MECHANISTIC STUDIES ON DIAMINE OXIDASE

A thesis presented in part fulfilment of the
requirements for the Degree of
Doctor of Philosophy

by

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To my wife Hazel and all my family,

One Love, One Blood.

ACKNOWLEDGEMENTS

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Lastly, I dedicate this thesis to those in my heart, my family. To my mother Yolanda and father Patrick for their many years of love and support. Most of all to my wife Hazel, for her endless encouragement and without whom my achievements would be meaningless.

ABBREVIATIONS

BSA	Bovine serum albumin
BSAO	Bovine serum amine oxidase
CBDC	Cyclobutanedicarboxylic acid
CID	Collision induced dissociation
DACCP	Diaminocyclohexylcarboxyphthalic acid Platinum (II)
DAO	Diamine oxidase
DBU	Diazabicyclo[5.4.0]undec-7-ene
DEAE	Diethylaminoethyl
DFMO	a-Difluoromethylornithine
DMAB	3-(Dimethylamino)benzoic acid
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenylhydrazine
DOPA	3,4-Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
ee	Enantiomeric excess
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
FAD	Flavine adenine dinucleotide
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
IR	Infrared
LSIMS	Liquid secondary ion mass spectrometry
K_M	Michaelis Menten constant

MBTH	3-Methyl-2-benzothiazolinone hydrazone
MGBG	Methylglyoxalbis(guanylhydrazone)
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
PQQ	Pyrroloquinoline quinone
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
THF	Tetrahydrofuran
TOPA	Topaquinone
UV	Ultraviolet
Vis	Visible
V_{max}	Maximum rate

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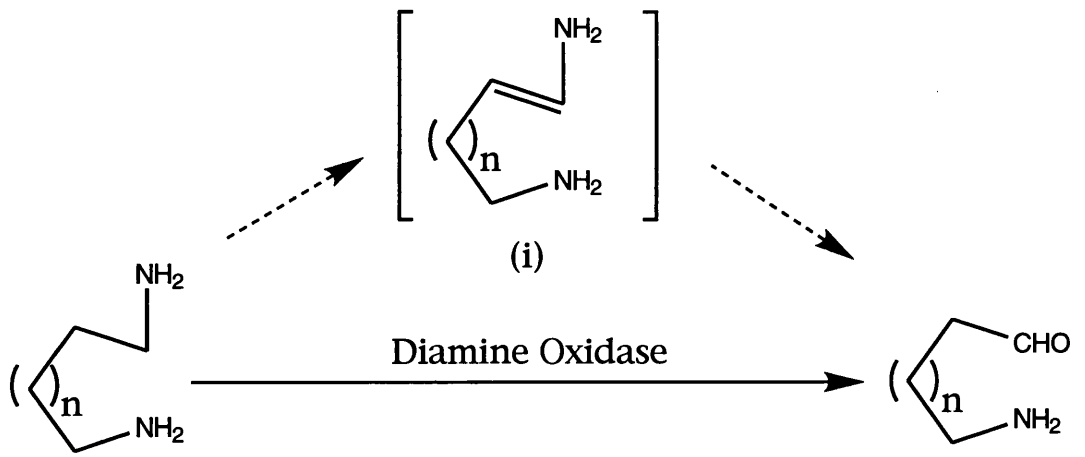
SUMMARY

To investigate the enzyme pea seedling diamine oxidase our studies concentrated on three main areas: 1. mechanistic studies of the oxidative deamination of primary diamines catalysed by diamine oxidase; 2. oxidation of aromatic compounds with amine side chains by pea seedling diamine oxidase; and 3. inhibition of pea seedling diamine oxidase. To make use of diamines synthesised within the research group a fourth area was studied: 4. synthesis of cisplatin analogues.

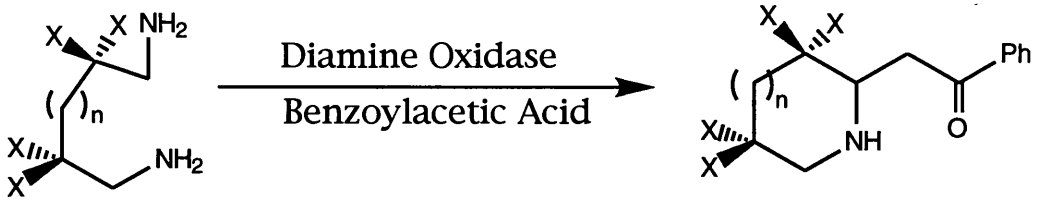
1. Mechanistic Studies of the Diamine Oxidase-Catalysed Deamination of Diamines

Diamine oxidase catalyses the oxidative deamination of diamines to their corresponding aminoaldehydes (Scheme A). To test the hypothesis that the diamine oxidase-catalysed oxidation proceeds via an enamine intermediate (i) we prepared a number of α,ω-diamines labelled with deuterium at the β-positions.



Scheme A

The [$^2H_4$]-labelled diamines and the corresponding unlabelled diamines were incubated with pea seedling diamine oxidase and the products were trapped with benzoylacetic acid *in situ.* (Scheme B). This gave substituted acetophenone products (ii) which were analysed by NMR and mass spectrometry. From a comparison of the spectroscopic data we were able to show that the enamine intermediate (i) is not involved in the enzymatic process.



X = H or D
n = 0-2

(ii)

Scheme B

2. Oxidation of Aromatic Compounds with Amine Side Chains by Pea Seedling Diamine Oxidase

A range of quinoline, pyridine, thiophene and pyrrole derivatives with amine side chains were synthesised and tested as substrates of pea seedling diamine oxidase using a spectrophotometric assay which measures the hydrogen peroxide by-product of the enzymatic reaction. From this assay V_{max} and K_M values were obtained for the oxidation of each substrate using diamine oxidase. The V_{max} is the maximal rate of oxidation and gives an indication of the oxidation rate for the various substrates. The K_M is a measure of the strength of the enzyme-substrate complex and determines the binding efficiency of the substrate to the enzyme. Analysis of this kinetic data provided information on

the enzymatic process and the nature of the pea enzyme's active site. Comparison of the kinetic data obtained from the various aromatic substrates enabled us to study the effect on the binding affinity and rate of oxidation from changes to the ring size of the substrates. The role of the second amine group was also explored using nitrogen heterocycles with amine side chains.

3. Inhibition of Pea Seedling Diamine Oxidase

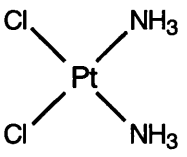
Polyamines are known to be essential in the growth and replication of cells, and diamine oxidase plays a key role in the polyamine metabolism with the oxidative deamination of diamines. Inhibitors of diamine oxidase should therefore have a considerable effect on the polyamine metabolism and hence cell growth. With this in mind inhibitors of diamine oxidase may possess important biological activity.

Compounds which were shown to be poor substrates but efficient binders of the pea seedling diamine oxidase from our initial studies were tested as inhibitors. These tests were carried out using the same spectrophotometric assay as before, but which had been modified to include the inhibitor. Most of the compounds tested were found to inhibit the diamine oxidase-catalysed deamination of putrescine and were shown to be competitive inhibitors.

4. The Synthesis of Cisplatin Analogues

Cisplatin (iii) is a widely used anticancer drug, but its therapeutic value is limited by the number of toxic side effects which it exhibits. To make use of the diamines available from other

studies, we attempted to make cisplatin analogues with various diamines used as bidentate ligands. As there were no examples of unsaturated diamines being used in cisplatin analogues we used *cis*-1,4-diaminobut-2-ene (iv) which had previously shown antibiotic activity. We made our target compound *cis*-1,4-diamino(dichloro)-platinum (II) (v), but were unable to make our second target *cis*-1,4-diaminobut-2-ene(1,1-cyclobutanedicarboxylato)platinum (II) (vi).



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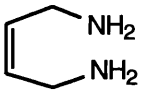

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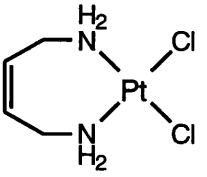

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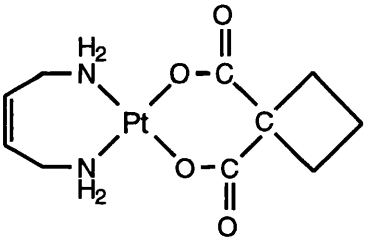

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CHAPTER 1

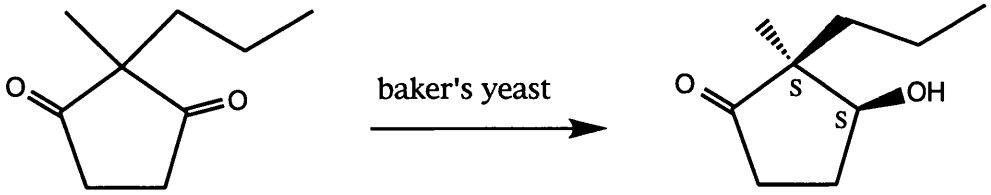
Introduction

1.1 The Growing Importance of Enzymes in Synthetic Applications

The significance of enzymes in organic synthesis has increased considerably over the past decade, with enzymatic reactions frequently now being employed to effect transformations that otherwise would be difficult to achieve by chemical means.[1]

Enzymes are exceptionally versatile catalysts and enzyme catalysed reactions often offer significant advantages over chemical methods. Not only do they allow possible control of stereochemistry and regiochemistry, but they are also extremely efficient in that they can increase the rate of reactions by over a million times. The ability of enzymes to catalyse a broad range of reactions in very mild conditions, at room temperature and in aqueous conditions at neutral pH make them an attractive option when dealing with fragile organic molecules.

The use of enzymes can provide methods for the preparation of optically active compounds. For example, the reduction of 2-methyl-2-propyl-1,3-cyclopentanedione (1) to (2*S*, 3*S*)-2-methyl-2-propyl-3-hydroxycyclopentanone (2) using baker's yeast,[2] gives an enantiomeric excess (ee) of 100% (Scheme 1.1). Reduction of (1) by $NaBH_4$ gives a mixture of the two racemates of 2-methyl-2-propyl-3-hydroxycyclopentanone in varying yields.



(1)

(2S, 3S): 100% ee

(2)

Scheme 1.1

Drug manufacturers find enzymes particularly beneficial in producing optically active compounds from achiral starting materials. They require to limit the synthesis of drugs to a single enantiomer, since only one enantiomer of a racemic mixture is generally responsible for the desired biological activity. The unwanted enantiomer may inhibit the desired effect of the active isomer and/or exhibit toxic side effects, as happened in the case of thalidomide. With the ever increasing demand for more selective drugs enantiomeric purity is becoming of vital importance and it seems that enzymes have an important role to play in future developments in this area.

There is an increasing number of commercially available biocatalysts which are capable of catalysing a wide range of biotransformations. Although use of isolated enzymes is generally favoured, whole organisms have been successfully employed as biocatalysts. The reduction of 2-methyl-2-propyl-1,3-cyclopentanedione (1) by baker's yeast in Scheme 1.1 is a typical example where *Saccharomyces cerevisiae* is the particularly versatile and easy to use micro-organism which is made up of a variety of dehydrogenase enzymes.[3a,b]

1.2 The Disadvantages of using Enzymes and Improvements using Immobilisation Techniques

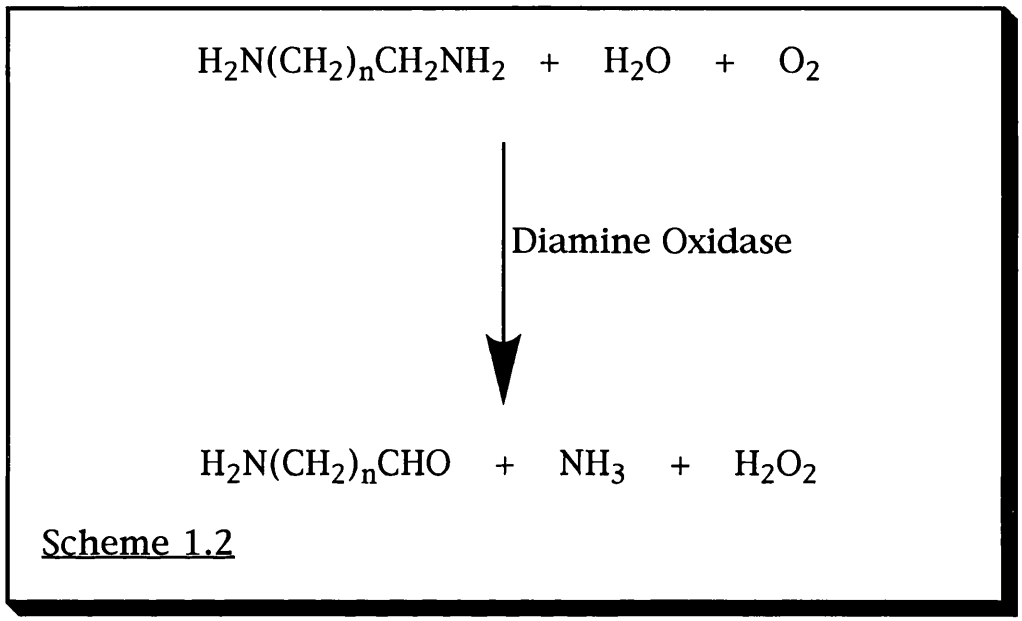
Although we are rapidly discovering more ways to exploit the unique properties of enzymes, there are some disadvantages in their use as organic catalysts, especially in industrial processes. Many enzymes are inactivated under extreme conditions, for example, at high temperatures or in highly acidic or basic solutions. Also, enzymes generally function optimally in aqueous solutions, rather than the organic solvents usually required to dissolve organic compounds. Moreover, enzymes are often inhibited by their substrates and their products at concentrations below those considered necessary for an economical process.

In recent years it has been shown that the stability of an enzyme can often be improved by making the molecule more rigid through multipoint attachment to a solid carrier.[4] This process is known as immobilisation and may be considered as the physical separation, during continuous operation, of the catalyst (enzyme) from the solvent in such a way that the substrate and product molecules may readily exchange between phases.[5] Separation of the biocatalyst from the solvent may be achieved by adsorption onto, or covalent binding to, insoluble organic or inorganic supports. Not only can the enzymes be made stable to the conditions of the industrial process but a batch reaction can more easily be terminated by the removal of an insoluble biocatalyst. Also, contamination of the organic product by the enzyme protein can normally be significantly reduced by immobilisation.

Various immobilisation techniques are available to the organic chemist, for example; chelation, adsorption and gel entrapment.

1.3 Diamine Oxidase

Diamine oxidases (DAO, EC 1.4.3.6) catalyse the oxidative deamination of a range of primary diamines to their corresponding aminoaldehydes (Scheme 1.2). The mechanism by which the deamination takes place is not fully understood. Current literature evidence will be reviewed in Chapter 2.



Scheme 1.2

Diamine oxidases are present in a wide variety of biological tissues, although two sources are particularly convenient. Pig kidney diamine oxidase is commercially available in a crude form and pea seedling diamine oxidase can be readily extracted from 10 day old pea seedlings.[6]

Diamine oxidases are copper-containing proteins. It was shown[7] that removal of the copper by dialysis with chelating agents caused deactivation of the enzyme and that the activity is restored on the subsequent addition of Cu^{2+}.

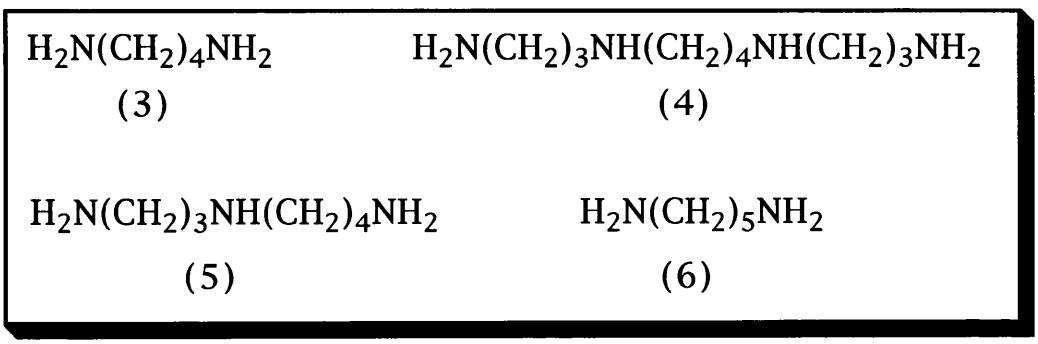
The identity of the organic cofactor has remained a mystery for many years. The fact that reagents capable of forming carbonyl

derivatives were shown to inhibit diamine oxidases[8] strongly suggested that the cofactor contained a carbonyl functionality. Early hypotheses centred around metal ions or pyridoxal phosphate as being the sole cofactor. However with the development of the hydrazine method,[9] opinions changed to consider the involvement of pyrroloquinoline quinone (PQQ). Recently strong evidence has been published which suggests that PQQ is not the cofactor and that topaquinone is the organic cofactor in a number of amine oxidases. The evidence relating to PQQ and other possible cofactors involved with amine oxidases will be discussed in Chapter 2.

1.4 Polyamine Metabolism and Cell Growth

Diamine oxidase has a significant role to play in the regulation of cellular levels of natural polyamines.

Crystals of polyamines were first described by Dutch microscopist Leeuwenhoek over three hundred years ago, and it took a further two centuries to identify the crystals as an organic base. This base was called spermine (4).[10] Since then the study of polyamines has fallen short when compared to many other biochemical topics. This is quite surprising since all plants and animals are thought to contain at least one polyamine, such as the widespread polyamines putrescine (3), spermine (4) and spermidine (5) and the less common polyamine cadaverine (6).



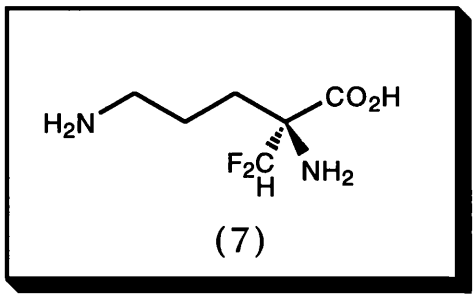
These natural polyamines are believed to have many key physiological roles to play. However it is their association with cell growth and replication that is the most important. The association between polyamines and cell growth stems from the ability of polyamines to undergo ionic binding to nucleic acids. At physiological pH, the protonated form of these polyamines bind strongly with the phosphate anions of the nucleic acids. This not only has a stabilising effect on DNA and RNA, but also speeds up every step of the transcription/translation sequence. This is the process where information coded by genes is used in the manufacture of proteins.[10]

There are two major pathways for polyamine biosynthesis. The first is the interconversion pathway which controls polyamine turnover by a cyclic process, and regulates intercellular polyamine levels. Decarboxylation of the simple amino acid ornithine by the enzyme ornithine decarboxylase (ODC) forms putrescine (3). Spermidine synthase then forms spermidine (5) from putrescine, and this is followed by the synthesis of spermine (4) from spermidine by the enzyme spermine synthase. These polyamines are then degraded by a process called catabolism. This occurs by a process of *N*-acetylation followed by oxidative cleavage which converts spermine back into spermidine, and spermidine back into putrescine.

The second major pathway is terminal polyamine catabolism which is catalysed by Cu^{2+} dependent amine oxidases. Each diamine can be converted into its corresponding aminoaldehyde by oxidative deamination of the primary amino group. The newly formed aminoaldehyde is further oxidised to the corresponding amino acid. These products and the *N*-acetylated polyamines are excreted in urine.

1.5 Inhibitors of Polyamine Biosynthesis

Development of inhibitors to polyamine biosynthesis in the late 1970s allowed biochemists to examine what happens to a system when the concentration of polyamines is significantly reduced. Merrell-Dow Pharmaceuticals synthesised α-difluoromethylornithine (DFMO) (7), which was found to bind specifically and irreversibly to ODC, thus inactivating it. This inhibition of ODC led to a significant reduction in the formation of polyamines which in turn inhibited cell proliferation in various cell cultures, for example leukaemia cells.[10] This work showed that polyamines are indeed involved in cell growth and replication, but the fact that this confirmed the importance of polyamines meant more research was required to realise the full potential of polyamine inhibition.



(7)

DFMO has subsequently been shown to possess very interesting antitumour activity due to its irreversible binding to ODC. Since tumour cells proliferate rapidly they have a higher demand for polyamines than normal cells, therefore inhibition of polyamine biosynthesis has a more detrimental effect on tumour cells.

Equally as interesting are the results obtained from experiments using plant systems. As well as the ODC pathway,

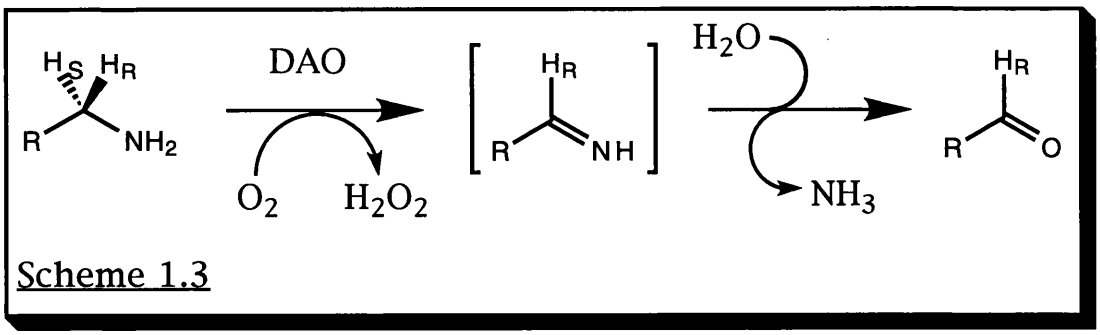
plants possess another pathway for polyamine biosynthesis via arginine and the enzyme arginine decarboxylase. However this is not the case for fungi which only possess the ODC pathway. So, if a particular fungus infects a plant (or crop), treatment with DFMO should lead to death of the fungus, while the plant (or crop) will be unaffected. This was shown to be the case when Rajam and Galston showed that DFMO had an inhibitory effect on the growth of several fungi on artificial media. Further work in collaboration with Weinstein showed that DFMO very effectively controlled rust infection in several types of beans.[10]

Since diamine oxidase is also involved in the determination of cellular levels of polyamines it would seem a reasonable assumption that inhibition of diamine oxidase may lead to useful antitumour and/or antifungal activity.

The kinetics of enzyme inhibition are discussed in Chapter 3, and the testing of various compounds as inhibitors of pea seedling DAO is described in Chapters 5 and 6.

1.6 Stereochemistry and Regiochemistry in Reactions Catalysed by Diamine Oxidase

Diamine oxidases are of low specificity acting upon a broad range of both monoamines and diamines. During deamination of an amine by DAO, a proton is removed from the prochiral methylene group adjacent to the nitrogen (Scheme 1.3). The absolute stereochemistry associated with this loss has been determined by various methods.



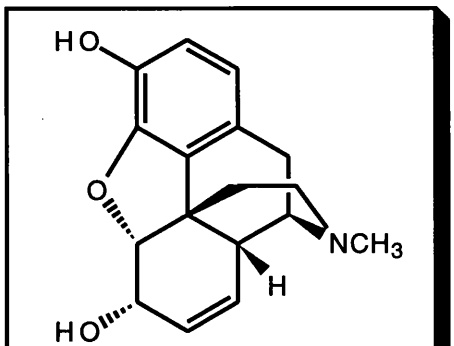
Scheme 1.3

Use of a wide range of substrates enantiomerically labelled with either deuterium or tritium has consistently demonstrated that the deamination of amines catalysed by DAO is accompanied by the loss of the pro-*S* hydrogen.[11-13]

Santaniello *et al.*[14] studied the regioselectivity of the DAO catalysed reaction. They showed that using pea seedling DAO, both (*R*)- and (*S*)-2-methylputrescine were regioselectively oxidised at the less hindered C-4 position. This is in contrast to pig kidney DAO where the regiochemistry differed depending on the stereochemistry of the substrate. These topics will be discussed further in Chapter 2.

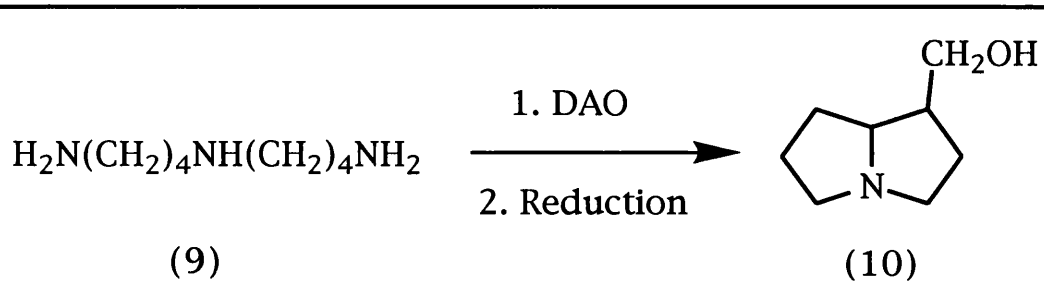
1.7 Alkaloid Biosynthesis

Alkaloids are organic compounds containing nitrogen, usually as part of a heterocyclic system, and they are of limited distribution among living organisms.[15] Alkaloids are found in plants and mosses, and some are known to have useful pharmaceutical properties, for example morphine (8) isolated from the opium poppy.



(8)

Pyrrolizidine alkaloids are bicyclic compounds which are wide-spread in plants, and homospermidine (9) has been shown to be a key intermediate in their biosynthesis. The initial steps in the conversion of homospermidine into pyrrolizidine alkaloids possibly involve oxidation of the primary amino groups. It has been shown that incubation of homospermidine with DAO and subsequent reduction of the likely product, 1-formylpyrrolizidine leads to the pyrrolizidine alkaloid trachelanthamidine (10) (Scheme 1.4).[16]



(9) (10)

Scheme 1.4

This use of diamine oxidase as an isolated enzyme may prove to be a convenient method in the synthesis of a wide range of new alkaloid analogues.

1.8 Work Described in this Thesis

A series of *N*-alkyl and *C*-alkylputrescines were tested as substrates of diamine oxidase by Frydman *et al.*[17] They suggested that oxidative deamination of putrescine (3) catalysed by DAO proceeds through an enamine intermediate. We decided to test this hypothesis by preparing α,ω-diamines strategically labelled with deuterium to monitor the reaction. The products of the enzyme reactions were coupled with benzoylacetic acid *in situ* to form phenacyl products. These compounds were analysed by NMR and MS, and the results are discussed in Chapter 4.

In order to obtain information on the active site of the DAO enzyme, a range of aromatic amines have been synthesised for testing as substrates and/or inhibitors of DAO. Following on from previous work involving pyridine derivatives, we synthesised a range of quinoline and pyridine derivatives with an alkylamine side chain. These were tested as substrates and/or inhibitors of pea seedling DAO and the results are described in Chapter 5.

Our studies then focused on the "π-excessive" aromatic systems of thiophenes and pyrroles. A range of thiophene and pyrrole derivatives with an alkylamine side chain have been synthesised and tested as substrates and/or inhibitors of pea seedling DAO. The results are discussed in Chapter 6.

In order to make use of the wide range of diamines synthesised within the research group, attempts were made to synthesise analogues of the well known antitumour drug cisplatin. It was hoped to use these diamines as bidentate ligands in a range of new platinum compounds. This work is described in Chapter 7.

CHAPTER 2

A Review of Diamine Oxidase

2.1 Isolation and Purification of Diamine Oxidase (DAO)

Although diamine oxidases are common in nature, there is at the present time only two convenient methods for their isolation. Crude DAO enzyme can be extracted from pig kidney and a purer form of the enzyme can be extracted from young pea seedlings. The DAO content of the pea seedlings is at a maximum between 7 and 16 days after germination,[18] although activity varies depending on the variety of pea.

A procedure for the purification of pea seedling DAO was developed by Hill,[6] where most of the unwanted material was removed by precipitation with a mixture of chloroform and ethanol (1:2). The enzyme was then isolated by precipitation with ammonium sulphate and then precipitation at pH 5 by the method of Tabor.[19] If highly pure enzyme is required it can be obtained by chromatography on hydroxyapatite DEAE-cellulose columns.

Diamine oxidase has been purified from pea epicotyls by the method of polyacrylamide gel electrophoresis (PAGE).[20] The DAO was purified to homogeneity by column chromatography on phosphocellulose and then MGBG-Sepharose, resulting in a 32 fold increase in specific activity. The purified enzyme showed absorption maximum at 280 nm and 500 nm. These procedures were developed using crude DAO which had been treated with 5% protamine and then concentrated with 65% saturated ammonium sulphate.[21]

The molecular weight of the purified DAO enzyme was found to be 180 000 which agreed with previously reported results. [21,22] Sodium dodecylsulphate (SDS) gel electrophoresis gave a single band of molecular weight 85 000. These results strongly suggest that the DAO enzyme consists of two identical subunits as in the other copper-containing amine oxidases.[23,24]

More recently a quick and easy method for purifying pea seedling DAO was developed.[25] The method was originally developed for studying the interaction of plant growth substances and cell wall polysaccarides[26] and was subsequently modified to purify DAO. This method involves packing the pea seedlings into a syringe barrel and attaching the barrel to a peristaltic pump to wash the seedlings with distilled water for 30 min. The seedlings are then vacuum infiltrated with 10 mM potassium phosphate buffer of pH 7 for 5 min. The DAO enzyme is isolated by buffer exchange into 20 mM potassium phosphate of pH 6 and 1 mM EDTA followed by FPLC on a cation exchange column.

2.2 The Role of Copper with the DAO Enzyme

Inhibition studies of diamine oxidase with various chelating ligands, such as cyanide, suggested that the enzyme is a metalloprotein and that inhibition takes place because these ligands act as carbonyl reagents.[27] Spectrographic analysis of highly purified pea seedling DAO showed that copper and manganese were the trace elements present in largest amounts.[28] The copper content was 0.08-0.09%, while the manganese was approximately 0.01%.

Mann[28] found that the DAO enzyme was inhibited by diethyldithiocarbamate because of the removal of copper.

Subsequent addition of Cu(II) ions led to reactivation of the enzyme.

Inhibition of DAO is also caused by chelating ligands which include salicylaldoxime and 8-hydroxyquinoline, although this inhibition can be reversed by the addition of a number of metal ions. Reversal appears to depend on the ability of the metal ion to displace the chelating ligand attached to the enzyme bound copper, leaving the active, copper-containing protein. These results suggest that copper may form part of the prosthetic group of the enzyme.

The debate on the role of copper in these enzyme reactions continues to be inconclusive. Electron paramagnetic resonance (EPR) experiments[29] failed to detect changes in the copper oxidation state during the enzymatic process. This has led to suggestions that Cu(II) may act as a Lewis acid;[30] that it has an indirect role in the catalysis;[31] or that it serves a structural role.[32]

It was thought that no Cu(I) was present in diamine oxidase,[33] however, recent new evidence suggests there may be a Cu(I) state present as a catalytic intermediate.[34] This area will be discussed later.

2.3 Early Proposals for Cofactors of Diamine Oxidase

The nature of the prosthetic groups involved with diamine oxidase has proved illusive to researchers over the last 30 years and studies continues to this day. Early studies[35] showed that diamine oxidase is inhibited by reagents capable of forming derivatives with carbonyl groups. Yamada and Yasunobu[8] showed complete inhibition of DAO by hydroxylamine and semicarbazide. This suggests that the active site contains some sort of carbonyl group. It was suggested by Mann[28] that this carbonyl group forms a

complex with copper and this complex makes up the prosthetic group of the DAO enzyme.

Previous evidence had suggested that both animal diamine oxidase[36] and pea seedling diamine oxidase[37] contain pyridoxal phosphate. However, since hydrogen peroxide is a by-product of the DAO catalysed reactions, it was thought that the enzymes were flavoproteins. Studies by Kapeller-Alder[38] and Goryachenkova[39] concluded that flavin adenine dinucleotide (FAD) was part of the prosthetic group of both animal and plant diamine oxidases. However, Mann[28] had concluded that the absorption spectrum of highly purified pea seedling DAO was not typical of an enzyme containing pyridoxal phosphate or FAD.

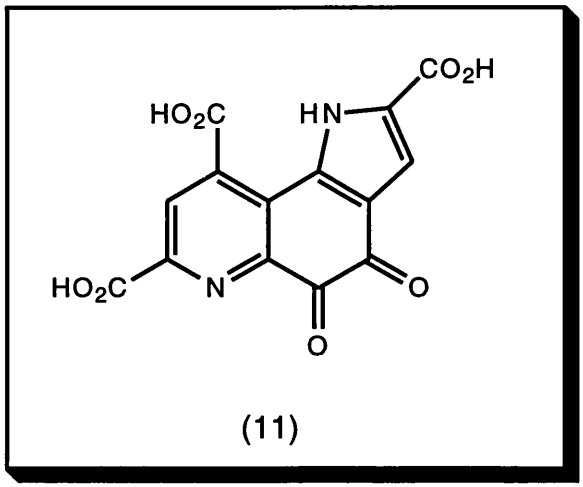
In 1984 two independent groups reported that bovine plasma amine oxidase contained pyrroloquinoline quinone (PQQ) as the cofactor.[9,40]

2.4 The History of Pyrroloquinoline Quinone (PQQ)

It was not until 1964 that substantial evidence for the involvement of PQQ (11) became available. During work on bacterial glucose dehydrogenase by Hauge,[41] a cofactor possessing different characteristics from well established cofactors was observed. Spectral studies by Hauge indicated that a naphthoquinone was present in the structure of the unknown cofactor.

Electron spin resonance (ESR) techniques used by Duine and co-workers indicated the presence of an organic free radical which had properties similar to those of an *o*-quinone. Examination of the fine structure of the ESR spectrum revealed the presence of two nitrogens. Suitable quantities were then isolated from methylotrophic bacteria for structure elucidation by Duine and co-

workers.[43] Confirmation that the cofactor was indeed PQQ was provided by X-ray characterisation of an acetone adduct of the cofactor.[44]



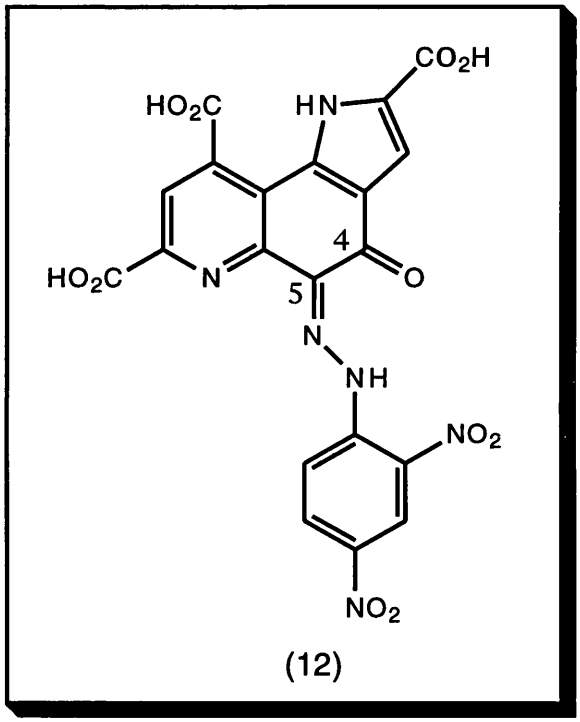
(11)

2.5 Pyrroloquinoline Quinone as a Cofactor for Copper Amine Oxidases

Indications that PQQ exists in a covalently bound form in amine oxidases resulted from work carried out on methylamine dehydrogenase.[45] The remarkable similarity between this enzyme and copper-containing amine oxidases led to the suggestion that PQQ may be a cofactor for this class of enzymes.

The two independent reports mentioned previously were by Lobenstein *et al.*[9] and by Ameyama *et al.*[40] The latter group found spectral similarities between PQQ and chromaphores isolated from bovine plasma amine oxidase. Lobenstein *et al.*[9] overcame problems of detaching the covalent cofactor from the protein by treating bovine serum amine oxidase with dinitrophenylhydrazine (DNPH) to give a stable adduct. This adduct was stable enough to survive enzyme proteolysis, and comparisons showed that the isolated product was identical to the hydrazone prepared from authentic

PQQ and DNPH. The derivatised product was shown to be homogenous by HPLC and it was found to have an identical retention time and absorption spectrum to that of the model PQQ-DNPH product. This evidence in fact showed pyridoxal phosphate was not the cofactor as the absorption spectrum of this adduct was quite different to that obtained by incubating pyridoxal phosphate with DNPH. Proton NMR studies on the derivatised product gave the structure as the monohydrazone (12) formed at the C-5 of PQQ.[46]



(12)

A fluorescing product was formed by degradation of the isolated product (12) in alkaline solution.[9] This fluorescing product had identical chromatographic properties to those of the compound obtained directly from PQQ. Subsequent applications of the "hydrazine method" suggest that PQQ is the second cofactor for a number of different amine oxidases.

The nature of the cofactor for pig kidney diamine oxidase was studied by Duine and co-workers.[47] They formed a hydrazone derivative with DNPH which after proteolytic degradation produced

a compound possessing the same chromatographic and spectroscopic properties as authentic derivatised PQQ. This approach has been used by Duine and co-workers to demonstrate that many mammalian oxidases contain PQQ, but has not been used for pea seedling diamine oxidase.

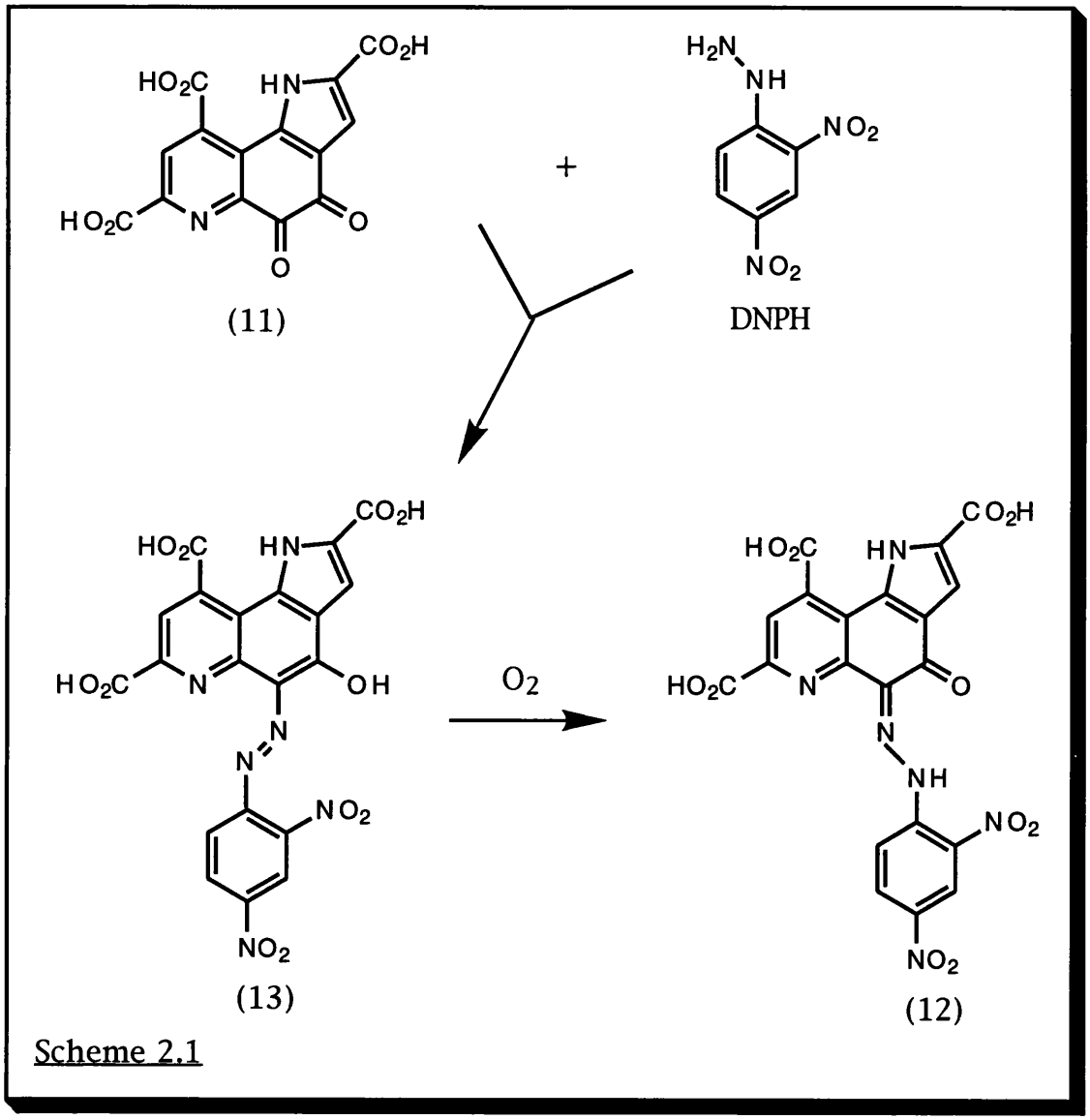
Glatz *et al.*[48] have however shown that pea seedling diamine oxidase has the ability to form derivatives with a number of reagents known to interact with PQQ. The fluorescence spectrum of purified DAO showed emissions consistent with that of a PQQ-containing enzyme. Addition of dimethoxyaniline to the enzyme showed the existence of a *o*-quinone by the characteristic absorbance (>500 nm), which is consistent with an *o*-quinone whose carbonyl groups are located on an aromatic ring.[49]

2.6 Using Oxygen to Improve the "Hydrazine Method"

Although the "hydrazine method" had proved useful, there were concerns over the extremely low yields of the isolated adduct. Initial studies[9] gave yields as low as 6% of the DNPH derivative and it was suggested that the hydrazone was the result of a reaction between DNPH and a PQQ impurity.

Reinvestigation by Duine and co-workers[46] found that the desired hydrazone was a minor product in the reaction between DNPH and the enzyme. The major product was a coloured compound possessing completely different properties from that of the hydrazone (12). Initially this compound was not identified, but it was discovered that, by carrying out the derivatisation under an oxygen atmosphere, the desired hydrazone was obtained at a yield 10 times that of normal conditions. After studying conditions for hydrazone formation, it was realised that treatment of the enzyme

with DNPH initially resulted in almost complete formation of the azo compound (13) and not hydrazone (12) (Scheme 2.1).



Scheme 2.1

However, this azo compound (13) can be converted into the desired hydrazone (12) at high concentrations of oxygen (Scheme 2.1).[50] Thus, by carrying out the initial derivatisation in an oxygen atmosphere the overall method can be significantly improved. Indeed, this improved method has been used to provide evidence of covalently bound PQQ in a wide range of enzymes.[51,52]

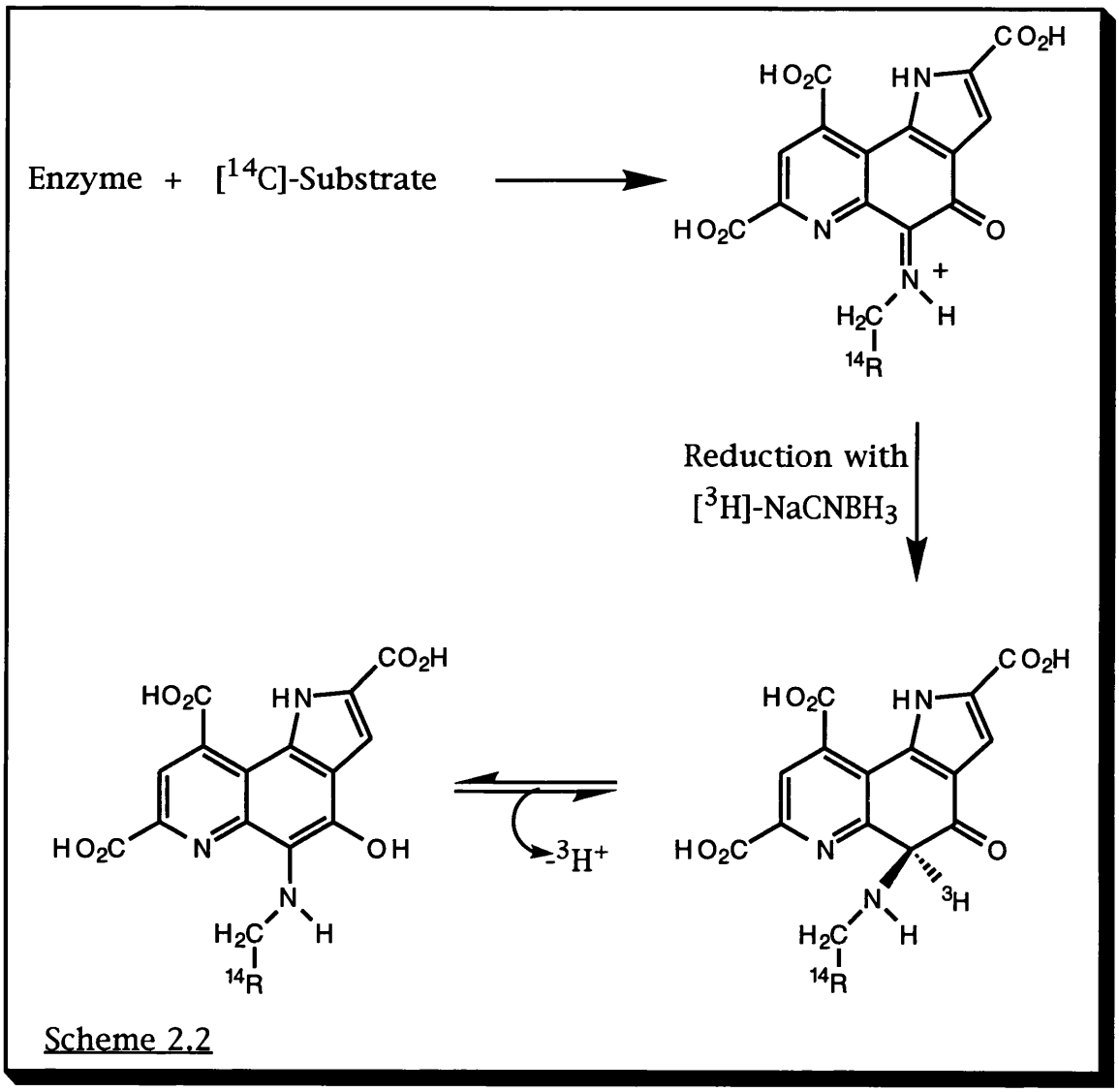
2.7 Alternative Methods for Detecting Enzyme Bound PQQ

A number of other approaches have been employed to detect PQQ in copper amine oxidases. Dooley and co-workers[53] carried out the first detailed structural characterisation for hydrazone derivatives of mammalian amine oxidases. Using resonance Raman spectroscopy techniques, comparisons were made between derivatives prepared directly from either PQQ or pyridoxal phosphate and those obtained from bovine and porcine plasma amine oxidases. The observed frequencies and relative intensities for these derivatives were in close agreement to those of the PQQ derivatives. These studies provided more evidence that PQQ or a derivative thereof is a cofactor for the amine oxidases, and that pyridoxal phosphate is not a cofactor.

Resonance Raman spectroscopy has been used by other groups for structure elucidation of cofactors.[54,55] However, these studies have been questioned because the absorption spectra obtained may have been due to the azo compound (see 2.6) and not the desired hydrazone.

Labelling studies examining the active site cofactor for bovine plasma amine oxidase were carried out by Hartmann and Klinman.[56] The enzyme was incubated with a high concentration of ^{14}C-labelled amine substrate followed by reduction of the Schiff base intermediate with [^{3}H]-labelled sodium cyanoborohydride. This resulted in stoichiometric incorporation of ^{14}C-labelled substrate into the enzyme, but no incorporation of ^{3}H. It was thought that these observations were consistent with the presence of PQQ. After reduction the substrate-cofactor complex would be labelled with tritium. However, the PQQ adduct possesses an acidic

proton at the C-5 position α to the C-4 carbonyl, and enolisation would lead to loss of the ^{3}H label (Scheme 2.2).



Scheme 2.2

Due to the apparent widespread occurrence of PQQ in enzymes there was a need for a new, fast analytical method for detection and quantitative analysis of PQQ in biological samples. A system was developed by Citro *et al.*[57] where antibodies could react with both free and protein bound PQQ to produce a specific antibody which allowed detection of PQQ in lentil amine oxidases.

Direct methods for detection of PQQ using carbonyl reagents were both insensitive and inaccurate according to Gallop and co-workers.[58] They developed a highly sensitive colourimetric assay

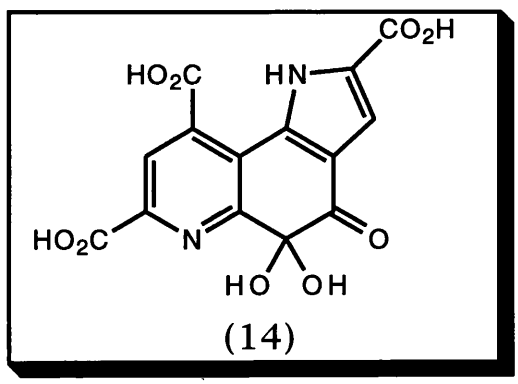
for detecting PQQ in cell extracts. If PQQ is present in greater than nanomolar quantities a significant background formazan colour is detected. This is due to reduction of PQQ by glycine to its hydroquinone and then reoxidation by nitroblue tetrazolium (NBT) which regenerates PQQ and formazan.

However, this method has been criticised[59] because of the reactivity of PQQ with certain amino acids leading to decarboxylation of the amino acid and formation of unreactive oxazole condensation products. Therefore, it is questionable whether the assay is quantitative for quinoproteins which require proteolysis to detach the cofactor.

2.8 Naturally Occurring Forms of PQQ

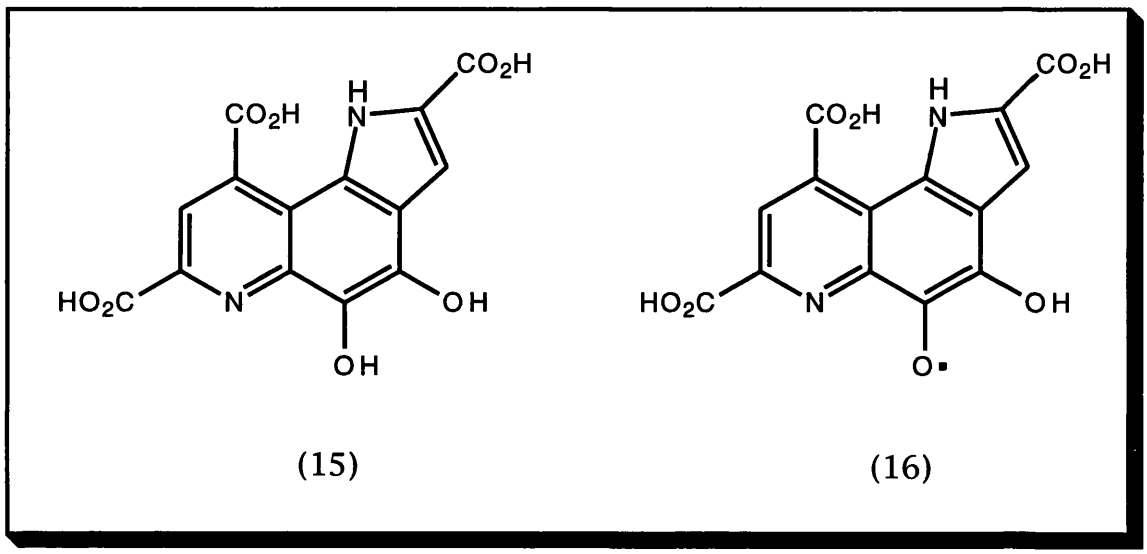
All mammalian quinoproteins so far investigated have been shown to contain PQQ in a covalently bound form. The cofactor is believed to be anchored to the protein by an amide or ester bond via a carboxylic acid grouping.

Spectroscopic studies of PQQ in aqueous solution have shown the presence of two interconverting species. Under these conditions, an equilibrium state is believed to exist between PQQ and its hydrated form (14), caused by the high reactivity of the C-5 carbonyl group towards nucleophiles.



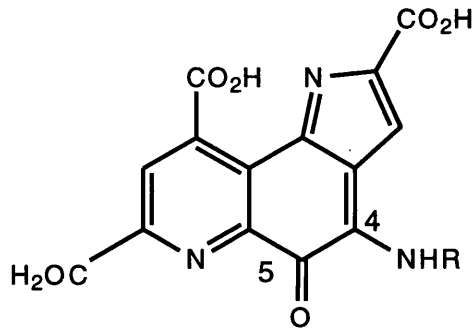
(14)

Two naturally occurring forms of PQQ have been discovered so far, the hydroquinone form PQQ(2H) (15) and a radical form PQQH· (16). It is unlikely that these forms will be present at physiological pH since reoxidation of these compounds can occur in aerobic conditions at pH >4.[60] However, they are able to exist when bound to proteins because of the increased stability associated with the protein environment and the shielding effect which prevents oxygen attack.



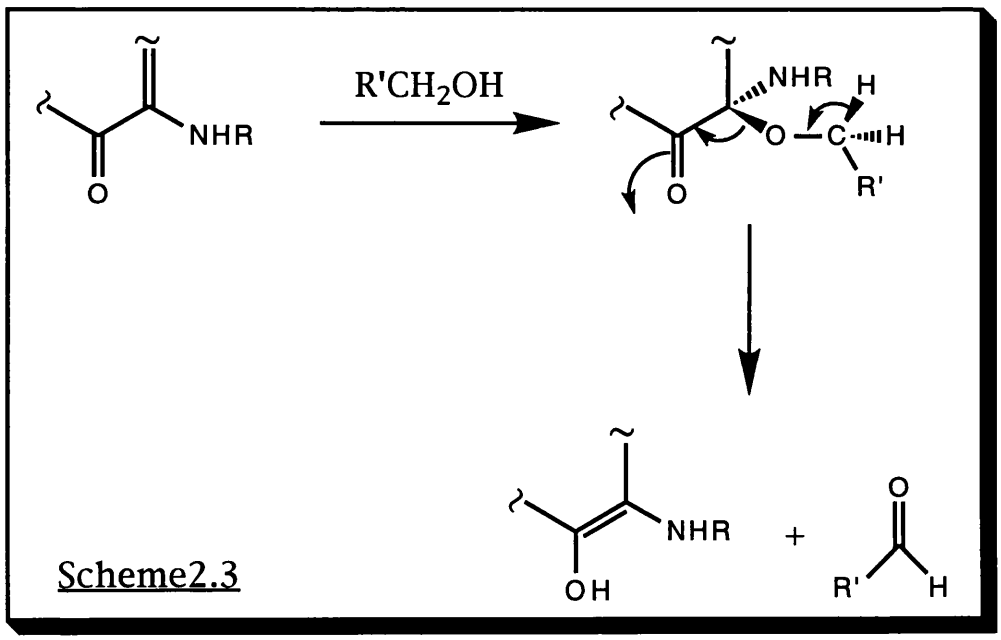
2.9 The Mechanisms Involved with PQQ

Early efforts to investigate the reaction mechanism in a PQQ-containing enzyme have mainly focused on methanol dehydrogenase. Covalent adducts have appeared consistently in studies of the mechanism and several research groups have reported activation of PQQ-dependent enzymes by either ammonia or primary amines.[61,62] This led to a proposed intermediate (17) being involved in the mechanism, formed via the interaction of an amine with PQQ.[63]



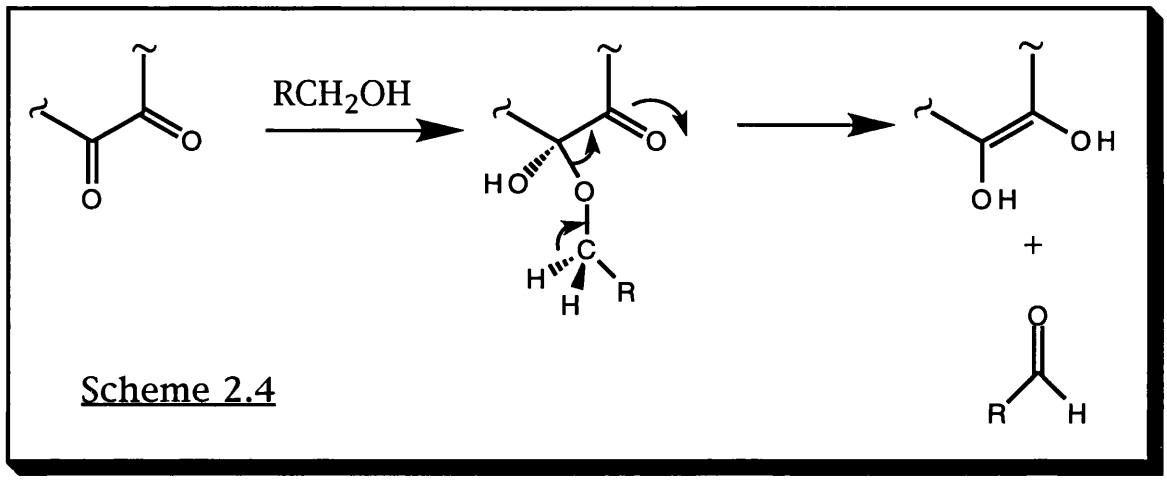
(17)

A reaction mechanism was suggested by Forrest *et al.*[63] in which attack of the amine intermediate (17) by an alcohol would lead to production of a carbinolamine derivative (Scheme 2.3). Subsequent oxidation of this derivative produces the desired aldehyde, as well as an aminoquinol containing nitrogen at the C-4 position, as the reduced form of the cofactor.



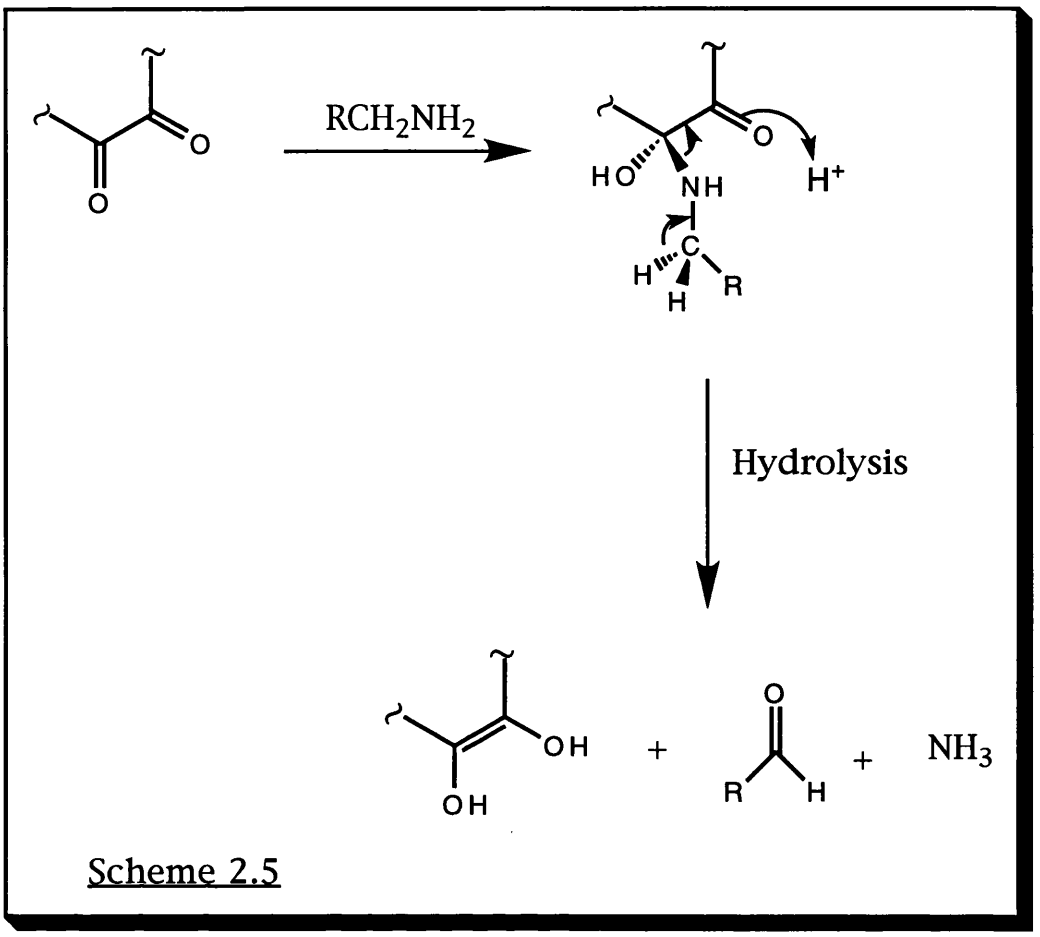
Scheme2.3

The above reaction scheme has one major drawback in that the reactive intermediate is formed via nucleophilic attack at the C-4 carbonyl. Existing studies[64] have shown that the C-5 carbonyl has a higher reactivity towards nucleophilic attack compared to the C-4 carbonyl. Taking this into consideration, an alternative mechanism can be written where initial formation of a hemiacetal at C-5, followed by substrate oxidation, leaves PQQ in a reduced quinol form (Scheme 2.4).



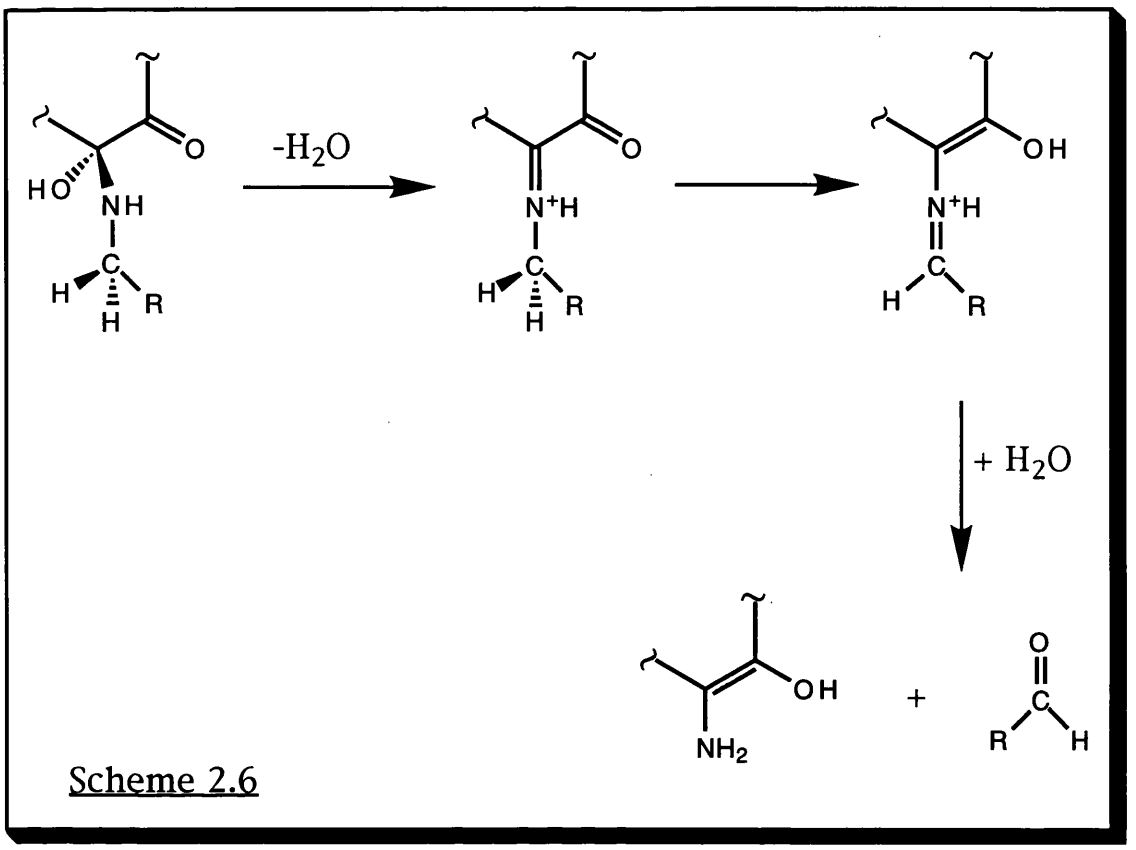
Scheme 2.4

An analogous mechanism has been proposed for amine oxidation which supports the observation that the quinol form of PQQ is the major product of the enzymatic reaction (Scheme 2.5).



Scheme 2.5

These similar mechanisms were thought to be an adequate representation for the role of PQQ in both classes of enzyme. However, there is a fundamental difference between amines and alcohols. Amines are able to generate stable Schiff base intermediates with PQQ, Bruice and co-workers[65] proposed a minor pathway for PQQ-catalysed amine oxidation (Scheme 2.6) with this fact in mind. Studies of the nitrogen transfer from substrate to cofactor during the cofactor reduction would be required to establish which of the two mechanisms occurs with amine oxidases.



Scheme 2.6

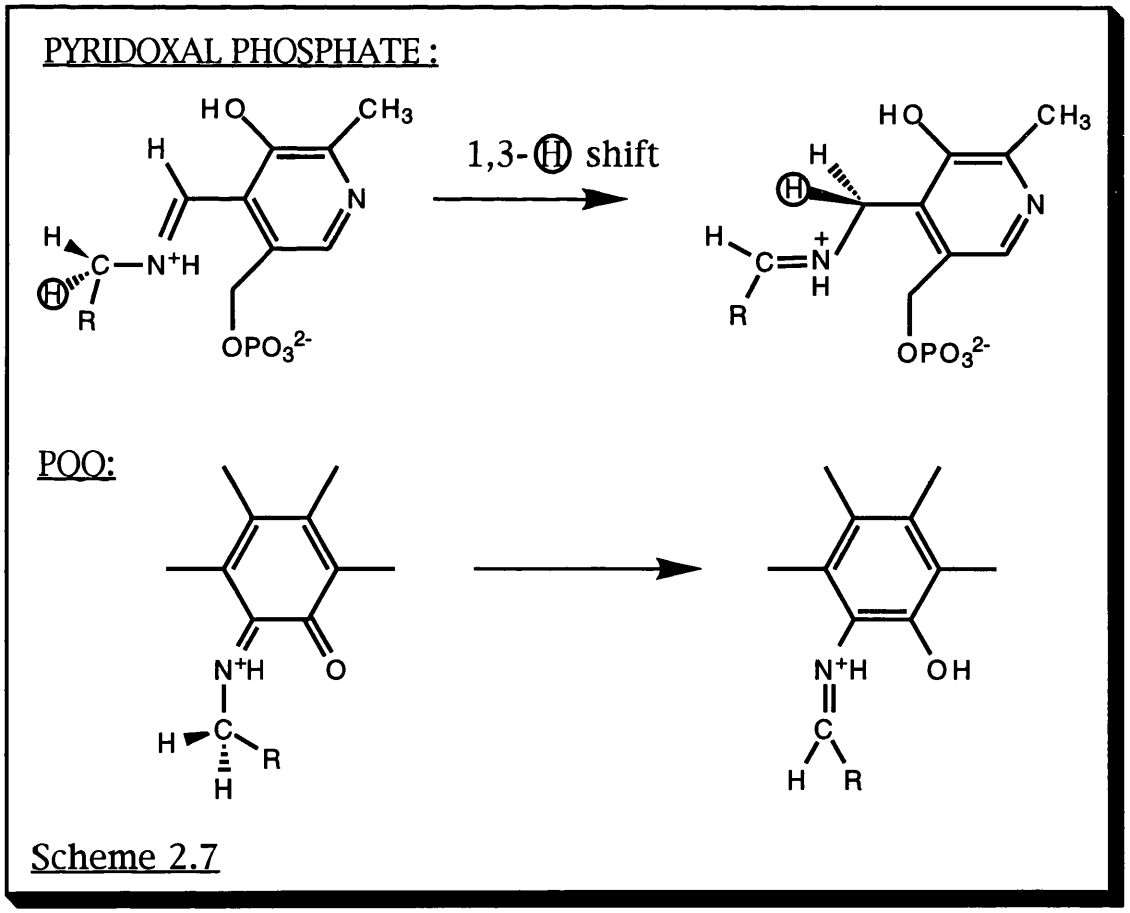
It has been reported by Taylor *et al.*[66] that under anaerobic conditions, amine oxidations using porcine plasma amine oxidase produce a burst of ammonia. This result appeared to rule out the latter suggestion of an aminotransferase mechanism (Scheme 2.6), since there is no ammonia produced from this pathway. However, later studies on the comparison between native enzyme and inhibited enzyme during the oxidation process showed no significant difference in ammonia release.[67] This suggests that it is non-specifically bound ammonia that is being released during the enzymic process.

Ruis *et al.*[68] used a quench technique and a sensitive assay for ammonia detection to show clearly that ammonia release, during the enzymic oxidation of benzylamine, correlated with the enzymic re-oxidation and not benzaldehyde formation. From these results it is deemed that the aminotransferase mechanism (Scheme 2.6)

provides a more legitimate description of how PQQ is involved in the oxidation of amines by enzymes.

2.10 A Further Insight into the Chemistry of PQQ

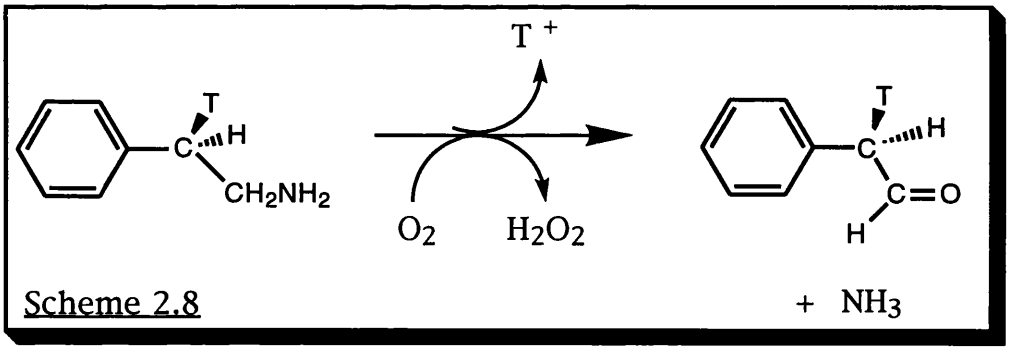
The mechanisms described so far, for the interaction of amines with PQQ, appear to be of a similar nature to that of known interactions of amines with pyridoxal phosphate. In both cases the Schiff base is formed and the proton is then removed from C-1 by a base catalysed reaction. A possible difference in the two mechanisms may be in the fate of the α-hydrogen during substrate oxidation (Scheme 2.7).



Scheme 2.7

The pyridoxal phosphate-substrate complex undergoes a 1,3-prototropic shift whereas the α-dicarbonyl structure of PQQ promotes proton transfer to the oxygen of the C-4 carbonyl.

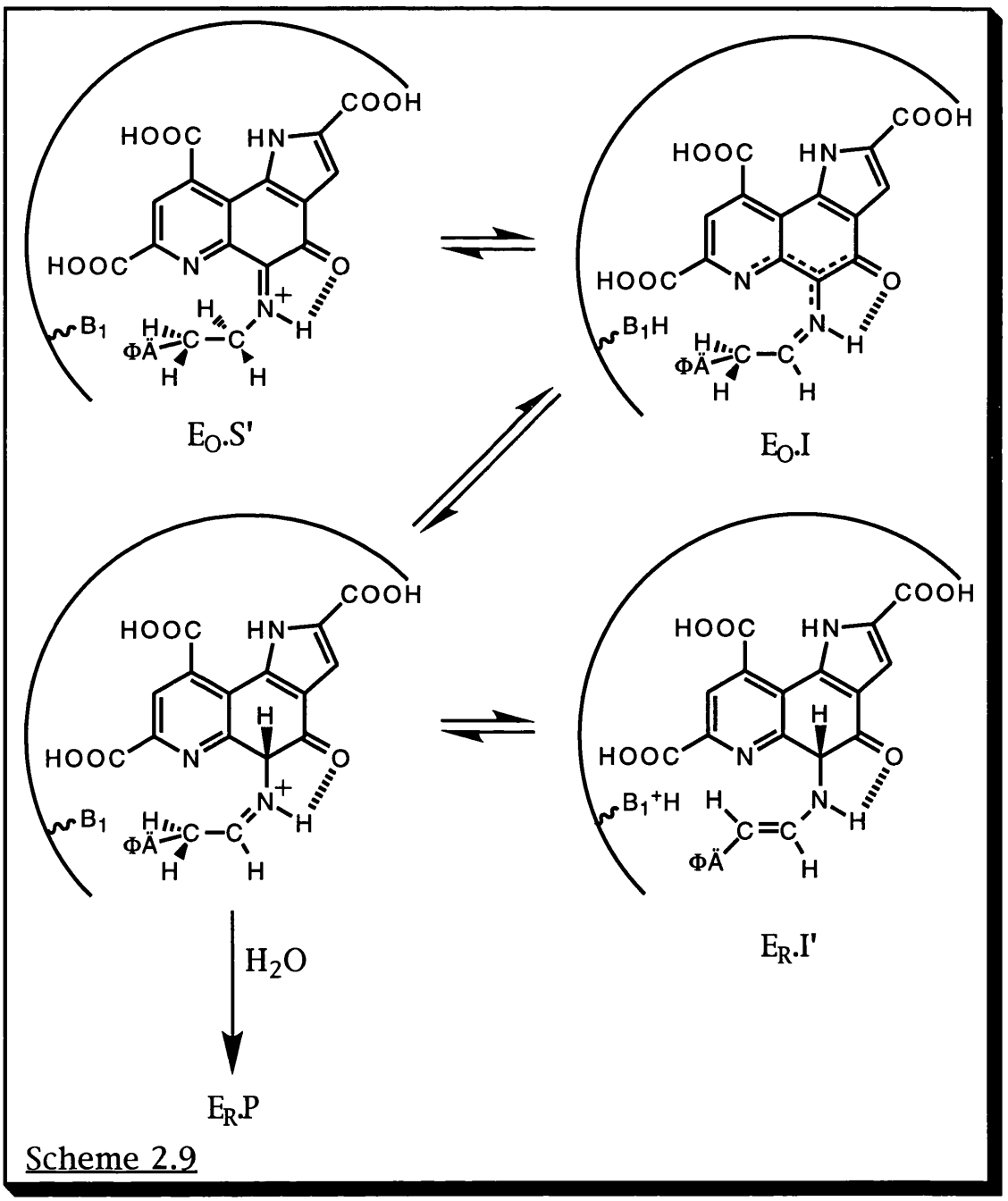
Further experiments carried out to investigate this concept focused on bovine plasma amine oxidase. Lovenberg and Beaven[69] noted that enzymic oxidation of phenylethylamines by plasma amine oxidases gave rise to a product which had undergone β-hydrogen exchange (Scheme 2.8). It was found that this exchange was kinetically rapid compared to the rate of the cofactor reduction. Further kinetic studies showed that the transfer of reducing equivalents from substrate to cofactor requires to be reversible and, that a subsequent step to cofactor reduction is partially rate limiting. As a consequence of the kinetic inequality, it was proposed that the bovine plasma amine oxidase functioned via a two base mechanism.



Scheme 2.8

Subsequent studies on pH dependency of bovine plasma amine oxidase led to the observation that identical pK_a values existed for both the exchange process and the substrate oxidation process.[70] This result implies strongly that an identical residue was involved in both steps. Strong support for this theory was recently provided by a series of stereochemical probes into the reaction.[71] If the two base theory is to be discounted then another explanation for the kinetic inequality must be found.

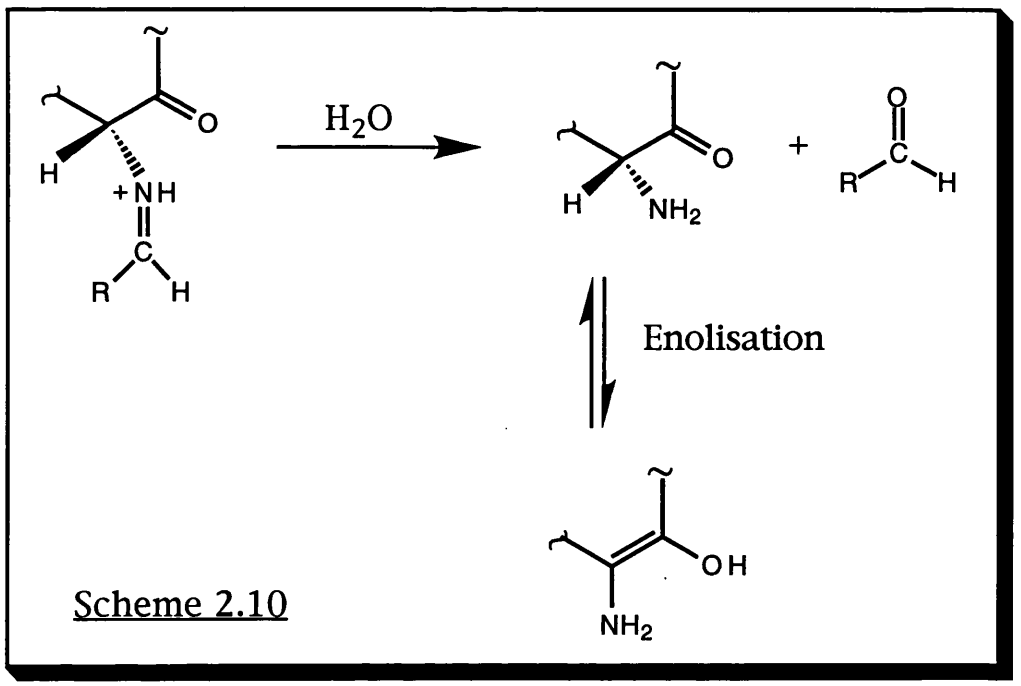
Current theories have returned to a 1,3-prototropic shift similar to that observed with pyridoxal phosphate. This proposal has been incorporated into a mechanism in which a Schiff base complex is formed between the substrate and PQQ (Scheme 2.9); followed by oxidation of the substrate via a proton abstraction mechanism; and transfer of both hydrogen and nitrogen from C-1 of the substrate to cofactor in the reductive half reaction.



Scheme 2.9

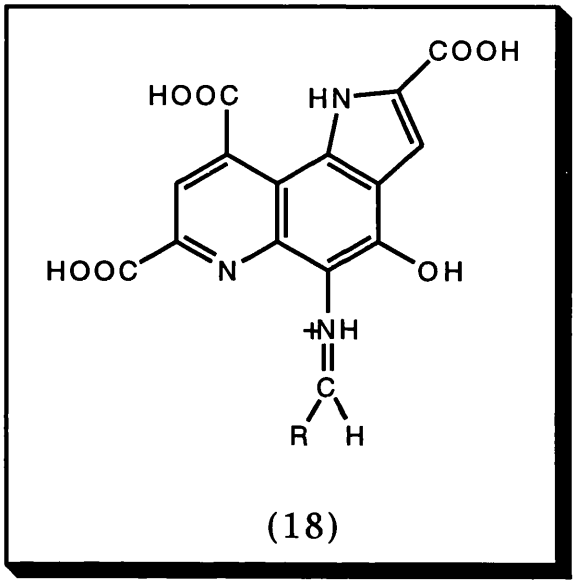
Scheme 2.9 shows the proposed mechanism for bovine plasma amine oxidase, in which a single active site residue, EB1 catalyses both substrate oxidation and exchange. $E_O.S'$ is the Schiff base complex between the amine substrate and C-5 of the cofactor; $E_O.I$ is the transiently formed carbanionic intermediate; $E_R.P$ is the product Schiff base, involving 1,3-prototropic shift from substrate to cofactor; $E_R.I'$ is the enamine formed in the course of hydrogen exchange from the β-carbon.[70]

Although this mechanism seemed best equipped to explain reported observations, certain aspects of it had to be justified. Namely, the postulated mechanism seems at first to contradict the results of Hartmann and Klinmann[56] (Section 2.7), who noted the loss of a tritium label from C-5 through a proposed enolisation process. It has been suggested that enolisation is a slow process within the product-imine complex and only occurs after hydrolysis of this intermediate (Scheme 2.10). This is in agreement with the proposed mechanism.



Scheme 2.10

Another feature of the mechanism which had to be confirmed was the protonation at the C-5 position. This process appears to be unfavourable, preventing the formation of the stable aromatic product (18) that would be formed from the reaction of the amine substrate and PQQ. However, the formation of this product might be expected to reduce significantly the rate of hydrolysis, contrary to the observed kinetics which show that the rate of hydrolysis is fast relative to other steps.



(18)

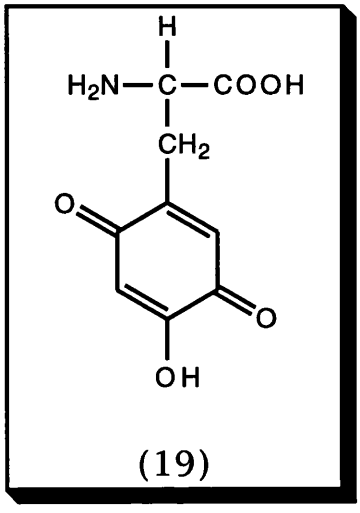
2.11 Arguments Against PQQ as a Cofactor for Amine Oxidases

Although there is much evidence in support of PQQ as the organic cofactor in enzymes, there was a concern about the lack of direct evidence for the presence of PQQ at the active site of a mammalian protein. An active site cofactor-containing peptide has been isolated from pig kidney diamine oxidase,[46] however the extremely poor yield obtained (0.1%) limited the characterisation to amino acid sequencing.

These results have subsequently been questioned and recent work by Hol and co-workers has cast doubt over the nature of a number of cofactors previously reported to be PQQ.[72] X-Ray diffraction studies on methylene dehydrogenase from *Thiobacillus versutus* have produced diffraction patterns which indicate the presence of an active site dicarbonyl which lacks the pyridine ring found in PQQ. This contradicts the earlier work of Duine and co-workers on methylamine dehydrogenase.[50]

In 1991 Ito *et al.* reported the crystal structure of galactose oxidase, previously claimed to contain PQQ.[73] They observed a crystal structure which showed all electron density could be accounted for by the known primary structure of the protein and solvent ions in solution. There was no density observed which corresponded to PQQ.

In 1990 Janes *et al.*[74] obtained direct evidence supporting the argument that PQQ was not the cofactor in mammalian protein. They isolated an active site cofactor-containing protein from bovine serum amine oxidase (BSAO). With a high yield (40%) and relatively small size of protein, a complete structural analysis was achieved. The result of this analysis led to the suggestion that the organic cofactor for BSAO was topaquinone (TOPA) (19), and not PQQ.



(19)

The cofactor-containing peptide was derivatised, as before, with phenylhydrazine to yield the stabilised phenylhydrazone. Mild enzymic proteolysis of the derivatised BSAO and purification by HPLC produced the desired peptide in 40% yield. The sequence of the purified peptide was shown to be;

-Leu-Asn-X-Asp-Tyr-

Since only one amino acid was detected at each round of peptide sequencing, it was concluded that the cofactor had a single, stable point of attachment to the protein. The sequence was verified in subsequent peptide preparations and for all the spectroscopic characterisations. The spectroscopic techniques used included (a) mass spectrometry; (b) UV/Vis spectroscopy; and (c) ^{1}H NMR spectroscopy.

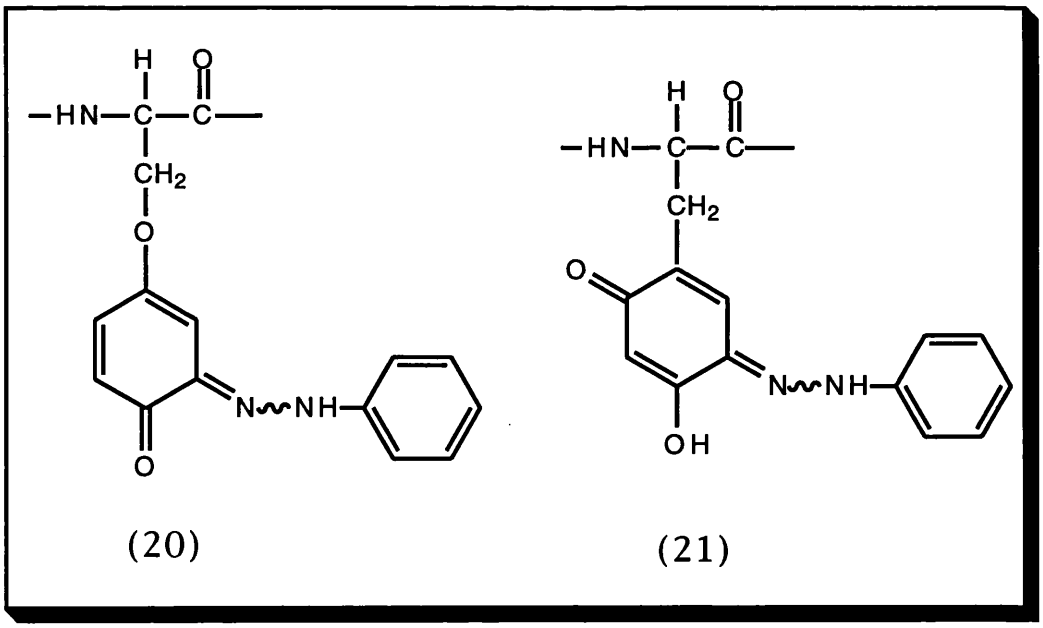
(a) Peptide characterisation by mass spectrometry (MS)

Studies using liquid secondary ion mass spectrometry (LSIMS) gave a molecular ion (MH^+) of *m/z* 807.5 for the phenylhydrazone pentapeptide isolated from BSAO. If the cofactor was initially derivatised with [1-^{15}N]-phenylhydrazine, then a molecular ion of *m/z* 808.5 was obtained. This result indicates that the phenylhydrazone moiety is preserved in the isolated peptide. Derivatisation of the carboxylic acid groups with acidic hexanol produced a new MH^+ at *m/z* 975.5. Since derivatisation of each acid group should give rise to an increase of 84.1 daltons, the observed difference of 168.2 daltons corresponds exactly to the formation of two ester linkages. Therefore, it would appear that two carboxylic acid groups are present in the isolated peptide. As both of these groups are present in the pentapeptide backbone (at Asp and at the carboxyl terminus) it would appear that no more free carboxyl

groups are present in the cofactor. Although one of the carboxylic acid groups could conceivably be involved in an amide linkage with the peptide itself, the absence of the remaining two groups would seem to rule out the presence of PQQ as the cofactor in this enzyme.

Subsequent studies on the dihexyl derivative of the penta-peptide led to an accurate mass measurement of *m/z* 974.5123, which on subtraction of the two hexyl derivatives, left a mass of 806.3245 for the peptide. Further subtraction of the accurate masses for the known amino acid components of the peptide gave a value of 283.0967 for X. This is not consistent with a PQQ type structure.

Computer permutations of the elemental composition gave five empirical formulae for X which were within ±5 ppm of this value. However, only one value, $C_{15}H_{13}N_3O_3$, was compatible with both the UV/Vis absorbance properties of the active site cofactor and the presence of a phenylhydrazone in X. Two possible structures (20) and (21) are compatible with this empirical formula.



High energy collision induced dissociation (CID) mass spectroscopy was used to examine the derivatised pentapeptide in an attempt to distinguish between the two structures above. The results obtained from the spectra allowed a tentative assignment of the active site cofactor in BSAO to be TOPA (19).

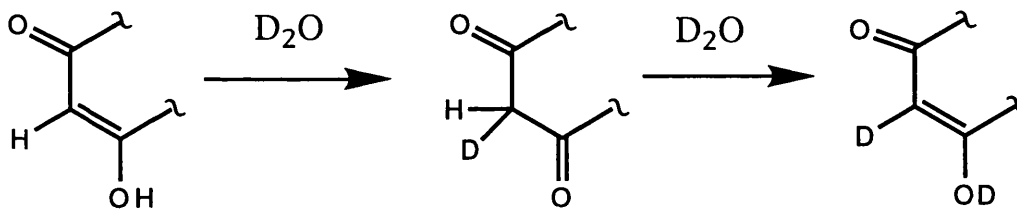
(b) Peptide characterisation by UV/Vis spectroscopy

Janes *et al.* observed λ_{max} at 448 nm for the phenylhydrazone-derivatised bovine serum amine oxidase, which was consistent with previous work.[74] This value correlates with the expected value for the phenylhydrazone derivative of PQQ, and has been cited in the past as evidence for the presence of PQQ. However, Janes *et al.* have shown that there is a shift of *ca.* 14 nm to 434 nm for the λ_{max} of the isolated peptide derivative. This new value correlated with the observed λ_{max} for the phenylhydrazone derivative of topaquinone. It was suggested that the effect of the protein side chain is to shift the value of λ_{max} to an absorbance which, by coincidence, corresponds to that obtained for PQQ.

(c) Peptide characterisation by NMR spectroscopy

As final confirmation of the structure of the active site cofactor, proton NMR studies of the pentapeptide were undertaken. Initially in D_2O, the spectrum produced was consistent with a pentapeptide backbone and tyrosine side chain. In addition to these were three further resonances at δ 6.9, 7.2 and 7.5, which had an integration ratio of 1:1:4.5 respectively. The equivalent protons were initially believed to be due to the phenylhydrazone ring and the remaining two protons were associated with the cofactor.

However, decoupling experiments using a nitrophenylhydrazone derivative showed that the signal at δ 7.2 could be attributed to the phenylhydrazone ring. Thus only one proton resonance was observed for the cofactor when the sample was run in D_2O. This is not surprising since the C-5 proton of TOPA lies between an enol and a ketone, which in the presence of D_2O would undergo tautomerism to form a deuteriated cofactor with a single proton resonance (Scheme 2.11).

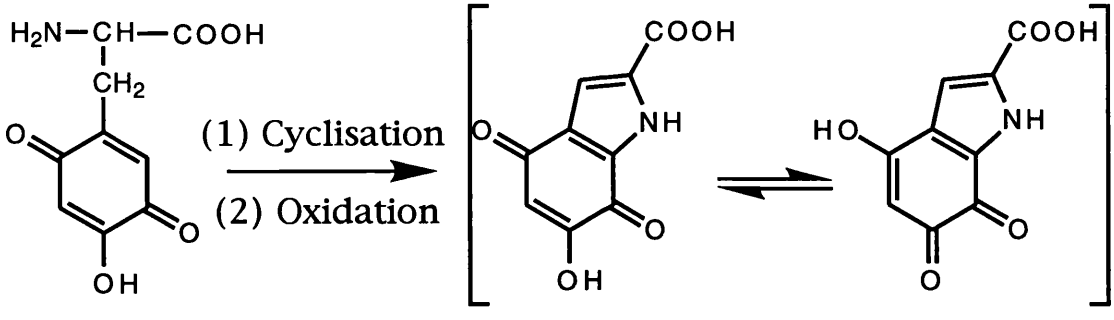


Scheme 2.11

Confirmation was given by running the pentapeptide sample in H_2O, providing a spectrum which produced an unambiguous characterisation of the cofactor structure. These findings have far reaching consequences in relation to other enzymes considering it is likely that all copper amine oxidases have a common cofactor. If TOPA is in fact the common cofactor, how was its structure confused with that of PQQ?

To prove the presence of PQQ as the cofactor evidence had relied on the isolation of phenylhydrazone derivatives obtained from the studied enzyme which coeluted on HPLC with authentic samples of PQQ phenylhydrazones. Janes *et al.* suggested that a derivative of TOPA phenylhydrazone, formed from BSAO during proteolysis, may coelute with the phenylhydrazone of PQQ.[75] This may occur because topaquinones undergo a rapid intramolecular

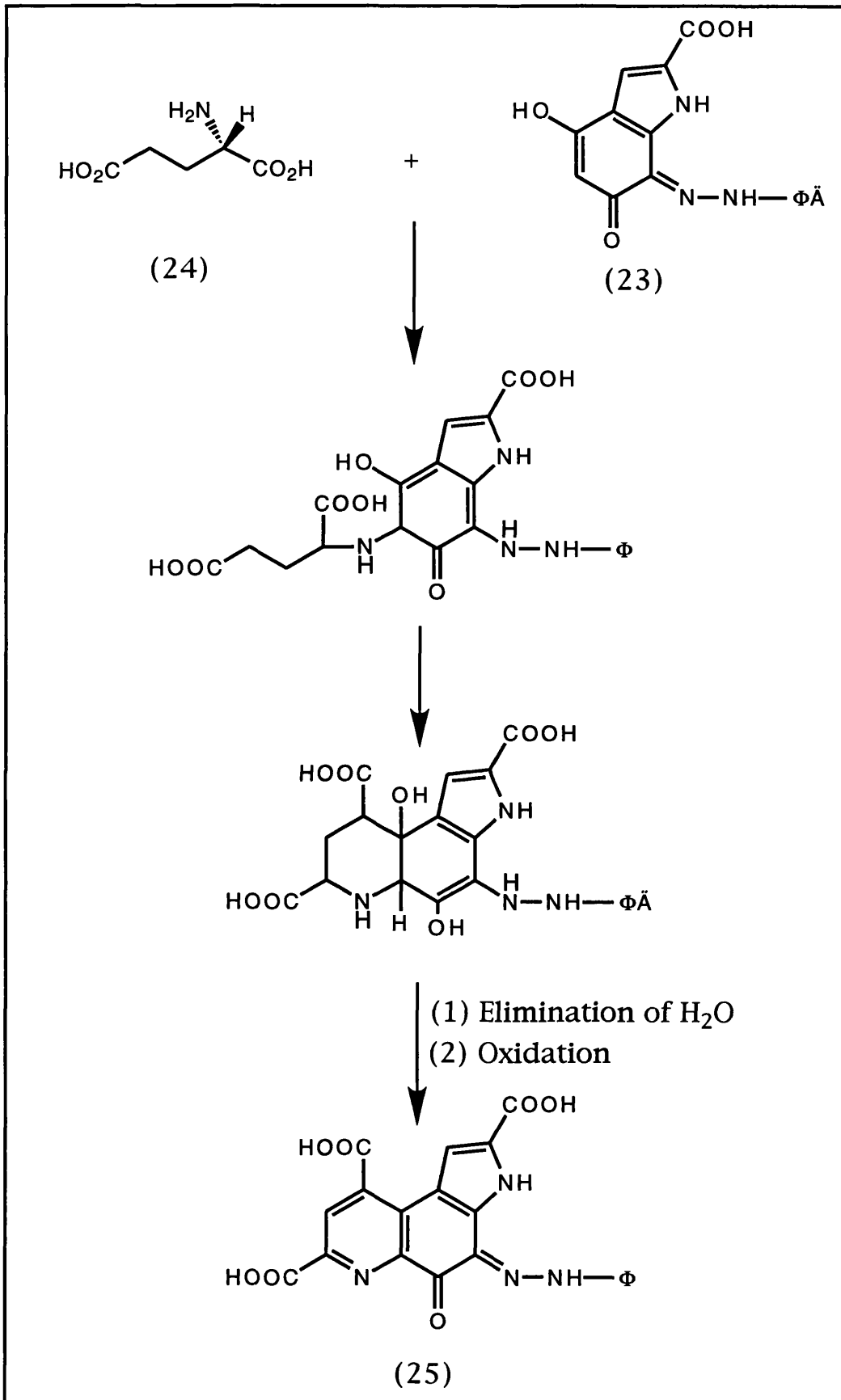
cyclisation giving rise to products (22) and (23) which closely resemble two of the rings of the PQQ structure (Scheme 2.12).



(19) (22) (23)

Scheme 2.12

These compounds closely resemble the structure of PQQ, but it would be unlikely that their phenylhydrazone derivatives would coelute with PQQ derivatives. However, these compounds are highly reactive and are expected to form Michael adducts with a range of nucleophilic compounds. Since proteolysis of oxidatively damaged proteins results in formation of high concentrations of glutamate (24), it may be possible that after such a process sufficient amounts of glutamate would be present to form a Michael adduct with compound (23). Also, the fact that there are two nucleophilic sites in glutamate may promote the formation of a stable six membered ring via a second Michael addition. This compound, after subsequent elimination of H_2O and oxidation produces a compound which would be anticipated to possess matching properties to that of PQQ (Scheme 2.13).



Scheme2.13

Janes *et al.*[75] suggested that proteolysis of phenylhydrazone derivatives of copper amine oxidases would result in the formation of compound (25). This would have similar retention times on HPLC to PQQ phenylhydrazone derivatives, and provides an explanation for the confusion which has surrounded the nature of the organic cofactor for copper amine oxidases for many years.

In subsequent studies Janes *et al.*[75] investigated the identity of the active site cofactor in porcine plasma, porcine kidney and pea seedling amine oxidases. Using resonance Raman spectroscopy on radiolabelled *p*-nitrophenylhydrazone derivatives of all three enzymes, the intensity of the signals and the relative peak position of the isolated peptides were superimposable on those of a model derivatised topaquinone.

On the basis of these findings and the earlier results regarding BSAO,[74] Janes concluded that topaquinone is likely to be the carbonyl-containing cofactor in all copper amine oxidases.

2.12 Current Understanding of the Nature of the Cofactor

The work by Janes *et al.*[74, 75] has significantly shifted the bias towards TOPA as the cofactor in copper amine oxidases. However, some doubts still exist over the presence of this compound at the active site.

Firstly, TOPA is neurotoxic[76] and since this toxicity has been traced to the redox properties of the compound, it would seem unusual that the same properties would enable it to perform as an active site cofactor.

Secondly, how could TOPA arise in the enzyme? It could conceivably be incorporated via the oxidation of an active site tyrosine, or since DOPA is a naturally occurring amino acid, via

direct incorporation into the growing chain. If tyrosine oxidation is the mechanism for TOPA formation, is it performed by a second enzyme or alternatively, do the active site metal atoms in the copper containing amine oxidases play a catalytic role in the event?

In light of the evidence discussed, it would appear that some questions remain unanswered in relation to the nature of the cofactor. Further purification and crystallisation of these enzymes is required and X-ray studies need to be carried out on the purified enzymes. Until this is done confirmation of the identity of the active site cofactor cannot take place.

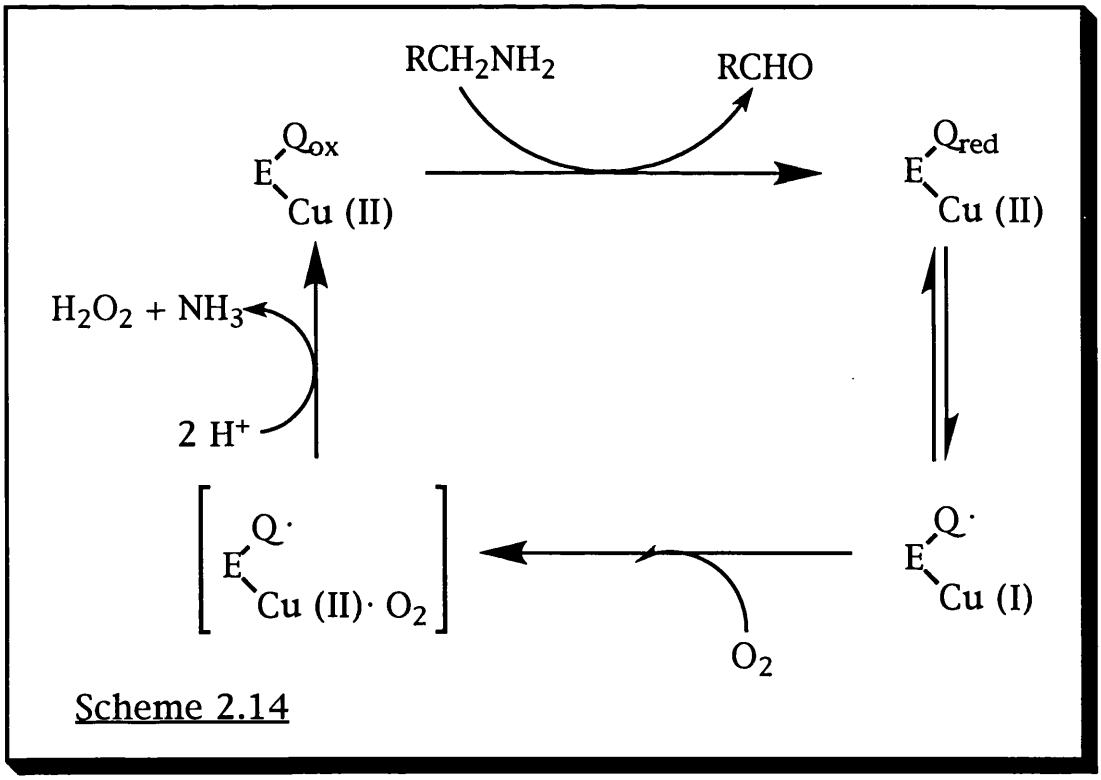
2.13 Interaction of the Prosthetic Groups in Diamine Oxidase

Although the structure of the active site cofactor for diamine oxidase has not been confirmed, it appears to contain a redox cofactor. The involvement of copper in amine oxidases was discussed earlier. In 1991 new evidence[77] suggested the function of copper in these enzymes was to catalyse a two electron oxidation of substrates by molecular oxygen. However, it is unlikely that a single copper centre, only capable of undergoing a one electron change (Cu^{II}/Cu^{I}), can catalyse a two electron transfer from a substrate to O_2.

Also in 1991 evidence was presented for the appearance of a Cu(I)-semiquinone from the substrate reduction of amine oxidases under anaerobic conditions.[34] This Cu(I)-semiquinone was proposed as the catalytic intermediate which reacts directly with the O_2 and is capable of undergoing a two electron oxidation.

EPR spectral changes accompanying the addition of appropriate amines to several amine oxidases, including pea and pig

diamine oxidases, were observed and shown to be similar. Since the EPR spectrum is independent of the enzyme and substrate used, the radical must be associated with a moiety that is conserved among the amine oxidases examined. Addition of cyanide to the reduced form of the enzyme produced a significant enhancement of this signal. This observation is in accordance with the presence of the proposed intermediate since cyanide would have a stabilising effect on the Cu(I)-semiquinone through its interaction with Cu(I). These results have led to the development of a plausible reaction mechanism which presents well-precedented roles for both copper and the quinone (Scheme 2.14).



Scheme 2.14

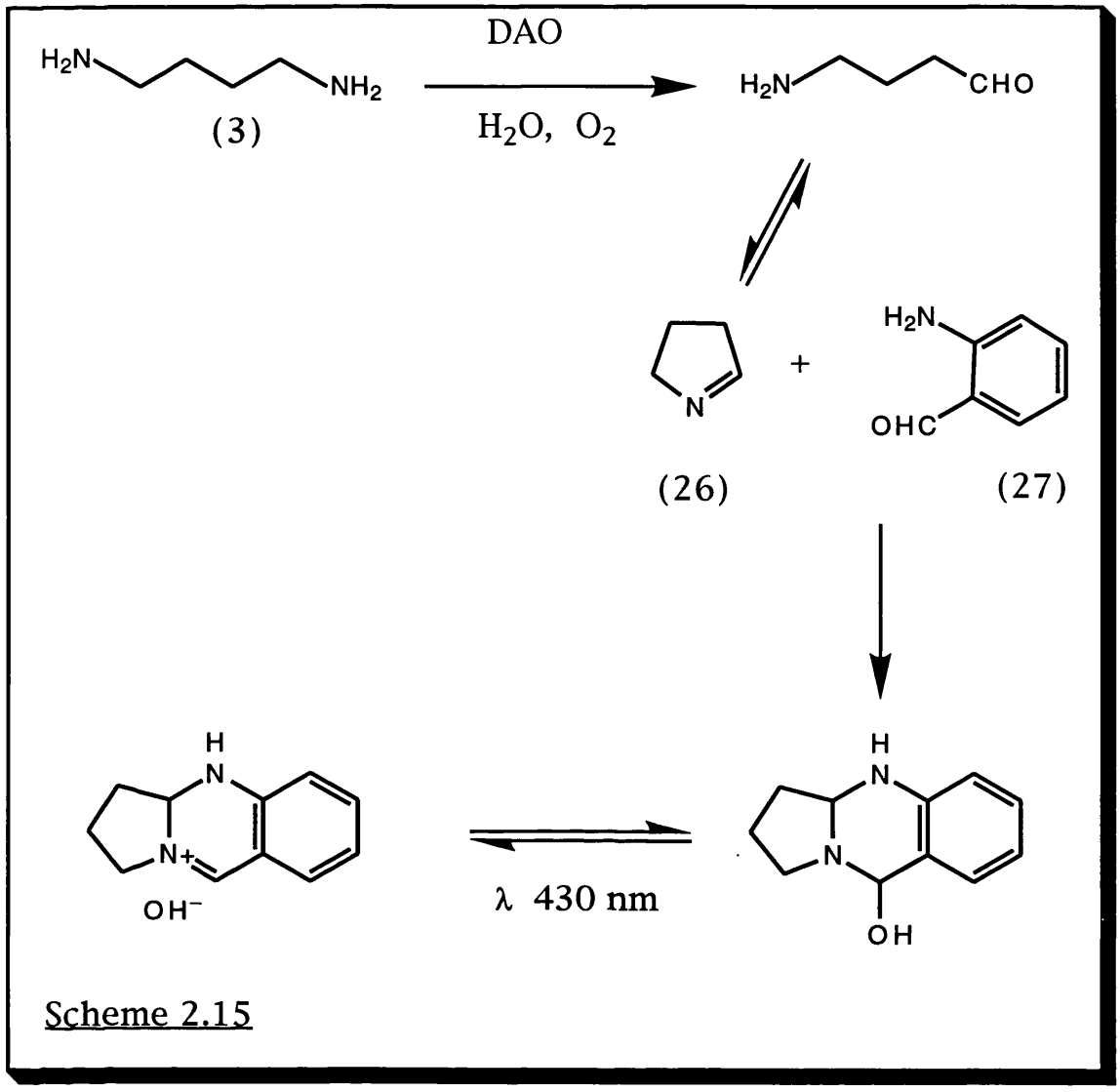
In Scheme 2.14 the species in the brackets is a hypothetical intermediate, shown to emphasise the possibility of a sequential one electron step in the reduction of O_2. Q_{ox} is the oxidised quinone; Q is the semiquinone; Q_{red} is the two electron reduced quinone.

The Cu(I)-semiquinone state of amine oxidase has not been detected before because this state is apparently in equilibrium with the Cu(II)-reduced quinone. Internal electron transfer from copper to semiquinone is favoured by low temperatures, and since previous work used low temperature EPR spectroscopy to study the copper centre, the semiquinone was missed. Further study is needed to gain an insight into whether the quinone is actually bonded to the copper or whether the substrates interact at the metal centre.

2.14 Assay Systems Used in the Determination of Diamine Oxidase Activity

During the oxidative deamination of diamines to their corresponding aminoaldehydes, the rate of conversion provides a direct measure of the activity of the enzyme. This technique was developed over a number of years, and a variety of assay systems have been used for this purpose.[78-81] The uptake of oxygen during the enzymic reaction was the centre of early studies, and systems were developed to determine diamine oxidase activity by the manometric measurement of the oxygen uptake.[82]

Several other methods were developed which relied on trapping the organic product from the enzymic reaction. Using the production of (1-pyrroline) (26) from the oxidative deamination of putrescine (3) (Scheme 2.15), Holmstedt and Tham[83] determined the activity of the DAO enzyme. The addition of *o*-aminobenzaldehyde (27) to the enzymic mixture, giving a yellow compound, allows the rate of formation of 1-pyrroline to be measured spectrophotomerically (Scheme 2.15).



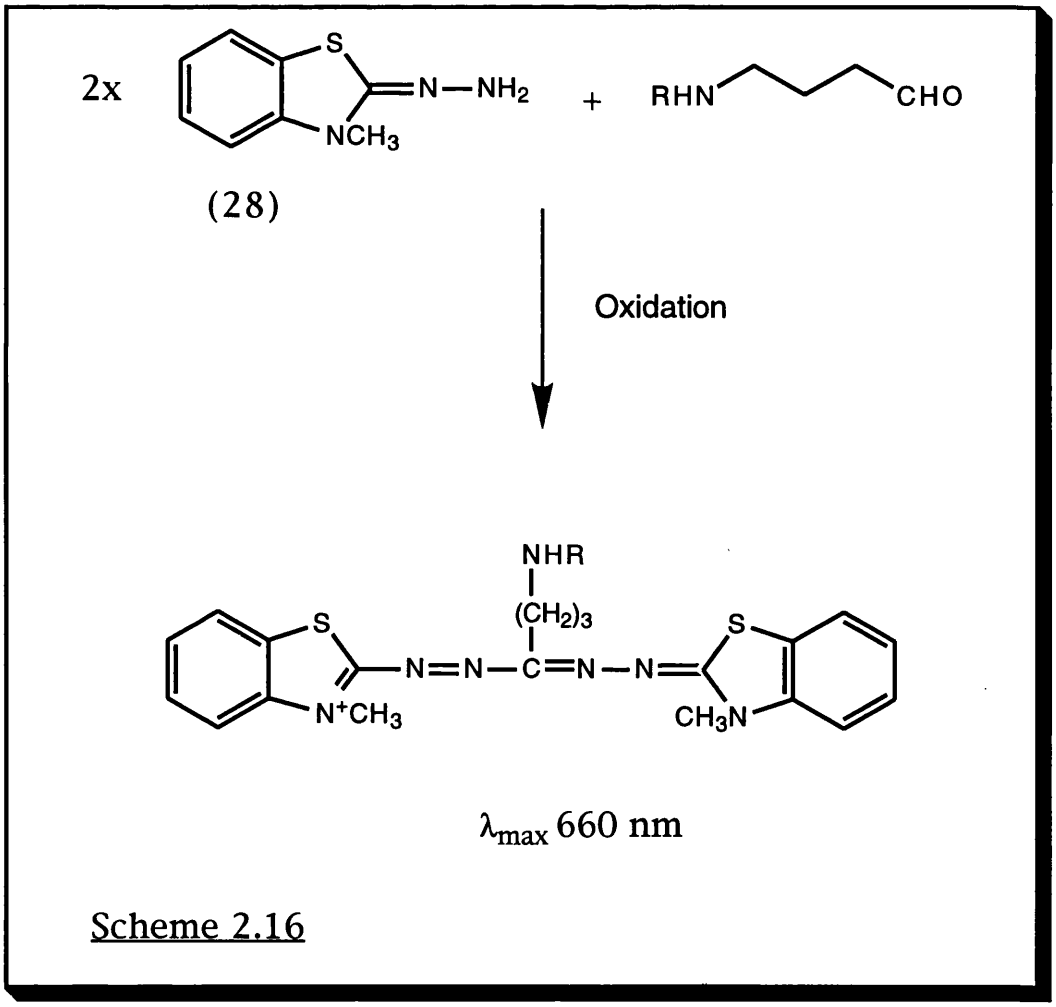
Scheme 2.15

Holmstedt *et al.*[84] subsequently calibrated this assay using known quantities of γ-aminobutanal, in the more stable acetal form. This procedure allowed the activity measurements to be calculated in units of micromoles per mg of enzyme per hour. This allowed comparisons to be made between this method and those dependent on oxygen uptake.

A later, more rapid and sensitive colourimetric method for the assay of DAO was described by Naik *et al.*[85] This procedure involved the reaction of 1-pyrroline (26) with ninhydrin reagent, in acidic medium, to form a coloured complex with λ_{max} of 510 nm. Pec and Pavlikova[86] augmented this work using cadaverine

(producing 1-piperideine) to calculate DAO activity. However, there is a drawback to these procedures in that substrates are limited to putrescine and cadaverine in order to get the required products of 1-pyrroline and 1-piperideine.

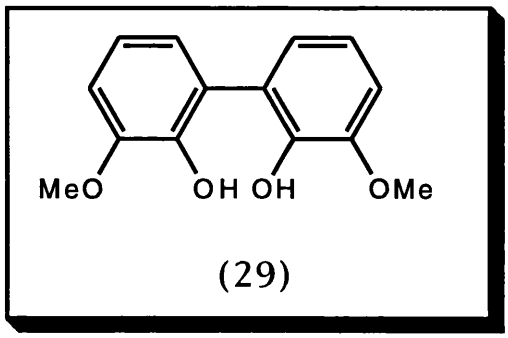
Frydman *et al.*[17] developed an assay system for determining the rates of oxidation of *N*-alkylputrescines by DAO. This method involved trapping the oxidation products (i.e. the aminoaldehydes) with 3-methyl-2-benzothiazolinone (MBTH) (28) to yield a bis-hydrazone cation having a λ_{max} at 660 nm (Scheme 2.16).



Scheme 2.16

Procedures have been developed for the measurement of hydrogen peroxide, a common product which is formed during all DAO reactions. A peroxide coupling reaction was developed by

Booth and Saunders[87] in which hydrogen peroxide, in the presence of peroxidase, rapidly oxidises guaiacol leading to the production of a brown/red solid from which 2,2'-dihydroxy-3,3'-dimethoxy-biphenyl (29) can be isolated.

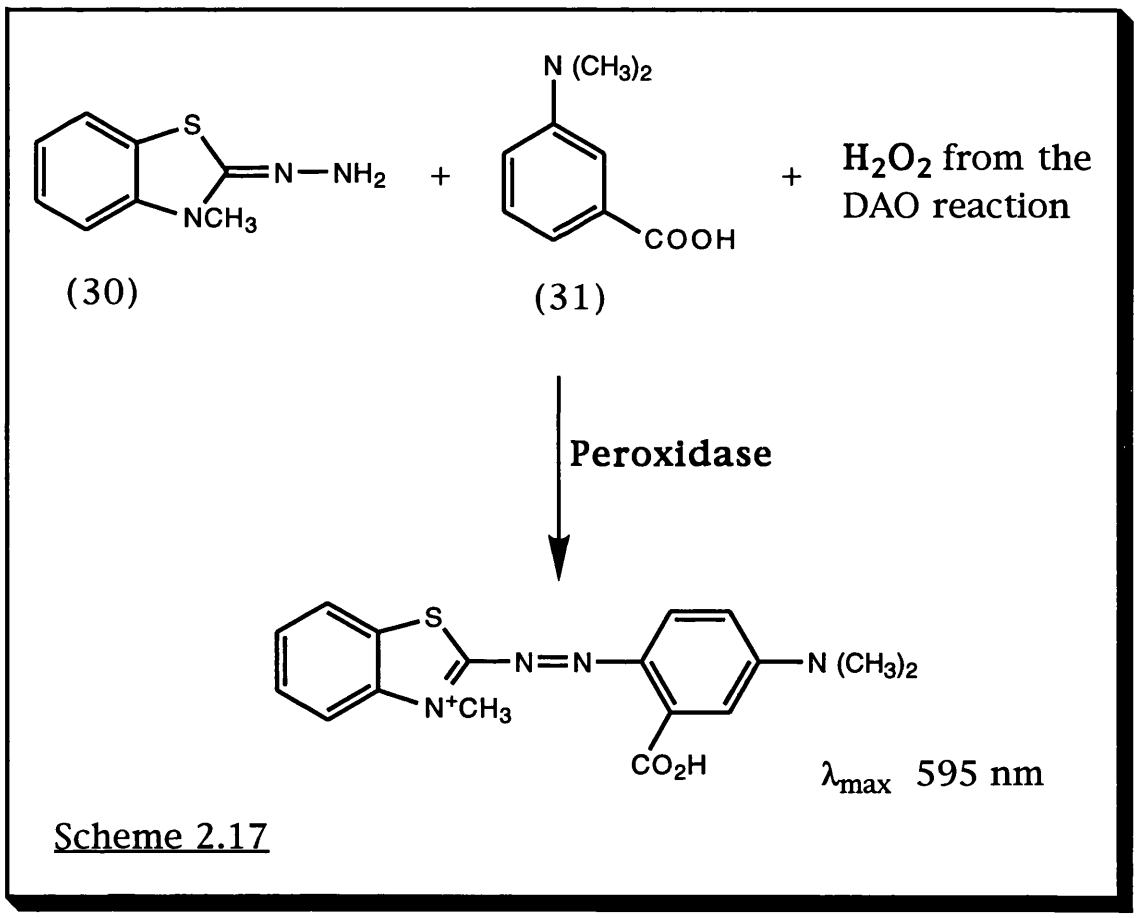


(29)

Smith[88] used this reaction to determine the activity of diamine oxidases, developing a colourimetric procedure utilising the peroxidase/guaiacol assay which had been adapted to determine the hydrogen peroxide formed in the course of amine oxidation. However, there are complications associated with this assay. The guaiacol oxidation products include certain quinones which are highly reactive.[87] These may combine with other compounds in the reaction mixture changing the chromagen, enzyme or substrate and could seriously inhibit the enzymic process. It is also known that 1-pyrroline, formed by incubation of putrescine with DAO, may be oxidised further in the presence of peroxidase. Despite these potential complications the stoichiometry obtained with the various substrates suggests that the method provides a reliable estimate of DAO activity.

In 1985 Stoner[89] reported an improved spectrophotometric assay for the measurement of DAO activity. This procedure, again based on the amount of hydrogen peroxide formed during diamine oxidation, involved a peroxidase-coupled assay. In the presence of hydrogen peroxide, 3-methyl-2-benzothiazolinone hydrazone

(MBTH) (30) reacts with 3-(dimethylamino)benzoic acid (DMAB) (31) producing a purple indamine dye with a characteristic absorbance maximum at 595 nm (Scheme 2.17). Stoner has shown that this assay is efficient for the measurement of diamine oxidase activity with histamine as substrate. However it has since been shown to be effective in activity determinations using a wide range of substrates.[90, 91] The kinetic studies discussed later in this thesis were carried out using this assay system.



Scheme 2.17

2.15 Substrate Specificity and the Active Site

In order to gain information on the active site of both pea seedling and pig kidney diamine oxidase, it is necessary to examine the substrate specificity of these enzymes. Although both enzymes have shown a broad substrate tolerance during studies by various

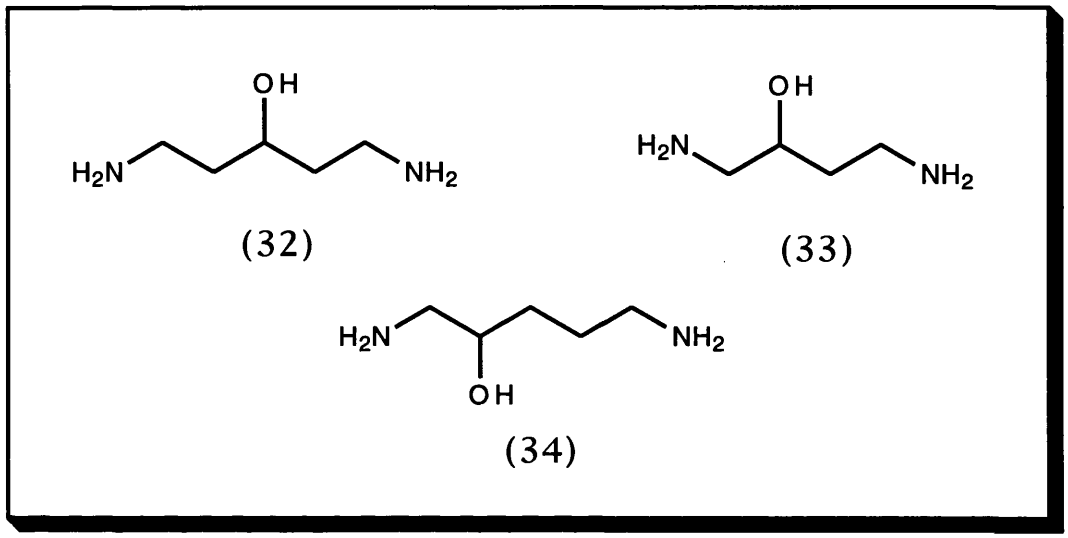
groups, it appears that there are some differences regarding the active site geometries of the two enzymes.

Mann[92] has shown that partially purified preparations of pea seedling diamine oxidase catalyse the oxidation of a wide range of substrates including the amino acids lysine and ornithine. These observations were confirmed by Werle *et al.*,[93] by showing that these compounds among others were oxidised by highly purified DAO. However, Costa *et al.*[94] failed to observe the oxidation of lysine by pig kidney diamine oxidase.

Frydman *et al.*[17] studied the oxidation of a range of *N*- and *C*-alkylated putrescine derivatives by both plant and animal diamine oxidases. These studies showed that *N*-ethyl-, *N*-propyl- and *N*-butylputrescine were all efficiently oxidised by both plant and mammalian forms of the enzyme, whereas *N*-methylputrescine surprisingly showed a very low rate of oxidation in both cases. The *C*-alkylputrescines were generally poorer substrates than the corresponding *N*-alkyl-derivatives with 1,4-dimethylputrescine failing to be oxidised by either enzyme. The specificity of diamine oxidase towards 1-propylputrescine was significantly different for the two enzyme forms, with the substrate being oxidised well by pea seedling diamine oxidase and poorly oxidised by the mammalian form. This result, combined with the fact that 1-methylputrescine and 2-methylputrescine are oxidised at very different rates by plant and mammalian DAO, appears to imply that differences exist at the active sites of these two enzyme forms.

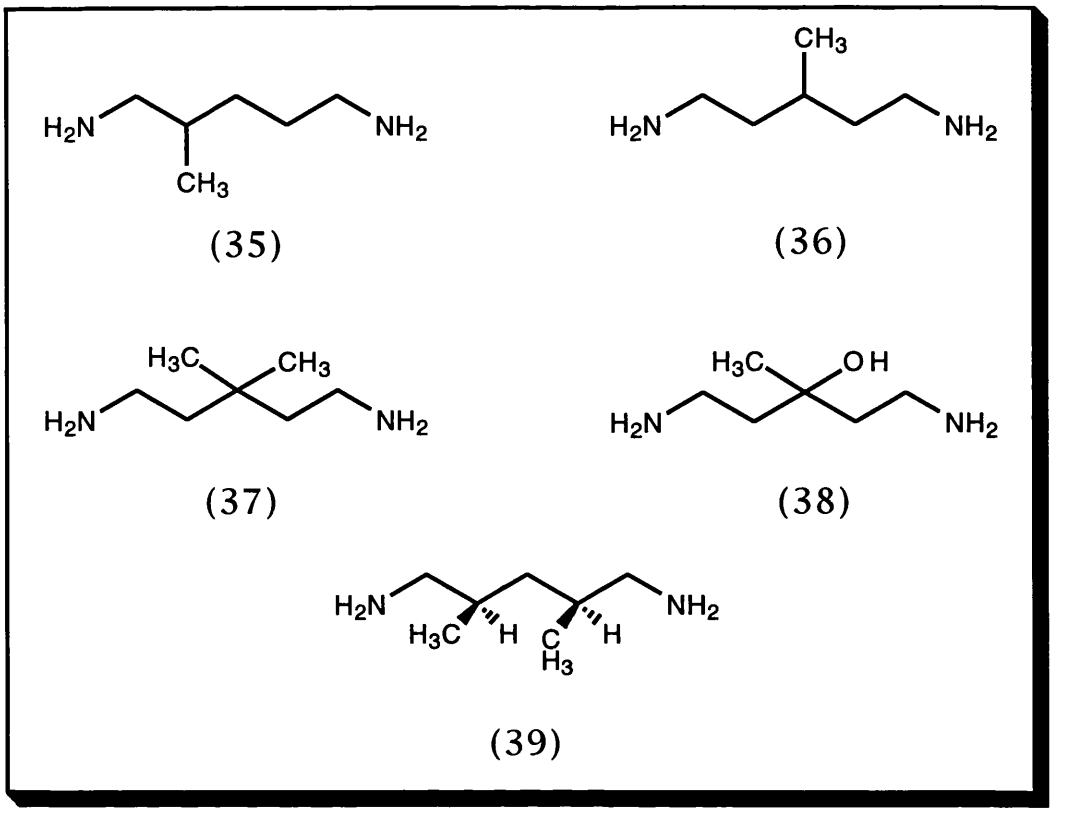
Macholan[95] used a series of experiments involving hydroxydiamines in an attempt to highlight the apparent differences in the behaviour of the two enzyme forms. He showed that the binding affinities for 3-hydroxypentane-1,5-diamine (32), 2-hydroxybutane-1,4-diamine (33) and 2-hydroxypentane-1,5-

diamine (34) were significantly different towards the two forms of DAO. He also found that 3-hydroxypentane-1,5-diamine (32) binds with the lowest affinity of the three substrates to pea seedling DAO, but has the highest affinity of the three with pig kidney DAO. These results demonstrate that the active site of the two forms may have structural differences.



The studies carried out by Frydman *et al.*[17] have since been repeated by Robins and co-workers[90] using the improved spectrophotometric assay of Stoner.[89] Contrary to the findings of Frydman, Robins and co-workers found that *N*-methylputrescine was a good substrate for both plant and animal DAO. In fact, the *N*-methylputrescine was oxidised significantly faster than the *N*-ethyl and *N*-propyl derivatives.

The work of Robins and co-workers was extended to the area of cadaverine derivatives.[91] They showed that 2-methylcadaverine (35), 3-methylcadaverine (36), 3,3-dimethylcadaverine (37), 3-hydroxy-3-methylcadaverine (38) and *meso*-2,4-dimethylcadaverine (39) are all substrates of partially purified pea seedling DAO.



There appears to be a progressive decrease in the rate of oxidation as the size of the substituents increases. It was found that 3-hydroxy-3-methylcadaverine (38) was a better substrate than 3,3-dimethylcadaverine (37). From this result it was felt that incorporation of a polar substituent into the backbone of the substrate leads to a more efficient enzymic process.

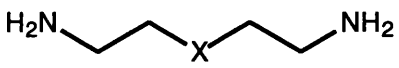
It has also been shown by Equi *et al.*[96] that a series of α,ω-diamines with chain lengths varying from 2 to 12 are oxidatively deaminated by pea seedling DAO. An association drawn between chain length and binding affinity suggested that the most efficient binders possessed carbon chain lengths between 4 and 7, with the best binders being the natural substrates (putrescine C4 and cadaverine C5). The rate of oxidation was also shown to be strongly dependent on chain length, with the natural substrates being most efficiently oxidised.

2.16 Diamine Analogues Containing Group VI Atoms (O, S, Se)

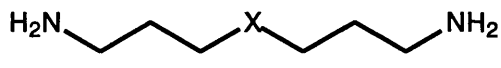
A range of thiodiamines and their oxygen analogues have been shown by Cragg *et al.*[97] to be oxidised by DAO. Pea seedling DAO was found to oxidise lanthionamine (40), whereas pig kidney DAO oxidises homocystamine (41) and homolanthionamine (42).

Corda *et al.*[98] examined a range of seleno-analogues as substrates of pig kidney DAO. They observed that selenocystamine (43) and selenolanthionamine (44) were efficiently oxidised by the enzyme. Seleno-homocystamine (45) and seleno-homolanthionamine (46) were also shown to be substrates of pig kidney DAO, although their rates of oxidation were found to be significantly lower than that of selenolanthionamine (44).

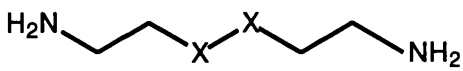
Monoamines such as benzylamine are also substrates of DAO, although rates of oxidation observed for these compounds are lower than for the corresponding diamines. It is believed that the presence of the second amine group is essential for efficient reaction. However, these results together with the work discussed in 2.15 highlight the broad substrate tolerance of DAO.



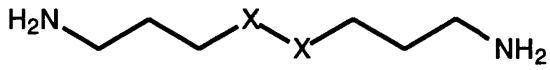
X = S (40)
X = Se (44)



X = S (42)
X = Se (46)



X = Se (43)

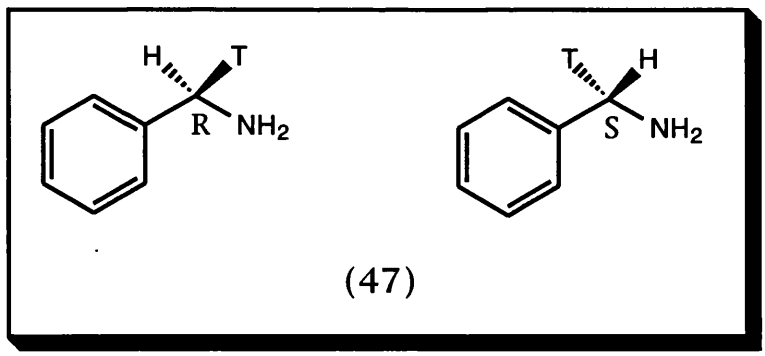


X = S (41)
X = Se (45)

2.17 Stereochemistry Involved in Reactions catalysed by DAO

It is not only important to understand the mechanism for the DAO catalysed reaction, but also the stereochemistry involved. The absolute stereochemistry of the abstraction of a hydrogen atom from the prochiral methylene group has been determined by a variety of methods. An important factor in all these methods was the availability of substrates which were labelled stereospecifically with tritium or deuterium at the methylene group and whose absolute stereochemistry had been determined by correlation with compounds of known chirality.

In 1974 Battersby *et al.*[12] synthesised (*R*)- and (*S*)-[methylene-$^3H_1$]benzylamines (47) in greater than 95% *ee* for both. These were used in three experiments involving the incubation of pea seedling DAO with (a) 100% *(R)*-[methylene-$^3H_1$]benzylamine, (b) 100% *(S)*-[methylene-$^3H_1$]benzylamine and (c) a 50:50 mixture of the above isomers.

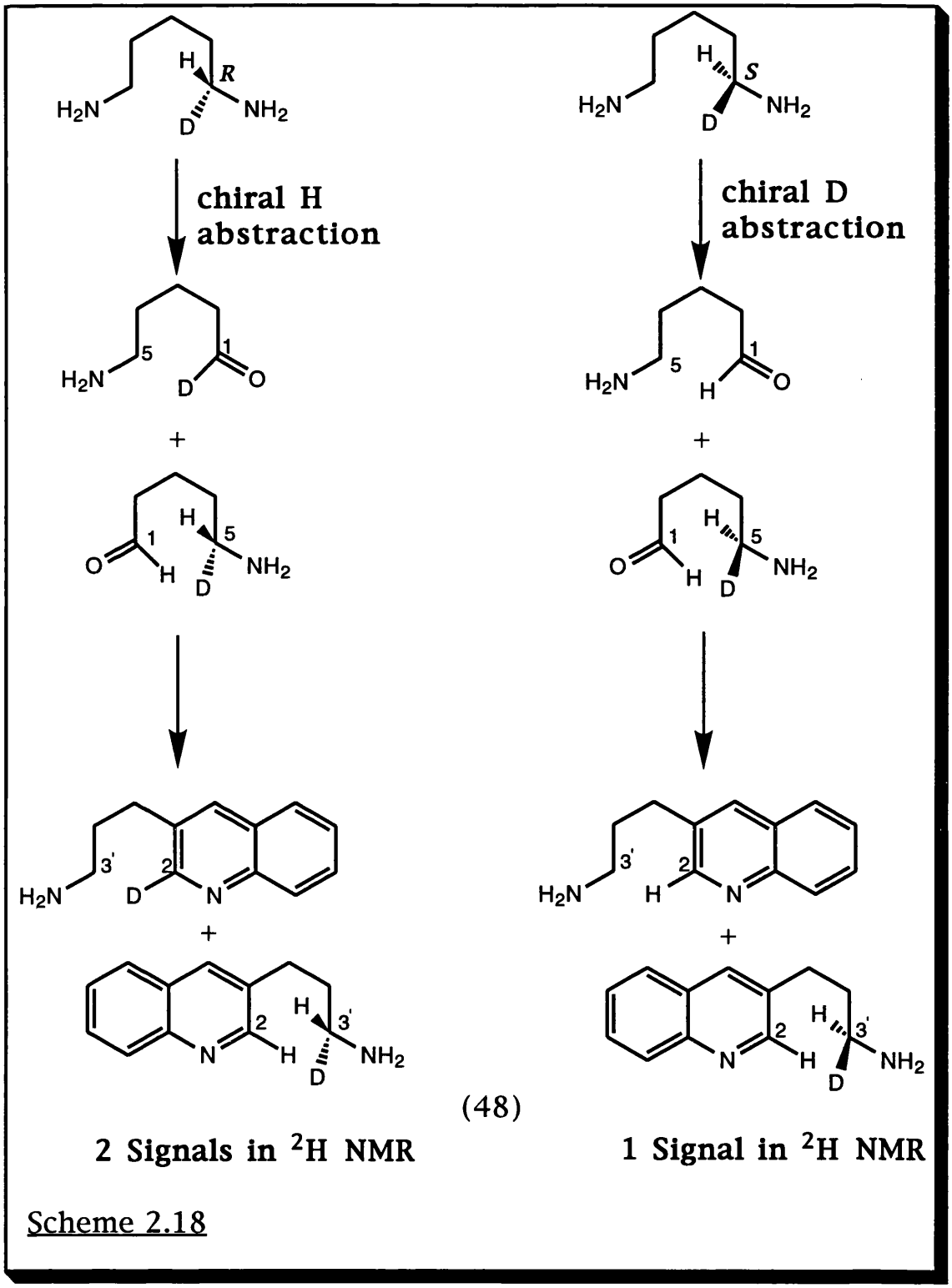


(47)

The product isolated from experiment (a) showed complete retention of the tritium label whereas in experiment (b) only a fraction of the original tritium label remained. The result of experiment (c) was consistent with the previous two experiments, with approximately 50% of the tritium label still retained. These

results showed that pea seedling DAO abstracts the *pro-S* hydrogen from the methylene group of benzylamine. Since these studies were carried out many groups have set out to try and understand the stereochemistry behind the DAO catalysed deamination reaction.

Using deuterium NMR spectroscopy Richards and Spenser[11] carried out investigations of the stereochemistry involved in the deamination of cadaverine by pig kidney DAO. They prepared *(S)*-[1-$^2H_1$]cadaverine, *(R)*-[1-$^2H_1$] cadaverine and [1,1-$^2H_2$]cadaverine in high configurational purities. The products from the enzyme catalysed oxidation of these substrates were trapped with *o*-aminobenzaldehyde and led to the formation of 3-(3'-aminopropyl)quinone (48) (Scheme 2.18). The products obtained from using *(R)*-[1-$^2H_1$] cadaverine and [1,1-$^2H_2$]cadaverine as substrates both showed two signals in their deuterium NMR spectra, whereas with *(S)*-[1-$^2H_1$]cadaverine as substrate only one signal was observed for the product. From these studies it was clear that the *pro-S* hydrogen from C-1 of cadaverine was lost during the oxidative deamination with DAO.



Scheme 2.18

Battersby *et al.*[99] also studied the stereochemistry involved in the oxidative deamination of cadaverine using pea seedling DAO. [1-^{3}H]-Labelled cadaverine was incubated with pea seedling DAO, and the aminoaldehyde product was converted into the more stable 5-aminopentan-1-ol in the presence of an alcohol dehydrogenase. This

aminoalcohol was also a substrate of DAO and was further deaminated and then reduced, leading to pentane-1,5-diol.

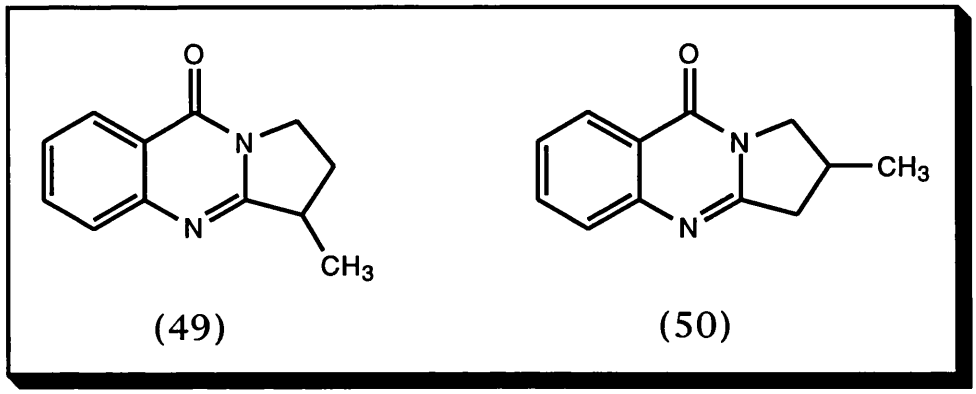
This resolved the problem arising with the symmetry of the substrate, which means either of the two amino groups can be oxidised initially by the DAO enzyme. Since stereospecific loss of the methylene hydrogen through two deaminations should lead to either complete loss or retention of the tritium label, depending on its stereochemistry, the results were easily interpreted. With the tritium label occupying the *S*-position on C-1 of cadaverine, Battersby's group observed only trace amounts of tritium in the final product. The complementary result was obtained with the label occupying the *R*-position on C-2 of cadaverine, with complete retention of the tritium label in the final product. Again on the basis of these results, Battersby and co-workers concluded that the *pro-S* hydrogen is indeed abstracted during the oxidation process.

Subsequent to these findings, it has been shown that loss of the *pro-S* hydrogen is associated with the DAO catalysed oxidation of a wide range of substrates,[13,100] for example (*S*)-1-amino[1-^{3}H]-heptane.[101] This stereochemical consistency with such a wide range of substrates has led to the assumption that all DAO catalysed oxidations result in the loss of the *pro-S* hydrogen.

2.18 Regioselectivity and Stereoselectivity Involved with the DAO-Catalysed Reaction

Santaniello *et al.* studied the regioselectivity for the DAO-catalysed oxidation of racemic 2-methylputrescine using both pig kidney and pea seedling DAO.[14] As discussed earlier the oxidation product could be trapped with *o*-aminoaldehyde and subsequent

oxidation would lead to formation of 1'- or 2'-methyl-2,3-trimethylene-4(3*H*)-quinazolone (49) or (50).



(49) (50)

The ^{1}H NMR spectrum of the product from the pig kidney DAO reaction showed two doublets at δ 1.24 and 1.46 of the same intensity, whereas the product from the pea seedling DAO reaction gave one doublet at δ 1.24. On the basis of this observation, Santaniello and co-workers concluded that pea seedling DAO catalyses the oxidation of 2-methylputrescine in a regioselective manner, whereas the pig kidney DAO lacks this regioselectivity.[14]

Santaniello and co-workers then studied the stereospecificity of the reaction using (*R*)- and (*S*)-2-methylputrescines as substrates for both pea seedling and pig kidney DAO. The (*R*)- and (*S*)-2-methylputrescines were synthesised from (*R*)- and (*S*)-3-methyladipic acid respectively.[102] The products were trapped with *o*-aminobenzaldehyde as before, and a quantitative analysis was performed using HPLC.

From the results it was found that pea seedling DAO catalyses the deamination of 2-methylputrescine at the less hindered amino group, independent of the configuration of the substrate used, since for both isomers, compound (50) was essentially the only detectable product. With pig kidney DAO however, the oxidation is dependent upon the chirality of the substrate. With the (*R*)- isomer, it is the C-

1 amine which is oxidised to form the corresponding aminoaldehyde (95%), whereas with the (*S*)-isomer it is the less hindered amine that is oxidised. This result suggests that for pig kidney DAO, the active site is more sensitive to the stereochemical configuration of the substrate, and in fact the pig kidney DAO is stereoselective.

It is possible therefore to conclude that the active sites of diamine oxidases from plant and animal sources are certainly different. This area would benefit from further work in order to understand fully the differences between these two enzymes.

2.19 The Necessity of Polyamines in Cell Growth and Replication

Polyamines are required for optimal growth in all living cells which have so far been examined. Many studies have shown that rapidly growing cells have higher levels of polyamines than slowly growing or inactive cells. It is also known that the polyamine content of cells increases before an increase in DNA, RNA and protein content.

After administration of inhibitors of polyamine biosynthesis, the levels of putrescine (3) and spermidine (5) fall rapidly. This decrease is noticed especially in rapidly replicating cells where there is a dramatic inhibition of growth and replication. It is clear therefore that polyamines are important to growth and replication of all living cells and that inhibitors of polyamine biosynthesis can have a dramatic effect on the concentration of polyamines in rapidly proliferating cells.[10,103]

2.20 Inhibitors of Diamine Oxidase

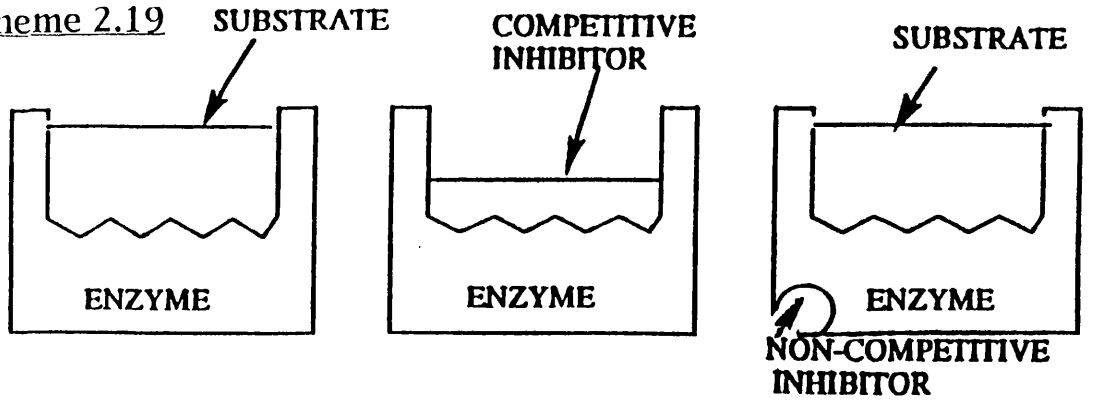
The inhibition of enzyme activity by specific compounds is important because it serves as a major control mechanism in many metabolic pathways. In fact the action of many drugs is to inhibit enzymic pathways. Inhibition can also provide an insight into the mechanistic action of enzymes.

There are two main types of inhibition, reversible and irreversible. In irreversible inhibition the inhibitor is either covalently linked to the enzyme or is so tightly bound in some other way that it releases very slowly. In contrast to this, reversible inhibition involves a rapid binding/dissociation equilibrium of the inhibitor and enzyme.

There are two main forms of reversible inhibition and the simplest form is competitive inhibition. In competitive inhibition the inhibitor mimics the substrate and binds to the active site of the enzyme, thus preventing the substrate from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzymic molecules that have a bound substrate.[104]

The other form of reversible inhibition is non-competitive inhibition. In this form the inhibitor and substrate can both bind to the same enzyme molecule. Since there is no overlap of binding sites, a non-competitive inhibitor decreases the turnover number of an enzyme rather than reducing the proportion of enzyme molecules that have a bound substrate (Scheme 2.19).

Scheme 2.19



There are also more complex types of inhibition that may be encountered. "Mixed inhibition" shows features of both competitive and non-competitive models, and coupling (acompetitive) inhibition is a specific form of mixed inhibition occurring when the inhibitor is bound into the same subsite as the substrate but the inhibitor binds only to the enzyme-substrate complex and not with the free enzyme. Partial non-competitive inhibition occurs where the presence of the inhibitor does not affect the binding of the substrate, but the rate of breakdown of the enzyme-inhibitor-substrate complex is slower than the breakdown of the enzyme-substrate complex.[147]

A study of inhibitors of DAO could effectively shine light on the physiological role of the DAO enzyme and possibly lead to a variety of drug candidates. Also measuring the rates of reaction at different concentrations of substrate and inhibitor would help to distinguish between the inhibition mechanisms of the various candidates.

There are six different types of compounds that are known to inhibit DAO. These types are:

1. enzyme inactivators;
2. copper chelating agents;
3. substrate analogues;

3. substrate analogues;

4. substrate inhibitors;

5. product inhibitors;

6. suicide substrates/inhibitors.

The first three are the most common and will be discussed in greater depth.

1. Enzyme inactivators: These inhibitors normally act by irreversible inhibition and lead to partial or total loss of enzyme activity. The mode of deactivation of the enzyme depends on the ability of the inhibitor to react with the carbonyl functionality of the active site of the enzyme. Phenylhydrazine, hydroxylamine and semicarbazide have all been shown to inhibit DAO by this mechanism.[28]

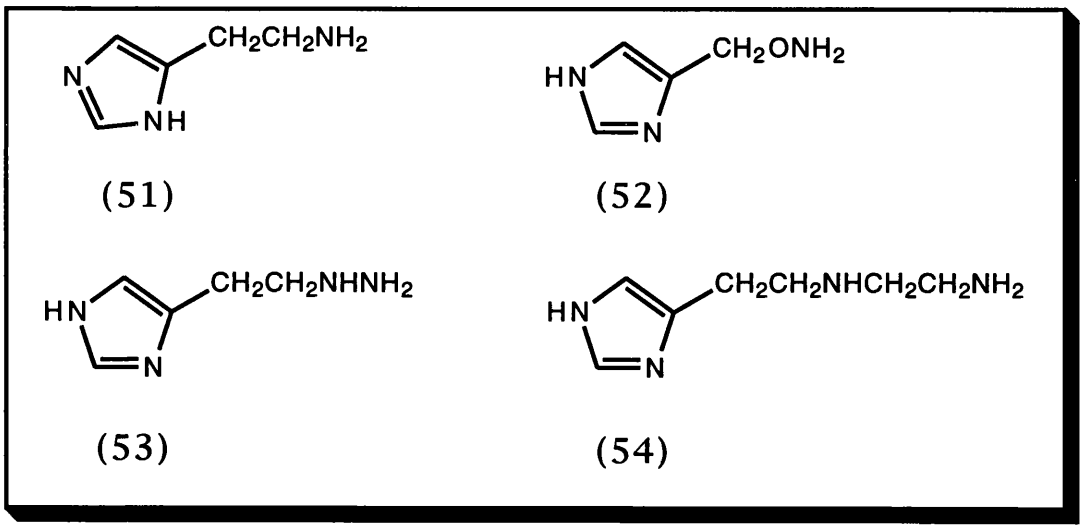
2. Copper chelating agents: It has been shown that by the addition of substrates capable of disrupting the catalytic function of Cu(II) that both plant and animal DAO have been inhibited. These chelating agents bind to the copper present at the active site of the enzyme. 8-Hydroxyquinoline, sodium diethyldithiocarbamate, 2,2-bipyridyl, 1:10-phenanthroline and sodium cyanide act in this manner.

Also Pec and Haviger[106] have shown that sodium azide inhibited pea seedling DAO. They concluded that the azide forms an inactive complex with the enzyme substrate and suggested that the binding of the substrate to the active site of the enzyme gives access to the central Cu^{2+} ion on which the azide, as ligand, is bound.

3. Substrate analogues: Effective inhibitors often resemble the structure of the natural substrate. Comparison of structure activity relationships between substrates and inhibitors of DAO have shown

that a subtle change in the structure of a compound may have dramatic effects towards reactivity with the DAO enzyme.

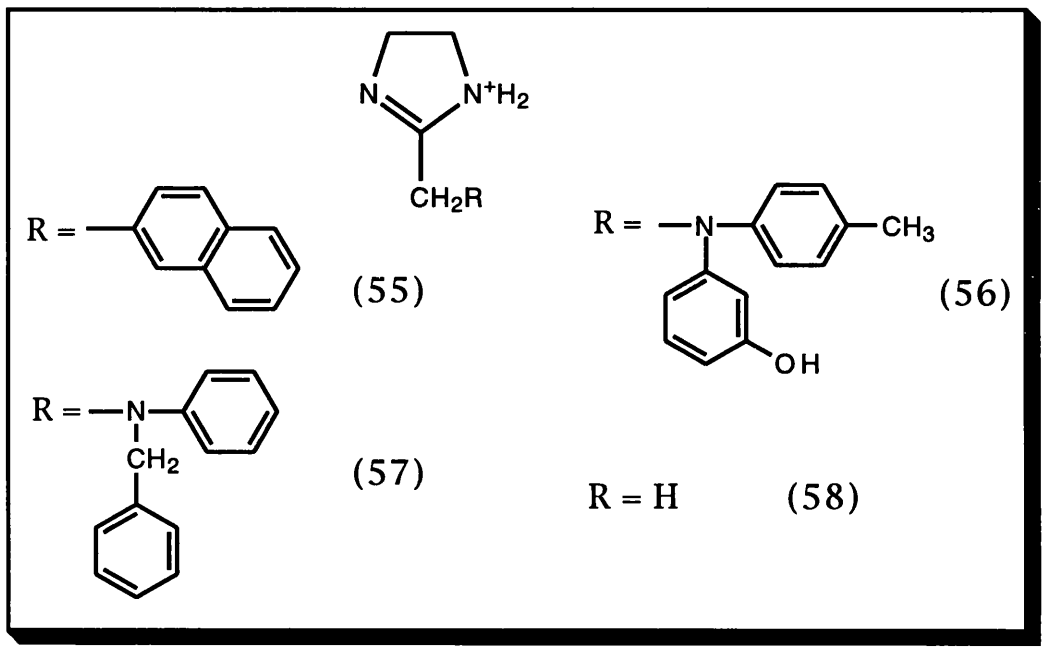
The structural relationships between DAO inhibitors and two substrates, putrescine (3) and histamine (51) were explored by Bieganski *et al.*[107] in the 1980s. They noted that histamine was a substrate for both pea seedling and pig kidney DAO and decided to examine the effects of histamine analogues on the oxidation process. These studies concentrated on two classes of compounds: (a) compounds with structures resembling histamine and having a further reactive amine group, for example (52) and (53); and (b) compounds combining a histamine structure and an aliphatic amine structure, for example (54).



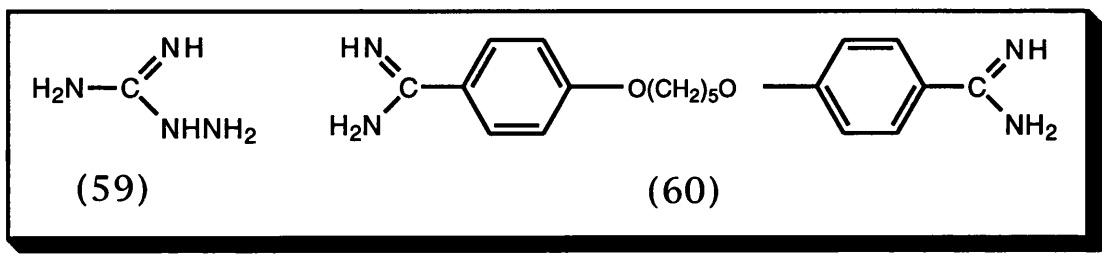
Compounds (52) and (53) were found to be potent inhibitors of both pea seedling and pig kidney DAO. This result showed that the presence of the imidazole ring could lead to inhibition of pea seedling DAO contrary to earlier work which suggested imidazole derivatives only inhibited mammalian DAO. Compound (54) showed selective inhibition of pea seedling DAO, and this was thought to be due to the presence of the aliphatic diamine side chain on the ring

system. This result again highlights the difference between the active site of the two enzymes.

The inhibitory effects of a range of 4,5-dihydroimidazole derivatives on the catalytic oxidation of 1,4-diamino-2-butene with pig kidney DAO was reported by Pec and Hlidkova.[108] They showed that natazolin (55), fentolamin (56) and anatazolin (57) are all non-competitive inhibitors of pig kidney DAO, whereas 2-methyl-4,5-dihydroimidazole (58) showed no inhibitory effects. This ruled out the possibility that the inhibition noted for the other analogues was due to Cu(II) chelation with the dihydroimidazole ring system.



Another group of compounds which are particularly potent inhibitors of both plant and animal DAO are the amidines, especially aminoguanidine (59). [109] The incorporation of an amidine grouping has led to a range of DAO inhibitors, such as MGBG (60).[110]



4. Substrate inhibitors: In many enzymes the rate of oxidation increases to a maximum value over a limited lower range of substrate concentrations, but at higher concentrations the rate decreases with the expected maximum rate not being achieved. The rate of reaction may actually drop at very high concentrations and this occurrence is known as substrate inhibition. This occurs with many different enzymes, including DAO.[111]

This may be due to a number of factors, for example high substrate concentrations may increase the ionic strength of the aqueous reaction mixture or may interfere with the binding of a coenzyme.

5. Product inhibitors: This occurs where the product of the enzyme reaction inhibits the forward process. In one type of product inhibition the reverse reaction, i.e. where the products are converted back into substrate, competes with the forward reaction so that product formation is reduced. Another type of product inhibition occurs when the product combines with the enzyme, or other reactive components of the system, and this slows the rate of forward reaction.

6. Suicide substrates/inhibitors: This is a type of inhibitor which uses the binding specificity and catalytic mechanism of the target enzyme for chemical activation. This transforms a normally harmless reversible inhibitor into a powerful irreversible inhibitor.

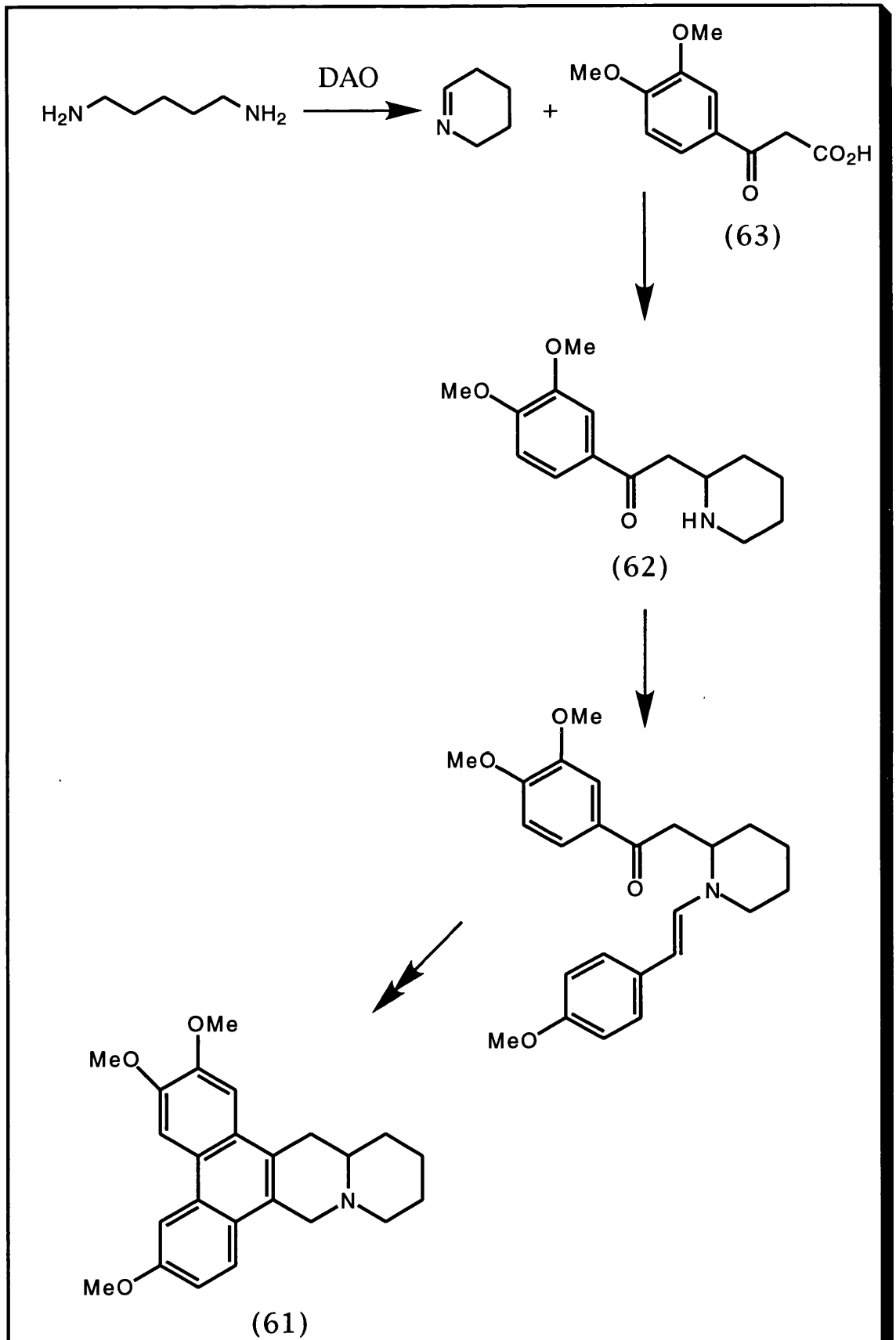
2.21 Synthetic Applications of DAO

The fact that DAO catalyses a functional group transformation which is extremely difficult to carry out chemically makes this enzyme increasingly important. An example of this is the convenient synthesis of a range of biologically active alkaloids by Cragg and Herbert.[112] They used pea seedling DAO as a catalyst in a key step during the synthesis of cryptopleurine (61) and other phenacyl derivatives.

Enzymatic oxidation of suitable diamines, followed by *in situ* condensation of the cyclic imine with a benzoylacetic acid derivative, led to a range of synthetically useful intermediates. 3',4'-Dimethoxy-2-(2-piperidinyl)acetophenone (62) found in *Boehmeria platyphylla* can be synthesised easily using DAO and is an important intermediate in the synthesis of cryptopleurine (61). The direct synthesis of this intermediate (62) was accomplished by condensation of 3,4-dimethoxybenzoylacetic acid (63) with Δ'-piperidine,[113] generated *in situ* from the catalytic oxidation of cadaverine with DAO (Scheme 2.20). Subsequent condensation of (62) with substituted phenylacetaldehydes and then several ring closures led to the formation of cryptopleurine (61).

Analogues of these alkaloids can be made easily by either using a different diamine, benzoylacetic acid derivative, or substituted phenylacetaldehyde derivative. These small alterations in the synthetic route may open the way to the preparation of new biologically active analogues.

The method of coupling DAO oxidation products with benzoylacetic acid derivatives was used in mechanistic studies during this project and is discussed in Chapter 4.


DAO
H2N
NH2
N
+
OMe
MeO
CO2H
O
(63)
OMe
MeO
O
HN
(62)
OMe
MeO
O
N
MeO
OMe
MeO
N
MeO
(61)

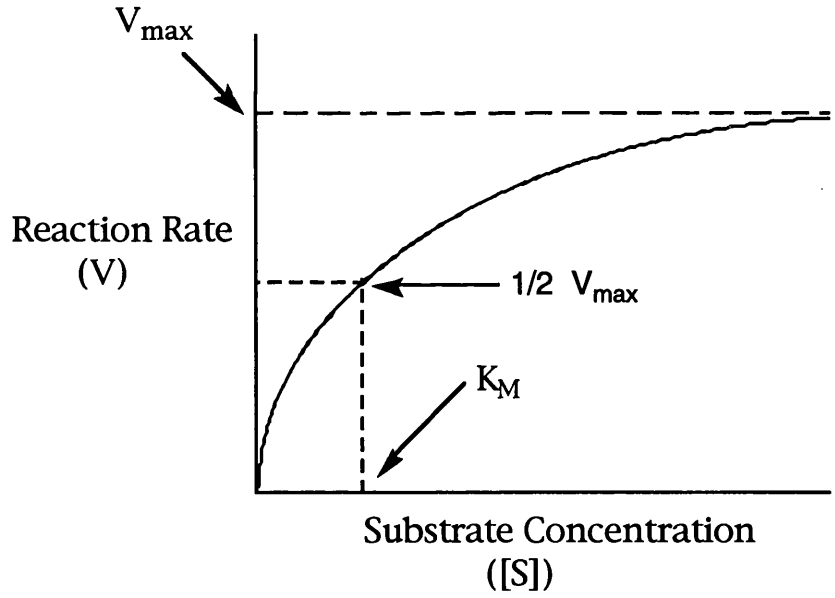
Scheme 2.20

CHAPTER 3

Enzyme Kinetics

3.1 Introduction

The rate of catalysis (V) for most enzyme reactions varies with substrate concentration ([S]) and almost all enzyme-catalysed reactions show a first order rate dependence providing that substrate concentrations are low. However, as substrate concentrations increase the rate of catalysis approaches a limit (V_{max}) which cannot be exceeded regardless of substrate concentrations (Graph 3.1).

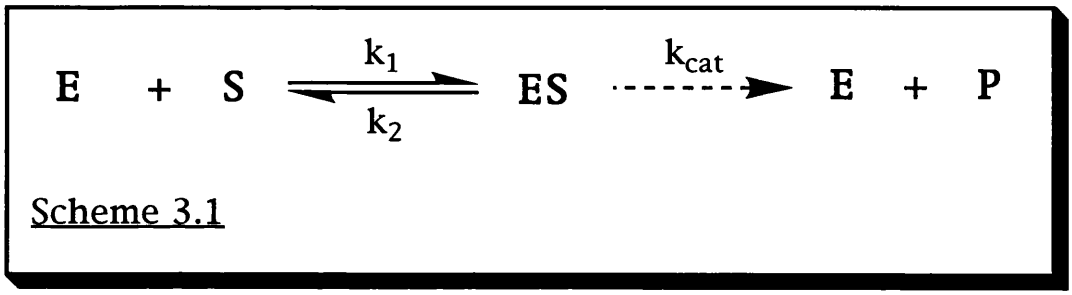


Graph 3.1 Reaction rate (V) *vs.* Substrate concentration ([S]).

3.2 Michaelis-Menten Kinetics

In 1913 Michaelis and Menten proposed a simple model to account for the kinetic characteristics described above. The main feature of this mechanism is the binding of the substrate (S) to the

enzyme (E) to form a necessary enzyme-substrate intermediate complex (ES) (Scheme 3.1). The ES complex can either dissociate to regenerate E and S with a rate constant k_2, or it can go on to form the product (P) with a rate constant k_{cat}. The dotted arrows in Scheme 3.1 indicate the possible complexity of several intermediate steps between ES and the formation of product P.



Scheme 3.1

The use of this simple model allows an expression to be obtained which relates the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps.

The model starts with the assumption that since the product is formed in the second step only, the rate of formation of product and hence the overall rate of reaction is given by:

$$V = k_{cat}[ES] \qquad (3.1)$$

This is followed by an expression of [ES] in terms of known quantities, and the rates of formation and breakdown of the ES complex are derived by:

$$\text{Rate of formation of ES} = k_1[E][S] \qquad (3.2)$$

$$\text{Rate of breakdown of ES} = (k_2 + k_{cat})[ES] \qquad (3.3)$$

Under steady state conditions the rate of formation of the ES intermediate is equal to the rate of breakdown, and the concentrations of intermediate ES remain constant while the concentrations of the starting material and products are changing. Thus:

$$k_1[E][S] = (k_2 + k_{cat})[ES] \quad (3.4)$$

This equation is rearranged to give an expression for [ES] as shown in (3.5), simplified by defining a new constant, the Michaelis constant (K_M), as shown in (3.6), and then substituting K_M into equation (3.5) as shown in (3.7).

$$[ES] = [E][S]/\{(k_2 + k_{cat})/k_1\} \quad (3.5)$$

$$K_M = (k_2 + k_{cat})/k_1 \quad (3.6)$$

$$[ES] = [E][S]/K_M \quad (3.7)$$

Provided that the concentration of enzyme is very much smaller than the substrate concentration, as is normal, the concentration of uncombined substrate [S] is very nearly equal to the total concentration of substrate. The concentration of free enzyme [E] is equal to the total enzyme concentration [E_T] less the concentration of the enzyme-substrate complex [ES]. Thus:

$$[E] = [E_T] - [ES] \quad (3.8)$$

Substituting equation (3.8) into equation (3.7) gives:

$$[ES] = ([E_T] - [ES])[S]/K_M \quad (3.9)$$

Solving this equation for [ES] gives:

$$[ES] = [E_T][S]/([S] + K_M) \quad (3.10)$$

Substituting the expression for [ES] into equation (3.1) gives:

$$V = k_{cat}[E_T][S]/([S] + K_M) \quad (3.11)$$

The maximal rate of catalysis, V_{max}, is achieved when the enzyme sites are saturated with substrate, that is when [S] is much greater than K_M so:

$$[S]/([S] + K_M) = 1 \quad (3.12)$$

This then gives the equation:

$$V_{max} = k_{cat}[E_T] \quad (3.13)$$

Substituting equation (3.13) into equation (3.11) leads to equation (3.14):

$$V = V_{max}[S]/([S] + K_M) \quad (3.14)$$

The shape of the curve in Graph 3.1 can now be explained by this equation. At low substrate concentrations, i.e. $[S] << K_M$, equation (3.14) becomes:

$$V = V_{max}[S]/K_M \quad (3.15)$$

This means the rate of reaction is directly proportional to the concentration of the substrate. However, this is not the case when the substrate concentration is high, i.e. when [S] >> K_M, equation (3.14) then becomes:

$$V = V_{max} \qquad (3.16)$$

At this stage the rate is at a maximal and is independent of the substrate concentration. From equation (3.14) we can achieve a definition of K_M. When [S] = K_M:

$$V = V_{max}/2 \qquad (3.17)$$

Therefore, [S] = K_M when the rate of reaction is at half of its maximal rate.

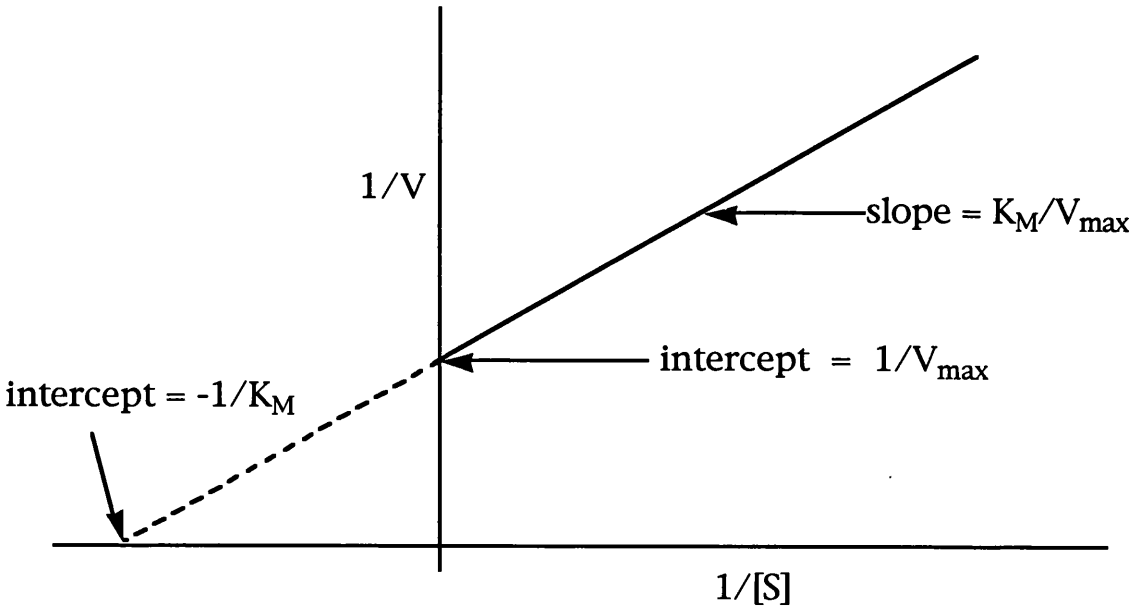
3.3 Determination of V_{max} and K_M by Experimental Methods

If the enzyme reaction obeys the kinetics shown in Scheme 3.1, then both V_{max} and K_M can be derived from the rates of catalysis measured at different substrate concentrations. However, Graph 3.1 of the Michaelis-Menten equation, rate (V) *vs.* substrate concentration [S], is not entirely satisfactory for the determination of V_{max} and K_M. If there are not three consistent points on the plateau of the curve at different substrate concentrations then accurate values cannot be obtained. Also, since the line is a curve and approaches V_{max} slowly, it is difficult to judge exactly where the limit is.

In 1934 Lineweaver and Burk[115] overcame this problem by simply inverting the original Michaelis-Menten equation, thus making the graph a straight line. The Michaelis-Menten equation (3.14) then becomes:

$$(1/V) = K_M/V_{max}(1/[S]) + 1/V_{max} \qquad (3.18)$$

The Lineweaver-Burk plot of 1/V *vs.* 1/[S] as shown in Graph 3.2 leads to a straight line with a gradient K_M/V_{max} and intercept on the y-axis $1/V_{max}$. Thus the kinetic parameters can be determined from the graph.



Graph 3.2 Lineweaver-Burk Plot, 1/V *vs.* 1/[S].

However, the accuracy of the Lineweaver-Burk plot has been questioned. A simple point of fact is that the graph often has to be redrawn because of unexpectedly long extrapolations. A more important point is that it gives undue weight to low substrate concentrations which are the least accurate values. Also, the double-reciprocal plot distorts experimental errors which occur in

the primary observations of V, making it difficult to judge which points are the most accurate.

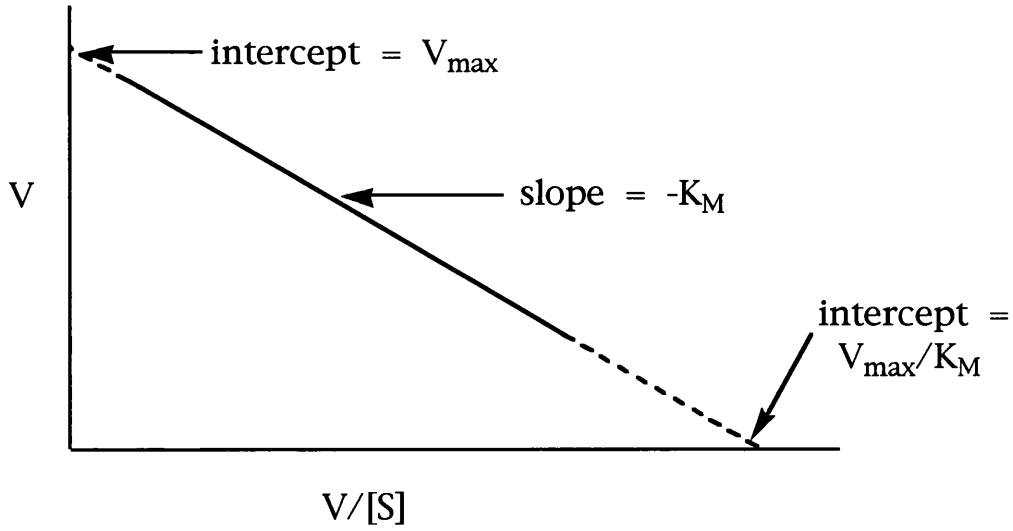
The Eadie-Hoftsee plot[116,117] is not entirely free from distortion, but is less severely affected than the double-reciprocal plot. This plot as shown in Graph 3.3 is derived by multiplying both sides of the Lineweaver-Burk equation (3.18) by $V.V_{max}$ giving:

$$V.V_{max}(1/V) = \{(K_M/V_{max})(1/[S])\}V.V_{max} + (1/V_{max})V.V_{max}$$

This equation can be simplified to give:

$$V = -K_M(V/[S]) + V_{max} \qquad (3.19)$$

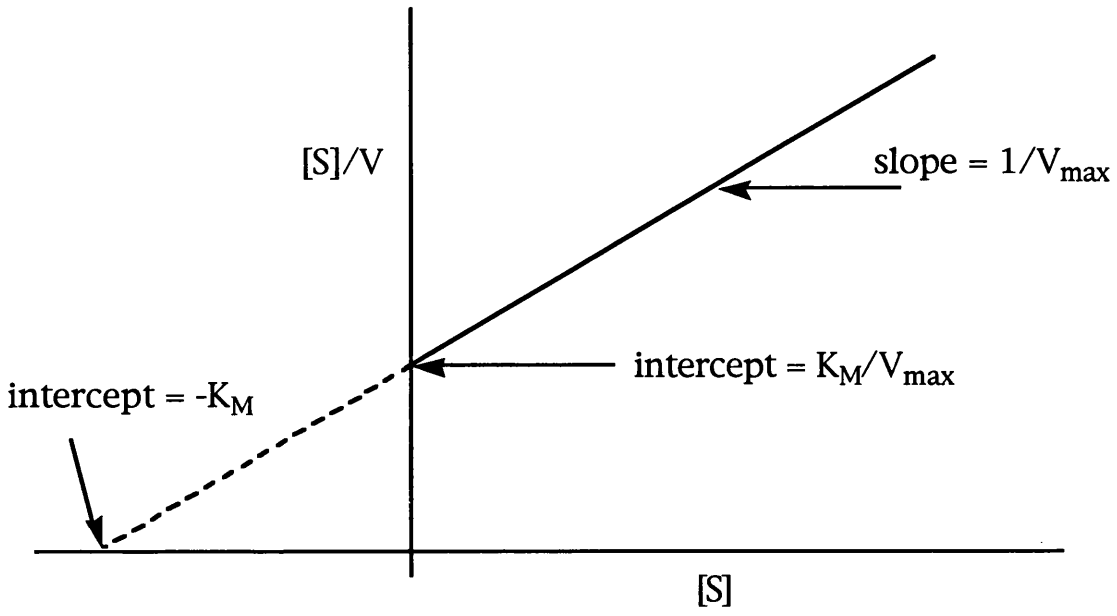
Again the plot from the equation is in the form of a straight line. A plot of V *vs.* V/[S] gives a gradient of $-K_M$ and the intercept on the y-axis, V_{max}.



Graph 3.3 Eadie-Hoftsee Plot, V *vs.* V/[S].

The Hanes plot[118] provides an additional check on accuracy of the data which are plotted. This is obtained by plotting [S]/V *vs.* [S] (Graph 3.4). This gives a straight line with a gradient $1/V_{max}$ and

an intercept on the y-axis of K_M/V_{max}. The required kinetic parameters can then be obtained directly from the graph.



Graph 3.4 The Hanes Plot, [S]/V *vs.* [S].

Experimentally it is best to consider values from all three linear plots in order to achieve the best estimate of V_{max} and K_M. A wide range of substrate concentrations is required to minimise errors and it has been found that substrate concentrations ranging from 3 times K_M to 1/8 of K_M achieve this most effectively.

3.4 The Kinetics Involved with Inhibition

As discussed earlier there are two main types of inhibition, reversible and irreversible, which can then be subdivided into more specific forms of inhibition. Reversible inhibition is split into competitive and non-competitive inhibition. It is possible to distinguish between competitive and non-competitive inhibition by measuring the rate of enzymic catalysis at various substrate and inhibitor concentrations.

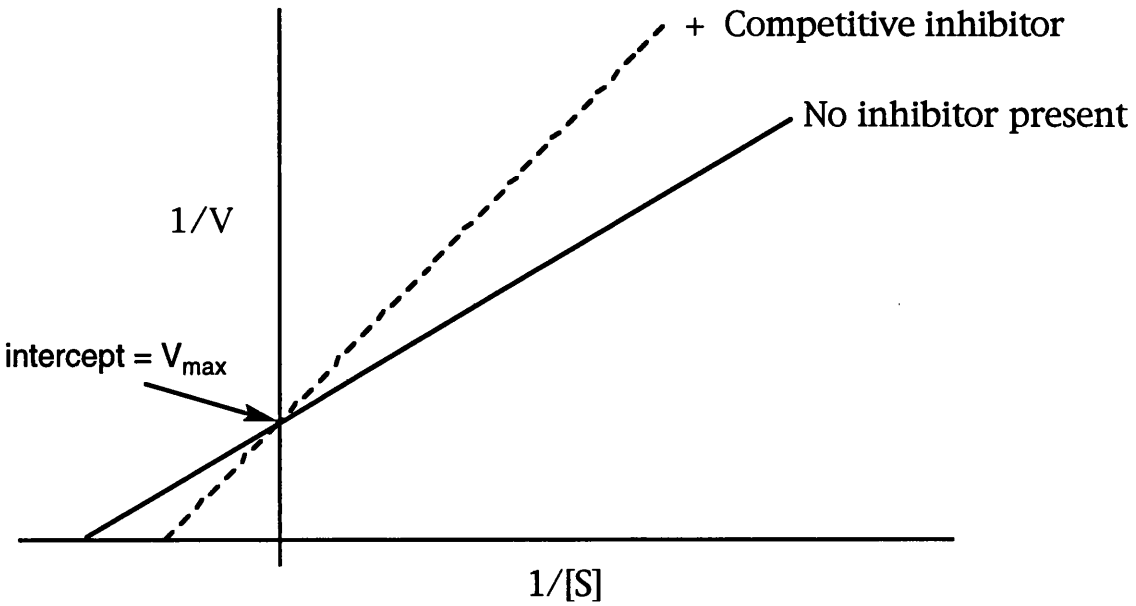
If we consider the Lineweaver-Burk equation (3.18) in an enzyme system which contains a competitive inhibitor, the equation changes to equation (3.20).

$$(1/V) = K_M/V_{max}(1/[S]) + 1/V_{max} \qquad (3.18)$$

$$(1/V) = K_M/V_{max}(1 + [I]/K_i)1/[S] + 1/V_{max} \qquad (3.20)$$

Where [I] is the inhibitor concentration and K_i is the dissociation constant of the enzyme-inhibitor complex.

The intercept on the y-axis of the plot of 1/V *vs.* 1/[S] (Lineweaver-Burk plot) is identical with or without the presence of inhibitor. The intercept of the y-axis corresponds to the V_{max} and it is therefore unaffected by a competitive inhibitor (Graph 3.5). This shows that competitive inhibition can be overcome by high substrate concentrations, because at this point virtually all active sites are filled by substrate.



Graph 3.5 How Competitive Inhibition Affects The Lineweaver-Burk Plot.

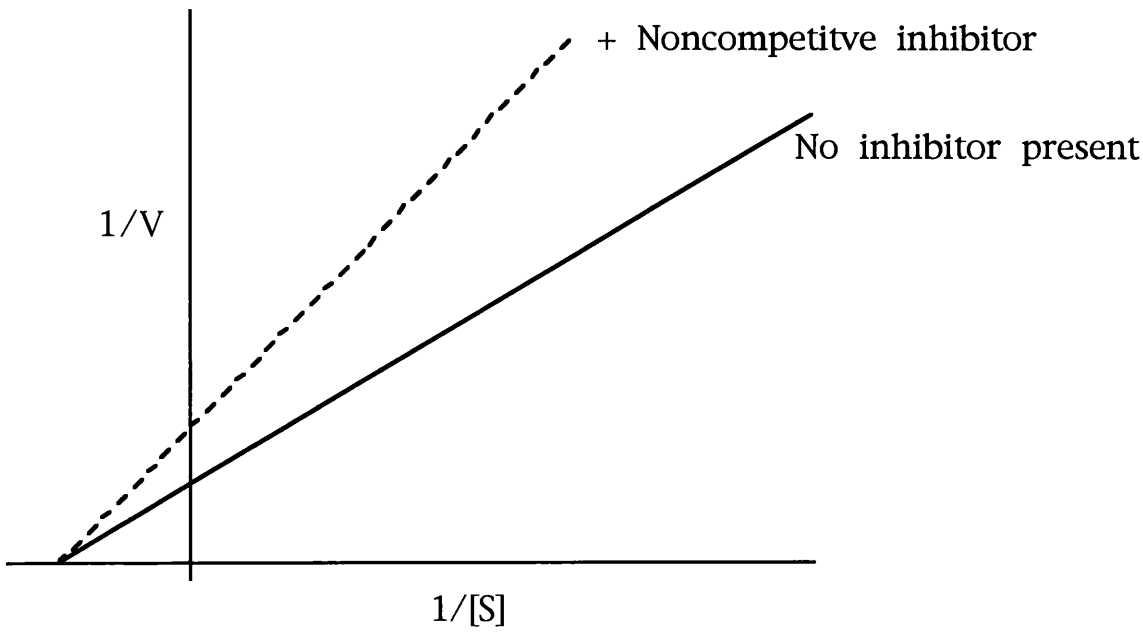
As shown in Graph (3.5) there is a difference in the gradients of the two lines. The gradient of the competitive inhibition plot is given by:

$$(\text{slope})^i/(\text{slope}) = 1 + [I]K_i \qquad (3.21)$$

$(\text{slope})^i$ = the gradient of the line when the competitive inhibitor is present

(slope) = the slope of the line without inhibitor

If we now consider the Lineweaver-Burk plot for an enzyme system which contains a non-competitive inhibitor, the V_{max} is decreased by V_{max}^i which gives an increase in the y-axis intercept (Graph 3.6). The gradient in the presence of inhibitor is equal to K_M/V_{max}^i, and differs from the gradient with no inhibitor present by a factor V_{max}^i. In the simplest cases K_M is not altered in the presence of non-competitive inhibitors.



Graph 3.6 How Non-Competitive Inhibition Affects The Lineweaver-Burk Plot.

CHAPTER 4

Mechanistic Studies on Diamine Oxidase

4.1 Introduction

Frydman *et al.*[17] studied the DAO-catalysed oxidative deamination of a series of *N*-alkyl- and *C*-alkyl-putrescines. With the surprising exception of *N*-methylputrescine (62), these putrescine derivatives were all found to be substrates of DAO and were oxidised to their corresponding aminoaldehydes. Cooper *et al.*[90] repeated this work using an improved assay procedure, and demonstrated that *N*-methylputrescine (62) was in fact a good substrate for both pea seedling and pig kidney DAO.

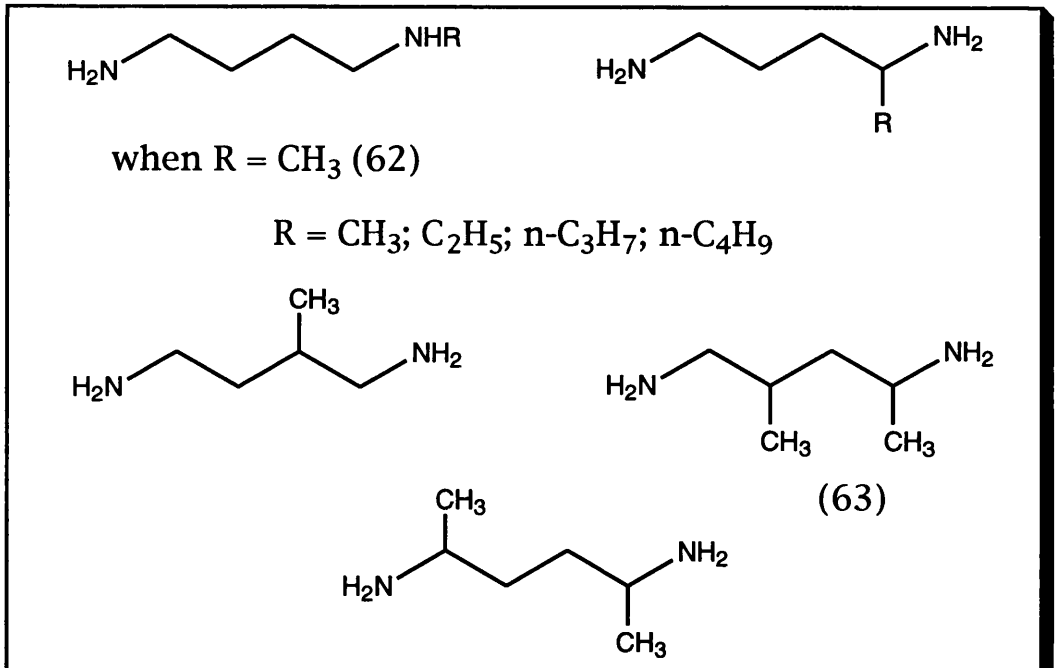
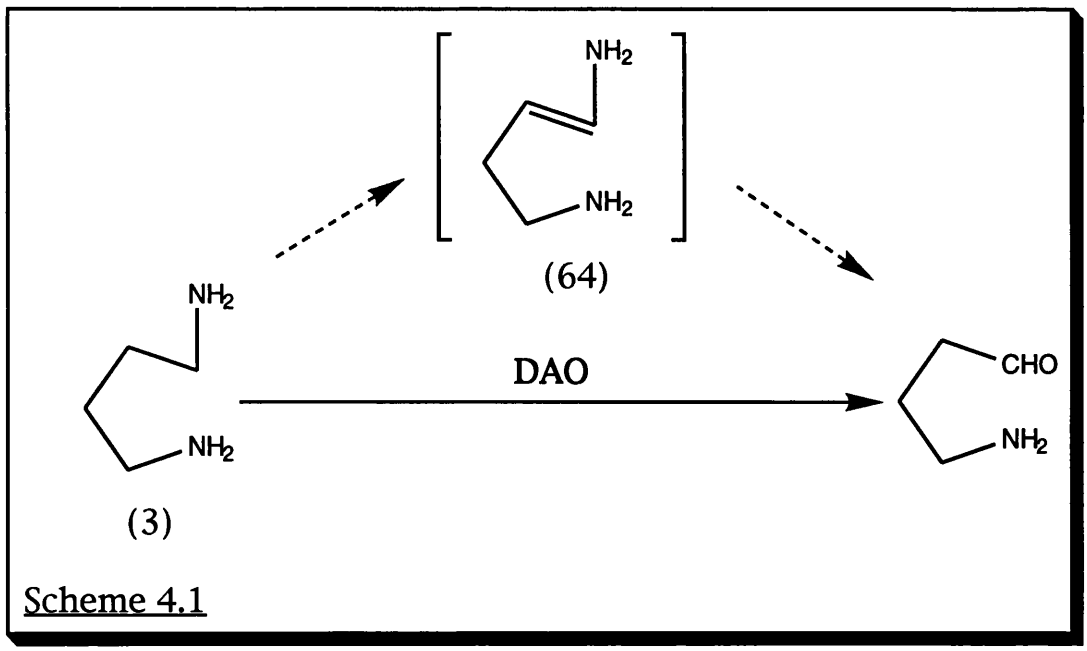


Figure 4.1 *N*-alkyl- and *C*-alkyl-putrescines used in the study of Frydman *et al.*[17]

Following this work Equi *et al.*[96] obtained kinetic data for the DAO catalysed oxidation of α,ω-diamines with chain lengths ranging

from 2-12 carbons. The highest rates of oxidation were observed for chain lengths 4-6, which combined with kinetic data suggested that after initial attachment of the substrate to the enzyme, the amine at the other end of the chain might also become attached to the enzyme thus creating a cyclic conformation for the diamine.

Frydman and co-workers noted that 1,3-dimethylputrescine (63) was a very poor substrate for pea seedling and pig kidney DAO. They suggested that oxidation of putrescine (3) proceeds via an enamine intermediate (64) (Scheme 4.1), which is formed by the abstraction of hydride from the C-2 of putrescine, with subsequent tautomerism and hydrolysis of the resulting imine producing the aldehyde product. They suggested that 1,3-dimethylputrescine (63) sterically hinders the formation of this intermediate (64), thus accounting for the poor substrate activity of (63) towards DAO.

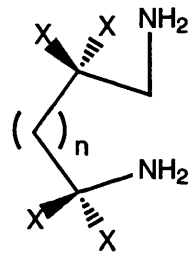


Scheme 4.1

To test this hypothesis we prepared α,ω-diamines containing deuterium labels at the β-positions, so that the loss or retention of deuterium in the products of the DAO-catalysed oxidative deamination could be followed by NMR and mass spectrometry.

4.2 Synthesis of Deuterium Labelled Diamines

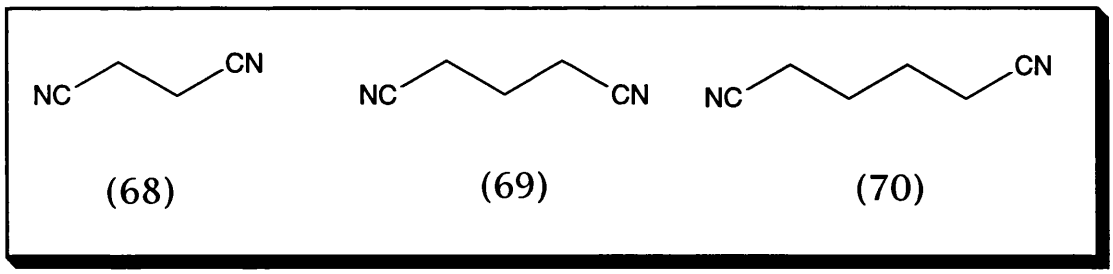
The starting point of the mechanistic study was to make the required [^{2}H4]-labelled diamine substrates and the corresponding unlabelled diamines, all as the dihydrochloride salts. These substrates were: [2,2,3,3-^{2}H4]-putrescine (65); [2,2,4,4-^{2}H4]-cadaverine (66); [2,2,5,5-^{2}H4]-1,6-hexanediamine (67); putrescine (3); cadaverine (6) and 1,6-hexanediamine (68).



n =1, x = H (3)	n =1, x = D (65)
n =2, x = H (6)	n = 2, x = D (66)
n =3, x = H (68)	n =3, x = D (67)

Unlabelled diamines (3), (6) and (68) were readily available as their free bases, and could be converted easily into their corresponding dihydrochloride salts by partitioning the free base between dichloromethane and 6M hydrochloric acid for two hours.

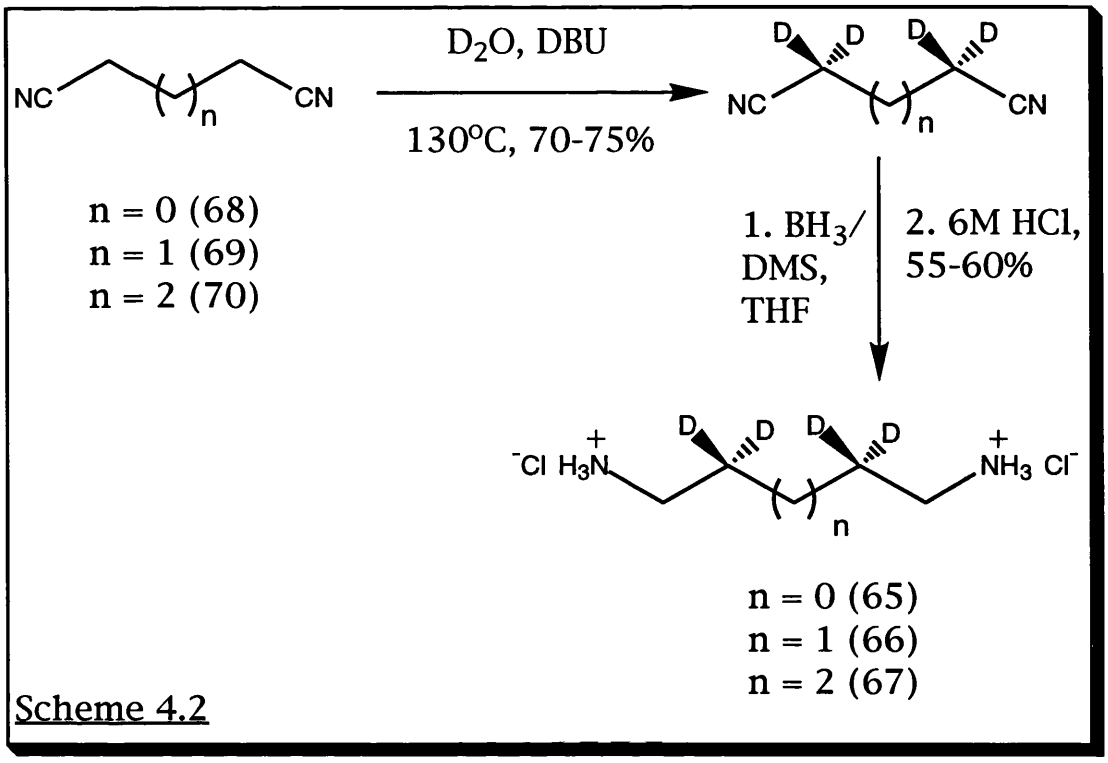
The three ^{2}H4-labelled α,ω–diamines (65), (66) and (67) were prepared by incorporation of the ^{2}H4-labels into the dinitrile starting materials succinonitrile (68), glutaronitrile (69) and adiponitrile (70). Subsequent reduction of the nitrile functionality followed by acidification gave the desired ^{2}H4-labelled diamine products.



(68) (69) (70)

The deuterium labels were incorporated into the dinitrile compounds by heating them at reflux with D_2O in the presence of a strong non-nucleophilic base, diazabicyclo[5.4.0]undec-7-ene, to exchange the α-protons (Scheme 4.2).[101] This process was repeated to ensure >95% deuterium [$^2H_4$] incorporation into the dinitriles. Monitoring of incorporation was achieved by using ^1H NMR and ^{13}C NMR spectroscopy, noting the loss of any relevant proton or change in carbon signals. For example, the ^1H NMR spectrum for glutaronitrile gave proton resonance at δ 2.15 (tt, 2H) and δ 2.65 (t, 4H), whereas the ^1H NMR spectrum of [$^2H_4$]-glutaronitrile gave a proton resonance of δ 2.15 (s, 2H), with no resonance at δ 2.65, showing clearly that [$^2H_4$]-labelling had taken place.

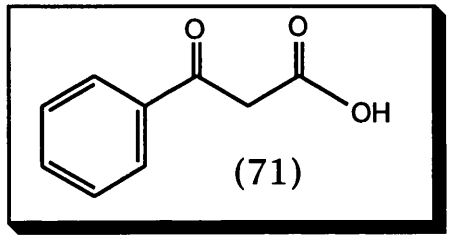
Reduction of the [$^2H_4$]-labelled dinitrile compounds to the corresponding [$^2H_4$]-labelled diamines (65), (66) and (67) was then carried out in a modification of a reported procedure,[119] with borane dimethylsulphide complex in THF, followed by heating to reflux in 6M HCl to form the dihydrochloride salts (Scheme 4.2). Incorporation of the label in each [$^2H_4$]-diamine product was estimated from the integrals of their corresponding ^1H NMR spectra and were found to be 98 ± 2% $^2H_4$ for [2,2,3,3-$^2H_4$]-putrescine (65), 96 ± 2% for [2,2,4,4-$^2H_4$]-cadaverine (66) and 93 ± 2% for [2,2,5,5-$^2H_4$]-1,6-hexanediamine (67). Deuterium incorporation was also confirmed by microanalysis and mass spectrometry.



Scheme 4.2

4.3 Results and Discussion

Once the diamine substrate has undergone oxidative deamination to the aminoaldehyde product, the amine functionality attacks the carbonyl and an intramolecular cyclisation occurs to form a cyclic imine.[120,121,122] This causes problems with characterisation and identification of the products as these cyclic imines have a tendency to trimerise in basic or neutral solution. In order to capture these cyclic imines a trapping agent, benzoylacetic acid (71), was employed.

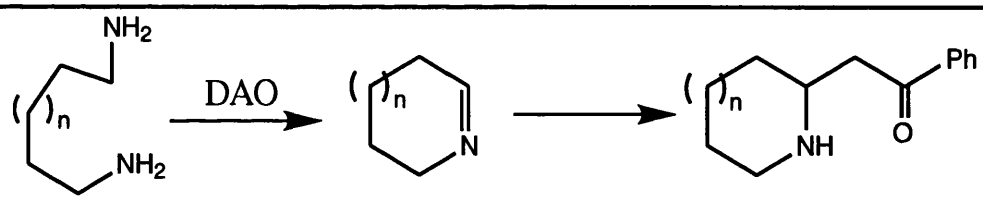


(71)

Benzoylacetic acid (71), a β-keto acid, was obtained from base hydrolysis of the β-ketoester ethyl benzoylacetate. However,

decarboxylation of the β-ketoacid (71) occurs readily above 25 °C, therefore care was taken to ensure that this did not occur. Synthesis of (71) immediately before use and storage in a freezer helped to minimise the amount of β-ketoacid lost by decarboxylation.

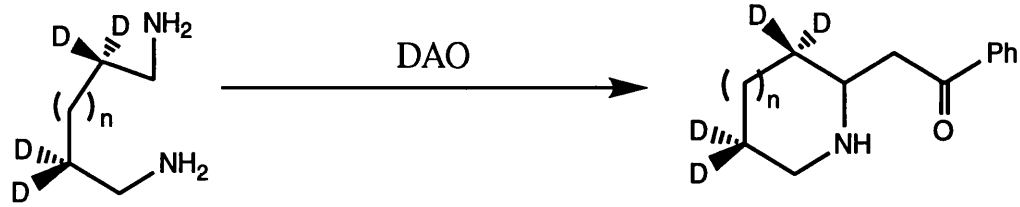
The isolation of diamine oxidase from pea seedlings is described in Chapter 5. Each unlabelled diamine (3), (6) and (68) and each corresponding [$^2H_4$]-labelled diamine (65), (66) and (67) was incubated with pea seedling diamine oxidase, catalase and benzoylacetic acid (71) in phosphate buffer (pH 7) at 25 °C (Schemes 4.3 and 4.4).[123] Catalase was required to remove hydrogen peroxide which exhibits inhibitory effects on DAO. Trapping of each corresponding cyclic imine with the benzoylacetic acid (71) *in situ* produced substituted acetophenones, either unlabelled (72), (73) and (74) or [$^2H_4$]-labelled (75), (76) and (77).



n = 0 (3)
n = 1 (6)
n = 2 (68)

n = 0 (72)
n = 1 (73)
n = 2 (74)

Scheme 4.3



n = 0 (65)
n = 1 (66)
n = 2 (67)

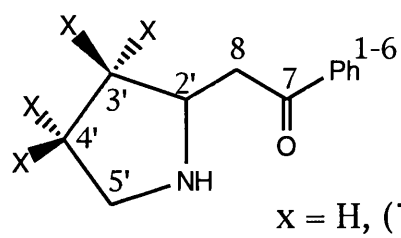
n = 0 (75)
n = 1 (76)
n = 2 (77)

Scheme 4.4

The percentage yield for these reactions varied widely, with no real pattern or reason being apparent. A possible reason may have been small changes in the pH at which the reaction was carried out, since the pH affects both the rate of deamination and the rate at which benzoylacetic acid decarboxylates. Although the pH was adjusted when necessary, the reaction was carried out over 24 hours and it was not possible to monitor the reaction constantly.

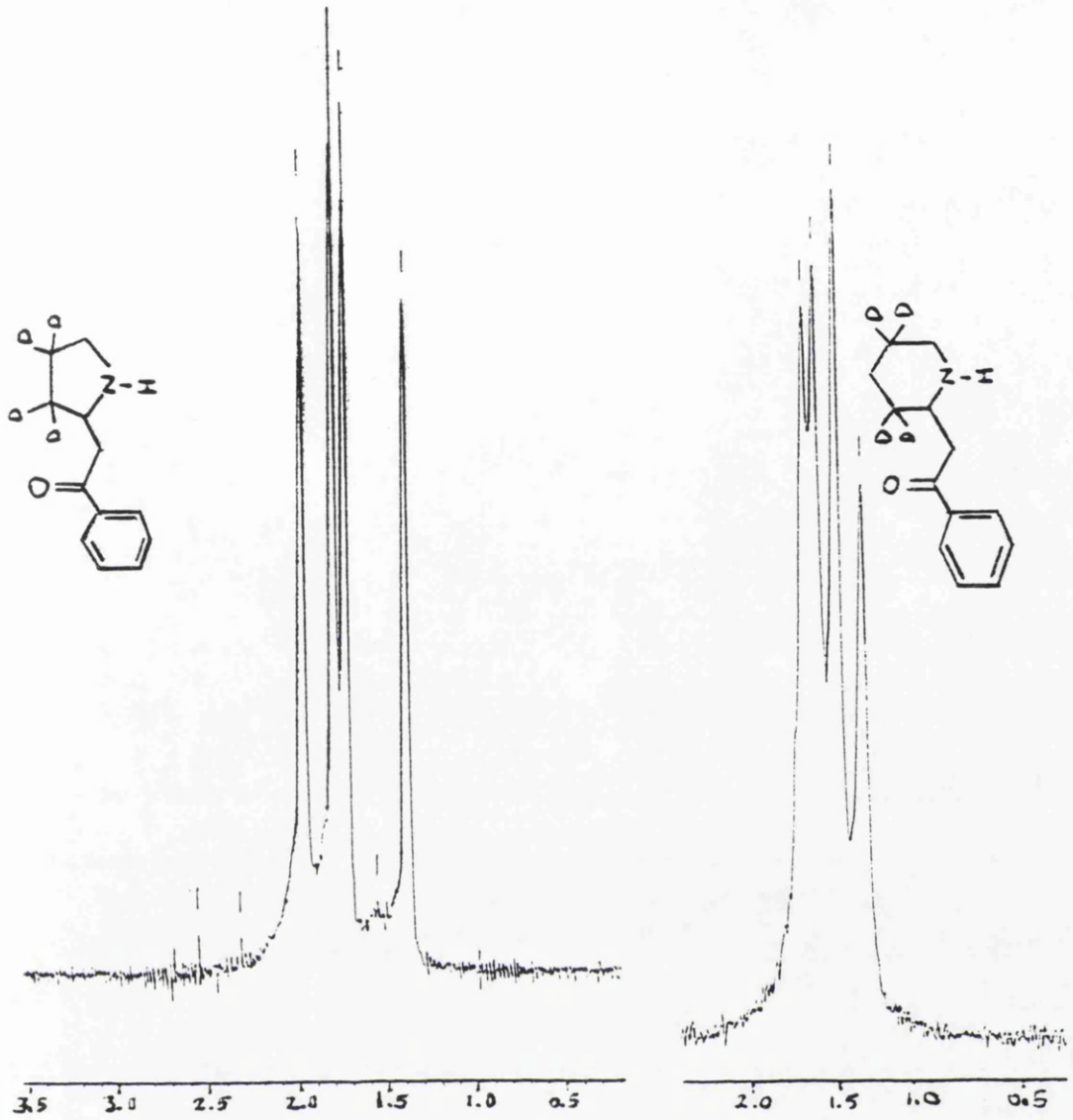
The product [3',3',4',4'-$^2H_4$]-2-pyrrolidin-2-ylacetophenone (75) (Figure 4.2) was produced in 76% yield from [$^2H_4$]-putrescine (65). The product (75) was purified by preparative thin layer chromatography and the purity was checked by HPLC. The $^2H_4$ content of the product (75) was estimated to be 96 ± 2% by analysis of the integrals for the protons in the ^1H NMR spectrum of (75).

For the ^1H NMR spectrum of the unlabelled product 2-pyrrolidin-2-ylacetophenone (72) a complex of signals was observed at δ 1.30-1.70 for the protons at the 3'- and 4'-positions of the pyrrolidinyl ring (Figure 4.2). Whereas the corresponding signals for the protons on the 3'- and 4'-positions of (75) (Figure 4.2) were barely visible at δ 1.30-1.90. The protons at the 5'-position of (75) were observed as a doublet of doublets at δ 2.98. Even the 2'-H protons and 8-H protons, which were not on the pyrrolidinyl ring, had been greatly simplified to an ABX system made up of eight lines at δ 3.25 and a doublet of doublets at δ 3.64.



x = H, (72)
x = D, (75)

Figure 4.2



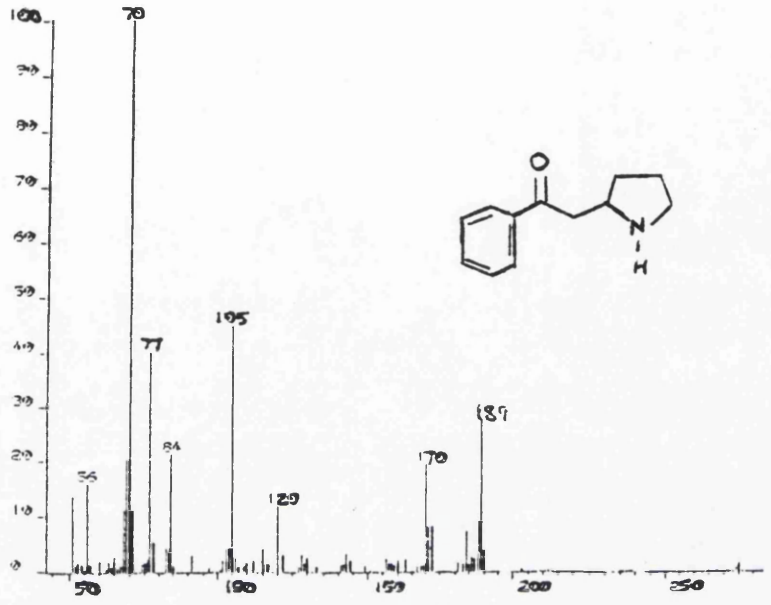
a. b.

Figure 4.3: a. ^{2}H NMR spectrum for (75); b. ^{2}H NMR spectrum for (76).

Furthermore, evidence that all four deuterium were still present after oxidation was strengthened by the ^{2}H NMR spectrum of the labelled product (75) (Figure 4.3a). This showed four separate signals at δ 1.38, 1.72, 1.79 and 1.96, for the $^{2}H_4$ label, which were all of almost equal intensity. Finally, comparison of the mass spectra for (72) and (75) (Figures 4.4a and 4.4b) confirmed the presence of the four deuterium atoms, with unlabelled (72)

having peaks at *m/z* 190 ($M^+ + 1$) and 189 (M^+), whereas labelled product (75) had major isotopic species with peaks at *m/z* 194 ($M^+ + 1$) and 193 (M^+).

a.



b.

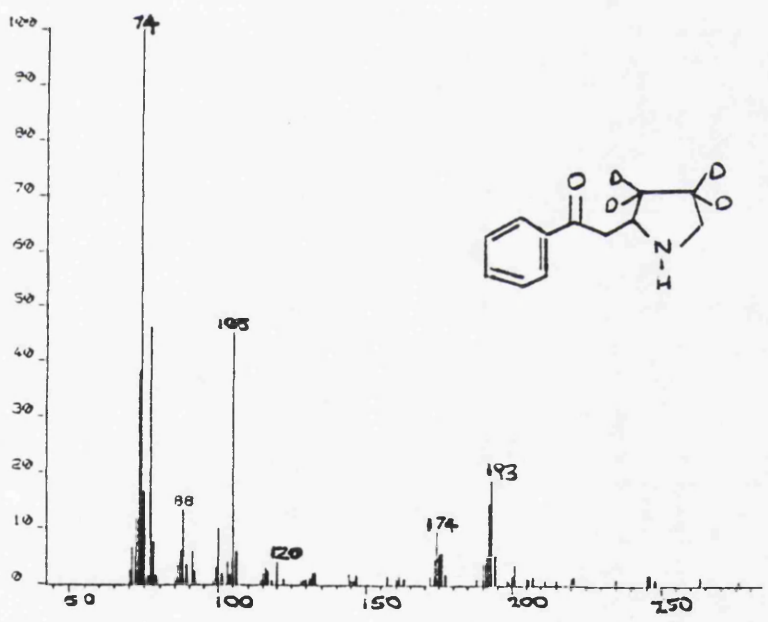
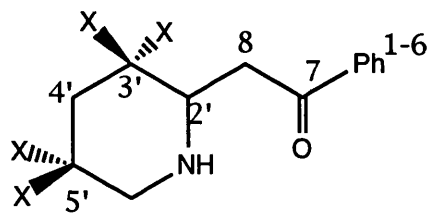


Figure 4.4: a. Mass Spectrum of (72), b. Mass Spectrum of (75).

The product [3',3',5',5'-$^2H_4$]-2-piperidin-2-ylacetophenone (76) (Figure 4.5) was produced in 70% yield from [$^2H_4$]-cadaverine (66). Again, the product (76) was purified by preparative thin layer chromatography and the purity was checked by HPLC. Comparison of the ^1H NMR spectra of the unlabelled product 2-piperidin-2-

ylacetophenone (73) and (76) indicated that the $^{2}H_4$ content of (76) was 97 ± 2%.

The ^{1}H NMR spectrum of unlabelled (73) showed a complex multiplet at δ 1.31-1.75 for the 6H at the 3'-, 4'- and 5'-positions of the piperidine ring (Figure 4.5 and Figure 4.6). Whereas, with the ^{1}H NMR spectrum of $^{2}H_4$-labelled (76) the protons at the 4'-position were present as an AB system at δ 1.25 and1.62, and the signals for the protons at the 3'- and 5'- positions were absent (Figure 4.5 and 4.7). Also the signal for the proton at the 2'-position which was present as a doublet of triplets for (73), split by the protons at the 3'- and 8- positions, was present as a doublet of doublets at δ 2.66 for (76). Signals for the remaining four protons of (76) were overlapping and were present as a complex multiplet at δ 2.99-3.14.



x = H, (73)
x = D, (76)

Figure 4.5

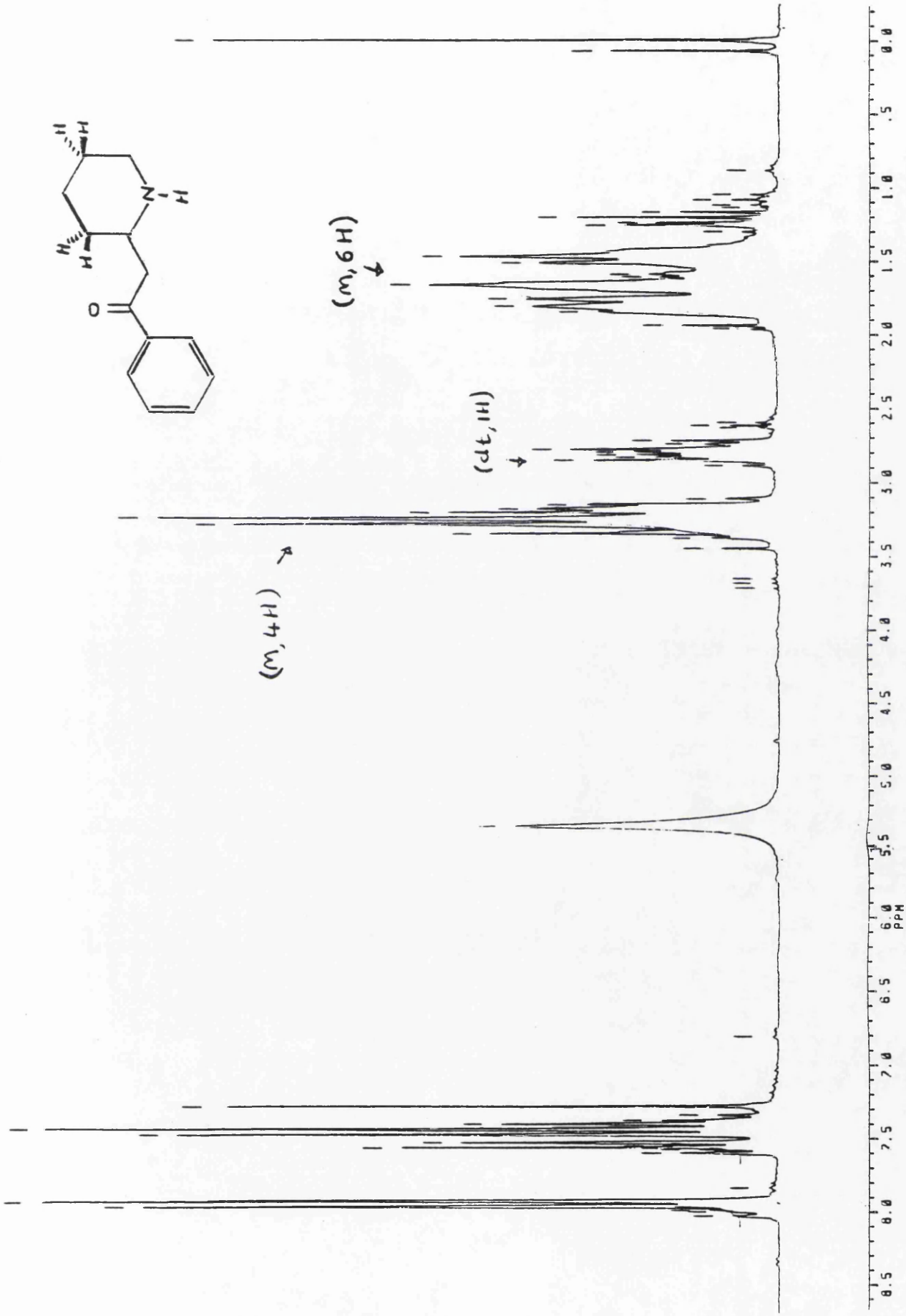


Figure 4.6: ^{1}H NMR spectrum of (73)

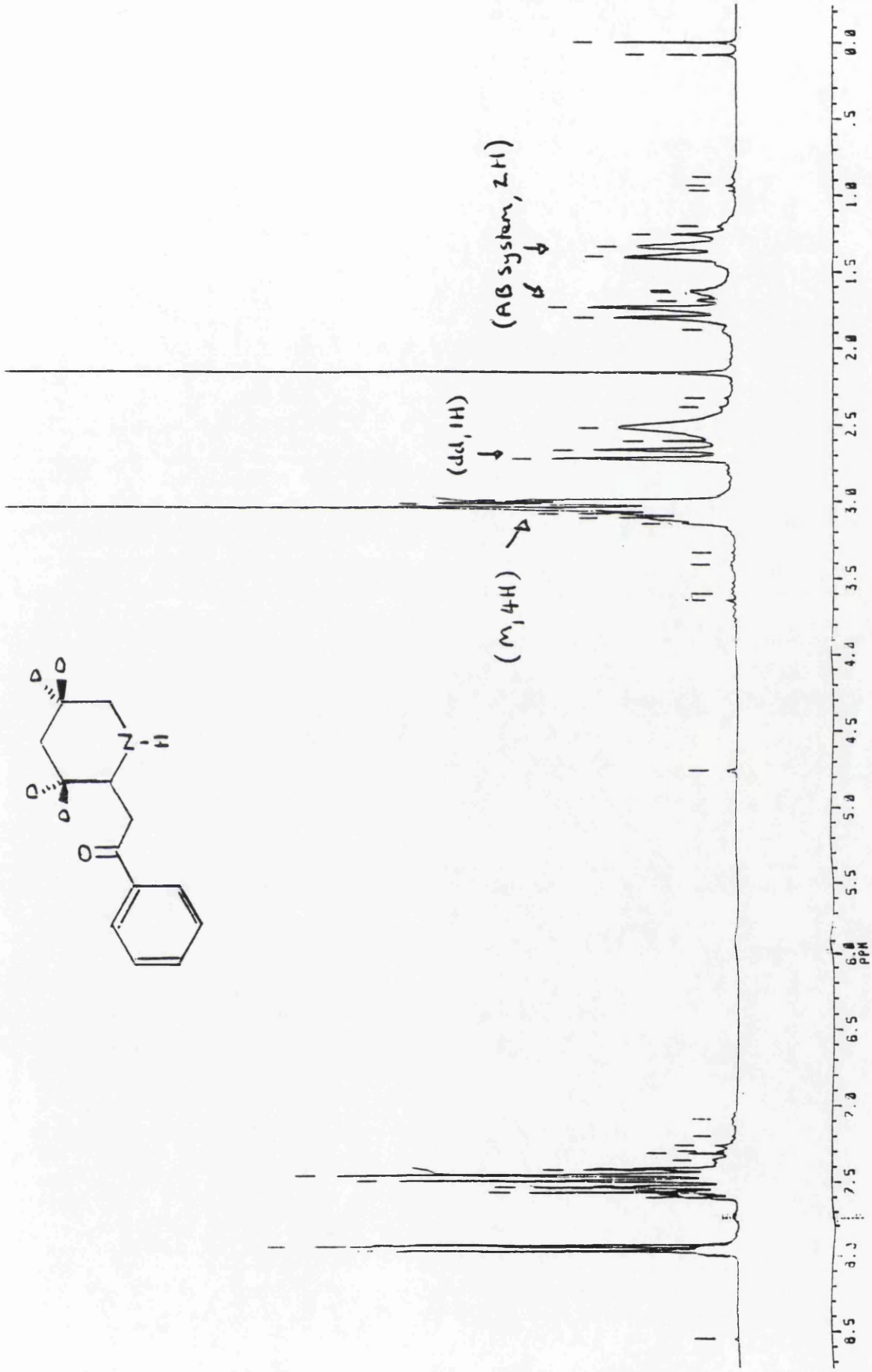


Figure 4.7: ^{1}H NMR spectrum of (76).

In the ^{2}H NMR spectrum of (76) (Figure 4.3b) four signals with approximately the same intensity were observed at δ 1.35, 1.49, 1.61 and 1.68. The mass spectrum of (76) also showed evidence of the four deuterium atoms with major isotopic species at *m/z* 208 (M^{+} + 1) and 207 (M^{+}), compared with unlabelled (73) having peaks at *m/z* 204 (M^{+} + 1) and 203 (M^{+}).

Finally, the product [3',3',6',6'-$^{2}H_4$]-2-azacycloheptanyl-acetophenone (77) was produced in 75% yield from [$^{2}H_4$]-1,6-hexanediamine (67). The product (77) was purified by preparative thin layer chromatography and the purity was checked by HPLC. The $^{2}H_4$ content of the product (77) was estimated to be 96 ± 2%.

The ^{1}H NMR spectra for both the unlabelled 2-azacycloheptanylacetophenone (74) and $^{2}H_4$-labelled (77) were complex in the region δ 1.0-1.60. However, in the spectrum of (77) the signals for this region integrated for four protons compared to eight in the corresponding spectrum of (74). The ^{2}H NMR spectrum for (77) showed four signals of almost equal intensity at δ 1.20, 1.27, 2.35 and 2.58. Comparison of the mass spectra of (74) and (77) confirmed the presence of the $^{2}H_4$-labelled species, with unlabelled (74) having major isotopic species at *m/z* 218 (M^{+} + 1) and 217 (M^{+}), compared with $^{2}H_4$-labelled (77) having peaks at *m/z* 222 (M^{+} + 1) and 221 (M^{+}).

Thus the enzyme-catalysed oxidative deamination of specifically tetradeuteriated samples (65), (66) and (67) by pea seedling diamine oxidase, and subsequent trapping of the products *in situ* using benzoylacetic acid (71) produced acetophenone derivatives (75), (76) and (77), which were all almost entirely tetradeuteriated. From these results it is clear that the oxidative deamination of α, ω-diamines catalysed by DAO cannot involve an enamine intermediate since at least some of the label would be lost

if this enamine was formed. The work described in this chapter has been published.[177]

This result helps to underline the need for more work on mechanistic studies of diamine oxidase. It also highlights the need for a more definite characterisation of the organic cofactor for diamine oxidase in order to gain a better understanding of the part which it plays in the enzymatic oxidation process.

CHAPTER 5

Oxidation of Substituted Quinolines and Pyridines By Pea Seedling Diamine Oxidase

5.1 Extraction and Partial Purification of Diamine Oxidase from Pea Seedlings

As discussed earlier, there are two convenient sources of DAO. However, due to the low specific activity of the commercial pig kidney product, accurate data are difficult to obtain using enzyme from this source. Therefore, only pea seedling DAO was used in this study.

Pea seedling DAO was extracted and purified using the method described by Hill.[6] Pea seedlings from the "Fillbasket" variety were grown for *ca.* 10 days. The extraction method depends on removing most of the unwanted material from the crude extract by precipitation with a 2:1 mixture of chloroform and ethanol. The enzyme was then precipitated using ammonium sulphate and contrary to the observations of Hill, separation of the solid occurred only after being left overnight. The procedure was then carried out as reported by Hill including further ammonium sulphate precipitations and dialysis. The purity of the enzyme at this stage was adequate for our purposes and further purification steps used by Hill were not undertaken. The protein obtained was taken up in phosphate buffer (pH 7) and stored in 0.5 ml aliquots in the freezer at *ca.* -20 ^{o}C. The enzyme was found to be stable for several months with very little loss of activity. Further details of the purification of the enzyme are discussed in Chapter 8.

5.2 The Determination of Protein Concentration

The determination of protein concentration was carried out using the method of Sedmak and Grossberg.[124] This method relies on the conversion of Coomassie brilliant blue G in dilute acid from a brown/orange colour into an intense blue colour with the addition of protein. The method was carried out using bovine serum albumin (BSA) as the protein standard (1 mg of BSA is equivalent to 1 mg of protein).

The absorbances of the mixture at A_{620} (blue) and A_{465} (brown/orange) were recorded for various concentrations of BSA and a standard graph was obtained by plotting A_{620}/A_{465} *vs.* protein concentration. The absorbance was measured approximately 20 minutes after the Coomassie reagent was added to the protein sample and the A_{620}/A_{465} was then measured for a range of enzyme samples of varying dilution. A standard graph was then used to determine the concentration of protein by measuring the absorbance ratios (Appendix).

This assay system for the determination of protein concentration was found to be highly reproducible and was shown to detect less than 1 μg of albumin.

5.3 The Assay Procedure

There have been many assay systems developed for the measurement of DAO activity, as discussed in Chapter 2.14. For our work it was necessary to be able to determine the rates of enzymatic reaction for a variety of substrates. Therefore we required an assay system which involved the measurement of a common factor and which was not restricted by the requirement of

1-pyrroline (or 1-piperideine) forming as the oxidation product. The spectrophotomeric assay developed by Stoner[89] was found to be a reliable method which was convenient for our studies.

The assay involves a coupled reaction with hydrogen peroxide, a by-product of the catalytic reaction, and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (30) with an acceptor, 3-(dimethylamino)benzoic acid (DMAB) (31) (see Chapter 2, Scheme 2.17). The MBTH is oxidatively coupled to DMAB in the presence of hydrogen peroxide and peroxidase, forming a stoichiometric amount of indamine dye having an absorption maximum at 595 nm (Scheme 2.17). The rates of reaction were determined directly from the spectrophotometer, with initial calibration carried out using standard solutions of hydrogen peroxide.

Stoner showed that MBTH (30) was an inhibitor of DAO and that this inhibition was both time and concentration dependent. However, he kept this inhibition to a minimum by controlling the concentration of the MBTH. In addition, it was found that this inhibitory effect could be further reduced by adding the substrate immediately after the addition of enzyme, thus reducing the incubation time of the enzyme with MBTH.

Using this spectrophotomeric assay system, most of the compounds tested were found to exhibit Michaelis-Menten behaviour.[114] Rate data were analysed for V_{max} and K_M by least square fitting of Lineweaver-Burk[115] (1/V *vs.* 1/[S]), Eadie-Hofstee[116,117] (V *vs.* V/[S]) and Hanes[118] ([S]/V *vs.* [S]) plots. The experiments were carried out three times using each substrate and the data are quoted as an average of the nine determinations.

5.4 Substituted Quinolines and Pyridines as Substrates for Pea Seedling DAO

5.4(a) Introduction

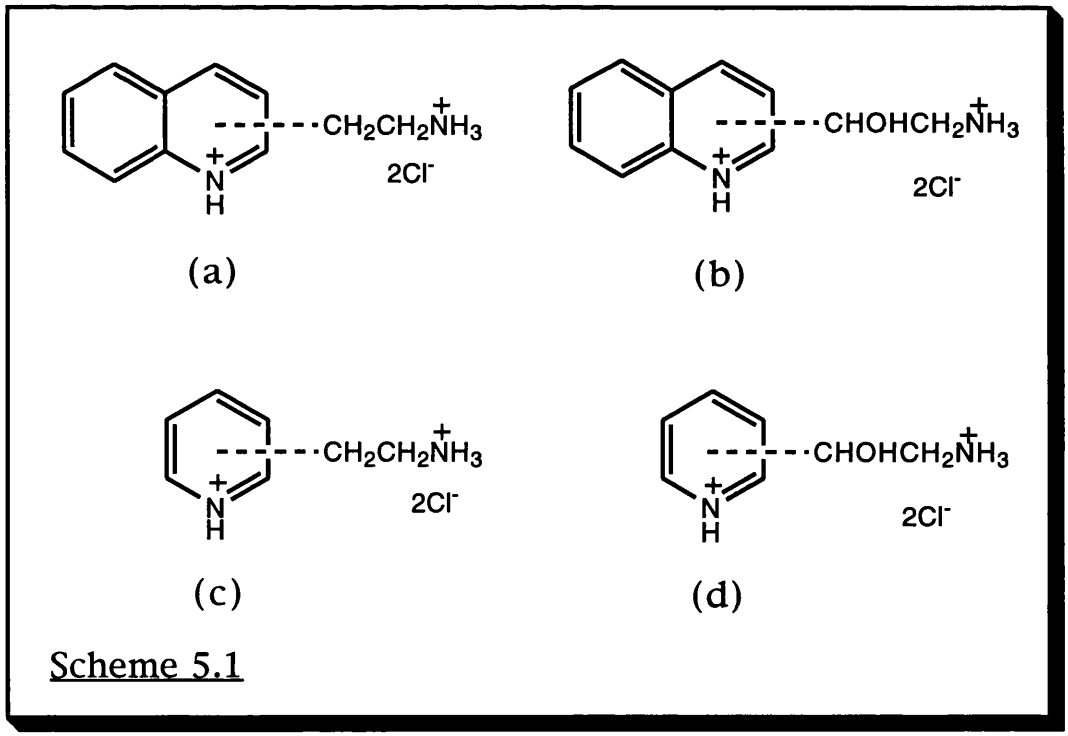
Diamine oxidase not only catalyses the oxidation of diamines to their corresponding aminoaldehydes, it also catalyses the oxidation of selected primary monoamines to the corresponding aldehydes.[94] The work carried out in our group has mainly concentrated on aliphatic primary diamines as substrates for pea seedling DAO. Equi *et al.*[96] studied the effects on the rate of oxidation and binding affinity when the chain length of the diamine substrate or the substituent groups on the diamine backbone were changed. However, more recently Barr studied aromatic diamines and compounds which contained only one primary amine group.[126] A range of compounds was studied, such as (aminoalkyl)pyridines, which contained two nitrogens, but only one primary amine was present capable of undergoing enzymatic oxidation.

Our aims were to expand the study and to undertake kinetic studies on a range of similar compounds in order to gain an insight into the role of the second amine group during the DAO catalysed oxidation. We also wished to find out if the nature of the second amine group has an effect on the binding affinity of the substrate, or if it plays a more important role in setting off the oxidative deamination process.

In order to carry out our aims we synthesised a number of substituted quinolines and pyridines, concentrating on methyl-, ethyl- and propyl-amine derivatives. We also synthesised a number of pyrrole and thiophene derivatives, and this work will be discussed in Chapter 6.

5.4(b) Synthesis of the Substituted Quinoline and Pyridine Derivatives

To begin with we decided to attempt the synthesis of 2-, 3- and 4-quinolylethylamine (Scheme 5.1a), or if this failed to synthesise 2-hydroxy-2'-, 2-hydroxy-3'- and 2-hydroxy-4'-quinolylethylamine (Scheme 5.1b). The reason behind this decision was that Barr had attempted to make 2-, 3- and 4-pyridylethylamine (Scheme 5.1c) and had failed.[126] However, it was found that 2-hydroxy-2'-, 2-hydroxy-3'- and 2-hydroxy-4'-pyridylethylamine (Scheme 5.1d) were suitable alternatives for the study on the role of the second amine in the deamination process. We also decided to attempt the synthesis of 2-, 3- and 4-pyridylpropylamine and 2-, 3- and 4-quinolylpropylamine.



Scheme 5.1

The starting materials for the synthesis of all the substituted quinolines were the corresponding quinolinecarboxaldehydes and either nitromethane or nitroethane. Condensation of nitroethane

with 2-quinolinecarboxaldehyde in the presence of a base, potassium fluoride, gave 2-hydroxy-2-(2'-quinolyl)nitroethane (78) as a racemic mixture in the form of an orange/red solid (Scheme 5.2).

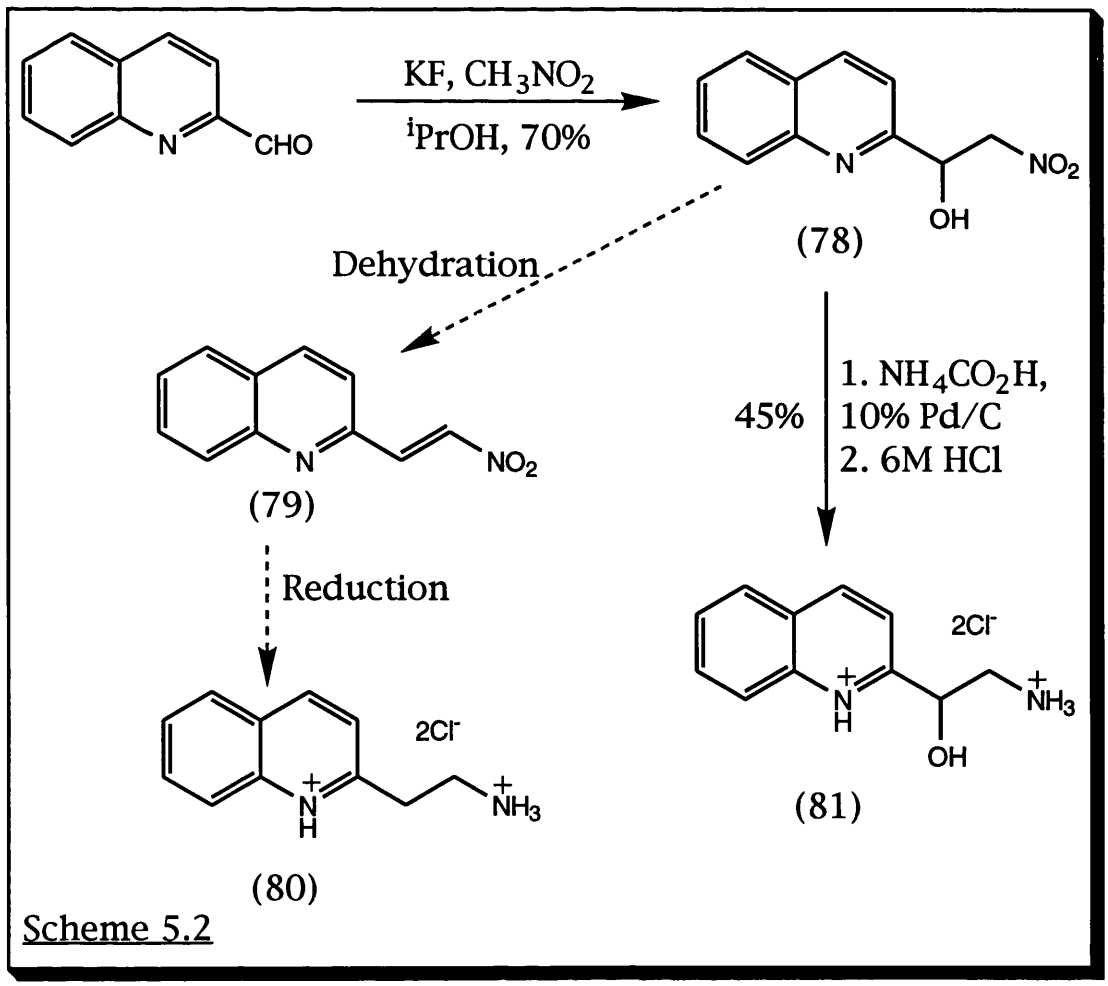
At this stage attempts were made to try and induce dehydration in the nitro-alcohol (78) to yield the corresponding nitro-alkene (79) which could then be reduced in acidic conditions to give 2-(2'-quinolyl)ethylamine dihydrochloride (80) (Scheme 5.2). Our first attempt was to use the procedure which Barr had found gave some indications of nitro-alkene formation by ^{1}NMR spectroscopy.[126] This procedure was to treat a solution of the nitro-alcohol (78) with acetic anhydride and 4-dimethylamino-pyridine (DMAP) over a period of 20 hours. However, the resulting product was a dark green solid which rapidly decomposed on standing and showed no indication of being the nitro-alkene (79).

The next attempt to induce dehydration involved heating the nitro-alcohol (78) at reflux in an acid solution in the presence of activated molecular sieves. This procedure yielded the starting nitro-alcohol as the corresponding hydrochloride salt. Other attempts using $POCl_3$ or base-catalysed elimination with NaOH also failed to yield nitro-alkene (79).

We then decided to try known procedures[127,128] which had been successful in the synthesis of thiophene nitro-alkenes and which we had used to make several thiophene derivatives (see Chapter 6). The first attempt using NaOH to facilitate the base condensation between 2-quinolinecarboxaldehyde and nitro-methane and then acid-catalysed elimination from the intermediate proved unsuccessful, giving the hydrochloride salt of nitro-alcohol (78). Changing the base to *N*-amylamine and storing the reaction

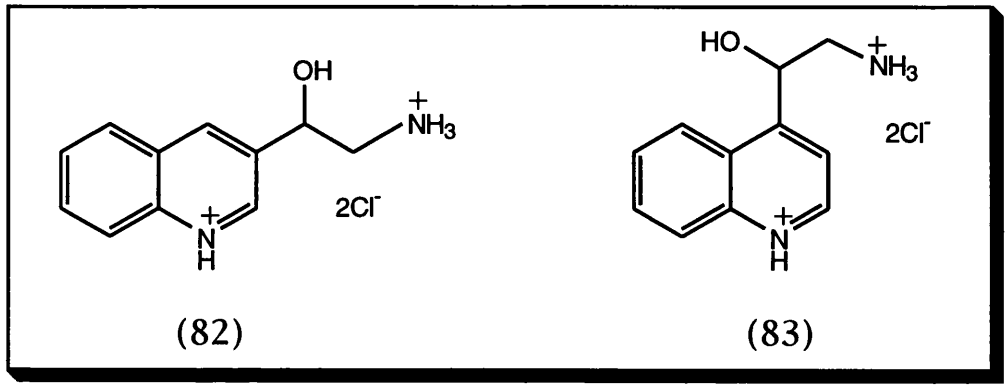
mixture in darkness for 14 days gave starting materials after separation on a silica gel column.

Having made several unsuccessful attempts at synthesising the nitro-alkene (79) and therefore being unable to use the proposed route to make the required product 2-(2'-quinolyl)ethylamine dihydrochloride (80) (Scheme 5.2), it was then decided to reduce (±)-2-hydroxy-2-(2'-quinolyl)nitroethane (78) to the corresponding diamine and examine this as a substrate for DAO. The catalytic reduction of (78) to yield (±)-2-hydroxy-2-(2'-quinolyl)ethylamine was carried out using anhydrous ammonium formate as a source of hydrogen *in situ*, with 10% palladium on carbon as a catalyst.[129] The crude amine was then partitioned between dichloromethane and 6M HCl to give (±)-2-hydroxy-2-(2'-quinolyl)ethylamine as the dihydrochloride salt (81) (Scheme 5.2).



Scheme 5.2

In similar procedures 3-quinolinecarboxaldehyde and 4-quinolinecarboxaldehyde were converted into the dihydrochloride salts of (±)-2-hydroxy-2-(3'-quinolyl)ethylamine (82) and (±)-2-hydroxy-2-(4'-quinolyl)ethylamine (83) respectively.

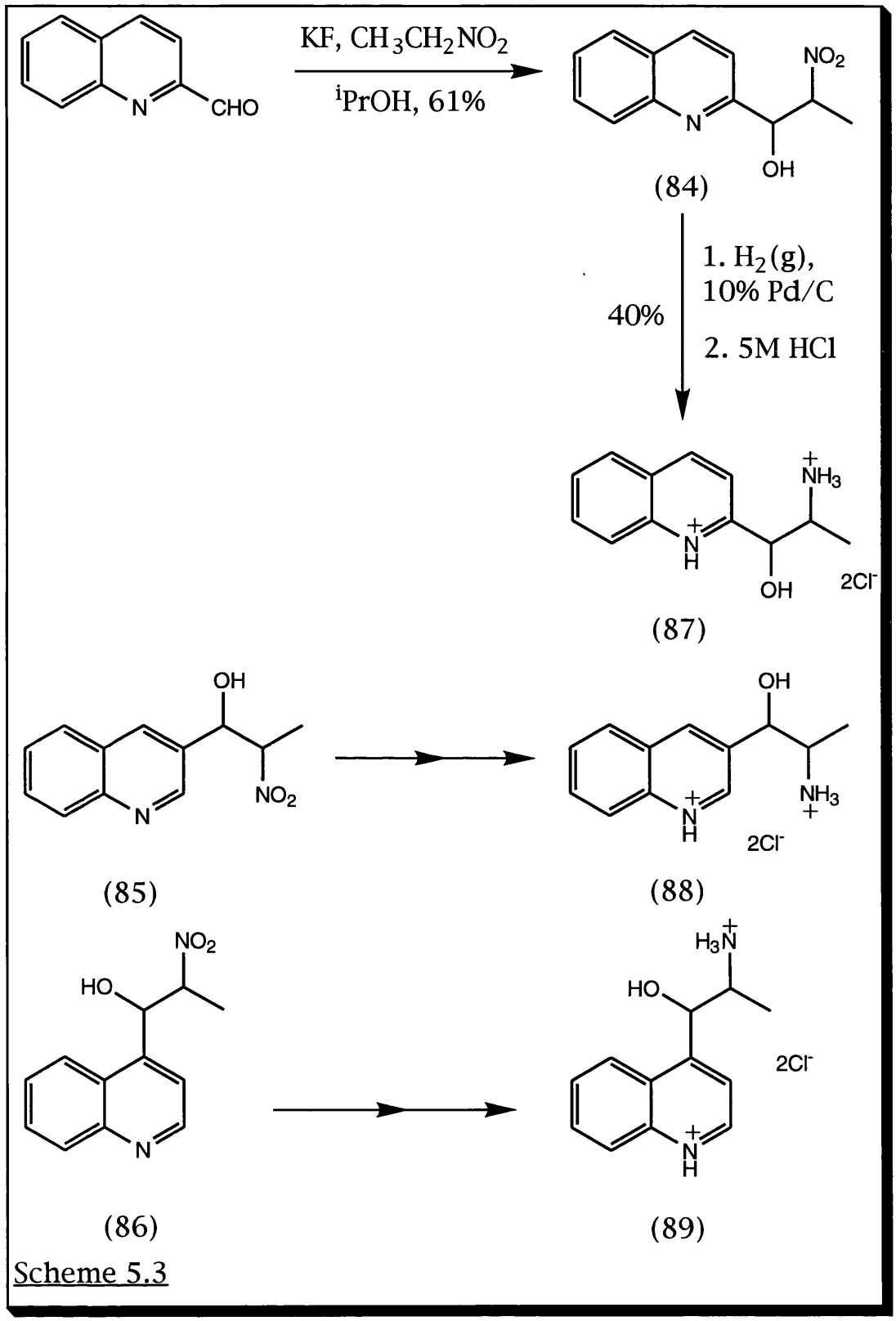


(82) (83)

The condensation of nitroethane with 2-, 3- and 4-quinolinecarboxaldehyde gave (±)-3-hydroxy-3-(2'-quinolyl)-2-nitropropane (84), (±)-3-hydroxy-3-(3'-quinolyl)-2-nitropropane (85) and (±)-3-hydroxy-3-(4'-quinolyl)-2-nitropropane (86), respectively, all as mixtures of diastereoisomers (Scheme 5.3). Due to the differing reactivities of the quinolinecarboxaldehydes and the differing physical forms of the product nitro-alcohols the reaction procedure for condensation of each quinoline-carboxaldehyde with nitroethane was altered as required.

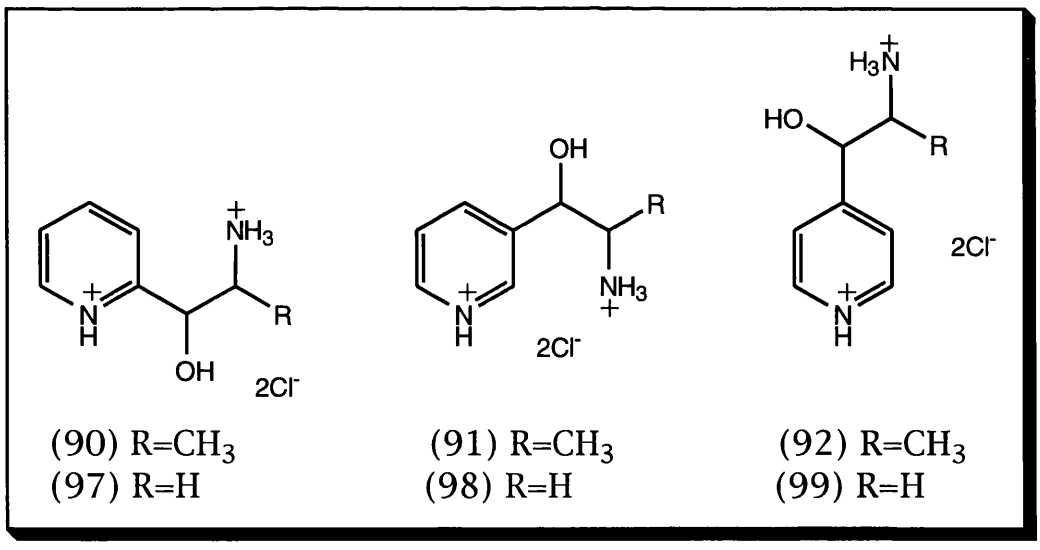
The reduction of nitro-alcohols (84), (85) and (86) was carried out using medium pressure catalytic hydrogenation, the pressure between *ca.* 40-60 p.s.i. depending on the reactivity of the nitro-alcohol and 10% Pd/C was used as the catalyst. The crude amines were filtered to remove the catalyst and 5M HCl was added to each filtrate yielding (±)-3-hydroxy-3-(2'-quinolyl)propyl-2-amine (87), (±)-3-hydroxy-3-(3'-quinolyl)-propyl-2-amine (88) and (±)-3-hydroxy-3-(4'-quinolyl)propyl-2-amine (89) all as dihydrochloride salts and mixtures of diastereoisomers (Scheme 5.3). Products (87)

and (88) had ratios of diastereoisomers of approximately 3:1 estimated from their ^{13}C NMR spectra, while the ^{13}C NMR spectrum for product (89) showed a single racemate.



Scheme 5.3

The condensation of nitroethane with 2-, 3- and 4-pyridine-carboxaldehyde gave the nitro-alcohols (±)-3-hydroxy-3-(2'-pyridyl)-2-nitropropane, (±)-3-hydroxy-3-(3'-pyridyl)-2-nitro-propane and (±)-3-hydroxy-3-(4'-pyridyl)-2-nitropropane respectively, which were subsequently reduced using catalytic hydrogenation to yield (±)-3-hydroxy-3-(2'-pyridyl)propyl-2-amine (90), (±)-3-hydroxy-3-(3'-pyridyl)propyl-2-amine (91) and (±)-3-hydroxy-3-(4'-pyridyl)propyl-2-amine (92) respectively as their dihydrochloride salts after treatment with HCl. These products were mixtures of two racemates and the ratios of each were estimated from the corresponding ^{13}C NMR spectra. Product (90) was estimated to be a 3:1 mixture, product (91) was estimated to be a 3:2 mixture and product (92) showed one racemate with only traces of the other racemate.



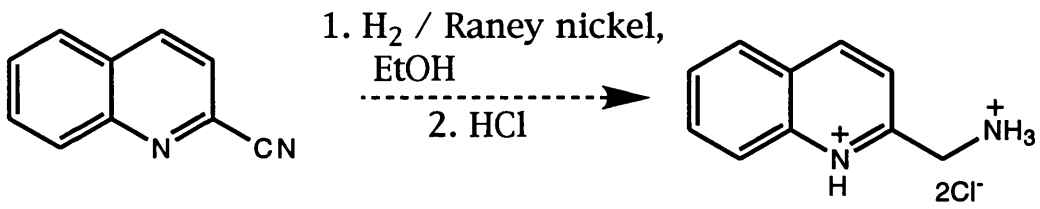
(90) R=CH_3 (97) R=H

(91) R=CH_3 (98) R=H

(92) R=CH_3 (99) R=H

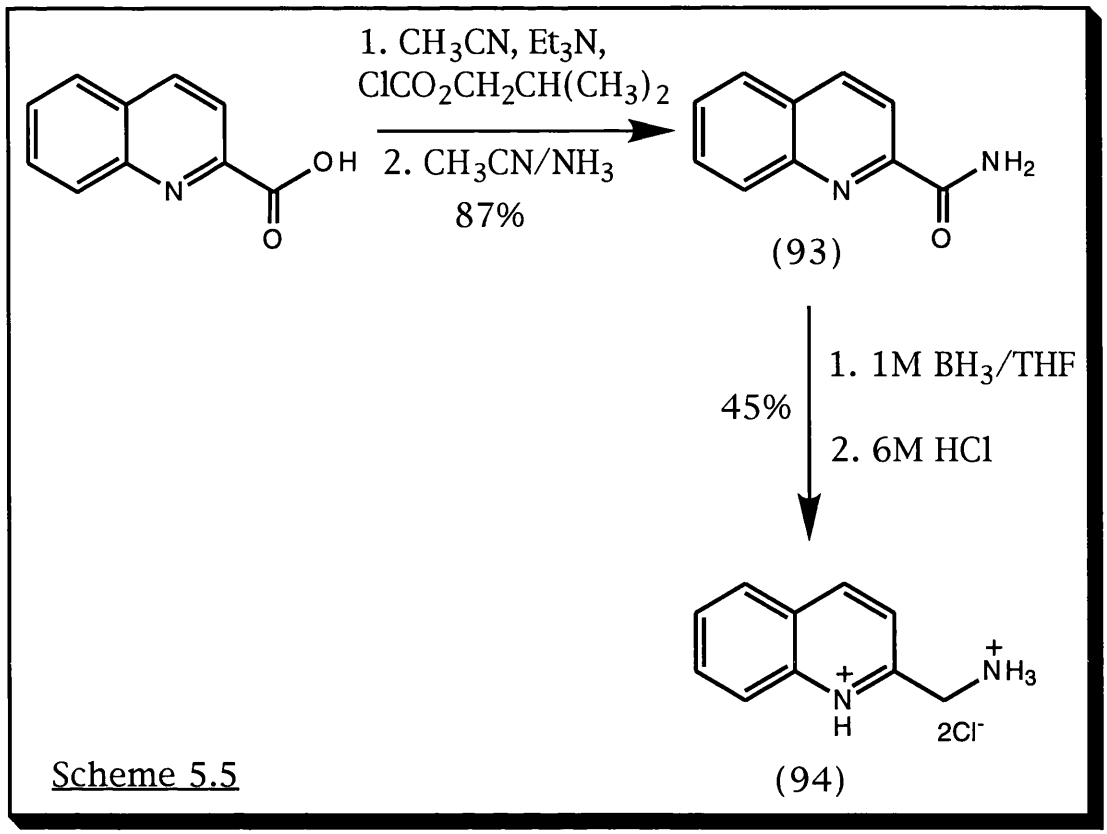
We also synthesised (±)-2-hydroxy-2-(2'-pyridyl)ethylamine (97), (±)-2-hydroxy-2-(3'-pyridyl)ethylamine (98) and (±)-2-hydroxy-2-(4'-pyridyl)ethylamine (99) in order to use them as a comparison in the kinetic studies. These compounds were synthesised from their corresponding pyridinecarboxaldehydes using the same procedure as before.

To synthesise the quinolylmethylamines we first attempted to reduce the corresponding readily available quinolinecarbonitriles. However the procedure used by Barr[126] to reduce pyridinecarbonitriles, namely catalytic hydrogenation at *ca.* 60 p.s.i. in the presence of Raney nickel,[130] produced the hydrochloride salt of each quinolinecarbonitrile after acidification (Scheme 5.4).



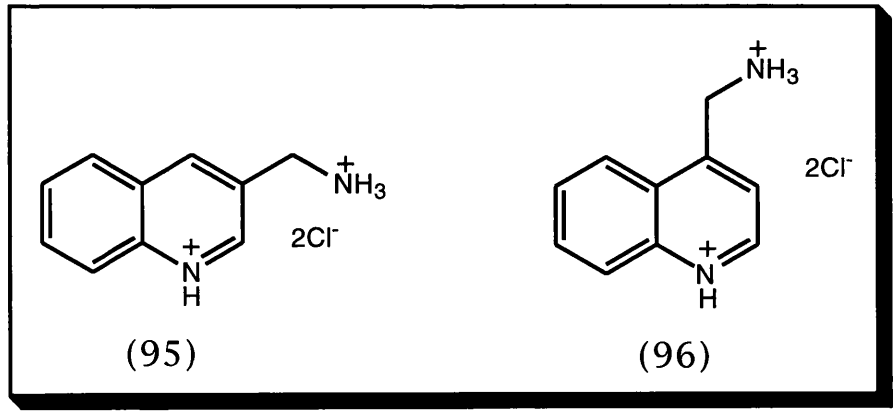
Scheme 5.4

An alternative route via the amide was used in place of the carboxylic acid. Isobutylchloroformate and triethylamine were added to a solution of 2-quinolinecarboxylic acid in acetonitrile. This formed an anhydride *in situ* which upon addition of acetonitile saturated with ammonia gave 2-quinolinecarboxamide (93) in 87% yield (Scheme 5.5).[131] Reduction of the 2-quinolinecarboxamide (93) was carried out by heating at reflux with 1M BH_3 solution in THF to give the crude amine.[132] Treatment of the crude amine with 6M HCl gave 2-quinolylmethylamine dihydrochloride (94) in 45% yield (Scheme 5.5).



Scheme 5.5

Using the same procedure with 3-quinolinecarboxylic acid and 4-quinolinecarboxylic acid as starting materials gave 3-quinolylmethylamine dihydrochloride (95) and 4-quinolylmethylamine dihydrochloride (96) respectively.



5.4 (c) Results and Discussion

Using Stoner's spectrophotomeric assay system[89], kinetic parameters for the enzymatic deamination of each substrate were obtained, and the data are summarised in Table 5.1. Errors for each substrate are quoted as an average of computer generated errors calculated from nine determinations of kinetic data.

Substrate	K_M	V_{max}
Putrescine (3)	1.21 (± 0.40)	1157 (± 200)
Cadaverine (6)	0.23 (± 0.06)	2325(± 390)
(±)-2-hydroxy-2-(2'-quinolyl)ethylamine (81)	0.30 (± 0.07)	1.8 (± 0.2)
(±)-2-hydroxy-2-(3'-quinolyl)ethylamine (82)	0.35 (± 0.09)	1.4 (± 0.2)
(±)-2-hydroxy-2-(4'-quinolyl)ethylamine (83)	1.05 (± 0.19)	1.7 (± 0.2)
(±)-2-hydroxy-2-(2'-pyridyl)ethylamine (97)	0.24 (± 0.04)	3.8 (± 0.2)
(±)-2-hydroxy-2-(3'-pyridyl)ethylamine (98)	0.27 (± 0.05)	0.9 (± 0.2)
(±)-2-hydroxy-2-(4'-pyridyl)ethylamine (99)	0.90 (± 0.10)	3.6 (± 0.2)
2-quinolylmethylamine (94)	0.10 (± 0.02)	4.0 (± 0.1)
3-quinolylmethylamine (95)	0.63 (± 0.10)	7.8 (± 0.2)
4-quinolylmethylamine (96)	0.16 (± 0.03)	5.5 (± 0.2)

Table 5.1- Formulae numbers refer to the dihydrochloride salts of each substrate, the form in which they were tested. K_M values are in units of mM and V_{max} values are in units of μmol $mg^{-1}h^{-1}$.

The compounds (±)-3-hydroxy-3-(2'-quinolyl)-, (±)-3-hydroxy-3-(3'-quinolyl)- and (±)-3-hydroxy-3-(4'-quinolyl)-propyl-2-amine dihydrochloride, (87), (88) and (89) respectively, were all found to be extremely poor substrates of the pea seedling DAO. (±)-3-Hydroxy-3-(2'-pyridyl)-, (±)-3-hydroxy-3-(3'-pyridyl)- and (±)-3-hydroxy-3-(4'-pyridyl)propyl-2-amine dihydrochloride, (90), (91) and (92)) respectively, were also found to be poor substrates of the pea enzyme. As such they did not display classical Michaelis-Menten behaviour; therefore we were unable to make accurate determinations of K_M and V_{max}.

From the data in Table 5.1 it is obvious that all the compounds tested are oxidised at rates which are much slower than those of the natural substrates, putrescine (3) and cadaverine (6). However, the K_M values are somewhat different, with all the substrates binding strongly to pea seedling DAO and having a greater binding affinity for the pea enzyme than putrescine. Surprisingly, 2-quinolylmethylamine (94) and 4-quinolylmethylamine (96) both have a greater binding affinity for the pea enzyme than cadaverine.

This result strengthens the suggestion by Barr[126] that the presence of a second primary amine group is not required for substrate binding, and that K_M values are dependent only on the initial interaction of one primary amine group with the pea enzyme and are independent of the nature of the second amine group.

From a comparison of the kinetic parameters of the hydroxy-2-pyridylethylamines and hydroxy-2-quinolylethylamines, it can be seen that although the rate of oxidation has decreased by about a half with the 2'- and 4'- quinoline derivatives, the binding affinities of the pyridine derivatives are only marginally lower than that of the quinoline derivatives. This is also the case when

comparing the kinetic parameters of the quinolylmethylamine substrates with those of pyridylmethylamines prepared and tested by Barr (Table 5.2).[126]

Substrates	K_M	V_{max}
2-pyridylmethylamine	0.04 (± 0.01)	8.6 (± 0.2)
3-pyridylmethylamine	0.40 (± 0.06)	19 (± 2)
4-pyridylmethylamine	0.06 (± 0.03)	10 (± 2)

Table 5.2- Results for pyridylmethylamines prepared and tested by Barr.[126] K_M values are in units of mM and V_{max} values are in units of $\mu mol\ mg^{-1}h^{-1}$.

It might be expected that the steric effect of the extra aromatic ring in the quinoline derivatives (81)-(83) and (94)-(96) would make binding to the active site of the enzyme more difficult. However, as seen from Tables 5.1 and 5.2, the decrease in binding affinity appears to be relatively small when comparing the quinoline derivatives with the corresponding pyridine derivatives. This result suggests that the active site cofactor for the pea seedling DAO is contained within an enzyme cavity which is large in nature and therefore can accommodate the larger ring size without affecting the binding.

Comparisons between the kinetic parameters of the methylamines as a group to that of the hydroxyethylamines as a group, show that the methylamines are generally oxidised at a faster rate and bind to the pea enzyme more efficiently than the hydroxyethylamines. Barr showed that the hydroxyl group has no influence on the catalytic process and therefore is not the cause of these differences.[126] A possible reason could lie in chain lengths of

the substrates. As discussed in Chapter 4.1 the highest rates of oxidation occur with linear diamines of carbon chain length 4-6. However, with these pyridine and quinoline derivatives, the highest rates appear to occur when the distance between the nitrogens is three carbons. From this result it seems unlikely that the creation of a cyclic conformation, as suggested by Equi *et al.*[96] for linear diamines, occurs in the catalytic process of these pyridine and quinoline derivatives. This suggests that although these aromatic substrates bind to the same active site as linear diamines, they may experience a different catalytic process.

5.5 Inhibition of Diamine Oxidase by Quinoline and Pyridine Derivatives

5.5 (a) Introduction

The assay system used for the inhibition studies was the same peroxidase-coupled procedure developed by Stoner *et al.* as we had used earlier.[89] The method was altered slightly by the addition of inhibitor to the assay mixture. Each study consisted of four experiments at various concentrations of inhibitor, the first of which was a blank experiment with no inhibitor present to provide a standard for comparison. The assay without inhibitor was initiated with the addition of enzyme immediately followed by substrate. In the assay with inhibitor present, the inhibitor was added after the addition of enzyme and before the addition of the substrate. This avoided oxidation of the substrate occurring before the addition of the inhibitor could take place.

The kinetics of inhibition were discussed earlier in Chapter 3. In competitive inhibition the intercept on the y-axis (V_{max}) of the

Lineweaver-Burk plot (1/V *vs.* 1/[S]) is independent of inhibitor concentrations, showing that V_{max} is unaffected by competitive inhibition. The difference in gradient between the plots with inhibitor and without inhibitor gives an indication of the efficiency in the binding of the substrate when the inhibitor is present. This can be represented by the equation,

$$K_M^*/K_M=1+[I]K_i \qquad (5.1)$$

where [I] is a fixed concentration of inhibitor, K_i is the inhibition constant and K_M^* is the apparent binding constant when a competitive inhibitor is present.

The apparent binding constant K_M^* was obtained at various concentrations of substrate at a fixed concentration of inhibitor [I] and then substituted into equation 5.1. This gave a value for the inhibition constant K_i which is a measure of the strength of the enzyme-inhibitor complex and thus shows the effectiveness of the particular inhibitor being studied. For these studies three different concentrations of inhibitor were used to calculate K_M^* values and K_i is the average of nine determinations.

There are many different types of inhibition, as discussed in Chapter 2, and each type has its own characteristics. Compounds which are oxidised by the pea enzyme at a slow rate but which bind well to the active site possess the necessary characteristics to be effective competitive inhibitors. Both the hydroxyquinolylethylamine substrates (81)-(83) and the quinolylmethylamine substrates (94)-(96) possess these characteristics and thus were chosen for our inhibition studies. We also repeated the inhibition studies of Barr on the hydroxypyridylethylamines (97)-(99).[126]

5.5 (b) Results and Discussion

Each system exhibited Michaelis-Menten kinetics when a fixed concentration of inhibitor was used in the peroxidase-coupled assay with putrescine as the substrate. The initial experiment in which putrescine was oxidised with no inhibitor present was followed by three further experiments in the presence of a fixed concentration of inhibitor. The concentrations chosen were determined by the binding affinity of the inhibitor and were generally equal to the K_M, 2 x K_M and 4 x K_M of the inhibitor. K_i values were calculated as discussed in Chapter 5.5 (a) and are reported as an average of nine determinations. Errors for K_i are quoted as an average of the computer generated errors calculated for each concentration of inhibitor.

Table 5.3 shows the K_i values in mM for the compounds studied. The lower the value of K_i the better the inhibition properties of the particular inhibitor.

Inhibitor	K_i
(±)-2-hydroxy-2-(2'-quinolyl)ethylamine (81)	2.09 (± 0.69)
(±)-2-hydroxy-2-(3'-quinolyl)ethylamine (82)	2.38 (± 0.84)
(±)-2-hydroxy-2-(4'-quinolyl)ethylamine (83)	2.99 (± 0.89)
(±)-2-hydroxy-2-(2'-pyridyl)ethylamine (97)	1.60 (± 0.46)
(±)-2-hydroxy-2-(3'-pyridyl)ethylamine (98)	1.93 (± 0.53)
(±)-2-hydroxy-2-(4'-pyridyl)ethylamine (99)	2.65 (± 0.85)
2-quinolylmethylamine (94)	1.02 (± 0.28)
3-quinolylmethylamine (95)	2.27 (± 0.77)
4-quinolylmethylamine (96)	1.53 (± 0.45)
2-pyridylmethylamine	0.32 (± 0.09)
3-pyridylmethylamine	1.55 (± 0.46)
4-pyridylmethylamine	0.54 (± 0.11)

Table 5.3- K_i values are reported in units of mM and are the values obtained when inhibitors were tested on the pea seedling DAO catalysed deamination of putrescine. Formulae numbers refer to the dihydrochloride salts of each substrate, the form in which they were tested. Samples of 2-, 3- and 4-pyridylmethylamine were synthesised and tested by Barr.[126]

All of the compounds tested were found to be competitive inhibitors of the pea seedling DAO oxidation of putrescine. The V_{max} of each oxidation was relatively unaffected by the presence of inhibitor, whereas the K_M values increased with increasing concentrations of inhibitor.

The quinolylmethylamines (94)-(96) had the lowest K_i values as a group apart from the pyridylmethylamines. With the quinolylmethylamines as inhibitors there was a notable reduction in the rate of oxidation of putrescine at low substrate concentrations. Although the reduction of the rate was not as great as that obtained for the pyridylmethylamines which were tested by Barr,[126] this can be explained by the differences in binding affinities. Since the pyridylmethylamines have lower K_M values (greater binding affinities to DAO) than those of the quinolylmethylamines, the pyridylmethylamines are more likely to show a greater affinity for the pea enzyme when competing against putrescine for the active site.

This difference in K_i values due to the binding affinities of the inhibitors can also be seen within the quinolylmethylamine group. 3-Quinolylmethylamine (95) had the lowest binding affinity for the pea seedling DAO and was found to be the poorest inhibitor of the DAO-catalysed oxidation. 2-Quinolylmethylamine (94) was the best inhibitor of the group and had the highest binding affinity for the pea enzyme. Another reason for the poor inhibition showed by 3-quinolylmethylamine (95) could lie in the fact that it is a better substrate for DAO than either 2-quinolylmethylamine (94) or 4-quinolylmethylamine; thus the overall rate of oxidation could be slightly increased by the oxidation of 3-quinolylmethylamine.

Comparison of the data obtained from study of the hydroxy-2-quinolylethylamines and the hydroxy-2-pyridylethylamines

show similar results to those of the quinolyl- and pyridyl-methylamines. 2-Hydroxy-2-(2'-pyridyl)ethylamine (97) and 2-hydroxy-2-(3'-pyridyl)ethylamine (98) are better inhibitors than the three hydroxy-2-quinolylethylamines (81), (82) and (83). Also the two compounds which had the lowest binding affinities for the pea DAO, 2-hydroxy-2-(4'-quinolyl)ethylamine (83) and 2-hydroxy-2-(4'-pyridyl)ethyl-amine (99) were the poorest inhibitors.

Looking at the data in Tables 5.1 and 5.3 obtained from the quinoline and pyridine derivatives, it can be seen that the extra steric bulk of the quinoline ring plays a part in the decrease of both substrate activity and inhibition properties. The fact that the steric bulk has less influence on the K_M and K_i values than the V_{max} values again suggests it is the catalytic process which is affected by the change in size and not the binding affinity.

CHAPTER 6

Oxidation of Substituted Thiophenes and Pyrroles By Pea Seedling Diamine Oxidase

6.1 Introduction

Our aim in this part of the study was to compare the binding affinities and rates of oxidation of substituted thiophenes and pyrroles with those of the pyridine and quinoline derivatives tested earlier. From these kinetic studies we could see if the change in electronic properties from a "π-deficient" aromatic ring to a "π-excessive" aromatic ring has a bearing on how the substrate binds to the enzyme, and if the rate of oxidation is affected by electronic effects. The steric effects of the different aromatic ring sizes can also be compared between the quinoline, pyridine and pyrrole derivatives.

A comparison of thiophene and pyrrole derivatives could help in the efforts to find out if the nature of the second amine group has an important role to play in the binding and oxidation of the substrate. The direct substitution of nitrogen in the pyrroles for sulphur in thiophenes means that we are able to see if a second amine is required for efficient binding and oxidation of the substrate.

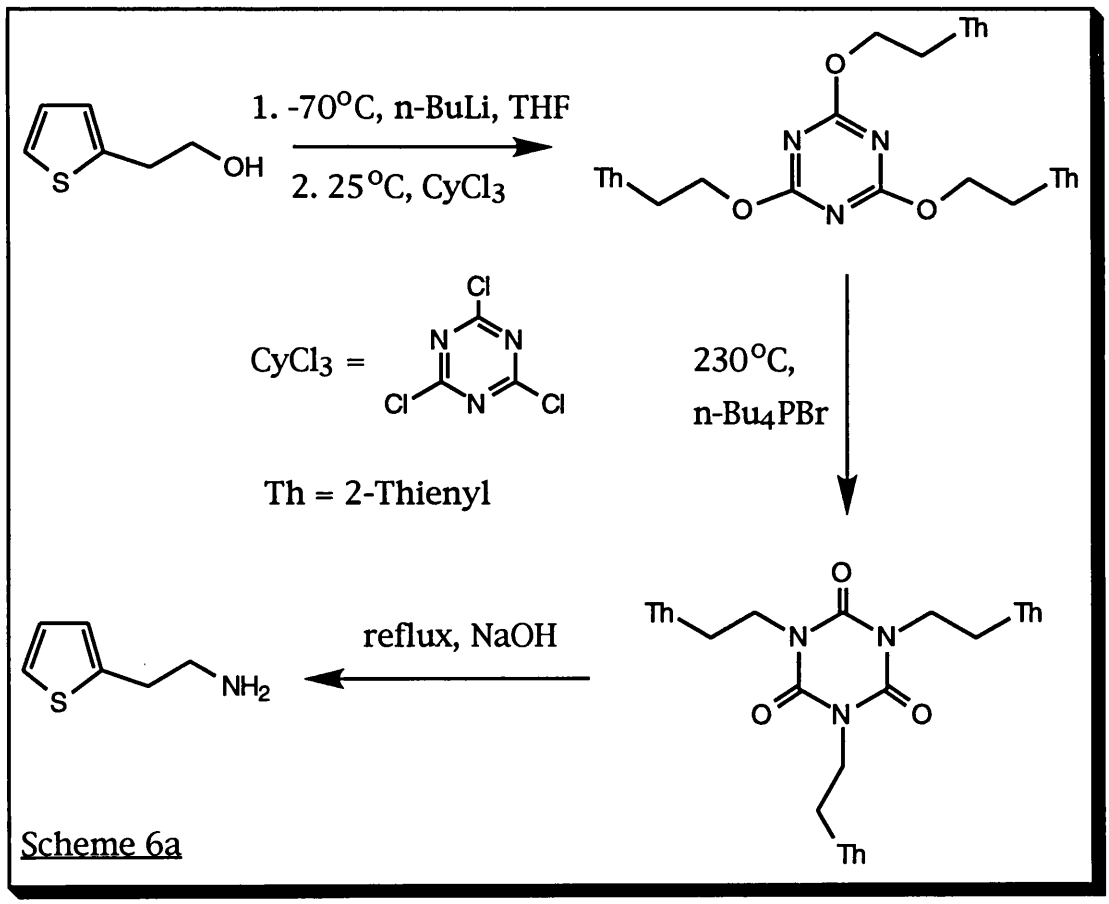
6.2 Synthesis of Substituted Thiophenes and Pyrroles

6.2(a) Synthesis of Substituted Thiophenes

2-(2'-Thienyl)ethylamine is a very important component in a number of different biologically active materials.[133] These materials

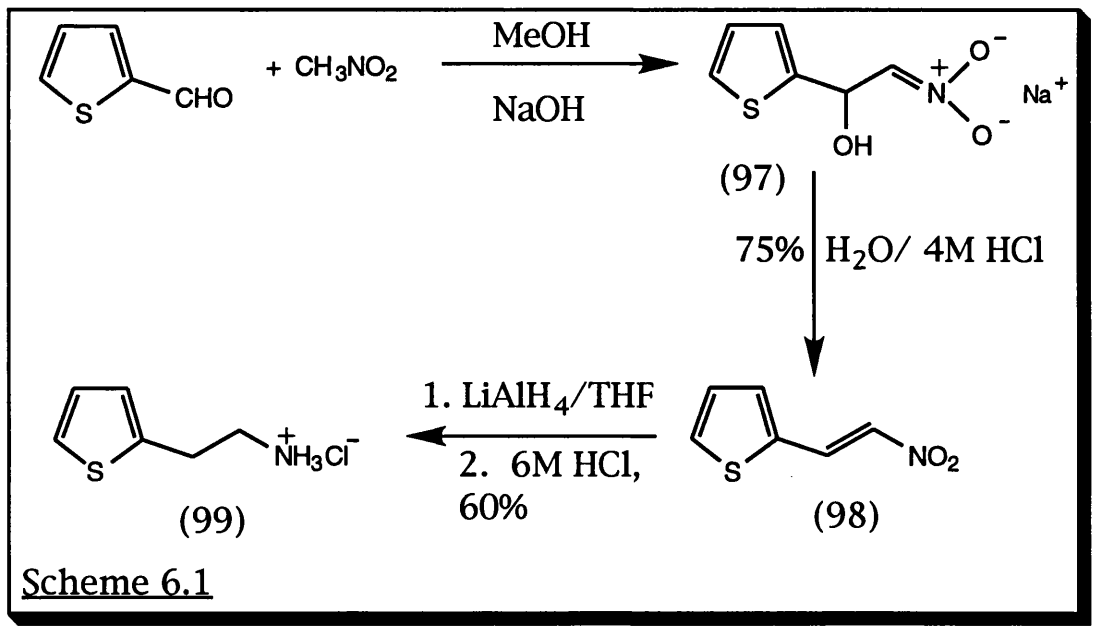
possess antibacterial,[134] antifungal and anti-hypertensive[135] activity among others. Due to this interest there has been a number of different synthetic routes described for the synthesis of 2-(2'-thienyl)ethylamine.

The most recent route was by Harrington and Sanchez[133] in 1993 which was based on the oxygen to nitrogen migration of a thienylalcohol linked to a cyanuric ring (Scheme 6a). Base hydrolysis of the isocyanurate and subsequent decarboxylation liberated the thienylamine. However, this method was not convenient due to the high temperatures that were used in the migration stage of the reaction. Instead we decided to use an older method developed by King and Nord in 1949[136] which is based on a similar reaction of an aldehyde to that used for the synthesis of the substituted pyridines and quinolines in Chapter 5.



Scheme 6a

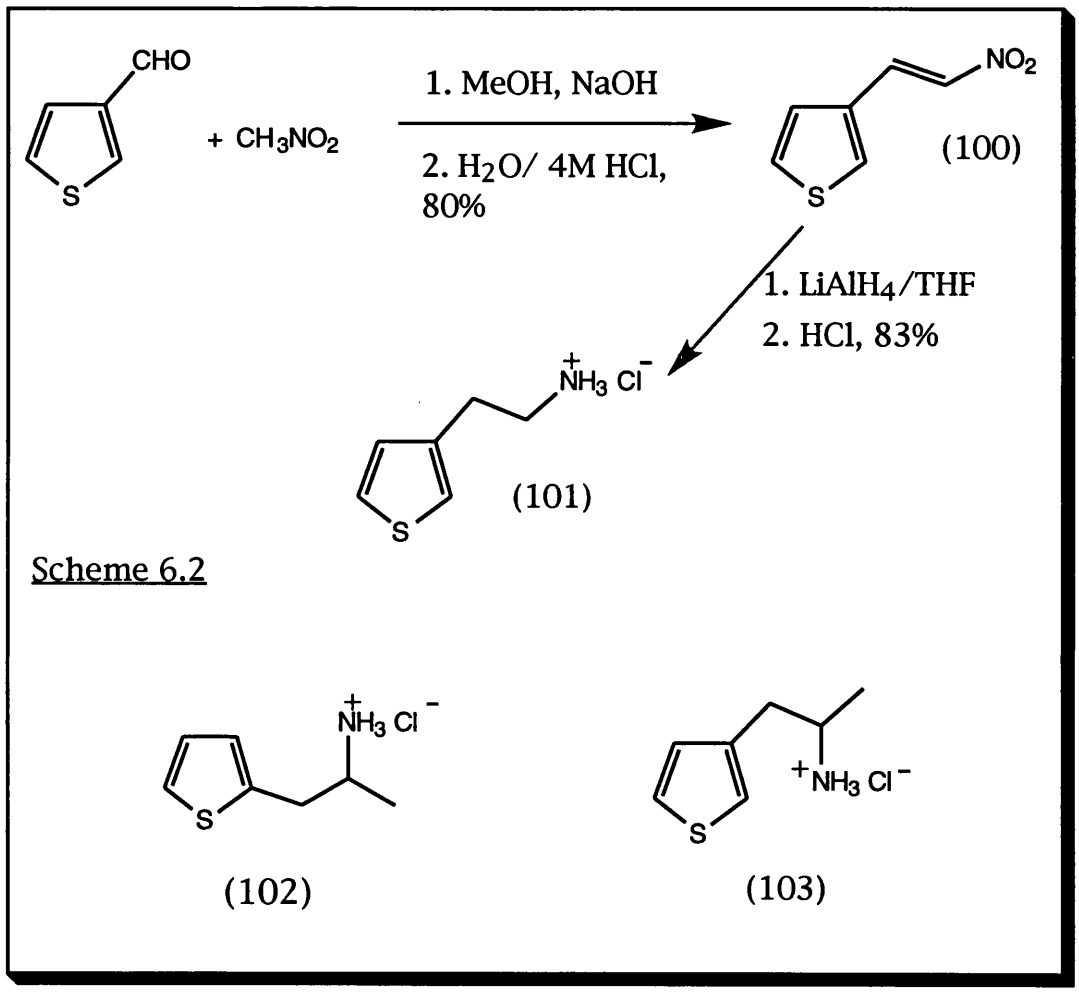
The starting material for this synthesis of substituted thiophenes was the corresponding thiophenecarboxaldehyde and again either nitromethane or nitroethane. Reaction of 2-thiophenecarboxaldehyde and nitroethane, with sodium hydroxide as the base, gave the sodium salt of the 2-thiophenenitroalcohol intermediate (97) (Scheme 6.1). Treatment with ice/water and 4M HCl caused dehydration of intermediate (97) to give the unsaturated compound 2-(2'-thienyl)nitroethene (98) as yellow crystals in 75% yield.[136] Reduction of 2-(2'-thienyl)nitroethene (98) with 1M $LiAlH_4$ in THF gave a crude amine which was partitioned between dichloromethane and 6M HCl to produce 2-(2'-thienyl)ethylamine (99) as the hydrochloride salt in 60% yield (Scheme 6.1).



Scheme 6.1

Using the same procedure 3-thiophenecarboxaldehyde was converted into 2-(3'-thienyl)nitroethene (100) and subsequently reduced with $LiAlH_4$ to give 2-(3'-thienyl)ethylamine hydrochloride (101) in an overall yield of 81.5% (Scheme 6.2). Similarly, 2- and 3-thiophenecarboxaldehyde were condensed with

nitropropane and the nitroalkenes were then reduced to give (±)-3-(2'-thienyl)propyl-2-amine (102) and (±)-3-(3'-thienyl)propyl-2-amine (103), respectively. Both products were isolated as their hydrochloride salts and as racemic mixtures.



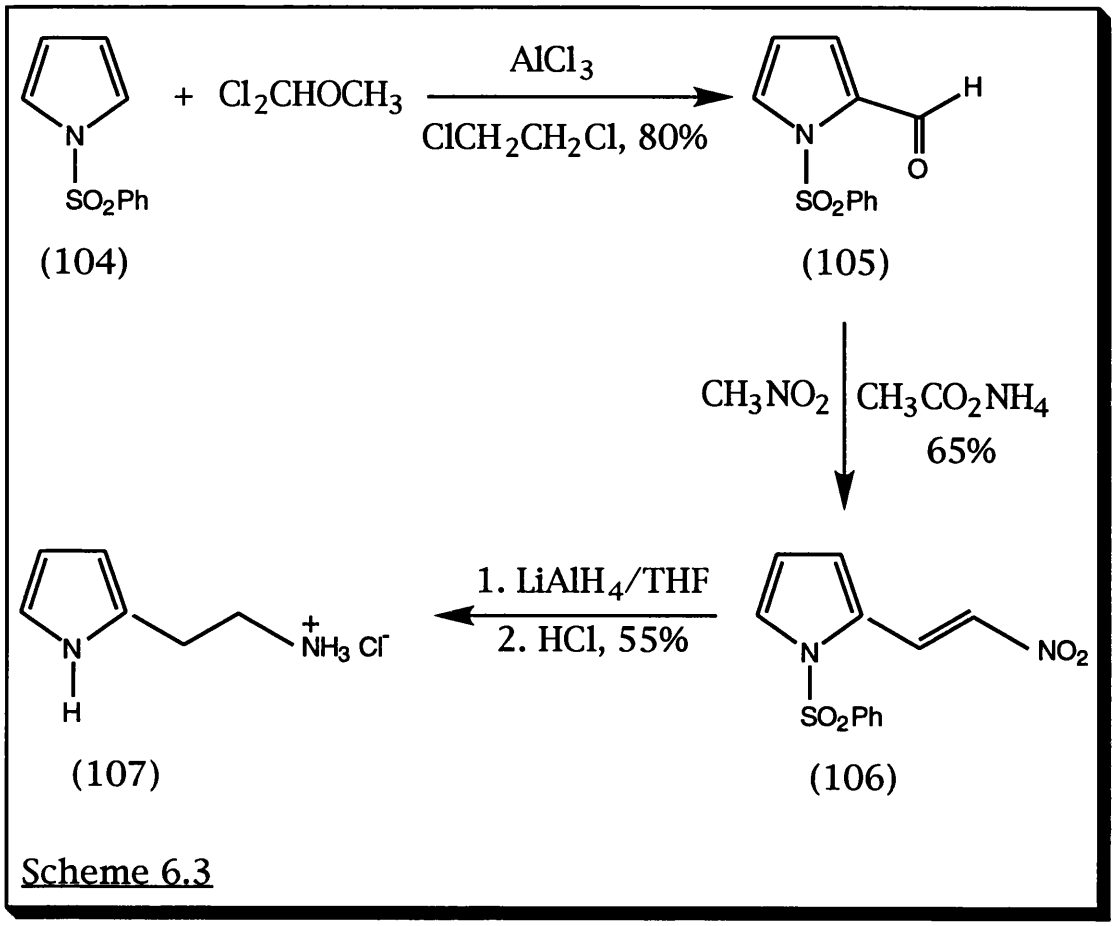
Scheme 6.2

6.2(b) Synthesis of Substituted Pyrroles

In order to make the alkylpyrroles required for our studies we first had to make the pyrrolecarboxaldehydes which would be condensed with the nitroalkanes. The regioselective synthesis of pyrrole derivatives has been the subject of much work in past years.[137,138] The regioselective introduction of 2-acyl substituents into pyrroles can be achieved by a number of common routes, for example Vilsmeier-Haack formylation[139] or direct electrophilic

substitution.[140] However, this is not the case for synthesis of 3-acyl derivatives which has proved to be more difficult. Typical routes involve the introduction of an electron withdrawing group at C-2 followed by electrophilic substitution at C-4 and subsequent removal of the 2-substituent.[141, 142] Some 1-alkyl-3-acylpyrroles were also obtained in moderately good yields by acid catalysed isomerisation of the corresponding 2-substituted pyrroles.[143] A more recent synthesis by Kakushima *et al.*[144] involving an $AlCl_3$-catalysed Friedel-Crafts acylation of 1-(phenylsulphonyl)pyrrole (104) was carried out with high yields and high regioselectivity at both the 2- and the 3-position of pyrrole. However, attempts by Kakushima *et al.* to apply their method to introduce formyl groups selectively had failed.

We adopted the method of Kakushima *et al.*[144] to make 1-(phenylsulphonyl)pyrrole-2-carboxaldehyde (105) (Scheme 6.3). Using 1-(phenylsulphonyl)pyrrole (104) and 1,1-dichloromethyl methyl ether in a Friedel-Crafts acylation gave (105) as white needles after recrystallisation in 80% yield. Condensation of nitromethane with 1-(phenylsulphonyl)pyrrole-2-carboxaldehyde (105) in the presence of ammonium acetate crystals gave 1'-(phenylsulphonyl)-2-(2'-pyrrolyl)nitroethene (106) in 65% yield.[178] Reduction of (106) by 1M $LiAlH_4$ in THF and subequent addition of HCl to the reaction mixture gave 2-(2'-pyrrolyl)ethylamine hydrochloride (107) in 55% yield (Scheme 6.3).

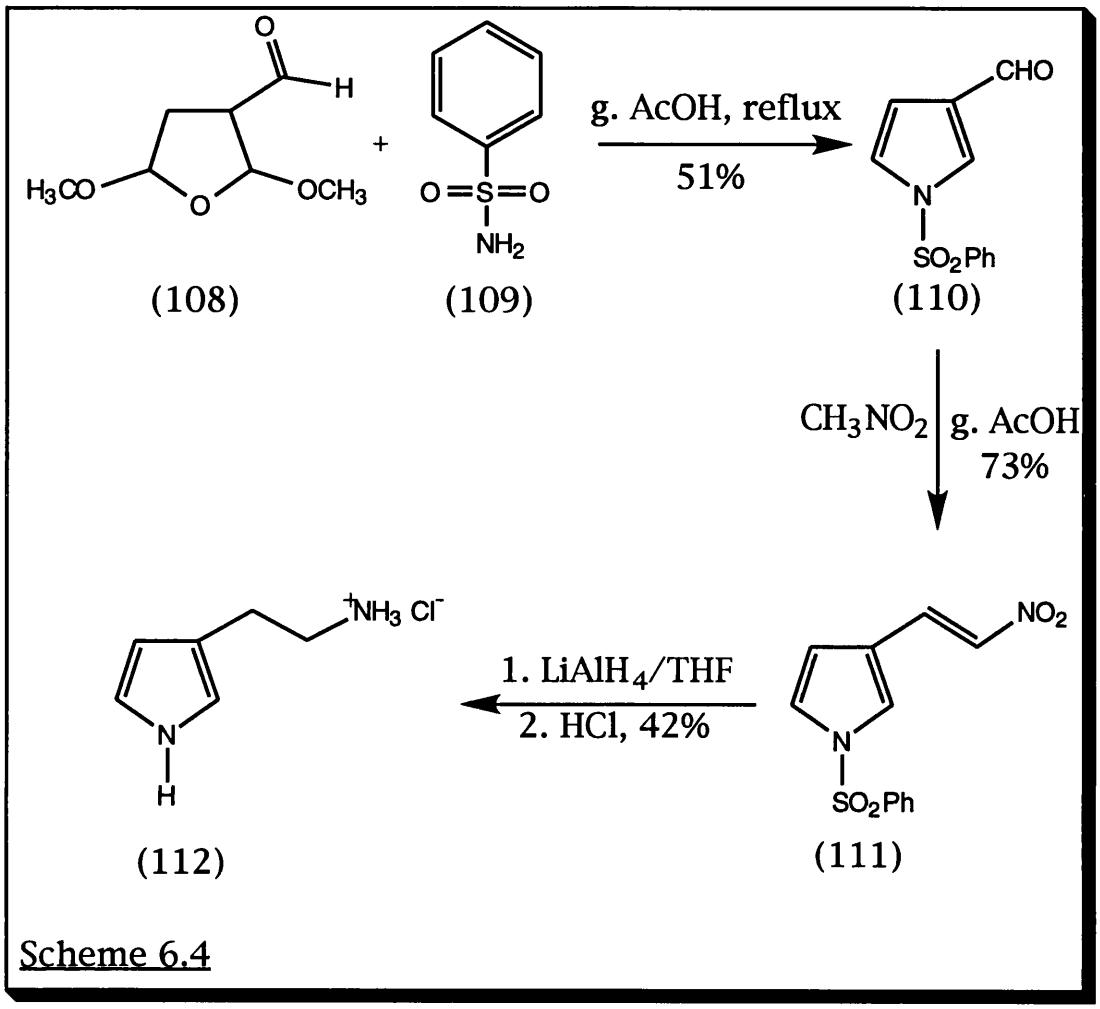


Scheme 6.3

The Clauson-Kaas method[145] is used to convert 2-acyl-2,5-dimethoxytetrahydrofurans into 2-acyl-1-phenylpyrroles. We used the method of Hamdan and Wasley[179] who adapted this procedure, starting with 2,5-dimethoxy-3-formyltetrahydrofuran (108) and heating at reflux with benzenesulphonamide (109) and glacial acetic acid to give the 3-acyl product, 1-(phenylsulphonyl)pyrrole-3-carboxaldehyde (110) in 51% yield (Scheme 6.4). Condensation of (110) with nitromethane in the presence of glacial acetic acid gave 1'-(phenylsulphonyl)-2-(3'-pyrrolyl)nitroethene (111) as yellow crystals in 73% yield. Reduction of (111) using 1M LiAlH4 in THF produced a crude amine, which after treatment with HCl gave 2-(3'-pyrrolyl)ethylamine hydrochloride (112) in 42% (Scheme 6.4).

Condensation of 1-(phenylsulphonyl)pyrrole-2-carboxaldehyde (105) and 1-(phenylsulphonyl)pyrrole-3-carboxaldehyde

(110) with nitroethane failed to produce the corresponding pyrrolylnitropropenes.



Scheme 6.4

6.3 Results and Discussion

6.3 (a) Substituted Thiophenes and Pyrroles as Substrates for Pea Seedling DAO

Using the same spectrophotomeric assay method as was used with the quinoline and pyridine derivatives, i.e. Stoner's method,[89] we obtained kinetic parameters for the DAO oxidative deamination of each substrate. The data are summarised in Table 6.1.

Experiments were carried out three times using each substrate and the data are quoted as an average of the nine determinations. Errors are quoted as an average of computer generated errors calculated from nine determinations of kinetic data.

Substrate	K_M	V_{max}
Putrescine (3)	1.21 (± 0.40)	1157 (± 200)
Cadaverine (6)	0.23 (± 0.06)	2325(± 390)
2-(2'-thienyl)ethylamine (99)	0.42 (± 0.09)	5.2 (± 0.3)
2-(3'-thienyl)ethylamine (101)	0.66 (± 0.1)	3.3 (± 0.2)
2-(2'-pyrrolyl)ethylamine (107)	0.35 (± 0.06)	18 (± 3)
2-(3'-pyrrolyl)ethylamine (112)	0.49 (± 0.09)	15 (± 2)

Table 6.1- Formulae numbers refer to the hydrochloride and dihydrochloride salts for thienyl and pyrrolyl substrates respectively, the form in which they were tested. K_M values are in units of mM and V_{max} values are in units of μmol $mg^{-1}h^{-1}$.

As observed with the corresponding quinoline and pyridine derivatives, (±)-3-(2'-thienyl)propyl-2-amine (102) and (±)-3-(3'-thienyl)propyl-2-amine (103) were found to be very poor substrates of pea seedling DAO. This meant they did not display classical Michaelis-Menten behaviour and we were unable to make accurate determinations of K_M and V_{max}.

Again all of the compounds tested were oxidised by the pea enzyme at a rate which was much slower than that of the natural substrates. The binding affinities for all of the active substrates

were lower than that of cadaverine, but higher than that of putrescine. As with the pyridyl substrates (97)-(99) and quinolyl substrates (81)-(83) and (94)-(96), the optimum distance between heteroatoms for highest oxidation rate appears to be three carbons. 2-(2'-Thienyl)ethylamine (99) has a slightly higher rate of oxidation than 2-(3'-thienyl)ethylamine (101) and 2-(2'-pyrrolyl)ethylamine (107) has about the same rate as 2-(3'-pyrrolyl)ethylamine (112). This is again in contrast to the optimum chain length of four to six carbons for linear primary diamines.

It is clear from the data in Table 6.1 that the rate of oxidation of the pyrrolyl substrates is approximately four times that of the thienyl substrates. However, the binding affinities of the pyrrolyl substrates are about the same as those of their thienyl counterparts. This shows that although the presence of a second nitrogen is important in the overall catalytic process, the absence of a second nitrogen has little affect on the binding of the substrate to the active site of the pea enzyme. Because a second nitrogen is not required for efficient binding to the DAO enzyme, this opens up many new areas for study into potential inhibitors of DAO with the chance of finding biologically active compounds.

From a comparison of the kinetic parameters in Tables 5.1 and 6.1 it is also clear that the pyrrolylethylamines are oxidised by pea DAO about ten times faster than the rates of oxidation for hydroxy-2-quinolylethylamines and approximately four times faster than the rates of oxidation for hydroxy-2-pyridylethylamines. The reason for these differences in oxidation rates could be due to either the decrease in steric bulk going from quinolyl substrates to pyrrolyl substrates, or it may be due to the change in electronic properties from a "π-deficient" aromatic ring to a "π-excessive" aromatic ring. However, since there is also a general

decrease in oxidation rate when comparing the pyridyl substrates with the quinolyl substrates, it seems more likely that the reduction in steric bulk plays a larger part in the increased oxidation rate of the pyrrolyl substrates than the electronic properties of its aromatic ring.

6.3 (b) Substituted Thiophenes and Pyrroles as Inhibitors of Pea Seedling DAO

The assay system used for the inhibition studies was a modified version of Stoner's method[89] and was the same method used for inhibition studies of the quinoline and pyridine derivatives. Each system exhibited Michaelis-Menten kinetics for the peroxidase-coupled assay with putrescine as the substrate. K_i values were calculated as discussed in Chapter 5.5 (a) and are reported as an average of nine determinations. Errors for K_i are quoted as an average of the computer generated errors calculated for each concentration of inhibitor.

Inhibitor	K_i
2-(2'-thienyl)ethylamine (99)	1.43 (± 0.42)
2-(3'-thienyl)ethylamine (101)	1.91 (± 0.55)
2-(2'-pyrrolyl)ethylamine (107)	1.33 (± 0.36)
2-(3'-pyrrolyl)ethylamine (112)	1.56 (± 0.48)

Table 6.2- K_i values are reported in units of mM and are the values obtained when inhibitors were tested on the pea seedling DAO catalysed deamination of putrescine. Formulae numbers refer to the dihydrochloride salts of each substrate, the form in which they were tested.

As with the quinoline and pyridine derivatives, the thiophene and pyrrole derivatives were found to be competitive inhibitors of the DAO oxidation of putrescine.

There was a notable reduction in the rate of oxidation of putrescine at low substrate concentrations for all the substrates tested. The best results were achieved for 2-(2'-pyrrolyl)ethylamine (107) and 2-(2'-thienyl)ethylamine (99). This result was to be expected since the binding affinities of these two substrates were higher than those of 2-(3'-pyrrolyl)ethylamine (112) and 2-(3'-thienyl)ethylamine (101). It is also worth noting that the K_i values for these compounds may have been affected by their own oxidation during the assay procedure. This means they may have lower K_i values than those recorded.

Comparison of the kinetic data in Tables 5.3 and 6.2 shows that overall the methylamines are the best inhibitors of all the substrates tested. Their high binding affinity with the pea enzyme would account for this result. The reason behind their higher binding affinities and thus their increased inhibition properties appears to be the smaller side arm of the substituent methyl group compared to the ethyl group. Due to time restraints we were unable to synthesise and test the thienyl- and pyrrolyl-methylamines. By considering previous results these compounds are likely to be better inhibitors than the corresponding thienyl- and pyrrolyl-ethylamines.

CHAPTER 7

Use of Diamines in the Synthesis of Cisplatin Analogues

7.1 Introduction

The classic coordination complex *cis*-diamminedichloroplatinum (II) (113), better known as cisplatin, has been the subject of much attention over recent years because of its activity towards certain types of tumours.[146, 147] Details of the mechanism of action of this antitumour drug are still not fully understood, but there is evidence to indicate that the biological activity is due to binding of cisplatin to DNA thus inhibiting replication.

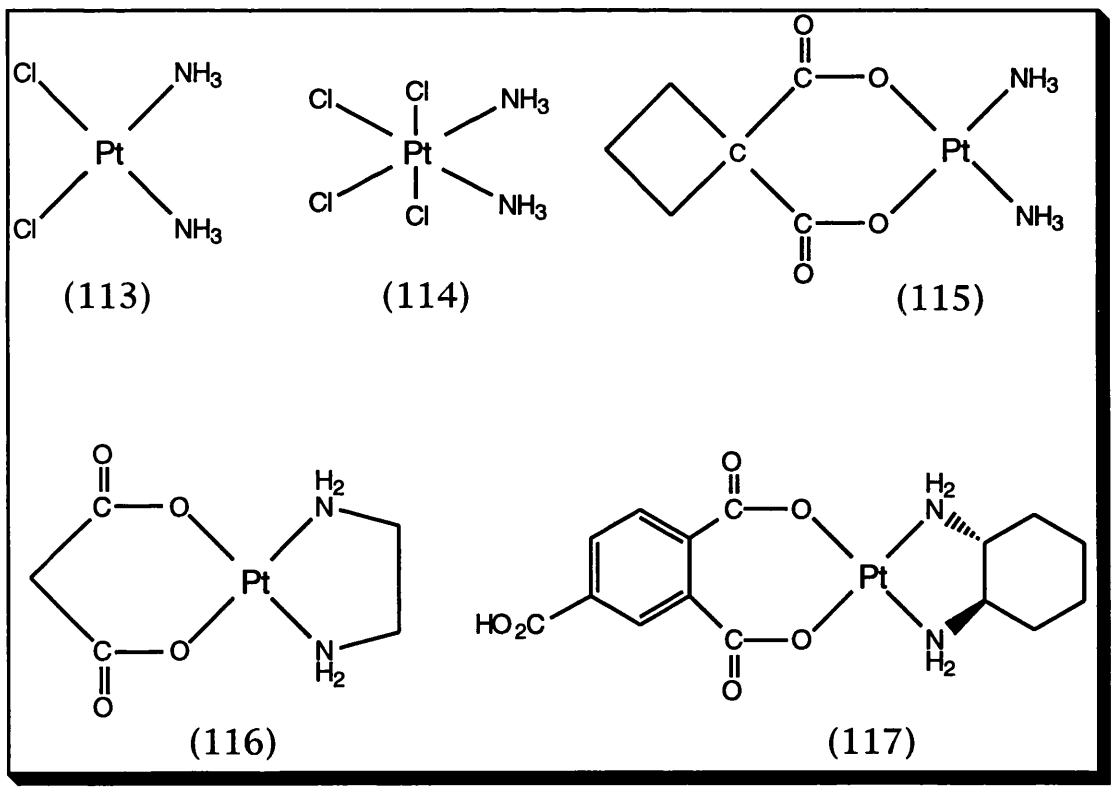
During studies in the early 1960s, Rosenberg *et al.*[148] noticed a curious phenomenon when an electric field was applied across platinum electrodes immersed in *E. coli* cells which were growing in the presence of ammonium chloride. The bacteria did not divide normally but grew into filaments up to 300 times their normal length. An electrolysis product from the electrodes, shown to be *cis*-$[Pt(NH_3)_2Cl_4]$ (114), was responsible for this effect.[149] Subsequently, other platinum complexes, including cisplatin, were found to induce filamentous growth in bacteria.

It was suggested that since these active platinum compounds suppressed cell division without killing the bacteria, then they may halt the rapid growth of tumour cells with little toxicity to the host animal.[150,151] On testing cisplatin was found to be particularly active against a wide variety of tumours and clinical trials were subsequently started.

Now one of the most widely used anticancer drugs in the world, both by itself and in combination chemotherapy,[147] cisplatin

has been used successfully in the treatment of bladder, lung, head and neck, and especially testicular and ovarian cancers.[152,153] However, severe toxic side effects from cisplatin have limited its use in chemotherapy.

Efforts to reduce toxicity and to give a broader range of therapeutic activity led to *cis*-diammine(1,1-cyclobutane-dicarboxylato)platinum (II) (115), known commonly as carboplatin, which has similar activity to that of cisplatin, but is less toxic.[154] Other second generation drugs include malonatoplatinum (116) and DACCP (117).



7.2 Mechanism of Action of Cisplatin

Both cisplatin and the corresponding *trans*-isomer of diamminedichloroplatinum (II) have two labile chloride ligands and two amine ligands that are inert to substitution under biological conditions.[155] However, *trans*-diamminedichloroplatinum (II) is

inactive, as are the *trans*-isomers of most of the biologically active platinum complexes. Monofunctional complexes, for example chloro-diethylenetriamineplatinum (II), containing just one labile ligand are also inactive towards most tumours. These findings suggest that chemical reactions of the platinum complexes responsible for antitumour activity require bifunctional attachment to biological molecules.

Other agents which produce filamentous growth in bacteria, such as hydroxyurea and UV radiation, are known to inhibit DNA synthesis.[156] Thus, a similar mechanism was suggested for platinum antitumour compounds. This theory was strengthened by experiments measuring the rates of synthesis of DNA and RNA in cells treated with cisplatin by monitoring the incorporation of radiolabelled precursors. At therapeutic doses of cisplatin, DNA synthesis was preferentially inhibited over RNA and protein synthesis.[157,158] The inactive *trans*-isomer inhibited DNA synthesis to only a minor extent at similar doses. These results suggest that selective inhibition of DNA synthesis is responsible for cisplatin's antitumour activity. Specifically, cisplatin is thought to bind directly to DNA, rendering it unsuitable as a template for replication.

Possible reasons behind the inactivity of *trans*-isomers and the activity of the *cis*-isomers are thought to be due to differential cellular uptake rates for the two isomers and/or repair of their DNA adducts. Another possibility is that adducts formed on DNA by *cis*-isomers are inherently more effective at inhibiting replication than those of the *trans*-isomers.

7.3 Interactions of Platinum Compounds with DNA

The surface of the DNA double helix is characterised by major and minor grooves and is composed of repeating deoxyribose phosphodiester units attached to alternating purine bases, adenine and guanine, and pyrimidine bases, cytosine and thymine. The double helix is stabilised by hydrogen bonds between guanine and cytosine and between thymine and adenine (Figure 7.1). The major groove of DNA is at the top of each base pair and the minor groove is at the bottom.

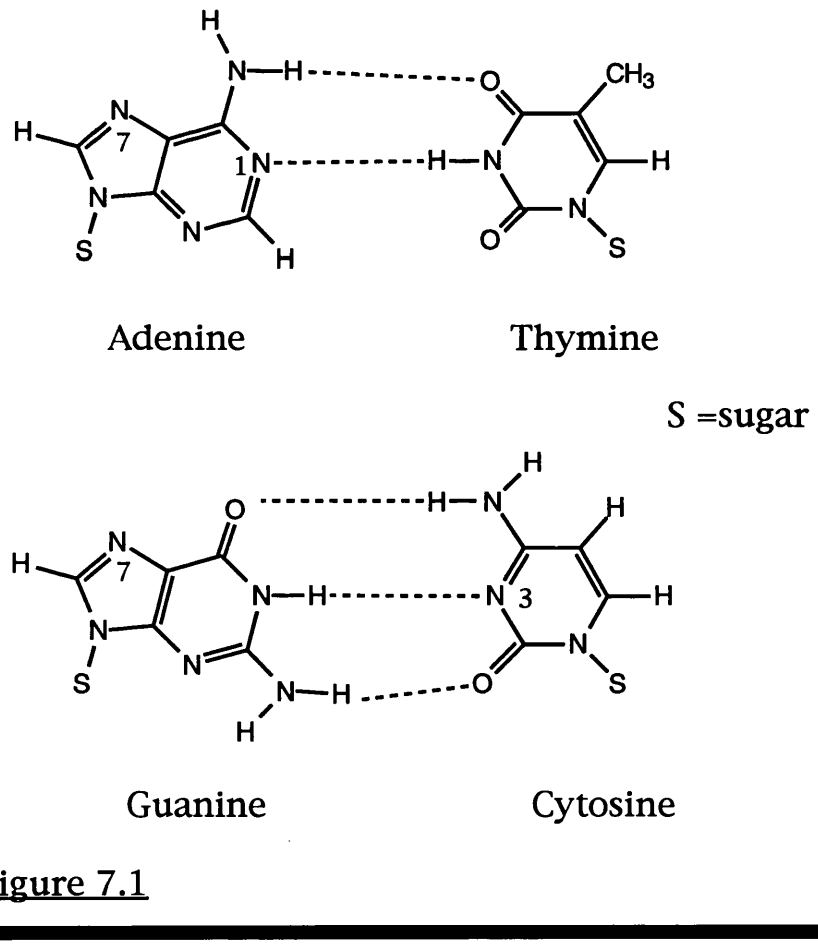


Figure 7.1

Under neutral conditions, platinum binds to the N-7 atom of guanine, the N-7 and N-1 atoms of adenine, and the N-3 atom of cytosine (Figure 7.1).[159] The atoms which are involved in base

pairing, i.e. N-1 of adenine and N-3 of cytosine are less available for binding than the more exposed sites in the grooves. Also electrostatic potential calculations indicate that the guanine carbonyl group enhances the basicity of the N-7 atom, while the amino group in adenine reduces the relative basicity of the N-7 atom.[160,161] In conclusion the N-7 atom of guanine is exposed on the surface of the major groove making it very accessible to platinum binding, and it is also the most basic nitrogen relative to the other bases.

Once the platinum has bound to a base there are several different ways in which bifunctional binding can take place. Bifunctional binding to two sites on a single base, cross-links between two bases on opposite strands of the helix, DNA-protein cross-links and intrastrand cross-links between two bases on the same DNA strand have all been proposed as being responsible for the antitumour activity of cisplatin. Interstrand cross-linking of DNA was thought to be the most likely of these binding modes to explain the activity of cisplatin.[162] However, during experiments to correlate interstrand cross-linking with cytotoxicity of cisplatin, none of these studies measured the amount of intrastrand cross-linking among the cisplatin-DNA adducts.[163] Also long term studies of cancer patients suggested that patients who benefited from cisplatin treatment had measurable intrastrand cross-links compared with patients who were not cured with cisplatin.[164] It has been suggested that the intrastrand cross-linking affects the DNA base sequences, thus inhibiting DNA replication, leading to cell death.[165] Further work on the chemical and structural nature of DNA cross-linking with platinum compounds is required to help find the cause of cisplatin antitumour activity.

7.4 Synthesis of Cisplatin Analogues

7.4(a) Introduction

A cisplatin analogue can be described by a structure (Figure 7.2), where X is a leaving group and A is an amine or other firmly bound ligand. The X group is mainly responsible for solubility of the complex. When A is kept unchanged, the nature of X determines the rate of its substitution, and thus the antitumour effectiveness of the analogue.[166,167] Useful antitumour properties are normally associated with intermediate lability of the Pt-X bonds. Complexes with highly labile ligands are very toxic, while strongly bound ligands give rise to kinetically inert complexes. The exception to this rule is the bidentate dicarboxylate ligands. Also, the lability of a leaving group depends, in part, on the nature of the ligand *trans* to it (due to the "*trans* -effect").[168]

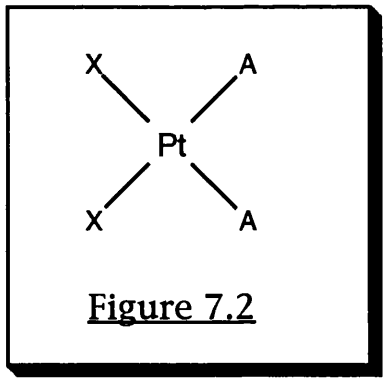


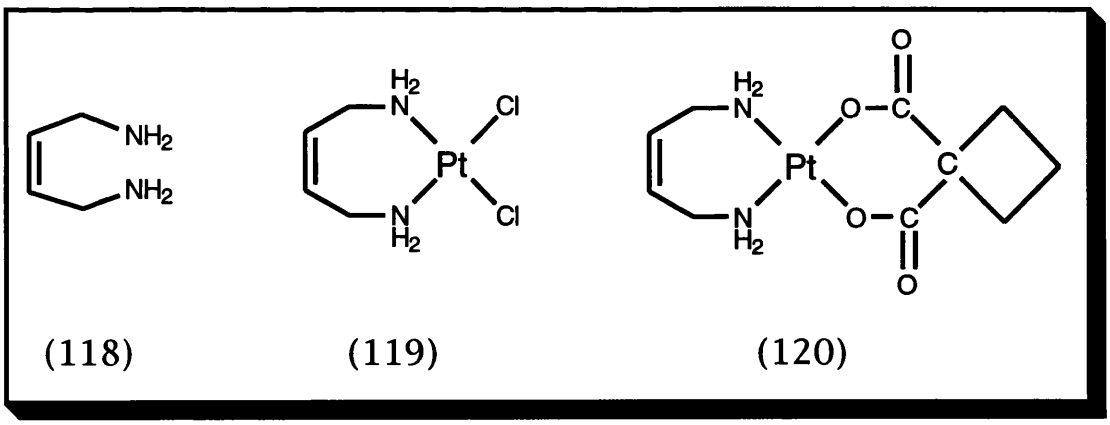
Figure 7.2

The role of the two A groups is less clear, but since they are relatively difficult to replace it is assumed that they accompany the platinum atom to the biological target. This means that they represent a major factor in governing the pharmacokinetic behaviour, the penetration into the cells and the interaction with

DNA. A number of non-leaving groups have been used, mainly amines, from simple ammonia to cyclic aliphatic amines.

Few cisplatin analogues incorporating linear diamines had been reported, and these were mainly restricted to 5- or 6-membered rings when bidentately bonded to platinum. Nowatari prepared some 7-membered chelates and several of these, particularly those containing substituted putrescines, showed high therapeutic ratios towards L1210 cells in mice.[169]

The aim of our work was to use putrescine derivatives which had already been shown to possess antibiotic activity as therapeutic ligands in platinum complexes. With no examples of unsaturated diamines being used in platinum complexes, we decided to use *cis*-1,4-diaminobut-2-ene (118) (unsaturated putrescine) as a starting point for our synthesis of cisplatin analogues. Our target compounds were therefore, *cis*-1,4-diaminobut-2-ene(dichloro)platinum (II) (119) and *cis*-diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)-platinum (II) (120).



(118) (119) (120)

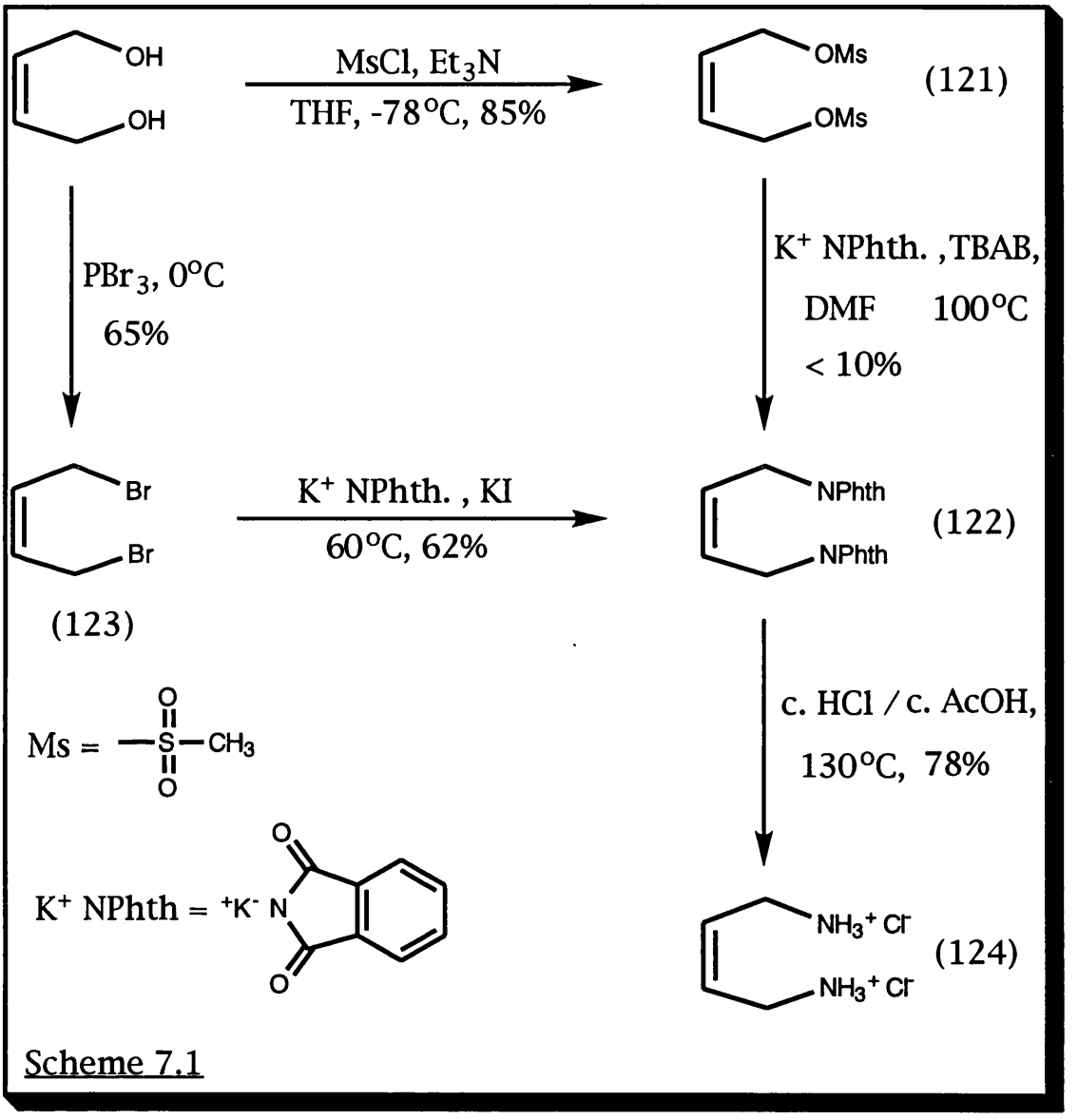
7.4(b) Synthesis of *cis*-1,4-Diaminobut-2-ene

Previous syntheses of putrescine and cadaverine derivatives had been carried out within our research group by Equi[125] by converting dimesylate compounds into diazides, and then catalytic reduction of the diazides to yield the diamines. However, in this work we used a more convenient method which did not involve the use of unstable azides.

Methanesulphonyl chloride was added to a solution of *cis*-but-2-ene-1,4-diol in THF in the presence of triethylamine to give *cis*-1,4-dimethylsulphonylbut-2-ene (121) in 85% yield (Scheme 7.1). Reaction of dimesylate (121) with potassium phthalimide gave *cis*-diphthalimidobut-2-ene (122) in very poor yields of less than 10% (Scheme 7.1). Problems with the insolubility of potassium phthalimide were slightly improved by using the phase transfer catalyst tetramethylammonium bromide, however this failed to improve the yield. This reaction was carried out at 100 ^{o}C and since the dimesylate (121) was unstable and decomposed quickly at room temperature it is likely that the high temperature of reaction was responsible for the poor yields.

We then used the method of Feigenbaum and Lehn,[170] which again uses potassium phthalimide, but starts with a dibromide. To make the dibromide, phosphorus tribromide was stirred with *cis*-but-2-ene-1,4-diol at 0 ^{o}C giving *cis*-1,4-dibromobut-2-ene (123) as a colourless oil in 81% yield (Scheme 7.1). Treatment of dibromide (123) with potassium phthalimide and a catalytic amount of potassium iodide gave *cis*-diphthalimidobut-2-ene (122) in 62% yield. Cleavage of the phthalimide groups was achieved by heating at reflux with a 1:1 mixture of concentrated HCl and glacial

acetic acid to give *cis*-1,4-diaminobut-2-ene in 78% yield as the dihydrochloride salt (124).



Scheme 7.1

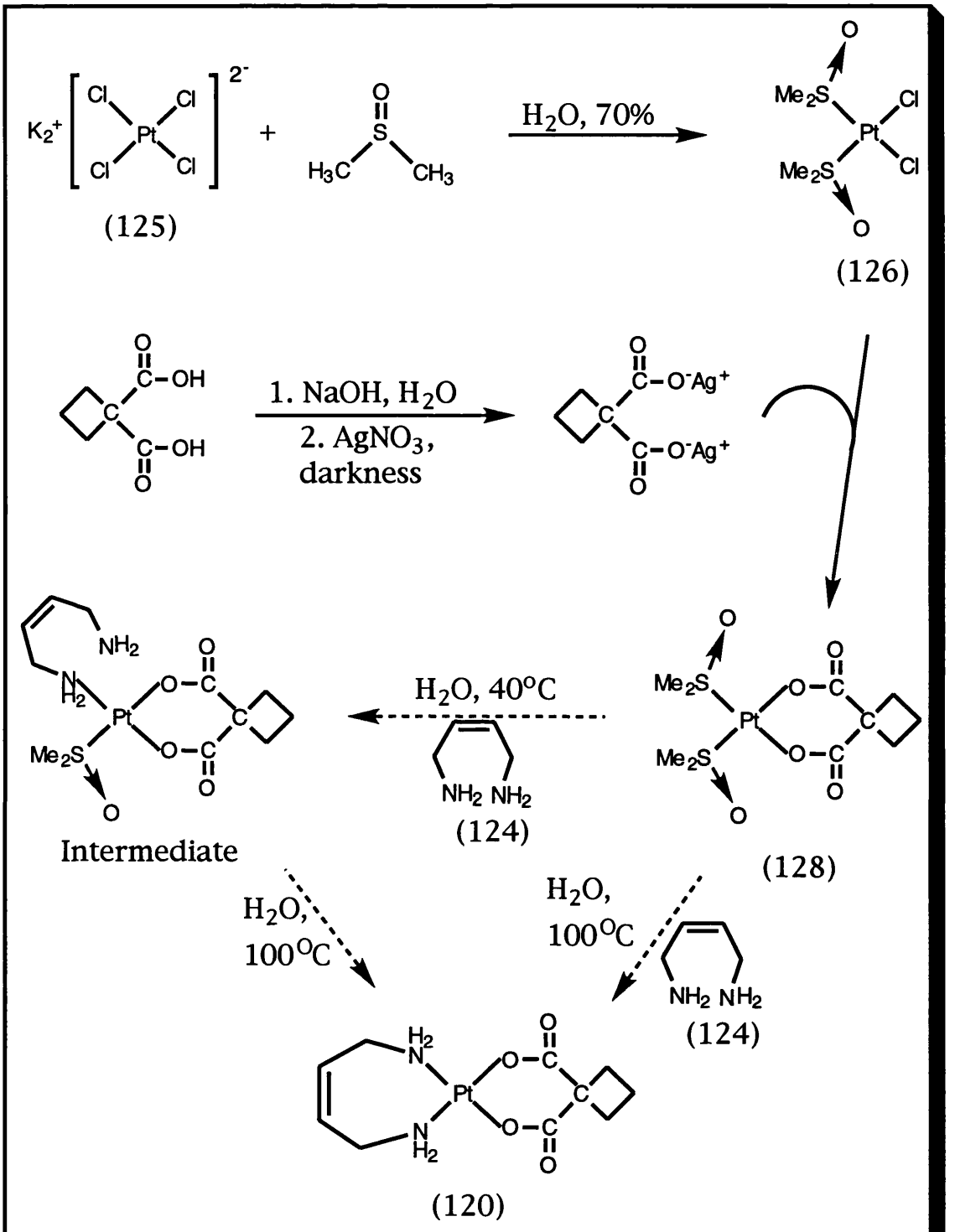
7.4(c) Synthesis of Platinum Precursors and Cisplatin Analogues

To make the cisplatin analogues there are two or three common platinum complexes which are all combined with diamines by various methods to yield the analogues. The common starting material for these precursors is potassium tetrachloroplatinate (II) (125).

An aqueous solution of potassium tetrachloroplatinate (125) and dimethylsulphoxide was allowed to stand until the solution turned yellow and yellow crystals appeared. These yellow crystals were filtered then washed with water to give *cis*-dichloro-bis-(dimethylsulphonyl)platinum (II) (126) in 70% yield (Scheme 7.2).[171] The silver salt of 1,1-cyclobutanedicarboxylic acid (127) was prepared by stirring 1,1-cyclobutanedicarboxylic acid with NaOH resulting in the sodium salt, which was subsequently stirred in darkness with $AgNO_3$ for 24 hours.

Reaction of *cis*-dichloro-bis-(dimethylsulphonyl)platinum (II) (126) with the silver salt of 1,1-cyclobutanedicarboxylic acid (CBDC) (127) in darkness for 24 hours gave the platinum complex *cis*-bis-dimethylsulphonyl(1,1-cyclobutanedicarboxylato)platinum(II) (128) as colourless crystals in 59% yield (Scheme 7.2). Two attempts were then made to complex (128) with *cis*-1,4-diaminobut-2-ene (124). The first attempt was to add the diamine (124) to a hot aqueous solution of platinum precursor (128) and stir the mixture at reflux.[172] The resulting product was a black solid which appeared to be the platinum complex *cis*-1,4-diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)platinum(II) (120) from the infra-red and mass spectra (Scheme 7.2). However, this solid contained platinum metal impurities and was insoluble in all common solvents making purification extremely difficult.

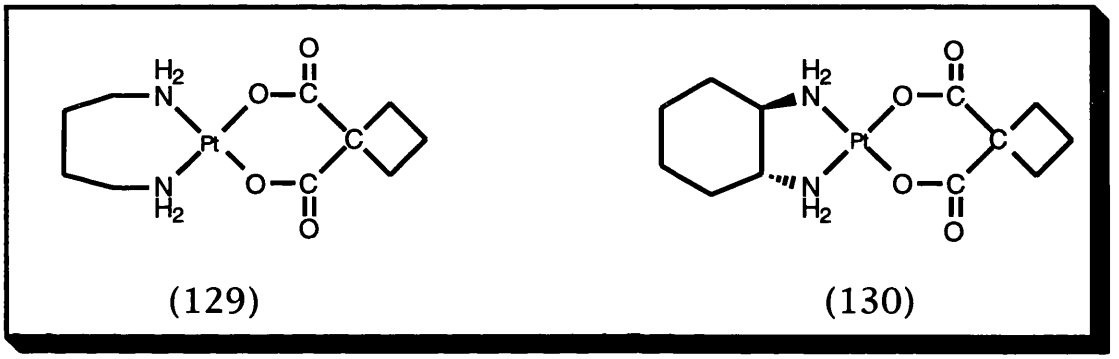
The second attempt was to heat the platinum precursor (128) with diamine (124) at 40 ^{o}C. This should form a monodentate intermediate[172] which would then be heated at reflux for 6 hours to form the product *cis*-1,4-diaminobut-2-ene(CBDC)platinum(II) (120) (Scheme 7.2). This method proved unsuccessful, with starting materials being retrieved and no evidence of product (120) by spectroscopic analysis.



Scheme 7.2

In an attempt to find out if these problems were caused by the *cis*-1,4-diaminobut-2-ene (124) or whether it was the experimental procedure, we repeated the first method combining the platinum precursor (128) with 1,4-diaminobutane (putrescine) (3) and with *trans*-(-)-1,2-diaminocyclohexane. Reacting putrescine

(3) and (128) gave colourless crystals of *cis*-1,4-diaminobutane-(1',1'-cyclobutanedicarboxylato)platinum (II) (129), but in a very poor yield of 10%. *trans*-(-)-1,2-Diaminocyclohexane also gave colourless crystals in 67% yield when combined with (128), which were shown to be the literature compound *cis*-(*trans*-(-)-1,2-diaminocyclohexane)(1',1'-cyclobutanedicarboxylato)platinum (II) (130).[172] However both of these products were only sparingly soluble in water and were also difficult to purify.

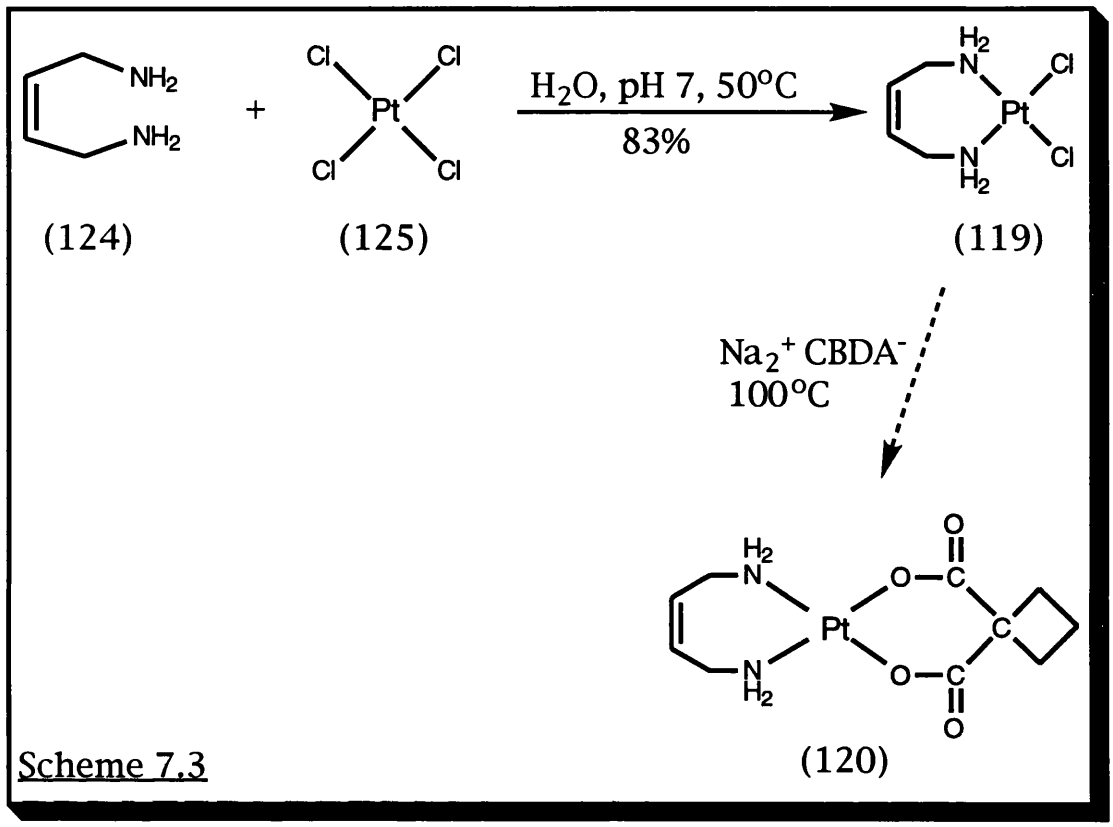


(129) (130)

Although attempts to make the required complex *cis*-1,4-diaminobut-2-ene (CBDC)Pt (II) (120) had proved unsuccessful, analysis of the black product had appeared to suggest that (120) was present along with other platinum impurities. Encouraged by these signs and the success in making (130), we adopted a different approach to the synthesis of (120). We decided to add the *cis*-1,4-diaminobut-2-ene (124) directly to the platinum salt instead of via the dimethylsulphoxide precursor (128).

Again starting with potassium tetrachloroplatinate (II) (125), we heated *cis*-1,4-diaminobut-2-ene (124) to 50 ^{o}C, maintaining a neutral pH with NaOH until no further adjustment was required. As the reaction mixture cooled to room temperature an orange/brown precipitate of *cis*-dichloro(1,4-diaminobut-2-ene)platinum (II) (119) was formed in 83% yield (Scheme 7.3).[173] This compound was only sparingly soluble in water and could not be purified further. So

the crude solid was suspended in water and heated to 50 °C with the sodium salt of 1,1-cyclobutanedicarboxylic acid, and then the reaction mixture was heated at reflux for 15 hours. The product on cooling was a similar black solid to that of the product from the first two methods; it was insoluble and therefore impossible to purify.



Scheme 7.3

The insolubility of the target compounds (119) and (120) had not only caused problems with purification methods, but their insolubility meant that they were of limited use as potential antitumour agents. In order to progress any further with these compounds we had to address the insolubility problem and the easiest way to do this was to find an alternative leaving group.

Kawai *et al.*[174] have shown that the enol form of 2,4-dioxo-pentanoic acid (131) (Figure 7.3) acts as a bidentate ligand with platinum compounds. Also there are two possibilities for binding of 2,4-dioxopentanoic acid to platinum, giving either the

acetylacetonate form (Figure 7.3a) or the α-hydroxycarboxylate form (Figure 7.3b). The acetylacetonate form showed high water solubility due to the existence of a carboxylate ion.

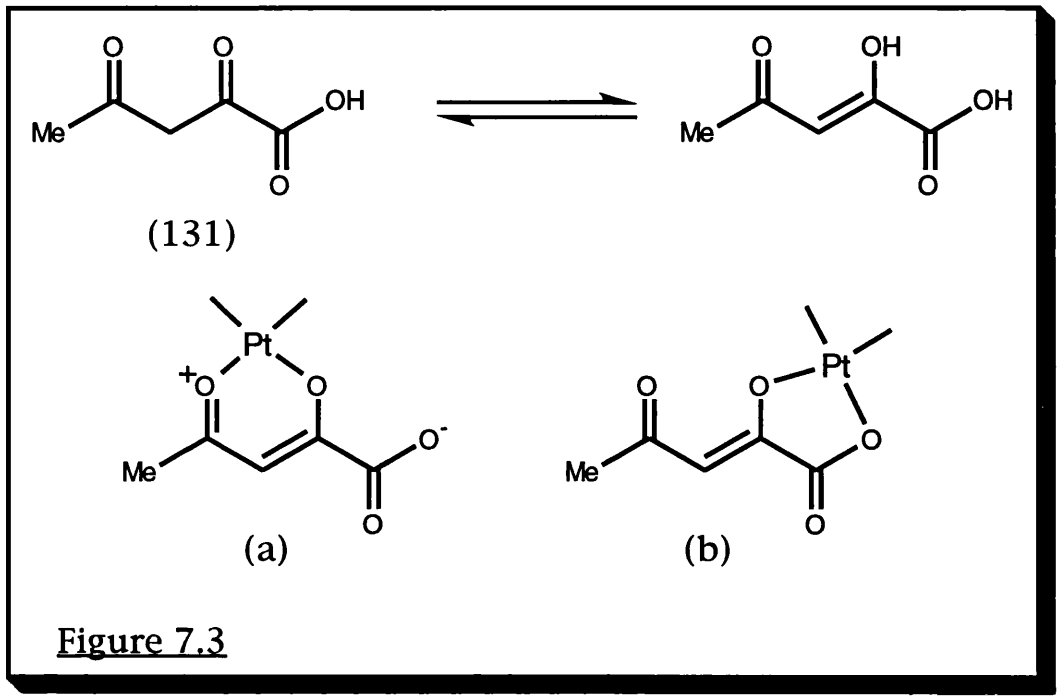


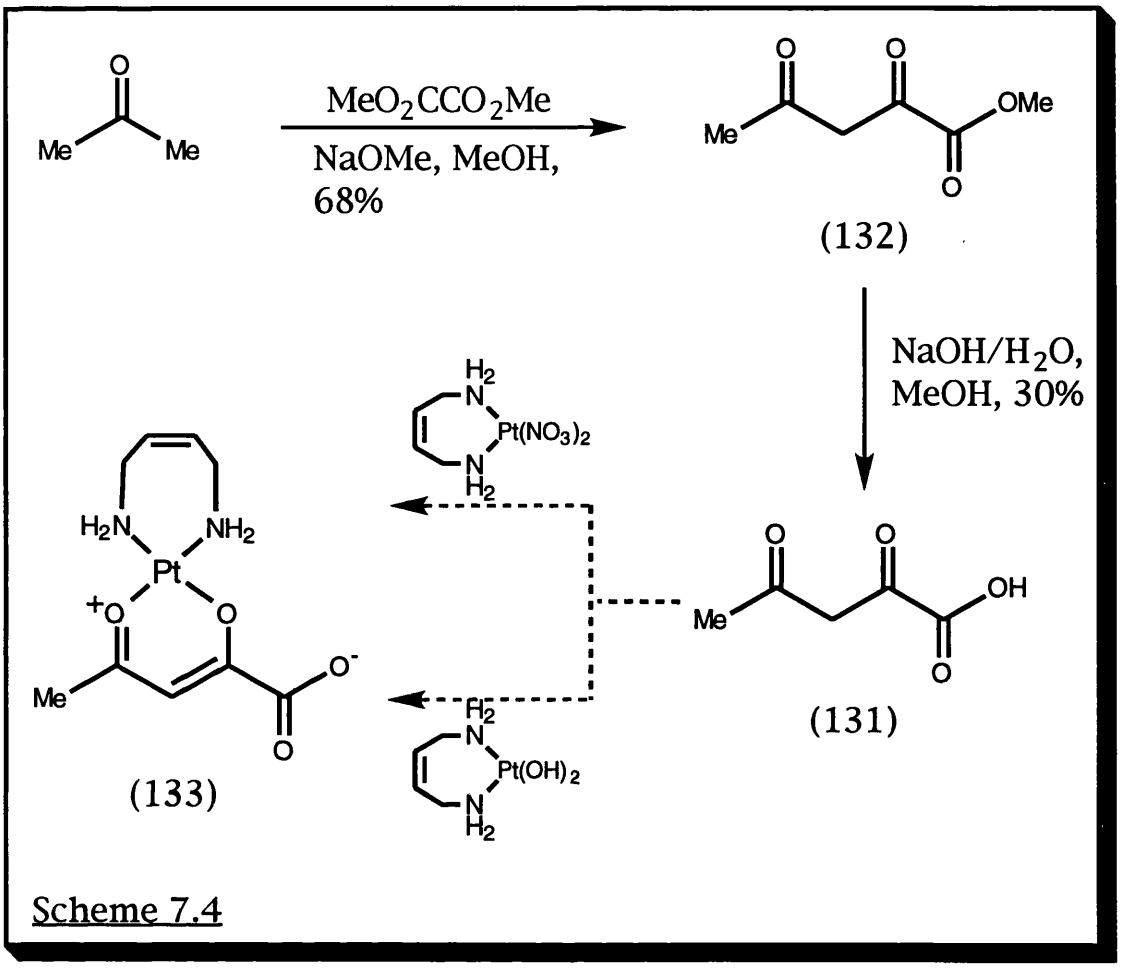
Figure 7.3

We decided to synthesise 2,4-dioxopentanoic acid (131) and then combine it with the platinum complexes containing the diamine *cis*-1,4-diaminobut-2-ene (124) thus improving the solubility.

2,4-Dioxopentanoic acid (131) was obtained from acetone in two steps. Condensation of acetone with dimethyl oxalate gave methyl 2,4-dioxopentanoate (132) (Scheme 7.4).[175] Base hydrolysis of methyl ester (132) with NaOH afforded the product 2,4-dioxopentanoic acid (131) in 30% yield.

At this stage the desired platinum complex *cis*-1,4-diaminobut-2-ene(2-hydroxy-4-oxo-2-pentanoato)platinum (II) (133) could be prepared by treatment of either dinitrato(1,4-diaminobut-2-ene)platinum (II) or dihydroxo(1,4-diaminobut-2-ene)platinum

(II) (Scheme 7.4). However, due to time constraints we were unable to continue with the synthesis.



Scheme 7.4

Although insolubility of the target compounds (119) and (120) caused problems and hindered the synthesis of suitable drug candidates, there is plenty of scope for future development. There are a number of diamines made within the group by Cook *et al.*[176] which have shown antifungal activity and can be used as alternative ligands to *cis*-1,4-diaminobut-2-ene (124). With this in mind and with the importance of finding less toxic antitumour drugs than those available today, studies should continue into trying these diamines as possible ligands for incorporation into cisplatin analogues.

CHAPTER EIGHT

Experimental

8.1 General Details

Melting points were measured on a Kofler hot-stage apparatus or a Gallenkemp melting point apparatus. Boiling points refer to the oven temperature using a Kugelrohr apparatus. Infra red spectra were obtained on Perkin Elmer 580 and P1000 spectrometers. Nuclear magnetic resonance spectra were recorded on a Perkin Elmer R32 spectrometer operating at 90 MHz, or a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H) or 50.3 MHz (δ_C). The multiplicities of the ^{13}C NMR spectrum were determined using DEPT spectra with pulse angles of $\theta = 90^o$ and $\theta = 135^o$. Spectra were recorded with either tetramethylsilane at 0 ppm or the NMR solvent as the internal standard and J values are approximations measured from the corresponding NMR spectra. Electron impact mass spectra were obtained using Kratos MS 12 or MS 902 spectrometers. Elemental analyses were performed with a Carlo-Erba 1106 elemental analyser.

Analytical and preparative thin layer chromatography were carried out in the solvent stated on Merck Kieselgel 60F plates of 0.25 mm and 2 mm thickness respectively. Diamine dihydrochlorides were detected using ninhydrin and all other compounds by iodine, vanillin/2M H_2SO_4/EtOH or the Dragendorff reagent. Chromatographic purification was carried out by flash column chromatography using Merck Kieselgel 60 (70-230 mesh) or ICN Silica 32-66 (60 mesh). High Performance Liquid

Chromatography (HPLC) was carried out on Spectra-Physics SP8800 and P4000 pumps and detection was obtained on UV2000 and Spectra 100 ultraviolet detectors. The HPLC solvent system used was methanol and water (70:30) on a reverse phase silica column with an octadecylsilane bonded phase.

All solvents were purified using standard techniques.[180] Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium-benzophenone immediately before use, and dichloromethane was dried by distillation over calcium hydride; all these distillations were carried out under nitrogen. Organic solvents were dried using either anhydrous sodium sulphate or anhydrous magnesium sulphate, and solvents were removed under reduced pressure below 50 ^{o}C.

8.2 Extraction of Diamine Oxidase (EC 1.4.3.6) from Pea Seedlings[6]

Step 1

Pea seeds (500 g), variety 'Fillbasket', were soaked in tap water for 24 h. The water was changed *ca.* 4 times. The pea seeds were then sown thickly in Perlite (4-6 cm deep) and covered in Perlite (1-2 cm). They were allowed to germinate and grow in the dark for 10-14 d until the shoots were 5-10 cm tall. Note: the Perlite was kept moist throughout but not too wet since this was found to reduce germination. The shoots were stripped of their roots, washed free of growing medium, drained and weighed (1-1.5 kg). The harvested shoots were kept cool throughout the following operation. The peas were minced using a pre-cooled Waring

blender. They were then strained through cotton mesh and the juice was squeezed out. The solid residue was mixed with 0.1 M potassium phosphate buffer (pH 7, 1 ml/g of material) and the juice was squeezed out as before. A second extraction using the same potassium phosphate buffer (0.5 ml/g of material) was performed. The total extract (2-3 litres) was cooled to < 5 ^{o}C.

Step 2

Ethanol/chloroform (2:1 v/v, 30 ml per 100 ml of extract) was cooled to -10 ^{o}C and added to the extract over 30 min. Care was taken to ensure that the temperature of the extract did not rise above 5 ^{o}C during this addition. The mixture was allowed to stand for *ca.* 1 h at 0-5 ^{o}C after which the inactive precipitate was removed by centrifugation at 3000-4000 g for 20 min. The supernatant liquid was collected and saturated with ammonium sulphate (45 g/100 ml) and the temperature was allowed to rise to 10 ^{o}C. A solid separated and floated. The lower liquid was siphoned off and discarded. The slurry was centrifuged at 3000 g for 10-15 min. The curd collected was mixed with 0.02 M phosphate buffer (pH 7, 400-500 ml) and allowed to stand overnight.

Step 3

The solution was stirred for 1.5 h at 15-18 ^{o}C and the precipitate was removed by centrifugation at 3000-4000 g for 20 min. The supernatant was again saturated with ammonium sulphate (200-300 g) and left for 1.5 h at 8-10 ^{o}C. It was then centrifuged at 3000-4000 g for 20 min. The curd was mixed with 0.2 M phosphate buffer (pH 7, 20 ml). The dialysis tubing was pre-

soaked in distilled water for *ca.* 2h. The solution was dialysed in a 30 cm tube (diameter 15 mm) for 2-3 h with cold running water. Dialysis was then carried out with 0.005 M phosphate buffer (pH 7, 1 litre) over 36 h at 0-4 ^{o}C. The buffer was changed twice during this period.

Step 4

The dialysed material was centrifuged at 3000 g for 10-20 min to remove inactive precipitate. The supernatant liquid was adjusted to pH 5 by slow addition of 0.05 M acetic acid at *ca.* 5 ^{o}C then allowed to stand for 1 h at 0-4 ^{o}C. The precipitate was collected by centrifugation and triturated with water (20 ml). The pH was adjusted to pH 7 using 0.05 M potassium hydroxide to dissolve the precipitate and then to pH 5 with 0.05 M acetic acid. The solution was left for 1 h and centrifuged to collect the precipitate. The precipitate obtained was taken up in 0.01 M phosphate buffer (pH 7, 1 ml/100 g of seedlings harvested). It was stored in the freezer (in 0.5 ml batches) at *ca.* -20 ^{o}C and was stable for many months.

The yield was *ca.* 30 mg per kg of peas. Protein concentration was *ca.* 8 mg per ml of enzyme solution [See Appendix for calculation].

<u>Determination of Protein Concentration</u>[124]

Coomassie brilliant blue G was prepared as a 0.06% (w/v) solution in 3% perchloric acid. The solution was stirred overnight and filtered to remove any undissolved material.

A standard graph was determined using Bovine Serum Albumin (BSA, 1 mg/ml phosphate buffer pH 6.3).

A typical cuvette contained;

1 ml	Dye
1000 µl - x µl	Distilled water
x µl	BSA

where x = 5 to 50 µl

The experiment was carried out twice and the average plot was used to determine the protein concentration of the unknown DAO samples (replacing BSA with DAO in the cuvette) [See Appendix].

Spectrophotometric Assay[89]

The kinetics of the DAO-catalysed oxidation of putative substrates were determined according to the method of Stoner.[89] This involved a peroxidase-coupled assay (horseradish peroxidase, EC 1.11.1.7, from Sigma) to monitor continuously the hydrogen peroxide released during diamine oxidation at 25 ^{o}C; using 70 mM phosphate buffer (pH 6.3), in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB). Oxidative coupling generated stoichiometric quantities of an indamine dye with a characteristic absorbance maximum at 595 nm. Rates were determined directly in the spectrophotometer.

Stock solutions were prepared as follows;

DMAB	18 mM (29.7 mg/10 ml phosphate buffer pH 6.3)
MBTH	0.6 mM (12.9 mg/100 ml distilled water)
Peroxidase	0.68 mg/2 ml phosphate buffer pH 6.3
Pea Seedling DAO	0.03-0.06 mg/ml phosphate buffer pH 6.3

A typical 1 cm path length cuvette contained;

2.5 ml	Phosphate buffer pH 6.3
100 µl	MBTH
170 µl	DMAB
50 µl	peroxidase
25 µl	pea seedling DAO
300 µl	substrate of varying concentrations

Method for making up substrates of varying concentrations:

From a stock solution, typically 5 ml of 40 mM substrate solution in phosphate buffer.

Required Conc.	Amount of Sample	Amount of Buffer
40mM	500µl of 40mM	0µl
30mM	375µl of 40mM	125µl
20mM	250µl of 40mM	250µl
10mM	5x125=625µl of 40mM	5x375=1875µl
8mM	400µl of 10mM	100µl
6mM	300µl of 10mM	200µl
4mM	200µl of 10mM	300µl

2mM	100µl of 10mM	400µl
1mM	5x50=250µl of 10mM	5x450=2250µl
0.8mM	400µl of 1mM	100µl
0.6mM	300µl of 1mM	200µl
0.4mM	200µl of 1mM	300µl
0.2mM	100µl of 1mM	400µl
0.1mM	50µl of 1mM	450µl

The reaction was initiated by the addition of standard enzyme to the thermally equilibrated reaction mixture, followed immediately by substrate addition, therefore minimising the possible inhibitory effects of extensive preincubation of DAO with the chromogenic agents.[89] Initial rates were determined over a range of substrate concentrations from the linear absorbance changes observed during the first minute of the reaction. Rate data were analysed for K_M and V_{max} by least squares fitting of Eadie-Hofstee[116,117] (V against V/[S]), Lineweaver-Burk[115] (1/V against 1/[S]) and Hanes ([S]/V against [S]) plots.

All experiments were carried out three times with all data quoted being the mean of nine determinations.

The validity of the assay system had previously been checked,[125] with the formation of the indamine dye being calibrated using standard solutions of hydrogen peroxide.

Inhibition Studies

The assay system used for inhibition studies was the same method as above but with the addition of various concentrations of inhibitor. The potential inhibitor was added to the reaction mixture

after DAO addition but always before the addition of the substrate. Also for inhibitor studies 100µl of distilled H_2O was added to the first run only. This is to compensate for the extra 100µl of inhibitor added when running the rest of the inhibitor studies.

The typical reaction mixture in the cuvette was :

2.5 ml	Phosphate buffer pH 6.3
100 µl	MBTH
170 µl	DMAB
50 µl	peroxidase
25 µl	pea seedling DAO
100 µl	inhibitor (added before substrate)
300 µl	substrate of varying concentrations

8.3 Experimental for Chapter Four

General Procedure (A) for the Synthesis of [$^2H_4$]-labelled Dinitriles from Non-labelled Species.[101]

Deuterium oxide (35 ml, 99.3 atom % ^2H), 1,4-dioxane (10 ml) and diazabicyclo[5.4.0]undec-7-ene (364 mg, 4.56 mmol) were added to the dinitrile (45.6 mmol) in a 100 ml round bottom flask. The mixture was stirred and heated to reflux for 24 h. The reaction mixture was then cooled and the solvent was removed *in vacuo* to yield an amorphous white solid that solidified on standing. 1 M Hydrochloric acid was added to dissolve the amorphous white solid and to make the resulting solution acidic. The acidic solution was extracted with chloroform (5 x 25 ml), dried (Na_2SO_4), filtered, and the solvent was removed *in vacuo* to yield a second amorphous white solid. The solid was recrystallised from hot benzene to yield the product as a white material. This process was repeated giving 93-98% [$^2H_4$] incorporation.

[2,2,3,3-$^2H_4$]-Succinonitrile

Succinonitrile (68) was labelled with deuterium to give [2,2,3,3-$^2H_4$]-succinonitrile using general procedure (A). White crystals were obtained, 75%; R_f 0.25 ($CHCl_3$); υ_{max} ($CHCl_3$) 3010, 2250, 1460 and 1070 cm^{-1}; δ_C ($CDCl_3$) 116.42 (2$\underline{C}$N); *m/z* 84 (M^+), 82, 56 (100%), 54 and 42; Found: M^+, 84.0617. $C_4D_4N_2$ requires M, 84.0625.

[2,2,4,4-$^2H_4$]-Glutaronitrile

Glutaronitrile (69) was labelled with deuterium to give [2,2,4,4-$^2H_4$]-glutaronitrile using general procedure (A). White crystals were obtained, 72%; R_f 0.30 ($CHCl_3$); υ_{max} ($CHCl_3$) 2990, 2880, 2250, 1450 and 1050 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.15 (s, 2H); δ_C ($CDCl_3$) 33.11 ($\underline{C}H_2$), 116.42 (2$\underline{C}$N); *m/z* 98 (M^+), 96, 70 (100%), 46, 38 and 28; Found: M^+, 98.0780. $C_5H_2D_4N_2$ requires M, 98.0782.

[2,2,5,5-$^2H_4$]-Adiponitrile

Adiponitrile (70) was labelled with deuterium to give [2,2,5,5-$^2H_4$]-adiponitrile using general procedure (A). White crystals were obtained, 70%; R_f 0.32 ($CHCl_3$); υ_{max} ($CHCl_3$) 3005, 2990, 2250, 1450 and 1070 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.80 (s,4H); δ_C ($CDCl_3$) 27.72 (2$\underline{C}H_2$), 116.44 (2$\underline{C}$N); *m/z* 112 (M^+), 110, 86, 61, 55, 44 (100%) and 28; Found: M^+, 112.0940. $C_6H_4D_4N_2$ requires M, 112.0939.

<u>General Procedure (B) for the Synthesis of [$^2H_4$]-labelled Diamine Dihydrochloride Salts from [$^2H_4$]-labelled Dinitriles</u>[119]

An oven dried, 50 ml round bottom flask containing a septum capped inlet and a magnetic stirring bar was equipped with a 12" Vigreux column. A 10 ml round bottom flask was fitted to the end of the receiver. The whole system was assembled under nitrogen, with the outlet connected to a source of nitrogen to maintain an

inert atmosphere. The flask was charged with the [$^2H_4$]-labelled dinitrile (15 mmol) and dry tetrahydrofuran (1.5 ml), and brought to reflux. Then borane-dimethyl sulphide complex in THF (16 ml, 32 mmol) was added dropwise over a period of 10 min. The dimethyl sulphide distilled off and was collected in the 10 ml round bottom flask. This gave a clear residue which was left for 15 minutes and then allowed to cool to room temperature. 6 M Hydrochloric acid (18 ml, 41 mmol) was added dropwise at first (care was taken because H_2 gas was evolved essentially immediately following each addition of acid) until no more H_2 was evolved. The reaction mixture was then heated under reflux for 30 min. The resultant clear solution was cooled to 0 ^oC giving a white precipitate of boric acid. The solution was filtered, and the filtrate was concentrated to approximately 5 to 10 ml giving a second precipitate of boric acid which was filtered off. MeOH (10 ml) and conc. HCl (2-3 drops) were added to the filtrate in order to remove any boric acid as the borate methyl ester. The resulting solution was evaporated to dryness giving a creamy white solid of diamine dihydrochloride. The solid was crystallised from 95% aqueous ethanol.

[2,2,3,3-$^2H_4$]-Butane-1,4-diamine (Putrescine) Dihydrochloride (65)

[2,2,3,3-$^2H_4$]-Succinonitrile was reduced to [2,2,3,3-$^2H_4$]-1,4-putrescine dihydrochloride (65) using general procedure B. White crystals were obtained, 55%; m.p. >250 ^oC; υ_{max} (KBr disc) 3075, 3025, 2010, 1605, 1473, 1395, 1335, 1170 and 1145 cm^{-1}; δ_H(200 MHz, D_2O) 2.85 (br s, 4H); δ_C (D_2O) 23.86 (quintet, 2$\underline{C}D_2$), 39.57

(2$\underline{C}H_2$); Found: C, 29.08; H, 6.20; D, 4.90; N, 16.98%. $C_4H_{10}D_4N_2Cl_2$ requires: C, 29.10; H, 6.12; D, 4.87; N, 16.97%.

[2,2,4,4-$^2H_4$]-Pentane-1,5-diamine (Cadaverine) Dihydrochloride (66)

[2,2,4,4-$^2H_4$]-Glutaronitrile was reduced to [2,2,4,4-$^2H_4$]-1,5-cadaverine dihydrochloride (66) using general procedure B. White crystals were obtained, 60%; m.p. >250 ^oC; υ_{max} (KBr disc) 3080, 2000, 1600, 1475, 1160 and 1145 cm^{-1}; δ_H (200 MHz, D_2O) 1.25 (br s, 2H), 2.79 (br s, 4H); δ_C (D_2O) 23.08 ($CD_2\underline{C}H_2CD_2$), 26.28 (quintet, 2$\underline{C}D_2CH_2N$), 39.85 (2$\underline{C}H_2N$); Found: C, 33.61; H, 6.73; D, 4.48; N, 15.72%. $C_5H_{12}D_4N_2Cl_2$ requires: C, 33.52; H, 6.70; D, 4.47; N, 15.64%.

[2,2,5,5-$^2H_4$]-Hexane-1,6-diamine Dihydrochloride (67)

[2,2,5,5-$^2H_4$]-Adiponitrile was reduced to [2,2,5,5-$^2H_4$]-1,6-hexanediamine dihydrochloride (67) using general procedure B. White crystals were obtained, 57%; m.p. >250 ^oC; υ_{max} (KBr disc) 3080, 3020, 2000, 1600, 1475, 1175 and 1145 cm^{-1}; δ_H (200 MHz, D_2O) 1.25 (s, 4H), 2.83 (s, 4H); δ_C (D_2O) 25.67 ($CD_22\underline{C}H_2CD_2$), 26.59 (quintet, 2$\underline{C}D_2CH_2N$), 40.07 ($\underline{C}H_2N$); Found: C, 37.30; H, 7.26; D, 4.02; N, 14.54%. $C_6H_{14}D_4N_2Cl_2$ requires: C, 37.31; H, 7.25; D, 4.14; N, 14.51%.

General Procedure (C) for the Conversion of Diamines (free base) into Diamine Dihydrochloride Salts

The free base of each diamine (6 mmol) was suspended between dichloromethane (80 ml) and 6 M hydrochloric acid (50 ml) for 2 h. The aqueous layer was separated from the dichloromethane and washed with a further 150 ml of dichloromethane before being evaporated to dryness leaving the dihydrochloride salt as a white solid. The dihydrochloride was crystallised from aqueous ethanol (95%) and acetone.

Butane-1,4-diamine (Putrescine) Dihydrochloride (3)

The free base of putrescine was converted into the corresponding dihydrochloride using general procedure C. White crystals were obtained, 85%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3400-3300, 3100-2900, 2560, 2040, 1470 and 1450 cm^{-1}; δ_H (200 MHz, D_2O) 1.60-1.90 (complex, 4H) and 2.95-3.10 (complex, 4H); *m/z* 89 (M^+ +1), 88 (M^+), 72 and 30 (100%); Found: C, 29.84; H, 8.82; N, 17.43%. $C_4H_{14}N_2Cl_2$ requires: C, 29.81; H, 8.70; N, 17.39%.

Pentane-1,5-diamine (Cadaverine) Dihydrochloride (6)

The free base of cadaverine was converted into the corresponding dihydrochloride using general procedure C. White crystals were obtained, 95%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3600-3400, 3200-2800, 2570, 2030, 1600 and 1475 cm^{-1}; δ_H (200 MHz, D_2O) 1.35 (complex, 2H), 1.60 (complex, 4H) and 2.90(t, 4H); δ_C (D_2O) 23.50 ($N(CH_2)_2\underline{C}H_2$), 27.70 ($NCH_2\underline{C}H_2$) and 40.04 ($N\underline{C}H_2$); *m/z*

103 (M^+ +1), 102 (M^+), 86 and 30 (100%); Found: C, 34.09; H, 9.04; N, 16.07%. $C_5H_{16}N_2Cl_2$ requires: C, 34.28; H, 9.14; N, 16.00%.

Hexane-1,6-diamine Dihydrochloride (68)

The free base of hexane-1,6-diamine was converted to the corresponding dihydrochloride using general procedure C. White crystals were obtained, 79%; m.p. >250 °C ; δ_H (200 MHz, D_2O) 1.26 (tt, 4H), 1.51 (tt, 4H), 2.83 (t, 4H) δ_C (D_2O) 26.24 (2$\underline{C}H_2CH_2CH_2N$), 27.63 (2$\underline{C}H_2CH_2N$), 40.53 (2$\underline{C}H_2N$); *m/z* 117 ($M^+$ +1), 116 (M^+), 101 and 30 (100%); Found: C, 37.99; H, 9.63; N, 14.77%. $C_6H_{18}N_2Cl_2$ requires: C, 38.09; H, 9.52; N, 14.81%.

Benzoylacetic Acid (71)

Ethyl benzoylacetate (1.92 g, 10.0 mmol) was stirred in 2.5% aqueous potassium hydroxide solution (175 ml) for 48 h. The solution was washed with ether (3 x 45 ml), cooled to 5 °C and acidified with dilute H_2SO_4 and further extracted with diethyl ether (6 x 75 ml). The latter combined ether extracts were dried (Na_2SO_4), filtered, and evaporated to dryness under reduced pressure at room temperature to yield a light yellow solid of benzoylacetic acid, 75%, which was stored at below 0 °C and used without purification, υ_{max} (nujol) 3100-2600, 1750, 1670, 1600, 1520, 1420, 1280, 1145 and 1025 cm^{-1}; δ_H ($CDCl_3$) 3.99 (s, 2H), 7.35-7.55 (m, 3H), 7.95 (dd, 2H); *m/z* 121, 120, 115 (100%) and 77.

General Procedure (D) for Oxidative Deamination of Diamines using Diamine Oxidase and the Subsequent Coupling with Benzoylacetic Acid[123]

A solution of benzoylacetic acid (320 mg, 1.96 mmol), diamine hydrochloride (0.1 M aqueous solution, 20 ml) and 0.02 M sodium phosphate buffer (pH 7, 7 ml) was prepared. The pH was adjusted to 7. Catalase (0.2 mg) and pea seedling DAO (300 µl, *ca.* 200 mg, enzyme activity 1200 units per mg) were added. The solution was incubated on a shaker at 25 ^{o}C for 24 h. The pH of the reaction mixture was monitored and readjusted to neutrality as necessary. The solution was then acidified with dilute sulphuric acid and washed with diethyl ether (4 x 30 ml) to remove any excess benzoylacetic acid. The aqueous solution was basified with conc. ammonia and extracted with chloroform (3 x 30 ml). The combined organic extracts were dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure yielding the product as an oil. The oils were purified by preparative TLC in the solvents stated and the purity of each was checked by HPLC.

Note: Yields for these reactions varied greatly and so attempts were made to optimise the conditions. The times were varied between 12-48 h and the pH was adjusted using sodium dihydrogen phosphate buffer.

2-Pyrrolidin-2-ylacetophenone (72)

Using putrescine dihydrochloride (3) as the substrate in general procedure D gave 2-pyrrolidin-2-ylacetophenone (72) as a yellow oil, 61%; R_f 0.40 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.85 min (MeOH/H_2O, 70:30); υ_{max} (film) 3680, 3020, 2400, 1680, 1635, 1600, 1525 and 1215 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.30-1.70 (complex, 4H), 2.35 (br s, 1H), 3.50 (complex, 5H), 7.35-7.60 (complex, 3H) and 7.93 (dd, 2H); δ_C ($CDCl_3$) 23.92 ($\underline{C}H_2(CH_2)_2N$), 30.74 ($\underline{C}H_2CH_2N$), 41.93 ($\underline{C}H_2CO$), 45.06 ($\underline{C}H_2N$), 55.21 ($\underline{C}HN$), 128.08 (2Ar-C), 128.57 (2Ar-C), 133.36 (Ar-C), 136.20 (Ar-C) and 197.70 (C=O); *m/z* 190 (M^+ +1), 189 (M^+), 170, 105, 84, 70 (100%) and 28; Found: M^+, 189.1154. $C_{12}H_{15}NO$ requires M, 189.1155.

2-Piperidin-2-ylacetophenone (73)

Using cadaverine dihydrochloride (6) as the substrate in general procedure D gave 2-piperidin-2-ylacetophenone (73) as a yellow oil, 87%; R_f 0.43 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.73 min (MeOH/H_2O, 70:30); υ_{max} (film) 3690, 3020, 2400, 1685, 1600, 1520, 1215, 1020 and 930 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.31-1.75 (complex, 6H), 2.68 (dt, 1H), 3.03-3.14 (complex, 4H), 3.43 (br s, 1H), 7.35-7.51 (complex, 3H) and 7.92 (dd, 2H); δ_C ($CDCl_3$) 24.37 ($\underline{C}H_2(CH_2)_2N$), 25.47 ($\underline{C}H_2CH_2N$), 32.15 ($\underline{C}H_2CHCH_2CO$), 45.03 ($\underline{C}H_2CO$), 46.57 ($\underline{C}H_2N$), 127.97 (2 Ar-C), 128.53 (2Ar-C), 133.18 (Ar-C), 136.85 (Ar-C) and 199.15(C=O); *m/z* 204 (M^+ +1), 203 (M^+), 202 (M^+ - 1), 186, 105, 98, 84 (100%), 77, 43 and 28; Found: M^+, 203.1289. $C_{13}H_{17}NO$ requires M, 203.1310.

2-Azacycloheptan-2-ylacetophenone (74)

Using hexane-1,6-diamine dihydrochloride (68) as the substrate in general procedure D gave 2-azacycloheptan-2-ylacetophenone (74) as a light brown oil, 50%; R_f 0.40 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.70 min (MeOH/H_2O, 70:30); υ_{max} (film) 3670, 3010, 2395, 1680, 1600, 1515, 1215, 1015 and 925 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 0.91-1.56 (complex, 8H), 2.09 (br s, 1H), 2.31-2.71 (complex, 3H), 2.95-3.00 (complex, 1H), 7.21-7.48 (complex, 3H), 7.85 (dd, 2H); δ_C 25.44 ($\underline{C}H_2(CH_2)_2N$), 27.25 ($\underline{C}H_2(CH_2)_3N$), 30.86 ($\underline{C}H_2CH_2N$), 36.54 ($\underline{C}H_2CHCH_2CO$), 46.16 ($\underline{C}H_2CO$), 46.90($\underline{C}H_2N$), 55.16 ($\underline{C}HN$), 128.21 (2Ar-C), 128.50 (2Ar-C), 133.03 (Ar-C), 136.80 (Ar-C) and 199.05 (C=O); *m/z* 218 ($M^+ + 1$), 217 (M^+), 200, 113, 105 (100%), 98, 43 and 28; Found: M^+, 217.1470. $C_{14}H_{19}NO$ requires M, 217.1466.

[3',3',4',4'-$^2H_4$]-2-Pyrrolidin-2-ylacetophenone (75)

Using [2,2,3,3-$^2H_4$]-1,4-putrescine dihydrochloride (65) as the substrate in general procedure D gave [3',3',4',4'-$^2H_4$]-2-pyrrolidin-2-ylacetophenone (75) as a brown oil, 76%; R_f 0.42 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.87 min (MeOH/H_2O, 70:30); υ_{max} (film) 3690, 3020, 2400, 2225, 1680, 1620, 1600, 1520, 1420, 1215, 1020 and 930 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.98 (dd, 2H), 3.25 (ABX system, octet, 2H), 3.64 (dd, 1H), 4.00 (br s, 1H), 7.45 (complex, 3H) and 7.94 (dd, 2H); δ_C ($CDCl_3$) 44.20 ($\underline{C}H_2N$), 45.69 ($\underline{C}H_2CO$), 54.48 ($\underline{C}HN$), 127.90 (2Ar-C), 128.01 (2Ar-C), 133.11 (Ar-C), 136.72 (Ar-C) and 199.15 (C=O); δ_D ($CHCl_3$) 1.38, 1.72, 1.79 and 1.96; *m/z* 194 ($M^+ + 1$), 193 (M^+), 192 ($M^+ - 1$), 174, 105, 88, 74

(100%), 73 and 28; Found: M^+, 193.1404. $C_{12}H_{11}D_4NO$ requires M, 193.1404.

[3',3',5',5'-$^2H_4$]-2-Piperidin-2-ylacetophenone (76)

Using [2,2,4,4-$^2H_4$]-cadaverine dihydrochloride (66) as the substrate in general procedure D gave [3',3',5',5'-$^2H_4$]-2-piperidin-2-ylacetophenone (76) as a brown oil, 70%; R_f 0.42 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.85 min (MeOH/H_2O, 70:30); υ_{max} (film) 3690, 3020, 2400, 1680, 1600, 1525, 1420, 1220, 1015 and 930 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.25 (AB system, 1H), 1.62 (AB system, 1H), 2.52 (br s, 1H), 2.66 (dd, 1H), 2.99-3.14 (complex, 4H), 7.40-7.59 (complex, 3H) and 7.94 (dd, 2H); δ_C ($CDCl_3$) 24.22 ($\underline{C}H_2CD_2CH_2N$), 45.50($\underline{C}H_2N$), 46.60 ($\underline{C}H_2CO$), 52.55($\underline{C}HCH_2CO$), 127.94 (2Ar-C), 128.51 (2Ar-C), 133.11 (Ar-C), 136.94 (Ar-C) and 199.50 (C=O); δ_D ($CHCl_3$) 1.35, 1.49, 1.61 and 1.68; *m/z* 208 ($M^+ + 1$), 207 (M^+), 190, 105, 88 (100%), 77, 43 and 28; Found: M^+, 207.1558. $C_{13}H_{13}D_4NO$ requires M, 207.1561.

[3',3',6',6'-$^2H_4$]-2-Azacycloheptan-2-ylacetophenone (77)

Using [2,2,4,4-$^2H_4$]-hexane-1,6-diamine dihydrochloride (67) as the substrate in general procedure D gave [3',3',6',6'-$^2H_4$]-2-azacycloheptan-2-ylacetophenone (77) as a brown oil, 75%; R_f 0.38 ($CHCl_3$/MeOH/c.NH_3, 80:19:1), R_t 2.69 min (MeOH/H_2O, 70:30); υ_{max} (film) 3680, 3015, 1680, 1600, 1525, 1215, 1020 and 930 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.17-1.67 (complex, 4H), 2.21 (br s, 1H), 2.38-2.73 (complex, 3H), 3.05 (dd, 1H), 3.15 (complex, 1H), 7.41-7.55 (complex, 3H) and 7.93 (dd, 2H); δ_C ($CDCl_3$) 25.16 ($\underline{C}H_2CD_2CH_2N$), 27.00 ($\underline{C}H_2CD_2CHN$), 46.19 ($\underline{C}H_2CO$), 46.78 ($\underline{C}H_2N$),

55.02 (CHN), 127.97 (2Ar-C), 128.51 (2Ar-C), 133.00 (Ar-C), 137.18 (Ar-C) and 199.69 (C=O); δ_D ($CHCl_3$) 1.00-1.80 (complex); *m/z* 222 (M^+ + 1), 221 (M^+ +1), 215, 117, 105 (100%), 102, 77, 43 and 28; Found: M^+, 221.1718. $C_{14}H_{15}D_4NO$ requires M, 221.1717.

8.4 Experimental for Chapter Five

Synthesis of Quinolyl and Pyridyl Ethylamines

(±)-2-Hydroxy-2-(2'-quinolyl)ethylamine Dihydrochloride (81)

(±)-2-Hydroxy-2-(2'-quinolyl)nitroethane (78)

2-Quinolinecarboxaldehyde (730 mg, 4.67 mmol) was dissolved in isopropanol (20 ml) with vigorous stirring and to this solution was added potassium fluoride (12.5 mg) and nitromethane (0.56 ml, 9.35 mmol). After stirring at room temperature for 4 h the solution was heated to 40-50 ^{o}C for a further 1 h. The solvent was removed *in vacuo* below 50 ^{o}C to yield (±)-2-hydroxy-2-(2'-quinolyl)nitroethane (78) as an orange/red solid. Purification on a silica gel column (hexane/ethyl acetate, 2:3) produced the pure product as a light orange solid, 70%; υ_{max} (KBr disc) 3500-3100, 1600, 1550 (strong), 1380, 1100 and 830 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.85 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 5.28 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H), 7.56-8.05 (complex, 5H) and 8.37 (d, 1H); δ_C ($CDCl_3$) 72.16 ($\underline{C}HOH$), 81.45 ($\underline{C}H_2NO_2$), 119.52 (Ar-C), 127.51 (Ar-C), 128.49 (Ar-C), 128.65 (Ar-C), 129.62 (Ar-C), 130.62 (Ar-C), 138.04 (Ar-C), 147.74 (Ar-C) and 159.96 (Ar-C); *m/z* 219 (M^+ +1), 218 (M^+), 158, 143 (100%), 128, 115, 102, and 75; Found: M^+, 218.0687. $C_{11}H_{10}N_2O_3$ requires M, 218.0683.

(±)-2-Hydroxy-2-(2'-quinolyl)ethylamine Dihydrochloride (81)

(±)-2-Hydroxy-2-(2'-quinolyl)nitroethane (78) (1 g, 4.59 mmol) was dissolved in dry methanol (50 ml) in a three necked round bottom flask equipped with a nitrogen balloon. To the solution was added 10% Pd/C catalyst (0.4 g) and anhydrous ammonium formate (1.45 g, 23.0 mmol). The mixture was stirred under nitrogen at 25 ^{o}C for 15 h. The mixture was then diluted with diethyl ether (50 ml), filtered through a Celite pad and evaporation of the solvent *in vacuo* gave the crude amine. The crude amine was partitioned between dichloromethane (50 ml) and 6 M HCl (30 ml) and stirred for 2 h. The aqueous layer was separated, washed with CH_2Cl_2 (100 ml) and evaporated to dryness to yield the dihydrochloride salt (81) as a brown solid which was recrystallised from ethanol and acetone, 45%; m.p. >250^{o}C; υ_{max} (KBr disc) 3625, 3150-2600, 1620, 1560 and 835 cm^{-1}; δ_H (200 MHz, D_2O) 3.37 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H), 5.53 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 7.52-8.01 (complex, 5H) and 8.64 (d, 1H); δ_C (D_2O) 44.58 ($\underline{C}H_2NH_2$), 67.65 ($\underline{C}HOH$), 120.28 (Ar-C), 120.75 (Ar-C), 129.93 (Ar-C), 130.13 (Ar-C), 130.53 (Ar-C), 136.33 (Ar-C), 138.09 (Ar-C), 148.93 (Ar-C) and 157.29 (Ar-C); *m/z* 262 (M^+ +1), 232, 129 (100%) and 36.

(±)-2-Hydroxy-2-(3'-quinolyl)ethylamine Dihydrochloride (82)

(±)-2-Hydroxy-2-(3'-quinolyl)nitroethane

3-Quinolinecarboxaldehyde (730 mg, 4.67 mmol) was dissolved in isopropanol (25 ml) with vigorous stirring and to this solution was added potassium fluoride (12.5 mg) and nitromethane (0.56 ml, 9.35 mmol). The solution was stirred at room temperature, and after 5 h a white precipitate appeared. The solution was allowed to stir for a further 21 h after which the white precipitate was filtered and the filtrate was concentrated to 5-10 ml to give a second white precipitate, which was filtered. The two batches of (±)-2-hydroxy-2-(3'-quinolyl)nitroethane were combined and washed with ether, and then used without further purification, 85%; υ_{max} (KBr disc) 3400-3000, 1570 (strong), 1530, 1380, 1090, 790 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.89 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 5.75 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H), 7.57-8.07 (complex, 4H), 8.41 (d, 1H) and 9.03 (d,1H); δ_C ($CDCl_3$) 69.69 ($\underline{C}HOH$), 82.03 ($\underline{C}H_2NO_2$), 127.70 (Ar-C), 128.52 (2Ar-C), 128.92 (Ar-C), 129.94 (Ar-C), 130.34 (Ar-C), 134.02 (Ar-C), 148.88 (Ar-C) and 150.11 (Ar-C); *m/z* 219 (M^+ +1), 218 (M^+), 157, 143, 128 (100%), 115, 101, and 75; Found: M^+, 218.0736. $C_{11}H_{10}N_2O_3$ requires M, 218.0688.

(±)-2-Hydroxy-2-(3'-quinolyl)ethylamine Dihydrochloride (82)

(±)-2-Hydroxy-2-(3'-quinolyl)nitroethane (580 mg, 2.66 mmol) was dissolved in dry methanol (75 ml) in a three necked

round bottom flask equipped with a nitrogen balloon. To the solution was added 10% Pd/C catalyst (0.3 g) and anhydrous ammonium formate (832 mg, 13.2 mmol). The mixture was stirred under nitrogen at 25 ^{o}C for 24 h. The mixture was diluted with diethyl ether (50 ml) and then filtered through a Celite pad and evaporation of the solvent *in vacuo* gave the crude amine. The crude amine was partitioned between dichloromethane (40 ml) and 6 M HCl (25 ml) and stirred for 2 h. The aqueous layer was separated, washed with CH_2Cl_2 (75 ml) and evaporated to dryness to yield the dihydrochloride salt (82) as a dark red solid which was recrystallised from ethanol and acetone, 38%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3500-3200, 3100-2650, 1615, 1550 and 835 cm^{-1}; δ_H (200 MHz, D_2O) 3.47 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 5.53 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 7.62-8.09 (complex, 4H), 8.95(d, 1H) and 9.01 (d, 1H); δ_C (D_2O) 45.67 ($\underline{C}H_2NH_2$), 67.36 ($\underline{C}HOH$), 120.79 (Ar-C), 130.24 (Ar-C), 131.27 (Ar-C), 135.29 (2Ar-C), 136.30 (Ar-C), 137.75 (Ar-C), 143.27 (Ar-C) and 145.49 (Ar-C); *m/z* 262 (M^+ +1), 129 (100%) and 36.

(±)-2-Hydroxy-2-(4'-quinolyl)ethylamine Dihydrochloride (83)

(±)-2-Hydroxy-2-(4'-quinolyl)nitroethane

4-Quinolinecarboxaldehyde (730 mg, 4.67 mmol) was dissolved in isopropanol (20 ml) with vigorous stirring and to this solution was added potassium fluoride (12.5 mg) and nitromethane (0.56 ml, 9.35 mmol). The solution was stirred at room temperature for 48 h after which time a white precipitate appeared. The

precipitate was filtered and the filtrate was concentrated to 5-10 ml to give a second white precipitate, which was filtered. The two batches of (±)-2-hydroxy-2-(4'-quinolyl)nitroethane were combined and washed with ether, then used without further purification, 55%; υ_{max} (KBr disc) 3600-2900, 1590, 1550 (strong), 1370, 1100, 870 and 760 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.76 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 6.32 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 7.64-7.87 (complex, 3H), 8.00-8.24 (dd, 2H) and 8.92 (d,1H); δ_C ($CDCl_3$) 67.86 ($\underline{C}HOH$), 81.66 ($\underline{C}H_2NO_2$), 119.46 (Ar-C), 123.53 (Ar-C), 125.72 (Ar-C), 127.91 (Ar-C), 130.05 (Ar-C), 131.13 (Ar-C), 145.98 (Ar-C), 148.60 (Ar-C) and 151.21 (Ar-C); *m/z* 219 (M^+ +1), 218 (M^+), 157, 143, 129, 115, 101, and 75 (100%); Found: M^+, 218.0623. $C_{11}H_{10}N_2O_3$ requires M, 218.0701.

(±)-2-Hydroxy-2-(4'-quinolyl)ethylamine Dihydrochloride (83)

(±)-2-Hydroxy-2-(4'-quinolyl)nitroethane (240 mg, 1.10 mmol) was dissolved in dry methanol (20 ml) in a three necked round bottom flask equipped with a nitrogen balloon. To the solution was added 10% Pd/C catalyst (0.1 g) and anhydrous ammonium formate (377 mg, 5.99 mmol). The mixture was stirred under nitrogen at 25 ^oC for 15 h. The mixture was diluted with diethyl ether (40 ml) and then filtered through a Celite pad and evaporation of the solvent *in vacuo* gave the crude amine as a yellow oil. The crude amine was partitioned between dichloromethane (50 ml) and 6 M HCl (25 ml) and stirred for 2 h. The aqueous layer was separated, washed with CH_2Cl_2 (75 ml) and evaporated to dryness to yield the dihydrochloride salt (83) as a light green solid which was recrystallised from ethanol and acetone,

precipitate was filtered and the filtrate was concentrated to 5-10 ml to give a second white precipitate, which was filtered. The two batches of (±)-2-hydroxy-2-(4'-quinolyl)nitroethane were combined and washed with ether, then used without further purification, 55%; υ_{max} (KBr disc) 3600-2900, 1590, 1550 (strong), 1370, 1100, 870 and 760 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.76 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 6.32 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 7.64-7.87 (complex, 3H), 8.00-8.24 (dd, 2H) and 8.92 (d,1H); δ_C ($CDCl_3$) 67.86 ($\underline{C}HOH$), 81.66 ($\underline{C}H_2NO_2$), 119.46 (Ar-C), 123.53 (Ar-C), 125.72 (Ar-C), 127.91 (Ar-C), 130.05 (Ar-C), 131.13 (Ar-C), 145.98 (Ar-C), 148.60 (Ar-C) and 151.21 (Ar-C); *m/z* 219 (M^+ +1), 218 (M^+), 157, 143, 129, 115, 101, and 75 (100%).

(±)-2-Hydroxy-2-(4'-quinolyl)ethylamine Dihydrochloride (83)

(±)-2-Hydroxy-2-(4'-quinolyl)nitroethane (240 mg, 1.10 mmol) was dissolved in dry methanol (20 ml) in a three necked round bottom flask equipped with a nitrogen balloon. To the solution was added 10% Pd/C catalyst (0.1 g) and anhydrous ammonium formate (377 mg, 5.99 mmol). The mixture was stirred under nitrogen at 25 °C for 15 h. The mixture was diluted with diethyl ether (40 ml) and then filtered through a Celite pad and evaporation of the solvent *in vacuo* gave the crude amine as a yellow oil. The crude amine was partitioned between dichloromethane (50 ml) and 6 M HCl (25 ml) and stirred for 2 h. The aqueous layer was separated, washed with CH_2Cl_2 (75 ml) and evaporated to dryness to yield the dihydrochloride salt (83) as a light green solid which was recrystallised from ethanol and acetone, 54%; m.p. >250 °C; υ_{max} (KBr disc) 3600-3150, 3100-2750, 1615,

1530, 850 and 780 cm^{-1}; δ_H (200 MHz, D_2O) 3.21 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 5.81 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 7.66-8.14 (complex, 5H) and 8.89 (d, 1H); δ_C (D_2O) 45.13 ($\underline{C}H_2NH_2$), 66.52 ($\underline{C}HOH$), 119.78 (Ar-C), 121.87 (Ar-C), 124.73 (Ar-C), 126.49 (Ar-C), 131.41 (Ar-C), 135.75 (Ar-C), 137.74 (Ar-C), 144.72 (Ar-C) and 159.11 (Ar-C); *m/z* 188, 159, 130 (100%) and 36.

(±)-2-Hydroxy-2-(2'-pyridyl)ethylamine Dihydrochloride (97)

(±)-2-Hydroxy-2-(2'-pyridyl)nitroethane

2-Pyridinecarboxaldehyde (1.0 g, 9.35 mmol) was dissolved in isopropanol (10 ml) and to this solution was added KF (25 mg) and nitromethane (1.12 ml, 18.7 mmol). After stirring at room temperature for 3 h the solution was heated to 40 °C for a further 1 h. The solvent was removed *in vacuo* to yield (±)-2-hydroxy-2-(2'-pyridyl)nitroethane as a red oil. Purification on a silica gel column (hexane/ethyl acetate 2:3) produced the pure product as a orange solid, 80%; υ_{max} (KBr disc) 3600-2960, 1600, 1548, 1440 and 1377 (strong) cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.70 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 5.11 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H) and 7.29-8.54 (complex, 4H); δ_C ($CDCl_3$) 71.91 ($\underline{C}HOH$), 81.57 ($\underline{C}H_2NO_2$), 121.58 (Ar-C), 123.80 (Ar-C), 137.90 (Ar-C), 149.51 (Ar-C) and 159.76 (Ar-C); *m/z* 168 (M^+), 121, 103, 79 (100%), 52; Found: C, 50.00; H, 4.76; N, 16.67%. $C_7H_8N_2O_3$ requires: C, 50.01; H, 4.74; N, 16.60%.

used without further purification, 90%; υ_{max} (KBr disc) 3610-3150, 2980-2810, 1600, 1575 (strong), 1380 and 620 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.60 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 5.43 (ABX, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H) and 7.76-8.58 (complex, 4H); δ_C ($CDCl_3$) 70.50 (CHOH), 80.71 (CH_2NO_2), 120.86 (Ar-C), 123.23 (Ar-C), 137.27 (Ar-C), 148.85 (Ar-C) and 156.67 (Ar-C); *m/z* 168 (M^+), 121 and 36 (100%).

(±)-2-Hydroxy-2-(3'-pyridyl)ethylamine Dihydrochloride (98)

(±)-2-Hydroxy-2-(3'-pyridyl)nitroethane (1.2 g, 7.15 mmol) was dissolved in methanol (120 ml). To this was added 10% Pd/C catalyst (0.48 g) and the mixture was hydrogenated at *ca.* 60 p.s.i. at 25 °C for 14 h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (50 ml) was then added. Evaporation of the solvent gave the crude dihydrochloride salt (98) which was recrystallised from ethanol and acetone, 65%; m.p. > 250 °C; υ_{max} (KBr disc) 3490-3280, 3050-2740, 1630, 1600 and 640 cm^{-1}; δ_H (200 MHz, D_2O) 3.18 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 5.14 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H) and 7.94 (complex, 1H) and 8.25-8.80 (complex, 3H); δ_C (D_2O) 45.45 (CH_2NH_2), 67.16 (CHOH), 128.40 (Ar-C), 140.03 (Ar-C), 141.48 (Ar-C), 141.90 (Ar-C) and 145.59 (Ar-C); *m/z* 139 (M^+ +1), 109 (100%), 78 and 36; Found: C, 39.67; H, 5.56; N, 13.33%. $C_7H_{12}N_2OCl_2$ requires: C, 39.81; H, 5.69; N, 13.27%.

(±)-2-Hydroxy-2-(4'-pyridyl)ethylamine Dihydrochloride (99)

(±)-2-Hydroxy-2-(4'-pyridyl)nitroethane

4-Pyridinecarboxaldehyde (2.0 g, 18.7 mmol) was dissolved in isopropanol (20 ml) and to this solution was added KF (25 mg) and nitromethane (2.24 ml, 37.4 mmol). The mixture was stirred for 24 h at room temperature giving rise to a yellow solution. The solvent was removed *in vacuo* leaving (±)-2-hydroxy-2-(4'-pyridyl)-nitroethane as a red/orange oil. Purification on a silica gel column (ethyl acetate 100%) gave an orange solid which was used without further purification, 70%; υ_{max} (KBr disc) 3550-2720, 1605, 1550 (strong), 1380 and 610 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.54 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 5.48 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 7.40 (d, with fine splitting, 2H) and 8.57 (d, with fine splitting, 2H); δ_C ($CDCl_3$) 69.41 ($\underline{C}HOH$), 81.10 ($\underline{C}H_2NO_2$), 121.12 (2Ar-C) 148.63 (2Ar-C) and 149.92(Ar-C); *m/z* 168 (M^+), 121 (100%) and 36.

(±)-2-Hydroxy-2-(4'-pyridyl)ethylamine Dihydrochloride (99)

(±)-2-Hydroxy-2-(4'-pyridyl)nitroethane (1.0 g, 5.96 mmol) was dissolved in methanol (75 ml). To this was added 10% Pd/C catalyst (0.4 g) and the mixture was hydrogenated at *ca.* 40 p.s.i. at 25 °C for 25 h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (40 ml) was added. Evaporation of the solvent *in vacuo* gave the crude dihydrochloride salt (99) which was recrystallised from ethanol and acetone, 75%; m.p. > 250 °C;

υ_{max} (KBr disc) 3320-3200, 3070-2600, 1630, 1605, 1465, 800 and 670 cm^{-1}; δ_H (200 MHz, D_2O) 3.15 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz, 2H), 5.16 (dd, J_{AX} 4 Hz J_{BX} 9 Hz, 1H), 8.02 (d, with fine splitting, 2H) and 8.60 (d, with fine splitting, 2H); δ_C (D_2O) 45.11 ($\underline{C}H_2NH_2$), 68.79 ($\underline{C}HOH$), 125.64 (2Ar-C), 142.32 (2Ar-C) and 161.68 (Ar-C); *m/z* 139 (M^+ +1) and 109 (100%), 80, 36; Found: C, 39.90; H, 5.75; N, 13.30%. $C_7H_{12}N_2OCl_2$ requires: C, 39.81; H, 5.69; N, 13.27%.

Synthesis of Quinolyl and Pyridyl Propylamines

General Procedure (E) for the Synthesis of Nitropropanes

Aromatic carboxaldehyde (18.7 mmol) was dissolved in isopropanol and to this solution was added KF (25 mg) and nitropropane (2.28 g, 37.4 mmol).

(i) The mixture was stirred for 4 h at 55-60 ^oC and subsequently for 1 h at room temperature producing a yellow solution. The solvent was removed *in vacuo* to yield the aromatic nitropropane as an oil. The oil was then purified on a silica gel column (hexane/ethyl acetate 2:3).

(ii) The mixture was stirred at room temperature for 24 h, the solvent was removed *in vacuo* to yield the aromatic nitropropane as an oil. The oil was then purified on a silica gel column (hexane/ethyl acetate 1:1).

(iii) The mixture was stirred at room temperature for 24 h after which time a white precipitate appeared. The solution was stirred for a further 24 h and then filtered. The filtrate was concentrated to 5-10 ml yielding a second precipitate which was filtered. The

two batches of aromatic nitropropane were combined and washed with ether, then used without further purification.

(±)-3-Hydroxy-3-(2'-quinolyl)-2-nitropropane (84)

Using 2-quinolinecarboxaldehyde in general procedure E(i) gave a racemic mixture of (±)-3-hydroxy-3-(2'-quinolyl)-2-nitropropane (84), in a 3:1 ratio as an orange oil, 61%; υ_{max} (KBr disc) 3400-2945, 1620, 1555 (strong), 1390, 1110, 790 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) Major Diastereoisomer, 1.39 (complex, 3H), 4.60 (complex, 1H), 5.11 (complex, 1H) and 7.49-8.21 (complex, 6H); δ_C ($CDCl_3$) Major Diastereoisomer, 16.04 ($\underline{C}H_3CHNO_2$), 74.94 ($\underline{C}HOH$), 86.95 ($\underline{C}HNO_2$), 118.75 (Ar-C), 122.13 (Ar-C), 129.72 (Ar-C), 129.82 (Ar-C), 130.76 (Ar-C), 131.04 (Ar-C), 137.90 (Ar-C), 147.80 (Ar-C) and 157.00 (Ar-C); δ_H (200 MHz, $CDCl_3$) Minor Diastereoisomer, 1.42 (complex, 3H), 4.71 (d, 1H), 5.28 (complex, 1H) and 7.49-8.21 (complex, 6H); δ_C ($CDCl_3$) Minor Diastereoisomer, 16.18 ($\underline{C}H_3CHNO_2$), 74.99 ($\underline{C}HOH$), 87.01 ($\underline{C}HNO_2$), 118.80 (Ar-C), 122.18 (Ar-C), 129.81 (Ar-C), 129.90 (Ar-C), 130.82 (Ar-C), 131.10 (Ar-C), 137.94 (Ar-C), 148.01 (Ar-C) and 157.08 (Ar-C); *m/z* 233 (M^+ +1), 232 (M^+), 157, 128 (100%), 115, 102, and 75.

(±)-3-Hydroxy-3-(3'-quinolyl)-2-nitropropane (85)

Using 3-quinolinecarboxaldehyde in general procedure E(ii) gave a racemic mixture of (±)-3-hydroxy-3-(3'-quinolyl)-2-nitropropane (85), in a 3:1 ratio as an orange oil, 88%; υ_{max} (KBr disc) 3590-2930, 1620, 1560 (strong), 1390, 1055, 1035, 790 and 760 cm^{-1}; δ_H (200 MHz, $CDCl_3$) Major Diastereoisomer, 1.35

(complex, 3H), 4.69 (d, 1H), 5.70 (complex, 1H), 7.70-9.04 (complex, 6H); δ_C ($CDCl_3$) Major Diastereoisomer, 16.10 ($\underline{C}H_3$-$CHNO_2$), 73.52 ($\underline{C}HOH$), 87.18 ($\underline{C}HNO_2$), 127.61 (Ar-C), 128.21 (Ar-C), 128.43 (Ar-C), 128.98 (Ar-C), 130.06 (Ar-C), 131.72 (Ar-C), 134.05 (Ar-C), 148.91 (Ar-C) and 150.12 (Ar-C); δ_H (200 MHz, $CDCl_3$) Minor Diastereoisomer, 1.40 (complex, 3H), 4.72 (d, 1H), 5.73 (complex, 1H), 7.70-9.04 (complex, 6H); δ_C ($CDCl_3$) Minor Diastereoisomer, 16.16 ($\underline{C}H_3$-$CHNO_2$), 73.62 ($\underline{C}HOH$), 87.23 ($\underline{C}HNO_2$), 127.69 (Ar-C), 128.33 (Ar-C), 128.50 (Ar-C), 129.09 (Ar-C), 130.16 (Ar-C), 131.78 (Ar-C), 134.12 (Ar-C), 149.00 (Ar-C) and 150.19 (Ar-C); *m/z* 233 (M^+ +1), 232 (M^+), 157 (100%), 128, 115, 101, and 75; Found: M^+, 232.0797. $C_{12}H_{12}N_2O_3$ requires M, 232.0871.

(±)-3-Hydroxy-3-(4'-quinolyl)-2-nitropropane (86)

Using 4-quinolinecarboxaldehyde in general procedure E(iii) gave (±)-3-hydroxy-3-(4'-quinolyl)-2-nitropropane (86) as a white powder, 65%; υ_{max} (KBr disc) 3590-3150, 1620, 1560 (strong), 1380, 1070, 1035 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.35 (d, J 2 Hz, 3H), 4.65 (d, J 6 Hz, 1H), 5.74 (complex, 1H) and 7.60-8.16 (complex, 4H), 8.45 (d, 1H) and 9.12 (d,1H); δ_C ($CDCl_3$) 16.16 ($\underline{C}H_3$-$CHNO_2$), 71.20 ($\underline{C}HOH$), 87.85 ($\underline{C}HNO_2$), 120.08 (Ar-C), 124.00 (Ar-C), 125.64 (Ar-C), 127.38 (Ar-C), 129.48 (Ar-C), 130.17 (Ar-C), 145.79 (Ar-C), 148.09 (Ar-C) and 150.47 (Ar-C); *m/z* 233 (M^+ +1), 232 (M^+), 157, 129 (100%), 102, and 75; Found: M^+, 232.0838. $C_{12}H_{12}N_2O_3$ requires M, 232.0846.

(±)-3-Hydroxy-3-(2'-pyridyl)-2-nitropropane

Using 2-pyridinecarboxaldehyde in general procedure E(i) gave a racemic mixture of (±)-3-hydroxy-3-(2'-pyridyl)-2-nitropropane, in a 3:1 ratio as an orange oil, 74%; υ_{max} (KBr disc) 3560-2945, 1600, 1555 (strong), 1395, 1110, 795 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) Major Diastereoisomer, 1.44 (complex, 3H), 4.80 (d, 1H), 5.42 (complex, 1H) and 7.26-7.80 (complex, 4H); δ_C ($CDCl_3$) Major Diastereoisomer, 15.58 ($\underline{C}H_3$-$CHNO_2$), 74.96 ($\underline{C}HOH$), 87.56 ($\underline{C}HNO_2$), 122.16 (Ar-C), 123.84 (Ar-C), 137.39 (Ar-C), 149.16 (Ar-C) and 156.60 (Ar-C); δ_H (200 MHz, $CDCl_3$) Minor Diastereoisomer, 1.47 (complex, 3H), 4.88 (d, 1H), 5.50 (complex, 1H) and 7.26-7.80 (complex, 4H); δ_C ($CDCl_3$) Minor Diastereoisomer, 15.63 ($\underline{C}H_3$-$CHNO_2$), 75.02 ($\underline{C}HOH$), 87.64 ($\underline{C}HNO_2$), 122.25 (Ar-C), 123.95 (Ar-C), 137.45 (Ar-C), 149.28 (Ar-C) and 156.68 (Ar-C); *m/z* 183 (M^+ +1), 136, 118, 107, and 79 (100%).

(±)-3-Hydroxy-3-(3'-pyridyl)-2-nitropropane

Using 3-pyridinecarboxaldehyde in general procedure E(ii) gave a racemic mixture of (±)-3-hydroxy-3-(3'-pyridyl)-2-nitropropane, in a 3:2 ratio as an orange oil, 81%; υ_{max} (KBr disc) 3600-3000, 1590, 1555 (strong), 1390, 1095 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) Major Diastereoisomer, 1.47 (d, 3H), 4.83 (d, 1H), 5.53 (complex, 1H), 7.33-8.19 (complex, 4H); δ_C ($CDCl_3$) Major Diastereoisomer, 15.32 ($\underline{C}H_3$-$CHNO_2$), 72.68 ($\underline{C}HOH$), 87.75 ($\underline{C}HNO_2$), 123.61 (Ar-C), 135.15 (Ar-C), 147.04 (Ar-C), 148.31 (Ar-C) and 153.24 (Ar-C); δ_H (200 MHz, $CDCl_3$) Minor Diastereoisomer, 1.50 (d, 3H), 4.87 (d, 1H), 5.60 (complex, 1H), 7.33-8.19 (complex, 4H); δ_C ($CDCl_3$) Minor Diastereoisomer, 15.40 ($\underline{C}H_3$-$CHNO_2$), 72.80 ($\underline{C}HOH$),

87.83 ($\underline{C}HNO_2$), 123.72 (Ar-C), 135.21 (Ar-C), 147.09 (Ar-C), 148.39 (Ar-C) and 153.31 (Ar-C); *m/z* 183 (M^+ +1), 182 (M^+), 135, 117, 107 (100%) and 78; Found: M^+, 182.0641. $C_8H_{10}N_2O_3$ requires M, 182.0684.

(±)-3-Hydroxy-3-(4'-pyridyl)-2-nitropropane

Using 4-pyridinecarboxaldehyde in general procedure E(iii) gave (±)-3-hydroxy-3-(4'-pyridyl)-2-nitropropane as a white powder, 83%; υ_{max} (KBr disc) 3620-3005, 1605, 1555 (strong), 1395, 1075, 830 and 620 cm^{-1}; δ_H (200 MHz, $CDCl_3$), 1.32 (d, J 4 Hz, 3H), 4.87 (d, J 6 Hz, 1H), 5.38 (complex, 1H), 7.45 (d, 2H) and 8.59 (d, 2H); δ_C ($CDCl_3$) 15.78 ($\underline{C}H_3$-$CHNO_2$), 73.74 ($\underline{C}HOH$), 87.72 ($\underline{C}HNO_2$), 122.33 (2Ar-C), 149.68 (Ar-C) and 149.76 (2Ar-C); *m/z* 183 (M^+ +1), 182 (M^+), 135, 117, 107 (100%), 94 and 78; Found: M^+, 182.0615. $C_8H_{10}N_2O_3$ requires M, 182.0680.

<u>General Procedure (F) for the Reduction of Nitropropanes to Propylamines</u>

The aromatic nitropropane (2.98 mmol) was dissolved in methanol (50 ml). To this was added 10% Pd/C catalyst (0.2 g) and the mixture was hydrogenated at *ca.* 40-60 p.s.i. at 25 ^oC for 15-24 h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (25 ml) was added. Evaporation of the solvent *in vacuo* gave the crude dihydrochloride salt and recrystallisation was attempted from ethanol and acetone.

(±)-3-Hydroxy-3-(2'-quinolyl)propyl-2-amine Dihydrochloride (87)

Using (±)-3-hydroxy-3-(2'-quinolyl)-2-nitropropane (84) in general procedure F gave a racemic mixture of (±)-3-hydroxy-3-(2'-quinolyl)propyl-2-amine dihydrochloride (87), in a 3:1 ratio as a brown solid, 40%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3600, 3100-2740, 1605, 1550 and 840 cm^{-1}; δ_H (200 MHz, D_2O) Major Diastereoisomer, 0.94 (d, 3H), 3.40 (complex, 1H), 5.40 (d, 1H) and 7.41-8.09 (complex, 6H); δ_C (D_2O) Major Diastereoisomer, 16.08 ($\underline{C}H_3$-CHNH$_2$), 49.54 ($\underline{C}$HNH$_2$), 71.02 ($\underline{C}$HOH), 118.34 (Ar-C), 121.29 (Ar-C), 130.12 (Ar-C), 130.38 (Ar-C), 131.61 (Ar-C), 133.10 (Ar-C), 135.52 (Ar-C), 143.99 (Ar-C) and 158.71 (Ar-C); δ_H (200 MHz, D_2O) Minor Diastereoisomer, 0.99 (d, 3H), 3.42 (complex, 1H), 5.45 (d, 1H) and 7.41-8.09 (complex, 6H); δ_C (D_2O) Minor Diastereoisomer, 16.17 ($\underline{C}H_3$-CHNH$_2$), 49.60 ($\underline{C}$HNH$_2$), 71.10 ($\underline{C}$HOH), 118.39 (Ar-C), 121.38 (Ar-C), 130.16 (Ar-C), 138.47 (Ar-C), 131.70 (Ar-C), 133.15 (Ar-C), 135.63 (Ar-C), 144.11 (Ar-C) and 158.78 (Ar-C); *m/z* 202, 130 (100%) and 36.

(±)-3-Hydroxy-3-(3'-quinolyl)propyl-2-amine Dihydrochloride (88)

Using (±)-3-hydroxy-3-(3'-quinolyl)-2-nitropropane (85) in general procedure F gave a racemic mixture of (±)-3-hydroxy-3-(3'-quinolyl)propyl-2-amine dihydrochloride (88), in a 3:1 ratio as a cream solid, 35%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3610, 3075-2770, 1600, 1545 and 785 cm^{-1}; δ_H (200 MHz, D_2O) Major Diastereoisomer, 1.00 (d, 3H), 3.45 (complex, 1H), 5.46 (d, 1H), 7.67-7.85 (complex, 3H), 8.61 (dd, 2H) and 8.88(d, 1H); δ_C (D_2O) Major

Diastereoisomer, 17.56 ($\underline{C}H_3$-$CHNH_2$), 51.39 ($\underline{C}HNH_2$), 66.21 ($\underline{C}HOH$), 128.44 (Ar-C), 128.99 (Ar-C), 129.23 (Ar-C), 129.78 (Ar-C), 130.86 (Ar-C), 131.11 (Ar-C), 134.20 (Ar-C), 149.13 (Ar-C) and 160.06 (Ar-C); δ_H (200 MHz, D_2O) Minor Diastereoisomer, 1.12 (d, 3H), 3.51 (complex, 1H), 5.50 (d, 1H), 7.67-7.85 (complex, 3H), 8.67 (dd, 2H) and 8.93(d, 1H); δ_C (D_2O) Minor Diastereoisomer, 17.62 ($\underline{C}H_3$-$CHNH_2$), 51.47 ($\underline{C}HNH_2$), 66.30 ($\underline{C}HOH$), 128.53 (Ar-C), 129.03 (Ar-C), 129.30 (Ar-C), 129.86 (Ar-C), 130.95 (Ar-C), 131.24 (Ar-C), 134.30 (Ar-C), 149.18 (Ar-C) and 160.10 (Ar-C); *m/z* 203, 130 (100%) and 36.

(±)-3-Hydroxy-3-(4'-quinolyl)propyl-2-amine Dihydrochloride (89)

Using (±)-3-hydroxy-3-(4'-quinolyl)-2-nitropropane (86) in general procedure F gave (±)-3-hydroxy-3-(4'-quinolyl)propyl-2-amine dihydrochloride (89) as a white solid, 77%; m.p. >250 °C; υ_{max} (KBr disc) 3650-3620, 3100-2600, 1620, 1545 and 780 cm^{-1}; δ_H (200 MHz, D_2O) 0.92 (d, 3H), 3.38 (complex, 1H), 5.35 (d, 1H), 7.49-7.89 (complex, 4H), 8.01 (dd, 1H) and 8.72(d, 1H); δ_C (D_2O) 16.11 ($\underline{C}H_3$-$CHNH_2$), 53.13 ($\underline{C}HNH_2$), 70.43 ($\underline{C}HOH$), 120.76 (Ar-C), 122.01 (Ar-C), 125.34 (Ar-C), 127.15 (Ar-C), 131.37 (Ar-C), 135.88 (Ar-C), 138.11 (Ar-C), 144.66, (Ar-C) and 158.86 (Ar-C); *m/z* 202, 130 (100%) and 36.

(±)-3-Hydroxy-3-(2'-pyridyl)propyl-2-amine Dihydrochloride (90)

Using (±)-3-hydroxy-3-(2'-pyridyl)-2-nitropropane in general procedure F gave a racemic mixture of (±)-3-hydroxy-3-(2'-

pyridyl)propyl-2-amine dihydrochloride (90), in a 3:1 ratio as a light green solid, 75%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3620, 3100-2500, 1620, 1550 and 830 cm^{-1}; δ_H (200 MHz, D_2O) Major Diastereoisomer, 1.20 (d, 3H), 3.57 (complex, 1H), 5.04 (d, 1H), 7.84-8.50 (complex, 3H) and 8.59 (d, 1H); δ_C (D_2O) Major Diastereoisomer, 15.79 ($\underline{C}H_3$-$CHNH_2$), 52.48 ($\underline{C}HNH_2$), 71.14 ($\underline{C}HOH$), 126.59 (Ar-C), 127.97 (Ar-C), 142.52 (Ar-C), 148.13 (Ar-C) and 153.90 (Ar-C); δ_H (200 MHz, D_2O) Minor Diastereoisomer, 1.26 (d, 3H), 3.60 (complex, 1H), 5.11 (d, 1H), 7.84-8.50 (complex, 3H) and 8.64 (d, 1H); δ_C (D_2O) Minor Diastereoisomer, 15.83 ($\underline{C}H_3$-$CHNH_2$), 52.55 ($\underline{C}HNH_2$), 71.18 ($\underline{C}HOH$), 126.66 (Ar-C), 128.04 (Ar-C), 142.60 (Ar-C), 148.21 (Ar-C) and 153.98 (Ar-C); *m/z* 152, 123, 109 (100%), 93, 80 and 36.

(±)-3-Hydroxy-3-(3'-pyridyl)propyl-2-amine Dihydrochloride (91)

Using (±)-3-hydroxy-3-(3'-pyridyl)-2-nitropropane in general procedure F gave a racemic mixture of (±)-3-hydroxy-3-(3'-pyridyl)propyl-2-amine dihydrochloride (91), in a 3:2 ratio as a creamy white solid, 32%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3300-3560, 3200-2700, 1600, 1560 and 770 cm^{-1}; δ_H (200 MHz, D_2O) Major Diastereoisomer, 0.91 (d, 3H), 3.28 (complex, 1H), 4.98 (d, 1H), 7.71 (complex, 1H) and 8.13-8.59 (complex, 3H); δ_C (D_2O) Major Diastereoisomer, 15.64 ($\underline{C}H_3$-$CHNH_2$), 51.70 ($\underline{C}HNH_2$), 72.56 ($\underline{C}HOH$), 130.37 (Ar-C), 141.47 (Ar-C), 141.58 (Ar-C), 141.96 (Ar-C) and 144.23 (Ar-C); δ_H (200 MHz, D_2O) Minor Diastereoisomer, 0.98 (d, 3H), 3.33 (complex, 1H), 5.06 (d, 1H), 7.80 (complex, 1H) and 8.13-8.68 (complex, 3H); δ_C (D_2O) Minor Diastereoisomer, 15.71 ($\underline{C}H_3$-$CHNH_2$), 51.80 ($\underline{C}HNH_2$), 72.62 ($\underline{C}HOH$), 130.46 (Ar-C), 141.50 (Ar-C),

141.63 (Ar-C), 142.09 (Ar-C) and 144.30 (Ar-C); *m/z* 151, 123, 109, 93, 80 (100%) and 36.

(±)-3-Hydroxy-3-(4'-pyridyl)propyl-2-amine Dihydrochloride (92)

Using (±)-3-hydroxy-3-(4'-pyridyl)-2-nitropropane in general procedure F gave (±)-3-hydroxy-3-(4'-pyridyl)propyl-2-amine dihydrochloride (92) as a sticky green solid, 69%; υ_{max} (KBr disc) 3710-3320, 3200-2600, 1605, 800 and 670 cm^{-1}; δ_H (200 MHz, D_2O) 0.86 (d, 3H), 3.21 (complex, 1H), 4.63 (d, 1H), 7.35 (d, 2H) and 8.39 (d, 2H); δ_C (D_2O) 15.72 ($\underline{C}H_3$-$CHNH_2$), 52.86 ($\underline{C}HNH_2$), 73.10 ($\underline{C}HOH$), 126.31 (2Ar-C), 142.29 (2Ar-C) and 161.40 (Ar-C); *m/z* 152, 123, 109, 80 (100%) and 36.

Synthesis of Quinolylmethylamine Dihydrochlorides

General Procedure(G) for the Synthesis of Amides from Acids[131]

NH_3(g) was bubbled through acetonitrile (approx. 20 ml) until the acetonitrile was saturated (15-20 min). The quinoline carboxylic acid (1.34 g, 7.74 mmol) was partially dissolved in acetonitrile (60 ml) (not the basified solution) and triethylamine (1.2 equivalents, 9.29 mmol) was added as the acid dissolved. Isobutylchloroformate (1.2 equivalents, 9.29 mmol) was added at -5 ^{o}C, slowly over a few min. This forms the anhydride *in situ.* After waiting for 5 min the acetonitrile saturated with NH_3 (20 ml) was added giving a white precipitate. This mixture was left at 0 ^{o}C for 2 h. The white precipitate was filtered and the filtrate was

concentrated to dryness giving a creamy white solid. The solid was taken up in ethyl acetate (25 ml) and washed with dilute sodium bicarbonate. The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness to yield a white solid, which was used without further purification.

2-Quinolinecarboxamide (93)

Using 2-quinolinecarboxylic acid in general procedure G gave 2-quinolinecarboxamide (93) as a creamy white powder, 86%; υ_{max} (KBr disc) 3430, 3200-3050, 1690, 1620, 1560, 875 and 770 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.78 (br s, 2H), 7.37-7.84 (complex, 5H) and 8.49 (d, 1H); δ_C ($CDCl_3$) 120.31 (Ar-C), 125.48 (Ar-C), 126.20 (Ar-C), 126.53 (Ar-C), 127.60 (Ar-C), 127.95 (Ar-C), 135.37 (Ar-C), 143.85 (Ar-C), 155.76 (Ar-C) and 169.74 (C=O); *m/z* 173 (M^+ +1), 172 (M^+), 156, 128, 79 (100%) and 28; Found: M^+, 172.0611. $C_{10}H_8N_2O$ requires M, 172.0633.

3-Quinolinecarboxamide

Using 3-quinolinecarboxylic acid in general procedure G gave 3-quinolinecarboxamide as a white powder, 80%; υ_{max} (KBr disc) 3435, 3150-3050, 1690, 1615, 1550 and 690 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.69 (br s, 2H), 7.36-7.78 (complex, 4H), 8.31 (d, 1H) and 9.00 (d, 1H); δ_C ($CDCl_3$) 126.42 (Ar-C), 127.26 (Ar-C), 127.32 (Ar-C), 127.99 (Ar-C), 128.34 (Ar-C), 132.60 (Ar-C), 134.17 (Ar-C), 147.53 (Ar-C), 149.74 (Ar-C) and 169.92 (C=O); *m/z* 172 (M^+), 156, 128, 79 (100%) and 28; Found: M^+, 172.0645. $C_{10}H_8N_2O$ requires M, 172.0638.

4-Quinolinecarboxamide

Using 4-quinolinecarboxylic acid in general procedure G gave 4-quinolinecarboxamide as a white powder, 89%; υ_{max} (KBr disc) 3435, 3150-3050, 1690, 1615, 1550, 875 and 760 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.60 (br s, 2H), 7.41-7.60 (complex, 3H), 7.82-7.97 (dd, 2H) and 8.73 (d,1H); δ_C ($CDCl_3$) 118.80 (Ar-C), 120.69 (Ar-C), 122.06 (Ar-C), 125.83 (Ar-C), 128.82 (Ar-C), 129.47 (Ar-C), 143.59 (Ar-C), 146.30 (Ar-C) and 149.07 (Ar-C) and 169.02 (C=O); *m/z* 173 (M^+ +1), 172 (M^+), 157, 128, 79 (100%) and 28; Found: M^+, 172.0580. $C_{10}H_8N_2O$ requires M, 172.0630.

General Procedure (H) for the Synthesis of Amines from Amides[132]

To a solution of 1 M BH_3 in THF (23.2 ml, 23.2 mmol) was added the quinolinecarboxamide (1 g, 5.81 mmol) in THF over a 15 min period, maintaining the temperature at 0 ^oC during the addition. The solution was brought to reflux and maintained for 24 h, then the solution was allowed to cool. Hydrochloric acid (6 M) was added slowly (dropwise at first) until no more H_2 was given off and the solution was at pH 1. The THF was removed at atmospheric pressure and the H_2O was removed under reduced pressure to yield a white solid. MeOH (10 ml) and conc. HCl (2-3 drops) were added to the filtrate in order to remove any boric acid as the borate methyl ester. The resulting solution was evaporated to dryness giving a creamy white solid of diamine dihydrochloride. The solid was crystallised from 95% aqueous ethanol.

2-Quinolylmethylamine Dihydrochloride (94)

Using 2-quinolinecarboxamide (93) in general procedure H gave 2-quinolylmethylamine dihydrochloride (94) as white crystals, 45%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3340-3200, 2950, 1605, 1575, 1490, 870 and 770 cm^{-1}; δ_H (D_2O) 4.61 (s, 2H), 7.64-7.98 (complex, 5H) and 8.32 (d, 1H); δ_C (D_2O) 41.66 ($\underline{C}H_2N$), 120.52 (Ar-C), 124.21 (Ar-C), 125.97 (Ar-C), 127.01 (Ar-C), 128.46 (Ar-C), 129.78 (Ar-C), 137.12 (Ar-C), 147.52 (Ar-C) and 158.38 (Ar-C); *m/z* 158, 143, 129, 79 (100%) and 28; Found: C, 51.91; H, 5.22; N, 12.13%. $C_{10}H_{12}N_2Cl_2$ requires: C, 51.95; H, 5.19; N, 12.12%.

3-Quinolylmethylamine Dihydrochloride (95)

Using 3-quinolinecarboxamide in general procedure H gave 3-quinolylmethylamine dihydrochloride (95) as white crystals, 37%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3290-3170, 2950, 1610, 1570, 1490, 790 and 770 cm^{-1}; δ_H (D_2O) 4.49 (2H, s), 7.36-7.81 (complex, 4H), 8.26 (d, 1H) and 8.87 (d, 1H); δ_C (D_2O) 40.42 ($\underline{C}H_2N$), 123.27 (Ar-C), 123.96 (2Ar-C), 124.55 (2Ar-C), 125.51 (Ar-C), 126.72 (Ar-C), 130.07 (Ar-C), 143.63 (Ar-C) and 145.40 (Ar-C); *m/z* 231 (M^++1), 157, 143, 128, 79 (100%) and 28; Found: C, 51.94; H, 5.24; N, 12.10%. $C_{10}H_{12}N_2Cl_2$ requires: C, 51.95; H, 5.19; N, 12.12%.

4-Quinolylmethylamine Dihydrochloride (96)

Using 4-quinolinecarboxamide in general procedure H gave 4-quinolylmethylamine dihydrochloride (96) as white crystals, 52%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3390-2960, 1620, 1580, 1485 and 620 cm^{-1}; δ_H (D_2O) 4.59 (2H, s), 7.49-7.61 (complex, 3H), 7.93-8.02 (dd, 2H) and 8.85 (d, 1H); δ_C (D_2O) 42.83 ($\underline{C}H_2N$), 120.43 (Ar-C), 121.51 (Ar-C), 123.18 (Ar-C), 125.38 (Ar-C), 129.94 (Ar-C), 134.16 (Ar-C), 136.75 (Ar-C), 143.42 (Ar-C) and 156.93 (Ar-C); *m/z* 231 (M^+ +1), 158, 128, 79 (100%) and 28; Found: C, 51.90; H, 5.21; N, 12.14%. $C_{10}H_{12}N_2Cl_2$ requires: C, 51.95; H, 5.19; N, 12.12%.

<u>Attempted Synthesis of 2-(2'-Quinolyl)nitroethene (79)</u>

1st Attempt: From 2-Quinolinecarboxaldehyde using NaOH as Base

2-Quinolinecarboxaldehyde (1.73 g, 11 mmol) and nitromethane (693 mg, 11 mmol) in MeOH (20 ml) were kept at 10-15 ^{o}C while NaOH solution (460 mg in 1.6 ml of H_2O, 7 M) was added very slowly dropwise. The reaction mixture went dark red immediately, turning yellow/brown after being allowed to stand for 15 min. Ice water was added to the solution, and the solution was then added to HCl (approx. 20 ml of 4 M) giving a green solution. The acidic solution was concentrated to 5 ml yielding a light creamy solid which was filtered off. Total weight of solid was 1.66 g; [note the white solid appeared to be the HCl salt of (±)-2-hydroxy-2-(2'-quinolyl)nitroethane (78), soluble in H_2O and not in $CHCl_3$]; δ_H (200 MHz, D_2O) 4.98 (ABX system, J_{AB} 11 Hz J_{AX} 3 Hz J_{BX} 7 Hz, 2H), 5.86

(dd, J_{AX} 3 Hz J_{BX} 7 Hz, 1H), 7.58-8.01 (complex, 5H) and 8.76 (d, 1H); δ_C (D_2O) 68.04 ($\underline{C}H_2NO_2$), 79.60 ($\underline{C}HOH$), 119.99 (Ar-C), 120.85 (Ar-C), 129.01 (Ar-C), 129.89 (Ar-C), 130.88 (Ar-C), 136.12 (Ar-C), 138.22 (Ar-C), 148.37 (Ar-C) and 157.09 (Ar-C); *m/z* 219 (M^+ +1), 218 (M^+), 171, 158, 143, 129 (100%), 115, 101, and 77.

2nd Attempt: From 2-Quinolinecarboxaldehyde using N-Amylamine as Base

2-Quinolinecarboxaldehyde (849 mg, 5.40 mmol) was dissolved in MeOH (20 ml), then nitromethane (329 mg, 5.40 mmol) and *N*-amylamine (47 mg, 0.54 mmol) were added. The mixture was stored in a dark place at room temperature for 14 d giving a dark coloured solution. The solvent was removed *in vacuo* to yield a dark oil. Purification on a silica gel column (hexane/ethyl acetate 2:3) gave starting materials.

3rd Attempt: Dehydration of 2-Hydroxy-2-(2'-quinolyl)nitroethane (78) using Acetic Anhydride and 4-Dimethylaminopyridine

2-Hydroxy-2-(2'-quinolyl)nitroethane (78) (0.65 g, 2.98 mmol) was dissolved in acetone (30 ml) and to this was added acetic anhydride (0.31 g, 3.04 mmol) and DMAP (5 mg). The solution was stirred at room temperature for 20 h, then filtered and evaporated under reduced pressure leaving a dark solid. Purification on a silica gel column (hexane/ethyl acetate 1:1) gave a dark green solid which decomposed immediately on standing leaving a black tar which was insoluble in all common organic solvents.

4th Attempt: Dehydration of 2-Hydroxy-2-(2'-quinolyl)-nitroethane (78) using Molecular Sieves and Hydrochloric Acid

2-Hydroxy-2-(2'-quinolyl)nitroethane (78) (900 mg, 4.13 mmol) was dissolved in dry THF (50 ml) which contained activated molecular sieves. The solution was heated at reflux overnight. The solution was then cooled and HCl (50 ml, 5 M) was added. The stirred solution was again heated at reflux for a further 4 h. After cooling the solution was filtered and evaporated to dryness under reduced pressure leaving only the hydrochloride salt of the starting material.

5th Attempt: Dehydration of 2-Hydroxy-2-(2'-quinolyl)-nitroethane (78) using Sodium Hydroxide

2-Hydroxy-2-(2'-quinolyl)nitroethane (78) (0.32 g, 1.2 mmol) was dissolved in THF (30 ml) and NaOH (15 ml, 5 M) was then added. The mixture was then heated at reflux for 24 h after which it was cooled and the solvent was removed *in vacuo* leaving an orange oil. Purification on a silica gel column (hexane/ethyl acetate, 2:3) gave an orange solid. This solid was starting material.

6th Attempt: Dehydration of 2-Hydroxy-2-(2'-quinolyl)-nitroethane (78) using Phosphorus Oxychloride

A solution of 2-hydroxy-2-(2'-quinolyl)nitroethane (78) (0.32 g, 1.2 mmol) in pyridine (12 ml) was cooled to 0 ^{o}C and $POCl_3$ (20 ml, 21.4 mmol) was added dropwise. The mixture was stirred overnight at room temperature and then decomposed by the

cautious addition of H_2O (10 ml). The solution was extracted with diethyl ether (2 x 20 ml), and washed with H_2O (2 x 20 ml) then brine (2 x 20 ml). After drying (Na_2SO_4) the solution was evaporated to dryness leaving a black sticky solid which was insoluble in all the common solvents. TLC showed at least six different compounds. Purification was not undertaken because of the insolubility of the solid.

8.5 Experimental for Chapter Six

Synthesis of Thienyl and Pyrryl Ethylamines and Propylamines

General Procedure (J) for the Synthesis of Nitro Vinyl-Thiophenes[136]

In a 3-necked round bottom flask fitted with a thermometer, mechanical stirrer and a separatory funnel, the thiophenecarboxaldehyde (3.70 g, 33 mmol) and nitroalkane (33 mmol) were dissolved in MeOH (10 ml). The solution was stirred and the temperature was kept between 10-15 ^{o}C while a solution of NaOH (1.4 g in 5 ml of H_2O, 7 M) was added slowly. A bulky precipitate formed during the addition of base. After 15 min standing the pasty mass was converted into a clear solution by the addition of ice/water. This clear solution was then slowly added to a beaker of HCl (20 ml, 4 M) giving a coloured precipitate. The precipitate was filtered and recrystallised from hexane.

2-(2'-Thienyl)nitroethene (98)

2-Thiophenecarboxaldehyde and nitromethane (2.00 g, 33 mmol) were used in general procedure J to give 2-(2'-thienyl)nitroethene (98) as yellow crystals, 75%, m.p. 77 ^{o}C (lit.,[136] m.p. 79-80 ^{o}C); υ_{max} (KBr disc) 3105, 3090, 1620, 1520, 1320, 1180, 970, 950 and 725 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 7.14 (dd, J 4Hz, J 5Hz, 1H), 7.44 (d, J 14Hz, 1H), 7.45 (ddd J 0.7Hz, J 4Hz, J 1Hz, 1H), 7.56 (dt, J 5Hz, J 1Hz, 1H) and 8.12 (dt, J 14Hz, J 0.7Hz, 1H); δ_C

($CDCl_3$) 128.90 (Ar-C), 131.71 (<u>C</u>HNO_2), 132.15 (Ar-C), 133.73 (Ar-C), 134.74 (Ar-C) and 135.28 (<u>C</u>$HCHNO_2$); *m/z* 156 (M^+ +1), 155 (M^+), 109, 97, 84 and 28 (100%); Found: M^+, 155.0021. $C_6H_5NO_2S$ requires M, 155.0029; Found: C, 46.38; H, 3.16; N, 9.03%. $C_6H_5NO_2S$ requires: C, 46.45; H, 3.23; N, 9.03%.

2-(3'-Thienyl)nitroethene (100)

3-Thiophenecarboxaldehyde and nitromethane (2.00 g, 33 mmol) were used in general procedure J to give 2-(3'-thienyl)nitroethene (100) as yellow crystals, 80%, m.p. 88-89 °C; υ_{max} (KBr disc) 3105, 1630, 1525, 1320, 1165, 970, 960 and 790 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 7.27 (dd, J 5Hz, J 3Hz, 1H), 7.41 (ddd, J 0.6Hz, J 5Hz, J 1Hz, 1H), 7.46 (d, J 14Hz, 1H), 7.73 (dd, J 3Hz, J 1Hz, 1H) and 7.98 (dd, J 14Hz, J 0.6Hz, 1H); δ_C ($CDCl_3$) 124.99 (Ar-C), 128.15 (Ar-C), 132.31 (CH<u>C</u>HNO_2), 132.92 (Ar-C), 132.57 (Ar-C) and 136.66 (<u>C</u>$HCHNO_2$); *m/z* 156 (M^+ +1), 155 (M^+), 108, 97, 84 and 45 (100%); Found: M^+, 155.0025. $C_6H_5NO_2S$ requires M, 155.0031; Found: C, 46.16; H, 3.16; N, 9.03%. $C_6H_5NO_2S$ requires: C, 46.45; H, 3.23; N, 9.03%.

3-(2'-Thienyl)-2-nitroprop-2-ene

2-Thiophenecarboxaldehyde and nitropropane (2.48 g, 33 mmol) were used in general procedure J to give 3-(2'-thienyl)-2-nitroprop-2-ene as yellow crystals, 60%, m.p. 65 °C (lit.,[136] m.p. 68.5 °C); υ_{max} (KBr disc) 3105, 3090, 1640, 1515, 1330, 1300, 975, 930 and 715 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.53 (s, 3H), 7.17 (dd, J 4Hz, J 5Hz, 1H), 7.42 (d, J 4Hz, 1H), 7.63 (d, J 5Hz, 1H) and 8.28 (s, 1H); δ_C ($CDCl_3$) 14.19 (<u>C</u>H_3), 127.27 (Ar-C), 128.23 (Ar-C), 131.89 (Ar-C),

134.90 (CHCCH3NO2), 135.14 (CHCCH3NO2), and 144.23 (Ar-C); *m/z* 170 (M^++ 1), 169 (M^+), 112, 97, 84 and 45 (100%); Found: M^+, 169.0202. $C_7H_7NO_2S$ requires M, 169.0207; Found: C, 46.62; H, 4.12; N, 8.21%. $C_7H_7NO_2S$ requires: C, 46.70; H, 4.14; N, 8.28%.

3-(3'-Thienyl)-2-nitroprop-2-ene

3-Thiophenecarboxaldehyde and nitropropane (2.48 g, 33 mmol) were used in general procedure J to give 3-(3'-thienyl)-2-nitroprop-2-ene as light brown crystals, 68%, m.p. 67-68 °C; υ_{max} (KBr Disc) 3105, 1630, 1520, 1315, 975, 940 and 775 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.51 (s, 3H), 7.24 (dd, J 3Hz, J 5Hz, 1H), 7.49 (d, J 3Hz, 1H), 7.80 (d, J 5Hz, 1H) and 8.12 (s, 1H); δ_C ($CDCl_3$) 15.07 (CH3), 123.36 (Ar-C), 124.17 (Ar-C), 131.45 (Ar-C), 133.78 (CHCCH3NO2), 135.25 (CHCCH3NO2), and 145.29 (Ar-C); *m/z* 170 (M^+ +1), 169 (M^+), 123 112, 97 and 45 (100%); Found: M^+, 169.0214. $C_7H_7NO_2S$ requires M, 169.0206; Found: C, 46.58; H, 4.15; N, 8.19%. $C_7H_7NO_2S$ requires: C, 46.70; H, 4.14; N, 8.28%.

General Procedure (K) for the Reduction of Nitro Vinyl-Thiophenes and Pyrroles to Amines using LiAlH4

A flame dried 50 ml round bottom flask, under nitrogen, was charged with 1 M LiAlH4 in THF (20 ml, 20 mmol). A solution of the nitro vinyl thiophene or pyrrole (1.65 mmol) in THF (25 ml) was added slowly over a few min so as to cause gentle reflux. The reaction was stirred (1 h for thiophenes, and 24 h for pyrroles) and then a few drops of H20 were added cautiously. Sodium potassium tartrate (35 ml, 20% aqueous soln.) was added to break up the salts. The organic layer was separated and the aqueous layer was washed

with ether (5 x 50 ml). The combined organic extracts were dried (Na_2SO_4), filtered and the solvent was removed *in vacuo* to give the free amine as an oil. The oil was partitioned between dichloromethane and HCl as in general procedure C to give the hydrochloride salt. Recrystallisation was from absolute alcohol with the addition of ether until the solution became cloudy, followed by boiling until the solution became clear and subsequent cooling to allow crystallisation.

2-(2'-Thienyl)ethylamine Hydrochloride (99)

Using 2-(2'-thienyl)nitroethene (98) in general procedure K gave 2-(2'-thienyl)ethylamine hydrochloride (99) as dark green crystals, 60%, m.p. 201-203 ^{o}C (lit.,[136] m.p. 200-202 ^{o}C); υ_{max} (KBr disc) 3300-2600 (strong), 1600, 1390, 785 and 770 cm^{-1}; δ_H (200 MHz, D_2O) 2.74 (t, 2H), 3.01 (t, 2H), 6.89 (d, 1H), 7.02 (d, 1H), 7.18 (d, 1H); δ_C (D_2O) 27.63 ($\underline{C}H_2CH_2NH_2$), 40.24 ($\underline{C}H_2NH_2$), 127.58 (Ar-C), 131.85 (Ar-C), 132.92 (Ar-C) and 134.74 (Ar-C); *m/z* 127, 109, 98 (100%), 45 and 30; Found: C, 44.13; H, 6.22; N, 8.49%. $C_6H_{10}ClNS$ requires: C, 44.04; H, 6.12; N, 8.56%.

2-(3'-Thienyl)ethylamine Hydrochloride (101)

Using 2-(3'-thienyl)nitroethene (100) in general procedure K gave 2-(3'-thienyl)ethylamine hydrochloride (101) as dark green crystals, 83%, m.p. 213-214 ^{o}C; υ_{max} (KBr disc) 3250-2560 (strong), 1600, 1420, 1395, 790 and 760 cm^{-1}; δ_H (200 MHz, D_2O) 2.82 (t, 2H), 3.13 (t, 2H), 6.92 (d, 1H), 7.10 (d, 1H), 7.29 (d, 1H); δ_C (D_2O) 28.21 ($\underline{C}H_2CH_2NH_2$), 40.87 ($\underline{C}H_2NH_2$), 123.96 (Ar-C), 127.95 (Ar-C), 128.92 (Ar-C) and 137.57 (Ar-C); *m/z* 127, 110, 98, 45 and

30 (100%); Found: C, 43.89; H, 6.29; N, 8.43%. $C_6H_{10}ClNS$ requires: C, 44.04; H, 6.12; N, 8.56%.

(±)-3-(2'-Thienyl)propyl-2-amine Hydrochloride (102)

Using 3-(2'-thienyl)-2-nitroprop-2-ene in general procedure K gave (±)-3-(2'-thienyl)propyl-2-amine hydrochloride (102) as dark brown crystals, 51%, m.p. 139-140 °C (lit.,[136] m.p. 143-144.5 °C); υ_{max} (KBr disc) 3210-2700 (strong), 1605, 1490, 1475, 1390, 790 and 770 cm^{-1}; δ_H (200 MHz, D_2O) 2.69 (ABX system, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H), 2.99 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 6.84 (d, 1H), 7.10 (d, 1H), 7.23 (d, 1H); δ_C (D_2O) 27.63 ($\underline{C}H_2CHNH_2$), 40.24 ($\underline{C}HNH_2$), 126.36 (Ar-C), 131.19 (Ar-C), 132.27 (Ar-C) and 133.84 (Ar-C); *m/z* 141, 128, 98 (100%), 58, 45 and 30; Found: C, 47.15; H, 6.60; N, 8.01%. $C_7H_{12}ClNS$ requires: C, 47.31; H, 6.81; N, 7.88%.

(±)-3-(3'-Thienyl)propyl-2-amine Hydrochloride (103)

Using 3-(3'-thienyl)-2-nitroprop-2-ene in general procedure K gave (±)-3-(3'-thienyl)propyl-2-amine hydrochloride (103) as brown crystals, 62%, m.p. 149-150 °C; υ_{max} (KBr disc) 3260-2500 (strong), 1600, 1500, 1390, 790 and 770 cm^{-1}; δ_H (200 MHz, D_2O) 2.73 (ABX system, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 8 Hz, 2H), 3.09 (dd, J_{AX} 4 Hz J_{BX} 8 Hz, 1H), 6.90 (d, 1H), 7.12 (d, 1H), 7.34 (d, 1H); δ_C (D_2O) 29.27 ($\underline{C}H_2CHNH_2$), 41.77 ($\underline{C}HNH_2$), 124.69 (Ar-C), 128.50 (Ar-C), 129.16 (Ar-C) and 138.78 (Ar-C); *m/z* 141, 127, 98, 58, 45 and 30 (100%); Found: C, 47.23; H, 6.59; N, 7.97%. $C_7H_{12}ClNS$ requires: C, 47.31; H, 6.81; N, 7.88%.

1-(Phenylsulphonyl)pyrrole-2-carboxaldehyde (105)

To a stirred ice-cooled mixture of 1-phenylsulphonyl pyrrole (104) (2.59 g, 12.5 mmol) and aluminium chloride (3.93 g, 29.5 mmol) in 1,2-dichloroethane was added dropwise 1,1-dichloromethyl methyl ether (2.00 g, 17.5 mmol). The resulting solution was stirred at 0 °C for 3 h and then poured into a mixture of ice and water. The organic layer was separated and the aqueous layer was washed with dichloromethane (3 x 50 ml). The combined organic extracts were washed with water and then brine, dried over $MgSO_4$, and concentrated under reduced pressure to give a dark oil which solidified on standing overnight. Purification on a silica gel column (hexane/$CHCl_3$, 20:80, R_f 0.40) followed by recrystallisation (ethyl acetate/hexane, 95:5) gave white needles, 80%; m.p. 79 °C (lit.,[144] m.p. 78-79 °C); υ_{max} (KBr disc) 3140, 1670, 1480, 1190, 1140, 750, 725 and 590 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.39 (dd, 1H), 7.14 (complex, 2H), 7.46-7.66 (complex, 3H), 7.82-7.95 (complex, 2H) and 9.94 (s, 1H); δ_C ($CDCl_3$) 113.71 (Ar-C), 120.72 (Ar-C), 124.99 (Ar-C), 126.67 (Ar-C), 127.39 (Ar-C), 129.35 (Ar-C), 129.48 (Ar-C), 129.57 (Ar-C), 134.58 (Ar-C), 135.86 (Ar-C) and 178.69 (C=O); *m/z* 236 (M^+ +1), 235 (M^+), 207, 141, 115 and 77 (100%); Found: M^+, 235.0301. $C_{11}H_9NO_3S$ requires M, 235.0304.

1-(Phenylsulphonyl)pyrrole-3-carboxaldehyde (110)

2,5-Dimethoxy-3-formyltetrahydrofuran (108) (2.00 g, 12.5 mmol), benzenesulphonamide (109) (1.96 g, 12.5 mmol) and glacial acetic acid (25 ml) were combined and heated at reflux vigorously at 130 °C for 4 h and the solution turned dark. The acetic acid was then removed *in vacuo* leaving a black oil. To remove any traces of

acid the oil was dissolved in diethyl ether, washed with 5% $NaHCO_3$ solution, water and then brine. The solution was dried (Na_2SO_4), filtered, treated with charcoal, filtered through Celite and evaporated under reduced pressure to yield a yellow oil. The oil was recrystallised (diethyl ether/hexane, 1:1) 51%, m.p. 58-59 °C (lit.,[178] m.p. 57-58 °C); υ_{max} (KBr Disc) 3120, 1680, 1480, 1180, 1060, 725, 620 and 590 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.56 (dd, 1H), 7.10 (complex, 1H), 7.34-7.49 (complex, 4H), 7.80-7.95 (complex, 2H) and 9.71 (s, 1H); δ_C ($CDCl_3$) 110.78 (Ar-C), 122.39 (Ar-C), 127.13 (Ar-C), 127.72 (Ar-C), 128.41 (Ar-C), 129.12 (Ar-C), 129.41 (Ar-C), 129.78 (Ar-C), 134.83 (Ar-C), 134.95 (Ar-C) and 185.35 (C=O); *m/z* 236 (M^+ +1), 235 (M^+), 141, 116 and 77 (100%); Found: M^+, 235.0305. $C_{11}H_9NO_3S$ requires M, 235.0306.

General Procedure (L) for the Synthesis of Nitro Vinyl-Pyrroles[179]

(i) (Phenylsulphonyl)pyrrolecarboxaldehyde (10 mmol), ammonium acetate crystals (7.00 mmol) and nitroalkane (10 ml) were combined and heated at reflux for 3 h. The excess nitroalkane was then evaporated under reduced pressure giving a black residue. The residue was dissolved in ethyl acetate (25 ml) and washed with dilute $NaHCO_3$ solution and brine. The organic layer was separated, dried (Na_2SO_4) and filtered, then treated with charcoal, filtered through Celite and evaporated under reduced pressure to yield a yellow oil. The oil was then recrystallised from diethyl ether.

(ii) (Phenylsulphonyl)pyrrolecarboxaldehyde (10 mmol) and nitroalkane (15 mmol) were dissolved in glacial acetic acid (20 ml) and heated at reflux overnight. The solvent was then evaporated

under reduced pressure and the residue was worked up as with procedure (i).

1'-(Phenylsulphonyl)-2-(2'-pyrrolyl)nitroethene (106)

1-(Phenylsulphonyl)pyrrole-2-carboxaldehyde (105) and nitro- methane (0.915 g, 15 mmol) were used in general procedure L(ii) to give 1'-(phenylsulphonyl)-2-(2'-pyrrolyl)nitroethene (106) as yellow crystals, 65%; m.p. 126 ^{o}C (lit.,[179] m.p. 126-128 ^{o}C); υ_{max} (KBr disc) 3140, 1625, 1540, 1370, 1190, 1170, 1150, 1140 and 990 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.28 (complex, 1H), 7.02 (complex, 3H), 7.40-7.59 (complex, 3H) and 7.80-7.89 (complex, 3H); δ_C ($CDCl_3$) 112.65 (Ar-C), 119.72 (Ar-C), 120.08 ($\underline{C}HNO_2$) 122.17 (Ar-C), 125.35 (Ar-C), 126.48 (Ar-C), 127.22 (Ar-C), 128.96 (Ar-C), 129.08 (Ar-C), 130.61 ($\underline{C}HCHNO_2$), 132.13 (Ar-C) and 133.27 (Ar-C); *m/z* 279 (M^+ +1), 278 (M^+), 262, 207, 141 and 77 (100%); Found: M^+, 278.0350. $C_{12}H_{10}N_2O_4S$ requires M, 278.0353.

1'-(Phenylsulphonyl)-2-(3'-pyrrolyl)nitroethene (111)

1-(Phenylsulphonyl)pyrrole-3-carboxaldehyde (110) and nitromethane (0.915 g, 15 mmol) were used in general procedure L(i) to give 1'-(phenylsulphonyl)-2-(3'-pyrrolyl)nitroethene (111) as yellow crystals, 73%; m.p. 125-126 ^{o}C (lit.,[179] m.p. 125-127 ^{o}C); υ_{max} (KBr disc) 3125, 1540, 1380, 1185, 1175, 1095, 1065 and 980 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 6.51 (complex, 1H), 7.06 (complex, 2H), 7.29-7.43 (complex, 4H) and 7.78-7.93 (complex, 3H); δ_C ($CDCl_3$) 109.26 (Ar-C), 119.47 ($\underline{C}HNO_2$), 120.14 (Ar-C), 126.02 (Ar-C), 127.19 (Ar-C), 127.84 (Ar-C), 128.95 (Ar-C), 129.36 (Ar-C), 129.91 (Ar-C), 132.70 ($\underline{C}HCHNO_2$), 133.11 (Ar-C) and 134.18 (Ar-C); *m/z*

278 (M^+), 262, 207, 141 and 77 (100%); Found: M^+, 278.0321. $C_{12}H_{10}N_2O_4S$ requires M, 278.0357.

Attempted Synthesis of 1'-(Phenylsulphonyl)-3-(2'-pyrrolyl)-2-nitroprop-2-ene

1-(Phenylsulphonyl)pyrrole-2-carboxaldehyde (105) and nitroethane (1.125 g, 15 mmol) were used in general procedure L(i). The product was a brown oil which was shown to be the starting aldehyde; δ_H (200 MHz, $CDCl_3$) 9.94 (s, 1H); *m/z* 235 (M^+), 207, 141, 115 and 77 (100%). [Note: the temperature and time of reflux were increased, but again the starting aldehyde was the major product. Conditions in general procedure L(ii) were also tried, but failed.]

Attempted Synthesis of 1'-(Phenylsulphonyl)-3-(3'-pyrrolyl)-2-nitroprop-2-ene

1-(Phenylsulphonyl)pyrrole-3-carboxaldehyde (110) and nitroethane (1.125 g, 15 mmol) were used in general procedure L(i). The product was a yellow oil which showed two spots by TLC (hexane/ $CHCl_3$, 20:80, R_f 0.26 and R_f 0.38). Purification by silica gel column with the same solvent system gave the starting aldehyde as the major product; δ_H (200 MHz, $CDCl_3$) 9.71 (s, 1H); *m/z* 235 (M^+), 207, 141, 115 and 77 (100%).

2-(2'-Pyrrolyl)ethylamine Hydrochloride (107)

Using $LiAlH_4$ to reduce 1'-(phenylsulphonyl)-2-(2'-pyrrolyl)nitroethene (106) in general procedure K gave 2-(2'-pyrrolyl)ethylamine hydrochloride (107) as a light brown solid, 55%; υ_{max} (KBr disc) 3380, 3220-3100, 1580, 725 and 590 cm^{-1}; δ_H (200 MHz, D_2O) 2.81 (t, 2H), 3.12 (t, 2H), 6.34 (complex, 1H), 7.17 (d, 1H), 7.25 (d, 1H), 7.93 (s,1H); *m/z* 111, 94, 80 and 69 (100%).

2-(3'-Pyrrolyl)ethylamine Hydrochloride (112)

Using $LiAlH_4$ to reduce 1'-(phenylsulphonyl)-2-(3'-pyrrolyl)nitroethene (111) in general procedure K gave 2-(3'-pyrrolyl)ethylamine hydrochloride (112) as a brown solid, 42%; υ_{max} (KBr disc) 3360, 3200-3100, 1570, 750 and 600 cm^{-1}; δ_H (200 MHz, D_2O) 2.73 (t, 2H), 3.09 (t, 2H), 6.84 (d, 1H), 7.11 (s, 1H), 7.20 (d, 1H), 7.89 (s,1H); *m/z* 111, 94, 80 and 69 (100%).

8.6 Experimental for Chapter Seven

Synthesis of Cisplatin Analogues

Synthesis of *cis*-1,4-Diaminobut-2-ene (119)

cis-*1,4-Dimethylsulphonylbut-2-ene (121)*

cis-But-2-ene-1,4-diol (2.29 g, 0.026 mol) was dissolved in dry THF (50 ml) and the solution was cooled to -78 ^{o}C under nitrogen. Methanesulphonyl chloride (4 ml, 0.052 mol) was added with stirring followed by the slow addition of triethylamine (5.25 g, 0.052 mol) over 5 min. The mixture was allowed to reach room temperature and left stirring overnight giving a thick white solution. The mix was poured onto ice/water (150 ml) and extracted with dichloromethane (3 x 75 ml). The combined organic extracts were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* leaving a light brown oil. White crystals of *cis*-1,4-dimethylsulphonylbut-2-ene (121) crystallised from the oil, were recrystallised from diethyl ether and stored at 0 ^{o}C, 85%, υ_{max} (KBr disc) 2950, 1615, 1350 and 1175 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.05 (s, 6H), 4.80 (d, 4H), 5.95 (complex, 2H); δ_C ($CDCl_3$) 29.82 (2$\underline{C}H_3$), 38.09 (2$\underline{C}H_2CH$), 127.63 (2$\underline{C}HCH_2$); *m/z* 96 (100%), 77 and 56.

cis-*1,4-Dibromobut-2-ene (123)*

Phosphorus tribromide (10 ml, 0.11 mol) was cooled to 0 ^{o}C and *cis*-but-2-ene-1,4-diol (12 g, 0.13 mmol) was added slowly over a 3 h period. This gave a dark brown solution which was

stirred overnight. The mixture was cooled to 0 °C and cold water (50 ml) was added slowly to hydrolyse any excess phosphorus tribromide. The organic layer was separated and diluted with diethyl ether (50 ml). After washing with $NaHCO_3$ solution (3 x 20 ml) the organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to give *cis*-1,4-dibromobut-2-ene (123) as a colourless oil, 81%; υ_{max} (thin film) 2950, 1630 and 690 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.00 (d, 4H) and 5.88 (complex, 2H); δ_C ($CDCl_3$) 35.30 (2$\underline{C}H_2$CH), 127.35 (2$\underline{C}$HCH$_2$); *m/z* 134, 104 (100%), 76 and 54.

cis-*1,4-Diphthalimidobut-2-ene (122)*

A solution of potassium phthalimide (8.5 g, 46 mmol) in DMF (100 ml) was heated to 50 °C and potassium iodide (0.40 g, 2.41 mmol) was added. With the reaction stirring rapidly *cis*-1,4-dibromobut-2-ene (123) (5.00 g, 23 mmol) was added slowly. The suspension was stirred at 60 °C for 16 h then poured onto ice/water (150 ml) and extracted with large quantities of dichloromethane (7 x 100 ml). The dichloromethane was dried, filtered and evaporated under reduced pressure, leaving behind residual DMF from which *cis*-1,4-diphthalimidobut-2-ene (122) precipitated after 2 h at 0 °C. The solid was filtered, washed free of DMF with diethyl ether (20 ml) and dried, leaving *cis*-1,4-diphthalimidobut-2-ene (122) as a white powder, 62%; υ_{max} (KBr disc) 3200-2950, 1765, 1710, 1620, 1150 and 720 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.54 (d, 4H) and 5.70 (complex, 2H); δ_C ($CDCl_3$) 34.61 ($\underline{C}H_2N$), 123.30 (Ar-C), 123.61 (Ar-C), 127.00 ($\underline{C}$HCH$_2$), 132.43 (Ar-C), 133.95 (Ar-C), 134.33 (Ar-C) and 167.87 (N$\underline{C}$O); *m/z* 346 (M^+), 204, 132, 104, 76 (100%) and 54.

cis-*1,4-Diaminobut-2-ene Dihydrochloride (124)*

A 1:1 mixture of concentrated hydrocloric acid (60 ml) and glacial acetic acid (60 ml) were added to *cis*-1,4-diphthalimidobut-2-ene (122) (5.00 g, 14 mmol) with stirring. The mixture was heated at reflux for 30 h after which time it was allowed to cool to room temperature. This resulted in a precipitate of phthalic acid which was removed by filtration. The filtrate was concentrated (5-10 ml) by evaporation yielding a second precipitate of phthalic acid which was again filtered off. The remaining filtrate was diluted with glacial acetic acid (50 ml) and addition of diethyl ether (50 ml added in 10 ml portions) gave a white precipitate of *cis*-1,4-diaminobut-2-ene dihydrochloride (124). The precipitate was washed with ether and crystallised from aqueous ethanol, 78%; m.p. >250 ^{o}C; υ_{max} (KBr disc) 3500-3300, 3100-3200, 1620, 1600, 1060 and 780 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.82 (d, 4H), 5.94 (complex, 2H) and 7.75-7.95 (complex, 8H); δ_C ($CDCl_3$) 37.05 ($\underline{C}H_2N$) and 127.84 ($\underline{C}HCH_2$); *m/z* 87, 85, 69 (100%) and 54.

Synthesis of Platinum Precursors

cis-*Dichloro-bis(dimethylsulphonyl)platinum(II) (126)*

Potassium tetrachloroplatinate(II) (125) (1.04 g, 3.00 mmol) was dissolved in water (10 ml) giving a red solution, and dimethyl sulphoxide (0.70 g, 9.00 mmol) was then added. The solution was allowed to stand and turned yellow over a period of 15 min followed by the growth of yellow crystals after 40 min. The crystals were filtered off and then washed with water, ethanol and ether.

The crystals were then placed on a vacuum pump to dry for 4 h, 70%; υ_{max} (KBr disc) 3040, 3015, 2920, 1157, 1134, 1020, 430, 380, 335 and 310 cm^{-1}; δ_H (200 MHz, D_2O) 3.70 (s, 12H); *m/z* 199, 78, 63 (100%) and 45; Found: C, 11.26; H, 2.89; S, 15.11%. $Cl_2PtC_4H_{12}O_2S_2$ requires: C, 11.37; H, 2.84; S, 15.17%.

Silver Salt of 1,1-Cyclobutanedicarboxylic Acid (127)

1,1-Cyclobutanedicarboxylic acid (2.00 g, 14 mmol) was dissolved in water (50 ml) and NaOH (1.12 g, 28 mmol) was added with stirring. When all the NaOH had dissolved (approx. 5 min), silver nitrate (4.76 g, 28 mmol) was added and the mixture was left stirring in darkness overnight. When the reaction was opened to light a white precipitate had formed which was filtered off, dried and quickly used without further purification, 47%; υ_{max} (KBr disc) 1670, 1650, 1320 and 1300 cm^{-1}; δ_H (200 MHz, D_2O) 1.86 (complex, 2H) and 2.69 (t, 4H).

cis-*Bis-dimethylsulphonyl(1,1-cyclobutanedicarboxylato)-platinum(II) (128)*[171]

cis-Dichloro-bis(dimethylsulphonyl)platinum(II) (126) (0.88 g, 2.1 mmol) and the silver salt of 1,1-cyclobutanedicarboxylic acid (127) (0.75 g, 2.1 mmol) were stirred in water (150 ml) at room temperature and left in darkness for 22 h giving a creamy precipitate. The solid was filtered off and the filtrate concentrated to *ca.* 5 ml giving the product (128) as colourless crystals, 59%; m.p. 200 ^oC dec (lit.,[171] m.p. 201 ^oC dec); υ_{max} (KBr disc) 3000, 2940, 2920, 1660, 1610, 1410, 1340, 1330, 1140, 1115 and 1025 cm^{-1}; δ_H (200 MHz, D_2O) 1.90 (complex, 2H), 2.79 (t, 4H) and 3.58 (s,

12H); δ_C (D_2O) 16.10 ($CH_2\underline{C}H_2CH_2$), 30.95 ($2\underline{C}H_2C$), 44.02 ($4\underline{C}H_3$), 57.38 ($\underline{C}C{=}O$) and 181.24 ($2\underline{C}{=}O$); Found: C, 24.24; H, 3.86; S, 12.86%. $PtC_{10}H_{18}O_6S_2$ requires: C, 24.34; H, 3.65; S, 12.98%.

cis-*1,4-Diaminobut-2-ene(dichloro)platinum(II) (119)*

cis-1,4-Diaminobut-2-ene (124) was partially dissolved in water (10 ml) and the solution taken to pH 7 with dilute HCl. Potassium tetrachloroplatinate(II) (125) was dissolved in water (10 ml) and added to the neutral solution. The mixture was heated to 50 ^oC and the pH of the solution kept at 7 by the addition of dilute NaOH. When there was no further change in the pH the reaction was allowed to cool to room temperature. This gave *cis*- 1,4-diaminobut-2-ene(dichloro)platinum (II) (119) as an orange/brown solid which was then washed with water, ethanol and ether. The solid was only sparingly soluble in water and was insoluble in all common organic solvents, 83%; υ_{max} (KBr disc) 3400-3100, 1640, 1620, 710, 335 and 310 cm^{-1}; *m/z* 87, 85, 71 and 54 (100%).

General Procedure (M) for the Synthesis of *cis*-Diamino (1,1-cyclobutanedicarboxylato)platinum(II) Complexes[172]

(i) To a hot aqueous solution (10 ml) of *cis*-bis-dimethylsulphonyl(1',1'-cyclobutanedicarboxylato)platinum(II) (128) (0.25 g, 0.50 mmol) was added the diamine (0.50 mmol) in water (3 ml). The mixture was stirred at 100 ^oC for 6 h then left to cool yielding a precipitate, which was filtered and dried.

(ii) Diamine (0.68 mmol) was added to an aqueous solution (40 ml) of *cis*-bis-dimethylsulphonyl(1',1'-cyclobutanedicarboxylato)-platinum(II) (128) (0.33 g, 0.68 mmol) and the mixture was stirred

at 40 °C for 1 h. The water was removed *in vacuo* at 40 °C leaving a solid which was washed with ethanol and then ether. The solid was then dissolved in water (10 ml) and heated at 100 °C for 6 h. After cooling to room temperature the product precipitated.

cis-*1,4-Diaminobutane(1',1'-cyclobutanedicarboxylato)-platinum(II) (129)*

Using 1,4-diaminobutane (3) in general procedure M(i) gave *cis*-1,4-diaminobutane(1',1'-cyclobutanedicarboxylato)-platinum(II) (129) as colourless crystals, 10%, m.p. >250 °C; υ_{max} (KBr disc) 3600-3300, 1650, 1615, 1380, 910 and 780 cm^{-1}; δ_H (200 MHz, D_2O) 1.62-1.87 (complex, 4H), 1.90 (complex, 2H), 2.79 (t, 4H) and 2.91-3.06 (complex, 4H); *m/z* 142, 114 (100%), 82 and 45.

cis-*(trans-(-)-1,2-Diaminocyclohexane)(1',1'-cyclobutane-dicarboxylato)platinum(II) (130)*

Using *trans*-(-)-1,2-diaminocyclohexane in general procedure M(i) gave *cis*-(*trans*-(-)-1,2-diaminocyclohexane)(1',1'-cyclobutane dicarboxylato)platinum(II) (130) as colourless crystals, 67%, m.p. 278-280 °C dec (lit.,[172] m.p. 280 °C dec); υ_{max} (KBr disc) 3600-3200, 1640, 1370, 1180, 1120, 1070, 1025, 910 and 780 cm^{-1}; δ_H (D_2O) 1.09-1.38 (complex, 4H), 1.60 (d, 2H), 1.88 (complex, 2H), 2.12 (d, 2H), 2.40 (complex, 2H) and 2.84 (t, 4H).

Attempted Synthesis of cis-1,4-Diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)platinum(II) (120) using General Procedure M (i)

Using *cis*-1,4-diaminobut-2-ene (124) in general procedure M(i) gave a black solid which appeared to be *cis*-1,4-diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)platinum(II) (120) by spectroscopic analysis, but was insoluble in all common solvents and further purification was not possible; υ_{max} (KBr disc) 1640, 1615, 1380 and 910 cm^{-1}; *m/z* 142, 114, 87, 82, 69 and 54 (100%).

Attempted Synthesis of* cis-*1,4-Diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)platinum(II) (120) using General Procedure M (ii)

Using *cis*-1,4-diaminobut-2-ene (124) in general procedure M(ii) gave a brown precipitate which was shown to be *cis*-bis-dimethylsulphonyl(1',1'-cyclobutanedicarboxylato)platinum(II) (128) by spectroscopic analysis; δ_H (200 MHz, D_2O) 1.90 (complex, 2H), 2.79 (t, 4H) and 3.58 (s, 12H).

Attempted Synthesis of* cis-*1,4-Diaminobut-2-ene(1',1'-cyclobutanedicarboxylato)platinum(II) (120) from* cis-*1,4-Diaminobut-2-ene(dichloro)platinum(II) (119)

A suspension of *cis*-1,4-diaminobut-2-ene(dichloro)platinum (II) (119) (0.35 g, 1.00 mmol) in water (50 ml) was heated to 50 °C and a solution of the sodium salt of 1,1-cyclobutanedicarboxylic acid (127) (0.19 g, 1.00 mmol) in water (10 ml) was added with stirring. The mixture was refluxed for 15 h and then allowed to

cool. A black solid was filtered and washed with water. The solid was insoluble in all common solvents and so was not purified further; υ_{max} (KBr disc) 1630, 1615, 1370, 910 and 710 cm^{-1}.

Synthesis of 2,4-Dioxopentanoic Acid[175]

Methyl 2,4-dioxopentanoate (132)

Acetone (8.5 ml) and dimethyloxalate (13.7 g, 0.12 mol) were gradually added to a stirred dry methanol solution (100 ml) of sodium methoxide (9.41 g, 0.174 mmol) and the mixture was heated at reflux for 3 h. After the mixture was cooled to room temperature the solvent was concentrated under reduced pressure and the resulting residue acidified with dilute HCl. The acidic solution was extracted with ethyl acetate (3 x 75 ml) and the combined organic extracts were washed with water and brine, dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* to give a light coloured residue which was crystallised from ethanol/hexane, 68%; m.p. 96 °C (lit.,[175] m.p. 98 °C); υ_{max} (KBr disc) 1730, 1640, 1440, 1280, 1024 and 975 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.30 (s, 3H), 3.90 (s, 3H), 6.39 (s, 1H), 14.42 (br s, 1H); Found: C, 50.12; H, 5.60%. $C_6H_8O_4$ requires: C, 50.00; H, 5.56%.

2,4-Dioxopentanoic Acid (131)

Methyl 2,4-dioxopentanoate (132) (2.57 g, 16.2 mmol) was dissolved in methanol (30 ml) and 2 M NaOH solution (20 ml) was added. The mixture was stirred at room temperature for 5 h and the solvent was then removed under reduced pressure. The resulting residue was acidified with 9 M HCl to pH 1 and then

extracted with diethyl ether and the solvent removed *in vacuo*. The residue was recrystallised from carbon tetrachloride to give colourless crystals, 30%; m.p. >250 ^{o}C (lit.,[175] m.p. 282 ^{o}C dec); υ_{max} (KBr disc) 3300-3200, 1690, 1640, 1280, 660 cm^{-1}; *m/z* 130 (M^{+}), 113, 85 and 57 (100%).

Appendix

See Experimental Section for method

Table 1 (a): Standard Protein Concentrations and A_{620}/A_{465} Readings (1)

Protein Content (mg)	A_{465}	A_{620}	A_{620}/A_{465} - A_{620}/A_{465} (Blank)
50	0.317	0.971	2.54
40	0.366	0.886	1.90
30	0.476	0.783	1.12
25	0.512	0.740	0.92
20	0.526	0.623	0.66
15	0.555	0.638	0.63
10	0.606	0.550	0.39
5	0.637	0.479	0.23
Blank	0.613	0.320	0.00

Appendix (cont.)

See Experimental Section for method

Table 1 (b): Standard Protein Concentrations and A_{620}/A_{465} Readings (2)

Protein Content (mg)	A_{465}	A_{620}	A_{620}/A_{465} - A_{620}/A_{465} (Blank)
50	0.424	1.003	1.80
40	0.445	0.920	1.50
30	0.463	0.868	1.31
25	0.490	0.782	1.03
20	0.504	0.737	0.90
15	0.589	0.662	0.56
10	0.598	0.578	0.40
5	0.682	0.486	0.23
Blank	0.681	0.384	0.00

Appendix (cont.)

See Experimental Section for method

Table 1 (c): Standard Protein Concentrations and Average A_{620}/A_{465} Readings from Table 1 (a) and 1 (b)

Protein Content (mg)	A_{620}/A_{465} - A_{620}/A_{465} (Blank)
50	2.17
40	1.70
30	1.22
25	0.98
20	0.78
15	0.60
10	0.40
5	0.23
Blank	0.00

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