20) as eluent to give a pale pink solid in 8% yield. Mass spectrometry confirmed the structure of the product (**98**) and the nmr spectrum showed the presence of major and minor diastereomers in a ratio of 3:1. The two α-CH protons are seen in the nmr spectrum as doublets at $\delta = 4.01$ pmm (major) and 4.12 ppm (minor). The 3-methine was not as well resolved, but the major diastereomer was seen as a doublet of triplets at $\delta = 3.37$ ppm. The 2-methylene was seen at $\delta = 4.31$ ppm as a doublet.

2.3.4 Comparison of Routes to Chromanone Derivative

Of the four glycine equivalents employed it was found that O'Donnell's reagent (**113**) gave the highest yield (40%) of the desired target before chromatography. The product obtained from the coupling with diethyl acetamidomalonate (**70**) was difficult to purify as the many byproducts made separation difficult. The reaction with *N*-Boc glycine methyl ester (**108**) gave a good yield of the desired product, but required more deprotection steps and was less pure. The dibenzylated analogue (**112**) did not react with α-bromochromanone (**104**) under the standard conditions used for the other reagents and gave a mixture of products which were inseparable by column chromatography. The reaction with O'Donnell's reagent (**113**) was thus the best synthesis as it had fewer byproducts, was easy to purify using column chromatography and was easily deprotected in one step to give the desired target (**98**) in high yield and purity. Synthesis *via* a cationic glycine equivalent (**106**) and the enolate anion of chromanone (**107**) did give a bicyclic product but the byproducts of the reaction made it difficult to isolate.

2.4 SYNTHESIS OF 4-KETO-2,3-DIHYDROQUINOLINE DERIVATIVE

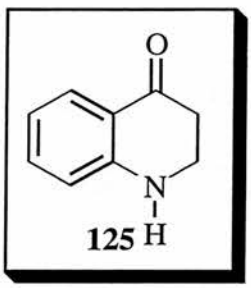
The originally envisaged procedure for the synthesis of the dihydroquinoline derivative (**99**) did not work altogether smoothly and several methods of cyclisation had to be attempted before the product was obtained (Scheme 2.11).



Scheme 2.11: Synthesis of Dihydroquinoline Derivative

The coupling of aniline and methyl acrylate occurred in 53% yield giving the methyl-β-anilinopropionate (**121**) as yellow crystals.[145] At first it was attempted to deprotect the ester protecting group with potassium hydroxide in methanol but the product could not be

isolated from the aqueous phase as this was practically an amino acid in structure (**122**).[145] The remainder of the methyl-β-anilinopropionate (**121**) was therefore tosylated in 80% yield with *p*-toluenesulfonyl chloride in pyridine giving the product (**123**) as a thick orange syrup.[145] It is worth noting the positions of the methylene groups as these shift during the subsequent reactions. The tosylate (**123**) was then de-esterified to give the free acid (**124**) in 81% yield.[145] At this point cyclisation was attempted using PPA at 120°C.[146] This gave the product below, (**125**). The harshly acidic conditions of the reaction removed the tosyl protection group as well as achieving the desired cyclisation.



125

With this in mind cyclisation was attempted in boron trifluoride diethyl etherate and although this showed some cyclised product by tlc (visualised using a polyphosphomolybdic acid dip) impurities were also present so the reaction was not followed up. Attempts were then made to activate the acid (**124**) as the acid chloride (**126**) using phosphorous pentachloride followed by cyclisation with stannic chloride.[147] The acid chloride however, did not form under these conditions and so no cyclised product was obtained with stannic chloride. Finally activation was facilitated by heating in neat oxalyl chloride at reflux overnight, this product was then isolated and washed several times with toluene to remove impurities and oxalyl chloride residues. The acid chloride (**126**) was then immediately used without further purification (88% yield). An attempt to cyclise using stannic chloride[147] was unsuccessful but it was discovered that cyclisation was facilitated by adding 3 to 4 equivalents of aluminium trichloride and stirring at room temperature for 48 hours. The product (**127**) was obtained as a thick brown oil in 96% yield.

Various attempts were made to brominate the product, [(4-methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**127**). The method employing cupric bromide in ethyl acetate and chloroform, used successfully for both indanone, tetralone and chromanone derivatives, gave no bromination by nmr or mass spectrometry in the case of the quinoline derivative. Bromine in methanol gave the dibromide, the structure of which was confirmed by nmr and mass spectrometry. Bromination with *N*-bromosuccinimide gave a brown oil which was difficult to identify. The mass spectrum of the compound however, showed that none of the desired bromide had been formed. Bromine was also added to a solution of **127** pre-treated with butyl lithium, but again ^{1}H-nmr spectroscopy gave no evidence for bromide formation. The mass spectrum of the crude product was however, indicative of the dibromide.

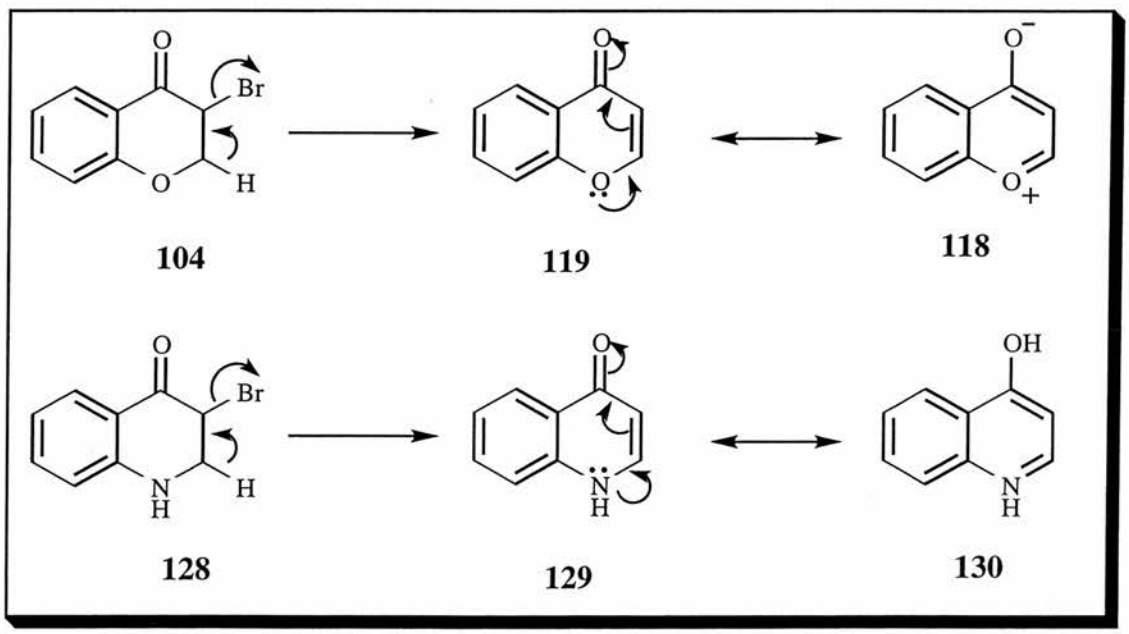
As with chromanone, an attempt was made to couple the cationic glycine equivalent (**106**) formed from bromoglycine (**109**) with the quinolinone (**127**). The bromoglycine was dehydrobrominated using triethylamine in THF at 78 °C to form the active imine (**106**). The enolate of the dihydroquinoline (**127**) was formed by stirring in THF with butyl lithium at -78 °C. The enolate was then added by cannula to the flask containing the imine and the solution stirred at -78 °C for 4 hours, then at room temperature overnight. Acidic work-up and extraction gave a yellow oil which was still a mixture of products after purification by column chromatography (pet. ether: diethyl ether; 1:1). Analysis by ^{1}H-nmr spectroscopy showed no formation of the desired product.

2.5 SYNTHETIC PROBLEMS WITH COMPETITIVE INHIBITORS

Originally it was felt that the low yield in the coupling reaction with chromanone was due to side reactions involving the anion of diethyl acetamidomalonate. This prompted the search for alternatives to diethyl acetamidomalonate (**70**) which had always given low

yields (30–45%) even for the work carried out by Pellicciari.[60] Where this was acceptable in the indanone (**96**) and tetralone (**97**) derivatives it was necessary to improve yields for the chromanone (**98**) and dihydroquinoline (**99**) derivatives. However, as shown above, none of the alternatives dramatically improved the yield of the coupling reaction. O'Donnell's reagent (**113**) did significantly reduce the amount of byproducts seen by tlc and this allowed for easy purification by column chromatography and the deprotected product required very little purification.

As alterations to the glycine moiety made no impact on yield or purity it was then concluded that the brominated bicycles themselves could be eliminating hydrogen bromide to give stable, aromatised compounds. This is especially true of the dihydroquinoline compound (**128**) as no bromination was seen and some double bond character was evident in the ^{1}H-nmr spectrum (Scheme 2.12).



Scheme 2.12: Elimination and Aromatisation of Heteroatom Containing Bicycles

In the case of the chromanone some of the α-brominated compound is available for coupling and only a portion is eliminated to give (**119**). With the dihydroquinoline however, the bromide (**128**) is not seen as it is eliminated very quickly to give (**129**) and

its stable tautomer (**130**). Neither the tetralone or the indanone can undergo elimination to give an aromatic compound.

2.6 BIOLOGICAL RESULTS

As previously discussed, kynureninase represents an important target for drug action in the treatment of some brain disorders.[22] Much of the research carried out to date on this enzyme has been on bacterial enzyme from both *Pseudomonas marginalis* and *Pseudomonas fluorescens*, with only a limited amount of research on mammalian forms of the enzyme.[148] Although kynureninase has been successfully isolated and purified from rat liver,[27] until recently the human enzyme was not available in sufficient quantities to conduct thorough kinetic studies. As a result most of our understanding to date of the properties and mechanism of kynureninase has come from studies on the bacterial enzyme. The cloning and expression of human kynureninase has been achieved within our research group.[149] This should lead to a greater understanding of the kynurenine pathway and enable more efficient design of drugs to target the pathway and its enzymes. The availability of human kynureninase is especially important as the bacterial and mammalian forms of the enzyme show different substrate preferences.

The indanone (**96**), tetralone (**97**) and chromanone (**99**) derivatives described previously were all tested as inhibitors against both bacterial kynureninase (from *Pseudomonas fluorescens*) and human recombinant kynureninase. Preliminary studies involved screening the compounds for their ability to reduce the rate of reaction at 1 mM of inhibitor. Values were then determined for the K_i by assaying the activity of the enzyme in the presence of appropriate concentrations of inhibitor and at a range of substrate concentrations spanning the K_m values of the enzymes (bacterial enzyme, $K_m = 44.2 \pm 1.1$ μM; human enzyme, $K_m = 9.39 \pm 1.78$ μM). The reactions were measured by fluorimetry at wavelengths of emission and excitation of 330 nm and 410 nm to measure the formation of hydroxyanthranilic acid (**9**) from hydroxykynurenine (**3**) for the human

enzyme and 310 nm and 410 nm respectively, to measure anthranilic acid (**8**) formation from kynurenine (**5**) for the bacterial enzyme.

Plots of 1/v against 1/S for each inhibitor concentration gave families of Lineweaver–Burk plots, where intersection of the plots on the x-axis indicated competitive inhibition. A graph of the slopes of these plots against inhibitor concentration gives a straight line graph with an x-axis intercept equal to $-K_i$. The Lineweaver-Burk plots and graphs of K_i determinations for both the bacterial and human enzyme are shown at the end of this section.

The table below gives the results for the 3 inhibitors studied with both bacterial and human kynureninase (Table 2.1).

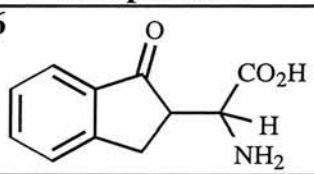
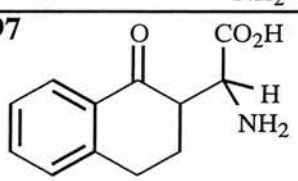
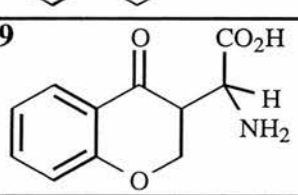
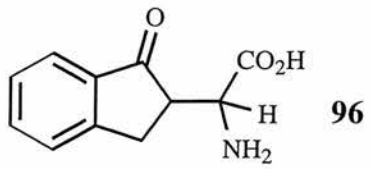
Compound	Bacterial Enzyme†	Human Enzyme†
96 	35 ± 10 μM	45 ± 12 μM
97 	170 ± 24 μM	227 ± 47 μM
99 	162 ± 31 μM	77 ± 23 μM

Table 2.1 K_i Values for the Inhibition of Induced Kynureninase from *P. fluorescens* and Recombinant Human Kynureninase Expressed in SF9 Cells, by Bicyclic Kynurenine Analogues

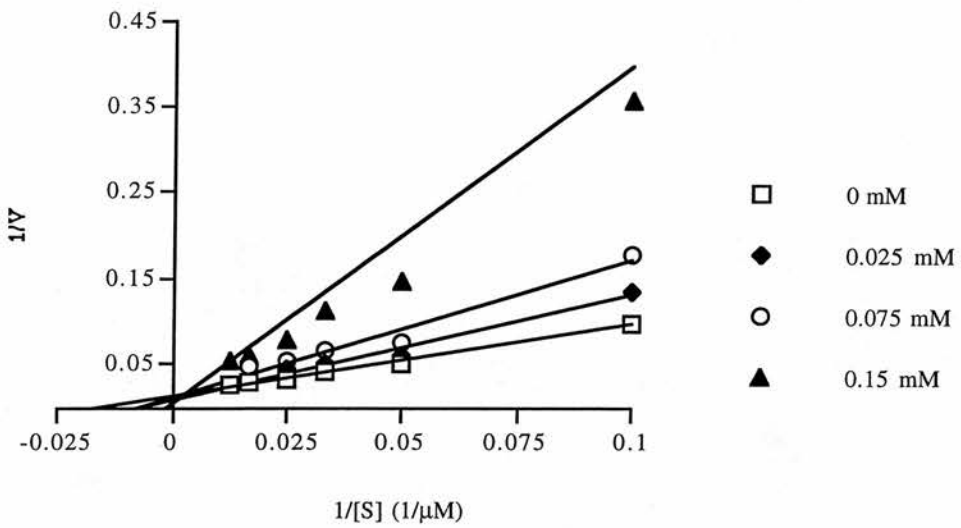
†The final K_i values quoted are the mean ± standard error from three separate determinations

It is important to emphasise that these inhibitors are racemic and as such there are four possible diastereomers of which at least two (the two 2*R* diastereomers) should have no

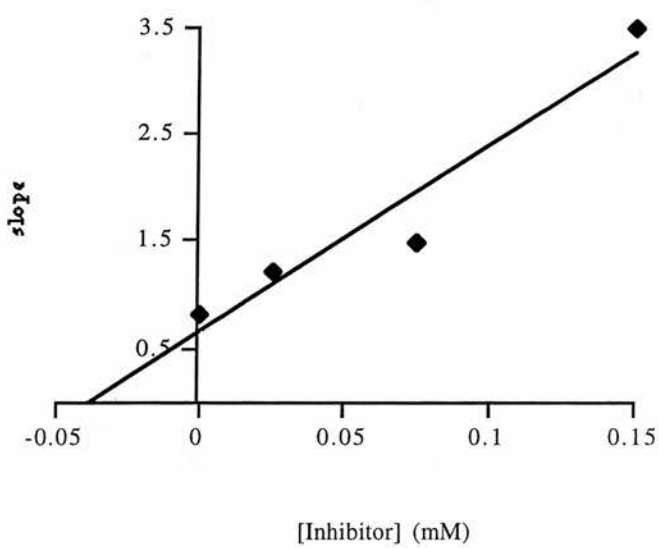
effect on the enzyme. Therefore observed K_i values could effectively be halved. Of the three inhibitors the indanone (**96**) was the most effective inhibitor of both bacterial and human enzymes with K_i values of 35 ± 10 and 45 ± 12 μM respectively. These are approximately five fold lower than those determined for the tetralone derivative (**97**). There was no significant difference in the K_i values found for the chromanone derivative (**98**) and the tetralone derivative towards the bacterial enzyme. However, the chromanone derivative was threefold more effective as an inhibitor of the human enzyme (K_i 77 ± 23 μM compared with 227 ± 47 μM).



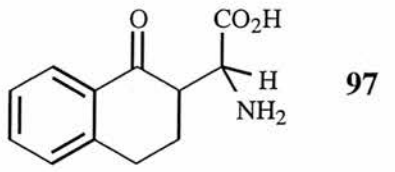
96



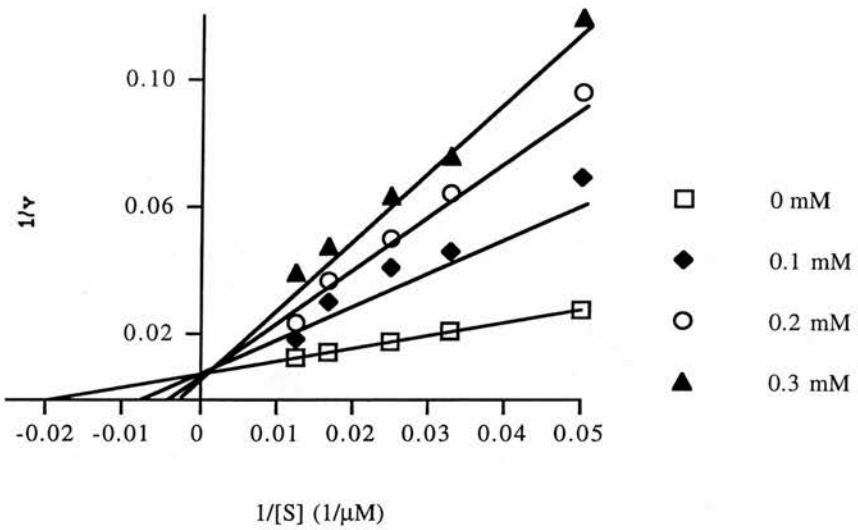
Graph 2.1: Lineweaver–Burk Plot for Inhibition of Bacterial Kynureninase with the Indanone Derivative



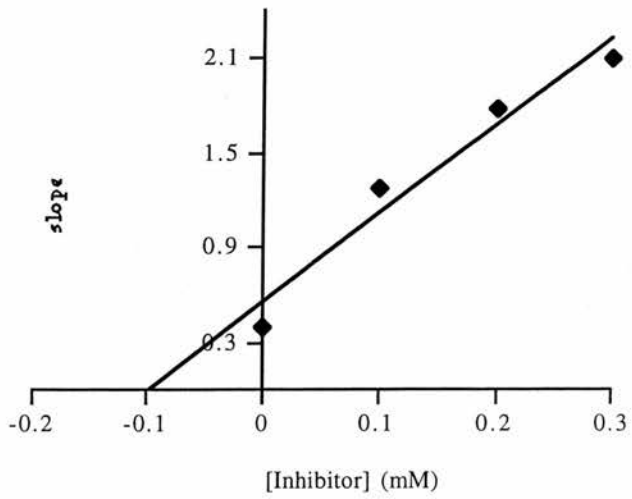
Graph 2.2: Secondary Plot of Slope *versus* Inhibitor Concentration for Bacterial Kynureninase and the Indanone Derivative



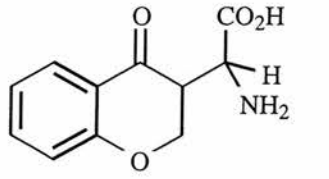
97



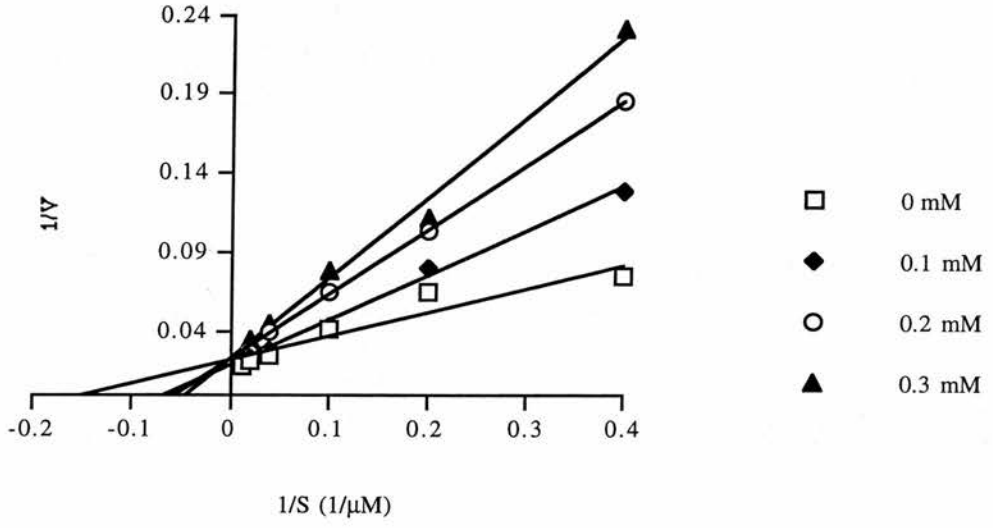
Graph 2.3: Lineweaver–Burk Plot of Inhibition of Bacterial Kynureninase with the Tetralone Derivative



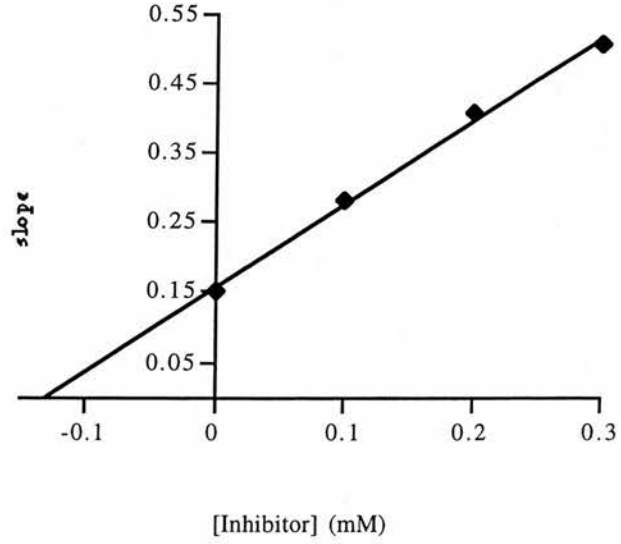
Graph 2.4: Secondary Plot of Slope *versus* Inhibitor Concentration for Bacterial Kynureninase and the Tetralone Derivative



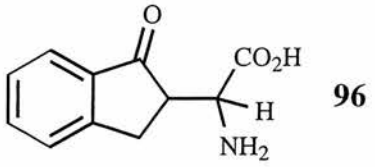
98



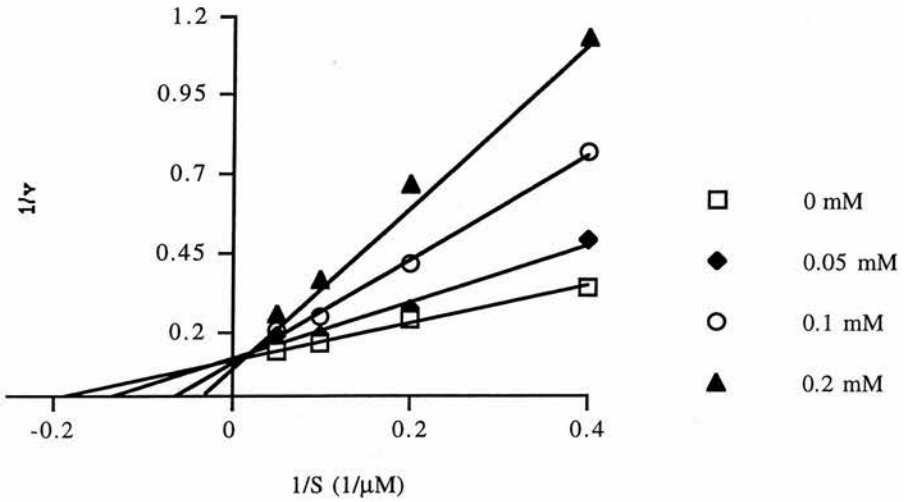
Graph 2.5: Lineweaver–Burk Plot of Inhibition of Bacterial Kynureninase with the Chromanone Derivative



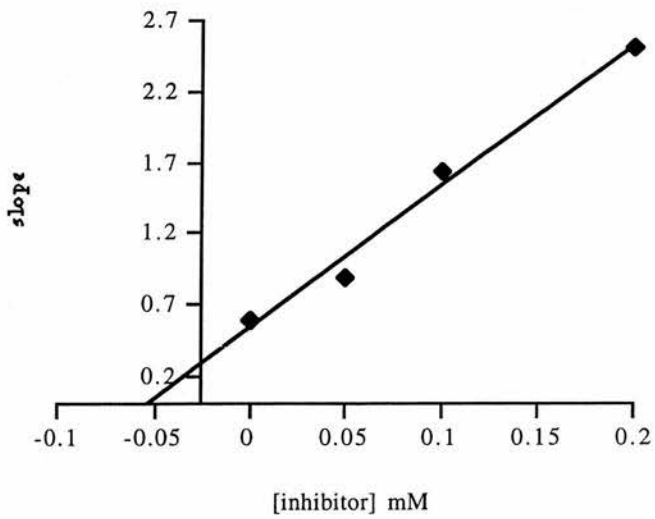
Graph 2.6: Secondary Plot of Slope *versus* Inhibitor Concentration for Bacterial Kynureninase and the Chromanone Derivative



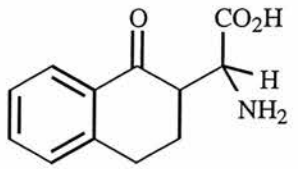
96



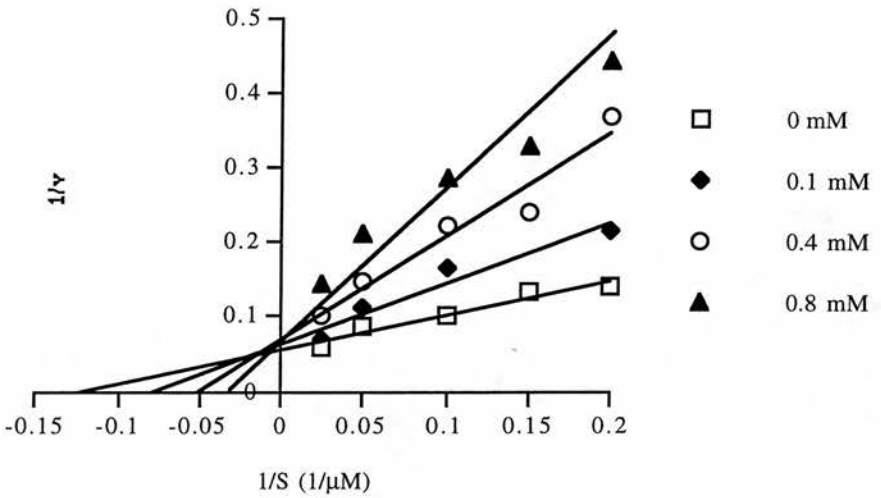
Graph 2.7: Lineweaver–Burk Plot of Inhibition of Human Kynureninase by the Indanone Derivative



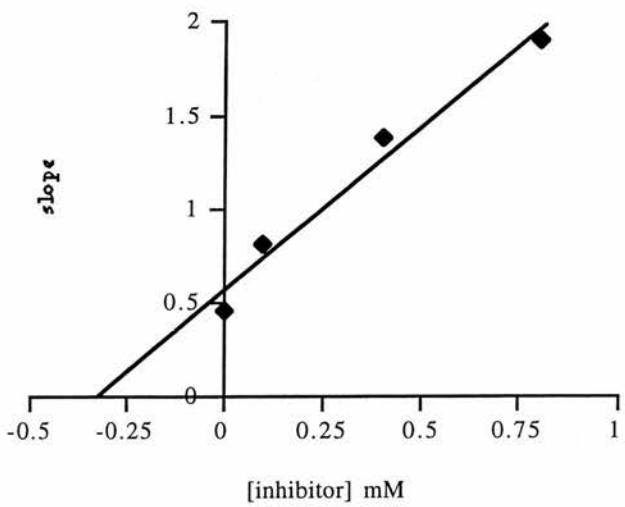
Graph 2.8: Secondary Plot of Slope *versus* Inhibitor Concentration for Human Kynureninase and the Indanone Derivative



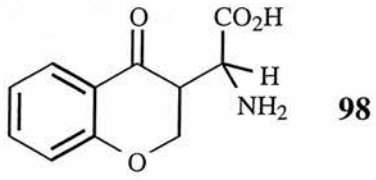
97



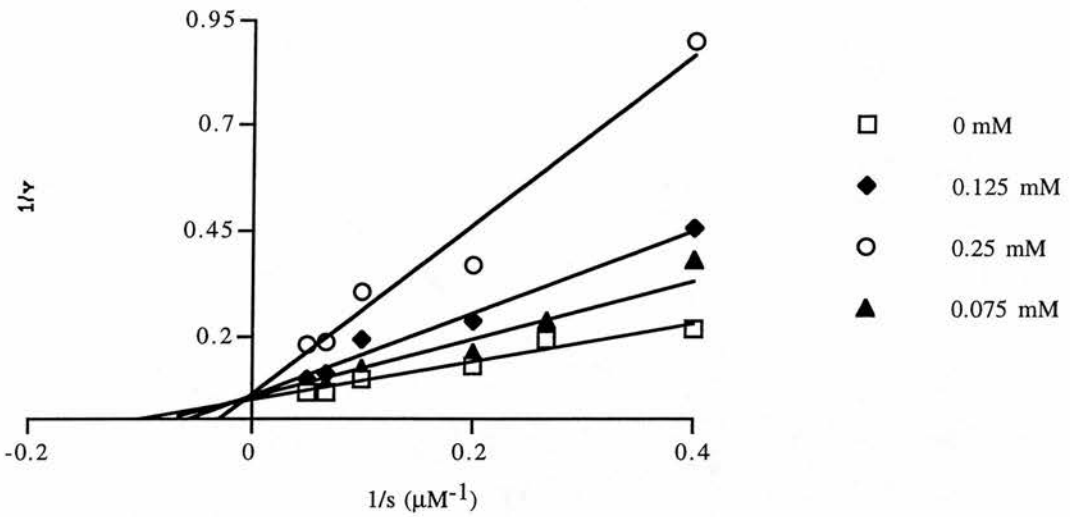
Graph 2.9: Lineweaver–Burk Plot of the Inhibition of Human Kynureninase by the Tetralone Derivative



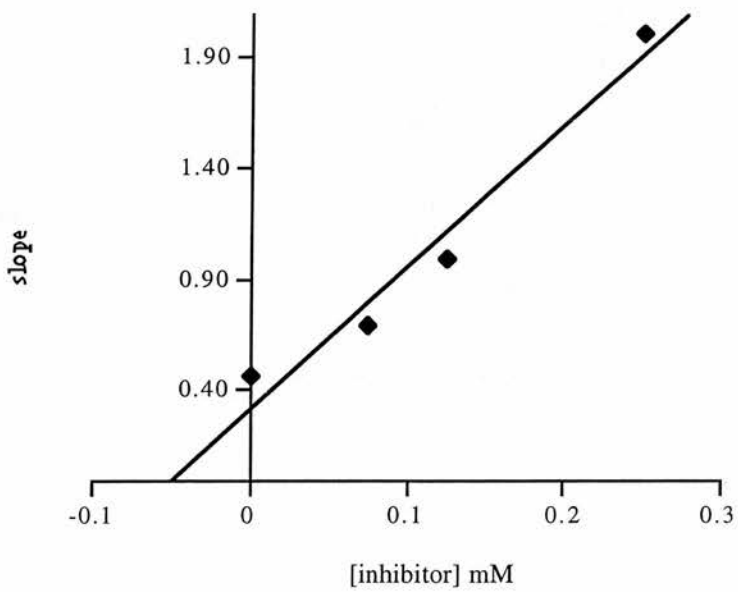
Graph 2.10: Secondary Plot of Slope *versus* Inhibitor Concentration for Human Kynureninase and the Tetralone Derivative



98

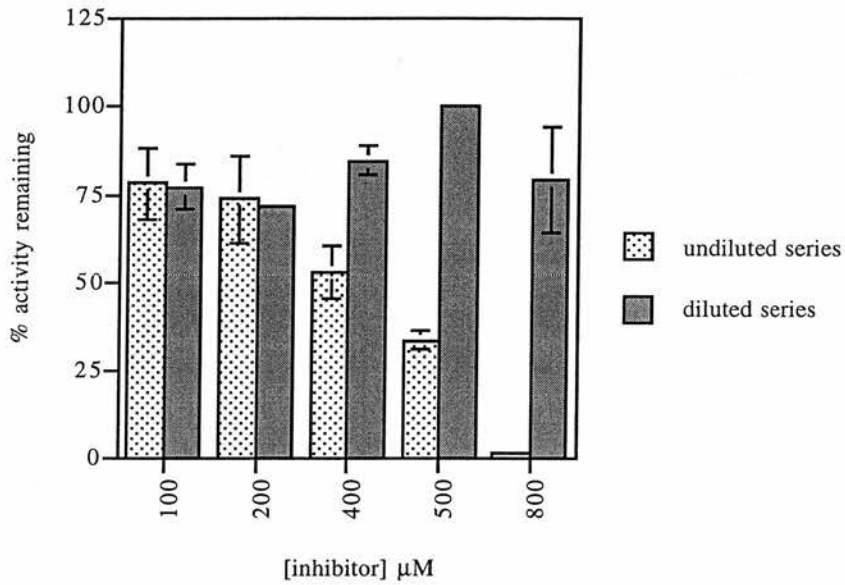


Graph 2.11: Lineweaver–Burk Plot of the Inhibition of Human Kynureninase by the Chromanone Derivative

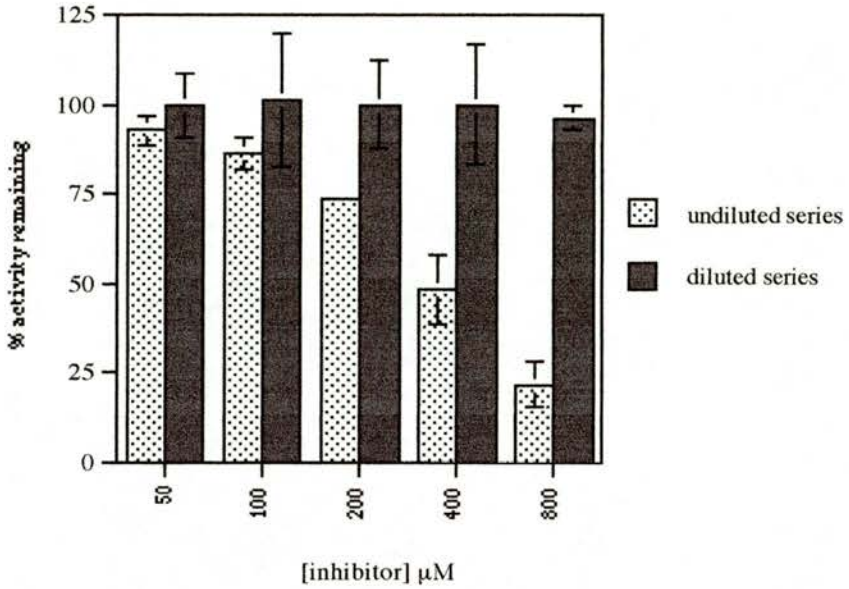


Graph 2.12: Secondary Plot of Slope *versus* Inhibitor Concentration for Human Kynureninase and the Chromanone Derivative

The inhibitors were also tested to show that as competitive inhibitors they were also reversible. This was verified by pre-incubating the enzyme, at 300 times the final assay concentration, with a range of inhibitor concentrations for ten minutes and then diluting 300-fold into the assay. An undiluted series was set up in which the enzyme was assayed as normal in the presence of inhibitor. Percentage inhibition was plotted for each series. As shown below, the activity of the enzyme returned to near 100% in the diluted series, indicating the reversibility of inhibition.



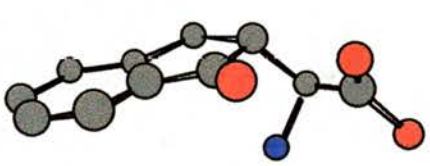
Graph 2.13: Reversibility of Inhibition of Kynureninase by the Indanone Derivative **96**

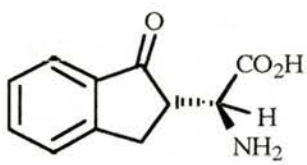


Graph 2.14: Reversibility of Inhibition of Kynureninase by the Tetralone Derivative **97**

2.7 DISCUSSION

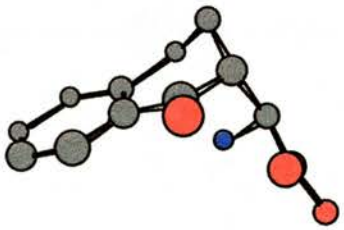
The arrangement of the active site of kynureninase is as yet unknown, but it would seem that from the data shown in 2.6 we can assume that the relative position of the carbonyl group and its relationship to the amino acid centre and the coenzyme is instrumental in determining its activity as a inhibitor. 3D-Chemdraw structures below give some idea of the relative spatial arrangements in the indanone (**96**), tetralone (**97**) and chromanone (**98**) derivatives for the 2*S* diastereomers formed in the reaction.

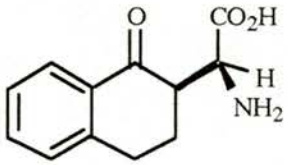




96

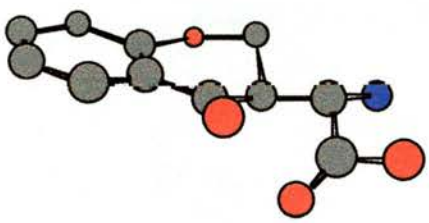
(2*S*, 3*R*)-Indanone derivative

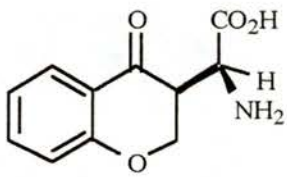




97

(2*S*, 3*S*)-Tetralone derivative





98

(2*S*, 3*S*)-Chromanone derivative

From this we can see that the (2*S*) indanone derivative (**96**) has a fairly flat shape and this derivative shows good inhibition of both bacterial and mammalian kynureninase. In the tetralone (**97**) the six membered ring is puckered, this is also true of the chromanone (**98**), which may account for their poorer binding at the active site of the bacterial enzyme. The indanone rings remain flat and this suggests that the reduced inhibition shown by the tetralone and chromanone derivatives compared to the indanone is perhaps due to the space occupied by the second ring. The active site may not be large enough to accommodate the 'kink' in the tetralone ring. This 'kink' is less prominent in the chromanone derivative which may explain its slightly better inhibition. The large difference in the K_i values determined towards the human and the bacterial enzymes for the indanone and tetralone derivatives may be attributed to changes in the relative positions of the carbonyl group and the benzene ring, the structural change achieved by increasing the ring size could reduce the spatial 'fit' of the compound in the active site.

The addition of the oxygen atom into the ring was designed to mimic the effect of the *ortho*-amino group of kynurenine which has been proven to be important for binding. The chromanone (**98**) should have a similar ring size and relative spatial configuration to

the tetralone (**97**) but the oxygen may shift the relative positions of the carbonyl and benzene ring moieties and this could account for the better K_i values observed for the human enzyme. The chromanone shows improved binding with the human enzyme, this implies that addition of oxygen overcomes problems with ring size. As the human enzyme prefers 3-hydroxykynurenine, the chromanone oxygen may be able to bind to groups present at the active site for binding to the hydroxyl of the substrate.

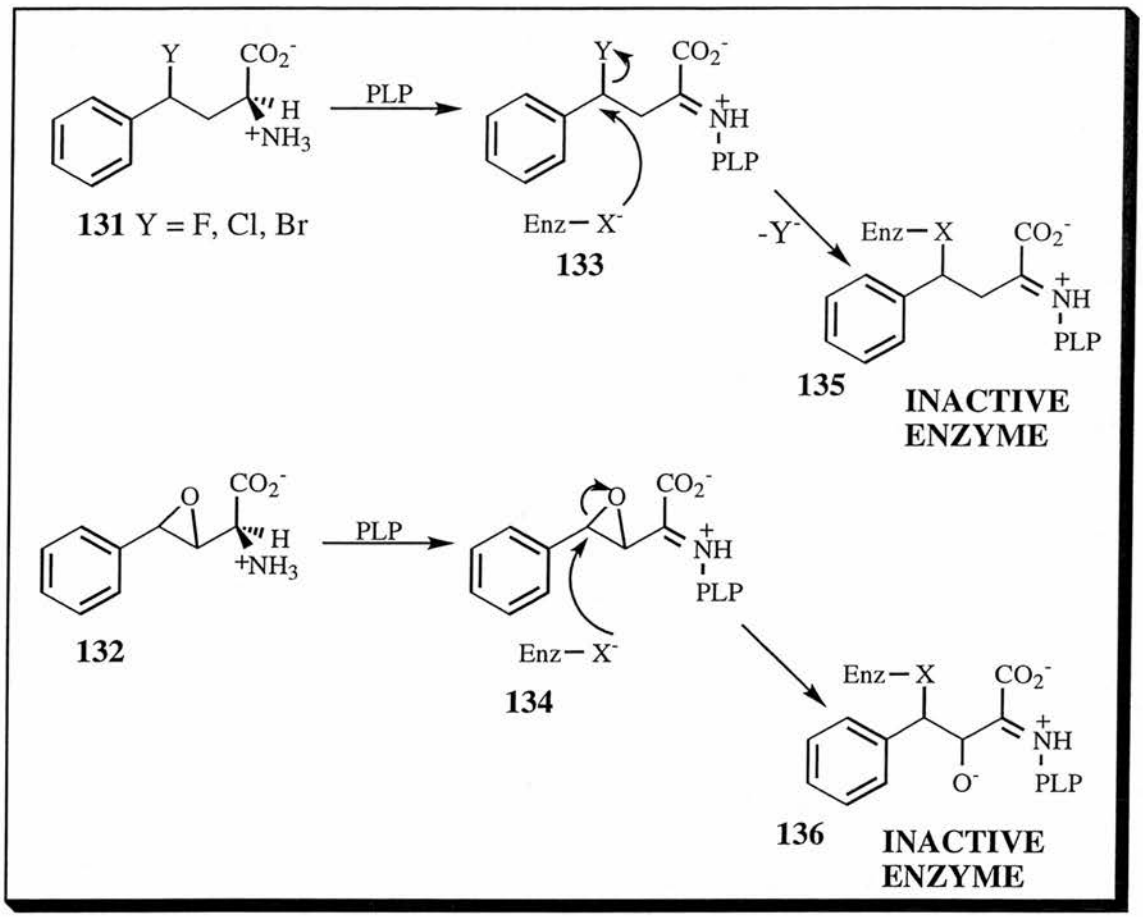
It is difficult to draw accurate conclusions from these models as only a crystal structure of the enzyme and its active site could give such details. It would seem however, that the active site has a narrow, flat binding site which is less able to accommodate the bulkier tetralone than the relatively flat indanone derivatives. The ring oxygen of the chromanone derivative imparts more stability to the ring system and the heteroatom may have a similar effect to the amino group of kynurenine and allow better binding within the active site. Research into the use of other heteroatoms in place of the oxygen may warrant further investigation.

CHAPTER 3

3 SYNTHESIS OF IRREVERSIBLE INHIBITORS OF KYNURENINASE

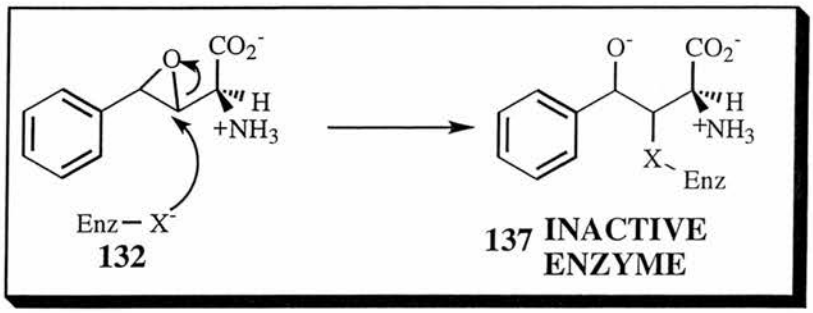
As previously described the mechanism for the hydrolysis of kynurenine by kynureninase is not fully understood. One possible mechanism is that described in Scheme 1.7 which is mediated by an enzymic nucleophile. The ketimine intermediate is attacked by an enzymic nucleophile at the γ-carbonyl group. Cleavage of the β,γ-carbon—carbon bond then takes place, giving an α-aminoacrylate derivative and a covalent intermediate. The enzymic nucleophile is regenerated when this intermediate forms anthranilate upon reaction with water.

The design of inhibitors to alkylate the putative active site nucleophile is one method of probing for a nucleophile mediated mechanism. Examples of these are compounds with good leaving groups such as fluorides, chlorides or bromides (**131**) and epoxides (**132**) whose ring opening leaves the nucleophile attached to the inhibitor. These compounds should easily form the Schiff's base (**133**, **134**) with the coenzyme but upon attack by the enzymic nucleophile a covalent bond is formed which irreversibly inactivates the enzyme (**135**, **136**, Scheme 3.1). Radioactively labelled derivatives of these inhibitors would then allow the structure of the enzyme active site to be elucidated. Covalent attachment of these affinity labels to the protein and digestion of the inactive enzyme should give labelled peptides which could be analysed to allow identification of the amino acid residues present at the active site. As no structural information on the active site of kynureninase exists this would be a useful tool in the design of effective inhibitors.



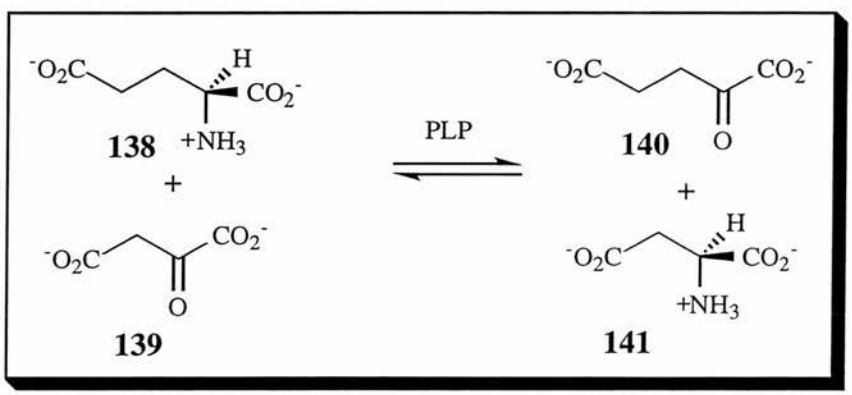
Scheme 3.1: Nucleophile Mediated Inactivation of Enzyme by Irreversible Inhibitors

The formation of the Schiff's base complex with PLP is important in that it directs the inhibitor to the correct site for reaction with the enzyme. The epoxides (**132**) however, may not require the presence of the coenzyme and an enzymic nucleophile may be sufficient to give ring opening of the epoxide and form a covalent bond with the enzyme (**137**, Scheme 3.2).



Scheme 3.2: Enzyme Inactivation by Epoxides Without PLP

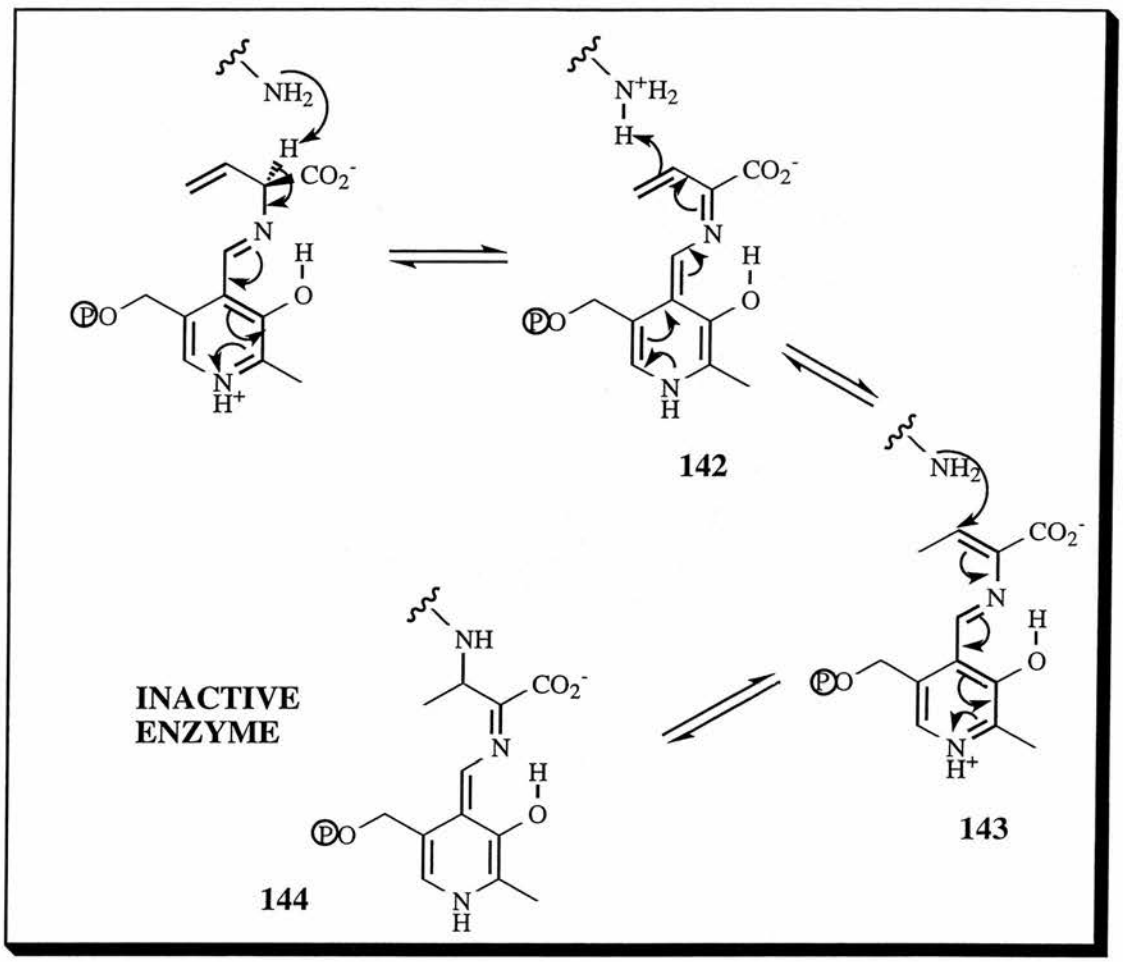
One route to the epoxide type derivatives discussed above is *via* appropriate alkenes. It has already been shown that many alkenes inhibit PLP dependent enzymes, one example is vinyl glycine which is an inhibitor of aspartate aminotransferase. Aspartate aminotransferase catalyses the transfer of the amino group from glutamate (**138**) to oxaloacetic acid (**139**) to give α-ketoglutaric acid (**140**) and aspartate (**141**), Scheme 3.3.



Scheme 3.3: Reaction of Aspartate Aminotransferase

The inhibitor, vinyl glycine forms a Schiff's base complex with PLP (**142**) and this lies in the same orientation as that of the natural substrate, glutamate. The α-hydrogen is abstracted to give the quinoid intermediate, but protonation occurs at the double bond instead of at C-4'. The Michael acceptor (**143**) formed in this step, undergoes conjugate addition resulting in inactivation of the enzyme (Scheme 3.4). Further protonation at C-4' or at α-C yields a product in which the inhibitor is irreversibly bound to the enzyme (**144**).

It is thus clear that as well as being important intermediates in the synthesis of the proposed epoxides, the alkenes may also be irreversible inhibitors of kynureninase.

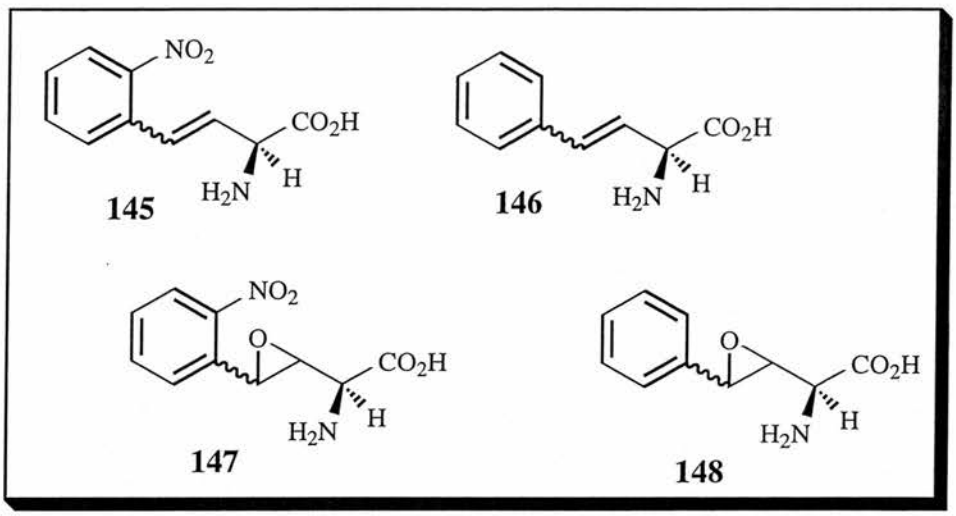


Scheme 3.4: Reaction of Vinyl Glycine on Aspartate Aminotransferase

3.1 AIMS

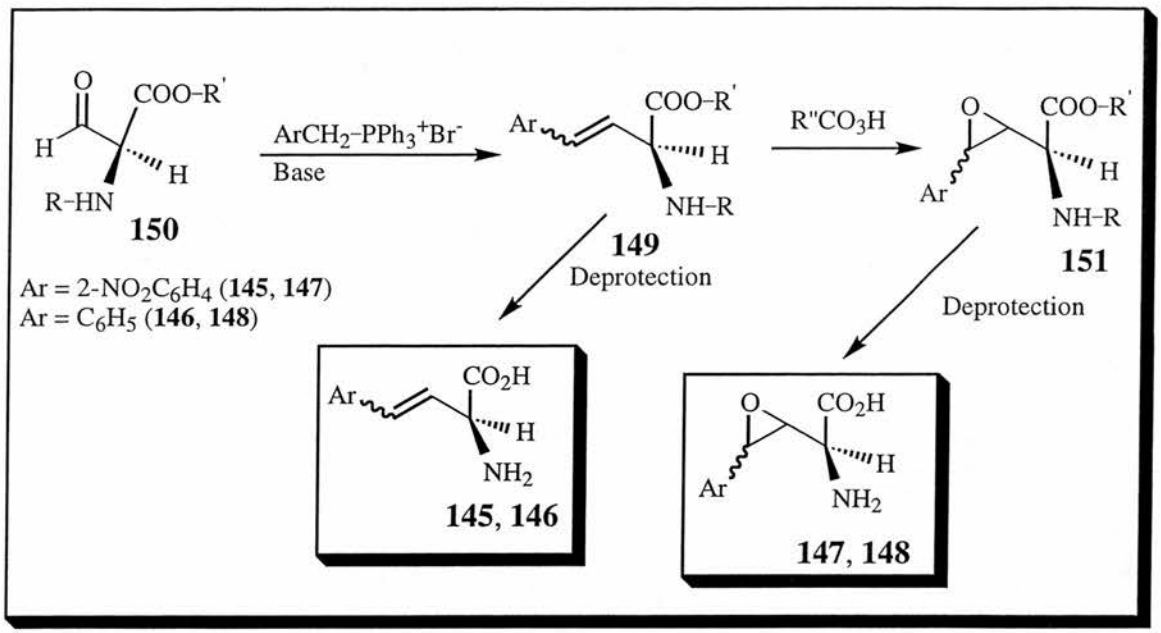
It was therefore our aim to synthesise the following alkenes and their corresponding epoxides (**145–148**). It is proposed that both the alkene and epoxide analogues would react with PLP to form the Schiff's base and that attack by the enzymic nucleophile would inactivate the enzyme by reprotonating at the double bond or ring opening the epoxide forming a covalent bond to the enzyme. The nitro containing alkene derivative may not form the Michael acceptor shown in Scheme 3.4 as the nitro group imparts extended

conjugation and this may be more stable than the final product, if this is the case it may show more competitive than irreversible inhibition.

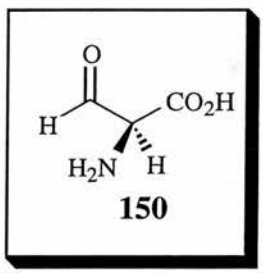


3.1.1 SYNTHETIC ROUTES TO THE IRREVERSIBLE INHIBITORS

It was decided to synthesise the inhibitors described above in only the (2*S*) form. One of the key intermediates in the formation of these analogues is the alkene. Formation of the alkene (**149**) could be most effectively achieved using Wittig and Horner–Emmons chemistry between the appropriately substituted alkyltriphenylphosphonium ylid and an aldehyde. The synthon thus required is a suitably protected serine aldehyde (**150**) as the carbonyl fragment for the Wittig reaction. Epoxidation of the double bond could then be achieved by reaction with meta-chloroperoxybenzoic acid to give the final targets (**151**, Scheme 3.5).

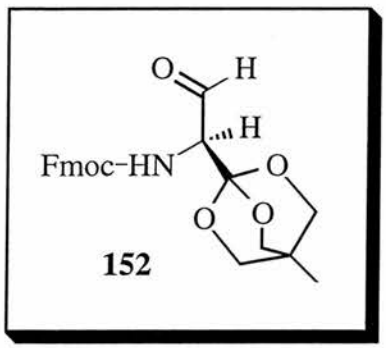


Scheme 3.5: Proposed Synthetic Route to Alkene and Epoxide Inhibitors



A number of serine aldehyde equivalents are known, which have been employed in the synthesis of chiral amino acids.[150] One major consideration with these is racemisation at the chiral centre. The acidity of the α-proton of serine means serine has a tendency to enolise and epimerise and this results in the loss of optical activity. Several protecting group strategies have been employed to reduce the acidity of this α-proton, these include the *ortho*ester derivative and Garner aldehyde described below.

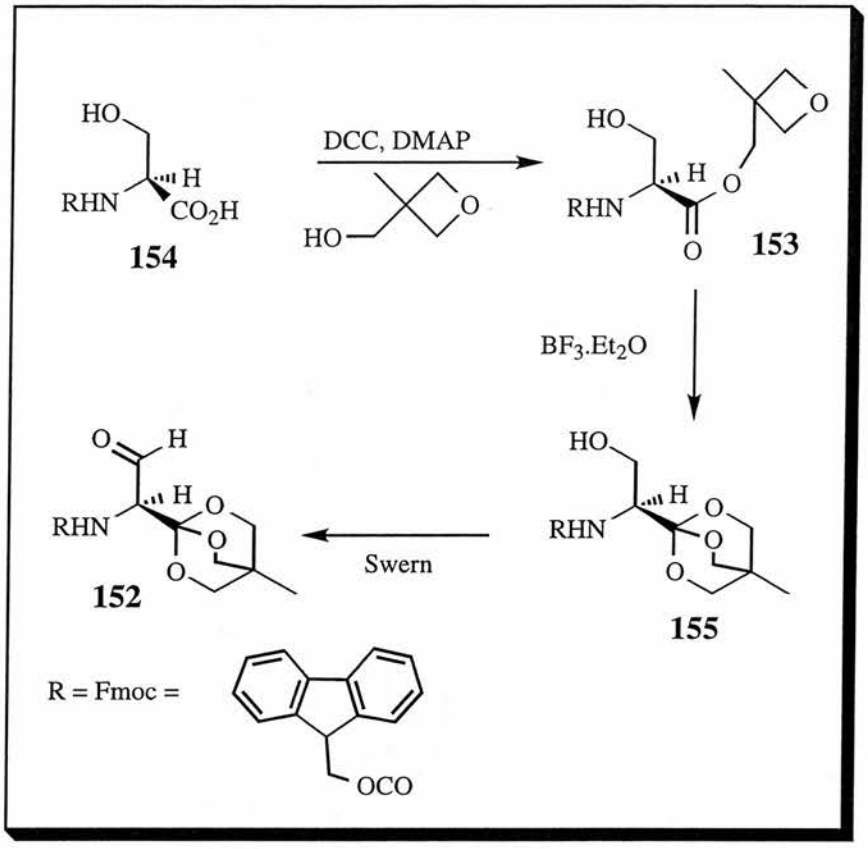
3.1.1.1 Fmoc-*S*-Serine Ortho Ester



152

Blaskovich and Lajoie[151] synthesised a base stable cyclic *ortho*-ester (**152**) in order to alleviate the racemisation problems observed under Wittig conditions. The acidity of the α-proton is sufficiently reduced in the *ortho*-ester to prevent loss of chirality at the α-centre. The amine is protected as the N^{α}-9-fluorenyl-methoxycarbonyl (Fmoc) derivative because of its stability towards the Lewis acid conditions required for the formation of the *ortho*-ester, and also because many Fmoc amino acids are crystalline. The synthesis involved formation of an intermediate oxetane ester (**153**), prepared in 80% yield from Fmoc-2*S*-serine (**154**) and 3-methyl-3-(hydroxymethyl)-oxetane using 1,3-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) to effect the coupling. The oxetane ester (**153**) was then converted to the *ortho*-ester (**155**) using boron trifluoride diethyl etherate and the aldehyde (**152**) formed under Swern conditions (Scheme 3.6).[152]

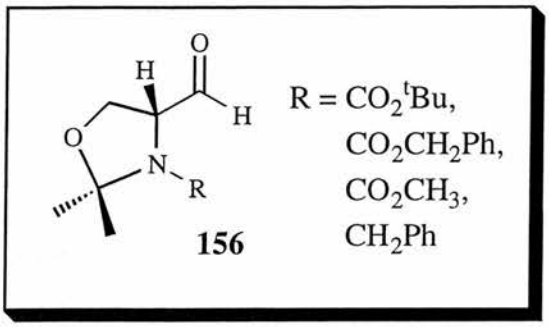
The protected serine aldehyde (**152**) was found to be very reactive towards a variety of classical reagents used for addition to aldehydes. Grignard additions carried out on the *ortho*-ester with both methyl magnesium bromide and phenyl magnesium bromide gave 2*S*-threonine and 2*S*-phenylserine in 77 and 85% yield respectively. Reoxidation of the β-hydroxy intermediates using Swern conditions gave the corresponding ketones in high yields without racemisation of the chiral centre. The *ortho*-ester has also been employed in both Reformatsky addition and Wittig reactions.[151]



Scheme 3.6: Synthesis of Serine Ortho-Ester

3.1.1.2 The Garner Aldehyde

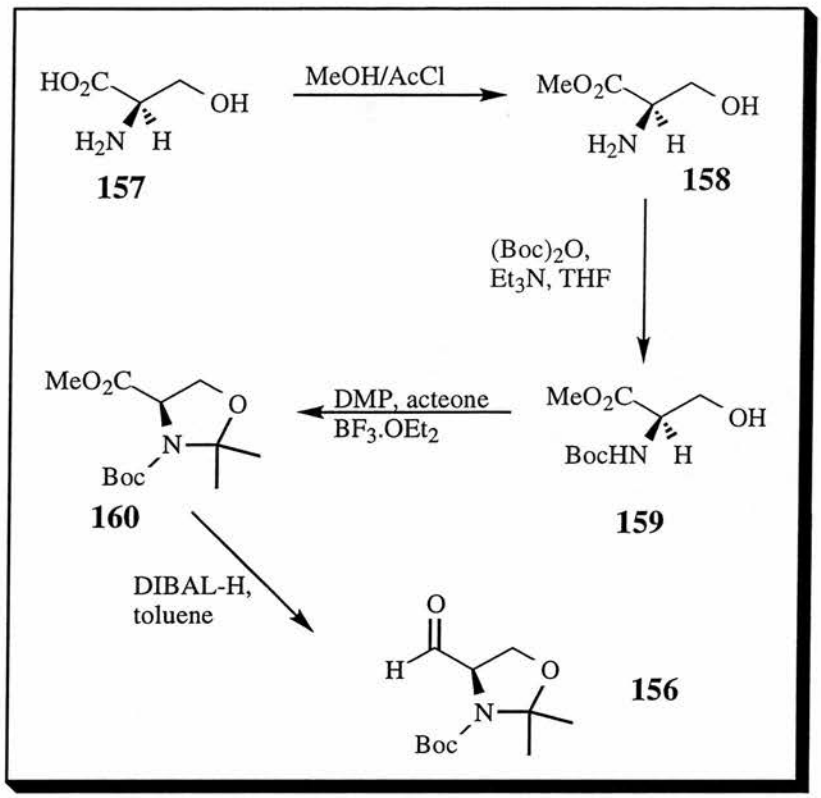
The most widely used serine aldehyde equivalent is the Garner aldehyde (**156**) which has been prepared with various nitrogen protecting groups. This derivative is noted for its synthetic versatility.[153-155] The tethering together of the serine OH and the amino function mean less synthetic steps and easier deprotections. In the synthesis the original carboxylic acid of serine becomes the aldehyde, and on deprotection what was originally the alcohol is converted into the carboxylic acid of the new amino acid. Therefore it is important to note that in order to prepare the *S*-amino acid derivative, *R*-serine must be used as the starting material, as the stereochemistry is effectively inverted through the procedure.



156

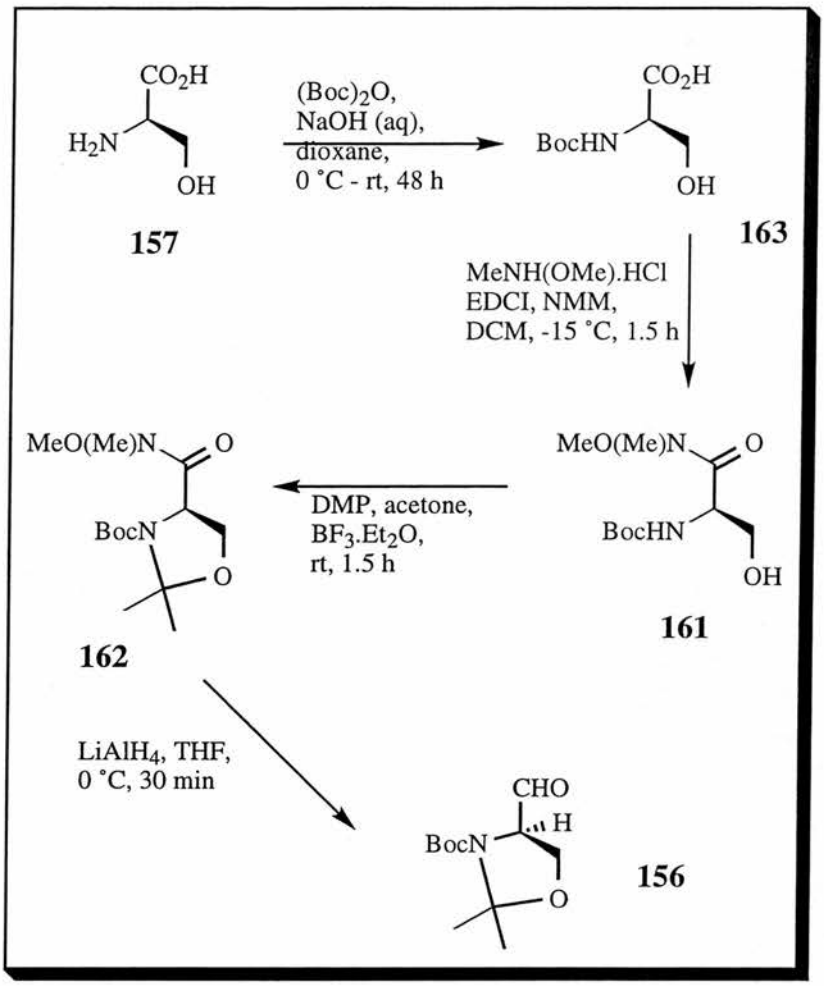
The original synthesis of the Garner aldehyde involved the conversion of *N*-Boc-serine into the corresponding methyl ester using diazomethane or methyl iodide, and then benzene as solvent for the cyclisation to form the oxazolidine.[156] Garner used this serine aldehyde equivalent in the synthesis of *threo*-β-hydroxy-2*S*-glutamic acid. However, Branquet *et al.* substituted Mitsunobu conditions for diazomethane/methyl iodide in their high yielding esterification of *N*-Boc-serine.[157]

More recently McKillop *et al.* carried out the esterification step prior to the nitrogen protection.[158] The reaction of serine (**157**) with methanol/acetyl chloride occurs quantitatively and the resulting ester (**158**) is then converted into the *N*-Boc protected form (**159**) in high yield. The oxazolidine (**160**) is formed using 2,2-dimethoxypropane (DMP) in THF/triethylamine in acetone with boron trifluoride diethyl etherate as catalyst. Following Garner's procedure, the aldehyde (**156**) is obtained by reduction of the ester using diisobutylaluminium hydride (DIBAL-H) as shown in Scheme 3.7. This has been our chosen route to the Garner aldehyde.



Scheme 3.7: Synthesis of the Garner Aldehyde

Many other routes to the Garner aldehyde have been developed. Dondoni and Perrone reported an alternative to the DIBAL reduction step,[159] which had been reported to be difficult, especially on a small scale.[160] They introduced a reliable 2-step alternative involving a lithium aluminium hydride reduction followed by a modified Swern oxidation using Hünig's base.[159] Subsequently Datta[161] and Avenoza and Cativiela[162] have converted less expensive amino acids into the Garner aldehyde, all of these methods however, increase the number of steps to the Garner aldehyde. Recent work by Taylor *et al.* has re-investigated the use of a Weinreb amide **161/162** (Scheme 3.8).[163] Previously this route was shown to give only 38% yield from Boc-serine.[164,165] The route used by Taylor was adapted from the procedure by Guanti *et al.*[165] which gave the Garner aldehyde in four steps without the need for reactions at low temperature and only one chromatographic separation. It was possible to prepare the Garner aldehyde on a ten gram scale in a single day using this procedure.

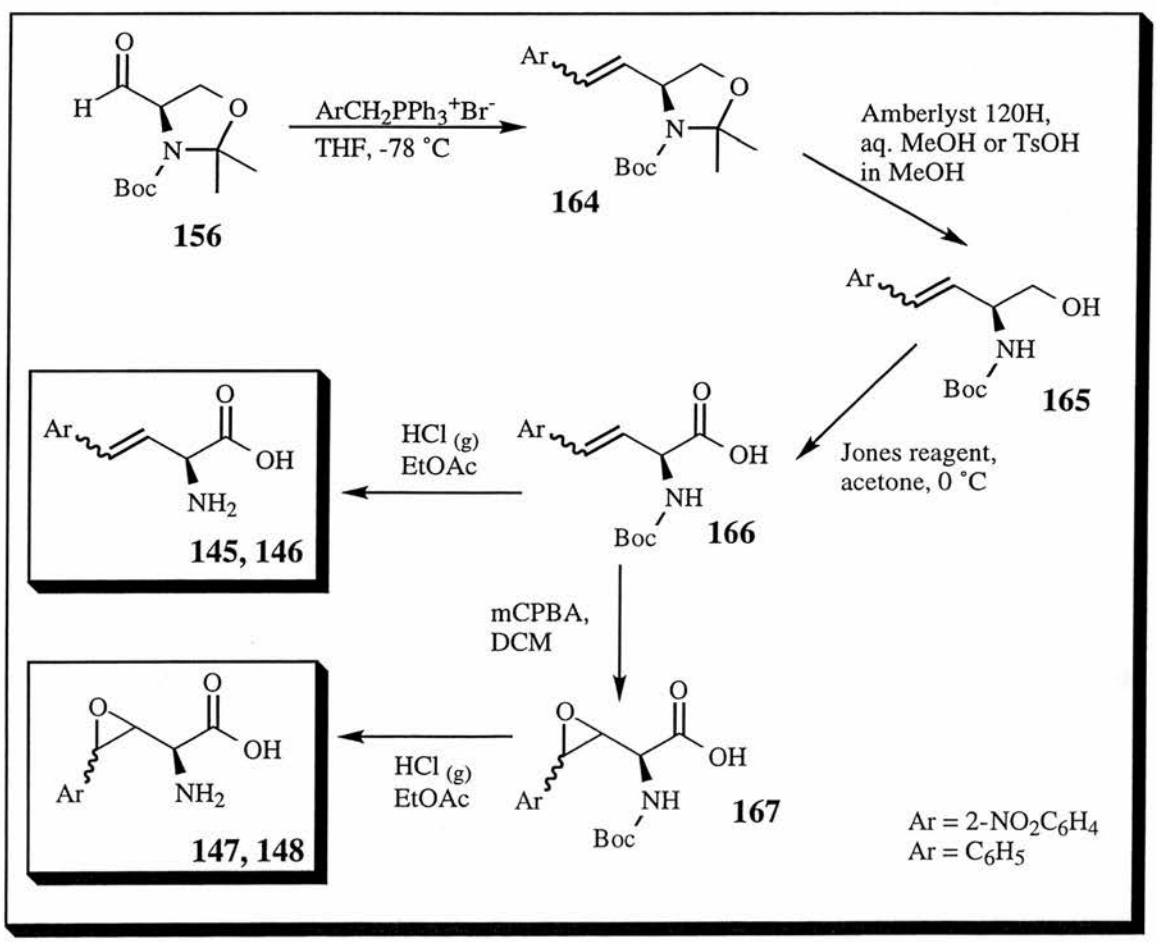


Scheme 3.8: Synthesis of the Garner Aldehyde via a Weinreb Amide

Having decided on a route to the serine aldehyde equivalent, the next step was finding a method for the Wittig coupling and the subsequent unmasking of the serine aldehyde part of the molecule to give the free amino acid. McKillop found that Wittig olefination using the hindered base potassium bis(trimethylsilyl)amide (KHMDS) in THF at -78 °C gave good yields and high optical purity.[158] Unmasking of the amino acid function is achieved in three steps, first with the removal of the ketal (**164**) to give the alcohol (**165**), then oxidation of the alcohol to the carboxylic acid (**166**) and finally deprotection of the amine (**167**). Deprotection to the alcohol (**165**) was reported to be possible by two methods, Amberlyst 120H in aqueous methanol[158] and tosic acid in methanol at room temperature.[156] Oxidation to the acid (**166**) requires the amine protecting group to be intact at this stage to avoid unwanted side reactions, also the choice of reagents must take

into account the presence of the double bond. Pyridinium dichromate in DMF,[166,167] catalytic ruthenium oxide in sodium periodate,[154] and Jones reagent[168,169] have all been used with varying degrees of success on compounds containing double bonds and other sensitive groups. Jones reagent however, is the reagent of choice for the oxidation of alcohols to carboxylic acids in the presence of double bonds. Unmasking of the amine (**145,146**) can be achieved easily using standard (HCl gas in ethyl acetate) or mild Boc deprotection methods such as $Mg(ClO_4)_2$, tin(II)chloride[170] or tin(IV)chloride.[171] The milder conditions of tin(IV)chloride have been shown to be applicable to peptide syntheses especially with thioamide linkages.

Conversion of the alkenes to their corresponding epoxides (**167**) using mCPBA or other equivalent reagents is probably more easily achieved prior to the deprotection of the amine (**168**, Scheme 3.9).



156 **164** **165** **166** **167** **145, 146** **147, 148**

Scheme 3.9: Synthetic Route to the Alkene and Epoxide Derivatives

3.2 SYNTHESIS OF THE GARNER ALDEHYDE

The first objective was synthesis of the Garner aldehyde using literature procedures. Firstly the esterification of *R*-serine (**157**) as outlined in Scheme 3.7 was achieved in 97% yield and the product (**158**), a white crystalline solid, was carried on to the second step without further purification. The melting point and spectral data were consistent with those in the literature[158] and results previously obtained in the group.[172]

Butoxycarbonyl protection of the amine moiety in THF and triethylamine occurred in 51% yield and again the spectral data for the compound (**159**) were consistent with those in the literature. The methyl protons from the ^{t}butyl group were clearly visible at 1.4 ppm in the nmr spectrum. The prochiral protons of the methylene group have different signals and appear as two double doublets spilt by each other and the α-proton. The yellow oil was not purified. Optical rotation, mass spectrometry and IR spectroscopy confirmed the structure of the compound.

Ketal protection of methyl-*N*-butyloxycarbonyl-D-serine (**159**) was facilitated by reaction with 2,2-dimethoxypropane in dry acetone with boron trifluoride diethyl etherate as catalyst. This reaction went smoothly in 75% yield after purification by distillation. The clear syrup was found to be the oxazolidine ester (**160**). It is interesting to note that in the proton spectrum there are two sets of peaks for each signal (apart from the methyl ester) at slightly different shifts. These are in a 60:40 ratio and are due to there being two forms in solution as observed by nmr studies. Again the β-methylene is split into two signals for H_A and H_B, however these were not as distinguishable as those for the methyl-*N*-Boc-serinate as they overlap to give a multiplet. In the carbon spectrum two peaks are observed for the ^{t}butyl quaternary carbon at 80.6 and 81.2 ppm and for the formyl quaternary carbon ($O\underline{C}(CH_3)_2N$) at δ = 94.7 and 95.3 ppm. Spectral data were consistent with those in the literature.[158]

The final step in this sequence involved reduction of the methyl ester (**160**) to give the *N*-Boc protected Garner aldehyde (**156**), with DIBAL-H following Garner's method. This was achieved in 84% yield.[153,155] The product (**156**) was purified by distillation and was pure by tlc (silica; hexane:ethyl acetate; 5:1) and nmr. The spectral data were consistent with those in the literature.[153,155] The proton nmr spectrum was significantly different to that for the oxazolidine methyl ester (**160**) having a set of sharper peaks for the β-methylene which was not as heavily split. There was a characteristic signal at δ = 9.52 and 9.57 ppm representing the aldehyde functional group, this was confirmed in the carbon spectrum with a large peak appearing at δ = 119.8 ppm. Although difficulties had been reported for this reaction on a small scale, it was found to work equally well using between 0.5 g and 10 g of the oxazolidine methyl ester.[159]

3.3 SYNTHESIS OF THE VINYL DERIVATIVES

3.3.1 PREPARATION OF (2*S*)-2-AMINO-4-(2-NITROPHENYL)-3-BUTENOIC ACID, 145

The triphenylphosphonium ylid of 2-nitrobenzyl bromide was formed by reaction with triphenylphosphine in toluene in 96% yield. The product (**168**) required no further purification and was clean by nmr spectroscopy. The melting point agreed with that given in the literature.[173]

The ylid (**168**) was stirred with potassium bis(trimethylsilyl)amide (KHMDS) and the Garner aldehyde (**156**) added at -78 °C. After work-up the product required purification by column chromatography to remove the triphenylphosphine byproduct. Analysis by nmr spectroscopy showed the product (**170**) in both *Z*- (**170a**) and *E*- (**170b**) forms in a 1:1 ratio. The alkene protons appeared at δ = 5.8 and 6.77 ppm (J = 10.8 Hz) for the *Z*-form and at δ = 6.15 and 6.97 (J = 15.5 Hz) for the *E*-form. It was also interesting to

note that the ring proton adjacent to the nitro group was split into two doublets. The carbon spectrum showed loss of the aldehyde group and addition of the nitrophenyl substituent. The *Z* and *E* double bond isomers ran very close to one another both on tlc (silica, pet. ether:ethyl acetate; 5:1) and on silica columns and at first separation was difficult. It was found that by using hexane instead of petroleum ether that the *Z* and *E* double bond isomers could be separated on small scale columns. This was used to further assign the proton and carbon spectra obtained for the separate isomers. In the carbon nmr spectrum for the *Z* isomer two sets of signals were seen for the methylene carbon and the ketal of the oxazolidine at $\delta = 66$ and 68 and $\delta = 94$ and 95 ppm respectively. The optical rotation was seen to change from negative in the Garner aldehyde to positive in the coupled vinyl analogue.

A similar coupling reaction was attempted using 2-nitrobenzyldiethyl phosphonate and the Garner aldehyde (**156**) in a Horner–Emmons reaction. Although the byproduct to this reaction is water soluble and can be removed by extractive work-up, it was found that the product still required purification by column chromatography. The ratio of *Z* to *E* products in this reaction was found to be almost exclusively *E*, but due to the low yield the previously used Wittig olefination was used in preference.

Removal of the ketal protecting group was first attempted in aqueous methanol using Amberlyst 120H. This was reported to be mildly acidic, removing only the ketal and not the Boc protection.[158] However, stirring with Amberlyst, even for short periods of time removed both the ketal and Boc protecting groups. As described previously it is important that the nitrogen remains protected as this could undergo unwanted oxidation in the next step.

The method of choice suggested by Garner was tosic acid in methanol at room temperature.[156] It was found that reaction at room temperature was not sufficient for removal of the ketal, but heating under reflux for five hours surprisingly removed the ketal

whilst leaving the Boc protection intact. The optical rotation was again positive indicating that no racemisation had occurred at the α-proton. Analysis by nmr spectroscopy confirmed the loss of the ketal group, with a broad singlet visible at δ = 3.6 ppm due to the free alcohol (**170**). Mass spectrometry (chemical ionisation) confirmed the presence of MH^+ at 309.

Oxidation of the alcohol (**170**) to the carboxylic acid (**171**) was attempted with pyridinium dichromate with no success, but Jones reagent effectively oxidised the alcohol to the acid in 85% yield. It was found that the reaction worked best when a slight excess of Jones reagent was used. This did not effect the double bond but less of the corresponding aldehyde was present after column chromatography. Reaction of the nitro compound with a slight excess of Jones reagent in acetone at 0 °C, followed by stirring for 5 hours at room temperature, gave an orange syrup after extraction into diethyl ether and removal of solvent. This orange syrup was not soluble in chloroform while the starting material was. The product (**171**) also ran close to the base line in tlc in neat ethyl acetate. The optical rotations in the *Z* and *E* double bond isomers of the carboxylic acid were very different +22 and +60° respectively. In the carbon nmr spectrum a peak was seen at δ = 174 ppm due to the carboxylic acid (**171**).

In the final step of the synthesis the Boc protection was removed by dissolving the acid (**171**) in dry ethyl acetate and bubbling HCl gas through the reaction mixture. After removal of the solvent under reduced pressure the product was recrystallised from methanol to give a pale brown solid (**145**) in quantitative yield. Nmr spectroscopy confirmed the loss of the nitrogen protecting group and that the double bond was still intact.

Mosher's amide derivatives were made of both the *Z* and *E* alkene products. These confirmed that only one enantiomer was present and that no racemisation had taken place during the synthesis (see Figure 3.1)

3.3.2 PREPARATION OF (2*S*)-2-AMINO-4-PHENYL-3-BUTENOIC ACID, 146

Benzyl triphenylphosphonium bromide (**172**) was formed in an analogous manner to the nitrobenzyl phosphonium ylid in 90% yield and the product, a white crystalline solid, also required no purification. The melting point agreed with that given in the literature and spectral data confirmed the structure.

Coupling of the benzyltriphenyl phosphonium ylid (**172**) and the Garner aldehyde (**156**) was achieved in 49% yield after purification by column chromatography following reaction of the phosphonium ylid with KHMDS in dry THF and then addition of the aldehyde at -78 °C. The yield was significantly lower than that obtained for the nitro derivative. The solution, in contrast to the purple of the nitrobenzyl derivative, was orange. As with the nitro compound two signals were seen for all proton nmr signals except the methyl of the Boc protecting group. Interestingly the coupled benzyl product (**173**) was less polar than the triphenyl phosphine byproduct as shown by tlc, whereas the nitro product (**169**) was more polar. As with the nitro derivative the optical rotation was positive. The *Z* and *E* double bond isomers of the phenyl derivative ran even closer together by tlc than those of the nitro derivative and as such no separation was possible at this stage of the synthesis.

Deprotection of the ketal (**173**) in tosic acid and methanol gave the free alcohol (**174**) in 93% yield. The double bond isomers were still inseparable by tlc and column chromatography. Separation of the isomers by crystallisation was attempted but proved to be unsuccessful.

Oxidation using Jones reagent furnished the desired carboxylic acid (**175**) along with some of the aldehyde. However, after separation from the acid, the aldehyde and any

remaining alcohol were recycled. At this point it was possible to separate the two double bond isomers, although they ran very close to the base line on tlc. Considerably more of the *Z* isomer was isolated than the *E* isomer. Nmr spectroscopy confirmed the structure of both products and the optical rotation was +10° for the *E* and +64° for the *Z*. The final removal of the Boc protection was analogous to that for the nitro analogue. The *E* isomer was deprotected in 76% yield but the *Z* isomer gave only 37% of the desired free amine (**146a,b**). The optical rotations were again shown to be positive and nmr analysis and mass spectrometry showed both to be the free amino acids. Mosher's amide derivatives of both compounds were synthesised to show that no racemisation had taken place at the chiral centre (see Figure 3.2).

3.3.3 FORMATION OF MOSHER'S AMIDES

In order to show that no racemisation had taken place it was necessary to form the MTPA-amides of the amino acids.[174,175] The ethyl esters of the free amino acids (**145**, **146**) were formed by heating in ethanol and thionyl chloride. Meanwhile reaction of *R*-(+)Mosher's acid with thionyl chloride gave the *S*-(+)Mosher's chloride.[174] The acid chloride was then dissolved in dry pyridine and carbon tetrachloride, and added to a sample of the amino acid ester and left to stand for 20 minutes. The reaction was then quenched by adding an excess of 3-dimethylamino-1-propylamine and extracting into diethyl ether.[175] After washing with first aqueous acid then base, the organic phases were dried and the solvent removed to give a yellow oil. The fluorine nmr spectra of the compounds were then analysed. The CF_3 group of Mosher's acid appears at -72 ppm and as no fluorine atoms are present in the compounds to be tested it was expected that a single peak would be observed. Note: all spectra contain traces of the Mosher's acid. The Mosher's amide of *R,S*-kynurenine and 2*S*-kynurenine were run as standards to demonstrate that the racemate would indeed show two peaks for the amide (Table 3.1). Two nmr traces are shown overleaf.

Compound	**Mosher's amide peak(s), ppm**
DL-kynurenine	-69.98 & -70.02
L-kynurenine	-70.01
145b (*E* nitro)	-69.97
145a (*Z* nitro)	-69.83
146a (*E* phenyl)	-69.95
146b (*Z* phenyl)	-69.72

Table 3.1: Fluorine Nmr Data for Mosher's Amides of Alkene Amino Acids

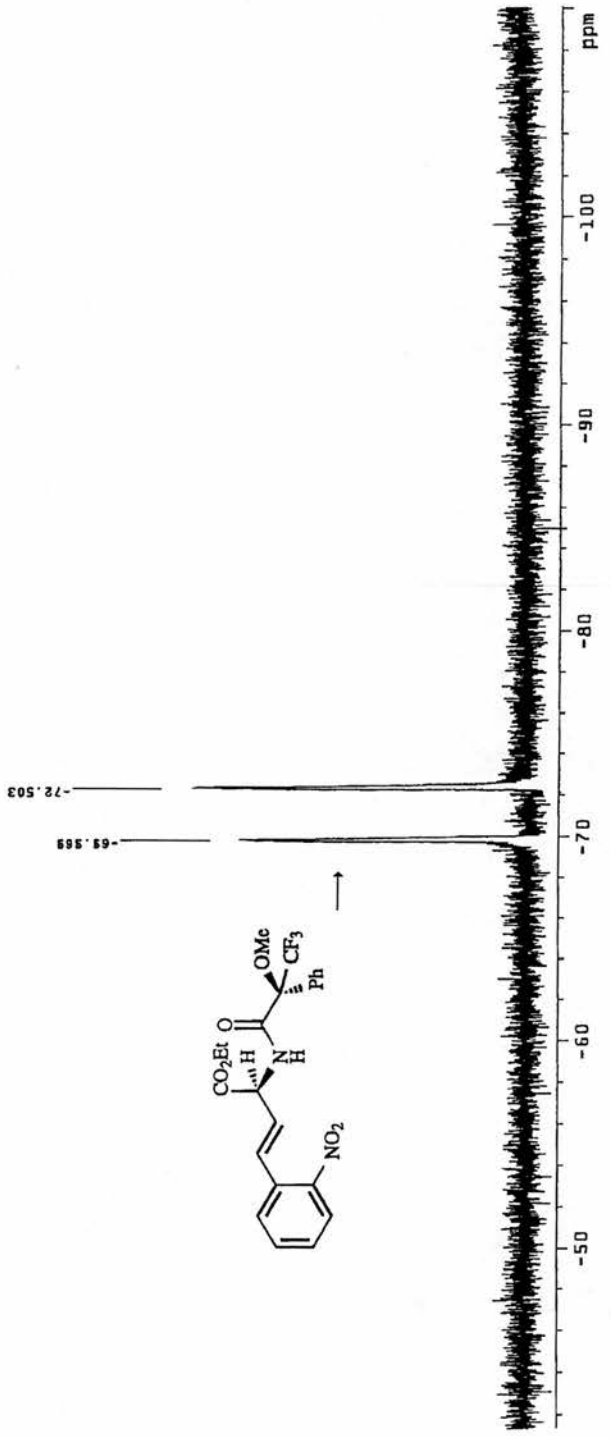


Figure 3.1: Mosher's Amide of *E* (2*S*)-2-Amino-4-(2-nitrophenyl)-3-butenoic Acid

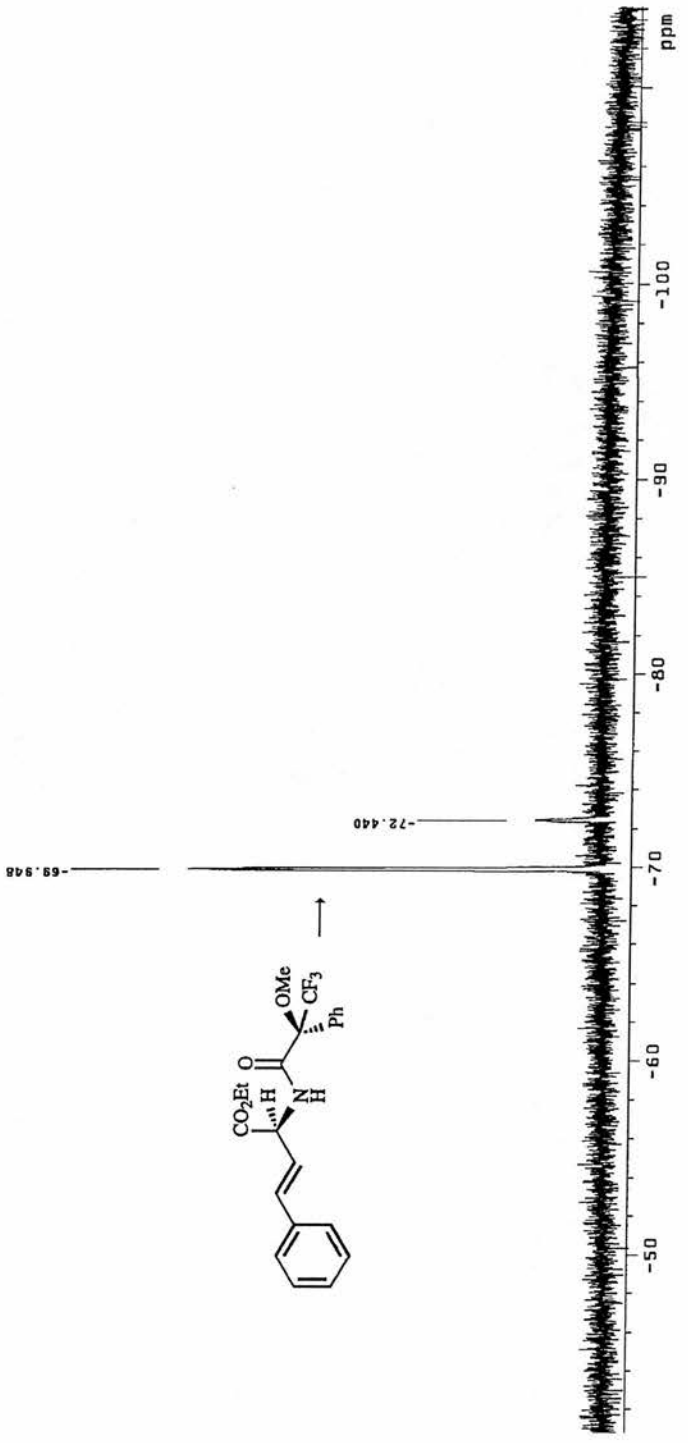


Figure 3.2: Mosher's Amide of *E* (2*S*)-2-Amino-4-phenyl-3-butenoic Acid

3.4 ATTEMPTED SYNTHESIS OF THE EPOXIDE DERIVATIVES

Having isolated the alkene analogues (**145**, **146**) for use as potential inhibitors, attempts were made to epoxidise the double bond to give compounds that would be able to alkylate the active site. It was proposed that the epoxides could be furnished by reacting the Boc protected alkenes with mCPBA in dichloromethane using a basic work-up. These reagents should give stereospecific addition products (i.e., the *E* double bond gives a *E* epoxide and analogously for the *Z* double bond). This reagent however, did not give the desired products with either the nitrophenyl or the phenyl alkenes. The nmr spectra in both cases showed only the starting material which was recovered. It was felt that the presence of water in the reagents may have resulted in ring opening of the epoxides, but this was not evident in the nmr spectra. Attempts were then made to form the epoxide using anhydrous peroxyacetic acid. This too gave no conversion of the double bond to the epoxide with the double bond character strongly evident in the ^{1}H-nmr spectrum. Hydrogen peroxide was then proposed as an alternative reagent, having been shown to epoxidise α,β-unsaturated ketones in base, whereas in acid it forms the epoxide which is ring opened to give the anti diol. It was felt that if the epoxide could not be formed the alcohol or diol analogues may also show activity with the enzyme. The reaction in 30% hydrogen peroxide did not give the epoxide and double bond character was still evident in the nmr spectrum. Reaction of the nitrophenyl derivative (**145**) in a premixed solution of ethanol, sodium carbonate and hydrogen peroxide resulted in a colour change from brown to yellow and after work-up the yellow oil showed loss of double bond character by nmr spectroscopy. A reverse phase tlc in acetonitrile:water (90:10) showed a single spot at the same R_f as the starting material. None of the epoxide was seen but it was not possible to fully assign the nmr spectrum as the reaction was on a very small scale. This was probably the most promising reagent tested.

Although the study has been by no means exhaustive it was found that mCPBA, anhydrous peroxyacetic acid, hydrogen peroxide and hydrogen peroxide in the presence of base gave no conversion to the epoxide. Further experiments need to be carried out to find a suitable route to the epoxide. Also the formation of the diol by ring opening of the epoxide may furnish further potential inhibitors. The work however, is limited by the availability of the starting alkenes which were only isolated in low overall yields from serine.

3.5 PRELIMINARY BIOLOGICAL TESTING OF THE ALKENES

3.5.1 MODEL UV STUDIES ON ALKENE DERIVATIVES

The reaction of amino acids with PLP, both enzyme catalysed and non-enzyme catalysed, can be readily monitored by UV spectroscopy. For example studies on *S*-aryl-L-cysteine *S,S*-dioxides[33] and *S*-(*ortho*-nitrophenyl)-L-cysteine[41] monitored the reaction between the inhibitor, PLP and kynureninase. In these, an increase in absorbance in the UV spectrum at 500 nm[33] or 470 nm[41] due to the formation of the stable quinoid carbanionic intermediate (Schiff's base) and a concomitant reduction of the peak at 420 nm[33] or 370 nm[41] due to enzymic bound pyridoxal 5'-phosphate was observed. Research by Phillips *et al.* on the mechanism of kynureninase showed the Schiff's base complex between PLP and 2*S*-kynurenine to be visible at 420 nm.[37] Model studies have also been carried out on reactions of PLP without the presence of an enzyme. One example involved monitoring the reactivity of α-keto-β-methylvaleric acid with pyridoxal, rather than pyridoxal 5'-phosphate, in the presence of metal ions (Al^{3+}, Cu^{2+}) at a range of pHs. The UV absorption at 365 nm was measured and this gave the rates of transamination.[176]

It was thus decided to examine the reaction of the alkene based inhibitors with PLP by UV spectroscopy. Formation of the Schiff's base between PLP, kynurenine, phenylalanine

and the inhibitors (**145**, **146**) were examined. Firstly, to establish the reaction conditions, the UV spectra of kynurenine (100 μM) on its own and with one equivalent of PLP in 10 mM phosphate buffer at pH 7.4 were measured. The absorbance at 370 nm was observed and seen to increase on addition of PLP (Figure 3.3). However, a similar experiment with phenylalanine showed a decrease in absorbance. 2*S*-2-Amino-4-phenyl-3-butenoic acid (**146**) showed an increase in absorbance at 370 nm and a slight decrease at 320 nm (100 μM, pH 7.4), Figure 3.4. The nitro analogue 2*S*-2-amino-4-(2-nitrophenyl)-3-butenoic acid (**145**) also showed an increase in absorbance at 370 nm and a decrease at 320 nm under the same conditions (Figure 3.5).

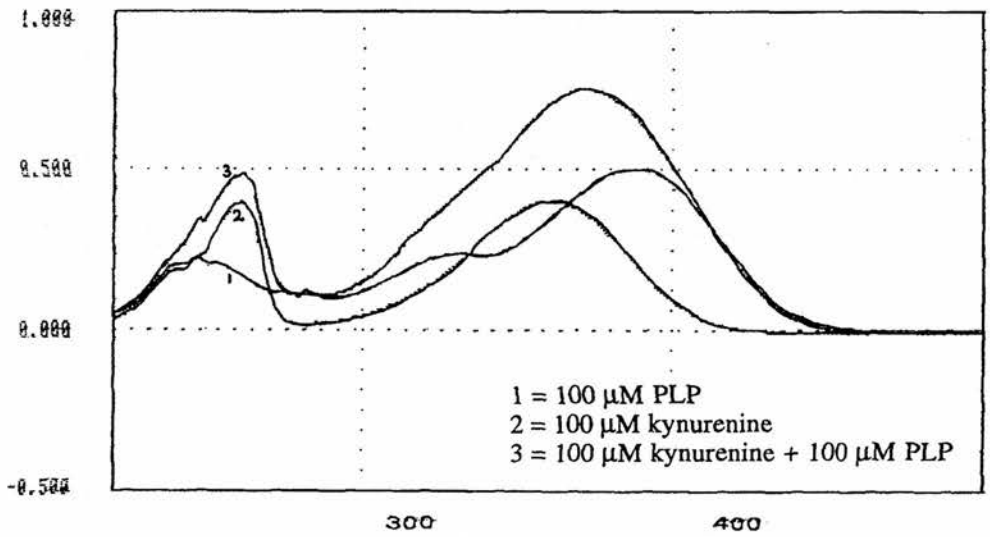


Figure 3.3: UV Spectrum of Kynurenine–PLP Complex

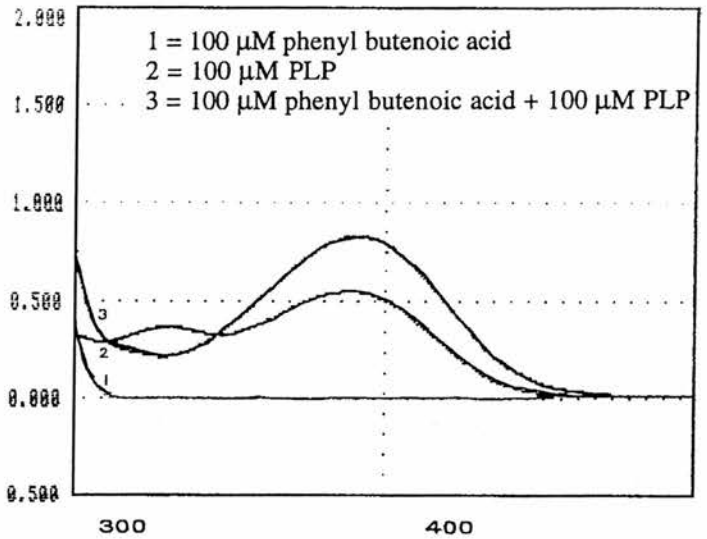


Figure 3.4: UV Spectrum of 2*S*-2-Amino-4-phenyl-3-butenoic Acid–PLP Complex (**146**)

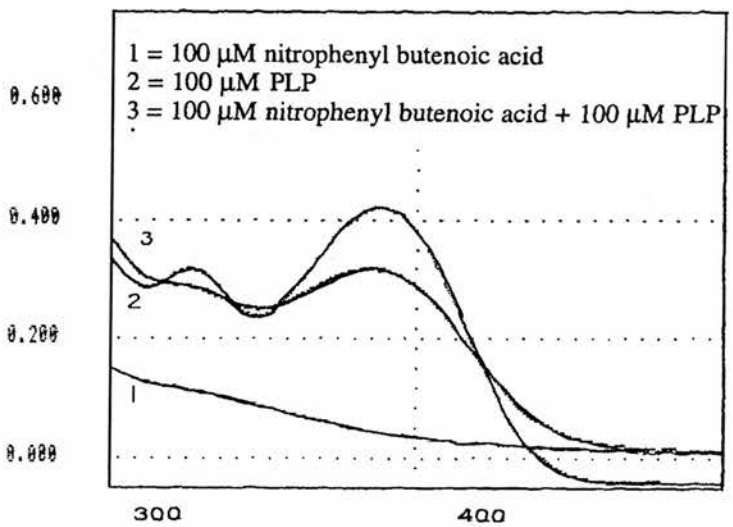
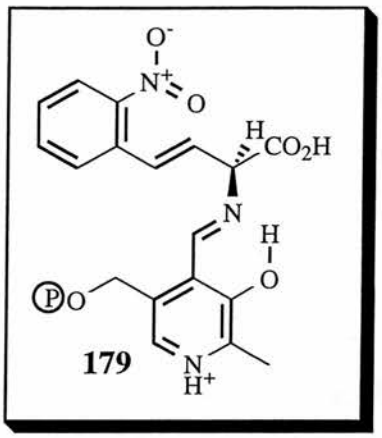


Figure 3.5: UV Spectrum of 2*S*-2-Amino-4-(2-nitrophenyl)-3-butenoic Acid–PLP Complex (**145**)

The measurements obtained for both the alkene inhibitors were repeated at basic (8.5) and acidic (5.3) pHs and no significant change in absorbance was evident. Measurements over a range of concentrations (30 μM–200 μM) for the alkenes showed the highest absorbance at 370 nm was for a one to one reaction. The change in absorbance upon mixing with PLP was immediate and was shown to be stable over time. The increase in absorbance at 370 nm is in direct opposition to the results shown in the literature, which all reported a decrease at similar wavelengths.[33,41]

It had been thought that the reaction of 2*S*-2-amino-4-(2-nitrophenyl)-3-butenoic acid (**145**) with PLP may give rise to a highly conjugated system (**179**) which might have contributed to inhibition of the enzyme. Unfortunately no large changes in absorbance were observed for either of the alkenes at 370 nm.

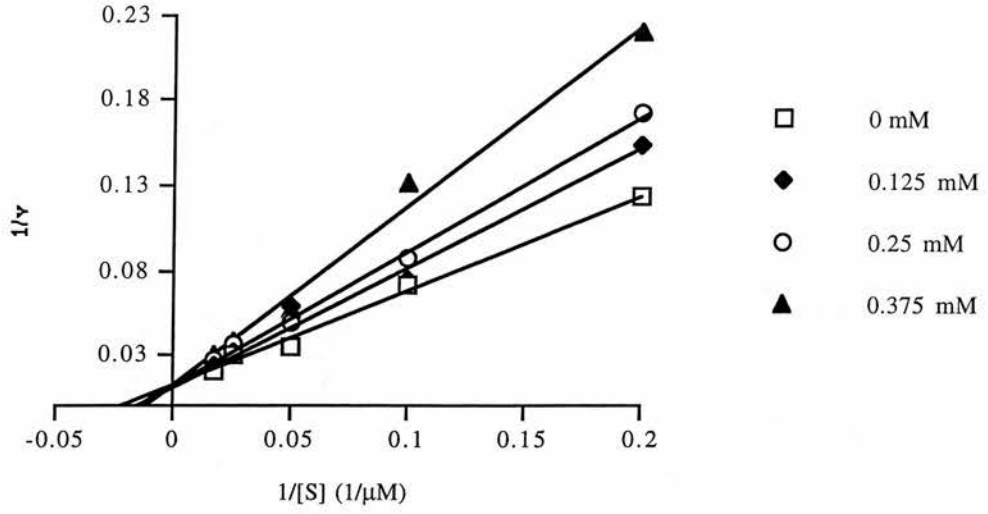


179

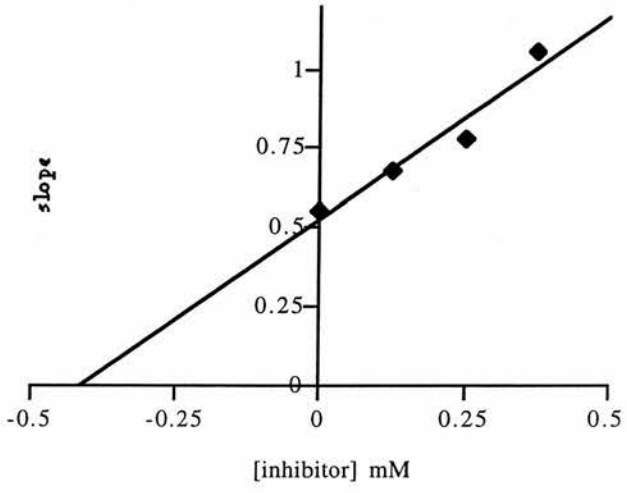
It is difficult to conclude anything from these results other than the alkenes form stable, pH and concentration independent complexes with PLP. Whether the increase rather than decrease in absorbance is significant is unclear.

3.5.2 INHIBITION OF KYNURENINASE

The *Z* (**146b**)and *E* (**146a**) double bond analogues of 2*S*-2-amino-4-phenyl-3-butenoic acid were tested as inhibitors of bacterial kynureninase. The *Z*-(2*S*)-2-amino-4-phenyl-3-butenoic acid showed weak inhibition at concentrations of 125–375 μM. The inhibition observed was reversible competitive inhibition and the K_i was found to be 413 μM (Graph 3.1 and 3.2). This is higher than those found for the indanone (**96**), tetralone (**97**) and chromanone (**98**) derivatives described in Chapter 2. It would thus appear that *Z*-(2*S*)-2-amino-4-phenyl-3-butenoic acid binds more poorly than the inhibitors described previously. A more thorough study of inhibition could not be carried out as the inhibitor concentration range was so high and there was not enough of the compound available for further testing.



Graph 3.1: Lineweaver–Burk Plot for *Z*-(2*S*)-2-Amino-4-phenyl-3-butenoic Acid (**146b**) with Bacterial Kynureninase

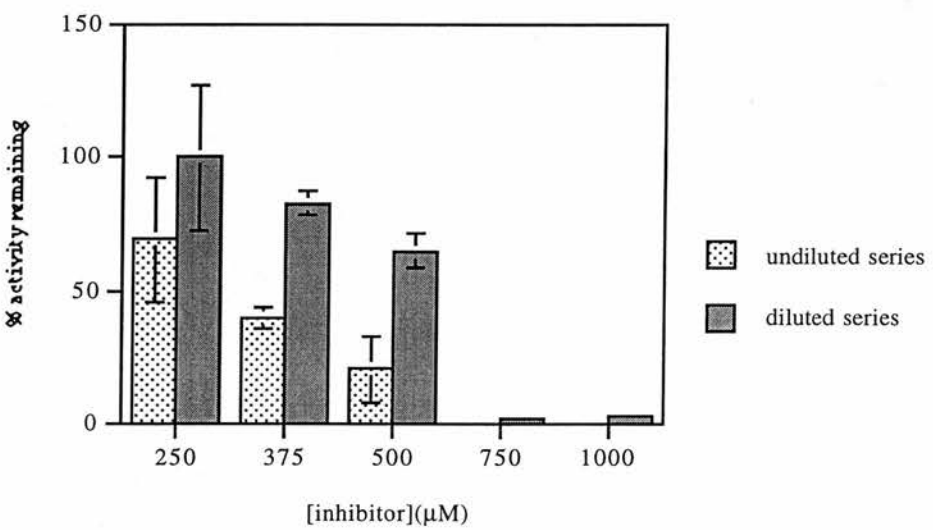


Graph 3.2: Secondary Plot of Slope Versus Inhibitor for *Z*-(2*S*)-2-Amino-4-phenyl-3-butenoic Acid (**146b**) with Bacterial Kynureninase

For the human enzyme, *Z*-(2*S*)-2-amino-4-phenyl-3-butenoic acid (**146b**) gave 90% inhibition at 1mM and at this concentration the inhibition was irreversible. However, there was evidence that at a lower concentration (250μM) it was reversible. Preliminary studies using 10μM hydroxykynurenine were not sufficient to determine an accurate K_i over the high inhibitor concentration range (125–375 μM) given previously for the

bacterial enzyme. Preliminary studies at higher concentrations, using dilution techniques, for the *E*-(2*S*)-2-amino-4-phenyl-3-butenoic acid (**146a**) however, showed that the inhibition becomes irreversible at concentrations over 0.5 mM (Graph 3.3). Preliminary kinetic studies for *E*-(2*S*)-2-amino-4-phenyl-3-butenoic acid at lower concentrations did not give reproducible or suitable data, and limited supplies of material precluded further inhibitor studies.

(Note: Due to the low yields of both the *Z*- and *E*-(2*S*)-2-amino-4-phenyl-3-butenoic acids obtained, more thorough studies at both high and low concentrations could not be carried out.)



Graph 3.3: Irreversibility of the Reaction of *E*-(2*S*)-2-Amino-4-phenyl-3-butenoic Acid (**146a**) with Bacterial Kynureninase

The *Z*-(2*S*)-2-amino-4-phenyl-3-butenoic acid showed less activity with the enzyme than was hoped. The *E* derivative (**146a**) was shown to be an irreversible inhibitor at high concentrations, although the *Z* derivative was a poor inhibitor of the enzyme at low concentrations. At low concentrations the reaction with the enzyme was reversible. It is postulated that for these inhibitors inactivation events are infrequent.

3.5.3 CONCLUSION

The data collected so far on both the phenyl and nitrophenyl derivatives are not sufficient to make any accurate conclusions about the potency or irreversibility of these inhibitors. Initial studies however, have shown that the phenyl alkenes are inhibitors of the enzyme if somewhat less potent than envisaged. However, as both the phenyl and nitrophenyl alkene derivatives were difficult to purify and much of the overall yield was lost due to purification by column chromatography, it would be better to optimise the reaction conditions further before carrying out more assays with the enzyme. As the epoxides have not yet been successfully synthesised they were unavailable for testing as inhibitors.

CHAPTER 4

4 SYNTHESIS OF 5-FLUOROKYNURENINE AND 6-FLUOROKYNURENIC ACID

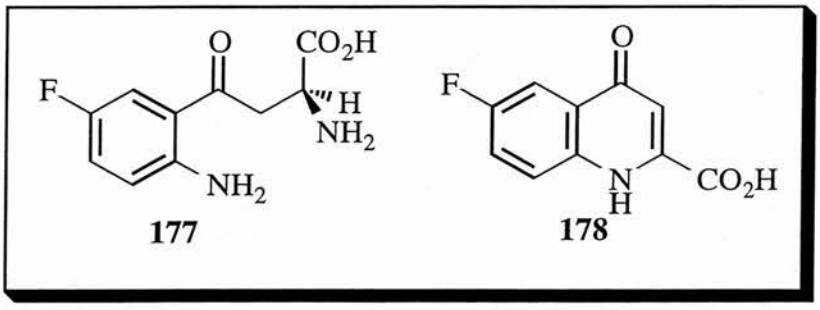
4.1. IMAGING USING ^{19}F-NMR TECHNIQUES

Nmr spectroscopy offers the possibility of non-invasive measurement of metabolism *in vivo*.[177] The sensitivity of ^{19}F-nmr spectroscopy is high when compared to ^{1}H and the chemical shift range of ^{19}F is much wider (50 to -300ppm). There are few background signals in humans and animals because there is little or no source in the diet, the main use being fluoride in dental treatments and drinking water. Also, as there is only one isotope of fluorine, it has 100 percent abundance. Single peaks are observed for the fluorine although there is some coupling to protons (as is the case in proton spectra which are split by ^{19}F–^{1}H coupling). As few methods have been developed for the direct evaluation of amino acids non-invasively, ^{19}F-nmr of fluoro-amino acids could be used to evaluate the metabolic functions of amino acids. Harada studied the metabolism of 5-fluorotryptophan (**176**) to 5-fluorokynurenine (**177**) as catalysed by kynureninase in rat liver.[177] The study showed that after approximately 40 minutes the injected peak for 5-fluorotryptophan decreased and a neighbouring peak, shifted by about 1.6 ppm, due to 5-fluorokynurenine began to emerge and steadily increase in size. This work was restricted to *in vivo* studies on rat liver but since then magnetic resonance imaging has been used to detect ^{17}O and ^{19}F in cat brain[178] and halothane in rabbit brain *in vivo*.[179] These techniques could be applied to the enzyme catalysed reaction of kynurenine (**5**) to kynurenic acid (**2**).

4.1.1 IMAGING OF THE KYNURENINE PATHWAY

Schwarcz *et al.* from the University of Maryland believe that the conversion of 2*S*-kynurenine (**5**) into kynurenic acid (**2**) during excitatory brain damage can be plotted on a graph and that after a certain level of kynurenic acid is released into the brain the damage is irreversible.[87]

One way of monitoring this change would be to use fluoro analogues and observe their metabolism by kynurenine aminotransferase *in vivo*. Also, there is the possibility of developing it into an assay to show the turnover of kynurenine into kynurenic acid in a number of neurodegenerative disorders. 6-Fluorokynurenic acid (**178**) is required as a standard to show how much kynurenine has been metabolised to kynurenic acid. Whether 5-fluorokynurenine (**177**) will be shown to be an inhibitor of KAT is unknown but it is hoped that it will be turned over at a similar rate to 2*S*-kynurenine.

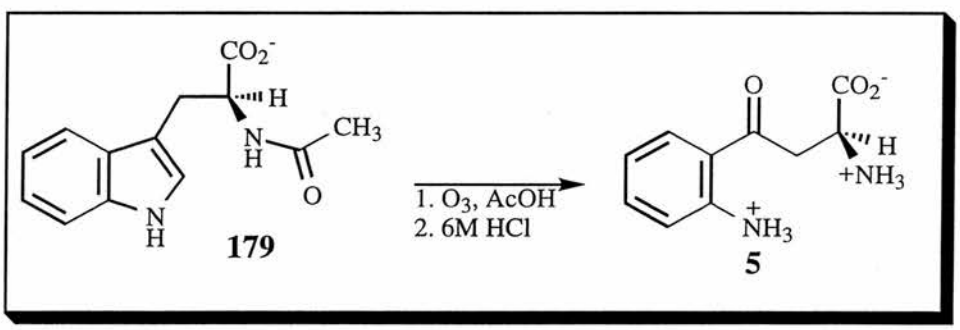


Previous studies of kynurenic acid analogues have shown that many 5-, 6-, 7-, and 8-substituted and disubstituted species show activity as selective antagonists of the glycine modulatory site on the NMDA receptor complex.[117] Of these it has been shown that the fluoro analogues are the least active. As yet, the synthetic precursor 5-fluorokynurenine (**177**) has not been synthesised by non-enzymatic routes. Harada however, reports that 5-fluorokynurenine can be synthesised by modifying the method of Warnell and Berg[180] and ozonolysing protected 5-fluorotryptophan, although no experimental data was given.[177] The closeness in structure of 5-fluorokynurenine to kynurenine and the comparable sizes of the covalent and van der Waals radii of fluorine and hydrogen would possibly not effect binding to kynurenine aminotransferase and thus its conversion into 6-fluorokynurenic acid (**178**). Replacing a hydrogen in a biologically active molecule with a fluorine atom often results in the formation of a compound which mimics the interactions of the natural substrate with biological molecules. However, exchanging a proton for a fluorine also affects the electron distribution within the molecule as well as electric

repulsive/attractive interactions within the intra/intermolecular environment and these changes could significantly affect enzyme–substrate interactions, receptor–substrate interactions and other systems.[181] It is to be hoped that the introduction of fluorine into kynurenine will not affect its interaction with either KAT or kynureninase. Soda and Tanizawa reported that 5-fluorokynurenine (prepared enzymatically by the method described by Snell)[182] was 91% as active a substrate as 2*S*-kynurenine for the enzyme from *P. marginalis*.[24]

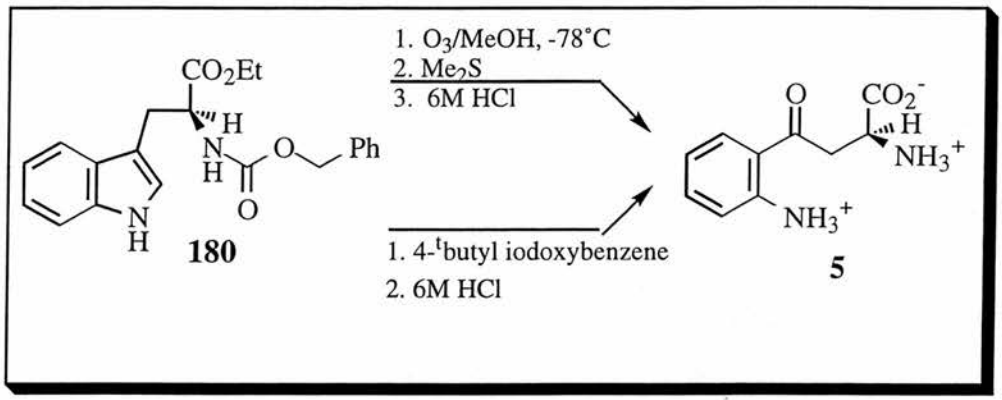
4.2. SYNTHETIC ROUTES TO 5-FLUOROKYNURENINE AND 6-FLUOROKYNURENIC ACID

The original literature synthesis[180,183] of kynurenine involves the ozonolysis of *N*-acetyl tryptophan (**179**) in glacial acetic acid, followed by the hydrolysis of the formyl and acetyl groups to give kynurenine (**5**, Scheme 4.1). However, this procedure has resulted in poor yields of the product which is difficult to purify.



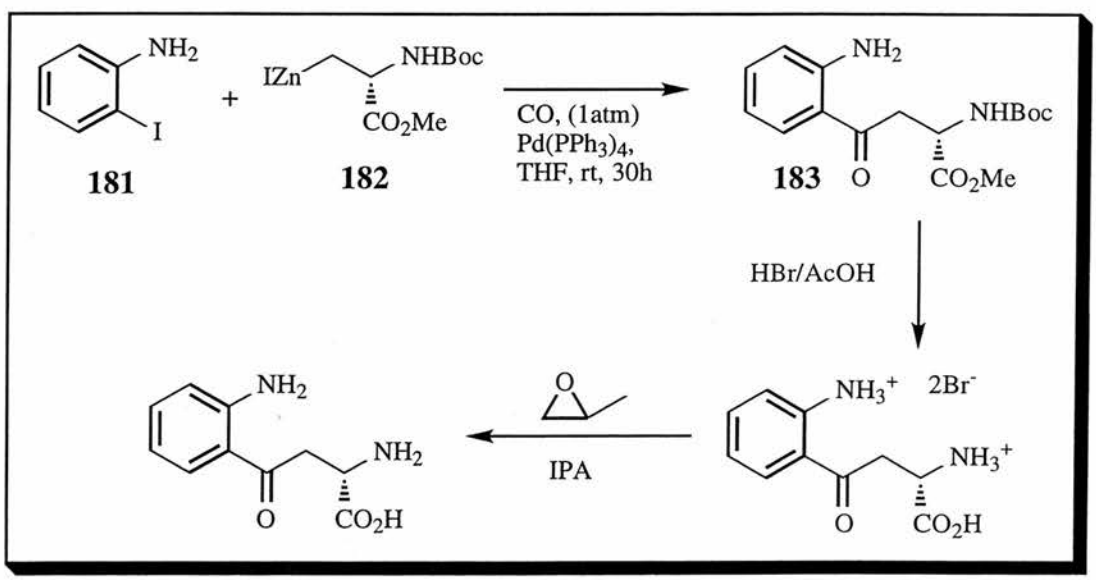
Scheme 4.1: Synthesis of Kynurenine from N*-Acetyltryptophan*

Other literature alternatives involve modification of the ozonolysis procedure by using a benzyloxycarbonyl protected nitrogen and protecting the acid as the ethyl ester (**180**). The reaction is then carried out at -78 °C in methanol. This is followed by quenching with dimethyl sulfide and deprotection to give kynurenine. A second route uses the same protected tryptophan (**180**) but employs 4-^{t}butyliodoxybenzene[184] in chlorobenzene at reflux to facilitate the oxidative cleavage of the indole double bond (Scheme 4.2).



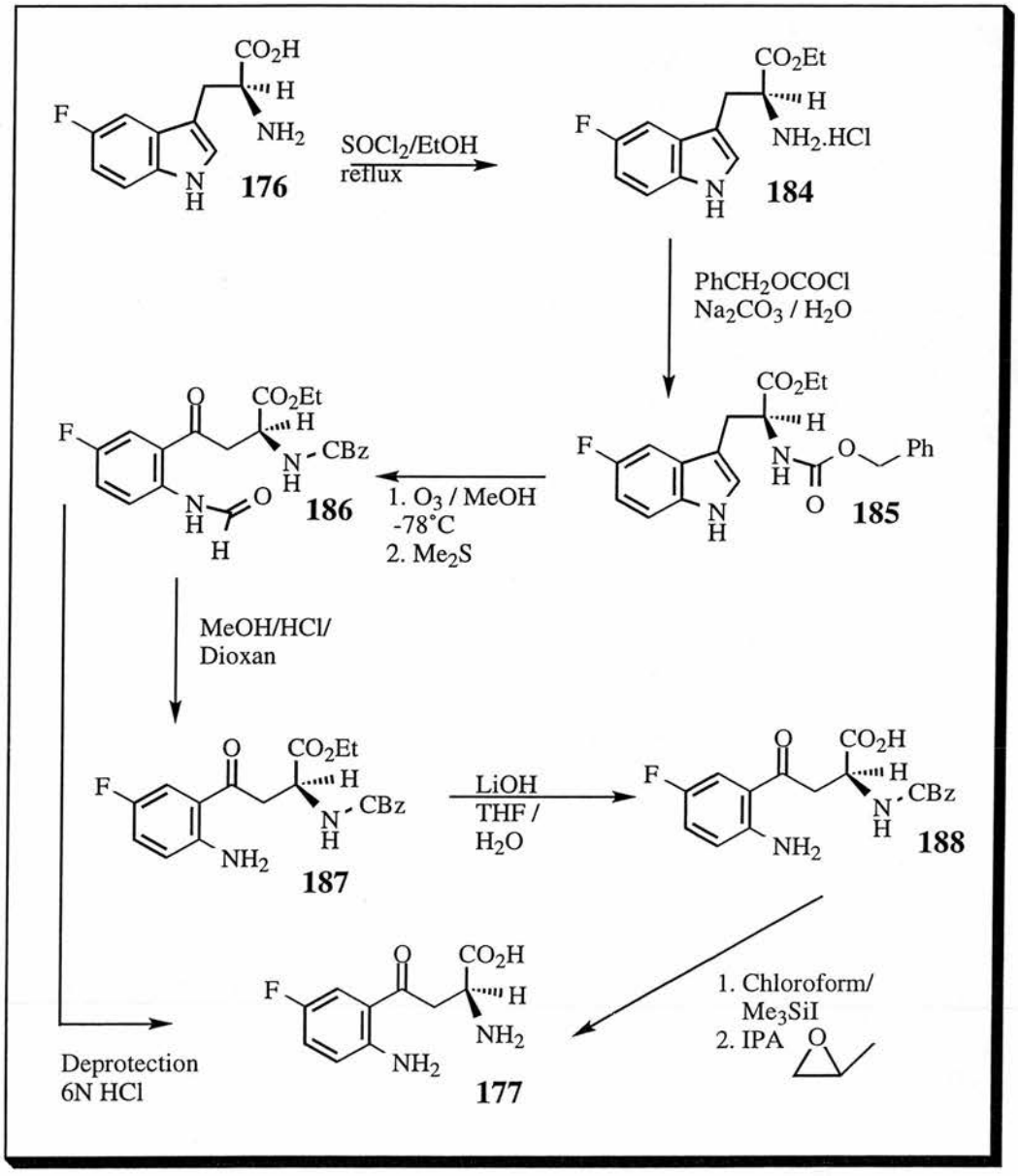
Scheme 4.2: Alternative Routes to Kynurenine

A more recent synthetic route to kynurenine is *via* a carbonylative coupling of an amino acid-derived organozinc reagent with functionalised aryl iodides.[185] 2-Iodoaniline (**181**) was reacted with a suitable serine-derived zinc reagent (**182**) in THF under a carbon monoxide atmosphere (1 atm, balloon) in the presence of (tetrakis)triphenylphosphine palladium(0), [$Pd(Ph_3P)_4$] at room temperature to give the protected kynurenine (**183**) in 52% yield. This was then deprotected using hydrogen bromide in acetic acid (Scheme 4.3). Whether a similar strategy would work for fluoroiodoaniline is unknown.



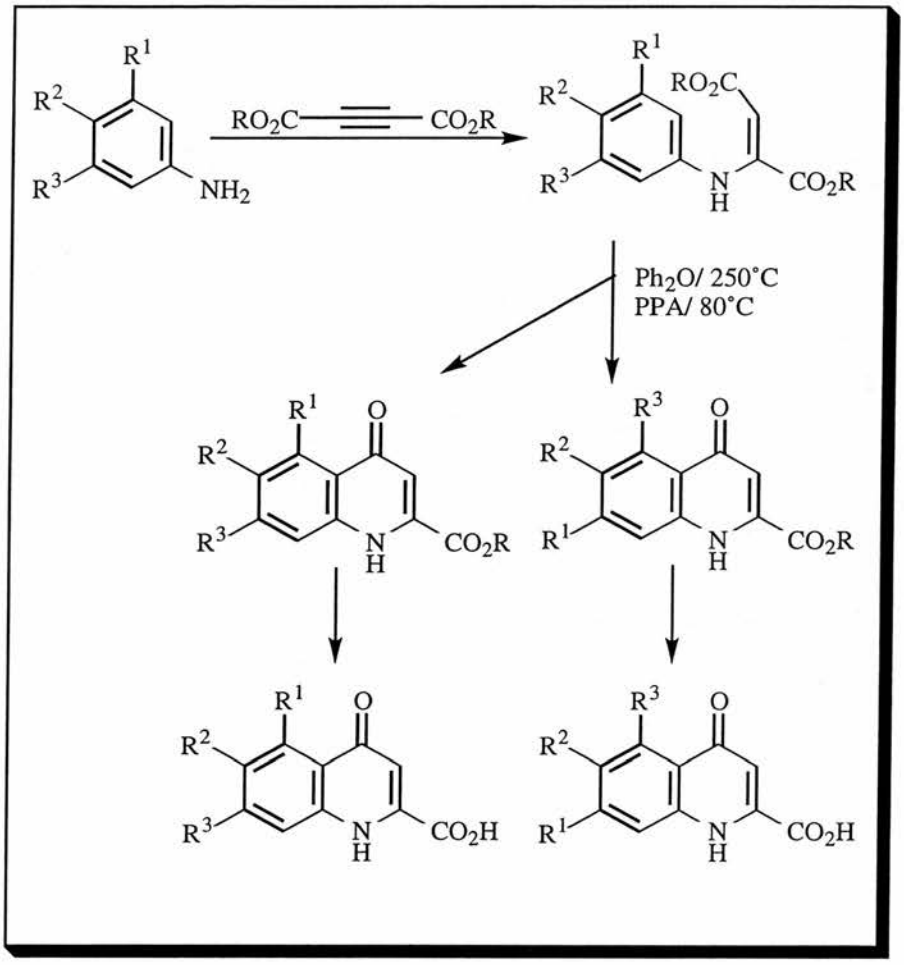
Scheme 4.3: Synthesis Of Kynurenine from an Organozinc Reagent and an Aryl Iodide

It was thus decided to synthesise 5-fluorokynurenine (**177**) using an ozonolysis strategy in methanol at -78 °C starting with 5-fluorotryptophan (**126**), which is commercially available (Scheme 4.4). This involves the protection of 5-fluorotryptophan and then the cleavage of the indole double bond *via* the ozonolysis reaction. The initial protection stages were easily carried out on a gram scale.



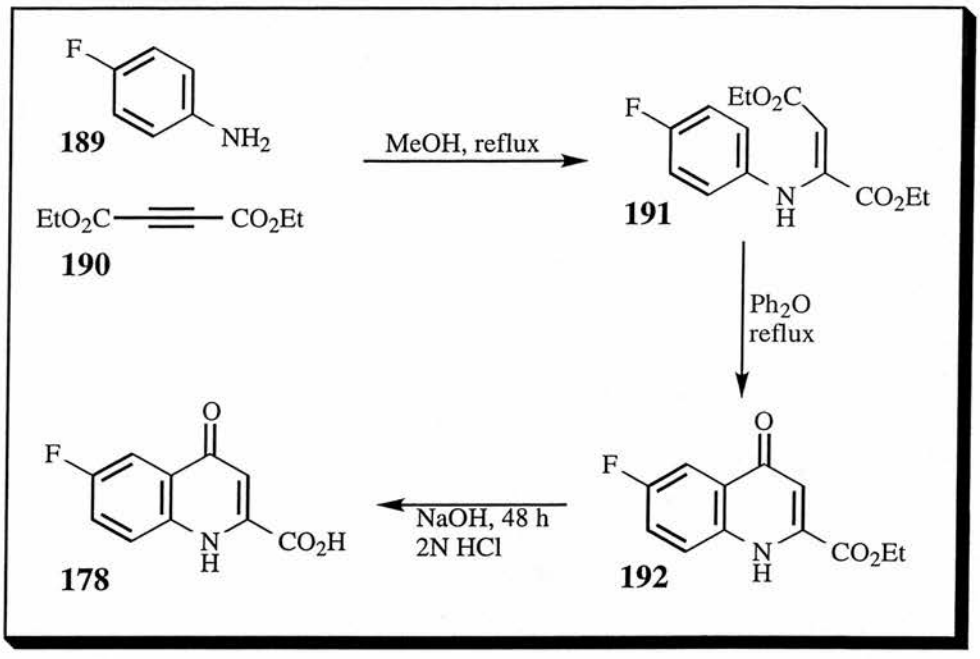
Scheme 4.4: Proposed Synthesis of 5-Fluorokynurenine from 5-Fluorotryptophan

The synthesis of kynurenic acid analogues is well documented. There are established routes such as the Conrad–Limpach synthesis (Scheme 4.5) and related procedures.[186,187]



Scheme 4.5: Conrad–Limpach Synthesis

As this series of reactions has been proven, it was possible to start with the commercially available 4-fluoroaniline (**189**) and diethyl acetylenedicarboxylate (**190**). The proposed synthetic route is shown in Scheme 4.6.



Scheme 4.6: Synthesis of 6-Fluorokynurenic Acid

4.3 SYNTHESIS OF 5-FLUOROKYNURENINE FROM 5-FLUOROTRYPTOPHAN

Initially, the ethyl ester of 5-fluorotryptophan was prepared by reacting 5-fluorotryptophan with ethanol and thionyl chloride under reflux conditions, giving the product (**184**) in 98% yield. The product precipitated out of solution and was easily filtered and purified. A second and third crop were also recovered by evaporating the ethanol solution under reduced pressure. Microanalysis and ^{1}H-nmr spectroscopy in D_2O confirmed the structure of the product and it required little further purification or recrystallisation. The ^{1}H-nmr spectrum was very clean showing the ester at $\delta = 1.1$ and 4.1 ppm, for the methyl and methylene protons respectively, and distinct ^{19}F–^{1}H splitting patterns for the three ring protons (2 double doublets and a double triplet).

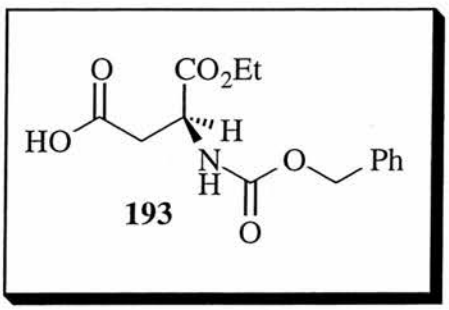
Subsequently the preparation of *N*-CBz 5-fluorotryptophan ethyl ester (**185**) was achieved on treatment of 5-fluorotryptophan ethyl ester (**184**) with a slight excess of benzyl chloroformate and sodium carbonate in aqueous solution. It was found that the reaction gave higher yields when the reaction mixture was more dilute as this prevented the *N*-CBz 5-fluorotryptophan ethyl ester from becoming sticky and thus more difficult to purify. This reaction was particularly good as the ethyl ester of 5-fluorotryptophan is fairly soluble in water and the benzyloxycarbonyl derivative is not, allowing easy separation by filtration. ^{1}H-Nmr spectroscopy and microanalysis showed this to be the correct product with no product remaining in solution. The yields were between 68–97%. The characteristic peak of the methylene of the CBz group was found at $\delta = 5.11$ ppm.

The protected 5-fluorotryptophan was then ozonolysed as outlined in Scheme 4.4. After ozonolysis for one hour the solution was quenched with dimethyl sulfide. Column chromatography did not yield a crystalline product as in the previous ozonolysis of protected tryptophan.[45] Fluorine nmr spectroscopy showed the loss of the fluorine-containing ring, as only small traces of fluorine were found in the spectrum. The brown tar-like oil produced was analysed by ^{1}H-, ^{13}C-, ^{19}F-nmr and by mass spectrometry. Results from the MS showed there to be a major peak at 296 (M + H).

The reaction was carried out a second time and was monitored more closely by tlc every ten minutes. The reaction was found to be complete after ten minutes with no further change in the chromatogram for one hour. Fresh dimethyl sulfide was used to quench the reaction and the product was extracted and chromatography carried out as before. Once again mass spectrometry, ^{1}H-, ^{13}C-, and ^{19}F-nmr spectroscopy showed no trace of the expected kynurenine analogue (**186**).

It appears that the product isolated is in fact the protected aspartic acid derivative (**193**) and a possible mechanism is given below (Scheme 4.7). The 5-fluoro-2-aminophenol

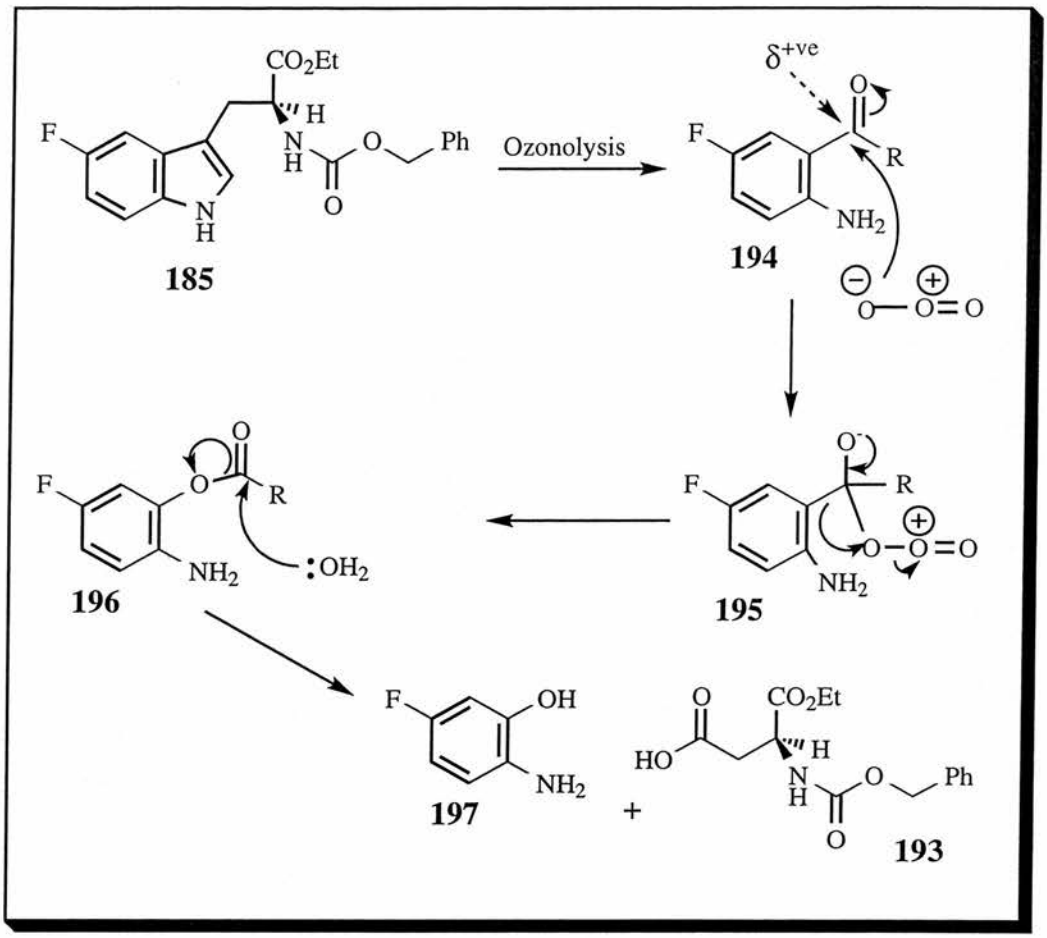
produced in this reaction is believed to have been extracted in the aqueous layer during the work-up.



193

4.3.1 PROPOSED MECHANISM FOR A BAEYER–VILLIGER TYPE INSERTION REACTION

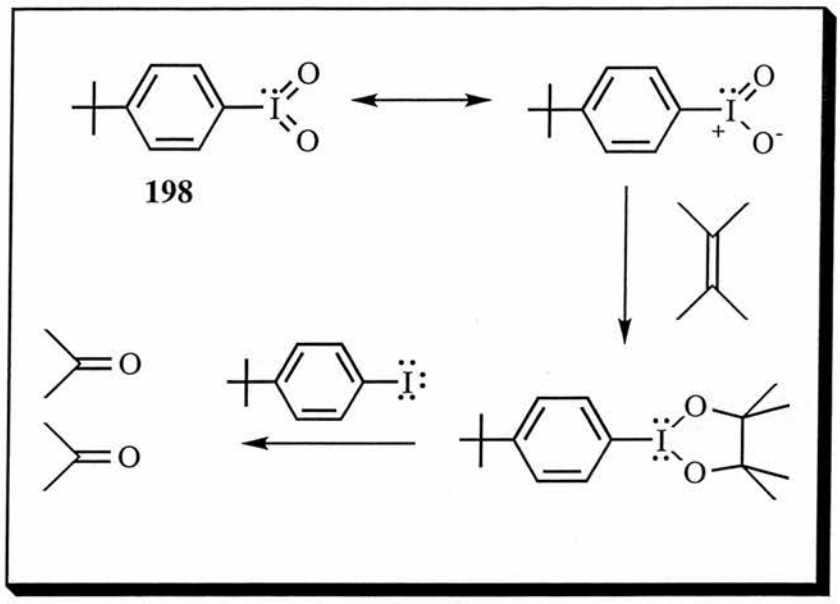
It has been proposed that the fluorine containing benzene ring, including the formyl protected nitrogen from the 5-fluorotryptophan ozonolysis, has been completely cleaved from the protected amino acid (**185**). This has been confirmed by nmr spectroscopic data which contain no proton, carbon or fluorine shifts for that ring and by mass spectrometry which shows the molecular weight of the molecule to be 295. A possible mechanism for the reaction is as follows (Scheme 4.7). It is believed that ozonolysis takes place almost immediately but then excess ozone allows the molecule to take part in a Baeyer–Villiger type insertion reaction. The fluorine containing group is then hydrolysed off during work-up. The Baeyer–Villiger reaction appears to be faster than oxidative cleavage as it was not possible to stop reaction after the first step by using shorter reaction times. Interestingly this is not observed in the ozonolysis of unsubstituted tryptophan. It is possible that the electron withdrawing effect of fluorine increases the reactivity of the carbonyl formed in the first step of the reaction.



Scheme 4.7: Proposed Baeyer–Villiger Insertion Reaction

4.3.2 ALTERNATIVES TO OZONOLYSIS

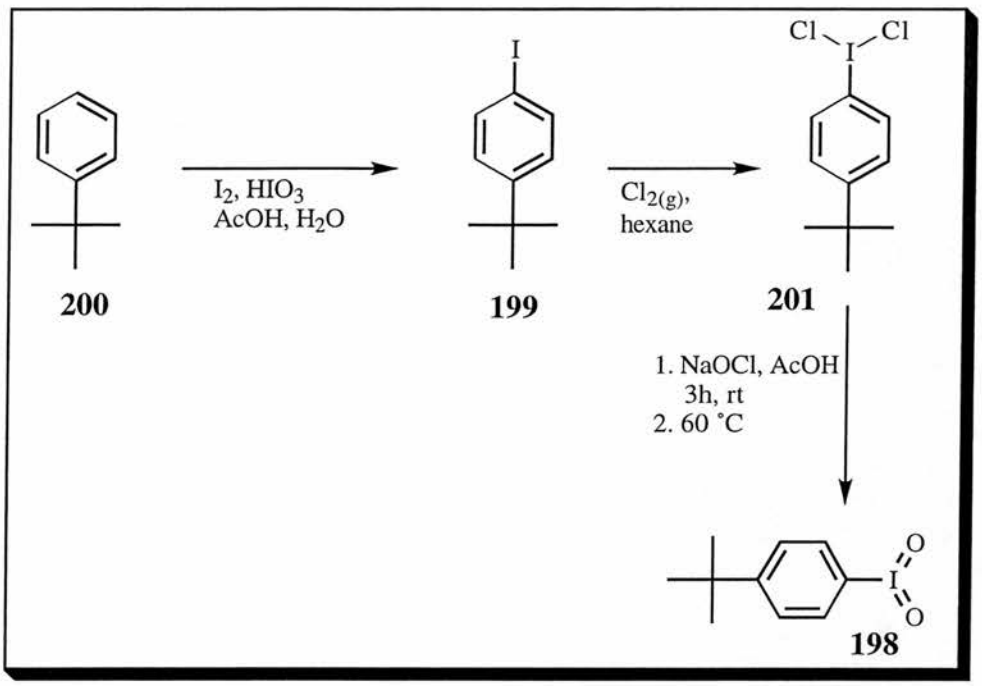
As ozone very effectively cleaved off the fluorine-containing aromatic ring in the molecule, an alternative reagent was sought. Ranganathan and co-workers identified 4-^{t}butyliodoxybenzene (**198**) as a reagent of promise.[184] This is synthesised from 4-^{t}butylbenzene and can be recycled from the oxidation reaction. A severe limitation is its insolubility in most non-polar solvents and thus there is the possibility that the protected 5-fluorotryptophan molecule would not dissolve in similar media. Also 4-^{t}butyliodoxybenzene is explosive, although careful handling should minimise this risk. The reaction with 4-^{t}butyliodoxybenzene is carried out in chlorobenzene[184] under reflux conditions which may increase the risk of explosion, but an earlier paper by Ranganathan on iodoxybenzene also shows the reaction to be viable in chloroform.[188]



Scheme 4.8: Proposed Mechanism of 4-^{t}Butyliodoxybenzene Action

4-^{t}Butyliodoxybenzene (**198**) is isoelectronic to ozone and it is thought that its reactions proceed *via* pathways remarkably similar to those of ozone.[188] In the scheme above a putative reaction pathway is given (Scheme 4.8). It is believed that the iodoxybenzene undergoes cycloaddition to the alkene and then collapses to form two carbonyl groups and regenerated iodobenzene which can be recycled (Scheme 4.8). An advantage of using this reagent is that one equivalent of iodoxybenzene can be easily measured which is not the case with ozone. If only one equivalent is used it should eliminate the possibility of the subsequent Baeyer–Villiger reaction taking place.

4-^{t}Butyliodoxybenzene (**198**) could be synthesised in a three step reaction as shown in Scheme 4.9 below, following a procedure by Ranganathan *et al.*[184]



Scheme 4.9 Synthesis of 4-^{t}Butyliodoxybenzene

4.3.3 SYNTHESIS OF 4-^{t}BUTYLIODOXYBENZENE

4-^{t}Butyliodobenzene (**199**) was synthesised from ^{t}butylbenzene (**200**) in 70% yield as a red syrupy oil. Nmr spectroscopic data confirmed the structure of the compound and it was used without further purification in the next stage.

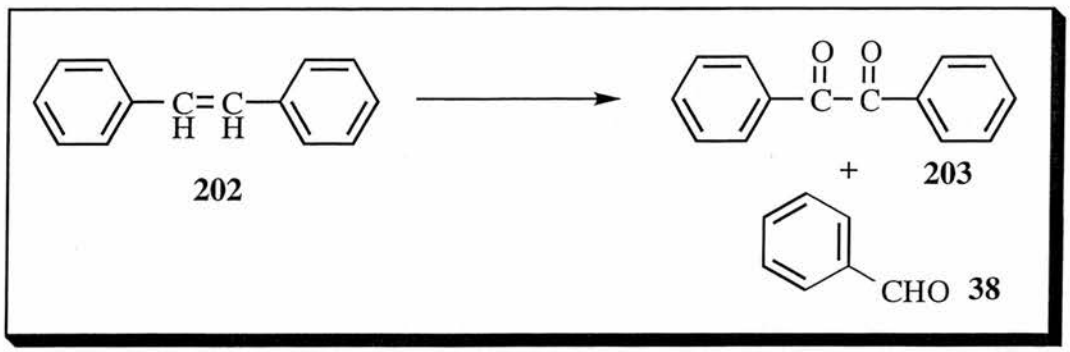
The formation of the dichloride (**201**) was carried out in hexane with chlorine gas passed through a sinter tipped glass tube. The pink/red solution turned yellow almost immediately and a fine yellow powder precipitated out in 78% yield, which required no further purification. Analysis by nmr spectroscopy showed the product to be the dichloride (**201**). The aromatic protons gave two doublets at δ = 7.48 and 8.08 ppm and these signals corresponded to those given in the paper.[184]

The final stage of the reaction was carried out in bleach solution with a catalytic amount of acetic acid. The solution was stirred at room temperature and yielded a pale yellow solid. This was then heated to give the final product (**198**) as a white solid in 91% yield. It proved difficult to dry yet required no purification. The melting point corresponded with that given in the paper[184] (it explodes at 220°C) and spectral data corresponded to that given in the literature. It was interesting to note that in the proton nmr spectrum the aromatic protons shift from δ = 7.48 and 8.08 ppm in the dichloride (**201**) to δ = 7.62 and 7.87 ppm in the final product (**198**).

4.3.3.1 OXIDATION OF 5-FLUOROTRYPTOPHAN USING 4-^{t}BUTYLIODOXYBENZENE

Unfortunately the iodoxybenzene did not oxidatively cleave the indole double bond as hoped. No change was seen by tlc (5:1 dichloromethane:ethyl acetate). Preliminary studies by nmr showed no significant change in the spectrum for the protected 5-fluorotryptophan even after column chromatography. Ranganathan *et al.* suggested that carbobenzyloxy protection of the nitrogen was likely to result in elimination of the protecting group and the use of benzoyl protection was advised.[189] In our case the *N*-CBz group had not been eliminated and yet no significant changes were observed in the proton spectrum to indicate that any reaction had taken place. Two experiments were carried out using both one and two equivalents of iodoxybenzene to starting material.

Attempts were also made to oxidise the double bond in stilbene (**202**) (as indicated in Ranganathan's paper). This however, also did not yield the desired products, benzaldehyde (**38**) and benzil (**203**) (Scheme 4.10). Nmr spectroscopy showed that only starting material was recovered which was contaminated with ^{t}butyliodobenzene.

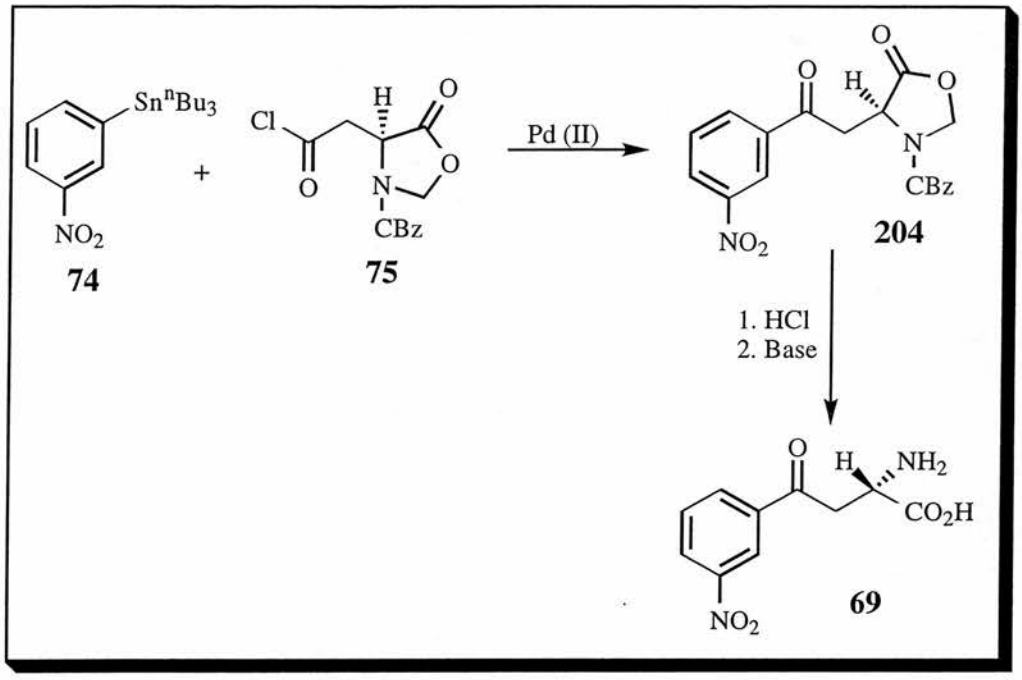


Scheme 4.10 Oxidation of E-*Stilbene*

As we were unable to repeat the experimental results of Ranganathan *et al.* or to oxidise the double bond of 5-fluorotryptophan using 4-^{t}butyliodoxybenzene it was decided that an alternative route to 5-fluorokynurenine (**181**) would have to be devised.

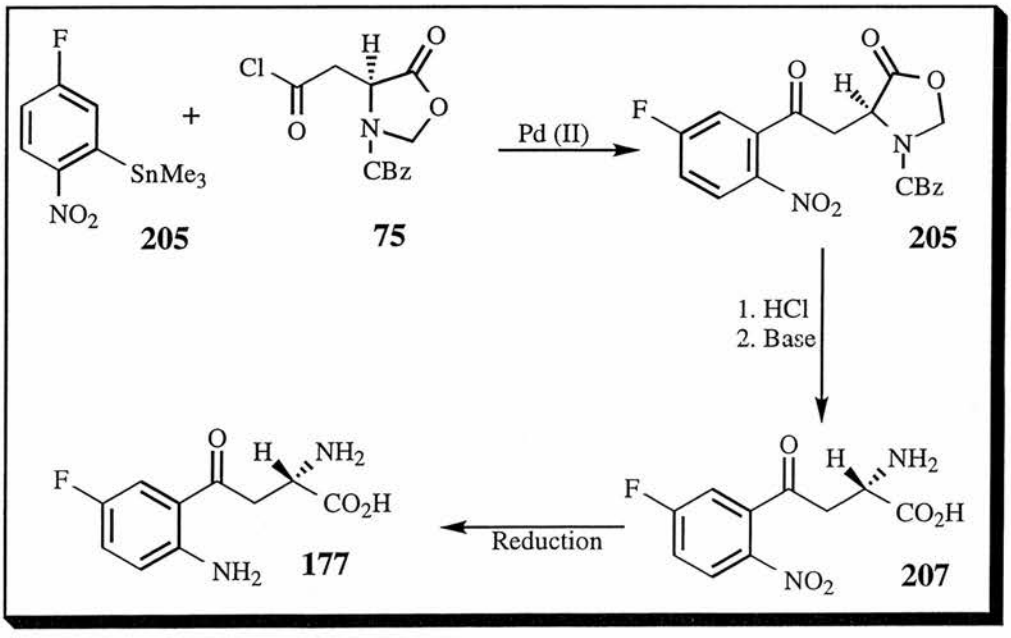
4.4 OTHER ROUTES TO 5-FLUOROKYNURENINE

As the original strategy for the synthesis of 5-fluorokynurenine from 5-fluorotryptophan proved unsuccessful, alternative routes to 5-fluorokynurenine were sought. Pellicciari *et al.*[61,190] described the synthesis of kynurenine analogues from a stannylated aromatic derivative and protected aspartyl chloride derivative (**75**) using a palladium-catalysed Stille cross-coupling reaction to give a protected kynurenine analogue.[190] Subsequent deprotection yielded the free base as a single enantiomer (provided a single enantiomer of aspartic acid was used) as shown in Scheme 4.11.



Scheme 4.11: Synthesis of a Kynurenine Analogue via a Palladium-Catalysed Cross-Coupling Reaction

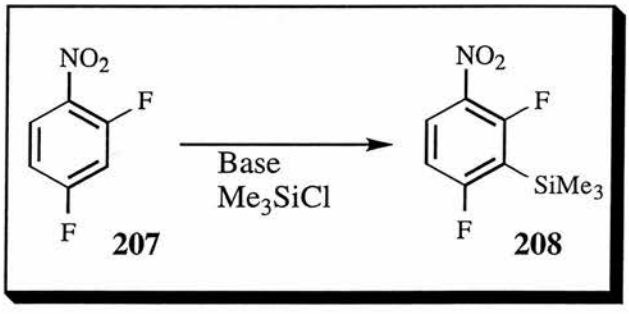
This forms the basis of the second strategy. Relative directing effects of the fluoro- and nitro- substituents should result in addition of the stannyl group at position 2 as shown in Scheme 4.12.



Scheme 4.12: Synthesis of 5-Fluorokynurenine from 4-Fluoronitrobenzene

A recent paper by Black *et al.*[191] reported the effect of base on metallation and gave substitution patterns for fluoronitrobenzenes treated with trimethyltin chloride and trimethylsilyl chloride in the presence of hindered bases. This provides a second method for the synthesis of tin substituted nitrobenzenes. The nitro group should enhance the acidity of the nearby aromatic protons significantly and allow the use of milder bases which would prevent the decomposition normally observed with alkyllithium bases. The intermediate (nitroaryl)lithium species is also unstable and this requires an electrophile which can be used *in situ* to minimise the lifetime of the intermediate.[192,193]

Fluorine has been shown to be a strong *ortho*-directing group[194] but in the presence of alkyllithium bases Black showed that many products were formed.[191] The conversion of 2,4-difluoronitrobenzene (**208**) to 2,4-difluoro-3-trimethylsilylnitrobenzene (**209**) was achieved in 91% and 100% yields respectively by using KHMDS and NaHMDS as base at -78 °C (Scheme 4.13).

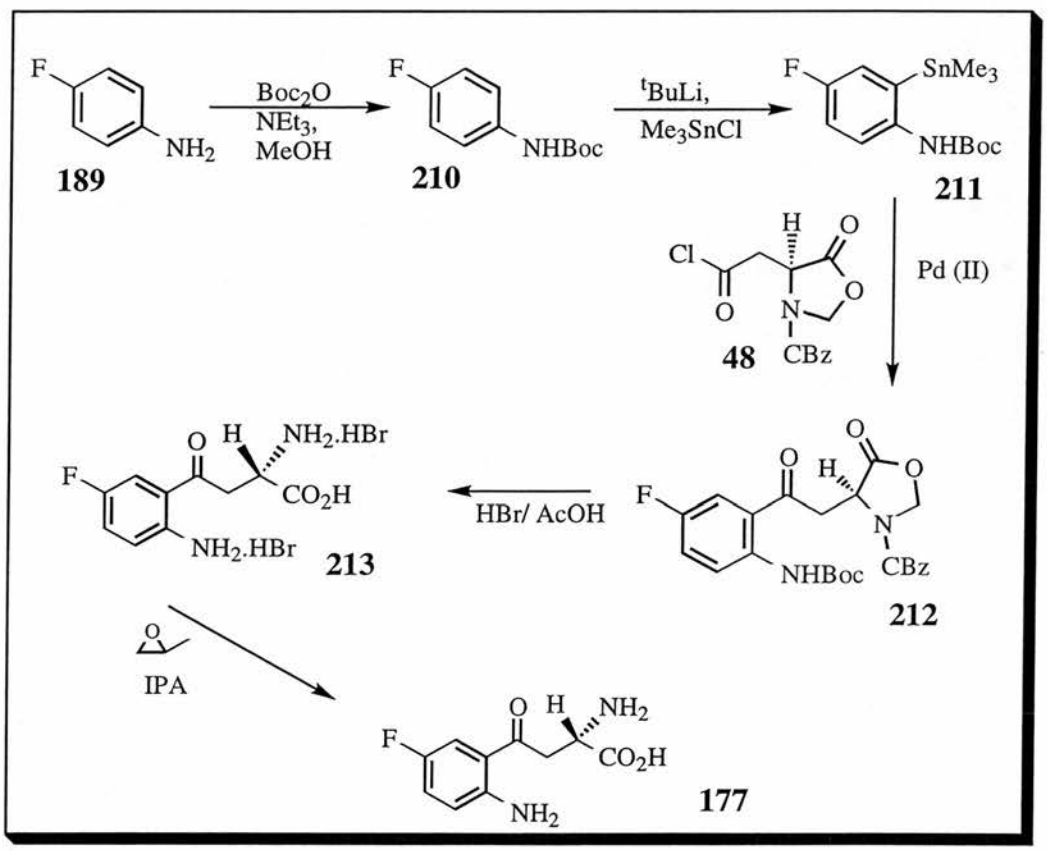


Scheme 4.13: Silylation of Fluoronitrobenzenes

Monofluorinated substrates such as 3-fluoronitrobenzene only gave the desired silylation product 3-fluoro-2-trimethylsilylnitrobenzene in 10% yield with NaHMDS.[191] 4-Fluoronitrobenzene was unreactive under these conditions.[191] It was postulated that trimethyltin chloride could be used under similar conditions to provide entry to palladium catalysed coupling reactions. The base lithium (^{t}butyldimethylsilyl)-^{t}butylamide (LiBSBA) was shown to give the best results for the difluoronitrobenzene described above, but no details were given for 4-fluoronitrobenzene or any other derivatives.

Snieckus also reported that fluorine substituted benzenes gave *ortho*-substituted products after treatment with an alkyllithium base and an electrophile.[195]

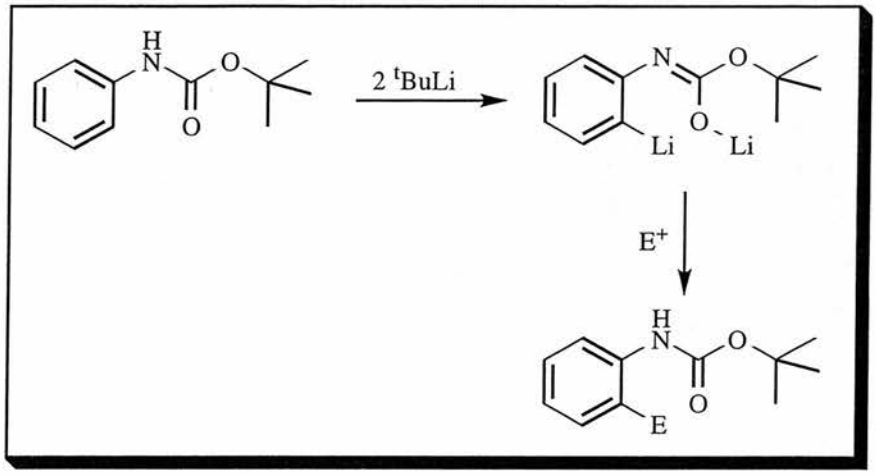
A third strategy is the use of aniline derivatives (**189**)[196] to form 5-fluorokynurenine. This involves the *N*-butoxycarbonyl (Boc) protection[197] of 4-fluoroaniline, subsequent stannylation and coupling with the protected aspartic acid residue as shown previously (Scheme 4.14).[196]



Scheme 4.14: Synthesis of 5-Fluorokynurenine from 4-Fluoroaniline

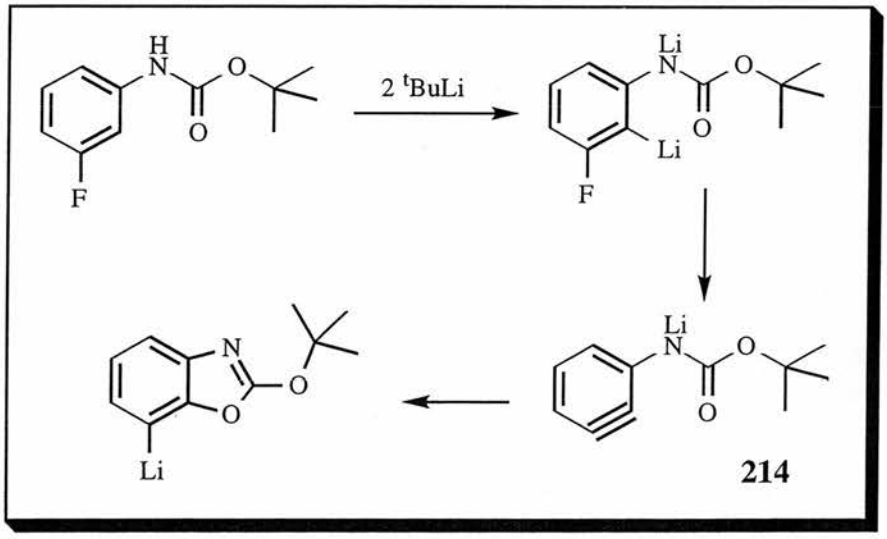
As this synthesis has already proved to give the correctly substituted stannane derivative for a direct analogue of kynurenine it has been suggested that this synthesis should take precedence over a synthesis from nitrobenzene derivatives. It is also attractive as no hydrogenation step is required to convert the nitro group to the ring amine of kynurenine.

Work by Muchowski showed that the Boc protection of the amino function of aniline directed lithiation *ortho* to the heteroatom and that subsequent treatment with an electrophile exclusively gave *ortho*-substitution, (Scheme 4.15).[198]



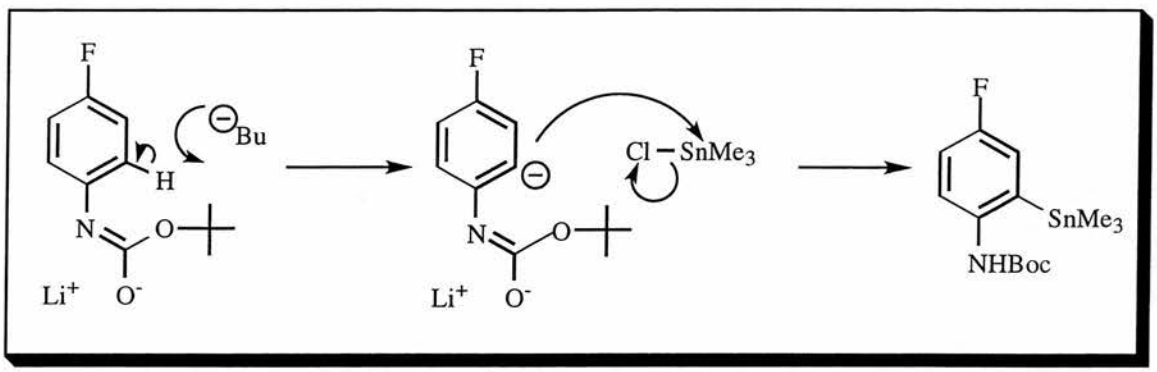
Scheme 4.15: Directed Lithiation by Boc-protection

Lithiation of Boc protected 3-fluoroaniline, however, gave rise to elimination of fluoride to give a benzyne (**214**), which then reacted with the carbonyl of the protecting group to give a rearranged product as shown in Scheme 4.16.[199,200]



Scheme 4.16: Benzyne Formation in Lithiation of Fluoroaniline

Our strategy involved the use of 4-fluoroaniline. It was assumed that the Boc protection would be sufficient to direct lithiation to the 2-position (Scheme 4.17). This species should not be capable of benzyne formation.

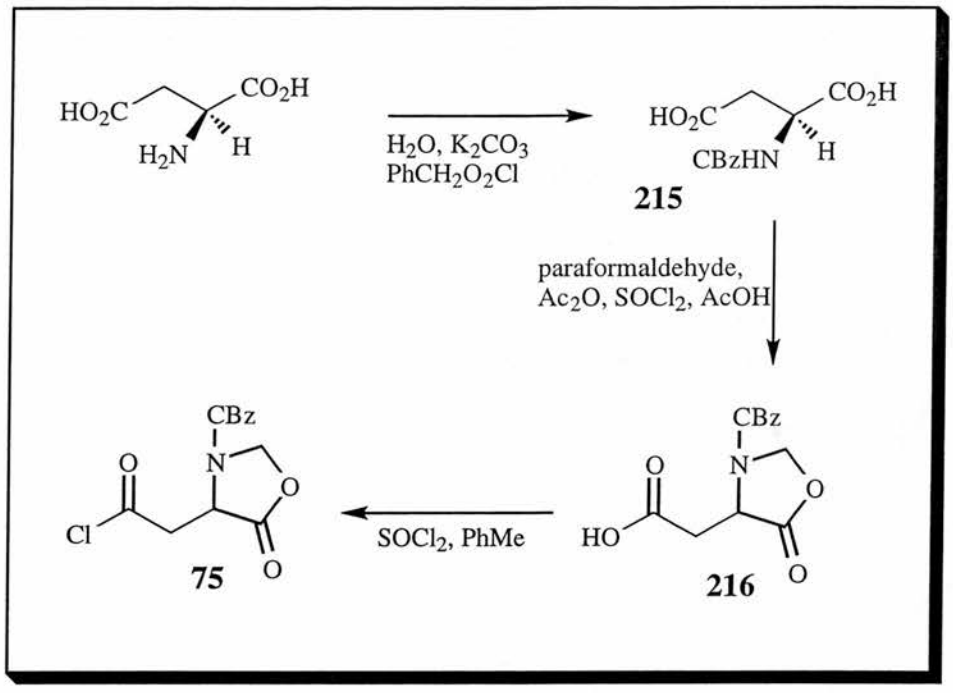


Scheme 4.17: Synthesis of 4-Fluoro-2-trimethylstannylaniline

The stannane would than be used in a Stille palladium-catalysed cross-coupling reaction as described previously for the nitrobenzene and pyridine nuclei.[61,190]

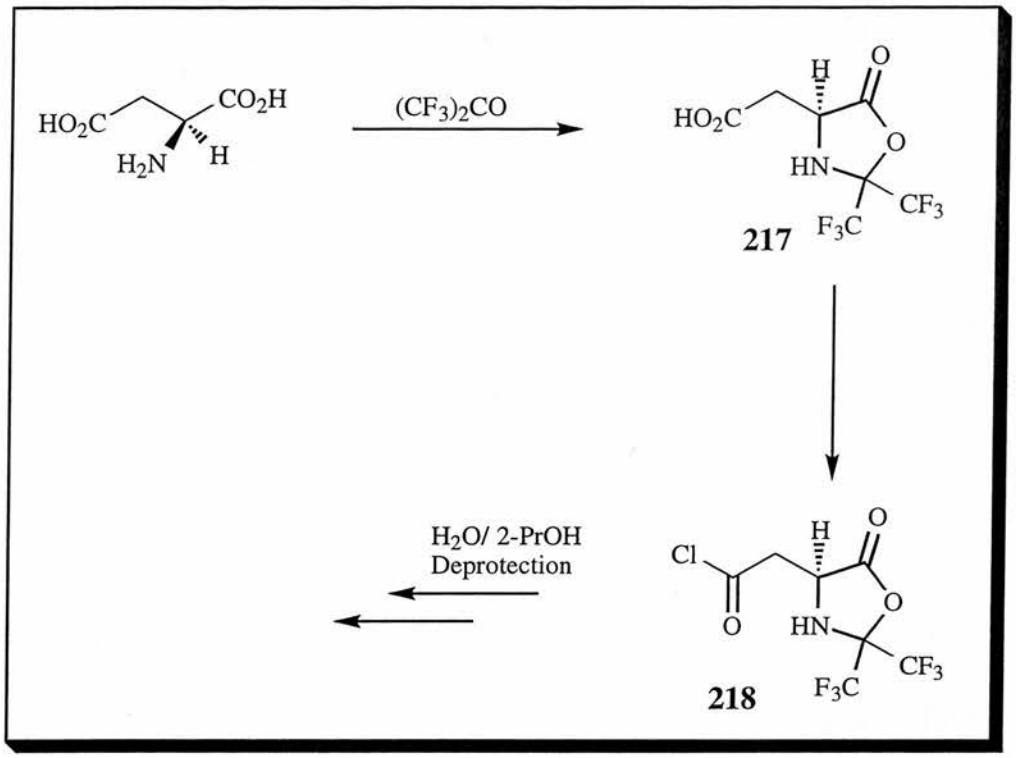
The proposed route for the synthesis of the protected aspartic acid is given in Scheme 4.18, and is one of two literature methods.[201,202]

Protection of the nitrogen with a CBz group (**215**) followed by protection of both the acid and the amine by condensation with paraformaldehyde affords a simple route to the fully protected aspartic acid (**216**). The acid must be activated to the acid chloride (**75**) before the coupling reaction with the stannane derivatives can take place. This type of acid/amine protection is easily removed in a one step reaction with hydrogen bromide in acetic acid and then propylene oxide and 2-propanol as shown in the coupling reaction in Scheme 4.14. The acid chloride is relatively unstable compared to the acid and therefore must be made as required before use. The stannane derivative to which it is coupled should be more stable.



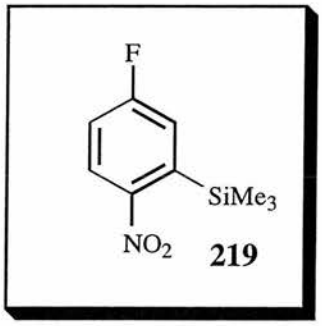
Scheme 4.18: Synthesis of Protected Aspartic Acid

The second literature method for synthesis of the protected aspartic acid involves hexafluoroacetone (Scheme 4.19).[202] This in fact eliminates the need to protect the amine function with a carbobenzyloxy group prior to condensation. Unfortunately this procedure requires gaseous hexafluoroacetone which is very difficult to handle. The acid chloride (**218**) would be made in the same way as for the *N*-CBz protected acid but deprotection would not be as simple.



Scheme 4.19: Hexafluoroacetone Protection of Aspartic Acid

4.4.1 SYNTHESIS OF SILYLATED 4-FLUORONITROBENZENE

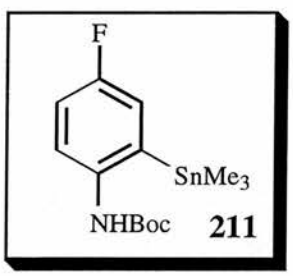


4-Fluoronitrobenzene was silylated with trimethylsilyl chloride using potassium bis(trimethylsilyl)amide (KHMDS) to remove the ring proton. A yellow liquid was formed in what seemed to be over 100% yield. Studies by nmr spectroscopy proved inconclusive. The proton nmr spectrum showed a 2:1 ratio for the aromatic protons, whereas in the starting material this relationship is 1:1. Expected values for each proton and carbon were calculated, but these were completely different to those observed. It is thought that the protons at low field in the spectrum are those closest to the nitro function.

Thin layer chromatography however, showed two spots which were difficult to separate by flash column chromatography. Increasing the ratio of trimethylsilyl chloride to starting material may improve yields, although separation would still be a problem.

As it was impossible to determine on to which carbon the trimethylsilane group had been substituted, and because the spectra were difficult to assign, it was decided that the use of the aniline derivative strategy was more desirable.

4.4.2 SYNTHESIS OF STANNYLATED 4-FLUOROANILINE



211

4-Fluoroaniline was *N*-butoxycarbonyl protected in the first step of this reaction in 90% yield as a yellow-white solid (see Scheme 4.14). This was done under standard conditions (triethylamine in methanol with di-*tert*-butyl dicarbonate). The aromatic protons appear as two signals at $\delta = 6.9$ and 7.3 ppm and these are coupled to the fluorine. A large singlet was also observed at $\delta = 1.50$ ppm due to the ^{t}butyl group.

Stannylation was facilitated by reaction of the Boc protected 4-fluoroaniline (**210**) with 2 equivalents of ^{t}butyl lithium and then trimethyltin chloride. A brown/orange oil (**211**) was isolated in 52% crude yield. The ^{1}H nmr spectrum showed the addition of the stannyl group but the ratio to that of the Boc group (also 9 protons) implied that the reaction had not gone to completion. However, the ^{19}F-nmr spectrum showed little fluorine to be present. It would seem that both starting material and product are present which would account for the various splitting patterns and broadened peaks. Thin layer chromatography confirmed the presence of two spots, the upper of which is the starting

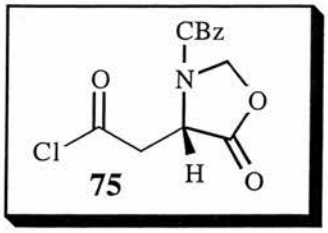
material. It was proposed that this could easily be purified by column chromatography and the reaction conditions optimised by using more than one equivalent of trimethyltin chloride.

Further reactions were carried out on the Boc protected 4-fluoroaniline (**210**) using different reaction times and equivalents of tin to starting material. The most effective of these employed 2 molar equivalents of trimethyltin chloride reacted for 5 hours at -20°C, and showed a significant increase in the ratio of product to starting material. Two sets of peaks occurred at $\delta = 6.5$ ppm for the NH proton. The lower of the two sets is not present in the starting material, the ratio of being approximately 2:1 (product:starting material). The remainder of the proton nmr spectrum is difficult to assign as the signals for the ring protons overlap. Mass spectroscopy of the crude product revealed a very small peak (4%) at 375 representing $[M+H]^+$ for the desired product. However, this does not elaborate on the position of substitution, or quantity of product. The next most significant peak is at 304 (89, $[M-{}^t\text{BuOH}]^+$ which may be significant.

Another strategy involved the addition of the trimethyltin chloride prior to the base. This however, only yielded starting material. It was hoped that this would trap the *N*-Boc fluoroaniline dianion in situ and that this would aid product formation.

It was later concluded that *N*-Boc-fluoroaniline underwent lithium–halogen exchange in the presence of the base (butyl lithium) and that the trimethyltin complex did add to the ring *para* to the amine at the carbon previously bonded to the fluorine. This would explain why little or no fluorine was seen in the ^{19}F-nmr spectrum, why two spots were visible by tlc and it would also account for the various changes seen in the proton spectrum.

4.4.3 SYNTHESIS OF PROTECTED 2*S*-ASPARTIC ACID CHLORIDE



75

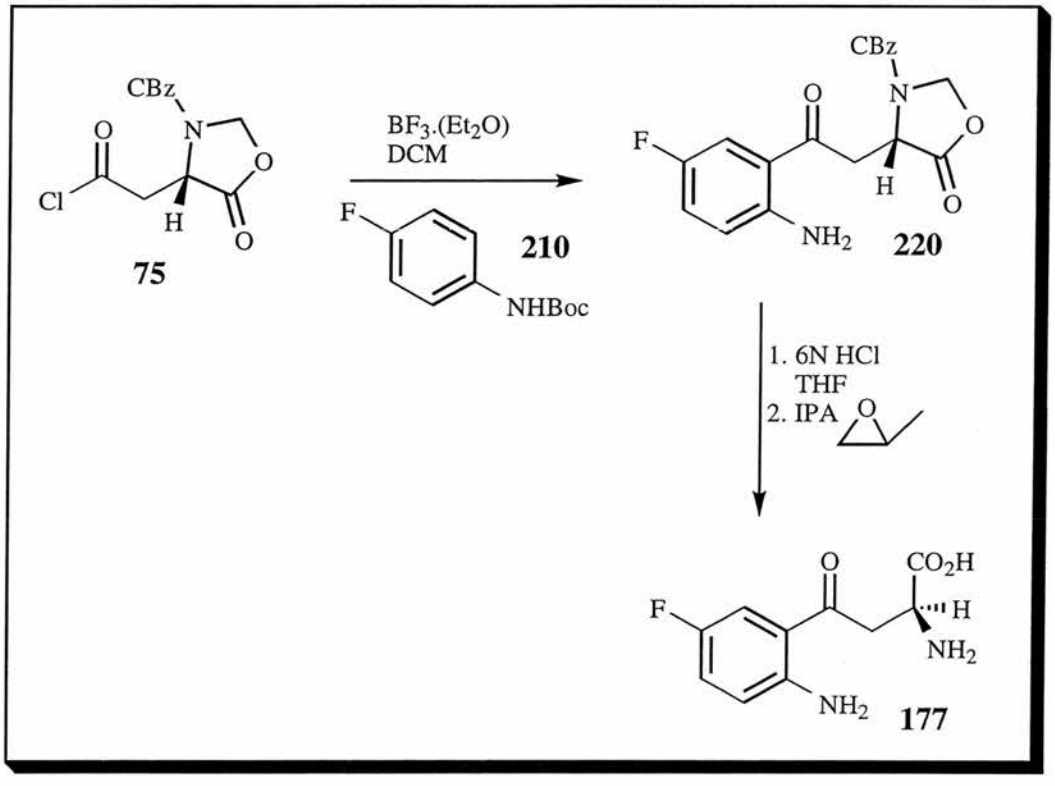
The carbobenzyloxy protection of the amine of 2*S*-aspartic acid was achieved in 74% yield (see Scheme 4.18). This reaction was carried out in water with base and the CBz protected product (**215**) formed after reaction overnight. Analysis by nmr spectroscopy confirmed the structure and presence of the *N*-CBz protecting group.

In the second step of this reaction the amine undergoes a condensation reaction with paraformaldehyde in acetic anhydride, thionyl chloride and acetic acid. The intermediate then rapidly cyclises on to the α-acid group with the loss of water. The product (**216**) was purified by flash column chromatography. The nmr spectrum showed the β-methylene to have moved from $\delta = 2.66$ ppm in the protected acid (**215**) to $\delta = 2.97$ ppm in the cyclised product (**216**). The syrup was used without further purification to prepare the activated acid chloride (**75**) prior to coupling.

The activation of the amino acid to the acid chloride (**75**) was facilitated by heating under reflux in toluene and oxalyl chloride for 2 hours. The solvent was then removed and the residue washed several times with toluene to remove traces of oxalyl chloride before coupling. The spectral data confirmed the structure of the product which was used without further purification.

4.4.4 SYNTHESIS OF 5-FLUOROKYNURENINE VIA A FRIEDEL–CRAFTS ACYLATION

As the lithiation strategy was unsuccessful an alternative was sought. Perhaps an even simpler route to 5-fluorokynurenine (**177**) involves the Friedel–Crafts acylation. This had previously been overlooked as it was assumed that the reaction between fluoroaniline and similar acid chlorides had been previously attempted and failed. This however was found not to be the case. The scheme below outlines the final strategy for the synthesis of 5-fluorokynurenine (Scheme 4.20).



Scheme 4.20: 5-Fluorokynurenine from a Friedel–Crafts Acylation

The acid chloride (**75**) from the protected aspartic acid described above, was dissolved in dichloromethane and boron trifluoride diethyl etherate was added. After stirring at room temperature for 20 minutes, *N*-Boc 4-fluoroaniline (**210**) (as prepared for the stannylation and silylation reactions) was added and the reaction mixture stirred overnight. Acidic

work-up and extraction gave the product as a brown oil which, upon purification by column chromatography, gave an orange oil in 63% yield. ^{1}H-Nmr spectroscopy showed that the Boc-protection on the fluoroaniline had been lost under the mildly acidic conditions of the reaction. The methylene group of the CBz protection was seen at δ = 5.25 ppm, and it seemed most likely that the aspartic acid had been added *ortho* to the amine. Accurate mass measurement showed that the product (**220**) had a mass of 373.120473, MH^+, which is consistent with the expected value ($C_{18}H_{18}N_2O_5F$ requires 373.119975)

Deprotection was afforded not by using hydrogen bromide in acetic acid as suggested in Scheme 4.14, but by heating under reflux in THF and 6M hydrochloric acid. The product was then stirred overnight in isopropyl alcohol and propylene oxide to give the free base.[203] The resulting orange oil was purified first by chromatography on reverse phase silica and then by reverse phase HPLC (water:acetonitrile; 90:10; 1.8 ml min^{-1}) to give the product (**177**) in 33% yield. Nmr spectroscopy showed the β-methylene protons at δ = 3.08 with signals for the α-proton and the three aromatic methines falling at δ = 4.5 and 7.3 ppm respectively. The ^{19}F-nmr signal for the fluorine atom was observed at δ = -115.7 ppm.

In order to show that no racemisation had taken place at the amino acid centre, it was necessary to form an MTPA-amide and analyse this by fluorine nmr spectroscopy. As Mosher's chloride itself contains fluorine it was expected that two signals would be seen in the amide. The ethyl ester of 5-fluorotryptophan was prepared by heating in ethanol and thionyl chloride at reflux. The Mosher's amide was prepared by reacting *R*-(+)Mosher's acid in thionyl chloride to form the *S*-(+)Mosher's chloride. The acid chloride was then dissolved in dry pyridine and carbon tetrachloride. The mixture was then added to a sample of 5-fluorokynurenine ethyl ester and left to stand for 20 minutes, quenched by adding an excess of 3-dimethylamino-1-propylamine and extracted into diethyl ether. After an acid and base wash, the organic phases were dried and the solvent

removed to give a yellow oil. The signal for the CF_3 group in Mosher's acid usually comes at approximately -71.1 ppm in the fluorine nmr spectrum. The peak at -78.1 ppm represents the CF_3 of the Mosher's amide and the signal for the fluorine of 5-fluorokynurenine (**177**) is observed at -113.6 ppm. There was an impurity seen at -127 ppm but there is a small peak in the normal spectrum of 5-fluorokynurenine at -125 ppm. From this it can be concluded that no racemisation had taken place over the course of this five step synthesis (Figure 4.1).

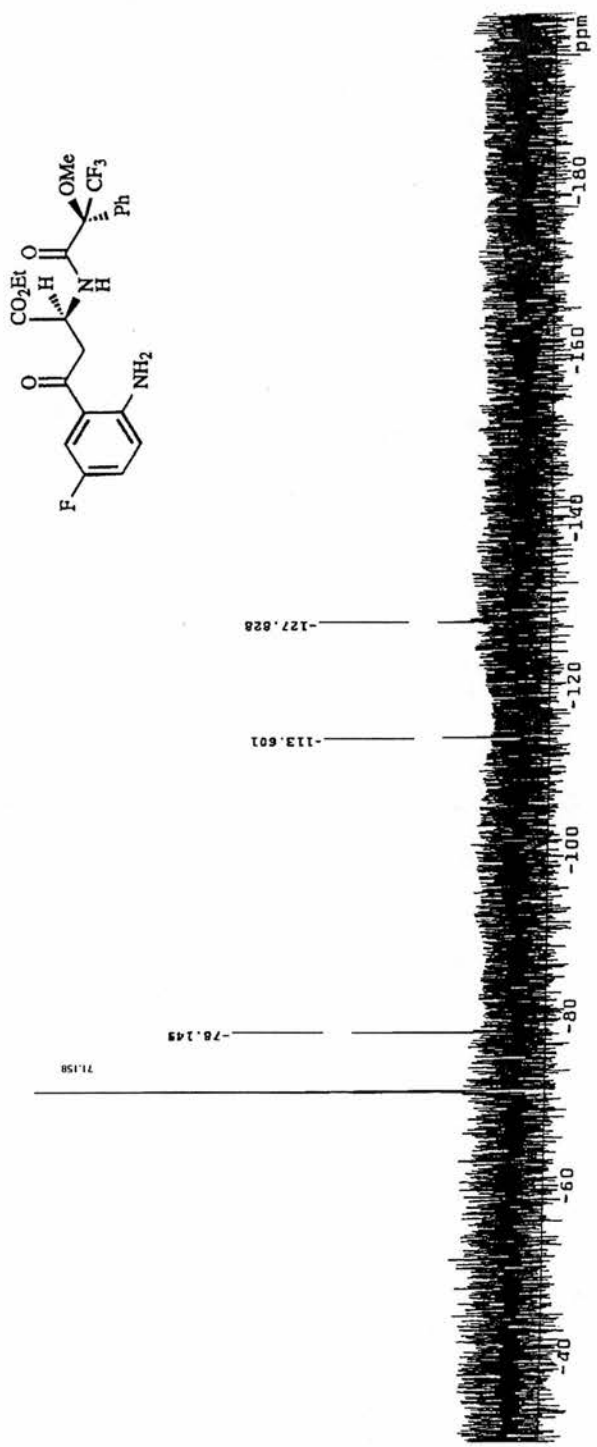
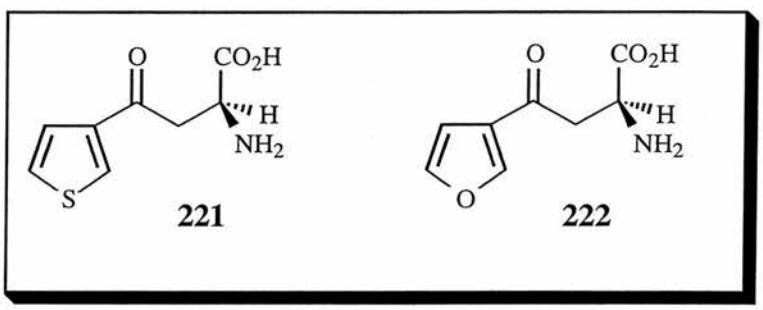


Figure 4.1: ^{19}F-Nmr Spectrum of the Mosher's Amide of 5-(2*S*)-Fluorokynurenine

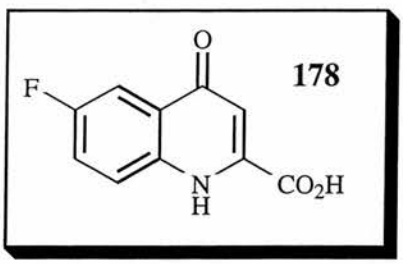
4.4.5 ATTEMPTED SYNTHESIS OF THIOPHENE AND FURAN DERIVATIVES

The Friedel–Crafts acylation reaction was also employed in the attempted synthesis of a thiophene (**221**) and a furan derivative (**222**). In the reaction with thiophene, tin tetrachloride was used as the Lewis acid and the reaction was carried out in toluene. Analysis of the compound after acidic work-up showed no conversion to the desired product. Similarly furan was reacted in boron trifluoride diethyl etherate and toluene with the acid chloride (**75**) and no product was seen by nmr spectroscopy.



It is unclear why these reactions did not give the desired products as thiophene has been shown to acylate readily under similar conditions. Pearson and Buehler report that the acylation of both furan and thiophene can be achieved with little or no catalyst.[204] The Lewis acid of choice was zinc chloride[205] in the presence of catalytic iodine in amounts as low as 4 $x10^{-4}$ mol and 0.08 mol per mole of acylating reagent for furan and thiophene respectively.[204] These figures however, are for reactions with anhydrides and acylations with acid chlorides required greater amounts of both Lewis acid and catalyst per mole of acylating agent. The use of a different Lewis acid may give the desired products but the yields reported by Pearson were very low and required the reactions to be carried out at high temperatures.

4.5 SYNTHESIS OF 6-FLUOROKYNURENIC ACID



The three step synthesis proposed in Scheme 4.6 was very successful. In the first reaction diethyl acetylenedicarboxylate (**190**) was coupled to 4-fluoroaniline (**189**) in methanol under reflux to give an orange oil in 91% yield. The structure of diethyl 2-[(4-fluorophenyl)amino]-2-butenedioate (**191**) was confirmed by ^{1}H-nmr spectrometry, showing a singlet at δ = 5.39 ppm for the alkene proton. However, as the compound was difficult to purify further (it would not crystallise) it was carried straight through into the second step.

The butenedioate was refluxed in diphenyl ether and a tan powder was obtained, which was recrystallised from ethyl acetate. Nmr spectroscopy confirmed the structure. The spectra were significantly different for the butenedioate and the quinoline carboxylate (**192**). Whereas in the butenedioate there were two methyl signals at δ = 1.10 and 1.29 ppm, and two methylene signals at δ = 4.16 and 4.24 ppm there was only one set of signals in the cyclised kynurenic acid analogue (δ = 1.44 and 4.48 ppm). Also, the singlet for the alkene proton shifts from δ = 5.39 ppm to 6.97 ppm in the cyclised product. Mass spectroscopy confirmed the structure as that of the quinoline carboxylate (**192**). Microanalysis showed the product to be pure.

In the final step the ethyl ester was hydrolysed in base to yield the acid (**178**). Reaction with sodium hydroxide forms the sodium salt which is then reacted with hydrochloric acid to yield 6-fluorokynurenic acid in 84% crude yield. The addition of acid was carried out

slowly as the product precipitated out readily and became more difficult to filter when excess acid was added. It was washed in ethanol to remove any water and acid residues and then with petroleum ether which dried the product further. Purification was carried out by recrystallisation from water. The proton nmr spectrum confirmed the structure and the fluorine nmr spectrum showed a peak significantly lower than that of the protected tryptophan (**185**). It was observed at -117.4 compared to -46.53 ppm for the tryptophan.

4.6 DISCUSSION

4.6.1 ^{19}F-NMR DATA

The following fluorine nmr data were obtained for compounds synthesised and for various starting materials and hydrolysis products. 4-Fluoroaniline was run as a standard and 5-fluoroanthranilic acid was run to give an indication of the frequency for the enzymatic degradation product of 5-fluorokynurenine when reacted with kynureninase.

The small difference (1.7 ppm) between the 5-fluorokynurenine (**177**) and 6-fluorokynurenic acid (**178**) is probably sufficient for monitoring the turnover of the substrate as catalysed by kynurenine aminotransferase. In his studies Harada reported a shift of about 1.6 ppm between 5-fluorotryptophan and 5-fluorokynurenine, but gave no peak frequencies and this difference was sufficient to allow the metabolism by kynureninase to be investigated.[177] A ^{19}F-nmr spectrum of a mixture of the two compounds is shown in Figure 4.2 clearly showing the two distinct signals. The assay would be carried out by injecting 5-fluorokynurenine into rat brain and monitoring its turnover to 6-fluorokynurenic acid by nmr spectroscopy. The 6-fluorokynurenic acid analogue was synthesised so that it can serve as a standard.

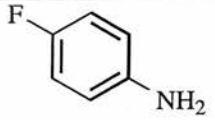
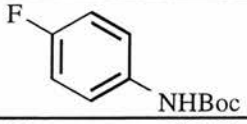
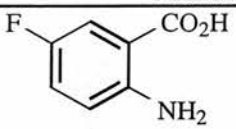
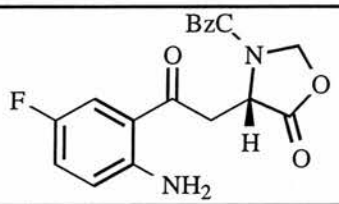
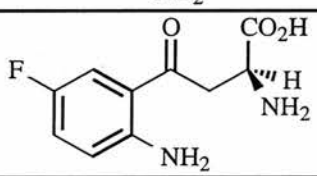
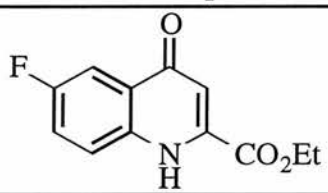
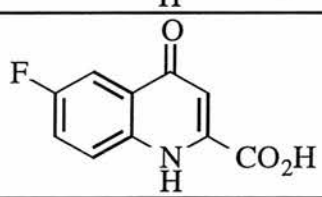
Compound	Structure	^{19}F Peak Freq.
Fluoroaniline **189**		-130.02
210		-121.28
Fluoro anthranilic acid **223**		-132.35†
220		-118.5
177		-115.72†
192		-116.24
178		-117.43§

Table 4.1: Fluorine Peak Frequencies for Standards and Compounds Synthesised

All spectra were run in $CDCl_3$ unless otherwise stated; † in MeOD, § in d^6-DMSO

An experiment was carried out to monitor the kynureninase catalysed hydrolysis of 5-fluorokynurenine by ^{19}F-nmr spectroscopy. Thus 5-fluorokynurenine (**177**, 10 mg) was dissolved in phosphate buffer (10 mM, pH 7.4) containing pyridoxal 5'-phosphate (20 μM) and placed in an nmr tube. A spectrum of 5-fluorokynurenine was run before any enzyme was added, which showed a single peak at -125 ppm due to 5-fluorokynurenine in water. A portion of the bacterial kynureninase (2 μl) was then added and an nmr spectrum taken every ten minutes for two hours. After approximately one hour the peak at -125 ppm began to diminish and a broader peak at -127.5 ppm began to appear. The sample was left overnight after adding another portion of enzyme and a further spectrum run after 24 hours. In this the peak at -125 ppm had completely disappeared while the small broad peak, slightly shifted to -128 ppm, had increased in size. In order to confirm that the peak at -128 ppm was due to 5-fluoroanthranilic acid (**223**) an nmr experiment was run under identical conditions. This showed an authentic sample of 5-fluoroanthranilic acid (**223**) to have a fluorine signal at -127.9 ppm in water. Nmr spectra for this series of reactions are shown in Figure 4.3.

There were also two larger peaks at -119.2 ppm and -121.7 ppm which had not been present in any of the spectra seen previously. It was thought that these might be degradation products. 6-Fluorokynurenic acid (**178**) was ruled out by running a spectrum of a pure sample under the same conditions, the peak in the fluorine nmr spectrum was seen at -116.7 ppm in water. The preparation of bacterial enzyme used in these experiments is not pure and so the other peaks are probably caused by other enzyme catalysed reactions. They may in fact result from further steps in the pathway, i.e. 3-hydroxylation, cleavage and on towards fluoroquinolinic acid.

This experiment confirmed that the 5-fluorokynurenine synthesised was authentic and that it had undergone reaction with kynureninase to give 5-fluoroanthranilic acid (**223**). A similar set of experiments should be able to show the reaction of 5-fluorokynurenine to 6-fluorokynurenic acid as catalysed by kynurenine aminotransferase.

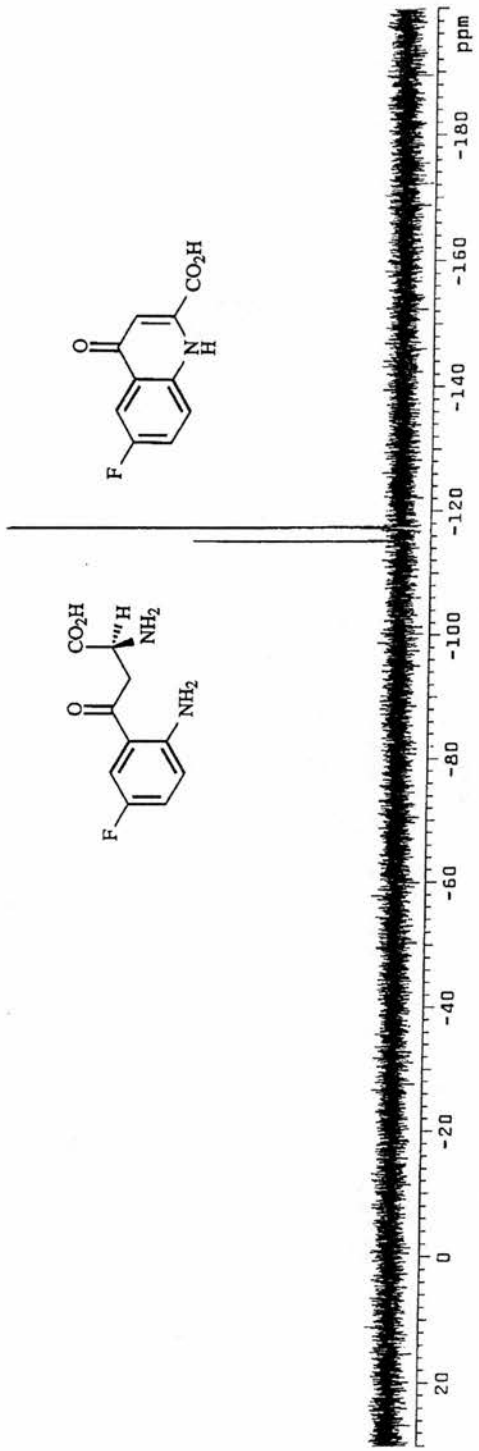


Figure 4.2: ^{19}F-Nmr Spectrum of 5-Fluorokynurenine and 6-Fluorokynurenic Acid

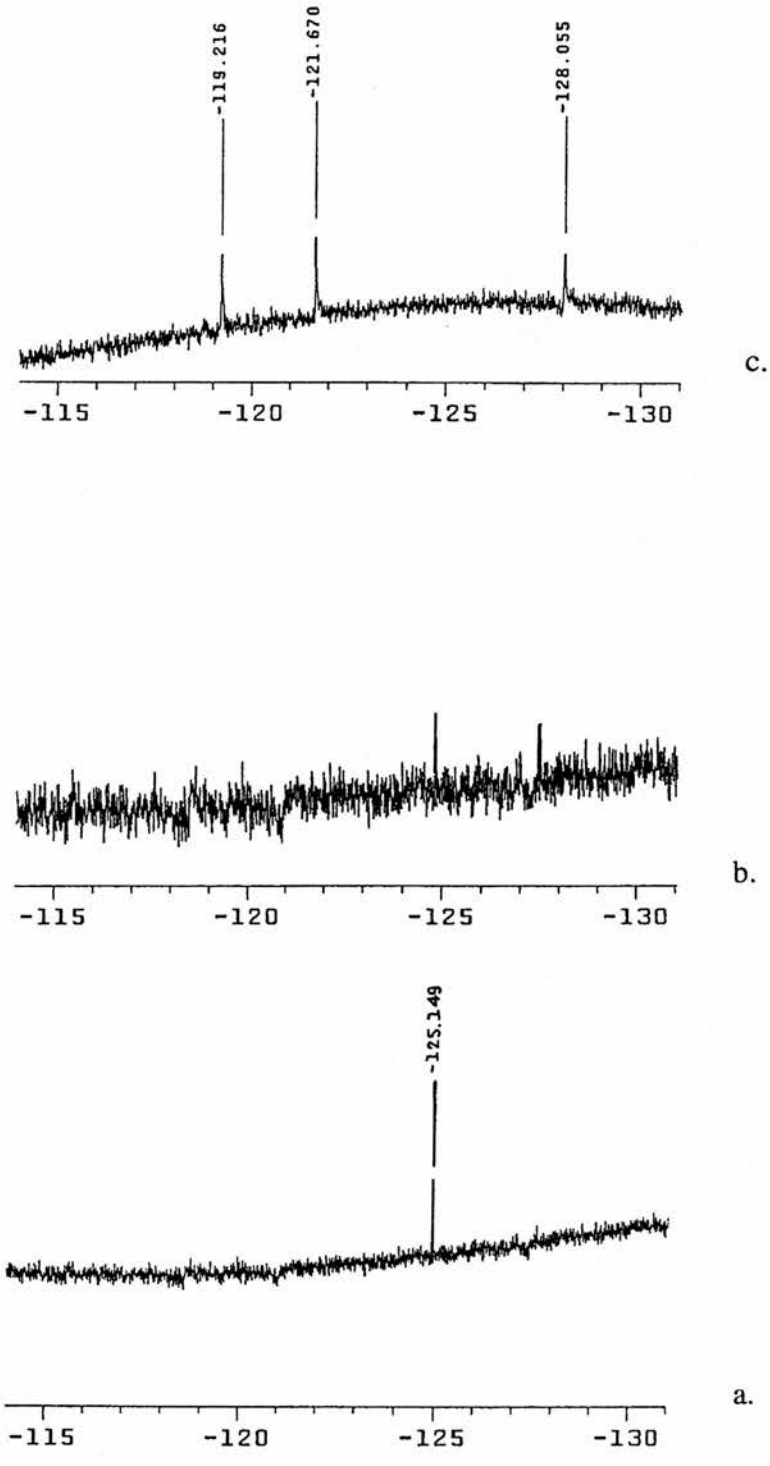


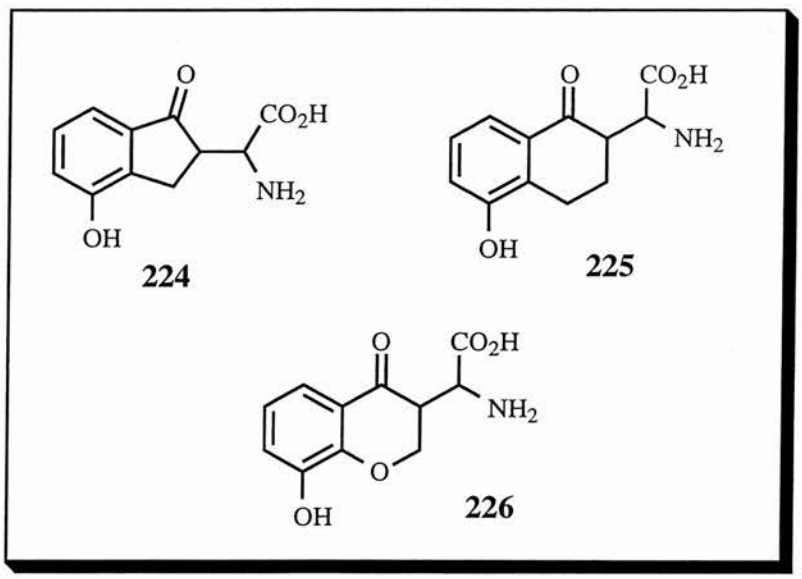
Figure 4.3: ^{19}F-Nmr of the Reaction Between Kynureninase and 5-Fluorokynurenine

a: time = 0 h; b: time = 1 h; c: time =24 h

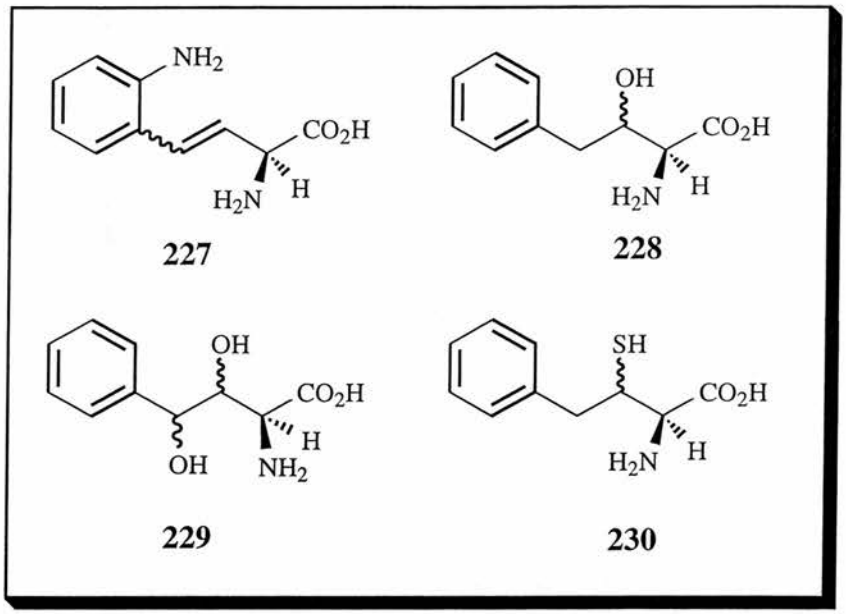
CHAPTER 5

5. FUTURE WORK

The promising preliminary results observed with the competitive inhibitors described in chapter 2, in particular the indanone derivative (**96**), offer much scope for future work. One possibility is the stereospecific synthesis of the 2*S*-isomers. Future work on the substitution of heteroatoms into the ring and other routes to the dihydroquinoline derivative (**99**) also need to be investigated. The introduction of a hydroxyl moiety into the benzene ring (**224–226**) to mimic 3-hydroxykynurenine (**3**) and thus increase selectivity with mammalian forms of kynureninase, is also worth investigation.



Although the results for the irreversible inhibitors were poor, further investigation of their reaction with bacterial and human kynureninase may better determine their efficacy as inhibitors. The overall synthesis needs to be optimised to achieve this. Further investigation of the substitution of other groups into the ring may yield other potential inhibitors such as the aniline (**227**). The epoxidation of the alkenes or alternatively the formation of alcohols, diols or thiols (**228–230**) should also yield potential inhibitors.



The initial nmr study of the reaction of 5-fluorokynurenine with kynureninase showed that there is a good possibility that a similar study of the reaction between 5-fluorokynurenine and kynurenine aminotransferase would be possible.

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 micro analytical laboratory.

Optical rotations were measured at room temperature using an Optical Activity Ltd. AA 1000 polarimeter. 10 cm path-length cells were used. The measurements are given in 10^{-1} deg cm^2 g^{-1}.

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

Nmr spectra were recorded on a Varian Gemini f.t. spectrometer (^1H, 300 MHz; ^{19}F, 298 MHz; ^{13}C, 74.76 MHz) and a Varian Gemini f.t. spectrometer (^1H, 200 MHz; ^{13}C, 50.31 MHz). ^1H and ^{13}C Nmr spectra were referenced on chloroform, TMS, methanol or DMSO. ^{19}F Nmr were referenced on fluorotrichloromethane. Nmr spectra are described in parts per million downfield shift from TMS and are reported consecutively as position (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 br - broad), coupling constants ($J_{X,Y}$ in Hertz, if applicable) and assignment.

Mass spectra and accurate mass measurements were recorded on a Kratos MS50 within the department. Major fragments are given as percentages of the base peak intensity.

Flash chromatography was performed according to the procedure of Still[206] using Sorbisil C60 (40-60mm) silica gel, Kieselgel 60 and BakerBond C18 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, phosphomolybdic acid in ethanol or ninhydrin.

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

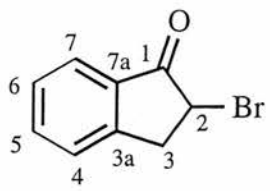
Sonication was carried out using a Heat Systems-Ultrasonics Inc. Europa W-220F sonicator.

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[207]

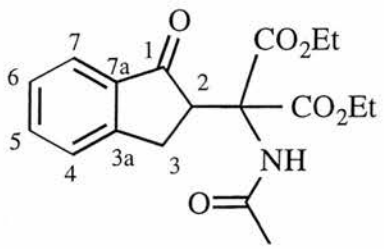
6.2 COMPETITIVE INHIBITORS

2-Bromo-indan-1-one (100)



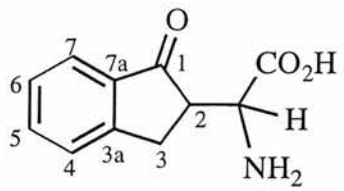
Cupric bromide (6.788 g, 30.4 mmol, 2 eq) was heated at reflux in ethyl acetate (10 ml) with stirring. To this was added indanone (2.015 g, 15.24 mmol) in chloroform (10 ml). The reaction was heated at reflux for a further 5 hours and then cooled. Copper bromide and cupric bromide residues were filtered off, the filtrate decolourised with activated charcoal, filtered through a bed of Celite and washed with ethyl acetate (4 x 50 ml) and the solvent removed under reduced pressure to give an orange oil which was dried further under vacuum (3.08 g, 96%); ν_{max} (nujol)/cm^{-1} 1710 (C=O); δ_H (300 MHz, $CDCl_3$) 3.43 (1H, d, $J_{3',3''}$ 18, H_A of 3-C$\underline{H}_2$), 3.85 (1H, dd, $J_{3',3''}$ 18, $J_{2,3''}$ 7.7, H_B of 3-C$\underline{H}_2$), 4.48 (1H, d, $J_{2,3''}$ 7.7, C$\underline{H}$Br), 7.46 (2H, m, 4-C$\underline{H}$ & 6-C$\underline{H}$), 7.67 (1H, t, $J_{4,5} = J_{5,6}$ 7.5, 5-C$\underline{H}$), 7.85 (1H, d, $J_{6,7}$ 7.5, 7-C$\underline{H}$); δ_C (74.76 MHz, $CDCl_3$) 37.9 (3-$\underline{C}H_2$), 44.1 ($\underline{C}$HBr), 125.0 (7-$\underline{C}$), 126.3 (4-$\underline{C}$), 128.1 (5-$\underline{C}$), 133.4 (7a-$\underline{C}$), 135.8 (6-$\underline{C}$), 150.9 (3a-$\underline{C}$), 199.4 ($\underline{C}$=O); *m/z* (CI) 213 & 211 ([M^+], 100% & 99%), 133 (36, (M-C_6H_5]$^+$), 131 (34, [M-Br]$^+$), 103 (4, [M-HBr-CO]$^+$).

Diethyl 2-(acetylamino)-2-[1-oxo-2,3-(1*H*)-dihydroinden-2-yl]-malonate (101)



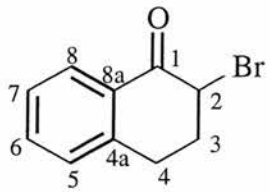
Sodium hydride (1.49 g, 37.4 mmol, 2.5 eq) 60% in mineral oil was suspended in dry DMF (4 ml) and a solution of diethyl acetamidomalonate (4.88 g, 22.5 mmol, 1.5 eq) in dry DMF (14 ml) added. The solution was stirred at 0 °C under a nitrogen atmosphere for 3 hours until the anion had formed. A solution of 2-bromo-1-indanone (**100**, 3.162 g, 14.98 mmol) in dry DMF (10 ml) was added and the solution warmed to room temperature and stirred overnight under nitrogen. The mixture was poured into distilled water (100 ml), acidified to pH 3 with 1M hydrochloric acid in an ice-bath and extracted into diethyl ether (4 x 70 ml). The organic phases were washed with brine (2 x 50 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to a brown oil. Purification by column chromatography (silica; diethyl ether) gave an orange oil (2.41 g, 44%); (Acc. Mass Found 348.144000, Calc. for $C_{18}H_{22}NO_6$, 348.144713); ν_{max} (nujol)/cm^{-1} 3360 (NH), 1750 (C=O), 1720 (CO ester), 1685 (CO amide); δ_H (200 MHz, $CDCl_3$) 1.32 (6H, t, *J* 7.1, $C\underline{H}_3CH_2CO_2$), 2.15 (3H, s, $C\underline{H}_3CO$), 3.51 - 3.83 (2H, m, 3-$C\underline{H}_2$), 3.9 - 4.12 (1H, m, 2-C<u>H</u>), 4.23 (4H, q, *J* 7.1, $CH_3C\underline{H}_2CO_2$), 7.37 (2H, m, 4-C<u>H</u> & 6-C<u>H</u>), 7.51 (1H, t, $J_{5,6} = J_{6,7}$ 7.5, 5-C<u>H</u>), 7.79 (1H, d, $J_{6,7}$ 7.5, 7-C<u>H</u>); δ_C (50.31 MHz, $CDCl_3$) 14.5 ($\underline{C}H_3CH_2CO_2$), 23.1 ($\underline{C}H_3CO$), 36.7 (3-$\underline{C}H_2$), 39.9 (2-<u>C</u>H), 56.8 ($CH_3\underline{C}H_2CO_2$), 123.2 (7-<u>C</u>), 125.1 (4-<u>C</u>), 126.4 (5-<u>C</u>), 131.7 (7a-<u>C</u>), 133.9 (6-<u>C</u>), 148.6 (3a-<u>C</u>), 167.3 ($CH_3CH_2\underline{C}O_2$), 170.3 ($CH_3\underline{C}O$), 190.1 (<u>C</u>=O); *m/z* (CI) 348 ([*M*+H], 100%), 218 (73, [*M*+H-C_9H_6)]$^+$), 174 (22, [M+H-$(EtO_2C)CNH_2$]$^+$), 133 (62, [218-CCO_2Et]$^+$).

Amino-[1-oxo-2,3-(1*H*)-dihydroinden-2-yl]-acetic acid (96)



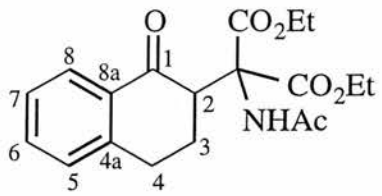
Diethyl 2-(acetylamino)-2-[1-oxo-2,3-(1*H*)-dihydroinden-2-yl]-malonate (**101**, 1.43 g, 4.1 mmol) was dissolved in 1,4-dioxane (50 ml) and 6M hydrochloric acid (70 ml) added. The reaction was heated under reflux for 7 hours until no starting material was visible by tlc (silica; pet. ether: ethyl acetate; 1:1). The solution was cooled and washed with ethyl acetate (50 ml). The aqueous phase was then concentrated under reduced pressure to a yellow green liquid which was triturated with acetone to a white solid. The solid was taken up in isopropyl alcohol (25 ml) and propylene oxide (10 ml) added, the solution was stirred overnight and the solvent removed under reduced pressure to give an off-white solid as a mixture of diastereomers in the ratio 1.43:1 [major:minor] (0.187 g, 22%); mp 152-158 °C (decomp.); (Acc. Mass Found 206.082462 Calc. for $C_{11}H_{12}NO_3$, 206.081718); ν_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (CO acid); δ_H (300 MHz, D_2O) 2.89 - 3.0 (1H, m, 3-C<u>H</u>$_2$), 3.12 - 3.26 (1H, m, 3-C<u>H</u>$_2$) 3.37 - 3.48 (1H, m, 2-C<u>H</u>), 4.30 (minor, 1H, d, *J* 3, α-C<u>H</u>), 4.38 (major, 1H, d, *J* 3, α-C<u>H</u>), 7.46 (1H, t, $J_{5,6}$=$J_{6,7}$ 7.5, 6-C<u>H</u>), 7.67 (1H, t, $J_{4,5}$=$J_{5,6}$ 7.5, 5-C<u>H</u>), 7.68 - 7.78 (2H, m, 4- & 7-C<u>H</u>); δ_C (73.76 MHz, D_2O) 26.3 & 27.4 (3-<u>C</u>H$_2$), 41.2 & 45.6 (2-<u>C</u>H), 51.7 & 53.8 (α-<u>C</u>H), 122.0 (7-<u>C</u>), 124.8 (4-<u>C</u>), 125.9 (5-<u>C</u>), 133.4 (7a-<u>C</u>), 134.1 (6-<u>C</u>), 152.4 (3a-<u>C</u>), 169.5 (<u>C</u>O$_2$H), 205.5 (<u>C</u>=O); *m/z* (CI) 206 ([*M*+H], 15%), 191 (76, [*M*+H-NH]$^+$), 151 (45, [*M*+H-$NH_2CHCO]^+$), 76 (99, [*M*+H-Ar]$^+$), 43 (100, CO_2).

2-Bromo-3,4-dihydro-1-(2*H*)-naphthalenone (102)



Cupric bromide (6.126 g, 27.4 mmol, 2 eq) was heated at reflux in ethyl acetate (10 ml) with stirring. To this was added 3,4-dihydro-1-(2*H*)-naphthalenone (2.091 g, 14.3 mmol) in chloroform (10 ml). The reaction was heated at reflux for a further 5 hours and then cooled. Copper bromide and cupric bromide residues were filtered off, the filtrate decolourised with activated charcoal and filtered through a bed of Celite and washed with ethyl acetate (4 x 50 ml). The solvent was removed under reduced pressure to give an orange oil which was dried further under vacuum (2.9 g, 90%); ν_{max} (nujol)/cm^{-1} 1720 (C=O); δ_H (200 MHz, $CDCl_3$) 2.49 (2H, t, $J_{3,4}$ 4, 4-C$\underline{H}_2$), 2.93 (1H, dt, $J_{2,3}$ 9.7, $J_{3,4}$ 4, H_A, 3-C$\underline{H}_2$), 3.26 - 3.41 (1H, m, H_B, 3-C$\underline{H}_2$), 4.74 (1H, s, C$\underline{H}$Br), 7.29 (1H, d, *J* 7.7, 5-C$\underline{H}$), 7.38 (1H, t, *J* 7.7, 7-C$\underline{H}$), 7.54 (1H, t, *J* 7.7, 6-C$\underline{H}$), 8.10 (1H, d, *J* 7.7, 8-C$\underline{H}$); δ_C (50.31 MHz, $CDCl_3$) 26.6 (4-$\underline{C}H_2$), 32.5 (3-$\underline{C}H_2$), 51.1 (2-$\underline{C}$H), 112.8 (7-$\underline{C}$), 127.7 (5-$\underline{C}$), 129.2 (8-$\underline{C}$), 134.7 (6-$\underline{C}$), 137.5 (8a-$\underline{C}$), 143.2 (4a-$\underline{C}$), 191.1 ($\underline{C}$=O); *m/z* (CI) 227 & 225 ([*M*+H], 98% & 100%), 147 (83, $[C_{10}H_{10}O]^+$), 118 (12, $[C(O)ArCH_3+H]^+$).

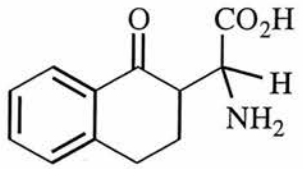
Diethyl 2-(acetylamino)-2-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl) malonate (103)



Sodium hydride (1.39 g, 34.9 mmol, 2.5 eq) 60% in mineral oil was suspended in dry DMF (4 ml) and a solution of diethyl acetamidomalonate (4.565 g, 21 mmol, 1.5 eq) in dry DMF (14 ml) added. The solution was stirred at 0 °C under a nitrogen atmosphere for 3 hours until the anion had formed. A solution of 2-bromo-3,4-dihydro-1-(2*H*)–naphthalenone (**102**, 3.148 g, 13.9 mmol) in dry DMF (10 ml) was added and the solution warmed to room temperature and stirred overnight under nitrogen. The mixture was poured into distilled water (100 ml), acidified to pH 3 with 1M hydrochloric acid in an ice-bath and extracted into diethyl ether (4 x 70 ml). The organic phases were washed with brine (2 x 50 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give an orange oil, which was purified by column chromatography (silica; diethyl ether) (1.86 g, 37%); (Acc. Mass Found 361.151867 Calc. for $C_{19}H_{23}NO_6$ 361.152538); ν_{max} (nujol)/cm^{-1} 3350 (NH), 1760 (C=O), 1725 (CO ester), 1690 (CO amide); δ_H (300 MHz, $CDCl_3$) 1.23 (6H, t, *J* 7.2, $C\underline{H}_3CH_2CO_2$), 1.99 (3H, s, $C\underline{H}_3CO$), 2.83 - 3.26 (4H, m, 3-$C\underline{H}_2$ & 4-$C\underline{H}_2$), 3.93 (1H, dd, J_1 9.7, J_2 3.75, 2-C<u>H</u>), 4.19 - 4.30 (4H, m, $CH_3C\underline{H}_2CO_2$), 6.86 (1H, br s, N<u>H</u>), 7.26 - 7.31 (2H, m, 6-C<u>H</u> & 5-C<u>H</u>), 7.47 (1H, t, *J* 7.7, 7-C<u>H</u>), 7.93 (1H, d, *J* 7.7, 8-C<u>H</u>); δ_C (73.76 MHz, $CDCl_3$) 13.7 ($\underline{C}H_3CH_2CO_2$), 23.1 ($\underline{C}H_3CO$), 26.7 (4-$\underline{C}H_2$), 29.7 (3-$\underline{C}H_2$), 55.9 ($CH_3\underline{C}H_2CO_2$), 63.0 (2-CH), 66.1 (α-<u>C</u>), 126.7 (7-<u>C</u>), 127.4 (5-<u>C</u>), 128.9 (8-<u>C</u>), 132.3 (6-<u>C</u>), 134.0 (8a-<u>C</u>), 144.7 (4a-<u>C</u>), 168.6 ($CH_3CH_2\underline{C}O_2$), 169.8 ($CH_3\underline{C}O$), 198.1 (<u>C</u>=O); *m/z* (EI) 361 ([M^+], 19%), 316 (10, [M- $CH_3CH_2O]^+$), 288 (16, [M-$CO_2CH_2CH_3]^+$), 246 (100, [M-$AcHNCCO_2H]^+$),

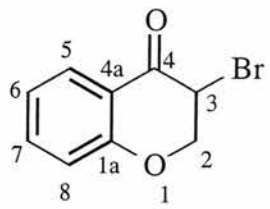
217 (64, [M-AcHNCCO$_2$Et+H]$^+$), 171 (70, [$C_{11}H_9NO$]$^+$), 145 (55, [$C_{10}H_9O$]$^+$), 129 (30, [AcHNCCO$_2$CH$_2$]$^+$), 115 (24, [AcHNCCO$_2$H]$^+$).

Amino-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-acetic acid (97)



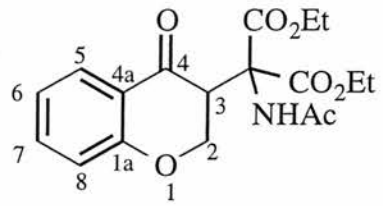
Diethyl 2-(acetylamino)-2-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-malonate (**103**, 1.63 g, 4.5 mmol) was dissolved in 1,4-dioxane (50 ml) and 6M hydrochloric acid (70 ml) added. The reaction was heated under reflux for 8 hours until no starting material was visible by tlc (silica; pet. ether: ethyl acetate; 1:1). The solution was then cooled and washed with ethyl acetate (50 ml). The aqueous phase was concentrated under reduced pressure to give a brown liquid which was triturated with acetone to produce a grey solid. The solid was taken up in isopropyl alcohol (25 ml) and propylene oxide (10 ml) added, the solution was stirred overnight and the solvent removed under reduced pressure to give an off-white solid as a mixture of diastereomers in the ratio 3:1 (0.147 g, 15%); mp 186-192 °C; (Found: C, 65.90; H, 6.09; N, 6.31. Calc. for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.97; N, 6.38%); ν_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (C=O); δ_H (200 MHz, D_2O) 2.04 (2H, t, *J* 6.25, 4-C$\underline{H}_2$), 3.01 (2H, d, *J* 6.25, 3-C$\underline{H}_2$), 3.13 - 3.38 (1H, m, 2-C$\underline{H}$), 3.90 - 4.09 (1H, m, α-C$\underline{H}$), 4.25 - 4.37 (1H, m, α-C$\underline{H}$), 7.29 (2H, m, 7-C$\underline{H}$ & 5-C$\underline{H}$), 7.52 (1H, t, *J* 6.25, 6-C$\underline{H}$), 7.88 (1H, d, *J* 6.25, 8-C$\underline{H}$); δ_C (50.31 MHz, D_2O) 27.8 (4-$\underline{C}H_2$), 30.1 (3-$\underline{C}H_2$), 64.2 (2-CH), 66.5 (α-$\underline{C}$), 126.5 (7-$\underline{C}$), 127.1 (5-$\underline{C}$), 128.5 (8-$\underline{C}$), 132.5 (6-$\underline{C}$), 133.7 (8a-$\underline{C}$), 144.2 (4a-$\underline{C}$), 173.4 ($\underline{C}O_2H$), 197.0 ($\underline{C}$=O); *m/z* (CI) 220 ([*M*+H], 25%), 203 (100, [*M*H-OH]$^+$), 147 (22, [$C_{10}H_{10}O$]$^+$).

3-Bromo-2,3-(4*H*)-dihydrochromen-4-one (104)



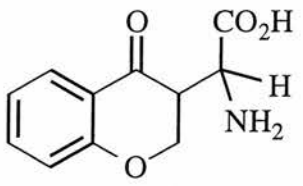
Cupric bromide (1.51 g, 6.75 mmol) was heated under reflux in ethyl acetate (4.2 ml) and then 4-chromanone (0.5 g, 3.37 mmol) in chloroform (4.2 ml) was added and the solution heated under reflux for a further 5 hours. Copper bromide and cupric bromide residues were filtered off and the filtrate decolourised with charcoal. The solution was then filtered and washed several times with ethyl acetate. The solvent was removed under reduced pressure to give yellow oily liquid, further drying on a vacuum pump gave the product as a pale yellow oily solid (0.63 g, 81%); ν_{max} (nujol)/cm^{-1} 1720 (C=O); δ_H (200 MHz; $CDCl_3$) 4.65 (3H, m, 2-C$\underline{H}_2$ and 3-C$\underline{H}$Br), 7.03 (1H, d, *J*7.7, 8-C$\underline{H}$), 7.12 (2H, t, *J* 7.7, 6-C$\underline{H}$), 7.57 (1H, t, *J* 7.7, 7-C$\underline{H}$), 7.96 (1H, d, *J* 7.7, 5-C$\underline{H}$); δ_C (50.31 MHz; $CDCl_3$) 45.92 (2-$\underline{C}$), 71.78 (3-$\underline{C}$), 118.52 (8-$\underline{C}$), 119.26 (7-$\underline{C}$), 122.86 (6-$\underline{C}$), 128.78 (5-$\underline{C}$), 137.26 (4a-$\underline{C}$), 161.0 (1a-$\underline{C}$), 185.76 ($\underline{C}$=O); *m/z* (EI) 228 & 226 ([*M*], 11% & 10%], 148 (14, $[C_9H_8O_2]^+$), 120 (100, $[C_9H_8O_2\text{-}CO]^+$), 92 (98, $[ArO]^+$).

Diethyl 2-(acetylamino)-2-[4-oxo-3,4-(2*H*)-dihydrochromen-3-yl]-malonate (105)



Sodium hydride (0.47 g, 2.5 eq, 18 mol) 60% in mineral oil was suspended in dry DMF (1.2 ml). A solution of diethylacetamidomalonate (1.44 g, 1.5 eq, 6.6 mmol) in dry DMF (7 ml) was added at 0 °C under a nitrogen atmosphere. After stirring for 3 hours the anion was formed (solution turned yellow). A solution of 3-bromo-2,3-(4*H*)-dihydrochromen-4-one (**104**, 1.07 g, 4.7 mmol) in dry DMF (2 ml) was added and the solution allowed to warm to room temperature and stirred over night under nitrogen. The mixture was poured onto distilled water (25 ml) and acidified to pH 3 with 1M HCl in an ice bath (solution goes from red to yellow/orange). The product was then extracted into diethyl ether (4 x 50 ml), washed with brine (2 x 25 ml), the organic layers dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give a brown oil (0.987 g, 55%). Purification by column chromatography (silica; diethyl ether) yielded the product as a sticky semi-crystalline oil (0.55 g, 31%); ν_{max} (nujol)/cm^{-1} 3360 (NH), 1760 (C=O), 1725 (CO ester), 1685 (CO amide); δ_H (200MHz, $CDCl_3$) 1.25 (6H, t, *J* 7.2, $C\underline{H}_3CH_2CO_2$), 2.01 (3H, s, $C\underline{H}_3CO$), 4.16 - 4.35 (6H, m, $CH_3C\underline{H}_2CO_2$ & 2-$C\underline{H}_2$), 4.81 (1H, m, 3-C<u>H</u>), 7.39 - 7.56 (2H, m, 6 & 8-C<u>H</u>), 7.79 (1H, dt, J_1 7.5, J_2 2.5, 7-C<u>H</u>), 8.02 (1H, dd, J_1 7.5, J_2 2.5, 5-C<u>H</u>); δ_C (50.31 MHz, $CDCl_3$) 14.2 ($\underline{C}H_3CH_2CO_2$), 22.8 ($\underline{C}H_3CO$), 58.1 (3-<u>C</u>H), 62.4 ($CH_3\underline{C}H_2CO_2$), 64.7 (2-$\underline{C}H_2$), 89.8 (α-<u>C</u>), 117.8 (8-<u>C</u>), 121.5 (4a-<u>C</u>), 123.2 (6-<u>C</u>), 128.4 (5-<u>C</u>), 134.8 (7-<u>C</u>), 163.7 (1a-<u>C</u>), 171.2 ($CH_3CH_2\underline{C}O_2$), 173.4 ($CH_3\underline{C}O$), 198.1 (4-<u>C</u>); *m/z* (EI) 363 (M^+, 19%), 259 (32, [*M*-ArCO]$^+$), 217 (44, [*M*-(AcHN)CH($CO_2Et)_2$]$^+$), 171 (74, [($EtCO_2$)C(NH)CHCO)]$^+$), 147 (100, [$C_9H_7O_2$]$^+$), 121 (70, [OCArOH]$^+$).

Amino-[4-oxo-3,4-(2*H*)-dihydrochromen-3-yl]-acetic acid (98)



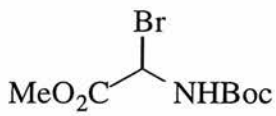
Diethyl 2-(acetylamino)-2-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-malonate (**105**, 0.2 g, 0.5 mmol) was dissolved in THF (20 ml) and 6M hydrochloric acid (25 ml) added. The reaction was heated at reflux for 8 hours until no starting material was visible by tlc (silica; pet. ether: ethyl acetate; 1:1). The solution was cooled and washed with ethyl acetate (50 ml). The aqueous phase was concentrated under reduced pressure to give a brown liquid which was triturated with acetone to give a reddish solid. The solid was taken up in isopropyl alcohol (10 ml) and propylene oxide (2 ml) added, the solution was stirred overnight and the solvent removed under reduced pressure to a pink solid (6 mg, 5%). ^{1}H-Nmr was consistent with that for compound (**98**) although the spectrum was weak and showed impurities; δ_H (200 MHz, DCl/D_2O) 3.26 - 3.53 (1H, m 3-C<u>H</u>), 3.91 - 4.17 (1H, m, α-C<u>H</u>), 4.27 (2H, d, $J_{2,3}$ 9.2, 2-C<u>H</u>$_2$), 6.60 (1H, d, $J_{7,8}$ 7.9, 8-C<u>H</u>), 6.66 (1H, t, $J_{5,6}$ 7.9, 5-C<u>H</u>), 7.17 (1H, dt, $J_{7,8}$ 7.9, $J_{6,7}$ 1.7, 7-C<u>H</u>), 7.35 (1H, dd, $J_{5,6}$7.9, $J_{6,7}$ 1.7, 6-C<u>H</u>).

Methyl *N*-(*tert*-butoxycarbonyl)-glycinate (108)

Glycine methyl ester hydrochloride (1.1 g, 7.96 mmol) was suspended in dry THF (20 ml) and dry triethylamine (1.2 ml, 8.76 mmol) added. The solution was stirred at 0 °C, and then a solution of di-tert-butyl dicarbonate (Boc_2O) (1.2 ml, 4.78 mmol, 0.6 eq) in dry THF (20 ml) was added. The reaction mixture was stirred for 2 hours, heated at reflux for a further two hours and then stirred overnight at room temperature. The solvent

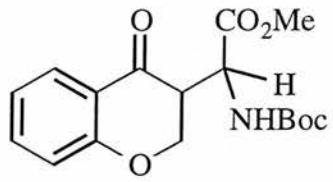
was removed under reduced pressure and the residue partitioned between diethyl ether (75 ml) and water (75 ml). The aqueous layer was washed with diethyl ether (2 x 50 ml). The combined organic phases were then washed with 3% hydrochloric acid (1M, 75 ml), 5% sodium hydrogen carbonate solution (75 ml) and brine (100 ml). The organic layers were dried ($MgSO_4$) and the solvent removed under reduced pressure to give the product as a pale yellow oil which required no further purification, (1.21 g 72%); δ_H (200 MHz, $CDCl_3$) 1.38 (9H, s, $(C\underline{H}_3)_3C$), 3.68 (3H, s, $C\underline{H}_3CO_2$), 3.85 (2H, d, *J* 7.5, $C\underline{H}_2$), 5.19 (1H, br s, N$\underline{H}$); δ_C (50.31 MHz, $CDCl_3$) 28.7 ($(\underline{C}H_3)_3C$), 42.7 ($\underline{C}H_3CO_2$), 52.6 (CH_2), 80.3 ($(CH_3)_3\underline{C}$), 156.3 ($CH_3\underline{C}O_2$), 171.9 ($\underline{C}O_2{}^tBu$); *m/z* (EI) 189 ([M^+], 4%), 57 (100, $[(CH_3)_3C]^+$).

Methyl 2-bromo-2-*N*-(*tert*-butoxycarbonyl)-glycinate (109)



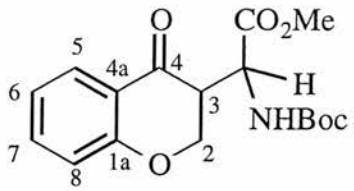
N-Bromosuccinimide (1.056 g, 5.93 mmol, 1.1 eq), methyl *N*-(*tert*-butoxycarbonyl)-glycinate (**108**, 1.026 g, 5.42 mmol) and benzoyl peroxide (6 mg, 1 g/mol) were suspended in carbon tetrachloride (20 ml) and stirred at room temperature for 15 minutes. The pale yellow solution was then heated gently to reflux and stirred for a further 3 hours. The reaction was filtered to remove succinimide, the solvent was removed under reduced pressure and the residue dried under vacuum to give a yellow syrup which was refrigerated (1.14 g, 78%); δ_H (200 MHz, $CDCl_3$) 1.45 (9H, s, $(C\underline{H}_3)_3C$), 3.68 - 3.91 (4H, m, $C\underline{H}_3CO_2$ & C$\underline{H}$Br); δ_C(50.31 MHz, $CDCl_3$) 28.7 ($(\underline{C}H_3)_3C$), 30.1 ($\underline{C}H_3CO_2$), 54.1 ($\underline{C}$HBr), 80.2 ($(CH_3)_3\underline{C}$), 132.5 ($CH_3\underline{C}O_2$).

Attempted coupling of 4-chromanone and 2-bromo-2-*N*-(^{t}butoxycarbonyl)-glycine methyl ester (110)



A solution of 4-chromanone (0.276 g, 1.86 mmol) was dissolved in dry THF (10 ml) and ^{n}butyl lithium (1.2 ml, 1.86 mmol, 1.6M in hexanes) was added at -78 °C under nitrogen and stirred for 30 minutes. Dry triethylamine (0.3 ml, 1.86 mmol) was added to a solution of methyl 2-bromo-2-*N*-(*tert*-butoxycarbonyl)-glycinate (**109**, 0.521 g, 1.86 mmol) in dry THF (10 ml) at -78 °C in an acetone-dry ice bath under a nitrogen atmosphere and also stirred for 30 minutes. The dihydro-chromenone enol was added by cannula to the preformed glycine imine and the solution stirred at -78 °C for 4 hours. The solution was warmed to room temperature, stirred overnight, poured into distilled water (50 ml) and acidified to pH 3 with 1M hydrochloric acid. The mixture was extracted into diethyl ether (50 ml), the aqueous phases washed with diethyl ether (2 x 30 ml). The combined organic phases were washed with brine (2 x 30 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a yellow oil. Purification by column chromatography (silica; pet. ether: diethyl ether; 1:1) gave a bicycle containing fragment which may be product (0.275 g, 44%). Analysis by ^{1}H-nmr spectroscopy showed the product to be similar to that for compound (**110**) as synthesised from 3-bromo-2,3-(4*H*)-dihydrochromen-4-one and diethylacetamidomalonate.

Methyl[(*tert*-butoxycarbonyl)amino]-[4-oxo-3,4-(2*H*)-dihydrochromen-3-yl]-acetate (110)

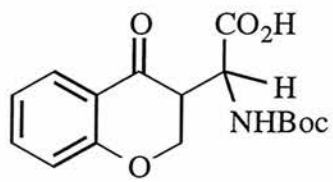


Sodium hydride (0.366 g, 9.15 mmol, 2.5 eq) 60% in mineral oil was suspended in dry DMF (5 ml) and stirred at 0 °C. A solution of methyl *N*-(*tert*-butoxycarbonyl)-glycinate (**108**, 1.05 g, 5.55 mmol, 1.5 eq) in dry DMF (15 ml) was added and the solution stirred at 0 °C for 2 hours under a nitrogen atmosphere. A solution of 3-bromo-2,3-(4*H*)-dihydrochromen-4-one (**104**, 0.84 g 3.67 mmol) in dry DMF (10 ml) was then added and the reaction stirred at room temperature overnight under nitrogen. The reaction mixture was poured into distilled water (100 ml) and acidified to pH 3 with 1M hydrochloric acid. The aqueous phase was extracted into diethyl ether (5 x 30 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a semi-crystalline yellow oil (0.97 g, 79.2%); δ_H (200 MHz, $CDCl_3$) 1.45 (9H, s, $(C\underline{H}_3)_3C$), 3.89 (3H, s, $C\underline{H}_3CO_2$), 4.49 - 4.87 (2H, m, 2-CH_2), 5.05 - 5.18 (2H, m, NH & 3-C$\underline{H}$), 5.67 - 5.75 (1H, m, α-C$\underline{H}$), 6.97 (1H, t, *J* 7.5, 6-C$\underline{H}$), 7.06 (1H, d, *J* 7.5, 8-C$\underline{H}$), 7.49 (1H, t, *J* 7.5, 7-C$\underline{H}$), 7.85 (1H, d, *J* 7.5, 5-C$\underline{H}$); δ_C (50.31 MHz, $CDCl_3$) 28.8 ($(\underline{C}H_3)_3C$), 30.2 (2-$\underline{C}H_2$), 32.3 (3-$\underline{C}$H), 37.3 ($\underline{C}H_3CO_2$), 42.7 (α-$\underline{C}$H), 80.6 ($(CH_3)_3\underline{C}$), 113.4 (8-$\underline{C}$), 118.7 (4a-$\underline{C}$), 125.8 (6-$\underline{C}$), 126.3 (5-$\underline{C}$), 134.4 (7-$\underline{C}$), 156.3 (1a-$\underline{C}$), 163.7 ($CH_3\underline{C}O_2$), 173.6 ($\underline{C}O_2{}^tBu$), 178.6 ($\underline{C}$=O).

A similar reaction was conducted using methyl *N*-(*tert*-butoxycarbonyl)-glycinate (**108**, 0.998 g, 5.3 mmol, 1.5 eq) in dry THF (10 ml) at 0 °C under nitrogen. To this was added ^{n}butyl lithium (5.4 ml, 8.6 mmol, 2.5 eq), the solution was stirred in an ice-bath for 30 minutes before 3-bromo-2,3-dihydro-4*H*-chromen-4-one (**104**, 0.786 g, 3.45

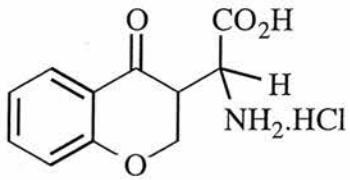
mmol) in dry THF (10 ml) was added. The solution stirred for 5 hours at room temperature, poured into distilled water (50 ml) and acidified to pH 3 with 1M hydrochloric acid, extracted into diethyl ether (5 x 20 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to yield an orange oil which by ^{1}H-nmr spectroscopy was neither product nor starting material.

[(*tert*-Butoxycarbonyl)amino]-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetic acid (120)



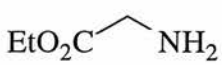
Methyl[(*tert*-butoxycarbonyl)amino]-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetate (**110**, 0.97 g, 2.8 mmol) was dissolved in aqueous potassium hydroxide solution (10% w/v, 25 ml) and THF (10 ml) added to aid dissolution. The reaction was stirred overnight and then the basic solution was washed with diethyl ether (50 ml). The aqueous phase was then acidified and extracted into diethyl ether (4 x 50 ml). The organic phases were dried ($MgSO_4$) and the solvent removed under reduced pressure to give a yellow/orange oil (0.59 g, 64%); δ_H (300 MHz, $CDCl_3$) 1.42 (9H, s, $(C\underline{H}_3)_3C$), 3.86 - 4.01 (3H, m, 2-$C\underline{H}_2$ & 3-C$\underline{H}$), 5.12 (1H, br s, α-C$\underline{H}$), 7.41 - 8.06 (4H, m, aromatic C$\underline{H}$); δ_C (50.31 MHz, $CDCl_3$) 28.7 ($(\underline{C}H_3)_3C$), 42.6 (2-$\underline{C}H_2$), 43.8 (3-$\underline{C}$H), 66.4 (α-$\underline{C}$H), 80.9 ($(CH_3)_3\underline{C}$), 118.8 (8-$\underline{C}$), 122.2 (4a-$\underline{C}$), 126.9 (6-$\underline{C}$), 131.2 (5-$\underline{C}$), 133.3 (7-$\underline{C}$), 156.5 (1a-$\underline{C}$), 165.0 ($\underline{C}O_2H$), 174.9 ($\underline{C}O_2{}^t$Bu), 186.3 ($\underline{C}$=O); *m/z* (EI) 290 ([*M*-CH_2OH]$^+$, 21%0, 262 (8, [*M*-CH_2CO_2H]$^+$), 121 (46, [ArC(O)OH]$^+$), 57 (100, [$(CH_3)_3C$]$^+$).

Amino-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetic acid hydrochloride (98)



[(*tert*-Butoxycarbonyl)amino]-(4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetic acid (**120**, 0.59 g, 1.86 mmol) was dissolved in dry ethyl acetate (20 ml) and HCl gas bubbled through the solution until saturated. The solid formed (118 mg) was filtered off and later found not to be product by ^{1}H-nmr spectroscopy. The solvent was removed under reduced pressure and the residue partitioned between ethyl acetate (20 ml) and water (20 ml). The aqueous phase was washed with chloroform (10 ml) and then freeze dried to give the product as an extremely hygroscopic pale yellow solid (0.13 g, 11%). Nmr spectroscopy showed the compound to be identical with the free base (**98**); ν_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (CO acid); δ_H (200 MHz, DCl/D_2O) 3.32 (1H, dt, $J_{2,3}$ 9.2, $J_{3,9}$ 2.5, 3-C<u>H</u>), 4.01 (1H, m, α-C<u>H</u>), 4.33 (2H, d, $J_{2,3}$ 9.2, 2-C<u>H</u>$_2$), 6.60 (1H, d, $J_{7,8}$ 7.9, 8-C<u>H</u>), 6.66 (1H, t, $J_{5,6}$ 7.9, 5-C<u>H</u>), 7.17 (1H, t, $J_{7,8}$ 7.9, 7-C<u>H</u>), 7.35 (1H, d, $J_{5,6}$7.9, 6-C<u>H</u>)

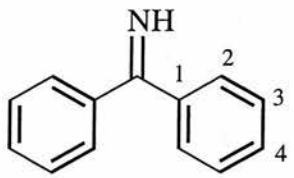
Glycine ethyl ester hydrochloride



Acetyl chloride (21 ml, 290 mmol) was added dropwise over a period of 10 minutes to dry redistilled ethanol (100 ml) and stirred at 0 °C in an ice bath for 15 minutes. Glycine (10.1 g, 134.7 mmol) was added and the mixture heated at reflux for 5 hours and then cooled. The solvent was removed under reduced pressure to yield the product as a white solid which was used without further purification (18.6 g, 99%), mp: 130 - 133 °C

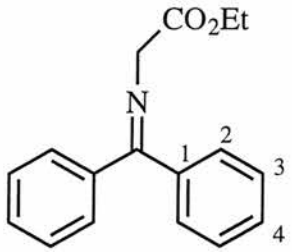
(lit.[208] 144 °C); δ_H (300 MHz, D_2O) 1.17 (3H, t, *J* 7.2, $C\underline{H}_3CH_2O$), 3.79 (2H, s, α-$C\underline{H}_2$), 4.18 (2H, q, *J* 7.2, $CH_3C\underline{H}_2O$); δ_C (74.76 MHz, D_2O) 9.6 ($\underline{C}H_3CH_2O$),36.9 ($CH_3\underline{C}H_2O$), 60.1 (α-$\underline{C}H_2$), 165.1 ($\underline{C}O_2Et$).

Diphenylmethanimine (114)



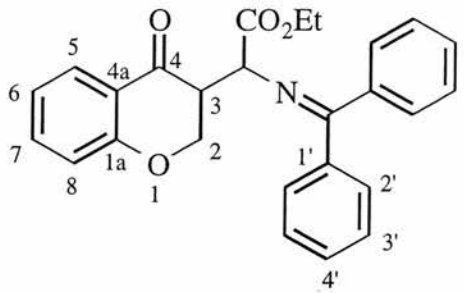
Magnesium turnings (0.776 g, 31.8 mmol, 1.1 eq) were suspended in dry diethyl ether (150 ml) under a nitrogen atmosphere. To this was added bromobenzene (3.35 ml, 31.8 mmol, 1.1 eq) in order to maintain a gentle reflux. Once the reaction had abated, the solution was heated under reflux for 30 minutes. The solution was then cooled to room temperature and benzonitrile (2.95 ml, 28.9 mmol) was added slowly in order to maintain a gentle reflux. The reaction was heated at reflux for 4 hours and then cooled, quenched by slowly adding dry methanol (40 ml) and stirred for 30 minutes. The solvents were removed under reduced pressure and the residue distilled on a Vigreux column (151 °C at 10 atm) to yield the product as a yellow liquid (3.54 g, 67.5%); (Acc. Mass Found 182.096348 Calc. for $C_{13}H_{12}N$ 182.096974); δ_H (300 MHz, $CDCl_3$) 7.33 - 7.69 (10H, m, 2 x $C_6\underline{H}_5$), 9.71 (1H, br s, N$\underline{H}$); δ_C (74.76 MHz, $CDCl_3$) 125.2 (4-$\underline{C}$), 126.0 (3-$\underline{C}$), 127.2 (2-$\underline{C}$), 129.1 (1-$\underline{C}$), 175.3 (C=NH); *m/z* (CI) 182 ([*M*+H]$^+$, 57%), 167 (100, [*M*+H-NH]$^+$), 104 (25, [*M*+H-C_6H_5]$^+$), 77 (20, [C_6H_5]$^+$).

Ethyl *N*-(diphenylmethylene)glycinate (113)



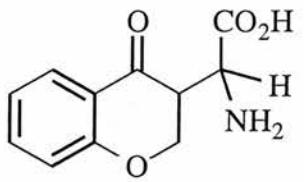
Diphenylmethanimine (**114**, 4.26 g, 23.3 mmol) was dissolved in dry dichloromethane (30 ml) and glycine ethyl ester hydrochloride (3.32 g, 23.3 mmol) added. The solution was stirred at room temperature overnight under a nitrogen atmosphere, filtered and the filtrate concentrated under reduced pressure. The residue was taken up in diethyl ether (40 ml), washed with water (2 x 30 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give yellow crystals. Recrystallisation from hexane/diethyl ether gave the product as white crystals (3.47 g, 55%), mp 51.8 - 52.6 °C (lit.[143] 51 - 52 °C); (Acc. Mass Found 268.133277 Calc. for $C_{17}H_{18}NO_2$ 268.133754); δ_H (300 MHz, $CDCl_3$) 1.26 (3H, t, *J* 7.2, $C\underline{H}_3CH_2CO_2$), 4.20 (4H, m, $CH_3C\underline{H}_2CO_2$ & α-$C\underline{H}_2$), 7.16 - 7.19 (2H, m, $C_6\underline{H}_5$), 7.32 - 7.36 (3H, m, $C_6\underline{H}_5$), 7.39 - 7.46 (3H, m, $C_6\underline{H}_5$), 7.63 - 7.67 (2H, m, $C_6\underline{H}_5$); δ_C (74.76 MHz, $CDCl_3$) 14.3 ($\underline{C}H_3CH_2CO_2$), 55.8 ($CH_3\underline{C}H_2CO_2$), 60.1 (α-$\underline{C}H_2$), 128.3 (2C, 4-$\underline{C}$), 129.1 (4C, 3-$\underline{C}$), 130.7 (4C, 2-$\underline{C}$), 136.3 (1-$\underline{C}$), 139.6 (1-$\underline{C}$), 170.9 ($\underline{C}$=NPh), 172.1 ($CH_3CH_2\underline{C}O_2$); *m/z* (CI) 268 ([*M*+H], 100%), 194 (64, [*M*-$CH_3CH_2CO_2$]$^+$), 183 (29, [*M*-$CH_3CH_2CO_2CH$]$^+$).

Ethyl-[(diphenylmethylene)amino](4-oxo-3,4-(2*H*)-dihydrochromen-3-yl)-acetate (117)



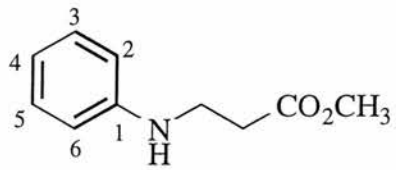
Ethyl *N*-(diphenylmethylene)glycinate (**113**, 1.79 g, 6.72 mmol), was dissolved in dry THF (20 ml) and cooled to -78 °C in an acetone bath. Lithium diisopropylamide (3.7 ml, 7.39 mmol, 1.1 eq, 2M in hexane/THF) was added slowly and the solution stirred for 2 hours at -78 °C under nitrogen. A solution of 3-bromo-2,3-(4*H*)-dihydrochromen-4-one (**104**, 1.53 g, 6.72 mmol) in dry THF (20 ml) was added and the reaction stirred for 1 hour at -78 °C. The solution was warmed to room temperature and stirred overnight then poured into 1M hydrochloric acid (20 ml) and extracted into diethyl ether (4 x 30 ml). The organic fractions were dried ($MgSO_4$) and the solvent removed under reduced pressure to give an orange oil. Purification by column chromatography (silica; pet. ether: diethyl ether; 5:1) gave the product as a yellow syrup (0.45 g, 16%); (Acc. Mass Found 414.171609 Calc. for $C_{26}H_{24}NO_4$, 414.170534); δ_H (300 MHz, $CDCl_3$) 1.24 (3H, t, *J* 7.1, $C\underline{H}_3CH_2CO_2$), 3.68 (1H, dt, J_1 9.6, J_2 3.8, 3-C$\underline{H}$), 4.18 (2H, q, *J* 7.1, $CH_3C\underline{H}_2CO_2$), 4.75 (2H, dd, J_1 9.6, J_2 3.8, 2-C$\underline{H}_2$), 4.84 (1H, d, *J* 3.8, α-C$\underline{H}$), 6.90 - 6.98 (3H, m, 2'-C$\underline{H}$ & 8-C$\underline{H}$), 7.22 - 7.59 (8H, m, 3' & 4'-C$\underline{H}$, 6- & 7-C$\underline{H}$), 7.75 - 7.88 (3H, m, 2'C$\underline{H}$ & 5-C$\underline{H}$); δ_C (74.76 MHz, $CDCl_3$) 14.1 ($\underline{C}H_3CH_2CO_2$), 48.7 (3-$\underline{C}$H), 61.4 ($CH_3\underline{C}H_2CO_2$), 62.3 (α-$\underline{C}$H), 117.9 (8-C), 121.3 (4a-$\underline{C}$), 127.3 (6-$\underline{C}$), 128.1 (4'-$\underline{C}$), 128.5 (3'-$\underline{C}$), 128.9 (2'-$\underline{C}$), 130.1 (5-$\underline{C}$), 135.9 (7-$\underline{C}$), 139.3 (1'-$\underline{C}$), 161.9 (1a-$\underline{C}$), 170.5 ($CH_3CH_2\underline{C}O_2$), 172.9 ($\underline{C}$=N), 191.7 ($\underline{C}$=O); *m/z* (CI) 414 ([*M*+H], 100%), 340(27, $[M-CO_2CH_2CH_3]^+$), 265 (59, $[M-C_9H_7O_2+H]^+$), 238 (20, $[M-C_9H_7O_2CNH_2]^+$), 220 (15, $[M-Ph_2C{=}NCH]^+$) 193 (38, $[Ph_2C{=}NCH]^+$), 165 (24, $[Ph_2C]^+$).

Amino-[4-oxo-3,4-(2*H*)-dihydrochromen-3-yl]-acetic acid (98)



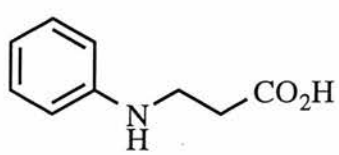
Ethyl [(diphenylmethylene)amino][4-oxo-3,4-(2*H*)-dihydrochromen-3-yl]-acetate (**117**, 0.466 g, 1.12 mmol) was dissolved in THF (5 ml) and 6N hydrochloric acid (10 ml) added. The solution was heated at reflux for 5 hours. The solvent was then removed under reduced pressure, the residue taken up in water (10 ml) and washed with ethyl acetate (10 ml). The aqueous phase was freeze dried to a red solid, taken up in isopropyl alcohol (10 ml) and propylene oxide (5 ml) added. The emulsion was stirred overnight and the solvent removed under reduced pressure to give a pink solid (100 mg, 40.1%). Purification by column chromatography (reverse phase silica; acetonitrile:water; 80:20) gave the product as a pale pink solid (20 mg, 8%) [product as a mixture of diastereomers in ratio 3:1], mp 138-140 °C [decomp.]; (Acc. Mass Found 244.059856 Calc. for $C_{11}H_{11}NO_4Na$, 244.058578); ν_{max} (nujol)/cm^{-1} 1690 (C=O), 1650 (CO acid); δ_H (300 MHz, DCl/D_2O) 3.37 (major, 1H, dt, $J_{2,3}$ 9.3, $J_{3,9}$ 2.6, 3-C<u>H</u>), 3.43 - 3.48 (minor, 1H, m, 3-C<u>H</u>), 4.01 (major, 1H, d, $J_{3,9}$ 2.6, α-C<u>H</u>), 4.11 (minor, 1H, d, $J_{3,9}$ 2.6, α-C<u>H</u>), 4.31 (2H, d, $J_{2,3}$ 9.3, 2-C<u>H</u>$_2$), 6.60 (1H, d, $J_{7,8}$ 7.9, 8-C<u>H</u>), 6.66 (1H, t, $J_{5,6}$ 7.9, 5-C<u>H</u>), 7.17 (1H, dt, $J_{7,8}$ 7.9, $J_{6,7}$ 1.7, 7-C<u>H</u>), 7.35 (1H, dd, $J_{5,6}$7.9, $J_{6,7}$ 1.7, 6-C<u>H</u>); δ_C (300 MHz, DCl/D_2O) 43.9 (3-<u>C</u>H), 47.8 (α-<u>C</u>H), 66.3 (2-<u>C</u>H$_2$), 115.8 (8-<u>C</u>), 120.0 (6-<u>C</u>), 124.7 (5-<u>C</u>), 135.7 (7-<u>C</u>), 159.5 (4a-<u>C</u>), 167.7 (1a-<u>C</u>), 171.7 (<u>C</u>O$_2$H), 191.6 (<u>C</u>=O); *m/z* (CI) 244 ([*M*+H+Na], 19%), 205 (100, [*M*-NH_2]).

Methyl 3-anilinopropanoate (121)



Equimolar quantities of redistilled aniline (21.46 g, 23 mmol) and methyl acrylate (20.1 g, 23 mmol) were refluxed with acetic acid (0.5 ml) for 15 hours. The yellow reaction mixture turned crimson red. The thick gel was distilled under reduced pressure (13-14 mmHg) and after the initial forerun a fraction came over at 156-160 °C. This yellow product crystallised on cooling. The product was dried under vacuum and used without further purification in the next step as yellow crystals (21.9 g, 53%); mp 37.6 - 39.2 °C (lit.,[145] 36 - 38 °C); δ_H (200 MHz; $CDCl_3$) 2.64 (2H, t, *J* 10.3, C<u>H</u>$_2$), 3.48 (2H, t, *J* 10.3, C<u>H</u>$_2$), 3.71 (3H, s, C<u>H</u>$_3$), 4.0 (1H., br s, N<u>H</u>), 6.64 (2H, t, *J* 10.3, 2 and 6-C<u>H</u>), 6.74 (1H, t, *J* 10.3, 4-<u>H</u>), 7.22 (2H, d, *J* 10.3, 3 and 5-C<u>H</u>); δ_C (50.31 MHz; $CDCl_3$) 34.18 (<u>C</u>H$_3$), 39.80 (<u>C</u>H$_2$), 113.53 (3 and 5-C), 118.26 (4-<u>C</u>), 129.86 (2 and 6-<u>C</u>), 148.07 (1-C), 173.5 (<u>C</u>=O).

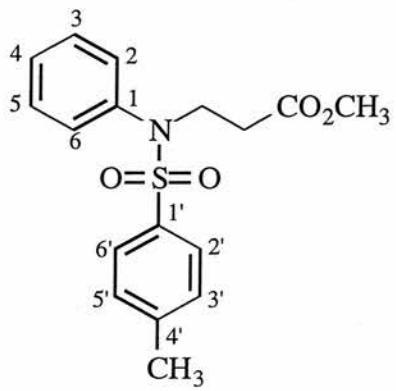
3-Anilinopropanoic acid (122)



Methyl 3-anilinopropanoate (**121**, 9.9 g, 55.5 mmol) was stirred in 80% methanol (100 ml) and 10% potassium hydroxide solution (25 ml) was added with stirring. The solution was stirred at room temperature for 16 hours. The solution was diluted with excess water (250 ml) and then poured with stirring into excess cold dilute hydrochloric acid (1M). The solvent was removed under reduced pressure and the residue dissolved in saturated sodium hydrogen carbonate solution with heating, filtered and acidified. No product

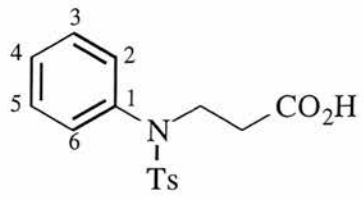
precipitated and so attempts were made to obtain the product from solution using ion exchange chromatography (Amberlite resin IR120 [H]). Nmr spectroscopy of the crude product showed the presence of the free acid; δ_H (200 MHz; $CDCl_3$) 2.39 (2H, t, *J* 7.7, CH_2), 3.25 (2H, t, *J* 7.7, CH_2), 6.35 (1H, d, *J* 7.6, NH), 6.85 (3H, m, 2-, 6-, and 4-CH), 7.21 (2H, t, *J* 7.9, 3- and 5--CH), 8.68 (1H, br s, OH).

Methyl 3-{[(4-methylphenyl)sulfonyl]anilino}propanoate (123)



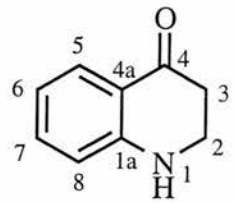
To methyl 3-anilinopropanoate (**121**, 11.1 g, 62 mmol) in dry pyridine (100 ml) was added *p*-toluenesulfonyl chloride (13.0 g, 68 mmol, 1.1 eq) in small increments. After formation of the pyridine hydrochloride salt, the solution was heated to steam-bath temperatures on an oil bath and then to boiling for 15 minutes. After cooling the solution was quenched with water (125 ml) and extracted into diethyl ether (2 x 70 ml). The organic phases were washed with 10% hydrochloric acid solution (100 ml), water (100 ml) and brine (150 ml). The organic phases were dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give a thick orange syrup, (16.5 g, 80%); δ_H (200 MHz; $CDCl_3$) 2.4 (3H, s, PhC$\underline{H}_3$), 2.53 (2H, t, *J* 7.5, C$\underline{H}_2$), 3.57 (3H, s, $CO_2C\underline{H}_3$), 3.83 (2H, t, *J* 7.5, C$\underline{H}_2$), 6.97 - 7.06 (2H, m, 3' and 5'-H), 7.20 - 7.34 (5H, m, $C_6\underline{H}_5N$), 7.48 (2H, d, *J* 10.5, 2' and 6'-H); δ_C (50.31 MHz; $CDCl_3$) 22.08 (Ph$\underline{C}H_3$), 24.47 ($CO_2\underline{C}H_3$), 47.29 ($\underline{C}H_2$), 128.19, 128.72, 129.38, 129.63, 129.94, 130.0 (tosyl aromatics), 136.37 (3 and 5-C), 139.41 (4-C), 144.13 (2 and 6-C), 150.24 (1-C), 171.08 (C=O).

3-{[(4-Methylphenyl)sulfonyl]anilino}propanoic acid (124)



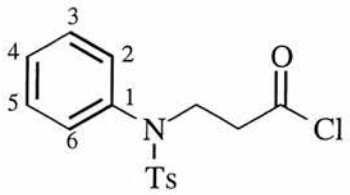
Methyl 3-{[(4-methylphenyl)sulfonyl]anilino}propanoate (**123**, 7.7 g, 23 mmol) was stirred in 80% methanol (50 ml) and 10% potassium hydroxide solution (12.5 ml) was added with stirring. The solution was stirred at room temperature for 16 hours. The solution was then diluted with excess water (200 ml) and poured, with stirring, into excess cold dilute hydrochloric acid (1M, 200 ml). The solvent was removed under reduced pressure and the residue dissolved in saturated sodium hydrogen carbonate solution with heating, filtered and acidified. The solid product was filtered off and dried (P_2O_5 in desiccator overnight). The product was obtained as a white crystalline powder (5.9 g, 81%), mp 139-42 °C; δ_H (200 MHz; $CDCl_3$) 2.41 (3H, s, PhC$\underline{H}_3$), 2.59 (2H, t, *J* 7.7, C$\underline{H}_2$), 3.83 (2H, t, *J* 7.7, C$\underline{H}_2$), 6.97 - 7.06 (2H, m, 3' and 5'-$\underline{H}$), 7.20 - 7.35 (5H, m, $C_6\underline{H}_5N$), 7.47 (2H, d, *J* 10.5, 2' and 6'-$\underline{H}$); δ_C (50.31 MHz; $CDCl_3$) 34.2 (Ph$\underline{C}H_3$), 47.31 ($\underline{C}H_2$), 128.19, 128.72, 129.38, 129.63, 129.94, 130.0 (<u>tosyl</u> aromatics), 135.67 (3 and 5-$\underline{C}$), 138.98 (4-$\underline{C}$), 142.07 (2 and 6-$\underline{C}$), 149.83 (1-$\underline{C}$), 176.05 ($\underline{C}$=O); *m/z* (CI) 320 ([*M*+H], 35%), 166 (100, $[ArNH(CH_2)_2CO_2H+H]^+$), 157 (77, $[TsH+H]^+$).

2,3-dihydro-4-(1*H*)-quinolinone (125)



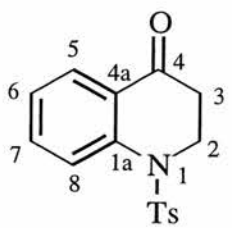
Polyphosphoric acid (33 g) was warmed to 120 °C with stirring and 3-{[(4-methylphenyl)sulfonyl]anilino}propanoic acid (**124**, 2 g, 6.2 mmol) was added slowly. The solution turned from clear to yellow through to cherry red as it became more liquid. Once 120 °C had been reached the solution was maintained at this temperature for 20 minutes. After cooling to 80 °C it was poured onto iced water (150 ml) and stirred. The precipitate was collected by filtration. The aqueous layer was extracted into ethyl acetate (3 x 50 ml), dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The product was obtained as an orange oil (0.85 g, 45%); δ_H (300 MHz; $CDCl_3$) 2.63 (2H, t, *J* 7.0, 3-C$\underline{H}_2$), 3.50 (2H, t, *J* 7.0, 2-C$\underline{H}_2$), 3.91 (1H, br s, N$\underline{H}$), 6.67 (2H, dd, J_1 7.0, J_2 2, 6 & 8-$\underline{H}$), 7.24 (1H, t, *J* 7.0, 7-$\underline{H}$), 7.76 (1H, d, *J* 7.0, 5-$\underline{H}$); δ_C (74.76 MHz; $CDCl_3$) 37.8 (2-$\underline{C}H_2$), 41.96 (3-$\underline{C}H_2$), 116.9 (6-$\underline{C}$), 118.1 (7-$\underline{C}$), 119.28 (4a-$\underline{C}$), 127.57 (5-$\underline{C}$), 135.45 (8-$\underline{C}$), 151.14 (1a-$\underline{C}$), 194.63 ($\underline{C}$=O), *m/z* (CI) 148 ([*M*+H], 41%), 85 (100, $[CHNH(CH_2)_2CO+H]^+$).

3-{[(4-Methylphenyl)sulfonyl]anilino}propanoyl chloride (126)



3-{[(4-Methylphenyl)sulfonyl]anilino}propanoic acid (**124**, 2 g, 6.4 mmol) was refluxed in oxalyl chloride (25 ml) overnight. After cooling the solvent was removed under reduced pressure and the product washed several times with dry toluene (3 x 10 ml) and finally dried under vacuum. The acid chloride was used without further purification as a yellow solid (1.9 g, 88%); δ_H (200 MHz; $CDCl_3$) 2.43 (3H, s, PhC<u>H</u>$_3$), 2.58 (2H, t, *J* 7.5, C<u>H</u>$_2$COCl), 3.81 (2H, t, *J* 7.5, C<u>H</u>$_2$NTs), 6.97 - 7.07 (2H, m, 3' and 5'-<u>H</u>), 7.20 - 7.32 (5H, m, C_6<u>H</u>$_5$N), 7.47 (2H, d., *J* 7.5, 2' and 6'-<u>H</u>).

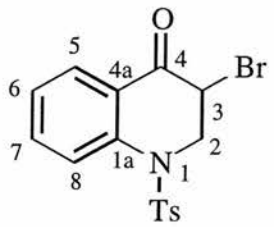
[(4-Methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (127)



3-{[(4-Methylphenyl)sulfonyl]anilino}propanoyl chloride (**124**, 1.87 g, 5.5 mmol) was dissolved in sodium dried toluene (30 ml) and aluminium chloride was added (2.53 g, 19 mmol, 3.4 eq). The reaction mixture was stirred at room temperature for 48 hours, quenched in dilute acid (30 ml) and extracted into ethyl acetate (2 x 30 ml). The organic phases were dried ($MgSO_4$), filtered and solvent removed under reduced pressure to give a thick brown oil, (1.6 g, 96%); δ_H (200 MHz; $CDCl_3$) 2.35 (3H, s, PhC<u>H</u>$_3$), 3.25 (2H, t, *J* 7.0, 3-C<u>H</u>$_2$), 4.0 (2H, t, *J* 7.0, 2-C<u>H</u>$_2$), 7.07 - 7.39 (5H, m, tosyl C<u>H</u> and 8-C<u>H</u>), 7.45 - 7.55 (1H, m, 6-C<u>H</u>), 7.70 - 7.86 (2H, m, 7- and 5-C<u>H</u>); δ_C (50.31 MHz; $CDCl_3$) 22.1 (Ph<u>C</u>H$_3$), 38.67 (2-<u>C</u>H$_2$), 47.39 (3-<u>C</u>H$_2$), 127.9 - 131.5 (6C, aromatics), 134.23

(6-C), 135.25 (7-C), 139.98 (4a-C), 144.14 (5-C), 144.47 (8-C), 144.79 (1a-C), 198.16 (C=O); *m/z* (CI) 247 ([*M*+H - $OCCH_2C$]$^+$, 100%), 211 (8, [$C_9H_8NO_3S$]$^+$), 155 (7, [$SO_2C_6H_4CH_3$]$^+$), 147 (21 [C_9H_8NO]$^+$).

Attempted Bromination of [(4-methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (128)



Cupric bromide (0.76 g, 3.32 mmol) was heated at reflux in ethyl acetate (5 ml). To this was added [(4-methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**124**) (0.75 g, 2.4 mmol) in chloroform (5 ml). The reaction was heated at reflux for a further 5 hours and then cooled. The copper bromide and cupric bromide residues were filtered off, the filtrate decolourised with activated charcoal, filtered through a bed of Celite, washed with ethyl acetate (4 x 50 ml) and the solvent removed under reduced pressure to give a brown oil which was dried further under vacuum (0.3 g, 28%). Analysis by ^{1}H-nmr spectroscopy and mass spectrometry showed that none of the desired bromide had been formed.

[(4-Methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**124**) (2.04 g, 6.76 mmol) was dissolved in dry redistilled methanol (60 ml) and bromine (0.8 ml, 15.4 mmol, 2.3 eq) in dry methanol (10 ml) was added slowly dropwise under cooling in an ice-bath. The reaction temperature was maintained at 5-6 °C for 2 hours then warmed to room temperature. Distilled water (50 ml) was added and the reaction mixture stirred overnight. The solution was extracted with diethyl ether (5 x 70 ml), the organic phases washed thoroughly with 10% potassium carbonate solution (w/w, 2 x 50 ml) and brine (2 x 70

ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give an orange/brown solid (2.52 g, 97.8%). This was found by ^{1}H-nmr spectroscopy to be the dibromide.

[(4-Methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**124**, 0.53 g, 1.76 mmol) was dissolved in dry THF (10 ml) and stirred in ice under a nitrogen atmosphere. ^{n}-Butyl lithium (1.2 ml, 1.82 mmol, 1.1 eq, 1.6M solution in hexanes) was added and the solution stirred for 30 minutes. *N*-Bromosuccinimide (0.335 g, 1.82 mmol) was added and the solution warmed to room temperature and stirred overnight under a nitrogen atmosphere and in foil. The reaction mixture was poured into ice water (75 ml), extracted into diethyl ether (2 x 100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a brown oil. Nmr spectroscopic analysis was inconclusive and mass spectrometry showed that no bromination had occurred.

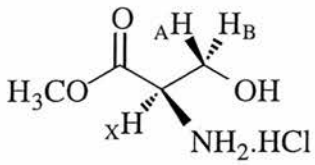
[(4-Methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**124**, 0.252 g, 0.8 mmol) was dissolved in dry THF (10 ml) and stirred in ice under a nitrogen atmosphere. ^{n}-Butyl lithium (1.2 ml, 1.82 mmol, 1.1 eq, 1.6M solution in hexanes) was added and the solution stirred for 30 minutes. Bromine (0.05 ml, 0.9 mmol) was added and the solution warmed to room temperature and stirred overnight. The reaction was poured into distilled water (75 ml) and extracted into diethyl ether (3 x 50 ml). The organic phases were washed with 10% potassium carbonate solution (75 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a brown oil. ^{1}H-Nmr spectroscopy showed that none of the bromide had been formed but mass spectrometry indicated the presence of some of the dibromide.

As with the dihydro-chromenone derivative an attempt was made to couple a positive glycine analogue with the enolate of the dihydroquinolinone as described below. A solution of [(4-methylphenyl)sulfonyl]-2,3-dihydro-4-(1*H*)-quinolinone (**124**, 0.58 g, 1.86 mmol) was dissolved in dry THF (10 ml) and ^{n}butyl lithium (1.2 ml, 1.86 mmol,

1.6M in hexanes) was added at -78 °C under nitrogen and stirred for 30 minutes. Dry triethylamine (0.3 ml, 1.86 mmol) was added to a solution of methyl 2-bromo-2-*N*-(*tert*-butoxycarbonyl)-glycinate (**109**, 0.556 g, 1.86 mmol) in dry THF (10 ml) at -78 °C in an acetone-dry ice bath under a nitrogen atmosphere and also stirred for 30 minutes. The enol was added by cannula to the preformed glycine imine and stirred at -78 °C for 4 hours. The solution was warmed to room temperature, stirred overnight, poured into distilled water (50 ml) and acidified to pH 3 with 1M hydrochloric acid. The mixture was extracted into diethyl ether (50 ml) and the aqueous phases washed with diethyl ether (2 x 30 ml). The combined organic phases were washed with brine (2 x 30 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a yellow oil. Purification by column chromatography (silica; pet. ether: diethyl ether; 1:1) gave a mixture of products (0.18 g, 20%). Analysis by ^{1}H-nmr spectroscopy showed no conversion to the desired product.

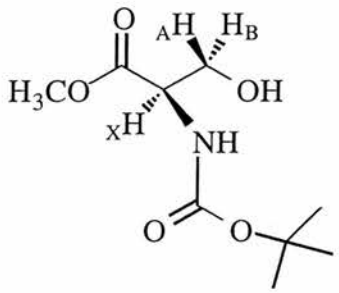
6.3 IRREVERSIBLE INHIBITORS

Methyl D-serinate hydrochloride (158)



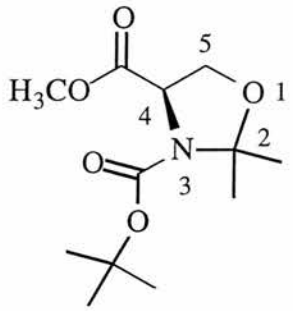
Acetyl chloride (20.8 ml, 290 mmol) was added dropwise over 10 minutes to dry, redistilled methanol (150 ml) at 0 °C and stirred for 5 minutes. D-Serine (10.62 g, 100 mmol) was added and the mixture heated to reflux for 8 hours until full conversion to product was observed by tlc (cellulose; isopropyl alcohol: concentrated ammonia: water; 26:12:10). The reaction mixture was cooled and concentrated by evaporation under reduced pressure to give crude methyl serinate hydrochloride as a white crystalline solid (15.2 g, 97%), mp 155-6 °C (lit.[209], 152-7 °C); $[\alpha]^{20}_{D}$ -8.8 (c = 1, MeOH), (lit.[209], $[\alpha]^{20}_{D}$ -9 (c = 4, MeOH)); ν_{max} (nujol)/cm^{-1} 3666 - 3533 (NH_2 and OH), 1700 (ester CO); δ_H (200 MHz; D_2O) 3.80 (3H, s, CH_3), 3.94 (1H, dd, $J_{A,B}$ 12, $J_{A,X}$ 3.5, $\underline{H}_A$ of ABX), 4.01 (1H, dd, $J_{A,B}$ 12 Hz, $J_{B,X}$ 3.5, $\underline{H}_B$ of ABX), 4.24 (1H, t, J 3.5, $\underline{H}_X$ of ABX); δ_C (50.31 MHz; D_2O) 56.5, (β-$\underline{C}H_2$), 57.5 ($CO_2\underline{C}H_3$), 62.1 (α-$\underline{C}H$), 171.7 ($\underline{C}O_2Me$); *m/z* (EI) 88 ([*M*-CH_2OH]$^+$, 47%), 60 (100, [*M*-CO]$^+$).

Methyl *N*-(*tert*-butoxycarbonyl) D-serinate (159)



Methyl D-serinate hydrochloride (**159**, 15.03 g, 96 mmol) was suspended in dry THF (200 ml) and dry triethylamine (18 ml, 130 mmol) was added. A solution of di-tert-butyl dicarbonate (Boc_2O) (13.44 g, 14.15 ml, 61.5 mmol) in dry THF (85 ml) was added at 0 °C dropwise over 30 minutes. The mixture was warmed to room temperature and stirred overnight. Then the suspension was heated to 50 °C and stirred for a further 2 hours. The solvent was removed under reduced pressure and the residue partitioned between diethyl ether (150 ml) and water (150 ml). The aqueous layer was washed with diethyl ether (2 x 120 ml) and the combined organic layers washed with 3% hydrochloric acid (120 ml), then 5% sodium hydrogen carbonate solution (120 ml) and brine (150 ml). The organic layers were dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to give the product as a yellowish oil (10.86 g, 51%); (Acc. Mass Found 220.117677 Calc. for $C_9H_{18}NO_5$, 220.118498); $[\alpha]^{20}_D$ -20.8 (c = 1, MeOH), [lit.,[158] $[\alpha]^{20}_D$ -18.9 (c = 5, MeOH)]; ν_{max} (neat)/cm^{-1} 3666 - 3466 (NH and OH), 1700 (carbamate and ester CO); δ_H (200 MHz; $CDCl_3$) 1.37 (9H, s, $C(C\underline{H}_3)_3$), 3.65 (3H, s, $CO_2C\underline{H}_3$), 3.77 (1H, dd, $J_{A,B}$ 12, $J_{A,X}$ 3.6, $\underline{H}_A$ of ABX), 3.88 (1H, dd, $J_{A,B}$ 12, $J_{B,X}$ 3.6, $\underline{H}_B$ of ABX), 4.28 (1H, t, J 3.6, $\underline{H}_X$ of ABX); δ_C (50.31 MHz, $CDCl_3$) 28.1 ($C(\underline{C}H_3)_3$), 52.9 ($CO_2\underline{C}H_3$), 58.2 (α-$\underline{C}H$), 63.4 (β-$\underline{C}H_2$), 80.0 ($\underline{C}(CH_3)_3$), 156.3 ($N\underline{C}O_2{}^tBu$), 172.0 ($\underline{C}O_2CH_3$); *m/z*(CI) 220 (M^+, 2%), 164 (100, [M+H-$C(CH_3)_3$]), 120 (44, [M+H-$CO_2C(CH_3)_3$]), 58 (22, [$C(CH_3)_3$+H]).

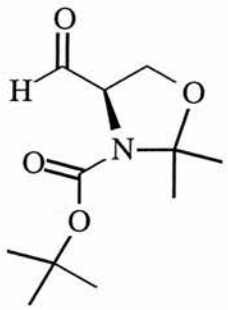
3-*tert*-Butyl-4-methyl-(4*R*)-2,2-dimethyl-1,3-oxazolidine-3,4-dicarboxylate (160)



Boron trifluoroetherate ($BF_3.OEt_2$) (0.05 ml, 0.4 mmol) was added to a solution of methyl *N*-(*tert*-butoxycarbonyl)-D-serinate (**159**, 3.01 g, 13.7 mmol) and 2,2-dimethoxypropane (9.4 ml, 76.5 mmol) in dry, redistilled acetone (38 ml). The solution was stirred for 4 hours at room temperature and then the solvent evaporated under reduced pressure to give a yellow syrup which turned red on standing. The residue was taken up in dichloromethane (40 ml), washed with saturated sodium hydrogen carbonate solution and water (1:1, 40 ml), then brine (40 ml). The organic phases were dried ($MgSO_4$) and the solvent evaporated under reduced pressure to give a clear, pale yellow syrup. Distillation using the Kugelrohr apparatus gave a clear syrup (2.69 g, 75.5%); (Found: C, 55.5; H, 8.4; N, 5.4. Calc. for $C_{12}H_{21}NO_5$: C, 55.6; H, 8.2; N, 5.4%); $[\alpha]^{20}_D$ -59.3 (c = 1, $CHCl_3$), [lit.,[153] $[\alpha]^{20}_D$ -57 (c = 1.3, $CHCl_3$)]; ν_{max} (neat)/cm^{-1} 3733 - 3573 (NH and OH), 1750 - 1650 (carbamate and ester CO); δ_H (200 MHz; $CDCl_3$) 1.39 and 1.47 (9H, s, $C(C\underline{H}_3)_3$), 1.51 and 1.65 (6H, s, $C(C\underline{H}_3)_2$), 3.75 (3H, s, $CO_2C\underline{H}_3$), 4.03 and 4.13 (2H, m, 5-$C\underline{H}_2$), 4.36 and 4.47 (1H, dd, J_1 6.9, J_2 3.1, 4-C$\underline{H}$); δ_C (50.31 MHz; $CDCl_3$) 24.7 ($\underline{C}H_3$), 25.3 ($\underline{C}H_3$), 28.6 ($C(\underline{C}H_3)_3$), 52.7 ($CO_2\underline{C}H_3$), 59.6 (4-$\underline{C}$H), 66.6 (5-$\underline{C}H_2$), 80.6 and 81.2 ($\underline{C}(CH_3)_3$), 94.8 and 95.4 ($\underline{C}(CH_3)_2$), 151.6 ($NCO_2{}^tBu$), 172.1 ($\underline{C}O_2CH_3$); *m/z* (EI) 244 ([*M*-CH_3]$^+$, 13%), 186 (9, [*M*-$OC(CH_3)_3$]$^+$), 144 (76, [*M*-$C(CH_3)_2$]$^+$), 128 (8, [*M*-$(CH_3)_2CO$]$^+$), 100 (12, [*M*-MeOC(O)C(N)CH_2]$^+$), 57 (100, $[C(CH_3)_3]^+$). NB: In solution this compound gives two sets of nmr signals, one

corresponding to the compound shown above and the other representing its hydrogen bonded form.

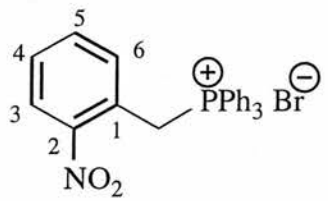
tert-Butyl-4-formyl-(4*R*)-2,2-dimethyl-1,3-oxazolidine-3-carboxylate (**156**)



3-*tert*-Butyl-4-methyl-(4*R*)-2,2-dimethyl-1,3-oxazolidine-3,4-dicarboxylate (**160**, 3.5 g, 13.5 mmol) was dissolved in anhydrous toluene (200 ml) and diisobutylaluminium hydride (25% w/w in toluene, 17 ml, 24.7 mmol) was added at -78 °C under a nitrogen atmosphere. The solution was stirred for 2 hours and then dry methanol (8 ml) was slowly added and the reaction warmed to room temperature. The mixture was poured into aqueous potassium sodium tartrate solution (31.5 g in 120 ml water) and the biphasic mixture stirred vigorously for 2 hours. The phases were separated and the aqueous layer extracted into diethyl ether (2 x 70 ml). The combined organic layers were dried ($MgSO_4$) and the solvent evaporated under reduced pressure to give a clear oil. The product was obtained as a clear oil after distillation (2.6 g, 84%); $[\alpha]^{20}_D$ -100.7 (c = 1, $CHCl_3$), [lit.,[153] $[\alpha]^{20}_D$ -91.7 (c = 1.34, $CHCl_3$)]; δ_H (200 MHz; $CDCl_3$) 1.41 and 1.49 (9H, s, $C(C\underline{H}_3)_3$), 1.53 and 1.62 (6H, s, $C(C\underline{H}_3)_2$), 4.03 - 4.06 (2H, m, 5-$C\underline{H}_2$), 4.16 - 4.18 and 4.28 - 4.36 (1H, m, 4-C<u>H</u>), 9.52 and 9.57 (1H, s, C<u>H</u>O); δ_C (50.31 MHz; $CDCl_3$) 24.5 and 25.0 ($\underline{C}H_3$), 26.3 and 27.1 ($\underline{C}H_3$), 28.7 ($C(\underline{C}H_3)_3$), 63.9 and 64.5 (4-<u>C</u>H), 65.2 and 65.8 (5-$\underline{C}H_2$), 81.6 and 81.8 ($\underline{C}(CH_3)_3$), 94.7 and 95.5 ($\underline{C}(CH_3)_2$), 152.6 ($NCO_2{}^tBu$), 199.8 (<u>C</u>HO); *m/z* (EI) 200 ([*M*-CHO]$^+$, 13%), 156 (11, [*M*-$(CH_3)_3CO$]$^+$), 144 (8, [*M*-$(CH_3)_3COC$]$^+$), 114 (6, [*M*-$(CH_3)_3CCO_2N$]$^+$), 100 (22, [*M*-^{t}BocNCH$_2$]$^+$),

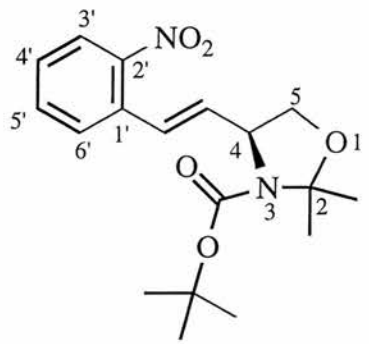
84 (16, $(CH_3)_2COCH_2CH^+$), 57 (100, $(CH_3)_3C^+$). NB: In solution this compound gives two sets of nmr signals, one corresponding to the compound shown above and the other representing its hydrogen bonded form.

2-Nitrobenzyltriphenylphosphonium Bromide (168)



Triphenylphosphine (3.9 g, 23 mmol) was dissolved in toluene (70 ml) and 2-nitrobenzyl bromide (4.97 g, 23 mmol) was added. The mixture was warmed gently for two hours and the solvent removed under reduced pressure. The resulting product was washed with diethyl ether to remove traces of 2-nitrobenzyl bromide. The product was obtained as a cream coloured crystalline solid (6.9 g, 63%), mp 275 °C [decomp] (lit:[173] 285 - 289 °C); δ_H (200 MHz; $CDCl_3$) 6.11 (2H, d, $J_{P,H}$ 14.8, C$\underline{H}_2$P), 7.56 - 7.82 (19H, m, Aromatic C$\underline{H}$); δ_C (50.31 MHz; $CDCl_3$) 29.1 (d, $J_{C,P}$ 49, $\underline{C}H_2P$), 116.8 (3-$\underline{C}$), 118.5 (5-$\underline{C}$), 124.8 (4-$\underline{C}$), 126.6 (6-$\underline{C}$), 130.5 (1-$\underline{C}$), 130.8 (3C, d, $J_{C,P}$ 12.7, $Ph_3\underline{C}P$), 134.7 (6C, d, $J_{C,P}$ 10.1, $\underline{Ph}_3P$), 135.4 (2-$\underline{C}$), 135.6 (3C, d, $J_{C,P}$ 12.2, $\underline{Ph}_3P$), 135.7 (6C, d, $J_{C,P}$ 9.6, $\underline{Ph}_3P$); *mz* (CI) 320 ([*MH*-PhBr]$^+$, 10%), 279 (100, [*MH*-Ph_2PCH_2Br]$^+$), 263 (69, [PPh_3+H]$^+$).

tert-Butyl-(4*S*)-2,2-dimethyl-4-[2-(2-nitrophenyl)-ethenyl]-1,3-oxazolidine-3-carboxylate (169)



2-Nitrobenzyltriphenylphosphonium bromide (**168**, 3.7 g, 7.72 mmol) was suspended in dry THF (70 ml) under a nitrogen atmosphere at room temperature. Potassium bis(trimethylsilyl)amide (0.5M in toluene, 22.2 ml, 11.1 mmol) was added and the resulting purple/red solution was stirred for 2 hours at room temperature. A solution of *tert*-Butyl-4-formyl-(4*R*)-2,2-dimethyl-1,3-oxazolidine-3-carboxylate (**156**, 1.0 g, 4.4 mmol) in THF (28 ml) was added dropwise at -78 °C, the solution warmed to room temperature and stirred overnight. The reaction was quenched with methanol (8.8 ml) and then poured into a mixture of potassium sodium tartrate and water 1:1 (150 ml). The aqueous phase was extracted into diethyl ether (2 x 70 ml), the combined organic layers dried ($MgSO_4$) and the solvent evaporated under reduced pressure to give an orange oil. Purification was carried out by flash column chromatography (silica; hexane: ethyl acetate, 5:1) giving the product as a yellow oil containing both *Z* and *E* isomers (1.0 g, 89%); (Acc. Mass Found 349.175477 Calc. for $C_{18}H_{25}N_2O_5$, 349.176347); $[\alpha]^{20}_D$ +21.2 (c = 1, $CHCl_3$); ν_{max} (thin film)/cm^{-1} 1660 (C=O), 1520 & 1350 (NO_2), 960 (*E*, ArCH=CHR), 770 (*o*-subst. C_6H_5), 710 (*Z*, ArCH=CHR); δ_H (200 MHz; $CDCl_3$) 1.44 (9H, s, $C(C\underline{H}_3)_3$), 1.51 (6H, s, $C(C\underline{H}_3)_2$), 4.02 - 4.13 (2H, m, 5-$C\underline{H}_2$), 4.46 - 4.56 (1H, m, 4-C<u>H</u>), 5.80 (1H, t, *J* 10.8, *Z*, ArCH=C<u>H</u>R), 6.15 (1H, dd, J_1 15.5 and J_2 6.5, *E*, ArCH=C<u>H</u>R), 6.77 (1H, d, *J* 10.8, *Z*, ArC<u>H</u>=CHR), 6.97 (1H, br d, *J* 15.5, *E*, ArC<u>H</u>=CHR), 7.27 - 7.48 (1H, m, 4'-C<u>H</u>), 7.52 - 7.67 (2H, m, 5'-C<u>H</u> and 6'-C<u>H</u>), 7.87 and 8.00 (1H, 2 x d, *J* 8.4, 3'-C<u>H</u>); δ_C (50.31 MHz; $CDCl_3$) 21.4 ($C(\underline{C}H_3)_2$), 28.8

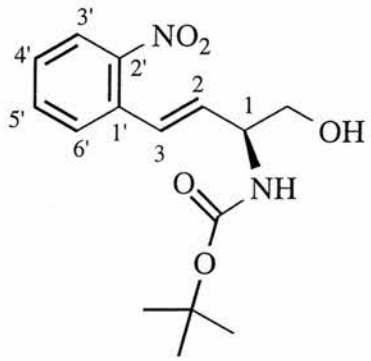
($C(\underline{C}H_3)_3$), 55.4 (4-$\underline{C}H$), 65.7 (5-$\underline{C}H_2$), 80.6 ($\underline{C}(CH_3)_3$), 94.6 ($\underline{C}(CH_3)_2$), 124.9 (3'-$\underline{C}$), 126.8 (Ar$\underline{C}$H=CHR), 128.6 (4'-$\underline{C}$), 128.8 (1'-$\underline{C}$), 129.0 (6'-$\underline{C}$), 132.5 (5'-$\underline{C}$), 133.4 (ArCH=$\underline{C}$HR), 152.1 (2'-$\underline{C}$), 171.5 ($\underline{C}$=O); *m/z* (CI) 349 ([*M*+H], 10 %), 293 (84, [*M*+H - $(CH_3)_3C$]$^+$), 263 (34, [*M*+H - $(CH_3)_3C$-CO]$^+$), 85 (85, [$(CH_3)_3COC$]$^+$), 71 (100, [CHCH(N)CH_2OH]$^+$).

A further reaction yielded 0.57g (35%) of product which was separated into the *Z* (0.28 g) and *E* (0.29 g) double bond isomers.

Spectral details for the *Z* isomer **169a** are as follows: δ_H (300 MHz; $CDCl_3$) 1.33 (3H, s, $C(C\underline{H}_3)_2$), 1.47 (9H, s, $C(C\underline{H}_3)_3$), 1.64 (3H, s, $C(C\underline{H}_3)_2$), 3.65 (1H, br s, 5-$C\underline{H}_2$), 3.88 (1H, s, 5-$C\underline{H}_2$), 4.56 (1H, heptet, *J* 3.6, 4-$C\underline{H}$), 5.83 (1H, br s, ArCH=C$\underline{H}$R), 6.80 (1H, d, *J* 10.7, Ar$C\underline{H}$=CHR), 7.42 (1H, t, *J* 7.1, 4'-$\underline{H}$), 7.62 (1H, t, *J* 7.1, 5'-$\underline{H}$), 7.78 - 7.90 (1H, m, 6'-$\underline{H}$), 8.04 (1H., d, *J* 7.1, 3'-$\underline{H}$); δ_C (74.76 MHz; $CDCl_3$) 24.2 ($C(\underline{C}H_3)_2$), 25.0 ($C(\underline{C}H_3)_2$), 28.s ($C(\underline{C}H_3)_3$), 54.8 (4-$\underline{C}H$), 66.1 and 68.1 (d, *J* 50.9, 5-$\underline{C}H_2$), 80.0 ($\underline{C}(CH_3)_3$), 94.2 and 94.8 (d, *J* 50.9, $\underline{C}(CH_3)_2$), 124.44 (3'-$\underline{C}$), 124.3 and 127.0 (d, *J* 135.7, Ar$\underline{C}$H=CHR), 128.0 (4'-$\underline{C}$), 131.6 (1'-$\underline{C}$), 131.9 (6'-$\underline{C}$), 132.0 and 134.3 (d, *J* 152.9, ArCH=$\underline{C}$HR), 132.9 (5'-$\underline{C}$), 147.9 (2'-$\underline{C}$), 172.1 ($\underline{C}$=O). Signals in the aromatic region were fully assigned using HMQC spectra (or ^1H-^{13}C COSY).

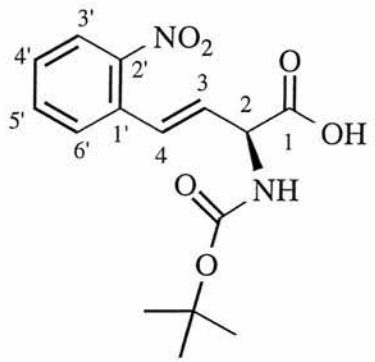
Spectral details for the *E* isomer **169b** are as follows: δ_H (200 MHz; $CDCl_3$) 1.44 (9H, s, $C(C\underline{H}_3)_3$), 1.52 (3H, s, $C(C\underline{H}_3)_2$), 1.67 (3H, s, $C(C\underline{H}_3)_2$), 3.85 (1H, d, $J_{A,B}$ 7.7, 5-$C\underline{H}_2$), 4.13 (1H, t, $J_{A,B}$ 7.7, 5-$C\underline{H}_2$), 4.4 - 4.7 (1H, m, 4-$C\underline{H}$), 6.18 (1H, dd, J_1 15.4 and J_2 7.7, *E,* ArCH=C$\underline{H}$R), 7.0 (1H, br d, $J_1$15.4, *E,* Ar$C\underline{H}$=CHR), 7.39 (1H, d, *J* 9.2, 4'-$\underline{H}$), 7.5 - 7.65 (2H, m, 5'-$\underline{H}$ and 6'-$\underline{H}$), 7.92 (1H., d, *J* 7.1, 3'-$\underline{H}$); δ_C (50.31 MHz; $CDCl_3$) 24.7 ($C(\underline{C}H_3)_2$), 28.1 ($C(\underline{C}H_3)_2$), 29.5 ($C(\underline{C}H_3)_3$), 60.1 (4-$\underline{C}H$), 69.2 (5-$\underline{C}H_2$), 81.3 ($\underline{C}(CH_3)_3$), 95.0 ($\underline{C}(CH_3)_2$), 125.5 (3'-$\underline{C}$), 127.4 (s, Ar$\underline{C}$H=CHR), 129.5 (4'-$\underline{C}$), 130.2 (1'-$\underline{C}$), 134.5 (6'-$\underline{C}$), 135.8 (s, ArCH=$\underline{C}$HR), 148.7 (5'-$\underline{C}$), 155.3 (2'-$\underline{C}$), 173.1 ($\underline{C}$=O).

***tert*-Butyl-(1*S*)-1-(hydroxymethyl)-3-(2-nitrophenyl)-2-propenyl Carbamate (170)**



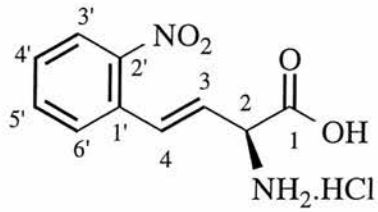
tert-Butyl-(4*S*)-2,2-dimethyl-4-[2-(2-nitrophenyl)-ethenyl]-1,3-oxazolidine-3-carboxylate (**169**, 0.52 g, 1.5 mmol) was dissolved in methanol (20 ml) and *p*-toluenesulfonic acid (0.05 g, 0.3 mmol) was added. The mixture was heated under reflux for 5 hours and then cooled. The solvent was removed under reduced pressure and the residue taken up in ethyl acetate (20 ml) and washed with saturated sodium hydrogen carbonate solution (20 ml). The organic fractions were dried ($MgSO_4$) and the solvent removed under reduced pressure. Three spots were visible by tlc under UV and $KMnO_4$ dip. The base line spot was separated by flash column chromatography (silica, hexane: ethyl acetate 2:1, graduated to 1:1), and the product obtained as an orange syrup (0.28 g, 60%); $[\alpha]^{20}_D$ +19 (c = 0.5, MeOH); ν_{max} (thin film)/cm^{-1} 3000 (br OH), 1660 (C=O), 1520 & 1350 (NO_2), 960 (*E*, ArCH=CHR), 770 (*o*-subst. C_6H_5), 710 (*Z*, ArCH=CHR); δ_H (200 MHz; $CDCl_3$) 1.31 (9H, s, $C(C\underline{H}_3)_3$), 3.6 (2H, br s, $C\underline{H}_2OH$), 4.15 - 4.35 (1H, m, 1-C<u>H</u>), 5.27 and 5.48 (1H, d, *J* 9, N<u>H</u>), 5.63 (1H, t, *J* 10, *Z*, 2-C<u>H</u>), 6.1 (1H, dd, J_1 16, J_2 6, *E*, 2-C<u>H</u>), 6.66 (1H, d, *J* 22.5, *Z*, 3-C<u>H</u>), 6.88 (1H, d, *J* 17, *E*, 3-C<u>H</u>),7.23 - 7.53 (3H, m, aromatic <u>H</u>), 7.73 and 7.88 (1H, 2 x d, *J* 8.7, 3'<u>H</u>); δ_C (74.76 MHz; $CDCl_3$) 28.66 ($C(\underline{C}H_3)_3$), 51.0 (1-<u>C</u>), 65.19 ($\underline{C}H_2OH$), 79.92 ($\underline{C}(CH_3)_3$), 124.86 (3'-<u>C</u>), 126.39 (3-<u>C</u>), 128.68 (4'-<u>C</u>), 131.25 (1'-<u>C</u>), 132.57 (6'-<u>C</u>), 133.78 (2-<u>C</u>), 148.21 (5'-<u>C</u>), 155.92 (2'-<u>C</u>), 171.56 (<u>C</u>=O); *m/z* (CI) 309 [*M*+H, 42%], 293 (6, $[M\text{-}CH_3]^+$), 277 (8, $[M\text{-}CH_2OH]^+$), 253 (100, $[M\text{-}C(CH_3)_3]^+$), 209 (50, $[M\text{-}^tBoc+H]^+$), 164 (61, $[M\text{-}NO_2]^+$) 74 (93, $[M\text{-}^tBocNH+H]^+$).

(2*R*)-2-[(*tert*-Butoxycarbonyl)amino]-4-(2-nitrophenyl)-3-butenoic Acid (171)



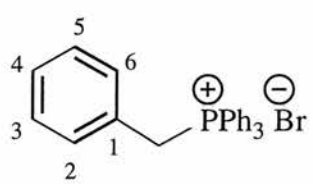
tert-Butyl-(1*S*)-1-(hydroxymethyl)-3-(2-nitrophenyl)-2-propenyl carbamate (**170**, 145 mg, 0.49 mmol) was dissolved in acetone (20 ml) and cooled to 0 °C in an ice bath. Jones reagent (0.5 ml, 0.5 mmol) was added and the solution allowed to warm to room temperature and stirred for 5 hours. The reaction mixture was quenched with methanol (10 ml) a little water added and extracted into ethyl acetate. The organic phase was separated, dried ($MgSO_4$) and the solvent removed under reduced pressure. The product was obtained as an orange syrup (0.736 g, 85%); (Found: C, 56.1; H, 5.9; N, 8.2. Calc. for $C_{15}H_{18}N_2O_6$: C, 55.9; H, 5.6; N, 8.7%); *Z* $[\alpha]^{20}_D$ +22 (c = 0.02, MeOH); *E* $[\alpha]^{20}_D$ +60 (c = 0.02, MeOH); ν_{max} (thin film)/cm^{-1} 3000 (br OH), 1780 (CO_2H), 1660 (C=O), 1520 & 1350 (NO_2), 960 (*E* ArCH=CHR), 770 (*o*-subst. C_6H_5), 710 (*Z* ArCH=CHR); δ_H (200 MHz; CD_3OD) 1.53 (9H, s, $C(C\underline{H}_3)_3$), 4.71(1H, d, *J* 10.2, 1-C<u>H</u>), 5.79 (1H, t, *J* 10.2, *Z*, 3-C<u>H</u>), 6.40 (1H, dd, J_1 15.4, J_2 5, *E*, 3-C<u>H</u>), 7.05 (1H, d, *J* 10.2, *Z*, 4-C<u>H</u>), 7.11 - 7.18 (1H, m, *E*, 4-C<u>H</u>), 7.42 - 8.13 (4H, m, aromatics); δ_C (50.31 MHz; CD_3OD) 28.97 (s, $C(\underline{C}H_3)_3$), 53.6 (s., 1-<u>C</u>), 81.0 (s, $\underline{C}(CH_3)_3$), 125.72 (2-<u>C</u>), 126.02 (3'-<u>C</u>), 128.35 (6'-<u>C</u>), 130.04 (4'-<u>C</u>), 130.10 (1'-<u>C</u>), 130.31 (5'-<u>C</u>), 133.09 (3-<u>C</u>), 134.82 (2'-<u>C</u>), 149.79 ($\underline{C}O_2{}^tBu$), 174.25 ($\underline{C}O_2H$); *m/z*. (CI) 323 ([*M*+H], 11%), 267 (82, *M*+H-$C(CH_3)_3$]$^+$), 237 (57, *M*+H-$(CH_3)_3CO]^+$), 223 (54, [*M*+H-^{t}Boc]$^+$), 194 (95, [*M*+H-^{t}BocNHCH]$^+$), 176, (100, ^{t}BocNHCH$_2$CO$_2$H+H), 168 (29, [*M*+H-^{t}BocNH$(CH)_3]^+$), 146 (47, [*M*+H-ArH]$^+$), 134 (27, $CArNO_2$), 118, (44, [*M*+H-CO]$^+$).

(2*S*)-2-Amino-4-(2-nitrophenyl)-3-butenoic acid (145)



To dry ethyl acetate (10 ml) was added (2*R*)-2-[(*tert*-Butoxycarbonyl)amino]-4-(2-nitrophenyl)-3-butenoic acid (**171**, 94 mg, 0.29 mmol) in dry ethyl acetate (5 ml). The solution was saturated with HCl gas and stirred for two hours, then resaturated and stirred overnight. The reaction was followed by tlc (silica, 2:1; ethyl acetate: diethyl ether and cellulose, isopropyl alcohol: ammonia: water; 26:6:10). The solvent was removed under reduced pressure and the solid residue recrystallised from methanol and water to give a pale brown solid (72 mg, 95%); mp 168-172 °C (decomp.); *Z* $[\alpha]^{20}_{D}$ +140 (c = 0.02, MeOH); $[\alpha]^{20}_{D}$ -155 (c = 0.02, MeOH); δ_H (200 MHz, CD_3OD) 4.58 (1H, d, *J* 12.7, 1-C<u>H</u>), 5.86 (1H, t, *J* 12.7, *Z*, 2-C<u>H</u>), 6.26 (1H, dd, J_1 11.5, J_2 2 5, *E*, 2-C<u>H</u>), 7.24 - 7.46 (2H, m, *Z* & *E*, 3-C<u>H</u>), 7.54 - 7.84 (4H, m, <u>Ar</u>NO_2); δ_C (50.31 MHz, CD_3OD) 64.8 (1-<u>C</u>), 123.1 (2-<u>C</u>), 126.5 (3'-<u>C</u>), 130.3 (6'-<u>C</u>), 131.1 (4'-<u>C</u>), 132.8 (1'-<u>C</u>), 133.7 (5'-<u>C</u>), 135.5 (3-<u>C</u>), 141.5 (2'-<u>C</u>), 170.8 (<u>C</u>O_2H); *mz* (CI) 257 (14%, [*M* - H]$^+$), 227 (7, [*M*+H-OOH]$^+$), 146 (29, [$NO_2Ar(CH)_2$]$^+$), 57 (100, [$(CH_3)_3C$]$^+$).

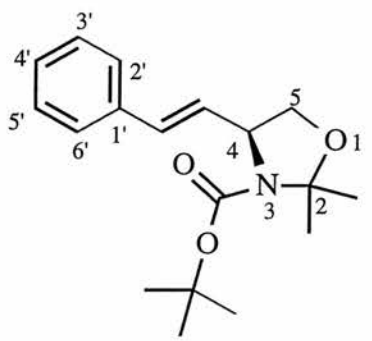
Benzyltriphenylphosphonium Bromide (172)



Triphenylphosphine (6.07 g, 23 mmol) was dissolved in toluene (70 ml) and benzyl bromide (3.97 g, 23 mmol) was added. The mixture was warmed gently for two hours and the solvent then removed under reduced pressure. The resulting product was washed

with diethyl ether to remove any traces of benzyl bromide. The product was obtained as a white crystalline solid, (9.05 g, 90%) mp 294 - 297 °C (lit.,[210] 288 °C); δ_H (200 MHz; $CDCl_3$) 5.4 (2H, d, $J_{P,H}$ 18, C<u>H</u>$_2$P), 7.3 (5H, m, C$_6$<u>H</u>$_5$), 7.7 (10H, m., (C$_6$<u>H</u>$_5$)$_3$); δ_C (50.31 MHz; $CDCl_3$) 31.3 (d, $J_{C,P}$ 47, <u>C</u>H$_2$P), 117.3 (4-<u>C</u>), 118.9 (1-<u>C</u>), 128.8 (3-<u>C</u> & 5-<u>C</u>), 129.3 (2-<u>C</u> & 6-<u>C</u>), 130.6 (6C, d, $J_{C,P}$ 12.6, <u>Ph</u>$_3$P), 131.9 (3C, s, Ph$_3$<u>C</u>P), 134.7 (3C, d, $J_{C,P}$ 9.6, <u>Ph</u>$_3$P), 135.5(6C, s, <u>Ph</u>$_3$P); *m/z* (CI) 279 (69%, [*MH*-Ph$_2$PCH$_2$Br]$^+$), 263 (100, [PPh$_3$ +H]$^+$).

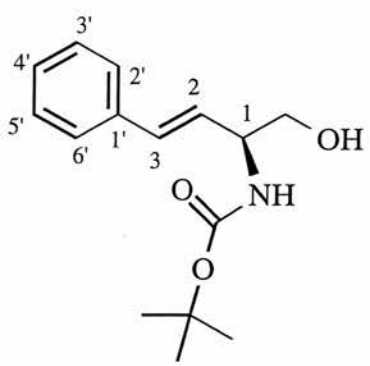
tert-Butyl-(4*S*)-2,2-dimethyl-4-(2-phenylethenyl)-1,3-oxazolidine-3-carboxylate (173)



Benzyltriphenylphosphonium bromide (**172**, 3.15 g, 7.37 mmol) was suspended in dry THF (70 ml) under a nitrogen atmosphere at room temperature. Potassium bis(trimethylsilyl)amide (0.5M in toluene, 21.2 ml, 10.6 mmol) was added and the resulting orange solution was stirred for 2 hours at room temperature. A solution of *tert*-Butyl-4-formyl-(4*R*)-2,2-dimethyl-1,3-oxazolidine-3-carboxylate (**156**, 1.0 g, 4.4 mmol) in THF (28 ml) was added dropwise at -78 °C, the solution warmed to room temperature and left overnight. The reaction was quenched with methanol (8.8 ml) and then poured onto a mixture of potassium sodium tartrate and water 1:1 (150 ml). The aqueous phase was extracted into diethyl ether (2 x 70 ml), the combined organic layers dried ($MgSO_4$) and the solvent evaporated under reduced pressure. Purification was carried out by flash column chromatography (silica; hexane: ethyl acetate; 5:1) giving the product as a pale

yellow oil (0.65 g, 49%); (Acc. Mass Found: 304.191758 Calc. for $C_{18}H_{26}NO_3$, 304.191269); $[\alpha]^{20}_D$ +75.5 (c = 1, $CHCl_3$); ν_{max} (thin film)/cm^{-1} 3000 (br OH), 1770 (C=O), 950 (*E*, ArCH=CHR), 720 (*Z*, ArCH=CHR); δ_H (200 MHz; $CDCl_3$) 1.5 (9H, s, $C(C\underline{H}_3)_3$), 1.52 (3H, s, $C(C\underline{H}_3)_2$), 1.64 (3H, s, $C(C\underline{H}_3)_2$), 3.85 (2H, dd, J_1 8.8, J_2 2.5, *E*, 5-$C\underline{H}_2$), 4.15 (2H, m, 5-$C\underline{H}_2$), 4.5 (*E*) and 4.9 (*Z*) (1H, br s, 4-C<u>H</u>), 5.7 (1H, t, *J* 10.5, *Z*, ArCH=C<u>H</u>R), 6.15 (1H, dd, J_1 16.5, J_2 7, *E*, ArCH=C<u>H</u>R), 6.45 (1H, d, *J* 10.5, *Z*, Ar<u>CH</u>=CHR), 6.55 (1H, d, *J* 16.5, *E* , Ar<u>CH</u>=CHR), 7.19 - 7.39 (5H, m, $C_6\underline{H}_5$); δ_C (50.31 MHz; $CDCl_3$) 24.1 ($C(\underline{C}H_3)_2$), 26.7 ($C(\underline{C}H_3)_2$), 28.5 ($C(\underline{C}H_3)_3$), 59.5 (4-<u>C</u>), 68.3 and 69.2 (d, *J* 33.5, 5-<u>C</u>), 79.8 ($\underline{C}(CH_3)_3$), 94.2 ($\underline{C}(CH_3)_2$), 126.4 (ArCH=<u>C</u>HR), 127.1 (6'-<u>C</u>), 127.6 (2'-<u>C</u>), 128.3 (4'-<u>C</u>), 128.6 (5'-<u>C</u>), 128.8 (3'-<u>C</u>), 133.0 (Ar<u>C</u>H=CHR), 136.7 (1'-<u>C</u>), 152.0 (<u>C</u>=O); *m/z* (CI) 304 ([*M*+H], 27%), 248 (100, $[M+H - C(CH_3)_3]^+$), 204 (19, $[M+H - {}^tBuO_2C^+]$).

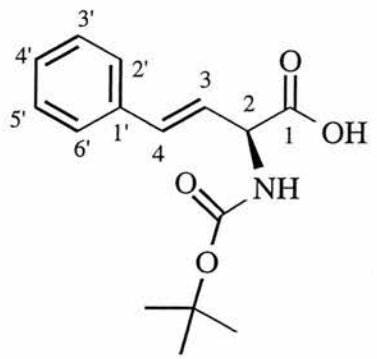
tert-Butyl-(1*S*)-1-(hydroxymethyl)-3-phenyl-2-propenyl Carbamate (174)



tert-Butyl-(4*S*)-2,2-dimethyl-4-(2-phenylethenyl)-1,3-oxazolidine-3-carboxylate (**173**, 0.31 g, 1.0 mmol) was dissolved in methanol (20 ml) and *p*-toluenesulfonic acid (0.05 g, 0.3 mmol) was added. The mixture was heated under reflux for 5 hours and then cooled. The solvent was removed under reduced pressure and the residue taken up in ethyl acetate (20 ml) and washed with saturated sodium hydrogen carbonate solution (20 ml). The organic fractions were dried ($MgSO_4$) and the solvent removed under reduced pressure. Three spots were observed by tlc, the base line spot was separated by flash column

chromatography (silica, hexane: ethyl acetate 2:1, then 1:1), and the product obtained as an off-white syrup (0.25 g, 93%); (Acc. Mass Found 278.138321 Calc. for $C_{15}H_{20}NO_4$, 278.139233); $[\alpha]^{20}_D$ +2.3 (c = 1, $CHCl_3$); ν_{max} (thin film)/cm^{-1} 2950 (br OH), 1770 (C=O), 950 (*E*, ArCH=CHR), 720 (*Z*, ArCH=CHR); δ_H (200 MHz; $CDCl_3$) 1.45 (9H, s, $C(C\underline{H}_3)_3$), 3.62 - 3.79 (2H, m, $C\underline{H}_2OH$), 4.31 - 4.45 (1H, m, 1-C<u>H</u>), 5.01 and 5.17 (1H, 2 x d, *J* 7.9, N<u>H</u>), 5.57 (1H, t, *J* 10.5, *Z*, 2-<u>H</u>), 6.14 (1H, dd, J_1 15.7, J_2 5.3, *E*, 2-<u>H</u>), 6.51 - 6.58 (1H, m, *Z*, 3-<u>H</u>), 6.6 (1H, d, *J* 15.7, *E*, 3-<u>H</u>), 7.18 - 7.72 (5H, m, $C_6\underline{H}_5$); δ_C (50.31 MHz; $CDCl_3$) 28.9 ($C(\underline{C}H_3)_3$), 55.1 (1-<u>C</u>), 65.6 ($\underline{C}H_2OH$), 80.3 ($\underline{C}(CH_3)_3$), 126.9 (2-<u>C</u>), 127.5 (6'-<u>C</u>), 127.6 (2'-<u>C</u>), 128.1 (4'-<u>C</u>), 129.0 (5'-<u>C</u>), 129.2 (3'-<u>C</u>), 132.6 (3-<u>C</u>), 137.1 (1'-<u>C</u>), 156.6 (<u>C</u>=O); *m/z* (CI) 264 [*M*+H, 8%], 248 (11, [*M*-CH_3]$^+$), 232 (17, [*M*-CH_2OH]$^+$), 208 (100, [*M*-$(CH)_2NH(CH_2)$]$^+$), 180 (56, [*M*-$(CH)_2NHCO(CH_2)$]$^+$), 147 (77, [*M*- ^{t}BocNH]$^+$) 132 (69, [*M*-$Ar(CH)_3CH_2$+H]$^+$), 57 (55, $(CH_3)C^+$).

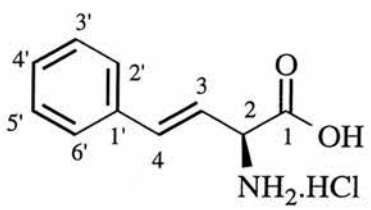
(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid (175)



tert-Butyl-(1*S*)-1-(hydroxymethyl)-3-phenyl-2-propenyl carbamate (**174**, 2.046 g, 9.13 mmol) was dissolved in acetone (50 ml) and cooled to 0 °C in an ice bath. Jones reagent (9.5 ml, 9.5 mmol) was added and the solution allowed to warm to room temperature and stirred for 5 hours. The reaction mixture was quenched with methanol (50 ml) and water (100 ml) and extracted into ethyl acetate (3 x 50 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. Purification by column chromatography (silica, 2:1; pet. ether: ethyl acetate) gave the product as an orange syrup (0.697 g, 85%) of which

E double bond isomer constituted 0.24 g and *Z* 0.45 g; (Acc. Mass Found 278.138321 Calc. for $C_{15}H_{20}NO_4$ 278.139233); *E* $[\alpha]^{20}_D$ +10.3 (c = 0.3, MeOH), *Z* $[\alpha]^{20}_D$ +64.2 (c = 0.5, MeOH); ν_{max} (thin film)/cm^{-1} 3000 (br OH), 1770 (C=O), 950 (*E*, ArCH=CHR), 720 (*Z*, ArCH=CHR); *E* δ_H (300 MHz, $CDCl_3$) 1.47 (9H, s, $C(C\underline{H}_3)_3$), 4.3 - 4.53 (1H, m, 2-C<u>H</u>), 5.01 - 5.4 (1H, br m, N<u>H</u>), 6.4 (1H, d, *J* 15.9, *E*, 4-C<u>H</u>), 7.38 - 7.58 (5H, m, $C_6\underline{H}_5$), 7.79 (1H, d, *J* 15.9, *E*, 3-C<u>H</u>); *Z* δ_H (200 MHz, $CDCl_3$) 1.45 (9H, s, $C(C\underline{H}_3)_3$), 4.63 - 4.77 (1H, m, 2-C<u>H</u>), 5.21 (1H, d, *J* 10, N<u>H</u>), 5.53 (1H, t, *J* 12.5, *Z*, 3-C<u>H</u>), 6.59 (1H, d, *J* 15, *Z*, 4-C<u>H</u>), 7.24 - 7.37 (5H, m, $C_6\underline{H}_5$); *E* δ_C (74.76 MHz, $CDCl_3$) 28.2 ($C(\underline{C}H_3)_3$), 66.3 (2-<u>C</u>H), 80.0 ($\underline{C}(CH_3)_3$), 117.6 (3-<u>C</u>H), 128.5 (2'-<u>C</u> & 6'-<u>C</u>), 129.1 (3'-<u>C</u> & 5'-<u>C</u>), 130.3 (4'-C), 130.8 (4-<u>C</u>H), 133.8 (1'-<u>C</u>), 147.1 ($\underline{C}O_2{}^tBu$), 172.5 ($\underline{C}O_2H$); *Z* δ_C (50.31 MHz, $CDCl_3$), 28.7 ($C(\underline{C}H_3)_3$), 66.0 (2-<u>C</u>H), 80.5 ($\underline{C}(CH_3)_3$), 126.8 (3-<u>C</u>H), 127.3 (2'-<u>C</u> & 6'-<u>C</u>), 128.0 (3'-<u>C</u> & 5'-<u>C</u>), 128.8 (4'-<u>C</u>), 132.5 (4-<u>C</u>H), 136.8 (1'-C), 147.3 ($\underline{C}O_2{}^tBu$), 171.9 ($\underline{C}O_2H$); *m/z* (CI) 278 ([*M*+*H*], 5%), 264 (72, $[M\text{-}CH_2]^+$), 232 (19, $[M+H\text{-}CO_2H]^+$), 222 (32, $[M+H\text{ -}(CH_3)_3C]^+$), 208 (100, $[M\text{-}(CH)_2CO_2H]^+$) 57 (53, $CH_3)_3C^+$).

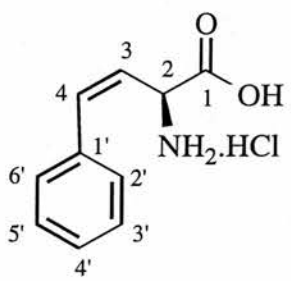
(3*E*)-2-Amino-4-phenyl-3-butenoic acid hydrochloride (146a)



To dry ethyl acetate (10 ml) was added (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid (151 mg, 0.54 mmol) in dry ethyl acetate (5 ml). The solution was saturated with HCl gas and stirred for two hours, then resaturated and stirred overnight. The reaction was followed by tlc (silica, 2:1; ethyl acetate: diethyl ether and cellulose, isopropyl alcohol: ammonia: water; 26:6:10). The solvent was removed under reduced pressure and the solid residue recrystallised from ethanol and water to give an off-white solid (89 mg, 76.5%); mp 128-130 °C; $[\alpha]^{20}_D$ +2.4 (c = 0.25, MeOH), δ_H (200 MHz,

CD_3OD) 6.47 (1H, d, *J* 17.5, 2-C<u>H</u>), 7.35 - 7.61 (5H, m, $C_6\underline{H}_5$), 7.68 (1H, d, *J* 17.5, 3-C<u>H</u>); δ_C (50.31 MHz, CD_3OD) 62.1 (1-<u>C</u>H), 119.6 (2-<u>C</u>H), 129.5 (2'-<u>C</u>), 129.8 (6'-<u>C</u>), 130.1 (5'-<u>C</u>), 130.3 (3'-<u>C</u>), 131.8 (4'-<u>C</u>), 134.4 (3-<u>C</u>H), 136.0 (1'-<u>C</u>), 170.7 (s., <u>C</u>O_2H); *m/z* (EI) 213 ([M^+], 7%), 148 (73, [M-$CHNH_2$.HCl]$^+$), 69 (100, [$CH(NH_2)CHCO]^+$).

(3*Z*)-2-Amino-4-phenyl-3-butenoic acid hydrochloride (146b)



This was prepared in an analogous manner to the *(E)*-isomer above (105 mg, 0.38 mmol) yielding a pale yellow oily solid (30 mg, 37.1%); $[\alpha]^{20}_D$ +51.3 (c = 0.15, MeOH), δ_H (200 MHz, CD_3OD) 5.73 (1H, t, *J* 10, 2-C<u>H</u>), 6.48 (1H, d, *J* 17.5, 3-C<u>H</u>), 7.31 - 7.72 (5H, m, $C_6\underline{H}_5$); δ_C (50.31 MHz, CD_3OD) 61.9 (1-<u>C</u>H), 120.5 (2-<u>C</u>H), 128.3 (2'-<u>C</u>), 129.5 (6'-<u>C</u>), 129.9 (5'-<u>C</u>), 130.1 (3'-<u>C</u>), 130.4 (4'-<u>C</u>), 131.8 (3-<u>C</u>H), 139.4 (1'-<u>C</u>), 173.3 (<u>C</u>O_2H); *m/z* (EI) 213 ([M^+], 7%), 148 (73, [M-$CHNH_2$.HCl]$^+$), 69 (100, [$CH(NH_2)CHCO]^+$).

Preparation of Mosher's amides of 2-amino-4-phenyl-3-butenoic acid hydrochloride and 2-amino-4-(2-nitrophenyl)-3-butenoic acid

The following procedure was used for the small scale preparation of MTPA derivatives for nmr studies. (*R*)-(+)-α-Methoxy-α-trifluoromethylphenylacetic acid [(+)-MTPA] was converted to the (*S*)-(+)-MTPA-Cl by heating with thionyl chloride under reflux for 48 hours.[174] Dry pyridine (0.3 ml) was added to a dry sample vial and (+)-

MTPA-Cl (15 mg, 0.06 mmol) was injected into the solution and carbon tetrachloride was added (0.3 ml). The solution was shaken and then added to the amine [(3*E*) or (3*Z*)-2-amino-4-phenyl-3-butenoic acid (10 mg, 0.047 mmol); (3*E*) or (3*Z*)-2-amino-4-(2-nitrophenyl)-3-butenoic acid (10 mg, 0.038 mmol)] and shaken again. The reaction was left to stand at room temperature for 20 minutes then excess 3-dimethylamino-1-propylamine (0.5 ml) was added. The solution was then quenched with diethyl ether (10 ml). The organic phases were washed with dilute hydrochloric acid (10 ml), saturated sodium carbonate solution (10 ml), brine (10 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to an oil. ^{1}H-Nmr and ^{19}F-nmr spectroscopy was then used to show that no racemisation had taken place at the 2-position.

Attempted epoxidation of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid and (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-(2-nitrophenyl)-3-butenoic acid

To (3*Z*)-(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid (35 mg, 0.13 mmol) was added *m*-chloroperoxybenzoic acid (44 mg, 0.13 mmol) dissolved in dichloromethane (10 ml). The solution was stirred overnight under a nitrogen atmosphere and the solution filtered and solvent removed under reduced pressure. ^{1}H-Nmr spectroscopy revealed that none of the desired product had been synthesised.

A similar reaction was carried out on (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-(2-nitrophenyl)-3-butenoic acid (20 mg, 0.06 mmol) using an equimolar quantity of *m*-CPBA (20 mg, 0.06 mmol). None of the desired epoxide was visible by ^{1}H-nmr.

(3*E*)-(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid (31 mg, 0.11 mmol) was suspended in 1M anhydrous peroxyacetic acid (2 ml) and stirred under a nitrogen atmosphere for 48 hours. The solvent was removed under reduced pressure to yield an

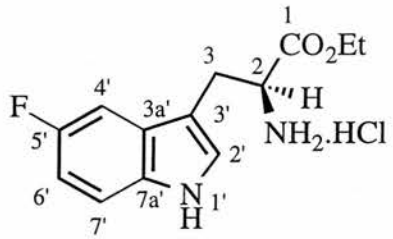
orange oil. Analysis by ^{1}H-nmr spectroscopy showed that none of the desired epoxide product had been formed.

A further experiment was carried out on (3*E*)-(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-phenyl-3-butenoic acid (33 mg, 0.11 mmol) in neat hydrogen peroxide (1 ml). The reaction was heated at reflux for 3 hours, cooled and the solvent removed under reduced pressure. The residue was taken up in water (5 ml) and extracted into ethyl acetate (2 x 5 ml). The organic phases were dried ($MgSO_4$) and the solvent removed under reduced pressure. ^{1}H-Nmr spectroscopy in $CD_3OD/CDCl_3$ showed no conversion to product.

A similar experiment in basic peroxide was carried out as described below. To (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-4-(2-nitrophenyl)-3-butenoic acid (33 mg, 0.1 mmol) in warm ethanol (10 ml) was added sodium carbonate (0.2 g, 1.8 mmol) and a premixed solution of hydrogen peroxide (2 ml) and water (5 ml). The solution changed colour from brown to yellow. The solution was stirred for 48 hours and the solvent removed under reduced pressure. The residue was taken up in water (5 ml) and extracted into diethyl ether (2 x 5 ml). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure to give a yellow oil. Reverse phase tlc (acetonitrile: water; 90:10) gave a single spot of the same R_f as the starting material. ^{1}H-Nmr spectroscopy in $CD_3OD/CDCl_3$ showed loss of double bond character, but no epoxide was observed.

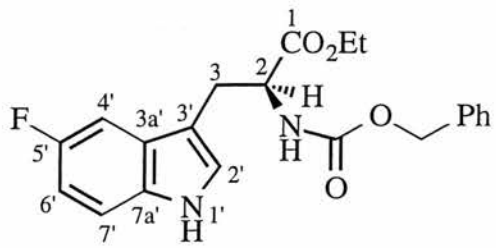
6.4 FLUORO-KYNURENINE AND FLUORO-KYNURENIC ACID

(2*S*)-5-Fluorotryptophan ethyl ester hydrochloride (184)



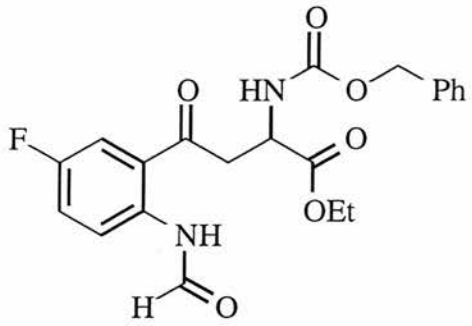
5-Fluorotryptophan (**176**, 1 g, 4.50 mmol) was dissolved in dry redistilled ethanol (20 ml) and cooled in ice. Redistilled thionyl chloride (0.35 ml, 4.80 mmol, 1.1 eq) was added and the solution heated at reflux for 1 hour at approximately 100 °C. The resulting solution was then evaporated at reduced pressure to obtain a white solid which was recrystallised from ethanol to give white crystals (1.26 g, 98%), mp 228–230° C; (Found: C, 54.24; H, 5.42; N, 9.75. Calc. for $C_{13}H_{16}ClFN_2O_2$: C, 54.45; H, 5.62; N, 9.77%); ν_{max} (nujol)/cm^{-1} 3000 (NH), 1725 (ester CO); δ_H (200 MHz; D_2O) 1.13 (3H, t, *J* 7.1, $CO_2CH_2C\underline{H}_3$), 3.35 (1H, d, $J_{2,3}$ 6.5, 3-C$\underline{H}_2$), 4.16 (2H, q, *J* 7.1, $CO_2C\underline{H}_2CH_3$), 4.33 (2H, t, $J_{2,3}$ 6.5, 2-C$\underline{H}$), 6.97 (1H, dt, $J_{5',6'} = J_{6',7'}$ 9.1 and $J_{4',6'}$ 2.5, 6'-C$\underline{H}$), 7.21 (1H, dd, $J_{4',5'}$ 9.1 and $J_{4',6'}$ 2.5, 4'-C$\underline{H}$), 7.27 (1H, s, 2'$\underline{H}$), 7.39 (1H, m, 7'-C$\underline{H}$); δ_C (50.31 MHz; D_2O) 15.84 ($CO_2CH_2\underline{C}H_3$), 28.55 (3-$\underline{C}H_2$), 56.19 (2-$\underline{C}$H), 66.47 ($CO_2\underline{C}H_2CH_3$), 105.31 (3'-$\underline{C}$), 105.79 (7'-$\underline{C}$), 112.97 (5'-$\underline{C}$), 113.49 (6'-$\underline{C}$), 115.61 (4'-$\underline{C}$), 115.79 (2'-$\underline{C}$), 129.86 (7a'-$\underline{C}$), 135.62 (3a'-$\underline{C}$), 172.82 (1-$\underline{C}O_2$Et); *m/z* (CI) 251 ([*M*+H], 100%), 235 (35, [*M*+H-NH_2]$^+$), 148 (55, [C_9H_7NF]$^+$).

(2*S*)-{[(Benzyloxy)carbonyl]amino}-5-fluorotryptophan ethyl ester (185)



Sodium carbonate (0.852 g, 8.04 mmol, 2.2 eq) and benzyl chloroformate (0.748 g, 4.38 mmol, 1.2 eq) were added to 5-fluorotryptophan ethyl ester hydrochloride (**184**, 1.047 g, 3.65 mmol) in water (200 ml) and stirred at room temperature overnight. Precipitation occurred immediately upon the addition of benzyl chloroformate. The white product which had precipitated out of solution was filtered off. The product was obtained as white crystals following recrystallisation in ethanol (1.367 g, 97%), mp 69-71 °C; (Found: C, 65.73; H, 5.26; N, 7.22. Calc.for $C_{21}H_{21}FN_2O_4$: C, 65.61; H, 5.50; N, 7.29%); ν_{max} (nujol)/cm^{-1} 3030 (NH), 1650 (carbamate CO); δ_H (200 MHz; $CDCl_3$) 1.24 (3H, t, *J* 7, $OCH_2C\underline{H}_3$), 3.25 - 3.28 (2H, m, 3-C$\underline{H}_2$), 4.12 (2H, q, *J* 7, $OC\underline{H}_2CH_3$), 4.6 - 4.75 (1H, m, 2-C$\underline{H}$), 5.11 (2H, s, C$\underline{H}_2$Ph), 5.41 (1H, br d, *J* 8, amide NH), 6.92 - 6.98 (2H, m, 6'H and 2'H), 7.12 - 7.24 (2H, m, 4'H and 7'H), 7.34 (5H, s, CBz aromatic), 8.36 (1H, br s, indole NH); δ_F (298 MHz; $CDCl_3$) -46.53; δ_C (50.31 MHz; $CDCl_3$) 14.55 ($CO_2CH_2\underline{C}H_3$), 28.46 (3-$\underline{C}H_2$), 58.96 (2-$\underline{C}$H), 62.16 ($CO_2\underline{C}H_2CH_3$), 67.52 ($\underline{C}H_2$Ph), 103.81 (3'$\underline{C}$), 104.28 (7'$\underline{C}$), 110.82 (5'$\underline{C}$), 111.35 (6'$\underline{C}$), 112.3 (4'$\underline{C}$), 112.49 (2'$\underline{C}$), 125.2 (7a'$\underline{C}$), 128.63, 128.70, 129.05 (3C, aromatic), 133.06 (quat. aromatic), 136.5 (3a'$\underline{C}$), 156.25 (NH$\underline{C}$O), 160.7 ($\underline{C}OCH_2$Ph), 172.38 ($\underline{C}O_2$Et); *m/z* (CI) 386 ([*MH*+H], 73%), 342 (100, [*M*+H-CO_2]$^+$), 278 (71, [*M*+H-PhCH_2OH]$^+$), 252 (57, [*M*+H-C_8H_5NF]$^+$), 148 (51, [C_9H_7NF]$^+$), 91 (37, [$PhCH_2$]$^+$).

Attempted Ozonolysis of (2*S*)-{[(Benzyloxy)carbonyl]amino}-5-fluorotryptophan ethyl ester (185)

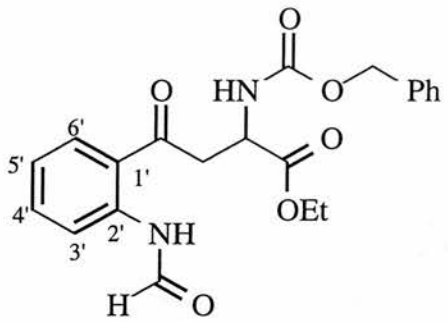


(2*S*)-{[(Benzyloxy)carbonyl]amino}-5-fluorotryptophan ethyl ester (**185**, 0.974 g, 2.54 mmol) was dissolved in dry distilled methanol (110 ml). The solution was cooled to -78 °C with stirring and then treated with ozone for one hour. The reaction was then quenched with dimethyl sulphide (10 ml). The reaction was checked by tlc (silica, ethyl acetate: dichloromethane, 1:9) and left to stir for a further hour at -78 °C. The solution was then evaporated at reduced pressure to give a brown/orange oil. The oil was dissolved in dichloromethane (20 ml) and washed with water (3 x 50 ml) and brine (2 x 50 ml). The solution was then dried ($MgSO_4$) and the solvent removed at reduced pressure to give a brown oil. Purification was carried out by column chromatography (silica, ethyl acetate: petroleum ether, 1:2). The product was obtained as an orange oil.

The procedure was repeated as above taking tlc samples at ten minute intervals. The product was found to contain only traces of fluorine with the following ^{1}H and ^{13}C NMR data confirming the structure proposed (**193**) in the results and discussion; δ_H (200 MHz; $CDCl_3$) 1.25 (3H, t, *J* 4.9, $CO_2CH_2C\underline{H}_3$), 2.93 - 3.07 (2H, m, 3-$C\underline{H}_2$), 4.23 (2H, q, *J* 4.9, $CO_2C\underline{H}_2CH_3$), 4.60 - 4.67 (1H, m, 2-C<u>H</u>), 5.15 (2H, s, $C\underline{H}_2Ph$), 7.27 - 7.36 (5H, m, CBz aromatic); δ_C (50.31 MHz; $CDCl_3$) 14.45 ($OCH_2\underline{C}H_3$), 36.78 (3-$\underline{C}H_2$), 50.79

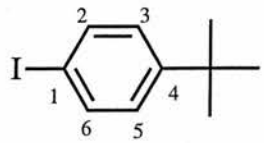
(2-CH), 62.41 (OCH_2CH_3), 67.57 (CH_2Ph), 128.54-128.97 (3C, aromatic), 136.6 (quat. aromatic), 156.67 (NHCO), 171.28 (CO_2Et), 174.78 (CO_2H); *m/z* (CI) 296 ([*M*+H], 43%), 278 (9, [*M*-H_2O]$^+$), 252 (54, [*M*-CO_2]$^+$), 147 (89, [CBz]$^+$), 91 (100, [$PhCH_2$]$^+$).

_N_'Formyl, _N_-(benzyloxycarbonyl))-(2S)-kynurenine ethyl ester



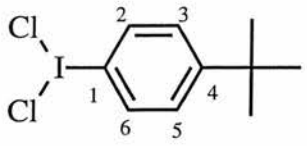
This was synthesised following the same protocol as for the fluoro-analogue. The brown oil obtained was purified by column chromatography (silica; ethyl acetate: petroleum ether; 1: 2) and evaporated to a yellow oil (30%); ν_{max} (nujol)/cm^{-1} 3305 (indole NH),1725 (ester CO), 1680 (amide CO); δ_H (200 MHz; d^6-DMSO) 1.20 (3H, t, *J* 7, OCH_2CH_3), 3.50 (2H, d, *J* 7, β-CH_2), 4.10 (2H, q, *J* 7, OCH_2CH_3), 4.60 (1H, m, α-CH), 5.10 (2H, s, CH_2Ph), 7.20 (1H, t, *J* 7, 5'-CH), 7.50 (5H, m, CH_2Ph), 7.65 (1H, t, *J* 7, 4'-CH), 7.80 (1H, d, *J* 7, 3'-CH), 8.05 (1H, d, *J* 7, 6'-CH), 8.50 (1H, m, NHCOH); δ_C (74.76 MHz, d^6-DMSO) 13.9 ($CO_2CH_2CH_3$), 41.3 (β-CH_2), 50.0 (α-C), 60.9 ($CO_2CH_2CH_3$), 65.6 (CH_2Ph), 121.2 (3'-C), 123.4 (5'-C), 123.9 (1'-C), 127.64, 127.8, 128.3 (CH_2Ph), 130.8 (6'-C), 134.3 (4'-C), 136.9 (quat. CH_2Ph), 137.9 (2'-C), 155.9 ($PhCH_2OCO$), 161.2 (NHCOH).

4-*tert*-Butyliodobenzene (199)



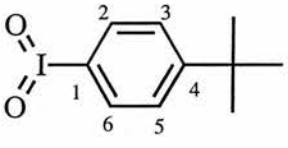
4-*tert*-Butylbenzene (7.906 g, 59 mmol), iodine (6.345 g, 25 mmol) and iodic acid (19.52 g, 111 mmol) were mixed and dissolved in glacial acetic acid (18 ml) and water (13 ml). The resulting solution was heated at reflux for 8 hours and the liquid phases decanted off. Water (30 ml) and potassium hydroxide in methanol (30 ml of a 20% solution) were added to the decanted mixture. The admixed solution was then heated at reflux for 30 minutes and allowed to cool. The lower, dark red portion of the mixture was taken up in dichloromethane (70 ml) to allow easy separation of the layers and then washed with water (3 x 50 ml), dried ($MgSO_4$), filtered and evaporated under reduced pressure to give a dark red syrupy liquid. This was further distilled using a Kugelrohr apparatus at 0.1 atm, 110 °C (lit.,[184] 114 °C/0.05 torr), (10.8 g, 70%). δ_H (200 MHz; $CDCl_3$) 1.31 (9H, s, $(C\underline{H}_3)_3$), 7.15 (2H, d, *J* 5.6, 3-<u>H</u> & 5-<u>H</u>), 7.62 (2H, d, *J* 5.6, 2-<u>H</u> & 6-<u>H</u>).

4-*tert*-Butyl dichloroiodobenzene (201)



4-*tert*-Butyliodobenzene (**199**, 7.813 g, 30.05 mmol) was added to hexane (15 ml) and stirred. A chlorine gas generator was set up as follows: concentrated hydrochloric acid (55 ml) was dropped slowly on to potassium permanganate (8.808 g) in a round bottomed flask and the resulting gas passed into the reaction mixture using a fritted glass tube. Excess gas was then bubbled through a 4M solution of sodium hydroxide. A four fold excess of chlorine gas was used in the reaction, the reagents above yield 9.88 g of chlorine gas (require 8.4 g). The solution turned from dark pink to yellow almost immediately. The yellow precipitate was washed several times with hexane to yield fine yellow crystals (7.8 g, 78%), mp 82.6 °C (lit.[184] 84 °C); δ_H (200 MHz; $CDCl_3$) 1.35 (9H, s, $(C\underline{H}_3)_3$), 7.48 (2H, d, $J_{2,3} = J_{5,6}$ 8.9, 3-C<u>H</u> & 5-C<u>H</u>), 8.08 (2H, d, $J_{2,3} = J_{5,6}$ 8.9, 2-C<u>H</u> & 6-C<u>H</u>); δ_C (50.31 MHz; $CDCl_3$) 31.60 ($C(\underline{C}H_3)_3$), 35.77 ($\underline{C}(CH_3)_3$), 78.19 (1-<u>C</u>), 129.44 (3-<u>C</u> & 5-<u>C</u>), 134.11 (2-<u>C</u> & 6-<u>C</u>), 156.69 (4-<u>C</u>).

4-*tert*-Butyliodoxybenzene (198)



4-*tert*-Butyl dichloroiodobenzene (**201**, 3.31 g, 10 mmol) was stirred with sodium hypochlorite (8% available Cl_2, 54 ml) and glacial acetic acid (2 ml) at room temperature for three hours to give a pale yellow solid. The solution was heated to 60-70 °C for 30 minutes to give a white solid. This was washed with water (5 x 50 ml) and dichloromethane (3 x 50 ml) and dried ($MgSO_4$). The product was obtained as a clean

white crystalline solid, (2.65 g, 91%), mp 219-221 °C [explodes] (lit.[184] 217-221 °C); δ_H (200 MHz; d^6-DMSO) 1.30 (9H, s, (C<u>H</u>$_3$)$_3$), 7.62 (2H, d, $J_{2,3} = J_{5,6}$ 8.5, 3-C<u>H</u> & 5-C<u>H</u>), 7.87 (2H, d, $J_{2,3} = J_{5,6}$ 8.5, 2-C<u>H</u> & 6-C<u>H</u>); δ_C (50.31 MHz, d^6-DMSO) 31.24 (C(<u>C</u>H$_3$)$_3$), 35.01 (<u>C</u>(CH$_3$)$_3$), 126.19 (3-<u>C</u> & 5-<u>C</u>), 126.69 (2-<u>C</u> & 6-<u>C</u>), 147.96 (4-<u>C</u>), 154.78 (1-<u>C</u>).

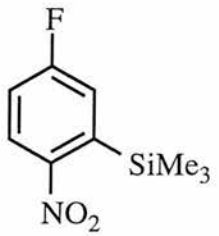
Attempted Oxidation of (2*S*)-{[(Benzyloxy)carbonyl]amino}-5-fluorotryptophan ethyl ester (185) using Iodoxybenzene

(2*S*)-{[(Benzyloxy)carbonyl]amino}-5-fluorotryptophan ethyl ester (0.50 g, 1.3 mmol) and *tert*-butyliodoxybenzene (0.381 g, 1.3 mmol) were dissolved in chlorobenzene (15 ml) and heated under reflux for 3 hours. A solid remained throughout the reaction, this was filtered off, and the solvent removed under reduced pressure. The nmr spectrum showed no significant change although the iodoxybenzene had been converted to iodobenzene. This reaction was carried out again in a similar manner using 1.5 equivalents of *tert*-butyliodoxybenzene, the solution was heated at reflux for twenty hours. No change was observed in the nmr spectrum.

Attempted Oxidation of Stilbene using Iodoxybenzene

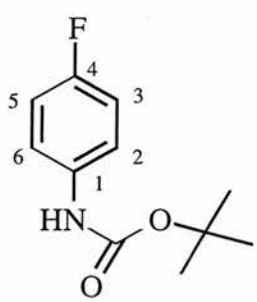
E-Stilbene (0.099 g, 0.5 mmol) and *tert*-butyliodoxybenzene (0.294, 1 mmol) were added together and suspended in chlorobenzene (8 ml). The solution was heated under reflux for 20 hours. The solvent was removed on a Kugelrohr apparatus at 0.05 torr at 100 °C. The resulting solid was examined by nmr spectroscopy and no change was observed in the spectrum compared with that of stilbene.

Attempted Synthesis of 4-Fluoro-2-trimethylsilyl-nitrobenzene (219)



4-Fluoronitrobenzene (1.411 g, 10 mmol) was stirred in dry THF (30 ml) at -78 °C and to this was added trimethylsilyl chloride (3.80 ml, 30 mmol, 3 eq). Potassium bis(trimethylsilyl)amide (0.5 M in toluene, 40 ml, 20 mmol, 2 eq) was then added dropwise keeping the internal temperature of the solution below -75 °C. The resulting solution was stirred at -78 °C for 30 minutes and then partitioned between 1M HCl (50 ml) and diethyl ether (50 ml). The aqueous phase was separated and extracted with diethyl ether (3 x 50 ml). The combined organic phases were then washed with 1M HCl (3 x 50 ml) followed by brine (70 ml). The combined organic fractions were then dried ($MgSO_4$), filtered and concentrated under reduced pressure. The product was obtained as a yellow liquid. No product was observed by nmr spectroscopy.

***N*-Butyloxycarbonyl-4-fluoroaniline (210)**



4-Fluoroaniline (1.112 g, 10 mmol) was added to a 10% solution of triethylamine in methanol (15 ml). The mixture was stirred vigorously and di-tert-butyl dicarbonate (4.635 g, 20 mmol, 2 eq) was added. The mixture was heated at 60 °C for 30 minutes and the reaction was followed by tlc (dichloromethane: methanol; 19:1). This was stirred

for a further 45 minutes and the solvent removed under reduced pressure. The residue was stirred with hydrochloric acid (10 ml, pH 2.15) for 10 minutes and then extracted into ethyl acetate (5 x 40 ml), The ethyl acetate fractions were dried ($MgSO_4$), filtered and evaporated to dryness. The product was isolated as yellow white crystals (1.73 g, 90%), mp 112.1-114.3 °C; ν_{max} (nujol)/cm^{-1} 3000 (NH), 1680 (carbamate CO); δ_H (200 MHz; $CDCl_3$) 1.50 (9H, s, $(CH_3)_3$), 6.45 (1H, br s, NH), 6.97 (2H, t, $J_{2,3} = J_{3,4}$ 9, 3-H & 5-H), 7.31 (2H, dd, $J_{2,3}$ 9, $J_{2,4}$ 4.7, 2-H & 6-H); δ_F (282.3 MHz, $CDCl_3$) -121.28; δ_C (50.31 MHz; $CDCl_3$) 27.87 ($(\underline{C}H_3)_3$), 81.06 ($\underline{C}(CH_3)_3$), 115.75 (<u>C</u>-2 & C-6), 116.20 (<u>C</u>-3 & <u>C</u>-5), 120.81 (d, $J_{C,F}$ 7.4, <u>C</u>-4), 134.80 (<u>C</u>-1), 153.44 ($\underline{C}O_2{}^tBu$); *m/z* (EI) 221 [M^+, 9%], 155 (28, [M-^{t}Bu]$^+$), 111 (28, [M-Boc]$^+$). 57 (100, [^{t}Bu]$^+$).

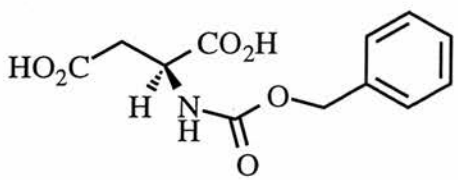
Attempted Stannylation of *N*-butyloxycarbonyl-4-fluoroaniline

N-Butyloxycarbonyl-4-fluoroaniline (1 mmol, 200 mg), was dissolved in dry THF (**210**, 5 ml). The system was sealed and flushed with nitrogen. The solution was stirred at -78 °C and ^{t}butyl lithium (2.1 mmol, 1.24 ml of a 1.7M solution in pentane) was added dropwise. Precautions were taken to exclude all moisture. The solution was stirred for 15 minutes at -78 °C then warmed to -20 °C and stirred for two hours. Trimethyltin chloride (1 mmol, 1 ml of a 1.0 M solution in THF) was added at -20 °C and the reaction stirred for a further 2.5 hours. The orange brown solution was slowly poured into 50 ml of water and extracted into ethyl acetate. The organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give an orange oil.

Studies by nmr spectroscopy showed that a product had been formed but large amounts of starting material remained. The experiment was repeated using 2 mmol of trimethyltin chloride and the reaction was stirred at -20 °C for 5 hours after addition of the tin. This showed a significant increase in the ratio of product to starting material. The amount of tin was doubled again to 4 mmol and the experiment run again for 5 hours, although no significant increase in product was observed by nmr spectroscopy. The experiment was

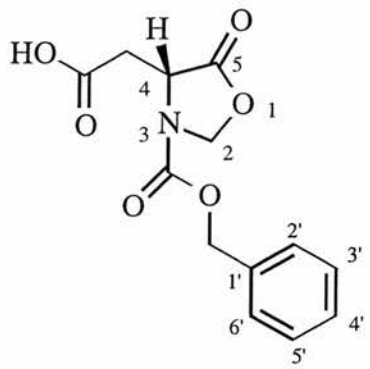
carried out a fourth time using 2 mmol of tin and run overnight (~18 hours), this also showed no significant increase in product formation. A fifth attempt was made adding the trimethyltin chloride (2 mmol) to the *N*-butyloxycarbonyl-4-fluoroaniline before adding the ^{t}butyl lithium. This afforded no product and the starting material was recovered with slight impurities from the tin.

_N_-[(Benzyloxy)carbonyl]-L-aspartic acid (215)



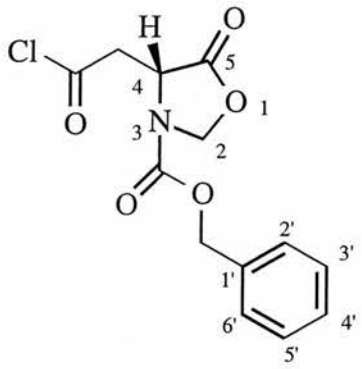
L-Aspartic acid (5 g, 38 mmol) was dissolved in distilled water (60 ml) containing potassium carbonate (10 g, 56.2 mmol). Benzyl chloroformate (6 ml, 48 mmol) was added in small portions over 15 minutes and the reaction mixture left to stir overnight. The solution was extracted into diethyl ether (2 x 50 ml) and the aqueous layer was acidified to pH 1 with HCl before extracting again with diethyl ether (3 x 50 ml). The second diethyl ether fractions were washed with brine (40 ml) and dried ($MgSO_4$) and evaporated under reduced pressure to a yellow oil which solidified in the fridge to off white sticky crystals. The crystals were washed with diethyl ether and recrystallised from ethyl acetate and petroleum ether (40-60 °C). The product was isolated as a white powder (7.429 g, 74%), mp 114 - 115.5 °C (lit.,[211] 116 °C); $[\alpha]^{20}_{D}$ +16.5 (c = 1, EtOAc), [lit.,[211] $[\alpha]^{20}_{D}$ +9.6 (c = 4, AcOH)]; ν_{max} (nujol)/cm^{-1} 3030 (NH), 1680 (carbamate CO); δ_H (200 MHz; d^6-DMSO) 2.66 (2H, 2x dd, J_1 17.3, J_2 6.9, β-C$\underline{H}_2$), 4.31 - 4.38 (1H, m, α-$\underline{H}$), 5.05 (2H, s, OC$\underline{H}_2$Ph), 7.36 (5H, s, Ph), 7.66 (1H, d, *J* 8.4, NH); δ_C (50.31 MHz; d^6-DMSO) 36.26 (β-$\underline{C}H_2$), 50.77 (α-$\underline{C}$), 65.75 (O$\underline{C}H_2$Ph), 127.98 (2-$\underline{C}$H), 128.09 (4-$\underline{C}$H), 128.62 (3-$\underline{C}$H), 137.17 (OCH$_2\underline{C}$Ph), 156.13 (NH$\underline{C}$O), 171.93 ($\underline{C}O_2H$), 172.98 ($\underline{C}O_2H$); *m/z* (EI) 267 [M^+, 7.5%], 249 (11, [M-H_2O]$^+$), 108 (51, [PhCH$_2$OH]$^+$), 91 (100, [PhCH$_2$]$^+$), 79 (15, [M-CO]$^+$).

(4*S*)-*N*-Carbobenzyloxy-5-oxo-4-oxazolidine acetic acid (216)



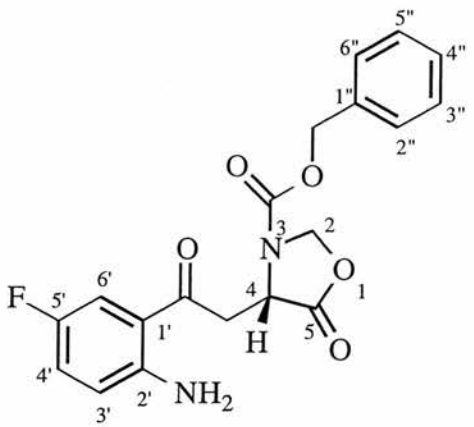
A mixture of *N*-carbobenzyloxy-L-aspartic acid (**215**, 5.35 g, 0.02 mol), paraformaldehyde (1.8 g), acetic anhydride (4.0 g, 0.04 mol) and thionyl chloride (0.3 ml) was added to glacial acetic acid (80 ml). This solution was heated at 100 °C on an oil bath for 4 hours. The solvent was then removed under reduced pressure to give a yellow/orange syrup. This was taken up in ethyl acetate (50 ml) and extracted into a 5% solution of sodium hydrogen carbonate (95 ml). The aqueous layer was acidified, under ice-cooling, with 6M HCl to pH 1 to give a yellow precipitate. This was then extracted with ethyl acetate (100 ml) and washed with water (2 x 100 ml) and brine (100 ml). The organic layers were dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give a pale yellow syrup. Purification by column chromatography (silica; DCM: ethyl acetate; 3:1), gave the product as a yellow syrup (3.77 g, 67%); $[\alpha]^{20}_D$ +186.3 (c = 1.1, $CHCl_3$), [lit.,[201] not given]; ν_{max} (neat)/cm^{-1} 3000 (br. OH), 1700 (carbamate CO); δ_H (200 MHz; $CDCl_3$) 2.95 - 3.19 (2H, m, β-C$\underline{H}_2$), 4.44 - 4.52 (1H, m, 4-C$\underline{H}$), 5.17 (2H, s, OC$\underline{H}_2$Ph), 5.22 (1H, s, 2-$\underline{H}_A$), 5.47 (1H, br s, 2-$\underline{H}_B$), 7.39 (5H, s, Ph), 9.66 (1H, br s, CO_2H); δ_C (50.31 MHz; $CDCl_3$) 51.76 (β-$\underline{C}H_2$), 67.01 (4-$\underline{C}$), 78.32 (O$\underline{C}H_2$Ph), 86.82 (2-$\underline{C}$), 127.89 (3' & 5'-$\underline{C}$), 128.28 (4'-$\underline{C}$), 128.67 (2' & 6'-$\underline{C}$), 136.31 (1'-$\underline{C}$), 152.90 (N$\underline{C}$O), 170.55 ($\underline{C}O_2H$), 171.72 ($CH_2\underline{C}O_2$); *m/z* (EI) 279 [M^+, 4.9%], 235 (22, [M-CO_2]$^+$), 144 (11, [M-CO_2-$PhCH_2$]$^+$), 126 (9, [M-CO_2-$PhCH_2$-H_2O]$^+$), 107 (36, [$PhCH_2O$]$^+$), 91 (100, [$PhCH_2$]$^+$).

(4*S*)-*N*-Carbobenzyloxy-5-oxo-4-oxazolidineacetyl chloride (75)



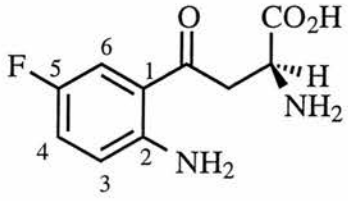
(4*S*)-*N*-Carbobenzyloxy-5-oxo-4-oxazolidine acetic acid (**216**, 0.937 g, 3.35 mmol) was dissolved in toluene (5 ml) and oxalyl chloride (10 ml) added. The solution was heated under reflux for two hours, the solvent removed under reduced pressure. The residue was washed several times with toluene to remove traces of oxalyl chloride and used without further purification. The product was obtained as an orange oil (0.72 g, 72%); δ_H (200 MHz, $CDCl_3$) 3.55 - 3.93 (2H, m, β-C<u>H</u>$_2$), 4.37 (1H, s, α-C<u>H</u>), 5.23 (2H, s, OC<u>H</u>$_2$Ph), 5.36 (1H, t, *J* 5.4, H_A of 2-C<u>H</u>$_2$), 5.52 (1H, br s, H_B of 2-C<u>H</u>$_2$), 7.41 (5H, s, OCH$_2$<u>Ph</u>); δ_C (50.31 MHz, $CDCl_3$) 52.9 (β-<u>C</u>H$_2$), 69.4 (α-<u>C</u>H), 79.7 (O<u>C</u>H$_2$Ph), 88.2 (2-<u>C</u>H$_2$), 129.7 (3' & 5'-<u>C</u>), 130.3 (4'-<u>C</u>), 130.6 (2' & 6'-<u>C</u>), 136.2 (1'-<u>C</u>), 153.8 (N<u>C</u>O), 171.5 (CH$_2$<u>C</u>O$_2$).

4-Fluoro-2-[(*4S*)-*N*-carbobenzyloxy-5'-oxo-4'-oxazolidinoacetyl]-aniline (220)



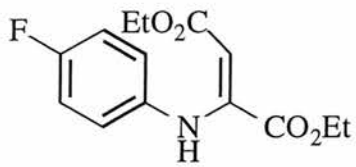
(*4S*)-*N*-Carbobenzyloxy-5-oxo-4-oxazolidineacetyl chloride (**75**, 1.747 g, 7.85 mmol, 1.1 eq) was dissolved in dry dichloromethane (30 ml) and boron trifluoride etherate ($BF_3.Et_2O$), (1.3 ml, 10.5 mmol, 1.8 eq wrt acid chloride) added. The solution was stirred at room temperature for 20 minutes before adding *N*-(^{t}butoxycarbonyl)-4-fluoroaniline (**210**, 1.011 g, 5.26 mmol). The reaction was stirred at room temperature overnight, poured into 1M hydrochloric acid (30 ml), extracted into dichloromethane (3 x 20 ml), and the organic fractions dried ($MgSO_4$) and concentrated under reduced pressure to a red-brown oil. Purification by column chromatography (silica; pet. ether: ethyl acetate; 2:1 graduating to 1:1 and collected in the ethyl acetate wash) gave the product as an orange oil (1.23 g, 63%); (Acc. Mass Found 373.120473 Calc. for $C_{18}H_{18}N_2O_5F$, 373.119975); $[\alpha]^{20}_D$ +157.1 (c = 0.27, $CHCl_3$); δ_H (200 MHz, $CDCl_3$) 3.05 (2H, d, *J* 10.7, β-C$\underline{H}_2$), 4.31 (1H, br s, α-C$\underline{H}$), 5.02 - 5.18 (2H, m, 2-C$\underline{H}_2$) 5.25 (2H, s, OC$\underline{H}_2$Ph), 6.88 (1H, t, $J_{3',4'}$ 8.9, 4'-C$\underline{H}$), 7.03 -7.21 (2H, m, 3' & 6'-C$\underline{H}$) 7.32 (5H, s, $OCH_2\underline{Ph}$); δ_F (282.3 MHz, $CDCl_3$) -118.53; δ_C (50.31 MHz, $CDCl_3$) 52.3 (β-C$\underline{H}_2$), 68.4 (4-C$\underline{H}$), 78.8 (OC$\underline{H}_2$Ph), 88.1 (2-C$\underline{H}_2$), 127.9 (3" & 5"-C), 128.3 (4"-C), 128.6 (2" & 6"-C), 128.8 (4'-C), 128.9 (6'-C), 129.1 (1'-C), 129.2 (3'-C), 133.9 (1"-C), 153.3 (NCO), 157.9 (2'-C), 163.2 (5'-C), 167.8 (5-CO), 191.9 (CO); *m/z* (CI) 373 ([*M*+H], 13%), 343 (72, [*M*+H-CH_2O]$^+$), 236 (49, [*M*+H-$PhCH_2OCO$]$^+$), 192 (53, [*M*+H-$PhCH_2OCO$-NCH_2O]$^+$), 91 (100, [$PhCH_2$]$^+$).

(2*R*)-2-Amino-4-(2-amino-5-fluorophenyl)-4-oxobutanoic acid (177)



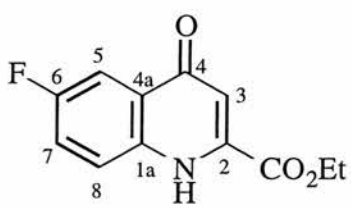
To 4-fluoro-2-[(*4S*)-*N*-carbobenzyloxy-5'-oxo-4'-oxazolidinoacetyl]-aniline (**220**, 0.3 g, 0.8 mmol) dissolved in THF (5 ml) was added 6M hydrochloric acid (15 ml) and the solution heated under reflux for 3 hours. After cooling the reaction mixture was washed with diethyl ether (2 x 10 ml) and the aqueous phases concentrated under reduced pressure to give a brown/orange oil. Purification by column chromatography (C18 reverse phase silica; acetonitrile:water; 1:1) followed by reverse phase HPLC separation of the resulting oil (water: acetonitrile; 90:10; flow rate: 1.8 ml min^{-1}) gave the product (60 mg, 33%); Acc. Mass Found 228.0911 Calc. for $C_{10}H_{11}N_2O_3F$+2H, 228.2227; $[\alpha]^{20}_D$ + 6 (c = 0.2, MeOH); ν_{max} (neat/cm^{-1}) 3420 - 3315 (OH), 1720 (CO), 1650 (acid CO); δ_H (300 MHz, CD_3OD) 3.08 (2H, d, *J* 10.7, β-C<u>H</u>$_2$), 4.52 - 4.61 (1H, m, α-C<u>H</u>), 7.30 - 7.38 (3H, m, 3, 4, & 6-<u>H</u>); δ_F (282.3 MHz, CD_3OD) -115.766; δ_C (75.4 MHz, CD_3OD) 45.4 (β-<u>C</u>H$_2$), 66.0 (α-CH), 129.0 (4-<u>C</u>), 129.2 (6-<u>C</u>), 129.7 (3-<u>C</u>), 130.1 (1-<u>C</u>), 137.9 (2-C), 158.5 (5-<u>C</u>), 171.7 (<u>C</u>O$_2$H), 191.2 (<u>C</u>O); *m/z* (Maldi-TOF) 268 ([*M*+2Na], 48%), 250 (24, [*M*+Na+H]), 228 (27, [*M*+2H]).

Diethyl (2*E*)-2-(4-fluoroanilino)-2-butenedioate (191)



Diethyl acetylenedicarboxylate (1.886 g, 11 mmol) was added slowly to a stirred solution of 4-fluoroaniline (1.114 g, 10 mmol) in dry redistilled methanol (15 ml). The solution was heated under reflux for 2 hours and then cooled to room temperature. The reaction was followed by tlc (silica; dichloromethane) and heated at reflux for a further 2.5 hours with no further change in the chromatogram. The resulting solution was evaporated at reduced pressure to give an orange oil (2.57 g, 91%) which was used without further purification in the next step. δ_H (200 MHz; $CDCl_3$) 1.10 (3H, t. *J* 7.1, $OCH_2C\underline{H}_3$), 1.29 (3H, t, *J* 7.1, $OCH_2C\underline{H}_3$), 4.08-4.16 (2H, m, $OC\underline{H}_2CH_3$), 4.18-4.24 (2H, m, $OC\underline{H}_2CH_3$), 5.39 (1H, s, C<u>H</u>), 6.86-7.03 (4H, m, aromatic), 9.59 (1H, br s, N<u>H</u>);

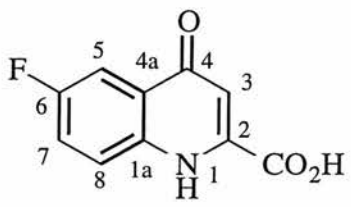
Ethyl 6-fluoro-4-oxo-1,4-dihydro-2-quinoline carboxylate (192)



Diethyl (2*E*)-2-(4-fluoroanilino)-2-butenedioate (**191**, 3.112 g, 11 mmol) was added to warm diphenyl ether (100 ml). The solution was stirred continuously and heated to reflux (250 °C). The solution darkened from pale orange to brown. After 45 minutes at reflux the condenser water supply was switched off to aid the removal of ethanol. After 30 minutes the solution was allowed to cool. The beige product was filtered off, washed several times with diethyl ether and recrystallised from ethyl acetate (1.55 g, 72%), mp 235-8 °C (lit.,[117] 239-240 °C); (Found: C, 61.46; H, 4.50; N, 5.96. Calc. for $C_{12}H_{10}FNO_3$: C, 61.28; H, 4.29; N, 5.95%); ν_{max} (nujol)/cm^{-1} 2920 (NH), 1720 (ester

CO); δ_H (200 MHz; $CDCl_3$) 1.44 (3H, t, *J* 7.1, $OCH_2C\underline{H}_3$), 4.48 (2H, q, *J* 7.1, $OC\underline{H}_2CH_3$), 6.97 (1H, s, 3-<u>H</u>), 7.46 (1H, dt, $J_{6,8}$ 9.1, $J_{5,8}$ 2.2, 8-<u>H</u>), 7.54 (1H, dd, $J_{5,6}$ 9.1, $J_{5,7}$ 3.5, 5-<u>H</u>), 8.00 (1H, dd, $J_{6,7}$ 8.7, $J_{5,7}$ 3.5, 7-<u>H</u>); δ_F (298 MHz; $CDCl_3$) -116.2; δ_C (50.31 MHz; d^6-DMSO) 19.56 ($CO_2CH_2\underline{C}H_3$), 68.35 ($CO_2\underline{C}H_2CH_3$), 114.35 (8-<u>C</u>), 127.07 (5-<u>C</u>), 127.58 (7-<u>C</u>), 128.83 ($J_{C,F}$ 8.5, 6-<u>C</u>), 142.95 (3-<u>C</u>), 144.39 (2-<u>C</u>), 162.15 (<u>C</u>O_2Et), 166.99 (4a-<u>C</u>), 167.86 (1a-<u>C</u>), 181.56 (4-<u>C</u>); *m/z* (EI) 235 (M^+, 67%), 189 (34, [*M*-EtOH]$^+$), 161 (100, [*M*-EtCO_2H]$^+$).

6-Fluoro-4-oxo-1,4-dihydro-2-quinoline carboxylic acid (178)



Ethyl 6-fluoro-4-oxo-1,4-dihydro-2-quinoline carboxylate (**192**, 1 g, 4.25 mmol) was dissolved in methanol (70 ml) and 1M sodium hydroxide (12 ml) added. The solution was stirred at room temperature for 48 hours. The resulting solution was then filtered to remove insoluble materials and the solvent removed at reduced pressure to give an off white solid. The residue was taken up in distilled water (45 ml) and stirred. 2M Hydrochloric acid solution was then added dropwise and the resulting white solid was filtered, dried in a desiccator (P_2O_5) and then crushed to a fine powder. Recrystallisation from water gave white flaky crystals (0.74 g, 84% crude), mp 279.8 °C; (Acc Mass Found: 208.045193, Calc. for $C_{10}H_7FNO_3$, 208.040996); ν_{max} (nujol)/cm^{-1} 2920 (NH), 1720 (ketone CO), 1590 (acid CO); δ_H (200 MHz; d^6-DMSO) 6.72 (1H, s, C<u>H</u>), 7.61-7.77 (2H, m, 8-C<u>H</u> and 5-C<u>H</u>), 8.04 (1H, dd, $J_{6,7}$ 9.1, $J_{5,7}$ 4.7, 7-C<u>H</u>); δ_F (298 MHz; d^6-DMSO) -117.4; δ_C (50.31 MHz; D_2O) 110.05 (8-<u>C</u>), 124.58 (5-<u>C</u>), 125.09 (7-<u>C</u>), 128.06 ($J_{C,F}$ 8.5, 6-<u>C</u>), 148.14 (3-<u>C</u>), 159.59 (2-<u>C</u>), 164.46 (<u>C</u>O_2H), 167.12 (4a-<u>C</u>), 169.41 (1a-<u>C</u>), 181.89 (4-<u>C</u>); *m/z* (CI): 208 (*M*+H, 18%), 164 (17, [*M*+H-CO_2]$^+$), 57 (100, [CCO_2H]$^+$).

Attempted Synthesis of Benzyl (4*S*)-5-oxo-4-[2-oxo-2-(3-thienyl)ethyl]-1,3-oxazolidine-3-carboxylate (221)

Thiophene (0.8 ml, 1 mmol) was stirred in dry toluene (5 ml) at 0 °C and tin tetrachloride (0.12 ml, 1 mmol) was added. The solution was stirred at 0 °C for 30 minutes and then a solution of (4*S*)-*N*-carbobenzyloxy-5-oxo-4-oxazolidineacetyl chloride (0.3 g, 1 mmol) in dry toluene (5 ml) was added, the solution warmed to room temperature and stirred for a further 2 hours. The reaction was quenched with 1M hydrochloric acid (10 ml), extracted into ethyl acetate (3 x 20 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a brown oil. Analysis of this compound by nmr spectroscopy showed no conversion to the desired product, although no starting material was evident either.

Attempted Synthesis of Benzyl (4*S*)-4-[2-(3-furyl)-2-oxoethyl]-5-oxo-1,3-oxazolidine-3-carboxylate (222)

Furan (1 ml, 2.3 mmol) and (4*S*)-*N*-carbobenzyloxy-5-oxo-4-oxazolidineacetyl chloride (0.391 g, 1.3 ml) were dissolved in dry toluene (10 ml) and boron trifluoroetherate (0.05 ml) added. The reaction was stirred at room temperature overnight and then quenched in 1M hydrochloric acid (10 ml), extracted into ethyl acetate (2 x 20 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to give a green liquid. Analysis by ^{1}H and ^{13}C-nmr spectroscopy showed no conversion to the desired product and the spectrum could not be assigned.

REFERENCES

REFERENCES

1. G. W. Beadle, H. K. Mitchell and J. F. Nyc, *Proc. Natl. Acad. Sci. USA*, (1947), **33**, 155 - 158.

2. J. C. Peters, *Adv. Exp. Med. Biol.*, (1991), **294**, 345 - 358.

3. H. Wolf, *Scand. J. Clin. Lab. Invest.*, (1974), **136** (Suppl.), 1 - 186.

4. A. Ellinger, *Z. Physiol. Chem.*, (1904), **43**, 325 - 337.

5. R. K. Gholson, I. Ueda, N. Ogasawara and L. M. Henderson, *J. Biol. Chem.*, (1964), **239**, 1208 - 1214.

6. T. W. Stone and M. N. Perkins, *Eur. J. Neuropharm.*, (1981), **72**, 411 - 412.

7. A. Freese, K. J. Swartz, M. J. During and J. B. Martin, *Neurology*, (1990), **40**, 691 - 695.

8. D. Jauch, E. M. Urbanska, P. Guidetti, E. D. Bird, J.-P. G. Vonsattel, W. O. Whetsell, Jr. and R. Schwarcz, *J. Neuro. Sci.*, (1995), **130**, 39 - 47.

9. L. Prado De Carvalho, P. Bochet and J. Rossier, *Neurochem. Int.*, (1996), **28**, 445 - 452.

10. R. Schwarcz, A. C. Foster and K. Iwai, *Abstr. Soc. Neurosci.*, (1983), **9**, 327.

11. T. W. Stone, *Pharm. Rev.*, (1993), **45**, 309 - 379.

12. A. C. Foster, A. Vezzani, E. D. French and R. Schwarcz, *Neuroscience Lett.*, (1984), **48**, 273 - 278.

13. C. L. Eastman and T. R. Guilarte, *Brain Res.*, (1989), **495**, 225 - 231.

14. S. Okuda, N. Nishiyama, H. Saito and H. Kaysuki, *J. Neurochem.*, (1998), **70**, 299 - 307.

15. S. Okuda, N. Nishiyama, H. Saito and H. Katsuki, *Proc. Natl. Acad. Sci. USA*, (1996), **93**, 12553 - 12558.

16. G. Curzon and P. J. Knott, *CRC Crit. Rev. Toxical.*, (1977), **5**, 145 - 187.

17. A. Freese, K. J. Swartz and M. During, *Ann. Intern. Med.*, (1988), **108**, 312 - 313.

18. J. D. Fernstrom and R. J. Wurtman, *Science*, (1971), **173**, 149 - 152.

19. J. D. Fernstrom and R. J. Wurtman, *Science*, (1971), **174**, 1023 - 1025.

20. E. M. Gál and A. D. Sherman, *Neurochem. Res.*, (1980), **5**, 223 - 239.

21. G. K. Aghajanian and I. M. Asher, *Science*, (1971), **172**, 1159.

22. N. P. Botting, *Chem. Soc. Rev.*, (1995), **24**, 401 - 411.

23. D. A. Bender In *Quinolinic Acid and the Kynurenines*; T. W. Stone, Ed., CRC Press, Boca Raton, (1989), 3-38.

24. K. Soda and K. Tanizawa, *Biochem. J.*, (1979), **86**, 1199 - 1209.

25. S. V. Koushik, R. A. McGraw and R. S. Philips, *Faseb Journal*, (1995), **9**, pA372.

26. M. Moriguchi, T. Yamamoto and K. Soda, *Biochem. Biophys. Res. Commun.*, (1971), **44**, 752 - 757.

27. W. Knox, *Biochem. J.*, (1953), **53**, 379 - 385.

28. E. Okuno and R. Kido In *Kynurenine and Serotonin Pathways*; R. Schwarcz, S. N. Young, R. R. Brown, Eds., Plenum Press, New York, (1991), 167 - 176.

29. K. Tanizawa and K. Soda, *Biochem. J.*, (1979), **85**, 901 - 906.

30. D. Albertigiani, R. Buchli, M. Malherbe, C. Broger, G. Lang, C. Kohler, H. W. Lahm and A. M. Cesura, *Eur. J. Biochem.*, (1996), **239**, 460 - 468.

31. F. Takeuchi, T. Tsubouchi, M. Yoshino and Y. Shibata, *Biochem. Biophys. Acta*, (1995), **1252**, 185 - 188.

32. M. Moriguchi, T. Yamamoto and K. Soda, *Biochemistry*, (1973), **12**, 2969.

33. R. K. Dua, E. W. Taylor and R. S. Phillips, *J. Am. Chem. Soc.*, (1993), **115**, 1264 - 1270.

34. J. Inada, E. Okuno, M. Kimura and R. Kido, *Int. J. Biochem.*, (1984), **16**, 623 - 628.

35. O. Hayaishi In *A Symposium on Amino Acid Metabolism*; W. D. McElroy, H. B. Glass, Eds., John Hopkins Press, Baltimore, (1955), 914 - 929.

36. R. S. Phillips and R. K. Dua, *J. Am. Chem. Soc.*, (1991), **113**, 7385 - 7388.

37. R. S. Phillips, B. Sundararaju and S. V. Koushik, *Biochemistry*, (1998), **37**, 8783 - 8789.

38. S. V. Koushik, J. A. Moore III, B. Sundararaju and R. S. Phillips, *Biochemistry*, (1998), **37**, 1376 - 1382.

39. M. M. Palcic, M. Antoun, K. Tanizawa, K. Soda and H. G. Floss, *J. Biol. Chem.*, (1985), **260**, 5248 - 5251.

40. H. C. Dunathan, *Proc. Natl. Acad. Sci. USA*, (1966), **55**, 712 - 716.

41. G. M. Kishore, *J. Biol. Chem.*, (1984), **259**, 10669 - 10674.

42. F. C. Ross, N. P. Botting and P. D. Leeson, *Bioorg. Med. Chem. Lett.*, (1996), **6**, 2643 - 2646.

43. J. P. Whitten, C. L. Barney, E. W. Huber, P. Bey and J. R. McCarthy, *Tetrahedron Lett.*, (1989), **30**, 3649 - 3652.

44. F. C. Ross, N. P. Botting and P. D. Leeson, *Bioorg. Med. Chem. Lett.*, (1996), **6**, 875 - 878.

45. F. C. Ross, PhD, University of St. Andrews, St. Andrews, 1996.

46. G. S. Bild and J. C. Morris, *Arch. Biochem. Biophys.*, (1984), **235**, 71 - 75.

47. V. P. Vassilev, T. Uchiyama, T. Kajimoto and C.-H. Wong, *Tetrahedron Lett.*, (1995), **36**, 5063 - 5064.

48. T. Miura, N. Masuo, Y. Fusamae, T. Kajimoto and Y. Ida, *Synlett*, (1998), 631 - 633.

49. F. Takeuchi, H. Otsuka and Y. Shibata, *Biochem. Biophys. Acta*, (1983), **743**, 323 - 330.

50. R. Kido In *Kynurenine and Serotonin Pathways*; R. Schwarcz, S. N. Young, R. R. Brown, Eds., Plenum Press, New York, (1991), 201 - 205.

51. E. Okuno, Y. Minatogawa, M. Nakamura, N. Kamoda, J. Nakanishi, M. Makino and R. Kido, *Biochem. J.*, (1980), **189**, 581 - 590.

52. H. Baran, E. Okuno, R. Kido and R. Schwarcz, *J. Neurochem.*, (1994), **62**, 730 - 738.

53. E. Okuno, W. Schmidt, D. A. Parks, M. Nakamura and R. Schwarcz, *J. Neurochem.*, (1991), **57**, 533 - 540.

54. S. J. Pearson, A. Meldrum and G. P. Reynolds, *J. Neural Transm, [Gen. Sect.]*, (1995), **102**, 67 - 73.

55. H. Baran, G. Amman, B. Lubec and G. Lubec, *Pediatric Research*, (1997), **41**, 404-410.

56. J. S. Wiseman and J. S. Nichols, *Anal. Biochem.*, (1990), **184**, 55.

57. S. S. Tate and A. Meister, *Adv. Enzymol.*, (1974), **35**, 503 - 543.

58. L. D. Arnold, T. H. Kalantar and J. C. Vederas, *J. Am. Chem. Soc.*, (1985), **107**, 7105 - 7109.

59. M. J. Drysdale and J. F. Reinhard, *Bioorg. Med. Chem. Lett.*, (1998), **8**, 133 - 138.

60. R. Pellicciari, B. Natalini, G. Constantino, M. R. Mahmoud, L. Mattoli, B. M. Sadeghpour, F. Moroni, A. Chiarugi and R. Carpenedo, *J. Med. Chem.*, (1994), **37**, 647 - 655.

61. B. Natalini, M. Mattoli, R. Pellicciari, R. Carpenedo, A. Chiarugi and F. Moroni, *Bioorg. Med. Chem. Lett.*, (1995), **5**, 1451-1454.

62. A. Chiarugi, R. Carpenedo, M. T. Molina, L. Mattoli, R. Pellicciari and F. Moroni, *J. Neurochem.*, (1995), **65**, 1176 - 1183.

63. A. Chiarugi, R. Carpenedo and F. Moroni, *J. Neurochem.*, (1996), **67**, 692 - 698.

64. A. Giordani, P. Pevarello, M. Cini, R. Bormetti, F. Greco, S. Toma, C. Speciale and M. Varasi, *Bioorg. Med. Chem. Lett.*, (1998), **8**, 2907 - 2912.

65. C. J. Parli, P. Krieter and B. Schmidt, *Arch. Biochem. Biophys.*, (1980), **203**, 161.

66. J. L. Walsh, W. P. Toss, B. K. Carpenter and R. Schwarcz, *Biochem. Pharm.*, (1991), **42**, 985.

67. D. R. Curtis and J. C. Watkins, *J. Physiol.*, (1963), **166**, 1 - 14.

68. J. A. Kemp, A. C. Foster and E. H. F. Wong, *Trends Neurosci.*, (1987), **10**, 294 - 298.

69. R. P. M. Bruyn and J. C. Stoof, *J. Neurol. Sci.*, (1990), **95**, 29-38.

70. S. A. Lipton and P. A. Rosenberg, *New Engl. J. Med.*, (1994), **330**, 613 - 622.

71. S. A. Lipton, Y.-B. Choi and Z.-H. Pan, *Nature*, (1993), **364**, 626 - 632.

72. M. F. Beal, *Ann. Neurol.*, (1992), **31**, 119 - 130.

73. S. D. Taylor-Robinson, R. A. Weeks, D. J. Bryant, J. Sargentoni, C. D. Marcus, A. E. Harding and D. J. Brooks, *Movement Disorders*, (1996), **11**, 167 - 173.

74. N. J. Sucher, S. Z. Lei and S. A. Lipton, *Brain Res.*, (1991), **551**, 297 - 302.

75. N. J. Sucher, *Brain Res.*, (1991), **559**, 179.

76. M. F. Beal, N. W. Kowall, K. J. Swartz, R. J. Ferrante and J. B. Martin, *J. Neurosci.*, (1988), **8**, 3901 - 3908.

77. R. Schwarcz, E. Okuno, R. J. White, E. D. Bird and W. O. Whetsell, Jr., *Proc. Natl. Acad. Sci. USA*, (1988), **85**, 4079 - 4081.

78. D. T. Monaghan, R. J. Bridges and C. W. Cotman, *Annu. Rev. Pharmacol. Toxicol.*, (1989), **29**, 365 - 402.

79. I. P. Lapin, *TIPS*, (1980), 410 - 413.

80. M. F. Beal, N. W. Kowall, D. W. Ellison, M. F. Mazurek, K. J. Swartz and J. B. Martin, *Nature*, (1986), **321**, 168 - 171.

81. M. Wolfensberger, U. Amsler, M. Cuenod, A. C. Foster, W. O. Whetsell, Jr. and R. Schwarcz, *Neuroscience Lett.*, (1983), **38**, 85 - 90.

82. R. Schwarcz, *Biochem. Soc. Trans.*, (1993), **21**, 77 - 82.

83. S. Fukui, R. Schwarcz, S. I. Rapoport, Y. Takada and Q. R. Smith, *J. Neurochem.*, (1995), **56**, 2007 - 2017.

84. F. Du, E. Okuno, W. O. Whetsell, Jr., C. Kohler and R. Schwarcz, *Neuroscience*, (1991), **42**, 397 - 406.

85. D. A. Bender In *Quinolinic Acid and the Kynurenines*; T. W. Stone, Ed., CRC Press, Boca Raton, (1989), 241 - 262.

86. A. C. Foster, W. C. Zinkand and R. Schwarcz, *J. Neurochem.*, (1985), **44**, 446 - 454.

87. A. C. Foster, W. O. Whetsell, Jr., E. D. Bird and R. Schwarcz, *Brain Res.*, (1985), **336**, 207 - 214.

88. M. P. Heyes, P. Hutto and S. P. Markey, *Neurochem. Int.*, (1988), **13**, 405 - 408.

89. M. N. Perkins and T. W. Stone, *Brain Res.*, (1983), **259**, 172 - 176.

90. M. N. Perkins and T. W. Stone, *J. Pharmacol. Exp. Ther.*, (1983), **226**, 551 - 557.

91. M. P. Heyes, K. Saito, E. O. Major, S. Milstein, S. P. Markey and J. H. Vickers, *Brain*, (1993), **116**, 1425 - 1450.

92. D. Holzman, *Science*, (1993), **259**, 25 - 26.

93. M. P. Heyes, K. Saito, J. S. Crowley, L. E. Davis, M. A. Demitrack, M. Der, L. A. Dilling, J. Elia, M. J. P. Kruesi, A. Lacner, S. A. Larsen, K. Lee, H. L. Leonard, S. P. Markey, A. Martin, S. Milstein, M. M. Mouradian, M. R. Pranzatelli, B. J. Quearry, A. Salazar, M. Smith, S. E. Strauss, T. Sunderland, S. W. Swedo and W. W. Tourtellotte, *Brain*, (1992), **115**, 1249 - 1273.

94. S. Feldblum, A. Rougier, P. Loiseau, F. Cohadon, P. L. Morselli and K. G. Lloyd, *Epilepsia*, (1988), **29**, 523 - 529.

95. F. Moroni, G. Lombardi, G. Moneti and C. Aldinio, *Neuroscience Lett.*, (1984), **47**, 51 - 55.

96. F. Moroni, G. Lombardi, V. Carla, D. Pellegrini, G. L. Carassale and C. Cortesini, *J. Neurochem.*, (1986), **46**, 869 - 874.

97. A. M. Sardar, J. E. Bell and G. P. Reynolds, *J. Neurochem.*, (1995), **65**, 932 - 935.

98. S. A. Lipton, *TINS*, (1992), **15**, 75 - 79.

99. F. Moroni, G. Lombardi and V. Carla In *Quinolinic Acid and Kynurenines*; T. W. Stone, Ed., CRC Press, Boca Raton, (1989), 53 - 63.

100. C. Heidelberger, M. E. Gullberg, A. F. Morgan and S. Lepkovsky, *J. Biol. Chem.*, (1949), **179**, 143 - 150.

101. T. W. Stone and J. H. Connick, *Neuroscience*, (1985), **15**, 597 - 617.

102. R. Schwarcz, A. C. Foster, E. D. French, W. O. Whetsell, Jr. and C. Köhler, *Life Sci.*, (1984), **35**, 19 - 32.

103. M. N. Perkins and T. W. Stone, *Brain Res.*, (1982), **247**, 184 - 187.

104. P. Birch, C. J. Grossman and A. G. Hayes, *Eur. J. Pharmacol.*, (1988), **154**, 85 - 87.

105. W. Danysz, E. Fadda, J. T. Wroblewski and E. Costa, *Neuroscience Lett.*, (1989), **96**, 340 - 344.

106. A. C. Foster and J. A. Kemp, *Nature*, (1989), **338**, 377 - 378.

107. F. Moroni, P. Russi, V. Carla and G. Lombardi, *Neuroscience Lett.*, (1988), **94**, 145 - 150.

108. K. J. Swartz, M. J. During, A. Freese and M. F. Beal, *J. Neurosci.*, (1990), **10**, 2965 - 2973.

109. F. Moroni, P. Russi, V. Carla, G. De Luca and V. Politi In *Kynurenine and Serotonin Pathways*; R. Schwarcz, S. N. Young, R. R. Brown, Eds., Plenum Press, New York, (1991), 299 - 308.

110. I. A. Pearce, M. A. Cambray-Dekin and R. D. Burgoyne, *FEBS Lett.*, (1987), **233**, 143 - 147.

111. F. Moroni, D. E. Pellegrini-Giampietro, M. Alesiani, G. Cherici, F. Mori and A. Galli, *Eur. J. Pharmacol.*, (1989), **163**, 123 - 126.

112. J. W. Johnson and P. Ascher, *Nature*, (1987), **325**, 529 - 531.

113. W. A. Turski, J. B. P. Gramsbergen, H. Traitler and R. Schwarcz, *J. Neurochem.*, (1989), **52**, 1629 - 1636.

114. H.-Q. Wu, U. Ungerstedt and R. Schwarcz, *Eur. J. Pharmacol.*, (1992), **213**, 375 - 380.

115. J. B. P. Gramsbergen, W. Turski and R. Schwarcz In *Kynurenine and Serotonin Pathways*; R. Schwarcz, S. N. Young, R. R. Brown, Eds., Plenum Press, New York, (1991), 587 - 590.

116. H.-Q. Wu, H. Baran, U. Ungerstedt and R. Schwarcz, *Eur. J. Neurosci.*, (1992), **4**, 1264 - 1270.

117. P. D. Leeson, R. Baker, R. W. Carling, N. R. Curtis, K. W. Moore, B. J. Williams, A. C. Foster, A. E. Donald, J. A. Kemp and G. R. Marshall, *J. Med. Chem.*, (1991), **34**, 1243-1252.

118. J. A. Kemp, A. C. Foster, P. D. Leeson, T. Priestley, R. Tridgett, L. L. Iversen and G. N. Woodruff, *Proc. Natl. Acad. Sci. USA*, (1988), **85**, 6547 - 6550.

119. J. C. Jaen, E. Laborde, R. A. Busch, B. W. Caprathe, R. J. Sorenson, J. Fergus, K. Speigel, J. Marks, M. R. Dickerson and R. E. Davis, *Journal of Medicinal Chemistry*, (1995), **38**, 4439 - 4445.

120. T. Ishii, H. Iwahashi, R. Sugata and R. Kido, *Arch. Biochem. Biophys.*, (1992), **294**, 616 - 622.

121. T. Ogawa, W. R. Matson, M. F. Beal, R. H. Myers, E. D. Bird, P. Milbury and S. Saso, *Neurology*, (1992), **42**, 1702 - 1706.

122. J. T. Coyle and P. Puttfarcken, *Science*, (1993), **262**, 689 - 695.

123. R. Schwarcz, W. O. Whetsell, Jr. and R. M. Mangano, *Science*, (1983), **219**, 316 - 318.

124. C. A. Tamminga, *Am. J. Psychiatry*, (1997), **154**, 725.

125. M. E. MacDonald and T. H. s. D. C. R. Group, *Cell*, (1993), **72**, 971 - 983.

126. J. F. Gusella, N. S. Wexler, P. M. Conneally, S. L. Naylor, M. A. Anderson, R. E. Tanzi, P. C. Watkins, K. Ottina, M. R. Wallace, A. Y. Sakaguchi, A. B. Young, I. Shoulson, E. Bonilla and J. B. Martin, *Nature*, (1983), **306**, 234 - 238.

127. S. J. Pearson and G. P. Reynolds, *Neuroscience Lett.*, (1992), **144**, 199 - 201.

128. H. Baran and K. Jellinger, *Eur. J. Neurosci.*, (1998), **10**, 3209.

129. I. P. Lapin, *Epilepsia*, (1981), **22**, 257 - 265.

130. L. Turski, B. S. Meldrum and J. F. Collins, *Brain Res.*, (1985), **336**, 162 - 166.

131. S. M. Dursun, G. Farrar, S. L. Handley, H. Rickards, T. Betts and J. A. Corbett, *Mol. Chem. Neuropathol.*, (1994), **21**, 55-60.

132. H. Baran, N. Cairns, B. Lubec and G. Lubec, *Life Sci.*, (1996), **58**, 1891 - 1899.

133. O. Billker, V. Lindo, M. Panico, A. E. Etienne, T. Paxton, A. Dell, M. Rogers, R. E. Sinden and H. R. Morris, *Nature*, (1998), **392**, 289 - 292.

134. M. K. Manthey, J. F. Jamie and R. J. W. Truscott, *J. Org. Chem.*, (1999), **64**, 3930 - 3933.

135. R. van Heyningen, *Nature*, (1971), **230**, 393.

136. M. Varasi, A. Della Torre, F. Heidempergher, P. Pevarello, C. Speciale, P. Guidetti, D. R. Wells and R. Schwarcz, *Eur. J. Med. Chem.*, (1996), **31**, 11 - 21.

137. E. R. Glazier, *J. Org. Chem.*, (1962), **27**, 2937.

138. E. R. Glazier, *J. Org. Chem.*, (1962), **27**, 4397 - 4399.

139. R. Kober, W. Hammes and W. Steglich, *Angew. Chem., Int. Ed. Engl.*, (1982), **21**, 203 - 204.

140. R. Kober and W. Steglich, *Leibigs Ann. Chem.*, (1983), 599 - 609.

141. R. Kober, K. Papadopoulos, W. Miltz, D. Enders and W. Steglich, *Tetrahedron*, (1985), **41**, 1693 - 1701.

142. M. J. O'Donnell, J. M. Boniece and S. E. Earp, *Tetrahedron Lett.*, (1978), **30**, 2641 - 2644.

143. M. J. O'Donnell and R. J. Polt, *J. Org. Chem.*, (1982), **47**, 2663 - 2666.

144. P. L. Pickard and T. L. Tolbert, *Org. Synth.*, (1964), **44**, 51 - 53.

145. W. S. Johnson, E. L. Woroch and B. G. Buell, *J. Am. Chem. Soc.*, (1949), **71**, 1901 - 1905.

146. J. Koo, *J. Org. Chem.*, (1963), **28**, 1134 - 1135.

147. W. S. Johnson and H. J. Glenn, *J. Am. Chem. Soc.*, (1949), **71**, 1092 - 1096.

148. K. Soda and K. Tanizawa, *Adv. Enzymol.*, (1979), **49**, 1 - 40.

149. D. H. Fitzgerald and N. P. Botting, "Unpublished results," University of St.Andrews, 1999.

150. R. O. Duthaler, *Tetrahedron*, (1994), **50**, 1539 - 1650.

151. M. A. Blaskovich and G. A. Lajoie, *J. Am. Chem. Soc.*, (1993), **115**, 5021 - 5030.

152. E. J. Corey and N. Raju, *Tetrahedron Lett.*, (1983), **24**, 5571 - 5574.

153. P. Garner and J.-M. Park, *J. Org. Chem.*, (1987), **52**, 2361 - 2364.

154. P. Garner and J.-M. Park, *J. Org. Chem.*, (1990), **55**, 3772 - 3787.

155. P. Garner and J.-M. Park, *Org. Synth.*, (1991), **70**, 18 - 25.

156. P. Garner, *Tetrahedron Lett.*, (1984), **25**, 5855 - 5858.

157. E. Branquet, P. Durand, L. Vo-Quang and F. Le Goffic, *Synth. Commun.*, (1993), **23**, 153 - 156.

158. A. McKillop, R. J. K. Taylor, R. J. Watson and N. Lewis, *Synthesis*, (1994), 31 - 33.

159. A. Dondoni and D. Perrone, *Synthesis*, (1997), 527 - 529.

160. W. R. Roush and J. A. Hunt, *J. Org. Chem.*, (1995), **60**, 798.

161. J. S. Ravi Kumar and A. Datta, *Tetrahedron Lett.*, (1997), **38**, 6779.

162. A. Avenosa, C. Cativiela, F. Corzane, J. M. Peregrina and M. M. Zurbano, *Synthesis*, (1997), 1146.

163. A. D. Campbell, T. M. Raynham and R. J. K. Taylor, *Synthesis*, (1998), 1707 - 1709.

164. G. Bold, T. Allmendinger, P. Herold, L. Moesch, H.-P. Schär and R. O. Duthaler, *Helv. Chim. Acta*, (1992), **75**, 865.

165. G. Ageno, L. Banfi, G. Cascio, G. Guanti, E. Manghisi, R. Riva and V. Rocca, *Tetrahedron*, (1995), **51**, 8121.

166. S. Raghavan, M. Ishida, H. Shinozaki, K. Nakanishi and Y. Ohfune, *Tetrahedron Lett.*, (1993), **34**, 5765 - 5768.

167. I. Jako, P. Uiber, A. Mann and C.-G. Wermuth, *J. Org. Chem.*, (1991), **56**, 5729 - 5733.

168. R. Zibuck and J. M. Streiber, *J. Org. Chem.*, (1989), **54**, 4717 - 4719.

169. K. Shimamoto and Y. Ohfune, *Tetrahedron Lett.*, (1990), **31**, 4049 - 4052.

170. J. A. Stafford, M. F. Brackeen, D. S. Karanewsky and N. L. Valvano, *Tetrahedron Lett.*, (1993), **34**, 7873 - 7876.

171. R. Frank and M. Schutowski, *J. Chem. Soc., Chem. Commun.*, (1996), 2509 - 2510.

172. D. Moothoo, A. A. B. Robertson and N. P. Botting, "Unpublished results," University of St.Andrews, 1996.

173. F. Kröhnke, *Chem. Ber.*, (1950), **83**, 291 - 296.

174. J. A. Dale, D. L. Dull and H. S. Mosher, *J. Org. Chem.*, (1969), **34**, 2543 - 2549.

175. J. A. Dale and H. S. Mosher, *J. Am. Chem. Soc.*, (1973), **95**, 512 - 519.

176. D. E. Metzler and E. E. Snell, *J. Am. Chem. Soc.*, (1952), **74**, 979 - 983.

177. M. Harada, H. Nishitani, K. Koga, I. Miura and R. Kido, *Acta Radiologica*, (1992), **33**, 258 - 261.

178. J. Pekar, T. Sinnwell, L. Ligeti, A. S. Chesnick, J. A. Frank and A. C. McLaughlin, *Journal of Cerebral Blood Flow and Metabolism*, (1995), **15**, 312 - 320.

179. Y. J. Shen, P. N. Venkatasubramanian and A. M. Wyrwicz, *Anesthesiology*, (1994), **81**, A901.

180. J. L. Warnell and C. P. Berg, *J. Am. Chem. Soc.*, (1954), **76**, 1708 - 1709.

181. W. Wu, K. Saeki and Y. Kawazoe, *Heterocycles*, (1996), **43**, 1937 - 1942.

182. E. E. Snell and O. Hayaishi In *Biochemical Preparations*; , John Wiley & Sons, New York, (1953), 108 - 111.

183. R. R. Brown and J. M. Price, *J. Am. Chem. Soc.*, (1955), **77**, 4158 - 4159.

184. S. Ranganathan, D. Ranganathan and S. K. Singh, *Tetrahedron Lett.*, (1985), **26**, 4955 - 4956.

185. R. F. W. Jackson, D. Turner and M. H. Block, *J. Chem. Soc., Chem. Commun.*, (1995), 2207 - 8.

186. A. R. Surrey and H. F. Hammer, *J. Am. Chem. Soc.*, (1946), **68**, 113 - 116.

187. A. R. Surrey and H. F. Hammer, *J. Am. Chem. Soc.*, (1946), **68**, 1244 - 1246.

188. S. Ranganathan, D. Ranganathan and P. Ramachandran, *Tetrahedron Lett.*, (1984), **40**, 3145 - 3151.

189. S. Ranganathan, D. Ranganathan, S. Singh and D. Bhattacharyya, *J. Chem. Soc., Chem. Commun.*, (1987), 1887-1888.

190. R. Pellicciari, M. A. Gallo-Mezo, B. Natalini and A. M. Amer, *Tetrahedron Lett.*, (1992), **33**, 3003 - 3004.

191. W. C. Black, B. Guay and F. Scheuermeyer, *J. Org. Chem.*, (1997), **62**, 758 - 760.

192. T. D. Krizan and J. C. Martin, *J. Am. Chem. Soc.*, (1983), **105**, 6155 - 6157.

193. E. J. Corey and A. W. Gross, *Tetrahedron Lett.*, (1984), **25**, 495 - 498.

194. A. J. Bridges, A. Lee, E. C. Maduakor and C. E. Schwartz, *Tetrahedron Lett.*, (1992), **33**, 7495 - 7498.

195. P. Beak and V. Snieckus, *Acc. Chem. Res.*, (1982), **15**, 306 - 312.

196. F. G. Salituro and I. A. McDonald, *J. Org. Chem.*, (1988), **53**, 6138 - 6139.

197. E. Ponnusamy, U. Fotadar, A. Spisni and D. Fiat, *Synthesis*, (1986), 48-49.

198. J. M. Muchowski and M. C. Venuti, *J. Org. Chem.*, (1980), **45**, 4798 - 4801.

199. V. Snieckus, *Chem. Rev.*, (1990), **90**, 879 - 933.

200. R. D. Clark and J. M. Caroon, *J. Org. Chem.*, (1982), **47**, 2804 - 2806.

201. M. Itoh, *Chem. Pharm. Bull.*, (1969), **17**, 1679 - 1686.

202. K. Burger, M. Gold, H. Neuhauser and M. Rudolph, *Chem. Ztg.*, (1991), **115**, 77 - 82.

203. D. Ben-Ishai and A. Berger, *J. Org. Chem.*, (1952), **17**, 1564 - 1570.

204. D. E. Pearson and C. A. Buehler, *Synthesis*, (1972), 533 - 542.

205. H. D. Hartough and A. I. Kosak, *J. Am. Chem. Soc.*, (1947), **69**, 1012 - 1013.

206. W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, (1978), **43**, 2923 - 2925.

207. D. D. Perrin, W. L. F. Armarego and D. R. Perrin,*Purification of Laboratory Chemicals*; Pergamon Press, Oxford, (1980).

208. *Dictionary of Organic Compounds*; 5 ed.; J. Buckingham, Ed., Chapman & Hall, New York, (1982), Vol. 3.

209. I. Gimesi, S. Geri, G. Toth and S. Bajusz, 155,047 (Cl,C07c)/1969, *(Chem. Abs.*, (1969), **70**, P4605u).

210. G. W. Fenton and C. K. Ingold, *J. Chem. Soc.*, (1929), **1929**, 2342 - 2357.

211. M. Bergmann and L. Zervas, *Chem. Ber.*, (1932), **65**, 1192 - 1201.

APPENDIX

APPENDIX 1

Assay Conditions for Competitive Inhibitor Studies

Assay of kynureninase

Enzyme activity was determined fluorimetrically at 37 °C, according to the method of Shetty and Gaertner (1973). The rate of formation of product was measured at wavelengths of excitation and emission of 310 nm and 417 nm (anthranilic acid) and 330 nm and 410 nm (hydroxyanthranilic acid) using a Perkin Elmer luminescence spectrometer (MODEL LS50B) connected to a GRANT circulating water bath. The reaction mixture contained 20 μM pyridoxal-5-phosphate (PLP), 100 mM Tris-HCl buffer or 40 mM potassium phosphate buffer, pH 7.5 and an appropriate amount of enzyme in a final volume of 3 ml. The reaction was initiated by the addition of L-kynurenine or DL-hydroxykynurenine. Preliminary assays were performed to confirm the linear relationship between product formation and both time and protein concentration under these conditions. The amount of product formed was measured by reference to standard curves of fluorescence intensity against anthranilate/hydroxyanthranilate concentration.

Kinetic parameters were calculated using the *MacCurveFit* computer programme (Version 1.2) using non-linear regression analysis of the experimental data.

Inhibitor Studies

Preliminary studies involved screening compounds for their ability to reduce the rate of reaction at 1 mM inhibitor. K_i values were then determined by assaying the activity of the enzyme as above, but in the presence of appropriate concentrations of inhibitor and at a range of substrate concentrations spanning the K_m value. For inhibitor studies, the reaction was initiated with enzyme rather than substrate.

The values of 1/V were plotted against the reciprocal concentration of substrate, for each of the inhibitor concentrations studied. Intersection of the plots on the x-axis indicates competitive inhibition. A graph of the slopes of these plots versus inhibitor concentration gives a straight line with an intercept on the x-axis equal to $-K_i$.

Reversibility studies

Reversibility of inhibition was examined by dilution techniques. Enzyme, at 300 times its normal assay concentration was incubated for 10 min with inhibitor, at a range of concentrations, based on the K_i value determined. The enzyme/inhibitor reaction solution was then diluted 300 fold into the assay buffer and the activity measured by the addition of substrate. A control series was also set up in which enzyme was incubated alone for 10 min and diluted into the assay before adding inhibitor to give the diluted concentration. If inhibition was reversible, then the activity was restored following dilution, when compared to an undiluted series, in which enzyme was assayed in the presence of the same concentrations of inhibitor.

APPENDIX 2

List of Schemes, Tables, Graphs and Figures

Chapter 1 **Page**

Chapter 3

Chapter 4